

Septoria Leaf Spot of Banana: A Newly Discovered Disease Caused by *Mycosphaerella eumusae* (Anamorph *Septoria eumusae*)

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ABSTRACT

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A previously undescribed leaf spot disease of banana has been discovered in southern and Southeast Asia. The fungus identified as the causal agent of this leaf spot has a *Mycosphaerella* teleomorph stage and a *Septoria* anamorph stage. Isolation and reinoculation of the fungus to

banana reproduced symptoms and confirmed its pathogenicity. Phylogenetic analysis based on sequences of the internal transcribed spacer and 5.8S ribosomal DNA regions from the different leaf spot pathogens of bananas was consistent with the definition of a new species. *M. eumusae* (anamorph *S. eumusae*) is the name proposed for the causal agent and *Septoria* leaf spot as the name for the disease. The presence of the pathogen has been confirmed in leaf specimens from southern India, Sri Lanka, Thailand, Malaysia, Vietnam, Mauritius, and Nigeria.

Two related ascomycete fungi cause the most important leaf spot diseases of banana. *Mycosphaerella fijiensis* (anamorph *Paracercospora fijiensis*) is the causal agent of black leaf streak disease (black Sigatoka) and *M. musicola* (anamorph *Pseudocercospora musae*) is the causal agent of Sigatoka (15). Another *Mycosphaerella* species, *M. musae*, causes a leaf speckle disease that is considered of little importance except in Australia (14). Many other fungal pathogens attack the foliage of banana, but most are of minor importance (14). One of these, *Phaeoseptoria musae*, is the cause of Phaeoseptoria leaf spot (12). This fungus, which produces symptoms very similar to Sigatoka, was first described in India and has been detected in other countries (11,12). Serious losses due to this minor foliar disease have not been reported (11).

M. fijiensis is currently spreading to new banana-growing areas and replacing *M. musicola*, which has an almost worldwide distribution on banana, as the dominant and most serious leaf spot pathogen. Although this spread is well documented in the Australasian, Pacific, African, Latin American, and Caribbean regions (8) it is not as well understood in southern and Southeast Asia because of contradictory reports and conflicting evidence (7).

Between 1992 and 1995, as part of a survey to determine the distribution of black leaf streak and Sigatoka diseases in southern and Southeast Asia, specimens of banana leaf spot were collected from southern India, Sri Lanka, western Malaysia, Thailand, and Vietnam for pathogen identification. Most specimens were an undetermined, pathogenic *Mycosphaerella* species with *Septoria* as the anamorph stage. A specimen of this new leaf spot was also collected later in Mauritius in 1997. An analysis of leaf spot fungi in strain collections and diseased leaf samples collected from Nigeria in 1999 revealed that the pathogen has been present in that country since at least 1989. *M. eumusae* (anamorph *S. eumusae*) is the name proposed for the fungus, which is described for the first time in this report.

Details of the isolation, culture, characterization, and pathogenicity of *M. eumusae* isolated from banana leaf specimens collected in southern and Southeast Asia are presented. The phylogenetic relationship between *M. eumusae* and other *Mycosphaerella* species attacking banana was also investigated using nucleotide sequences of internal transcribed spacers (ITS) and 5.8S ribosomal DNA (rDNA) regions. This region has been used to analyze relationships within a variety of fungi (10).

MATERIALS AND METHODS

Diseased tissue collection. Specimens of Sigatoka-like leaf spots were collected in countries in southern and Southeast Asia between 1992 and 1997 (Table 1). Leaf tissue with advanced symptoms of leaf spot were cut into squares of approximately 20 × 10 cm. These tissues were dried between sheets of newspaper and dispatched by courier for diagnosis within 1 week of collection.

Microscopic observations of diseased tissues. Necrotic lesions found on diseased leaf specimens collected in southern and Southeast Asia were cleared in lactophenol and mounted in lactophenol-cotton blue for microscopic examination. Fruiting bodies associated with these lesions were described, and the fungus was identified.

Fungal isolation, culture, conservation, and in vitro sporulation techniques. Diseased banana leaves with necrosis were dried at room temperature for 48 h and soaked in distilled water for 15 min. Sections of leaves were secured to the underside of the lids of petri dishes containing 3% water agar. Ascospores were discharged overnight on the agar surface, and single ascospores were transferred to fresh potato dextrose agar medium the next morning. Cultures were incubated at 25°C. Mycelium fragments from developing colonies were placed in 10% glycerol, kept for 2 h at 4°C, and transferred to liquid nitrogen for long term storage at -80°C. Conidial sporulation was induced by culturing on modified V8-juice medium (100 ml of V8 juice, 0.2 g of CaCO₃, and 20 g of agar per liter of medium, pH 6). Three to five 5-mm-diameter agar plugs were sonicated for 1 min in 5 ml of water and spread on the medium. Cultures were incubated at 22°C for 10 to 14 days

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under 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of continuous, cool-white, fluorescent light (9).

Fungal isolates. The isolates studied are listed in Table 1. Fifteen isolates of *M. eumusae* were chosen to represent all of the locations where this new pathogen was detected. The isolates of *M. eumusae* from Nigeria isolated in 1989 and 1990 were originally identified as *M. musicola* or were unclassified. The other isolates in Table 1 belong to previously identified species of leaf spot pathogens of bananas: *M. fijiensis*, *M. musicola*, *M. musae*, and *P. musae*. All cultures were isolated from infected leaves of bananas and derived from single ascospores.

Inoculations. Isolates were artificially inoculated on cv. Grande Naine (AAA, Cavendish subgroup) approximately 10 weeks after plants were removed from tissue culture. Water was used to dislodge conidia from the cultures and the resulting suspension was adjusted to 10^4 spores per ml. Approximately 1 ml of suspension was sprayed on the lower surface of the youngest leaves of test plants using an artist's airbrush (Sagola 150, Vitoria, Spain). Three plants per isolate were inoculated. Inoculated plants were placed in a saturated atmosphere and incubated at 25°C. After 1 week, the relative humidity was reduced to 80%. Thereafter, plants were examined for a 10-week period, two times per week. The type of symptoms were observed, and the appearance of first and final symptoms with necrosis were recorded.

DNA isolation. Isolates were grown on V8 juice medium (300 ml of V8 juice, 3 g of CaCO_3 and 20 g of agar per liter, pH 6.0) for 2 weeks at 25°C. Mycelia were harvested and dried for 2 days in an oven at 50°C. Genomic DNA was extracted using a modification of the method described by Carlier et al. (3). Approximately 50 to 100 mg of dried mycelium was ground with sand, dispersed in 0.8 ml of cetyltrimethylammonium bromide (CTAB) extraction buffer (1% CTAB, 0.7 M NaCl, 50 mM Tris-HCL [pH 8.0], 10 mM EDTA [pH 8.0], and 1% β -mercaptoethanol) in a 2-ml centrifuge tube, and placed in an oven at 65°C for 60 to 90 min with continuous, gentle rocking. An equal volume of chloroform/isoamyl alcohol (24:1) was added, and the contents were mixed by inversion to form an emulsion. The emulsion was spun for 20 to 30 min at $2,000 \times g$ and the upper aqueous phase was transferred to a clean 2-ml tube. An equal volume of CTAB precipitation buffer (1% CTAB, 50 mM Tris-

HCL pH 8.0, 10 mM EDTA [pH 8.0]) was added, the contents were mixed by inversion, and the mixture was spun at $2,000 \times g$ for 5 min. The supernatant was discarded, and the pellet was resuspended in 0.6 ml of high-salt TE buffer (1 M NaCl, 10 mM Tris HCL [pH 8.0], 1 mM EDTA [pH 8.0]). DNA was precipitated by adding 2.5 volumes of absolute ethanol at -20°C and centrifuged at $2,000 \times g$ for 5 min. The pellet was resuspended in 0.3 ml of TE buffer containing RNase A at $100 \mu\text{g ml}^{-1}$, and the tube was incubated for 30 min at 37°C. DNA was precipitated again by adding 30 ml of 5 M NaCl and 2.5 volumes of absolute ethanol at -20°C, spun at $2,000 \times g$ for 5 min, washed by adding 2 ml of 70% ethanol, dried, and resuspended in 100 μl of TE buffer. The yield was from 20 to 40 μg of DNA per extraction.

ITS amplification and sequencing. The ITS region was amplified and sequenced using primers ITS1 and ITS4 (18). Amplifications were performed in 25- or 50- μl final volumes containing 1 ng of fungal DNA as template, 0.4 μM of each primer, 1.5 mM MgCl_2 , 0.2 mM dNTPs, 67 mM Tris-HCL [pH 8.8], 16.6 mM $(\text{NH}_4)_2\text{SO}_4$, 0.1% Tween 20 and 0.01 $\text{U}\mu\text{l}^{-1}$ of *Taq* polymerase (Eurobio, Les Ulis, France). After an initial denaturation step of 7 min at 94°C, 30 cycles (= 1 min at 94°C, 1 min at 60°C, 2 min at 72°C) were performed, followed by a final extension step for 10 min at 72°C. Polymerase chain reactions (PCR) were performed in a PTC100 thermal cycler (MJ Research, Watertown, US). Amplification products were analyzed by electrophoresis in 2% agarose Tris-borate-EDTA gels. Direct sequencing was carried out by the chain termination technique with a 370A nucleotide sequence analyzer (Applied Biosystems, Foster City, CA). Sequencing was done on both strands from the ITS1 and ITS4 primers, and ambiguities were corrected by comparisons of the electrophoregrams using Sequence Navigator software (Perkin-Elmer, Norwalk, CT).

Sequence analysis. Sequences were aligned using the Clustal X program (17). A dissimilarity matrix based on the proportion of different nucleotide sites was calculated from the alignment, with transitions and transversions given the same weight (deletions were ignored). A tree was constructed from the distance matrix by the neighbor-joining method, and a bootstrap analysis (1,000 replicates) was carried out using the Clustal X program (17). Phylogenetic analysis was also under-

TABLE 1. Geographic origin, genomic group of banana host, year of collection, and source of isolates studied

Isolate code	Country	Location	Genomic group ^a	Year	Source ^b
<i>Mycosphaerella eumusae</i>					
385	India	Bangalor	Grande Naine, AAA	1992	1
535	India	Kannara	Grande Naine, AAA	1995	1
554	Sri Lanka	Gannoruwa	Cavendish, AAA	1995	1
563	Sri Lanka	Nugahena	Anamala, AAA	1994	1
482	Thailand	Sukothai	Grande Naine, AAA	1994	1
484	Thailand	Surat Thani	Williams, AAA	1994	1
485	Thailand	Thong Ka Wat	Grande Naine, AAA	1994	1
487	Thailand	Tha Yang	Kluai Hom Tong, AAA	1994	1
458	Malaysia	Johor State	Pisang Kapas, AAB	1993	1
670	Vietnam	Mekong Delta	Pisang Mas, AA	1995	1
744	Mauritius		Grande Naine, AAA	1997	1
162	Nigeria	Unknown	AAB	1990	1
BF735	Nigeria	Onne	AAB	1989	2
<i>M. fijiensis</i>					
009	Gabon	Ntoun	Unknown	Unknown	1
01A	Philippines				1
<i>M. musicola</i>					
090	Colombia	Armenia	AAB	1988	1
121	Indonesia, West Java		AAA	1989	2
<i>M. musae</i>					
122	Cook Islands	Unknown	AAA	1989	2
<i>Phaeoseptoria musae</i>					
IMI 271341	Honduras	Unknown	Unknown	1982	3

^a Cultivar's name or genomic group of banana host.

^b 1 = CIRAD (France); 2 = B. A. Fullerton (Horticulture and Food Research Institute, New Zealand); 3 = International Mycological Institute (England).

taken with parsimony method and bootstrap resampling using the PHYLIP package (4).

RESULTS

Field observations. Disease symptoms observed initially were first visible as faint brown streaks. When infection density was low, streaks developed into oval or ellipsoidal lesions with gray

centers. However, when infection density was high, developing lesions coalesced, and large areas of leaf tissue became necrotic (Fig. 1A). These symptoms, which closely resembled those of other leaf spot diseases, were observed on cultivars belonging to different genomic groups (Table 1).

Fungal description and identification. Two types of fruiting structures, pycnidia and perithecia, were closely associated in all of the specimens. These structures were more prevalent in lesions

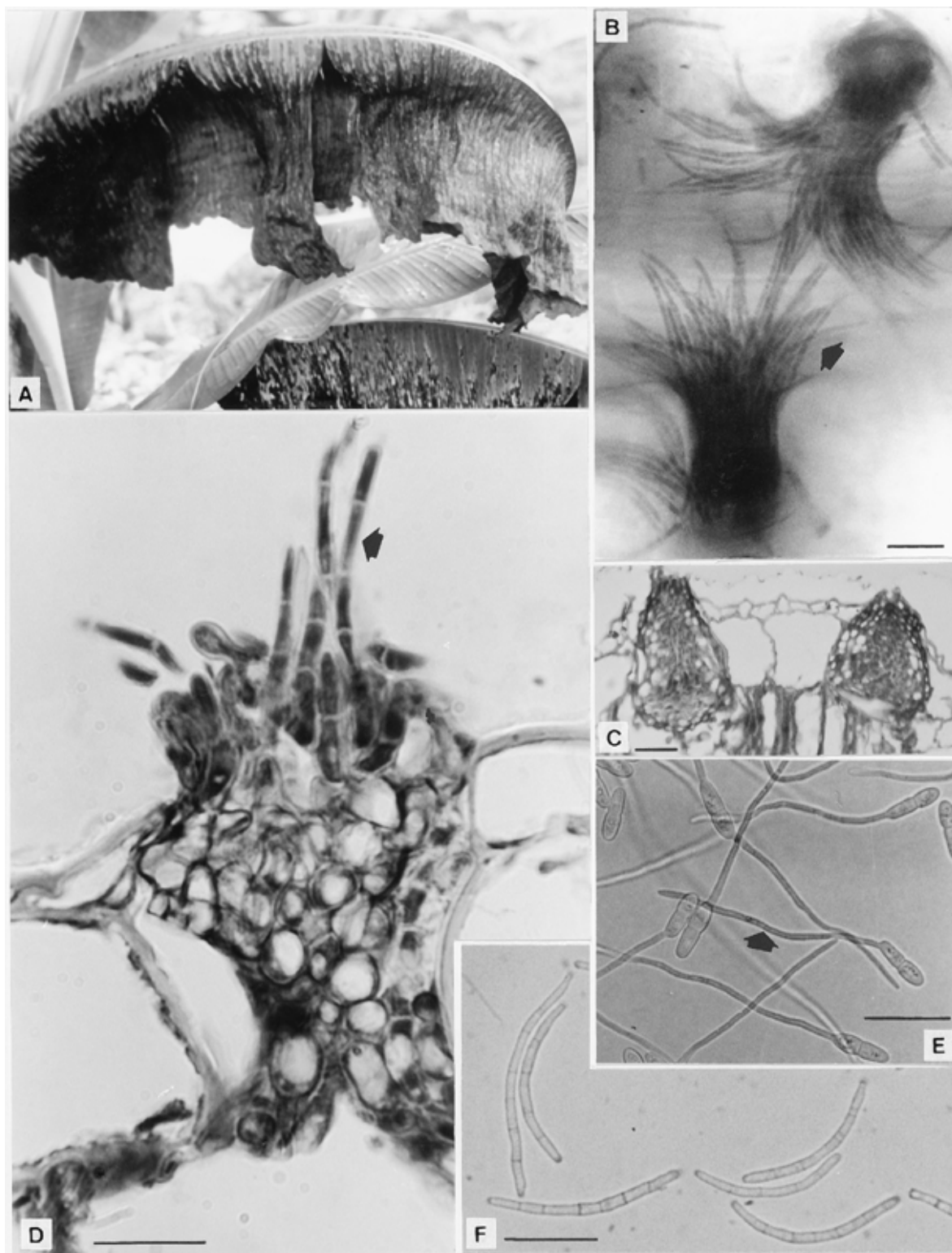


Fig. 1. Photographs of *Mycosphaerella eumusae* (anamorph *Septoria eumusae*), cause of Septoria leaf spot of banana. **A**, Symptoms on naturally infected banana leaves; **B**, view of pycnidia and conidia (arrow) from naturally infected leaves; **C**, view of two young, immersed pycnidial bodies; **D**, section of an acervular-like pycnidium and conidia (arrow) in a necrotic leaf spot; **E**, germinating ascospores on V8-juice medium. Usually ascospores produce only one germ tube after 24 h in culture; **F**, conidia produced from a single-ascospore culture on V8-juice medium. Bars = 20 μ m.

on upper leaf surfaces. They were characterized from the Mauritius leaf sample. Pycnidia were pear-shaped, 31 to 42 µm wide (15 observed), immersed, more or less erumpent, and ostiolated when young (Fig. 1B and C) but often acervular-like when mature (Fig. 1D). Fusiform, hyaline conidia, which were three to five septate and measured between 21.2 and 41.6 µm long (20 observed), could be seen in the pycnidia (Fig. 1B and D). This imperfect state of the fungus was identified as *Septoria*. The conidia were different than those of *Phaeoseptoria musae*, which are pale brown, obclavate, and smaller in length (22 to 30 µm [11]). Perithecia were dark brown, 42 to 51 µm in diameter (15 observed), amphigenous, scattered, and globose, with a short, protruding ostiole. They had a well-defined dark wall and contained oblong asci. Ascospores were two-celled and measured 12.0 to 16.5 × 3.0 to 4.5 µm (30 observed; Fig. 1E). These observations allowed us to identify the perfect state of the fungus as *Mycosphaerella*, which was indistinguishable from the perfect states of *M. musicola* and *M. fijiensis*. The fungus was given the name *M. eumusae* (anamorph *S. eumusae*).

Single-ascospore isolation and culture. Cultures of the pathogen, initiated from single *Mycosphaerella* ascospores ejected from perithecia, had black stroma-like structures and appeared similar to cultures of *M. fijiensis*. After 1 week of incubation in V8-juice-modified medium, pycnidial structures and numerous conidia were produced (Fig. 1F). All the cultures produced hyaline, straight, or slightly flexuous, three- to five-celled conidia. Conidia produced in culture by several isolates were measured (40 conidia observed per isolate; Table 2). Their average size was 40.9 × 2.1 µm (range 24.5 to 53.9 × 1.8 to 2.7 µm). The conidia produced on agar media generally were broader and longer than those observed in leaf specimens and were longer than those of *Phaeoseptoria musae* in culture (25 to 33 × 2.5 to 3.5 µm) as described by Punithalingam (11). They were also different from those of *M. fijiensis* and *M. musicola*.

Pathogenicity. *M. eumusae* isolates 458, 487, 535, and 670 inoculated on banana cv. Grande Naine (AAA, Cavendish subgroup) were pathogenic. For each isolate, symptoms were observed in all repetitions, and mature lesions were produced in at least one repetition. Initial symptoms were small yellow specks that appeared approximately 20 to 34 days after inoculation. These lesions enlarged and turned pale brown or yellowish gray. Mature lesions, which developed 57 to 82 days after inoculation, were dark brown and usually surrounded by narrow yellow halos. Our objective was to reproduce disease by artificial inoculation. Due to the small number of repetitions for each isolate, statistical analyses were not calculated, and the pathogenicity of isolates were not compared.

ITS sequence analysis. ITS sequence analysis was conducted with two isolates each of *M. eumusae*, *M. fijiensis*, and *M. musicola* and one isolate each of *M. musae* and *P. musae*. A single PCR product between 530 and 550 pb was observed for all of the isolates studied using ITS1 and ITS4 primers. Sequencing resolved the exact lengths of the ITS regions, which were similar among species (Table 3). The alignment of the sequences of the ITS region is presented in Figure 2. Comparison with the EMBL database using the BLAST algorithm (1) showed that the ITS sequence from *P. musae* was more related to those from asco-

mycete fungi belonging to the *Leptosphaeria* and *Phaeosphaeria* genera. *Phaeoseptoria* is one of the anamorphs corresponding to these genera (5). The previously published sequence from *L. microscopica* (Genbank Accession no. U04234) was also included in the analysis. The alignment of the ITS region contained 266 variable sites, 132 of which were phylogenetically informative. From this alignment, a dissimilarity matrix (Table 4) and corresponding tree with bootstrap analysis (Fig. 3) were calculated. Within the *Mycosphaerella* species, the similarity of the sequences between the two isolates of each of the species used was between 100% for *M. eumusae* and 98% for *M. musicola*. The similarity of the sequences between two *Mycosphaerella* species was between 97.8% for *M. eumusae* with *M. fijiensis* and 87.5% for *M. eumusae* or *M. musicola* with *M. musae*. The *P. musae* isolate was more related to *L. microscopica* (similarity of 89%) compared with the other *Mycosphaerella* species (similarity <79%). Bootstrap analysis indicated that all the branches of the tree were supported at a 95% confidence level, except the node joining *M. fijiensis* and *M. eumusae*. Similar results were obtained by the parsimony method with bootstrap resampling.

Nigerian isolate 162 (Table 1) that originally could not be identified did not correspond to either *M. fijiensis* or *M. musicola* when analyzed. In the study of the global population structure of *M. musicola*, Nigerian isolates formed a group very distant from other African isolates (H. L. Hayden, E. A. B. Aitken, and J. Carlier; unpublished data). The digested patterns of the ITS regions of the Nigerian isolates using several restriction enzymes were identical to the pattern of one of the *M. eumusae* isolates. Observation of the morphology of some of these isolates in culture (including isolates 162 and BF735; Table 1) and sequencing of their ITS regions confirmed that they belonged to *M. eumusae* species. These isolates came from two collections and were isolated in 1989 and 1990 (Table 1). A diseased leaf sample from the same Nigerian location (Onne) was analyzed recently. Fifty plants from one field were sampled in 1999. *M. eumusae* and *M. fijiensis* were isolated from 5 and 28 samples, respectively.

DISCUSSION

The results presented in this paper show that most specimens of Sigatoka-like leaf spots collected on banana in southern India, Sri Lanka, western Malaysia, Thailand, and Vietnam during the mid-1990s were caused by a previously undescribed pathogenic fungal species. Although a *Mycosphaerella* stage was present in the leaf specimens, the anamorph stage observed was very different from others previously described on banana, including those of *M. fijiensis* and *M. musicola*, which are the most important banana leaf spot pathogens (15). The anamorph stage belonged to the genus *Septoria*. It had pycnidial conidiomata and fusiform, hyaline, and septate conidia with unthickened scars (16). Identical anamorphic fungal structures were produced in pure cultures initiated from single *Mycosphaerella* ascospores ejected from perithecia in lesions on the leaf specimens, confirming the link

TABLE 2. Average size and range of conidia^a produced in culture by *Septoria eumusae* isolates

Isolate	Average size (µm)	Range (µm)
487	41.6	29.0–51.4
535	39.6	24.5–53.9
670	43.1	29.4–49.1
744	39.5	28.9–49.0

^a Estimations based on 40 conidia.

TABLE 3. Size of the internal transcribed spacer (ITS) region of the fungal species studied

Isolate	Species	ITS region size (bp)
90	<i>Mycosphaerella musicola</i>	451
121	<i>M. musicola</i>	452
458, 487	<i>M. eumusae</i>	457
9, 1A	<i>M. fijiensis</i>	446
122	<i>M. musae</i>	449
IMI 271341	<i>Phaeoseptoria musae</i>	447
Lm	<i>Leptosphaeria microscopica</i> ^a	504

^a GenBank Accession no. U04234.

	10	20	30	40	50	60	70	80
<i>M. musicola</i> (90)	CATTACCGAG	TGAGG*****	GCTCACCCCC	G*AC***CTC	CAA*CCC***	***TT*****	**TG*TGAAC	**CA**CA*C
<i>M. musicola</i> (121)	-----	-----	-----	-*-----	---*-----	***-----	**-----	***-----*
<i>M. eumusae</i> (458)	-----T---	-----	-----G---	-*-----	---*-----	***-C*****	**-----	***-----*
<i>M. eumusae</i> (487)	-----T---	-----	-----G---	-*-----	---*-----	***-C*****	**-----	***-----*
<i>M. fijiensis</i> (9)	-----	-----	-----G---	-*-----	---*-----	***-----	**-----	***-----A-
<i>M. fijiensis</i> (1A)	-----	-----	-----G---	-*-----	---*-----	***-----	**-----	***-----A-
<i>M. musae</i> (122)	-----C---	-----	-----CGG---	-*-----	---*-----	***-----	**-----T	***--*A-A-
<i>P. musae</i>	-----*-T	-C-----	**--A**G---	**--G***--*	***-*****	***-----	**--*--T-T*	***--**C*-
<i>L. microscopica</i>	-----A*-T	-C--TAGCTT	--ACTGTCA	-G-GGTG--G	T--G--GCA	TAG--TAATT	AC--A-G-G-	GG--GG-CCT
	90	100	120	130	140	150	160	170
<i>M. musicola</i> (90)	CTGTT*G***	*CTT**CGGG	GGCGACCTG	CCGGCGAGCT	CGTCGCCG*G	GC**GCCC*C	CGGA*GGTCT	CCC***TC-C
<i>M. musicola</i> (121)	-----*---	*-----	-----	-----A-A-	T-----*	-----*	-----*	--T***-A-
<i>M. eumusae</i> (458)	T-----*---	*-----	-----	-----A-	C-----*	-----*	-----*	T-T***AA--
<i>M. eumusae</i> (487)	T-----*---	*-----	-----	-----A-	C-----*	-----*	-----*	T-T***AA--
<i>M. fijiensis</i> (9)	T-----*---	*-----	-----*	-----*	**--G--*	-----*	-----*--C-G	T-T***AA--
<i>M. fijiensis</i> (1A)	T-----*---	*-----	-----*	-----*	**--G--*	-----*	-----*--C-G	T-T***AA--
<i>M. musae</i> (122)	T-----*---	*-----	-----	-----*	TCG--G--C	-----*	-----*--AAA	T-T***AA--
<i>P. musae</i>	T-----*---	*-----*TTT*	*---TA-T-A	T--TT***-	CC--G--*	--TT--TG-	--TT--A-A	A-TTTA-A--
<i>L. microscopica</i>	-----T-TAC	C---GT-TCC	T---CA-*C	AT-TT***-	CC--G--*	--TTGCC-G-	--ATT--A-A	AAACTA-A--
	180	190	200	210	220	230	240	250
<i>M. musicola</i> (90)	ACTG*****	CATC**TCTG	CGTCGGAGTA	TGAAACAAAT	CGGACAAAAC	TTTCAACAAC	GGATCTCTTG	GTTCTGGCAT
<i>M. musicola</i> (121)	-----	-----*	-----A---T	CC-----	-----	-----	-----	-----
<i>M. eumusae</i> (458)	-----	-----**T--	-----T	CA-----	--A-----	-----	-----	-----
<i>M. eumusae</i> (487)	-----	-----**T--	-----T	CA-----	--A-----	-----	-----	-----
<i>M. fijiensis</i> (9)	-----	-----**T--	-----T	-C-----	--A-----	-----	-----	-----
<i>M. fijiensis</i> (1A)	-----	-----**T--	-----T	-A-----	--A-----	-----	-----	-----
<i>M. musae</i> (122)	-----	-----G--AA-T--	T-----	CTTGTT--*	A*A-----	-----	-----	-----
<i>P. musae</i>	CT-TTTAAAT	-T--AA-A-	-----T--A--	AT-T-----*	A*ATT-C--	-----	-----	-----
<i>L. microscopica</i>	*--ATTCAAT	TT--AA-A-	-----T--*--	AA-CTT--*	A*ATT-C--	-----	-----	-----
	260	270	280	290	300	310	320	330
<i>M. musicola</i> (90)	CGATGAAGAA	CGCAGCGAAA	TGCGATAAGT	AATGTGAATT	GCAGAATTCA	GTGAATCATC	GAATCTTTGA	ACGCACATTG
<i>M. musicola</i> (121)	-----	-----	-----	-----	-----	-----	-----	-----
<i>M. eumusae</i> (458)	-----	-----	-----	-----	-----	-----	-----	-----
<i>M. eumusae</i> (487)	-----	-----	-----	-----	-----	-----	-----	-----
<i>M. fijiensis</i> (9)	-----	-----	-----	-----	-----	-----	-----	-----
<i>M. fijiensis</i> (1A)	-----	-----	-----	-----	-----	-----	-----	-----
<i>M. musae</i> (122)	-----	-----	-----	-----	-----	-----	-----	-----
<i>P. musae</i>	-----	-----	-----	-----G-----	-----	-----	-----	-----
<i>L. microscopica</i>	-----	-----	-----	-----G-----	-----	-----	-----	-----
	340	350	360	370	380	390	400	410
<i>M. musicola</i> (90)	CGCCCTTTGG	TATTCGGAAG	GGCATGCCTG	TTCGAGCGTC	ATTC*ACCA	CTCAAGCCTG	GCTTGGTATT	GGG*****
<i>M. musicola</i> (121)	-----	-----	-----	-----	-----*	-----*	-----*	-----*
<i>M. eumusae</i> (458)	-----	-----	-----	-----	-----*--C-	-----	-----	-----*
<i>M. eumusae</i> (487)	-----	-----	-----	-----	-----*--C-	-----	-----	-----*
<i>M. fijiensis</i> (9)	-----	-----	-----	-----	-----*--C-	-----	-----	-----*
<i>M. fijiensis</i> (1A)	-----	-----	-----	-----	-----*--C-	-----	-----	-----*
<i>M. musae</i> (122)	-----G---	-----CG-	-----	-----	-----*--C-	-----G--T--	A--C-----	-----*
<i>P. musae</i>	-----CT---	-----ATG-	-----	-----	-----GT--CT	*-----T-T	-----G--	-----TG*****
<i>L. microscopica</i>	-----CT---	-----ATG-	-----	-----	-----GT--C*	-----CTCT	-----G--	-----TGTTTGT
	420	430	440	450	460	470	480	490
<i>M. musicola</i> (90)	*C*GCCGCG	G---TCCG*C	GCGCCCTPAA	GT**TCCCGG	CTGAGCC*GT	CCGTC**TC*	T*AAGCGTTG	TGG*ATTTT
<i>M. musicola</i> (121)	*-----*	-----*	-----*	-----*	-----*	-----*	-----*	-----*
<i>M. eumusae</i> (458)	*---T---	-T---T--*	-----T---	-----*--T---	-----*--T---	-----*--C-	*--G---TT-	---*---C--
<i>M. eumusae</i> (487)	*---T---	-T---T--*	-----T---	-----*--T---	-----*--T---	-----*--C-	*--G---TT-	---*---C--
<i>M. fijiensis</i> (9)	*---T---	-TTC-T--*	-----T---	-----*--*---	-----*--*---	-----*--*---	*-----TT-	---*---C--
<i>M. fijiensis</i> (1A)	*---T---	-TTC-T--*	-----T---	-----*--*---	-----*--*---	-----*--*---	*-----TT-	---*---C--
<i>M. musae</i> (122)	*---T---	TTTCGAT--*	-----T---	-----*--*---	-----GA--*	-----*--*---	C*G---TT-	---C--C-G-
<i>P. musae</i>	*-TT-T-TTT	TT---AA-A-	T-A--TC--	---A-T***-	***---A--	GTT-TGG-AG	*-----CA-	CAC*-----G
<i>L. microscopica</i>	C-TCT--T-T	-C---TG-A-	T---TC--	-CAA-T***-	***---A--	GTT-TGG-AT	-G-----CA-	CAC*-----G
	500	510	520	530	540	550		
<i>M. musicola</i> (90)	CAATTCGCTC	CGG*AGT*GC	GGGTGGCCGC	GGCCGTTAAA	TCCT***CA	AAGGTTGACC		
<i>M. musicola</i> (121)	-----	-----*	-----	-----	-----T-----*	-----		
<i>M. eumusae</i> (458)	-----T---	-----*	-----C-----	-----	-----T-TATTG-	-----		
<i>M. eumusae</i> (487)	-----T---	-----*	-----C-----	-----	-----T-TATTG-	-----		
<i>M. fijiensis</i> (9)	-----T---	-----*	-----	-----	-----T-TATTG-	-----		
<i>M. fijiensis</i> (1A)	-----T---	-----*	-----	-----	-----T-TATTG-	-----		
<i>M. musae</i> (122)	-----A	G--*--C--	--AG---GTT	-----	-----CACCCATC-	-----		
<i>P. musae</i>	-GTC-T-G--	-C***T-AA-	A-****-A--	AT--A-C--G	C-A-TTCTC	-CTT-----		
<i>L. microscopica</i>	-G---TGG-	-TATAA-*A-	TT*****-	GT--A-*--G	C-TATTTTC-	-CTT-----		

Fig. 2. Aligned sequences of the internal transcribed spacer (ITS) region of rDNA of the fungal species studied. ITS1 include base pairs 6 to 216, the 5.8S coding region extends from base pair 217 to 274, and the ITS 2 extends from base pair 275 to 550. The strain of *Phaeoseptoria musae* corresponds to IMI Accession no. 271341, and the sequence of *Leptosphaeria microscopica* corresponds to GenBank Accession no. U04234. - = base homologous with isolate 90. * = possible deletion. *M.* = *Mycosphaerella*.

between the *Mycosphaerella* and *Septoria* stages. Symptoms similar to those observed in the field were produced after artificial inoculation with several of these isolates. The fungus identified, thus, was the causal agent of the leaf spots sampled. The name *M. eumusae* (anamorph *S. eumusae*), the cause of *Septoria* leaf spot disease of banana, is proposed, because this new pathogen was isolated from *Eumusae*-derived banana cultivars. Type material of banana leaves was deposited in July 1999 at the Laboratoire de Cryptogamie, Museum d'Histoire Naturelle, Paris.

Because *Phaeoseptoria* leaf spot has been reported in southern India and eastern Malaysia, it was first thought that this and *Septoria* leaf spot may have been the same disease (2). However, this initial assumption was erroneous, because the causal fungus described in this paper was discovered to be morphologically distinct from *Phaeoseptoria musae*.

Phylogenetic analysis based on sequences of the ITS region of the rDNA from *M. eumusae*, *M. musicola*, *M. fijiensis*, *M. musae*, and *Phaeoseptoria musae* confirmed that all of these pathogens are different species. The level of homology between isolates within the species studied was high (>98%) and similar to that previously published for other species (13). The level of homology between the two *M. musicola* isolates was lower compared with that of *M. fijiensis* and *M. eumusae*. A greater variability in the ITS1 sequences has been observed within *M. musicola* compared with *M. fijiensis*, using eight isolates of each species from different origins (6). This result suggests a longer period of divergence within *M. musicola*. Low genetic divergence was found between *M. fijiensis*, *M. musicola*, and *M. eumusae*. These species formed a significant monophyletic group, suggesting that the major leaf spot pathogens of banana may have been derived from a common ancestor. Phylogenetic analysis using more *Mycosphaerella* species isolated from bananas and other plants has been conducted (J. Carlier and M. F. Zapater, unpublished data). From these ITS sequences, a molecular diagnostic method for the identification of the banana leaf spot pathogens has been developed, using

digestion of PCR-amplified ITS regions with restriction enzymes. Application of this method confirmed that all the *M. eumusae* strains isolated from the various countries belong to the same species (data not shown).

Fungi causing banana leaf spots have been collected and identified in many countries during the 20th century, but *M. eumusae* had not been described until now. The distribution of *M. eumusae*, thus, may be restricted to the areas where it has recently been found (Table 1). However, further surveys are necessary to determine exactly where this new leaf spot pathogen occurs. The occurrence of the pathogen in Nigeria at an apparently low frequency suggests that it might also be present in other locations where *M. fijiensis* or *M. musicola* are the dominant leaf spot pathogens.

Evidence suggests that the *Septoria* leaf spot of bananas, first described in this paper, has most likely been mistaken for Sigatoka or black leaf streak in the past based on visual symptoms. Severely diseased plants were observed in the field in southern and Southeast Asia, indicating that the pathogen might cause significant damage. Assessments of the threat that this disease poses to banana cultivation need to be made and quarantine authorities alerted to its existence. Research on all the aspects of the biology of *M. eumusae* is also needed, including studies on host-pathogen interactions and population structure analysis.

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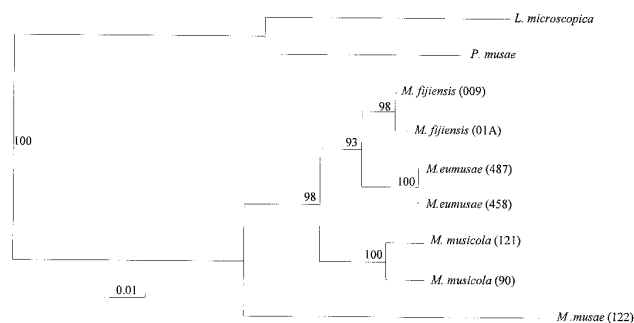


Fig. 3. Dendrogram generated by neighbor-joining analysis of the alignment of the internal transcribed spacer sequences shown in Figure 2. Bootstrap confidence levels, based on 1,000 resamples, are given at the appropriate branches.

TABLE 4. Dissimilarity matrix of the internal transcribed spacer region for fungal species studied

Isolate	Species	90	121	458	487	9	1A	122	Pm ^a
90	<i>Mycosphaerella musicola</i>								
121	<i>M. musicola</i>	0.020							
458	<i>M. eumusae</i>	0.049	0.046						
487	<i>M. eumusae</i>	0.049	0.046	0.000					
9	<i>M. fijiensis</i>	0.049	0.049	0.027	0.027				
1A	<i>M. fijiensis</i>	0.046	0.049	0.022	0.022	0.005			
122	<i>M. musae</i>	0.122	0.125	0.125	0.125	0.110	0.112		
Pm ^a	<i>Phaeoseptoria musae</i>	0.210	0.215	0.210	0.210	0.210	0.208	0.247	
Lm	<i>Leptosphaeria microscopica</i> ^b	0.220	0.222	0.225	0.225	0.225	0.220	0.254	0.110

^a Isolate code IMI 271341.

^b GenBank Accession no. U04234.

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