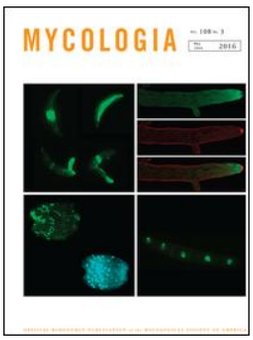

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A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*

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Abstract: *Magnaporthe oryzae* is described as a new species distinct from *M. grisea*. Gene trees were inferred for *Magnaporthe* species using portions of three genes: actin, beta-tubulin, and calmodulin. These gene trees were found to be concordant and distinguished two distinct clades within *M. grisea*. One clade is associated with the grass genus *Digitaria* and is therefore nomenclaturally tied to *M. grisea*. The other clade is associated with *Oryza sativa* and other cultivated grasses and is described as a new species, *M. oryzae*. While no morphological characters as yet distinguish them, *M. oryzae* is distinguished from *M. grisea* by several base substitutions in each of three loci as well as results from laboratory matings; *M.oryzae* and *M. grisea* are not interfertile. Given that *M. oryzae* is the scientifically correct name for isolates associated with rice blast and grey leaf spot, continued use of *M. grisea* for such isolates would require formal nomenclatural conservation.

Key Words: gray leaf spot, *Pyricularia grisea*, *Pyricularia oryzae*, rice blast

INTRODUCTION

Magnaporthe grisea (Hebert) Barr is the causal agent of rice blast and gray leaf spot of grasses. Blast is the most important fungal disease of rice worldwide due to its widespread occurrence and destructiveness under conducive conditions. *M. grisea* has been reported to occur on more than 50 grass species (Ou 1987). Borromeo and coworkers (1993) identified four major, highly distinct groups of *Pyricularia* (anamorph of *Magnaporthe*, see below) isolates by means of RFLPs: (i) isolates from *Digitaria ciliaris* (Retz.) Koeler and *Eragrostis* sp., (ii) a single isolate from *Cenchrus echinatus* L., (iii) isolates from *Cyperus brevifolius* (Rottb.) Endl. ex Hassk and *Cyperus rotundus* L., and (iv) isolates from *Oryza sativa* L. and other grasses. Shull and Hamer (1994) reviewed unpublished data for mtDNA RFLPs which indicated that pathogens of *Digitaria* and *Pennisetum* are distinct from other host specific forms of *Pyricularia grisea*. Combined data for rDNA, mtDNA, and nuclear DNA haplotypes identified two groups among isolates from species of *Digitaria* and two groups among isolates from species of *Pennisetum*. (Shull and Hamer 1994). Our studies focus on species delimitation among isolates associated with *Digitaria* and *Oryza*.

Host association appears to define fundamentally distinct groups within *M. grisea*: species, populations, and clonal lineages. Several host range studies have been performed in the laboratory or greenhouse, with conflicting results (reviewed by Ou 1987). Such laboratory studies may not reflect host range in nature. The realized host range is likely better understood through sampling from field populations. Clonal lineages from *O. sativa* in the USA and Colombia, identified by DNA fingerprinting using repetitive elements, have been found to be associated with distinct assemblages of rice cultivars (Levy et al 1991, 1993, Correa Victoria et al 1994). DNA fingerprinting and RFLP studies have identified genetically distinct, host-specific populations of *M. grisea* (Borromeo et al 1993, Hamer et al 1989, Kato et al 2000). RFLP and DNA sequencing studies have resolved highly divergent lineages within *M. grisea* from *Digitaria* sp. and *O. sativa* (Borromeo et al 1993, Bunting et al 1996, Kato et al 2000). Borromeo et al (1993) and Kato et al (2000) suggested that the divergent lineages from *Digitaria* and rice represent distinct species. No new species have been described.

Hebert (1971) described *Ceratosphaeria grisea* based on a cross between isolates of the anamorph, *Pyricularia grisea* (Cook) Sacc., from *Digitaria sanguinalis* (L.) Scop. (crabgrass). A teleomorph produced as a result of a cross between isolates from *D. sanguinalis* (*D. sanguinalis* × *D. sanguinalis*) was reported to be morphologically identical to the teleomorphs produced in crosses of isolates from other grasses [*O. sativa* × *O. sativa* (Kato and Yamaguchi 1982), *Eleusine indica* (L.) Gaertn. × *E. indica* (Yaegashi and Udagawa 1978), *E. coracana* (L.) Gaertn × *E. coracana* (Yaegashi and Udagawa 1978), *O. sativa* × *E. indica* (Yaegashi and Udagawa 1978), and *O. sativa* × *E. coracana* (Ueyama and Tsuda 1976)]. As

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a consequence of observations made of these crosses, no teleomorph distinct from that described by Hebert from isolates from *Digitaria* has been described for isolates from rice or other grasses.

Yaegashi and Udagawa (1978) produced a teleomorph consistent with Hebert's description and morphologically identical to authentic material of *C. grisea*, from a cross between *Pyricularia* isolates from *E. indica* (C10 and T28). They provided a detailed description and accompanying illustration of the teleomorph produced from these isolates. Their comparison of this teleomorph with *M. salvinii* (Cattaneo) Kraus and Webster, the type species of *Magnaporthe*, led them to transfer *C. grisea* to *Magnaporthe*. This transfer was, however, subsequent to that of Barr, who did not provide an illustrated description (Barr 1977).

Two form-species names have been applied to the anamorph of *M. grisea*. *Pyricularia grisea* was described from *D. sanguinalis*, and *P. oryzae* Cavara was described from *O. sativa*. *Pyricularia oryzae* was distinguished from *P. grisea* based on its sparse, usually nonseptate hyphae and larger, biseptate conidia (reviewed by Ou 1987). The usage of the names *P. grisea* and *P. oryzae* has generally reflected the host from which the fungus was isolated rather than any morphological differences, with the name *P. oryzae* applied to isolates from rice and *P. grisea* to isolates from cereals and other grasses (Sprague 1950). The morphological similarity of *Pyricularia* isolates from different grass hosts has led to the view that *P. oryzae* and *P. grisea* are synonymous (Sprague 1950, Ou 1987, Rossman et al 1990). Rossman et al (1990) confirmed the morphological similarity after examination of the type specimens of *P. grisea* and *P. oryzae*. Fully fertile matings between isolates from rice and isolates from other grasses were interpreted as evidence for the existence of a single biological species (Yaegashi and Udagawa 1978). However, no successful crosses of isolates from *Digitaria* and from *Oryza* have been reported (Hebert 1971, Kato et al 2000). Based on overlap in conidial morphology and interfertility among isolates from rice and other grasses, Rossman et al (1990) synonymized *P. oryzae* under *P. grisea*. The synonymizing of these names may have been premature and is evaluated in the present study.

The objective of this study was to investigate the phylogenetic relationship among *M. grisea* isolates from different grass hosts using a multilocus gene genealogy of DNA sequences from portions of three genes. If *M. grisea* isolates from *Digitaria* species and rice belong to diagnosably distinct monophyletic clades, a new species epithet is required for the clade including isolates from rice. Alternatively, if polyphyletic or paraphyletic groups were inferred, isolates from both *Digitaria* and *Oryza* should continue to be accommodated under *M. grisea*. If isolates representing these clades are not interfertile, the segregation of a new species from *M. grisea*, as both a phylogenetic and a biological species, is further indicated.

MATERIALS AND METHODS

Fungal isolates.—All isolates (TABLE I) were maintained on potato dextrose agar (PDA) (Difco, Sparks, Maryland). Storage of isolates was on filter paper inoculated and colonized by the fungus, then desiccated and maintained at −20 C. Matings of *M. grisea* isolates were performed on oatmeal agar [20 g of oats in 1 L of distilled water was heated to 70 C for 1 h, then filtered through cheesecloth, then adjusted to 1 L, to which 18 g of agar (Difco, Sparks, Maryland) was added before autoclaving]. Isolates were inoculated approximately 2.5 cm apart and incubated under fluorescent lights at a distance of 52.5 cm. Matings were first performed between the isolates 8465 and 8470 in order to test their fertility, then between each of these fertile isolates and JP34, Py-D, JP37, BK-6, K76–79, RW12, G48, and G8. Isolates of *M. poae* Landschoot & Jackson, and *M. rhizophila* Scott & Deacon were grown on PDA and incubated under fluorescent lights to induce production of anamorphs or teleomorphs for microscopic observation (Scott and Deacon 1983).

DNA extraction.—Isolates were grown in 100 mL of liquid Fries medium (30 g sucrose, 5 g ammonium tartrate, 1.0 g NH_4NO_3, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.0 g KH_2PO_4, 0.1 g NaCl, 0.13 g $CaCl_2 \cdot 2H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 1.0 g yeast extract, per liter of H_2O) on a rotary shaker at 100 rpm for 4–5 d. Mycelium was harvested by filtration through Miracloth (Calbiochem, La Jollia, California), dried in a Savant Speed Vac (Savant Instruments, Inc., Farmingdale, New York) and stored at −20 C. DNA was extracted from approximately 15 mg of dried mycelium following the procedure of Zolan and Pukkila (1986).

Polymerase chain reaction (PCR) and DNA sequencing.—DNA amplification reactions were performed in either a Perkin-Elmer 9600 or 9700 thermocycler (Perkin Elmer, Foster, City, California) following the protocol provided with PCR core reagents (Roche Molecular Systems Inc., Branchburg, New Jersey). The reaction volume was 20 μL containing 10 μL of template DNA at a 1:200 dilution of the DNA extraction. The PCR primers ACT-512F, ACT-783R, Bt1a, Bt1b, CAL-228F, and CAL-737R were used to amplify portions of the actin, beta-tubulin, and calmodulin genes, respectively. Sequences for these primers have been published previously (Carbone and Kohn 1999, Glass and Donaldson 1995). The PCR conditions used for all primers were as follows: an initial denaturation step at 95 C for 8 min, 30 cycles of 95 C for 30 s, 55 C for 20 s, 72 C for 1 min and a final extension at 72 C for 5 min. PCR products were purified using QIAquick spin columns (Qiagen Inc. Mississauga, Ontario) following the manufacturer's instructions.

Both forward and reverse strands were sequenced using

TABLE I. *Magnaporthe* isolates used in this study

Species[a]	Collection	Host	Geographic Origin	Collector
Magnaporthe grisea	*Py-D*	*Digitaria horizontalis*	Brazil	Prabhu
	JP34	*D. smutsii*	Japan	Notteghem
	81T4	*Digitaria* sp.	USA	Marchetti
	91T16	*Digitaria* sp.	USA	Marchetti
	94-118-1a[b,c]	*Digitaria* sp.	China	
	94-118-1c[b]	*Digitaria* sp.	China	
	94-118-2b[b]	*Digitaria* sp.	China	
	94-118-4b[b]	*Digitaria* sp.	China	
	93-14-1b[b]	*Digitaria* sp.	China	
	93-14-1e[b]	*Digitaria* sp.	China	
	RW 12[c]	*Eleusine coracana*	Rwanda	Notteghem
	1122[c]	*E. coracana*	India	Kumar
	94-4-1d[b,c]	*E. coracana*	China	
	94-115-1a[b,c]	*E. coracana*	China	
	94-115-3a[b,c]	*E. coracana*	China	
	94-117-1b[b,c]	*E. coracana*	China	
	94-115-2a[b,c]	*E. coracana*	China	
	MAFF305522[c,d](C10)	*Eleusine indica*	Japan	Yaegashi
	MAFF 305523[c,d] (T28)	*E. indica*	Japan	Yaegashi
	NI909[c]	*Eragrostis curvula*	Japan	Kato
	K76-79[c]	*E. curvula*	Japan	Yaegashi
	365[c]	*Lolium perenne*	KS, USA	Tisserat
	330[c]	*L. perenne*	KS, USA	Tisserat
	A598[c]	*Oryza sativa*	AR, USA	Correll
	A264[c]	*O. sativa*	AR, USA	Correll
	A119[c]	*O. sativa*	AR, USA	Correll
	A347[c]	*O. sativa*	AR, USA	Correll
	BK-6[c]	*O. sativa*	Bandrakoti, India	Milgroom
	BK-19[c]	*O. sativa*	Bandrakoti, India	Milgroom
	Guy 11[c]	*O. sativa*	French Guyana	Notteghem
	ML-56[c]	*O. sativa*	Matli, India	Milgroom
	ML-91[c]	*O. sativa*	Matli, India	Milgroom
	R694-2b[c]	*O. sativa*	Ranichauri, India	Kumar
	R707-1E[c]	*O. sativa*	Ranichauri, India	Kumar
	R93-1-1b[b,c]	*O. sativa*	China	
	R93-2-1a[b,c]	*O. sativa*	China	
	R93-10-1a[b,c]	*O. sativa*	China	
	R93-11-1a[b,c]	*O. sativa*	China	
	R95-4-1a[b,c]	*O. sativa*	China	
	R96-1-1a[b,c]	*O. sativa*	China	
	R96-2-1a[b,c]	*O. sativa*	China	
	R97-101-1a[b,c]	*O. sativa*	China	
	R97-102-1b[b,c]	*O. sativa*	China	
	R97-105-1a[b,c]	*O. sativa*	China	
	R97-107-1a[b,c]	*O. sativa*	China	
	G-48[c]	*Setaria* sp.	WV, USA	Latterell
	1152[c]	*Setaria* sp.	India	Kumar
	8465[e]		NA	FGSC[f]
	8470[e]		NA	FGSC[f]
M. poae	ATCC 64411	*Triticum aestivum*		Landschoot
M. rhizophila	ATCC 58114	*T. aestivum*	South Africa	Deacon
	ATCC96043	*Poa pratensis*	PA, USA	Landschoot
M. salvinii	MS-1	*O. sativa*	TX, USA	Marchetti

[a] Species epithet under which specimen was accessioned by collector.

[b] Isolates used to test PCR-RFLP diagnostic. Isolates provided by Zonghua Wang, Fujian Agricultural University.

[c] Isolate determined to be *M. oryzae* as a result of the present study.

[d] National Institute of Agrobiological Resources, Tskuba, Japan.

[e] Mapping strains reported in Nitta et al (1997).

[f] Strains available through the Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center.

the same primers used in the amplification reactions. Sequencing reactions were performed using an ABI Prism Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, California), following the manufacturer's instructions, with the exception that only 1/4 of the recommended volume of terminator mix was used. Sequencing reactions were subjected to capillary electrophoresis on an ABI 310 Genetic Analyzer. Electropherograms were interpreted with Sequence Analysis Software version 3.3 (Applied Biosystems, Foster City, California).

DNA sequence alignment.—Analyzed sequences were imported into Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, Michigan), checked visually and placed in contigs. All sequences were deposited in GenBank, with accession numbers AF395947 to AF396033 and AY063734 to AY063739. Sequences were aligned using CLUSTAL W Version 1.74 (Thompson et al 1994) and edited manually using Sequence Alignment Editor version 1.0 alpha 1 (http://evolve.zoo.ox.ac.uk/software/Se-Al/Se-Al.html). The intron and exon positions were identified by aligning *Magnaporthe* sequences with the following sequences: beta-tubulin 1 from *Neurospora crassa* Shear and Dodge (GenBank accession number M13630), calmodulin from *M. grisea* (AF104986), and actin from *Acremonium chrysogenum* (Thirumalachar and Sukapure) Gams (AF056976). Percentage divergence was calculated by dividing the number of variable positions in the aligned sequence by the total length of the consensus sequence.

Phylogenetic analysis.—Phylogenetic analysis was first performed using only *M. grisea* isolates. Phylogenies were inferred from each of the three genes individually, then from the combined data for all three genes. A second analysis was performed using all *Magnaporthe* species. All phylogenetic analyses were performed using PAUP 4.0b3 (Swofford 1998). Trees and DNA sequence alignments were deposited in TreeBASE, accession number SN1015. Heuristic searches were performed using the optimality criterion of maximum parsimony. Starting trees were obtained via stepwise addition with a simple addition sequence using *M. salvinii* as a reference taxon. The branch swapping algorithm used was tree-bisection-reconnection. In the analysis of *M. grisea* isolates, unrooted trees were produced which were then rooted using isolates from species of *Digitaria*. In the analysis of all *Magnaporthe* species, trees were left unrooted. The molecular clock hypothesis was tested using the one degree of freedom method of Tajima (1993). To assess the support for each branch, bootstrapping (Felsenstein 1985) was performed with 500 replicates using the heuristic search option. Phylogenies were inferred from each of the three genes individually and then for the combined data for the beta-tubulin and calmodulin genes. Phylogenetic congruence was determined by the partition homogeneity test [(PHT); (Huelsenbeck et al 1996)], performed with 100 replicates, and Templeton's summed ranks test (Templeton 1983), both implemented in PAUP 4.0b3 (Swofford 1998).

Maximum likelihood analysis.—Heuristic searches were also performed using the optimality criterion of maximum likelihood (Felsenstein 1981). Nucleotide frequencies were calculated from the data, all sites were assumed to evolve at the same rate and a molecular clock was not enforced.

PCR-RFLP diagnostic for species identification.—The beta-tubulin region was amplified as described above. PCR reactions were precipitated using sodium acetate and ethanol then resuspended in 50 μL of H_2O. Five μL of DNA was digested with *Hpa* II (New England Biolabs, Boston, Massachusetts) according to the manufacturer's instructions and separated on a 1.5% agarose gel containing ethidium bromide and visualized under UV light.

RESULTS

Cultural studies and crosses.—Characteristic anamorphs were observed in isolates of *M. grisea* and *M. salvini* (*Sclerotium oryzae* Cattaneo), but not *M. rhizophila* or *M. poae*. Teleomorphs were not observed in the two isolates of *M. rhizophila* but did develop in the following crosses: 8465 × 8470 (TABLE I), 8465 × RW12 (*Eleusine coracana*), 8470 × NI909 (*Eragrostis curvula* (Schrad.) Nees), and 8470 × G48 (*Setaria* sp.). In these successful crosses, perithecia containing asci with ascospores were observed; ascospores were germinable. Attempts to germinate ascospores from the type specimen of *Ceratosphaeria grisea* (BPI 625033) were unsuccessful.

Morphological examination.—The teleomorph produced in the 8465 × 8470 cross, as well as the type specimen of *M. grisea* (see taxonomic part below) and all anamorphs were examined. Ascospores and conidia were measured and perithecial morphology was compared among specimens and against published descriptions. Consistent with the literature, no morphological differences were observed between materials associated with *Digitaria* and those associated with *Oryza* and other non-*Digitaria* grasses.

DNA extraction, sequencing and alignment.—Due to the paucity of perithecia in the type specimen of *Ceratosphaeria grisea* (BPI 625033), DNA extraction for PCR amplification was not attempted. DNA extraction was successful from as few as 20 freeze-dried perithecia from the cross of isolates 8465 × 8470 (designated in the present study as the holotype of *M. oryzae*), but was unsuccessful from 5 or 10 perithecia. The actin, beta-tubulin and calmodulin genes could be amplified from all *Magnaporthe* species. The exon sequences for these genes were easily alignable across all species. Alignments of the intron sequences were unambiguous within species, but were more complicated among species. As a result, intron sequences were eliminated from the phylogenetic analyses in which all species were included. The intron sequences from *M. grisea* isolates were alignable with each

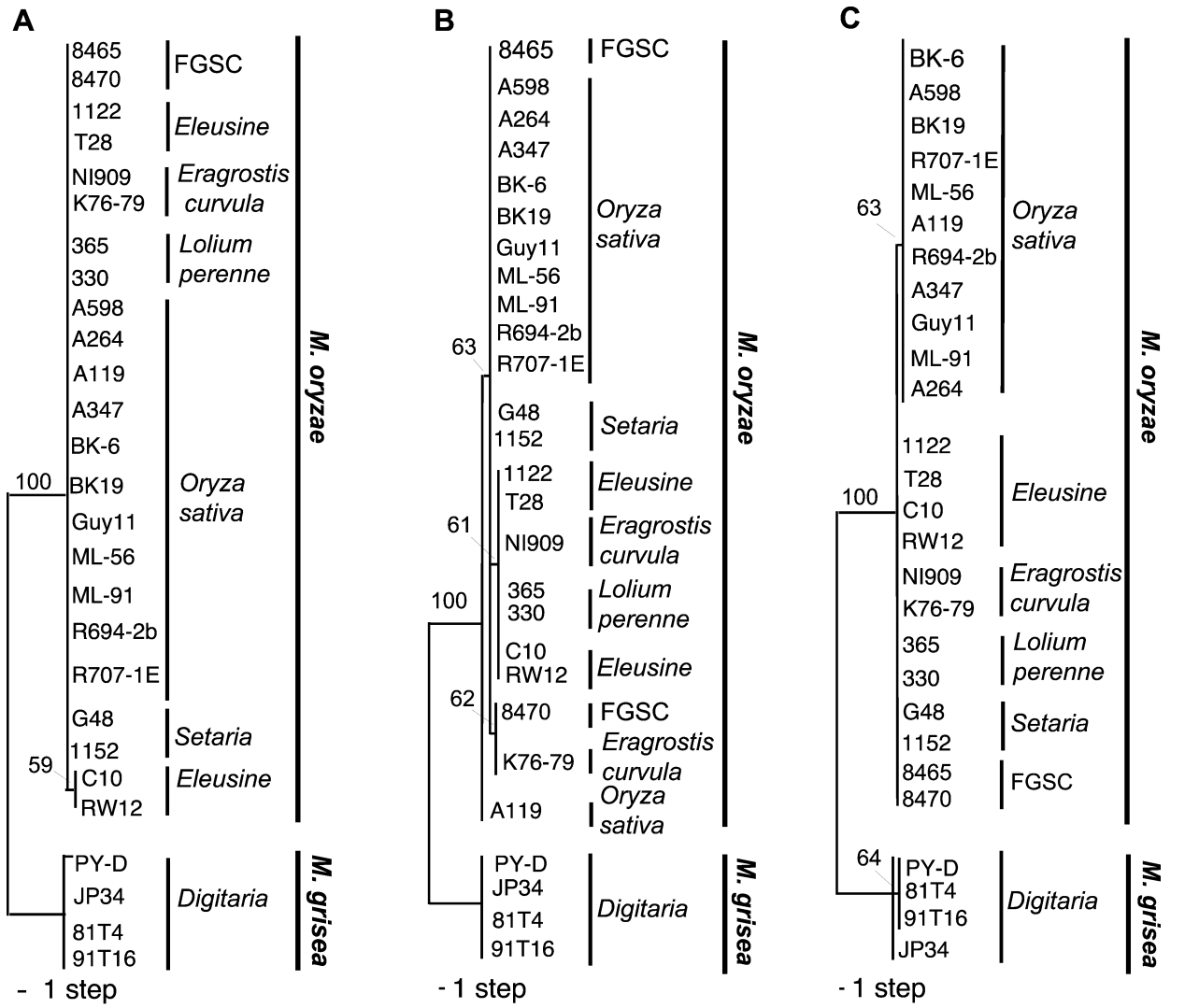

FIG. 1. Phylogenies of *M. grisea* isolates inferred from three genes: actin, beta-tubulin and calmodulin. Labels on the phylogeny are, from left to right: isolate number, origin of each strain as either the host from which it was isolated or the Fungal Genetics Stock Center (FGSC), and the species determination from the present study as *M. oryzae* or *M. grisea*. Where host species epithets are not provided, either multiple species within the genus were sampled or the host species was unreported. Bootstrap values, based on 500 replicates, are indicated above the branches appearing in the consensus tree. **A.** The single most parsimonious tree produced based on the actin gene. The tree length is 20 and the consistency index (CI) = 1.0. **B.** The single most parsimonious tree based on the beta-tubulin gene. The tree length is 34 and the CI = 1.0. **C.** The single most parsimonious tree based on the calmodulin gene. The tree length is 75 and the CI = 1.

other and were included in the phylogenetic analysis restricted to *M. grisea* isolates.

Phylogenetic analysis of M. grisea.—Single most parsimonious trees were inferred for *M. grisea* isolates based on data from each of the three genes (FIG. 1). In each phylogeny, isolates from *Digitaria* sp. were distinct from the isolates from *O. sativa* and other grasses. The isolates C10 and T28 [the voucher isolates from *Eleusine indica* used in Yaegashi and Udagawa's cross (1978)] clustered within the clade containing isolates from *O. sativa*. In the PHT, no significant incongruence ($P = 0.7$) was found among phylogenies inferred from the actin, beta-tubulin and calmodulin genes. Similar results were obtained with Templeton's signed rank test ($P > 0.3$ for all tests). As a result, data from each of these genes could be combined. Five most parsimonious trees, one step longer than the minimum length tree, were inferred from the combined data; one representative tree is presented (FIG. 2). These trees each have a consistency index (CI) of 0.992 and differ from one another only in the branching order within the clade containing isolates from *O. sativa*. Isolates from *Digitaria* sp. are separated from the other isolates by a total of

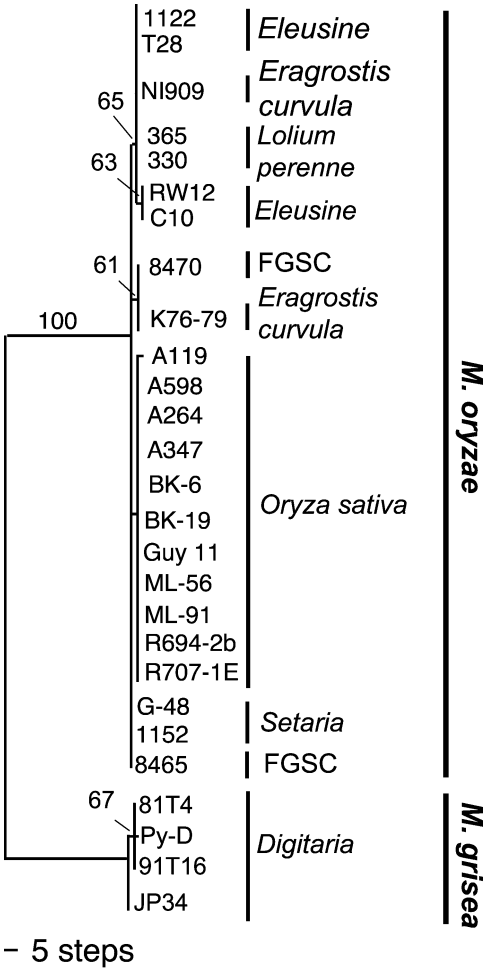

FIG. 2. One of five most parsimonious trees inferred for *Magnaporthe grisea* isolates from the combined data for the actin, beta-tubulin and calmodulin genes. Labels on the phylogeny are, from left to right: isolate number, origin of each strain as either the host from which it was isolated or the Fungal Genetics Stock Center (FGSC), and the species determination from the present study as *M. oryzae* or *M. grisea*. Where host species epithets are not provided, either multiple species within the genus were sampled or the host species was unreported. Bootstrap values, based on 500 replicates, are indicated above the branches appearing in the consensus tree. The tree length is 130, one step longer than the minimum length tree and the consistency index is 0.992. The host from which the isolates were derived from is indicated in brackets.

123 steps. Polymorphisms distinguishing *Digitaria* isolates from *O. sativa* isolates are presented in TABLES II, III and IV.

Phylogenetic analysis of Magnaporthe *species.*—Two clades within *M. grisea* are resolved in phylogenies inferred for *Magnaporthe* species based on the beta-tubulin and calmodulin genes (FIG. 3). A single most parsimonious tree was inferred from the beta-tubulin gene (FIG. 3A). A single most parsimonious, minimum length tree was inferred from the data for the calmodulin gene (FIG. 3B). Both trees have the same topology as the trees generated using maximum likelihood. The evolution of both the beta-tubulin and calmodulin genes in *M. grisea* was consistent with a molecular clock ($P > 0.25$, $P > 0.999$ respectively). The exon sequences from the actin gene which were alignable across all *Magnaporthe* species do not contain characters which resolve the relationships among isolates of *M. grisea* from different hosts. Consequently, the actin region was not used for phylogenetic analysis among all *Magnaporthe* species.

A single most parsimonious tree was produced for the combined datasets of the beta-tubulin and calmodulin genes (FIG. 4). The tree has a length of 87, which is 5 steps longer than the minimum length tree (CI = 0.943) and is equal to the sum of tree lengths for each region separately. Since the phylogeny inferred from the combined dataset is not longer than the sum of the tree lengths for each individual dataset, it is appropriate to combine the datasets for both genes (Farris et al 1995). The maximum likelihood tree produced for this dataset has the same topology as the maximum parsimony tree. In this phylogeny, two clades are clearly resolved within *M. grisea*.

PCR-RFLP.—A PCR-RFLP assay (Chehab et al 1987) was developed as a simple, rapid diagnostic for distinguishing members of the two clades within *M. grisea*. The G-A transition at position 160 and the G-C transversion at position 161 in the beta-tubulin gene

TABLE II. Polymorphic sites in the actin gene which differentiate *M. grisea* isolates from *Digitaria* spp. and *Oryza sativa*. Numbers at the top of columns indicate base positions in the alignment

	1111112222 5555677901239935 66 7014821713877595707
Digitaria spp.	TCAACTTCCTTTCATTGTC
Oryza sativa	C--TACCTG-CA--C-CGG

TABLE III. Polymorphic sites in the beta-tubulin gene which differentiate *M. grisea* isolates from *Digitaria* spp. and *Oryza sativa*. Numbers at the top of columns indicate base positions in alignment

Host	1111111111111111111222222233 2442455556666777777990000123 7 3942806123601361235896903891679
Digitaria spp.	TGCCTCAACCGACTAACCAAGTCG-CCCCGT
Oryza sativa	CTTTCTGTTTAGGCTGTTGGACTACTATTCC

TABLE IV. Polymorphic sites in the calmodulin gene which differentiate *M. grisea* isolates from *Digitaria* spp. and *Oryza sativa*. Numbers at the top of columns indicate base positions in alignment

	111111
	111112222333334445556666677888234446
	356734568045912389123289034797836797268O
Digitaria spp.	-TGGCTTCCCCTATCGGCAGAGACGGTAAGATCCAAGTTG
Oryza sativa	TG-----GTAG-GCGAAGG--CTTTACGTAGCTGTCACCA
	11111111111222222222223344444444444444444
	66677778889122335555661200111122334455 6
	13623680193716122679121926456847694615 6
Digitaria spp.	CAGCG-TTCCGTATTGT--GATGTATGCGAGTACATA-T
Oryza sativa	TCAGTTACGTCAGCCACACCTACCCCAAACT-CTTGGTC

result in the absence of the *Hpa* II restriction site in isolates from *Digitaria*. Consequently, isolates from *Digitaria* can be distinguished from isolates from rice and other grasses based on the PCR-RFLP phenotype for the beta-tubulin region (FIG. 5). Twenty-two Chinese isolates from three hosts, *Digitaria* sp. (6 isolates), *Eleusine coracana* (5 isolates), and *O. sativa* (11 isolates), were used to evaluate this assay. Twenty-one isolates were found to have the predicted PCR-RFLP phenotype. One isolate (94–118–1a), reportedly from *Digitaria*, possessed a PCR-RFLP phenotype characteristic of isolates from *O. sativa*. To confirm the identity of this isolate the beta-tubulin gene was sequenced and was found to be identical to isolates from *O. sativa*. This suggests that the isolate was either mislabeled and is not from *Digitaria*, or that *M. oryzae* may occur rarely on *Digitaria*.

DISCUSSION

Based on the inferred phylogenies (FIGS. 1–4), *M. grisea* contains two distinct lineages, one associated with *Digitaria*, and the other associated with *O. sativa* and other grasses. Four lines of evidence indicate that these lineages represent two distinct species: (i) they constitute two well supported, monophyletic clades; (ii) they have only one shared polymorphism against a preponderance of fixed differences; (iii) they are associated with different groups of hosts; (iv) they are reproductively isolated and have been so for a long time. This is supported by infertility in laboratory crosses (the present study, as well as Hebert 1971, Kato et al 2000). These lines of evidence also indicate that the two lineages are not divergent populations within the same species.

The large number of base substitutions separating these two lineages within *M. grisea* is consistent with a long period of isolation. When data from all three genes were combined in analysis of isolates of *M. grisea*, 123 steps separated the clade including isolates from *Digitaria* from the clade composed of isolates from *O.sativa* and other grasses (FIGS. 1, 2). In order to evaluate the monophyly of these groups, species-level phylogenies (including all *Magnaporthe* species) were constructed (FIGS. 3, 4). In this analysis, the two lineages were clearly resolved as monophyletic and separated by a total of 13 steps.

In the species-level phylogenies *M. rhizophila*, represented by two isolates, was paraphyletic. One isolate was from the United States and one was from South Africa. These isolates may represent distinct species.

The ecological and reproductive isolation of the two lineages within *Magnaporthe grisea* has resulted in what appear to be multiple fixed DNA sequence differences between the lineages. Only one polymorphism is shared by the two lineages within *M. grisea*. At position 48 in the beta-tubulin gene the rice isolate A119 and the isolates from *Digitaria* have a C, the remaining isolates have a T at this position. This shared polymorphism may represent a character reversal.

The differentiation of population divergence from speciation events has been addressed recently by Carbone and Kohn (2001). Nested cladistic and coalescent analyses at both the population and species levels were used to order population divergence within *Sclerotinia sclerotiorum* (Lib.) de Bary and speciation events within *Sclerotinia*. Species were distinguished from populations based on differences in coalescence times; populations coalesce before species. Within *S. sclerotiorum* the most divergent populations differed by 22 base pairs out of a total of 4000, or 0.55%. In the species level analysis, the two closely related species *S. minor* Jagger and *S. trifoliorum* Erikss. differed by 22 base pairs out of a total of 1250, or 1.76%. In comparison, based on data from three genes, the divergence between the two lineages resolved within *M. grisea* was 9.7%. In the species level analysis, the divergence between the two lineages within *M. grisea* was 2.21% (substitution rates were

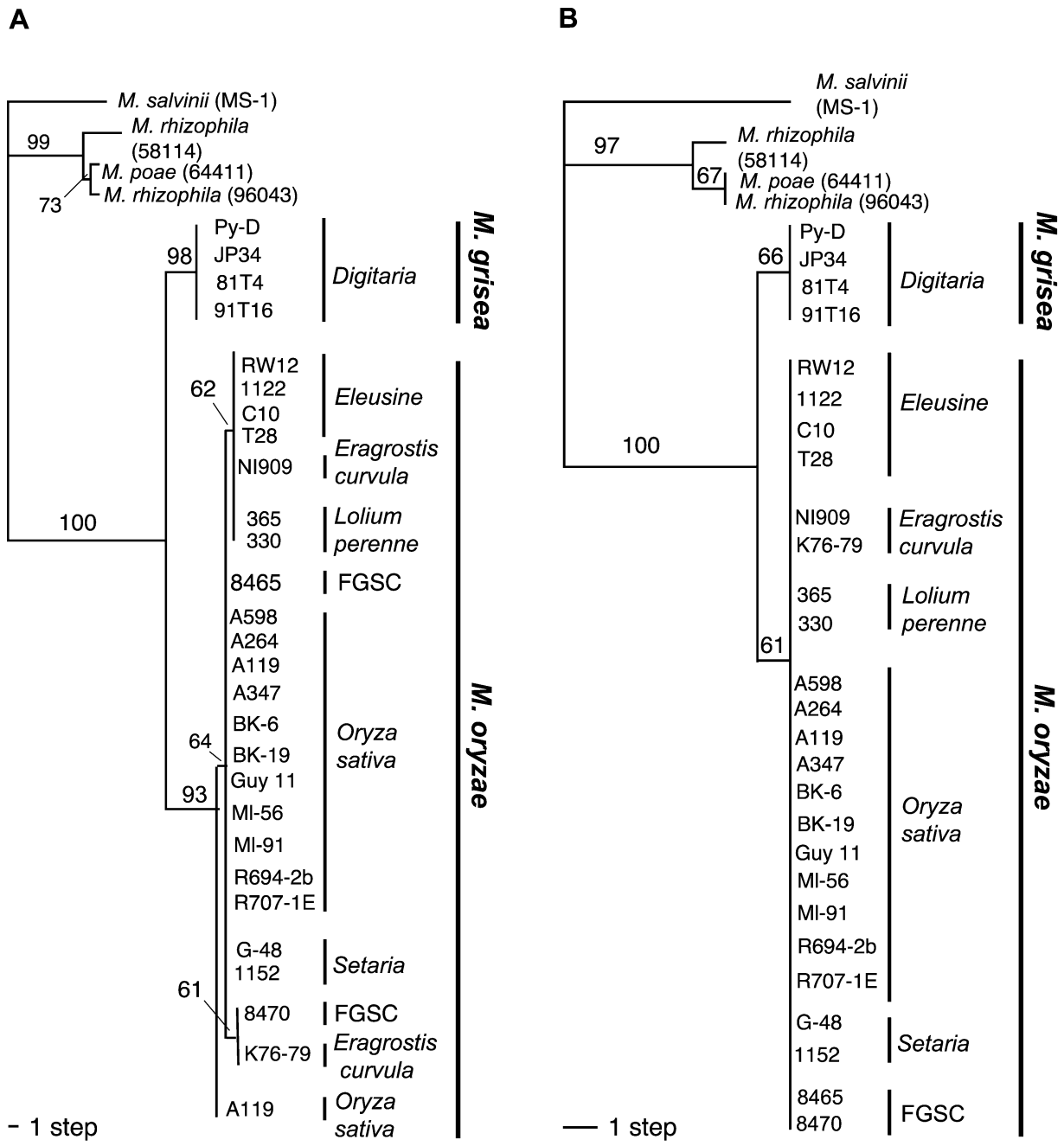

FIG. 3. Phylogenies of *Magnaporthe* species inferred from beta-tubulin and calmodulin genes. Labels on the phylogeny are, from left to right: isolate number, origin of each strain as either the host from which it was isolated or the Fungal Genetics Stock Center (FGSC), and the species determination from the present study as *M. oryzae* or *M. grisea*. Where host species epithets are not provided, either multiple species within the genus were sampled or the host species was unreported. Bootstrap values, based on 500 replicates, are indicated above the branches appearing in the consensus tree. **A.** The single most parsimonious tree produced based on the beta-tubulin gene. The tree length is 66, 5 steps longer than the minimum length tree and the CI is 0.924. **B.** The single most parsimonious, minimum length, tree produced based on the calmodulin gene. The tree length is 21 and the CI is 1.

clock-like). This level of divergence is comparable to the species level divergence found in *Sclerotinia*. Considering both the level of divergence between the two clades within *M. grisea* and their monophyly, they could be considered phylogenetic species under Cracraft's (1983) definition in which a phylogenetic species is recognized as a diagnosably distinct, monophyletic, basal group of organisms.

The association of each clade with different hosts indicates ecological specialization. *Digitaria* species

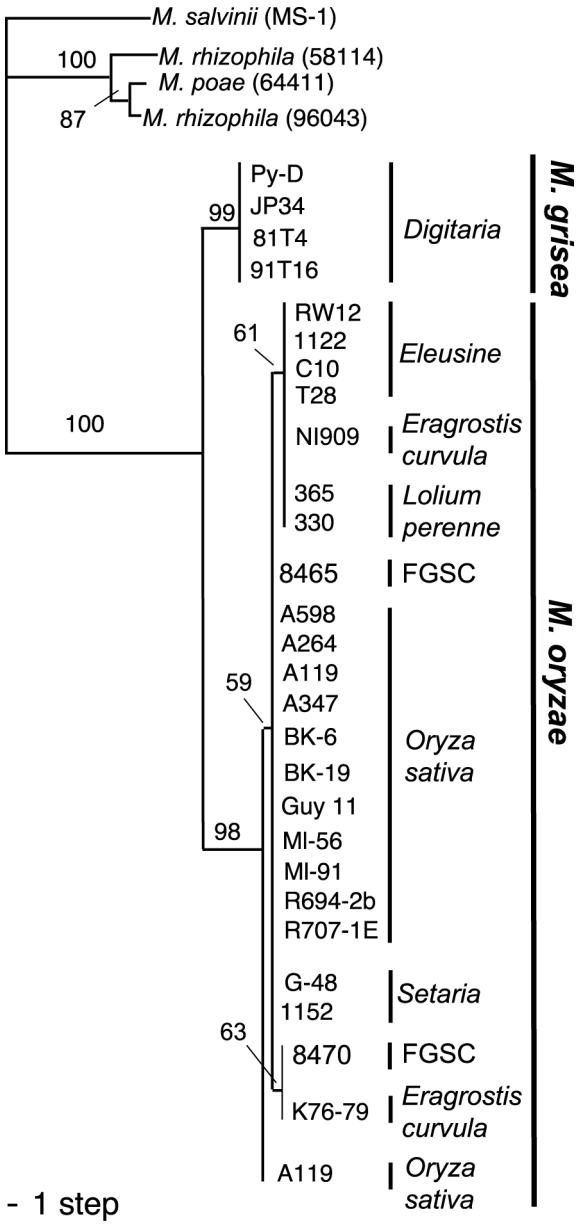

FIG. 4. A single most parsimonious tree inferred for *Magnaporthe* species based on combined data for the beta-tubulin and calmodulin genes. Labels on the phylogeny are, from left to right: isolate number, origin of each strain as either the host from which it was isolated or the Fungal Genetics Stock Center (FGSC), and the species determination from the present study as *M. oryzae* or *M. grisea*. Where host species epithets are not provided, either multiple species within the genus were sampled or the host species was unreported. Bootstrap values, based on 500 replicates, are indicated above the branches appearing in the consensus tree. The tree length is 87 and the consistency index is 0.943.

are ubiquitous weeds and often occur adjacent to rice cultivation, yet host association is maintained. This host association may be maintained by ecological factors such as the life history or microhabitat occupied by the host. Alternatively, host association may be maintained by the inability to overcome resistance genes present in other grass hosts.

Interfertility between isolates from *Digitaria* and isolates from *Oryza* and other grasses has not been reported (Hebert 1971, Kato et al 2000). In contrast, there have been fertile crosses between isolates from *Digitaria* (Hebert 1971, Kato et al 2000), and between isolates from *Oryza* (*Oryza* × *Oryza*) (Kato and Yamaguchi 1982), from *Eleusine* (*Eleusine* × *Eleusine*) (Yaegashi and Udagawa 1978), as well as between isolates from both grass genera (*Oryza* × *Eleusine*). This further supports the reproductive isolation of *Digitaria* isolates from isolates from rice and other hosts evident in the phylogenetic analyses. Biological species boundaries are congruent with phylogenetic species boundaries.

Based on our own as well as published observations, there are no known morphological characters that distinguish isolates from *Oryza* or other grasses from *Digitaria* isolates (Rossman et al 1990, Yaegashi and Udagawa 1978). When Yaegashi and Udagawa (1978) mated the isolates from *Eleusine*, C10 and T28, they concluded that based on morphology, the teleomorph was consistent with Hebert's description of *C. grisea* and that their teleomorph matched those preserved by Hebert. In this study, based on DNA sequence data, the isolates C10 and T28 could be distinguished from isolates from species of *Digitaria* and therefore from Hebert's concept of *M. grisea* which was based on isolates from *Digitaria*. Although these two lineages within *M. grisea* do not seem to be morphologically differentiated, they are differentiated by molecular characters and host association. Unfortunately, no authentic living material attributable to Hebert seems to exist and ascospores preserved in his type specimen of *C. grisea* are no longer viable.

Describing a new species based upon DNA sequence differences and host of origin makes identification difficult if DNA sequencing facilities are not available or the host of origin is not known. To address this difficulty, we have developed the diagnostic PCR-RFLP test and demonstrated its utility.

This study conclusively demonstrates that there are two species within the present circumscription of *M. grisea*. The two lineages have been detected in previous studies using other molecular methods (Borromeo et al 1993, Shull and Hamer 1994, Bunting et al 1996, Kato et al 2000). Because the name *M. grisea* is nomenclaturally tied to isolates from *Digitaria*, the

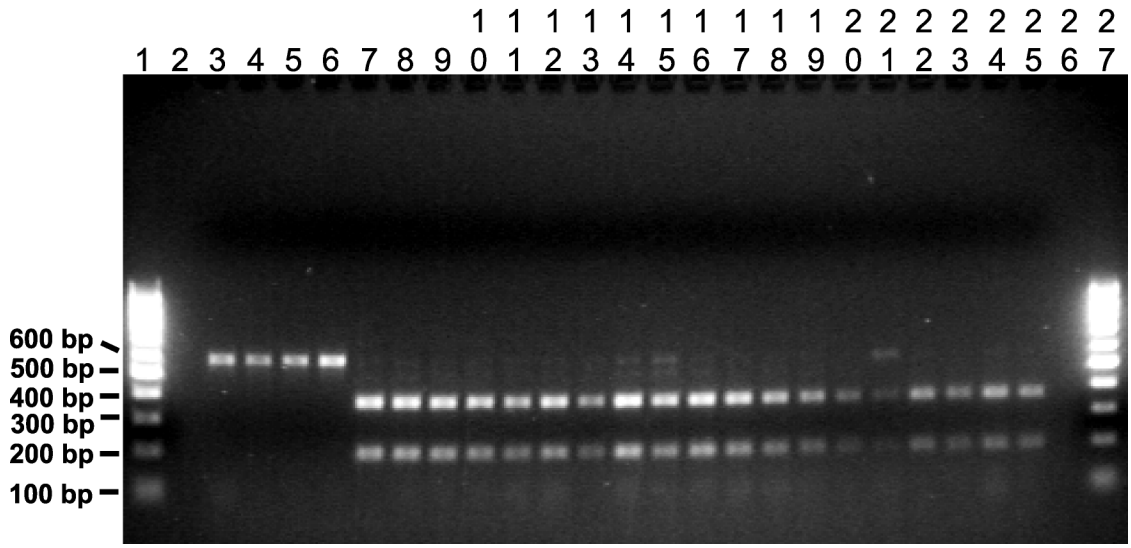

FIG. 5. PCR-RFLP of the beta-tubulin gene. The beta-tubulin gene was amplified from 23 isolates and digested with *Hpa* II: lanes 3–6 contain isolates from *Digitaria*, 81T4, Py-D, 91T16, and JP34, lanes 7 and 8 contain *Lolium perenne* isolates 330, 365, lanes 9 and 10 contain *Setaria* isolates G-48, 1152, lanes 11 and 12 contain *Eleusine coracana* isolates RW 12, 1122, lanes 13 and 14 contain *Eragrostis curvula* isolates NI909, K76–79, lanes 15–25 contain *Oryza sativa* isolates, R694–2b, ML-56, R707–1E, ML-91, Guy 11, BK-6, BK-19, A598, A347, A264, and A119. Lanes 1 and 27 contain the Mass Ruler size standard (MBI Fermentas, Flamborough, Ontario), the sizes of size standard fragments are indicated on the left. Lanes 2 and 26 were not loaded.

scientifically correct name for isolates from *Oryza* and other grasses is *M. oryzae*. If the user community desires to continue applying the name *M. grisea* in a scientifically accurate way for isolates associated with rice blast and grey leaf spot, formal nomenclatural conservation through proposals to the International Committee on Fungal Nomenclature will be required. If successful, a new epithet for isolates from *Digitaria* would be required.

Description of species.—A new species is described for the strains of *Magnaporthe* from *O. sativa* and closely related isolates from other grasses, which are phylogenetically distinct from isolates from *Digitaria*.

Magnaporthe oryzae B. Couch sp. nov.
FIG. 1, Yaegashi and Udagawa (1978);
FIG. 5, Couch and Kohn (this paper).

= *Magnaporthe grisea* sensu Yaegashi and Udagawa, Can. J. Bot. 56:180–183 (1978)

Teleomorphus ut in *Magnaporthe grisea* simili sed ab hac specie differt: in gramina *Eragrostis curvula* et *Eleusine coracana* et *Lolium perenne* et *Setaria* spp. et *Oryza sativa* sed haud *Digitaria* spp. genitus; gens beta-tubulin vocata locis 160 et 161 situ rumpenti *Hpa* II vocato exemplis PCR-Bt1a et PCR-Bt1b amplicata exhibens; PCR-RFLP enzymato *Hpa* II vocato fragmenta duo 188 fasciarum et 362 fasciarum praebens. Anamorphus est *Pyricularia oryzae* vocatus.

Teleomorph similar to *Magnaporthe grisea* but differs from that species: parasitic on grasses *Eragrostris curvula*, *Eleusine coracana*, *Lolium perenne*, *Setaria* spp., and *Oryza sativa*, but not *Digitaria* spp. Among many polymorphisms in each of three gene loci, those polymorphisms in the beta-tubulin gene at positions 160 and 161, when amplified using primers Bt1a and Bt1b, result in the addition of an *Hpa* II restriction site. PCR amplification followed by restriction digestion with *Hpa* II yields two DNA fragments, one of 188 base pairs and the other 362 base pairs. Anamorph is *Pyricularia oryzae*.

Type specimen: GUYANA and OTHER LOCALITIES: cross of the mapping strains 8465 by 8470 (Nitta 1997) which were the progeny of a cross of Guy11, from *Oryza sativa* in Guyana, and 2539, a fertile lab strain derived from several fertile strains crossed successively to increase fertility, originally isolated from *Eleusine* and *Oryza sativa* from unreported localities (HOLOTYPE—BPI 841383; duplicate, TRTC 52742; strains available from Fungal Genetics Stock Center, Department of Microbiology, University of Kansas Medical Center Kansas City, Kansas 66160-7420 USA).

Etymology. Latinized from *oryza*, Greek = rice, referring to the name of the anamorph, *Pyricularia oryzae*, which was first described from rice.

Anamorph. Pyricularia oryzae Cavara, Fungi Longobardiae Exsiccati No. 49. 1892.

= *Dactylaria oryzae* (Cavara) Sawada, Trans. Nat. Hist. Soc. Taiwan 6:242. 1916.

Specimens examined. UNITED STATES. NORTH CARO-

LINA: Barley grain and rice straw, 1971 Jan 00, *T. T. Hebert,* BPI 625033 (Holotype of *Ceratosphaeria grisea*).

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