CHAPTER 6

MOLECULAR AND MORPHOLOGICAL IDENTIFICATION

A. *Pyricularia*

6.1A Introduction

The genus *Pyricularia* (Cooke) Sacc. (anamorphic Magnaporthaceae) was established by Saccardo (1880) with the type species, *P. grisea* (Cooke) Sacc., which was originally described from crabgrass (*Digitaria sanguinalis* L.). The name "pyricularia" refers to the pyriform shape of the conidia. Subsequently, Cavara (1892) described *P. oryzae* Cav. from rice (*Oryza sativa* L.), a taxon with very 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 synonymised with *P. grisea* and grouped these two anamorphs under the teleomorph *Magnaporthe grisea* (Hebert) Barr. Recent molecular genetic analyses have, however, 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 *Pyricularia* isolates from *Digitaria* 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 *Digitaria*).

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 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 are usually cut off by a septum to form a separating cell (rhexolytic secession, Figure 6.1) and the conidia are mostly obpyriform. In *Dactylaria* there is no separating cell in the denticles (schizolytic secession, Figure 6.1) 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.*, 2003b; Paulus *et al.*, 2003; Luo *et al.*, 2004).

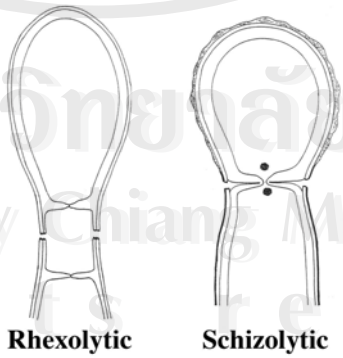

Figure 6.1 Conidial secession: rhexolytic (involving the circumscissile splitting of the periclinal wall of the cell below the basal conidial septum rather than the septum itself) and schizolytic (involving a splitting of delimiting septum).

Analysis of ribosomal DNA has frequently been used in mycological investigations (Bruns *et al.*, 1991; Hibbett, 1992; Lee and Taylor, 1992; Li, 1997; Okane *et al*., 2003; Pandey *et al*., 2003; Tomita, 2003; Menkis *et al*., 2004; Rodrigues *et al*., 2004). In the present 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 establish relationships with *Nakataea*, *Pyriculariopsis* and *Tumularia*, genera with species originally 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 by various authors (Guadet *et al.*, 1989; Rehner and Samuels, 1994, 1995; Kuhls *et al.*, 1997). A further aim of this study was to establish if molecular techniques can determine anamorph-teleomorph relationships of species of *Dactylaria* or *Pyricularia*.

6.2A Materials and methods

6.2.1A Fungal isolates and morphology

Fungal isolates used in this study are listed in Table 6.1. 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

Table 6.1 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 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 leaf	Australia
Pyricularia angulata Hashioka	NBRC9625	*Musa sapientum*	rotten leaf	Japan
P. costina Sarbajna	ICMP14436	*Amomum siamense*	healthy leaf	Thailand
P. costina	ICMP14437	*Alpinia malaccensis*	healthy leaf	Thailand
P. costina	ICMP14609	*Alpinia malaccensis*	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	*Amomum siamense*	healthy leaf	Thailand
P. variabilis Bussaban	ICMP14487	*Amomum 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

during the present studies (Chapters 3–5). The fungi were recovered as endophytes, as saprobes on dead or decaying plant tissues, or as parasites from plants with symptoms of leaf blast, leaf spot, or leaf streak. For morphological study, mounts were prepared in lactophenol, examined using both differential interference phase contrast and bright-field phase contrast microscopy as previously described (Chapters 3–5).

6.2.2A Extraction of genomic DNA

Genomic DNA was extracted by an 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 days. 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 at 65 °C for 30 min. 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 then adjusted to 1.4 M, and 1/10 volume of 10% CTAB buffer (10% CTAB, 500 mM Tris-HCl, 100 mM EDTA, pH 8.0) was added. The solution was then thoroughly mixed and incubated at 65 °C for 10 min. After cooling at 15 °C for 2 min, an equal volume of chloroform isoamyl alcohol (24:1 v/v) was added, thoroughly mixed, and the tube was centrifuged at 14,000 rpm for 15 min. 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 for 15 min at 14,000 rpm, at 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 at 37 °C for 1 h (Liou and Tzean, 1997). The suspension was stored at –20 °C pending use for PCR amplification.

6.2.3A 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 the following 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 visualized by staining with ethidium bromide. Residual nucleotides and primers were removed using High Pure PCR Product Kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The 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 using sequencing primers, ITS1 forward and ITS4 reverse (White *et al.*, 1990).

6.2.4A DNA sequence alignment and phylogenetic analysis

Sequences were assembled using Sequencher 3.1.1 for Macintosh (Applied Biosystems). Sequences were submitted to http://bioweb.pasteur.fr/seqanal/clustalw, for multiple alignment using CLUSTAL W 1.82 (Thompson *et al.*, 1994), and manually adjusted using 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 6.2). Phylogenetic trees were inferred using PAUP*4.0b10 (Swofford, 2002). Heuristic searches were performed using 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, consistency index, retention index, rescaled consistency index, and homoplasy index (TL, CI, RI, RC and HI) were also 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.

6.3A Results

6.3.1A 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 in Table 6.2. 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 very consistent: 157 (±1) bp for strains investigated. No sequence variation is detectable within species, and is low among species within both the genera *Pyricularia* and *Gaeumannomyces*.

6.3.2A Molecular phylogeny

Of 680 total characters in the aligned sequence data, maximum parsimony 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 using the Kishino-Hasegawa test (Figure 6.2). Those parsimony-informative characters in the alignment were also analyzed by means of the NJ method using 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 family Magnaporthaceae with high bootstrap support (MP=99%, Figure 6.1), 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 to the Magnaporthaceae with 82% bootstrap support (Figure 6.1). The Magnaporthaceae is comprised of 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, *P. higginsii* Luttr., *P. juncicola* MacGarvie and *Pyricularia* sp. (ICMP14468, ICMP14469). The *Gaeumannomyces* clade is comprised of four *Gaeumannomyces* species, *Harpophora graminicola* (Deacon) W. Gams, *Pyricularia zingiberis* Nishik., and *P. longispora* Bussaban with 90% bootstrap support. *Gaeumannomyces amomi* Bussaban and *Pyricularia zingiberis* isolates from zingiberaceous plants were closely related with 100% bootstrap support. The *Gaeumannomyces* isolates from grass were also closely related to the zingiberaceous isolates. *Gaeumannomyces cylindrosporus* D. Hornby, Slope, Gutter. & Sivan. and *Phialophora graminicola* clustered with 100% bootstrap support.

Table 6.2 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
Hapophora 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

Table 6.2 (Continued).

Genus and species	ITS1	5.8S	ITS2	Total	GenBank accession no.
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

The remaining taxa were distantly related 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 & Arx and *D. purpurella* (Sacc.) Sacc. are clustered with 100% bootstrap support and formed a sister cluster to this former cluster with 75% bootstrap support. *Pyricularia variabilis* seems to be a completely different taxon to 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 to 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.

Figure 6.2 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 bold.

6.4A Discussion

6.4.1A Molecular phylogeny and relationships of *Pyricularia* and related genera

Species of the anamorphic fungus *Pyricularia* are typically plant pathogens, e.g., *P. oryzae* is the 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). Morphologically similar pathogens, *Pyricularia penniseti* Prasada & Goyal and *P. setariae* Nishik. 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.* (2001b, d, 2003b) reported several *Pyricularia* species, including *P. costina*, living as endophytes in healthy wild gingers.

The criterion used by Ellis (1971, 1976) for separating *Pyricularia* from *Dactylaria* was 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 already occupied, by a different fungus, *D. juncicola* (MacGarvie) G.C. Bhatt & W.B. Kendr., thus Ellis (1976) proposed the new name, *D. junci*. *Nakataea fusispora* was also 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 transferred to *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* should therefore be maintained in *Pyricularia.* Likewise, *Nakataea fusispora* should also be maintained in *Nakataea*, rather than accepting the transfer to *Pyricularia*, since this species has distinctive, fusiform, verrucose conidia and it formed a sister cluster to 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 phylogenetically unrelated to the other *Pyricularia* species studied, but more related to *Dactylaria*, *Tumularia* or *Ochroconis* species. Further work is needed to establish the appropriate placement of this taxon and determine its relationships.

The results of the present study indicate that the other *Dactylaria* and species of *Pyriculariopsis*, *Tumularia* and *Ochroconis* are distinct from the Magnaporthaceae, and that the genus *Dactylaria* is polyphyletic. *Dactylaria* is a form genus and includes species with conidia grouped at the apical region of conidiophores on cylindrical or tapering denticles. Conidia are, however, variously shaped and the different taxa may be unrelated. The *Dactylaria* type of conidiogenesis may have evolved more than once, and the 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 clustered with *Ochroconis humicola* with 100% bootstrap support. *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* were originally 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, rostrate

conidia. The exclusion of these taxa from *Pyricularia* is, therefore, justified and supported by morphological and molecular data.

6.4.2A 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 uses three categories of information to identify taxa (Kirk *et al.*, 2001): conidiomatal types, Saccardo 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 using molecular techniques has been advocated (e.g., 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* were thought to differ from *Pyricularia* in the absence of a separating cell in the denticles (Ellis, 1971, 1976). The conidia also are of various shapes, usually fusiform, naviculate, or more or less cylindrical. Teleomorphs of *Dactylaria* species have not been commonly reported. 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.* (2002b) reported a teleomorph-anamorph connection between an unidentified ascomycete and *Dactylaria*.

Carbone and Kohn (1993) confirmed 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 the present study, phylogenies showed *Pyricularia zingiberis* and *Gaeumannomyces amomi* isolated from zingiberaceous plants were strongly grouped and closely related to other *Gaeumannomyces* species from grasses. The 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 specialisation (Table 6.1) 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* was also 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). In the present study, results are consistent with the probability that *P. zingiberis* may 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). They have also provided information on the genetic diversity among different populations 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). A combination of morphological characters (e.g., spore morphology) and molecular characters (ITS ribosomal DNA sequences data) may confidently allow us to distinguish *Pyricularia* from *Dactylaria* species, especially if sexual structures are not readily produced in culture. It would be concluded that conidial shape could be used as a primary character to distinguish *Pyricularia* species from related genera such as *Dactylaria*.

B. *Myrothecium*

6.1B Introduction

The genus *Myrothecium* was described by Tode (1790), with *M. inundatum* Tode as the type species. Thirty-six available type materials were examined and compared with morphologically similar genera by Tulloch (1972). She also provides a key to species of 13 accepted *Myrothecium* species. The fruiting body consists of a sporodochium with differentiated marginal hyphae. Conidia are produced from phialides and accumulate in a hyaline, olivaceous or black slimy head. The appearance of the fructifications is varied since sporodochia display different degrees of complexity. Hyaline or dark setae are sometimes present, arising from the basal stroma. *Myrothecium dimorphum* Ts. Watanabe, which has straight hyaline setae and two kinds of conidia, was recently described and differentiated from other *Myrothecium* species with setose or hyphal sporodochia (Watanabe *et al*., 2003). *Myrothecium acadiense* Seifert & G. Sampson, which produces sporodochial conidiomata but apparently proliferates percurrently was also recently described based on studies of morphology and phylogenetic analysis of partial sequences of large subunit ribosomal DNA (Seifert *et al*., 2003).

The genus *Solheimia* described by Morris (1967) is characterized by synnemata and phialides, which produce single unicellular conidia with longitudinal ridges (Morris, 1967; Varghese and Rao, 1977; Bills *et al*., 1994; Matsushima, 1995). The differentiation between *Solheimia* and other synnematous fungi, including *Myrothecium*, is based only on its unbranched conidiophores (Morris, 1967; Seifert, 1985). However, *Solheimia kamatii* Varghese & V.G. Rao has double dichotomously branched conidiophores (Varghese and Rao, 1977). Bills *et al.* (1994) examined the

conidiomata and spore ornamentation and shape of *M. cinctum* (Corda) Sacc. and *S. costaspora* E.F. Morris growing on agar media and on autoclaved banana leaves, and found that all characters of these two fungi were very similar.

During current studies a fungus with morphological characters, both on natural substrates and on agar media, which fit *Myrothecium cinctum* was recovered from zingiberaceous plants. This fungus was examined and compared to *M. pandanicola* from *Pandanus penetrans*. *Myrothecium cinctum* was also morphologically similar to *Solheimia costaspora* from *Licuala longicalycata* and *S. kamatii* from *Musa acuminata*. The aim of the present study was to report if *M. cinctum* and these *Solheimia* isolates were clumped based on traditional morphological characters and ITS rDNA sequence information.

6.2B Materials and methods

6.2.1B Fungal isolates and morphology

Myrothecium cinctum was obtained from dead tissues of the zingiberaceous plants by single spore isolation (Chapter 4). *M. pandanicola* from *Pandanus penetrans*, *Solheimia costaspora* from *Licuala longicalycata* and *S. kamatii* from *Musa acuminata*, were obtained from BIOTEC Culture Collection, Bangkok, Thailand. Hyphal tips of fungal isolates were transferred to potato dextrose agar (PDA). For morphological study, mounts were prepared in lactophenol, examined using either differential interference phase contrast or bright-field phase contrast microscopy. The number of ridges on the conidia was observed by viewing the conidia from the end and refers to the total number surrounding each conidium. Radial growth was determined from isolates grown on corn meal agar (CMA) and

PDA at room temperature (27–30 °C). Each isolate was also subcultured on CMA and PDA, containing autoclaved strips of host leaf tissue. The description of colony morphology was made from the same cultures.

6.2.2B DNA extraction, PCR amplification and sequencing

Fungal DNA was extracted from freeze-dried mycelium using the SDS-CTAB method as described in section 6.2.2A. Amplification of the rDNA ITS region containing ITS1 and ITS2 and the intervening 5.8S rRNA gene, analysing of PCR products and sequencing of amplified products were completed as descibed in section 6.2.3A. The raw data were used to assemble the sequences of the regions using Sequencher 3.1.1 for Macintosh (Applied Biosystems).

6.2.3B DNA sequence alignment and phylogenetic analysis

The sequences were submitted to http://bioweb.pasteur.fr/seqanal/clustalw, for multiple alignment using CLUSTAL W 1.82 (Thompson *et al*., 1994), and manually adjusted using GeneDoc 2.6.002 (Nicholas and Nicholas, 1997). The GenBank accession numbers of the sequences are in Table 6.3. Phylogenetic trees were inferred using PAUP*4.0b10 (Swofford, 2002). Due to the large size of the data set, parsimony trees were constructed using heuristic methods. Heuristic searches were performed using the random addition sequence option and the tree bisection reconnection branch-swapping algorithm. The parsimony tree scores including tree length, consistency index, retention index, rescaled consistency index, and homoplasy index (TL, CI, RI, RC and HI) were also calculated. All molecular characters were unordered and given equal weight during analysis. The robustness of internal

branches was estimated with 1000 bootstrap replications (Felsenstein, 1985) using the same heuristic search options described above. Anamorphic Pezizaceae, *Peziza vesiculosa* Sacc. and *P. echinospora* P. Karst. were used to root for phylogenetic tree. DNA sequence alignment and trees were deposited in TreeBASE, accession number SN1790.

Table 6.3 Isolates used in the phylogenetic analyses or morphological studies.

Isolate	Strain no.	GenBank accession no.	Original substrate	Geographic origin
Clonostachys rosea		AF358234		
Giocladium penicillioides		AF048733		
Graphium erubescens		AF198247		
Koorchaloma spartinicola		AF422963		
Myrothecium atroviride	(Wb256)	AJ302002		
M. atroviride	CBS224.78 (BBA71016)	AF455507	Stem of *Picea* sp.	Germany
M. cinctum	(BBA65670)	AJ301996		
M. cinctum	(BBA69182)	AJ301997		
M. cinctum[b]	ATCC18947	DQ135997	Forest soil	Canada
M. cinctum[b]	ATCC32918	DQ135998	Pathogen of *Saccharum officinarum*	Taiwan
M. cinctum	CBS479.85 (BBA71018)	AJ302004	Leaf of palm	Japan
M. cinctum[a]	BCC8249	DQ135995	Dead pseudostems of *Amomum siamense*	Thailand
M. inundatum		AJ302005		
M. leucotrichum	CBS131.64 (BBA71014)	AJ302000	Uncultivated soil	India
M. pandanicola[a]	BCC9829	DQ135992	Dead leaves of *Pandanus penetrans*	Thailand
M. roridum	(BBA62764)	AJ301993		
M. roridum	(BBA63372)	AJ301994		
M. roridum	(BBA67679)	AJ301995		
M. roridum	CBS212.92 (BBA71015)	AJ302001	Water from nursery	Netherlands
M. verrucaria	(BBA70749)	AJ302003		
M. verrucaria	CBS328.52 (BBA71017)	AJ301999	Deteriorated baled cotton	USA
Pesotum cupulatum		AF198230		
Pesotum fragrans		AF198248		
Peziza echinospora		AF491573		
Peziza vesiculosa		AF491623		
Solheimia costaspora[b]	AR2737	DQ135996	Litter	Costa Rica
S. costaspora[a]	BCC11170	DQ135994	Dead petioles of *Licuala longicalycata*	Thailand
S. kamatii[a]	BCC9775	DQ135993	Decaying petioles of *Musa acuminata*	Thailand
Stilbella byssiseda		AF335453		
Tubercularia sp.		AF422980		
Verticillium dahliae		AF363998		
V. dahliae		AF364005		
Virgatospora echinofibrosa[b]	AR2824	DQ135999	Litter	Mexico
Volutella ciliata		AJ301967		

[a]Isolate with complete ITS region sequenced in present study, [b]Isolate with complete ITS region sequenced from A.Y. Rossman

6.3B Results

6.3.1B Taxonomy

Myrothecium cinctum (Corda) Sacc., *Syll. Fung*. 4: 751, 1886 (Figure 6.3a, b, c)

=*Solheimia costaspora* E.F. Morris, *Mycopath. Mycol. Appl.* 33: 181 (1967)

=*Solheimia kamatii* Varghese & V.G. Rao, *Curr. Sci.* 46: 23 (1977)

Colonies on natural substratum in the form of synnemata, scattered, determinate, composed of dark-olivaceous stipes, becoming pale olivaceous towards the black slimy head; stipes up to 350 µm high, 23 µm diam, cylindrical; conidiophores unbranched or branched dichotomously two times, synnematous hyphae 2–2.3 µm wide, parallel, flexuous, septate, verruculose, dark olivaceous. Conidiogenous cells monophialidic, smooth, hyaline to pale olivaceous. Conidia 9–12 × 3–3.7 µm ($\bar{x}$ = 10.2 × 3.1 µm, n = 30), unicellular, fusiform, apex acute, truncate at the base, olivaceous to dark olivaceous, with 8 mostly straight longitudinal ridges.

Colonies on PDA reaching 6 cm diam after 14 days, velvety, yellowish white, edge regular, not staining agar, producing synnemata and spores in the center within 2 months; on CMA reaching 5 cm diam after 14 days, velvety, yellowish-white, edge regular, producing synnemata and spores only when sterile plant tissue included.

Specimens examined: Thailand, Chiang Mai, Doi Suthep-Pui National Park, on dead pseudostem of *Amomum siamense*, 15 Oct. 2000, *B. Bussaban* (PDD74128, living culture in BCC8249, ICMP14537); Phayao, on dead leaves of *Zingiber officinale*, 3 Sep. 2001, *B. Bussaban*; Mae Hong Son, Huay Ticha Village, on decaying petioles of *Musa acuminata*, 23 Nov. 2000, W. Pothita (PDD74447, living culture in BCC9775, ICMP14539); Narathiwat, Sirindhorn Peat Swamp Forest, on

dead petioles of *Licuala longicalycata*, May 2001, *U. Pinruan* (PDD74446, living culture in BCC11170, ICMP14538).

Known distribution: Costa Rica, Ecuador, India, Panama, Peru, Thailand.

Notes: Initially, the morphological characters of the two specimens (PDD74446 and PDD74447) were matched to those of the original descriptions of *Solheimia costaspora* and *S. kamatii*, respectively, while that of PDD74128 matched the description of *Myrothecium cinctum*. However, the spore shape and size of *Myrothecium cinctum* from Zingiberaceae, *S. costaspora* from *L. longecalycata*, *S. kamatii* from *M. acuminata* and of previously described *Myrothecium cinctum* (Morris, 1967; Tulloch, 1972; Varghese and Rao, 1977; Bills *et al.*, 1994; Matsushima, 1995) overlap (Table 6.4). The conidiomata and spore ornamentation and shape of *M. cinctum and S. costaspora* growing on agar media and on autoclaved banana leaves, was previously demonstrated to be identical (Bills *et al.*, 1994). In the present study, the molecular data (ITS ribosomal DNA sequences) also supported this conjecture. Because of these findings it is considered better to retain one generic and specific epithet for these *Myrothecium* fungi with striate spores.

Myrothecium cinctum is similar to *M. pandanicola* (Figure 6.3d) in having striate spores. *Myrothecium cinctum*, however, has distinctly shorter conidia than those of *M. pandanicola* (9–12 × 3–3.7 µm vs. 14–28 × 2–4.5 µm). The mean length to width ratio for the conidia of *M. cinctum* is 3:1, compared to 6:1 for *M. pandanicola*. *Myrothecium cinctum* also has more longitudinal ridges (7–9 vs. 6–7 for *M. pandanicola*). The morphological separation of *M. cinctum* and *M. pandanicola* was strongly supported by the molecular data (ITS ribosomal DNA sequences) (section 6.3.2B).

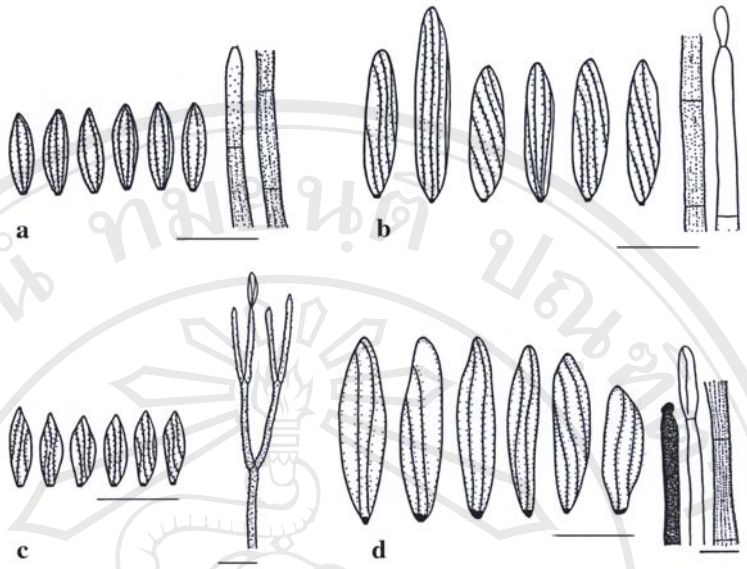

Figure 6.3 Diagrammatic representation of conidiophores, conidiogenous cells and conidia of *Myrothecium cinctum* isolated from Zingiberaceae (a), *Licuala longicalycata* (b), *Musa acuminata* (c) and *M. pandanicola* from *Pandanus penetrans* (d).

Table 6.4 Comparison of morphological characters of *Myrothecium* isolated from monocotyledonous plants in Thailand.

Characters	***Amomum siamense***	***Licuala longicalycata***	***Musa acuminata***	***Pandanus penetrans***
• On natural substrate				
Stipes	up to 350 µm high, 23 µm diam	stipes up to 1500 µm high, 30 µm diam	up to 460 µm high, 15 µm diam	up to 850 µm high, 75 µm diam
Conidiophores	unbranched or double dichotomously branched	mostly unbranched	double dichotomously branched	mostly unbranched
Conidial size	9–12 × 3–3.7 µm	15–19 × 3.7–5 µm	7–11 × 2–3 µm	13.9–27.8 × 2.3–4.6 µm
Striation	8 mostly straight longitudinal ridges	7–9 straight, curved or spiral ridges	6–8 spiral ridges	6–7 straight or mostly curved ridges
• Colony diam on PDA after 14 days	6 cm	5 cm	7 cm	7.5 cm
• Colony diam on CMA after 14 days	5 cm	4 cm	4.5 cm	6 cm

6.3.2B Molecular phylogeny

The alignment generated 757 characters, 384 of which were potentially phylogenetically informative. Parsimony analysis of the ITS sequence using the options of a heuristic search generated 6 equally parsimonious trees with a total length of 1458 steps (CI = 0.6598, RI = 0.7082, RC = 0.4673 and HI = 0.3402) (Figure 6.3). All of the *Myrothecium* taxa were sorted into a large cluster, forming two sister groups, clade I and clade II (75% bootstrap support). Clade I included the type of the genus, *M. inundatum* and *M. atroviride*, *M. verrucaria*, *M. roridum*, *M. leucotrichum*, *M. pandanicola* and *Didymostilbe echinofibrosa* (69% bootstrap support). In this clade, *M. atroviride* and *M. verrucaria* were closely related with 99% bootstrap support. *Myrothecium cinctum* with striate conidia, isolated from various hosts, and two species previously described as *Solheimia* that also have striate conidia, fell within the same clade (II, 100% bootstrap support). Members in *Myrothecium* cluster with the taxa that have a pale-coloured conidial mass and belong to Hypocreales or Sordariales *Incertae Sedis* (*Tubercularia* sp., *Koorchaloma spartinicola*, *Gliocladium penicillioides*, *Clonostachys rosea*, *Stilbella byssiseda*, *Volutella ciliata* and *Verticillium dahliae*) rather than to the remaining taxa that have a dark-coloured conidial mass and belong to Ophiostomatales (*Pesotum cupulatum*, *P. fragran*s, *Graphium erubescens*) (Figure 6.4).

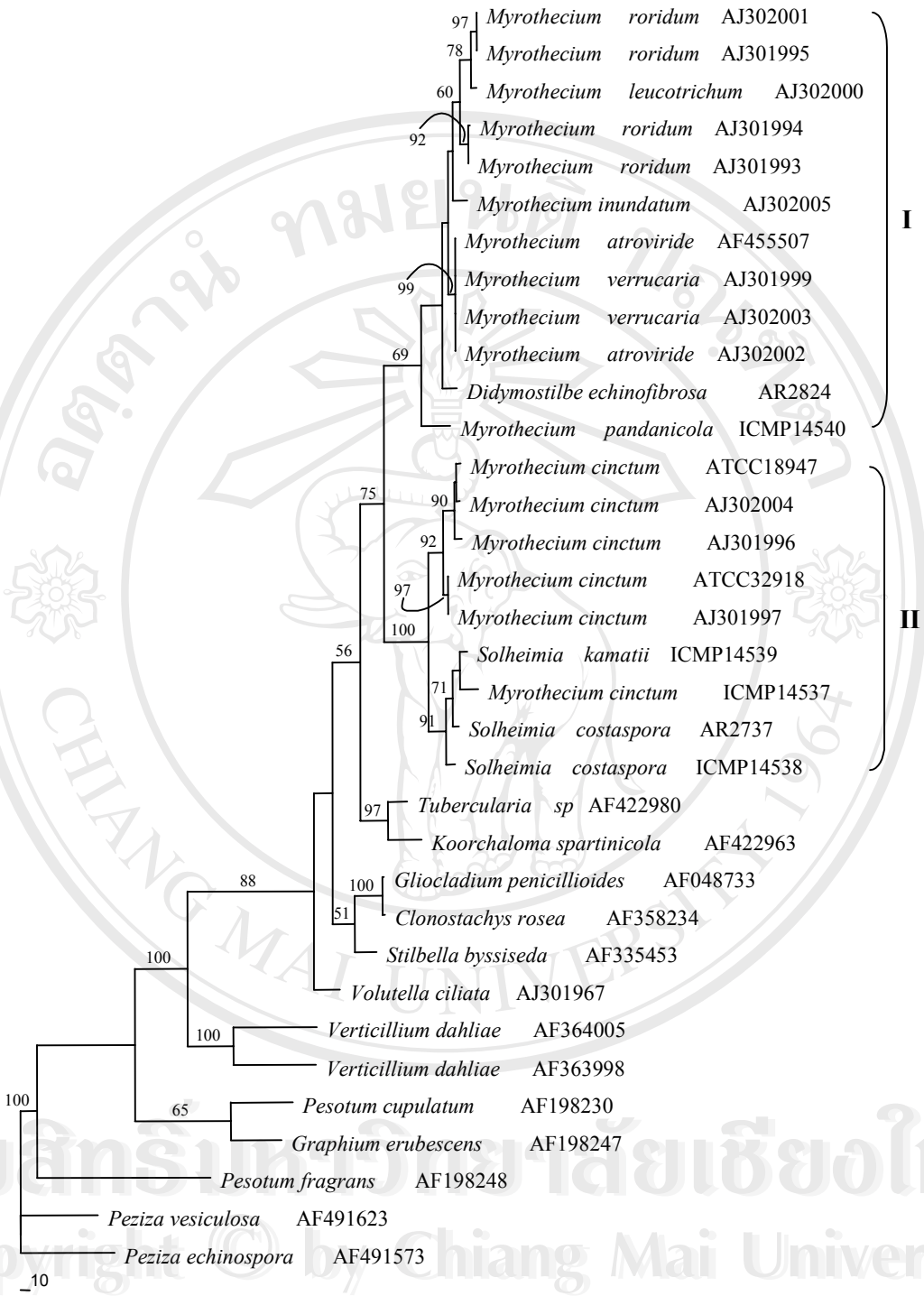

Figure 6.4 One of 6 most parsimonious trees inferred from a heuristic search of the ITS1-5.8S-ITS2 rDNA sequence alignment of 34 isolates of *Myrothecium* and related genera. *Peziza echinospora* and *P. vesiculosa* were used to root the tree. The size of the branches is indicated with a scale bar. Branches with strong support (≥ 95%) are in bold, with bootstrap values ≥ 50% indicated above the branches.

6.4B Discussion

There are 69 names for *Myrothecium* and two names for *Solheimia* species recorded in IndexFungorum (http://www.indexfungorum.org/Names/Names.asp) but only a few comprehensive generic diagnoses have been provided. Tulloch (1972) recognised the difficulty in delimiting *Myrothecium* from other genera. Her decision to select the colour of the conidial mass as the main characteristic was criticised by Nag Raj (1995). The differentiation between *Solheimia* and other synnematous fungi including *Myrothecium*, is based only on the unbranched conidiophores of *Solheimia* (Morris, 1967; Seifert, 1985). *Solheimia kamatii* Varghese & V.G. Rao, however, was described under this genus with two times dichotomously branched conidiophores (Varghese and Rao, 1977). In addition, *Virgatospora natarajensis* with striate conidia resembling those of *Myrothecium* or *Solheimia* species, was described for synnematous fungi producing slimy, non-septate, phialidic conidia with distinct striation (D'Souza *et al*., 2002). It is, therefore, difficult to delimit *Myrothecium* from other genera such as *Solheimia*, *Virgatospora*.

Tulloch (1972) revised *Myrothecium* Tode and accepted 13 species. The broad generic limits include cupulate, sporodochial and synnematal forms. Cupulate conidiomata are most typical of *M. gramineum* Lib., *M. leucotrichum* (Peck) Tulloch and *M. atroviride* (Berk. & Br.) Tulloch. In all conidiomata, however, differential marginal hyphae are present. Hyaline or dark setae are also sometimes formed. The marginal hyphae surround the central mass of slimy green to black conidia, which become hard when dried. Conidia may or may not be pigmented but are formed holoblastically from phialides (Sutton, 1980) which sometimes percurrently proliferate.

The genus *Solheimia* is a synnematous hyphomycete with large, slimy, dark green, striate conidia borne on phialides. This genus is morphologically similar to *Myrothecium cinctum* and *Virgatospora echinofibrosa* (syn. *Didymostilbe echinofibrosa*), the latter having 3-septate striate conidia (Bills *et al.*, 1994; Chang, 1998). Bills *et al.* (1994) have demonstrated the conidiomata and spore ornamentation and shape of *M. cinctum* and *S. costaspora* growing on agar media and on autoclaved banana leaves, and indicated that all characters of these two fungi were identical. In our study, *Solheimia costaspora*, *S. kamatii* and *Myrothecium cinctum* with striate conidia also appear to have a close phylogenetic relationship based on ITS including 5.8S rDNA sequence analysis. These close correlation of morphological characteristics have also been observed in other fungi using ITS sequence analysis (e.g. *Sepedonium*) (Sahr *et al.*, 1999).

The differences in composition and structure of the polysaccharides of the F1SS fraction of *Myrothecium* species were demonstrated by Ahrazem *et al.* (2000). The results of the ^{1}H-NMR spectra of the polysaccharides of the FlSS fraction isolated from the *M. atroviride* and four strains of *M. verrucaria* were very similar, and similar to those of *M. inundatum*, *M. setiramosum*, *M. prestonii*, *M. tongaense* and *M. roridum*. Moreover, the ^{1}H-NMR spectra of the polysaccharides of the FlSS fraction from *M. cinctum* were completely different from those species investigated. In the ITS sequence analysis *M. atroviride* and *M. verrucaria* were clustered with 99% bootstrap support and clustered in the same clade (I) of *M. inundatum*, *M. roridum*, *M. leucotrichum*, and *M. pandanicola*. Clade I is also sister to clade II, which comprises all isolates of *M. cinctum*.

Analysis of ribosomal DNA has frequently been used in mycological investigations (Bruns *et al*., 1991; Hibbett, 1992; Lee and Taylor, 1992; Li, 1997; Pandey *et al*., 2003; Tomita, 2003; Menkis *et al*., 2004; Rodrigues *et al*., 2004; Callac and Guinberteau, 2005; Didukh *et al*., 2005), and it can be potentially used to classify fungi. Including traditional morphological data and chemotaxonomic data with sequence information can greatly increase the resolving power of the tree, delineating or clumping species in individual data-set analyses.