A molecular analysis of species boundaries and relationships in *Pythium*

by

Andrew Michael Schurko

A thesis submitted to the Faculty of Graduate Studies in partial fulfillment of the requirement for the degree Doctor of Philosophy

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A MOLECULAR ANALYSIS OF SPECIES BOUNDARIES AND RELATIONSHIPS IN *PYTHIUM*

BY

ANDREW MICHAEL SCHURKO

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

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ABSTRACT

Restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA (rDNA) intergenic spacer (IGS) was used to resolve relationships among over 360 isolates representing 90 *Pythium* species. Multiple isolates of the same species usually exhibited minimal intraspecific variation in their restriction fragment profiles, while a high degree of interspecific variation was usually observed when comparing fragment patterns between different species. In some cases though, different isolates of the same species had very different fragment profiles. This may be indicative of genetic variation within a species, or of the existence of cryptic speciation. However, some groups of morphologically similar species shared similar restriction fragment profiles. For such groups, RFLP analysis revealed that isolates representing different species often formed monophyletic clusters. The clustering of these species was usually supported by a high degree of morphological similarity among the species. This suggested that certain species may in fact be conspecific. These clusters included as few as two conspecific species (e.g. *P. aristosporum* and *P. arrhenomanes*) to as many as four (e.g. *P. dissotocum, P. diclinum, P. lutarium,* and *P. tumidum*). In total, 14 clusters of conspecific species, comprised of 34 total species, were revealed using RFLP analysis of the IGS. These results suggested that the morphological species concept alone may not be sufficient to delimit species in *Pythium* and that the application of a phylogenetic species concept is crucial for resolving species boundaries in this genus.

Pythium insidiosum, the only species in the genus capable of infecting animals, is the etiological agent of pythiosis, a disease characterized by cutaneous and subcutaneous lesions and some vascular diseases. RFLP analysis of the IGS for 28 isolates of *P.*

insidiosum from a variety of animal hosts and geographic origins revealed the existence of three genetic clusters which exhibited a high degree of geographic isolation. These results were strengthened by a sequence analysis using rDNA internal transcribed spacer (ITS). This phylogenetic analysis produced similar results to those obtained from RFLP analysis, and also revealed the relationships of *P. insidiosum* isolates relative to other *Pythium* species and isolates of *Lagenidium giganteum* and *Phytophthora megasperma.* The isolates of *P. insidiosum* were more closely related to each other than to any other *Pythium* species or genus, but the three genetic clades of isolates were evident. Clade I consisted of isolates from North and South America, while clade II contained isolates from Asia and Australia. Clade III was comprised of isolates from Thailand and the United States. The results from RFLP analysis and ITS sequencing therefore provided evidence for geographic variants within *P. insidiosum,* or for cryptic speciation in this species.

Accurate diagnosis of pythiosis and identification of its causal agent are often inconsistent with current diagnostic methods. The presence of other pathogenic organisms that produce symptoms similar to pythiosis in their hosts complicates matters further. A species-specific DNA probe was constructed using a 530 bp *Hin*fI fragment from the IGS of *P. insidiosum.* When the probe was incubated with dot blots of genomic DNA from 104 *Pythium* species, it hybridized only to the DNA of *P. insidiosum* and *P. destruens,* two species which are assumed to be conspecific. The probe also hybridized to DNA from all the isolates of *P. insidiosum* tested, regardless of their geographic origin or animal host. When tested against genomic DNA from other pathogenic organisms (*Aspergillus fumigatus, Basidiobolus ranarum, Conidiobolus coronatus, Lagenidium*

giganteum, Paracoccidioides brasiliensis, and *Prototheca wickerhamii*), no cross-hybridization was detected. The capacity of this probe to hybridize to genomic DNA of all the *P. insidiosum* isolates and not cross-react with DNA from other *Pythium* species or pathogens that causes similar symptoms in its host, makes it an additional and powerful tool for the diagnosis of pythiosis and has the potential for clinical and environmental diagnostic applications.

ACKNOWLEDGEMENTS

I would like to express gratitude to my supervisor, Dr. Glen Klassen, for his faith in my abilities, patience, guidance, and influence on me as a scientist and a person. The comfortable working environment and many opportunities provided, as well as his dedication to me and my work, are very much appreciated. I would like to thank my examining committee for their support and advice throughout this project. Thanks to Dr. Georg Hausner, for his assistance with phylogenetic work and words of encouragement, and to Drs. Deborah Court and Anita Brûlé-Babel for their guidance and helpful suggestions. Thank you also to Dr. Frank Martin for his involvement as the external examiner, and to Dr. Peter Maeba whose retirement preceded the completion of this thesis. I am also grateful to Dr. Arthur de Cock for providing DNA samples and much help with the morphological analysis, and to Drs. André Lévesque and Leonel Mendoza who made time in their busy schedules for me to visit their laboratories and complete parts of my project there.

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Catalyst" Narayansingh, George "The Clincher" Golding, and Justin Rivard. I also appreciate the good nature of Patrick Chong, who put up with my constant joking and pranks (usually without retaliation), Prashen Chelikani's "visits" to my lab and faith in my driving abilities, Tamiko Hisanaga's often unique outlook on life in general and many humorous times, Matt Young's frequent "welcome back" or "farewell" parties, Yongqiang "Paul" Chen's inquisitive nature and generosity, and Tammy Stuart's friendship and "Ode to *Pythium*".

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LIST OF ABBREVIATIONS

A	adenine
AFLP	amplified fragment length polymorphism
avg.	average
bp	base pair (s)
BSC	biological species concept
C	cytosine
CFA	culture filtrate antigen
CMA	cornmeal agar
CMV	cell mass vaccine
*cox*II	cytochrome oxidase II
CTAB	hexadecyltrimethyl ammonium bromide
d	day (s)
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
ddUTP	2',3'-dideoxyuridine-5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
diam.	diameter
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
dUTP	2'-deoxyuridine 5'-triphosphate
EDTA	ethylenediamine-tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
ETS	external transcribed spacer
Fig (s).	figure (s)
FISH	fluorescent in situ hybridization
g	gram (s)

G	guanine
h	hour (s)
ID	immunodiffusion
IGS	intergenic spacer
IPTG	isopropylthiogalactoside
ITS	internal transcribed spacer
kb	kilobase pair (s)
kDa	kiloDalton (s)
L	litre (s)
LB	Luria-Bertani
LSU	large subunit
M	Molar
min	minute (s)
mL	millilitre (s)
mm	millimetre (s)
mM	millimolar
MSC	morphological species concept
mtDNA	mitochondrial DNA
nm	nanometres
NOR	nucleolar organizer region
ng	nanograms
NTS	nontranscribed spacer
PCR	polymerase chain reaction
pmol	picomole
PSC	phylogenetic species concept
RAPD	random amplified polymorphic DNA
rDNA	ribosomal DNA
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease

rpm	revolutions per minute
rRNA	ribosomal RNA
s	second (s)
S	Svedberg unit
SDS	sodium dodecyl sulphate
sp.	species (singular)
spp.	species (plural)
SSC	Saline sodium citrate
SSU	Small subunit
T	thymine
TE	tris EDTA buffer
T_m	melting temperature
Trizma/Tris	tris(hydroxymethyl)aminomethane
U	Units
µg	microgram (s)
µL	microlitre (s)
µM	micromolar
UPGMA	unweighted pair groups method with averages
UV	ultraviolet
var.	variety
V/cm	volts/centimetre
v/v	volume/volume
vol	volume (s)
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside

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INTRODUCTION

The morphological species concept defines a species based on a number of essential morphological characteristics. The use of this concept in mycology is very widespread and of great historical importance. However, the extreme levels of morphological, genetic, and physiological diversity among organisms often exposes the limitations of this species concept. Therefore, the application of other concepts, such as the biological or phylogenetic species concepts, is often necessary to resolve species boundaries.

Pythium is a genus in the class Oomycetes comprised of approximately 120 described species which includes many economically important plant pathogens. The identification and classification of *Pythium* species based on morphological characters is often complicated by the variation in or the absence of structures in culture. The use of molecular markers and DNA sequences can therefore provide alternative techniques for identifying *Pythium* species and examining species relationships.

The ribosomal RNA (rRNA) genes are a multigene family arranged in tandem arrays of repeated ribosomal DNA (rDNA) units. Each unit encodes the small subunit (SSU), 5.8S, and large subunit (LSU) rRNA genes. In *Pythium,* the 5S rRNA gene is found either linked to the rDNA repeat unit within the intergenic spacer (IGS), or unlinked elsewhere in the genome. The rRNA genes evolve via concerted evolution which results in sequence similarity among rDNA repeat units that is greater within than among species. Therefore, rDNA sequences are often representative of a species, making them an effective target for species identification and for the application of the phylogenetic species concept. The coding regions in the rDNA evolve slowly and their

sequences are often highly conserved between closely related species, so they are useful for resolving relationships between groups of species or genera. However, the spacer regions (the internal transcribed spacer [ITS] and IGS) evolve more rapidly and are therefore most useful for species identification and for inferring phylogenetic relationships among species.

Molecular markers that have previously been used to identify *Pythium* species include those derived from restriction fragment length polymorphisms (RFLPs) of mitochondrial DNA (mtDNA) (Martin, 1990), RFLPs of rDNA (Chen *et al.,* 1992a), random amplified polymorphic DNA (RAPDs) (Herrero and Klemsdal, 1998), and DNA probes (Klassen *et al.,* 1996). Phylogenetic studies of *Pythium* species have also been done using sequence analysis of the rDNA ITS (Matsumoto *et al.,* 1999) and the mtDNA cytochrome oxidase II gene (Martin, 2000). One of the primary goals of this thesis was to use polymerase chain reaction (PCR) amplification of the rDNA IGS and subsequent restriction endonuclease digestions of PCR products to produce restriction fragment profiles to identify over 360 isolates representing 90 *Pythium* species from around the world. It was hoped that RFLP analysis would reveal clustering patterns of species and individual isolates. This would allow for the examination of the genetic relationships among these species and the correlation between the morphological and phylogenetic species definitions in the genus. These methods will also be useful for confirming previous species assignments of isolates based on morphological observations.

Pythium insidiosum (de Cock *et al.,* 1987) is the only species in the genus capable of infecting animal hosts, including humans. *P. insidiosum* is the etiological agent of pythiosis, a disease prevalent in tropical and subtropical regions characterized by

cutaneous and subcutaneous lesions and some vascular diseases. While serological studies have suggested that all isolates of *P. insidiosum* from around the world are antigenically identical (Mendoza *et al.,* 1987), other studies have shown physiological variation among isolates (McMeekin and Mendoza, 2000). Despite the ability of *P. insidiosum* to cause potentially fatal infections, very little molecular work has been done on this organism. Another aim of this work was to pursue RFLP analysis of the rDNA IGS and also ITS sequence analysis for several isolates of *P. insidiosum* from a variety of animal hosts and geographic origins. This was done to reveal phylogenetic relationships and intraspecific variability among *P. insidiosum* isolates and also relationships to related *Pythium* species and genera. In addition, the growth of several isolates of *P. insidiosum* was measured over a range of temperatures to compare growth rates among isolates from different regions and hosts. This was done in an attempt to uncover a physiological trait that corresponded to the genetic relationships revealed from the RFLP and sequencing studies.

Accurate and precise diagnosis of pythiosis is important so that the appropriate therapeutic measures can be taken. Current diagnostic methods often produce conflicting results. As well, the diagnosis of the disease can be complicated by the presence of other organisms which mimic the symptoms of pythiosis. The development of a species-specific DNA probe for *P. insidiosum* was attempted using a *Hin*fI restriction fragment from the IGS. The specificity of this probe for *P. insidiosum* was tested with genomic DNA from over 100 *Pythium* species, as well as with DNA from several *P. insidiosum* isolates and pathogens which produce symptoms similar to pythiosis. Such a powerful molecular tool would provide an additional diagnostic method for pythiosis.

CHAPTER 1

LITERATURE REVIEW

I. The ribosomal RNA multigene family

A. Eukaryotic ribosomal RNA gene organization

In eukaryotes, the nuclear ribosomal RNA (rRNA) genes are arranged as a single transcription unit repeated in tandem arrays in a head-to-tail configuration within the nucleolar organizer region (NOR). The rRNA genes encode for rRNA molecules characterized in sedimentation velocity units (S). Each ribosomal DNA (rDNA) repeat unit is comprised of a large subunit (LSU; 25 to 28S) rRNA gene, 5.8S rRNA gene, and a small subunit (SSU; 17 to 18S) rRNA gene. Two internal transcribed spacers (ITS-1 and ITS-2) separate the three rRNA genes from one another. Adjacent rDNA units are further separated by the intergenic spacer (IGS) which is comprised of a nontranscribed spacer (NTS) and the external transcribed spacer (ETS) just upstream of the SSU rRNA gene (Fig. 1.1) (reviewed by Hillis and Dixon, 1991). In higher eukaryotes, the 5S rRNA genes are commonly found in clusters of tandem arrays unlinked to the rDNA unit elsewhere in the genome, as in *Xenopus* (Carroll and Brown, 1976), *Drosophila* (Hershey *et al.,* 1977), humans (Sorensen and Frederiksen, 1991), plants (Hemleben and Grierson, 1978), and some fungi such as *Neurospora crassa* (Selker *et al.,* 1981). However, in fungi the 5S rRNA genes are also found linked to the rDNA repeat unit within the IGS as in *Saccharomyces cerevisiae* (Rubin and Sulston, 1973), *Coprinus* spp. (Pukkila and Cassidy, 1987), and *Candida* spp. (Magee *et al.,* 1987). In *Pythium,* the 5S rRNA genes have been found linked to rDNA in the IGS, and also unlinked in tandem arrays (Belkhiri *et al.,* 1992). Bedard (2002) showed the orientation of linked 5S rRNA genes was inverted with respect to the direct of rRNA transcription in some species and non-

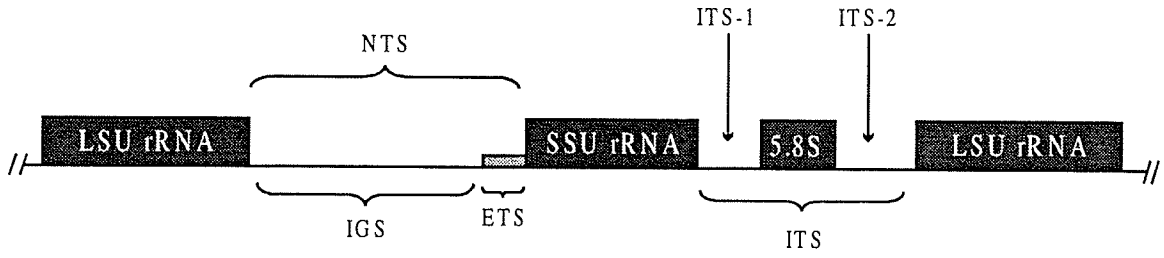


Figure 1.1. Organization of rRNA genes in a typical eukaryotic rDNA repeat unit. Rectangles indicate LSU (large subunit), SSU (small subunit), and 5.8S rRNA genes. Abbreviations: NTS, nontranscribed spacer; IGS, intergenic spacer; ETS, external transcribed spacer; ITS, internal transcribed spacer.

inverted in others. In three species (*P. anandrum, P. tracheiphilum,* and *P. ostracodes*), both the linked and unlinked orientations were detected. Transcription of the rRNA genes by RNA polymerase I commences from a promoter upstream of the ETS and terminates downstream of the LSU rRNA gene. The 5S rRNA gene is transcribed separately by RNA polymerase III.

Some variation from this rRNA gene organization exists in eukaryotes. In *Drosophila* (Roiha *et al.,* 1981) and *Linum* spp. (Agarwal *et al.,* 1992), rDNA units are present in tandem arrays which are frequently interrupted by short non-rDNA sequences, so the rDNA units are arranged in short blocks of arrays rather than one continuous tandem array. For many protozoa, while many have chromosomally integrated rRNA genes in tandem arrays, rDNA units are also present as extrachromosomal DNA in some organisms. Tandem repeats of rDNA units are found in linear and circular extrachromosomal molecules in *Paramecium tetraurelia* (Findly and Gall, 1978) and as extrachromosomal linear and circular monomers in hypotrichous ciliates (Lipps and Steinbrück, 1978) and *Euglena gracilis* (Ravel-Chapuis *et al.,* 1985), respectively.

Circular and linear palindromic rDNA unit dimers are found in *Physarum polycephalum* (Vogt and Braun, 1976) and *Entamoeba histolytica* (Huber *et al.,* 1989), respectively, and it has been shown there are no integrated chromosomal copies of rRNA genes in *Entamoeba* (Bagchi *et al.*, 1999).

Hypervariability in chromosomal location of rDNA loci is unusual, but has been reported in amphibians (Nardi *et al.,* 1977), reptiles (Trajtengertz *et al.,* 1995), salmonid species (Zhou *et al.,* 1995), and plants (Schubert and Wobus, 1985). In lake trout, while the chromosomal location of one NOR is invariable, a number of variable NORs are found at different chromosomal locations in different individuals (Phillips *et al.,* 1989), suggesting the movement of NORs is an ongoing process in lake trout (Zhou *et al.,* 1995). Schubert and Wobus (1985) found rDNA loci at different chromosomal locations in different cells of an individual onion, suggesting these units were mobile, similar to transposons.

B. Copy number and dosage repetition of rRNA genes

Copy number of rRNA genes is not necessarily characteristic of a species and there may be much variation among individuals. In fungi with haploid nuclei, such as *S. cerevisiae*, rDNA is generally encoded on a single chromosome (Petes, 1979), although there are exceptions. In the Oomycetes, the numbers and locations of rDNA loci vary among species and occasionally between individuals of the same species. Ribosomal DNA is encoded on 1 to 5 chromosomes in *Pythium* (Martin, 1995) and on 1 to 6 chromosomes in *Phytophthora* (Tooley and Carras, 1992).

Ribosomal RNA genes exhibit dosage repetition as several copies per nucleus are present to provide large numbers of gene products to the cell that a single gene copy could not produce in the appropriate time. Copy number of the rDNA repeat unit in eukaryotes varies from as few as one, as in *Tetrahymena* (Yao and Gall, 1977), to several hundred or thousand (reviewed by Long and Dawid, 1980). Copy number variation may also exist between individuals of the same species. In the newt (*Triturus vulgaris*), copy number of rDNA loci within an individual ranges from 2,000 to 20,000 copies per haploid genome (Andronico *et al.,* 1985). Variation in rDNA copy number in newts has even been described within different tissues of the same individual (Lohman and Kraus, 1986). A minimum copy number, however, may be necessary for the viability of some organisms. For example, *Drosophila melanogaster* contains 200 to 250 copies of the rDNA unit per NOR (Ritossa, 1976), and while individuals with 30 to 100 copies have been shown to survive, their development was slowed compared to those with 100 copies or more (Shermoen and Kiefer, 1975).

C. Length heterogeneity in the IGS of the rDNA repeat unit

Length variation among rDNA repeat units in a species is common in eukaryotes and is often due to variation in the number of repeated sequences within the IGS. Intraspecific variation in IGS size due to insertions or deletions of subrepeated sequences is very common in plants (reviewed by Rogers and Bendich, 1987) and in many metazoans including humans (Gonzalez and Sylvester, 1995), *Xenopus laevis* (Wellauer *et al.,* 1976), mouse (Arnheim and Kuehn, 1979), *Drosophila* (Wellauer and Dawid, 1978), and *Herdmania curvata* (Degnan *et al.,* 1998). In fungi, variation in the number of

subrepeats is less common, although it has been described in *Yarrowia lipolytica* (Van Heerikhuizen *et al.,* 1985), *Puccinia graminis* (Kim *et al.,* 1992), *Verticillium* spp. (Morton *et al.,* 1995), *Laccaria bicolor* (Martin *et al.,* 1999), and also in the Oomycete *Pythium* (Klassen and Buchko, 1990; Martin, 1990b). The IGS of *Pythium ultimum* is comprised of several regions of repetitive sequences. The A and B repeats, downstream of the large subunit rRNA gene, are two regions of length heterogeneity comprised of 62 and 40 bp subrepeats, respectively. Further upstream, the C repeats are 366 bp subrepeated sequences which are an additional source of length heterogeneity in the IGS (Buchko, 1996). The size of individual subrepeats varies widely, from as large as 540 to 990 bp in the white spruce *Picea glauca* (Beech and Strobeck, 1993) to as small as 21 bp in *Brassica nigra* (Bhatia *et al.,* 1996). Variation in the number of subrepeats can also occur in the ITS as in *Peronosclerospora* (Yao *et al.,* 1992).

Some size variation among rDNA repeat units may also be due to the insertion of non-rDNA sequences into the LSU rRNA coding regions, such as the R1 and R2 elements of *D. melanogaster* (Wellauer and Dawid, 1977) and *Bombyx mori* (Eickbush and Robins, 1985), and the R1 elements in *Calliphora erythrocephala* (Smith and Beckingham, 1984). Variation in the number and size of inserted introns in the rRNA genes of *Physarum polycephalum* (Nomiyama *et al.,* 1981), *Cladonia chlorophaea* (DePriest and Been, 1992), and Deuteromycetes (Rogers *et al.,* 1993) contribute to rDNA length variation as well.

D. Evolution of repetitive sequences in the IGS

In many cases, IGS subrepeat-related satellite sequences have been discovered. In *Chironomus thummi thummi,* the genome contains highly homologous subrepeats (*Cla*-elements) present in both the IGS and as satellite DNA. The same sequence is present only as satellite DNA in *C. thummi piger,* the phylogenetically older species, suggesting that these repeated elements had been transposed into the IGS (Schmidt, 1984). A similar case was reported for *Vigna faba,* where an IGS subrepeat was thought to be derived from a homologous satellite DNA element (Maggini *et al.,* 1991). The opposite is suspected in species of *Triturus.* Subrepeats are confined to the IGS, except in *T. vulgaris* where they are present both in the IGS and as satellite elements, suggesting the subrepeats were transposed from the IGS into other regions of the genome (De Lucchini *et al.,* 1988). Satellite sequences related to IGS subrepeats have also been described in *Drosophila simulans* (Lohe and Roberts, 1990), *Vigna radiata* (Unfried *et al.,* 1991), and *Phaseolus vulgaris* (Falquet *et al.,* 1997). The transposition of subrepeats into or from the IGS is a possibility since some share similarities with transposons. In *V. faba,* tandem arrays of subrepeats are surrounded by 150 bp direct repeats (Kato *et al.,* 1985).

E. Influences on the size of the IGS

Several studies have suggested natural or directional selection influences the differentiation of rDNA repeat variation and frequency of specific IGS length variants. In *Hordeum vulgaris*, Saghai-Maroof *et al.* (1984) demonstrated directional selection for specific rDNA length variants as dramatic changes in frequencies of rDNA alleles were observed through several generations. In barley populations in Israel and Iran, a

correlation between rDNA allele frequencies and numerous environmental factors, such as climate, has also been observed (Saghai-Maroof *et al.,* 1990; Gupta *et al.,* 2002). Similar situations have been observed in *Triticum dicoccoides,* where rDNA alleles were correlated with climatic factors (Flavell *et al.,*1986), and in oats where IGS length variation was related to breeding and environmental adaptive responses (Polanco and De la Vega, 1997). Therefore, natural selection driven by climatic or environmental factors can operate on rDNA, possibly providing an organism with a genotype specific for various ecological adaptations. A relationship between IGS length variants and artificial selection for specific developmental characteristics has also been demonstrated in *D. melanogaster* where changes in rDNA spacer length compositions underwent frequent cycles of selection for development rate (Cluster *et al.,* 1987). Rocheford *et al.* (1990) also showed that selecting maize for high yield resulted in a reduction in the frequency of the shortest IGS length variant and an increase in the largest one. Therefore, selection may operate on rDNA differentiation which may provide genotypes with various ecological adaptations.

F. Functional role of the IGS

While the IGS physically separates individual rDNA units from one another in an array, it also has many other functional roles. Despite length and sequence variation in the IGS between different species, higher eukaryotes share several generally conserved structural features in the IGS such as subrepeats, repetitive enhancer elements, duplicated promoters, and transcription termination sequences. Baldridge *et al.* (1992) demonstrated that extensive regions of self-complementary sequences which can generate cruciform

DNA structures or extensive secondary structure exist in the IGS among higher eukaryotes. This conservation of higher order structure may reflect evolutionary and functional constraints in the IGS on chromatin organization, rRNA processing, and transcription regulation (Baldridge *et al.,* 1992).

G. Enhancers of Transcription in *Xenopus*

The general organization and functions of individual IGS sequence elements are perhaps best understood for *Xenopus laevis* (Fig. 1.2). The RNA polymerase I promoter (gene promoter) is found upstream of the 18S rRNA gene (positions -142 to +6 relative to the transcription start site +1) (Moss, 1983). Upstream of the promoter, there are several regions which contain repeated elements. Multiple blocks of 60/81 bp repeats alternate with spacer promoters. The 60-/81-bp repeats are 90% homologous with a 42 bp domain of the gene promoter (Reeder, 1984) and act as RNA polymerase I transcription enhancers on the gene promoter (Moss, 1983; Labhart and Reeder, 1984). Spacer promoters, which are imperfect copies of the gene promoter (Boseley *et al.*, 1979), can direct transcription at a low efficiency and also stimulate transcription from the adjacent gene promoter (DeWinter and Moss, 1986). However, the spacer promoter alone does not stimulate transcription from the gene promoter, but rather amplifies the enhancement from the 60/81-bp repeats (DeWinter and Moss, 1987). The fact that the 60-/81-bp repeats can enhance rRNA transcription in the absence of spacer promoters provides further evidence the spacer promoter only amplifies their enhancing effects (DeWinter and Moss, 1987). Further upstream are "0" repeats (34 bp repeated elements) and "1" repeats (100 bp elements) repeated about six to nine times per spacer. Mougey *et al.*

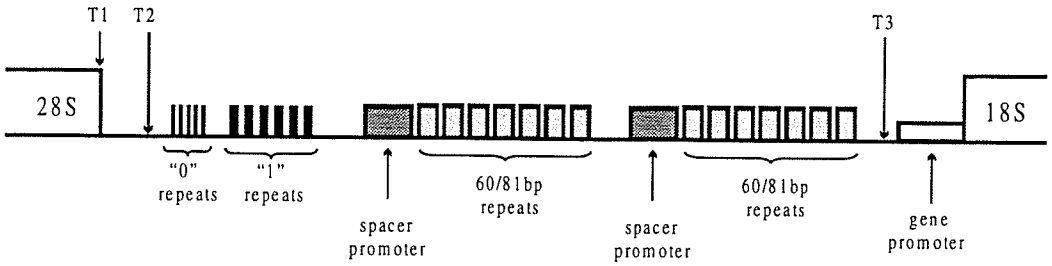


Figure 1.2. Organization of repetitive sequence elements in the IGS of *Xenopus laevis.* The 60/81 bp repeats, spacer promoters, "0" repeats, and "1" repeats are all involved in enhancement of transcription. The T1, T2, and T3 sites play a role in transcription termination.

(1996) suggested that the "0" repeats, "1" repeats, and combined "0/1" repeats serve as enhancers of both spacer promoter and gene promoter transcription. However, Robinett *et al.* (1997) found these repeats basically organize the rRNA genes into single-expression units, restricting activation by the 60-/81-bp repeats to the proximal gene promoter, and preventing them from activating an upstream promoter. Therefore, virtually the entire *Xenopus* IGS consists of repetitive elements that enhance transcription of rRNA genes.

H. Transcription enhancers in other eukaryotes

Sequence elements within the IGS that serve as transcription enhancers have been characterized in other eukaryotes as well. In *Arabidopsis,* repetitive 113 bp spacer promoters, which share sequence homology to the gene promoter, are present between arrays of 20-/21-bp repeats (the *Sal*I repeats) (Gruendler *et al.,*1991). These spacer promoters, when cloned in place of analogous spacer elements in *X. laevis* oocytes, replace them as functional enhancers of transcription (Doelling *et al.,* 1993) suggesting that repetitive enhancer elements are highly conserved in function (although not sequence) among eukaryotes (Doelling and Pikaard, 1995).

The IGS of *D. melanogaster* is comprised of three different arrays of repeated DNA sequences which consist of 95, 330, and 240 bp subrepeats (Coen and Dover, 1982). Each 240 bp repeat contains a 42 bp sequence homologous to a region of the gene promoter (Coen and Dover, 1982), and the 330 bp repeats are actually made up of different portions of the 95 bp and 240 bp repeats (Simeone *et al.,* 1985). Grimaldi and Di Nocera (1988) showed that transcriptional activity of the gene promoter was directly correlated with the number of 240 bp repeats present, as well as sequences upstream of the 240 bp repeats, suggesting the duplicated promoter regions within the 240 bp and 330 bp repeats stimulate transcription.

In the rat, a 130 bp repetitive element has been shown to exhibit enhancer functions *in vitro* (Ghosh *et al.,* 1993). As well, a 174 bp enhancer element stimulates transcription from the gene promoter 10 to 20-fold compared to the gene promoter alone (Dixit *et al.,*1987). An additional sequence element in the IGS with 13 bp homologous to the gene promoter sequence supported transcription initiation *in vitro,* but overall was less efficient than the gene promoter (Cassidy *et al.,* 1987). Imperfect duplications of the gene promoter within the IGS have also been characterized for *Oryza* (Cordesse *et al.,* 1992) and *Daucus carota* (Suzuki *et al,* 1996). While the mechanism by which spacer promoters augment transcription is unclear, it has been suggested that they act as a site of entry for transcription factors for the formation of the transcription initiation complex (Schmitz *et al.,* 1989).

The mouse 140 bp repeats in the IGS have no obvious sequence similarity to the mouse gene promoter (Kuehn and Arnheim, 1983). However, Pikaard *et al.* (1990) showed they act as transcription enhancers, analogous to the role of the 60-/81-bp repeats

in *Xenopus*. They also showed the 140 bp repeats enhanced a frog ribosomal gene promoter in frog oocyte injection assays. Conversely, *Xenopus* enhancers can also function across species boundaries in a mouse cell-free transcription system (Kuhn *et al.*, 1990). A similar situation exists in *Acanthamoeba castellanii* where repeated elements, with an average size of 140 bp, act as enhancers in *in vitro* assays, but show no apparent sequence homology to the gene promoter (Yang *et al.*, 1994).

The yeast IGS lacks repetitive enhancer elements or promoter-like sequences, but a complex 190 bp sequence upstream of the transcription initiation site was shown to stimulate transcription (Elion and Warner, 1984; 1986). This sequence, which lacks homology to the gene promoter (Skryabin *et al.*, 1984), overlaps the 22 bp spacer promoter which is sufficient for transcription initiation *in vitro* (Swanson *et al.*, 1985). As well, sequences near the 5' end of the 190 bp enhancer also mediate RNA polymerase I-dependent termination of transcription (Mestel *et al.*, 1989). Thus, the 190 bp enhancer in yeast contains sequences that appear to act as enhancers of transcription from the gene promoter and shows involvement in transcription termination.

I. Transcription Termination

In many eukaryotes, transcription termination signals are usually at the extreme ends of the IGS, either downstream of the large subunit rRNA gene or upstream of the gene promoter. In *Xenopus,* three sites (T1, T2, and T3) are involved in rRNA processing and transcription termination (Fig. 1.2). The 3' end of mature rRNA is processed at the T1 site, but transcription continues through to the T2 site where the transcript becomes less stable. The rRNA is eventually cleaved at T2 and a 12 bp inverted repeat at the 3'

end forms a hairpin to act as a barrier to exonuclease degradation. Synthesis of unstable RNA continues and terminates 215 bp upstream of the gene promoter at the T3 site, which shares a conserved sequence block with T2 (Labhart and Reeder, 1986). It is unclear whether the function of T3 is promoter occlusion (releasing RNA polymerase I to prevent it from dislodging the initiation complex of the adjacent gene promoter), as a fail-safe terminator, or for preservation of RNA polymerase I on the rRNA gene to pass it from T3 to the adjacent gene promoter without losing it to the free pool (McStay and Reeder, 1986). Similarly, in *Vigna radiata*, 174 bp repeated elements in the IGS contain termination sequences similar to the T2 and T3 sites in *Xenopus* which allow the formation of a stem-loop at the 3' end of each subrepeat. Transcription in *V. radiata* was in fact shown to terminate 20 bp downstream of the stem-loop of the first 174 bp subrepeat (Schiebel *et al.,* 1989).

In the mouse, Grummt *et al.* (1986b) identified a repetitive set of termination sequences (*Sal*I boxes) 565 bp downstream from the 28S rRNA gene. A repeated 18 bp sequence motif was shown to mediate the binding of a nuclear factor to stop the movement of RNA polymerase I along the rDNA template and allow the release of pre-rRNA. An additional *Sal*I box was also located 171 bp upstream of the transcription initiation site was also shown to terminate transcription *in vitro* (Grummt *et al.,* 1986a; Henderson and Sollner-Webb, 1986).

In contrast, Tautz and Dover (1986) suggested there is no regular termination of transcription in *D. melanogaster* at any particular point within the IGS and that transcription may proceed through the promoter of the following rDNA unit. Termination in *Herdmania curvata* is thought to be similar as no termination signal was present in the

IGS and transcription often proceeded through to the next rDNA unit (Degman *et al.*, 1998).

J. Additional functional roles of IGS sequences

In addition to involvement in transcription enhancement, initiation, and termination, sequence elements in the IGS may also mediate chromosome pairing. McKee *et al.* (1992) showed that the IGS of *D. melanogaster* functioned in *X-Y* meiotic chromosome pairing in males. More specifically, Ren *et al.* (1997) showed that the 240 bp repeats are effective in promoting pairing of nonhomologous *X* and *Y* chromosomes. Origins of replication of chromosomal DNA have also been located within the IGS of *Lytechinus variegates* (Botchan and Dayton, 1982), yeast (Linskens and Huberman, 1988), and even within the extrachromosomal rRNA genes of *Physarum polycephalum* (Benard *et al.*, 1995).

K. Concerted Evolution of rRNA genes

Concerted evolution is the nonindependent evolution of repetitive DNA sequences resulting in a sequence similarity of repeating units that is greater within than among species (Arnheim, 1983). Concerted evolution is the result of molecular drive, the fixation and homogenization of sequence variants in a population via mechanisms such as unequal crossing over between repeating units, replicative transposition, gene amplification, and gene conversion (Dover, 1982). Concerted evolution of rDNA was first detected in the rRNA genes of *Xenopus* (Brown *et al.*, 1972). While the 18S and 28S rRNA gene sequences between *X. laevis* and *X. mulleri* were virtually identical, the IGS regions between the two species varied greatly. In essence, multiple copies of rDNA units

do not evolve independently due to molecular drive and concerted evolution. Even rDNA arrays on five nonhomologous chromosomes, as in humans, have been shown to evolve in a concerted fashion (Ohta and Dover, 1983). Therefore, relative homogeneity of multigene families within species can be maintained despite their divergence between species.

In early studies with *Drosophila,* Coen and Dover (1983) showed that interchromosomal exchanges between *X* and *Y* were responsible for the evolution of subrepeat arrays in the IGS leading to species-specific homogenization (i.e. concerted evolution). However, new rDNA variants are often slow to spread within a species resulting in intraspecific variants, or rDNA copies may be converted by gene conversion so that sequence homology is attained on nonhomologous chromosomes (Dvorak *et al.,* 1987). The latter has been demonstrated with lizards where one rDNA type was selectively favored over the other due to biased gene conversion (Hillis *et al.,* 1991).

In sexually reproducing populations, mechanisms responsible for concerted evolution lead to the fixation of a single rDNA genotype. In *Fusarium oxysporum,* variable IGS haplotypes among populations of non-pathogenic isolates were reported (Appel and Gordon, 1995; 1996). These results are consistent with sexual reproduction being absent or rare in *F. oxysporum,* as IGS mutations were confined to the clonal lineages from which they arose. But even if sexual reproduction is rare, rDNA units can become homogenized in a individual through mitotic gene conversion (Jackson and Fink, 1981) or translocations during mitotic divisions (Szostak and Wu, 1980). As well, recombination suppression during meiosis is thought to occur in fungi such as *S. cerevisiae* (Petes and Botstein, 1977), *Coprinus cinereus* (Cassidy *et al.,* 1984), and

Schizophyllum commune (James *et al.,* 2001) which results in a lack of recombination between arrays of homologous chromosomes at meiosis and intrachromosomal homogenization of variant sequences. When recombination is active, multiple rDNA repeat units can co-exist within an array, such as in *Pythium* (Martin, 1990b). However, both unequal crossing-over (Szostak and Wu, 1980) and gene conversion (Jackson and Fink, 1981) can occur in mitosis. Szostak and Wu (1980) noted that although mitotic recombination is slow in the genome, rDNA seems to be the exception, likely due to intrachromosomal recombination.

Different regions of the rDNA repeat unit may be subjected to different mechanisms of molecular drive. Polanco *et al.* (1998) proposed that exchanges between IGS regions in *Drosophila* only occur in *X-Y* chromosome pairing, which would explain the shared distribution of IGS variants between *X* and *Y,* while the ITS appears to evolve along haplotypic lineages with variants specific to either *X* or *Y* (Schlötterer and Tautz, 1994). This may be due to the presence of topoisomerase I sites in the IGS that may permit frequent interchromosomal exchanges of IGS blocks (Polanco *et al.,* 1998). In fact, Polanco *et al.* (2000) showed that only the 240 bp subrepeats in the IGS were involved in genetic exchanges between *X* and *Y,* which would allow the 240 bp subrepeats, promoters, and ETS to be exchanged, while the rest of the rDNA unit (including the ITS) evolves along a separate chromosomal lineage.

Ganley and Scott (1998) suggested that unequal crossing-over occurs not only between entire rDNA units, but also between IGS subrepeats generating variation in the copy number of subrepeats within an array. Thus, a paradox exists whereby the same system which plays a role in homogenization also generates high levels of variation.

II. Defining a fungal species

A. Application of species concepts to fungi

In mycology, species recognition is essential for scientific communication, assessing fungal biodiversity, and issues of environmental and socio-economic importance such as the diagnosis of pathogens, quarantine legislation, and patenting (Brasier, 1997). As of 1995, there were approximately 72,000 described fungal species (Hawksworth *et al.,* 1995), although the magnitude of fungal diversity is estimated to be over 1.5 million fungal species (Hawksworth, 2001). Therefore, with such a variety in morphology, genetic and breeding systems, and evolutionary histories, it is often difficult to apply a single species concept to fungi. Mayden (1997) recognized 22 species concepts that are applied to a wide range of organisms. However, three species concepts have been widely used in mycology: the morphological, biological, and phylogenetic species concepts.

The morphological species concept (MSC) defines a species based on a number of essential morphological characteristics. Regan (1926) defined a morphological species as "a community, or a number of related communities, whose distinctive morphological characters are, in the opinion of a competent systematist, sufficiently definite to entitle it, or them, to a specific name". The MSC is of particular importance in mycology as it is often simple to apply and so widespread that comparisons can be made between new and existing taxa. Some limitations of the MSC are the parallel evolution of characters, the possibility that useful morphological characters have not yet accumulated, the small number of characters available, absence of sexual structures, and difficulty finding structures in the field or laboratory (Brasier, 1997).

According to Mayr (1940), the biological species concept (BSC) defines a species as "groups of actually or potentially interbreeding natural populations which are reproductively isolated from other groups". Therefore, a species begins at the origin of reproductive isolation, from where different evolutionary lineages are formed. *Armillaria mellea* was thought to be a single morphological species, but Hintikka (1973) demonstrated the fungus was capable of outcrossing and since then, over a dozen biological species of *A. mellea* have been described (Anderson and Ullrich, 1979; Anderson and Satsovski, 1992). The BSC can be used to test fungal intersterility barriers in culture and has become a standard system in mycology for defining species. However, the BSC does have its limitations. It cannot be applied to asexual or homothallic fungi, and some heterothallic fungi will not mate in culture under certain conditions. It has also been questioned as to whether intersterility actually defines a species. Mating tests do not distinguish actual gene flow in nature from potential gene flow, and may lump together groups of fungi which are genetically isolated in nature but have retained the ancestral character of interbreeding (Taylor *et al.,* 2000). Therefore, the BSC may be overly simplistic as it doesn't consider the independent evolution of genetically isolated populations and may not reflect evolutionary relationships (Vilgalys, 1991).

The phylogenetic species concept (PSC) defines a species as the "smallest aggregation of populations (sexual) or lineages (asexual) diagnosable by a unique combination of character states in comparable individuals" (Nixon and Wheeler, 1990). For example, based on rDNA sequence analysis, Vilgalys and Sun (1994) showed that eight biological species of *Pleurotus* where actually comprised of more than one phylogenetic species. They suggested that the PSC, in which monophyletic geographic

populations represent a species, is more desirable for developing species concepts in fungi since intersterility groups may be paraphyletic. The PSC can be applied to asexual and homothallic fungi, and also to genetically isolated groups recognizing geographically isolated groups as possible unique species. A drawback is that although the PSC groups individuals well, it can be difficult to objectively decide where to place the limit on what a species is (Taylor *et al.*, 2000).

The three species concepts attempt to recognize evolutionary species, but once progeny species evolve from an ancestor, gene sequence changes can occur and be recognized before changes in morphology or mating behaviour are recognized (Brasier, 1997). Therefore, the PSC appears to be most appropriate for fungi, although a combination of the three species concepts would be most effective.

B. Applications of rDNA for species identification in fungi

Molecular biology techniques are useful for the identification of fungi as they provide a greater degree of objectivity not always possible with morphological identification methods. The need to produce characteristic structures in pure culture is eliminated as DNA can be extracted from any living mycelium. Nuclear rDNA is especially useful for phylogenetic studies as it evolves in a concerted fashion, so sequences of an individual are generally representative of a species (Hillis and Davis, 1986). Ribosomal DNA sequences are repetitive in the genome, so multiple copies are present which is advantageous in sequence amplification or hybridization procedures. As well, rDNA contains both slowly evolving (the SSU, 5.8S, and LSU rRNA genes) and

more rapidly evolving regions (the ITS and IGS) so that phylogenetic information at different resolution levels can be recovered and analyzed.

The spacer regions (ITS and IGS) appear to be the most useful for species and strain differentiation, as well as for inferring phylogenetic relationships. Among the spacer regions, the IGS appears to be the most rapidly evolving region followed by the ITS (Hillis and Dixon, 1991). It has been suggested that the IGS would be too variable for studying relationships of closely related species. Hsiao *et al.* (1995) even suggested the IGS would be less valuable than the ITS because the IGS supposedly only gives random noise. However, restriction fragment length polymorphism (RFLP) and sequence analysis of the IGS has been very informative for phylogenetic and population studies. In fact, Sallares and Brown (1999) showed that ITS sequences had a limited capacity for studying closely related species in *Triticum* and *Aegilops* while the IGS provided a greater level of resolution. Therefore, both regions may provide valuable information when studying closely related species or strains in a population.

C. Restriction site mapping and RFLP analysis

Restriction site mapping of the entire rDNA repeat unit has been used to estimate species relationships on the basis of the shared presence or absence of restriction sites. By comparing rDNA restriction site maps, Anderson *et al.* (1989) divided the biological species of *Armillaria* into six classes, which corresponded to classes based on morphological similarities. Restriction site maps have also been used to examine variation among *Gaeumannomyces graminis* varieties (Tan *et al.,* 1994) and for studying evolutionary relationships, population studies, and monitoring hybrid genomes in plants

including *Solanum* spp. (Borisjuk *et al.,* 1994), *Bromus* (Pillay, 1996), and maize (Zimmer *et al.,* 1988).

RFLPs were the first DNA markers used for fungal evolutionary biology. Alterations in the restriction endonuclease recognition sequence due to nucleotide substitutions, insertions, deletions, or methylation of nucleotides can affect the activity of an enzyme at a specific site and subsequently change the fragment pattern (Taylor *et al.,* 1999). The use of rDNA probes hybridized with restriction digested genomic DNA has been used to differentiate and identify fungal species of *Candida* (Magee *et al.,* 1987), *Verticillium* (Typas *et al.,* 1992), *Metarhizium* (Pipe *et al.,* 1995), and *Cylindrocladium* (Crous *et al.,* 1997). Variation in RFLP patterns has also revealed intraspecific variation among strains of *Histoplasma capsulatum* (Vincent *et al.,* 1986), *Aspergillus fumigatus* (Spreadbury *et al.,* 1990), *Cenococcum geophilum* (LoBuglio *et al.,* 1991), and *Verticilluim albo-atrum* (Griffen *et al.,* 1997), and has also differentiated biological species of *Armillaria mellea* (Anderson *et al.,* 1987) and *Laccaria laccata* (Gardes *et al.,* 1990).

D. PCR-RFLP analysis of rDNA

Highly conserved sequences in the rRNA genes of a broad spectrum of fungi have allowed for the construction of universal primers for polymerase chain reaction (PCR) amplification of numerous regions of the rDNA repeat unit (White *et al.,* 1990). PCR products can be treated with restriction endonucleases for RFLP analysis. The ITS has been used to identify fungal species and to examine intraspecific variation within species using PCR-RFLP analysis in many cases (Table 1.1). While the IGS is the most

Table 1.1. Examples of PCR-RFLP analysis of the rDNA ITS and/or IGS for strain and species identification in fungi, and for the establishment of phylogenetic relationships.

PCR-RFLP analysis of the ITS	Reference
Arthrobotrys spp.	Persson *et al.* (1996)
Candida spp.	Williams *et al.* (1995)
Gaeumannomyces graminis and *Phialophora graminicola*	Ward and Akrofi (1994)
Glomales	Redecker *et al.* (1997)
Laccaria spp.	Gardes *et al.* (1991)
Lentinus spp.	Hibbett and Vilgalys (1991)
Penicillium nodositatum	Sequerra *et al.* (1997)
Phytophthora spp.	Lee and Taylor (1992); Ristaino *et al.* (1998)
Trichophyton rubrum	Jackson *et al.* (1999)

PCR-RFLP analysis of the IGS	Reference
Armillaria spp.	Harrington and Wingfield (1995), (IGS-1); Banik *et al.* (1996), (IGS-1); Volk *et al.* (1996); White *et al.* (1998), (IGS-1 & IGS-2)
Cronartium flaccidum & *Peridermium pini*	Moricca *et al.* (1996)
Cylindrocladium spp.	Risède and Simoneau (2001)
Fusarium oxysporum	Appel and Gordon (1995); Woudt *et al.* (1995); Edel *et al.* (1997; 2001)
Hebeloma cylindrosporum	Guidot *et al.* (1999)
Laccaria proxima	Albee *et al.* (1996)
Paecilomyces farinosus	Chew *et al.* (1997)
Pyrenophora graminea	Pecchia *et al.* (1998)
Saccharomyces spp.	Molina *et al.* (1993), (IGS-1)

IGS and ITS regions	Reference
Armillaria spp.	Chillali *et al.* (1998), (IGS-1)
Ascochyta spp., *Phoma medicaginis*	Faris-Mokaiesh *et al.* (1996)
Chytridiomycota	Hausner *et al.* (2000)
Fusarium lutarium	Hyun and Clark (1998)
Laccaria spp.	Henrion *et al.* (1992), (IGS-1)
Morels	Buscot *et al.* (1996)
Saccharomyces cerevisiae	Montrocher *et al.* (1998)
Tuber spp.	Henrion *et al.* (1994), (IGS-1)
Tylospora fibrillose	Erland *et al.* (1994), (IGS-1)
Verticillium chlamydosporium	Arora *et al.* (1996)

rapidly evolving of the spacers, it has also been used as a target for species and strain differentiation using PCR-RFLP analysis for many fungi (Table 1.1). While the entire IGS may be used for PCR-RFLP analysis, difficulties occasionally arise in amplifying the entire region due to its relatively large size. The presence of the 5S gene within the IGS in some fungi permits PCR amplification and RFLP analysis of either IGS-1 (between the LSU and 5S rRNA genes) or IGS-2 (between the 5S and SSU rRNA genes), regions that are smaller in size and easier to amplify, yet still informative (Table 1.1).

E. Accuracy of RFLP analysis for species identification

The assignment of relatedness based solely on fragment banding patterns, without any knowledge of their mutational basis, could yield erroneous estimates of genetic similarity. While the presence of shared restriction sites or fragments between different individuals may be identical by descent, critics argue that this may not be the case for absent sites since it is easier to lose a site than to gain one. Since the loss of a site may occur several ways (e.g. base substitutions, length mutations), missing sites which arise by different routes complicate the evolutionary analysis (Taylor *et al.,* 1999). The assumption is made that if two individuals share a particularly sized fragment, they must also share flanking cleavage sites. This generally is true for closely related individuals or intraspecific comparisons, but the likelihood that two samples have fragments that are the same size but are produced by different cleavage sites increases as the sequences become more different (Dowling *et al.,* 1990). In fact, Upholt (1977) suggested that restriction fragment comparisons be used only for sequences that differ by less than 15%.

F. Ribosomal DNA sequencing studies (ITS, IGS, ETS)

Nucleotide sequences often provide the highest level of resolution for phylogenetic studies. Analysis of PCR-amplified ITS sequences for inferring phylogenetic relationships among fungi has been done for Oomycete species in *Phytophthora* (Cooke and Duncan, 1997; Cooke *et al.,* 2000) and *Pythium* (Matsumoto *et al.,* 1999). However, Anderson and Stasovski (1992) found ITS sequences among Northern Hemisphere isolates of *Armillaria* too uniform, and instead IGS-1 sequences were more effective for resolving relationships among isolates. Sequence analysis of IGS regions has also been done to resolve intraspecific relationships among isolates of *F. oxysporum* (Appel and Gordon, 1996) and *Armillaria mellea* (Coetzee *et al.,* 2000).

Although not frequently used for fungi, the ETS is another potential target for sequence analysis and has been used in angiosperm phylogenies. Linder *et al.* (2000) found ETS sequence data more informative than the ITS for resolving relationships among Asteraceae. ETS sequence analysis has also been used for relationships within *Brassica* and *Raphanus* (Bennett and Smith, 1991) as well as *Nicotiana* and *Solanum* (Volkov *et al.,* 1996). Exploitation of the ETS for phylogenetic studies is hampered, however, by a lack of universal primers to amplify the region across a broad range of taxa as well as an unclear understanding how concerted evolution operates in that region (Linder *et al.*, 2000).

G. DNA Probes using rDNA regions

DNA probes are particularly useful for the identification of pathogenic fungal agents because probes can often detect DNA in a mixed culture, so pure cultures are

unnecessary. In fact, commercially available probes for *Blastomyces dermatitidis, Coccidioides immitis, Cryptococcus neoformans,* and *Histoplasma capsulatum* have been evaluated (Stockman *et al.,* 1993). Genomic DNA fragment probes have been used with RFLPs to identify *H. capsulatum* (Keath *et al.,* 1989) and with genomic dot blots to identify *Candida albicans* (Oren *et al.,* 1991). More specific genomic regions include a clone containing the nuclear-encoded acid proteinase gene as a probe for *C. albicans* (Ganesan *et al.,* 1991) and a mitochondrial DNA (mtDNA) fragment specific for *C. krusei* (Manavathu *et al.,* 1996).

Ribosomal DNA is an especially useful target for DNA probes as it is a highly abundant target in cells, representing a pre-amplification step that increases test sensitivity and produces a strong hybridization signal. As well, regions within rDNA can be selected which are specific for species or groups of organisms. A clone comprised of two fragments from the rDNA IGS has been used as a diagnostic probe to identify *Candida krusei* (Carlotti *et al.,* 1996; 1997a). Oligonucleotide probes from the SSU rRNA gene have also been designed to identify fungal species in blood samples (Einsele *et al.,* 1997) and for fluorescent *in situ* hybridization (FISH) in infected tissue and blood (Lischewski *et al.,* 1996; 1997). The LSU rRNA gene has also served as a source of oligonucleotide probes for over 20 fungal species representing eight genera (Sandhu *et al.,* 1995; 1997). As well, oligonucleotide probes from ITS-2 were designed for several *Candida* species (Elie *et al.,* 1998), *Trichophyton rubrum* (El Fari *et al.,* 1999), and for use in microtitration plate enzyme immunoassays (EIA) to specifically detect amplified DNA of several fungal pathogens (Fujita *et al.,* 1995; Lindsley *et al.,* 2001). Recently, the

effectiveness of peptide nucleic acid (PNA) probes with FISH has been evaluated for the differentiation of *C. albicans* and *C. dubliniensis* (Oliveira *et al.,* 2001).

Species-specific DNA probes are also useful for the identification of fungal species that infect plant, insects, and food products. Genomic DNA clones were designed to detect *Beauveria bassiana* in grasshoppers (Hegedus and Khachatourians, 1993; 1996) and *Polymyxa betae* within infected sugar beet roots (Mutasa *et al.,* 1993). Mitochondrial DNA probes have been used to identify the Ascomycetes *Phoma tracheiphila* from infected lemon seedling roots (Rollo *et al.,* 1987) and *Gaeumannomyces graminis,* which causes take-all disease in wheat (Henson, 1989). Small subunit rRNA gene oligonucleotides have also been created to detect the Ascomycetes *Aureobasidium pullulans* and *Cladosporium herbarum* (Li *et al.,* 1996) as well as eleven species of yeasts involved in the spoilage of yoghurt (Kosse *et al.,* 1997). Species-specific probes for *Debaryomyces hansenii* have also been designed using conserved fragments from random amplified polymorphic DNA (RAPD) amplifications (Corredor *et al.,* 2000).

Species specific probes have also been constructed for several pathogenic Oomycetes, including genomic probes for *Phytophthora parasitica* (Goodwin *et al.,* 1989; 1990a), *Ph. citrophthora* (Goodwin *et al.,* 1990b), and *Ph. cinnamomi* (Judelson and Messenger-Routh, 1996), a mtDNA probe for *Personosclerospora sorghi* (Yao *et al.,* 1991), and oligonucleotide probes from ITS-1 for four *Phythophthora* species (Lee *et al.,* 1993). Species-specific probes for *Ph. cinnamomi* have also been designed using fragments from RAPD amplifications (Dobrowolski and O'Brien, 1993) and the cinnamomin gene sequence for use as a capture probe in microtitration plate immunoassays (Coelho *et al.,* 1997).

H. Detection of fungi using PCR amplification of rDNA

The sensitivity of PCR makes it an effective molecular tool to detect and identify fungi in plants and soils. Species-specific primers were designed from genomic DNA clones to specifically amplify DNA of *Phytophthora parasitica* and *P. citrophthora* (Ersek *et al.,* 1994). However, these non-defined, poorly characterized target sequences may or may not be present in all isolates of the same species. To overcome such a problem, rDNA provided a more specific target for PCR detection of fungi. Nazar *et al.* (1991) designed species-specific primers within the ITS to detect and differentiate *Verticillium albo-atrum* and *V. dahliae* in infected alfalfa crops. Trout *et al.* (1997) similarly designed ITS primers to specifically detect *Phytophthora infestans* in infected tomatoes. PCR primers from sequences of IGS-2 in *Phytophthora* species were also designed to detect *Ph. medicaginis* from infected plant tissue (Liew *et al.,* 1998).

I. PCR fingerprinting using repetitive DNA sequences

PCR can be used to sample polymorphic loci or repetitive DNA sequences. For epidemiological studies of pathogenic fungi, PCR amplification of polymorphic regions in the IGS has been used as a DNA fingerprinting technique to identify and differentiate strains of *Candida krusei* (Carlotti *et al.,* 1997b) and *Aspergillus fumigatus* (Radford *et al.,* 1998) from various patients. Such techniques can also be used with repetitive DNA sequences outside of the rDNA unit. Probes originally designed to detect mini- and microsatellite sequences were used as PCR primers to amplify genomic fragments flanked by microsatellite DNA to examine DNA polymorphisms among species of morels (Buscot *et al.,* 1996) and to characterize isolates of *Cenococcum geophilum*

(Portugal *et al.,* 2001). REPs (repetitive extragenic palindromic sequences; Higgins *et al.,* 1982) and ERICs (enterobacterial repetitive intergenic consensus sequences; Hulton *et al.,* 1991) are repetitive extragenic sequences present in prokaryotic genomes, but are also present in fungi such as *F. oxysporum* (Edel *et al.,* 1995), *Aspergillus* spp. (Van Belkum *et al.,* 1993), and *Verticillium chlamydosporium* (Arora *et al.,*1996). These sequences have been used for PCR fingerprinting to detect and differentiate strains of these species.

In general, such fingerprinting techniques are useful for discriminating strains from one another but not necessarily useful for estimating phylogenetic relationships among isolates or species. This is because homology among individual fragments is difficult to establish since alleles may not be identical by descent (i.e. inherited from a common ancestor) (Taylor *et al.,* 1999).

J. PCR techniques using regions other than rDNA

i) RAPDs

Random amplified polymorphic DNA (RAPD) analysis (Welsh and McClelland, 1990; Williams *et al.,* 1990) often detects variation among isolates at a higher resolution than RFLPs (Taylor *et al.,* 1999). However, while Cooke *et al.* (1996) found that RAPDs were more useful than ITS sequence for distinguishing among group I *Phytophthora* species, Thorman *et al.* (1994) found RAPDs less reliable than RFLPs for estimating genetic relationships in cruciferous species. Nonetheless, RAPDs have been used to analyze many groups of fungi, including *Fusarium* spp. (Kelly *et al.,* 1994; Bentley *et al.,* 1995), *Metarhizium* spp. (Cobb and Clarkson, 1993; Fegan *et al.,* 1993),

entomopathogenic fungi (Strongman *et al.,* 1993; Bidochka *et al.,* 1994), *Verticillium albo-atrum* (Barasubiye *et al.,* 1995), and dermatophytes (Liu *et al.,* 1996; Gräser *et al.,* 1998). RAPDs analyze a larger portion of the genome compared to RFLP analysis, which makes it useful for strain differentiation. However, one of the biggest drawbacks of RAPDs involves its lack of reproducibility, not only among laboratories, but within laboratories over time which makes the development of a standard database and protocol difficult (Soll, 2000). As well, bands of equal mobility may not actually be identical by descent as was the case in PCR fingerprinting.

ii) AFLPs

Amplified fragment length polymorphisms (AFLPs) (Vos *et al.,* 1995) are similar to RFLPs in that they rely on differences in restriction sites. However, they produce more fragments than RFLPs from random locations in the genome and the results are generally more reproducible than RAPDs (Taylor *et al.,* 1999). Although not as frequently used in fungal systematics as the other molecular methods, AFLP fingerprinting has been employed to estimate genetic diversity among isolates of *Cladosporium fulvum* and *Pyrenopeziza brassicae* (Majer *et al.,* 1996) and *Phytophthora infestans* (Purvis *et al.,* 2001). In fact, Bakkeren *et al.* (2000) found AFLP fingerprints more effective in distinguishing closely related *Ustilago* species compared to ITS sequencing. A shortcoming of AFLP analysis is the tendency to underestimate variation since two independent events may lead to fragments of the same size, such that two differences are only scored as one change. Variation can also be overestimated as alleles (co-dominant markers) are not recognized by AFLPs, so allelic fragments may be scored as

independent when in reality they are not (Majer *et al.,* 1996). Thus, AFLPs are more applicable to strain typing and detection of intraspecific variation.

III. Biology and identification of *Pythium* species

A. Taxonomic status of Oomycetes

Oomycetes may initially seem indistinguishable from true fungi based on their morphology (the production of hyphae and complex thalli), absorptive mode of nutrition, and parasitic or saprobic ecological roles in nature. However, many characteristics differentiate them from true fungi, and thus they are classified separately from the Kingdom Fungi.

Pringsheim (1858) noted early on that Oomycetes were related to certain algae, and de Bary (1866) suggested Oomycetes may have evolved from xanthophyte algae. Leedale (1974) first proposed the kingdom name "Heterokonta", which included the Oomycetes and heterokont algae, but without proper Latin description. Cavalier-Smith (1981) later established the kingdom "Chromista", distinct from the Kingdom Fungi, to include these organisms.

Most recently, the Oomycetes were classified as members of the Kingdom Straminipila which also includes chromophytic algae (possessing chlorophylls *a* and *c*), thraustochytrids, labyrinthulids, and hyphochytriomycetes (Patterson, 1989). Despite the diversity of its members, rRNA gene sequencing (Gunderson *et al.,* 1987; Förster *et al.,* 1990; Leipe *et al.,* 1994; Van de Peer *et al.,* 1996), mtDNA sequencing (Lang *et al.,* 1999), and nuclear-encoded protein sequence data (Bhattacharya *et al.,* 1991; Baldauf

and Palmer, 1993) supported the monophyly of these organisms in the Kingdom Straminipila. These organisms possess tripartite tubular hairs (stramenopiles) on their anteriorly directed flagellum on the zoospore. Evidence tends to suggest that Oomycetes diverged before one of their relatives acquired a photosynthetic cell with chlorophylls *a* and *c,* leading to the radiation of stramenopile algae. Based on rDNA sequencing, the Oomycetes are basal to algae in the Kingdom Straminipila (Berbee and Taylor, 1999) and there is no evidence of the plastid or plastid-associated membranes in Oomycetes which would be evidence of an algal ancestry (Barr, 1992).

The Oomycetes are a class comprised of over 65 genera and about 500 to 800 described species (Dick, 1990b). They form a cosmopolitan group of organisms found in freshwater, salt water, and terrestrial environments. While many aquatic forms are saprobic on dead plant and animal matter, some are parasitic on algae and small aquatic animals. The majority of terrestrial Oomycetes are facultative or highly specialized parasites of vascular plants causing diseases such as late blight of potato (e.g. *Phytophthora infestans*), root rot (e.g. *Phytophthora sojae*), downy mildews (e.g. *Plasmopara viticola*), and damping off of seedlings (e.g. *Pythium ultimum*).

Oomycetes possess many physiological, biochemical, and morphological characteristics which differentiate them from true fungi. The life cycle of Oomycetes is based on vegetative diploidy (Dick, 1995) and the ploidy cycle is haplomitotic B, with mitosis confined to the diploid phase (Dick, 1987). So unlike in many true fungi, haploid mitosis does not occur. Rather, a diploid thallus is produced and meiosis occurs in the developing gametangia.

Many Oomycetes do not synthesize sterols, although they may be required for sexual reproduction. Among those that do synthesize sterols, fucosterol is most abundant, in contrast to true fungi where ergosterol is the characteristic sterol. Polyene antiobiotics, which target membrane-bound sterols and are effective against true fungi, are subsequently ineffective against Oomycetes. The biflagellate zoospore of Oomycetes has straminipilous ornamentations on the anterior flagellum and a smooth posterior whiplash flagellum. The cell walls contain cellulose rather than chitin as in true fungi (Aronson, 1965), although there may be greater diversity in cell wall composition as chitin has been reported in *Achlya* (Campos-Takaki *et al.,* 1982) and *Saprolegnia* (Bulone *et al.,* 1992). The mitochondria of Oomycetes contain tubular cristae while true fungi have flattened cristae as do animals. As well, the Golgi bodies consist of multiple flattened cisternae compared to the relatively poorly developed single cisternal element in true fungi. Oomycetes also possess unique dense body vesicles in the protoplasm which may be involved in the storage of polysaccharides or phosphates (Wang and Bartnicki-Garcia, 1974).

But perhaps the most significant biochemical indication that Oomycetes do not share a common origin with fungi is their mode of lysine biosynthesis. Oomycetes synthesize lysine via the diaminopimelic acid (DAP) synthetic pathway (as in plants and bacteria) while true fungi utilize the α-aminoadipic acid (AAA) pathway (Vogel, 1960). It is thought that the DAP synthetic pathway may interfere with the pathway for chitin synthesis (LéJohn, 1972).

B. The Genus *Pythium*

Pythium is a genus in the class Oomycetes, order Pythiales, and family Pythiaceae, that is comprised of over 120 described species (Van der Plaats-Niterink, 1981; Dick, 1990a). The genus was established by Pringsheim (1858) based on descriptions of *P. monospermum* and *P. entophytum.* The latter species was transferred to *Lagenidium* leaving *P. monospermum* as the type species of the genus. *Pythium* was placed in the Saprolegniaceae by Pringsheim (1858) and later in the Peronosporaceae by de Bary (1881) where it currently is the type genus of the Pythiaceae. *Pythium* species live in water and soil as saprophytes on plant and animal substrates, but they can also be parasitic. Most parasitic species are plant pathogens causing diseases such as post- and pre-emergence damping-off and root rot. Some species, such as *P. oligandrum,* are also mycoparasites of other fungi and even other *Pythium* species (Deacon, 1976). *Pythium insidiosum* is the only species in the genus that infects mammalian hosts and is the etiological agent of pythiosis (de Cock *et al.,* 1987).

C. Asexual reproduction

Zoosporangia are the structures in which zoospores are produced, although the cleavage of zoospores occurs in a vesicle that protrudes from the zoosporangium. The released zoospores encyst and germinate via a germ tube. There is a great deal of morphological diversity among zoosporangia. Some species produce filamentous zoosporangia which may be strongly inflated forming lobate or toruloid structures (e.g. *P. torulosum*), slightly inflated forming dendroid structures (e.g. *P. aquatile*), or not inflated at all (e.g. *P. diclinum*). Other species form globose zoosporangia (e.g. *P. hypogynum*)

while a few form proliferating zoosporangia whereby a new zoosporangium forms from within the initial zoosporangium (e.g. *P. multisporum*). However, not all species have been observed to produce zoosporangia. For example, *P. ultimum* var. *ultimum* only produces undifferentiated hyphal swellings (Van der Plaats-Niterink, 1981).

D. Sexual reproduction

The majority of *Pythium* species are homothallic (self-fertilizing). However, after Campbell and Hendrix (1967) first described heterothallism (outcrossing) in *P. sylvaticum,* there have been six other species with similar heterothallic tendencies described (Van der Plaats Niterink, 1981). Nonetheless, even some homothallic species are thought to be capable of outcrossing, such as *P. ultimum* (Francis and St. Clair, 1993).

Sexual reproduction in *Pythium* occurs via gametangial contact between the oogonium and antheridium. Oogonia are globose, ellipsoid, or lemon shaped with spiny ornamentations on the wall in some species. The oogonia may have a terminal or intercalary location on a hypha. Antheridia are club-shaped hyphal tips which are stalked or sessile. The number of antheridia present per oogonium varies among species, from one to several. The antheridial arrangement is described as being monoclinous (originating from the oogonial stalk), diclinous (originating from a different hypha than the oogonium), or hypogynous (a proximate part of the oogonial stalk becomes the antheridium). The oospore forms as a result of sexual reproduction, and after a dormant phase it germinates via a germ tube. The diameter of the oospore wall varies among species. Plerotic oospores fill the entire oogonium while a space is present between the oogonial wall and oospore wall in aplerotic oospores.

E. Morphological classification of *Pythium* species

The identification of *Pythium* species has traditionally been based on morphological characters, such as the sexual and asexual structures described above. The first identification keys of the genus by Matthews (1931) and Middleton (1943) focused on zoosporangial morphology. Waterhouse (1967) later developed a key which separated species based on reproductive structure size. Hendrix and Papa (1974) compiled a list of "species-complexes" in *Pythium,* placing species into groups based on morphological characteristics. Van der Plaats-Niterink (1981) then produced a key using oogonial wall character and the presence of reproductive structures as primary criteria. In the most recent key of the genus, Dick (1990a) used zoosporangial morphology and characteristics of oogonia and antheridia to produce a Venn-diagram key, updating one published earlier by Dick and Ali-Shtayeh (1986).

Identification of *Pythium* species using these keys requires a great deal of time and expertise, and is often complicated by the limited number of morphological characters to delimit species. Variability in morphological characters between isolates of the same species is common and can be a result of the growth media used, temperature, and the age of the culture (Hendrix and Papa, 1974). As well, some isolates fail to produce sexual structures in culture and are placed into general groupings of isolates based on the descriptions of their zoosporangia or hyphal swellings (Van der Plaats-Niterink, 1981). Due to these difficulties, several attempts have been made using biochemical, physiological, immunological, and molecular data to identify *Pythium* species and define species boundaries.

F. Protein and isozyme analysis

The earliest attempts using biochemical data for species identification in *Pythium* compared starch gel electrophoresis patterns of total mycelial proteins between species (Clare, 1963). However, Clare *et al.* (1968) found electrophoretic patterns produced by histochemical staining of specific enzymes of greater value than total protein electrophoresis to characterize taxonomic groups. Chen *et al.* (1988) also noted that the total protein electrophoresis banding patterns may change over time. In a comparison of isozyme polymorphism analysis with total protein electrophoresis, Chen *et al.* (1991; 1992b) found both methods could distinguish morphologically distinct *Pythium* species, but were less effective at distinguishing morphologically similar species. Intraspecific variation was also higher in starch gel eletrophoretic patterns compared to isozyme patterns. Isozyme variation among different loci was used by Barr *et al.* (1997a) to differentiate isolates representing six heterothallic *Pythium* species and to distinguish isolates of *P. irregulare* from closely related taxa (Barr *et al.,* 1997b). This technique was also a beneficial aid to identify unknown isolates that do not produce oospores in culture Barr *et al.* (1996).

Among other protein-based identification techniques, Adaskaveg *et al.* (1988) used isoelectric focusing to differentiate *Pythium* species. While many bands were common among species, some bands were species-specific and could be used to distinguish among species. Takenaka and Kawasaki (1994) also found that lectin analysis of cell wall glycoproteins provided sufficient variation for species identification.

G. Serological identification methods

Serological techniques have been used for species comparisons in *Pythium.* With immunochemotaxonomic techniques, Krywienczyk and Dorworth (1980) were able to divide species into serological groupings. White *et al.* (1994) used the enzyme-linked immunosorbent assay (ELISA) to assess the extent of antibody-binding with cell wall fractions of several *Pythium* species and create serological profiles that were unique to each species. Lyons and White (1992) used polyconal antibodies and competition ELISA to detect *P. violae* and *P. sulcatum* in carrots. Similarly, polyclonal antibodies were raised against zoospore surface antigens of *P. porphyrae* to detect this species in *Porphyra yezoensis* (Addepalli and Fujita, 2001). Monoclonal antibodies have also been produced for detection and identification of *P. graminicola* (White, 1975), *P. ultimum* (Yuen *et al.,* 1993), *P. sulcatum* (Kageyama *et al.,* 2002), and the zoospores and cysts of *P. aphanidermatum* (Estrada-Garcia *et al.,* 1989).

H. Molecular identification of *Pythium* species

i) Species identification using RFLPs and PCR

Initial molecular biology techniques to identify *Pythium* species utilized RFLPs to detect interspecific polymorphisms. Klassen *et al.* (1987) used restriction site maps of rDNA units to differentiate *Pythium* species from each other and from closely related genera. Mitochondrial DNA RFLPs were later used by Martin and Kistler (1990) to distinguish several species from one another. Low levels of intraspecific variation in fragment patterns were observed, although some species (e.g. *P. sylvaticum* and *P. heterothallicum*) showed relatively high levels of similarity in their fragment patterns.

Comparisons of mtDNA restriction fragment patterns were also used to show *Pythium* type HS isolates (Martin, 1990a) and type G isolates (Huang *et al.,* 1992) were asexual forms of *P. ultimum.* RFLPs of total genomic DNA have been used by Lévesque *et al.* (1993) to find a correlation between certain fragment profiles of *P. ultimum* and the soil type from which they were isolated. Similarly, Descalzo *et al.* (1996) used total genomic DNA restriction fragment patterns to group isolates from glyphosate-treated bean seedlings in an attempt to correlate genotype with glyphosate synergists.

The utilization of PCR to amplify specific regions makes rDNA a commonly used target for delimiting species in *Pythium.* Chen (1992) used RFLP analysis of PCR amplicons of the ITS and regions of the SSU rRNA and LSU rRNA genes to distinguish among three heterothallic species. The ITS provided the best resolution, and fragment analysis subsequently revealed that heterothallism evolved polyphyletically in *Pythium.* Similarly, PCR-RFLP analysis of the ITS and SSU rRNA gene distinguished five *Pythium* species (Chen *et al.,* 1992a), although only the RFLPs of the ITS were species-specific. Chen and Hoy (1993) also used RFLPs of the ITS to show that *P. arrhenomanes* and *P. graminicola* were distinct species, even though protein electrophoresis and isozyme polymorphisms failed to distinguish the two species (Chen *et al.,* 1991, 1992b). Rafin *et al.* (1995) showed a high level of similarity among *Pythium* type F isolates using RFLPs of the ITS, and that fragment patterns of type F isolates were identical to those of *P. aqautile* and *P. coloratum.* Wang and White (1997) used ITS RFLPs to distinguish and identify 36 *Pythium* species, and most species could be distinguished, except *P. aphanidermatum* and *P. butleri.* Cluster analysis based on fragment profile separated the species into two main branches: i) species with filamentous zoosporangia and smooth

oogonia, and ii) species with globose zoosporangia and smooth or ornamented oogonia. RFLPs of the ITS have also been employed to investigate levels of intraspecific variation among isolates of *P. irregulare* (Matsumoto *et al.,* 2000) and *P. ultimum* (Chee and Kim, 2000).

Other molecular biology techniques using PCR have been used to identify *Pythium* species. With isolates of *P. aphanidermatum,* RAPDs revealed a low degree of intraspecific variation and distinguished them from other species (Herrero and Klemsdal, 1998). RAPDs have also been used to examine intraspecific variation among isolates of *P. irregulare* (Matsumoto *et al.,* 2000). The construction of PCR primers to amplify species-specific sequences in the ITS has been applied to detect *P. ultimum* in diseased plants (Kageyama *et al.,* 1997) and the zoospores of *P. porphyrae* in seawater (Park *et al.,* 2001).

ii) DNA probes

Among *Pythium* species, species-specific probes have been constructed for several species using different target regions. Probes based on mtDNA fragments were specific for *P. oligandrum* and *P. sylvaticum* (Martin, 1991). The 5S rRNA gene spacer provided an additional source of species-specific probes for eight *Pythium* species (Klassen *et al.,* 1996). A restriction fragment from ITS-1 was specific for *P. ultimum* (Lévesque *et al.,* 1994), and the ITS-1 region has also served as a source of oligonucleotides probes in reverse dot blot hybridizations (Lévesque *et al.,* 1998), and as species-specific capture probes in PCR-ELISA assays (Bailey *et al.,* 2002).

iii) Sequence analysis

Sequence analysis is useful for examining phylogenetic relationships among *Pythium* species. It has also frequently been used to confirm the species identifications of isolates from nature (e.g. Galland and Paul, 2001) and in the descriptions of new species (e.g. Paul, 2001). However, as a result of phylogenetic studies, sequence analysis has also provided insight as to which morphological characters may carry more taxonomic weight than others in the diagnostic keys. Briard *et al.* (1995) used sequences from the variable D2 domain of the LSU rRNA gene to examine relationships in the Pythiaceae. The eight *Pythium* species were divided into two groups, one containing species with spherical zoosporangia and the other comprised of species with filamentous zoosporangia. These clusters did not agree with the morphological groupings of Waterhouse (1967) and Van der Plaats-Niterink (1981) which emphasized reproductive structure size and oogonial wall characters, respectively. Sequencing of the ITS by Matsumoto *et al.* (1999) revealed similar clustering patterns. Two clusters were formed and designated as the F-group (species with filamentous and lobulate zoosporangia), the S-group (species with spherical zoosporangia or hyphal swellings). A phylogenetic analysis using the mitochondrial cytochrome oxidase II gene also resulted in the formation of three clades which generally reflected zoosporangial morphology (Martin, 2000). Therefore, there appears to be a correlation between molecular clusters and zoosporangial groups, suggesting an ancient differentiation of this character, and that perhaps zoosporangial form may have more hierarchical importance among morphological characters, as suggested by Middleton (1943).

IV. *Pythium insidiosum*: the etiological agent of pythiosis

A. Pythiosis

Pythiosis is a granulomatous disease which occurs in temperate, tropical, and subtropical areas of the world. The disease has been reported in horses, dogs, cats, cattle, and a captive spectacled bear with symptoms including cutaneous and subcutaneous infections (Miller *et al.,* 1985; Dykstra *et al.*, 1999), bone lesions (Mendoza *et al.,* 1988; Alfaro and Mendoza, 1990), esophagitis (Patton *et al.,* 1996), gastrointestinal disease (Allison and Gillis, 1990; Buergelt, 2000), and pulmonary infections (Goad, 1984). Pythiosis also occurs in humans, causing arteritis (inflammation of arteries) (Sathapatayavongs *et al.,* 1989; Thitithanyanont *et al.,* 1998), keratitis (corneal inflammation) (Virgile *et al.,*1993; Badenoch *et al.,* 2001), and cutaneous or subcutaneous infections (Shenep *et al.,* 1998). In studies on experimental pythiosis, both normal and cortisone-treated rabbits were equally susceptible to infection by zoospores (Miller and Campbell, 1983), which does not support a hypothesis that an impaired immune response is an important predisposing factor to pythiosis. Sathapatayavongs *et al.* (1989) suggested that thalassemia hemoglobinopathy syndrome may be an important predisposing factor in human pythiosis cases, but the factors which predispose thalassemic patients to pythiosis are not known.

B. History of *Pythium insidiosum*

While the term "pythiosis" was first introduced by Chandler *et al.* (1980), reported cases of pythiosis date back to the nineteenth century. Jackson (1842), Smith

(1884), and Drouin (1896) reported cases of equine granulomatous lesions sharing an etiological agent with a mycelial nature. The disease was initially named "hyphomycosis destruens" by de Haan and Hoogkamer (1901), but later extended to "hyphomycosis destruens-equi" (de Haan, 1902). However, the etiological agent could not be identified as fruiting bodies did not form.

Bridges and Emmons (1961) described isolates from several equine cases in Texas based on colony and mycelial morphology, and named the isolate *Hyphomyces destruens.* Although they could not induce sporulation, they speculated the isolate was related to the Mortierellaceae of the Zygomycetes. Amemiya and Nishiyana (1966) made similar observations with an isolate from an equine in Japan, concluding it was a species of the class Phycomycetes in the Family Mortierellaceae. However, the binomial name *H. destruens* was proposed without a Latin description and the type was not designated which violated articles in the *International Code of Botanical Nomenclature* (Voss *et al.,* 1983).

Austwick and Copland (1974) observed zoosporangia in an equine isolate from Papua New Guinea and biflagellate zoospores were produced. They concluded that it was a Phycomycete and should be included in the genus *Pythium* in the order Peronosporales and Family Pythiaceae. Later, Ichitani and Amemiya (1980) isolated a *Pythium* isolate from an equine in Japan and equated it with *P. gracile* Schenk. However, others never confirmed this identification. De Cock *et al.* (1987) observed the development of oogonia in several mammalian strains of *Pythium* and described the new species *Pythium insidiosum,* the etiological agent of pythiosis. Around the same time, Shipton (1987) described isolates from equines in Australia as members of the new species *P. destruens.*

These isolates were essentially identical to *P. insidiosum* and speculation arose as to whether the etiological agent of pythiosis represented a single species or several.

C. Antigenic relationships

Mendoza *et al.* (1987) studied antigenic relationships among isolates of *P. insidiosum* from around the world. In immunodiffusion (ID) precipitin tests, a *P. insidiosum* antiserum created against antigens of the ex-type strain and adsorbed with antigens from *P. diclinum* was specific for all the isolates of *P. insidiosum.* In addition, a fluorescein isothiocyanate-conjugated rabbit antiglobulin to *P. insidiosum* stained cellular elements of all *Pythium* species tested, but stained only isolates of *P. insidiosum* when pre-adsorbed with hyphal elements of *P. diclinum* and *P. graminicola.* This showed that while all *Pythium* species shared antigens with *P. insidiosum,* the antigenic differences were sufficient to distinguish *P. insidiosum* from other species. As well, all human and animal *P. insidiosum* isolates were antigenically identical to the ex-type strain.

Using similar ID techniques, Mendoza and Marin (1989) showed that antigens and antisera of the ex-type strains of *P. insidiosum* and *P. destruens* shared at least three precipitin bands in common. When the antiserum of each species was reacted against other strains from animals and humans, three common precipitin bands were observed as well.

Based on the antigenic relationships among mammalian and human isolates by Mendoza *et al.* (1987) and Mendoza and Marin (1989), and also on sexual morphological similarities, it was suggested that all isolates of *P. insidiosum* and *P. destruens,* including the isolate *P. gracile* sensu Ichitani and Amemiya, were the same species.

D. *Pythium insidiosum* Life Cycle

Miller (1983) first suggested a life cycle for *P. insidiosum* and demonstrated that zoospores were strongly attracted to the hairs of equines, cows, rabbits, mice, and humans. However, it was unclear what the natural host or substrate for the vegetative state was and how animals were infected. Since many *Pythium* species are plant pathogens, an aquatic plant would make an ideal natural host and animals could become infected when in close contact with swampy waters.

Mendoza *et al.* (1993) proposed a more detailed life cycle which considered the asexual cycle and explained the *in vivo* events leading up to the invasion of plant and animal tissues. Strains developed filamentous zoosporangia from an undifferentiated hyphal tip on pieces of grass and water lilies. Upon release from the zoosporangial vesicle, zoospores were chemotactically attracted to bits of grass, water lily leaves, hair, and horse hide. After zoospores encysted, they became spherical and formed a nipple-like projection leading to germ tube formation, but no invasive pegs or appressoria were detected. Ravishankar *et al.* (2001) demonstrated that *P. insidiosum* hyphae do not exert sufficient pressure to penetrate undamaged skin by mechanics alone, and concluded that hyphae must also secrete proteinases which would reduce tissue strength and allow penetration. Zoospores which encysted on leaves, hair, and skin were covered with an amorphous material. This may act as an adhesive substance to attach zoospores to host surfaces and explain how zoospores bind and remain attached to damaged plant and animal tissues. Secretion of an adhesive cell coat during encystment to bind cells to host surfaces is common among pythiaceous zoospores (Sing and Bartnicki-Garcia, 1972).

The sexual cycle and production of oospores was not observed, but it was suggested that sexual reproduction may occur in nature, producing oospores to serve as resistant spores.

It was therefore assumed that zoospores are the invasive agent in pythiosis due to their chemotactic attraction to animal tissues and their ability to produce adhesive substances to maintain tight contact with the host during the initial stages of infection. Therefore, if an animal enters the ecosystem, zoospores are attracted by injured tissue and can invade the host causing pythiosis.

E. Diagnosis of pythiosis

Since pythiosis often responds poorly to available treatments, early diagnosis is essential to assure timely and effective treatment. However, clinically and histologically, *P. insidiosum* infections mimic infections caused by members of the class Zygomycetes (Kaufman *et al.,* 1990). Therefore, it is important to differentiate pythiosis and *P. insidiosum* from these similar diseases and their etiological agents. The clinical signs of pythiosis may also resemble symptoms of other illnesses. For example, the presence of tissue masses in dogs often leads to a misdiagnosis of neoplasia (Buergelt, 2000).

i) Culture characteristics

Clinical specimens (pus, tissue biopsies, etc.) can be grown in culture and examined microscopically. In fresh or smear preparations, *P. insidiosum* hyphae appear short and flat with branching hyphae perpendicular to the main stem, and septae are usually present in older colonies. Isolates have been shown to produce oogonia only on cornmeal agar (de Cock *et al.,* 1987). Perhaps the most important diagnostic cultural

feature is the production of motile biflagellate zoospores, which can be induced in water cultures using grass blades and Ca^{2+}, Mg^{2+}, and K^{+} ions (Mendoza and Prendas, 1988). However, inducing zoospore formation in some cultures may take about a week and some isolates will not produce zoospores in culture.

ii) Histopathology

Tissue sections can be stained to examine histopathologic features of the disease. Hyphae usually stain poorly or not at all with periodic acid-Schiff (PAS) methods commonly used for fungal infections, since the cell wall consists of cellulose (Thainprasit *et al.,* 1996). With haematoxylin-eosin (HE) staining, hyphae are visible, but not very distinct. Gomori's methenamine-silver nitrate stain (GMS) is the superior method for staining *P. insidiosum* hyphae in tissue. With the GMS stain, the short, rarely septate hyphae with perpendicular branching are visible, but members of the Zygomycetes have a similar appearance in histological sections (Dykstra *et al.,* 1999).

Numerous "kunkers" (yellow-white, coral-like necrotic masses of tissue which contain hyphae) are often present in cutaneous and subcutaneous lesions (Thianprasit *et al.,* 1996). These lesions usually exhibit an intense eosinophilic inflammatory reaction, but macrophages, neutrophils, giant cells, lymphocytes, and plasma cells are usually also found. The Splendore-Hoeppli phenomenon (eosinophilic sleeves of antigen-antibody complexes and cell debris surrounding hyphae) is also often observed (Miller *et al.,* 1985). These eosinophil and antigen-antibody complex precipitates are usually narrower in pythiosis compared to those in conidiobolomycosis, basidiobolomycosis, and

cutaneous habronemiais, and may be a useful character for differentiating *P. insidiosum* from other Zygomycetes in tissue (Patton *et al.,* 1996).

iii) Serological Methods

Numerous serological methods have been developed to diagnose and monitor pythiosis in animals and humans. Miller and Campbell (1982) first developed a complement fixation test, but it only detected pythiosis in about 82% of infected equines and sometimes yielded false positives for healthy equines. Brown *et al.* (1988) developed an immunoperoxidase test to provide an immunohistochemical method for staining *P. insidiosum* in tissues when histological examination was not conclusive. Via an indirect peroxidase staining technique, rabbit anti-*P. insidiosum* antibodies specifically stained *P. insidiosum* hyphae in tissue sections. A fluorescein isothiocyanate-conjugated rabbit antiglobulin to *P. insidiosum* is also used to stain hyphal elements of *P. insidiosum* in tissue (Mendoza *et al.,* 1987).

Immunodiffusion tests have been the most widely used serological diagnostic methods for pythiosis. Miller and Campbell (1982) first developed an ID test using a trypsinized antigen (TPA) from a *P. insidiosum* strain which detected one precipitin in infected horse serum. Mendoza *et al.* (1986) later developed a more complex and more stable antigen using culture filtrate antigen (CFA). Using antiserum from infected equines, CFA consistently detected 3 to 6 precipitins. While the number of precipitins did not correlate with the duration of illness or its severity, treated equines tended to show declines in the number of precipitins, and horses with zero to one precipitins could be considered clinically cured. Therefore, the CFA demonstrated a capacity for both

diagnosing and monitoring pythiosis during treatment. A practical ID test was later developed using a rabbit reference anti-*P. insidiosum* serum to detect *P. insidiosum* antibodies. Infected horse serum reacting with CFA and producing one or more line of identity with the reference system was considered positive for pythiosis. The ID test has also been evaluated for diagnosing pythiosis in human cases of subcutaneous and systemic infections (Imwidthaya and Srimuang, 1989) and arteritis (Pracharktam *et al.*, 1991).

While the ID test is entirely specific for pythiosis, Pracharktam *et al.* (1991) showed that the ID test failed to detect pythiosis in patients with ocular pythiosis, suggesting corneal infections may be inaccessible to immunological defense mechanisms. Thitithanyanont *et al.* (1998) also reported a negative ID test from a boy with a proven arteritic infection due to *P. insidiosum.* To improve diagnosis of pythiosis, an ELISA test was developed by Mendoza *et al.* (1997) using soluble antigen from broken hyphae (SABH) of *P. insidiosum.* Out of 13 human and animal pythiosis cases tested, only eight were positive for pythiosis with the ID test while all 13 were positive using ELISA. Grooters *et al.* (2002) also developed an ELISA test using soluble mycelial extract which is very effective in diagnosing canine pythiosis and monitoring responses to therapy. Therefore, ELISA is a sensitive and accurate method for detecting pythiosis in early and chronic stages of the disease, and is especially useful when the ID test fails.

F. Therapy for pythiosis

If pythiosis is not treated early, the disease commonly progresses to a chronic stage leading to severe destruction of tissues and organs and often results in amputation

or fatality. While no single therapy is available which is curative in 100% of cases, several treatments are available including surgery, chemotherapy, and immunotherapy.

i) Surgery

Surgical removal of tissues containing *P. insidiosum* is commonly used in veterinary medicine. However, it is sometimes impractical as lesions may be too extensive or may involve critical anatomical structures. Often it is difficult to determine how much healthy tissue must also be removed and lesions often reappear if the kunkers are not completely removed. The use of a neodymium:yttrium-aluminum-garret laser has been used in conjunction with surgery to kill any organisms which may infiltrate surrounding healthy tissue and, thus, the treatment reduces the risk of recurrence (Sedrish *et al.,* 1997).

ii) Chemotherapy

While *P. insidiosum* is not susceptible to potassium iodide (KI) i*n vitro,* the treatment of pythiosis with a saturated solution of potassium iodide (SSKI) has produced beneficial results in two human cases (Sathapatayavongs *et al.,* 1989) and in two other human cases of subcutaneous pythiosis (Thainprasit, 1990) causing lesions to subside and disappear. Therefore, SSKI is an agent worth trying in certain cases. Antifungal agents that target ergosterol in the cell membrane or interfere with ergosterol synthesis should be ineffective in treating pythiosis. Nonetheless, Sekhon *et al.* (1992) showed that *P. insidiosum* was susceptible to fluconazole, ketoconazole, and miconazole *in vitro.* Other agents were effective, but only at concentrations that would be toxic to humans. In a

feline with a nasal and retrobulbar infection, treatment with ketoconazole resulted in temporary clinical improvement (Bissonnette *et al.,* 1991), and Triscott *et al.* (1993) successfully treated two human cases of subcutaneous pythiosis with a combination of amphotericin B, 5-fluorocytosine, and hydrocortisone. In a child with a deeply invasive infection of the head and neck, Shenep *et al.* (1998) demonstrated successful synergistic treatment using the combination of itraconazole and terbinafine.

Streptomycin has been shown to inhibit the growth of some Oomycetes (Gottlieb and Shaw, 1970), however, McMeekin and Mendoza (2000) showed that streptomycin had varying effects on the growth of *P. insidiosum.* Growth of isolates from Florida, Tennessee, and Costa Rica was inhibited or not significantly affected, while the growth of a human strain from Thailand was stimulated by streptomycin. Pythiosis is often treated with antibiotics for bacterial infections prior to the detection of *P. insidiosum* in infected tissues, so the indiscriminate prescription of streptomycin or other antibiotics may actually be deleterious to patients with undiagnosed pythiosis.

iii) Immunotherapy

Immunotherapy was first applied by Miller (1981) using a cell mass vaccine (CMV) created from sonicated hyphal elements of *P. insidiosum.* While the vaccine cured 53% of treated equines, complications including severe inflammation and abscesses were common (Miller *et al.,* 1983). Mendoza and Alfaro (1986) developed a second vaccine using CFA that resulted in a less severe inflammatory reaction and no abscesses. The only complications reported were inflammation in affected tissues by *P. insidiosum* and secondary bacterial contamination in surrounding tissues. Inflammation

supposedly indicates that the host immune system is functioning and predicts that the horse will be cured. It was believed that immunotherapy showed the best results in the early stages, but not in chronic forms of the disease as tissues were already destroyed and lesions heavily contaminated with bacteria (Mendoza *et al.,* 1988).

In a comparison of the CMV and CFA vaccines (Mendoza *et al.,* 1992b), no statistical difference in each vaccine's capacity to cure horses was found, but the inflammatory reactions were less severe with the CFA vaccine. Horses that have lesions one month or younger showed a quick response to each vaccine, but those with lesions two months or older did not respond and died. Miller *et al.* (1983) suggested that after vaccination, kunkers containing dead hyphae degenerate and act as foreign bodies causing tissue necrosis. Mendoza *et al.* (1992b) pointed out that kunkers are heavily contaminated with bacteria in chronic cases, so while horses that have lesions 1.5 months or older show an initial improvement after vaccination (degeneration of kunkers), the bacteria present in the infected tissue may continue the infection. While the vaccine is effective in treating pythiosis in horses, it has also been used to cure a human case of pythiosis in a boy with arteritis (Thitithanyanont *et al.,* 1998).

In general, both vaccines cured pythiosis in over 50% of pythiosis cases, but always failed in chronic cases. Immunoblotting experiments with immunoglobulin G (IgG) from sera of horses with pythiosis revealed three immunodominant antigens with sizes of 28, 30, and 32 kDa (Mendoza *et al.,* 1992a). The antibodies for these proteins persisted for long periods of time in horses that had been successfully vaccinated, suggesting that these three proteins may possess protective and therapeutic features. Dixon *et al.* (1998) reported that supplementing the CFA vaccine with these three

purified proteins enhanced the curative properties of the vaccine from 53% to 70% and was successful in some chronic cases that were 60 days or older.

There is much speculation about the mechanism by which the vaccine functions. Cohen (1994) suggested that the vaccine boosts the immune system of the infected horse, but Dixon *et al.* (1998) believed the mechanism might be due to the presentation of epitopes previously shielded in the necrotic tissue masses. Mendoza *et al.* (1992b) noted that in the ID test, the number of precipitin bands did not increase after vaccination, but rather decreased over time in cured cases. This may suggest that cellular immunity, rather than humoral immunity, is involved in the elimination of infection. This is also supported by the fact that after vaccination, the initial eosinophilic inflammatory reaction gradually changes to a mononuclear immunoresponse replacing the eosinophils with an infiltrate with neutrophils, cytotoxic T lymphocytes, plasma cells, and macrophages.

V. Thesis objective

The objective of this thesis was to apply the phylogenetic species concept using RFLP analysis of the rDNA IGS to identify *Pythium* species and examine species relationships, thus overcoming the difficulties encountered with the morphological species concept. This technique, in addition to ITS sequence analysis, was also used to study intraspecific variation and phylogenetic relationships among isolates of *P. insidiosum* and related species and genera. Lastly, a region of the IGS was used as a species-specific DNA probe to differentiate *P. insidiosum* from other *Pythium* species and organisms that cause symptoms similar to pythiosis.

CHAPTER 2

Materials and methods

Culture conditions and DNA isolation

All *Pythium* isolates used in this study are listed in Tables 3.1, 4.1, and 5.1. *Pythium* strains were subcultured from agar slants onto cornmeal agar (CMA) at 25°C for up to 7 d. For each strain, approximately five to six agar plugs from these actively growing cultures were transferred into sterile petri dishes containing pea-broth medium (200 g of frozen peas boiled in 1.0 L of water, then filter decanted, to which 5.0 g glucose was added) and incubated at 25°C from 2 to 3, or up to 10, days. The mycelial mat was harvested by vacuum-filtration using Whatman No. 1 filter paper (Fisher Scientific, Nepean, Ontario), washed twice with distilled water, and frozen at –20°C. Frozen mycelial mats were freeze-dried overnight and subsequently stored at –20°C until needed for DNA extraction. For strains of *P. insidiosum,* agar plugs containing mycelia were used to inoculate 100 mL Sabouraud broth (2.0 % glucose, 1.0 % peptone) which was incubated at 37°C while being rotated at 150 rpm for 5 d. Mycelia were harvested by vacuum-filtration and stored as described above.

DNA was extracted from mycelia according to the method of Möller *et al.* (1992) with some modifications. Approximately 30 mg of freeze-dried mycelia were extracted in 1 mL of TES buffer [100 mM Tris (pH 8.0), 10 mM EDTA, 2 % sodium dodecyl sulphate (SDS)] with the addition of 100 to 200 mg of proteinase K (Sigma-Aldrich Corp., St. Louis, Missouri) and then incubated at 55°C for 30 min with occasional agitation. To this was added 280 µL 5 M NaCl and 138 µL cetyltrimethylammonium bromide (CTAB)/NaCl (10 % / 0.7 M) and the mixture was incubated at 65°C for 10 min. The solution was then centrifuged at 14,000 x g for 10 min at 4°C to remove cell debris and the supernatant was extracted in an equal volume of chloroform:isoamyl alcohol

(24:1) followed by a 30 min incubation on ice. The mixture was centrifuged at 14,000 x g for 10 min at 4°C and the supernatant was again removed and extracted in 450 µL of 5 M ammonium acetate followed by an incubation on ice for 30 min. The mixture was centrifuged at 14,000 x g for 10 min at 4°C and DNA was precipitated from the supernatant by adding 715 µL of isopropanol and centrifuging the sample at 14,000 x g for 15 min. The DNA pellet was washed for 15 min in 70% ethanol, dried, and resuspended in 100 µL TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Amplification of DNA for RFLP analysis and construction of DNA probes

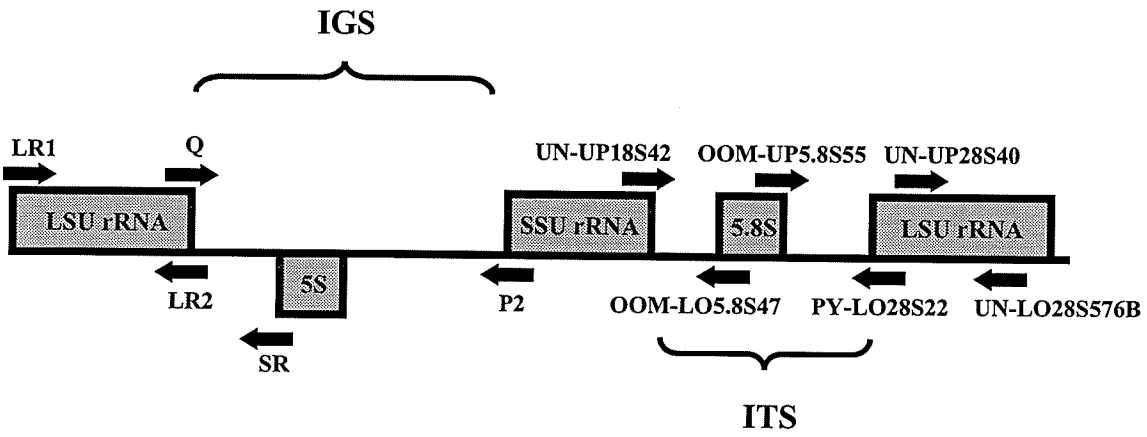
Regions of genomic DNA were amplified using the polymerase chain reaction (PCR) (Saiki *et al.,* 1988). To amplify the rDNA IGS and regions within, reactions were carried out in 50 µL volumes containing 5 µL *Taq* Extender 10x reaction buffer (Stratagene, La Jolla, California), 200 µM each of dATP, dCTP, dGTP, and dTTP, 1.5 mM $MgCl_2$, 20 pmol of each primer, 10 ng DNA template, 1.25 U *Taq* DNA polymerase, and 1.25 U *Taq* Extender (Stratagene). Primers used for amplifications are listed in Table 2.1 and their genomic locations are shown in Fig. 2.1. Amplifications were done using a Techne Unit Genius Thermocycler (Techne Incorporated, Princeton, New Jersey) with the following temperature cycling parameters: denaturation at 93°C for 3 min for the first cycle and 1 min for subsequent cycles, annealing for 1 min at 50°C, and elongation for 10 min at 65°C for primers Q and P2, 5 min at 72°C for primers LR1 and LR2, and 2 min at 72°C for primers Q and SR. The parameters were repeated for 20 cycles for primers Q and P2, 25 cycles for LR1 and LR2, and 30 cycles for Q and SR followed by a final elongation at the respective elongation temperature for 8 to 10 min.

Table 2.1. List of oligonucleotide primer sequences used for PCR amplifications and DNA sequencing reactions. Locations of primers within the rDNA repeat unit are indicated in Fig. 2.1.

Primer	Sequence (5' to 3')	Reference
Q	ACGCCTCTAAGTCAGAATC	Belkhiri *et al.* (1992)
P2	ATACTTAGACATGCATGGC	Belkhiri *et al.* (1992)
SR	GAAGCCCGGGTGCTGTCTAG	Klassen *et al.* (1996)
LR1	GCATATCAATAAGCGGAGGA	Henrion *et al.* (1992)
LR2	GACTTAGAGGCGTTCAG	Henrion *et al.* (1992)
UN-UP18S42	CGTAACAAGGTTTCCGTAGGTGAAC	Bakkeren *et al.* (2000)
UN-LO28S576B	CTCCTTGGTCCGTGTTTCAAGACG	Bakkeren *et al.* (2000)
PY-LO28S22	GTTTCTTTTCCTCCGCTTATTAATATG	Man in't Veld *et al.* (2002)
OOM-LO5.8S47	ATTACGTATCGCAGTTCGCAG	Man in't Veld *et al.* (2002)
OOM-UP5.8S55	TGCGATACGTAATGCGAATT	Man in't Veld *et al.* (2002)
UN-UP28S40	GCATATCAATAAGCGGAGGAAAAG	this study
T3[a]	AATTAACCCTCACTAAAGGG	Stratagene
T7[a]	GTAATACGACTCACTATAGGGC	Stratagene

[a]Used for sequencing reactions of insert DNA in the vector pPCR-Script Amp SK(+).

Figure 2.1. Schematic representation of primers used for PCR amplification and DNA sequencing of regions within the rDNA repeat unit. Primer sequences are listed in Table 2.1. Arrows indicate the approximate locations and orientations of the primers. Open boxes represent the LSU (large subunit), 5.8S, SSU (small subunit), and 5S rRNA genes. The IGS (intergenic spacer) and ITS (internal transcribed spacer) regions are also indicated.


IGS
LR1
Q
UN-UP18S42
OOM-UP5.8S55
UN-UP28S40
LSU rRNA
SSU rRNA
5.8S
LSU rRNA
LR2
5S
P2
OOM-LO5.8S47
PY-LO28S22
UN-LO28S576B
SR
ITS

Restriction endonuclease digestions and agarose gel electrophoresis

Restriction endonuclease digestions of PCR-amplified DNA were carried out in total volumes of 15 µL containing 2.5 U of restriction endonuclease (Invitrogen, Carlsbad, California), 1.5 µL of the appropriate 10x REact buffer (Invitrogen), and 8 to 12 µL of amplified DNA. Restriction digestions were performed using *Alu*I, *Cfo*I, *Hae*III, *Hha*I, *Hin*cII, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I. Restriction digestions were incubated at 37°C for all enzymes, with the exception of *Taq*I which was incubated at 65°C, and the reactions were stopped after 2 h with the addition of 3.0 µL gel loading buffer [40% (w/v) sucrose, 0.25% bromophenol blue, 20 mM EDTA] to each reaction tube.

Electrophoresis of PCR products and of restriction digested DNA fragments was carried out in 1x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 20 mM EDTA) in 0.8 to 1.5 % agarose (Invitrogen) horizontal submarine gels at 2 to 10 V/cm. Agarose gels were stained with ethidium bromide and DNA bands were visualized under ultraviolet (UV) light (310 nm) on a transilluminator (Fotodyne Incorporated, Mississauga, Ontario) and photographed using Polaroid 667 film (Polaroid Corp., Waltharn, Massachusetts). The lengths of DNA fragments were estimated using the 1 kb Plus DNA Ladder (Invitrogen).

DNA sequencing of the rDNA ITS

Sequencing template preparation

All primers used for sequencing reactions are in Table 2.1 and their locations within the rDNA repeat unit are indicated in Fig. 2.1. PCR using primers UN-UP18S42

and UN-LO28S576B amplified a region of the rDNA repeat unit including the 3' end of the 18S rRNA gene, ITS-1, 5.8S rRNA gene, ITS-2, and approximately 580 bp of the 5' end of the 28S rRNA gene (Fig. 2.1). Amplifications were carried out in 20 µL volumes containing 0.1 to 10 ng genomic DNA, 0.1 mM dNTPs, 4.0 mM $MgCl_2$, 0.08 µM of each primer, and 1.0 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl]. Amplifications were done using a Techne Unit Genius Thermocycler (Techne Incorporated) with the following temperature cycling parameters: denaturation at 95°C for 3 min for the first cycle and 1 min for subsequent cycles, annealing for 45 s at 68°C, and elongation for 1 min 45 s at 72°C with a total of 30 cycles followed by a final extension for 10 min at 72°C. To assess the efficiency of the amplification, 2 µL aliquots of PCR products were electrophoresed in a 1.0% agarose gel in 1x TBE buffer to observe the quantities of amplicons produced by the reaction. The remaining volumes of the PCR amplicons were used directly for DNA sequencing.

DNA sequencing

Direct sequencing reactions of PCR products was performed using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 1.0 (Applied Biosystems, Foster City, California, USA). Each reaction was performed in a 20 µL total volume containing 2 µL sequencing mix diluted 1:4 in sequencing buffer [200 mM Tris (pH 9.0), 5 mM $MgCl_2$], 3.2 µM primer, and 5 to 20 ng of PCR template. Primers UN-UP18S42 and OOM-UP5.8S55 were used to obtain the forward sequences and primers PY-LO28S22 and OOM-LO5.8S47 for the reverse sequences (Table 2.1 and Fig. 2.1). Reactions were performed using a Robocycler Gradient 96 (Stratagene) with the

following parameters: 20 s denaturation at 95°C, 30 s annealing at 60°C, and 2 min elongation at 60°C for a total of 30 cycles. Automated sequencing was carried out using an ABI Prism 310 Genetic Analyzer and analyzed using the software Sequencing Analysis, version 3.0 (Applied Biosystems).

Phylogenetic analysis

RFLP analysis

Restriction fragment polymorphism (RFLP) analysis of rDNA IGS amplicons was used to estimate the genetic relationships among *Pythium* isolates and species. Amplicons of the IGS amplified using primers Q and P2 were restriction digested with *Alu*I, *Cfo*I, *Hae*III, *Hha*I, *Hin*cII, *Hin*fI, *Mbo*I, *Msp*I, *Rsa*I, and *Taq*I. The resulting restriction fragments were electrophoresed in a 1.5% agarose gel in 1x TBE buffer and the length of each restriction fragment was estimated using the 1 kb Plus DNA Ladder (Invitrogen). The presence or absence of each restriction fragment from each digestion was scored as "1" or "0", respectively, in a binary matrix for each isolate examined. Genetic distances were calculated for all pairwise comparisons of the isolates and used for the construction of a distance matrix according to Nei and Li (1979). This was used to produce phenograms with the unweighted pair groups method with averages (UPGMA) (Sneath and Sokal, 1973) and neighbor-joining methods (Saitou and Nei, 1987) using the Genetic Data Environment (GDE) (Smith *et al.,* 1994) through the Biological Research Computer Hierarchy (BIRCH) at the University of Manitoba (Fristensky, 1999).

ITS sequence analysis

Overlapping sequences from ITS sequencing reactions were assembled into contigs using SEQMAN (DNASTAR Inc., Madison, Wisconsin, USA). Sequences were aligned using CLUSTAL X (Thompson *et al.,* 1997) and the alignments were edited manually to improve the number of aligned sites using GeneDoc (Nicholas and Nicholas, 1997). Phylogenetic analysis using distance and parsimony methods was carried out using programs in PHYLIP, version 3.5c (Felsenstein, 1993). Pairwise distances between sequences were calculated with the DNADIST program using Kimura's two-parameter model (Kimura, 1980). The resulting distance matrix data was used for phylogenetic tree construction with the neighbor-joining algorithm (Saitou and Nei, 1987) using the NEIGHBOR program. Parsimony analysis (Fitch, 1971) was performed with the program DNAPARS using a subtree pruning and re-grafting branch swapping algorithm. For both distance and parsimony analysis, the sequence input order was randomized using the jumble option. To test the reliability of the inferred trees, bootstrap re-sampling (Felsenstein, 1985) with 1000 replicates was done using SEQBOOT. A majority-rule consensus tree was obtained using CONSENSE and visualized using TREETOOL.

_Hin_fI partial restriction digestion of *P. insidiosum* IGS-1 region

The IGS-1 region of *P. insidiosum* was amplified with PCR using primers Q and SR (Fig. 2.1 and Table 2.1). Partial digestions of Q-SR amplicons were carried out in a total volume of 15 µL. In eight separate reaction tubes, approximately 8 to 10 µL of PCR product was mixed with 1.5 µL REact 2 buffer (Invitrogen) and brought to a final volume of 14.5 µL with sterile distilled water. To this was added 5 U (0.5 µL) of *Hin*fI restriction

endonuclease (Invitrogen) and the tubes were incubated at 37°C. One sample was removed from the waterbath at time intervals of 10 s, 30 s, 1, 3, 5, 10, 30, and 60 min to which 4.0 μL of gel loading buffer [40% (w/v) sucrose, 0.25% bromophenol blue, 20 mM EDTA] was added to stop the reaction and the sample was placed on ice. Digested DNA fragments were separated in a 1.0% agarose gel in 1x TBE buffer for Southern blotting and hybridization with the Q-primer DNA probe for the construction of the *Hin*fI restriction site map of IGS-1.

Isolation of DNA fragments from agarose gels

Fragments of DNA were excised and purified from agarose gel using the "freeze-squeeze" technique (Tautz and Renz, 1983). This was done for LR1-LR2 PCR products from the LSU rRNA gene and a 530 bp *Hin*fI fragment from IGS-1 of *P. insidiosum.* Fragments were cut from the agarose gel and incubated at –60°C for at least 30 min. The frozen agarose plug was placed between two pieces of Parafilm (Amersham National Can, Chicago, Illinois) and pressure was applied using the fingers. As the agarose plug thawed, the liquid was collected in a microcentrifuge tube. An equal volume of 2% CTAB / 2.0 M NaCl was added and the sample was incubated at 55°C for 10 min. Two chloroform extractions were performed and the DNA was precipitated in 2 vol 95% ethanol. The DNA pellet was washed in 70% ethanol, dried, and resuspended in 20 μL sterile distilled water for digoxigenin labelling reactions and the construction of DNA probes.

DIG-labelling of DNA probes and DNA-DNA hybridizations

Labelling of DNA probes and blot preparation

Restriction fragment and PCR product DNA probes were random-primed labelled with Digoxigenin (DIG)-11-dUTP using the DIG DNA Labelling Kit (Roche Diagnostics GmbH, Mannheim, Germany). Oligonucleotide primer probes were 3'-end labelled with DIG-11-ddUTP using the DIG Oligonucleotide 3'-End Labelling Kit (Roche Diagnostics GmbH). Both labelling procedures were carried out according to the manufacturer's specifications for use in DNA-DNA hybridizations with Southern blots and genomic DNA dot blots.

Spot blots of the appropriate dilutions of genomic DNA were prepared by spotting 1.0 µL of denatured DNA (heated at 100°C for 10 min and immediately chilled on ice) on a Hybond-N membrane (Amersham Corporations, Piscataway, New Jersey). Southern blots of partially digested DNA were prepared according to the method of Southern (1975). The DNA was fixed to the membranes from both dot blots and Southern blots by cross-linking under UV light on a transilluminator for 4 min.

DNA-DNA hybridizations

For hybridizations using DNA probes, membranes were incubated for 1 h with constant agitation in 15 mL pre-hybridization solution (1% SDS, 1 M NaCl) at 42°C for oligonucleotide probes and 60°C for restriction fragment or PCR product probes. Subsequently, the DNA probe was denatured at 100°C for 10 min and added directly to

the pre-hybridization solution after the 1 h incubation at a final concentration of 5 to 25 ng/mL. The hybridization reaction was left to proceed overnight at 42°C (for oligonucleotide probes) or 60°C (for DNA fragment or PCR product probes) with constant agitation. After hybridization was complete, membranes were washed twice, 5 min per wash, in 2x wash solution [2x SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 0.1 % SDS] at room temperature. Two additional washes were then done for 15 min each in 0.1x wash solution (0.1x SSC, 0.1% SDS) at the temperature used for the initial hybridization reaction.

Chemiluminescent Detection of DIG-labelled probes

Following the post-hybridization washes, membranes were equilibrated in buffer 1 (100 mM maleic acid, 150 mM NaCl, pH 7.5) for 1 min and then washed in buffer 2 [1% blocking reagent (Roche Diagnostics GmbH) in buffer 1] for 30 min with gentle agitation. The membrane was subsequently incubated in a solution of a 1:10,000 dilution of Anti-digoxigenin-alkaline phosphatase Fab fragments (Roche Diagnostics GmbH) in buffer 2 for 30 min. Two 15 min washes in buffer 1 were then done followed by a final 2 min wash in buffer 3 (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$). Membranes were then coated with 100 to 500 µL of a 1:100 dilution of a 25 mM solution of CDP-*Star* (Roche Diagnostics GmbH) in buffer 3 and sealed in plastic sheets. Sealed membranes were exposed to Kodak X-Omat x-ray film (Eastman Kodak Co., Rochester, New York) for 1 to 30 min to detect the chemiluminescent reaction.

Subsequent to chemiluminescent detections, membranes were stripped to remove DNA probes by rinsing the membrane in water for 2 min followed by two 15 min

incubations at 37°C in stripping solution (0.2 M NaOH, 0.1% SDS). Membranes were then washed 2 to 3 times in 2x SSC and stored at 4°C in 2x SSC until ready for use.

Cloning of the *P. insidiosum* species-specific probe DNA fragment

DNA fragments that had been purified using the "freeze squeeze" technique were subsequently cloned into the pPCR-Script Amp SK(+) vector (Stratagene) using the PCR-Script AMP Cloning Kit. The polishing, ligation, and transformation protocols are described below.

Polishing DNA fragments and DNA ligation

The ends of restriction fragments were polished by adding 10 µL purified DNA fragment, 1.0 µL 10 mM dNTP mix (2.5 mM each of dATP, dCTP, dGTP, and dTTP), 1.3 µL 10x polishing buffer, and 0.5 U of cloned *Pfu* DNA polymerase to a microcentrifuge tube. The reaction mixture was overlaid with 20 µL of mineral oil and incubated at 72°C for 30 min.

The ligation of the polished fragment to the pPCR-Script Amp SK (+) vector was performed by adding the following components to a 0.5 mL microcentrifuge tube: 1.0 µL pPCR-Script Amp SK(+) cloning vector (10 ng/µL), 1.0 µL PCR-Script 10x reaction buffer, 0.5 µL 10 mM rATP, 2 to 4 µL polished DNA fragment, 1.0 µL *Srf*I (5 U/µL), 1.0 µL T4 DNA ligase (4 U/µL) and distilled water to a final volume of 10 µL. The ligation reaction was incubated at room temperature for 1 h and then heated at 65°C for 10 min to stop the reaction.

Transformation

The transformation of the pPCR-Script Amp SK(+) vector and insert was performed using Epicurian Coli XL 10-Gold Kan ultracompetent cells (Stratagene). Subsequent to thawing on ice, 40 µL of cells and 1.6 µL XL 10-Gold ß-mercaptoethanol were mixed in a Falcon polypropylene tube and incubated on ice for 10 min with periodic mixing. To this mixture was added 2 µL of the vector-insert ligation reaction followed by a 30 min incubation on ice. The reaction mixture was heat pulsed at 42°C for 30 to 45 sec and placed on ice for 2 min. 0.45 mL preheated NZY^+ broth (Stratagene) was added and the reaction was incubated with rotation at 37°C for 1 h. Approximately 200 µL of the transformation mixture was spread plated onto LB-ampicillin agar plates (50 µg/mL ampicillin) that were pre-coated with 100 µL 2% X-gal and 100 µL 10 mM IPTG. Plates were incubated overnight at 37°C.

Screening transformants

Resulting transformant (white) colonies were pick-plated onto fresh LB-ampicillin plates and 2 µL of a 10 mM IPTG/2% X-gal (10%:75%, v:v) was spotted onto each transferred colony. Plates were incubated overnight at 37°C. White colonies were screened for the presence of the insert by purifying plasmid DNA. With a sterile toothpick, colonies were scraped off into a 0.5 mL microcentrifuge tube to which 40 µL of GTE [50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0)] was added. After vortexing and a 1 min centrifugation, the supernatant was transferred to a fresh tube to which 2 µL RNase (20 µg/mL) was added. Samples were electrophoresed on a 1.0%

agarose gel which was Southern blotted and hybridized with the *P. insidiosum* 530 bp probe. Colonies from the respective plasmids that hybridized with the probe were used to inoculate 5 mL LB broth + ampicillin (50 µg/mL) which were incubated overnight at 37°C. Plasmid DNA was purified from the cultures using the QIAprep Spin Miniprep Kit (QIAGEN Inc, Mississauga, Ontario) and screened for the presence of the insert by restriction digestion with a combination of *Bam*HI and *Not*I and subsequent agarose gel electrophoresis. A plasmid containing the insert was sequenced in both directions using the primers T3 and T7, carried out using a 377XL ABI DNA Sequencer (University Core DNA Services, University of Calgary, Calgary, Alberta).

Cultivation of *P. insidiosum* isolates for growth rate studies

Isolates of *P. insidiosum* and *P. destruens* were stored on CMA slants at 37°C until required. Sabouraud agar plates (1.0 % glucose, 0.5 % peptone) were stored at 4°C until ready for use. Prior to incubation, plates were always incubated overnight at their respective temperatures to avoid the lag time for equilibration at that temperature. For each isolate, small pieces of CMA agar containing mycelia were placed onto Sabouraud agar plates and incubated at 37°C for 24 to 48 h. Agar plugs were cut from the growing edge of each colony and placed in the center of fresh Sabouraud agar plates. Three plates of each isolate were incubated at 22, 25, 28, 31, 34, 37, 40, and 43°C. After 24 h, the diameter of radial growth was marked at two perpendicular angles and the plates were incubated for an additional 24 h. After 48 h of total incubation time, radial growth was measured again. Measurements of the diameter of radial growth after 24 and 48 h were

made with a ruler and used for the construction of growth curves.

CHAPTER 3

RFLP analysis of the rDNA IGS to resolve species boundaries in *Pythium*

INTRODUCTION

There are approximately 120 described *Pythium* species, the majority of which are saprophytic or pathogenic to plants (Van der Plaats-Niterink, 1981; Dick, 1990a). The morphological species concept has traditionally been used to define species in *Pythium* while the biological species concept has been of little use since there are only seven reported heterothallic species in the genus. Comparisons using the dimensions and nature of sexual and asexual structures have been used to delimit species. However, the limited number of available morphological features and widespread variability and overlap among these characters often complicates species identification. In *Pythium,* while morphological characteristics (and to a lesser extent, mating tests) present significant traits that can be observed and measured, they do not always provide the desired level of objectivity necessary to define and identify species. This shortcoming is mainly due to morphological similarities among many species, and a lack of observable or distinguishable traits in certain isolates.

The phylogenetic species concept has been used recently to resolve species boundaries in *Pythium.* Molecular biology techniques provide a more objective approach compared to morphological observations to define species and identify isolates. Molecular methods which have been used in *Pythium* taxonomy include RFLPs of mtDNA (Martin and Kistler, 1990) and nuclear rDNA (Chen *et al.,* 1992a; Wang and White, 1997), RAPDs (Herrero and Klemsdal, 1998), DNA probes (Martin, 1991; Klassen *et al.,* 1996; Lévesque *et al.,* 1998), and DNA sequencing studies (Matsumoto *et al.,* 1999; Martin, 2000).

In this chapter, RFLP analysis of the rDNA IGS was used to provide a molecular biology technique to identify isolates of *Pythium* to the species level. For each group of species, the rDNA IGS was amplified using PCR with primers Q and P2. The resulting amplicons were treated with seven restriction endonucleases producing restriction fragment patterns to be used for RFLP analysis. This allowed for the examination of genetic relationships among morphologically similar species and the correlation between the morphological and phylogenetic species definitions in *Pythium.* Initially, RFLP analysis of the IGS was done for a worldwide collection of *P. ultimum* isolates to assess the level of intraspecific variation among restriction fragment patterns in that taxonomically well-studied species. RFLP analysis was then used to examine genetic relationships among over 360 isolates representing 90 species (summarized in Table 3.1).

In this analysis, species were divided into four main groups based on their zoosporangial morphology. These groups included species with i) globose non-proliferating zoosporangia, ii) spherical or ellipsoid proliferating zoosporangia, iii) filamentous inflated zoosporangia, and iv) filamentous noninflated zoosporangia. Species within each zoosporangial group were also subdivided into smaller groupings according to shared morphological features. The genetic clusters of species will be discussed with reference to their common morphological characteristics and the features that distinguish one species from another. However, several groups of morphologically similar species formed monophyletic clusters which likely represent conspecific species. The significance of these clusters with respect to morphological and molecular data will also be discussed.

I. RFLP analysis of the IGS in *Pythium ultimum*

Introduction to *Pythium ultimum*

Pythium ultimum is one of the most commonly reported *Pythium* species from soil because it is an important plant pathogen causing diseases such as root rot, post- and pre-emergence damping off, and leak in potatoes. *P. ultimum* is a taxonomically sound species with relatively little morphological variation. However, two varieties of *P. ultimum* can be distinguished.

Pythium ultimum var. *ultimum* forms globose hyphal swellings but rarely forms zoosporangia, and zoospores are only produced at 5°C. Oogonia are globose, smooth, and terminally located on hyphae. There is usually one sac-like monoclinous antheridium per oogonium, and oospores are aplerotic (Van der Plaats-Niterink, 1981). While little variation exists among isolates, Tojo *et al.* (1998) reported two morphological groups of *P. ultimum* var. *ultimum* from vegetable fields in Japan that were distinguished based on the dimensions of their sexual structures. *P. ultimum* var. *sporangiiferum* produces subglobose zoosporangia with zoospores readily produced at 20°C. Its other morphological characters are essentially identical to those of *P. ultimum* var. *ultimum* (Van der Plaats-Niterink, 1981). So the two varieties of *P. ultimum* essentially differ in their production of zoosporangia and capacity to release zoospores at room temperature. Barr *et al.* (1996) reported that isolates of *P. ultimum* var. *sporangiiferum* had a large vacuole in the zoosporangium which they postulated may be associated with zoospore production.

Pythium ultimum is a homothallic species and self-fertilization can take place via a monoclinous antheridium. However, evidence has suggested that *P. ultimum* may exist as both self-fertile and self-sterile forms. Campbell and Sleeth (1946) found an equal proportion of sexual and asexual reproduction among *P. ultimum* isolates from soil. Some isolates also occasionally form diclinous antheridia (Van der Plaats-Niterink, 1981) and many *Pythium* type HS isolates exist (isolates which produce hyphal swellings but do not complete the sexual cycle in culture) that are closely related to *P. ultimum.* Using a monoclonal antibody specific for *P. ultimum,* Yuen *et al.* (1993) found common reactivity of the antibody with both isolates of *P. ultimum* and self-sterile *Pythium* type HS isolates. Martin (1990) compared mtDNA restriction fragment patterns of *P. ultimum* and *Pythium* type HS isolates and found the majority of them to be nearly identical. Kageyama *et al.* (1998) found similar results using ITS RFLPs and RAPDs.

In general, *Pythium* type HS isolates are thought to be *P. ultimum* isolates that have lost the ability to reproduce sexually (Martin, 1990). But according to the Knight-Darwin rule, no organism capable of sexual reproduction will strictly inbreed (Bell, 1982). Using molecular markers, Francis and St. Clair (1993) showed that *Pythium* type HS isolates appeared to act as males in crosses with *P. ultimum* isolates. Saunders and Hancock (1994) provided further evidence of sexual reproduction in *P. ultimum* by showing that *Pythium* type HS isolates produced antheridia that fused with oogonia of *P. irregulare,* although the oospores were abortive. These studies suggested that mating systems may be equally complex for homothallic *Pythium* species as for heterothallic ones, and that outcrossing could have a dramatic effect on genetic variation in *P. ultimum* populations and genomes.

Several studies have suggested that the two varieties of *P. ultimum* are genetically distinct. Francis *et al.* (1994) used RFLPs and RAPDs to show that significant heterogeneity existed among isolates of *P. ultimum* var. *ultimum, P. ultimum* var. *sporangiiferum,* and *Pythium* type HS strains since isolates did not cluster according to their morphological type. Isoelectric focusing revealed similar patterns among isolates of all varieties and this technique could not distinguish isolates from each other (Adaskaveg *et al.,* 1988). A species-specific probe from ITS-1 for *P. ultimum* also could not distinguish *P. ultimum* var. *ultimum* from *P. ultimum* var. *sporangiiferum* (Lévesque *et al.,* 1994). These results suggested a close genetic relationship among the two varieties which could be a reflection of interbreeding among isolates. As well, they cast doubt upon the validity of using zoospore production as a trait to separate varieties since zoospore production is also variable within other species, such as *P. intermedium* (Adaskaveg *et al.,* 1988).

However, there is also evidence that has supported the separation of *P. ultimum* into two varieties. Isozyme analysis showed that isolates of *P. ultimum* var. *sporangiiferum* had a genotype distinct from isolates of *P. ultimum* var. *ultimum* (Barr *et al.,* 1996). A DNA probe constructed from the 5S rRNA gene spacer of *P. ultimum* var. *ultimum* hybridized to genomic DNA from all isolates of that species and also to the DNA from two isolates of *P. ultimum* var. *sporangiiferum.* However, the probe for *P. ultimum* var. *sporangiiferum* only hybridized to genomic DNA from isolates of that species (Klassen *et al.,* 1996). As well, an oligonucleotide probe for *P. ultimum* var. *ultimum* hybridized strongly to DNA from isolates of *P. ultimum* var. *ultimum* but very faintly to isolates of *P. ultimum* var. *sporangiiferum.* Mitochondrial *cox*II gene sequence

analysis also showed that isolates of both varieties formed separate groupings (Martin, 2000).

Results

RFLP analysis of the rDNA IGS was done for several isolates representing both varieties of *P. ultimum* to examine the degree of genetic variation within the species and between varieties, and to assess the applicability of RFLP analysis of the IGS as a tool for species identification in *Pythium.* Restriction digestions of rDNA IGS amplicons were done for 32 isolates of *P. ultimum* var. *ultimum* and six isolates of *P. ultimum* var. *sporangiiferum,* and also for isolates of *P. heterothallicum, P. irregulare, P. prolatum,* and *P. splendens* (Table 3.1) using *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 3.1). A total of 316 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the UPGMA phenogram is presented in Figure 3.2. Isolates of *P. heterothallicum* and *P. splendens* were included as outgroups since these two species were most closely related to *P. ultimum* based on ITS sequence analysis (C.A. Lévesque, personal communication) and mitochondrial *cox*II gene sequencing (Martin, 2000). *P. heterothallicum* and *P. splendens* are heterothallic species that produce hyphal swellings and aplerotic oospores, and they have larger oogonia compared to *P. ultimum* (Van der Plaats-Niterink, 1981). *P. irregulare* and *P. prolatum* were also included as outgroups. *P. irregulare* produces hyphal swellings but occasionally forms globose zoosporangia. It also has smaller oogonia with projections on the oogonial wall. *P. prolatum* produces ovoid, elongate

Table 3.1. List of *Pythium* isolates used in Chapter 3 for RFLP analysis of the IGS.

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
P. acanthicum Drechsler	71[d]	CBS 284.31	USA
	148	CBS 431.68	Netherlands
	151	CBS 227.94	France
	253	CBS 429.68	Netherlands
	332	CBS 377.34	Sweden
P. acanthophoron Sideris	52	CBS 337.29	Hawaii, USA
	4000b	APCC 4000b	USA
P. acrogynum Yü	69[d]	CBS 549.88	China
	152	CBS 226.94	France
	385	adc 97.70	Netherlands
P. adhaerens Sparrow	25	CBS 520.74	Netherlands
P. amasculinum Yü	298	CBS 552.88	China
P. anandrum Drechsler	1[d]	CBS 258.31	USA
	254	CBS 394.61	Netherlands
	295	CBS 259.94	Netherlands
	4401d	IMI 308112	United Kingdom
P. angustatum Sparrow	27[e]	CBS 522.74	Netherlands
	153	CBS 229.94	Togo
	357	CBS 676.95	Norway
P. aphanidermatum (Edson) Fitzp.	135[f]	CBS 118.80	France
	BR206		Ontario, Canada
P. apleroticum Tokunaga	70[d]	CBS 772.81	Netherlands
P. aquatile Höhnk	21[f]	CBS 215.80	United Kingdom
	154	CBS 231.94	France
	184	CBS 214.80	Netherlands
	243	adc 94.18	Netherlands
	255	CBS 216.80	United Kingdom
	386	adc 97.64	Netherlands
P. aristosporum Vanterpool	2[d]	CBS 263.38	Canada
	149	CBS 162.88	Ohio, USA
	BR136		Canada
	BR166		Indiana, USA
	BR608		PEI, Canada
P. arrhenomanes Drechsler	3[d]	CBS 324.62	Wisconsin, USA
	137	CBS 294.32	Canada
	138	CBS 325.62	Hawaii, USA
	139	CBS 163.68	Ohio, USA
	170	CBS 430.86	Netherlands
	256	CBS 293.22	?
	4201a	IMI 145456	Australia
	4201b	APCC 4201b	Australia
	4201c	APCC 4201c	Australia
	4201d	APCC 4201d	Australia
	BR607		Manitoba, Canada
P. boreale Duan	79[d]	CBS 551.88	China
P. buismaniae Van der Plaats-Niterink	63[d]	CBS 288.31	Netherlands
P. capillosum Paul	73[g]	CBS 222.94	France
P. catenulatum Matthews	80[e] (a)	CBS 843.68	South Carolina, USA
	81 (b)	CBS 842.68	South Carolina, USA
	257	CBS 461.75	India
P. chamaehyphon Sideris	82[d]	CBS 259.30	Hawaii, USA
	359	adc 95.24	Netherlands

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
P. chondricola de Cock	87[d]	CBS 203.85	Netherlands
	213	CBS 208.85	Netherlands
P. coloratum Vaartaja	22[d]	CBS 154.64	South Australia
	172	CBS 732.94	British Columbia, Canada
	258	CBS 648.79	Canada
	BR176		Newfoundland, Canada
	BR180		Ontario, Canada
	BR323		New Zealand
	BR401		Ontario, Canada
	BR465	ATCC 16514	South Australia
	BR621		Alberta, Canada
	BR632		Canada
P. conidiophorum Jokl	259	CBS 224.88	United Kingdom
P. cucurbitacearum Takimoto	337	CBS 748.96	Australia
P. diclinum Tokunaga	30[f]	CBS 664.79	Netherlands
	179	adc 94.46	Netherlands
	260	CBS 573.75	Iran
	261	CBS 217.80	United Kingdom
	262	CBS 774.81	Netherlands
	306	CBS 526.74	Netherlands
	4110a	APCC 4110a	?
P. dimorphum Hendrix & Campbell	31[d]	CBS 406.72	Louisiana, USA
P. dissimile Vaartaja	32[d]	CBS 155.64	Australia
	263	CBS 523.74	Netherlands
	264	CBS 431.91	Germany
	4204b	IMI 308135	United Kingdom
	4204c	IMI 308136	United Kingdom
	4204e	IMI 308138	United Kingdom
	BR160		Ontario, Canada
P. dissotocum Drechsler	4	CBS 166.68	Ohio, USA
	265[d]	CBS 260.30	Hawaii, USA
	266	CBS 379.34	Japan
	267	CBS 378.52	?
	268	CBS 524.74	Netherlands
	269	CBS 525.74	Netherlands
P. drechsleri Rajagopalan & Ramakrishnan	75[d]	CBS 221.94	?
P. echinulatum Matthews	33[e]	CBS 281.64	Australia
	308	CBS 238.79	United Kingdom
	309	CBS 239.79	United Kingdom
	352	CBS 556.67	France
	4306a	IMI 308139	?
	4306b	IMI 308140	?
	4306d	IMI 308142	?
P. erinaceus Robertson	34[d]	CBS 505.80	New Zealand
	78621		?
	78622		?
Pythium type F	238	adc 94.25	Netherlands
	239	adc 94.26	Netherlands
P. flevoense Van der Plaats-Niterink	84[d] (f)	CBS 234.72	Netherlands
	85[e] (m)	CBS 236.72	Netherlands
	310	CBS 233.72	Netherlands
P. folliculosum Paul	76[d]	CBS 220.94	Switzerland
P. graminicola Subramaniam	5[f]	CBS 327.62	Jamaica

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
	140	CBS 328.62	Hawaii, USA
	4205a	IMI 034768	?
	4205g	APCC 4205g	Australia
	4205h	APCC 4205h	Australia
	4205i	APCC 4205i	Australia
	BR506		Saskatchewan, Canada
P. grandilobatum	210	CBS 739.94	South Africa
	211[d]	CBS 738.94	South Africa
	212	CBS 740.94	South Africa
P. grandisporangium Fell & Master	54[d]	CBS 286.79	Florida, USA
	214	CBS 211.85	Netherlands
	232	CBS 606.81	British Columbia, Canada
	391	CBS 212.85	Netherlands
P. helicandrum Drechsler	40[g]	CBS 393.54	USA
	271	CBS 527.74	Netherlands
	272	CBS 134.86	Netherlands
	311	CBS 694.79	Netherlands
	398	CBS 650.79	Netherlands
P. helicoides Drechsler	50[e]	CBS 286.31	USA
	273	CBS 293.35	Japan
	274	CBS 167.68	Florida, USA
	275	CBS 343.72	?
	278	CBS 597.68	Zaire
P. heterothallicum Campbell & Hendrix	23[d] (m)	CBS 450.67	Canada
	24[d] (f)	CBS 451.67	Canada
	187	CBS 143.69	Netherlands
	188	CBS 207.68	Netherlands
	189	CBS 208.68	Costa Rica
	384	adc 97.67	Netherlands
P. hydnosporum (Mont.) Schröter	90[e]	CBS 253.60	Germany
	434a	IMI 147441	?
P. hypogynum Middleton	55	CBS 692.79	Alberta, Canada
	345	CBS 234.94	France
	BR260		Ontario, Canada
	BR389	IMI 241681	Alberta, Canada
	BR393		Quebec, Canada
	BR635	IMI 242092	?
P. indigoferae Butler	351[e]	CBS 261.30	India
P. inflatum Matthews	86[e]	CBS 168.68	Louisiana, USA
	156	CBS 230.94	Benin
	276	CBS 647.79	Canada
	387	adc 97.69	Netherlands
P. irregulare Buisman	67[f]	CBS 250.28	Netherlands
P. iwayamai S. Ito	29[e]	CBS 156.64	Australia
	64	CBS 697.83	Japan
P. jasmonium	395	CBS 101876	?
P. lutarium Ali-Shtayeh & Dick	36[d]	CBS 222.88	United Kingdom
P. macrosporum Vaartaja & Van der Plaats-Niterink	7[d] (+)	CBS 574.80	Netherlands
	8[d] (-)	CBS 575.80	Netherlands
	143 (-)	CBS 579.80	Ontario, Canada
P. mamillatum Meurs	9[e]	CBS 251.28	Netherlands
P. marinum Sparrow	94	CBS 312.93	Washington, USA
	236	CBS 313.93	Washington, USA

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
	333	CBS 750.96	United Kingdom
P. mastophorum Dreschsler	57[e]	CBS 375.72	United Kingdom
	313	CBS 243.86	Netherlands
	376	CBS 100412	Netherlands
P. middletonii Sparrow	35[e]	CBS 528.74	Netherlands
	314	CBS 293.37	Sudan
	354	CBS 679.95	Norway
P. minus Ali-Shatyeh & Dick	37[d]	CBS 226.88	United Kingdom
P. monospermum Pringsheim	10[f]	CBS 158.73	United Kingdom
	277	CBS 381.79	Netherlands
	355	CBS 677.95	Norway
	356	CBS 790.95	Norway
P. multisporum Poitras	53[d]	CBS 470.50	USA
P. myriotylum Drechsler	11[f]	CBS 254.70	Israel
	125	CBS 315.33	Netherlands
	129	CBS 114.77	Netherlands
	150	CBS 695.79	Malaysia
P. nagaii S. Ito & Tokunaga	4321a	IMI 308183	United Kingdom
	4321b	IMI 308184	United Kingdom
P. oedochilum Drechsler	38[g]	CBS 292.37	USA
	316	CBS 252.70	Netherlands
	362	adc 96.24	Netherlands
	399	adc 99.47	Netherlands
P. okanaganense Lipps	59[d]	CBS 315.81	Wisconsin, USA
	317	CBS 701.83	Japan
P. oligandrum Drechsler	12[e]	CBS 382.34	United Kingdom
	136	CBS 149.84	Netherlands
	162	CBS 530.74	Netherlands
	163	CBS 531.74	Netherlands
	292	CBS 217.46	?
	381	adc 97.57	Netherlands
	4410b	IMI 308324	United Kingdom
P. ostracodes Drechsler	49[e]	CBS 768.73	Spain
P. pachycaule Ali-Shtayeh & Dick	157	CBS 224.94	France
	158	CBS 225.94	France
	247[d]	CBS 227.88	United Kingdom
	4117b	APCC 4117b	United Kingdom
	338315	IMI 339315	Australia
P. paddicum Hirane	46	CBS 698.83	Japan
P. paroecandrum Drechsler	68[e]	CBS 157.64	Australia
	115	CBS 203.79	Netherlands
P. parvum Ali-Shtayeh & Dick	42[d]	CBS 225.88	United Kingdom
	4009b	IMI 308302	United Kingdom
P. periilum Drechsler	26[e]	CBS 169.68	Florida, USA
	159	CBS 232.94	Togo
	160	CBS 233.94	Algeria
	279	CBS 218.80	India
P. periplocum Drechsler	91[d]	CBS 289.31	USA
	318	CBS 170.68	Louisiana, USA
	4461a	APCC 4461a	United Kingdom
	4461b	APCC 4461b	United Kingdom
P. perplexum Kouyeas & Theohari	319	CBS 674.85	?
P. pleroticum T. Ito	51	CBS 776.81	Netherlands
	320	CBS 685.79	Quebec, Canada

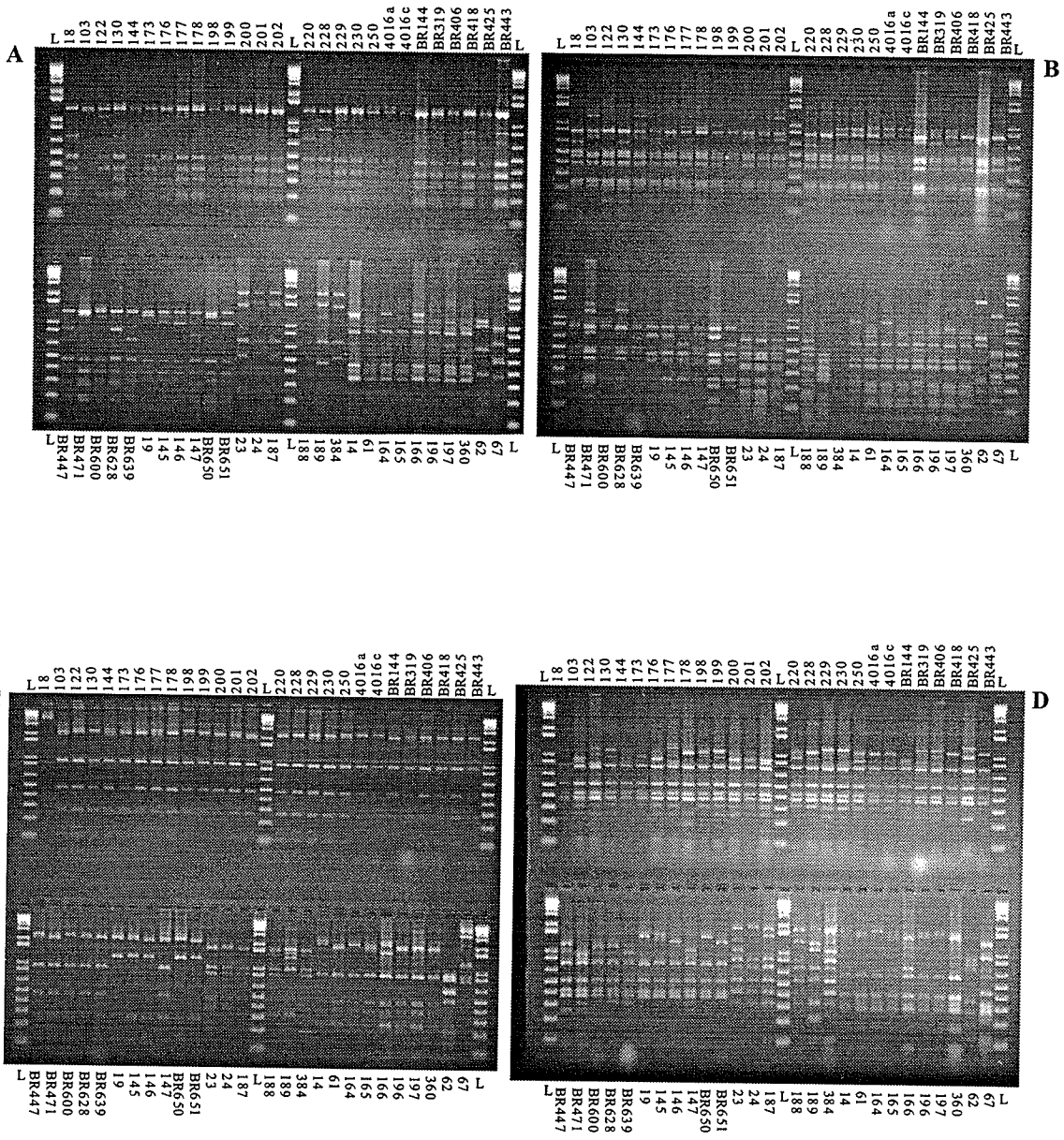
Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
P. polymastum Drechsler	93[e]	CBS 811.70	Netherlands
P. porphyrae Takahashi & Sasaki	215[e]	CBS 369.79	Japan
	350	CBS 517.82	Japan
P. prolatum Hendrix & Campbell	62[d]	CBS 845.68	Georgia, USA
	321	CBS 844.68	Georgia, USA
P. pyrilobum Vaartaja	43[d]	CBS 158.64	Australia
P. radiosum Paul	77[d]	CBS 217.94	France
P. rostratum Butler	88[f]	CBS 533.74	Netherlands
	192	CBS 172.68	United Kingdom
	4329f	IMI 308318	United Kingdom
P. salpingophorum Drechsler	39[e]	CBS 471.50	United Kingdom
	280	CBS 472.50	Poland
	323	CBS 326.62	Germany
	324	CBS 165.68	Ohio, USA
	1092	APCC 4331e	United Kingdom
P. scleroteichum Drechsler	89[g]	CBS 294.37	USA
P. spinosum Sawada	13[e]	CBS 275.67	Netherlands
	4012e	IMI 308287	United Kingdom
P. splendens Braun	14[e] (-)	CBS 462.48	USA
	61[e] (+)	CBS 266.69	Belgium
	164	CBS 191.25	?
	165	CBS 265.69	Costa Rica
	166	CBS 338.29	Hawaii, USA
	196	CBS 269.69	Congo
	197	CBS 462.93	USA
	360	adc 96.22	?
Pythium sp.	282	CBS 677.85	?
	303	CBS 86.016	?
	327	CBS 632.85	?
	328	CBS 633.85	?
	330	CBS 676.85	?
P. sulcatum Pratt & Mitchell	44[d]	CBS 603.73	Wisconsin, USA
	281	CBS 292.76	Canada
	331	CBS 604.73	Florida, USA
	365	adc 97.21	Netherlands
	BR113	IMI 197679	Ontario, Canada
	BR146		?
	BR157		Quebec, Canada
	BR652		?
P. torulosum Coker & Patterson	17[e]	CBS 316.33	Netherlands
	169	CBS 406.67	Netherlands
	283	CBS 329.62	Hawaii, USA
	284	CBS 553.88	China
	4212b	IMI 308266	United Kingdom
P. tracheiphilum Matta	92[d]	CBS 323.65	Italy
	285	CBS 870.72	Netherlands
	286	CBS 519.77	Netherlands
	363	adc 96.25	Netherlands
P. tumidum	78[d]	CBS 223.94	?
P. ultimum var. *ultimum* Trow	18[f]	CBS 398.51	Netherlands
	103	CBS 488.86	Poland
	122	CBS 114.79	Spain
	130	CBS 305.35	Netherlands
	144	CBS 378.34	Netherlands

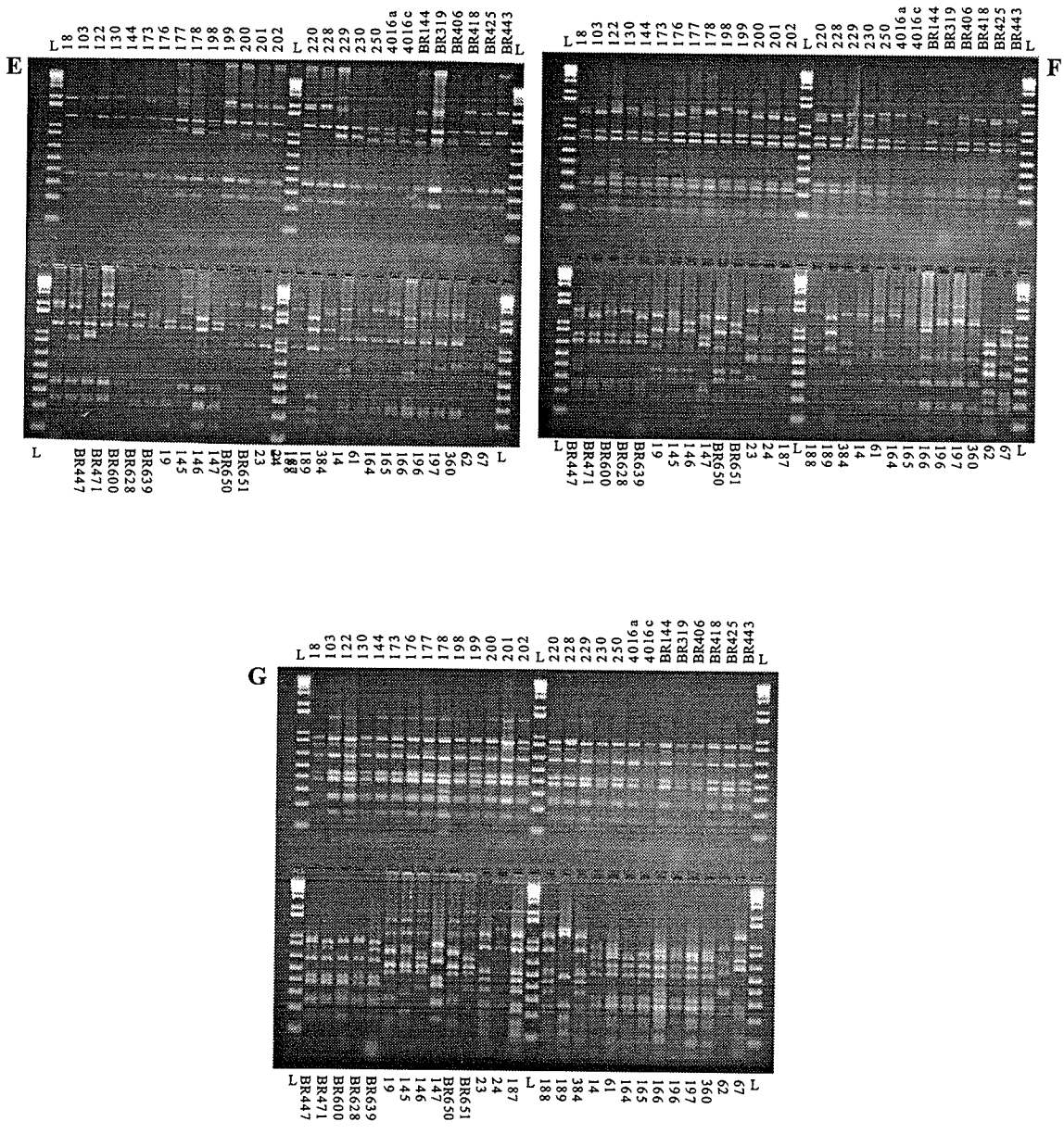
Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
	173	CBS 730.94	Canada
	176	CBS 726.94	Canada
	177	CBS 728.94	Canada
	178	CBS 729.94	Canada
	198	CBS 249.28	Netherlands
	199	CBS 264.38	Netherlands
	200	CBS 291.31	?
	201	CBS 296.37	United Kingdom
	202	CBS 656.68	Netherlands
	220	adc 94.06	Netherlands
	228	CBS 489.86	Poland
	229	CBS 490.86	Poland
	230	CBS 491.86	Poland
	250	adc 94.19	Netherlands
	4016a	IMI 082512	Australia
	4016c	IMI 308273	United Kingdom
	BR144		Ontario, Canada
	BR319		California, USA
	BR406		?
	BR418		Quebec, Canada
	BR425		Alberta, Canada
	BR443		Saskatchewan, Canada
	BR447		Alberta, Canada
	BR471		USA
	BR600		British Columbia, Canada
	BR628		?
	BR638		Alberta, Canada
P. ultimum var. *sporangiiferum* Drechsler	19[d]	CBS 219.65	USA
	145	CBS 111.65	Lebanon
	146	CBS 114.79	Spain
	147	CBS 171.68	United Kingdom
	BR650		Maryland
	BR651		Spain
P. uncinulatum Van der Plaats-Niterink & Blok	203[d]	CBS 518.77	Netherlands
	301	CBS 515.77	Netherlands
P. undulatum H.E. Petersen	48[f]	CBS 157.69	Alabama, USA
	251	adc 94.49	Netherlands
	252	adc 94.50	Netherlands
	287	CBS 346.69	Canada
	288	CBS 113.79	United Kingdom
	289	CBS 114.91	Netherlands
	293	CBS 323.47	USA
	400	adc 99.29	Netherlands
	401	adc 99.35	Netherlands
P. vanterpoolii V. Kouyeas & H. Kouyeas	58[d]	CBS 295.37	United Kingdom
	161	CBS 228.94	France
	204	CBS 115.77	Netherlands
	205	CBS 293.76	Ontario, Canada
	206	CBS 700.83	Japan
	4213b	IMI 280170	Japan
P. vexans de Bary	20[e]	CBS 119.80	Iran

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
	123	CBS 339.29	Hawaii, USA
	207	CBS 270.38	Iowa, USA
	208	CBS 334.36	India
	209	CBS 455.62	Netherlands
	4017a	IMI 308244	United Kingdom
	4017b	IMI 308245	United Kingdom
	4017c	IMI 308246	United Kingdom
	4017d	IMI 308247	United Kingdom
	80346	IMI 80346	Madagascar
	132189	IMI 132189	Netherlands
P. violae Chesters & Hickman	28[e]	CBS 159.64	Australia
	126	CBS 178.86	Netherlands
	369	adc 97.26	Netherlands
	375	adc 97.39	Netherlands
P. volutum Vanterpool & Truscott	47	CBS 699.83	Japan
P. zingiberis Takahashi	41	CBS 216.82	Japan
	290	CBS 217.82	Japan

[a]Numbers by which isolates are referred to in this study. BR isolates were obtained from the Biosystematics Research Centre, Ottawa, Ontario. For heterothallic species, mating types are designated as (a), (b), (m) (male), (f) (female), or (+) and (-) (opposite mating types).
[b]Accession numbers of isolates in the following culture collections: CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), APCC (Aquatic Phycomycete Culture Collection, Reading, England), IMI (International Mycological Institute, Egham, United Kingdom), ATCC (American Type Culture Collection, Manassas, Virginia), adc (personal collection of A.W.A.M. de Cock).
[c]?=unknown geographic origin.
[d]Ex-type strain.
[e]Isolate used by Van der Plaats-Niterink (1981) for species description.
[f]Isolate designated as the neotype strain because all ex-type material is missing.
[g]Authentic strain, identified by the author of the species.

Figure 3.1. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. heterothallicum, P. irregulare, P. prolatum, P. splendens, P. ultimum* var. *sporangiiferum,* and *P. ultimum* var. *ultimum* with **(A)** *Alu*I, **(B)** *Cfo*I, **(C)** *Hae*III, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L
18
103
122
130
144
173
176
177
178
198
199
200
201
202
L
220
228
229
230
250
4016a
4016c
BR144
BR319
BR406
BR418
BR425
BR443
L
67
62
360
197
196
166
165
164
61
14
384
189
188
L
187
24
23
BR651
BR650
147
146
145
19
BR639
BR628
BR600
BR471
BR447
L
B
C
D


E
L
18
103
122
130
144
173
176
177
178
198
199
200
201
202
L
220
228
229
230
250
4016a
4016c
BR144
BR319
BR406
BR418
BR425
BR443
L
L
BR447
BR471
BR600
BR628
BR639
19
145
146
147
BR650
BR651
23
14
61
164
165
166
196
197
360
62
67
L
F
L
18
103
122
130
144
173
176
177
178
198
199
200
201
202
L
220
228
229
230
250
4016a
4016c
BR144
BR319
BR406
BR418
BR425
BR443
L
L
BR447
BR471
BR600
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BR639
19
145
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BR650
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23
24
187
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14
61
164
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62
67
L
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18
103
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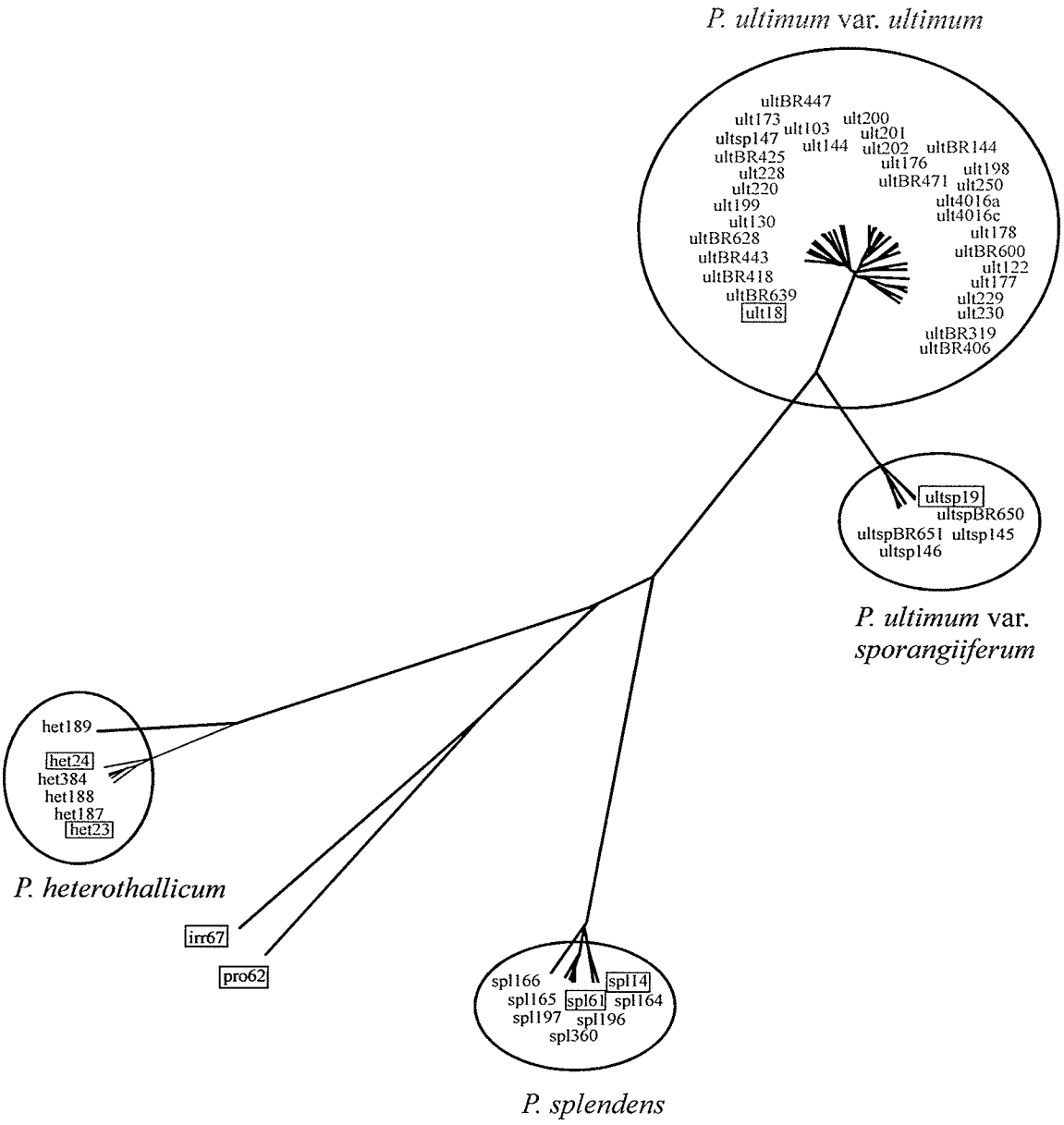
4016a
4016c
BR144
BR319
BR406
BR418
BR425
BR443
L
L
BR447
BR471
BR600
BR628
BR639
19
145
146
147
BR650
BR651
23
24
187
188
189
384
14
61
164
165
166
196
197
360
62
67
L

Figure 3.2. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.1. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: ult, *P. ultimum* var. *ultimum*; ultsp, *P. ultimum* var. *sporangiiferum*; het, *P. heterothallicum*; spl, *P. splendens*; irr, *P. irregulare*; pro, *P. prolatum.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. ultimum var. ultimum
ultBR447
ult173
ult103
ult200
ultsp147
ult144
ult201
ult202
ultBR144
ultBR425
ult176
ult198
ult228
ultBR471
ult250
ult220
ult4016a
ult199
ult4016c
ult130
ult178
ultBR628
ultBR600
ultBR443
ult122
ultBR418
ult177
ultBR639
ult229
ult18
ult230
ultBR319
ultBR406
ultsp19
ultspBR650
ultspBR651
ultsp145
ultsp146
P. ultimum var. sporangiiferum
het189
het24
het384
het188
het187
het23
P. heterothallicum
irr67
pro62
spl166
spl14
spl165
spl61
spl164
spl197
spl196
spl360
P. splendens
.10

zoosporangia which distinguishes it from all the other species in this analysis (Van der Plaats-Niterink, 1981).

All 32 isolates of *P. ultimum* var. *ultimum* from a wide geographic distribution formed a monophyletic cluster distinct from all other species (Fig. 3.2). Five isolates of *P. ultimum* var. *sporangiiferum* also formed a cluster that was more closely related to the *P. ultimum* var. *ultimum* cluster than to the other species. One isolate of *P. ultimum* var. *sporangiiferum* (147) was present within the cluster of *P. ultimum* var. *ultimum* isolates which suggested it was most likely a *P. ultimum* var. *ultimum* isolate which had been misidentified. Isolates representing *P. heterothallicum* and *P. splendens* also formed individual clusters. The average genetic distance between the two varieties of *P. ultimum* was 0.0506 while genetic distance values were 0.1200 and greater when comparing *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum* isolates to the outgroup species.

Conclusions

The RFLP data from the IGS supported studies that suggest *P. ultimum* var. *ultimum* and *P. ultimum* var. *sporangiiferum* are genetically distinct. Each variety formed its own cluster and genetic distances between the clusters were significantly lower compared to distances between *P. ultimum* varieties and the outgroup species. This suggested that the classification of varieties in *P. ultimum* based on the production of zoospores and zoosporangia is suitable and that the varieties may not represent distinct species since the pairwise genetic distances between the varieties was significantly smaller in comparison to distances between isolates of *P. ultimum* and the outgroup

species. *P. ultimum* is a taxonomically sound species and the low levels of morphological variation were reflected by the low levels of intraspecific variation in the restriction fragment patterns among isolates. This was also true for *P. heterothallicum* and *P. splendens.* These results therefore validated the use of RFLP analysis of the IGS for investigating species relationships among other morphologically similar *Pythium* species, and as a tool for species identification.

II. RFLP analysis of *Pythium* species with globose non-proliferating zoosporangia

According to Van der Plaats-Niterink (1981) and Dick (1990a), approximately 30 *Pythium* species possess spherical or ellipsoid zoosporangia that do not exhibit proliferation. Several of these species also produce globose oogonia with spiny ornamentations on their oogonial walls, while others form smooth-walled oogonia. The following sections describe work in which RFLP analysis of the rDNA IGS was used to examine relationships among three groupings of morphologically similar species that possess spherical non-proliferating zoosporangia.

A. Molecular comparison of mycoparasitic *Pythium* species with globose non-proliferating zoosporangia and ornamented oogonia

Introduction

Drechsler (1930) described three species with spines on their oogonial walls: *P. acanthicum* and *P. periplocum* from decayed fruits of *Citrullus vulgaris*, and *P. oligandrum* from discolored rootlets of *Pisum sativum.* These three species possess spiny

oogonia very similar in size and morphology. In fact, Hendrix and Campbell (1970) did not separate *P. oligandrum* and *P. acanthicum,* and referred to these species as the "*P. acanthicum-P. oligandrum* complex". *P. oligandrum* and *P. acanthicum* produce subglobose zoosporangia that form complexes, but the complexes are less complicated in *P. acanthicum.* The main difference between these species is the plerotic oospores in *P. acanthicum* and aplerotic oospores in *P. oligandrum.* However, the heavy oogonial spine covers often make it difficult to observe this character. Ribeiro and Butler (1995) also reported considerable variation in the nature of the oospore among *P. oligandrum* isolates. *P. oligandrum* can also be distinguished from *P. acanthicum* by its longer oogonial spines. Drechsler (1946) suggested that the size of reproductive structures would be useful for separating the two species, but Ribeiro and Butler (1995) found the dimensions of oogonial spines to be a more reliable character. *P. periplocum* can be distinguished from the other two species by its filamentous lobate zoosporangia. In fact, *P. periplocum* is the only species in the genus with ornamented oogonia and filamentous inflated zoosporangia (Van der Plaats-Niterink, 1981).

Pythium hydnosporum and *P. amasculinum* are morphologically similar to *P. oligandrum.* These three species possess globose oogonia with similar long, slender oogonial spines, although the projections are more slender in *P. hydnosporum.* The average oospore diameter ranges from 20.5 to 22.0 µm in all three species. The nature of the oospores is variable for *P. amasculinum* and *P. hydnosporum,* but aplerotic for *P. oligandrum* (Van der Plaats-Niterink, 1981). The most significant differences between these species are among the zoosporangia and antheridia.

In *P. oligandrum,* zoosporangia are subglobose and often form irregular aggregates of one or more subglobose elements connected by hyphal parts that produce complicated complexes. *P. amasculinum* forms "sporangia-like swellings" or "contiguous sporangia" which do not produce zoospores (Yü, 1973). While the production of zoospores or zoosporangia has not been shown in *P. hydnosporum,* Van der Plaats-Niterink (1981) presented illustrations from the ex-type material of Montagne (1845) that indicated the presence of hyphal swellings in a contiguous orientation, and even refers to them as "sporangia" in the figure legend. In *P. oligandrum,* antheridia are rare, but diclinous when present. Antheridia are hypogynous in *P. hydnosporum,* but hypogynous antheridia may be a misinterpretation of the oogonial stalk (A.W.A.M. de Cock, personal communication). Antheridia have not been observed in *P. amasculinum* (Yü, 1973).

Therefore, *P. oligandrum, P. hydnosporum,* and *P. amasculinum* share contiguous structures (zoosporangia or hyphal swellings), which may or may not develop zoospores, and globose oogonia with spiny ornamentations. The presence or absence of antheridia is also notable, but using the absence of a morphological structure to classify a species can often be problematic.

Results

RFLP analysis of the rDNA IGS was done to investigate molecular relationships among isolates of *P. acanthicum, P. amasculinum, P. hydnosporum, P. oligandrum,* and *P. periplocum* (Table 3.1). The outgroup species *P. anandrum, P. erinaceus,* and *P. mamillatum* were chosen based mainly on their similar oogonial ornamentations, however the oogonial spines of *P. mamillatum* are blunter and shorter compared to those of *P.*

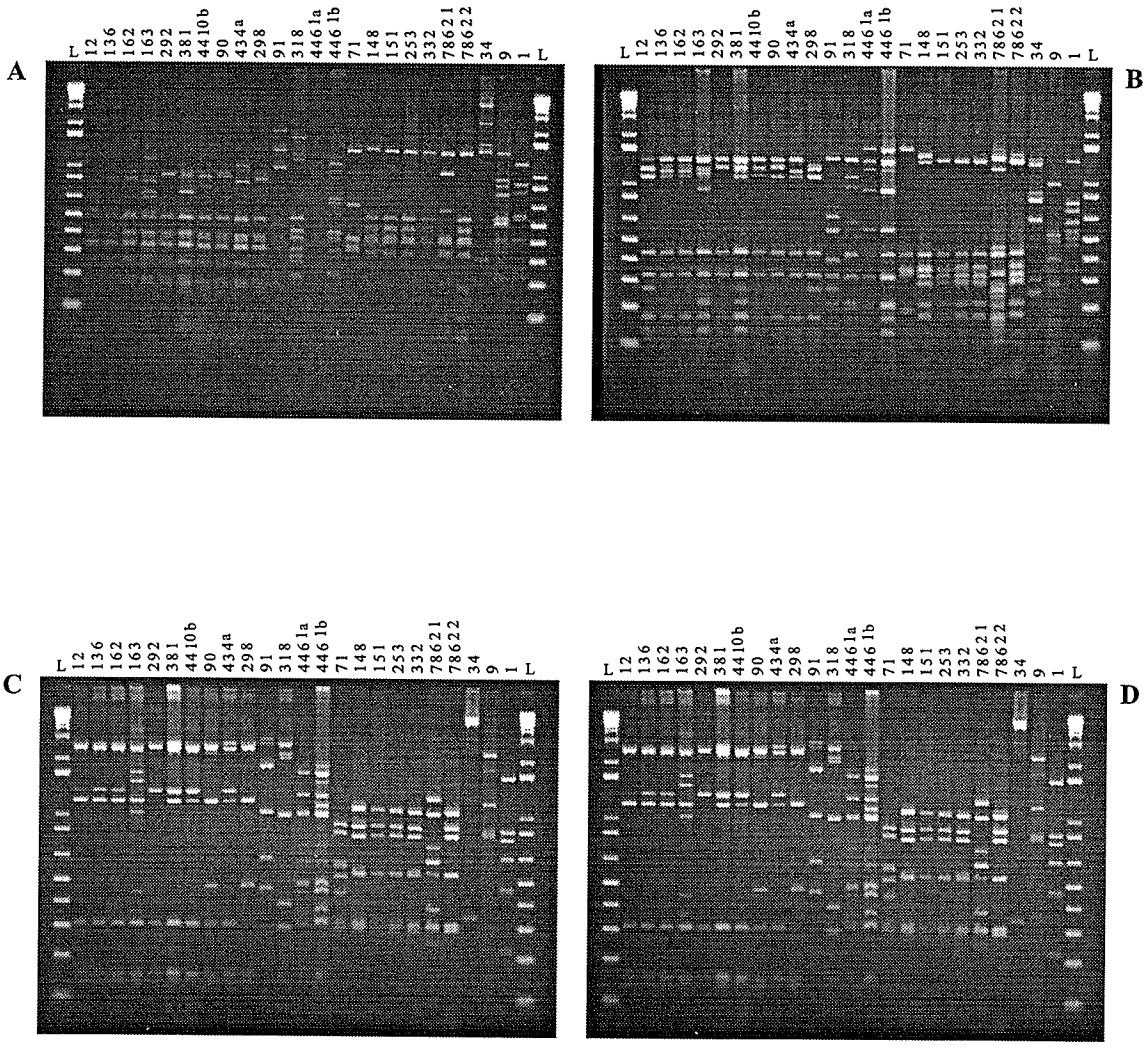
hydnosporum. *P. anandrum* produces ellipsoid zoosporangia which are occasionally proliferating, while *P. mamillatum* and *P. erinaceus* form globose zoosporangia. All three outgroup species also produce plerotic oospores (Van der Plaats-Niterink, 1981). Restriction endonuclease digestions were performed on PCR products of the IGS using *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 3.3). A total of 339 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the neighbor-joining phenogram is presented in Figure 3.4.

Isolates of *P. oligandrum, P. hydnosporum,* and *P. amasculinum* formed a monophyletic cluster separated from all other species (Fig. 3.4). There was a low level of intraspecific variation among fragment patterns of the ten isolates in the cluster, despite the range of geographic origins of the isolates. The genetic relationship among the four isolates of *P. periplocum* was not as evident. Isolates 91 and 318 were present on separate branches, while isolates 4461a and 4461b were closely related but on an individual branch. Isolates of *P. acanthicum* formed a cluster, although the ex-type culture (71) was more distant from the other four isolates. Two putative isolates of *P. erinaceus* (78621 and 78622) were also present within the *P. acanthicum* cluster, and genetically distant from the ex-type culture of *P. erinaceus* (34). The outgroup species *P. anandrum* and *P. mamillatum* were also present on individual branches.

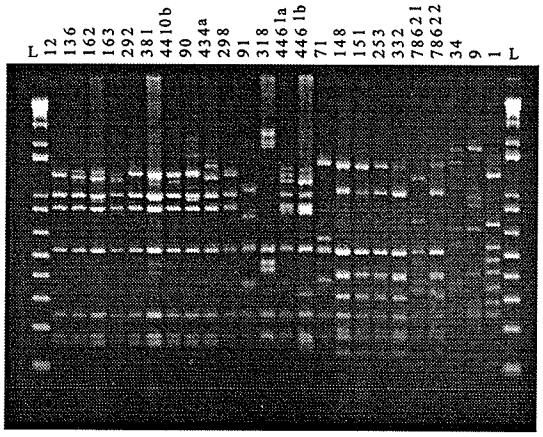
Conclusions

Therefore, *P. oligandrum, P. hydnosporum,* and *P. amasculinum* may in fact be conspecific species based on their molecular relationship and comparable morphologies.

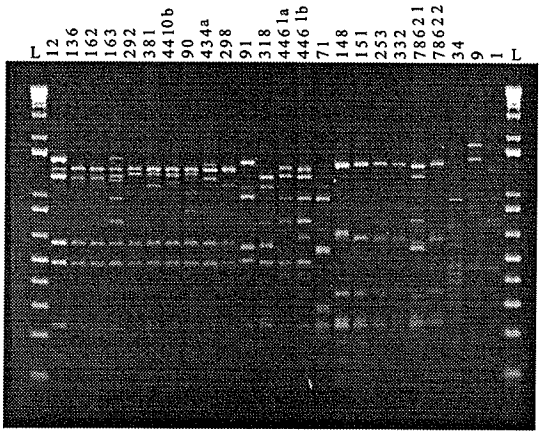
Figure 3.3. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. acanthicum, P. amasculinum, P. anandrum, P. erinaceus, P. hydnosporum, P. mamillatum, P. oligandrum,* and *P. periplocum* with **(A)** *Alu*I, **(B)** *Cfo*I, **(C)** *Hae*III, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 12 136 162 163 292 381 4410b 90 434a 298 91 318 446 1a 446 1b 71 148 151 253 332 78621 78622 34 9 1 L
B
L 12 136 162 163 292 381 4410b 90 434a 298 91 318 446 1a 446 1b 71 148 151 253 332 78621 78622 34 9 1 L
C
L 12 136 162 163 292 381 4410b 90 434a 298 91 318 446 1a 446 1b 71 148 151 253 332 78621 78622 34 9 1 L
D
L 12 136 162 163 292 381 4410b 90 434a 298 91 318 446 1a 446 1b 71 148 151 253 332 78621 78622 34 9 1 L

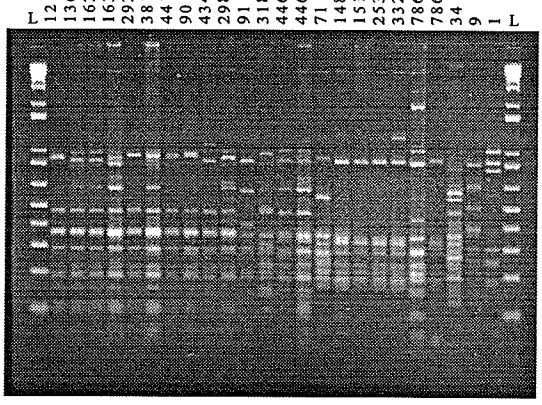
E


L
12
136
162
163
292
381
4410b
90
434a
298
91
318
4461a
446 1b
71
148
151
253
332
78621
78622
34
9
1
L

F


L
12
136
162
163
292
381
4410b
90
434a
298
91
318
4461a
446 1b
71
148
151
253
332
78621
78622
34
9
1
L

G


L
12
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4461a
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253
332
78621
78622
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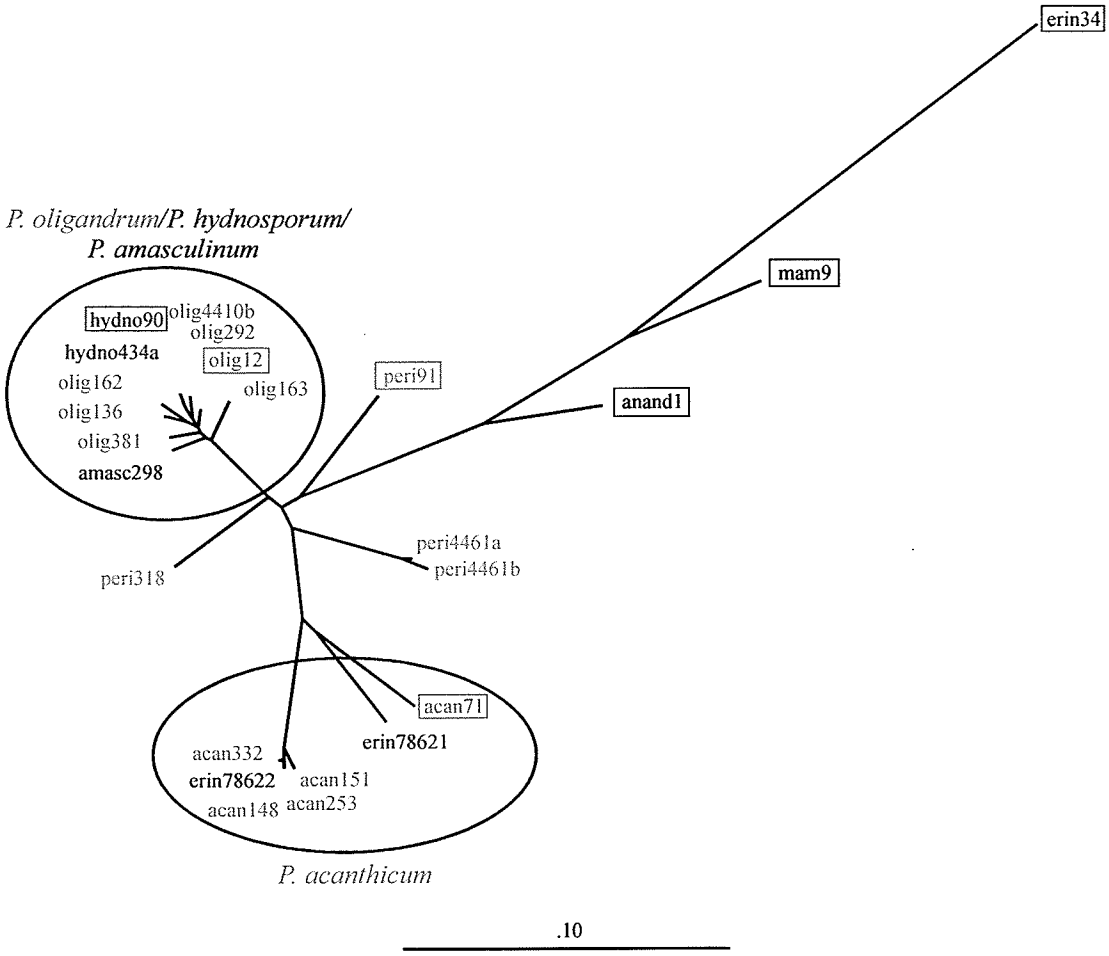
1
L

Figure 3.4. Unrooted neighbor-joining phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.3. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: acan, *P. acanthicum*; amasc, *P. amasculinum*; anand, *P. anandrum*; erin, *P. erinaceus*; hydno, *P. hydnosporum*; mam, *P. mamillatum*; olig, *P. oligandrum*; peri, *P. periplocum.* Boxed species names represent the ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


erin34
mam9
anand1
peri91
P. oligandrum/P. hydnosporum/
P. amasculinum
hydno90
olig4410b
olig292
hydno434a
olig12
olig162
olig163
olig136
olig381
amasc298
peri318
peri4461a
peri4461b
acan71
erin78621
acan332
erin78622
acan151
acan148
acan253
P. acanthicum
.10

The main morphological characters that differentiate these species (the more slender spines of *P. hydnosporum* and absence of antheridia in *P. amasculinum*) are insignificant compared to the shared morphological features. Van der Plaats-Niterink (1981) noted that an isolate from wilting poppies and *Antirrhinum* sp. in South Africa was originally identified as *P. hydnosporum* (Wager, 1931), but later re-identified as *P. oligandrum* (Wager, 1941). *P. amasculinum* was first isolated from soil in China by Yü (1973), but no other records of this species are known and little is known about its pathogenicity. So, similar errors in identifying these morphologically similar species may be more common in literature than reported.

There was genetic variation among the isolates of *P. periplocum* as they did not form a single species cluster. This may reflect the morphological variation which is common within this species. Paul and Masih (2000) reported a high degree of variation in the plerotic/aplerotic nature of the oospores and in the number of oospores per oogonium among isolates of *P. periplocum* from the Canary Islands. Additional morphological examination of these isolates would be necessary to draw conclusions about their taxonomic status. Isolates 78621 and 78622, originally identified as *P. erinaceus,* were most likely misidentified since they were present within the *P. acanthicum* cluster. *P. acanthicum* and *P. erinaceus* are morphologically very similar due to their comparable oogonial ornamentations, globose zoosporangia, and plerotic oospores. The main distinguishing feature between these two species is the shorter oogonial spines in *P. acanthicum* (Van der Plaats-Niterink, 1981).

It is important to be able to accurately distinguish among these species with spiny oogonia as several of them are mycoparasites that have potential as biocontrol agents. *P.*

oligandrum, P. acanthicum, and *P. periplocum* are broad spectrum mycoparasites, but sporadic reports of mycoparasitism in other *Pythium* species with spiny oogonia include *P. acanthophoron* (Lodha and Webster, 1990) and *P. radiosum* (Paul, 1999). Mycoparasitism has also been reported in *P. nunn* and *P. mycoparasiticum,* species which possess smooth oogonia (Jones and Deacon, 1995). *P. aphanidermatum* has also been shown to antagonize *P. oligandrum* (Berry *et al.,* 1993). However, *P. oligandrum* has been the most extensively studied mycoparasitic *Pythium* species.

Pythium oligandrum exhibits mycoparasitic interactions with a wide range of susceptible fungi including *Pythium* spp. (Berry *et al.,* 1993), *Verticillium dahliae* (Al-Rawahi and Hancock, 1998) and *Phytophthora infestans* (Picard *et al.,* 2000a). Benhamou *et al.* (1999) showed that *P. oligandrum* could synthesize cellulases to facilitate the mycoparasitic process. However, *P. oligandrum* does not produce volatile inhibition compounds (Foley and Deacon, 1986), although it is thought to produce antibiotics to inhibit growth of certain fungi (Whipps, 1987). Extensive hyphal coiling around the host is often observed, and is thought to indicate host resistance by some (Foley and Deacon, 1986) while others view it as an indication of host susceptibility (Ali-Shtayeh and Saleh, 1999).

Pythium oligandrum has the potential to be useful as a protectant against a range of fungal root invaders as it can persist in the rhizosphere for extended periods of time. *P. periplocum* has also been evaluated as a biocontrol agent for damping-off of cucumber seedlings caused by *P. aphanidermatum* (Hockenhell *et al.,* 1992). Attempts have been made using the oospores of *P. oligandrum* as a biocontrol inoculant to protect crop plants from seedling diseases by using manual seed-coating with oospores or by the

incorporation of oospores into sowing sites (Martin and Hancock, 1987; McQuilken *et al.,* 1990). One limitation is that newly formed oospores germinate slowly, requiring 8 to 12 hours, while a pathogen such as *P. ultimum* can infect a plant within a few hours.

Pythium oligandrum was also shown to induce resistance in host tomato plants upon challenge with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Benhamou *et al.,* 1997). This may be due to the production of oligandrin, a low molecular weight protein secreted by *P. oligandrum,* which has been shown to induce plant resistance to cell invasion by *Phytophthora parasitica* (Picard *et al.,* 2000b) and *Fusarium oxysporum* (Benhamou *et al.,* 2001). Oligandrin may act as an elicitin, a fungal proteinaceous molecule with signalling properties that induce hypersensitive reactions and resistance against fungi and bacteria. Elicitins are also produced by *Phytophthora* spp., in which they act as inducers of tobacco defense responses, and in the *Pythium* species *P. vexans, P. oedochilum,* and *P. marsipium* (Panabières *et al.,* 1997).

In conclusion, *P. oligandrum, P. hydnosporum,* and *P. amasculinum* may be conspecific due to their morphological similarities and genetic relationships revealed from RFLP analysis.

B. Molecular relationships among non-mycoparasitic species with globose non-proliferating zoosporangia and ornamented oogonia

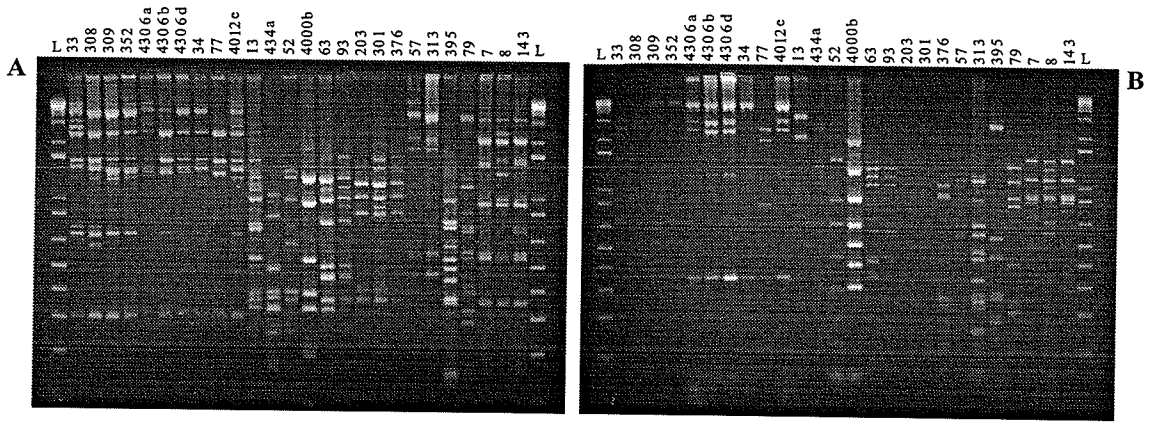
Results and Discussion

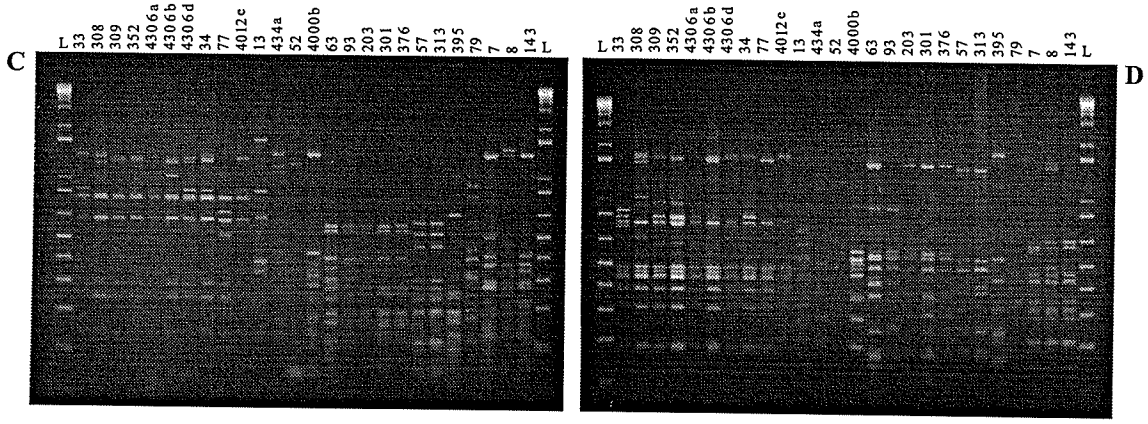
The interspecific relationships among additional subsets of species with spherical zoosporangia and ornamented oogonia were examined using RFLP analysis of the rDNA

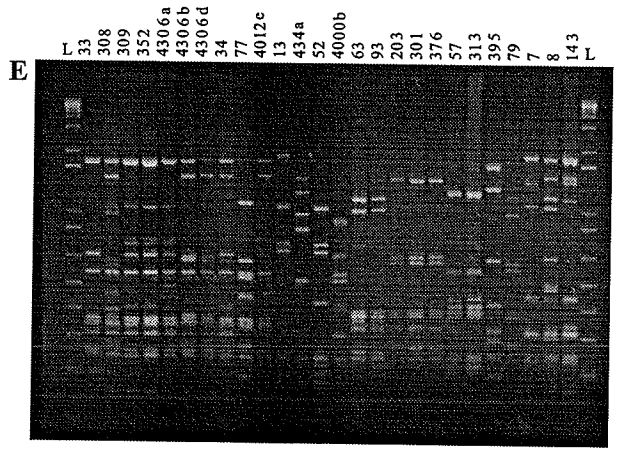
IGS. Restriction endonuclease digestions of the amplified IGS using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I were performed for several isolates of *P. acanthophoron, P. buismaniae, P. echinulatum, P. erinaceus, P. hydnosporum, P. jasmonium, P. mastophorum, P. polymastum, P. radiosum, P. spinosum,* and *P. uncinulatum* (Fig. 3.5). Isolates of *P. macrosporum* and *P. boreale,* which do not produce ornamented oogonia, were also included in the analysis. A total of 399 restriction fragments were used for RFLP analysis. The UPGMA and neighbor-joining phenograms had identical topologies, and the UPGMA phenogram is presented in Figure 3.6.

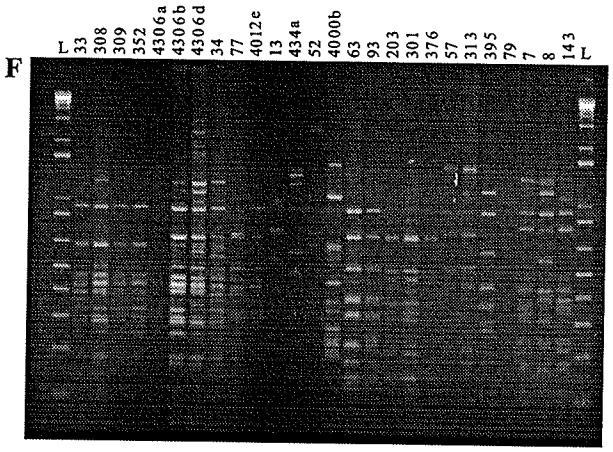
Pythium echinulatum, P. erinaceus, P. radiosum, and *P. spinosum* 4012e formed a discrete cluster which included the ex-type cultures of *P. erinaceus* (34) and *P. radiosum* (77) (Fig. 3.6). The ex-type culture of *P. echinulatum* was unavailable, but the ex-type culture of *P. spinosum* (13) was distantly related to this cluster on a separate branch. *P. echinulatum* and *P. erinaceus* are morphologically similar as they possess globose zoosporangia (which are occasionally arranged in chains in *P. echinulatum*), globose oogonia with similar average diameters (21.0 and 22.0 µm, respectively), and comparable conical oogonial projections. *P. echinulatum* produces aplerotic or plerotic oospores while the oospores of *P. erinaceus* are described as "plerotic or nearly so" (Van der Plaats-Niterink, 1981). The main differences between these species are the antheridial origins (hypogynous or monoclinous in *P. echinulatum* and diclinous in *P. erinaceus*) and the broad contact of the antheridium with the oogonium in *P. erinaceus* (Van der Plaats-Niterink, 1981). *P. radiosum* is similar to *P. echinulatum* and *P. erinaceus* as it also forms globose zoosporangia with conical ornamentations. However, the oogonia of *P.*

Figure 3.5. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. acanthophoron, P. buismaniae, P. echinulatum, P. erinaceus, P. hydnosporum, P. jasmonium, P. mastophorum, P. polymastum, P. radiosum, P. spinosum,* and *P. uncinulatum* with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 33 308 309 352 4306a 4306b 4306d 34 77 4012e 13 434a 52 4000b 63 93 203 301 376 57 313 395 79 7 8 143 L
B
L 33 308 309 352 4306a 4306b 4306d 34 77 4012e 13 434a 52 4000b 63 93 203 301 376 57 313 395 79 7 8 143 L


C
L 33 308 309 352 4306a 4306b 4306d 34 77 4012e 13 434a 52 4000b 63 93 203 301 376 57 313 395 79 7 8 143 L
D
L 33 308 309 352 4306a 4306b 4306d 34 77 4012e 13 434a 52 4000b 63 93 203 301 376 57 313 395 79 7 8 143 L


E
L
33
308
309
352
4306a
4306b
4306d
34
77
4012e
13
434a
52
4000b
63
93
203
301
376
57
313
395
79
7
8
143
L


F
L
33
308
309
352
4306a
4306b
4306d
34
77
4012e
13
434a
52
4000b
63
93
203
301
376
57
313
395
79
7
8
143
L

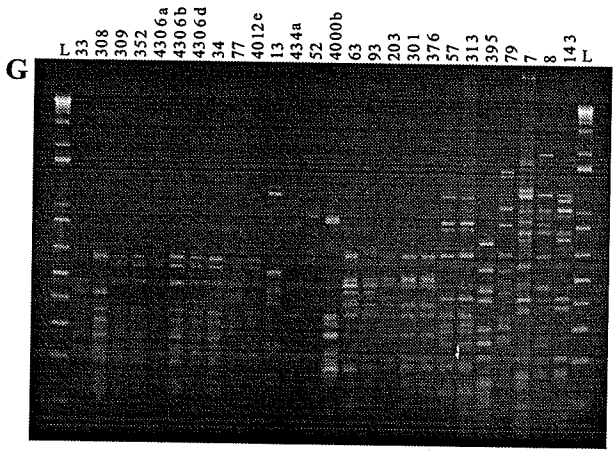
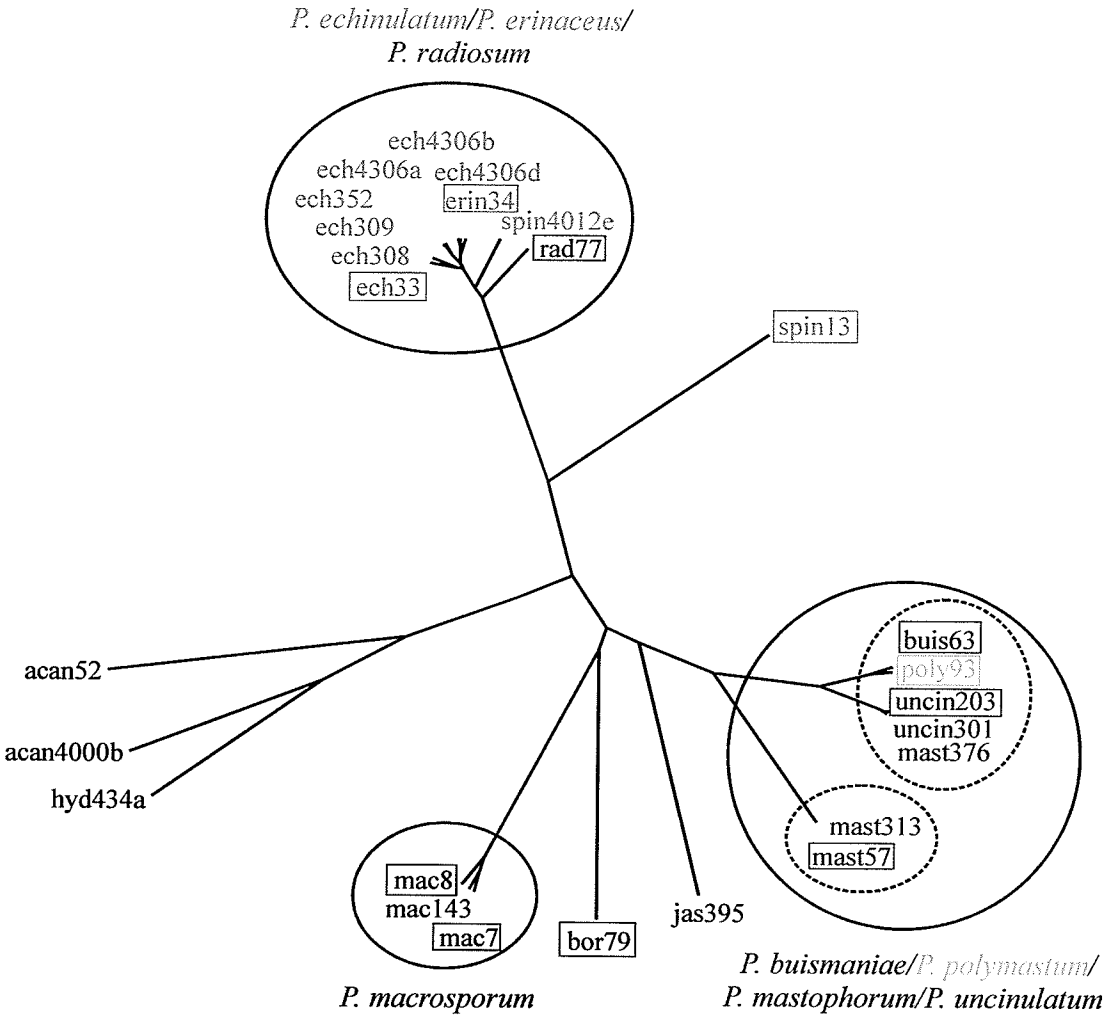

G
L
33
308
309
352
4306a
4306b
4306d
34
77
4012e
13
434a
52
4000b
63
93
203
301
376
57
313
395
79
7
8
143
L

Figure 3.6. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.5. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: acan, *P. acanthophoron*; bor, *P. boreale*; buis, *P. buismaniae*; ech, *P. echinulatum*; erin, *P. erinaceus*; hyd, *P. hydnosporum*; jas, *P. jasmonium*; mac, *P. macrosporum*; mast, *P. mastophorum*; poly, *P. polymastum*; rad, *P. radiosum*; spin, *P. spinosum*; uncin, *P. uncinulatum.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. echinulatum/P. erinaceus/
P. radiosum
ech4306b
ech4306a
ech4306d
ech352
erin34
ech309
spin40f12e
ech308
rad77
ech33
spin13
acan52
acan4000b
hyd434a
buis63
poly93
uncin203
uncin301
mast376
mast313
mast57
mac8
mac143
mac7
bor79
jas395
P. macrosporum
P. buismaniae/P. polymastum/
P. mastophorum/P. uncinulatum

.10

radiosum are somewhat larger (up to 35 µm in diameter) and have longer oogonial spines. When present, the antheridia of *P. radiosum* are hypogynous, as in *P. echinulatum,* but the aplerotic oospores of *P. radiosum* do not distinguish this species from the others (Paul, 1992).

Since the ex-type culture of *P. echinulatum* was not available, morphological identifications of the other *P. echinulatum* isolates had previously been made based on comparisons to isolate 33. The ex-type culture of *P. erinaceus* (34) was present in this analysis, but no other records of this species are known. Since these two species share many morphological similarities and a close genetic relationship, these species may be conspecific. If the single reported isolate of *P. erinaceus* is in fact a strain of *P. echinulatum,* the species name "*erinaceus*" could be ignored. Notably, isolates 33 and 34 are also geographically related as they were isolated from soil in Australia and New Zealand, respectively.

The ex-type culture of *P. radiosum* (77) is the only reported isolate of this species (Paul, 1992). It was very similar to *P. echinulatum* and *P. erinaceus* based on RFLP analysis of the IGS. The main morphological difference is the relatively larger oogonia of *P. radiosum.* This isolate of *P. radiosum* was shown to be an aggressive mycoparasite of *Botrytis cinerea* (Paul, 1999), but mycoparasitism has not been exhibited in isolates of *P. echinulatum* or *P. erinaceus.* The acquisition of additional isolates of *P. radiosum,* or the exhibition of mycoparasitism in *P. echinulatum* or *P. erinaceus,* would support this evidence of the close genetic relationship among these three species. The only other mycoparasite examined in this analysis was *P. acanthophoron.* This species can be distinguished from the others as it does not form zoosporangia and has shorter oogonial

spines with rounded tips (Lodha and Webster, 1990). Isolates 52 and 4000b were not closely related in the phenogram, though. This culture of *P. acanthophoron* is currently sterile so further morphological comparisons between these two isolates are not possible.

Isolate 4012e, which was also present within the *P. echinulatum/P. erinaceus/P. radiosum* cluster, was most likely incorrectly identified as *P. spinosum* since *P. spinosum* (13), the culture used by Van der Plaats-Niterink (1981) in the species description, was present on an individual branch. *P. spinosum* is morphologically similar to *P. echinulatum, P. erinaceus,* and *P. radiosum* as it also forms globose ornamented oogonia, monoclinous and diclinous antheridia, and plerotic or aplerotic oospores. The main difference is the finger-like spiny oogonial ornamentations with blunt tips of *P. spinosum* in comparison to the conical spines of the other species. *P. spinosum* also does not form zoosporangia, but rather produces hyphal swellings (Van der Plaats-Niterink, 1981). It is difficult to discern whether isolate 4012e is more closely related to *P. radiosum* or to *P. echinulatum/P. erinaceus* due to its intermediate position between these species in the phenogram (Fig. 3.6).

Isolates of *P. buismaniae, P. mastophorum, P. polymastum,* and *P. uncinulatum* formed a monophyletic cluster which included ex-type cultures of *P. buismaniae* (63) and *P. uncinulatum* (203) (Fig. 3.6). *P. buismaniae* and *P. polymastum* are morphologically similar (see Table 3.2) as they produce very large oogonia that are covered with spines of similar length and width. Both species also form very large aplerotic oospores and diclinous antheridia that usually entangle the oogonial stalk. The key difference between these species is that zoosporangia are not formed in *P. buismaniae.*

Pythium uncinulatum and *P. mastophorum* are similar to *P. buismaniae* and *P. polymastum* due to their comparable oogonial spine dimensions, zoosporangial form, diclinous antheridia, and aplerotic oospores (Table 3.2). However, they can be differentiated from *P. buismaniae* and *P. polymastum* by their relatively smaller oospores and oogonia, which are still rather large compared to those of most *Pythium* species (Van der Plaats-Niterink, 1981). *P. uncinulatum* and *P. mastophorum* share similarly sized oospores and antheridial cells that are broadly applied to the oogonial surface. *P. mastophorum* can be differentiated from *P. uncinulatum* by its sometimes plerotic oospores, slightly larger oogonia, and mammiform oogonial spines.

In the UPGMA phenogram (Fig. 3.6), the cluster comprised of *P. buismaniae, P. mastophorum* 376, *P. polymastum,* and *P. uncinulatum* was divided into two smaller groupings (dashed circles), one containing *P. buismaniae* 63 and *P. polymastum* 93, and the other comprised of *P. uncinulatum* (isolates 203 and 301) and *P. mastophorum* 376. While the large cluster reflects the morphological similarities among these four species, the smaller groupings within demonstrated which species, or isolates, were more closely related to one another. The isolate of *P. polymastum* (93) was the culture used by Van der Plaats-Niterink (1981) for the species description. Due to the close genetic and morphological relationships of the ex-type cultures of *P. buismaniae* (63) and *P. polymastum* (93), these species are most likely conspecific. Studies of isolates of each species from various locations would strengthen this conclusion.

Pythium uncinulatum may be genetically and morphologically related to *P. buismaniae* and *P. polymastum,* but isolates 203 and 301 were present on a separate branch which supports the distinction of this species from the others based on its smaller oogonia and

Table 3.2. Comparison of several morphological characteristics between *P. buismaniae, P. mastophorum, P. polymastum,* and *P. uncinulatum* based on the descriptions by Van der Plaats-Niterink (1981).

Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. buismaniae	Not formed	Terminal on short side branches, avg. diam 58.0 µm; conical spines around 7 µm long and 4.5 µm at the base	Diclinous, cells often lobed, 1—3 per oogonium; antheridial stalks sometimes entwine the oogonium	Aplerotic (avg. diam 51.5 µm; wall 2—4 µm)
P. polymastum	Subglobose, intercalary or terminal	Terminal on short side branches, avg. diam. 53.0 µm; conical or mammiform spines, from 5—8 µm long and 5—6 µm at the base	Diclinous and lobate or with projections, 1—4 antheridia per oogonium; usually entangle the oogonial stalk and basal part of the oogonium	Aplerotic (avg. diam 43.5 µm; wall 2—5 µm thick)
P. uncinulatum	Globose, terminal, occasionally intercalary	Terminal, avg. diam 34.0 µm; conical and acute spines, from 7—10µm long and 4—5 µm at the base; spines slightly bent	Diclinous, one stalk per oogonium bearing 1—8 antheridial cells; antheridia broadly applied to the oogonium near its stalk	Aplerotic (avg. diam. 30.5 µm; wall 3—4 µm thick)
P. mastophorum	Subglobose, terminal or intercalary	Terminal on short side branches, avg. diam 38.5µm; conical or mammiform spines, from 2—8 µm long and 2—6 µm at the base	Diclinous, one per oogonium; lobate cells, often with projections, broadly applied to the oogonium	Aplerotic or plerotic (avg. diam 34.5 µm; wall up to 4 µm thick)

oospores. Two isolates of *P. mastophorum* (57 and 313) were more distantly related to *P. uncinulatum* and *P. mastophorum* 376. No ex-type culture is available for *P. mastophorum*, but the description by Van der Plaats-Niterink (1981) was based on isolate 57. Therefore, due to its close genetic relationship to the ex-type culture of *P. uncinulatum* (203) and the distance from other isolates of *P. mastophorum*, isolate 376 is probably an isolate of *P. uncinulatum* incorrectly identified as *P. mastophorum.*

The outgroup species *P. hydnosporum* (ornamented oogonia but lacking zoosporangia), *P. boreale* (globose zoosporangia and smooth oogonia), and *P. jasmonium* were genetically distinct from the other species, although *P. hydnosporum* was somewhat related to the isolates of *P. acanthophoron,* a species that also has ornamented oogonia and lacks zoosporangia. The three isolates of *P. macrosporum* (7, 8, and 143), a heterothallic species with smooth oogonia and globose zoosporangia, formed a cluster separate from all other species. The isolate of *P. jasmonium (nomen nudum)* was related to *P. buismaniae* and *P. uncinulatum* based on ITS sequence analysis (C.A. Lévesque, personal communication), but little is known about this species as no descriptions are available to date. RFLP analysis of the IGS showed that *P. jasmonium* was distinct from all of the compared species.

C. Relationships between *P. conidiophorum, P. hypogynum,* and *P. salpingophorum*; species with globose non-proliferating zoosporangia and smooth oogonia

Introduction

There are between 12 to 15 *Pythium* species with globose non-proliferating zoosporangia that also possess smooth-walled oogonia (Dick, 1990a). In the previous two

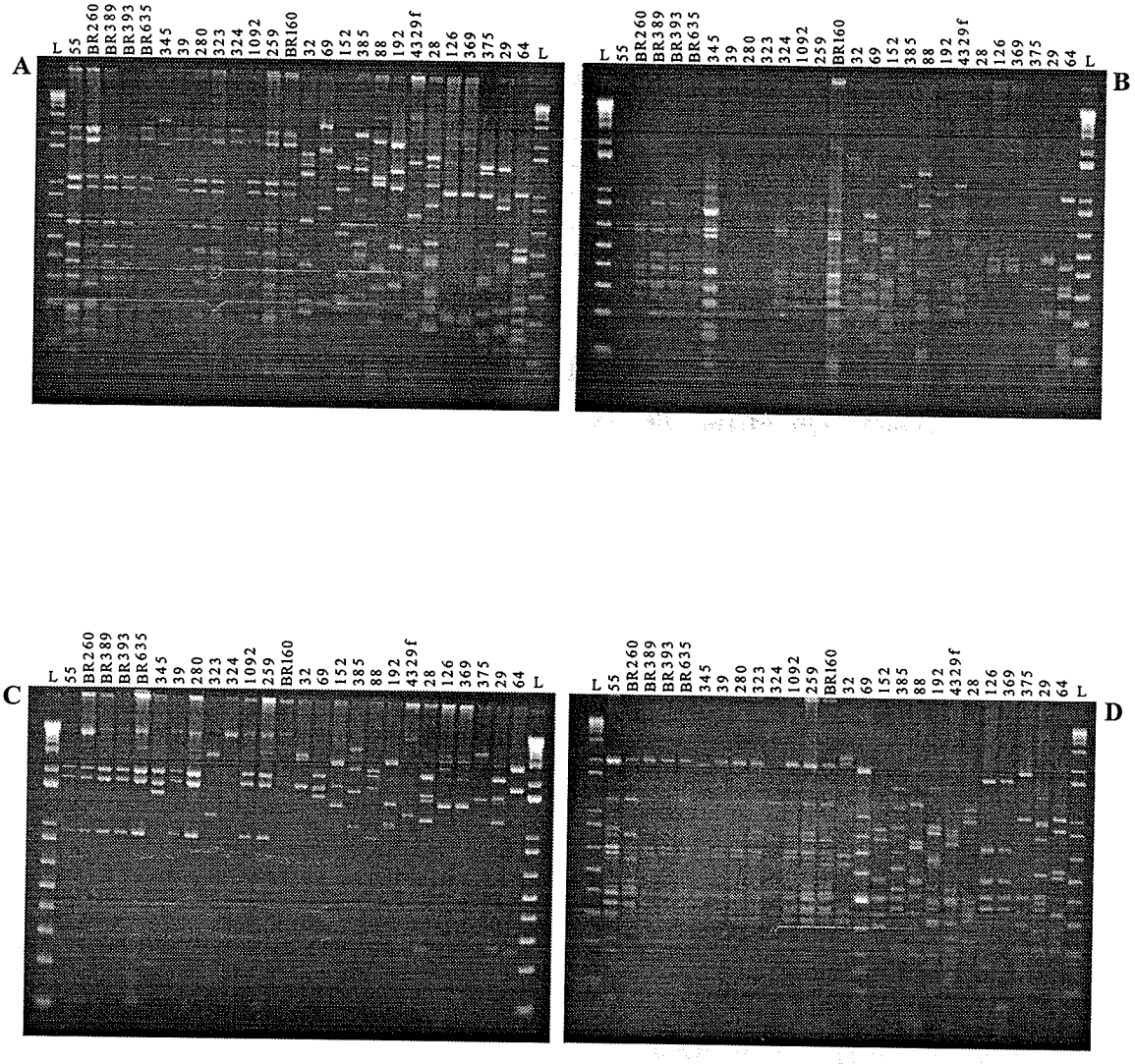
examples of groups of species with ornamented oogonia and globose zoosporangia, isolates of the same species tended to form species clusters. When more than one species was present within a cluster, those species had a majority of morphological features in common. The following example illustrates a situation where several isolates of different species (*P. conidiophorum, P. hypogynum,* and *P. salpingophorum*) formed a genetic cluster, despite significant morphological differences among them, and some species (*P. acrogynum, P. iwayamai, P. rostratum,* and *P. violae*), whose isolates exhibited such heterogeneity in their restriction fragment patterns , were scattered throughout the phenogram.

Results and Discussion

Restriction endonuclease digestions of amplicons of the IGS were done for isolates of *P. acrogynum, P. conidiophorum, P. dissimile, P. hypogynum, P. iwayamai, P. rostratum, P. salpingophorum,* and *P. violae* using *Alu*I, *Hae*III, *Hha*I, *Hin*cII, *Hin*fI, *Msp*I, and *Rsa*I (Fig. 3.7). A total of 327 restriction fragments were used for RFLP analysis. The UPGMA and neighbor-joining phenogram topologies were identical, and the UPGMA phenogram is presented in Figure 3.8.

A monophyletic cluster containing isolates of *P. conidiophorum, P. hypogynum,* and *P. salpingophorum* was produced (Fig. 3.8), and isolate BR160 of *P. dissimile* was also present. *P. conidiophorum* was classified as a doubtful species by Van der Plaats-Niterink (1981) and there has been a great deal of uncertainty in the classification of isolates with this species name. In fact, isolates 323 and 324 were originally identified as

Figure 3.7. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. acrogynum, P. conidiophorum, P. dissimile, P. hypogynum, P. iwayamai, P. rostratum, P. salpingophorum,* and *P. violae* with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hinc*II, **(E)** *Hin*fI, **(F)** *Msp*I, and **(G)** *Rsa*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 55 BR260 BR389 BR393 BR635 345 39 280 323 324 1092 259 BR160 32 69 152 385 88 192 4329f 28 126 369 375 29 64 L
B
L 55 BR260 BR389 BR393 BR635 345 39 280 323 324 1092 259 BR160 32 69 152 385 88 192 4329f 28 126 369 375 29 64 L
C
L 55 BR260 BR389 BR393 BR635 345 39 280 323 324 1092 259 BR160 32 69 152 385 88 192 4329f 28 126 369 375 29 64 L
D
L 55 BR260 BR389 BR393 BR635 345 39 280 323 324 1092 259 BR160 32 69 152 385 88 192 4329f 28 126 369 375 29 64 L

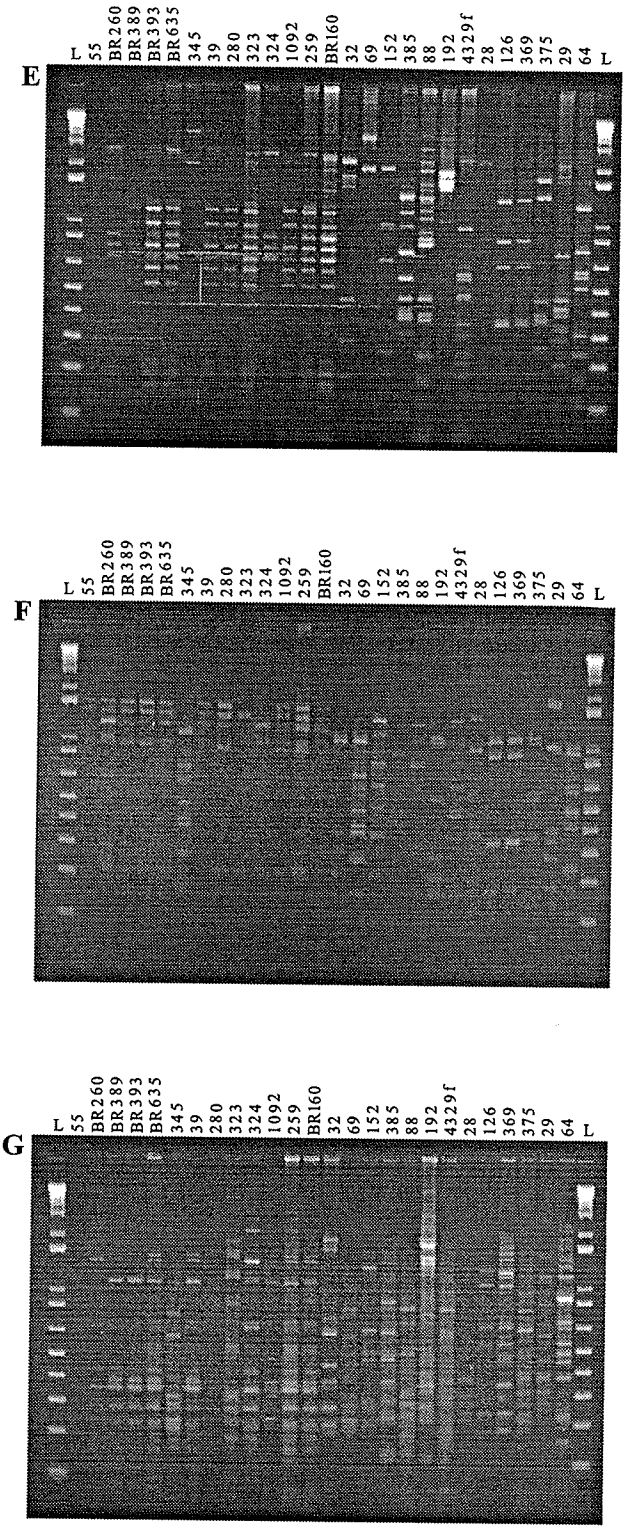
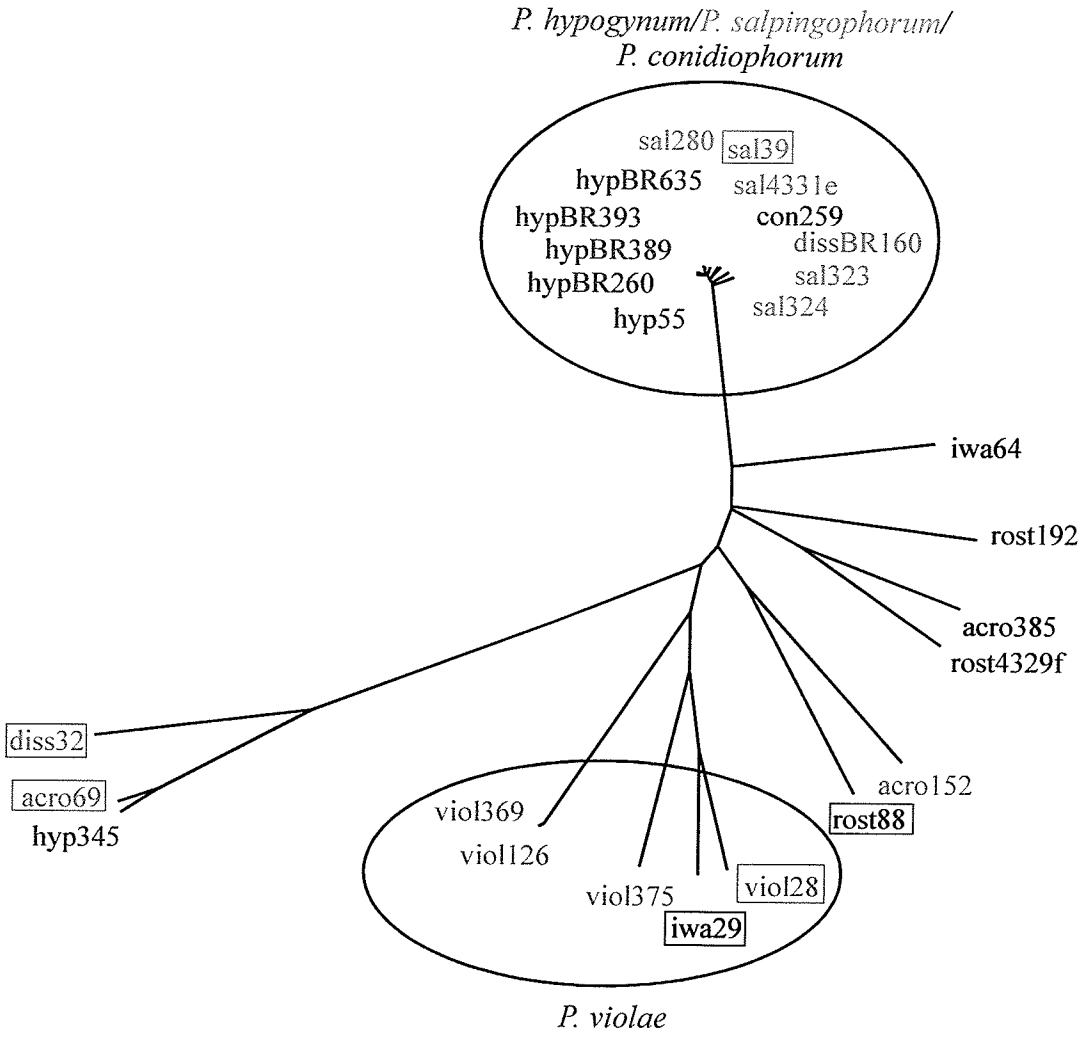

E
L
55
BR260
BR389
BR393
BR635
345
39
280
323
324
1092
259
BR160
32
69
152
385
88
192
4329f
28
126
369
375
29
64
L
F
L
55
BR260
BR389
BR393
BR635
345
39
280
323
324
1092
259
BR160
32
69
152
385
88
192
4329f
28
126
369
375
29
64
L
G
L
55
BR260
BR389
BR393
BR635
345
39
280
323
324
1092
259
BR160
32
69
152
385
88
192
4329f
28
126
369
375
29
64
L

Figure 3.8. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.7. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: acro, *P. acrogynum*; con, *P. conidiophorum*; diss, *P. dissimile*; hyp, *P. hypogynum*; iwa, *P. iwayamai*; rost, *P. rostratum*; sal, *P. salpingophorum*; viol, *P. violae.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. hypogynum/P. salpingophorum/
P. conidiophorum
sal280
sal39
hypBR635
sal4331e
hypBR393
con259
dissBR160
hypBR389
sal323
hypBR260
sal324
hyp55
iwa64
rost192
acro385
rost4329f
diss32
acro69
hyp345
viol369
viol126
viol375
viol28
iwa29
acro152
rost88
P. violae
.10

P. conidiophorum by IMI, but were later re-identified as *P. salpingophorum.* To confuse matters further, the ex-type culture of *P. conidiophorum* (CBS 223.88; RFLPs not included in this study) was determined to be the same strain as *P. salpingophorum* 323, and is now present in the IMI collection under the name *P. salpingophorum* (A.W.A.M. de Cock, personal communication). The main difference between the two species is the production of proliferating zoosporangia by *P. salpingophorum* and hyphal swellings or "conidia" by *P. conidiophorum* (Van der Plaats-Niterink, 1981). Based on its genetic relationship with *P. hypogynum* and *P. salpingophorum,* and the frequent uncertainty in the identification of isolates, the species name *P. conidiophorum* should therefore be considered a synonym of *P. salpingophorum* and *P. hypogynum.*

Pythium hypogynum and *P. salpingophorum* are actually quite morphologically distinct from one another. Both species produce subglobose zoosporangia which can be terminal or intercalary. However, proliferation of zoosporangia is sometimes observed in *P. salpingophorum*, so variation of this character within the species could result in the misidentification of isolates. The oogonia of *P. salpingophorum* are smaller than those of *P. hypogynum,* and are often present in chains of 2 to 5 and connected by hyphal parts. Antheridia in *P. hypogynum* are strictly hypogynous, while *P. salpingophorum* may have monoclinous or diclinous antheridia, or lack them altogether. Both species do, however, share plerotic oospores of similar size (Van der Plaats-Niterink, 1981; Dick, 1990a).

Using partial ETS sequences, Belkhiri (1994) showed that *P. hypogynum* was the only species with globose zoosporangia which had an ETS sequence that could be aligned with corresponding sequences from species with filamentous zoosporangia. Based on ITS sequence analysis, C.A. Lévesque (personal communication) showed that *P.*

conidiophorum and *P. salpingophorum* were closely related to each other and located within a clade comprised of species with filamentous zoosporangia. This molecular positioning of *P. conidiophorum, P. hypogynum,* and *P. salpingophorum* among species with filamentous zoosporangia may be an indication that the zoosporangial form in these species is not homologous with that of species with globose or spherical zoosporangia, which was suggested by Belkhiri (1994). However, the morphologies of the isolates and species in question would have to be re-examined in order to draw conclusions about the close genetic relationships between *P. hypogynum* and *P. salpingophorum* since significant morphological differences are evident when these species are compared.

Pythium dissimile BR160 was also present in the cluster with *P. hypogynum* and *P. salpingophorum,* but the ex-type culture (32) was located on an individual branch. *P. dissimile* is similar to *P. hypogynum* and *P. salpingophorum* as it produces terminal or intercalary globose zoosporangia and plerotic oospores with a comparable wall thickness. Antheridia are often lacking (as in *P. salpingophorum*) but are hypogynous when present (like in *P. hypogynum*). The main difference is the smaller oogonia of *P. dissimile* (Van der Plaats-Niterink, 1981). Since isolate BR160 was more closely related to *P. hypogynum* and *P. salpingophorum* than to the ex-type culture of *P. dissimile,* it was most likely misidentified.

The three isolates of *P. acrogynum* (69, 152, and 385) were genetically diverse as they were present on individual branches (Fig. 3.8). However, *P. hypogynum* 345 was closely related to the ex-type culture of *P. acrogynum* (69) and distinct from the *P. hypogynum/P. salpingophorum* cluster. These species are quite similar as they produce subglobose zoosporangia, oogonia with an average diameter of 21 to 22 µm, hypogynous

antheridia, and plerotic thin-walled oospores. The differences are the larger antheridia and papillate oogonia of *P. acrogynum* (Van der Plaats-Niterink, 1981). It is most probable that isolate 345 is an isolate of *P. acrogynum* mistakenly classified as *P. hypogynum.* ITS sequence analysis also showed that *P. hypogynum* 345 was almost identical to *P. acrogynum* 69 (C.A. Lévesque, personal communication). The other isolates of *P. acrogynum* (152 and 385) were distantly related to each other and to the ex-type culture (69). This may be indicative of morphological variability among these three isolates or the existence of entirely different species altogether. More detailed morphological examinations would be necessary.

A similar situation was evident for *P. rostratum* as isolates 88, 192, and 4329f did not form a genetic cluster (Fig. 3.8). Isolates 88 (the neotype culture) and 4329f were present on branches with *P. acrogynum* 152 and 385, respectively, but the large branch lengths separating the isolates indicated the lack of a close genetic relationship. These species are similar in that they produce subglobose zoosporangia, hypogynous antheridia, plerotic oospores, and have similarly sized oogonia (Van der Plaats-Niterink, 1981). Additional morphological examinations of these isolates are necessary to better understand their relationship. Dick (1990a) suggested that *P. rostratum* may be close to, if not conspecific with, *P. hypogynum.* However, *P. rostratum* has monoclinous antheridia and thicker oospore walls that differentiate it from *P. hypogynum* (Van der Plaats-Niterink, 1981), and this was supported by the lack of clustering of these species in Figure 3.8.

Isolates of *P. violae* formed a cluster, but there was a high degree of genetic diversity among isolates due to the long branch lengths separating the isolates from each

other (Fig. 3.8). The culture used by Van der Plaats-Niterink (1981) in the species description (isolate 28) was from soil in Australia while the remaining isolates (126, 369, and 375) were from carrots in Europe. Morphologically, these three isolates were also distinct from isolate 28 and may in fact represent new species (A.W.A.M. de Cock, personal communication) or geographical variants of *P. violae.* Zoosporangia in *P. violae* are unknown but hyphal swellings are produced. ITS sequence analysis showed that *P. violae* 28 was present in a clade among species with filamentous zoosporangia, while isolate 126 was located in another clade among isolates with globose zoosporangia or hyphal swellings (C.A. Lévesque, personal communication). Therefore, morphological variation may exist among these isolates of *P. violae,* reflecting the lack of clustering of the isolates in the phenogram.

The two isolates of *P. iwayamai* (29 and 64) were also sufficiently distinct from one another, and isolate 29 was present among isolates of *P. violae. P. iwayamai* is similar to *P. violae* in that both species produce hyphal swellings, but *P. iwayamai* also forms globose zoosporangia. Both species have terminal or intercalary oogonia of similar diameters and monoclinous or diclinous antheridia. However, the number of antheridia per oogonium is lower in *P. iwayamai* which also has smaller oospores (Van der Plaats-Niterink, 1981).

III. RFLP analysis of *Pythium* species with proliferating zoosporangia

Approximately 18 *Pythium* species produce spherical or ellipsoid zoosporangia which are proliferating (Dick, 1990a). Internal proliferation may occur where a new zoosporangium forms inside the old zoosporangial wall. However, a filament may also

grow out from within the old zoosporangium and form a new zoosporangium externally (Van der Plaats-Niterink, 1981). The following section reports studies using RFLP analysis of the rDNA IGS to examine relationships among three subgroups of *Pythium* species with proliferating, or occasionally proliferating, zoosporangia.

A. Molecular comparison of *Pythium* species with globose proliferating zoosporangia

Results and Discussion

Pythium helicoides, P. middletonii, P. oedochilum, and *P. ostracodes* (Table 3.3) produce globose to subglobose proliferating zoosporangia and smooth globose oogonia, but the average oogonial diameter is smallest for *P. middletonii.* Antheridial origins are monoclinous in these species, although sometimes variable in *P. ostracodes* and *P. middletonii.* Coiling of the antheridial stalk around the oogonial branch occurs only in *P. oedochilum* and *P. helicoides,* and broad contact of antheridia with oogonia is common in these species except in *P. middletonii.* As well, *P. middletonii* has the smallest oospores among these four species, and only *P. ostracodes* forms plerotic oospores.

To examine relationships among *P. helicoides, P. middletonii, P. oedochilum,* and *P. ostracodes,* RFLP analysis of the IGS was done using several isolates. Three isolates of *P. grandilobatum (nomen nudum)* were also included. The outgroup species used were *P. nagaii, P. multisporum,* and *P. marsipium* which also produce proliferating zoosporangia, but the zoosporangia of *P. nagaii* and *P. multisporum* are often pyriform and the oogonia and oospores of *P. nagaii* are smaller compared to those of the above

Table 3.3. Comparisons of several morphological traits of *P. helicoides, P. middletonii, P. oedochilum,* and *P. ostracodes* based on descriptions by Van der Plaats-Niterink (1981).

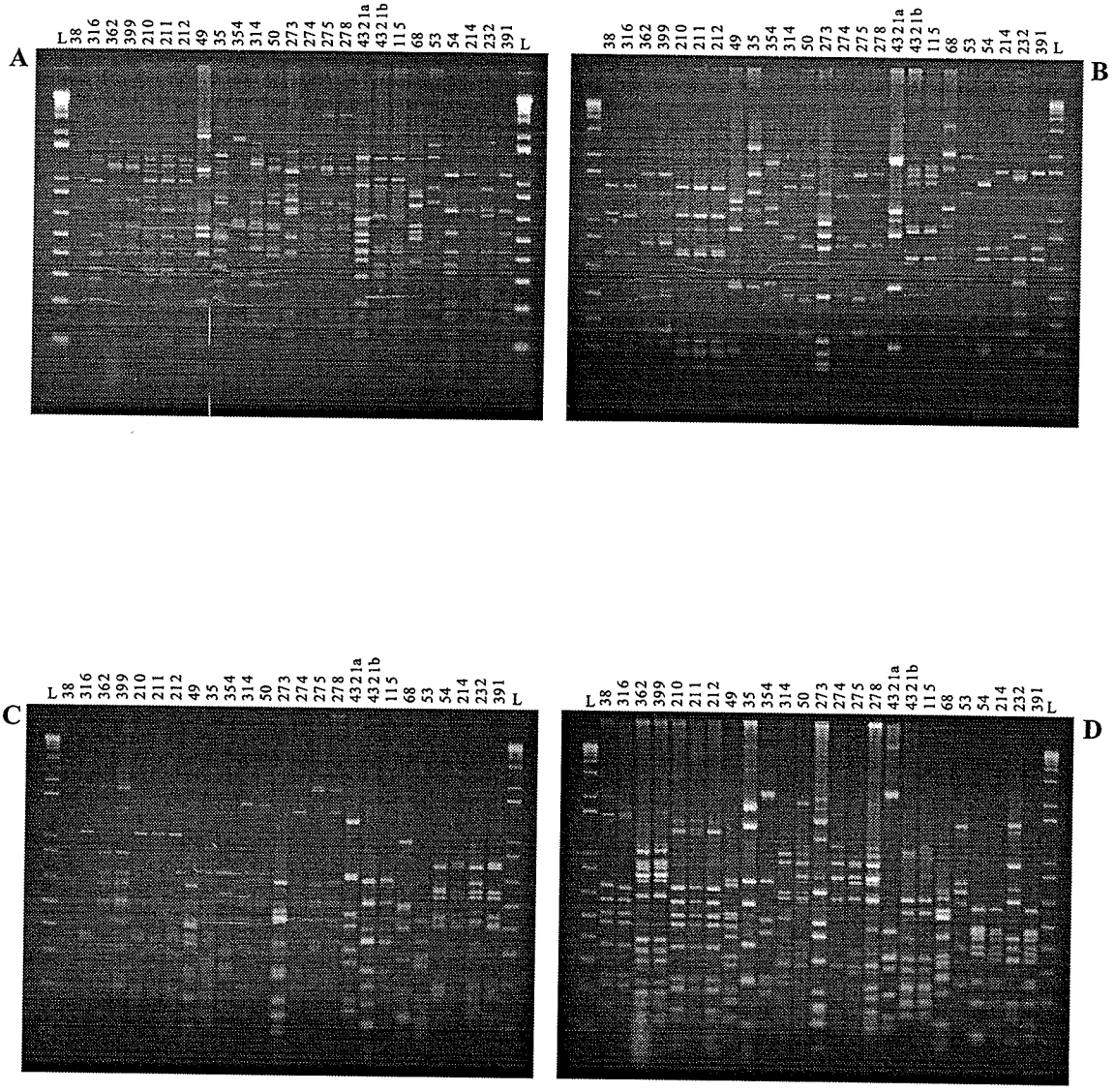
Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. helicoides	Subglobose or ovoid, proliferating	Terminal, lateral, or intercalary; avg. diam. 33.5 µm	Monoclinous, sometimes winding around oogonial stalk or other hyphae; from 1—4 per oogonium	Aplerotic, often yellowish; avg. diam. 30.5 µm; wall 4—6 µm thick
P. middletonii	Globose or ovoid, proliferating	Mostly intercalary, occasionally terminal; avg. diam. 23.0 µm	Monoclinous and stalked or sessile, originating immediately below oogonium; also diclinous or hypogynous	Aplerotic; avg. diam. 20.5 µm; wall 1.5—2 µm thick
P. oedochilum	Subglobose, proliferating	Terminal, occasionally intercalary; avg. diam. 32.8 µm	Mostly diclinous, some monoclinous; cells curved or elongate and applied lengthwise to oogonium; 1—2 (up to 4) per oogonium	Aplerotic; avg. diam. 30.3 µm; wall 1.4—5 µm thick
P. ostracodes	Subglobose, proliferating	Intercalary or terminal; avg. diam. 35.0 µm	Monoclinous, occasionally diclinous with long antheridial cells often laterally applied to oogonium; from 1—2 per oogonium	Plerotic, or nearly plerotic but not fused with oogonial wall; avg. diam. 32.5 µm; wall 4—5 µm thick

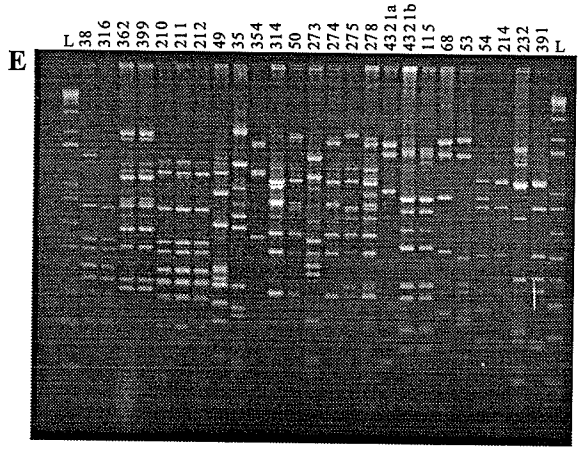
species. *P. multisporum* forms plerotic oospores with more than one oospore in an oogonium, while *P. marsipium* produces papillate zoosporangia and diclinous antheridia (Van der Plaats-Niterink, 1981). Two isolates of *P. paroecandrum* and *P. grandisporangium* were also included in the analysis. Restriction endonuclease digestions of the PCR-amplified IGS were performed for isolates of the above named species using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Msp*I, *Rsa*I, and *Taq*I (Fig. 3.9). A total of 347 restriction fragments were used for cluster analysis and the UPGMA phenogram is presented in Figure 3.10.

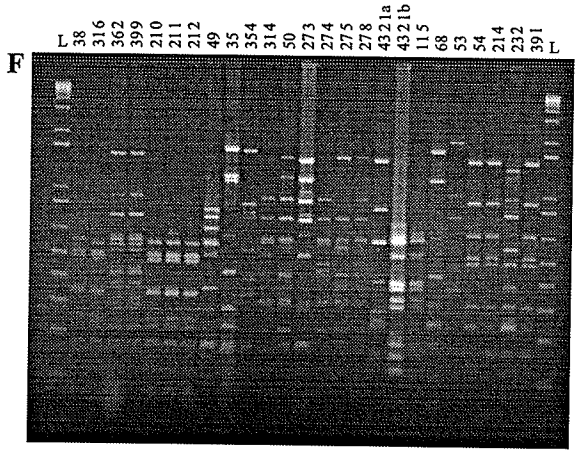
Isolates of *P. grandilobatum, P. helicoides, P. middletonii, P. oedochilum,* and *P. ostracodes* formed two monophyletic clusters (Fig. 3.10). Two isolates of *P. oedochilum* (isolate 38, the authentic strain, and isolate 316) were present in one cluster with isolates (210, 211, and 212) of *P. grandilobatum.* These isolates (210, 211, and 212) were originally deposited as *P. grandilobatum* and were to be described as a new species, but no description was ever published and morphological examinations revealed no differences when compared to isolates 38 and 316 of *P. oedochilum* (A.W.A.M. de Cock, personal communication). Based on morphological and molecular evidence, isolates 210, 211, and 212 all represent *P. oedochilum* and therefore, *P. grandilobatum* should be considered a synonym of *P. oedochilum.*

Four isolates of *P. helicoides* (50, 274, 275, and 278) were present in another cluster with two isolates of *P. oedochilum* (362 and 399) and *P. middletonii* 314 (Fig. 3.10). *P. helicoides* and *P. oedochilum* are morphologically very similar (Table 3.3). The main difference is the wavy contour of the antheridia in *P. oedochilum* and the non-furrowed contour in *P. helicoides* (Van der Plaats-Niterink, 1981). Due to the extent of

Figure 3.9. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. grandisporangium, P. helicoides, P. middletonii, P. multisporum, P. nagaii, P. oedochilum, P. ostracodes, P. paroecandrum,* and *P. grandilobatum (nomen nudum)* with (**A**) *Alu*I, (**B**) *Hae*III, (**C**) *Hha*I, (**D**) *Hin*fi, (**E**) *Msp*I, (**F**) *Rsa*I, and (**G**) *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 4321a 4321b 115 68 53 54 214 232 391 L
B
38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 4321a 4321b 115 68 53 54 214 232 391 L
C
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 4321a 4321b 115 68 53 54 214 232 391 L
D
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 4321a 4321b 115 68 53 54 214 232 391 L


E
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 432 1a 432 1b 115 68 53 54 214 232 391 L


F
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 432 1a 432 1b 115 68 53 54 214 232 391 L

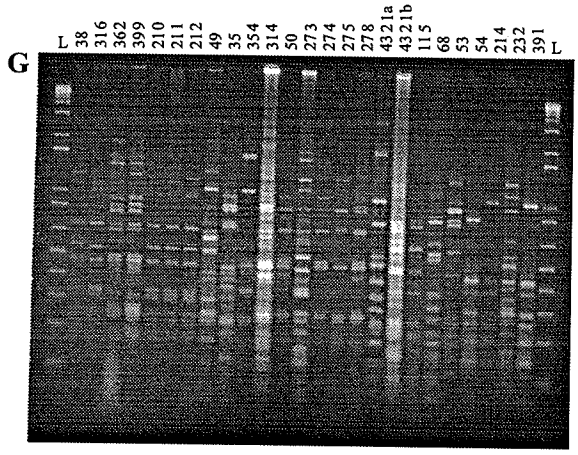
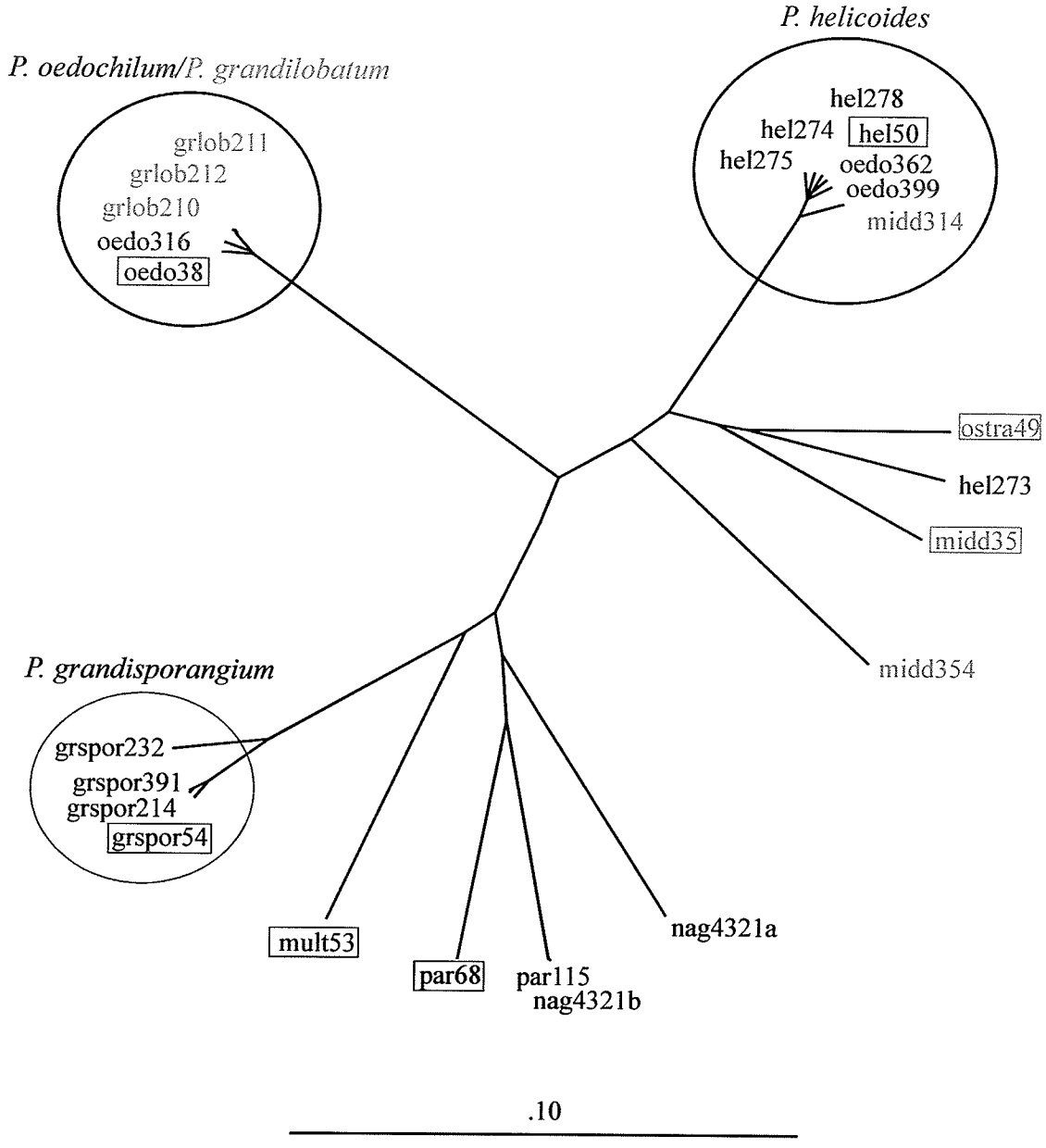

G
L 38 316 362 399 210 211 212 49 35 354 314 50 273 274 275 278 432 1a 432 1b 115 68 53 54 214 232 391 L

Figure 3.10. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.9. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: grlob, *P. grandilobatum (nomen nudum)*; grspor, *P. grandisporangium*; hel, *P. helicoides*; midd, *P. middletonii*; mult, *P. multisporum*; nag, *P. nagaii*; oedo, *P. oedochilum*; par, *P. paroecandrum.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. helicoides
P. oedochilum/P. grandilobatum
hel278
hel274
hel50
hel275
oedo362
oedo399
midd314
grlob211
grlob212
grlob210
oedo316
oedo38
ostra49
hel273
midd35
midd354
P. grandisporangium
grspor232
grspor391
grspor214
grspor54
mult53
par68
par115
nag4321b
nag4321a
.10

morphological similarities between *P. helicoides* and *P. oedochilum,* and the fact that *P. oedochilum* 38 (the authentic strain) was present in another cluster, isolates 362 and 399 were most likely *P. helicoides* isolates that were incorrectly identified as *P. oedochilum. P. middletonii* 314 was also present in this cluster, distinct from *P. middletonii* (35), the isolate used by Van der Plaats-Niterink (1981) in the species description. Isolate 314 may have been misidentified, but *P. middletonii* is morphologically quite distinct from *P. helicoides* and *P. oedochilum* (Table 3.3). Notably, isolate 314 is from Sudan while other isolates of *P. middletonii* are of European origin.

Pythium helicoides (273), *P. ostracodes* (49), and *P. middletonii* (35) were present on branches separated by long branch lengths. *P. ostracodes* differs from *P. middletonii* by its oogonial, oospore, and antheridial characteristics (Table 3.3). The main differences between *P. ostracodes* and *P. helicoides* are the antheridia which wind around the oogonial stalk in *P. helicoides* and the aplerotic oospores of *P. helicoides*. However, the oospores of *P. ostracodes* may be "nearly plerotic", not totally fused with the oogonial wall, and therefore may appear aplerotic (Van der Plaats-Niterink, 1981). An additional isolate of *P. middletonii* (354), located on its own branch, was distantly related to all the other *P. middletonii* isolates. *P. middletonii* may represent a heterogeneous species as the three isolates were scattered throughout the phenogram. This may be a reflection of the morphological variation common in *P. middletonii*. The antheridial origin in *P. middletonii* is reported to be monoclinous, diclinous, and hypogynous (Van der Plaats-Niterink, 1981). Variation in the size of zoosporangia and discharge tubes has also been reported (Dissmann, 1927). Isolate 354 may also represent a different species due to its lack of a genetic relationship with other isolates of *P. middletonii* and other species (Fig.

3.10). Studies involving additional isolates of *P. middletonii* would be useful to help resolve the taxonomic status of this species.

The outgroup species appeared to be genetically distinct. The four isolates of *P. grandisporangium* formed a monophyletic cluster and *P. multisporum* 53 was present on an individual branch. However, isolates 4321a and 4321b of *P. nagaii* were quite different from one another, and *P. paroecandrum* 115 was closely related to *P. nagaii* 4321b, yet distinct from *P. paroecandrum* 68 (Fig. 3.10). These two species produce globose oogonia of similar size, monoclinous antheridia, and aplerotic oospores with similar dimensions. However, only *P. nagaii* forms pyriform or globose proliferating zoosporangia while the zoosporangia of *P. paroecandrum* are non-proliferating (Van der Plaats-Niterink, 1981). Isolate 115 may have been mistakenly identified as *P. paroecandrum* if the proliferating nature of the zoosporangium was not observed. Hendrix and Papa (1974) reported that proliferation is not always shown in culture and they did not consider it as a useful taxonomic character.

B. Molecular comparison of *Pythium* species with elongated proliferating zoosporangia

Results and Discussion

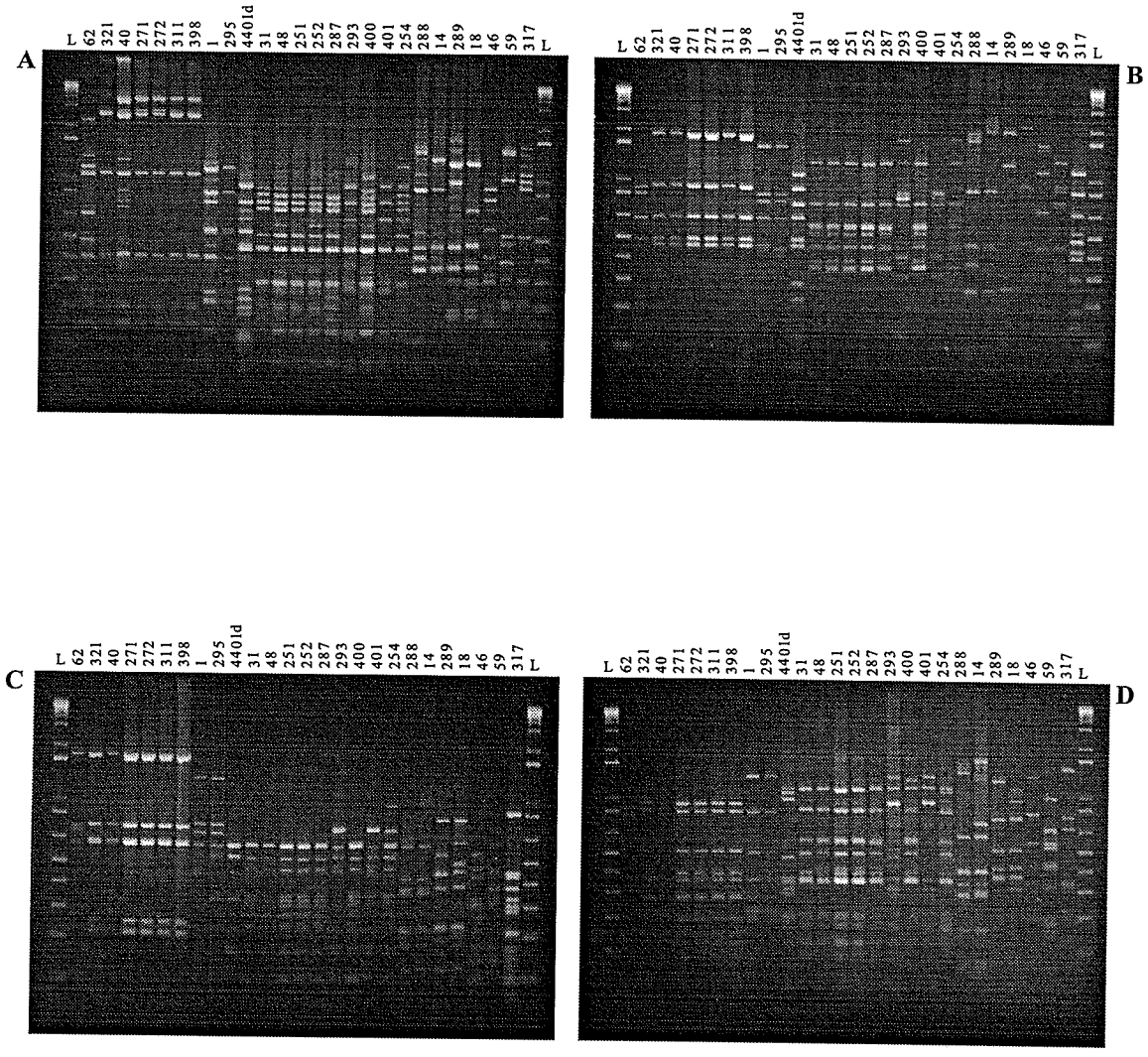
Pythium anandrum, P. dimorphum, P. helicandrum, P. prolatum, and *P. undulatum* are a group of species characterized by elongate to ellipsoidal zoosporangia that are usually proliferating. These species share a number of additional morphological

similarities with one another, but there has also been speculation as to whether *P. undulatum* is a member of *Pythium* or *Phytophthora.*

Restriction endonuclease digestions of amplicons of the rDNA IGS were done for isolates of *P. anandrum, P. dimorphum, P. helicandrum, P. okanaganense, P. paddicum, P. prolatum, P. ultimum* var. *ultimum,* and *P. undulatum* using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 3.11). A total of 336 restriction fragments were used for cluster analysis. The UPGMA and neighbor-joining phenogram topologies were identical, and the UPGMA phenogram is presented in Figure 3.12.

Isolates of *P. helicandrum* and *P. prolatum* formed a monophyletic cluster distinct from all other species (Fig. 3.12). The ex-type culture of *P. prolatum* (62) was more distant from the other isolates of *P. helicandrum* and *P. prolatum*, but was still within the same cluster. *P. prolatum* and *P. helicandrum* produce elongated ellipsoid zoosporangia, but they are occasionally proliferating in *P. prolatum.* The oogonia of *P. helicandrum* and *P. prolatum* have similar average diameters (29.0 µm and 26.0 µm, respectively) and contain spiny ornamentations of similar length with a wide base. Single diclinous antheridia which entwine the oogonial stalk and make broad contact with the oogonium are usually present in both species, and they have aplerotic oospores with similar dimensions (Van der Plaats-Niterink, 1981). The key differences between these species are that the oogonial projections cover the oogonium more densely and the antheridial side branches often form a tangled mass in *P. prolatum,* while zoosporangial proliferation is not observed in *P. helicandrum* (Van der Plaats-Niterink, 1981). However, the morphological similarities among the oogonia, antheridia, and oospores support the genetic clustering of these two species. Since *P. prolatum* 62 (the ex-type culture) was

Figure 3.11. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. anandrum, P. dimorphum, P. helicandrum, P. okanaganense, P. paddicum, P. prolatum, P. ultimum* var. *ultimum,* and *P. undulatum* with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L
B
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L
C
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L
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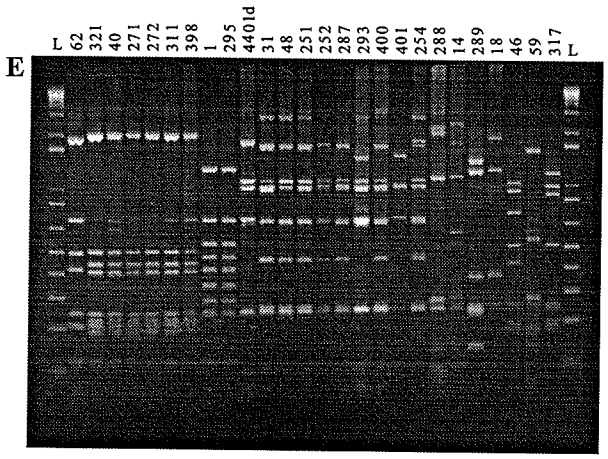
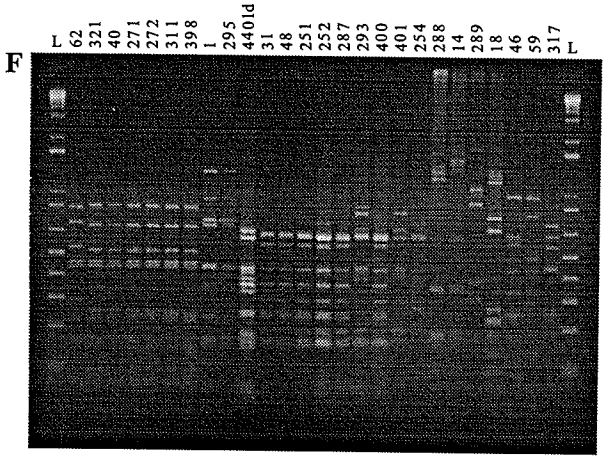

E
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L


F
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L

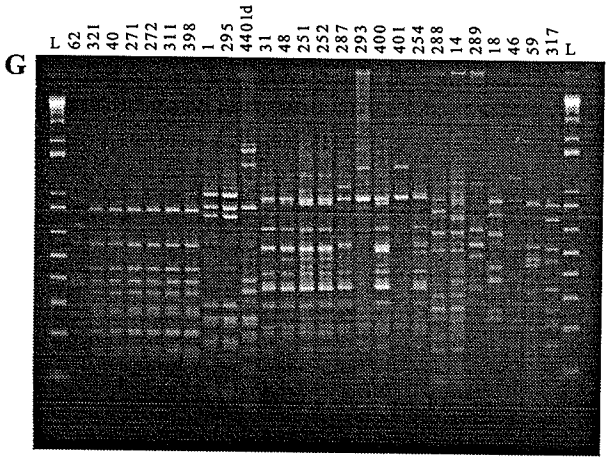
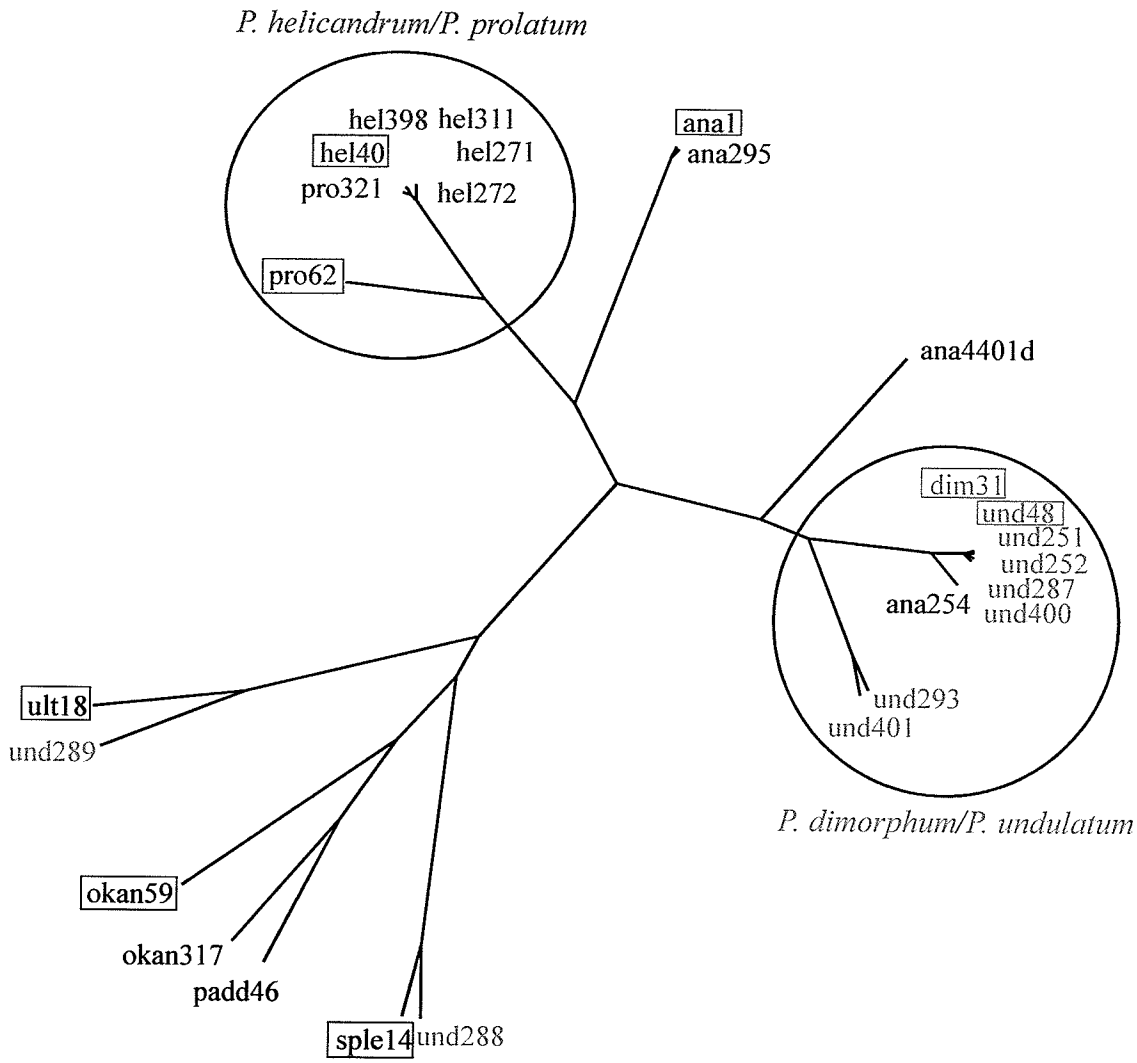

G
L 62 321 40 271 272 311 398 1 295 440Id 31 48 251 252 287 293 400 401 254 288 14 289 18 46 59 317 L

Figure 3.12. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.11. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: ana, *P. anandrum*; dim, *P. dimorphum*; hel, *P. helicandrum*; okan, *P. okanaganense*; padd, *P. paddicum*; pro, *P. prolatum*; ult, *P. ultimum* var. *ultimum*; und, *P. undulatum.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. helicandrum/P. prolatum
hel398
hel311
hel40
hel271
pro321
hel272
pro62
ana1
ana295
ana4401d
dim31
und48
und251
und252
ana254
und287
und400
und293
und401
P. dimorphum/P. undulatum
ult18
und289
okan59
okan317
padd46
sple14
und288
.10

more distant from the other isolates, isolate 321 may actually be an isolate of *P. helicandrum.* Nonetheless, these two species are very closely related morphologically and genetically. These species were also genetically distinct from the four isolates of *P. anandrum.* In *P. anandrum,* the zoosporangia are more regular in shape and frequently proliferating, and the oogonial spines are thinner and more widely spaced (Van der Plaats-Niterink, 1981).

Pythium undulatum is an interesting case. This species produces internally proliferating zoosporangia that sometimes have papillae. Dark yellow chlamydospores with thick walls are also produced (Van der Plaats-Niterink, 1981). Originally described by Petersen (1909), *P. undulatum* was transferred to *Pythiomorpha* by Apinis (1929) and was renamed *Pythiomorpha undulata* because zoospores were sometimes formed within the zoosporangium. However, Dissman (1927) suggested that this phenomenon was caused by very slow germination of the zoospores due to external conditions. Van der Plaats-Niterink (1981) had occasionally observed the formation of zoospores within the zoosporangia of other *Pythium* species, such as *P. tracheiphilum* and *P. uncinulatum,* so this phenomenon was not unique to *P. undulatum.* Sparrow (1960) therefore suggested there was no reason to transfer *P. undulatum* to *Pythiomorpha.*

However, speculation continued to arise as to whether *P. undulatum* was a *Pythium* species or a *Phytophthora* species. Blackwell *et al.* (1941) regarded *Pythiomorpha* as a synonym of *Phytophthora,* and Beneke and Schmitt (1961) referred to *P. undulatum* as "*Phytophthora undulatum* (*Pythiomorpha undulata*)" without commenting on this grouping of species names. No sexual reproduction has been observed in *P. undulatum*, so classification of this taxon has relied on zoosporangial

morphology. *P. undulatum* resembles *Phytophthora* by its sometimes papillate zoosporangia and zoospore formation within the zoosporangium rather than externally in a vesicle. DNA data of Belkhiri and Dick (1988) indicated that *P. undulatum* did not show the characteristic rDNA bands of other *Pythium* species. Subsequently, Dick (1989) transferred *P. undulatum* to *Phytophthora* based on zoosporangial morphology and DNA data, and renamed the species *Phytophthora undulata comb. nov.*. Additional studies later supported this move. Responses of *P. undulatum* isolates to hymexazol and metalaxyl more closely resembled those of *Phytophthora* species than *Pythium* species (Kato *et al,* 1990). Panabières *et al.* (1997) also showed that patterns of elicitin peptides for *P. undulatum* were identical to those of other *Phytophthora* species and different from *P. marsipium, P. oedochilum,* and *P. vexans* (the only other *Pythium* species that produce elicitins).

However, additional DNA analyses and comparisons using a larger subset of *Pythium* species indicated that this move may have been premature. ITS sequence analysis revealed that *P. undulatum* was present in a clade with *P. dimorphum, P. prolatum, P. helicandrum,* and isolate 254 (*P. anandrum*) and part of a larger clade of *Pythium* species with globose zoosporangia or hyphal swellings. Several *Phytophthora* species included in this analysis formed a clade genetically separate from all *Pythium* species (C.A. Lévesque, personal communication). Similar observations were made in an analysis of rRNA LSU gene sequences of several Oomycetes (Riethmüller *et al.*, 2002). *P. undulatum* was present in the *Pythium-Lagenidium* clade among species of Pythiales, while all *Phytophthora* species formed a clade within the Peronosporaceae.

In this study, RFLP analysis of the IGS showed that seven isolates of *P. undulatum* were part of a monophyletic cluster with the ex-type culture of *P. dimorphum* (31) and isolate 254 of *P. anandrum* (Fig. 3.12). *P. dimorphum* and *P. undulatum* both produce elongate zoosporangia that are internally proliferating. Most notable is the production of large chlamydospores with comparable sizes by both species. These species have been shown to produce chlamydospores as large as 75 to 80 μm in diameter (Hendrix and Campbell, 1971; Van der Plaats-Niterink, 1981). Since sexual structures have not been observed in *P. undulatum,* further morphological comparisons between *P. dimorphum* and *P. undulatum* cannot be made. However, the oogonia of *P. dimorphum* contain bluntly conical projections, antheridia are absent and monoclinous or hypogynous when present, and oospores may be plerotic or aplerotic (Hendrix and Campbell, 1971). *P. undulatum* appears to be genetically related to certain *Pythium* species despite evidence suggesting a close affiliation with *Phytophthora.* The species *P. dimorphum* and *P. undulatum* may in fact be conspecific, and observing sexual structures in *P. undulatum* and comparing them with those of *P. dimorphum* would strengthen this conclusion.

Isolate 254, identified as *P. anandrum,* was also present in the *P. undulatum/P. dimorphum* cluster and distinct from *P. anandrum* isolates 1 (the ex-type culture), 295, and 4401d (Fig. 3.12). *P. anandrum* shares morphological characteristics with *P. dimorphum* and *P. undulatum* such as proliferating, sometimes papillate zoosporangia, and also ornamented oogonia (Van der Plaats-Niterink, 1981). However, in *P. anandrum* antheridia are normally lacking, oospores are plerotic and smaller compared to *P. dimorphum,* and chlamydospores are not produced. Nonetheless, the significant morphological similarities and lack of the formation of taxonomically useful structures in

P. undulatum may account for the incorrect identification of isolate 254 as *P. anandrum.* Isolate 4401d was located on a branch separate from the ex-type culture (1) and isolates 295 and 254, so its taxonomic status is uncertain, although it showed a closer affinity for the *P. undulatum/P. dimorphum* cluster than for the ex-type culture of *P. anandrum.*

Isolates 293 and 401 of *P. undulatum* were located on a branch distinct from the *P. undulatum/P. dimorphum* cluster (Fig. 3.12). These isolates may have been misidentified or could represent a new species, but since their fragment patterns were distinct from all other examined species, and due to the morphological similarities between *P. undulatum* and *Phytophthora,* these isolates could also represent a *Phytophthora* species. Closer morphological examinations are necessary.

Isolates 288 and 289 appeared to have been incorrectly identified as *P. undulatum* since they showed a close relationship with *P. splendens* (14) and *P. ultimum* var. *ultimum* (18), respectively (Fig. 3.12). The lack of a large number of comparable morphological traits for *P. undulatum* could explain hasty identifications of isolates because *P. undulatum. P. splendens* and *P. ultimum* var. *ultimum* do not produce zoosporangia, but they do form large terminal or intercalary hyphal swellings (Van der Plaats-Niterink, 1981) which could be mistaken for the large chlamydospores of *P. undulatum.*

The outgroup species *P. okanaganense* and *P. paddicum* were sufficiently distinct from the other isolates examined. Both of these species produce proliferating zoosporangia, but differ from other such species due to the smooth oogonia of *P. okanaganense,* and the smaller zoosporangia and less complex antheridia of *P. paddicum* (Van der Plaats-Niterink, 1981). Isolates 59 (the ex-type culture) and 317 of *P.*

okanaganense were genetically distinct. This may be a reflection of geographic variation between these two isolates since isolate 59 is from Wisconsin, USA and isolate 317 is from Japan. On the other hand, isolate 317 may have been misidentified or may represent a new species. Detailed re-examination of morphology may resolve this issue.

C. Classification of *P. vexans* and other related species using RFLP analysis

Introduction

Pythium vexans is characterized by its subglobose zoosporangia, which are occasionally proliferating, and its monoclinous bell-shaped antheridia that exhibit broad apical attachment to oogonia (Van der Plaats-Niterink, 1981). However, this species has traditionally presented many taxonomic problems. There has been speculation, based on morphological, physiological, and molecular data, that *P. vexans* differs from other *Pythium* species and may be part of another genus. Dick (1990a) noted that some isolates of *P. vexans* tended to resemble *Phytophthora* in vitamin requirements and tolerance to certain fungicides. In a comparison of growth inhibition by metalaxyl and hymexazol, the response of *P. vexans* was unlike that of any other *Pythium* species tested, but very close to other *Phytophthora* species (Kato *et al.,* 1990). However, *P. vexans* lacks morphological characters that would enable its transfer to *Pythophthora.* In *Phytophthora,* zoospore differentiation occurs in the cytoplasm of the zoosporangium from where they are released, while in *Pythium* a vesicle is first produced in which zoospores are formed. In addition, *Phytophthora* species produce well-differentiated

zoosporangia on distinct sporangiophores and many species produce amphigynous antheridia (Brasier and Hansen, 1992).

Based on comparative DNA studies, Belkhiri and Dick (1988) suggested that *P. vexans* was incorrectly assigned to the genus *Pythium* and that this species may be part of an "intermediate" genus. Sequence analysis of the D2 region of the LSU rRNA gene showed a clear distinction between *Pythium* and *Phytophthora* species as each genus formed a separate clade (Briard *et al.,* 1995). The exception was *P. vexans* which was present on an individual branch, separate from the *Pythium* and *Phytophthora* clades. Briard *et al.* (1995) therefore suggested that *P. vexans* should be placed in a new genus, and speculated on the creation of a third independent genus in the Pythiaceae rather than an "intermediate" one between *Pythium* and *Phytophthora.*

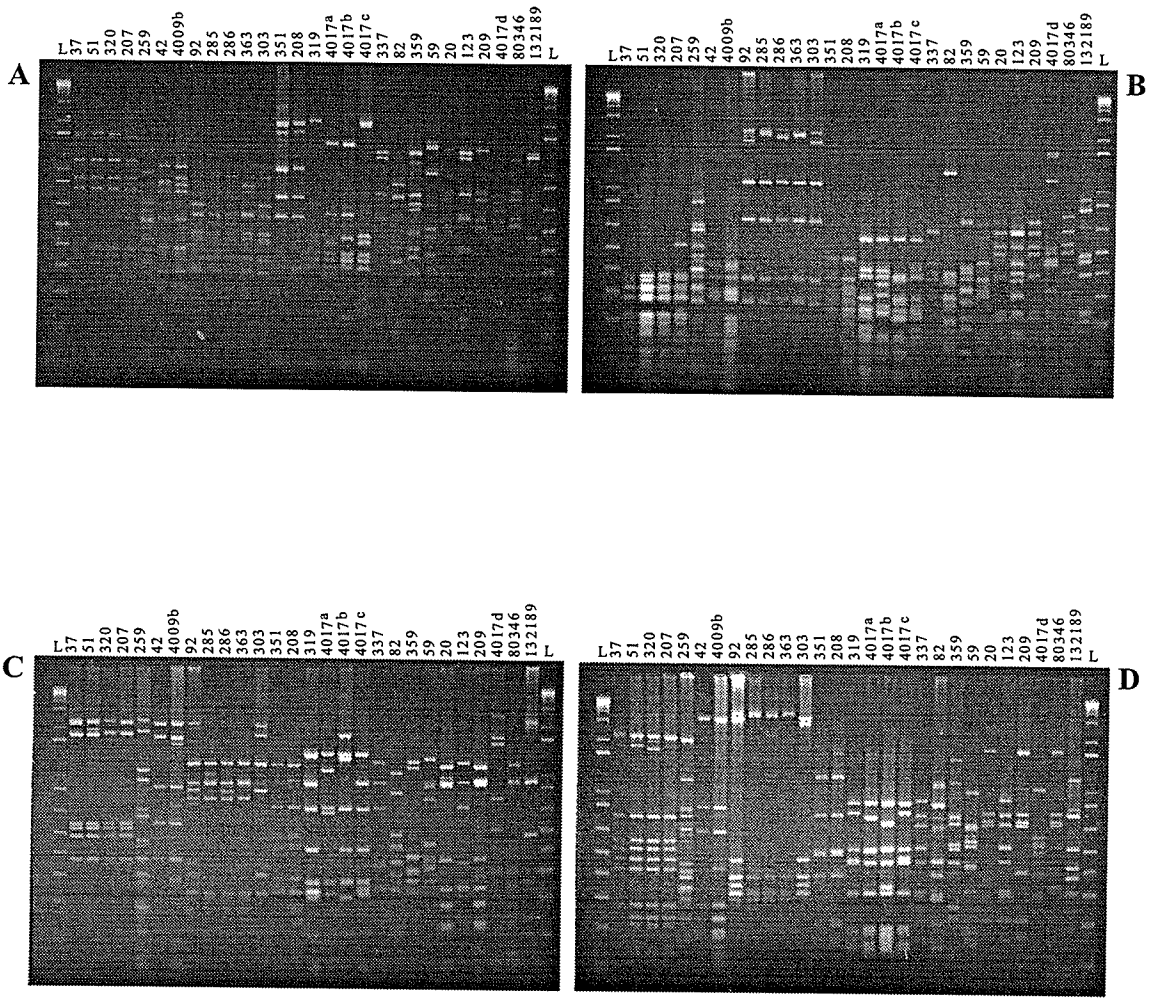
The production of elicitins is another property that distinguishes *P. vexans* from many other *Pythium* species. Elicitins are secreted proteins which are thought to be major determinants of the resistance response of *Nicotiana* spp. against *Phytophthora* spp. (Yu, 1995). While most *Phytophthora* species secrete elicitins, *P. vexans, P. marsipium,* and *P. oedochilum* are the only *Pythium* species that have been shown to produce elicitins (Panabières *et al.,* 1997). Phylogenetic analysis using elicitin protein sequences showed that *Pythium* species clustered with *Phytophthora* species. Furthermore, Panabières *et al.,* (1997) suggested that *P. vexans, P. oedochilum,* and *P. marsipium* may be the "missing link" between species of *Phytophthora* and *Pythium,* and that these species should be separated from these two genera.

Results and Discussion

RFLP analysis of the IGS was done for several isolates of *P. vexans* and for many other species with a variety of zoosporangial forms and morphological variations. Restriction digestions of IGS amplicons were done using *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I for isolates of *P. chamaehyphon, P. conidiophorum, P. cucurbitacearum, P. indigoferae, P. minus, P. okanaganense, P. parvum, P. perplexum, P. pleroticum, P. tracheiphilum,* and *P. vexans* (Fig. 3.13). A total of 432 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the UPGMA phenogram is presented in Figure 3.14.

Several clusters of isolates, often comprised of more than one species, were observed in the phenogram (Fig. 3.14). Cluster A contained several smaller groupings of species and isolates within. *P. vexans* (20), the culture used by Van der Plaats-Niterink (1981) in the species description, was present in cluster A on a branch with isolates 209 and 80346. *P. vexans* 123 and *P. cucurbitacearum* 337 were also closely related to each other as they formed a separate grouping within cluster A. The morphological distinction between these two species is based on the antheridial attachment to the oogonium. The antheridium attaches to the oogonium just below the oogonial stalk in *P. cucurbitacearum,* while in *P. vexans,* antheridia exhibit broad apical attachment to the oogonium (Dick, 1990a). *P. vexans* 208 and *P. indigoferae* 351 (both from India) also formed a discrete grouping within cluster A. These species are morphologically similar as they share aplerotic oospores with similar average diameters and monoclinous, rarely diclinous, antheridia. The inflated filamentous zoosporangia and smaller oogonia of *P. indigoferae* distinguish it from *P. vexans* (Van der Plaats-Niterink, 1981).

Figure 3.13. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. chamaehyphon, P. conidiophorum, P. cucurbitacearum, P. indigoferae, P. minus, P. okanaganense, P. parvum, P. perplexum, P. pleroticum, P. tracheiphilum,* and *P. vexans* with **(A)** *Alu*I, **(B)** *Cfo*I, **(C)** *Hae*III, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 37 51 320 207 259 42 4009b 92 285 286 363 303 351 208 319 4017a 4017b 4017c 337 82 359 59 20 123 209 4017d 80346 132189 L
B
L 37 51 320 207 259 42 4009b 92 285 286 363 303 351 208 319 4017a 4017b 4017c 337 82 359 59 20 123 209 4017d 80346 132189 L
C
L 37 51 320 207 259 42 4009b 92 285 286 363 303 351 208 319 4017a 4017b 4017c 337 82 359 59 20 123 209 4017d 80346 132189 L
D
L 37 51 320 207 259 42 4009b 92 285 286 363 303 351 208 319 4017a 4017b 4017c 337 82 359 59 20 123 209 4017d 80346 132189 L

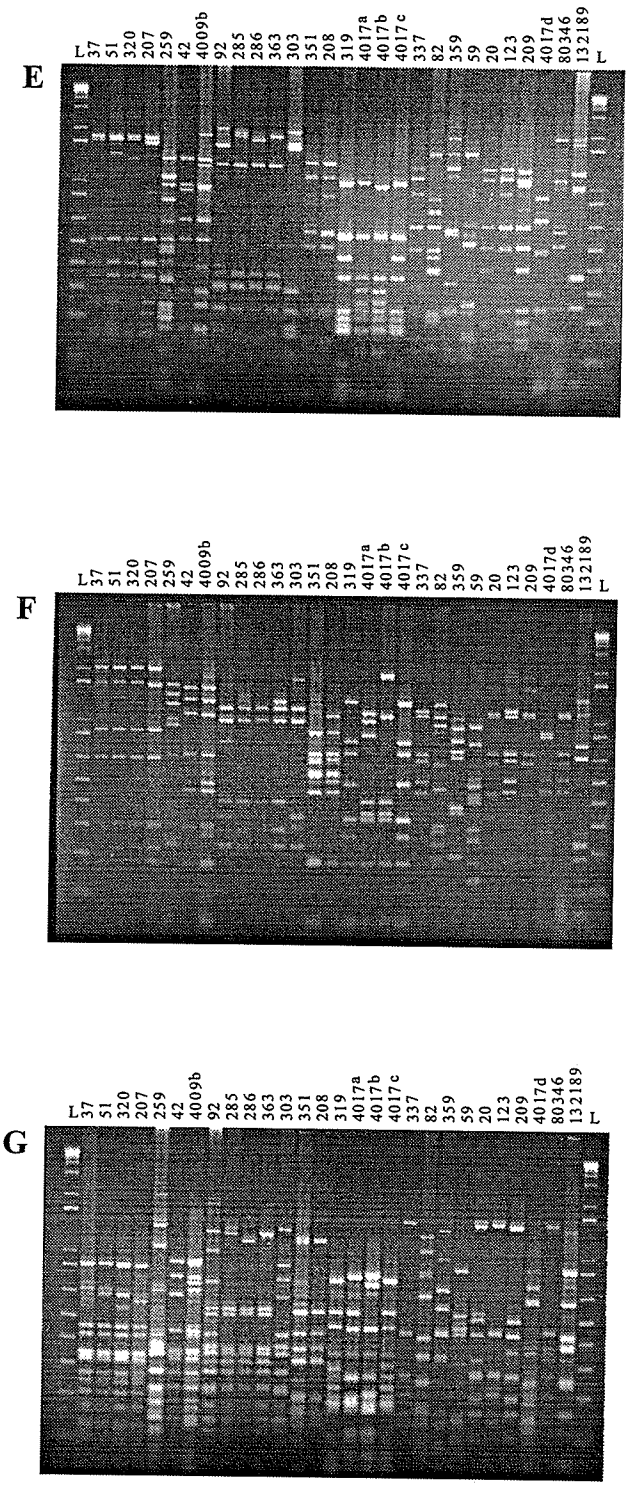
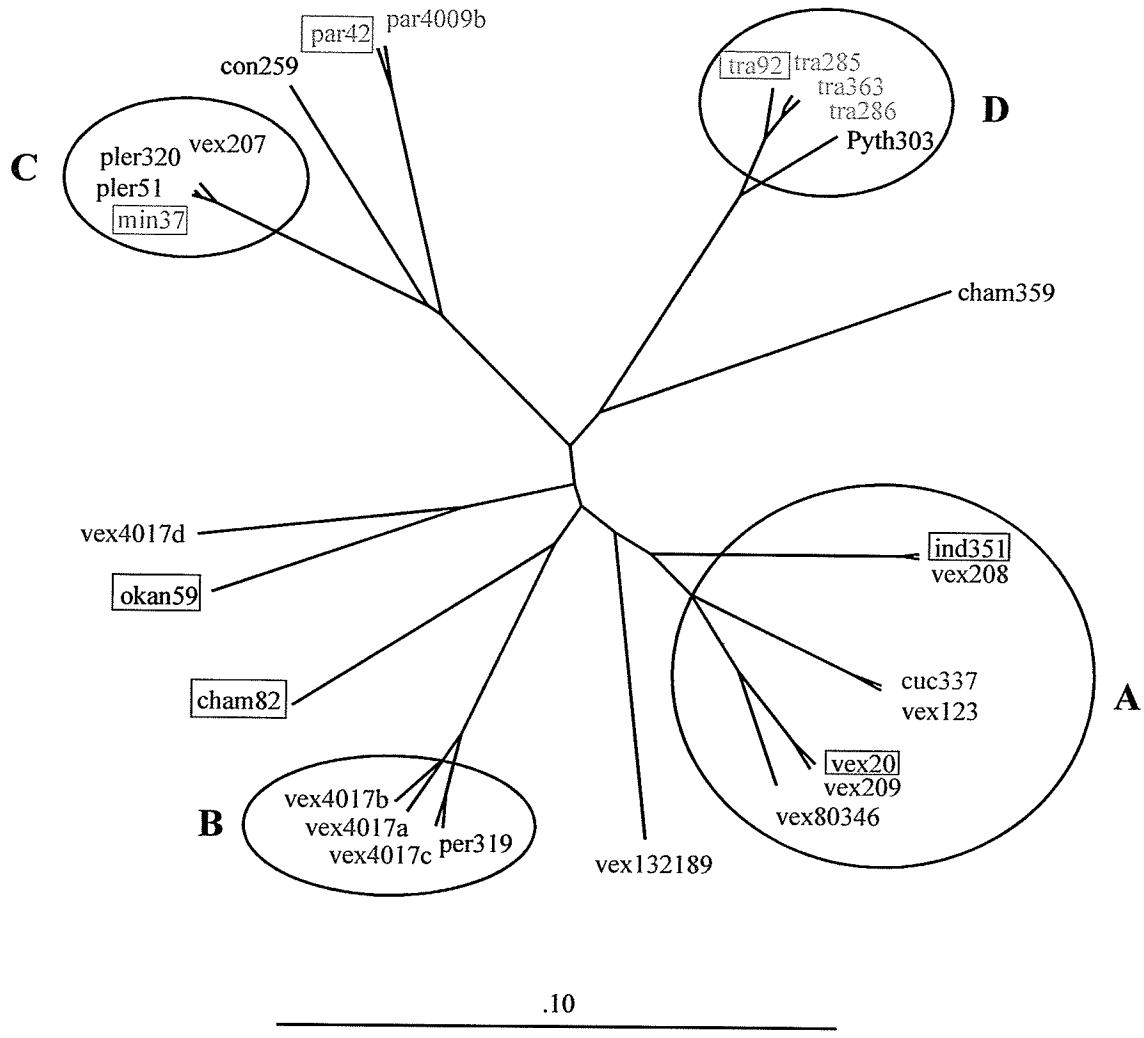

E
L
37
51
320
207
259
42
4009b
92
285
286
363
303
351
208
319
4017a
4017b
4017c
337
82
359
59
20
123
209
4017d
80346
132189
L
F
L
37
51
320
207
259
42
4009b
92
285
286
363
303
351
208
319
4017a
4017b
4017c
337
82
359
59
20
123
209
4017d
80346
132189
L
G
L
37
51
320
207
259
42
4009b
92
285
286
363
303
351
208
319
4017a
4017b
4017c
337
82
359
59
20
123
209
4017d
80346
132189
L

Figure 3.14. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.13. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: cham, *P. chamaehyphon*; con, *P. conidiophorum*; cuc, *P. cucurbitacearum*; ind, *P. indigoferae*; min, *P. minus*; okan, *P. okanaganense*; par, *P. parvum*; per, *P. perplexum*; pler, *P. pleroticum*; Pyth, *Pythium* sp.; tra, *P. tracheiphilum*; vex, *P. vexans*. Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


par42
par4009b
con259
tra92
tra285
tra363
tra286
Pyth303
D
C
pler320
vex207
pler51
min37
cham359
vex4017d
okan59
ind351
vex208
cham82
cuc337
vex123
A
vex20
vex209
vex80346
B
vex4017b
vex4017a
vex4017c
per319
vex132189
.10

Three additional isolates of *P. vexans* (4017a, 4017b, 4017c) and an isolate of *P. perplexum* (319) formed cluster B (Fig. 3.14). These species possess from 1 to 2 monoclinous, bell-shaped antheridia per oogonium and aplerotic oospores. The key morphological differences between these two species are the lack of lobed antheridia in *P. perplexum* and its larger oogonia and oospores. In addition, the zoosporangia of *P. vexans* are occasionally proliferating while they are non-proliferating in *P. perplexum* (Van der Plaats-Niterink, 1981). *P. perplexum* had been misidentified as *P. vexans* by Butler (1907), and Van der Plaats-Niterink (1981) suggested that these two species were probably confused in the genus key of Middleton (1943). However, ITS-1 sequence analysis of *P. perplexum* isolates from soil by Galland and Paul (2001) showed these two species are distinct from one another. Therefore, these three isolates (4017a, 4017b, and 4017c) appeared to be isolates of *P. perplexum* mistakenly identified as *P. vexans.*

Pythium vexans 132189 was quite divergent from the other *P. vexans* isolates. However, little is known about this isolate. *P. vexans* 4017d was also distantly related to all other isolates of *P. vexans.* In an examination of various dimensions of oogonia and oospores, Shahzad *et al.* (1992) showed that the measurements for isolate 4017d were more divergent compared to those of 4017a and 4017b which were similar to one another. This morphological variation in isolates 4017d compared to the others may be reflected in Fig. 3.14. Isolate 4017d was present on a branch with *P. okanaganense* 59, but these species were separated by long branch lengths. *P. okanaganense* is morphologically similar to *P. vexans* as it also forms occasionally proliferating subglobose zoosporangia, aplerotic oospores, and monoclinous antheridia that originate far below the oogonium, but it possesses larger oogonia than *P. vexans* (Van der Plaats-Niterink, 1981). The two

isolates of *P. chamaehyphon* were genetically distinct from all other species, but the ex-type culture (82) was also distinct from isolate 359. *P. chamaehyphon* is similar to *P. vexans,* but it possesses larger oogonia. In *P. chamaehyphon,* the antheridial attachment to the oogonium is near the oogonial stalk, like in *P. cucurbitacearum,* but these species can be distinguished by the papillate zoosporangia of *P. cucurbitacearum* (Van der Plaats-Niternink, 1981).

In this example, RFLP analysis of the IGS illustrated the taxonomic uncertainty that accompanies the use of morphology to identify isolates as *P. vexans.* Despite the significant morphological differences that delimit *P. vexans, P. cucurbitacearum, P. perplexum,* and *P. indigoferae,* molecular data from IGS RFLP analysis suggests a close genetic relationship among these species. Dick (1990a) suggested that *P. cucurbitacearum* should probably be considered a *Phytophthora* species due to its papillate zoosporangia and broad apical attachment of antheridia, so the taxonomic status of this species, like *P. vexans,* is questionable. The inflated, filamentous zoosporangia of *P. indigoferae* are quite distinct from the subglobose zoosporangia of *P. vexans.* Considering that *P. indigoferae* 351 was the isolate used by Van der Plaats-Niterink (1981) in the species description, it is quite likely that *P. vexans* 208 was misidentified. The similar situation was also true for isolate 123 which was most likely an isolate of *P. cucurbitacearum* misidentified as *P. vexans.* Since no ex-type culture of *P. vexans* was available, the isolates which may best represent *P. vexans* are isolates 209 and 80346 which are similar to isolate 20 which was used in the species description by Van der Plaats-Niterink (1981).

Pythium minus and *P. pleroticum* shared almost identical restriction fragment profiles (Fig. 3.13) and were present in cluster C (Fig. 3.14). These species do not produce zoosporangia in culture, although *P. pleroticum* produces large hyphal swellings (Jacobs, 1982). Both species produce 4 to 5 antheridia per oogonium which are monoclinous, occasionally diclinous, and arise at some distance from the oogonium, but the antheridial branches of *P. minus* sometimes entwine the oogonial stalk. The oogonia of *P. pleroticum* are slightly larger than those of *P. minus,* and while both species have plerotic oospores with thin walls, *P. minus* has been shown to produce 2 to 6 oospores per oogonium while only single oospores are present in *P. pleroticum* (Van der Plaats-Niterink, 1981; Ali-Shtayeh and Dick, 1985). *P. minus* and *P. pleroticum* therefore appeared to be very closely related and may in fact be conspecific. The morphological characters that differ between these species, notably the production of multiple oospores by *P. minus* and slightly larger oogonia in *P. pleroticum,* may not be significant to define these as separate species considering the extent of common morphological characters. Due to the limited number of reported isolates and morphological descriptions of these species, the morphological variation reported may in fact be representative of the species.

Pythium vexans 207 was also present in cluster C. The morphological differences when comparing between *P. vexans* to *P. minus* and *P. pleroticum* are quite striking since *P. vexans* produces subglobose, proliferating zoosporangia, aplerotic oospores, and relatively larger oogonia. Morphological similarities between these three species that may result in confusion when identifying isolates include the origin of antheridia far from the oogonium and their monoclinous, rarely diclinous, antheridia. Therefore, isolate 207 is most likely an isolate of *P. minus* or *P. pleroticum* due to its genetic relationship to

. cluster C. However, the apparent misidentification of isolate 207 as *P. vexans* is difficult to account for due to the considerable morphological differences among these species (A.W.A.M. de Cock, personal communication).

Pythium minus and *P. pleroticum* were genetically distinct from *P. parvum* and *P. conidiophorum* (Fig. 3.14). While *P. parvum* also lacks zoosporangia and produces hyphal swellings, it possesses hypogynous antheridia, larger oogonia, and smaller plerotic oospores (Ali-Shtayeh and Dick, 1985). In *P. conidiophorum,* antheridia are absent and the hyphal swellings are smaller compared to those in *P. pleroticum* and *P. minus* (Van der Plaats-Niterink, 1981). Four isolates of *P. tracheiphilum* formed a cluster separate from all other species (cluster D). *P. tracheiphilum* is distinct from the other species in this analysis due to its globose, nonproliferating zoosporangia and plerotic oospores. *P. tracheiphilum* also produces chlamydospores which are absent in the others species examined (Van der Plaats-Niternink, 1981). An unidentified *Pythium* species (isolate 303) was closely related to cluster D isolates and its morphology requires re-examination to confirm its likely classification as *P. tracheiphilum.*

IV. Comparison of species with filamentous inflated zoosporangia

There are approximately 18 to 20 *Pythium* species that produce filamentous zoosporangia with lobulate or toruloid inflated elements (Dick, 1990a). In many species, the zoosporangia may also form contiguous complexes. The following section is a study in which RFLP analysis of the IGS was used to examine genetic relationships among three subgroups of *Pythium* species with filamentous inflated zoosporangia.

A. Genetic relationships between *P. folliculosum, P. torulosum,* and other species with filamentous inflated zoosporangia

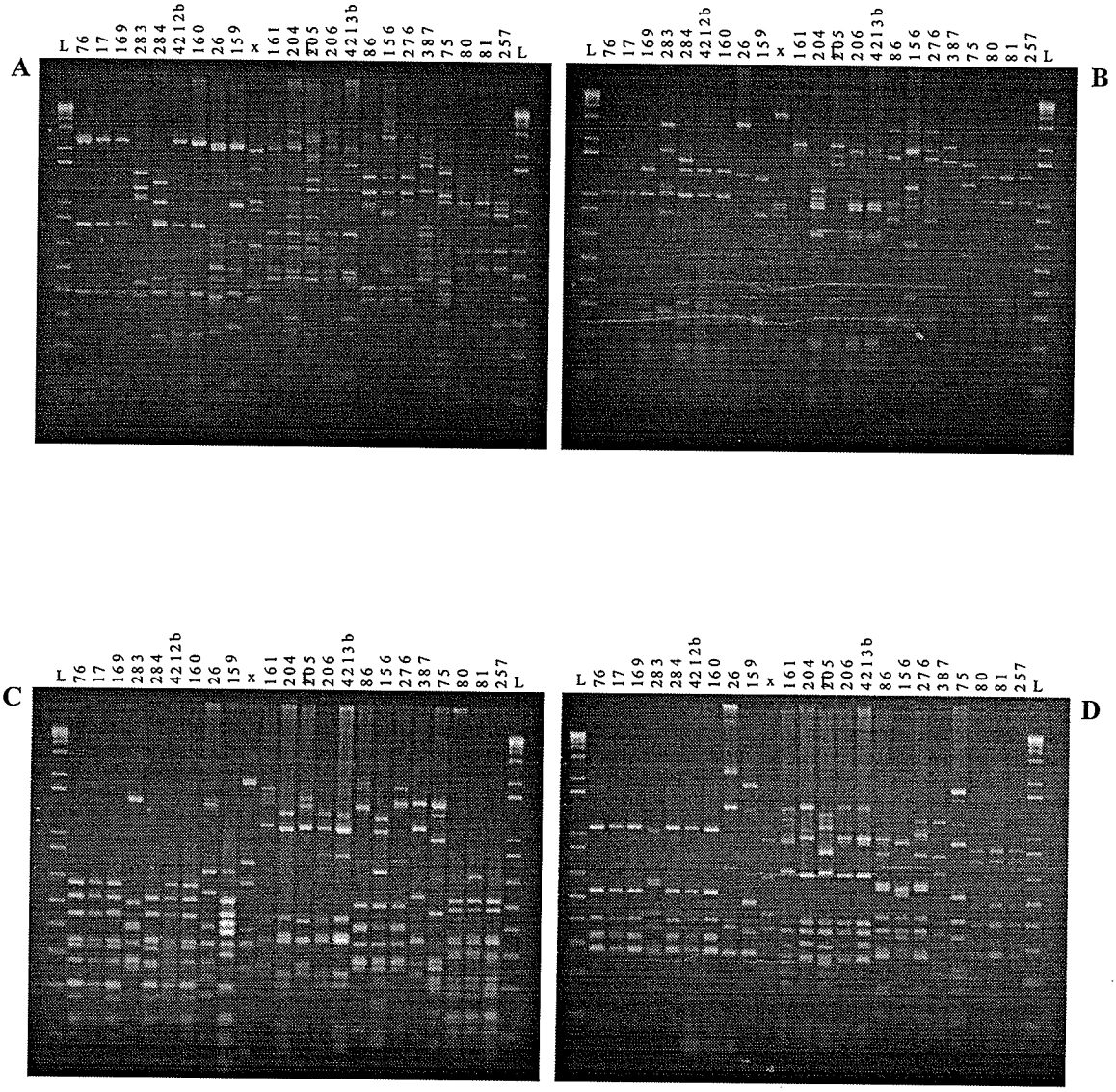
Results and Discussion

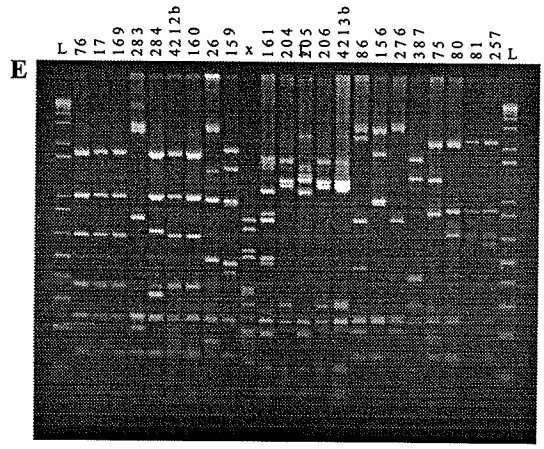
Pythium folliculosum and *P. torulosum* possess filamentous zoosporangia with toruloid inflated elements that form contiguous structures (Van der Plaats-Niterink, 1981; Paul, 1991). However, their oogonial characteristics distinguish these species from one another. *P. torulosum* produces smooth globose oogonia with an average diameter of 15.0 µm. While the oogonia of *P. folliculosum* can be spherical, they are also pear-shaped to elongate and form sac-like structures. The average diameter of spherical oogonia is 19.5 µm, but elongate oogonia can be as long as 70 µm with an average diameter of 32.7 µm (Paul, 1991). It is these relatively large sac-like oogonia that distinguish *P. folliculosum* from other species with filamentous inflated zoosporangia.

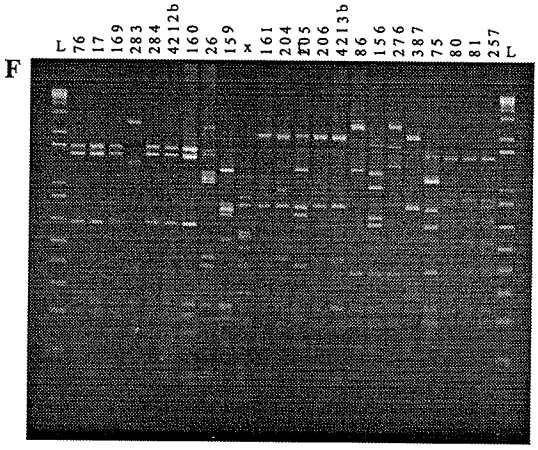
To examine the genetic relationships between isolates of *P. folliculosum, P. torulosum,* and other species with filamentous inflated zoosporangia, restriction endonuclease digestions were done for several isolates of *P. catenulatum, P. drechsleri, P. folliculosum, P. inflatum, P. periilum,* and *P. torulosum* with *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 3.15). A total of 338 fragments were used for UPGMA and neighbor-joining analysis, and the resulting phenogram topologies were identical. The UPGMA phenogram is presented in Figure 3.16.

Four isolates of *P. torulosum* (17, 169, 284, and 4212d) were present in a cluster along with the ex-type culture of *P. folliculosum* (76) and *P. periilum* 160 (Fig. 3.16). These three species are morphologically related by their inflated filamentous

Figure 3.15. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. catenulatum, P. drechsleri, P. folliculosum, P. inflatum, P. periilum, P. torulosum,* and *P. vanterpoolii* with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder. "x" refers to a sample that was errantly included in the gel, but disregarded in the RFLP analysis.


A
L 76 17 169 283 284 4212b 160 26 159 x 161 204 205 206 4213b 86 156 276 387 75 80 81 257 L
B
L 76 17 169 283 284 4212b 160 26 159 x 161 204 205 206 4213b 86 156 276 387 75 80 81 257 L
C
L 76 17 169 283 284 4212b 160 26 159 x 161 204 205 206 4213b 86 156 276 387 75 80 81 257 L
D
L 76 17 169 283 284 4212b 160 26 159 x 161 204 205 206 4213b 86 156 276 387 75 80 81 257 L


E
L
76
17
169
283
284
4212b
160
26
159
x
161
204
TD5
206
4213b
86
156
276
387
75
80
81
257
L


F
L
76
17
169
283
284
4212b
160
26
159
x
161
204
TD5
206
4213b
86
156
276
387
75
80
81
257
L

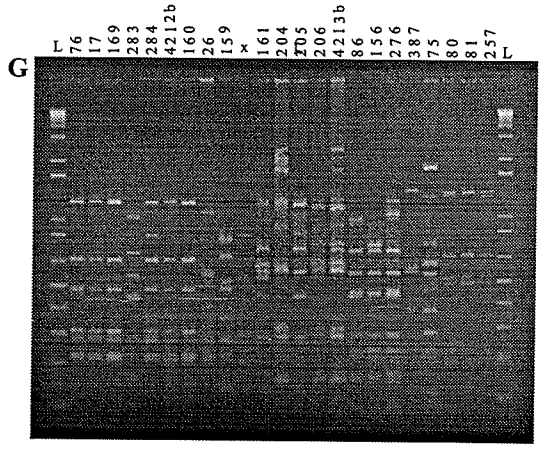
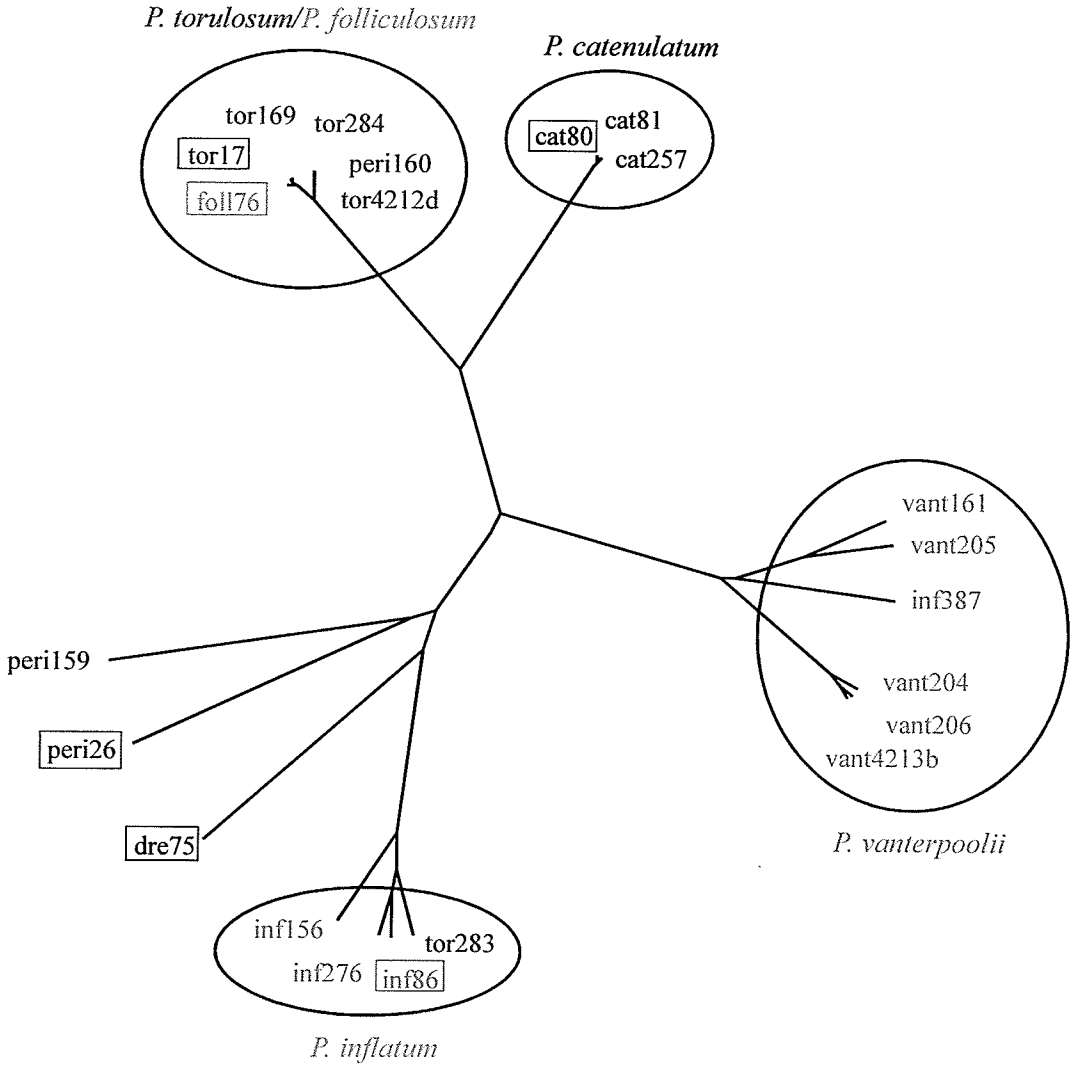

G
L
76
17
169
283
284
4212b
160
26
159
x
161
204
TD5
206
4213b
86
156
276
387
75
80
81
257
L

Figure 3.16. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.15. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: cat, *P. catenulatum*; dre, *P. drechsleri*; foll, *P. folliculosum*; inf, *P. inflatum*; peri, *P. periilum*; tor, *P. torulosum*; vant, *P. vanterpoolii.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. torulosum/P. folliculosum
P. catenulatum
tor169
tor284
tor17
peri160
foll76
tor4212d
cat80
cat81
cat257
vant161
vant205
inf387
peri159
vant204
peri26
vant206
vant4213b
dre75
P. vanterpoolii
inf156
tor283
inf276
inf86
P. inflatum
.10

zoosporangia and plerotic oospores. However, the large sac-like and elongate or pear-shaped oogonia distinguish *P. folliculosum* from the other species (Paul, 1991). The antheridia of *P. periilum* often entangle the oogonium and oogonial stalk, which is not observed in *P. folliculosum* or *P. torulosum.* As well, the antheridial cells in *P. periilum* make broad apical contact with the oogonium which is sometimes also observed in *P. folliculosum. P. periilum* 26, the culture used by Van der Plaats-Niterink (1981) in the species description, was distantly related to this cluster, so isolate 160 was probably incorrectly identified as *P. periilum* based on its morphological similarities and genetic relationships with *P. torulosum* and *P. folliculosum.*

In spite of the tremendous contrast in oogonial shapes and sizes between *P. folliculosum* and *P. torulosum,* isolates of these species were genetically very similar. The variation in oogonial size and shape is so extreme in *P. folliculosum* that a single shape or size range is not available for simple comparisons (Paul, 1991), but other morphological characteristics of *P. folliculosum* are quite similar to those of *P. torulosum.* The presence of large oogonia which occasionally form may be diagnostic for this species, but on the other hand, such oogonial variation may also exist in *P. torulosum* but was not observed among the isolates in this study or has not been observed in this species to date. It is therefore difficult to draw conclusions about the relationship between *P. folliculosum* and *P. torulosum,* although RFLP analysis of the IGS suggested they were more closely related to each other than to any other species with similar characteristics and may in fact be conspecific. Additional isolates of *P. folliculosum* would strengthen these observations and conclusions.

Isolate 283 of *P. torulosum* was present within a cluster of *P. inflatum* isolates. This isolate (283) may have been incorrectly identified since *P. inflatum* shares many features with *P. torulosum,* including filamentous inflated zoosporangia, globose smooth oogonia, and plerotic oospores. *P. inflatum* has diclinous antheridia, but diclinous antheridia are occasionally observed in *P. torulosum* (Van der Plaats-Niterink, 1981). Isolate 387 of *P. inflatum* was present among isolates of *P. vanterpoolii,* so its morphology will have to be re-examined.

Pythium vanterpoolii is morphologically similar to *P. torulosum* and there has been past confusion in distinguishing these two species. Vanterpool (1938) described an isolate which was thought to be a geographic variant of *P. torulosum,* but he later observed that the zoosporangia contained subglobose and ellipsoid elements different from the filamentous branched outgrowths of *P. torulosum.* This isolate was later identified as *P. vanterpoolii* (Kouyeas and Kouyeas, 1963). The features that distinguish *P. vanterpoolii* from *P. torulosum* are its subglobose zoosporangia sometimes arranged in chains, and the larger distance of the monoclinous antheridial stalk from the oogonium (Van der Plaats-Niterink, 1981). The six isolates of *P. vanterpoolii* formed a genetic cluster separate from *P. torulosum,* supporting the separation of these two species based on morphology. However, isolates 161 and 205 were on a separate branch from isolates 204, 206, and 4213b.

The outgroup species *P. drechsleri* and *P. catenulatum* were distinct from all other species examined. Isolate 75 is morphologically very similar to *P. salinum* which forms subglobose zoosporangia (A.W.A.M. de Cock, personal communication), but a culture of *P. salinum* was not available. The three isolates of *P. catenulatum* formed a

cluster distinct from all other species. *P. catenulatum* is heterothallic and produces filamentous inflated zoosporangia, but it also forms hyphal swellings and from 5 to 12 diclinous antheridia per oogonium (Van der Plaats-Niterink, 1981).

B. The genetic relationship between *P. arrhenomanes* and *P. graminicola* using RFLP analysis

Introduction

Pythium arrhenomanes and *P. graminicola* are morphologically similar species that are important pathogens of graminaceous plants (Table 3.4). Both species possess inflated filamentous zoosporangia that may form irregular complexes, and plerotic oospores. These species can be distinguished primarily by the larger oogonia and greater number of antheridia per oogonium in *P. arrhenomanes*.

Intraspecific morphological variation is common within these species and this makes unambiguous identification of isolates difficult. Chen and Hoy (1993) could not conclusively differentiate isolates using the morphological criteria of Van der Plaats-Niterink (1981). Overlap in a number of morphological characters between both species is common and thus, these two species are often placed into a species complex (Gilbert *et al.,* 1995). Nonetheless, Shahzad *et al.* (1992) reported that oospore and oogonial dimensions distinguished *P. arrhenomanes* from *P. graminicola.* Gilbert *et al.* (1995) used growth curve data at different temperatures to confirm the identity of non oogonia-forming isolates of *P. arrhenomanes,* but they were unable to show a clear distinction between *P. arrhenomanes* and *P. graminicola.*

Table 3.4. Morphological comparison of *P. arrhenomanes, P. aristosporum, P. graminicola,* and *P. periilum* based on the descriptions by Van der Plaats-Niterink (1981).

Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. arrhenomanes	Filamentous and inflated, forming complicated structures	Subglobose, terminal and occasionally intercalary; avg. diam. 32.5 µm	Diclinous; 12—15 per oogonium	Plerotic, or nearly so; avg. diam. 27.0; wall up to 2 µm thick
P. aristosporum	Filamentous with inflated elements; globose hyphal swellings often present	Subglobose, terminal and occasionally intercalary; avg. diam. 32.7 µm	Monoclinous or diclinous; up to 8 per oogonium	Aplerotic; avg. diam 26.8 µm; wall up to 2 µm thick
P. graminicola	Filamentous and inflated, forming irregular complexes	Globose, terminal and intercalary; avg. diam. 22.3 µm	Monoclinous, occasionally diclinous; 1—3 (up to 6) per oogonium	Plerotic; wall up to 3 µm thick
P. periilum	Partly strictly filamentous, partly consisting of inflated elements	Globose, terminal or intercalary; avg. diam. 20.0 µm	Monoclinous or diclinous; 2—5 per oogonium	Plerotic or nearly so, slightly yellowish; wall 1—2 µm thick

Results from total protein electrophoresis and isozyme polymorphisms suggested that *P. arrhenomanes* and *P. graminicola* do not represent distinct species since isolates of both species formed two overlapping clusters (Chen *et al.,* 1991; Chen *et al.,* 1992b). RFLP analysis of rDNA ITS and SSU rRNA gene sequences revealed two distinct clusters of *P. graminicola,* one close to *P. arrhenomanes* and the other distant from *P. arrhenomanes* (Chen *et al.,* 1992a). Chee and Kim (2000) produced identical RFLP patterns for *P. graminicola* and *P. arrhenomanes* using the ITS and with M-13 PCR markers.

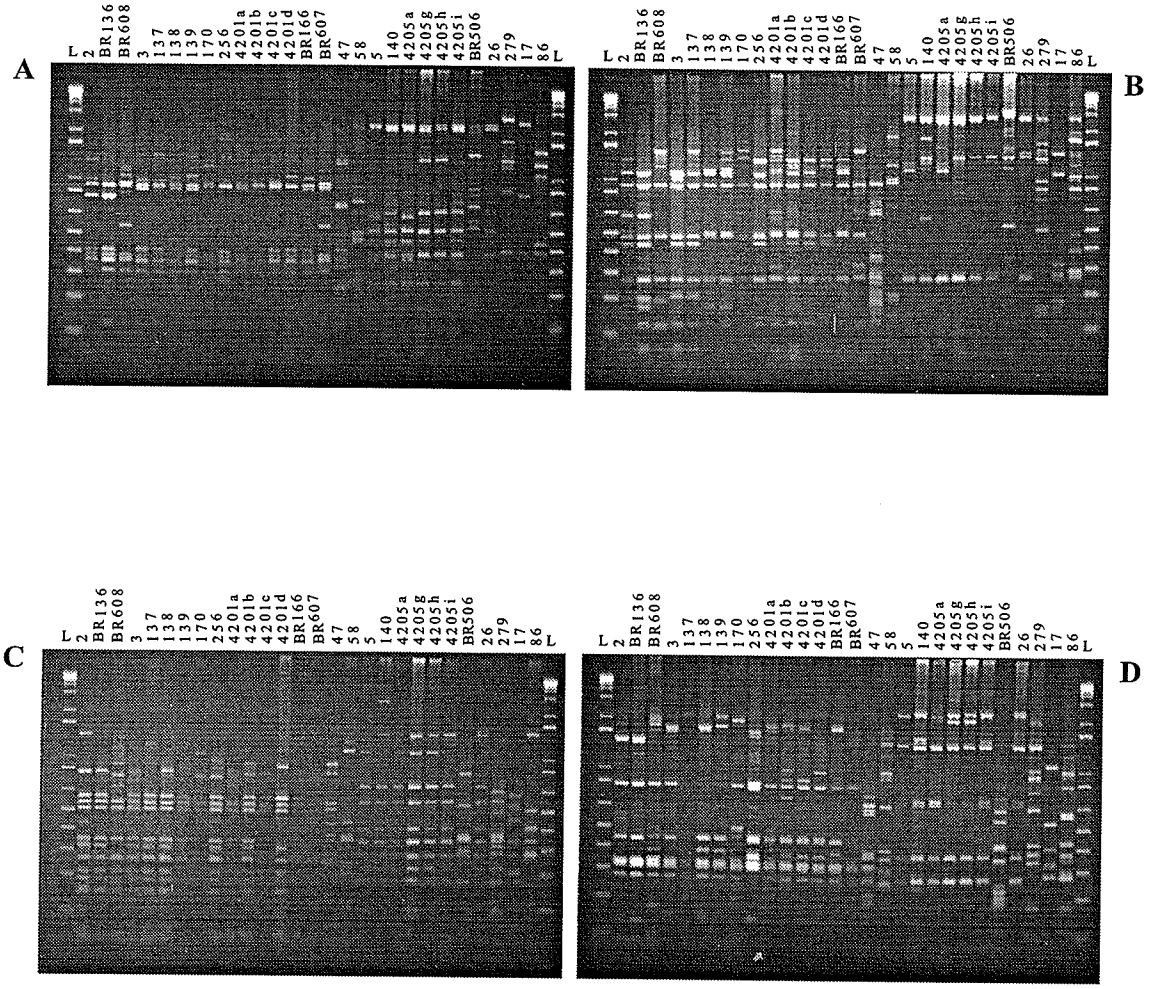
However, RFLP analysis of mtDNA showed that only 20% of restriction fragments were co-migrating between these two species, but only one isolate of each species was examined (Martin and Kistler, 1990). With a larger number of isolates, RFLPs of the ITS and LSU rRNA gene showed that *P. arrhenomanes* and *P. graminicola* were genetically distinct species (Chen and Hoy, 1993). ITS sequence analysis of 30 *Pythium* species showed that *P. arrhenomanes* and *P. graminicola* were very closely related (Matsumoto *et al.,* 1999), but an additional ITS sequence analysis with a larger subset of species showed that *P. arrhenomanes* and *P. aristosporum* were present in a clade with *P. volutum*, while *P. graminicola* was more distantly related in a separate clade with *P. inflatum* (C.A. Lévesque, personal communication). Mitochondrial *cox*II gene sequencing also showed that *P. arrhenomanes* and *P. volutum* clustered together, while *P. graminicola* was located on a separate clade (Martin, 2000).

Results and Discussion

To examine the genetic relationships among *P. graminicola, P. arrhenomanes,* and *P. aristosporum,* restriction endonuclease digestions of PCR products of the rDNA IGS were done using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I for these species, as well as isolates of *P. inflatum, P. periilum, P. torulosum, P. vanterpoolii,* and *P. volutum* (Fig. 3.17). A total of 439 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the neighbor-joining phenogram is presented in Figure 3.18. The outgroup species were selected based on molecular relationships (discussed above) and morphological similarities to *P. arrhenomanes, P. graminicola,* and *P. aristosporum. P. volutum* shares filamentous and inflated zoosporangia, similar sized oogonia, and diclinous antheridia with *P. arrhenomanes* and *P. aristosporum,* while both *P. inflatum* and *P. graminicola* possess plerotic oospores, inflated filamentous zoosporangia, diclinous antheridia, and similar oogonial diameters (Van der Plaats-Niterink, 1981). *P. vanterpoolii* is distinct from these species as its zoosporangia form catenulate complexes of subglobose or ellipsoidal unbranched elements. *P. torulosum* was included as an outgroup as its zoosporangial system contains a combination of inflated and noninflated elements, similar to those of *P. periilum* which was also included (Van der Plaats-Niterink, 1981).

Two genetic clusters are seen in the neighbor-joining phenogram (Fig. 3.18). One cluster was comprised of isolates of *P. aristosporum* and *P. arrhenomanes,* including the ex-type cultures for each species (isolates 2 and 3, respectively). The other cluster consisted of six isolates of *P. graminicola* and *P. periilum* (26), the culture used by Van der Plaats-Niterink (1981) in the species description. Additional isolates of *P.*

Figure 3.17. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. aristosporum, P. arrhenomanes, P. graminicola, P. inflatum, P. periilum, P. torulosum, P. vanterpoolii,* and *P. volutum* with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.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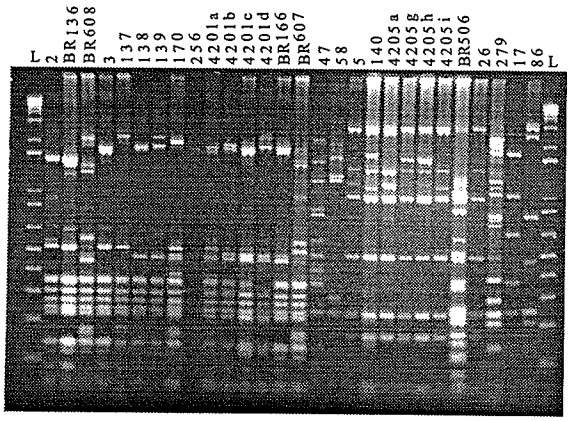
B
C
D
L
2
BR136
BR608
3
137
138
139
170
256
4201a
4201b
4201c
4201d
BR166
BR607
47
58
5
140
4205a
4205g
4205h
4205i
BR506
26
279
17
86
L

E


L
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BR136
BR608
3
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170
256
4201a
4201b
4201c
4201d
BR166
BR607
47
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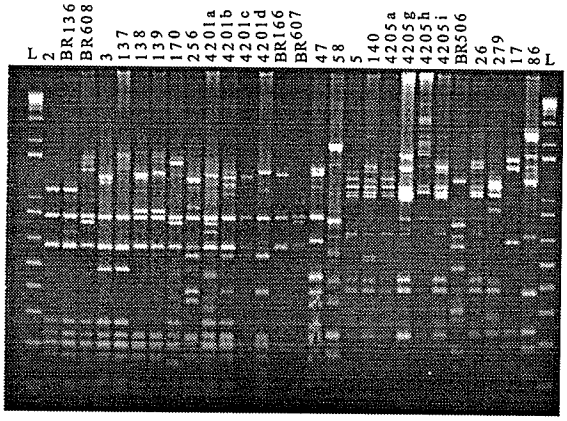
5
140
4205a
4205g
4205h
4205i
BR506
26
279
17
86
L

F


L
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BR136
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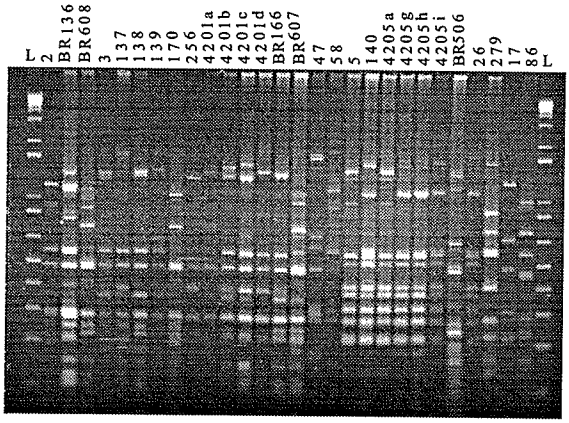
4201a
4201b
4201c
4201d
BR166
BR607
47
58
5
140
4205a
4205g
4205h
4205i
BR506
26
279
17
86
L

G


L
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4201b
4201c
4201d
BR166
BR607
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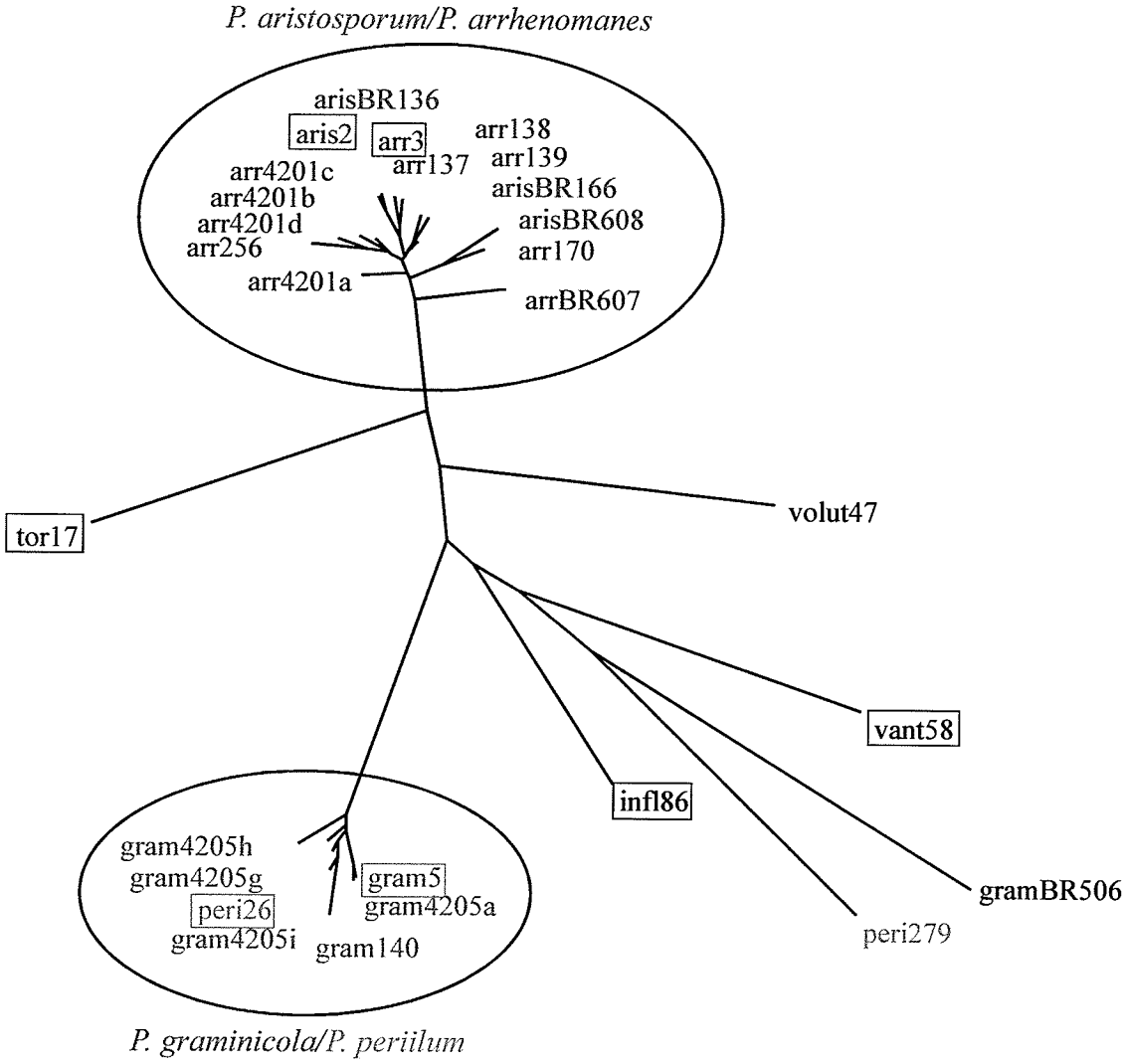
4205a
4205g
4205h
4205i
BR506
26
279
17
86
L

Figure 3.18. Unrooted neighbour joining phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.17. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: aris, *P. aristosporum*; arr, *P. arrhenomanes*; gram, *P. graminicola*; infl, *P. inflatum*; peri, *P. periilum*; tor, *P. torulosum*; vant, *P. vanterpoolii*; volut, *P. volutum.* Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. aristosporum/P. arrhenomanes
arisBR136
aris2
arr3
arr138
arr139
arr137
arr4201c
arr4201b
arisBR166
arr4201d
arisBR608
arr256
arr170
arr4201a
arrBR607
tor17
volut47
vant58
infl86
gram4205h
gram4205g
gram5
peri26
gram4205a
gram4205i
gram140
gramBR506
peri279
P. graminicola/P. periilum
.10

graminicola (BR506) and *P. periilum* (279) were distantly related to all other isolates and were present on their own individual branches. The remaining outgroup species (*P. inflatum, P. torulosum, P. vanterpoolii,* and *P. volutum*) were genetically distinct from each other and from the main clusters.

RFLP analysis of the rDNA IGS suggested that *P. arrhenomanes* and *P. graminicola* are genetically distinct species, which would agree with the results obtained from RFLPs of mtDNA (Martin and Kistler, 1990) and rDNA (Chen and Hoy, 1993), and also sequencing studies (Martin, 2000). The main distinguishing features between these two species are the larger oogonia and the larger number of antheridia per oogonium in *P. arrhenomanes* (as many as 20 per oogonium) compared to *P. graminicola.* The number of antheridia per oogonium is a character which Chen and Hoy (1993) suggested should actually be given more weight when distinguishing these two species.

Isolate 26 of *P. periilum*, the culture used by Van der Plaats-Niterink (1981) in the species description, was present within the cluster of *P. graminicola* isolates and genetically distinct from *P. periilum* 279. Morphologically, *P. periilum* is more similar to *P. graminicola* than is *P. arrhenomanes* (Table 3.4). The major difference between *P. periilum* and *P. graminicola* is that the zoosporangia of *P. periilum* contain a combination of inflated and noninflated elements, similar to *P. torulosum* and *P. myriotylum.* Otherwise, both species have similar oogonia, antheridia, and plerotic oospores (Van der Plaats-Niterink, 1981). Like *P. graminicola, P. periilum* is a common pathogen of graminaceous species, causing root rot and crown rot (Abad *et al.,* 1994). Both species also cause root rot of sugar cane (Rands and Dopp, 1938). Therefore, these species may in fact be conspecific based on the molecular data and comparable morphological

features. However, it may also be possible that isolate 26 was actually a *P. graminicola* isolate misidentified as *P. periilum* and that perhaps isolate 279 is a more suitable representative isolate for *P. periilum.* Further morphological examinations would be necessary to draw conclusions.

Four isolates of *P. aristosporum* were present in the cluster with *P. arrhenomanes* isolates (Fig. 3.18), suggesting a genetic relationship between these two species. *P. arrhenomanes* and *P. aristosporum* are morphologically very similar as both form zoosporangia with inflated elements and possess oogonia and oospores with similar sizes (Table 3.4). Antheridia are monoclinous or diclinous in *P. aristosporum* while they are strictly diclinous in *P. arrhenomanes,* but both species possess several antheridia per oogonium. In *P. aristosporum,* oospores are described as being aplerotic, but in a description by Sideris (1931), they were described as being plerotic, resembling those of *P. arrhenomanes.* Thus, many characteristics are common between these two species. The RFLP analysis of the IGS reflected the close morphological relationship of these two species. They are also similarly pathogenic to graminaceous plants. Hodges and Coleman (1985) described a disease of bentgrass infections on golf greens with high sand content, naming it "*Pythium* root dysfunction" and describing the pathogenicity of *P. arrhenomanes* and *P. aristosporum* to the bentgrass roots. In a comparison of the pathogenicity of several *Pythium* species from turfgrass showing symptoms of root and crown rot, *P. arrhenomanes* and *P. aristosporum* were the most aggressively pathogenic species (Abad *et al.,* 1994).

It appears that oogonial diameter is the most useful morphological feature to differentiate these species from one another. *P. arrhenomanes* and *P. aristosporum*

possess much larger oogonia than do *P. graminicola* and *P. periilum.* The number of antheridia per oogonium is a highly variable character within these species and perhaps should not be given as much taxonomic weight as oogonial diameter to delimit these species. Based on the large number of common morphological features and the molecular data presented, it appears *P. aristosporum* and *P. arrhenomanes* may in fact be conspecific. Since isolates of *P. graminicola* and *P. periilum* were present in the same cluster and are morphologically similar, they may be conspecific species as well since the ex-type culture of *P. graminicola* and culture used by Van der Plaats-Niterink (1981) to describe *P. periilum* were present in the same cluster. However, a larger sample size of *P. periilum* isolates would be necessary to draw such a conclusion in this case since an additional isolate of *P. periilum* (279) was genetically distinct. This isolate (279) may be a more appropriate representation of *P. periilum* than is isolate 26, but further morphological examination is necessary.

C. Examination of relationships between *P. myriotylum* and *P. zingiberis* in comparison to other species with filamentous inflated zoosporangia

Results and Discussion

Pythium myriotylum, P. zingiberis, P. aristosporum, and *P. pachycaule* are a group of morphologically similar species that share filamentous inflated zoosporangia, similar sized oogonia, and monoclinous or diclinous antheridia. A detailed comparison of some morphological characters of these species is in Table 3.5. To examine the

Table 3.5. Comparison of several morphological traits between *P. myriotylum, P. zingiberis, P. aristosporum,* and *P. pachycaule.*

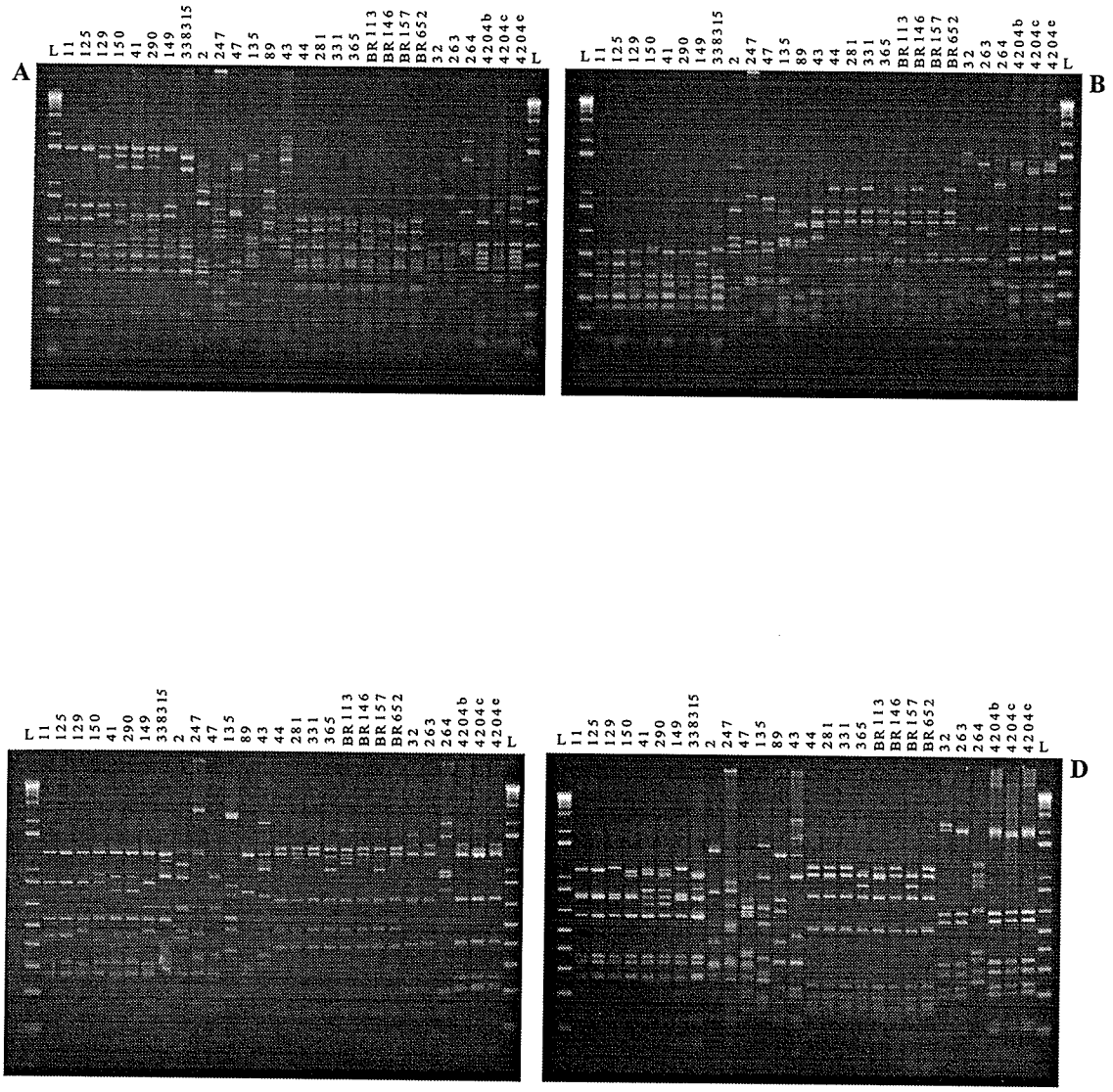
Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. myriotylum[a]	Filamentous, with inflated lobulate elements	Subglobose, terminal or intercalary; avg. diam. 29.0 µm	Diclinous, occasionally monoclinous; 3—6 per oogonium, making apical contact with oogonium	Aplerotic; avg. diam. 24.5 µm; wall up to 2 µm thick
P. zingiberis[b]	Filamentous with lobulate or toruloid inflated elements	Globose, terminal; avg. diam. 33 µm	Diclinous, occasionally monoclinous; 1—5 per oogonium, making apical contact with oogonium	Plerotic, avg. diam. 28.5 µm; wall from 4—8 µm thick
P. aristosporum[a]	Filamentous with inflated elements; globose hyphal swellings often present	Subglobose, terminal or occasionally intercalary; avg. diam. 32.7 µm	Monoclinous or diclinous; up to 8 per oogonium	Aplerotic, avg. diam. 26.8 µm; wall from 2—3 µm thick
P. pachycaule[c]	Filamentous and slightly inflated	Globose or sac-/trumpet-shaped; terminal and intercalary; avg. diam 26.6 µm	Monoclinous or diclinous ; 1—3 per oogonium	Aplerotic; avg. diam 22.2; avg. wall thickness 1.9 µm

[a]Based on description by Van der Plaats-Niterink (1981).
[b]Based on description by Takahashi (1954).
[c]Based on description by Ali-Shtayeh and Dick (1985).

relationships among these species and other related species, restriction endonuclease digestions of the PCR-amplified IGS were done for several isolates of *P. aphanidermatum, P. aristosporum, P. dissimile, P. myriotylum, P. pachycaule, P. pyrilobum, P. scleroteichum, P. sulcatum, P. volutum,* and *P. zingiberis* using *Alu*I, *Cfo*I, *Hae*III, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 3.19). A total of 436 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the UPGMA phenogram is presented in Figure 3.20.

Isolates of *P. myriotylum* and *P. zingiberis* were present in a cluster along with single isolates of *P. pachycaule* (338315) and *P. aristosporum* (149) (Fig. 3.20). The main differences between *P. myriotylum* and *P. zingiberis* are the slightly thicker oospore wall and larger oogonia and oospores in *P. zingiberis* (Table 3.5). *P. myriotylum* also occasionally produces clusters of appressoria which have not been observed in *P. zingiberis* (Van der Plaats-Niterink, 1981). As stated by Van der Plaats-Niterink (1981), "the description of *P. zingiberis* is somewhat confusing and does not agree with the figures", and she concluded that *P. zingiberis* was possibly a synonym of *P. volutum* due to their comparable zoosporangia, oogonia, and oospores. However, RFLP analysis showed that *P. volutum* (47) was not related to the *P. myriotylum/P. zingiberis* cluster. Moreover, *P. volutum* can be differentiated from those species by its antheridia that entwine the oogonial stalk (Van der Plaats-Niterink, 1981). It is likely that *P. myriotylum* and *P. zingiberis* are conspecific based on morphological comparisons and molecular data. However, the key morphological differences that separate these species are the larger oogonia, plerotic oospores, and thicker oospore wall of *P. zingiberis* (Takahashi,

Figure 3.19. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. aphanidermatum, P. aristosporum, P. dissimile, P. myriotylum, P. pachycaule, P. pyrilobum, P. scleroteichum, P. sulcatum, P. volutum,* and *P. zingiberis* with **(A)** *Alu*I, **(B)** *Cfo*I, **(C)** *Hae*III, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.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BR113
BR146
BR157
BR652
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263
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4204b
4204c
4204e
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BR113
BR146
BR157
BR652
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BR113
BR146
BR157
BR652
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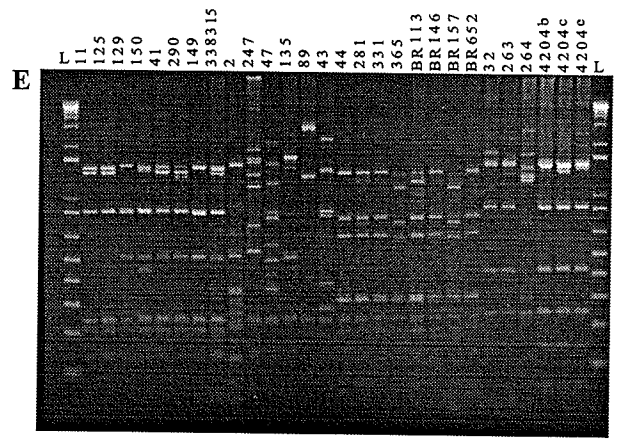
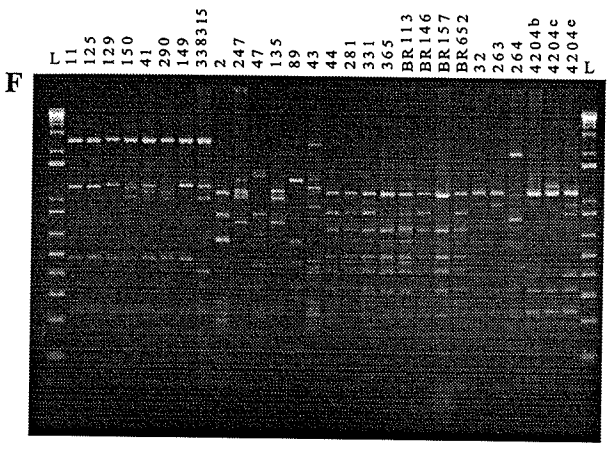
264
4204b
4204c
4204e
L


E
L
11
125
129
150
41
290
149
338315
2
247
47
135
89
43
44
281
331
365
BR113
BR146
BR157
BR652
32
263
264
4204b
4204c
4204e
L


F
L
11
125
129
150
41
290
149
338315
2
247
47
135
89
43
44
281
331
365
BR113
BR146
BR157
BR652
32
263
264
4204b
4204c
4204e
L

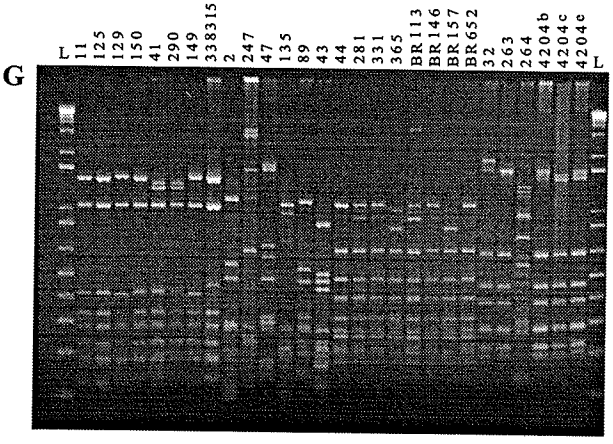
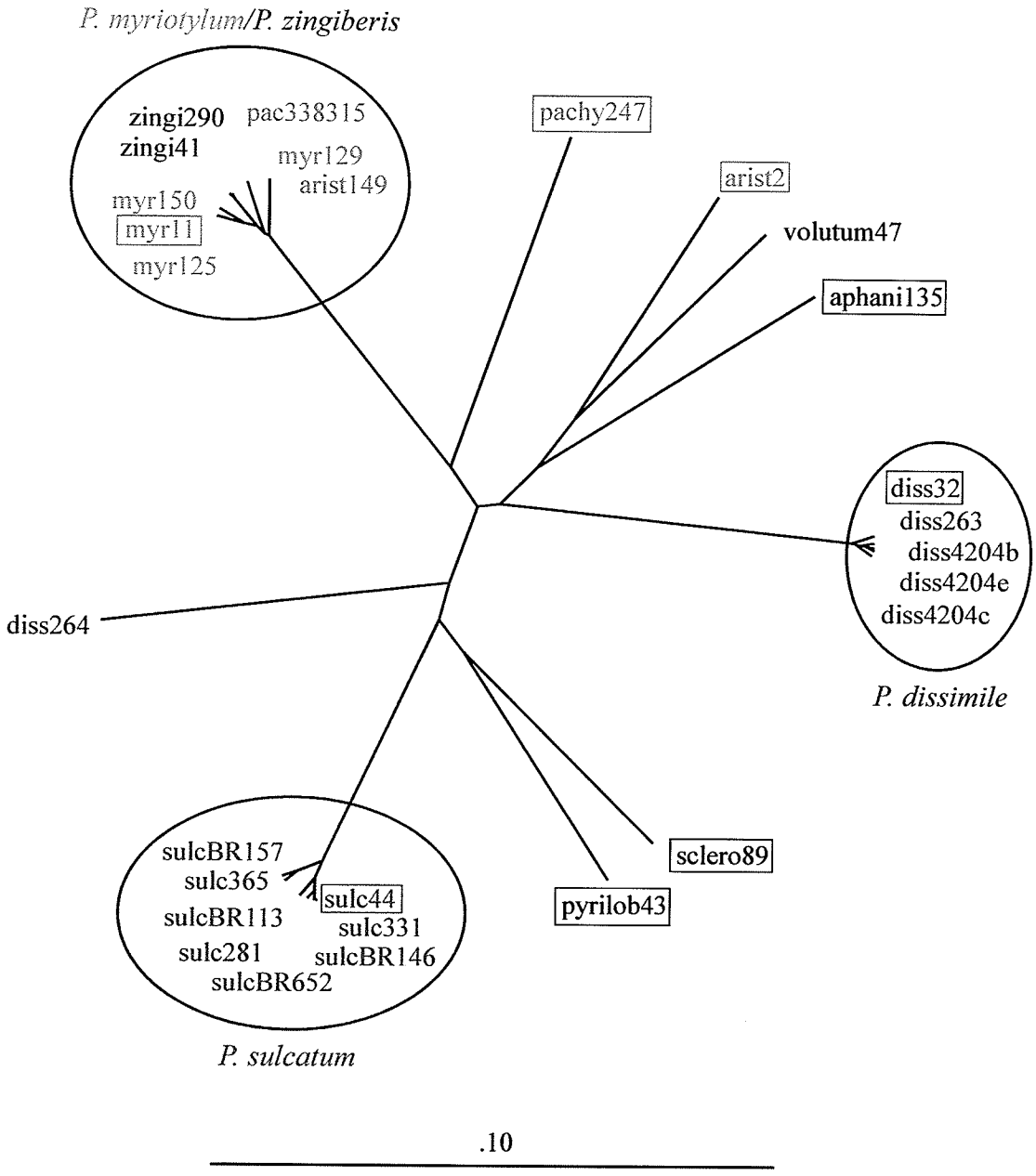

G
L
11
125
129
150
41
290
149
338315
2
247
47
135
89
43
44
281
331
365
BR113
BR146
BR157
BR652
32
263
264
4204b
4204c
4204e
L

Figure 3.20. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.19. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: aphani, *P. aphanidermatum*; arist, *P. aristosporum*; diss, *P. dissimile*; myr, *P. myriotylum*; pachy, *P. pachycaule*; pyrilob, *P. pyrilobum*; sclero, *P. scleroteichum*; sulc, *P. sulcatum*; volutum, *P. volutum*; zingi, *P. zingiberis*. Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


P. myriotylum/P. zingiberis
zingi290
pac338315
zingi41
myr129
arist149
myr150
myr11
myr125
pachy247
arist2
volutum47
aphani135
diss32
diss263
diss4204b
diss4204e
diss4204c
P. dissimile
diss264
sulcBR157
sulc365
sulc44
sulcBR113
sulc331
sulc281
sulcBR146
sulcBR652
P. sulcatum
sclero89
pyrilob43
.10

1954). These differences are significant, so it is difficult to draw a conclusion about the conspecificity of these species since no ex-type culture of *P. zingiberis* is available. The neotype culture of *P. myriotylum* (11) was included in the analysis, so it may be more probable that isolates 141 and 290 are actually isolates of *P. myriotylum* that were misidentified as *P. zingiberis.* The inclusion of an ex-type culture of *P. zingiberis* in the analysis would strengthen these conclusions.

The same was most likely true for *P. aristosporum* 149 and *P. pachycaule* 338315 which were present in the *P. myriotylum/P. zingiberis* cluster. The ex-type culture of *P. aristosporum* (2) was genetically distinct from this cluster, and this species can be differentiated from *P. myriotylum* and *P. zingiberis* by its larger number of antheridia per oogonium and presence of hyphal swellings. Isolate 247 of *P. pachycaule,* the ex-type culture, was also present on a branch distinct from isolate 338315 that was within the *P. myriotylum/P. zingiberis* cluster. *P. pachycaule* can be differentiated from these species by its smaller oospores and smaller sac- or trumpet-shaped oogonia (Ali-Shtayeh and Dick, 1985).

RFLP analysis of the IGS also showed that *P. myriotylum* is genetically distinct from the other outgroup species. Five isolates of *P. dissimile* formed a cluster, but isolate 264 was present on an individual branch. *P. dissimile* can be differentiated from the other species by its smaller oogonia, zoosporangia comprised of complexes of globose and filamentous outgrowths, and hypogynous antheridia (Van der Plaats-Niterink, 1981). Single isolates of *P. pyrilobum* and *P. scleroteichum* were also genetically distinct. *P. pyrilobum* has similar sexual organs to *P. myriotylum* and *P. aristosporum,* but its zoosporangia consist of globose and filamentous elements, similar to those of *P.*

oligandrum and *P. acanthicum* (Van der Plaats-Niterink, 1981). Zoosporangia in *P. scleroteichum* have never been observed, and its furrowed antheridial stalks distinguish it from all other *Pythium* species (Van der Plaats-Niterink, 1981). Eight isolates of *P. sulcatum* formed a monophyletic cluster and this species can be differentiated from the others in this analysis by its filamentous noninflated zoosporangia and the formation of hyphal swellings (Van der Plaats-Niterink, 1981). *P. aphanidermatum* was included in the analysis due to its high maximum growth temperature (more than 40°C), which is the same as for *P. myriotylum,* but it was genetically distinct from the *P. myriotylum/P. zingiberis* cluster, and can also be distinguished by its smaller oogonia and oospores and intercalary antheridia (Van der Plaats-Niterink, 1981).

V. Species with filamentous noninflated zoosporangia

There are approximately 15 to 20 species that possess filamentous noninflated, or slightly inflated, zoosporangia and smooth-walled oogonia. Isolates with these characteristics that do not produce oogonia in culture are classified as *Pythium* type F isolates. According to Rafin *et al.* (1995), approximately 75% of the total isolates forming filamentous noninflated zoosporangia were grouped as *Pythium* type F isolates by Van der Plaats-Niterink (1981). However, *Pythium* type F isolates share a large number of morphological similarities with defined species that have filamentous noninflated zoosporangia. The following section reports the use of RFLP analysis of the IGS to evaluate species boundaries among *Pythium* species with filamentous noninflated zoosporangia.

A. Comparison of morphologically similar species with filamentous zoosporangia (the "*P. diclinum* complex")

Introduction

The species *P. aquatile, P. angustatum, P. coloratum, P. diclinum, P. dissotocum, P. lutarium,* and *P. pachycaule* share many morphological similarities in addition to filamentous noninflated zoosporangia. The significant morphological characters which distinguish these species from one another are summarized in Table 3.6. Only *P. dissotocum, P. coloratum,* and *P. pachycaule* produce zoosporangia that are slightly inflated while the other species have strictly filamentous zoosporangia. All species produce globose or subglobose oogonia with a similar diameter ranging from 20.0 to 22.7 µm. The only exception is *P. pachycaule* which may produce larger sac- or trumpet-shaped oogonia. The antheridial arrangement is variable between species, but the range in the number of antheridia per oogonium is similar. All species, except *P. lutarium,* produce aplerotic oospores with a similar range in diameter and wall thickness, although the average oospore diameter of *P. pachycaule* is larger compared to the other species. It should be noted that in the description of *P. dissotocum,* oospores were described as aplerotic or nearly plerotic (Van der Plaats-Niterink, 1981), which may suggest that this character is variable among this group of species.

Results and Discussion

To examine relationships among these morphologically similar species with filamentous noninflated zoosporangia, RFLP analysis of the IGS was done for 43 isolates, which

Table 3.6. Comparison of several morphological characteristics among *Pythium* species with filamentous zoosporangia and smooth oogonia.

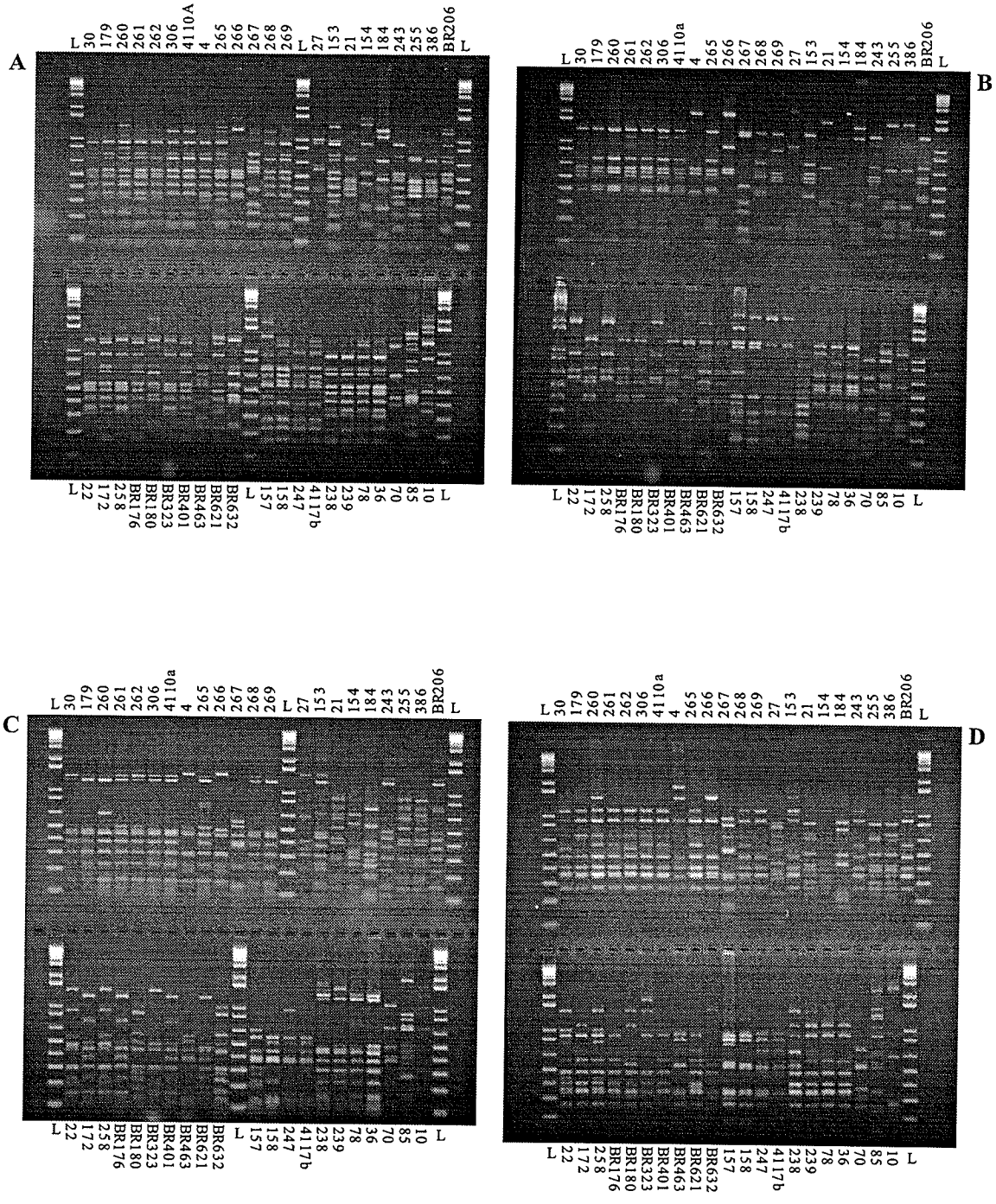
Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. diclinum[a]	Filamentous, noninflated; branched or unbranched	Spherical or ovoid; terminal or occasionally intercalary; avg. diam. 20.5 µm	Diclinous; 1—2 per oogonium	Aplerotic; avg. diam. 17.5 µm; avg. wall 3 µm thick
P. dissotocum[a]	Filamentous, forming slightly inflated, dendroid structures	Subglobose; terminal or intercalary; avg. diam. 22.5 µm	Monoclinous or diclinous; 1—3 per oogonium	Aplerotic, or nearly plerotic; avg. diam. 19.8 µm; wall from 1—3 µm thick
P. coloratum[a]	Filamentous, occasionally slightly inflated forming dendroid structures	Subglobose or pyriform, sometimes with a papilla; terminal or occasionally intercalary; avg. diam. 22.7 µm	Monoclinous or diclinous; 1—5 per oogonium	Aplerotic with yellowish contents and a lilac-colored wall; avg. diam. 18.9 µm; wall from 2—4 µm thick
P. aquatile[a]	Filamentous or slightly inflated, forming rectangular dendroid structures	Globose; terminal or intercalary, often clustering in small groups; avg. diam. 21.0 µm	Monoclinous; 1—2 per oogonium	Aplerotic; avg. diam. 17.9 µm; wall from 2—3 µm thick
P. angustatum[a]	Strictly filamentous and noninflated	Subglobose; terminal or intercalary; avg. diam. 20.0 µm	Monoclinous or diclinous; 1—5 per oogonium	Aplerotic; avg. diam. 18.0 µm; wall from 1.0—1.8 µm thick
P. lutarium[b]	Filamentous and noninflated, often branched	Globose; intercalary and terminal; avg. diam. 21.6 µm	Diclinous; 1—3 per oogonium	Plerotic; avg. diam. 19.6 µm; avg. wall 2.3 µm thick
P. pachycaule[b]	Filamentous and slightly inflated	Globose or sac-/trumpet-shaped; terminal and intercalary; avg. diam. 26.6 µm	Monoclinous or diclinous; 1—2 per oogonium	Aplerotic (22.2 µm; 1.9 µm)

[a]Based on descriptions by Van der Plaats-Niterink (1981).
[b]Based on descriptions by Ali-Shtayeh and Dick (1985).

included 37 isolates representing *P. angustatum, P. aquatile, P. coloratum, P. diclinum, P. dissotocum, P. lutarium,* and *P. pachycaule.* Two *Pythium* type F isolates were also included, as well as an isolate of *P. tumidum* (*nomen nudum*).No description of *P. tumidum* has been published, but it is morphologically similar to the species described in Table 3.6 (A.W.A.M. de Cock, personal communication). Single isolates of the outgroup species *P. apleroticum, P. flevoense,* and *P. monospermum* were included in the analysis. *P. apleroticum* and *P. flevoense* produce filamentous noninflated zoosporangia, however, *P. apleroticum* possesses plerotic oospores and has smaller oogonia and a thinner oospore wall compared to these species, while *P. flevoense* is heterothallic and has more complex antheridial arrangements. *P. monospermum* has filamentous zoosporangia, but it also has smaller oogonia and plerotic oospores (Van der Plaats-Niterink, 1981). Restriction digestions of the PCR-amplified IGS for all 43 isolates were done using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Hpa*II, *Rsa*I, and *Taq*I and are presented in Figure 3.21. A total of 316 restriction fragments were used for RFLP analysis. The topologies of the UPGMA and neighbor-joining phenograms were identical, and the UPGMA phenogram is presented in Figure 3.22.

Four prominent monophyletic clusters of isolates from the RFLP analysis were evident (Fig. 3.22). Cluster A was most extraordinary as it contained 21 isolates, with at least one isolate representing *P. angustatum, P. aquatile, P. coloratum, P. diclinum, P. dissotocum, P. lutarium,* and *P. tumidum* present. The neotype culture of *P. diclinum* (30) and the ex-type cultures of *P. dissotocum* (265) and *P. lutarium* (36) were present in cluster A. Two *Pythium* type F isolates (238 and 239) and an isolate of *P.*

Figure 3.21. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. angustatum, P. apleroticum, P. aquatile, P. coloratum, P. diclinum, P. dissotocum, P. flevoense, P. lutarium, P. monospermum, P. pachycaule, P. tumidum, Pythium* type F, and *P. aphanidermatum* BR206 with (**A**) *Alu*I, (**B**) *Hae*III, (**C**) *Hha*I, (**D**) *Hin*fI, (**E**) *Hpa*II, (**F**) *Rsa*I, and (**G**) *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L
30
179
260
261
262
306
4110A
4
265
266
267
268
269
L
27
153
21
154
184
243
255
386
BR206
L
L
22
172
258
BR176
BR180
BR323
BR401
BR463
BR621
BR632
41l7b
247
158
157
L
239
238
78
36
70
85
10
L
B
L
30
179
260
261
262
306
4110a
4
265
266
267
268
269
27
153
21
154
184
243
255
386
BR206
L
L
22
172
258
BR176
BR180
BR323
BR401
BR463
BR621
BR632
41l7b
247
158
157
L
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238
78
36
70
85
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L
C
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179
260
261
262
306
4110a
4
265
266
267
268
269
L
27
153
21
154
184
243
255
386
BR206
L
L
22
172
258
BR176
BR180
BR323
BR401
BR463
BR621
BR632
41l7b
247
158
157
L
239
238
78
36
70
85
10
L
D
L
30
179
260
261
262
306
4110a
4
265
266
267
268
269
27
153
21
154
184
243
255
386
BR206
L
L
22
172
258
BR176
BR180
BR323
BR401
BR463
BR621
BR632
41l7b
247
158
157
L
239
238
78
36
70
85
10
L

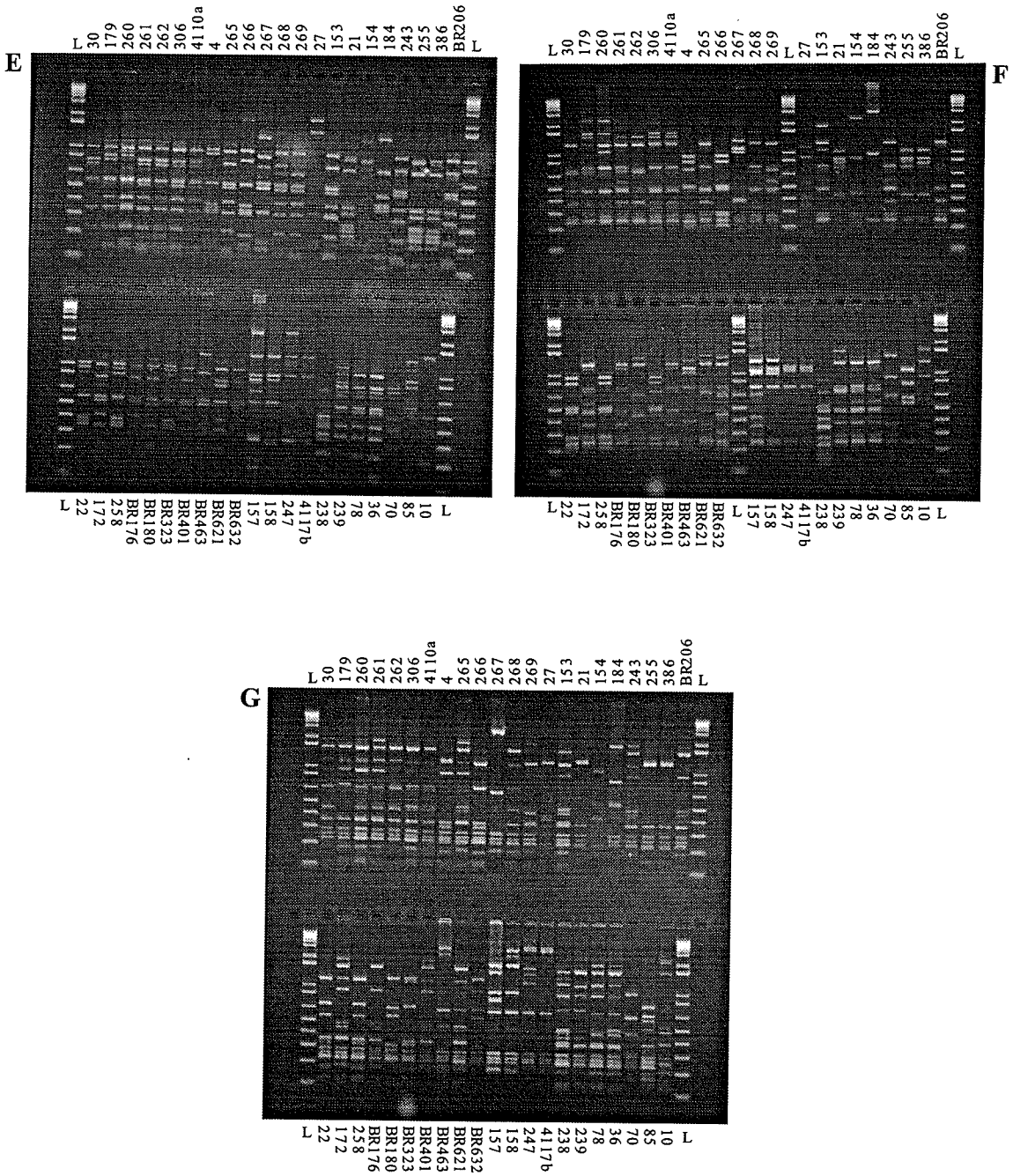
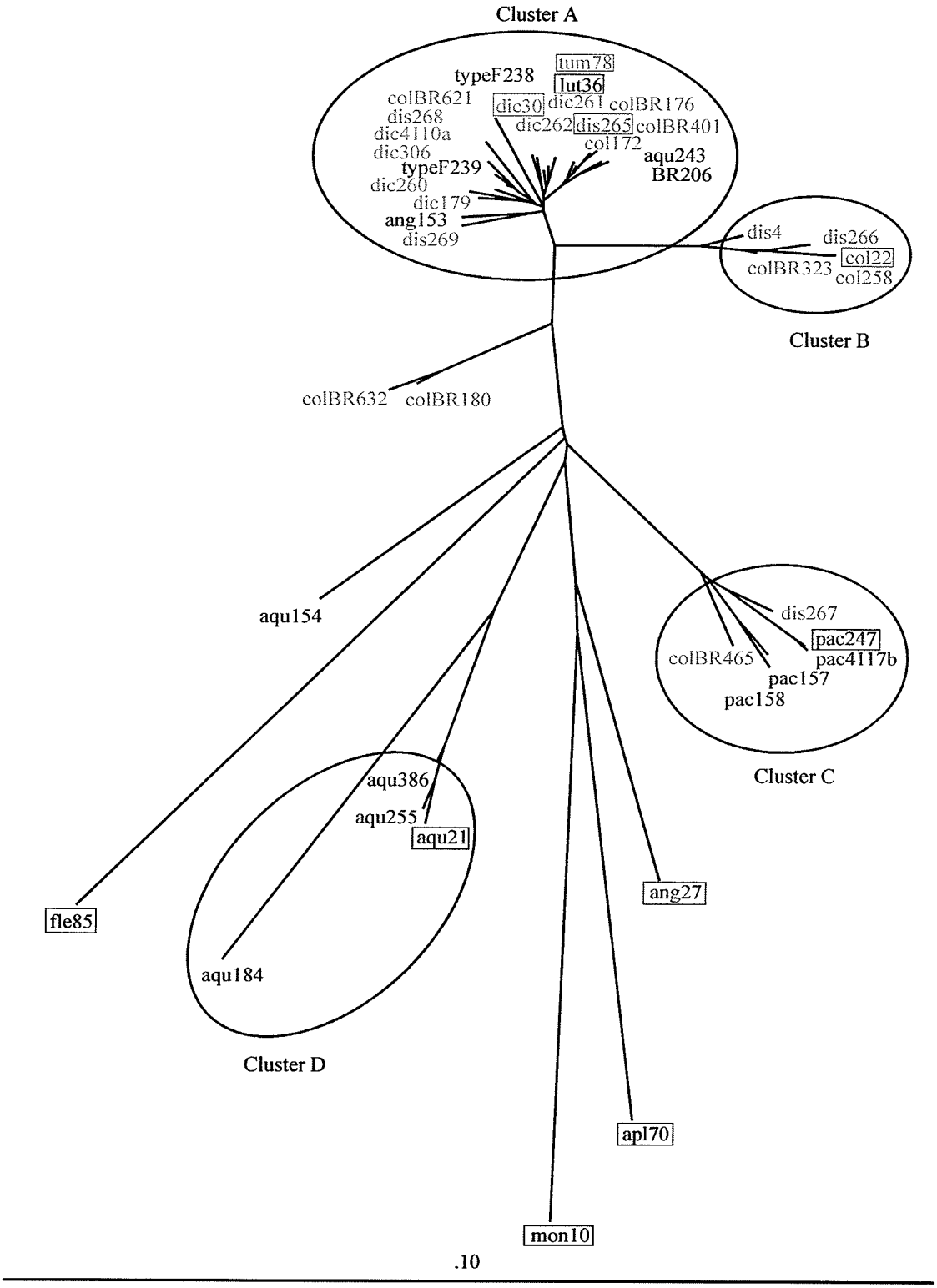

E
L 30 179 260 261 262 306 4110a 4 265 266 267 268 269 27 153 21 154 184 243 255 386 BR206 L
L 22 172 258 BR176 BR180 BR323 BR401 BR463 BR621 BR632 10 85 70 36 78 239 238 4117b 247 158 157 L
F
L 30 179 260 261 262 306 4110a 4 265 266 267 268 269 L 27 153 21 154 184 243 255 386 BR206 L
L 22 172 258 BR176 BR180 BR323 BR401 BR463 BR621 BR632 L 157 158 247 4117b 238 239 78 36 70 85 10 L
G
L 30 179 260 261 262 306 4110a 4 265 266 267 268 269 27 153 21 154 184 243 255 386 BR206 L
L 22 172 258 BR176 BR180 BR323 BR401 BR463 BR621 BR632 157 158 247 4117b 238 239 78 36 70 85 10 L

Figure 3.22. Unrooted UPGMA phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.21. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: ang, *P. angustatum*; apl, *P. apleroticum*; aqu, *P. aquatile*; col, *P. coloratum*; dic, *P. diclinum*; dis, *P. dissotocum*; fle, *P. flevoense*; lut, *P. lutarium*; mon, *P. monospermum*; pac, *P. pachycaule*; tum, *P. tumidum*; typeF, *Pythium* type F; BR206, *P. aphanidermatum* isolate BR206. Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


Cluster A
tum78
typeF238
lut36
colBR621
dic30
dic261
colBR176
dis268
dic262
dis265
colBR401
dic4110a
col172
dic306
aqu243
typeF239
BR206
dic260
dic179
ang153
dis269
dis4
dis266
col22
colBR323
col258
Cluster B
colBR632
colBR180
aqu154
dis267
pac247
colBR465
pac4117b
pac157
pac158
Cluster C
aqu386
aqu255
aqu21
ang27
fle85
aqu184
Cluster D
apl70
mon10
.10

aphanidermatum (BR206) were also present in this cluster. Cluster B contained three isolates of *P. coloratum,* including the ex-type culture (22), and two isolates of *P. dissotocum.* Two additional isolates of *P. coloratum* (BR180 and BR632) were more distantly related on an individual branch. The four isolates of *P. pachycaule* were present in cluster C along with *P. coloratum* BR465 and *P. dissotocum* 267. Cluster D contained four isolates of *P. aquatile* (21, 184, 255, and 386), but *P. aquatile* 184 was more distantly related to the other three isolates (including the neotype culture 21) which formed a smaller grouping within cluster D. An additional isolate of *P. aquatile* (154), *P. angustatum* (27) (the isolate used by Van der Plaats-Niterink [1981] in the species description), and the outgroup species *P. apleroticum, P. flevoense,* and *P. monospermum* were present on individual branches.

The main distinguishing morphological features among species in cluster A are the diclinous antheridia of *P. diclinum* and *P. lutarium,* formation of plerotic (or nearly plerotic) oospores of *P. lutarium* and *P. dissotocum,* and the yellow colored oospores of *P. coloratum* (Table 3.6). The monoclinous antheridia of *P. dissotocum* originate immediately below the oogonium, which is also unique among the species in cluster A, and *P. lutarium* is the only species that produces hyphal swellings (Van der Plaats-Niterink, 1981; Ali-Shtayeh and Dick, 1985). However, empty oogonia may sometimes be misinterpreted as hyphal swellings (A.W.A.M. de Cock, personal communication). A species description for *P. tumidum* has not been published to date, and due to the close molecular relationships to the other species in cluster A, its status as a species is doubtful and should be ignored (A.W.A.M. de Cock, personal communication). The original identification of isolate BR206 as *P. aphanidermatum* was probably incorrect as this

isolate was present in cluster A and its restriction fragment patterns were distinct from other isolates of *P. aphanidermatum* (not shown). Morphologically, *P. aphanidermatum* resembles the species in cluster A with respect to oogonial size, antheridial arrangement, and oospore characteristics. The most striking contrast are the zoosporangia of *P. aphanidermatum* which consist of terminal complexes of swollen hyphal branches forming toruloid structures (Van der Plaats-Niterink, 1981). These could have been mistaken for the filamentous slightly-inflated zoosporangia common to some species in cluster A, leading to this misidentification.

Isolates of *P. coloratum* were present within clusters A, B, and C, and two other isolates (BR180 and BR 632) were on an individual branch (Fig. 3.22). This may be a reflection of morphological variation within the species, such as the occasional presence of papilla on oogonia and the yellow color of the oospores (Van der Plaats-Niterink, 1981). On the other hand, this may indicate that isolates which possess filamentous slightly inflated zoosporangia are commonly misclassified as *P. coloratum* due to the overlap of common morphological characteristics among these species.

Isolates of *P. pachycaule* formed a monophyletic cluster (cluster C) well separated from the other species. The morphological characteristics that distinguish *P. pachycaule* from the other species are the larger oogonia and oospores and the sac- or trumpet-shaped oogonia. *P. coloratum* BR465 and *P. dissotocum* 267 were most likely isolates of *P. pachycaule* that were misidentified since they were present in cluster C.

The identification of *P. aquatile* and *P. angustatum* is often complicated by shared morphological features, although these species can be differentiated by the strictly filamentous zoosporangia of *P. angustatum* and monoclinous antheridia of *P. aquatile.*

As well, the oogonia of *P. aquatile* often form clusters and the oospore wall is thinner in *P. angustatum*. While *P. aquatile* (21) and *P. angustatum* (27) were present on separate branches, single isolates of each species were also present within cluster A. The majority of morphological characteristics described in Table 3.6 are similar when comparing *P. angustatum* and *P. aquatile* to the other species, with the exception of the monoclinous antheridia of *P. aquatile* and the strictly filamentous zoosporangia of *P. angustatum.* Nonetheless, based on RFLP analysis, the majority of *P. aquatile* and *P. angustatum* isolates (including the cultures used for species descriptions) were genetically distinct from cluster A.

There appears to be confusion identifying *Pythium* species with filamentous noninflated zoosporangia since isolates of several species were scattered throughout phenogram, with the exception of *P. pachycaule* and *P. diclinum.* The isolates and species in cluster A may represent a species complex (the "*P. diclinum* complex") due to the large number of shared morphological features among the species within the cluster, and the presence of the neotype culture of *P. diclinum* and ex-type cultures of *P. dissotocum* and *P. lutarium.* Isolates of *P. coloratum* were scattered throughout the phenogram, but the ex-type culture (22) was in cluster B, distinct from the *"P. diclinum* complex". *P. aquatile* and *P. angustatum* were genetically distinct from each other and from the "*P. diclinum* complex" since isolates were present on separate branches and in different clusters. *P. pachycaule* was also a genetically distinct species, and this is supported by the key morphological differences discussed above.

This group of *Pythium* species with filamentous noninflated zoosporangia perhaps most effectively illustrates the complications that arise in classifying *Pythium* species

based solely on morphological characteristics. The fact that the zoosporangia are often undifferentiated from vegetative hyphae can complicate morphological analyses as well. With respect to these species, molecular markers would aid in their identification and classification, and would assist in determining species boundaries and deciphering which morphological characters are most practical for differentiating one species from another.

B. Molecular relationships among other species with filamentous noninflated zoosporangia

Introduction

There are several other species with filamentous noninflated zoosporangia that share many morphological characteristics. Morphological comparisons between *P. capillosum, P. chondricola, P. flevoense, P. marinum,* and *P. porphyrae* are presented in Table 3.7. *P. capillosum* and *P. flevoense* are morphologically similar as they both possess filamentous noninflated zoosporangia, diclinous antheridia that entwine oogonia, and aplerotic or plerotic thick-walled oospores with similar diameters. The oogonia of *P. capillosum* distinguish this species from *P. flevoense* as they are larger and sometimes produce 1 to 3 papillae on the oogonial surface (Paul, 1987). As well, *P. flevoense* is distinctive as it is heterothallic while *P. capillosum* is homothallic (Van der Plaats-Niterink, 1972).

Pythium chondricola, P. marinum, and *P. porphyrae* possess spherical smooth oogonia with similar diameters, diclinous antheridia, and plerotic oospores. *P. chondricola* can be differentiated from the *P. marinum* and *P. porphyrae* as it produces

Table 3.7. A comparison of select morphological characteristics among some *Pythium* species with filamentous noninflated zoosporangia.

Species	Zoosporangia	Oogonia	Antheridia	Oospores
P. capillosum[a]	Filamentous, noninflated or slightly inflated	Spherical, but sometimes barrel-shaped (avg. diam. 23.09 µm); intercalary, rarely terminal, and smooth, but at times with 1—3 papillae	Diclinous, rarely monoclinous; branched with 1—6 per oogonium; often completely encircle the oogonium making a knot	Aplerotic and plerotic (avg. diam. 19.68 µm; avg. wall 2.36 µm thick)
P. flevoense[b]	Filamentous and noninflated, not differing from vegetative hyphae	Spherical, smooth, and mostly terminal (avg. diam. 19.0 µm); only formed in dual isolates of compatible isolates	Diclinous, 1 to several per oogonium; antheridial branches often entwine the oogonium	Aplerotic, occasionally plerotic (avg. diam. 17.7 µm; wall 2—4 µm thick)
P. chondricola[c]	Filamentous and noninflated; hyphal swellings present	Spherical and smooth (diam. 16—24 µm), but with an undulate wall if aplerotic oospores present; usually terminal, at times intercalary	Diclinous, 1—3 (up to 4) per oogonium; sac- to club-shaped antheridial cell broadly attached to oogonium	Plerotic, occasionally aplerotic (wall up to 1.5 µm thick)
P. marinum[b]	Filamentous and noninflated	Spherical and smooth, but occasionally forming a small apical papilla; terminally located	Diclinous; 1 per oogonium	Plerotic (wall 1.5—2 µm thick)
P. porphyrae[b]	Filamentous and noninflated	Globose and smooth (diam. 14.5—19.5 µm); intercalary and rarely terminal	Diclinous, 1—2 (up to 4) per oogonium; cells are globose or clavate	Plerotic (diam. 13.2—17.5 µm with thick walls)

[a]Based on description by Paul (1987).
[b]Based on descriptions by Van der Plaats-Niterink (1981).
[c]Based on description by de Cock (1986).

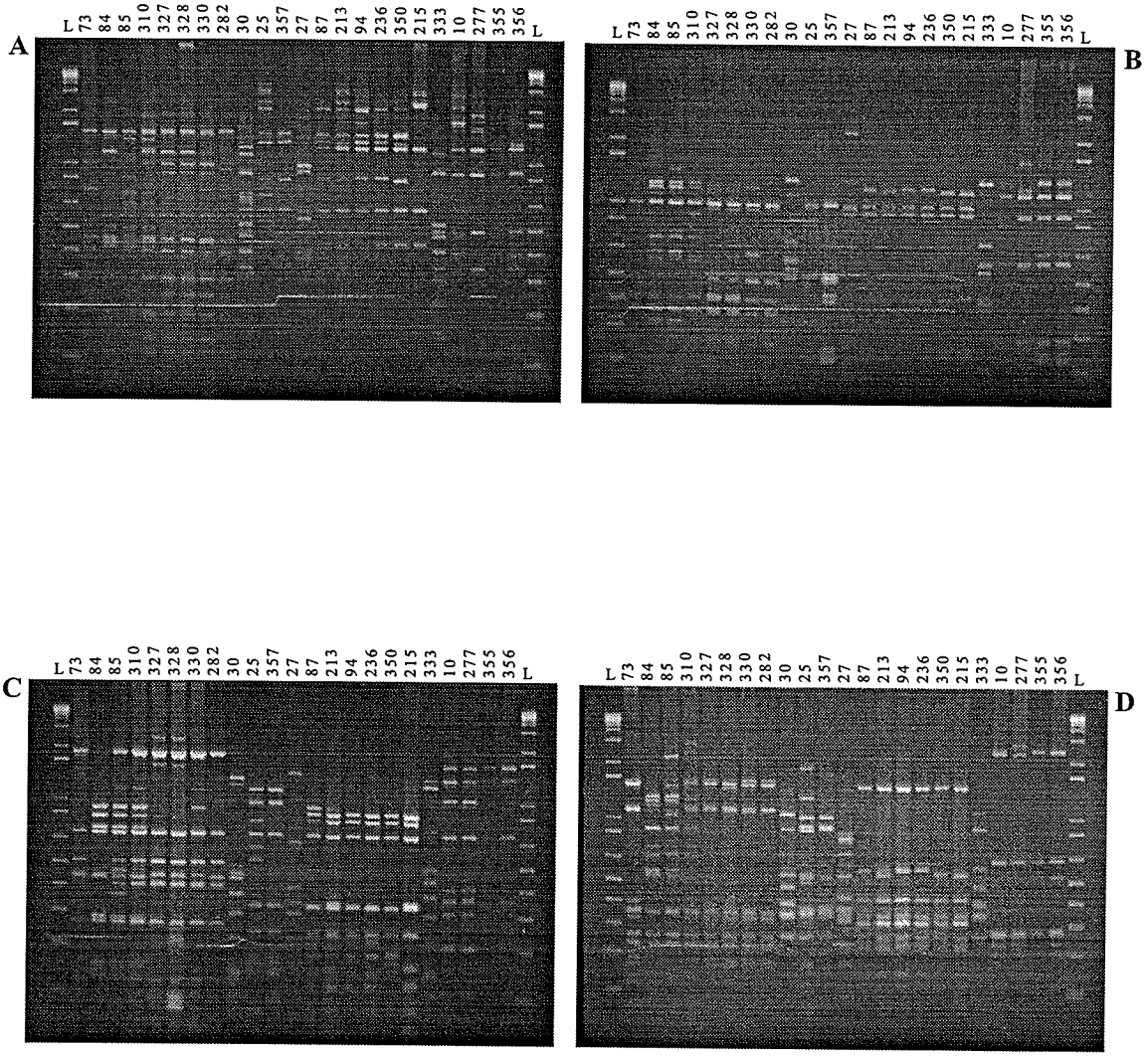
hyphal swellings and occasionally forms aplerotic oospores. *P. marinum* produces a single antheridium per oogonium while more than one antheridium per oogonium is often present in the other species. The oogonia of *P. porphyrae* are often intercalary and smooth, while in *P. chondricola* and *P. marinum* they are usually terminal and may have an undulate or papillate wall (Van der Plaats-Niterink, 1981; de Cock, 1986).

Results and Discussion

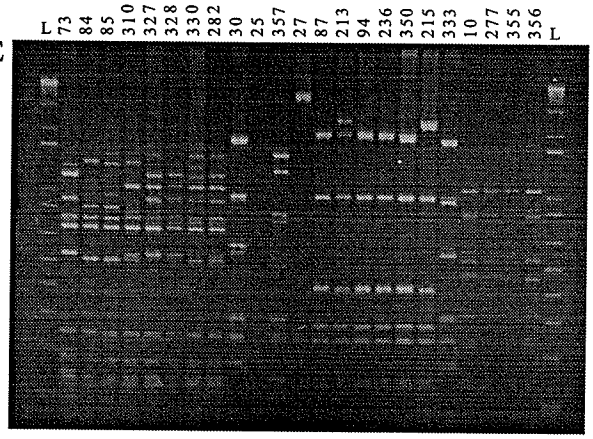
To examine the relationships among these morphologically similar species, restriction endonuclease digestions of rDNA IGS amplicons using *Alu*I, *Hae*III, *Hha*I, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I were done for several isolates of *P. adhaerens, P. angustatum, P. capillosum, P. chondricola, P. diclinum, P. flevoense, P. marinum, P. monospermum,* and *P. porphyrae* (Fig. 3.23). Four unclassified *Pythium* spp. were also included in the analysis. A neighbor-joining phenogram constructed from RFLP analysis of a total of 306 fragments is presented in Figure 3.24 and its topology was identical to the UPGMA phenogram (not shown).

Isolates of *P. capillosum* and *P. flevoense* formed monophyletic cluster, but also present within this cluster were four unidentified *Pythium* isolates (282, 327, 328, and 330) (Fig. 3.24). The three isolates of *P. flevoense* (84, 85, and 310) were genetically very similar, but *P. capillosum* was present on a separate branch. The key difference between *P. capillosum* and *P. flevoense* is that *P. flevoense* is heterothallic, but otherwise these species are morphologically and genetically very closely related. Isolates 327, 328, and 330 (*Pythium* spp.) were expected to be *P. flevoense* based on morphology (A.W.A.M. de Cock, personal communication), but they were more closely related to *P. capillosum* than

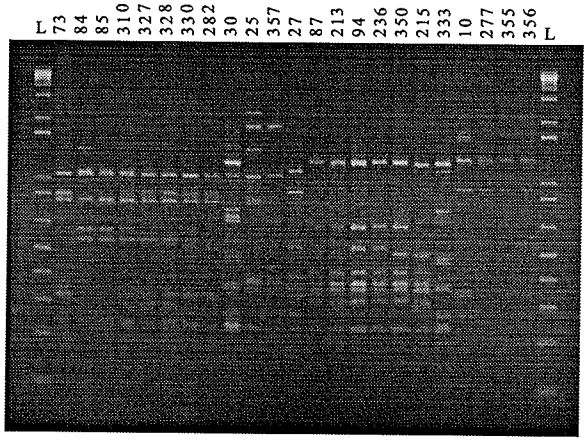
Figure 3.23. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. adhaerens, P. angustatum, P. capillosum, P. chondricola, P. diclinum, P. flevoense, P. marinum, P. monospermum, P. porphyrae,* and *Pythium* sp. with **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 3.1. L stands for 1 kb Plus DNA Ladder.


A
L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L
B
L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L
C
L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L
D
L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L

E


L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L

F


L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L

G

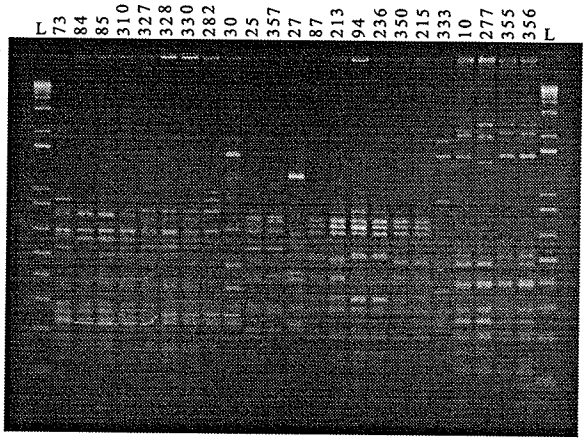
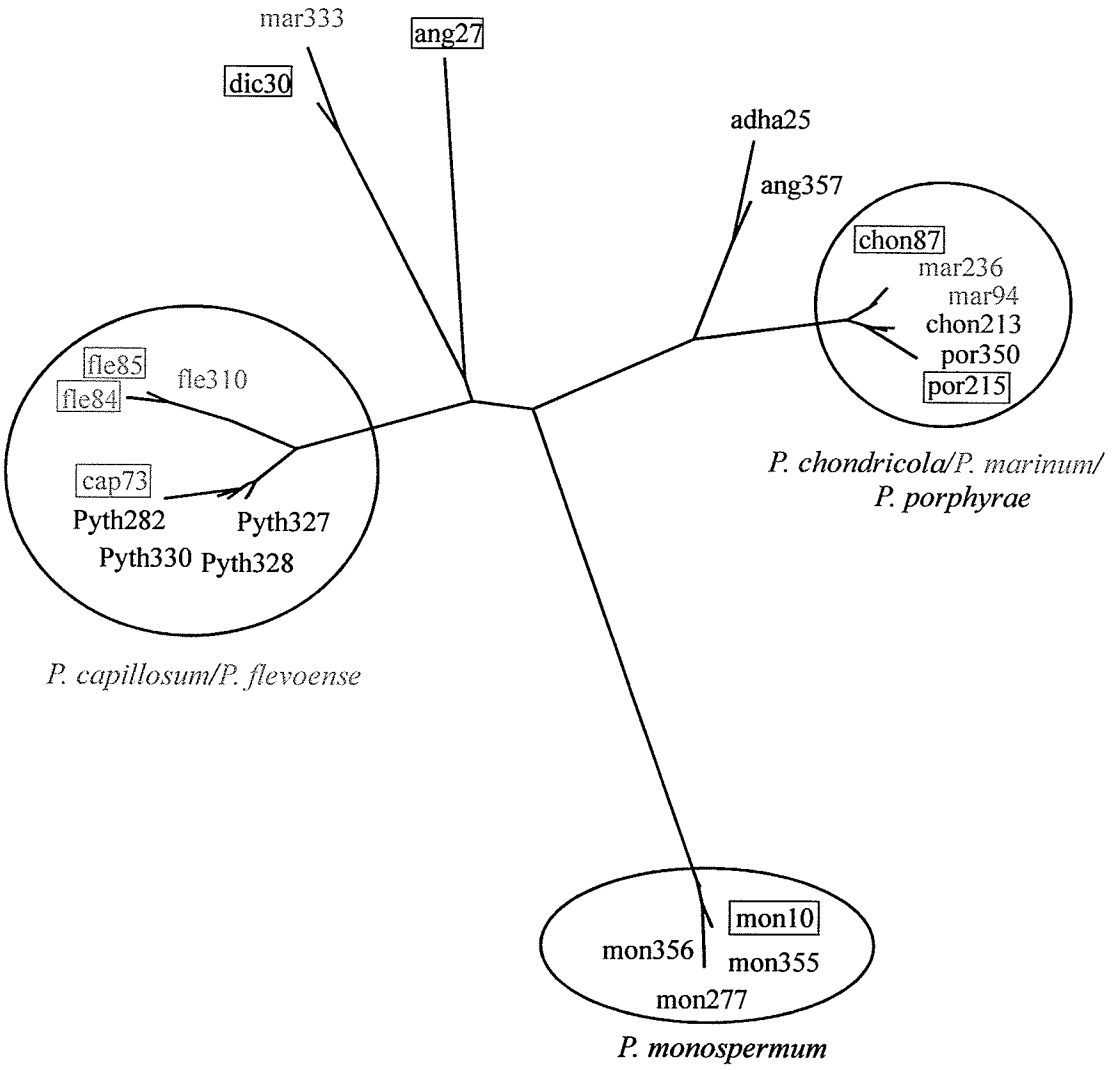

L 73 84 85 310 327 328 330 282 30 25 357 27 87 213 94 236 350 215 333 10 277 355 356 L

Figure 3.24. Unrooted neighbour-joining phenogram constructed using RFLP analysis of the rDNA IGS with data from Figure 3.23. Numbers correspond to isolate designations in Table 3.1, and species abbreviations are as follows: adha, *P. adhaerens*; ang, *P. angustatum*; cap, *P. capillosum*; chon, *P. chondricola*; dic, *P. diclinum*; fle, *P. flevoense*; mar, *P. marinum*; mon, *P. monospermum*; por, *P. porphyrae*; Pyth, *Pythium* sp.. Boxed species names represent ex-type, neotype, or authentic strains, or the culture used in the species description by Van der Plaats-Niterink (1981) (see Table 3.1). The scale represents the genetic distance calculated by the method of Nei and Li (1979).


mar333
ang27
dic30
adha25
ang357
chon87
mar236
mar94
chon213
por350
por215
fle85
fle84
fle310
cap73
Pyth282
Pyth327
Pyth330
Pyth328
P. capillosum/P. flevoense
P. chondricola/P. marinum/
P. porphyrae
mon10
mon356
mon355
mon277
P. monospermum
.10

to *P. flevoense*. Interestingly, these isolates were isolated from mosquito larvae while the other isolates of *P. flevoense* were from soil. *P. flevoense* has been known to infect *Daphnia* sp. as well (Van der Plaats-Niterink, 1968). Morphological information on isolate 282 was unavailable, but its presence in a cluster among isolates of *P. capillosum*and *P. flevoense* suggests a close relationship to these species. Based on the clustering of these isolates, *P. capillosum* may simply represent a homothallic strain of *P. flevoense*. The *Pythium* sp. isolates and the isolate of *P. capillosum* have not exhibited heterothallism, so perhaps RFLP analysis of the IGS has the capability to distinguish heterothallic isolates from homothallic isolates of the same species.

The *P. capillosum/P. flevoense* cluster was genetically separated from other morphologically similar species with filamentous noninflated zoosporangia (*P. adhaerens, P. angustatum,* and *P. diclinum*) (Fig. 3.24). *P. angustatum* and *P. diclinum* differ from *P. capillosum* and *P. flevoense* as their antheridia do not encircle the oogonium. While the antheridia of *P. adhaerens* do encircle the oogonium, the oogonia and oospores are smaller compared to *P. capillosum* and *P. flevoense* (Van der Plaats-Niterink, 1981). Isolate 357, originally identified as *P. angustatum,* was closely related to *P. adhaerens* 25 and was distinct from the ex-type culture of *P. angustatum* (27). Isolate 357 was most likely an isolate of *P. adhaerens* misidentified as *P. angustatum* as these species are morphologically similar, both possessing filamentous noninflated zoosporangia.

Isolates of *P. chondricola, P. marinum,* and *P. porphyrae* also formed a monophyletic cluster (Fig. 3.24). The ex-type culture (87) of *P. chondricola* was present in the cluster, but the ex-type culture of *P. porphyrae* was not available. However,

isolates 215 and 350 of *P. porphyrae* showed a close relationship to *P. chondricola,* and isolate 215 was the isolate described by Van der Plaats-Niterink (1981). The morphological differences between *P. chondricola* and *P. porphyrae* are considered to be insignificant compared to their similarities and these species can be considered synonymous (A.W.A.M. de Cock, personal communication). Two isolates of *P. marinum* (94 and 236) were also present within the cluster, but neither one was the ex-type culture. Isolate 94 was a sterile culture, so the misidentification of this isolate as *P. marinum* is understandable since comparisons using sexual structures could not be made. However, the presence of an additional isolate (236) in the cluster suggested that a closer relationship among *P. marinum, P. chondricola,* and *P. porphyrae* may exist. Comparisons using more isolates, especially the ex-type culture, is required to confirm the molecular evidence of this relationship presented here. Isolate 333 was most likely misidentified as *P. marinum* since it clustered with the neotype culture of *P. diclinum,* a morphologically similar species. However, this is not to say that *P. marinum* should be considered a member of the "*P. diclinum* species complex" since the other two isolates of *P. marinum* (94 and 236) were genetically distinct from *P. diclinum.* Again, comparisons using the ex-type culture of *P. marinum* would resolve this issue. The four isolates of *P. monospermum* (10, 277, 355, and 356) formed a monophyletic cluster and can be distinguished from the other species by their monoclinous and diclinous antheridia that arise at various distances from the oogonium (Van der Plaats-Niterink, 1981).

CONCLUSIONS

The RFLP analysis of the rDNA IGS provides a molecular biological method that uncovers a degree of interspecific variation sufficient to distinguish morphologically distinct *Pythium* species from one another. Relatively low levels of intraspecific variation among restriction fragment patterns were often observed between isolates of the same species, even for geographically diverse isolates of the same species. In the case of *P. ultimum,* this technique detected varieties of the species as well. For *P. capillosum* and *P. flevoense,* it was suggested that variation in fragment patterns may have differentiated homothallic strains from heterothallic ones. Such a molecular tool is of great importance as traditional morphological identifications of *Pythium* species are often inconsistent and prone to error and conflicting classifications. As well, because *Pythium* species are important plant pathogens, it is important to be able to accurately identify a species (and the correct genus as well) so that disease epidemics can be promptly and efficiently dealt with.

However, not all species exhibited minimal intraspecific variation in fragment patterns. Table 3.8 lists *Pythium* species for which more than one unique fragment pattern was present. While these examples may represent situations where this technique was insufficient to identify a species, it could also reflect variation within a species with respect to morphological diversity or geographic isolation within a species. On the other hand, isolates of a species which produced fragment patterns unique from those of their respective ex-type culture and from other species in the collection may also be representative of species not yet described or species which were not examined in this study.

Table 3.8. Summary of *Pythium* isolates which produced restriction fragment profiles unique from other isolates of that species and from all other species examined in this study. Isolate numbers refer to those listed in Table 3.1.

Species	Type culture material[a]	Isolate(s) with unique fragment pattern[b]
P. acanthophoron	52	4000b
P. acrogynum	69	152, 385
P. anandrum	1	4401d
P. aquatile	21	154, 184
P. chamaehyphon	82	359
P. dissimile	32	264
P. graminicola	5	BR506
P. helicoides	50	273
P. inflatum	86	387
P. iwayamai	29	64
P. middletonii	35	354
P. nagaii	n/a	4321a, 4321b
P. okanaganense	59	317
P. periilum	26	159/279[c]
P. periplocum	91	318, 4461a/4461b[c]
P. rostratum	88	192, 4329f
P. undulatum	48	293/401[c]
P. vexans	20	4017d, 132189
P. violae	28	126/369[c], 375

n/a, no ex-type culture available.

[a]refers to the ex-type culture, authentic strain, or isolated used in the description of Van der Plaats-Niterink (1981).

[b]refers to isolates with restriction fragment profiles unique from all other isolates (including the ex-type culture) of that species and all other species in the analysis.

[c]isolates with similar restriction fragment profiles that were unique from their respective ex-type or neotype culture and from all other isolates in the analysis.

Upon screening over 360 *Pythium* isolates using RFLP analysis of the IGS, several isolates appeared to have been originally classified with the incorrect species name. These initial identifications had been based on morphological comparisons with published descriptions, and RFLP analysis was used to confirm these identifications. From comparisons of restriction fragment patterns and phenograms in this study, a total of 48 *Pythium* isolates appeared to have been classified with the incorrect species name (Table 3.9). In the majority of cases, morphological comparisons between the species name originally assigned to the isolate and the species name suspected to be "correct" based on RFLP analysis revealed that most morphological characteristics were similar. Table 3.9 illustrates the taxonomic confusion that can arise when using morphology alone to identify *Pythium* species.

There were also several groups of morphologically related species which shared similar restriction fragment profiles. For such groups, RFLP analysis revealed that isolates of different species often formed monophyletic clusters. Table 3.10 summarizes the 14 groupings of *Pythium* species which shared similar restriction fragment patterns. These genetic relationships among morphologically similar species suggested that the species within these groupings may be synonymous. For the most part, morphological differences between species in each grouping were insignificant compared to the majority of shared features.

RFLP analysis of the IGS suggested that the phylogenetic species concept may be the most appropriate species concept to define *Pythium* species. However, it would not be proper to be dependent on one particular species concept as the standard for defining and classifying all isolates and species. It is important to combine information from more than

Table 3.9. The classification of certain *Pythium* isolates based on RFLP analysis compared to their initial identification based on morphological descriptions. Isolate numbers correspond to those listed in Table 3.1.

Isolate No.	Species classification based on morphology	Species classification based on RFLP analysis
4, 266	*P. dissotocum*	*P. coloratum*
36	*P. lutarium*	*P. diclinum* complex
78	*P. tumidum*	*P. diclinum* complex
115	*P. paroecandrum*	*P. nagaii*
123	*P. vexans*	*P. cucurbitacearum*
147	*P. ultimum* var. *sporangiiferum*	*P. ultimum* var. *ultimum*
149	*P. aristosporum*	*P. myriotylum/P. zingiberis*
153	*P. angustatum*	*P. diclinum* complex
160	*P. periilum*	*P. folliculosum/P. torulosum*
172	*P. coloratum*	*P. diclinum* complex
207	*P. vexans*	*P. minus/P. pleroticum*
208	*P. vexans*	*P. indigoferae*
210, 211, 212	*P. grandilobatum*	*P. oedochilum*
243	*P. aquatile*	*P. diclinum* complex
254	*P. anandrum*	*P. undulatum/P. dimorphum*
267	*P. dissotocum*	*P. pachycaule*
282, 327, 328, 330	*Pythium* sp.	*P. capillosum/P. flevoense*
283	*P. torulosum*	*P. inflatum*
288	*P. undulatum*	*P. splendens*
289	*P. undulatum*	*P. ultimum* var. *ultimum*
303	*Pythium* sp.	*P. tracheiphilum*
314	*P. middletonii*	*P. helicoides*
321	*P. prolatum*	*P. helicandrum*
333	*P. marinum*	*P. diclinum*
345	*P. hypogynum*	*P. acrogynum*
362, 399	*P. oedochilum*	*P. helicoides*
357	*P. angustatum*	*P. adhaerens*
376	*P. mastophorum*	*P. uncinulatum*
4012e	*P. spinosum*	*P. echinulatum/P. erinaceus/P. radiosum*
4017a, 4017b, 4017c	*P. vexans*	*P. perplexum*
338315	*P. pachycaule*	*P. myriotylum/P. zingiberis*
78621, 78622	*P. erinaceus*	*P. acanthicum*
BR160	*P. dissimile*	*P. conidiophorum/P. salpingophorum/P. Hypogynum*
BR176	*P. coloratum*	*P. diclinum* complex
BR206	*P. aphanidermatum*	*P. diclinum* complex
BR401	*P. coloratum*	*P. diclinum* complex
BR465	*P. coloratum*	*P. pachycaule*
BR621	*P. coloratum*	*P. diclinum* complex

Table 3.10. Summary of groupings of *Pythium* species which shared similar restriction fragment patterns and may in fact be conspecific based on RFLP analysis and discussed morphological comparisons. The species name listed first is the species with the earliest description date.

Species grouping	Fig. no. in this study
P. arrhenomanes, P. aristosporum	Fig. 3.18
P. buismaniae, P. polymastum	Fig. 3.6
P. dissotocum, P. diclinum, P. lutarium, P. tumidum	Fig. 3.22
P. echinulatum. P. erinaceus, P. radiosum	Fig. 3.6
P. flevoense, P. capillosum	Fig. 3.24
P. graminicola, P. periilum	Fig. 3.18
P. oedochilum, P. grandilobatum	Fig. 3.10
P. hydnosporum, P. oligandrum, P. amasculinum	Fig. 3.4
P. myriotylum, P. zingiberis	Fig. 3.20
P. pleroticum, P. minus	Fig. 3.14
P. porphyrae, P. chondricola, P. marinum	Fig. 3.24
P. salpingophorum, P. hypogynum, P. conidiophorum	Fig. 3.8
P. torulosum, P. folliculosum	Fig. 3.16
P. undulatum, P. dimorphum	Fig. 3.12

one species concept and not rely too heavily on a single species definition. The concurrence of data and conclusions from more than one species concept would strengthen the definition of a species, and would compensate for the shortcomings one species concept might have compared to another. For example, from the RFLP analysis in this study, it was possible to determine which morphological characters carried greater taxonomic weight than others within different groups of species.

Zoosporangial morphology is a character which can be used to differentiate some species from another. The filamentous zoosporangia of *P. periplocum* differentiate it from all other species with ornamented oogonia, and proliferating zoosporangia are an important feature in many species. But in many cases, zoosporangia are not formed, or they do not provide sufficient morphological features to distinguish one species from another. In the case of the "*P. diclinum* complex", zoosporangial form is of little taxonomic value. Oogonial size and the nature of the oogonial wall are useful characters for differentiating closely related species. In most cases, different species with similar restriction fragment patterns usually had oogonia very similar in size. The large and similar sized oogonia of *P. buismaniae* and *P. polymastum* reflect the close genetic relationship between these two species, and distinguish these two species from the morphologically similar species *P. uncinulatum* and *P. mastophorum* which have smaller oogonia and different restriction fragment patterns. However, with reference to the results from RFLP analysis, the slightly larger oogonia of *P. pleroticum* do not distinguish this species from *P. minus.* Oogonial spines, when present, can be of great taxonomic value. While the thickness of the spines is not always of great significance (e.g. *P. amasculinum, P. hydnosporum,* and *P. oligandrum),* the length and shape may be

sometimes useful (e.g. the longer oogonial spines of *P. radiosum* compared to those of *P. echinulatum* and *P. erinaceus*). The monoclinous or diclinous origin of antheridia can be useful to distinguish species, but often a species will have both monoclinous and diclinous antheridia, or lack antheridia altogether. *P. hypogynum* has strictly hypogynous antheridia, but it was very closely related to *P. salpingophorum* which has monoclinous or diclinous antheridia or does not form them at all. The plerotic or aplerotic nature of the oospores is often variable within species and ambiguous in species descriptions, so it should not be given a great degree of taxonomic weight in comparison to the other features discussed. Because of the variability in the presence and nature of characters within and between species, molecular data are now commonly used to supplement descriptions of new *Pythium* species. For example, in the species description of *P. megacarpum* sp. nov., Paul (2000) provided alignments of ITS-1 sequences with related species, in addition to morphological comparisons, to strengthen the conclusion that this new species was both morphologically and genetically unique.

In conclusion, RFLP analysis of the PCR-amplified IGS is a rapid, inexpensive, and effective molecular tool which can be used to identify *Pythium* species, confirm the species classifications of previously identified isolates, and reveal genetic relationships among related species. While often efficient, the classification of *Pythium* species based solely on morphology can result in misidentifications and descriptions of new species that may simply be morphological or geographic variants of previously described species. The supplementation of morphological data with molecular data, such as the RFLPs presented here, strengthens the definition of a *Pythium* species.

CHAPTER 4

A molecular phylogeny of *Pythium insidiosum*

INTRODUCTION

Pythium insidiosum (de Cock *et al.,* 1987) and *P. destruens* (Shipton, 1987) are the only species in the genus capable of infecting animal hosts while the other species are saprophytic or pathogenic to plants. These two species are widely considered to be synonymous (Mendoza and Marin, 1989) and the name *P. insidiosum* has priority. *P. insidiosum* possesses filamentous, noninflated zoosporangia which produce biflagellate zoospores that are chemotactically attracted to skin tissue, equine and human hair, and leaves of water lilies and grasses (Mendoza *et al.,* 1993).

Pythium insidiosum is the etiological agent of pythiosis, a granulomatous disease that occurs in tropical and subtropical areas of the world, with some cases having also been reported in more temperate regions. Pythiosis is characterized by cutaneous and subcutaneous infections, bone lesions, esophagitis, gastrointestinal diseases, and pulmonary infections in mammals including equines, canines, felines, cattle, and a captive spectacled bear (*Tremarctos ornatus*). In humans, symptoms such as arteritis, keratitis, and cutaneous or subcutaneous infections have been reported. *P. insidiosum* has also been isolated from a mosquito larva (isolate 296 in this study; CBS strain 777.84).

Pythium insidiosum is difficult to identify morphologically as it usually only develops hyphae, which may even be septate as in true fungi (de Cock *et al.,* 1987). The ability to distinguish *P. insidiosum* from other *Pythium* species and from organisms that cause similar symptoms in the host is crucial for treatment of the disease. Serological tests currently available to diagnose pythiosis and identify the etiological agent include complement fixation tests (Miller and Campbell, 1982), fluorescent antibodies (Mendoza *et al.,* 1987), immunoperoxidase staining (Brown *et al.,* 1988), immunoblot analysis

(Mendoza *et al.,* 1992a), enzyme-linked immunosorbent assays (ELISA) (Mendoza *et al.,* 1997; Grooters *et al.,* 2002), and immunodiffusion (ID) tests (Mendoza *et al.,* 1986; Imwidthaya and Srimuang, 1989; Pracharktam *et al.,* 1991).

Despite the ability of *P. insidiosum* to cause potentially fatal infections in animals, very little molecular work has been reported on the identification and phylogeny of the species. In a phylogenetic analysis of *Pythium* species based on the mitochondrial *cox*II gene, two isolates of *P. insidiosum* formed a clade within a clade of species that possess filamentous to lobate zoosporangia, but they did not form a distinct lineage separate from the *Pythium* plant pathogens (Martin, 2000). This suggested that *P. insidiosum* may be an opportunistic pathogen on the verge of true animal pathogenicity.

Although results from fluorescent antibody and ID tests supported the idea that isolates of *P. insidiosum* from around the world were antigenically identical (Mendoza *et al.*, 1987), other studies have revealed the existence of physiological variation among isolates. For instance, McMeekin and Mendoza (2000) showed varying effects of streptomycin on the growth of *P. insidiosum* isolates *in vitro.* The presence of streptomycin inhibited or had no effect on the growth of isolates from Costa Rica or the USA, while it stimulated the growth of a human strain from Thailand.

The objectives of this chapter are threefold. RFLP analysis of the rDNA IGS was used to examine relationships among 28 isolates of *P. insidiosum* from a variety of animal hosts and geographic origins. Secondly, the rDNA internal transcribed spacer (ITS) was sequenced for 23 isolates of *P. insidiosum* to investigate the phylogenetic relationships between clades of isolates. This method, which has been used for *Phytophthora* and other Oomycetes (Cooke *et al.,* 2000), allows for the construction of a

bootstrapped phylogenetic tree showing estimates of genetic distances between clades of *P. insidiosum* isolates and their relationships to the outgroup species *Phytophthora megasperma* Drechsler and *Lagenidium giganteum* Couch. Lastly, the growth of a select number of *P. insidiosum* isolates was measured over a range of temperatures to compare growth rates of isolates representing the genetic clusters from the molecular analysis.

RESULTS

RFLP analysis of the rDNA IGS

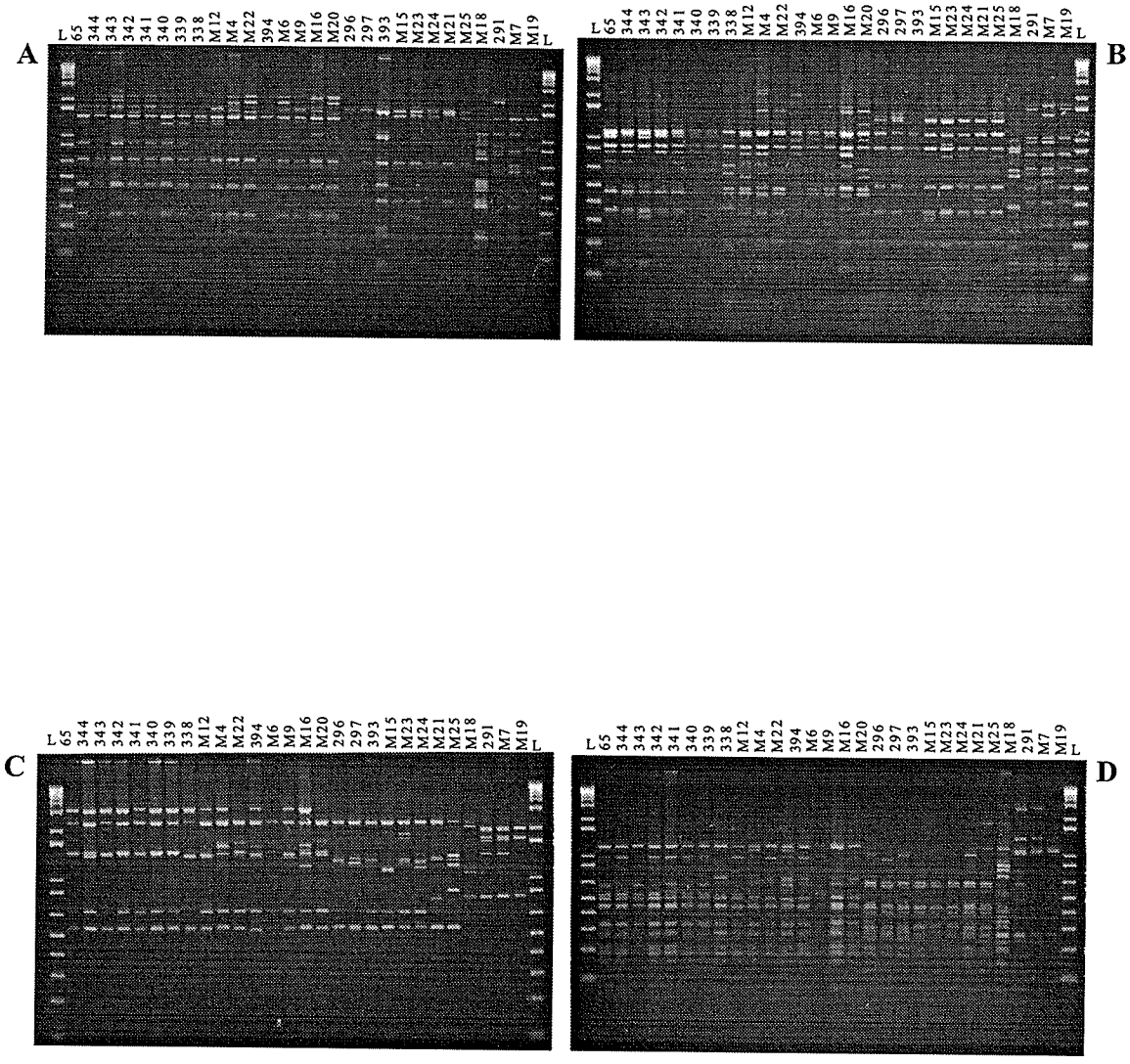
Twenty-eight isolates of *P. insidiosum* from a variety of animal hosts representing a wide geographic distribution were studied (Table 4.1). No significant morphological differences had been observed among these isolates (A.W.A.M de Cock, personal communication). Amplification of the IGS using primers Q and P2 (Fig. 2.1) yielded a single PCR product ranging in size from 4.8 to 5.2 kb for all 28 isolates. Restriction digestions of the amplicons were done using *Alu*I, *Hae*III, *Hin*cII, *Hin*fI, *Mbo*I, *Rsa*I, and *Taq*I (Fig. 4.1). The ex-type cultures of *P. aphanidermatum, P. deliense,* and *P. grandisporangium* were used as outgroup species in this analysis. *P. aphanidermatum* and *P. deliense* are morphologically very similar to *P. insidiosum,* and the former species shares the extraordinarily high optimum growth temperature of 37°C with *P. insidiosum* while most other *Pythium* species grow best at 25–30°C (Van der Plaats-Niterink, 1981). Phylogenetic analysis of the mitochondrial *cox*II gene also indicated that *P. aphanidermatum, P. deliense,* and *P. insidiosum* were very closely related (Martin, 2000). Sequence analysis of the internal transcribed spacer (ITS) of the rDNA repeat unit indicated that, although *P. aphanidermatum* and *P. deliense* were closely related to *P. insidiosum, P. grandisporangium* was even closer (C.A. Lévesque, personal communication). Restriction digestions of the amplicons of the outgroup species *P. aphanidermatum, P. deliense,* and *P. grandisporangium* were done as well and compared to the fragment patterns of *P. insidiosum* isolates on separate agarose gels (not shown).

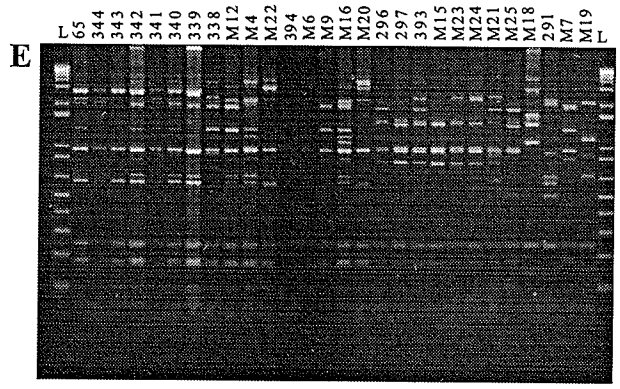
Table 4.1. List of *P. insidiosum* isolates and outgroup species used in Chapter 4.

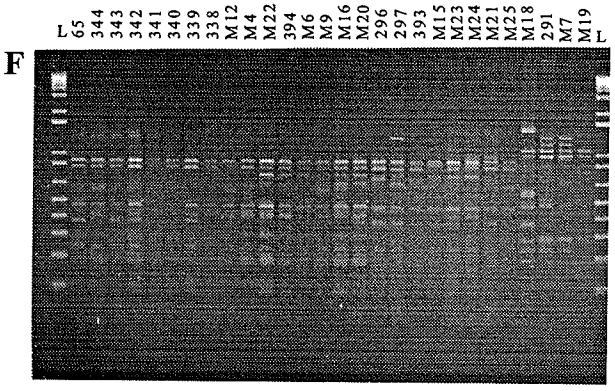
Species	Isolate	Source and Accession No.[b]	Host	Country of Origin	GenBank No.[d]
P. insidiosum de Cock	65[a]	CBS 574.85/ATCC 58643	Equine	Costa Rica	AY151157
P. insidiosum	338	CBS 573.85/ATCC 58644	Equine	Costa Rica	AY151158
P. insidiosum	339	CBS 575.85/ATCC 58642	Equine	Costa Rica	AY151159
P. insidiosum	340	CBS 576.85/ATCC 58641	Equine	Costa Rica	
P. insidiosum	341	CBS 577.85/ATCC 58640	Equine	Costa Rica	AY151160
P. insidiosum	342	CBS 578.85/ATCC 58639	Equine	Costa Rica	AY151161
P. insidiosum	343	CBS 579.85/ATCC 58638	Equine	Costa Rica	AY151162
P. insidiosum	344	CBS 580.85/ATCC 58637	Equine	Costa Rica	
P. insidiosum	394	CBS 101555	Equine	Brazil	AY151163
P. insidiosum	M4	undeposited[c]	Feline	Florida, USA	AY151164
P. insidiosum	M6	ATCC 200269	Human	Tennessee, USA	
P. insidiosum	M9	undeposited[c]	Canine	Louisiana, USA	
P. insidiosum	M12	undeposited[c]	Equine	Costa Rica	AY151165
P. insidiosum	M16	ATCC 76049	Human	Haiti	AY151166
P. insidiosum	M20	ATCC 200268	Canine	North Carolina, USA	AY151167
P. insidiosum	M22	undeposited[c]	Canine	Wisconsin, USA	AY151168
P. insidiosum	296	CBS 777.84	Mosquito larva	India	AY151169
P. insidiosum	297	CBS 702.83/ATCC 46947	Equine	Japan	AY151170
P. insidiosum	393	CBS 101039	Human	India	
P. insidiosum	M15	ATCC 28251	Equine	Papua New Guinea	AY151171
P. insidiosum	M21	undeposited[c]	Human	Pennsylvania, USA	AY151172
P. insidiosum	M25	undeposited[c]	Human	Thailand	AY151173
P. destruens Shipton	M23[a]	ATCC 64221	Equine	Australia	AY151174
P. destruens	M24	ATCC 64218	Equine	Australia	AY151175
P. insidiosum	291	CBS 673.85	Human	Thailand	AY151176
P. insidiosum	M7	undeposited[c]	Human	Thailand	AY151177
P. insidiosum	M18	ATCC 90478	*Tremarctos ornatus*	South Carolina, USA	AY151178
P. insidiosum	M19	ATCC 90586	Human	Texas, USA	AY151179
P. aphanidermatum	135[a]	CBS 118.80	Unknown	France	AY151180
P. deliense Meurs	66[a]	CBS 314.33	*Nicotiana tabacum*	Indonesia	AY151181
P. grandisporangium	54[a]	CBS 286.79/ATCC 28295	*Distichlis spicata*	Florida, USA	AY151182
Lagenidium giganteum	36492[e]	ATCC 36492	Mosquito larva	North Carolina, USA	AY151183

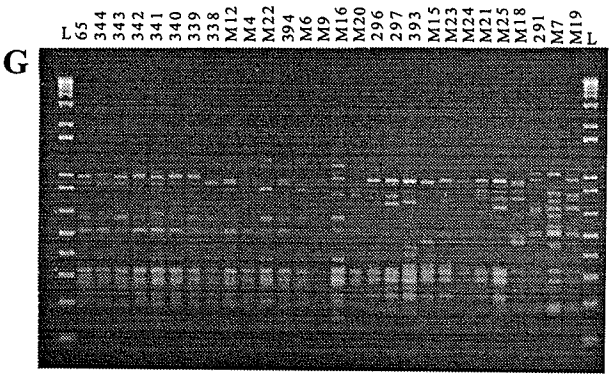
[a] ex-type or neotype culture.
[b] CBS= Centraalbureau voor Schimmelcultures, Utrecht, Netherlands; ATCC= American Type Culture Collection, Manassas, Virginia, USA.
[c] Personal collection of L. Mendoza.
[d] Indicates isolates used for ITS sequence analysis in this study and sequences were deposited in GenBank with their designated accession numbers.
[e] DNA provided by M. Hudspeth, Northern Illinois University, DeKalb, Illinois, USA.

Figure 4.1. Restriction endonuclease digestions of PCR products of the rDNA IGS for isolates of *P. insidiosum* and *P. destruens* using **(A)** *Alu*I, **(B)** *Hae*III, **(C)** *Hha*I, **(D)** *Hin*fI, **(E)** *Mbo*I, **(F)** *Rsa*I, and **(G)** *Taq*I. Lane numbers correspond to isolate designations in Table 4.1. L stands for 1 kb Plus DNA Ladder.


A
L 65 344 343 342 341 340 339 338 M12 M4 M22 394 M6 M9 M16 M20 296 297 393 M15 M23 M24 M21 M25 M18 291 M7 M19 L
B
L 65 344 343 342 341 340 339 338 M12 M4 M22 394 M6 M9 M16 M20 296 297 393 M15 M23 M24 M21 M25 M18 291 M7 M19 L
C
L 65 344 343 342 341 340 339 338 M12 M4 M22 394 M6 M9 M16 M20 296 297 393 M15 M23 M24 M21 M25 M18 291 M7 M19 L
D
L 65 344 343 342 341 340 339 338 M12 M4 M22 394 M6 M9 M16 M20 296 297 393 M15 M23 M24 M21 M25 M18 291 M7 M19 L


E
L
65
344
343
342
341
340
339
338
M12
M4
M22
394
M6
M9
M16
M20
296
297
393
M15
M23
M24
M21
M25
M18
291
M7
M19
L


F
L
65
344
343
342
341
340
339
338
M12
M4
M22
394
M6
M9
M16
M20
296
297
393
M15
M23
M24
M21
M25
M18
291
M7
M19
L

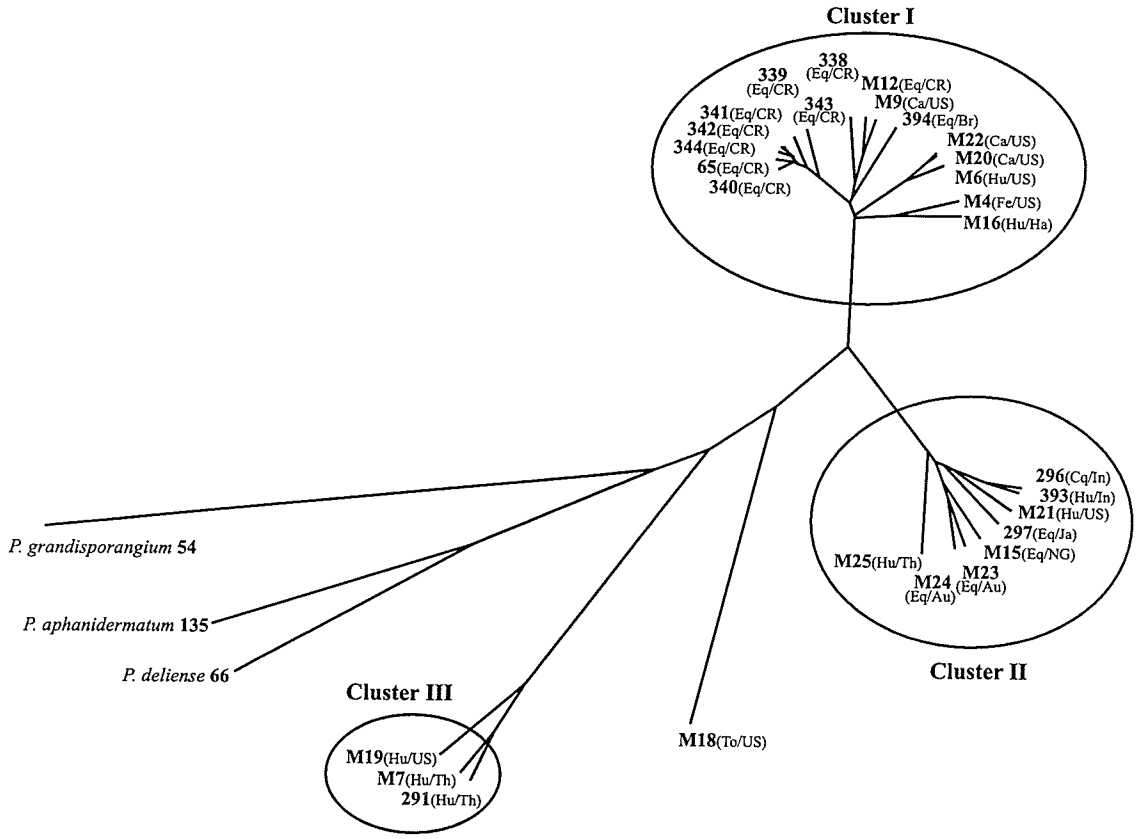

G
L
65
344
343
342
341
340
339
338
M12
M4
M22
394
M6
M9
M16
M20
296
297
393
M15
M23
M24
M21
M25
M18
291
M7
M19
L

The unrooted UPGMA and neighbor-joining phenograms (Fig. 4.2) were produced by combining data from all seven restriction endonucleases. In each phenogram, three major clusters (I, II, and III) of isolates were observed, and one isolate (M18) was excluded from the clusters. Cluster I contained 16 isolates of *P. insidiosum,* including the ex-type culture 65. Seven out of nine isolates (65, 339, 340, 341, 342, 343, and 344) were isolated from equines on the same ranch in Guanacaste, Costa Rica, and they formed a single cluster. Two other isolates (338 and M12), from different regions of Costa Rica, clustered with isolate M9 from a canine in Louisiana and isolate 394 from an equine in Brazil in the UPGMA phenogram (Fig. 4.2A). In the neighbor-joining analysis (Fig. 4.2B), isolate 394 was more closely related to isolates M4 and M16. Isolates M20 and M22, from canines in North Carolina and Wisconsin, respectively, were almost identical and clustered with human isolate M6 from Tennessee. Isolate M4, from a feline in Florida, clustered with human isolate M16 from Haiti, two geographically close regions.

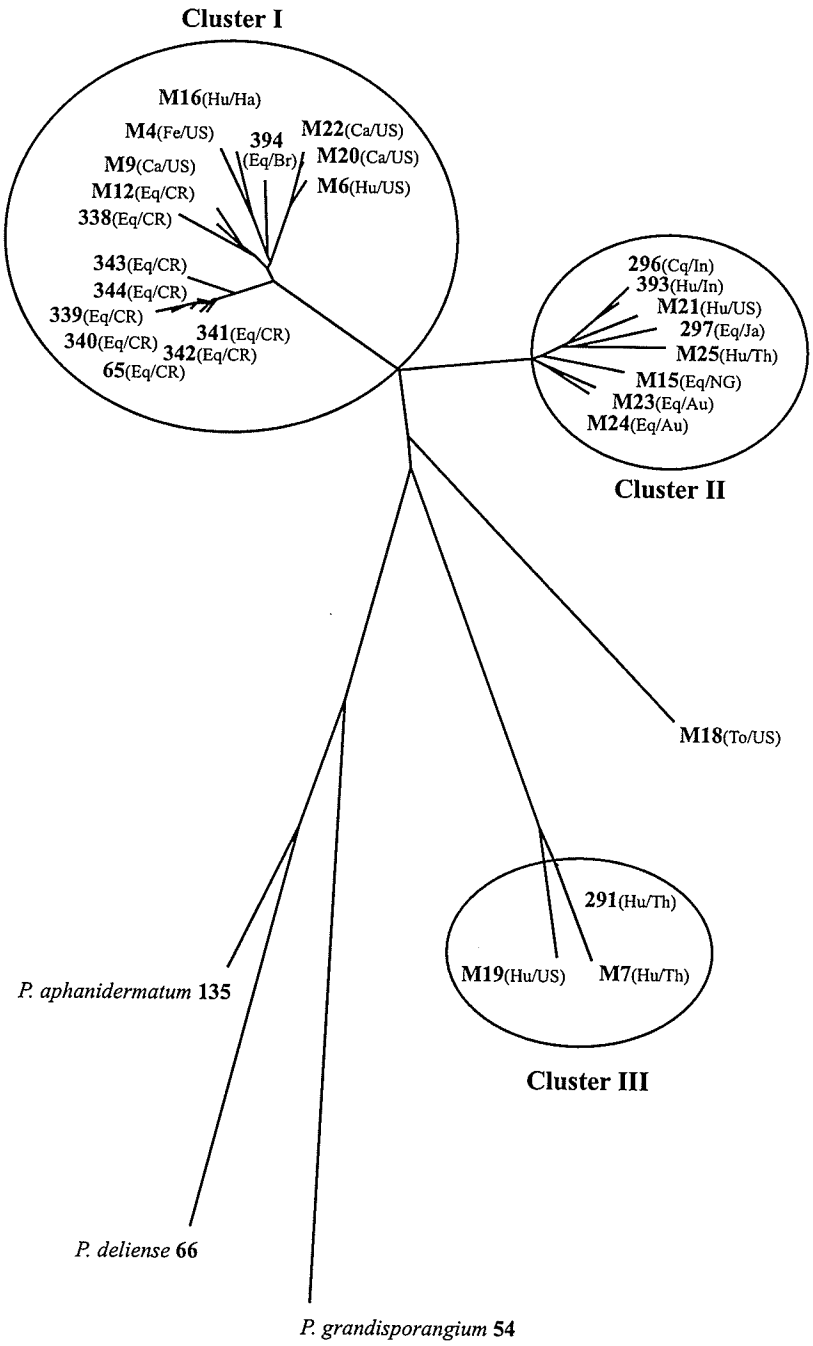
Cluster II contained eight isolates which formed two smaller groupings. *P. insidiosum* isolates 296 and 393, India isolates from a mosquito larva and human, respectively, were very similar and clustered with human isolate M21 from Pennsylvania and equine isolate 297 from Japan. The ex-type culture of *P. destruens* (M23) clustered with the other *P. destruens* isolate (M24) (both from equines in Australia), and also with *P. insidiosum* M15 from an equine in Papua New Guinea. An additional *P. insidiosum* isolate, M25 from a human in Thailand, was the most divergent member of this cluster in the UPGMA phenogram (Fig. 4.2A), but closer to the India and Japan isolates in the neighbor-joining phenogram (Fig. 4.2B).

Figure 4.2. Unrooted (**A**) UPGMA and (**B**) neighbor-joining phenograms showing relationships among isolates of *P. insidiosum*. Isolate numbers are in bold and correspond to those presented in Table 4.1. The host and country of origin are indicated in parentheses (Host/Country) with the following abbreviations: Host - Ca, canine; Cq, *Culex quinquefasciatus* larva; Eq, equine; Fe, feline; Hu, human; To, *Tremarctos ornatus* (Spectacled bear); Country - AU, Australia; Br, Brazil; CR, Costa Rica; Ha, Haiti; In, India; Ja, Japan; NG, New Guinea; Th, Thailand; US, United States.

A


Cluster I
338 (Eq/CR)
339 (Eq/CR)
M12(Eq/CR)
M9(Ca/US)
343 (Eq/CR)
394(Eq/Br)
341(Eq/CR)
342(Eq/CR)
344(Eq/CR)
65(Eq/CR)
340(Eq/CR)
M22(Ca/US)
M20(Ca/US)
M6(Hu/US)
M4(Fe/US)
M16(Hu/Ha)
296(Cq/In)
393(Hu/In)
M21(Hu/US)
297(Eq/Ja)
M15(Eq/NG)
M25(Hu/Th)
M24 (Eq/Au)
M23 (Eq/Au)
Cluster II
P. grandisporangium 54
P. aphanidermatum 135
P. deliense 66
Cluster III
M19(Hu/US)
M7(Hu/Th)
291(Hu/Th)
M18(To/US)
.10

B


Cluster I
M16(Hu/Ha)
M4(Fe/US)
394
(Eq/Br)
M22(Ca/US)
M20(Ca/US)
M9(Ca/US)
M6(Hu/US)
M12(Eq/CR)
338(Eq/CR)
343(Eq/CR)
344(Eq/CR)
339(Eq/CR)
341(Eq/CR)
340(Eq/CR)
342(Eq/CR)
65(Eq/CR)
296(Cq/In)
393(Hu/In)
M21(Hu/US)
297(Eq/Ja)
M25(Hu/Th)
M15(Eq/NG)
M23(Eq/Au)
M24(Eq/Au)
Cluster II
M18(To/US)
291(Hu/Th)
M19(Hu/US)
M7(Hu/Th)
Cluster III
P. aphanidermatum 135
P. deliense 66
P. grandisporangium 54

.10

Cluster III contained three human *P. insidiosum* isolates (291, M7, and M19). Two isolates, 291 and M7, were from patients in Thailand, while isolate M19, from a patient in San Antonio, Texas, was located on its own branch. An additional *P. insidiosum* isolate, M18, was isolated from a bone lesion on the paw of a spectacled bear (*Tremarctos ornatus*) in a South Carolina zoo. The isolate failed to group within clusters I, II, or III.

The average genetic distances between isolates within each cluster were significantly lower than average distances between clusters (Table 4.2). Clusters I and II were more closely related to each other than either one was to cluster III or isolate M18. The single divergent isolate, M18, was more closely related to clusters I and II than were the outgroup species, but cluster III was not significantly closer to any of the clusters than were the outgroups.

ITS Sequence analysis

Twenty-three isolates of *P. insidiosum* and *P. destruens,* and a single isolate of *Lagenidium giganteum,* were used for sequence analysis of the ITS and sequences were deposited in GenBank (Table 4.1). PCR amplification of the rDNA ITS, including 580 bp of the 5' end of the LSU rRNA gene, with primers UN-UP18S42 and UN-LO28S576B (Fig. 2.1) yielded a single product of approximately 1600 bp for all isolates. The ITS region between the SSU and LSU rRNA genes was sequenced in both directions using the primers indicated in Fig. 2.1. For a select number of isolates, the 580 bp of the LSU rRNA gene was also sequenced, but this region was subsequently omitted from the

Table 4.2. Pairwise comparison of genetic distance values [calculated according to Nei and Li (1979)] among isolates of *P. insidiosum* and related species based on RFLP analysis of the IGS. Clusters I, II, and III refer to genetic clusters of isolates in Fig. 4.2.

	***Pythium* Isolate(s)**						
	Cluster I[a]	**Cluster II[b]**	**Cluster III[c]**	**M18**	**54**	**66**	**135**
Cluster I	0.0000						
Cluster II	0.0553	0.0000					
Cluster III	0.0921	0.1035	0.0000				
***P. insidiosum* M18**	0.0793	0.0737	0.0974	0.0000			
***P. grandisporangium* 54**	0.1349	0.1107	0.1356	0.1315	0.0000		
***P. deliense* 66**	0.1144	0.1310	0.1479	0.1504	0.1059	0.0000	
***P. aphanidermatum* 135**	0.0976	0.0836	0.1171	0.1016	0.1172	0.0638	0.0000

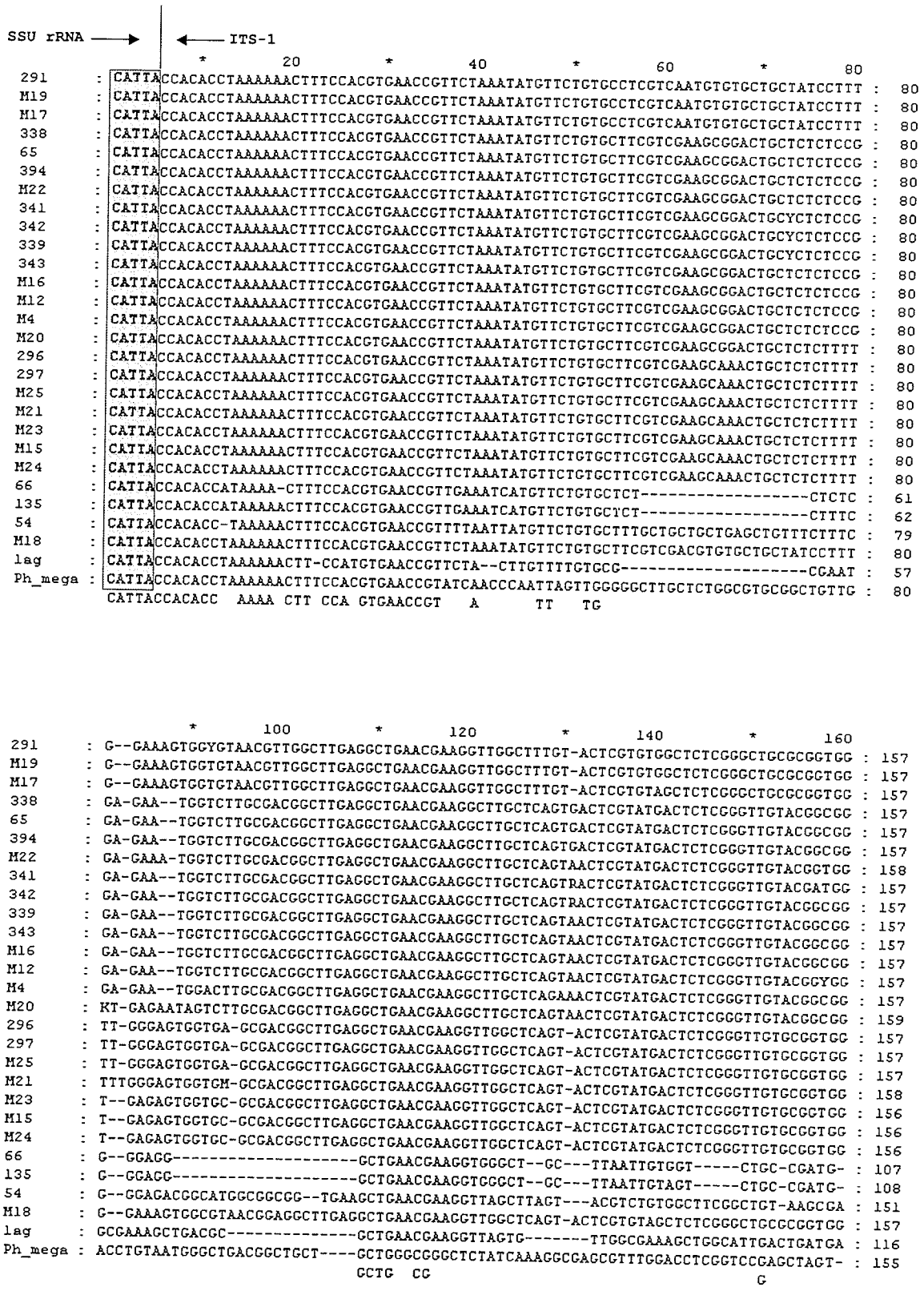
[a]Average distance within Cluster I: 0.0196
[b]Average distance within Cluster II: 0.0200
[c]Average distance within Cluster III: 0.0215

overall analysis due to the low level of sequence divergence among isolates. The alignment of the ITS sequences for isolates of *P. insidiosum,* which also included isolates of *P. aphanidermatum, P. deliense, P. grandisporangium,* and *L. giganteum,* is presented in Fig. 4.3. The sequence for *Phytophthora megasperma* (818 bp) was obtained from GenBank (L41381) and included in the alignment.

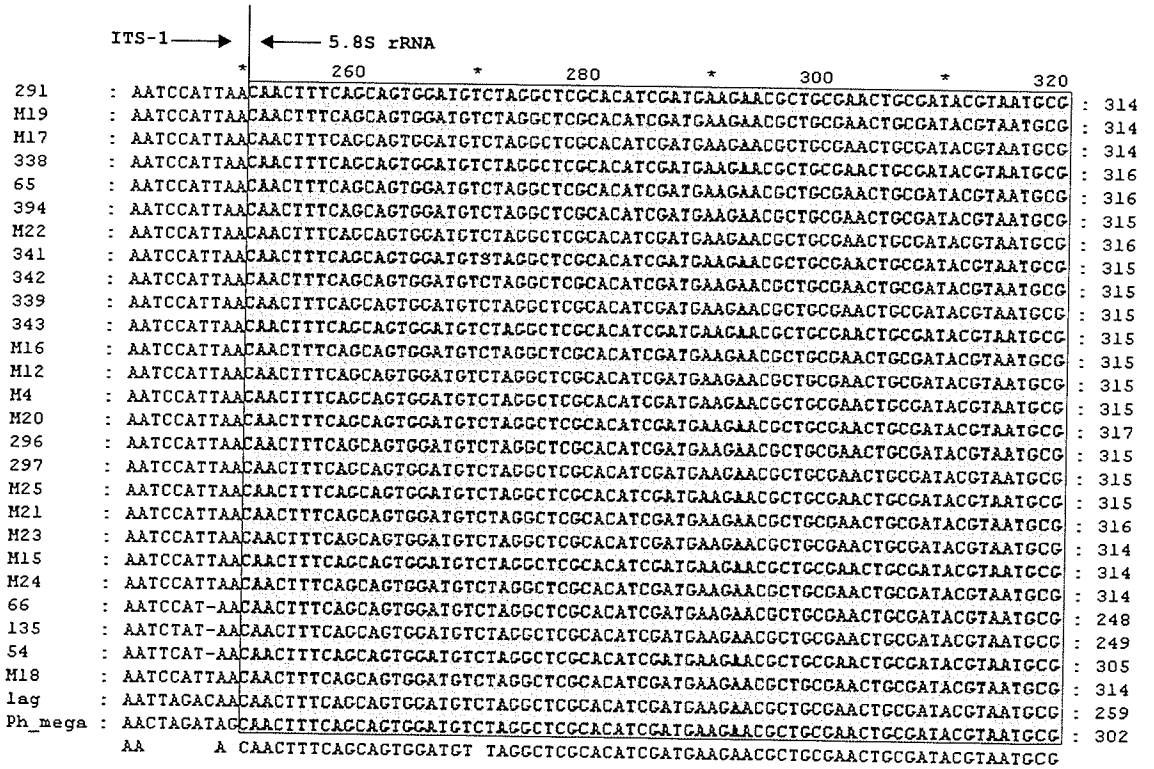
The length of the sequence containing ITS-1, the 5.8S rRNA gene, and ITS-2 showed little size variation among *P. insidiosum* isolates and *P. grandisporangium* 54, ranging in size from 833 to 842 bp. The same region was slightly shorter for *P. deliense, P. aphanidermatum,* and *L. giganteum,* with sizes of 782, 785, and 791 bp, respectively. Sequences were identical for the following three pairs of isolates: *P. insidiosum* M15 and *P. destruens* M24, *P. insidiosum* M12 and M16, and *P. insidiosum* 296 and M25. Most of the variation among aligned ITS sequences of *P. insidiosum* was due to transitions, but there were also some transversions and short (1 to 3 bp) insertion and deletion events. Larger insertions and deletions mainly occurred when isolates of other *Pythium* species, *Ph. megasperma,* or *L. giganteum,* were added to the alignment.

The neighbor-joining and parsimony analyses generated phylogenetic trees with nearly identical topologies (Fig. 4.4). *Ph. megasperma* was used to root the trees in each analysis, as it was the most distant species relative to the other isolates based on pairwise distance values (Table 4.3). The *Pythium* species formed a monophyletic clade distinct from isolates of *L. giganteum* and *Ph. megasperma.* The isolates of *P. insidiosum* and *P. destruens* also formed a clade that was well separated from the other *Pythium* species used in the analysis. This clade was further divided into three smaller clades of isolates

Figure 4.3. Sequence alignment obtained using CLUSTAL X of rDNA ITS sequences. Shaded regions and arrows indicate coding regions of the SSU, 5.8S, and LSU rRNA genes. Locations of ITS-1 and ITS-2 are indicated with arrows as well. Isolate numbers correspond to those in Table 4.1. Conserved base positions are given below the alignment. Abbreviations for *L. giganteum* 36492 and *Ph. megasperma* (GenBank L41381) are "lag" and "Ph_mega", respectively.


SSU rRNA
ITS-1
291 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCCTCGTCAATGTGTGCTGCTATCCTTT : 80
M19 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCCTCGTCAATGTGTGCTGCTATCCTTT : 80
M17 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCCTCGTCAATGTGTGCTGCTATCCTTT : 80
338 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCCTCGTCAATGTGTGCTGCTATCCTTT : 80
65 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
394 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
M22 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
341 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCYCTCTCCG : 80
342 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCYCTCTCCG : 80
339 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCYCTCTCCG : 80
343 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCYCTCTCCG : 80
M16 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
M12 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
M4 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
M20 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTCCG : 80
296 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCGGACTGCTCTCTTTT : 80
297 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
M25 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
M21 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
M23 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
M15 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
M24 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGAAGCAAACTGCTCTCTTTT : 80
66 : CATTACCACACCATAAAAA-CTTTCCACGTGAACCGTTGAAATCATGTTCTGTGCTCT----------CTCTC : 61
135 : CATTACCACACCATAAAAACTTTCCACGTGAACCGTTGAAATCATGTTCTGTGCTCT----------CTTTC : 62
54 : CATTACCACACC-TAAAAACTTTCCACGTGAACCGTTTTAATTATGTTCTGTGCTTTGCTGCTGCTGAGCTGTTTCTTTC : 79
M18 : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTTCTAAATATGTTCTGTGCTTCGTCGACGTGTGCTGCTATCCTTT : 80
lag : CATTACCACACCTAAAAAACTT-CCATGTGAACCGTTCTA--CTTGTTTTGTGCG----------CGAAT : 57
Ph_mega : CATTACCACACCTAAAAAACTTTCCACGTGAACCGTATCAACCCAATTAGTTGGGGGCTTGCTCTGGCGTGCGGCTGTTG : 80
CATTACCACACC AAAA CTT CCA GTGAACCGT A TT TG
291 : G--GAAAGTGGYGTAACGTTGGCTTGAGGCTGAACGAAGGTTGGCTTTGT-ACTCGTGTGGCTCTCGGGCTGCGCGGTGG : 157
M19 : G--GAAAGTGGTGTAACGTTGGCTTGAGGCTGAACGAAGGTTGGCTTTGT-ACTCGTGTGGCTCTCGGGCTGCGCGGTGG : 157
M17 : G--GAAAGTGGTGTAACGTTGGCTTGAGGCTGAACGAAGGTTGGCTTTGT-ACTCGTGTGGCTCTCGGGCTGCGCGGTGG : 157
338 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTGACTCGTATGACTCTCGGGTTGTACGGCGG : 157
65 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTGACTCGTATGACTCTCGGGTTGTACGGCGG : 157
394 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTGACTCGTATGACTCTCGGGTTGTACGGCGG : 157
M22 : GA-GAAA-TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGTGG : 158
341 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTRACTCGTATGACTCTCGGGTTGTACGATGG : 157
342 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
339 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
343 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
M16 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
M12 : GA-GAA--TGGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGYGG : 157
M4 : GA-GAA--TGGACTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGAAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
M20 : KT-GAGAATAGTCTTGCGACGGCTTGAGGCTGAACGAAGGCTTGCTCAGTAACTCGTATGACTCTCGGGTTGTACGGCGG : 157
296 : TT-GGGAGTGGTGA-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTACGGCGG : 159
297 : TT-GGGAGTGGTGA-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTCGCGGTGG : 157
M25 : TT-GGGAGTGGTGA-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTCGCGGTGG : 157
M21 : TTTGGGAGTGGTGM-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTCGCGGTGG : 157
M23 : T--GAGAGTGGTGC-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTCGCGGTGG : 158
M15 : T--GAGAGTGGTGC-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTGCGGTGC : 156
M24 : T--GAGAGTGGTGC-GCGACGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTATGACTCTCGGGTTGTGCGGTGG : 156
66 : G--GGAGG----------GCTGAACGAAGGTGGGCT--GC---TTAATTGTGGT-----CTGC-CGATG- : 107
135 : G--GGAGG----------GCTGAACGAAGGTGGGCT--GC---TTAATTGTAGT-----CTGC-CGATG- : 108
54 : G--GGAGACGGCATGGCGGCGG--TGAAGCTGAACGAAGGTTAGCTTAGT---ACGTCTGTGGCTTCGGCTGT-AAGCGA : 151
M18 : G--GAAAGTGGCGTAACGGAGGCTTGAGGCTGAACGAAGGTTGGCTCAGT-ACTCGTGTAGCTCTCGGGCTGCGCGGTGG : 157
lag : GCGAAAGCTGACGC----------GCTGAACGAAGGTTAGTG-------TTGGCGAAAGCTGGCATTGACTGATGA : 116
Ph_mega : ACCTGTAATGGGCTGACGGCTGCT----GCTGGGCGGGCTCTATCAAAGGCGAGCGTTTGGACCTCGGTCCGAGCTAGT- : 155
GCTG CG
G


* 180 * 200 * 220 * 240
291 : AACAGCTTACCGATGTCTTTTTCAAACCCATTA---ACTAAATACTGATCCTTACTCCGAGGACGAAAGTCTTTGGTTTT : 234
M19 : AACAGCTTACCGATGTCTTTTTCAAACCCATTA---ACTAAATACTGATCCTTACTCCGAGGACGAAAGTCTTTGGTTTT : 234
M17 : AACAGCTTACCGATGTCTTTTTCAAACCCATTA---ACTAAATACTGATCCTTACTCCGAGGACGAAAGTCTTTGGTTTT : 234
338 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTTT-ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 236
65 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTTT-ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 236
394 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M22 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 236
341 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
342 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
339 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
343 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M16 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M12 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M4 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M20 : AACTGCTGGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
296 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 237
297 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M25 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 235
M21 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 236
M23 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 234
M15 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 234
M24 : AACAGCTTGCCGATGTCTTTTTCAAACCCATTTT--ACTAAACACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 234
66 : -------------TAT--TTTTCAAACCCATTT---ACCTAATACTGATCTATACTCCAAAAACGAAAGTTTCTGGTTTT : 169
135 : -------------TAT--TTTTCAAACCCATTT---ACCTAATACTGATCTATACTCCAAAAACGAAAGTTTATGGTTTT : 170
54 : AAAAGCTTTCCGATGT--TTTTCAAACCCATTT---ACTAAATACTGATCTATACTCCAAAGACGAAAGTTTTTGGTTTT : 226
M18 : AACGGCTTACCGATGTCTTTTTCAAACCCATTT---ACTAAATACTGATCTATACTCCGAGGACGAAAGTCTTTGGTTTT : 234
lag : A-----------------CTTTTAAACCCCTTCATTCCTAATTACTGATAAATACTCTAGGAACGAAAGTTCTTAGTTTT : 179
Ph_mega : A---GCT------TTACTTTTTTAAACCCATTCT---TTACATACTGA-ATATACTGTGGGGACGAAAGTCTCTGCTTTT : 222
TTT AAACCC TT ACTGA TACT ACGAAAGT T TTTT


ITS-1
5.8S rRNA
* 260 * 280 * 300 * 320
291 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
M19 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
M17 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
338 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 316
65 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 316
394 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M22 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 316
341 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
342 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
339 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
343 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M16 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M12 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M4 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M20 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 317
296 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
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M25 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 315
M21 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 316
M23 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
M15 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
M24 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
66 : AATCCAT-AACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 248
135 : AATCTAT-AACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 249
54 : AATTCAT-AACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 305
M18 : AATCCATTAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 314
lag : AATTAGACAACAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 259
Ph_mega : AACTAGATAGCAACTTTCAGCAGTGGATGTCTAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG : 302
AA A CAACTTTCAGCAGTGGATGT TAGGCTCGCACATCGATGAAGAACGCTGCGAACTGCGATACGTAATGCG


* 340 * 360 * 380 * 400
291 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
M19 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
M17 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
338 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 396
65 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 396
394 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M22 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 396
341 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
342 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
339 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
343 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M16 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M12 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M4 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M20 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
296 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 397
297 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M25 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M21 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 395
M23 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 396
M15 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
M24 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
66 : AATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 328
135 : AATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCACATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 329
54 : AATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 385
M18 : AATTGCAGGATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCGGGTTATGCCTGGAAGTATGCCTGTATCA : 394
lag : AATTGCAGAATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTTCCGGGTTATGCCTGGGAGTATGCCTGTATCA : 339
Ph_mega : AATTGCAGCATTCAGTGAGTCATCGAAATTTTGAACGCATATTGCACTTCCGGGTTAGTCCTGGGAGTATGCCTGTATCA : 382
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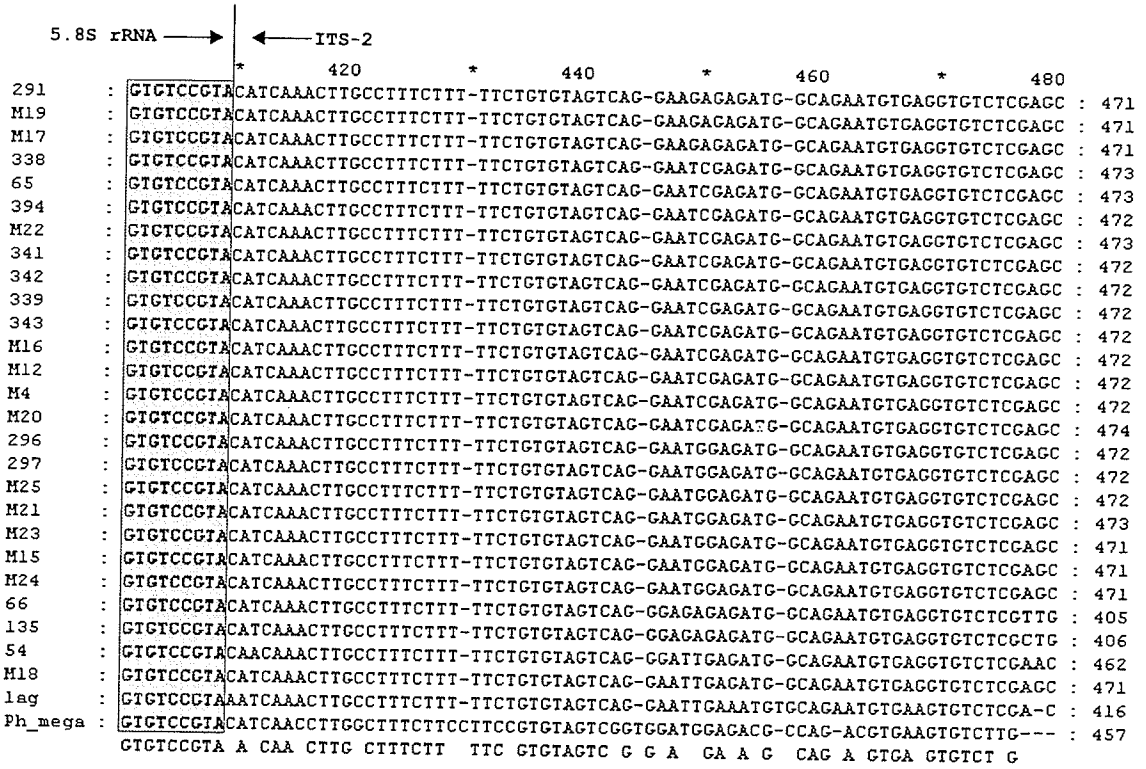
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291 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAAGAGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
M19 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAAGAGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
M17 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAAGAGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
338 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 473
65 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 473
394 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M22 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 473
341 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
342 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
339 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
343 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M16 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M12 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M4 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M20 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATCGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 474
296 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
297 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M25 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 472
M21 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 473
M23 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
M15 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
M24 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATGGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
66 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GGAGAGAGATG-GCAGAATGTGAGGTGTCTCGTTG : 405
135 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GGAGAGAGATG-GCAGAATGTGAGGTGTCTCGCTG : 406
54 : GTGTCCGTACAACAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GGATTGAGATG-GCAGAATGTGAGGTGTCTCGAAC : 462
M18 : GTGTCCGTACATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATTGAGATG-GCAGAATGTGAGGTGTCTCGAGC : 471
lag : GTGTCCGTAAATCAAACTTGCCTTTCTTT-TTCTGTGTAGTCAG-GAATTGAAATGTGCAGAATGTGAAGTGTCTCGA-C : 416
Ph_mega : GTGTCCGTACATCAACCTTGGCTTTCTTCCTTCCGTGTAGTCGGTGGATGGAGACG-CCAG-ACGTGAAGTGTCTTG--- : 457
GTGTCCGTA A CAA CTTG CTTTCTT TTC GTGTAGTC G G A GA A G CAG A GTGA GTGTCT G

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                  *       500         *       520         *       540         *       560
291       : CGTCCCCATCT---GGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGTAGAAGTGTAA : 545
M19       : CGTCCCCATCT---GGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGTAGAAGTGTAA : 545
M17       : CGTCCCCATCT---GGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGTAGAAGTGTAA : 545
338       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 549
65        : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 549
394       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M22       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 549
341       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
342       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
339       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
343       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M16       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M12       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M4        : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M20       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 550
296       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
297       : CGTCCCCTCTT--TGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 546
M25       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M21       : CGTCCCCTCTTT-TGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 548
M23       : CGTCCCCTCTTTTGGGAAAATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 547
M15       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 547
M24       : CGTCCCCTCTTTTTGGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGT-GAAGTGTAA : 547
66        : ACTCCCTTTTCG-GAGGAGAAGACGCGAGTCCCTTTAAATGTAC--GTTCGCTCTTTCTTGTGTCTAAGATGAAGTGTGA : 482
135       : GCTCCCTTTTCG-GAGGAGAAGACGCGAGTCCCTTTAAATGTAC--GTTCGCTCTTTCTTGTGTCTAAGATGAAGTGTGA : 483
54        : TGTCCAATTTTA-ATTGCGATGGGGCGAGTCCCTTTAAATGTAC--GTTCGCTCTCTCTTGTGTTTAAGTAGAAGTGTGA : 539
M18       : CGTCCCCTTTTT--GGGAGATAGCACGAGTCCCTTTAAATGTAC--GTT-GATCTCTCTTGTGTCTTAGTAGAAGTGTAA : 546
lag       : TTTCTATTCATTTGAATAGA-GATTTGAGTCCTTTTAAATCGAC--ACTGTCTCTCTATATAGTTACTGT-GATTTGCTT : 492
Ph_mega   : CTGCTGGTCTTTCGAGTCCG-GCGGTGAGTCCTTTGAAATGTACTGAACTGTACTTCTCTTTGCTCAAAAAGCGTGGCGT : 536
               C                        GAGTCC TT AAAT  AC          CT        G         G    G
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                  *       580         *       600         *       620         *       640
291       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAGAGGACATTAAGGAGG-CGACCTCT : 623
M19       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAGAGGACATTAAGGAGC-CGACCTCT : 623
M17       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAGAGGACATTAAGGAGG-CGACCTCT : 623
338       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 627
65        : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 627
394       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
M22       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 627
341       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
342       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
339       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
343       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGCAGA-TGACCTCT : 626
M16       : TGCTCGAA-CGCAGTGATCTGTTCACATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGCAGA-TGACCTCT : 626
M12       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
M4        : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
M20       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 628
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297       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 624
M25       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
M21       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 626
M23       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 625
M15       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 625
M24       : TGCTCGAA-CGCAGTGATCTGTTCAGATTGCTTTGCGCTGGTGGGCGACTTCGGAAAGGACATTAAGGAGA-TGACCTCT : 625
66        : TTCTCGAATCGCAGTGATCTGTTTGGATCGCTTTGCGCATTYGGGCGACTTCGGTTAGGACATTAAAGGAAGCAACCTCT : 562
135       : TTCTCGAATCGCGGTGATCTGTTTGGATCGCTTTGCGCATTTGGGCGACTTCGGTTAGGACATTAAAGGAAGCAACCTCT : 563
54        : TGCTCGAA-CGCGGTAATCTATTTGGATTGCTTTGCGCTGGTGGGCGACTTCGGAAGAAACATTAAGGGGA-CAACCTCT : 617
M18       : TGCTCGAA-CGCAGTGATCTGTTCAGATCGCTTTGCGCTGGTGGGCGACTTCGGAGAGGACATTAAGGAGGCGAACCTCT : 625
lag       : TGCATGAA-CGCGGTGATCTGCT--GATCG-TTTGCGCATGTTGGCGACTTCGGTAAGAACATATAGGGGA--AACCTCA : 566
Ph_mega   : TGCTGGTT--GTGG-AGCCTGCCTGCGTGGCCAGTCGGCGACCGGTTTGTCTCTTGTGGCGTTTAATGGAGGAGTGTTCG : 613
             T C  G    G  G    CT       T G     CG      GG    T            T  A           TC
```

```
                 *       660         *       680         *       700         *       720
291      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGACG-GGGTGTTGTTTTCCGTTCTTTCCTTGA--GGTGTAC : 699
M19      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGACGAGGGTGTTGTTTTCCGTTCTTTCCTTGA--GGTGTAC : 700
M17      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGACG-GGGTGTTGTTTTCCGTTCTTTCCTTGA--GGTGTAC : 699
338      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 702
65       : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 702
394      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
M22      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 702
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343      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGACAGGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 702
M16      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
M12      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
M4       : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
M20      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
296      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 703
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M25      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 699
M21      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 701
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M15      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 700
M24      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTT-CCGTTCTTTCCTTGA--GGTGTAC : 700
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135      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-AGAGTGTGGTTTTCTGTTCTTTCCTTGA--GGTGTAC : 639
54       : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGAGTGGTGTTT-CCGTTTCTTCCTTGA--GGTGTAC : 692
M18      : ATTGGCGGTATGTTA-GGCTTCGGCCCGACGTTGCAGCTGAC-GGGGTGTTGTTTTTCCGTTCTTTCCTTGA--GGTGTAC : 701
lag      : ATTTGCGGTATGTTGTGGCTTCGGCTCGACAATCTTGCTTAT-TGTGTGTGGCCT-CTGTT-TTTATTCGA--GGTGTAC : 641
Ph_mega  : ATTCGCGGTATGGTT-GGCTTCGGCTGAACAATGCGCTTATTGAATGTTTTTCCTGCTGTGGCCGGTACGAACTGGTGAAC : 692
           ATT GCGGTATG T  GGCTTCGGC   AC  T     T       GT      T C GT          A  GGTG AC
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```
                 *       740         *       760         *       780         *       800
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M19      : CTGTCGTGTGTGAGGT--CGARCTGGGCGC-TGGTTATTGTGTAGTAGAGTATTGCTGCTCTTTG-ACGCC-----TTCG : 771
M17      : CTGTCGTGTGTGAGGT--CGAACTGGGCGC-TGGTTATTGTGTAGTAGAGTATTGCTGCTCTTTG-ACGCC-----TTCG : 770
338      : CTGTCGTGTGTGAGGT--C-AACTGGACGC-TGGT-ATTGTGTAGTA-AGTATTGCTGCGCTTTG-ACGCC-----TTCG : 770
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341      : CTGTCGTGTGTGAGGT--CGAACTGGACGC-TGGTTATTGTGTAGTAGAGTATTGCTGCGCTTTG-ACGCC-----TTCG : 772
342      : CTGTCGTGTGTGAGGT--CGAACTGGACGC-TGGTTATTGTGTAGTAGAGTATTGCTGCGCTTTG-ACGCC-----TTCG : 772
339      : CTGTCGTGTGTGAGGT--CGAACTGGACGC-TGGTTATTGTGTAGTAGAGTATTGCTGCGCTTTG-ACGCC-----TTCG : 772
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M21      : CTGTCGTGTGTGAGGT--CGAACTGGACGC-TGGTTATTGTGTAGTAGAGTATTGCTGCGCTTTG-ACGCC-----TTCG : 772
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54       : CTA--TCGTGTGAGGCT-TGATCTG---ATGTGGGAGCTGTGTAGTAGAGTATTACTGCTCTTAG-ACGCC-TGTTTTCG : 764
M18      : CTGTCGTGTGTGAGGT--TGAACTGGGCGC-CGGTTATTGTGTAGTACAGTGTTGCTGCTCTTTG-ACGCC-----TTCG : 772
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Ph_mega  : CGTAGCTGTGTGTGGCTTGGCTTTTGAACCGGCTTTGCTGTTT-GCGAAGTAGACCGGC---------GGC-----TTCG : 757
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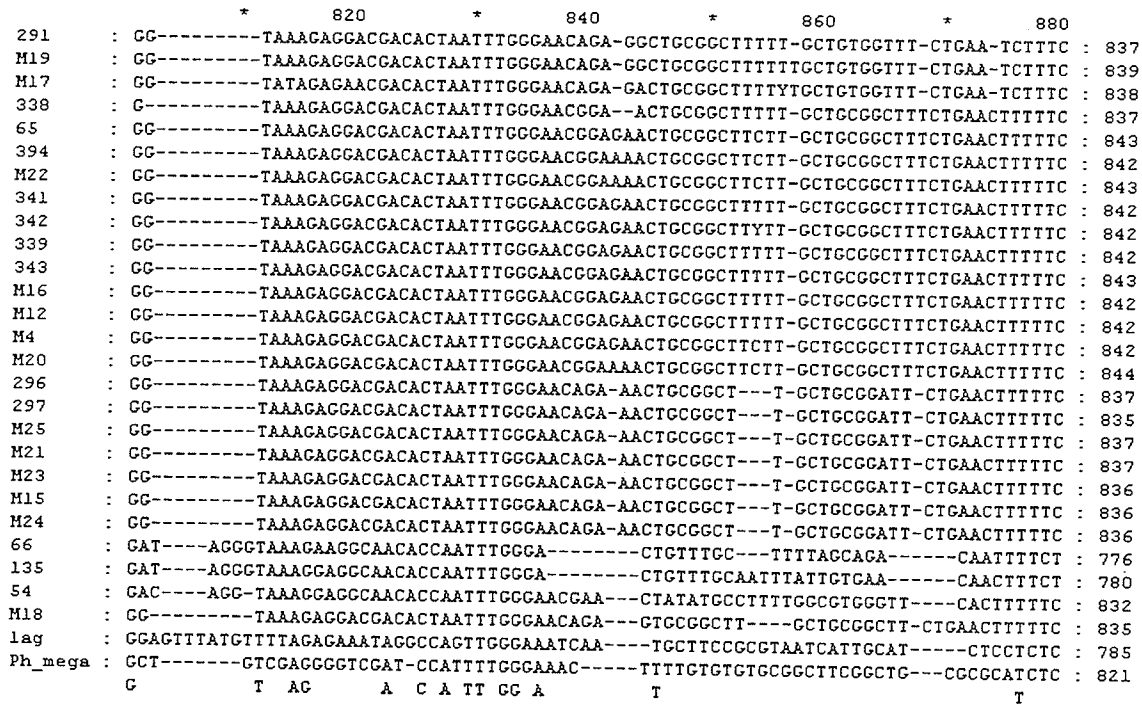
820
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860
880
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M19 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-GGCTGCGGCTTTTTTGCTGTGGTTT-CTGAA-TCTTTC : 839
M17 : GG----------TATAGAGAACGACACTAATTTGGGAACAGA-GACTGCGGCTTTTYTGCTGTGGTTT-CTGAA-TCTTTC : 838
338 : G-----------TAAAGAGGACGACACTAATTTGGGAACGGA--ACTGCGGCTTTTT-GCTGCGGCTTTCTGAACTTTTTC : 837
65 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTCTT-GCTGCGGCTTTCTGAACTTTTTC : 843
394 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAAAACTGCGGCTTCTT-GCTGCGGCTTTCTGAACTTTTTC : 842
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339 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTTTT-GCTGCGGCTTTCTGAACTTTTTC : 842
343 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTTTT-GCTGCGGCTTTCTGAACTTTTTC : 843
M16 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTTTT-GCTGCGGCTTTCTGAACTTTTTC : 842
M12 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTTTT-GCTGCGGCTTTCTGAACTTTTTC : 842
M4 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAGAACTGCGGCTTCTT-GCTGCGGCTTTCTGAACTTTTTC : 842
M20 : GG----------TAAAGAGGACGACACTAATTTGGGAACGGAAAACTGCGGCTTCTT-GCTGCGGCTTTCTGAACTTTTTC : 844
296 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 837
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M25 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 837
M21 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 837
M23 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 836
M15 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 836
M24 : GG----------TAAAGAGGACGACACTAATTTGGGAACAGA-AACTGCGGCT---T-GCTGCGGATT-CTGAACTTTTTC : 836
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135 : GAT----AGGGTAAACGAGGCAACACCAATTTGGGA--------CTGTTTGCAATTTATTGTGAA------CAACTTTCT : 780
54 : GAC----AGG-TAAACGAGGCAACACCAATTTGGGAACGAA---CTATATGCCTTTTGGCGTGGGTT----CACTTTTTC : 832
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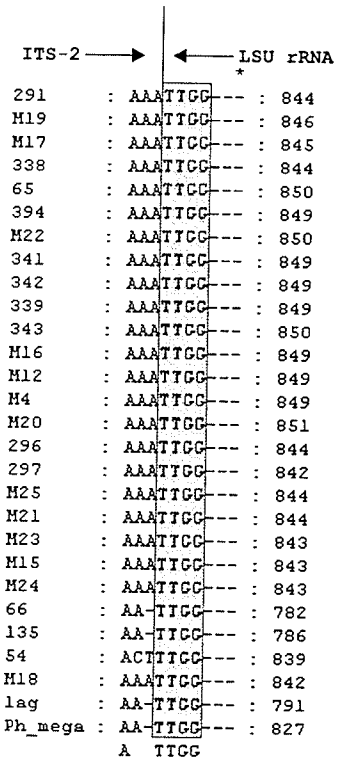
ITS-2
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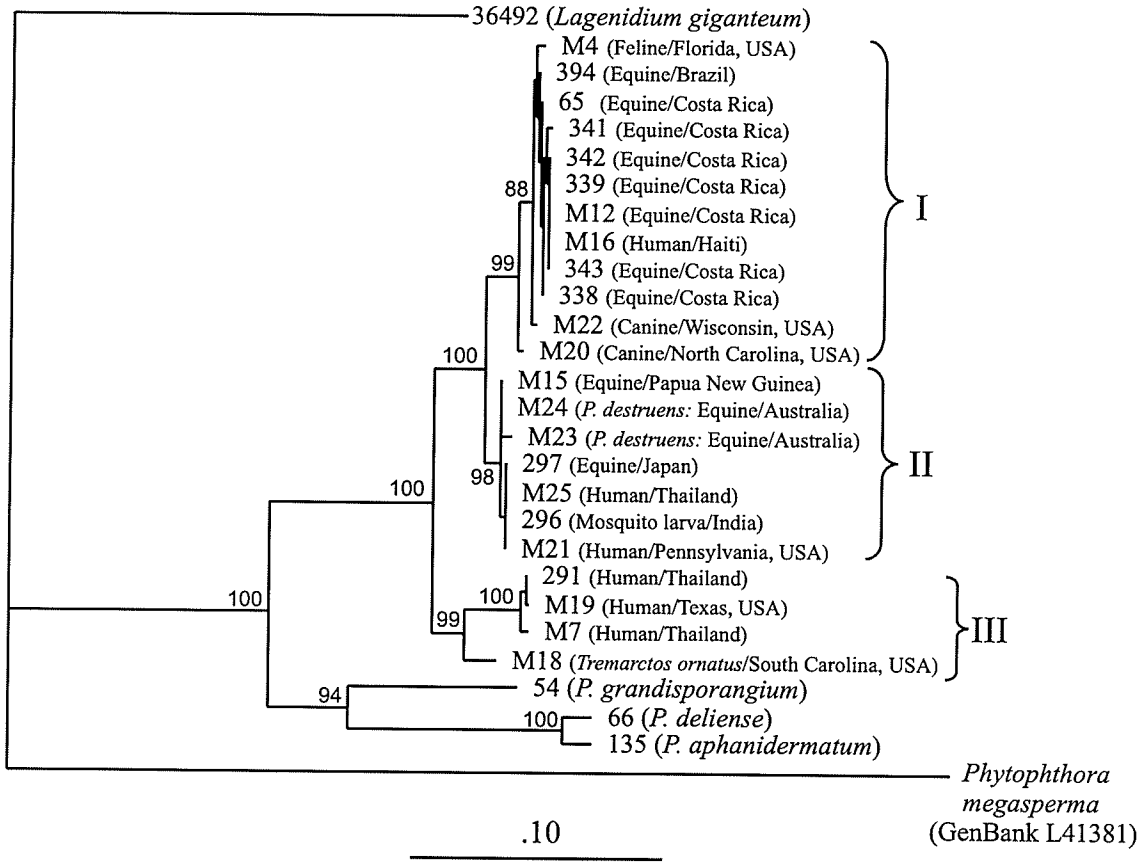
M17 : AAATTGG--- : 845
338 : AAATTGG--- : 844
65 : AAATTGG--- : 850
394 : AAATTGG--- : 849
M22 : AAATTGG--- : 850
341 : AAATTGG--- : 849
342 : AAATTGG--- : 849
339 : AAATTGG--- : 849
343 : AAATTGG--- : 850
M16 : AAATTGG--- : 849
M12 : AAATTGG--- : 849
M4 : AAATTGG--- : 849
M20 : AAATTGG--- : 851
296 : AAATTGG--- : 844
297 : AAATTGG--- : 842
M25 : AAATTGG--- : 844
M21 : AAATTGG--- : 844
M23 : AAATTGG--- : 843
M15 : AAATTGG--- : 843
M24 : AAATTGG--- : 843
66 : AA-TTGG--- : 782
135 : AA-TTGG--- : 786
54 : ACTTTGG--- : 839
M18 : AAATTGG--- : 842
lag : AA-TTGG--- : 791
Ph_mega : AA-TTGG--- : 827
A TTGG

Figure 4.4. Phylogenetic trees constructed using **(A)** neighbor-joining and **(B)** parsimony analysis of rDNA ITS sequences showing relationships among isolates of *P. insidiosum, P. destruens,* and outgroup species. Isolate numbers correspond to those in Table 4.1. The host and country of origin are in parentheses. Bootstrap values, expressed in percentages based on 1000 replicates, are present at their corresponding clades.

A


36492 (Lagenidium giganteum)
M4 (Feline/Florida, USA)
394 (Equine/Brazil)
65 (Equine/Costa Rica)
341 (Equine/Costa Rica)
342 (Equine/Costa Rica)
339 (Equine/Costa Rica)
M12 (Equine/Costa Rica)
M16 (Human/Haiti)
343 (Equine/Costa Rica)
338 (Equine/Costa Rica)
M22 (Canine/Wisconsin, USA)
M20 (Canine/North Carolina, USA)
I
M15 (Equine/Papua New Guinea)
M24 (P. destruens: Equine/Australia)
M23 (P. destruens: Equine/Australia)
297 (Equine/Japan)
M25 (Human/Thailand)
296 (Mosquito larva/India)
M21 (Human/Pennsylvania, USA)
II
291 (Human/Thailand)
M19 (Human/Texas, USA)
M7 (Human/Thailand)
M18 (Tremarctos ornatus/South Carolina, USA)
III
54 (P. grandisporangium)
66 (P. deliense)
135 (P. aphanidermatum)
88
99
100
98
100
100
100
99
94
100
Phytophthora megasperma (GenBank L41381)
.10

B

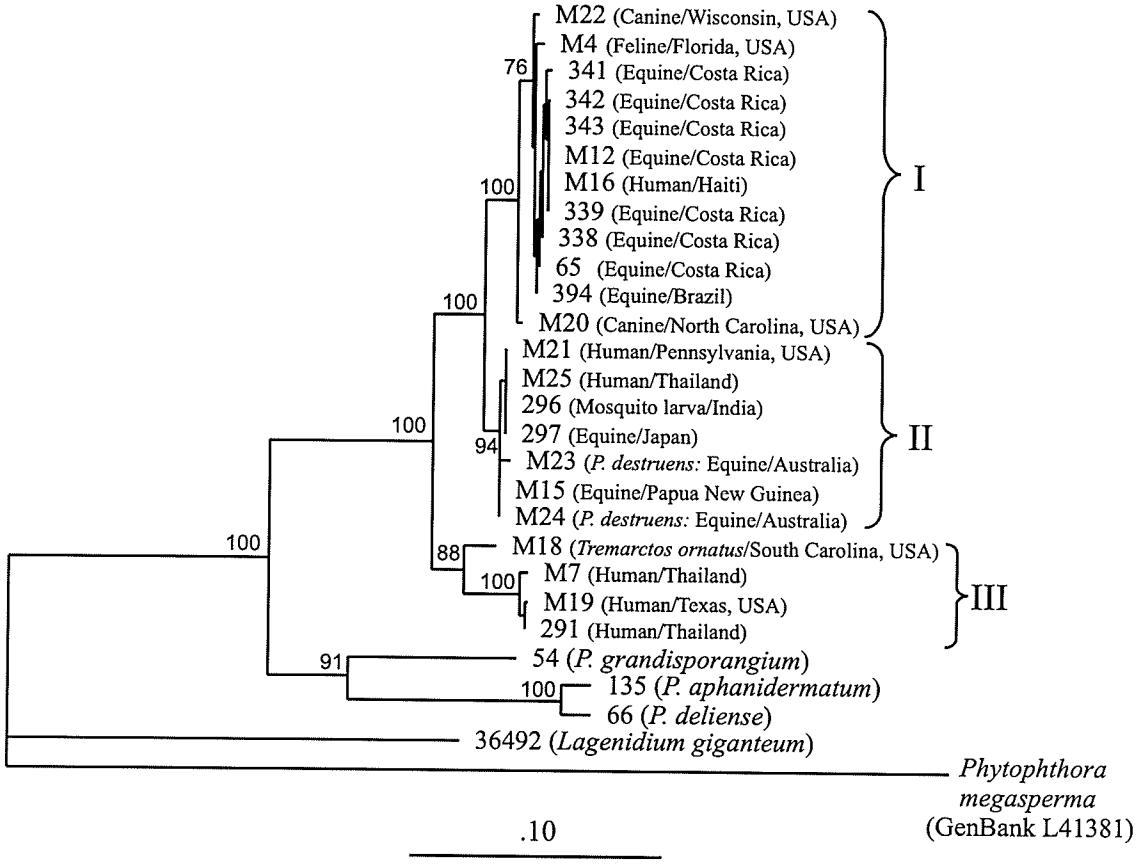

M22 (Canine/Wisconsin, USA)
M4 (Feline/Florida, USA)
341 (Equine/Costa Rica)
342 (Equine/Costa Rica)
343 (Equine/Costa Rica)
M12 (Equine/Costa Rica)
M16 (Human/Haiti)
339 (Equine/Costa Rica)
338 (Equine/Costa Rica)
65 (Equine/Costa Rica)
394 (Equine/Brazil)
M20 (Canine/North Carolina, USA)
I
M21 (Human/Pennsylvania, USA)
M25 (Human/Thailand)
296 (Mosquito larva/India)
297 (Equine/Japan)
M23 (P. destruens: Equine/Australia)
M15 (Equine/Papua New Guinea)
M24 (P. destruens: Equine/Australia)
II
M18 (Tremarctos ornatus/South Carolina, USA)
M7 (Human/Thailand)
M19 (Human/Texas, USA)
291 (Human/Thailand)
III
54 (P. grandisporangium)
135 (P. aphanidermatum)
66 (P. deliense)
36492 (Lagenidium giganteum)
Phytophthora megasperma (GenBank L41381)
76
100
100
100
94
100
88
100
91
100
.10

Table 4.3. Pairwise comparison of genetic distance values of *P. insidiosum* isolates and related species from ITS sequence analysis calculated using Kimura's two-parameter model. Clades I, II, and III refer to the clades resulting from neighbor-joining and parsimony analysis in Fig. 4.4.

	Isolate(s)							
	Clade I[a]	**Clade II[b]**	**Clade III[c]**	**54**	**66**	**135**	***L. giganteum***	***Ph. megasperma***
Clade I	0.0000							
Clade II	0.0283	0.000						
Clade III	0.0604	0.0513	0.0000					
***P. grandisporangium* 54**	0.1648	0.1641	0.1751	0.0000				
***P. deliense* 66**	0.1712	0.1675	0.1663	0.1409	0.0000			
***P. aphanidermatum* 135**	0.1816	0.1713	0.1692	0.1416	0.0221	0.0000		
L. giganteum	0.3158	0.3124	0.3103	0.3280	0.3233	0.3235	0.0000	
Ph. megasperma	0.5147	0.5126	0.5191	0.5163	0.5303	0.5227	0.5249	0.0000

[a]Average genetic distance within Clade I: 0.0036
[b]Average genetic distance within Clade II: 0.0022
[c]Average genetic distance within Clade III: 0.0190

that resembled the three clusters formed from RFLP analysis of the IGS (Fig. 4.2). The branching patterns of the clades were strongly supported by high bootstrap values. Clade I contained 12 isolates of *P. insidiosum,* including the ex-type culture (65), representing a variety of mammalian hosts from Costa Rica, Haiti, Brazil, and the USA. Clade II contained seven isolates from various regions of Australia, Southeast Asia, and the United States. The ex-type culture for *P. destruens* (M23) was present and very similar to *P. destruens* M24 and to *P. insidiosum* M15. Two human *P. insidiosum* isolates (M21 and M25), from Pennsylvania and Thailand, respectively, were also present, as well as isolates from a mosquito larva in India (296) and an equine from Japan (297). Clade III contained two human isolates from Thailand (291 and M7) as well as a third human isolate from Texas, USA (M19). A fourth isolate (M18), from a spectacled bear in South Carolina, was present on a separate branch in clade III.

The pairwise genetic distance measurements among isolates of *P. insidiosum* using the Kimura two-parameter model ranged from 0.0000 to 0.0678 (values not shown). Average genetic distances among isolates were low within clades I and II (0.0036 and 0.0022, respectively), but relatively higher within clade III (0.0190). Comparisons of distance values between the three clades showed that clades I and II were more closely related to each other than to clade III (Table 4.3). In comparison, genetic distance ranges were greater when comparing *P. insidiosum* isolates to other *Pythium* species (from 0.1641 to 0.1816 when compared to *P. deliense, P. aphanidermatum,* and *P. grandisporangium*), *L. giganteum* (0.3103 to 0.3158), and *Ph. megasperma* (0.5126 to 0.5191) (Table 4.3).

Comparison of growth rates of *P. insidiosum* isolates

The growth rates of nine isolates of *P. insidiosum* (representing genetic clusters I, II, and III) over a range of temperatures were determined by measuring radial growth on Sabouraud agar over a 48 h period. Isolate M4 (cluster I) had the highest growth rate at all eight temperatures while isolate M7 (cluster III) tended to grow the slowest, except at 22°C where isolate M9 had the slowest growth rate (Table 4.4). At 40°C, isolates M7 and M9 had the same rate of growth and neither grew at 43°C. In a comparison of growth curves of all nine isolates (Fig. 4.5), the curve peaks, representing the optimum growth temperatures, were all very similar, ranging from 35—38°C, and a dramatic decrease in growth rate was observed at temperatures 40°C and higher. An almost exponential increase in growth rate was observed from 22—30°C for most isolates, and a plateau was usually evident from 30—35°C where the growth rate remained relatively constant with a slight increase.

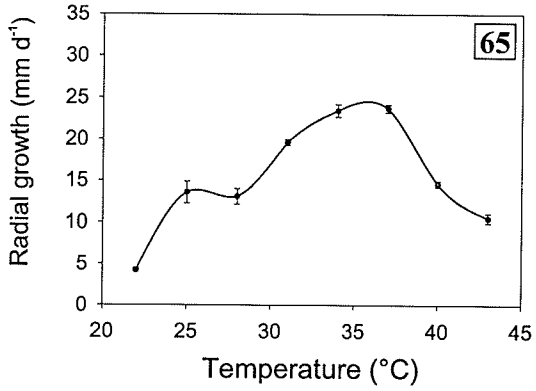
DISCUSSION

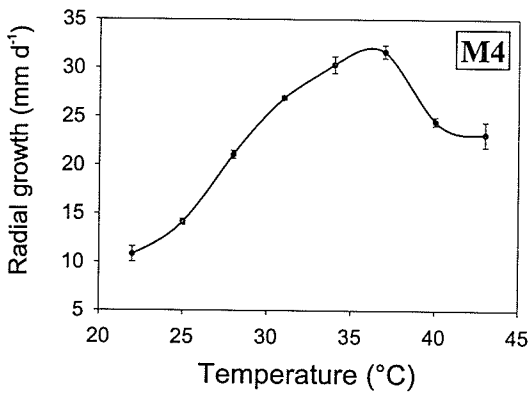
The identification of *P. insidiosum* based on morphology can be difficult due to its restricted morphological differentiation in culture. In the description of the species (de Cock *et al.,* 1987), no significant morphological differences were reported among isolates from infected animals and humans from various geographic locations. Fluorescent antibody and ID tests (Mendoza *et al.,* 1987) also revealed no antigenic differences among *P. insidiosum* isolates, regardless of their geographic origin or host. However, at the DNA level, there were significant differences between isolates in their IGS and ITS regions which tended to correspond to their geographic origins.

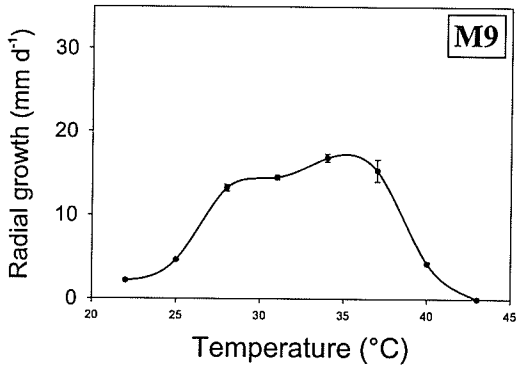
Table 4.4. Average radial growth rates (mm d^{-1}) of nine *P. insidiosum* isolates incubated over a range of temperatures on Sabouraud agar for 48 hours. The corresponding genetic clade for each isolate (with reference to Figs. 4.2 and 4.4) is indicated in parentheses.

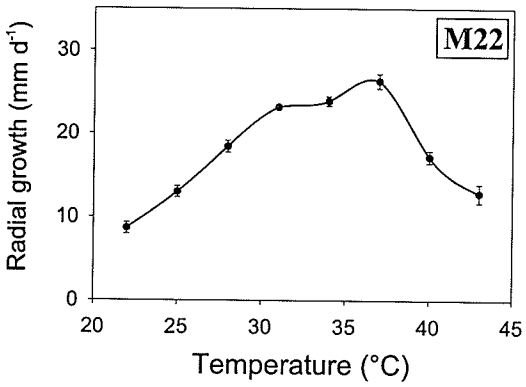
	Temperature							
Isolate	**22°C**	**25°C**	**28°C**	**31°C**	**34°C**	**37°C**	**40°C**	**43°C**
65 (I)	4.3 ± 0.3	13.6 ± 1.3	13.1 ± 0.9	19.6 ± 0.4	23.4 ± 0.8	23.7 ± 0.5	14.6 ± 0.4	10.5 ± 0.6
M4 (I)	10.8 ± 0.8	14.2 ± 0.3	21.1 ± 0.4	26.9 ± 0.2	30.3 ± 0.9	31.7 ± 0.7	24.5 ± 0.4	23.2 ± 1.3
M9 (I)	2.2 ± 0.1	4.7 ± 0.2	13.3 ± 0.4	14.5 ± 0.3	16.8 ± 0.5	15.3 ± 1.3	4.3 ± 0.2	0.0 ± 0.0
M22 (I)	8.7 ± 0.7	13.0 ± 0.7	18.4 ± 0.7	23.2 ± 0.3	23.9 ± 0.6	26.3 ± 0.9	17.2 ± 0.8	12.8 ± 1.1
M23 (II)	4.5 ± 0.7	7.7 ± 0.4	12.2 ± 0.3	13.3 ± 0.3	16.3 ± 0.3	16.3 ± 0.7	14.0 ± 0.6	9.2 ± 0.6
M24 (II)	6.6 ± 0.8	6.8 ± 0.5	13.7 ± 0.6	16.8 ± 0.6	18.5 ± 0.6	21.2 ± 1.1	13.2 ± 0.5	10.3 ± 1.1
M15 (II)	9.7 ± 1.0	14.0 ± 0.5	17.4 ± 0.3	20.5 ± 0.5	20.8 ± 0.4	24.8 ± 0.5	15.0 ± 0.7	15.0 ± 1.3
M21 (II)	3.8 ± 0.8	7.5 ± 0.4	14.8 ± 0.4	19.7 ±0.5	19.8 ± 0.5	24.7 ± 0.6	18.2 ± 1.2	15.2 ± 2.4
M7 (III)	2.8 ± 0.2	4.0 ± 0.4	8.0 ± 0.2	10.0 ± 0.4	11.0 ± 0.4	12.3 ± 0.4	4.3 ± 0.5	0.0 ± 0.0

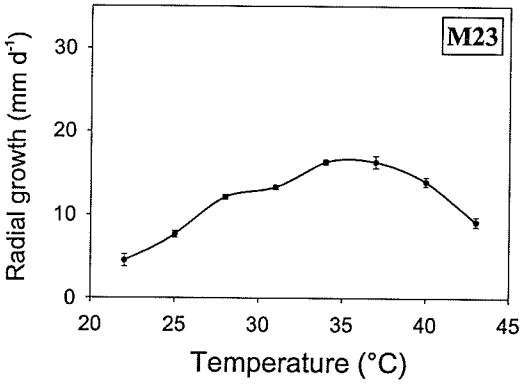
Figure 4.5. Comparison of the rate of growth (mm d^{-1}) of different *P. insidiosum* isolates at various temperatures on Sabouraud agar over a 48 h incubation period. Actual growth rate values are presented in Table 4.4. Isolate numbers are indicated in the top right corner of each plot and correspond to the following species and genetic clusters indicated in Figs. 4.2 and 4.4: 65, *P. insidiosum* (cluster I); M4, *P. insidiosum* (cluster I); M9, *P. insidiosum* (cluster I); M22, *P. insidiosum* (cluster I); M23, *P. destruens* (cluster II); M24, *P. destruens* (cluster II); M15, *P. insidiosum* (cluster II); M21, *P. insidiosum* (cluster II); M7, *P. insidiosum* (cluster III). Error bars indicate standard error.

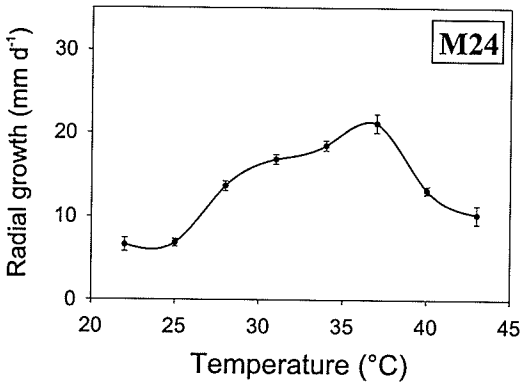

65
Radial growth (mm d-1)
Temperature (°C)

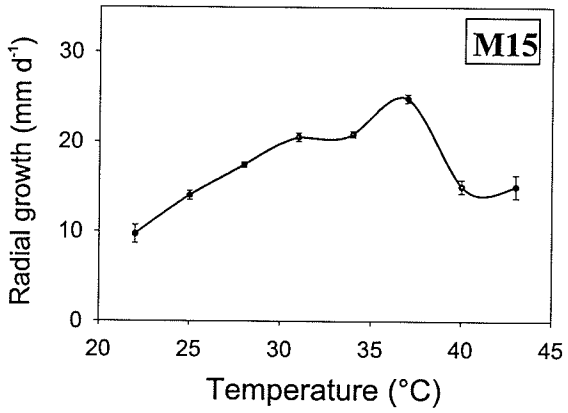

M4
Radial growth (mm d-1)
Temperature (°C)

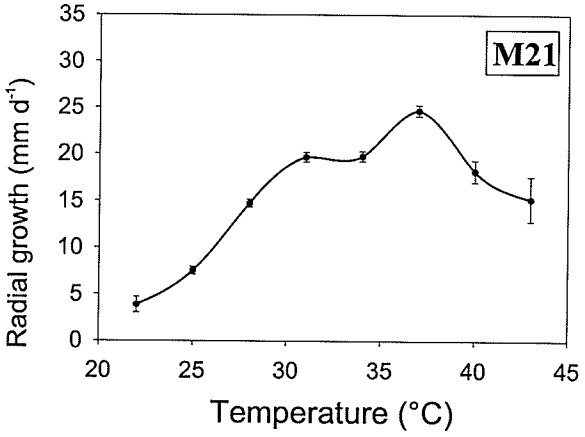

M9
Radial growth (mm d-1)
Temperature (°C)

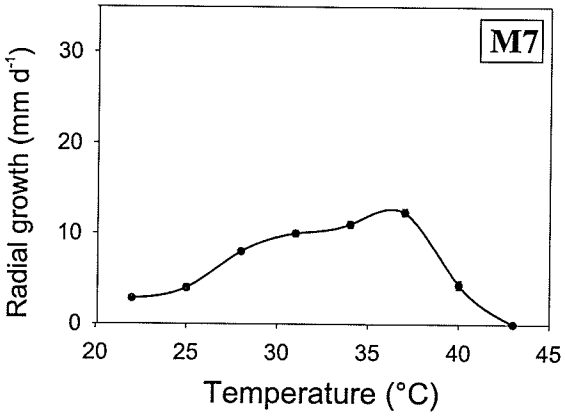

M22
Radial growth (mm d-1)
Temperature (°C)


M23
Radial growth (mm d-1)
Temperature (°C)


M24
Radial growth (mm d-1)
Temperature (°C)


M15
Radial growth (mm d-1)
0
10
20
30
20
25
30
35
40
45
Temperature (°C)


M21
Radial growth (mm d-1)
0
5
10
15
20
25
30
35
20
25
30
35
40
45
Temperature (°C)


M7
Radial growth (mm d-1)
0
10
20
30
20
25
30
35
40
45
Temperature (°C)

Examination of restriction fragment patterns of IGS amplicons revealed four general profiles among the 28 isolates of *P. insidiosum* and *P. destruens* used in this study (Fig. 4.1). RFLP analysis produced phenograms that revealed three clusters, each comprised of isolates from specific geographic locations, and a separate branch for isolate M18 (Fig. 4.2). The outgroup species were distantly related to the three main clusters on individual branches.

Cluster I consisted of isolates from North, Central, and South America, predominantly from tropical and subtropical regions. The smaller groupings of isolates generally corresponded to more proximate geographic origins. Seven isolates from Costa Rica formed a tight cluster. The remaining isolates in cluster I formed three additional groupings, one of which consisted of isolates from the USA, while the isolates in the other two groupings were more geographically diverse. No correspondence could be demonstrated between host-specificity and clustering patterns.

Cluster II consisted of isolates from Australia and Southeast Asia, as well as one isolate from the USA. Again, no clear correlation between host-specificity and branching patterns was observed. Two groupings within the cluster were evident. The first consisted of isolates from India, Japan, and the USA. Isolate 297, from an equine in Japan, was actually originally identified as *P. gracile* by Ichtani and Amemiya (1980), but was later classified as *P. insidiosum* by de Cock *et al.* (1987). *P. insidiosum* M21 was isolated from a human with keratitis in Pennsylvania. The patient was originally from Afghanistan, so the source of the infection was uncertain. The grouping of this isolate with those from India and Japan, and the fact that there are no other reported cases of human pythiosis from this region of the USA, suggested that the origin of infection may be the Middle

East and the man could have acquired the disease upon reception of food products from Afghanistan.

The second grouping within cluster II consisted of *P. destruens* and *P. insidiosum* isolates from Australia and Papua New Guinea, respectively. *P. destruens* was described by Shipton (1987) based on isolates from equines in Australia. Results from fluorescent antibody and immunodiffusion tests (Mendoza *et al.*, 1987; Mendoza and Marin, 1989) concluded that these two *Pythium* species are antigenically and morphologically similar and that *P. insidiosum* and *P. destruens* are conspecific. In this study, isolates of *P. destruens* were present in cluster II with *P. insidiosum* isolates from India, USA, and Japan, and were most closely related to *P. insidiosum* M15 from an equine in Papua New Guinea. The fact that isolates of *P. destruens* were present in this cluster supported evidence that *P. destruens* and *P. insidiosum* are conspecific. However, it could also mean that all isolates in cluster II should be considered to be *P. destruens* since the ex-type culture for *P. destruens* was present.

Pythium insidiosum M25 was from a patient in Thailand and was present among Asian isolates in cluster II. However, two other human isolates from Thailand (M7 and 291) were within cluster III. This may indicate that there are two different populations of *P. insidiosum* present in Thailand which infect humans, one of which is very similar to the human and animal isolates in cluster II. Although it was believed that the patient who was the source of isolate M25 was infected in Thailand, the possibility of infection from neighboring regions by way of imported foods or visitors cannot be excluded.

Cluster III consisted of three human isolates, two from Thailand and one from the USA. With only three isolates present, it is difficult to draw conclusions about the genetic

relationship between cluster III and the other isolates. The average genetic distances between cluster III and clusters I and II were relatively large and in the same range as the values for the outgroups (Table 4.2). This suggested that cluster III may represent a subspecies of *P. insidiosum* or a different species altogether. Additional isolates representative of cluster III are needed to arrive at firm conclusions. Isolate M18, from a spectacled bear in a South Carolina zoo, was divergent from clusters I, II, and III, and average genetic distances between M18 and the clusters were large, yet slightly less than the values for the outgroups (Table 4.2). This isolate may therefore represent a variant form of *P. insidiosum* or a different species.

The RFLP analysis of the IGS thus pointed to the existence of at least three clusters of *P. insidiosum* isolates with a high degree of geographical isolation, with clusters I and II showing the greatest affinity (Fig. 4.2). The IGS of the rDNA repeat unit therefore may also be useful for resolving intraspecific relationships within a species since variations in restriction fragment patterns reflected the geographic origins of *P. insidiosum* isolates. There was, however, no apparent relationship between the host and clustering patterns.

The sequence analysis using the ITS1-5.8S-ITS2 region was done to examine phylogenetic relationships among 23 isolates of *P. insidiosum* from a variety of hosts and geographic origins, and it provided a higher level of molecular resolution than RFLP analysis. The ex-type strains of *P. aphanidermatum, P. deliense,* and *P. grandisporangium* were also included in the analysis. *L. giganteum* and *Ph. megasperma* represent genera in the order *Pythiales* closely related to *Pythium* and were also used as outgroup species in this study. Phylogenetic analysis of ITS sequences separated 23

isolates of *P. insidiosum* into three clades supported by high bootstrap values (Fig. 4.4). Clades I, II, and III corresponded to the clusters I, II, and III, respectively, previously obtained from RFLP analysis of the IGS (Fig. 4.2).

Clade I contained *P. insidiosum* isolates from North, Central, and South America from hosts including equines, canines, a feline, and a human. Clade II contained isolates from Australia, Southeast Asia, and the USA. Two isolates of *P. destruens* (M23, the ex-type culture, and M24) were present among the five other *P. insidiosum* isolates supporting the suggestion from RFLP analysis that *P. destruens* and *P. insidiosum* are conspecific, or that all the isolates in clade II should be considered *P. destruens*. Isolate M25, from a human in Thailand, was distinct from two other human Thailand isolates in clade III. Isolate M21, from a patient in Pennsylvania who was originally from Afghanistan, was also present in clade II. Its presence among Asian isolates may indicate an origin of infection from that region, as previously discussed.

Pythium insidiosum isolate 296, from a mosquito larva in India, was unique to clade II as it is the only one isolated from a non-mammalian host. *Lagenidium* is a genus in the family Pythiaceae (Dick, 2001) that contains species which are parasitic on algae, water molds, other Oomycetes, and microscopic animals (Sparrow, 1973). *L. giganteum* is a facultative parasite that attacks and kills mosquito larvae; this makes it a potential biological control agent of mosquitoes (Berbee and Kerwin, 1993). In this study, *P. insidiosum* 296 was present in clade II with other *P. insidiosum* isolates from mammalian hosts, and distinct from the *L. giganteum* isolate from a mosquito larva in North Carolina. The ITS sequence analysis therefore supported the identification of isolate 296 as *P. insidiosum* and provided evidence that *P. insidiosum* has the potential to parasitize non-

mammalian hosts such as mosquitoes and plants. It has been suggested that *P. insidiosum* parasitizes aquatic plants, such as water lilies, as part of its life cycle (Mendoza *et al.*, 1993). Furthermore, because *P. insidiosum* can easily be cultivated and induced to sporulate in pure culture (Mendoza and Prendas, 1988), it is likely that a saprobic stage may in fact be part of its life cycle. Relative to this study, the fact that phylogenetically related strains of *P. insidiosum* can infect both mammals and insects (as in clade II) suggests that the organism does not exhibit host specialization. The zoospores, which are attracted to hairs and tissues of mammals (Miller, 1983), are most likely simply opportunistic invaders of these animals.

Clade III contained three human isolates, two from Thailand and one from the USA, and an isolate (M18) from a spectacled bear in South Carolina. RFLP analysis of the rDNA showed that isolate M18 was divergent from clades I, II, and III (Fig. 4.2), but ITS sequence analysis showed it had a significant affinity for isolates in clade III. Clade III formed a clade which was distant from clades I and II, but still part of the larger clade of *P. insidiosum* isolates separate from other *Pythium* species. The fact that clade III appeared to be the most distantly related of the three clades indicates that it may represent a subspecies of *P. insidiosum* or a different species altogether.

In a comparison of genetic distance values among isolates, the distances within clades I and II were low in comparison to those within clade III (Table 4.3). The relatively high distances within clade III were most likely due to the presence of isolate M18, which was relatively divergent from isolates M7, M19, and 291. In a pairwise comparison of genetic distances among the three clades, clades I and II were most closely related while clade III was most distant (Table 4.3). However, when comparing each of

the three clades to the outgroup *Pythium* species, distances were much greater, and even greater when the clades were compared to *L. giganteum* or *Ph. megasperma*. Relative to genetic distances from RFLP analysis (Table 4.2), the distances from ITS sequence analysis were lower when comparing clades to one another, yet much larger when comparing clades and the outgroup species (Tables 4.2 and 4.3).

Overall, clades I, II, and III of *P. insidiosum* isolates were more closely related to each other than to the other *Pythium* species in this study. These three clades were part of a larger clade containing three closely related *Pythium* species (*P. aphanidermatum, P. deliense,* and *P. grandisporangium*). This larger clade of *Pythium* species was well separated from isolates of *L. giganteum* and *Ph. megasperma.* The genera *Pythium* and *Phytophthora* are distinguished from each other mainly by their mode of zoospore differentiation. In *Phytophthora,* zoospores develop within the zoosporangium while in *Pythium* they develop in an external vesicle outside of the zoosporangium. It has been suggested that *Pythium* is ancestral to *Phytophthora* and this view has been supported by sequencing studies by Briard *et al.* (1995) and Cooke *et al.* (2000). *Lagenidium* species are similar to *P. insidiosum* based on their comparable septation or segmentation of hyphae, and their formation of zoospores in a vesicle, but they differ as their sexual structures are less well differentiated (de Cock *et al.,* 1987).

While *P. insidiosum* isolates and other *Pythium* species in this study were well separated from *L. giganteum* and *Ph. megasperma,* the *Pythium* clade appeared to be more closely related to *L. giganteum* than to *Ph. megasperma.* The average pairwise genetic distances of *Pythium* isolates compared to *L. giganteum* and *Ph. megasperma* were 0.3188 and 0.5193, respectively. Recent studies tend to support this result. For

instance, in a *cox* II molecular phylogeny (Hudspeth *et al.,* 2000), *Pythium* and *Lagenidium* were located in a clade separate from *Phytophthora.* Dick *et al.* (1999) showed that *Lagenidium* was a sister to the *Pythium* and *Phytophthora* lineage, and Dick (2001) later placed *Pythium, Phytophthora,* and *Lagenidium* together in the order Pythiales in the family Pythiaceae. However, Peterson and Rosendahl (2000) showed that *Phytophthora* species clustered more closely with *Peronospora* than with *Pythium* and *Lagenidium,* and suggested that *Phytophthora* be removed from the Pythiales and placed in the Peronosporales. Most recently, Riethmüller *et al.* (2002) used sequence analysis of the LSU rRNA gene to show that *Phytophthora* was more closely related to the Peronosporaceae than to *Pythium,* and that *L. chthamalophilum* was present within a clade of *Pythium* species. Nonetheless, the ITS sequence data presented here showed that the isolates from the reported cases of pythiosis were all *P. insidiosum* and that they were not a species in a closely related genus such as *Phytophthora* or *Lagenidium.*

While RFLP analysis of the IGS provided a technique for estimating the intraspecific variability among isolates, ITS sequence analysis allowed for the examination of phylogenetic relationships, not only among *P. insidiosum* isolates, but also between *P. insidiosum* and other *Pythium* species and closely related genera. This task was not possible within the limitations of the RFLP technique. The sequencing results in general corresponded to those obtained from RFLP analysis, thus verifying the use of RFLP analysis as a technique to rapidly screen isolates and show relationships. The main difference was that RFLP analysis could not definitely show to which cluster isolate M18 was most closely related to, while ITS sequencing showed that isolate M18 was present within clade III. The sequence data also provides an abundance of genetic

information which is useful for molecular diagnosis. Badenoch *et al.* (2001) used ITS sequence analysis to confirm the identification of a strain of *P. insidiosum* isolated from a human keratitis case. Grooters and Gee (2002) applied ITS sequence data to develop a nested PCR assay to detect *P. insidiosum* from clinical cases of pythiosis. Furthermore, the ITS sequence data provides a potential target for the development of a species-specific probe, a technique which has been used to identify and detect other Oomycetes (Lévesque *et al.,* 1998) and clinically important fungi (e.g. Elie *et al.,* 1998; El Fari *et al.,* 1999).

To complement the molecular data, an attempt was made to uncover a physiological trait that would differentiate isolates into the same groupings as the genetic studies. The growth rates at eight temperatures were measured to construct and compare growth curves for nine isolates (four from each of clusters I and II, and one isolate from cluster III). Gilbert *et al.* (1993) had previously used growth curves to distinguish *P. arrhenomanes, P. aristosporum, P. myriotylum,* and *P. volutum* from one another.

In the present study, no obvious relationship between growth rates or growth curves and genetic clusters was evident. All isolates had an optimum temperature in the range of 35—38°C, but the overall growth rates did not correspond to specific clusters. Isolate M4 (cluster I) appeared to have the fastest growth rate at all temperatures, but isolate M9 (also from cluster I) often grew much more slowly than isolates from clusters I and II (Table 4.4). Isolates M4 and M22 (cluster I) usually had higher growth rates than isolates in cluster II. However, the growth rates of the ex-type culture of *P. insidiosum* (65) were not distinct from those of isolates in either cluster I or II. Three isolates from cluster II (M15, M21, and M24) had similar growth curves, but isolate M23 (cluster II)

grew relatively slower at most temperatures. The most significant observation was that the sole isolate from cluster III (M7) had an overall growth rate significantly slower than all other isolates. Therefore, the most genetically distinct isolate could be distinguished from the others by its relatively slower growth rate at various temperatures.

So, while isolates M4 and M7 produced growth curves (two extremes) that could be distinguished from all other isolates, those of the remaining isolates were difficult to differentiate from one another. Variation in growth rates among isolates may be common to this species as it has not been studied in great detail. On cornmeal agar (CMA), the daily growth rate of *P. insidiosum* 65 at 24°C and 34°C was reported to be 8 mm and 12.5 mm, respectively, while on brain heart infusion agar it was 11 mm and 18.5 mm, respectively (de Cock *et al.,* 1987). The growth rates reported here on Sabouraud agar were considerably higher (13.6 mm and 23.4 mm at 25°C and 34°C, respectively).

In general, growth rates at different temperatures failed to show a clear distinction among genetic clusters. A comparison of morphological characteristics between isolates within each cluster was another consideration, but there are limitations to such an analysis. Zoosporangia are filamentous and not distinguishable from vegetative hyphae, and oogonia and oospores rarely form, although they had been known to form sporadically on CMA. The animal hosts infected or the clinical symptoms of each isolate also did not correlate with the genetic clusters.

Nonetheless, the data from ITS sequencing and RFLP analysis of the IGS were in agreement, supporting the existence of three genetic clades of *P. insidiosum* that exhibit a high degree of geographic isolation. All isolates were more closely related to each other than to other closely related *Pythium* species or Oomycetes. This molecular study

therefore supported the view that geographically isolated populations of *P. insidiosum* may exist that could be endemic to various regions of the world, or that *P. insidiosum* may consist of more than one species and may represent a recently diverging lineage of *Pythium* species with the ability to infect mammals. An understanding of intraspecific variation in *P. insidiosum* may be crucial for the successful diagnosis and treatment of pythiosis in animals and humans.

It is important to identify *P. insidiosum* accurately since infections caused by this pathogen often mimic the symptoms caused by other organisms. The ID test has been widely used to detect pythiosis, but it sometimes fails to detect pythiosis in canines and humans (Chetchotisakd *et al.,* 1992; Wanachiwanawin *et al.,* 1993; Mendoza *et al.,* 1997; Thitithanyanont *et al.,* 1998). Many clinical laboratories are not properly equipped to identify *P. insidiosum* quickly and this causes delays in the treatment of the disease. RFLP analysis of the IGS or ITS sequencing provide additional techniques for identifying the causative agent in cases of pythiosis once the organism has been isolated from the host. These methods may also provide valuable information on the geographic origin of an isolate and the source of an infection.

The unresponsiveness of an infected host to the *P. insidiosum* vaccine could depend not only on the immunological state of the host, as suggested by Mendoza *et al.* (1992b), but by the strain of *P. insidiosum* causing the infection. Early identification would allow timely treatment or less invasive surgery. Therefore, knowledge of intraspecific variability in *P. insidiosum* may be important for the management of pythiosis in mammals.

CHAPTER 5

Characterization of a species-specific DNA probe for *Pythium insidiosum*

INTRODUCTION

Timely and accurate diagnosis of pythiosis is crucial to ensure that proper therapeutic measures are taken. However, diagnosis of the disease is often complicated by the presence of other pathogenic fungi, algae, and bacteria in infected tissue which mimic the symptoms of pythiosis. The diagnosis of pythiosis and detection of *P. insidiosum* in serum and blood samples with current available methods frequently produce inconsistent results. Histopathological staining of infected tissues is often done, but some members of the Zygomycetes produce results similar to those obtained for *P. insidiosum.* Several serological techniques have been developed, including an immunoperoxidase test (Brown *et al.,* 1988), fluorescent antibodies (Mendoza *et al.,* 1987), and immunodiffusion (ID) tests (Mendoza *et al.,* 1986). While the ID test has been most frequently used, it has often failed to detect pythiosis in proven cases of the disease. The ELISA test provides an additional serological detection method with a greater level of sensitivity (Mendoza *et al.,* 1997; Grooters *et al.,* 2002). The observation of cultural characteristics is also important, but this may take several weeks and requires a pure culture.

Due to the often contradictory results from serological tests, molecular biology techniques present alternative methods for the diagnosis of pythiosis and detection of *P. insidiosum*. RFLP analysis of the IGS and ITS sequence analysis (Chapter 4) were able to distinguish isolates of *P. insidiosum* from all other *Pythium* species and closely related genera, and also demonstrated intraspecific variation among isolates. In addition, Grooters and Gee (2002) developed a nested PCR assay using primers in ITS-1 to detect

P. insidiosum in infected animals. This chapter describes the development of a species-specific DNA probe from the IGS of the ex-type culture of *P. insidiosum* which can i) distinguish *P. insidiosum* from all other *Pythium* species, ii) hybridize to all geographically isolated strains of *P. insidiosum* from a variety of animal hosts, and iii) selectively hybridize to genomic DNA of *P. insidiosum* and not cross-react with DNA from other fungal and algal pathogens which produce symptoms that resemble pythiosis. The potential applications of the DNA probe are also discussed.

RESULTS

Construction and characterization of the *P. insidiosum* species-specific DNA probe

The region between the LSU rRNA gene and 5S rRNA gene (IGS-1) was PCR-amplified for *P. insidiosum* (isolate 65) using primers Q and SR (Fig. 5.1A) producing a 1900 bp product. A *Hin*fI partial digestion of the Q-SR amplicon was hybridized with a DIG-11-ddUTP-labelled Q-primer oligonucleotide probe for the construction of the *Hin*fI restriction site map of IGS-1 (Fig. 5.1A). The central 530 bp fragment was selected for further characterization and use as a species-specific DNA probe for *P. insidiosum.*

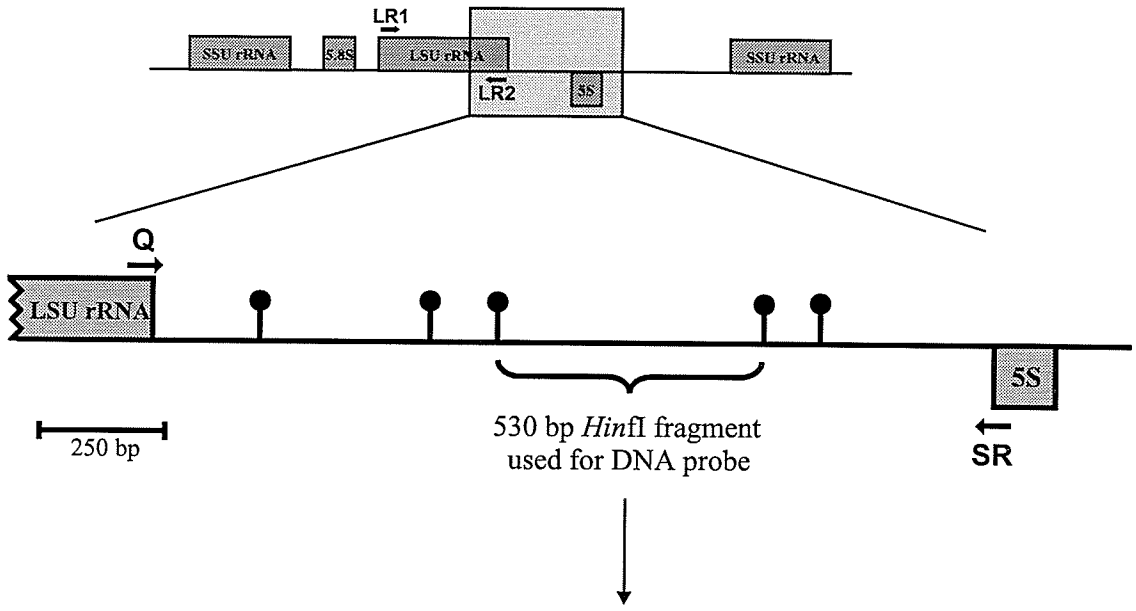
A complete *Hin*fI digestion of the Q-SR product was subsequently done and the 530 bp fragment was excised and purified from agarose gel using the freeze-squeeze method. The fragment was cloned into the pPCR-Script Amp SK(+) vector and sequenced (Fig. 5.1B). The resulting sequence had a G+C ratio of 44.34% and a T_m of 82.14°C. The fragment was labelled with DIG-11-dUTP using the random-primed labelling method for use in hybridization tests with genomic DNA of several *Pythium* species.

Confirmation of probe specificity among *Pythium* species

Spot blots of genomic DNA representing 104 *Pythium* species (Table 5.1) distributed on two separate membranes (blots I and II) were prepared (Fig. 5.2). Before the blots were made, the amount of DNA to be spotted had to be optimized. To do so, a DNA probe was prepared by amplifying and DIG-labelling a 2500 bp region of the LSU

Figure 5.1. **(A)** *Hin*fI restriction site map of IGS-1 for *P. insidiosum* 65. ↑ refers to locations of *Hin*fI restriction sites. Rectangles represent coding regions for the LSU (large subunit), SSU (small subunit), 5.8S, and 5S rRNA genes. Locations and orientations of the primers LR1, LR2, Q, and SR are indicated with arrows. **(B)** Nucleotide sequence of the 530 *Hin*fI fragment from IGS-1 selected as a species-specific probe for *P. insidiosum.*

A



B

```
1     CCAATCGCGG ACAGACGATT AGTAGATGTG TAGCTCGCAG CAGCGCGCAT ACAATGATAC
61    CAAACACAAC ACCATACCAC TAATAACAAC ACAGATAAAC AATGACCTAG CAACGACAAC
121   ACTACTGTGA AACGCAAAAG GCAAATAAAC CCTGATAATT TCCAGTTTAC AGCCTGTACA
181   CAATTTTACA CTCCACCTCA CCACTTGACA GTGCACACAC ACATTACCAG TATAAATACC
241   TACCCTTTGA CGTCAGTCGC ATTTTCATAC CTAGTCTAAC GGCAGAGATA TTCAATGCCC
301   TAGCGCCGCC TTTTTAGGGT ATTTAATTGG AATTTTTGGG CGTTAGTTTT TGACCCCCCT
361   TGGCAGGACT AAGACGCCAG GCACCCAGTC TATCATCAGT CTATAGTCTA TCCAAGTCAT
421   TTCTTGGACA CTCGGCCGTC CAAACACCAA GTCCACAGCC CAACCACTAT ACTGAAACAA
481   AGTATATAAT CAGACACTTG TCCTTTTCGG CTTACGACTG CTTACGACTG
```

Table 5.1. List of organisms used in Chapter 5.

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
Pythium acanthicum	71[d]	CBS 284.31	USA
P. acanthophoron	52	CBS 337.29	Hawaii, USA
P. acrogynum	69[d]	CBS 549.88	China
P. adhaerens	25	CBS 520.74	Netherlands
P. amasculinum	298	CBS 552.88	China
P. anandrum	1[d]	CBS 258.31	USA
P. angustatum	27[e]	CBS 522.74	Netherlands
P. aphanidermatum	135[f]	CBS 118.80	France
P. apleroticum	70[d]	CBS 772.81	Netherlands
P. aquatile	21[f]	CBS 215.80	United Kingdom
P. aristosporum	2[d]	CBS 263.38	Canada
P. arrhenomanes	3[d]	CBS 324.62	Wisconsin, USA
P. australe Shahzad	aus2	IMI 332970	Australia
P. boreale	79[d]	CBS 551.88	China
P. buismaniae	63[d]	CBS 288.31	Netherlands
P. capillosum	73[g]	CBS 222.94	France
P. catenulatum	80[e] (a)	CBS 843.68	South Carolina, USA
	81 (b)	CBS 842.68	South Carolina, USA
P. chamaehyphon	82[d]	CBS 259.30	Hawaii, USA
P. chondricola	87[d]	CBS 203.85	Netherlands
P. coloratum	22[d]	CBS 154.64	South Australia
P. conidiophorum	259	CBS 224.88	United Kingdom
P. cucurbitacearum	337	CBS 748.96	Australia
P. cylindrosporum Paul	74[d]	CBS 218.94	Germany
P. debaryanum Hesse	336	CBS 752.96	United Kingdom
P. deliense	66[f]	CBS 314.33	Sumatra
P. destruens	M23[d]	ATCC 64221	Papua New Guinea
	M24	ATCC 64218	Papua New Guinea
P. diclinum	30[f]	CBS 664.79	Netherlands
P. dimorphum	31[d]	CBS 406.72	Louisiana, USA
P. dissimile	32[d]	CBS 155.64	Australia
P. dissotocum	4	CBS 166.68	Ohio, USA
P. drechsleri	75[d]	CBS 221.94	?
P. echinulatum	33[e]	CBS 281.64	Australia
P. erinaceus	34[d]	CBS 505.80	New Zealand
P. flevoense	84[d] (f)	CBS 234.72	Netherlands
P. folliculosum	76[d]	CBS 220.94	Switzerland
P. graminicola	5[f]	CBS 327.62	Jamaica
P. grandilobatum	211	CBS 739.94	South Africa
P. grandisporangium	54[d]	CBS 286.79	Florida, USA
P. helicandrum	40[g]	CBS 393.54	USA
P. helicoides	50[e]	CBS 286.31	USA
P. heterothallicum	23[d] (m)	CBS 450.67	Canada
	24[d] (f)	CBS 451.67	Canada
P. hydnosporum	90[e]	CBS 253.60	Germany
P. hypogynum	55	CBS 692.79	Alberta, Canada
P. indigoferae	351[e]	CBS 261.30	India
P. inflatum	86[e]	CBS 168.68	Louisiana, USA
P. insidiosum	65[d]	CBS 574.85	Costa Rica
	291	CBS 673.85	Thailand

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
	296	CBS 777.84	India
	297	CBS 702.83	Japan
	339	CBS 575.85	Costa Rica
	344	CBS 580.85	Costa Rica
	393	CBS 101039	India
	394	CBS 101555	Brazil
	M4	undeposited[c]	Florida, USA
	M6	ATCC 200269	Tennessee, USA
	M7	undeposited[c]	Thailand
	M9	undeposited[c]	Louisiana, USA
	M15	ATCC 28251	Papua New Guinea
	M16	ATCC 76049	Haiti
	M18	ATCC 90478	South Carolina, USA
	M19	ATCC 90586	Texas, USA
	M20	ATCC 200268	North Carolina, USA
	M21	undeposited[c]	Pennsylvania, USA
	M22	undeposited[c]	Wisconsin, USA
	M25	undeposited[c]	Thailand
P. intermedium de Bary	6	CBS 266.38	Netherlands
P. irregulare	67[f]	CBS 250.28	Netherlands
P. iwayamai	29[e]	CBS 156.64	Australia
P. jasmonium	395	CBS 101876	?
P. kunmingense Yü	60[d]	CBS 550.88	China
P. lutarium	36[d]	CBS 222.88	United Kingdom
P. macrosporum	7[d] (+)	CBS 574.80	Netherlands
	8[d] (-)	CBS 575.80	Netherlands
P. mamillatum	9[e]	CBS 251.28	Netherlands
P. marinum	94	CBS 312.93	Washington, USA
P. marsipium Drechsler	96	CBS 773.81	Netherlands
P. mastophorum	57[e]	CBS 375.72	United Kingdom
P. middletonii	35[e]	CBS 528.74	Netherlands
P. minus	37[d]	CBS 226.88	United Kingdom
P. monospermum	10[f]	CBS 158.73	United Kingdom
P. multisporum	53[d]	CBS 470.50	USA
P. myriotylum	11[f]	CBS 254.70	Israel
P. nagaii	4321a	IMI 308183	United Kingdom
P. nodosum	396	adc 99.30	Netherlands
P. nunn Lifshitz, Stanghellini & Baker	361[d]	CBS 808.96	Colorado, USA
P. oedochilum	38[g]	CBS 292.37	USA
P. okanaganense	59[d]	CBS 315.81	Wisconsin, USA
P. oligandrum	12[e]	CBS 382.34	United Kingdom
P. orthogonon Ahrens	346[d]	CBS 376.72	Lebanon
P. ostracodes	49[e]	CBS 768.73	Spain
P. pachycaule	247[d]	CBS 227.88	United Kingdom
P. paddicum	46	CBS 698.83	Japan
P. paroecandrum	68[e]	CBS 157.64	Australia
P. parvum	42[d]	CBS 225.88	United Kingdom
P. periilum	26[e]	CBS 169.68	Florida, USA
P. periplocum	91[d]	CBS 289.31	USA
P. perplexum	319	CBS 674.85	?
P. pleroticum	51	CBS 776.81	Netherlands
P. plurisporium Abad, Shew, Grand & Lucas	388[d]	CBS 100530	North Carolina, USA

Species	Ref. no[a]	Accession No.[b]	Country of Origin[c]
P. polymastum	93[e]	CBS 811.70	Netherlands
P. polymorphon Sideris	334	CBS 751.96	United Kingdom
P. porphyrae	215[e]	CBS 369.79	Japan
P. prolatum	62[d]	CBS 845.68	Georgia, USA
P. pyrilobum	43[d]	CBS 158.64	Australia
P. radiosum	77[d]	CBS 217.94	France
P. rostratum	88[f]	CBS 533.74	Netherlands
P. salpingophorum	39[e]	CBS 471.50	United Kingdom
P. scleroteichum	89[g]	CBS 294.37	USA
P. spinosum	13[e]	CBS 275.67	Netherlands
P. splendens	14[e] (-)	CBS 462.48	USA
P. sulcatum	44[d]	CBS 603.73	Wisconsin, USA
P. sylvaticum Campbell & Hendrix	15[d] (m)	CBS 452.67	USA
	16[d] (f)	CBS 453.67	USA
P. torulosum	17[e]	CBS 316.33	Netherlands
P. tracheiphilum	92[d]	CBS 323.65	Italy
P. tumidum	78[d]	CBS 223.94	?
P. ultimum var. *ultimum*	18[f]	CBS 398.51	Netherlands
P. ultimum var. *sporangiiferum*	19[d]	CBS 219.65	USA
P. uncinulatum	203[d]	CBS 518.77	Netherlands
P. undulatum	48[f]	CBS 157.69	Alabama, USA
P. vanterpoolii	58[d]	CBS 295.37	United Kingdom
P. vexans	20[e]	CBS 119.80	Iran
P. violae	28[e]	CBS 159.64	Australia
P. volutum	47	CBS 699.83	Japan
P. zingiberis	41	CBS 216.82	Japan
Aspergillus flavus	Af	undeposited[c]	?
Basidiobolus ranarum	Br	undeposited[c]	?
Conidiobolus coronatus	Cc	undeposited[c]	?
L. giganteum[h]	Lg	ATCC 36492	North Carolina, USA
Paracoccidioides brasiliensis	Pb	undeposited[c]	?
Prototheca wickerhamii	Pw	undeposited[c]	?

[a]Numbers by which isolates are referred to in this study. For heterothallic species, mating types are designated as a, b, m (male), f (female), or + and - (opposite mating types).
[b]Accession numbers of isolates in the following culture collections: CBS (Centraalbureau voor Schimmelcultures, Utrecht, Netherlands), IMI (International Mycological Institute, Egham, United Kingdom), ATCC (American Type Culture Collection, Manassas, Virginia), adc (personal collection of A.W.A.M. de Cock).
[c]?=unknown geographic origin.
[d]Ex-type strain.
[e]Isolate used by Van der Plaats-Niterink (1981) for species description.
[f]Isolate designated as the neotype strain because all ex-type material is missing.
[g]Authentic strain, identified by the author of the species.
[h]DNA provided by M. Hudspeth, Northern Illinois University, DeKalb, Illinois, USA.

Figure 5.2. Positions of 108 genomic DNA spots representing 104 *Pythium* species on membranes used for DNA hybridizations in Fig. 5.3. Isolate numbers refer to those indicated in Table 5.1.

Blot I

- **71** - *P. acanthicum*
- **52** - *P. acanthophoron*
- **69** - *P. acrogynum*
- **25** - *P. adhaerens*
- **298** - *P. amasculinum*
- **1** - *P. anandrum*
- **27** - *P. angustatum*
- **135** - *P. aphanidermatum*
- **70** - *P. apleroticum*
- **21** - *P. aquatile*
- **2** - *P. aristosporum*
- **3** - *P. arrhenomanes*
- **aus2** - *P. australe*
- **79** - *P. boreale*
- **63** - *P. buismaniae*
- **73** - *P. capillosum*
- **80** - *P. catenulatum*
- **81** - *P. Catenulatum*
- **82** - *P. chamaehyphon*
- **87** - *P. chondricola*
- **22** - *P. coloratum*
- **259** - *P. conidiophorum*
- **337** - *P. cucurbitacearum*
- **74** - *P. cylindrosporum*
- **336** - *P. debaryanum*
- **66** - *P. deliense*
- **M23** - *P. destruens*
- **30** - *P. diclinum*
- **31** - *P. dimorphum*
- **32** - *P. dissimile*
- **265** - *P. dissotocum*
- **75** - *P. drechsleri*
- **33** - *P. echinulatum*
- **34** - *P. erinaceus*
- **84** - *P. flevoense*
- **76** - *P. folliculosum*
- **5** - *P. graminicola*
- **211** - *P. grandilobatum*
- **54** - *P. grandisporangium*
- **40** - *P. helicandrum*
- **50** - *P. helicoides*
- **23** - *P. heterothallicum*
- **24** - *P. heterothallicum*
- **90** - *P. hydnosporum*
- **55** - *P. hypogynum*
- **351** - *P. indigoferae*
- **86** - *P. inflatum*
- **6** - *P. intermedium*
- **67** - *P. irregulare*
- **29** - *P. iwayamai*
- **395** - *P. jasmonium*
- **60** - *P. kunmingense*

Blot II

- **36** - *P. lutarium*
- **7** - *P. macrosporum*
- **8** - *P. macrosporum*
- **9** - *P. mamillatum*
- **94** - *P. marinum*
- **96** - *P. marsipium*
- **57** - *P. mastophorum*
- **35** - *P. middletonii*
- **37** - *P. minus*
- **10** - *P. monospermum*
- **53** - *P. multisporum*
- **11** - *P. myriotylum*
- **4321a** - *P. nagaii*
- **396** - *P. nodosum*
- **361** - *P. nunn*
- **38** - *P. oedochilum*
- **59** - *P. okanaganense*
- **12** - *P. oligandrum*
- **346** - *P. orthogonon*
- **49** - *P. ostracodes*
- **247** - *P. pachycaule*
- **46** - *P. paddicum*
- **68** - *P. paroecandrum*
- **42** - *P. parvum*
- **26** - *P. periilum*
- **91** - *P. periplocum*
- **319** - *P. perplexum*
- **51** - *P. pleroticum*
- **65** - *P. insidiosum*
- **388** - *P. plurisporium*
- **93** - *P. polymastum*
- **334** - *P. polymorphon*
- **215** - *P. porphyrae*
- **62** - *P. prolatum*
- **43** - *P. pyrilobum*
- **77** - *P. radiosum*
- **88** - *P. Rostratum*
- **39** - *P. salpingophorum*
- **89** - *P. scleroteichum*
- **13** - *P. spinosum*
- **14** - *P. splendens*
- **44** - *P. sulcatum*
- **15** - *P. sylvaticum*
- **16** - *P. sylvaticum*
- **92** - *P. tracheiphilum*
- **17** - *P. torulosum*
- **78** - *P. tumidum*
- **18** - *P. ultimum* var. *ultimum*
- **19** - *P. ultimum* var. *sporangiiferum*
- **203** - *P. uncinulatum*
- **48** - *P. undulatum*
- **58** - *P. vanterpoolii*
- **20** - *P. vexans*
- **28** - *P. violae*
- **47** - *P. volutum*
- **41** - *P. zingiberis*

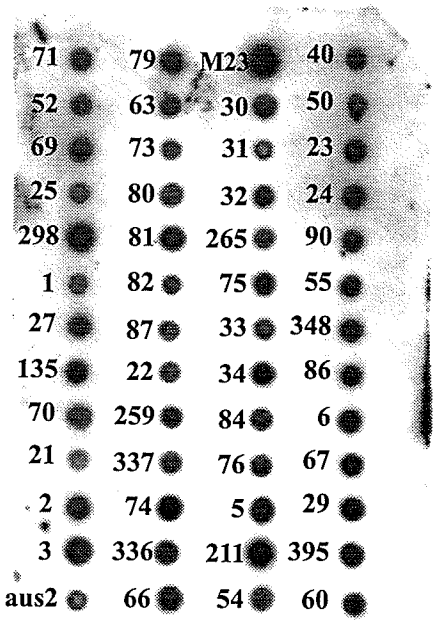
rRNA gene of *P. insidiosum* 65 with the primer pair LR1-LR2 (Fig. 5.1A). This probe was then hybridized to spot blots containing a range of genomic DNA concentrations from each species (data not shown). Concentrations of genomic DNA that produced hybridization signals of approximately equal intensities were determined for each of the 108 isolates, and these were spotted onto two membranes (blots I and II). The blots were then hybridized with the LSU rRNA gene probe at 60°C to ensure the hybridization signals were relatively equal (Fig. 5.3A). The membranes were then stripped and re-hybridized with the 530 bp *P. insidiosum Hin*fI fragment probe (Fig. 5.3B). A hybridization temperature of 60°C was selected on the basis that the hybridization should be performed at a temperature 20 to 25°C below the T_m for DNA-DNA hybridizations (Anderson, 1999). The probe hybridized to genomic DNA of *P. insidiosum* 65 and *P. destruens* M23, but did not cross-react with genomic DNA of any of the other 102 *Pythium* species.

Sensitivity of probe for detecting *P. insidiosum* isolates from a diverse range of animal hosts and geographic origins

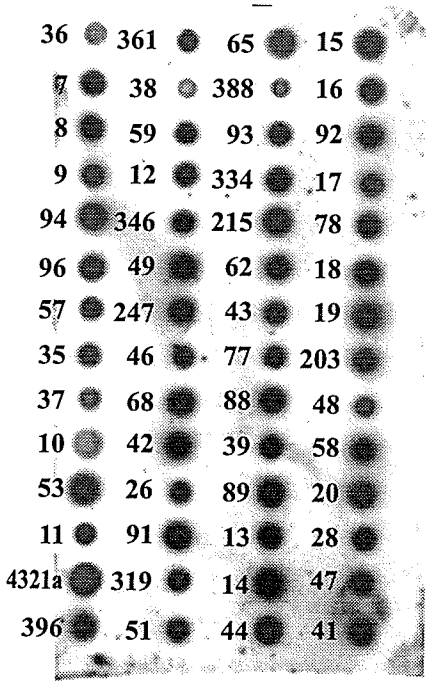
To test if the *P. insidiosum Hin*fI fragment probe would hybridize to genomic DNA of *P. insidiosum* isolates from various animal hosts and geographic origins, spot blots were prepared using genomic DNA from 22 isolates of *P. insidiosum* and *P. destruens* representing isolates from the genetic clusters I, II, and III discussed in Chapter 4. The concentrations of genomic DNA were normalized using the LSU rRNA gene probe of *P. insidiosum* 65 (Fig. 5.4A). The membranes were then stripped and re-

Figure 5.3. Dot blot hybridizations of genomic DNA representing 104 *Pythium* species with (**A**) a LSU rRNA gene probe (LR1-LR2 PCR amplicon) from *P. insidiosum,* and (**B**) a 530 bp *Hin*fI fragment probe from IGS-1 of *P. insidiosum.* Subsequent to hybridization with the LR1-LR2 probe, membranes in (A) were stripped and re-hybridized with the *Hin*fI fragment probe in (B). Hybridization conditions were identical for both probes. Numbers correspond to isolate designations in Table 5.1 and the species order is illustrated in Fig. 5.2.

A

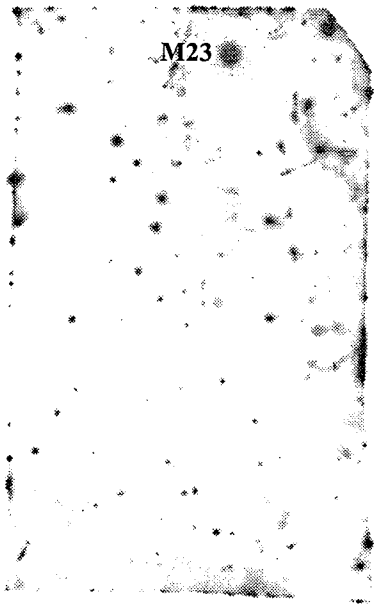


Blot I



Blot II

B

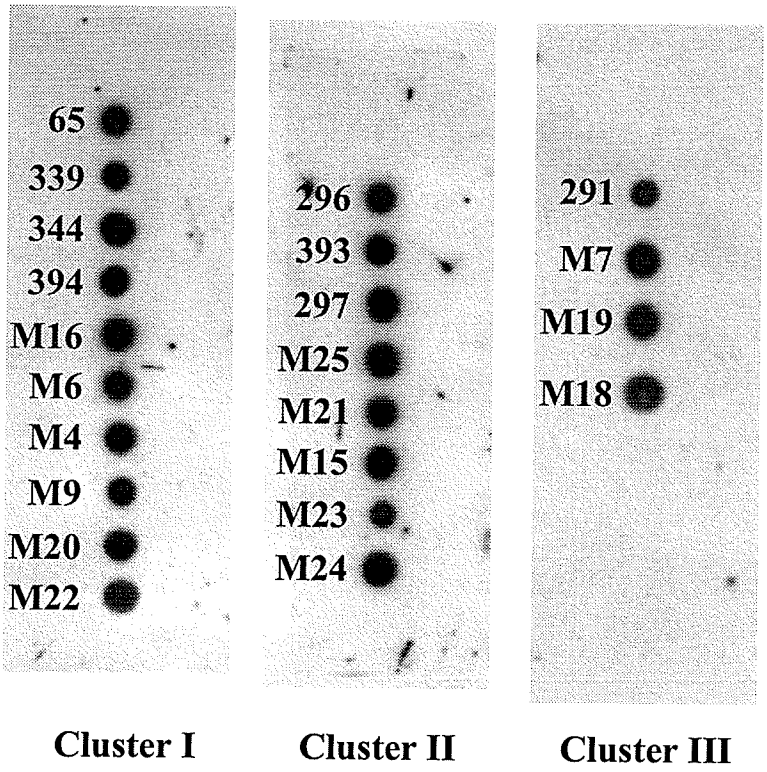


Blot I

Blot II

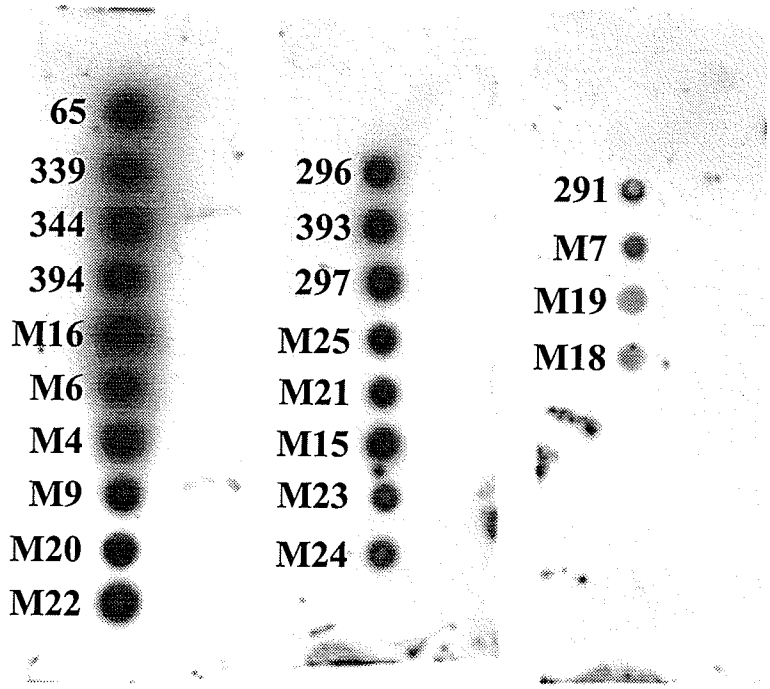
Figure 5.4. Dot blot hybridizations of *P. insidiosum* genomic DNA from isolates representing the genetic clusters (clusters I, II, and III) discussed in Chapter 4. Blots were hybridized with the **(A)** LSU rRNA gene probe (LR1-LR2 amplicon) from *P. insidiosum,* and the **(B)** 530 bp *Hin*fI fragment probe from *P. insidiosum.* Subsequent to hybridization with the LR1-LR2 probe, membranes in (A) were stripped and re-hybridized with the *Hin*fI fragment probe in (B). Isolate numbers correspond to those listed in Table 5.1.

A



Cluster I Cluster II Cluster III

B



Cluster I Cluster II Cluster III

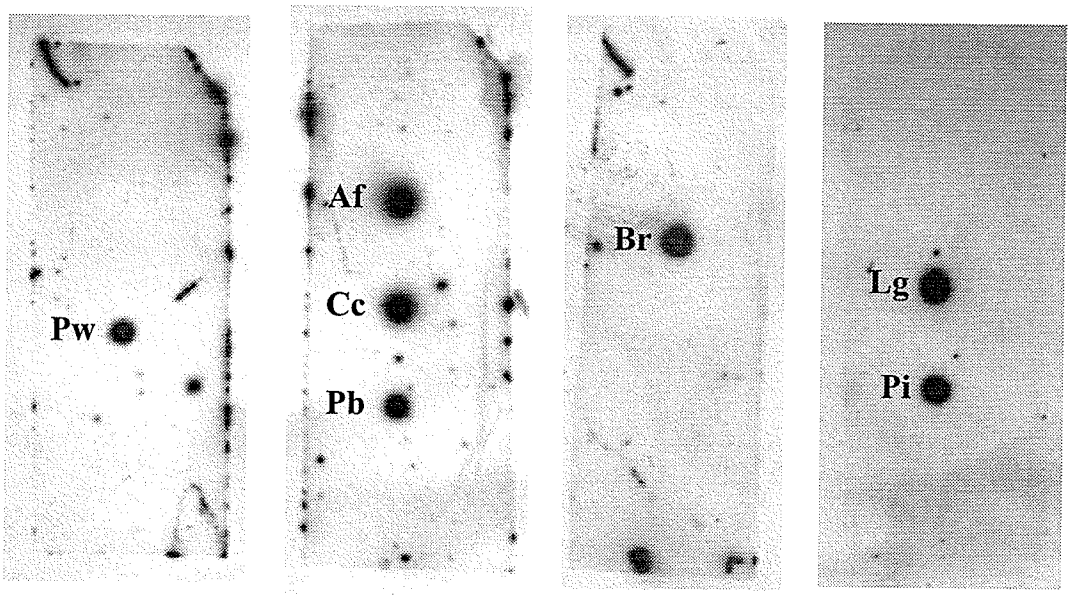
hybridized with the *P. insidiosum Hin*fI fragment probe (Fig. 5.4B). The probe hybridized with the DNA from all 22 isolates, but the hybridization signal was most intense with isolates from cluster I. The intensity of the hybridization signal decreased with cluster II isolates and was weakest with the four cluster III isolates (291, M7, M19, and M18) (Fig. 5.4B).

Confirmation of probe specificity compared to other pathogenic organisms

The specificity of the *P. insidiosum Hin*fI fragment probe was tested against genomic DNA from several pathogenic fungi and algae that produce symptoms similar to those of pythiosis in infected hosts. Spot blots were prepared using genomic DNA of *Aspergillus flavus* (Ascomycota), *Basidiobolus ranarum* (Zygomycota), *Conidiobolus coronatus* (Zygomycota), *Paracoccidioides brasiliensis* (Ascomycota), and *Prototheca wickerhamii* (Chlorophyta). An isolate of *Lagenidium giganteum* (Oomycota) was also included along with the ex-type culture of *P. insidiosum.* The blots were first hybridized with LSU rRNA gene probes for each class of organisms to ensure that the hybridization signals of the tested species were approximately equal (Fig. 5.5A). The membranes were stripped and re-hybridized with the *P. insidiosum Hin*fI fragment probe which only hybridized to genomic DNA of *P. insidiosum* 65 and did not cross-react with DNA from any of the other pathogens (Fig. 5.5B).

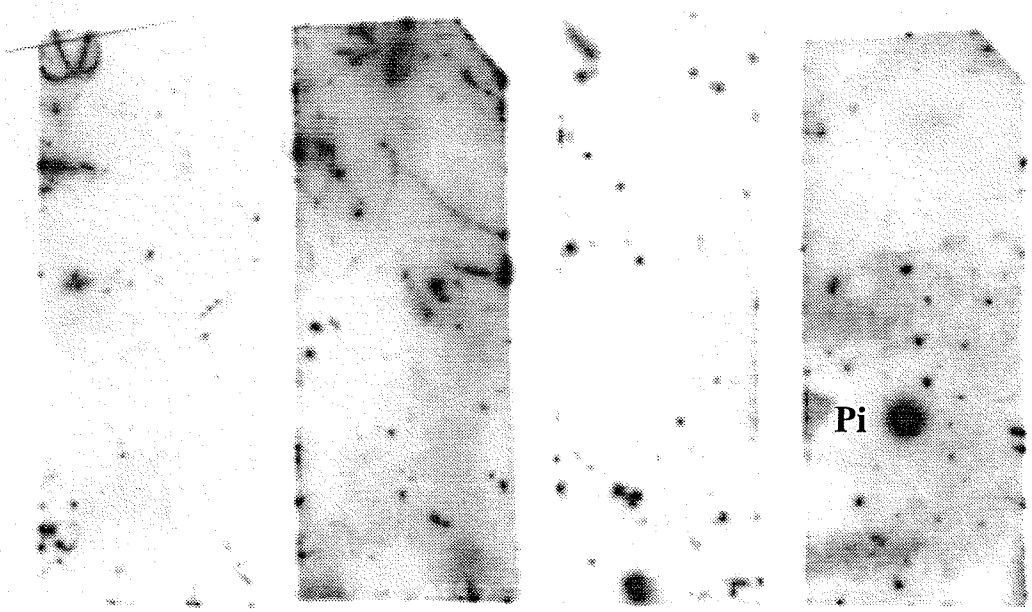
Figure 5.5. Dot blot hybridizations of genomic DNA from organisms that produce symptoms similar to pythiosis. **(A)** Membranes were hybridized with the LSU rRNA gene probes (LR1-LR2 amplicons) amplified from the following organisms: *Prototheca wickerhamii* (blot 1), *Aspergillus flavus* and *Basidiobolus ranarum* (1:1 mixture of both probes; blots 2 and 3), and *P. insidiosum* (blot 4). **(B)** Membranes hybridized with 530 bp *Hin*fI fragment probe from *P. insidiosum.* Subsequent to hybridization with the LR1-LR2 probes, membranes in (A) were stripped and re-hybridized with the *Hin*fI fragment probe in (B). Species abbreviations as follows: Af, *Aspergillus flavus*; Br, *Basidiobolus ranarum*; Cc, *Conidiobolus coronatus*; Lg, *Lagenidium giganteum*; Pi, *Pythium insidiosum*; Pw, *Prototheca wickerhamii.*

A



Blot1 Blot 2 Blot 3 Blot 4

B



Blot1 Blot 2 Blot 3 Blot 4

DISCUSSION

Species-specific probes have been constructed for other *Pythium* species including *P. oligandrum* and *P. sylvaticum* (Martin, 1991), *P. ultimum* (Lévesque *et al.*, 1994), and several other *Pythium* species using 5S rRNA gene spacer probes (Klassen *et al.*, 1996), oligonucleotide probes (Lévesque *et al.*, 1998), and capture probes in ELISA assays (Bailey *et al.*, 2002). Ribosomal DNA is a commonly used target for DNA probes as it is present in multiple copies which increases the sensitivity of the hybridization and produces a strong hybridization signal. In comparison to regions of the ITS and rRNA genes, the IGS has not been commonly exploited as a source of species-specific probes, although a diagnostic probe for *Candida krusei* was constructed using two fragment from the IGS (Carlotti *et al.*, 1996; 1997).

A species-specific probe for *P. insidiosum* was constructed using a 530 bp *Hin*fI fragment from the IGS-1 between the LSU and 5S rRNA genes of *P. insidiosum.* When tested with genomic DNA of 104 *Pythium* species, the probe specifically hybridized to DNA of the ex-type cultures of *P. insidiosum* (65) and *P. destruens* (M23). This also suggested that a genetic relationship exists between these two species, supporting the evidence that these two species are synonymous from ID tests (Mendoza and Marin, 1989) and RFLP analysis of the rDNA IGS and ITS sequencing studies (Chapter 4). The probe also hybridized to DNA from 22 isolates of *P. insidiosum* from a variety of animal hosts and geographic origins. Despite the genetic variation that is present within this species (Chapter 4), the probe exhibited a sufficient degree of sensitivity as it hybridized to genomic DNA from *P. insidiosum* regardless of the host or geographic origin of an

isolate. This level of sensitivity has not always been evident with other diagnostic tests for pythiosis, such as the ID and immunoperoxidase tests. With reference to the genetic clusters discussed in Chapter 4, the hybridization signal was most intense for isolates from cluster I and had a weaker signal for isolates from clusters II and III. Since the probe hybridized to DNA from all 22 isolates and did not cross-react with DNA from other *Pythium* species, this supported the evidence from Chapter 4 that the isolates representative of clusters I, II, and III are genetically related and distinct from all other *Pythium* species. However, the varying intensities of the hybridization signals also agreed with the conclusions from Chapter 4 that the isolates within the three clusters are, to some extent, genetically distinct from each other.

The probe was able to distinguish *P. insidiosum* from other pathogenic organisms that cause similar symptoms (summarized in Table 5.2). These diseases are characterized by the presence of cutaneous and subcutaneous lesions and frequent infections of internal organs, arteries, body cavities, and bones. Histopathological examinations do not always distinguish these species from one another. For example, the observations of eosinophilic inflammatory reactions, the Splendore-Hoeppli reaction, and necrotic areas with broad, hyaline, and aseptate hyphae are common in pythiosis, basidiobolomycosis, and conidiobolomycosis. The presence of kunkers, or necrotic masses that resemble kunkers, are common in pythiosis, aspergillosis, basidiobolomycosis, and conidiobolomycosis. Therefore, the misdiagnosis of these diseases may be frequent due to their common symptoms and histopathological

Table 5.2. Compilation of fungal and algal species that did not cross-react with the *P. insidiosum Hin*fI fragment probe, but whose symptoms resemble those of pythiosis.

Disease	Etiological agent	Classification	Symptoms of disease	Hosts and geographic distribution of the disease
Aspergillosis[a]	*Aspergillus flavus*	Ascomycota	Invasive inflammatory and granulomatous disease of the lungs and other organs (e.g. brain, kidneys); often produces "fungus balls" and lesions within infected lung tissue; cutaneous lesions are also common, leading to necrosis of the skin	Humans and animals in all parts of the world
Basidiobolomycosis (aka. subcutaneous zygomycosis)[a]	*Basidiobolus ranarum*	Zygomycota	Disease begins with subcutaneous nodules (like kunkers) that increase in size, and may spread to the shoulder, arms, face, neck, or legs; can also infect underlying organs (e.g. liver, intestines).	Humans and animals (e.g. horses, dogs); predominant in Africa and Indonesia, with cases in Southeast Asia, Costa Rica, and USA.
Conidiobolomycosis (aka. chronic rhinofacial zygomycosis, rhinophycomycosis)[a]	*Conidiobolus coronatus*	Zygomycota	Infection starts in nose causing nasal swelling and disfigurement of overlying tissues with little tendency to disseminate; lesions are hard, pale grey to yellow, and ulcerated resembling kunkers.	Humans and animals including horses, dolphins, chimpanzees.
Paracoccidioidomycosis[a]	*Paracoccidioides brasiliensis*	Ascomycota	Primary pulmonary infection disseminates to form granulomatous lesions of the facial, nasal, and gastrointestinal mucosa; infections may also involve the lymph nodes, cutaneous tissues, bone, and other organ systems.	Humans, but natural infection of animals is unknown; restricted to South and Central America
Protothecosis[b]	*Prototheca wickerhamii*	Chlorophyta	Lesions to skin and underlying tissues, often forming subcutaneous nodules and granulomatous eruption; may also cause bursitis and form nodular lesions in the peritoneal cavity, forehead, and nose.	Humans and animals (especially cattle and dogs) worldwide.

[a]Rippon (1988).
[b]Kaplan (1980).

characteristics. This can be especially problematic in the case of pythiosis because the misdiagnosis of pythiosis as a disease caused by a Zygomycete or Ascomycete would likely result in the prescription of antifungal agents. These would have no curative effect since *P. insidiosum* is not a member of the true fungi and does not respond to common antifungal agents.

Among the organisms listed in Table 5.2, species-specific probes have been constructed for *Pa. brasiliensis* (Sandhu *et al.,* 1997; Lindsley *et al.,* 2001) and *A. flavus* (Sandhu *et al.,* 1995), but the cross-hybridization of these probes with *P. insidiosum* had not been examined. The isolate of *L. giganteum* included in the analysis was from a mosquito larva in North Carolina, USA. Similarly, *P. insidiosum* 296 was from a mosquito larva in India. The hybridization of the probe to DNA from *P. insidiosum* 296 and not DNA from *L. giganteum* supports the distinction of these two isolates as separate species, despite their similar hosts, and also confirms the ability of *P. insidiosum* to infect insect hosts as well as mammalian hosts. It also indicated that the probe does not cross-react with a closely related genus, although only one species was tested. *Lagenidium* is closely related to *Pythium* based on *cox*II gene sequencing (Hudspeth *et al.,* 2000) and LSU rRNA gene sequencing (Riethmüller *et al.,* 2002). Lagenidiosis, a disease in canines similar to pythiosis and zygomycosis, is caused by a *Lagenidium* species, although not necessarily *L. giganteum.* It will be important to test the *P. insidiosum* probe with DNA from this species to confirm a lack of cross-hybridization.

The *P. insidiosum Hin*fI fragment probe may have several practical applications in clinical and diagnostic settings. The probe can be used to classify an organism as *P.*

insidiosum from suspected cases of pythiosis, and distinguish it from pathogenically related Zygomycetes, Ascomycetes, and algae, provided a pure culture of the infectious agent is available. It can also be useful to confirm the positive diagnosis of pythiosis from previous histopathological examinations and ID or ELISA tests, which have been shown to produce conflicting results.

However, it may take several days to obtain a pure culture from an infection. The ability of the probe to detect *P. insidiosum* in infected tissue sections would be of great significance as this would avoid the need to obtain pure cultures or perform DNA extractions. As well, the probe would be very useful if it could monitor a patient's response to therapy by detecting the presence or absence of the organism in blood or tissue samples. Fluorescent in situ hybridization (FISH) using an 18S rRNA gene oligonucleotide probe has been shown to have the ability to identify *Candida* species in experimentally infected tissues and blood of humans and mice (Lischewski *et al.,* 1997). The ability of the probe to detect *P. insidiosum* DNA in total DNA isolated from infected tissues or blood would also be advantageous. For this to be practical though, the specificity of the probe would have to be tested against genomic DNA from bacteria and other pathogenic fungi that may be present in an infection, as well as against the DNA from the animal host, to ensure cross-hybridization of the probe does not occur. These culture-independent methods for detecting *P. insidiosum* with the DNA probe would decrease the time required for diagnosis of the disease, and allow the appropriate therapy to proceed without lengthy delays.

The fact that the *P. insidiosum* probe did not cross-react with other *Pythium* species is significant as well. A common route of infection of *P. insidiosum* is by zoospores through small cuts or body cavities of individuals who spend time in or drink from stagnant swampy waters or fields. Many fungal species, including *Pythium* species, could thrive in such environments. The probe could therefore serve as a diagnostic tool to detect *P. insidiosum* in such environments and to differentiate it from other *Pythium* species that may be present and harmless to animal hosts. Commercial kits are available that use ELISA to detect *Pythium* and *Phytophthora* zoospores in irrigation water (Ali-Shtayeh *et al.,* 1991). As well, polyclonal antibodies (Addepalli and Fujita, 2001) and competitive PCR (Park *et al.,* 2001) have been used to detect the zoospores of *P. porphyrae* in seawater from *Porphyra* cultivation farms. Similarly, the DNA probe for *P. insidiosum* could provide a method for detecting *P. insidiosum* zoospores in contaminated water sources which are frequented by humans and animals.

The identification of a pathogenic species is essential so than an appropriate clinical decision can be made concerning the significance of the isolate, the therapeutic approach to be employed, and the dosage and duration of the appropriate therapy. The species-specific probe for *P. insidiosum* presented here provides a molecular biological diagnostic method which can be used, in conjunction with present clinical tools, to diagnose cases of pythiosis with greater confidence and accuracy. As well, the probe has the potential to be used as a tool to screen tissue and blood samples and environment sources that may harbour the infectious organism itself. The IGS therefore is a region of rDNA that is a potential source of species-specific probes and should not be overlooked.

CONCLUSIONS

This thesis entailed an exhaustive molecular survey of over 90 *Pythium* species using RFLP analysis of the IGS to identify species and reveal species relationships. RFLP analysis of the IGS provided an inexpensive and rapid molecular biological technique that can be used to screen isolates for species identification. In the process, relationships among morphologically similar species were uncovered and misidentified isolates were revealed. The IGS not only provided a region with a low level of intraspecific variation, but also a region with sufficient sequence variation to distinguish morphologically similar species from one another. However, the discovery of 14 groupings of species which appeared to be conspecific based on RFLP analysis suggests that the morphological species concept alone may not be sufficient to delimit species in *Pythium.* Instead, a phylogenetic species concept may be more applicable. That is not to say, however, that morphology should be ignored as a criterion to classify species in this genus. Rather, the genetic relationships presented here often identified which morphological features were of greater taxonomic value than others among morphologically similar species. Morphology, on the other hand, is also important as it can be used with the molecular data to determine objectively where to place the limit on what a phylogenetic species is within the genus. When both species concepts are applied in conjunction, they can complement each other and compensate for each other's shortcomings.

The molecular analysis of isolates of *P. insidiosum* using RFLP analysis of the IGS and ITS sequencing uncovered the existence of three genetic clusters of *P. insidiosum* isolates which exhibited a high degree of geographic isolation. The sequence

data also showed the phylogenetic relationships of *P. insidiosum* isolates relative to other *Pythium* species and related genera. The fact that no morphological variation had previously been observed among these isolates, and that no additional physiological variability is known, underlines the importance of using the phylogenetic species concept in this genus. The results from this study suggest the existence of geographic variants within *P. insidiosum,* or the existence of cryptic speciation within this species. An understanding of intraspecific variation in *P. insidiosum* is crucial for the successful diagnosis and treatment of pythiosis in animals and humans. Despite the intraspecific genetic variation within this species, the species-specific probe constructed in this thesis provided a diagnostic tool that hybridized to the genomic DNA of any isolate of *P. insidiosum*. The specificity of the probe for *P. insidiosum,* and the lack of cross-hybridization with DNA from other *Pythium* species and taxa which cause symptoms similar to pythiosis, suggests that attempts to use this probe as a diagnostic tool in clinical trials or to screen environmental water samples should be successful.

The identification of *Pythium* isolates to the species level is crucial as many species are pathogenic to economically important crops, animals, and even humans. The data accumulated from this thesis helps to clarify species boundaries within the genus and to establish groupings of species that are related both morphologically and phylogenetically. The work presented here will have a major impact on future taxonomic keys and species descriptions in an upcoming edition of the genus monograph, and will also influence how a *Pythium* species is defined.

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