Mycologia, 97(5), 2005, pp. 1002–1011.

Molecular and morphological characterization of *Pyricularia* and allied genera

B. Bussaban
S. Lumyong[1]
Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

P. Lumyong
Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand 50200

T. Seelanan
Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand

D.C. Park
E.H.C. McKenzie
Landcare Research, Private Bag 92170, Auckland, New Zealand

K.D. Hyde
Centre for Research in Fungal Diversity, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, China

Abstract: The phylogenetic relationships of *Pyricularia* species and species from related genera were established from sequences of the internal transcribed spacer ribosomal RNA gene. Phylogenetic analysis disclosed a consistent correlation with spore morphology. Most *Pyricularia* species studied, and two species of *Dactylaria* that have obpyriform conidia, fell within the Magnaporthaceae cluster with high bootstrap support. *Pyricularia variabilis* was more related to *Dactylaria*, *Tumularia* or *Ochroconis* species than to the Magnaporthaceae. *Dactylaria* and species of *Nakataea*, *Ochroconis*, *Pyriculariopsis* and *Tumularia* were distinct from the Magnaporthaceae, and the genus *Dactylaria* is polyphyletic. The combination of morphological and molecular characters, such as spore morphology and ITS ribosomal DNA sequences data, suggested that conidial shape could be a primary character to distinguish *Pyricularia* from related genera.

Key words: *Dactylaria*, ITS rDNA, phylogeny, systematics, 5.8S rDNA

INTRODUCTION

The genus *Pyricularia* (Cooke) Sacc. (anamorphic Magnaporthaceae) was established by Saccardo (1880) with the type species, *P. grisea* (Cooke) Sacc., which originally was described from crabgrass (*Digitaria sanguinalis* L.). The name ''Pyricularia'' refers to the pyriform shape of the conidia. Cavara (1892) subsequently described *P. oryzae* Cav. from rice (*Oryza sativa* L.), a taxon with similar morphology to *P. grisea*. Despite the lack of obvious morphological differences, these two taxa have been maintained as separate species. Rossman et al (1990) argued that *P. oryzae* should be synonymized with *P. grisea* and grouped these two anamorphs under the teleomorph *Magnaporthe grisea* (Hebert) Barr. Recent molecular genetic analyses, however, have indicated that *Pyricularia* species isolated from different hosts are genetically distinct (Borromeo et al 1993, Shull and Hamer 1994, Kato et al 2000). Based on RFLP and DNA sequence analysis, Borromeo et al (1993) and Kato et al (2000) suggested that the *Pyricularia* isolates from *Digitaria* sp. and rice represent distinct species. Using a molecular approach based on three genes (actin, beta-tubulin and calmodulin), Couch and Kohn (2002) described the teleomorph *Magnaporthe oryzae* B. Couch (associated with *Oryza sativa* and other cultivated grasses) as a species distinct from *M. grisea* (associated with the grass genus *Digitaria* Haller).

Pyricularia has been well circumscribed (Ellis 1971, 1976), although the distinction between it and some *Dactylaria* species is not always clear (Goh and Hyde 1997). The conidiogenous cells of *Dactylaria* and *Pyricularia* are polyblastic, integrated on the conidiophores, and are sympodial, cylindrical, geniculate and denticulate. The conidia are solitary, dry, acropleurogenous, simple, variously shaped, and hyaline to pale brown (Ellis 1971, 1976). In *Pyricularia*, however, denticles usually are cut off by a septum to form a separating cell (rhexolytic secession) and the conidia are mostly obpyriform. In *Dactylaria* there is no separating cell in the denticles (schizolytic secession) and the conidia are of various shapes, usually fusiform, naviculate or more or less cylindrical (Ellis 1976). The presence of a separating cell and cylindrical denticles in *Pyricularia* were characteristics used by Ellis (1976) to delineate *Pyricularia* from *Dactylaria*. Furthermore *Pyricularia* species are important pathogens, while *Dactylaria* species are usually saprobes (Cai et al 2002, Ho et al 2002, Bussaban et al 2003, Paulus et al 2003, Luo et al 2004).

Analysis of ribosomal DNA frequently has been used in mycological investigations (Bruns et al 1991).

Accepted for publication 29 August 2004.
[1] Corresponding author. E-mail: scboi009@chiangmai.ac.th

TABLE I. The sources of *Pyricularia* isolates and allied genera used for ITS1-5.8S-ITS2 rDNA sequence analysis

Genus and species	Strain No.[a]	Original substrate	Habitat	Geographic origin
Dactylaria ampulliformis (Tubaki) G.C. Bhatt & W.B. Kendr.	ICMP3660	*Cocos nucifera*	leaf streak	Japan
D. appendiculata Cazau, Aramb. & Cabello	ICMP14617	*Uncinia* sp.	dead culm	New Zealand
D. purpurella (Sacc.) Sacc.	NBRC9336	*Castanopsis cuspidata* var. *sieboldii*	submerged balsa wood	Japan
Dactylaria sp.	P24	*Cortaderia* sp.	dead leaf	New Zealand
Dactylaria sp.	ICMP14618	*Uncinia* sp.	dead culm	New Zealand
Gaeumannomyces amomi Bussaban	ICMP14650	*Alpinia malaccensis*	healthy pseudostem	Thailand
G. amomi	ICMP14648	*Amomum siamense*	healthy leaf	Thailand
Nakataea fusispora (Matsush.) Matsush.	MUCL39228	*Myricus* sp.	—	Cuba
N. fusispora	MUCL40987	—	decaying leaf	Venezuela
Ochroconis humicola (G.L. Barron & L.V. Busch) de Hoog & Arx	ICMP14434	*Cryptocarya mackionianna*	decaying leaves	Australia
Pyricularia angulata Hashioka	NBRC9625	*Musa sapientum*	rotten leaf	Japan
P. costina Sarbajna	ICMP14436	*Am. siamense*	healthy leaf	Thailand
P. costina	ICMP14437	*Al. malaccensis*	healthy leaf	Thailand
P. costina	ICMP14609	*Al. malccensis*	leaf spot	Thailand
P. higginsii Luttr.	ICMP14707	*Microleana avenacea*	leaf spot	New Zealand
P. higginsii	ICMP14620	*M. avenacea*	dead leaf	New Zealand
P. juncicola MacGravie	ICMP14625	*Carex* sp.	dead leaf	New Zealand
P. juncicola	P17	*Uncinia* sp.	dead panicle	New Zealand
P. longispora Bussaban	ICMP14608	*Am. siamense*	healthy leaf	Thailand
P. variabilis Bussaban	ICMP14487	*Am. siamense*	healthy leaf	Thailand
P. zingiberis Nishik.	NBRC9624	*Zingiber mioga*	—	Japan
P. zingiberis	MUCL9449	*Z. officinale*	—	Japan
Pyricularia sp.	ICMP14468	*Stenotaphrum secundatum*	leaf spot	New Zealand
Pyricularia sp.	ICMP14469	*Digitaria sanguinalis*	leaf spot	New Zealand
Pyriculariopsis parasitica (Sacc. & Berl.) M.B. Ellis	MUCL9450	*Phyllachora graminis*	—	USA
Tumularia aquatica (Ingold) Marvanová & Descals	MUCL28096	*Quercus* sp.	—	UK

[a] ICMP: International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; NBRC: National Institute of Technology and Evaluation Biological Resource Center, Osaka, Japan; MUCL: Mycotheque de l' Universite Catholique de Louvain, Belgium.

In this study the phylogenetic relationships among 41 isolates of *Pyricularia* and related genera were determined by analyzing complete sequences of the ITS regions (including 5.8S rRNA gene). The aims were to determine whether the morphological characters used to distinguish between *Dactylaria* and *Pyricularia* are supported by molecular data and also to establish relationships with *Nakataea*, *Pyriculariopsis* and *Tumularia*, whose species originally were described in or transferred to *Pyricularia*. The potential of rDNA sequences in the analysis of anamorph-teleomorph relationships at the generic level or using sequence analysis of rDNA combined with PCR-fingerprinting to prove the connection between an anamorph species and an ascomycete has been demonstrated (Guadet et al 1989, Rehner and Samuels 1994, 1995, Kuhls et al 1997). A further aim of this study, therefore, was to establish whether molecular techniques can determine anamorph-teleomorph relationships of species of *Dactylaria* or *Pyricularia*.

MATERIALS AND METHODS

Fungal isolates and morphology.—Fungal isolates used in this study are listed (TABLE I). They were obtained from culture collections (International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand, ICMP; Mycotheque de l' Université Catholique de Louvain, Louvain-la-Neuve, Belgium, MUCL; National Institute of Technology and Evaluation Biological Resource Center, Osaka, Japan, NBRC) or from case studies (Bussaban et al 2001a, b, c, 2003). These isolates were recovered from healthy plants, dead or decaying plant tissues, or those exhibiting symptoms of leaf blast, leaf spot, or leaf streak. For morphological

study, mounts were prepared in lactophenol, examined with both differential interference phase contrast and bright field phase contrast microscopy.

Extraction of genomic DNA.—Genomic DNA was extracted by a SDS-CTAB (sodium dodecyl sulfate-cetyltrimethylammonium bromide) method (Kim et al 1990). All isolates were grown in Nobles broth (1.2% malt extract) at 22 C for 10 d. Mycelia including conidia were harvested, freeze dried, frozen in liquid nitrogen and ground into a fine powder with a mortar and pestle. About 15 mg of powdered mycelia including conidia were suspended in 1 mL of ice-cold lysis buffer (150 mM NaCl, 50 mM EDTA, 10 mM Tris-HCl, pH 7.4, 30 μg/mL proteinase K), transferred into 1.5 mL Eppendorf tube and kept at 4 C to prevent endonuclease activity during rehydration of the sample. SDS was added to a final concentration of 2%, vortexed and incubated 30 min at 65 C. After centrifugation for 15 min at 14 000 rpm, the supernatant was transferred to a new sterile 1.5 mL Eppendorf tube. The volume of supernatant was measured and the NaCl concentration was adjusted to 1.4 M and one-tenth volume of 10% CTAB buffer (10% CTAB, 500 mM Tris-HCl, 100 mM EDTA, pH 8.0) was added. The solution was thoroughly mixed and incubated 10 min at 65 C. After cooling 2 min at 15 C, an equal volume of chloroform isoamyl alcohol (24 : 1 v/v) was added, thoroughly mixed and the tube was centrifuged 15 min at 14 000 rpm. The extraction was repeated until the interface was clear. The supernatant was removed to a new Eppendorf tube, containing 2 volumes of cold 100% ethanol. After DNA precipitation, the pellet was centrifuged 15 min at 14 000 rpm, 4 C. Then the pellet was washed with 70% ethanol and dried at room temperature. It was resuspended in 100 μL of 0.002% RNase (5 μg/mL) in TE buffer and incubated 1 h at 37 C (Liou and Tzean 1997). The suspension was stored at −20 C pending use for PCR amplification.

PCR amplification and sequencing.—The internal transcribed spacer (ITS) regions 1 and 2, including 5.8S rDNA, were amplified in a 25 μL reaction on a GeneAmp 9700 thermal cycler (Applied Biosystems) under these reaction conditions: 1 μL of template DNA at a 1 : 20 dilution of the DNA extraction, 0.2 mM each dNTP, 0.2 μL of FastTaq (Applied Biosystems), 0.2 μM each of primers ITS1 and ITS4, 2.5 μL of the supplied 10× PCR buffer with $MgCl_2$, and sterile water to bring volume to 25 μL. Thermal cycling was initiated by denaturation at 95 C for 4 min. This was followed by 35 cycles of denaturation at 94 C for 1 min, annealing at 45 C for 1 min, and extension at 72 C for 1 min, with a final extension at 72 C for 7 min. PCR products were analyzed by electrophoresis on 1% agarose gels in TAE buffer (20 mM Tris-Acetate, 1 mM EDTA, pH 8.0) (Sambrook et al 1989) and viewed by staining with ethidium bromide. Residual nucleotides and primers were removed with High Pure PCR Product Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. Amplified products were sequenced with BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) on an ABI PRISM 310 or ABI PRISM 377 automated DNA sequencer. Sequences were determined on both strands with sequencing primers, ITS1 forward and ITS4 reverse (White et al 1990).

DNA sequence alignment and phylogenetic analysis.—Sequences were assembled with Sequencher 3.1.1 for Macintosh (Applied Biosystems). Sequences were submitted to http://bioweb.pasteur.fr/seqanal/clustalw, for multiple alignment with Clistal W 1.82 (Thompson et al 1994) and manually adjusted with GeneDoc 2.6.002 (Nicholas and Nicholas 1997). Calculation of base pair (bp) differences was carried out by pairwise comparison of strains from the alignment. Twenty-six new sequences were deposited in GenBank with accession numbers AY265315-AY265340. Fifteen previously published sequences were obtained from GenBank for inclusion in analyses (TABLE II). Phylogenetic trees were inferred with PAUP*4.0b10 (Swofford 2002). Heuristic searches were performed with the criterion of maximum parsimony (MP) with tree-bisection-reconnection-branch swapping algorithm. Starting trees were obtained via stepwise addition with 100 random sequence input orders. The parsimony tree scores, including tree length and consistency, retention, rescaled consistency and homoplasy indices (TL, CI, RI, RC and HI), also were calculated. The neighbor joining (NJ) method based on a Kimura two-parameter distance measurement also was used to infer a phylogenetic tree. All molecular characters were unordered and given equal weight during analysis. Relative branch support was estimated with 1000 bootstrap replications (Felsenstein 1985) for NJ and MP analyses. Anamorphic Orbiliaceae, *Arthrobotrys amerospora* S. Schenck, W.B. Kendr. & Pramer, *A. musiformis* Drechsler and *Dactylella cylindrospora* (R.C. Cooke) A. Rubner were used to root for phylogenetic tree. DNA sequence alignment and trees were deposited in TreeBase, accession number SN1368.

RESULTS

DNA extraction, sequencing and alignment.—The ITS region (covering ITS1 region, 5.8S gene and ITS2 region) were amplified from all *Pyricularia* and related species, and the sizes of these regions are listed (TABLE II). Boundaries of the ITS1 and ITS2 regions were determined by comparison with published sequences of the ITS region. ITS regions varied in length from 402 to 623 bp. The length of the 5.8S gene was consistent: 157(±1) bp for strains investigated. No sequence variation is detectable within species and low among species within both the genera *Pyricularia* and *Gaeumannomyces*.

Molecular phylogeny.— Of 680 total characters in the aligned sequence data, maximum parsimony

TABLE II. The PCR product size (bp) and GenBank sequence accession numbers of ITS1-5.8S-ITS2 of *Pyricularia* and allied fungi

Genus and species	ITS1	5.8S	ITS2	Total	GenBank accession no.
Arthrobotrys amerospora	214	156	193	563	AF106533
A. musiformis	203	157	167	527	U51948
Dactylaria ampulliformis ICMP3660	151	157	209	517	AY265336
D. appendiculata ICMP14617	220	158	230	608	AY265339
D. dimorphospora	159	157	147	463	U51980
D. lanosa	155	157	162	474	U51979
D. purpurella NBRC9336	242	156	225	623	AY265335
Dactylaria sp. P24	201	157	199	557	AY265332
Dactylaria sp. ICMP14618	220	158	230	608	AY265338
Dactylella cylindrospora	185	156	207	548	AF106538
D. cylindrospora	185	156	183	524	U51953
Gaeumannomyces amomi ICMP14648	142	157	185	484	AY265318
G. amomi ICMP14650	140	157	187	484	AY265317
G. caricis	164	157	174	495	AJ010030
G. cylindrosporus	174	157	174	505	AJ010029
G. cylindrosporus	175	157	174	506	U17211
G. graminis	137	157	172	466	AJ010034
G. graminis var. *tritici*	156	157	171	484	AF087684
Magnaporthe grisea	124	157	174	455	U17329
M. grisea	124	157	174	455	U17328
Nakataea fusispora MUCL39228	64	157	184	405	AY265330
N. fusispora MUCL40987	64	157	181	402	AY265331
Ochroconis humicola ICMP14434	248	156	214	618	AY265334
Phialophora graminicola	175	157	174	506	U17218
Pyricularia angulata NBRC9625	113	157	231	501	AY265322
P. costina ICMP14436	128	157	191	476	AY265327
P. costina ICMP14437	125	157	192	474	AY265328
P. costina ICMP14609	159	157	228	544	AY265329
P. higginsii ICMP14707	153	157	225	535	AY265326
P. higginsii ICMP14620	149	157	219	525	AY265325
P. juncicola ICMP14625	151	157	225	533	AY265320
P. juncicola P17	152	157	225	534	AY265321
P. longispora ICMP14608	195	157	186	538	AY265319
P. variabilis ICMP14487	180	157	182	519	AY265333
P. zingiberis MUCL9449	133	157	193	483	AY265315
P. zingiberis NBRC9624	129	157	186	472	AY265316
Pyricularia sp. ICMP14468	154	157	232	543	AY265323
Pyricularia sp. ICMP14469	157	157	236	550	AY265324
Pyriculariopsis parasitica MUCL9450	228	158	194	580	AY265340
Tumularia aquatica MUCL28096	165	157	208	530	AY265337
T. aquatica	134	157	149	440	AY148101

analysis was conducted for 442 potentially phylogenetically informative characters. Forty sites ambiguously aligned were excluded from the analysis to avoid fragmentary ambiguities. A total of 90 equally most parsimonious trees (TL = 1967, CI = 0.519, RI = 0.698, RC = 0.370 and HI = 0.481) were obtained and compared for the best topology with the Kishino-Hasegawa test (FIG. 1). Those parsimony informative characters in the alignment also were analyzed by means of the NJ method with the Kimura two-parameter distance measurement, assuming equal base frequencies of entire sequences across taxa, and unequal transition to transversion ratio. Supports for grouping in NJ trees were evaluated with 1000 bootstrap replications, which produced a similar tree topology, giving high bootstrap values for the relevant clades.

In the parsimony and distance analyses, most taxa were sorted into a large cluster, belonging to the

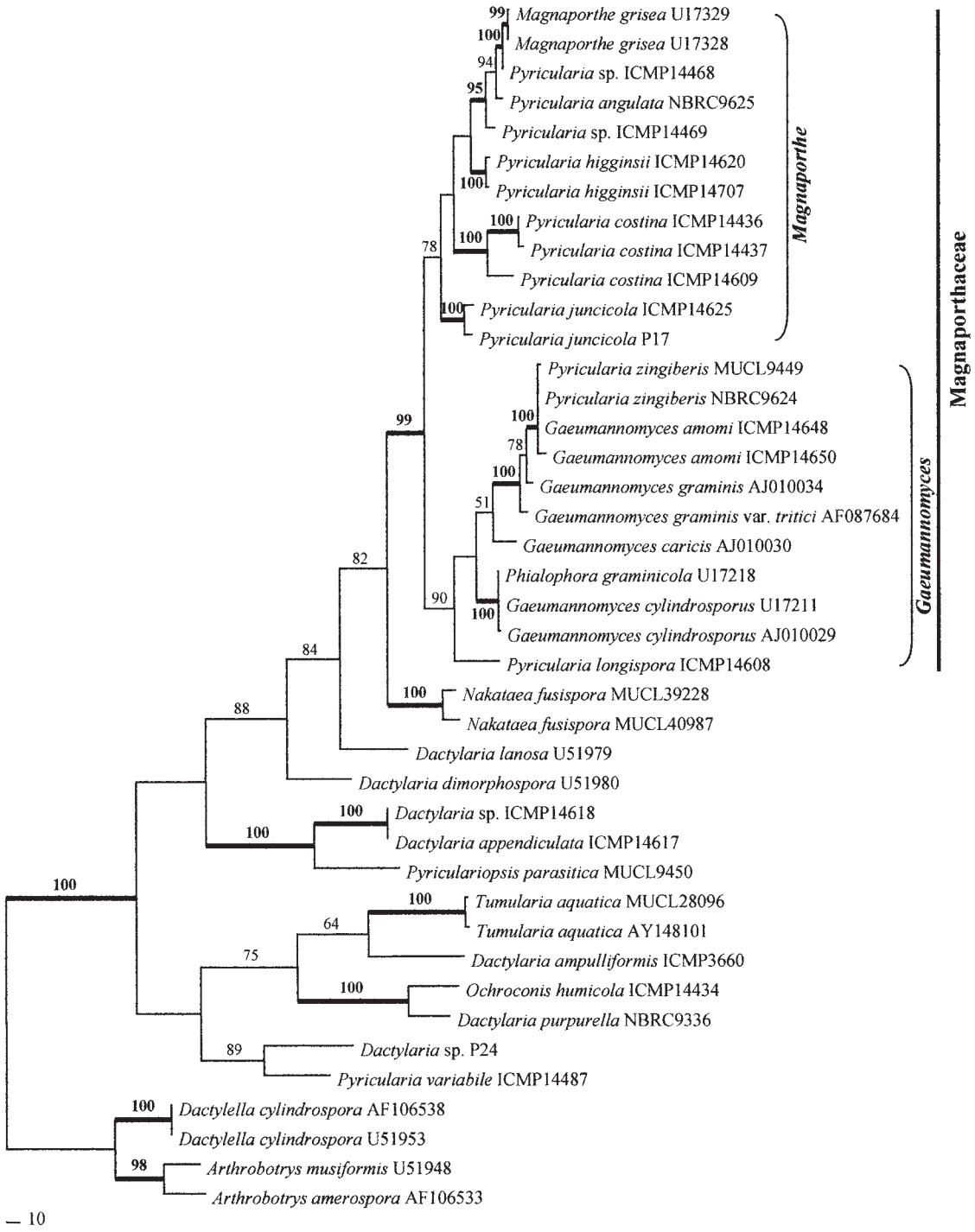

FIG. 1. One of 90 equally most parsimonious trees inferred from a heuristic search of the ITS1-5.8S-ITS2 rDNA sequence alignment of 41 isolates of *Pyricularia* and related genera. *Dactylella* and *Arthrobotrys* were used to root the tree. The size of the branches is indicated with a scale bar. The bootstrap values representing 1000 bootstrap replications are given (when more than 50%) above or below the branches. Branches ≥95% are strongly supported (Felsenstein 1985) and are shown in boldface.

family Magnaporthaceae with high bootstrap support (MP = 99%, FIG. 1), while the remaining taxa were basal to this group. With the exception of *P. variabilis* Bussaban, all *Pyricularia* species studied fell within the Magnaporthaceae. In the MP tree *Nakataea fusispora* (Matsush.) Matsush. formed a sister taxon of the Magnaporthaceae with 82% bootstrap support (FIG. 1). The Magnaporthaceae comprises two sister taxa corresponding to the teleomorph genera *Magnaporthe* and *Gaeumannomyces*. The *Magnaporthe*

clade included the type of the genus, *M. grisea* and the anamorphic species, *P. angulata* Hashioka, *P. costina* Sarbajna, *Pyricularia higginsii* Luttr., *P. juncicola* MacGarvie and *Pyricularia* sp. (ICMP14468, ICMP14469). The *Gaeumannomyces* clade comprised four *Gaeumannomyces* species, *Phialophora graminicola* (Deacon) J. Walker, *Pyricularia zingiberis* Nishik. and *P. longispora* Bussaban with 90% bootstrap support. Also in this clade, *Gaeumannomyces amomi* Bussaban and *Pyricularia zingiberis* isolates from Zingiberaceae plants were related closely with 100% bootstrap support. The *Gaeumannomyces* isolates from grass also were related closely to the Zingiberaceae isolates. *Gaeumannomyces cylindrosporus* D. Hornby, Slope, Gutter. & Sivan. and *Phialophora graminicola* clustered with 100% bootstrap support, and *Pyricularia longispora* occurred separately.

The remaining taxa were related distantly to the Magnaporthaceae. In the MP analysis *Pyriculariopsis parasitica* (Sacc. & Berl.) M.B. Ellis formed a closely related cluster (100% bootstrap support) with *Dactylaria appendiculata* Cazau, Aramb. & Cabello and *Dactylaria* sp. ICMP14618. *Tumularia aquatica* (Ingold) Marvanová & Descals and *D. ampulliformis* (Tubaki) G.C. Bhatt & W.B. Kendr. are clustered but with low bootstrap support (64%). *Ochroconis humicola* (G.L. Barron & L.V. Busch) de Hoog & von Arx and *D. purpurella* (Sacc.) Sacc. are clustered with 100% bootstrap support and formed a sister cluster of this former cluster with 75% bootstrap support. *Pyricularia variabilis* seems to be a taxon different from the other *Pyricularia* species studied. This species clustered with *Dactylaria* sp. isolate P24 (89% bootstrap support). They showed 24.4% of sequence variation and formed a sister group of members including the type species of *Dactylaria*, *D. purpurella*. However this branch lacked bootstrap support. Similar clusters resulted in the NJ tree but with differing bootstrap support.

DISCUSSION

Molecular phylogeny and relationships of Pyricularia and related genera.—Species of the anamorphic fungus *Pyricularia* are typically plant pathogens (e.g., *P. oryzae* is a serious rice blast pathogen (Ou 1987)). *Pyricularia grisea* is the cause of gray leaf spot of St Augustine grass (Malca and Owen 1957). Other *Pyricularia* species cause diseases on members of Cannaceae, Commelinaceae, Marantaceae, Musaceae and Zingiberaceae (Meredith 1963; Asuyama 1965; Hashioka 1971, 1973; Kotani and Kurata 1992; Pappas and Paplomatas 1998). *Pyricularia penniseti* Prasada & Goyal and *P. setariae* Nishik., morphologically similar pathogens, have been reported on cereals and grasses (Nishikado 1917, Sprague 1950, Malca and Owen 1957, Bailey and van Eijnatten 1961, Asuyama 1965, Wells et al 1969, Prasada and Goyal 1970). *Pyricularia zingiberis*, *P. costina*, *P. curcumae* Rathaiah and *P. distorta* Hashioka are pathogenic on Zingiberaceae (Nishikado 1917, Hashioka 1971, Rathaiah 1980, Sarbajna 1990). Bussaban et al (2001a, b, 2003) reported several *Pyricularia* species, including *P. costina*, living as endophytes in healthy wild ginger.

The criterion used by Ellis (1976) for separating *Pyricularia* from *Dactylaria* is that the conidia of *Pyricularia* secede in a rhexolytic manner, with the denticle acting as a separating cell, and with a protruding hilum on the conidia, whereas those of *Dactylaria* secede in a schizolytic manner. However subsequent revisions of *Dactylaria* (de Hoog 1985, Goh and Hyde 1997) are not consistent with this method of conidiogenesis in *Dactylaria*. Two species originally described in *Pyricularia* (*P. higginsii* Luttr. and *P. juncicola* MacGarvie) that secede in a schizolytic manner, were transferred to *Dactylaria* (Ellis 1976). The name *Dactylaria juncicola* was occupied already by a different fungus, *D. juncicola* (MacGarvie) G.C. Bhatt & W.B. Kendr., thus Ellis (1976) proposed the new name *D. junci*. *Nakataea fusispora* also was transferred to *Pyricularia fusispora* because of its rhexolytic conidial secession (Zucconi et al 1984). However the phylograms inferred from ITS sequence data presented here did not disclose any consistent correlation with the type of conidial secession. Nonetheless it did reveal an interesting correlation between this clade and conidial morphology. With the exception *of P. variabilis*, all *Pyricularia* species studied including two species of *Pyricularia* (*P. higginsii* and *P. juncicola*, previously renamed *Dactylaria*) that have obpyriform conidia were grouped within the family Magnaporthaceae with high bootstrap support. This suggested the clade might represent a monophyletic lineage of species with obpyriform conidia. Following this conidial morphology criterion, the originally named *Pyricularia higginsii* and *P. juncicola* therefore should be maintained in *Pyricularia*. Likewise *Nakataea fusispora* also should be maintained in *Nakataea* because this species has distinctive, verrucose, fusiform conidia and it formed a sister cluster of members in Magnaporthaceae.

Pyricularia variabilis was the only species of *Pyricularia* studied that did not group in the Magnaporthaceae. This taxon has swollen, terminal and intercalary nodes on the conidiophores, and variously shaped conidia. Analyses of ITS sequence data showed that *P. variabilis* was unrelated phylogenetically to the other *Pyricularia* species studied but

more closely related to *Dactylaria*, *Tumularia* or *Ochroconis* species. Further work is needed to establish the appropriate placement of this taxon and determine its relationships.

Our results indicated that the rest of *Dactylaria* and species of *Pyriculariopsis*, *Tumularia* and *Ochroconis* were distinct from the Magnaporthaceae and that the genus *Dactylaria* is polyphyletic. *Dactylaria* is a form genus and includes species with many conidia born at the apical region of conidiophores on cylindrical or tapering denticles. Conidia, however, are shaped variously and therefore taxa may be unrelated. Furthermore this type of conidiogenesis might have evolved more than once. Our results support this conjecture.

Dactylaria purpurella is the type species of *Dactylaria* and has short conidiophores with cylindrical or tapering denticles bearing navicular conidia. In the ITS sequence analysis it was clustered with *Ochroconis humicola* with 100% BS. *Ochroconis humicola* was described originally as a species of *Scolecobasidium* and subsequently included in *Ochroconis* (de Hoog and von Arx 1973). *Ochroconis* and *Scolecobasidium* are members of the *Dactylaria* complex characterized by rhexolytic conidium secession and pale brown conidia. Most species of *Ochroconis* have ellipsoidal, clavate or fusiform conidia, while *Scolecobasidium* species have trilobate conidia (de Hoog and von Arx 1973, de Hoog 1985). The other species of *Dactylaria* appear to be unrelated to *D. purpurella* and further work is needed to understand this genus complex.

Tumularia aquatica and *Pyriculariopsis parasitica* originally were described in *Pyricularia* and later accommodated in newly introduced genera (Ingold 1943, Hughes 1958, Ellis 1971, Marvanová and Descals 1987). *Tumularia aquatica* differs from *Pyricularia* in lacking denticles and having lemon-shaped conidia. *Pyriculariopsis parasitica* differs in having straight or curved, obclavate and rostrate conidia. The exclusion of these taxa from *Pyricularia*, therefore, is justified and supported by morphological and molecular data.

Anamorph-teleomorph connections.—Anamorphic fungi that have not been linked to any teleomorphs make up a large proportion of known fungi. The inability to identify such links lies in inherent difficulties in experimentally proving anamorph-teleomorph connections and the fact that many fungi will not sporulate in culture. The present classification system for anamorphic genera therefore uses three categories of information to identify taxa (Kirk et al 2001): conidiomatal types, Saccardo's spore groups and conidiogenous events. Relationships suggested by such information, however, do not necessarily reflect evolutionary relationships (Hawksworth et al 1995), and the need for identifying relationships of anamorphs with their teleomorphs with molecular techniques has been advocated by Rossman et al (2001). *Dactylaria* and *Pyricularia* have similar types of conidiogenesis and spore types and are hyphomycetes (Ellis 1976). Species of *Pyricularia* have been characterized by morphological, physiological or molecular information (Ellis 1971, 1976; Matsuyama et al 1977; Kato et al 2000; Couch and Kohn 2002) and have been linked to *Magnaporthe* teleomorphs (Hebert 1971, Kato et al 1976). Species of *Dactylaria* differ from *Pyricularia* in the absence of a separating cell in the denticles, and the conidia also are of various shapes, usually fusiform, naviculate, or more or less cylindrical (Ellis 1976). Teleomorphs of *Dactylaria* species have not been reported commonly. Carmichael et al (1980) mentioned the occurrence of an anamorph for *Acrospermum compressum* Tode. This anamorph bears some similarity to *Subulispora minima* P.M. Kirk and to *Dactylaria graminicola* Årsvoll. Sivichai et al (2002) reported a teleomorph-anamorph connection between an unidentified teleomorph and *Dactylaria*.

Carbone and Kohn (1993) demonstrated the confirmations of anamorph-teleomorph connection by comparative sequence analysis of amplified products of *Sclerotinia* and *Sclerotium*, which showed 98% sequence homology in the ITS region of rDNA. Kuhls et al (1997) established the connection between *Trichoderma* anamorphs and *Hypocrea* teleomorphs where five *Trichoderma-Hypocrea* connections were supported by 100% identity in ITS1 and ITS2 sequences. Egger and Sigler (1993) investigated the ex type strains of the anamorph *Scytalidium vaccinii* Dalpé, Litten & Sigler and the ascomycete *Hymenoscyphus ericae* (D.J. Read) Korf & Kernan. They found 1.2–3.5% divergence in the ITS1 and ITS2 regions and concluded from these data and morphological observations that *S. vaccinii* and *H. ericae* are anamorph and teleomorph of a single taxon. In our study phylogenies showed *Pyricularia zingiberis* and *Geaumannomyces amomi* isolated from Zingiberaceae plants were grouped strongly and closely related to other *Gaeumannomyces* species from grasses. Our isolates from Zingiberaceae contained up to six nucleotide differences in the entire ITS sequences, while nucleotide sequences of *P. zingiberis* isolate NBRC9624 and *G. amomi* isolate ICMP14648 were identical. Geographical separation or host specialization (TABLE I) could be an explanation for the nucleotide sequence differences present in *P. zingiberis* from Japan, isolates MUCL9449 (*Zingiber*

officinale Rosc.) and NBRC9624 (*Zingiber mioga* Rosc.), in comparison with *G. amomi* isolates ICMP14650 (*Alpinia malaccensis* (Burm.) Rosc.) and ICMP14648 (*Amomum siamense* Craib.) from Thailand. However information from a larger number of isolates is required to confirm this. According to ITS sequences, and a distinct morphological character of sickle-shaped conidia, *Harpophora* W. Gams, a genus comprising phialidic anamorphs of the Magnaporthaceae was introduced with *H. radicicola* (Cain) W. Gams (=*Phialophora radicicola* Cain) as type (Ward and Bateman 1999, Gams 2000). A connection between *H. graminicola* (Deacon) W. Gams (=*P. graminicola* (Deacon) J. Walker) and *Gaeumannomyces cylindrosporus* also was supported by ITS sequences similarity (Walker 1980, Bryan et al 1995). Likewise Couch and Kohn (2002) extracted DNA directly from freeze dried perithecia of *Magnaporthe* and mycelia of anamorphic *Pyricularia* isolates and the result, based on three genes (actin, beta-tubulin and calmodulin), supported the anamorph-teleomorph connection demonstrated by Hebert (1971) and Yaegashi (1977). Our results are consistent with the possibility that heterogenous *P. zingiberis* might be the anamorph state of *Gaeumannomyces amomi*.

Molecular studies have suggested that *Pyricularia* spp. isolated from different hosts are genetically distinct (Borromeo et al 1993, Shull and Hamer 1994, Kato et al 2000, Couch and Kohn 2002, Goodwin et al 2003) or provided information on the genetic diversity among different population of rice blast fungi, *Pyricularia grisea* or *P. oryzae* (Lebrun et al 1991, Levy et al 1991, Zhu et al 1992, Chen et al 1995, George et al 1998). In our study the combination of morphological characters (e.g., spore morphology) and molecular characters (ITS ribosomal DNA sequences data) may confidently let us distinguish *Pyricularia* from *Dactylaria* species, especially if sexual structures are not readily produced in culture. We conclude that conidial shape can be used as a primary character to distinguish *Pyricularia* species from related genera such as *Dactylaria*.

ACKNOWLEDGMENTS

This work was supported by The Thailand Research Fund (Royal Golden Jubilee Ph.D. Program 4B4M/43/D1, BGJ 4680011) and The Biodiversity and Training Program (BRT 142006). International Collection of Microorganisms from Plants (ICMP), National Institute of Technology and Evaluation Biological Resource Center (NBRC). The Mycotheque de l' Universite Catholique de Louvain-la-Neuve (MUCL) and B. Paulus provided some isolates of Pyricularia and related genera. J.M. Young, M. Fletcher and R. Kodsueb are thanked for culture arrangement. Landcare Research, Auckland provided facilities and resources for B. Bussaban to carry out much of this work.

LITERATURE CITED

Asuyama H. 1965. Morphology, taxonomy, host range, and life cycle of *Piricularia oryzae*. In: Chandler RF Jr., ed. The rice blast disease. USA: Johns Hopkins Press. p 9–22.

Bailey AG, van Eijnatten C. 1961. Corn gray spot caused by *Pyricularia grisea*. Phytopathology 51:197–198.

Borromeo ES, Nelson RJ, Bonman JM, Leung H. 1993. Genetic differentiation among isolates of *Pyricularia* infecting rice and weed hosts. Phytopathology 83: 393–399.

Bruns TD, White TJ, Taylor JW. 1991. Fungal molecular systematics. Ann Rev Ecol Syst 22:525–564.

Bryan GT, Daniels MJ, Osbourn AE. 1995. Comparison of fungi within the *Gaeumannomyces-Phialophora* complex by analysis of ribosomal DNA sequences. Appl Environm Microb 61:681–689.

Bussaban B, Lumyong S, Lumyong P, McKenzie EHC, Hyde KD. 2001a. Endophytic fungi from *Amomum siamense*. Canad J Microbiol 47:943–948.

———, ———, ———, ———, ———. 2001b. Endophytic fungi from Zingiberaceae: *Alpinia malaccensis*. Phytopathology 91:S104. Publication P-2001-0023-MSA.

———, ———, ———, ———, ———. 2001c. Two new species of endophytes (ascomycetes) from Zingiberaceae sporulating in culture. Nova Hedw 73:487–493.

———, ———, ———, ———, ———. 2003. Three new species of *Pyricularia* are isolated as Zingiberaceous endophytes from Thailand. Mycologia 95:521–526.

Cai L, Tsui CKM, Zhang K, Hyde KD. 2002. Aquatic fungi from Lake Fuxian, Yunnan, China. Fungal Diversity 9:57–70.

Carbone I, Kohn LM. 1993. Ribosomal DNA sequence divergence within internal transcribed spacers 1 of the Sclerotiniaceae. Mycologia 85:415–427.

Carmichael JW, Kendrick WB, Conners IL, Sigler L. 1980. Genera of Hyphomycetes. Alberta, Canada: University of Alberta Press. 386 p.

Cavara F. 1892. Contributzione alla micologia lombarda. Atti Ist Bot Univer Lab Crittogam Pavia Ser 2, 2:207–292.

Chen D, Zeigler RS, Leung H, Nelson RJ. 1995. Population structure of *Pyricularia grisea* at two screening sites in the Philippines. Phytopathology 85:1011–1020.

Couch BC, Kohn LM. 2002. A multilocus gene genealogy concordant with host preference indicates segregation of a new species, *Magnaporthe oryzae*, from *M. grisea*. Mycologia 94:683–693.

de Hoog GS. 1985. Taxonomy of the *Dactylaria* complex, IV. *Dactylaria*, *Neta*, *Subulispora* and *Scolecobasidium*. Stud Mycol 26:1–60.

———, von Arx JA. 1973. Revision of *Scolecobasidium* and *Pleurophragmium*. Kavaka 1:55–60.

Egger KN, Sigler L. 1993. Relatedness of the ericoid endophytes *Scytalidium vaccinii* and *Hymenoscyphus*

ericae inferred from analysis of ribosomal DNA. Mycologia 85:219–230.

Ellis MB. 1971. Dematiaceous hyphomycetes. Kew, England: Commonwealth Mycological Institute. 680 p.

———. 1976. More dematiaceous hyphomycetes. Kew, England: Commonwealth Mycological Institute. 507 p.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791.

Gams W. 2000. *Phialophora* and some similar morphologically little-differentiated anamorphs of divergent ascomycetes. Stud Mycol 45:187–199.

George MLC, Nelson RJ, Zeigler RS, Leung H. 1998. Rapid Population analysis of *Magnaporthe grisea* by using rep-PCR and endogenous repetitive DNA sequences. Phytopathology 88:223–229.

Goh TK, Hyde KD. 1997. A revision of *Dactylaria*, with description of *D. tunicata* sp. nov. from submerged wood in Australia. Mycol Res 101:1265–1272.

Goodwin SB, Levy M, CavalettoJR, Tian Y. 2003. Phylogenetic analyses place *Magnaporthe grisea* in the order Diaporthales and suggest rapid speciation. 8th International Congress of Plant Pathology. Christchurch, New Zealand, Abstracts, vol vol. 2, p 349.

Guadet J, Julien J, Lafay JF, Brygoo Y. 1989. Phylogeny of some *Fusarium* species as determined by large-subunit rRNA sequence comparison. Mol Biol Evol 6:227–242.

Hashioka Y. 1971. Notes on *Pyricularia* I. Three species parasitic to Musaceae, Cannaceae and Zingiberaceae. Trans Mycol Soc Japan 12:126–135.

———. 1973. Notes on *Pyricularia* II. Four species and one variety parasitic to Cyperaceae, Gramineae and Commelinaceae. Trans Mycol Soc Japan 14:256–265.

Hawksworth DL, Kirk MP, Sutton BC, Pegler DN. 1995. Dictionary of the fungi. 8th ed. Surrey, England: CAB International. 616 p.

Hebert TT. 1971. The perfect stage of *Pyricularia grisea*. Phytopathology 61:83–87.

Ho WH, Yanna, Hyde KD, Hodgkiss IJ. 2002. Seasonality and sequential occurrence of fungi on wood submerged in Tai Po Kau Forest Stream, Hong Kong. Fungal Diversity 10:21–43.

Hughes SJ. 1958. Revisiones hyphomycetum aliquot cum appendice de nominibus rejiciendis. Can J Bot 36:727–836.

Ingold CT. 1943. Further observations on aquatic hyphomycetes of decaying leaves. Trans Brit Mycol Soc 26: 105–115.

Kato H, Yamaguchi T, Nishihara N. 1976. The perfect stage of *Pyricularia oryzae* Cav. in culture. Ann Phytopath Soc Japan 42:507–510.

———, Yamamoto M, Yamaguchi-Ozaki T, Kadouchi H, Iwamoto Y, Nakayashiki H, Tosa Y, Mayama S, Mori N. 2000. Pathogenicity, mating ability and DNA restriction fragment length polymorphisms of *Pyricularia* populations isolated from Gramineae, Bambusideae and Zingiberaceae plants. J Gen Plant Pathol 66:30–47.

Kim WK, Mauthe W, Hausner G, Klassen GR. 1990. Isolation of high molecular weight DNA and double-stranded RNAs from fungi. Can J Bot 68:1898–1902.

Kirk PM, Cannon PF, David JC, Stalpers JA. 2001. Dictionary of the fungi. 9th ed. Surrey, England: CAB International. 655 p.

Kotani S, Kurata M. 1992. Black blotch of rhizome by *Pyricularia zingiberi* Nishikado. Ann Phytopath Soc Japan 58:469–472.

Kuhls K, Lieckfeldt E, Samuels GJ, Meyer W, Kubicek CP, Börner T. 1997. Revision of *Trichoderma* sect. *Longibrachiatum* including related teleomorphs based on analysis of ribosomal DNA internal transcribed spacer sequences. Mycologia 89:442–460.

Lebrun MH, Capy MP, Garcia N, Dutertre M, Brygoo Y, Notteghem JL, Vales M. 1991. Biology and genetics of *Pyricularia oryzae* and *P grisea* populations: current situation and development of RFLP markers. In: Rice genetics 2. Proceedings of the Second International Rice Genetics Symposium. IRRI. Los Banos, Laguna, Philippines, p 487–497.

Levy M, Ramao J, Marchetti MA, Hamer JE. 1991. DNA fingerprinting with a dispersed repeated sequence resolves pathotype diversity in the rice blast fungus. Plant Cell 3:95–102.

Liou GY, Tzean SS. 1997. Phylogeny of the genus *Arthrobotrys* and allied nematode-trapping fungi based on rDNA sequences. Mycologia 89:876–884.

Luo J, Yin JF, Cai L, Zhang KQ, Hyde KD. 2004. Freshwater fungi in Lake Dianchi, a heavily polluted lake in Yunnan, China. Fungal Diversity 16:93–112.

Malca MI, Owen JH. 1957. The gray leaf spot disease of St Augustine grass. Plant Dis Rep 41:874–875.

Marvanová L, Descals E. 1987. New taxa and new combinations of ''aquatic hyphomycetes''. Trans Brit Mycol Soc 89:499–507.

Matsuyama N, Kato H, Yamaguchi T. 1977. Comparison of the isozyme patterns of extracellular enzymes in *Pyricularia* strains from gramineous and zingiberaceous plants. Ann Phytopath Soc Japan 43:419–425.

Meredith DS. 1963. *Pyricularia grisea* (Cooke) Sacc. causing pitting disease of bananas in central America. I. Preliminary studies on pathogenicity. Ann Appl Biol 52:453–463.

Nicholas KB, Nicholas HB Jr. 1997. GeneDoc: a tool for editing and annotating multiple sequence alignments. http://www.psc.edu/biomed/genedoc

Nishikado Y. 1917. Studies on the rice blast fungus. I. Ber Ohara Inst Landwirtsch Forsch 1:171–217.

Ou SH. 1987. Rice diseases. 2nd ed. Kew, England: CAB International. 380 p.

Pappas AC, Paplomatas EJ. 1998. *Pyricularia* leaf spot: a new disease of ornamental plants of the family Marantaceae. Plant Dis 82:465–469.

Paulus B, Gadek P, Hyde KD. 2003. Two new species of *Dactylaria* (anamorphic fungi) and an update of species in *Dactylaria* sensu lato. Fungal Diversity 14:157–170.

Prasada R, Goyal JP. 1970. A new species of *Pyricuaria* on Bajra. Curr Sci 39:287–288.

Rathaiah Y. 1980. Leaf blast of turmeric. Plant Dis 64: 104–105.

Rehner SR, Samuels GJ. 1994. Taxonomy and phylogeny of

Gliocladium analyzed by large subunit rDNA sequences. Mycol Res 98:625–634.

———, ———. 1995. Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. Can J Bot 73:S816–S823.

Rossman AY, Howard RJ, Valent B. 1990. *Pyricularia grisea*, the correct name for the rice blast disease fungus. Mycologia 82:509–512.

———, Farr DF, Castlebury LA. 2001. Phylogenetic relationships among obscure microfungi: Why does it matter? Phytopathology 91:S164. Publication P-2001-0119-MSA.

Saccardo PA. 1880. Fungorum extra-europaeorum Pugillus. Michelia 2:136–149.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. 2nd ed. New York, USA: Cold Spring Harbor Press. 1592 p.

Sarbajna KK. 1990. New species of *Mycovellosiella* and *Pyricularia* from West Bengal. J Mycopath Res 28: 159–164.

Shull V, Hamer JE. 1994. Genomic structure and variability in *Pyricularia grisea*. In: Zeigler R, Leong SA, Teng PS.., eds. Rice blast disease. Wallingford, England: CAB International. p 65–86.

Sivichai S, Jones EBG, Jones NLH. 2002. Lignicolous freshwater higher fungi with reference to their teleomorph and anamorph stages. In: Watling R, Frankland JC, Ainsworth AM, Isaac S, Robinson CH. eds. Tropical mycology. Wallingford, England: CAB International. p 41–49.

Sprague R. 1950. Disease of cereals and grasses in North America. New York, USA: Ronald Press. 538 p.

Swofford DL. 2002. PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods), beta version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nuc Acids Res 22:4673–4680.

Walker J. 1980. *Gaeumannomyces*, *Linocarpon*, *Ophiobolus* and several other genera of scolecospored ascomycetes and phialophora conidial states, with a note on hyphopodia. Mycotaxon 11:1–129.

Ward E, Bateman GL. 1999. Comparison of *Gaeumannomyces*- and *Phialophora*-like fungal pathogens from maize and other plants using DNA methods. New Phytol 141:323–331.

Wells HD, Burton GW, Powell JB. 1969. *Piricularia* leaf spot of pearl millet. Phytopathology 59:1057.

White TJ, Bruns TD, Lee S, Taylaor JW. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ., eds. PCR protocols: a guide to methods and applications. San Diego: Academic Press. p 315–322.

Yaegashi H. 1977. On the sexuality of blast fungi, *Pyricularia* spp. Ann Phytopath Soc Japan 43:432–439.

Zhu H, Jiang W, Wang J, Lei C, Ling Z, Zhu L. 1992. DNA fingerprint and pathotype characterization of *Pyricularia oryzae* isolates. Mycosystema 5:89–98.

Zucconi L, Onofri S, Persiani AM. 1984. Rare or interesting hyphomycetes from tropical forest litter. II. *Pyricularia fusispora* comb. nov., a new species combination for the species *Nakataea fusispora*. Mic Ital 2:7–10.