CHAPTER 3

MOLECULAR PHYLOGENY OF GENERA OF THE TRUE CERCOSPOROID FUNGI BASED ON ITS RIBOSOMAL DNA REGION SEQUENCE ANALYSIS

3.1. Introduction

In the cercosporoid fungi, until present time, only a few molecular phylogenetic analyses have been published worldwide. One of the first significant phylogenetic analyses was arguably published by Stewart *et al.* (1999) who reported the monophyletic of *Cercospora* Fresen., *Passalora* Fr., and *Pseudocercospora* Speg. based on ITS region of partial rDNA sequence analysis, and pointed out that *Ramulispora* Miura and *Mycocentrospora* Deighton are not related to *Mycosphaerella* Johanson teleomorph. Stewart *et al.* (1999) also reduced *Paracercospora* Deighton as synonym of *Pseudocercospora*. However, because of limited taxa were included in their analysis, it was not possible to determine the phylogenetic relationship of the cercosporoid species to other anamorphs genera within *Mycosphaerella* teleomorph.

Similar to the cercosporoid fungi, the taxonomy and phylogenetic of *Mycosphaerella* teleomorph is also complicated (von Arx, 1983; Crous *et al.*, 2000). Due to a large number of associated anamorphs, Crous and Wingfield (1996) pointed out that *Mycosphaerella* was a polyphyletic assemblage of presumably monophyletic anamorph genera. On the other hand, Goodwin *et al*. (2001), based on the analysis of a large number of anamorphs of *Mycosphaerella* using ITS region of rDNA sequence, also found that the genus *Mycosphaerella* was not monophyletic. Furthermore,

Goodwin *et al*. (2001) noticed that many anamorph genera within *Mycosphaerella* were polyphyletic. In order to test the polyphyletic of genus *Mycosphaerella*, Crous *et al*. (2007) analyzed Large Sub Unit (LSU) region of ribosomal DNA (28SrDNA) sequence, and reaffirmed the polyphyletic of *Mycosphaerella*. A new family, *Teratosphaeriaceae* Crous and U. Braun, was proposed to accommodate many extreme-tolerant species Crous *et al*. (2007).

Although the *Mycosphaerella* complex encompasses thousands of names, the study regarding the phylogenetic relationships among taxa in this group remains a few. This is partly due to the fact that these organisms are isolated and cultivated with plenty of difficulties, and in fact, the first to address the taxonomy of this complex based on DNA sequence data was only relatively recently published (Stewart *et al*., 1999). Fortunately, significant results have still been successfully produced from the relatively limited publications in the *Mycosphaerella* complex, such as the synonymous of *Paracercospora*, *Phaeoisariopsis* Ferraris, *Stigmina* Sacc., and *Cercostigmina* U. Braun to *Pseudocercospora*, *Mycovellosiella* Rangel and *Phaeoramularia* Muntk.-Cvetk. to *Passalora*. In the phylogenetic study of genus *Cercospora* alone, several authors reported the monophyletic of this genus (Stewart *et al*., 1999; Goodwin *et al*., 2001). Interestingly, Goodwin *et al*. (2001) noted that the *Cercospora* species with ability on producing toxin cercosporin was suggested to have a single evolutionary origin. On the other hand, Stewart *et al*. (1999) reported the monophyletic of genera *Passalora* and *Pseudocercospora* albeit with limited samples in the analysis. In addition, further extensive studies that include larger samples of taxa also suggested that the genus *Pseudocercospora* is monophyletic within *Mycosphaerella* teleomorph (Hunter *et al*., 2006; den Breeÿen *et al*., 2006;

Burgess *et al.*, 2007). However, the monophyletic of genus *Passalora* as indicated by Stewart *et al.* (1999) has been in uncertainty due to several results in contradictory, for example, Hunter *et al.* (2006) and den Breeÿen *et al.* (2006) indicated the polyphyletic of *Passalora*, but the recent progress of Burgess *et al.* (2007) supported the finding of Stewart *et al.* (1999) regarding the monophyletic of *Passalora*. Therefore, further intensive analysis is definitely necessary to resolve the uncertainty of this genus.

In relation to the morphological structure of the cercosporoid fungi, Crous and Braun (2003) suggested that conidial catenulation, septation, and proliferation of conidiogenous cells were less importance in separating species at generic level. On the other hand, Crous and Braun (2003) also affirmed several morphological characters that significant to the molecular phylogenetic analysis at generic level, such as pigmentation (*Cercospora* vs. *Passalora*), scar structure (*Passalora* vs. *Pseudocercospora*), and verruculose superficial hyphae (*Stenella* vs. *Passalora*). All these findings indicated that, in some cases, generic concepts of anamorphs based on morphology and conidium ontogeny particularly in the cercosporoid fungi, conform well with phylogenetic relationships, albeit this is not true in all cases because of convergence evolution (Crous *et al*., 2007). Unfortunately, no decision has been made regarding *Stenella* (verrucose conidia and mycelium), *Stigmina* (distoseptate conidia), and several other less well-known genera such as *Asperisporium* Maubl., *Denticularia* Deighton, *Distocercospora* N. Pons and B. Sutton, *Prathigada* Subram., *Ramulispora, Pseudocercosporidium* Deighton, *Stenellopsis* B. Huguenin and *Verrucisporota* D.E. Shaw and Alcorn, due to the unavailability of cultures.

In the present study, we carried out ITS phylogenetic analysis of taxa belong to the true cercosporoid fungi fide Crous and Braun (2003) in a larger dataset than the previous study. Other taxa related to this group such as *Stigmina* and *Phaeosariopsis* were included in this analysis. This analysis was conducted to address one of the goal in this thesis, that is, to assess the robustness of previously defined clades that had relatively weak statistical support. This study was also aimed to determine the relationships of some species with their host relatives. ITS region was employed in this study because of this region evolve faster than the coding regions of rRNA genes and much more divergence, therefore, they are useful for studying closely related organisms, such as species within a genus or among populations (Bruns *et al.*, 1991; O'Donell, 1992). The results generated from Neighbor Joining (NJ), Maximum Parsimony (MP), and Bayesian Inference (BI) analyses, largely corroborated the phylogenetic relationships described by Stewart *et al.* (1999), but with larger taxa.

3.2. Materials and Methods

Collection Sites and Morphological Examination

The cercosporoid fungi were collected at several locations in northern Thailand up to 2008. Specimens with disease symptoms of the cercosporoid fungi on leaves were collected during the course of field trips by using a 10× and 20× magnifying lens. Detailed observations of morphological characters were carried out by means of an OLYMPUS BX51 (OLYMPUS®, Japan) light microscope using oil immersion (1000×). Specimens for microscopic observation were prepared by hand sectioning. Water and Shear's solution were used as mounting media. Thirty conidia, hila, conidiophores, conidiogenous loci, and 10 stromata were measured for each

specimen. Line drawings were prepared at a magnification of 400× and 1000×. Single spore isolation of each new fungus encountered were attempted refer to Choi *et al.* (1999) with a modification. Voucher specimen has been deposited at BIOTEC Bangkok Herbarium (BBH), Thailand. Cultures isolated from the specimens have also been deposited at BIOTEC Bangkok Culture Collection (BCC), Thailand, and Molecular laboratory of Department of Plant Pathology, Chiang Mai University, Chiang Mai, Thailand.

Molecular Characterization

DNA Extraction, Polymerase Chain Reaction (PCR) and Sequencing

In this study, total genomic DNA was extracted from fungal mycelia cultured on Malt Extract Agar (Difco, USA) following a 2 × cetyltrimethylammoniumbromide (CTAB) protocol (Rogers and Bendich, 1994). DNA amplification of ITS region of nrDNA was performed by polymerase chain reaction (PCR) using ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTA ACAAGG-3′) primers (White *et al.*, 1990) to generate approximately 500 nucleotides from the complete ITS, including 5.8S rDNA region. The amplification condition was performed in a 50 ml reaction volume as follows: 1 × PCR buffer, 0.2 mM each dNTP, 0.3 mM of each primer, 1.5 mM $MgCl_2$, 0.8 units Amplitaq Taq Polymerase (Perkin-Elmer, Foster City, California, USA), and 10 ng DNA. PCR parameters for all the regions were performed as follows: initial denaturation at 94ºC for 3 min, followed by 30 cycles of 94ºC for 1 min, 52ºC for 50 s, 72ºC for 1 min, and final extension of 72ºC for 10 min.

The characterization of PCR products was performed via agarose gel electrophoresis on a TAE 1% agarose gel containing Ethidium Bromide (EtBr) as the staining agent. The PCR product was purified using Qiaquick purification kit (Qiagen) and DNA concentration of the PCR products was subjected to automatic sequencing (ABI PRISM Dye Terminator Cycle Sequencing and ABI PRISM Sequencer model 377, Perkin Elmer). In total, 105 sequences covered ITS and 5.8S regions of cercosporoid fungi are included in the analyses. The new ribosomal DNA sequences in the analyses are listed in Table 6, and the sequences retrieved from NCBI GenBank database are listed in Table 7

Table 6 New sequences generated in this study.

Name	Code	Host Family
Cercospora neobougainvilleae	BBH 23759	*Nyctaginaceae*
Cercospora christellae	BBH 23574	*Thelypteridaceae*
Cercospora citrullina	BBH 23754	*Cucurbitaceae*
Cercospora durantae-erectae	BBH 23619	*Verbenaceae*
Cercospora zinniicola	BBH 23563	*Asteraceae*
Cercospora lactucae-sativae	BBH 23572	*Asteraceae*
Cercospora caricola	BBH 23732	*Caricaceae*
Pseudocercospora prunicola	BBH 23727	*Rosaceae*
Pseudocercospora platycerii	BBH 23735	*Polypodiaceae*

Table 6 (continued)

Name	Code	Host Family
Pseudocercospora abelmoschi	BBH 23709	*Malvaceae*
Pseudocercospora justiciae	BBH 23710	*Acanthaceae*
Pseudocercospora centrosematicola	BBH 32487	*Fabaceae*
Pseudocercospora clitoriae	BBH 23765	*Fabaceae*
Pseudocercospora jahnii	BBH 23695	*Bignoniaceae*
Pseudocercospora oroxyli	BBH 23590	*Bignoniaceae*
Pseudocercospora jatrophae	BBH 23736	*Euphorbiaceae*
Pseudocercospora euphorbiae-pubescentis	BBH 23588	*Euphorbiaceae*
Pseudocercospora kopsiae-fruticosae	BBH 23584	*Apocynaceae*
Pseudocercospora marsdeniae	BBH 23720	*Asclepiadaceae*
Pseudocercospora lythracearum	BBH 23706	*Lythraceae*
Pseudocercospora butleri	BBH 23767	*Oleaceae*
Pseudocercospora quisqualidis	BBH 23743	*Combretaceae*
Pseudocercospora phyllitidis	BBH 23700	*Lomariopsidaceae*
Pseudocercospora mori	BBH 23711	*Moraceae*
Pseudocercospora fici	BBH 23581	*Moraceae*

Table 7 List of sequences retrieved from NCBI GenBank database.

Name	Code	Host Family
Cercospora fukushiana strain KACC 42268	EF600954	*Balsaminaceae*
Cercospora asparagi	AF297229	*Liliaceae*
Cercospora penzigii strain CPC 4002	DQ835072	*Rutaceae*
Cercospora canescens isolate CCA19	AY266164	*Fabaceae*
Cercospora zeae-maydis	AF291709	*Poaceae*
Cercospora polygonacea voucher KACC 42432	EF535652	*Polygonaceae*
Cercospora sorghi f. *maydis* Kenya 1	AF297232	*Poaceae*
Cercospora piaropi strain CBS 113127	DQ835075	*Pontederiaceae*
Cercospora apii strain CBS 252.67	DQ233318	*Apiaceae*
Cercospora bizzozeriana	EU031780	*Brassicaceae*
Cercospora rodmanii strain CBS 113130	DQ835082	*Pontederiaceae*
Cercospora populicola STE-U 1051	AY260069	*Salicaceae*
Cercospora zebrina STE-U 3958	AY260080	*Fabaceae*
Cercospora physalidis strain CBS 570.69	DQ835074	*Fabaceae*
Cercospora agavicola isolate HCe001	AY647237	*Liliaceae*
Cercospora beticola strain CBS 539.71	DQ233323	*Chenopodiaceae*
Cercospora physalidis strain CBS 131.32	DQ835073	*Fabaceae*
Cercospora capsici voucher KACC 42531	EF535687	*Solanaceae*
Cercospora hayi isolate CH5	AY266163	*Musaceae*

Table 7 (continued)

Name	Code	Host Family
Cercospora acaciae-mangii strain CPC 10550	AY752139	*Fabaceae*
Cercospora kikuchii voucher BRCK 179	AY633838	*Fabaceae*
Passalora loranthi strain CBS 122465	EU514279	*Loranthaceae*
Passalora loranthi strain CBS 122466	EU514280	*Loranthaceae*
Passalora loranthi	AY348311	*Loranthaceae*
Passalora sp. CPC 11147	AY752162	*Fabaceae*
Passalora sp. CPC 11150	AY752163	*Fabaceae*
Passalora henningsii	AF284389	*Euphorbiaceae*
Passalora daleae strain CBS 113031	EU040236	*Fabaceae*
Passalora sp. CBS 113384	DQ676524	*Asteraceae*
Passalora ampelopsidis strain CBS 249.67	AY293063	*Vitaceae*
Passalora ampelopsidis	AF362053	*Vitaceae*
Passalora sp. CBS 113613	DQ676525	*Asteraceae*
Phaeoisariopsis griseola strain CPC 12241	DQ289827	*Fabaceae*
Phaeoisariopsis griseola strain CPC 10463	DQ289824	*Fabaceae*
Phaeoisariopsis griseola strain CPC 10474	DQ289825	*Fabaceae*
Phaeoisariopsis griseola strain CPC 10479	DQ289826	*Fabaceae*
Pseudocercospora cydoniae voucher KACC 42644	EF535716	*Rosaceae*

Table 7 (continued)

Name	Code	Host Family
Pseudocercospora lythracearum voucher KACC 42649	EF535720	*Myrtaceae*
Pseudocercospora abelmoschi voucher KACC 42648	EF535719	*Malvaceae*
Pseudocercospora callicarpae voucher KACC 42637	EF535709	*Verbenaceae*
Pseudocercospora paraguayensis strain CBS 111286	DQ267602	*Myrtaceae*
Pseudocercospora fatouae strain CPC 11648	DQ303076	*Moraceae*
Pseudocercospora humuli voucher KACC 42529	EF535685	*Cannabaceae*
Pseudocercospora humuli voucher KACC 42522	EF535682	*Cannabaceae*
Pseudocercospora lythri voucher KACC 42641	EF535713	*Myrtaceae*
Pseudocercospora platylobii VPRI 21698	AF488744	*Fabaceae*
Pseudocercospora platylobii VPRI 22656	AF488742	*Fabaceae*
Pseudocercospora eucalyptorum clone STE-U-17	AF309599	*Myrtaceae*

Table 7 (continued)

Name	Code	Host Family
Pseudocercospora eucalyptorum clone STE-U-17	AF309598	*Myrtaceae*
Pseudocercospora robusta	AF309597	*Myrtaceae*
Pseudocercospora syzygiicola	AF309600	*Myrtaceae*
Pseudocercospora paraguayensis	AF309596	*Myrtaceae*
Ramularia coleosporii voucher KACC 42484	EF535673	*Asteraceae*
Ramularia sp. KACC 42481	EF535671	-
Ramularia coleosporii voucher KACC 42483	EF535672	*Asteraceae*
Ramularia pratensis var. *pratensis* strain CPC 11294	EU019284	*Polygonaceae*
Ramularia aplospora strain CBS 545.82	EU040238	*Rosaceae*
Ramularia proteae strain CBS 112161	EU707899	*Proteaceae*
Ramularia eucalypti strain CPC 13304	EF394862	*Myrtaceae*
Ramularia eucalypti strain CPC 13046	EF394861	*Myrtaceae*
Ramularia lamii var. *lamii* voucher KACC 42523	EF535683	*Lamiaceae*
Ramularia lamii var. *lamii* voucher KACC 42534	EF535688	*Lamiaceae*
Stenella citri-grisea strain X743	EU514229	*Rutaceae*

Table 7 (continued)

Name	Code	Host Family
Stenella citri-grisea strain CBS 122456	EU514228	*Rutaceae*
Stenella musicola strain CBS 122479	EU514294	*Poaceae*
Stenella queenslandica strain CBS 122475	EU514295	*Musaceae*
Stenella musae strain MSY64	FJ441662	*Poaceae*
Stenella musae strain msy104	FJ441627	*Poaceae*
Stenella xenoparkii strain CBS 111185	DQ303028	*Myrtaceae*
Stenella xenoparkii strain CBS 111089	DQ303027	*Myrtaceae*
Stenella pseudoparkii strain CBS 111049	DQ303025	*Myrtaceae*
Stenella pseudoparkii strain CBS 111000	DQ303024	*Myrtaceae*
Stenella araguata	AF362066	*Fabaceae*
Stenella araguata strain CBS 105.75	EU019250	*Fabaceae*
Stigmina eucalypti strain CPC 13384	EF394866	*Myrtaceae*
Stigmina eucalypti	AF362061	*Myrtaceae*
Stigmina platani strain STE-U 4299	AY260090	*Platanaceae*
Stigmina platani	AF222849	*Platanaceae*
Cladosporium oxysporum isolate HKB25	EF029816	-
Cladosporium cladosporioides AHS-511-43	DQ026006	-

Sequence Alignment and Phylogenetic Analysis

Sequence obtained from the respective primers (ITS5 and ITS4) was aligned in ClustalX (Thomson *et al.*, 1997) and BioEdit (Hall, 1999). The sequences alignments were also refined by direct examination. Regions designated as ambiguously aligned were excluded from the analyses. Gaps were treated as missing data. Phylogenetic analyses were performed in PAUP version 4.0b10 (Swofford, 2002). Trees generated from all analysis were figured in TreeView (Page, 1996). *Cladosporium cladosporioides* and *C. oxysporum* were assigned as outgroup in all analysis.

Neighbor Joining or distance analysis was performed because of its simple and efficiency in computational analysis, and also consistent under many models of evolution (Saitou and Nei, 1987). In this analysis, the most appropriate evolution was determined for a given data set using PAUP* and Modeltest 3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004). Likelihood scores were calculated for 56 alternative models of evolution by PAUP*. The output file was then imported to Modeltest to compare the models by the Akaike Information Criterion (AIC) (Akaike, 1974). Once a model of evolution was chosen, it was used to construct phylogenetic trees with the NJ method in PAUP*. The HKY85 model (Hasegawa *et al.*, 1985) was used as the substitution model for the calculation. The strength of the internal branches from the resulting trees was tested by bootstrap analysis using 1000 replications (Felsenstein, 1985).

Maximum parsimony analysis was performed in this study because of it is fast and consistent (Felsenstein, 1978; Maddison *et al.*, 1999). Trees were inferred using the heuristic search option with 100 random sequence additions. Maxtrees were

limited to 100 and all multiple parsimonious trees were saved. Tree bisection reconnection (TBR) branch swapping and the MULPARS option in effect, and zero-length branches were collapsed. Descriptive tree statistics (tree length [TL]. consistency index [CI], retention index [RI], related consistency index [RC], homoplasy index [HI], and log likelihood [-ln L]) were calculated for trees generated under different optimality criteria. The Kishino-Hasegawa (KH) likelihood test (Kishino and Hasegawa, 1989) was carried out using PAUP to compare the best tree topology obtained by the nucleotide sequence data with a constrained tree. All sites were treated as unordered and unweighted. The HKY85 model (Hasegawa *et al.*, 1985) was used as the substitution model for the calculation. Clade stability was assessed in bootstrap analyses with 1000 replicates, each with 100 replicates of random stepwise addition of taxa. Random sequence addition was used in the bootstrap analyses. Bootstrap supports equal to and above 50% were regarded as significant (Tuffley and Steel, 1997; Maddison *et al.*, 1999).

Bayesian Inference was selected in this study because of its efficient simulation algorithms schemes (Yang and Rannala, 1997). In the Bayesian analysis, the best-fit model of evolution was determined by Modeltest3.7 (Posada and Crandall, 1998; Posada and Buckley, 2004) which calculated likelihood scores for 56 alternative models of evolution. Posterior probabilities (PP) (Rannala and Yang 1996; Zhaxybayeva and Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), using above estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100th generations (resulting 10,000 total trees). The first 1,000 trees that represented the burn-in phase of the

analyses were discarded and the remaining 9,000 were used for calculating posterior probabilities (PP) in the majority rule consensus rule tree. The analyses were repeated five times starting from different random trees to ensure trees from the same tree space were being sampled during each analysis. Posterior probabilities equal to and above 95% were regarded as significant (Huelsenbeck and Ronquist, 2001).

3.3. Results

The ITS data set consists of 105 sequences, of which 28 from the genus *Cercospora*, 11 from the genus *Passalora*, four from the genus *Phaeoisariopsis*, 34 from the genus *Pseudocercospora*, 10 from the genus *Ramularia*, 12 from the genus *Stenella*, four from the genus *Stigmina*, and two from the genus *Cladosporium* as outgroups. Most of the sequences used in this study to construct phylogenetic tree belong to genus *Pseudocercospora* (table 6 and 7), and the members of this largest group in this analysis were isolated from 23 families of host plants (see chapter 2). The second largest group was *Cercospora*, and the sequences of this genus were isolated from 19 families of host plants (see chapter 2).

Alignment of the ITS sequences of the cercosporoid fungi and outgroups resulted in matrix of 580 characters, of which 73 characters were excluded from the analysis. Of the remaining 507 included characters, 194 characters were constant, 64 variable characters were parsimony uninformative, and 249 characters were parsimony informative. From seven best equally MP trees retained from the analysis, the best parsimonious tree selected by using KH test was generated in 1072 steps (CI = 0.475, RI = 0.815, RC = 0.387, HI = 0.525).

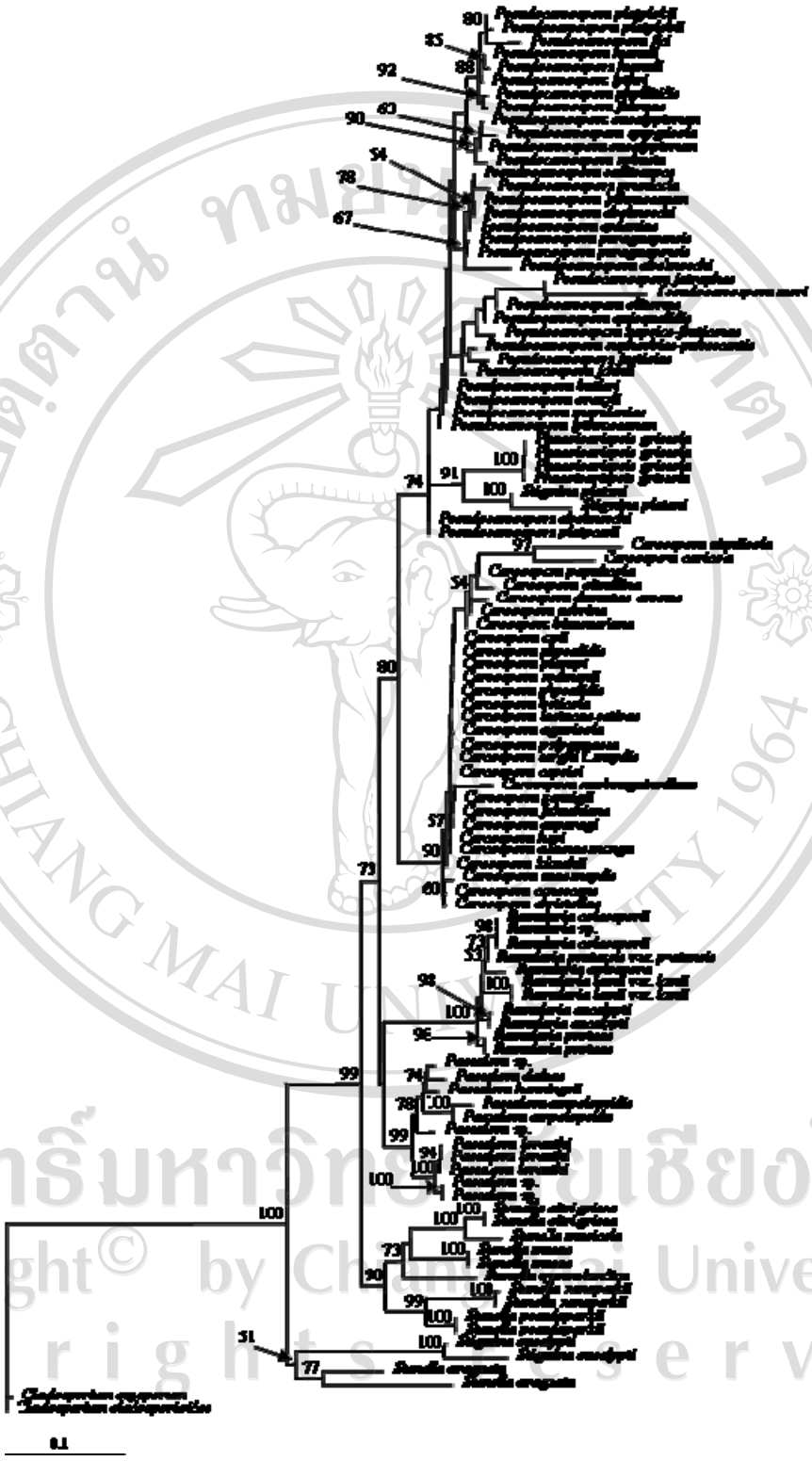

Figure 155 Phylogenetic tree based on NJ analysis of ITS nrDNA sequence data representing relationships of members of the true cercosporoid fungi within the *Mycosphaerella* anamorphs. Bootstrap values ≥ 50% from the analysis are shown above internodes.

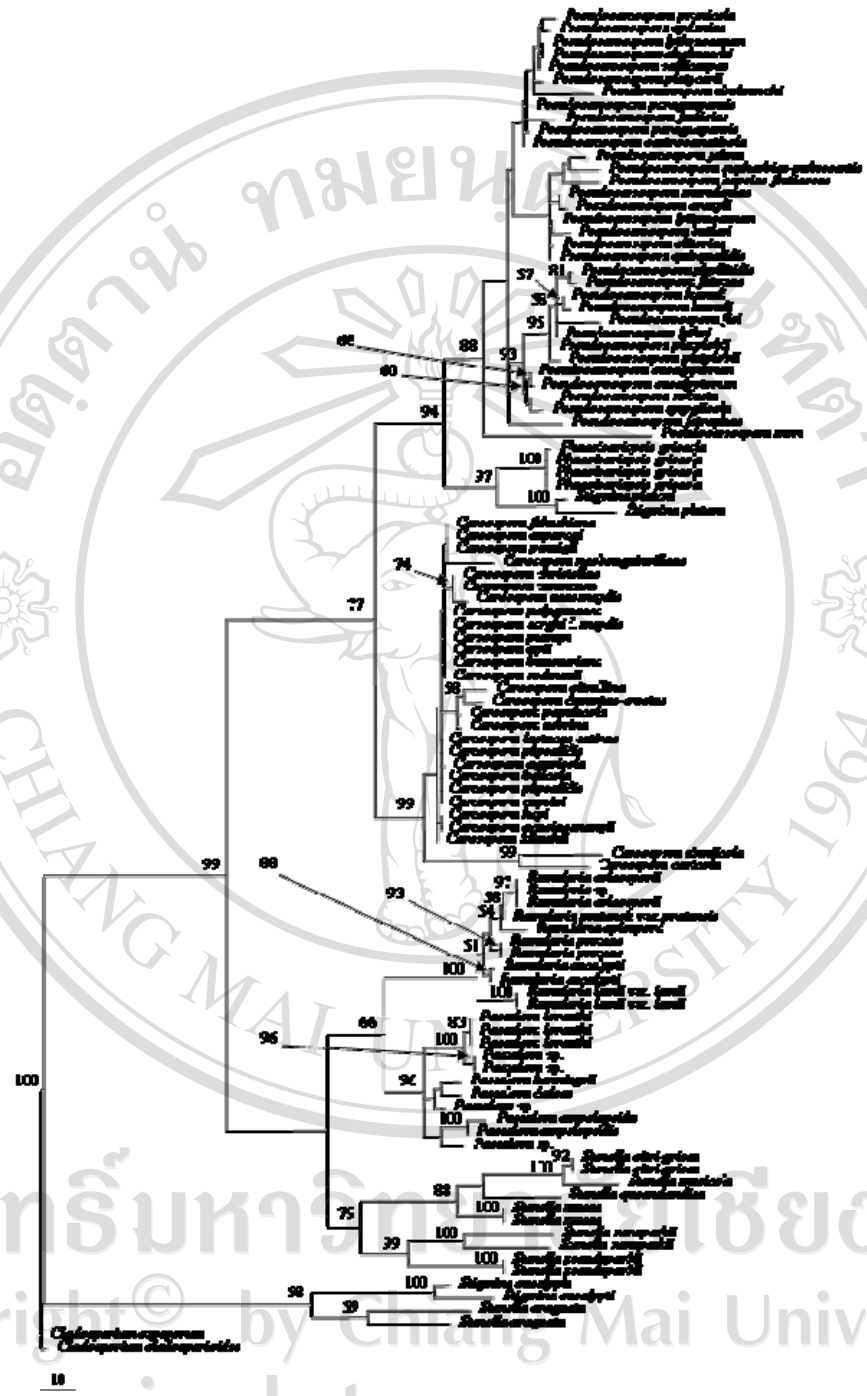

Figure 156 Phylogenetic tree based on MP analysis of ITS nrDNA sequence data representing relationships of members of the true cercosporoid fungi within the *Mycosphaerella* anamorphs. Bootstrap values ≥ 50% from the analysis are shown above internodes.

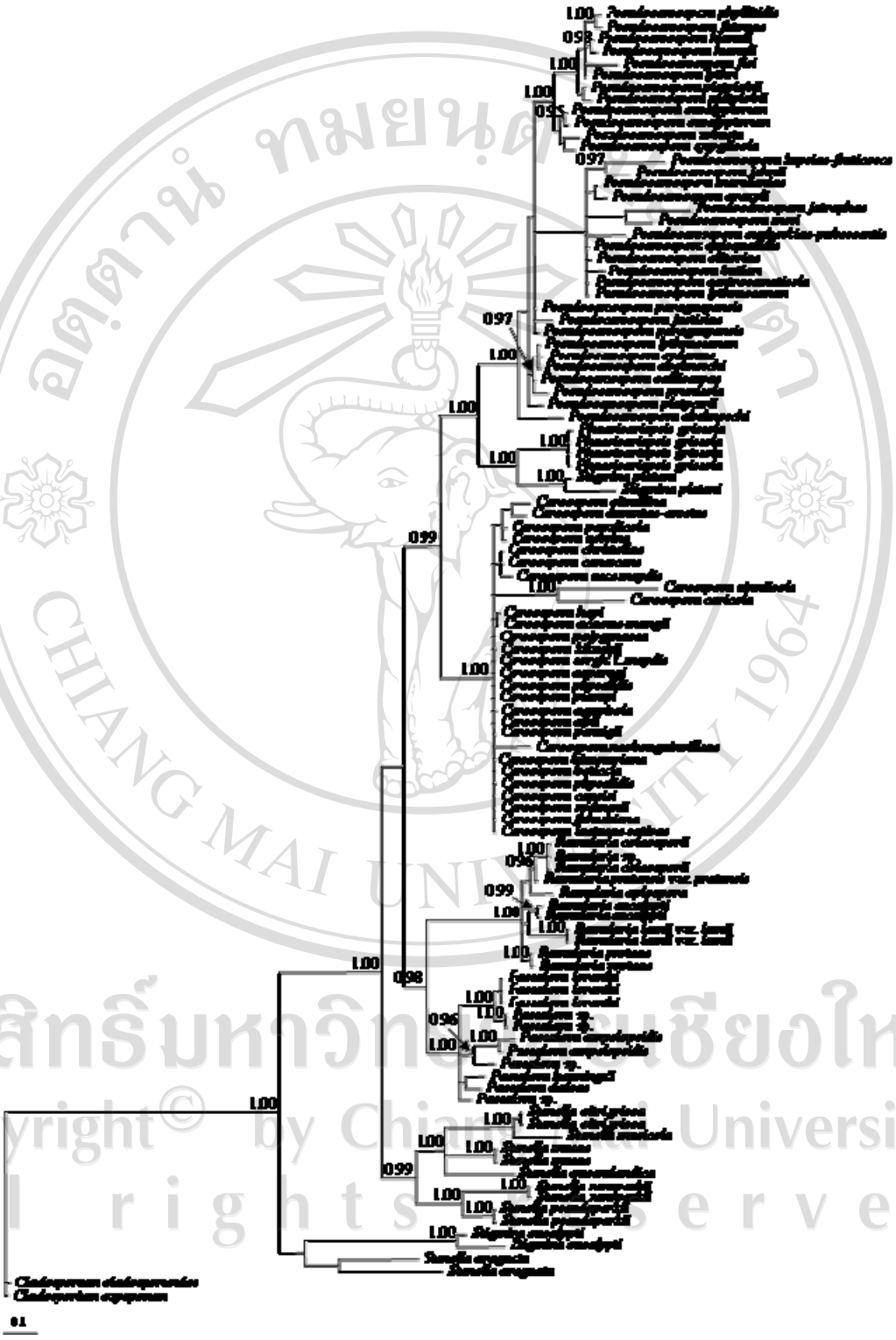

Figure 157 Phylogenetic tree based on Bayesian analysis of ITS nrDNA sequence data representing relationships of members of the true cercosporoid fungi within the *Mycosphaerella* anamorphs. Posterior probabilities values ≥ 95% from the analysis are shown above internodes.

The tree topology generated from MP analysis was very similar to Bayesian inference (figures 157 and 158), but NJ tree was slightly different to both MP and Bayesian trees due to *Pseudocercospora* species are polyphyletic within a large clade of *Pseudocercospora-Phaeoisariopsis-Stigmina platani* complex (figure 155). In general, the sequences generated from the NJ, MP and Bayesian analyses could be divided into five monophyletic groups, viz, group I consisted of the members of *Pseudocercospora* (88% in MP and 1.00 in Bayesian analysis), group II consisted of *Cercospora* species (90% in NJ, 99% in MP, and 1.00 in Bayesian analysis), group III consisted of *Ramularia* species (100% in NJ and MP, 1.00 in Bayesian analysis), group IV consisted of *Passalora* species (99% in NJ, 96% in MP, and 1.00 in Bayesian analysis), and group V consisted of almost all of *Stenella* species except *Stenella araguata* (90% in NJ, 79% in MP, and 0.99 in Bayesian analysis). Sequences of the genus *Stigmina* which were represented by only four sequences of two species formed a polyphyletic group. Another small group, *Phaeoisariopsis* clade, was monophyletic (100% in NJ and MP, 1.00 in Bayesian analysis), but this clade was only represented by a single species *Ph. griseola*, therefore, addition of more sequences/species is necessary in order to determine the monophyletic of this genus.

3.4. Discussion

This study provides strong supportive evidence for the distinction between *Cercospora*, *Passalora*, *Pseudocercospora*, and *Stenella* as separate genera based on analysis of ITS region of rDNA sequence. From all analysis in this study, it can be noted that except genus *Stenella*, the other genera of the true cercosporoid fungi fide Crous and Braun (2003) are monophyletic as previously suggested (Stewart *et al*.,

1999; Burgess *et al.*, 2007). This result is in contradictory with Hunter *et al*. (2006) and den Breeÿen *et al.* (2006) who indicated the polyphyletic of *Passalora*. In fact, the four genera morphologically are well-separated based on presence/absence of conidia and conidiophores scars, pigmentation of conidia, and appearance of secondary superficial hypha (smooth or verrucose) (Crous and Braun, 2003). Genus *Cercospora* is easily recognized by thickness of conidiophores and conidia scars, and hyaline acicular conidia (see chapter 1, figure 5), genus *Pseudocercospora* differs by unthickened and not darkened conidia and conidiophores scars (see chapter 1, figure 7), *Passalora* is most similar to *Cercospora* but the conidia is pigmented (mostly light brown or brown) (see chapter 1, figure 6), and *Stenella* is most similar to *Cercospora* and *Passalora* in having distinct and thick conidia and conidiophores scars, but the secondary superficial hypha is verruculose and some species have a verruculose conidia (see chapter 1, figure 8). However, the molecular phylogeny analysis in this study does not support the morphological elucidation of genus *Stenella*, and therefore, it is questionable whether morphological characteristics such as verruculose superficial hypha is phylogenetically significant.

The concept and circumscription of *Cercospora*, which is introduced by Fresenius in 1863 for *Passalora*-like fungi with pluriseptate conidia, was subsequently widened, and culminated in the treatment of the genus by Chupp (1954), which included almost all cercosporoid hyphomycetes. Recently, the morphological concept of this genus is well defined by Crous and Braun (2003) who reaffirmed the structure of the conspicuously thickened and darkened conidiogenous loci (scars), and conidial hila are very important for the identification of *Cercospora* spp. This definition will not be well-performed without the contribution of David (1997). David

(1997) examined scar structures of cercosporoid hyphomycetes by means of SEM and proposed the term '*Cercospora*-type' for planate scars. However, despite comprehensive taxonomic treatments, the systematics of the genus *Cercospora* is still controversial and often confusing to the non-specialist. Therefore, most of the identification refers to the host plant which *Cercospora* species associated with.

In addition, Crous and Braun (2003) proposed *C. apii s. lat.* as a 'compound species' to accommodate all cercosporoid hyphomycetes indistinguishable from the *Cercospora* from *Apium graveolens*, therefore, introduction of new names for morphologically indistinguishable *Cercospora* collections detected on new host genera and families, respectively, should be avoided, and should simply be referred to *C. apii s. lat.* Even though this concept was successfully reduced the number of species in genus *Cercospora*, however, it is still some problems remains to be done such as a highly variation in morphological characteristics of *Cercospora s. str*. Fungi are an organism, and therefore, they are growth and developed in space and time. Their growth is also affected by many environmental factors. Therefore, it is often difficult to determine from direct observation whether the specimen under examination is immature or already mature. The age of fungi and environmental factors directly or indirectly have a significant effect to the size of vegetative structures, and probably pigmentation in several taxa (Deacon, 2006). Therefore, the proposal of new taxa in this genus is still progressing because of those factors.

In this study, members of the genus *Cercospora* clustered together with a high statistical support in all analyses (90% in NJ, 99% in MP, and 1.00 in Bayesian analysis). The monophyletic of the genus *Cercospora* was also reported by several previous authors (Stewart *et al*., 1999; Goodwin *et al*., 2001; Hunter *et al*., 2006; den

Breeÿen *et al.*, 2006; Burgess *et al.*, 2007). Goodwin *et al*. (2001) in the study on the *Mycosphaerella* anamorphs relationships revealed that taxa within *C. apii s. lat.* are polyphyletic, and emphasized that species of *Cercospora* capable of producing chemical compound called '*cercosporin*' nested together in a large clade of *Cercospora* to form a monophyletic clade. In contrast, Ayala-Escobar *et al.* (2005) suggested the monophyletic of *C. apii s. lat.* by combining five loci in their phylogenetic analysis, viz, rDNA, elongation factor 1-α gene (EF), actin gene (ACT), calmodulin gene (CAL), histone H3 gene (HIS), however, the robustness of their study is still questionable due to the number of sequences used were very small. Only six sequences of *C. apii s. lat.* and three sequences of *Cercospora s. str.* non *C. apii s. lat.* were included to construct phylogenetic tree in their analysis (Ayala-Escobar *et al.*, 2005).

In this study, the monophyletic of *C. apii s. lat.* and also the relationship of *Cercospora* species based on their host relationship were tested by examining ITS region including 5.8S rDNA. The consensus trees generated from NJ, MP, and Bayesian analyses showed that taxa belong to *C. apii s. lat.* such as, *C. citrullina*, *C. fukushiana*, *C. canescens*, etc. (Crous and Braun, 2003), are polyphyletic as they nested together with other *Cercospora* species non *C. apii s. lat.* such as *C. agavicola*, *C. asparagi*, *C. caricola*, etc. (Crous and Braun, 2003). It is also clear that *Cercospora* species used in this analysis do not show a host-specificity indication due to species from different host (up to family level) nested together in the *Cercospora* clade, and species from the same host family are separated, for example, *C. canescens* (*Fabaceae*) clustered together with *C. zeae-maydis* (*Poaceae*) and *C. christellae* (*Thelypteridaceae*) with high statistical support (60% in NJ, and 74% in MP), but

separated from other species from family *Fabaceae* such as *C. physalidis*, *C. zebrina, C. kikuchii*. The polyphyletic of *C. apii s. lat.* and the fact that most of *Cercospora* species used in this study do not indicate a host-specificity are probably true even if the molecular analysis executed by using more genes loci, either from other regions of nuclear rDNA such as 28S and 18S regions (Crous *et al.*, 2007), or protein genes such as Elongation Factor 1-α gene (EF), Actin gene (ACT), Calmodulin gene (CAL), and Histone H3 gene (HIS) (Ayala-Escobar *et al.*, 2005). Therefore, at present time, it is not possible to determine which species are specific to their host, and which species have a multi-hosts relationship. Sequences availability limitation of *Cercospora*-complex species in the web-based sequence database such as NCBI and DDBJ genbank is also another major obstacle that hampers the progress in the phylogenetic and molecular study of this group of fungi. On the other hand, even though lacking of molecular data, Crous and Braun (2003) and Aptroot (2006) assumed that the genus *Mycosphaerella* and its anamorphs (*Cercospora*-complex) encompass both saprobic and parasitic life forms. Crous and Braun (2003) and Aptroot (2006) also believed that the parasitic species are supposed to be host-specific, albeit in some cases experimental evidence exists of the contrary. The saprobic species, in the past often described repeatedly from different hosts, are generally accepted to be less host-specific.

Another genus of the true cercosporoid fungi, *Pseudocercospora*, showed a monophyletic clade with high statistical supports in the trees generated from all analyses (74% in NJ, 88% in MP, and 1.00 in Bayesian analysis). *Pseudocercospora* accommodates a wide range of cercosporoid hyphomycetes with pigmented conidiophores, and inconspicuous, unthickened and not darkened conidiogenous loci.

The early morphological data from Stewart *et al.* (1999) suggested that *Pseudocercospora* is monophyletic, but Crous *et al.* (2001) contrarily showed *Pseudocercospora* to be polyphyletic within *Mycosphaerella*, having evolved more than once from different *Mycosphaerella* holomorphs, and in several occasions having lost the teleomorph. However, the recent studies suggested that the genus *Pseudocercospora* is monophyletic within *Mycosphaerella* (Hunter *et al*., 2006; den Breeÿen *et al.*, 2006; Burgess *et al.*, 2007). In addition, based on the host relationship analysis in this study, the consensus trees generated from all analyses showed that most of taxa of *Pseudocercospora* species are also not host-specific due to sequences from a single family does not form a subgroup within the large monophyletic clade of *Pseudocercospora*. For example, eight sequences of six *Pseudocercospora* species which belong to five different plant families, viz, *P. phyllitidis* (*Lomariopsidaceae*), *P. fici* and *P. fatouae* (*Moraceae*), *P. humuli* (*Cannabaceae*), *P. lythri* (*Myrtaceae*), *P. platylobii* (*Fabaceae*), nested together to form a monophyletic subgroup (88% in NJ, 95% in MP analysis, and 1.00 in Bayesian analysis). The other species are paraphyletic within *Pseudocercospora* clade. This data indicated that proposal of new names in *Pseudocercospora* based on host and morphological characteristics are possibly not sufficient enough. Therefore, another alternative such as pathogenicity test and molecular phylogenetic analysis are necessary, but these alternative ways are also very difficult to carry out to all the cercosporoid taxa since the isolation and sporulation of cultures in this group proved to be difficult.

Genus *Passalora* is morphologically highly variable, comprise of taxa characterized by single or catenate conidial formation, superficial secondary mycelium with solitary or fasciculate, branched or unbranched, straight or decumbent

conidiophores (Crous and Braun, 2003). Stewart *et al*. (1999) was the first to characterize *Passalora* phylogenetically based on ITS region of rDNA sequence analysis. However, the monophyletic of genus *Passalora* as indicated by Stewart *et al.* (1999) has been in uncertainty due to contrary results reported by Hunter *et al*. (2006) and den Breeÿen *et al.* (2006) that indicated the polyphyletic of *Passalora*, but the recent progress of Burgess *et al.* (2007) supported the finding of Stewart *et al.* (1999) regarding the monophyletic of *Passalora*. The phylogenetic analyses in this study also support the monophyletic of the genus *Passalora* as previously suggested by Stewart *et al.* (1999) and Burgess *et al.* (2007), due to *Passalora* sequences used in the analysis nested together to form a monophyletic clade with high statistical supports (99% in NJ, 96% in MP, and 1.00 in Bayesian analyses). Moreover, the *Passalora* clade appears as a sister group to *Ramularia* clade with 66% bootstrap support in MP and 0.98 in Bayesian analyses. The similarity of morphology characteristics between *Passalora* and *Ramularia* were also discussed by Braun (1998) and Crous and Braun (2003).

Stenella Sydow (1930) which is differentiated from other genera of the true cercosporoid fungi, particularly genus *Passalora*, based on the formation of verruculose superficial hyphae and usually rough-walled, catenate conidia (Crous and Braun, 2003), is polyphyletic in all analysis of this study. In the previous molecular analysis of genus *Stenella*, Crous *et al.* (2000, 2001) also suggested that this genus is polyphyletic due to *S. araguata* clustered separately from other species of *Stenella*. Although *Stenella* is not monophyletic, however, several previous authors suggested that this genus should be retained as a separate genus from *Passalora* and similar taxa (Pretorius *et al.*, 2003; Taylor *et al.,* 2003). This is probably because of the other

similar taxa, such as *Cercospora*, *Passalora*, and *Pseudocercospora* are well-established morphologically and phylogenetically. This study also supports the separation of *Stenella* to *Passalora* and similar taxa, and supports the morphological elucidation of David (1997) regarding the pileate scars of *Stenella* differs from the planate *Cercospora*-type scars. Further investigation with larger dataset is necessary to carry out in order to clarify the uncertainty of this genus.

Stigmina was circumscribed by Sutton and Pascoe (1989) to represent foliicolous species allied to *S. platani* with pigmented structures, percurrently proliferating conidiogenous cells, and transversely, occasionally longitudinally distoseptate conidia (figures 159a-b). In this study the members of *Stigmina* which are represented by four sequences of two species, *S. platani* and *S. eucalypti*, are polyphyletic. However, due to the limited sequences included in this analysis, therefore, it is very difficult to elucidate the relationship of this genus with other cercosporoid fungi. From the trees generated in this study, *S. platani* clade, appeared as a sister group to *Phaeoisariopsis griseola* clade (91% in NJ, 97% in MP, and 1.00 in Bayesian analysis). Morphologically, both species share similar characteristics in having mostly multi septate and obclavate conidia with truncate and unthickened hila, sometimes verruculose, and composed of transverse and longitudinal septate. Despite both species are separated due to synnematous conidiophores of *Phaeoisariopsis* (figure 160), however, conidiophores in *Stigmina* actually also in packed closely together forming a pulvinate sporodochia (synnematous-like, but very short in size) (figure 159a). Therefore, further molecular analysis with more sequences included is also needed to determine the relationship between the two taxa. In addition, both genera indicate the close relationship with genus *Pseudocercospora,* as the three

genera nested together to form a monophyletic clade with high statistical supports (74% in NJ, 94% in MP, and 1.00 in Bayesian analysis). In fact, the three genera also share similarity in morphology characteristics by having unthickened and not darkened conidiogenous loci and conidia hila. This data indicated that scars of conidiophores and conidia (related to conidiogenesis process during conidial separation) are important characters to delimit taxa at genus level as previously suggested by Crous and Braun (2003).

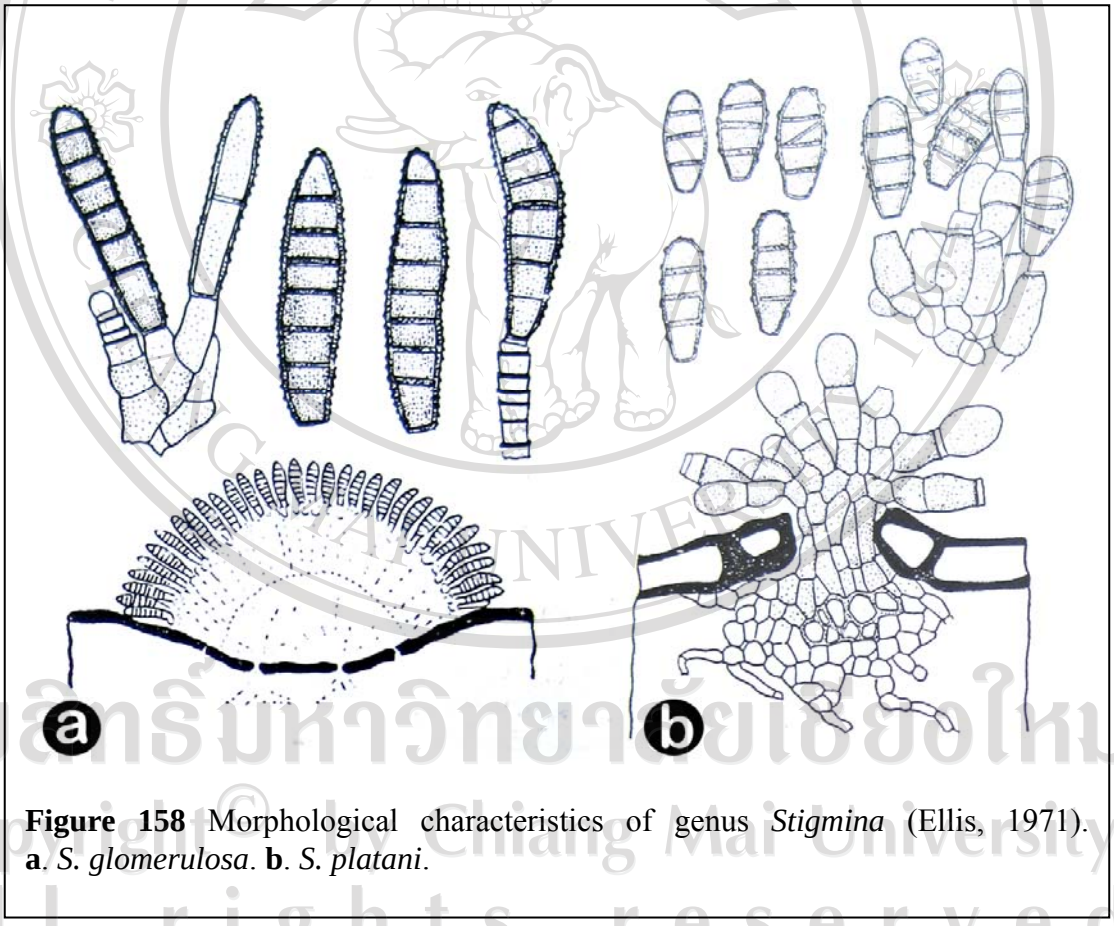

Figure 158 Morphological characteristics of genus *Stigmina* (Ellis, 1971). **a**. *S. glomerulosa*. **b**. *S. platani*.

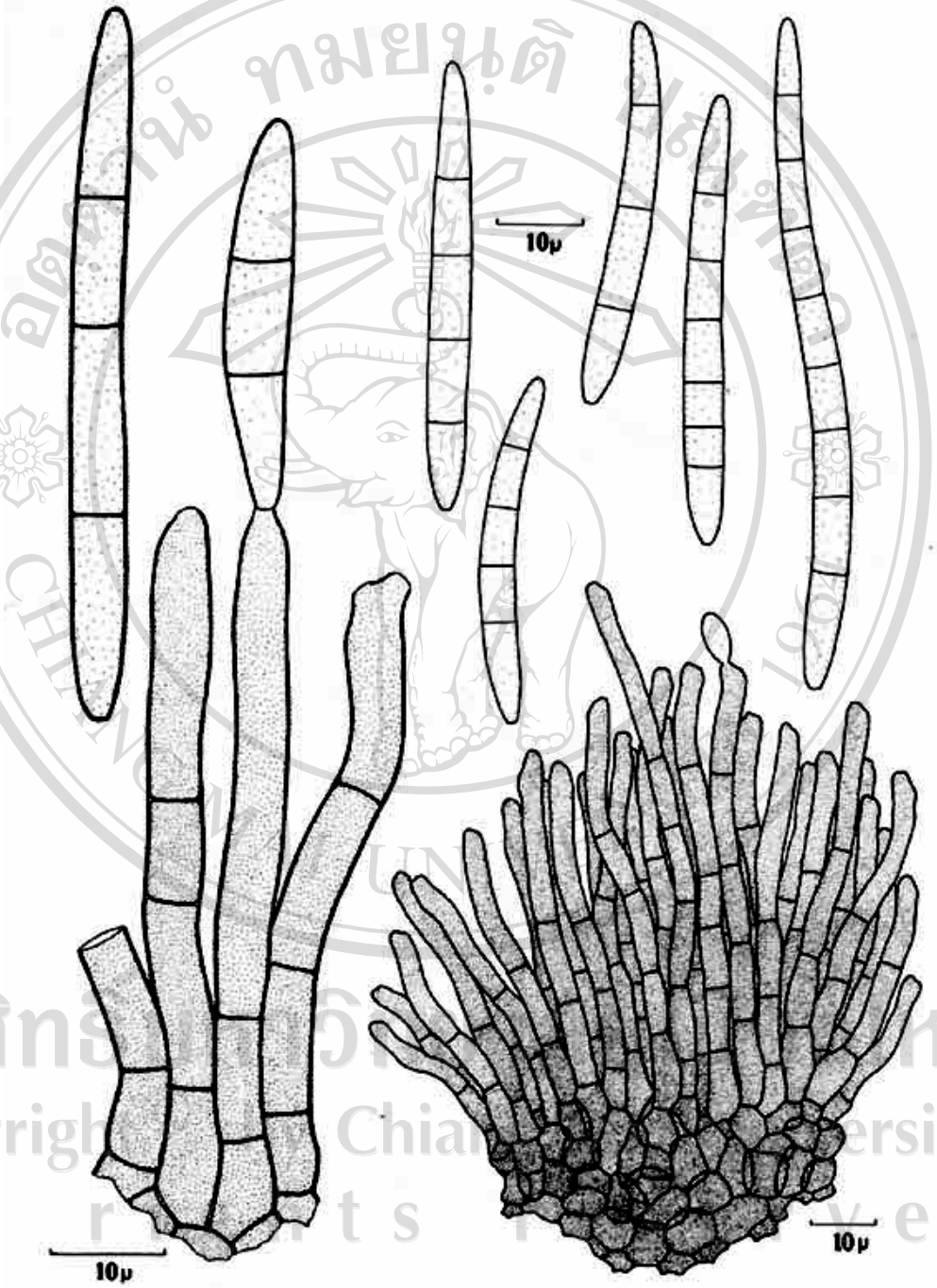

Figure 159 Morphological characteristics of *Phaeoisariopsis sphaeroidea* (Ellis, 1971).

3.5. Conclusions

Based on ITS region of rDNA sequence data analysis using NJ, MP and Bayesian approaches, it is clear that *Cercospora*, *Passalora*, and *Pseudocercospora*, are well defined morphologically and phylogenetically as previously suggested (Stewart *et al*., 1999; Goodwin *et al*., 2001; Crous and Braun, 2003; Hunter *et al*., 2006; den Breeÿen *et al*., 2006; Burgess *et al*., 2007). However, further molecular investigation of genus *Stenella*, which is polyphyletic in this study, with larger dataset is necessary in order to clarify the placement of this genus in the classification scheme as previously suggested (Crous *et al*., 2000; 2001).

Most of the members of genus *Cercospora* and *Pseudocercospora* are possibly not host-specific based on ITS region of rDNA sequence analysis. This is probably true because of the genus *Mycosphaerella* and its anamorphs (*Cercospora*-complex) encompass both saprobic and parasitic life forms (Aptroot, 2006). At present time, however, it is still difficult to determine the host-specificity in this group of fungi. The parasitic species are supposed to be host-specific, but in some cases the experimental evidence exists of the contrary (Crous and Braun, 2003).

3.6. References

Akaike, H. 1974. A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19**: 716–723.

Aptroot, A. 2006. Mycosphaerella and Its Anamorphs: 2. Conspectus of *Mycosphaerella*. *CBS Biodiversity Series* **5**. Utrecht, Netherlands.

Arx, von J. A. 1983. *Mycosphaerella* and its anamorphs. *Proc. K. Nederl. Akad. Wet., Ser. C* **86**: 15-54.

Ayala-Escobar, V., Yañez-Morales, M. J., Braun, U., Groenewald, J. Z., and Crous, P. W. 2005. *Cercospora agavicola* – a new foliar pathogen of *Agave tequilana* var. *Azul* from Mexico. *Mycotaxon* **93**: 115-121.

Braun, U. 1998. A monograph of *Cercosporella*, *Ramularia* and Allied Genera (phytopathogenic hyphomycetes). Vol. 2. IHW-Verlag, Eching, Germany.

Breeÿen, den A., Groenewald, J. Z., Verkley, G. J. M., and Crous, P. W. 2006. Morphological and molecular characterisation of *Mycosphaerellaceae* associated with the invasive weed, *Chromolaena odorata*. *Fungal Diversity.* **23**: 89-110.

Bruns, T. D., White, T. J., and Taylor, J. W. 1991. Fungal molecular systematics. *Annual Review of Ecology and Sytematics* **22**: 525-564.

Burgess, T. I., Barber, P. A., Sufaati, S., Xu, D., Hardy, G. E. StJ., and Dell, B. 2006. *Mycosphaerella* spp. on *Eucalyptus* in Asia, new species, new hosts and new records. *Fungal Divers.* **24**: 135-157.

Choi, Y. W., Hyde, K. D., and Ho, W. H. 1999. Single spore isolation of fungi. *Fungal Divers.* **3**: 29-38

Chupp, C. 1954. *A Monograph of the Fungus Genus Cercospora.* Published by the author, Ithaca, New York. USA.

Crous, P. W. and Wingfield, M. J. 1996. Species of *Mycosphaerella* and their anamorphs associated with leaf blotch disease of *Eucalyptus* in South Africa. *Mycologia* **88**: 441-458.

Crous, P. W., Aptroot, A., Kang, J. C., Braun, U., and Wingfield, M. J. 2000. The genus *Mycosphaerella* and its anamorphs. *Stud. Mycol.* **45**: 107-121.

Crous, P. W., Kang, J. C., and Braun, U. 2001. A phylogenetic redefinition of anamorph genera in *Mycosphaerella* based on ITS rDNA sequences and morphology. *Mycologia* **93**: 1081-1101.

Crous P. W. and Braun, U. 2003. *Mycosphaerella* and Its Anamorphs: 1. Names published in *Cercospora* and *Passalora*. *CBS Biodiversity Series* **1**. Utrecht, Netherlands.

Crous, P. W., Braun, U., and Groenewald, J. Z. 2007. *Mycospaherella* is polyphyletic. *Stud. Mycol.* **58**: 1-32.

David, J. C. 1997. A contribution to the systematics of *Cladosporium*. Revision of the fungi previously referred to *Heterosporium. Mycol. Pap.* **172**: 1-157.

Deacon, J. 2006. *Fungal Biology*. 4th ed. Blackwell Publishing Ltd., Oxford, England.

Ellis, M. B. 1971. *Dematiaceous Hyphomycetes*. CMI, Kew, Surrey, England.

Felsenstein, J. 1978. Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology* **27**:401-410.

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

Fresenius, G. 1863. Beitrage zur Mikologie. *Frankfurt* **3**: 91.

Goodwin, S. B., Dunkle, L. D., and Zismann, V. L. 2001. Phylogenetic analysis of *Cercospora* and *Mycosphaerella* based on the ITS region of ribosomal DNA. *Phytopathology* **91**: 648-658.

Hall, T. A. 1999. BioEdit: a user–friendly biological sequence alignment editor and analyses program for Windows 95/98/NT. - *Nucleic Acids Symposium Series* **41**: 95-98.

Hasegawa, M., Kishino, H. and Yano, T. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA, *J. Mol. Evol*. **22**: 160-174.

Huelsenbeck, J. P. and Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**: 754-755.

Hunter, G. C., Wingfield, B. D., Crous, P. W., and Wingfield, M. J. 2006. A multigene phylogeny for species of *Mycosphaerella* occurring on *Eucalyptus* leaves. *Stud. Mycol.* **55**: 147-161.

Kishino, H. and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data, and the branching Order in Hominoidea. *J. Mol. Evol.* **29**: 170-179.

Maddison, D. R., Baker, M. D., and Ober, K. A. 1999. Phylogeny of carabid beetles as inferred from 18S ribosomal DNA (*Coleoptera: Car*abidae). *Syst. Entom.* **24**: 1-36.

O'Donnell, K. 1992. Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*). *Current Genetics* **22**: 213-220.

Page, R. D. M. 1996. TreeView, an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**: 357-358.

Posada, D. and Crandall, K. A. 1998. Modeltest: testing the model of DNA substitution. *Bioinformatics* **14**: 817-818.

Posada, D. and Buckley, T. R. 2004. Model selection and model averaging in phylogenetics: advantages of the AIC and Bayesian approaches over likelihood ratio tests. *Systematic Biology* **53**: 793-808.

Pretorius, M. C., Crous, P. W., Groenewald, J. C., and Braun, U. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. *Sydowia* **55**: 286-305.

Rannala, B. and Yang, Z. 1996. Probability distribution of molecular evolutionary trees: a new method of phylogenetic inference. *J. Mol. Evol.* **43**: 304-311.

Rogers, S. O. and Bendich, A. J. 1994. Extraction of total cellular DNA from plants, algae and fungi. In: *Plant molecular biology manual* , 2nd Edition, (eds. Gelvin, S. B. and Schilperoort, R. A.), Kluwer Academic Press, Dordrecht, Netherlands, **D1**: 1-8.

Saitou N. and Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Stewart, E. L., Liu, Z., Crous, P. W., and Szabo, L. 1999. Phylogenetic relationships among some cercosporoid anamorphs of *Mycosphaerella* based on rDNA sequence analysis. *Mycol. Res.* **103**: 1491–1499.

Sutton. B. C. and Pascoe, I. 1989. Reassessment of *Peltosoma*, *Stigmina* and *Batcheloromyces* and description of *Hyphotyrium* gen. nov. *Mycol. Res.* **92**: 210-222.

Swofford, D. L. 2002. *PAUP*: Phylogenetic analyses using parsimony (* and other methods), version 4.0b 10*. Sinauer Associates, Sunderland, Massachusetts.

Taylor, J. E., Groenewald, J. Z., and Crous, P. W. 2003. A phylogenetic analysis of *Mycosphaerellaceae* leaf spot pathogens of *Proteaceae*. *Mycol. Res.* **107**: 653-658.

Thomson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The Clustal_X windows interface: Flexible strategies for multiple sequences alignment aided by quality analyses tools. *Nucleic Acids Res.* **25**: 4876-4882.

Tuffley, C. and Steel, M. 1997. Links between maximum likelihood and maximum parsimony under a simple model of site substitution. *Bull. Math. Bio.* **59**: 581-607.

White, T. J., Burns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: *PCR Protocols: a Guide to Methods and Application*, (eds. Innis, M. A., Gelfand, D. H., Sninsky, J. J., and White, T. J.), Academic Press, San Diego, USA, pp. 315-322.

Yang, Z. and Rannala, B. 1997. Bayesian phylogenetic inference using DNA sequences: a Markov chain Monte Carlo method. *Molecular Biology and Evolution* **14**:717-724.

Zhaxybayeva, O. and Gogarten, J. P. 2002. Bootstrap, Bayesian probability and maximum likelihood mapping exploring new tools for comparative genome analyses. *Genomics* **3**: 1-15.