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# Effets de fondation et différenciation génétique aux échelles continentale et locale chez *Mycosphaerella fijiensis*, champignon responsable de la maladie des raies noires du bananier

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# INTRODUCTION GENERALE

L'évolution des populations de champignons phytopathogènes entraîne généralement un contournement ou une érosion des résistances variétales dont l'efficacité pour le contrôle des épidémies est perdue ou réduite au cours du temps (McDonald & Linde 2002; Mundt *et al.* 2002). Cette évolution conditionne ainsi la durabilité des résistances variétales. Les principaux objectifs de l'étude des populations de ces champignons sont de décrire leur structure et de comprendre leur évolution. Décrire la structure des populations d'une espèce donnée revient à estimer le niveau et la distribution de la diversité au sein des populations dans l'espace et le temps. La compréhension de l'évolution de ces populations passe par une évaluation de l'importance relative des différents facteurs évolutifs (mutation, recombinaison, dérive génétique, flux de gènes, sélection). La génétique des populations de champignons phytopathogènes s'est développée surtout au cours de la dernière décennie. Ces modèles biologiques présentent des particularités telles que : des populations à larges effectifs, des capacités de dispersion pouvant être importantes, des évènements d'extinction et de colonisation fréquents, une reproduction sexuée et/ou asexuée, une forte sélection due à l'interaction avec un ou des hôte(s) (Burdon 1992; McDonald & Linde 2002).

L'étude des populations pathogènes dans les systèmes agronomiques a été abordée au travers de deux approches complémentaires. La première approche consiste à analyser la structure spatiale des populations à différentes échelles géographiques, à l'aide de marqueurs moléculaires neutres. Les structures observées résultant de l'action combinée des facteurs évolutifs, de telles études renseignent sur l'importance relative de certains facteurs avec en particulier : la recombinaison la dérive génétique et les flux de gènes. La plupart des travaux portant sur des populations de champignon phytopathogènes, publiés au cours de la dernière décennie, ont été réalisés selon cette approche (McDonald & Linde 2002). C'est à partir du pathosystème *Mycosphaerella graminicola*-blé que les études les plus complètes ont été publiées à ce jour (Zhan *et al.* 2002). La seconde approche correspond au suivi de l'évolution de populations pathogènes au cours du temps sur des peuplements hôtes résistants. Les échantillons sont analysés à l'aide d'une évaluation du pouvoir pathogène et par utilisation ou non de marqueurs moléculaires. De telles études apportent plus spécifiquement des renseignements sur l'effet de la sélection par l'hôte sur l'évolution des populations. Des travaux réalisés selon cette approche ont été publiés récemment (Cowger & Mundt 2002; Zhan *et al.* 2002).

Une grande majorité des travaux réalisés concerne donc des milieux cultivés correspondant souvent à des cultures monovariétales sur de grandes surfaces. Il n'existe que quelques exemples d'études de populations pathogènes, dans le temps et dans l'espace, en milieu naturel (Sicard *et al.* 1997; Thrall & Burdon 2002). Ces études, menées à partir de contextes écologiques très différents par rapport au milieux cultivés, apportent des informations utiles pour la recherche de résistances et pour la compréhension des mécanismes impliqués dans l'évolution des populations pathogènes (Burdon 1993).

L'étude des populations des agents phytopathogènes auront des implications pour la création et la gestion de variétés résistantes. La superposition de la structure spatiale des populations d'un parasite donné et de son hôte en milieu naturel permet de localiser les zones géographiques où la diversité est importante pour les deux partenaires. Ces zones géographiques pourraient correspondre à des zones de co-évolution, et ainsi à des sources potentielles de résistances (Lenné & Wood 1991; Linde *et al.* 2002). La localisation de telles sources de résistances, et l'exploitation de la biodiversité de l'hôte apparaissent incontournables dans l'avenir pour une gestion dans le temps et l'espace d'un certain nombre de résistances différentes. Les résistances doivent aussi être sélectionnées telles qu'elles soient efficaces, au moins vis-à-vis de certaines populations pathogènes. La description de la structure spatiale des populations permettra de choisir des sites pour mener cette sélection. Pour un agent pathogène donné, sa diversité au sein de ces sites devra être représentative de celle existante dans la ou les zones où les résistances seront utilisées (Leung *et al.* 1993).

En tenant compte du potentiel adaptatif des populations pathogènes, il devrait être possible de définir des stratégies de gestion des résistances assurant la durabilité d'une culture dans un lieu donné. Cette gestion se baserait sur une utilisation de différentes résistances dans l'espace et le temps, l'efficacité de ces résistances pouvant varier selon ces deux dimensions. Nous tenterons ainsi de limiter ou de canaliser l'évolution des parasites en jouant indirectement sur l'importance de certains facteurs. Par exemple, le maintien de discontinuités dans le paysage devrait limiter la dispersion et les flux de gènes entre populations pathogènes établies, limitant ainsi la diffusion de gènes impliqués dans le contournement ou l'érosion des résistances. La sélection par l'hôte pourrait varier dans le temps et l'espace en fonction de la distribution des résistances selon ces deux dimensions.

Le parasite étudié dans notre étude est le champignon ascomycète *Mycosphaerella fijiensis* (forme imparfaite *Paracercospora fijiensis*) responsable de la maladie des raies noires du bananier. Les épidémies de ce parasite dans toute la zone de production tropicale se

sont déclarées dans la deuxième moitié du siècle précédent et sont donc récentes (Pasberg-Gauhl *et al.* 2000). La maladie des raies noires constitue aujourd'hui l'une des principales contraintes de la culture du bananier (Jones 2000). Le contrôle de cette maladie se fait aujourd'hui uniquement à l'aide de fongicides en plantations industrielles destinées à l'exportation. Les nombreuses applications de produits nécessaires ont des effets néfastes sur l'environnement et la santé humaine. Mais surtout, les fongicides ne peuvent être utilisés par les petits producteurs à cause de leur coût élevé, alors que cette production représente plus de 80% de la production mondiale de banane et qu'elle constitue la base alimentaire de nombreux pays tropicaux (Lescot 1999).

L'intégration de bananiers résistants dans les systèmes de culture apparaît donc indispensable, voire même incontournable. Des programmes d'amélioration génétique du bananier sont en cours et de nouvelles variétés résistantes sont, soit déjà diffusées, soit en cours de validation (Bakry 2001). Des recherches sur l'interaction hôte-parasite à différents niveaux d'intégration biologique reste cependant nécessaire aussi bien pour la création de bananiers résistants efficaces, que pour la définition d'une gestion durable des résistances utilisées. Les études de populations présentées dans ce travail sont orientées vers cette dernière application.

*M. fijiensis* présente un cycle asexué avec la production de conidie (forme imparfaite) et un cycle sexué avec la production d'ascospore (forme ascomycète). Il est haploïde et hétérotallique ce qui sous-entend une recombinaison génétique importante (Carlier *et al.* 2000). La dissémination de la maladie s'effectue par les conidies, les ascospores et le mouvement de matériel végétal infecté (Gauhl *et al.* 2000). L'importance relative de ces différents modes de dissémination dépend de la distance. Les conidies sont dispersées sur des distances très courtes, les ascospores sur des distances courtes et moyennes alors que le matériel végétal infecté peut être transporté d'un continent à l'autre. La structure des populations de *M. fijiensis* a été décrite l'échelle mondiale (Carlier *et al.* 1996). Des échantillons provenant des différentes zones de production (Sud Est Asiatique, Afrique, Amérique Latine et Pacifique) ont été analysés à l'aide de marqueurs mono-locus RFLP (restriction fragments length polymorphism). Cette étude a permis de mettre en évidence chez *M. fijiensis* : (i) un centre de diversité et d'origine situé dans le Sud Est Asiatique comme son hôte (ii) le maintient d'une importante diversité génétique, (iii) l'existence de populations en partie issus d'une reproduction sexuée et panmictique, (iv) l'existence d'une différenciation génétique entre populations résultant d'effets de fondation intervenus lors de la dissémination

de la maladie à travers le monde. Ces résultats supportent l'hypothèse d'une dissémination de la maladie à l'échelle mondiale au travers de mouvements occasionnels de matériel végétal infecté. La structure des populations, les flux de gènes et les processus de dispersions ne sont pas connus à des échelles géographiques inférieures.

Les objectifs principaux du présent travail sont de décrire la structure de populations de *M. fijiensis* aux échelles continentales et locales, et d'appréhender les flux gènes et les processus de dispersion de cet agent pathogène à ces deux d'échelles.

Le premier chapitre présentera en détail le pathosystème étudié. Le deuxième correspond aux études réalisées à l'échelle continentale en Afrique et en Amérique Latine/ Caraïbes, à partir d'échantillons provenant de plusieurs pays pour chacune des régions et à l'aide de marqueurs moléculaires CAPS (Caractérized Amplified Polymorphic Sequences) et microsatellites. L'existence d'une différenciation génétique importante ayant été mise en évidence à l'échelle continentale, le troisième chapitre présentera une étude réalisée à l'échelle d'un pays, au Costa Rica. Des échantillons provenant de différentes localités ont été analysés à l'aide des même marqueurs moléculaires et nous avons tenté d'estimer plus précisément la dispersion et les flux de gènes à l'aide d'un modèle génétique.

# CHAPITRE 1

## BLACK LEAF STREAK DISEASE OF BANANAS CAUSED BY THE FUNGUS *MYCOSPHAERELLA FIJIENSIS*. A RECENT DESTRUCTIVE EPIDEMIC AT GLOBAL SCALE

### Introduction

With a total world production of 88 million tonnes, bananas and plantains is one of the most important global crop for food security. Plantains, other cooking bananas and various types of dessert bananas, are a basic food source in many regions around the world (Sharrock and Frison 1999). Pest and disease pressures affecting bananas have increased considerably in recent years, and a number of important pathogens are causing severe yield losses worldwide. Black leaf streak disease, caused by the fungus *Mycosphaerella fijiensis* is considered the most serious constraint to plantain and banana production globally. This pathogen can cause severe leaf necrosis, reducing yields by 30-50% and many important and widely grown cultivars are susceptible (Stover and Simmonds 1987).

Chemical control of black Sigatoka is not only expensive and beyond the means of most small-scale producers, but it is also extremely damaging to the environment. Moreover, the harmful effects of chemical use on plantation workers health is of great concern. In Costa Rica, over-use of chemicals for export bananas production has resulted in the development of fungicide-resistant pathogen populations. Furthermore, small-scale plantain production has been reduced by 40% as a result of this disease as farmers are unable to afford high costs of fungicides needed to control it (Frison and Sharrock 1998).

In the future, development of sustainable banana and plantain production will utilize resistant varieties. Use of resistant varieties not only reduces production costs, but it also benefits the environment as pesticides polluting effects are reduced and workers themselves are not exposed to health-damaging chemicals (Rosales and Pocasangre 2002).

## 1.The genus *Musa*

### 1.1 Importance

Bananas are grown in every humid tropical region and constitute the 4th largest fruit crop in the world, following grapes, citrus fruits and apples (FAO 2000). Although it is viewed only as a dessert or an addition to breakfast cereal in most developed countries, it is actually a very important agricultural product. After rice, wheat and milk, it is the fourth most valuable food. Banana harvested area in the world reaches approximately 10 million hectares, with an annual production of almost 88 million metric tonnes. As export crop, it ranks fourth among all agricultural commodities and it is the most significant of all fruits, with an annual world trade totaling $2.5 billion. Yet, only 10% of the annual 88 million tons global output enters the international market. Subsistence farmers for local markets in Africa, America and Asia produce much of the remaining harvest. For most of these domestic consumers, banana and plantain are staple foods that represent major dietary sources of carbohydrates, fibber, vitamins A, B6 and C, potassium, phosphorus and calcium (Rosales *et al.* 1998).

The 20 top banana and plantain producers in 1999 are showed in Table 1. Banana and plantain production at global level is characterized by diversity. This diversity manifests itself in the varieties produced, the way they are prepared, eaten and marketed, and the systems in which they are cultivated (Sharrock & Frison 1999). In Africa, this crop provides more than 25% of food energy requirements for around 70 million people. East Africa alone produces around 15 million tons annually and in this region bananas reach their greatest importance as staple food crop (Table 2). In countries such as Uganda, Burundi and Rwanda annual per capita consumption has been estimated in 220-440 kg, the highest in the world. Bananas and plantains are also a staple food crop in much of West and Central Africa. Around 10 million tonnes of the crop are produced in this region annually, 99% of which is consumed or traded locally.

In the Asia-Pacific region, bananas are the most widely produced fruit in India, Philippines, Indonesia, China and Thailand (Table 2). Ninety five percent of the region's production, some 25 million tons annually, is consumed or marketed locally. In the Pacific, although the banana industry is small in absolute terms, in some countries its importance is great considering the national economy and welfare of individual growers.

**Table 1**

The 20 largest banana and plantain producers in 1999 (metric Tonnes) in the world (FAO 2000).

| Country | Production (mT) | Country | Production (mT) |
|---|---|---|---|
| India | 13 900 000 | Rwanda | 2 212 250 |
| Uganda | 10 143 000 | CongoDemocratic Republic of | 2 112 000 |
| Ecuador | 7 291 724 | Ghana | 2 061 240 |
| Brazil | 6 339 350 | Nigeria | 1 902 000 |
| China | 4 812 531 | Mexico | 1 802 278 |
| Colombia | 4 259 000 | Thailand | 1 720 000 |
| Philippines | 4 155 668 | Côte d'Ivoire | 1 646 458 |
| Indonesia | 3 376 660 | Venezuela | 1 551 300 |
| Costa Rica | 2 790 000 | Burundi | 1 513 997 |
| Cameroon | 2 253 000 | Peru | 1 414 900 |

**Table 2**

Banana and plantain production and exports 1999 (metric Tonnes) in Africa, Asia and Latin America (FAO 2000).

| Continent/Country | Total production 1999, MT | Total export 1999, MT |
|---|---|---|
| *Africa* | | |
| Uganda | 10 143 000 | 752 |
| Cameroon | 2 253 000 | 165 000 |
| Rwanda | 2 212 250 | - |
| Congo Dem. Republic of | 2 112 000 | - |
| Ghana | 2 061 240 | 2 844 |
| Nigeria | 1 902 000 | - |
| Côte d'Ivoire | 1 646 458 | 215 000 |
| Burundi | 1 513 997 | - |
| Tanzania | 1 304 756 | - |
| Kenya | 580 000 | - |
| *Asia* | | |
| India | 13 900 000 | 8 111 |
| Philippines | 4 155 668 | 1 319 632 |
| Indonesia | 3 376 660 | 76 087 |
| China | 4 812 531 | 57 274 |
| Thailand | 1 720 000 | 6 795 |
| *Latin America* | | |
| Ecuador | 7 291 724 | 4 056 141 |
| Costa Rica | 2 790 000 | 2 557 000 |
| Colombia | 4 259 000 | 1 865 675 |
| Panama | 918 382 | 596 900 |
| Guatemala | 802 545 | 576 900 |
| Mexico | 1 802 278 | 174 131 |
| Honduras | 702 578 | 155 200 |

Bananas grown for export are produced in Latin America and the Caribbean and this crop is of major socio-economic importance to the region. Even in this region, export production constitutes only around 30% of total production, and much of this is still grown by small-scale farmers. In the Caribbean, three of the poorest countries, Cuba, Haiti and the Dominican Republic produce some 1.5 million tonnes of bananas and plantains annually, 64% of the sub-region's total, of which less than 1% is exported. Ecuador, Costa Rica and Colombia are the major exporting countries (Table 2).

Bananas produced for export are almost exclusively from one cultivar – 'Cavendish' (AAA) – it accounts for a little more than 13% of global banana and plantain production. The remaining 87% or so is made up of a very wide range of varieties, each adapted to a specific eco-region and selected for specific eating or cooking qualities. These include the true plantains (AAB) of West Africa and Central and South America, which are cooked by frying, boiling or roasting them when they are green or ripe; the highland bananas (AAA) of East Africa, which are generally steamed to make 'Matooke', but are also used for beer distilling; the cooking bananas (ABB) and sweet acid dessert bananas (AAB) of Southeast Asia and the Americas; and the Pacific Maia Maoli/Popoulu (AAB) type of cooking-banana (Fig. 1) (Frison & Sharrock 1998).

### 1.2 Biology

The banana plant, often erroneously referred to as a "tree", is a large herb, with succulent, very juicy stem (properly "pseudostem") which is a cylinder of leaf-petiole sheaths, reaching a height of 6-7.5 m arising from a fleshy rhizome or corm (Fig.2) (Morton 1987). Cultivated banana exhibits parthenocarpic fruit development, a marked degree of sterility, and vegetative propagation. Wild banana presents sexual reproduction and vegetative propagation by ratooning (Horry *et al.* 1997).

### 1.3 Systematic

Banana plant is a large monocotyledonous herb from the Musaceae family and Zingiberales order that originated in Southeast Asia. Virtually all grown cultivars are thought to have been selected as naturally occurring hybrids in this region by the earliest of farmers (Champion 1963). The genus *Musa* is divided in five sections: Australimusa, Callimusa,

**Figure 1**

Main varieties of bananas grown worldwide (Sharrock & Frison 1999).

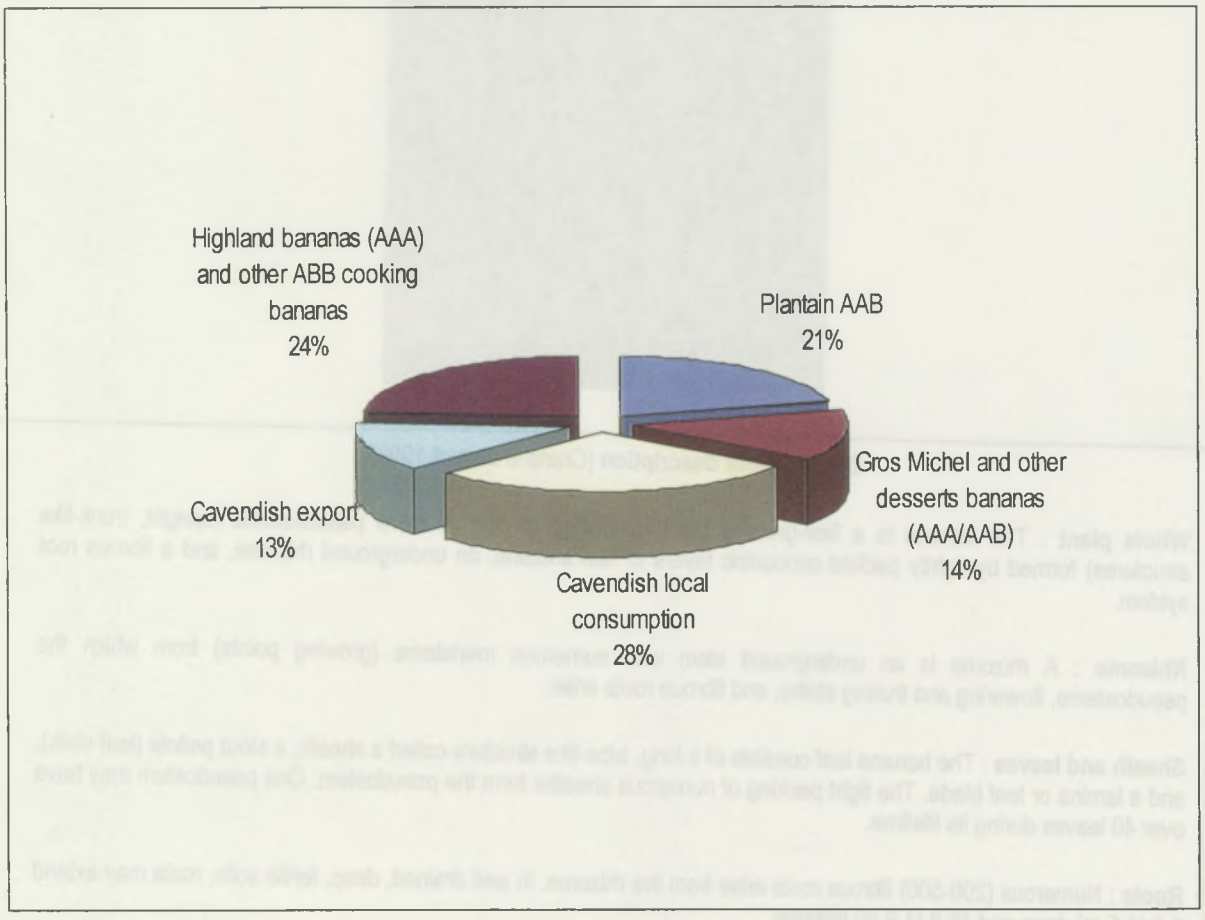


Figure 2

Horn plantain (AAB) growing in Costa Rica (photo: G.G. Rivas, CATIE).

**Banana plant description** (Crane & Balerdi 1998)

**Whole plant** : The banana is a fast-growing plant consisting of one or more pseudostems (upright, trunk-like structures) formed by tightly packed concentric layers of leaf sheaths, an underground rhizome, and a fibrous root system.

**Rhizome** : A rhizome is an underground stem with numerous meristems (growing points) from which the pseudostems, flowering and fruiting stalks, and fibrous roots arise.

**Sheath and leaves** : The banana leaf consists of a long, tube-like structure called a sheath, a stout petiole (leaf stalk), and a lamina or leaf blade. The tight packing of numerous sheaths form the pseudostem. One pseudostem may have over 40 leaves during its lifetime.

**Roots** : Numerous (200-500) fibrous roots arise from the rhizome. In well drained, deep, fertile soils, roots may extend 5 ft (1.5 m) deep and 16 ft (4.9 m) laterally.

**Flowers and fruit** : The banana inflorescence (flowering stalk) emerges from the center of the pseudostem 10 to 15 months after planting; by this time 26 to 32 leaves have been produced. The process of banana flowering is called shooting. The flowers appear spirally along the axis of the inflorescence in groups of 10 to 20, covered by purplish-to-greenish fleshy bracts which shed as flowering development progresses. The first flowers to emerge are functionally female. In the edible cultivars, the rapidly growing ovaries develop parthenocarpically (without pollination) into clusters of fruits, called "hands." Although most banana cultivars produce seedless fruit, some are fertile and can set seed. The last flowers to emerge are functionally male. In plantains, the male part of the inflorescence and/or male flowers may be absent or greatly reduced. The time from shooting to fruit harvest depends upon temperature, cultivar, soil moisture, and cultural practices and ranges from 80 to 180 days.

Rhodochlamys, Eumusa and Ingentimusa (Table 3). Eumusa comprises 13 to 15 species, they are diploids with a basic number=11 of chromosomes. Three of these species are of particular importance: *M. acuminata* and *M. balbisiana* are the source of all cultivars and *M. schizocarpa* could be involved in the evolution of certain cultivars (Horry 1993). Most cultivated bananas and their wild relatives belong to section Eumusa of the genus *Musa.*

Eumusa cultivars are derived from two infertile species, *M. acuminata* and *M. balbisiana*, which supply the A and B genomes, respectively. Although diploid AA and AB forms exist, triploid forms (AAA, AAB and ABB) are more common. The cultivated tetraploid forms usually originate from breeding programs. The three groups of triploid cultivars are: AAA, only *acuminata* characters; AAB, predominantly *acuminata* characters; and ABB, predominantly *balbisiana* characters (Horry 1993).

### 1.4 Geographic distribution and genetic diversity

The evolution of main genomic groups of edible banana cultivars of Eumusa series is showed in figure 3 and figure 4 shows the geographical distribution of *Musa* spp. in the world. Southeast Asia is the origin centre of the genus *Musa* (Simmonds 1962). *Rhodochlamys* is restricted to Assam, Thailand, Burma; *Callimusa* to Borneo, Malaysia and Indochina; *Australimusa* to Borneo and Indonesia, The Philippines, Australia and the Pacific; and *Eumusa* to the whole area (Fig. 5 and 6). Genetic diversity in wild and cultivated bananas is the result of inter and intraspecific crosses, vegetative multiplication, and a long domestication process (Champion 1963).

Bananas diversity is greatest in South East Asia, where the crop had its origin. It is here that wild parents of cultivated banana are found. The banana belongs to a group of probably more than 30 species known scientifically under the generic name of *Musa*. In their wild state, they are distributed in forest habitats in a range that extends from Australia and the Pacific to northern India. Little is known about the diversity and status of wild species, mainly because some of the areas where they are at their richest are poorly explored by botanists, and somewhat remote and dangerous to visit. Perhaps, the best known is the parent species of the cultivated bananas, *Musa acuminata* and *M. balbisiana*. The first has been divided into nine distinct subspecies, centered in Indonesia and Malaysia. It is thought that they appeared as a

**Table 3**

Systematics of the family *Musaceae* (Horry *et al.* 1997).

| Family | Genus | Sections | Species | Subspecies |
|---|---|---|---|---|
| Musaceae | Musa | Callimusa (2n=20) (Indochina, Malasya, Myanmar) | *beccari* | |
| | | | *borneensis* | |
| | | | *coccinea* | |
| | | | *gracilis* | |
| | | Australimusa (2n=20) (Queensland to Philippines) | *textilis* | |
| | | | *angustigemma* | |
| | | | *bukensis* | |
| | | | *jackeyi* | |
| | | | *lolodensis* | |
| | | | *maclayi* | |
| | | | *peekelii* | |
| | | Eumusa (2n=22) (Southern India to Japan and Samoa) | *acuminata* | *a. burmanica* |
| | | | | *a.burmanicoides* |
| | | | | *a. siamea* |
| | | | | *a. malaccensis* |
| | | | | *a. truncata* |
| | | | | *a. microcarpa* |
| | | | | *a. banskii* |
| | | | | *a. errans* |
| | | | | *a. zebrina* |
| | | | *balbisiana* | |
| | | | *basjoo* | |
| | | | *cheesmani* | |
| | | | *flaviflora* | |
| | | | *halabanensis* | |
| | | | *itinerans* | |
| | | | *nagensium* | |
| | | | *schizocarpa* | |
| | | | *sikkimensis* | |
| | | | *sumatrana* | |
| | | Rhodochlamys (2n=22) (India to Indo-China) | *laterita* | |
| | | | *ornata* | |
| | | | *sanguinea* | |
| | | | *velutina* | |

**Figure 3**

Evolution of edible banana cultivars of the Eumusa series
( After Simmonds 1962, Carreel, 1995).

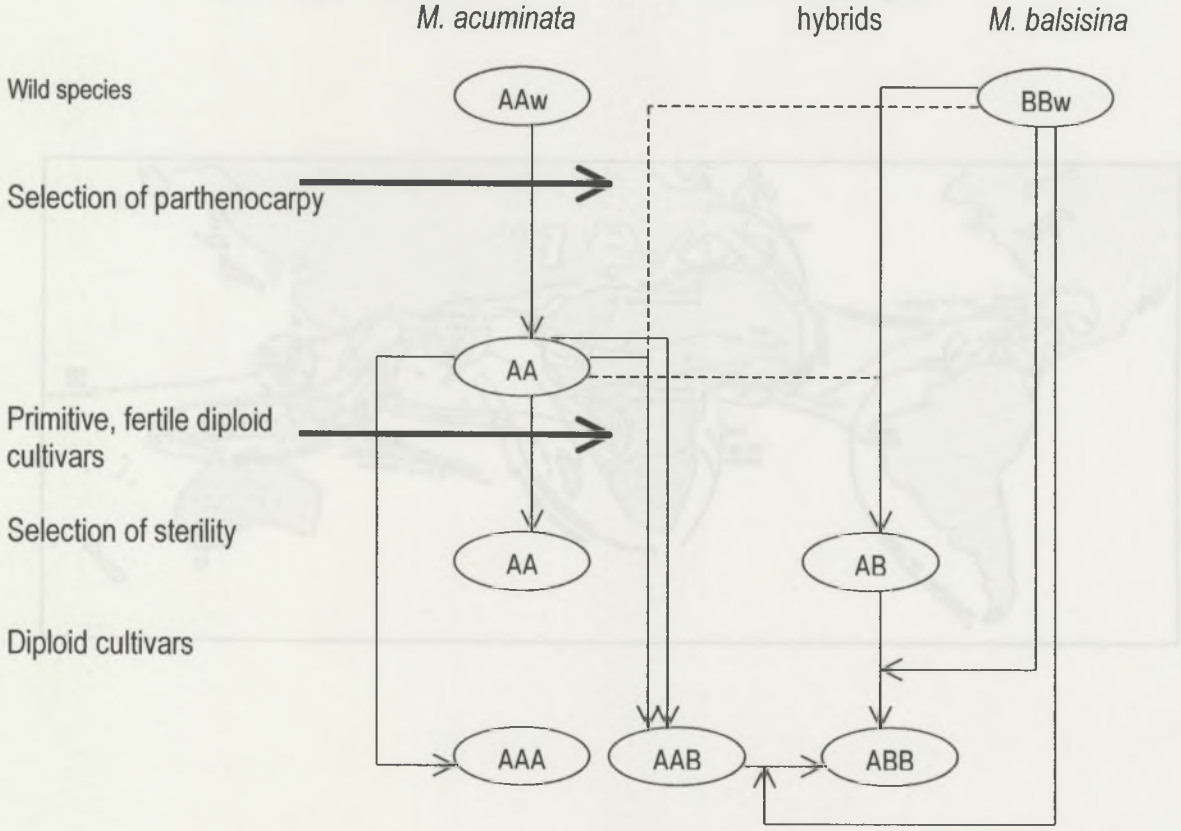


**Figure 4**

Dispersion of bananas in the world (After Horry 1989).

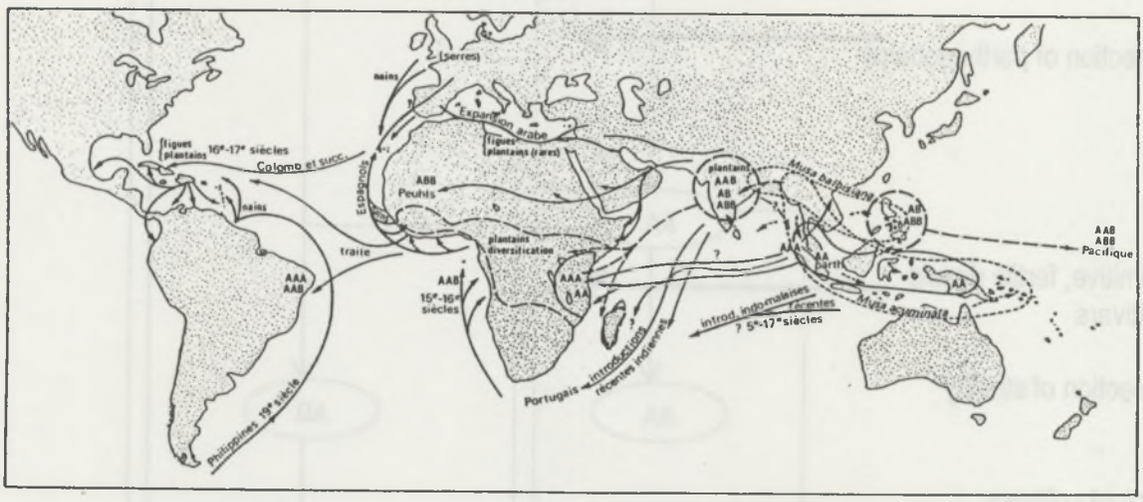


**Figure 5**

Geographic distribution of the *Musa acuminata* subspecies

(Simmonds 1962, Horry *et al.* 1997).

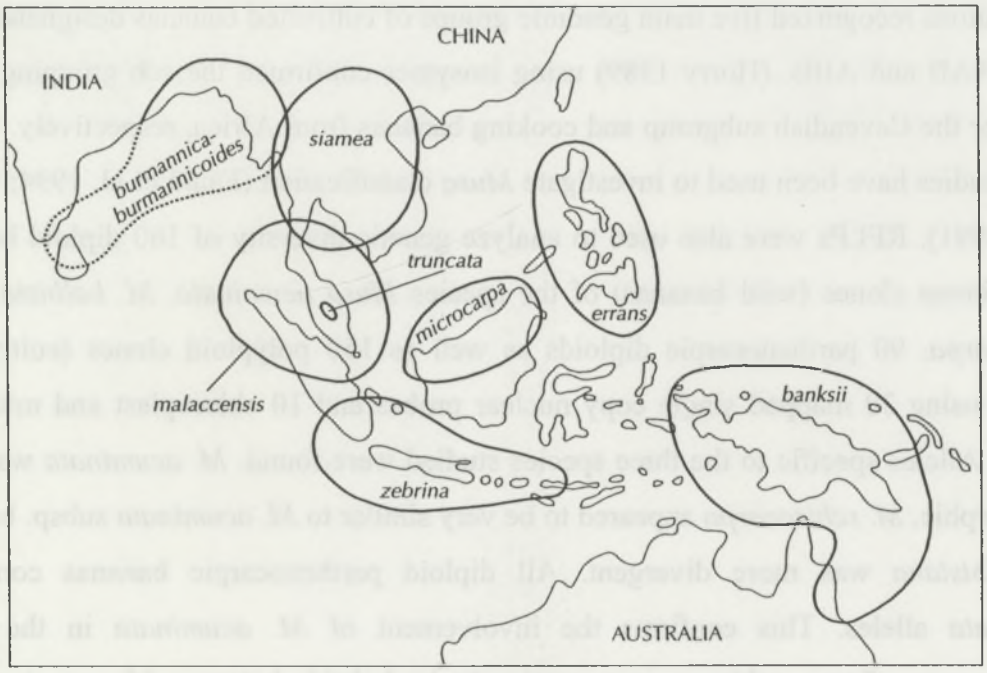
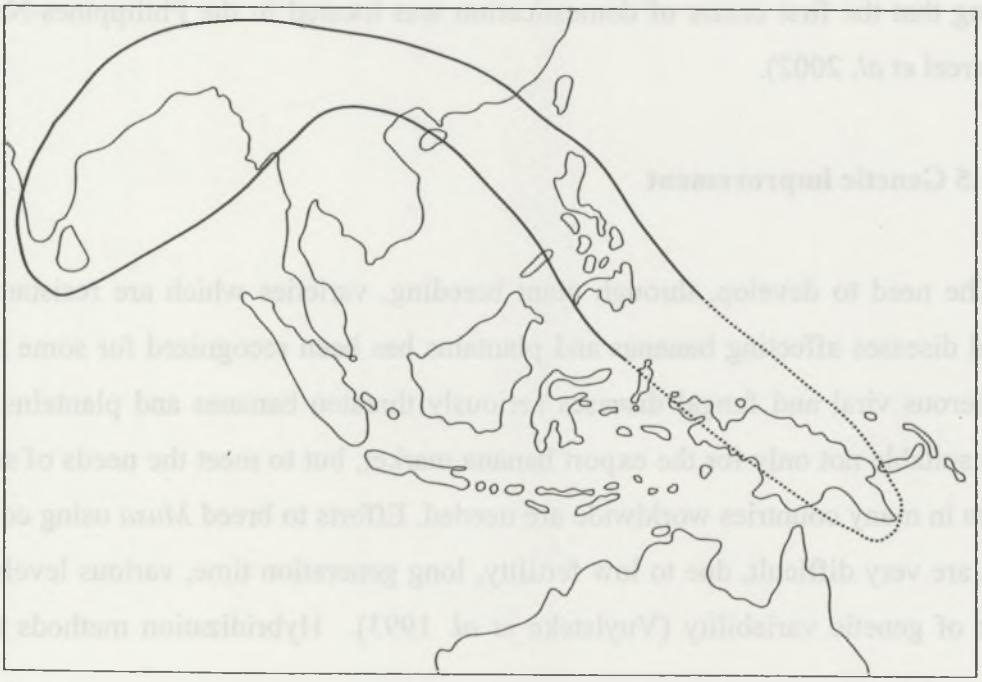



**Figure 6**

Geographic distribution of the *Musa balbisiana* subspecies

(Simmonds 1962).



result of the development of natural reproductive barriers within species, causing several subspecies to diverge genetically (Horry *et al.* 1997).

Simmonds and Shepherd (Simmonds & Shepherd 1955), using morphological observations recognized five main genomic groups of cultivated bananas designated AA, AB, AAA, AAB and ABB. (Horry 1989) using isosymes confirmed the sub grouping AAA and AAB for the Cavendish subgroup and cooking bananas from Africa, respectively. Cytoplasm DNA studies have been used to investigate *Musa* classification (Fauré et al. 1994; Gawel and Jarret 1991). RFLPs were also used to analyze genetic diversity of 160 diploid bananas: 70 seminiferous clones (wild bananas) of the species *Musa acuminata*, *M. balbisiana* and *M. schizocarpa*, 90 parthenocarpic diploids as well as 150 polyploid clones (cultivars) were studied using 30 mapped single copy nuclear probes and 10 chloroplast and mitochondrial probes. Alleles specific to the three species studied were found. *M. acuminata* was the most polymorphic. *M. schizocarpa* appeared to be very similar to *M. acuminata* subsp. banksii, and *M. balbisiana* was more divergent. All diploid parthenocarpic bananas contained *M. acuminata* alleles. This confirms the involvement of *M. acuminata* in the origin of parthenocarpy. Some cultivars were proven to be hybrids between *M. acuminata* and *M. schizocarpa*. "Starchy" cultivars were found to be closely associated with *M. acuminata* subsp. *banksii*, whereas "sweet" cultivars were close to *M. acuminata* subsp. *malaccensis* (Carreel 1995). A mitochondrial DNA RFLP analyses of banana showed that parthenocarpic varieties of *Musa* are linked to *M. acuminata banksii* and *M. acuminata errans*, thus suggesting that the first centre of domestication was located in the Philippines-New Guinea area (Carreel *et al.* 2002).

### 1.5 Genetic improvement

The need to develop, through plant breeding, varieties which are resistant to major pests and diseases affecting bananas and plantains has been recognized for some time. Pests and numerous viral and fungal diseases seriously threaten bananas and plantains. Resistant varieties suitable not only for the export banana market, but to meet the needs of smallholder producers in many countries worldwide are needed. Efforts to breed *Musa* using conventional methods are very difficult, due to low fertility, long generation time, various levels of ploidy and lack of genetic variability (Vuylsteke *et al.* 1993). Hybridization methods for banana

improvement used actually produce either triploid or tetraploid varieties (Fig; 7 a and b)(Bakry 2001).

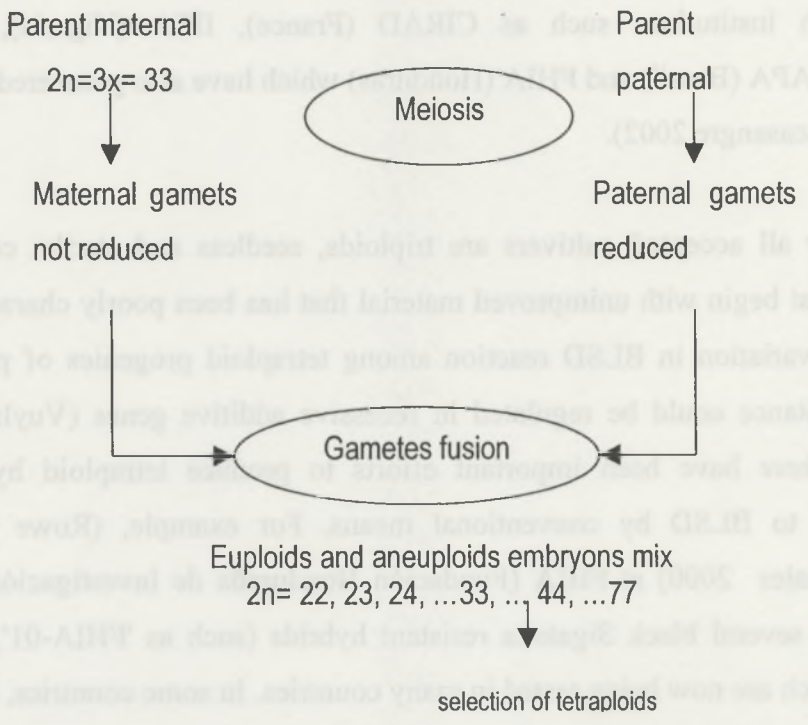
The main objective of improvement programs is to develop cultivars resistant to pest and diseases such as black Sigatoka, Panama disease, Moko, bacterial withering, nematodes and others. Besides that, it is sought to obtain high fruit yielding varieties with characteristics attractive to the market. Development of Musa improvement programs continues as a priority to several research institutions such as CIRAD (France), IITA (Nigeria), CARBAP (Cameroon) EMBRAPA (Brazil) and FHIA (Honduras) which have also pioneered this line of work (Rosales & Pocasangre 2002).

Since nearly all accepted cultivars are triploids, seedless and sterile, conventional breeding efforts must begin with unimproved material that has been poorly characterized for genetic traits. The variation in BLSD reaction among tetraploid progenies of plantain has suggested that resistance could be regulated in recessive additive genes (Vuylsteke *et al.* 1993). However, there have been important efforts to produce tetraploid hybrids with resistance/tolerance to BLSD by conventional means. For example, (Rowe & Rosales 1993)(Rowe & Rosales 2000) at FHIA (Fundación Hondureña de Investigación Agrícola, Honduras) released several black Sigatoka resistant hybrids (such as 'FHIA-01', 'FHIA-18' and 'FHIA-21'), which are now being tested in many countries. In some countries, farmers are already cultivating these hybrids on a wide scale, but acceptance for export markets has not been possible and their use by local people has had mixed results. Similarly, BLSD-resistant plantain hybrids have been developed by IITA (International Institute of Tropical Agriculture, Nigeria) and widely distributed for evaluation in Africa (Ortiz *et al.* 1995). In spite of these results, genetic improvement of bananas by conventional means remains a slow task, and considering the scale and diversity of problems facing banana growers worldwide, *Musa* genetic improvement efforts are still too small (Vuylsteke *et al.* 1993).

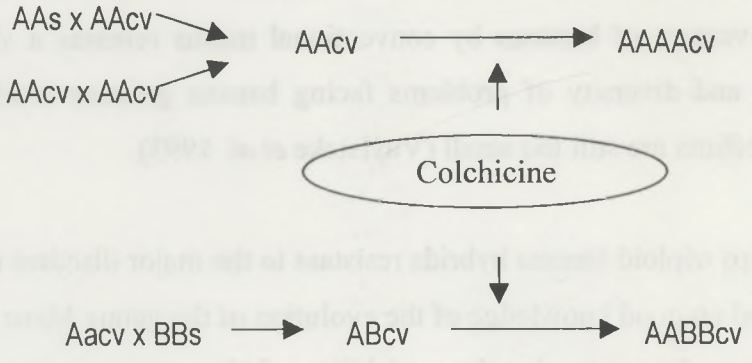
CIRAD develops triploid banana hybrids resistant to the major diseases (Bakry 2001). This procedure is based on good knowledge of the evolution of the genus *Musa* leading to the present natural varieties. For example, the variability of the *acuminata* genome permits variation in the fruit type and plant obtained, whether dessert or cooking. The triploids hybrids are obtained by simple hybridisation between a diploid parent and a tetraploid parent. The tetraploid parent has previously been obtained by doubling with colchicine an ancestral

**Figure 7**

Breeding scheme for improvement bananas a) tetraploids varieties by restitution of maternal gametes in triploid varieties, and b) triploid varieties from diploid genitors (s= wild, cv= cultivated) (Bakry 2001).




a)




b)

diploid or an improved diploid. The potential for domestic and export markets of the new triploid hybrides remains to be defined.

Other option for banana genetic improvement is genetic transformation. Two categories of transformation systems can be distinguished. The first includes indirect transformation systems of *Agrobacterium* meditated transformation (AMT). Direct gene transfer techniques fall into the second group which comprises, among other techniques, particle bombardment and protoplast electroporation. Two main systems are now routinously used for *Musa* genetic transformation at different institutions around the world: KULeueven, Belgium (Hernandez *et al.* 1999; Sagi *et al.* 1995), QUT(Australia), DNAP, USA (Higgs & May 1999).

## 2. Black leaf streak disease

### 2.1 Taxonomy and diagnosis

Black leaf streak disease (black Sigatoka) and Sigatoka disease (yellow Sigatoka) are the major banana leaf spot diseases. An ascomycete fungus *Mycosphaerella* causes both diseases. BLSD is caused by *Mycosphaerella fijiensis* Morelet (Stover 1963) an haploid and heterothallic fungus (Mourichon & Zapater 1990) and yellow Sigatoka by *M. musicola* (Stover 1963). The anamorphs of both fungi are *Paracercospora fijiensis* and *Pseudocercospora musae* (Deighton 1976; Pons 1990).

Other banana leaf spot diseases were reported recently by (Carlier *et al.* 2000). This is eumusae leaf spot disease of banana caused by *Mycosphaerella eumusae* (anamorph *Pseudocercospora eumusae*) (Crous & Mourichon 2002). At present, this pathogen causes severe plants damage and could become an important new banana disease in the tropics. This disease is present in southern India, Sri Lanka, Thailand, Malaysia, Vietnam, Mauritius and Nigeria.

Conidia of *P. fijiensis* are in average longer and more flexuous than those of *P. musae* and present a thickened basal hilum. Conidiophores are formed singly or in small groups (2-5) on lower leaf surface. They can be straight or bent, 0-3 septate and occasionally branched,

slightly thickened spore scars and conidia taper from base to apex, 1-6 septate, distinct basal scars (Pons 1990).

Conidiophores of *P. musae* are usually short and bottle shaped while *P. fijiensis* are elongated, often bent and with conspicuous conidial scars. Conidiophores formed in dense clusters (sporodochia) on dark stromata on both leaf surfaces, are straight, usually nonseptate and unbranched, no spore scars conidia uniform thickness for full length, 1-5 septate, no distinct basal scars (Pons 1990).

Because of their morphological similarities, accurate identification of these two *Mycosphaerella* species is difficult (Table 4). Pathogen isolation in pure culture is not only time consuming, but it could be confounded by the presence of other closely related fungi on the leaf. Development of reliable and safe methods for *Mycosphaerella* complex diagnosis is an important strategy for epidemiological research, particularly in surveying disease programs. An accurate and relatively simple technique known as Polymerase Chain Reaction (PCR) has been developed to identify these organisms in simple leaf extracts (Johanson & Jeger 1993). This technique uses the fungus ribosomal DNA and uses specific primers that discriminate between *Mycosphaerella* species (Johanson 1994). Amplification of ribosomal genes is used for genetic analysis of many organisms because of their ease and relatively high gene copy number; although nucleotide sequences of mature RNAs are highly conserved (Nazar *et al.* 1991). Ribosomal DNAs are composed of variously conserved and variable regions which have proven to be useful for inferring fungal relationships at different taxonomic levels (Bruns *et al.* 1991). The internal transcribed spacers (ITS) are often variable within and between species (Lee & Taylor 1991). DNA sequences which are polymoprphic between fungal species, such as ITSs are good candidates to detect a species excluding the rest (Edel 1998). For *M. fijiensis* and *M. musicola* the ITS 1 region (between the 18S and 5.8S rDNA subunits) was sequenced from different isolates by PCR using standard primers (White et al. 1990). The primers MF137 and MM137 were constructed respectively for both *Mycosphaerella* species (Johanson 1994). Analysis of sequence data has allowed to develop phylogenetic analysis of *Cercospora* and *Mycosphaerella* genera (Crous *et al.* 2001; Goodwin *et al.* 2001; Stewart *et al.* 1999). In banana plants, the ITS study has shown the relationships of *Mycosphaerella* species present in this host (Carlier *et al.*, data unpublished) and the need to develop specific primers to discriminate between analyzed species. Since ITS regions are conserved, new highly polimorphic markers such as microsatellites will facilitate future

**Table 4**

Morphological characteristics of the anamorphs of *Mycosphaer*ella leaf spot pathogens of bananas (Carlier *et al.* 2000, Crous & Mourichon 2002).

| Species (anamorph) | Conidiophores | Conidia |
|---|---|---|
| *Paracercospora fijiensis* | First appearance at early streak stage (Fouré's stages 2 to 3). | Obclavate to cylindro-obclavate or curved, Hyaline to very pale olivaceous, 1 to 10 septate, distinct basal hilum (scar). |
| | Mainly lower leaf surface. | |
| | Emerge singly or in small groups(2 to 6), sporodochia and stomata absent. | Between 30 to 132x2.5 to 5 µm. |
| *Pseudocercorspora musae* | Straight or bent geniculate; pale to ligth brown 0-5 septate, occasionaly branched, sligthly thickned spore-scars (between 16.5 to 62.5x4 to 7µm). | Cylindric to obclavate-cylindric, straigth or curved, pale to very pale olivaceous, 0 to 8 septate, no distinct basal hilum. |
| | First appearance at spot satge. | |
| | Abundant on both leaf surface. | Between 10 to 109 X 2 to 6 µm. |
| | In dense fascicles (sporodochia) on dark stromata. | |
| *Pseudocercospora eumusae* | Straigth, hyaline? Mostly without septation and geniculation; no spore scars (between 5 to 25 x 2 to 5 µm). | Fusiform, hyaline, cylindrical and curved, 3 to 5 septate. |
| | First appearance at spot stage. | Between 21.2 to 41.6 x 2.5 µm). |
| | Mainly on the upper leaf surface. | |
| | Pear-shaped, immersed, more or less erumpent, ostiolated when young but often acervular-like when mature(31 to 42 µm). | |

diagnosis of these pathogens group and of new species such as *M. eumusae* recently discovered in Asia (Carlier *et al.* 2000; Crous & Mourichon 2002).

### 2.2 Distribution and economic importance

Sigatoka or yellow Sigatoka (*M. musicola*) was first observed in Java in 1902 and in Fiji in 1913. It was diagnosed in Australia in 1924 and in China and India in 1925. Its widespread occurrence throughout south Asia suggests that the fungus had been around long time before it was discovered. *M. musicola* arrived to Central America in the late 1930s, and a few years later it was found in the African continent (Jeger *et al.* 1995).

BLSD was first recognized in the Sigatoka Valley of Fiji in 1963, but it was probably widespread in Southeast Asia and the South Pacific by that time (Stover 1980). Subsequently, the disease was reported throughout the Pacific (Torres Strait & Cape York Peninsula region of Australia, Papua New Guinea, Solomon Islands, Vanuatu, New Caledonia, Norfolk Island, Federated States of Micronesia, Tonga, Western Samoa, Niue, Cook Islands, Tahiti and Hawaii). BLSD has also been found in Asia (Bhutan, Taiwan, southern China including Hainan Island, Vietnam, Philippines, West Malaysia and Sumatra in Indonesia). However, distribution of this disease in southeast Asia still needs clarification especially in the Indonesian archipelago. In the Western Hemisphere, it first appeared in 1972 in Honduras and it now occurs on the mainland from central Mexico south to Bolivia and northwestern Brazil, and in the Caribbean basin, Cuba, Jamaica, the Dominican Republic and southern Florida. In Africa, the disease was first recorded in Zambia in 1973 and has since spread throughout the sub-Saharan portions of that continent. In most areas, black Sigatoka has now replaced yellow Sigatoka becoming the predominant banana leaf spot disease (Jones 2000). Present distribution of this disease is shown in Figure 8.

BLSD does not kill plants immediately, but crop losses increase gradually with plantations age. Destruction of most mature leaves by severe streak decreases functional leaf area resulting in a reduction of fruit quality and quantity (Stover & Simmonds 1987; Stover 1983). Leaf infection by the fungus leads to necrosis, decrease of photosynthetic rate and marked yield loss in the field (Mobambo *et al.* 1993). In West Africa yield losses due to BLSD are 33 and 76% during the first and second cropping cycles (Mobambo *et al.* 1996).

**Figure 8**

Worldwide distribution of *M. fijiensis*. ● countries where BLSD has been reported. (After Mourichon & Fullerton 1990, Jones 2000, Marin *et al*. 2003).

## 2.3 Infection Cycle and epidemiology

*M. fijiensis* produces repeated infection cycles more or less continuously, although in those regions with a well-defined dry season, the infection rate diminishes substantially during this period. Sexual cycles and asexual cycles occur simultaneously in both *Mycosphaerella* species , but the asexual cycle is more prominent in *M. musicola*, and the sexual cycle in *M. fijiensis* (Jeger *et al.* 1995).

BLSD development needs a relative humidity > 90% and temperature near to 25-30 C (Jacome & Schuh 1992). Climate conditions are major factors that influence BLSD development (Gauhl 1994). During the infection cycle, three successive events happen: infection (germination of conidium or ascospore and penetration through stoma), incubation (development of first symptoms) and symptoms evolution. These stages occur in combination with the sexual and asexual reproduction of *M. fijiensis* (Fouré & Moreau 1992; González 1975) (Figures 9 and 10).

### 2.3.1 Infection

Conidia or ascospores start the infection and usually germinate within 2-3 h of deposition on a moist leaf surface. Germ-tube from both conidia or ascospore penetrates through stomata after 48-72h. Grown germ-tube is epiphyllic (Fouré & Moreau 1992). Humidity and temperature influence this step. The minimum, optimum and maximum temperatures for development of ascospore/conidia germ-tubes are 12° C, 27° C and 36° C, respectively (Jacome *et al.* 1991; Jacome & Schuh 1992; Porras & Pérez 1997).

### 2.3.2 Incubation period

After infection and initial invasion of leaf tissue, one or more vegetative *M. fijiensis* hyphae emerge from stomata on the lower leaf surface developing into conidiophores or growing across the leaf surface parallel to the veins to infect adjacent stomata (Vásquez *et al.* 1990). During this stage, mycelial hyphae develops and is found in the spongy mesophyll or palisade parenchyma (Beveraggi *et al.* 1993). The incubation period occurs between infection

**Figure 9**

Infectious cycle with asexual and sexual reproduction of the *Mycosphaerella* leaf spot pathogens of bananas (after González, 1975).

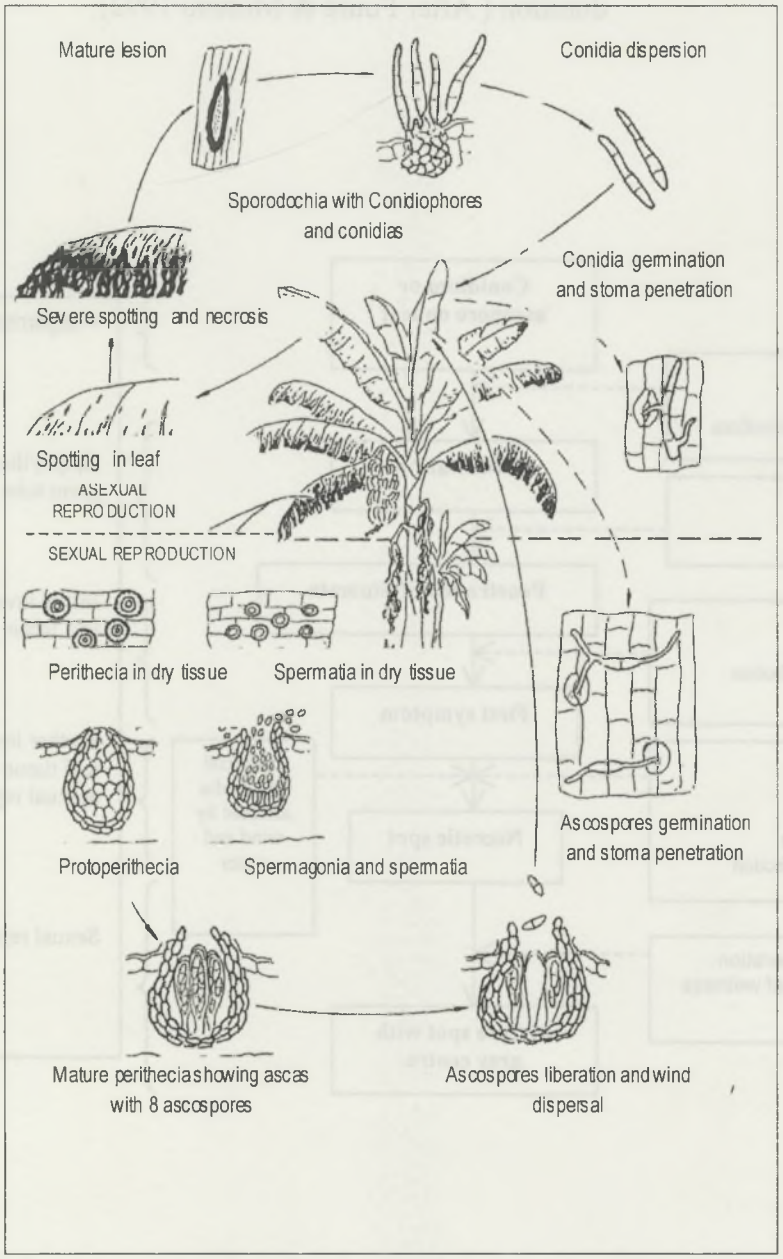
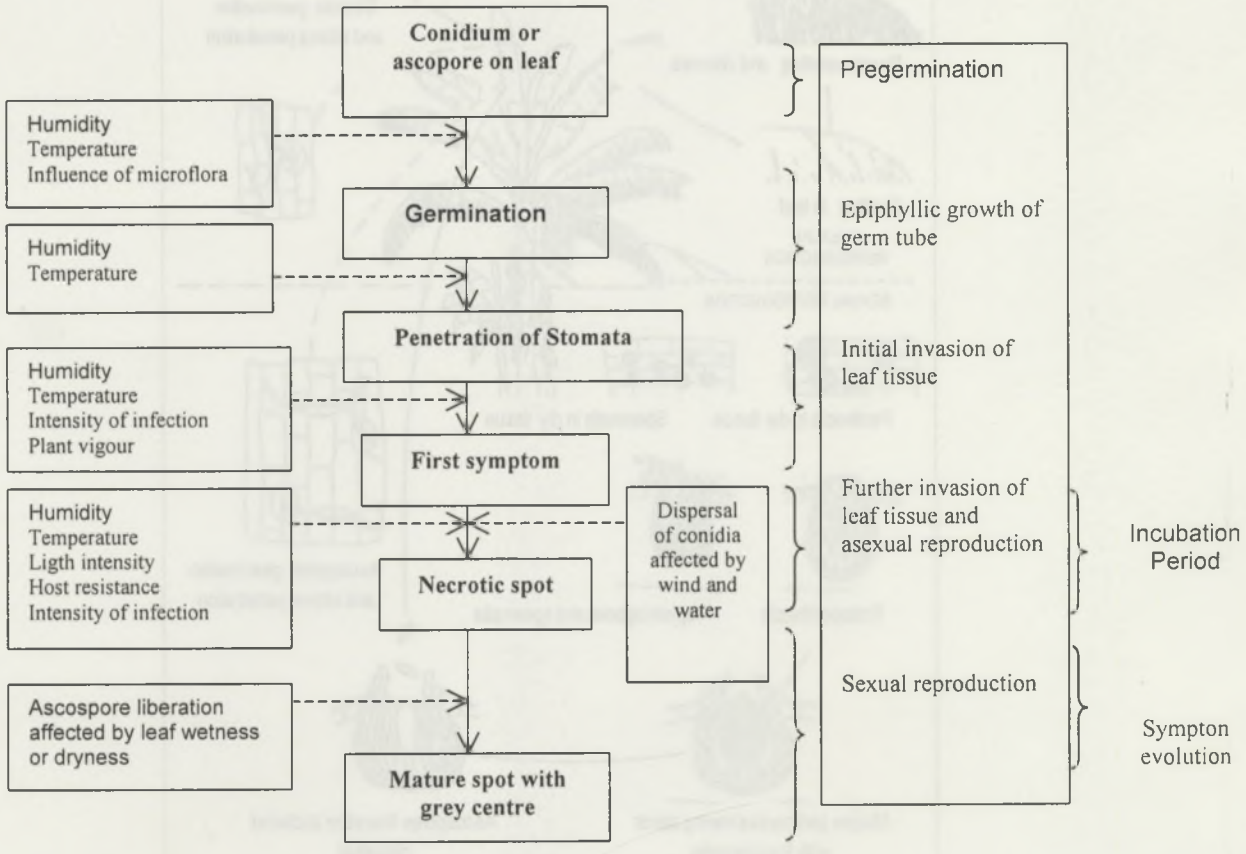


**Figure 10**

Stages in the development of black leaf streak symptoms and factors that influence their duration ( After Fouré & Moreau 1992).



and appearance of the first specks on the leaf, that is about 10 – 15 days under ideal disease development conditions (Fouré & Moreau 1992; Jacome *et al.* 1991).

### 2.3.3 Symptoms evolution

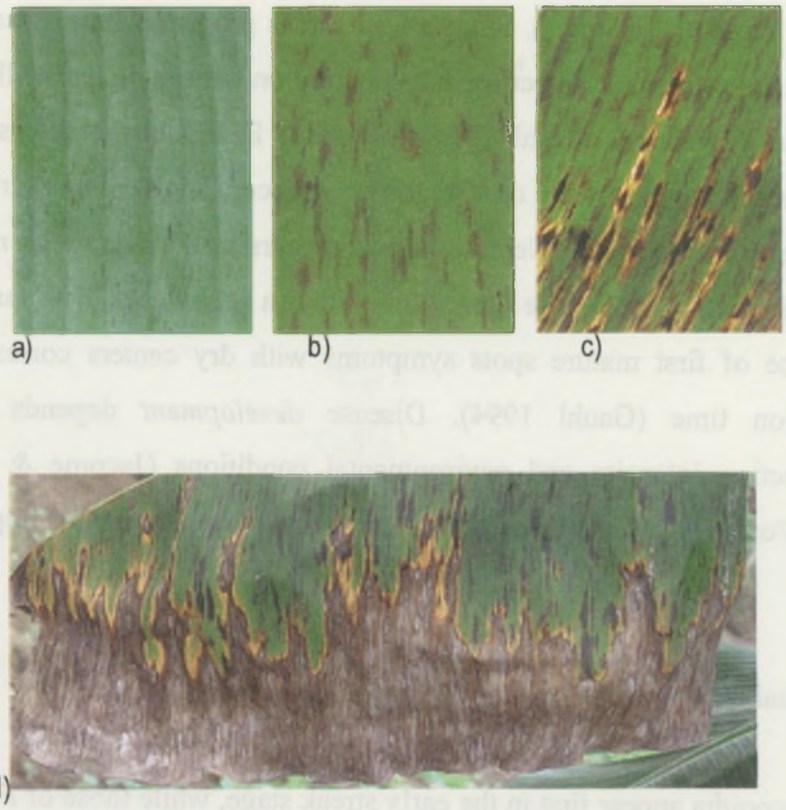
The optimum temperature for Sigatoka development is between 26 and 28° C, and growth is inhibited below 15 and above 35° C. Fungus growth is invisible to the unaided eye for 2-4 weeks after the initial infection. Since a new leaf is produced approximately every 7-10 days, the first visible streaks of infection that occurred on the candle leaf will appear when that leaf is in 2 to 4 position (Gauhl 1994). The first BLSD observable symptoms are appearance of reddish-brown streaks on the lower surface, lengthening of reddish-brown streaks that enlarge to mature spots. Necrotic tissue appears as a black streak mass (Jeger *et al.*. 1995). In severe infections, entire leaves die within a few weeks. The number of days between appearance of first mature spots symptoms with dry centers corresponds to the symptoms evolution time (Gauhl 1994). Disease *development* depends on cultivars susceptibility, infection intensity and environmental conditions (Jacome & Schuh 1992; Meredith 1970). (Fouré 1982) identified six main stages of symptoms development (Figure 11).

### 2.3.4 Asexual reproduction

*P. fijiensis* conidia appear first in the early streak stage, while those of *P.musae* appear later as mature spots begin to develop. Conidia form daily during high humidity periods , even in the absence of rain. In *P. fijiensis* conidia are formed on clusters of 2-5 short conidiophores that emerge through the stomata on leaf underside (Fouré *et al.* 1984). Conidia development in conidiophores are dispersed by wind and dripping or splashing water. Conidia are generally the most important means of local disease spread (González 1975; Stover 1980). Conidia are associated mostly with local disease spread mainly during high humidity periods, frequent heavy dews, and intermittent showers.

**Figure 11**

Symptoms of black leaf streak disease of bananas caused by *Mycosphaerella fijiensis*: stages a) 1, b) 2, c) 4 and d) 6 according to Fouré 1982 (Photos: G.G.Rivas, CATIE).




Stage 1 is the first external symptom of the disease. It appears as a small whitish or yellow coloured spot that resembles the first stage of Sigatoka/yellow Sigatoka disease. These symptoms are not visible in transmitted light and can be observed only on the underside of the leaf (Figure 11 a).

Stage 2 appears as a stripe, generally brown in colour and visible on the underside of the leaf; later the symptom also appears on the upper part of the limb as a stripe, the yellow colour of which resembles the stripe at Stage 1 of Sigatoka/yellow Sigatoka. The colour of this stripe will change progressively to brown and later to black on the upper side of the leaf, but will retain the brown colour on the underside (Figure 11 b).

Stage 3 differs from the previous one in its dimensions. The stripe becomes longer, wider and, in certain conditions (weak inoculums and unfavourable climatic conditions), can reach a length of 2 or 3cm.

Stage 4 appears on the underside as a brown spot and on the upper side as a black spot (Figure 11 c).

Stage 5 is when the elliptical spot is totally black and has spread to the underside of the leaf. It is surrounded by a yellow halo with the centre beginning to flatten out.

Stage 6 is when the centre of the spot dries out, turns light grey, and is surrounded by a well-defined black ring, which is, in turn, surrounded by a bright yellow halo. These spots remain visible after the leaf has dried out because the ring persists (Figure 11 d).

### 2.3.5 Sexual reproduction

The sexual cycle begins with the production of tiny, flask-shaped spermagonia in the necrotic centres of mature spots. The precise role of the spermagonia has not been studied, but it is presumed that spermatia landing on the receptive hyphae of opposite mating types lead to nuclear fusion and initiation of perithecia formation (Stover 1980). Spermatia are discharged from mature spermogonies (Mourichon & Fullerton 1990). Perithecia are abundant in matures lesions (Fouré & Mouliom Pefoura 1988). Mature perithecia need to be impregnated with water before ascospores can be discharged (Fouré & Moreau 1992).

The perithecia develop in the necrotic centers of mature spots and in the large patches of dried tissues formed by coalesced spots. Within each perithecium, the asci matures gradually, one at a time, even during periods of dry weather. Under favourable conditions, ascospores can be produced 2 weeks. A few minutes after the leaves have been wet by rain, ascospores are forcibly discharged from the mature ascus and disseminated by air currents. Ascospore release continues for about 2 hours once the leaves are wet (Jeger *et al.* 1995).

A high ascospores concentration was found by (Gauhl 1994) in Costa Rica´s banana producing areas; this concentration occurred in the wettest month. The minimum amount of rainfall necessary to induce ascospores release is as low as 0.1 mm h $m^{-1}$ . The highest ascospore concentration per cubic meter of air was 6876 $m^{-3}$. The highest ascospore concentration per month was 17 595 $m^{-3}$ registered in December, 1985. Another factor that affected ascospores production was temperature. Temperatures below 20° C inhibited *M. fijiensis* germ tube growth (Stover 1983). Ascospores are spread by air currents which are responsible for the long-distance BLSD dissemination (Gauhl 1994).

## 2.4 Host-pathogen interaction

Host-pathogen interactions are of two types: compatible and incompatible. When a compatible interaction occurs, the disease develops faster for sensitive banana plants than for those showing partial resistance, however the final necrotic stage is reached in both cases (Mourichon *et al.* 2000). Partial resistance can be controlled by several genes.. Incompatible

interaction with total resistance is related to an over-sensitiveness reaction with fast and simultaneous death of host cells and pathogenic cells just after stomatal penetration. Symptoms evolution is blocked at early stages and sporulation does not occur This resistance type can be controlled only by one or by a small group of resistance genes confering resistance to the plant which can be easily overcomed by changing pathogenic populations (Mourichon *et al.* 2000).

Characterization of partial resistance components was assessed by dissecting the fungus infectious cycle under field and controlled conditions (Abadie *et al.* 2003). The *in vitro* inoculation method used banana leaf pieces maintained survive on agar medium. Partial resistance of 10 bananas was characterized under natural infestation in field and under controlled conditions with 15 *M. fijiensis* isolates. The estimated parameters were: spore efficacy (number of lesions), incubation time, surface and growth rate of lesions, latency period and amount of asexual reproduction. Significant differences between varieties were observed at all stages of infectious cycle. Epidemiological roles of some resistance components were studied under field conditions. Disease development was observed on a susceptible and two resistant varieties (*Pisang berlin* and *Pisang madu*) which differ for two resistance components: spore efficacy and sexual sporulation capacities; *P. berlin* produced more lesions and perithecia than *P. madu*. The results showed that partial resistance can control efficiently BLSD in comparison with susceptible plot. Differences in severity between resistant varieties could be explained by their differences in sexual sporulation capacities and/or a modification within years of *P. madu* physiology enhancing its resistance. These two hypothesis are tested at present.

### 2.5 Population genetic structure and variability of *M. fijiensis*

RFLP markers were used to study the genetic structure of global *M. musicola and M. fijiensis* populations (Carlier *et al.* 1996), Hayden *et al.* unpublished data) Random single-locus probes were applied to samples from Southeast Asia, Australia, the Pacific Islands, Africa, Latin America and the Caribbean and features common to both pathogens were observed.

Southeast Asia has the highest gene diversity level and most alleles found in this region were also present in the other regions. This supports the hypothesis that pathogens originated in Southeast Asia. Founder events accompanying pathogens introduction to other regions have led to a genetic diversity reduction in comparison with Southeast Asia. Nevertheless, genetic diversity is maintained in all populations. Ecological conditions favor disease development as does banana production almost year-round in most growing areas. Low genetic drift in large pathogen populations can maintain the high levels of genetic diversity observed. Therefore, certain pathogenicity variability level might also be maintained in pathogen populations, allowing pathogens to attack newly introduced resistant genotypes, as observed with *M. fijiensis* on 'Paka' and 'T8' in Rarotonga, Cook Islands (Fullerton & Olsen 1995). Occurrence of specific interactions between the host and *M.fijiensis* isolates was suggested for highly resistant genotypes. Aggressiveness variability was evaluated in two *M. fijiensis* samples from Cameroon and the Philippines by inoculating five partially resistant cultivars using a leaf piece assay (El Hadrami 2000). Variability was similar but low for both countries, however, genetic diversity in the Philippines was much higher (Carlier *et al.* 1996). Specific interactions between the isolates and the cultivar were not detected. Only susceptible hosts are cultivated in these countries, and lack of selection pressure by the hosts on the pathogens could explain the results. The potential of pathogen populations to overcome partial resistance should be analyzed by following their evolution on fields of resistant hosts (Abadie *et al.* unpublished data).

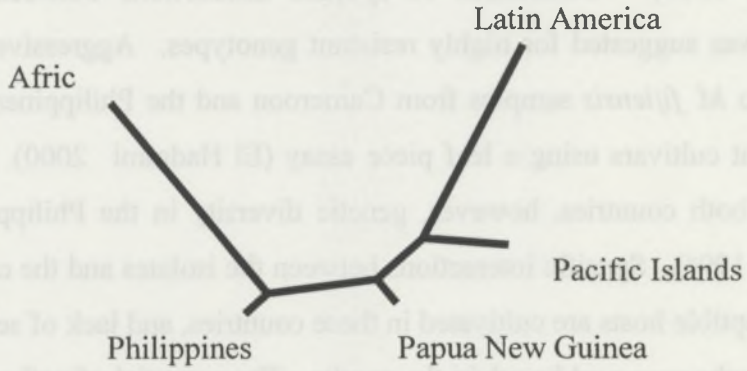
Genetic recombination plays an important role for *M. musicola* and *M. fijiensis* genetic structures. Genetic markers were statistically independent, therefore, pathogenicity characteristics could not be related to RFLP genotypes. With regards to breeding programs, introducing specific resistance genes in individual cultivars (pyramiding) may not be a good strategy for durable resistance in banana. Mixing varieties or partially resistant hosts could be more appropriate .

A high genetic differentiation level was observed between populations of *M. musicola* and *M. fijiensis* from different regions (Figure 12). The $F_{st}$ parameter (Wright 1951) estimated for all loci between pairs of populations varied between 0.14 and 0.58 for *M. fijiensis* and between 0.025 and 0.55 for *M. musicola*. But, whereas the African *M. fijiensis* populations were significantly different from the Latin American/Caribbean ones ($F_{st} = 0.49$), no significant differences were observed between the African and the Latin American *M.*

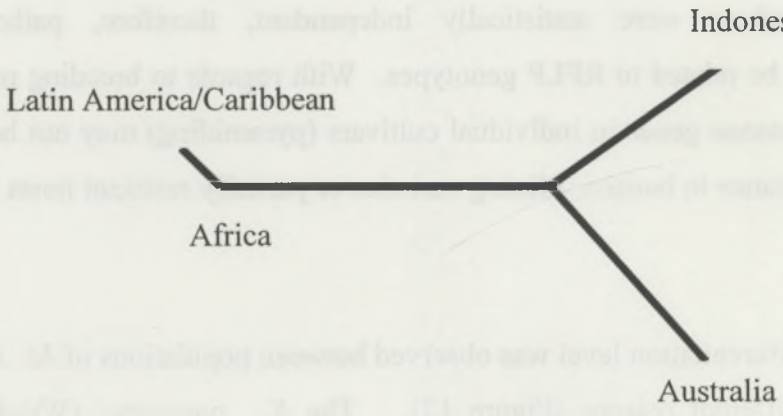
*musicola* populations ($F_{st}$ = 0.025, not significant). This suggests a separate introduction of *M. fijiensis* in each region but a common one for *M. musicola*.

**Figure 12**

Global population structure of : a) *M. fijiensis* (Carlier *et al.* 1996) and b) *M. musicola* (Hayden 2002). Additives trees constructed from estimates of Wrigth's $F_{st}$ among pairs of geographical populations.




a)




b)

On the other hand, the high genetic differentiation levels observed between Australian and African *M. musicola* populations ($F_{st}$ = 0.47) do not support Stover's hypothesis (1962) whereby *M. musicola* spores were carried by air currents from Australia to Africa. In general, the high genetic differentiation level of both pathogens at a global scale suggests occasional migration events between continents. Long distance disease dissemination around the world was more likely to have occurred by movement of infected plant material, as proposed by (Mourichon & Fullerton 1990).

Population structures at a continental scale were studied in Australia for *M. musicola* (Hayden 2000). Isolate collections from twelve sites along the east coast were analyzed using 15 RFLP markers. Gene diversity level (Nei 1978), varied between 0.14 and 0.37. Most plant lesions corresponded to a unique haplotype, showing diversity presence at a fine scale. Low to high genetic differentiation levels were observed between populations ($F_{st}$ = 0.04 to 0.45). There was no apparent correlation between genetic and geographical distances as high genetic differentiation levels were observed among neighboring populations and low levels in populations separated by long distances.

Pathogen population evolution was studied on plots constituted with partial resistant varieties to evaluate the effect of the selection pressure exerted by the host on these populations. Samples of isolates were collected after seven and 25 months of cultivation from the plots of the susceptible (*Grande naine*) and resistant (*Pisang berlin* and *Pisang madu*) varieties used also to evaluate efficiency partial resistance components. The samples were compared with molecular markers and aggressiveness measures. The results suggested a low genetic drift due to founder effects accompanying the establishment of pathogen populations on plots. A decrease in genetic differentiation between plots observed during time might be explain by gene flow between them an/or from environment. Only few strains isolated from resistant bananas were more aggressive than all others, leading to higher mean surfaces of lesion. This result needs to be confirmed within time to conclude on a selective effect of banana resistance on pathogen populations. However, gene flow suggested by genetic analysis could counteract this selection.

## 2.6 Disease management

Several methods have been developed to control BLSD such as cultural practices, fungicide applications, and resistant varieties utilization. The objective of cultural practices is to reduce farms inoculum levels. Leaves with extensive necrosis are removed from plants and small areas of necrotic leaf tissue excised as soon as they appear (Romero & Sutton 1997; Stover 1987). Another practice is to reduce relative humidity inside the crop. This can be achieved by an efficient drainage system and optimum plant populations are enough to control (Stover 1987).

BLSD can be chemically controlled but it demands many applications per year. Up to 36 spray cycles per year can be required for commercial plantations (Gauhl 1994; Romero & Sutton 1997). However, this excessive use of agrochemicals has resulted in the development of resistant fungus strains. In many countries such as Mexico and Costa Rica, 40-60% of all pesticide consumption is applied to control black Sigatoka disease. Nowadays, the annual cost of fungicide applications range from USD 1000-1300 per hectare. All cultivated banana varieties are susceptible to the fungus causing black Sigatoka, therefore, food supply for millions of people is seriously endangered in Latin America, Africa and Asia where bananas are a staple food (Jacome 1998; Orozco-Santos 1998; Romero 1997). Chemical control is a very expensive practice since it includes the use of airplanes or helicopters, permanent landing strips and facilities for mixing and loading fungicides, besides the high recurring expense of the spray materials themselves. In total, it has been estimated that these costs are ultimately responsible for 27% of the final fruit retail price for the importing countries (Stover and Simmons 1987). The extremely high cost of these control measures make them essentially unavailable to smallholders , who are therefore, the most affected by this important disease. Chemical control of black Sigatoka is not only expensive and beyond the means of most small-scale producers, but it is also extremely damaging to the environment. Moreover, the harmful effects of chemical use on plantation workers health are of great concern. In Costa Rica, over-use of chemicals for f export bananas production has resulted in the development of fungicide-resistant pathogen populations . Furthermore, small-scale plantations have been reduced by 40% as a result of this disease as farmers are unable to afford fungicides high cost needed to control it (Carlier *et al.* 2000).

Another option to obtain BLSD resistance is *Musa* genetic breeding. This approach involves identification of resistance genes in traditional cultivars or related wild species and

incorporation of this resistance into commercially acceptable varieties. This can be achieved through conventional breeding using hybridization techniques, which in the case of banana is particularly difficult as most cultivated varieties are sterile and do not produce seeds, and/or through genetic transformation using molecular techniques. These approaches are complementary and *Musa* improvement programs are presently using both. The first hybrids released in large scale were produced by FHIA (Fundación Hondureña de Investigación Agrícola) Honduras, and these are now being tested in national evaluation programs in more than 50 countries. In some countries farmers are already cultivating these hybrids on a wider scale. Similarly, black Sigatoka-resistant plantain hybrids have been developed by IITA (International Institute of Tropical Agriculture) Nigeria, and these have been widely distributed for evaluation by national programs in Africa. Other breeding programs such as those of CIRAD (France), CARBAP(Cameroon) and EMBRAPA (Brazil) have also made good progresses in recent years. Nevertheless, banana and plantain genetic improvement remains an expensive and slow task, and, considering the scale and diversity of problems facing banana and plantain growers world-wide, *Musa* genetic improvement efforts are still too small (Rosales & Pocasangre 2002).

# CHAPITRE 2

## STRUCTURE DES POPULATIONS DE *MYCOSPHAERELLA FIJIENSIS* A L'ECHELLE CONTINENTALE

**Résumé**

L'épidémie à l'échelle mondiale provoquée par le champignon *Mycosphaerella fijiensis* sur le bananier s'est développée récemment à partir du Sud Est Asiatique. Les effets de fondation détectés dans la structure mondiale de *M.fijiensis* reflètent une invasion des différents continents au travers d'un ou de quelques événements d'introduction de matériel végétal infecté. Le principal objectif de ce travail était d'appréhender les processus de dispersion et les flux de gènes chez *M. fijiensis* dans des régions récemment envahies, à partir d'une étude de la structure des populations à l'échelle continentale. Des échantillons d'isolats ont été collectés à partir de bananeraies dans 13 pays de la région Amérique Latine-Caraïbes et d'Afrique. Ces isolats ont été analysés avec des marqueurs moléculaires CAPS (Cleaved Amplified Polymorphic Sequences) et microsatellites. Les résultats obtenus montrent l'existence, à l'échelle même d'une plante, d'un haut niveau de diversité génétique et d'une recombinaison génétique importante. Des effets de fondations sont de nouveau détectés à l'échelle continentale et à celle d'un pays. Ces résultats suggèrent une dissémination stochastique de la maladie à ces échelles géographiques qui peut être aussi bien due à une dispersion limitée des ascospores, sur des distances de quelques centaines de kilomètres, qu'à des mouvements de matériel végétal infecté. Les limites de dispersion et les flux de gènes chez *M. fijiensis* devront être précisés sur des distances inférieures à quelques centaines de kilomètres.

Les échantillons provenant de la région Amérique Latine-Caraïbes ont été analysés dans le cadre de ce travail de thèse. Les structures des populations de *M. fijiensis* étant similaires dans cette région et en Afrique, l'ensemble des résultats obtenus par l'équipe pour les deux régions ont été regroupés et sont présentés dans la publication ci-après.

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# Founder effects and stochastic dispersal at the continental scale of the fungal pathogen of bananas *Mycosphaerella fijiensis*

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**Abstract**

**The worldwide destructive epidemic of the fungus *Mycosphaerella fijiensis* on banana started recently, spreading from South-East Asia. The founder effects detected in the global population structure of *M. fijiensis* reflected rare migration events among continents through movements of infected plant material. The main objective of this work was to infer gene flow and dispersal processes of *M. fijiensis* at the continental scale from population structure analysis in recently invaded regions. Samples of isolates were collected from banana plantations in 13 countries in Latin America and the Caribbean and in Africa. The isolates were analysed using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and microsatellite molecular markers. The results indicate that a high level of genetic diversity was maintained at the plantation and the plant scales. The loci were at gametic equilibrium in most of the samples analysed, supporting the hypothesis of the existence of random-mating populations of *M. fijiensis*, even at the plant scale. A low level of gene diversity was observed in some populations from the Africa and Latin America–Caribbean regions. Nearly half the populations analysed showed a significant deviation from mutation-drift equilibrium with gene diversity excess. Finally, a high level of genetic differentiation was detected between populations from Africa ($F_{ST}$ = 0.19) and from the Latin America–Caribbean region ($F_{ST}$ = 0.30). These results show that founder effects accompanied the recent invasion of *M. fijiensis* in both regions, suggesting stochastic spread of the disease at the continental scale. This spread might be caused by either the limited dispersal of ascospores or by movements of infected plant material.**

*Keywords*: bananas, founder effects, genetic population structure, long-distance dispersal of fungi, Mycosphaerella fijiensis

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## Introduction

Rapid aerial dispersal of pathogens at the global and continental scales have extreme consequences on plant diseases (Brown & Hovmøller 2002). These authors made a distinction between two forms of disease spreading from an inoculum source. The first is a rare, unpredictable single-step pathogen invasion involving the transport of spores for very long distances, even between continents. This stochastic form of disease spread could also result from the transport of infected plant material and tends to occur more at a global scale. The second form consists of a gradual expansion of the range of pathogen population within continents through normal pathogen dispersal processes. However, single-step invasion may also be involved in the spread of disease at a continental scale. This question can be investigated by the analysis of population genetic structure because the two forms of disease spread may have different impacts on population structure. Founder effects should be more marked in single-step invasions than in gradual expansion.

The spread of the ascomycete fungus *Mycosphaerella fijiensis* Morelet [anamorph *Paracercospora fijiensis* (Morelet)

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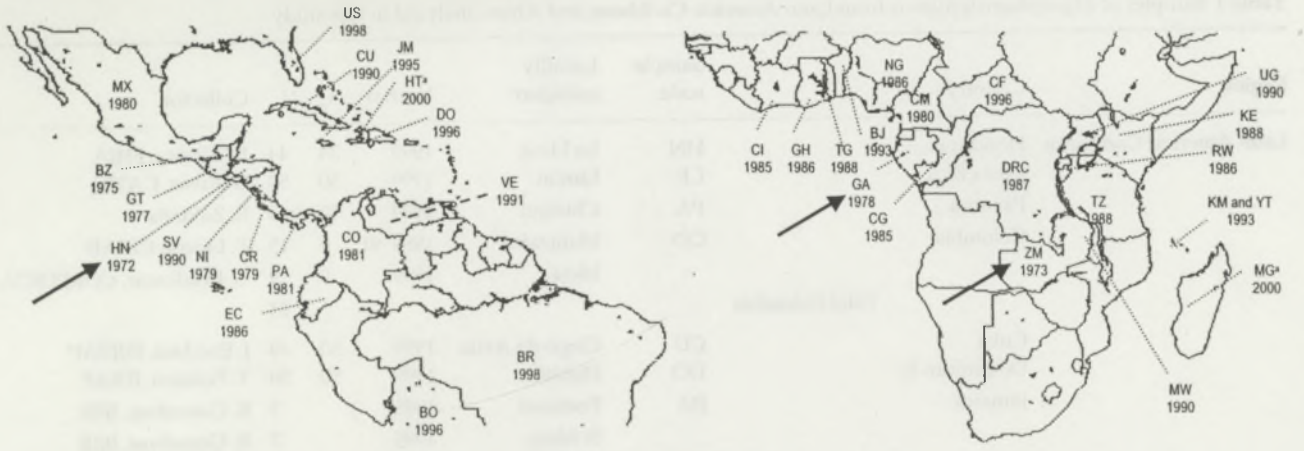


**Fig. 1** Chronology of first report of black leaf streak disease (BLSD) of bananas caused by the fungus *Mycosphaerella fijiensis* in Latin America–Caribbean and African regions (after Mourichon & Fullerton 1990; Pasberg-Gauhl *et al.* 2000; [a]X. Mourichon & M. F. Zapater, unpublished results). The arrows indicate the countries where the disease was first observed in each continent. *M. fijiensis* was first identified in Latin America in 1972 in Honduras and it spread northwards and southwards to Florida and Brazil in 1998. In the Caribbean, *M. fijiensis* was first observed in Cuba in 1990 and subsequently in Jamaica, the Dominican Republic and more recently in Haiti. It threatens the rest of the Caribbean. The earliest report in Africa was in Zambia in 1973 but this outbreak remains unconfirmed. The first authenticated reports were in Gabon in 1978. *M. fijiensis* spread along the west coast to Côte d'Ivoire. The disease is present in the Congo and its eastward spread was probably via the Democratic Republic of Congo to Kenya. Introduction occurred on the island of Pemba around 1987 and BLSD spread from there to Zanzibar and coastal areas of Kenya and Tanzania. BLSD has also been detected in Malawi, the Comoro Islands and recently in Madagascar.

Deighton] causing the very destructive black leaf streak disease (BLSD) of banana is recent in the tropical area (Mourichon & Fullerton 1990; Pasberg-Gauhl *et al.* 2000). The chronology of *M. fijiensis* records around the world suggests that it originated in South-East Asia, like *Mycosphaerella musicola* Leach that causes Sigatoka disease, another banana leaf spot disease. South-East Asia is also the centre of origin of the host genus *Musa* (Stover & Simmonds 1987). *M. fijiensis* is still spreading to new banana-growing areas and replacing *M. musicola*, which has almost worldwide distribution on banana, as the dominant most serious leaf spot pathogen (Fig. 1). *M. fijiensis* is an haploid, heterothallic fungus (Carlier *et al.* 2000). Both anamorphs and telomorphs are present on infected leaves and the ascospores produced during the sexual stage play an important epidemiological role through windborne dispersal (Gauhl *et al.* 2000).

The global genetic structure of *M. fijiensis* populations was studied using molecular markers (Carlier *et al.* 1994; Carlier *et al.* 1996). Gametic disequilibrium tests between loci were mostly nonsignificant, suggesting that random sexual reproduction plays an important role in the genetic structure of *M. fijiensis* populations. The highest level of diversity was detected in South-East Asia. Founder effects accompanying the introduction of the pathogen in other regions have led to a reduction of genetic diversity in comparison with South-East Asia. A high level of genetic differentiation was observed between populations from different regions. These results supported the hypothesis that the pathogen originated in South-East Asia and suggested single-step invasion in the other regions. In contrast with rust fungi, wind dispersal of viable spores over distances greater than a few hundred kilometres is unlikely (Parnell *et al.* 1998; Brown & Hovmøller 2002). Thus, the original cause of single-step *M. fijiensis* invasion around the world was probably the movement of infected plant material. Knowledge of the distribution of genetic diversity and the levels of gene flow related to dispersal processes are lacking at the local and continental scales. This knowledge is needed for the refinement of disease resistance management strategies.

The main objective of the present study was to infer gene flow and dispersal processes of *M. fijiensis* at the continental scale from analysis of population genetic structures in the recently invaded Latin America–Caribbean and Africa regions. Samples from different countries in each region were characterized using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and microsatellite markers, and genetic analysis was performed on these data. This analysis was used to test the hypothesis that, after introduction via infected plants in a few places, the pathogen spread gradually in continents with expansion of an epidemic front by substantial dispersal of ascospores. From this hypothesis, few or no founder effects in the comparison of the genetic structure of different populations within continents were expected. Alternatively, marked founder effects might be accompanied by single-step invasions of the pathogen within continents through limited dispersal.

**Table 1** Samples of *Mycosphaerella fijiensis* from Latin America–Caribbean and Africa analysed in this study

| Region | Country | Sample code | Locality collected | Year(s) | $n_p$ | $N$ | Collector |
|---|---|---|---|---|---|---|---|
| Latin America–Caribbean | Honduras | HN | La Lima | 1999 | 34 | 44 | M. Rivera, FHIA |
| | Costa Rica | CR | Limón | 1999 | 50 | 50 | G. Rivas, CATIE |
| | Panama | PA | Chiriquí | 1999 | 13 | 43 | E. Zaldaña |
| | Colombia | CO | Manizales | 1989–91 | 5 | 15 | T. Lescot, CIRAD |
| | | | Meta | 1999 | 9 | 9 | S. Belalcazar, CORPOICA |
| | Total Colombia | | | | | 24 | |
| | Cuba | CU | Ciego de Avila | 1999 | 50 | 49 | J. Escalant, INIBAP |
| | Dominican R. | DO | Dajabón | 1999 | 50 | 50 | T. Polanco, IDIAF |
| | Jamaica | JM | Portland | 1995 | | 3 | R. Gonsalves, BBR |
| | | | St Mary | 1995 | | 7 | R. Gonsalves, BBR |
| | | | Hanover | 1995 | | 12 | R. Gonsalves, BBR |
| | | | Westmoreland | 1995 | | 6 | R. Gonsalves, BBR |
| | | | Trelawny | 1995 | | 5 | R. Gonsalves, BBR |
| | Total Jamaica | | | | | 33 | |
| Total Latin America–Caribbean | | | | | | 293 | |
| Africa | Côte d'Ivoire | CI | Km17 | 1999 | 15 | 44 | C. Abadie, CARBAP |
| | Nigeria | NG | Onne | 1999 | 30 | 44 | A. Tenkouano, IITA |
| | Cameroon | CM-Nj-F | Njombé | 1996 | 26 | 30 | J. Carlier, CIRAD |
| | | CM-Nj-P | Njombé | 1996 | 1 | 28 | J. Carlier, CIRAD |
| | | CM-Pm | Penda-Mboko | 1999 | 49 | 49 | C. Abadie, CARBAP |
| | | CM-Kr | Kribi | 1999 | 43 | 43 | C. Abadie, CARBAP |
| | | CM-Mb | Mbalmayo | 1999 | 42 | 42 | C. Abadie, CARBAP |
| | Total Cameroon | | | | | 192 | |
| | Gabon | GA | Bibolou | 1998 | | 4 | J. Guyot, CIRAD |
| | | | WeligaNdjore | 1998 | | 13 | J. Guyot, CIRAD |
| | | | Mitzic M. | 1998 | | 7 | J. Guyot, CIRAD |
| | | | Mitzic A. | 1998 | | 9 | J. Guyot, CIRAD |
| | Total Gabon | | | | | 33 | |
| | Uganda | UG | Namulonge | 1998 | 37 | 49 | D. Vuylsteke, IITA |
| | Comoros | KM | Moroni | 2000 | 21 | 50 | T. Lescot, CIRAD |
| Total Africa | | | | | | 412 | |
| Total sample | | | | | | 705 | |

$n_p$ = number of plants collected; $N$ = sample size; Dominican R. = Dominican republic; Mitzic M. = Mitzic Muleemekone; Mutzic A. = Mutzic Akinetome; BBR = Banana Board; CARBAP = Centre africain de recherches sur bananiers et plantains; CATIE = Centro Agronómico Tropical de investigación y Enseñanza; CIRAD = Centre de coopération internationale en recherche agronomique pour le developpement; CORPOICA = Corporación colombiana de investigación agropecuaria; FHIA = Fundación hondureña de investigación agrícola; IDIAF = Instituto dominicano de investigaciones agropecuarias y forestales; IITA = International Institute of Tropical Agriculture; INIBAP = International Network for the Improvement of Banana and Plantain.

## Materials and methods

### *Sampling of* M. fijiensis *isolates*

The geographical origins of the 705 isolates used are shown in Table 1. Most banana hosts were susceptible clones belonging to the AAA and AAB genomic groups (Stover & Simmonds 1987). Most of the samples were collected over a 5-year period (1995–2000) in seven countries in Latin America and the Caribbean and six countries in Africa. Samples were from a single locality in each country except for Jamaica, Colombia, Gabon and Cameroon. The samples from Jamaica, Colombia and Gabon were grouped as a single sample for each country for the analysis because sample sizes per locality were small and localities were geographically close. The samples from the four localities in Cameroon were considered separately in the analysis. In each locality in all the countries, infected leaves were collected from a single banana plantation and, as far as possible, cultures were isolated from different plants

distributed in the plantation or from different leaf fragments from the same plants. The cultures of the sample from Njombé in Cameroon, noted CM-Nj-P (Table 1), were from different leaf fragments collected from a single plant in the middle of the plantation from which the sample noted CM-Nj-F was prepared. All cultures were derived from a single ascospore, identified as belonging to the species *M. fijiensis* and stored using the methods described in Carlier *et al.* (2002).

### *DNA extraction*

The genomic DNA extraction method was derived from those described by Sweigard *et al.* (1990) and Rogers & Bendich (1988). Mycelium from each isolate was produced in six wells of tissue culture plates containing 3 mL 2-YEGK medium (2 g yeast extract, 10 g glucose, 3 g $KNO_3$ and 2 g $KH_2PO_4$ per litre, pH 6.0) maintained for 10–15 days at 24 °C in the dark. Mycelium was harvested, dried on absorbent paper and placed in 2 mL microcentrifuge tubes. It was incubated at room temperature for 2 h in 1.5 mL Glucanex (Novo Nordisk) solution (30 mg/mL in NaCl 0.7 M, pH 6.0). Digested mycelium was harvested by centrifugation and resuspended in 800-µL extraction buffer (1% cetylytrimethyl ammonium bromide (CTAB), 0.7 M NaCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 1% β-mercaptoethanol) at 65 °C and 0.3 g glass beads (Sigma G8772) was added. The tubes were vortexed for 2 min and placed in an oven at 65 °C for 45 min with continuous gentle rocking. An equal volume of chloroform:isoamyl alcohol (24:1) was added and the contents mixed by inversion to form an emulsion. The emulsion was spun for 15 min at 10 000 *g* and the upper aqueous phase was transferred to a fresh 2-mL microcentrifuge tube. One mL CTAB precipitation buffer was added (1% CTAB, 50 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0), mixed by inversion and placed at 4 °C for 15 min. The precipitated DNA was then spun for 5 min at 10 000 *g*. The supernatant was discarded and the pellet dissolved in 500 µL high-salt TE (1 M NaCl, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) at 65 °C. DNA was precipitated by adding 2 volumes cold absolute ethanol, mixed by inversion and incubated at –20 °C for 10 min. The precipitated DNA was spun for 5 min at 10 000 *g*, washed twice with ethanol 70% and then dissolved in 100 µL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0). RNA was digested with RNAse A at a final concentration of 100 µg/mL for 30 min at 37 °C. The yield was from 5 to 10 µg DNA per extraction.

### *Molecular markers*

Nine PCR-RFLP markers were used as described by M. F. Zapater *et al.* (2004). Eight of these markers (*Mf*RFLP-29/*Hae*III, *Mf*RFLP-62/*Taq*I, *Mf*RFLP-64/*Hin*fI, *Mf*RFLP-99/*Hin*fI, *Mf*RFLP-99/*Msp*I, *Mf*RFLP-172/*Xho*I, *Mf*RFLP-203/*Hin*fI, *Mf*RFLP-213/*Hin*fI) were defined in anonymous and single-copy nuclear DNA sequences from *M. fijiensis* and one (*Mf*RFLP-ITS/*Dra*I) in the internal transcribed spacer and 5.8S ribosomal DNA sequence. PCR products obtained with locus-specific primer pairs were digested with a single restriction enzyme detecting polymorphism (noted for each locus as follows: *Mf*RFLP-locus number/polymorphism detecting enzyme). The different RFLP patterns detected with one enzyme for each locus were treated as alleles. Seven of the nine PCR-RFLP loci were analysed on all samples. The two other loci *Mf*RFLP-172/*Xho*I and *Mf*RFLP-203/*Hin*fI were analysed only on the samples from Cameroon and the Latin America–Caribbean regions, respectively. The four microsatellite loci (*Mf*SSR05, *Mf*SSR025, *Mf*SSR061, *Mf*SSR137) published by Neu *et al.* (1999) were used on the samples from the Latin America–Caribbean region as follows.

PCR reactions were performed in a 20-µL volume containing 10–20 ng of genomic DNA of *M. fijiensis*, 1 X reaction buffer (Eurobio), 200 µM of each dNTP (Sigma), 1.5 mM $MgCl_2$, 0.33 µM forward and reverse primers and 0.03 U of *Taq*-DNA polymerase (Eurobio). PCRs were performed in a PTC-100 thermocycler (MJ Research) with initial denaturation at 94 °C (7 min), 35 cycles at 94 °C (1 min), 55 °C (1 min), 72 °C (3 min) and final elongation at 72 °C (10 min). The forward primers were end-labelled with ($\gamma$-$^{33}$P) ATP. PCR products were separated on a 5% denaturing acrylamide gel in 0.5 X TBE buffer and exposed for 24–72 h to X-ray film (X-Omat AR; Kodak). Examples of both marker types are shown in Fig. 2.

### *Data analysis*

The allele frequencies, the mean number of alleles per locus, the number of polymorphic loci and unbiased Nei estimation (Nei 1978) gene diversity ($H_E$) were calculated for all the samples using the BIOSYS-1 program (Swofford & Selander 1981). The significance of non-random association or gametic disequilibrium among pairs of loci in each population was tested with Fisher's exact test using the GENEPOP program (version 3.3; Raymond & Rousset 1995b). The Bonferroni sequential test procedure was used to adjust the significance level for a 'table-wide' 5% level (Rice 1989). Gametic disequilibrium was also tested in each population using the multilocus data sets. The index of multilocus linkage disequilibrium $\bar{r}_d$ was estimated and the null hypothesis $\bar{r}_d = 0$ (gametic equilibrium) was tested by randomization (1000 replicates) using the MULTILOCUS program (Agapow & Burt 2001). The index $\bar{r}_d$ is a modification of the index of association $I_A$ defined by Maynard Smith *et al.* (1993) to remove dependency on the number of loci (Agapow & Burt 2001). Recent founder effects or

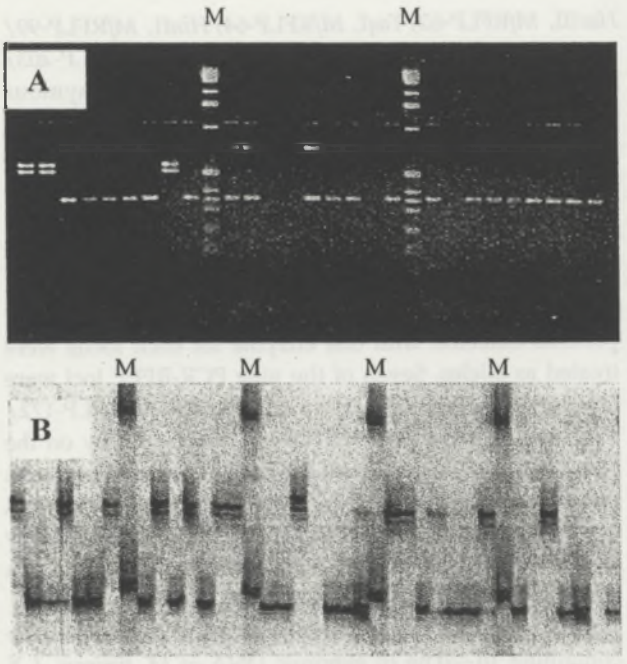


**Fig. 2** Examples of polymorphic CAPS and microsatellite markers used to analyse the population structure of *Mycosphaerella fijiensis*. The lanes noted M were DNA molecular weight markers. Each other lane corresponded to a *M. fijiensis* isolate. (A) PCR-RFLP locus *Mf*RFLP-29/*Hae*III. Amplified products of homologous DNA regions among isolates were digested with the restriction enzyme *Hae*III. The different restriction patterns were treated as alleles. (B) Microsatellite locus *Mf*SSR025. Amplified products of homologous DNA regions among isolates with different molecular sizes were treated as alleles.

bottlenecks can be detected with the method described by Cornuet & Luikart (1996) and the BOTTLENECK program (version 1.2, Piry *et al.* 1999) as follows. Populations that have experienced a recent reduction of their effective size exhibit a correlative reduction of the allele number and gene diversity ($H_E$) at polymorphic loci. However, the allele number is reduced faster than $H_E$. Thus $H_E$ becomes larger than the gene diversity expected at mutation-drift equilibrium ($H_{EQ}$) because $H_{EQ}$ is calculated from the observed number allele using mutation models: infinite allele model (IAM), stepwise mutation model (SMM) or two-phase model (TPM). Wilcoxon's test for gene diversity excess ($H_E > H_{EQ}$) across all the loci was conducted for each sample.

The genetic differentiation between subdivisions (populations or subpopulations) was estimated and tested using the GENEPOP program (Raymond & Rousset 1995b) as follows. Genetic differentiation described by $F_{ST}$ (Wright 1951) was estimated using Weir & Cockerham's (1984) estimator $\hat{\theta}$. To test genetic differentiation, an exact test for differences in allele frequencies was performed for each locus and multilocus statistics were obtained using Fisher's combined probability test (Raymond & Rousset 1995a). The significance levels were adjusted subsequently using the sequential Bonferroni correction method (Rice 1989). Hierarchical analysis of genetic differentiation was conducted using the TFPGA program (version 1.3, Miller 1997) to estimate $F_{ST}$ between the whole samples from Latin America/Caribbean and Africa. An unrooted neighbour-joining cluster analysis was performed with Cavalli-Sforza & Edwards (1967) chord distance ($D_c$) using POPULATIONS version 1.2.28 (O. Langella, Centre National de la Recherche Scientifique, Laboratoire Populations, Génétique et Evolution, Gif sur Yvette; http://www.cnrs-gif.fr/pge/bioinfo/populations) and visualized using TREEVIEW (Page 1996). For closely related populations, the distance $D_c$ was shown to provide a higher probability of obtaining the correct topology than other distance measures (Takezaki & Nei 1996). The resulting tree was bootstrapped among loci (1000 permutations).

## Results

### *Population structure at local and country scales*

Gametic disequilibrium (GD) was tested in each sample between all pairs of polymorphic loci and using multilocus data sets (Table 2). No significant GD was generally observed among pairs of loci and measures of multilocus linkage disequilibrium ($\bar{r}_d$) did not differ significantly from zero. These results support the hypothesis of the existence of random-mating populations of *M. fijiensis*. GDs were detected only in the two samples from Colombia and Jamaica: 1/28 and 6/28 pairs of loci displayed significant GD, respectively, and the value of $\bar{r}_d$ differed significantly from 0 for both samples. As these samples consisted of isolates from different localities (Table 1), GD may have been caused by genetic differentiation between the subpopulations from these localities. Structuring within samples (subpopulations) leads to an underestimation of between-sample structuring (Balloux & Logon-Moulin 2002). Pooling samples from different localities in Colombia, Jamaica or Gabon might thus lead to an underestimation of genetic differentiation levels between these samples and the others. However, such underestimation would not change the main conclusions of this study because the results were interpreted overall and the relations between specific pairs of populations are not considered.

As the isolates were cloned from ascospores and *M. fijiensis* is an heterothallic fungus, isolates with the same haplotype could not be the progeny of either asexual or sexual reproduction by selfing. The haplotype frequencies expected in random mating populations correspond to the product of allele frequencies between the loci studied and thus depend on the allele frequencies at each locus and on the number of loci studied. The haplotypes found several times in samples correspond to those with the highest expected frequencies. This feature was verified in all samples

**Table 2** Gametic disequilibrium analysis among polymorphic loci in *Mycosphaerella fijiensis* populations from Latin America–Caribbean and Africa

| Location | Sample code | $N$ | $n_d$ | $\bar{r}_d$ |
|---|---|---|---|---|
| Latin America–Caribbean | | | | |
| Honduras | HN | 44 | 0/36 | 0.010 |
| Costa Rica | CR | 50 | 0/45 | 0.010 |
| Panama | PA | 43 | 0/27 | 0.031 |
| Colombia | CO | 24 | 1/28 | 0.107* |
| Cuba | CU | 49 | 0/10 | 0.009 |
| Dominican R. | DO | 50 | 1/8 | −0.064 |
| Jamaica | JM | 33 | 6/28 | 0.273* |
| Africa | | | | |
| Côte d'Ivoire | CI | 44 | 0/8 | 0.007 |
| Nigeria | NG | 44 | 0/10 | 0.000 |
| Cameroon | | | | |
| Njombé | CM-Nj-F | 30 | 0/14 | 0.014 |
| Njombé | CM-Nj-P | 28 | 0/16 | 0.036 |
| Penda-Mboko | CM-Mp | 49 | 0/15 | 0.024 |
| Kribi | CM-Kr | 43 | 0/10 | −0.022 |
| Mbalmayo | CM-Mb | 42 | 0/15 | 0.017 |
| Gabon | GA | 33 | 0/10 | 0.041 |
| Uganda | UG | 49 | 0/10 | 0.09 |
| Comoros | KM | 50 | 0/6 | −0.019 |

*Values that differ significantly from zero at the 'table-wide' 5% level (Rice 1989). $N$ = sample size; $n_d$ = number of pairs of polymorphic loci showing significant, at the 'table-wide' 5% level (Rice 1989), gametic disequilibrium using Fisher's exact test (Raymond & Rousset 1995b); $\bar{r}_d$, index of multilocus linkage disequilibrium (Agapow & Burt 2001). Dominican R. = Dominican Republic.

**Table 3** Gene diversity in *Mycosphaerella fijiensis* populations from Latin America–Caribbean and Africa

| Location | Sample code | $N$ | $n_l$ | $n_a$ | $n_{pl}$ | $H_E$ |
|---|---|---|---|---|---|---|
| Latin America–Caribbean | | | | | | |
| Honduras | HN | 44 | 12 | 2.0 | 75.0 | 0.36 |
| Costa Rica | CR | 50 | 12 | 2.2 | 83.3 | 0.41 |
| Panama | PA | 43 | 12 | 2.1 | 66.7 | 0.22 |
| Colombia | CO | 24 | 12 | 1.8 | 66.7 | 0.27 |
| Cuba | CU | 49 | 12 | 1.4 | 41.7 | 0.15 |
| Dominican R. | DO | 50 | 12 | 1.5 | 50.0 | 0.15 |
| Jamaica | JM | 33 | 12 | 1.8 | 66.7 | 0.21 |
| Total | | 293 | 12 | 2.5 | 83.3 | 0.35 |
| Africa | | | | | | |
| Côte d'Ivoire | CI | 44 | 7 | 1.7 | 71.4 | 0.16 |
| Nigeria | NG | 44 | 7 | 1.9 | 71.4 | 0.30 |
| Cameroon | | | | | | |
| Njombé | CM-Nj-F | 30 | 8 | 1.9 | 75.0 | 0.31 |
| Njombé | CM-Nj-P | 28 | 8 | 1.9 | 75.0 | 0.36 |
| Penda-Mboko | CM-Pm | 49 | 7 | 1.9 | 85.7 | 0.29 |
| Kribi | CM-Kr | 43 | 7 | 1.7 | 62.5 | 0.19 |
| Mbalmayo | CM-Mb | 42 | 8 | 1.9 | 75.0 | 0.19 |
| Gabon | GA | 33 | 7 | 1.9 | 71.4 | 0.30 |
| Uganda | UG | 49 | 7 | 2.0 | 71.4 | 0.28 |
| Comoros | KM | 50 | 7 | 1.6 | 57.1 | 0.13 |
| Total | | 412 | 8 | 2.1 | 75.0 | 0.31 |

$N$ = sample size; $n_l$ = number of loci studied; $n_a$ = mean number of alleles per locus; $n_{pl}$ = percentage of polymorphic loci; $H_E$ = gene diversity (Nei 1978); Dominican R. = Dominican Republic.

in which haplotypes were found several times (data not shown). Because identical multilocus haplotypes cannot be treated as clones, gametic disequilibrium analysis on clone-corrected data sets were not conducted. Furthermore, estimations of genetic and genotypic diversities are correlated positively from such samples. Thus, the samples in this study were compared only for gene diversity.

Samples from four Cameroon localities were analysed (Table 1). For the Njombé locality, one sample was from different plants distributed in a field (noted CM-Nj-F) and another from three different leaves of one plant (noted CM-Nj-P) in the middle of this field. Gene diversity levels were similar (Table 3). Estimates of $F_{ST}$ for all pairs of populations are shown in Table 4. No genetic differentiation was detected between CM-Nj-F and CM-Nj-P samples. The levels of gene diversity were also similar and no genetic differentiation was detected between the central localities Njombé and Penda Mboko (approximately 30 km apart) or between the southern localities Kribi and Mbalmayo (approximately 200 km apart). The level of genetic diversity was lower for the two southern localities. Significant ($P < 0.001$) genetic differentiation ($F_{ST} = 0.13–0.21$) was detected between the central and southern localities that were approximately 300 km apart. Overall estimation of $F_{ST}$ was 0.11 ($P < 0.001$) in Cameroon. This means that approximately 10% of total gene diversity in Cameroon is distributed among plantations from different localities and 90% within plantations.

### *Population structure within regions*

Different levels of gene diversity were detected among samples from the countries in each region (Table 3). The highest levels of gene diversity in the Latin America–Caribbean region were found in the populations from Costa Rica and Honduras and the lowest levels were observed in the Caribbean populations. In the Africa region, gene diversity levels were similar between samples from the different countries, with the exception of Côte d'Ivoire and the Comoros for which the levels were approximately half. Detection of recent historical bottlenecks was conducted on each sample using Wilcoxon's test for gene diversity excess (Table 5). A significant excess of gene diversity on average across loci was detected in 8/17 samples from both regions when either the IAM, TPM or SMM was assumed. The estimated mean $H_E$ was 0.31–0.41

**Table 4** Genetic differentiation between *Mycosphaerella fijiensis* populations from Latin America–Caribbean and Africa

| Sample code | CR | JM | DO | CO | CU | PA | HN | CM-Nj-F | CM-Nj-P | CM-Mb | CM-Kr | CM-Mp | CI | GA | NG | UG | KM |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CR | — | 0.21 | 0.32 | 0.19 | 0.32 | 0.24 | 0.15 | 0.53 | 0.51 | 0.58 | 0.58 | 0.53 | 0.57 | 0.54 | 0.51 | 0.59 | 0.64 |
| JM | 0.17* | — | 0.25 | 0.14 | 0.25 | 0.19 | 0.19 | 0.57 | 0.55 | 0.53 | 0.53 | 0.59 | 0.63 | 0.59 | 0.57 | 0.63 | 0.67 |
| DO | 0.38* | 0.47* | — | 0.25 | 0.07 | 0.38 | 0.27 | 0.53 | 0.51 | 0.50 | 0.50 | 0.52 | 0.59 | 0.55 | 0.53 | 0.59 | 0.63 |
| CO | 0.14* | 0.17* | 0.33* | — | 0.22 | 0.21 | 0.13 | 0.61 | 0.59 | 0.57 | 0.57 | 0.62 | 0.67 | 0.63 | 0.61 | 0.66 | 0.70 |
| CU | 0.37* | 0.43* | 0.03* | 0.32* | — | 0.34 | 0.25 | 0.53 | 0.52 | 0.50 | 0.50 | 0.55 | 0.60 | 0.56 | 0.53 | 0.60 | 0.63 |
| PA | 0.19* | 0.35* | 0.58* | 0.32* | 0.55* | — | 0.18 | 0.60 | 0.60 | 0.60 | 0.58 | 0.61 | 0.66 | 0.63 | 0.61 | 0.65 | 0.68 |
| HN | 0.09* | 0.16* | 0.31* | 0.11* | 0.31* | 0.16* | — | 0.56 | 0.55 | 0.54 | 0.53 | 0.54 | 0.62 | 0.59 | 0.56 | 0.62 | 0.66 |
| CM-Nj-F | 0.51* | 0.64* | 0.56* | 0.61* | 0.56* | 0.71* | 0.54* | — | 0.05 | 0.14 | 0.14 | 0.07 | 0.12 | 0.06 | 0.14 | 0.16 | 0.22 |
| CM-Nj-P | 0.45* | 0.58* | 0.49* | 0.55* | 0.50* | 0.67* | 0.48* | 0.00 | — | 0.14 | 0.15 | 0.06 | 0.15 | 0.05 | 0.09 | 0.16 | 0.23 |
| CM-Mb | 0.58* | 0.66* | 0.58* | 0.64* | 0.58* | 0.75* | 0.58* | 0.15* | 0.15* | — | 0.05 | 0.20 | 0.17 | 0.15 | 0.22 | 0.23 | 0.24 |
| CM-Kr | 0.58* | 0.66* | 0.58* | 0.64* | 0.58* | 0.74* | 0.57* | 0.13* | 0.13* | 0.00 | — | 0.20 | 0.15 | 0.14 | 0.20 | 0.22 | 0.23 |
| CM-Mp | 0.53* | 0.65* | 0.56* | 0.62* | 0.58* | 0.71* | 0.52* | 0.01 | 0.00 | 0.21* | 0.17* | — | 0.17 | 0.08 | 0.16 | 0.19 | 0.23 |
| CI | 0.62* | 0.76* | 0.69* | 0.74* | 0.70* | 0.81* | 0.68* | 0.15* | 0.17* | 0.35* | 0.30* | 0.16* | — | 0.14 | 0.18 | 0.21 | 0.16 |
| GA | 0.49* | 0.61* | 0.54* | 0.58* | 0.54* | 0.69* | 0.52* | 0.01 | 0.00 | 0.16* | 0.13* | 0.02* | 0.11* | — | 0.09 | 0.13 | 0.18 |
| NG | 0.44* | 0.57* | 0.50* | 0.55* | 0.50* | 0.65* | 0.50* | 0.14* | 0.05* | 0.32* | 0.30* | 0.12* | 0.26* | 0.06* | — | 0.17 | 0.24 |
| UG | 0.56* | 0.65* | 0.59* | 0.63* | 0.59* | 0.71* | 0.58* | 0.19* | 0.14* | 0.27* | 0.25* | 0.17* | 0.26* | 0.07* | 0.16* | — | 0.17 |
| KM | 0.68* | 0.78* | 0.72* | 0.77* | 0.72* | 0.83* | 0.71* | 0.32* | 0.30* | 0.41* | 0.34* | 0.28* | 0.25* | 0.18* | 0.35* | 0.18* | — |

*Significant genetic differentiation at the 'table-wide' 5% level (Rice 1989) using Fisher's combined probability test (Raymond & Rousset 1995a, 1995b). Values above the diagonal are the distance $D_c$ (Cavalli-Sforza & Edwards 1967) and below the diagonal are $F_{ST}$ (Weir & Cockerham 1984). See Table 1 for key to sample codes.

**Table 5** Test for mutation-drift equilibrium at polymorphic loci in *Mycosphaerella fijiensis* populations from Latin America–Caribbean and Africa. The gene diversity observed ($H_E$) was compared with the gene diversity expected at mutation-drift equilibrium ($H_{EQ}$) and calculated from the observed number of alleles under three mutation models: IAM = infinite allele model; TPM = two-phase model; SSM = stepwise mutation model (Cornuet & Luikart 1996). *N* = sample size, E/D number of loci showing an excess/deficit of gene diversity

| Location | Sample code | *N* | E/D | | |
|---|---|---|---|---|---|
| | | | IAM | TPM | SMM |
| Latin America–Caribbean | | | | | |
| Honduras | HN | 44 | 8/1* | 8/1* | 8/1* |
| Costa Rica | CR | 50 | 10/1* | 10/1* | 9/1* |
| Panama | PA | 43 | 4/4 | 4/4 | 4/4 |
| Colombia | CO | 24 | 6/2 | 6/2 | 6/2 |
| Cuba | CU | 49 | 4/1* | 4/1* | 3/2 |
| Dominican R. | DO | 50 | 3/3 | 3/3 | 3/3 |
| Jamaica | JM | 33 | 4/4 | 3/5 | 3/5 |
| Africa | | | | | |
| Côte d'Ivoire | CI | 44 | 2/3 | 2/3 | 3/2 |
| Nigeria | NG | 44 | 5/0* | 5/0* | 5/0* |
| Cameroon | | | | | |
| Njombé-F | CM-Nj-F | 30 | 5/1* | 5/1* | 5/1* |
| Njombé-P | CM-Nj-P | 28 | 6/0* | 5/1* | 5/1* |
| Penda Mboko | CM-Pm | 49 | 4/2* | 4/2* | 4/2* |
| Kribi | CM-Kr | 43 | 2/3 | 2/3 | 2/3 |
| Mbalmayo | CM-Mb | 42 | 2/4 | 2/4 | 2/4 |
| Gabon | GA | 33 | 5/0* | 5/0* | 5/0* |
| Uganda | UG | 49 | 5/0* | 5/0* | 5/0* |
| Comoros | KM | 50 | 2/2 | 2/2 | 2/2 |

*Significant ($P > 0.05$) gene diversity excess ($H_E > H_{EQ}$) on average across all polymorphic loci using Wilcoxon's test (Cornuet & Luikart 1996; Piry *et al.* 1999).

for these samples (Table 3). Significant excess of gene diversity in the sample from Cuba was detected under IAM and TPM and low probability was observed ($P < 0.08$) under SMM. The mean estimate of $H_E$ was 0.15. No significant excess of gene diversity was detected in either model for the 8/17 samples and the mean $H_E$ estimate was 0.13–0.27.

Overall estimates of $F_{ST}$ were 0.19 and 0.30 in the Africa and Latin America–Caribbean regions, respectively, indicating a high, significant ($P < 0.001$) level of genetic differentiation. This means that approximately 20% of the total gene diversity in Africa (30% in Latin America–Caribbean region) is distributed among plantations from different countries and 80% within plantations (70% for Latin America–Caribbean region). Any private allele was detected among samples from Africa. Among samples from the Latin American–Caribbean region, 7.2% (11/28) and 7.7% (11/26) private alleles were observed only for the samples from Costa Rica and Panama, respectively. A high, significant level of genetic differentiation was detected between most pairs of populations within each region (Table 4 and Fig. 3). $F_{ST}$ values varied between 0 and 0.41 and 0.03–0.58 for the Africa and Latin America–Caribbean regions, respectively.

### *Population structure among regions*

A similar level of gene diversity was detected in the total samples from the Latin America–Caribbean and Africa regions (Table 3). The gene diversity level was slightly different in the previous study ($H_E = 0.40$ and $H_E = 0.25$) for Latin America and Africa, respectively (Carlier *et al.* 1996) but estimations in the present study are more accurate because the sample sizes are more than 10-fold higher.

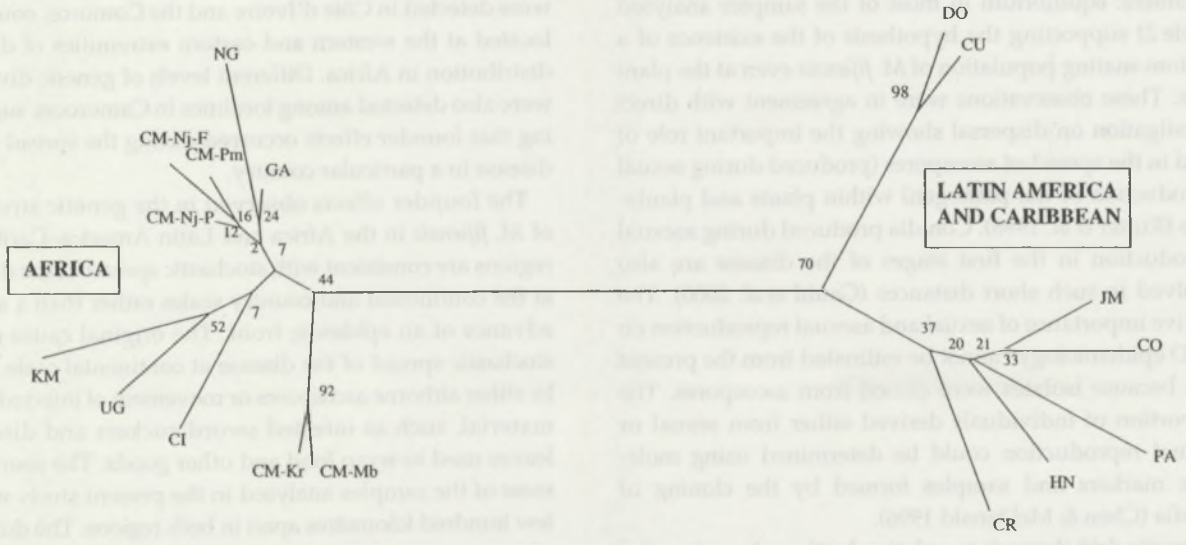


**Fig. 3** Genetic differentiation between *Mycosphaerella fijiensis* populations from Latin America–Caribbean and Africa. Unrooted neighbour-joining tree based on the distance $D_s$ (Cavalli-Sforza & Edwards 1967). The data were boostrapped 1000 times with replacement over loci. See Table 1 for key to sample codes.

A high, significant ($P < 0.001$) level of genetic differentiation was detected between continental populations from the Africa and Latin America–Caribbean regions with a $F_{ST}$ value of 0.52. This is very similar to the value estimated in the previous study between the same regions ($F_{ST} = 0.49$). This means that approximately 50% of the total gene diversity encompassing the two regions was distributed among plantations from different regions and 50% within plantations. The number of private alleles were 30.8% (4/13) and 40% (6/15) in the total samples from Africa and Latin America–Caribbean regions, respectively. Two main groups corresponded to populations from the Latin America–Caribbean and Africa regions (Fig. 3). The $F_{ST}$ values between pairs of populations from the Latin America–Caribbean and Africa regions were all significant (at the 'table-wide' 5% level, Rice 1989; Table 4) and ranged from 0.43 to 0.83 (Table 4). These values are higher than those estimated between pairs of populations either from the Latin America–Caribbean or from Africa except in three comparisons.

## Discussion

A high level of genetic diversity and the presence of random mating in *M. fijiensis* populations were previously suggested in the study of the global population structure of *M. fijiensis* (Carlier *et al.* 1996). However, the samples analysed consisted of isolates from different countries and/or localities and considered to represent continental populations. In the present work, most of the samples analysed were made up from a single plantation. Samples from one particular plantation and from one plant located in this plantation were also compared. The results indicate that a high level of genetic diversity was maintained at the plantation and the plant scales (Table 3). The loci were at gametic equilibrium in most of the samples analysed (Table 2) supporting the hypothesis of the existence of a random-mating population of *M. fijiensis* even at the plant scale. These observations were in agreement with direct investigation on dispersal showing the important role of wind in the spread of ascospores (produced during sexual reproduction of the pathogen) within plants and plantations (Rutter *et al.* 1998). Conidia produced during asexual reproduction in the first stages of the disease are also involved in such short distances (Gauhl *et al.* 2000). The relative importance of sexual and asexual reproduction on BLSD epidemiology cannot be estimated from the present data because isolates were cloned from ascospores. The proportion of individuals derived either from sexual or asexual reproduction could be determined using molecular markers and samples formed by the cloning of conidia (Chen & McDonald 1996).

Genetic drift through population bottlenecks or founder effects accompanying the spread of the disease may have been the main evolutionary factor in the shaping of population structures of *M. fijiensis* at the continental scale. Three observations expected from theoretical models supported this interpretation. First, founder effects may cause loss of genetic diversity (Nei *et al.* 1975; Maruyama & Fuerst 1985). A lower level of genetic diversity was detected in some populations in both Africa and Latin America–Caribbean regions (Table 3). Second, recent founder effects generate gene diversity excess at selectively neutral loci in comparison with gene diversity expected from the observed number of alleles under mutation-drift equilibrium (Cornuet & Luikart 1996). Significant gene diversity excess was detected in nearly half (9/17) of the populations analysed in the regions overall. Absence of significant excess of gene diversity in the other samples may be explained by lower statistical power as the number of loci and /or the mean observed gene diversity are smaller (Cornuet & Luikart 1996). Third, founder effects may cause genetic differentiation among populations (Boileau *et al.* 1992) as observed for *M. fijiensis* in the Africa and Latin America–Caribbean regions (Table 4 and Fig. 3).

The population structures observed in the Africa and Latin America–Caribbean regions reflect the history of the spread of *M. fijiensis* (Fig. 1). The highest levels of genetic diversity in the Latin America–Caribbean region were found in populations from Honduras and Costa Rica, supporting the hypothesis that the pathogen first entered the continent in this area (Fig. 1, Pasberg-Gauhl *et al.* 2000). In Africa, the levels of genetic diversity are similar in most countries. However, locating the place(s) where the pathogen first entered this continent is more ambiguous than for Latin America (Pasberg-Gauhl *et al.* 2000) and so, the total sample from Africa analysed might not include subsamples from or near the original(s) population(s). Nevertheless, lower levels of genetic diversity in this region were detected in Côte d'Ivoire and the Comoros, countries located at the western and eastern extremities of disease distribution in Africa. Different levels of genetic diversity were also detected among localities in Cameroon, suggesting that founder effects occurred during the spread of the disease in a particular country.

The founder effects observed in the genetic structure of *M. fijiensis* in the Africa and Latin America–Caribbean regions are consistent with stochastic spread of the disease at the continental and country scales rather than a steady advance of an epidemic front. The original cause of the stochastic spread of the disease at continental scale could be either airborne ascospores or movement of infected plant material, such as infected sword suckers and diseased leaves used to wrap food and other goods. The sources of most of the samples analysed in the present study were a few hundred kilometres apart in both regions. The duration of ascospore viability (about 6 h in continuous sunlight) does not preclude the possibility of transport over such

distances (Parnell *et al.* 1998). In Cameroon, genetic differentiation was detected between localities more than 300 km apart and not between localities less than 200 km apart. Thus, if ascospore dispersal is involved in the spread of the disease within continents and countries, the results of the present work suggest that a limited number of ascospores are transported for several hundred kilometres. These observations are supported by a preliminary epidemiological study showing the existence of a disease gradient from an inoculum source for a distance of several tens of meters (C. Abadie *et al.* unpublished results).

The genetic structure at the global, continental and country scales differed between *M. fijiensis* and the related wheat pathogen *M. graminicola* (Linde *et al.* 2002; Zhan *et al.* 2003). Comparable studies of population genetic structures were conducted for *M. fijiensis* and *M. graminicola* and the two pathogens display similar biological features, with the presence of both anamorphs and teleomorphs in the field. The structures are similar at field scale, with high levels of gene diversity maintained and the existence of random mating populations. However, the level of genetic differentiation between populations is much higher at other scales for *M. fijiensis*. This important difference could result from several factors. The first factor might be the different histories of the global spread of the pathogen. *M. fijiensis* has spread only recently, since the 1960s–1970s (Pasberg-Gauhl *et al.* 2000), in contrast with *M. graminicola* that is believed to have spread in the New World since European colonization (between 200 and 500 years ago) and in the Old World since the spread of agriculture (5000–8000 years ago, Zhan *et al.* 2003). Thus, founder effects following spread are still detectable today for *M. fijiensis* although such effects may have disappeared in time through mutation and gene flow for *M. graminicola*. A second factor might be a difference in the level of gene flow between the pathogens via movement of infected materials. In the case of *M. graminicola*, infected seed or straw could be a means of dispersal, particularly between continents (Linde *et al.* 2002). *M. fijiensis* can be carried only by infected sword suckers and diseased leaves used to wrap food or other goods. The reproduction of parthenocarpic cultivars of bananas is vegetative and there is substantial international trade in banana fruits but, in contrast with wheat grains, these fruits are not used for planting purposes. Tissue culture plants now used for planting cannot carry *M. fijiensis*. It is therefore reasonable to consider that gene flow via the movement of infected material is less important in the case of *M. fijiensis*. A third factor could be the difference in the frequencies of local population extinction and recolonization. Frequent extinction/recolonization events can be an important source of gene flow (Slatkin 1987). Such events should be more frequent for *M. graminicola* because the wheat is an annual plant in contrast with banana, which can be considered as a perennial crop.

An estimate of contemporary gene flow between pathogen populations is required for the development of sustainable disease resistance management strategies. The gene flow level based on the island model (Wright 1951) was not estimated because the immigrants reaching each subpopulation certainly do not arrive with equal probability from the other subpopulations. Furthermore, contemporary gene flow based on measurement of population subdivision (using $F_{ST}$) may be over- or underestimated, depending on historical gene flow or allele frequency divergences established during colonization, because populations could not reach genetic equilibrium (Slatkin 1987; Boileau *et al.* 1992). Isolation by distance models appear more realistic and some nonequilibrium patterns can be distinguished (Slatkin 1993; Rousset 2001). The sampling used in the present study was not suitable for the use of such models because the distance between samples appears to be too long. Estimation of gene flow and dispersal parameters under isolation by distance should be tested at the local scale (Rousset 2001) and compared with more direct estimates from disease gradient studies (McCartney & Fitt 1998). Phylogeography based on coalescent methods is another alternative to estimate gene flow with temporal resolution (Hare 2001; Neigel 2002).

The main result of the present work is the demonstration of the existence of founder effects and genetic differentiation in the fungus *M. fijiensis* at continental scale. This may reflect the recent stochastic spread of the pathogen in tropical areas. Expansion of the range of *M. fijiensis* populations within continents may result either from limited ascospores dispersal over few hundred kilometres or from the movement of infected plant material. Although the relative importance of these two dispersal processes could not be determined, improvement of quarantine measures might limit the risk of disease introduction in new areas and exchanges between existing populations in different countries. The sustainability of disease resistance management strategies will depend first on the effectiveness of quarantine measures. Disease management should also try to limit gene flow between pathogen populations via natural dispersal. Parameters corresponding to these evolutionary and epidemiological processes should therefore be estimated accurately.

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The data set from Latin America and Caribbean is a part of the PhD research (ENSAM Monpellier, France) of Gonzalo-Galileo Rivas. Marie-Françoise Zapater, Catherine Abadie and Jean Carlier have a research programme dealing with the host–pathogen interaction in banana leaf spot fungi. The main studies have been conducted last years on epidemiology and population genetics of *Mycosphaerella fijiensis*.

# CHAPITRE 3

# STRUCTURE DES POPULATIONS DE *MYCOSPHAERELLA FIJIENSIS* AU COSTA RICA

**Résumé**

Le champignon *Mycosphaerella fijiensis*, agent causal d'une grave maladie foliaire du bananier, se dissémine en Amérique Latine depuis 1972. Des effets de fondation, détectés à partir de la structure continentale des populations de *M. fijiensis*, suggère une dissémination stochastique du champignon à cette échelle géographique, au travers de mouvements de matériel végétal infecté et/ou d'une dispersion limitée des ascospores. Les objectifs de cette étude étaient de décrire la structure des populations de *M. fijiensis* dans une zone de culture et d'appréhender les flux de gènes ainsi que les processus de dispersion du parasite. Des échantillons d'isolats de *M. fijiensis* ont été constitués à partir de 17 sites au Costa Rica, la plupart distribués le long d'un transect d'environ 300 km recouvrant la zone de culture des bananiers. Ces isolats ont été analysés avec des marqueurs CAPS (Cleaved Amplified Polymorphic Sequences) et microsatellites. Des effets de fondation et une différenciation génétique entre sites sont de nouveau observés dans une même zone de culture. Ceux-ci sont toutefois moins importants par rapport aux échelles géographiques supérieures, suggérant des flux de gènes plus importants entre populations. La signification statistique du test d'isolement par la distance géographique dépend des populations de deux sites. Ce modèle génétique ne peut donc être utilisé dans cette étude pour estimer les flux de gènes et les limites de dispersion chez *M. fijiensis*. Ce résultat, qui pourrait être dû à l'hétérogénéité du milieu au Costa Rica, devra être comparé à d'autres obtenus à partir de milieux plus homogènes dans d'autres pays.

Les résultats de cette étude sont présentés dans la publication ci-après qui sera soumise avec des analyses complémentaires à la revue *Fungal Genetics and Biology*.

# Genetic differentiation and isolation by distance analysis in the Costa Rican populations of the fungus *Mycosphaerella fijiensis*

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## Abstract

The fungus *Mycosphaerella fijiensis*, that causes a very destructive leaf disease of bananas, has been spreading in Latin America since 1972. The founder effects detected in continental population structures suggested the stochastic spread of *M. fijiensis* at such a scale via the movement of infected plant material and/or limited wind dispersal of ascospores. The aim of this study was to describe *M. fijiensis* population structures within a production area and draw inferences with regard to gene flow and dispersal process of the pathogen from isolation by distance analysis. Samples of *M. fijiensis* were collected at 17 sites in Costa Rica, mainly on a transect some 300 km long across the banana production area. The isolates were characterised using CAPS (Cleaved Amplified Polymorphic Sequence) and microsatellite markers. Founder effects and genetic differentiation were still detected at this scale but were less important in comparison with higher geographical scales, suggesting higher levels of gene flow. The statistical significance of isolation by distance was associated with populations from two sites, preventing estimation of gene flow and dispersal limits using this model. As this result may be due to environmental heterogeneity in Costa Rica, it should be compared with others obtained from more homogenous environments in other areas.

## Introduction

Estimation of the relative importance of evolutionary factors in the evolution of fungal pathogens of plants is necessary for defining disease resistance management (McDonald & Linde 2002). Gene flows (and indirectly dispersal rate) balancing genetic drift can be estimated from population structure analysis using neutral molecular markers and genetic models (Rousset 2001). Gene flows in fungal plant pathogens were estimated using the island

model (Wright 1951). However, the main assumption of this model that immigrants in each subpopulation come with equal probability from the other subpopulations is certainly contradicted. Furthermore, contemporary gene flow based on measurement of population subdivision (using $F_{st}$) may be over or under-estimated depending on the historical gene flows or allele frequency divergences established during range expansion because populations could not be in equilibrium (Boileau *et al.* 1992; Slatkin 1987).

Isolation by distance models are an alternative to the island model for estimating gene flow and inferring dispersal rates (Rousset 2001). The mean assumption of such models that dispersal occurs preferentially between nearby subpopulations appears more realistic than the above assumption of the island model. However, it is also assumed that populations reach demographic equilibrium in isolation by distance models. Although this assumption is often not testable directly, some non-equilibrium patterns can be distinguished from isolation by distance analysis (Slatkin 1993). Most isolation by distance analysis of fungal plant pathogens were conducted using samples collected on large spatial scales with a thousand to several thousand kilometres, sometimes covering different continents (Et-touil *et al.* 1999; Linde *et al.*2002; McDonald 1999; Milgroom 1995). At such distances, the effect of demographic events on genetic differentiation is more likely to be observed (Rousset 2001). In particular, increases in fungal pathogen range through aerial dispersal or the movement of infected plant material has serious consequences on plant disease (Brown & Howwøller 2002). Other factors such as mutation or selection may also interfere with isolation by distance analysis at large spatial scales. For these reasons, it is recommended to conduct such analysis and to ask the question of whether demographic fluctuations are important at the local scale should be asked (Rousset 2001).

The spread from Southeast Asia of the ascomycete fungus *Mycosphaerella fijiensis* (anamorph *Paracercospora fijiensis*) that causes the very destructive black leaf streak disease (BLSD) of bananas is recent in the tropics (Mourichon & Fullerton 1990; Pasberg-Gauhl *et al.* 2000); *M. fijiensis* was identified in Latin America for the first time in 1972 in Honduras and it spread northwards and southwards to Florida and Brazil in 1998. The pathogen was detected in Costa Rica in 1979 and it gradually spread to the entire banana production area (González 1987). *M. fijiensis* is a haploid, heterothallic fungus (Carlier *et al.* 2000). Both anamorph and telomorph are present on infected leaves and ascospores produced during the

sexual stage through wind-borne dispersal play an important epidemiological role (Gauhl *et al.* 2000).

The global, continental and local genetic structure of *M. fijiensis* populations was studied using molecular markers (Carlier *et al.* 1996; Rivas *et al.* 2003). A high level of genetic diversity and random mating populations were maintained even at the plant scale. The highest level of diversity was detected in Southeast Asia, the centre of origin. Founder effects and genetic differentiation were detected at the global and continental scales, suggesting stochastic spread of the disease. The original cause of the spread of *M. fijiensis* around the world was probably the movement of infected plant material. Spread on continents may be caused either by limited ascospore dispersal over a few hundred kilometres or the movement of infected plant material. Most gene flow and natural dispersal of the pathogen might occur on distances of less than a few hundred kilometres. Only preliminary data were published from samples collected on such a distance.

The aim of this study is to describe *M. fijiensis* population structures within a production area and to make inferences concerning gene flow and dispersal processes using isolation by distance analysis. Samples were collected in Costa Rica mainly along a transect of about 300 km across the banana production area. These samples were characterised using CAPS (Cleaved Amplified Polymorphic Sequence) and microsatellite markers and genetic analysis of the data was performed.

## Material and methods

### *Sampling of M. fijiensis isolates*

The geographical origins of the 396 isolates used are shown in Table 1. The samples were collected in 2001 at 17 sites, each being a locality in Costa Rica. Sites 1 to 15 were distributed across the main banana production area of Costa Rica along the Caribbean coast following a transect some 300 km long (Figure 1). Sites 16 and 17 were on the other side of the Talamanca's range on the Pacific coast. The geographical co-ordinates of the sites were determined using the geographic positioning system (GPS). Along the transect, the distance between adjacent was 2 to 10 km (mean 7.5) from sites 1 to 5 and 8.0 to 77 km (mean 26.7) from sites 5 to 15. Samples of infected leaves from each site were collected in a single small

plot (approximately 30 x 30 m) where susceptible clones belonging to the AAA and AAB genomic groups were cultivated without fungicides. Twelve to 26 isolates at each site were isolated from different bananas randomly distributed within the plantation plot. All cultures were derived from a single ascospore and stored as described in (Rivas *et al.* 2003).

**Table 1**

Samples of *Mycosphaerella fijiensis* from Costa Rica used in this study.

| Province | Place | Site number | Location | Genotype banana host | Sample size |
|---|---|---|---|---|---|
| Alajuela | La Perla | 1 | 10°30'N 84°38'W | AAB | 26 |
| | Los Angeles | 2 | 10°28'N 84°34'W | AAB | 24 |
| | Muelle | 3 | 10°28'N 84°39'W | AAB | 26 |
| | Concepción | 4 | 10°28'N 84°38'W | AAB | 26 |
| | San Miguel | 5 | 10°28'N 84°33'W | AAB | 25 |
| Heredia | Sarapiquí | 6 | 10°25'N 83°59'W | AAB | 14 |
| | Horquetas | 7 | 10°16'N 83°56'W | AAB | 26 |
| Limón | Guácimo | 8 | 10°12'N 83°41'W | AAB | 24 |
| | 28 millas (La Lola) | 9 | 10°06'N 83°32'W | AAA | 24 |
| | Batán | 10 | 10°05'N 83°28'W | AAB | 23 |
| | 4 millas | 11 | 10°04'N 83°24'W | AAB | 23 |
| | Valle de la Estrella | 12 | 09°47'N 82°55'W | AAA | 24 |
| | Bribrí | 13 | 09°29'N 82°59'W | AAB | 24 |
| | Parque Margarita | 14 | 09°37'N 82°47'W | AAB | 24 |
| | Sixaola | 15 | 09°32'N 82°59'W | AAB | 20 |
| Puntarenas | Palmar Sur | 16 | 08°54'N 83°25'W | AAA | 22 |
| | Barrido | 17 | 08°32'N 83°04'W | AAB | 25 |
| Total sample | | | | | 396 |

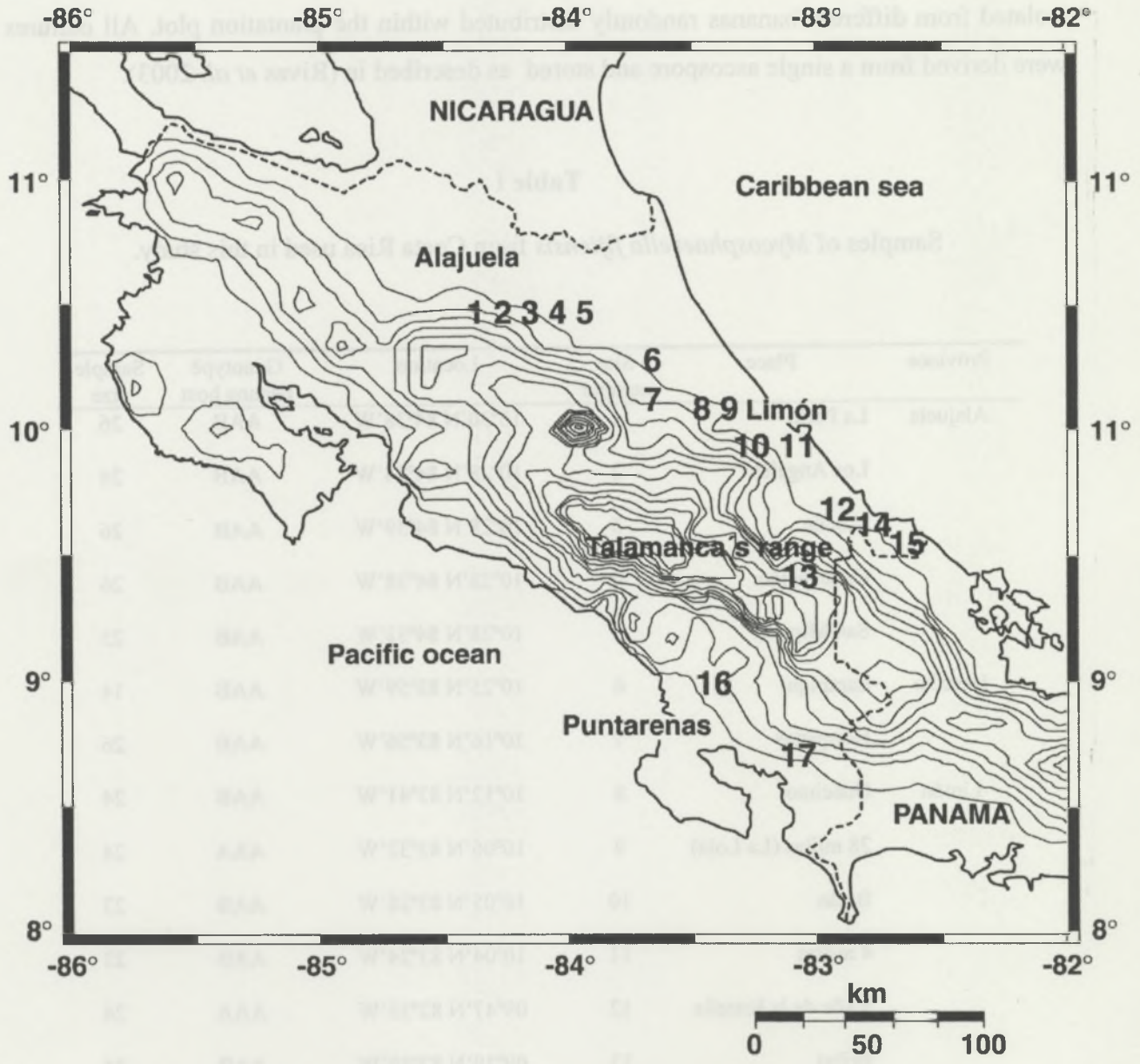


**Figure 1**. Map showing the sampling site locations for *Mycosphaerella fijiensis* isolates in Costa Rica. 1. Perla; 2. Los Angeles; 3. Muelle; 4. Concepción; 5. San Miguel; 6. Sarapiquí; 7. Horquetas; 8. Guácimo; 9. 28 Millas (La Lola); 10. Batán; 11. 4 Millas ; 12. Valle de la Estrella; 13. Bribrí ; 14. Parque Margarita; 15. Sixaola; 16. Palmar Sur; and 17. Barrido. Map source: OMC http://www.aquarius.geomar.de/omc/

*DNA extraction and molecular markers*

The genomic DNA of each isolates was extracted following the method described in Rivas et al (2003). Eight CAPS loci (*Mf*CAPS-29/*Hae*III, *Mf*CAPS-62/*Taq*I, *Mf*CAPS-64/*Hin*fI, *Mf*CAPS-99/*Hin*fI, *Mf*CAPS-99/*Msp*I, *Mf*CAPS-203/*Hin*fI, *Mf*CAPS-213/*Hin*fI, *Mf*CAPS-ITS/*Dra*I) were used as described by M. Zapater F., A. Rakotonantoandro, S. Cohen & J. Carlier (*manuscript in preparation)*. The four microsatellite loci (*Mf*SSR05, *Mf*SSR025, *Mf*SSR061, *Mf*SSR137) published by (Neu et al. 1999) were also used as described in (Rivas et al. 2003). Microsatellite PCR products were analysed for length variation on polyacrylamide gels using an DNA Analyser $IR^2$ (LICOR). Alleles were scored using the program SAGA. To check consistency of the results from gels, series of rerun sessions were made.

*Data analysis*

The allele frequencies, mean number of alleles per locus, number of polymorphic loci and unbiased Nei estimation (Nei 1978) gene diversity (H) were calculated for all the samples using the Genetix-4.01 program (Belkhir *et al.* 2002). Genotypic diversity was estimated according to (Stoddart & Taylor 1988). The significance of non-random association or gametic disequilibrium among pairs of loci in each population was tested with Fisher's exact test using the GENEPOP program (Version 3.3; (Raymond & Rousset 1995)). The Bonferroni sequential test procedure was used to adjust the significance level for a 'table-wide' 5% level (Rice 1989). Recent founder effects can be detected if a significant heterozygosity excess (in the sense of Nei's gene diversity) is observed at selectively neutral loci in comparison with expected heterozygosity from the number of alleles under mutation-drift equilibrium (Cornuet & Luikart 1996). Wilcoxon's test for heterozygosity excess overall loci was conducted with the BOTTLENECK program (version 1.2, (Piry *et al.* 1999). for each sample using three different mutation models: infinite allele model (IAM), stepwise mutation model (SMM) and two-phase model (TPM). A graphic descriptor of the shape of the allele frequency distribution is also proposed by the BOTTLENECK program. A characteristic mode-shift distortion was expected in populations that had recently suffered bottlenecks.

The genetic differentiation between arbitrary subdivisions (populations or subpopulations) described by $F_{st}$ (Wright 1951) was estimated using estimator of Weir and Cockerham's estimator (1984) (Weir & Cockerham 1984). The genetic differentiation among subdivisions was tested on all the loci with Fisher's combined probability test using the

GENEPOP program (Raymond & Rousset 1995). Isolation by geographic distance among populations was studied using the method described by (Rousset 1997) implemented in the GENEPOP program Version 3.3; (Raymond & Rousset 1995). The relationship between $F_{st}/(1-F_{st})$ and natural logarithm of geographic distance for pairs of population were plotted and regression calculated. Correlation between the matrices of genetic and geographic distances was tested using Mantel permutation procedure and 20.000 permutations.

**Results**

*Genetic diversity within plantations*

Gametic disequilibrium between all pairs of polymorphic loci was tested in each sample using Fisher's exact test. No significant gamete disequilibrium was detected. The level of genetic diversity (encompassing gene and genotypic diversity) was similar in the first 16 sites sampled (Table 2). Gene diversity (H) and percentage of maximum genotypic diversity (G/N) varied from 0.25 to 0.41 and from 70% to 100% respectively in these samples. Because isolates were cloned from ascospores and *M. fijiensis* is an heterothallic fungus, isolates with the same haplotype could not be the progeny of either asexual or sexual reproduction by selfing. The haplotype frequencies expected in random mating populations will correspond to the product of allele frequencies between all loci and will thus depend on the allele frequencies at each locus and on the number of loci studied. The haplotypes found several times in samples will correspond to those with the highest expected frequencies. This feature was verified in all samples in which haplotypes were found several times (data not shown).

The level of genetic diversity was lower for the last site (Barrido localities) near the frontier with Panama with H= 0.18 and G/N = 64.1%. This observation suggested founder effects accompanying the spread of *M. fijiensis* in the Costa Rican banana growing area. The detection of recent historical bottlenecks was conducted on each sample using Wilcoxon's test for heterozygosity excess (Table 3). A significant excess of heterozygosity on average across loci was detected in 11/17 samples when either the IAM or TPM was assumed. The test was not significant only for two of them when SMM was assumed. A bottleneck-induced distortion of allele frequency distribution, i.e. a characteristic mode-shift distortion, was observed for most of these samples. The number of polymorphic loci was $\geq 7$. The test was significant for half of the six other only with the IAM and no mode-shift distortion was observed for most of them. The number of polymorphic loci was $\leq 7$ for these samples.

**Table 2**

Genetic diversity in *Mycosphaerella fijiensis* populations from Costa Rica.

| Location | Site number | N | $n_l$ | $n_a$ | $n_{pl}$ | $n_g$ | H | G | G/N(%) |
|---|---|---|---|---|---|---|---|---|---|
| Perla | 1 | 26 | 9 | 2.0 | 75.0 | 26.0 | 0.35 | 26.0 | 100.0 |
| Los Angeles | 2 | 24 | 9 | 2.1 | 75.0 | 22.0 | 0.35 | 20.5 | 85.7 |
| Muelle | 3 | 26 | 9 | 2.1 | 75.0 | 22.0 | 0.35 | 18.8 | 72.2 |
| Concepción | 4 | 26 | 9 | 2.1 | 75.0 | 25.0 | 0.38 | 24.1 | 92.8 |
| San Miguel | 5 | 24 | 9 | 1.9 | 66.7 | 21.0 | 0.32 | 18.0 | 75.0 |
| Sarapiquí | 6 | 14 | 8 | 1.9 | 66.7 | 12.0 | 0.26 | 9.80 | 70.0 |
| Horquetas | 7 | 26 | 9 | 2.1 | 75.0 | 25.0 | 0.36 | 24.1 | 92.8 |
| Guácimo | 8 | 20 | 8 | 1.9 | 66.7 | 19.0 | 0.35 | 18.1 | 90.9 |
| La Lola | 9 | 25 | 9 | 2.4 | 75.0 | 25.0 | 0.36 | 25.0 | 100.0 |
| Batán | 10 | 24 | 9 | 2.5 | 75.0 | 24.0 | 0.41 | 24.0 | 100.0 |
| 4 Millas | 11 | 23 | 8 | 2.2 | 66.7 | 21.0 | 0.35 | 19.6 | 85.18 |
| Valle Estrella | 12 | 23 | 8 | 1.8 | 66.7 | 23.0 | 0.30 | 23.0 | 100.0 |
| Bribri | 13 | 24 | 9 | 1.7 | 58.3 | 21.0 | 0.27 | 19.8 | 80.0 |
| Parque Margarita | 14 | 24 | 7 | 1.7 | 58.3 | 23.0 | 0.25 | 22.1 | 92.3 |
| Sixaola | 15 | 20 | 9 | 2.2 | 75.0 | 17.0 | 0.37 | 15.38 | 76.9 |
| Palmar Sur | 16 | 22 | 9 | 2.2 | 75.0 | 22.0 | 0.34 | 22.0 | 100.0 |
| Barrido | 17 | 25 | 7 | 1.7 | 33.3 | 19.0 | 0.18 | 16.0 | 64.1 |
| Total | | 396 | 9 | 1.9 | 75.0 | 238 | 0.38 | 150.20 | 37.9 |

*N* = sample size; $n_l$ = number of loci studied; $n_a$ = mean number of alleles per locus; $n_{pl}$ = percentage of polymorphic loci; $n_g$ = number of genotypes; *H* = gene diversity (NEI, 1978); *G* = genotypic diversity and *G/N* (%) = percentage of maximum genotypic diversity possible (Stoddart & Taylor 1988).

*Genetic differentiation among plantations*

A moderate, significant level of genetic differentiation was detected on all populations ($F_{st}$ = 0.13, p<0.001). The $F_{st}$ values varied between 0.01 to 0.44 (Table 4) for population pairs and the genetic differentiation was significant (p<0.05) for 161/171 of the total possible combinations (Table 4). The population from Barrido (site 17) is very remote from all other populations ($F_{st}$ = 0.26 to 0.44, Figure 2). The two populations sampled in Puntarenas province (sites 16 and 17) are located on the other side of the Talamanca's range (Figure 1). Because gene flows between these two populations and the others is unlikely, samples from Puntarenas province were excluded in the isolation by distance analysis. Genetic differentiation ($F_{st}$ = 0.09) was still significant (p < 0.001) on all the remaining 15 samples distributed along the transect from Alajuela to Limon provinces. Significant isolation by geographical distance was detected (Mantel test, p < 0.05; Figure 3). This isolation remained significant (Mantel test, p < 0.05) when the 24 pairs of populations separated by less than 50

km were excluded from the analysis and not (Mantel test, p = 0.37) without the 10 pairs of populations separated by more than 250 km. These 10 pairs corresponded to combinations between samples from sites 1 to 5 and sites 14 to 15. Isolation by distance was not significant (Mantel test, p = 0.18) when the samples from sites 14 and 15 were excluded from the analysis although significant genetic differentiation ($F_{st}$ = 0.08, P < 0.001) was still detected on all the 13 remaining samples.

**Table 3**

Heterozygosity excess at polymorphic loci in *Mycosphaerella fijiensis* populations from Costa Rica.

| Location | Site number | N | He/Hd | | | Allele frequency Distribution |
|---|---|---|---|---|---|---|
| | | | IAM | TPM | SMM | |
| Perla | 1 | 26 | 9/0* | 8/1* | 7/2* | S |
| Los Angeles | 2 | 24 | 8/1* | 8/1* | 8/1* | S |
| Muelle | 3 | 26 | 8/1* | 8/1* | 7/2 | S |
| Concepción | 4 | 26 | 8/1* | 8/1* | 8/1 | S |
| San Miguel | 5 | 14 | 7/2* | 7/2 | 7/2 | S |
| Horquetas | 6 | 26 | 4/4 | 4/4 | 4/4 | L |
| Sarapiquí | 7 | 20 | 9/0* | 8/1* | 8/1* | S |
| Guácimo | 8 | 25 | 8/0* | 8/0* | 8/0* | S |
| La Lola | 9 | 24 | 6/3* | 6/3 | 6/3 | L |
| Batán | 10 | 23 | 9/0* | 8/1* | 7/2* | L |
| 4 Millas | 11 | 23 | 8/0* | 8/0* | 6/2* | L |
| Valle Estrella | 12 | 24 | 7/1* | 7/1* | 7/1 | S |
| Bribri | 13 | 24 | 7/2* | 7/2 | 5/4 | S |
| Parque Margatita | 14 | 24 | 7/0* | 6/1* | 4/3* | S |
| Sixaola | 15 | 20 | 8/1* | 8/1 | 6/3 | S |
| Palmar Sur | 16 | 22 | 7/2* | 6/3 | 6/3 | L |
| Barrido | 17 | 25 | 4/3 | 4/3 | 4/3 | L |

*N* = sample size, $H_e/H_d$ = ratio of the number of loci with a heterozygosity excess ($H_e$) to the number with a heterozygosity deficiency ($H_d$); IAM = infinite allele model; TPM = two-phase model; SSM = stepwise mutation model; S = mode-shift distribution and L = L-shaped distribution (Luikart *et al.*, 1998).

* Significant ($p<0.05$) heterozygosity excess on average across all polymorphic loci using Wilcoxon's test (Cornuet & Luikart 1996; Pyri *et al.*, 1999).

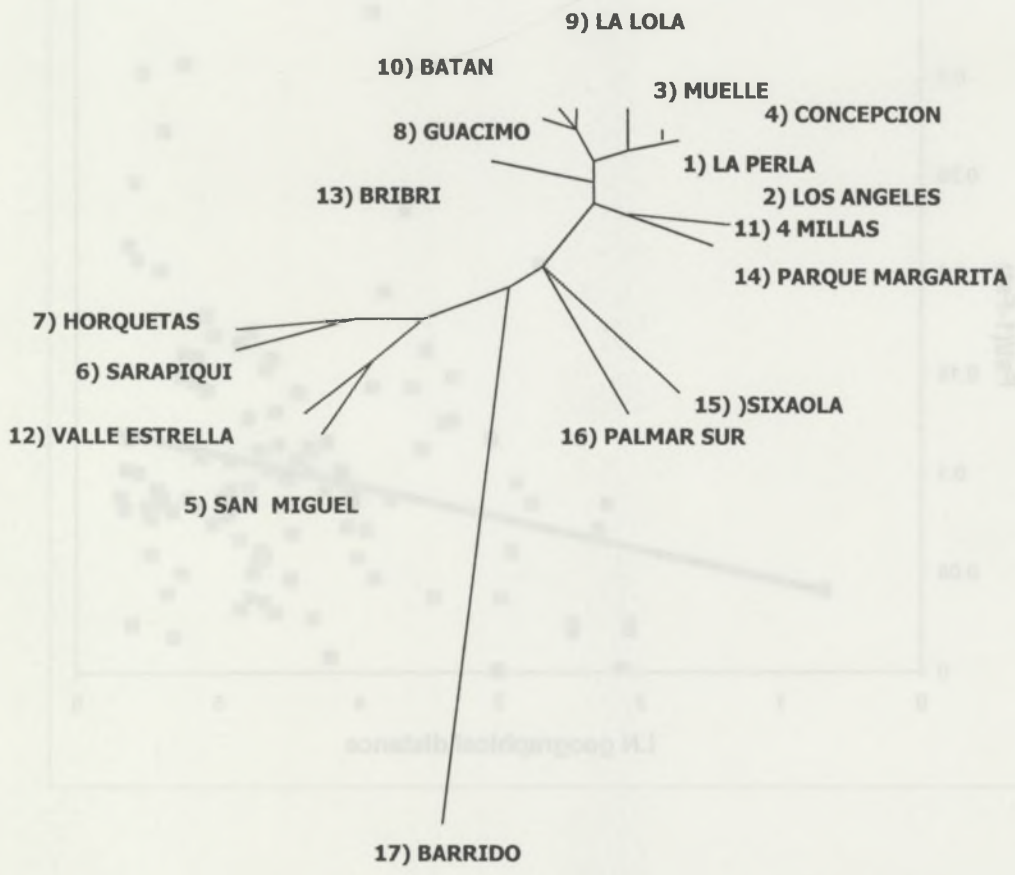


**Figure 2**. Additive tree constructed from estimates of Fst values among pairs of *Mycosphaerella fijiensis* populations from Costa Rica.

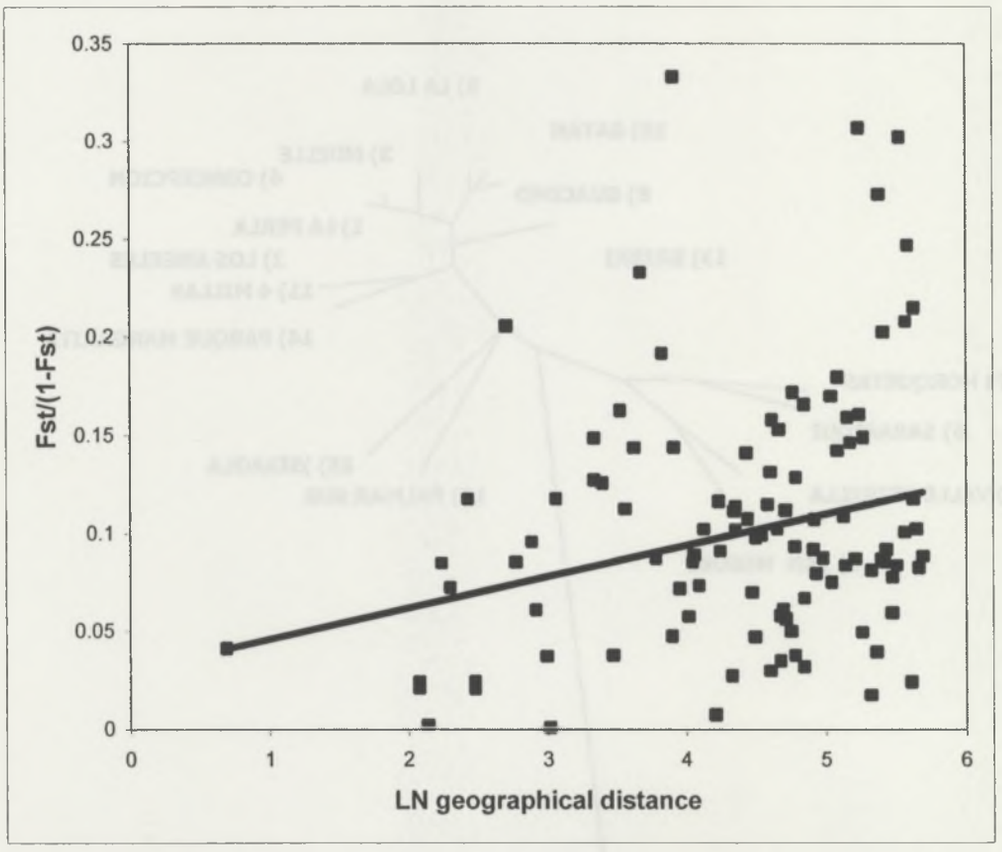


**Figure 3**. Differentiation among *Mycosphaerella fijiensis* populations in Costa Rica. Multilocus estimates of pairwise differentiation $(F_{st}/(1-F_{st})$ are plotted against the natural logarithm of pairwise geographic distances (in kilometres). The regression $y = 0.03 + 0.016x$ $(P=0.04)$ was estimated according to Rousset (1997).

## Table 4

Genetic differentiation between *Mycosphaerella fijiensis* populations from Costa Rica.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | - | 8.5 | 18.5 | 20.5 | 30.0 | 58.0 | 76.0 | 108.0 | 120.0 | 128.0 | 136.0 | 213.0 | 247.0 | 281.0 | 297.0 | - | - |
| 2 | 0.02 n.s. | - | 10.0 | 12.0 | 21.5 | 49.5 | 67.5 | 99.5 | 111.5 | 119.5 | 127.5 | 204.5 | 238.5 | 273.5 | 288.5 | - | - |
| 3 | 0.05 ** | 0.07 ** | - | 2.0 | 11.5 | 39.5 | 57.5 | 89.5 | 101.5 | 109.5 | 117.5 | 194.5 | 228.5 | 263.5 | 278.5 | - | - |
| 4 | 0.01 n.s. | 0.02 n.s. | 0.04 n.s. | - | 9.5 | 37.5 | 55.5 | 87.5 | 99.5 | 107.5 | 115.5 | 192.5 | 226.5 | 261.5 | 276.5 | - | - |
| 5 | 0.11 ** | 0.10 ** | 0.11 ** | 0.07 ** | - | 28.0 | 46.0 | 78.0 | 90.0 | 98.0 | 106.0 | 183.0 | 217.0 | 252.0 | 267.0 | - | - |
| 6 | 0.08 ** | 0.05 n.s. | 0.20 ** | 0.13 ** | 0.17 ** | - | 18.0 | 50.0 | 62.0 | 70.0 | 78.0 | 155.0 | 189.0 | 224.0 | 239.0 | - | - |
| 7 | 0.02 ** | 0.005 n.s. | 0.08 ** | 0.05 ** | 0.15 ** | 0.09 ** | - | 32.0 | 44.0 | 52.0 | 60.0 | 137.0 | 171.0 | 206.0 | 221.0 | - | - |
| 8 | 0.03 ** | 0.03 ** | 0.04 ** | 0.06 ** | 0.08 ** | 0.13 ** | 0.03 ** | - | 12.0 | 20.0 | 28.0 | 105 | 139.0 | 174.0 | 189.0 | - | - |
| 9 | 0.11 ** | 0.05 ** | 0.13 ** | 0.11 ** | 0.08 ** | 0.09 ** | 0.07 ** | 0.01 n.s. | - | 8.0 | 16.0 | 93.0 | 127.0 | 162.0 | 177.0 | - | - |
| 10 | 0.06 ** | 0.03 ** | 0.07 ** | 0.05 ** | 0.09 ** | 0.09 ** | 0.06 ** | 0.03 ** | 0.01 n.s. | - | 8.0 | 85.0 | 119.0 | 154.0 | 169.0 | - | - |
| 11 | 0.09 ** | 0.03 ** | 0.15 ** | 0.05 ** | 0.14 ** | 0.11 ** | 0.07 ** | 0.11 ** | 0.07 ** | 0.02 ** | - | 77.0 | 111.0 | 146.0 | 161.0 | - | - |
| 12 | 0.04 ** | 0.01 n.s. | 0.13 ** | 0.05 ** | 0.09 ** | 0.07 ** | 0.09 ** | 0.09 ** | 0.08 ** | 0.09 ** | 0.11 ** | - | 34.0 | 69.0 | 84.0 | - | - |
| 13 | 0.08 ** | 0.05 ** | 0.08 ** | 0.07 ** | 0.19 ** | 0.24 ** | 0.07 ** | 0.07 ** | 0.13 ** | 0.08 ** | 0.10 ** | 0.14 ** | - | 35.0 | 50.0 | - | - |
| 14 | 0.09 ** | 0.02 n.s. | 0.17 ** | 0.09 ** | 0.21 ** | 0.17 ** | 0.06 ** | 0.13 ** | 0.14 ** | 0.13 ** | 0.07 ** | 0.11 ** | 0.09 ** | - | 15.0 | - | - |
| 15 | 0.08 ** | 0.07 ** | 0.17 ** | 0.10 ** | 0.20 ** | 0.07 ** | 0.08 ** | 0.13 ** | 0.13 ** | 0.09 ** | 0.13 ** | 0.12 ** | 0.25 ** | 0.17 ** | - | - | - |
| 16 | 0.19 ** | 0.14 ** | 0.23 ** | 0.17 ** | 0.27 ** | 0.23 ** | 0.15 ** | 0.21 ** | 0.14 ** | 0.08 ** | 0.09 ** | 0.23 ** | 0.24 ** | 0.18 ** | 0.14 ** | - | - |
| 17 | 0.32 ** | 0.32 ** | 0.31 ** | 0.36 ** | 0.43 ** | 0.40 ** | 0.29 ** | 0.32 ** | 0.33 ** | 0.26 ** | 0.39 ** | 0.44 ** | 0.43 ** | 0.45 ** | 0.27 ** | 0.33 ** | - |

The upper matrix show geographical distance (in km) between populations used in isolation by distance analysis and the lower matrix show $F_{st}$ value for each pair of populations. See Table 1 for key to number site.

* P <0.05, ** P<0.001 using Fisher's combined probability test (RAYMOND and ROUSSET, 1995a; 1995b).

Fst overall= 0.13 **; 95% confidence interval= (0.09,0.17)

## Discussion

This study confirms from substantial sampling the existence of genetic diversity and a sexual panmictic population (random mating) in *M. fijiensis* at a local scale. These features and their implications were discussed in previous studies (Carlier *et al.* 1996; Rivas *et al.* 2003).

The structure of the *M. fijiensis* populations observed in Costa Rica suggests that founder effects occurred during the spread of the disease at the scale of a country. Theoretical models have shown that founder effects may cause loss of genetic diversity (Maruyama and Fuerst 1985; Nei *et al.* 1975). Smaller genetic diversity was observed only in the sample that was furthest from the others (site 17 at Barrido). Nevertheless, significant excess heterozygosity was detected in more than half of the samples with the two or three different mutation models used. As the spread of *M. fijiensis* in Costa Rica is recent (González 1987), the excess heterozygosity in these populations probably results from founder effects. The absence of significant excess heterozygosity in 6 of the other 17 samples with two or three mutation models may result from insufficient statistical power as the number of loci is smaller for these samples (Cornuet & Luikart 1996); Finally, theoretical models have shown that founder effects may cause genetic differentiation among populations (Boileau *et al.* 1992), as observed among the *M. fijiensis* populations analysed in the present work ($F_{st}$ = 0.13 on all populations). This level of differentiation is very close to that estimated on the same geographic scale in Cameroon using samples from four sites ($F_{st}$ = 0.11, (Rivas *et al.* 2003)).

Founder effects have also been suggested using population structure at the global and continental scales (Carlier *et al.* 1996; Rivas *et al.* 2003). However, a decrease in the level of genetic differentiation is observed between from global to country scales. $F_{st}$ values are 0.32 at the global scale between populations from different regions or continents (Carlier *et al.* 1996), 0.19 to 0.30 at the continental scale between the populations of different countries (Rivas et al. 2003) and 0.11 to 0.13 at the country scale between the populations at different sites (Rivas *et al.* 2003), this study). This is certainly the result of the increase of the two main modes of *M. fijiensis* dispersal when geographic scale decreases. The first mode is the movement of infected plant material. *M. fijiensis* can be carried by infected sword suckers and diseased leaves used to wrap food or other goods. The second dispersal mode is the wind-borne spread of ascospores produced during the sexual cycle. Wind dispersal of viable

ascospores over distances greater than a few hundred kilometres is unlikely (Parnell *et al.* 1998); At the global scale, the introduction of the disease in the different regions or continents is probably the result of rare movements of infected plant material causing marked founder effects and limited gene flows between established populations. The situation at the continental scale seems to be intermediate between global and country scales. The spread of the disease by the first mode could increase at continental scale and be complemented by ascospores dispersal. The latter mode is probably limited to distances of a few hundred kilometres, however. At the country scale, movements of plant material and spread by ascospores for distances of less than a few hundred kilometres would logically be much greater. The founder effects would be smaller and the gene flows between populations would increase at this scale. Preliminary epidemiological study suggested the existence of a disease gradient from an inoculum source for a distance of several tens meters (Abadie *et al.*, unpublished results). However, ascospores are certainly dispersed on larger distance following a tail of a probability distribution.

As dispersal of *M. fijiensis* mainly seems to take place for distances of less than a few hundred kilometres we used the isolation by distance model in order to estimate the gene flows at such a scale. The statistical test performed on all the samples collected along a transect of about 300 km was of low significance ($P < 0.05$). The statistical power of this test may be limited by the number of markers used and the variability of the latter. However, the significance of the test depends on two populations at one of the extremities of the transect (sites 14 and 15, Figure 1). The genetic structure was still significant, however, considering the remaining 13 sites although this structure has no geographic pattern. Such an ambiguous case does not allow reliable estimation of gene flows (Bossart & Prowell 1998). The structure observed may result from the recent spread of the disease in a varied environment such as Costa Rica. Indeed, large zones with no bananas often covered by tropical forest and hills lie along the transect between the sites sampled. The populations may not have attained equilibrium between the gene flows and genetic drift and reaching such an equilibrium may take more time following the founder effects observed and according to their importance.

This study enabled us to describe the structure of *M. fijiensis* populations at the scale of a country over a distance of several hundred kilometres. This structure reveals that foundation effects accompanied the spread of the disease and that there is genetic differentiation between pathogen populations at this scale. As the structure does not follow the isolation by distance model, the latter cannot be used to estimate gene flows. This may

partly result from the fact that the present study was conducted in a markedly heterogeneous environment. A similar study is in progress in Cameroon where the environment is more homogeneous. Nevertheless, the use of genetic models to estimate gene flows has many limits (Bossart & Prowell 1998). Results should be compared with more direct estimates from disease gradient studies (McCartney & Fitt 1998). Other methods of analysis such as phylogeography based on coalescence will be an alternative to such estimations (Hare 2001; Neigel 2002).

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# CONCLUSIONS ET PERSPECTIVES

La création de nouvelles variétés de banane 'dessert' ou 'à cuire' résistantes est aujourd'hui possible pour lutter, en particulier, contre les maladies foliaires (Bakry 2001). Elle se base sur la mise au point de schémas d'hybridation, sur une bonne connaissance de l'évolution du genre *Musa* et sur une caractérisation des ressources génétiques disponibles. De nouvelles variétés résistantes sont actuellement en cours de diffusion à Cuba (variétés tétraploïdes FHIA) ou en cours de validation en Guadeloupe (variétés triploïdes CIRAD). Cependant, afin d'optimiser la création variétale et d'aboutir à une gestion des résistances, la base génétique utilisée pour la transmission de la résistance nécessite d'être élargie et les connaissances sur les interactions hôte -parasites doivent être approfondies.

***Différentes espèces pathogènes***

Trois champignons ascomycètes apparentés sont considérés comme responsables des plus importantes maladies foliaires du bananier (Carlier *et al.* 2000; Crous & Mourichon 2002; Jones 2000): *Mycosphaerella fijiensis* (anamorphe *Paracercospora fijiensis*) responsable de la maladie des raies noires, *Mycosphaerella musicola* (anamorphe *Pseudocercospora musicola*) responsable de la maladie de Sigatoka et *Mycosphaerella eumusae* (anamorphe *Pseudocercospora eumusae*) responsable de l'ELSB (*eumusae* leaf spot of bananas). La distribution géographique des trois espèces dans les différentes régions de production est bien documentée à l'exception du Sud Est Asiatique. Depuis les années 1970, l'espèce la plus agressive *M. fijiensis* s'est disséminée en Afrique, en Amérique latine, dans les Caraïbes et dans les îles du Pacifique remplaçant *M. musicola* dans la plus grande partie des zones de productions bananières. *M. fijiensis* est aujourd'hui prédominante dans ces régions. L'espèce *M. eumusae* a été seulement observée dans le Sud Est Asiatique (à l'exception du Nigéria où elle semble très localisée depuis plusieurs années). Les efforts de recherche ont surtout été axés sur *M. fijiensis* au cours des deux dernières décennies. Cependant, les trois espèces pathogènes devront être considérées à l'avenir en particulier dans le Sud Est Asiatique où elles restent toutes très présentes.

Dans un premier temps, la répartition géographique des trois espèces pathogènes sur l'ensemble du Sud Est Asiatique devra être précisée. En effet, les interactions hôte - parasites

pourraient être différentes considérant les trois espèces. Ceci constitue une hypothèse que nous privilégions pour expliquer le maintien de ces espèces dans le Sud Est asiatique, puisque c'est dans cette région que l'on trouve la plus grande diversité de l'hôte. L'étude de la répartition des espèces pathogènes en fonction de l'hôte sera une première approche pour tester cette hypothèse. Les interactions hôte-parasite devront être ensuite comparées en condition contrôlées grâce notamment aux méthodes d'inoculation développées ces dernières années (Beveraggi *et al.* 1995 ; El Hadrami 2000). Si ces interactions sont différentes, les résistances qui seront utilisées pour créer de nouvelles variétés pourront être plus ou moins efficaces, voir même spécifiques, selon les espèces pathogènes. Ainsi leur utilisation devra être raisonnée en fonction de leur répartition géographique.

Un outil de diagnostic moléculaire serait cependant très utile pour préciser la distribution des espèces pathogènes plus particulièrement dans le Sud Est asiatique. L'identification des espèces se fait classiquement à partir de l'observation de la morphologie de la forme imparfaite. Le développement d'amorces spécifiques pourrait permettre d'identifier les espèces directement par PCR à partir de feuilles présentant des symptômes et donc sans isolement. Un plus grand nombre d'échantillons pourraient être ainsi analysés rapidement. De plus, les échantillons pour lesquels des formes imparfaites sont non observables peuvent être aussi étudiés. Les travaux réalisés à ce jour sur la taxonomie et les populations des parasites montrent qu'il existe un complexe d'espèces apparentées sur les feuilles du bananier et une importante diversité intra-spécifique. Le développement d'amorces doit se faire à partir d'un échantillon le plus représentatif possible des différentes espèces apparentées et de la diversité existante au sein de chaque espèce. Des études taxonomiques et de diversité sont encore nécessaires pour définir un tel échantillon.

***Sélection de variétés résistantes vis-à-vis de M. fijiensis***

Une forte diversité génétique est maintenue dans les populations de *M. fijiensis* qui sont en partie issue d'une reproduction sexuée panmictique (Carlier *et al.* 1996). L'existence d'une variabilité du pouvoir pathogène a été également mise en évidence (Fullerton & Olsen 1995 ; El Hadrami 2000). Ces caractéristiques suggèrent des capacités d'adaptation importantes qui doivent être prises en compte dans le choix d'une stratégie de création de variétés résistantes. Une stratégie de création tel que le 'pyramiding' (combinaison de plusieurs gènes de résistance spécifiques dans une même variété) n'est pas envisageable,

contrairement à des parasites asexués tel que *Magnaporthe grisea* (Carlier *et al. 2003*). Dans le cas d'un parasite présentant une reproduction sexuée tel que *M. fijiensis*, la probabilité d'apparition par recombinaison de génotypes cumulant plusieurs gènes de virulence sera certainement importante. Ainsi, les programmes d'amélioration génétique utilisent des résistances partielles que l'on suppose durables. Des travaux ont été entrepris sur la caractérisation de la résistance partielle pour orienter la sélection plus particulièrement vis-à-vis de l'espèce pathogène la plus importante *M. fijiensis* (Abadie *et al.* 2003a; 2003b). Des composantes de résistance agissant à différentes étapes du cycle infectieux ont été identifiées. Des études épidémiologiques et le développement d'un modèle sont en cours à l'échelle d'une plantation afin d'évaluer l'importance relative de ces composantes en terme d'efficacité. Parallèlement des études se poursuivent pour cartographier génétiquement des gènes de résistance ou des QTL impliqués dans l'expression des résistances dans le but de mettre en place une sélection assistée par marqueurs (Carreel *et al.* 1999). L'ensemble de ces travaux est pour le moment réalisé sur un nombre restreint de géniteurs. Ce nombre devra dans l'avenir être plus important afin de pouvoir générer une large gamme de variétés résistantes.

***Interactions hôte-pathogènes***

Aucuns travaux moléculaires sur les interactions hôte-parasites ont été publiés à ce jour. Des systèmes de transformation pour les deux partenaires sont toutefois disponibles (Balint-Kurti *et al.* 2001; Sagi 2000). Des banques EST (Expressed Sequence Tag) sont en cours de constitution à partir de feuille de bananier infecté par *M. fijiensis*. Cette ressource sera ensuite utilisée pour tenter d'isoler des gènes impliqués dans les réactions de défense et dans le pouvoir pathogène. Des cartes génétique et physique du génome de *M. fijiensis* sont également en cours de développement. Comme nous le ferons ci-dessous, l'ensemble de ces ressources devrait nous permettre de disposer prochainement d'information et de nombreux marqueurs moléculaires pour développer une approche plus globale, tenant compte simultanément des différents niveaux d'intégration biologique (du gène à la population).

***Structure des populations de M. fijiensis***

Les résultats du présent travail de thèse permettent tout d'abord de cerner le niveau et la distribution de la diversité génétique maintenue chez *M. fijiensis* en Afrique et dans la

région Amérique Latine - Caraïbes. Des effets de fondation ont été détectés à l'échelle d'un continent et d'un pays, reflétant l'expansion récente de *M. fijiensis* dans ces régions et entraînant une différenciation génétique importante entre populations pathogènes. De tels effets ont été mis en évidence auparavant lors d'une étude de la structure des populations de *M. fijiensis* à l'échelle mondiale (Carlier *et al.* 1996). Concernant la région Amérique Latine-Caraïbes, une diversité génétique plus importante a été détectée au Honduras, pays où la maladie a été observée pour la première fois et à l'origine de l'épidémie dans cette région (Pasberg-Gauhl *et al.* 2000). L'un des plus importants programmes d'amélioration génétique du bananier est conduit au Honduras (Jones 2000); il conviendrait donc de préciser la structure des populations pathogènes dans ce pays. Des localités où la diversité est maximale et représentative de l'ensemble de la région Amérique Latine - Caraïbes pourraient être ainsi identifiées comme sites de sélection des résistances génétiques.

Concernant l'Afrique, aucune zone ne se distingue des autres par un niveau de diversité plus importante chez *M. fijiensis*. Les informations sur la zone d'introduction de la maladie dans ce continent sont moins précises (Pasberg-Gauhl *et al.* 2000) et l'échantillonnage réalisé dans cette étude pourrait ne pas recouvrir une telle zone. Si des études ultérieures sont conduites, elles devront prendre en compte des échantillons plus importants provenant des zones supposées d'introductions (telles que le Gabon, la Zambie et Sao Tomé ; Pasberg-Gauhl *et al.* 2000). Cependant, nous doutons de l'intérêt et des retombées de cette démarche, en particulier si les moyens à mettre en œuvre sont importants. En effet, même si une population a subi un effet de fondation, le niveau de diversité sera rétabli au cours du temps grâce à la mutation (Cornuet & Luikart 1996). De plus, certaines mutations peuvent dès à présent exister dans de telles populations à faibles fréquences, lesquelles pourraient rapidement augmenter en réponse à une sélection exercée par des résistances génétiques.

La structure des populations à l'échelle mondiale de *M. fijiensis* ainsi que de *M. musicola* montre qu'il existe une diversité beaucoup plus importantes dans le Sud Est asiatique (Carlier *et al. 2003*). Cette région est très certainement le centre d'origine de ces espèces parasites. Le Sud Est Asiatique serait aussi le centre d'origine de l'espèce *M. eumusae* puisque, à l'exception d'une localité au Nigeria, elle n'a pas été détectée dans les autres régions, malgré un grand nombre d'échantillons analysés ces dernières années (Carlier *et al.* 2000). Enfin, le Sud Est Asiatique est également le centre d'origine et de diversité du bananier (Horry 1989). On y trouve des populations sauvages des deux espèces à l'origine des

cultivars, *Musa acuminata* et *Musa balbisiana*, ainsi qu'une importante diversité chez les cultivars parthénocarpiques. Pour les deux espèces *M. fijiensis* et *M. musicola*, très peu d'échantillons provenant d'Asie ont été analysés à ce jour et aucun pour l'espèce *M. eumusae*. En fonction de la répartition des populations sauvages de l'hôte, l'étude de la structure des populations pathogènes en Asie du Sud Est permettra de localiser pour les trois espèces des zones de co-évolution. De telles zones constitueraient des sources de résistance potentielles pour chacune des espèces et permettraient d'étudier l'évolution des populations pathogènes en milieu naturel.

***Dispersion, stratégie reproductive et flux de gènes chez M. fijiensis***

Les résultats obtenus dans cette étude montrent l'existence, à l'échelle même d'une plante, de populations de *M. fijiensis* présentant une importante diversité génétique et issue d'une reproduction sexuée panmictique. Aux échelles supérieures, une différentiation génétique entre populations pathogènes est mise en évidence ; cette différenciation augmentant avec la distance géographique. L'ensemble de ces résultats peut s'expliquer par une augmentation des flux de gènes lorsque l'échelle géographique diminue au travers des deux principaux modes de dissémination : le mouvement de matériel végétal infecté et les ascospores. A l'échelle mondiale, entre continents, le seul mode de dissémination serait le mouvement de matériel végétal infecté (Carlier *et al.* 1996; Mourichon & Fullerton 1990). Bien que ce mode de dissémination puisse augmenter aux échelles inférieures, son importance relative par rapport au mode de dissémination par les ascospores diminuerait. Ainsi, la dispersion des ascospores augmenterait lorsque la distance géographique diminue et serait très importante à l'échelle d'une parcelle. La situation à l'échelle continentale serait intermédiaire, avec une dissémination qui pourrait être due aussi bien à des mouvements de matériel végétal qu'à une dispersion des ascospores. Dans ce dernier cas, la dispersion des ascospores serait cependant limitée sur des distances de quelques centaines de mètres.

Nous avons tenté d'estimer les flux de gène et les limites de dispersion sur une distance de 300 km environ au Costa Rica par utilisation du modèle d'isolement par la distance géographique. A ce jour cette estimation n'a pas été possible et d'autres analyses sont nécessaires pour explorer d'avantage cette voie. Alternativement, d'autres méthodes d'analyses basées sur les méthodes de coalescences, en cours de développement, pourront être appliquées à notre modèle biologique (Hare 2001; Neigel 2002). Finalement, la concordance

des estimations de paramètres de dispersion obtenues de façon indirecte, à l'aide de modèle génétique, et par observation directe, au travers de l'étude de gradients de maladie, devra être vérifié (Rousset 2001).

Les résultats de cette étude ne nous ont pas permis d'appréhender l'importance de la dispersion par les conidies produites lors du cycle asexué. En effet, les isolats analysés sont tous issus d'un clonage monoascospore, donc directement d'une reproduction sexuée. Des observations directes suggèrent que la dispersion des conidies se fait à l'échelle de la plante et entre plantes contiguës (Pasberg-Gauhl *et al.* 2000). Suite à l'infestation des feuilles par les ascospores, les conidies participent à la ré-infestation et ainsi à une augmentation du niveau de maladie sur une plante. Il sera important de préciser, à cette échelle, l'importance relative de la dispersion des ascospores et des conidies, donc des reproductions sexuées et asexuées. En effet, des génotypes plus adaptés à une variété résistante donnée pourraient augmenter en fréquence par sélection et ainsi modifier la structure des populations pathogènes.

Quantifier l'importance relative de la dispersion par les ascospores et les conidies est très difficile à réaliser par observation directe. Cette difficulté pourrait être contournée indirectement par une analyse plus fine de la structure des populations pathogène à l'échelle de quelques plantes contiguës. Les échantillons seront constitués par des isolats issus d'un clonage monoconidie. Les isolats, issus d'une reproduction asexuée, présentant un même génotype, pourront être identifiés à l'aide de marqueurs moléculaires générant des empreintes génétiques. Les génotypes différents observés seront, par contre, issus d'une reproduction sexuée. L'importance relative des deux modes de dispersion et de reproduction pourra être ainsi estimée.

***Evolution des populations pathogènes***

Les travaux réalisés dans la présente thèse concerne l'analyse de la structure spatiale des populations de *M. fijiensis*. L'érosion d'une résistance partielle par adaptation de populations pathogènes a été récemment observée dans le cas du blé vis-à-vis du champignon *M. graminicola* (Cowger & Mundt 2002; Mundt *et al.* 2002). L'évaluation du potentiel adaptatif des populations de *M. fijiensis* vis-à-vis de composante de résistance partielle du bananier a été également abordée (Abadie *et al.* 2003a; Abadie *et al.* 2003b). L'évolution des populations pathogènes est suivie sur des parcelles monovariétales constituées par des génotypes partiellement résistants ou un génotype sensible. Des échantillons d'isolats

prélevés aux différents cycles de culture ont été analysés, conjointement avec des marqueurs moléculaires et une quantification de l'agressivité, de façon à estimer l'importance relative de la dérive génétique et de la sélection par l'hôte. Cette étude présente plusieurs limites. La première concerne le dispositif lui-même avec des parcelles non isolées, contiguës et situées dans une zone de culture bananière intensive. Les premiers résultats sur deux cycles de culture suggèrent des flux de gènes important entre parcelles ou provenant de l'environnement au travers de la dispersion par les ascospores. Ces flux de gènes pourrait contre balancer complètement un éventuel effet de la sélection par l'hôte (Lenormand 2002). Notons cependant qu'un faible effet de la dérive génétique a été observé suite au premier cycle de culture et donc lors de l'établissement des populations pathogènes. Cette dérive a entraîné des déséquilibres de liaison entre marqueurs moléculaires qui pourraient être exploités comme proposé plus loin. La deuxième principale limite est liée à l'imprécision de l'évaluation de l'agressivité des populations pathogènes par inoculation en conditions contrôlées. Du fait de la lourdeur d'une telle évaluation, le nombre d'isolats et de répétitions est restreint limitant ainsi la puissance des analyses statistiques.

Les difficultés exposées ci-dessus pourraient être contournées de la façon suivante : un dispositif avec une parcelle isolée des zones de culture et un apport extérieur d'une source d'inoculum a été mis en place récemment avec succès pour mesurer un gradient de maladie (Abadie *et al*, non publié). Un dispositif similaire avec des parcelles isolées, constitué par différents génotypes et soumis à une même source d'inoculum est aujourd'hui envisageable. Un tel dispositif permettrait de limiter les flux de gène entre parcelles et de pouvoir observer plus facilement un éventuel effet sélectif de l'hôte. Alternativement, l'évolution des populations pathogènes pourrait être suivie dans des régions où des cultivars partiellement résistants sont en cours de diffusion à grande échelle, comme à Cuba.

Il sera possible dans un avenir proche d'analyser des populations pathogènes avec un plus grand nombre de marqueurs en s'appuyant sur une carte génétique. Une telle approche a été récemment utilisée chez la drosophile, *Drosophila melanogaster* (Harr & Schlötterer 2002). Grâce aux déséquilibres créés dans un premier temps au hasard par la dérive génétique en début d'épidémie, des marqueurs moléculaires peuvent se trouver liés à des locus soumis à une pression de sélection. Les fréquences alléliques à ces marqueurs pourrait ainsi varier par sélection au travers de l'effet dit 'd'auto stop génétique'. La comparaison de la différenciation génétique entre parcelles pour des marqueurs entraînés par 'd'auto stop génétique' et 'neutres' et devrait nous permettre de quantifier plus précisément des pressions de sélection. Une

estimation de l'agressivité pourra être ensuite menée, de façon complémentaire, en tenant compte de la présence éventuelle de génotypes différents qui différeraient pour des régions génomiques soumises à une pression de sélection. Cette approche nous permettra aussi d'identifier des régions génomiques portant des gènes impliqués dans l'expression du pouvoir pathogène ouvrant la voie à d'autres démarches comme celle dite 'gènes candidats' avec l'exploitation des banques EST.

***Vers une gestion des résistances***

Une gestion des résistances devra être efficace en terme de contrôle de la maladie en tenant compte du potentiel évolutif des populations pathogènes. La mise en évidence de flux de gènes occasionnels à l'échelle mondiale nous a amené à proposer une gestion des résistances indépendantes pour chaque zone de production (Asie, Pacifique, Afrique, Amérique Latine – Caraïbes ; Carlier *et al.* 1996). Les résultats obtenus à ce jour ne permettent pas de préciser à quelles échelles géographiques la gestion des résistances sera raisonnée dans chaque région. Cependant, ces échelles seront supérieures à celle d'une localité ne permettant pas de tester expérimentalement différents scénarios de gestion. Le développement d'outils de modélisation et de simulation s'impose. Il s'agira de développer à la fois des modèles épidémiologiques, pour tester l'efficacité de différents scénarios, et des modèles génétiques, pour tenter de simuler et de prévoir les réponses adaptatives des populations pathogènes vis-à-vis de ces scénarios. Une validation de ces modèles et des réajustements résulteront d'observations réalisées en conditions réelles. La modélisation sera également très utile pour optimiser les expérimentations conduites à une échelle locale et pour mieux exploiter les données obtenues. La modélisation de l'évolution des populations pathogènes nécessite d'estimer des paramètres reliés aux différents facteurs évolutifs. Les résultats des études de la structure et de l'évolution des populations de *M. fijiensis* réalisées à ce jour nous ont déjà permis d'appréhender l'importance de certains facteurs. Les travaux qui seront développés les prochaines années tenteront plus précisément d'estimer des paramètres liés au régime de reproduction, au flux de gènes et à la sélection par l'hôte.

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# Annexe

Sous-chapitre d'un ouvrage sous presse

A paraître dans *Mycosphaerella leaf spot diseases of bananas: Present status and outlook.* (eds Jacome L, Lepoivre P, Marin R, Ortiz R, Romero RA & Escalant JV), INIBAP, Montpellier.

## Genetic differentiation in *Mycosphaerella* leaf spot pathogens

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### Abstract

Black leaf streak disease and Sigatoka disease, caused respectively by two related fungi, *Mycosphaerella fijiensis* and *M. musicola,* are the most important leaf spot diseases of bananas. Understanding the genetic structure of the populations and the evolution of these pathogens is an important aid in breeding and managing disease resistance. The population structure of each pathogen was analysed using molecular markers mainly at the global and continental scales. Features common to both were observed: 1) the centre of diversity is located in Southeast Asia and founder events accompanying the introduction of the pathogens in other regions led to a reduction of genetic diversity; 2) genetic diversity is maintained in all populations and is also present at the scale of the plant; 3) genetic recombination played an important role in the genetic structure of both pathogens; 4) genetic differentiation exists between populations from the global to the local level. The main difference between the two species had to do with the measures of genetic differentiation. Whereas the African populations of *M. fijiensis* were significantly different from the Latin American/Caribbean ones, no significant difference was observed between the African and Latin American/Caribbean populations of *M. musicola.* This suggests independent introductions of *M. fijiensis* but not of *M. musicola.* Except for this situation, the genetic differentiation observed between populations at the global and continental scales indicate an important effect of genetic drift and limited gene flow.

## Resumen

### Diferenciación genética en los patógenos de la mancha foliar *Mycosphaeralla*

Las enfermedades de la mancha foliar de los bananos más importantes se deben a dos hongos relacionados: *Mycosphaerella fijiensis* y *Mycosphaerella musicola,* los agentes causales de la enfermedad de la raya negra de la hoja y de la enfermedad de la Sigatoka, respectivamente. El entendimiento, tanto de la estructura genética de la población, como de la evolución de estos patógenos proporciona información importante para brindar asistencia al mejoramiento y manejo de la resistencia a la enfermedad. La estructura de la población de ambos patógenos fue analizada utilizando marcadores moleculares a escalas global y continental. Se observaron las siguientes características comunes: 1) el centro de la diversidad está localizado en el Sudeste de Asia y los eventos de colonización que acompañaron la introducción de los patógenos en otras regiones han llevado a una reducción de la diversidad genética en comparación con el Sudeste de Asia; 2) la diversidad genética se mantiene en todas las poblaciones y se distribuye a escala de la planta, 3) la recombinación genética desempeña un papel importante en la estructura genética de ambos patógenos; 4) existe una diferenciación genética entre las poblaciones a escalas de global a local. La principal diferencia observada es la existencia de una diferenciación genética entre las poblaciones africanas y las poblaciones latinoamericanas y caribeñas de *M. fijiensis* pero no de *M. musicola*. Este resultado sugiere introducciones independientes de *M. fijiensis* pero no de *M. musicola*. Con excepción de la situación descrita arriba, la diferenciación genética observada en ambos patógenos entre las poblaciones a escalas global y continental indica una derivación genética importante y un flujo de genes bajo.

## Résumé

### Différentiation génétique chez les *Mycosphaerella* pathogènes

La maladie des raies noires et la maladie de Sigatoka, respectivement causées par deux champignons voisins, *Mycosphaerella fijiensis* et *M. musicola,* sont les maladies de taches des feuilles les plus importantes chez le bananier. La compréhension de la structure génétique des populations et de l'évolution de ces pathogènes représente une aide importante pour l'amélioration et la gestion de la résistance à ces maladies. La structure de la population de

chaque pathogène a été analysée en utilisant des marqueurs moléculaires, principalement à l'échelle globale et continentale. Des caractéristiques communes aux deux pathogènes ont été observées : 1) leur centre de diversité est localisé en Asie du Sud-est et des événements fondateurs accompagnant l'introduction des pathogènes dans d'autres régions ont conduit à une réduction de la diversité génétique ; 2) la diversité génétique est maintenue dans toutes les populations et est également présente à l'échelle de la plante ; 3) la recombinaison génétique a joué un rôle important dans la structure génétique des deux pathogènes ; 4) une différentiation génétique existe entre populations, du niveau global au niveau local. La principale différence entre les deux espèces concerne les mesures de la différentiation génétique. Alors que les populations africaines de *M. fijiensis* sont significativement différentes de celles d'Amérique latine/Caraïbes, aucune différence significative n'a été observée entre les populations de *M. musicola* originaires d'Afrique et d'Amérique latine/Caraïbes. Ceci suggère des introductions indépendantes de *M. fijiensis*, mais pas de *M. musicola*. A part cette situation, la différentiation génétique observée entre populations à l'échelle globale et continentale indique un effet important de la dérive génétique et des flux géniques limités.

**Introduction**

Black leaf streak disease, caused by *Mycosphaerella fijiensis,* and Sigatoka disease, caused by *M musicola,* are the most important leaf spot diseases of bananas (Jones, 2000). The fungi are haploid and heterothallic. The anamorph and teleomorph stages are both present on infected leaves, and the ascospores produced during the sexual stage play an important role in epidemics. The first species to be described was *M. musicola,* in Java in 1902. The rapid dissemination of Sigatoka disease round the world in the 1930s, led to speculations that the spores were carried by air currents between continents: from Asia to the Pacific, from the Pacific to Australia, from Australia to Africa and from Africa to Latin America (Stover, 1962). In 1962, *M. musicola* was present in all banana-producing regions, making Sigatoka disease one of the most important plant diseases. Black leaf streak disease was reported in Fiji in 1964 and since then has been reported throughout the Pacific and Asia. The chronology of records suggests that *M. fijiensis* originated, as *M. musicola,* in Southeast Asia (Mourichon and Fullerton, 1990), which is also the centre of origin of the host genus *Musa*. Starting in the 1970s, *M. fijiensis* spread to Africa, Latin America and the Caribbean. Being more aggressive, *M. fijiensis* replaced *M. musicola* as the dominant leaf spot pathogen in many areas. Although the distribution of both pathogens is well documented in Australia, the

Pacific region, Africa, Latin America and the Caribbean, it is still not well understood in Southeast Asia.

**Why and how to analyse populations of pathogens?**

Knowledge of the genetic population structure and evolution of the pathogens is an important aid in breeding and managing disease resistance. The main objective of such study is to provide information on the level and distribution of variability. Molecular markers are often used to analyse population structure at different geographical scales. It should make it possible to identify potential sources of resistance, which are expected to be in areas where the diversity of pathogens and host are high. It should also ensure that the diversity of pathogens used when screening for resistance is representative of the one in the regions where resistant hosts are intended to be used. Pathogens can evolve to break down total resistance or erode partial resistance. The evolution of pathogen populations depends on mutation, recombination, genetic drift, gene flow and the selection pressure exerted by the host. It should be possible to limit and restrict the evolution of pathogenicity by varying host resistance in space and time. Such strategies should improve the durability of the types of resistance used and ensure the durability of the culture.

Another objective of pathogen population studies is to evaluate the relative importance of the evolutionary factors on the evolution of pathogens. Such information would make it possible to model and test the effect of different management strategies on the evolution of the pathogen. Analysing population structure through space allows us to evaluate the effects of genetic recombination, genetic drift and gene flow on the evolution of the pathogen. This paper reviews the results obtained at global and local scales for *M. musicola* and *M. fijiensis*. A second approach consists in studying the evolution of the pathogen in fields of resistant hosts by using molecular markers and by characterizing pathogenicity. This allows us to evaluate the effect of the selection pressure exerted by the host on the pathogen. This second approach is described in another paper (see Abadie *et al.* in this volume).

**Global population structure**

RFLP markers were used to study the genetic structure of the global population of *M. musicola* and of *M. fijiensis* (Carlier *et al.*, 1996; Hayden *et al.*, in prep.) Random single-

locus probes were used on samples from Southeast Asia, Australia, the Pacific Islands, Africa, Latin America and the Caribbean. Features common to both pathogens were observed.

Southeast Asia has the highest level of gene diversity (table 1) and the majority of alleles found in this region were also present in the other regions. This supports the hypothesis that the pathogens originated in Southeast Asia. Founder events accompanying the introduction of the pathogens to other regions have led to a reduction in genetic diversity in comparison with Southeast Asia. Nevertheless, genetic diversity is maintained in all populations. Ecological conditions being favourable for disease development and banana cultivation almost year round in most growing areas, low genetic drift in large pathogen populations can maintain the high levels of genetic diversity observed. Therefore, a certain level of variability in pathogenicity might also be maintained in pathogen populations, allowing the pathogen to attack newly introduced resistant genotypes, as has been observed with *M. fijiensis* on 'Paka' and 'T8' in Rarotonga, Cook Islands (Fullerton and Olsen, 1995). The existence of specific interactions between the host and *M. fijiensis* isolates was suggested for highly resistant genotypes (Fullerton and Olsen, 1995). Variability in aggressiveness was evaluated for two *M. fijiensis* samples from Cameroon and the Philippines by inoculating five partially resistant cultivars using a leaf piece assay (El Hadrami, 2000). Variability was similar but low for both countries, however genetic diversity in the Philippines was much higher (Carlier *et al.,* 1996). Specific interactions between the isolates and the cultivar were not detected. Only susceptible hosts are cultivated in these countries, and the lack of a selection pressure being exerted by the host on the pathogens could explain the results. The potential of pathogen populations to overcome partial resistance should be analysed by following their evolution in fields of resistant hosts (see Abadie *et al.* in this volume).

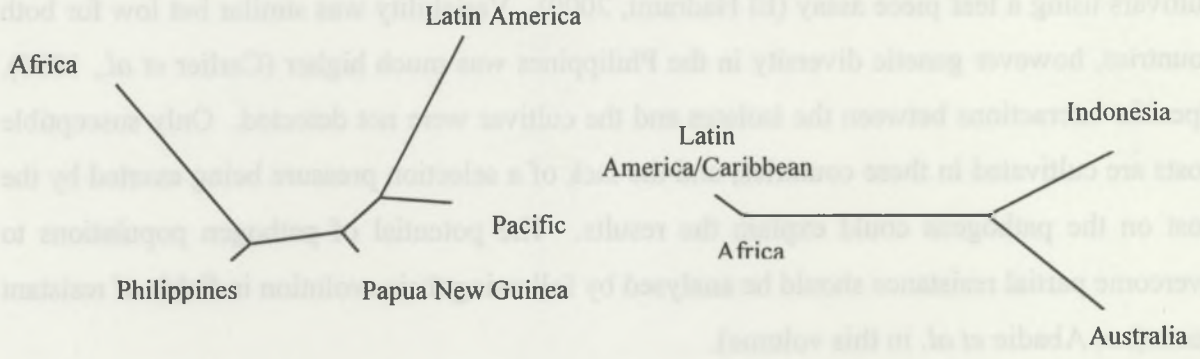
Genetic recombination plays an important role in the genetic structure of *M. musicola* and *M. fijiensis*. Genetic markers were statistically independent therefore characteristics of pathogenicity could not be related to RFLP genotypes. With regards to breeding programmes, introducing specific resistance genes in individual cultivars (pyramiding) may not be a strategy for durable resistance in banana. Mixing varieties or using partially resistant hosts might be more appropriate.

A high level of genetic differentiation was observed between populations of *M. musicola* and *M. fijiensis* from different regions (figure 1). The $F_{st}$ parameter (Wright, 1951) estimated for

**Table 1**. Estimates of Nei (1978) gene diversity for populations of *M. fijiensis* (Carlier *et al.*, 1996) and *M. musicola* (Hayden *et al.*, in prep.) from different geographical regions.

| Species | Asia | Africa | Latin America and Caribbean | Australia and Pacific |
|---|---|---|---|---|
| *M. fijiensis* | 0.57 | 0.25 | 0.40 | 0.28 |
| *M. musicola* | 0.41 | 0.27 | 0.21 | 0.33 |

all loci between pairs of populations varied between 0.14 and 0.58 for *M. fijiensis* and between 0.025 and 0.55 for *M. musicola*. But whereas the African populations of *M. fijiensis* were significantly different from the Latin American/Caribbean ones ($F_{st}$ = 0.49), no significant difference was observed between the African and Latin American populations of *M. musicola* (Fst = 0.025, not significant). This suggests a separate introduction of *M. fijiensis* in each region but a common one for *M. musicola*.




a) b)

**Figure 1.** Global population structure of a) *M. fijiensis* (Carlier *et al.*, 1996) and b) *M. musicola* (Hayden *et al.*, in prep.). Additive trees constructed from estimates of Wright's Fst among pairs of geographical populations.

On the other hand, the high levels of genetic differentiation observed between Australian and African populations of *M. musicola* ($F_{st}$ = 0.47) does not support the hypothesis of Stover (1962) whereby spores of *M. musicola* were carried by air currents from Australia to Africa. In general, the high level of genetic differentiation of both pathogens at a global scale suggests occasional migration events between continents. Long distance dissemination of the disease around the word was more likely to have occurred by movement of infected plant material, as proposed by Mourichon and Fullerton (1990).

**Continental and local population structures**

Population structures at a continental scale were studied in Australia for *M. musicola* (Hayden, 2000). Collections of isolates from twelve sites along the east coast were analysed using 15 RFLP markers. The level of gene diversity (Nei, 1978), varied between 0.14 and 0.37. On a plant, the pathogen isolated from a given lesion would often be a haplotype not found in the other lesions, meaning that diversity is also present at a fine scale. Low to high levels of genetic differentiation were observed between populations ($F_{st}$ = 0.04-0.45). There was no apparent correlation between genetic and geographical distances as high levels of genetic differentiation were observed between neighbouring populations and low levels were observed in populations separated by long distances.

The population structure of *M. fijiensis* was analysed in Africa, Latin America and the Caribbean (Rivas *et al.*, submitted.). Samples from different countries were characterized using CAPS (Cleaved Amplified Polymorphic Sequence) markers (Zapater *et al.*, subm.). The results obtained for both continents were similar. The value of gene diversity varied between 0.19 and 0.38 for Africa, and 0.16 and 0.36 for the Latin America/Caribbean region. The low levels detected in some populations suggest that founder effects occurred during the spread of the disease on both continents. In the Latin America and Caribbean region, the highest levels are observed in populations from Honduras and Costa Rica, supporting the suggestion that the pathogen entered the continent in this area. In one locality in Cameroon, the values of gene diversity estimated at the scale of the field and of the plant are similar suggesting, as with *M. musicola*, that diversity is distributed at a fine scale.

Important levels of genetic differentiation were detected between most of the populations (Fst = 0.04-0.45 for Africa and 0.01-0.56 for Latin America/Caribbean). There is sufficient

differentiation between populations in the Caribbean islands to support the hypothesis that there was more than one introduction from Latin America. In Cameroon, genetic differentiation was detected between localities 300 km apart but not between localities 200 km apart.

Finally, the genetic structure of *M fijiensis* was studied in the Australasia/Pacific region using RFLP markers (Hayden *et al.,* subm.). Genetic differentiation was detected between the Torres Straits Islands, Pacific Islands and Papua New Guinea. At a local scale, there was no differentiation between two sites in the small Mer island.

The levels of genetic differentiation observed at the continental scale for both pathogens suggest an important effect of genetic drift on population structure and limited gene flow. Thus, spread of the diseases within continents could be due to the movement of infected plants and/or very restricted ascospore dispersal. As populations are probably not in genetic equilibrium, gene flow resulting from ascospore dispersal may be underestimated. However, preliminary results from an epidemiological study of black leaf streak disease suggests that dispersal of the pathogen is more restricted than previously thought. The results suggest a dispersal gradient of about 25 m from an inoculum source (see Abadie *et al.* in this volume).

**Conclusion and perspectives**

The population structures of *M. musicola* and *M. fijiensis* are now better known at different geographical scales. However, at a regional scale few samples from Southeast Asia have been analysed. A new pathogen, *Mycosphaerella eumusae,* was recently discovered and detected mainly in Southeast Asia (Carlier *et al.*, 2000). Southeast Asia is not only the centre of origin of all three pathogens but also of the host genus *Musa.* The distribution of the pathogens and their population structure should now be determined in detail for this region. Host-pathogen interactions could differ for each pathogen. One hypothesis to explain the continued presence of the three pathogens in Southeast Asia is the high diversity of host species. The hypothesis could be tested by surveying the fungal species in relation to host diversity. If host-pathogen interactions differ, the resistance genes introduced to produce new varieties could be more or less efficient depending on the pathogen they are exposed to. Their utilization should take into account the distribution of pathogen species. Zones of co-evolution for the three pathogens could be localized in Southeast Asia. This area is a

potential source of resistance, therefore a study of pathogen populations in natural systems should provide us with information to complement evaluations of the relative importance of the different evolutionary forces.

The results to date suggest that genetic drift has an important effect on the structure of pathogen populations and that gene flow is limited. The limit of ascospore dispersal should be estimated indirectly at a country scale using genetic models such as the isolation by distance model (Rousset, 1997). However, we can already predict that the improvement of quarantine measures at the continental scale might limit the risk of introducing the disease to new areas, and limit the exchange between existing pathogen populations from different countries. At a country and local level, geographical obstacles could also limit exchange between populations from different fields by playing on gene flow. Such a measure could have an impact on the durability of the resistances and of the management strategies.

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**RESUME :**

Le champignon *Mycosphaerella fijiensis* est un agent pathogène du bananier. Les objectifs de ce travail étaient de décrire la structure de populations de *M. fijiensis* aux échelles continentales et locales, et d'appréhender les flux gènes et les processus de dispersion de ce champignon à ces deux échelles. Des échantillons d'isolats collectés en Amérique Latine-Caraïbes et en Afrique ont été analysés à l'aide de marqueurs moléculaires neutres. Les populations de *M. fijiensis* présentent un haut niveau de diversité génétique et une recombinaison génétique importante à l'échelle même d'une plante. A l'échelle continentale, la dissémination de *M. fijiensis* serait stochastique et pourrait être aussi bien due à des mouvements de matériel végétal infecté, qu'à une dispersion limitée des ascospores sur des distances de quelques centaines de kilomètres. La différenciation génétique entre populations diminue ensuite avec l'échelle géographique suggérant une augmentation des flux de gènes par la dispersion des ascospores.

**Founder effect and genetic differentiation at continental and local scales in *Mycosphaerella fijiensis*, fungus causing black leaf streak disease of banana**

**ABSTRACT:**

The fungus *Mycosphaerella fijiensis* is a pathogen of banana. The objective of this work was to describe populations structure of *M. fijiensis* at continental and local scales and to infer gene flow and dispersal processes of this fungus at these two sales. Samples of isolates collected in Latin America-Caribbean region and in Africa were analyzed using neutral molecular markers. A high level of genetic diversity and recombination were maintained in *M. fijiensis* populations even at the plant scale. At continental scale, the spread of *M fijiensis* might be stochastic and could be due either to movement of infected plant material or to limited ascospore dispersal for distance of several hundred kilometers. The level of genetic differentiation between populations decreased after with geographic scale suggesting an increase of gene flow through ascospores dispersal.

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**Mots-clefs:** Bananier, maladie des raies noires, *Mycosphaerella fijiensis*, structure des populations, effets de fondation, flux de gènes, dispersion, marqueurs moléculaires

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