CHAPTER 4

IDENTIFICATION OF MYCELIA STERILIA FUNGI BY MOLECULAR TECHNIQUES

Classifications systematic of organisms are historically based on observable or morphological characteristics. Fungal systematic is still based mainly on morphological criteria. The classical light microscopic methods have been enhanced by Nomarski differential interference contrast, fluorescence, cytochemistry, and the development of new staining techniques such as those for ascus apical structures. Unfortunately, during growth, some fungi show only the vegetative phase (absence of sporulation); in host tissue, only hyphal elements or other nonspecific structures are observed. Non-sporulating fungi were delimited by Bill (1996) as mycelia sterilia and grouped them into morphospecies. Although the pigmentation and shape of these hyphae and the presence or absence of septa can give us an idea of their identity, fungal culture is required for accurate identification.

Numerous alternative approaches have been developed, including nutritional and physiological studies, serologic tests, secondary metabolites, ubiquinone systems, and fatty acids. Although some of these are very useful for identifying poorly differentiated fungi such as yeasts and black yeasts, they are only complementary tools of morphological data in most cases. Molecular biology techniques, especially the analysis of rRNA sequences, are currently used for reliable phylogenetic studies, which enable a more natural classification system to be established. Comprehensive

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and detailed reviews of the use of molecular techniques in fungal systematics have been provided by Bruns *et al.* (1991), Hibbett (1992) and Kohn (1992).

Molecular techniques have been successfully used for the phylogenetic placement of non-spore forming fungi. For example, Guo *et al.* (2003) identified 18 white morphotype strains of mycelia sterilia from *Pinus tabulaeformis* to various taxonomic levels based on nuclear ribosomal DNA (nrDNA) sequence analysis. Lacap *et al.* (2003) compared nucleotide sequence similarities of ITS regions and 5.8S gene and identified six morphotypes of mycelia sterilia based on rDNA sequence analysis, all of which have been identified to genus level. Two years later, Promputtha *et al.* (2005) identified 31 morphospecies to generic level based on ITS and 5.8S regions.

During the study of filamentous fungi on brown rice (including paddy rice) (Chapter 3), numerous sterile mycelia were encountered and grouped into 12 morphospecies. To determine the phylogenetic position of the mycelia sterilia, 28S gene and ITS regions were used. Because the 28S gene is highly conserved, this region is used for the phylogenetic analysis of higher taxonomic level, whereas the highly variable ITS regions are used for analysis of lower taxonomic level.

The objectives of this study were 1) to identify non sporulating fungi and classify them to familial and generic level and 2) to determine their phylogenetic relationships with related species.

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4.1 MATERIALS AND METHODS

SOURCES OF FUNGAL CULTURES

The fungi were isolated from several rice varieties outlined in Chapter 3. The cultures were examined periodically and identified when isolates sporulated. The remaining isolates which failed to sporulate after promote sporulation were treated as mycelia sterilia. All mycelia sterilia were separated into morphospecies differentiated by culture characteristics such as colony surface, texture, hyphal pigmentation and growth rate on half PDA. The non-sporulating fungi were divided into 12 morphospecies and prefixed with MS (Table 4.1). They were identified following DNA sequence analysis. LSU and ITS sequences data were generated from these morphotypes to enable their identification.

4.1.1 DNA EXTRACTION

Fungal cultures were grown on PDA plates for 5-20 days and total DNA was extracted from fresh mycelium using the modified protocols of Lacap *et al*. (2003). Mycelium were directly scraped off the culture plates and transferred into a 1.5 ml centrifuge tube. Mycelium was mixed with 0.2 g of sterile white quartz sand and 600 μl of preheated (60 °C) 2X CTAB buffer [2% v/w CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, pH 8.0]. Mycelium was ground with a plastic pestle for 5-10 min and incubated at 60 °C for 40 min with occasional gentle swirling every 10 min. The solution was then extracted with an equal volume of chloroform : isoamyl alcohol (24:1) at 13000 rpm for 30 min two or three times or until no interface was visible. Two volumes of absolute cold ethanol were added and the tube was inverted gently and stored overnight at -20 °C to precipitate DNA. Contents were then centrifuged at

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Table 4.1 Cultural characteristics of 12 morphospecies (MS) of mycelia sterilia

Code	Colony on PDA after 7 days	Colony diameter after 7 days(cm)	Colony characters
MS1		5.5	White-grey, cotton, entire
MS2		5.2	Grey to pale brown, velvety, crenated
MS3		1.5 (3.5 cm after 14 days)	White to yellowish-green, reverse yellowish-green, fluffy, fimbriate
MS4		6.0	White, velvety, entire

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Table 4.1 (continued).

Code	Colony on PDA after 7 days	Colony diameter after 7 days(cm)	Colony characters
MS5		5.0	Greenish, fluffy, entire
MS6		4.2	Greenish-brown, fluffy, entire
MS7		7.0	Pinkish, velvety, entire
MS8		3.0	White, fluffy, entire

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Table 4.1 (continued).

Code	Colony on PDA after 7 days	Colony diameter after 7 days(cm)	Colony characters
MS9		5.5	Pale yellow at middle and white at outer, fluffy, entire
MS10		7.0	Pinkish-orange, flat, entire
MS11		5.2	Greenish-brown, fluffy, entire
MS12		6.5	Grey at middle, white at outer, flat, light radial fold

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11000 rpm for 30 min at 4 °C. The DNA pellet obtained was washed with 70% cold ethanol twice and dried under vacuum. The pellet was resuspended in suitable volume of TE buffer [10 mM Tris-HCl, 1 mM EDTA, pH 8] containing 1 mg/ml RNase A. In addition some tissues specimens were extracted for DNA by using DNA extraction Kits (NucleoSpin® Plants II, Macherey Nagel, Catalog no. 740770.50) following manufacturer's protocol. DNA samples were checked for purity by electrophoresis in 1% (w/v) agarose stained with ethidium bromide (10 mg/ml).

4.1.2 PCR AMPLIFICATION AND SEQUENCING OF ITS AND 28S rDNA

Partial sequences from the different regions of the rDNA molecule were amplified. The primer pair LROR (5′-ACCCGCTGAACTTAAGC-3′) and LR5 (5′-TCCTGAGGGAAACTTCG-3′), as defined by Vilgalys and Hester (1990), were used to amplify a segment of the large 28S subunit approximately 850 nucleotides. In addition, primer pairs ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′), as defined by White *et al.* (1990), were used to amplify the complete ITS (including 5.8S) rDNA regions (about 500 nucleotides). Amplification was carried out in a 50 µl reaction volume using illustra hot start master mix kit (GE Healthcare, Catalog no. 25-1500-01) following the manufacturer's protocol. The thermal cycles consisted of 5 min initial denaturation at 95°C, followed by 30 cycles of 1 min denaturation at 95°C, 1 min primer annealing at 55°C, 1 min extension at 72°C, and a final 10 min extension at 72°C. Size and purify of PCR products were examined by electrophoresis in 1% (w/v) agarose gel with ethidium bromide (10 mg/ml). PCR products were purified using GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Catalog no. 27-9602-01)

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following the manufacturer's protocol. The purified PCR products were directly sequenced in an automated sequencer at Macrogen Sequencing System, Korea. The PCR primers mentioned above were used as sequencing primer.

4.1.3 PHYLOGENETIC ANALYSIS

Each sequence of the 12 morphospecies was used as query sequence to blast search for similar sequences in GenBank (http://www.ncbi.nlm.nih.gov/blast/). Nucleotide sequences of the 28S rDNA and ITS were initially aligned using BioEdit (Hall, 1999), Clustal X 1.83 with default parameter settings (Chenna *et al.,* 2003). Alignments were manually edited where necessary. Maximum parsimony (MP) and Neighbor joining (NJ) analyses were conducted using MEGA4 (Tamura *et al*., 2007). Tree searches were carried out using the heuristic method with a random stepwise addition and tree bisection and reconstruction branch-swapping algorithm. The topological analysis was performed with 1000 bootstrap replicates.

4.2 RESULTS AND DISCUSSION

4.2.1 DNA SEQUENCES OF 12 MYCELIA STERILIA

The ITS region (covering ITS1 region, 5.8S gene and ITS2 region) and LSU were amplified from 12 morphospecies of mycelia sterilia fungi. The sequences of all mycelia sterilia were submitted to GenBank. The GenBank accession number and length of each sequence are listed in Table 4.2.

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Table 4.2 Sequence length of 12 morphospecies of mycelia setrilia

Mycelia sterilia	Isolates	Sequence length (base pair) / GenBank accession number	
		ITS	LSU
MS1	BR354	548 / FJ971840	863 / FJ971833
MS2	BR479	563 / FJ971841	843 / FJ971834
MS3	BR159	564 / FJ971842	852 / FJ971835
MS4	BR311	614 / GQ141700	929 / FJ971836
MS5	BR386	551 / GQ141701	855 / GQ141697
MS6	BR393	560 / FJ971843	863 / FJ971837
MS7	BR488	508 / GQ141702	850 / AJ358496
MS8	BR439	537 / GQ141703	849 / AJ358495
MS9	BR329	526 / GQ141704	851 / FJ971838
MS10	BR522	534 / GQ141705	845 / GQ141698
MS11	BR550	538 / GQ141706	865 / GQ141699
MS12	BR307	517 / GQ141707	840 / FJ971839

4.2.2 IDENTIFICATION OF MYCELIA STERILIA MS1-MS12

The blast search for similar 28S rDNA and ITS regions in GenBank of 12 non-sporulating isolates showed that MS1-MS3, MS5-MS7, MS9 and MS11 are bitunicate ascomycetes, MS4 is basidiomycetes, while MS8, MS10 and MS12 are unitunicate ascomycetes.

The 28S rDNA sequences were used to clarify its order and/or familial placement. The phylogeny trees of 28S rDNA sequences were generated from a maximum parsimony analysis of 77 taxa. *Ganoderma lucidum* was used as out group. The consensus tree of 38 parsimonious trees is shown in Figure 4.1. The tree had a length (TL) of 594, a consistency index (CI) of 0.488, a retention index (RI) of 0.886, and the composite index of 0.444.

Maximum parsimony analyses reveal that MS1-MS3, MS6-MS7, MS9 and MS11 are species of *Pleosporaceae* (*Pleosporales*) while MS5 clusters with

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Massarina igniaria (*Massarinaceae, Pleosporales*) with 96% bootstrap support. MS4 clustered with species of *Polyporaceae* (*Polyporales*). MS8 claded with species of *Persiciospora* (Ceratostomataceae, Melanosporales) with high bootstrap support (99%). MS10 and MS12 grouped within species of Diaporthales.

Some of those taxa could be further identified to lower taxonomic level based on ITS and 5.8S gene sequences. Taxonomic placements of bitunicate, unitucate and basidiomycetes mycelia sterilia were constructed.

Taxonomic placement of bitunicate mycelia sterilia MS1-MS3, MS5-MS7, MS9 and MS11

After 28S rDNA analyses, MS1 clustered with MS3 but no bootstrap support. Both are the species of *Pleosporaceae.* MS1 and MS3 were further analyses to clarify its generic placement using ITS (including 5.8S) rDNA regions. The maximum-parsimony tree was generated from a maximum parsimony analysis of 26 taxa. *Gibberella thapsina* was used as out group. The tree obtained with bootstrap support is shown in Figure 4.2 [tree length (TL) of 280, a consistency index (CI) of 0.691, a retention index (RI) of 0.852, and the composite index of 0.657. Sequence analysis and percentage of nucleotide similarities indicated that MS3 belong to the genus *Alternaria* (Pleosporaceae). MS3 clustered with *Alternaria longissima* (=*Prathoda longissima*) with high bootstrap support of 99%. Simmons (2007) established *A. longissima* as a new genus (*Prothoda longissima*) based on morphological characteristics but this name still argued. Moreover, 28S reference sequences are limited in data base. However, the current taxonomic status of *A. longissima* needs further investigation as it is found to be distantly phylogenetically related to other

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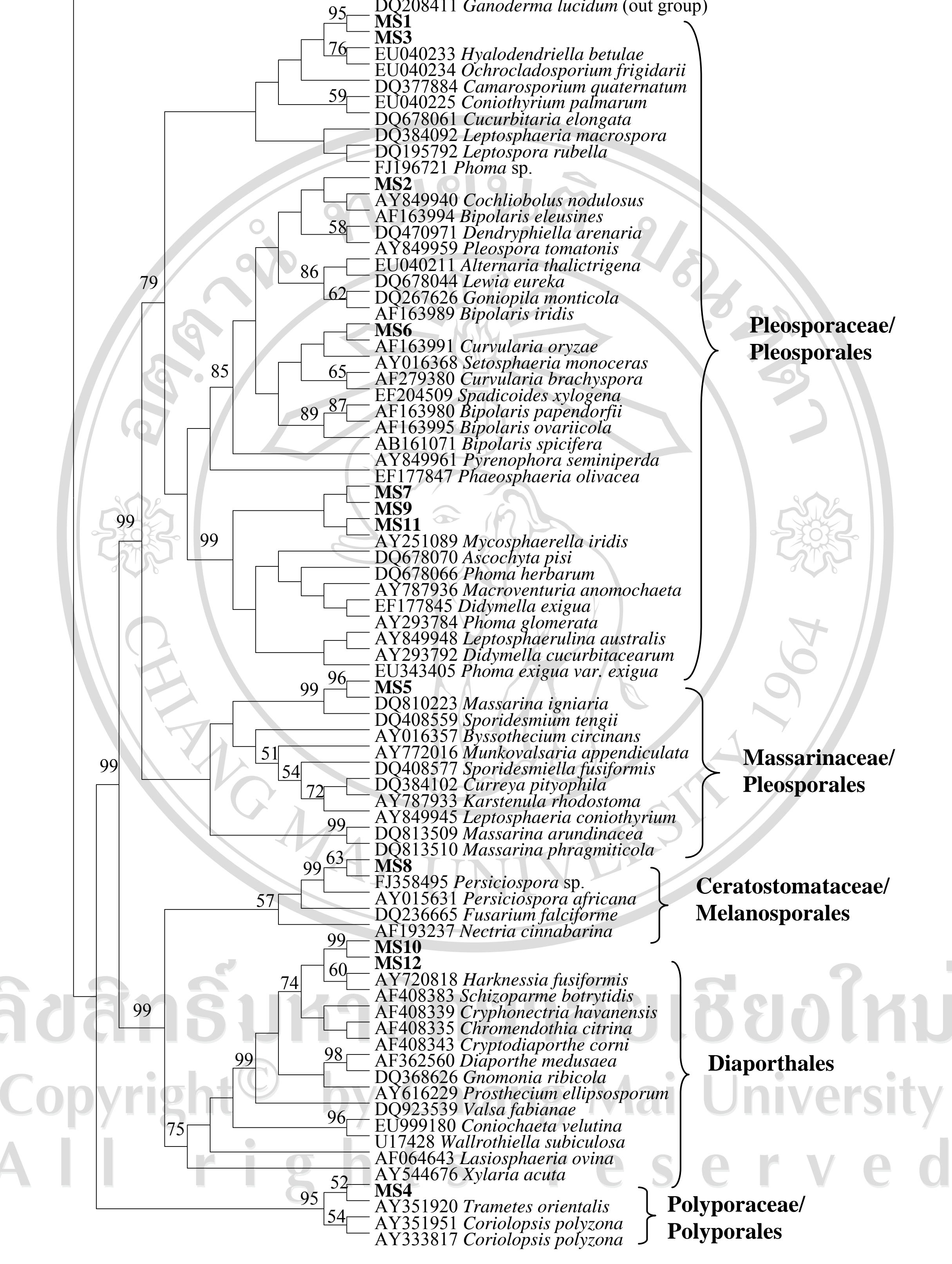

Figure 4.1 Maximum-parsimony tree generated from the 28rDNA sequences of 77 taxa show in the relationship of MS1-MS12 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches

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Alternaria species (Pryor and Gilbertson, 2000). MS1 clustered with *Dendriphiella* sp. with low bootstrap support of 63%. MS1 cannot place to species level because the reference sequences in data base are limited. MS1 need more further study such as other new gene/region.

MS2 belongs to family *Pleosporaceae* after 28S sequence analyses. Identification of MS2 to the lower taxonomic level based on ITS and 5.8S gene sequences was performed. The heuristic search under the maximum parsimony criterion yielded a tree (Figure 4.3) with tree length (TL) of 107, a consistency index (CI) of 0.560, a retention index (RI) of 0.607, and the composite index of 0.482. MS2 clusters with *Bipolaris oryzae* (*Pleosporaceae*) with moderate bootstrap support of 77%.

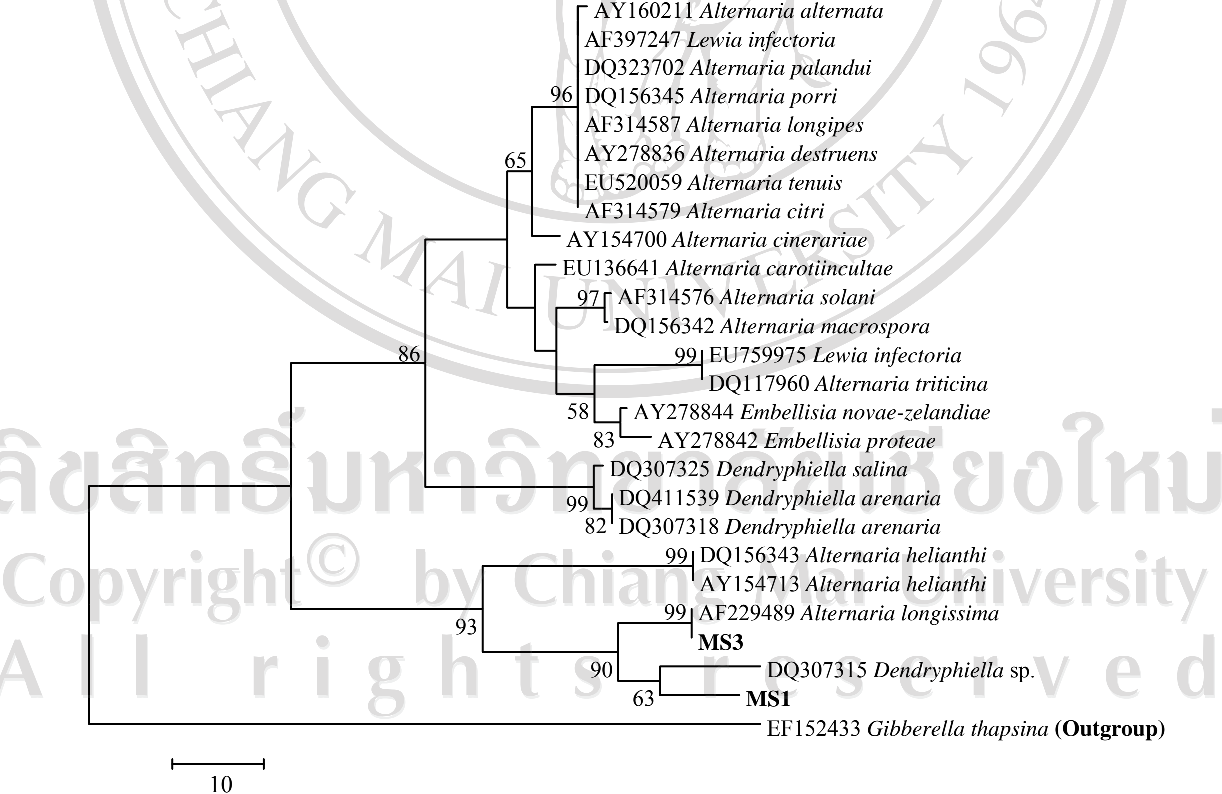

Figure 4.2 Maximum-parsimony tree generated from ITS1-5.8S-ITS2 sequence of 26 taxa showing the relationship of MS1 and MS3 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

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MS5 belongs to family *Massarinaceae* and further analyses showed that MS5 belongs to the genus *Massarina*. The parsimonious tree had a tree length (TL) of 417, a consistency index (CI) of 0.522, a retention index (RI) of 0.651, and the composite index of 0.368 (Figures 4.1, 4.4). There were a total of 262 positions in the final dataset, out of which 105 were parsimony informative. MS5 clustered with *M. eburnean* with low bootstrap support of 51%. From 28S sequence analysis, MS5 clustered with *M. igniaria* with high bootstrap support of 96% but it dose not have the similar ITS sequence from GenBank. Moreover, this genus was polyphyletic. Similar to Aptroot (1998) that he commented that the accepted species of *Massarina* probably do not form a monophyletic group. Liew *et al*. (2002) reported *Massarina* species should be transferred to other families, such as *Lophiostomataceae*. Currently, the family *Massarinaceae* should be circumscribed to include *Massarina, Keissleriella, Saccharicola, Helminthosporium* and *Aquaticheirospora* (Kodsueb *et al*., 2007). However, whether characteristics that significant in delineating, the *Lophiostomataceae* and *Massarinaceae* is still obscure at present. Thus, the study to assess the monophyletic of this genus is needed.

Identification of MS6 to the lower taxonomic level based on ITS and 5.8S gene sequences was performed. The heuristic search under the maximum parsimony criterion yielded a tree (Figure 4.5) with tree length (TL) of 161, a consistency index (CI) of 0.648, a retention index (RI) of 0.834, and the composite index of 0.606. MS6 clusters with *Curvularia* species with high bootstrap support of 99% and formed subclade with *C. oryzae* and *B. portulacae*. Both *Curvularia* and *Bipolaris* were polyphyletic (Berbee *et al*., 1999).

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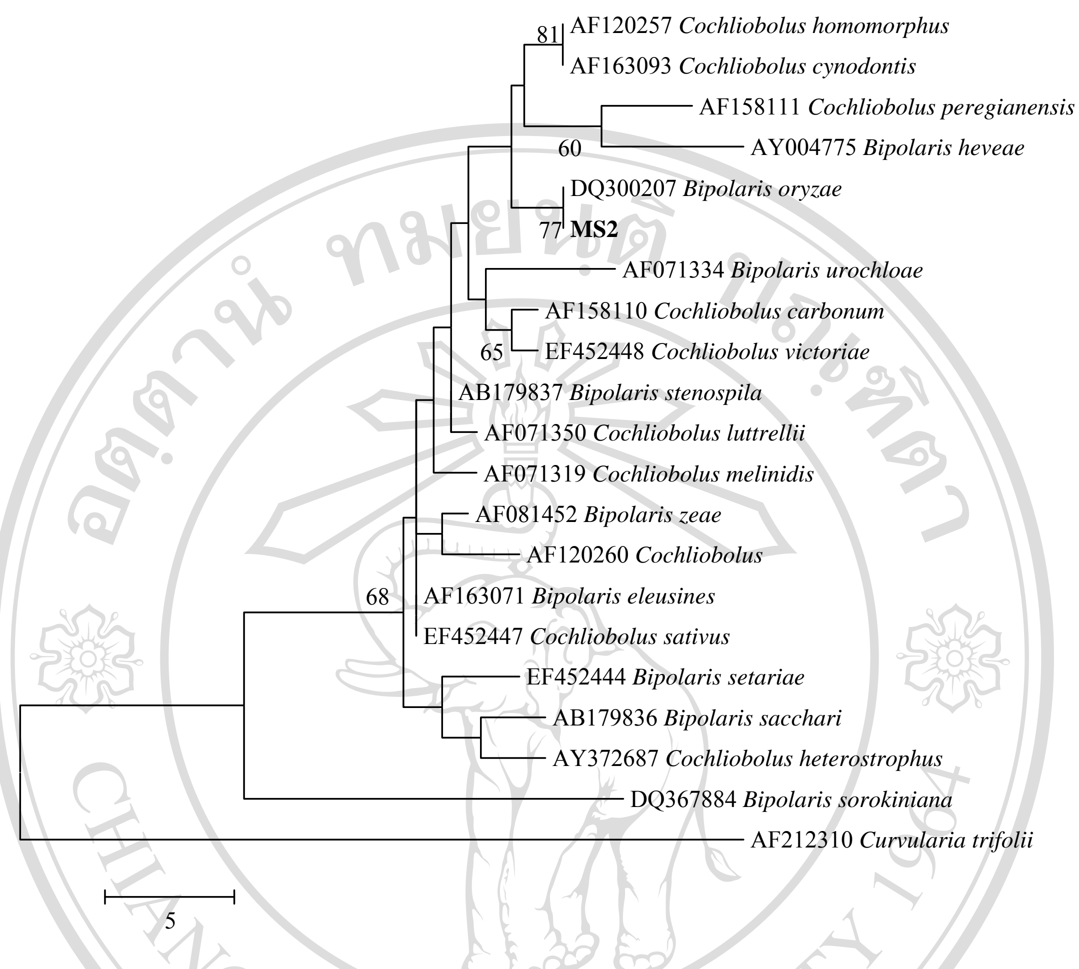

Figure 4.3 Maximum-parsimony tree generated from ITS1-5.8S-ITS2 sequence of 21 taxa showing the relationship of MS2 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

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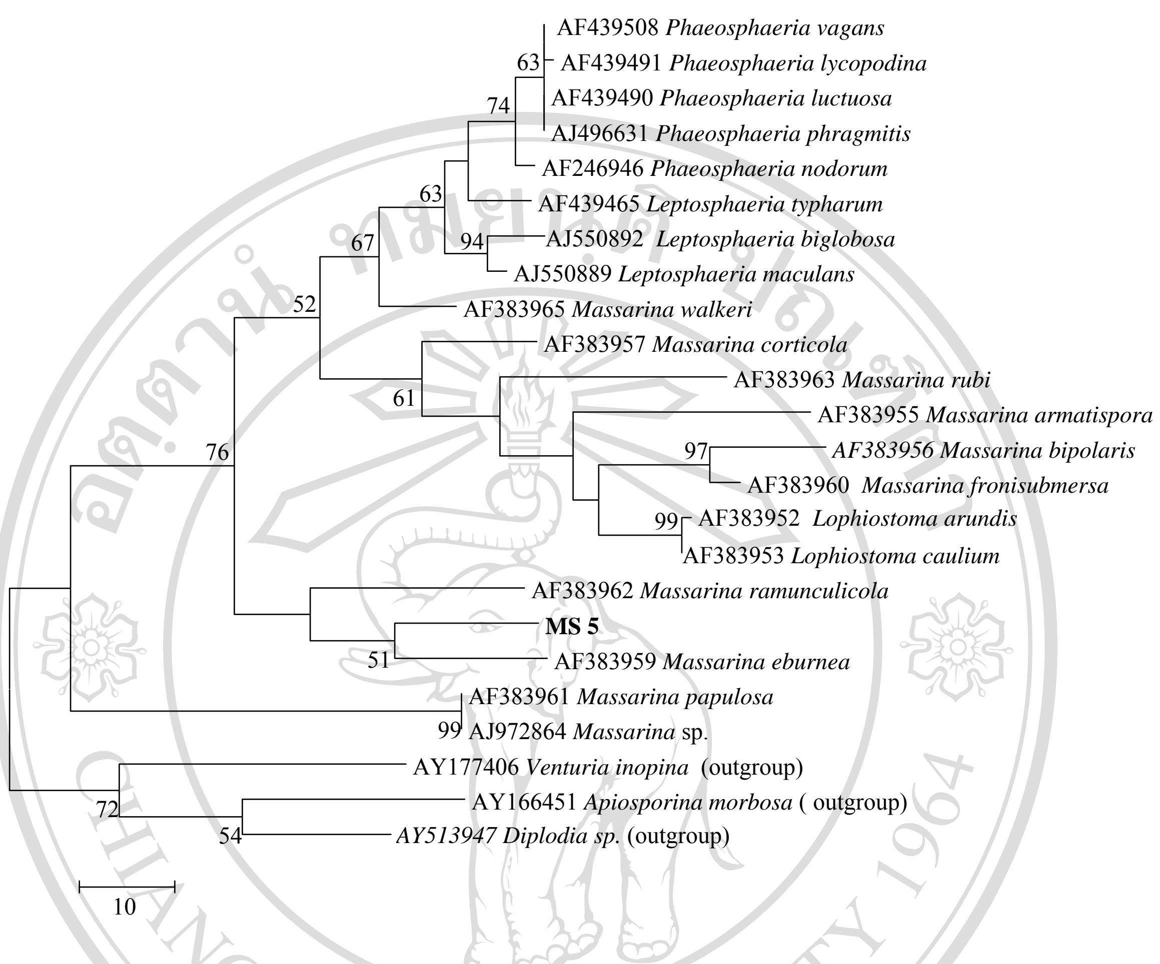

Figure 4.4 Maximum-parsimony tree generated from ITS1-5.8S-ITS2 sequence of 24 taxa showing the relationship of MS5 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

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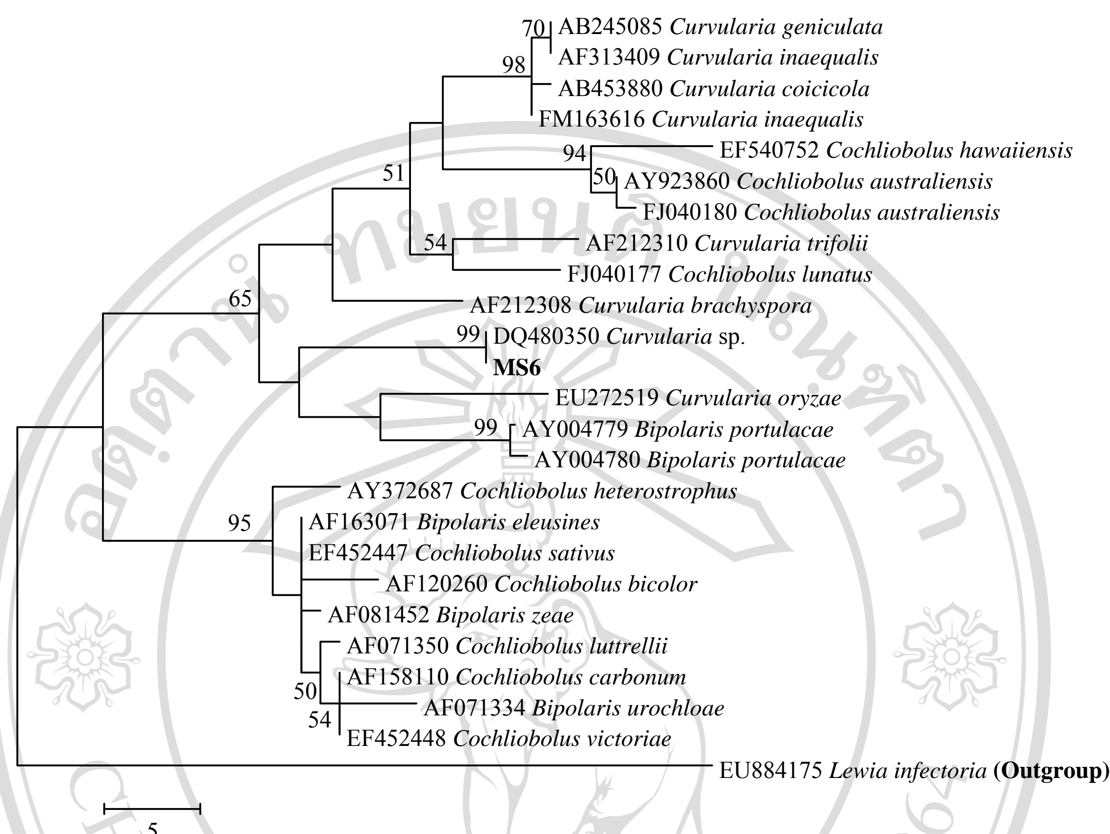

Figure 4.5 Maximum-parsimony tree generated from ITS1-5.8S-ITS2 sequence of 25 taxa showing the relationship of MS6 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

Blast search results indicated that MS7, MS9 and MS11 were highly similar to species of *Pleosporales* (97-99%). Phylogenetic analyses indicated that MS11 grouped with *Mycosphaerella iridis* (Figure 4.1) but no bootstrap support. Unfortunately, all those MS could not be further identified to lower taxonomic level because it dose not have reference ITS sequences in GenBank. MS7, MS9 and MS11 need to further study such as other new gene or new regions to get better solution.

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Taxonomic placement of unitunicate mycelia sterilia MS8, MS10, and MS12

MS8 belongs to family *Ceratostomataceae* (*Melanosporales*). Maximum parsimony analysis generated from 28S revealed that MS8 clusters with *Persiciospora* species with bootstrap support 99% and it was the subclade of *Fusarium falciforme* (Figure 4.1). MS8 could not be further identified to species level because only few reference sequences had been found from blast search (1 sequence of 18S and 2 sequences of 23S rDNA). *Persiciospora* was first described by Doguet (1955) as *Melanospora moraui.* Nowaday, *Perisiciospora* including four species, *P. moreaui* and *P. masonii* (Cannon and Howksworth, 1982), *P. japonica* from Japan (Horie *et al.,* 1986) and *P. africana* from South Africa (Krug, 1988). Zhang and Blackwell (2002) reported most of *Melanospora* are parasitic on or closely associated with other fungi. The wilt disease fungus *F. oxysporum* is one of the most common hosts, and some *Melanospora* species belongs to species of fuse with the host protoplasts to obtain nutrients, an interaction called fusion biotrophism. MS8 need to further study such as new gene or cultured it in the presence of *Fusarium* species some metabolites produced from *Fusarium* to induce its sporulation as recommended by Harveson and Kimbrough (2000).

MS10 and MS12 belong to the species of *Diaporthales.* Maximum parsimony analysis of 28S rDNA indicated that MS10 clusters with MS12 with high bootstrap support of 99%. They formed subclade with *Harknessia fusiformis* and *Schizoparme botrytidis*, but no bootstrap support. MS8 could not be further identified to genera or species level because the reference sequences in data base are limited. It need further study.

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Taxonomic placement of basidiomycetes mycelia sterilia MS4

MS4 belongs to the species of *Polyporaceae* (*Polyporales*) and further analyses showed that MS4 belongs to the genus *Coriolopsis*. Maximum parsimony analysis of aligned ITS1 & 2 and 5.8S sequences of 24 taxa were performed. The tree obtained with bootstrap support is shown in Figure 4.6 [tree length (TL) of 166, a consistency index (CI) of 0.550, a retention index (RI) of 0.783, and the composite index of 0.504]. MS4 clustered with *Coriolopsis polyzona* with bootstrap support of 97%.

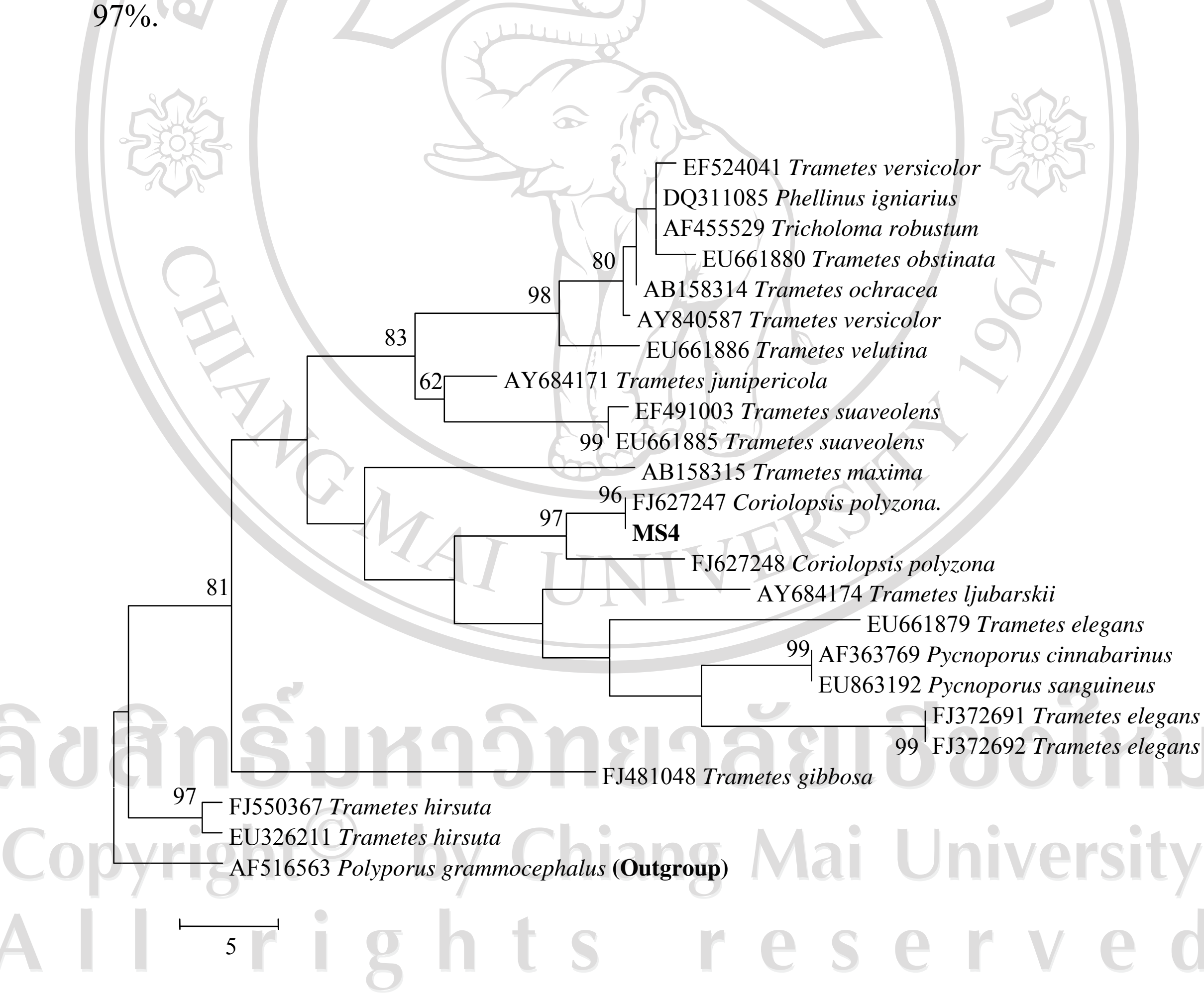

Figure 4.6 Maximum-parsimony tree generated from ITS1-5.8S-ITS2 sequence of 24 taxa showing the relationship of MS4 with reference taxa. Bootstrap values higher than or equal to 50% (1000 replicates) are shown at each branches.

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Sequence analyses of the 28S rDNA, 5.8S gene and ITS regions of rDNA have been widely used in taxonomic placement of fungi at different levels in recent years. Guo *et al.* (2003) identified 18 morphotypes from *Pinus tabulaeformis* to various taxonomic levels based on nrDNA sequence analysis. Lacap *et al.* (2003) compared nucleotide ITS and 5.8S rDNA sequence similarities and identified 6 morphotypes to genus and verified 'morphotypes' as taxonomic groups. Promputtha *et al.* (2005) identified 31 morphospecies to generic level based on ITS and 5.8S regions. Dela Paz *et al*. (2006) verified taxonomic placement of *B. oryzae* based on ITS and 5.8S rDNA to support the spore germination evidence. Kodsueb *et al.* (2006) redefined the family *Tubeufiaceae* based on 28S rDNA supported the morphological characteristic. Tang *et al*. (2007) used four gene regions (LSU rDNA, SSU rDNA, β-tubulin and RPB2) to supported phylogenetic analysis previously that *Boliniaceae, Chaetosphaeriaceae* and *Coniochaetaceae* were separated from *Sordariales* and placement of *Coronophorasles* in *Hypocreomycetidae*.

Although a degree of certainty exists in assigning morphotypes to genera based only on the sequence similarity comparison, cluster analysis of the 5.8S and ITS sequences of the rDNA gave results that agreed well with initial mycelia sterilia morphotype groupings. Strains within each morphotypes clearly grouped together as taxonomic units, whereas these different morphotypes being distinct taxa is unequivocal. In fact, it appears that fungal numbers estimated by designating each morphotype into a separate species may even be an underestimation. Although the morphotypes in this study have been shown to be valid taxonomic units, the approach of not conducting phylogenetic analysis precludes the assignment of these morphotypes into definite species. One of the greatest limitations in phylogenetic

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analyses in this regard is the restricted number of available sequences that could be used as reference taxa, which are crucial to taxonomic placements of test sequences (Guo *et al*., 2000).

4.3 CONCLUSION

This study has employed molecular techniques to increase our knowledge of fungal diversity. A similar approach was used to identify non-sporulating fungi from rice seeds. Forty taxa in 21 genera were identified following the identification of sporulating and colony characteristics (Chapter 3). A further 12 morphotypes were recognised for the non-sporulating isolates. Molecular analysis showed these morphotypes belong to 4 order (*Diaporthales*, *Melanosporales*, *Pleosporales* and *Polyporales*), 4 families (*Ceratostomataceae*, *Massarinaceae*, *Pleosporaceae* and *Polyporaceae*). Some of MS could be identified to the generic and/or species levels i.e. *Dendryphiella* (MS1), *Bipolaris* (MS2), *A. longissima* (MS3), *C. polyzona* (MS4), *Massarina* (MS5), *Curvularia* (MS6) and *Persiciospora* (MS8). The remaining MS need further study to place them to the real taxonomic level.

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