

**12-Month Progress Report
(August 2006 – July 2007)**

For: National Center for Genetic Engineering and Biotechnology (BIOTEC)
Biodiversity Research and Training Program (BRT)

Project Title: The Phylogenetic Relationships of Selected Coelomycete Genera

BRT Project Code: BRT R_249005

Principal Investigator: Prof. E. B. Gareth Jones

Co Investigators: Dr. Jariya Sakayaroj
Dr. Sayanh Somrithipol

Research Assistant Mr. Nattawut Rungjindamai

This report consists of two parts:

A: Twelve month progress report of research undertaken (Page 2)

B: Appendices

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Part A: Twelve-month progress report

1. Summary of report

Coelomycetes are anamorphic fungi producing asexual spores (conidia) (Sutton, 1980; Nag Raj, 1993) and play an important role in terrestrial ecosystems: as saprobes, or parasites of higher plants, fungi, lichens and vertebrates and may occur as endophytes (Kirk *et al.*, 2001). Over the past 12 months, the small subunit (SSU), large subunit (LSU) and internal transcribed spacer (ITS) rDNA were analyzed to continue to resolve the taxonomic position of selected coelomycetes. The investigation of the phylogeny of basidiomycetous coelomycete: *Chaetospermum*, *Giulia* and *Mycotribulus* species has been completed, and a manuscript has been submitted to Mycological Research (see Appendix 1, page 27).

The rDNA of coelomycetes with fusiform-conidia comprising *Robillarda sessilis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea*, was examined to determine their taxonomic position. Preliminary results suggest that *Robillarda sessilis*, and *Xepiculopsis graminea* are classified within the Xylariales and Hypocreales (Sordariomycetes), while *Pseudorobillarda siamensis* nestled within the Pleosporales (Dothideomycetes).

2. Project objectives

1) To continue the analyses of entire rDNA sequences (SSU, LSU and ITS regions) of 13 selected coelomycete strains: *Chaetospermum camelliae*, *Chaetospermum artocarpi*, *Giulia tenuis*, *Infundibulomyces cupulata*, *Infundibulomyces oblongisporus*, *Mycotribulus mirabilis*, *Robillarda sessilis*, *Satchmopsis brasiliensis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea* (Table 1)

2) To evaluate the phylogenetic relationships of a further 19 coelomycetes for the third year, using all regions of the rRNA gene including SSU, LSU and ITS. Six of these are marine coelomycete species from Dr. Jariya's study of marine fungi, six unidentified coelomycetes and seven selected coelomycetes from Dr. Sayanh's study of leaf litter coelomycetes (Table 2).

3. Research methods

3.1 Growth of fungi:

The fungi were maintained on freshwater potato dextrose agar. Fungi were grown in liquid broth on a rotary shaker at 200 rpm at a temperature of 25 °C. The fungal biomass was harvested by filtration and washed with sterile distilled water. The biomass was then frozen at -20 °C overnight.

3.2 Genomic DNA extraction: (Applied from O'Donnell *et al.*, 1997)

A fine powder of fungal mycelia of 50-100 mg was placed into 400 µl lysis buffer and incubated at 65 °C for 1 hour. Then an equal volume of phenol-chloroform-isoamyl alcohol was added. The upper liquid phase was transferred to a new microtube containing chilled absolute ethanol and 7.5 M ammonium acetate. The mixture was kept at -20 °C for at least 30 minutes, or until the DNA precipitated out, and centrifuged at 14K, 4 °C, for 10 minutes. The DNA pellet was washed twice with chilled 75 % ethanol and air dried. Finally, DNA was resuspended in 50 µl nanopure water and checked for the quantity and quality in a 1% agarose gel electrophoresis.

3.3 PCR amplification:

rDNA was amplified with Taq DNA polymerase from DyNAzyme™ II DNA Polymerase Kit, FINNZYMES, Finland. Different regions of ribosomal DNA were amplified using PCR in a MJ Research DNA Engine DYAD ALD 1244 thermal cycler. Primers used for amplification the rRNA gene follows White *et al.* (1990) and Landvik (1996).

3.4 PCR product purification:

The PCR product was purified directly following the manufacturer's instructions of NucleoSpin^R Extract (MACHEREY-NAGEL). The purified PCR product was used directly for DNA sequencing.

3.5 DNA Sequencing:

PCR products were directly sequenced by Macrogen Inc. (Korea) using primers NS1, NS3, NS5, NS6, NS8, JS1, JS5, JS8, LROR, LR7, NL3, NL4, NL4R, ITS1, ITS4 and ITS5 (White *et al.*, 1990; Landvik, 1996).

3.6 Phylogenetic analysis:

The sequences were aligned along with selected sequences obtained from the GenBank database and with suitable outgroup taxa. Sequences was aligned in Clustal W 1.6 program (Thompson, *et al.*, 1994) and refined visually in BioEdit version 6.0.7 (Hall, 2004). Alignment was entered into PAUP, version 4.0b10 (Swofford, 2002). Phylogenetic trees were generated using maximum parsimony using PAUP version 4.0b10. The statistical support, the maximum parsimony, was calculated to evaluate the robustness of the phylogenetic trees.

4. Results

4.1 Fungal DNA extraction and PCR amplification of selected 13 coelomycetous fungi

Please see the BRT R_249005's 6-month progress report. Over the last 6 months, DNA of nine coelomycetes have been sequenced for the ITS regions. The updated status of PCR amplification and rDNA sequencing of coelomycetes studied over the last six months are shown in Table 1. The new 19 selected coelomycetes for the third year project are listed in Table 2. The new selected fungi consist of three groups: 6 isolates are marine coelomycetes (from Dr. Jariya), 6 are unidentified coelomycetes and 7 are selected coelomycetes (from Dr. Sayanh). The six marine coelomycetes have been analysed and preliminary trees obtained, but further taxa are required for a complete evaluation. These preliminary findings place them in the Pleosporales.

Table 1. The updated status of DNA extraction, PCR amplification and rDNA sequencing of coelomycetes studied over the last six months.

Group of fungi	No	Coelomycetes names	Original SFC code	BCC code	DNA extracted	rDNA sequences			BLAST	Sequence alignment	Phylogenetic analysis
						SSU	LSU	ITS			
Cupulate-conidiomata fungi	1	<i>Infundibulomyces cupulata</i>	0943	11929	/	/	/	/			
	2	<i>Infundibulomyces siamensis</i>	0981	13400	/	/	/	/	Completed	Completed	Completed
	3	<i>Satchmopsis brasiliensis</i>	1901	18579	/	/	/	/			
Basidiomycetous fungi	4	<i>Chaetospermum camelliae</i>	1625	13401	/	/	/	/			
	5	<i>Chaetospermum camilliae</i>	1909	18582	/	/	/	/			
	6	<i>Chaetospermum camilliae</i>	1925	18604	/	/	/	/			
	7	<i>Chaetospermum artocarpi</i>	1904	18581	/	/	/	/	Completed	Completed	Completed
	8	<i>Mycotribulus mirabilis</i>	0852	13341	/	/	/	/			
	9	<i>Mycotribulus mirabilis</i>	1922	18601	/	/	/	/			
Fusiform-conidial fungi	10	<i>Guilia tenuis</i>	0865	13066	/	/	/	/			
	11*	<i>Pseudorobillarda siamensis</i>	0795	12531	/	/	/	/			
	12	<i>Robillarda sessilis</i>	0858	13393	/	/	/	/	Completed	Completed	Completed
	13	<i>Xepiculopsis graminea</i>			/	/	/	/			

* = The phylogenetic investigation of *Pseudorobillarda siamensis* using LSU and ITS regions need to be further studied by compared with other strains of *Pseudorobillarda*.

Table 2. The updated status of DNA extraction, PCR amplification and rDNA sequencing of coelomycetes studied over the last six months.

Group of fungi	No	Coelomycetes names	Original code	BCC code	Culture	DNA extraction	Preliminary study
Marine coelomycete	1	Marine coelomycete	KH0087	25065	/	/	
	2	Marine coelomycete	KH0088	25066	/	/	Pleosporales
	3	Marine coelomycete	KH0089	25067	/	/	Plepsporomycetidae
	4	Marine coelomycete	KH0090	25068	/	/	Dothideomycetes
	5	Marine coelomycete	KH0091	25069	/	-	
	6	Marine coelomycete	KH0092	25070	/	-	
Unidentified coelomycete	7	Unidentified sp. 1	SFC1912	18583	/	-	-
	8	Unidentified sp. 2	SFC1920	18586	/	-	-
	9	Unidentified sp. 3	SFC1940*	-	-	-	-
	10	Unidentified sp. 4	SFC1941	20494	/	-	-
	11	Unidentified sp. 5	SFC1946	20812	/	-	-
	12	Unidentified sp. 6	SFC2109	21373	/	-	-
Selected coelomycete	13	<i>Vermiculariopsiella</i> sp.	SFC2075	22244	/	-	-
	14	<i>Lauriomyces</i> sp. 1	SFC1649	18576	/	-	-
	15	<i>Lauriomyces</i> sp. 1	SFC1649	18577	/	-	-
	16	<i>Wiesnesriomyces</i> sp. 2	SFC1689*	-	-	-	-
	17	<i>Wiesneriomyces</i> sp. 1	SFC1929	18608	/	-	-
	18	<i>Wiesneriomyces laurinus</i>	SFC1930	18609	/	-	-
	19	<i>Falcocladium</i> sp. 1	SFC2101	22055	/	-	-

* = Culture is in preparation for BCC deposition.

- = Analysis in progress

4.2) Phylogenetic analysis:

In order to analyze the phylogenetic relationships, the 13 coelomycetes were divided into three groups comprising: cupulate-conidiomata, basidiomycetous and fusiform-conidia based on the morphological and sequence data of these fungi (Table 1). The first group includes the coelomycetes that produce funnel-shaped cupulate-conidiomata that are superficial on the substratum. Basidiomycetes with coelomycete anamorphs are rare. The final group comprises coelomycetes with fusiform to ellipsoidal conidia.

Each dataset was independently analyzed comprising the SSU, LSU and ITS sequence data. The SSU and LSU rDNA sequences that contain highly conserved regions were suitable to investigate the ordinal and familial level classification, while the ITS region is a hyper variable region and used for the identification of genera and species. The rDNA sequences of our coelomycetes were incorporated with other sequences retrieved from the GenBank database with suitable outgroup taxa.

4.3) Phylogenetic relationships:

A) Phylogeny of *Infundibulomyces cupulata*, *Infundibulomyces siamensis* and *Satchmopsis brasiliensis*

The draft manuscript entitled “The significance of cupulate-conidiomata and the phylogenetic relationship of the coelomycete genera, *Infundibulomyces* and *Satchmopsis*” of this investigation has been submitted into *Mycologia*, a journal published by the Mycological Society of America. The manuscript has been reviewed and a major revision has been suggested and this is in progress.

Abstract: “The significance of cupulate-conidiomata and the phylogenetic relationship of the coelomycete genera, *Infundibulomyces* and *Satchmopsis*”

Two coelomycete genera (*Infundibulomyces* and *Satchmopsis*) with cupulate-conidiomata were assessed for their phylogenetic relationships from ribosomal DNA sequences: partial small subunit (SSU), partial large subunit (LSU) and complete internal transcribed spacer (ITS) regions using maximum parsimony and Bayesian analysis. Neither genus has a known teleomorph. *Infundibulomyces* species form a monophyletic group in the Chaetosphaeriaceae

(Sordariomycetidae) with *Dictyochoeta simplex* as a sister clade. There is no previous report of the Chaetosphaeriaceae with a coelomycete anamorph. *Satchmopsis brasiliensis* grouped within the Helotiales (Leotiomyetidae), but its familial position could not be resolved. Cupulate-conidiomata are not phylogenetically significant in the taxonomy of the coelomycetes and have evolved independently in the taxa evaluated. A new species *Infundibulomyces oblongisporus*, based on morphological and phylogenetic evidence, is described from leaf litter collected at Khao Yai National Park, Thailand.

B) Phylogeny of *Chaetospermum camelliae*, *Chaetospermum atrocarki*, *Giulia tenuis* and *Mycotribulus mirabilis*.

The draft manuscript of this phylogenetic investigation has been prepared entitled “Putative basidiomycete teleomorphs and phylogenetic placement of the coelomycete genera: *Chaetospermum*, *Giulia* and *Mycotribulus* based on nuclear rDNA sequences” and was submitted to Mycological Research, a journal published by the British Mycological Society (BMS). See the further detail in draft of this manuscript (Appendix 1, page 28).

Abstract: “Putative basidiomycete teleomorphs and phylogenetic placement of the coelomycete genera: *Chaetospermum*, *Giulia* and *Mycotribulus* based on nuclear rDNA sequences”

Few basidiomycetes are known to have a coelomycete anamorph. The partial small subunit and large subunit of nuclear rDNA of three coelomycete genera (*Chaetospermum*, *Giulia*, *Mycotribulus*) were sequenced to determine their phylogenetic relationship. *Mycotribulus mirabilis* was well placed in the marasmioid clade (Marasmiaceae and Physalacriaceae), Agaricales, while *Giulia tenuis* clusters with the Corticiaceae, Corticiales. *Chaetospermum camelliae* and *Chaetospermum atrocarki* form a closely relationship with the Sebacinaceae, Sebaciniales. Although morphologically these coelomycetes are pycnidial and with appendaged conidia, they show no consistency in their phylogenetic relationship, belonging to disparate major taxonomic groups with putative teleomorphs in the Agaricales, Corticiales and Sebaciniales. Further molecular studies of coelomycetes may be rewarding to evaluate their phylogenetic affinities.

C) Phylogeny of fusiform-conidia coelomycetes: *Robillarda sessilis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea*

Among these coelomycetes, they share character of fusiform to ellipsoidal shape of conidia and were then classified into the same group. Their phylogenetic observation was conducted in order to prove the correlation between the morphology and phylogeny. The entire rDNA sequence were analyzed and the phylogenetic tree were constructed.

SSU phylogeny of fusiform-conidia coelomycetes

Following the reclassification of the kingdom Fungi (Spatafora *et al.*, 2006) and the recent released rDNA sequences from the GenBank database, the phylogenetic investigations of these coelomycetes were reanalyzed in order to resolve and improve the taxonomic position of our fungi. The dataset of the fusiform-conidia coelomycetes was updated with latest sequences. The SSU sequences of *Robillarda sessilis*, *Xepiculopsis graminea* and *Pseudorobillarda siamensis* were aligned along with several members of the Ascomycota. The result demonstrates that these coelomycetes fall within the 3 subclasses: Hypocreomycetidae, Xylariomycetidae (Sordariomycetes) and Pleosporomycetidae (Dothideomycete) respectively (Figure 1). The phylogenetic tree consists of three families of the Hypocreomycetidae (Microascales, Halosphaeriales and Hypocreales), the Xylariales, an only one order within the Xylariomycetidae, and Plesporales, a single order of the Pleosporomycetidae. The other three families of the Sordariomycetidae (Diaporthales, Ophiostomatales, Sordariales) and three families of the Dothideomycetes (Botryosphaeriales, Dothideales, Hysteriales) were employed in this examination in order to improve the tree topology whereas two taxa of the Pezizales (Pezizomycetes) were used as the outgroup.

The SSU rDNA sequence of *Robillarda sessilis* is placed within the Xylariales (Xylariomycetidae) with moderate statistical support (72% bootstrap value, Figure 2). *Robillarda sessilis* groups together with several species of *Discostroma* and *Seiridium* (Amphisphaeriaceae, Xylariales) with moderate support (84% bootstrap values). *Xepiculopsis graminea* is nestled within the Hypocreales (Hypocreomycetidae, Sordariomycetes) with high statistical support (93% bootstrap value), and a phylogenetic affinity with various *Myrothecium* species (*Incertae sedis*, Hypocreales). It forms a sister clade with *Peethambara spirostriata* and *Didymostilbe echinofibrosa* (Bionectriaceae, Hypocreales). *Pseudorobillarda siamensis* clustered with the

Pleosporales (Pleosporomycetidae, Dothidiomycete) with five *Cenococcum geophilum* strains and single sequence of *Helicoma isiola* (Pleosporales) as a sister group, although statistical support is low. Further analysis of each coelomycete genus is required to resolve their phylogenetic placement.

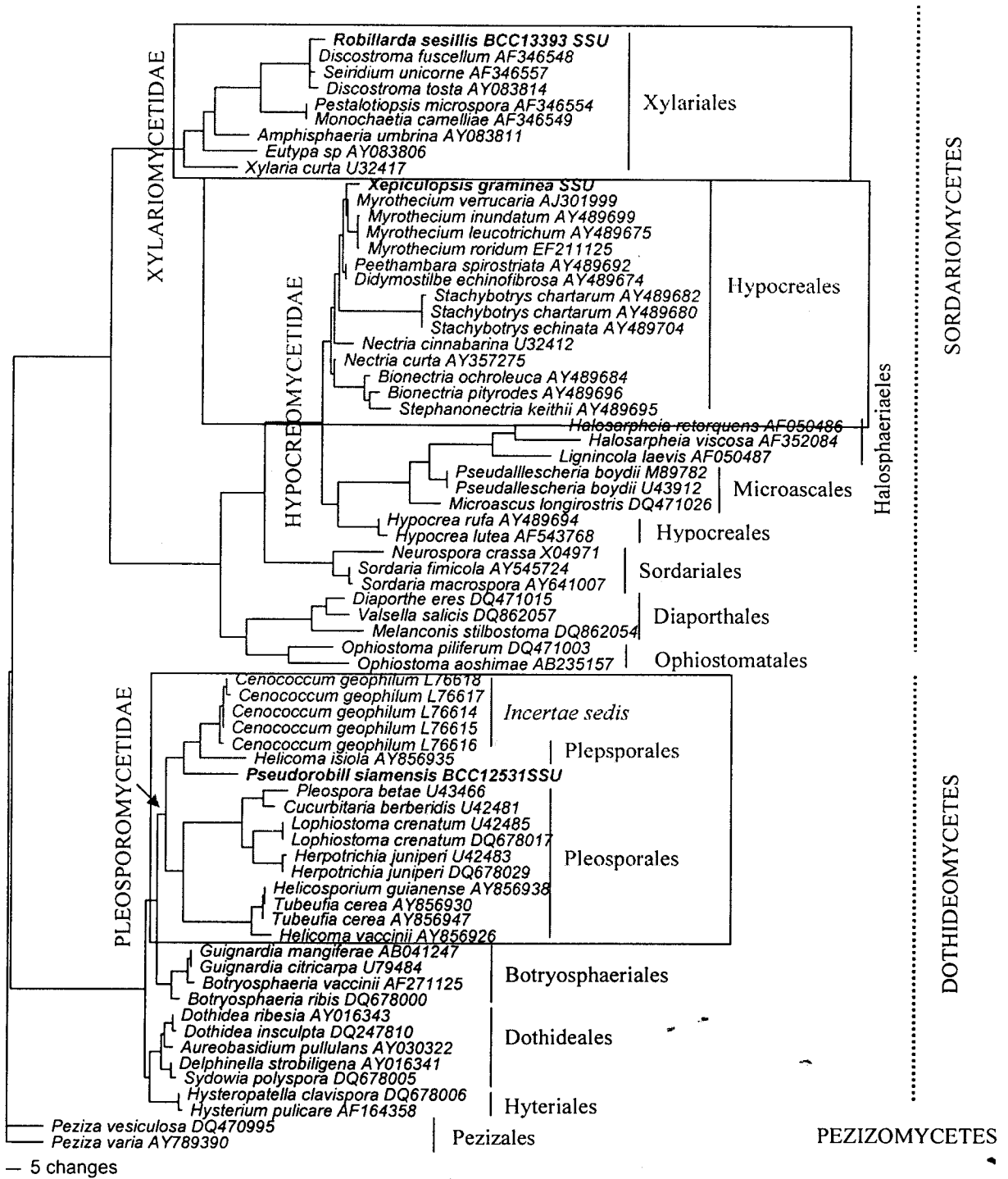


Figure 1. One of the 888 MPTs inferred from SSU rDNA sequences of *Robillarda sesillis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea*.

Bootstrap

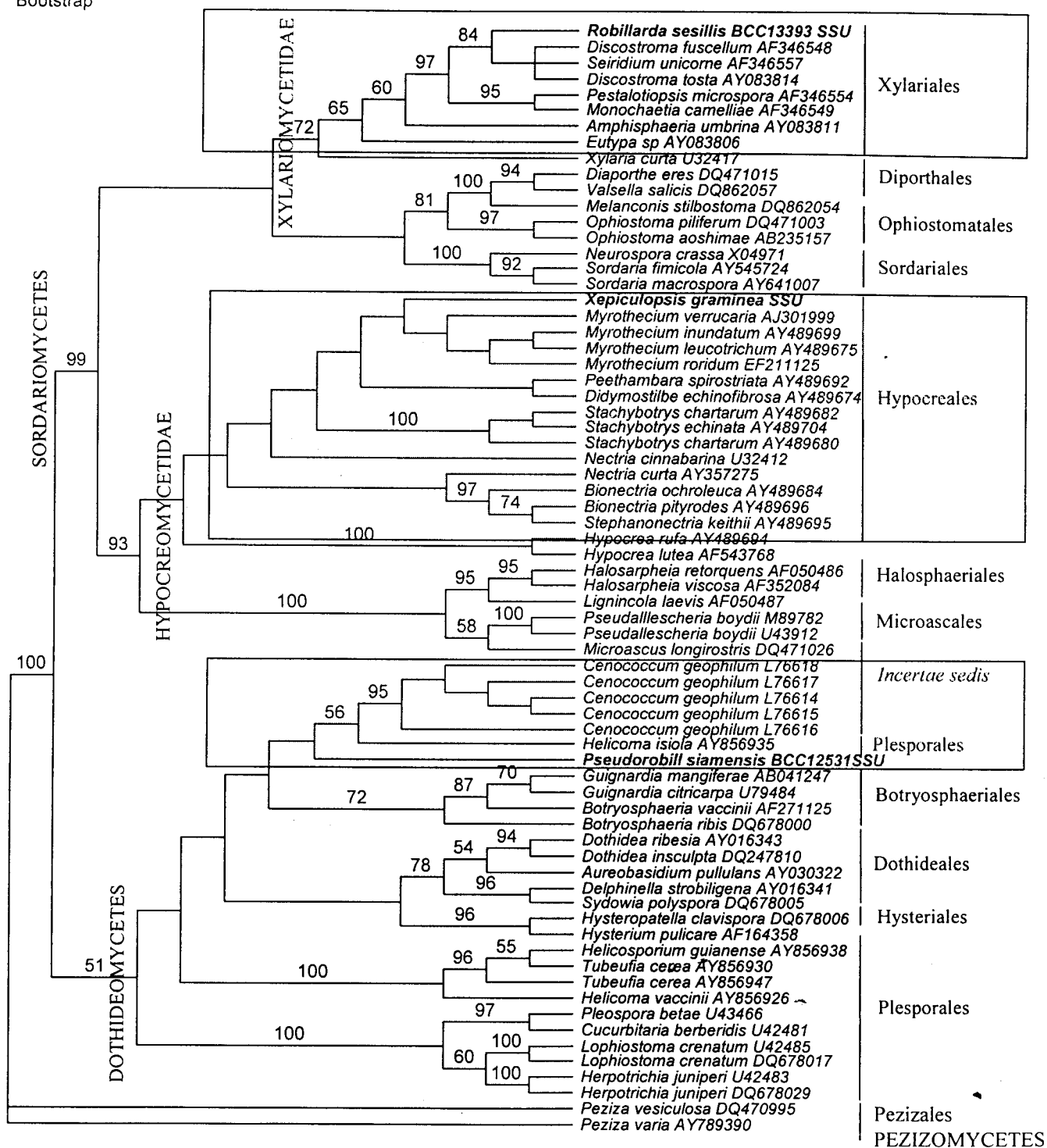


Figure 2. A strict consensus tree inferred from SSU rDNA sequences of *Robillarda sesillis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea*. Maximum parsimony bootstrap values greater than 50% are indicated above the branches.

LSU phylogeny of *Robillarda sessilis*

The phylogenetic investigation of this coelomycete was performed with 27 members of the Xylariales using *Diaporthales arctii* and *Diaporthe eres* (Diaporthales) as the outgroup. LSU rDNA sequence of *Robillarda sessilis* falls within the Xylariales (Figures 3-4). In order to clarify its familial level, the four major families of the Xylariales consisting of the Amphisphaeriaceae, Cainiaceae, Diatrypaceae and Xylariaceae and minor families consisting of the Clypeosphaeriaceae and Hyponectriaceae were aligned along with LSU sequence of *R. sessilis*. *Robillarda sessilis* clustered with several members of the Amphisphaeriaceae with good support (100% bootstrap value), even though it did not form a close relationship with any sequence in this family. They form a sister subclade with the Clypeosphaeriaceae and Hyponectriaceae, while the other three families representing Cainiaceae, Diatrypaceae and Xylariaceae were separated into basal lower subclade.

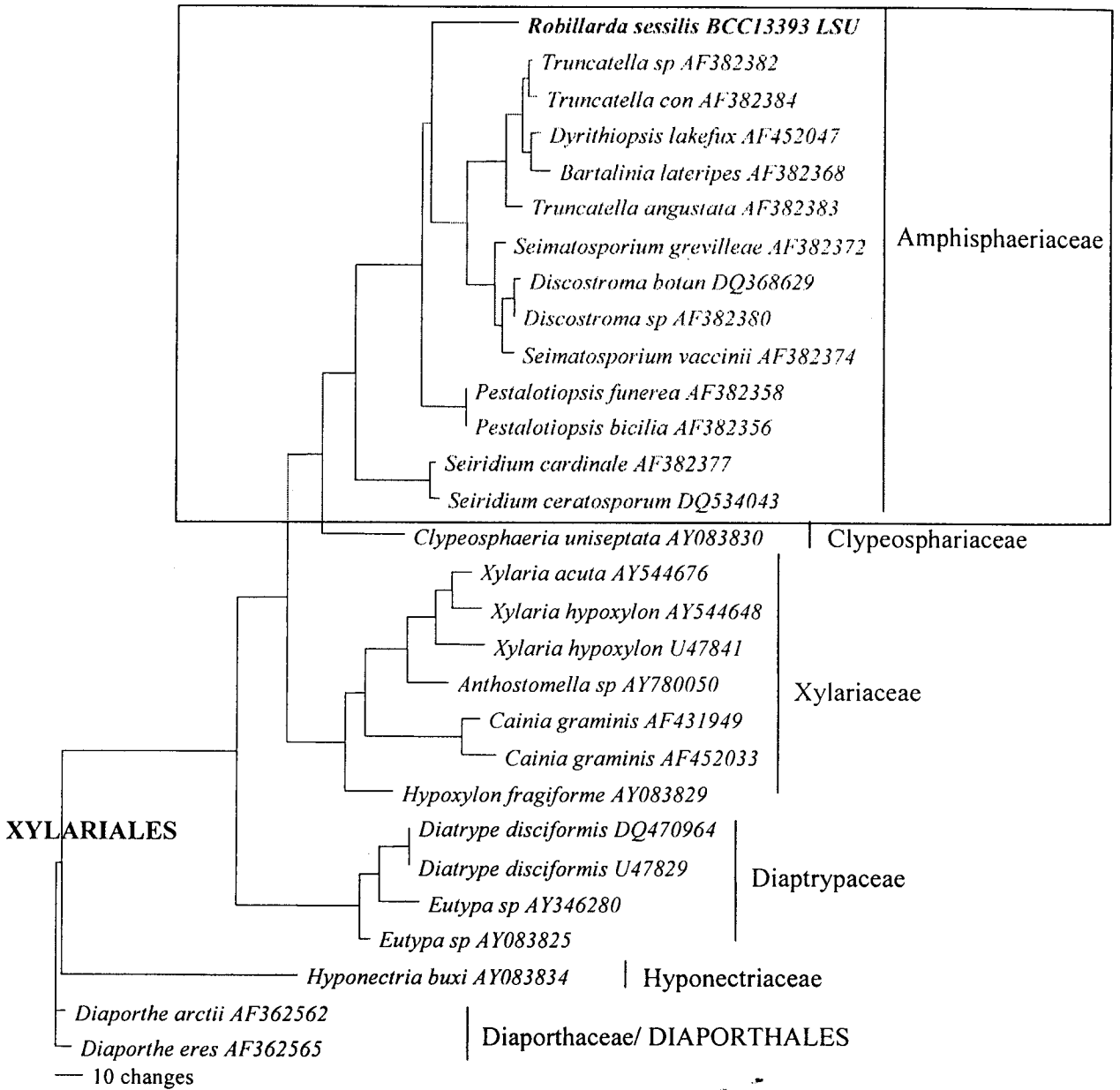


Figure 3. One of the 12 MPTs inferred from LSU rDNA sequences of *Robillarda sessilis* within the Xylariales.

Bootstrap

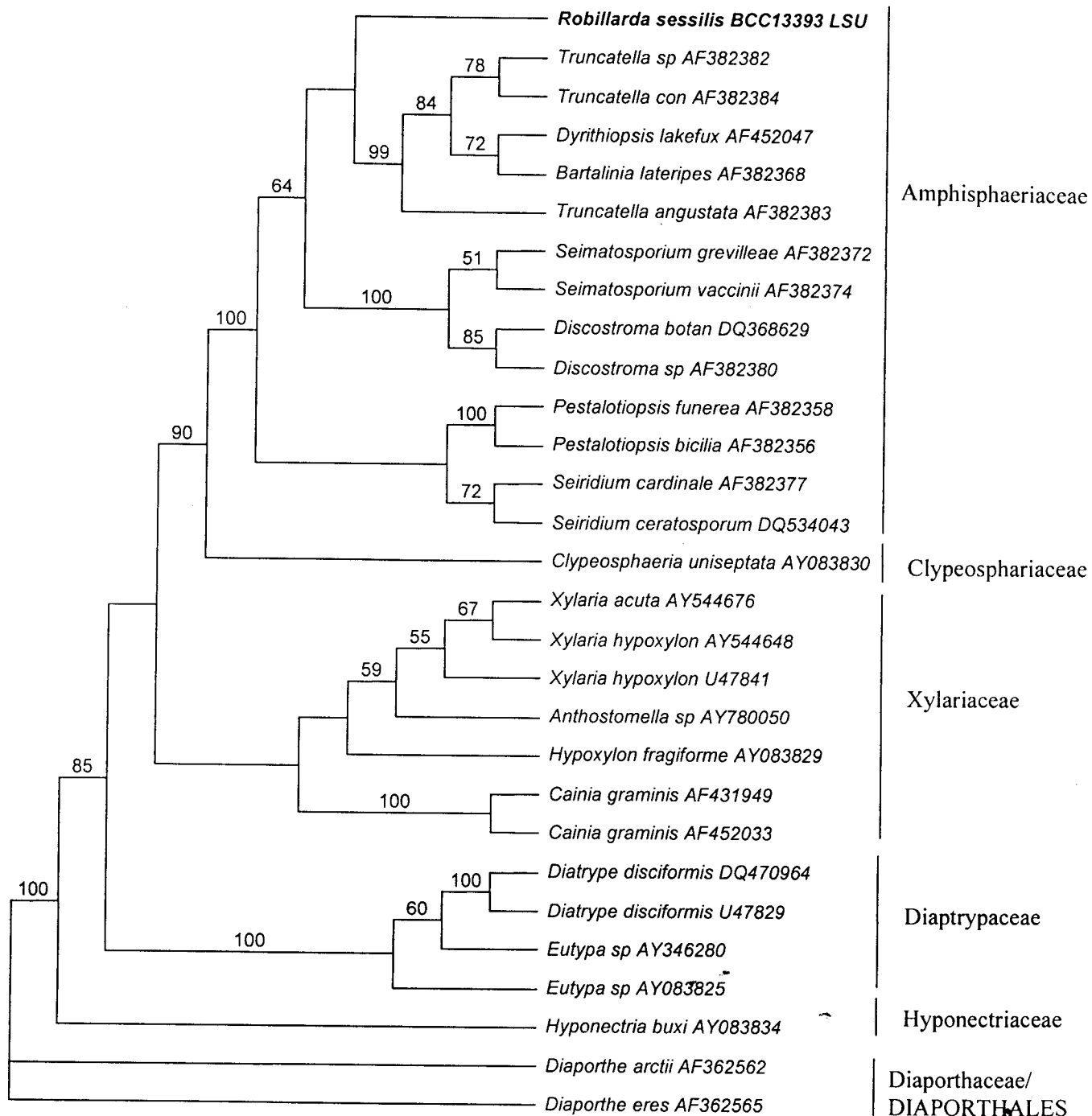


Figure 4. A strict consensus tree inferred from SSU rDNA sequences of *Robillarda sessilis* within the Xylariales. Maximum parsimony bootstrap values greater than 50% are indicated above the branches.

ITS phylogeny of *Robillarda sessilis*

The familial or lower taxonomic level of *Robillarda sessilis* was determined and improved using ITS rDNA sequences. The dataset comprise 32 taxa from four families of the Xylariales (Amphisphaeriaceae, Clypeosphaeriaceae, Diatrypaceae and Xylariaceae) with two taxa of the Diaporthales as the outgroup (Figure 5). ITS sequence of *Robillarda sessilis* is well placed with in the Xylariales with 100% bootstrap values and xxx (Figure 6). *Robillarda sessilis* groups within the Amphisphaeriaceae with 100% statistical support and clustered with several species of *Seiridium*, *Discostroma* and *Discosia*, while its closest relationship is with *Ellurema indica* AF009816 (Amphisphaeriaceae) with moderate statistical support (68% bootstrap values). This subclade forms a sister group with the species of *Pestalotiopsis* and *Monochaetia*. While the families of Acanthorhynchaceae, Cainiaceae, Diatrypaceae and Xylariaceae are placed in a basal subclade.

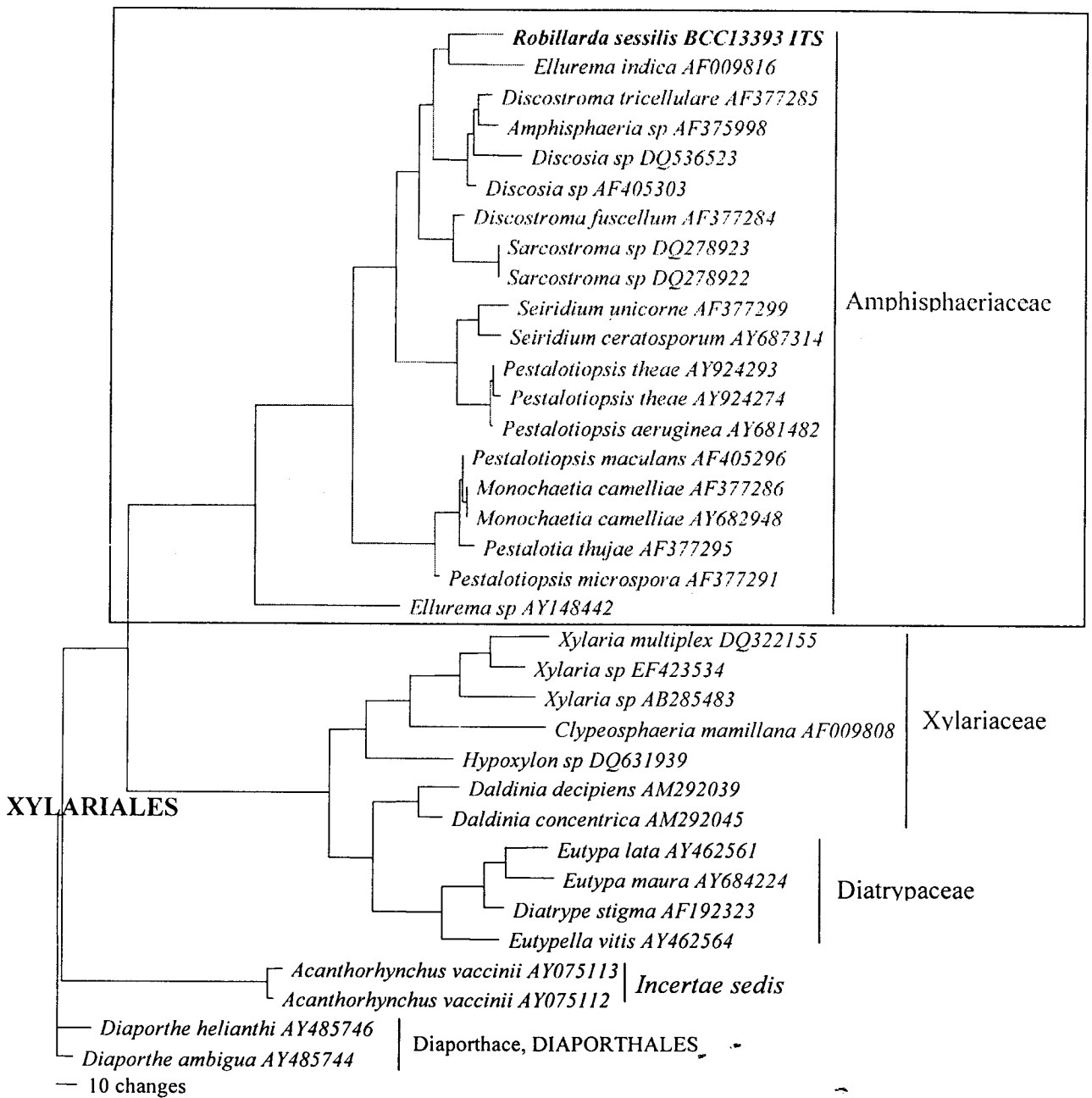


Figure 5. One of the 32 MPTs inferred from LSU rDNA sequences of *Robillarda sessilis* within the Xylariales.

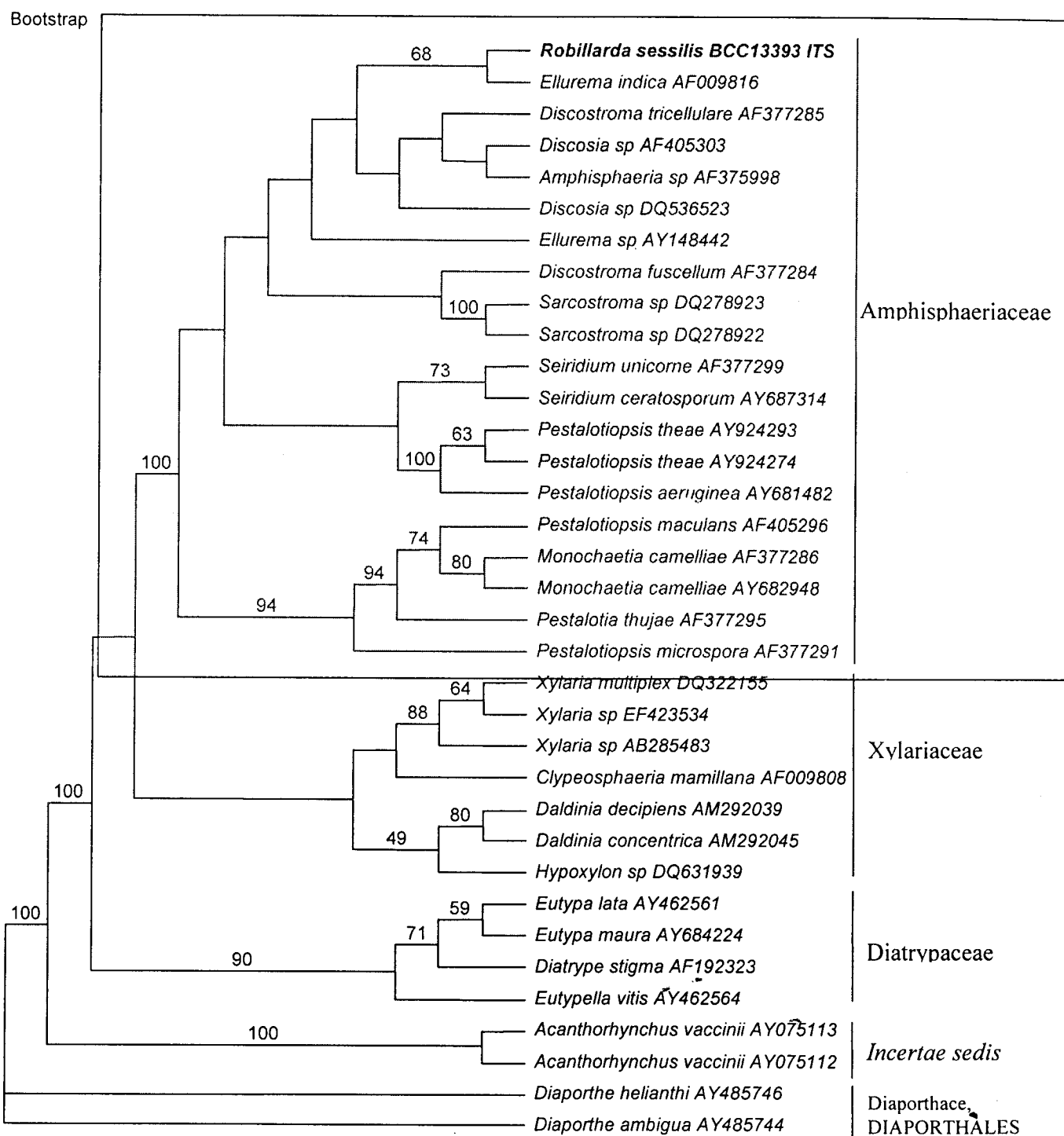


Figure 6. A strict consensus tree inferred from ITS rDNA sequences of *Robillarda sessilis* within the Xylariales. Maximum parsimony bootstrap values greater than 50% are indicated above the branches.

LSU phylogeny of *Xepiculopsis graminea*

LSU rDNA sequence of *Xepiculopsis graminea* were integrated with 45 representative taxa from the Hypocreales and two representatives from the Phyllachorales used as the outgroup. *Xepiculopsis graminea* groups with the Hypocreales with 100% statistical support. The dataset comprise five families of the Hypocreales (Bionectriaceae, Clavicipitaceae, Nectriaceae, Niessliaceae and Hypocreaceae) and two uncertain taxonomic groups (*Incertae sedis*, Hypocreales and *Incertae sedis*, Ascomycota, Figures 7-8). The LSU sequence of *X. graminea* exhibits a strong phylogenetic relationship with six species of *Myrothecium* (*Incertae sedis*, Hypocreales), but with low statistical support. The subclade of *Xepiculopsis graminea* and the *Myrothecium* forms a sister clade with species of the *Didymostilbe* and *Peethambara* (Bionectriaceae) which are polyphyletic. They also have a phylogenetic affinity with three species of the *Stachybotrys* (*Incertae sedis*, Ascomycota).

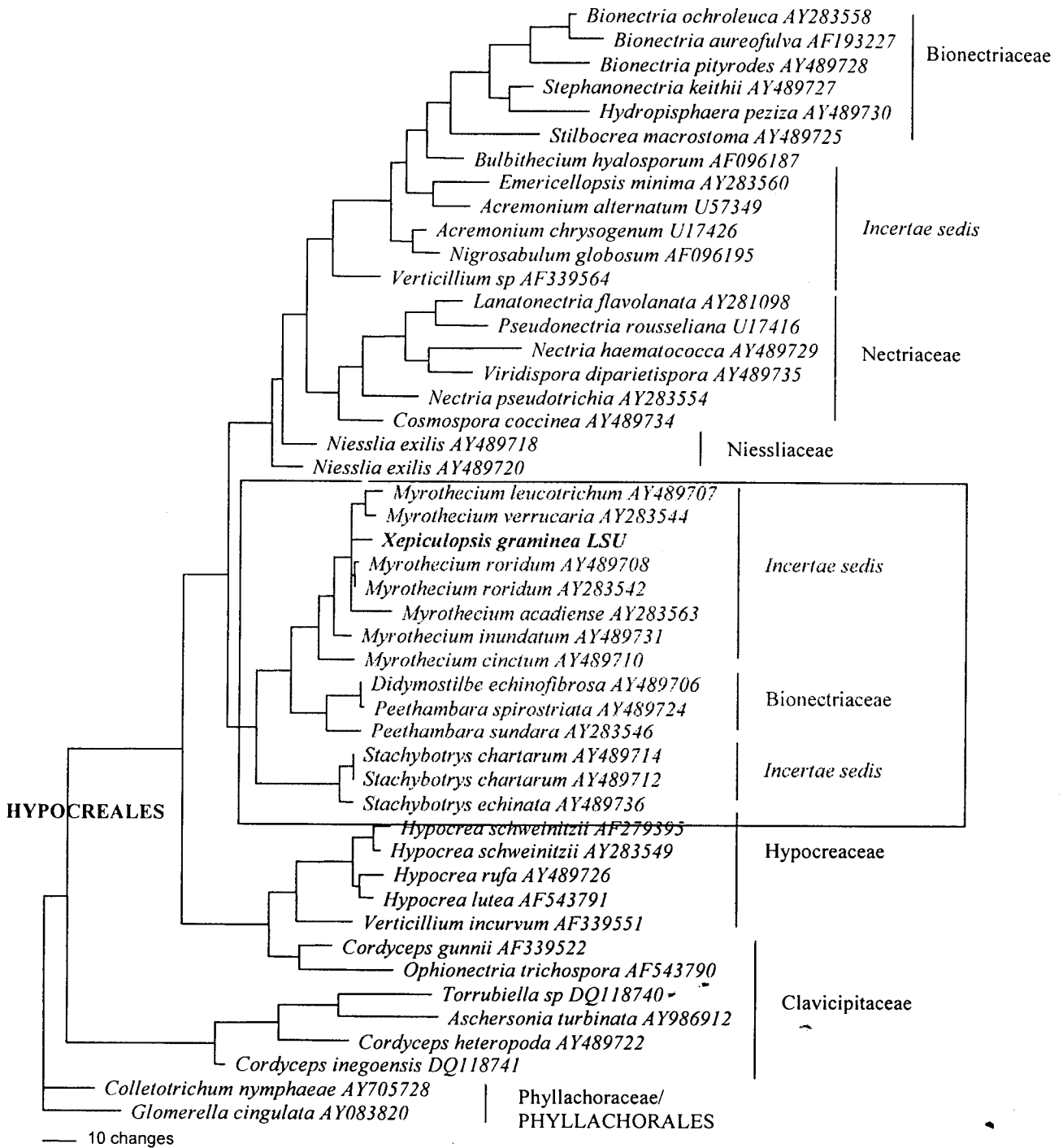


Figure 7. One of the 36 MPTs inferred from LSU rDNA sequences of *Xepiculopsis graminea* within the Hypocreales.

Bootstrap

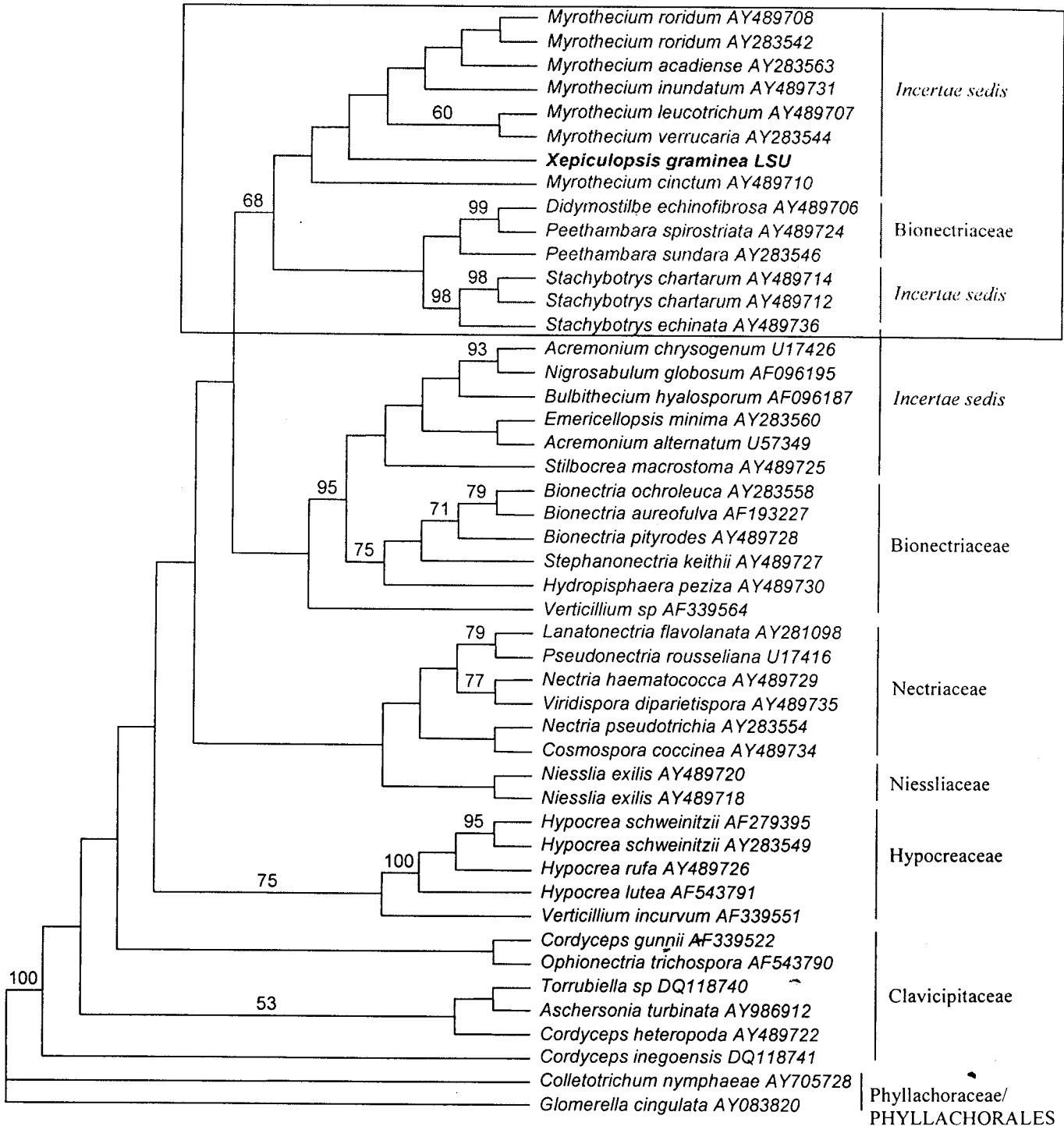


Figure 8. A strict consensus tree inferred from LSU rDNA sequences of *Xepiculopsis graminea* within the Hypocreales. Maximum parsimony bootstrap values greater than 50% are indicated above the branches.

ITS phylogeny of *Xepiculopsis graminea*

ITS rDNA sequence of *Xepiculopsis graminea* was incorporated with related sequences comprising 10 taxa of the Bionectriaceae, Nectriaceae and Niessliaceae (Hypocreales) and 16 taxa from several species of *Stachybotrys* and *Myrothecium* (*Incertae sedis*, Hypocreales) and *Trichoderma hamatum* (Hypocreaceae, Hypocreales) was used as the outgroup (Figure 9). The *Xepiculopsis graminea* sequence groups with a *Myrothecium* clade with moderate support (83% bootstrap values, Figure 10). Furthermore, *Xepiculopsis graminea* groups with *Myrothecium roridum* AY303605 with 100% bootstrap support. The Niessliaceae and Bionectriaceae are a sister clade to the *Myrothecium* clade.

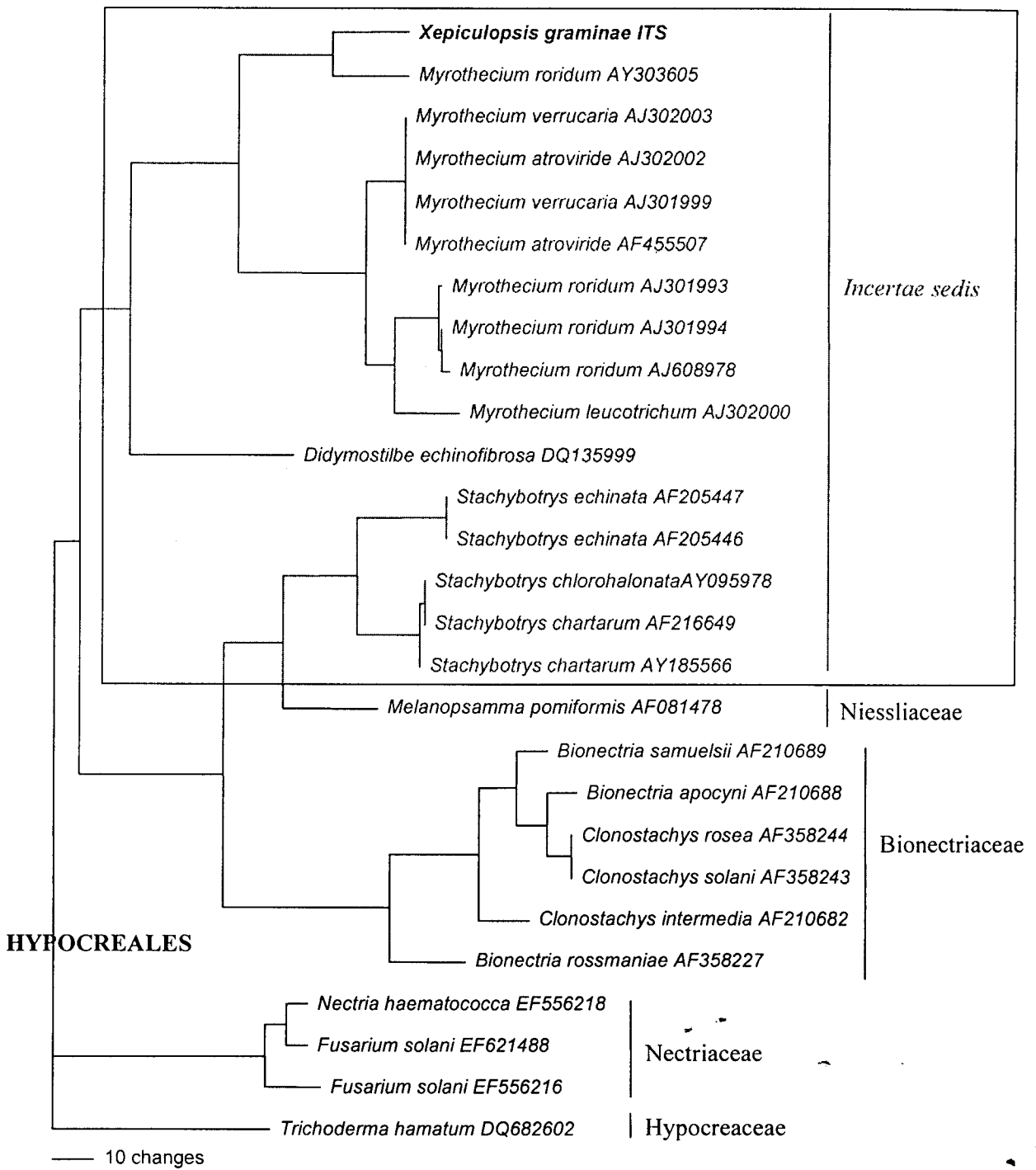


Figure 9. One of the 4 MPTs inferred from ITS rDNA sequences of *Xepiculopsis graminea* within the Hypocreales.

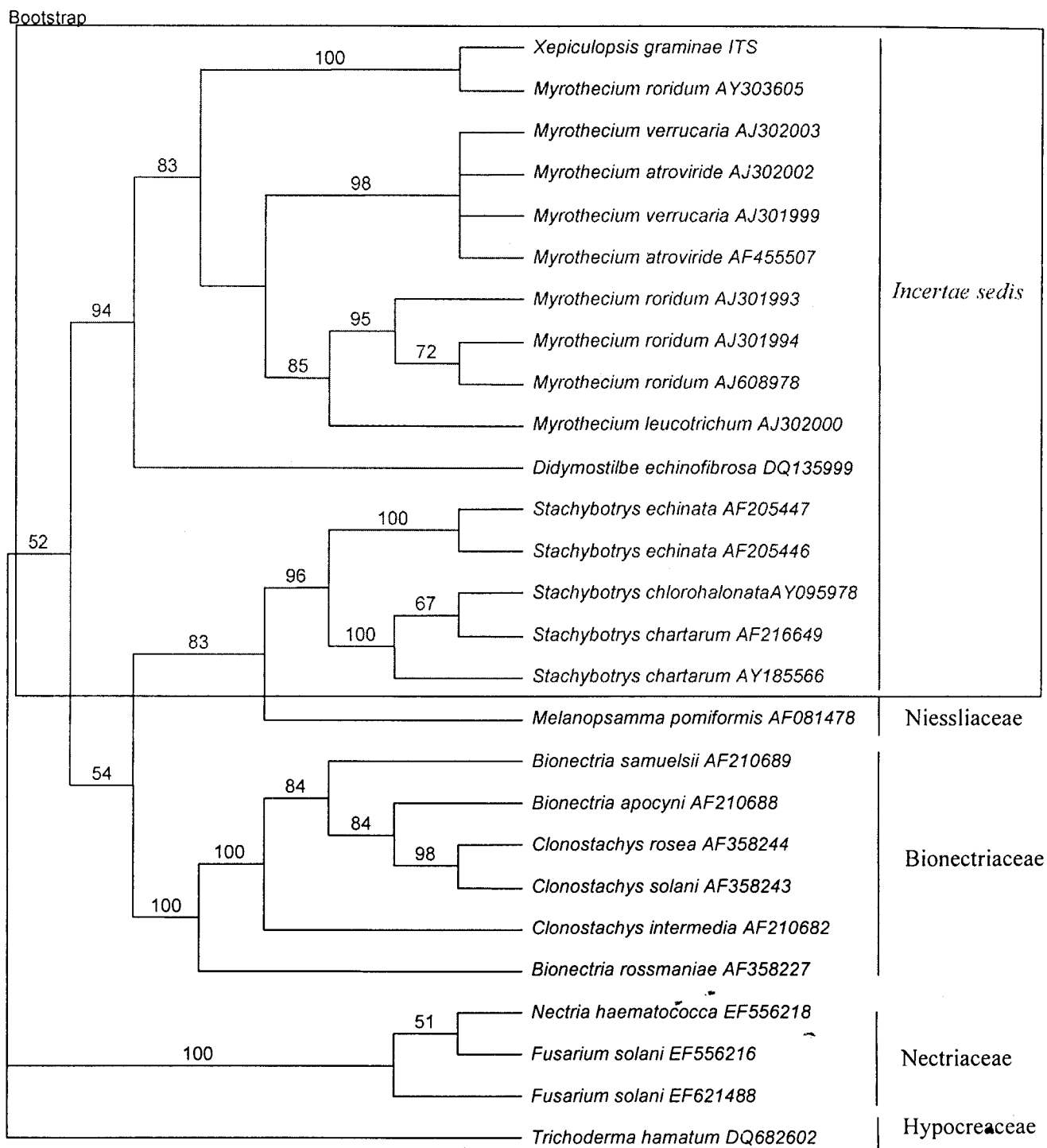


Figure 10. A strict consensus tree inferred from ITS rDNA sequences of *Xepiculopsis graminea* within the Hypocreales. Maximum parsimony bootstrap values greater than 50% are indicated above the branches.

LSU and ITS phylogeny of *Pseudorobillarda siamensis*

Although the preliminary study of LSU and ITS rDNA sequences of this coelomycete demonstrate an affinity with the Dothideomycetes- the largest class of the Ascomycota. Further analysis did not support such a relationship. *Pseudorobillarda siamensis* does not group with any other taxa indicating its uncertain taxonomic position. Further sequences of related taxa are required to resolve its taxonomic position. *Pseudorobillarda sojiae* and *Pseudorobillarda texana* obtained from BIOTEC Culture Collection (BCC) will be sequenced and then analyzed together with the previous *Pseudorobillarda siamensis* data set.

5. Conclusions

1. Phylogenetically, *Chaetospermum camelliae*, *Chaetospermum artrocarpi*, *Guilia tenuis* and *Mycotribulus mirabilis* group within the Basidiomycota. This is the first report of these fungi as the members of the Basidiomycota.

2. The teleomorph of *Mycotribulus mirabilis*, *Guilia tenuis* and *Chaetospermum* species are phylogenetically related to the Agaricales, Corticiales and Sebaciales, respectively.

3. Although morphologically these coelomycetes are pycnidial and with appendaged conidia, they show no consistency in their phylogenetic relationship, belonging to disparate major taxonomic groups with putative teleomorphs in the Agaricales, Corticiales and Sebaciales.

4. The teleomorph of fusiform-conidial coelomycetes, consisting of *Robillarda sessilis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea* are located in the Xylariales, Pleosporales and Hypocreales, respectively.

5. The teleomorph of *Robillarda sessilis* is to be found in the Amphisphaeriaceae (Xylariales, Xylariomycetidae, Sordariomycetes).

6. The teleomorph of *Xepiculopsis graminea* is to be in the Hypocreales. Phylogenetically, the teleomorph of *Xepiculopsis graminea* could be *Myrothecium* species (*Incertae sedis*, Hypocreales, Hypocreomycetidae, Sordariomycetes).

7. Sequences of *Robillarda sessilis*, *Pseudorobillarda siamensis* and *Xepiculopsis graminea* indicate they are not related, therefore possession of fusiform appendaged conidia is not phylogenetically significant.

6. Future Plans

1. The manuscript on the phylogenetic observation of the fusiform-conidia coelomycetes is being drafted.

2. The rDNA sequence comprising SSU, LSU and ITS for new 19 selected coelomycetes will be sequenced in order to continue the third year project of the phylogenetic relationship of selected coelomycete genera (BRT R_249005).

7. Outputs

1. The draft manuscript entitled “The significance of cupulate-conidiomata and the phylogenetic relationship of the coelomycete genera, *Infundibulomyces* and *Satchmopsis*” submitted to *Mycologia*, a journal published by the Mycological Society of America. The manuscript has been reviewed and a major revision has been suggested and this is in progress.

2. The draft manuscript entitled “Putative basidiomycete teleomorphs and phylogenetic placement of the coelomycete genera: *Chaetospermum*, *Giulia* and *Mycotribulus* based on nuclear rDNA sequences”, was submitted to *Mycological Research*- a journal published by British Mycological Society (BMS).

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Part B: Appendix

Appendix 1

1) Draft manuscript entitle “Putative basidiomycete teleomorphs and phylogenetic placement of the coelomycete genera: *Chaetospermum*, *Giulia* and *Mycotribulus* based on nuclear rDNA sequences”, was submitted to Mycological Research- a journal published by British Mycological Society (BMS).

MYCOLOGICAL RESEARCH

Putative basidiomycete teleomorphs and phylogenetic placement of the coelomycete genera: *Chaetospermum*, *Giulia* and *Mycotribulus* based on nuclear rDNA sequences

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ABSTRACT

Few basidiomycetes are known to have a coelomycete anamorph. The partial small subunit and large subunit of nuclear rDNA of three coelomycete genera (*Chaetospermum*, *Giulia*, *Mycotribulus*) were sequenced to determine their phylogenetic relationship. *Mycotribulus mirabilis* was well placed in the marasmioid clade (Marasmiaceae and Physalacriaceae),

Agaricales, while *Giulia tenuis* clusters with the Corticiaceae, Corticiales. *Chaetospermum camelliae* and *Chaetospermum artocarp*i form a closely relationship with the Sebacinaceae, Sebaciniales. Although morphologically these coelomycetes are pycnidial and with appendaged conidia, they show no consistency in their phylogenetic relationship, belonging to disparate major taxonomic groups with putative teleomorphs in the Agaricales, Corticiales and Sebaciniales. Further molecular studies of coelomycetes may be rewarding to evaluate their phylogenetic affinities.

INTRODUCTION

Over 9,000 coelomycetes in *circa* 700 genera have been described (Kirk *et al.* 2001), from a wide range of substrata and geographical locations (Sutton 1980, Nag Raj 1993). A small number have been linked to their teleomorphs, with affinities to ascomycetes while a few are basidiomycetes (Nag Raj 1978, 1980; Dykos & Sutton 1979; Cole & Samson 1979; Nag Raj *et al.* 1989). Most of these connections have been established by culture techniques or morphological observation of clamp connections. Sequencing techniques have enabled an alternative approach to determine the putative teleomorphs of anamorphic fungi (Fernández *et al.* 1999; Liu *et al.* 2002).

A survey of coelomycetes in Thailand was embarked upon as they are poorly documented for the principality. Studies included their isolation, ultrastructure of selected species with appendaged conidia (Plaingam *et al.* 2004), and their ability to produce bioactive compounds (Seephonkai *et al.* 2002). New taxa include the genus *Infundibulomyces* (Plaingam *et al.* 2003; Somrithipol *et al.* 2007) and *Pseudorobillarda siamensis* (Plaingam *et al.* 2005).

Molecular sequencing of selected species was performed to determine their putative teleomorphs, resulting in three species being assigned to the Basidiomycota.

MATERIALS AND METHODS

Specimen collection and culture maintenance

Selected coelomycetes included three strains of *Chaetospermum camelliae*, one of *Chaetospermum artocarp*i, two strains of *Mycotribulus mirabilis* and a single isolate of *Giulia tenuis*. Details of collection sites and substratum, and isolate accession codes are listed in Table 1. All strains are deposited in the BIOTEC Culture Collection (BCC).

Fungal cultivation

Fungi were grown on potato dextrose agar (PDA) and transferred into potato dextrose broth (PDB) without agitation at 25 °C for 2 weeks for DNA extraction. Mycelium was harvested, washed with warm sterilized distilled water, frozen at -80 °C for 1-2 hours, and the fungal biomass ground into fine powder with sterilized mortar and pestle.

DNA extraction and PCR amplification

DNA was extracted using CTAB lysis buffer (O'Donnell *et al.* 1997) and incubated at 65 °C for 1 hour. The mixture was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1). The upper liquid phase was precipitated with 7.5 M ammonium acetate and absolute ethanol and kept at -20 ° for at least 30 min. Extracted DNA was washed twice with 70% ethanol, air dried and the DNA resuspended in 50 µl TE buffer.

Partial small subunit (SSU) and large subunit (LSU) regions of rDNA were amplified with gene specific primers: NS1, NS6 and LROR, LR7, respectively (White *et al.* 1990; Bunyard *et al.* 1994) using FINNZYMES, DyNAzyme™ II DNA polymerase kit (Cat No F-551S, Finnzymes, Finland). The amplification cycles were performed following White *et al.* (1990) and Bunyard *et al.* (1994) with a DNA Engine DYAD ALD 1244 thermocycler (MJ Research, Inc). The PCR products were purified with NucleoSpin® Extract DNA purification kit (Cat. No. 740 609.50, Macherey-Nagel, Germany) following the manufacturer's instruction and then sequenced by Macrogen Inc. (Korea) using the same primers as for amplification.

Sequence alignment and phylogenetic analysis

A BLAST search was employed to obtain the closest matched sequences in the GenBank database (Altschul *et al.* 1990). The SSU and LSU rDNA sequences were multiple aligned along with other related sequences obtained from GenBank using Clustal W 1.6 (Thompson *et al.* 1994) and adjusted manually where necessary using BioEdit 7.5.0.3 (Hall 2006).

The aligned dataset was subsequently analysed using maximum parsimony in PAUP* 4.0b10 (Swofford 2002), for the most parsimonious trees (MPTs). Heuristic searches algorithm with tree-bisection-reconnection (TBR) branch swapping, 100 replicates of random stepwise sequence addition, were performed. Gaps were treated as missing data and given equal weight. The tree length, consistency indices (CI) and retention indices (RI) were calculated for each tree generated. The Kishino-Hasegawa (K-H) test was used for estimation of the best tree topology (Kishino & Hasegawa 1989).

Bayesian phylogenetic inference was calculated with MrBayes 3.0b4 with general time reversible (GTR) model of DNA substitution and a gamma distribution rate variation across sites

(Huelsenbeck & Ronquist 2001). Four Markov chains were run from random starting trees for 2 000 000 generations and sampled every 100 generations. The first 100 000 generations were discarded as burn-in of the chain. A majority rule consensus tree of all remaining trees was calculated.

Statistical support for the internal branches was estimated by bootstrapping analysis (Felsenstein 1985) with 1000 replications (10 replicates of random stepwise sequence addition, TBR branch swapping) and posterior probabilities were performed. The maximum parsimony bootstrap values ($\geq 50\%$) and Bayesian posterior probabilities (≥ 0.95) are shown above and below the tree branches, respectively. The rDNA sequences, consisting of SSU and LSU were submitted into the GenBank database (Table 1). The accession numbers for all sequences derived from the GenBank database are included in the phylogenetic trees. The new sequences generated for *Chaetospermum*, *Giulia* and *Mycotribulus* are shown in Table 1. The alignments are deposited in TreeBase: xxxx, xxxx.

RESULTS

SSU phylogeny

The SSU rDNA sequences of seven of coelomycete isolates were incorporated with 55 taxa from the Agaricales, Corticiales, Polyporales, Sebaeinales and Tremellales (Agaricomycotina) (Hibbett *et al.* 2007). Two taxa from the Taphrinales (Taphrinomycotina) were selected as the outgroup. The seven coelomycete isolates nestled within the Basidiomycota with high statistical support (100% bootstrap value and 1.00 posterior probabilities) (Figure 1). Two strains of *Mycotribulus mirabilis* and *Giulia tenuis* are nestled in the Agaricales and

Corticiales (Agaricomycetidae), respectively, while *Chaetospermum* species showed phylogenetic affinity with members of the Sebacinales (Tremellomycetidae).

The two *Mycotribulus mirabilis* strains are monophyletic with 97% bootstrap value and 1.00 posterior probabilities and well placed within the Agaricales with 88% bootstrap value and 1.00 posterior probabilities (Figure 1 subclade A). In an analysis of eight families of the Agaricales, *Mycotribulus mirabilis* formed a sister clade with various genera of the Marasmioid clade (Marasmiaceae and Physalacriaceae) with 85% bootstrap value and 1.00 posterior probabilities.

Giulia tenuis falls within the Corticiales, but with low statistical support. *Giulia tenuis* sequences were aligned with selected taxa from the Corticiales (Figure 1 subclade B). The closest relationship was with *Tretopileus sphaerophorus* (Basidiomycota *Incertae sedis*) (Okada *et al.* 1998) with high statistical support (86% bootstrap value and 1.00 posterior probabilities), with *Marchandiomyces aurantiacus* (*Incertae sedis*, Ceratobasidiales, Agaricomycetidae) and *Galzinia incrustans* (Agaricales) as sister groups.

All the *Chaetospermum* strains group within the Sebacinales and formed a monophyletic group with *Sebacina* species as a sister group (Figure 1 subclade C).

LSU phylogeny

Seven LSU rDNA sequences were aligned with 75 representative taxa from several families of the Agaricales, Corticiales, Polyporales, Sebacinales and Tremellales with the Taphrinales as the outgroup. LSU sequences confirm that the selected coelomycetes fall firmly within the Basidiomycota with 100% bootstrap value and 1.00 posterior probabilities (Figure 2).

Mycotribulus mirabilis shows the greatest phylogenetic affinity with members of the Agaricales, and are monophyletic with 100% bootstrap value and 1.00 posterior probabilities (Figure 2 subclade A). They formed a sister group to *Physalacria orinoceansis* and *Cylindrobasidium laeve* (Physalacriaceae) and three *Marasmius* species, *Flammulina velutipes*, *Oudemansiella radicata* and *Xerula furfuracea*, but with low support. Nevertheless, *Mycotribulus mirabilis* still shows phylogenetic affinity with *Cylindrobasidium laeve* as in the SSU phylogeny.

The LSU rDNA sequence of *Giulia tenuis* displayed a phylogenetic relationship within the Corticiales and groups within a clade comprising the *Corticaceae* (Figure 2 subclade B).

Chaetospermum species are monophyletic with a close relationship between *C. camelliae* and *C. artocarpi* in LSU and SSU phylogeny. The *Chaetospermum* subclade forms a sister taxon with *Craterocolla cerasi* (Sebacinaceae) (92% bootstrap value and 1.00 posterior probabilities) (Figure 2 subclade C), but the subclade with *Chaetospermum artocarpi* and *Craterocolla cerasi* is weakly supported.

DISCUSSION

In this discussion, the classification proposed by Hibbett *et al.* (2007) is followed. A wide range of basidiomycetes have been shown to have hyphomycete anamorphs (Kendrick & Watling 1979; Walther *et al.* 2005; Walther & Weiß 2006) and these appear to be more common in certain groups than others e.g. the Bolbitiaceae (Walther & Weiß 2006). Previously only seven basidiomycete genera were known with coelomycete anamorphs: *Cenangiomycetes luteus* (Dykos & Sutton 1979), *Ellula guadaue* (Nag Raj 1980), *Fibulocoela indica* (Nag Raj 1978) *Helicogloea angustispora* (Kirschner 2004), *Pycnovellomyces folliicola* (Nag Raj *et al.* 1989),

Basidiopycnis hyalina and *Proceropycnis pinicola* (Oberwinkler *et al.* 2006) and their taxonomic position is summarized in Table 2.

Sporodochial coelomycetes for which molecular phylogenetic data is available include *Helicogloea angustispora*, *Leucogloea compressa*, *Basidiopycnis hyalina* and *Proceropycnis pinicola*, all genera assigned to the Atractiellales (Microbotryomycetes). *Infundibura adhaerens*, a sporodochial species, has basal asymmetrical conidial appendages, initially enrobed in an inconspicuous matrix, the terminal appendages are knob-like, becoming empty at maturity (Nag Raj & Kendrick 1981). The fungus also produces setae adjacent to the conidia. The teleomorph is *Helicogloea angustispora*, supported by cultural and sequence data (Kirschner 2004). A related taxon is *Leucogloea compressa* designated by sequence data based on the sporodochial anamorph *Pleurocolla compressa* (Kirschner 2004).

Basidiopycnis hyalina (with an unnamed anamorph) produces synnemata, while *Proceropycnis pinicola* (unknown teleomorph) has conidia formed in pycnidia. Sequence data also places both genera in the Atractiellales (Oberwinkler and Bandoni 1982; Oberwinkler and Bauer 1989), with typical genera such as *Atractiella*, *Succoblastia*, *Helicogloea* and *Phleogena* (Hibbett *et al.* 2007).

Of the coelomycetes *C. tuteus*, *E. guadaue*, *F. indica* and *P. folliicola* designation as basidiomycota is based on the production of clamp connections (Nag Raj 1993). No teleomorph has been linked to them and no sequence data is available.

The three genera treated in this study, all have conidiomata with appendaged conidia (Figures 3-8), but phylogenetically are unrelated. *Mycotribulus mirabilis* groups within the Agaricales (Agaricomycetes, Agaricomycotina) (Hibbett *et al.* 2007) in the marasmioid clade comprising basidiomycetes with widely different morphologies, e.g. those with gills,

cyphellaceous, and gasteroid basidiomes (Binder *et al.* 2006). To this can now be added *M. mirabilis* a coelomycete anamorph with pycnidia. Within the marasmioid clade, *M. mirabilis* shows greatest affinity with *Physalacria orinocensis* and *Cylindrobasidium*.

The *Chaetospermum* species nestle in the Sebaciniales along with the anamorphic genera *Flahaulita* and *Opadorhiza*, a basal group in the Agaricomycotina with weak support. Hibbett *et al.* (2007) refers the order to the Agaricomycetes *Incertae sedis*, basidiomycetes with longitudinal septate basidia, gelatinous coral-like sporocarps, nonperforated septal pore cap (SPC), with clampless hyphae and a poorly understood group (Weiß *et al.* 2004). The absence of clamp connection in the Sebaciniales strengthens the link to *Chaetospermum*, as no clamp connections have been observed in that genus. However, members of the Sebaciniales are generally mycorrhizal, although there is a report of some producing basidiomata on soil (Wells & Oberwinkler 1982)

Giulia tenuis groups within a subclade comprising *Corticium salmonicolor*, *C. roseum* and *Erythricium laetum* (Corticaceae, Corticiales, Agaricomycetes *Incertae sedis*) (Hibbett *et al.* 2007). This is an order with effused or discoid basidiomata, smooth hymenophore, a monomitic hyphal system with clamped, rarely single septate hyphae (Hibbett *et al.* 2007), and including the genera *Corticium*, *Vaillieminia* and *Punctularia*. Anamorphs include *Rhizoctonia* and *Tretopileus*.

This study has linked further coelomycetes species with basidiomycete teleomorphs, bringing the total to thirteen (Table 2). For ten species no known basidiomycete teleomorph has been established and further cultural studies are required. However, four of these species can now be assigned to a basidiomycete order/family based on sequence data. It is interesting that many of these species have appendaged conidia (Figures 3-8) and the examination of further

coelomycetes may be rewarding. Of the four species studied here, they show no consistency in their phylogenetic relationship, belonging to disparate major taxonomic groups: Agaricales, Corticiales and Sebaciniales. Despite the fact that these few species are pycnidial and have appendaged conidia, they are not phylogenetically related, further proving the incongruity of molecular and morphological data.

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LEGENDS

Table 1. Collection site, substrata and GenBank accession number of *Chaetospermum camelliae*, *Chaetospermum artocarpi*, *Mycotribulus mirabilis* and *Giulia tenuis* sequenced in this study.

Table 2. Taxonomic affinities of coelomycetes with basidiomycete teleomorphs.

Figure 1. One of 60 MPTs inferred from SSU rDNA sequences of *Chaetospermum camelliae*, *C. artocarpi*, *Giulia tenuis* and *Mycotribulus mirabilis*. The maximum parsimony bootstrap values ($\geq 50\%$) and Bayesian posterior probabilities (≥ 0.95) are shown above and below the branches, respectively (tree length = 975 steps, CI = 0.481, RI = 0.739). Coelomycetes sequenced in this study are printed in bold. The scale bar represents the number of changes per nucleotide position.

Figure 2. One of 12 MPTs inferred from LSU rDNA sequences of *Chaetospermum camelliae*, *C. artocarpi*, *Giulia tenuis* and *Mycotribulus mirabilis*. The maximum parsimony bootstrap values ($\geq 50\%$) and Bayesian posterior probabilities (≥ 0.95) are shown above and below the branches, respectively (tree length = 2,529 steps, CI = 0.329, RI = 0.693). Coelomycetes sequenced in this study are printed in bold. The scale bar represents the number of changes per nucleotide position.

Figures 3-8. Mature conidia of three coelomycetes used in this investigation. Figures 3-4. Mature conidia of *Chaetospermum camelliae* with 8 subpolar appendages. Bars = 10 μm . Figures 5-6. Mature conidia of *Giulia tenuis*, cylindrical with an obtuse apex and truncate base, straight or curved, unicellular, colourless, bearing 4-7 apical extra-cellular appendages; filiform, flexuous.

Bars = 20 μm . Figures 7-8. Mature conidia of *Mycotribulus mirabilis*, fusiform with a truncate base and acute apex, unicellular, colourless, bearing 3 basal appendages and a single apical appendage. Bars = 5 μm .

Table 1. Collection site, substrata and GenBank accession number of *Chaetospermum camelliae*, *Chaetospermum artocarpi*,

Mycotribulus mirabilis and *Giulia tenuis* sequenced in this study.

Taxa	Source*	Substratum and geographical origin	GenBank accession number	
			SSU	LSU
<i>Chaetospermum artocarpi</i> Nag Raj	BCC18581	Dead leaf of a broad leaf tree, Nakon Nayok, Thailand	EF589728	EF589735
<i>Chaetospermum camelliae</i> Agnihotr	BCC18582	Dead leaf of <i>Typha angustifolia</i> , Pathum Thani, Thailand	EF589729	EF589736
<i>C. camelliae</i> Agnihotr	BCC18604	Leaf litter, Bangkok, Thailand	EF589730	EF589737
<i>C. camelliae</i> Agnihotr	BCC13401	<i>Pandanus</i> leaf, Nam Nao Park, Phetchabun, Thailand	EF589731	EF589738
<i>Giulia tenuis</i> (Sacc.) Tassi ex Sacc.&D.Sacc.	BCC13066	Sheath of <i>Bambusa arundinacea</i> , Thailand	EF589732	EF589739
<i>Mycotribulus mirabilis</i> Nag Raj & W.B.Kendr.	BCC13341	Dead leaf of <i>Eucalyptus camaldulensis</i> , Bangkok, Thailand	EF589733	EF589740
<i>M. mirabilis</i> Nag Raj & W.B.Kendr.	BCC18601	Leaf litter, Bangkok, Thailand	EF589734	EF589741

*BCC = BIOTEC Culture Collection, Pathum Thani, Thailand

Table 2. Taxonomic affinities of coelomycetes with basidiomycete teleomorphs.

Classification	Teleomorph	Anamorph	Clamp	Substratum	Reference
Basidiomycota	No teleomorph known	<i>Cenangiomycetes luteus</i>	+	Pine needles, UK	Dyko & Sutton (1979)
Basidiomycota	No teleomorph known	<i>Ellula guadaue</i>	Cupulate	Dead culms of <i>Guadua</i> sp., Brazil	Nag Raj (1980)
Basidiomycota	No teleomorph known	<i>Fibulocoela indica</i>	Pycnidial	Rotting leaves of <i>Bambusa</i> sp.,	Nag Raj (1978)
Basidiomycota	No teleomorph known	<i>Helicogloea angustispora</i>	?	<i>Bucida palustris</i> , Cuba, India	Kirschner (2004)
Attractiellales		<i>Infundibura adhaerens</i>		Cones of <i>Pinus sylvestris</i> on ground	Kirschner (2004)
Platyglloeaceae?					Nag Raj & Kendrick (1981)
	No teleomorph known	<i>Pycnovellomyces</i>	+	Dead leaves of <i>Mimusops</i>	Nag Raj <i>et al.</i> (1989)
		<i>foliicola</i>	Pycnidial	<i>commersonii</i> ,	
				Cuba	
Basidiomycota	<i>Leucogloea compressa</i>	<i>Pleurocolla compressa</i>	Sprodochial	Dead wood	Kirschner (2004)
Attractiellales				Taiwan, USA	
Basidiomycota	<i>Basidiopycnis hyalina</i>	Unnamed synnematosus	-	Bark beetles in bark of <i>Picea abies</i>	Oberwinkler <i>et al.</i> (2006)
Attractiellales		fungus	Pycnidial	Germany, Italy, Switzerland	

Table 2. (Cont.)

Classification	Teleomorph	Anamorph	Clamp	Substratum	Reference
Basidiomycota	No known teleomorph	<i>Proceromyces pinicola</i>	-	Wood of <i>Pinus pinea</i> and <i>P. pinaster</i>	Oberwinkler <i>et al.</i> (2006)
Atractiellales			Pycnidial		
Basidiomycota	<i>Hyalopycnis</i>	<i>Heterogastridium</i>	Pycnidial	Spain, China, Taiwan	Oberwinkler & Bauer
Microbotryomycetes	<i>blepharistoma</i>	<i>pycnidiodeum</i>		One collection produced basidiospore	(1990)
<i>Incertae sedis</i>					
Heterogastridiales					
Basidiomycota	No teleomorph known	<i>Chaetospermum</i>	-	Leaves, petioles of various plants,	Nag Raj (1993)
Sebacinales		<i>artocarp</i> ,	Pycnidial	Wide distribution	
Sebaciniaceae		<i>C. camelliae</i>			
Basidiomycota	No teleomorph known	<i>Giulia tenuis</i>	-	Dead stems of <i>Lepidium</i>	Nag Raj (1993)
Corticiales			Pycnidial	<i>graminifolium</i>	
Corticaceae				Thai material? Italy	
Basidiomycota	No teleomorph known	<i>Mycotribulus mirabilis</i>	?	Rotting leaves <i>Eucalyptus</i> spp.,	Nag Raj & Kendrick (1970)
Agaricales			Pycnidial	<i>Mangifera indica</i>	
Marasmioid clade				Cuba, India, West Indies	
Physalacriaceae					

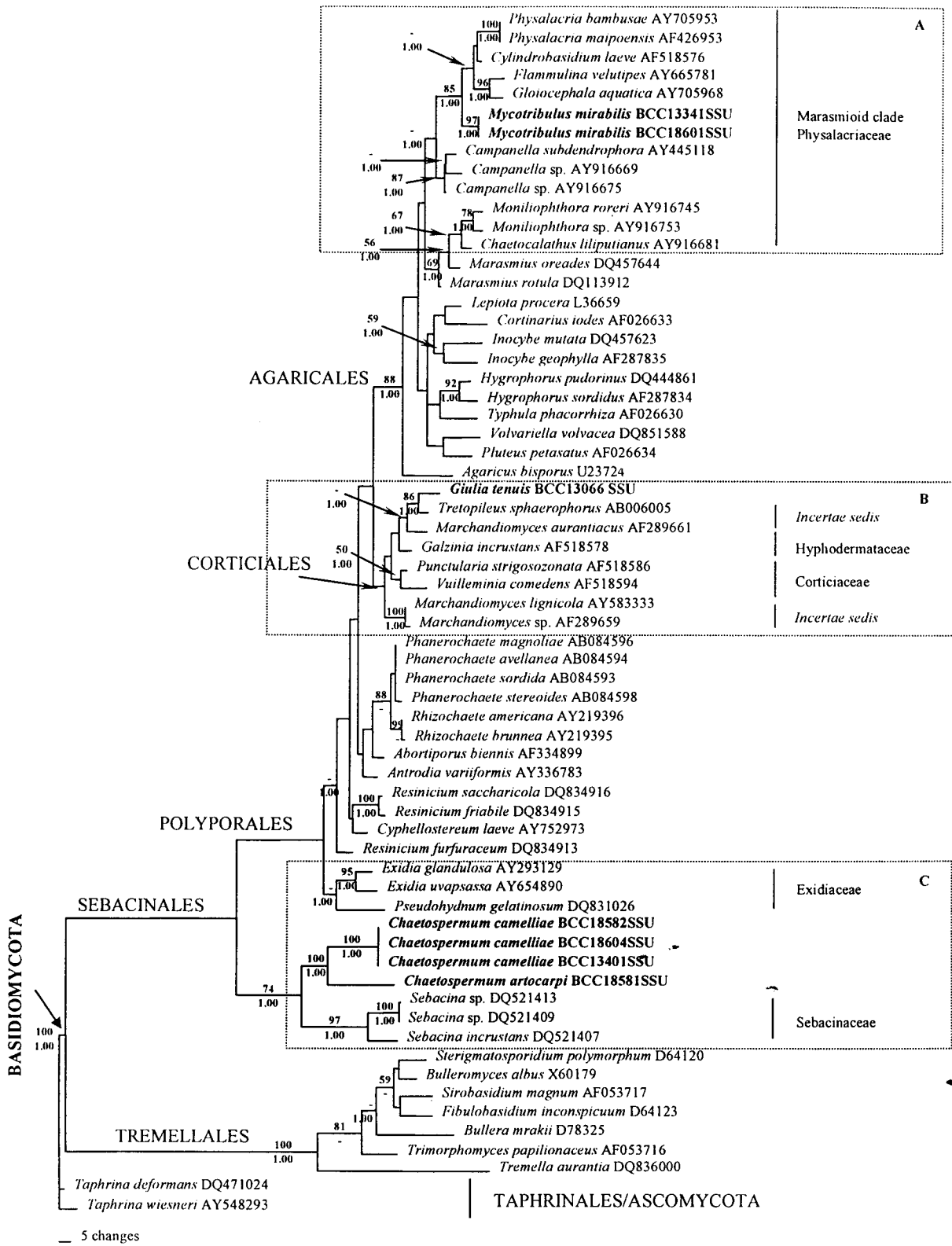


Figure 1.

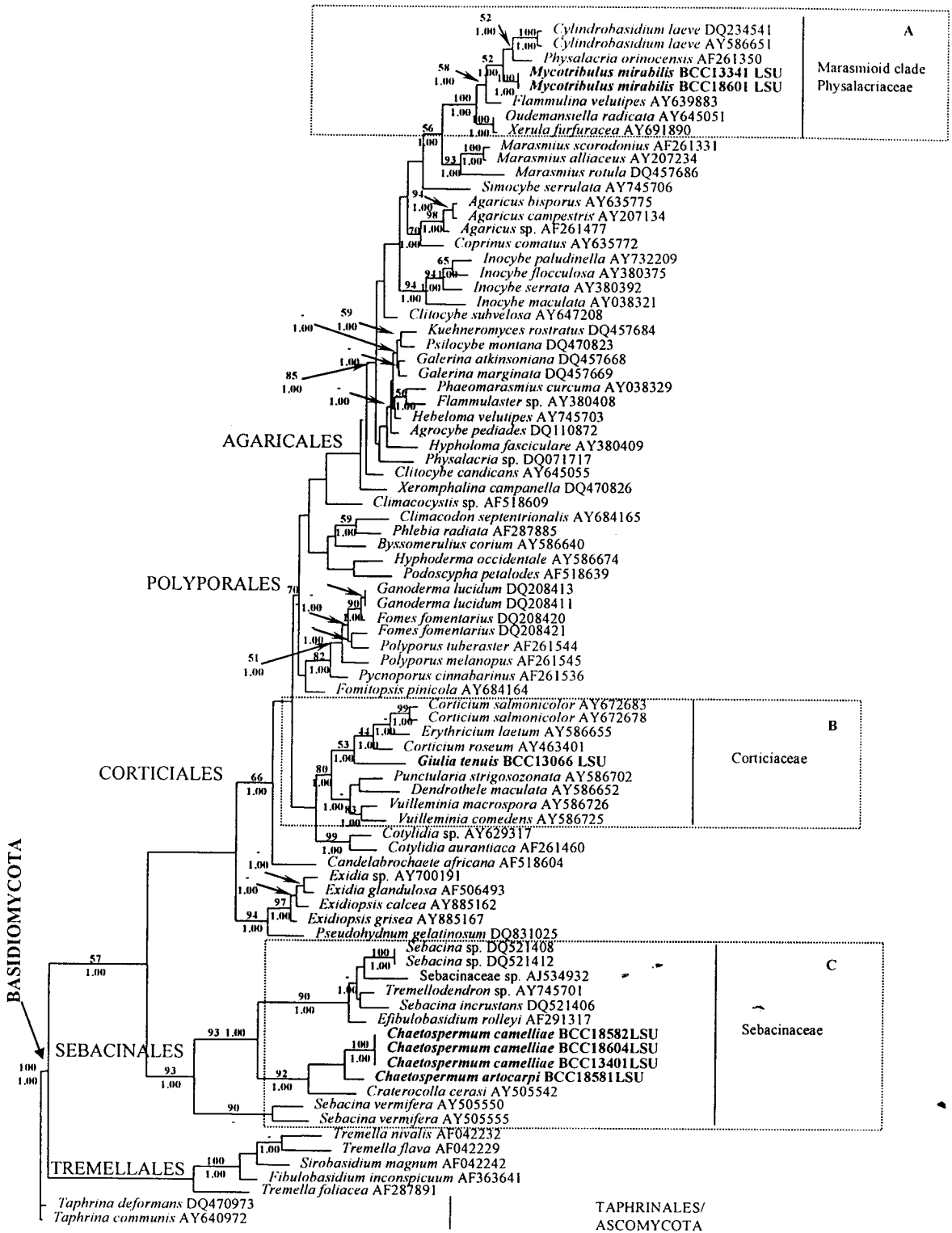
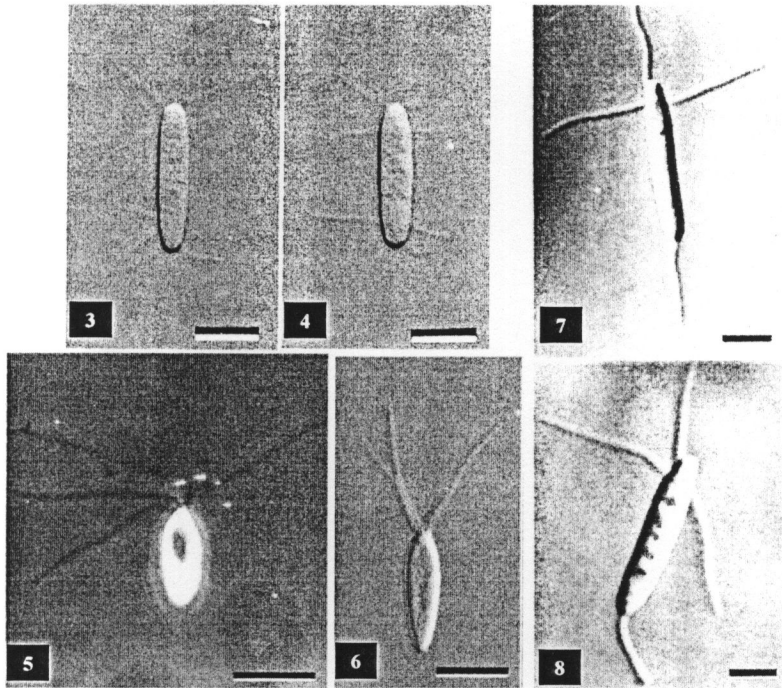


Figure 2.



Figures 3-8