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Bacteria Associated with Rice Seed from Philippine Farmers' Fields

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"Geluk is wanneer wat je denkt, wat je zegt en wat je doet in overeenstemming zijn."
Mahatma Gandhi

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Contents

Preface

The remarkable increase in rice (*Oryza sativa*) yields during the 1960s and 1970s, popularly referred to as the "Green Revolution", was accomplished through the development and dissemination of high-yielding, semi-dwarf rice cultivars, expanded irrigated areas with abundant water, high input of chemical fertilizers and pesticides, and mechanization. Land conversion and intensification of rice production, however, also altered the biotic interactions and patterns of resource availability in ecosystems and has resulted in a change in the disease scenario in rice fields with newly emerged insect, disease, and weed problems. The emergence of new diseases or diseases previously not considered important such as blast, sheath blight, bacterial blight, grain rot, and sheath brown rot, has posed a major challenge to sustain the increases in rice production needed to meet the anticipated demands of rapidly escalating populations in Asia. Disease and pest management tactics have relied primarily on new resistant cultivars and the use of pesticides. Host plant resistance is an effective and ecologically sound approach for disease control, however, current commercial rice cultivars either carry no adequate resistance toward many existing diseases, or resistance genes have yet to be identified and incorporated. Moreover, the widespread and continuous deployment in monoculture of genetically uniform plants that accompanies agricultural intensification, often carries the risk of being susceptible to devastating epidemics. Currently, the maximum dependence has been on chemical control and, over the years, the use of chemicals has increased enormously. As a result, pesticide resistance has become a ubiquitous problem, as have the environmental and human health threats associated with pesticide transfers to water and air. Further, pesticides severely reduce biological diversity by destroying a wide array of susceptible species in the ecosystem. For example, spiders are an important group of predators commonly found in rice fields but are very susceptible to insecticides. Fungicidal control of blast and sheath blight is increasingly used in many rice intensification areas but can also be highly toxic to natural enemies in the rice ecosystem. There is little doubt that also microbial communities are sensitive to changes in the environment. Agricultural practices, such as tillage, intercropping, rotation, drainage, and uses of fertilizers, can have significant implications on plant-associated microbial communities (Bossio et al., 1998; Buckley and Schmidt, 2001; McLaughlin and Mineau, 1995). Other comparative studies using DNA-based community profiling methods have documented the effects of pesticides, herbicides, and fungicides on microbial communities (El Fantroussi et al., 1999; Johnsen et al., 2001; Sigler and Turco, 2002).

The growing concern about the environment has caused a shift in crop protection approaches in agriculture. The present trend is not to eliminate a pest organism but to reduce and keep it below damaging levels by employing strategies that take advantage of ecological interactions within agricultural systems and complement or substitute for pesticides. This approach to achieving an ecologically sound and sustainable agriculture is the principle behind Integrated Pest Management (IPM), which advocates the use of host plant resistance, biological control, crop rotations, and soil and water management. Biological control includes the exploitation of nonpathogenic plant-associated microorganisms to suppress

plant diseases. The great variability reported for microbial biocontrol agents often indicated them to be host and site specific, and suggests that the best possibilities for biological control might lie in the use or stimulation of indigenous bacteria naturally present in the same ecological niche as the pathogen. Early research identified the presence of many microbial antagonists in association with the rice ecosystem and in particular with the seed (Rosales et al., 1993; Sakthivel and Gnanamanickam, 1987; Xie et al., 1997). Hence, a long-term research objective of the Plant Pathology Division at IRRI has been to develop biocontrol agents for disease suppression of rice sheath blight and blast, caused by *Rhizoctonia solani* AG-1 and *Pyricularia grisea*, respectively. However, few potential biocontrol agents that showed promising results in laboratory and greenhouse studies have lived up to their promises when tested in the field. The reasons behind this are obviously a lack of knowledge on factors that determine the success or failure of introduced biocontrol agents in the rice fields. In nature microorganisms live in complex associations whose compositions vary depending on the environmental conditions. The activities of individual members of such associations are strongly influenced both by the prevailing conditions in the environment and by the activities of the other members. Beneficial microorganisms developed for biocontrol applications and introduced into nature must also be able to integrate into the indigenous microbial communities that inhabit the same niche, or at least tolerate them, in order to be able to survive and function properly. The most obvious and well-studied group of plant-associated bacteria are the pathogens. For instance, the extensive studies made on bacterial blight caused by *Xanthomonas oryzae* pv. o*ryzae*, and blast caused by *Pyricularia grisea*, have shown that detailed knowledge of pathogen population biology can advance the development of disease management strategies, such as host resistance deployment (Leung et al., 1993). In contrast, much less is known about the vast majority of nonpathogenic and potentially beneficial plant-associated bacteria. This is in part due to the fact that in developing biocontrol, much attention has been devoted to testing mass application of microorganisms, and less emphasis has been placed on studying their ecological context. Therefore, microbial diversity studies are important to come to a better understanding of microbial ecology in agricultural systems. It is obvious that a sound understanding of bacterial communities will require more than merely identifying some of the "key" microbial components but the whole bacterial community structure. And this also requires greater emphasis on research areas of microbial systematics and physiology, which provide the foundation and tools for microbiological studies.

Traditionally, taxonomic classification of bacteria has been determined based on few metabolic, morphological, and physiological traits (Holt et al., 1994). The development of molecular typing methods together with enhanced computer-aided cluster analysis techniques for the assessment of microbial diversity, have caused a shift to a polyphasic bacterial taxonomy, aiming at the integration of different kinds of data (phenotypic, genotypic, and phylogenetic) on microorganisms (Vandamme et al., 1996). Diversity among bacteria is expressed in numerous different molecules, which explains why monophasic classification systems are bound to fail. Any character that reveals part of this diversity is therefore useful and can be considered. The number of different molecules that have been applied in taxonomic studies is large, and their applications as markers are manifold. These include nucleic acids, protein and carbohydrate-containing macromolecules, and lipids. Each of these molecules has advantages over the others in specific uses and resolution level. It is however essential to be able to integrate different studies based on different markers.

A spectacular development in the past 15 years has been the analysis and sequencing of ribosomal RNA (rRNA) genes, which has revolutionized our understanding of bacterial phylogeny and taxonomy. Woese and colleagues compiled a molecular sequence-based phylogenetic tree showing that the main diversity of life is microbial (Woese, 1987; Woese et al., 1990). Comparative analysis of ribosomal DNA (rDNA) sequences indicated that all cellular life is distributed among three primary relatedness groups or domains: Archaea, Bacteria, and Eucarya. The rRNA genes have been recognized for their utility as molecular chronometers. Due to the high degree of structural and functional conservation, and the ubiquity of ribosomal RNA in all cellular life forms, comparative analysis of their sequences can be universally applied to infer relationships among organisms. As a consequence of the increased interest in rRNA gene sequences during the past years, and the development of automated DNA sequencing systems, rapidly increasing amounts of rDNA sequence data have been accumulated in international databases. For example, the Ribosomal Database Project II (RDP-II) (Maidak et al., 2001) and the European database on small subunit ribosomal RNA (Wuyts et al., 2002) now contain several thousand sequences, and represent an invaluable resource. By comparison of the more variable regions of the rDNA molecule, it is possible to design oligonucleotides of varying phylogenetic resolution. As such, domain-, phylum-, division-, subdivision-"signature" sequences (Amann et al., 1995; Manz et al., 1992; Roller et al., 1994), as well as some genus-, and species-specific motifs have been identified. Recently, a database containing more than 700 published rRNA-targeted oligonucleotide probe sequences has been compiled and made available through the internet (Loy et al., 2003). The advances in molecular phylogeny not only brought a phylogenetic perspective into microbiology, but also allowed the development of the necessary technologies for isolating RNA or DNA from an environment, and inferring the sequences of individual molecules (Olsen et al., 1986). This began what is now recognized as molecular microbial ecology. The first culture-independent estimate of prokaryotic abundance in soil by Torsvik et al. (1990) revealed a single gram of soil to contain more than 4,000 distinct genomes. That estimate was determined by the reassociation time of total community DNA extracted from soil compared with a standard curve of reassociation kinetics of a known number of cultured genomes. Since then, a number of culture-independent analyses have been used to record microbial diversity in various environments and also to assess the impact of agricultural practices and interactions with plants and other microorganisms on mostly soil and rhizosphere microbial communities. Several recent reviews have been published on the topic (DeLong and Pace, 2001; Johnsen et al., 2001; Kent and Triplett, 2002). Conversely, little is known on epiphytes from a culture-independent perspective as this has only been studied on citrus (Yang et al., 2001) and the seagrass *Halophia stipulacea* (Weidner et al., 2000).

Methods for classifying bacterial diversity

A wide variety of phenotypic and genotypic methods have been applied in taxonomic analyses of bacteria. The advent of molecular biology has caused a significant shift in the types of approaches used to characterize and identify bacteria. Currently, genotypic techniques are more frequently applied than the phenotypically oriented approaches in both taxonomy and ecology studies. It should be emphasized that limitations exist in the applicability of each technique depending on the group of bacterial isolates to be categorized. A

single, ideal identification technique does not exist. The different methods can be ordered according to their taxonomic resolution levels and their technical complexity. Therefore, when a collection of isolates needs to be examined, it is important to make a choice of methods that are most appropriate for the group of bacteria under investigation. Screening methods such as whole-cell fatty acid analysis allow to compare large numbers of isolates, whereas methods such as DNA-DNA hybridization or DNA sequencing remain laborious and will rather be restricted to a representative set of isolates. Some of the most common methods used for studying bacteria, and recently developed culture-independent methods, are given below. A more complete overview of taxonomic techniques required to study bacteria at different taxonomic levels can be found in Vandamme et al. (1996).

Phenotypic methods

Historically, a variety of phenotypic characteristics of microorganisms have been exploited to identify and classify bacteria such as morphological, physiological, and biochemical features. The morphology of a bacterium includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (color, dimensions, form) characteristics (Vandamme et al., 1996). Recently, a computer-aided system called CMEIAS (Center for Microbial Ecology Image Analysis System) has been developed for image analysis of the morphological diversity in complex microbial communities revealed by phase-contrast microscopy (Liu et al., 2001). The CMEIAS automatically categorizes each cell into one of 11 bacterial morphotypes, including cocci, spirals, curved rods, U-shaped rods, regular straight rods, unbranched filaments, ellipsoids, clubs, rods with extended prostheca, rudimentary branched rods, and branched filaments. Although CMEIAS can significantly enhance the ability to quantitate bacterial morphotype diversity without cultivation, it is obvious that a diversity assessment of microbial communities based on cellular morphology would be rather limited and provide little information about taxonomic diversity. (In this work, colony morphotype diversity observed within a particular cellular type already exceeded 11 types.) Direct microscopic examination of microbial communities by confocal laser scanning microscopy (CLSM) combined with fluorescence *in situ* hybridization (FISH) using rRNA-targeted oligonucleotide probes, has been used for quantitative analyses of bacterial community composition (Daims et al., 2001; Dang and Lovell, 2002). Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes has also been combined with microautoradiography (MAR) to simultaneously determine phylogenetic identification and *in situ* nutrient uptake patterns of various cultivable or uncultivable bacteria (Lee et al., 1999). Automated phenotypic fingerprinting systems such as Biolog, which analyzes substrate utilization profiles, have been popularly used for the identification of isolates as well as for assessing community-level metabolic functions (Grimont et al, 1996; Jones et al., 1993; Heuer and Smalla, 1997). However, Biolog has the inherent biases of culture-based approaches and therefore, may not give a metabolic fingerprint that is representative for the natural microbial community (Øvreås and Torsvik, 1998; Smalla et al., 1998). Fatty acid methyl ester (FAME) content is a stable parameter and has proven very useful for the characterization of bacterial taxa (Stead, 1992; Welch, 1991; Yang et al., 1993). The method is cheap, rapid, and highly automated. FAME analysis of environmental samples has been used widely to characterize the composition of soil microbial communities (Bossio et al., 1998; Ibekwe and Kennedy, 1998).

Genotypic methods

Genotypic methods can be divided roughly into PCR amplification-dependent and -independent genomic analysis approaches. One of the most fundamental direct genomic analysis methods is based on total DNA-DNA homology, or DNA-DNA re-association kinetics, to compare bacterial isolates pairwise. In fact, the 70% criterion of total genomic DNA-DNA relatedness has been accepted (Wayne et al., 1987), and currently been reconfirmed (Stackebrandt et al., 2002), as the gold standard for defining a bacterial species. Another direct genomic analysis method based on the digestion of total genomic DNA with restriction enzymes and analysis of the resulting banding patterns after hybridization with specific genomic probes is restriction fragment length polymorphism (RFLP). A typical example of such approach is the ribotyping method, which uses rRNA gene-specific oligonucleotide probes.

The advent of the PCR methodology has led to the development of a vast array of PCR-based DNA-typing methods, which have attracted much interest because of their simplicity and general applicability. PCR-based genomic fingerprinting methods include arbitrarily primed (AP-PCR) (Welsh and McClelland, 1990) or randomly amplified polymorphic DNA (RAPDs) (Williams et al., 1990), repetitive sequence-based rep-PCR (Rademaker et al., 1998; Versalovic et al., 1991), and amplified fragment length polymorphism (AFLP) (Vos et al., 1995) analysis. The AFLP-method is a combination of restriction enzyme analysis and PCR-based typing that screens for amplified fragment length polymorphisms by selective amplification of restriction fragments. Due to its simplicity and robustness, rep-PCR has been used most extensively to characterize phytobacterial populations. Rep-PCR genomic fingerprinting refers to the protocols known as REP-, ERIC- and BOX-PCR, which respectively use primers binding to conserved repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements, distributed in the genomes of diverse bacteria. Each of these protocols generates a collection of genomic fragments via PCR, which are resolved as banding patterns that provide the highest level of taxonomic resolution currently achievable by PCR methods. Of these protocols, BOX-PCR has often been found to be the most useful. The term repetitive sequence-based PCR genomic fingerprinting generally refers to any method using primers that anneal to repetitive DNA sequences.

The principle of enhanced specificity using restriction enzyme analysis is applicable for any amplified product and generically may be referred to as PCR-RFLP. PCR-RFLP applied to ribosomal gene sequences is known as amplified ribosomal DNA restriction analysis (ARDRA) (Heyndrickx et al., 1996; Vaneechoutte et al., 1992), in which universal primers are used to amplify bacterial rDNA sequences (with or without spacer regions), followed by digestion with frequently cutting restriction endonucleases and gelelectrophoresis, to characterize bacterial isolates at the genus and species level. The ARDRA technique is most frequently used on isolates but has recently also been used to probe community structure, either applied directly on the mixture of amplified rDNA PCR-products or on clone libraries (in E. coli) constructed from these PCR products (Øvreås and Torsvik, 1998). The internally transcribed spacer (ITS) regions between the 16S and 23S rRNA genes appear to be under less selection pressure and therefore more variable than the 16S and 23S rRNA genes themselves. PCR-based fingerprinting techniques that target the ITS regions are known as ITS-PCR analysis (Maes et al., 1996; Tan et al., 2001), or ITS-restriction fragment length polymorphism (ITS-RFLP) analysis (Schmidt, 1994).

Culture-independent PCR-based profiling methods

Comparative sequence analysis of rRNA gene sequences has become the gold standard to infer prokaryotic phylogeny and is widely used in molecular microbial ecology. The occurrence of phylogenetic types of organisms, "phylotypes," and their distributions in natural communities can be surveyed by cloning and sequencing rRNA genes obtained from DNA isolated directly from environmental samples. The ribosomal DNA operon in bacteria comprises three functionally and evolutionary conserved genes, the small subunit 16S rRNA gene, the large subunit 23S rRNA gene, and 5S rRNA gene, interspersed with variable spacer regions. Culture-independent approaches utilizing PCR-amplified 16S rDNA fragments from DNA extracted from the microbial community, followed by cloning and sequence analysis have been used to explore the diversity of soil microbial communities (Borneman et al., 1996; McCaig et al., 1999; Weber et al., 2001). Because of the complexity of microbial communities, comparative community analyses based solely on sequencing are laborious and financially not possible. Other techniques that provide a means to rapidly screen communities have recently been developed, applying phylogenetic markers such as 16S rDNA sequences and novel separation techniques to the PCR amplified gene. More detailed information on these methodologies currently used for microbial community analyses can be found in recent reviews by Head et al. (1998), Marsh (1999), Tiedje et al. (1999), and van Elsas et al. (1998). The initial steps include the extraction of community DNA followed by PCR amplification of fragments or nearly entire 16S rRNA genes using universal or group specific primers. The resulting PCR products are separated in different ways, depending on the technique. Such methods as denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993), temperature gradient gel electrophoresis (TGGE) (Muyzer and Smalla, 1998), and single-strand-conformation polymorphism (SSCP) (Schwieger and Tebbe, 1998) involve the separation of PCR-amplified DNA products of the same length but of different primary sequence composition based on electrophoretic mobility. Alternatively, length polymorphisms can be assayed directly, as in ribosomal intergenic spacer analysis (RISA) (Ranjard et al., 2000), or by digesting the amplified products with selected restriction enzymes and then characterizing the resulting fragment length polymorphisms (i. e. variants of ARDRA). A number of approaches have been developed to simplify the banding patterns and to improve fragment analysis by utilizing fluorescently labeled oligonucleotide primers for PCR amplification that can be detected in an automated sequencer. These techniques include automated ribosomal intergenic spacer analysis (ARISA) (Fisher and Triplett, 1999) and terminal restriction fragment length polymorphism (T-RFLP) (Liu et al., 1997). Currently, T-RFLP has become very popular for analysis of bacterial communities (Kitts, 2001; Marsh et al., 2000; Osborne et al., 2000) and exploits the observation that the length of the terminal restriction fragment of the amplified 16S rRNA gene is specific to different phylogenetic groups. Because the analysis is based on the size of the terminal fragments carrying the fluorescently labeled primer, direct comparisons can be made with expected terminal restriction length fragments from known 16S rRNA gene sequences in the sequence database of ribosomal RNA. Most studies using these culture-independent methods typically target the 16S rRNA gene for microbial community analyses. Other genes can be used as phylogenetic markers including housekeeping genes (Marsh, 1999) and genes involved in nitrogen metabolism (Braker et al., 2000; Briones et al., 2002; Poly et al., 2001). Functional genes associated with methane oxidation have been used for community analyses of paddy soil methanotrophs (Eller and Frenzel, 2001; Henckel et al., 1999; Horz et al.,

2001; van Bodegom et al., 2001). Several limitations in relation to the extraction of nucleic acids from natural samples, and biases associated with PCR amplification and cloning of amplified products have been recognized for these PCR-based methods (Niemi et al., 2001). To date, these community profiling methods have mostly been used to assess differences in community composition between environments or treatments, or to evaluate relative changes in microbial communities over time. When specific phylogenetic information about community members is desired, a clone library from the amplified phylogenetic markers is constructed and subsequently screened by sequencing, restriction analysis (ARDRA), or dot blot hybridization procedures using rRNA-targeted oligonucleotide probes of defined phylogenetic resolution (Dojka et al., 2000). Clone libraries have proven useful for identification of previously undescribed phylotypes, or for developing target-specific PCR primers, as was done to identify 2,4-diacetylphloroglucinol producing *Pseudomonas* biocontrol strains (McSpadden Gardener et al., 2001).

Virtually all molecular phylogenetic surveys of natural microbial ecosystems have resulted in the discovery of novel types of rRNA sequences, often representing new lineages only distantly related to known ones (Derakshani et al., 2001; Dojka et al., 2000). The term "*Candidatus*" has been used to denote organisms with no cultivated representatives. However, assigning function to new microbial groups remains the challenge of this new technology (DeLong and Pace, 2001). The rDNA-based techniques allow recognition of molecular diversity but the corresponding phenotype of the organisms cannot be circumscribed. Consequently, descriptions of newly identified bacterial divisions such as the *Acidobacterium* and *Verrucomicrobia*, are presently confounded by too few cultivated representatives and only rudimentary descriptions of the strains (Hugenholtz et al., 1998). The application of these culture-independent methods has undoubtedly revealed a wealth of novel diversity and confirmed that our view of microbial diversity obtained by culture-based approaches was limited, but there are still several reasons why isolates remain important. First, the level of biological characterization and identification can be much greater with isolates. Second, cultures are necessary for evaluation of specific functions such as production of metabolites, or pathogenic and antagonistic activities. And last, bacterial culture collections obtained through isolation and culture characterization are a valuable asset to integrate a larger research community through the exchange of strains. While comparative analysis of 16S rDNA sequences allowed to infer the phylogeny of bacteria, the conserved nature of the molecule and the presence of multiple 16S rRNA genes in bacteria has shown that the application of these genes for species level identification is not straightforward (Fox et al., 1992). As many important ecological characteristics of bacteria, such as pathogenicity, competitiveness, substrate range, and bioactive molecule production, vary below the species level, methods with higher resolution than that of 16S rDNA sequencing are needed. One approach, that most likely will have an enormous potential for the simultaneous identification of functional diversity and taxonomic diversity in microbial communities, involves the use of DNA microarray technology. rRNA-based phylogenetic DNA microarrays consisting of collections of oligonucleotide probes that detect the target microorganisms at multiple taxonomic levels of specificity are now increasingly being developed (Reyes-Lopez et al., 2003; Small et al., 2001; Wilson et al., 2002). Functional gene arrays (Wu et al., 2001) or genome fragments of reference strains (Cho and Tiedje, 2001) may be used to survey functional gene composition or species content in microbial communities. In addition, whole-genome DNA microarrays specific for a single organism can be used to analyze

closely related organisms (Akman and Aksoy, 2001; Murray et al., 2001). As more whole bacterial genome sequences become available, it should be possible to identify genes that are conserved across taxa, or to associate specific genes to bacteria that inhabit the same niche (Van Sluys et al., 2002).

Aim of this study

The selection of the rice seed in this study was based on the fact that its bacterial diversity had not previously been investigated. The seed is of particular interest because of its importance as planting material, and as a potential vehicle for transmission of pathogenic bacteria as well as for delivery of biocontrol agents. It is also believed that the diverse communities of nonpathogenic microorganisms carried by the seed represent a largely untapped potential for disease suppression in the field.

This work has to be placed within the framework of the collaborative project "Managing rice diseases through seed health and rice seed associated antagonistic bacteria – a key component of Integrated Pest Management (IPM)", which aimed at improving crop management practices by increasing the efficiency of input use in rice production with special emphasis on the seed and seed-associated microorganisms.

The major rationale for this work is based on the anticipation that a better understanding of the identity, relative abundance, and biological activity of the different components of the bacterial communities associated with rice seed should lead to (i) a more efficient, knowledge-based deployment of bacterial populations for disease management and crop health; (ii) providing a rational foundation for seed health certification programs and management of seedborne diseases.

The overall objective of this study was to provide a basic understanding of the composition of culturable bacterial communities that inhabit rice seeds. The specific objectives were:

1. To isolate and characterize rice seed-associated bacteria and to determine the predominant groups of bacteria.
2. To classify the isolates in phenotypic groups based on similarities in cellular and colonial characteristics and whole-cell fatty acid composition.
3. To assess the genetic diversity among isolates by genomic DNA fingerprinting and to identify frequently occurring genotypes.
4. To identify the proportion of pathogenic and antagonistic bacteria in the collection of isolates.
5. To give special emphasis to particular groups of pathogenic bacteria.

The methods used for the identification of isolates (FAME, Biolog, and BOX-PCR fingerprinting) were selected on the basis of their general applicability to screen a large collection of heterogeneous aerobic bacteria. Preliminary amplifications using REP, ERIC and BOX primers had shown that, among the three primer sets, BOX-PCR generated the patterns of highest resolution from a random set of gram-positive and gram-negative isolates.

Since the start of this work, a number of high throughput, culture independent community profiling methods have been developed that are nowadays popularly used for microbial community analyses. In this work, we have used the culture-based approach for the practical purpose to allow further investigation and applications of the obtained isolates. For instance,

a practical application of our work was to provide antagonistic bacteria that could be further tested for their potential as biological control agents by a different research group.

As the study proceeded and more different samples were analyzed, statistical analysis was attempted to see if specific groups or genotypes could be correlated with different rice cultivars or environments. No correlation between the variables and the high genetic diversity in bacterial communities between samples could be found primarily because several statistical requirements had not been planned in the initial experimental design. The statistical significance of the difference in genetic diversity between samples could not be tested since there was only one collection of strains that was analyzed per field and the variances of the diversity estimates were not known.

This work is divided into four chapters: the first chapter addresses a wide range of issues related to rice in general and seed-associated bacteria in particular. The second chapter describes the diversity of culturable bacteria associated with rice seeds collected from farmers' fields in the Philippines. The third chapter focuses on the characterization of seed-associated pathogenic *Burkholderia* populations, the complex group of *Pseudomonas* spp. that are implicated in sheath browning and grain discoloration of rice in the Philippines, and the development of a PCR-based method for detection of *X. oryzae* pv. *oryzae* in rice seeds. Finally, the last chapter provides a summary and conclusion of this work.

Chapter 1

Introduction

This introductory chapter starts by citing a number of economic, social, historical and cultural facts that highlight the importance of rice cultivation in many Asian societies. General information is presented about the morphology and growth of the rice plant, the varietal types, the ecosystems under which it is grown, as well as some recent scientific breakthroughs in rice research.

Then, to put context to the practical implications of our work, we provide a brief general description of the rice seed and what is known about the types of bacteria that inhabit the seed. To clarify the possible role of seed-associated bacteria for plant health management, we address concepts and issues related to the application of beneficial bacteria as seed treatment, as well as issues about the significance of seedborne pathogens as viewed from a plant quarantine perspective.

We conclude this introductory chapter with a description of four prevalent diseases of rice, including two fungal diseases of which the causal organisms were targets in the screening of our isolates for antagonism, and two bacterial diseases that were subjects of separate studies in this work.

General Information on Rice

Economic importance

Rice (*Oryza sativa* L.) is the principal staple food for more than two billion people, most of whom live in rural and urban areas of tropical and subtropical Asia. Rice provides about 40 to 70% of the calories consumed by these people (Hossain and Fischer, 1995). It is also the most important source of employment and income in the rural areas of Asia (Hossain, 1998).

Of the total world rice production of 592,831,326 tons (FAO Production Yearbook, 2000), more than 90% is produced and consumed in Asia. Rice is grown on millions of small farms with an average size ranging from 0.4 to 3.5 ha, primarily to meet family needs. China and India account for roughly 50% of the world's total rice area and jointly produce 55% of world's rice. Other major rice-growing countries are Indonesia, Bangladesh, Vietnam, and Thailand, which produce respectively nine, six, five, and four percent of world's rice. The only major rice producer outside of Asia is the United States with a production of 8.9 million tons in 1994, accounting for 1.5% of total world production. In the European Union, rice is grown in Italy, Spain, France, Portugal, and Greece, which jointly produced 2.9 million tons in 1997 or nearly 0.5% of total world production. Most rice is consumed in the countries where it is produced, less than 5% of world rice production is traded internationally. Major rice exporters are Thailand, USA, and Vietnam, which export about five, three and two million tons a year, respectively.

In less developed Asian countries, socioeconomic development and industrial growth are linked closely to a sustainable supply of rice at low, stable prices. The first sign of civil unrest is often traced to rising rice prices. It is no wonder therefore that rice occupies such an important position in many Asian societies and that self-sufficiency in rice production is a political objective of many Asian governments (Anderson and Hayami, 1986). However, sustaining or just achieving self-sufficiency is becoming more difficult, mainly because of rapid population growth at 1.5 to 2.8% per year in most Asian countries (IRRI, 1998). To meet the growing demand for food and to sustain food security for low-income people in low-income countries, rice production has to be raised by more than 40% over the next 20 years. Rapidly increasing population means not only more mouth to feed but also less available land and water for rice production as urbanization diverts agricultural lands and

irrigation water for human settlements and industrial use. Rice production considerably needs more water than any other cereal crop; it can take up to 5000 liters of water to grow just 1 kg of rice. With the projected growth of Asian cities and industries and their increased need for fresh water, water scarcity will be a serious constraint to rice production in the coming years. This is compounded by increasing environmental constraints such as soil erosion, desertification, intrusion of saline water, and loss of biodiversity.

Uses of rice

Rice is grown primarily for human consumption. After harvest, paddy rice undergoes milling to remove the hull, which yields brown rice that is further polished to remove the thin brown pericarp tissues and aleurone layer (bran part) to produce white rice, the form widely preferred for consumption (Fig. 1). Despite the dramatic food value losses resulting from milling, most rice is consumed in its polished state. Brown rice is more nutritious than white (polished) rice but not as popular because white rice is considered tastier, easier to cook, and the oil-rich aleurone layer of brown rice easily turns rancid during storage.

The common or starchy types of rice are used in various dishes, or are further processed into cakes, soups, pastries, breakfast foods, and noodles; the glutinous types are often prepared in sweetened form for deserts or special foods used for religious or ceremonial occasions (IRRI, 1997). Alcoholic beverages made from rice are found throughout the rice-producing countries. The most common is a rice beer produced by crushing and boiling the hulled rice, adding some yeast cake, and allowing the mixture to ferment. [Ten percent of the rice grown in the U.S. each year goes into beer (Budweiser).] Sake, a wine-like beverage produced from milled rice is popularly known in Japan, as is wang-tsiu in China.

The high value by-products derived from the milling process (rice hulls, bran, aleurone layer, and germ) can be used to develop new rice products. Rice bran (germ, pericarp tissues, and oil-rich aleurone layer) can be made into oil for cosmetics, or added to “health foods” for its high fiber and nutrient content. The hulls are used as animal feed, fuel for cooking, packing material, or insulation around ice blocks. Also, rice straw after harvest is utilized in various ways as fuel, or stacked and preserved for cattle fodder, braided into rope, molded into building bricks, and made into paper.

Fig. 1. Milling and polishing of harvested paddy rice (left) removes the hull and the thin brown surface layers that enclose the grain to produce white rice (right), the form preferred for consumption.

Historical and cultural aspects of rice

Rice is among the first cultivated crops in human history. Archaeological evidence points to the early domestication of rice in mainland Asia. Pottery shards dating from at least 4000 years B.C. bearing imprints of *O. sativa* seeds were discovered at Non Nok Tha in the Korat area of Thailand (IRRI, 1997). The earliest and most convincing archeological evidence was discovered at Hemudu in Zhejiang Province, China, because, next to rice grains, it also yielded bone spades, hoe blades, and cooking utensils that demonstrated a well-developed culture supported by rice cultivation dating from 7000 years B.C. (White, 1994). This evidence not only pushed back the documented origin of cultivated rice but also suggested that agriculture itself may be older than was previously thought.

Linguistic evidence also points to the early origin of cultivated rice in Asia. In several Asian languages the words for rice and food, or for rice and agriculture are synonymous. For example in Japanese, the terms for breakfast, lunch, and dinner are *asa gohan* (morning rice), *hiru gohan* (afternoon rice), and *ban gohan* (evening rice). The words for rice—*oriza* in Greek and *oryza* in Latin may have been derived from *arisi*, the Tamil word for rice. The word seems to have been adopted into their local language by the sea-faring traders along with rice-culture. Thus *arisi* became *erus* in Malay and *al-ruz* or *arruz* in Arabic. When the Arabic traders carried it to Europe, it became *arroz* in Spanish, *riso* in Italian, *riz* in French, *reis* in German, *rijst* in Dutch and *rice* in modern English.

From ancient times to modern history, rice has consistently been a part of Asian culture. Its cultivation was considered as the basis of the social order and occupied a major place in Asian religions and customs. The grain and the plant are traditional motifs in Oriental art. Many ceremonies have arisen in connection with planting and harvesting rice. Both Hindu and Buddhist scriptures make frequent reference to rice, and in both religions the grain is used as a major offering to the gods. In China and Bangladesh, a polite way to greet a visitor is to ask: “Have you eaten your rice today?” To have a steady job is referred to in China as “to have an iron rice bowl.” Even in the automobile industry, rice remains a symbol of success. The characters for Toyota mean “bountiful rice field,” and Honda means “main rice field.”

Structure and growth of the rice plant

Cultivated rice is considered a semi-aquatic annual grass. At maturity the rice plant has a main stem and a number of tillers (Fig. 2). Each productive tiller bears a terminal flowering head or panicle. Plant height varies according to cultivar and environmental conditions, ranging from approximately 0.4 m to more than 5.0 m in some deepwater rice types.

The growth duration of the rice plant ranges from 3 to 6 months from germination to maturity, depending on the cultivar and the environment under which it is grown. During this period, rice completes basically three sequential growth stages: vegetative, reproductive, and ripening (Fig. 3). The duration of the vegetative stage differs with the rice cultivar, while the duration of the reproductive and ripening stages are approximately the same for most cultivars (Yoshida, 1981). For example, a 120-day cultivar planted in a tropical environment spends about 60 days in the vegetative stage, 30 days in the reproductive stage, and 30 days in the ripening stage.

The vegetative stage refers to a period from germination to the initiation of panicle primordia, and is characterized by active tillering and gradual increase in plant height. Tillers are branches that develop from the leaf axils at each unelongated node of the main

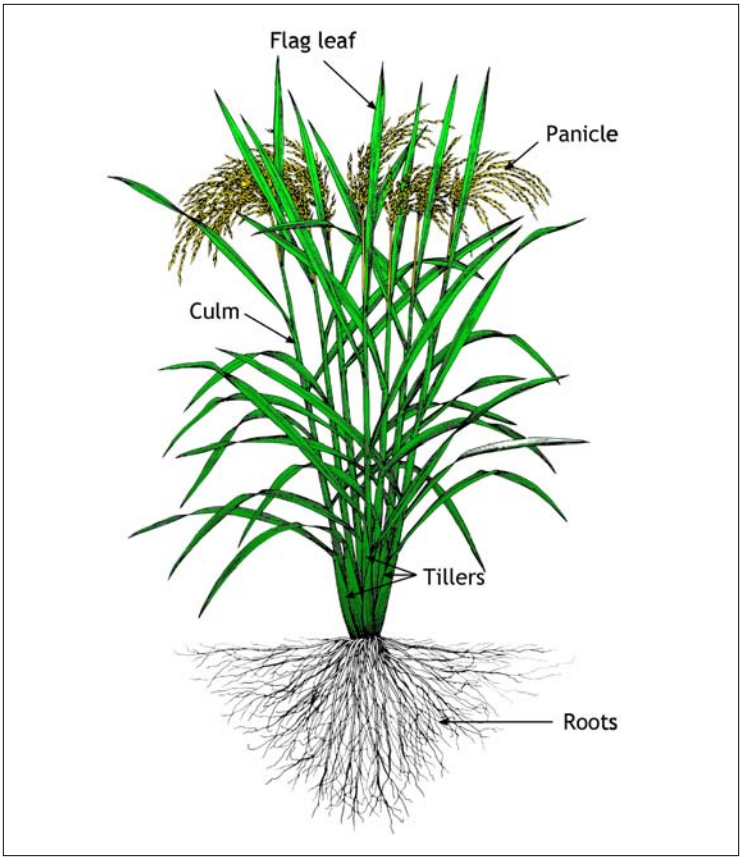

Fig. 2. Structure of the rice plant.

shoot or from other tillers during vegetative growth. Active tillering refers to a stage when the increase in tiller number is high. The maximum tiller number stage is when tiller number per plant is at maximum. A single plant can have from 16 to 25 tillers at the maximum tillering stage depending on the cultivar, light, spacing, nutrient supply, and cultural practices. The tillers remain attached to the plant at maturity even as the individual tillers produce their own roots.

The reproductive stage spans the period from panicle primordia initiation to flowering, and is characterized by culm elongation, emergence of the flag leaf (the last leaf), booting, heading (panicle emergence), and flowering.

The ripening stage refers to the period from flowering to maturity, and is characterized by leaf senescence and grain growth, which may be subdivided based on grain texture and color into milky, dough, yellow-ripe, and maturity stages.

Classification and varietal types

Rice belongs to the genus *Oryza*, subfamily *Oryzoideae*, of the family *Poaceae* (Vaughan, 1989). Cultivated rice belongs to two species, *O. sativa* L. and *O. glaberrima* Steud. Of the two cultivated species, *O. sativa* is by far the more widely utilized and is grown worldwide. *O. glaberrima* is grown on a small scale in a few countries in West Africa and is fast

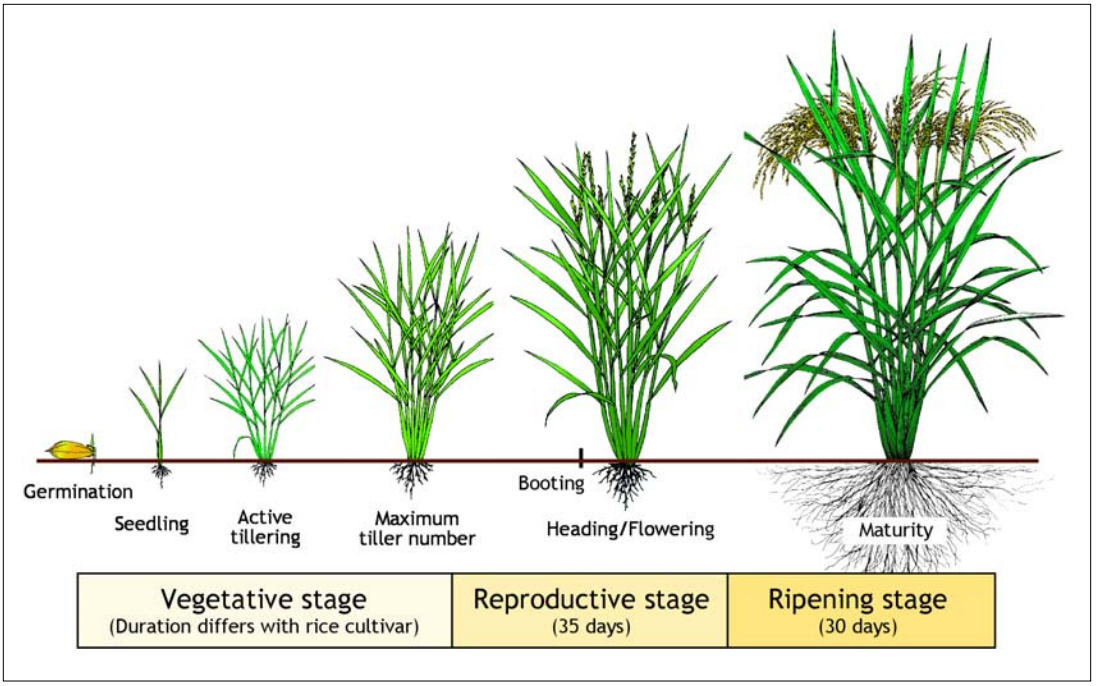

Fig. 3. Growth stages of the rice plant.

disappearing from the farmers' fields. *O. sativa* is estimated to have more than 140,000 varieties (Jackson, 1995). The diverse varietal types of *O. sativa* are generally classified into *indica* and *japonica* groups or subspecies. The differences between these two evolved both geographically and culturally over thousands of years as farming groups relocated to different ecosystems. *Indica* rice is usually grown in tropical climates. The grains are long and tend to break easily. *Japonica* rice is usually grown in temperate climates. The grains are round and do not easily crack or break, and when cooked, this rice is more sticky and moist.

The rice germplasm collection conserved in the International Rice Genebank at the International Rice Research Institute (IRRI), Los Baños, Philippines, comprises more than 80,000 accessions of cultivated rice and wild species. More than 76,000 of these accessions belong to *O. sativa*, 1,250 accessions are *O. glaberrima*, and nearly 3,000 accessions are wild species. A safety backup of the IRRI collection is stored at the USDA National Seed Storage Laboratory (NSSL) in the vaults at Fort Collins, Colorado, USA. The rice genetic resources maintained in the genebank at IRRI are held in trust for the world community (Cantrell and Reeves, 2002).

Rice production environments

Rice is grown worldwide in very different production environments in terms of topography, soil type, water regime and climatic factors (IRRI, 1997). In Asia, rice is grown in four different ecosystems, namely: the irrigated lowlands, the rainfed lowlands, the deepwater and flood-prone environments, and the upland systems. These ecosystems mainly differ in terms of hydrology and water control (Fig. 4). The rice cultivars that are grown are different for each ecosystem. The modern semi-dwarf, high-yielding cultivars that launched the Green Revolution in rice were developed for the irrigated and favorable rainfed lowlands.

Table 1 shows the areas, average yields, and the relative contribution to global rice production from each of the ecosystems.

Irrigated rice ecosystem. Irrigated rice is grown in leveled, bunded fields with water control. High cropping intensity and intensive use of agrochemicals characterize this rice ecosystem. The area under intensive irrigated rice is the main food basket of Asia. Of the 148 million ha of agricultural land planted to rice worldwide, the irrigated rice environment covers about 79 million ha, representing 53% of the world's rice area (IRRI, 1995). More than 75% of the world's rice supply is produced in irrigated ricelands, and yields average 4.9 t ha^{-1}. The possibility of further increases in rice production from irrigated ecosystems is constrained by the high cost of irrigation facilities, the shifting to other crops with higher value, and environmental concerns (Pingali et al., 1997).

Rainfed lowland rice ecosystem. Rainfed lowland rice is grown in bunded fields that are non-continuously flooded with water depths not exceeding 0.5 m for more than 10 consecutive days. Conditions are diverse and unpredictable. Soil transitions from flooded and anaerobic to droughted and aerobic, are important as rainfall fluctuates. Drought stress is common, and yields average 2.3 t ha^{-1}. The rainfed lowland ecosystem covers approximately 36 million ha or about 25% of the world's total rice land, and contributes 17% of the global rice supply. It is believed that an increase in productivity in rainfed lowlands holds the key to improved food security in the future (Pandey, 1998).

Upland rice ecosystem. In the upland ecosystem, rice is direct seeded on dry or well-drained soil on flat to steeply sloping fields. Upland rice is low yielding and generally prone to serious soil erosion and soil degradation problems. The upland rice ecosystem covers

Fig. 4. The four rice ecosystems are mainly characterized by different water regimes under which rice is grown. In bunded fields with regular water supply (irrigated ecosystem, A); in bunded fields with irregular water supply (rainfed lowland ecosystem, B); in well-drained unbunded fields (upland ecosystem, C); and in deeply flooded fields (flood prone ecosystem, D).

Table 1. Contribution to global rice production, average yield, and area covered by each rice ecosystem (IRRI, 1995).

Ecosystem	Area (million ha)	Average yield (t ha^{-1})	Contribution to rice production
Irrigated	79	4.9	75%
Rainfed lowland	36	2.3	17%
Upland	19	1.0	4%
Flood-prone	14	1.3	4%

about 19 million ha accounting for 13% of the world's total rice area. It contributes 4% of the global rice supply with an average yield of 1 t ha^{-1}. The crop is grown alone or in diverse mixtures in shifting or permanent fields. Shifting cultivation, also known as slash and burn farming was common in Indonesia, Laos, Philippines, northern Thailand, and Vietnam, and in the forested areas of Latin America and West Africa but is now declining because of reduced availability of land.

Flood-prone rice ecosystem. Rice in the flood-prone ecosystem is grown in unbunded fields subject to temporary (less than 1 month) or long periods (1 to 5 months) of submergence in floodwater ranging from 0.3 to 4.0 m deep. The deepwater and floating rice cultivars grown in this ecosystem are tolerant to submergence and have the ability to elongate rapidly so that they can rise above or float on the water. Deepwater rice is adapted to water depths of up to 1.0 m, and floating rice is adapted to floodwaters as deep as 4.0 m. The flood-prone areas in South and Southeast Asia are mostly in the delta areas of large rivers such as the Mekong of Vietnam and Cambodia, the Irrawaddy of Myanmar, the Chao Phraya of Thailand, and the Ganges of India and Bangladesh. They occupy around 14 million ha of ricelands representing 9% of the world's rice area and contribute about 4% of the global rice supply. Rice yields in this environment are low, averaging a little over 1 t ha^{-1}. There is a potential to increase yield in this ecosystem with the development of improved cultivars of deepwater rice that can yield up to 4.7 t ha^{-1}.

Recent highlights in rice research

The new plant type. A new plant type (NPT), optimistically dubbed as "Super Rice," was designed that could yield up to 25% more (yield potential of 12 to 13 t ha^{-1}) than the current modern semi-dwarf cultivars. The advantageous characteristics of the NPT (Fig. 5) include fewer (8 to 10) tillers with large panicles bearing 200 to 250 grains each, a sturdier stem and deeper root system that prevent the plant from lodging, and thick, erect leaves that increase photosynthesis efficiency (Khush, 1995). The NPT has a short stature (90 cm tall), and is early maturing with growth duration of 100 to 130 days. The NPT would be more amenable to dense planting and therefore would also increase land productivity.

The NPT was conceptualized because of the concern that rice productivity is showing signs of decline, and land area for growing rice is being lost to other purposes. The yield level in many intensive rice-growing areas is close to reaching the yield potential of the current commercial semidwarf cultivars. The modern semidwarf cultivars produce as many as 25 tillers but only 14 to 15 of these produce small panicles each containing about 100 grains and the rest remain unproductive. Scientists reasoned that it should be possible to increase the efficiency of the rice plant by reducing the number of tillers but increasing the panicle size. The breeding work for the NPT led by Gurdev Khush began in 1989, and a

prototype was created in 1995. Since then, breeders have continued to improve the characteristics of the NPT, overcoming problems of poor grain filling, and incorporating traits such as good grain quality and resistance to insect pests and diseases. Advanced lines of the NPT are already under field evaluation and IRRI is optimistic that cultivars with the NPT will be ready for distribution to farmers by the year 2005.

The NPT is the second major modification of the rice plant architecture since the early 1960s when IRRI developed the prototype of the modern semi-dwarf cultivars that enabled the Green Revolution in rice. Traditional cultivars commonly planted before were tall and leafy, lodged easily when vegetative growth was stimulated by nitrogen fertilization, were late maturing (6 to 7 months), and had poor yields (1 to 2 t ha^{-1}). In contrast, the modern semi-dwarf cultivars were shorter and high-tillering, efficiently converted nitrogen fertilizer into grain and did not lodge easily, were early maturing (100 to 120 days), and had higher yields (3 to 10 t ha^{-1}). The first modern semi-dwarf cultivar IR8 was released in 1966. Under optimal conditions, this cultivar can yield up to 10 t ha^{-1} and allows for at least two crops per year. It dramatically doubled grain yield at a time when populations in Asia were growing alarmingly, and was hailed as "Miracle Rice." Since then, a large number of IR-parented cultivars with improved resistance to insect pests and diseases have been released by national rice research institutions in Asia.

Nutritional enhancement of rice. Increasing the nutritional value of rice is an important target in rice improvement. A predominant rice diet promotes vitamin A deficiency because milled rice contains neither beta-carotene (provitamin A) nor any of its immediate C40

Fig. 5. Comparative sketches illustrating the modifications in plant structure made by conventional breeding for improving yield potential. Left, tall conventional plant type. Center, modern high-tillering, semi-dwarf plant type that permitted the Green Revolution in rice. Right, new plant type with low tillering but sturdier stems, and with a larger number of grains per panicle. (Adopted from Khush, 1995)

carotenoid precursors in its endosperm. Recently, rice has been genetically engineered to produce beta-carotene into its endosperm tissue, and is popularly called "Golden Rice" because of the yellow color imparted by beta-carotene in the rice endosperm.

Immature rice endosperm synthesizes the carotenoid precursor geranyl geranyl diphosphate. In order to convert geranyl geranyl-pyrophosphate to beta-carotene, two genes (encoding phytoene synthase and lycopene beta-cyclase**)** from *Narcissus pseudonarcissus* and a bacterial phytoene desaturase gene from *Erwinia uredovora*, which encode enzymes in the beta-carotene biosynthetic pathway, were introduced into rice endosperm (Ye et al., 2000). Three more introduced genes allow rice grains to accumulate extra iron in a form that the human body can better absorb. The first gene, from *Aspergillus fumigatus*, encodes an enzyme known as phytase that breaks down a sugarlike molecule in the rice endosperm, which prevents the human body from absorbing iron. The second gene, from *Phaseolus vulgaris*, encodes the iron-storage protein ferritin; and the third gene, from basmati rice, encodes a protein that enhances iron absorption in the human digestive system (Lucca et al., 2001). Currently, IRRI is crossbreeding the genes into popular indica cultivars for field-testing in the tropics, and will take the task to investigate further the safety and effectiveness of Golden Rice. After acceptance, these new cultivars producing provitamin A and higher levels of iron in the endosperm will be freely available to farmers in developing countries, who will have unrestricted rights to them.

Vitamin A and iron deficiencies are serious public health problems in developing countries. In Southeast Asia, 70% of children under the age of five suffer from vitamin A deficiency, leading to vision impairment and increased susceptibility to disease. Iron deficiency is the leading nutritional disorder in the world, affecting particularly women, leaving them weakened by anemia and susceptible to complications during childbirth. Hence, bioengineered rice producing provitamin A and higher levels of iron may ameliorate these malnutrition problems suffered by billions of people in developing nations who depend on rice as their staple food.

The rice genome sequence. The completion of draft sequences of the complete rice genome represents another milestone in rice science. One team, based at the Beijing Genomics Institute (BGI) China, has sequenced a rice strain of the subspecies *indica* (Yu et al., 2002); whereas a US-based group working for the biotech company Syngenta, has sequenced a strain of the subspecies *japonica* (Goff et al., 2002). The publication of rice genome sequences opens many opportunities to investigate genetic control of biological functions.

Rice is the first crop plant to have its full genome sequenced. A wealth of fundamental information about important aspects of crop biology can be learned from the genome sequences of rice. Rice is a model for learning about yield, hybrid vigor, and disease resistance. Rice is also a model for adaptive responses as it is grown in widely diverse environments, from tropical flooded environment to temperate dry land. The genome sequence will be useful for determining the function of thousands of rice genes and identifying which ones might be used to improve nutritional value, increase yield, or confer resistance to diseases and environmental stresses. Plant biologists expect that knowledge of the rice genome will accelerate the breeding of improved cultivars not only of rice but also of other major crops. The rice genome is significantly smaller compared to other cereals but the vast majority of rice genes will also have close relations in the other cereals. Assuming functional conservation, the extensive sequence similarity between rice and other cereals could provide a short

cut to the isolation of genes of agronomic importance in other cereals. The availability of the rice genome sequence will provide a rich resource for understanding the biological processes of plants and promises to positively impact cereal crop production (Ronald and Leung, 2002).

The two published draft genome sequences of rice still contain many gaps and errors but they provide the first look at the genetic blueprint of rice. The publicly funded International Rice Genome Sequence Project (IRGSP) is set to deliver a more complete sequence in 2004.

The Rice Seed

The seed, is not only the carrier of the genetic potential of the plant, but is also a vehicle for the transmission of beneficial and deleterious microorganisms and an excellent food basis for a diversity of microorganisms.

Structure of the rice seed

Like for most cereals, the rice seed is synonymous to the grain. The grain with the intact hull is commonly referred to as paddy rice (Fig. 6). The hull consists of the palea, lemma and rachilla, which are remnant parts of the flower. Removing the hull exposes the true fruit (caryopsis), which is commonly referred to as brown rice. Brown rice consists mainly of the embryo and endosperm enclosed in a thin brown pericarp. A single intact grain weighs about 10 to 45 mg at 0% moisture content. Grain length, width, color, and thickness may widely vary among cultivars (Fig. 7).

Microorganisms associated with rice seeds

The rice seed provides a habitat for a rich diversity of microorganisms consisting of bacteria, fungi, microscopic algae, as well as members of the microfauna such as plant nematodes. This study focuses on the bacterial component of rice seed-associated microbial communities.

The majority of bacteria carried by the seed are located on the surface, or slightly more deeply seated under the hull. Because there is little known on the identities of total bacterial populations associated with rice seeds, plant pathologists often group bacteria by the effect they may have on the plant into categories of neutral, beneficial, or deleterious.

- The category “neutral” refers to those bacteria whose importance is not known, and basically comprises the vast majority of the rice seed-associated bacteria. Thus, the least is know about the most common group of bacteria, and this situation represents a significant gap in our understanding of the microbial ecology of the rice seed.
- Beneficial bacteria are those that stimulate seedling emergence, promote plant growth, fix atmospheric nitrogen, enhance nutrient uptake, and suppress disease. Mechanisms contributing to disease suppression by beneficial bacteria include antibiosis, resource competition, parasitism, and induced systemic resistance in the host (van Peer et al., 1991; Wei et al., 1991). Production of antibiotics (Anjaiah et al., 1998; Rosales et al., 1995; Thomashow and Weller, 1988), siderophores (Kloepper et al., 1980; Leeman et al., 1996; Press et al., 2001), and other metabolites such as lipopolysaccharides (Leeman et al., 1995) or salicylic acid (De Meyer and Höfte, 1997) by beneficial bacteria is considered important for their disease suppressive ability. Resource competition for seed exudate fatty acids between the biological control bacterium *E.*

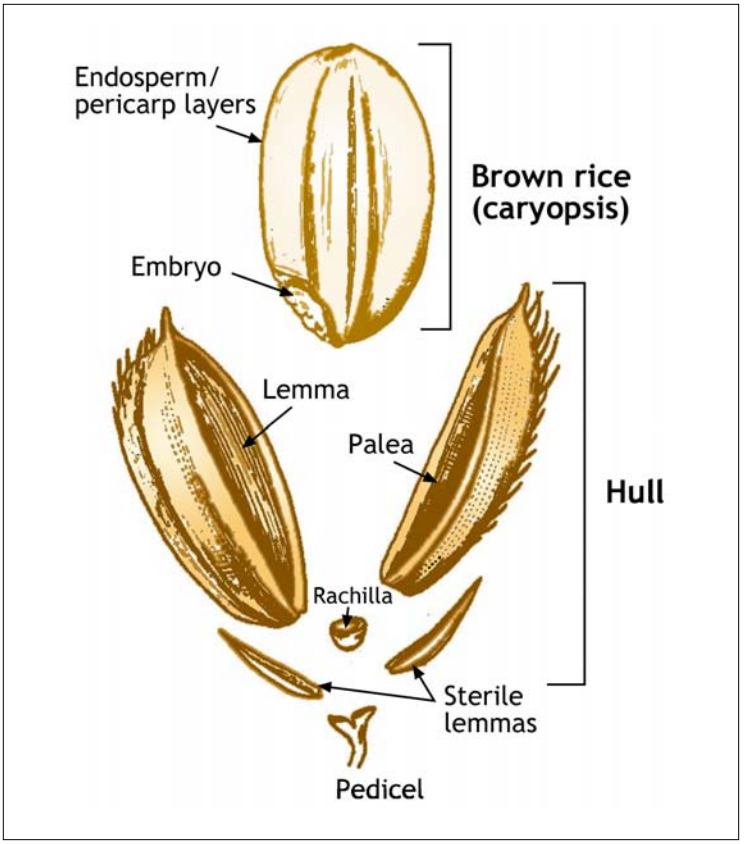

Fig. 6. Structure of the rice seed.

cloacae and the plant-pathogenic fungus *Pythium ultimum* was demonstrated to result in the suppression of *Pythium* seed rot in cotton (van Dijk and Nelson, 2000).

- Deleterious bacteria are those that show plant growth-reducing effects by inhibiting seedling emergence or producing diseased seedlings; or, as defined in this study, those that are able to cause disease symptoms in rice seedlings after artificial inoculation. They are the most obvious and well-studied group of rice seed-associated bacteria, apparently because of their economic importance. However, caution is needed when defining pathogenicity of bacterial strains by artificial inoculation methods because the choice of host cultivar and inoculation method including inoculum density might influence the test results. There are no standard guidelines and it is difficult to know when an inoculum density is too high to be considered a fair test of a strain's possible pathogenicity. Usually inoculum densities ranging from 10^6 to 10^8 cfu/ml are used in pathogenicity testing of bacteria—even with well-known bacterial plant pathogens—which is not a likely population density for a single bacterial strain to occur on plants in nature.

The seed microflora has implications for disease management strategies. On one hand, seed is a potential vehicle for transmitting plant pathogens through local and international seed trading activities. On the other hand, beneficial microorganisms can be applied onto seeds to

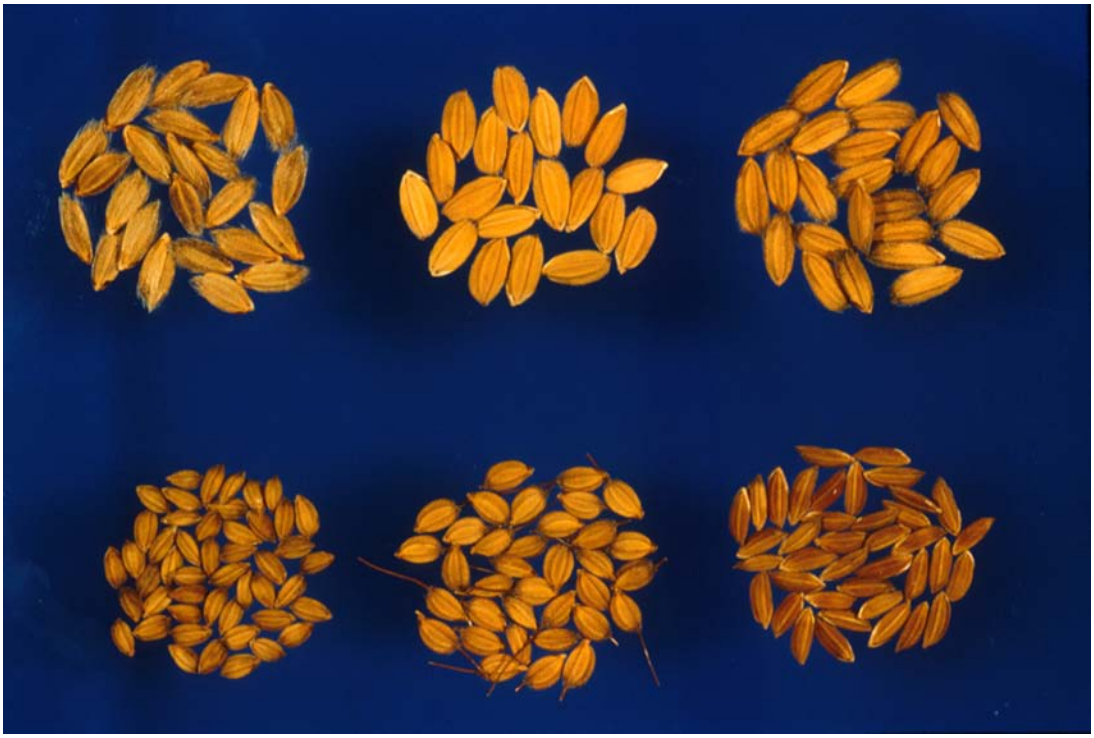

Fig. 7. Grains of different rice cultivars can greatly vary in size, color, and shape.

deliver the organisms using the seed as vehicle. Treatment of seeds before sowing to protect against diseases and to enhance crop development is a commonly practiced crop protection measure.

The seed as delivery system for beneficial bacteria

The application of beneficial microorganisms to seeds for use in agriculture has been practiced for many years in much of the world. The scientific interest in biological seed treatments for plant health management greatly increased since the demonstration by Merriman et al. (1974) that applications of bacteria to seed resulted in increased plant growth in the field. Kloepper et al. (1980) coined the term "Plant Growth Promoting Rhizobacteria" (PGPR) to describe bacteria that colonized roots and caused plant growth promotion, and it was subsequently demonstrated by Weller and Cook (1983) that a major disease, take-all of wheat, could be controlled by application of fluorescent pseudomonads to seed. Biological seed treatments are generally viewed favorably because of the combination of their specific effect and limited environmental impact in comparison with agrochemicals. A common trend is the co-formulation of biological products with reduced doses of conventional chemical pesticides.

Microorganisms can be applied to seeds to perform specific functions on the plant, such as nitrogen fixation, plant-growth promotion, and biological control of plant pathogens. Overall, there are two major rationales for the application of beneficial microorganisms: (i) A first one is to promote general plant growth and nutrition by various mechanisms, of which inoculation with nitrogen-fixing bacteria to legume crops and fungi called mycor-

rhizae in tree nurseries, have long histories of practical use. Nitrogen-fixing bacteria and mycorrhizae live in close symbiotic associations with their host-plant. Mycorrhizae concentrate rare nutrients, including phosphorus, which they exchange with plants in return for carbohydrates. Seed inoculation of legumes such as soybeans and alfalfa with beneficial strains of rhizobia (belonging to the genera: *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Sinorhizobium*), either before or at the time of sowing, is a widespread commercial practice for ensuring that effective nitrogen-fixing nodules are formed (Eaglesham, 1989).
(ii) A second rationale is to suppress diseases caused by seedborne or soilborne plant pathogens. The known disease-suppressive plant-associated microorganisms are a very diverse group, and bacteria in numerous genera have been implicated as potential biocontrol agents (Kloepper et al., 1988; Weller, 1988). Most research has focused on bacterial strains in the genera *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Enterobacter*. Seed treatment with *Bacillus subtilis* to stimulate plant growth and for protection against soilborne pathogens has been a subject of research interest for over 30 years (Merriman et al., 1974; Mahaffee and Backman, 1993; Pusey and Wilson, 1984). Also *Pseudomonas* spp. have been under investigation for several years as potential biological control agents (Bull et al., 1991; Callan et al., 1990; Paulitz et al., 1992; Shah-Smith and Burns, 1996; Weller and Cook, 1983). Strains of *Burkholderia cepacia* have been widely used as biological control agents (Hebbar et al., 1992; Parke, 1990; Parke et al., 1991), and commercial formulations are available as seed treatments against phytopathogenic fungi and nematodes. Strains of *Enterobacter cloacae* and *Pantoea agglomerans* (previously *Erwinia herbicola*) applied as seed treatments have been reported as potentially useful for the control of phytopathogenic fungi on different crops (Kempf and Wolf, 1989; Nelson, 1988).

Seed treatment with microbial antagonists naturally present in the rice environment has been attempted for biological control of rice pathogens. During the past years, numerous bacteria isolated from paddy fields and different parts of the rice plant were screened by *in vitro* dual culture analysis for their ability to inhibit mycelial growth of major fungal rice pathogens. These studies revealed that antagonistic bacteria, mostly belonging to *Pseudomonas* and *Bacillus* species, are widely distributed in the rice ecosystem (Mew and Rosales, 1986; Xie et al., 1997). Applied as seed treatments, some antagonistic strains protected rice seedlings from infection by *Rhizoctonia solani* and *Fusarium moniliforme*; others promoted the germination and seedling growth as measured by increased root and shoot length (Rosales et al., 1993 and 1997). However, many bacteria have been shown to suppress plant pathogens in laboratory and greenhouse experiments, yet few produce similar results when tested in the field. The reasons behind this are mainly due to a lack of understanding of the molecular processes that control plant-bacterium interactions and of microbial ecology in agricultural ecosystems. Ecological knowledge should be the foundation for biological control in any system. The use of living organisms to combat other living organisms presupposes a thorough knowledge of their ecology and a good understanding on how introduced bacterial biocontrol agents affect the indigenous microbial community structure.

Other central issues in developing effective biocontrol products include strain selection, cost-efficient production and formulation. Strategies for strain selection of suitable biological control agents for application to seeds should be based on criteria of competitive ability and colonizing competence in order to provide effective protection to the plant (Kim et al., 1997; Rhodes, 1993; Weller, 1988). There is also the need for monitoring the induced changes in the structure and composition of the microbial community, however, current

experimental techniques do not enable the monitoring of more than a few specific groups of microorganisms that make-up the total microbial community. Finally, the challenge of production and formulation of biocontrol agents remains, with each organism bringing its own set of problems. Strategies for formulation of microbial biocontrol products and applications of seed treatments have been reviewed (Burges (ed.), 1998; Martin (ed.), 1994). In general, bacteria that produce spores have better shelf-life qualities than non-sporeforming gram-negative bacteria, which are more sensitive to drying or heat and pose particular challenges for formulation as seed treatments (Weller, 1988). Additives to seed treatments, such as specific food bases or materials to control pH, can assist the survival and expansion of beneficial organism populations on seed during environmental stress. For example, Kloepper and Shroth (1981) reported that application of plant-growth promoting rhizobacteria to seed in a powder formulation of talc with xanthan gum provided protection from dessication and extended the viability.

A broad definition of biological control was given in a report from the National Academy of Sciences (1987) as "the use of organisms, genes, or gene products to control undesirable organisms (pests) and favor desirable organisms such as crops, trees, and beneficial microorganisms and insects." As proposed in the definition, biological control agents are not only useful for the formulation of biopesticides but are also considered potential sources of beneficial gene products useful in the defense against pathogens. Well-identified genes that code for agriculturally important traits may eventually be transferred into plant-associated microorganisms, or directly into plants. The classical examples of such transgenic plants are those engineered with the *Bacillus thuringiensis* (*Bt*) gene for production of insect toxins (Vaeck et al., 1987). However, it is known that for bacterial plant pathogens many genes are involved for successful pathogenesis to occur. Accordingly, it is expected that plant growth promotion, ecological competence, and the successful control of a pathogen by a biocontrol agent reflects the action of many genes. Hence, it might be difficult to transfer such multigenic traits, and until then we will continue to depend on the naturally occurring microorganisms for biological control of plant pathogens. Nevertheless, with increasing public awareness of the potential environmental hazards of agrochemicals, and the advances in biotechnology to improve performance of microbial products, applications of beneficial microorganisms to seed are likely to increase in the future. Whether biological treatments are viable commercially in resource-poor rice growing countries will depend on logistical and economic factors. Disease control is usually and correctly justified economically on the basis of the higher yield and presumably higher profit for the farmer (Cook, 1986). Biological seed treatments will find their place where an acceptable effectiveness, reliability, environmental safeness and competitive pricing can be provided.

The significance of seedborne pathogens

Rice diseases have always been a significant factor in rice supply. Considering the large area of rice produced in the world, even a conservative estimate of 1 to 5% annual loss would translate into tons of rice and billions of dollar income loss to farmers. Although it is recognized that diseases cause significant yield losses, there are very few reliable estimates of yield losses and the data are variable because of differences in crop loss assessment methods. In particular, losses caused by seedborne pathogens or through seed transmission of diseases have not been quantified (James and Teng, 1979). This is, at least in part, due to the fact that there is little understanding on the transmission rate and spread in the field for the majority of seedborne pathogens (McGee, 1997).

The term “seedborne pathogens” includes all plant pathogenic bacteria, fungi, nematodes, and other microorganisms, and viruses that can be carried in, on, or with seeds (Agarwal and Sinclair, 1996). Most seedborne pathogens are located on or under the seed hull and do not infect the embryo; they are thus relatively easily controlled by physical, chemical, or biological seed treatments.

Seed transmission of disease is defined as the transfer of a seedborne pathogen from infected or contaminated seeds to the emerging seedlings with an inoculum level capable of causing disease on the new generation of the host. Methods for the detection of seedborne pathogenic fungi and bacteria, and their mechanisms of seed transmission have been reviewed by Agarwal and Sinclair (1996), Maude (1996), and Neergaard (1977). All seedborne pathogens may be seed transmitted by more than one means. But it is by no means certain that the seedborne pathogen will produce an infected seedling, therefore one should differentiate between transport of the pathogen from place to place, or from season to season, and its successful transmission to a new generation of the host. However, it is often assumed, without conclusive evidence, that the presence of a pathogen on or in the seed assures transmission.

There is considerable concern about seedborne pathogens because diseases, or new virulent strains of a pathogen, might spread to other regions through the movement of seeds. Plant pathogens have spread locally in this way with planting material since crop cultivation began. In modern times, seeds are exchanged throughout the world by commercial seed trading activities and through germplasm exchange activities of public and private institutes concerned with crop improvement. Consequently, the worldwide seed trading activities enhance the risks for transmitting seedborne pathogens, which might cause disease in the planting field and lead to economic losses. The worldwide movement of seeds is regulated by plant quarantine rules that impose restrictions on seedborne pathogens. Seed health tests have been developed and evaluated by the Plant Disease Committee of the International Seed Testing Association (ISTA). The drawback of current seed health testing is that emphasis is more on detection of target pathogens in seed lots rather than on the epidemiological significance of seedborne inoculum that could cause potential yield loss in the planting field. Also, complaints have been raised that severely restrictive quarantine regulations have significant impact on the interests of the seed industry, germplasm banks, and international research organizations, and consequently could do more harm than good to the economy of a country (McGee, 1995). It has been recognized that the quarantine significance of a seedborne pathogen should not only be related to its potential for seed transmission, but as well to the potential yield loss that could be caused by the seedborne inoculum under the average environmental conditions in which the seed is sown. As long as there is no epidemiological information available on the quarantine significance of a seedborne pathogen, plant quarantine officers tend to take the cautious approach of “better safe than sorry”, and seeds may be needlessly rejected. For this purpose, it has been recommended that inoculum threshold levels should be established by correlation between seed infection levels based on seed testing and disease ratings in field plantings (Kuan, 1988). Inoculum thresholds of seedborne pathogens are defined as the levels of infection on, or in seed that will significantly affect disease development and result in economic loss. The establishment of inoculum threshold levels for each host-pathogen combination can provide realistic guidelines for seed certification programs and for the implementation of disease control programs in seed production fields. In this view, the term “clean seeds,” i.e., seeds with a

pathogen level that will not result in economic loss, are preferred over "pathogen-free seeds" in control programs (Kuan, 1988).

Economically important rice diseases

More than 100 pathogens including bacteria, fungi, nematodes, and viruses have been reported to attack rice, however not all of these pathogens are economically important or of seed health quarantine significance (Khan, 1988; Ou, 1985). Of the more than 12 genera of plant parasitic nematodes attacking rice, only the white tip nematode *Aphelenchoides besseyi* is known to be seed transmitted. Most rice virus diseases are transmitted by arthropod vectors, only wrinkled stunt and witches' broom are reported to be seedborne. The bacterial pathogens of rice presented in Table 2 are those that are well documented in the literature. Also other bacteria have been reported to cause rice diseases about which little is known or for which the correct nomenclature of the purported pathogen is unclear (Ou, 1985; Webster and Gunnell, 1992). Fungi have been reported to cause more diseases of rice than any other group of pathogens. There are 56 fungal pathogens of rice and all of them can be seedborne (Mew and Gonzales, 2002). The most important ones are presented in Table 3.

Xanthomonas oryzae pv. *oryzae* and *Pseudomonas fuscovaginae*, the causal organisms of respectively bacterial blight and sheath brown rot, are the subject of separate studies presented further in this work (see Chapter 3). While the causal organisms of blast and sheath blight, respectively *Pyricularia grisea* and *Rhizoctonia solani* AG-1, have been included as test isolates in the dual culture screenings of our isolates for antagonism. As no durable host resistance is known for these fungal diseases, they have been research targets for biocontrol strategies at the Plant Pathology Division (IRRI) for the past 20 years. Blast, sheath blight, bacterial blight, and sheath brown rot are among the economically most important rice diseases worldwide. Brief descriptions are given here of these diseases.

Blast

Pyricularia grisea (Cooke) Sacc., teleomorph *Magnaporthe grisea* (T. T. Hebert) Barr, is the cause of rice blast. The name *P. oryzae* Cavara has been used widely in the rice literature for the fungus attacking rice. *P. oryzae* is morphologically indistinguishable from *P. grisea*, which attacks other grasses, and both have the same teleomorph, and therefore, are regarded as synonymous. According to the rules of nomenclature, the earliest name *P. grisea*, is the correct name for the rice blast pathogen (Rossman et al., 1990).

Rice blast is one of the most devastating diseases occurring throughout the rice world but is particularly destructive in the temperate irrigated lowland and the tropical upland environments (Pinnschmidt et al., 1994). Estimates of yield losses range from negligible to as high as 40%. Disease development is favored by factors such as long duration of leaf wetness due to dew deposition, increased fertilizer use, and intermittent drought stress in rainfed rice cultivation. Blast symptoms commonly appear on the leaves as elliptical or spindle-shaped lesions (0.5 to 1.5 × 0.3 to 0.5 cm), with pointed ends and gray or white centers, dark-green to reddish-brown margins, sometimes with a yellow halo (Fig. 8A). In cases of severe infection, lesions coalesce, leaves whither and the whole plant might die. Severely infected fields have a scorched appearance. Rice blast is differentiated as leaf, node and neck (panicle) blast based on the presence of lesions on different plant parts. Conidia are pro-

Table 2. Bacterial pathogens of rice that can be found in association with seeds

Pathogen	Synonym	Disease	References
Acidovorax avenae subsp. *avenae*	*Pseudomonas avenae, Pseudomonas setariae*	Bacterial brown stripe	Shakya et al (1985)
Burkholderia glumae	*Pseudomonas glumae*	Grain rot Bacterial seedling rot	Goto and Ohata (1956) Uematsu et al (1976)
Burkholderia plantarii	*Pseudomonas plantarii*	Seedling blight	Azegami et al (1987)
Pantoea agglomerans	*Erwinia herbicola*	Palea browning	Azegami et al (1983)
Pectobacterium chrysanthemi	*Erwinia chrysanthemi*	Foot rot	Goto (1979)
Pseudomonas fuscovaginae		Bacterial sheath brown rot	Miyajima et al (1983)
Pseudomonas syringae pv. *syringae*	*Pseudomonas oryzicola*	Bacterial sheath rot	Klement (1955), Ou (1985)
Pseudomonas syringae pv. *oryzae*		Bacterial halo blight	Kuwata (1985)
Xanthomonas oryzae pv. *oryzae*	*Xanthomonas campestris* pv. *oryzae*	Bacterial blight	Ou (1985)
Xanthomonas oryzae pv. *oryzicola*	*Xanthomonas campestris* pv. *oryzicola*	Bacterial leaf streak	Ou (1985)

Table 3. Fungal pathogens of rice that can be found in association with seeds

	Pathogen	Synonym	Disease[a]
Foliar diseases	*Alternaria padwickii*	*Trichoconis padwickii*	Stackburn
	Bipolaris oryzae, teleomorph *Cochliobolus miyabeanus*	*Drechslera oryzae, Helminthosporium oryzae*	Brown spot
	Cercospora janseana, teleomorph *Sphaerulina oryzina*	*Cercospora oryzae*	Narrow brown leaf spot
	Microdochium oryzae, teleomorph *Monographella albescens*	*Gerlachia oryzae*, *Rhynchosporium oryzae* *Metasphaeria albescens*	Leaf scald
	Pyricularia grisea, teleomorph *Magnaporthe grisea*	*Pyricularia oryzae*	Blast
Leaf sheath, culm and root diseases	*Fusarium fujikuroi*, teleomorph *Gibberella fujikuroi*	*Fusarium moniliforme*	Bakanae, foot rot
	Magnaporthe salvinii, sclerotial state: *Sclerotium oryzae*, conidial state: *Nakataea sigmoidea*	*Leptosphaeria salvinii*	Stem rot
	Rhizoctonia solani AG-1, teleomorph *Thanatephorus cucumeris*		Sheath blight
	Sarocladium oryzae	*Acrocylindrium oryzae*, *Sarocladium attenuatum*	Sheath rot
Inflorescence and grain diseases	*Curvularia lunata*, teleomorph *Cochliobolus lunatus*		Black kernel
	Gibberella zeae, anamorph *Fusarium graminearum*	*Gibberella saubinetii*, *Botryosphaeria saubinetii*	Scab
	Nigrospora oryzae, teleomorph *Khuskia oryzae*		Minute leaf and grain spot
	Phoma sorghina, teleomorph *Mycosphaerella holci*	*Phoma glumarum*, *Phyllosticta glumarum*, *Phyllosticta oryzina*	Glume blight
	Tilletia barclayana	*Tilletia horrida*, *Neovossia horrida*	Kernel smut
	Ustilaginoidea virens, teleomorph *Claviceps oryzae-sativae*		False smut

[a]A detailed description of disease symptoms and epidemiology can be found in Ou (1985). An illustration description of the pathogens' cell and colony morphology can be found in Mew and Gonzales (2002).

duced on lesions on both sides of the leaf, and the rate of sporulation increases with increasing humidity. The disease spreads through the dispersal of abundantly produced spores. Seedborne inoculum, infected crop residue, and other crops of rice are important sources of primary infection (Manandhar et al., 1998).

P. grisea has a broad host range that includes rice, wheat, barley and many weed species (Ou**,** 1985). However, little is known of the importance of inoculum from weed hosts under natural conditions. *P. grisea* isolates have often been found only to infect the host species from which they were isolated. Isolates collected from rice and weed species (e.g., *Echinocloa colona, Digitaria ciliaris,* and *Leersia hexandra*) in a single ricefield, revealed to represent distinct genetic populations that do not cross-infect (Borromeo et al**.,** 1993). The high variability of the blast pathogen has been described based on virulence typing against a set of differential cultivars (Bonman et al**.,** 1986), and genomic typing by molecular markers (George et al., 1998; Levy et al., 1991).

Foliar applications of systemic fungicides have been proven effective for blast control in a number of cases. Host plant resistance is widely used to control the disease but is often short-lived because pathogen populations quickly adopt and overcome the deployed resistance (Bonman et al., 1992). Recent work indicated that appropriate crop diversification strategies, such as using multilines and varietal mixtures, might be the most effective approach to counteract the capability of the pathogen to adapt quickly to host resistance. The planting of multilines consisting of 13 near-isogenic lines with single resistance genes against blast proved highly effective to controlling the disease in Japan (Koizumi, 2001). Research undertaken by IRRI and Yunnan Agricultural University demonstrated that the interplanting of disease-susceptible glutinous rice with resistant hybrid rice markedly reduced the severity of rice blast by 94% and increased the yield of the highly valued glutinous cultivar by 90% (Zhu et al., 2000). Varietal diversity may limit the build-up of a dominant pathotype, creates a different microclimate that may be less favorable to the pathogen, and the disease-resistant crop can act as a physical barrier to the spread of disease spores.

Sheath blight

The causal agent of sheath blight is *Rhizoctonia solani* Kühn, teleomorph *Thanatephorus cucumeris* (A. B. Frank) Donk. The fungus belongs to anastomosis group AG-1, intraspecific group I-A of *R. solani*. The disease occurs throughout temperate and tropical rice production areas worldwide and is second to rice blast in economic importance (Lee and Rush, 1983). The increasing importance of rice sheath blight has been associated with the rapid intensification of rice production since the widespread adoption of high-yielding cultivars in tropical Asia (Cu et al., 1996). The high-yielding rice cultivars require increased nitrogen fertilizer inputs, are grown in denser stands and their high tillering abilities produce a closed canopy; all are factors that create a favorable microclimate for sheath blight development (Savary et al., 1995). For example, the area affected by sheath blight in Vietnam has increased tenfold between 1985 and 1990 (IRRI, 1993). Estimates of yield losses can be as high as 25% when the infection is well distributed and severe in a field. Heavily infected plants produce poorly filled grains and additional losses in yield result from increased lodging as a result of death of the culm.

Symptoms of sheath blight usually appear when the crop reaches its full vegetative growth at maximum tillering. The disease causes lesions on the leaf sheaths and the leaf

blades. Typical lesions on the leaf sheaths are at first ellipsoid or ovoid, somewhat irregular, greenish-grey, ranging in size from 1 to 3 cm long (Ou, 1985). The center of the lesion typically turns greyish-white with a brown margin (Fig. 8B). Lesions may coalesce to encompass entire leaf sheaths and stems. Sclerotia are produced superficially on infected tissue and are loosely attached and easily dislodged from the plant when they are mature. The disease spreads through the dispersal of sclerotia in soil, or floating on irrigation water. Interplant spread occurs through leaf-to-leaf contacts, which serve as bridges for mycelial growth to spread from a diseased to a healthy plant (Savary et al., 1997). Although *R. solani* is commonly believed a soilborne pathogen, disease spread through the canopy seems to be more important in sheath blight epidemiology in the tropics (Banniza et al., 1999; Savary et al., 1995).

R. solani is a generalist pathogen that infects several weed species commonly found in rice fields, which are considered important sources of primary inoculum in the tropics.

Fig. 8. Disease symptoms produced by *Pyricularia grisea*, causal organism of blast (A); *Rhizoctonia solani*, causal organism of sheath blight (B); *Xanthomonas oryzae* pv. *oryzae*, causal organism of bacterial blight (C); and *Pseudomonas fuscovaginae*, causal organism of sheath brown rot (D).

Infected seeds may also be a potential source of inoculum; however, the seedborne nature of the disease needs to be further investigated and does not seem to play an important role in disease establishment. The diversity of sheath blight populations has been studied using primarily morphological characters, anastomosis grouping, pathogenicity, and only more recently, molecular markers (Banniza et al., 1999). Only very few basic studies on the ecology of the fungus have been done in the tropics.

Control of sheath blight relies mostly on foliar applications of fungicides (IRRI, 1993). No host resistance is known for rice sheath blight despite various efforts to look for sources of genetic resistance (Lee and Rush, 1983). The use of crop rotation is also ineffective because inoculum is maintained on many weed species and alternate crops in the absence of rice. A number of *Pseudomonas* and *Bacillus* species isolated from the rice ecosystem are known to be antagonistic to the pathogen. Foliar applications of antagonistic bacteria over several seasons in large-scale field experiments in Jiangsu province (China), Pathum Thani province (Thailand), and Mekong Delta (Vietnam) have shown promising results for controlling rice sheath blight (pages 47, 55, 61 *in* Mew and Cottyn, 2001); and may offer an alternative to help reduce the heavy reliance on fungicide. Recently, rice plants have been engineered with chitinase genes, and are being tested for resistance to sheath blight in IRRI's transgenic greenhouse.

Bacterial blight

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most important bacterial diseases of rice. The disease was first reported in 1884 from Japan, and emerged as a serious problem in tropical Asia in the 1970s following the widespread cultivation of modern, high yielding but susceptible rice cultivars such as TN1 and IR8 (Ou, 1985). The disease is prevalent during the rainy season in irrigated or rainfed lowland rice production systems (Mew, 1987). Although the high epidemic potential of bacterial blight is well documented, only few estimates of yield losses are available, and range from 20 to 30% when infection was moderate and above 60% in case of severe infection (Mizukami and Wakimoto, 1969).

Bacterial blight symptoms usually develop in the field at the tillering stage and the disease incidence increases with plant growth, peaking at the flowering stage. Lesions on the leaf blade are initially water-soaked and typically associated with the leaf tips and edges. Lesions gradually enlarge, turn yellow and may coalesce to cover the entire leaf blade. Older lesions appear as bleached, white to straw-colored necrotic areas (Fig. 8C), and severely infected leaves wither quickly. Seedlings are the most susceptible, and a severe form of the disease termed "kresek" may develop if roots or leaves are damaged and infected during transplanting. Such early infection usually results in seedling death (Mew et al., 1993).

The present taxonomic status of *Xanthomonas oryzae* pv. *oryzae* (*ex* Ishiyama 1922) Swings et al. 1990, is the result of integrated phenotypic and genotypic analyses (Swings et al., 1990). Earlier classifications were "*Pseudomonas oryzae*" Uyeda and Ishiyama 1926, "*Xanthomonas oryzae*" (Uyeda and Ishiyama 1926) Dowson 1943, and "*Xanthomonas campestris* pv. *oryzae*" (Ishiyama 1922) Dye 1978. The species *X. oryzae* includes the two pathovars of pv. *oryzae* and pv. *oryzicola*, the causal organism of bacterial leaf streak of rice (Fang et al., 1957). Aside from the different symptoms produced on rice, the two pathogens also can be differentiated by few phenotypic features (Vera Cruz et al., 1984), whole-cell

fatty acid and protein profiles (Vauterin et al., 1992), reaction to monoclonal antibodies (Benedict et al., 1989), rRNA gene restriction patterns (Berthier et al., 1993), genomic DNA restriction fragment length polymorphisms (RFLP) (Leach et al., 1990), and rep-PCR DNA fingerprints (Louws et al., 1994).

Traditionally, *X. o. oryzae* populations have been characterized by virulence typing on a set of differential cultivars carrying different resistance genes, thus establishing races or pathotypes (Mew, 1987). In the Philippines, ten races of *X. o. oryzae* have been defined based on a set of near-isogenic lines (called IRBB lines) carrying 12 individual bacterial blight resistance genes in the common genetic background of IR24 (Mew and Vera Cruz, 1979; Mew, 1987; Nelson et al., 1994; Vera Cruz et al., 1996; Finckh and Nelson, 1999). Analysis of genomic variation of the pathogen was initiated with the development of DNA fingerprinting techniques in the 1980s, and the discovery of repetitive DNA elements in *X. o. oryzae* (Leach et al., 1990). Several repetitive elements such as insertion sequences (IS*1112* and IS*1113*), transposable elements (TNX6 and TNX7), and avirulence genes have been identified in copy numbers varying from 3 to 80 in the genome of *X. o. oryzae*. A high genomic diversity within *X. o. oryzae* was detected by DNA fingerprinting using the repetitive elements and avirulence genes as markers, which combined with virulence typing allowed for describing *X. o. oryzae* populations across major rice growing regions and for monitoring changes in the pathogen population structure (Adhikari et al., 1995; George et al., 1997; Leach et al., 1992; Nelson et al., 1994; Vera Cruz et al., 1996).

X. o. oryzae is a vascular pathogen and infects the plant through hydathodes or wounds (Mew et al., 1984). Upon entering the leaf through a hydathode, the pathogen multiplies in the mesophyll intercellular spaces and gains access to the xylem vessels (Tabei, 1977). Its multiplication and spread within leaf-lesions is more extensive in compatible (susceptible) than incompatible (resistant) interactions (Barton-Willis et al., 1989). Histological studies of diseased leaf tissue indicated that in resistant interactions the pathogen is enveloped by plant polymers resulting in densely packed bacterial cells that are unable to colonize the intercellular spaces (Horino et al., 1993). More recently, genes encoding peroxidases that are predominantly expressed in rice leaves during resistant interactions have been identified, and increased peroxidase has been correlated with accumulation of lignin-like phenolic compounds (Chittoor et al., 1997; Young et al., 1995). The pathogen multiplies rapidly in the susceptible reaction and drops of bacterial ooze produced on the surface of leaf-lesions, can be passed directly from plant-to-plant by contact, or indirectly via irrigation water. Secondary spread of *X. o. oryzae* in the tropics is associated strongly with occurrence of typhoons, that not only disperse inoculum but also cause wounds that promote infection (Ou, 1985).

The interaction between rice and *X. o. oryzae* races follows the classic gene-for-gene model (Flor, 1971), in which resistance is conditioned by the recognition between an avirulence gene in *X. o. oryzae* and a corresponding resistance gene in rice. The three avirulence genes (*avrxa5*, *avrXa7*, and *avrXa10*) isolated from *X. o. oryzae* belong to the *avrBs3* gene family (Hopkins et al., 1992), a common type of avirulence gene found in *Xanthomonas* pathogens of other crop plants. The central portion of these avirulence genes consists of directly repeated 102 bp DNA sequences; the number and arrangement of repeats are responsible for host cultivar specificity and virulence (Leach and White, 1996). Functional analysis of avirulence genes with mutations that conferred the loss of both avirulence and aggressiveness functions (Bai et al., 2000; Vera Cruz et al., 2000), suggested that the

durability of a resistance gene could be predicted by assessing the fitness penalty in the corresponding avirulence gene (Leach et al., 2001). Over 25 genes that confer resistance to specific races or clusters of races of *X. o. oryzae* have been identified from cultivated and wild rice species (Lin et al., 1996; Ogawa, 1996; Zhang et al., 1996). Two resistance genes, *Xa21* and *Xa1*, have been cloned and molecularly characterized (Song et al., 1995; Wang et al., 1996; Yoshimura et al., 1998).

Chemical control of the disease by antibiotics such as streptomycin and its derivatives, or by mercuric and copper compounds, applied as seed treatments and field sprayings, has not proven effective (Mizukami and Wakimoto, 1969). Biological control with strains of *Pantoea agglomerans* (previously *Erwinia herbicola*), or bacteriocinogenic strains of *X. o. oryzae* has been explored (Hsieh and Buddenhagen, 1974; Sakthivel and Mew, 1991). Cultural practices such as avoiding excessive application of nitrogen fertilizer, removing of diseased rice straw, and eradication of alternate host plants around paddy fields, are useful but often difficult to implement. The deployment of host resistance has been the most effective approach for controlling bacterial blight. However, the widespread deployment in monoculture of single-gene resistant cultivars often led to the breakdown of resistance in one to a few years as a result of shifts in the pathogen population and the emergence of new, virulent races. For example, long term surveys of *X. o. oryzae* populations in the Philippines revealed that predominant race 1 had shifted to race 2 as a result of the widespread deployment of resistance gene *Xa*4 (Mew et al., 1992). Strategic deployment of individual race-specific resistance genes or pyramiding of genes that have complementary resistance against multiple pathogen races may limit the build-up of particular races.

The integration of knowledge of *X. o. oryzae* population genetics into breeding and deployment strategies for resistant cultivars may provide the foundation to design gene-based disease management strategies. The principle behind such a population approach is that use of host plant resistance should be guided by knowledge of the pathogen population structure over time and across geographical regions.

Sheath rot complex and grain discoloration

Sheath rot complex and grain discoloration of rice describes the disease syndrome involving a brown discoloration or rot of the flag leaf sheath and discoloration of the grain. The grain symptoms are not necessarily combined with sheath symptoms, and grain discoloration with little or no sheath symptoms can be commonly observed in tropical rice fields. The syndrome is more prevalent during the rainy season in both lowland and upland areas, and has become widespread in tropical Asia since the introduction of modern, high-yielding rice cultivars.

In the past, symptoms of sheath rot and grain discoloration were generally ascribed to rice pathogenic fungi including *Bipolaris oryzae*, *Fusarium* spp., and particularly *Sarocladium oryzae*, which causes sheath rot (Ou, 1985). Studies on the etiology of browning and rot of the flag leaf sheath and discoloration of grains from different geographic areas, pointed out that the syndrome is mainly bacterial in nature (Duveiller et al., 1988; Miyajima et al., 1983; Zeigler et al., 1987). Published rice pathogenic bacteria including *Acidovorax avenae* subsp. *avenae*, *Burkholderia glumae*, and *Erwinia* spp., described in the literature as causing distinct diseases, have been implicated in grain discoloration and sheath rot of rice (Azegami et al., 1983; Goto, 1965 and 1979; Goto and Ohata, 1956; Ou, 1985; Zeigler and Alvarez, 1990). *Pseudomonas marginalis* has been associated to some extent with sheath rot of rice (Goto, 1965; Zeigler and Alvarez, 1987).

Pseudomonas fuscovaginae and *Pseudomonas syringae* pv. *syringae*, reported as causing almost identical symptoms on rice, are described as the principal causal agents of rice sheath rot and grain discoloration. *P. syringae* pv. *syringae* (junior synonym: *Pseudomonas oryzicola*), the causal agent of bacterial sheath rot of rice, has been recorded in Hungary, China, Australia, and Chile (Cother, 1974; Klement, 1955; Ou, 1985; Zeigler et al., 1987).

Pseudomonas fuscovaginae, the causal agent of sheath brown rot, was first reported from Japan (Tanii et al., 1976; Miyajima et al., 1983), and subsequently has been isolated from sheath brown rot of rice in Central Africa, Latin America, and Madagascar (Duveiller et al., 1988; Rott et al., 1989; Zeigler et al., 1987). The disease was initially related to cold stress in temperate rice growing environments until it was also found in tropical highlands. Because *P. fuscovaginae* favors cool temperatures (17 to 23ºC) and high humidity for disease development at booting stage (Miyajima, 1983), the cool night temperatures and high rainfall in the humid tropical highland areas might predispose the plants to outbreaks (Autrique and Maraite, 1983). *P. fuscovaginae* was also reported as a pathogen of wheat, sorghum, and maize (Duveiller et al., 1989; Duveiller and Maraite, 1990), and has a broad host range among wild grasses (Miyajima et al., 1983). The widespread distribution of *P. fuscovaginae* on various continents might, at least in part, be due to the tremendous increase in international seed exchange that has occurred in the last two decades, but might as well reflect the ubiquitous nature of the pathogen.

The symptoms caused by *P. fuscovaginae* commonly include brown necrotic lesions ranging from small specks to large brown blotches on the flag leaf sheath or extensive necrosis of the sheath, poor panicle emergence, grain discoloration and sterility (Fig. 8D). Grains on affected tillers may be completely discolored to nearly symptomless with only small brown spots. The most important yield loss caused by *P. fuscovaginae* is grain sterility. Further, discolored and poorly filled grain directly affects grain quality and not only earns discounted prices in the market but is also considered poor quality planting material.

Isolation and identification of *P. fuscovaginae* is often difficult because various fluorescent pseudomonads, most of them saprophytes, are isolated from rice sheath rots and discolored grains. The combination of pathogenicity and eight biochemical tests was proposed for identification and differentiation of *P. fuscovaginae* from other oxidase and arginine dihydrolase positive fluorescent pseudomonads (Rott et al., 1991).

The pathogen is seedborne and considered seedtransmitted. Measures for controlling the pathogen are scarce and are mostly directed towards the use of clean seeds. Seed treatment by heat therapy at 65ºC for six days has been recommended to eradicate the pathogen (Zeigler and Alvarez, 1987). Chemical control with antibiotics such as kasugamycin, or a mixture of streptomycin and oxytetracycline, applied as field sprayings at heading was proven effective in Japan (Miyajima, 1983). Host plant resistance has not been investigated.

P. fuscovaginae is probably the principal causal agent of sheath brown rot in tropical high altitude areas. Known *P. fuscovaginae* strains from different geographic origins were shown to react differently with antisera but have identical biochemical profiles based on the eight characteristics (Rott et al., 1989). However, other fluorescent pseudomonads able to induce sheath rot symptoms on rice were isolated from low and high elevations in Madagascar, Burundi, and Columbia (Duveiller et al., 1988; Rott et al., 1989; Zeigler and Alvarez, 1990). So far, experimental evidence suggests that the rice sheath rot complex and grain discoloration involves a complex group of fluorescent pseudomonads, including *P. fuscovaginae* and other *Pseudomonas* with unknown affiliation.

CHAPTER 2

TOTAL CULTURABLE BACTERIA ASSOCIATED WITH RICE SEED

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- Cottyn, B., B. Lanoot, E. Regalado, T. W. Mew, J. Swings (2003): Characterization of bacteria from rice seed PSBRc14 in Philippine farmers' fields. In preparation for submission to Microbial Ecology.

Microbial diversity is a significant component of a sustainable biosphere, and many species likely play key roles in sustaining efficient agricultural ecosystems (Tiedje, 1995). Microbial interactions with plant communities range from disease-producing pathogens to beneficial microorganisms that promote plant health by suppressing disease, enhancing nutrient uptake, fixing atmospheric nitrogen, and invigorating plant growth. A promising application of beneficial plant-microbe interactions is microbial biocontrol, the use of indigenous microorganisms to suppress plant pathogens. Numerous microbial antagonists form part of the natural resources of the rice ecosystem and are potentially useful for protection against rice diseases (Mew and Rosales, 1986). However, the challenge remains how to develop strategies that allow the management of microbial communities such that they remain beneficial to plants. Studies on the population biology of plant pathogens in relation to the deployment of host resistance in the field, have indicated that a population approach can lead to better disease management (Leung et al., 1993). Likewise, it can be expected that knowledge of the whole microbial community structure could lead to a better management of bacterial communities in order to maximize the effectiveness of populations of beneficial microorganisms. The understanding of bacterial communities, and functioning of the different component groups, will require more than merely identifying some of the "key" microbial components but the identification and elucidation of the activities of all individual isolates. Our aim was to provide more insight into the community composition and structure of the culturable fraction of rice seed-associated bacteria. The seed is of particular interest for obvious reasons. In respect to disease management strategies, concerns are often raised about seed transmission of diseases; on the other hand, the seed is also a preferred means for the application of biocontrol agents. Despite its importance, relatively little is known about the microbial ecology of rice seed. Research in the past has focused primarily on the seedborne pathogens, whereas identification of the large other portion of nonpathogenic, saprophytic bacteria has, for the most part, not received much attention.

In this study, we have characterized total bacterial populations isolated from paddy rice seed in an attempt to assess its bacterial diversity. A general scheme of the experimental approach and the used methods is shown in Figure 1. Rice seed samples were collected from farmers' fields; each sample was obtained from a different field. Collected samples were stored at 4ºC in the Seed Health Unit of the International Rice Research Institute. Extracts were prepared from individual samples either by seed soaking or partial maceration, and dilution series were plated on agar medium. The agar medium (tryptic soy agar) used in this study is considered nonselective and is widely accepted as a general medium for isolation of diverse bacterial populations from environmental samples. Bacterial colonies were isolated after 3 days of growth at 28ºC, and additionally after 7 days in order to detect slowly growing organisms. Per sample, all distinct colony types observed on isolation plates were enumerated and picked. The isolates were purified and stock cultures were maintained at –70ºC in nutrient broth with 15% glycerol.

A first coarse level of characterization was used to fractionate the large number of isolates into morphotype groups based on Gram-morphology, basic biochemical characteristics, and colony appearance on two different media (tryptic soy agar and King's medium B). Morphotype groups sorted in this manner were then subjected to redundancy reduction by repetitive sequence-based polymerase chain reaction (rep-PCR) genomic fingerprinting of individual isolates using the BOX primer. Rep-PCR genomic fingerprinting refers to the

respective protocols of REP-PCR, ERIC-PCR and BOX-PCR, in which primers are used that correspond to the prokaryotic repeats known as repetitive extragenic palindromic (REP) sequences, enterobacterial repetitive intergenic consensus (ERIC) sequences, and BOX elements, that are dispersed throughout the genome of most bacteria (Martin et al., 1992; Versalovic et al., 1991). The primers are designed to amplify intervening genomic regions between the repeat elements, leading to the selective amplification of DNA fragments, which after gel electrophoresis yield a genomic fingerprint profile that permits differentiation at the species, subspecies or strain level. Rep-PCR genomic fingerprinting has been

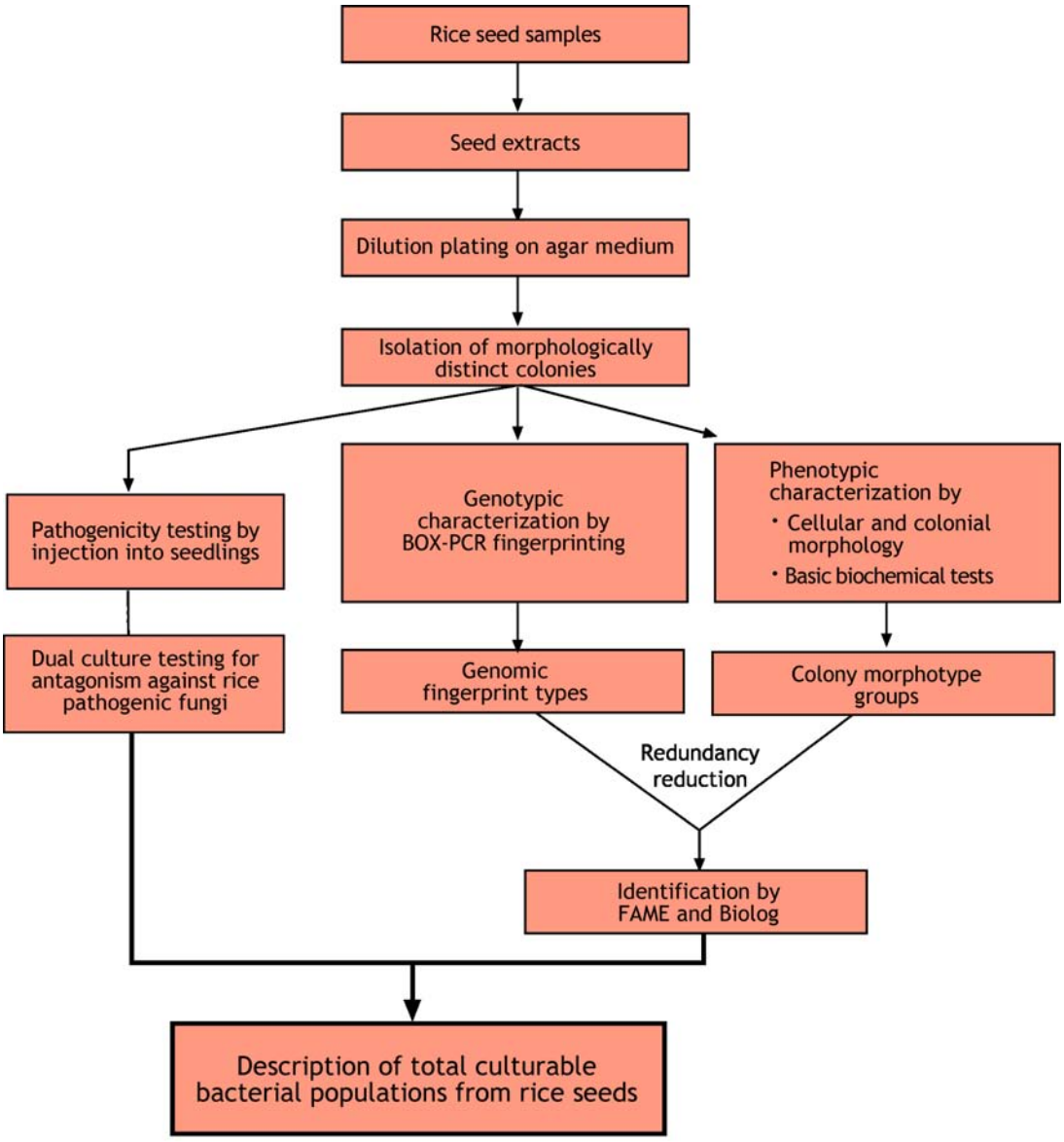

Fig. 1. General scheme of the approach used for isolation and characterization of bacteria from rice seed.

extensively used to assess the diversity of phytobacterial populations, and several reviews have discussed the technical aspects and practical applications of rep-PCR (Louws et al., 1999, Rademaker and de Bruijn, 1997; Rademaker et al., 1998; Versalovic et al., 1994). The BOX-PCR genomic fingerprinting method, combined with computer-assisted pattern analysis using the GelCompar software, was useful not only to remove redundancy but also provided a fine level of characterization to reveal the extent of genetic diversity present in the collection of isolates. Subsequently, isolates representative for each defined BOX-PCR fingerprint type (FPT) were identified by whole-cell fatty acid methyl ester (FAME) analysis using the MIDI Microbial Identification System (MIS). The FAME-MIS is a fully automated, computerized, high-resolution gas chromatography system that has a database for the identification of a large variety of different microorganisms. A second rapid commercial system for identification of bacteria, which was especially used for the further identification of isolates belonging to *Enterobacteriacea* and *Pseudomonadaceae*, is the Biolog GN MicroPlate system. The system is based on the utilization profile of 95 carbon substrates by the organism tested; the ability to utilize substrates is detected by reduction of a tetrazolium dye. The accuracy of these automated identification systems is highly dependent on the organisms tested, and both methods have similar database limitations for the identification of environmental isolates (Stager and Davis, 1992; Welch, 1991). Alternatively, we have constructed a database containing BOX-PCR fingerprint profiles of 137 LMG reference strains and over 2000 rice seed isolates to facilitate identification of rice seed bacteria. This database has proven useful in several instances. For example, prevalent isolates from the genera *Burkholderia* and *Pantoea* often were identified at species level with a low likelihood or remained unidentified in FAME-MIS and Biolog, however, database comparison revealed them to possess genomic fingerprint patterns nearly identical to generated profiles of type strains for the species. As with any database for identification of bacteria, progressive expansion of the number of patterns with those of newly characterized isolates will increase the reliability of the constructed database for identifying new isolates.

In addition, all isolates were screened for their ability to induce disease symptoms upon inoculation in rice seedlings, and for in vitro inhibition of fungal growth of selected rice pathogens that are a target of the biocontrol research group at Plant Pathology Division, IRRI.

The scheme in Figure 1 shows the flow of methods from top to bottom, but it is equally important that the information obtained from one method is integrated into the knowledge obtained from all other methods in order to achieve an efficient grouping of isolates.

An overview of the origin of the samples analyzed in three separate surveys of bacteria associated with rice seed from farmers' fields is given in Tables 1, 2 and 3. The characterization of total bacterial populations associated with rice seeds obtained from different cultivars and production environments could provide fundamental information on the composition and variability of the bacterial communities. The two manuscripts presented hereinafter describe the types of bacteria isolated from rice seeds collected from farmers' fields in the Philippines. In the first study, rice seeds were collected from farmers' fields at different locations of rainfed lowland cropping systems on the island of Iloilo. In the second study, rice seeds were collected from farmers' fields at one location (the municipality of Jalajala) that forms part of the irrigated intensive rice cropping systems of southern Luzon. Both surveys were initially designed to include four successive crops of 30 farmers' fields, i. e. two dry and two wet seasons' harvests. As rice is a self-pollinated crop, farmers in the

Table 1. Collected rice seed samples from farmers' fields on the island of Iloilo, Philippines (Total number of isolated bacteria = 1,029).

Sample code	Rice cultivar		Field location
	DS95[a]	WS95	
1-1	IR64	IR64	San Miguel
2-1	IR64	–[b]	San Miguel
2-7	IR64	IR64	San Miguel
3-7	IR64	IR64	San Miguel
4-2	IR64	–	Oton
5-1	PSBRc10	–	Oton
6-4	IR36	–	Oton
7-1	IR64	–	Dumangas
8-8	IR66	–	Dumangas
9-6	IR68	–	Dumangas
10-1	IR64	IR64	Zarraga
11-5	PSBRc10	–	Zarraga
12-4	PSBRc8	–	Zarraga
13-2	C46	–	Dingle
14-8	IR36	IR38	Dingle
15-9	IR64	–	Dingle
16-2	IR64	–	Mina
17-6	IR64	–	Mina
18-5	IR65	–	Tolarucan
19-9	Local cv.	IR64	Ajuy
20-6	IR60	IR60	Ajuy
21-1	PSBRc10	–	Ajuy
22-1	C96	–	Barotac Nuevo
23-7	IR36	IR36	Barotac Nuevo
24-6	Cipag	–	Barotac Nuevo
25-4	PSBRc4	IR36	Concepcion
26-9	PSBRc10	–	Concepcion
27-2	IR36	–	Concepcion
28-9	PSBRc14	PSBRc14	San Dionisio
30-8	Bordagol	Bordagol	San Dionisio

[a]DS95, seeds collected from the dry season harvest in May 1995; WS95, seeds collected from the wet season harvest in October 1995.
[b]The crop was devastated by super typhoon "Rosing".

Table 2. Collected rice seed samples from Vietnamese farmers' fields (received from Mrs. Mai Thi Vinh in October 1996) (Total number of isolated bacteria = 160)

Sample code	Rice cultivar	Date harvested	Field location
MtvA	3A	04/08/96	Long An
MtvB	MTL110	07/09/96	Long An
MtvC	KSB460-37	21/08/96	Ho Chi Minh City
MtvD	IR64	10/08/96	Ho Chi Minh City
MtvE	IR29723	07/09/96	Ho Chi Minh City

Philippines commonly use seed from their previous harvest for planting the next season's crop. It was hypothesized that the analysis of successive seed generations could additionally provide information on the dynamics of particular populations between cropping seasons.

The survey in Iloilo was prematurely disrupted during the second cropping season by the occurrence of super typhoon "Rosing" that had devastated the crop before harvest in 19 of the 30 farmers' fields. Eleven fields had been harvested before appearance of the typhoon,

Table 3. Collected rice seed samples from farmers' fields at the municipality of Jalajala, Rizal Province, Luzon, Philippines (Total number of isolated bacteria = 5,017)

Sample code	Rice cultivar			Farmer participant
	WS96[a]	DS97	WS97	
BPJ-1	PSBRc14	PSBRc14	PSBRc20	Lamberto Campo
BPJ-2*	PSBRc14	PSBRc14	PSBRc14	Wilfredo Campo
BPJ-3	PSBRc14	PSBRc14	M30	Rogelio Carlos
BPJ-4*	PSBRc14	PSBRc14	PSBRc14	Rodolfo Villones
BPJ-5*	PSBRc14	PSBRc14	PSBRc14	Cecilio Teodoro
BPJ-6	PSBRc14	PSBRc14	M30	Zaldy Panguito
BPJ-7*	BS1	BS1	BS1	Rolando Rivera
BPJ-8*	PSBRc14	PSBRc14	PSBRc14	Teodoro Bueza
BPJ-9*	PSBRc14	PSBRc14	PSBRc14	Vicente Villariño
BPJ-10*	PSBRc14	PSBRc14	PSBRc14	Crispin Panguito
BPJ-11*	PSBRc14	PSBRc14	PSBRc14	Marcelino Ramos
BPJ-12*	PSBRc14	PSBRc14	PSBRc14	Michael Villones
BPJ-13*	PSBRc14	PSBRc14	PSBRc14	Domingo Villones
BPJ-14	Ri10	Ri10	PSBRc14	Basilio Sisante
BPJ-15*	PSBRc14	PSBRc14	PSBRc14	Bernardo Sisante
BPJ-16*	PSBRc20	PSBRc20	PSBRc20	Juanito Recilio
BPJ-17*	PSBRc14	PSBRc14	PSBRc14	Julian Balakit
BPJ-18	PSBRc14	PSBRc14	PSBRc20	Mario Escamillan
BPJ-19	PSBRc20	PSBRc20	BH-1	Bienvenido Escamillan
BPJ-20*	PSBRc20	PSBRc20	PSBRc20	Caesar Escamillan
BPJ-21	PSBRc14	PSBRc20	PSBRc18	Claudio Escamillan
BPJ-22	R1040	PSBRc20	Bordagol	Romulo Reyes
BPJ-23	PSBRc20	PSBRc14	IR74	Fernando Sta. Ana
BPJ-24	PSBRc14	PSBRc14	PSBRc18	Floro San Jose
BPJ-25	PSBRc12	PSBRc14	PSBRc14	Florante Villaran
BPJ-26*	PSBRc14	PSBRc14	PSBRc14	Alfredo Villaran
BPJ-27	R1040	PSBRc14	PSBRc14	Ladino San Juan
BPJ-28	PSBRc14	PSBRc14	PSBRc18	Eliseo Enguito
BPJ-29*	PSBRc22	PSBRc22	PSBRc22	Lucio de Chavez
BPJ-30*	BS1	BS1	BS1	Marcelo de Chavez

[a]WS96, seeds collected from the wet season harvest in November 1996; DS97, seeds collected from the dry season harvest in May 1997; WS97, seeds collected from the wet season harvest in December 1997.
*Samples from 17 fields continuously planted to the same cultivar over three cropping seasons, of which the isolated bacteria formed the subject of the study on the characterization of *Burkholderia* populations (see p. 86).

and collected seed samples from these fields were analyzed (Table 1). The first paper presented describes the bacterial populations isolated from these samples obtained from six different cultivars and seven locations in Iloilo.

The sampling done at the site in Jalajala covered three successive crops of 30 farmers' fields beginning with the 1996 wet season crop (Table 3). The farmers had not planted the fourth crop of the 1998 dry season due to occurrence of the "El Niño-Southern Oscillation" phenomenon in the transition of 1997-98, which is associated with an extended period of severe drought throughout Southeast Asia. In contrast to the first paper, the second paper aimed to evaluate the variability in bacterial populations among samples obtained from a single cultivar (PSBRc14) grown in adjacent fields at Jalajala.

Bacterial Populations Associated with Rice Seed in the Tropical Environment

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ABSTRACT

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During the 1995 wet season, harvested rice seed was collected from farmers' fields at different locations in Iloilo, Philippines. Bacterial isolations from crushed seed yielded 428 isolates. The isolates were characterized by BOX-polymerase chain reaction fingerprinting of total genomic DNA and represented 151 fingerprint types (FPT). Most FPTs were found on a single occasion, although matching fingerprints for isolates from different samples also were found. Identifications were made by cellular fatty acid methyl ester analysis and additional use of Biolog GN/GP MicroPlates and API 20E/50CHE systems. The predominant bacteria were *Enterobacteriaceae* (25%), *Bacillus* spp. (22%), and *Pseudomonas* spp. (14%). Other bacteria regularly present were identified as *Xanthomonas* spp., *Cellulomonas flavigena,* and *Clavibacter michiganense.* Of the total number of isolated bacteria, 4% exhibited in vitro antifungal activity against *Rhizoctonia solani* or *Pyricularia grisea.* Two percent of isolates were pathogens identified as *Burkholderia glumae* and *Burkholderia gladioli.* Five percent of isolates induced sheath necrosis on only 50 to 90% of inoculated plants and were related to *Bacillus pumilus, Paenibacillus* spp., *Pseudomonas* spp., and *Pantoea* spp.

Additional keywords: coryneform bacteria, nonpathogenic xanthomonads.

Rice *(Oryza sativa* L.) is the primary food grain consumed by almost half of the world's population, making it the most important food crop currently produced. Current integrated pest management (IPM) strategies in tropical rice production emphasize the use of host-plant resistance, cultural practices, and biological control for maintaining low pest populations. The diverse communities of nonpathogenic, plant-associated microorganisms are a largely untapped resource for protection against plant disease (6). Microbial biological control of plant diseases is often crop- and site-specific; therefore, it is also believed that its real potential may be in the use of many different locally adapted strains (7). Understanding the bacterial

community composition on rice seed would be a first step toward exploiting bacteria for biological control.

The seed, being the planting material and the carrier of the genetic potential of the crop, is considered the starting point for achieving sustainable high yields. However, in contrast to the rice rhizosphere, about which interest in biological nitrogen fixation has led to ample characterization of free-living and endophytic diazotrophic bacteria (30,41), little is known about the composition of bacterial communities of rice seed. Characterization of bacterial populations on the rice phylloplane has commonly been restricted to those bacteria that cause disease (2,16,26,43,50), in much the same way that studies on rice seed microflora have been confined to seedbome pathogens (10,29,39).

Several studies have demonstrated the usefulness of repetitive sequence-based polymerase chain reaction (rep-PCR) to fingerprint a large variety of bacteria and to study microbial diversity in natural ecosystems (21,47). The rep-PCR method (referring collectively to REP-, ERIC-, and BOX-PCR) uses oligonucleotide primers matching interspersed repetitive DNA sequences and the rep-PCR to generate a genomic fingerprint that can distinguish bacteria at a fine level. These interspersed repetitive elements are conserved in diverse genera of bacteria and, therefore, enable single primer sets to be used for DNA fingerprinting of many different microorganisms (21,47). In the present study, we used the BOXAIR primer corresponding to the boxA-subunit sequences (22) for PCR-based DNA fingerprinting of bacteria isolated from rice seed. In addition, whole-cell fatty acid analysis, the Biolog MicroPlate system (Biolog Inc., Hayward, CA), and API systems (BioMerieux, La Balme-les-Grottes, France) were used for species identification of the isolates. These phenotypic typing methods are relatively simple and automated, and have been widely used both for taxonomic studies and identification (11,12,27,40). However, the resolution level of these techniques depends on the group of bacteria studied (48).

The purpose of this study was to (i) assess the genetic diversity of the culturable aerobic bacterial isolates of rice seed by BOX- PCR genomic fingerprinting; (ii) identify the strains represented by defined BOX-PCR fingerprint types; (iii) classify the isolates into functional groups of saprophytic, antagonistic, and pathogenic bacteria; and (iv) assess the influence of seed germination on the composition of bacterial communities.

Materials and methods

Bacterial strains. For each BOX-PCR fingerprint type (FPT) defined in this study, a representative strain has been deposited in the Belgian Coordinated Collections of Microorganisms/Laboratorium Microbiologie Gent (BCCM/LMG) culture collection. In addition, genomic DNA of 137 reference strains was included in the BOX-PCR fingerprinting. These reference strains were obtained from the BCCM/LMG culture collection and mainly consisted of type strains of various species of the genera *Acidovorax, Acinetobacter, Agrobacterium, Aureobacterium, Bacillus, Burkholderia, Cellulomonas, Chryseobacterium, Clavibacter, Curtobacterium, Enterobacter, Erwinia, Flavimonas, Klebsiella, Pantoea, Pseudomonas, Salmonella, Sphingomonas,* and *Xanthomonas*.

Isolation of bacteria from seed. During the 1995 wet season, 1-kg samples of harvested rice were purchased from 11 farmers in Iloilo (Philippines). An overview of the collected samples, their respective cultivar, and location of origin is given in

Table 1. Rice seed lots collected during the 1995 wet-season harvest in Iloilo, Philippines

Cultivar	Collection number[a]	Field location	No. of isolates[b]
IR64	1-1	San Miguel	25
	2-7	San Miguel	19
	3-7	San Miguel	22
	10-1	Zarraga	45
	19-9	Ajuy	41
IR36	23-7	Barotac Nuevo	52
	25-4	Concepcion	47
IR38	14-8	Dingle	43
IR60	20-6	Ajuy	50
RC14	28-9	San Dionisio	46
Bordagol	30-8	San Dionisio	38

[a]Remnant seed from the collected seed lots are stored at the Seed Health Unit of the International Rice Research Institute, Los Baños,Philippines.
[b]All visually distinct colony types, with two to three arbitrarily picked for abundant types, were isolated.

Table 1. From each of the 11 samples, a 10-g batch of seed was processed. The seed was partially crushed (until approximately 80% of the seed was broken) with a sterilized mortar and pestle and suspended in l00 ml of sterile phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with 0.025% Tween 20 (Sigma-Aldrich, St. Louis). The seed suspension was incubated for 2 h at 4°C. Then, l00 µl of 10-fold serial dilutions (10^{-2}, 10^{-3}, and 10^{-4}) of the suspension in sterile saline (0.85% NaCl) was plated in duplicate on King's medium B (KMB) (14) and glucose yeast chalk agar (GYCA) (37) supplemented with 0.01% cycloheximide (Sigma-Aldrich). The duplicate dilution plates were incubated at 28°C for 4 days. All visually distinct colony types, with two to three arbitrarily picked for abundant types, were isolated from each sample. The isolates were purified on nutrient agar (NA; Difco Laboratories, Detroit) and maintained at –70°C in nutrient broth (NB; Difco Laboratories) with 15% glycerol.

To assess the impact of seed germination on the composition of bacterial communities, additional isolations were done from soaked germinated seed from each sample. Seed (10 g) was incubated in 50 ml of sterile distilled water in plastic germination boxes (14 by 17 by 4.5 cm), precleaned with 10% sodium dodecyl sulfate (SDS) and 70% ethanol, for 3 days at 28°C until ≈40% of the seed was germinated. Germination was indicated by the appearance of the white tip of the coleoptile. On the fourth day, an additional 50 ml of sterile distilled water with 0.025% Tween 20 was added, and the germination boxes were shaken at 50 rpm on an orbital shaker for 1 h at room temperature. Portions of 100 µl of 10-fold serial dilutions (10^{-4}, 10^{-5}, and l0^{-6}) of the seed soak in sterile saline (0.85% NaCl) were plated in duplicate on KMB and GYCA supplemented with 0.0 1% cycloheximide. The plates were incubated at 28°C for 4 days. Picking and purification of colonies was performed as described above.

Remaining seed of the 11 samples was stored at 4°C in the Seed Health Unit of the International Rice Research Institute (IRRI), Los Baños, Philippines. BOX-PCR fingerprinting was performed for all picked colonies from all crushed seed samples, and from two arbitrarily chosen genninated seed samples.

Pathogenicity tests. All bacteria isolated from crushed seed were tested for pathogenicity by inoculation of 21-day-old rice seedlings of cv. IR24 grown in the greenhouse under natural light with day and night temperatures of ≈35 and 25°C, respectively, and with relative humidity ranging from 40 to 65%. Seed was disinfected for 5 min in 70% ethanol and rinsed five times with sterile distilled water, then soaked for 12 h under running tap water and planted in autoclaved soil. For each bacterial isolate, four seedlings were inoculated by injecting the culm ≈2 cm above the soil with 1 ml of an overnight-grown NB culture (≈3×10^8 CFU/ml.

Plants inoculated with sterile NB and cultures of known virulent strains *(X. oryzae* pv. *oryzae* strain PXO81, and *Burkholderia glumae* strain 2056) served as negative and positive control, respectively. The plants were examined for symptoms 3 and 10 days after inoculation. Plants were scored as negative when no obvious symptoms or a hypersensitive-like reaction localized at the point of inoculation occurred. Elongation of a brown necrotic zone of tissue away from the point of inoculation, often extended up to the third leaf, was scored as a positive reaction. Isolates that produced a positive reaction on at least two out of four seedlings in the first experiment were inoculated two more times on four plants in a second and third experiment.

Bacteria were re-isolated from selected plants to confirm that symptoms were caused by the inoculated isolate. The pathogenic isolates were differentiated on the basis of the consistency of the caused reaction between the three inoculation experiments. Bacterial isolates were considered pathogens if all plants inoculated showed a positive reaction; whereas, isolates that caused symptoms on at least 50% of inoculated plants in the three experiments were considered pathogens with low disease potential.

Screening for antifungal activity in vitro. All bacteria isolated from crushed seed were tested by the dual culture method as previously described (24) for antagonistic activity against four rice fungal pathogens: *Rhizoctonia solani, Pyricularia grisea, Sarocladium oryzae,* and *Fusarium moniliforme.* Cultures of the fungal pathogens collected from rice in the Philippines were maintained at the IRRI. Five mycelial plugs (5 mm in diameter) were taken from an actively growing culture of *R. solani* on potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, and 18 g of agar per liter). One plug was placed at the center and four plugs at opposite sides toward the edge of the plate on pigment production medium (PPM; 20 g of proteose peptone, 20 g of glycerol, 5 g of NaCl, 1 g of KNO_3, and 15 g of agar per liter, pH 7.2). Four bacterial cultures, grown for 48 h on PPM, were placed between the four peripheral agar plugs of *R. solani.* For antagonism tests against *Pyricularia grisea, Sarocladium oryzae,* and *Fusarium moniliforme,* 50 ml of an aqueous conidial suspension (50,000 conidia per ml) of each fungal pathogen was added to 1 liter of melted PPM at 45°C. The inoculated PPM was poured into petri plates and allowed to congeal for 4 h, after which bacterial isolates to be tested were placed in four equidistant places in the plates. Each fungal-bacterial combination was replicated three times. All dual- culture plates were incubated at 28°C for 2 to 3 days and scored for inhibition of fungal growth. The diameter of the inhibition zone around the bacterial colony was measured.

DNA isolation. Bacteria were grown in 8.0 mI of NB on a rotary shaker at 150 rpm for 24 h at 28°C. Extraction of total DNA was done as described previously (13). The DNA concentrations were estimated visually by comparison with λ DNA standards in an agarose gel.

BOX-PCR fingerprinting. The primer BOXAIR [5'-CTA-CGGCAAGGCGACGCTGACG-3'] sequence corresponding to BOXA, a subunit of the BOX element (22), was synthesized by Operon Technologies Inc. (Alameda, CA). Each 25-µl PCR reaction contained 2 µM BOXAIR primer, ≈40 to 80 ng of genomic DNA, 1.25 mM each dNTP (United States Biochemical, Cleveland), 2 units of *Taq* DNA polymerase (Pharmacia Biotech, Asia Pacific, Hong Kong), 10% (vol/vol) DMSO (Sigma- Aldrich), and bovine serum albumin at 0.16 mg/ml (Boehringer Mannheim, Far East, Singapore) in 1× Gitschier buffer (15). PCR

amplifications were performed in a DNA thermal cycler (480; Perkin-Elmer, Norwalk, CT) with an initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 65°C for 8 min, and a final extension at 65°C for 15 min. Aliquots (7 µl) of amplified PCR products were separated by gel electrophoresis on gels composed of 0.8% Synergel (Diversified Biotech, Boston, MA) and 0.8% agarose (United States Biochemical) in 0.5x Tris-borate-EDTA (TBE) (35) at 75 V for 14 h. As standard, a 1-kb size marker (Life Technologies, Pacific, Hong Kong) was run in the second, sixteenth, and thirtieth lane. The gels were stained with ethidium bromide and photographed on an UV transilluminator with Polaroid Type 55 film (Polaroid Corp., Cambridge, MA). The photographs were scanned on a flatbed scanner (Sharp JX-610) at 200 dots per inch (dpi) resolution. Normalization of BOX-PCR patterns and cluster analysis was achieved with the commercially available GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). Similarity between BOX-PCR fingerprints was calculated by the Pearson correlation coefficient, and clustering was done by the unweighted pair group method using arithmetic averages (UPGMA). Additional interpretation of the DNA fingerprints was carried out by visual examination. We defined an FPT as a set of strains with identical or nearly identical BOX-PCR DNA patterns. Patterns were considered nearly identical when variation was limited to two or three faint DNA fragments.

Reproducibility of DNA fingerprints. The level of reproducibility of BOX-PCR fingerprinting was determined by including DNA of isolate G237 in each PCR assay; its resulting fingerprint was used as an internal standatd during each electrophoresis run. Generated BOX-PCR fingerprints of examined isolates were repeated at least twice, and gel runs had been separated over a period of 9 months. Intergel reproducibility as derived from the similarity scale in the dendrogram generated by the UPGMA clustering analysis software of GelCompar was 89%. Although this could be considered a rather low reproducibility, it was mainly attributed to differences in electrophoresis times and variations in background intensities because, visually, no differences in the obtained banding patterns could be seen. When reproducibility was determined by obtaining repeated patterns of six strains in three PCR experiments and amplification products were separated on the same gel, the intragel correlation between these patterns was above 93%.

Phenotypic identification. The following features were examined for all isolates: description of colonial and cellular morphology, Gram stain, fluorescent pigment production on KMB, Kovac's oxidase reaction, nitrate reduction, and reaction on Hugh and Leifson's oxidation-fermentation medium (17,37). Fatty acid methyl ester (FAME) analysis, in combination with Biolog and API-strips, was performed on at least two isolates per defined FPT unless the FPT was unique to a single isolate.

FAME analysis. All examined isolates were grown on trypticase soy agar (TSA) (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) at 28°C for 24 h. Extraction and preparation of the cellular fatty acid methyl esters were performed accotding to Sasser (36). FAMEs were analyzed with a Hewlett- Packard HP5890A gas-liquid chromatograph on a 5% phenylmethyl silicone-coated glass capillary column. The generated profiles were identified by using the Microbial Identification System (MIS version 4.15; Microbial ID Inc., Newark, DE). The same software was also used for calculating the

dissimilarity between profiles using the Euclidean distance coefficient, and for UPGMA clustering. The correlation between a profile from an unknown isolate and a library entry was expressed as a similarity index (SI) on a numeric scale of 0 to 1. MIS identifications at the species level were considered reliable when the first choice had an SI value of ≥0.5, and the numerical difference between the first and second choice SI value was ≥0.1. Cellular fatty acid profiles of gram-negative and gram-positive isolates were clustered in distinct dendrograms. FAME clusters were arbitrarily delineated at approximately 12 Euclidean distance units. Only clusters containing at least three isolates (as the number of isolates per FPT had been determined by the comparison of BOX-PCR fingerprints) were assigned as a group in the generated dendrograms. Single or less than three delineated isolates were ranked as ungrouped. Duplicate analysis of individual isolates clustered at a level of less than 5 Euclidean distance units.

Biolog and API systems. Whenever automatic comparison of the generated fatty acid profiles to the commercial TSBA library (version 4.15; Microbial ID Inc.) failed to give a reliable identification, further identification was done using the Biolog GN/GP MicroPlate systems (Biolog Inc.). Isolates identified by Biolog with a low similarity coefficient as belonging to the *Enterobacteriaceae* were further investigated by API 20E and 50 CHE galleries according to the manufacturer's instructions (API Systems, Biomérieux). Isolates for Biolog analysis were grown on TSA for 24 h at 28°C. The Biolog MicroPlates were inoculated with a bacterial suspension in sterile saline (0.85% NaCl), adjusted in density to the Biolog MicroPlate system's turbidity standard, and incubated for 24 h at 30°C. The plates were read on an automated microplate reader, and results were analyzed with Microlog software (version 3.50).

SDS-polyacrylamide gel electrophoresis of whole cell proteins. Whole-cell protein profiles were generated for those isolates that were thought to belong to the genus *Burkholderia.* Cultivation of bacterial cultures, preparation of whole-cell protein extracts, and SDS-polyacrylamide gel electrophoresis (PAGE) were performed as described previously (32). The obtained protein profiles were visually compared with the patterns in the database of the genus *Burkholderia* created by Vandamme et al. (45).

Results

A total of 428 bacterial isolates, consisting of 244 gram-negative and 184 gram-positive bacteria, were isolated from crushed seed of 11 farmers' rice seed samples. On the basis of BOX-PCR fingerprinting of genomic DNA, 82 FPTs were distinguished among the gram-negative bacteria and 69 FPTs among the gram-positive bacteria. If isolates within one FPT can be considered members of a single population, then at least 151 bacterial populations occurred in the seed samples. No FPT was found in all 11 samples. The majority of FPTs (64%) were found just once, with 76 of these 96 FPTs delineated for a single isolate. The remaining 55 FPTs were found in more than one sample. The BOX-PCR fingerprint patterns consisted of 10 to 23 PCR products ranging in size from ≈200 bp to 4.5 kb. Minimal to no BOX-PCR amplification was observed for 90% of isolates identified as *Chryseobacterium indologenes* and *Acinetobacter* spp. Isolates identified as *Bacillus* spp. (especially *Bacillus cereus)* often revealed a minimal BOX-PCR pattern of only one to six PCR

products. Such minimal amplification patterns were also obtained from corresponding LMG reference strains.

Resolution level of the BOX-PCR technique. BOX-PCR genomic fingerprinting of 137 LMG reference strains was used to determine the resolution level of the technique. The BOX-PCR technique clearly distinguished different species but the diagnostic value of the patterns (i.e., the degree of variability observed among multiple reference strains of a single species) varied according to species (data not shown). Within some species, nearly identical fingerprint patterns were produced for multiple reference strains: *Burkholderia glumae* (LMG 2196, LMG 10905, and LMG 10906), *Burkholderia plantarii* (LMG 9035 and LMG 10908), *Pseudomonas luteola* (LMG 5946 and LMG 7041), *Pseudomonas tolaasii* (LMG 2342 and LMG 6635), *X. hyacinthi* (LMG 7419 and LMG 8042), and *Pantoea ananatis* (LMG 2665 and LMG 2676). Within other species, polymorphic patterns sharing multiple bands of equal mobility could be distinguished: *Pseudomonas fuscovaginae* (pattern 1: LMG 2158 and LMG 2192, pattern 2: LMG 5097, pattern 3: LMG 5742), *Pseudomonas viridiflava* (pattern 1: LMG 5285tl, LMG 5285t2, and LMG 5400; pattern 2: LMG 2359), *Acidovorax avenae* spp. *avenae* (pattern 1: LMG 1806 and LMG 10904; pattern 2: LMG 2117), *Pantoea agglomerans* (pattern 1: LMG 1286, pattern 2: LMG 2595, pattern 3: LMG 2565 and LMG 2578), and *Aureobacterium testaceum* (pattern I : LMG 16144, pattern 2: LMG 16344). Furthermore, high variability among multiple reference strains was observed within some species: *Sphingomonas paucimobilis* (pattern 1: LMG 11151, pattern 2: LMG 1227), *Pseudomonas stutzerii* (pattern 1: LMG 1228, pattern 2: LMG 11199), *Pseudomonas fulva* (pattern 1: LMG 11722, pattern 2: LMG 11723), *Pseudomonas fragi* (pattern 1 : LMG 2191, pattern 2: LMG 5919), *Pseudomonas putida* (pattern 1: LMG 2257, pattern 2: LMG 5835), *Bacillus pumilus* (pattern 1: LMG 3455, pattern 2: minimal amplification of only six bands for LMG 10642), *Bacillus megaterium* (pattern 1: LMG 7127, pattern 2: LMG 7132, pattern 3: LMG 11162; all showing minimal amplification of 6 to 11 bands), and *Brevibacillus laterosporus* (pattern 1: a minimal pattern for LMG 16000 and LMG 6931, pattern 2: good amplification for LMG 6932). BOX-PCR of *Bacillus cereus* strains (LMG 6923, LMG 17605, and LMG 17613) produced only a single PCR product, distinctive for each reference strain.

Pathovars within the genera *Pseudomonas* and *Xanthomonas* were clearly differentiated by the BOX-PCR technique, although *Pseudomonas marginalis* pv. *marginalis* (LMG 2210) only differed in the presence of two additional bands from the pattern of *Pseudomonas marginalis* pv. *alfalfae* (LMG 2214). Polymorphism was observed within *X. arboricola* pv. *pruni* (pattern 1: LMG 854, pattern 2: LMG 860t1) and also, consistent with Louws et al. (19), within *Pseudomonas syringae* pv. *syringae* (pattern 1: LMG 2230 and LMG 2231, pattern 2: LMG 5570) and *Pseudomonas syringae* pv. *oryzae* (pattern 1: LMG 10917 and LMG 10920, pattern 2: LMG 10912 and LMG 10915). On the other hand, no polymorphism was observed between two strains of *X. translucens* pv. *graminis* (LMG 713 and LMG 726).

At the subspecies level, distinct BOX-PCR fingerprint patterns were observed between *Clavibacter michiganense* subsp. *insidiosum* (LMG 3663) and *Clavibacter michiganense* subsp. *michiganense* (LMG 7333), consistent with Louws et al. (18).

Generated BOX profiles of 12 strains belonging to the genera *Clavibacter, Cellulomonas, Curtobacterium, Arthrobacter,* and *Aurefobacterium* were distinct but shared some bands in common.

Qualitative composition of bacterial communities of rice seed. The 428 bacteria isolated from the 11 crushed seed samples were analyzed by BOX-PCR fingerprinting of total genomic DNA. Isolates with a nearly identical BOX-PCR pattern were defined as one FPT and considered as representing one bacterial population. Consequently, fatty acid analysis was done on representative isolates for each BOX-PCR FPT. An abridged dendrogram obtained by UPGMA analysis of the whole-cell FAME patterns for the gram-negative bacteria is shown in Figure 1. The predominant gram-negative bacteria were *Enterobacteriaceae,* nonfluorescent *Pseudomonas* spp., and *Xanthomonas* spp. The *Enterobacteriaceae* were identified by FAME-MIS, in combination with Biolog and API galleries, as *Pantoea* spp., *Enterobacter cloacae, Enterobacter sakazakii, Salmonella choleraesuis,* and *Kluyvera ascorbata* (Table 2). The identification for many isolates, however, remained doubtful. A single isolate identified as *Erwinia carotovora* (now *Pectobacterium carotovorum)* remained ungrouped on the basis of FAME analysis. The other isolates formed seven distinct subgroups delineated at 10 Euclidean distance units within FAME group N8. Four subgroups were occupied by isolates identified as *Pantoea* spp. Enterobacterial isolates were found in nearly all samples, except sample 2-7. The BOX-PCR fingerprinting differentiated 23 FPTs among the enterobacterial isolates. Six FPTs matched isolates found in four or more samples. Isolates within the two FPTs delineated for LMG 18570 and LMG 18571 were found in 8 of the II samples. The two FPTs, only dissimilar in the absence of one prominent band in the profile of LMG 18571, were highly similar to the pattern generated for the *Pantoea stewartii* subsp. *stewartii-type* strain LMG 2715 (previously *Erwinia stewartii)* (data not shown).

Nonfluorescent pseudomonads were differentiated by 17 BOX-PCR FPTs. Based on fatty acid analysis, two groups (N9 and N10) were delineated at 12 Euclidean distance units and one isolate (LMG 18604) remained ungrouped (Table 2). The isolates within the predominant FAME group N10 tightly grouped together at less than 5 Euclidean distance units, except for six isolates (associated with FPTs defined for LMG 18568, LMG 18573, and LMG 18641) that clustered at 10 Euclidean distance units. FAME-MIS identifications as *Flavimonas oryzihabitans* were congruent with the phenotypic characteristics determined for the isolates. However, identifications as *Pseudomonas aeruginosa,* though obtained with high similarity value indices (0.8 to 0.9), appeared unacceptable because the isolates were nonfluorescent, negative for oxidase, and lacked the typical colony morphology of *Pseudomonas aeruginosa.* Also, Biolog analysis of those isolates provided unsatisfactory identifications with low similarity indices to *Pseudomonas syringae* or *Pseudomonas corrugata.* Nonfluorescent pseudomonads were found in nearly all samples, except samples 1-1 and 3-7. The *Pseudomonas* populations represented by the FPTs delineated for LMG 18605 and LMG 18616 were found in five and four samples, respectively.

Xanthomonads were found in 9 of the 11 samples and were not pathogenic on rice in our pathogenicity tests. They possessed the three characteristic fatty acids of the genus *Xanthomonas* (46): 11:0 iso, 11:0 iso 3OH, and 13:0 iso 3OH. The isolates remained unidentified in FAME-MIS,

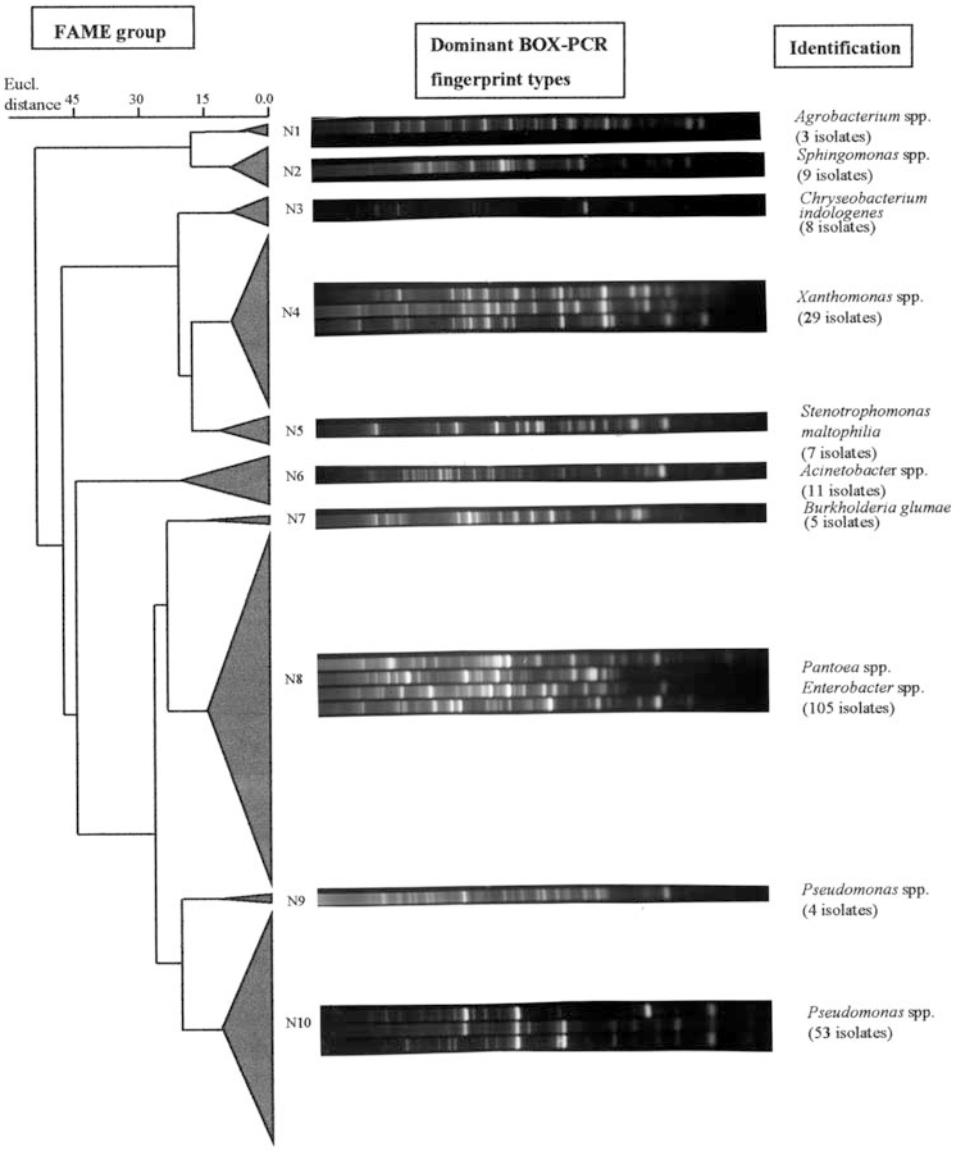

Fig. 1. Abridged dendrogram obtained by unweighted pair group average linkage of Euclidean distance values between fatty acid methyl ester (FAME) profiles of gram-negative bacteria isolated from rice seed. Ungrouped isolates were not included. Dominant polymerase chain reaction (BOX-PCR) fingerprint types delineated among the isolates within each FAME group illustrate the genetic heterogeneity present in each FAME cluster. Identifications were obtained by FAME-MIS, Biolog GN MicroPlates, and API 20E/50CHE systems.

whi1e identifications as *X. campestris* pv. *dieffenbachia* or *X. campestris* pv. *strelitzia* were obtained in Biolog. Additionally, the fatty acid profi1es of TSA cultures grown for 48 h were compared with the profiles contained in a database of the genus *Xanthomonas* created by Yang et a1. (49), and did not fit into any of the recognized *Xanthomonas* spp. BOX-PCR fingerprinting differentiated 16 FPTs within this group of nonpathogenic xanthomonads. The generated profiles did not share more than two or three bands of equal mobility and clustered at an overall similarity of less

Table 2. Gram-negative bacteria from rice seed[a]

FAME group[b]	Identification[c]	No. of FPTs (LMG no.)[d]	Origin (no. of isolates)[e]
N 1	*Agrobacterium* spp.	3 FPTs (18631, 18633, 18565)	2 seed lots (3)
N 2	*Sphingomonas paucimobilis*	4 FPTs (18624, 18625, 18618, 18575)	4 seed lots (8)
	Sphingomonas capsulata	1 FPT (18601)	1 seed lot (1)
N 3	*Chryseobacterium indologenes*	3 FPTs (18594, 18595, 18636)	1 seed lot (8)
N 4	*Xanthomonas campestris* pv. *dieffenbachia* (B), pv. *strelitzia* (B)	16 FPTs (18567, 18578, 18582, 18589, 18610, 18612, 18613, 18614, 18617, 18622, 18623, 18630, 18635, 18637, 18643, 18645)	9 seed lots (29)
N 5	*Stenotrophomonas maltophilia*	2 FPTs (18581, 18646)	2 seed lots (7)
N 6	*Acinetobacter baumannii*	3 FPTs (18599, 18603, 18644)	6 seed lots (9)
	Acinetobacter calcoaceticus	2 FPTs (18592, 18598)	2 seed lots (2)
N 7	*Burkholderia glumae* (SDS)	1 FPT (18634)	4 seed lots (5)
N 8	*Enterobacter cloacae* (B)	3 FPTs (18577, 18607, 18626)	5 seed lots (9)
	Enterobacter sakazakii (B)	2 FPTs (18584, 18640)	4 seed lots (20)
	Kluyvera ascorbata	2 FPTs (18588, 18627)	2 seed lots (3)
	Pantoea agglomerans	4 FPTs (18576, 18579, 18597, 18621)	3 seed lots (12)
	Pantoea ananatis	1 FPT (18639)	1 seed lot (1)
	Pantoea spp. (API)	5 FPTs (18570, 18571, 18572, 18638, 18642)	8 seed lots (38)
	Salmonella choleraesuis	5 FPTs (18580, 18587, 18608, 18609, 18620)	7 seed lots (22)
N 9	*Pseudomonas* spp. (B)	2 FPTs (18586, 18611)	2 seed lots (4)
N 10	*Flavimonas oryzihabitans*	4 FPTs (18583, 18596, 18605, 18628)	6 seed lots (15)
	Pseudomonas aeruginosa	7 FPTs (18574, 18585, 18591, 18600, 18616, 18619, 18629)	6 seed lots (32)
	Pseudomonas spp. (B)	3 FPTs (18568, 18573, 18641)	4 seed lots (6)
Ungrouped	*Burkholderia gladioli* (SDS)	1 FPT (18569)	2 seed lots (2)
	Burkholderia multivorans (SDS)	1 FPT (18606)	1 seed lot (1)
	Methylobacterium rhodesianum	1 FPT (18593)	1 seed lot (1)
	Pectobacterium carotovorum (API)	1 FPT (18632)	1 seed lot (1)
	Pseudomonas putida	2 FPTs (18566, 18615)	2 seed lots (2)
	Pseudomonas spp.	1 FPT (18604)	1 seed lot (1)
	Sphingomonas paucimobilis (B)	2 FPTs (18590, 18602)	2 seed lots (2)

[a]Bacterial isolations from crushed seed of II farmer' seed lots yielded 244 gram-negative bacteria. Eighty-two fingerprint types (FPTs) were differentiated among these isolates on the basis of polymerase chain reaction (BOX-PCR) fingerprinting of genomic DNA. The FPTs were arranged according to the groups from the cluster analysis of fatty acid profiles of the isolates.

[b]Refers to the groups determined by unweighted pair group method using arithmetic averages analysis of Euclidean distances calculated from fatty acid methyl ester (FAME) data of the 244 gram-negative rice seed isolates. Ungrouped: isolates with fatty acid profiles that remained unclustered at a level of 12 Euclidean distance units.

[c]Identifications obtained by: FAME-MIS (version 4.15); B = Biolog GN MicroPlate system (version 3.50); API = API 20E / 50CHE system; SDS = comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis whole-cell protein profiles with the predetermined *Burkholderia* library from Vandamme et al. (45).

[d]Indicates the number of BOX-PCR FPTs delineated among the isolates associated with the respective identifications within each FAME group. Numbers in parentheses refer to the accession number of deposited representative isolates for each FPT in the culture collection of the Laboratorium Microbiologie Gent (LMG), Gent, Belgium.

[e]Indicates the number of seed lots, out of II seed lots examined, from which the isolates within the respective FPTs were found; number in parentheses refers to the actual number of isolates found within the respective FPTs.

than 60%. They were unique compared with fingerprint profiles generated from 11 Philippine strains of *X. oryzae* and 24 reference LMG strains of other *Xanthomonas* spp. (data not shown).

An abridged dendrogram obtained by UPGMA analysis of the whole-cell FAME patterns for the gram-positive bacteria is shown in Figure 2. The dendrogram consisted of two distant groupings, one related to *Bacillus* spp. and the other to coryneform bacteria. Isolates identified as *Bacillus* spp. were contained in FAME groups P7 to P11, with respective identifications of *Bacillus licheniformis*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus pumilus*, and *Bacillus cereus*. Eleven *Bacillus-like* isolates remained ungrouped on the basis of their fatty acid compositions (Table 3). The isolates within FAME group P8 were

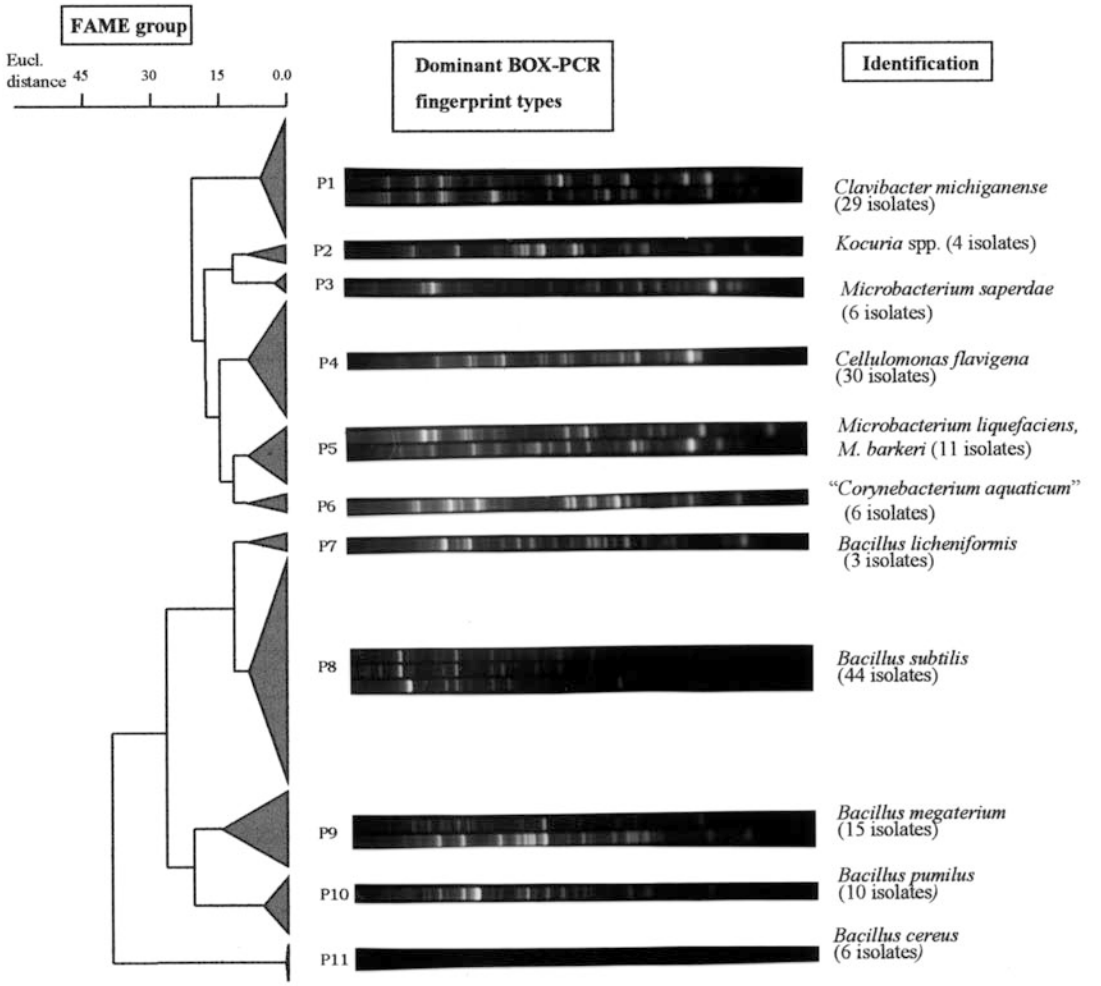

Fig. 2. Abridged dendrogram obtained by unweighted pair group average linkage of Euclidean distance values between fatty acid methyl ester (FAME) profiles of gram positive bacteria isolated from rice seed. Ungrouped isolates were not included. Dominant polymerase chain reaction (BOX-PCR) fingerprint types delineated among the isolates within each FAME group illustrate the genetic heterogeneity present in each FAME cluster. Identifications were obtained by FAME-MIS (version 4.15).

equally identified in comparison with the commercial TSBA library to *Bacillus amyloliquefaciens* and *Bacillus subtilis,* often with a numerical difference of less than 0.1 between the first and second choice similarity index value. Hence, for convenience, this group was named *Bacillus subtilis* group (sensu stricto).

The coryneform isolates were not a1ways clearly differentiated by FAME-MIS. The generated BOX-PCR fingerprints, though very distinct, often shared few bands in common among isolates identified to different coryneform species. The isolates within FAME group P1 formed two subgroups delineated at seven Euclidean distance units. One subgroup was identified to *Clavibacter michiganense* subsp. *insidiosum,* whereas the isolates within the other subgroup showed low similarity indices to the library profile for this species. The isolates within each

Table 3. Gram-positive bacteria from rice seed[a]

FAME group[b]	Identification[c]	No. of FPTs (LMG no.)[d]	Origin (no. of isolates)[e]
P 1	*Clavibacter michiganense*	6 FPTs (18656, 18657, 18661, 18695, 18697, 18701)	9 seed lots (29)
P 2	*Kocuria kristinae*	3 FPTs (18651, 18680, 18681)	3 seed lots (4)
P 3	*Microbacterium saperdae*	2 FPTs (18654, 18664)	5 seed lots (6)
P 4	*Cellulomonas flavigena*	8 FPTs (18647, 18649, 18655, 18665, 18669, 18706, 18711, 18712)	8 seed lots (30)
P 5	*Microbacterium barkeri*	1 FPT (18662)	2 seed lots (3)
	Microbacterium liquefaciens	1 FPT (18700)	2 seed lots (7)
	"Corynebacterium aquaticum"	1 FPT (18699)	1 seed lot (1)
P 6	*"Corynebacterium aquaticum"*	1 FPT (18713)	2 seed lots (2)
	Unidentified	3 FPTs (18648, 18677, 18683)	3 seed lots (4)
P 7	*Bacillus licheniformis*	1 FPT (18685)	2 seed lots (3)
P 8	*Bacillus subtilis*-group (sensu stricto)	11 FPTs (18652, 18671, 18679, 18684, 18691, 18694, 18703, 18704, 18707, 18709, 18715)	8 seed lots (44)
P 9	*Bacillus megaterium*	7 FPTs (18670, 18686, 18687, 18688, 18705, 18710, 18714)	6 seed lots (15)
P 10	*Bacillus pumilus*	2 FPTs (18658, 18676)	6 seed lots (10)
P 11	*Bacillus cereus*	3 FPTs (18653, 18675, 18698)	4 seed lots (6)
Ungrouped	*Arthrobacter atrocyaneus*	1 FPT (18689)	1 seed lot (1)
	"Bacillus filicolonicus"	1 FPT (18692)	1 seed lot (1)
	Bacillus sphaericus	1 FPT (18663)	1 seed lot (1)
	Bacillus spp.	3 FPTs (18650, 18668, 18696)	3 seed lots (3)
	Brevibacillus brevis	1 FPT (18667)	1 seed lot (1)
	Brevibacillus laterosporus	1 FPT (18659)	1 seed lot (1)
	Brevibacterium spp.	1 FPT (18682)	1 seed lot (1)
	"Corynebacterium aquaticum"	2 FPTs (18702, 18708)	2 seed lots (2)
	Curtobacterium spp. (B)	1 FPT (18672)	1 seed lot (1)
	Exiguobacterium acetylicum	1 FPT (18674)	1 seed lot (1)
	Microbacterium arborescens	1 FPT (18693)	1 seed lot (1)
	Paenibacillus macerans	1 FPT (18690)	1 seed lot (1)
	Paenibacillus polymyxa	1 FPT (18678)	1 seed lot (1)
	Paenibacillus spp.	2 FPTs (18660, 18666)	2 seed lots (2)
	Staphylococcus saprophyticus	1 FPT (18673)	1 seed lot (2)

[a]Bacterial isolations from crushed seed of II farmers' seed lots yielded 184 gram positive bacteria. Sixty-nine fingerprint types (FPTs) were differentiated among these isolates on the basis of polymerase chain reaction (BOX-PCR) fingerprinting of genomic DNA. The FPTs were arranged according to the groups from the cluster analysis of fatty acid profiles of the isolates.

[b]Refers to the groups determined by unweighted pair group method using arithmetic averages analysis of Euclidean distances calcu lated from fatty acid methyl ester (FAME) data of the 184 gram-positive rice seed isolates. Ungrouped: isolates with fatty acid profiles that re-mained unclustered at a level of 12 Euclidean distance units.

[c]Identifications obtained by: FAME-MIS (version 4.15); B = Biolog GP MicroPlate system (Microlog version 3.50); names in quotation marks are not validated.

[d]Indicates the number ofBOX-PCR FPTs delineated among the isolates associated with the respective identifications within each FAME group. Numbers in parentheses refer to the accession number of deposited representative isolates for each FPT in the culture collection of the Laboratorium Microbiologie Gent (LMG), Gent, Belgium.

[e]Indicates the number of seed lots, out of II seed lots examined, from which the isolates within the respective FPTs were found. The number in parentheses refers to the actual number of isolates found within the respective FPTs.

identified *Microbacterium* sp. were homogeneous both in FAME and in BOX-PCR analysis. For example, five of the six *Microbacterium saperdae* isolates within FAME group P3, though isolated from four different samples, produced identical BOX-PCR profiles. The single isolate LMG 18654 was different from the other five isolates both in BOX-PCR and FAME analysis. The *Microbacterium liquefaciens* and *Microbacterium barkeri* isolates formed two subgroups delineated at 5 Euclidean distance units within FAME group P5, and produced a single BOX-PCR FPT within each subgroup. Isolates identified as *Corynebacterium aquaticum* were

heterogeneous both in BOX-PCR fingerprints and in fatty acid composition (Table 3).

In addition, FAME and BOX-PCR analyses were done for all 83 bacterial colonies that were picked from soak extracts of two arbitrarily chosen in vitro germinated seed samples 23-7 and 28-9. BOX-PCR fingerprinting differentiated 25 FPTs among the 83 isolates from the two germinated seed samples. Eleven FPTs matched FPTs previously found in the ungerminated crushed seed (in order of occurrence in Table 2: LMG 18631, LMG 18618, LMG 18623, LMG 18630, LMG 18634, LMG 18576, LMG 18584, LMG 18571, LMG 18642, LMG 18591, and LMG 18573). Of the 83 isolates from the germinated seed, 41 were identified to *Enterobacteriaceae.* BOX-PCR fingerprinting differentiated 10 FPTs among these enterobacterial isolates; only 4 matched previously found FPTs in the ungerminated crushed seed. A representative strain was deposited in the LMG culture collection for isolates identified by Biolog as *Enterobacter agglomerans* biogroup 7 (LMG 18791), biogroup 5 (LMG 18792), biogroup 2B (LMG 18790), and *Enterobacter cloacae* (LMG 18793). Four FPTs were differentiated among six isolates identified as *Sphingomonas paucimobilis.* Two FPTs of nonfluorescent pseudomonads that were previously found in ungerminated seed matched the patterns generated from 16 isolates in the germinated seed. A new pseudomonad FPT (represented by deposited isolate LMG 18794) was generated from seven isolates identified as *Pseudomonas stutzerii,* and revealed a profile nearly identical to the profile from the *Pseudomonas stutzerii-type* strain LMG 11199 (data not shown). Two FPTs were delineated among fluorescent pseudomonads identified as *Pseudomonas putida.* Also, two FPTs of nonpathogenic xanthomonads were found in the genninated seed. One FPT was defined for a single isolate identified as *Alcaligenes xylosoxydans.* Only two FPTs for gram-positive bacteria were found, one from each germinated sample, and both were identified as *Paenibacillus polymyxa.* A general observation based on the results of the phenotypic tests performed on all isolates from the other nine germinated samples was that gram-positive bacteria were nearly absent in the soak extracts from germinated seed.

Assuming each BOX-PCR FPT is a single population, the number of FPTs in a sample can be used as a measure of the population diversity. The results from the BOX-PCR fingerprinting, although only one-time isolations were done from the samples, suggested that considerable differences in population diversity can be found among samples (Fig. 3).

Pathogenicity tests. Among the 428 isolates from crushed seed, 7 isolates consistently caused symptoms on all 12 plants that were inoculated, and 22 isolates caused variable symptoms on 6 to 11 of inoculated plants. The remaining 399 isolates induced no symptoms, or induced a hypersensitive-like reaction localized at the point of inoculation.

The seven pathogenic isolates were found in six samples and were clearly virulent on rice. Five isolates (represented by deposited strain LMG 18634) had uniform BOX-PCR fingerprints similar to the generated profile from *Burkholderia glumae-type* strain LMG 2196, and two isolates (represented by deposited strain LMG 18569) had a BOX-PCR profile similar to the type strain of *Burkholderia gladioli* pv. *gladioli* LMG 2216 (data not shown). The two groups of isolates also were clearly differentiated on the basis of their fatty acid composition (Table 2). Comparison of their whole-cell protein profiles with the profiles in the

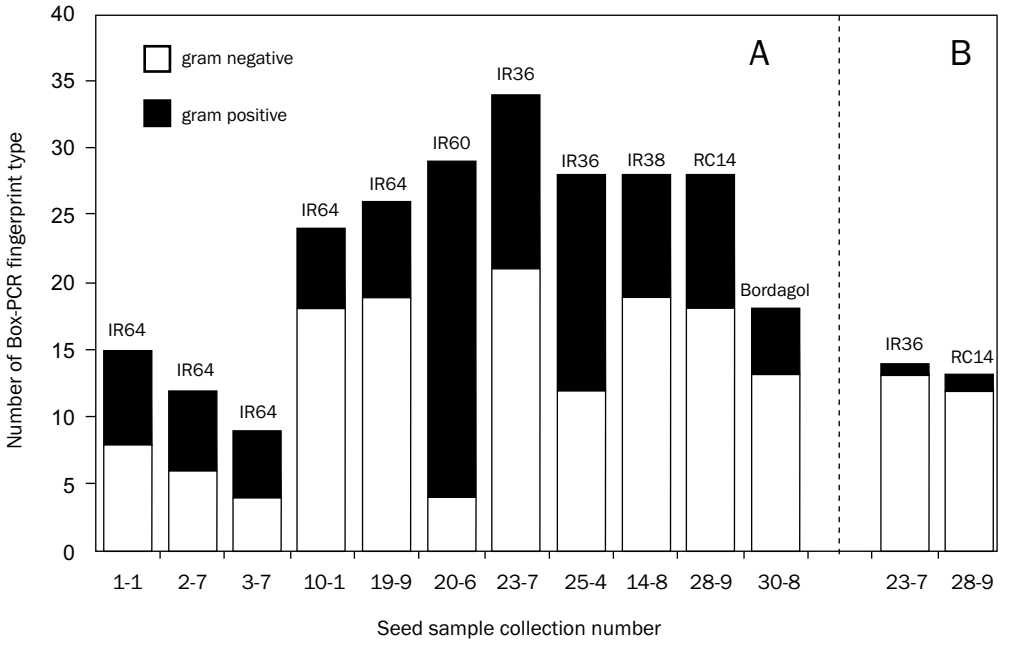

Fig. 3. Bacterial population diversity found in rice seed samples on the basis of polymerase chain reaction (BOX-PCR) fingerprint types delineated among the number of colonies isolated from each sample collected during the 1995 wet-season harvest in Iloilo, Philippines. Isolations were done once from each sample. The respective rice cultivar is indicated on top of each bar. A, Number of bacterial populations found in crushed 10-g rice seed samples; and B, number of bacterial populations found in two soaked 10-g rice seed samples after in vitro germination.

Burkholderia database generated by Vandamme et al. (45) positively identified five isolates to *Burkholderia glumae* and two isolates to *Burkholderia gladioli.* All seven isolates induced similar symptoms when inoculated by injection into rice plants, except that the *Burkholderia gladioli* isolates appeared less aggressive. Infection was rapid and characterized by the development of brown necrotic lesions on the sheath plus apparent chlorosis of the second and third leaves within 3 days after inoculation. The leaves gradually wilted and brown rot developed on the sheath; the plant usually collapsed. Infection with *Burkholderia glumae* isolates always led to death of the entire plant.

Twenty-two isolates caused symptoms of sheath necrosis to a variable extent on only 50 to 90% of inoculated plants and were considered pathogens with low disease potential. Seven isolates were identified as *Bacillus pumilus* by FAME-MIS, two as *Paenibacillus* spp., nine were nonfluorescent pseudomonads, and four were *Pantoea* spp. They were delineated by 10 BOX-PCR FPTs, which, however, often also comprised isolates without disease potential. The reaction of the *Bacillus pumilus* and *Paenibacillus* isolates on the inoculated plants was constant among the three inoculation experiments. They induced water-soaked blotches and streaks on the sheath and along the margins of the third leaf; the second leaf often appeared curled and grayish-green. After 10 days, the sheath showed brown necrotic zones and affected leaves were wilted. The reactions elicited by the *Pantoea* spp. and pseudomonad isolates on inoculated plants were variable in severity of caused symptoms between the first and the following two inoculation experiments, making it difficult to determine if they were virulent

or not. Clearly, further work is needed to establish their real pathogenic potential.

Antifungal activity. Screening of all 428 rice bacterial isolates yielded 17 bacteria that exhibited in vitro antifungal activity against one of the four tested fungal rice pathogens. *R. solani* was the most sensitive fungal pathogen and was inhibited by 14 isolates; *Pyricularia grisea* was inhibited by three isolates (Table 4). None of the isolates inhibited *Fusarium moniliforme* or *Sarocladium oryzae.* These 17 antagonists, found in 6 of the 11 samples, were associated with 13 BOX-PCR FPTs, and 9 of these FPTs included both producers and nonproducers of antifungal compounds. Two of the five FPTs associated with eight isolates identified as *Bacillus subtilis* showed different inhibition spectra.

Discussion

Studies on bacterial populations associated with the rice plant have traditionally focused on the organisms of obvious interest (i.e., pathogens or biological control agents) and have commonly neglected the other large fraction of saprophytic, opportunistic, and symbiotic bacteria. The aim of this study was to provide more insight into the qualitative composition of bacterial communities associated with rice seed. The seed was of particular interest because of its importance as planting material and its potential as vehicle for transmission of beneficial or deleterious bacteria. Single bacterial isolates were characterized by BOX-PCR fingerprint typing of genomic DNA. This technique allowed the comparison of

Table 4. Rice seed isolates with *in vitro* antifungal activity

Identification[a]	Collection no.[b]	Inhibition zone (mm)[c]	
		R. solani	*P. grisea*
Pantoea spp. (API)	G2	11	0
	G4	11.7	0
	G193	8	0
Enterobacter cloacae (B)	G5 (LMG 18607)	7.5	0
Stenotrophomonas maltophilia	G266 (LMG 18581)	7.7	0
Xanthomonas spp. (B)	G314 (LMG 18637)	10.3	0
Acinetobacter baumannii	G6	7.5	0
Paenibacillus macerans	G429 (LMG 18690)	0	33
Cellulomonas flavigena	G7 (LMG 18647)	6.7	0
Bacillus subtilis	G229 (LMG 18671)	9	0
	G460	10.3	0
	G396	0	26
	G425 (LMG 18707)	0	21.5
	G364	8.3	0
	G329	12.0	0
	G412	8.3	0
	G452 (LMG 18715)	9.5	0

[a]Identifications obtained by fatty acid methyl ester (FAME)-MIS (version 4.15); B = Biolog GN MicroPlate system (Microlog version 3.50); API =API 20E/50CHE system.
[b]Accession numbers refer to the collection of the Entomology and Plant Pathology Division at the International Rice Research Institute, Los Baños, Philippines. Numbers in parentheses refer to the accession numbers of duplicate cultures deposited in the culture collection of the Laboratorium Microbiologie Gent (LMG), Gent, Belgium.
[c]Values represent the mean diameter of mycelial growth inhibition zones on *Rhizoctonia solani* and *Pyricularia grisea* for three replicates measured after 3 days of incubation at 28°C. Inhibition was determined on pigment production medium.

fingerprints of individual isolates between samples. Several studies have reported the usefulness of the rep-PCR technique to fingerprint a large variety of bacteria, and its ability to differentiate bacteria at the species, pathovar, or strain level (19,20,38,47). The results of the BOX-PCR genomic fingerprinting revealed a large variety of bacteria with unique fingerprints and groups of bacteria with nearly identical fingerprints that were isolated from different samples. In this study, very few fingerprints of identified isolates matched the profiles of corresponding LMG reference strains. Previous studies using DNA fingerprinting have demonstrated considerable variability within bacterial taxonomic groups (18-20,47). Also, the preliminary BOX-PCR analysis in this study of selected LMG reference strains indicated that the degree of variability found within a species varied among species. The revealed diversity in BOX-PCR fingerprints complicated the interpretation of relatedness between the large number of seed isolates. Therefore, phenotypic characterization and whole-cell fatty acid analysis was useful to achieve relevant phenotypic groupings in order to facilitate the interpretation of the obtained variability in BOX-PCR profiles. The results of this study revealed a high diversity of seed-associated bacteria despite the known limitations of traditional cultivation techniques (1,42). In addition, the pure culture approach allowed for further testing and characterization of pathogenic and antagonistic isolates.

On the basis of FAME and BOX-PCR analysis, *Enterobacteriaceae* were the most diverse group of isolates among the gram-negative bacteria found in rice seed. The enterobacterial populations as determined by distinct BOX-PCR fingerprints may reflect a differentiation at the subspecies or even strain level (47). *Pantoea* populations, followed by *Enterobacter* populations related to *Enterobacter cloacae* and *Enterobacter sakazakii,* dominated the group of *Enterobacteriaceae.* Identifications at the species level, however, remained doubtful for many isolates. In previous studies, the extreme heterogeneity of the species *Enterobacter agglomerans* was shown and has been amended into several *Pantoea* spp. (4,9,23). Therefore, obtained identifications of *Enterobacter agglomerans* from the taxonomically older entries in the API library were for convenience given as *Pantoea* spp. The diversity of enterobacterial species associated with rice seed has been largely overlooked in the past, because only *Erwinia herbicola* (subjective synonym of *Pantoea agglomerans)* is reported to be commonly isolated from infected and healthy rice seed (3,25, 29,50). In this study, two frequently found enterobacterial FPTs (represented by LMG 18570 and LMG 18571) had highly similar patterns to the pattern generated for the *Pantoea stewartii* subsp. *stewartii*-type strain LMG 2715 (previously *Erwinia stewartii).*

Pseudomonads can commonly be isolated from healthy and discolored rice seed and are primarily considered saprophytes, although some are biocontrol agents (24,33,34) or rice pathogens (2,16,26,29). Several have been associated with the seed discoloration and leaf sheath rot syndrome of rice (8,50). In this study, mainly nonfluorescent pseudomonads were found that were homogeneous in fatty acid composition but clearly heterogeneous on the basis of BOX-PCR analysis. Identification of these pseudomonads by FAME-MIS and Biolog often gave inaccurate results; only 26% of isolates were correctly identified with these methods. FAME-MIS identifications as *Flavimonas oryzihabitans* were acceptable, but identifications as *Pseudomonas aeruginosa* were suspicious.

Since the last decade, the occurrence of nonpathogenic xanthomonads occasionally has been reported from rice seed as well as from other crops (13,25,46). The present results clearly indicate that nonpathogenic xanthomonads are commonly associated with rice seed. These isolates formed a homogeneous group on the basis of fatty acid composition but revealed a high diversity in BOX-PCR fingerprints. They did not fit into any recognized *Xanthomonas* sp. in the fatty acid database created by Yang et al. (49) and, thus, seem to occupy a unique position within the genus *Xanthomonas.* The only known *Xanthomonas* sp. on rice is *X. oryzae*, comprising the two pathovars *oryzae* and *oryzicola,* causing bacterial leaf blight and bacterial leaf streak on rice, respectively (29). These nonpathogenic xanthomonads can be confused with *X. oryzae* based on similar colony appearance (25) and, therefore, may be incorrectly identified as *X. oryzae.* However, they certainly did not belong to *X. oryzae* both in FAME and BOX-PCR analyses.

An unexpected finding was the large diversity of bacilli and coryneform bacteria that can be found associated with rice seed. These populations have hardly been explored in the past; hence, little is known about their presence and significance as a component of the rice seed microflora. To our knowledge, this is the first report of coryneform bacteria in rice seed. These populations should not be neglected, because they may represent not only an untapped resource of beneficial properties but also a source of unknown disease potential. For instance, some isolates exhibited antifungal activity and others were able to induce symptoms of sheath necrosis on rice.

Two percent of the isolates were seedbome pathogens identified as *Burkholderia* spp. and were found in 55% of the samples. *Burkholderia glumae* is recognized as an important rice pathogen causing both seed rot (10) and seedling rot (43). On the other hand, reports on *Burkholderia gladioli* as a rice pathogen are limited and come only from Japan (44). *Burkholderia plantarii,* another *Burkholderia* sp. known as a rice pathogen causing seedling blight (2), was not found in the seed samples. Furthermore, an ill-defined group comprising 5% of the total number of isolates caused symptoms of sheath necrosis to a variable extent on 50 to 90% of inoculated plants. These isolates were identified as *Bacillus pumilus, Paenibacillus* spp., *Pseudomonas* spp., and *Pantoea* spp. They were considered pathogens with low disease potential and were different from true pathogens on the basis of lower aggressiveness and inconsistency in producing symptoms. The choice of inoculation method may have been inappropriate for assessing the true virulence of these isolates; however, the majority of isolates did not induce symptoms on rice plants upon inoculation. The disease potential on rice of *Pantoea agglomerans* strains (3), synanamorph *Erwinia herbicola* (4,9), and strains related to saprophytic *Pseudomonas* spp. (8,50), has previously been reported. Usually regarded as saprophytes, these bacteria have not been given much attention in the past. However, such isolates were regularly recovered from rice seed in subsequent isolations, and further work to establish their disease potential is in progress.

In the present study, 4% of the bacteria isolated showed in vitro antifungal activity. The majority (82%) inhibited the mycelial growth of *Rhizoctonia solani,* whereas the other isolates inhibited *Pyricularia grisea.* Nearly half (47%) of the antagonists were identified as *Bacillus subtilis;* the other isolates with antifungal

activity belonged to various taxa. Strains of *Bacillus subtilis* (24,33), *Enterobacter cloacae* (28), and *Pantoea agglomerans* (synanamorph *Erwinia herbicola)* (28,33) have been used as biological control agents against rice fungal diseases. The potential use of *Stenotrophomonas maltophilia* as a biological control agent has been explored (5,51). However, in vitro inhibition of mycelial growth does not guarantee that these strains will be effective biocontrol agents (24), and further work to establish their potential in vivo is needed.

To assess the impact of seed germination on the composition of bacterial communities, BOX-PCR and FAME analyses were performed on isolates obtained from two arbitrarily chosen germinated seed samples. It is assumed that the large majority of bacteria are localized in and under the rice hull and, therefore, can be released from the seed by soaking. Results from recent experiments have shown that isolations by seed soaking give comparable results and, often, a larger number of colony morphotypes compared with isolations by seed crushing (B. Cottyn, *unpublished data)*. FAME analysis of the isolates from the two germinated samples suggested that the main phenotypic groups of gram-negative bacteria found in ungerminated seed were also present after germination. The overall diversity of populations as reflected by the number of BOX-PCR FPTs was lower. The most apparent result was the near absence of gram-positive bacteria. Only isolates identified as *Paenibacillus polymyxa* were found and, interestingly, this was also the single gram-positive species isolated from rice seedlings in the study by Mukhopadhyay et al. (28). Further, a qualitative shift in the composition of enterobacterial populations was found Populations not isolated before germination dominated the populations that persisted after germination. It is suggested that exudates from germinating seed create a different environment that can inhibit or induce the proliferation of certain bacteria (31). Second, the increase in one population of the community can likewise affect other populations.

In conclusion, BOX-PCR analysis of a collection of rice seed isolates revealed a high heterogeneity within the bacterial communities associated with rice seed. However, the function of this genetic diversity among populations of bacteria in relation to environmental variables, including farmers' cultural practices, is not clear. Further research that elucidates the mechanisms eliciting this genetic diversity is needed. An understanding of the ecology of natural microbial communities should lead to a more efficient deployment of bacterial populations for disease management.

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Characterization of Bacteria from Seeds of Rice Cultivar PSBRc14

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ABSTRACT
Cottyn, B., Lanoot, B., Regalado, E., Mew, T. W., and Swings, J. 2002. Characterization of bacteria from rice seed PSBRc14 in Philippine farmers' fields.

Seed of rice cultivar PSBRc14 was collected from farmers' fields at one location of irrigated lowland in southern Luzon, Philippines. Isolations of distinct colonies, representative for the observed morphological diversity on dilution plates from seed washes of 12 samples and three arbitrarily chosen germinated samples, yielded 498 isolates. Classification of the isolates according to similarities in cellular characteristics, whole-cell fatty acid composition, and colony appearance differentiated 101 morphotype groups aside from 18 *Actinomycetes* spp. and 18 ungrouped isolates. Predominant bacteria were *Coryneform* spp., *Pantoea* spp., and *Pseudomonas* spp. Other bacteria regularly present were *Actinomycetes* spp., *Bacillus pumilus*, *Bacillus subtilis*, *Burkholderia glumae*, *Enterobacter cloacae*, *Paenibacillus polymyxa*, *Staphylococcus* spp., and *Xanthomonas* spp. Sixteen percent of isolates inhibited in vitro the mycelial growth of the pathogens *Rhizoctonia solani* and *Pyricularia grisea*. Four percent of isolates were pathogens identified as *Burkholderia glumae*, *Burkholderia gladioli*, and *Acidovorax avenae* subsp. *avenae*. The genetic diversity among isolates was assessed by BOX-polymerase chain reaction fingerprinting of genomic DNA and represented 284 fingerprint types (FPTs). Most FPTs (78%) were not shared among samples, while eight FPTs occurred frequently in the samples. Seven of these FPTs also occurred frequently in a previous collection made from rainfed lowlands of Iloilo island, Philippines.

Additional keywords: Biolog, FAME-MIS, pathogens with low disease potential, nonpathogenic xanthomonads.

Rice (*Oryza sativa* L.) is the staple food for nearly half the world's people, making it the principal food crop currently produced. Studies on bacterial populations associated with the rice plant have traditionally focused on the bacteria of obvious interest, such as pathogens (2, 11, 19, 26, 29, 40, 41, 42, 43, 50), biological control agents (24, 33, 34) or diazotrophic bacteria (30, 39); and have commonly neglected the other large fraction of saprophytic and symbiotic bacteria. The seed is of particular interest because of its importance as planting material and its potential as a vehicle for transmission of beneficial or deleterious bacteria. There is still a considerable need for extensive inventory studies on rice seed-associated bacteria to understand the ecological distribution of populations and their multilevel interactions on the host plant, before efficient deployment of bacterial populations for disease management will be possible.

Our earlier study (5), which described culturable bacteria associated with rice seed obtained from six cultivars and seven locations of rainfed lowlands of Iloilo island, Philippines, revealed a broad genetic diversity of bacteria inhabiting the seed. A considerable genetic variability in bacterial populations among samples was observed, while the presence of few frequently occurring populations might indicate preferential associations with the seed. It is not clear whether this variability is inherent to the seed bacterial community, or has an ecological significance in relation to plant genotype, agroecological environment, or farmers' crop management practices. Because production of rainfed lowland rice depends on erratic rainfall, farming conditions are more diverse than in irrigated continuous rice-cropping systems. In an attempt to further verify this variability in bacterial populations, we assessed the diversity of bacteria associated with seed of a single rice genotype collected from 12 farmers' fields at one location of irrigated lowland of southern Luzon island, Philippines. The cultivar PSBRc14, which contains moderate or partial resistance to major fungal and bacterial rice diseases, was released by the Philippine Seed Board (PSB; Rc stands for rice) in 1992. The cultivar is high yielding with short growth duration of 110 days, and is drought tolerant. Our present study also differed from the previous study (5) in the method used to isolate bacteria from the seed. Because it is assumed that the large majority of bacteria are localized in and under the rice hull, and can be released from the seed by soaking, isolations were performed by seed washing instead of seed crushing. In our previous study (5), we used whole-cell fatty acid methyl ester (FAME) analysis to classify the phenotypic diversity among isolates. The established FAME-groups often comprised isolates of distinct colony appearance, moreover, revealed a high genetic variation among isolates reflected by low linkage levels among DNA fingerprints ranging from 10 to 30% similarity. Therefore, a more detailed representation of phenotypic diversity based on morphotype groups might correspond better to the observed genetic diversity. Whole-cell fatty acid analysis by the MIDI Microbial Identification System (MIS; Microbial ID Inc., Newark, DE) and Biolog GN MicroPlates (Biolog, Inc., Hayward, CA) were used for species identification of the isolates. These phenotypic typing methods are relatively simple and automated, and have been proven useful for identification of bacteria (14, 38, 48).

Analogous to our previous study (5), genomic DNA fingerprinting of individual isolates by the BOXA1R primer and the polymerase chain reaction (BOX-PCR; 23) was used to evaluate genetic diversity among the isolates. The efficiency of

repetitive DNA-based polymerase chain reaction genomic fingerprinting to assess the genetic diversity of phytobacterial communities has been demonstrated (8, 12, 21, 39), and its applications to environmental microbiology have been reviewed (22, 47). The characterization of single bacterial isolates by BOX-PCR genomic fingerprinting allowed for the comparison of fingerprints with those previously described for isolates from six rice cultivars in rainfed farmers' fields (5). In fact the two studies are complementary and resulted in the construction of a library database with BOX-PCR genomic fingerprint profiles of rice seed-associated bacteria.

The aims of this study were to (i) describe culturable bacteria associated with seed of rice cultivar PSBRc14 collected from one location of irrigated lowland, (ii) provide an expanded list of the nature and predominance of rice seed-associated bacterial morphotype groups, (iii) assess the genetic variability among isolates obtained from seed of a single cultivar and one geographical location, and (iv) identify common rice seed-associated populations by comparison of the BOX-PCR patterns with those previously generated for bacteria from rice seed of six cultivars and seven locations of rainfed lowlands of Iloilo island, Philippines (5).

Materials and methods

Bacterial strains. A representative isolate for each defined morphotype group in this study has been deposited in the Belgian Coordinated Collections of Microorganisms/Laboratory Microbiology Gent (BCCM/LMG) Bacteria Collection, University Gent, Gent, Belgium. The BOX-PCR patterns of 428 isolates from rice seed of six cultivars and seven locations of rainfed lowlands of Iloilo island, Philippines, and the generated patterns of 137 reference strains from the BCCM/LMG Bacteria Collection (5), were included in comparative analyses of BOX-PCR fingerprints.

Isolation of bacteria from seed. Seed was collected in November 1996 from 12 farmers' fields cropped continuously to irrigated rice at the municipality of Jalajala in Rizal province of southern Luzon island, Philippines. The location forms part of the irrigated intensive rice cropping systems of Luzon. The selected farmers had all planted rice cultivar PSBRc14, and commonly use seed from their previous harvest for planting the next season's crop. An overview of the collected seed samples and number of isolates per sample are given in Table 1. For each of the 12 samples, a 50-g batch was processed by dilution plating of seed washes. Seed (50-g) was immersed in 100 ml of sterile phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with 0.025% Tween 20 (Sigma-Aldrich, St. Louis, MO), and soaked at ambient temperature (ca. 22 to 25°C) for 2 h on an orbital shaker at 130 rpm. Aliquots (0.1 ml) of a 1000-fold dilution of the

Table 1. Collected seed samples of rice cultivar PSBRc14

Collection no.[a]	No. of isolates[b]	
	Dry seed	Germinated seed
BPJ-2	42	42
BPJ-4	32	
BPJ-5	36	
BPJ-8	26	23
BPJ-9	31	
BPJ-10	26	
BPJ-11	23	
BPJ-12	28	41
BPJ-13	46	
BPJ-15	36	
BPJ-17	29	
BPJ-26	37	

[a]Seed samples were collected during the wet season of 1996 from 12 adjacent farmers' fields at Jalajala in southern Luzon island, Philippines.
[b]Visually distinct colonies were isolated from each sample and three arbitrarily chosen germinated samples by dilution plating of washes from 50-g seed onto tryptic soy agar medium.

original seed wash prepared in sterile saline (0.85% NaCl) were plated in duplicate onto tryptic soy agar (TSA; Sigma-Aldrich) amended with 0.01% cycloheximide (Sigma-Aldrich) to prevent fungal contamination. The appropriate dilution that yielded approximately 30 to 300 colonies per dilution plate was established in preliminary experiments. The duplicate dilution plates were inspected after 3 and 7 days of incubation at 28°C and all colonies were closely examined visually and under the dissecting microscope at 12-power magnification. Each distinct colony type was picked and enumerated. The isolates were purified on TSA and maintained at –70°C in nutrient broth (NB; Difco Laboratories, Detroit, MI) with 15% glycerol. Remaining seed of the 12 seed lots was stored at 4°C in the Seed Health Unit of the International Rice Research Institute (IRRI), Los Baños, Philippines.

In addition, isolations were done from germinated seed of three arbitrarily chosen seed lots. Seed (50-g) was incubated in 50 ml of sterile distilled water in plastic germination boxes (14 by 17 by 4.5 cm) for 3 days at 28°C until about 40% of the seeds were germinated. Germination was indicated by the appearance of the white tip of the coleoptile. On the fourth day, 100 ml of PBS with 0.025% Tween 20 was added and the germinated seed were washed at ambient temperature (ca. 22° to 25°C) for 2 h on an orbital shaker set at 130 rpm. Aliquots (0.1 ml) of a million-fold dilution of the seed washes prepared in sterile saline (0.85% NaCl) were plated in duplicate onto TSA supplemented with 0.01% cycloheximide. Isolation, enumeration, and purification of isolates were done as described above.

Phenotypic characterization. The following features were examined for all isolates: Gram morphology, Kovac's oxidase reaction, and reaction on Hugh and Leifson's oxidative-fermentative medium (20). Gram-negative oxidative bacteria were further tested for fluorescent pigment production on King's medium B (16). The colony morphology on TSA medium was closely examined visually and under the dissecting microscope at 12-power magnification, and the isolates were grouped according to similarities in colony shape, texture, and color. The colony appearance of each type was described for 4-day-old TSA cultures grown at 28°C. The colony pigmentation was evaluated against a gray background by using a color standard guide (13). All tests were repeated at least twice for each isolate to assess the reliability of the test results. The isolates were classified into morphotype groups on the basis of similarities in cellular characteristics, whole cell fatty acid methyl ester (FAME) composition, and colony appearance. Identifications were made by FAME analysis and additional use of Biolog GN MicroPlates.

FAME analysis. All isolates were grown in highly standardized conditions on TSA (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD) at 28°C for 24 h. Extraction and preparation of the cellular fatty acid methyl esters were performed according to Sasser (36). FAMEs were analyzed with a Hewlett-Packard HP5890A gas-liquid chromatograph on a 5% phenylmethyl silicone-coated glass capillary column. The generated profiles were identified by using the Microbial Identification System (MIS version 4.15; Microbial ID Inc., Newark, DE). The same software was also used for calculating the dissimilarity between profiles using the Euclidean distance coefficient, and for cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA). Duplicate analysis of individual isolates clustered at a level of less than 5 Euclidean distance

units. The correlation between a profile from an unknown isolate and a library entry was expressed as a similarity index (SI) on a numeric scale of 0 to 1. MIS identifications at the species level were considered reliable when the first choice had an SI value of ≥0.5, and the numerical difference between the first and second choice SI value was ≥0.1.

Biolog. Whenever FAME-MIS failed to give a reliable identification for gram-negative isolates, further identification was done using the Biolog GN MicroPlate system (Biolog Inc., Hayward, CA). Isolates for Biolog analysis were grown on TSA for 24 h at 28°C. The Biolog MicroPlates were inoculated with a bacterial suspension in sterile saline (0.85% NaCl), adjusted in density to the Biolog MicroPlate system's turbidity standard, and incubated for 24 h at 30°C. The plates were read on an automated microplate reader, and results were analyzed with Microlog software (version 3.50).

SDS-polyacrylamide gel electrophoresis of whole-cell proteins. Whole-cell protein profiles were generated for those isolates that were thought to belong to the genus *Burkholderia*. Cultivation of bacterial cultures, preparations of whole-cell protein extracts, and SDS-polyacrylamide gel electrophoresis (PAGE) were performed as described previously (32). The obtained protein profiles were visually compared with the patterns in the database of the genus *Burkholderia* created by Vandamme et al. (44).

Community structure analysis. Rank-abundance (dominance-diversity) curves plot the abundance of taxa against decreasing rank (4). A rank-abundance curve was applied to defined morphotype groups found in the 12 dry seed samples. The 99 morphotype groups, representing 84 identified groups along with a group of *Actinomycetes* spp. and 14 single unidentified gram-negative bacteria, were sorted in descending order of mean abundance estimated from total colony counts of the isolates within each group taken over 12 samples, then plotted on a log scale of abundance against rank. In a second analysis, morphotype group abundance and frequency of occurrence were given equal weights and used as measures of morphotype group dominance. Morphotype groups were given ranks in descending order of abundance and in descending order of frequency of occurrence, and the sum of ranks for each morphotype group was taken as dominance score.

DNA isolation. Bacterial cells were grown on TSA for 24 h at 28°C, harvested with a sterile plastic spatula and washed once in 0.5 ml of saline buffer (150 mM NaCl, 10 mM EDTA, pH 8.0) in a 1.5 ml Eppendorf centrifuge tube. After centrifugation for 2 min at 11000 rpm, cells of gram-negative bacteria were resuspended in 100 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and kept on ice. Cells of gram-positive bacteria were resuspended in 100 ml of TE buffer containing lysozyme (50 mg/ml) and incubated at 37°C for 30 min. Extraction of total genomic DNA was done as described by Pitcher et al. (31). DNA samples were redissolved overnight at 4°C in 100 ml of TE buffer. The quality of the DNA was checked by electrophoresis in 0.8% agarose gels in 0.5x Trisborate-EDTA (TBE) (35), and stained with ethidium bromide. The DNA concentrations were estimated visually by comparison with λ DNA standards in an agarose gel. All DNA preparations were stored at –20°C.

BOX-PCR fingerprinting. All isolates were analyzed by polymerase chain reaction using the BOXA1R primer [5'-CTACGGCAAGGCGACGCTGACG-3'] as described before (5). PCR amplifications were performed in 25-ml reaction volumes

containing 2 mM of BOXA1R primer, approximately 40-80 ng of genomic DNA, 1.25 mM of each dNTP (United States Biochemical, Cleveland, OH), 2 units of *Taq* DNA polymerase (Pharmacia Biotech, Asia Pacific, Hong Kong), 10% (vol/vol) DMSO (Sigma-Aldrich) and 0.16 mg/ml bovine serum albumin (Boehringer Mannheim, Far East, Singapore) in 1x Gitschier buffer (18). PCR amplifications were performed in a DNA thermal cycler (480; Perkin-Elmer, Norwalk, CT) with an initial denaturation at 95°C for 7 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 65°C for 8 min, and a final extension at 65°C for 15 min. Aliquots (7 ml) of amplified PCR products were separated by gel electrophoresis on gels composed of 0.8% Synergel (Diversified Biotech, Boston, MA) and 0.8% agarose (United States Biochemical) in 0.5x TBE at 75V for 14 h. The two outer wells were not used to avoid distortion of the profiles. As standard, a 1-kb size marker (Life Technologies, Pacific, Hong Kong) was run in the second, sixteenth, and thirtieth lane. The gels were stained with ethidium bromide and photographed on an UV transilluminator with Polaroid Type 55 film (Polaroid Corp., Cambridge, MA). The photographs were scanned on a flatbed scanner (Sharp JX-610) at 200 dots per inch (dpi) resolution and saved as TIFF files. Normalization of BOX-PCR patterns and cluster analysis was achieved with the commercially available GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). Background was subtracted using the rolling disk method as described by the manufacturer of GelCompar. Levels of similarity between the BOX-PCR patterns were calculated by using the Pearson product moment correlation coefficient, and were expressed as percentages of similarity for convenience. Dendrograms were generated by the unweighted pair group method using arithmetic averages (UPGMA) of correlation coefficients for the BOX-PCR patterns. Generated BOX-PCR fingerprints of examined isolates were repeated at least twice. A fingerprint type (FPT) was defined as one bacterial isolate with a particular BOX-PCR pattern or a set of isolates with nearly identical BOX-PCR patterns arbitrarily delineated at a level of 70% similarity.

Reproducibility of DNA fingerprints. The level of reproducibility of BOX-PCR fingerprinting was determined by including DNA of isolate G237 in each PCR assay; its resulting fingerprint was used as an internal standard during each electrophoresis run. Sixty-one gel runs had been separated over a period of 11 months. Intergel reproducibility of the internal standard pattern as derived from the similarity scale in the dendrogram generated by the UPGMA clustering analysis software of GelCompar, was 86%. However, visually no differences were observed in the obtained profiles of amplified fragments. Variations were mainly attributed to differences in electrophoresis times and in the intensities of amplified bands. Large bands (greater than 4 kb) and faint bands were not always amplified to the same extent or were not equally visible after ethidium bromide staining. When reproducibility was determined by obtaining repeated patterns of four strains in three PCR experiments conducted at separate dates, the intergel correlation between these patterns was above 95%.

Pathogenicity tests. All isolates were tested for pathogenicity by inoculation of 21-day-old rice seedlings of cv. IR24 grown in the greenhouse under natural light with day and night temperatures of about 35 and 25°C, respectively, and with relative humidity ranging from 40 to 65%. Cultivar IR24 plants were used in the pathogenicity

tests because they are known to be susceptible to most rice diseases. Seed was disinfected for 5 min in 70% ethanol and rinsed five times with sterile distilled water, then soaked for 12 h under running tap water and planted in autoclaved soil. Inoculum consisted of aqueous suspensions of 12-h-old bacterial cultures from TSA slant tubes (approximately 3 x 10^8 CFU/ml). An aliquot (0.1-ml) of each bacterial suspension was injected into the plant at the base of the culm about 2 cm above the soil using a disposable syringe and hypodermic needle. For each bacterial isolate, four seedlings were inoculated in a first screening conducted from January to March 1997. Isolates that produced a positive reaction on at least two of four seedlings in the first experiment were inoculated two more times on four plants in a second and third experiment conducted from July to September 1997. Plants inoculated with sterile distilled water served as negative control. The plants were examined for symptoms 3 and 10 days after inoculation. Plants were scored as negative when no obvious symptoms or a hypersensitive-like reaction localized at the point of inoculation occurred. Elongation of a brown necrotic zone of tissue away from the point of inoculation, often extended up to the third leaf, was scored as a positive reaction. Bacterial isolates were considered pathogens if all plants inoculated produced disease symptoms; whereas, isolates that caused symptoms on at least 50% of inoculated plants in the three experiments were considered pathogens with low disease potential. Bacteria were re-isolated from selected plants to confirm that symptoms were caused by the inoculated isolate. The identity of the colonies was verified by comparing the BOX-PCR fingerprint with the fingerprint profile of the inoculated isolate.

Screening for antifungal activity in vitro. All isolates were tested by the dual culture method as previously described (24) for antagonistic activity against four rice fungal pathogens: *Rhizoctonia solani* AG1, *Pyricularia grisea* PO6, *Sarocladium oryzae*, and *Fusarium moniliforme*. Cultures of the fungal pathogens collected from rice in the Philippines were maintained at the Seed Health Unit of IRRI. Five mycelial plugs (5 mm in diameter) were taken from an actively growing culture of *R. solani* AG1 on potato dextrose agar (PDA; 200 g of potato, 20 g of dextrose, and 18 g of agar per liter). One plug was placed at the center and four plugs at opposite sides toward the edge of the plate on pigment production medium (PPM; 20 g of proteose peptone, 20 g of glycerol, 5 g of NaCl, 1 g of KNO3, and 15 g of agar per liter, pH 7.2). Four bacterial cultures, grown for 48 h on PPM, were placed between the four peripheral agar plugs of *R. solani* AG1. For antagonism tests against *P. grisea* PO6, *S. oryzae* and *F. moniliforme*, 50 ml of an aqueous conidial suspension (50,000 conidia/ml) of each fungal pathogen was added to 1 liter melted PPM at 45°C. The inoculated PPM was poured into petri plates and allowed to congeal for 4 h, after which bacterial isolates to be tested were placed in four equidistant places in the plates. Each fungal-bacterial combination was replicated three times. All dual-culture plates were incubated at 28°C for 2 to 3 days and scored for inhibition of fungal growth. The diameter of the inhibition zone around the bacterial colony was measured.

Results

Phenotypic diversity. Isolations of visually distinct colonies by dilution plating of seed washes from 12 samples yielded 392 isolates and 106 isolates from three arbitrarily chosen germinated samples. The 498 isolates consisted of 261 gram-negative bacteria (52%) and 237 gram-positive

bacteria (48%). The isolates were classified into morphotype groups based on similarities in cellular characteristics, whole-cell fatty acid composition, and colony appearance. Gram-negative bacteria represented 47 morphotype groups aside from 18 diverse rod-like isolates that remained ungrouped, and were arranged in Table 2 in order of frequency of occurrence within groups of *Enterobacteriaceae* (26%), *Pseudomonas* (26%), other aerobic rods (36%), helical aerobic bacteria (5%), and 18 ungrouped isolates (7%). Gram-positive bacteria represented 54 morphotype groups and a group of *Actinomycetes* spp. with aerial mycelium, and were arranged in Table 3 in order of frequency of occurrence within groups of cocci (18%), coryneform bacteria (48%), endospore-forming rods (27%), and *Actinomycetes* spp. (7%). Mean abundances of morphotype groups found on the dry seed, estimated from colony counts of each type on isolation plates and averaged over 12 samples, ranged from 10^3 to $2x10^5$ CFU/ml seed wash on a total cultured mean abundance of $1.24x10^6$ CFU/ml seed wash. Mean abundances of morphotype groups found on germinated seed, averaged over 3 samples, ranged from $3.3x10^6$ to $2.6x10^8$ CFU/ml seed wash on a total cultured mean abundance of $9.66x10^8$ CFU/ml seed wash (Table 2 and 3).

Genetic diversity based on BOX-PCR genomic fingerprinting. BOX-PCR amplifications were performed on all isolates and resulted in specific fingerprint patterns consisting of 10 to 23 PCR products ranging in size from about 300 bp to 4.5 kb. Computer-assisted numerical analysis of the generated BOX-PCR patterns revealed a high dissimilarity among isolates. Separate cluster analyses were performed independently on the gram-negative bacteria and gram-positive bacteria, and additionally on each defined phenotypic group. A total of 284 fingerprint types (FPTs) were delineated at an arbitrary level of 70% similarity. The 261 gram-negative bacteria represented 133 FPTs, and the 237 gram-positive bacteria represented 151 FPTs (Table 2 and 3). Assuming each FPT is a single population, then at least 284 bacterial populations occurred in the seed samples. The majority of FPTs (221 FPTs or 78%) was not shared among samples, while eight FPTs (3%) were found in five to all samples. The frequently occurring FPTs were: *Pantoea stewartii* LMG 20115, found in all samples; *Curtobacterium flaccumfaciens* LMG 20194, found in 10 samples; *Pantoea dispersa* LMG 20116, found in seven samples; *Burkholderia glumae* LMG 20138 and *Bacillus pumilus* LMG 20162, found in six samples; *Clavibacter michiganense* LMG 20187, *Pseudomonas aeruginosa* LMG 20125, and *Methylobacterium radiotolerans* LMG 20139, found in five samples.

The linkage level of BOX-PCR patterns generated for isolates within each morphotype group is a measure for the degree of genetic variation within the defined morphotype groups. Linkage levels above 50% similarity were found for 61 (i.e., 60%) of the 101 defined morphotype groups. Twenty-one morphotypes were defined for single isolates. Much more distinct fingerprint patterns with linkage levels ranging from 17 to 45% similarity were found for 19 morphotype groups identified as: *Acinetobacter calcoaceticus*, *Agrobacterium radiobacter*, *Agrobacterium rhizogenes*, *Arthrobacter citreus*, *Azospirillum brasilense*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus sphaericus*, *Bacillus subtilis*, *Brevibacterium epidermidis*, *Enterobacter cloacae*, *Microbacterium esteraromaticum*, *Paenibacillus polymyxa*, *Sphingomonas paucimobilis*, *Staphylococcus gallinarum*, *Staphylococcus simulans*, *Staphylococcus xylosus*,

Table 2. Gram-negative bacteria from rice seed of cv. PSBRc14 in irrigated farmers' fields at Jalajala in southern Luzon island, Philippines[a]

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e] Dry seed samples CFUx10^3/ml (± SE)	Mean abundance[e] Germinated samples CFUx10^6/ml	Matching FPTs[f]
Enterobacteriaceae					
Pantoea stewartii, BOX (20115)	Butyrous yellow (1/23)	12	180.0 (± 47.53)	6.7	LMG 18570, 18571, 2715^T
Pantoea dispersa, B (20116)	Thin pale yellow (3/15)	9	30.0 (± 9.29)	20.0	LMG 18609 , 2603^T
Enterobacter cloacae, B (20117)	Butyrous greenish white (5/12)	8	43.3 (± 21.93)	36.7	LMG 18577, 18626, 2783^T
Pantoea agglomerans (20118)	Pale yellow (1/3)	3	3.3 (± 1.88)	-	LMG 18621
Enterobacter agglomerans biogroup 3B, B (20119)	Butyrous pale yellow (3/4)	3	9.2 (± 6.68)	-	
Klebsiella mobilis, B (20120)	Mucoid butyrous white (1/2)	2	7.5 (± 5.92)	-	
Pantoea ananatis (20121)	Translucent pale greenish yellow (1/2)	2	5.0 (± 4.17)	-	LMG 18572 , 18642, 2665^T
Enterobacter sakazaki, BOX (20122)	Butyrous cream yellow (1/3)	2	4.2 (± 2.88)	-	LMG 18584, 5740^T
Escherichia vulneris, B (20123)	Butyrous brownish cream (1/2)	2	-	23.3	LMG 18627
"*Enterobacter aerogenes*" (20124)	Butyrous yellowish white (1/2)	1	-	10.0	LMG 18620
Nonfluorescent pseudomonads					
Pseudomonas aeruginosa (20125)	Glossy chrome yellow (5/22)	12	73.3 (± 24.29)	50.0	LMG 18585, 18591, 18596, 18600, 18605
Pseudomonas oryzihabitans, B (20126)	Wrinkled light chrome yellow (3/10)	9	32.5 (± 14.62)	23.3	LMG 7040^T
Pseudomonas stutzeri, B (20127)	Wrinkled light orange brown (3/7)	6	15.0 (± 4.69)	-	LMG 18794
Pseudomonas aeruginosa (20128)	Semi-wrinkled chrome yellow (1/2)	2	1.7 (± 1.67)	6.7	
Fluorescent pseudomonads					
Pseudomonas aeruginosa (20129)	Translucent pale straw yellow (1/5)	4	1.7 (± 1.12)	10.0	LMG 18641
Pseudomonas putida, B (20130)	Translucent yellowish gray +greenish diffusible pigment (1/9)	2	-	263.3	
Pseudomonas putida, B (20131)	Butyrous light cream (4/5)	2	-	60.0	
Pseudomonas citronellolis, B (20132)	Mucoid grayish white + brownish diffusible pigment (2/3)	2	-	10.0	

continued...

Table 2 continued.

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e] Dry seed samples CFUx10^3/ml (± SE)	Mean abundance[e] Germinated samples CFUx10^6/ml	Matching FPTs[f]
Pseudomonas aeruginosa, B (20133)	Mucoid pinkish white + brownish diffusible pigment (1/1)	1	-	16.7	
Pseudomonas aeruginosa, B (20134)	Translucent ecru beige (1/1)	1	-	6.7	LMG 1242^T
Pseudomonas putida, B (20135)	Translucent wrinkled white (1/1)	1	-	6.7	
Pseudomonas putida (20136)	Butyrous straw yellow (1/1)	1	-	6.7	LMG 18566
Other aerobic rods					
Xanthomonas spp., B (20137)	Mucoid pale yellow (9/10)	8	17.5 (± 5.66)	13.3	LMG 18567, 18578, 18645
Burkholderia glumae, SDS (20138)	Glossy ivory yellow (1/13)	6	130.8 (± 40.81)	6.7	LMG 18634, 2196^T
Agrobacterium radiobacter (20140)	Butyrous pale brownish white (5/5)	5	7.5 (± 4.46)	10.0	LMG 18631
Methylobacterium radiotolerans (20139)	Reddish pink (1/5)	5	5.0 (± 1.95)	-	
Chryseobacterium indologenes, B (20141)	Glossy reddish yellow (4/7)	5	1.7 (± 1.12)	53.3	
Sphingomonas paucimobilis (20142)	Glossy deep yellow (5/5)	4	5.8 (± 4.17)	10.0	LMG 18618
Stenotrophomonas maltophilia, B (20143)	Translucent light olive gray (7/9)	4	4.2 (± 2.88)	23.3	
Acinetobacter calcoaceticus, B (20144)	Mucoid yellowish white (5/6)	4	4.2 (± 2.88)	33.3	
Burkholderia gladioli, SDS (20145)	Shiny yellowish white + yellow-green diffusible pigment (2/5)	3	11.7 (± 9.99)	23.3	LMG 18569, 2216^T
"*Sphingomonas capsulata*" (20146)	Dull deep yellow (4/4)	3	6.7 (± 3.76)	-	
"*Chryseobacterium meningosepticum*" (20147)	Mucoid reddish yellow (2/3)	3	0.8 (± 0.83)	10.0	
Agrobacterium rhizogenes, B (20148)	Mucoid brownish white (3/3)	3	-	10.0	
Acinetobacter radioresistent, B (20149)	Translucent yellowish gray (2/2)	2	5.8 (± 4.34)	-	
Chryseobacterium indologenes, B (20150)	Glossy yellowish brown(1/2)	2	0.8 (± 0.83)	3.3	

continued...

Table 2 continued.

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e] Dry seed samples CFUx10^3/ml (± SE)	Mean abundance[e] Germinated samples CFUx10^6/ml	Matching FPTs[f]
Acidovorax avenae ss *avenae*, B (20151)	Glossy yellowish gray (2/2)	2	-	10.0	
Sphingobacterium multivorum (20152)	Yellowish cream (2/2)	2	-	10.0	
Burkholderia cepacia, SDS (20153)	Wrinkled olive yellow (1/2)	2	-	6.7	
Burkholderia cepacia, SDS (20154)	Glossy yellowish white (1/3)	1	-	13.3	
Achromobacter xylosoxidans (20155)	Butyrous brownish white (2/2)	1	-	30.0	
Ochrobactrum anthropi (20156)	Scarlet red (1/1)	1	2.5 (± 2.50)	-	LMG 18593
Acinetobacter sp., B (20157)	Thin yellowish gray (1/1)	1	0.8 (± 0.83)	-	
Brevundimonas vesicularis, B (20158)	Brilliant olive yellow (1/2)	1	0.8 (± 0.83)	-	
Brevundimonas vesicularis (20159)	Translucent light cinnamon (1/1)	1	-	3.3	
Helical aerobic bacteria					
"*Azospirillum brasilense*" (20160)	Translucent pinkish white (8/10)	2	0.8 (± 0.83)	43.3	
Herbaspirillum rubrisubalbicans (20161)	Translucent pale cream (2/3)	2	0.8 (± 0.83)	16.7	
Ungrouped	Assorted morphotypes (18/18)	10	15.0 (± 3.59)	13.3	

[a]Isolations of visually distinct colonies by dilution plating of washes from 12 seed samples and three arbitrarily chosen germinated seed samples yielded 261 gram-negative bacteria. The phenotypic diversity represented 47 morphotype groups aside from 18 isolates that remained ungrouped. The genetic diversity among isolates was assessed by BOX-polymerase chain reaction (BOX-PCR) analysis and represented 133 fingerprint types (FPTs).

[b]Represent best fitting identifications of isolates within defined morphotype groups obtained by FAME-MIS (version 4.15); B = Biolog GN MicroPlates (version 3.50); BOX = with matching BOX-PCR patterns to the pattern generated for the type-strain of the species; SDS = with matching sodium dodecyl sulfate-polyacrylamide gel electrophoresis whole-cell protein profiles to profiles in the predetermined *Burkholderia* library from Vandamme et al. (44). Identifications in quotation marks were obtained with a similarity index value < 0.50 to the profile in the Microbial Identification System (MIS)-database. Ungrouped = unidentified isolates with distinct morphotypes and genomic FPTs. Numbers in parentheses refer to the LMG accession number of representative isolates deposited in the BCCM/LMG Bacteria Collection, Gent, Belgium.

[c]Morphotype groups were defined based on similarities in cellular characteristics, whole-cell fatty acid composition, and colony appearance. The colony pigmentation of 4-day-old cultures grown at 28°C on tryptic soy agar (TSA) was described against a gray background using a color standard guide (13). Numbers in parentheses indicate the number of defined BOX-PCR FPTs on the total number of isolates comprised within the morphotype group.

[d]Indicates the number of samples, out of 12 samples examined, from which the isolates were found.

[e]Values are mean abundances, averaged over 12 samples (over 3 samples for germinated seed), of colony-forming units (CFU) on TSA medium plated with 0.1 ml of 10^{-3} dilutions (10^{-6} dilutions for germinated samples) of washes from 50-g seed. Calculated standard error is given in parentheses, no estimate of standard error is given for mean abundances after seed germination due to insufficient number of examined samples.

[f]LMG numbers refer to isolates representative for BOX-PCR FPTs of bacteria from rice seed of six cultivars in rainfed lowlands of Iloilo island, Philippines (5); ^{T} = LMG reference type strain of the species; with matching patterns to FPT patterns differentiated among the isolates within the particular morphotype group.

Table 3. Gram-positive bacteria from rice seed of cv. PSBRc14 in irrigated farmers' fields at Jalajala in southern Luzon island, Philippines[a]

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e]		Matching FPTs[f]
			Dry seed samples CFUx10^3/ml (± SE)	Germinated samples CFUx10^6/ml	
Cocci					
Staphylococcus gallinarum (20176)	Dull pale yellow orange (7/7)	5	29.2 (± 13.51)	-	
Staphylococcus simulans (20177)	Glossy grayish white (5/12)	5	17.5 (± 7.89)	-	
Micrococcus luteus (20178)	Dull sulphur yellow (4/6)	5	16.7 (± 8.38)	6.7	
Kocuria kristinae (20179)	Dull light yellow orange (2/3)	3	12.5 (± 7.3)	-	
Staphylococcus xylosus (20180)	Glossy pale beige (4/4)	3	5.8 (± 3.58)	-	
Micrococcus lylae (20181)	Dull pale sulphur yellow (3/3)	3	5.8 (+ 3.58)	-	
Staphylococcus arlettae (20182)	Glossy yellowish white (2/4)	2	6.7 (± 4.7)	-	
Kytococcus sedentarius (20183)	Dull pale yellow (2/2)	2	2.5 (± 1.79)	-	
Staphylococcus hominis (20184)	Glossy pale yellow (1/1)	1	2.5 (± 2.50)	-	
Coryneform bacteria					
Microbacterium esteraromaticum (20185)	Butyrous yellowish white (3/4)	2	9.2 (± 7.53)	-	
Microbacterium barkeri (20186)	Butyrous grayish white (1/2)	2	5.0 (± 3.59)	-	
Shades of translucent yellow					
"*Curtobacterium flaccumfaciens*" (20194)	Translucent pale olive yellow (3/18)	12	105.0 (± 22.88)	-	LMG 18661
Clavibacter michiganense (20195)	Translucent pale yellow (3/5)	5	16.7 (± 10.89)	3.3	LMG 18697
Curtobacterium flaccumfaciens (20196)	Translucent yellow (2/8)	5	40.8 (± 20.94)	-	
Curtobacterium flaccumfaciens (20197)	Translucent straw yellow (1/3)	3	14.2 (± 10.03)	-	
Clavibacter michiganense (20198)	Translucent light straw yellow (3/3)	3	13.3 (± 8.65)	-	LMG 18695
"*Clavibacter michiganense*" (20199)	Translucent pale yellowish brown (2/2)	2	2.5 (± 1.79)	-	
No Match (20200)	Thin pale yellow (2/2)	2	0.8 (± 0.83)	6.7	
Shades of reddish yellow					
Clavibacter michiganense (20187)	Light apricot yellow (3/9)	7	14.2 (± 4.68)	13.3	LMG 18647, 18649, 18665
Cellulomonas flavigena (20188)	Light reddish yellow (4/8)	6	36.7 (± 13.45)	-	LMG 18669

continued...

Table 3 continued.

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e] Dry seed samples CFUx10^3/ml (± SE)	Mean abundance[e] Germinated samples CFUx10^6/ml	Matching FPTs[f]
Microbacterium sp. (20189)	Pale yellowish brown (3/4)	4	15.0 (± 8.92)	-	
Microbacterium imperiale (20190)	Pale yellow orange (2/2)	2	5.8 (± 4.34)	-	
"*Microbacterium lacticum*" (20191)	Dull yellow orange (2/2)	2	2.5 (± 1.79)	-	LMG 18713
Clavibacter michiganense (20192)	Naples yellow (1/1)	1	1.7 (± 1.67)	-	LMG 18712
Microbacterium lacticum (20193)	Glossy yellowish orange (1/1)	1	2.5 (± 2.50)	-	
Shades of green yellow					
"*Cellulomonas flavigena*" (20201)	Citron yellow (2/3)	3	3.3 (± 2.25)	6.7	
No Match (LMG 20202)	Pale citron yellow (1/2)	2	2.5 (± 1.79)	-	
Cellulomonas flavigena (LMG 20203)	Translucent pale yellow green (1/1)	1	2.5 (± 2.50)	-	
No Match (20204)	Glossy light yellow green (1/1)	1	1.7 (± 1.67)	-	
Microbacterium saperdae (20205)	Glossy citron yellow (1/1)	1	1.7 (± 1.67)	-	
No Match (20206)	Thin greenish yellow (1/1)	1	0.8 (± 0.83)	-	
Rod-coccus cycle					
No Match (20207)	Glossy pink (3/6)	5	8.3 (± 3.22)	-	
"*Arthrobacter citreus*" (20208)	Pale greenish yellow (5/5)	4	17.5 (± 9.93)	-	
Brevibacterium epidermidis (20209)	Glossy brownish white+ yellow streaks (5/7)	3	7.5 (± 5.09)	-	
"*Arthrobacter agilis*" (20210)	Wrinkled pale orange (2/3)	3	7.5 (± 4.46)	-	
Exiguobacterium acetylicum (20211)	Glossy golden yellow (2/2)	2	5.8 (± 4.34)	-	LMG 18674
No Match (20212)	Glossy brownish white (2/2)	2	2.5 (± 1.79)	-	
Brevibacterium linens (20213)	Glossy white (2/2)	1	2.5 (± 2.50)	-	
Brevibacterium mcbrellneri (20214)	Deep citron yellow (2/2)	1	2.5 (+2.50)	-	
Arthrobacter ramosus (20215)	Mucoid lemon yellow (2/2)	1	2.5 (± 2.50)	-	
Endospore-forming rods					
Bacillus pumilus (20162)	Wrinkled yellowish white (5/15)	7	40.8 (± 22.7)	-	LMG 18676

continued...

Table 3 continued.

Identification (LMG no.)[b]	Morphotype group[c] (no. of FPTs/ no. of isolates)	Frequency of occurrence[d]	Mean abundance[e] Dry seed samples CFUx10^3/ml (± SE)	Mean abundance[e] Germinated samples CFUx10^6/ml	Matching FPTs[f]
Bacillus subtilis (20163)	Rough brownish white (8/11)	6	22.5 (± 10.81)	-	LMG 18652, 18691, 18703, 18707, 18709
"*Paenibacillus polymyxa*" (20164)	Mucoid thin yellow (6/9)	6	10.0 (± 3.48)	-	LMG 18660
Bacillus cereus (20165)	Frosted dull white (4/7)	4	8.3 (± 4.05)	6.7	LMG 18675
Bacillus megaterium (20166)	Cream yellow (4/4)	4	6.7 (± 3.55)	3.3	LMG 18686
Bacillus sphaericus (20167)	Yellow beige (6/8)	4	6.7 (± 4.97)	16.7	
Paenibacillus lentimorbus (20168)	Heaped mucoid brownish white (2/2)	2	4.2 (± 2.88)	-	LMG 18690
Bacillus gibsonii (20169)	Dull pale yellow (1/1)	1	1.7 (± 1.67)	-	
Bacillus licheniformis (20170)	Dull powdery white (1/1)	1	1.7 (± 1.67)	-	
Paenibacillus pabuli (20171)	Spreading brownish white (1/1)	1	2.5 (± 2.50)	-	
No Match (20172)	Mucoid pale cinnamon pink (1/1)	1	2.5 (± 2.50)	-	
No Match (20173)	Pale yellowish brown (1/1)	1	2.5 (± 2.50)	-	
No Match (20174)	Glossy dark yellow (1/1)	1	0.8 (± 0.83)	-	
No Match (20175)	Thin grayish white (1/1)	1	-	3.3	
***Actinomycetes* spp. with aerial mycelium**	Rough compact grayish white (11/18)	6	46.7 (± 16.48)	-	

[a] Isolations of visually distinct colonies by dilution plating of washes from 12 seed samples and three arbitrarily chosen germinated seed samples yielded 237 gram-positive bacteria. The phenotypic diversity represented 54 morphotype groups and a group of *Actinomycetes* spp. The genetic diversity among isolates was assessed by BOX-polymerase chain reaction (BOX-PCR) analysis and represented 151 fingerprint types (FPTs).

[b] Represent best fitting FAME-MIS (version 4.15) identifications of isolates within defined morphotype groups; identifications in quotation marks were obtained with a similarity index value < 0.50 to the profile in the Microbial Identification System (MIS)-database. Numbers in parentheses refer to the LMG accession number of representative isolates deposited in the BCCM/LMG Bacteria Collection, Gent, Belgium.

[c] Morphotype groups were defined based on similarities in cell morphology, whole-cell fatty acid composition, and colony appearance. The colony pigmentation of 4-day-old cultures grown at 28°C on tryptic soy agar (TSA) was described against a gray background using a color standard guide (13). Numbers in parentheses indicate the number of defined BOX-PCR FPTs on the total number of isolates comprised within the particular morphotype group.

[d] Indicates the number of samples, out of 12 samples examined, from which the isolates were found.

[e] Values are mean abundances, averaged over 12 samples (over 3 samples for germinated seed), of colony-forming units (CFU) on TSA medium plated with 0.1 ml of 10^{-3} dilutions (10^{-6} dilutions for germinated samples) of washes from 50-g seed. Calculated standard error is given in parentheses, no estimate of standard error is given for mean abundances after seed germination due to insufficient number of examined samples.

[f] LMG numbers refer to isolates representative for BOX-PCR FPTs of bacteria from rice seed of six cultivars in rainfed lowlands of Iloilo island, Philippines (5), with matching patterns to FPT patterns differentiated among the isolates within the particular morphotype group.

Stenotrophomonas maltophilia, and *Xanthomonas* spp.

Qualitative composition of the bacterial community. A rank-abundance curve was applied to the morphotype groups found in the 12 dry seed samples for analyzing the bacterial community structure (Fig. 1). The cumulative plots of ranked-abundances for 99 morphotype groups indicated that 26 groups captured 80% of the total cultured abundance, 42 groups captured 90% and 58 groups captured 95%. Three morphotype groups were present in all samples, and twelve morphotype groups were present in six to nine samples. Rare morphotype groups found in one or two samples numbered 53, which included 14 single unidentified gram-negative isolates. The frequency of occurrence and abundance were used as measures of morphotype group dominance to rank the morphotype groups that fell within the 80% abundance threshold in descending order of dominance (Table 4). These 26 groups represented all morphotype groups found in at least half of the examined samples along with 11 morphotype groups found in three to five samples. The morphotype group of *Pantoea stewartii* LMG 20115 ranked with highest dominance followed by the groups of *Curtobacterium flaccumfaciens* LMG 20194, and nonfluorescent *Pseudomonas aeruginosa* LMG 20125. Seventeen common morphotype groups belonged to gram-positive bacteria that mainly represented coryneform bacteria (nine morphotype groups).

Predominant *Enterobacteriaceae* were *Pantoea stewartii*, *Pantoea dispersa*, and *Enterobacter cloacae*; ranked first, eighth and fifth, respectively, in dominance (Table 4). All morphotype groups identified as *Pantoea* spp. were yellow pigmented colonies distinguishable from one another by slight differences in shade and fullness of yellow pigmentation. The morphotype group of *Pantoea stewartii* LMG 20115 was found in all samples and displayed

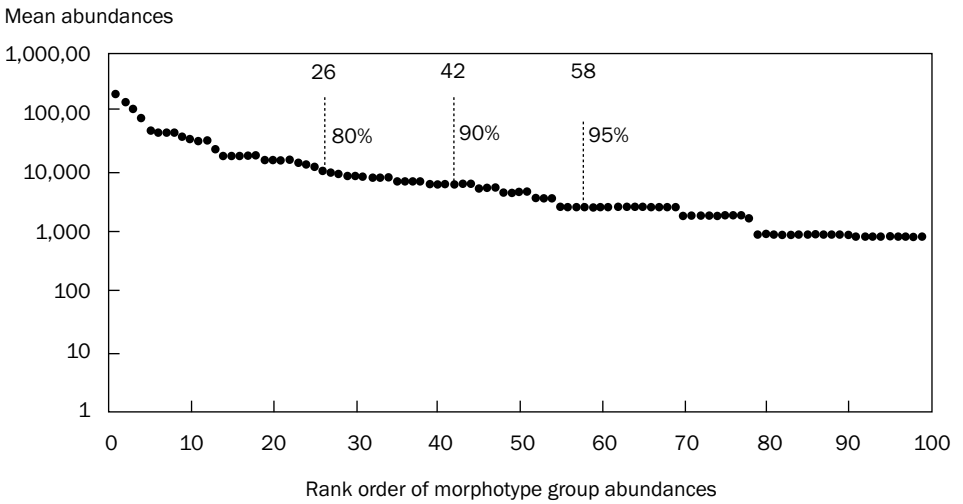

Fig. 1. Rank-abundance curve of bacterial morphotype groups, averaged over 12 samples, from rice seed of cv. PSBRc14 in irrigated farmers' fields at Jalajala in southern Luzon, Philippines (99 morphotype groups, 392 isolates representing a total mean abundance of 1.24×10^6 CFU/ml wash from 50-g seed). Dots represent mean abundances of each morphotype group, taken over 12 samples, plotted against the rank mean abundance for the group on the x-axis. Dashed vertical lines are abundance thresholds that identify morphotype group ranks that captured 80, 90 and 95% of the total cultured abundance. The top row of numbers indicates the number of morphotype groups comprising each abundance threshold.

Table 4. Rank-dominance of common bacterial morphotype groups from rice seed of cv. PSBRc14 in irrigated farmers' fields at Jalajala in southern Luzon island, Philippines

Dominance rank	Morphotype group[a]	Rank[b]		
		Frequency of occurrence	Mean abundance	Dominance score[c]
1	*Pantoea stewartii* (LMG 20115)**	1	1	2
2	"*Curtobacterium flaccumfaciens*" (LMG 20194) **	1	3	4
3	*Pseudomonas aeruginosa* (LMG 20125) **	1	4	5
4	*Burkholderia glumae* (LMG 20138) **	5	2	7
5	*Enterobacter cloacae* (LMG 20117) **	3	6	9
6	*Actinomycetes* spp.	5	5	10
7	*Pseudomonas oryzihabitans* (LMG 20126)	2	9	11
	Bacillus pumilus (LMG 20162) **	4	7	11
8	*Pantoea dispersa* (LMG 20116) **	2	10	12
9	*Cellulomonas flavigena* (LMG 20188) **	5	8	13
	Curtobacterium flaccumfaciens (LMG 20196)	6	7	13
10	*Xanthomonas* spp. (LMG 20137) **	3	13	16
11	*Bacillus subtilis* (LMG 20163) **	5	12	17
	Staphylococcus gallinarum (LMG 20176)	6	11	17
12	*Staphylococcus simulans* (LMG 20177)	6	13	19
13	*Clavibacter michiganense* (LMG 20187) **	4	16	20
	Pseudomonas stutzeri, B (LMG 20127) **	5	15	20
	Clavibacter michiganense (LMG 20195) **	6	14	20
	Micrococcus luteus (LMG 20178)	6	14	20
	"*Arthrobacter citreus*" (LMG 20208)	7	13	20
14	*Microbacterium* sp. (LMG 20189)	7	15	22
15	*Curtobacterium flaccumfaciens* (LMG 20197)	8	16	24
16	"*Paenibacillus polymyxa*" (LMG 20164) **	5	20	25
	Clavibacter michiganense (LMG 20198) **	8	17	25
17	*Kocuria kristinae* (LMG 20179)	8	18	26
18	*Burkholderia gladioli* (LMG 20145) **	8	19	27

[a]The 26 morphotype groups that fell within the 80% abundance threshold (Fig. 1) were sorted in descending order of dominance. Morphotype group identifications in quotation marks were obtained with a similarity index value < 0.50 to the profile in the FAME-MIS database. Numbers in parenthesis refer to LMG accession numbers of representative isolates deposited in the BCCM/LMG Bacteria Collection, Gent, Belgium. Morphotype groups marked with ** shared at least one population (i.e., BOX-PCR FPT) in common with populations found in seed samples from six rice cultivars in rainfed lowlands of Iloilo island, Philippines (5).

[b]Frequency of occurrence ranks represent the number of samples from which the morphotype group was found: 1, all 12 samples; 2, nine samples; 3, eight samples; 4, seven samples; 5, six samples; 6, five samples; 7, four samples; 8, three samples. Mean abundance ranks represent morphotype group abundances ranging from 10^4 to $2x10^5$ CFU/ml wash from 50-g seed.

[c]Dominance score was calculated as the sum of ranks for frequency of occurrence and abundance of each morphotype group.

yellow colonies with a convex elevation, entire margins and a glossy surface with strongest yellow color in the center (Fig. 2, A). Biolog analysis resulted in identifications with low similarity value indices to *Pantoea agglomerans*, while FAME-MIS yielded alternate identifications to *Pantoea ananatis* and *Pantoea agglomerans*. When analyzed by BOX-PCR genomic fingerprinting and subjected to database comparison, these isolates were found to have genomic fingerprint patterns nearly identical to the type strain for *Pantoea stewartii* LMG 2715. BOX-PCR fingerprinting analysis distinguished three subgroups among the 23 isolates with linkage levels of 76, 79, and 84% similarity, respectively, interrelated at 72% similarity into a single FPT. The morphotype group of *Pantoea dispersa* LMG 20116 was found in nine samples and displayed pale yellow colonies with raised elevation, smooth margins, and contained inner granules visible under 12-power magnification (Fig. 2, B). Isolates identified as *Pantoea agglomerans* displayed similar colonies but the growth of *Pantoea dispersa* isolates on TSA medium was more thin and semi-liquid. BOX-PCR

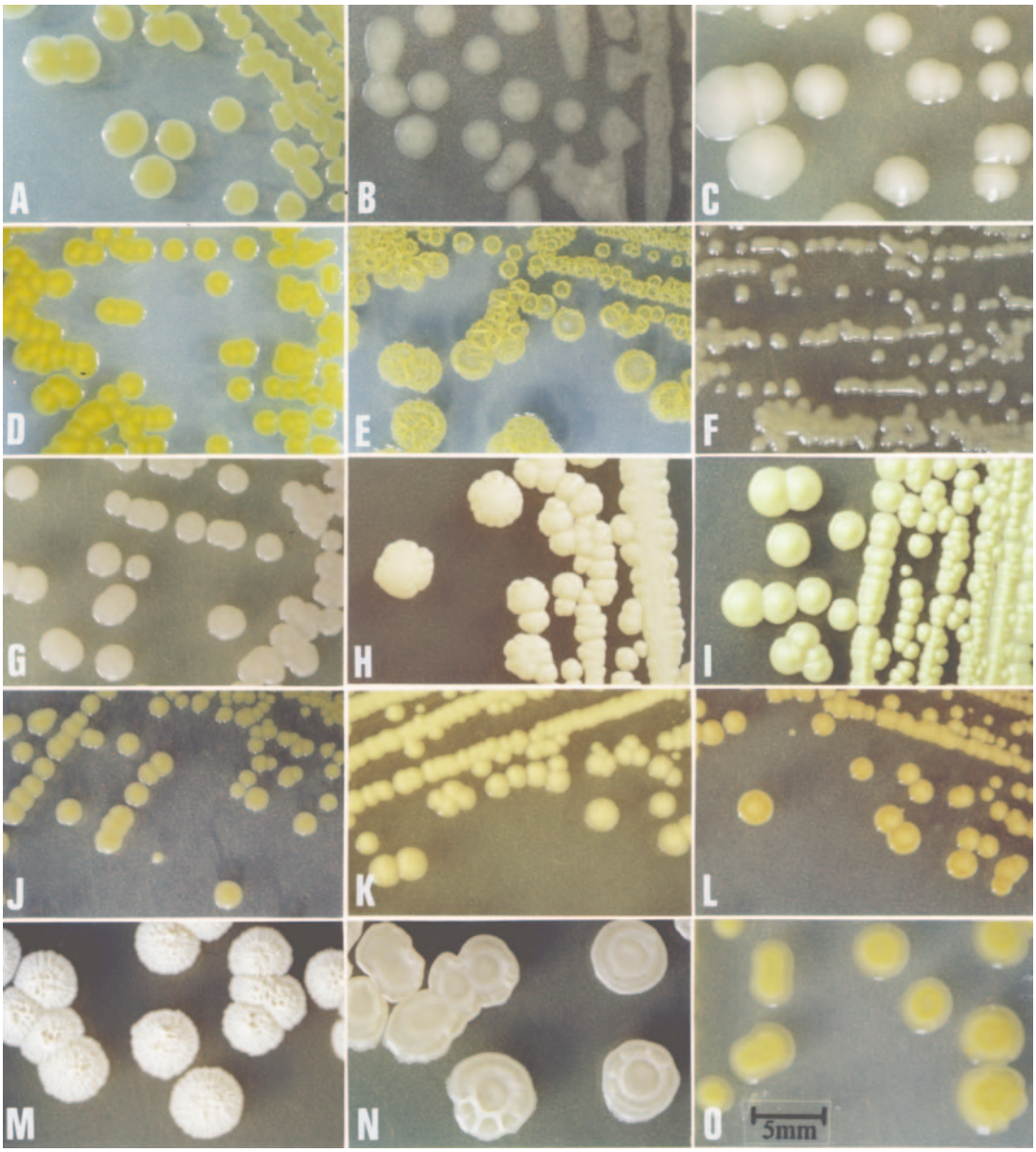

Fig. 2. Colony appearance of selected isolates representative for common bacterial morphotype groups from rice seed of cv. PSBRc14. A, *Pantoea stewartii* LMG 20115; B, *Pantoea dispersa* LMG 20116; C, *Enterobacter cloacae* LMG 20117; D, *Pseudomonas aeruginosa* LMG 20125; E, *Pseudomonas oryzihabitans* LMG 20126; F, *Xanthomonas* sp. LMG 20137; G, *Burkholderia glumae* LMG 20138; H, *Staphylococcus gallinarum* LMG 20176; I, *Micrococcus luteus* LMG 20178; J, *Curtobacterium flaccumfaciens* LMG 20194; K, *Clavibacter michiganense* LMG 20187; L, *Cellulomonas flavigena* LMG 20188; M, *Bacillus pumilus* LMG 20162; N, *Bacillus subtilis* LMG 20163; and O, *Paenibacillus polymyxa* LMG 20164. Cultures were grown for 4 days at 28°C on tryptic soy agar medium and photographed against a gray background with a Nikon Micro-Nikkor 55 mm (1:2.8) lens under daylight. LMG numbers refer to the accession number of deposited isolates in the BCCM/LMG Bacteria Collection, Gent, Belgium.

analysis differentiated three FPTs among the 15 *Pantoea dispersa* isolates linked at a level of 54% similarity. The FPT of LMG 20116 represented 11 isolates from seven samples and was highly similar to the pattern generated for the *Pantoea dispersa* type strain LMG 2603. The morphotype group of *Enterobacter cloacae* LMG 20117 was found in eight samples and displayed larger greenish-white colonies with convex elevation and smooth margins (Fig. 2, C). BOX-PCR analysis indicated a high genetic variation among the 12 isolates differentiated into five FPTs linked at a low level of 35% similarity. One FPT defined for three isolates, distinct from the FPT of LMG 20117, was nearly identical to the pattern generated for the *Enterobacter cloacae* type strain LMG 2783.

Predominant pseudomonads were nonfluorescent isolates classified into two morphotype groups identified as *Pseudomonas aeruginosa* and *Pseudomonas oryzihabitans*; ranked third and seventh, respectively, in dominance (Table 4). The two morphotype groups were only distantly related at a level of 15% similarity based on BOX-PCR fingerprinting. The morphotype group of *Pseudomonas aeruginosa* LMG 20125 was found in all samples and displayed chrome yellow colonies with convex elevation, smooth margins, and a glossy surface; however, some colonies may also wrinkle (Fig. 2, D). No reliable identification for these isolates was obtained in Biolog. The identification as *Pseudomonas aeruginosa*, though obtained with high similarity value indices (0.8 to 0.9) to the profile in the commercial database of FAME-MIS, remained suspicious because these isolates were negative for Kovac's oxidase reaction, nonfluorescent, and atypical in colony appearance. BOX-PCR fingerprinting analysis distinguished five subgroups (FPTs) among the 22 isolates with linkage levels of 70, 72, 75, 78, and 70% similarity, respectively. Four FPTs were interrelated at a level of 53% similarity and linked to a fifth FPT, comprising only two isolates, at a level of 38% similarity. The FPT of LMG 20125 represented seven isolates from five samples. The morphotype group of *Pseudomonas oryzihabitans* LMG 20126 was found in nine samples and displayed lighter chrome yellow pigmented colonies adherent to the medium and with a characteristic wrinkled appearance (Fig. 2, E). BOX-PCR fingerprinting analysis differentiated three FPTs among the 10 isolates linked at a level of 57% similarity. The FPT of LMG 20126 represented four isolates from four samples and was nearly identical to the pattern generated for the *Pseudomonas oryzihabitans* type strain LMG 7040.

Other bacteria regularly present were *Burkholderia glumae* and saprophytic xanthomonads. The morphotype group of *Xanthomonas* sp. LMG 20137 was found in eight samples and ranked tenth in dominance (Table 4). Colonies were glossy pale yellow with a convex elevation, smooth margins, and became mucoid after 3 days of incubation at 28°C (Fig. 2, F). They contained the three fatty acids characteristic for the genus *Xanthomonas* (49): 11:0 iso, 11:0 iso 3OH and 13:0 iso 3OH but were not further identified to species level by FAME-MIS. Biolog analyses yielded identifications as *X. campestris* pv. *dieffenbachiae*, *X. campestris* pv. *strelitzia*, and *X. oryzae* pv. *oryzae* F. They did not cause disease symptoms when artificially inoculated into the rice plant. BOX-PCR fingerprinting analysis differentiated nine FPTs among the 10 isolates linked at a low level of 33% similarity. Only the FPT of LMG 20137 was found in two samples. The morphotype group of *Burkholderia glumae* LMG 20138 was found in six samples and ranked fourth in dominance (Table 4). Their whole-cell protein profiles matched

the profile of *B. glumae* in the *Burkholderia* database generated by Vandamme et al. (44). The colonies were ivory yellow in pigmentation, convex in elevation, smooth with narrow translucent margins, and glossy in appearance (Fig. 2, G). No apparent yellow-green diffusible pigments were produced on TSA medium, unlike for the *Burkholderia gladioli* isolates. BOX-PCR fingerprinting analysis revealed the 13 isolates to be comprised of a homogeneous population represented by a single FPT that was nearly identical to the pattern for the type strain of *B. glumae* LMG 2196.

Gram-positive bacteria consisted of cocci, coryneform bacteria, endospore-forming rods, and *Actinomycetes* spp. The 18 *Actinomycetes* isolates produced an aerial mycelium and were not further identified; as group they ranked sixth in dominance (Table 4). Only 20 % of the cocci yielded FAME-MIS identifications with a similarity value index > 0.5; the remaining cocci were not identified or were named with low similarity value indices to other species within a same genus. Predominant cocci found in five samples were *Staphylococcus gallinarum*, *Staphylococcus simulans* and *Micrococcus luteus*; ranked eleventh, twelfth and thirteenth, respectively, in dominance (Table 4). The morphotype group of *Staphylococcus gallinarum* LMG 20176 displayed pale yellow orange colonies, raised in elevation, with lobate margins, and a dull surface (Fig. 2, H). BOX-PCR analysis revealed disparate genomic profiles among the seven isolates linked at a level of 22% similarity. The morphotype group of *Staphylococcus simulans* LMG 20177 displayed grayish white colonies with low convex elevation, smooth margins, and a glossy surface (not shown). BOX-PCR analysis differentiated five FPTs among the 12 isolates linked at a level of 35% similarity. All *Staphylococcus* isolates exhibited spherical cells occurring singly or in irregular clusters, while *Micrococcus luteus* isolates displayed spherical cells occurring in tetrades or cubical packets. The morphotype group of *Micrococcus luteus* LMG 20178 displayed sulphur yellow pigmented colonies with a drop-like elevation, smooth margins, and a dull surface (Fig. 2, I). BOX-PCR analysis differentiated four FPTs linked at a level of 51% similarity. The FPT of LMG 20178 represented three isolates; the other three FPTs were defined for single isolates.

Among the 114 coryneform isolates, 83 isolates representative for 22 morphotype groups shared a coryneform morphology without marked rod-coccus cycle. Two morphotype groups of distinct whitish colonies were identified as *Microbacterium esteraromaticum* and *Microbacterium barkeri* (Table 3). The other 20 morphotype groups comprised various yellow pigmented colonies classified according to colony pigmentation into shades of translucent yellow, shades of reddish yellow, and a smaller group of diverse greenish yellow colonies (Table 3). The main groups of translucent yellow and reddish yellow colonies, represented by 41 and 27 isolates, respectively, were each comprised of seven morphotype groups. The two groups were homogeneous on the basis of fatty acid composition, however, revealed a high heterogeneity across morphotype groups on the basis of BOX-PCR fingerprinting reflected by overall linkage levels of 17 and 22% similarity, respectively. The morphotype group represented by LMG 20194 among the translucent yellow colonies, was found in all samples and ranked second in dominance (Table 4). Colonies were translucent pale olive yellow with raised to low convex elevation, smooth margins, and a stronger yellow color in the center (Fig. 2, J). FAME-MIS yielded first choice identifications to *Bacillus coagulans* with similarity value

indices ranging from 0.51 to 0.65, and second choice identifications with low similarity value indices (0.36 to 0.47) to *Curtobacterium flaccumfaciens*. BOX-PCR analysis differentiated three FPTs among the 18 isolates linked at a level of 64% similarity. One FPT represented 15 isolates from 10 samples, a second FPT represented two isolates from two samples, and a third FPT was defined for a single isolate. The morphotype group represented by isolate LMG 20187 among the reddish yellow colonies, was found in seven samples and ranked thirteenth in dominance (Table 4). The colonies were light apricot yellow with convex elevation, smooth translucent margins, and a glossy surface (Fig. 2, K). FAME-MIS yielded alternate first choice identifications to *Clavibacter michiganense*, *Kocuria varians*, or *Microbacterium* spp. BOX-PCR analysis differentiated three FPTs among the nine isolates linked at a level of 55% similarity. The FPT of LMG 20187 represented five isolates from five samples, a second FPT represented three isolates from two samples, and a third FPT was defined for a single isolate. Another common morphotype group represented by LMG 20188, was found in six samples and ranked ninth in dominance (Table 4). The colonies were light reddish yellow with low convex elevation, smooth margins, a glossy surface, and typically turned paler yellow in the margin and center (Fig. 2, L). These isolates were equally identified in FAME-MIS to *Cellulomonas flavigena* and *Paenibacillus macerans* with a numerical difference of less than 0.1 between first and second choice similarity index value. BOX-PCR analysis differentiated four FPTs among the eight isolates linked at a level of 52% similarity.

Thirty-one isolates exhibited a rod-coccus cycle; young cultures (1 to 2 days old) occurred as diphteroidal rods or as filaments that fragmented into rods and coccoid forms, while older cultures (3 to 5 days old) were mainly composed of coccoid cells. FAME-MIS yielded identifications to *Exiguobacterium acetylicum*, *Arthrobacter* spp., and *Brevibacterium* spp.; however, no reliable identifications were obtained for 16 isolates comprised within four morphotype groups.

Predominant endospore-forming bacteria were *Bacillus pumilus*, *Bacillus subtilis*, and isolates thought to be *Paenibacillus polymyxa*; ranked seventh, eleventh and sixteenth, respectively, in dominance (Table 4). The morphotype group of *Bacillus pumilus* LMG 20162 was found in seven samples, and displayed slightly yellowish white colonies with a low convex elevation, ciliated margins, and a wrinkled surface (Fig. 2, M). BOX-PCR analysis differentiated five FPTs among the 15 isolates with four FPTs delineated for single isolates. The FPT of LMG 20162 represented 11 isolates from 6 samples. The morphotype group of *Bacillus subtilis* LMG 20163 was found in six samples, and displayed grayish-to-brownish white colonies, flat in elevation, with irregular raised margins, and a rough surface (Fig. 2, N). BOX-PCR analysis differentiated eight FPTs among the 11 isolates linked at a low level of 26% similarity. The morphotype group represented by isolate LMG 20164 was found in six samples, and displayed yellow colonies that were flat in elevation with stronger yellow pigmentation in the center and translucent mucoid margins (Fig. 2, O). FAME-MIS yielded alternate identifications with low similarity value indices (0.26 to 0.42) to *Paenibacillus polymyxa* and *Cellullomonas turbata*. BOX-PCR analysis differentiated six FPTs among the nine isolates linked at a low level of 30% similarity.

The analysis of the 106 isolates obtained from three arbitrarily chosen germinated

seed samples suggested germination to result in a qualitative and quantitative shift in the bacterial community, especially in a decrease of gram-positive populations (Table 3). Gram-negative morphotype groups that were dominant in the 12 dry seed samples persisted on the germinated seed, moreover, fewer but identical populations of these morphotype groups were recovered after seed germination. Morphotype groups that were initially not found or in low populations on the dry seed, but clearly increased during seed germination belonged to fluorescent pseudomonads, *Chryseobacterium indologenes*, *Acinetobacter calcoaceticus*, *Stenotrophomonas maltophilia*, and helical bacteria. Four distinct morphotype groups among fluorescent pseudomonads were identified as *Pseudomonas putida*, and three distinct morphotype groups as *Pseudomonas aeruginosa* (Table 2). Only isolate LMG 20134 displayed the typical colony morphology of *Pseudomonas aeruginosa*, and revealed a BOX-PCR pattern nearly identical to the pattern of the type strain of *Pseudomonas aeruginosa* LMG 1242.

Comparison of bacterial populations between two rice environments. Comparative analysis of the BOX-PCR FPTs with those previously defined for rice seed bacteria from six cultivars in rainfed lowlands of Iloilo island, Philippines (5), revealed the presence of common populations between the two bacterial collections. Forty-two (15%) of the 284 FPTs matched 47 (31%) of the previously defined 151 FPTs with a value above 70% similarity. The discrepancy in number of matching FPTs was due to the different choice of cutoff level used to define a FPT in the two studies. Eighteen shared FPTs were found in a single sample, 17 shared FPTs were found in two to four samples, and seven shared FPTs were found in five to all 12 samples. Thus, except for the FPT of *Methylobacterium radiotolerans* LMG 20139, all frequently found FPTs in this study were shared by the collection from rainfed rice (5) and likewise represented more frequently found populations. These 42 shared FPTs belonged to 32 morphotype groups (Table 2 and 3). Only four shared FPTs belonged to rare morphotype groups found in one sample. The majority of shared FPTs (26, or 62%) belonged to 16 of the 26 morphotype groups that fell within the 80% abundance threshold, established in the rank-abundance curve (Fig. 1), and ranked in descending order of dominance in Table 4. All morphotype groups found in six or more samples had at least one population (FPT) in common with the bacterial collection from the rainfed environment, except for the groups of *Pseudomonas oryzihabitans* and *Actinomycetes* spp.

Pathogenicity tests. Among the 498 isolates, 20 isolates (4%) caused disease symptoms on all 12 seedlings that were inoculated, 36 isolates caused symptoms on 6 to 11 of inoculated plants. The remaining 442 isolates did not induce any symptoms, or induced a hypersensitive-like reaction localized at the point of inoculation. Thirteen of the 20 pathogenic bacteria were identified as *Burkholderia glumae*, five as *Burkholderia gladioli*, and two as *Acidovorax avenae* subsp. *avenae*. The *Burkholderia glumae* isolates induced sheath necrosis and chlorosis of the leaves within three days after inoculation; followed by withering and soft rot of the whole seedling. The reactions induced by the *Burkholderia gladioli* isolates were similar to those caused by the *Burkholderia glumae* isolates, except for two isolates that did not cause withering. The *Acidovorax avenae* subsp. *avenae* isolate LMG 20151 caused a distinct brown stripe on the leaf sheath that usually extended further up into

the leaf blade leading to browning and withering of the entire leaf blade.

Thirty-six isolates (7%) identified as *Bacillus pumilus*, *Paenibacillus polymyxa*, *Paenibacillus lentimorbus*, *Burkholderia cepacia*, fluorescent pseudomonads, and *Pantoea stewartii* affected only 50 to 90% of the 12 inoculated seedlings and therefore, were considered pathogens with low disease potential. Eleven of the 15 *Bacillus pumilus* isolates induced symptoms on 10 to 12 seedlings; the other four isolates induced symptoms on 7 to 9 of inoculated seedlings. The infection was rapid and elongated brown streaks were produced on the sheath within three days, the emerging leaf became grayish-green and curled, chlorosis appeared along the margin of the third leaf and brownish spots with a gray center that coalesced into larger blotches developed on the leaf blade. After 10 days, the affected leaves became withered and dry. The five *Burkholderia cepacia* isolates produced a brown streak that extended throughout the entire leaf sheath and often further up into the leaf blade on 7 to 10 of the 12 inoculated seedlings. Three fluorescent pseudomonads identified as *P. aeruginosa* and *P. putida* induced symptoms on 10 to 11 of the 12 inoculated seedlings. The *P. aeruginosa* isolates LMG 20133 and LMG 20134 produced elongated brown lesions on the sheath and chlorosis along the margins of the third leaves, which became brown and dried. The *P. putida* isolate LMG 20136 produced a distinct reddish brown stripe that extended throughout the entire leaf sheath and further up into the midrib of the leaf blade. Seven of the nine *Paenibacillus polymyxa* isolates induced moderate symptoms on 8 to 10 of the 12 seedlings that were inoculated; two isolates did not elicit any reactions. They caused elongated water-soaked greenish-brown lesions on the sheath and the newly emerged leaf became dark green, curled, and withered. The two *Paenibacillus lentimorbus* isolates produced similar symptoms on 10 of the 12 inoculated seedlings. Only three of the 23 *Pantoea stewartii* isolates induced moderate sheath browning on six of the 12 seedlings that were inoculated. Clearly, further fine characterization of these isolates and their pathogenic potential is needed.

Antifungal activity. All 498 bacterial isolates were tested in vitro for inhibition of mycelial growth of *Rhizoctonia solani* AG1, *Pyricularia grisea* PO6, *Fusarium moniliforme*, and *Sarocladium oryzae* by the dual culture test. None exhibited in vitro antifungal activity against *F. moniliforme* or *S. oryzae*. Twenty isolates (4%) inhibited the growth of *Pyricularia grisea* PO6, 19 isolates (4%) inhibited the growth of *Rhizoctonia solani* AG1, and 41 isolates (8%) inhibited the growth of both fungal pathogens (Table 5). Further research on their effect on seed germination, plant growth enhancement, and potential to suppress blast disease in the field is in progress.

Discussion

The composition of the bacterial community associated with seed of a single rice genotype collected from farmers' fields at one location of irrigated lowland in southern Luzon island, Philippines, was assessed by phenotypic classification of isolates into morphotype groups and by genomic DNA fingerprinting analysis to evaluate genetic variation among isolates. Isolations and enumeration of all visually distinct colonies from each sample by dilution plating of seed washes yielded 392 isolates from 12 samples and 106 isolates from three arbitrarily chosen germinated samples. The isolates were assumed to be representative for the morphological diversity observed on isolation plates. It was often difficult to

Table 5. Bacteria from seed of rice cv. PSBRc14 that suppressed in vitro the mycelial growth of the rice pathogens *Rhizoctonia solani* AG1 and *Pyricularia grisea* P06

Identification[a]	No. of isolates		Inhibition zone (mm)[b]	
	Tested	Antagonists	*R. solani*	*P. grisea*
Enterobacteriaceae				
Pantoea stewartii	23	3	-	18.3
		2	8.2	-
		1	14.7	26.7
Enterobacter cloacae	12	1	7.7	26.3
Pantoea dispersa	15	3	-	18.1
		1	8.0	-
		1	7.7	29.0
Enterobacter agglomerans biogroup 3B	4	2	7.0	-
Klebsiella mobilis	2	1	14.3	31.0
Pantoea agglomerans	3	1	13.7	36.7
Pseudomonads				
Nonfluorescent *Pseudomonas aeruginosa*	22	2	10.2	18.5
		1	-	25.0
		1	10.0	-
Pseudomonas oryzihabitans	10	3	-	19.8
Pseudomonas stutzeri	7	1	-	15.0
Pseudomonas putida	9	9	13.6	28.5
Other gram-negative aerobic rods				
Burkholderia glumae	13	4	6.1	19.3
		2	3.1	-
		2	-	12.8
		1	6.3	-
Xanthomonas spp.	10	1	9.0	-
Agrobacterium radiobacter	5	1	10.0	-
"Sphingomonas capsulata"	4	1	11.0	-
Acinetobacter calcoaceticus/baumannii	6	1	12.3	27.0
Acinetobacter sp.	1	1	-	17.3
Gram-positive cocci				
Staphylococcus simulans	12	3	12.2	22.6
Staphylococcus arlettae	4	1	-	23.7
Micrococcus lylae	3	1	14.0	25.0
		1	-	22.3
Coryneform bacteria				
"Curtobacterium flaccumfaciens"	18	1	9.7	27.7
"Arthrobacter citreus"	5	1	-	11.7
Microbacterium esteroaromaticum	4	2	7.7	-
Brevibacterium epidermidis	7	1	14.3	41.0
Unidentified *Coryneform* sp.	1	1	-	11.0
Endospore-forming rods				
Bacillus pumilus	15	1	7.0	-
		1	-	30.0
Bacillus subtilis	11	10	12.0	25.0
Bacillus cereus	7	1	14.7	29.7
		1	8.7	-
Bacillus sphaericus	8	1	-	13.7
		1	12.3	-
Paenibacillus lentimorbus	2	1	10.7	21.3
		1	17.3	-
Actinomycetes *spp.* with aerial mycelium	*18*	*2*	*12.3*	*34.5*
		1	7.0	-

[a]Identifications in quotation marks were obtained in FAME-MIS with a similarity index value <0.5.
[b]Values represent the mean diameter of mycelial growth inhibition zones on *Rhizoctonia solani* AG1 and *Pyricularia grisea* P06 for three replicates measured after 3 days of incubation at 28°C. Inhibition was determined on pigment production medium.

draw clear lines of distinction between single colonies on the isolation plates, hence, after comparison of pure cultures it was noticed that some colony types had been isolated more than once per sample. The classification of isolates into morphotype groups based on similarities in cellular characteristics, whole-cell fatty acid composition and colony appearance, supported the observation that there is a high morphological diversity of rice seed-associated bacteria.

A rank-abundance curve was applied to the morphotype groups defined for the isolates found in 12 dry seed samples to analyze the bacterial community structure. Twenty-six morphotype groups captured 80% of the total cultured abundance. Over half of the morphotype groups (53 of 99) recorded for the 12 samples were rare groups that comprised nearly 10% of the total cultured abundance. The use of abundance thresholds (i.e., number of morphotype groups that constitute 80, 90, or 95% of total abundance) provide practical guidelines for determining which and how many taxa an ecologist could focus on in future community-level investigations. Abundance and frequency of occurrence were used as measures of morphotype group dominance. The dominance ranking of morphotype groups that fell within the 80% abundance threshold, established in the rank-abundance curve (Fig. 1), indicated the morphotype group of *Pantoea stewartii* to be dominant and the majority of common groups (17 of 26) to be gram-positive bacteria that mainly represented *Coryneform* spp. (Table 4). FAME-MIS and Biolog analyses were used for species identification of the isolates.

The BOX-PCR genomic fingerprinting complemented the phenotypic approaches to classify and assess the diversity among isolates. The efficacy of the repetitive DNA-based polymerase chain reaction genomic fingerprinting technique to assess the genetic diversity within bacterial populations has been demonstrated (8,12,21,46). BOX-PCR analysis differentiated 284 FPTs among 498 isolates from seed of a single rice genotype. The majority of FPTs (78%) was not shared among samples, while seven FPTs (3%) were shared among five to ten samples, and one FPT was found in all 12 samples. This supports the earlier finding on the variability of populations among seed samples from six rice cultivars (5), and further suggests that there is a high genetic variability in bacterial populations among samples from different farmers' fields. The number of FPTs differentiated among isolates of a particular morphotype group together with their distribution among the seed samples indicates that phenotypic similar isolates can differ in genomic fingerprint profiles from one sample to the next. The linkage level of BOX-PCR patterns generated for isolates within each morphotype group is an indication for the degree of genetic variation within defined morphotype groups. Linkage levels above 50% similarity were shown for 61 (60%) of the 101 defined morphotype groups. Twenty-one morphotype groups (21%) were defined for single isolates. Considerable genetic variability, as reflected by linkage levels below 50% similarity, was found within 19 morphotype groups (19%). The taxonomic level of this genetic variation among phenotypic related isolates, however, remains to be answered. It is known that the resolution level of the rep-PCR technique allows the differentiation of bacteria at the species, subspecies or strain levels (46). Further, the degree of genetic variability found within bacterial taxonomic groups based on genomic profiles from PCR amplification of repetitive elements has been demonstrated to vary considerably depending on the taxon (5, 12,

21, 22). Nevertheless, conclusions concerning the phylogenetic relatedness of isolates with low similarity in BOX-PCR patterns require other genetic tools such as sequencing of 16S rDNA and genomic DNA-DNA hybridization analyses (22, 45, 47).

The analysis of populations from three germinated samples suggested seed germination to result in a qualitative and quantitative shift in the bacterial community, especially in a decrease of gram-positive populations; however, dominant gram-negative populations appeared to persist on the germinated seed.

Comparative analysis of the BOX-PCR FPTs with those previously defined for rice seed-associated bacteria from six cultivars in rainfed lowlands of Iloilo island, Philippines (5), revealed that 42 FPTs (15%) were shared in common by the two bacterial collections. The shared FPTs represented single as well as frequently found populations. Certain populations that were frequently found in the samples from both rice environments appeared to be shared in common between different rice cultivars and different geographical locations, therefore might represent common rice seed-associated populations. At least one population within all common morphotype groups that were found in six or more samples was shared between the two bacterial collections, except for the groups of *Pseudomonas oryzihabitans* and *Actinomycetes* species. Colonies of *Actinomycetes*, however, were observed and picked on the isolation plates after seven days of incubation; and this might have been the reason for their absence in our previous study that performed isolations after four days of incubation.

Predominant *Enterobacteriaceae* were *Pantoea stewartii*, *Pantoea dispersa*, and *Enterobacter cloacae*. A similar predominance of *Pantoea* spp. was found associated with rice seed in our previous study (5). Whereas whole-cell fatty acid analysis did not provide a clear distinction among *Enterobacteriaceae*, they were differentiated into distinct morphotype groups recognizable by colony appearance in this study. Although *Pantoea* spp. are ubiquitous epiphytes on most plant species, the rice seed may provide a more selective environment for certain *Pantoea* spp. than that provided by other plant surfaces. In the past, only *Erwinia herbicola* (subjective synonym of *Pantoea agglomerans*) (10) is reported to be frequently isolated from rice seed (3, 25, 29, 50).

Dominant pseudomonads were nonfluorescent isolates identified as *Pseudomonas aeruginosa* and *Pseudomonas oryzihabitans*. The two morphotype groups displayed rather similar colonies but were only distantly related on the basis of BOX-PCR fingerprinting analysis. The identification of the main group as *P. aeruginosa*, though obtained with high similarity value indices in FAME-MIS, remains questionable. Identical populations were also commonly found in the seed samples from the rainfed rice environment (5), whereas the populations of *P. oryzihabitans* appeared to be unique for the present samples from the irrigated rice environment. Other nonfluorescent pseudomonads regularly found were *Pseudomonas stutzerii*, recognizable by a distinctive colony appearance. Fluorescent pseudomonads were negligible on the dry seed, but appeared to proliferate on the germinated seed. Pseudomonads can be commonly isolated from rice seed (6, 25, 50); however, most of them are considered saprophytes and have not been given much attention in the past. The rice pathogens *Pseudomonas fuscovaginae* (26) and *Pseudomonas syringae* pv. *oryzae* (19) were not found in the samples. Several seedborne pathogens formerly described as *Pseudomonas* spp. (2, 41, 42) have been

reclassified into new genera by recent taxonomic revisions (17).

Xanthomonas-like bacteria, which do not cause apparent disease symptoms when artificially inoculated into the rice plant, were frequently found in the seed samples though in low populations. This supports previous findings on the association of nonpathogenic xanthomonads with rice seed (5, 25). They appeared to be comprised of genetically heterogeneous organisms on the basis of BOX-PCR genomic fingerprinting. Their ecological role in the microbial seed community and their relationship to characteristic pathogenic *Xanthomonas* needs to be further investigated. *Xanthomonas*-pathogens of rice include *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, causing bacterial blight and bacterial leaf streak, respectively (40). Direct isolation of *X. oryzae* pv. *oryzae* from seed has been difficult to demonstrate, and the widespread occurrence of nonpathogenic xanthomonads may have contributed to the thought that *X. oryzae* pv. *oryzae* is seed transmitted (25).

Gram-positive bacteria of rice seed have been largely neglected in the past; however, they accounted for 48% of the total number of isolates and represented 53% of the total number of defined BOX-PCR FPTs. They consisted of cocci, coryneform bacteria, endospore-forming rods, and *Actinomycetes* species with an aerial mycelium. Regularly present cocci were *Staphylococcus* spp. and *Micrococcus luteus*. Their ecological role in the microbial seed community is unknown, yet 14% of cocci inhibited in vitro the mycelial growth of the rice pathogens *Rhizoctonia solani* AG1 and *Pyricularia grisea* PO6. Coryneform bacteria appeared to be most prevalent and exhibited a variety of yellow, reddish yellow and greenish yellow pigmented colonies. They were also commonly found in the seed samples from the rainfed rice environment (5). The usage of the term "coryneform" follows that of Davis (7) in that it describes gram-positive, non-acid fast, non-spore-forming, pleomorphic, rod-shaped bacteria without regard to their generic classification. FAME-MIS often yielded ambiguous identifications, or no identification of these coryneform bacteria. Colonies with similar pigmentation, distinguishable from one another by slight differences in shade and fullness of pigmentation, shared a similar fatty acid composition but clearly formed different genotypic groups on the basis of BOX-PCR fingerprinting. Although the usefulness of colony appearance in classification is largely unknown, Seiler (37) also found it to be a useful characteristic in differentiating coryneform bacteria. Their presence and ecological role in the microbial seed community of rice is largely unknown. Several coryneform genera include plant pathogens. Although none of the isolates expressed pathogenic potential on rice in our pathogenicity tests, a recent report (15) claiming a coryneform bacterium to be the causal organism for red stripe of rice, suggests that they may represent an unknown source of disease potential. Five percent of coryneform isolates inhibited in vitro the mycelial growth of *Rhizoctonia solani* AG1 and *Pyricularia grisea* PO6; hence they may as well represent an untapped resource of beneficial properties.

Predominant endospore-forming bacteria were *Bacillus pumilus*, *Bacillus subtilis*, and *Paenibacillus polymyxa*. Strains of these species have been reported as putative endophytes of rice seedlings (27, 39). *Bacillus pumilus* and *Bacillus subtilis* were also commonly found in the seed samples from the rainfed rice environment, while *Paenibacillus polymyxa* was previously only sporadically found (5).

Pathogenic bacteria, which caused apparent symptoms on all plants inoculated, accounted for 4% of isolates and were

identified as *Burkholderia glumae* (65%), *Burkholderia gladioli* (25%), and *Acidovorax avenae* subsp. *avenae* (10%). The results corroborate our previous findings on pathogens associated with rice seed (5), and imply that *Burkholderia glumae* and *Burkholderia gladioli* are prevalent seedborne pathogens in the tropical irrigated and rainfed rice environment. *B. glumae*, the bacterial cause of grain rot (11) and seedling rot (42), has become the most important bacterial pathogen of rice in Japan since the adoption of mechanical transplanting of rice seedlings grown in nursery boxes. Reports on *B. gladioli* as a rice pathogen, however, are limited (5,43). The closely related rice pathogen *Burkholderia plantarii*, only recorded in Japan as the bacterial cause of rice seedling blight (2), was not found. *Acidovorax avenae* subsp. *avenae*, which causes brown stripe of rice, is known to occur in most rice growing countries (41). In addition, 7% of the isolates caused various symptoms of sheath necrosis and were different from 'true pathogens' on the basis of lower disease potential. They were related to *Bacillus pumilus*, *Paenibacillus* spp., *Burkholderia cepacia*, *Pseudomonas* spp. and *Pantoea stewartii*. Similar isolates were also found in the seed samples from six rice cultivars from the rainfed environment (5). So far we have not observed the described symptoms, artificially induced by inoculation, on rice crops in the field. We assume that eventually they could emerge as pathogens under specific rice cropping practices. Furthermore, the choice of inoculation method may have influenced the expression of their pathogenicity, or they might only show disease potential when present at high inoculum concentration. Nevertheless, they deserve further investigation. Strains with a similar low disease potential have been reported for *Erwinia herbicola* causing palea browning of rice (3), and several saprophytic *Pseudomonas* spp. associated with the sheath rot syndrome and grain discoloration of rice (6, 50).

A variety of bacterial species, accounting for 16% of the total number of isolates, exhibited in vitro mycelial growth inhibition of the rice pathogens *Rhizoctonia solani* AG1 and *Pyricularia grisea* PO6. The potential to suppress fungal growth was common among isolates of *Bacillus subtilis*, *Paenibacillus lentimorbus*, *Pseudomonas putida*, *Klebsiella mobilis*, and *Burkholderia glumae*. Strains of *Burkholderia glumae* are also known to produce antibiotics and have been explored as biocontrol agent against *Ralstonia solanacearum* on tomato (9). The species is, however, a pathogen of rice and indicates the importance of determining the interactions between antagonistic bacteria, the plant host, and the target pathogen. The antagonistic activity of these bacteria in the seed environment may partially allow rice seed to resist fungal attack on the plant and in the soil environment. The high genetic diversity of antagonistic bacteria associated with rice seed complicates the selection of strains, or may as well emphasize the use of strain mixtures for devising biocontrol strategies. Bacterial antagonism toward fungal pathogens of rice has been reported for strains of *Bacillus subtilis* (24, 33), *Pseudomonas* spp. (24, 33, 34), *Enterobacter cloacae* (27), and *Pantoea agglomerans* (33).

In conclusion, the diversity of isolates illustrates the complex nature of bacteria associated with seed of a single rice genotype in farmers' fields. The classification of isolates according to morphotype groups and generated BOX-PCR fingerprints supported the observation that there is a high morphological and genetic diversity. The comparative analysis of BOX-PCR FPTs between two bacterial collections

from seed of different rice cultivars and two rice-cropping environments indicated that frequently occurring populations within dominant phenotypic groups are shared in common, and therefore may represent rice seed adapted bacteria. The bacterial community structure consisted of a large number of rare populations and few frequently occurring populations. The high variability in bacterial populations among samples may contain critical information about the dynamics of the seed microenvironment, which might be dictated by changing environmental conditions within the particular fields such as farmers' cropping practices. In order to attain better knowledge about the criteria that influence this variability in seed bacterial populations, it would be interesting to examine patterns of variation among farmers' fields, among seasons, or among agroecological environments for rice cultivation. This would require more intensive sampling and the application of appropriate statistical tools for microbial ecology. Despite its established value, the pure culture approach has many limitations and culturable bacteria probably only represent a fraction of the diversity actually present on the seed (1). Culture-independent molecular techniques based on the PCR-mediated amplification of rDNA sequences provide a way for fast comparative analyses of total bacterial community DNA among samples (28). The obtained information on the culturable bacterial communities of rice seed provides a framework that will aid in the selection of representative isolates or their DNA signature sequences for further ecological and taxonomic studies.

This study has provided an improved insight and an expanded list of the culturable bacteria associated with rice seed in the tropical environment. Detailed knowledge of the impact of the genetic variability of populations on their relevant properties (such as pathogenicity, antagonism and plant growth promotion), and further research to establish the prevalence of specific populations on other parts of the rice plant, is still much needed to devise efficient bacterial deployment strategies for plant health management.

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CHAPTER 3

RICE SEED-ASSOCIATED BACTERIA WITH PATHOGENIC POTENTIAL

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Interest in plant-associated bacteria has been largely centered on the bacteria that caused the various diseases. The economic impact of diseases on crop production continues to provide a major motivation for research on the phytopathogenic bacteria. Seedborne pathogens are of particular interest because of the need for seed purity in germplasm exchange, and the general concern that pathogens, or new virulent strains of a pathogen, might spread to other regions through the worldwide movement of seeds.

Due to rice cropping intensification in the past decades, several new or previously not considered important diseases have emerged as more threatening to rice productivity from the change of rice cultivation, cultivars, and other management practices. Among these diseases are bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*; grain rot, caused by *Burkholderia glumae*; and the sheath rot complex and grain discoloration, caused by a complex group of pseudomonads including *P. fuscovaginae*.

The first part of this chapter describes the occurrence of *Burkholderia* species as predominantly detected pathogenic bacteria on apparently healthy rice seeds. The second part of this chapter discusses the complex group of pathogenic bacteria involved in rice sheath rot and grain discoloration symptoms in the major rice-growing districts in the Philippines. And the last part of this chapter describes the development of a seed PCR-assay for the detection of *Xanthomonas oryzae* pv. *oryzae*, which is especially difficult to detect and diagnose in seed by culture-based methods

A. Pathogenic *Burkholderia* populations

The majority (90%) of true pathogenic bacteria obtained in the surveys made on the bacterial diversity of rice seed (see Chapter 2) were *Burkholderia* species identified as *B. glumae* and *B. gladioli*. *B. glumae* is a well-known rice pathogen causing both grain rot and seedling rot of rice. Bacterial grain rot was first reported in the Kyushu district of Japan by Goto and Ohata (1956). The pathogen was described as a new species, which was named *Pseudomonas glumae* (Kurita and Tabei 1967), and later reclassified as *Burkholderia glumae* (Urakami et al., 1994). Bacterial grain rot was initially a minor disease in Japan with limited areas affected until the early 1970s when the disease became prevalent in the southern and central regions of Japan. The increased incidence of rice grain rot coincided with the introduction of mechanical transplanting technology and the consequent shift from raising seedlings in traditional lowland nurseries to plastic nursery boxes specifically designed for the transplanting machines. In 1976, *B. glumae* was also identified as the causal agent of seedling rot, which became a serious problem of plants raised in nursery boxes used for machine transplanting (Uematsu et al., 1976). Seedling rot had rarely been observed in traditional lowland nurseries. The seedlings for machine transplanting are raised indoors under conditions of high seeding rates, high temperature and high humidity that are conducive to bacterial diseases. Seeds infected in the pre-emergence stage result in rotting before they can germinate, while seedlings raised from seeds infected during germination often rot and die. In the past two decades, *B. glumae* has emerged as the most important bacterial pathogen of rice in Japan, Korea and Taiwan, causing serious yield loss due to grain sterility or poor ripening. Although the pathogen has been implicated in the sheath rot complex and grain discoloration of rice in the tropics, the specific diseases of

grain rot and seedling rot caused by *B. glumae* are not considered major constraints to rice production in the Philippines. The widespread occurrence of *B. glumae* as epiphytic populations on apparently healthy seeds collected from farmers' fields in the Philippines, indicates that the pathogen resides on the surfaces of seeds as a latent infection (Sinclair, 1991), which could have implications for seed certification programs. Natural infection of *B. glumae* has until now only been observed on rice. Because the bacterium is seedborne, use of healthy seeds has been advocated as the primary method of control.

The frequent detection of *Burkholderia gladioli* in association with rice seeds and its identification as a rice-pathogenic bacterium was rather unexpected, as this species has hardly been reported in rice pathology literature. Short reports from Japan have described the isolation of this species from diseased rice (Miyagawa and Kimura, 1989; Ura et al., 1996). Because strains of *B. gladioli* cause similar symptoms on rice as those caused by *B. glumae*, they might have been mistaken for *B. glumae* in the past. Initially named *Pseudomonas gladioli* (synonym *P. marginata*) and subsequently transferred to the new genus *Burkholderia* (Yabuuchi et al., 1992), the species was originally described as a phytopathogenic pseudomonad causing disease of *Gladiolus* species (McCulloch, 1921; Severini, 1913).

In addition, isolates identified as *Burkholderia cepacia* were found among those isolates defined as pathogens with low disease potential. These *B. cepacia* isolates revealed phenotypic and genetic heterogeneity but their genomovar status had not been determined. *B. cepacia* is an extraordinarily versatile bacterium commonly found in soil, water, and the rhizosphere of plants. However, strains of *B. cepacia* as well as *B. gladioli* have received increased attention in clinical microbiology with the numerous reports describing these species as opportunistic human pathogens in cystic fibrosis and immunocompromised patients (Govan et al., 1996; Khan et al., 1996; LiPuma, 1998; Ross et al., 1995). Strains of *B. cepacia* have been widely used in agriculture for biological control of plant diseases, but, because some strains have been implicated in causing pulmonary infections of humans with cystic fibrosis, the potential risks of its use in agriculture are being carefully evaluated.

The observation that a phenotypic and genetic diversity of isolates from the genus *Burkholderia* were prominent in the collection of seedborne pathogenic bacteria, along with their widespread occurrence on symptomless rice seeds, prompted a more detailed characterization of this group. In the study presented, we characterized all *Burkholderia* isolates in the collection of pathogenic isolates obtained from seed samples collected over three successive cropping seasons from 17 farmers' fields at one site in the Philippines (origin of the 51 samples is shown in Table 3, p. 30). The isolates were evaluated by various taxonomic techniques including BOX-PCR genomic fingerprinting analysis, SDS-PAGE of whole cell proteins, and 16S rDNA and *recA* based RFLP analysis.

Burkholderia Populations Associated with Seed of Tropical Irrigated Rice

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ABSTRACT
Isolations of distinct colonies from washes of 51 rice seed samples collected over three successive cropping seasons from 17 farmers' fields at Jalajala in southern Luzon, Philippines, yielded 1,938 isolates. Artificial inoculation of rice seedlings revealed 196 isolates (10%) that induced symptoms of sheath necrosis. They were differentiated based on aggressiveness into pathogens (4%) and pathogens with low disease potential (6%), and grouped according to colony appearance and BOX-PCR genomic fingerprints. Comparison of whole-cell protein profiles with those of *Burkholderia* reference strains identified 100 isolates from five BOX-PCR fingerprint groups as *B. glumae* (45%), *B. gladioli* (31%), *B. cepacia* complex (23%), and *B. vietnamiensis* (1%). 16S rDNA and *recA* based PCR assays and RFLP analyses identified the two BOX-PCR groups differentiated among the *B. cepacia* complex isolates as genomovar I (10 isolates) and genomovar III (13 isolates), respectively. The morphological diversity, as reflected by colony appearance, among *Burkholderia* isolates corresponded to the groups differentiated by BOX-PCR analysis. *B. glumae* and *B. gladioli* isolates represented 92% of detected pathogens. The *B. cepacia* complex isolates represented 21% of detected pathogens with low disease potential to rice seedlings. Our results revealed that *B. cepacia* genomovar III, a genomic species associated with "cepacia syndrome" in cystic fibrosis patients, constituted about half of detected *B. cepacia* isolates. The present study also demonstrated that *Burkholderia* species can be effectively differentiated by BOX-PCR fingerprinting and suggests that the technique could be a useful tool for the differentiation of *B. cepacia* genomovars I and III.

Keywords: *Burkholderia glumae*, *Burkholderia gladioli*, *Burkholderia cepacia* complex, BOX-PCR fingerprinting

Introduction

Rice (*Oryza sativa* L.) is the principal staple food for nearly half of the world's population, most of them living in Asia. Diversity studies of plant-associated bacteria provide fundamental knowledge of the ecology and the interactions between bacterial populations and their plant host. The seed is of particular interest because of its importance as planting material and its potential as a vehicle for transmission of bacterial populations from one plant generation to the next. Recently, we charac-

terized total culturable bacterial populations associated with rice seed collected from Philippine farmers' fields in an attempt to assess its bacterial diversity (12). Predominant bacteria were *coryneforms, Enterobacteriaceae, Pseudomonas* and *Bacillus* species. Other bacteria regularly present belonged to the genera *Xanthomonas and Burkholderia*. The isolates were classified into functional groups of saprophytic, antagonistic, and pathogenic bacteria. Two percent of isolates were pathogens and all were identified as *Burkholderia* species.

The genus *Burkholderia* includes mainly soil- and plant-associated bacteria that are known as saprophytes, pathogens, nitrogen fixers, and biocontrol and bioremediation agents (3, 5, 14, 17, 29, 32). However, some also occur as human pathogens in the clinical environment (10, 19, 21, 44). Well-known rice pathogens are *Burkholderia glumae* and *Burkholderia plantarii*. *Burkholderia plantarii,* the causal agent of seedling blight of rice has only been reported from Japan (2). *Burkholderia glumae*, the bacterial cause of grain rot (17) and seedling rot (41), has emerged in recent years as the most important bacterial pathogen of rice in the temperate environment of Japan and Korea. In the tropics, the pathogen has been associated with the sheath rot complex and grain discoloration syndrome of rice (13, 47). Strains of *Burkholderia cepacia* from the rice environment have been reported as potential biocontrol agents against the rice sheath blight pathogen (36), others as endophytes capable of biological dinitrogen fixation (4). A strain of the N2-fixing species of *Burkholderia vietnamiensis* with plant-growth promoting ability has been demonstrated to increase rice yield in field studies (40).

Little is known about the predominance of *Burkholderia* populations on rice seed, or their inherent qualitative and quantitative variability. In the present study, we performed bacterial isolations from newly harvested rice seed collected during three successive cropping seasons from farmers' fields at the municipality of Jalajala in southern Luzon island, Philippines. As rice is a self-pollinated crop, Philippine rice farmers commonly save the seeds from their previous harvest for planting the next crop. The collected samples from three successive crops of 17 farmers' fields represented three seed generations of the modern rice cultivars released by the Philippine Seed Board: PSBRc14, PSBRc20, and PSBRc22 that are high yielding, early maturing, and contain partial resistance to most insect pests and major diseases; and one local cultivar BS1 with unknown genetic background. All isolates were routinely tested for pathogenicity by artificial inoculation of rice seedlings immediately after isolation and purification.

The aim of this study was to characterize all *Burkholderia* isolates in the collection of isolates that induced symptoms of sheath necrosis on inoculated seedlings by BOX-PCR fingerprinting of genomic DNA and whole-cell protein electrophoresis. *Burkholderia cepacia* complex isolates were further identified using 16S rDNA and *recA* based restriction fragment length polymorphism (RFLP) analysis and PCR assays.

Materials and methods

Reference strains and bacterial isolations from seed. Type and reference strains of *Burkholderia* species were obtained from the Belgian Coordinated Collections of Microorganisms / Laboratory Microbiology Gent (BCCM/LMG) Bacteria Collection, University Gent, Gent, Belgium, and the Entomology and Plant Pathology Division of the International Rice Research Institute, Los Baños, Philippines. The reference

strains used in the BOX-PCR fingerprinting analysis are listed in Table 1. Bacterial isolations were done from newly harvested rice seed of the 1996 and 1997 wet season crops and the intermediate 1997 dry season crop from 17 irrigated farmers' fields at the municipality of Jalajala in Rizal province of southern Luzon island, Philippines. The collected seed samples comprised successive generations of four rice cultivars planted on the 17 sampled fields over the three seasons: PSBRc14 (12 fields), PSBRc20 (two fields), PSBRc22 (one field), and a local cultivar BS1 (two fields). Fifty gram of seed (approximately 1,500 seeds) was immersed in 100 ml of sterile phosphate-buffered saline solution (PBS; 137 mM NaCl, 2.7 mM KCl, 0.01 M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) with 0.025% Tween 20 (Sigma-Aldrich, St. Louis), and soaked at ambient temperature (ca. 22 to 25°C) for 2 h on an orbital shaker at 130 rpm. The wash extract was serially diluted to 10^{-3} in sterile saline (0.85% NaCl) and aliquots of 0.1 ml were plated with a sterile L-shaped glass rod in duplicate on tryptic soy agar (TSA; Sigma-Aldrich) amended with 0.01% cycloheximide (Sigma-Aldrich) to prevent fungal contamination. After incubation for 4 days at 28°C, all visually distinct colonies per sample were enumerated on isolation plates and a representative colony for each type was picked. The isolates were purified on TSA and maintained at –70°C in nutrient broth (NB; Difco Laboratories, Detroit, MI) with 15% glycerol. Remaining seed of the collected samples was stored at 4°C in the Seed Health Unit of the International Rice Research Institute (IRRI), Los Baños, Philippines.

Pathogenicity tests. All isolates obtained from the seed samples collected over three successive cropping seasons from 17 farmers' fields were tested for pathogenicity on 21 day-old IR24 seedlings grown in autoclaved soil in a greenhouse under natural light with day and night temperatures of approximately 35 and 25°C, respectively, and with relative humidity

Table 1. Reference strains of *Burkholderia* species included in the BOX-PCR fingerprinting analysis.

Species	Strain[a]	Other designation[b]	Source (reference)
Burkholderia glumae	LMG 2196 ^{T}	NCPPB 2981 ^{T}	*Oryza sativa*, Japan
	LMG 10905	ICMP 3727	*Oryza sativa*, Japan
	LMG 10906	ICMP 3729	*Oryza sativa*, Japan
	LMG 18634		Rice seed, Philippines (12)
	IRRI 1858		Rice sheath, Philippines (13)
	IRRI 2056		Rice seed, Philippines (12)
Burkholderia gladioli	LMG 2216 ^{T}	ATCC 10248^T	*Gladiolus* sp., United States
	LMG 18569		Rice seed, Philippines (12)
Burkholderia plantarii	LMG 9035 ^{T}	ICMP 9424	*Oryza sativa*, Japan
	LMG 10908	ICMP 9426	*Oryza sativa*, Japan
Burkholderia cepacia	LMG 1222 ^{T}	ATCC 25416 ^{T}	Allium cepa
Burkholderia multivorans	LMG 18606		Rice seed, Philippines (12)
Burkholderia vietnamiensis	LMG 10929^T	TVV75 ^{T}	Rice rhizosphere, Vietnam

[a] LMG, Bacteria Collection of the Laboratory of Microbiology, University Gent, Belgium; IRRI, Entomology and Plant Pathology Division, International Rice Research Institute, Los Baños, Philippines. ^{T}, type strain.
[b] ATCC, American Type Culture Collection, Rockville, Maryland; ICMP, International Collection of Microorganisms from Plants, Plant Diseases Division, DSIR Mount Albert Research Center, Auckland, New Zealand; NCPPB, National Collection of Plant Pathogenic Bacteria, Harpenden Laboratory, Hertfordshire, U.K.; TVV, collected by Tran Van V., Centre de Pédologie Biologique, Université de Nancy, France.

ranging from 40 to 65%, as described previously (12). The rice cultivar IR24 was used for pathogenicity testing because it is known to be susceptible to most rice diseases. The inocula consisted of aqueous suspensions of 12-h-old bacterial cultures from TSA slant tubes (approximately 10^8 CFU/ml). Aliquots of 0.1 ml of each bacterial suspension were injected into the culm at 2 cm above soil level with a hypodermic needle and syringe. For each bacterial isolate, four seedlings were inoculated in a first test and isolates that produced symptoms on at least two of four seedlings were inoculated two more times on four plants in a second and third experiment. Seedlings injected with sterile distilled water served as negative control. The seedlings were examined for symptoms 3 and 10 days after inoculation. A negative score was given when no obvious symptoms or a hypersensitive-like reaction localized at the point of inoculation occurred. Elongation of a brown necrotic zone of tissue away from the point of inoculation often extended up to the third leaf was scored as a positive reaction. Isolates that induced symptoms on all plants that were inoculated, were considered pathogens; whereas, isolates that induced symptoms on at least 50% of inoculated plants in the three experiments were considered pathogens with low disease potential. Bacteria were reisolated from selected plants to confirm that symptoms were caused by the inoculated isolate.

Isolates that induced a positive reaction in the seedling inoculation tests were analyzed by BOX-PCR genomic fingerprinting and selected isolates from each BOX-PCR fingerprint group were further evaluated for reproducibility of symptom expression using dilution series of inoculum concentration. Aqueous suspensions of 12-h-old bacterial cultures from TSA slant tubes were serially diluted to 10^7, 10^6,10^5 and 10^4 CFU/ml in sterile saline (0.85% NaCl), and suspension concentrations were confirmed by dilution plate counting on TSA. Aliquots of 0.1 ml from each dilution series were injected between the leaf sheaths of eight seedlings.

Representative isolates of *B. glumae* and *B. gladioli* were also inoculated on IR24 plants at booting and heading stage in a growth chamber with day and night temperatures of 29 and 21°C, respectively, and relative humidity ranging from 70 to 95%. Aliquots of 1 ml of an aqueous bacterial suspension (approximately 10^8 CFU/ml) were injected into boots of tillers from five plants. Plants at heading stage were inoculated by spraying individual emerged panicles with 2-ml of an aqueous bacterial suspension (approximately 10^8 CFU/ml).

Phenotypic characterization. All isolates that induced symptoms in the seedling inoculation tests were examined for Gram morphology and reaction on Hugh and Leifson's oxidative-fermentative medium according to Lelliott and Stead (24). Gram-negative oxidative bacteria were further tested for fluorescent pigment production on King's medium B (KB; 22). Pure cultures grown for 4 days at 28°C on TSA and KB were examined visually and under the dissecting microscope at 12-power magnification for description of colony shape, texture and pigmentation. The tests were repeated at least twice for each isolate to assess the reliability of the test results. The isolates were grouped on the basis of similarities in cellular characteristics and colony appearance.

DNA isolation. Bacterial cells were grown on TSA for 24 h at 28°C, harvested with a sterile plastic spatula and washed once in 0.5 ml of saline buffer (150 mM NaCl, 10 mM EDTA, pH 8.0) in a 1.5 ml Eppendorf centrifuge tube. After centrifugation for 2 min at 11000 rpm, cells were resuspended in 100 µl TE buffer (10 mM

Tris-HCl, 1 mM EDTA, pH 8.0). Extraction of total genomic DNA was done as described by Pitcher et al. (33). DNA samples were redissolved in 100 µl of TE buffer. The DNA concentrations were estimated visually by comparison with λ DNA standards in an agarose gel. All DNA preparations were stored at -20°C.

BOX-PCR genomic fingerprinting analysis. Selected *Burkholderia* reference strains and all isolates that induced symptoms in the seedling inoculation tests were analyzed by genomic DNA fingerprinting based on PCR using the primer BOXA1R [5'-CTACGGCAAGGCGACGCTGACG-3'] corresponding to the BOX element (28), and synthesized by Operon Technologies Inc. (Alameda, CA). BOX-PCR analysis and electrophoresis conditions were as described previously (12). Briefly, approximately 40 to 80 ng of genomic DNA was used as template in a 25-µl reaction mixture containing 2 µM of BOXA1R primer, 1.25 mM deoxynucleoside triposphates (United States Biochemical, Cleveland), 2 units of *Taq* DNA polymerase (Pharmacia Biotech, Asia Pacific, Hong Kong), 10% (vol/vol) dimethyl sulfoxide (Sigma-Aldrich) and 0.16 mg/ml bovine serum albumin (Boehringer Mannheim, Far East, Singapore) in 1x Gitschier buffer (23). PCR mixtures were overlaid with 25 µl of sterile mineral oil (Sigma-Aldrich). PCR amplifications were performed in a DNA thermal cycler (480; Perkin-Elmer, Norwalk, CT) with an initial denaturation at 95°C for 7 min, 30 cycles consisting of 94°C for 1 min, annealing at 53°C for 1 min, extension at 65°C for 8 min, and a single final extension at 65°C for 15 min, followed by cooling at 4°C. After the amplification process, 7-µl of each reaction mixture was electrophoresed on gels composed of 0.8% Synergel (Diversified Biotech, Boston, MA) and 0.8% agarose (United States Biochemical) in 0.5×TBE (Tris borate-EDTA) (38) at 75V for 14 h. The two outer wells were not used to avoid distortion of the profiles. As standard, a 1-kb size marker (Life Technologies, Pacific, Hong Kong) was run in the second, sixteenth, and thirtieth lane. The gels were stained with ethidium bromide and photographed on an UV transilluminator using Polaroid Type 55 film (Polaroid Corp., Cambridge, MA). The photographs were scanned on a flatbed scanner (Sharp JX-610) at 200 dots per inch (dpi) resolution and saved as TIFF files. Normalization of BOX-PCR patterns and cluster analysis was achieved with the commercially available GelCompar software (version 4.0; Applied Maths, Kortrijk, Belgium). Background was subtracted using the "rolling disk" method. Levels of similarity between BOX-PCR patterns were calculated by the Pearson correlation coefficient, and were expressed as percentages of similarity for convenience. Cluster analysis was done by the unweighted pair group method using arithmetic averages (UPGMA). In addition, the interpretation of the DNA fingerprints was carried out by visual examination.

Reproducibility of DNA fingerprints. The level of reproducibility of BOX-PCR fingerprinting was determined by including DNA of strain G237 in each PCR assay; its resulting fingerprint was used as an internal standard during each electrophoresis run. Intergel reproducibility as derived from the similarity scale in the dendrogram generated by the UPGMA clustering analysis software of GelCompar was at least 89%. Visually no differences were observed in the obtained profiles of amplified fragments. Variations were mainly attributed to differences in electrophoresis times and in the intensities of amplified bands. When reproducibility was determined by obtaining repeated patterns of four isolates in replicate PCR amplifications and gel electrophoresis conducted at three separate

dates, no differences were observed in the patterns of each of these isolates among the three replicates and the intergel correlation between these patterns was above 95% similarity.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins. All isolates thought to belong to *Burkholderia* species were grown on nutrient agar (Oxoid CM3) supplemented with 0.04% (wt/vol) KH_2PO_4 and 0.24% (wt/vol) Na_2HPO_4*12 H_2O (pH 6.8) and incubated for 48 h at 28°C. Preparation of whole-cell proteins and SDS-PAGE was performed as described previously (34). Densitometric analysis, normalization and interpolation of the protein profiles, and numerical analysis using the Pearson product moment correlation coefficient were performed using the GelCompar software package (version 4.2; Applied Maths). All whole-cell protein profiles were compared with over 3000 profiles in a database comprising all presently known *Burkholderia, Ralstonia* and *Pandoraea* species (7, 8, 9, 42, 44, 45; P. Vandamme, unpublished data).

16S rDNA and *recA* based RFLP analyses and PCR assays. 16S rDNA and *recA* based PCR assays and RFLP analyses of *Burkholderia cepacia* complex isolates were performed using procedures described previously (27).

Results

Isolations of visually distinct colonies from individual washes of 51 seed samples collected over three successive cropping seasons from 17 farmers' fields at one location in southern Luzon island, Philippines, yielded 1,938 isolates. All isolates were tested for pathogenicity by artificial inoculation into rice seedlings. The majority of isolates (90%) did not induce symptoms in the seedling inoculation tests. One hundred ninety-six isolates (10%) produced diverse symptoms of sheath browning on inoculated seedlings, and were further grouped according to similarities in cellular characteristics, colony appearance, and BOX-PCR genomic fingerprints. Among these 196 isolates, 83 isolates (4%) consistently produced symptoms on all inoculated seedlings and were gram-negative oxidative rods that did not produce fluorescent pigments on KB. Seven of these 83 isolates produced the brown stripe symptom characteristic for *Acidovorax avenae* (30), the other 76 isolates caused severe sheath browning or soft rot of inoculated seedlings and were thought to be *Burkholderia glumae*. The remaining 113 isolates (6%) produced variable symptoms of sheath browning on 50 to 90% of seedlings that were inoculated in three separate experiments, and were considered pathogens with low disease potential. They consisted of 65 gram-positive endospore-forming rods, 8 gram-negative fermentative rods, and 40 gram-negative oxidative rods of which 16 isolates produced fluorescent pigments on KB. Preliminary whole-cell fatty acid analysis using the commercially available Microbial Identification System (Microbial ID Inc., Newark, DE) as described previously (12), tentatively identified the majority of isolates with low disease potential as *Bacillus pumilus* (37 isolates), *Paenibacillus polymyxa* (28 isolates), *Pseudomonas aeruginosa* (16 isolates), and *Pantoea* species (8 isolates). The pathogenic bacteria that produced the typical brown stripe symptom were confirmed as *Acidovorax avenae*. No reliable identifications were obtained for the remaining 24 gram-negative oxidative isolates with low disease potential that formed three distinct BOX-PCR fingerprint groups, and for the 76 pathogenic bacteria thought to be *Burkholderia glumae* that formed two main BOX-PCR fingerprint groups. Analysis by

means of whole-cell protein electrophoresis of representative isolates for each of these five BOX-PCR fingerprint groups identified their protein profiles as typical for *Burkholderia* species (see below). Subsequently, all 100 isolates belonging to these five groups were included in the protein electrophoretic analyses.

BOX-PCR fingerprinting analysis. BOX-PCR genomic fingerprinting analysis distinguished five fingerprint groups among the 100 *Burkholderia* isolates. Figure 1 is the result of a computer-assisted numerical comparison of the BOX-PCR fingerprints of a selection of isolates representing the genetic variability observed within the five fingerprint groups.

The 24 isolates with low disease potential to rice seedlings formed three BOX-PCR fingerprint groups. The first group included 10 isolates represented by LMG 19589 and LMG 19590, and displayed highly similar genomic fingerprint patterns linked at a level of 76% similarity. Although several polymorphisms were observed, these isolates shared multiple bands of equal mobility in common with the pattern generated for the type strain of *B. cepacia* LMG 1222 (not shown). The second group included 13 isolates represented by LMG 19587 and LMG 19588, and showed a linkage level of 72% similarity. The isolates from the two fingerprint groups were distinguishable by colony appearance (see below) and only distantly related to each other based on BOX-PCR fingerprinting analysis. In fact, they were found to represent different *B. cepacia* genomovars classified as genomovar I and genomovar III (see below). The third group included the fingerprint pattern of the single isolate LMG 19591 that was matched at a level of 51% similarity to the pattern of the type strain for *B. vietnamiensis* LMG 10929 (not shown).

The 76 pathogenic bacteria that were thought to be *B. glumae* formed two main BOX-PCR fingerprint groups identified as *B. glumae* and *B. gladioli*, respectively, by the inclusion of the type and reference strains belonging to these species. The 45 isolates identified as *B. glumae*, represented by LMG 19582 and LMG 19583, displayed highly similar but not identical genomic fingerprints linked at a level of 64% similarity. The genomic variability among these isolates was limited to two or three DNA fragments, and their patterns were nearly identical to those of the type and reference strains for *B. glumae* listed in

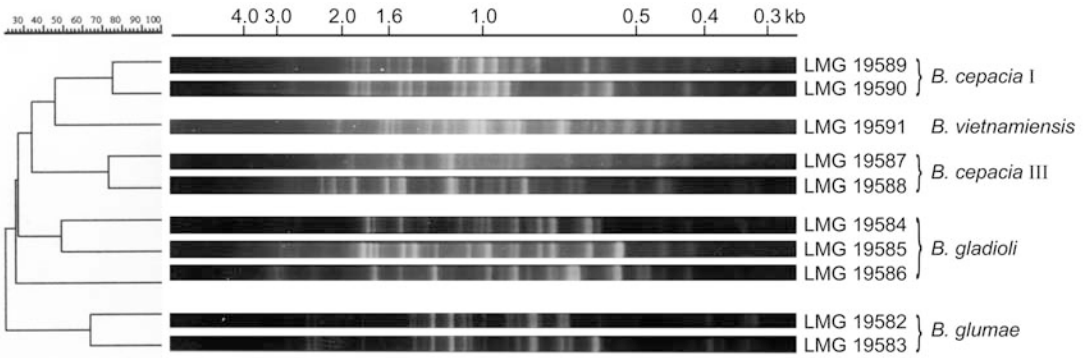

FIG. 1. Dendrogram obtained by computer assisted numerical analysis of BOX-PCR genomic fingerprints of a selection of *Burkholderia* isolates representing the genetic variability observed within the four delineated fingerprint groups and the unique *B. vietnamiensis* isolate. The scale on top represents the DNA molecular size marker (1-kb ladder, Life Technologies). LMG numbers refer to the accession number of deposited isolates in the Bacteria Collection of the Laboratory of Microbiology, University Gent, Belgium.

Table 1 (data not shown). In contrast, the 31 isolates identified as *B. gladioli* displayed more diverse genomic fingerprints but shared several comigrating bands in common, and cluster analysis revealed three subgroups within the population. Moreover, the isolates from each subgroup were distinguishable by colony appearance (see below). The first subgroup included 26 isolates represented by LMG 19584 and displayed fingerprint patterns that were similar to the pattern of the type strain for *B. gladioli* LMG 2216 (not shown). The second subgroup included three isolates represented by LMG 19585, and the third subgroup included two isolates represented by LMG 19586.

Whole cell protein electrophoresis. The whole-cell protein profiles of all 100 *Burkholderia* isolates were compared with those of *Burkholderia* reference strains. The identification of the two BOX-PCR fingerprint groups differentiated among the 76 pathogenic bacteria as *B. glumae* and *B. gladioli*, respectively, was confirmed. The 24 isolates with low disease potential to rice seedlings, represented by two BOX-PCR fingerprint groups and a single isolate (LMG 19591) with a unique BOX-PCR fingerprint, were identified as members of the *B. cepacia* complex. Twenty-three of them had whole-cell protein profiles that resembled those of *B. cepacia* genomovar I and III strains, but discrimination at the genomovar level was equivocal. The single isolate LMG 19591 with a unique BOX-PCR fingerprint was identified as *B. vietnamiensis.* Figure 2 shows the whole-cell protein profiles of a random selection of isolates representing the four BOX-PCR fingerprint groups and the unique *B. vietnamiensis* isolate.

16S rDNA and *recA* based RFLP analyses and PCR assays. The identification of the single *B. vietnamiensis* isolate was confirmed by the generation of a *B. vietnamiensis* specific 16S rDNA RFLP pattern (data not shown). Since the latter

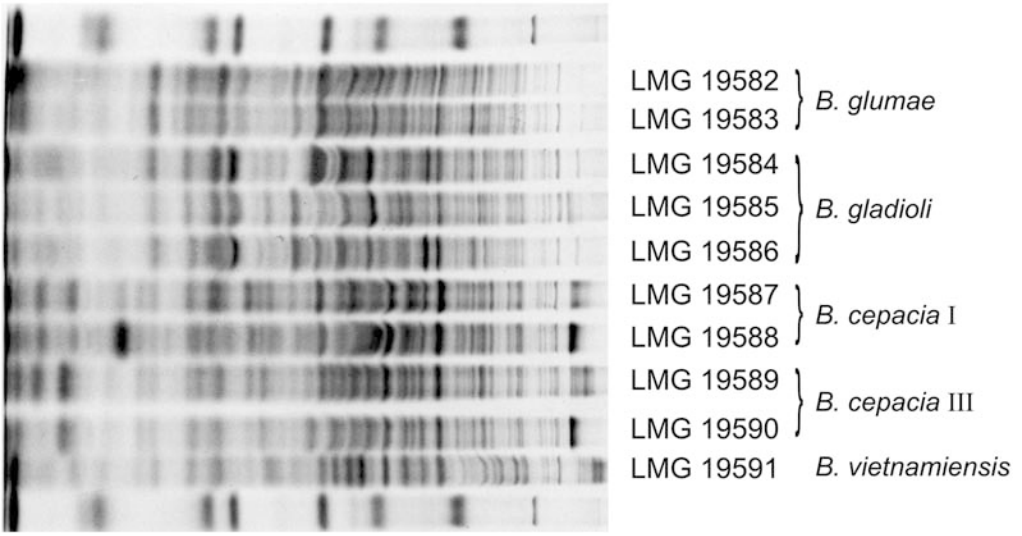

FIG. 2. Patterns obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of whole-cell protein extracts of *Burkholderia* isolates representing the four main BOX fingerpint patterns and the unique *B. vietnamiensis* isolate. The molecular weight markers used (lanes at top and bottom) were (from right to left) b-galactosidase (116,000), bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and lysozyme (14,200).

approach is not sensitive enough to distinguish between *B. cepacia* genomovars I and III (27), we performed genomovar specific *recA* based PCR tests and *Hae*III-*recA* RFLP analyses to identify the *B. cepacia* complex isolates to the genomovar level. The two BOX-PCR fingerprint groups distinguished among the 23 *B. cepacia* isolates were identified as *B. cepacia* genomovars I and III, respectively (data not shown). Of the 13 *B. cepacia* genomovar III isolates, 10 were identified as genomovar IIIA, none as genomovar IIIB. The remaining three isolates did not react with either one of the genomovar III PCR assays but were identified by their genomovar III specific *recA* RFLP profile (data not shown).

Cultural characteristics and population sizes of *Burkholderia* isolates associated with rice seed. The colony appearance of *Burkholderia* isolates differed markedly on TSA and KB surfaces after 4 days of incubation at 28°C. The isolates from each delineated BOX-PCR fingerprint group were distinguishable by colony appearance.

Isolates identified as *B. cepacia* genomovar I displayed dull, wrinkled, olive yellow colonies on TSA, 2 to 4 mm in diameter, raised to umbonate in elevation with smooth to irregular margins. On KB, colonies were dull, pale greenish-yellow, 2 to 3 mm, umbonate in elevation with smooth to wavy margins. Isolates identified as *B. cepacia* genomovar III displayed two distinct colony morphotypes. Ten isolates, represented by LMG 19587, produced creamy white colonies on TSA, 2 to 3 mm in diameter, butyrous, translucent, convex in elevation with smooth pale margins. On KB, colonies were whitish and mucoid, 3 to 4 mm in diameter, low convex in elevation with smooth margins. Three isolates, represented by LMG 19588, produced colonies on TSA and KB with a similar greenish pigmentation as those produced by genomovar I isolates but the colonies on TSA did not wrinkle and were glossy, 3 to 4 mm in diameter, translucent, convex in elevation with smooth margins. On KB, colonies were mucoid, 3 to 4 mm in diameter, convex with smooth translucent margins. The colonies of both *B. cepacia* genomovars occasionally produced slime on the media. The single isolate LMG 19591 identified as *B. vietnamiensis* produced light brownish white colonies on TSA medium, 2 to 3 mm in diameter, glossy, droplike in elevation with smooth margins. On KB, colonies were grayish white, 1.5 to 2 mm, glossy, droplike in elevation with wavy margins. The isolate initially displayed raspberry-shaped colonies on isolation plates but lost this characteristic morphology after sub-culturing.

Burkholderia glumae isolates displayed ivory yellow colonies on TSA medium, 1.5 to 2.5 mm in diameter, glossy, convex in elevation with smooth narrow translucent margins, and only weakly produced a faint yellow diffusible pigment in the medium. On KB, colonies were similar but 1 to 1.5 mm in diameter, mucoid, and produced a nonfluorescent yellowish-green diffusible pigment.

Burkholderia gladioli isolates displayed three distinct colony morphotypes each representing a distinct BOX-PCR fingerprint subgroup. The 26 isolates represented by LMG 19584 produced yellowish white colonies on TSA medium, 2 to 2.5 mm in diameter, butyrous, translucent, convex in elevation with smooth pale margins. Colonies on KB were similar but mucoid and glossy. They produced nonfluorescent yellowish-green diffusible pigments on TSA and KB. The three isolates represented by LMG 19585 produced creamy white colonies on TSA medium, 2 to 3 mm in diameter, butyrous, translucent, convex in elevation with smooth to wavy, paler margins; older colonies typically displayed

a convex center with raised, extended, translucent margins. On KB, two colony morphology variants were observed; most colonies were yellowish-white, 3 mm in diameter, glossy, convex with wavy margins and rough surfaces, whereas others were translucent and raised in elevation with irregular margins. Nonfluorescent yellowish-green diffusible pigments were produced on KB but not on TSA medium. The two isolates represented by LMG 19586, produced wrinkled white colonies on TSA and KB, 2 to 4mm in diameter, translucent, raised in elevation with wavy to lobate margins and rough surfaces. Nonfluorescent yellowish-green diffusible pigments were produced on TSA and KB.

Table 2 gives an overview of the morphological diversity among the *Burkholderia* isolates and their frequency of detection in the examined samples per cropping season. On the total of 51 samples examined over the three cropping seasons, *B. glumae* isolates were found in 61% of the samples, *B. gladioli* in 43%, *B. cepacia* genomovar I in 20% and *B. cepacia* genomovar III in 22%. The enumeration data for each colony type on isolation plates were log transformed to achieve normality and allowed for an estimation of mean population sizes, averaged over 17 samples per cropping season (Fig. 3). *B. glumae* was the most frequently isolated *Burkholderia* species from rice seed. The sizes of the populations of *B. gladioli*, though consistently lower than those of *B. glumae*, appeared steadily to increase over the three cropping seasons. *B. cepacia* genomovars I and III had more or less comparable population sizes.

Pathogenicity tests. One hundred *Burkholderia* isolates were identified among the 196 isolates that caused symptoms of sheath browning in the seedling inoculation tests. The other 1,742 isolates, or sterile distilled water used as negative control, did not induce symptoms upon injection into rice seedlings.

The 23 isolates identified as *Burkholderia cepacia* genomovars I and III, and the single isolate identified as *B. vietnamiensis* produced, within three days

Table 2. Morphological diversity and frequency of detection of *Burkholderia* species in rice seed samples collected during three successive cropping seasons from Philippine farmers' fields.

Identification[a]	No. of isolates (LMG no.)[b]	Colony appearance on TSA[c]	Frequency of detection[d]		
			WS96	DS97	WS97
B. glumae	45 (19582, 19583)	Glossy ivory yellow	9	8	14
B. gladioli	26 (19584)	Butyrous yellowish white (yellowish-green diffusible pigments)	4	7	10
	3 (19585)	Butyrous creamy white	1	-	2
	2 (19586)	Translucent wrinkled white (yellowish-green diffusible pigments)	-	-	2
B. cepacia I	10 (19589, 19590)	Wrinkled olive yellow	4	3	3
B. cepacia III	10 (19587)	Butyrous creamy white	2	3	3
	3 (19588)	Glossy olive yellow	2	-	1
B. vietnamiensis	1 (19591)	Glossy light brownish white	1	-	-

[a] Roman numerals indicate *Burkholderia cepacia* genomovars.
[b] Numbers in parentheses refer to the LMG accession number of representative isolates deposited in the Bacteria Collection of the Laboratory of Microbiology, University Gent, Belgium.
[c] After 4 days of incubation at 28°C.
[d] Indicates the number of samples, out of 17 examined samples per cropping season, from which the isolates were found. WS96, wet season 1996; DS97, dry season 1997; WS97, wet season 1997.

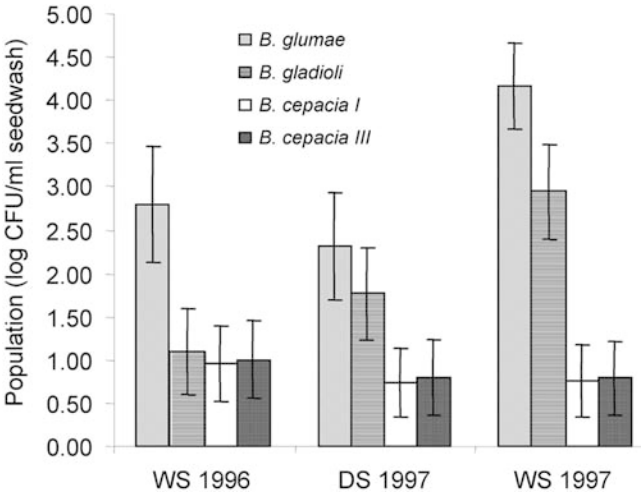

FIG. 3. Mean population sizes, averaged over 17 samples per cropping season, of *Burkholderia* isolates detected in rice seed samples collected from farmers' fields at Jalajala in southern Luzon, Philippines. Bars represent standard error of the mean. WS, wet season crop harvested during, respectively, November 1996 and December 1997; DS, dry season crop harvested during May 1997.

after inoculation, an elongated water-soaked brown streak on the sheaths of 8 to 10 of 12 seedlings that were inoculated in three separate experiments. After 10 days, the lesion appeared as a dark brown necrotic streak (Fig. 4B). The symptoms caused by these isolates were indistinguishable; however, the extent of sheath browning and the number of affected seedlings varied among isolates and inoculation experiments. In a first and second trial, three to four of four inoculated seedlings developed sheath browning, while in a third trial only two to three seedlings were affected. Also, the induction of sheath browning by the *B. cepacia* complex isolates was dependent on inoculum concentration (data not shown). Sheath browning was produced only when aliquots of 0.1 ml from suspensions containing 10^8 or 10^7 CFU/ml were inoculated, while inoculation of suspensions containing 10^6 CFU/ml produced only mild sheath browning on four of eight inoculated seedlings. No symptoms were observed on seedlings

FIG. 4. Symptoms on IR24 rice seedlings artificially inoculated with representative *Burkholderia* isolates, ten days after inoculation. A, Control plant inoculated with sterile distilled water. B, Longitudinal brown necrotic stripe on the sheath caused by isolate 1230 (LMG 19588) identified as *B. cepacia* genomovar III. C and D, Soft rot of the seedlings (upheld by noninoculated tillers) caused by isolates 4323 (LMG 19586) and 1879 (LMG 19582) identified as *B. gladioli* and *B. glumae*, respectively.

injected with 0.1-ml aliquots from suspensions containing 10^5 or 10^4 CFU/ml.

The 45 isolates identified as *B. glumae* and the 31 isolates identified as *B. gladioli* consistently produced symptoms on all seedlings that were inoculated in three separate experiments. However, the severity of symptom expression differed among *B. gladioli* isolates. The 29 isolates comprised within the BOX-PCR fingerprint subgroups represented by *B. gladioli* LMG 19584 and LMG 19585, induced water-soaked brown blotches on the sheath and chlorosis of leaves within three days after inoculation. The blotches developed into lesions with a grayish center and dark brown margin within 10 days after inoculation; inoculated seedlings often collapsed (data not shown). The two isolates from the third BOX-PCR fingerprint subgroup represented by *B. gladioli* LMG 19586, induced rapid and severe symptoms closely resembling those caused by *B. glumae* isolates and inoculated seedlings wilted within 10 days after inoculation (Fig. 4C). The *B. glumae* isolates caused brown discoloration and soft rot of the sheath accompanied by chlorosis and shriveling of leaves, and rapid death of inoculated seedlings (Fig. 4D). The symptom expression of *B. gladioli* isolates was dependent on the inoculum concentration (data not shown). Inoculation of 0.1-ml aliquots from suspensions containing 10^8, 10^7 or 10^6 CFU/ml produced the described symptoms on all inoculated seedlings. Inoculation of 0.1-ml aliquots from suspensions containing 10^5 CFU/ml produced only mild sheath necrosis on four to five of eight inoculated seedlings, and the seedlings were able to outgrow the symptoms. No symptoms were observed on seedlings injected with 0.1-ml aliquots from suspensions containing 10^4 CFU/ml. *B. glumae* isolates produced the described symptoms at inoculum concentrations of 10^8, 10^7, 10^6 or 10^5 CFU/ml, whereas inoculation of 0.1-ml aliquots from suspensions containing 10^4 CFU/ml caused brown discoloration of the sheath but did not affect the leaves and the seedlings did not wilt (data not shown).

Injection of *B. glumae* isolates into rice plants at booting stage produced severe sheath browning and up to 70% yellowish-gray unfilled grains and completely brown discolored grains on the panicles, which often only partially emerged from the boots (data not shown). Injection of representative *B. gladioli* isolates into the boots produced only slight brown discoloration of the sheath and considerably less brown discolored grains on the panicles but up to 40% yellowish-gray unfilled grains (data not shown). *B. glumae* and *B. gladioli* isolates produced similar symptoms upon spray-inoculation of rice plants at heading stage. The panicles showed up to 40% yellowish-gray unfilled grains and spotted brown discolored grains, no symptoms were produced on the sheath (data not shown).

Discussion

The aim of the present study was to characterize and identify 100 *Burkholderia* isolates in a collection of 1,938 isolates from rice seed collected over three successive cropping seasons from 17 farmers' fields at the municipality of Jalajala in southern Luzon island, Philippines. The location forms part of the irrigated intensive rice cropping systems of Luzon island. The *Burkholderia* isolates represented 51% of isolates that induced symptoms upon artificial inoculation of rice seedlings. The large majority of the 100 *Burkholderia* isolates was identified as *B. glumae* (45%) and *B. gladioli* (31%), found in 61 and 43 percent of the examined samples, respectively. Aside from seven isolates identified as *Acidovorax avenae*, *B. glumae* and *B.*

gladioli represented 92% of detected seedborne rice pathogens. *B. plantarii*, another rice pathogen causing seedling blight (2), was not found. The *B. cepacia* complex isolates comprised 24% of the *Burkholderia* isolates and were identified as *B. cepacia* genomovar I (10%) and genomovar III (13%), and a single *B. vietnamiensis* isolate. They represented 21% of isolates considered pathogens with low disease potential to rice seedlings, which were differentiated from true pathogens based on lower aggressiveness and inconsistency in producing symptoms.

The detection of *B. glumae*, as predominant seedborne pathogen on rice seed is not surprising as it is a well-known pathogen responsible for both grain rot (17) and seedling rot of rice (41). The incidence of grain rot has increased in recent years in Japan and Korea, and the disease results in serious yield loss in the field (1). Seedling rot mainly occurs in seedling boxes prepared for mechanical transplanting in the northern part of Japan. In the tropics, *B. glumae* is commonly associated with the sheath rot complex and grain discoloration syndrome of rice, and causes symptoms on inoculated rice plants at the booting stage that resemble sheath brown rot caused by *Pseudomonas fuscovaginae* (13, 47). The frequent detection of *B. glumae* on apparent healthy rice seed as a latent pathogen suggests that its disease development may require particular conditions of host susceptibility, inoculum density, and climatic factors. Strains of *B. glumae* are also known to produce antibiotics and have been explored as potential biocontrol agents against *Ralstonia solanacearum* on tomato (16).

B. gladioli on the contrary, is primarily known as an organism responsible for soft rot in onions and *Gladiolus* and *Iris* species (31), and has only since the last decade been recognized as a rice pathogen in Japan (1). In seedling pathogenicity tests, the *B. gladioli* isolates induced similar symptoms as those caused by *B. glumae*, however only when inoculated at relative high concentrations, and might have been mistaken for *B. glumae* in the past. *B. gladioli* is also known as a potential biocontrol organism against *Botrytis cinerea*, a plant pathogen that causes grey mould on various host plants including numerous commercial crops (46), and as an opportunistic pathogen in compromised humans (6, 21, 37).

The *B. cepacia* complex isolates exhibited low disease potential upon injection into rice seedlings. They share this low disease potential with several fluorescent pseudomonads (13, 20) and other rice isolates identified as *Pantoea* species, *Bacillus pumilus*, and *Paenibacillus polymyxa* (12). *B. cepacia* complex strains too, are traditionally known as wound pathogens of onions causing sour skin, a rot of onion bulbs (5). *B. cepacia* strains can commonly be isolated from rice bed soil in nursery boxes in Japan, and have been reported to slightly retard the seedling growth when applied as seed inoculum (1). On the other hand, strains of *B. cepacia* isolated from rice seed produced the antifungal metabolite pyrrolnitrin and were explored as biological control agents against *Rhizoctonia solani*, the rice sheath blight pathogen (36). During the 1980s, however, *B. cepacia* emerged as a life-threatening human pathogen particularly in compromised patient groups such as cystic fibrosis patients (19, 25). Recently, integrated genotypic and phenotypic analyses (11, 44) showed that isolates presumptively identified as *B. cepacia* comprise at least nine genomic species, or genomovars, i. e. *B. cepacia* (genomovar I), *B. multivorans* (formerly known as *B. cepacia* genomovar II), *B. cepacia* genomovar III, *B. stabilis* (formerly known as *B. cepacia* genomovar IV), *B. vietnamiensis* (also known as *B.*

cepacia genomovar V), *B. cepacia* genomovar VI, *Burkholderia ambifaria* (genomovar VII) (9), *Burkholderia anthina* (genomovar VIII), and *Burkholderia pyrrocinia* (genomovar IX) (43). Altogether these nine genomovars are referred to as the *B. cepacia* complex. In the absence of straightforward biochemical tests for the identification of *B. cepacia* genomovars I, III, and VI, the latter two genomic species were not formally classified as novel *Burkholderia* species (genomovar I contains the type strain and therefore retains the name *B. cepacia)*. Among these, *B. cepacia* genomovar III has received most interest because it comprises the majority of the cystic fibrosis isolates world wide, and because of its strong association with epidemic spread and poor clinical outcome. The integration of strict segregation policies has reduced epidemic spread to a considerable extent but did not prevent regular new cases of *B. cepacia* colonization, the majority of which involve genetically unrelated strains with unique DNA fingerprints (18). Since the primary habitats of *B. cepacia* complex bacteria include river sediments, soil, and the plant rhizosphere, it seems reasonable to assume that these must be the reservoirs for infections with *B. cepacia* genomovars III as well. This was first demonstrated in a study by Balandreau et al. (3) who reported genomovar III strains to be predominant *B. cepacia* complex bacteria in the rhizosphere of maize and wheat, and in the tissues of wheat and lupine in France and Australia. These findings were confirmed and extended by Fiore et al. (15**)** who reported genomovars I, III, and VII, to be dominant *B. cepacia* complex species in the maize rhizosphere in Italy. Our results show that *B. cepacia* genomovar III isolates represented about half of the found *B. cepacia* complex isolates and substantiate this further. The identification of *B. cepacia* complex bacteria as organisms with low disease potential to rice seedlings mirrors their role as opportunistic pathogens for compromised onions (healthy or undamaged onions are not affected by *B. cepacia* complex bacteria) and for the immune compromised humans such as cystic fibrosis patients.

The identification of the *B. vietnamiensis* isolate as an organism with low disease potential on rice seedlings is a remarkable finding as *B. vietnamiensis* is primarily known as a nitrogen fixer with growth promoting characteristics for rice (40). It is possible that there were additional *B. vietnamiensis* isolates in the collection of 1,742 non-pathogenic isolates. These would not have been included in the present analyses of *Burkholderia* isolates as the first selection criterion was the ability to produce symptoms upon artificial inoculation in rice seedlings. Nevertheless, this finding suggest that genes responsible for growth promoting effects and pathogenicity may not be common to all *B. vietnamiensis* isolates, and that statements about risks associated with biotechnological use of individual isolates cannot be extrapolated to the entire species.

It is apparent from this study that the morphological diversity, expressed by distinct colony appearances, among the *Burkholderia* isolates detected on rice seed can be useful to distinguish or identify these organisms in combination with other tests, such as pathogenicity and BOX-PCR DNA fingerprinting. Each BOX-PCR fingerprint group corresponded to a distinct colony morphotype, while three morphotypes were distinguished within the population of *B. gladioli* and two within the population of *B. cepacia* genomovar III. The usefulness of repetitive sequence-based DNA polymerase chain reaction fingerprinting methods (rep-PCR) to characterize phytobacterial populations has been demon-

strated (35, 39), and its applications to environmental microbiology have been reviewed (26). Depending on the bacteria studied, BOX-PCR fingerprinting analysis seems to have the potential to differentiate bacteria at the species, subspecies, or even strain level. In a previous study (12), we demonstrated that this method generated highly conserved fingerprints within *B. glumae.* This was confirmed and extended in the present study by the inclusion of additional *B. glumae* isolates. The population of *B. gladioli* appeared to be more heterogeneous, and is comprised of three distinct lineages based on several BOX-PCR fingerprint polymorphisms but isolates share numerous comigrating bands, and the majority display a fingerprint pattern highly similar to the pattern of the *B. gladioli* type strain. *B. cepacia* complex isolates were effectively separated by BOX-PCR analysis into three fingerprint groups with respective identifications as *B. cepacia* genomovar I and genomovar III, and *B. vietnamiensis*. The straightforward differentiation of *B. cepacia* genomovars I and III isolates by means of this approach suggests that BOX-PCR analysis might be a valuable alternative for the differentiation of *B. cepacia* complex bacteria. The present study confirmed that whole-cell protein electrophoresis is not always discriminatory enough to distinguish isolates of *B. cepacia* genomovars I and III (10). It also revealed that *recA* PCR tests for the detection of genomovar III isolates may lack sensitivity (three isolates out of 13 were not identified) and reinforced the value of the *recA* RFLP approach.

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B. Sheath Rot Complex and Grain Discoloration

Historically, a bacterial sheath rot of rice caused by *Pseudomonas oryzicola* was first described in Hungary by Klement (1955), but the name was later shown to be a synonym of *Pseudomonas syringae* pv. *syringae* van Hall 1902 (Luketina and Young, 1979). In the 1960's, a similar disease characterized by brown discoloration of the sheath and discoloration of the grain was reported in China (Fang and Ren, 1960) and Japan (Funayama and Hirano, 1963; Goto, 1965), and the causal organism was generally referred to as *Pseudomonas marginalis*. In 1976, an epidemic of sheath brown rot with symptoms identical to those caused by *P. syringae* pv. *syringae* was reported in Hokkaido (Japan); the causal organism was described as a new species, *Pseudomonas fuscovaginae* (Miyajima et al., 1983; Tanii et al., 1976). Subsequently, the isolation of *P. fuscovaginae* was reported from rice sheath rot symptoms and discolored grains in Latin America and Africa.

Autrique and Maraite (1983) isolated *P. fuscovaginae* from discolored grains and sheaths of rice plants at booting stage in altitude swamps at 1600 m elevation in Burundi. Also, *P. fuscovaginae* was isolated from brown rot lesions on leaf sheaths of maize and on flag leaf sheaths of sorghum, suggesting its widespread occurrence in Burundi (Duveiller et al., 1989). Zeigler et al. (1987) reported the widespread occurrence of *P. fuscovaginae* in Latin America; whereas *P. syringae* pv. *syringae* was only found in Chile. Because the rice cultivars grown in most Latin American countries were introduced primarily from Asia, and the rice cultivars grown in Chile from Europe where *P. syringae* pv. *syringae* was first described, it was believed that the movement of germplasm had been the primary source of the spread of the disease. There's no conclusive evidence, however, to support this speculation.

Aside from *P. fuscovaginae*, which is considered the principal fluorescent pseudomonad causing sheath rot and grain discoloration of rice, other fluorescent pseudomonads able to induce similar symptoms on rice were isolated and considered as species different from *P.fuscovaginae* (Duveiller et al., 1988; Zeigler and Alvarez 1987). In the study by Zeigler et al. (1987), pathogenic fluorescent pseudomonads were isolated from discolored rice grains and flag leaf sheath samples received from the Philippines. Further, *P. fuscovaginae* was isolated from seedlings derived from seeds imported from the Philippines (IRRI) to Burundi (ISABU) (Duveiller et al., 1988). These seeds were cold-tolerant cultivars (84IRCTN) that were multiplied at Victoria in Laguna province, before sending to ISABU in 1984. At that time, *P. fuscovaginae* had not been reported in the Philippines.

Inquiries for information on the occurrence and identity of fluorescent pseudomonads associated with grain discoloration and leaf sheath rot of rice in the major rice-growing districts of the Philippines prompted the survey described in the next two papers. Although the sheath rot complex and grain discoloration syndrome of rice has become widespread in the tropics, it is currently not considered a major constraint to rice production in the Philippines. Tropical upland rice production areas, however, may be subject to outbreaks of sheath rot and grain discoloration when conditions are favorable to disease development, such as cool night temperatures and high humidity at booting stage. This might have been the case for the rare severe attack observed in an upland rice field plot at Siniloan, Philippines. In the third paper, we report on the isolation and characterization of *P. fuscovaginae*-like isolates obtained from discolored seeds as the causal agent for the severe outbreak of sheath rot and grain discoloration at Siniloan in January 2000.

Bacterial Diseases of Rice. I. Pathogenic Bacteria Associated with Sheath Rot Complex and Grain Discoloration of Rice in the Philippines

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ABSTRACT

Cottyn, B., Cerez, M. T., Van Outryve, M. F., Barroga, J., Swings, J., and Mew, T. W. 1996. Bacterial diseases of rice. I. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80:429-437.

Surveys were made to determine if bacterial pathogens were associated with grain discoloration and leaf sheath rot of rice in the major rice-growing districts in the Philippines. In 1988 and 1989, 304 diseased plant and grain samples were collected from 16 different provinces, and bacterial pathogens were found in 64 samples representing 12 of the provinces. In other cases, sheath rot or grain discoloration was attributed to fungal disease, insects, or abiotic factors. Pathogenic bacteria (204 strains) represented 3.6% of the total number of strains isolated and tested. Inoculations into the leaf sheaths of seedlings or the flag leaf sheaths produced symptoms characteristic of infection by *Burkholderia glumae* (formerly *Pseudomonas glumae)* and *Pseudomonas fuscovaginae.* None of the pathogens could be associated with distinctive symptoms. Strains of putative *P. fuscovaginae* were isolated from samples collected from the tropical lowland provinces of Laguna, Palawan, and Davao, and from the tropical highland (above 950 m) province of Ifugao. Since bacterial pathogens were isolated from 21% of the collections with sheath rot or grain discoloration, bacteria appear important in these disease complexes in the Philippines.

Sheath rot complex and grain discoloration describe the disease of rice *(Oryza sativa* L.) that appears as brown discoloration or rot of the flag leaf sheath and discoloration of the grain. The disease seems widespread and more prevalent in tropical Asia since the introduction of modern semidwarf and photoperiod-insensitive cultivars (5). The disease is especially apparent during the rainy season, and the intensity of infection varies from mild to severe (8).

The causal agent(s) of this disease have not been determined with certainty. Grain discoloration has been ascribed to rice bugs in West Africa and to adverse abiotic factors (21). Recently, several fluorescent and nonfluorescent pseudomonads, including *Pseudomonas glumae,* reclassified as *Burkholderia glumae* (20), and *Pseudomonas fuscovaginae,* have been associated with sheath rot and grain discoloration in the tropics (3,15,23). *B. glumae,* which

causes both seedling rot and grain rot, also known as glume blight (grayish discoloration of the grain), was first reported in Japan and later in other Asian countries (4, 19). Seedling rot on seedlings raised in nursery boxes for mechanical transplanting is common in northeast Japan; while grain rot, occurring at the flowering stage under high temperature and high humidity, is common in southwest Japan (S. Takaya, *personal communication). P. fuscovaginae*, which causes sheath brown rot, was first reported by Tanii et al. (17) in Hokkaido, the northern part of Japan. Initially, the disease was related to cold stress in temperate rice growing environments until *P. fuscovaginae* was found in Columbia, Latin America (23); and in Burundi and Madagascar, Africa (3,15). It was also detected from seeds sent to Burundi from the Philippines (3).

Sheath rot is a fungal disease of rice caused by *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksworth, described here for convenience as Sarocladium sheath rot, which is well documented in the literature (13). *S. oryzae* and other fungal pathogens, such as *Bipolaris oryzae* (Breda de Haan) Shoemaker and *Fusarium* spp., were also isolated from discolored seeds (9). The frequency of isolating *S. oryzae* from discolored seeds was lower than 10% (7). In the tropics, the symptoms of sheath rot complex, bacterial sheath brown rot, and Sarocladium sheath rot are difficult to differentiate based on descriptions in the literature. The typical bacterial sheath brown rot symptoms described by Tanii et al. (17) have not been observed on tropical rice.

At a workshop held at IRRI, scientists experienced with bacterial sheath brown rot found plants with symptoms of the disease. Both *Pseudomonas* spp. and *S. oryzae* were readily isolated from sheath tissues showing brown to dark discoloration and rotting on plants with Sarocladium sheath rot.

B. glumae and *P. fuscovaginae* appear to cause similar symptoms in the tropics. *Pseudomonas avenae*, reclassified as *Acidovorax avenae* subsp. *avenae* (22), causes bacterial stripe and has long been recorded in the tropics (13,18). Although the above-mentioned bacterial pathogens are described in the literature as causing distinct symptoms, the differentiation between these symptoms is not clear. Since pathogens cannot be uniquely associated with symptoms, the present study was designed to investigate the occurrence of pathogenic bacteria involved in the sheath rot complex and in grain discoloration. The specific objective was to determine if *P. fuscovaginae* and other pathogenic pseudomonads were indeed present in the Philippines.

Materials and methods

Isolation of bacteria from plant material. Plants in the ripening stage with sheath rot and grain discoloration were collected throughout the Philippines during the wet seasons of 1988 and 1989. The number of samples collected in a particular province corresponded with the availability of sampling sites at that location. Sheath and grain material collected from 10 hills per field composed one sample. The collected samples contained a wide range of sheath and/or grain symptoms, varying from translucent to brown dots to brown blotches to brown streaks to a completely brown sheath, and/or clear to brown spots to brown blotches to completely dark discolored seeds. A total of 304 samples were collected from rice grown in 16 different provinces. The discolored sheaths and grains were examined under a stereomicroscope, and material infested with

fungi was discarded, as those discolorations were attributed to *S. oryzae.* (Only seven samples were excluded for that reason.) Small segments of sheath from the region between discolored and adjacent healthy tissue were plated directly on King's medium B (6) and incubated at 28°C for 1 to 2 days. Any bacterial growth was restreaked to separate colonies.

To provide additional assays of grain in field samples, about 20 seeds from each sample were crushed in 10 ml of sterile water. The suspension was shaken for 5 min at room temperature. Loopfuls of the suspension were streaked onto King's medium B. Two grain samples of 1 kg each of the varieties IR54 (Los Baños, dry season 1989) and IR8866 (Banaue, same season) were sorted into discolored and symptomless seeds. Samples (100 g) of each were macerated and thoroughly mixed in 500 ml of sterile distilled water containing 0.025% Tween 20 and shaken for 2 h at room temperature. Portions (100 µl) from decimally diluted seed-soak were streaked on nutrient agar and incubated at 28°C for 2 to 3 days.

Three to five predominant colony types and all fluorescent colony types were purified by repeated streaking and kept on nutrient agar (Difco) slants. For long-term storage, the strains were suspended in 10% skimmed milk at 4°C or in nutrient broth with 30% glycerol at –70°C. All pathogenic strains were also lyophilized.

Biochemical characteristics. The pathogenic strains were divided into fluorescent and nonfluorescent groups based on fluorescent pigment production on King's medium B. One-day-old nutrient agar cultures were used for the Gram staining, Kovac's oxidase reaction, and for the inoculation of Hugh and Leifson's OF medium (2).

Pathogenicity test. Inoculations were made on greenhouse-grown rice seedlings.

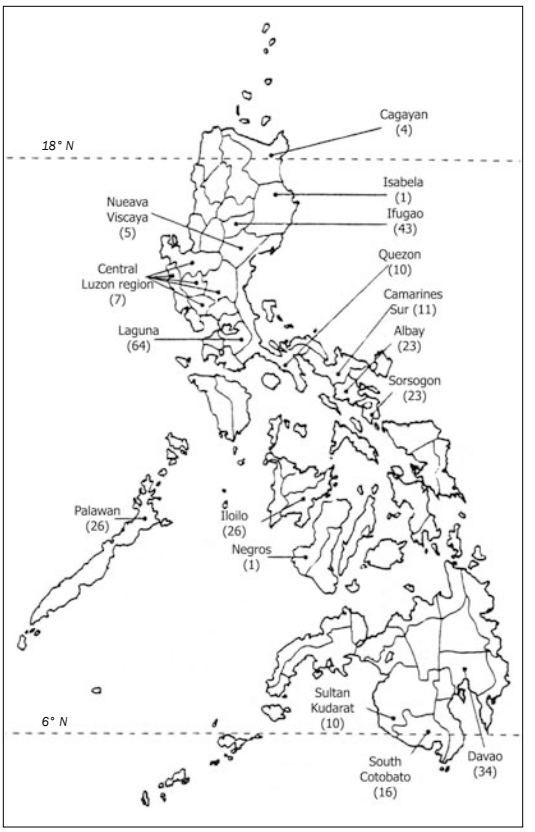

Fig. 1. Sampling sites and respective number of samples collected over a period of 2 years in the major rice growing provinces of the Philippines.

Seeds of TN1 were washed for 30 s in 70% ethanol and then rinsed three times in sterile distilled water. Seeds were germinated on wet paper towels in a growth chamber at 28°C under artificial light (16 h/day). After 1 week, the seedlings were transplanted into plastic containers filled with sterilized soil. After transplanting, plants were grown in the greenhouse under natural light, with day/night temperatures of about 30/20°C and a relative humidity of 40 to 65%.

The first screening of the strains consisted of injecting an overnight-grown nutrient broth culture into the leaf sheath of 21-day-old seedlings. In later screenings, bacterial cells collected by centrifugation of

a nutrient broth culture were resuspended in sterile water to a concentration of ca. 10^8 CFU/ml. For the control, sterile water was injected into the plant sheaths. Symptoms were observed 1 week after inoculation. Only strains that produced symptoms on two of three seedlings in a first trial and four of four to eight seedlings in a second trial were retained and tested in three subsequent pathogenicity tests.

The pathogenic strains were also tested on booting cultivar TN1 about 65 days after sowing. Bacteria from an 18-h nutrient broth culture suspended in nutrient broth in sterile water to a concentration of ca. 10^8 CFU/ml were injected in the flag leaf sheath, and sterile water was injected as a control. Symptoms were observed 2 weeks after inoculation. *A. a.* subsp. *avenae* was detected by the method of Shakya and Chung (16); 5 g of TN1 seeds were added to 50 ml of nutrient broth inoculated with a loopful of freshly grown bacteria scraped from nutrient agar plates of each nonfluorescent strain. The suspension was shaken for 18 h at 28°C. The seeds were then germinated in petri dishes on three layers of filter paper saturated with 230 ppm urea solution. One set was watered afterwards with 225 ppm urea solution; an equivalent set was watered with distilled water only. The seedlings were observed 10 days after sowing.

Further characterization of the pathogenic strains identified by the Biolog system (1) as related to *P. fuscovaginae, B. glumae,* and *A. a.* subsp. *avenae* was made by seed-soak inoculation of rice seedlings of cultivars TN1 and IR24. The rice seeds, which had been washed for 30 s in 70% ethanol and rinsed three times in sterile distilled water, were soaked in inoculum (10^8 CFU/ml) at 25°C for 2 h. For the control, surface sterilized seeds were soaked in sterile distilled water. The seeds were then sown on autoclaved garden soil in plastic containers (14 × 17 × 4.5 cm). Two trays with two rows per tray and 25 seeds per row were used to test each strain. Inoculated plants were incubated in a dew chamber (near 100% RH, 28°C) for 24 h and were transferred to the greenhouse. They were observed 2 weeks after sowing. Strains identified by Biolog (I) as *P. fuscovaginae* and *B. glumae* were injected until runoff into the flag leaf sheath of cultivars IR24 and IR64 at maximum tillering stage (about 50 days after sowing) and at booting stage (about 70 days after sowing), respectively. At maximum tillering, five hills with four tillers per hill were inoculated with each strain. At booting, two hills with two tillers per hill were inoculated with each strain. The inoculum and controls were prepared as before. Symptoms were observed until grain maturity.

Results

Over 5,600 bacterial strains were isolated from 304 samples collected in 16 provinces (Fig. 1) and tested for pathogenicity on 21-day-old TNl seedlings. Out of 64 samples distributed over 12 provinces, 204 pathogenic strains were obtained, comprising 150 strains from plants showing symptoms of sheath rot and grain discoloration, 14 strains from diseased seedlings, and 40 strains from two grain samples of 1 kg. The seed of cultivar IR54 contained 227 g of discolored seed and yielded eight pathogens, four of them from discolored seed. Thirty-two pathogens were obtained from the IR8866 seed. Eight of these strains came from discolored seed, which weighed 223 g. Distribution and isolation frequency of the 204 strains, grouped by subsequent Biolog clustering (1), are presented in Table 1. From four provinces (Negros, Quezon, Sorsogon, and Central Luzon region), no pathogenic forms were obtained

from the 525 strains isolated from 41 rice collections. Some strains were related to *Erwinia herbicola* (14 strains) and *Stenotrophomonas maltophilia* (four strains), but these were not included in the present study.

Biochemical characteristics. The 204 pathogenic strains isolated from Philippine-grown rice were oxidative, Gram-negative rods. The nonfluorescent group contained 19 strains, and 185 strains produced fluorescent pigment on King's medium B. Kovac's oxidase reaction was variable among strains.

Pathogenicity and symptoms. Initial pathogenicity of the 204 strains was based on seedling symptoms ranging from localized browning around the inoculation point to brown streaks all over the sheath and extending to the midrib of the youngest leaf. More severely infected seedlings wilted. All strains were tested on 24 to 33 TN1 seedlings (discrepancy due to non-germinated seeds) over five trials (Table 2). Most of the 204 strains did not cause consistent symptoms. Although the observed symptoms were generally the same, the severity of the produced lesions greatly varied among the strains. Variability in virulence among the 204 strains appeared later to correspond more or less to the different groups in the Biolog clustering (1). Table 3 shows the mean percentage of plants affected by the strains grouped according to the Biolog clusters (1) together with the lowest and highest percentage of diseased plants. The *A. a.* subsp. *avenae* (cluster E) and *B. glumae* (cluster Dl) strains were able to induce symptoms on more than 95% of the total number of seedlings tested. Their pathogenicity was obvious and clear-cut. With the modified method of Shakya and Chung (16), the characteristic brown stripe associated with *A. a.* subsp. *avenae* could not unequivocally be identified among the browning patterns. Symptoms were the same on seedlings treated with 230 ppm urea solution or with

Table 1. Origin and isolation frequency of pathogenic strains associated with sheath rot complex and grain discoloration of rice in the Philippines

Province	Pathogens (total)[a]	Samples (total)[b]	Biolog cluster[c]								
			A_2	A_3	A_4	A_5	A_6	B_1	B_2[d]	D_1	E
Albay	1(358)	1(23)	0	0	1	0	0	0	0	0	0
Cagayan	5(91)	1(4)	0	0	0	0	0	0	0	0	5
Camarines Sur	3(128)	2(11)	0	0	0	0	0	0	0	3	0
South Cotabato	1(400)	3(16)	0	0	1	0	0	0	0	0	0
Davao	9(373)	7(34)	0	0	5	2	1	1	0	0	0
Ifugao	69(983)	18(43)	2	0	4	0	0	4	59	0	0
	32(289)*		0	3	19	1	7	2	0	0	0
Iloilo	39(389)	15(26)	0	0	4	0	1	0	34	0	0
Isabela	2(7)	1(1)	0	0	0	0	0	0	0	2	0
Laguna	17(780)	9(64)	0	0	3	0	0	2	3	0	9
	8(710)*		1	0	3	1	2	0	1	0	0
Nueva Viscaya	9(111)	3(5)	0	0	1	0	0	0	8	0	0
Palawan	5(256)	3(26)	0	0	2	0	1	2	0	0	0
Sultan Kudarat	1(283)	1(10)	0	0	0	0	1	0	0	0	0

[a]Number of pathogenic strains compared to (the total number of bacteria isolated). Asterisks refer to the isolates from the two batches of one kilogram seed of IR54 (Los Baños, Laguna) and IR8866 (Banaue, Ifugao).
[b]Number of samples from which the pathogenic strains were isolated, compared to (the total number of samples collected).
[c]Number of isolates grouped according to the numerical analysis of the Biolog data. Clusters A_2, A_3, A_4 and A_6 remained unidentified as they did not contain reference strains. Cluster A_5 was identified as *P. aeruginosa* and cluster B_1 as *P. fuscovaginae*. Cluster B_2 was related to *P. aureofaciens*, *P. corrugata*, *P. fluorescens* and *P. marginalis*. Cluster D_1 was identified as *P. glumae* and E as *A. avenae* subsp. *avenae*.
[d]Three strains pertaining to cluster B_2 are not available anymore, hence not included.

Table 2. Tissue of origin of the pathogenic strains and their reactions over five successive pathogenicity tests on 21-day-old TN1 seedlings and on TN1 plants at the booting stage in the IRRI greenhouse

Biolog cluster[a]	IRRI number	Tissue origin[b]	S1[c]	S2	S3	S4	S5	P (Total)[d]	BT[e]
A_2	4937	sheath^2	2	4	0	0	0	6(29)	4
	5405	sheath^0	2	4	0	0	0	6(29)	0
	5440	seed^0	3	4	0	0	0	7(33)	4
A_3	6239	seed^2	2	4	6	0	0	12(33)	0
	6244	seed^2	2	4	0	0	0	6(33)	0
	6269	seed^2	2	4	0	0	0	6(33)	0
A_4	2128	seed^3	3	7	16	0	0	26(33)	4
	3474	seed^2	3	8	8	0	0	19(33)	0
	3680	seed^3	2	8	6	0	0	16(33)	0
	3953	seed^3	3	8	8	0	0	19(32)	0
	4008	seed^3	2	8	10	0	0	20(33)	0
	4142	seed^2	2	8	5	0	0	15(32)	0
	4184	seed^2	3	2	12	0	0	17(33)	0
	4276	seed^2	3	8	6	0	0	17(33)	0
	4704	sheath^2	2	8	5	3	3	21(25)	0
	4707	sheath^2	3	8	7	0	0	18(33)	0
	4735	sheath^2	3	8	3	0	0	14(33)	0
	4736	sheath^2	3	8	15	3	3	32(33)	0
	5268	sheath^2	2	4	0	0	0	6(29)	0
	6464	sheath^2	3	4	9	0	0	16(29)	0
	6591	sheath^2	3	4	5	0	0	12(29)	0
	6593	sheath^2	3	4	0	0	0	7(29)	0
	6594	sheath^2	2	4	3	0	0	9(28)	0
	6595	sheath^2	3	4	16	0	0	23(29)	0
	6625	sheath^2	3	4	0	0	0	7(29)	0
	6687	seed^2	2	4	16	0	0	22(29)	0
	6717	seed^1	3	4	4	0	0	11(29)	0
	6720	seed^3	3	4	16	0	0	23(29)	4
	6726	sheath^1	3	4	16	0	0	23(29)	0
	6730	sheath^1	3	0	13	0	0	16(29)	0
	7073	seed^0	3	4	0	0	0	7(33)	0
	7160	seed^0	2	4	16	0	0	22(33)	0
	7161	seed^0	3	4	16	0	0	23(33)	0
	7164	seed^0	2	4	7	0	0	13(33)	0
	7173	seed^0	3	4	15	0	0	22(33)	0
	7174	seed^0	3	4	16	0	0	23(33)	0
	7245	seed^0	2	4	16	0	0	22(33)	0
	7252	seed^0	3	4	6	0	0	13(33)	0
	7277	seed^0	3	4	0	0	0	7(33)	0
	7285	seed^0	3	4	16	0	0	23(33)	0
	7308	seed^0	3	4	16	0	0	23(33)	0
	7392	seed^0	3	4	0	0	0	7(33)	0
	7406	seed^0	3	4	0	0	0	7(33)	0
	7407	seed^0	3	4	16	0	0	23(33)	0
	7470	seed^0	3	4	16	0	0	23(33)	0
	7471	seed^0	3	4	0	0	0	7(33)	0
	7475	seed^0	3	4	16	0	0	23(33)	0
	7478	seed^0	3	4	16	0	0	23(33)	0
	7479	seed^0	3	4	16	0	0	23(33)	0
A_5	5459	seed^0	3	4	0	0	0	7(33)	0
	7342	seed^2	3	3	16	0	0	22(29)	0
	7343	seed^2	3	4	0	0	0	7(29)	0
	7358	seed^0	3	4	0	0	0	7(33)	0
A_6	3678	seed^2	2	6	6	0	0	14(33)	4
	4185	seed^2	3	8	7	0	0	18(32)	1

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Table 2 continued.

Biolog cluster[a]	IRRI number	Tissue origin[b]	S1[c]	S2	S3	S4	S5	P (Total)[d]	BT[e]
	5164	seed^0	3	4	0	0	0	7(33)	4
	6245	seed^2	2	4	0	0	0	6(33)	0
	6251	seed^2	2	4	4	0	0	10(33)	0
	6257	seed^2	2	4	0	0	0	6(32)	0
	6535	sheath^2	3	4	0	0	0	7(29)	0
	6702	seed^1	2	4	0	0	0	6(28)	0
	6827	sheath^1	3	4	0	0	0	7(29)	0
	7104	seed^0	2	4	1	0	0	7(33)	0
	7270	seed^0	3	4	16	0	0	23(33)	0
	7346	seed^0	3	4	0	0	0	7(33)	0
	7391	seed^0	3	4	0	0	0	7(33)	0
B_1	4521	sheath^3	3	8	8	0	0	19(33)	0
	4605	sheath^3	3	8	16	3	2	32(33)	0
	5793	seed^2	2	4	5	0	0	11(28)	3
	5801	seed^2	3	4	16	3	3	29(29)	6
	5803	seed^2	3	4	16	3	3	29(29)	0
	6031	seed^2	3	4	3	0	0	10(29)	0
	6202	seed^2	3	4	0	0	0	7(33)	1
	6235	seed^2	3	4	3	0	0	10(33)	0
	6609	sheath^2	3	4	16	3	3	29(29)	3
	7007	sheath^2	3	4	16	0	0	23(29)	0
	7008	sheath^2	3	4	16	3	3	29(29)	2
B_2[f]	4784	sheath^2	3	4	15	0	0	22(28)	4
	4790	sheath^1	3	4	15	0	0	22(29)	4
	4792	sheath^1	2	4	14	0	0	20(27)	4
	4794	sheath^1	3	4	0	0	0	7(29)	2
	4797	sheath^1	3	4	13	3	3	26(27)	4
	4799	sheath^1	3	4	15	0	0	22(29)	2
	4807	sheath^2	3	4	15	0	0	22(28)	4
	4808	sheath^1	2	4	14	0	0	20(27)	3
	4809	sheath^2	3	4	13	0	0	20(27)	4
	4830	sheath^2	3	4	15	2	3	27(28)	2
	4831	sheath^2	3	4	11	0	0	18(28)	4
	4832	sheath^1	3	4	1	0	0	8(28)	4
	4833	sheath^2	2	4	0	0	0	6(29)	4
	4834	sheath^1	2	4	0	0	0	6(29)	4
	4836	sheath^2	3	4	0	0	0	7(29)	2
	4840	sheath^2	3	4	0	0	0	7(29)	4
	4841	sheath^2	3	4	1	0	0	8(29)	4
	4842	sheath^2	3	4	0	0	0	7(29)	4
	4845	sheath^2	3	4	0	0	0	7(29)	4
	4846	sheath^2	3	4	0	0	0	7(29)	4
	4847	sheath^2	3	4	0	0	0	7(29)	4
	4849	sheath^2	3	4	1	0	0	8(29)	4
	4855	sheath^2	3	4	0	0	0	7(29)	4
	4863	sheath^2	2	4	0	0	0	6(29)	3
	4866	sheath^2	2	4	2	0	0	8(29)	4
	4868	sheath^2	3	4	0	0	0	7(29)	4
	4882	sheath^2	3	4	0	0	0	7(29)	4
	4902	sheath^2	3	4	0	0	0	7(29)	4
	4909	sheath^2	3	4	0	0	0	7(29)	4
	4915	sheath^2	3	4	0	0	0	7(29)	1
	4924	sheath^2	3	4	0	0	0	7(29)	4
	4928	sheath^2	3	4	0	0	0	7(29)	4
	4967	sheath^2	3	4	0	0	0	7(29)	3
	5067	sheath^1	3	4	0	0	0	7(29)	2
	5068	sheath^2	3	4	0	0	0	7(29)	1
	5191	sheath^0	2	4	1	0	0	7(27)	4

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Table 2 continued.

Biolog cluster[a]	IRRI number	Tissue origin[b]	S1[c]	S2	S3	S4	S5	P (Total)[d]	BT[e]
	5200	sheath^{0}	3	4	0	0	0	7(29)	0
	5229	sheath^{0}	3	4	0	0	0	7(29)	0
	5232	sheath^{0}	3	4	0	0	0	7(29)	0
	5237	sheath^{2}	3	4	1	0	0	8(29)	0
	5244	sheath^{2}	3	4	0	0	0	7(29)	0
	5245	sheath^{1}	3	4	0	0	0	7(29)	0
	5247	sheath^{2}	3	4	0	0	0	7(29)	0
	5256	sheath^{0}	3	4	0	0	0	7(29)	4
	5263	sheath^{2}	3	4	2	0	0	9(29)	0
	5270	sheath^{2}	3	4	16	0	0	23(29)	0
	5273	sheath^{0}	3	4	0	0	0	7(29)	0
	5274	sheath^{0}	3	4	0	0	0	7(29)	0
	5276	sheath^{0}	3	4	0	0	0	7(29)	0
	5280	sheath^{0}	3	4	0	0	0	7(29)	0
	5301	sheath^{0}	2	4	0	0	0	6(29)	0
	5310	sheath^{2}	3	4	0	0	0	7(29)	0
	5313	sheath^{0}	3	4	0	0	0	7(29)	0
	5314	sheath^{0}	3	4	0	0	0	7(29)	0
	5317	sheath^{2}	3	4	0	0	0	7(29)	0
	5318	sheath^{2}	3	4	0	0	0	7(29)	0
	5321	sheath^{0}	3	4	0	0	0	7(29)	0
	5328	sheath^{2}	3	4	0	0	0	7(29)	0
	5329	sheath^{2}	3	4	0	0	0	7(29)	0
	5337	sheath^{2}	3	4	16	2	3	28(29)	0
	5371	sheath^{2}	3	4	0	0	0	7(29)	0
	5372	sheath^{0}	3	4	0	0	0	7(29)	0
	5373	sheath^{2}	2	3	0	0	0	5(28)	0
	5395	sheath^{0}	3	4	1	0	0	8(24)	0
	5397	sheath^{0}	2	4	1	0	0	7(26)	0
	5418	sheath^{2}	3	4	1	0	0	8(28)	0
	5463	seed^{0}	3	4	0	0	0	7(33)	0
	5614	seed^{2}	3	4	0	0	0	7(29)	0
	6182	sheath^{1}	3	4	0	3	3	13(29)	0
	6183	sheath^{1}	2	4	13	3	3	25(29)	0
	6190	sheath^{0}	2	4	0	0	0	6(29)	0
	6192	sheath^{1}	2	4	13	2	3	24(28)	0
	6193	sheath^{1}	2	4	16	3	0	25(29)	0
	6194	sheath^{0}	2	4	16	0	0	22(29)	0
	6217	sheath^{0}	2	4	16	0	0	22(29)	0
	6221	sheath^{1}	3	4	2	0	0	9(29)	0
	6285	sheath^{2}	3	4	10	0	0	17(29)	0
	6287	sheath^{0}	3	4	0	0	0	7(29)	0
	6288	sheath^{2}	3	4	16	0	0	23(29)	0
	6291	sheath^{1}	3	4	16	0	0	23(29)	0
	6309	sheath^{1}	3	4	16	0	0	23(29)	0
	6316	sheath^{1}	3	4	16	0	0	23(29)	0
	6318	sheath^{1}	2	4	16	0	0	22(29)	0
	6333	sheath^{1}	2	4	0	0	0	6(29)	0
	6334	sheath^{1}	3	4	0	0	0	7(29)	0
	6347	sheath^{1}	2	4	0	0	0	6(28)	0
	6348	sheath^{1}	3	4	15	0	0	22(29)	0
	6356	sheath^{1}	3	4	0	0	0	7(29)	0
	6357	sheath^{1}	3	4	16	0	0	23(29)	0
	6358	sheath^{1}	3	4	0	0	0	7(29)	0
	6362	sheath^{1}	3	4	0	0	0	7(29)	0
	6368	sheath^{1}	2	4	16	0	0	22(29)	0
	6369	sheath^{1}	2	4	16	0	0	22(29)	0
	6370	sheath^{1}	3	4	0	0	0	7(29)	0
	6371	sheath^{1}	3	4	16	0	0	23(29)	0

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Table 2 continued.

Biolog cluster[a]	IRRI number	Tissue origin[b]	S1[c]	S2	S3	S4	S5	P (Total)[d]	BT[e]
	6372	sheath[1]	3	4	0	0	0	7(29)	0
	6375	sheath[1]	3	4	6	0	0	13(29)	0
	6376	sheath[1]	2	4	16	2	3	27(29)	0
	6400	sheath[1]	2	4	0	0	0	6(29)	0
	6402	sheath[1]	3	4	16	0	0	23(29)	0
	6408	sheath[1]	3	4	16	0	0	23(29)	0
	6415	sheath[2]	2	4	16	0	0	22(29)	0
	7246	sheath[1]	2	3	16	0	0	21(28)	0
	7253	sheath[1]	2	4	16	3	2	27(29)	0
	7405	sheath[1]	2	4	16	0	0	22(29)	0
D_1	1857	sheath[2]	3	7	14	3	3	30(33)	4
	1858	sheath[2]	3	7	16	3	3	32(33)	7
	2056	seed[0]	2	8	16	3	3	32(33)	7
	2057	seed[0]	3	8	16	3	3	33(33)	3
	2076	seed[0]	3	8	16	3	3	33(33)	7
E	1837	seedling[1]	3	6	16	3	3	31(33)	0
	1840	seedling[1]	2	1	16	3	3	25(33)	5
	1845	seedling[1]	3	6	16	3	3	31(33)	0
	1851	seedling[1]	3	7	16	3	3	32(33)	1
	1891	seedling[1]	2	7	16	3	3	31(33)	2
	7010	seedling[1]	3	4	16	3	3	29(29)	2
	7012	seedling[1]	3	4	16	3	3	29(29)	0
	7014	seedling[1]	2	4	16	3	3	28(28)	0
	7015	seedling[1]	2	4	16	3	3	28(28)	0
	7017	seedling[1]	3	4	16	3	3	29(29)	0
	7018	seedling[1]	2	4	16	3	3	28(28)	1
	7019	seedling[1]	3	4	16	3	3	29(29)	1
	7021	seedling[1]	3	4	16	3	3	29(29)	0
	7023	seedling[1]	3	4	16	3	3	29(29)	1

[a]From the numerical analysis of Biolog data. No reference strains were grouped in clusters A_2, A_3, A_4, and A_6. Cluster A_5 was identified as *Pseudomonas aeruginosa* and B_1 as *P. fuscovaginae*. Cluster B_2 was related to *P. aureofaciens, P. corrugata, P. fluorescens,* and *P. marginalis.* Cluster D_1 was identified as *Burkholderia glumae* and cluster E as *Acidovorax avenae* subsp. *avenae.*
[b]With symptom description: 0 = symptomless; 1 = slight infection (few small lesions); 2 = coalesced lesions to browning all over; 3 = no description available.
[c]Number of plants with leaf sheath necrosis, observed 1 week after inoculation for: S1 = first inoculation on three seedlings; S2 = second inoculation on four or eight seedlings; S3 = third inoculation on 16 seedlings; S4 = fourth inoculation on three seedlings; S5 = fifth inoculation on three seedlings.
[d]Total positive reactions (total of tested plants) over the five seedling inoculation tests.
[e]Number of plants showing leaf sheath necrosis on a total of seven plants, observed 2 weeks after inoculation at the booting stage.
[f]Three strains pertaining to cluster B_2 are not available anymore, hence are not included.

distilled water (results not shown). The mean infection rate of the fluorescent pathogens of clusters A and B was low, as they infected less than 50% of the total number of seedlings tested (Table 3). The *P. fuscovaginae* group (cluster B1) showed a higher virulence, but five strains produced symptoms on over 95% of the test plants, whereas six strains were pathogenic on less than 40% of the plants. Some of the fluorescent strains may have lost their pathogenicity, as they were able to induce symptoms in the first two trials only (Table 2). This was especially the case for strains belonging to the A clusters.

Only a few of the 204 pathogenic strains induced symptoms on TNl plants inoculation at the booting stage. Moreover, most were only slightly pathogenic in our experimental conditions, as slightly less than half of the inoculated plants developed symptoms of sheath necrosis. All sus-

Table 3. Percentage of plants showing symptoms after inoculation

Biolog cluster	Mean % ± SD[a]	Range
A2	21.0%	21%
A3	24.2±10.5%	18–36%
A4	54.6±20.9%	21–97%
A5	35.6±26.9%	21–76%
A6	30.0±16.2%	18–70%
B1	69.0±32.7%	21–100%
B2	42.4±26.5%	21–100%
D1	97.0± 3.7%	91–100%
E	96.8± 6.6%	76–100%

[a]The average is for all strains per Biolog cluster.

pected *B. glumae* strains caused blotching and brown necrosis on the flag leaf sheath. Only 45% of the suspected *P. fuscovaginae* strains and almost 35% of the strains from cluster B2 produced leaf sheath symptoms. Some of these strains caused severe discoloration and rotting of the flag leaf sheath. The other strains, including suspected *A. a.* subsp. *avenae* strains, caused only slight discoloration of the sheath. The symptoms ranged from a restricted dark brown zone around the inoculation site, to small brown spots that occasionally coalesced to form large indistinct blotches on the flag leaf sheath.

Suspected *B.* glumae, *A. a.* subsp. *avenae*, and *P. fuscovaginae* strains were further characterized with the seed-soaking method of inoculation (Table 4). Both cultivars TNl and IR24 gave the same results. None of the *P. fuscovaginae* strains induced any symptom on the seedlings by this inoculation method. *A. a.* subsp. *avenae* strains induced browning of the sheaths, with the youngest leaf becoming dry or having a brown stripe on the midrib (Fig. 2B). *B. glumae* strains reduced the germination rate by causing soft rotting of the soaked seeds. Resulting seedlings also were smaller and mostly displayed brown rotting sheaths. Twisting, curling, and whitening of the leaves were also observed 1 to 2 weeks after inoculation. However, the virulence of these *B. glumae* strains was considerably different; strains 2056, 2057, and 2076 (isolated from apparently symptomless seeds from a field in the province of Camarines) were consistently virulent, while 1857 and 1858 (isolated from discolored sheath material in the province of Isabela) displayed a less severe type of lesions.

IR24 plants injected with suspected *B. glumae* and *P. fuscovaginae* strains at maximum tillering stage developed severe dark browning of the sheath. On IR64 plants in the booting stage, very distinct brown discolorations and dry rotting of the flag leaf sheath were observed (Fig. 3A).

Table 4. Symptoms induced on IR24 seedlings by selected strains 14 days after seed-soak inoculation of 100 seeds per strain.

IRRI no.	Biolog identity at sowing	Soft rotting of seeds at sowing	Brown striping of sheath and leaf midrib	Sheath brown, leaves curled, whitened
1840	*A. avenae*	–	+	–
1851	*A. avenae*	–	+	–
7015	*A. avenae*	–	+	–
1857	*P. glumae*	+	–	+
1858	*P. glumae*	+	–	+
2056	*P. glumae*	+	–	+
2057	*P. glumae*	+	–	+
2076	*P. glumae*	+	–	+
6031	*P. fuscovaginae*	–	–	–
6235	*P. fuscovaginae*	–	–	–
7008	*P. fuscovaginae*	–	–	–

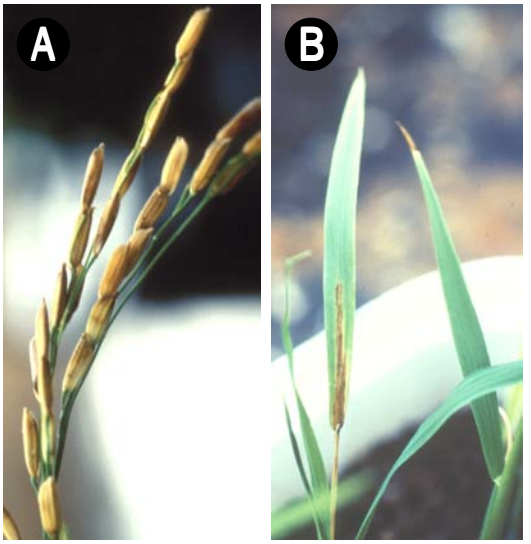

Fig. 2. Disease symptoms induced by selected nonfluorescent strains: (A) brown striping (arrows) on the grains of IR64 observed at maturity after injection of the flag leaf sheath at booting stage with strain 2056 (suspected Burkholderia glumae); (B) water-soaked brown lesion along the midrib of TNI seedlings observed 2 weeks after seed-soak inoculation with strain 1840 (suspected *Acidovorax avenae).*

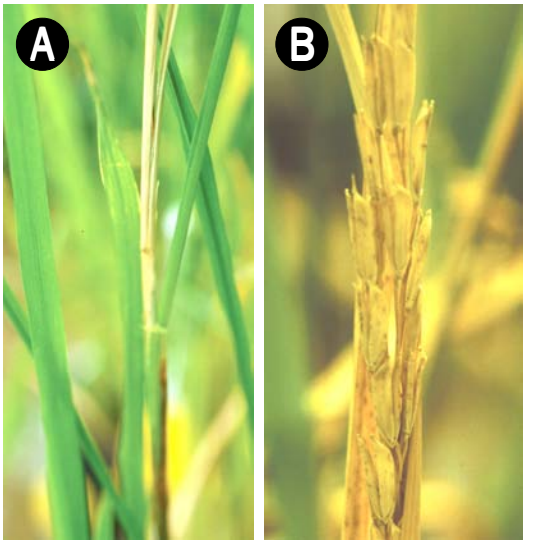

Fig. 3. Disease symptoms induced by suspected *'Pseudomonas fuscovaginae* strains on IR64 rice plants at the booting stage: (A) necrotic leaf 8 days after inoculation of the flag leaf sheath with strain 7008; (B) spikelet sterility observed 14 days after inoculation with strain 6031.

The symptoms appeared as early as 2 to 3 days after inoculation. Also, spikelet sterility appeared (Fig. 3B). At maturity, the grains showed brown discoloration ranging from small spots on the hull to discoloration of the whole grain for both *P. fuscovaginae* and *B. glumae* strains inoculated (Table 5). Some grains, however, displayed the characteristic brown streak on the hulls typical for *B. glumae* (Fig. 2A).

Discussion

It is clear that the sheath rot complex and grain discoloration syndrome of rice involves a complex group of *Pseudomonads* spp. in addition to *S. oryzae*, other fungal pathogens, insects, and abiotic factors (7,13,21,23,24). Sarocladium sheath rot causes sheath discoloration to rotting (dry rot) of the sheath tissues and abortion of the panicle exertion (13). *S. oryzae* may be isolated from lesions of sheath browning, but sheath browning does not always produce *S. oryzae* (T. W. Mew, *unpublished).* In recent years, several pathogenic pseudomonads have been reported to cause a similar syndrome of sheath rot complex and grain discoloration (3,10,15,17,23,24). The distinct features of bacterial sheath brown rot of *P. fuscovaginae* reported by Tanii et al. (17) were not observed throughout our field survey in the Philippines. However, the bacterial pathogen has been isolated from seed obtained from this country (3).

Our survey throughout the major rice growing districts in the Philippines was conducted from 1988 to 1990. Of the total collected bacterial strains from rice plants with the sheath rot complex and grain discoloration, only 3.6% was pathogenic. Detailed studies to characterize the large remaining portion of isolated nonpatho-

Table 5. Symptoms produced by suspected *Burkholderia glumae* and *Pseudomonas fuscovaginae* strains inoculated into rice plants at maximum tillering stage/booting stage

IRRI no.	Biolog identity	Discolored grain (%)[a]	Sheath browning	
			MT[b]	BT[c]
1857	*Burkholderia glumae*	50.1	+	+
1858	*B. glumae*	28.8	+	+
2056	*B. glumae*	47.0	+	+
2057	*B. glumae*	18.1	+	+
2076	*B. glumae*	19.5	+	+
6202	*Pseudomonas fuscovaginae*	40.8	+	+
4521	*P. fuscovaginae*	64.9	+	+
4605	*P. fuscovaginae*	51.5	+	+
5793	*P. fuscovaginae*	24.1	+	+
5801	*P. fuscovaginae*	17.8	+	+
5803	*P. fuscovaginae*	39.7	+	+
6031	*P. fuscovaginae*	36.3	+	+
6235	*P. fuscovaginae*	41.9	+	+
6609	*P. fuscovaginae*	19.7	+	+
7007	*P. fuscovaginae*	37.0	+	+
7008	*P. fuscovaginae*	19.6	+	+

[a]On a total of 200 grains.
[b]Observed 7 to 14 days after inoculation. Each strain was inoculated onto 20 plantsof cultivar IR24 at the maximum tillering (MT) stage.
[c]Observed 7 to 14 days after inoculation up to grain maturity. Each strain was inoculated onto four plants of cultivar IR64 at the booting (BT) stage.

genic bacteria, some showing antagonistic properties against rice fungal pathogens, are in progress. Although there might have been a bias toward isolation of fluorescent strains on King's medium B, the group of 19 nonfluorescent and a majority of 185 fluorescent strains. All were pathogenic on rice seedlings by the inoculation method of injection, and they induced the development of leaf sheath necrosis or browning (Table 2). The pattern of leaf sheath necrosis differed among the strains, but the syndrome was very similar, consequently preventing diagnosis based solely on symptomatology, as was also reported in earlier studies (23,24). The nonfluorescent strains were generally very consistent in producing leaf sheath necrosis or browning in the different pathogenicity tests. *A. a.* subsp. *avenae* was only isolated from diseased seedlings. *A. a.* subsp. *avenae* is seedborne and has been detected in seed lots from many countries (16). Its role in the sheath rot complex is not clear, as opposed to an earlier report (25). *B. glumae*, which also infects seedlings (19), was obtained from discolored leaf sheaths and apparently healthy seeds. The strains grouped into *B. glumae* produced very consistent sheath browning (Tables 4 and 5). The severity, however, varied among strains at the seedling, maximum tillering, and booting stages.

The fluorescent strains caused indistinguishable symptoms by the methods used for the pathogenicity tests. The extent of sheath necrosis or browning, by the injection method either at booting or at seedling stage, varied among the strains. There is a marginal relationship between the degree of leaf sheath necrosis or browning and the obtained classification into Biolog clusters. The individual strains within a Biolog cluster may produce a diversity in leaf sheath browning patterns and rotting. The capability of each cluster to cause sheath discoloration is reflected in the mean percentage of affected plants (Table 3).

Generally, most of the strains from seeds were grouped in the A clusters (Table 2). Strains derived from leaf sheath samples were mainly grouped in cluster B2. Cluster B1 contained a small number of strains derived both from seeds and sheaths. Cluster B2, with 108 strains, represented more than half of the obtained pathogenic forms and was closely related to cluster B1 (1). Cluster B2 strains produced a wide range of sheath browning and rotting and, based on the types of lesions produced, were indistinguishable from cluster B1. However, the mean percentage of plants affected by cluster B2 strains was lower than that for cluster B1 (Table 3). Furthermore, cluster B2 was phenotypically distinct from cluster B1 (1).

Among the pathogenic forms isolated from leaf sheaths, seeds, and seedlings, only a small portion clustered with *P. fuscovaginae* (11 strains), *B. glumae* (five strains), and *A. a.* subsp. *avenae* (14 strains). In fact, the majority (85%) of the pathogenic forms was grouped by the Biolog identification system into either unidentified clusters or clusters containing *Pseudomonas* spp. usually regarded as saprophytes (1). In the literature, these *Pseudomonas* spp. are not considered pathogens (3,10,14,24); whether they are opportunists needs further verification. Their role in the development of the disease syndrome has not been recognized so far.

In our survey, 52 pathogenic strains were obtained from apparently symptomless leaf sheaths or grains (28 strains from the two seed batches and 24 strains from seven of the 26 samples containing symptomless material in the total of 304 samples). This appeared to confirm the finding of Miyajima and Akita (11) that rice plants harbor many pathogenic bacteria that reside for a long time on or in the leaf sheath and cause disease at the booting stage. Pathogenic bacteria were also isolated from both discolored and healthy seed in a single seed batch. It is difficult to ascertain if there had been contamination during seed harvesting and processing, or if these strains were naturally associated with the seed. In the present survey, no pathogenic strains were isolated from more than 70% of leaf sheaths with browning or rotting and discolored seeds. It is premature to assume that pathogenic bacteria are involved in all cases of the sheath rot complex and grain discoloration syndrome. Earlier reports recognized the difficulty of recovering pathogenic pseudomonads from rice tissues (10,25). The large number of samples that did not produce any pathogenic strain could also be related to the efficiency of the isolation method used in the current study. A recent endeavor to improve the method of isolation of these bacteria from rice seed produced a higher recovery (G. L. Xie, *personal communication).*

Although there were strains resembling *P. fuscovaginae,* they were few in number but originated from a wide array of locations in the Philippines representing both tropical lowland (Laguna, Palawan, Davao) and tropical highland areas (Banaue, Ifugao at an elevation of more than 950 m above sea level). Although clustered with *P. fuscovaginae* by the Biolog system, none of the strains was identical to the type culture of *P. fuscovaginae* described by Miyajima et al. (1,12). In addition, pathogenicity tests did not produce the typical symptoms of bacterial sheath brown rot caused by *P. fuscovaginae* (17). Strains clustered with *A. a.* subsp. *avenae* produced the typical brown stripe lesions on leaves of rice seedlings. Strains clustered with *B. glumae,* however, did not produce the typical seedling rot, nor the grain rot or glume blight caused by *B. glumae* (19). Strains of *A. a.* subsp. *avenae* and *B. glumae* all originated from tropical lowland areas.

Further specification of *P. fuscovaginae* and *B. glumae* in relation to the sheath rot complex and grain discoloration is in progress. The influence of environmental factors and rice group, i.e., the indica (under tropical climate) and the japonica (under temperate climate), on the pathogenicity of these bacteria is an important area to be addressed in further studies. Because a large number of pseudomonads considered non-pathogenic in the literature were isolated from tissues with the sheath rot complex and grain discoloration, their roles in the syndrome, especially in relation to the pathogenic forms, need further research effort.

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Bacterial Diseases of Rice. II. Characterization of Pathogenic Bacteria Associated with Sheath Rot Complex and Grain Discoloration of Rice in the Philippines

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ABSTRACT

Cottyn, B., Van Outryve, M. F., Cerez, M. T., De Cleene, M., Swings, J., and Mew, T. W. 1996. Bacterial diseases of rice. II. Characterization of pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80:438-445.

From over 5,600 bacteria isolated from rice plants with sheath rot complex and grain discoloration syndrome, and two batches of 1 kg of rice seed (cultivars IR54 and IR8866), 204 pathogens were initially characterized by phenotypic tests, serology, and growth on selective media, and further distinguished by API 20NE, Biolog, and cellular fatty acid methyl ester-fingerprints. The best differentiation was obtained by the Biolog system. The nonfluorescent pathogens were represented by clusters DI *(Burkholderia glumae,* formerly *Pseudomonas glumae)* and E *(Acidovorax avenae* subsp. *avenae,* formerly *Pseudomonas avenae).* Seven clusters were distinguished among the fluorescent strains associated with sheath rot complex and grain discoloration. Cluster A5 was identified as *Pseudomonas aeruginosa,* and cluster BI as *P. fuscovaginae.* Cluster B2 is related to *Pseudomonas aureofaciens, P. corrugata, P. fluorescens,* and *P. marginalis.* Clusters B1 and B2 were only slightly different. The strains identified as *P. fuscovaginae* were different from the type strains in 2-ketogluconate production.

Sheath rot complex and grain discoloration of rice *(Oryza sativa* L.) generally describes the disease syndrome involving a brown discoloration or rot of the flag leaf sheath and discoloration of the grain. Both fungi and bacteria (mainly *Pseudomonas)* are reported to be associated with the disease syndrome (3,12,16,17,33). Several fluorescent and nonfluorescent pathogenic *Pseudomonas* spp. have been isolated from rice plants with sheath rot complex and grain discoloration in Latin America, Africa, and Asia (5,13,20,33,36). *Pseudomonas syringae* pv. *oryzae,* causal agent of halo blight (9), and *P. plantarii,* reclassified as *Burkholderia plantarii* (29), causal agent of seedling blight (1), have been reported so far only in Japan. *Pseudomonas avenae,* reclassified as *Acidovorax avenae* subsp. *avenae* (31), causes bacterial stripe, also

known as brown stripe, and has long been recorded in the tropics (16,26). *Pseudomonas glumae,* reclassified as *Burkholderia glumae* (29), the cause of both seedling rot (28) and grain rot (6), was first reported in Japan and later in other Asian countries. *Pseudomonas fuscovaginae,* which causes sheath brown rot, was first reported in Hokkaido, North Japan (25). The species *P. fuscovaginae* was of special interest, as it was isolated from seedlings issued from seeds of cold-tolerant rice cultivars supplied by IRRI (Philippines) to ISABU (Burundi) (5). Reports on the occurrence of this pathogen in tropical Asia were lacking.

A prior study described the survey that was conducted to sample bacterial pathogens associated with grain discoloration and leaf sheath rot syndromes throughout the major rice growing districts in the Philippines (3). Pathogenicity tests identified 204 pathogenic forms out of the total pool of collected strains. The symptoms caused by these 204 bacterial pathogens were indistinguishable based on symptomatology induced by artificial inoculation (3); hence, the etiology of the disease syndrome could not be determined from symptoms only. Various selective media are reported to distinguish the different pathogenic pseudomonads of rice (11,13,18,27,34). In this study, we attempt to apply and confirm the efficiency of these media. The characterization and identification of *Pseudomonas* spp. is a long process when determined from cytological, morphological, and biochemical characteristics. As more techniques become available to meet the need for fast, automated, and reliable identification of bacteria, e.g., the Biolog GN MicroPlate system, API 20NE, and cellular fatty acid methyl ester (FAME) analysis, we plan to compare the applicability of these techniques. Also, the suggested combination of diagnostic tests for identification of *P. fuscovaginae* by Rott et al. (19) was applied on the Philippine strains. The objectives of this study were to determine the bacterial pathogens associated with sheath rot complex and grain discoloration in the Philippines, clarify the frequency of occurrence of the different *Pseudomonas* spp. involved, evaluate the potential of different identification methods for identification of bacterial pathogens of rice, and investigate the prevalence of *P. fuscovaginae* in the Philippines as reported (5,36) but never confirmed.

Materials and methods

Isolation of bacteria and pathogenicity. A survey was conducted to sample bacterial pathogens associated with grain discoloration and leaf sheath rot complex syndromes throughout the major rice growing districts in the Philippines during the wet seasons of 1988 and 1989. Isolation of bacteria from rice plants with the sheath rot complex and grain discoloration was done as described in the previous study (3). Based on pathogenicity tests, 204 pathogenic forms out of the total pool of collected strains were identified (3). The symptoms caused by these pathogenic strains, using inoculation methods of injection at seedling and booting stages, were indistinguishable. All pathogenic strains were lyophilized. The type and reference strains were obtained from the LMG Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium.

Selective media. The two media for detection of *B. glumae,* S-PG and PPGA + 0.1% $CaCl_2$, were prepared as described, respectively, by Tsushima et al. (27) and Matsuda et al. (11). Medium KBS of Rott et al. (18), arginine differential medium of Zeigler and Alvarez (34), and Miyajima's selective medium (13) were used for detection of *P. fuscovaginae.*

Serology. An antiserum for *P. fuscovaginae* "A" was supplied by K. Mijayima (13), whereas antisera to *B. glumae* NIAES 1169^T and *B. plantarii* NIAES 1723^T were supplied by K. Azegami (1). Serological relationships were observed by the Ouchterlony double diffusion method using standard procedures (10). The wells had a diameter of 4 mm, and the distance between the centers of the wells was 9 mm. The agar plates loaded with the antigens and antisera were placed in a humidified plastic box covered with plastic wrap and kept at room temperature.

Biochemical characteristics. The pathogenic strains were divided into two groups according to fluorescent pigmentation on King's medium B (8). One-day-old nutrient agar cultures were used for the Gram staining, Kovac's oxidase reaction, and the inoculation of Hugh and Leifson's OF medium (4). Nitrate reduction, levan from sucrose, arginine dihydrolase, and 2-ketogluconate production were determined according to the methods described by Schaad (22) and Lelliot and Stead (10). Acid production from sucrose, inositol, sorbitol, and trehalose was tested on the medium of Ayers et al. (10).

The API 20NE galleries were used according to the manufacturer's specifications (API Systems Bio Merieux SA 69280 Marcy-l'Etoile, France). The results were recorded after 48 h incubation at 28°C. The Biolog GN microplates (Biolog Inc., 3447 Investment Blvd., Suite 3, Hayward, CA 94545) were inoculated with a bacterial suspension (OD at 590 nm was about 0.250). The plates were incubated at 30°C for 48 h. Results were analyzed with Biolog GN database version 2.00 to determine the identity of each strain. Numerical analysis was performed using the simple matching coefficient and unweighted arithmetic average clustering. Growth conditions of cultures and preparation of the fatty acid methyl esters were performed according to Sasser (21). The generated profiles were analyzed using the Standard Aerobe Library provided by the Microbial Identification System (MIS version 3.2, Microbial ID Inc., Newark, DE). The same software was also used for unweighted, arithmetic average clustering and principal component analysis.

Results

The 204 Philippine strains pathogenic for rice were aerobic Gram-negative rods (3). They were divided into two groups based on fluorescent pigmentation on King's medium B. The nonfluorescent group contained 19 strains, while 185 strains belonged to the diverse group of fluorescent pseudomonads. Twelve biochemical characteristics were determined for the 204 strains, as well as growth on 65 different carbon sources (results not shown). When the eight biochemical tests recommended for identification of *P. fuscovaginae* by Rott et al. (19) were used for comparison, none of the 204 Philippine strains entirely fitted the biochemical profile. The 11 strains identified by the Biolog system as *P. fuscovaginae* differed from Rott's *P. fuscovaginae* profile only for production of 2-ketogluconate (Table 1). Moreover, the biochemical profile of these 11 strains was unique among the 185 fluorescent strains. They were oxidase and arginine dehydrolase positive and produced acid from trehalose. They did not produce acids from inositol, sorbitol, or sucrose (with the exception of strain 4521). They were, however, positive for production of 2-ketogluconate. The biochemical profiles of the nonfluorescent strains identified by Biolog as *B. glumae* and *A. avenae* conformed to the phenotypic features of the species (1, 2); there was a weak or positive Kovac's oxidase reaction for some of the *A.*

avenae strains (1,35). The results obtained by Biolog, API, and FAME-MIS are shown in Table 2 for the type and reference strains and in Table 3 for the 204 rice strains.

Biolog. The 45 reference strains were correctly identified except for *B. glumae, B. plantarii, P. aureofaciens,* and the pathovars of *P. syringae* (Table 2). The first two did not occur in the Biolog GN Data Base version 2. Table 3 shows the results of the identification of the 204 rice strains grouped according to the numerical analysis of the Biolog data. A simplified dendrogram of these results is represented in Figure 1. The majority of the rice strains associated with sheath rot and grain discoloration was grouped into five A clusters and two B clusters delineated at 90% similarity. The clusters A2, A3, A4, and A6 did not contain reference strains. Cluster A5 was related to *P. aeruginosa.* Cluster B1 contained 19 strains, including all *P. fuscovaginae* reference strains. Cluster B1, although displaying a unique phenotypic profile, was closely related to cluster B2, which contained 108 pathogenic rice strains and eight reference strains belonging to the species *P. marginalis, P. corrugata, P. fluorescens,* and *P. aureofaciens.* The homogeneous cluster Dl contained the *B. glumae* reference strains together with five strains; whereas cluster D2 contained the *B.*

Table 1. Selected biochemical characteristics of the strains pathogenic to rice identified by the Biolog GN MicroPlate system as *Acidovorax avenae, Burkholderia glumae,* and *Pseudomonas fuscovaginae*

IRRI number	Biolog identity	Biochemical tests[a]									
		NI	FL	ARG	KOV	LEV	SOR	SUC	INO	TRE	2–KG
1837	*A. avenae*	+[b]	–	–	–	–	+	–	–	–	–
1840	*A. avenae*	+	–	–	–	–	+	–	–	–	–
1845	*A. avenae*	+	–	–	–	–	+	–	–	–	–
1851	*A. avenae*	+	–	W	–	–	+	–	–	–	–
1891	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7010	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7012	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7014	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7015	*A. avenae*	+	–	W	+	–	+	–	–	–	–
7017	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7018	*A. avenae*	+	–	–	–	–	+	–	–	–	–
7019	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7021	*A. avenae*	+	–	–	+	–	+	–	–	–	–
7023	*A. avenae*	+	–	–	+	–	+	–	–	–	–
1857	*B. glumae*	+	–	–	W	–	+	–	+	+	–
1858	*B. glumae*	+	–	–	W	–	+	–	+	+	–
2056	*B. glumae*	+	–	–	–	–	+	–	+	+	–
2057	*B. glumae*	+	–	–	–	–	+	–	+	+	–
2076	*B. glumae*	+	–	–	–	–	+	–	+	+	–
4521	*P. fuscovaginae*	+	+	+	+	–	–	+	–	+	+
4605	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5793	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5801	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
5803	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6031	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6202	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6235	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
6609	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
7007	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+
7008	*P. fuscovaginae*	–	+	+	+	–	–	–	–	+	+

[a]NI = nitrate reduction test; FL = fluorescence on King's medium B; ARG = arginine dihydrolase test; KOV = Kovac's oxidase test; LEV = levan production from sucrose; SOR = acid production from sorbitol; SUC = acid production from sucrose; INO = acid production from inositol; TRE = acid production from trehalose; 2–KG = production of 2–ketogluconate. [b]+ = positive reaction; – = negative reaction; W = weak reaction.

Table 2. Identification and characterization of the type and reference strains

Type and reference strains	BIOLOG		API 20 NE	FAME-MIS
	Cluster	Identification (similarity)	Identification (profile)	Identification (similarity)
Pseudomonas aeruginosa LMG[a] 1242[Tb]	A5	*P. aeruginosa* (0.689)	*P. aeruginosa* (1154575)	*P. aeruginosa* (0.758)
P. aureofaciens LMG 1245[T]	B2	*P. fluorescens* C (0.350)	*Pseudomonas* (1157557)	*P. aureofaciens* (0.700)
P. aureofaciens LMG 5832	B2		*Pseudomonas* (0157557)	*P. aureofaciens* (0.740)
Acidovorax avenae subsp. *avenae* LMG 1806	E	*A. avenae* (0.448)	No match (1205464)	*P. facilis* (0.550)
A. avenae subsp. *avenae* LMG 2117[T]	E	*A. avenae* (0.534)	No match (1205454)	*P. facilis* (0.580)
A. avenae subsp. *avenae* LMG 2118	E	*A. avenae* (0.501)	No match (1204464)	*P. facilis* (0.655)
A. avenae subsp. *avenae* LMG 6516	E	*A. avenae* (0.665)	No match (1205464)	*P. facilis* (0.656)
A. avenae subsp. *avenae* LMG 6517	E	*A. avenae* (0.680)	No match (1205460)	*P. facilis* (0.592)
A. avenae subsp. *avenae* LMG 10904	E	*A. avenae* (0.835)	No match (1245474)	*P. facilis* (0.856)
P. corrugata LMG 2172[T]	B2	*P. corrugata* (0.765)	*Pseudomonas* (0057555)	
P. fluorescens LMG 1794[T]	B2	*P. fluorescens* A (0.612)	*P. fluorescens* (0147555)	*P. fluorescens* B (0.856)
P. fuscovaginae LMG 2158[T]	B1	*P. fuscovaginae* (0.642)	*Pseudomonas* (0157455)	*P. aureofaciens* (0.603)
P. fuscovaginae LMG 2192	B1	*P. fuscovaginae* (0.626)	*Pseudomonas* (0147455)	*P. aureofaciens* (0.353)
P. fuscovaginae LMG 5097	B1	*P. fuscovaginae* (0.627)	*Pseudomonas* (0147455)	*P. aureofaciens* (0.580)
P. fuscovaginae LMG 5742	B1	*P. fuscovaginae* (0.778)	*Pseudomonas* (0157455)	*P. aureofaciens* (0.320)
P. fuscovaginae LMG 12427	B1	*P. fuscovaginae* (0.825)	*P. fluorescens* (0146555)	*P. aureofaciens* (0.379)
P. fuscovaginae LMG 12428	B1	*P. fuscovaginae* (0.163)	*P. fluorescens* (0147555)	*P. putida* A (0.312)[b]
P. fuscovaginae LMG 12424	B1	*P. fuscovaginae* (0.501)	*Pseudomonas* (0047455)	*P. fluorescens* A (0.612)
P. fuscovaginae LMG 12425	B1	*P. fuscovaginae* (0.676)	*P. fluorescens* (0147555)	*P. aureofaciens* (0.360)
Burkholderia glumae LMG 1277	D1		No match (1477551)	No match
B. glumae LMG 2196[T]	D1		No match (1077051)	*P. cepacia* (0.748)
B. glumae LMG 10905	D1		No match (1077451)	*P. cepacia* (0.671)
B. glumae LMG 10906	D1		No match (1477451)	
P. marginalis pv. *marginalis* LMG 2210	B2	*P. marginalis* (0.756)	No match(0547555)	*P. fluorescens* C (0.531)
P. marginalis pv. *alfalfae* LMG 2214	B2		No match (1557555)	*P. fluorescens* C (0.806)
P. marginalis pv. *marginalis* LMG 2215	B2		*Pseudomonas* (1157555)	*P. fluorescens* A (0.670)
P. marginalis pv. *pastinacae* LMG 2238	B2	*P. marginalis* (0.700)	*Pseudomonas* (1157555)	*P. fluorescens* B (0.843)
B. plantarii LMG 9035[T]	D2	*P. caryophylli* (0.329)	*Pseudomonas* (1077555)	
B. plantarii LMG 10907	D2		*Pseudomonas* (0077555)	
B. plantarii LMG 10908	D2		No match (1077656)	*P. gladioli* (0.324)
B. plantarii LMG 10909	D2	*P. caryophylli* (0.325)	*Pseudomonas* (1077555)	
B. plantarii LMG 10910	D2		No match (1077554)	
B. plantarii LMG 10911	D2		No match (1077554)	
P. putida LMG 2257[T]	A1	*P. putida* B (0.798)	*P. putida* (0140457)	*P. aureofaciens* (0.321)
P. putida LMG 5835	A1	*P. putida* B (0.627)	*P. putida* (0140457)	
P. syringae pv. *oryzae* LMG 10912	C	*P. syringae* pv. *pisi* (0.451)	No match (0447477)	*P. syringae* (0.658)
P. syringae pv. *oryzae* LMG 10913	C	*P. syringae* pv. *aptata* (0.569)	*P. chlororaphis* (0447451)	*P. syringae* (0.545)
P. syringae pv. *oryzae* LMG 10914	C	*P. syringae* pv. *aptata* (0.628)	*P. chlororaphis* (0447441)	*P. syringae* (0.595)
P. syringae pv. *oryzae* LMG 10915	C	*P. syringae* pv. *pisi* (0.405)	*P. chlororaphis* (0447441)	*P. syringae* (0.592)
P. syringae pv. *oryzae* LMG 10916	C	*P. syringae* pv. *aptata* (0.668)	No match (0447450)	*P. syringae* (0.314)
P. syringae pv. *oryzae* LMG 10917	C	*P. syringae* pv. *pisi* (0.451)	*P. chlororaphis* (0447451)	*P. syringae* (0.640)
P. syringae pv. *oryzae* LMG 10918	C		*P. chlororaphis* (0447441)	*P. syringae* (0.599)
P. syringae pv. *oryzae* LMG 10919	C	*P. syringae* pv. *tabaci* (0.412)	*P. chlororaphis* (0447441)	*P. syringae* (0.394)
P. syringae pv. *oryzae* LMG 10920	C	*P. syringae* pv. *pisi* (0.322)	No match (0447440)	*P. syringae* (0.385)
P. syringae pv. *panici* LMG 2367	C	*P. syringae* pv. *aptata* (0.547)	*P. chlororaphis* (0447455)	*P. syringae* (0.825)

[a]LMG = Culture Collection of the Laboratorium voor Microbiologie, Universiteit Gent.
[bT] Type strain.
[c]Second choice.

plantarii reference strains only. Cluster E comprised the *A. a.* subsp. *avenae* reference strains and 14 rice strains. The Biolog clustering reflected the phylogenetic relatedness between the different species involved in this study. All groups containing strains related to *P. fluorescens (Pseudomonas fluorescens* rRNA group of superfamily II) (30) or gamma subdivision (32) gathered into one cluster separate from the strains of rRNA superfamily III (30) or beta subdivision (32). The latter, comprising the former *P. glumae* and the former *P. plantarii*, belongs to the *solanacearum* rRNA complex, which has been transferred to the new genus *Burkholderia* (29), and *A. a*. subsp. *avenae* belongs to the *acidovorans* rRNA complex (31).

Table 3. Identification and characterization of the pathogenic strains associated with the "sheath rot complex" and "grain discoloration" syndrome

Number and origin of pathogenic strains[a]	BIOLOG		API 20 NE	FAME-MIS
	Cluster	Identification (similarity)[b]	Identification (profile)	Identification (similarity)[b]
1 seed isolate (IR54)	A2	*Pseudomonas fluorescens* C (0.682-0.862)	*Pseudomonas* (0156557)	*P. aureofaciens* (0.270-0.527)
2 ShR[c] & GD[d] isolates				
3 seed isolates (IR8866)	A3	*P. fluorescens* C (0.638-0.768)	*Pseudomonas* (1152555)	
22 seed isolates (IR54, IR8866)	A4	*P. fulva* (0.234-0.451)	No match (0054455)	*P. putida* A (0.181-0.755)
21 ShR & GD isolates		*P. putida* A (0.137-0.790)	*Pseudomonas* (0056451)	
			Pseudomonas (0056455)	
			Pseudomonas (0154455)	
			P. fluorescens (0156455)	
2 seed isolates (IR54, IR8866)	A5	*P. aeruginosa* (0.834-0.826)	*P. aeruginosa* (0154475)	*P. aeruginosa* (0.660-0.739)
2 Shr & GD isolates			*P. aeruginosa* (1154475)	
9 seed isolates (IR54, IR8866)	A6	*P. fluorescens* C (0.093-0.697)	*Pseudomonas* (0156457)	No match
4 ShR & GD isolates			*Pseudomonas* (0156557)	
2 seed isolates (IR8866)	B1	*P. fuscovaginae* (0.711-0.917)	*P. fluorescens* (0147555)	No match
9 ShR & GD isolates			*P. fluorescens* (0157455)	*P. putida* A (0.381)
			Pseudomonas (0157555)	
3 seed isolates (IR54, IR8866)	B2	*P. corrugata* (0.334-0.424)	*P. fluorescens* (0147555)	*P. aureofaciens* (0.135-0.796)
105 ShR & GD isolates		*P. marginalis* (0.090-0.838)	*P. fluorescens* (0156555	*P. fluorescens* B (0.106-0.802)
		P. fluorescens B (0.747)	*Pseudomonas* (0157555)	*P. putida* A (0.197-0.769)
			No match (0357555)	
5 ShR & GD isolates	D1		No match (1077051)	No match
			No match (1077451)	
			No match (1477551)	
14 seedling isolates	E	*Acidovorax avenae* (0.188-0.900)	No match (1205474)	*P. facilis* (0.145-0.509)
			No match (1205464)	*Hydrogenophaga pseudoflava* (0.123-0.457)
			No match (1205460)	
			No match (1247577)	

[a]Seed isolates were obtained from 1-kg seed batches.
[b]Minimum and maximum similarity is mentioned when more isolates gave same identification.
[c]ShR = sheath rot.
[d]GD = grain discoloration.

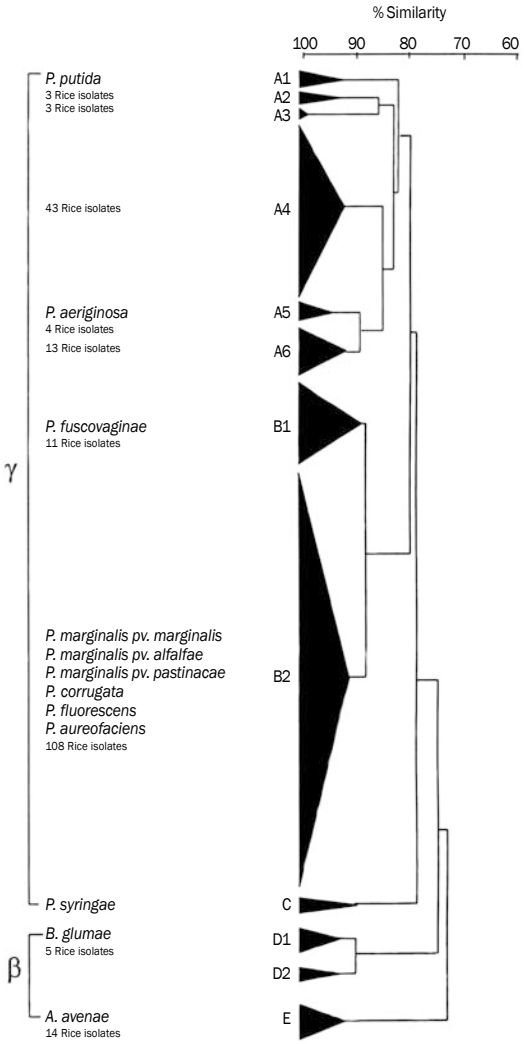

Fig. 1. Simplified dendrogram showing the Biolog clusters of the 204 pathogenic strains and 40 reference strains. The species names refer to the type strains and/or reference strains that were included. The designations β and γ refer to the subdivisions distinguished by Woese (32).

API. Many API codes either did not occur in the API profile index or identified the strain at genus level only (Tables 2 and 3). Strains with API code identical to the type or the reference strains were tentatively identified. This was the case for the strains belonging to *A. a.* subsp. *avenae* and *B. glumae* and for two strains related to *P. fuscovaginae.*

FAME-MIS. Identification of *A. a.* subsp. *avenae, B. glumae,* and *B. plantarii* was hampered by the absence of their profiles in the Standard Aerobe Library (version 3.2). Cluster analysis and principal component analysis revealed six homogeneous groups related to *P. syringae, P. aeruginosa, B. plantarii, A. a.* subsp. *avenae,* and (two groups) *B. glumae.* The mean fatty acid profiles of these groups are shown in Table 4. The majority of the strains and reference strains belonging to the fluorescent pseudomonads constituted a heterogeneous group. A mean profile for this group is also shown in Table 4. It is only indicative and should be considered with care. The hydroxy fatty acids have discriminative value (15,24). They constituted the only qualitative differences between the fluorescent pseudomonads and *P. syringae* and *P. aeruginosa* on one hand, and *B. glumae* and *B. plantarii* on the other hand. The hydroxy fatty acids 12:0 2-OH and 12:0 3-OH were characteristic of the former group, while 16:1 2-OH, 18:1 2-OH, and summed feature 3 were indicative of the latter. *A. a.* subsp. *avenae* contained fewer fatty acids but was characterized by significantly higher amounts of 16:1 *cis* 9. *B. glumae* and *B. plantarii* differed from each other in the amount of 17:0 cyclo, 19:0 cyclo, and summed feature 7. The differences between the two groups of *B. glumae* were only quantitative. The amount of the following fatty acids was significantly different among *P. syringae, P. aeruginosa,* and the other fluorescent pseudomonads: 12:0 2-OH, 12:0, 17:0 cyclo, 16:1 cis 9, 16:0 3-OH, and summed feature 7.

Ouchterlony double diffusion tests. None of the II strains resembling *P. fuscovaginae* reacted with the antiserum to *P. fuscovaginae* "A" provided by K. Mijayima. Antigenic variability from the Japanese strain may be the reason. Also, a

Table 4. Fatty acid profiles of the Biolog clusters

	A1,A2,A3,A4, A6,B1,B2[a]	C	A5	D1		D2	E
10:0 3-OH	6.0 (4.1)[b]	5.4 (1.0)	4.9 (0.5)	tr			6.2 (1.0)
12:0	1.5 (0.9)	5.3 (0.4)	3.2 (0.1)			3.4 (0.5)	
12:0 2-OH	7.2 (1.5)	3.4 (0.3)	4.5 (0.2)				
12:1 3-OH			0.4 (0.3)				
12:0 3-OH	5.5 (1.0)	4.9 (0.6)	4.5 (0.2)	tr			
14:0	0.3 (0.1)	tr	0.6 (0.1)	4.7 (0.7)	3.7 (0.0)	4.6 (0.3)	2.2 (0.5)
? 14.503				1.6 (1.2)			
15:0			tr			0.8 (1.5)	
16:1 *cis* 9	23.8 (5.9)	39.1 (0.8)	14.4 (3.5)	6.3 (2.6)	16.29 (2.9)	4.6 (1.2)	45.7 (2.1)
16:0	28.0 (3.3)	25.0 (1.2)	23.3 (1.6)	18.9 (3.8)	22.67 (0.2)	23.7 (2.0)	28.8 (2.0)
17:0 cyclo	7.4 (4.9)		1.0 (0.7)	10.61 (2.3)	3.6 (3.15)	20.0 (3.1)	
16:1 2-OH				tr	1.2 (0.0)	0.4 (0.4)	
16:0 2-OH				tr	0.9 (0.0)	tr	
16:0 3-OH		1.1 (0.8)		5.5 (0.2)	4.55 (0.0)	6.6 (0.6)	
18:0	0.7 (0.2)	0.6 (0.1)	0.5 (0.1)		1.1 (0.2)	0.8 (0.5)	
19:0 cyclo C11-12	0.4 (0.4)		1.5 (1.0)	6.4 (2.0)	2.4 (2.0)	10.0 (0.9)	
18:1 2-OH				4.7 (0.9)	3.0 (0.5)	5.1 (0.4)	
SUM 3[c]				13.7 (6)	5.4 (0.1)	8.3 (4.3)	
SUM 7[d]	16.2 (3.4)	14.4 (1.5)	38.3 (1.0)	25.5 (3.5)	34.7 (3.2)	14.6 (2.3)	12.3 (2.0)

[a]Referring to the Biolog clusters.
[b]Percent fatty acid; standard deviation in parenthesis.
[c]Summed features 16:1 *iso* and 14:0 3-OH.
[d]Summed features 18:1 *cis* 11 and 18:1 *trans* 11.

negative serological reaction was previously reported by Rott et al. (20) as not exclusive for identification of *P. fuscovaginae.* Of the 19 nonfluorescent strains, only five (1857, 1858, 2056, 2057, and 2076) reacted with the antiserum to *B. glumae* NIAES 1169^T, and none formed precipitin bands with the antiserum to *B. plantarii* NIAES 1723^T (Table 5).

Selective media. An overview of the tested selective media described in the literature for differentiating bacterial pathogens related to sheath rot and grain discoloration is shown in Table 6. The diverse group of fluorescent strains, comprising 90% of the 204 collected pathogens, were all tested on *P. juscovaginae*-selective media, since no other selective media for fluorescent rice pathogens were noted in the literature. Although KBS medium was suggested as a semiselective medium to isolate *P. fuscovaginae,* all fluorescent strains except seven grew on it. Also, 44 strains were tentatively identified as *P. fuscovaginae* on the arginine differential medium. Only the medium described by Miyajima proved to be more selective. Three strains (6202, 6235, 6031) showed the characteristic cream colonies with greenish center after incubation (Fig. 2). However, eight strains identified by Biolog as *P. fuscovaginae* did not show the typical colony morphology on this medium.

Differentiation of the nonfluorescent strains was made by use of selective media for *B. glumae.* No calcium oxalate crystals, a typical characteristic for *B. glumae,* were produced by any of the nonfluorescent strains after incubation on potato-peptone-glucose agar (PPGA) medium supplemented with 0.1% $CaCl_2$. Growth on the arginine differential medium (34) appeared not to conform with the identities obtained by the Ouchterlony double diffusion tests (ODD) and by Biolog, as shown in Table 5. On S-PG medium, both colony types A (reddish brown) and B (purplish pink) were observed. While colonies identified as *B. glumae* type B could actually be *A. a.* subsp. *avenae,* five strains (Table 5) were

Table 5. Characterization of the nonfluorescent pathogenic strains by selective media and serology

IRRI number	Biolog identity	SPG medium[a]	Arginine medium	Serological reaction[b]	
				P. glumae NIAES 1169^T	*P. plantarii* NIAES 1723^T
1857	*Burkholderia glumae*	*B. glumae* A	*B. glumae*	+	–
1858	*B. glumae*	*B. glumae* A	*B. glumae*	+	–
2056	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
2057	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
2076	*B. glumae*	*B. glumae* B	*B. glumae*	+	–
1837	*Acidovorax avenae*	*B. glumae* B	*B. glumae*	–	–
1840	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1845	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1851	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
1891	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7010	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7012	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7014	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7015	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7017	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7018	*A. avenae*	*B. glumae* B	*B. glumae*	–	–
7019	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7021	*A. avenae*	*B. glumae* B	*A. avenae*	–	–
7023	*A. avenae*	*B. glumae* B	*B. glumae*	–	–

[a]Type A colonies = reddish brown; type B colonies = opalescent purple.
[b]Ouchterlony double diffusion test (ODD) where + = formation of precipitin bands and – = absence of precipitin bands.

Table 6. Evaluation of selective media for the detection of *Burkholderia glumae* and fluorescent pathogenic pseudomonads of rice

Group	Selective media	Diagnostic value
Nonfluorescent	S-PG medium for *B. glumae*	+[a]
	Potato-peptone-glucose agar (PPGA) with 0.1 % $CaCl_2$ for *B. glumae*	–
Fluorescent	Miyajima's selective medium for *Pseudomonas fuscovaginae*	+
	KBS semi-selective medium for *P. fuscovaginae*	–
	Arginine differential medium	–

[a]+ = satisfactory; – = poor.

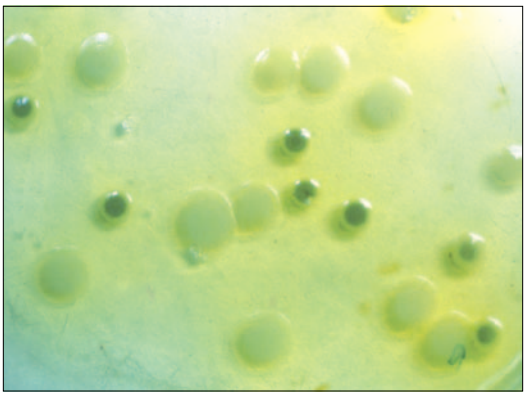

Fig. 2. Typical greenish colorations in the center of colonies of strain 6031 on the selective medium of Miyajima for *Pseudomonas fuscovaginae*.

positively identified as *B. glumae* in combination with the ODD tests. This procedure was recommended by Tsushima et al. (27).

Discussion

The previous study confirmed the presence of different bacterial pathogens associated with sheath rot complex and grain discoloration in the Philippines (3). Of the total number of strains collected from rice tissues with sheath rot complex and grain discoloration syndrome, only 3.6% was

found pathogenic (3). In agreement with earlier reports, it is known that various pseudomonads may be isolated from rice plants displaying the disease syndrome, and that apart from the small proportion of pathogenic species, the majority of these bacteria are saprophytic (19, 20, 33, 35). The symptoms caused by these strains were similar (3), preventing determination of the etiology from symptoms alone. More than half of the 204 pathogenic strains were grouped by Biolog into cluster B2, together with eight reference strains of the species, *P. marginalis, P. corrugata, P. fluorescens,* and *P. aureofaciens.* The cluster B1 containing *P. fuscovaginae* produced lesions rapidly, but was indistinguishable from cluster B2 for the types of lesions produced (3). However, all strains of cluster B2 differed phenotypically from cluster B1 for at least three biochemical characteristics, generally being positive for acid production from inositol, sorbitol, and sucrose. Although these *Pseudomonas* spp. are usually regarded as saprophytes, their possible role in the development of the disease syndrome has not been recognized so far. They were, however, identified in this study as pathogenic forms able to induce leaf sheath browning. *P. marginalis* has been involved to some extent in sheath rot of rice (7, 20, 33, 35). Therefore, we feel that their identity needs to be further clarified and established.

P. syringae was not detected among the Philippine strains. All phenotypical characteristics suggested the absence of this species, and none of the strains was identified as such by Biolog or FAME. Of the bacterial pathogens commonly reported to be involved in sheath rot complex and grain discoloration, three were encountered in this study. They were *P. fuscovaginae, B. glumae,* and *A. a.* subsp. *avenae.* However, they represented only 15% of the collected pathogenic strains associated with the disease syndrome in the Philippines. *A. a.* subsp. *avenae* strains that produced the brown stripe on the leaf midrib of inoculated seedlings were the only pathogens recognizable by a typical symptom expression in the pathogenicity tests (3). However, because it was isolated only from seedlings, the role of *A. a.* subsp. *avenae* in sheath rot complex and grain discoloration in the Philippines was not clear. Five strains were identified as *B. glumae* by the appearance of the typical colony formation on S-PG medium in combination with a positive serological reaction to *B. glumae* NIAES l169^T antiserum, as was recommended by Tsushima et al. (27). They also fit the biochemical profile of the species (1, 2) and were congruent with the Biolog identification. Although phenotypically related to *B. glumae* (1), none of the strains reacted with the *B. plantarii* NIAES l723^T antiserum, and none produced the reddish brown pigment associated with tropolone production in the media (1). This suggested the absence of *B. plantarii* in the collected pathogens. The species *P. fuscovaginae* was of special interest, as it was suspected to occur in the Philippines (5,35); whereas a study on the occurrence of this pathogen in tropical Asia was lacking. *P. fuscovaginae* is reported to be widespread and is considered the principal causal organism of sheath browning and grain discoloration (35). It has been described by Miyajima et al. (14) and confirmed by Duveiller et al. (5) as a fluorescent pseudomonad that is positive for the arginine dehydrolase and oxidase reactions and that can be distinguished from other species within this group by the simultaneous lack of 2-ketogluconate production and presence of acid production from trehalose but not from inositol. Further, Rott et al. (19) reported that the combination of pathogenicity and a biochemical profile of eight characteristics permitted the identification of *P.*

fuscovaginae, while a serological negative reaction was not exclusive. Several authors reported serological variability within the *P. fuscovaginae* species (5, 19, 20, 35). From the 185 Philippine fluorescent pathogenic strains, only 11 strains were clustered with *P. fuscovaginae* by Biolog. All of these 11 strains were pathogenic on rice (3), and all except one conformed to seven of the eight characteristics in the biochemical profile of Rott et al. (19). However, they were positive for production of 2-ketogluconate (Table 1). Strain 4521 was different from the others in being positive for both nitrate reduction and acid production from sucrose, which has also been reported for *P. fuscovaginae* strains from Colombia (33). On the medium of Miyajima, only three strains appeared positive by the appearance of the characteristic cream colonies with greenish center (Fig. 2). None of the 11 strains gave a positive serological reaction with the antiserum to *P. fuscovaginae* "A" from Miyajima, suggesting that the Philippine strains were serologically different from that of Japan.

Notwithstanding the use of three commercially available identification techniques—API Systems, Biolog GN MicroPlate System, and FAME-MIS—and the use of reference strains, only 23 pathogenic rice strains were unambiguously identified as either *P. aeruginosa, B. glumae,* or *A. a.* subsp. *avenae.* For the remaining 181 pathogens, the results were conflicting, although API and Biolog were comparable. However, these techniques provided an extensive characterization and constituted a real advantage, since a large number of characteristics could be checked in a short time. When identification was hampered by the lack of certain species in the library, cluster analysis of the data was very helpful. Identification was also determined by the type of characteristics used. The number of fatty acids produced by the species in this study varied around 10, apparently not always enough to allow differentiation. As demonstrated (17,24), they were not enough for differentiating most of the fluorescent pseudomonads.

Except for *P. syringae* and *P. aeruginosa,* differentiation of most bacteria was mainly based on metabolic activity. Nutritional characteristics have long been an important differentiating tool for this group of bacteria (23). The two identification systems based on metabolic activity, API and Biolog, matched well. Each Biolog cluster was characterized by a unique set of API codes, except for the closely related clusters B1 and B2, which both contained strains with the API 20NE codes 0147555 and 0157555.

Although the pathogenic species reportedly associated with the disease syndrome are separately described in the literature as causing distinct symptoms, the differentiation between these symptoms was not clear. In addition, as might be expected, there are no symptoms described in the literature for the large group of encountered pseudomonad saprophytes. Hence, the etiology of sheath rot complex and grain discoloration syndrome could not be determined from symptoms alone. The use of selective media to differentiate the various pathogens proved to be of limited value. The characterization and identification of pseudomonads by conventional procedures of cytology, morphology, and biochemical characteristics is time-consuming and greatly hampered by the implicit complexity and problems associated with the present taxonomy of the pseudomonads. From the three automated systems (Biolog GN MicroPlate, FAME, and API) applied, the Biolog GN MicroPlate system in combination with cluster analysis was the most helpful to differentiate the various pathogens. The Biolog library was, how-

ever, limited in the number of bacterial pathogens of rice. The efficiency of the system was improved by including several reference strains. Notwithstanding the application of the Biolog system with incorporation of several reference strains and cluster analysis of the data, the majority of the 204 strains still could not be identified accurately. Definitive phenotypic differentiation of these pathogenic strains may require the use of more than the 95 tests available in the Biolog GN MicroPlate system.

Surprisingly, considering that these Biolog groups were associated with sheath rot complex and grain discoloration in the Philippines, the most frequently isolated pathogens were either related to saprophytic *Pseudomonas* spp. or remained unidentified (3). Their identity and specific role in the development of the disease syndrome needs to be further established. Serological (19, 20), as well as phenotypical, variability (5, 35) within the species *P. fuscovaginae* has been reported, suggesting intraspecies differences and questioning its designation as a defined species. The Philippine strains, clustered with *P. fuscovaginae* by the Biolog GN MicroPlate system, were few in number. None produced the typical brown sheath symptoms described by Tanii et al. (25). Further, they were serologically different from the *P. fuscovaginae* strain of Miyajima, and differed phenotypically from Miyajima et al.'s (14) and Rott et al.'s (19) biochemical profile for 2-ketogluconate production. These results also are in contradiction with the characteristics earlier attributed to the *P. fuscovaginae* strains that were suspected to occur in the Philippines (5, 35). As the cluster containing *P. fuscovaginae* was only slightly different from the group containing the majority of the fluorescent pseudomonads, including the type strains of *P. marginalis* and *P. fluorescens*, other techniques are needed to improve our insight into the genomic relatedness of these pathogens.

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Characterization of Rice Sheath Rot from Siniloan, Philippines

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We document here a rare incidence of rice sheath brown rot in the fourth crop of an upland rice successive cropping experiment (two crops per year) planted in October 1999 with elite upland indica line IR55423-01 and lowland hybrids Mestizo and Magat (crop management details available from T. George). This crop grew normally until flowering after which disease symptoms started to develop on all cultivars in January 2000. The plants showed dark brown lesions on flag leaf sheaths, poor panicle emergence, spikelet sterility and brownish-black discolored seed. The symptoms resembled bacterial sheath brown rot caused by *Pseudomonas fuscovaginae*. We characterized the causal agent and performed a genotypic comparison of the isolates with a previous collection of rice sheath rot isolates from the Philippines (Cottyn et al. 1996), *Pseudomonas fuscovaginae* strains from Japan and Burundi (Duveiller et al. 1988; Miyajima et al. 1983), and other *Pseudomonas* strains related to the disease (Jaunet et al. 1995) by BOX-PCR fingerprinting (Table 1).

Isolations were done by partially crushing 100 discolored seeds in 20 ml sterile water, and ten-fold dilutions in sterile saline (0.85% NaCl) were streaked

Table 1. Rice sheath rot isolates from the Philippines, reference strains of *Pseudomonas fuscovaginae* and other fluorescent pseudomonads used in the comparative analysis of BOX-PCR fingerprints.

Species	Origin		Year	Habitat	Isolate/strain number[a]
P. fuscovaginae-like	Philippines	Siniloan	2000	Rice seed	S-E1, S-E2, S-E3
		Rizal	1997	Rice seed	BPJ 3744
		Banaue	1989	Rice seed	IRRI 5793, IRRI 5801, IRRI 5803, IRRI 6031, IRRI 6202, IRRI 6235
		Laguna	1989	Rice seed	IRRI 4605
		Davao	1988	Rice sheath	IRRI 6609
		Palawan	1988	Rice sheath	IRRI 7007, IRRI 7008
P. fuscovaginae	Japan	Hokkaido	1971	Rice sheath	LMG 2192 (Miyajima 7103)
			1970	Rice sheath	LMG 5097 (Miyajima BM-1)
			1968	Rice sheath	LMG 2158^T (Miyajima 6801)
	Burundi	Gisha	1982	Rice sheath	LMG 5742 (Maraite HMB264)
P. syringae pv. *syringae*	Hungary		1957	Rice sheath	LMG 2230 (Klement A289)
P. putida bv.A	U.S.		1973	Soil	LMG 2257^T (ATCC12633)

[a]S-E, BPJ and IRRI numbers refer to Philippine rice sheath rot isolates maintained at the Entomology and Plant Pathology Division, IRRI, Los Baños, Philippines. LMG numbers refer to reference strains received as genomic DNA from the BCCM/LMG Bacteria Collection, Laboratory of Microbiology, University Gent, Belgium. Alternate strain numbers are given in parentheses. T = type strain.

onto King's medium B (KB). After incubation for 2 days at 28°C, three colonies for each abundant type were arbitrarily picked and purified on tryptic soy agar (TSA). The 18 isolates were tested for pathogenicity on 21-day-old rice seedlings of cv. IR24 grown in the greenhouse. For each isolate, eight seedlings were injected into the culm with 0.1 ml of an aqueous bacterial suspension (approximately 10^8 cells/ml). Isolates that produced a positive reaction were further inoculated on 10 plants at booting and heading in a growth chamber with day/night temperatures of 29/21°C, and relative humidity ranging from 70 to 95%. The boots were injected with 0.5-ml of aqueous bacterial suspensions; individual panicles at heading were sprayed with 2-ml of bacterial suspensions. BOX-PCR genomic fingerprinting analysis was done as described previously (Cottyn et al. 2001).

Of the 18 isolates, three isolates (S-E1 to S-E 3) representing a fluorescent colony type that was found with high frequency (approximately 10^6 CFU/ml of seed suspension) on isolation plates induced symptoms on all inoculated IR24 plants at seedling, booting, and heading stage. Their colonies were similar to those of the sheath rot isolates previously described from the Philippines (Cottyn et al. 1996). On TSA, 2-days-old colonies were creamy white, 1 to 2 mm in diameter, glistening, convex with pale smooth margins and darker color in the center. On KB, the colonies first appeared mucoid whitish, 2 to 3 mm, convex with smooth margins, and produced a pale green fluorescent pigment. After 5 days incubation, the colonies turned yellowish with greenish shades and produced an orange-brown pigment; some colony variants were translucent, flat with irregular margins. Inoculated seedlings produced water-soaked patches on the sheath that quickly developed into brown lesions with grayish necrotic centers, and often died after 10 days. Plants inoculated at the booting stage often showed inhibition of panicle emergence and symptoms were similar to those observed in the field (Fig. 1). Spray-inoculation at heading induced dark brown patches on the panicle necks and 50% discolored seed.

Based on BOX-PCR fingerprinting analysis, two clusters only distantly related at a level of 38% similarity were distinguished; one comprising all rice sheath rot isolates from the Philippines, and the other the *P. fuscovaginae* reference strains (Fig. 2). This indicates that the Philippine *P. fuscovaginae*-like isolates not only differ serologically and biochemically from the *P. fuscovaginae* type strain (Cottyn et al. 1996) but also constitute a genetically distinct population. A further taxonomic evaluation of these isolates is required to

Fig. 1. Symptoms on IR24 at booting stage 10-days after artificial inoculation by injection of *P. fuscovaginae*-like isolate S-E1.

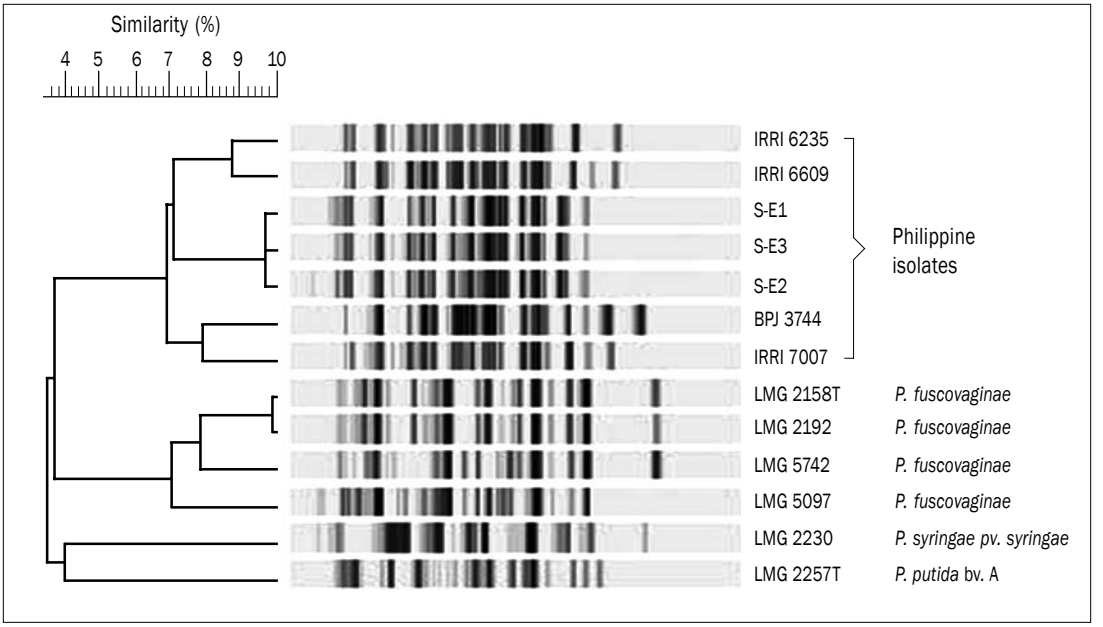

Fig. 2. Dendrogram derived from the unweighted pair group average linkage method of Pearson correlation coefficients between the BOX-PCR patterns of the isolates from discolored seed collected from the field in Siniloan (S-E), selected Philippine rice sheath rot isolates from previous collections (IRRI and BPJ), and reference strains of *Pseudomonas fuscovaginae*, *Pseudomonas syringae* pv. *syringae* (described as causing similar sheath brown rot symptoms on rice), and *Pseudomonas putida* bv. A (described as closest neighbor to the Philippine isolates based on PCR-RFLP analysis of 16S-rDNA by Jaunet et al. (1995).

establish their true relationship to *P. fuscovaginae*. In the tropics, however, bacterial sheath brown rot has overall low incidence and is not a major constraint to rice production. Previous detection of this pathogen in temperate environment or at high altitudes in the tropics pointed out the favorable influence of low temperatures on symptom development. The crop at Siniloan was exposed to minimum and maximum temperatures in the last week of December 1999 and in January 2000 of about 18/21°C and 21/25°C, respectively. It is possible that these low temperatures favored the disease development. In any case, the severe attack is evidence that the disease should not be ignored.

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C. Detection of *Xanthomonas oryzae* pv. *oryzae* in Rice Seed

Bacterial blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) became a problem to rice production in tropical Asia during the 1970s due to the widespread deployment of modern semi-dwarf cultivars and the increased use of nitrogen fertilizer (Mew, 1987). Host plant resistance has been the primary control strategy for bacterial blight; however, cultivars with single-gene resistances deployed over large areas in monoculture have been vulnerable to genetic adaptation by the pathogen population (Mew et al., 1992). Substantial research has been carried out on *Xoo* (see p. 21) and knowledge of the pathogen population structure is being actively integrated into resistance breeding and disease management. Strategies for controlling the dissemination or transmission of *Xoo* require complete understanding of how the pathogen survives in absence of the primary host, however, the uncertainty regarding the role of seed contamination for disease transmission has long impeded the design of effective control measures. The pathogen can be carried on rice seeds, but the extent to which the pathogen survives on the seed and subsequently may infect the next generation of plants is still controversial (Mew et al., 1989). Several studies indicated that seed transmission of bacterial blight is unlikely to occur in the tropics (Kauffman and Reddy, 1975; Mizukami and Wakimoto, 1969; Murty and Devadath, 1984). Infected plant debris, alternate weed hosts, or irrigation water are considered more important sources of initial inoculum for bacterial blight epidemics (Ou, 1985). There are, however, claims for seed transmission of bacterial blight (Reddy, 1983; Sakthivel et al., 2001; Singh et al., 1983).

Isolation of *Xoo* from rice seeds is especially difficult because of the low seed populations of the pathogen, its slow growth in culture and its poor competitive ability relative to other seed-associated bacteria (Gnanamanickam et al., 1994; Ming et al., 1991). The extreme difficulty to detect *Xoo* presumably has contributed to contradictory reports regarding the significance of its seedborne phase. In our experience, *Xoo* was not isolated either from seeds analyzed in the extensive surveys of rice seed-associated bacteria (see Chapter 2), nor from seeds collected from plants with bacterial blight symptoms in the following study. On the other hand, nonpathogenic xanthomonads of unknown affiliation with colony appearances similar to those of *Xoo* were frequently found in association with rice seeds. The differentiation of *Xoo* from those similar appearing nonpathogenic xanthomonads remains problematic (Ming et al., 1991). *Xoo* is considered of quarantine importance by seed regulation agencies and in case nonpathogenic xanthomonads cannot be differentiated from real pathogens, seed lots may needless be rejected. Hence, rapid and sensitive methods for detection and diagnosis of *Xoo* are critical to effective seed regulatory programs.

In the study presented, a PCR assay for detection of *Xoo* in rice seeds was developed using primers derived from the multicopy insertion sequence IS*1112* cloned from *Xoo*. A preliminary report on the primers has been published (Cottyn et al., 1994). The PCR assay was further applied to investigate whether transmission of the pathogen from contaminated seeds to seedlings can be detected.

Detection of *Xanthomonas oryzae* pv. *oryzae* in Rice Seed using the Polymerase Chain Reaction and Evaluation of its Seedborne Phase

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ABSTRACT
The polymerase chain reaction (PCR)-mediated amplification of an internal DNA segment of the insertion sequence IS*1112* is described for the detection of the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in rice seed. Two oligonucleotide primers, derived from the IS*1112* sequence, amplified the expected size fragment of 497 bp and an additional fragment of 574 bp from total DNA extracts of the pathogen. Southern hybridization suggested the presence of a specific sequence variation of 77 bp in the amplified region among copies of the IS*1112* element in *X. o. oryzae*. The limit of detection for the 574-bp fragment by ethidium bromide staining was approximately 10 cells for a pure culture of the pathogen. DNAs from 68 strains of other bacteria, including the closely related rice pathogen *X. o. oryzicola* and rice associated nonpathogenic xanthomonads, did not produce the 574-bp fragment. The primer pair differentiated Asian strains of *X. o. oryzae* from African and North American strains. The pathogen was detected against background bacterial microflora greater than 10^5 cfu per ml in washes from naturally contaminated seed that failed to yield the pathogen by plating on XOS medium. The pathogen was not detected in seed transmission tests, providing further supporting evidence that seed inoculum might not be significant for disseminating the disease.

Key words: *Xanthomonas oryzae* pv. *oryzae*, rice seed (*Oryza sativa* L.), insertion sequence IS*1112*, polymerase chain reaction, nonpathogenic xanthomonads

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (Swings et al., 1990), is a major rice disease prevalent throughout Asia (Ou, 1985). Yield losses in infected fields are estimated to range from 10 to 20% (Mew et al., 1993). Deployment of resistant cultivars is the preferred means for managing the disease, however, variation in the spectrum of virulence among strains has been reported and the emergence of new virulent races or pathotypes of *X. o. oryzae* has continued to be a constraint to rice production (Mew, 1987; Vera Cruz et al., 1996).

X. o. oryzae is a vascular leaf pathogen and enters the plant through hydathodes or wounds (Tabei, 1977). Drops of bacterial ooze are formed on the surface of leaf lesions and the bacteria from these drops can be passed directly to other plants by contact, or indirectly via the irrigation water (Ou, 1985). The source of initial

inoculum for bacterial blight epidemics is thought to originate from infected plant debris, irrigation water, weed hosts, or seed (Gonzales et al., 1991; Mizukami and Wakimoto, 1969; Ou, 1985). The presence of *X. o. oryzae* on rice seed has been demonstrated but is not marked by perceptible seed symptoms (Hsieh et al., 1974; Mizukami and Wakimoto, 1969). The extent to which the pathogen survives on the seed and its significance for disease transmission to the next generation of plants is still controversial (Mew et al., 1993). Numerous investigators (Eamchit and Ou, 1970; Kauffman and Reddy, 1975; Mizukami and Wakimoto, 1969; Murty and Devadath, 1984) consider seed transmission of bacterial blight unlikely to occur in normal conditions, although there are claims that seedlings are infected through seed (Reddy, 1983; Singh et al., 1983). Despite conclusive evidence for seed transmission, several countries have phytosanitary restrictions for seedborne *X. o. oryzae* to avoid introduction of the pathogen.

The significance of the seedborne phase has been difficult to assess due to the low populations of the pathogen on contaminated seed and its poor competitive ability relative to the large background rice seed microflora (Eamchit and Ou, 1970; Gnanamanickam et al., 1994). Current seed assays to detect *X. o. oryzae* include dilution plating on semiselective media and immunoassays. Serological assays such as enzyme-linked immunosorbent assay (ELISA) and immunofluorescence colony staining have been useful for detection of *X. o. oryzae* (Benedict et al., 1989; Gnanamanickam et al., 1994) but still require cultivation of the pathogen and no single monoclonal antibody has yet been obtained that reacted to all strains of the pathogen. Of the available semiselective media (Gonzales et al., 1991; Ming et al., 1991; Yuan, 1990), XOS medium was demonstrated the most effective for isolation of *X. o. oryzae* (Gnanamanickam et al., 1994). However, it is still inadequate for detecting low levels of the pathogen, and recovery of *X. o. oryzae* on XOS medium was only successful when artificially added to seed extracts at concentrations higher than 10^5 cfu/ml (Gnanamanickam et al., 1994). Additionally, the semiselective medium supports the growth of contaminants in seed extracts and differentiation of *X. o. oryzae* from similar appearing nonpathogenic xanthomonads remains problematic (Benedict et al., 1989: Gnanamanickam et al., 1994, Jones et al., 1989; Ming et al., 1991). The frequent occurrence of nonpathogenic xanthomonads was confirmed in a recent study on total culturable bacterial populations associated with rice seed (Cottyn et al., 2001). Although nonpathogenic xanthomonads can be differentiated from *X. o. oryzae* strains by whole-cell fatty acid analysis (Jones et al., 1989), serological assays (Benedict et al., 1989; Gnanamanickam et al., 1994), and BOX-PCR fingerprinting (Cottyn et al., 2001); morphologically they may be confused with *X. o. oryzae*. Thus, a rapid and sensitive method for the accurate identification of the low pathogen level in contaminated rice seed is needed.

Leach et al. (1990) identified an insertion sequence IS*1112* present in approximately 81 copies in the genome of *X. o. oryzae.* The IS*1112* element has been widely used for analyses of the population structure and genetic diversity of *X. o. oryzae* strains (Adhikari et al., 1995; Leach et al., 1992; Ochiai et al., 2000; Vera Cruz et al., 1996; Yashitola et al., 1997). However, the presence of IS*1112* sequences in strains of the closely related rice pathogen *X. o. oryzicola*, the causal agent of bacterial leaf streak, prevented its use as a probe for

detection of *X. o. oryzae* in dot blot hybridization procedures (Leach et al., 1990). The objective of this study was to determine if *X. o. oryzae* associated with rice seed could be detected by PCR amplification using primers directed at internal sequences of the IS*1112* repetitive element. Wash extracts from artificially and naturally contaminated seeds were assayed for the presence of *X. o. oryzae* by PCR as well as by dilution plating on XOS medium. The method was also used to investigate whether transmission of the pathogen from contaminated seed to emerging seedlings can be detected.

Materials and methods

Bacterial strains and DNA extraction. The bacterial strains used in evaluating the PCR assay are listed in Table 1. Strains collected from rice in the Philippines comprised 28 strains of *X. o. oryzae*, 27 strains of *X. o. oryzicola*, and six nonpathogenic xanthomonads maintained at the Entomology and Plant Pathology Division of the International Rice Research Institute, Los Baños, Philippines. The *X. o. oryzae* strains were previously characterized with regard to their phylogeny and virulence (Leach et al, 1992; Vera Cruz et al., 1996). The strains were revived and cultured at 28° C for 72 h on peptone sucrose agar (PSA; sodium glutamate 1.0 g/l, peptone 10.0 g/l, sucrose 10.0 g/l, and agar 17.0 g/l). Other reference strains used were 45 strains of *X. o. oryzae* from different geographical origins, 18 strains of other *Xanthomonas* species, and 17 strains of non-*Xanthomonas* species. These strains were provided as total genomic DNA by the Kansas State University collection of plant pathogenic bacteria (Manhattan, KS, USA); the Miyazaki University collection of *X. o. oryzae* strains (Miyazaki, Japan); and the Laboratory of Microbiology Gent (LMG) bacteria collection (University Gent, Belgium).

Bacterial genomic DNA was extracted by the cetyltrimethylammonium bromide (CTAB) method (Ausubel et al., 1990). Bacterial cells grown overnight in 5 ml nutrient broth (Difco Laboratories, Detroit, MI) were pelleted, redissolved in 200 µl of Tris-EDTA buffer (10 mM Tris pH 8.0, and 1 mM EDTA), lysed with 25 µl of 10% sodium dodecyl sulfate (SDS) and 5 µl of proteinase K at 10 mg/ml, and incubated at 37° C with gentle shaking for 1 h. After addition of 45 µl of 5 M NaCl and 40 µl of CTAB solution (10% CTAB in 0.7 M NaCl), the mixture was incubated at 65° C for 20 min. Samples were extracted with chloroform/isoamyl alcohol (24:1) and DNA was precipitated with isopropanol and redissolved in 100 µl of modified Tris-EDTA buffer (10 mM Tris pH 8.0, and 0.1mM EDTA). The DNA concentrations were estimated using the DNA-specific dye Hoechst 33258 and a TKO-100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA). All DNA samples were kept at –20 °C.

Primer design. Initially seven oligonucleotide primers were designed based on the nucleotide sequence of IS*1112* (Yun, 1991), which amplify internal DNA fragments of the *X. o. oryzae* repetitive element. The oligonucleotides were synthesized by Operon Technologies Inc. (Alameda, CA). The optimum annealing temperature was calculated using software PC/GENE (IntelliGene, Mountain View, CA). The selected primers designated Xo1 (5'-AGCGTGGTTGAGCAGCGAAG-3') and Xo2 (5'-TCAACTCTGATTCGCAACGC-3') delineated a 497-bp fragment of the insertion sequence IS*1112*. The annealing site of primer Xo1 at positions 551 to 570 is located well within the insertion sequence,

Table 1. Bacterial strains tested and results obtained with the polymerase chain reaction method.

Strain	Origin	Source[a]	Amplified DNA fragments[b] 574 bp	497 bp	Other bands
Xanthomonas oryzae pv. *oryzae*					
PX01, PX020, PX026, PX035, PX077, PX0170, PX0188, PX0241 (Race 1); PX063, PX078, PX086, PX0137, PX0168, PX0169, PX0172, PX0190, PX0306 (Race 2); PX081, PX0143, PX0340 (Race 3); PX071 (Race 4); PX0112, PX0237 (Race 5); PX0127 (Race 6); PX0154 (Race 7); PX0208 (Race 8); PX088, and PX0142 (Race 9)	Philippines	IRRI	+	+	–
LMG 641; C1, C4, and C8	China	LMG; KSU	+	+	–
LMG 5047; A3842, A3850, and A3867	India	LMG; KSU	+	+	–
93-023, 93-147, and 93-173	Indonesia	KSU	+	+	–
LMG 795; T7174, T7147, T7133, and H75373	Japan	LMG; MU	+	+	–
89031, 8766, and 8709	Korea	KSU	+	+	–
MX097, MX0192, and MX0274	Malaysia	KSU	+	+	–
NX0101, NX0149, and NX0334	Nepal	KSU	+	+	–
LMG 6518; Af1947, Af1948, Af1949, and Af1951	Africa	LMG; KSU	–	(+)	–
Am7, Am12, Am14, Am15, AmX7-20, AmX13-3A, AmX13-5C, AmX57-5, AmX200-1, AmX207-K1, and AmX212-2-3	U.S.A.	KSU	–	+	290 bp, 590 bp, 630 bp
Am2 and Am17	U.S.A.	KSU	+	+	290 bp, 630 bp
Am16 and Am18	U.S.A.	KSU	–	+	290 bp, 320 bp, 720 bp
Xanthomonas oryzae pv. *oryzicola*					
LMG 665, LMG 793; BLS253, BLS270, BLS286, BLS288, BLS289, BLS291, BLS292, BLS293, BLS303, BLS305, BLS309, BLS310, BLS311, BLS312, BLS315, BLS319, BLS321, BLS328, BLS341, BLS343, BLS344, BLS353, BLS354, BLS367, and BLS372	Philippines	LMG; IRRI	–	+	1030 bp
Nonpathogenic xanthomonads					
X18, X25, X52, X61, X105, and X160	Philippines	IRRI	–	(+)	some weak bands
Other *Xanthomonas* species					
X. fragariae LMG 708, *X. hyacinthi* LMG 8042, *X. campestris* pv. *vesicatoria* type B LMG 919 [*X. vesicatoria*][c], *X. c. citri* D LMG 9182 [*X. a. aurantifolii*][c], *X. c. holcicola* LMG 8276 [*X. vasicola* pv. *holcicola*][c], *X. c. vasculorum* typeB LMG 896 [*X. v. vasculorum*][c], *X. c. graminis* LMG 726 [*X. translucens* pv. *graminis*][c], and *X. translucens* pv. *translucens* LMG 5262		LMG	–	+	–
X. campestris pv. *campestris* LMG 568, *X. campestris* pv. *cerealis* LMG 679 [*X. translucens* pv. *cerealis*][c], *X. c. phleipratensis* LMG 843 [*X. t. phleipratensis*][c], and *X. c. poae* LMG 728 [*X. t. poae*][c]		LMG	–	(+)	3 to 4 weak bands
X. axonopodis LMG 538 [*X. a. axonopodis*][c], *X. campestris* pv. *citri* LMG 682 [*X. a. citri*][c], *X. c. phaseoli* LMG 7455 [*X. a. phaseoli*][c], *X. c. graminis* LMG 8269 [*X. bromi*][c], *X. cucurbitae* LMG 7481, and *X. pisi* LMG 847		LMG	–	–	–

continued. . .

Table 1 continued.

Strain	Origin	Source[a]	Amplified DNA fragments[b] 574 bp	497 bp	Other bands
Non-*Xanthomonas* species					
Pseudomonas aeruginosa LMG 1242, *P. fluorescens* LMG 1794, *P. marginalis* LMG 2210, *P. mendocina* LMG 1223, *P. oryzihabitans* LMG 7040, *P. putida* LMG 2257, and *P. stutzerii* LMG 11199		LMG	–	(+)	4 to 5 weak bands
Bacillus pumilus LMG 7132, *B. subtilis* LMG 7135, *Burkholderia gladioli* LMG 2216, *B. glumae* LMG 2196, *Pantoea agglomerans* LMG 2578, *P. ananatis* LMG 2665, *P. stewartii* subsp. *stewartii* LMG 2715, *P. s. indologenes* LMG 2632, *Pseudomonas fuscovaginae* LMG 2158, and *Stenotrophomonas maltophilia* LMG 958		LMG	–	–	–

[a] IRRI, Entomology and Plant Pathology Division collection of rice bacteria, International Rice Research Institute, Los Baños, Philippines; LMG, Laboratory of Microbiology University Gent Bacteria Collection, Gent, Belgium; KSU, Kansas State University collection of plant pathogenic bacteria, Manhattan, Kansas, U.S.A.; MU, Miyazaki University *X. o. oryzae* strain collection, Miyazaki, Japan.
[b] +, fragment amplified ; (+), fragment amplified in low yield; -, fragment not amplified.
[c] Proposed name by Vauterin et al. (1995).

while primer Xo2 anneals at positions 1029 to 1048 near the 3'-end of the determined 1053 nucleotide sequence of IS*1112*.

Southern blot hybridizations. Approximately 5 µg of genomic DNA of selected reference strains was digested overnight at 37° C with 25 U of *Eco*RI or *Pst*I according to conditions specified by the manufacturer (Boehringer Mannheim, Far East, Singapore). Digested fragments were separated by electrophoresis in 0.7% agarose gels at 45 V for 16 h before alkali transfer to Hybond-N membrane according to the supplier's specifications (Amersham, Far East, Hong Kong). Phage λ *Pst*I-digested DNA fragments were included in gels as molecular standards. The entire repetitive DNA element IS*1112* contained in plasmid pBS101 (Leach et al., 1990), or the *X. o. oryzae* specific DNA fragments amplified from strain PXO86 were labeled by random priming with digoxigenin-labeled deoxyuridine triphosphate (Boehringer Mannheim), and hybridized to Southern blots according to manufacturer's instructions. Hybridized bands were detected on X-ray film (Amersham Hyperfilm) by chemiluminescence with 3-(2'-spiroadamatane)-4-methoxy-4-(3"-phosphoryloxy)-phenyl-1,2-dioxetane (AMPPD) as the substrate for alkaline phosphatase (Boehringer Mannheim).

Processing of leaf samples. Leaves with blight lesions were collected from infected plants in farmer's fields in Luzon island, Philippines. Crude leaf-lesion extracts were used as source of template for amplification of the *X. o. oryzae* DNA fragments from infected leaves. A leaf fragment of 0.5 cm^2 was cut along the advancing tip of the lesion with flame-sterilized scissors and partially crushed in 100 µl of sterile distilled water (dH_2O) in an eppendorf tube. Aliquots of 5 µl from the crude extract were used as template DNA for PCR amplification.

Processing of seed samples. Seeds were harvested from plants with bacterial blight symptoms in Philippine farmer's fields in Luzon island during four consecutive cropping seasons. In addition, IRBB10 seeds obtained from the International Rice

Research Institute were artificially contaminated by soaking individual batches of 25-g seed in a 100 ml aqueous suspension of *X. o. oryzae* strain PXO340 (approximately 3×10^8 cfu/ml or 2×10^5 cfu /ml). The suspensions were prepared from 72-h grown cultures of PXO340 on PSA slant tubes diluted in sterile dH_2O, and suspension concentrations were confirmed by dilution plate counting on PSA. After soaking for 12 h, the seeds were dried for 6 h on tissue paper in a laminar flow hood prior to processing.

Extraction of bacteria from naturally and artificially contaminated seeds was done by washing individual batches of 10-g seed (approximately 300 seeds) in 50 ml of sterile phosphate-buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 0.01M Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 2 h at room temperature on an orbital shaker at 125 rpm. For single seed PCR, randomly taken seeds from each sample were soaked individually in 100 µl of PBS for 2 h at room temperature. Aliquots (5-µl) of the wash extracts from 10-g seed or single seeds were used directly as template for PCR amplification.

The persistence of *X. o. oryzae* DNA that was coated onto seeds for PCR amplification was assessed. Nutrient broth cultures of PXO86 (approximately 10^8 CFU/ml) were subjected to three freeze-thaw cycles at –70° C and 65° C, and the resulting lysates were checked for cell-death by plating aliquots of 0.1 ml in triplicate on PSA medium. Pathogen-free seeds were soaked per batches of 10 g in the lysed PXO86 cultures for 8 h. The seeds were dried for 48 h at 37.8° C in a Precision convection incubator 6LM (Fisher Scientific, Springfield, NJ) and stored at room temperature. Individual batches of 10-g coated seed were assayed as described above after one week, 6 months, and 1 year.

Dilution plating on XOS medium. Recovery of *X. o. oryzae* and non-target bacteria on XOS medium was assayed from wash extracts of naturally and artificially contaminated seeds. Aliquots of 0.1 ml from diluted 10-g seed washes in sterile saline (0.85% NaCl) were plated in triplicate on XOS medium (Ming et al., 1991). Plates were incubated at 28° C and colony-forming units were recorded from day 3 to day 7. Colonies suspected of being *X. o. oryzae* were initially identified based on colony morphology compared to known *X. o. oryzae* strains, and confirmed by the PCR assay and pathogenicity testing.

PCR amplification. DNA fragments were amplified in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µM of each deoxynucleotide triphosphate (United States Biochemical, Cleveland, OH), 0.4 µM of each primer, and 2 units of *Taq* DNA polymerase (Pharmacia Biotech, Asia Pacific, Hong Kong). Standard PCR reactions were performed in a 20 µl reaction volume that typically contained 20 ng of bacterial genomic DNA as template. Aliquots (5 µl) of DNA extracts from plant tissue in the seed transmission tests were amplified in 100 µl reaction volume. Aliquots (5 µl) of extracts from seeds or leaf-pieces were incubated with GeneReleaser reagent (BioVentures, Inc., Murfreesboro, TN) according to the manufacturer's guidelines prior to amplification in a reaction volume of 100 µl. Pre-PCR addition of the cell lysis reagent GeneReleaser is claimed by the manufacturer to improve amplification by sequestering potential inhibitors of DNA polymerase. A positive control (total DNA from PXO86) and a negative control (no template DNA) were included in each PCR run. The reaction mixture was overlaid with one drop of mineral oil (Perkin-Elmer,

Norwalk, CT). PCR amplifications were performed in a DNA thermal cycler (480; Perkin-Elmer), with an initial denaturation at 94° C for 2 min followed by 40 cycles of denaturation at 94° C for 1 min, annealing at 62° C for 1 min, extension at 72° C for 2 min, and a final extension at 72° C for 5 min. Half of the PCR products were separated by gel electrophoresis on gels composed of 0.75% agarose and 0.8% Synergel (Diversified Biotech, Boston, MA) at 10V/cm in 0.5× Tris-borate-EDTA buffer (Sambrook et al., 1989). Gels were stained with ethidium bromide and photographed on an UV transilluminator with Polaroid Type 55 film (Polaroid Corp., Cambridge, MA). Amplification reactions were routinely done in four replications. Amplification of the 497-bp and 574-bp DNA fragments was scored as positive for presence of *X. o. oryzae*, no amplification or amplification of a 497-bp product only was scored as negative.

Sensitivity of detection. Dilution series in sterile saline (0.85% NaCl) were prepared to estimate the limit of detection for *X. o. oryzae* strain PXO86. Aliquots of 5 µl from each dilution were used as template DNA in three replicate PCR assays. The GeneReleaser protocol was used for direct lysis of the bacterial cells prior to DNA amplification. Aliquots of the dilution series were plated on PSA medium in 10 replicates to estimate the average number of colony-forming units per reaction.

Seed transmission tests. Transmission of *X. o. oryzae* from seed to seedlings was tested with 300 seeds harvested from infected IR24 plants at Calauan, and 1200 IRBB10 seeds artificially contaminated by soaking in suspensions of strain PXO340 as described above. The combination of *X. o. oryzae* strain PXO340 (race 3, lineage C) and the susceptible cultivar IRBB10 is known to represent a highly compatible pathogen system (C.M. Vera Cruz, *personal communication*). The seeds were germinated for 5 days on moistened filter paper, planted in autoclaved soil and seedlings were raised in a growth chamber under conditions favorable for disease development (Ou, 1985) at day/night temperature of 29/21°C and a relative humidity of 75 to 90%.

Individual plants grown from the naturally and artificially contaminated seeds were sampled for PCR amplification in replicates of 10 plants at 5, 7, 10, 14, 21 and 28 days after planting. For each plant, the root and stem/leaves portion was processed separately for DNA extraction by a modification of the procedure of Volossiouk et al. (1995). The roots were shortly rinsed in sterile water to minimize interference from soil debris and separated from the above ground portion. The individual root and stem/leaves samples were ground in liquid nitrogen and the powdered plant tissue was suspended in 0.5 ml of skim milk powder solution (4 mg/ml in dH_2O) by vigorous vortexing. The suspension was centrifuged at 12,000 rpm for 10 min and the supernatant was mixed with 0.5 ml of extraction buffer (0.6 % SDS, 0.24 M NaCl and 0.1 M sodium acetate). Each sample was then extracted with an equal volume of a 1:1 equilibrated phenol and chloroform/isoamyl alcohol (24:1) mixture. The DNA was precipitated with isopropanol, washed with 70% ethanol and redissolved in 50 µl of modified Tris-EDTA buffer. A positive control, i.e. root or stem/leaves samples from control plants to which approximately 10^5 cells of *X. o. oryzae* was added prior to grinding and DNA extraction, was included in each PCR run. Five microliter aliquots of each DNA extract were assayed for PCR amplification.

Results

Specificity of the primers. The primer pair Xo1-Xo2 amplified the expected size fragment of 497 bp from plasmid DNA of pBS101 containing the IS*1112* element (Fig. 1, lane 1). When total DNA extracts of *X. o. oryzae* LMG 641 and *X. o. oryzicola* LMG 793 were used as templates, an additional fragment of 574 bp was amplified from *X. o. oryzae* and a different fragment of 1030 bp from *X. o. oryzicola*, aside from the expected 497-bp fragment (Fig. 1, lanes 2 and 3). The optimal primer concentration, the amount of template DNA, and the thermal profile were examined in preliminary experiments to establish the reproducibility of the PCR amplification. To confirm the *X. o. oryzae* amplification products, amplifications were done using total DNA extracts from 73 strains of *X. o. oryzae* from diverse geographic origins (Table 1). All *X. o. oryzae* strains from Asia comprising 28 strains representing the nine established races in the Philippines, and 25 strains from other Asian origins, produced the 497-bp and 574-bp fragments (Fig. 2, lanes 1 to 8). The exceptional strains were from Africa and the United States. The five African strains produced a faint 497-bp band only (Fig. 2, lane 9). The 15 strains from the United States were heterogeneous and produced three distinct amplification patterns that shared fragments of 290 bp and 497 bp in common. Two patterns amplified for 13 strains contained an additional 630-bp band and only differed in the presence of a faint 590-bp band in the pattern produced by 11 strains and a 574-bp band in the pattern produced by two strains (Fig. 2, lanes 10 and 11). The third pattern produced by two strains contained an additional 320-bp band and a faint 720-bp band (Fig. 2, lane 12). The 27 strains of *X. o. oryzicola*, collected from different locations in the Philippines

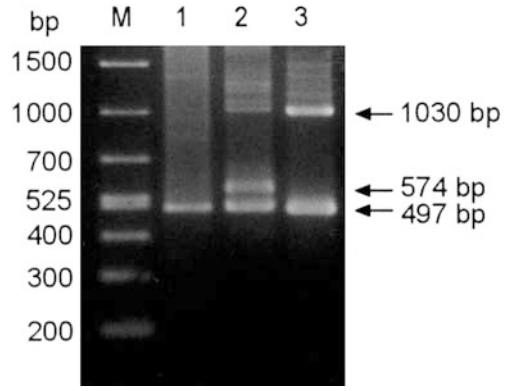

Fig. 1. Ethidium-bromide stained gel of PCR-amplification products directed by primers Xo1 and Xo2 derived from IS*1112*, an insertion sequence isolated from *Xanthomonas oryzae* pv. *oryzae*. Template DNA (20 ng) was plasmid pBS101 containing the element IS*1112* (lane 1), and total DNA extracts from X. o. oryzae LMG 641 (lane 2) and *X. o. oryzicola* LMG 793 (lane 3). Molecular weight standard (BioMarker EXT, BioVentures, TN) was run in lane M, and numbers on the left indicate sizes in base pairs. The arrows on the right denote the positions of the expected fragment of 497 bp, the additional fragment of 574 bp from *X. o. oryzae* and the fragment of 1030 bp from *X. o. oryzicola*.

(Table 1), yielded amplification products of 497 bp and 1030 bp (not shown).

The specificity of the primer pair was further evaluated on total DNA extracts of 18 reference strains of other *Xanthomonas* species, 6 nonpathogenic *Xanthomonas* isolates from rice, and 17 reference strains of non-*Xanthomonas* species (Table 1). None produced the *X. o. oryzae* specific DNA fragment of 574 bp upon amplification. A single 497-bp fragment, however, was also amplified from eight strains belonging to *X. fragariae*, *X. hyacinthi*, and pathovars of *X. campestris* and *translucens* listed in Table 1. Another four strains representing pathovars of *X. campestris* and *translucens* produced a weak amplification pattern of four to five bands including a faint 497-bp band (data not shown). Amplification from total DNA extracts of seven *Pseudomonas* reference strains given in Table 1 also produced a faint 497-bp band along with various additional faint bands (Fig. 3, lanes 3 to 6). The six nonpatho-

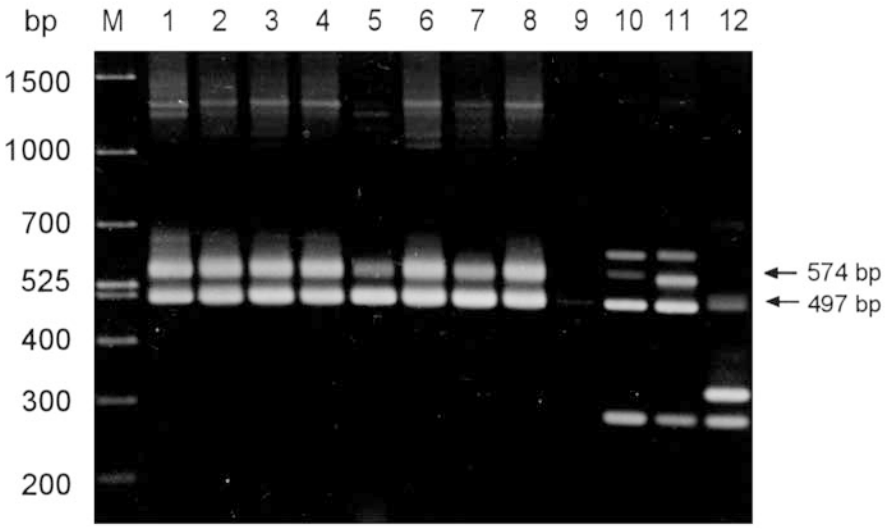

Fig. 2. Ethidium-bromide stained gel of PCR-amplification products directed by primers Xo1 and Xo2 from total DNA (20 ng) of *Xanthomonas oryzae* pv. *oryzae* strains representing different geographic areas. Lane 1, PX086 (the Philippines); lane 2, 93-023 (Indonesia); lane 3, MX097 (Malaysia); lane 4, LMG 641 (China); lane 5, T7174 (Japan); lane 6, 8709 (Korea); lane 7, NX0101 (Nepal); lane 8, LMG 5047 (India); lane 9, Af1947 (Africa); lanes 10 to 12, Am7, Am2 and Am16 (United States). Lane M contains the molecular weight standard (BioMarker EXT, BioVentures, TN), and numbers on the left indicate sizes in base pairs. The arrows on the right mark the positions of the *X. o. oryzae* specific amplification products observed for all strains from Asia.

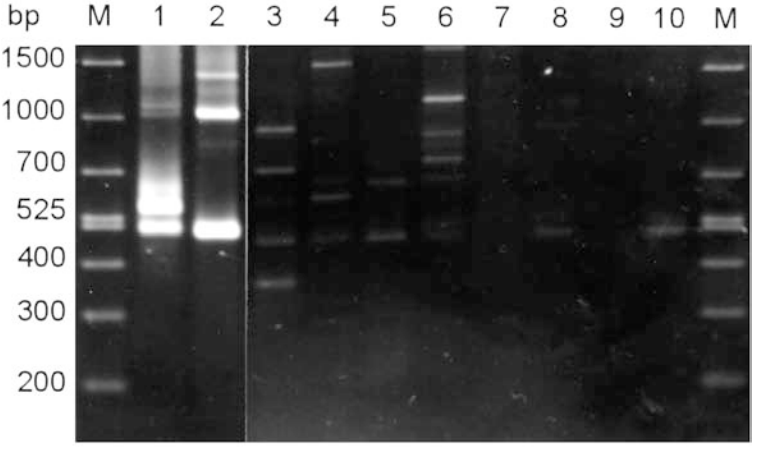

Fig. 3. Ethidium-bromide stained gel of nonspecific PCR products observed upon amplification with primer pair Xo1-Xo2 from total DNA extracts of saprophytic bacteria presumably associated with rice seed. Control DNA (20 ng) was from *Xanthomonas oryzae* pv. *oryzae* LMG 795 (lane 1) and *X. o. oryzicola* LMG 665 (lane 2). Template DNAs (20 ng) were from *Pseudomonas putida* LMG 2257 (lane 3); *Pseudomonas aeruginosa* LMG1242 (lane 4); *Pseudomonas oryzihabitans* LMG 7040 (lane 5); *Pseudomonas mendocina* LMG 1223 (lane 6); *Pantoea agglomerans* LMG 2578 (lane 7); and nonpathogenic xanthomonads X105, X18, and X52 (lanes 8 to 10). The left and right lanes (M) contain molecular weight standards (BioMarker EXT, BioVentures, TN), and numbers on the left indicate sizes in base pairs.

genic xanthomonads produced either no amplification or weak amplification of a 497-bp band along with other faint nonspecific bands (Fig. 3, lanes 8 to 10). Tested strains of the genera *Bacillus*, *Burkholderia*, *Pantoea*, and *Stenotrophomonas* did not produce any discrete bands upon amplification with the primer pair on ethidium bromide stained agarose gel (data not shown).

Hybridization of either the entire IS*1112* element or the 497-bp and 574-bp amplification products from *X. o. oryzae* PXO86 as probes to *Pst*I or *Eco*RI digested total DNA from strains of *X. o. oryzae* and *X. o. oryzicola*, resulted in identical banding patterns. Each strain displayed a distinct hybridization pattern, however, the pattern of *X. o. oryzae* strain LMG 6518 from Africa contained fewer hybridizing fragments compared to the *X. o. oryzae* strains from Asia (Fig. 4). The IS*1112* element or the individual *X. o. oryzae* DNA fragments did not hybridize to *Eco*RI digested genomic DNA from *Pseudomonas aeruginosa* LMG 1242, *P. fluorescens* LMG 1794, *P. marginalis* LMG 2210, or *P. putida* LMG 2257 (data not shown).

Sensitivity threshold. A dilution series of cultured cells of strain PXO86 yielded a limit of detection for the 574-bp product on ethidium bromide stained agarose gel of approximately 10 cfu per reaction in replicate amplifications (actual colony-forming units in replicate platings on PSA medium were 8, 11, 13, 7, 14, 9, 8, 13, 12,

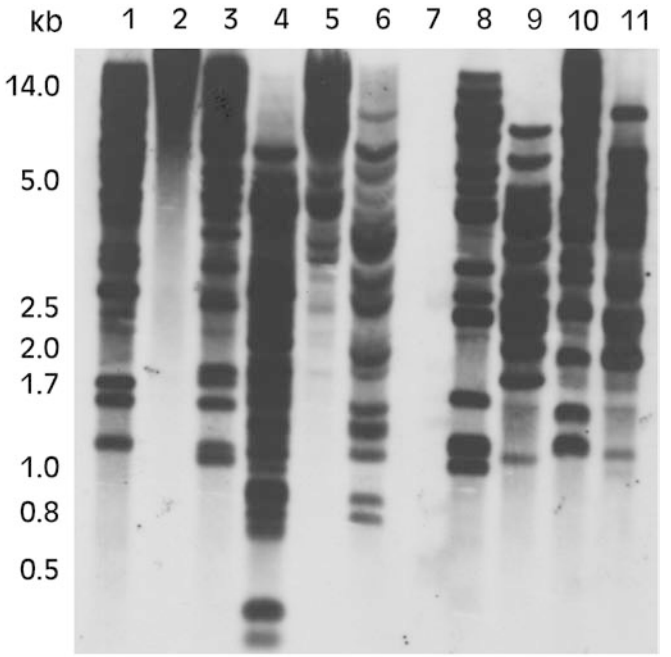

Fig. 4. Southern hybridization profiles of *Eco*RI (lanes 1, 3, 5, 8, and 10) and *Pst*I (lanes 2, 4, 6, 9, and 11) digested DNA from strains of *Xanthomonas oryzae* pv. oryzae LMG 795 from Japan (lanes 1 and 2), LMG 5047 from India (lanes 3 and 4), LMG 6518 from Africa (lanes 5 and 6), and strains of *X. o. oryzicola* LMG 665 (lanes 8 and 9) and LMG 793 (lanes 10 and 11), probed with the digoxigenin-labeled fragment of 497 bp amplified with primer pair Xo1-Xo2 from *X. o. oryzae* strain PXO86. Hybridization using the 574-bp amplification product or the entire IS*1112* element as probe resulted in identical banding patterns. Molecular weight standard (phage λ DNA digested with *Pst*I) was run in lane 7, and numbers on the left indicate sizes in kilobases.

and 8). When the reaction contained approximately 1 cfu (actual colony-forming units in replicate platings were 2, 1, 1, 2, 0, 1, 1, 1, 1, and 1), the 574-bp product was detected on ethidium bromide stained agarose gel in only two of three replicate amplifications. The 497-bp band was clearly amplified from all dilution series, whereas the intensity of the 574-bp band markedly decreased when the reaction contained less than 100 cells (Fig. 5), indicative for a lower copy number of IS*1112* sequences containing the 77-bp sequence variation.

Detection of *X. o. oryzae* in crude leaf extracts. The primer pair successfully mediated the amplification of PCR products of the expected size from crude extracts of bacterial blight lesions on rice leaves, and no products were observed from control leaf tissue (data not shown). The PCR detection of *X. o. oryzae* in bacterial blight lesions on rice leaves, however, would be of little practical relevance because the high pathogen populations can readily be isolated and confirmed by pathogenicity tests, this in contrast to the low populations in seeds.

Detection of *X. o. oryzae* in seed washes. The primer pair successfully mediated the amplification of the *X. o. oryzae* DNA fragments from wash extracts of 10 gram or single IRBB10 seeds artificially contaminated by soaking in suspensions of *X. o. oryzae* strain PXO340. The 497-bp and 574-bp DNA fragments were not amplified from the IRBB10 seeds if cells of *X. o. oryzae* were not added (data not shown). Amplification of the *X. o. oryzae* DNA fragments from wash extracts of 10-g seed occurred in spite of background bacterial microflora of up to 10^5 cfu/ml recovered on XOS medium (Table 2).

Wash extracts from 10-g seed from the samples harvested from bacterial blight

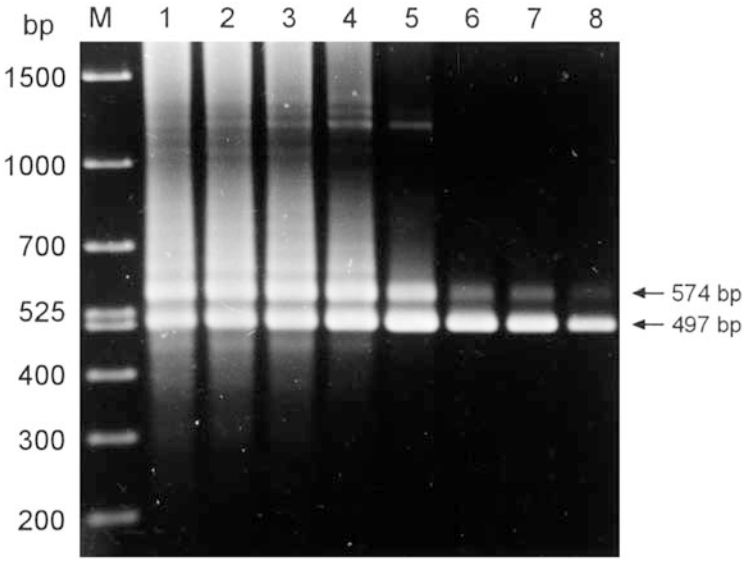

Fig. 5. Detection limit of the PCR assay for dilution series of cultured cells of *Xanthomonas oryzae* pv. *oryzae* strain PXO86. Aliquots (5 µl) of each dilution were added directly to the PCR mixture for a 40-cycle amplification run. Lanes 1 to 5 contained 1.4×10^7 to 1.4×10^3 cfu per reaction; lanes 6 to 8 contained 98, 10, and 1 cfu per reaction. Lane M contains molecular weight standard (BioMarker EXT, BioVentures, TN), and numbers on the left indicate sizes in base pairs. The arrows on the right denote the expected positions of *X. o. oryzae* amplification products.

Table 2. Comparison of dilution plating on XOS medium and the polymerase chain reaction (PCR) method for detection of *Xanthomonas oryzae* pv. *oryzae* in wash extracts from seeds harvested from plants with bacterial blight symptoms in Philippine farmers' fields and artificially contaminated seeds.

Seed samples	Mean cfu/ml of seed wash recovered on XOS [a]		Amplification of *X. o. oryzae* DNA fragments [b]	
	Other bacteria	*X. o. oryzae*	10-g seed	Single seeds
Collected from bacterial blight infested fields				
Quezon (cv. C22)	3.0×10^5	0	(+)	0/10
Calauan (cv. IR24)	5.3×10^5	0	+	10/10
Mabitac (cv. IR76)	6.0×10^5	0	-	0/10
Pila (cv. unknown)	3.7×10^5	0	+	2/10
Artificially contaminated IRBB10 seeds				
Soaked in 2×10^5 cfu/ml of strain PXO340	2.7×10^4	0	+	10/10
Soaked in 3×10^8 cfu/ml of strain PXO340	1.7×10^4	8.0×10^5	+	10/10

[a] Washes from 10-g seed in 50 ml phosphate-buffered saline were diluted to 10^{-3} and 10^{-4} in sterile saline (0.85% NaCl), and aliquots of 0.1 ml were plated on XOS medium. Values of colony-forming units are averages of triplicate platings counted after 7 days incubation at 28°C.
[b] Aliquots of 5 µl of original wash extracts from 10-g seed or individual seeds were used as source of template DNA in the PCR amplification assay. +, Presence; (+), presence in two of four replicate amplifications; or -, absence of the 497-bp and 574-bp DNA fragments. Numbers indicate the number of single seed extracts that produced the 497-bp and 574-bp amplification products on a total of 10 individual seeds tested.

infected plants in farmer's fields at Calauan and Pila were scored positive for the *X. o. oryzae* DNA fragments in replicate amplifications (Fig. 6, lanes 19 and 20). Wash extracts from 10-g seed from the sample collected in Quezon were scored weak positive on the basis of amplification of a faint 574-bp band along with a clear 497-bp band in two of four replicate amplifications (data not shown). The sample from Mabitac was scored negative on the basis of amplification of a faint 497-bp band in one of the replicate amplifications and no detectable products in the other three replicate amplifications. PCR amplification from single seed extracts revealed dissimilar proportions of contaminated seeds among the collected samples. Wash extracts from individual seeds randomly chosen from the sample collected in Calauan were scored positive on the basis of amplification of a clear 497-bp product and a 574-bp product that sometimes differed in intensity (Fig. 6, lanes 1 to 18). When 100 seeds were tested, 97 extracts produced the 497-bp and 574-bp amplification products, two extracts produced the 497-bp band only, and one extract did not produce any amplification products. For ten individual seeds tested from the sample collected in Pila, five extracts were scored positive for the *X. o. oryzae* amplification products, three extracts produced the 497-bp band only, and two extracts did not produce any amplification products. None of ten individual seeds from the sample collected in Quezon produced detectable amplification products.

The long-term stability of bacterial DNA for PCR detection was demonstrated from seeds coated with a lysed culture of *X. o. oryzae*. Wash extracts from the coated seeds produced the *X. o. oryzae* DNA fragments upon amplification with the primer pair one week, six months, and one year after seed coating (data not shown).

Dilution plating of seed washes on XOS medium. To compare the relative efficiency of the PCR assay versus plating on semiselective XOS medium, the wash extracts from 10-g batches of the naturally or artificially contaminated seeds were additionally assayed by dilution plating on XOS medium for presence of *X. o. oryzae*.

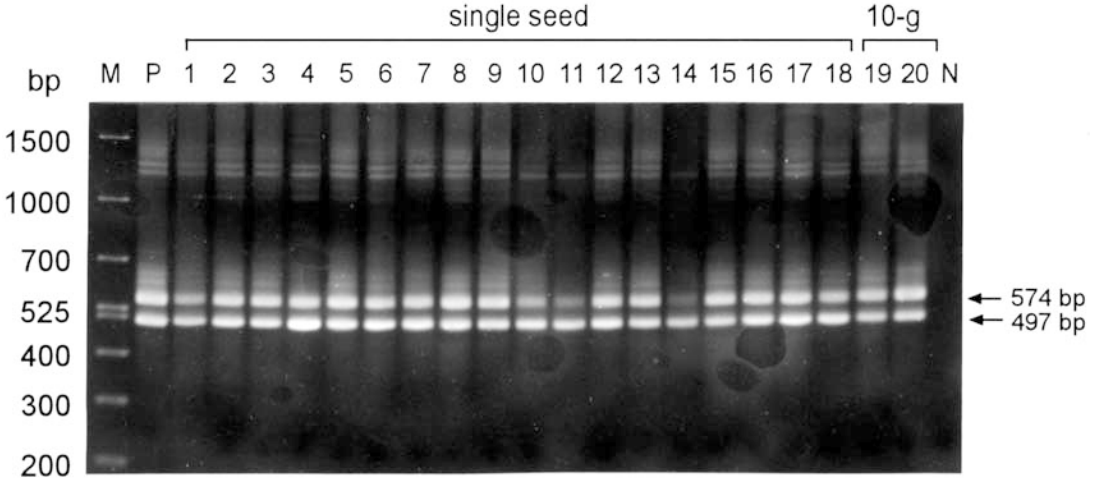

Fig. 6. PCR-mediated amplification with primer pair Xo1-Xo2 for detection of *Xanthomonas oryzae* pv. *oryzae* in wash extracts from naturally contaminated rice seeds harvested from bacterial blight infected plants in farmer's field. Aliquots (5 μl) from single seed wash extracts (lanes 1 to 18), and from 10-g seed wash extracts (lanes 19 and 20) were added directly to the PCR mixture as source of template DNA. Lane N contains the no-DNA control, and lane P contains *X. o. oryzae* strain PXO86 as positive control. Lane M contains the molecular weight standard (BioMarker EXT, BioVentures, TN), and numbers on the left indicate sizes in base pairs. The arrows on the right denote the expected positions of *X. o. oryzae* amplification products.

Colonies of *X. o. oryzae* strain PXO340 were recovered on XOS medium from the wash extracts of IRBB10 seeds soaked in a PXO340 suspension of 3×10^8 cfu/ml but not from the wash extracts of IRBB10 seeds soaked in a PXO340 suspension of 2×10^5 cfu/ml (Table 2). The attempts to recover PXO340 cells on XOS medium after germination of the artificially contaminated IRBB10 seeds were unsuccessful.

The pathogen was not detected on XOS medium by dilution plating of wash extracts from seed samples harvested from rice plants with bacterial blight symptoms in farmers' fields. Dilution plating of seed washes on XOS medium resulted in the growth of various contaminant bacteria (approximately 10^5 cfu/ml) associated with rice seed (Table 2). Several colonies that were morphologically similar to *X. o. oryzae* were found on the medium after 7 days of incubation. Of 12 *Xanthomonas*-like colonies picked randomly from isolation plates, all were scored negative in the PCR assay and were nonpathogenic upon inoculation of IR24 plants by the leaf clipping method (Kauffman et al., 1973).

Assessment of seed transmission. To test transmission of the pathogen from contaminated seeds to emerging seedlings, 1,200 artificially contaminated IRBB10 seeds soaked in suspensions of strain PXO340 and 300 seeds harvested from bacterial blight infected IR24 plants in farmer's field at Calauan, were grown under conditions favorable for disease development. Individual plants were sampled in 10 replicates at 5, 7, 10, 14, 21, and 28 days after planting and DNA was extracted separately from the root portion and from the above ground portion, i. e. stem and leaves.

The root or stem/leaves DNA extracts of 60 individual plants derived from naturally contaminated seeds did not produce any detectable PCR products upon amplification with the primer pair on ethidium

bromide stained agarose gels. For assayed plants derived from artificially contaminated seeds, DNA extracts from roots sampled at the earliest days after planting occasionally yielded the *X. o. oryzae* amplification products but at no time was a positive amplification for *X. o. oryzae* observed from stem/leaves DNA extracts (data not shown). Five of ten root DNA extracts from day 5 and day 7 after planting, and four of ten root extracts from day 10 produced the *X. o. oryzae* amplification products of 497 bp and 574 bp, the other extracts produced the 497-bp band only. At 14 days and 21 days after planting, respectively, eight and four of ten root extracts produced the 497-bp band only and the other extracts did not produce any amplification products. At 28 days after planting, none of the root extracts from 10 replicate plants yielded any amplification products, indicating that prior positive amplification likely represented detection of the initial seed inoculum. None of the DNA extracts from the above ground portion of individual plants sampled during the 28 days after planting produced the *X. o. oryzae* DNA fragments upon amplification. Although a faint 497-bp band, which might be due to nonspecific amplification from non-target bacteria, was produced from four and two of ten DNA extracts of stem/leaves portions sampled at day 10 and day 14, respectively, no detectable amplification occurred from all other assayed stem/leaves extracts.

In comparison, DNA extracts from control plant tissue to which approximately 10^5 cells of *X. o. oryzae* were added consistently produced the *X. o. oryzae* amplification products of 497 bp and 574 bp. At no time were disease symptoms observed on the plants during the seven weeks that they were allowed to grow.

Discussion

The significance of the seedborne phase of *X. o. oryzae* has been difficult to assess because detection of low levels of the pathogen is masked by numerous faster growing seed contaminants (Eamchit and Ou, 1970; Hsieh et al., 1974; Mew et al., 1993). The available methods developed for diagnosis of *X. o. oryzae*, such as semiselective media and immunoassays are valuable but not sufficiently precise for seed detection of the pathogen (Gnanamanickam et al., 1994; Ming et al., 1991). A more sensitive assay that can avoid the problems commonly associated with cultivation methods is needed. PCR-based methods have been described for detection of diverse groups of plant pathogens and can meet the criteria of specificity and sensitivity required for diagnostic procedures (Henson and French, 1993). In the present study we report the PCR-mediated amplification of an internal fragment of IS*1112*, a multicopy insertion sequence isolated from *X. o. oryzae* (Leach et al., 1990), for detection of *X. o. oryzae* in contaminated rice seed. Multi-copy target sequences are known to allow for greater sensitivity, and have been desirable targets for pathogen population studies and PCR detection methods (Adhikari et al., 1995; Audy et al., 1994; Berthier et al., 1994; Leach et al., 1992; Samac et al., 1998).

Aside from a group of ill-defined nonpathogenic xanthomonads, the only *Xanthomonas* species reported from rice is *X. oryzae*, which includes the pathovars *oryzae* and *oryzicola* (Cottyn et al., 2001; Swings et al., 1990). The pathovars of *X. oryzae* share over 90% of DNA homology and occupy a unique position within the genus *Xanthomonas* (Vauterin et al., 1992). The insertion sequence IS*1112* was demon-

strated in high copy number in both pathovars (Leach et al., 1990). Several primers derived from the determined nucleotide sequence of IS*1112* by Yun (1991) were initially tested to find a suitable combination of primers that could differentiate *X. o. oryzae* from *X. o. oryzicola*. All primer combinations directed the amplification of the expected size fragments from strains of *X. o. oryzae* as well as *X. o. oryzicola*. Only combinations with primer Xo2, which anneals to a region near the 3'-end of the IS*1112* sequence, consistently directed the amplification of an additional 77-bp larger DNA fragment aside from the expected size fragments exclusively from strains of *X. o. oryzae*. As such, the selected primer pair Xo1-Xo2 directed the amplification of a 497-bp fragment and an additional 574-bp fragment from all Asian strains of *X. o. oryzae*. Southern hybridization to *X. o. oryzae* DNA using either the whole IS*1112* element or the *X. o. oryzae* amplification products as probes resulted in identical banding patterns. We postulate that there is a specific sequence variation of 77 bp in the amplified region among copies of IS*1112* in the genome of Asian *X. o. oryzae* strains. The diagnostic 574-bp fragment was not amplified from genomic DNA of 27 strains of *X. o. oryzicola*, 18 strains of *Xanthomonas* mainly representing pathovars of cereals and grasses, six nonpathogenic *Xanthomonas* rice isolates, and 17 strains of other species presumably associated with rice seed. On the other hand, the 497-bp fragment characteristic of the IS*1112* element was also amplified from strains of *X. o. oryzicola* and different *Xanthomonas* pathovars. In the study by Leach et al. (1990), seven of 24 tested pathovars of *X. campestris* showed hybridization patterns of less than 10 bands with the IS*1112* element, which indicated that several *Xanthomonas* pathovars may possess a related repetitive element in lower copy number. These *Xanthomonas* pathovars are not known to occur on rice and are unlikely to cause problems in the practical application of the method, although caution is warranted. The IS*1112* element did not hybridize to DNA from nonpathogenic xanthomonads and *Pseudomonas* strains in this study or by Leach et al. (1990). However, weak amplification of a comparable 497-bp product was also observed in nonpathogenic xanthomonads and *Pseudomonas* strains often along with various faint bands, seen only when DNA from pure cultures was used for amplification but not from seed washes. Hence, it is possible that nonspecific amplification products could be produced by untested bacterial strains with the primers described here. However, if observed, these could be readily distinguished from specific amplification products on the basis of band intensity, size, and hybridization analyses.

The primer pair differentiated Asian strains of *X. o. oryzae* from strains of Africa and North America. The African and North American strains were previously shown exceptional in that they contain fewer copies of the IS*1112* element (Leach et al., 1990; Ryba-White et al., 1995), their distinct amplification patterns described here additionally suggests that they contain a more distantly related repetitive element. Although the *X. o. oryzae* strains from Africa clearly hybridized with the IS*1112* element (Ryba-White et al., 1995), they produced only a faint 497-bp band upon amplification with the primer pair, which could be due to the target sequences recognized by the primers. The strains from North America produced three distinct amplification patterns. The North American strains also can be differentiated from Asian strains by their low virulence to rice and distinct fatty acid profiles (Jones et al., 1989), by growth on selective medium

MXO (Ming et al., 1991), and by monoclonal antibodies (Benedict et al., 1989). The increasing evidence for their distinct nature prompts for a taxonomic reevaluation of these strains as *X. o. oryzae*.

The detection limit of the *X. o. oryzae* specific amplification product of 574 bp by ethidium bromide staining was approximately 10 cells per reaction for a pure culture of the pathogen. Although the presence of a large background microflora is a major limitation in the detection of the pathogen by plating on semiselective media (Ming et al., 1991), the PCR assay successfully detected *X. o. oryzae* in contaminated seeds against background bacterial microflora larger than 10^5 cfu/ml of seed wash. In preliminary experiments, a step for 10-fold or 50-fold concentration of the wash extracts was included; however, nonconcentrated wash extracts gave the same results as the concentrated extracts. Because excessive concentration of extracts can concomitantly also increase the presence of potential DNA polymerase inhibitors in seed extracts, amplification was routinely performed from the original seed washes without the need for concentration. Of the assayed seed lots that were harvested during four cropping seasons from bacterial blight infected fields, two were scored positive for the *X. o. oryzae* fragments in all replicate amplifications, one scored positive in only some of the replicate amplifications, and one seed lot was scored negative. If the number of individual seeds that tested positive by PCR may be correlated with the apparent concentration of the pathogen in the seed lot, then dissimilar proportions of seed contamination occurred among seed lots. Although the number of extracts tested from individual seeds is not large, it is interesting to note that a higher rate of seed contamination occurred for the seeds from Calauan and Pila that were collected during wet seasons. Mundt et al. (1999) considered splash dispersal to be the most likely explanation for disease spread of bacterial blight in infested fields. Because the pathogen multiplies rapidly on the surface of leaf lesions, secondary spread of bacterial blight in the tropics is associated strongly with the occurrence of typhoons, which disperse inoculum from leaf-to-leaf and from plant-to-plant (Mizukami and Wakimoto, 1969; Ou, 1985). As such, infected upper leaves might easily provide inoculum sources for seed contamination of the panicles, which would also corroborate previous data on the superficial nature of seed contamination (Hsieh et al., 1974; Mizukami and Wakimoto, 1969). The suggested virtual absence of seed contamination during the dry season, when typhoons are rare, might be attributed to lack of dispersal. However, further work is needed to prove this hypothesis correct.

Detection of *X. o. oryzae* associated with contaminated seed by the PCR procedure was compared with plating on semiselective XOS medium and seed transmission tests. We did not detect *X. o. oryzae* from naturally contaminated seeds by dilution plating on XOS medium, which is consistent with recent studies that also failed to detect *X. o. oryzae* from seeds harvested from bacterial blight infested fields on semiselective media (Gnanamanickam et al., 1994; Jones et al., 1989; Ming et al., 1991). On the other hand, strain PXO340 of *X. o. oryzae* was recovered on XOS medium from artificially contaminated seeds only when present in high levels in comparison to other bacteria in seed washes. This corroborates the findings of Gnanamanickam et al. (1994) regarding the inadequacy of XOS medium for detection of low populations of *X. o. oryzae*. Attempts to recover PXO340 on XOS medium after germination of the artificially contaminated seeds, however, were unsuccessful. This might be due to

interference by numerous faster growing contaminants on the germinating seeds, or to failure of *X. o. oryzae* to multiply actively on the seed. Kauffman and Reddy (1975) also reported that *X. o. oryzae* populations rapidly decline during seed imbibition and eventually might die before the seed germinates. Despite the semiselectivity of XOS medium, plating of seed washes resulted in the growth of background bacterial microflora and similar appearing nonpathogenic xanthomonads among other yellow contaminants of rice seed such as *Pseudomonas* and *Pantoea* species were found, which increases the difficulty of detecting *X. o. oryzae*. The presence of nonpathogenic xanthomonads in rice seed lots could present a potential identification problem for regulatory agencies, and may have contributed to conflicting reports regarding the seed transmission of bacterial blight (Ming et al., 1991).

Transmission of the pathogen from contaminated seeds to emerging seedlings could not be determined conclusively. At no time was a positive PCR amplification for presence of *X. o. oryzae* obtained from stem/leaves DNA extracts of plants derived from artificially or naturally contaminated seeds. Amplification of a single 497-bp product in low yield was occasionally detected, which might be due to nonspecific amplification from epiphytic *Pseudomonas* and nonpathogenic *Xanthomonas*. Furthermore, no disease symptoms developed on the plants during the seven weeks they were observed. Our results support previous studies on seed transmission of the bacterial blight pathogen (Eamchit and Ou, 1970; Kauffman and Reddy, 1975; Murty and Devadath, 1984), which suggested that the seedborne phase is a weak point in the life cycle of the pathogen and unlikely to be important for disease spread. Several investigators have emphasized the greater significance of other sources of primary inoculum for bacterial blight epidemics such as plant debris, weed hosts, or irrigation waters (Hsieh and Buddenhagen, 1975; Mew et al., 1993; Mizukami and Wakimoto, 1969; Ou, 1985). However, seed transmission of phytopathogens often is complex and depends on the host, pathogen, environment, and their interaction over time. Assessment of seed transmission should preferably be done in the field crop using large numbers of naturally contaminated seeds sown under optimal conditions for disease expression to obtain the desired level of confidence in the data. Further, it must be shown in experimental demonstration of transmission that no alternate sources of inoculum are present. No studies on the seed transmission of *X. o. oryzae* have fulfilled all these requirements yet.

The epidemiological significance of seedborne *X. o. oryzae* would in first instance depend on whether the pathogen remains viable on the seed until planting. The viability of *X. o. oryzae* on seed is believed to be short under the high temperature and relative humidity of the tropics (Eamchit and Ou, 1970), however, there is still uncertainty regarding the length of survival of *X. o. oryzae* on seed and estimates range from two months (Kauffman and Reddy, 1975) up to six months (Murty and Devadath, 1984). A disadvantage of the PCR-based method is that a positive result based solely on the detection of DNA fragments cannot ascertain whether the pathogen is still viable on the seed. The positive PCR amplifications obtained up to one year after seed coating with a lysed culture of *X. o. oryzae* demonstrated the long-term persistence of DNA for PCR detection. On the other hand, an immunocapture PCR assay that uses the *X. o. oryzae* specific monoclonal antibodies (Benedict et al., 1989) to concentrate the

pathogen from seed samples prior to PCR with the primers described here, could demonstrate viability by plating a subsample of the immunocaptured cells. With the added advantage that the identity of detected cells could be confirmed by fingerprinting and screening for pathogenicity on differential rice cultivars, which would allow to track the spread of different strains through a host plant community.

The simple, yet sensitive, PCR assay described here has the potential to detect low levels of the rice bacterial blight pathogen that could not be detected on XOS medium. The advantage of using oligonucleotide probes with the PCR is that the tests are easy to perform and the results are readily visualized on agarose gels. Additionally, the primer pair can differentiate *X. o. oryzae* from *X. o. oryzicola* and nonpathogenic xanthomonads whenever doubtful yellow-pigmented colonies are isolated. The detection of *X. o. oryzae* relied on the amplification of an internal IS*1112* region that appears to contain a specific 77-bp sequence variation among copies in the genome of *X. o. oryzae*. The design of a "nested" set of primers that would amplify only the 77-bp sequence variation in the targeted region might further improve the specificity of the method. Although the PCR assay can be used to detect the pathogen in contaminated seed lots, and further supported the opinion that the seedborne phase might rather be a random event, additional research is needed to exclude the significance of seedborne *X. o. oryzae* for disease spread of bacterial blight.

Acknowledgments

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CHAPTER 4

SUMMARY AND CONCLUSIONS

With increasing public awareness of the environmental hazards of agrochemicals, current trends in crop protection are directed towards ecologically based ap proaches that rely on management rather than eradication of deleterious organisms (Jacobsen, 1997; Lewis et al., 1997). Microorganisms can play varied roles in plant development, which are of immediate significance to farmers' production systems. Applications of microbial biocontrol agents as seed treatments to suppress disease or enhance plant growth have since long been employed in agriculture. A long-term goal of plant pathologists is to improve crop health and yield through effective management of indigenous plant-associated microbial communities. Research in the last decades has presented hints of the enormous potential that exploitation of plant-associated microorganisms might bring for managing plant health. However, before this potential can be fully realized, it will first be necessary to obtain full knowledge of microbial ecology in agricultural ecosystems. The characterization of plant-associated bacterial populations is just one first step toward understanding its microbial ecology.

The aim of this study was to characterize total culturable bacterial populations associated with rice seeds in an attempt to provide more insight into its bacterial diversity, in order to be able to develop knowledge-based strategies of microbial applications for disease management. The selection of the rice seed was based on the fact that the microbial diversity associated with this ecological niche had not previously been investigated. Bacterial isolations were done by dilution plating of seed extracts on agar medium. All morphologically distinct colony types observed per sample were picked and each type was enumerated on the dilution plates. In this work, 136 seed samples harvested from tropical rice fields were examined and more than 6,000 bacterial colonies isolated. The bacterial collection is kept in the Plant Pathology Division of IRRI at –70°C in nutrient broth with 15% glycerol, and representative isolates were lyophilized. All 6,000 isolates were described for colony morphology, and screened for the ability to induce disease symptoms in rice seedlings and for in vitro antagonism towards major fungal rice pathogens. A more detailed phenotypic and genotypic description was made for isolates from selected samples by characterization of cellular and colonial morphology, whole-cell fatty acid composition, and BOX-PCR genomic fingerprints. A first study characterized the bacterial communities isolated from seed samples obtained from six rice cultivars and seven different locations of rainfed rice production environments. A second study characterized the types of bacteria isolated from seed samples of a single rice cultivar grown in farmers' fields at one location of irrigated rice production systems. The bacteria isolated from rice seeds roughly consisted of 52% gram-negative bacteria and 48% gram-positive bacteria. Predominant bacteria were identified as strains of *coryneforms, Enterobacteriaceae,* nonfluorescent *Pseudomonas* and *Bacillus* species. Other bacteria regularly found were nonpathogenic *Xanthomonas* sp. and pathogenic bacteria of the genus *Burkholderia*. An interesting array of other gram-negative and gram-positive species was identified at lower frequencies. One characteristic of the bacterial communities associated with rice seed was the abundance of pigmented forms. Chromogenic species are reported common to the phyllosphere, and has led to the speculation that pigment production may confer a selective advantage to bacteria that colonize a habitat that is exposed to solar radiation (Hirano and Upper, 2000). In our study, detailed characterization of colony appearance in combination with Gram-morphology was found useful for a first grouping of the isolates, especially for *coryneforms*. The coryneform bacteria isolated from rice seeds display a great variety in yellow pigmented colonies, are

relatively homogeneous on the basis of fatty acid composition but reveal a high genetic heterogeneity on the basis of BOX-PCR fingerprinting. Therefore, the classification into pigmentation groups, further subdivided into colony morphotype groups, was found useful in structuring the diversity of coryneform isolates, and conformed relatively well to the results of the BOX-PCR fingerprinting analysis (see Appendix 1, p. 190).

The collection of isolates was analyzed with phenotypic and genotypic methods that provide information at different resolution levels. BOX-PCR genomic fingerprinting of individual isolates revealed an overall high genetic diversity. The generated fingerprints were analyzed by computer-assisted pattern analysis to construct separate dendrograms linking DNA fingerprints to defined colony morphological types, and conclusions about the genetic diversity within morphotype groups as well as diversity among samples could be reached. A considerable variability in composition of bacterial communities was found among samples. No higher similarity of bacterial communities was observed among samples analyzed from a single rice cultivar. The majority (approximately 70%) of BOX-PCR fingerprint types (FPTs) was not shared among samples and only a few FPTs (approximately 5%) were frequently detected from different samples. When FPTs were compared between the two surveys, approximately 15% of FPTs were found to be shared among the isolates obtained from two different rice production environments. The FPTs shared in common belonged to rare as well as dominant colony morphotype groups, and might represent bacteria that are well adapted to the rice seed environment. Nine dominant FPTs that were frequently detected in both environments belonged to *Bacillus pumilus* LMG 20162, *Burkholderia glumae* LMG 20138, *Clavibacter michiganense* LMG 20187 and LMG 20195, *Curtobacterium flaccumfaciens* LMG 20194, *Enterobacter cloacae* LMG 20117, *Pantoea dispersa* LMG 20116, *Pantoea stewartii* LMG 20115, and nonfluorescent *Pseudomonas aeruginosa* LMG 20125. The general picture is that rice seed harbors a high diversity of bacterial populations and that some populations can be detected more frequently. The presence of a few dominant components with a large number of populations that occur at lower frequencies fits a common pattern observed in numerous studies of organismal community structure (Ricklefs and Miller, 2000).

The isolates were classified into functional groups in terms of their potential to induce disease symptoms upon injection in rice seedlings, or to inhibit in vitro the mycelial growth of rice-pathogenic fungi. In the pathogenicity tests, the majority (approximately 90%) of rice seed isolates had no observable effect after inoculation in seedlings as compared to controls. Respectively 2 and 4% of the isolates from the two surveys produced disease symptoms in all inoculated seedlings, and were considered true pathogens. They were identified as strains of *Burkholderia glumae*, *Burkholderia gladioli*, and *Acidovorax avenae* subsp. *avenae*. An additional 5 and 7% of isolates from the first and second survey, respectively, were able to produce weak disease symptoms in 50 to 90% of plants that were inoculated, hence were considered pathogens with low disease potential. They were identified as belonging to *Bacillus pumilus*, *Paenibacillus polymyxa*, *Burkholderia cepacia*, fluorescent *Pseudomonas* spp., and *Pantoea stewartii*. This group of pathogens with low disease potential has not previously been recognized because members of these species have not been observed yet as causing epidemics in the field. They have probably eluded detection in the past, at least in part, because screening of total bacteria from rice seed for pathogenicity has not previously been done. Also, the symptoms produced such as sheath browning, necrosis along the leaf margins and wilting of the newly emerging leaf, are subtle

and can easily escape notice in field conditions, especially because the plants commonly outgrow the symptoms. Their effect on plant growth may be comparable to previously reported "minor pathogens" (Salt, 1979; Suslow and Shroth, 1982). We assume that eventually they could emerge as more threatening to crop growth under certain environmental conditions and cropping practices. One could argue that injection of plants grown in the greenhouse may not always be a good indicator of the pathogenic potential of a strain because plant reactions may not resemble those encountered in the field and hence be difficult to interpret. However, there are no standard guidelines in phytobacteriology as to which inoculation method to use, or what inoculum dosage is too high to be considered a fair test of a strain's possible pathogenicity.

The screening of isolates by the dual culture test on agar medium for antagonism toward pathogenic fungi provided presumptive evidence for the occurrence of antifungal activity in the seed environment. Respectively 4 and 16% of isolates from the two surveys showed in vitro antagonism against the pathogenic fungi *Rhizoctonia solani* AG1 and/or *Pyricularia grisea*. These antagonistic bacteria belonged to a number of different genera and families including *Bacillus*, *Burkholderia*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, and *coryneforms*. Considering the great variety of antagonistic bacteria that can be isolated from rice seed, it would seem reasonable to hypothesize that introducing mixtures of compatible antagonists could have more sustainable effects than single strains for accomplishing disease control. Understanding the genetic diversity within populations of antagonistic bacteria with a common biocontrol trait further holds the promise of pairing specific genotypes with their most supportive environments to maximize host colonization and disease suppression (Weller et al., 2002). Presently, application of a single biocontrol agent for the control of a specific target disease is the most popular strategy. However, there are examples where combinations of antagonistic strains resulted in improved biological control of various bacterial and fungal diseases (Fukui et al, 1999; Janisiewicz, 1996; Raupach and Kloepper, 1998). Except in cases of induced systemic resistance (Liu et al., 1995), rapid and extensive colonization of plant surfaces, particularly seeds, by biocontrol agents that act by competition or antagonism is believed to be important for successful disease suppression (Bull et al., 1991; Kim et al., 1997; Parke, 1990; Weller, 1988). Resistance-inducing rhizobacteria offer an attractive alternative for controlling plant diseases. Unlike biological control mediated through bacterial antagonism, rhizobacteria-mediated induced systemic resistance (ISR) can be effective even if populations of inducing bacteria decline over time. Once activated, the natural resistance mechanisms of the host maintain an enhanced defensive capacity for prolonged periods and are effective against multiple pathogens. Two forms of induced resistance in plants, pathogen-induced systemic acquired resistance (SAR), and nonpathogenic rhizobacteria-mediated induced systemic resistance (ISR), have been demonstrated (van Loon et al., 1998). SAR is characterized by an accumulation of salicylic acid (SA) and pathogenesis-related proteins (PRs). Experiments with *nahG*-transformed plants indicated that SA is an essential signaling molecule in SAR induced by necrotizing pathogens. The *nahG* gene encodes salicylate hydroxylase, which converts SA into catechol, a product that does not induce resistance. ISR refers to resistance developed in plants following colonization by biocontrol rhizobacteria, and is mediated by jasmonic acid and ethylene and is not associated with expression of pathogenesis-related proteins (PRs) (Pieterse et al., 1996). Thus, ISR operates by a pathway distinct from that of SAR, without activation of PR protein genes. A regulatory gene in SAR signal transduction,

the *npr1* gene, has been discovered and encodes an ankyrin repeat-containing protein that is essential for defense gene activation (Buchanan et al., 2000). The NPR1 protein functions beyond the expression of PR-genes and is required for both pathogen-dependent and rhizobacteria-mediated systemic induced resistance. Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi (van Wees et al., 1997), bacteria (Press et al., 1997; van Peer et al., 1991; Wei et al., 1991), and viruses (Raupach et al., 1996).

Obviously, potential candidates for use as biological seed treatment should survive and be able to grow in the environment of germinating seed. To assess changes in the bacterial community structure in response to seed germination, additional isolations were done from in vitro germinated rice seed samples. Comparative BOX-PCR analyses of the isolates obtained from dry seeds with those obtained from germinated seeds suggested seed germination to result in a qualitative shift and reduction in diversity. Remarkably, almost no gram-positive bacteria were isolated after seed germination. Several new FPTs belonging to gram-negative colony morphotypes not recovered from dry seeds and fewer but identical FPTs belonging to groups of predominant gram-negative bacteria on dry seeds, were detected from germinating seeds.

Special emphasis was given to particular groups of pathogenic bacteria that have been implicated with newly emerged disease problems due to the intensification of rice production during the last decades.

- A detailed study was made on the characterization of isolates from the genus *Burkholderia* that were prominent in the collection of pathogenic bacteria obtained from rice seeds collected from farmers' fields in the Philippines. The results demonstrated the presence of a single strain of *B. vietnamiensis* and four major groups of *Burkholderia* species respectively identified as *B. glumae, B. gladioli*, and *B. cepacia* genomovars I and III. The detection of *B. glumae* as predominant seedborne pathogen on rice seed is not surprising, as it is a well-known rice pathogen. The detection of *B. gladioli* on the contrary, was rather unexpected as this species has been rarely reported from rice and is not mentioned in standard rice pathology works. The widespread occurrence on apparently healthy seeds of *B. glumae* and *B. gladioli* indicates that they can survive as epiphytic populations on the seed, and might provide inoculum for disease development whenever the environment becomes favorable. Both pathogens are also described as beneficial bacteria on other host plants (Furuya et al., 1991; Walker et al., 1996). *B. cepacia* is a common inhabitant of plants and is well known as biocontrol agent; however, concern has been raised since members of genomovar III were recognized as the major cause of respiratory tract infections in cystic fibrosis patients (Vandamme et al., 1997). Our results indicated that BOX-PCR fingerprinting has potential for identification of the pathogens *B. glumae* and *B. gladioli*, and for differentiating strains of *B. cepacia* genomovars I and III, while at the same time providing information on the subspecific genetic heterogeneity within these four groups (see Appendix 3, pp. 228 and 229).
- The studies made on the etiology of grain discoloration and the sheath rot syndrome of rice in the major rice growing districts of the Philippines suggest the involvement of a complex group of fluorescent pseudomonads. *Pseudomonas fuscovaginae*-like bacteria were repeatedly isolated from heavily discolored seeds collected from plants with sheath rot symptoms. Only on one occasion were such pathogenic pseudomonads isolated from apparently healthy seeds collected in the surveys made on rice seed-associated bacteria.

These fluorescent pseudomonads strongly resemble *P. fuscovaginae* in caused symptoms and various phenotypic characteristics but differ in serology and clearly constitute a separate group on the basis of BOX-PCR fingerprinting. Although isolate 6031 from the Philippines was reported to be closely related to the type strain of *P. putida* LMG 2257 on the basis of 16S-rDNA RFLP analysis (Jaunet et al., 1995), no such relationship could be found on the basis of BOX-PCR fingerprinting in our study. It is clear that the challenge in understanding the etiology of the grain discoloration and sheath rot syndrome is intimately tied with the complexity of the *Pseudomonas* taxonomy.

- A polymerase chain reaction (PCR) technique was developed for detection of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) in rice seed, and for evaluating its possible seed transmission. Bacterial blight, caused by *Xoo*, is one of the most important diseases of rice. Seedtransmission of *Xoo* is still a controversial issue. The pathogen can be seedborne but is especially difficult to isolate from seeds and therefore an ideal candidate for a PCR-based detection method. In our experience, *Xoo* was neither isolated in the extensive surveys made on rice seed-associated bacteria, nor from seeds collected from plants with bacterial blight symptoms. Conversely, similar appearing nonpathogenic xanthomonads were frequently isolated from rice seeds. This situation emphasizes the possibly that nonpathogenic xanthomonads associated with bacterial blight diseased plants might be mistaken for *Xoo* and the need for a reliable, specific diagnostic test for *Xoo*. The PCR assay developed can differentiate *Xoo* from *X. o. oryzicola* and nonpathogenic xanthomonads, and has the potential to detect low numbers of *Xoo* in seed extracts that could not be detected by plating on semi-selective medium. No transmission of the pathogen from contaminated seeds to emerging seedlings could be detected. Our results support the opinion that the seedborne phase of *Xoo* is unlikely to be important for disease spread.

The diversity analyses made in this study by BOX-PCR fingerprinting revealed the amount of genetic variation within populations of morphological types defined on the basis of similarities in cellular and colonial characteristics and fatty acid composition. At first sight, BOX-PCR analysis of rice seed-associated microbial populations appeared to reveal an almost limitless variety of FPTs. Subsequently, FPTs detected previously could be detected again and the more isolates that were found of a particular morphotype group, the more the group clustered closer together. Thus, it seems that the bacterial diversity within the found populations is not infinitely variable, and that populations of rice seed can probably be described with reasonable accuracy provided that a greater number of samples are being analyzed. The dendrograms (see Appendix 1, pp. 189-195) constructed from the BOX-PCR analysis illustrate the amount of genetic variation among isolates within predominant colony morphotype groups detected from rice seeds. Several morphotype groups, such as the group of nonpathogenic xanthomonads and *Bacillus subtilis*, revealed a high level of genetic diversity, while others, such as those identified as *Pantoea stewartii* and *Burkholderia glumae*, appeared to be much more uniform. It is clear that the amount of genetic variation can greatly vary among colony morphotype groups and serves as a caution for accepting a single colony on an isolation plate as typical of the complete population. The exact taxonomic levels of the revealed genetic variation within each morphotype group are not clear, however, the resolution of BOX-PCR fingerprinting relates to species, subspecies, and strain level. The genetic variability of bacterial populations can be considered as a possible mechanism for populations to

respond successfully to environmental changes. Processes of mutation, recombination and migration generate genetic variation within natural populations, whereas selection pressure and genetic drift act upon existing variation (Feil and Spratt, 2001; Ochman et al., 2000). Recombinational events are mediated by transformation (naked DNA from the environment), transduction (by a bacteriophage), and conjugation (physical contact between donor and recipient cells). The frequency of recombination depends on a multitude of biological and ecological factors. Bacteria also harbor a variety of mobile elements, such as plasmids, transposons, and insertion elements, that have at least the potential for considerable genetic exchange and also rearrangement of chromosomal genes. Recent studies have shown the considerable variation in the rates and impact of recombination in different species. BOX-PCR fingerprints are a reflection of genome structure (Louws et al., 1994), and selection for a specialized niche is assumed to affect genome organization, hence the distribution of repetitive sequences. The diversity analysis made by BOX-PCR fingerprinting in our study provides a provisional framework of the genetic structure of various rice seed-associated populations. Knowledge of the structure of indigenous bacterial populations that inhabit natural ecosystems is limited. Interest in the genetic structure of populations of plant-inhabiting bacteria has been largely centered on pathogenic bacteria, and information about pathogen population dynamics has shown useful in designing improved strategies for disease management and resistance breeding (Leung et al., 1993). It is theorized that the amount of genetic variation in pathogen populations reflects its evolutionary potential, and its capability to overcome disease management strategies (McDonald and Linde, 2002). A new molecular typing procedure, multilocus sequence typing (MLST), has recently been developed for analysis of bacterial population structure (Maiden et al., 1998). MLST characterizes the genetic relatedness of isolates by partial sequence analyses of seven housekeeping genes. Clonal complexes defined by MLST have been suggested to correspond to ecotypes within defined bacterial species (Cohan, 2002). Ecotypes are defined as populations of organisms occupying the same ecological niche, whose divergence is purged recurrently by natural selection. The introduction of innovative methods during the last decade which allow determination of inter- and intraspecies relatedness between organisms has provided new categories of information of potential taxonomic value. Bacterial taxonomy recognizes the diversity present within defined species and recommends to maintain the current parameters of DNA-DNA similarity for species delineation (Wayne et al., 1987) whilst integrating the new techniques and knowledge (Stackebrandt et al., 2002). Bacterial species can be considered as “condensed nodes” in a cloudy and confluent taxonomic space (Vandamme et al., 1996).

In general, the same genera or species of bacteria detected on rice seed, have also been found as epiphytes on a broad range of plants (Hirano and Upper, 2000). However, these species are themselves highly variable and the rice seed might provide a more selective environment for certain individuals, whereas other members of these species might be adapted to many plants. In this perspective, there has been an increased interest in the role that the host plant might play in shaping plant-associated microbial communities. Experimental evidence suggests that epiphytic microbial communities are similar on different individuals of the same plant species but might be unique on different plant species (Smalla et al., 2001; Yang et al., 2001). It is assumed that plants selectively stimulate certain microbial populations possibly by exudates that are different in different plant species, and hence, might support the growth of different bacteria. The observation of such high varia-

tion within bacterial populations and the fact that many of the economic properties of microorganisms, such as antibiotic production and pathogenesis, vary below the species level indicates that a high level of resolution is desirable for bacterial diversity studies. Plant pathogens are generally being defined as all members of a particular pathogenic bacterial species, but differences in aggressiveness between strains of the same pathogenic species can often be found, and not all strains of a pathogenic species may express pathogenicity. Often strains can express both deleterious and beneficial properties depending on particular conditions and host plant (Åström and Gerhardson, 1988; Gardner et al., 1984). This phenotypic variation is even higher among biocontrol agents, the potential to inhibit mycelial growth or induce plant growth promotion is found in several bacterial species but does not apply to all strains of these species. Studies have shown that the antagonistic activity and the antifungal mode of action of plant-associated bacteria is often strain specific (Keel et al., 1996; Minkwitz and Berg, 2001; Neiendam-Nielsen et al., 1998). Differential ability for colonization of the host has been shown among antagonistic strains (Hebbar et al., 1992; Landa et al., 2002; Raaijmakers and Weller, 2001). Several researchers have observed variation among plant species or cultivars for antibiotic production (Georgakopoulos et al., 1994), disease suppression (King and Parke, 1993; Smith et al., 1997), host competence (Berg et al., 2002), and induction of resistance (Liu et al, 1995: van Wees et al., 1997) by microbial biocontrol agents. Such host variation for response to beneficial microorganisms suggests that plant genes play a role in supporting these interactions (Smith et al., 1999). It is not known what the relationship is between host genes that affect pathogenic and beneficial plant-microbe interactions. However, there is increasing evidence suggesting the possibility of similar signaling for the different plant-microbe interactions. Perhaps one of the most intensively studied plant-bacterium systems is the interaction between legumes and rhizobia. The host-specific interaction between legumes and rhizobia shows similarity to the gene-for-gene relationships (Flor, 1971; Heath, 1991) observed in many pathogen systems. Recently, the plant receptor molecules required for mycorrhizal and *Rhizobium* symbiotic plant-microbe interactions have been identified, and surprisingly, it was the same receptor-like protein kinase involved in both the bacterial and fungal symbiosis (Endre et al., 2002; Stracke et al., 2002). Plant receptor kinases also play a role in the sensing of pathogen-derived factors, and similar proteins are found in animals that function in the innate immune system. Many aspects of induced plant defense appear to be conserved in other eukaryotes, perhaps indicating the existence of an ancient defense strategy against microbial attack. It is anticipated that advances in the understanding of the genetic basis of pathogenicity will likely contribute to the understanding of the interactions between plants and beneficial microbes. In this genomic era, as an ever-increasing number of whole bacterial genomes will become available, comparative and functional genomics will become extremely powerful.

Finally, this work represents one of the first studies in which total bacterial populations associated with rice seeds are described in detail. The results have provided a better understanding of the composition of bacterial communities associated with rice seed and the predominant culturable populations have been determined. A considerable phenotypic and genotypic bacterial diversity was detected despite the fact that what has been actually characterized is the fraction of culturable bacteria obtained by dilution plating of seed extracts on agar medium. Over a hundred bacterial types were detected and several of these, particularly gram-positives, have not been previously described from rice seed. However,

many questions still remain unanswered: What controls the distribution and abundance of members of the microbial community? How do these communities change with time in response to their environment? How geographically unique are particular populations? What genotypic and phenotypic characteristics influence competitive success in microbial populations and communities? What is the extent of microbial diversity in relation to the significance of the unculturable bacteria? The high variability in composition of bacterial communities among samples collected from different fields, rice environments and cropping seasons, may contain critical information about the dynamics of the seed environment. The statistical significance of the difference in genetic diversity between samples could not be tested because the analysis was based only on a one-time sampling per field and the variances of the diversity within the individual fields were not known. The extent to which reliance on culture-based methods limits the study of bacterial populations in association with rice seed remains unexplored. Analysis of microbial diversity must ultimately embrace the entire microbial community DNA, rather than DNA from culturable bacteria. Since the start of this work, several high-throughput PCR-based methods have been developed that can reveal changes in the composition of microbial communities over time and space (Duineveld et al., 2001; Smit et al., 2001). Culture-independent methods such as DGGE and T-RFLP are relatively rapid to perform, and many samples can be run simultaneously. Comparing culture-based and culture-independent analyses of bacterial diversity could contribute to a better understanding of microbial communities and actually provide complementary results to reveal an increased proportion of the microbial diversity. A disadvantage of these culture-independent methods is that analyses of microbial community structure are commonly restricted to a determination of whether microbial communities are similar or different between samples. Alternatively, the information provided in this study could serve as a framework for further molecular ecological studies to examine how specific microbial groups respond to environmental manipulation. Which could provide more “safe” information on the possible variations in bacterial community structure between ecosystems. Sequence analysis of representative isolates from “key” bacterial groups identified in this study would allow the identification of diagnostic sequence regions that are suitable for the recognition of these taxa, leading to the synthesis of probes. Such taxa-specific probes could then be used to investigate more rapidly the temporal and spatial changes of certain community members by using culture-independent molecular methods.

The microbial ecology of the seed environment, and population dynamics on the germinating seed, are extremely complex but still poorly understood. An improved understanding of the significance of the high diversity of populations on the functioning of microbial communities, their interactions with the host plant and the environment, is still needed in order to develop strategies that will allow the management of bacterial communities for improving crop health.

NEDERLANDSE SAMENVATTING EN CONCLUSIES

Met het algemeen groeiend milieubewustzijn, heeft zich ook in de landbouw de strekking ontwikkeld naar het gebruik van milieuvriendelijke methodes voor gewasbescherming die eerder gericht zijn op het natuurlijk onderdrukken van schadelijke organismen dan het gebruik van chemische bestrijdingsmiddelen (Jacobsen, 1997; Lewis et al., 1997). Micro-organismen kunnen diverse rollen vervullen in de plantengroei die van onmiddelijk belang zijn voor landbouwproduktiesystemen. De behandeling van zaden met microbiële biokontrole middelen voor bescherming tegen plant pathogenen of voor plantengroeibevordering, is een wijdverspreid gebruik in de landbouw. Onderzoek in de loop van de voorbije decennia heeft hoopgevende resultaten aangetoond van de enorme toepassingsmogelijkheden van plant-geassociëerde micro-organismen voor het bevorderen van de plantengroei. Het doel op lange termijn is om de opbrengst van landbouwgewassen te kunnen verbeteren door strategiën te ontwikkelen die toelaten om de met planten geassociëerde microbiële gemeenschappen gericht te exploiteren en te sturen. Echter, alvorens dit potentieel tenvolle zal kunnen benut worden, is het eerst nodig om een betere kennis te verwerven van de microbiële ecologie in landbouwsystemen. De karakterisering van individuele microbiële populaties is een eerste stap naar het beter begrijpen van plant-geassociëerde microbiële gemeenschappen.

De bedoeling van deze studie was om een inzicht te verwerven in de samenstelling en struktuur van de microbiële flora van rijstzaad. De keuze van rijstzaad als studieobject was gebaseerd op het feit dat de bacteriële diversiteit van dit niche nog niet eerder werd onderzocht. Bacteriële isolaties werden uitgevoerd van rijststalen geoogst van verschillende rijstvelden en lokaliteiten in de Filipijnen. De gevolgde strategie was hierbij om per staal alle verschillende kolonietypes te isoleren die kunnen verkregen worden na het uitplaten van verdunningen van rijstzaad suspensies op agar medium. De suspensies werden door wassen of homogenisatie van rijstzaden verkregen. En de aantallen van elk kolonietype werden geteld op de dilutie platen. In de totale studie werden 136 rijststalen onderzocht en meer dan 6000 bacteriële kolonies geïsoleerd. De isolaten werden geclassificeerd volgens koloniemorfologie, en zijn bewaard bij –70°C in groeimedium met 15% glycerol in de Afdeling voor Plant Pathologie van het IRRI. Alle isolaten werden gescreend op hun vermogen om ziektesymptomen te verwekken in rijstplanten en om in vitro de groei van belangrijke rijstpathogene schimmels te inhiberen. Voor isolaten van geselecteerde stalen werd de fenotypische en genotypische diversiteit beschreven op basis van gedetailleerde karakterisering van cel- en koloniemorfologie, gaschromatografische analyse van cellulaire vetzuren, en BOX-PCR genomische fingerprinting. In een eerste studie werd de samenstelling en variabiliteit onderzocht van bacteriële gemeenschappen geassociëerd met rijststalen afkomstig van zes variëteiten en zeven lokaliteiten van niet-geïrrigeerde rijstproduktiesystemen. In een tweede studie werden de bacteriële gemeenschappen onderzocht van stalen van rijstvariëteit PSBRc14 afkomstig van aanliggende velden in eenzelfde geïrrigeerd rijstproduktiesysteem. De bacteriën geïsoleerd van rijstzaden bestonden gemiddeld uit 52% Gram-negatieve bacteriën en 48% Gram-positieve bacteriën. Koloniemorfotypes die domineerden werden geïdentificeerd als behorende tot *Bacillus* species, coryneformen, *Enterobacteriaceae*, en niet-fluorescerende pseudomonaden. Andere

veel voorkomende bacteriën waren pathogene *Burkholderia* species en niet-pathogene xanthomonaden. Verder werd de bacteriële flora van rijstzaden nog gekenmerkt door een brede waaier van andere Gram-negatieve en Gram-positieve species die voorkwamen met lage frekwenties. Opvallend voor de bacteriële gemeenschappen geassociëerd met rijstzaden was de verscheidenheid aan gepigmenteerde kolonietypes. Chromogene species zijn typisch voor de fyllosphere, en heeft geleid tot de hypothese dat pigment produktie een selektief voordeel zou meebrengen voor bacteriën die een habitat koloniseren dat blootgesteld is aan UV-bestraling van de zon (Hirano and Upper, 2000). Koloniemorfologie, in combinatie met Gram-morfologie karakterisering, werd nuttig bevonden in onze studie als kenmerk voor een eerste classificatie van de isolaten, en dan voornamelijk voor de coryneformen. Coryneforme bacteriën werden gevonden in een verscheidenheid van geel-gepigmenteerde kolonies, en waren relatief homogeen in vetzuren samenstelling maar zeer heterogeen op basis van de BOX-PCR genomische fingerprinting. De doorgevoerde classificatie in pigmentatiegroepen, verder onderverdeeld in koloniemorfotypes, bleek nuttig om een struktuur te scheppen in de diversiteit van coryneformen, en stemde bovendien goed overeen met de resultaten van de BOX-PCR fingerprinting analyse (zie Appendix 1, pag. 190).

De genetische diversiteit van de isolaten werd onderzocht aan de hand van BOX-PCR genomische fingerprinting. Bij de classificatie van de isolaten werd de similariteit tussen hun genotypische patronen berekent door middel van computer-ondersteunde analyse, en werden zeer gelijkaardige patronen geklasseerd in eenzelfde fingerprinttype (FPT). Hierbij werd een grote diversiteit en variabiliteit in samenstelling van bacteriële gemeenschappen in de onderzochte stalen aangetoond. Bovendien kon geen grotere similariteit in bacteriële gemeenschappen waargenomen worden tussen de onderzochte stalen van eenzelfde rijstvariëteit. De meerderheid (ongeveer 70%) van de BOX-PCR fingerprinttypes (FPTs) werd gevonden in afzonderlijke stalen, en slechts een gering aantal FPTs (ongeveer 5%) werd frekwent gevonden in meerdere stalen. Een vergelijkende analyse van de FPTs tussen de twee studies toonde de aanwezigheid aan van gemeenschappelijke FPTs (ongeveer 15%) in de kollekties van isolaten afkomstig van verschillende rijst produktie systemen. De gemeenschappelijke FPTs behoorden zowel tot zelden voorkomende als dominante koloniemorfotypes. FPTs die frequent werden teruggevonden in de staalnames van beide rijstproduktiesystemen werden geïdentificeerd als *Bacillus pumilus* LMG 20162, *Burkholderia glumae* LMG 20138, *Clavibacter michiganense* LMG 20187 en LMG 20195, *Curtobacterium flaccumfaciens* LMG 20194, *Enterobacter cloacae* LMG 20117, *Pantoea dispersa* LMG 20116, *Pantoea stewartii* LMG 20115, en de niet-fluorescerende *Pseudomonas aeruginosa* LMG 20125. Men zou kunnen aannemen dat deze populaties een belangrijke komponent vormen van de zaadmicroflora en mogelijks efficiënt rijstzaden kunnen koloniseren. Het algemene beeld is dat rijstzaden een grote verscheidenheid aan bacteriële populaties herbergen en dat sommige populaties meer frekwent voorkomen. Het voorkomen van een gering aantal dominerende komponenten en een groot aantal populaties die voorkomen met lage frekwenties, is een typisch patroon dat zowel microbiële als macro-ecologische gemeenschappen kenmerkt (Ricklefs and Miller, 2000).

De isolaten werden verder ingedeeld in funktionele groepen naargelang van hun vermogen om ziektesymptomen te verwekken na injectie in rijstplanten, of om in vitro de groei van rijstpathogene schimmels te onderdrukken. Uit de pathogeniciteitstesten bleek dat de meeste isolaten (ongeveer 90%) na injectie geen observeerbaar effekt hebben op

rijstplanten. Respektievelijk 2 en 4% van de isolaten uit de twee afzonderlijke studies veroorzaakten symptomen in alle geïnokuleerde planten, en werden beschouwd als “echte” pathogenen. Een bijkomend aantal isolaten, die respektievelijk 5 en 7% uitmaakten van de isolaten uit de twee studies, waren in staat om symptomen te veroorzaken in 50 tot 90% van geïnokuleerde planten en werden beschouwd als pathogenen met zwak ziekteverwekkend vermogen. Zij werden geïdentificeerd als behorende tot *Bacillus pumilus*, *Paenibacillus polymyxa*, *Burkholderia cepacia*, *Pantoea stewartii*, en fluorescerende *Pseudomonas* sp. Deze groep van pathogenen met zwak ziekteverwekkend vermogen is niet eerder beschreven in de rijstpathologie omdat tot op heden nog geen stammen van die species epidemiën hebben veroorzaakt. Mogelijkerwijs zijn ze aan detectie ontsnapt in het verleden, deels omdat een screening van totale bacteriële populaties van rijstzaden nog niet eerder werd uitgevoerd. Bovendien zijn de veroorzaakte symptomen zoals bruinverkleuring van de bladschede, necrose verschijnselen langs de bladranden en het verwelken van het nieuwe blad in de groeitop, subtiel en in het veld gemakkelijk over het hoofd te zien, vooral ook omdat de planten meestal de symptomen ontgroeien. Hun effekt op de plantegroei zou kunnen vergeleken worden met de eerder beschreven “minor pathogens” (Salt, 1979; Suslow and Shroth, 1982). We veronderstellen dat ze eventueel, met de tijd en onder veranderende rijstproduktie methodes, als een probleem zouden kunnen opduiken.

Uit de screening van isolaten op in vitro inhibitorische activiteit tegen rijstpathogene schimmels, vertoonde respektievelijk 4 en 16% van de isolaten uit de twee studies antagonistische activiteit tegen *Rhizoctonia solani* AG1 en/of *Pyricularia grisea*. Deze vermoedelijk fungicide producerende bacteriën werden geïdentificeerd als behorend tot verschillende genera en families zoals *Bacillus*, *Burkholderia*, *Enterobacteriaceae*, *Pseudomonas*, *Staphylococcus*, en coryneformen. De grote verscheidenheid aan antagonistische bacteriën die natuurlijk aanwezig zijn op rijstzaad zou redelijkerwijs laten veronderstellen dat mengsels van kompatibele antagonisten als zaadbehandeling waarschijnlijk een stabieler en duurzamer effekt kunnen hebben voor de biologische bestrijding van rijstpathogene schimmels. Een beter begrijpen van de genetische diversiteit in populaties van antagonistische bacteriën met eenzelfde biokontrole eigenschap zou vervolgens toelaten om specifieke genotypes te verenigen met hun meest aangepaste milieu (Weller et al., 2002). Tot op heden is het gebruik van individuele biokontrole middelen voor het bestrijden van een bepaalde rijstpathogene schimmel de meest toegepaste strategie in biokontrole experimenten voor rijst. Er zijn nochtans voorbeelden voor andere planten waarbij combinaties van antagonistische stammen geleid hebben tot een verbeterde biokontrole van bacteriële en fungale plantenziekten (Fukui et al, 1999; Janisiewicz, 1996; Raupach and Kloepper, 1998). Over het algemeen wordt aangenomen dat het koloniserend vermogen van biokontrole middelen op zaad en vervolgens de kiemplant belangrijk is voor het effektief onderdrukken van ziekte (Bull et al., 1991; Kim et al., 1997; Parke, 1990; Weller, 1988), uitgezonderd in geval van geïnduceerde resistentie (Liu et al., 1995). Het is duidelijk dat potentiële biokontrole middelen voor gebruik als zaadbehandeling in staat moeten zijn om te overleven en aktief te groeien in de omgeving van kiemende zaden. Vergelijkende BOX-PCR analyses van isolaten afkomstig van droge rijstzaden met isolaten afkomstig van in vitro gekiemde zaden, toonde aan dat zaadkieming in het algemeen resulteerde in een kwalitatieve verandering en een afname van diversiteit. Het meest opmerkelijke resultaat was de drastische afname van gram-positieve bacteriën op kiemende rijstzaden. Verder werden nieuwe FPTs behorende tot gram-negatieve koloniemorfotypes die niet geisoleerd waren van droge zaden, en een geringer aantal maar identieke FPTs

behorende tot de gram-negatieve koloniemorfotypes die dominant waren op droge zaden, gevonden.

Sedert de laatste decennia zijn nieuwe ziekteproblemen opgedoken in de rijstproductiesystemen tengevolge van de verhoogde rijstproductiviteit en de daarmee gepaard gaande veranderingen in productiemethoden en rijstvariëteiten. Te voorschijn gekomen ziekteproblemen van bacteriële aard zijn "bacterial blight", "grain rot", en "sheath brown rot". De karakterisering van de causale bacteriële organismen en hun verspreiding op rijstzaad vormden onderwerp van aparte studies in dit werk.

- Een grondige studie was gewijd aan de karakterisering van *Burkholderia* populaties die dominant waren in de verzameling van pathogene bacteriën uit de isolaties gemaakt van rijstzaden afkomstig van 17 rijstvelden en drie opeenvolgende groeiseizoenen. De verzameling van *Burkholderia* isolaten bestond uit pathogene bacteriën geïdentificeerd als *B. glumae* (45%) en *B. gladioli* (31%), en pathogene bacteriën met zwak ziekteverwekkend vermogen geïdentificeerd als *B. vietnamiensis* (1%), *B. cepacia* genomovar I (10%) en genomovar III (13%). De resultaten toonden aan dat de BOX-PCR fingerprinting techniek een bruikbaar alternatief kan vormen voor het identificeren van *Burkholderia* species en voor het onderscheiden van *B. cepacia* genomovars I en III stammen, en bovendien informatie kan verschaffen over de genetische variatie binnen elk species of genomovar (zie Appendix 3, pag. 228 en 229). Het voorkomen van *B. glumae* als dominante pathogeen op rijstzaad was niet verwonderlijk gezien dit species welbekend is als een belangrijke rijstpathogeen verantwoordelijk voor "grain rot" en "seedling rot". Anderzijds was het onverwacht om *B. gladioli* zo frequent aan te treffen op rijstzaad gezien dit species tot hiertoe zelden, en enkel in Japan, vermeld werd als geïsoleerd van rijst, en in de literatuur niet beschreven is als een rijstpathogeen. Het wijdverspreid voorkomen op schijnbaar gezonde rijstzaden van deze pathogenen zonder symptomen expressie, toont aan dat ze als epifytische populaties overleven op rijstzaad en kunnen eventueel, onder bepaalde omstandigheden, dienen als inoculum voor ziekteontwikkeling in het veld. Naast hun rol als rijstpathogenen, zijn zowel *B. glumae* als *B. gladioli* ook beschreven als potentiële biologische bestrijdingsmiddelen tegen bacteriële en fungale pathogenen op andere waardplanten (Furuya et al., 1991; Walker et al., 1996). *B. cepacia* stammen zijn algemeen met planten geassocieerde bacteriën en alomgekend als biologische bestrijdingsmiddelen in de landbouw; echter, de bezorgdheid is gerezen sedert erkend werd dat voornamelijk *B. cepacia* genomovar III stammen de oorzaak zijn van luchtweginfecties in mucoviscidose patienten (Vandamme et al., 1997).
- Een studie op de etiologie van de gecompliceerde ziekteverschijnselen op rijst die gekenmerkt worden door een bruinverkleuring van de zaden en de bladschede van het laatste blad, werd uitgevoerd in de voornaamste rijstproducerende regionen van de Filipijnen. Uit de resultaten bleek dat een complexe groep van fluorescerende pseudomonaden mogelijks met het ziektesyndroom betrokken zijn. Eenzelfde type van pathogene bacteriën, vermoedelijke *Pseudomonas fuscovaginae*, werden herhaaldelijk gevonden in associatie met donkerbruinverkleurde zaden en rottend bladweefsel afkomstig van laag- en hoogland lokaties in de Filipijnen, maar werd slechts eenmaal gevonden op gezonde zaden in de studies op de bacteriële diversiteit van rijstzaden. De Filipijnse isolaten vertonen grote gelijkenis met de referentiestammen van *Pseudomonas fuscovaginae* qua opgewekte symptomen en fenotypische kenmerken, maar zijn duidelijk verschillend in serologische eigenschappen en op basis van BOX-PCR genomische fingerprinting. In de studie van Jaunet et al. (1995), bleek onze isolaat 6031 nauw verwant te zijn tot isolaten van

Madagascar en tot de type-stam van *P. putida* LMG 2257 op basis van 16S-rDNA restrictiefragment profilering; zulke verwantschap tot *P. putida* LMG 2257 kon echter niet bevestigd worden op basis van BOX-PCR analyse in onze studie. Het is duidelijk dat de opheldering van de etiologie van de bruinverkleuring en rotting van rijst nauw samenhangt met de complexiteit van de *Pseudomonas* taxonomie.

- Een DNA-amplificatiegebaseerde (PCR) detectiemethode was ontwikkeld voor het opsporen van *Xanthomonas oryzae* pv. *oryzae* in rijstzaad, en werd gebruikt om de mogelijke zaadoverdracht van de pathogeen te onderzoeken. De van nature lage *X. o. oryzae* populaties op rijstzaden maakt het uiterst moeilijk om de pathogeen uit rijstzaden te cultiveren. Het zij hierbij opgemerkt dat in onze ervaring, *X. o. oryzae* niet geïsoleerd werd in de uitgebreide studies op bacteriële diversiteit van rijstzaad, noch van rijstzaadstalen genomen van planten met "bacterial blight" ziekteverschijnselen. De ontwikkelde PCR-test is in staat om *X. o. oryzae* cellen rechtstreeks op te sporen in waswater van rijstzaden, daar waar aanwezigheid van de pathogeen niet kon aangetoond worden via het uitplaten van waswater verdunningen op selectief medium. Bovendien biedt de PCR-test de mogelijkheid om *X. o. oryzae* te identificeren en te onderscheiden van *X. o. oryzicola* en de talrijk voorkomende niet-pathogene xanthomonaden, in geval twijfelachtige gelijkaardige kolonies geïsoleerd worden. In geen enkel geval kon zaadoverdracht van *X. o. oryzae* van natuurlijk of kunstmatig geïnfecteerde zaden naar devolgende plantgeneratie aangetoond worden. In tegenstelling tot wat sommige auteurs beweren, bevestigden onze resultaten de meer verspreide mening dat de levensfase van *X. o. oryzae* op rijstzaad onbelangrijk lijkt voor ziekteoverdracht.

Bacteriën komen onder natuurlijke omstandigheden voor als heterogene populaties die veranderlijk zijn en beïnvloed worden door milieufactoren. De spontane processen van mutatie, recombinatie en migratie veroorzaken genetische verschuivingen in bacteriële populaties, waarbij natuurlijke selektiedruk het uiteindelijke resultaat van de variaties dicteert (Feil and Spratt, 2001; Ochman et al., 2000). De genetische variatie in bacteriële populaties kan beschouwd worden als een mogelijk mechanisme voor de populaties om zich doeltreffend aan te passen aan wisselende omstandigheden in het milieu. Wat men weet over de genetische struktuur van met planten geassocieerde bacteriële populaties, is voornamelijk afkomstig van de studie van plant pathogenen. De genetische struktuur van plantpathogene populaties wordt verondersteld een weerspiegeling te zijn van hun evolutief vermogen, en kan aldus een aanwijzing geven voor hun adaptief vermogen om bestrijdingsstrategiën zoals het gebruik van antibiotica en genetische resistentie te overkomen (McDonald and Linde, 2002). De diversiteitsanalyse van de isolaten in onze studie via BOX-PCR genomische fingerprinting, verschaft een voorlopig referentiekader van de genetische struktuur van rijstzaad-geassocieerde populaties. Over het algemeen worden dezelfde bacteriële genera of species die gevonden zijn op rijstzaad, ook teruggevonden als epifyten op tal van andere planten (Hirano and Upper, 2000). Het feit is echter dat deze species op zichzelf zeer variabel zijn, en dat rijstzaad eventueel een meer selektieve omgeving kan verschaffen voor bepaalde individuen terwijl andere stammen van deze species kunnen aangepast zijn aan verschillende plant species. De selektie van dendrogrammen (zie Appendix 1, pag. 189-195) verkregen uit de BOX-PCR fingerprinting analyse van rijstzaad isolaten geven een idee van de mate van genetische variatie binnen dominante koloniemorfotype groepen. Het is duidelijk dat de genetische variabiliteit enorm kan verschillen naargelang van koloniemorfotype groep, en dient als een waarschuwing voor het beschouwen van een enkele kolonie op een isolatie plaat als zijnde representatief

voor de ganse populatie. De precieze taxonomische niveaus van die genetische variatie binnen morfotype groepen zijn niet duidelijk, en zou verdere gedetailleerde taxonomische studies vereisen. Nog minder is geweten in hoeverre de genetische en taxonomische verscheidenheid een weerspiegeling zijn van funktionele diversiteit en ecosysteem karakteristieken.

Een pertinente vraag in bacteriële biodiversiteit studies is welk resolutieniveau het meest geschikt is voor het bestuderen van bacteriën? De species vormt de basiseenheid voor de bacteriële taxonomie; maar is echter niet praktisch in gebruik omwille van de complexiteit om een definitieve species identificatie te bekomen. Bovendien worden tal van economisch belangrijke eigenschappen van micro-organismen, zoals antibioticaproduktie en plantengroeibevorderende effecten, op stamniveau aangetroffen. Dergelijke intraspecifieke variatie voor expressie van belangrijke eigenschappen, zou eerder wijzen op de noodzaak om bacteriën op stamniveau te bestuderen. Plant pathogenen worden door de band gedefiniëerd als zijnde alle stammen van een bepaald plantpathogeen bacteriëel species. Nochtans zijn verschillen in aggressiviteit tussen stammen van eenzelfde pathogeen species een regelmaat, en vertonen niet alle stammen van een pathogeen species noodzakelijkerwijs pathogeniciteit. Zulke fenotypische variatie is nog meer uitgesproken voor microbiële biokontrole middelen. Zo kan het vermogen om schimmelgroei te inhiberen of om de plantengroei te bevorderen in verschillende bacteriële species gevonden worden, maar geldt niet voor alle stammen van die species. Verschillende studies hebben aangetoond dat de antagonistische activiteit en het antifungale mechanisme van plant-geassociëerde bacteriën meestal stamspecifiek is (Keel et al., 1996; Minkwitz and Berg, 2001; Neiendam-Nielsen et al., 1998). Het zij hierbij opgemerkt dat het klasseren van bacteriën als schadelijk of bevorderend voor de plantengroei van nature een eerder subjectieve benadering is; het feit is immers dat bacteriën dikwijls zowel een schadelijk als bevorderend effekt kunnen hebben afhankelijk van bepaalde kondities en de waardplant (Åström and Gerhardson, 1988; Gardner et al., 1984). Terwijl traditioneel de biokontrole research voornamelijk gericht was op de wisselwerking tussen pathogeen en antagonist, is er tegenwoordig meer belangstelling voor de rol van de waardplant in het ondersteunen en dirigeren van die microbiële wisselwerkingen. Mikrobieel ekologische studies van planten hebben aangetoond dat de bacteriële gemeenschappen gelijkaardig zijn op verschillende planten van eenzelfde plant species, maar verschillen tussen plant species (Smalla et al., 2001; Yang et al., 2001). Er wordt verondersteld dat de hoeveelheid en de samenstelling van exudaten verschilt tussen verschillende plant species, en aldus selectief de groei van verschillende bacteriën kan beïnvloeden. Verschillen tussen antagonistische bacteriën voor hun koloniserend vermogen van de waardplant zijn waargenomen (Hebbar et al., 1992; Landa et al., 2002; Raaijmakers and Weller, 2001). Maar ook verschillen tussen plant species en variëteiten voor het produceren van antibiotica (Georgakopoulos et al., 1994), het onderdrukken van plantenziekten (King and Parke, 1993; Smith et al., 1997), het koloniseren van de waardplant (Berg et al., 2002), en het induceren van resistentie (Liu et al, 1995: van Wees et al., 1997) door biokontrole middelen werden waargenomen. Dergelijke waardplant specificiteit voor het reageren op biokontrole middelen doet vermoeden dat plantengenen een rol spelen in het laten doorgaan van die interacties (Smith et al., 1999). Het is niet geweten of er een verband bestaat tussen plantengenen die pathogenische en biokontrole plant-bacterie wisselwerkingen beinvloeden. Maar er worden wel steeds meer aanwijzingen gevonden die gelijkaardige signaal uitwisseling en communicatie mechanismen voor de verschillende plant-bacterie interacties laten vermoeden. Ongetwijfeld een van de best

bestudeerde plant-bacterie interacties is de *Rhizobium*-legumineuzen symbiose. De waardplant specifieke interactie tussen *Rhizobium* en legumineuzen vertoont gelijkenis met de stapsgewijze gereguleerde genexpressie (Flor, 1971) waarneembaar in de meeste pathogeensystemen. Onlangs werden de plant receptor molecules geïdentificeerd die vereist zijn voor de symbiontische interacties van planten met mycorrhizae en *Rhizobium*, en precies dezelfde receptor kinase bleek verantwoordelijk te zijn voor zowel de bacteriële als fungale symbiose (Endre et al., 2002; Stracke et al., 2002). Plant receptor kinases vervullen ook een rol in de perceptie van pathogeen-geassociëerde faktoren, en gelijkaardige proteïnen worden tevens een rol toebedeeld in het immuunsysteem van dieren. Alzo wordt verondersteld dat vorderingen in het onderzoek naar de genetische mechanismen van pathogeniciteit ook zullen bijdragen tot een beter begrijpen van de wisselwerking tussen planten en microbiële biokontrole middelen.

Ten slotte is dit werk één van de eerste studies waarin bacteriële populaties van rijstzaad grondig worden beschreven. Een complexe microbiële gemeenschap werd aangetoond niettegenstaande het feit dat wat eigenlijk gekarakteriseerd werd de fraktie van kweekbare bacteriën is die kan verkregen worden door uitplaten van verdunningen van rijstzaad suspensies op een voedingsbodem. In hoeverre het betrouwen op cultuurmethoden de studie van bacteriële populaties beperkt, is nog niet onderzocht geweest voor rijstzaad. De grote variabiliteit in samenstelling van bacteriële gemeenschappen tussen de onderzochte stalen van verschillende velden, ecosystemen en groeiseizoenen, kan belangrijke informatie bevatten over de dynamiek van het zaadmilieu. Het zou van belang zijn om de mogelijke effecten van verschillende variëteiten, ecosystemen en rijstproductiemethoden op de samenstelling van bacteriële gemeenschappen te onderzoeken. Gezien voor dergelijke microbiëel ecologische studies grote aantallen stalen dienen onderzocht te worden om relevante verschillen in de struktuur van microbiële gemeenschappen te kunnen vaststellen, is de tijdrovende cultuurmethode hiervoor duidelijk niet geschikt. De moleculaire rDNA-technieken zoals de denaturerende gradient gel elektroforese (DGGE) verschaffen een manier om verschuivingen in complexe microbiële gemeenschappen sneller te onderzoeken (Duineveld et al., 2001; Muyzer et al., 1993; Smit et al., 2001; Yang et al., 2001). De informatie verschaft in deze studie kan dienen als een referentiekader voor het selecteren van oligonucleotide probes voor dergelijke moleculair ecologische studies. Anderzijds kan sequentie-analyse van representatieve isolaten voor de dominante populaties geïdentificeerd in deze studie toelaten om diagnostische sequentiegebieden te karakteriseren die kunnen gebruikt worden voor het aanmaken van probes. Dergelijke specifieke probes kunnen dan gebruikt worden om via DGGE op een vlugge manier de tijdelijke en ruimtelijke veranderingen van bepaalde componenten in de gemeenschap te onderzoeken.

De rijstzaad microflora vormt een complexe microbiële gemeenschap die bestaat uit honderden types die interageren met elkaar en met de waardplant. De microbiële ecology van rijstzaden, en de invloed van het kiemingsproces op de zaadmicroflora, zijn bijzonder ingewikkeld maar nog weinig gekend. Onderzoek naar de relatie tussen de diversiteit van de samenstellende populaties en het functioneren van microbiële gemeenschappen is dringend gewenst alvorens strategiën kunnen ontwikkeld worden die toelaten om gericht plant-geassociëerde microbiële gemeenschappen te sturen voor plantengroeibevordering. Het gaat daarbij niet alleen om de rol van individuele populaties, maar om de diversiteit van interacties tussen de volledige microbiële gemeenschap, de waardplant en het milieu in brede zin.

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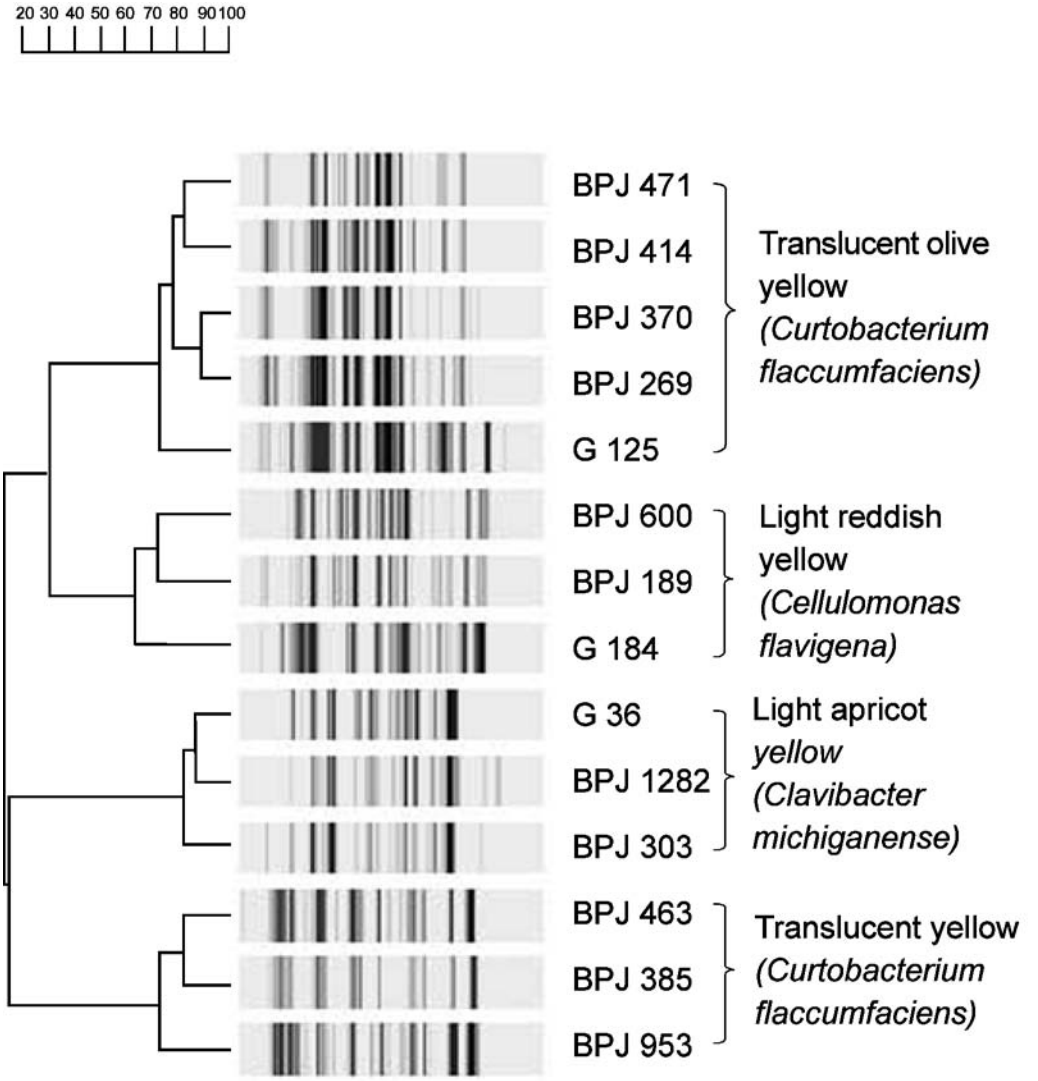
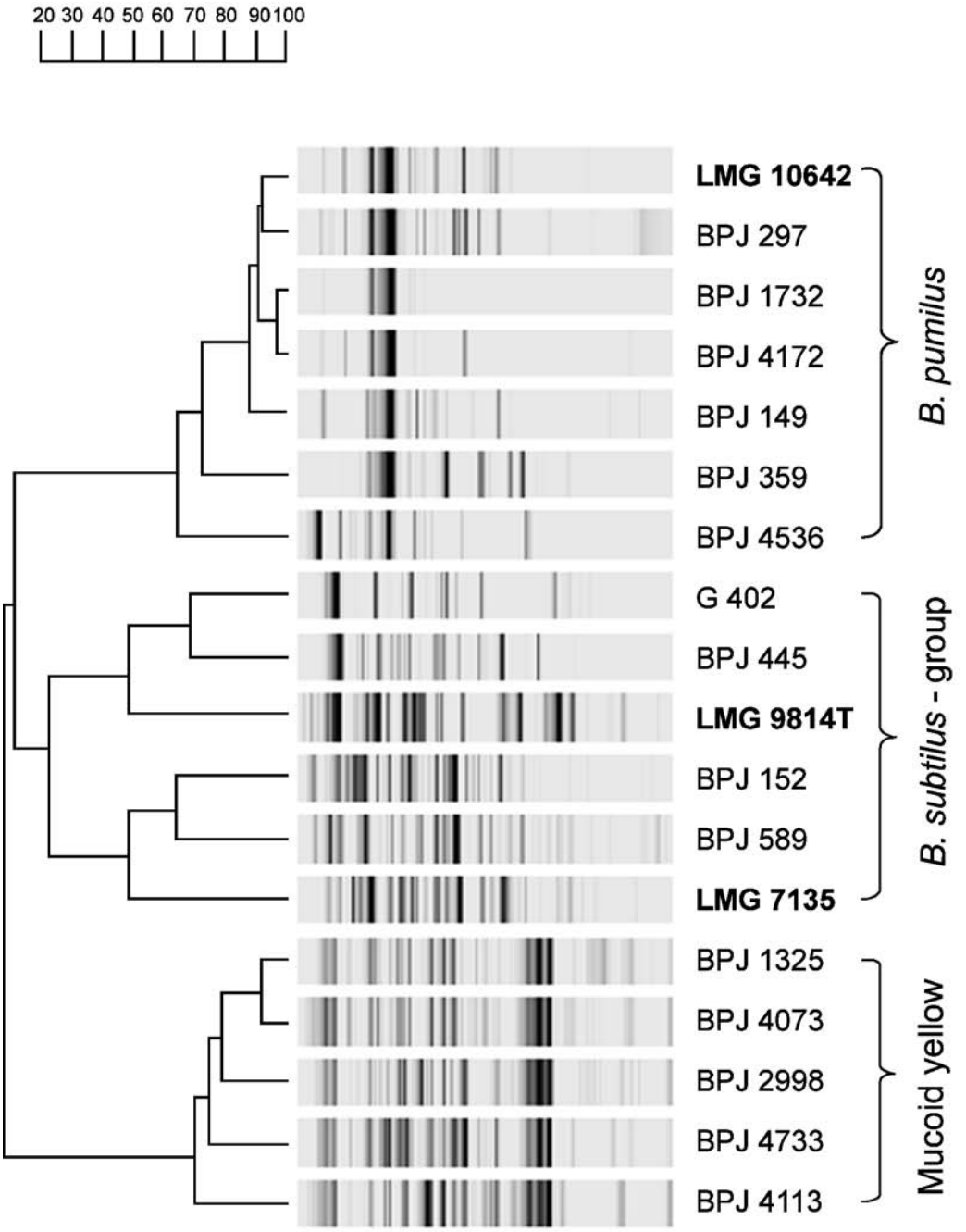
Appendix 1

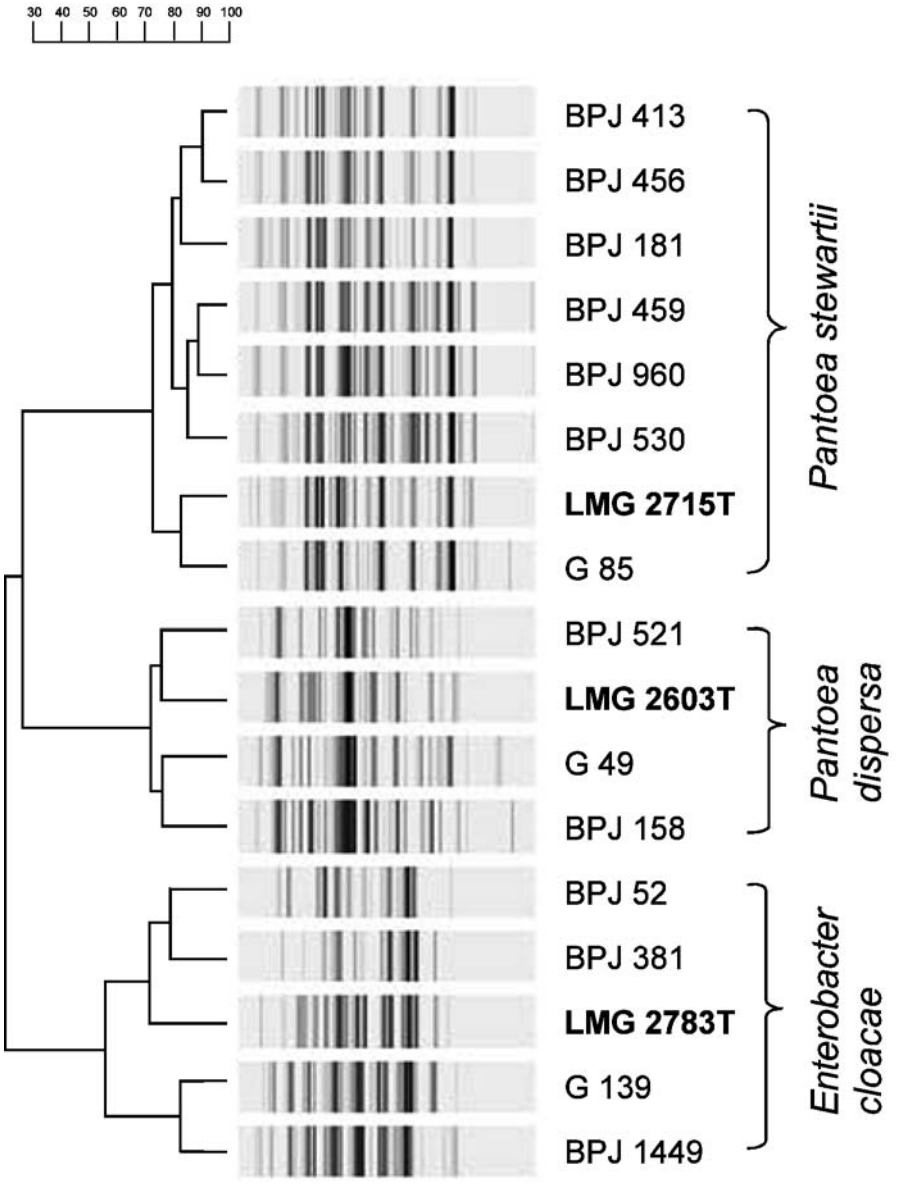
Comparative analyses of BOX-PCR genomic fingerprints for a selection of isolates representative for the most commonly detected bacterial groups from rice seed. Cluster analysis was performed by the unweighted pair group method with arithmetic averages (UPGMA) using the Pearson correlation coefficient; correlation levels are expressed in percentages of similarity in the dendrogram scale.



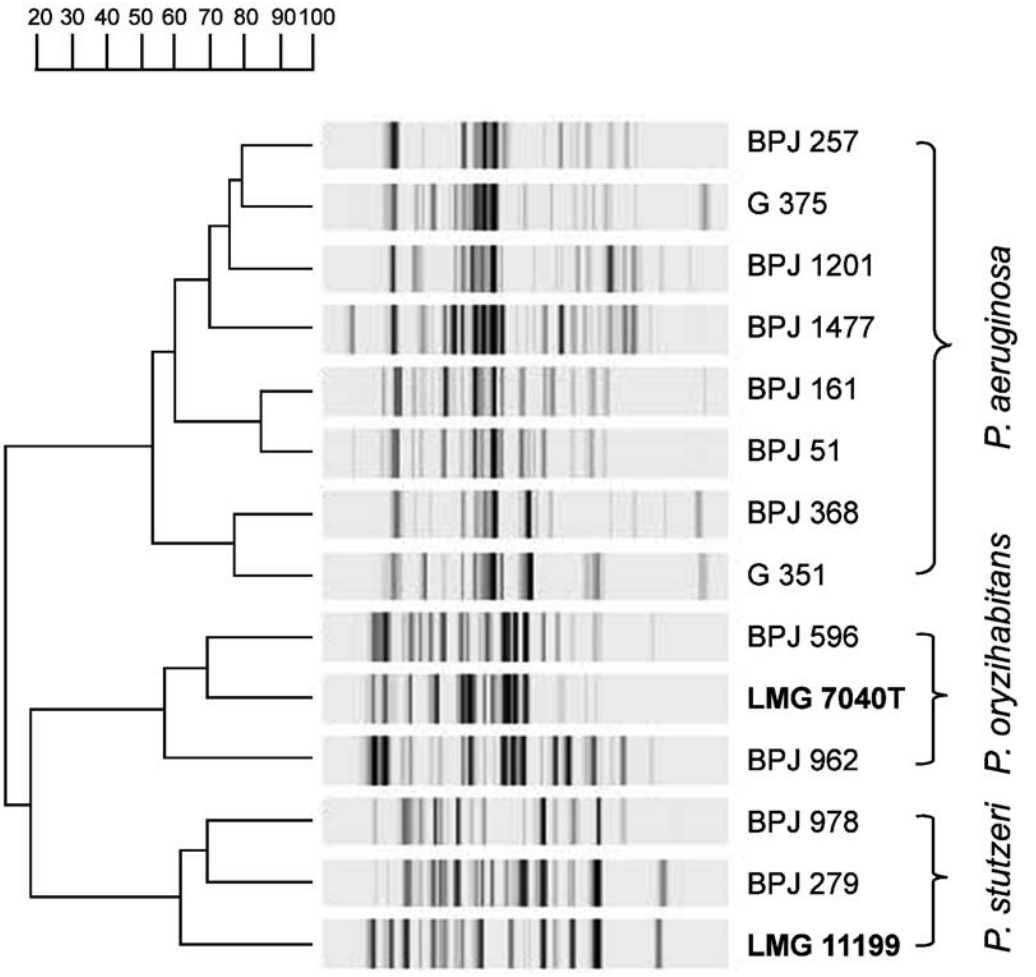
Coryneform bacteria



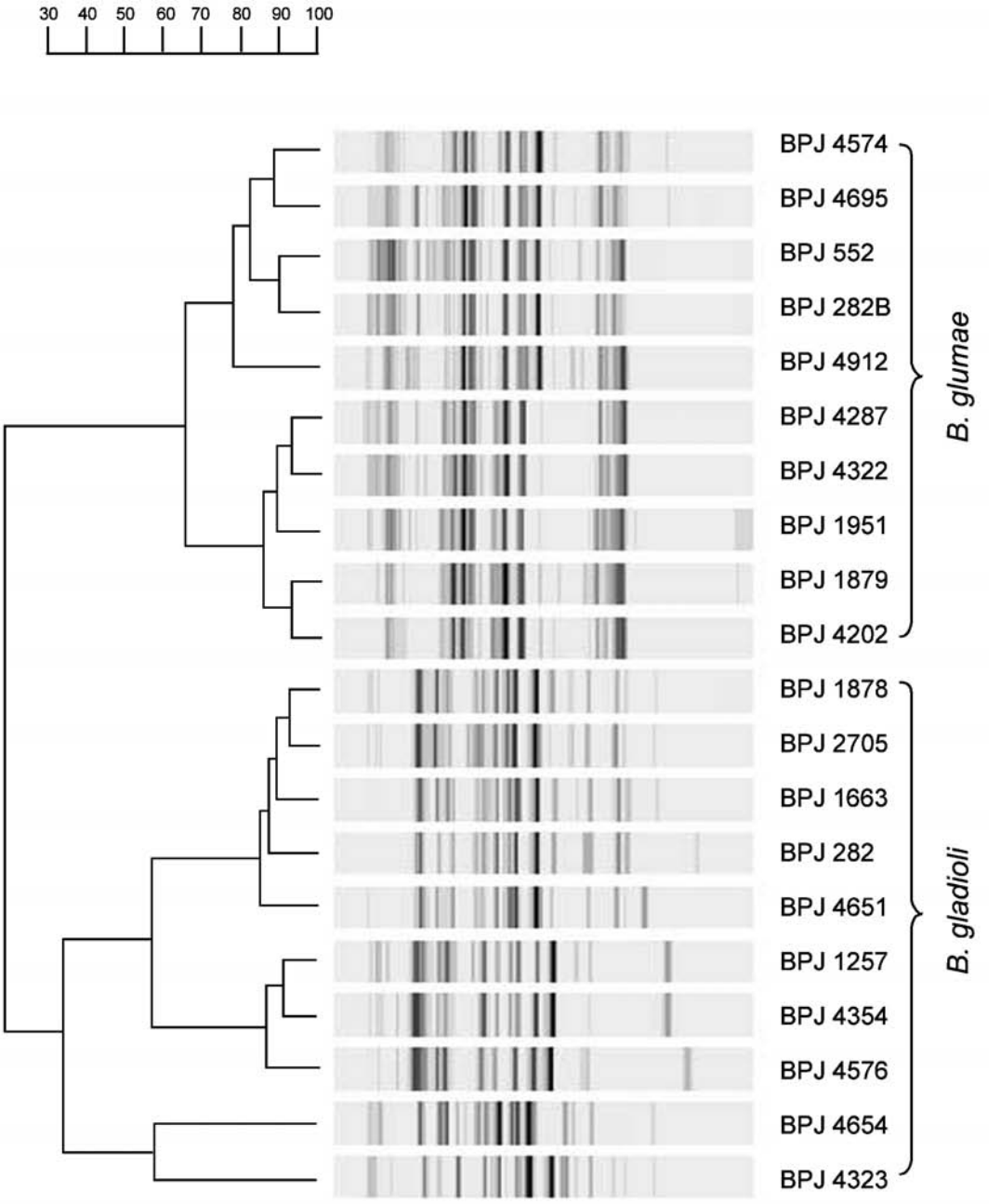
Endospore-forming bacilli



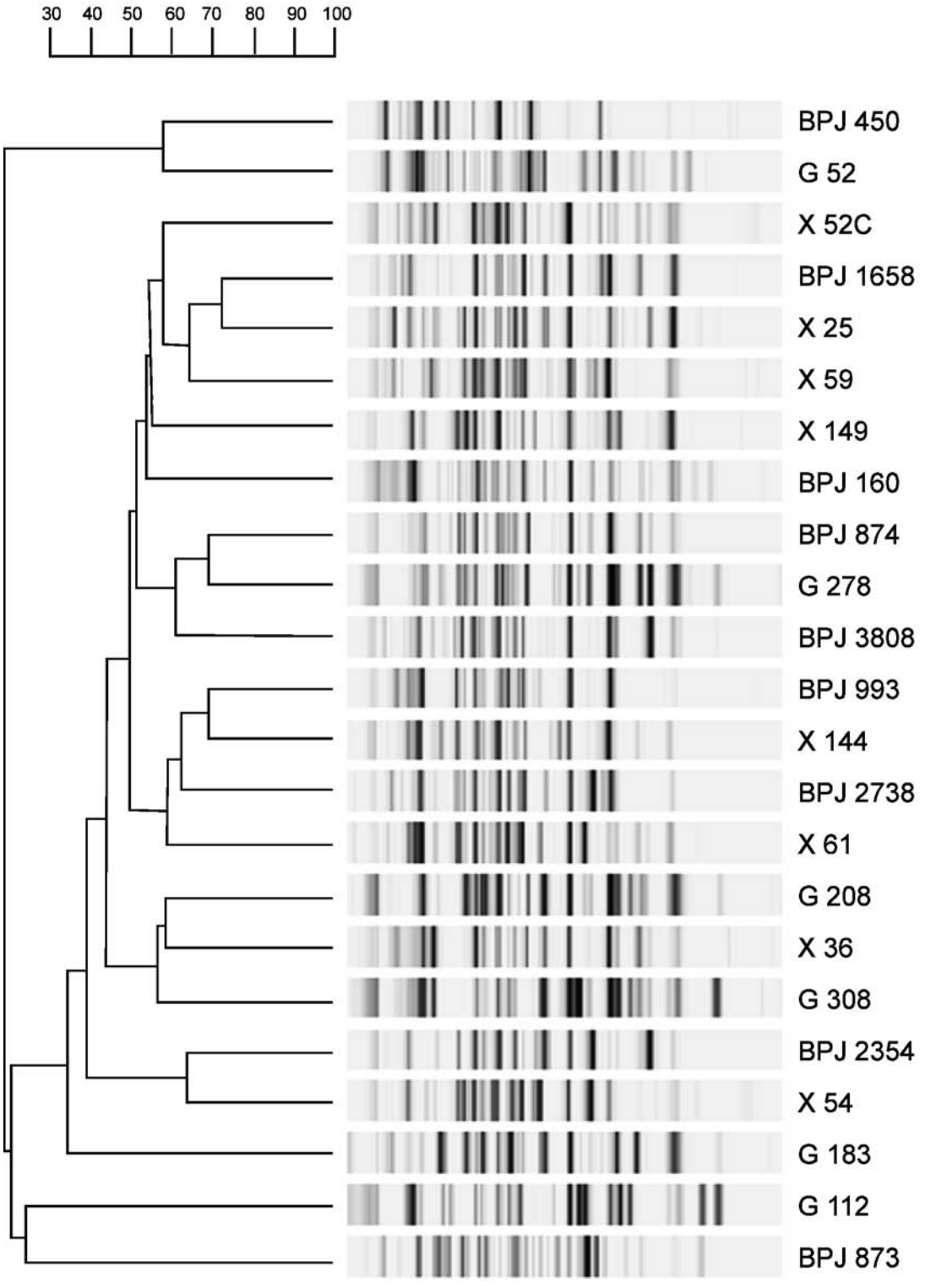
Enterobacteriaceae



Nonfluorescent pseudomonads



Pathogenic *Burkholderia* species



Nonpathogenic xanthomonads

Appendix 2

A selection of characterized isolates grouped according to colony appearance and BOX-PCR fingerprint types

Gram-positive bacteria

Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
ENDOSPORE-FORMING RODS											
Thin pale cinnamon pink		No match	20172	bpj 972	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Dull pale yellow		*Bacillus gibsonii*	20169	bpj 406	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Hairy dull white		*Brevibacillus brevis*	18667	g 233	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
Silvery white	1	*Brevibacillus brevis*		bpj 2769	TSA			PSBRc14	J-5G	Jalajala, Rizal	May '97
				bpj 2799	TSA			PSBRc14	J-11G	Jalajala, Rizal	May '97
	2	*Brevibacillus brevis*		bpj 3098	TSA			PSBRc14	J-17G	Jalajala, Rizal	May '97
Dull powdery white	1	*Bacillus licheniformis*	20170	bpj 177	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	2			bpj 3	TSA			PSBRc14	J-1	Jalajala, Rizal	Nov '96
Yellow beige	1	*Bacillus sphaericus*		bpj 1455	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	2			bpj 1239	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	3	[*Bacillus sphaericus*]		bpj 583	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	4	*Bacillus sphaericus*	20167	bpj 1251	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Translucent grayish white	1	*Bacillus sphaericus*	18663	g 162	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
		No match	18668	g 253	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
	2	*Bacillus filicolonicus*	18692	g 386	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Spreading brownish white	1	No match		bpj 3263	TSA			PSBRc22	J-29G	Jalajala, Rizal	May '97
				bpj 3540	TSA			BS1	J-30G	Jalajala, Rizal	May '97
				bpj 4861	TSA			PSBRc14	J-12G	Jalajala, Rizal	Dec '97
				bpj 5011	TSA			PSBRc22	J-29G	Jalajala, Rizal	Dec '97
	2	[*Paenibacillus pabuli*]		bpj 3036	TSA	LDP		PSBRc20	J-16G	Jalajala, Rizal	May '97
				bpj 3069	TSA			PSBRc14	J-13G	Jalajala, Rizal	May '97
	3	[*Paenibacillus pabuli*]		bpj 4671	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	Dec '97
				bpj 4693	TSA	LDP		PSBRc14	J-5G	Jalajala, Rizal	Dec '97
	4	[*Paenibacillus pabuli*]		bpj 4669	TSA			PSBRc14	J-4G	Jalajala, Rizal	Dec '97
	Outlier	No match	20175	bpj 1452	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	Outlier	[*Paenibacillus pabuli*]		bpj 2706	TSA	LDP		BS1	J-7G	Jalajala, Rizal	May '97
	Outlier	*Paenibacillus pabuli*	20171	bpj 357	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
Rough (mucoid) brownish white	1	*Bacillus subtilis*	20163	bpj 589	TSA		Rs/Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 152	TSA		Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
	2	*B. subtilis*		bpj 1152	TSA			BS1	J-30	Jalajala, Rizal	Nov '96
	3			bpj 584	TSA		Rs/Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
	4			bpj 949	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	5	*B. subtilis*		bpj 148	TSA		Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 281	TSA		Rs	PSBRc14	J-9	Jalajala, Rizal	Nov '96
	6	*B. amyloliquefaciens*	18704	g 441	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
				g 352	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
				g 365	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	Outlier	*B. subtilis*		bpj 592	TSA		Rs/Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
subgroup 2	1	*B. amyloliquefaciens*	18691	g 462	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
		B. amyloliquefaciens	18703	g 402	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				g 267	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 329	KMB		Rs	IR38	14-8	Dingle, Iloilo	Oct '95
				g 412	GYCA		Rs	IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				bpj 445	TSA		Rs/Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	2	*B. amyloliquefaciens*	18652	g 59	GYCA			IR64	2-7	San Miguel, Iloilo	Oct '95
		B. subtilis	18707	g 425	KMB		Pg	IR60	20-6	Ajuy, Iloilo	Oct '95
				g 45	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
				g 78	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
		B. amyloliquefaciens	18671	g 229	KMB		Rs	PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 396	KMB		Pg	IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				g 459	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	3	*B. amyloliquefaciens*	18679	g 346	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	4			bpj 447	TSA		Rs/Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	5	*B. amyloliquefaciens*	18684	g 417	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
subgroup 3	1	*Paenibacillus macerans*	18690	g 429	KMB		Pg	IR60	20-6	Ajuy, Iloilo	Oct '95
				bpj 151	TSA	LDP	Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
	2	[*Paenibacillus lentimorbus*]		bpj 4578	TSA	LDP		PSBRc22	J-29	Jalajala, Rizal	Dec '97
	3	*Paenibacillus lentimorbus*	20168	bpj 383	TSA	LDP	Rs	PSBRc14	J-11	Jalajala, Rizal	Nov '96
	Outlier	*B. amyloliquefaciens*	18715	g 452	KMB		Rs	IR60	20-6	Ajuy, Iloilo	Oct '95
subgroup 4	1	*B. amyloliquefaciens*	18709	g 432	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
		Paenibacillus macerans		bpj 356	TSA	LDP	Rs/Pg	PSBRc14	J-10	Jalajala, Rizal	Nov '96
	Outlier	*B. amyloliquefaciens*	18694	g 51	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
hairy dull white subgroup 5	Outlier	*Bacillus* sp.	18685	g 451	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	1	*Paenibacillus macerans*		bpj 4169	TSA		Pg	PSBRc14	J-10	Jalajala, Rizal	Dec '97
				bpj 4537	TSA		Pg	PSBRc14	J-26	Jalajala, Rizal	Dec '97
Mucoid thin yellow (presumably *Paenibacillus* sp.)	1	*Cellulomonas turbata*	18666	g 213	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				bpj 1782	TSA			PSBRc14	J-11	Jalajala, Rizal	May '97
				bpj 4113	TSA			PSBRc14	J-11	Jalajala, Rizal	Dec '97
				bpj 4187	TSA			PSBRc14	J-10	Jalajala, Rizal	Dec '97
				bpj 4558	TSA	LDP		PSBRc14	J-26	Jalajala, Rizal	Dec '97
	2	[*Cellulomonas cartae*]		bpj 3813	TSA	LDP		PSBRc14	J-2	Jalajala, Rizal	Dec '97
				bpj 2961	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	May '97
	3			bpj 562	TSA			PSBRc20	J-16	Jalajala, Rizal	Nov '96
				bpj 1126	TSA			PSBRc22	J-29	Jalajala, Rizal	Nov '96
	4			bpj 226	TSA	LDP		BS1	J-7	Jalajala, Rizal	Nov '96
				bpj 1325	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	Nov '96
				bpj 2515	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 2998	TSA	LDP		PSBRc14	J-15G	Jalajala, Rizal	May '97
				bpj 3907	TSA	LDP		PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 4048	TSA	LDP		PSBRc14	J-8	Jalajala, Rizal	Dec '97
				bpj 4073	TSA	LDP		PSBRc14	J-9	Jalajala, Rizal	Dec '97
				bpj 4733	TSA	LDP		PSBRc14	J-8G	Jalajala, Rizal	Dec '97

continued on next page

Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
	5	*Cellulomonas turbata*		bpj 266	TSA	LDP		PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 225	TSA	LDP		BS1	J-7	Jalajala, Rizal	Nov '96
				bpj 2839	TSA	LDP		PSBRc14	J-9G	Jalajala, Rizal	May '97
				bpj 3509	TSA	LDP		BS1	J-30G	Jalajala, Rizal	May '97
				bpj 4191	TSA	LDP		PSBRc14	J-10	Jalajala, Rizal	Dec '97
	6			bpj 3998	TSA	LDP		BS1	J-7	Jalajala, Rizal	Dec '97
	7			bpj 296	TSA	LDP		PSBRc14	J-9	Jalajala, Rizal	Nov '96
	8			bpj 299	TSA	LDP		PSBRc14	J-9	Jalajala, Rizal	Nov '96
subgroup 2	1	*Cellulomonas turbata*		bpj 267	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	2			bpj 532	TSA	LDP		PSBRc14	J-15	Jalajala, Rizal	Nov '96
subgroup 3	1	*Kocuria varians*	18660	g 310	KMB	LDP		IR64	19-9	Ajuy, Iloilo	Oct '95
	2			bpj 464	TSA	LDP		PSBRc14	J-13	Jalajala, Rizal	Nov '96
	3			bpj 386	TSA	LDP		PSBRc14	J-11	Jalajala, Rizal	Nov '96
	4	*Cellulomonas turbata*		c 74	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	5	*Paenibacillus polymyxa*		c 145	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	Outlier			bpj 1227	TSA			PSBRc14	J-5G	Jalajala, Rizal	Nov '96
	Outlier	[*Cellulomonas cartae*]		bpj 956	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	Outlier	*Cellulomonas turbata*	20164	bpj 955	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	Outlier	*Staphylococcus saprophyticus*	18673	g 192	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
opaque white	Outlier	*Paenibacillus polymyxa*	18678	g 284	GYCA	LDP		IR64	19-9	Ajuy, Iloilo	Oct '95
Frosted dull white	Minimal amplification	[*Bacillus cereus*]	18653	g 62	GYCA			IR64	2-7	San Miguel, Iloilo	Oct '95
		[*Bacillus cereus*]	18675	g 150	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
		Bacillus cereus	18698	g 227	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		No match	18696	g 196	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Bacillus cereus		g 404	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
		No match	18682	g 420	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
				bpj 2	TSA			PSBRc14	J-1	Jalajala, Rizal	Nov '96
		Bacillus cereus	20165	bpj 40	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
				bpj 42	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
				bpj 404	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 446	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 642	TSA			PSBRc20	J-18(2)	Jalajala, Rizal	Nov '96
		Bacillus cereus		bpj 742	TSA			PSBRc20	J-20	Jalajala, Rizal	Nov '96
				bpj 853	TSA			PSBRc20	J-23	Jalajala, Rizal	Nov '96
				bpj 948	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 1303	TSA			PSBRc14	J-4G	Jalajala, Rizal	Nov '96
				bpj 1417	TSA			PSBRc14	J-24G	Jalajala, Rizal	Nov '96
		[*Bacillus cereus*]		bpj 1470	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 1577	TSA			PSBRc14	J-1	Jalajala, Rizal	May '97
				bpj 1645	TSA			PSBRc14	J-4	Jalajala, Rizal	May '97
				bpj 1810	TSA			PSBRc14	J-12	Jalajala, Rizal	May '97
				bpj 2735	TSA			PSBRc14	J-2G	Jalajala, Rizal	May '97
				bpj 3782	TSA			PSBRc20	J-1	Jalajala, Rizal	Dec '97
				bpj 3889	TSA			PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 4176	TSA			PSBRc14	J-10	Jalajala, Rizal	Dec '97
				bpj 4608	TSA			BS1	J-30	Jalajala, Rizal	Dec '97
				bpj 4667	TSA		Pg	PSBRc14	J-4G	Jalajala, Rizal	Dec '97
				bpj 4703	TSA			PSBRc14	J-5G	Jalajala, Rizal	Dec '97
				bpj 4831	TSA		Pg	PSBRc14	J-17G	Jalajala, Rizal	Dec '97
				bpj 4913	TSA			BS1	J-30G	Jalajala, Rizal	Dec '97
Wrinkled yellowish white	1	*Bacillus pumilus*	20162	bpj 359	TSA	LDP		PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 1	TSA	LDP		PSBRc14	J-1	Jalajala, Rizal	Nov '96
				bpj 96	TSA			PSBRc14	J-3	Jalajala, Rizal	Nov '96
				bpj 100	TSA	LDP		PSBRc14	J-3	Jalajala, Rizal	Nov '96
				bpj 149	TSA	LDP	Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 297	TSA	LDP		PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 320	TSA	LDP		PSBRc14	J-6	Jalajala, Rizal	Nov '96
				bpj 408	TSA	LDP		PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 441	TSA	LDP	Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 586	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 587	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 1114	TSA	LDP		PSBRc22	J-29	Jalajala, Rizal	Nov '96
				bpj 1504	TSA			PSBRc14	J-17G	Jalajala, Rizal	Nov '96
				bpj 1676	TSA			PSBRc14	J-5	Jalajala, Rizal	May '97
				bpj 1732	TSA	LDP		BS1	J-7	Jalajala, Rizal	May '97
				bpj 1774	TSA	LDP		PSBRc14	J-11	Jalajala, Rizal	May '97
				bpj 2489	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 2837	TSA	LDP		PSBRc14	J-9G	Jalajala, Rizal	May '97
				bpj 2992	TSA	LDP		PSBRc14	J-15G	Jalajala, Rizal	May '97
				bpj 3034	TSA	LDP		PSBRc20	J-16G	Jalajala, Rizal	May '97
				bpj 3996	TSA	LDP		BS1	J-7	Jalajala, Rizal	Dec '97
				bpj 4129	TSA	LDP		PSBRc14	J-12	Jalajala, Rizal	Dec '97
				bpj 4172	TSA	LDP	Pg	PSBRc14	J-10	Jalajala, Rizal	Dec '97
				bpj 4320	TSA	LDP		PSBRc20	J-16	Jalajala, Rizal	Dec '97
				bpj 4352	TSA			PSBRc14	J-17	Jalajala, Rizal	Dec '97
				bpj 4575	TSA			PSBRc22	J-29	Jalajala, Rizal	Dec '97
				bpj 4822	TSA	LDP		PSBRc14	J-17G	Jalajala, Rizal	Dec '97
				g 178	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 225	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 313	GYCA	LDP		IR38	14-8	Dingle, Iloilo	Oct '95
				g 398	KMB	LDP		IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				g 418	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
	2	*Bacillus pumilus*		bpj 2422	TSA	LDP		BS1	J-30	Jalajala, Rizal	May '97
				bpj 3886	TSA	LDP		PSBRc14	J-4	Jalajala, Rizal	Dec '97
	3	*Bacillus pumilus*	18676	g 330	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	4			bpj 2714	TSA	LDP	Rs	BS1	J-7G	Jalajala, Rizal	May '97
	5	*Bacillus pumilus*		bpj 2732	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	May '97
				bpj 4536	TSA	LDP	Pg	PSBRc14	J-26	Jalajala, Rizal	Dec '97
				bpj 4866	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	Dec '97
	6	*Bacillus pumilus*		bpj 384	TSA	LDP		PSBRc14	J-11	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
	Outlier	*Bacillus pumilus*		bpj 604	TSA	LDP		PSBRc14	J-17	Jalajala, Rizal	Nov '96
	Outlier	*Bacillus pumilus*		bpj 358	TSA	LDP		PSBRc14	J-10	Jalajala, Rizal	Nov '96
	Outlier	*Bacillus pumilus*		bpj 1916	TSA	LDP		PSBRc20	J-16	Jalajala, Rizal	May '97
	Outlier	*Bacillus pumilus*		bpj 442	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	Outlier	*Bacillus pumilus*	18658	g 113	KMB	LDP		IR64	10-1	Zarraga, Iloilo	Oct '95
Cream yellow	1	*Bacillus megaterium*	20166	bpj 599	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
		Brevibacillus laterosporus	18686	g 449	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	2			bpj 93	TSA			PSBRc14	J-3	Jalajala, Rizal	Nov '96
	3			bpj 309	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	4			bpj 467	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	5			bpj 1277	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	6	*Brevibacillus laterosporus*	18659	g 156	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
	7	*Bacillus megaterium*	18670	g 212	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Bacillus megaterium	18714	g 457	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	8	*Bacillus megaterium*	18688	g 382	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				g 22	KMB			IR24	2-4	Calauan, Laguna	Sept '95
				g 257	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 428	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	9	*Bacillus megaterium*	18705	g 353	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	10			g 153	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
	11	[*Bacillus megaterium*]	18650	g 37	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
	12	*Bacillus megaterium*	18687	g 445	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	13	*Bacillus megaterium*	18710	g 439	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
	14	*Bacillus megaterium*		bpj 762	TSA			PSBRc14	J-21(2)	Jalajala, Rizal	Nov '96
COCCI											
Glossy grayish white	1	*Staphylococcus simulans*	20177	bpj 55	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
	2	No match		bpj 375	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
	3			bpj 426	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 476	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Dull pale yellow orange	1	*Staphylococcus gallinarum*	20176	bpj 307	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	2	No match		bpj 190	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	3			bpj 194	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	4	No match		bpj 601	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	5			bpj 602	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	6			bpj 536	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
Dull pink white	1	[*Staphylococcus saprophyticus*]		bpj 423	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
	2	No match		bpj 513	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
Dull pink white	1	[*Staphylococcus haemolyticus*]		bpj 409	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Glossy mauve-pink	1	*Staphylococcus sciuri*		bpj 72	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
Glossy pale beige	1	*Staphylococcus xylosus*	20180	bpj 360	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
Glossy pink white	2	*Staphylococcus xylosus*		bpj 475	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Glossy yellowish white	1	*Staphylococcus arlettae*	20182	bpj 192	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 175	TSA		Pg	PSBRc14	J-5	Jalajala, Rizal	Nov '96
Glossy pale yellow		*Staphylococcus hominis*	20184	bpj 196	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
Dull sulphur yellow	1	*Micrococcus luteus*	20178	bpj 399	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
	2	No match		bpj 533	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
	3	*Micrococcus lylae*		bpj 268	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	4	[*Micrococcus luteus*]		bpj 373	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 607	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 1203	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	Outlier			bpj 979	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Dull pale sulphur yellow	1	*Kocuria kristinae*		bpj 283	TSA		Pg	PSBRc14	J-9	Jalajala, Rizal	Nov '96
	2	No match		bpj 54	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
	Outlier	*Micrococcus lylae*	20181	bpj 470	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Dull light yellow orange	1	*Kocuria kristinae*	20179	bpj 512	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
	2			bpj 361	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 424	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Dull pale yellow	1	*Kytococcus sedentarius*	20183	bpj 77	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
CORYNEFORM BACTERIA											
Shades of White											
Butyrous glistening white		*Arthrobacter mysorens*		bpj 4034	TSA	LDP		PSBRc14	J-8	Jalajala, Rizal	Dec '97
Butyrous grayish white	1	*Microbacterium barkeri*	20186	bpj 509	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 403	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Butyrous yellowish white	1	*Microbacterium esteraromaticum*	20185	bpj 372	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 364	TSA		Rs	PSBRc14	J-10	Jalajala, Rizal	Nov '96
Butyrous white		*Kocuria varians*	18648	g 25	GYCA			IR24	2-4	Calauan, Laguna	Sept '95
Translucent white		No match	18683	g 430	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
Shades of Reddish Yellow											
Light reddish yellow	1	*Cellulomonas flavigena*	20188	bpj 189	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 600	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	2	*Cellulomonas flavigena*		bpj 272	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 63	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	3	*Paenibacillus macerans*	18669	g 184	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				bpj 64	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	4			bpj 968	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Naples yellow	1	*Clavibacter michiganense*	20192	bpj 62	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
			18712	g 280	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	2	*Cellulomonas flavigena*	18706	g 392	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	Outlier	*Cellulomonas flavigena*	18655	g 88	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
Light apricot yellow	1	*Kocuria varians*	20187	bpj 1282	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
		Clavibacter michiganense		bpj 1492	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 303	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 376	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 421	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 474	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
				g 30	GYCA			IR24	2-4	Calauan, Laguna	Sept '95
		Cellulomonas flavigena	18649	g 36	KMB			IR64	3-7	San Miguel, Iloilo	Oct '95
				g 68	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 90	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
		Clavibacter michiganense	18665	g 157	KMB	LDP		IR36	25-4	Concepcion, Iloilo	Oct '95
				g 263	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 274	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	2	*Cellulomonas flavigena*	18647	g 7	GYCA		Rs	IR64	1-1	San Miguel, Iloilo	Oct '95
				bpj 170	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 273	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	Outlier	No match	18681	g 424	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
	Outlier	*Microbacterium imperiale*	20190	bpj 535	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
Pale yellowish brown	1	*Nesterenkonia halobia*	20189	bpj 967	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	2	*Kocuria kristinae*		bpj 60	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	3	*Microbacterium barkeri*		bpj 285	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 422	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Glossy yellowish orange	1	*Microbacterium lacticum*	20193	bpj 977	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	2	*Corynebacterium aquaticum*	18702	g 354	GYCA			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	3	*Cellulomonas flavigena*	18711	g 388	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	4	*Corynebacterium aquaticum*	18699	g 136	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
Dull yellow orange	1	*Corynebacterium aquaticum*	18713	g 277	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 61	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	2	*Paenibacillus macerans*	20191	bpj 271	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	Outlier	*Microbacterium arborescens*	18693	g 57	GYCA			IR64	2-7	San Miguel, Iloilo	Oct '95
Shades of Translucent Yellow											
Translucent pale yellow	1	*Clavibacter michiganense*	20195	bpj 258	TSA	LDP		PSBRc14	J-8	Jalajala, Rizal	Nov '96
		Clavibacter michiganense	18697	g 141	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 21	KMB			IR24	2-4	Calauan, Laguna	Sept '95
	2	*Corynebacterium aquaticum*	18708	g 292	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
				g 189	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
	3	*Microbacterium barkeri*	18701	g 421	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
	4	*Clavibacter michiganense*		bpj 963	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	5	*Clavibacter michiganense*		g 199	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 160	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
				bpj 526	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 606	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 1480	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
	6	*Clavibacter michiganense*	18656	g 93	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 18	KMB			IR24	2-4	Calauan, Laguna	Sept '95
				g 385	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				g 411	GYCA			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Translucent pale olive yellow	1	*Clavibacter* sp.	18661	g 125	KMB	LDP	Pg	IR64	10-1	Zarraga, Iloilo	Oct '95
				g 10	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
				bpj 165	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 203	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
		Bacillus coagulans	20194	bpj 269	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 291	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 370	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 415	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 462	TSA		Rs/Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 595	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 954	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	2			bpj 395	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
	3			bpj 290	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 371	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
	4	No match		bpj 964	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	Outlier	*Bacillus coagulans*	18677	g 326	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
Translucent straw yellow	1	*Curtobacterium flaccumfaciens*	20197	bpj 292	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 542	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
	2			bpj 49	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	Outlier	No match	18672	g 325	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
Translucent yellow	1	*Curtobacterium flaccumfaciens*	20196	bpj 385	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
				bpj 183	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 260	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 394	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
				bpj 463	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 953	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Translucent light straw yellow	1	*Clavibacter michiganense*	20198	bpj 275	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	2	*Clavibacter michiganense*	18695	g 129	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 294	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
	3	*Bacillus coagulans*		bpj 171	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	Outlier	*Clavibacter michiganense*		bpj 401	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
Translucent pale yellowish brown	1	*Bacillus coagulans*	20199	bpj 304	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	2	*Clavibacter* sp.	18657	g 106	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				bpj 270	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
Thin pale yellow	1	No match - slow growth	20200	bpj 1275	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	2			bpj 950	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Shades of Green Yellow											
Citron yellow	1	*Paenibacillus macerans*	20201	bpj 1488	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 207	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
Pale citron yellow	1	No match	20202	bpj 43	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
				bpj 951	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Translucent citron yellow	1	*Kocuria kristinae*	18680	g 369	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Translucent green yellow	1	*Kocuria kristinae*	18651	g 48	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
	2	*Microbacterium saperdae*	18664	g 158	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
		Kocuria varians	18654	g 66	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 11	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
Greenish yellow	1	*Microbacterium barkeri*	18662	g 148	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 393	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Light yellow green	1	*Microbacterium liquefaciens*	20203	bpj 44	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
Translucent pale yellow green	1	*Microbacterium liquefaciens*	18700	g 170	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 450	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
Thin greenish yellow		No match - slow growth	20206	bpj 436	TSA		Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
Glossy citron yellow		*Microbacterium saperdae*	20205	bpj 434	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Clear Rod-Coccus Cycle (Pleomorphic)											
Glistening white	1	*Arthrobacter mysorens*		bpj 4034	TSA	LDP		PSBRc14	J-8	Jalajala, Rizal	Dec '97
Glistening white	2	No match		bpj 479	TSA	LDP		PSBRc14	J-13	Jalajala, Rizal	Nov '96
Glossy light yellow green	3	[*Arthrobacter ilicis*]	20204	bpj 204	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
Lemon yellow	1	*Arthrobacter atrocyaneus*	18689	g 333	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
Mucoid lemon yellow	2	[*Arthrobacter ramosus*]	20215	bpj 469	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Pale greenish yellow	1	[*Arthrobacter ramosus*]		bpj 185	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	2			bpj 511	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
	3			bpj 540	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
Glossy pink	1	No match	20207	bpj 278	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 310	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	2			bpj 78	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	3			bpj 205	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 306	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
Wrinkled pale orange	1	[*Arthrobacter* sp.]	20210	bpj 539	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 402	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
				bpj 425	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 477	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	2			bpj 975	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
Glossy dark yellow		No match	20174	bpj 468	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Glossy white	1	*Brevibacterium linens*	20213	bpj 541	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
	2			bpj 534	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
Glossy brownish white + yellow streaks	1	*Brevibacterium epidermidis*	20209	bpj 69	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	2			bpj 66	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
	Outlier			bpj 193	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	Outlier			bpj 301	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
Deep citron yellow		*Brevibacterium mcbrellneri*	20214	bpj 451	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
Glossy golden yellow	1	*Exiguobacterium acetylicum*	20211	bpj 472	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	2	*Exiguobacterium acetylicum*	18674	g 155	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
	3	No match		bpj 83	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
Pale greenish yellow		[*Arthrobacter citreus*]	20208	bpj 431	TSA		Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
Pale yellowish brown		No match	20173	bpj 79	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
Glossy brownish white		No match	20212	bpj 197	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
Unidentified Actinomycetes											
				bpj 30	TSA			PSBRc14	J-1	Jalajala, Rizal	Nov '96
				bpj 36	TSA			PSBRc14	J-1	Jalajala, Rizal	Nov '96
				bpj 103	TSA		Rs/Pg	PSBRc14	J-3	Jalajala, Rizal	Nov '96
				bpj 105	TSA		Rs/Pg	PSBRc14	J-3	Jalajala, Rizal	Nov '96
				bpj 178	TSA		Rs/Pg	PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 179	TSA		Pg	PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 206	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 213	TSA			BS1	J-7	Jalajala, Rizal	Nov '96
				bpj 251	TSA			BS1	J-7	Jalajala, Rizal	Nov '96
				bpj 327	TSA			PSBRc14	J-6	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 328	TSA			PSBRc14	J-6	Jalajala, Rizal	Nov '96
				bpj 410	TSA		Pg	PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 411	TSA		Rs/Pg	PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 518	TSA		Pg	PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 520	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 574	TSA			PSBRc20	J-16	Jalajala, Rizal	Nov '96
				bpj 575	TSA			PSBRc20	J-16	Jalajala, Rizal	Nov '96
				bpj 603	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 631	TSA		Pg	PSBRc14	J-18(1)	Jalajala, Rizal	Nov '96
				bpj 677	TSA			PSBRc20	J-18(2)	Jalajala, Rizal	Nov '96
				bpj 699	TSA			PSBRc20	J-19	Jalajala, Rizal	Nov '96
				bpj 752	TSA			PSBRc20	J-20	Jalajala, Rizal	Nov '96
				bpj 754	TSA			PSBRc20	J-20	Jalajala, Rizal	Nov '96
				bpj 842	TSA			R1040	J-22	Jalajala, Rizal	Nov '96
				bpj 895	TSA			PSBRc20	J-23	Jalajala, Rizal	Nov '96
				bpj 896	TSA			PSBRc20	J-23	Jalajala, Rizal	Nov '96
				bpj 1004	TSA			PSBRc14	J-24	Jalajala, Rizal	Nov '96
				bpj 1036	TSA			R1040	J-27	Jalajala, Rizal	Nov '96
				bpj 1037	TSA			R1040	J-27	Jalajala, Rizal	Nov '96
				bpj 1069	TSA			PSBRc14	J-28(1)	Jalajala, Rizal	Nov '96
				bpj 1070	TSA			PSBRc14	J-28(1)	Jalajala, Rizal	Nov '96
				bpj 1177	TSA			BS1	J-30	Jalajala, Rizal	Nov '96
				bpj 1179	TSA			BS1	J-30	Jalajala, Rizal	Nov '96
				bpj 1614	TSA			PSBRc14	J-2	Jalajala, Rizal	May '97
				bpj 1661	TSA			PSBRc14	J-4	Jalajala, Rizal	May '97
				bpj 1674	TSA			PSBRc14	J-5	Jalajala, Rizal	May '97
				bpj 1699	TSA			PSBRc14	J-6	Jalajala, Rizal	May '97
				bpj 1700	TSA			PSBRc14	J-6	Jalajala, Rizal	May '97
				bpj 1730	TSA			BS1	J-7	Jalajala, Rizal	May '97
				bpj 1731	TSA			BS1	J-7	Jalajala, Rizal	May '97
				bpj 1755	TSA			PSBRc14	J-8	Jalajala, Rizal	May '97
				bpj 1772	TSA			PSBRc14	J-11	Jalajala, Rizal	May '97
				bpj 1788	TSA			PSBRc14	J-11	Jalajala, Rizal	May '97
				bpj 1827	TSA			PSBRc14	J-12	Jalajala, Rizal	May '97
				bpj 1876	TSA			Ri10	J-14	Jalajala, Rizal	May '97
				bpj 1938	TSA			PSBRc20	J-16	Jalajala, Rizal	May '97
				bpj 2002	TSA			PSBRc14	J-17	Jalajala, Rizal	May '97
				bpj 2188	TSA			PSBRc20	J-20	Jalajala, Rizal	May '97
				bpj 2778	TSA			PSBRc14	J-5G	Jalajala, Rizal	May '97
				bpj 3761	TSA			PSBRc20	J-1	Jalajala, Rizal	Dec '97
				bpj 3798	TSA			PSBRc14	J-2	Jalajala, Rizal	Dec '97
				bpj 3843	TSA			M30	J-3	Jalajala, Rizal	Dec '97
				bpj 3888	TSA			PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 3920	TSA			PSBRc14	J-5	Jalajala, Rizal	Dec '97
				bpj 3963	TSA			M30	J-6	Jalajala, Rizal	Dec '97
				bpj 4037	TSA			PSBRc14	J-8	Jalajala, Rizal	Dec '97
				bpj 4089	TSA			PSBRc14	J-9	Jalajala, Rizal	Dec '97
				bpj 4125	TSA			PSBRc14	J-11	Jalajala, Rizal	Dec '97
				bpj 4161	TSA			PSBRc14	J-12	Jalajala, Rizal	Dec '97
				bpj 4178	TSA			PSBRc14	J-10	Jalajala, Rizal	Dec '97

Gram-negative bacteria

Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
ENTEROBACTERIACEAE											
Butyrous yellow	1	*Cedecea lapagei* (B)		bpj 162	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
		No match		bpj 4879	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	Dec '97
	2	*Pectobacterium carotovorum* (API)	18632	g 296	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
Butyrous brownish cream	1	*Escherichia vulneris* (B)	20123	bpj 1200	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
				bpj 1481	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
		Enterobacter agglomerans biogroup 2B (B)	18627	g 247	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
Butyrous cream (KMB)	1	*Escherichia hermannii* (B)		c 48	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				c 78	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	Outlier	*Salmonella choleraesuis*	18608	g 44	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
Mucoid butyrous white	1	*Enterobacter aerogenes* (B)	20120	bpj 176	TSA		Rs	PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 145	TSA		Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
Butyrous cream yellow	1	No match	20122	bpj 167	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
		Enterobacter sakazakii (B)	18584	g 341	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
				g 256	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 152	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
		Enterobacter sp. (API)		g 442	KMB			IR60	20-6	Ajuy, Iloilo	Oct '95
		Enterobacter sakazakii (B)		c 42	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				c 259	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	2	*Enterobacter* sp. (B)		g 270	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
	3	*Enterobacter sakazakii* (B)	18640	g 403	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
		Enterobacter sp. (API)		g 317	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
	4	*Enterobacter sakazakii* (B)		g 149	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
	5	*Enterobacter sakazakii* (B)		g 248	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 419	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
	Outlier	No match		bpj 286	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	Outlier	*Enterobacter sakazakii* (API)	18576	g 134	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
Translucent butyrous white	1	*Enterobacter cloacae* (B)	20117	bpj 1449	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
		Enterobacter cloacae (B)	18577	g 139	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 355	GYCA			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				bpj 547	TSA			PSBRc20	J-16	Jalajala, Rizal	Nov '96
		Serratia odorifera (B)		bpj 581	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	2	*Enterobacter cloacae* (B)		bpj 141	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	3	*Serratia marcescens* (B)		bpj 143	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	4			bpj 172	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
subgroup 2	1	*Enterobacter cloacae* (B)		c 1	KMB		Pg	IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	2			bpj 4988	TSA			PSBRc22	J-29G	Jalajala, Rizal	Dec '97
	3	*Salmonella choleraesuis*	18620	g 175	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 389	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				bpj 1238	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
subgroup 3	1	*Enterobacter cloacae* (B)		bpj 38	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
				bpj 52	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
				bpj 381	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
	2	*Enterobacter* sp. (API)	18588	g 144	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
	3	*Enterobacter cloacae* (B)	18607	g 5	KMB		Rs	IR64	1-1	San Miguel, Iloilo	Oct '95
subgroup 4	1	*Enterobacter cloacae* (B)	18626	g 238	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 176	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 299	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 433	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	2	*Enterobacter cloacae* (B)		g 410	GYCA			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	3			bpj 1184	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
subgroup 5	1	*Enterobacter cloacae* (B)	18793	c 4	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				c 129	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
Translucent dull greenish yellow =*Pantoea ananatis* (BOX-PCR)	1	*Serratia plymuthica* (B)	20121	bpj 965	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
		Pantoea ananatis		c 263	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				g 13	KMB		Rs	IR24	2-4	Calauan, Laguna	Sept '95
		Cedecea lapagei (B)	18572	g 100	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 297	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
		Pantoea ananatis (B)		g 324	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
		Enterobacter agglomerans (API)	18642	g 400	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Thin pale yellow + granules =*Pantoea dispersa* (BOX-PCR)	1	*Pantoea dispersa* (B)	20116	bpj 158	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 521	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 597	TSA		Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 1264	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1473	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				g 2	KMB		Rs	IR64	1-1	San Miguel, Iloilo	Oct '95
		Salmonella choleraesuis	18609	g 49	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
		Pantoea agglomerans (B)		g 132	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
				g 198	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 272	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	2			bpj 47	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
		Pantoea dispersa (B)		bpj 957	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	3	*Pantoea agglomerans* (B)		bpj 298	TSA		Pg	PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 4725	TSA				J-8G	Jalajala, Rizal	Dec '97
	4	*Salmonella choleraesuis*		g 202	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				bpj 365	TSA		Rs/Pg	PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 455	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	5	*Klebsiella pneumoniae* (API)		g 12	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
				bpj 1471	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
	6	[*Pantoea dispersa*] (B)		bpj 1472	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	7	*Salmonella choleraesuis*		g 338	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
				bpj 439	TSA		Pg	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	Outlier	*Salmonella choleraesuis*	18580	g 240	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
Butyrous yellow + center dot =*Pantoea stewartii* (BOX-PCR)	1	*Enterobacter* sp. (API)	18570	g 85	KMB			IR64	3-7	San Miguel, Iloilo	Oct '95
		Enterobacter sp. (API)	18571	g 87	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
				g 20	KMB			IR24	2-4	Calauan, Laguna	Sept '95
				g 97	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 159	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
		Pantoea ananatis		g 193	GYCA		Rs	PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 244	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
		Serratia plymuthica (B)	18597	g 306	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 53	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
		Klebsiella pneumoniae (B)		bpj 140	TSA		Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
		Enterobacter agglomerans biogroup 3B (B)		bpj 181	TSA	LDP		PSBRc14	J-5	Jalajala, Rizal	Nov '96
		Pantoea ananatis	20115	bpj 259	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 293	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 366	TSA		Rs	PSBRc14	J-10	Jalajala, Rizal	Nov '96
		Pantoea agglomerans		bpj 391	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
				bpj 413	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
		Pantoea ananatis		c 15	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
		No match		bpj 459	TSA	LDP	Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 530	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
		No match		bpj 598	TSA		Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 959	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 1154	TSA	LDP		BS1	J-30	Jalajala, Rizal	Nov '96
		Pantoea ananatis		bpj 1474	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 1596	TSA			PSBRc14	J-2	Jalajala, Rizal	May '97
				bpj 2844	TSA	LDP		PSBRc14	J-9G	Jalajala, Rizal	May '97
	2			bpj 155	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				g 26	GYCA			IR24	2-4	Calauan, Laguna	Sept '95
		Enterobacter sp. (API)		g 203	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 319	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
		Enterobacter sp. (API)		g 363	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	3			bpj 156	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	4			bpj 531	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
P. agglomerans group 1 Butyrous cream	1	*Enterobacter agglomerans* biogroup 5 (B)	18792	c 90	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				c 258	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	2	*Kluyvera ascorbata*		bpj 437	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
		Enterobacter agglomerans biogroup 2B (B)		c 36	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
P. agglomerans group 2 Butyrous yellow + granules	1	*Enterobacter agglomerans* biogroup 3B (B)	20119	bpj 263	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 392	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
	2	No match		bpj 454	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
	Outlier	*Salmonella choleraesuis*	18587	g 401	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
P. agglomerans group 3 Pale yellow + granules	1	*Pantoea agglomerans* (B)	18621	g 200	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Salmonella subsp. 1F(B)	20118	bpj 265	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 154	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 48	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
	Outlier	*Pantoea ananatis*	18639	g 342	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
P. agglomerans group 4 (Wrinkled) pale yellow	1	*Enterobacter agglomerans* (API)	18579	g 230	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Pantoea agglomerans (B)		g 215	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
P. agglomerans group 5 Butyrous yellow	1	*Enterobacter agglomerans* biogroup 2B (B)	18790	c 253	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				c 65	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	Outlier	*Pantoea agglomerans* (B)		bpj 458	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
P. agglomerans group 6 Butyrous white	1	*Enterobacter agglomerans* biogroup 7(B)	18791	c 24	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				c 143	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	Outlier	*Pantoea* sp. (B)	18638	g 332	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	Outlier	No match		bpj 508	TSA		Pg	PSBRc14	J-15	Jalajala, Rizal	Nov '96
	Outlier	No match		bpj 958	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	Outlier	No match	20124	bpj 1271	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
GRAM NEGATIVE AEROBIC RODS											
Scarlet red	1	*Ochrobactrum anthropi*	20156	bpj 274	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	2	*Methylobacterium rhodesianum*	18593	g 367	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Reddish pink	1	*Methylobacterium radiotolerans*	20139	bpj 81	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
		Ochrobactrum anthropi		bpj 543	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 427	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 482	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
		Methylobacterium mesophilicum		bpj 974	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	Outlier			bpj 380	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
Thin translucent pink		Gilardi pink gram negative rod (B)		bpj 82	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
		Gilardi pink gram negative rod (B)		bpj 737	TSA			PSBRc20	J-21(1)	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
Mucoid brownish white+ diffusible brown pigment	1	*Agrobacterium rhizogenes* (B)	20148	bpj 1466	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	2			bpj 1259	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	3			bpj 1185	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
	4	*Agrobacterium rhizogenes* (B)	18633	g 300	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
	5			bpj 432	TSA	LDP	Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
Brownish white+ diffusible brown pigment	1	*Agrobacterium radiobacter*	18631	g 293	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 1467	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	2			bpj 943	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	3	*Agrobacterium* sp. (B)		c 125	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	4	*Agrobacterium radiobacter*	20140	bpj 174	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
	Outlier			bpj 1241	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	Outlier	No match	18565	g 9	GYCA		Rs	IR64	1-1	San Miguel, Iloilo	Oct '95
Butyrous brownish white	1	*Achromobacter xylosoxidans*		c 245	KMB			Bordagol	30-8G	San Dionisio, Iloilo	Oct '95
				c 236	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	Outlier	*Achromobacter xylosoxidans*	20155	bpj 1458	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
Butyrous yellowish white	Minimal amplification	*Acinetobacter baumannii* (B)	20144	bpj 1260	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 147	TSA		Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 591	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
		Acinetobacter baumannii		bpj 1468	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				g 6	GYCA		Rs	IR64	1-1	San Miguel, Iloilo	Oct '95
				g 135	GYCA			IR36	25-4	Concepcion, Iloilo	Oct '95
		Acinetobacter baumannii	18603	g 336	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
		Acinetobacter baumannii	18644	g 371	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
		Acinetobacter genospecies 3		g 406	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
		Acinetobacter baumannii	18599	g 260	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
		Acinetobacter calcoaceticus	18598	g 323	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
		Acinetobacter calcoaceticus	18592	g 206	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Acinetobacter radioresistent (B)	20149	bpj 355	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 76	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
Thin yellowish gray		*Acinetobacter* sp. (B)	20157	bpj 80	TSA		Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
Yellowish cream	Minimal amplification	*Sphingobacterium multivorum*	20152	bpj 1193	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
		Sphingobacterium spiritivorum		bpj 1270	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Glossy reddish yellow	Minimal amplification	*Chryseobacterium indologenes* (B)	20141	bpj 1204	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
				bpj 1491	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	(2)	*Chryseobacterium gleum* (B)		bpj 188	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 525	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 1205	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
		Chryseobacterium indologenes		bpj 2974	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	May '97
Mucoid reddish yellow	(3)	[*Chryseobacterium meningosepticum*]	20147	bpj 465	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 1281	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Glossy yellowish brown	(4)	*Chryseobacterium indologenes* (B)	20150	bpj 970	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 1493	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	Outlier	*Chryseobacterium indologenes*		bpj 4720	TSA			PSBRc14	J-5G	Jalajala, Rizal	Dec '97
	Outlier	*Chryseobacterium indologenes*	18594	g 285	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	Outlier		18636	g 309	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
	Outlier	*Chryseobacterium indologenes*	18595	g 304	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
Glossy deep yellow	1	*Spingomonas paucimobilis* A (B)	18625	g 231	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 164	KMB			IR36	25-4	Concepcion, Iloilo	Oct '95
	2	*Sphingomonas paucimobilis*	18590	g 185	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
	3	*Spingomonas paucimobilis* A (B)		g 65	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 114	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
	4	*Sphingomonas paucimobilis*	18624	g 209	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
subgroup 2	1	*Sphingomonas paucimobilis*	18618	g 118	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
				c 173	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
		Sphingomonas paucimobilis	20142	bpj 461	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	2	*Sphingomonas paucimobilis*	18789	c 184	KMB		Pg	PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	3	*Sphingomonas paucimobilis* A (B)		bpj 1279	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	Outlier	*Sphingomonas capsulata*		bpj 528	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
subgroup 3	1	*Sphingomonas paucimobilis*	18602	g 464	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
	2	*Sphingomonas capsulata*	18601	g 348	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	3	*Sphingomonas paucimobilis*		c 119	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
Dull deep yellow	1	*Sphingomonas paucimobilis* (B)		c 241	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	2			bpj 429	TSA		Rs	PSBRc14	J-12	Jalajala, Rizal	Nov '96
	3	[*Sphingomonas capsulata*]	20146	bpj 420	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 300	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
	Outlier	*Sphingomonas paucimobilis* A (B)		bpj 1278	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	Outlier	*Spingomonas paucimobilis* A (B)	18575	g 130	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
Translucent light cinnamon	1	*Brevundimonas vesicularis*	20159	bpj 1283	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Brilliant olive yellow	2	*Brevundimonas vesicularis* (B)	20158	bpj 608	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
Cream yellow	1	*Comamonas testosteroni* (B)	18586	g 361	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Pale yellow	2	*Pseudomonas* sp.	18611	g 64	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 397	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Glistening yellowish gray	1	*Acidovorax konjaci*		bpj 1105		P		PSBRc22	J-29	Jalajala, Rizal	Nov '96
		Acidovorax avenae		bpj 4090	TSA	P		PSBRc14	J-11	Jalajala, Rizal	Dec '97
				bpj 4821	TSA	P		PSBRc14	J-17G	Jalajala, Rizal	Dec '97
		Acidovorax avenae		bpj 4897	TSA	P		BS1	J-30G	Jalajala, Rizal	Dec '97
	2			bpj 3254	TSA	P		PSBRc22	J-29G	Jalajala, Rizal	May '97
	3	*Acidovorax avenae* (B)	20151	bpj 1188	TSA	P		PSBRc14	J-8G	Jalajala, Rizal	Nov '96
				bpj 1291	TSA	P		PSBRc14	J-4G	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 3028	TSA	P		PSBRc20	J-16G	Jalajala, Rizal	May '97
				bpj 3095	TSA	P		PSBRc14	J-17G	Jalajala, Rizal	May '97
		Acidovorax avenae		bpj 4863	TSA	P		PSBRc14	J-12G	Jalajala, Rizal	Dec '97
	4	*Acidovorax avenae*		bpj 3528	TSA	P		BS1	J-30G	Jalajala, Rizal	May '97
	5	*Acidovorax avenae* (B)		7015	KMB	P			409	Los Banos, Laguna	Oct '88
	6			bpj 4652	TSA	P		PSBRc14	J-4G	Jalajala, Rizal	Dec '97
		Acidovorax avenae		bpj 4943	TSA	P		PSBRc20	J-16G	Jalajala, Rizal	Dec '97
	7	*Acidovorax avenae*		bpj 3531	TSA	P		BS1	J-30G	Jalajala, Rizal	May '97
	8			bpj 1375	TSA	P		PSBRc14	J-18(1)G	Jalajala, Rizal	Nov '96
	9	*Acidovorax avenae* (B)		1891	KMB	P		Wagwag	A03	Solana, Cagayan	Oct '89
	10	*Acidovorax avenae* (B)		7010	KMB	P			410	Los Banos, Laguna	Oct '88
	Outlier	*Acidovorax avenae* (B)		bpj 1240	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	Outlier	*Acidovorax avenae*		bpj 2979	TSA	LDP		PSBRc14	J-15G	Jalajala, Rizal	May '97
FLUORESCENT *PSEUDOMONAS* SPP.											
Translucent yellowish gray	1	*Pseudomonas putida* type A1 (B)	20130	bpj 1183	TSA		Rs/Pg	PSBRc14	J-8G	Jalajala, Rizal	Nov '96
				bpj 1190	TSA		Rs/Pg	PSBRc14	J-8G	Jalajala, Rizal	Nov '96
		Pseudomonas resinovorans (B)		bpj 1453	TSA		Rs/Pg	PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	Outlier	*Pseudomonas putida* type A1 (B)	18615	g 77	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
Butyrous light cream	1	*Pseudomonas putida* type A1 (B)		bpj 1244	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	2	*Acinetobacter* genospecies 15 (B)		bpj 1454	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	3	No match		bpj 4696	TSA			PSBRc14	J-5G	Jalajala, Rizal	Dec '97
	Outlier	*Pseudomonas viridae* (B)		6720	KMB	LDP		IR36	303	Zarraga, Iloilo	Oct '89
Dull butyrous straw yellow	1	*Pseudomonas putida*	18566	g 42	GYCA			IR64	1-1	San Miguel, Iloilo	Oct '95
				bpj 1316	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	Nov '96
		Pseudomonas putida		bpj 1523	TSA	LDP		PSBRc14	J-17G	Jalajala, Rizal	Nov '96
				bpj 2929	TSA	LDP		PSBRc14	J-10G	Jalajala, Rizal	May '97
		Pseudomonas putida		bpj 2956	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	May '97
				bpj 4683	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	Dec '97
				bpj 4729	TSA	LDP		PSBRc14	J-8G	Jalajala, Rizal	Dec '97
				bpj 4880	TSA	LDP	Pg	PSBRc14	J-12G	Jalajala, Rizal	Dec '97
				bpj 4980	TSA	LDP		PSBRc20	J-16G	Jalajala, Rizal	Dec '97
	2	*Pseudomonas putida*	20136	bpj 1276	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Translucent pale straw yellow	1	*Pseudomonas aeruginosa*	18641	g 463	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 393	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
		No match		bpj 952	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 1265	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1479	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
		Pseudomonas aeruginosa	20129	bpj 1482	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
Translucent wrinkled yellowish white	1	*Pseudomonas putida* type A1 (B)	20135	bpj 1465	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
		Pseudomonas putida		c 20	KMB			IR36	25-4G	Concepcion, Iloilo	Oct '95
Mucoid white (brownish diffusible pigment)	1	*Pseudomonas citronellolis* (B)	20132	bpj 1194	TSA	LDP		PSBRc14	J-8G	Jalajala, Rizal	Nov '96
		[*Pseudomonas mendocina*]		bpj 2760	TSA	LDP	Rs	PSBRc14	J-2G	Jalajala, Rizal	May '97
	2	No match		bpj 1249	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1252	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Light brownish white	1	No match		bpj 3744		P		PSBRc14	J-27G	Jalajala, Rizal	May '97
		Pseudomonas fuscovaginae (B)		6235	NA	P		IR8866	A15	Banaue, Ifugao	May '89
		Pseudomonas fuscovaginae (B)		6609	KMB	P			363	Davao City	Oct '89
		Pseudomonas fuscovaginae (B)		7007	KMB	P			390	Aborlan, Palawan	Oct '89
				S-E1	TSA	P		IR55423-01		Siniloan, Laguna	Jan '00
				S-E2	TSA	P		IR55423-01		Siniloan, Laguna	Jan '00
Group 8	1	*Pseudomonas putida* type A1 (B)		6625	KMB	LDP			359	Asuncion, Davao Norte	Oct '89
		Pseudomonas putida type A1 (B)		7161	NA	LDP		IR8866	A15	Banaue, Ifugao	May '89
		Pseudomonas fulva (B)		7406	NA	LDP		IR8866	A15	Banaue, Ifugao	May '89
Group 9	1	*Pseudomonas marginalis* (B)		4833	KMB	LDP			237	Banaue, Ifugao	Oct '89
		Pseudomonas corrugata (B)		5310	KMB	LDP		Pinidwa	211	Banaue, Ifugao	Oct '89
		Pseudomonas marginalis (B)		6193	KMB	LDP		IR72	304	New Lucena, Iloilo	Oct '89
Pinkish white (brownish diffusible pigment)	1	*Pseudomonas aeruginosa* (B)	20133	bpj 1262	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1211	TSA	LDP		PSBRc14	J-5G	Jalajala, Rizal	Nov '96
				bpj 2978	TSA	LDP		PSBRc14	J-15G	Jalajala, Rizal	May '97
	Outlier	*Pseudomonas putida* type A1 (B)	20131	bpj 1243	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Translucent ecru beige	1	*Pseudomonas aeruginosa* (B)	20134	bpj 1461	TSA	P		PSBRc14	J-12G	Jalajala, Rizal	Nov '96
		No match		bpj 1508	TSA	P		PSBRc14	J-17G	Jalajala, Rizal	Nov '96
		No match		bpj 2770	TSA	LDP	Rs	PSBRc14	J-5G	Jalajala, Rizal	May '97
				bpj 2942	TSA	P		PSBRc14	J-12G	Jalajala, Rizal	May '97
				bpj 4781	TSA	P		PSBRc14	J-2G	Jalajala, Rizal	Dec '97
		No match		bpj 4832	TSA	P	Rs/Pg	PSBRc14	J-17G	Jalajala, Rizal	Dec '97
		Pseudomonas aeruginosa		c 26	KMB	P	Pg	IR64	19-9G	Ajuy, Iloilo	Oct '95
				c 111	KMB	P	Pg	IR64	19-9G	Ajuy, Iloilo	Oct '95
		Pseudomonas aeruginosa (B)		5459	NA	LDP		IR54	A13	Los Baños, Laguna	May '89
	Outlier	*Pseudomonas putida* type A1 (B)		bpj 1248	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
NONFLUORESCENT *PSEUDOMONAS* SPP.											
Glossy translucent chrome yellow	1	*Pseudomonas oryzihabitans*	18596	g 315	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
				g 92	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
		Pseudomonas aeruginosa	18600	g 375	KMB	LDP		IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
		Pseudomonas aeruginosa		c 175	KMB	LDP		PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				bpj 46	TSA		Rs/Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 161	TSA		Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 184	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
		Pseudomonas aeruginosa	20125	bpj 257	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 289	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 389	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
		Pseudomonas aeruginosa		bpj 460	TSA		Rs	PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 524	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 961	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 1202	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
	2	*Pseudomonas* sp. (B)	18573	g 102	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
		Pseudomonas aeruginosa		c 58	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				c 108	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
		Pseudomonas mendocina		c 212	KMB			Bordagol	30-8G	San Dionisio, Iloilo	Oct '95
				bpj 1477	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	3	*Pseudomonas aeruginosa*	18585	g 351	KMB	LDP		IR38	14-8	Dingle, Iloilo	Oct '95
				g 101	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 413	GYCA			IR60	20-6	Ajuy, Iloilo	Oct '95
		Pseudomonas aeruginosa		bpj 368	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 416	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 1476	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
subgroup 2	1	*Pseudomonas aeruginosa*	18591	g 188	GYCA	LDP		PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 95	GYCA	LDP		IR64	10-1	Zarraga, Iloilo	Oct '95
				g 320	GYCA	LDP		IR38	14-8	Dingle, Iloilo	Oct '95
		Pseudomonas aeruginosa		c 23	KMB		Pg	PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				c 190	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				bpj 1268	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	2	*Pseudomonas* sp.	18604	g 399	KMB	LDP		IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	3	*Pseudomonas aeruginosa*		bpj 1649	TSA			PSBRc14	J-4	Jalajala, Rizal	May '97
				bpj 1766	TSA	LDP		PSBRc14	J-9	Jalajala, Rizal	May '97
				bpj 2512	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 4000	TSA	LDP		BS1	J-7	Jalajala, Rizal	Dec '97
	4	*Pseudomonas aeruginosa*		g 347	KMB	LDP		IR38	14-8	Dingle, Iloilo	Oct '95
	5	*Pseudomonas aeruginosa*		bpj 398	TSA			PSBRc14	J-11	Jalajala, Rizal	Nov '96
subgroup 3	1	*Pseudomonas aeruginosa*	20128	bpj 166	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	2	*Pseudomonas aeruginosa*		bpj 1269	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
subgroup 4	1	*Pseudomonas* sp. (B)	18568	g 71	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 17	KMB			IR24	2-4	Calauan, Laguna	Sept '95
		Pseudomonas aeruginosa	18616	g 96	GYCA			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 204	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 246	GYCA			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
		Pseudomonas aeruginosa	18583	g 339	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
	2	*Pseudomonas oryzihabitans*	18605	g 378	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
		Pseudomonas aeruginosa		g 234	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
				g 322	GYCA			IR38	14-8	Dingle, Iloilo	Oct '95
	3	*Pseudomonas aeruginosa*	18619	g 121	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
				g 31	GYCA			IR24	2-4	Calauan, Laguna	Sept '95
				g 182	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		Pseudomonas aeruginosa		g 343	KMB			IR38	14-8	Dingle, Iloilo	Oct '95
				bpj 594	TSA			PSBRc14	J-17	Jalajala, Rizal	Nov '96
	Outlier	*Pseudomonas aeruginosa*	18628	g 265	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
subgroup 5	1	*Pseudomonas aeruginosa*	18574	g 123	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
	2	*Pseudomonas aeruginosa*	18629	g 269	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
Wrinkled light chrome yellow	1	*Pseudomonas oryzihabitans* (B)	20126	bpj 596	TSA		Pg	PSBRc14	J-17	Jalajala, Rizal	Nov '96
				bpj 163	TSA		Pg	PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 180	TSA		Pg	PSBRc14	J-5	Jalajala, Rizal	Nov '96
				bpj 4882	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	Dec '97
	2			bpj 262	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
		Pseudomonas luteola		bpj 287	TSA			PSBRc14	J-9	Jalajala, Rizal	Nov '96
		Pseudomonas aeruginosa		bpj 457	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
				bpj 962	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
	3	*Pseudomonas oryzihabitans*		bpj 1266	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 419	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
Wrinkled light orange brown	1	*Pseudomonas stutzeri*	18794	c 192	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
				c 3	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
		Pseudomonas stutzeri (B)		bpj 75	TSA		Pg	PSBRc14	J-2	Jalajala, Rizal	Nov '96
		Pseudomonas stutzeri (B)	20127	bpj 279	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 369	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
	2	*Pseudomonas stutzeri* (B)		bpj 523	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 418	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
	3			bpj 367	TSA			PSBRc14	J-10	Jalajala, Rizal	Nov '96
				bpj 978	TSA			PSBRc14	J-26	Jalajala, Rizal	Nov '96
***BURKHOLDERIA* SPP.**											
Glossy ivory yellow	1	*Burkholderia glumae*		2056	KMB	P			A08	Baao, Camarines Sur	Oct '89
				g 119	KMB	P		IR64	10-1	Zarraga, Iloilo	Oct '95
				g 390	KMB	P		IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				c 208	KMB	P		PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				mtv 25	TSA	P		KSB460-37	V3	Ho Chi Ming City, Vietnam	Aug '96
				mtv 52	TSA	P		IR64	V4	Ho Chi Ming City, Vietnam	Aug '96
				mtv 87	TSA	P		IR29723	V5	Ho Chi Ming City, Vietnam	Sep '96
				bpj 90	TSA	P		PSBRc14	J-3	Jalajala, Rizal	Nov '96
				bpj 254	TSA	P	Rs/Pg	PSBRc14	J-8	Jalajala, Rizal	Nov '96
				bpj 308	TSA	P	Rs/Pg	PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 382	TSA	P	Rs	PSBRc14	J-11	Jalajala, Rizal	Nov '96
				bpj 405	TSA	P	Rs	PSBRc14	J-12	Jalajala, Rizal	Nov '96
		Burkholderia glumae (SDS)	20138	bpj 517	TSA	P	Rs/Pg	PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 1111	TSA	P		PSBRc22	J-29	Jalajala, Rizal	Nov '96

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 1149	TSA	P		BS1	J-30	Jalajala, Rizal	Nov '96
				bpj 1591	TSA	P		PSBRc14	J-2	Jalajala, Rizal	May '97
				bpj 1622	TSA	P	Rs	PSBRc14	J-3	Jalajala, Rizal	May '97
				bpj 1831	TSA	P		PSBRc14	J-13	Jalajala, Rizal	May '97
		Burkholderia glumae (SDS)	19582	bpj 1879	TSA	P		PSBRc14	J-15	Jalajala, Rizal	May '97
				bpj 1951	TSA	P	Pg	PSBRc14	J-17	Jalajala, Rizal	May '97
				bpj 2010	TSA	P		PSBRc14	J-18(1)	Jalajala, Rizal	May '97
				bpj 2385	TSA	P	Pg	PSBRc22	J-29	Jalajala, Rizal	May '97
				bpj 2500	TSA	P	Pg	PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 3184	TSA	P		PSBRc14	J-21(1)G	Jalajala, Rizal	May '97
				bpj 3220	TSA			PSBRc20	J-20G	Jalajala, Rizal	May '97
				bpj 3260	TSA	P		PSBRc22	J-29G	Jalajala, Rizal	May '97
				bpj 3792	TSA	P		PSBRc14	J-2	Jalajala, Rizal	Dec '97
				bpj 4031	TSA	P		PSBRc14	J-8	Jalajala, Rizal	Dec '97
				bpj 4092	TSA	P	Pg	PSBRc14	J-11	Jalajala, Rizal	Dec '97
				bpj 4132	TSA	P	Pg	PSBRc14	J-12	Jalajala, Rizal	Dec '97
				bpj 4202	TSA	P		PSBRc14	J-13	Jalajala, Rizal	Dec '97
				bpj 4287	TSA	P	Rs	PSBRc14	J-15	Jalajala, Rizal	Dec '97
				bpj 4322	TSA	P	Rs	PSBRc20	J-16	Jalajala, Rizal	Dec '97
				bpj 4868	TSA	P	Pg	PSBRc14	J-12G	Jalajala, Rizal	Dec '97
				bpj 4900	TSA	P	Pg	BS1	J-30G	Jalajala, Rizal	Dec '97
	2	*Burkholderia glumae* (SDS)	18634	g 307	KMB	P	Pg	IR64	19-9	Ajuy, Iloilo	Oct '95
				bpj 430	TSA	P	Pg	PSBRc14	J-12	Jalajala, Rizal	Nov '96
				bpj 946	TSA	P		PSBRc14	J-26	Jalajala, Rizal	Nov '96
	3	*Burkholderia glumae*		1858	KMB	P			A05	Roxas, Isabela	Oct '89
				bpj 282b	TSA	P		PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 552	TSA	P		PSBRc20	J-16	Jalajala, Rizal	Nov '96
				bpj 1464	TSA	P		PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 1706	TSA	P		BS1	J-7	Jalajala, Rizal	May '97
				bpj 3790	TSA	P		PSBRc14	J-2	Jalajala, Rizal	Dec '97
				bpj 3890	TSA	P		PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 3922	TSA	P		PSBRc14	J-5	Jalajala, Rizal	Dec '97
				bpj 3995	TSA	P		BS1	J-7	Jalajala, Rizal	Dec '97
				bpj 4534	TSA	P		PSBRc14	J-26	Jalajala, Rizal	Dec '97
				bpj 4574	TSA	P		PSBRc22	J-29	Jalajala, Rizal	Dec '97
				bpj 4607	TSA	P	Pg	BS1	J-30	Jalajala, Rizal	Dec '97
		Burkholderia glumae (SDS)	19583	bpj 4695	TSA	P		PSBRc14	J-5G	Jalajala, Rizal	Dec '97
				bpj 4912	TSA	P	Rs/Pg	BS1	J-30G	Jalajala, Rizal	Dec '97
Butyrous yellowish white (yellowish-green diffusible pigment)	1	*Burkholderia gladioli* (SDS)	18569	g 73	KMB	P		IR64	2-7	San Miguel, Iloilo	Oct '95
				g 122	KMB	P		IR64	10-1	Zarraga, Iloilo	Oct '95
				X 196	XMSM	P			leaves	Los Baños, Laguna	Dec '95
				mtv 53	TSA	P		IR64	V4	Ho Chi Ming City, Vietnam	Aug '96
		Burkholderia gladioli (SDS)	20145	bpj 947	TSA	P		PSBRc14	J-26	Jalajala, Rizal	Nov '96
				bpj 282a	TSA	P		PSBRc14	J-9	Jalajala, Rizal	Nov '96
				bpj 1254	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1296	TSA	P		PSBRc14	J-4G	Jalajala, Rizal	Nov '96
				bpj 1623	TSA	P		PSBRc14	J-3	Jalajala, Rizal	May '97
				bpj 1663	TSA	P		PSBRc14	J-5	Jalajala, Rizal	May '97
				bpj 1857	TSA	P		Ri10	J-14	Jalajala, Rizal	May '97
				bpj 1878	TSA	P		PSBRc14	J-15	Jalajala, Rizal	May '97
				bpj 2460	TSA	P		PSBRc14	J-1G	Jalajala, Rizal	May '97
				bpj 2647	TSA	P		PSBRc14	J-3G	Jalajala, Rizal	May '97
		Burkholderia gladioli (SDS)	19584	bpj 2705	TSA	P		BS1	J-7G	Jalajala, Rizal	May '97
				bpj 2990	TSA	P		PSBRc14	J-15G	Jalajala, Rizal	May '97
				bpj 3126	TSA	P		PSBRc14	J-17G	Jalajala, Rizal	May '97
				bpj 3172	TSA	P		PSBRc14	J-21(1)G	Jalajala, Rizal	May '97
				bpj 3291	TSA	P		PSBRc20	J-21(2)G	Jalajala, Rizal	May '97
				bpj 3372	TSA	P		PSBRc14	J-25G	Jalajala, Rizal	May '97
				bpj 3458	TSA	P		PSBRc20	J-19G	Jalajala, Rizal	May '97
				bpj 3689	TSA	P		PSBRc20	J-18(2)G	Jalajala, Rizal	May '97
				bpj 3884	TSA	P		PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 4030	TSA	P		PSBRc14	J-8	Jalajala, Rizal	Dec '97
				bpj 4206	TSA	P	Pg	PSBRc14	J-13	Jalajala, Rizal	Dec '97
				bpj 4248	TSA	P		PSBRc14	J-14	Jalajala, Rizal	Dec '97
				bpj 4651	TSA	P		PSBRc14	J-4G	Jalajala, Rizal	Dec '97
				bpj 4704	TSA	P		PSBRc14	J-5G	Jalajala, Rizal	Dec '97
				bpj 4864	TSA	P		PSBRc14	J-12G	Jalajala, Rizal	Dec '97
				bpj 4914	TSA	P	Pg	BS1	J-30G	Jalajala, Rizal	Dec '97
				bpj 4950	TSA	P		PSBRc20	J-16G	Jalajala, Rizal	Dec '97
	2			bpj 2491	TSA	P	Pg	PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 4783	TSA	P		PSBRc14	J-2G	Jalajala, Rizal	Dec '97
				bpj 4818	TSA	P		PSBRc14	J-17G	Jalajala, Rizal	Dec '97
	3			bpj 3068	TSA	P		PSBRc14	J-13G	Jalajala, Rizal	May '97
	4			bpj 4062	TSA	P		PSBRc14	J-9	Jalajala, Rizal	Dec '97
	5			bpj 1253	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Creamy white	1	*Burkholderia gladioli* (SDS)	19585	bpj 1257	TSA	P		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 4354	TSA	P		PSBRc14	J-17	Jalajala, Rizal	Dec '97
				bpj 4576	TSA	P		PSBRc22	J-29	Jalajala, Rizal	Dec '97
	2			bpj 1944	TSA	P		PSBRc14	J-17	Jalajala, Rizal	May '97
Wrinkled white (yellowish-green diffusible pigment)	1	*Burkholderia gladioli* (SDS)	19586	bpj 4323	TSA	P		PSBRc20	J-16	Jalajala, Rizal	Dec '97
	2			bpj 4654	TSA	P		PSBRc14	J-4G	Jalajala, Rizal	Dec '97
	Outlier			bpj 3601	TSA	P		PSBRc14	J-28(1)G	Jalajala, Rizal	May '97
Wrinkled olive yellow	1	*B. cepacia* genomovar I (SDS)	20153	bpj 1274	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 830	TSA	LDP		R1040	J-22	Jalajala, Rizal	Nov '96
				bpj 1029	TSA	LDP		R1040	J-27	Jalajala, Rizal	Nov '96
				bpj 1489	TSA	LDP		PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 1530	TSA	LDP		PSBRc14	J-17G	Jalajala, Rizal	Nov '96
				bpj 2524	TSA	LDP		PSBRc14	J-4G	Jalajala, Rizal	May '97
				bpj 2625	TSA	LDP		PSBRc14	J-3G	Jalajala, Rizal	May '97
		B. cepacia genomovar I (SDS)	19589	bpj 2818	TSA	LDP		PSBRc14	J-11G	Jalajala, Rizal	May '97
		B. cepacia genomovar I (SDS)	19590	bpj 3352	TSA	LDP		PSBRc14	J-26G	Jalajala, Rizal	May '97

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
				bpj 3570	TSA	LDP		PSBRc14	J-28(1)G	Jalajala, Rizal	May '97
				bpj 3912	TSA	LDP	Rs/Pg	PSBRc14	J-4	Jalajala, Rizal	Dec '97
				bpj 4673	TSA	LDP	Pg	PSBRc14	J-4G	Jalajala, Rizal	Dec '97
				bpj 4803	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Dec '97
				bpj 4970	TSA	LDP	Pg	PSBRc20	J-16G	Jalajala, Rizal	Dec '97
Butyrous creamy white	1	*B. cepacia* genomovar III (SDS)	20154	bpj 1247	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1007	TSA	LDP		R1040	J-27	Jalajala, Rizal	Nov '96
				bpj 1509	TSA	LDP		PSBRc14	J-17G	Jalajala, Rizal	Nov '96
				bpj 1641	TSA	LDP		PSBRc14	J-4	Jalajala, Rizal	May '97
				bpj 3226	TSA	LDP		PSBRc20	J-20G	Jalajala, Rizal	May '97
		B. cepacia genomovar III (SDS)	19587	bpj 3262	TSA	LDP		PSBRc22	J-29G	Jalajala, Rizal	May '97
				bpj 4917	TSA	LDP	Pg	BS1	J-30G	Jalajala, Rizal	Dec '97
				bpj 4989	TSA	LDP		PSBRc22	J-29G	Jalajala, Rizal	Dec '97
				bpj 4997	TSA	LDP		PSBRc22	J-29G	Jalajala, Rizal	Dec '97
Glossy olive yellow				6854	KMB	0/4	Rs/Pg		374	Brooke's Point, Palawan	Oct '89
Glossy olive yellow				6858	KMB	0/4	Rs/Pg		374	Brooke's Point, Palawan	Oct '89
Glossy olive yellow				bpj 829	TSA	LDP		R1040	J-22	Jalajala, Rizal	Nov '96
Glossy olive yellow		*B. cepacia* genomovar III (SDS)	19588	bpj 1230	TSA	LDP		PSBRc14	J-5G	Jalajala, Rizal	Nov '96
Glossy olive yellow				bpj 4594	TSA	LDP	Pg	PSBRc22	J-29	Jalajala, Rizal	Dec '97
	2			bpj 4956	TSA	LDP		PSBRc20	J-16G	Jalajala, Rizal	Dec '97
	3			bpj 1258	TSA	LDP		PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 3219	TSA	LDP		PSBRc20	J-20G	Jalajala, Rizal	May '97
Butyrous white		*Burkholderia multivorans* (SDS)	18606	g 360	KMB	LDP		IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
Glossy light brownish white		*Burkholderia vietnamiensis* (SDS)	19591	bpj 1213	TSA	LDP		PSBRc14	J-5G	Jalajala, Rizal	Nov '96
XANTHOMONAS SPP.											
Mucoid pale yellow	1	*X. c. dieffenbachia* A (B)	18630	g 278	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
				c 233	KMB			IR36	23-7G	Barotac Nuevo, Iloilo	Oct '95
	2	*Xanthomonas* sp. (B)		c 239	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
				c 202	KMB			PSBRc14	28-9G	San Dionisio, Iloilo	Oct '95
	3	*X. o. oryzae* F (B)		bpj 159	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
				bpj 3808	TSA			PSBRc14	J-2	Jalajala, Rizal	Dec '97
	4	*X. c. dieffenbachia* A (B)	18578	g 190	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
		X. o. oryzae E (B)		mtv 75	TSA			IR29723	V5	Ho Chi Ming City, Vietnam	Sep '96
	5	*X. c. dieffenbachia* A (B)	18582	g 276	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	6	*X. c. dieffenbachia* A (B)	18643	g 381	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	7	No match		X 59	XOS			IR36	23-7G	Barotac Nuevo, Iloilo	May '95
		No match		X 119	GYCA				roots	Pila, Laguna	Nov '95
				bpj 874	TSA			PSBRc20	J-23	Jalajala, Rizal	Nov '96
				bpj 3078	TSA			PSBRc14	J-13G	Jalajala, Rizal	May '97
	8	*X. c. dieffenbachia* A (B)		bpj 1837	TSA			PSBRc14	J-13	Jalajala, Rizal	May '97
	9	*X. c. dieffenbachia* A (B)		bpj 993	TSA			PSBRc14	J-24	Jalajala, Rizal	Nov '96
		No match		X 18	GYCA			IR64	17-6	Mina, Iloilo	May '95
		Xanthomonas sp. (B)		X 144	XOS			IR36	27-2	Concepcion, Iloilo	May '95
	10	*X. c. dieffenbachia* A (B)		X 36	GYCA			IR38	14-8	Dingle, Iloilo	May '95
		X. o. oryzae F (B)		X 88	XOS				leaves	Pila, Laguna	Nov '95
	11	No match		bpj 396	TSA		Rs	PSBRc14	J-11	Jalajala, Rizal	Nov '96
		No match		bpj 1779	TSA	LDP		PSBRc14	J-11	Jalajala, Rizal	May '97
		No match		bpj 1880	TSA	LDP		PSBRc14	J-15	Jalajala, Rizal	May '97
		No match		bpj 3271	TSA	LDP		PSBRc22	J-29G	Jalajala, Rizal	May '97
		No match		bpj 5006	TSA	LDP		PSBRc22	J-29G	Jalajala, Rizal	Dec '97
	12	*X. c. strelitzia* A (B)		bpj 1669	TSA			PSBRc14	J-5	Jalajala, Rizal	May '97
	13	*X. c. dieffenbachia* A (B)	18610	g 55	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
				X 25	GYCA			IR64	16-2	Mina, Iloilo	May '95
		X. c. strelitzia A (B)		bpj 1658	TSA			PSBRc14	J-4	Jalajala, Rizal	May '97
				bpj 1749	TSA			PSBRc14	J-8	Jalajala, Rizal	May '97
	14	*X. c. dieffenbachia* A (B)		X 149	XOS			IR24	2-4	Calauan, Laguna	Nov '95
	15	*X. c. dieffenbachia* A (B)	18567	g 46	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
		X. c. pilea A (B)		X 52c	XOS			IR64	19-9	Ajuy, Iloilo	Oct '95
	16			bpj 1112	TSA			PSBRc22	J-29	Jalajala, Rizal	Nov '96
	17	*X. c. dieffenbachia* A (B)	18613	g 72	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
				g 282	GYCA			IR64	19-9	Ajuy, Iloilo	Oct '95
	18	*X. c. dieffenbachia* A (B)	18623	g 208	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
			18637	g 314	GYCA		Rs	IR38	14-8	Dingle, Iloilo	Oct '95
	19	*X. c. dieffenbachia* A (B)	18589	g 183	GYCA			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
subgroup 2	1	*X. c. dieffenbachia* A (B)	18614	g 75	GYCA			IR64	3-7	San Miguel, Iloilo	Oct '95
		X. o. oryzae F (B)		X 160	GYCA				leaves	Mabitac, Laguna	Nov '95
				bpj 529	TSA			PSBRc14	J-15	Jalajala, Rizal	Nov '96
				bpj 2738	TSA			PSBRc14	J-2G	Jalajala, Rizal	May '97
	2	*X. o. oryzae* F (B)		X 23	GYCA			IR38	14-8	Dingle, Iloilo	May '95
		No match		X 54	XOS			IR36	23-7G	Barotac Nuevo, Iloilo	May '95
				bpj 784	TSA			PSBRc14	J-21(2)	Jalajala, Rizal	Nov '96
	3	*X. c. dieffenbachia* A (B)	18645	g 362	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
				bpj 2352	TSA			PSBRc14	J-28(1)	Jalajala, Rizal	May '97
				X 49	XOS			IR64	19-9	Ajuy, Iloilo	May '95
	4			bpj 1922	TSA			PSBRc20	J-16	Jalajala, Rizal	May '97
				bpj 2390	TSA			PSBRc22	J-29	Jalajala, Rizal	May '97
	5	*X. c. dieffenbachia* A (B)	18612	g 70	KMB			IR64	2-7	San Miguel, Iloilo	Oct '95
subgroup 3	1	*Xanthomonas* sp. (B)	20137	bpj 45	TSA			PSBRc14	J-2	Jalajala, Rizal	Nov '96
	2	*Kluyvera ascorbata* (B)		bpj 1273	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
				bpj 1483	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	3	*X. c. dieffenbachia* A (B)		bpj 160	TSA			PSBRc14	J-4	Jalajala, Rizal	Nov '96
	4			bpj 256	TSA			PSBRc14	J-8	Jalajala, Rizal	Nov '96
	5	*X. o. oryzae* F (B)		X 61	GYCA			IR36	23-7G	Barotac Nuevo, Iloilo	May '95
				bpj 2599	TSA			PSBRc14	J-8G	Jalajala, Rizal	May '97
	6			bpj 3114	TSA			PSBRc14	J-17G	Jalajala, Rizal	May '97
	Outlier	No match		bpj 2442	TSA	LDP		BS1	J-30	Jalajala, Rizal	May '97

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Colony appearance on TSA	BOX-FPTs[a]	Identification[b]	LMG no.	IRRI no.	Isolation medium[c]	Path[d]	Antag[e]	Cultivar	Sample no.[f]	Location	Date collected
subgroup 4	1	*X. c. dieffenbachia* A (B)	18617	g 112	KMB			IR64	10-1	Zarraga, Iloilo	Oct '95
	2	*X. c. dieffenbachia* A (B)	18635	g 308	KMB			IR64	19-9	Ajuy, Iloilo	Oct '95
	3	*X. o. oryzae* F (B)		bpj 182	TSA			PSBRc14	J-5	Jalajala, Rizal	Nov '96
subgroup 5 (< 50%)	1	*X. c. dieffenbachia* A (B)		g 52	KMB			IR64	1-1	San Miguel, Iloilo	Oct '95
	2	*X. c. strelitzia* A (B)		bpj 450	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
	Outlier			bpj 873	TSA			PSBRc20	J-23	Jalajala, Rizal	Nov '96
STENOTROPHOMONAS MALTOPHILIA											
Translucent light olive gray (yellowish center)	1	*S. maltophilia* (B)		bpj 1165	TSA			BS1	J-30	Jalajala, Rizal	Nov '96
				X 113	GYCA				roots	Pila, Laguna	Nov '95
				X 169	XMSM				roots	Mabitac, Laguna	Nov '95
		Xanthomonas sp. (B)		X 184	XOS				leaves	Los Baños, Laguna	Dec '95
	2	*S. maltophilia* (B)		bpj 3724	TSA			PSBRc14	J-23G	Jalajala, Rizal	May '97
	3	*S. maltophilia* (B)		bpj 1485	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
	4			X 200	KMB			IR24	2-4	Calauan, Laguna	Sept '95
	5			X 201	KMB			IR24	2-4	Calauan, Laguna	Sept '95
subgroup 2	1	*S. maltophilia* (B)		X 102	XMSM				roots	Pila, Laguna	Nov '95
				bpj 1528	TSA			PSBRc14	J-17G	Jalajala, Rizal	Nov '96
	2	*S. maltophilia* (B)		X 127	XMSM				roots	Pila, Laguna	Nov '95
subgroup 3	1	*S. maltophilia* (B)		bpj 1228	TSA			PSBRc14	J-5G	Jalajala, Rizal	Nov '96
				bpj 1525	TSA			PSBRc14	J-17G	Jalajala, Rizal	Nov '96
	2	*S. maltophilia* (B)		bpj 1305	TSA			PSBRc14	J-4G	Jalajala, Rizal	Nov '96
	3			X 130	XMSM				roots	Pila, Laguna	Nov '95
	4			bpj 1486	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
subgroup 4	1	No match		bpj 1794	TSA			PSBRc14	J-10	Jalajala, Rizal	May '97
Ochre yellow				bpj 2000	TSA			PSBRc14	J-17	Jalajala, Rizal	May '97
	2	*S. maltophilia* (B)		bpj 3572	TSA			PSBRc14	J-28(1)G	Jalajala, Rizal	May '97
	3	*S. maltophilia* (B)		bpj 407	TSA			PSBRc14	J-12	Jalajala, Rizal	Nov '96
	Outlier	*S. maltophilia* (B)		bpj 449	TSA			PSBRc14	J-13	Jalajala, Rizal	Nov '96
subgroup 5	1	*S. maltophilia*	20143	bpj 1484	TSA			PSBRc14	J-12G	Jalajala, Rizal	Nov '96
				bpj 1272	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	2			X 50	GYCA			IR64	19-9	Ajuy, Iloilo	May '95
		S. maltophilia (B)		X 112	XMSM				roots	Pila, Laguna	Nov '95
subgroup 6 (< 50%)	1	*S. maltophilia* (B)		X 96	XMSM				roots	Pila, Laguna	Nov '95
	2			X 111	XMSM				roots	Pila, Laguna	Nov '95
	Outlier	No match		bpj 1197	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
	Outlier			bpj 1199	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
subgroup 7	1	No ID		bpj 2267	TSA			PSBRc14	J-24	Jalajala, Rizal	May '97
Greenish ochre yellow	2	No ID		bpj 3766	TSA			PSBRc20	J-1	Jalajala, Rizal	Dec '97
	Outlier	*S. maltophilia* (B)		X 168	XMSM				roots	Mabitac, Laguna	Nov '95
subgroup 8	1	*S. maltophilia* (B)		X 67	GYCA				leaves	Pila, Laguna	Nov '95
Yellowish white (clear center)				X 116	XMSM				roots	Pila, Laguna	Nov '95
	2	*X. campestris* pv. T1 (B)		bpj 3492	TSA			BS1	J-30G	Jalajala, Rizal	May '97
	3	*S. maltophilia* (B)		X 89	GYCA				leaves	Pila, Laguna	Nov '95
subgroup 9	1	No match	18646	g 255	KMB			Bordagol	30-8	San Dionisio, Iloilo	Oct '95
Olive ochre yellow		*X. c. strelitzia* A (B)	18581	g 266	KMB		Rs	Bordagol	30-8	San Dionisio, Iloilo	Oct '95
				g 376	KMB			IR36	23-7	Barotac Nuevo, Iloilo	Oct '95
	Outlier	*X. c. xanthosoma* (B)	18622	g 207	KMB			PSBRc14	28-9	San Dionisio, Iloilo	Oct '95
	Outlier	*S. maltophilia* (B)		X 170	XMSM				roots	Mabitac, Laguna	Nov '95
subgroup 10 (< 50%)	1			bpj 1106	TSA			PSBRc22	J-29	Jalajala, Rizal	Nov '96
	2	*S. maltophilia* (B)		X 86	GYCA				leaves	Pila, Laguna	Nov '95
	3	*S. maltophilia* (B)		bpj 2549	TSA			PSBRc14	J-6G	Jalajala, Rizal	May '97
Aerobic, helical gram negative bacteria											
Translucent pale cream		*Herbaspirillum rubrisubalbicans*	20161	bpj 1256	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
Translucent pinkish white	1	[*Azospirillum brasilense*]	20160	bpj 1192	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
	2			bpj 1245	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96
	3			bpj 1191	TSA			PSBRc14	J-8G	Jalajala, Rizal	Nov '96
	4		bpj	1263	TSA			PSBRc14	J-2G	Jalajala, Rizal	Nov '96

[a] BOX-PCR fingerprint types (FPTs) were arbitrarily delineated at about 70% similarity, and groups at about 50% similarity. Levels of similarity between BOX-PCR patterns were calculated by the Pearson correlation coefficient, and clustering was done by the unweighted pair group method using arithmetic averages (UPGMA).

[b] Identifications were obtained by FAME-MIS (version 4.15) analysis; (B)= by Biolog (version 3.50) analysis; (API)= by the API 20E/50CHE systems; (SDS)= by comparison of sodium dodecyl sulfate-polyacrylamide gel electrophoresis whole-cell protein profiles to the profiles in the *Burkholderia* library from Vandamme et al., 1997. Identifications in parenthesis were obtained with a similarity index value < 0.50 to the profile in the Microbial Identification System (MIS) or Biolog database.

[c] TSA= tryptic soy agar, NA = nutrient agar, GYCA = glucose yeast chalk agar (Schaad, 1988); KMB = King's medium B (King et al., 1954); XOS = semiselective medium for isolation of *Xanthomonas oryzae* pv. *oryzae* (Ming et al., 1991); XMSM = selective medium for isolation of *Stenotrophomonas maltophilia* (Juhnke and Des Jardin, 1989).

[d] Pathogenic activity was determined by injection in 21-day-old IR24 seedlings. P= pathogens, are those isolates able to induce symptoms in all inoculated plants; LDP= pathogens with low disease potential, are those isolates able to induce symptoms in at least 50% of inoculated plants.

[e] Antagonistic activity against *Rhizoctonia solani* (Rs) and *Pyricularia grisea* (Pg) was determined in dual culture tests by the presence of mycelial growth inhibition zones on pigment production medium.

[f] Sample numbers refer to rice seed samples collected from farmers' fields from which the isolates were obtained; G= refers to isolations from germinated seed of the sample. Additional rice-leaf and -root samples that were examined are indicated as such.

Appendix 3

Assorted dendrograms derived from unweighted pair group average linkage of Pearson correlation coefficients (expressed as percentages of similarity) for BOX-PCR patterns of isolates representative for bacterial morphotype groups detected in rice seed samples collected from farmers' fields in the tropical environment. (Other regularly found morphological types identified as *B. cereus* and *Acinetobacter* spp., which resulted in minimal BOX-PCR amplification, are not included.)

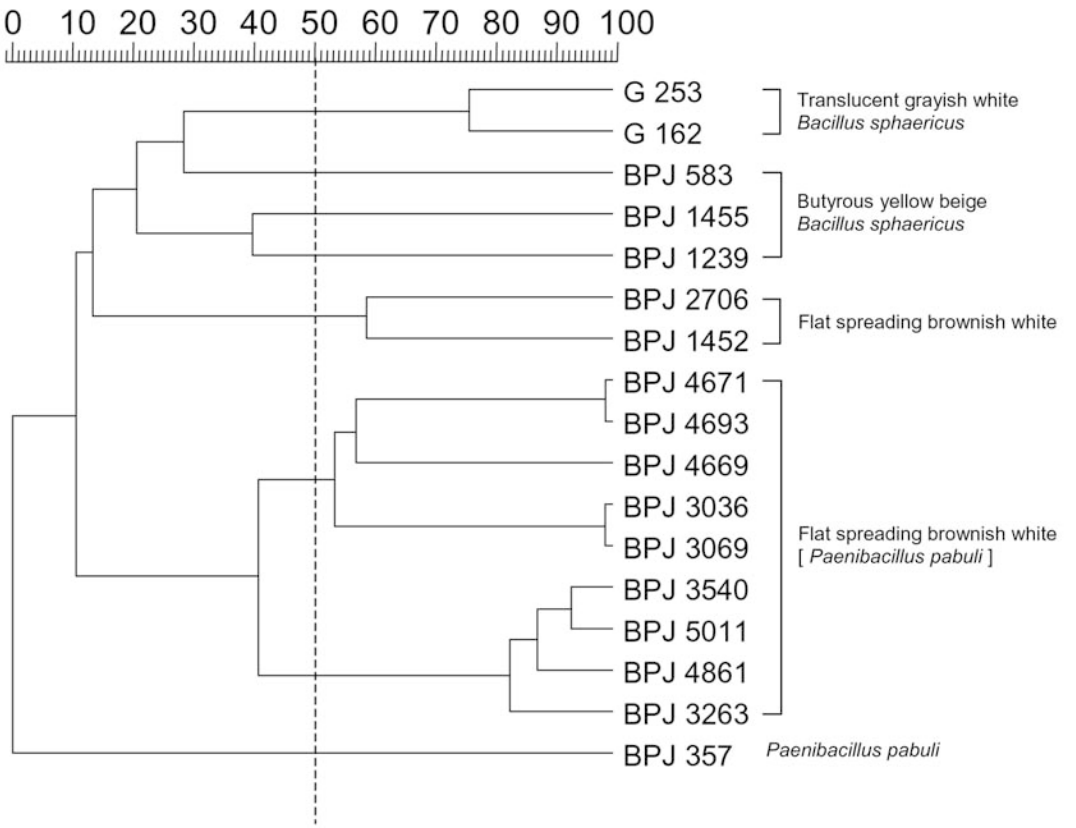
Bacillus spp.

Entries: 17

Correlation: Correlation - Fine

Zones: [5-394]

Clustering: UPGMA



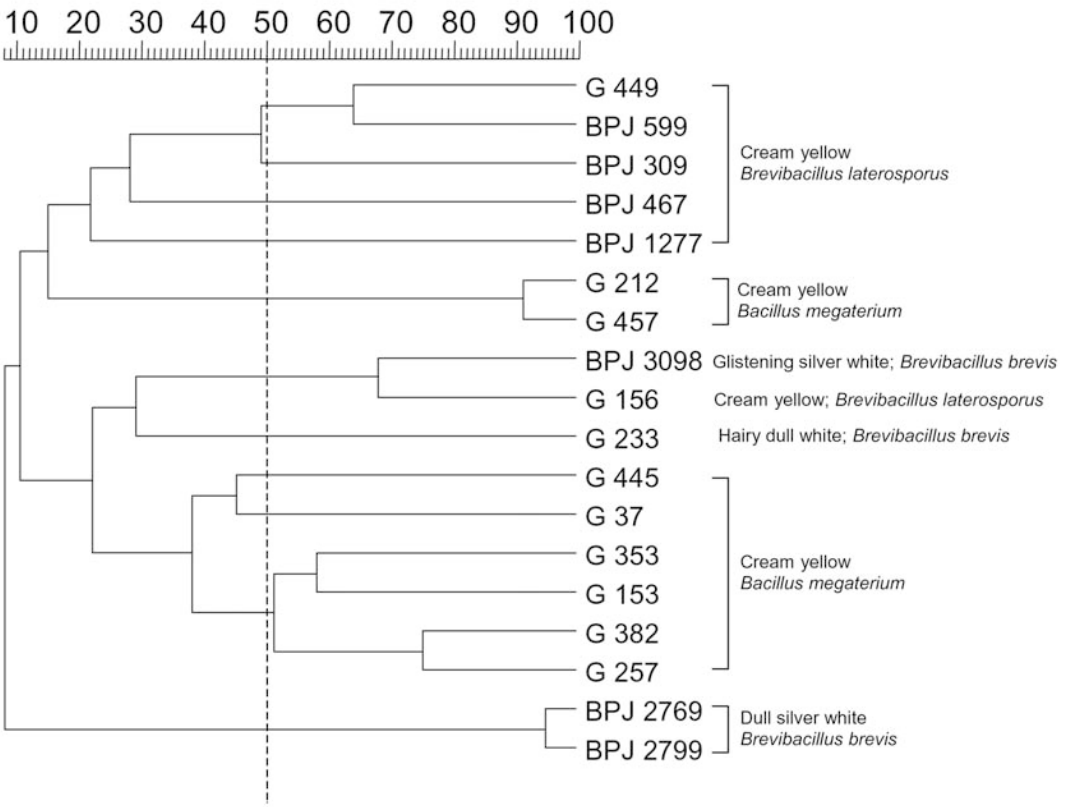
Bacillus spp.

Entries: 18

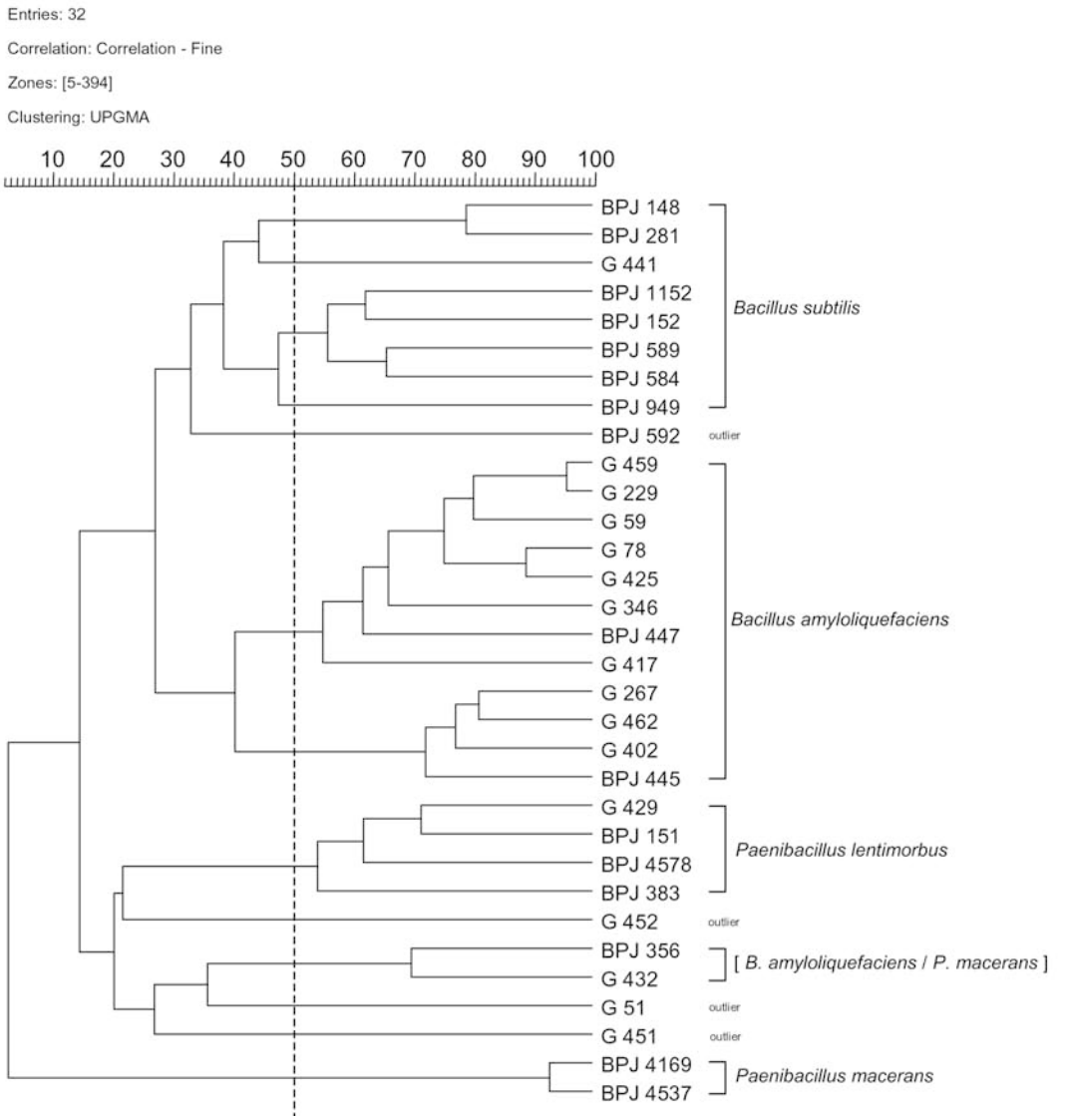
Correlation: Correlation - Fine

Zones: [5-394]

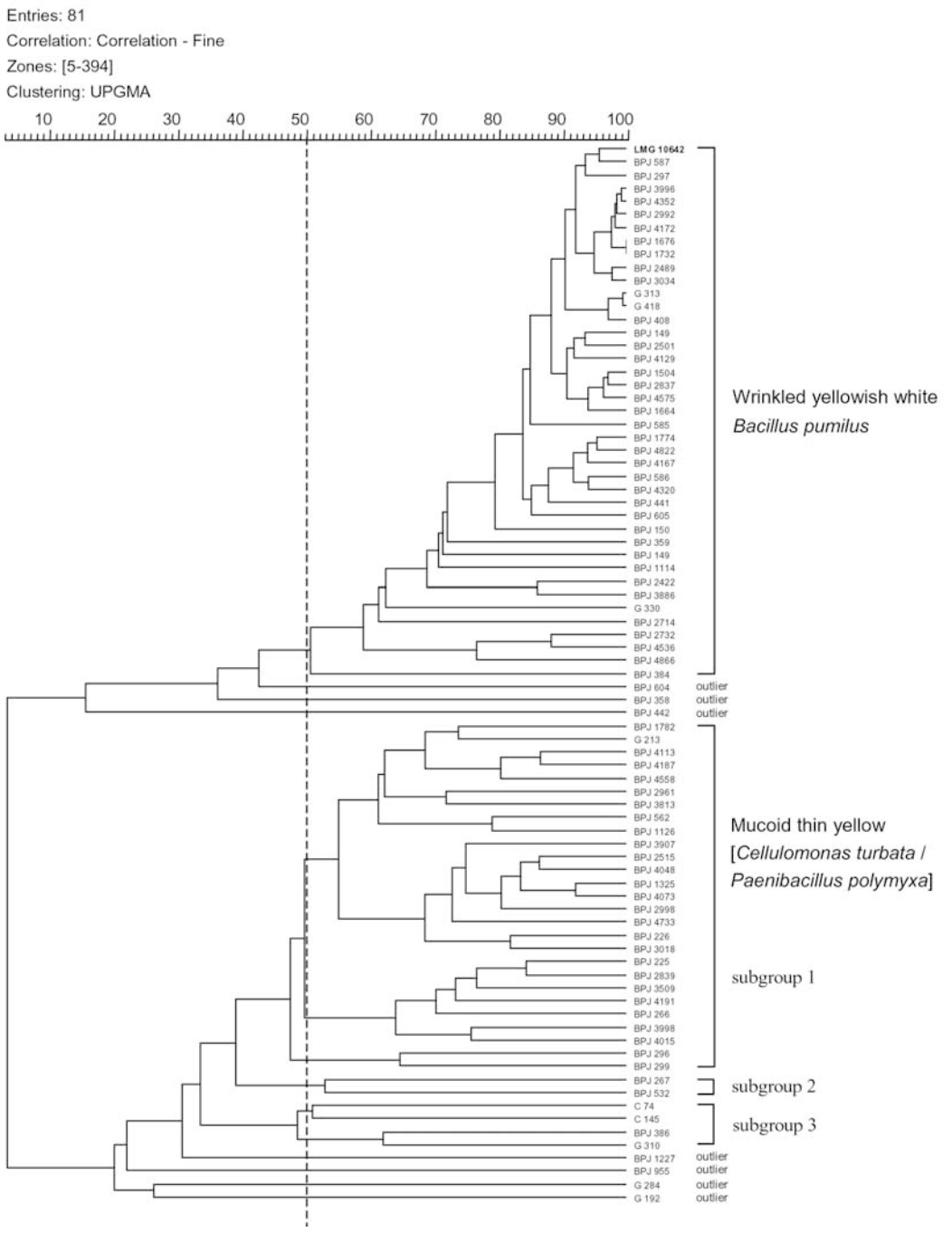
Clustering: UPGMA



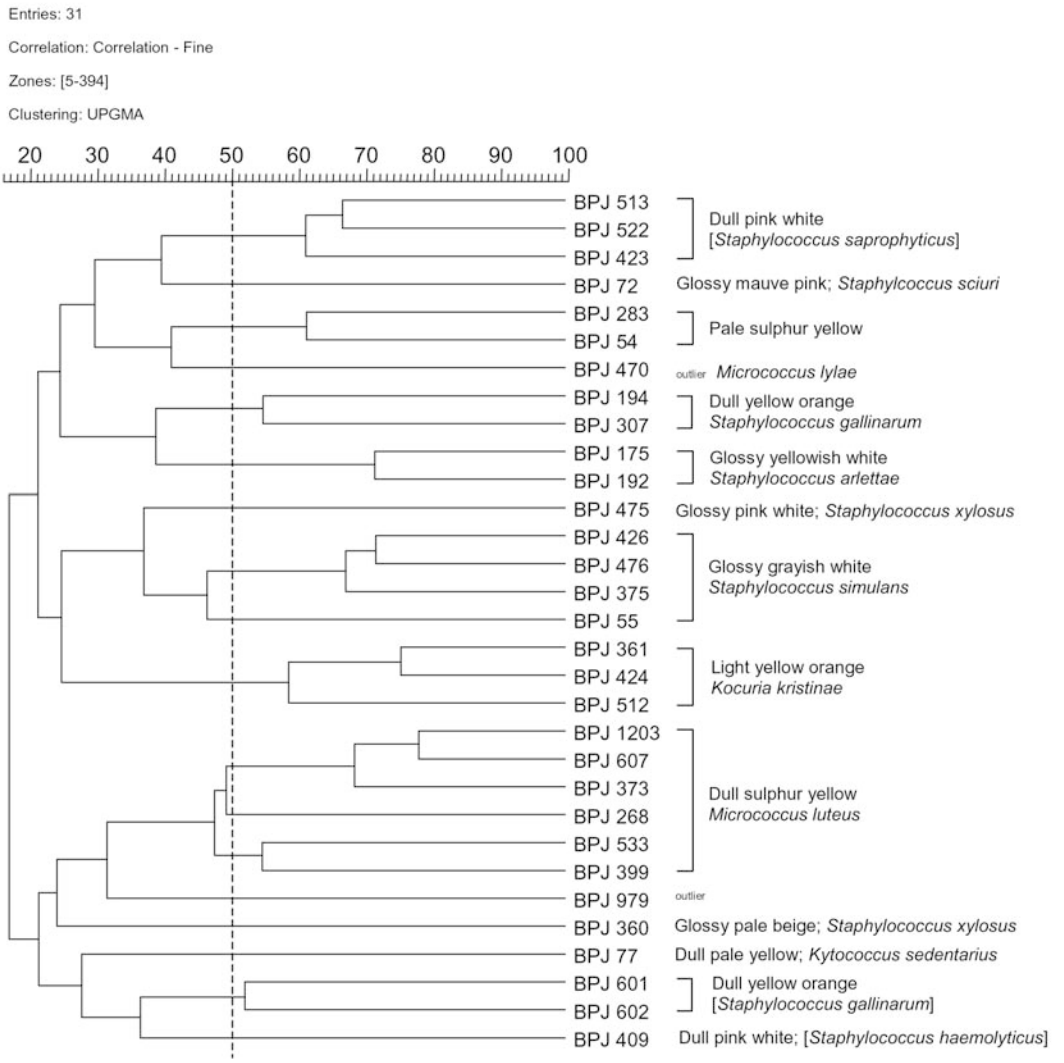
Bacillus subtilis-group
Rough (mucoid) brownish white



Bacillus spp. with low disease potential



Gram-positive cocci



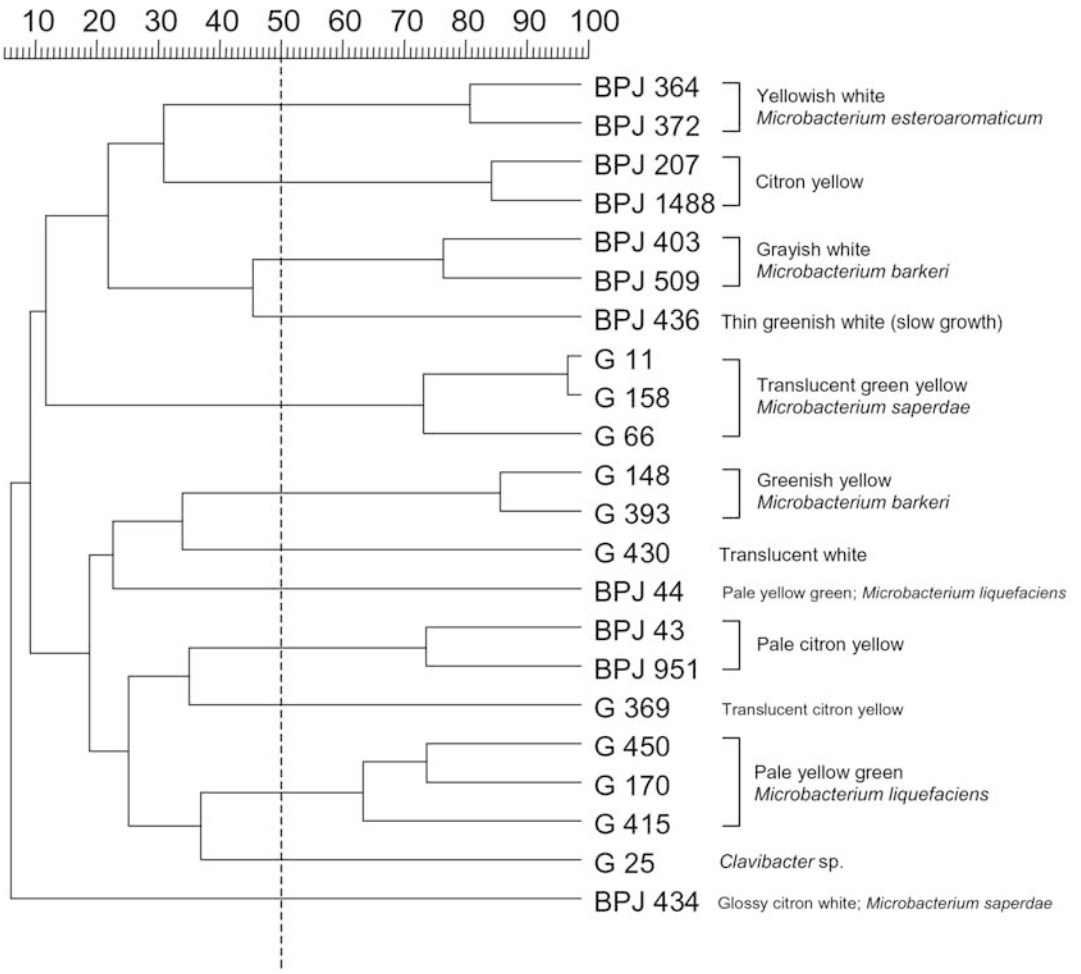
Coryneform bacteria
Shades of white and green

Entries: 22

Correlation: Correlation - Fine

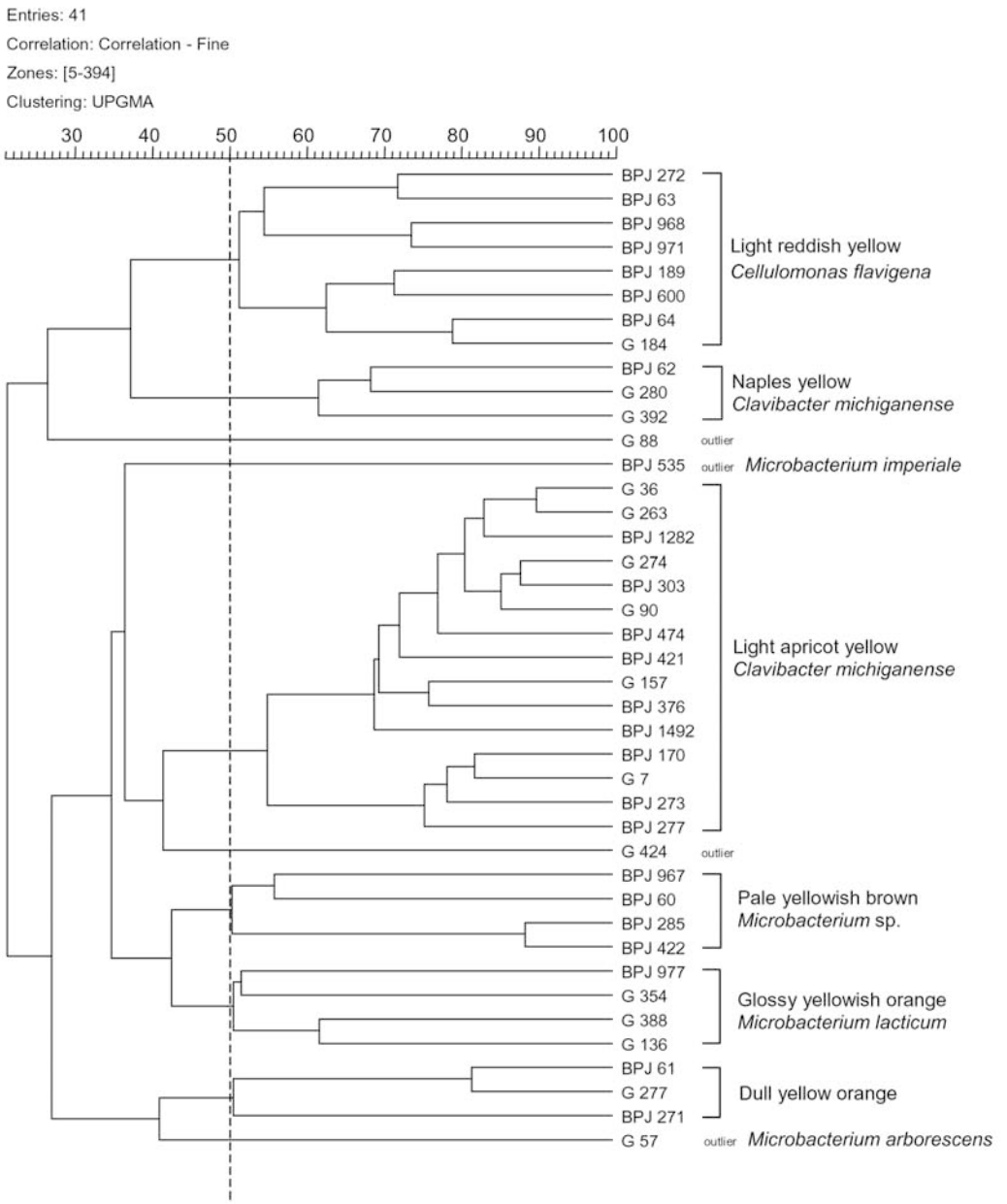
Zones: [5-394]

Clustering: UPGMA



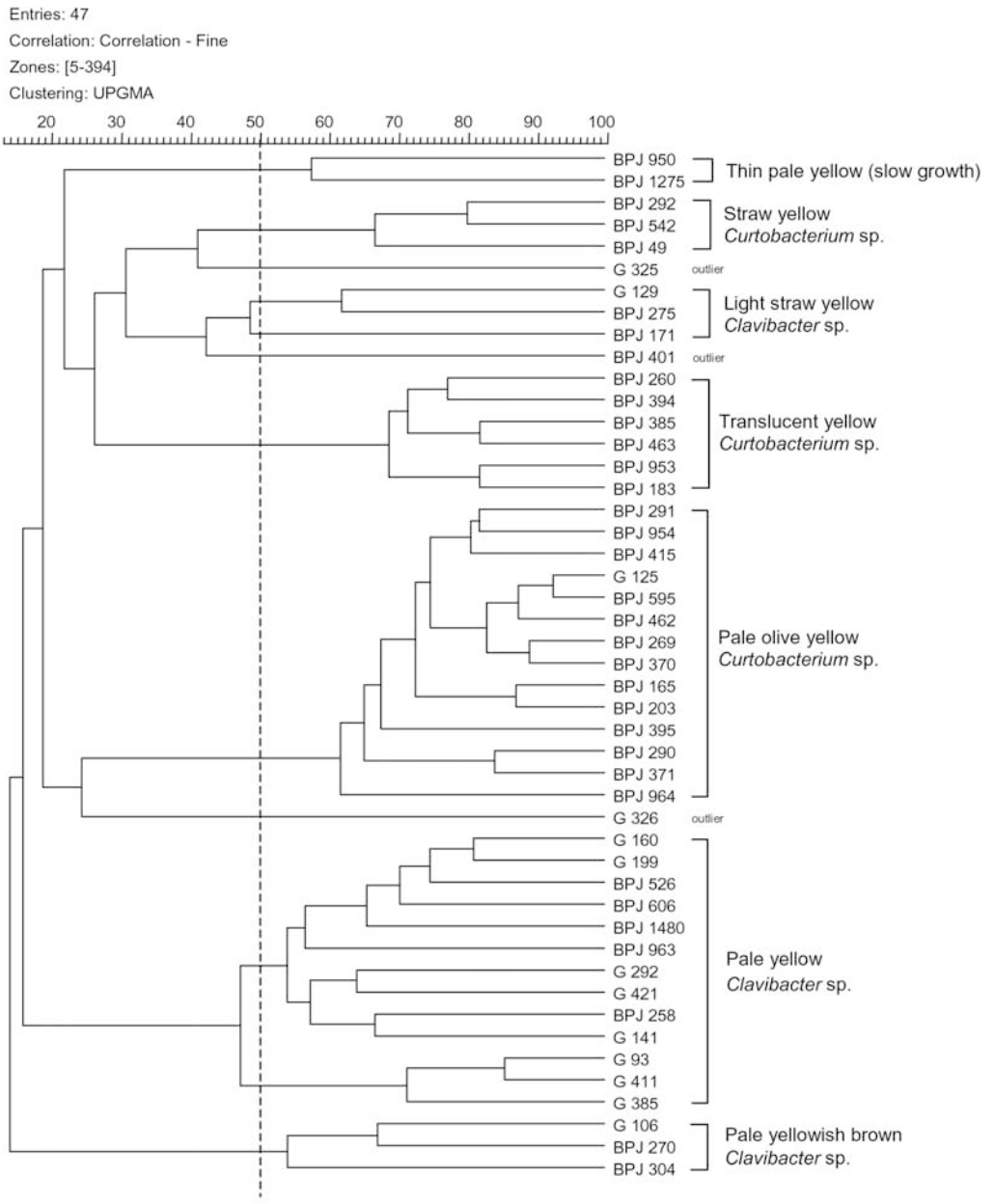
Coryneform bacteria

Shades of reddish yellow



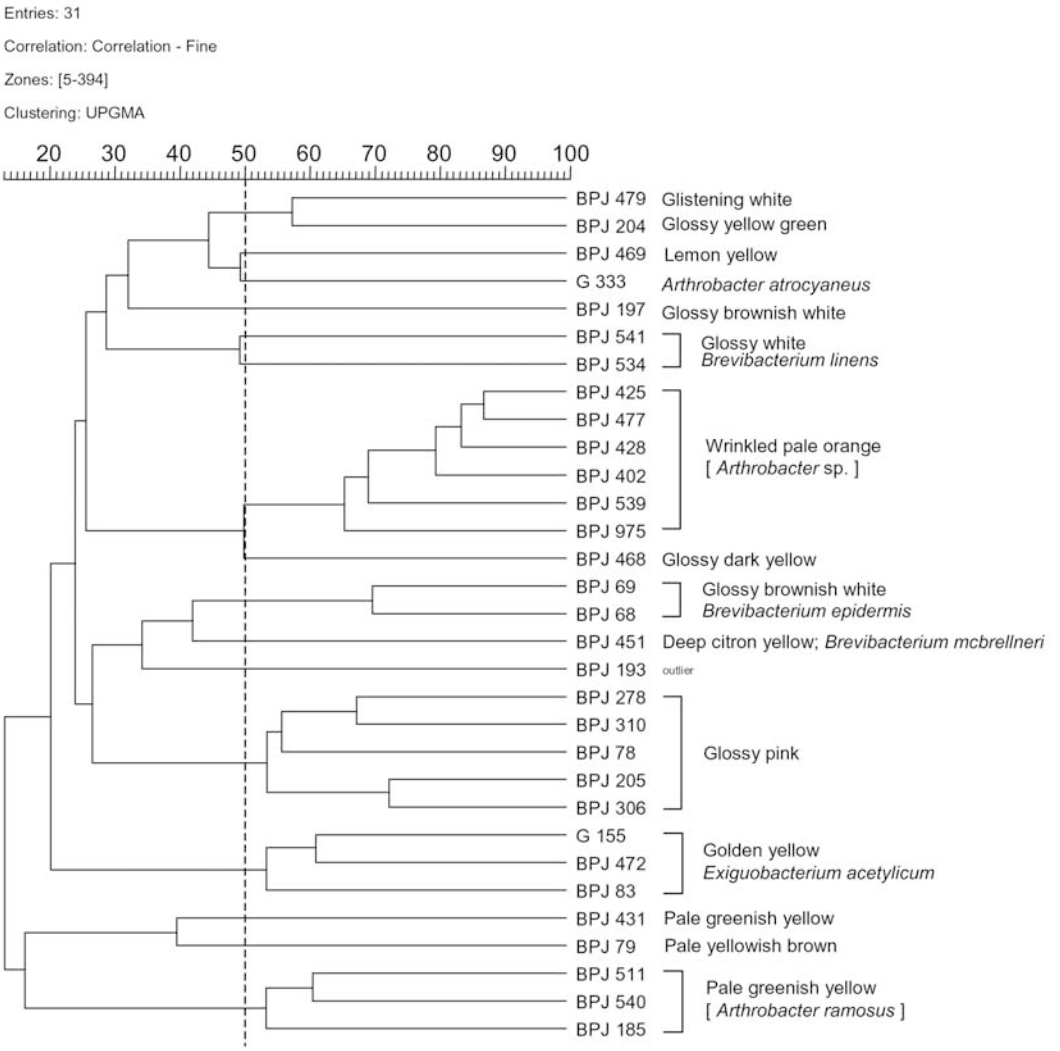
Coryneform bacteria

Shades of translucent yellow

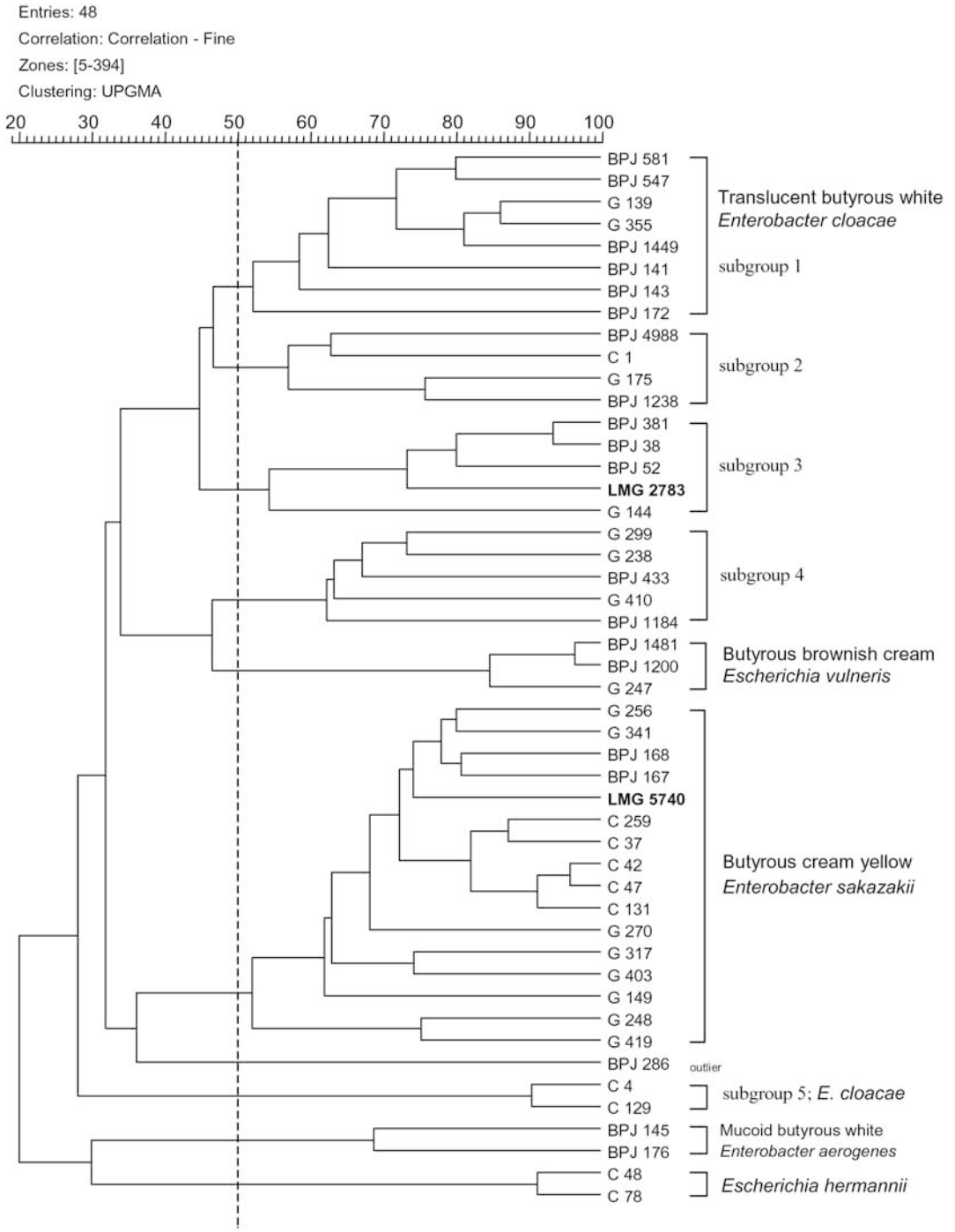


Coryneform bacteria

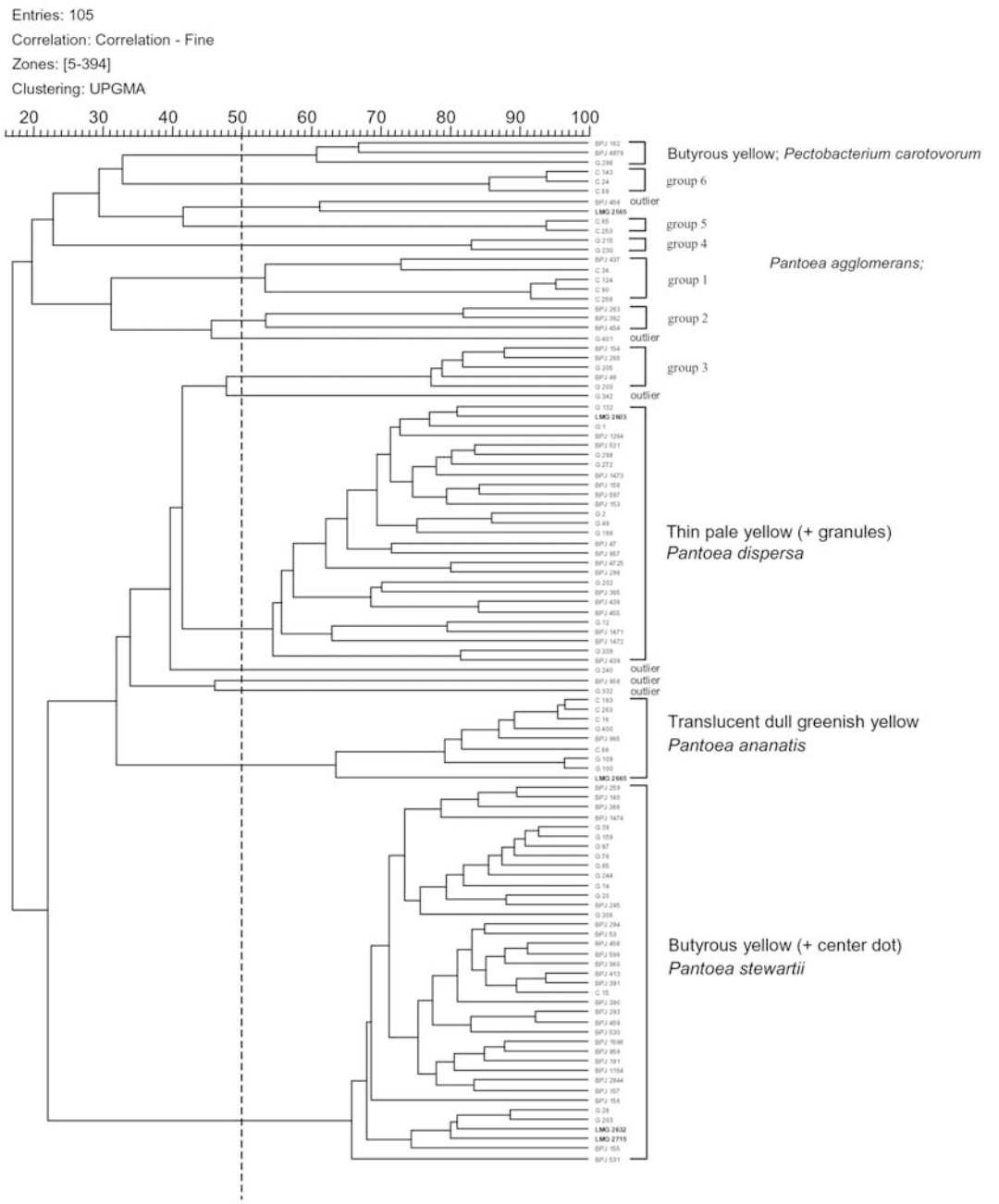
Rod-coccus cycle



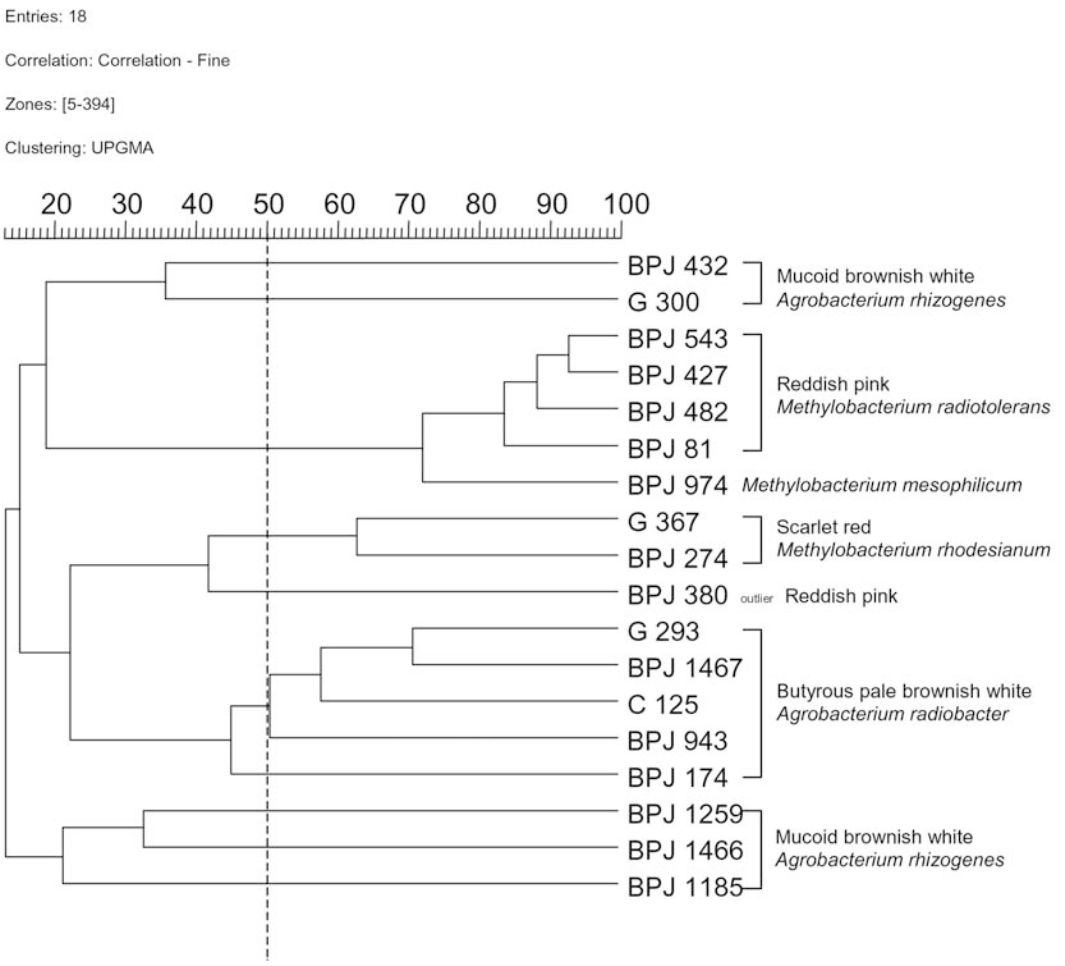
Enterobacter and *Escherichia* spp.

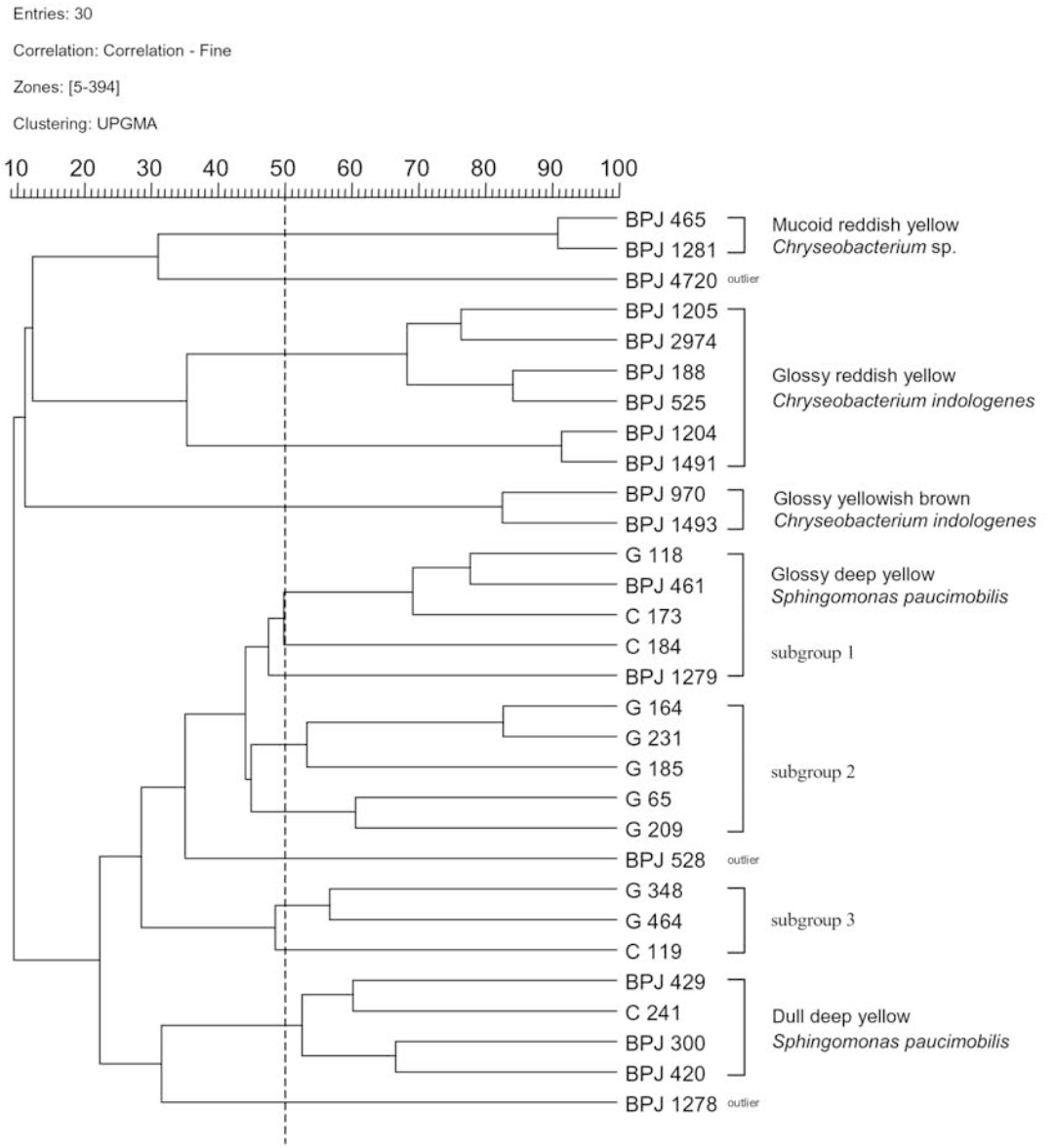


Pantoea and *Pectobacterium* spp.



Agrobacterium and *Methylobacterium* spp.




Entries: 30
Correlation: Correlation - Fine
Zones: [5-394]
Clustering: UPGMA
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BPJ 1281
BPJ 4720 outlier
BPJ 1205
BPJ 2974
BPJ 188
BPJ 525
BPJ 1204
BPJ 1491
BPJ 970
BPJ 1493
G 118
BPJ 461
C 173
C 184
BPJ 1279
G 164
G 231
G 185
G 65
G 209
BPJ 528 outlier
G 348
G 464
C 119
BPJ 429
C 241
BPJ 300
BPJ 420
BPJ 1278 outlier
Mucoid reddish yellow
Chryseobacterium sp.
Glossy reddish yellow
Chryseobacterium indologenes
Glossy yellowish brown
Chryseobacterium indologenes
Glossy deep yellow
Sphingomonas paucimobilis
subgroup 1
subgroup 2
subgroup 3
Dull deep yellow
Sphingomonas paucimobilis

Acidovorax avenae

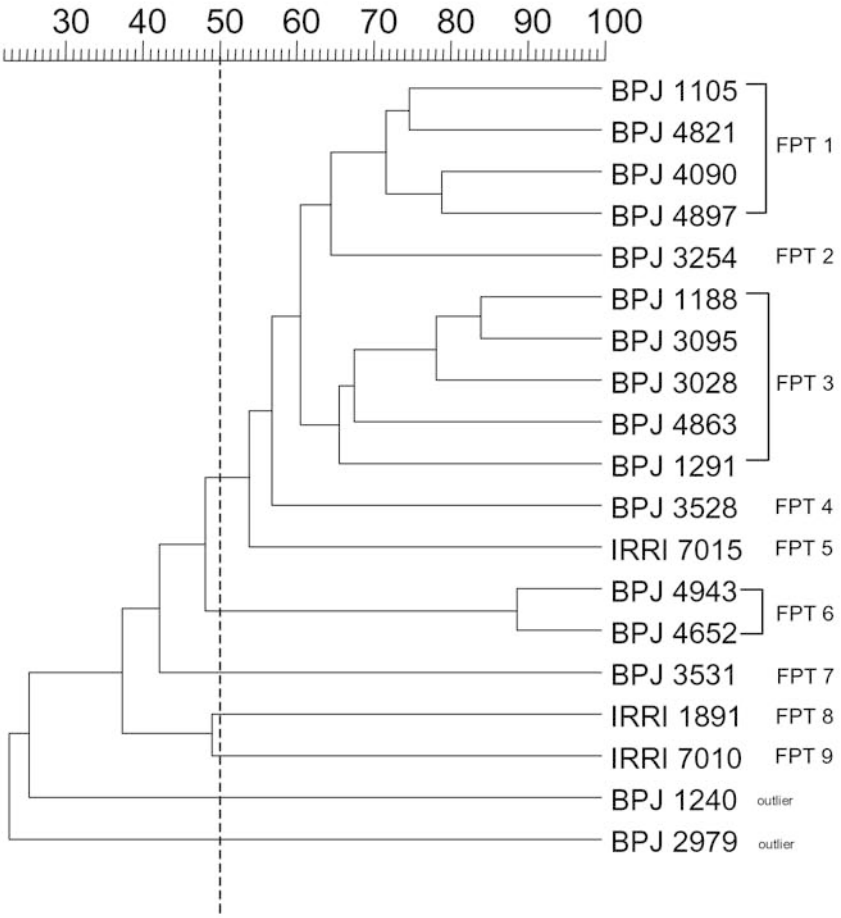
Glistening yellowish gray

Entries: 19

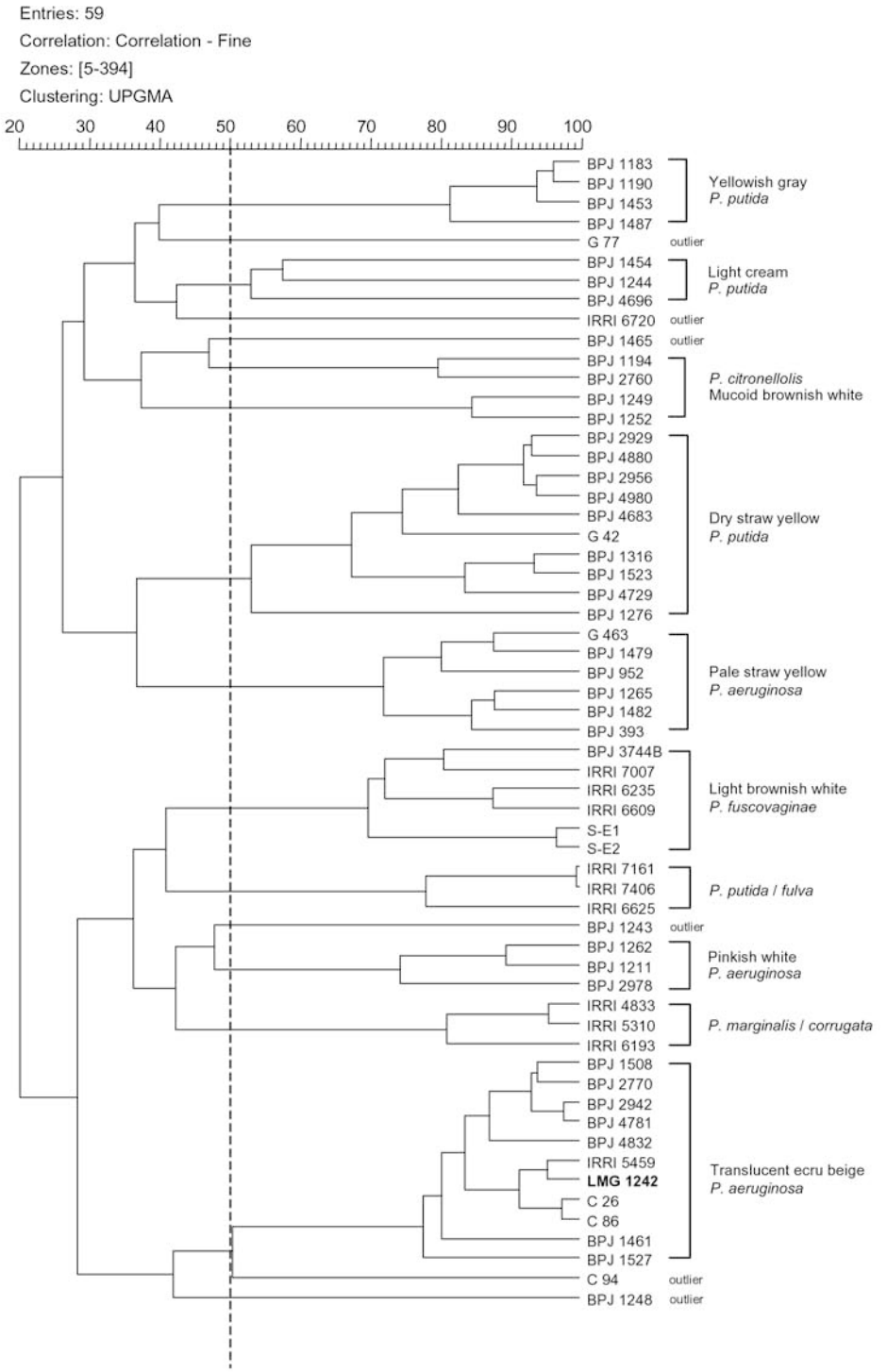
Correlation: Correlation - Fine

Zones: [5-394]

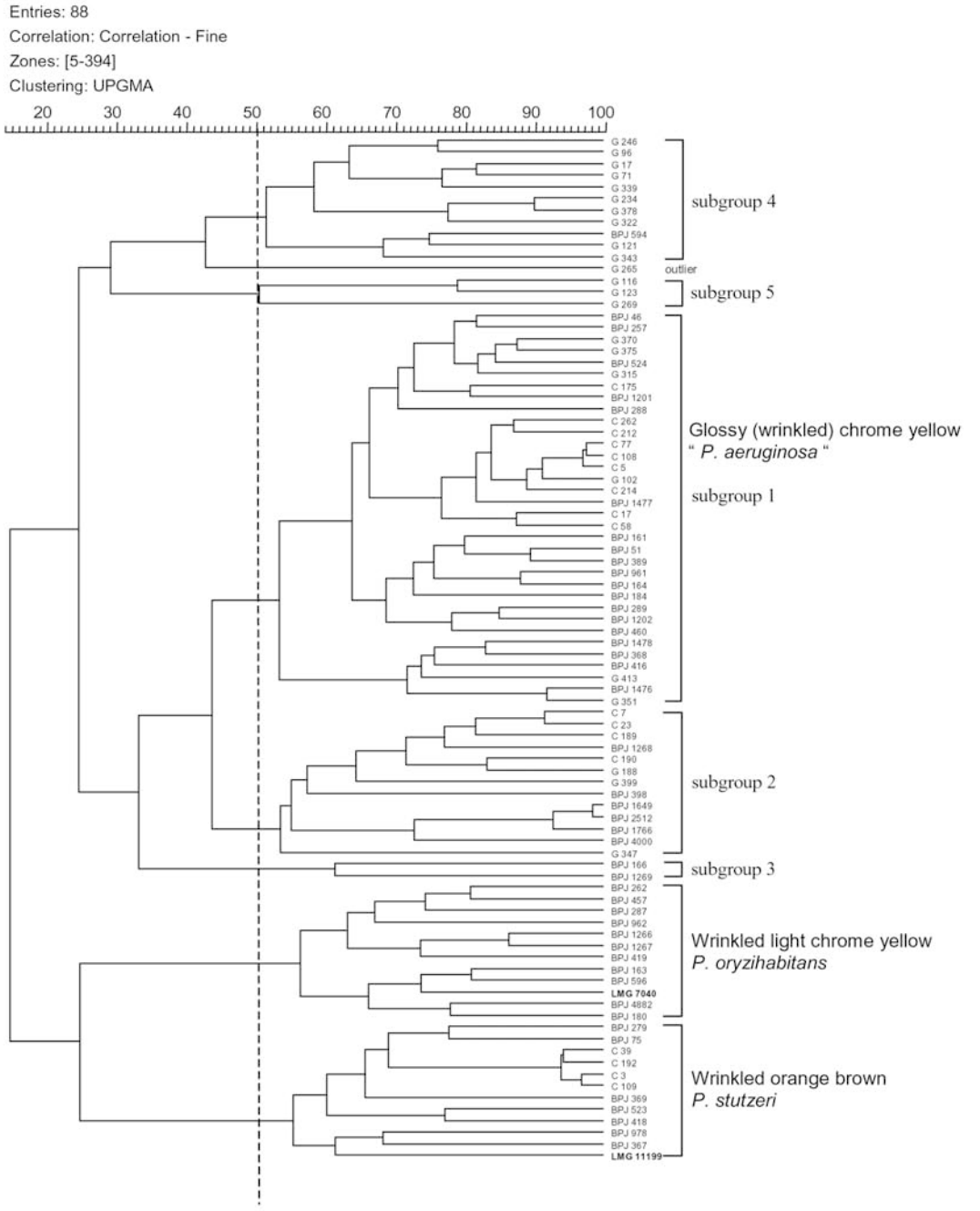
Clustering: UPGMA



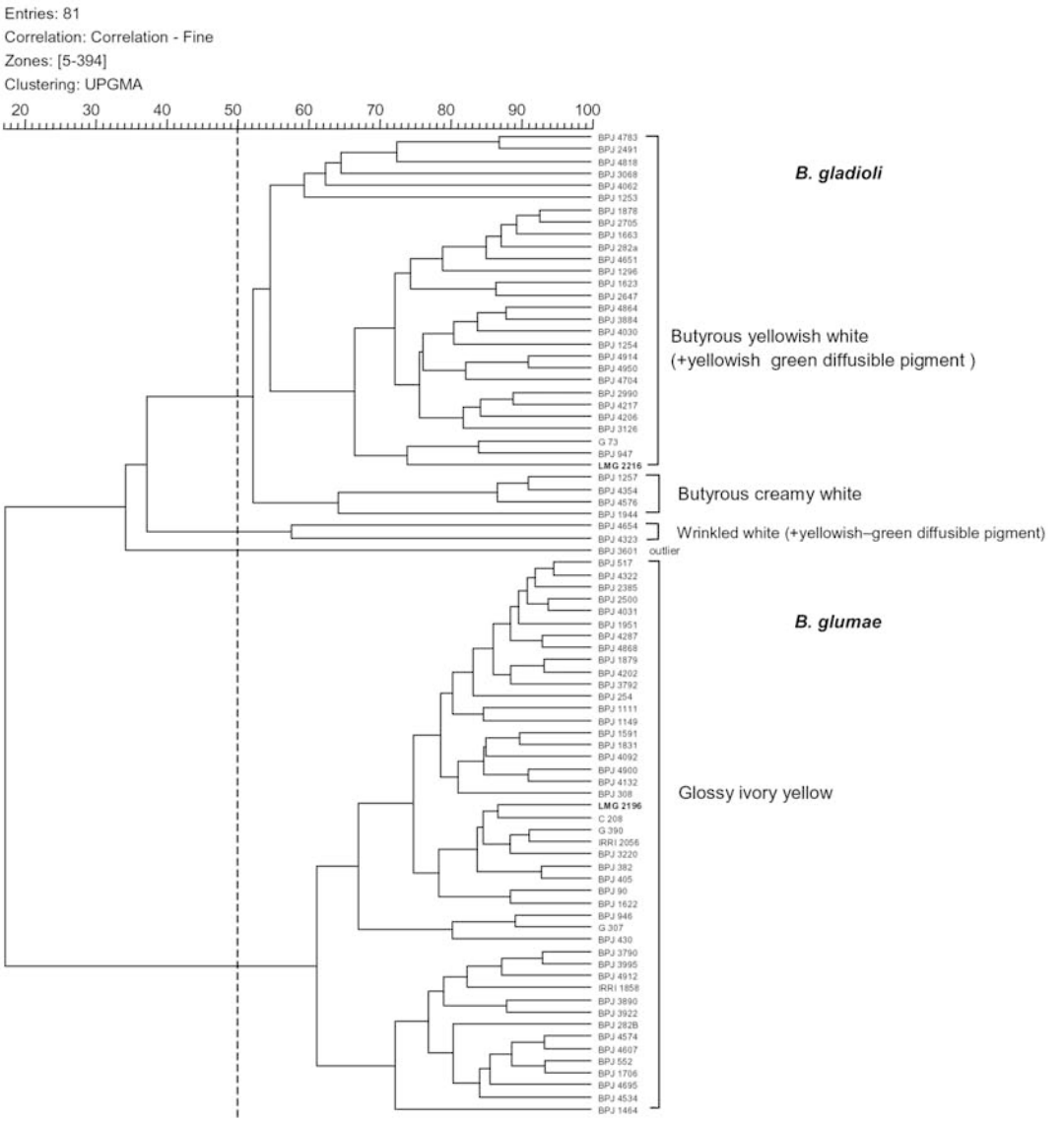
Fluorescent *Pseudomonas* spp.



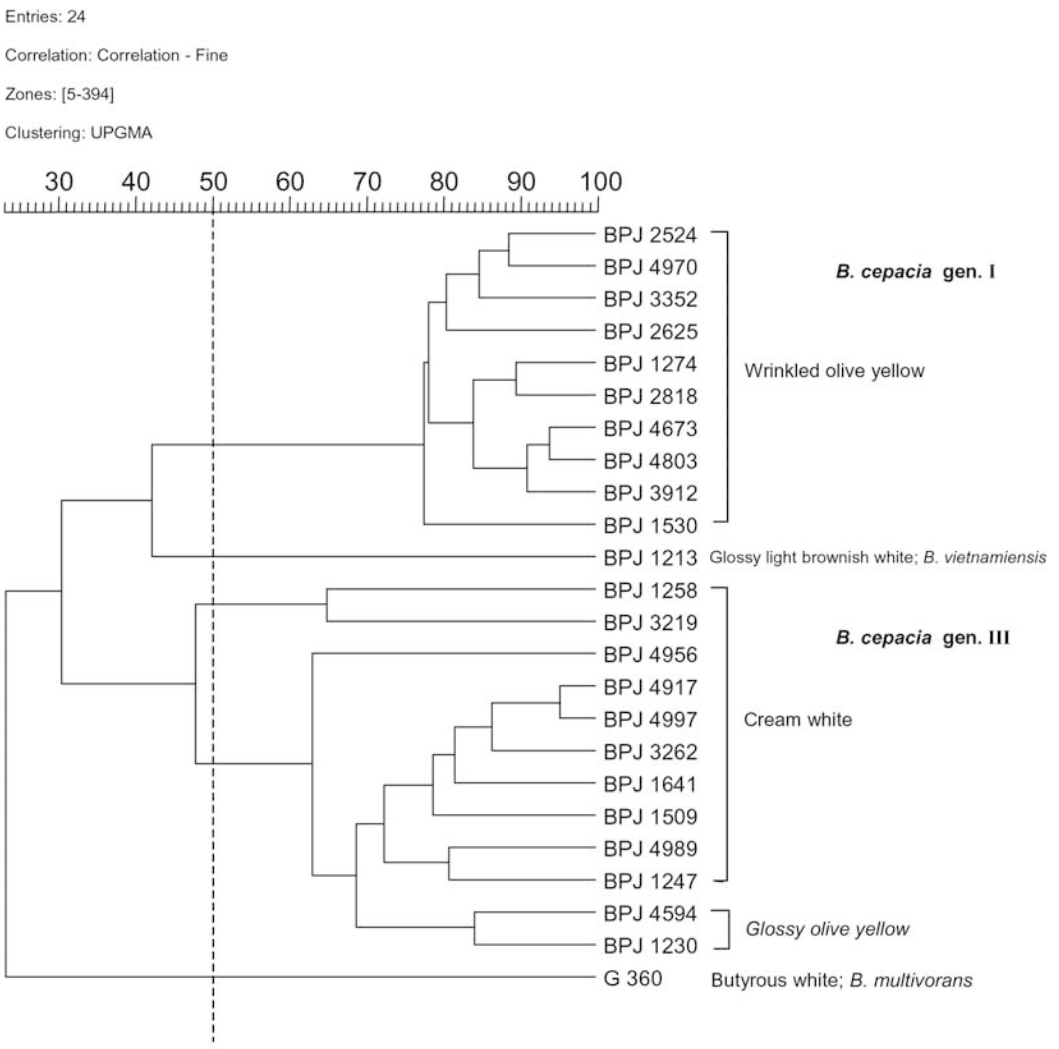
Nonfluorescent *Pseudomonas* spp.



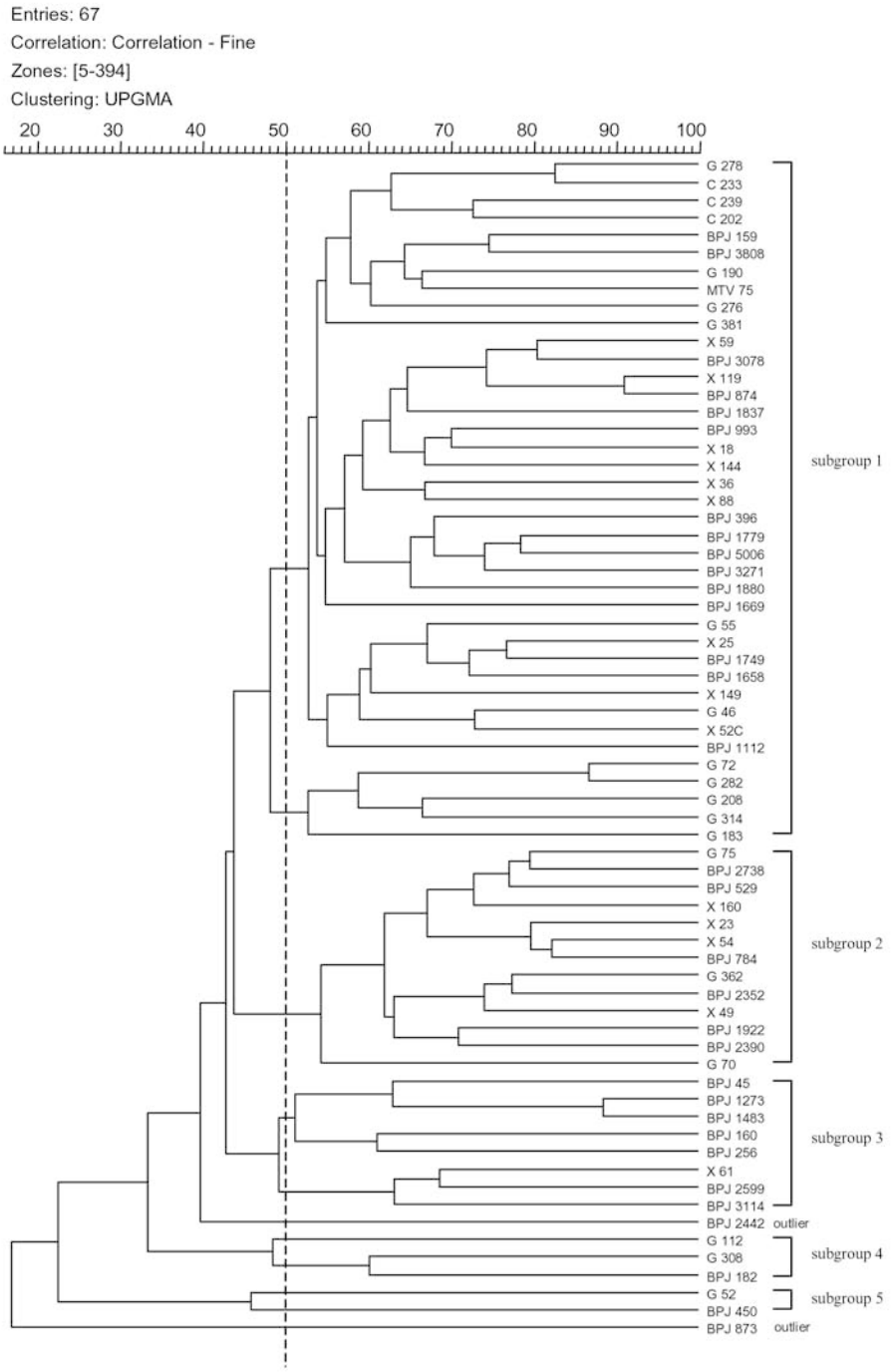
Burkholderia glumae / gladioli



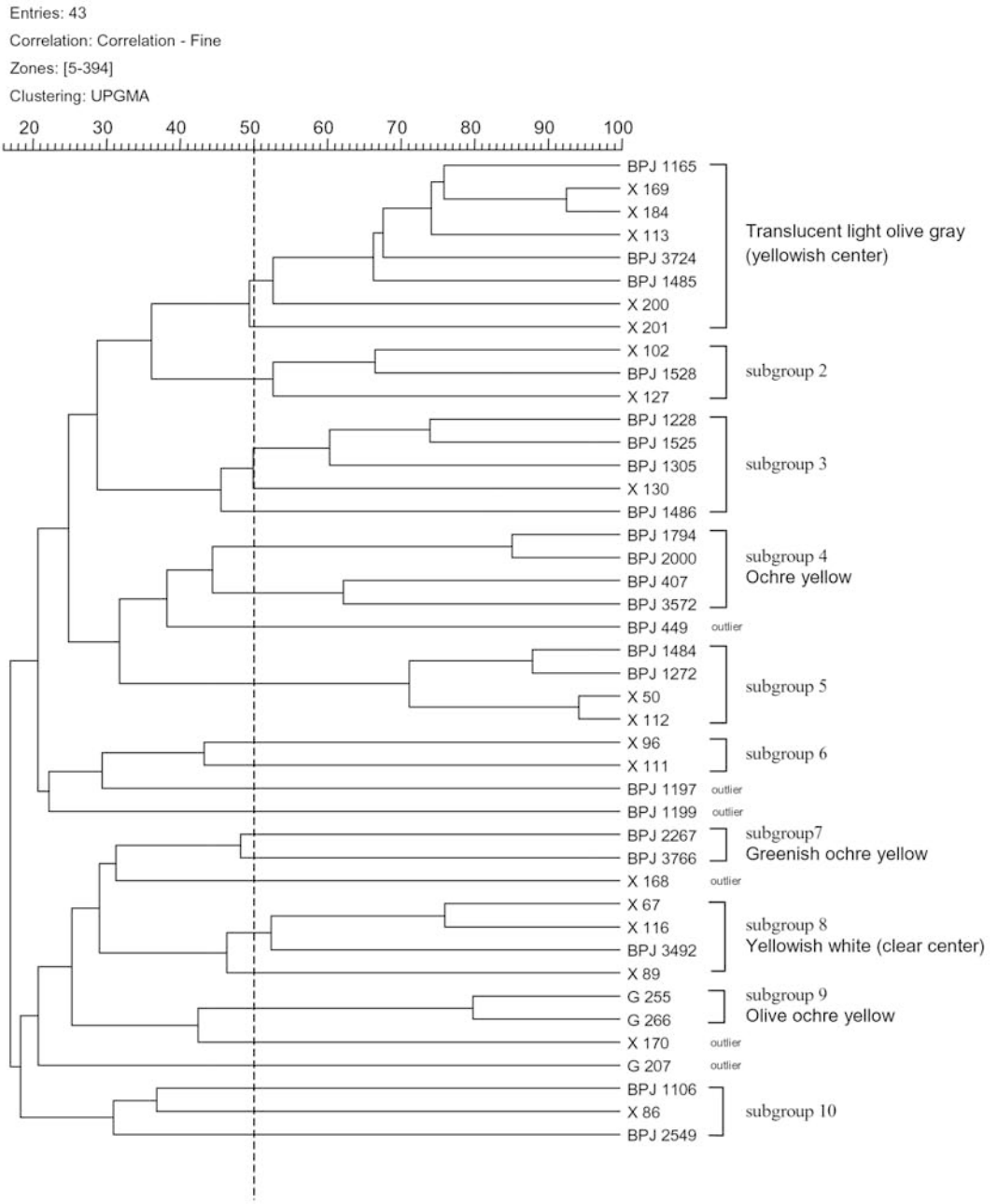
Burkholderia cepacia



Xanthomonas spp.
Mucoid pale yellow



Stenotrophomonas maltophilia



Appendix 4

PROJECT SUMMARY

The collaborative project "Managing rice diseases through seed health and rice seed associated antagonistic bacteria – a key component of Integrated Pest Management" was supported by grants from the Directorate General for International Cooperation (DGIC), Belgium.

The collaborative network established by the project between the International Rice Research Institute and the University Gent, in partnerships with collaborators from the national agricultural research and extension systems (NARES) in Southeast Asia, provided important links through which knowledge could be shared and technology disseminated directly to farmers via participatory field experiments.

Rationale

Seed quality is an important factor in attaining the genetic yield potential of modern rice cultivars. In most rice growing regions in Asia, only a small percentage of the total seed requirement is met through certified seeds. Farmers commonly use homegrown seeds for planting, or seeds exchanged with neighbors. Such seeds often are contaminated with insects, soil particles, weed seeds or pathogenic microorganisms, which not only can lead to poor yields but also increases the reliance on potentially dangerous and expensive pesticides and fungicides. Upholding seed health for improved planting material and utilization of biocontrol agents for disease management are environment friendly approaches that contribute to a reduction in pesticide use, while increasing rice yield and farmers' profit.

Research activities

The overall objective was to improve crop management practices by increasing the efficiency of input use in rice production, with emphasis on the seed. The research activities have focused on three key areas aimed at developing seed-based strategies for improved disease management:

1. Development and farmer adoption of improved seed management practices for rice crop production.
2. Characterization of seed-inhabiting fungi and bacteria in order to acquire basic knowledge on the structure of seed microbial communities, which should lead to a more rational exploitation of indigenous beneficial microorganisms and formulation of biological seed treatments for disease management.
3. Evaluation of rice-associated microbial antagonists as biocontrol agents for controlling rice sheath blight disease in farmers' fields.

Outputs and impact

1. Socioeconomic surveys to assess farmers' practices of seed management were conducted in the Philippines, Thailand and Vietnam, and translated into best-practice guidelines for improving seed management at the farm level. Using the farmer participatory approach, a significant increase in rice yields by 7 to 20% was demonstrated for farmers who performed seed cleaning procedures compared to those that did not. Other advantages included less disease problems and a higher germination percentage resulting in better (uniform) stand establishment, which allowed a reduction in seeding rate for direct-seeding from 250 kg/ha to 150 kg/ha, this alone already saved the participatory farmers US$50-75 per ha.
2. A total of 53 fungal species and over a hundred bacterial types (likely different species) were characterized from rice seeds in the Philippines. The project has constructed a fungal and bacterial culture collection of rice seed isolates, as well as a database of BOX-PCR genomic fingerprint patterns for identification of rice seed bacteria. The obtained information will become useful for seed health strategies that aim at deploying biological seed treatments or promoting the effectiveness of indigenous microbial components beneficial to plant growth.
3. Field experiments, conducted during ten consecutive cropping seasons in Pathum Thani province (Thailand), demonstrated that periodic applications of antagonistic strains *Pseudomonas* 90-321 and *Bacillus subtilis* 90-562 in mixture with the fungicide benomyl effectively prevented disease spread of sheath blight in the long run. The antagonistic strain *Bacillus subtilis* B-916, used in the long-term field experiments in Jiangsu province (China), proved to be an effective biocontrol agent not only against rice sheath blight but also against false smut. In the year 2000, *Bacillus subtilis* strain B-916 combined with a reduced dose of the fungicide Jingangmycin, has been commercialized by the Jiangsu Academy of Agricultural Sciences (JAAS) under the tradename "Wenquning" as a product for controlling rice sheath blight and false smut. By the year 2001, thousands of farms, covering almost 70,000 ha in Jiangsu -one of China's most important rice growing provinces- used Wenquning to protect their crops from sheath blight disease.

The impact of this technology is that it not only effectively controls rice diseases in a way that is environmentally more friendly, but also saves farmers money by reducing the necessity for expensive chemicals.

As 13% of the world's total harvested crop area is planted to rice, any success in helping rice farmers reduce the inappropriate use of pesticides promises to have a major impact on the environment, especially in Asia.

Appendix 5

CURRICULUM VITAE

The author was born and raised in the Belgian City of Bruges, often called "the Venice of the north". He is the third child in the family, and has one brother and three sisters. During his childhood he took a lively interest in exploring nature and has always been fond of travelling and camping. He has been a member of the youth organizations Chiro and KSA, and later joined camping activities of the Scout Association as quartermaster. At high school, he was a fervent member of the judo and rowing team, and earned one national and some regional medals.

He completed his secondary education, classical side (Latin-Sciences), at Sint Lodewijkscollege in Bruges. He obtained the degree of Licentiaat Biology, major in Botany with specialization in plant molecular genetics from the University Gent (UG), Belgium, in 1986.

After graduation, he fulfilled his military service with the Medical Forces of the Belgian Army in 1987.

He has a diverse working experience from taking on summer jobs as a waiter on the Belgian coast in Knokke and at the Spermalie Hotel School in Bruges, to handling quarters of cows as a laborer at Coldstar N.V., Kortrijk. He took a job with Philips Brugge as an assistant warehouse-clerk in electrical components from October 1983 to May 1984. In 1987, he also took on a one-month stint as a seasonal laborer in the banana plantations on Krete, Greece.

From 1988 –1990, he worked as a Research Assistant in the Laboratory of Genetics (Prof. M. van Montagu) at the University Gent, Belgium.

From September 1990 - December 1992, he worked as a Collaborative Research Fellow in the Plant Pathology Division at the International Rice Research Institute (IRRI), Los Baños, Philippines, under Year 3 & Year 4 of the ABOS (Belgium)-supported IRRI-UG collaborative project "Association of pathogenic bacteria with rice seed".

In 1993 (March – September), he took on an assignment for the duplication of an *Arabidopsis thaliana* genomic library at the Laboratory of Dr. H. M. Goodman, Department of Molecular Biology, Massachusetts General Hospital, Harvard Medical School, Boston, USA.

In 1994 he was hired as a Consultant in the Entomology and Plant Pathology Division (Dr. T. W. Mew, Head), IRRI.

From 1995 – 1997, he worked as a Project Scientist in the Entomology and Plant Pathology Division at IRRI under the DGIC (Belgium)-supported project "Managing rice diseases through seed health and rice-associated antagonistic bacteria – a key component of IPM" in collaboration with the Laboratory of Microbiology (Prof. dr. ir. J. Swings) at University Gent, Belgium.

From 1998 – 2001, he was given an appointment as an Affiliate Scientist in the Entomology and Plant Pathology Division at IRRI to continue the work under the same IRRI-UG collaborative project. During 2002, he extended his stay at IRRI to work on his doctoral dissertation.

Scientific Publications

Peleman, J., B. Cottyn, W. Van Camp, M. van Montagu, D. Inze (1991): Transient occurrence of extrachromosomal DNA of an *Arabidopsis thaliana* transposon-like element, *Tat1*. Proc. Natl. Acad. Sci. USA 88, 3618-3622.

Cottyn, B., A. T. Bautista, R. J. Nelson, J. Swings, T. W. Mew (1994): Polymerase chain reaction amplification of DNA from bacterial pathogens of rice using specific oligonucleotide primers. Int. Rice Res. Newsl. 19, 30-32.

Cottyn, B., M. T. Cerez, M. F. Van Outryve, J. Barroga, J. Swings, T. W. Mew (1996): Bacterial diseases of rice. I. Pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80, 429-437.

Cottyn, B., M. F. Van Outryve, M. T. Cerez, M. De Cleene, J. Swings, T. W. Mew (1996): Bacterial diseases of rice. II. Characterization of pathogenic bacteria associated with sheath rot complex and grain discoloration of rice in the Philippines. Plant Dis. 80, 438-445.

Cottyn, B., E. Regalado, B. Lanoot, M. De Cleene, T. W. Mew, J. Swings (2001): Bacterial populations associated with rice seed in the tropical environment. Phytopathology 91, 282-292.

Cottyn, B., H. Barrios, T. George, C. M. Vera Cruz (2002): Incidence of sheath rot and grain discoloration in Siniloan, Philippines. Int. Rice Res. Newsl. 27, 39-40.

Technical Publications and Conference Reports

Cottyn, B., M. T. Cerez, T. W. Mew (1994): Bacteria and Bacterial Pathogens, Chapters 7 & 15. In *A Manual of Rice Seed Health Testing*; Mew, T.W., Misra, J. K., eds.; International Rice Research Institute, Los Baños, Philippines, 113 pp.

Mew, T. W., and B. Cottyn, eds. (2001): Seed Health and Seed-Associated Microorganisms for Rice Disease Management, Limited Proceedings No. 6. International Rice Research Institute, Philippines.

Cottyn, B., and T. W. Mew (2002): Bacterial Blight of Rice. In *Encyclopedia of Plant & Crop Science*; Goodman, R. M., ed.; Marcel Dekker, Inc.: New York, *in press*.

Presentations

"PCR-based diagnosis of *Xanthomonas oryzae* pv. *oryzae* in rice seed" Poster presentation, Federation of Crop Science Societies of the Philippines (FCSSP), Annual Conference, Davao City, Philippines, May 20-24, 1996.

"Speciation and identification in bacterial systematics – polyphasic taxonomy" Oral presentation, Nanjing Agricultural University, Nanjing, Jiangsu, China, September 12, 1997.

"Bacterial communities associated with rice seed" Oral presentation, Exploiting Biodiversity for Sustainable Pest Management, ADB RETA 5711 Review Workshop; Multiple Cropping Center, Chiang Mai University, Chiang Mai, Thailand, October 14, 1998.

"Bacterial diversity in rice seed" Poster presentation, International Rice Congress, Beijing, China, September 16-20, 2002.

Lecturing and training

Training course on microbiological techniques, Department of Agriculture, Bangkhen, Bangkok, Thailand, June 28- July 7, 1997.

Issues of identification and classification of microbial biocontrol agents, Biodiversity Training Course, ADB RETA 5711, International Rice Research Institute, Los Baños, Philippines, March 24 – April 3, 1998.

Bacterial isolation, purification, and characterization, Rice Seed Health for Crop Management Course, International Rice Research Institute, Los Baños, Philippines, Yearly in July - September.