EXPLORING THE DIVERSITY OF ASCOMYCETE FUNGI: EVOLUTION OF MATING SYSTEMS IN *PLEOSPORA* AND DISCOVERY OF NEW LINEAGES IN THE DOTHIDEOMYCETES

by

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Statement of co-authorship

For Patrik Inderbitzin's PhD thesis:

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Chapter 4: *Decorospora*, a new genus for the marine ascomycete *Pleospora gaudefroyi*

This chapter was a slightly modified version of the publication:

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To this paper I contributed as follows: I collected the fungus in its natural habitat and initiated the research. I did the molecular work and phylogenetic analyses under the supervision of M. Berbee. The remaining two co-authors were responsible for the morphological part including the illustrations. I was responsible for the final version of the manuscript and its submission, under supervision of M. Berbee.

Chapter 5: Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata

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I contributed to this publication in the following ways: I participated in the collection of the fungi in Thailand together with M. Abdel-Wahab, and China where I initiated the collection effort. I was the main contributor to the morphological studies, being responsible for the characterization of the novel features of these fungi, and writing the descriptions of the new taxa. Together with M. Abdel-Wahab, I took the photographs.
I did the molecular lab work and the phylogenetic analyses supervised by S. Landvik and M. Berbee. I wrote up the manuscript and submitted it for publication, under supervision of M. Berbee.

Sincerely,

Patrik Inderbitzin

For the co-authors:

Dr. Mary Berbee

Vancouver, 23. September 2004

ABSTRACT

This thesis explored aspects of diversity in the ascomycete genus *Pleospora* and the new family Aliquandostipitaceae. In *Pleospora*, the main focus was on mating system evolution. I found that homothallism in *Pleospora* evolved in three different ways from heterothallism. One origin of homothallism resulted from a horizontal transfer across lineages involving a MAT locus.

The approach chosen to investigate mating system evolution in *Pleospora* was based on delimiting the polyphyletic genus *Pleospora* to *Pleospora* sensu stricto, inferring a robust species phylogeny of *Pleospora* sensu stricto and cloning and examining the master regulator locus of sexual development in ascomycetes, the MAT locus. Conclusions were then drawn by integration of the total evidence.

Research of mating system evolution in *Pleospora* was initiated by confirmation of the monophyly of *Pleospora* isolates with *Stemphylium* asexual states. This group contained the type of *Pleospora*, and was thus called *Pleospora* sensu stricto. Contributing towards a monophyletic genus *Pleospora*, the marine species *P. gaudefroyi* collected in British Columbia, and lacking a *Stemphylium* asexual state, was transferred to the new genus *Decorospora*. Phylogenetic 18S rDNA analyses and Shimodaira-Hasegawa and Kishino-Hasegawa tests rejected the null hypothesis of monophyly for the two taxa. Instead, *Pleospora gaudefroyi* grouped distantly from *P. herbarum*, at the base of the family *Pleosporaceae* confirming the importance of a *Stemphylium* asexual state for the definition of *Pleospora* sensu stricto in this case. Since no existing genus was available to accommodate *P. gaudefroyi*, the new genus *Decorospora* had to be erected.

To generate a species phylogeny of *Pleospora* sensu stricto, 114 ingroup taxa with *Stemphylium* asexual states were used. Phylogenetic analyses based on ITS, *GPD*, *EF-1alpha* and *vmaA-vpsA* DNA sequences with four different algorithms showed that *Pleospora* sensu stricto contained 22 phylogenetic species. Morphological species generally correlated well with phylogenetic species, except for the type *P. herbarum* whose phylogenetic species contained the four additional morphological species *P. alfalfae*, *S. vesicarium*, *P. tomatonis* and *P. sedicola*. Another conflict between morphological and phylogenetic species concept could arise in the phylogenetic species *S. xanthosomatis* that also contained an isolate of *S. lycopersici,* which, however, was not certain to represent the type of the species. Three phylogenetic species, one of which was exclusively collected in British Columbia, did not contain any morphological species and might be new to science.

The protein-coding gene *EF-1 alpha*, parts of which where used for phylogenetic inference in *Pleospora* sensu stricto, contained an unusual intron

in the phylogenetic species *S. lancipes*, *S. trifolii* and *Stemphylium sp.* strain P246. The intron was up to 1678 bp long, more than 1400 bp longer than introns in other species of *Pleospora,* it encoded a protein and was delimited at the 5'-end by the non-canonical splice site GGT, instead of GT. The ORF encoded by the introns of all three *Pleospora* species was most similar to a hypothetical zinc finger protein from the filamentous ascomycete *Gibberella zeae.* In case of experimental verification, this would be the first report of a 'parasitic' intron splice site in fungi.

To continue investigations of mating system evolution in *Pleospora* sensu stricto, the MAT locus was PCR amplified using primers targeting the conserved motives alpha box and HMG box on the *MAT1-1* and *MAT1-2* genes respectively. A chromosome walking approach was then used to recover the MAT flanking regions and neighboring genes. It was shown that *Pleospora* sensu stricto contained three kinds of MAT regions, comprising either a *MAT1-1* or *MAT1-2* idiomorph, or both *MAT1-1 and MAT1-2* idiomorphs fused end to end, with the inverted *MAT1-1* gene placed between ORF1 and *MAT1-2.* The genes flanking the MAT regions were ORF1 upstream, and *BGL1* downstream of the idiomorphs, as in the close relative *Cochliobolus.*
The idiomorphs of *Pleospora* sensu stricto were well delimited upstream of the MAT genes, terminating 16 or 17 amino acids inside ORF1 for respectively *MAT1-1* and *MAT1-2.* The downstream boundary of the idiomorphs of *Pleospora* sensu stricto was poorly defined.

There were no important differences between MAT genes from fused and separate MAT regions of *Pleospora* sensu stricto. *MAT1-1* genes from both separate and fused regions were 1193 bp in length and contained one intron of 53 bp inserted at position 218. *MAT1-2* genes were all 1093 bp long and comprised one intron of 55 bp inserted at position 491.

The fused MAT regions of *Pleospora* sensu stricto consisted of *MAT1-1* and *MAT1-2* idiomorphs fused end to end, with *MAT1-1* and flanking regions inverted. The gene arrangement found in the fused MAT regions was hypothesized to have evolved from ancestors with separate MAT regions by a crossover following the inversion of *MAT1-1.* The crossover was possibly facilitated by a short, 4 bp long stretch of DNA sequence similarity between *MAT1-1* and *MAT1-2* idiomorphs, resulting from the inversion of *MAT1-1* plus flanking regions.

MAT locus architecture and phylogenetic species correlated well. All phylogenetic species of *Pleospora* sensu stricto with more than one isolate contained both *MAT1-1* and *MAT1-2* isolates with separate MAT regions, or only isolates with fused *MAT* regions.

MAT regions also correlated with mating systems in *Pleospora* sensu stricto. Species with fused MAT regions were homothallic, whereas species with separate MAT regions were heterothallic except one group that was ho-

mothallic. In all homothallic isolates with separate MAT regions, only *MAT1-1* was detected.

To evaluate the number of times a switch between mating systems occurred in the evolution of *Pleospora* sensu stricto, the species phylogeny was compared to the MAT phylogenies, in conjunction with the results from structural analyses of the MAT loci. MAT data of *Pleospora* sensu stricto suggested a single origin of the fused MAT regions from separate MAT regions. The single origin was supported by the monophyly of the fused MAT regions in MAT phylogenies, as well as the complicated structure of the fused MAT regions that was unlikely to have evolved twice independently. Whereas combined MAT evidence suggested a single origin of the fused MAT regions, the species phylogeny suggested at least two independent origins of the fused MAT regions. The conflicting evidence between MAT data and the *Pleospora* sensu stricto species phylogeny was consistent with a single origin of the fused MAT regions followed by a horizontal transfer across lineages, by sexual or asexual means. The one time evolution and subsequent horizontal transfer of the fused MAT region constituted two different evolutionary origins of the homothallics with fused MAT regions.

A third origin of homothallism in *Pleospora* sensu stricto was in the group with a separate MAT locus containing a forward-oriented *MAT1-1* gene. Homothallism in this case may be do to unknown mutations in other than the *MAT* genes, as possibly in the homothallic *Neurospora africana*.

The last part of my thesis dealt with two new species in the new family Aliquandostipitaceae. Both *Aliquandostipite khaoyaiensis* and *A. sunyatsenii* were collected in Asia, and comprised several features not previously reported in ascomycetes. Novel morphological features were the presence of two types of fruitbodies side by side on the substrate, and the widest hyphae reported in ascomycetes. Overall morphological appearance suggested that species of *Aliquandostipite* were related to members of the order Pleosporales. However, molecular analyses of the 18S rDNA showed that species of *Aliquandostipite* did not belong to the Pleosporales, but instead grouped with uncertain affinity in the class Dothideomycetes.

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CHAPTER 1. General introduction

1.1. The ascomycetes

Fungi are important ecologically and economically, e. g. as wood degraders, mycorrhizal partners, endophytes, bread, beer, and antibiotics producers, and as human and plant pathogens. Despite these key roles, we know little about fungal diversity. Fungi are the second largest group of eukaryotes, after the insects (Hawksworth, 1991). Of an estimated 1.5 million fungal species, only 5% are currently known to science (Hawksworth, 1991). The knowledge of fungi in British Columbia is similarly poor. A six year survey of the ascomycete group Loculoascomycetes from wood found at least 25% new species in a restricted area in Sidney on Vancouver Island (Barr & Huhndorf, 2001).
The largest group of fungi are the ascomycetes with more than 32,000 described species (Hawksworth *et al.*, 1995). Well-known ascomycetes are Baker's yeast, *Neurospora*, *Penicillium*, athlete's foot fungus, the truffles and morels. Common to all ascomycetes is the ascus. This is a sac-like structure where karyogamy and meiosis take place, and the sexual spores are formed.

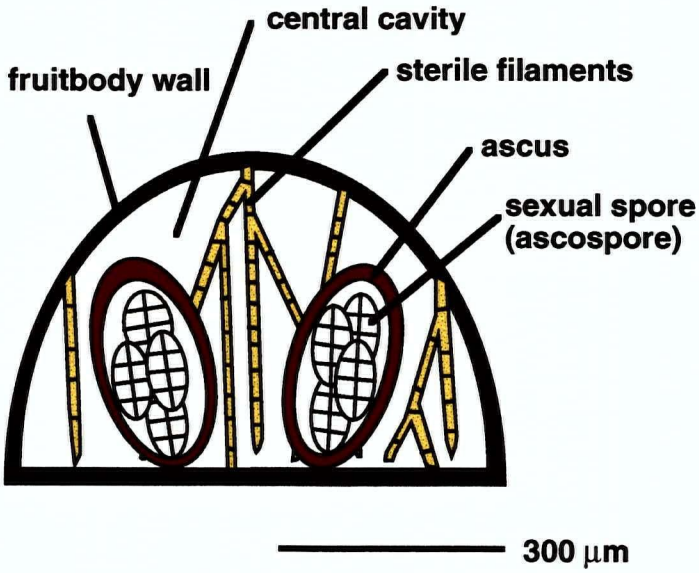


Figure 1-1. Diagram of vertical section through fruitbody of a microscopic ascomycete.

The asci containing the sexual spores or ascospores are placed within a small fruitbody made up of tightly interwoven, melanized hyphae (Figure 1-1). Ascospores can be hyaline or pigmented, smooth or ornamented, and can have several transverse and longitudinal septa. Interspersed among the asci are sterile hyphal filaments, the pseudoparaphyses. At maturity, the asci elongate, and the ascospores are forcibly discharged through a pore at the fruitbody apex. On the appropriate substrate, the ascospores will germinate and give rise to a mycelium that will form asexual spores or conidia. The morphology of the conidia-forming state or anamorph differs greatly from the sexual state or teleomorph. The two states can also be separated in space and time. For these reasons, the anamorph has often been given a separate name. However, if the sexual state of a fungus is known, its name refers to the entire fungus. This is confusing, and authors are discouraged from creating names for asexual states, if the connection to a sexual state is known (Greuter, 2000).

1.2. On species concepts in fungi

A species defined as 'the lowest principal rank in the nomenclatural hierarchy' (Hawksworth *et al.*, 1995), can be erected based on any variable character depending on the biological circumstances (Mishler & Donoghue, 1992), views of the taxonomist or requirements of the end user. In mycology, at least five different species concepts have been used (Hawksworth *et al.*, 1995). Three species concepts are relevant to this thesis and are briefly discussed below. These are the morphological, biological and phylogenetic species concepts.

Most fungal species have been described based on morphological characters (Hawksworth *et al.*, 1995). In the microscopic ascomycetes, examples of characters used for species definition are ascospore color, dimensions, morphology, or fruitbody size and ornamentation.

The biological species concept defines species as 'groups of interbreeding natural populations that are reproductively isolated from other such groups' (Mayr in Wiley (1981)). Biological species have only been defined in relatively few cases in fungi. Because reproductive isolation is difficult to observe in a natural setting, crossing experiments have been performed under laboratory conditions. In some cases several reproductively isolated groups within one morphological species were found (Korhonen, 1978; Kurtzman, 1993). This outcome is not unexpected given the relatively few morphological characters available for definition of morphospecies. However, occurrence of mating in the laboratory does not necessarily imply the existence of sexual reproduction in nature as sexual compatibility of geographically separate species can be a shared ancestral character.

The phylogenetic species concept has become popular in fungi with the easy accessibility to DNA sequence data. As opposed to morphological and biological species concepts, it is not based on a particular type of character, but corresponds to monophyletic groups inferred from any type of character (Donoghue, 1985). Phylogenetic species recognition is the methodology that uses phylogenetic analyses based on DNA data from several loci to detect phylogenetic species (Taylor *et al.*, 2000). Data from the different loci are combined for the inference of a phylogenetic tree. Phylogenetic species are then characterized by incongruence and low bootstrap supports within species due to sexual reproduction, and congruence and high bootstrap supports between species due to reproductive isolation. Phylogenetic species recognition is particularly suitable for fungi, since it overcomes drawbacks of morphological and biological species concepts, which respectively are few characters because of simple morphology, and the difficulties of detecting sex in nature. The method has been applied successfully a number of times, for example in the human pathogenic fungus *Coccidioides immitis* Stiles (Koufopanou *et al.*, 1997). The morphological species *C. immitis* was thought to be asexual. However, phylogenetic species recognition found that *C. immitis* contained two

groups with a history of reproductive isolation, both of which were reproducing sexually (Burt *et al.*, 1996). It was found that the two phylogenetic species were indistinguishable by morphological characters, but differed in growth rates (Fisher *et al.*, 2002), and might differ in pathogenicity and symptoms caused in patients with coccidioidomycosis (Taylor & Fisher, 2003). The sexual states are still unknown. Thus, neither morphological nor biological species concept would have detected the two species of *Coccidioides*.

1.3. The ascomycete genus *Pleospora*

Pleospora is a large, heterogenous genus with almost 1000 described species (Holm, 1962). Species of *Pleospora* are microscopic, to the naked eye the fruitbodies appear as black dots in the substrate (Figure 1-2). The main morphological character of *Pleospora* is the presence of both transverse and vertical septa in the ascospores, called 'muriform' ascospores (Wehmeyer, 1961) (Figure 1-3). Muriform ascospores are relatively rare in ascomycetes. However, it is easy to imagine that muriform ascospores originated repeatedly from the more common transversally septate ascospores, and that thus *Pleospora* is polyphyletic (Holm, 1962). To divide *Pleospora* into natural groupings, additional morphological characters have to be considered, both of the anamorph as well as the teleomorph (Crivelli, 1983; Eriksson, 1967).

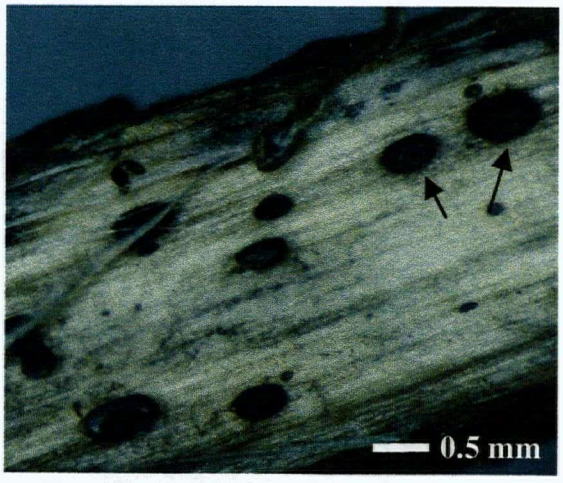


Figure 1-2. Fruitbodies of *Pleospora sp.* strain P60 on decaying plant material.

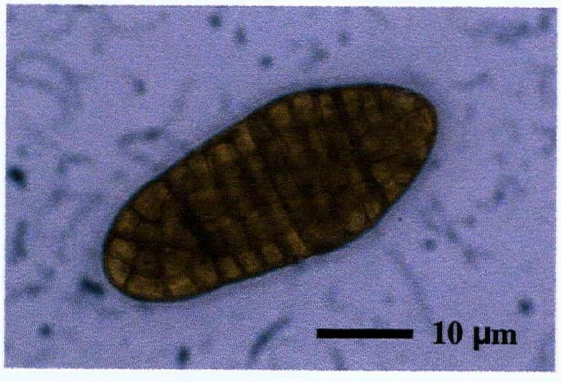


Figure 1-3. Muriform ascospore of *Pleospora gigaspora* strain P134.

The type species of *Pleospora*, *P. herbarum* (Pers.: Fries) Rabenhorst ex Cesati & de Notaris has a *Stemphylium* anamorph (Simmons, 1969; 1985; 1989; 2001). As opposed to *Pleospora*, *Stemphylium* is monophyletic, and exclusively produces *Pleospora* teleomorphs (Câmara *et al.*, 2002). Thus, based on the data currently available, *Pleospora* is monophyletic and well defined, if restricted to species forming a *Stemphylium* anamorph, which I will refer to as *Pleospora* sensu stricto.

However, for most of the *Pleospora* species little information is available, especially about their anamorphs, and this may be one reason that this generic concept has not been published.

A relatively small number of *Pleospora* species that differ morphologically from *Pleospora* sensu stricto have been transferred to other genera, most of them by Crivelli (1983) and Leuchtmann (1984) who transferred 29 and six species, respectively. However, together with *Pleospora* sensu stricto, the 'true' *Pleospora* species with *Stemphylium* anamorph, these total fewer than 100, so that most of the estimated nearly 1000 described species of *Pleospora* remain to be evaluated (Holm, 1962). In this thesis, unless specified otherwise, *Pleospora* refers to species with *Stemphylium* anamorph, *Pleospora* sensu stricto.

1.4. Mating type genes

In ascomycetes, sexual reproduction is controlled by the MAT locus, a master regulator of downstream gene expression (Turgeon *et al.*, 1993). There are two MAT alleles, and only strains with different MAT alleles can mate (Kronstad & Staben, 1997). In the genus *Cochliobolus* in the family Pleosporaceae as is *Pleospora*, the MAT alleles were shown to be necessary for mate recognition, fruitbody and sexual spore formation (Turgeon, 1998). However, exactly how the MAT locus controls gene expression is not yet known in filamentous ascomycetes, but is understood in considerable detail in the yeast *Saccharomyces cerevisiae* (Herskowitz, 1989).

As *Pleospora*, *Cochliobolus* contains different life histories. There are both outcrossing and selfing species. In fungi, the term used for enforced outcrossing is 'heterothallism', whereas 'homothallism' denotes self-fertility (Hawksworth *et al.*, 1995). In *Cochliobolus*, hetero- and homothallism is correlated with mating type gene arrangement: Heterothallic species either have a *MAT1-1* or a *MAT1-2* gene at their MAT locus, but homothallic species have both *MAT1-1* and *MAT1-2* genes. The mating type genes of homothallics in *Cochliobolus* are arranged in different ways, suggesting multiple evolutionary origins from heterothallics (Yun *et al.*, 1999). In some homothallic species, an unequal crossover between MAT alleles was thought to be responsible for the presence of both MAT regions end to end at the MAT locus (Yun *et al.*, 1999). Crossovers between MAT alleles are rare, since MAT alleles have entirely different DNA sequences, and are thus not alleles in the true sense. The term used to describe this situation is ' MAT idiomorphs' (Metzenberg & Glass, 1990).

Homothallic ascomycetes are not confined to selfing. *Gibberella zeae* (Schweinitz) Petch, in the subphylum Pezizomycotina as *Pleospora*, has both MAT genes present end to end in the genome, and can self (Yun *et al.*, 2000). However, the same fungus has been shown to outcross under laboratory con-

ditions, and probably does so naturally (Bowden & Leslie, 1999). Thus, the term 'homothallism' is not equivalent to 'selfing', but instead describes a condition in which selfing is possible but not necessarily obligate.

In ascomycetes, MAT gene arrangement does not always correlate with homothallism as in *Cochliobolus*, because homothallism can be achieved in alternative ways. For example, in the secondary homothallic or pseudohomothallic ascomycete *Neurospora tetrasperma* Shear & Dodge opposite mating types are packaged into one ascospore after meiosis (Metzenberg & Glass, 1990). And in the homothallic *N. africana* Huang & Backus, only one MAT idiomorph has been detected (Glass *et al.*, 1988) even though the species is self-fertile. The other MAT idiomorph might have escaped detection, or an alteration of the *MAT*-controlled regulatory cascade might allow the expression of the genes necessary to complete a sexual cycle.

1.5. Thesis theme and objectives

My thesis uses phylogenetic DNA analyses as well as morphological examination to answer questions of evolution, diversity and taxonomy in the ascomycete class Dothideomycetes. The first two chapters focus on *Pleospora* species with *Stemphylium* anamorphs. In chapter one, I establish a robust multigene phylogeny in order to group the *Pleospora* isolates into phylogenetic species. Morphological species of *Stemphylium* are represented by type strains or strains compared to type specimens, so that I can evaluate how well morphological species correspond to phylogenetic species.

In chapter two, I investigate evolution of hetero- and homothallism in *Pleospora*. To determine how many times a switch from hetero- to homothallism or vice versa occurred, I generate phylogenetic tree topologies from MAT loci, and contrast them to the organismal phylogeny from chapter one. Invoking standard mechanisms of DNA rearrangement, I provide a hypothesis for the origin of the MAT gene arrangement found in selfers.

In chapter 3, I contribute towards a monophyletic genus *Pleospora* by transferring the non–*Stemphylium* producing marine fungus *Pleospora gaudefroyi* Patouillard to the new genus *Decorospora*. Using ribosomal DNA sequences, I test if *Decorospora* and *Pleospora* are monophyletic, and determine the phylogenetic placement of *Decorospora*.

In the last chapter, I describe the new family Aliquandostipitaceae with the new genus *Aliquandostipite* for two novel and unusual fungi. Phylogenetic analyses with ribosomal DNA sequences are used to investigate the suspected close relationship of the two morphologically similar fungi, their relationship to the phenotypically somewhat similar genus *Tubeufia*, as well as to three orders in the class Dothideomycetes.

1.6. Bibliography

Barr, M. E. & Huhndorf, S. M. (2001). Loculoascomycetes. In *The Mycota VII Part A*, pp. 283-305. Edited by M. M. Lemke. Berlin, Heidelberg: Springer Verlag.

Bowden, R. L. & Leslie, J. F. (1999). Sexual recombination in *Gibberella zeae*. *Phytopathology* **89**, 182-188.

Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. (1996). Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 770-773.

Câmara, M. P. S., O'Neill, N. R. & Berkum, v., P (2002). Phylogeny of *Stemphylium* spp. based on ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **94**, 660-672.

Crivelli, P. G. (1983). Über die heterogene Ascomycetengattung *Pleospora* Rabenh.; Vorschlag für eine Aufteilung. Ph.D. thesis. Eidgenössische Technische Hochschule, Zürich, Switzerland.

Donoghue, M. J. (1985). A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist* **88**, 172-181.

Eriksson, O. (1967). *On graminicolous pyrenomycetes from Fennoscandia. 1. Dictyosporous species*. Stockholm: Almqvist & Wiksell.

Fisher, M. C., Koenig, G. L., White, T. J. & Taylor, J. W. (2002). Molecular and phenotypic description of *Coccidioides posadasii* sp. nov., previously recognized as the non-California population of *Coccidioides immitis*. *Mycologia* **94**, 73-84.

Glass, N. L., Vollmer, S. J., Staben, C., Grotelueschen, J., Metzenberg, R. L. & Yanofsky, C. (1988). DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. *Science* **241**, 570-573.

Greuter, W. J. (2000). *International Code of Botanical Nomenclature (Saint Louis Code) adopted by the Sixteenth International Botanical Congress St. Louis, Missouri, July - August 1999*. Königstein, Germany: Koeltz Scientific Books.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: Magnitude, significance, and conservation. *Mycological Research* **95**, 641-655.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995). *Dictionary of the Fungi*, 8 edn. Wallingford, Oxon, UK: CAB International.

Herskowitz, I. (1989). A Regulatory Hierarchy for Cell Specialization in Yeast. *Nature* **342**, 749-757.

Holm, L. (1962). Lewis E. Wehmeyer, a world monograph of the genus *Pleospora* and its segregates. *Svensk Botanisk Tidskrift* **56**, 377-381.

Korhonen, K. (1978). Interfertility and clonal size in the *Armillariella mellea* complex. *Karstenia* **18**, 31-42.

Koufopanou, V., Burt, A. & Taylor, J. W. (1997). Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitits*. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 5478-5482.

Kronstad, J. W. & Staben, C. (1997). Mating type in filamentous fungi. *Annual Review of Genetics* **31**, 245-276.

Kurtzman, C. P. (1993). The systematics of ascomycetous yeasts defined from ribosomal RNA sequence divergence: Theoretical and practical considerations. In *The Fungal holomorph: Mitotic, meiotic and pleomorphic speciation in Fungal systematics*, pp. 271-279. Edited by D. R. Reynolds & J. W. Taylor. Wallingford: CAB International.

Leuchtmann, A. (1984). Über *Phaeosphaeria* Miyake und andere bitunicate Ascomyceten mit mehrfach querseptierten Ascosporen. *Sydowia* **37**, 75-194.

Metzenberg, R. L. & Glass, N. L. (1990). Mating type and mating strategies in *Neurospora*. *BioEssays* **12**, 53-59.

Mishler, B. D. & Donoghue, M. J. (1992). Species concepts: a case for pluralism. In *The units of evolution: essays on the nature of species*, pp. 121-137. Edited by M. Ereshefsky. Cambridge, MS: MIT Press.

Simmons, E. G. (1969). Perfect states of *Stemphylium*. *Mycologia* **61**, 1-26.

Simmons, E. G. (1985). Perfect states of *Stemphylium*. II. *Sydowia, Annales Mycologici Ser. II.* **38**, 284-293.

Simmons, E. G. (1989). Perfect states of *Stemphylium*. III. *Memoirs of the New York Botanical Garden* **49**, 305-307.

Simmons, E. G. (2001). Perfect states of *Stemphylium*: IV. *Harvard Papers in Botany* **6**, 199-208.

Taylor, D. L., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M. C. (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**, 21-32.

Taylor, J. W. & Fisher, M. C. (2003). Fungal multilocus sequence typing - it's not just for bacteria. *Current Opinion in Microbiology* **6**, 351-356.

Turgeon, B. G. (1998). Application of mating type gene technology to problems in fungal biology. *Annual Review of Phytopathology* **36**, 115-137.

Turgeon, B. G., Christiansen, S. K. & Yoder, O. C. (1993). Mating type genes in ascomycetes and their imperfect relatives. In *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*, pp. 199-215. Edited by D. R. Reynolds & J. W. Taylor. Wallingford: CAB International.

Wehmeyer, L. E. (1961). *A world monograph of the genus Pleospora and its segregates*. Ann Arbor: The University of Michigan Press.

Wiley, E. O. (1981). *Phylogenetics: the theory and practice of phylogenetic analysis*. New York: John Wiley & Sons.

Yun, S. H., Berbee, M. L., Yoder, O. C. & Turgeon, G. (1999). Evolution of fungal self-fertile reproductive life style from self-sterile ancestors. *Proceedings of the National Academy of Sciences of the USA* **96**, 5592-5597.

Yun, S. H., Arie, T., Kaneko, I., Yoder, O. C. & Turgeon, B. G. (2000). Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. *Fungal Genetics and Biology* **31**, 7-20.

CHAPTER 2. Phylogenetic species of *Pleospora* sensu stricto

2.1. Introduction

2.1.1 *Pleospora* sensu stricto and hypotheses

Fungi in the genus *Pleospora* are small, their sexual fruitbodies are black, and generally less than one millimeter in diameter. They have a worldwide distribution, growing as saprotrophs on decaying vegetation, or as pathogens on living plants, causing damage in agriculture. Affected crops are alfalfa, clover, onions, garlic, asparagus, tomato, potato, ornamental flowers, and cotton (Câmara *et al.*, 2002). Sometimes the damage is severe. For example, in the 1990's in Brazil, *Pleospora* caused up to 100% field loss in cotton, requiring a change of the cotton cultivar (Mehta, 1998). Members of *Pleospora* produce both sexual and asexual spores. Sexual spores are muriform (Figure 2-1), and formed within asci that are produced inside a hard, melanized fruitbody. In *Pleospora*, asexual spores are muriform as well; however, they are not formed within a fruitbody, but at the tips of superficial hyphae (Figure 2-2). The sexual and asexual spores are often produced independently of each other in space and time. Due to these circumstances, separate names have been used for the sexual and asexual state of this organism: *Stemphylium* denotes the asexual state, whereas *Pleospora* refers to the entire fungus if a sexual state is known. Here, *Pleospora* is used to collectively refer to all ingroup isolates included in this study, i. e. *Pleospora* sensu stricto. Also, for strains without published names, *Pleospora* is used if the fungus was isolated from sexual spores, and *Stemphylium* for the strains derived from asexual spores.

The primary goal of this study was to generate a robust phylogenetic hypothesis for *Pleospora* strains with *Stemphylium* anamorphs. The second goal was to apply a phylogenetic species concept to species delimitation, focusing especially on the type, *P. herbarum*. A number of morphological similar species to *P. herbarum* have been published (Simmons, 1969; Simmons, 1985; Simmons, 2001). An earlier phylogenetic study based on two nuclear genes suggested that those species could not be distinguished from the type (Câmara *et al.*, 2002). I wanted to know if by using more loci and isolates, the morphological species concept would agree with the phylogenetic species concept.

2.1.2 Loci used for phylogenetic analyses

2.1.2.1 Ribosomal internal transcribed spacer region (ITS)

The ITS region situated between the 18S and 28S ribosomal genes, has been extensively used in fungal phylogenetics at the species level. It is present in several hundred copies, homogenized by concerted evolution. In some cases,

more than one ITS type per strain has been found (O'Donnell & Cigelnik, 1997).

2.1.2.2 *Glyceraldehyde-3-phosphate dehydrogenase* (*GPD*)

GPD is an enzyme involved in glycolysis. Intron-containing partial DNA sequences of *GPD* have previously been used by Berbee et al. (1999) for species level phylogenetics in *Cochliobolus*. No evidence for the presence of multiple *GPD* copies was found.

2.1.2.3 *Elongation factor-1 alpha* (*EF-1 alpha*)

EF-1 alpha plays an important role during protein synthesis, by catalyzing the GTP-dependent binding of aminoacyl-transferase RNA to ribosomes. In eukaryotes, it is the second most abundant protein after actin, constituting 1-2% of the total protein in normal growing cells (Condeelis, 1995). Exons of *EF-1 alpha* are highly conserved, and amino acid sequences have been used in eukaryote phylogenetics (Baldauf & Doolittle, 1997), whereas introns were used successfully to resolve relationships between species of *Fusarium* (O'Donnell *et al.*, 1998). No evidence for multiple copies of this gene has been found.

2.1.2.4 *vmaA–vpsA* intergenic spacer

During this study, I briefly had access to the *Cochliobolus heterostrophus* Drechsler genome sequence courtesy of Syngenta, through a collaboration with B. G. Turgeon and G. Saenz of the former Torrey Mesa Research Institute in San Diego. Greg Saenz provided me with a *C. heterostrophus*, single copy intergenic spacer region of ca. 700 bp in length that I could PCR amplify in *Pleospora* (see results). Blast searches at GenBank (Altschul *et al.*, 1997) revealed that the flanking genes were most similar to the respective *Aspergillus oryzae* (Ahlburg) Cohn gene for vacuolar membrane ATPase catalytic subunit A (Kuroki *et al.*, 2002), and *A. nidulans* (Eidam) Winter *vpsA* gene which is involved in vacuolar biogenesis (Tarutani *et al.*, 2001). The intergenic spacer between the *vmaA* and *vpsA* genes was used for phylogenetic analyses as a fourth locus.

2.2. Materials and methods

2.2.1 Fungal strains used

Most strains included were retrieved from culture collections. They were selected to cover the genetic diversity of *Pleospora* in culture collections, with an emphasis on strains similar to the type species. Only *Pleospora* strains derived from type specimens or considered representative of type specimens by E. G. Simmons were given complete names. Exceptions were *P. gigaspora* determined by P. G. Crivelli, *P. triglochinicola* and *S. astragali* collected by the re-

spective type authors and *S. callistephi* and *S. loti* possibly collected by their respective type authors (Table 2-1). For unnamed strains, the teleomorph name *Pleospora* was only used for strains derived from sexual spores. Unnamed strains derived from asexual spores were named *Stemphylium sp.*, regardless if they formed the sexual state or not (Table 2-2).

In total, 115 fungal strains were used for DNA sequencing, 111 were unique (Table 2-2), and four were duplicates included as controls. Most isolates were obtained from Dr. Emory Simmons, Crawfordsville, IN, USA. These included 99 strains of *Pleospora* or *Stemphylium*, as well as one strain of *Alternaria alternata* (Fries) Keissler as an outgroup taxon. Three strains of *S. loti* and *S. callistephi* were received from Dr. Nichole O'Neill from the US Department of Agriculture, Beltsville, MD, USA. Four unnamed strains isolated from diseased cotton were obtained from Dr. Y. R. Mehta, Instituto Agronômico do Paraná, Londrina, Brazil. The remaining eight strains were collected locally in British Columbia, Canada. These were *Pleospora sp.* strains P56, P93, P107, P301, P327, P338, P342 and P343.

For the following three isolates, duplicates were included as controls: Isolate EGS 36-118, *P. triglochinicola*, three replicates derived from three different ascospores, as strains P120, P123, and P130. Isolate EGS 08-174, *Stemphylium sp.*, two replicates as strains P201 and P202, and isolate EGS 48-095, *P. sedicola*, as strains P211 and P271. All duplicates were sequenced for at least one locus, but only one representative per replicate was used for analyses and submitted to GenBank (strains P123, P201 and P271).

Eight additional single ascospore isolates not listed in Table 2-2 and derived from the material of *Pleospora sp.* strain P56 were used to screen for *MAT1-2*.

2.2.2 Fungal isolation and culture conditions

Single, germinated conidia or ascospore were transferred from water agar to V8 agar plates (Hawksworth *et al.*, 1995). All cultures were grown on V8 agar plates at room temperature prior to DNA extraction, and stored on V8 agar slants at 4°C in the refrigerator. For long term storage, strains were grown on water agar, and three mycelium-containing agar cubes of ca. 1 mm^3 were placed in a tube with distilled water, and kept in the refrigerator at 4°C (Bandoni, personal communication; Croan et al. (1999)).

2.2.3 Molecular work

2.2.3.1 DNA extraction and PCR

Mycelium was scraped off the surface of a Petri plate, and DNA was extracted using a phenol-chloroform extraction method (Lee & Taylor, 1990). See chapter 7.1 for details. PCR reactions were performed with ReadyToGo PCR Beads,

or puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA), following the manufacturer's instructions. Or GIBCO BRL Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD) was used. See chapter 7.1 for details. For touchdown PCR, Expand High Fidelity PCR System Taq was used (Roche Diagnostics GmbH, Mannheim, Germany), on a Perkin Elmer DNA Thermal Cycler. Most other PCR reactions were done on a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA, USA). PCR conditions varied depending on the primers and loci and are described in detail below.

2.2.3.2 DNA sequencing

2.2.3.2.1 Set-up

DNA sequencing reactions were set up using 4 µl of Big Dye Terminator Cycle Sequencing Kit v2.0 or 3.0 (Applied Biosystems, Foster City, CA, USA), 100-200 ng of PCR product, and 3.2 pmol of sequencing primer per 20 µl final reaction volume. All PCR products were completely sequenced in both directions. The sequencing PCR program used was 5 minutes initial denaturation at 95°C, 25 cycles of 10 sec at 96°C, 5 sec at 50°C and 4 min at 60°C, on a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA, USA).

2.2.3.2.2 Trouble shooting

Poor DNA sequencing results were a major problem in this study. In case of ambiguous DNA sequence (mostly multiple peaks, weak signals), sequencing primers situated a few base pairs internal to the PCR primers were taken. If that did not help, new PCR and sequencing primers were designed in close proximity. If problems persisted, primer sites were moved 200 - 300 bp away from the initial site. With this approach, all sequencing problems were overcome. The reason for the sequencing anomalies remains unknown.

2.2.3.3 Primers and PCR conditions used

2.2.3.3.1 Internal transcribed spacer (ITS)

Ribosomal internal transcribed spacer regions (ITS) were PCR amplified using primers ITS5 and ITS4 (White *et al.*, 1990), and sequenced with the same primers, or the internal ITS1 (White *et al.*, 1990) or ITS871r (Table 2-3), 16 bp internal to ITS4 (Figure 2-3). If that did not work, then ITS5 was substituted by the external ITS1-F (Gardes & Bruns, 1993) for PCR with ITS4, and sequenced with ITS5 or ITS1, and ITS871r. PCR conditions used were 5 minutes initial denaturation at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 55°C and 30 sec (+4 sec/cycle) at 72°C, followed by a final extension of seven minutes at 72°C.

2.2.3.3.2 *Glyceraldehyde-3-phosphate dehydrogenase (GPD)*

Primers used were GPD1 and GPD2 (Berbee et al., 1999). A new forward primer designed from *Stemphylium GPD* sequences was GPD2033f (Table 2-3), 17bp downstream of GPD1 (Figure 2-3). Based on *Cochliobolus lunatus* (GenBank X58718) and *C. heterostrophus* (GenBank X63516) *GPD* sequences, the following additional primers were designed: GPD2669r, a reverse primer 28bp downstream of GPD2, and GPD3r, a reverse primer 163 bp downstream of GPD2 (Table 2-3).

PCR conditions used were 5 minutes initial denaturation at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 60°C and 30 sec (+4 sec/cycle) at 72°C, followed by a final extension of seven minutes at 72°C. PCR amplifications were done using GPD1 and GPD3r, and sequenced with GPD1 and GPD2, or GPD2033f and GPD2669r if the former primers did not sequence well.

2.2.3.3.3 *Elongation factor-1 alpha (EF-1 alpha)*

Primers used were designed based on *EF-1 alpha* DNA sequences of *Aureobasidium pullulans* (GenBank U19723) and *Podospora anserina* (GenBank X74799) (Table 2-3; Figure 2-3). Forward primers designed were EF446f, EF451f and EF462f at positions 78, 106, and 117 of *P. anserina*, respectively. Reverse primers were EF1598r, EF1473r, and EF3R at positions 886, 766, 654 of *P. anserina*, respectively. PCR products were obtained with combinations of EF446f/EF1473r or EF1598r. For reamplification of weak PCR bands, one initial PCR primer was substituted with an internal primer. PCR products were sequenced with EF451f or EF462f, and EF3r. In some cases other primer combinations for PCR and sequencing were used to obtain satisfactory DNA sequences. PCR conditions used were 5 minutes initial denaturation at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 52°C and 30 sec (+4 sec/cycle) at 72°C, followed by a final extension of seven minutes at 72°C. For reamplification, an annealing temperature of 55°C and 40 cycles were used.

For *S. lancipes* isolate P229, *S. trifolii* isolate P244, and *Stemphylium sp.* isolate P246, initial PCR reactions yielded weak bands of ca. 2.5 kbp. To obtain sufficient PCR product for DNA sequencing, two sets of touchdown PCR with hotstart conditions were employed. Each set consisted of 20 cycles with decreasing annealing temperature, and 20 cycles with stable annealing temperature. In the first set, an initial denaturation step (2 min at 94°C) was followed by 20 cycles of denaturation (10 sec at 94°C), annealing (20 sec first at 70°C, then -0.5° per cycle), and extension steps (first 30 sec, then +4 sec per cycle at 72°C), followed by 20 cycles of denaturation (10 sec at 94°C), annealing (20 sec at 60°C), and extension (first 1 min, then +4 sec per cycle at 72°C), and a final extension step of 7 min at 72°C. The second set used internal primers on a 100 times dilution of the PCR product from the first round, and the same PCR conditions as in the first round, except for 5°C lower an-

nealing temperatures, and a one minute longer extension time for the last 20 cycles.

A hotstart protocol was followed in both sets of PCR. The PCR cocktail was prepared without the addition of Taq polymerase. 12.5 µl of template were added, overlaid with a drop of mineral oil and the PCR program started. Once the tubes were heated to 94°C, 0.5 µl of Expand High Fidelity PCR System Taq was used (Roche Diagnostics GmbH, Mannheim, Germany), and the PCR program left to run as described above.

Primers used for PCR and DNA sequencing of *S. lancipes* isolate P229, *S. trifolii* isolate P244, and *Stemphylium sp.* isolate P246 were as follows: PCR of *S. lancipes* isolate P229 was with EF446f/EF1473r, reamplified with EF451f/EF1473r, sequenced with EF462f, EFP229f, EfaIIF2, EfaIIF, and reverse primers EF3R, EFLr, EFAIIr (Table 2-3). PCR of *S. trifolii* isolate P244 was with EF446f /EF1473r, reamplification with EF451f/EF3r, and sequenced with EF462f, EFLf, EFAIIf2, EFAIIf, EF3r, EfLr, EFAIIr. PCR of *Stemphylium sp.* isolate P246 was like *S. trifolii* isolate P244, sequencing with EF462f, EFP246f, EFAIIF2, EFAIIf, and EF3r, EFLr, EFAIIr.

The sequencing of outgroup strain *Alternaria alternata* isolate P95 was not straightforward. The 5'-end was obtained with primer pair EF451f/EF1473r, and sequenced with EF462f, EF1473r and EF2r (Table 2-3). However, the 3'-end of this sequence was not alignable with the other sequences, and it was assumed that during PCR, the reverse primer EF1473r bound upstream of its intended site, within the *A. alternata* isolate P95 intron. To obtain the 3'-end, the primer 95f was designed downstream of the intron 3'-end (Table 2-3; Figure 2-3), and used together for PCR and sequencing with Efr designed ca. 280 bp downstream of EF1598r.

2.2.3.3.4 *vmaA-vpsA* intergenic spacer

The DNA sequences of two adjacent *Cochliobolus heterostrophus* genes separated by a spacer of ca. 700 bp were obtained from the former Torrey Mesa Research Institute (G. Saenz and B. G. Turgeon, personal communication).

To design primers amplifying the intergenic spacer between the *vmaA* and *vpsA* homologs in *Pleospora*, portions of the adjacent gene flanks were amplified using primers designed based on regions conserved in *C. heterostrophus* and sequences retrieved from GenBank. These were the *A. oryzae vmaA* (GenBank AB073302), and *A. nidulans vpsA* genes (GenBank AB074883). For the *vmaA* homolog, an approximately 310 bp portion ca. 480 bp upstream of the 3'-end of the gene, was PCR amplified with primers VATP2949f, and VATP3238r (Table 2-3; Figure 2-3). For the *vpsA* homolog, approximately 170 bp downstream of the 5'-end, a ca. 530-bp fragment was PCR amplified with primers GTP446f and GTP980r. Subsequently, *Pleospora*-specific forward and reverse primers were designed on the sequences obtained. For *vmaA*,

VATP3195f and AiF were designed 235 and 218 bp upstream of the *A. oryzae* vmaA gene 3'-end, respectively. Then, based on VATP3195 *Pleospora* sequences, AN1 and ATPF2 were designed 179 and 113 bp upstream from the *A. oryzae vmaA* 3'-end, respectively. For the *vpsA* gene, GTP604r and GTPr were designed 244 and 57 bp downstream of the *A. nidulans* vpsA 5'-end, respectively.

Isolates were initially PCR amplified with primer pair VATP3195f/GTP604r, and sequenced with ATPF2/GTP604r. For isolates that did not sequence well, other primer combinations for PCR and sequencing were used, most successfully PCR with AiF/GTP604r, and sequencing with AiF, GTP604r or GTPr, as well as internal primers AGf, and its reverse complement AGr (Table 2-3; Figure 2-3).

2.2.4 Phylogenetic analyses

2.2.4.1 DNA sequences

DNA sequences were assembled using AutoAssembler version 1.4.0 (Applied Biosystems, Perkin Elmer Corp., Norwalk, Connecticut, USA) and DNA sequence alignments were generated by ClustalX (1.8) (Thompson *et al.*, 1997) using default settings, and manually optimized in Se-Al v1.d1 (Rambaut, 1995).

Phylogenetic analyses were performed using PAUP* 4.0b10 for parsimony, likelihood and Neighbor joining analyses (Swofford, 2002), and MrBayes v3.0b4 for Bayesian analyses of phylogeny (Huelsenbeck & Ronquist, 2003).

For the inference of most parsimonious trees, 30 heuristic searches using random taxon addition replicates were done with default settings, including gaps coded as missing characters. Bootstrap support for the branches was based on 500 replicates, using random taxon addition and otherwise default settings.

For likelihood analyses, necessary parameters such as base frequencies, transition – transversion ratio, proportion of invariable sites, and gamma shape parameter was estimated from a most parsimonious tree. The gamma distribution was approximated by four rate categories. Most likely trees were estimated using 30 heuristic searches with random taxa addition and otherwise default settings. Bootstrap support values were based on a varying number of random taxa addition replicates, with the number of branch rearrangements limited at times to overcome computational limitations.

Neighbor joining analyses were done using likelihood modeled distances with parameters estimated on a most parsimonious tree. Branch support was evaluated with 500 bootstrap replicates.

For Bayesian analyses, a general time reversible model of evolution was used. Rate heterogeneity across sites was modeled with a gamma distribution. Four chains starting with a random tree were run for one million generations,

retaining each 100th tree. The first 1000 trees of the 10,000 collected trees were discarded, and the subsequently calculated consensus trees were based on the remaining 9000 trees. The 50% majority rule consensus tree was calculated in PAUP*.

2.3. Results

2.3.1 DNA sequences

2.3.1.1 Ribosomal internal transcribed spacer region (ITS)

Pleospora sequences were submitted to GenBank (103 sequences, AY329168 - AY329270). Sequence length was between 482 and 493 bp. Eight homologous regions were retrieved from GenBank. *S. callistephi* (AF442783), two strains of *S. loti* (AF442789, AF442788), four *Stemphylium sp.* sequences (AF203451, AF203448, AF203449, AF203450), and *Alternaria alternata* (AF347031).

The resulting alignment thus contained 111 taxa, was 502 characters in length, and was mapped as follows: Positions 1-166 ITS1, 167-324 5.8S, 325-502 ITS2.

2.3.1.2 *Glyceraldehyde-3-phosphate dehydrogenase* (*GPD*)

Pleospora sequences were submitted to GenBank (107 sequences, AY316968 - AY316973, AY316975 - AY316978, AY316980, AY316982 - AY316989, AY316991 - AY317074, AY534637 - AY534640). The four *Stemphylium sp.* strains P406 – 409 sequenced were the same isolates for which ITS sequences were retrieved from GenBank. Sequences were between 500 and 508 bp in length, they corresponded to bases 1069 - 1578 of the *C. lunatus* DNA *GPD* sequence from GenBank (X58718). Four homologous regions were retrieved from GenBank for inclusion in the alignment: *Stemphylium callistephi* (AF443882), two strains of *S. loti* (AF443888, AF443887) and *A. alternata* (AY278808). The alignment thus contained 111 taxa and 511 characters. Intron-exon designations in the *GPD* alignment were by comparison to the *C. lunatus* sequence: Intron positions 1-37, exon 38-101, intron 102-216, exon 217-505.

2.3.1.3 *Elongation factor-1 alpha* (*EF-1 alpha*)

Pleospora and *A. alternata* sequences were submitted to GenBank (110 *Pleospora sequences,* AY324671 - AY324776, AY534633 - AY534636, and AY438647 5'-end and AY438648 3'-end of *A. alternata*). For *S. callistephi* strain P383, *S. loti* strains P384 and P385 and *A. alternata* strain P95 the same isolates were used for which ITS and *GPD* sequences were retrieved from GenBank. Sequences of *Pleospora* were between 684 bp and 2313 bp in

length. To designate position numbers and introns for the *Pleospora* sequences, homologous nucleotide positions and homologous introns from *Podospora anserina* (GenBank X74799) were specified. *Stemphylium* sequences started between positions 605 – 628 in intron number 3 (intron ends in *P. anserina* at position 792), across exon number 4 and intron number 4, to position 1307 in exon number 5. The outgroup *A. alternata* strain P95 sequence was 528 bp in length and intron number 4, along with 106 bp of exon number 5 were not sequenced. The alignment was 3145 characters in length.

The large difference in size among the *Pleospora EF-1 alpha* sequences were due to the introns present approximately at the position of intron number 4 in *P. anserina*, the internal intron in Figure 2-3. The introns at this position varied in size from 49 – 1678 bp (Table 2-4). Intron lengths were determined using amino acid translations of the adjacent coding regions, and assuming intron consensus splice sites of 5'-GT and AG-3'. Introns generally diverged little between closely related isolates, but were too divergent to be aligned between the groups (data not shown). For this reason, intron data could not be analyzed using phylogenetic methods. Introns were instead classified into groups based on their visually assessed similarity. There were eight different groups of intron sequences. Introns from each group were present in one to 67 isolates (Table 2-4). Introns were inserted at four positions, corresponding to *P. anserina* (GenBank X74799) positions 912 or 913 for two groups of isolates, and positions 929 or 932 for the remaining six groups (Table 2-4). Three of the introns contained ORFs from 141 – 1440 bp long. These are described below in detail.

2.3.1.3.1 Protein encoding *EF-1 alpha* introns

The intron of three not closely related *Stemphylium* isolates encoded a protein. These were the introns from *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246. The introns of these three isolates were all inserted at position 929 of GenBank sequence X74799 (Table 2-4), and were 1501 – 1678 bp in length, containing an ORF of 834 – 1440 bp, which at the 3'-end comprised a protein whose DNA sequence was 507 – 1389 bp in length (Table 2-5). The protein was similar in the three isolates, an alignment of the overlapping translated protein sequences was 160 amino acids in length, with 75% identical sites and two gaps (data not shown). All three proteins were most similar to a hypothetical protein from *Gibberella zeae* (GenBank XP_381390), with a random match probability equal or smaller than 10^{-14}, and at least 28% amino acid identity (Table 2-5). Using a PFAM protein online search (Bateman *et al.*, 2004), it was determined that the proteins contained a zinc finger domain of the C2H2 type, involved in nucleic acid binding. An *EF-1 alpha* gene was not present in *G. zeae* near the gene encoding the hypothetical protein (data not shown).

The large introns of *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246 also differed from all other sequenced *Pleospora* introns in that they were not delimited by the standard GT intron splice site at the 5'-end, but by 5'-GGT (data not shown). The 3'-end was a standard AG-3'. All attempts to splice the intron at a 5'-GT resulted in a reading frame shift and an early stop codon (data not shown). 5'-GGT splice sites are known from mammals (Burset *et al.*, 2000). However, experimental evidence for the splicing at 5'-GGT in the three *Stemphylium* isolates is lacking.

2.3.1.4 *vmaA–vpsA* intergenic spacer

Pleospora and *A. alternata* sequences were submitted to GenBank (53 *Pleospora sequences*, AY329271 - AY329323, and AY329324 from *A. alternata*). The final alignment was 718 characters in length. It contained the following features: Positions 1-42 *vmaA*, 43-718 intergenic spacer.

2.3.2 Verification of results

Mislabeling, contamination and mixing up of samples is a concern in phylogenetics. To check for human error, after preliminary phylogenetic analyses (data not shown), seven suspicious strains were reordered from E. G. Simmons, reisolated as single conidia, and resequenced for ITS, *GPD* and *EF-1 alpha loci*. The strains were *Stemphylium sp.* strain P210, *S. lancipes* strain P229, *S. solani* P240, *S. trifolii* strain P244, *Stemphylium sp.* strain P246, *P. paludiscirpi* strain P270, *Stemphylium sp.* strain P303. All sequences were identical to previously obtained ones (data not shown). Using GenBank Blast searches, 39 of my ITS and *GPD* sequences were compared to homologous sequences from GenBank from identical or conspecific strains. No evidence for major error was found. Some of my sequences differed from the GenBank sequences in single nucleotide positions. Rechecking my data did not require any changes (data not shown). Additionally, identical ITS, *GPD* and *EF-1 alpha* DNA sequences of *P. triglochinicola* strain P123 were independently generated from isolates P120 and P130 derived from three different ascospores from the same ascoma. This is a likely outcome in the case of progeny from the same parents. ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* sequences identical to *S. astragali* strain P201 were obtained from *S. astragali* strain P202, both of which derived from the same isolate, EGS 08-174. *Stemphylium sp.* strain P211 and *P. sedicola* strain P271 were both derived from isolate EGS 48-095, and gave identical ITS, *GPD* and *EF-1 alpha* DNA sequences (data not shown). Thus, no evidence for major sequencing errors was found.

2.3.3 Protein sequences

2.3.3.1 *Glyceraldehyde-3-phosphate dehydrogenase* (*GPD*)

There was one amino acid substitution in the *GPD* dataset. Amino acid number 1, a V is changed to I in *S. sarciniforme* strain P239, *Stemphylium sp.* strains P309 and P310, *S. sarciniforme* strain P247, *Stemphylium sp.* strain P306, *S. solani* strain P240, *Stemphylium sp.* strains P241, P252 and P253, and in *S. callistephi* strain P383 (data not shown; Figure 2-5).

2.3.3.2 *Elongation factor-1 alpha* (*EF-1 alpha*)

There was only one amino acid substitution in the *EF-1 alpha* dataset. Amino acid 84 changed from P to V in the group with *S. sarciniforme* strain P239, *Stemphylium sp.* strains P309 and P310, as well as in the group with *S. sarciniforme* strain P247, with *Stemphylium sp.* strain P306 (data not shown; Figure 2-5).

2.3.3.3 *vmaA-vpsA* intergenic spacer

Only for 14 predicted amino acids at the 3'-end of *vmaA* DNA sequence coverage was obtained. Out of the 14 amino acids, four were constant, five were parsimony-uninformative, and five were parsimony-informative (data not shown). However, the low number of characters did not allow the inference of a phylogeny with a well-supported branching order.

2.3.4 Phylogenetic analyses

2.3.4.1 Selection of representative isolates for *vmaA-vpsA* sequencing

ITS, *GPD* and *EF-1 alpha* datasets were combined and analyzed with parsimony, gaps were treated as fifth character. The combined dataset contained 111 *Pleospora* taxa and 1653 characters, the *EF-1 alpha* internal intron was excluded from analyses. The analysis was aborted after it ran out of memory (data not shown). In order for analyses to be completed, only one genotype from each clade with identical branch length was retained, resulting in a total of 39 unique multilocus genotypes (Table 2-6). The parsimony analysis was repeated with the 39 taxa and gaps were coded as missing data. The analysis resulted in 572 most parsimonious trees, of 471 steps each (CI = 0.735, RI = 0.893) (Figure 2-4).

2.3.4.2 Parsimony analyses of single datasets for representative isolates

From the 110 *Pleospora* isolates for which the ITS, *GPD* and *EF-1 alpha* loci were sequenced, 53 representatives were chosen for sequencing of the additional *vmaA-vpsA* locus (Figure 2-4). The resulting, reduced datasets were first analyzed separately for each locus, in each case using only unique geno-

types to speed up analyses. The reduced single datasets were then tested for combinability prior to pooling them for combined analyses using four different algorithms.

2.3.4.2.1 Ribosomal internal transcribed spacer region (ITS)

The reduced ITS dataset of 53 representative isolates contained all 19 ITS genotypes obtained for the 110 *Pleospora* isolates (Table 2-7). The alignment thus comprised 19 taxa, and was 502 characters in length. 45 characters were variable (9.0%), of which 13 were parsimony informative (2.6%). The search resulted in 56 most parsimonious trees, 54 steps each (CI = 0.889, RI = 0.864).

The bootstrap analysis was done with only the informative characters included. Only the grouping of *S. sarciniforme* strain P239 and *S. loti* strain P384 received more than 70% bootstrap support (81%) (Figure 2-5).

2.3.4.2.2 *Glyceraldehyde-3-phosphate dehydrogenase* (*GPD*)

The reduced *GPD* dataset of 53 representatives contained 27 genotypes, including all but three genotypes of the 110 *Pleospora* isolates (Table 2-8). The genotypes of *Stemphylium sp.* strains P212 and P221 were not included, they were represented by the closely related *Pleospora sp.* strain P107 (Figure 2-4). The genotype of the *Stemphylium sp.* strain P407 was represented by *S. solani* strain P240. The alignment used for phylogenetic analyses thus contained 27 taxa, and was 511 characters in length. 130 characters were variable (25.4%), of which 89 were parsimony informative (17.4%). The search resulted in 18 most parsimonious trees, 211 steps each (CI = 0.735, RI = 0.854).

The bootstrap analysis was done with only the informative characters included. Twelve groupings received more than 70% bootstrap support (Figure 2-5).

2.3.4.2.3 *Elongation factor-1 alpha* (*EF-1 alpha*)

The reduced *EF-1 alpha* dataset of 53 taxa contained 28 of the 30 *EF-1 alpha* genotypes from the 110 *Pleospora* isolates (Table 2-9), not considering the *EF-1 alpha* internal intron (Figure 2-3). Excluded were *Stemphylium sp.* strain P221 and *S. solani* strain P241, close relatives of the included *Pleospora sp.* strain P107 and *S. solani* strain P240, respectively. Thus, the dataset used for analyses contained 28 taxa and 640 characters. 123 were variable (19.2%), of which 70 were parsimony informative (10.9%). The search resulted in 64 most parsimonious trees, 191 steps long (CI = 0.738, RI = 0.837).

The bootstrap analysis was done with only informative characters included. Thirteen groupings received more than 70% bootstrap support (Figure 2-5).

2.3.4.2.4 *vmaA-vpsA* intergenic spacer

The 53 *Pleospora* isolates sequenced for *vmaA-vpsA* had 28 unique genotypes (Table 2-10). The alignment used in this single locus analysis thus contained 28 taxa and 718 characters. 247 characters were variable (34.4%), of which 184 were parsimony informative (25.6%). The search resulted in 24 most parsimonious trees, 388 steps long (CI = 0.771, RI = 0.933).

The bootstrap analysis was done with only informative characters included. Thirteen groupings received more than 70% bootstrap support (Figure 2-5).

2.3.4.3 Test for combinability of ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* datasets

A partition homogeneity test was used to assess the phylogenetic conflict between small ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* datasets. Due to limitations of available computational power, only the unique 35 multilocus genotypes (Table 2-11), and informative characters were included in the analyses. The internal *EF-1 alpha* intron was excluded. The dataset thus contained 35 taxa and 370 characters. The partition homogeneity test judged the four datasets not to be significantly different, as out of 1000 sets of resampled datasets, only 821 were longer than 655 steps, the sum of the single most parsimonious trees based on informative characters (P = 0.179).

2.3.4.4 Analyses of combined ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* dataset

A combined ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* dataset without the second *EF-1 alpha* introns was investigated for phylogenetic structure. The dataset contained 53 taxa and 2371 characters. For analyses, only unique multilocus genotypes were included, so that the number of taxa was reduced to 35 (Table 2-11). Four different phylogenetic methods were used. These were parsimony, likelihood, Bayesian analyses, and Neighbor joining.

2.3.4.4.1 Parsimony

In parsimony analyses, 24 most parsimonious trees of 860 steps each were found (CI = 0.748, RI = 0.886) (Figure 2-6). The most parsimonious trees differed by the disposition of branches with 52% or lower bootstrap support (data not shown). The position of the *P. gigaspora* strain P129 *and S. majusculum* strain P262 containing group was interchanged, and combined with rearrangements in the sister group to *Stemphylium sp.* strain P303. *Stemphylium sp.* strains P272, P273, P277 and the five isolates identical to *Stemphylium sp.* strain P235 were either polyphyletic or monophyletic, of varying topology. Branches supported by more than 70% of the bootstrap replicates are numbered in Figure 2-6, and support percentages are listed in Table 2-12.

2.3.4.4.2 Likelihood

One most likely trees was obtained (-ln likelihood = 8367.9). Its topology was identical to the most parsimonious tree in Figure 2-6. Bootstrap support for the branches was based on the evaluation of 124 replicates. Branch swapping was limited to 10,000 per replicate due to computational limitations. Branches supported by more than 70% of the bootstrap replicates are numbered in Figure 2-6, and support percentages are listed in Table 2-12.

2.3.4.4.3 Bayesian analyses

The Bayesian 50% majority rule consensus tree with branch lengths, based on 9000 trees, was identical in topology to one of the 24 most parsimonious trees (data not shown). Differences to the most parsimonious tree shown in Figure 2-6 involved the topology of the sister group to *Stemphylium sp.* strain P303, among which the monophyly of *Stemphylium sp.* strains P235 and identical taxa plus *Stemphylium sp.* strain P277 was supported by 74% posterior probability (data not shown). The remainder of the posterior probabilities above 70% are numbered in Figure 2-6, and values listed in Table 2-12.

2.3.4.4.4 Neighbor joining

In Neighbor joining analyses, a maximum likelihood model for distance calculations was used with the same parameters employed as in the likelihood analyses. The Neighbor joining tree differed from the most parsimonious tree illustrated in Figure 2-6 by the following alterations. These were rearrangements in the sister group of *Stemphylium sp.* strain P303, as well as *P. tarda* strain P1 plus identical taxa being sole sister group to the taxa ranging from *P. eturmiuna* strain P269 to *Stemphylium sp.* strain P235, and *S. lancipes* strain P229 being sister taxon to *S. solani* strain P240 plus identical taxa and *S. callistephi* strain P383. None of these branches was supported by more than 50% of the replicates in the Neighbor joining bootstrap analyses, and thus compatible with the most parsimonious trees. Branches supported by more than 70% of the bootstrap replicates are numbered in Figure 2-6 and the support percentages are listed in Table 2-12.

2.4. Discussion

2.4.1 Phylogenetic relationships in *Pleospora*

2.4.1.1 Comparison to other studies

Results obtained agreed with other studies of molecular evolution in *Pleospora* (Câmara *et al.*, 2002; Mehta *et al.*, 2002).

Câmara et al. (2002) used ITS and *GPD* DNA sequences to analyze phylogenetic relationships for 37 isolates of *Pleospora*, 20 of which were identical to isolates included in this study. We used 110 isolates that were se-

quenced for the three loci ITS, *GPD* and *EF-1 alpha*, as well as the additional *vmaA-vpsA* locus for 53 representative isolates. Since this study was based on more loci and isolates than that of Câmara et al.'s (2002) and an outgroup was not included, some novel or more highly supported clades were obtained.

Câmara et al. (2002) recognized five monophyletic groups in *Pleospora*, A – E. The five groups were also obtained here, each one supported by 100% of the bootstrap replicates in all the multilocus analyses (Figure 2-6). Relevant additions of this study to *Pleospora* phylogenetics are restricted to groups C, D and E, and are discussed below by comparison to the combined ITS/*GPD* phylogeny in Câmara et al. (2002).

Group C contained the type species of *Pleospora*, *P. herbarum*. It received 100% support in all combined four loci analyses in this study, and 72% in Câmara et al. (2002). My results added four additional representatives of morphological species (Figure 2-6). These were *P. tomatonis* strain P268, *P. sedicola* strain P271, *P. gigaspora* strains P129, and *P. eturmiuna* strain P269. *Pleospora tomatonis* strain P268 and *P. sedicola* strain P271 grouped with *P. herbarum* strain P2, *P. alfalfae* strain P81, *S. vesicarium* strain P238, as well as unnamed strains, with 100% support in all analyses, as compared to 89% obtained by Câmara et al. (2002). The sister group to the previous taxa was *P. gracilariae* strain P243 and *Stemphylium sp.* strain P315 with at least 86% bootstrap support (Figure 2-6). The next well supported node included all the previous taxa, plus *S. majusculum strain* P262 with unnamed *Stemphylium sp.* strain P311, *P. gigaspora* strains P122 and P129, and S. *astragali* strain P201 in the combined four loci analyses with at least 90% of the bootstrap replicates (Fig. 6). *Pleospora eturmiuna* strain P269, as well as unnamed taxa were the sister group to all the previous taxa, with 100% support in all combined four loci analyses, as opposed to 72% in Câmara et al. (2002).

Group D received 100% support in all combined four loci analyses (Figure 2-6), and 76% in Câmara et al. (2002).

Group E also received maximal support, and 89% in Câmara et al. (2002). Group E in this study is more diverse than previously, when it used to contain five lineages (Câmara *et al.*, 2002). My results added seven potential new lineages (Figure 2-6), represented by *P. paludiscirpi* strain P270, *Pleospora sp.* strains P56, P107, and P327, *Stemphylium sp.* strains P210 and P246, as well as *S. sarciniforme* strain P247. *Pleospora paludiscirpi* strain P270, *Pleospora sp.* strain P327 and another unnamed strain, as well as *Stemphylium sp.* strain P210 were monophyletic together with the previously included *P. triglochinicola* strain P123 with 100% support in all combined four loci analyses. *Pleospora sp.* strain P56 grouped with the previously included *Stemphylium sp.* strain P227, as well as unnamed isolates, with 100% bootstrap support in the ITS/*GPD*/ *EF-1 alpha* analyses (*vmaA-vpsA* was not sequenced for *Stemphylium sp.* strain P227) (Figure 2-4). *Stemphylium sarcini-*

forme strain P247 and unnamed isolates grouped with maximal support in all combined four loci analyses with *S. sarciniforme* strain P239 plus unnamed isolates. *Stemphylium sp.* strain P246 was the most distant one of the new group E taxa, as it did not show any clear affinity to a particular monophyletic group (Figure 2-6).

Although included in the Câmara et al. (2002) study, *S. lancipes* strain P229 was not attributed to any of the groups. In this multigene analyses, it was sister taxon to group B, with little support except in the Bayesian analysis (Figure 2-6). The phylogenetic position of *S. lancipes* strain P229 is thus doubtful, and for the sake of completeness, it is placed in its own group, group F.

The four *Stemphylium sp.* strains P406 to 409, have been used in a phylogenetic study of cotton pathogens in Brazil by Mehta et al. (2002). *Stemphylium sp.* strain P406 differed from the other three strains in RAPD pattern and ITS sequence. This correlated with our results that place *Stemphylium sp.* strain P406 into group A, whereas *Stemphylium sp.* strains P407 to P409 group with *S. solani* strain P240 in group D (Figure 2-4). The RAPD pattern of *Stemphylium sp.* strain P409 differed slightly from *Stemphylium sp.* strains P407 and P408 (Mehta *et al.*, 2002). This difference is consistent with results from this study, where *Stemphylium sp.* strains P407 and P408 have identical sequence for ITS, *GPD* and *EF-1 alpha*, differing by one substitution from *Stemphylium sp.* strain P409 (*vmaA-vpsA* was not sequenced for these isolates). Another difference between *Stemphylium sp.* strains P407 and P408, and *Stemphylium sp.* strain P409 are the host and lack of virulence in strain P409 (Mehta *et al.*, 2002).

2.4.1.2 Datasets and phylogenetic signal

Combination of the single datasets into one analysis increased the bootstrap support values of the branches, as can be expected (Huelsenbeck *et al.*, 1996). However, the addition of the *vmaA-vpsA* dataset did not notably improve the resolution: All the branches supported with 100% of the bootstrap replicates in the four loci analyses also were supported by 100% in the combined ITS/*GPD*/ *EF-1 alpha* parsimony analysis (Figure 2-4; Figure 2-6). In single dataset analyses, ITS was the only dataset where not all groups were recognized, but only groups B and E (Figure 2-5). This can be explained by the lower information content of ITS as compared to the other loci (Table 2-13).

2.4.1.3 Rooting of phylogenetic trees

Câmara et al. (2002) used several outgroup taxa to root the *Pleospora* phylogenies. They showed that the genus was monophyletic. In this study, rooting was attempted using the outgroup *Alternaria alternata* strain P95, a possible closest relative to the genus *Pleospora* (Berbee *et al.*, 1999). However, the *A.*

alternata strain P95 DNA sequences were difficult to align with the ingroup sequences, prolonged phylogenetic analyses, and lowered bootstrap support values of the branches (data not shown). Thus, the outgroup was omitted. The trees in Figure 2-4, Figure 2-5 and Figure 2-6 are presented as rooted, based on data from mating type phylogenies (see section on MAT protein phylogenies on page 81).

2.4.1.4 The internal protein encoding *EF-1 alpha* intron

It is puzzling that the internal *EF-1 alpha* intron encoded a similar protein in three not closely related isolates of *Stemphylium*. Additionally, the respective introns had other unique features in common, such as their unusual length, and delimitation by a non-standard intron splice site.

The internal intron of *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246 was 1501 – 1678 bp in length, much longer than the *S. callistephi* strain P383 intron with 199 bp, the longest *EF-1 alpha* internal intron of the remaining isolates (Table 2-4). *EF-1 alpha* introns were also much shorter in other fungi. In *Aureobasidium pullulans* and *Neurospora crassa*, also members of the subphylum Pezizomycotina, the introns in the *EF-1 alpha* gene were below 250 bp in length (Ichiishi & Inoue, 1995; Thornewell *et al.*, 1995). In *Saccharomyces cerevisiae* (Cottrelle *et al.*, 1985), there were no introns in the *EF-1 alpha* gene, and in the basidiomycete *Schizophyllum commune*, the longest *EF-1 alpha* intron was 55 bp in length (Wendland & Kothe, 1997).

Another shared feature of the internal intron of the three isolates was the non-canonical GTT splice site at the intron 5'-end. Splice sites of this kind are known from only nine cases in mammals (Burset *et al.*, 2000). They were nicknamed 'parasitic' splice sites, because the conventional 5'-GT was hypothesized to be 'exploited' by the 5'-GGT site for the assemblage of the splicing machinery (Burset *et al.*, 2000).

The third shared feature was the similar, intron encoded protein. It contained a zinc finger domain of the C2H2 type that is involved in nucleic acid binding and was most similar to an unknown protein in the ascomycete *Gibberella maydis* with a random match probability as low as 9×10^{-27} (Table 2-5). The *G. maydis* protein was not encoded in an *EF-1 alpha* intron. It is not known if the zinc finger protein is expressed in the three *Stemphylium* isolates, or what its origin is, especially since the isolates concerned are not closely related (Figure 2-6).

It might be suspected that the unusual intron length, the presence of the intron-encoded protein, and 'parasitic' intron splice site interfere with the expression of *EF-1 alpha*, the second most abundant protein in eukaryotes (Condeelis, 1995). However, the growth rates of cultures of *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246 were not obvi-

ously different from other *Pleospora* isolates. No evidence for more than one copy of *EF-1 alpha* was found in this study.

2.4.2 Phylogenetic species in *Pleospora*

In this study, 22 phylogenetic species were found using the phylogenetic species recognition approach (Table 2-14). This methodology has been used in mycology to detect reproductively isolated groups within morphological species (Taylor *et al.*, 2000). It involves the generation of gene phylogenies with a taxon sampling covering the genetic diversity of the species in question. Datasets are combined for phylogenetic analyses, and strains that group together with high support are designated phylogenetic species. Phylogenetic species are groups of individuals that have shared a history of sexual reproduction, as opposed to biological species that are groups of presently sexually compatible individuals (Mayr in Wiley (1981)). On the level of phylogenetic trees, a shared history of sexual recombination manifests itself in discordant topologies within phylogenetic species, and concordance of the branching order among phylogenetic species. New phylogenetic species have successfully been defined with, for instance, 17 – 46 isolates, and 4 –5 loci (Geiser *et al.*, 1998; Kasuga *et al.*, 1999; Koufopanou *et al.*, 1997).

In this study, 110 isolates were used for sequencing of three loci, of which 53 representatives were chosen for the sequencing of an additional fourth locus. However, the intent of the taxon sampling was not to cover the entire diversity of *Pleospora* in detail, but it was focused on the diversity around the type of *Pleospora*, *P. herbarum* strain P2. Thus, of the 22 phylogenetic species found, only four contained more than five isolates, and in five cases, single isolates were designated phylogenetic species (Table 2-14). These isolates were designated as separate phylogenetic species because they seemed too divergent to be included in phylogenetic species together with related isolates. In order to prove the status of most postulated phylogenetic species as constituting natural, reproductively isolated groups, additional studies with more isolates are necessary.

When investigating the relationships between morphological and phylogenetic species, it is crucial to include representatives of morphological species in the phylogenetic analyses. In this way, phylogenetic species can be tied to morphological species, preferably directly to type material. If type material of morphological species is not available for DNA sequencing, then a representative has to be chosen by morphological comparison with the original type material.

In agreement with Câmara et al. (2002), in *Pleospora* morphological and phylogenetic species correlated rather well. Of the 22 phylogenetic species, nine contained just one isolate representative of the type of a morphological species (Table 2-14). The phylogenetic species *P. herbarum* comprised five

types, and the phylogenetic species *S. xanthosomatis* might contain *S. lycopersici* which has no known type (E. G. Simmons, personal communication) (Table 2-14). Five phylogenetic species contained isolates that may or may not have been compared with the respective types (Table 2-15), and the remaining six phylogenetic species did not contain any named isolates (Table 2-14).

In Table 2-15, details are given on how the 16 isolates linking phylogenetic species to morphological species relate to the respective types. For the two phylogenetic species that might be in conflict with morphological species, isolates of *P. herbarum* and *S. xanthosomatis* were chosen as representatives, since *P. herbarum* is the type of *Pleospora* and no type species is known from *S. lycopersici* (Table 2-1).

2.4.3 Delimitation of *Pleospora herbarum*, the type of *Pleospora*

Pleospora herbarum could not be differentiated from closely related morphological species. Both *P. alfalfae*, *S. vesicarium*, *P. tomatonis*, *P. sedicola* as well as 42 unnamed isolates fell within the phylogenetic species *P. herbarum* (Figure 2-4, Figure 2-6).

Due to its central taxonomic role, *Pleospora herbarum* has been well studied. Its type material has been located, the link to its asexual state established, and representative cultures have been chosen (Simmons, 1985). At least four morphological species similar to *P. herbarum* in both anamorph and teleomorph characters have been described (Simmons, 1969; Simmons, 1985; Simmons, 2001). Câmara et al. (2002) included two of those species, *P. alfalfae* and *S. vesicarium* in phylogenetic analyses based on ITS and *GPD* datasets, in addition to the type species of *Pleospora*, *P. herbarum*. They could not differentiate *P. herbarum*, *P. alfalfae* and *S. vesicarium*, and concluded that molecular support was lacking for the distinction of those morphological species (Câmara *et al.*, 2002). Because the genetic circumscription of the type species of *Pleospora* is important, we wanted to investigate if we could find phylogenetic species underlying the morphological species *P. herbarum*, *P. alfalfae* and *S. vesicarium*. We included in our study 42 unnamed isolates with morphological affinity to *P. herbarum*, and sequenced the three loci ITS, *GPD* and *EF-1 alpha* for all of them. Results showed that all 42 unnamed isolates grouped together with the named isolates *P. herbarum* strain P2, *P. alfalfae* strain P81, *S. vesicarium* strain P238, as well as two additional representatives of morphological species, *P. tomatonis* strain P268 and *P. sedicola* strain P271 (Figure 2-4), yielding a most parsimonious topology requiring only three steps.

Based on the ITS, *GPD* and *EF-1 alpha* analyses, 17 isolates comprising all morphological species as well as covering the genetic diversity of the group were chosen for sequencing of the additional fourth locus *vmaA-vpsA*. This did not add any notable variation (Figure 2-6). As can be seen in the phylogenetic

trees for the isolates selected for *vmaA-vpsA* sequencing in Figure 2-5, the phylogenetic structure of the *EF-1 alpha* and *vmaA-vpsA* trees in the phylogenetic species *P. herbarum* is not identical. Thus, in addition to being genetically closely related, there seems to be no shared phylogenetic structure that corresponds to the described morphological species. On the other hand, the monophyly of the phylogenetic species *P. herbarum* is well supported in combined analyses of all four loci (Figure 2-6). This means that the phylogenetic species *P. herbarum* is genetically isolated from neighboring species, and due to the lack of concordant internal phylogenetic structure, represents thus only one phylogenetic species according to the phylogenetic species recognition approach (Taylor *et al.*, 2000).

It could be argued that the loci used in this study were not variable enough to differentiate between the morphological species. However, morphological characters supported the close phylogenetic relationships of *Pleospora herbarum*, *P. alfalfae*, *P. tomatonis*, *P. sedicola* and *S. vesicarium*. In all five species, the following morphological features fell within the respective range given: Sexual fruitbodies were between 0.35 – 1 mm in diameter, and contained yellow-brown sexual spores, 28 - 45 um long and 8 - 18 um wide, with seven to nine transverse septa with 1 – 3 vertical septa per cell. Asexual spores were 25 – 48 um long and 12 – 27 um wide, with 1-7 transverse septa and 1 – 3 vertical septa per cell, and were yellow-brown with a warted wall (Simmons, 1969; Simmons, 1985; Simmons, 2001). The morphological characters were not stable for one species. For example, the number of transverse septa of the asexual spores of *S. vesicarium* ranged from one to six (Simmons, 1969).

There is evidence outside *P. herbarum* that in *Pleospora* morphological groupings did not always correspond to phylogenetic entities. Câmara et al. (2002) mentioned the example of *Stemphylium sp.* strain P227 in group E. This isolate had morphological features similar to *S. globuliferum*, but did not group with the morphologically similar isolates which were placed in group B instead.

Thus, based on our data, the five morphological species *P. herbarum*, *P. alfalfae*, *P. sedicola*, *P. tomatonis* and *S. vesicarium* constitute one phylogenetic species. Additional investigations using a population genetics approach are needed to test for reproductive boundaries in the phylogenetic species *P. herbarum* (Zhan *et al.*, 2002), and whether they correspond to described morphological species.

2.4.4 Unidentified phylogenetic species

As many as six potentially new species were obtained. In six cases, strains or groups of strains did not cluster with a named isolate, or in the case of *S. sarciniforme* strain P247, might be sufficiently different from a named isolate

(Figure 2-6). Three of these phylogenetic species contained isolates collected in British Columbia. These were *Pleospora sp.* strains P56, P107, and P327.

To name all phylogenetic species, considerable research would be necessary, as up to 1000 species of *Pleospora* have been described (Holm, 1962). The slide collection vouchering Wehmeyer's work (1961), documenting about 1200 collections of *Pleospora* and *Pleospora*-like fungi, would be a starting point.

Table 2-1. Morphological species included in this study. The names of both anamorphs and teleomorphs are given together with their P strain numbers (see Table 2-2). Relations of the isolates to type specimens for both sexual and asexual states as well as literature references to the type descriptions are also given. 'Type' refers to collections judged equivalent to type material, 'ex type' are strains derived from type collections. See section on fungal strains on page 11 for rationale of applying names to strains.

Morphological species: Teleomorph	Morphological species: Anamorph	Representative strain	Relation to type	Reference
P. alfalfae Simmons	*S. alfalfae* Simmons	P81	ex type	Simmons (1985)
P. eturmiuna Simmons	*S. eturmiuna* Simmons	P269	ex type	Simmons (2001)
P. gigaspora Karsten	*Stemphylium sp.*	P129	unknown[1]	Karsten (1884)
P. gracilariae Simmons & Schatz	*S. gracilariae* Simmons	P243	ex type	Simmons (1989)
P. herbarum (Pers.: Fries) Rabenhorst ex Cesati & de Notaris	*S. herbarum* Simmons	P2	type / ex type	Simmons (1985)
P. paludiscirpi Simmons	*S. paludiscirpi* Simmons	P270	ex type	Simmons (2001)
P. sedicola Simmons	*S. sedicola* Simmons	P271	ex type	Simmons (2001)
P. tarda Simmons	*S. botryosum* Wallroth	P1	ex type / type	Simmons (1985)
P. tomatonis Simmons	*S. tomatonis* Simmons	P268	ex type	Simmons (2001)
P. triglochinicola Webster	*S. triglochinicola* Sutton & Pirozynski	P123	unknown[2]	Webster (1969)
Pleospora sp.	*S. astragali* Yoshii	P201	unknown[3]	Yamamoto (1960); Yoshii (1929)
Pleospora sp.	*S. majusculum* Simmons	P262	type[4]	Simmons (1969);
Pleospora sp.	*S. vesicarium* (Wallroth) Simmons	P238	type[4]	Simmons (1969)
unknown	*S. callistephi* Baker & Davis	P383	unknown[5]	Baker and Davis (1950)
unknown	*S. lancipes* (Ellis & Everhart) Simmons	P229	type[6]	Simmons (1969)
unknown	*S. loti* J. H. Graham	P384	unknown	Graham (1953)
unknown	*S. lycopersici* (Enjoji) W. Yamamoto	P242	type[7]	Yamamoto (1960)
unknown	*S. sarciniforme* F. Cavara	P239	type[8]	Cavara (1890)
unknown	*S. solani* G. F. Weber	P240	type[8]	Weber (1930)
unknown	*S. trifolii* J. H. Graham	P244	type[8]	Graham (1957)
unknown	*S. xanthosomatis* Huguenin	P232	ex type[9]	Huguenin (1965)

[1] Identified by Crivelli (1983).
[2] Sent to Simmons by J. Webster, author of type (E. G. Simmons, personal communication).
[3] Sent to Simmons by H. Yoshii, author of type (E. G. Simmons, personal communication).
[4] Simmons, personal communication.
[5] Collected by K. Baker (Câmara *et al.*, 2002) who might be identical to the author of the type.
[6] Excellent representative of type (E. G. Simmons, personal communication).
[7] Simmons' concept of this species, no type specimen known (E. G. Simmons, personal communication).
[8] Compared to type by Simmons (E. G. Simmons, personal communication).
[9] Isolated ex type by type author and sent to Simmons (E. G. Simmons, personal communication).

Table 2-2. Isolates used in this study, with substrates and locations of origin, grouped according to their P strain numbers. Regardless of origin, all isolates received a P number, which was used in the text throughout. Original strain identifiers, if existing, are listed as well. For strains collected in this study, P numbers also refer to my fungarium. Strain P95 is *Alternaria alternata*, the remaining strains are *Pleospora*. Specific epithets were only used for isolates for which a link to type material could be established (see Table 2-1 and p. 11). In the case of unnamed strains, the teleomorph name *Pleospora* was only used for strains derived from sexual spores. Unnamed strains derived from asexual spores were named *Stemphylium sp.*, regardless if they formed the sexual state or not.

P #	*Pleospora* teleo-morph	*Stem-phylium* anamorph	Strain identi-fiers	Substra-tum	Country	Locality
1	*tarda*	*botryosum*	EGS 08-069[1]	*Medicago sativa* L.	USA	NH
2	*herbarum*	*herbarum*	EGS 36-138[1]; IMI 276975[2]	*Medicago sativa* L.	India	Jhansi
56	*sp.*	*sp.*	SA1.17[3]	decaying leaf of *Phragmites* or similar plant	Canada	South Arm Marshes Wildlife Management Area, Ladner, BC[13]
81	*alfalfae*	*alfalfae*	EGS 36-088[1]; IMI 2696832[2]	*Medicago sativa* L.	Australia	WA
93	*sp.*	*sp.*	SA2.28[3]	decaying stalk of as-teraceous plant	Canada	South Arm Marshes Nature Reserve, Lad-ner, BC[14]
95	-	*alternata*	EGS 34-016[1]	-	-	-
107	*sp.*	*sp.*	N1.8[3]	decaying herbaceous dicot.	Canada	Along White Rapids Rd, South of Nanaimo, BC[14]
122	*gigaspora*	*sp.*	EGS 37-016[1]; ZT9127[4]	*Minuartia hybrida* (Vill.) Schischkin	Switzer-land	Lucomagno, TI
123	*triglochini-cola*	*triglochini-cola*	EGS 36-118[1]	*Triglochin maritima* L.	GB	Dawlish Warren, De-von
126	*sp.*	*sp.*	EGS 37-149[1]; ZT9122[4]	*Rumex acetosa* L.	Switzer-land	Meride, TI
129	*gigaspora*	*sp.*	EGS 37-017[1]; ZT9127[4]	*Minuartia hybrida* (Vill.) Schischkin	Switzer-land	Lucomagno, TI
201	-	*astragali*	EGS 08-174[1]	*Astragalus sp.*	Japan	Fukuoka
203	-	*sp.*	EGS 27-1941[1]	*Astragalus sinicus* L.	Japan	Tominaga, Tokushima
204	-	*sp.*	EGS 27-1942[1]	*Astragalus sinicus* L.	Japan	Tominaga, Tokushima
205	-	*sp.*	EGS 29-062[1]; IFO 7244[5]	*Astragalus sp.*	Japan	-
206	-	*sp.*	EGS 30-181[1]	*Medicago sativa* L.	New Zealand	Palmerston
207	-	*sp.*	EGS 48-074[1]	*Medicago sativa* L.	New Zealand	Auckland

208	-	*sp.*	EGS 48-075[1]	*Medicago sativa* L.	New Zealand	Auckland
209	-	*sp.*	EGS 48-087[1]	*Pistacia vera* L.	USA	Kern County, CA
210	-	*sp.*	EGS 48-089[1]	*Pistacia vera* L.	USA	Kern County, CA
212	-	*sp.*	EGS 48-097[1]	*Passiflora edulis* Sims	New Zealand	Auckland
213	-	*sp.*	EGS 48-099[1]	*Medicago lupulina* L.	New Zealand	-
214	-	*sp.*	EGS 48-101[1]	*Vicia sativa* L.	New Zealand	Auckland
215	-	*sp.*	EGS 48-102[1]	*Trifolium pratense* L.	New Zealand	Auckland
216	-	*sp.*	EGS 48-103[1]	*Medicago sativa* L.	New Zealand	Papakura
217	-	*sp.*	EGS 48-104[1]	*Medicago sativa* L.	New Zealand	Central Otago
218	-	*sp.*	EGS 48-105[1]	*Medicago sativa* L.	New Zealand	Central Otago
219	-	*sp.*	EGS 35-190[1]; IMI 265269[2]	*Pisum sativum* L.	GB	-
220	-	*sp.*	EGS 38-089[1]	*Cicer arietinum* L.	USA	WA
221	-	*sp.*	EGS 38-090[1]	*Lens culinaris* Medik.	USA	WA
222	-	*sp.*	EGS 38-091[1]	*Lens culinaris* Medik.	USA	WA
223	-	*sp.*	EGS 40-038[1]	*Medicago sativa* L.	USA	KS
224	-	*sp.*	EGS 41-194[1]	*Cheiranthus cheiri* L.	Italy	-
226	-	*sp.*	EGS 42-022[1]	*Euphorbia esula* L.	Canada	Saskatchewan
227	-	*sp.*	EGS 42-138[1]	*Malus sylvestris* (L.) Miller	Australia	WA
228	-	*sp.*	EGS 44-149[1]	*Malus x domestica* Borkh.	New Zealand	-
229	-	*lancipes*	EGS 46-182[1]	*Aquilegia sp.*	New Zealand	-
230	-	*sp.*	EGS 47-132[1]	*Passiflora edulis* Sims	New Zealand	-
231	-	*sp.*	EGS 47-135[1]; IMI 98083[2]	*Dianthus sp.*	USA	CA

232	-	*xanthoso-matis*	EGS 17-137[1]	*Xanthosoma sagittifo-lium* (L.) Schott	New Caledo-nia	-
233	-	*sp.*	EGS 35-163[1]	*Brassica oleracea* L.	USA	AZ
234	-	*sp.*	EGS 35-169[1]	*Digitalis purpurea* L.	GB	-
235	-	*sp.*	EGS 35-170[1]	*Digitalis purpurea* L.	GB	-
236	-	*sp.*	EGS 35-171[1]	*Digitalis purpurea* L.	GB	-
237	-	*sp.*	EGS 35-187[1]; IMI 264212[2]	*Trigonella foenum-graecum* L.	GB	-
238	*sp.*	*vesicarium*	EGS 37-067[1]; IMI 278459[2]	*Medicago sp.*	South Africa	-
239	-	*sarciniforme*	EGS 38-121[1]	*Trifolium pratense* L.	USA	MA
240	-	*solani*	EGS 41-135[1]	*Lycopersi-con escu-lentum* L.	USA	IN
241	-	*solani*	EGS 42-027[1]	*Lycopersi-con escu-lentum* L.	USA	IN
242	-	*lycopersici*	EGS 46-001[1]	*Lycopersi-con escu-lentum* L.	Domini-can Re-public	-
243	*gracilariae*	*gracilariae*	EGS 37-073[1]; ATCC 66972	*Gracilaria sp.*	Israel	-
244	-	*trifolii*	EGS 12-142[1]	*Trifolium repens* L.	USA	PA
245	-	*sp.*	EGS 29-161[1]	*Capsicum frutescens* L.	USA	FL
246	-	*sp.*	EGS 31-008[1]	*Chrysan-themum sp.*	New Zealand	-
247	-	*sarciniforme*	EGS 29-188[1]	*Cicer arietinum* L.	Iran	-
248	-	*sp.*	EGS 46-183[1]	*Salvia offi-cinalis* L.	New Zealand	-
249	-	*sp.*	EGS 47-197[1]	*Helianthus argophyllus* Torr. & Gray	Australia	QLD

250	-	*sp.*	EGS 45-031[1]	*Chrysanthemoides monilifera* (L.) Norlindh	Australia	NSW
251	-	*sp.*	EGS 45-036[1]	*Chrysanthemoides monilifera* (L.) Norlindh	Australia	NSW
252	-	*sp.*	EGS 42-055[1]	*Euphorbia marginata* Pursh	USA	KS
253	-	*sp.*	EGS 44-070[1]	*Capsicum frutescens* L.	USA	IN
262	*sp.*	*majusculum*	EGS 16-068[1]; IMI135459[2]	*Lathyrus maritimus* (L.) Bigelow	USA	NY
268	*tomatonis*	*tomatonis*	EGS 29-089[1]; IMI 386968[2]	*Lycopersicon esculentum* L.	USA	Central Valley, CA
269	*eturmiuna*	*eturmiunum*	EGS 29-099[1]; IMI 386969[2]	*Lycopersicon esculentum* L.	New Zealand	-
270	*paludiscirpi*	*paludiscirpi*	EGS 31-016[1]; IMI 386966[2]	*Scirpus sp.*	USA	Point Judith, RI
271	*sedicola*	*sedicola*	EGS 48-095[1]; IMI 386967[2]	*Sedum spectabile* Boreau	New Zealand	Auckland
272	-	*sp.*	EGS 48-163[1]; NY-To-1[6]	*Allium cepa* L.	USA	Ithaca, NY
273	-	*sp.*	EGS 48-165[1]; NY-Or: o[6]	*Allium cepa* L.	USA	Orange County, NY
274	-	*sp.*	EGS 48-167[1]; MX-SLP-p[6]	*Allium cepa* L.	Mexico	San Luis Potosi State
275	-	*sp.*	EGS 48-168[1]; MX-T: r[6]	*Allium cepa* L.	Mexico	Tamaulipas State
276	-	*sp.*	EGS 48-169[1]; MX-T: s[6]	*Allium cepa* L.	Mexico	Tamaulipas State
277	-	*sp.*	EGS 48-170[1]; NY-Os: Sb1[6]	*Allium cepa* L.	USA	Oswego County, NY
278	-	*sp.*	EGS 48-171[1]; NY-Os: Sb6[6]	*Allium cepa* L.	USA	Oswego County, NY
279	-	*sp.*	EGS 48-172[1]; NY-Os: Sb7[6]	*Allium cepa* L.	USA	Oswego County, NY

280	-	*sp.*	EGS 48-173[1]; NY-Os: u[6]	*Allium cepa* L.	USA	Oswego County, NY
281	-	*sp.*	EGS 48-175[1]; NY-Os: 700b[6]	*Allium cepa* L.	USA	Oswego County, NY
301	-	*sp.*	-	*Brassica oleracea* L.	Canada	Acadia Park Residences, UBC Campus, Vancouver, BC[15]
302	-	*sp.*	EGS 49-029[1]; IBT8213[7]; #1[8]	-	-	-
303	-	*sp.*	EGS 49-030[1]; IBT8214[7]; #2[8]	*Trigonella sp.*	Egypt	-
306	-	*sp.*	EGS 49-033[1]; IBT8217[7]; #5[8]	*Cicer arietinum* L.	Iran	-
307	-	*sp.*	EGS 49-034[1]; IBT8218[7]; #6[8]	*Medicago sativa* L.	Canada	Ontario
308	-	*sp.*	EGS 49-035[1]; IBT8220[7]; #7[8]	-	Italy	-
309	-	*sp.*	EGS 49-036[1]; IBT8221[7]; #8[8]	-	Iran	-
310	-	*sp.*	EGS 49-037[1]; IBT8222[7]; #9[8]	*Trifolium pratense* L.	USA	Lafayette, IN
311	-	*sp.*	EGS 49-038[1]; IBT8223[7]; #10[8]	*Lathyrus maritimus* (L.) Bigelow	USA	Suffolk County, NY
312	-	*sp.*	EGS 49-039[1]; IBT8224[7]; #11[8]	*Brassica napus* L.	Italy	-
313	-	*sp.*	EGS 49-040[1]; IBT8225[7]; #12[8]	*Medicago sativa* L.	Canada	Ontario
314	-	*sp.*	EGS 49-041[1]; IBT8226[7]; #13[8]	*Brassica napus* L.	Italy	-
315	-	*sp.*	EGS 49-042[1]; IBT8227[7]; #14[8]	*Brassica napus* L.	Italy	-
316	-	*sp.*	EGS 49-043[1]; IBT8228[7]; #15[8]	-	Indonesia	Bogor, Java
317	-	*sp.*	EGS 49-044[1]; IBT8231[7]; #18[8]	*Lycopersicon esculentum* L.	Greece	Crete
318	-	*sp.*	EGS 49-045[1]; IBT8232[7]; #19[8]	*Medicago sativa* L.	India	Uttar Pradesh
319	-	*sp.*	EGS 49-046[1]; #22[8]	*Brassica napus* L.	Italy	-

320	-	*sp.*	EGS 49-047[1]; #25[8]	*Brassica napus* L.	Italy	-
321	-	*sp.*	EGS 49-048[1]; #26[8]	*Brassica napus* L.	Italy	-
322	-	*sp.*	EGS 49-050[1]; #28[8]	-	Korea	-
323	-	*sp.*	EGS 49-051[1]; #30[8]	*Hordeum vulgare* L.	-	-
324	-	*sp.*	EGS 49-052[1]; #31[8]	-	Italy	-
325	-	*sp.*	EGS 49-053[1]; IBT7159[7]; #32[8]	*soil*	-	-
326	-	*sp.*	EGS 49-054[1]; #34[8]	-	Korea	-
327	*sp.*	*sp.*	-	*Salicornia sp.*	Canada	Near Roberts Bank Port, BC[16]
338	*sp.*	*sp.*	-	*Salicornia sp.*	Canada	On the beach of Cape Gurney, Hornby Island, BC[16]
342	*sp.*	*sp.*	MEBarr10382[9]	*Lactuca muralis* (L.) Gaertner	Canada	Sidney, BC[17]
343	*sp.*	*sp.*	MEBarr10380[9]	*Geum macro-phyllum* Willd.	Canada	Sidney, BC[17]
383	-	*callistephi*	NO 536[10]	-	USA	CA
384	-	*loti*	NO 770[10]	*Trifolium pratense* L.	USA	Geneva, NY
385	-	*loti*	NO 1364[10]	*Lotus sp.*	USA	Mifflin, PA
406	-	*sp.*	SS1[11]; T-1953[12]	*Lycopersi-con escu-lentum* L.	Brazil	Botucatu, SP
407	-	*sp.*	SS21[11]	*Gossypium sp.*	Brazil	Goioeré, PR
408	-	*sp.*	SS28[11]	*Gossypium sp.*	Brazil	Terra Nova d'Oeste, PR
409	-	*sp.*	SS31[11]	*Gossypium sp.*	Brazil	Goiàs

[1] refers to the E. G. Simmons private culture collection.
[2] refers to the fungal culture collection at CABI Bioscience, UK.
[3] refers to the P. Inderbitzin private fungarium.
[4] see Crivelli (1983).
[5] refers to NITE Biological Resource Center culture collection, Chiba, Japan.
[6] refers to *Stemphylium* isolates in de Jesus Yanez Morales (2001).
[7] refers to the fungal culture collection of the Technical University of Denmark, Lyngby.
[8] refers to *Stemphylium* isolates in Andersen (1995).

[9] refers to the M. E. Barr private herbarium.
[10] see Câmara et al. (2002).
[11] see Mehta (2001).
[12] see Mehta & Brogin (2000).
[13] collected by A. & R. Bandoni, S. Landvik and P. Inderbitzin.
[14] collected by P. Inderbitzin.
[15] collected and isolated by Jennifer Guojuan Zhang.
[16] collected by A. & R. Bandoni.
[17] collected by M. E. Barr.

Table 2-3. New primers designed for this study. '*v-v*' is locus *vmaA-vpsA*. For primer map see Figure 2-3.

Region	Primer name	Primer sequence
ITS	ITS871r	5'-GCT TAA GTT CAG CGG GTA-3'
GPD	GPD2033f	5'-TTG GCC GTA TCG TCT TCC GC-3'
GPD	GPD2669r	5'-CTT GTC GTG GAT GAC CTT GGC-3'
GPD	GPD3r	5'-ACC AGT GCT GCT GGG AATG-3'
EF-1 alpha	EF446f	5'-TCA CTT GAT CTA CAA GTG CGG TGG-3'
EF-1 alpha	EF451f	5'-GAC AAG CGT ACC ATC GAG AAG TTC G-3'
EF-1 alpha	EF462f	5'-CAT CGA GAA GTT CGA GAA GG-3'
EF-1 alpha	EF1598r	5'-CGT GGT GCA TCT CGA CGG-3'
EF-1 alpha	EF1473r	5'-CGA TCT TGT AGA CAT CCT GGA GG-3'
EF-1 alpha	EF3r	5'-CTT GGT CTC CTT CTC CC-3'
EF-1 alpha	EFP229f	5'-TGG AGG AGC TTT ACG ACG CC-3'
EF-1 alpha	EfallF2	5'-CGC CTC CGA GCC CGC TTC C-3'
EF-1 alpha	EfallF	5'-GTT CCG ATG CGA GKT CTG TG-3'
EF-1 alpha	EFLr	5'-TCA CAG AMC TCG CAT CGG AAC-3'
EF-1 alpha	EFAllr	5'-AGA GGT GCG TCG CTG ACC AC-3'
EF-1 alpha	EFLf	5'-TGR TGC TGC TGC CTC RGG AC-3'
EF-1 alpha	EFP246f	5'-TTC AGC ATG ATG CTG CTA CC-3'
EF-1 alpha	EF2r	5'-TAG TGA TAC CAC GCT CAC GC-3'
EF-1 alpha	95f	5'-CAA GAA CAT GAT CAC TGG TAC-3'
EF-1 alpha	Efr	5'-TCA CCA GAC TTG ATG AAC TTG GG-3'
v-v	VATP2949f	5'-TCG ATC AGT TAC AGC AAG TAC-3'
v-v	VATP3238r	5'-GCC TTC TGC GCT TCG TCG TGG-3'
v-v	GTP446f	5'-TTC GGT GCT TGA GAA CAT TG-3'
v-v	GTP980r	5'-GCC AAA TCG GTG TTG GCG GC-3'
v-v	VATP3195f	5'-CGC TCT GGA AGA CTG AGT GG-3'
v-v	AiF	5'-TGG ATG ATG AAG AAC ATG ATG-3'
v-v	AN1	5'-CAG AAG GCT GTC TCC CAA GG-3'
v-v	ATPF2	5'-CAG CTC CGA TCA ATG AAG TTC G-3'
v-v	GTP604r	5'-ATG AGC TGG AGA ATG AGC GG-3'
v-v	GTPr	5'-GGG TAA TTA GCC CCG GGT CG-3'
v-v	AGf	5'-CTG ACG ACG CTG TTT CGC GTC-3'

Table 2-4. *EF-1 alpha* internal introns in isolates of *Pleospora*. For gene map see Figure 2-3. Position is intron insertion site on *Podospora anserina EF-1 alpha* DNA sequence from GenBank (X74799). Intron length, phylogenetic distribution, variability, and presence of ORF and GenBank match are given. Isolates sharing a similar intron were generally close relatives (Figure 2-4), except for *S. loti strain* P384 with an intron as *P. triglochinicola* strain P123, *P. paludiscirpi* strain P270, plus three unnamed isolates, and the similar, long, protein encoding intron of *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246. Introns were excluded from phylogenetic analyses due to high divergence. For information on strain numbers see Table 2-2.

Position	Length (bp)	Isolates	Variability (bp or %)	ORF >100 bp	ORF - GenBank match
912	49	Group B	-	-	NA
913	59	Group C	5	-	NA
929	155	*S. sarciniforme* strain P239 plus four unnamed isolates (strains P247, P306, P309, P310)	2	141	-
929	199	*S. callistephi* strain P383	NA	183	-
929	1501 1567 1678	*S. lancipes* strain P229 *S. trifolii* strain P244 *Stemphylium sp.* strain P246	36.8%	1440 1077 834	Hypothetical *G. zeae* protein XP_381390 with zinc finger domain
932	52 / 53	Group A; *S. loti* strains P384, P385; *P. triglochinicola* strain P123, *P. paludiscirpi* strain P270, plus three unnamed isolates (strains P210, P327, P338)	13	-	NA
932	54	*Pleospora sp.* strain P56, plus six other unnamed isolates (strains P107, P212, P221, P227, P342, P343)	2	-	NA
932	119	Group D, except *S. callistephi* strain P383	1	-	NA

Table 2-5. Comparison of intron encoded zinc finger proteins. The internal *EF-1 alpha* intron of *S. lancipes* strain P229, *S. trifolii* strain P244 and *Stemphylium sp.* strain P246 contained a hypothetical zinc finger protein most similar to *Gibberella zeae* protein XP_381390 from GenBank. Expect values (number of hits expected by chance), identities (identical amino acids), and positives (similar amino acids) are from GenBank protein-protein Blast searches (Altschul *et al.*, 1997).

Isolates	Protein length (bp)	Expect Value	Identities	Positives
S. lancipes strain P229	1389	9e-27	98/297 (32%)	141/297 (47%)
S. trifolii strain P244	921	3e-24	85/259 (32%)	141/297 (47%)
Stemphylium sp. strain P246	507	1e-14	71/249 (28%)	110/249 (44%)

Table 2-6. Unique ITS/*GPD/EF-1 alpha* multilocus genotypes. A total of 39 combined multilocus genotypes were obtained from 110 isolates of *Pleospora*. Only one representative per genotype was included in phylogenetic analyses. For information on strain numbers see Table 2-2.

Representative isolates	Genotype groups
P. tarda strain P1	P1, P216, P307, P313
P. herbarum strain P2	P2, P81, P126, P206, P208, P209, P213, P215, P217, P219, P220, P222, P223, P226, P228, P231, P233, P234, P237, P238, P268, P271, P274, P275, P276, P278, P279, P280, P281, P308, P314, P318, P322, P324, P326
Pleospora sp. strain P56	P56
Pleospora sp. strain P107	P107
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201, P203, P204, P205
Stemphylium sp. strain P207	P207
Stemphylium sp. strain P210	P210
Stemphylium sp. strain P214	P214
Stemphylium sp. strain P212	P212
Stemphylium sp. strain P221	P221
S. lancipes strain P229	P229
S. xanthosomatis strain P232	P232, P249
Stemphylium sp. strain P93	P93, P218, P230, P235, P236, P277, P301, P323, P325
S. sarciniforme strain P239	P239, P309, P310
S. solani strain P240	P240, P252, P253, P409
S. solani strain P241	P241
Stemphylium sp. strain P242	P242, P245, P251, P316
P. gracilariae strain P243	P243, P315, P319
S. trifolii strain P244	P244
Stemphylium sp. strain P246	P246
S. sarciniforme strain P247	P247, P306
Stemphylium sp. strain P248	P248
Stemphylium sp. strain P250	P250, P406
S. majusculum strain P262	P262, P311
P. eturmiuna strain P269	P224, P269, P312, P317, P320, P321
P. paludiscirpi strain P270	P270
Stemphylium sp. strain P272	P272
Stemphylium sp. strain P273	P273
Stemphylium sp. strain P302	P302
Stemphylium sp. strain P303	P303
Pleospora sp. strain P327	P327
Pleospora sp. strain P338	P338
Pleospora sp. strain P342	P227, P342, P343
S. callistephi strain P383	P383
S. loti strain P384	P384
S. loti strain P385	P385
Stemphylium sp. strain P407	P407, P408

Table 2-7. Unique ITS genotypes. A total of 19 ITS genotypes were obtained from 110 isolates of *Pleospora*. Only one representative per genotype was included in phylogenetic analyses. For information on strain numbers see Table 2-2.

Representative isolates	Genotype groups
P. tarda strain P1	P1, P216, P307, P313
P. herbarum strain P2	P2, P81, P93, P126, P206, P208, P209, P213, P215, P217, P218, P219, P220, P222, P223, P226, P228, P230, P231, P233, P234, P235, P236, P237, P238, P268, P271, P272, P273, P274, P275, P276, P277, P278, P279, P280, P281, P301, P308, P314, P318, P322, P323, P324, P325, P326
Pleospora sp. strain P56	P56, P107, P212, P221, P227, P342, P343
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201, P203, P204, P205, P243, P262, P311, P315, P319
P. eturmiuna strain P269	P207, P214, P224, P269, P302, P312, P317, P320, P321
Stemphylium sp. strain P210	P210
S. xanthosomatis strain P232	P232, P242, P245, P248, P249, P250, P251, P316, P406
S. sarciniforme strain P239	P239, P247, P306, P309, P310
S. solani strain P240	P240, P229, P241, P252, P253, P407, P408, P409
S. trifolii strain P244	P244
Stemphylium sp. strain P246	P246
P. paludiscirpi strain P270	P270
Pleospora sp. strain P303	P303
Pleospora sp. strain P327	P327
Pleospora sp. strain P338	P338
S. callistephi strain P383	P383
S. loti strain P384	P384, P385

Table 2-8. Unique *GPD* genotypes. A total of 30 *GPD* genotypes were obtained from 110 isolates of *Pleospora*. Only one representative per genotype was included in phylogenetic analyses. Genotypes in bold were not sequenced for *vmaA-vpsA*, but were represented in combined analyses by the closely related *Pleospora sp.* strain P107 for *Stemphylium sp.* strains P212 and P221, or *S. solani* strain P240 for the *Stemphylium* sp. strain P407 genotype (Figure 2-4). For information on strain numbers see Table 2-2.

Representatives	Genotype groups
P. tarda strain P1	P1, P216, P307, P313
P. herbarum strain P2	P2, P81, P93, P126, P206, P208, P209, P213, P215, P217, P218, P219, P220, P222, P223, P226, P228, P230, P231, P233, P234, P235, P236, P237, P238, P268, P271, P272, P273, P274, P275, P276, P277, P278, P279, P280, P281, P301, P303, P308, P314, P318, P322, P323, P324, P325, P326
Pleospora sp. strain P56	P56
Pleospora sp. strain P107	P107
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201, P203, P204, P205
Stemphylium sp. strain P207	P207
Stemphylium sp. strain P210	P210
Stemphylium sp. strain P212	P212
Stemphylium sp. strain P221	P221
S. lancipes strain P229	P229
S. xanthosomatis strain P232	P232, P248, P249
S. sarciniforme strain P239	P239, P309, P310
S. solani strain P240	P240, P241, P252, P253, P409
P. gracilariae strain P243	P243, P315, P319
S. trifolii strain P244	P244
Stemphylium sp. strain P246	P246
S. sarciniforme strain P247	P247, P306
Stemphylium sp. strain P250	P242, P245, P250, P251, P316, P406
S. majusculum strain P262	P262, P311
P. eturmiuna strain P269	P214, P224, P269, P312, P317, P320, P321
P. paludiscirpi strain P270	P270
Stemphylium sp. strain P302	P302
Pleospora sp. strain P327	P327, P338
Pleospora sp. strain P342	P227, P342, P343
S. callistephi strain P383	P383
S. loti strain P384	P384
S. loti strain P385	P385
Stemphylium sp. strain P407	P407, P408

Table 2-9. Unique *EF-1 alpha* genotypes. A total of 30 unique *EF-1 alpha* genotypes were obtained from 110 *Pleospora* isolates. The internal intron was not considered. Genotypes in bold were not sequenced for *vmaA-vpsA*, and were represented in the combined analyses by the closely related *Pleospora sp.* strain P107 and *S. solani* strain P240, respectively (Figure 2-4). For information on strain numbers see Table 2-2.

Representatives	Genotype groups
P. tarda strain P1	P1, P216, P307, P313
P. herbarum strain P2	P2, P81, P126, P206, P208, P209, P213, P215, P217, P219, P220, P222, P223, P226, P228, P231, P233, P234, P237, P238, P268, P271, P274, P275, P276, P278, P279, P280, P281, P303, P308, P314, P318, P322, P324, P326
Pleospora sp. strain P56	P56
Pleospora sp. strain P107	P107, P212
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201, P203, P204, P205
Stemphylium sp. strain P210	P210
Stemphylium sp. strain P221	P221
S. lancipes strain P229	P229
S. xanthosomatis strain P232	P232, P249, P250, P406
Stemphylium sp. strain P235	P93, P218, P230, P235, P236, P277, P301, P323, P325
S. sarciniforme strain P239	P239, P309, P310
S. solani strain P240	P240, P252, P253, P407, P408, P409
S. solani strain P241	P241
P. gracilariae strain P243	P243, P315, P319
S. trifolii strain P244	P244
Stemphylium sp. strain P245	P242, P245, P248, P251, P316
Stemphylium sp. strain P246	P246
S. sarciniforme strain P247	P247, P306
S. majusculum strain P262	P262, P311
P. eturmiuna strain P269	P207, P224, P269, P312, P317, P320, P321
P. paludiscirpi strain P270	P270
Stemphylium sp. strain P272	P272
Stemphylium sp. strain P273	P273
Stemphylium sp. strain P302	P302, P214
Pleospora sp. strain P327	P327, P338
Pleospora sp. strain P342	P227, P342, P343
S. callistephi strain P383	P383
S. loti strain P384	P384, P385

Table 2-10. Unique *vmaA-vpsA* genotypes. A total of 28 unique *vmaA-vpsA* genotypes were obtained from 53 *Pleospora* isolates. For information on strain numbers see Table 2-2.

Representatives	Genotype groups
P. tarda strain P1	P1, P216
P. herbarum strain P2	P2, P238, P272, P273, P277, P303, P314, P318
Pleospora sp. strain P56	P56
Pleospora alfalfae strain P81	P81, P237, P268, P271
Pleospora sp. strain P107	P107
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201
Stemphylium sp. strain P207	P207, P269
Stemphylium sp. strain P210	P210
S. lancipes strain P229	P229
S. xanthosomatis strain P232	P232
Stemphylium sp. strain P235	P235, P236, P301, P323, P325
S. sarciniforme strain P239	P239, P310
S. solani strain P240	P240, P252, P253
P. gracilariae strain P243	P243
S. trifolii strain P244	P244
Stemphylium sp. strain P245	P245, P250, P316
Stemphylium sp. strain P246	P246
S. sarciniforme strain P247	P247, P306
S. majusculum strain P262	P262, P311
P. paludiscirpi strain P270	P270
Stemphylium sp. strain P302	P302
Stemphylium sp. strain P315	P315
Pleospora sp. strain P327	P327
Pleospora sp. strain P342	P342
S. callistephi strain P383	P383
S. loti strain P384	P384, P385

Table 2-11. Unique ITS/*GPD/EF-1 alpha/vmaA-vpsA* multilocus genotypes. A total of 35 unique multilocus genotypes were obtained from 53 isolates of *Pleospora*. Only one representative per genotype was included in phylogenetic analyses. For information on strain numbers see Table 2-2.

Representative	Genotype group
P. tarda strain P1	P1, P216
P. herbarum strain P2	P2, P238, P314, P318
Pleospora sp. strain P56	P56
Pleospora alfalfae strain P81	P81, P237, P268, P271
Pleospora sp. strain P107	P107
P. gigaspora strain P129	P122, P129
P. triglochinicola strain P123	P123
S. astragali strain P201	P201
Stemphylium sp. strain P207	P207
Stemphylium sp. strain P210	P210
S. lancipes strain P229	P229
S. xanthosomatis strain P232	P232
Stemphylium sp. strain P235	P235, P236, P301, P323, P325
S. sarciniforme strain P239	P239, P310
S. solani strain P240	P240, P252, P253
P. gracilariae strain P243	P243
S. trifolii strain P244	P244
Stemphylium sp. strain P245	P245, P316
Stemphylium sp. strain P246	P246
S. sarciniforme strain P247	P247, P306
Stemphylium sp. strain P250	P250
S. majusculum strain P262	P262, P311
P. eturmiuna strain P269	P269
P. paludiscirpi strain P270	P270
Stemphylium sp. strain P272	P272
Stemphylium sp. strain P273	P273
Stemphylium sp. strain P277	P277
Stemphylium sp. strain P302	P302
Stemphylium sp. strain P303	P303
Stemphylium sp. strain P315	P315
Pleospora sp. strain P327	P327
Pleospora sp. strain P342	P342
S. callistephi strain P383	P383
S. loti strain P384	P384
S. loti strain P385	P385

Table 2-12. Bootstrap support values above 70% for the branches of the *Pleospora* species phylogenies in Figure 2-5 and Figure 2-6 on pages 64 and 66, respectively. Node numbers listed here are present by the nodes of the ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* single loci phylogenies in Figure 2-5 and by the nodes of the combined four loci phylogeny in Figure 2-6. For the single gene phylogenies based on ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* in Figure 2-5, only parsimony bootstrap support percentages are given. For the four loci combined phylogeny in Figure 2-6, support percentages are given for parsimony, likelihood, Bayesian and Neighbor joining analyses in this order. All trees were congruent at a 70% support level, except a minor alteration within the phylogenetic species *P. herbarum* in the Bayesian analyses (see page 23), and node 28 that was only present in the *vmaA-vpsA* tree (Figure 2-5). Support in the combined analyses was generally higher than in the single dataset analyses. Branches receiving 100% support in the combined analyses and marked with an asterisk are due to the inclusion in Figure 2-6 of duplicate multilocus genotypes.

Node number	Single loci phylogenies support values (Figure 2-5, page 64)[1]				Combined four loci phylogeny support values (Figure 2-6, page 66)[2]	Node number
	ITS	*GPD*	*EF1-alpha*	*vmaA-vpsA*		
1	100	-	-	-	-/-/90/-	1
2	-	-	-	-	100*	2
3	-	100	97	99	100	3
4	100	100	100	100	100	4
5	-	-	77	-	92/86/100/99	5
6	-	-	-	-	100*	6
7	-	-	-	-	-/-/93/-	7
8	-	-	-	-	100*	8
9	-	-	78	-	76/-/98/85	9
10	-	-	-	-	90/94/100/92	10
11	-	96	-	99	100/100/100/99	11
12	-	-	77	98	100	12
13	-	-	-	-	100*	13
14	-	-	-	-	71/-/99/-	14
15	-	-	-	-	-/-/98/-	15
16	-	-	-	-	100*	16
17	-	99	83	99	100	17
18	-	-	-	-	74/-/84/-	18
19	-	84	-	-	-/-/99/74	19
20	-	99	88	92	100	20
21	-	95	85	95	100/100/100/89	21
22	100	99	97	100	100/100/100/-	22
23	-	-	-	-	100*	23
24	-	-	-	-	100*	24
25	100	100	100	100	100	25
26	100	100	100	100	100	26
27	-	-	-	-	-/-/99/-	27
28	-	-	-	91	-	28
29	-	-	-	-	90/-/92/-	29
30	-	-	-	-	97/98/100/99	30
31	-	-	-	-	-/88/100/-	31
32	-	100	89	100	100	32
33	-	100	98	100	100	33
34	-	-	-	-	94/98/100/99	34

[1] Support values are bootstrap support percentages.

[2] Support values are for parsimony, likelihood, Bayesian and Neighbor joining analyses in this order.

Table 2-13. Phylogenetic information content of the four loci used for the *Pleospora* species phylogeny. For the single, 53 taxa datasets (Figure 2-5), the percentages of variable and parsimony informative characters are given, as well as the groups resolved.

Dataset	Variable characters	Parsimony informative characters	Groups resolved
ITS	9%	2.6%	B, E
GPD	25.4%	17.4%	All
EF1-alpha	19.2%	10.9%	All
vmaA-vpsA	34.4%	25.6%	All

Table 2-14. Phylogenetic species of *Pleospora*. There were 22 phylogenetic species in *Pleospora*. For each phylogenetic species, the representative isolates and culture identifiers are given (Table 2-2), as well as the group affiliations, and the number of isolates (Figure 2-4) and morphological types contained (Table 2-15). See section on phylogenetic species on page 27 for naming of the two phylogenetic species potentially conflicting with morphological species (*P. herbarum*, *S. xanthosomatis*).

Representative isolate	Culture IDs	Group	Number of isolates	Number of types
S. xanthosomatis strain P232	EGS 17-137	A	9	2[1]
P. tarda strain P1	EGS 08-069	B	4	1
P. eturmiuna strain P269	EGS 29-099	C	9	1
P. gigaspora strain P129	EGS 37-016	C	2	-
P. gracilariae strain P243	EGS 37-073	C	3	1
P. herbarum strain P2	EGS 36-138	C	47	5
S. astragali strain P201	EGS 08-174	C	4	-
S. majusculum strain P262	EGS 16-068	C	2	1
S.callistephi strain P383	NO 0536	D	1	-
S. solani strain P240	EGS 41-135	D	7	1
P. paludiscirpi strain P270	EGS 31-016	E	1	1
P. triglochinicola strain P123	EGS 36-118	E	1	-
Pleospora sp. strain P107	P107	E	3	NA
Pleospora sp. strain P327	P327	E	2	NA
Pleospora sp. strain P56	P56	E	4	NA
S. loti strain P384	NO 0770	E	2	-
S. sarciniforme strain P239	EGS 38-121	E	3	1
S. sarciniforme strain P247	EGS 29-188	E	2	NA
Stemphylium sp. strain P210	EGS 48-089	E	1	NA
Stemphylium sp. strain P246	EGS 31-008	E	1	NA
S. trifolii strain P244	EGS 12-142	E	1	1
S. lancipes strain P229	EGS 46-182	F	1	1

[1]*S. lycopersici* strain P242 also fell within the phylogenetic species *S. xanthosomatis* (see Figure 2-4). However, *S. lycopersici* has no known type specimen (E. G. Simmons, personal communication) (see Table 2-1).

Table 2-15. Phylogenetic species containing morphological species. Sixteen of a total of 22 phylogenetic species could be linked to morphological species. For morphological species underlying the phylogenetic species, author names of teleomorphs and anamorphs are given, as well as the relation of the strains to the respective type material from Table 2-1. See section on fungal strains on page 11 for rationale of applying names to strains and section on phylogenetic species on page 27 for naming of the two phylogenetic species potentially conflicting with morphological species (*P. herbarum*, *S. xanthosomatis*). Publications refer to the original descriptions. For numbers of morphological species contained per phylogenetic species see Table 2-14.

Phylogenetic species: Teleomorph	Phylogenetic species: Anamorph	P #	Culture IDs	Relation to type	Reference
P. eturmiuna Simmons	*S. eturmiuna* Simmons	P269	EGS 29-099	ex type	Simmons (2001)
P. gigaspora Karsten	*Stemphylium sp.*	P129	EGS 37-016	unknown[1]	Karsten (1884)
P. gracilariae Simmons & Schatz	*S. gracilariae* Simmons	P243	EGS 37-073	ex type	Simmons (1989)
P. herbarum (Pers.: Fries) Rabenhorst ex Cesati & de Notaris	*S. herbarum* Simmons	P2	EGS 36-138	type / ex type	Simmons (1985)
P. paludiscirpi Simmons	*S. paludiscirpi* Simmons	P270	EGS 31-016	type	Simmons (2001)
P. tarda Simmons	*S. botryosum* Wallroth	P1	EGS 08-069	ex type / type	Simmons (1985)
P. triglochinicola Webster	*S. triglochinicola* Sutton & Pirozynski	P123	EGS 36-118	unknown[2]	Webster (1969)
Pleospora sp.	*S. astragali* Yoshii	P201	EGS 08-174	unknown[3]	Yamamoto (1960); Yoshii (1929)
Pleospora sp.	*S. majusculum* Simmons	P262	EGS 16-068	type[4]	Simmons (1969)
unknown	*S. callistephi* Baker & Davis	P383	NO 0536	unknown[5]	Baker and Davis (1950)
unknown	*S. lancipes* (Ellis & Everhart) Simmons	P229	EGS 46-182	type[6]	Simmons (1969)
unknown	*S. loti* Graham	P384	NO 0770	unknown	Graham (1953)
unknown	*S. sarciniforme* F. Cavara	P239	EGS 38-121	type[7]	Cavara (1890)
unknown	*S. solani* G. F. Weber	P240	EGS 41-135	type[7]	Weber (1930)
unknown	*S. trifolii* Graham	P244	EGS 12-142	type[7]	Graham (1957)
unknown	*S. xanthosomatis* Huguenin	P232	EGS 17-137	ex type[8]	Huguenin (1965)

[1] Identified by Crivelli (1983).
[2] Sent to Simmons by J. Webster, author of type (Simmons, personal communication).
[3] Sent to Simmons by H. Yoshii, author of type (Simmons, personal communication).
[4] Simmons, personal communication.
[5] Collected by K. Baker (Câmara *et al.*, 2002), who might be identical to the author of the type.
[6] Excellent representative of type (Simmons, personal communication).
[7] Compared to type by Simmons (Simmons, personal communication).
[8] Isolated ex type by type author and sent to Simmons (E. G. Simmons, personal communication).

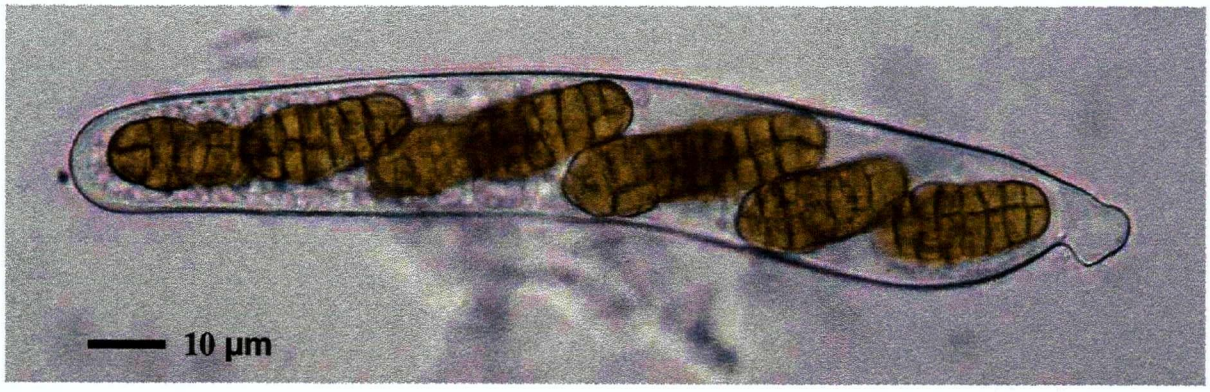


Figure 2-1. Ascus of *Pleospora sedicola* strain P271 containing eight muriform ascospores.

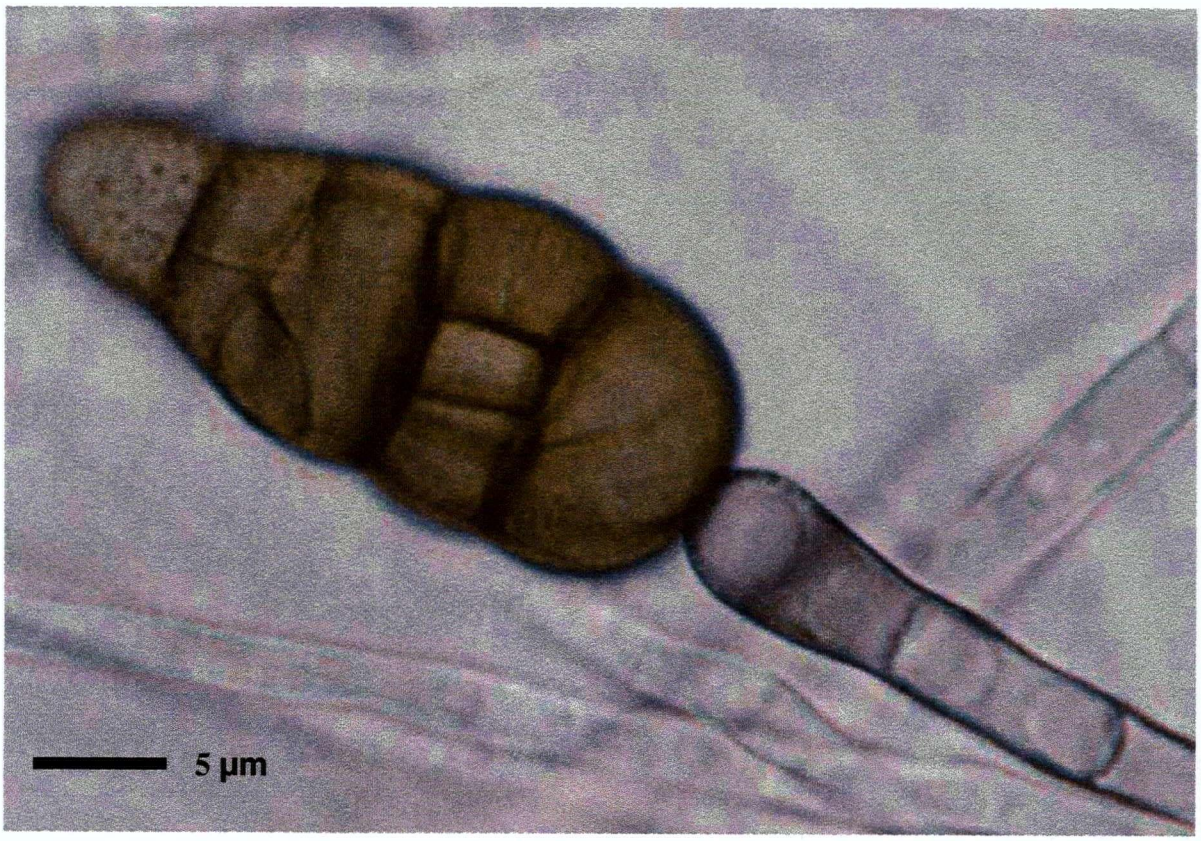


Figure 2-2. Asexual spore of *Stemphylium trifolii* strain P244 developing at the tip of a conidiogenous cell.

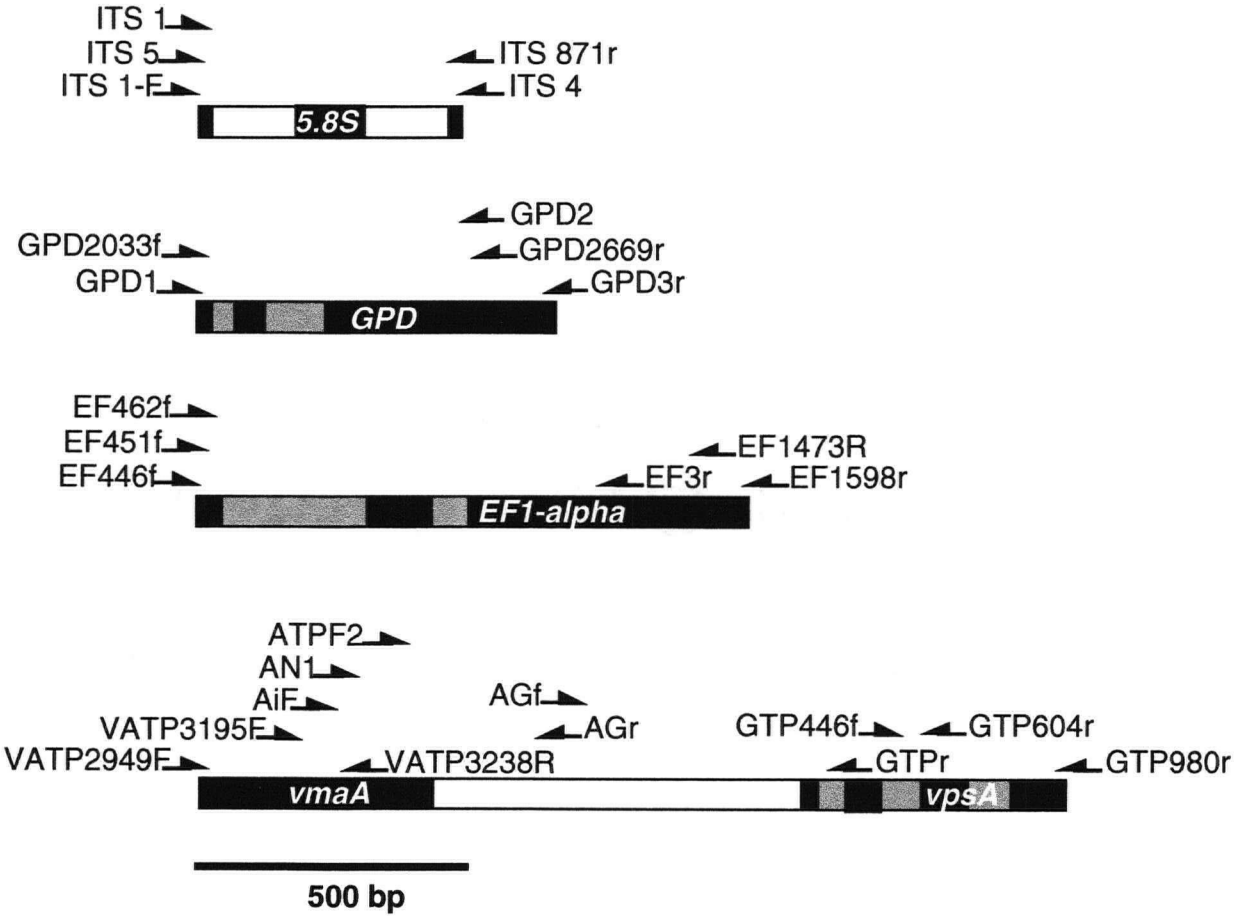


Figure 2-3. Positions of primers used for PCR amplification and DNA sequencing to generate *Pleospora* species phylogeny. Gene diagrams approximately to scale. Black boxes represent coding regions, gray boxes introns, and white boxes intergenic spacers. Specific primers designed for *Alternaria alternata*, *S. lancipes* isolate P229, *S. trifolii* isolate P244 and *Stemphylium sp.* isolate P246 are not shown. In *EF-1 alpha*, the small intron was excluded from analyses due to high divergence. In the text, the small intron is referred to as 'internal intron' since it was the only *EF-1 alpha* intron that was entirely sequenced.

Figure 2-4. One most parsimonious tree from combined ITS, *GPD* and *EF-1 alpha* DNA sequences. Analyses were done with 39 unique multilocus genotypes, the remaining isolates were added after analyses (Table 2-6). Values by the branches are bootstrap support percentages above 70. Branches in bold were supported by 100% of the bootstrap replicates. The 53 boxed isolates were chosen for sequencing of the additional *vmaA-vpsA* locus. Black vertical lines on the right indicate subgeneric groups following Câmara et al. (2002) and this study. For information on strain numbers see Table 2-2.

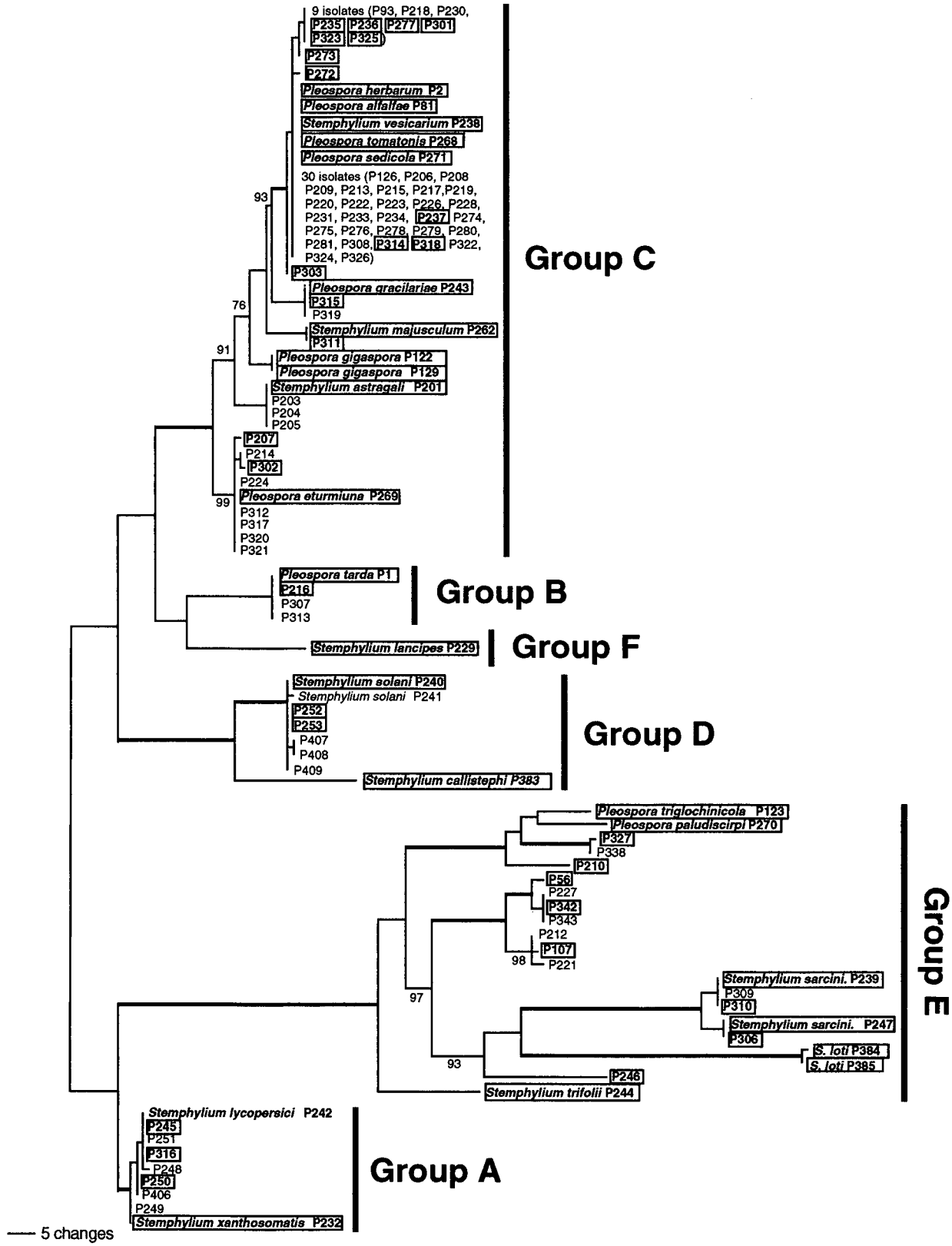
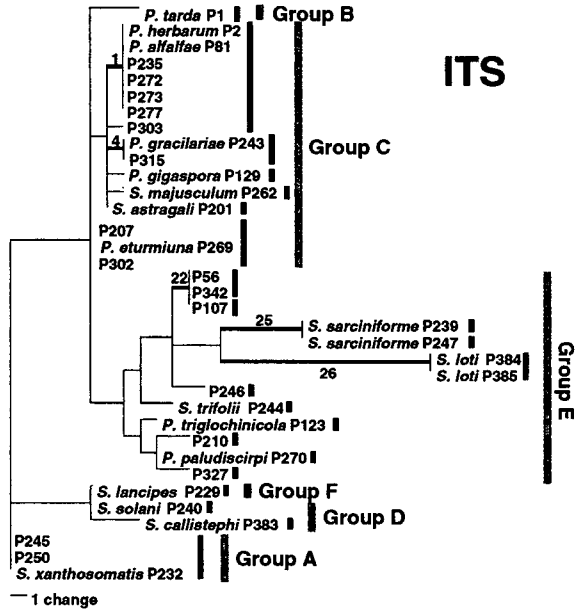
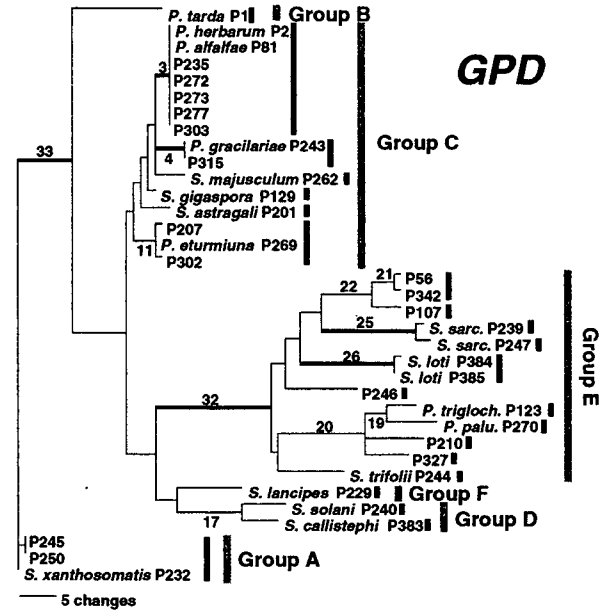
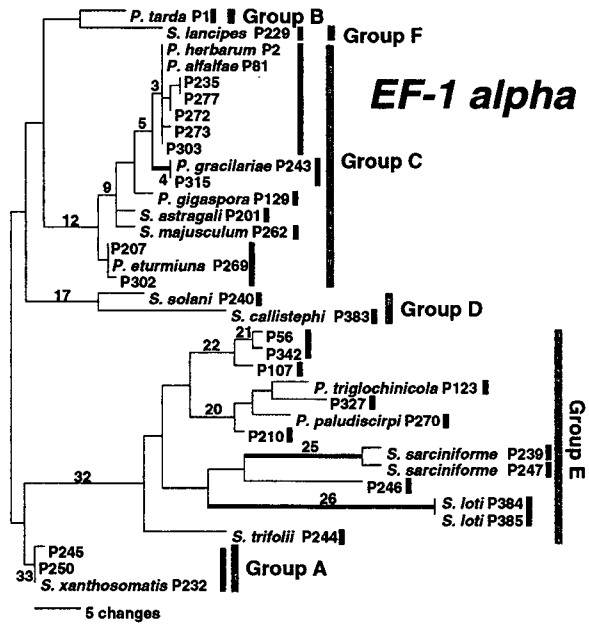

9 isolates (P93, P218, P230, P235 P236 P277 P301 P323 P325)
P273
P272
Pleospora herbarum P2
Pleospora alfalfae P81
Stemphylium vesicarium P238
Pleospora tomatonis P268
Pleospora sedicola P271
30 isolates (P126, P206, P208 P209, P213, P215, P217,P219, P220, P222, P223, P226, P228, P231, P233, P234, P237 P274, P275, P276, P278, P279, P280, P281, P308, P314 P318 P322, P324, P326)
P303
Pleospora gracilariae P243
P315
P319
Stemphylium majusculum P262
P311
Pleospora gigaspora P122
Pleospora gigaspora P129
Stemphylium astragali P201
P203
P204
P205
P207
P214
P302
P224
Pleospora eturmiuna P269
P312
P317
P320
P321
Group C
Pleospora tarda P1
P216
P307
P313
Group B
Stemphylium lancipes P229
Group F
Stemphylium solani P240
Stemphylium solani P241
P252
P253
P407
P408
P409
Stemphylium callistephi P383
Group D
Pleospora triglochinicola P123
Pleospora paludiscirpi P270
P327
P338
P210
P56
P227
P342
P343
P212
P107
P221
Stemphylium sarcini. P239
P309
P310
Stemphylium sarcini. P247
P306
S. loti P384
S. loti P385
P246
Stemphylium trifolii P244
Group E
Stemphylium lycopersici P242
P245
P251
P316
P248
P250
P406
P249
Stemphylium xanthosomatis P232
Group A
5 changes

Figure 2-5. Most parsimonious trees from separate ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* analyses. Analyses were performed using only unique genotypes for which *vmaA-vpsA* was sequenced (Table 2-11). Additional isolates were added to the trees after analyses. Numbers by the branches reflect bootstrap support percentages above 70, given in Table 2-12. Branches in bold were supported by 100% of the bootstrap replicates. Solid vertical lines represent phylogenetic species (Table 2-14). Gray vertical lines are groups A – F following Câmara et al. (2002) and this study. Note that phylogenetic species are congruent between all datasets. Phylogenetic trees have different scales, for a comparison of the information content between the datasets see Table 2-13. For information on strain numbers see Table 2-2.


ITS
P. tarda P1
P. herbarum P2
P. alfalfae P81
P235
P272
P273
P277
P303
P. gracilariae P243
P315
P. gigaspora P129
S. majusculum P262
S. astragali P201
P207
P. eturmiuna P269
P302
P56
P342
P107
S. sarciniforme P239
S. sarciniforme P247
S. loti P384
S. loti P385
P246
S. trifolii P244
P. triglochinicola P123
P210
P. paludiscirpi P270
P327
S. lancipes P229
S. solani P240
S. callistephi P383
P245
P250
S. xanthosomatis P232
Group A
Group B
Group C
Group D
Group E
Group F
1 change


GPD
P. tarda P1
P. herbarum P2
P. alfalfae P81
P235
P272
P273
P277
P303
P. gracilariae P243
P315
S. majusculum P262
S. gigaspora P129
S. astragali P201
P207
P. eturmiuna P269
P302
P56
P342
P107
S. sarc. P239
S. sarc. P247
S. loti P384
S. loti P385
P246
P. trigloch. P123
P. palu. P270
P210
P327
S. trifolii P244
S. lancipes P229
S. solani P240
S. callistephi P383
P245
P250
S. xanthosomatis P232
Group A
Group B
Group C
Group D
Group E
Group F
5 changes


EF-1 alpha
P. tarda P1
S. lancipes P229
P. herbarum P2
P. alfalfae P81
P235
P277
P272
P273
P303
P. gracilariae P243
P315
P. gigaspora P129
S. astragali P201
S. majusculum P262
P207
P. eturmiuna P269
P302
S. solani P240
S. callistephi P383
P56
P342
P107
P. triglochinicola P123
P327
P. paludiscirpi P270
P210
S. sarciniforme P239
S. sarciniforme P247
P246
S. loti P384
S. loti P385
S. trifolii P244
P245
P250
S. xanthosomatis P232
Group A
Group B
Group C
Group D
Group E
Group F
5 changes

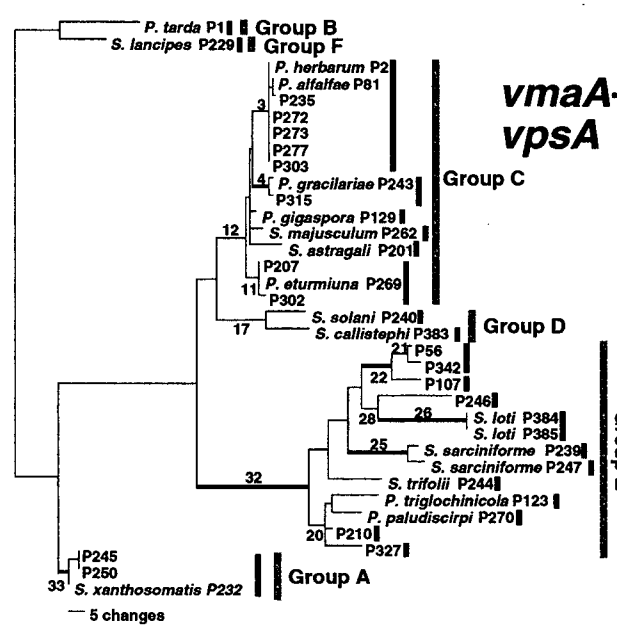
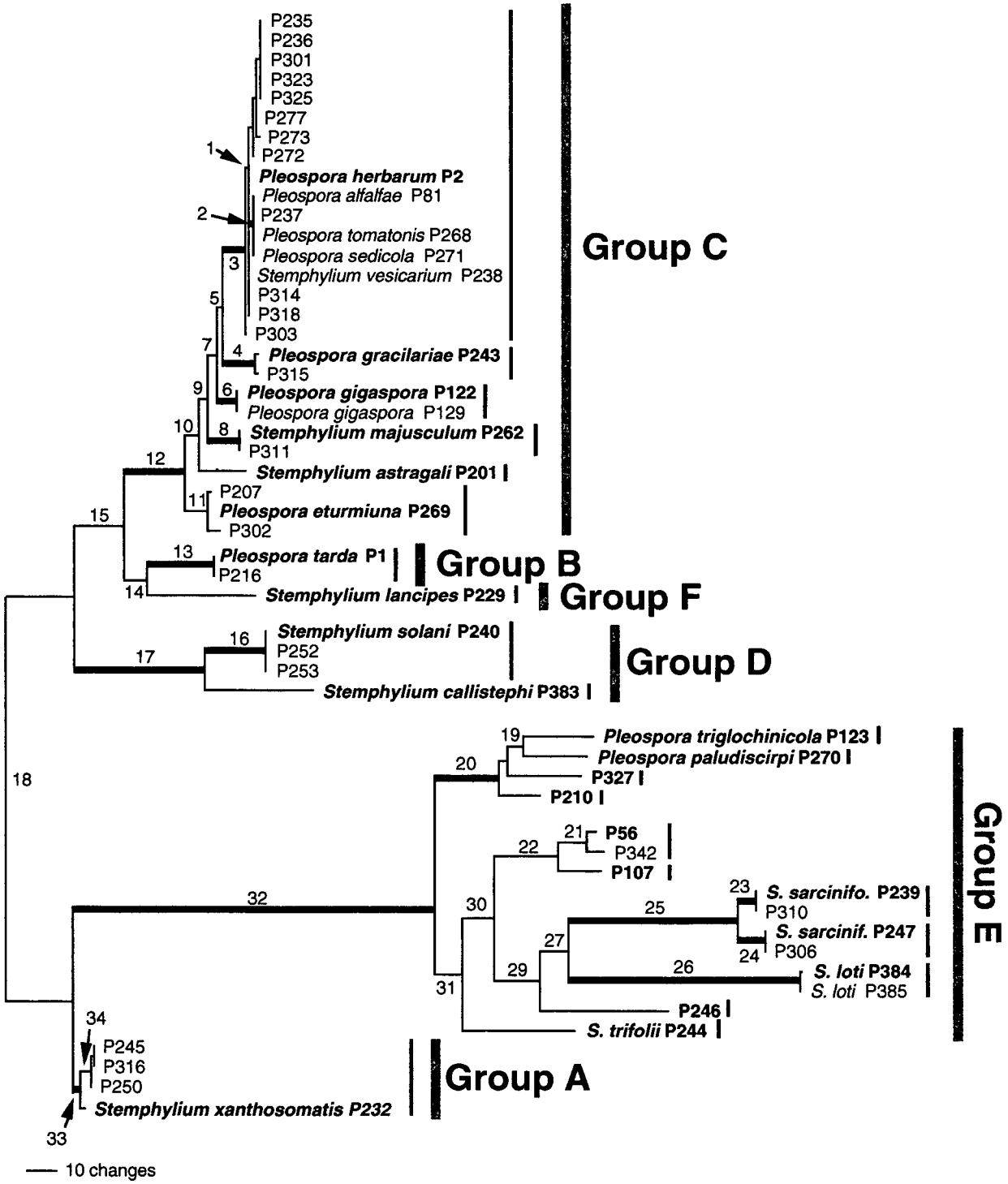

vmaA-vpsA
P. tarda P1
S. lancipes P229
P. herbarum P2
P. alfalfae P81
P235
P272
P273
P277
P303
P. gracilariae P243
P315
P. gigaspora P129
S. majusculum P262
S. astragali P201
P207
P. eturmiuna P269
P302
S. solani P240
S. callistephi P383
P56
P342
P107
P246
S. loti P384
S. loti P385
S. sarciniforme P239
S. sarciniforme P247
S. trifolii P244
P. triglochinicola P123
P. paludiscirpi P270
P210
P327
P245
P250
S. xanthosomatis P232
Group A
Group B
Group C
Group D
Group E
Group F
5 changes

Figure 2-6. One most parsimonious tree from combined ITS, *GPD*, *EF-1 alpha* and *vmaA-vpsA* analyses. Only 35 unique multilocus genotype were included in analyses (Table 2-11), the remaining isolates were added to the tree after completion of the analyses. Values by the branches indicate bootstrap support percentages above 70 for parsimony, likelihood, Bayesian and Neighbor joining analyses, and are given in Table 2-12. Branches in bold are supported by 100% of the replicates with all algorithms. Thin vertical lines on the right represent phylogenetic species (Table 2-14). Gray vertical lines are groups A – F following Câmara et al. (2002) and this study. For information on strain numbers see Table 2-2.


P235
P236
P301
P323
P325
P277
P273
P272
Pleospora herbarum P2
Pleospora alfalfae P81
P237
Pleospora tomatonis P268
Pleospora sedicola P271
Stemphylium vesicarium P238
P314
P318
P303
Pleospora gracilariae P243
P315
Pleospora gigaspora P122
Pleospora gigaspora P129
Stemphylium majusculum P262
P311
Stemphylium astragali P201
P207
Pleospora eturmiuna P269
P302
Pleospora tarda P1
P216
Stemphylium lancipes P229
Stemphylium solani P240
P252
P253
Stemphylium callistephi P383
Pleospora triglochinicola P123
Pleospora paludiscirpi P270
P327
P210
P56
P342
P107
S. sarcinifo. P239
P310
S. sarcinif. P247
P306
S. loti P384
S. loti P385
P246
S. trifolii P244
P245
P316
P250
Stemphylium xanthosomatis P232
Group C
Group B
Group F
Group D
Group E
Group A
10 changes

2.4.5 Bibliography

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389-3402.

Andersen, B. (1995). Characterization of cereal-borne *Alternaria* and *Stemphylium*. Ph.D. thesis. Ph.D. Thesis. The Mycology Group, Department of Biotechnology, Technical University of Denmark.

Baker, K. F. & Davis, L. H. (1950). *Stemphylium* leaf spot of China Aster. *Mycologia* **42**, 477-486.

Baldauf, S. L. & Doolittle, W. F. (1997). Origin and evolution of the slime molds (Mycetozoa). *Proceedings of the National Academy of Sciences of the United States of America* **94**, 12007-12012.

Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., Studholme, D. J., Yeats, C. & Eddy, S. R. (2004). The Pfam protein families database. *Nucleic Acids Research* **32**, D138-D141.

Berbee, M. L., Pirseyedi, M. & Hubbard, S. (1999). *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and *glyceraldehyde-3-phosphate dehydrogenase* gene sequences. *Mycologia* **91**, 964-977.

Burset, M., Seledtsov, I. A. & Solovyev, V. V. (2000). Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Research* **28**, 4364-4375.

Câmara, M. P. S., O'Neill, N. R. & Berkum, v., P (2002). Phylogeny of *Stemphylium* spp. based on ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **94**, 660-672.

Cavara, F. (1890). *Macrosporium sarcinaeforme* Cav. Nuovo parassita del trifoglio. *La Difesa dai Parassiti* **4**, 3-8.

Condeelis, J. (1995). Elongation factor 1alpha, translation and the cytoskeleton. *Trends Biochem Sci.* **20**, 169-170.

Cottrelle, P., Thiele, D., Price, V. L., Memet, S., Micouin, J.-Y., Marck, C., Buhler, J.-M., Sentenac, A. & Fromageot, P. (1985). Cloning, nucleotide sequence, and expression of one of two genes coding for yeast elongation factor 1alpha. *The Journal of Biological Chemistry* **260**, 3090-3096.

Crivelli, P. G. (1983). Über die heterogene Ascomycetengattung *Pleospora* Rabenh.; Vorschlag für eine Aufteilung. Ph.D. thesis. Eidgenössische Technische Hochschule, Zürich, Switzerland.

Croan, S. C., Burdsall, H. H. & Rentmeester, R. M. (1999). Preservation of tropical wood-inhabiting basidiomycetes. *Mycologia* **91**, 908-916.

de Jesus Yanez Morales, M. (2001). Molecular and morphological analyses of *Alternaria* and *Stemphylium* from onion. Ph.D. thesis. Cornell University.

Gardes, M. & Bruns, T. D. (1993). ITS primers with enhanced specificity of basidiomycetes: application to the identification of mycorrhizae and rusts. *Molecular Ecology* **2**, 113-118.

Geiser, D. M., Pitt, J. I. & Taylor, J. W. (1998). Cryptic speciation and recombination in the aflatoxin-producing fungus *Aspergillus flavus*. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 388-393.

Graham, J. H. (1953). A disease of Birdsfoot Trefoil caused by a new species of *Stemphylium*. *Phytopathology* **43**, 577-579.

Graham, J. H. (1957). A *Stemphylium* disease of Ladino white clover. *Phytopathology* **47**, 213-215.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995). *Dictionary of the Fungi*, 8 edn. Wallingford, Oxon, UK: CAB International.

Holm, L. (1962). Lewis E. Wehmeyer, a world monograph of the genus *Pleospora* and its segregates. *Svensk Botanisk Tidskrift* **56**, 377-381.

Huelsenbeck, J. P. & Ronquist, F. (2003). MrBayes: Bayesian analysis of phylogeny: Distributed by the authors.

Huelsenbeck, J. P., Bull, J. J. & Cunningham, C. W. (1996). Combining data in phylogenetic analysis. *Trends Ecol. Evol.* **11**, 152-158.

Huguenin, B. (1965). Mycromycètes du pacifique sud. Troisième contribution. Dématiées de Nouvelle-Calédonie. *Bullétin de la Société Mycologique de France* **81**, 694-698.

Ichiishi, A. & Inoue, H. (1995). Cloning, Nucleotide-Sequence, and Expression of *Tef-1*, the Gene Encoding *Translation Elongation-Factor 1-Alpha (Ef-1-Alpha) of Neurospora crassa. Japanese Journal of Genetics* **70**, 273-287.

Karsten, P. A. (1884). Fragmenta mycologica XI. *Hedwigia* **23**, 37-39.

Kasuga, T., Taylor, J. W. & White, T. J. (1999). Phylogenetic relationships of varieties and geographical groups of the human pathogenic fungus *Histoplasma capsulatum* Darling. *Journal of Clinical Microbiology* **37**, 653-663.

Koufopanou, V., Burt, A. & Taylor, J. W. (1997). Concordance of gene genealogies reveals reproductive isolation in the pathogenic fungus *Coccidioides immitits. Proceedings of the National Academy of Sciences of the United States of America* **94**, 5478-5482.

Kuroki, Y., Juvvadi, P. R., Arioka, M., Nakajima, H. & Kitamoto, K. (2002). Cloning and characterization of *vmaA*, the gene encoding a 69-kDa catalytic subunit of the vacuolar H+-ATPase during alkaline pH mediated growth of *Aspergillus oryzae. Fems Microbiology Letters* **209**, 277-282.

Lee, S. B. & Taylor, J. W. (1990). Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols*, pp. 282-287. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Mehta, Y. R. (1998). Severe outbreak of *Stemphylium* leaf blight, a new disease of cotton in Brazil. *Plant Disease* **82**, 333-336.

Mehta, Y. R. (2001). Genetic diversity among isolates of *Stemphylium solani* from cotton. *Fitopathologia Brasileira* **26**, 703-709.

Mehta, Y. R. & Brogin, R. L. (2000). Phytotoxicity of a culture filtrate produced by *Stemphylium solani* of cotton. *Plant Disease* **84**, 838-842.

Mehta, Y. R., Mehta, A. & Rosato, Y. B. (2002). ERIC and REP-PCR banding patterns and sequence analysis of the internal transcribed spacer of rDNA of *Stemphylium solani* isolates from cotton. *Current Microbiology* **44**, 323-328.

O'Donnell, K. & Cigelnik, E. (1997). Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* **7**, 103-116.

O'Donnell, K., Kistler, H. C., Cigelnik, E. & Ploetz, R. C. (1998). Multiple evolutionary origins of the fungus causing Panama disease of banana: Concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 2044-2049.

Rambaut, A. (1995). *Se-Al v1.d1*, Version 1.0 alpha edn. Oxford, U.K: Department of Zoology, University of Oxford.

Simmons, E. G. (1969). Perfect states of *Stemphylium. Mycologia* **61**, 1-26.

Simmons, E. G. (1985). Perfect states of *Stemphylium*. II. *Sydowia, Annales Mycologici Ser. II.* **38**, 284-293.

Simmons, E. G. (1989). Perfect states of *Stemphylium*. III. *Memoirs of the New York Botanical Garden* **49**, 305-307.

Simmons, E. G. (2001). Perfect states of *Stemphylium*: IV. *Harvard Papers in Botany* **6**, 199-208.

Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sunderland, Massachusetts: Sinauer Associates.

Tarutani, Y., Ohsumi, K., Arioka, M., Nakajima, H. & Kitamoto, K. (2001). Cloning and characterization of *Aspergillus nidulans vpsA* gene which is involved in vacuolar biogenesis. *Gene* **268**, 23-30.

Taylor, D. L., Jacobson, D. J., Kroken, S., Kasuga, T., Geiser, D. M., Hibbett, D. S. & Fisher, M. C. (2000). Phylogenetic species recognition and species concepts in fungi. *Fungal Genetics and Biology* **31**, 21-32.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. In *Nucleic Acids Research*, pp. 4876-4882: Distributed by the authors.

Thornewell, S. J., Peery, R. B. & Skatrud, P. L. (1995). Cloning and characterization of the gene encoding translation elongation factor 1-alpha from *Aureobasidium pullulans*. *Gene* **162**, 105-110.

Weber, G. F. (1930). Gray leaf spot of tomato caused by *Stemphylium solani*, sp. nov. *Phytopathology* **20**, 513-518.

Webster, J. (1969). The *Pleospora* state of *Stemphylium triglochinicola*. *Transactions of the British Mycological Society* **53**, 478-482.

Wehmeyer, L. E. (1961). *A world monograph of the genus Pleospora and its segregates.* Ann Arbor: The University of Michigan Press.

Wendland, J. & Kothe, E. (1997). Isolation of *tef1* encoding translation elongation factor EF1 alpha from the homobasidiomycete *Schizophyllum commune*. *Mycological Research* **101**, 798-802.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols*, pp. 315-322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Wiley, E. O. (1981). *Phylogenetics: the theory and practice of phylogenetic analysis.* New York: John Wiley & Sons.

Yamamoto, W. (1960). Synonymous species of *Alternaria* and *Stemphylium* in Japan [in Japanese]. *Transactions of the Mycological Society of Japan* **2**, 88-93.

Yoshii, H. (1929). A leaf spot disease of *Astragalus sinicus* L [in Japanese]. *Journal of Plant Protection* **16**, 533-537.

Zhan, J., Pettway, R. E. & McDonald, B. A. (2002). The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* **38**, 286-297.

CHAPTER 3. Mating system evolution in *Pleospora* sensu stricto

3.1. Introduction

3.1.1 The MAT locus in *Cochliobolus* and hypotheses

Sexual reproduction in ascomycetes is controlled by the MAT locus, a master regulator of downstream gene expression (Turgeon *et al.*, 1993b). To be sexually compatible, individuals of one species have to differ at their MAT locus (Turgeon & Yoder, 2000). Opposite mating types evolved early in ascomycete history, since they are shared among all major groups of ascomycetes. MAT loci consist of MAT genes and flanking regions. Since MAT alleles are too divergent to be aligned, they are not alleles in the true sense and are thus called idiomorphs instead (Metzenberg & Glass, 1990). In *Cochliobolus* which as *Pleospora* is in the family Pleosporaceae, there are two MAT idiomorphs, each containing a *MAT1-1* or *MAT1-2* gene. Mating type genes can be arranged in different ways in *Cochliobolus*, correlating with different life history strategies. Heterothallic species either have a *MAT1-1* or a *MAT1-2* gene at their MAT locus, whereas homothallic species have both. The MAT regions can either be fused end to end as in *C. luttrellii* or in reversed order in *C. homomorphus*, or their arrangement can be more complex. In *C. kusanoi MAT1-1* is fragmented, and in *C. cymbopogonis*, the MAT genes were still present in the same genome, but not near each other (Yun *et al.*, 1999). An unequal crossover between *MAT1-1* and *MAT1-2* strains was suspected to be responsible for the fusion of the adjacent MAT genes. For the more complicated MAT gene arrangements, no explanation was yet possible (Yun *et al.*, 1999).

Phylogenetic analyses showed that homothallism evolved several times in *Cochliobolus* (Berbee *et al.*, 1999), and most likely from heterothallism. Whereas it is theoretically possible that homothallism was the ancestral state in *Cochliobolus*, and heterothallism was derived, it is hard to imagine how the homothallic MAT gene arrangement could have broken down to yield the arrangement in the heterothallics (Yun *et al.*, 1999).

As in *Cochliobolus*, *Pleospora* contains both heterothallic and homothallic species, and is thus amenable to the same kind of studies as in *Cochliobolus*. In this chapter, I address the following mating type related questions. What is the architecture of the MAT locus in species of *Pleospora*? Do the mating type genes in *Pleospora* correlate with mating system as they do in *Cochliobolus*? How many times did a switch between heterothallics and homothallics occur in the evolution of *Pleospora*?

My approach was to first generate a robust species phylogeny based on four non-MAT loci (see Chapter 2). I then screened all isolates for the presence of *MAT1-1* and *MAT1-2* genes, and determined the mating type arrangement of representative isolates by DNA sequencing. To investigate a correlation between mating system and MAT locus architecture, I compared the mating type

arrangements in *Pleospora* with evidence for selfing and outcrossing for the phylogenetic species. Finally, to determine the number of mating system changes in the evolution of *Pleospora*, I generated mating type gene phylogenies, and compared them to the species phylogeny. Supported by MAT gene arrangement data, the results suggested the occurrence of a lateral gene transfer in the evolution of *Pleospora* and the evolution of three kinds of selfers from outcrossers.

3.2. Materials and methods

3.2.1 Fungal strains used

All 110 strains of *Pleospora* (Table 2-2) were screened for presence of mating type genes. A representative selection of 37 isolates was chosen for sequencing of the entire mating type region (Figure 3-1).

3.2.2 Conditions used for crosses

In crosses within phylogenetic species of *S. xanthosomatis* and *S. solani*, isolates of differing mating types were paired on V8 agar plates (Hawksworth *et al.*, 1995) to which autoclaved, previously dried alfalfa stems were added. Plates were sealed with parafilm, and left on a laboratory bench for three months, subject to both natural and artificial light during working hours and darkness at night.

3.2.3 Molecular work

3.2.3.1 DNA extraction, PCR and DNA sequencing

See page 12.

3.2.3.2 Primers and PCR conditions

3.2.3.2.1 Species phylogeny

The combined ITS, *GPD*, *EF-1 alpha*, and *vmaA-vpsA* multigene species phylogeny in Figure 3-1 was taken from Chapter 2 (Figure 2-6).

3.2.3.2.2 Screening for mating type gene arrangement

To screen *Pleospora* isolates for presence and arrangement of mating type genes, mating type specific primers in conserved regions were designed. Conserved regions were the DNA binding motives alpha box on *MAT1-1*, and the HMG box on *MAT1-2*. Primers were based on *Pleospora* sequences contained in the thesis of de Jesus Yanez Morales (2001). The *MAT1-1*, alpha box specific forward and reverse primers were Jen1f and Jen1r, and the *MAT1-2*, HMG box specific primers were Jen2f and Jen2r (Table 3-1 to Table 3-4). PCR conditions

used were 5 minutes initial denaturation at 95°C, 40 cycles of 10 sec at 95°C, 20 sec at 52°C and 30 sec (+4 sec/cycle) at 72°C, followed by a final extension of seven minutes at 72°C. For isolates that had both alpha and HMG boxes, the orientation of the mating type genes was probed by PCR amplification using combinations of alpha with HMG box primers, with identical PCR conditions.

3.2.3.2.3 Screening for the presence of *MAT1-2* in additional isolates of *Pleospora sp.* strain P56

Primer sets Alpha181f/Jen1ri and Jen2f/Jen2r (see Figure 3-2) were used to screen the eight additional single ascospore isolates from the material of *Pleospora sp.* strain P56 for *MAT1-1* and *MAT1-2*, respectively. The additional isolates were not listed in Table 2-2.

3.2.3.2.4 Generation of MAT gene DNA sequences and flanking regions

To determine DNA sequences of the entire mating type genes and flanking regions, the chromosome walking kit Vectorette was used (Sigma-Genosys, The Woodlands, Texas, USA). For detailed protocols, see chapter 7.2. The procedure involved a digest of the fungal DNA using the respective restriction enzymes *BamHI*, *EcoRI* or *ClaI* (Roche Diagnostics GmbH, Mannheim, Germany). Adapters were then ligated to the DNA fragments using a T4 DNA ligase (Roche Diagnostics GmbH, Mannheim, Germany). In PCR reactions, an adapter specific primer was used with a *Pleospora* specific primer to generate PCR fragments of up to ca. 6 kpb (Table 3-8).

For chromosome walking off the MAT genes, *Pleospora* primers were designed in the previously sequenced DNA binding motives of the mating type genes. Two or three primers pointing in the same direction were designed at least ca. 20 bp apart. In a first set of PCR reactions, the outer *Pleospora* primer was used together with the outer adapter primer, nicknamed 'Vec'. The resulting PCR product was used diluted 100 times in a second set of PCR reactions, generally with an internal MAT primer, and the internal adapter primers, 'VecN'. The resulting PCR products were sequenced with the PCR primers, or internal MAT primers, and additional forward and reverse sequencing primers were designed to reach the desired overlapping sequencing coverage in both directions. If necessary, chromosome walking was repeated to further extend sequencing coverage.

Hotstart and touchdown PCR protocols were followed in both sets of chromosome walking PCRs. The PCR cocktail was prepared without the addition of Taq polymerase. 12.5 µl of template were added, overlaid with a drop of mineral oil and the PCR program started. Once the tubes were heated to 94°C, 0.5 µl of Expand High Fidelity PCR System Taq was used (Roche Diagnostics GmbH, Mannheim, Germany), and the PCR program left to run with the follow-

ing parameters. At first, 20 cycles with decreasing annealing temperature, followed by 20 cycles with stable annealing temperature. The initial denaturation step (2 min at 94°C) was followed by 20 cycles of denaturation (10 sec at 94°C), annealing (20 sec first at 70°C, then -0.5° per cycle), and extension steps (first 30 sec then +4 sec per cycle at 72°C), followed by 20 cycles of denaturation (10 sec at 94°C), annealing (20 sec at 60°C), and extension (first 1 min, then +4 sec per cycle at 72°C), and a final extension step of 7 min at 72°C. The second set of PCR reactions used internal primers on a 100 times dilution of the PCR product from the first set, and the same PCR conditions as in the first set, except for 5°C lower annealing temperatures and a one minute longer extension time for the last 20 cycles, on a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA, USA).

On the DNA sequences obtained from chromosome walking in selected isolates, additional primers were designed in conserved sites to PCR amplify and sequence the MAT regions of additional isolates. A total of 93 different primers were used in PCR amplifications and sequencing. The positions of the most frequently used primers are shown on the gene diagrams in Figure 3-2. For exact positions of all primers and primer sequences, see Table 3-1 and Table 3-2 for the *MAT1-1* region, Table 3-3 and Table 3-4 for the *MAT1-2* region and Table 3-5 and Table 3-6 for the fused MAT regions. For primers and PCR conditions used for each isolate, see Table 3-7 for the *MAT1-1* regions, Table 3-8 for the *MAT1-2* regions, and Table 3-9 for the fused MAT regions. Conditions for conventional PCR were as follows, with the respective annealing temperature and number of cycles from Table 3-7 - Table 3-9. 5 minutes initial denaturation at 95°C, X cycles of 10 sec at 95°C, 20 sec at Y°C and 30 sec (+4 sec/cycle) at 72°C, followed by a final extension of seven minutes at 72°C, on a GeneAmp PCR System 9700 (PE Biosystems, Foster City, CA, USA).

3.2.3.3 Phylogenetic analyses

3.2.3.3.1 DNA

For DNA sequence analyses, see Chapter 2, page 16.

3.2.3.3.2 Proteins

For parsimony analyses, the PHYLIP 3.572 package was used (Felsenstein, 2001). Most parsimonious trees were inferred using PROTPARS. Default options were used, except that input order was randomized, and 30 random sequence additions were used (jumble set to 30). Strict consensus trees were calculated and drawn using PAUP* (Swofford, 2002). For bootstrap analyses, 500 random datasets were created using SEQBOOT with default settings, and analyzed in PROTPARS with one random taxon addition per dataset (jumble set to 1).

For distance trees, a likelihood estimated distance matrix was obtained with TREE-PUZZLE 5.0 (Strimmer & von Haeseler, 1996). Default settings were utilized, except that 10,000 puzzling steps were used with a HKY substitution model, the proportion of variable sites and transition-transversion ratio were estimated from the dataset, and the gamma distributed substitution rates were approximated by four rate categories. The resulting distance matrix was imported into the FITCH component of PHYLIP 3.572 (Felsenstein, 2001) to construct and optimize the tree topology using a Fitch-Margoliash method with default settings, except that the global optimization option was in effect and the input order was randomized with the jumble option set to 30. The resulting tree was manipulated in MacClade 4.03 (Maddison & Maddison, 2001), and printed in PAUP* (Swofford, 2002).

3.2.3.4 Defining idiomorphs by MAT region comparisons

To define idiomorph boundaries, pairs of *MAT1-1* and *MAT1-2* region DNA sequences were aligned for each species using ClustalX (1.8) (Thompson *et al.*, 1997). The beginning of the idiomorphs was expected to be marked by a sudden drop of DNA sequence similarity between *MAT1-1* and *MAT1-2* regions. The computer program SWAN (1.0b) (Proutski & Holmes, 1998) was used to plot the percentage similarity of the DNA sequences. Settings were default except for a window size of 10 bp and a shift of 10 bp.

3.3. Results

3.3.1 Mating type genes

3.3.1.1 Mating type screening to investigate MAT locus architecture

Out of the 110 isolates of *Pleospora* screened, 24 isolates had *MAT1-1* only, ten had *MAT1-2* only, and the remaining 76 isolates had both *MAT1-1* and *MAT1-2* genes (Table 3-10). All *Pleospora* isolates were initially screened with the *MAT1-1* and *MAT1-2* specific primer sets. For *MAT1-1*, the diagnostic fragment was expected to be ca. 206 bp in length, and the *MAT1-2* specific fragment ca. 225 bp (de Jesus Yanez Morales, 2001). In ten cases, primer Jen1F had to be replaced by primer BPHO4d, 15 bp upstream of the Jen1F 3'-end (data not shown; Table 3-1). For isolates that had both alpha and HMG boxes, the orientation of the mating type genes was probed by PCR amplification using all different combinations of alpha with HMG box primers. The successful combination was primers Jen1r and Jen2f yielding a PCR product of ca. 1.6 kb, indicating that the mating type genes were located in close proximity, and that one was reversed. It was determined that all isolates with both *MAT1-1* and *MAT1-2* diagnostic PCR fragments also had the ca. 1.6 kb Jen1r and Jen2f PCR fragment indicating that the MAT genes in homothallics were all ar-

ranged in the same way with respect to each other (data not shown). Mating type loci with both MAT genes are referred to as '*MAT1-1*; *MAT1-2*' (Lee *et al.*, 2003), as opposed to '*MAT1-1*' and '*MAT1-2*' where only one MAT gene is present at the MAT locus (Turgeon & Yoder, 2000).

3.3.1.2 Screening for *MAT1-2* in additional isolates of the homothallic *Pleospora sp.* strain P56

In all eight additional single ascospore isolates of *Pleospora sp.* strain P56, only *MAT1-1* was detected (data not shown).

3.3.1.3 MAT DNA sequences obtained

A total of 14 *MAT1-1*, six *MAT1-2*, and 17 *MAT1-1*; *MAT1-2* region DNA sequences were generated. Isolates were chosen to reflect the genetics of the isolates used in this study (Figure 3-1). This was done to confirm PCR MAT screening results, to find genes flanking the MAT loci, and to determine the orientation of the MAT genes with respect to neighboring genes. A chromosome walking PCR approach and DNA sequencing was used with the following isolates. *Pleospora sp.* strain P56, *S. lancipes* strain P229, *Stemphylium sp.* strain P245 and *S. lancipes* strain P247 for isolates with only *MAT1-1.* The sequences generated were between 8455 and 2831 bp in length. For isolates with only *MAT1-2*, chromosome walking was used for *S. xanthosomatis* strain P232 and *Stemphylium sp.* strains P250 and P252, with DNA sequences generated ranging in size from 4707 – 7248 bp. For isolates with both MAT genes, chromosome walking was used for *P. tarda* strain P1 and *P. herbarum* strain P2, where sequences of 6102 bp and 3997 bp were generated respectively. All DNA sequences were aligned to find conserved sequence regions common to all isolates suitable for primer design. The resulting primers were used to PCR amplify 10 more *MAT1-1* regions, three more *MAT1-2* regions, and 15 more fused MAT regions. The sequence lengths ranged from 2203 bp to 2475 bp in *MAT1-1* isolates, 2571 – 2607 bp in *MAT1-2* isolates, and 3851 – 4099 bp in isolates with both MAT genes. Thus, a total of 14 *MAT1-1*, six *MAT1-2*, and 17 *MAT1-1*; *MAT1-2* region DNA sequences were generated. Homologous DNA sequences were easily alignable between isolates with only *MAT1-1* or *MAT1-2*, and isolates with both MAT genes (data not shown).

3.3.1.4 Mapping of mating type regions

3.3.1.4.1 *MAT1-1* regions

The longest *MAT1-1* region DNA sequence obtained was the one of *Pleospora sp.* strain P56, 8455 bp in length. Using DNA sequence comparison to the homologous region in the closely related *Cochliobolus heterostrophus* (AF029913), it was determined that it contained the following genes (Figure 3-3). GTPase activating protein homolog *GAP1* (positions 1 - 637 in *Pleospora*

sp. strain P56), ORF1 (1184 - 1801), *MAT1-1* (2451 - 3643), and beta glucosidase homolog *BGL1* (8146 - 8455). Thus, the genes and gene arrangement in the *Pleospora sp.* strain P56 mating region were identical to the one in *C. heterostrophus* (Turgeon *et al.*, 1993a). The remaining 13 *MAT1-1* region DNA sequences (Figure 3-1) started between 34 and 280 bp inside the ORF1 and extended between 322 and 2373 bp beyond *MAT1-1.* In all isolates, the *MAT1-1* gene was 1193 bp in length, with an intron of 53 bp from position 218 – 270. Thus; the exon was 1140 bp in length.

3.3.1.4.2 *MAT1-2* regions

The longest *MAT1-2* region DNA sequence was from *S. xanthosomatis* strain P232, 7248 bp in length. Using DNA sequence comparison to the homologous regions in *C. heterostrophus* (AF027687), it was mapped as follows (Figure 3-3): ORF1 (positions 1-321), *MAT1-2* (1352 – 2444), beta glucosidase homolog *BGL1* (6958 – 7248). Thus, as in *MAT1-1*, the genes and gene arrangement in the *S. xanthosomatis* strain P232 mating region were identical to the one in *C. heterostrophus* (Turgeon *et al.*, 1993a). The remaining five isolates (Figure 3-1) extended 21 – 320 bp into ORF1, and 397 – 2265 bp beyond the *MAT1-2* gene. In all isolates, the *MAT1-2* gene was 1093 bp in length, with an intron of 55 bp from position 491 – 545. Thus, the exon was 1038 bp in length.

3.3.1.4.3 Fused mating type regions with both *MAT1-1* and *MAT1-2*

The longest DNA sequence containing both *MAT1-1* and *MAT1-2* genes was from *P. tarda* strain P1, and was 6102 bp long. It was compared to homologous regions from *C. heterostrophus* (AF029913, AF027687) and was mapped as follows (Figure 3-3). *GAP1* gene (positions 1-255), ORF1 618 bp in length (802-1419), inverted *MAT1-1* 1193 bp in length (2040-3232), and *MAT1-2*, 1093 bp in length (3710-4802). The sequences of the remaining 16 fused MAT regions (Figure 3-1) started 44 – 167 bp within the ORF1 and terminated 418 - 464 bp downstream of *MAT1-2.* No length variation within the mating type genes or introns was present. The MAT exons and genes were of equal lengths, and contained introns at the same positions as the MAT genes of the outcrossers. However, the fused MAT regions did fall into two groups lengthwise, due to a deletion of 201 bp 97 bp upstream of the *MAT1-2* 5'-end in isolates *P. tarda* strain P1, *P. herbarum* strain P2, *P. alfalfae* strain P81, *P. gigaspora* strain P129, *S. astragali* strain P201, *Stemphylium sp.* strain P207, *S. vesicarium* strain P238, *P. gracilariae* strain P243, *S. majusculum* strain P262, *P. tomatonis* strain P268, *P. eturmiuna* strain P269, *P. sedicola* strain P271, and *Stemphylium sp.* strain P303. Isolates *P. triglochinicola* strain P123, *Stemphylium sp.* strain P210, *P. paludiscirpi* strain P270 and *Pleospora sp.* strain P327 did not have this deletion (data not shown).

3.3.1.5 Phylogenetic information content in MAT regions

Homologous DNA sequences from separate and fused MAT regions were aligned, and the content of parsimony informative characters determined using PAUP* (Swofford, 2002) (Table 3-11). Sequences evaluated corresponded to the idiomorph portion of the inverted *MAT1-1* regions and the *MAT1-2* idiomorph present in the fused MAT regions.

3.3.1.6 Comparison between separate and fused MAT regions

The *MAT1-1* gene of the fused MAT regions was inverted. DNA sequence comparison of fused mating type regions to separate MAT regions showed that the fused MAT regions were arranged as shown in Figure 3-4. An inverted *MAT1-1* gene was present between the ORF1 and *MAT1-2*. In detail, the *MAT1-1* region was in the usual forward orientation found in separate *MAT1-1* idiomorphs, 326 - 335 bp downstream of ORF1, depending on the isolate. There it was cut upstream of an 'ATG' motive and fused to an inverted *MAT1-1* fragment of 1710 – 1797 bp in length, carrying the *MAT1-1* gene and flanking regions. The flanking regions were 204 - 288 and 310 - 316 bp in length, downstream and upstream of the *MAT1-1* gene, respectively. The inverted *MAT1-1* region was then fused to a forward *MAT1-2* region, 159-362 bp upstream of the *MAT1-2* gene. The *MAT1-2* region, from the fusion point to *MAT1-1* downstream, was as in separate *MAT1-2* regions.

In summary, the fused MAT regions contained two complete mating type genes. The *MAT1-1* region, spanning from the ORF1 ca. 2000 bp downstream, was as in separate homologs, except that the ORF1 distal ca. 1850 bp were inverted, and fused to a *MAT1-2* region.

3.3.1.7 Evolution of fused MAT regions

A possible scenario for the evolution of the fused MAT regions is based on the following observations. At the fusion junction between all *MAT1-1* and *MAT1-2* regions, the four nucleotide motive 'CCAT' was present (Figure 3-5). This motive was also present at homologous position in separate *MAT1-2* regions, and as reverse complement in separate *MAT1-1* regions. This allowed for the theoretical possibility of a crossover between separate MAT regions following an inversion in *MAT1-1*, leading to the fused MAT regions found in *Pleospora* (Figure 3-6). The 'CCAT' motive was not present in all the isolates, but in four out of the six *MAT1-2* regions sequenced (*S. xanthosomatis* strains P232, *Stemphylium sp.* strains P250 and P252, and *S. loti* strain P385), as a reverse complement in five out of 14 *MAT1-1* regions (*S. solani* strain P240, *S. trifolii* strain P244, *Stemphylium sp.* strains P245, P253, P316) and in all of the fused MAT regions (Figure 3-5).

3.3.1.8 Delimitation of the idiomorphs

MAT alleles are called idiomorphs for lack of any compelling DNA similarity (Metzenberg & Glass, 1990). To delimit the idiomorphs in *Pleospora*, all MAT regions were aligned. It was found that the similarity between *MAT1-1* and *MAT1-2* regions dropped to a random level 16 or 17 amino acids upstream of the ORF1 stop codon for *MAT1-2* and *MAT1-1* respectively. Thus, the 5'-end of the idiomorph was within the adjacent ORF1 (Figure 3-7).

The delimitation of the 3'-end of the idiomorph was less distinct, islands of similarity between *MAT1-1* and *MAT1-2* appeared gradually. The 3'-end of the idiomorph was chosen to be upstream of the motive TT(T/C)(T/C)(T/C), shared between *MAT1-1* and *MAT1-2* (Figure 3-7). In fused MAT regions, the 5'-end of this motive was 100 or 175 bp downstream of *MAT1-1* and 176 bp downstream of *MAT1-2*. In separate MAT regions, it was 175 bp downstream of *MAT1-1* and 173 – 176 bp downstream of *MAT1-2*, depending on the isolates.

The entire idiomorphs were sequenced for the following five separate *MAT1-1* regions. *Pleospora sp.* strain P56, *S. lancipes* strain P229, *S. sarciniforme* strain P247, and *Stemphylium sp.* strains P246 and P316, they were between 2029 – 2078 bp long. For the remaining nine isolates only the 3'-idiomorph border was reached. For the separate *MAT1-2* regions, complete idiomorphs were obtained for *S. xanthosomatis* strain P232 and strains *Stemphylium sp.* P250 and P252, all 2349 bp in length. For the remaining three isolates, only the 3'-idiomorph border was reached. For fused *MAT1-1* regions, the 5'-idiomorph border was reached in *P. tarda* strain P1, *P. herbarum* strain P2, *P. alfalfae* strain P81, *S. majusculum* strain P262, *P. paludiscirpi* strain P270 and *P. sedicola* strain P271. For the *MAT1-2 genes* from the fused regions, the 5'-idiomorph end was not present, due to fusion to the inverted *MAT1-1* region. The 3'-idiomorph border was reached in all 17 fused MAT regions. An idiomorph length as for separate MAT regions was not possible to define in the selfers, since the inverted portion of the *MAT1-1* region extended 108 - 114 bp beyond the idiomorph downstream of *MAT1-1* (Figure 3-7).

The choice of idiomorph boundary had implications on the outcome of phylogenetic idiomorph analyses. Including DNA characters beyond the idiomorphs where crossovers are not suppressed will introduce phylogenetic error. To examine the phylogenetic information present around the chosen idiomorph 3'-end, an alignment of all DNA sequences downstream of the MAT genes was made. Phylogenetic parsimony analyses showed that the MAT - idiomorph 3'-end region separated *MAT1-1* and *MAT1-2* with 100% bootstrap support. This is expected in the case of idiomorphs. The 162 positions from the 3' idiomorph end downstream to the end of the inverted *MAT1-1* region in selfers, separated *MAT1-1* and *MAT1-2* with only 66% bootstrap support (data not shown). This might be evidence for recombination beyond the idiomorph 3'-

end, gradually introducing a phylogenetic signal grouping according to species instead of mating types.

3.3.1.9 Phylogenetic analyses

Two sets of analyses were done. To test for monophyly of *Pleospora* and determination of the basal lineage within *Pleospora*, *MAT1-1* and *MAT1-2* protein sequences were analyzed. Amino acid comparisons showed that from the homologous sequences available in GenBank, only *Cochliobolus* and *Alternaria* protein sequences were similar enough to *Pleospora* to be aligned with confidence (data not shown). Thus, to test for monophyly of *Pleospora*, four representatives of *Cochliobolus*, and one of *Alternaria alternata* were chosen for inclusion in the analyses. In a second round of analyses, to increase the phylogenetic resolution within *Pleospora*, DNA sequences of MAT genes and flanking regions were used. The taxon sampling was restricted to *Pleospora*, and the trees were rooted based on results from protein analyses.

3.3.1.9.1 Mating type protein phylogenies

3.3.1.9.1.1 *MAT1-1* protein analyses

All 31 *MAT1-1* protein sequences from both separate and fused MAT regions were used for phylogenetic analyses with likelihood, parsimony, and distance algorithms. To test for monophyly of *Pleospora*, five taxa of the family Pleosporaceae retrieved from GenBank were added. These were *Alternaria alternata* (AB009451), *Cochliobolus cymbopogonis* (AF129744), *C. heterostrophus* (AF029913), *C. ellisii* (AF129746) and *C. sativus* (AF275373). Only one representative per amino acid sequence was included. Thus, excluded from analyses were the following five taxa: *S. sedicola* strain P271 and *Stemphylium sp.* strain P303 (both identical to *P. herbarum* strain P238), *P. tomatonis* strain P268 (identical to *P. alfalfae* strain P81), *Pleospora sp.* strain P207 (identical to *P. eturmiuna* strain P269), *Stemphylium sp.* strain P316 (identical to *Stemphylium sp.* strain P245). The alignment used for analyses thus contained 31 taxa and 429 characters.

Three different algorithms were used for inference of phylogenetic protein trees.

The Fitch-Margoliash method yielded a distance tree. It is illustrated in Figure 3-8, with the five excluded taxa added, and thus comprising all 31 ingroup taxa.

Parsimony analyses yielded 50 most parsimonious trees of 1012 steps each. Bootstrap support values above 70% are shown in Figure 3-8 by the branches in first position or in Table 3-12 and Table 3-13. On a 70% bootstrap support level, the most parsimonious trees were identical to the distance tree (data not shown).

In likelihood analyses using quartet puzzling one tree was obtained. Support for branches above 70% are shown by the branches in second position in Figure 3-8 or in Table 3-12 and Table 3-13. On a 70% bootstrap support level, the quartet puzzling tree was congruent with the distance tree (data not shown).

3.3.1.9.1.2 *MAT1-2* protein analyses

All 23 *MAT1-2* protein sequences were used for phylogenetic protein analyses. Other *MAT1-2* sequences from GenBank were used from the same five taxa as in the *MAT1-1* dataset. These were *A. alternata* (AB009452), *C. cymbopogonis* (AF129745), *C. heterostrophus* (AF027687), *C. ellisii* (AF129747), *C. sativus* (AF275374). Excluded from analyses were *S. vesicarium* strain P238, *P. tomatonis* strain P268, *P. sedicola* strain P271 and *Stemphylium sp.* P303 (all identical to *P. alfalfae* strain P81). Thus, the dataset contained 24 taxa and 365 characters.

The same three algorithms as for *MAT1-1* were used for inference of phylogenetic *MAT1-2* protein trees.

In distance analyses, a Fitch-Margoliash distance tree was obtained. It is illustrated in Figure 3-9, with the four excluded taxa added and thus comprising all 23 *MAT1-2* protein sequences obtained.

Parsimony analyses yielded 50 most parsimonious trees of 754 steps each. Bootstrap support percentages above 70% are given by the branches in first position in Figure 3-9 or in Table 3-12 and Table 3-13. The strict consensus tree of the most parsimonious trees was compatible with the topology of the distance tree in Figure 3-9, on a 70% bootstrap support level (data not shown).

Likelihood analyses with quartet puzzling yielded one tree. Support for compatible branches above 70% are shown in second position in the distance tree of Figure 3-9 or in Table 3-12 and Table 3-13. The quartet puzzling tree was compatible with the distance tree in Figure 3-9, except that the phylogenetic species *P. eturmiuna* and *P. tarda* were monophyletic with 76% branch support (data not shown).

3.3.1.9.2 Idiomorph DNA sequence analyses

3.3.1.9.2.1 *MAT1-1* idiomorph DNA sequence analyses

These analyses based on DNA sequences were aimed at improving the branch support within *Pleospora*. Only *Pleospora* taxa were used. The *MAT1-1* DNA sequences included for analyses corresponded to the inverted part of the *MAT1-1* idiomorph in fused MAT regions, and homologous regions in separate MAT regions. Excluded were *P. sedicola* strain P271, *Stemphylium sp.* strain P303 (both identical to *S. vesicarium* strain P238) and *Stemphylium sp.* strain

P316 (identical to *Stemphylium sp.* strain P245). Thus, the dataset used for analyses contained 28 taxa and 1688 characters.

For phylogenetic analyses, both likelihood, parsimony, Bayesian analyses and Neighbor joining were used.

Likelihood analyses yielded one most likely tree (-ln likelihood = 8500.90814). It is illustrated in Figure 3-10, with the three excluded taxa re-added. Bootstrap branch support percentages were based on 217 replicates and are listed in Table 3-12 and Table 3-13.

In parsimony analyses, four most parsimonious trees were obtained of 1145 steps each (CI = 0.768, RI = 0.851). Parsimony bootstrap supports above 70% are listed in Table 3-12 and Table 3-13. The most parsimonious trees differed among each other as follows. The phylogenetic species *S. astragali* was either sister group to *P. eturmiuna* or *P. gigaspora* plus ingroup, or the branching order of the three groups was unresolved. These rearrangements were combined with *P. gigaspora* and *S. majusculum* being monophyletic, or *S. majusculum* plus ingroup being sister to *P. gigaspora.* On a 70% bootstrap support level, the most parsimonious trees were compatible with the most likely tree in Figure 3-10.

In Bayesian analyses, one consensus phylogram was obtained. Except for failing to resolve the poorly supported branching order of *P. triglochinicola* strain P123, *Stemphylium sp.* strain P210 and *P. paludiscirpi* strain P270, its topology was identical to the most likely tree in Figure 3-10. The posterior probabilities above 70% are shown in Table 3-12 and Table 3-13.

One Neighbor joining tree was obtained. Neighbor joining bootstrap support above 70%, and compatible with the most likely tree, are listed in Table 3-12 and Table 3-13. The Neighbor joining tree differed from the most likely tree in Figure 3-10 in that *P. gigaspora* and *S. majusculum* were monophyletic supported with 77% of the bootstrap replicates. *Stemphylium trifolii* was sister taxon to the phylogenetic species *Pleospora sp.* strain P107 plus ingroup with 82% bootstrap support (data not shown). Otherwise, on a 70% bootstrap support level, the Neighbor joining tree was compatible with the most likely tree in Figure 3-10 (data not shown).

3.3.1.9.2.2 *MAT1-2* idiomorph DNA analyses

As for *MAT1-1*, analyses were restricted to *Pleospora.* The region used corresponded to the *MAT1-2* idiomorph present in fused MAT regions, and homologous regions in separate MAT regions. Excluded taxa were *P. tomatonis* strain P268, *P. sedicola* strain P271 (both identical to *P. alfalfae* P81) and *Stemphylium sp.* strain P303 (identical to *S. vesicarium* strain P238). Thus, the dataset used for analyses contained 20 taxa and 1632 characters.

For phylogenetic analyses, both likelihood, parsimony, Bayesian analyses and Neighbor Joining were used.

Likelihood yielded one most likely tree (- ln likelihood = 6301.35211). It is illustrated in Figure 3-11 with the three excluded taxa re-added. Bootstrap support percentages were based on 363 bootstrap replicates, and are listed in Table 3-12 and Table 3-13 for the ones above 70%.

In parsimony analyses, two most parsimonious trees were obtained of 750 steps each (CI = 0.854, RI = 0.877). One tree was identical in topology to the most likely tree in Figure 3-11, the other differed in the position of the phylogenetic species *P. eturmiuna* which was sister group to *S. astragali* plus ingroup (data not shown). The bootstrap support values of more than 70% are listed in Table 3-12 and Table 3-13.

In Bayesian analyses, the topology of the consensus phylogram from 9000 trees was identical in topology to the most likely tree in Figure 3-11 (data not shown). The posterior probabilities above 70% are listed in Table 3-12 and Table 3-13.

One Neighbor joining tree was obtained. Bootstrap supports of more than 70% are listed in Table 3-12 and Table 3-13. The Neighbor joining tree differed from the most likely tree in Figure 3-11 by *P. eturmiuna* and *S. astragali* plus sister group being monophyletic supported by 82% of the bootstrap replicates, and other branches supported by less then 70% (data not shown).

3.4. Discussion

These studies of mating system evolution showed that the three groups of homothallics in *Pleospora* evolved in three different ways. One homothallic lineage evolved by fusion of the *MAT1-1* and *MAT1-2* loci, a second homothallic clade originated by horizontal transfer of the fused MAT locus, and a third origin resulted in a clade of homothallics with only a single detectable forward-pointing *MAT1-1* idiomorph. The evolution of homothallism by horizontal transfer as in *Pleospora* has never been reported before. All findings are discussed in detail below.

3.4.1 The mating type locus of *Pleospora*

There were three different types of idiomorphs present at the MAT locus in *Pleospora*. They contained either a *MAT1-1* or *MAT1-2* gene, or both *MAT1-1* and *MAT1-2* genes (Figure 3-4). The organization of the MAT locus in *Pleospora* was thus similar to other fungi in the class Dothideomycetes, such as *Cochliobolus*. However, it differed from all of them in the length of the idiomorphs and MAT genes, as well as the architecture of the fused MAT regions.

3.4.1.1 Mating type genes

The mating type genes in *Pleospora* were similar to other fungi in the class Dothideomycetes (Table 3-14). There were no major differences within homolo-

gous mating type genes from separate and fused MAT regions. All *MAT1-1* genes were 1193 bp long, and had an intron of 53 bp inserted at position 218. This was similar to other members of the Dothideomycetes, where the *MAT1-1* genes were between 995 – 1368 bp in length, with one intron of 45 – 55 bp (Table 3-14). *Mycosphaerella graminicola* was the only species with an additional intron (see Table 3-14).

All *MAT1-2* genes in *Pleospora* were 1093 bp in length and had one intron of 55 bp inserted at position 491 (Table 3-14). This fell within the range of *MAT1-2* genes of other fungi in the Dothideomycetes, where *MAT1-2* genes ranged from 1083 – 1255 bp in length, with an intron of 48 – 55 bp.

3.4.1.2 Idiomorph delimitation

Sexual reproduction in ascomycetes is controlled by the MAT locus, a master regulator of downstream gene expression (Turgeon *et al.*, 1993b). MAT loci consist of MAT genes and flanking regions. Since MAT alleles are too divergent to be aligned, they are not alleles in the true sense and are thus called idiomorphs instead (Metzenberg & Glass, 1990).

As in all other ascomycetes, the mating type genes in *Pleospora* were embedded in idiomorphs. The idiomorphs were well delimited upstream of the MAT genes, and poorly delimited downstream of the MAT genes (Figure 3-7). The 5'-limits of the idiomorphs were clearly recognizable by abrupt return to high levels of sequence identity, and lay within the ORF1 gene, 51 or 54 bp upstream of the ORF1 3'-end in *MAT1-2* or *MAT1-1*, respectively.

Idiomorphs in the class Dothideomycetes varied widely in their extent at the 5'-end (Figure 3-12). Idiomorphs reaching into the upstream gene were also present in *P. nodorum* and in *L. maculans*. In *P. nodorum*, the idiomorph covered about half of ORF1, whereas in *L. maculans*, the idiomorph extended well beyond ORF1, 130 bp into *GAP1*. It is noteworthy that the amino acid sequence of ORF1 was more similar than the DNA sequence between idiomorphs, with respectively 12% and 30% variable sites (data not shown). This might indicate an evolutionary constraint for function in ORF1.

In other species of the Dothideomycetes, the idiomorph did not reach the upstream gene (Figure 3-12). This was the case for *A. alternata* and *C. heterostrophus*. Whereas the idiomorph of *A. alternata* stopped just 4 bp short of ORF1, the idiomorph in *C. heterostrophus* barely extended beyond the mating types genes, with 47 and 37 bp upstream of *MAT1-1* and *MAT1-2*, respectively. This might be due to the isogenic nature of these lab strains obtained by repeated inbreeding (Leach *et al.*, 1982).

The idiomorph was poorly delimited downstream of the MAT genes in *Pleospora*. Thus, the 3' idiomorph end was chosen conservatively close to the MAT genes, in order not to introduce error in phylogenetic idiomorph analyses. The 3'-end of the idiomorphs was thus selected upstream of the first stretch

of DNA sequence similarity between *MAT1-1* and *MAT1-2*, 100 - 176 bp downstream of the MAT gene 3'-end, depending on the isolate (Figure 3-7). Downstream of the chosen 3' idiomorph end, the DNA sequence dissimilarity between *MAT1-1* and *MAT1-2* isolates of one phylogenetic species gradually disappeared. It might be possible to define an 'idiomorph transition zone' between the idiomorph proper, and the intergenic spacer beyond the influence of recombination suppression. However, sequencing coverage of the entire spacer would be necessary.

The idiomorph end downstream of the mating type genes was also poorly delimited in other taxa of the Dothideomycetes. In *P. nodorum*, the idiomorph was defined as the region containing less then ca. 90% similar sequences between *MAT1-1* and *MAT1-2* (Bennett *et al.*, 2003). Using the program SWAN (Proutski & Holmes, 1998) as Bennett et al. (2003) did for *P. nodorum*, I found other poorly defined 3'-idiomorph boundaries in *A. alternata* and *L. maculans* (data not shown).

3.4.1.3 Conservation of gene order in the MAT locus

The genes flanking the mating type region in *Pleospora* were homologous with the ones in equivalent position in *Cochliobolus heterostrophus* (Turgeon *et al.*, 1993a) (Figure 3-12). A gene of unknown function, ORF1, and a GTPase activating protein homolog, *GAP1*, were upstream, and a beta glucosidase homolog *BGL1* was downstream. The three genes were not sequenced in all *Pleospora* isolates, but were only determined to be adjacent to the separate *MAT1-1* region in *Pleospora sp.* strain P56. However, the presence of *GAP1* upstream of ORF1 was confirmed in the fused MAT region of *P. tarda* strain P1, and *BGL1* downstream of the separate *MAT1-2* region in *S. xanthosomatis* strain P232 (Figure 3-3). The mating type region sequences of the remaining isolates were shorter, extending from 21 – 320 bp inside ORF1, to 322 – 2373 bp downstream of the 3'-end of the MAT genes. However, all DNA sequences were readily alignable beyond the idiomorphs, so that it is likely that all had flanking genes identical to *Pleospora sp.* strain P56 as shown in Figure 3-3.

Like species of *Pleospora*, *Alternaria alternata* and *Phaeosphaeria nodorum* had an ORF1 upstream of the MAT genes. However, the downstream genes in these two fungi are unknown (Arie *et al.*, 2000; Bennett *et al.*, 2003). Other members of the Dothideomycetes had different flanking genes (Figure 3-12). In *Leptosphaeria maculans*, downstream of the mating type genes was a DNA lyase, whereas the upstream gene was ORF1, as in *Pleospora* (Cozijnsen & Howlett, 2003). As opposed to *L. maculans*, in *Mycosphaerella graminicola* the upstream gene was a DNA lyase, so that in this fungus the MAT genes might have been inverted as compared to *L. maculans* (Waalwijk *et al.*, 2002). The MAT downstream gene in *M. graminicola* is unknown.

3.4.2 Architecture of the fused MAT regions

The fused MAT regions in *Pleospora* were unique, containing both *MAT1-1* and *MAT1-2* genes rearranged in the following way. In all cases, the sequence containing the *MAT1-1* gene plus flanking regions was inverted, and fused to a *MAT1-2* idiomorph, upstream of the *MAT1-2* gene (Figure 3-4). The idiomorph in selfers was thus delimited by the 5' and 3' ends of *MAT1-1* and *MAT1-2* idiomorphs, respectively (Figure 3-7). The 5' *MAT1-2* idiomorph end that was situated in a non-coding region was not present, since it was truncated by the fusion with the inverted *MAT1-1* idiomorph. The 3' *MAT1-1* idiomorph end was present, however, between ORF1 and *MAT1-1*, since the inverted *MAT1-1* region extended beyond the 3' idiomorph end. Thus, between ORF1 and the inverted *MAT1-1* gene, the idiomorph in selfers was interrupted on a stretch of 108 – 114 bp, depending on the isolate (Figure 3-7).

Fused MAT regions were also known in *Cochliobolus*. However, as opposed to *Pleospora*, the MAT genes in *Cochliobolus* were arranged in different ways (Yun *et al.*, 1999). For example, in both *C. luttrellii* and *C. homomorphus*, the *MAT1-1* and *MAT1-2* were fused, but in different order. In *C. kusanoi*, the arrangement was complex with *MAT1-1* being fragmented, and in *C. cymbopogonis*, both MAT regions were present in one genome, but not fused (Yun *et al.*, 1999).

3.4.3 A hypothesis for the evolution of the fused MAT regions

The fused MAT regions of *Pleospora* may have evolved by a gene inversion creating a short stretch of DNA sequence identity between *MAT1-1* and *MAT1-2*, allowing a crossover to occur (Figure 3-6).

In this hypothesis, the gene inversion took place in an ancestor and affected *MAT1-1* and flanking regions. Since the entire *MAT1-1* was inverted, the gene remained functional, and the ancestor retained the ability to outcross. During sexual reproduction, at meiosis an unequal crossover to a forward oriented *MAT1-2* region resulted in a fused MAT region passed on to progeny. The occurrence of a crossover is supported by a shared, 4 bp motive located between the fused *MAT1-1* and *MAT1-2* idiomorphs, and at homologous position in some *MAT1-1* and *MAT1-2* genes from separate MAT regions (Figure 3-5). The motive is only 4 nucleotides in length. However, in *S. cerevisiae*, four nucleotides similarity is sufficient for a crossover to occur (Schiestl & Petes, 1991). *Pleospora* isolates with separate *MAT1-1* regions carrying an inverted *MAT1-1* gene would support this evolutionary hypothesis, but none are known. A crossover is also thought to be responsible for fused MAT arrangements found in species of *Cochliobolus* (Yun *et al.*, 1999).

3.4.4 Phylogenetic information content of the MAT regions

As suspected by Turgeon & Berbee (1998), the mating type region was more variable than other regions, such as ITS or *GPD*. In this study, the idiomorph portion downstream of the *MAT1-1* gene contained about 17 times more parsimony informative characters on a percent basis than the ITS region, or almost 1.8 times as many as the *vmaA-vpsA* intergenic spacer region (Table 2-13, Table 3-11). However, the resolving power of this *MAT1-1* idiomorph region might not be superior to *vmaA-vpsA*, as the alignment comprised only 174 characters. Another drawback of using idiomorphs for phylogenetics is that separate analyses for each mating type are necessary.

3.4.5 Incongruence between MAT phylogeny and species phylogeny

All mating type phylogenies disagreed with the species phylogeny, but were congruent among each other for relevant nodes. Whereas in the species phylogeny fused MAT regions were found in separate groups (Figure 3-1), in the MAT phylogenies they were monophyletic (Figure 3-8 - Figure 3-11). All other changes with regard to the species phylogeny were either minor, or concerned poorly supported branches in the species phylogeny (Table 3-12, Table 3-13). Thus, the topologies of the mating type phylogenies were essentially congruent to a species phylogeny with all fused MAT genes constrained to be monophyletic within group E. Entirely congruent MAT and species phylogenies were found in *Fusarium* (O'Donnell *et al.*, 2004).

The rooted protein phylogenies both showed *Pleospora* to be monophyletic with high bootstrap support in all analyses. This agreed with results by Câmara et al. based on *ITS*, *GPD* and *EF-1 alpha* datasets (2002). In the outgroup, the genus *Cochliobolus* was monophyletic agreeing with Berbee et al. (1999), and *Alternaria alternata* was sister taxon to *Pleospora*. However, it was not clear if *Alternaria* was closer related to *Pleospora* than *Cochliobolus*, due to the exclusion from analyses of other close relatives, such as *Setosphaeria* and *Pyrenophora* (Berbee *et al.*, 1999). Thus, the closest relative of *Pleospora* was still uncertain.

3.4.6 MAT gene arrangements in phylogenetic species

Mating type gene arrangements correlated with phylogenetic species. Each of the 22 phylogenetic species either had only one MAT gene at the mating type locus, or the fused MAT gene arrangement instead (Figure 3-1).

3.4.7 Mating system in *Pleospora* species with fused MAT regions

In *Cochliobolus*, as *Pleospora* in the *Pleosporaceae*, species with both MAT regions combined into one genome are homothallic (Yun *et al.*, 1999).

In *Pleospora*, nine of the 11 phylogenetic species with fused MAT regions were homothallic (Table 3-15). The remaining two species, *Pleospora sp.*

strain P327 and *P. triglochinicola* did not form the sexual state in culture, but are known to form the sexual state in their natural habitats (Table 3-15).

Fruiting can be difficult to induce under laboratory conditions. Substrate type, temperature, light exposure and humidity all can play a role (Schmiedeknecht, 1962). Fungal strains can also lose their ability to reproduce sexually after frequent subculturing. Thus under appropriate conditions, *Pleospora sp.* strain P327 and *P. triglochinicola* might form the sexual states in the laboratory, as would be expected based on their MAT locus shared with species that do fruit in culture.

Homothallic ascomycetes are not restricted to selfing, but in some cases can also outcross, as has been shown in the filamentous ascomycete *Gibberella zeae* (Bowden & Leslie, 1999). Just as in homothallic species of *Pleospora*, the MAT locus of *G. zeae* contains all MAT genes found in heterothallics (Yun *et al.*, 2000). Is there evidence that homothallic species in *Pleospora* do outcross? The evidence for outcrossing is based on the single *EF-1 alpha* and *vmaA-vpsA* gene genealogies obtained in the last chapter (Figure 2-5). In clonal species, all genes are expected to give identical topologies, since all are effectively linked. In outcrossing species, the topologies from different genes vary within species due to recombination (Burt *et al.*, 1996). The topologies within group C of the *EF-1 alpha* and *vpsA-vmaA* trees obtained in Figure 2-5 are incongruent. In *EF-1 alpha*, *Stemphylium sp.* strain P235 has the same allele as *Stemphylium sp.* strain P277, whereas in *vmaA-vpsA*, *Stemphylium sp.* strain P235 is most similar to *P. alfalfae* strain P81, sharing one apomorphy. However, this is not conclusive proof, since the synapomorphy might instead be a homoplasy. To assess the contribution of outcrossing to the population structure of the homothallic species of *Pleospora*, population genetic studies are necessary (Milgroom, 1996; Zhan *et al.*, 2002).

3.4.8 Mating system in *Pleospora* species with separate MAT regions

Species of *Cochliobolus* with separate MAT regions are all heterothallic (Yun *et al.*, 1999). In *Pleospora*, unlike *Cochliobolus*, species with separate MAT regions are either heterothallic or homothallic. Homothallism in *Pleospora* is also possible in the presence of only one MAT gene, as in the filamentous ascomycete *Neurospora africana* (Glass *et al.*, 1988).

The *Pleospora* homothallics with separate MAT regions are the phylogenetic species *Pleospora sp.* strains P56 and P107 (Table 3-15). In both of them, only *MAT1-1* regions have been detected.

Assuming a randomly recombining population, mating type genes are expected to occur in equal proportions (Milgroom, 1996). But all seven isolates derived from sexual spores of the phylogenetic species *Pleospora sp.* strain P56 and *Pleospora sp.* strain P107 included in phylogenetic analyses had *MAT1-1* only. From eight additional single ascospore isolates from the phylo-

genetic species *Pleospora sp.* strain P56, only *MAT1-1* was found. Thus, a total of 15 isolates may contain only *MAT1-1*. Assuming that *MAT1-1* and *MAT1-2* genes segregated in equal proportions in the sexual fruitbodies, randomly selecting 15 ascospores with *MAT1-1* in a row would be expected to occur once in 32,768 attempts, a rare event ($P < 0.0001$). Alternatively, it would be possible that *MAT1-2* was present in the genome, but could not be detected by PCR. We know that *MAT1-2* was not close to the *MAT1-1* gene in *Pleospora sp.* strain P56. DNA sequence analysis of more than 2kpb upstream and 4.5 kpb downstream of the *MAT1-1* gene showed that *MAT1-2* was absent. Alternatively, *MAT1-2* could be located elsewhere in the genome, as in the homothallics *C. cymbopogonis* (Yun *et al.*, 1999) and *Aspergillus nidulans* (Dyer *et al.*, 2003).

The mating systems in the phylogenetic species *Pleospora sp.* strains P56 and P107 might be similar to the one in *N. africana*, as they all seem to lack *MAT1-2*. In *N. africana* only *MAT A*, the equivalent to *MAT1-1* in *Pleospora*, has been found (Glass *et al.*, 1988). Further studies are needed to investigate potential similarities between the mating systems of *Pleospora sp.* strains P56 and P107, and *N. africana*.

For the remaining *Pleospora* species with separate MAT regions, no sexual states have been reported, nor obtained in this study. However, there is evidence that they might reproduce sexually, and be heterothallics. All five phylogenetic species comprising more than one strain had both *MAT1-1* and *MAT1-2* isolates, which is expected in outcrossing species (Milgroom, 1996). Additional evidence for outcrossing was found in the phylogenetic species *S. solani*, where three of the four possible allele combinations between *GPD* and *EF1-alpha* were found, resulting in incongruent phylogenies (Figure 2-5). More research is needed to find the sexual states and confirm heterothallism in species where *MAT1-1* and *MAT1-2* idiomorphs are present in different isolates.

3.4.9 Mating system evolution in *Pleospora*

The *Pleospora* species phylogeny shows four groups of homothallics (Figure 3-1), suggesting four possible independent origins of homothallics from heterothallics. However, the MAT idiomorph phylogenies only show one or two groups of homothallics (Figure 3-10, Figure 3-11). So how many times did homothallism evolve in *Pleospora*?

In the closely related *Cochliobolus*, as in *Pleospora*, homothallics are polyphyletic (Yun *et al.*, 1999). Homothallism in *Cochliobolus* evolved independently multiple times from heterothallic ancestors through rearrangements at the master regulator locus of sexual development, the MAT locus. Combination of both *MAT1-1* and *MAT1-2* idiomorphs into one genome repeatedly led to homothallism in *Cochliobolus* (Yun *et al.*, 1999). In homothallics of *Pleospora*, there are two different types of MAT loci suggesting two independent

origins of homothallism from heterothallism (Figure 3-4). How can this be reconciled with the four groups of homothallics in the *Pleospora* species phylogeny (Figure 3-1) and the one or two groups of homothallics in the MAT idiomorph phylogenies (Figure 3-10, Figure 3-11)?

3.4.9.1 Single origin of homothallics with a separate *MAT1-1* region

Species and MAT phylogenies agree on the origin of the homothallics with a single, forward-oriented *MAT1-1* gene in group SP. Group SP is well-supported in the *Pleospora* species phylogeny (Figure 3-1), as well as in the *MAT1-1* idiomorph phylogeny (Figure 3-10). This is consistent with a single origin of the homothallics in group SP from heterothallic ancestors.

3.4.9.2 Horizontal transfer of the fused MAT region

The species phylogeny recognizes three groups of homothallics with fused MAT regions (Figure 3-1), whereas the MAT phylogenies only recognize one group of homothallics with fused MAT regions (Figure 3-10, Figure 3-11). What are the reasons for this topological conflict, and what are its implications on the evolution of homothallism in *Pleospora*?

The *Pleospora* species phylogeny shows homothallics with the fused MAT regions in groups B, C and TP (Figure 3-1). There is no support for independent origin of groups B and C. However, the closest relatives of group TP are isolates with separate MAT regions, and not groups B and C, so that the fused MAT regions appear to have evolved twice.

However, Avise (1994) lists seven explanations for the origin of discontinuous distributions of character states, such as the fused MAT regions in *Pleospora*. Not discussed in detail are the three possibilities of mistaken species phylogeny, extreme rate heterogeneity of loci used for phylogenetic analyses, and the use of paralogous loci for phylogenetic inference (Avise, 1994). There was no evidence in the data for extreme differences in substitution rates between isolates, nor were there any paralogous gene copies detected. This was supported by separate analysis of the four datasets used for *Pleospora* species phylogenies, which resulted in congruent trees (Figure 2-5). Also, phylogenetic species were defined based on phylogenetic analyses, and not a priori assumptions.

Following Avise (1994), this leaves four possibilities to account for the discontinuous distribution of the fused MAT regions in *Pleospora* which are convergent evolution, retention of ancestral polymorphisms and horizontal transfer by asexual or sexual means.

3.4.9.2.1 Convergent evolution

Convergent evolution is the independent, multiple origin of a character state. With regard to *Pleospora*, this implies that the fused MAT regions originated at

least twice: In an ancestor of groups B and C, and in an ancestor of the TP group (Figure 3-1). However, this scenario is not compatible with the MAT gene phylogenies. Both *MAT1-1* and *MAT1-2* analyses show that a switch between fused and separate MAT regions occurred only once (Figure 3-10, Figure 3-11). This is supported by the hypothesis proposed for the evolution of the fused MAT region (Figure 3-6). The inversion of the *MAT1-1* gene, and fusion to a *MAT1-2* region by means of a crossover is unlikely to occur more than once at exactly the same sites (Figure 3-5). As to the direction of evolution, the fused MAT regions most likely is derived from ancestors with separate MAT regions, because the opposite, the break up and distribution of fused MAT regions into separate individuals, together with the reconstitution of a forward orientated MAT region, lacks any credible mechanisms. It would also contradict the phylogenetic protein analyses, where the fused MAT genes are derived from separate MAT regions (Figure 3-8, Figure 3-9). Thus, convergent evolution does not seem likely, and alternative possibilities requiring only one switch from separate to fused MAT regions have to be considered.

3.4.9.2.2 Retention of an ancestral character state

Retention of an ancestral state implies a single origin of a character state that subsequently is retained in different lineages, resulting in a polyphyletic distribution pattern. For *Pleospora* this implies that the fused MAT regions evolved only once, and were driven to fixation in the lineages B, C and TP (Figure 3-1). Consequentially, in the lineages leading to these groups, both separate and fused MAT regions would have coexisted in recombining populations. Homothallics with fused MAT regions can recombine (Bowden & Leslie, 1999), but it is unknown if that included frequent outcrossing to isolates with separate MAT regions.

In any case, the data do not suggest retention of the fused MAT regions. Had it occurred, then the fused MAT regions would have evolved before the divergence of the lineages leading to groups B, C and E, thus at the base of the species phylogeny (Figure 3-1). In the phylogenetic MAT protein analyses, this early evolution would have resulted in a placement of the root half way between the separate and fused MAT regions. However, this is not the case. The root in the MAT protein trees was placed at a branch within the separate MAT regions (Figure 3-8, Figure 3-9). Furthermore, the maximal genetic distances within the fused and separate MAT region clades would be similar in case of a single evolution and retention of the fused MAT regions. However, the maximal genetic distance in the separate MAT region clade, for example between *S. callistephi* strain P383 and *S. trifolii* strain P244, was much larger than the maximal distance in the separate MAT region clade, for example between *P. paludiscirpi* strain P270 and *P. herbarum* strain P2 (Figure 3-10, Figure 3-11). Together with the evidence from the root placement in

the MAT trees, this indicates that the fused MAT regions were more likely derived from separate MAT regions after the divergence of the lineages B, C and TP in Figure 3-1.

3.4.9.2.3 Sexual or asexual horizontal transfer

The third and forth options considered to account for the discontinuous distribution of the fused MAT regions are horizontal transfer by sexual or asexual means. Hybridization, sexual recombination between lineages, resulting in transfer of genes, is known to occur in fungi occasionally, for example in *Fusarium* (O'Donnell *et al.*, 2000). However, if hybridization occurred in *Pleospora*, the hybrid progeny would be expected to receive half the genetic information from either parent. In phylogenetic analyses done separately for all genes, the hybrids would be expected to group with either parent approximately the same number of times. However, all genes sampled for the *Pleospora* species phylogeny were congruent for groups B, C and TP (Figure 2-5). This might be due to the small number of genes sampled, or a linkage between the genes. Alternatively, the particular chromosome combination of the hybrids might have been favorable to hybrid survival (Greig *et al.*, 2002).

Equal parental chromosome contribution would also be expected for 'parasexual hybridization'. Parasexuality is the random elimination of supernumerary chromosomes following nuclear fusion, leading to a return to the haploid state (Caten, 1981). However, parasexuality is not known to occur naturally.

Asexual transfer between lineages of a fragment of DNA containing the fused MAT regions is the last option. A fragment of at least 11 kb has been transferred between a bacterial endosymbiont and an insect host (Kondo *et al.*, 2002). The fused MAT region in *Pleospora* measured less than 10 kb and was thus not unreasonably long to be transferred asexually.

3.4.9.3 Three different origins of homothallics in *Pleospora*

To conclude, topological conflict between species and MAT phylogenies, as well as MAT region arrangements lead to the conclusion that homothallism in *Pleospora* evolved independently three times from heterothallic ancestors. Through unknown genetic alterations in ancestors of the group where only a forward-oriented *MAT1-1* was detected, through rearrangements at the MAT locus resulting in the fused MAT regions, and by horizontal transfer across lineages of the fused MAT region by sexual or asexual means.

Table 3-1. All *MAT1-1* region forward primers used. Primer names are given, together with the position of the 5'-end on the sequence of *Pleospora sp.* strain P56 (AY339851).

Name	5'-end	sequence 5'-> 3'
GAPf	-49	GTC ACC CGT CTT CAC ACG G
GAPF2	625	GTG GGG CGT TCA TTG CGG
ORF556f	1691	GTG GTC AAG GTC CGT GAC GG
ORF589f	1724	TGG AAG GGC CAG GTG CGA G
MAT1r	2231	AAT GCA CTG ACG CTC TCG CC
BPHO4d	2648	TGC BTT YGT YGG ATT YCG STG TAA G
Jen1F	2665	GCT GTA AGT CTA ATT CTT ACT GC
Alpha181f	2749	GCA ATG GCC CAT GAA GAA GC
Alpha191f	2767	GCT CTC CAA CCT CAT TGG GC
P2F3	3362	CRT TRC TCG ACC ACT GCT CC
P2F2	3399	CCA ATC CGT TCT ACG ACG G
Jen1Fa	3593	GCC AAC GAA GAT GTT ACT CTT CC
MP56f	4333	CTG CAG TTG GAA GGA TTT GG
M245f	4968	CCA CTT CTC CAT TGA CAG CC
MP56F2	5161	CGG TGT TGG TTT CTT GGC G
M245F1	5170	TGA CTT GGC GGA TGT CTT GC
MP56F3	5811	CTC CAT GGA GCG GTA CCA C
M245F2	ca. 5966	TTG CAT CAG ACC TAC ACG CC
Mat1	6296	CGC GAC ACC CGA ACA CGG C
Mat1af	6358	TTG ATT CCG CTG CGT CGC TG
Mat1F1	6398	GAC ACT AGC GGC GGT ATG GC
56f	7149	AGA TCA TAG GTC CAC TGA GC
56F2	7875	TTG CCT TGA CTA GCG CAT CC

Table 3-2. All *MAT1-1* region reverse primers used. Primer names are given, together with the position of the 5'-end on the sequence of *Pleospora sp.* strain P56 (AY339851).

Name	5'-end	sequence 5'-> 3'
Br	+44	CCG TCT TGC AAA CAG AGC TG
BGLr	7943	ACC CCG CAA CAC CAA GCC
BGLR2	7481	TGG TGT AGA TTG CGA TGG CG
BGLR3	6855	GAT CST TCT GCT TGC AGA CC
MP56r	5795	CAC TGA CTC ATT TGG TAC TGC
P245R2900	5376	TCA GTA TTC CAC CAC CAG AG
S3r	5364	TTA TCC CTT GCA GAA GAT AGG
MP56R2	5180	TCG CCA AGA AAC CAA CAC CG
S2R10	4816	TCG GTC ATR TGC GAC RCR AGC TG
Sr	4805	CGA CAT GAA CTG CAT CCA GCC
S2RN	4802	CAC RAG CTG CAT CCA GTC
Sr2	ca. 4794	GCG TCC AGT CCA GCT CGC TC
M245R1	4431	GCC AAG TCA CGA CCA TCC
MP56R3	4415	AAG CGC AGG TTA AGA GCA GG
Lr	4205	TAG CGT AGA TAG TAG TAG TCC
M247R1	3850	CGA GCC GAT CAA GTC GTG G
MP56r171	3806	CCA CGT CGA ACA TCG AGA GG
P2R	3615	GGA AGA GTA ACA TMT TCG TTG
P2R2	3614	GGA GAG TAA CAT ATT CAT TGG
M245R2	3590	CGA GAC GGA AAG CCT CAT GG
MP56R4	ca. 3222	TGT ACT ACC GAG GCA GAA CC
Jen1r	2871	TCC TTG CCG ATT TGA TCA CG
Jen1Ri	2865	CCG ATT TGA TCA CGA ATA GTC G
M1r	2250	GGC GAG AGC GTC AGT GCA TTC
Mat1f	2246	AGA GCG TCA GTG CAT TCA CC
cORF589f	1742	CTC GCA CCT GGC CCT TCC A
cORF556f	1710	CCG TCA CGG ACC TTG ACC AC
cORF413f	1567	GGA AGT CGA GTA CAT RTC TTC TCC A
ORFr	796	GCA GGT GAT GCT TTG GGC G

Table 3-3. All *MAT1-2* region forward primers used. Primer names are given, together with the position of the 5'-end on the sequence of *S. solani strain P232* (AY340940).

Name	5'-end	sequence 5'-> 3'
ORF556f	214	GTG GTC AAG GTC CGT GAC GG
P232F1015	647	CTG GTA CAT GAG CAC AYC TCG
P232F1681	1318	CTS TTC GTT GCA CAT ACA CC
Jen2F	1766	TGT TGG ATC ATC TTC CGA G
HMG85f	1782	GAG ATG TGA TGC ACA AGC AGC
HMG97f	1795	CAA GCA GCT CAA RGC TGA GC
2nf	2229	TCA ACG AGC TTT CAT GAG G
2F	2352	ACA TTC GCC TTG TTC AAT GAC G
P232F2844	2481	AAC GYT CCC GAA GCA TGT TC
P232f	3133	TCG CCA CCG ATG CCA CC
P232F2	3881	GAT TGA AAT GCT CAA TTC ACG G
Mat2f	4580	GTA CCT CAT TGG CGT TGT GC
Mat2F1	4593	GTT GTG CTT ACC ATG CCA CG
232f	5301	TGC CCC GAT GAC GCC ACG
232F2	6032	CAC AAC CGA GCC CGG TAG C
232F3	6803	CGC CCG TTC TGA GGG TTC GG

Table 3-4. All *MAT1-2* region reverse primers used. Primer names are given, together with the position of the 5'-end on the sequence of *S. solani strain P232* (AY340940).

Name	5'-end	sequence 5'-> 3'
Br	+62	CCG TCT TGC AAA CAG AGC TG
BGLr	6758	ACC CCG CAA CAC CAA GCC
BGLR2	6272	TGG TGT AGA TTG CGA TGG CG
BGLR3	5643	GAT CST TCT GCT TGC AGA CC
P232R4600	4074	TAT CAA TGC GCC GTC TCC GC
S3r	3429	TTA TCC CTT GCA GAA GAT AGG
P232R1	3356	GAT AAG GCG CCC CGT CGC
M2R	2950	TTG CAG ATG GAC CTT GCA GTG
P232R2b	2563	GCG AAY GAG TGA CGT TCG TG
2nr	2485	ACG TTC GTG AAT ACG AGG
2r	2373	CGT CAT TGA ACA AGG CGA ATG T
Jen2R	1990	CGA AGG TGC TCT TCT TTT GC
HMG97r	1832	GGA CTG TGA GAT TGG GGA GC
P232R1662	1337	GGT GTA TGT GCA ACG AAS AG
SMr	1266	GAA GAG AGA AGT GAG ATG ATT C
383r	1266	TAA GAG AGA AGT GAG ACG ATT G
P232R995	667	CGA GRT GTG CTC ATG TAC CAG

Table 3-5. All *MAT1-1*; *MAT1-2* region forward primers used. Primer names are given, together with the position of the 5'-end on the sequence of *P. tarda* strain P1 (AY335164).

Name	5'-end	sequence 5'-> 3'
GAPf	-362	GTC ACC CGT CTT CAC ACG G
GAPF2	243	GTG GGG CGT TCA TTG CGG
GAPF3	978	TTC ACC AGC CCG TCC AGC
ORF413f	1161	TGG AGA AGA YAT GTA CTC GAC TTC C
ORF556f	1309	GTG GTC AAG GTC CGT GAC GG
CHO13	1333	ATT GCA GAT TGG AAA GGC CAA GT[1]
MAT1825f	1435	GGA TTC GAC CGG TGC AAT GG
P1f	2112	GTC AAT CAG GTT CGA CAT GC
Jen1r	2812	TCC TTG CCG ATT TGA TCA CG
Jen1Ri	2818	CCG ATT TGA TCA CGA ATA GTC G
Mat1f	3441	AGA GCG TCA GTG CAT TCA CC
Mat1Af	3853	TCA AGA CAA CAT CCC ACA GC
Jen2f	4124	TGT TGG ATC ATC TTC CGA G
HMG85f	4140	GAG ATG TGA TGC ACA AGC AGC
HMG97f	4153	CAA GCA GCT CAA RGC TGA GC
2f	4710	ACA TTC GCC TTG TTC AAT GAC G
P1F2914	4908	CAT TAC TTA TTT GCG CAC GCC
P1Endf	5546	AAC GTC GCT GTT TGC TCC

[1]From Berbee et al. (2003)

Table 3-6. All *MAT1-1*; *MAT1-2* region reverse primers used. Primer names are given, together with the position of the 5'-end on the sequence of *P. tarda* strain P1 (AY335164).

Name	5'-end	sequence 5'-> 3'
S3r	5780	TTA TCC CTT GCA GAA GAT AGG
Mat4707r	5438	TAC AGA GCG TTT GAC CTC GG
M2r	5308	TTG CAG ATG GAC CTT GCA GTG
P1R2	4786	AAG AAC GCT GGC AGA GTT GC
2r	4731	CGT CAT TGA ACA AGG CGA ATG T
Jen2r	4348	CGA AGG TGC TCT TCT TTT GC
Mat1Ar	3869	GTG GGA TGT TGT CTT GAA GG
Mat1r	3456	AAT GCA CTG ACG CTC TCG CC
Jen1F	3018	GCT GTA AGT CTA ATT CTT ACT GC
Alpha181f	2934	GCA ATG GCC CAT GAA GAA GC
Alpha191f	2916	GCT CTC CAA CCT CAT TGG GC
Mat2642r	2265	ACT CAC TAA CCT CTT ATC CGG
P1r	2130	CAT GTC GAA CCT GAT TGA CC
Jen1Fa	2090	GCC AAC GAA GAT GTT ACT CTT CC
cORF589f	1360	CTC GCA CCT GGC CCT TCC A
cORF556f	1328	CCG TCA CGG ACC TTG ACC AC
cORF413f	1185	GGA AGT CGA GTA CAT RTC TTC TCC A
ORFr	414	GCA GGT GAT GCT TTG GGC G

Table 3-7. *MAT1-1* region primers and PCR conditions used. Primer pairs used in initial PCR for respective strains are given in column 'PCR 1', with reamplification primers under 'PCR 2'. PCR conditions employed are in columns 'Cond. 1' and 'Cond. 2', referring to PCR1 and PCR2, respectively. For standard PCR, conditions are annealing temperature/number of cycles. Chromosome walking PCR conditions are marked by 'V', followed by '1' and restriction enzyme for primary PCR, and '2' followed by approximate size of the resulting PCR product for secondary PCR. Respective forward and reverse sequencing primers are given in the last two columns. For complete PCR and sequencing conditions see text.

Strain	PCR 1	Cond. 1	PCR 2	Cond. 2	Sequ. Forward	Sequ. Reverse
P56	cORF589f, Vec	V1 EcoRI	cORF556f, VecN	V2/5K	GAPf, GAPF2	cORF413f, ORFr
P56	Jen1r, Vec	V1 BamHI	Jen1Ri, VecN	V2/1.8K	VecS, MAT1r	Jen1Ri, MAT1f
P56	Alpha181f, MP56r171	48/40	Alpha191f, MP56r171	V2	Alpha191f,	MP56R4
P56	Alpha181f, Vec	V1 EcoRI	Jen1Fa, VecN	V2/2.8K	Jen1Fa, MP56f, MP56F2, MP56F3	VecN, MP56r, MP56R2, MP56R3
P56	Mat1, Vec	V1 ClaI	Mat1af, VecN	V2/3.4K	Mat1F1, 56f, 56F2	Br, BGLr, BGLR2, BGLR3
P107	ORF556f, Jen1r	52/30	-	-	ORF589f, MAT1r	Jen1Ri, M1r
P107	Alpha181f, S2RN	60/40	Alpha191f, S2RN	60/40	Alpha191f, P2F2	S2RN, Lr, P2R
P229	Jen1R, Vec	V1 BamHI	Jen1Ri, VecN	V2/1.4K	VecN, MAT1r	Jen1Ri, MAT1f
P229	Alpha181f, S2R10	60/30	-	-	Alpha191f, P2F2	S2R10, MP56r171, P2R
P229	Alpha181f, Vec	V1 BamHI	Jen1Fa, VecN	V2/1.4K	Jen1Fa	VecN
P239	ORF556f, Jen1r	52/30	-	-	ORF556f, MAT1r	Jen1Ri, M1r
P239	Alpha181f, S2R10	60/30	-	-	Alpha191f, P2F3	S2R10, P2R
P240	ORF556f, Jen1r	52/30	-	-	ORF556f, MAT1r	Jen1R, M1r
P240	Alpha181f, S2R10	60/30	-	-	Alpha191f, P2F3	Sr2, P2R
P244	ORF556f, Jen1r	52/30	-	-	ORF589f, MAT1r	Jen1Ri, M1r
P244	Alpha181f, S2R10	55/30	-	-	Alpha181f, P2F3	S2R10, P2R
P245	ORF556f, Jen1r	52/40	-	-	ORF556f, MAT1r	Jen1Ri, MAT1f
P245	Alpha181f, Vec	V1 EcoRI	Alpha191f, VecN	V2/3.3K	Alpha191f, Jen1Fa, M245f, M245F1, M245F2	VecN, P245R2900, M245R1, M245R2
P246	ORF556f, Jen1r	52/40	-	-	ORF556f, MAT1r	Jen1Ri, M1r
P246	Alpha181f, S3r	55/40	-	-	Alpha191f, P2F3	Sr, P2R2

P247	ORF556f, Jen1r	52/30	-	-	ORF556f, MAT1r	Jen1Ri, M1r
P247	Alpha181f, Vec	V1 EcoRI	Alpha191f, VecN	V2/1.8K	Alpha191f, Jen1Fa	VecN, M247R1
P253	ORF556f, Jen1r	52/40	-	-	ORF589f, MAT1r	Jen1Ri, MAT1f
P253	Alpha181f, S2R10	60/30	-	-	Alpha191f, P2F3	S2R10, P2R
P316	ORF556f, Jen1r	52/40	-	-	ORF556f, MAT1r	Jen1Ri, M1r
P316	Alpha181f, S2R10	60/30	-	-	Alpha191f, P2F2	S2R10, P2R
P342	ORF556f, Jen1r	52/30	-	-	ORF589f, MAT1r	Jen1Ri, M1r
P342	Alpha181f, S2RN	60/40	Alpha191f, S2RN	60/40	Alpha191f, P2F2	S2RN, Lr, P2R
P383	ORF556f, Jen1r	52/40	ORF556f, Jen1Ri	52/40	ORF589f, MAT1r	Jen1Ri, MAT1f
P383	Alpha181f, S2R10	55/30	Alpha181f, S2R10	55/30	Alpha191f, P2F3	S2R10, P2R
P384	ORF556f, Jen1r	52/30	-	-	ORF589f, MAT1r	Jen1Ri, M1r
P384	Alpha181f, S2R10	55/40	Alpha181f, S2R10	55/30	Alpha191f, P2F3	S2R10, P2R

Table 3-8. *MAT1-2* region primers and PCR conditions used. Primer pairs used in initial PCR for respective strains are given in column 'PCR 1', with reamplification primers under 'PCR 2'. PCR conditions employed are in columns 'Cond. 1' and 'Cond. 2', referring to PCR1 and PCR2, respectively. For standard PCR, conditions are annealing temperature/number of cycles. Chromosome walking PCR conditions are marked by 'V', followed by '1' and restriction enzyme for primary PCR, and '2' followed by approximate size of the resulting PCR product for secondary PCR. Respective forward and reverse sequencing primers are given in the last two columns. For complete PCR and sequencing conditions see text.

Strain	PCR 1	Cond. 1	PCR 2	Cond. 2	Sequ. Forward	Sequ. Reverse
P232	Jen2R, Vec	V1 BamHI	HMG97r, VecN	V2/2K	VecN, P232F1015, P232F1681	HMG97r, P232R1662, P232R995
P232	HMG85f, Vec	V1 BamHI	HMG97f, VecN	V2/2.6K	HMG97f, P232F2844, P232f, P232F2	VecN, P232R4600, P232R1, P232R2b
P232	Mat2f, Vec	V1 ClaI	Mat2F1, VecN	V2/4.8K	Mat2F1, 232F, 232F2, 232F3	Br, BGLr, BGLR2, BGLR3
P250	Jen2R, Vec	V1 BamHI	HMG97R, VecN	V2/1.8K	VecN, P232F1015, P232F1681	HMG97r, P232R1662, P232R995
P250	HMG85f, Vec	V1 BamHI	HMG97f, VecN	V2/2.9K	HMG97f, P232F2844, P232f, P232F2	VecS, P232R4600, P232R1, P232R2b
P252	Jen2R, Vec	V1 BamHI	HMG97r, VecN	V2/2K	VecS, P232F1015, P232F1681	HMG97r, P232R1662, P232R995
P252	Jen2F, Jen2R	52/40	-	-	Jen2F	Jen2R
P252	HMG85f, Vec	V1 ClaI	HMG97f, VecN	V2/2.4K	HMG97f, P232F2844, P232F, P232F2	VecS, P232R1, P232R2b
P252	Jen2R, Vec	V1 ClaI	HMG97r, VecN	V2/6K	-	HMG97r
P306	ORF556f, Jen2R	52/40	-	-	ORF556f, P232F1015, P232F1681	Jen2R, SMr
P306	Jen2F, S3r	55/30	-	-	Jen2F, 2F	M2R, 2r
P310	ORF556f, Jen2R	52/40	-	-	ORF556f, P232F1015	Jen2R, SMr
P310	Jen2F, S3r	52/40	-	-	Jen2F, 2F	M2R, 2r
P385	ORF556f, Jen2R	52/40	-	-	ORF556f, P232F1015	Jen2R, 383r
P385	Jen2f, S3r	52/40	-	-	Jen2F, 2nf	M2R, 2nr

Table 3-9. Fused *MAT1-1*; *MAT1-2* region primers and PCR conditions used. Primer pairs used in initial PCR for respective strains are given in column 'PCR 1', with reamplification primers under 'PCR 2'. PCR conditions employed are in columns 'Cond. 1' and 'Cond. 2', referring to PCR1 and PCR2, respectively. For standard PCR, conditions are annealing temperature/number of cycles. Chromosome walking PCR conditions are marked by 'V', followed by '1' and restriction enzyme for primary PCR, and '2' followed by approximate size of the resulting PCR product for secondary PCR. Respective forward and reverse sequencing primers are given in the last two columns. For complete PCR and sequencing conditions see text.

Strain	PCR 1	Cond. 1	PCR 2	Cond. 2	Sequ. forward	Sequ. Reverse
P1	cORF589f, Vec	V1 EcoRI	cORF556f, VecN	V2/2.2K	GAPf, GAPF2, GAPF3	cORF413f, ORFr
P1	Alpha181f, Vec	V1 BamHI	Jen1Fa, VecN	V2/1K	VecN	Jen1Fa
P1	CHO13, Mat2642r	55/40	-	-	MAT1825f	-
P1	ORF413f, Alpha181f	55/40	ORF413f, Alpha191f	55/40	P1f	-
P1	ORF556f, Jen1F	55/40	-	-	-	Jen1F
P1	Jen1r, Jen2r	55/40	-	-	Jen1Ri	Jen2r
P1	Jen1r, Jen2r	55/40	Jen1Ri, Jen2r	55/40	Mat1f, Mat1Af	Mat1Ar, Mat1r
P1	HMG85f, Vec	V1 BamHI	HMG97f, VecN	V2/2K	HMG97f, P1F2914, P1Endf	VecS, Mat4707r, P1R2
P2	Alpha181f, Vec	V1 BamHI	Jen1Fa, VecN	V2/1K	VecS	Jen1Fa
P2	ORF413f, Alpha191f	55/40	-	-	ORF556f, P1f	Alpha191f, Mat2642r
P2	Jen1r, Jen2r	55/40	Jen1Ri, Jen2r	55/40	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P2	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P81	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P81	Jen1r, Jen2r	55/40	Jen1Ri, Jen2r	55/40	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P81	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P123	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P123	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P123	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P129	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P129	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P129	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P202	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P202	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P202	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P207	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P207	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f,	Jen2r, Mat1Ar,

					Mat1Af	Mat1r
P207	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P210	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P210	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P210	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P238	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P238	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P238	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P243	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P243	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P243	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P262	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P262	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P262	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P268	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P268	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P268	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P269	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P269	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P269	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P270	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Jen1f, P1r
P270	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P270	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P271	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P271	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P271	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r
P303	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P303	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f	Jen2r, Mat1Ar, Mat1r
P303	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r

P327	ORF413f, Alpha181f	50/40	ORF413f, Alpha191f	55/40	ORF556f, P1f	Alpha191f, P1r
P327	Jen1r, Jen2r	55/40	-	-	Jen1Ri, Mat1f, Mat1Af	Jen2r, Mat1Ar, Mat1r
P327	Jen2f, S3r	52/40	-	-	Jen2f, 2f	M2r, 2r

Table 3-10. MAT loci in phylogenetic species of *Pleospora*. For phylogenetic species with only one type of MAT locus and more than one isolate, numbers of isolates are given instead of P strain numbers. For groups, see Figure 3-1. For details on phylogenetic species, see Table 2-14.

Phylogenetic species	Group	*MAT1-1*	*MAT1-2*	*MAT1-1*; *MAT1-2*
P. eturmiuna	C	-	-	9
P. gigaspora	C	-	-	2
P. gracilariae	C	-	-	3
P. herbarum	C	-	-	47
P. paludiscirpi	E/TP	-	-	P270
Pleospora sp. strain P56	E/SP	4	-	-
Pleospora sp. strain P107	E/SP	3	-	-
Pleospora sp. strain P327	E/TP	-	-	2
P. tarda	B	-	-	4
P. triglochinicola	E/TP	-	-	P123
S. astragali	C	-	-	4
S .callistephi	D	P383	-	-
S. lancipes	F	P229	-	-
S. loti	E	P384	P385	-
S. majusculum	C	-	-	2
S. sarciniforme strain P239	E	P239	P309, P310	-
S. sarciniforme strain P247	E	P247	P306	-
S. solani	D	P240, P241, P253, P407, P408, P409	P252	-
Stemphylium sp. strain P210	E/TP	-	-	P210
Stemphylium sp. strain P246	E	P246	-	-
S. trifolii	E	P244		-
S. xanthosomatis	A	P245, P248, P316, P406	P232, P242, P249, P250, P251	-

Table 3-11. Comparison of parsimony informative characters. The MAT genes, was well as up and downstream regions are compared. The compared regions correspond to the inverted *MAT1-1* idiomorph portion, and the *MAT1-2* idiomorph present in the fused MAT regions.

Region	Upstream	MAT gene	Downstream
MAT1-1	24.3%	23.6%	45.4%
MAT1-2	24.2%	18.9%	36.6%

Table 3-12. Branch supports of mating type phylogenies compatible with species phylogeny. Numbers of branches taken from species phylogeny in Figure 3-1. For protein phylogenies, support values are for parsimony and likelihood. For idiomorphs, support values are likelihood, parsimony, Bayesian and Neighbor joining in this order. Values in bold were supported by 100% in all analyses. Supported groups were D (branch 17) and A (branch 33). Groups B and F were distinct, but only had one member. Groups C and E were not supported. Other supported branches were inside group C (branches 3-11), group D (branch 16) and group E (branches 21-30).

Branch	*MAT1-1* proteins (Figure 3-8)	*MAT1-2* proteins (Figure 3-9)	*MAT1-1* idiomorphs (Figure 3-10)	*MAT1-2* idiomorphs (Figure 3-11)	Supported clades
3	99/95	100/72	**100**	**100**	*P. herbarum*
5	-	-	89/93/100/-	-	Branch 3 plus *S. gracilariae*
9	84/76	-	99/97/100/94	95/93/100/82	Branch 5 plus *S. majusculum* and *S. gigaspora*
10	-	-	-	97/96/100/94	Branch 9 plus *S. astragali*
11	-	97/-	**100**	**100**	*P. eturmiuna*
16	100/100	-	**100**	-	*S. solani*
17	96/100	-	**100**	-	Group D
21	100/87	-	**100**	-	*Pleospora spp.* strains P56 and P342
22	98/78	-	**100**	-	Branch 21 plus *Pleospora sp.* strain P107
25	-	100/81	**100**	**100**	*S. sarciniforme* strain P239 and P247
27	-	-/96	-/-/-/82	**100**	Branch 25 plus *S. loti*
29	73/-	-	**100**	-	Branch 27 plus *Stemphylium sp.* strain P246
30	-	-	-/-/78/-	-	Branches 22 plus 29
33	-	100/77	**100**	**100**	Group A

Table 3-13. Branch supports of mating type phylogenies contradicting species phylogeny. For protein phylogenies (Figure 3-8 and Figure 3-9), support values are for parsimony and likelihood. For idiomorph phylogenies (Figure 3-10 and Figure 3-11), support values are likelihood, parsimony, Bayesian and Neighbor joining in this order. Values in bold were supported by 100% in all analyses. The genus *Pleospora* was monophyletic in the protein phylogenies where outgroups were included (branch 16). The MAT phylogenies differed from the species phylogeny mainly in the monophyly of the fused MAT regions (branch N12). Minor, frequently poorly supported topological differences were within group C (branches N1 – N8), or were in relation to the monophyly of the fused MAT regions (branches N9 – N13). The branching order of groups A, D and F with separate MAT regions was resolved as opposed to the species phylogeny (branches N14 – N15).

Branches	*MAT1-1* proteins Figure 3-8	*MAT1-2* proteins Figure 3-9	*MAT1-1* idiomorphs Figure 3-10	*MAT1-2* idiomorphs Figure 3-11	Supported clades
N1	-/87	-	-	-	*S. astragali*, *P. eturmiuna*, group B
N2	-/75	-	-/-/90/-	-	*P. herbarum*, *P. gracilariae*, *S. majusculum*
N3			-/-/94/-		within *P. herbarum*
N4			88/85/100/95		within *P. herbarum*
N5	-	-	-	**100**	within *P. herbarum*
N6	-	-	-	86/85/100/91	within *P. herbarum*
N7	-	-	-	-/-/87/-	within *P. herbarum*
N8	-	-	-	-/-/74/-	within *P. herbarum*
N9	-	-	**100**	**100**	Groups B and C
N10	-	-	-	89/78/99/78	*Stemphylium sp.* P210 plus branch N9
N11	-	-	-	-/71/90/-	*P. triglochinicola* plus branch N10
N12	100/99	98/95	**100**	**100**	fused MAT regions
N13	88/81	-/96	**100**	**100**	Groups E, C, B
N14	99/98	-/85	**100**	**100**	Groups A, D, F
N15	-	-	**100**	-	Groups D, F
N16	100/92	100/98	-	-	*Pleospora*

Table 3-14. Comparison of MAT genes and idiomorphs in *Pleospora* to other members of the Dothideomycetes. Gene, exon, intron and idiomorph lengths are given. For diagrams of the extents of the idiomorphs see Figure 3-12.

Species	Gene	Length bp	Exon bp	Intron I Length 5'-end		Intron II Length 5'-end		Idio-morph bp	Acc. No.
Alternaria alternata	*MAT1-1*	1217	1170	47	248	-	-	2285	AB009451
Cochliobolus heterostrophus	*MAT1-1*	1205	1152	52	218	-	-	1297	AF029913
Leptosphaeria maculans	*MAT1-1*	1368	1323	45	254	-	-	3723	AY174048
Mycosphaerella graminicola	*MAT1-1*	995	891	55	320	49	414	2851	AF440399
Phaeosphaeria nodorum	*MAT1-1*	1090	1035	55	221	-	-	4306	AY212018
Pleospora spp.	*MAT1-1*	1193	1140	53	218	-	-	2057 – 2106	-[1]
Alternaria alternata	*MAT1-2*	1083	1029	54	482	-	-	2599	AB009452
Cochliobolus heterostrophus	*MAT1-2*	1087	1032	55	485	-	-	1172	AF027687
Leptosphaeria maculans	*MAT1-2*	1255	1200	55	467	-	-	4227	AY174049
Mycosphaerella graminicola	*MAT1-2*	1233	1185	48	635	-	-	2783	AF440398
Phaeosphaeria nodorum	*MAT1-2*	1091	1038	53	491	-	-	4530	AY212019
Pleospora spp.	*MAT1-2*	1093	1038	55	491	-	-	2376	-[2]

[1] Isolates for which entire idiomorphs were sequences are *Pleospora sp.* strain P56 (AY339851), *S. lancipes* strain, P229 (AY339853), *Stemphylium sp.* strain P246 (AY339858), *S. sarciniforme* strain P247 (AY339859) and *Stemphylium sp.* strain P316 (AY339861).
[2] Isolates for which entire idiomorphs were sequenced are *S. xanthosomatis* strain P232 (AY340940), *Stemphylium sp.* strain P250 (AY340941) and *Stemphylium sp.* strain P252 (AY340942).

Table 3-15. MAT locus and teleomorph formation in phylogenetic species of *Pleospora*. Phylogenetic species, groups (see Figure 3-1), MAT locus arrangements, presence of the teleomorphs, and the capabilities to form teleomorphs in culture are given. All cultures were derived from single spores, so that teleomorph formation in culture is proof of homothallism. For phylogenetic species where teleomorph formation is reported for the first time, teleomorph-forming strains are listed.

Phylogenetic species	Group	MAT locus	Teleomorph	Teleomorph formation in culture
P. eturmiuna	C	fused	+[1]	+
P. gigaspora	C	fused	+[2]	+
P. gracilariae	C	fused	+[3]	+
P. herbarum	C	fused	+[4]	+
P. paludiscirpi	E/TP	fused	+[1]	+
Pleospora sp. strain P56	E/SP	*MAT1-1*	+	+[5]
Pleospora sp. strain P107	E/SP	*MAT1-1*	+	+[6]
Pleospora sp. strain P327	E/TP	fused	+[7]	-
P. tarda	B	fused	+[4]	+
P. triglochinicola	E/TP	fused	+[8]	-
S. astragali	C	fused	+	+[9]
S. callistephi	D	*MAT1-1*	-	-
S. lancipes	F	*MAT1-1*	-	-
S. loti	E	*MAT1-1* or *MAT1-2*	-	-
S. majusculum	C	fused	+[10]	+
S. sarciniforme strain P239	E	*MAT1-1* or *MAT1-2*	-	-
S. sarciniforme strain P247	E	*MAT1-1* or *MAT1-2*	-	-
S. solani	D	*MAT1-1* or *MAT1-2*	-	-
Stemphylium sp. strain P210	E / TP	fused	+	+[11]
Stemphylium sp. strain P246	E	*MAT1-1*	-	-
S. trifolii	E	*MAT1-1*	-	-
S. xanthosomatis	A	*MAT1-1* or *MAT1-2*	-	-

[1] Simmons (2001).
[2] Crivelli (1983).
[3] Simmons (1989).
[4] Simmons (1985).
[5] Teleomorph forming isolates were *Stemphylium sp.* strain P227 and *Pleospora sp.* strains P342 and P343.
[6] Teleomorph forming isolates were *Stemphylium sp.* strains P212 and P221.
[7] Teleomorph only obtained in natural habitat.
[8] Webster (1969).
[9] Teleomorph forming isolate was *Stemphylium sp.* strain P203.
[10] Simmons (1969).
[11] Teleomorph forming isolate was *Stemphylium sp.* strain P210.

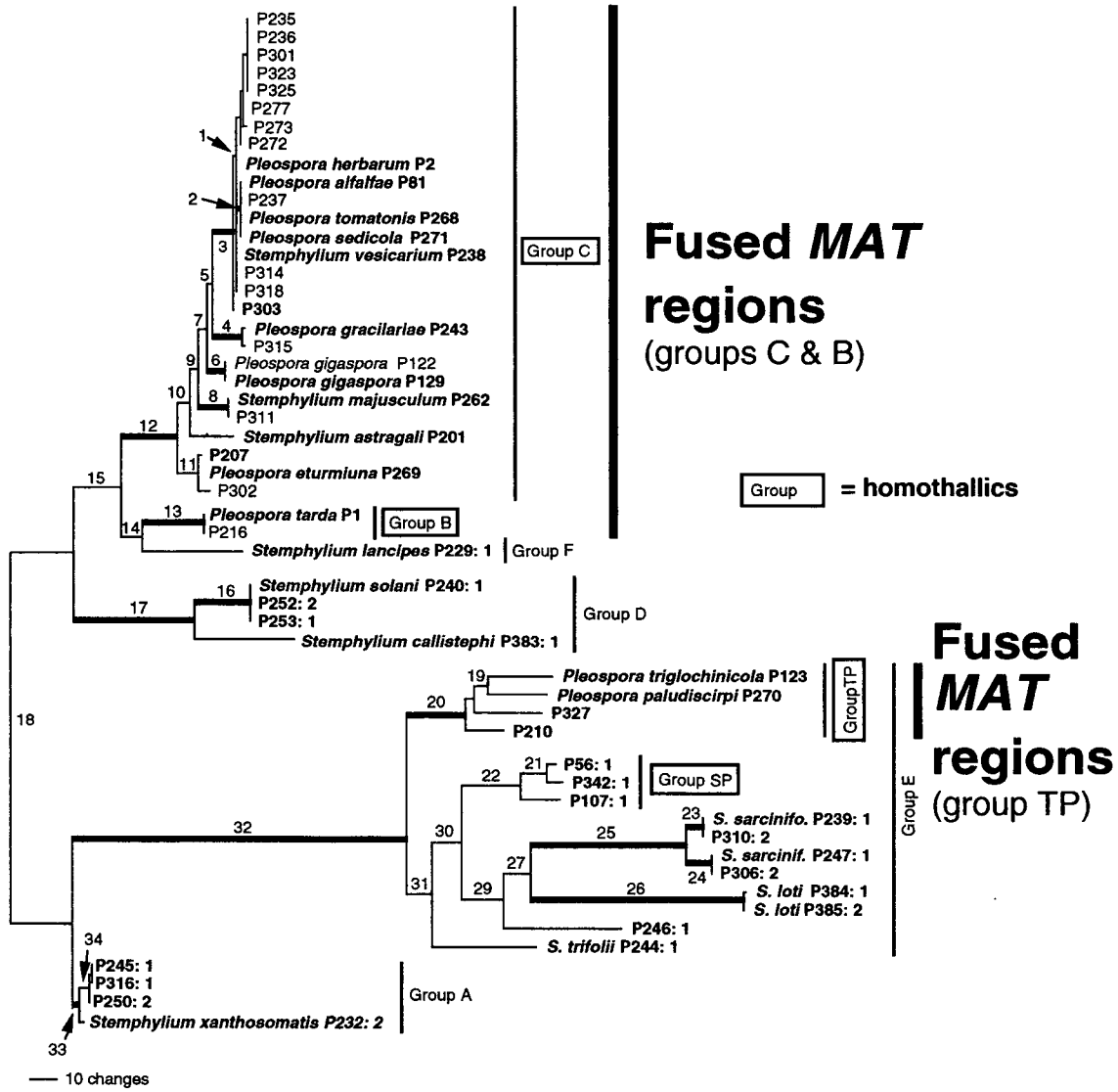


Figure 3-1. ***Pleospora*** **species phylogeny and distribution of MAT regions and mating systems. Tree based on four loci (see Figure 2-6). Numbers by the branches reflect bootstrap supports above 70%. Values are given in Table 2-12. Bold branches had maximal support in all analyses. Thin vertical lines on the right indicate groups according to Câmara et al. (2002) and this paper. Solid vertical lines indicate distribution of fused MAT regions. The remaining groups all have either** ***MAT1-1*** **or** ***MAT1-2*** **indicated behind the taxon name as '1' or '2'. For mating types of all isolates screened see Table 3-10. Boxed groups are homothallic. Taxa in bold were chosen for sequencing of the MAT region. For details on isolate numbers, see Table 2-1.**

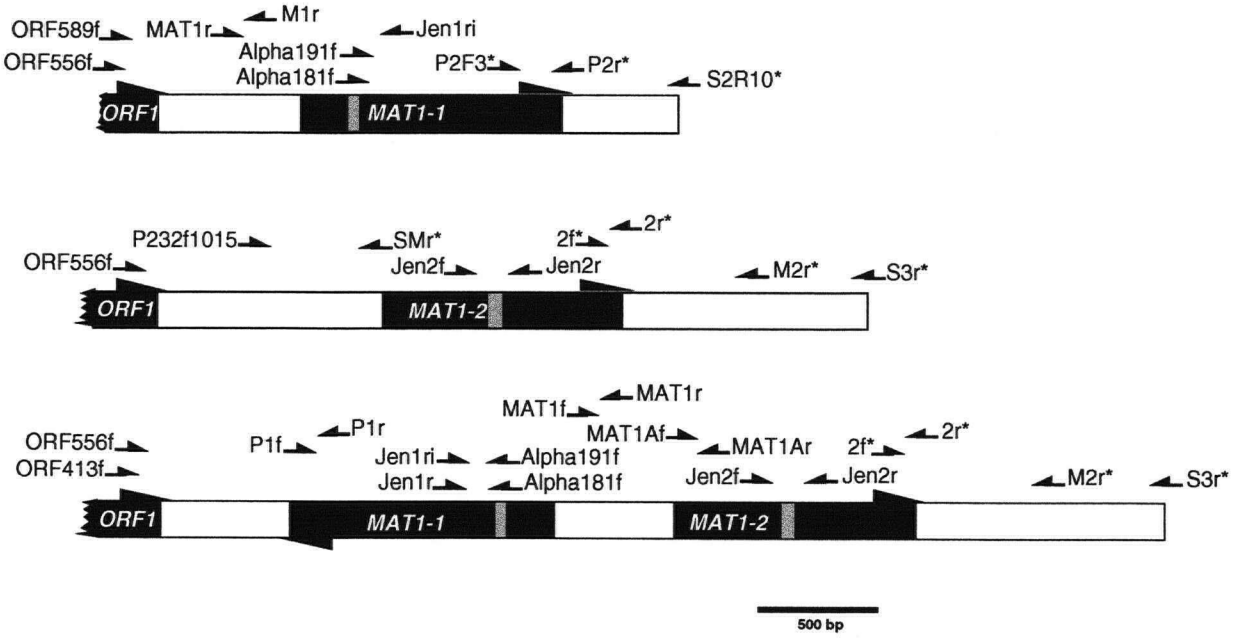


Figure 3-2. Main primers used for PCR amplification and DNA sequencing for *MAT1-1*, *MAT1-2* and fused MAT regions. Gene diagrams approximately to scale. Tips of arrows indicate approximate 3'-end of primers. Black boxes are coding sequences, gray boxes are introns and white boxes are intergenic spacers. Half arrow heads indicate the direction of transcription. Primers with asterisk had to be replaced by alternative primers in some isolates, due to high DNA sequence variability at the primer site. For a complete list of primers and primer sequences, including primers used for chromosome walking, see Table 3-1 - Table 3-6.

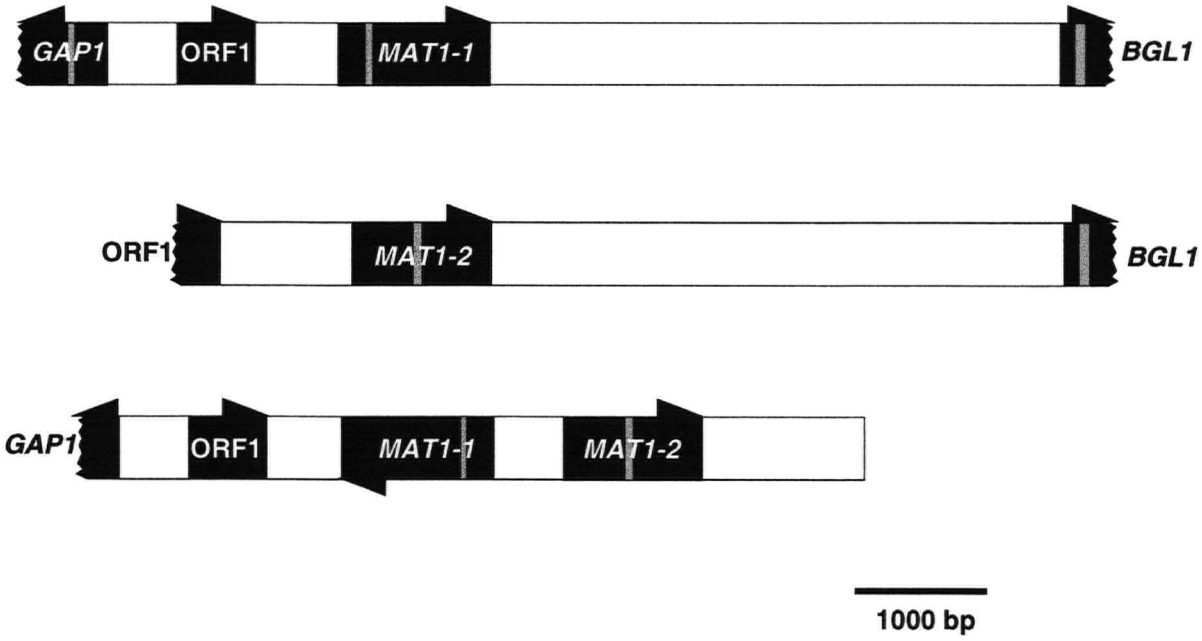


Figure 3-3. Longest MAT regions sequenced. These were from top to bottom *Pleospora sp.* strain P56, *S. xanthosomatis* strain P232 and *P. tarda* strain P1, respectively 8455 bp, 7248 bp and 6102 bp in length. Gene diagrams are to scale. Black boxes are coding regions, gray boxes are introns and white boxes are intergenic spacers. Half arrow heads indicate direction of transcription. Shark teeth delimit incompletely sequenced genes.

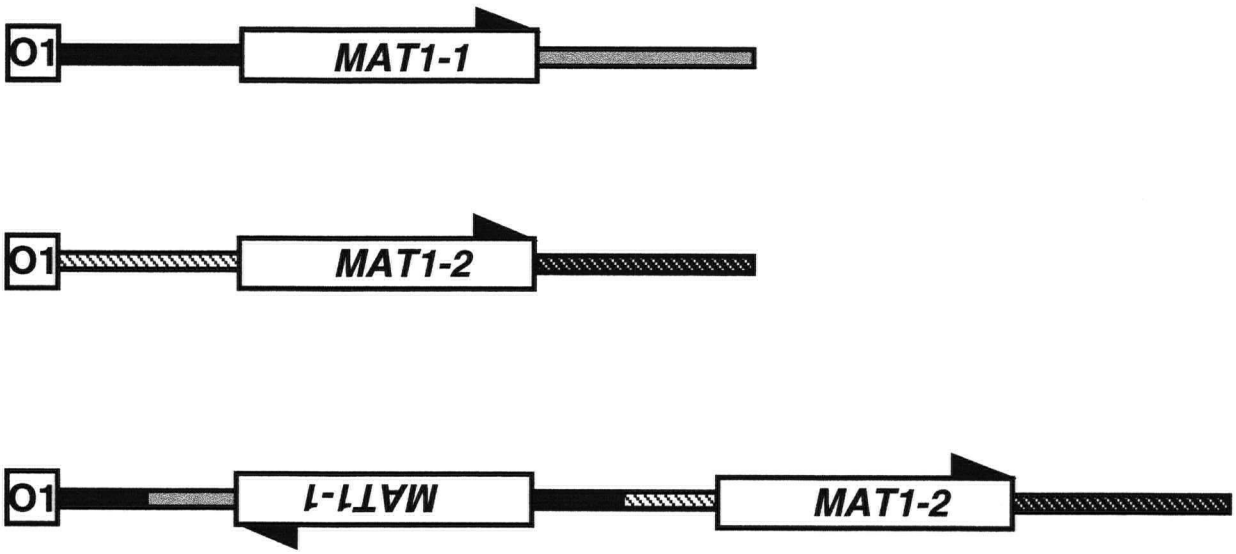
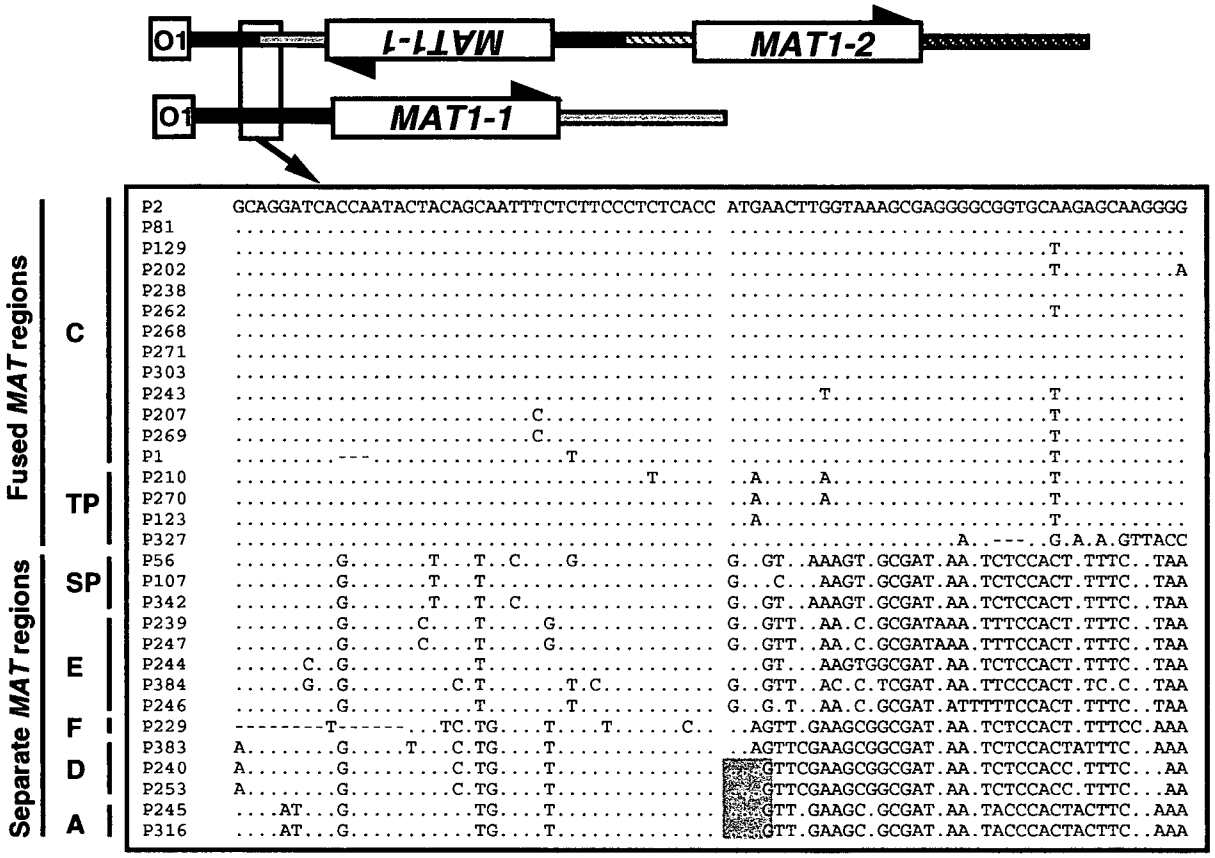
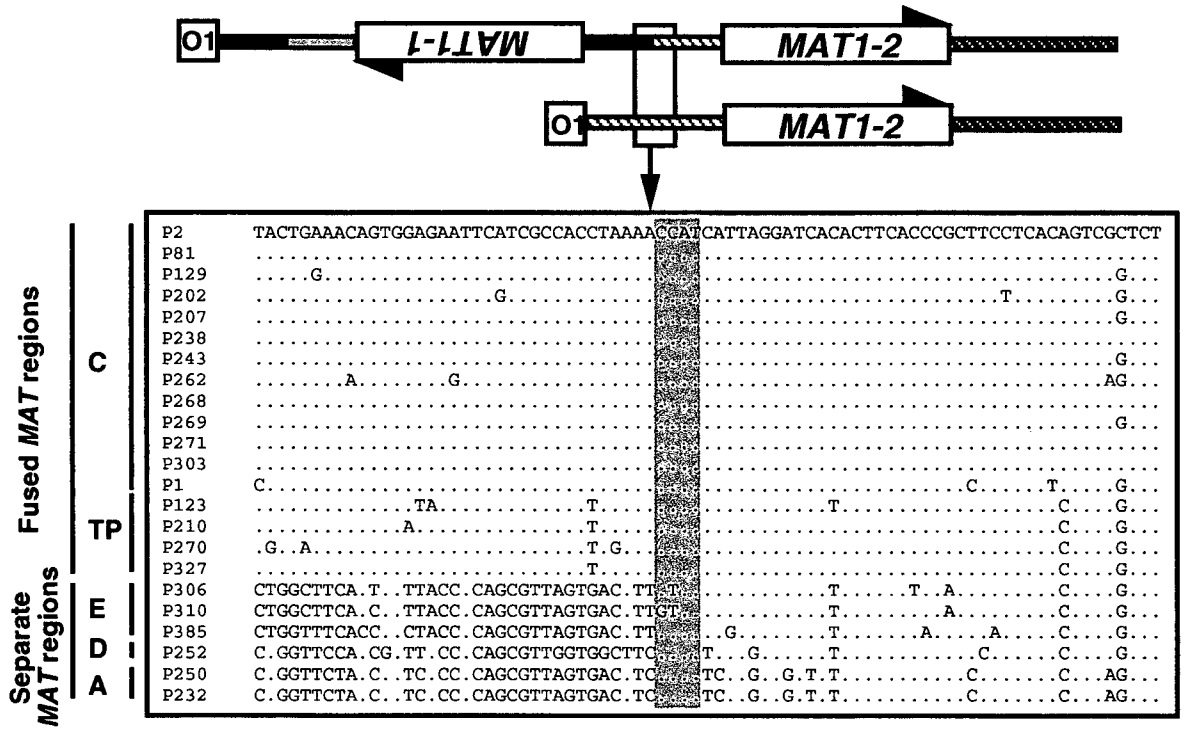


Figure 3-4. Schematic representation of the MAT loci. In the fused MAT region, the *MAT1-1* gene plus flanking region is inverted, and fused to a *MAT1-2* region. White boxes are genes, half arrow heads indicate direction of transcription. Narrow boxes are intergenic spacers which are pattern coded with shades of gray for the *MAT1-1* spacers, and lines for the *MAT1-2* spacers to illustrate the organization of the fused MAT region. 'O1' is flanking gene ORF1.

Figure 3-5. DNA sequences of *MAT1-1; MAT1-2* fusion areas. The ORF1 ('O1') proximal fusion region is at the top, the ORF1 distal region at the bottom. Vertical lines on the left mark MAT regions and phylogenetic groups. DNA sequences of fused and separate MAT regions are aligned. Dots indicate identical nucleotides to top rows. In the top diagram, a column of blanks separates forward and reverse MAT regions. Shaded nucleotides represent the putative crossover site, in forward orientation in the presumptive *MAT1-1* ancestors in the top diagram, in the bottom diagram in reverse orientation in four presumptive *MAT1-2* ancestors, as well as in all fused MAT regions.


O1
MAT1-1
MAT1-2
O1
MAT1-1
Fused MAT regions
Separate MAT regions
C
TP
SP
E
F
D
A
P2 GCAGGATCACCAATACTACAGCAATTTCTCTTCCCTCTCACC ATGAACTTGGTAAAGCGAGGGGCGGTGCAAGAGCAAGGGG
P81
P129
P202
P238
P262
P268
P271
P303
P243
P207
P269
P1
P210
P270
P123
P327
P56
P107
P342
P239
P247
P244
P384
P246
P229
P383
P240
P253
P245
P316


O1
MAT1-1
MAT1-2
O1
MAT1-2
Fused MAT regions
Separate MAT regions
C
TP
E
D
A
P2 TACTGAAACAGTGGAGAATTCATCGCCACCTAAAACGATCATTAGGATCACACTTCACCCGCTTCCTCACAGTCGCTCT
P81
P129
P202
P207
P238
P243
P262
P268
P269
P271
P303
P1
P123
P210
P270
P327
P306
P310
P385
P252
P250
P232

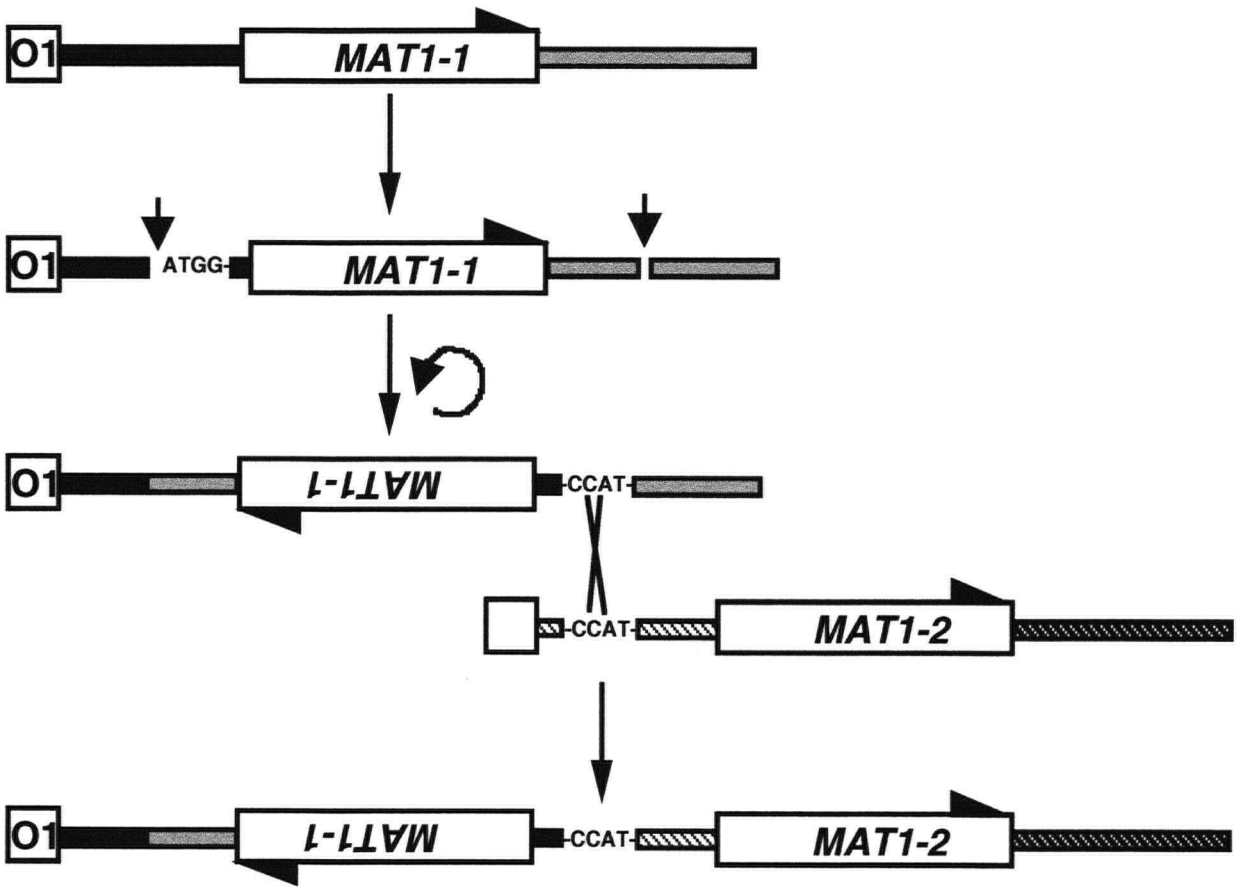


Figure 3-6. Evolution of fused MAT regions. In an ancestor, a portion of the idiomorph containing the entire *MAT1-1* gene is inverted. The 'ATGG' motive at the 5'-end of the inverted fragment is now present as inverse complement 'CCAT' at the ORF1 distal fusion site. Assuming that the inverted *MAT1-1* gene is functional, during mating a crossover to a separate *MAT1-2* gene at the shared 'CCAT' motive would yield the fused MAT locus.

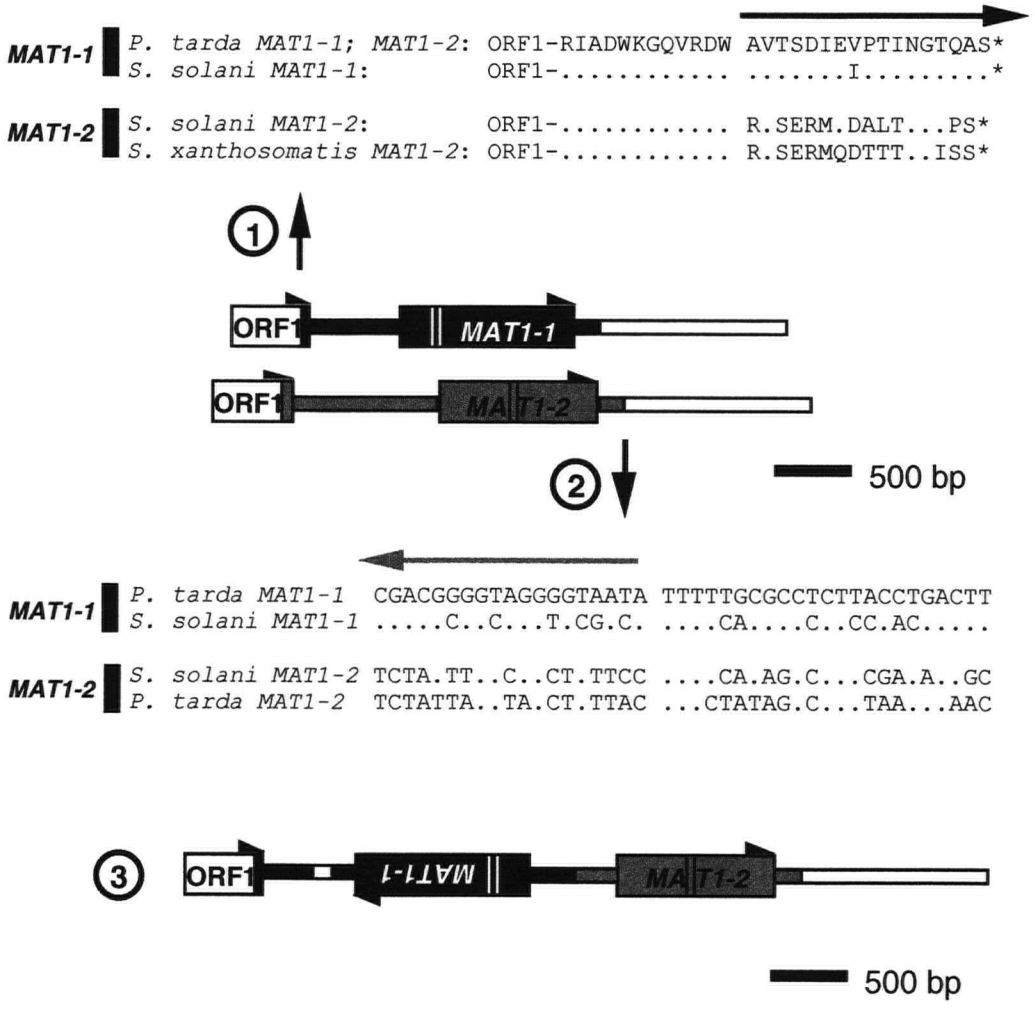
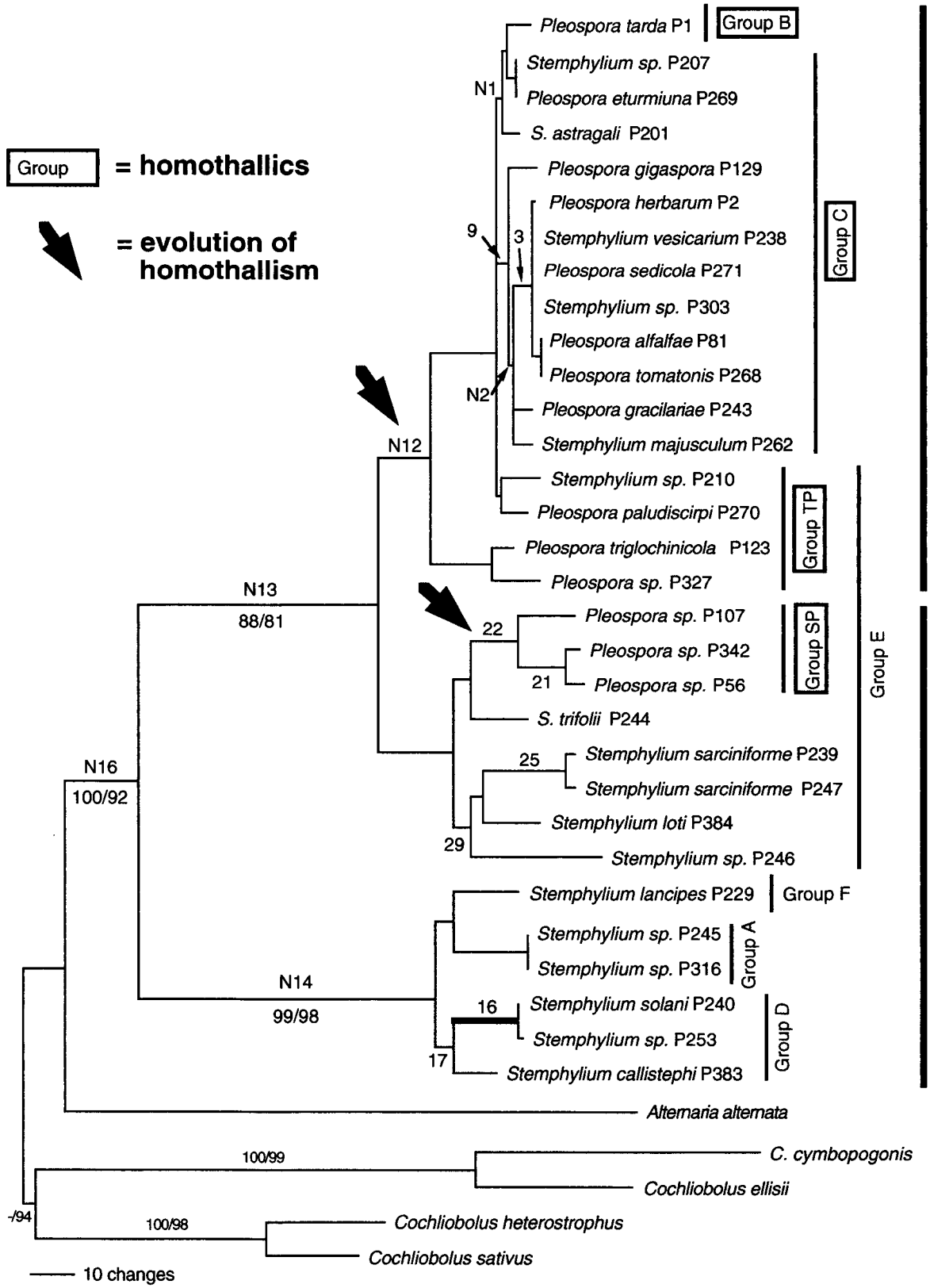


Figure 3-7. MAT idiomorphs. Gene diagrams approximately to scale. The dark portions are the idiomorphs comprising the MAT genes. The white portions are the idiomorph flanking regions, such as ORF1 and intergenic spacers. Sequences of MAT idiomorph boundaries are aligned, dots indicate nucleotide identity to top rows. Alignments are at (1) the 5'-end within ORF1, and at (2) the 3'-end downstream of the MAT genes. Horizontal arrows above alignments indicate the extension of the idiomorphs. At the 5'-end, the ORF1 amino acid translations are given, and DNA sequences at the 3'-end. The idiomorph was distinctly delimited at the 5'-end, regardless of separate or fused MAT regions, such as *P. tarda* strain P1. The downstream idiomorph border at (2) was chosen conservatively upstream of the first shared nucleotide motive between *MAT1-1* and *MAT1-2* regions. (3) Shows the idiomorphs in fused MAT regions. The idiomorph was not continuous, as the inverted portion of *MAT1-1* extended beyond the *MAT1-1* downstream idiomorph boundary.

Figure 3-8. *MAT1-1* protein phylogeny (Fitch-Margoliash distance tree). The tree is rooted with species of *Cochliobolus* and *Alternaria*. Vertical lines on the right mark phylogenetic groups and MAT regions. Boxed groups contain homothallics, and large black arrows indicate evolution of homothallism. For the ingroup, branches supported by more than 70% in either parsimony or likelihood (quartet puzzling) are numbered and listed in Table 3-12 and Table 3-13. Numbers preceded by 'N' indicate nodes that are not contained in the species phylogeny in Figure 3-1. For important nodes supporting the monophyly of *Pleospora* or contradicting the species phylogeny, branch supports are given.


Group = homothallics
= evolution of homothallism
Pleospora tarda P1
Group B
Stemphylium sp. P207
Pleospora eturmiuna P269
S. astragali P201
Pleospora gigaspora P129
Pleospora herbarum P2
Stemphylium vesicarium P238
Pleospora sedicola P271
Stemphylium sp. P303
Pleospora alfalfae P81
Pleospora tomatonis P268
Pleospora gracilariae P243
Stemphylium majusculum P262
Group C
Stemphylium sp. P210
Pleospora paludiscirpi P270
Group TP
Pleospora triglochinicola P123
Pleospora sp. P327
Pleospora sp. P107
Pleospora sp. P342
Pleospora sp. P56
Group SP
S. trifolii P244
Stemphylium sarciniforme P239
Stemphylium sarciniforme P247
Stemphylium loti P384
Stemphylium sp. P246
Group E
Stemphylium lancipes P229
Group F
Stemphylium sp. P245
Stemphylium sp. P316
Group A
Stemphylium solani P240
Stemphylium sp. P253
Stemphylium callistephi P383
Group D
Alternaria alternata
C. cymbopogonis
Cochliobolus ellisii
Cochliobolus heterostrophus
Cochliobolus sativus
10 changes
N1
N2
N12
N13
88/81
N14
99/98
N16
100/92
9
3
22
21
25
29
16
17
100/99
100/98
-/94
Fused MAT regions
Separate MAT regions

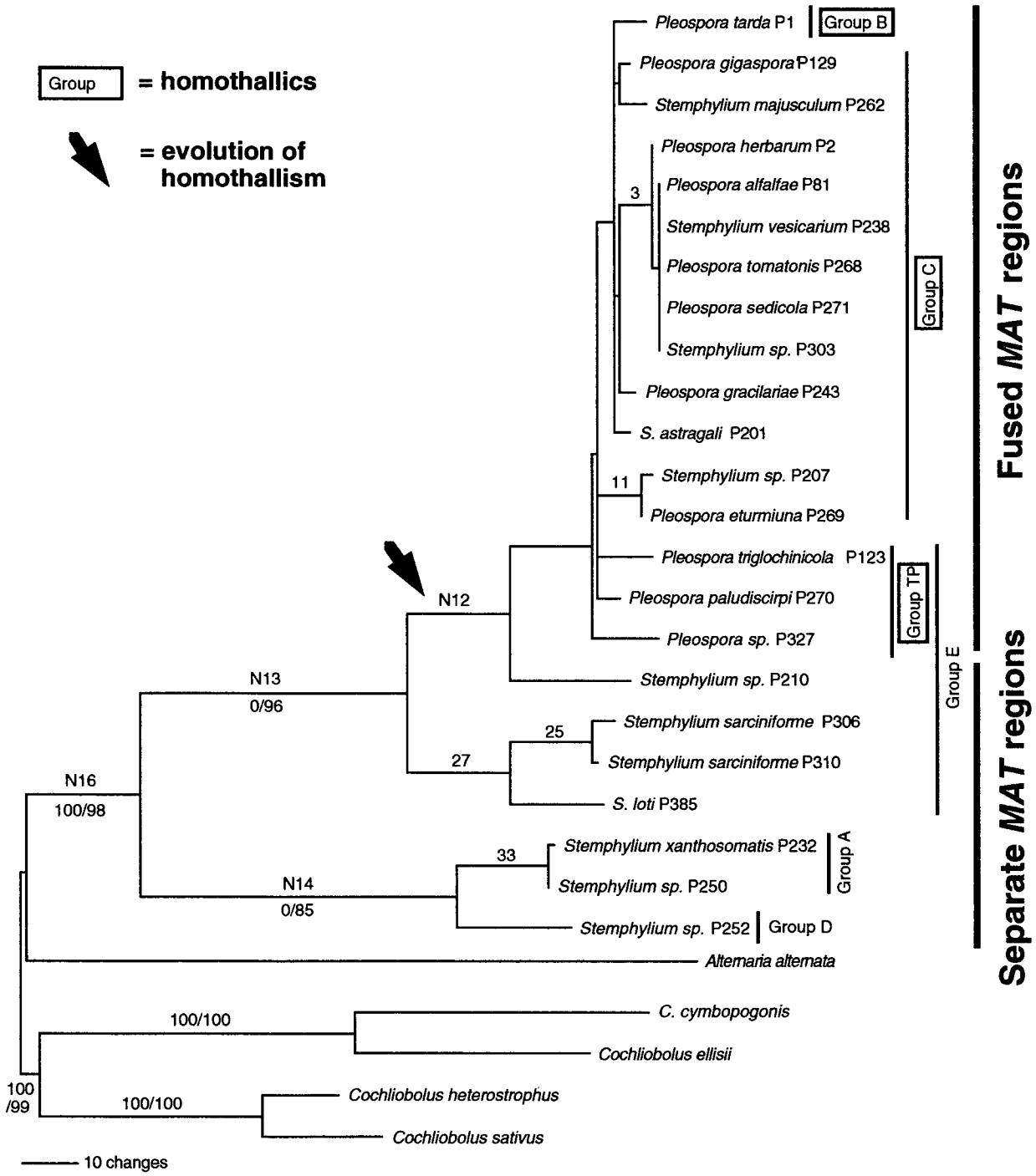


Figure 3-9. *MAT1-2* protein phylogeny (Fitch-Margoliash distance tree). The tree is rooted with species of *Cochliobolus* and *Alternaria*. Vertical lines on the right mark phylogenetic groups and MAT regions. Boxed groups are homothallics. Large black arrow marks evolution of homothallism. For the ingroup, branches supported by more than 70% in either parsimony or likelihood (quartet puzzling) are numbered and listed in Table 3-12 and Table 3-13. Numbers preceded by 'N' indicate nodes that are not contained in the species phylogeny in Figure 3-1. For important nodes supporting the monophyly of *Pleospora* or contradicting the species phylogeny, branch supports are given.

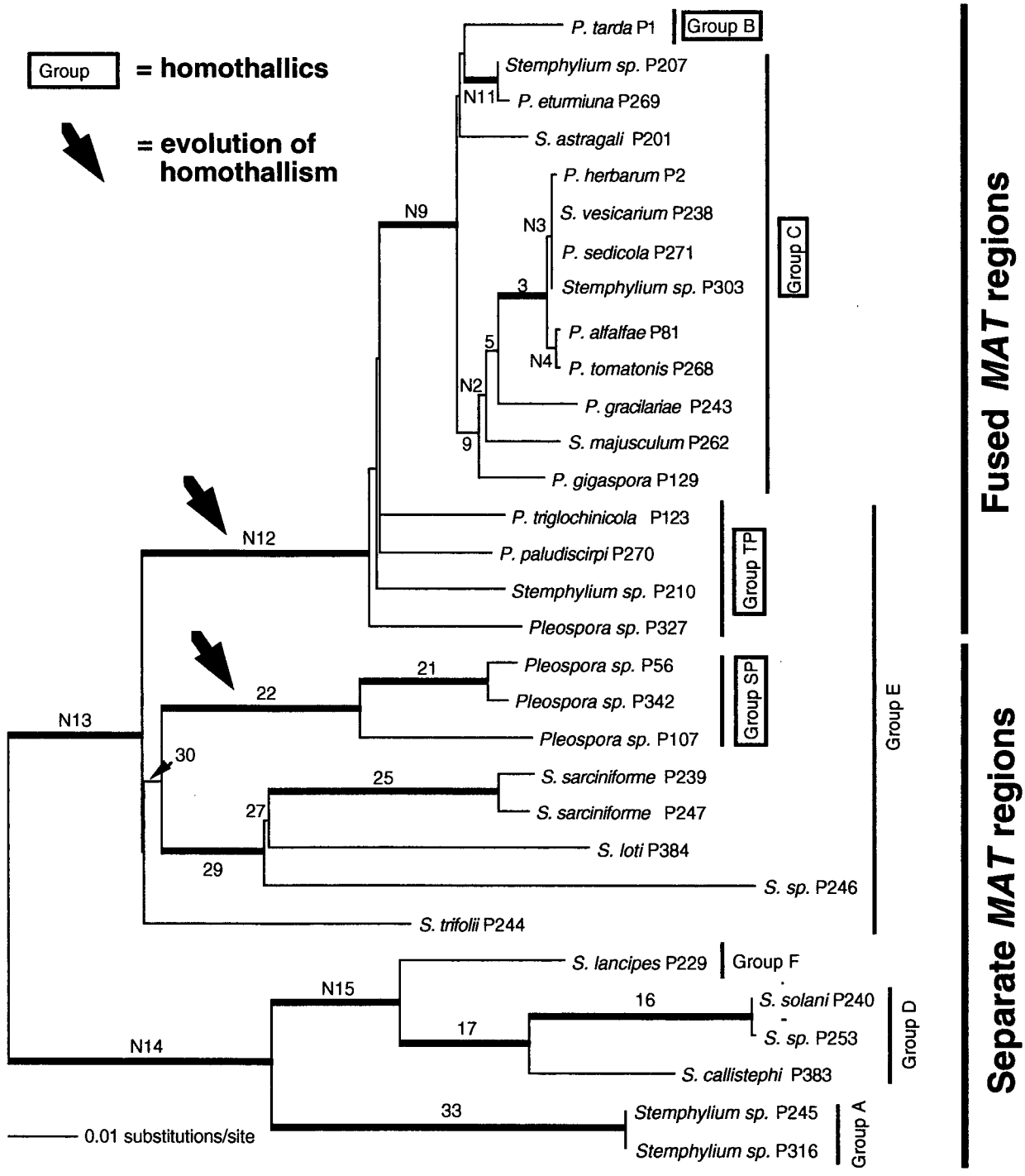


Figure 3-10. *MAT1-1* idiomorph most likely tree. The unrooted tree is presented as rooted according to the *MAT1-1* protein phylogeny in Figure 3-8. Vertical lines on the right mark phylogenetic groups and MAT regions. Boxed groups are homothallic. Large black arrows indicate the evolution of homothallism. Branches supported by more than 70% in either likelihood, parsimony, Bayesian or Neighbor joining analyses are numbered and listed in Table 3-12 and Table 3-13. Numbers preceded by 'N' indicate nodes that are not contained in the species phylogeny in Figure 3-1. Branches with 100% support in all analyses are in bold.

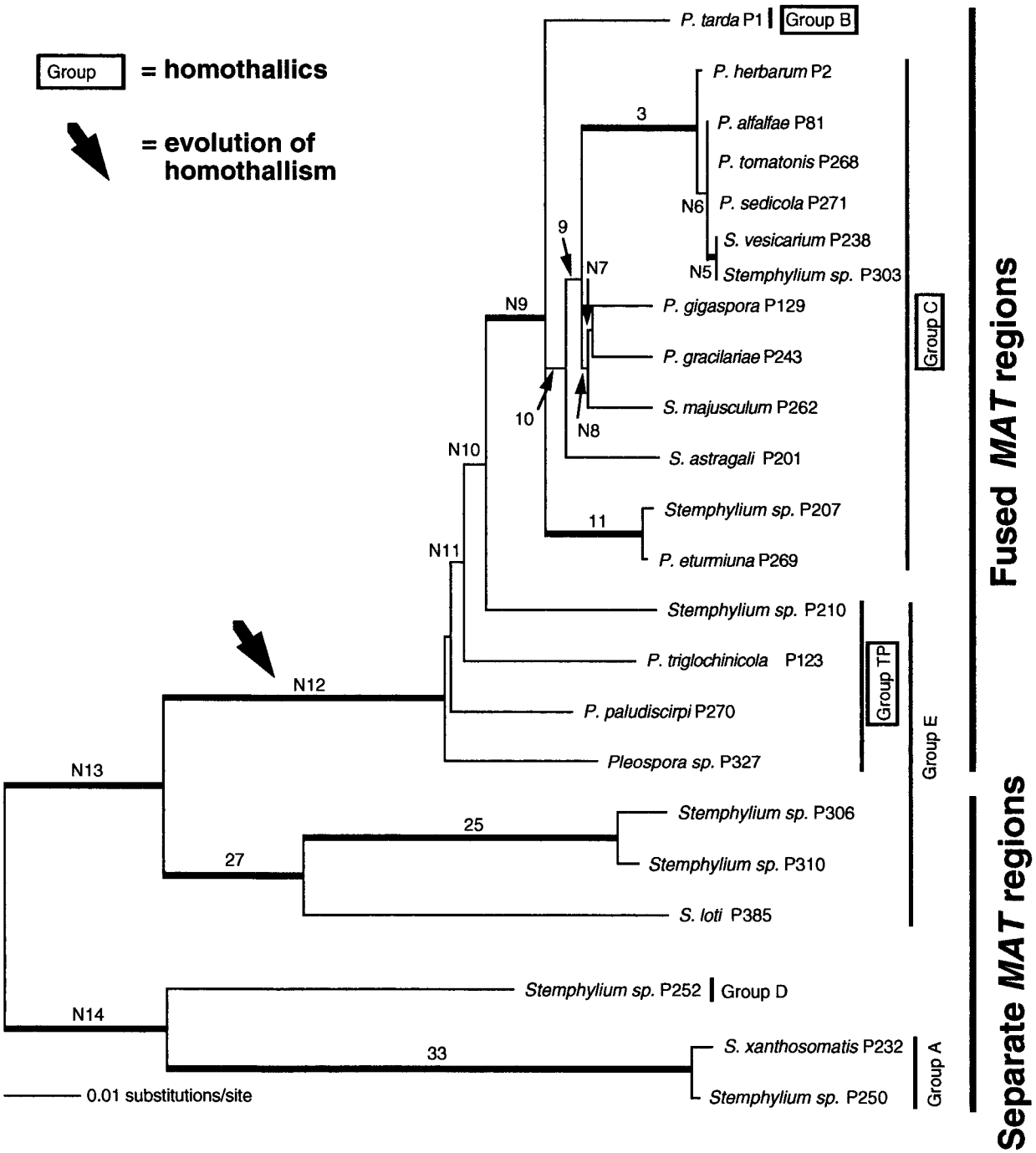
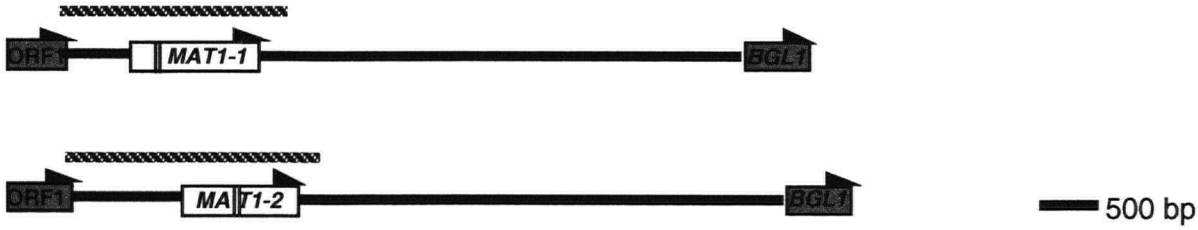


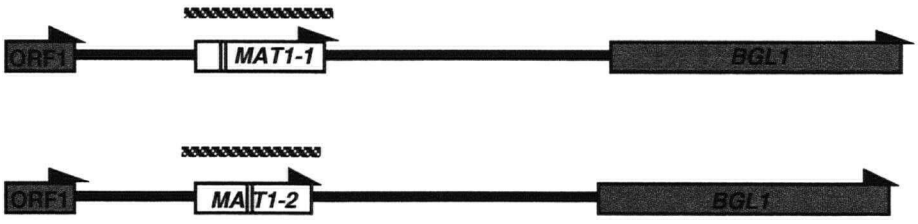
Figure 3-11. *MAT1-2* idiomorph most likely tree. The unrooted tree is presented as rooted according to the *MAT1-2* protein phylogeny in Figure 3-9. Vertical lines on the right mark phylogenetic groups and MAT regions. Boxed groups are homothallic. Large black arrow indicates evolution of homothallism. Branches supported by more than 70% in either likelihood, parsimony, Bayesian or Neighbor joining analyses are numbered and listed in Table 3-12 and Table 3-13. Numbers preceded by 'N' indicate nodes that are not contained in the species phylogeny in Figure 3-1. Branches with 100% support in all analyses are in bold.

Figure 3-12. The MAT loci and the extent of the idiomorphs in fungi of the Dothideomycetes. Gene diagrams of known DNA sequence regions that include the MAT genes, approximately to scale (for GenBank accession numbers see Table 3-14). White boxes are MAT genes, with positions of introns indicated by vertical lines, gray boxes are flanking genes, half arrow heads are directions of transcription and black boxes intergenic spacers. Hatched lines above the gene diagrams indicate the extent of the idiomorphs. The separate MAT locus in *Pleospora* illustrated here was most similar to *Cochliobolus heterostrophus*. The extent of the idiomorphs varied widely between the species of the Dothideomycetes.

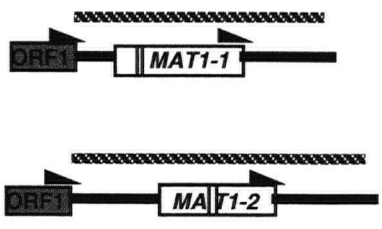
Pleospora spp.



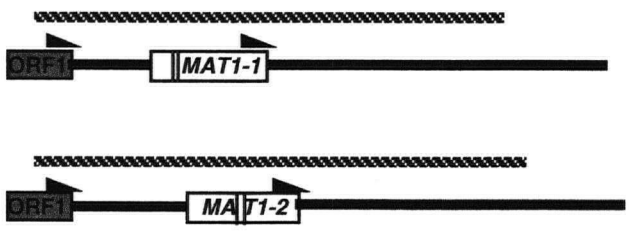
Cochlibolus heterostrophus



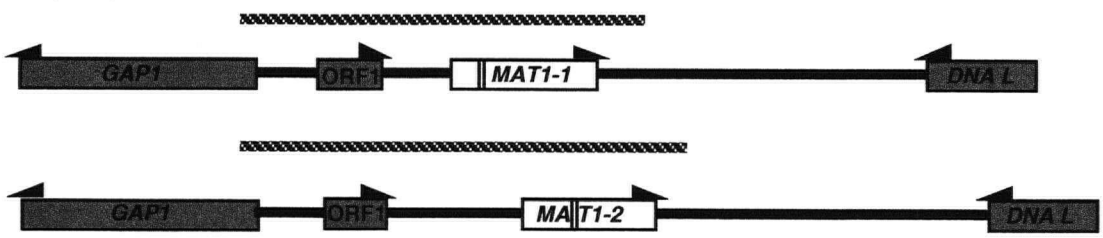
Alternaria alternata



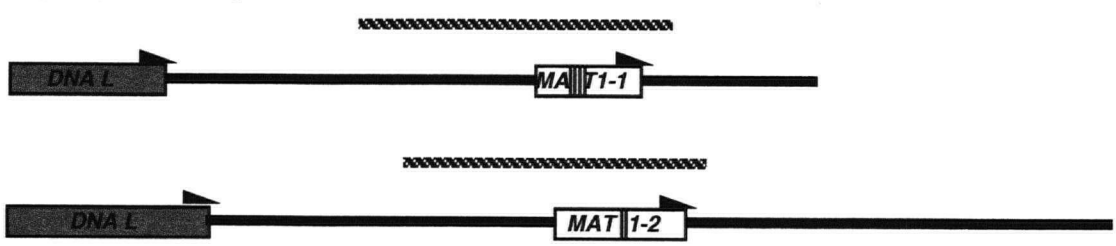
Phaeosphaeria nodorum



Leptosphaeria maculans



Mycosphaerella graminicola



3.5. Bibliography

Arie, T., Kaneko, I., Yoshida, T., Noguchi, M., Nomura, Y. & Yamaguchi, I. (2000). Mating-type genes from asexual phytopathogenic ascomycetes *Fusarium oxysporum* and *Alternaria alternata*. *Molecular Plant-Microbe Interactions* **13**, 1330-1339.

Avise, J. C. (1994). *Molecular markers, natural history & evolution*. New York: Chapman & Hall.

Bennett, R. S., Yun, S. H., Lee, T. Y., Turgeon, B. G., Arseniuk, E., Cunfer, B. M. & Bergstrom, G. C. (2003). Identity and conservation of mating type genes in geographically diverse isolates of *Phaeosphaeria nodorum*. *Fungal Genetics and Biology* **40**, 25-37.

Berbee, M. L., Pirseyedi, M. & Hubbard, S. (1999). *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and *glyceraldehyde-3-phosphate dehydrogenase* gene sequences. *Mycologia* **91**, 964-977.

Berbee, M. L., Payne, B. P., Zhang, G., Roberts, R. G. & Turgeon, B. G. (2003). Shared ITS DNA substitutions in isolates of opposite mating type reveal a recombining history for three presumed asexual species in the filamentous ascomycete genus *Alternaria*. *Mycological Research* **107**, 169-182.

Bowden, R. L. & Leslie, J. F. (1999). Sexual recombination in *Gibberella zeae*. *Phytopathology* **89**, 182-188.

Burt, A., Carter, D. A., Koenig, G. L., White, T. J. & Taylor, J. W. (1996). Molecular markers reveal cryptic sex in the human pathogen *Coccidioides immitis*. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 770-773.

Câmara, M. P. S., O'Neill, N. R. & Berkum, v., P (2002). Phylogeny of *Stemphylium* spp. based on ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **94**, 660-672.

Caten, C. E. (1981). Parasexual processes in fungi. In *The fungal nucleus*, pp. 191-214. Edited by K. Gull & S. G. Oliver. Cambridge: Cambridge University Press.

Cozijnsen, A. J. & Howlett, B. J. (2003). Characterisation of the mating-type locus of the plant pathogenic ascomycete *Leptosphaeria maculans*. *Current Genetics* **43**, 351-357.

Crivelli, P. G. (1983). Über die heterogene Ascomycetengattung *Pleospora* Rabenh.; Vorschlag für eine Aufteilung. Ph.D. thesis. Eidgenössische Technische Hochschule, Zürich, Switzerland.

de Jesus Yanez Morales, M. (2001). Molecular and morphological analyses of *Alternaria* and *Stemphylium* from onion. Ph.D. thesis. Cornell University.

Dyer, P. S., Paoletti, M. & Archer, D. B. (2003). Genomics reveals sexual secrets of Aspergillus. *Microbiology* **149**, 2301-2303.

Felsenstein, J. (2001). PHYLIP: phylogeny inference package. Seattle: Distributed by the author, Department of Genetics, University of Washington.

Glass, N. L., Vollmer, S. J., Staben, C., Grotelueschen, J., Metzenberg, R. L. & Yanofsky, C. (1988). DNAs of the two mating-type alleles of *Neurospora crassa* are highly dissimilar. *Science* **241**, 570-573.

Greig, D., Louis, E. J., Borts, R. H. & Travisano, M. (2002). Hybrid speciation in experimental populations of yeast. *Science* **298**, 1773-1774.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995). *Dictionary of the Fungi*, 8 edn. Wallingford, Oxon, UK: CAB International.

Kondo, N., Nikoh, N., Ijichi, N., Shimada, M. & Fukatsu, T. (2002). Genome fragment of *Wolbachia* endosymbiont transferred to X chromosome of host insect. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14280-14285.

Leach, J., Lang, B. R. & Yoder, O. C. (1982). Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*. *Journal of General Microbiology* **128**, 1719-1729.

Lee, J., Lee, T., Lee, Y.-W., Yun, S. H. & Turgeon, B. G. (2003). Shifting fungal reproductive mode by manipulation of mating type genes: obligatory heterothallism of *Gibberella zeae*. *Molecular Microbiology* **50**, 145-152.

Maddison, D. R. & Maddison, W. P. (2001). MacClade 4: Analysis of Phylogeny and Character Evolution. Version 4.03. Sunderland, Massachusetts, USA: Sinauer Associates.

Metzenberg, R. L. & Glass, N. L. (1990). Mating type and mating strategies in *Neurospora*. *BioEssays* **12**, 53-59.

Milgroom, M. G. (1996). Recombination and the multilocus structure of fungal populations. *Annual Review of Phytopathology* **34**, 457-477.

O'Donnell, K., Kistler, H. C., Tacke, B. K. & Casper, H. H. (2000). Gene genealogies reveal global phylogeographic structure and reproductive isolation among lineages of *Fusarium graminearum*, the fungus causing wheat scab. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 7905-7910.

O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C. & Aoki, T. (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**, 600-623.

Proutski, V. & Holmes, E. C. (1998). SWAN: sliding window analysis of nucleotide sequence variability. *Bioinformatics* **14**, 467-468.

Schiestl, R. H. & Petes, T. D. (1991). Integration of DNA fragments by illegitimate recombination in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **88**, 7585-7589.

Schmiedeknecht, M. (1962). Pseudothecien-Entwicklung bei *Pleospora papaveracea* (de Not.) Sacc. *Zeitschrift für Pilzkunde* **27 (1961)**, 62-67.

Simmons, E. G. (1969). Perfect states of *Stemphylium*. *Mycologia* **61**, 1-26.

Simmons, E. G. (1985). Perfect states of *Stemphylium*. II. *Sydowia, Annales Mycologici Ser. II.* **38**, 284-293.

Simmons, E. G. (1989). Perfect states of *Stemphylium*. III. *Memoirs of the New York Botanical Garden* **49**, 305-307.

Simmons, E. G. (2001). Perfect states of *Stemphylium*: IV. *Harvard Papers in Botany* **6**, 199-208.

Strimmer, K. & von Haeseler, A. (1996). Quartet puzzling: A quartet maximum likelihood method for reconstructing tree topologies. *Molecular Biology and Evolution* **13**, 964-969.

Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4.* Sunderland, Massachusetts: Sinauer Associates.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. In *Nucleic Acids Research*, pp. 4876-4882: Distributed by the authors.

Turgeon, B., Bohlmann, H., Ciuffetti, L., Christiansen, S., Yang, G., Schafer, W. & Yoder, O. (1993a). Cloning and analysis of the mating type genes from *Cochliobolus heterostrophus*. *Mol Gen Genet* **238**, 270 - 284.

Turgeon, B. G. & Berbee, M. L. (1998). Evolution of pathogenic and reproductive strategies in *Cochliobolus* and related genera. In *Molecular Genetics of host-specific toxins in*

plant disease. Edited by K. a. Y. Kohmoto, O C. The Netherlands: Kluwer Academic Publishers.

Turgeon, B. G., Christiansen, S. K. & Yoder, O. C. (1993b). Mating type genes in ascomycetes and their imperfect relatives. In *The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics*, pp. 199-215. Edited by D. R. Reynolds & J. W. Taylor. Wallingford: CAB International.

Turgeon, G. & Yoder, O. C. (2000). Proposed nomenclature for mating type genes of filamentous ascomycetes. *Fungal Genetics and Biology* **31**, 1-5.

Waalwijk, C., Mendes, O., Verstappen, E. C. P., de Waard, M. A. & Kema, G. H. J. (2002). Isolation and characterization of the mating-type idiomorphs from the wheat septoria leaf blotch fungus *Mycosphaerella graminicola*. *Fungal Genetics and Biology* **35**, 277-286.

Webster, J. (1969). The *Pleospora* state of *Stemphylium triglochinicola*. *Transactions of the British Mycological Society* **53**, 478-482.

Yun, S. H., Berbee, M. L., Yoder, O. C. & Turgeon, G. (1999). Evolution of fungal self-fertile reproductive life style from self-sterile ancestors. *Proceedings of the National Academy of Sciences of the USA* **96**, 5592-5597.

Yun, S. H., Arie, T., Kaneko, I., Yoder, O. C. & Turgeon, B. G. (2000). Molecular organization of mating type loci in heterothallic, homothallic, and asexual *Gibberella/Fusarium* species. *Fungal Genetics and Biology* **31**, 7-20.

Zhan, J., Pettway, R. E. & McDonald, B. A. (2002). The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* **38**, 286-297.

CHAPTER 4. *Decorospora*, a new genus for the marine ascomycete *Pleospora gaudefroyi*[1]

[1] This chapter is a slightly altered version of the article: **Inderbitzin, P., Volkmann-Kohlmeyer, B., Kohlmeyer, J. & Berbee, M. L. (2002).** *Decorospora*, a new genus for the marine ascomycete *Pleospora gaudefroyi*. *Mycologia* **94**, 651-659. It was a collaboration with Jan Kohlmeyer and Brigitte Volkmann-Kohlmeyer, Institute of Marine Sciences, University of North Carolina at Chapel Hill, Morehead City, North Carolina. The contributions of the co-authors are gratefully acknowledged.

4.1. Introduction

Pleospora gaudefroyi Patouillard is a marine ascomycete described in 1886 from the northern coast of France. Morphological characters of *P. gaudefroyi* include black ascomata becoming superficial on the substrate at maturity (Figure 4-1), septate and branched pseudoparaphyses (Figure 4-4), fissitunicate, clavate asci (Figure 4-3, Figure 4-4), as well as yellow-brown ascospores (Figure 4-2) with seven transverse septa and one to three longitudinal septa in each segment (Kohlmeyer, 1962). Ascospores in *P. gaudefroyi* produce a characteristic gelatinous sheath that is thought to be exosporial in origin, having a tripartite outer boundary (Yusoff *et al.*, 1993). Upon release from the ascus, this hyaline layer swells once in contact with water, and transforms into a thick sheath, generally bearing two extensions at each polar region of the ascospores. One pair of extensions is formed on either side of the pole in a plane through the long axis of the ascospore. The planes stand at a 90° angle to each other, so that in side view only three of the extensions are visible. They are about as wide and long as the ascospore, and taper towards the apex (Kohlmeyer, 1962).

At one point, *P. gaudefroyi* was considered to be a synonym of *Pleospora herbarum* (Pers.: Fr.) Rabenhorst ex Cesati & de Notaris, the type species of the genus *Pleospora* (Wehmeyer, 1961). However, even though *Pleospora herbarum* and *P. gaudefroyi* are morphologically similar, an anamorph is unknown for *P. gaudefroyi,* and *P. herbarum* ascospores lack the sheath characteristic of *P. gaudefroyi.* Kohlmeyer (1962) reestablished *P. gaudefroyi* arguing that the presence of the ascospore sheath with its apical extensions, as well as the marine habitat of *P. gaudefroyi*, are sufficient to keep it separate from *P. herbarum.*

In this study, we are using phylogenetic analyses of partial SSU and ITS ribosomal DNA sequences to investigate the following questions: Are *P. gaudefroyi* and *P. herbarum* conspecific, congeneric, or should they be placed in distinct genera? In case *P. gaudefroyi* could not be retained in *Pleospora*, in which genus should it be placed instead?

4.2. Materials and methods

4.2.1 Molecular work

The culture of *Pleospora gaudefroyi* used for DNA extraction was derived from specimen J.K. 817 (on *Salicornia*, France) illustrated and discussed by Kohlmeyer (1962). We obtained the culture from the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands (CBS 332.63), where it had been deposited in 1963 by J. Kohlmeyer. DNA was isolated with a standard phenol-chloroform extraction (Lee & Taylor, 1990) from mycelium scraped off

a PDA Petri dish. The SSU ribosomal DNA region was PCR amplified by NS1 and cITS5, the complement to ITS5 (White *et al.*, 1990). Sequencing reactions were performed with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems, Mississauga, Canada) using NS1, NS2, and cITS5 (White *et al.*, 1990), NS19 (Gargas & Taylor, 1992), MB1, MB2, and Bas3 (Inderbitzin *et al.*, 2001). The ITS ribosomal DNA region was PCR amplified and sequenced by ITS4 / ITS5 (White *et al.*, 1990). Sequences were determined automatically on an ABI 377XL Automatic Sequencer (Perkin Elmer Corp., Norwalk, USA), and assembled in AutoAssembler Version 1.4 (Applied Biosystems, Perkin Elmer Corp., Norwalk, USA).

4.2.2 Phylogenetic analyses of SSU rDNA datasets

In a BLAST search, the SSU ribosomal DNA (SSU rDNA) sequence of *Pleospora gaudefroyi* had highest percentage similarity to species of *Pyrenophora*, *Cochliobolus*, *Pleospora*, *Setosphaeria*, and *Alternaria* in that order. A representative of each of these genera was included in the phylogenetic analyses. The remainder of the taxa retrieved from GenBank were chosen to represent the monophyletic sister group to *Rhytidhysteron rufulum* (Liew *et al.*, 2000; Winka & Eriksson, 1998). Thus, a total of 27 partial SSU rDNA sequences were retrieved from GenBank (Table 4-1). The sequences were manually aligned with the homologous sequence of *Pleospora gaudefroyi* using Se-Al v1.d1 (Rambaut, 1995). The resulting data matrix contained 28 taxa and 1720 characters. The following 14 sequences were approximately 1060 bp in length: *Trematosphaeria hydrela*, *Mycosphaerella citrullina*, *Sporormiella australis*, *Pseudotrichia aurata*, *Pleomassaria siparia*, *Phaeodothis winteri*, *Montagnula opulenta*, *Massariosphaeria phaeospora*, *Melanomma pulvis-pyrius*, *Massarina australiensis*, *Massaria platani*, *Leptospora rubella*, *Didymella exigua*, and *Delitschia winteri*. The sequence of *Rhytidhysteron rufulum* was approximately 1600 bp in length, whereas the remaining 13 sequences, including *P. gaudefroyi*, were around 1700 bp long. The data matrix was analysed in PAUP* 4.0b3 (Swofford, 2001) using parsimony and Neighbor joining with default settings, unless noted otherwise. *Rhytidhysteron rufulum* was used as outgroup. This alignment was submitted to TreeBASE (M1158).

Parsimony trees were inferred in 30 heuristic searches with random addition of taxa. All characters were weighted equally, and gaps were treated as missing data. In Neighbor joining analyses, the Jukes-Cantor distance correction was used, since the estimated pairwise Jukes-Cantor distances between the taxa were around 0.05 substitutions per site (Kumar *et al.*, 1993): Out of 378 possible pairwise comparisons for the 28 taxa, 13 were above 0.05 substitutions per site. The maximal estimated pairwise distance was 0.058 substitutions per site between *P. gaudefroyi* and *Phaeodothis winteri*. Bootstrap support for the branches was based on 500 replicates with random taxon ad-

dition. The parsimony-based Kishino-Hasegawa test was applied using default settings in PAUP* (Swofford, 2001).

For computational reasons, a smaller dataset comprising the taxa of the Pleosporaceae (*Alternaria alternata*, *Cochliobolus sativus*, *Pleospora herbarum*, *Pyrenophora tritici-repentis*, *Setosphaeria rostrata*) and *P. gaudefroyi* was used in the likelihood-based, non-parametric Shimodaira-Hasegawa tests (SHT) as implemented in the program SHTests v1.0 (Rambaut, 2000). A Jukes-Cantor model of evolution was used. The number of bootstrap replicates was 500. The topology of the most likely tree needed for the SHT was obtained in PAUP* (Swofford, 2001) using a Jukes-Cantor model of evolution.

4.2.3 Phylogenetic analyses of the ITS rDNA dataset

Species of *Alternaria* were closest matches to the ITS ribosomal DNA (ITS rDNA) sequence of *P. gaudefroyi* in a BLAST search. Clustal W (1.74) (Thompson *et al.*, 1994) was used for aligning the *P. gaudefroyi* ITS rDNA sequence with homologous regions from the following taxa retrieved from GenBank (Table 4-1): *Alternaria alternata*, *Cochliobolus sativus*, two different sequences named *Leptosphaeria maculans*, *Pleospora herbarum*, *Pyrenophora tritici-repentis*, and *Setosphaeria rostrata*. The resulting dataset contained 8 taxa and 625 characters. Parsimony analyses were performed as described for the large SSU rDNA dataset. *Leptosphaeria maculans* (M96384) was used as outgroup.

4.3. Results

4.3.1 Phylogenetic analyses of the SSU rDNA dataset

The SSU ribosomal DNA dataset consisting of 28 taxa was subjected to parsimony analyses, with *Rhytidhysteron rufulum* as the outgroup. Out of the 1720 characters, 215 were variable (12.5%), of which 117 were parsimony informative (6.8%). One most parsimonious (MP) tree was obtained, measuring 348 steps (CI = 0.690, RI = 0.794).

This study focused on the placement of *P. gaudefroyi* which in both MP and Neighbor joining (NJ) trees was unambiguous: *Pleospora gaudefroyi* grouped with 100% support as the sister group to the Pleosporaceae (Figure 4-5). The monophyly of the Pleosporaceae without *P. gaudefroyi* was supported by 94% bootstrap support in both MP and NJ analyses. Outside the *P. gaudefroyi*-Pleosporaceae group, at a 60% bootstrap support-level, the branching order of the NJ tree did not contradict results obtained in other studies (data not shown) (Liew *et al.*, 2000; Winka & Eriksson, 1998). The MP tree differed by the disposition of *Massariosphaeria phaeospora*, which was sister taxon to a poorly supported clade, containing taxa from *Didymella exigua* to *P. herbarum* (Figure 4-5).

The topology obtained within the Pleosporaceae excluding *P. gaudefroyi* in MP and NJ tree was conflicting: Whereas *Alternaria alternata* grouped with 94% bootstrap support with *Pleospora herbarum* in the MP analyses (Figure 4-5), *A. alternata* was basal in the NJ tree (data not shown). The next taxa to branch off in the NJ tree were *Setosphaeria rostrata*, then *P. herbarum*, followed by *Cochliobolus sativus* and *Pyrenophora tritici-repentis*. The bootstrap supports ranged from 58 to 74% (data not shown).

The conflict between NJ and MP trees was consistent with previous work which also provided contradicting information about the branching order within the Pleosporaceae: Based on ITS and GPD sequences, Berbee et al. (1999) found that the relationship between species of *Pleospora* and *A. alternata* could not be resolved.

4.3.2 Kishino-Hasegawa test

To investigate the possibility of a monophyletic genus *Pleospora*, *P. gaudefroyi* was constrained to the group with *P. herbarum* in the large dataset. In this scenario, nine most parsimonious trees were obtained, measuring 359 steps each, 11 steps more than the MP tree. According to the parsimony-based Kishino-Hasegawa test (KHT), all of the constrained MP trees were significantly worse than the unconstrained MP tree ($P < 0.05$).

4.3.3 Shimodaira-Hasegawa tests

The KHT was designed to compare the fit of two *a priori* specified tree topologies to a dataset (Goldman *et al.*, 2000). However, we wanted to test if *a priori* topologies with a monophyletic genus *Pleospora* were significantly different from the best tree derived from the dataset. The appropriate test to use in this case was the likelihood-based non-parametric Shimodaira-Hasegawa tests (SHT) (Goldman *et al.*, 2000). This test allowed multiple comparisons of *a priori* hypotheses to the ML tree inferred from the dataset. For the small dataset containing six taxa, 15 *a priori* hypotheses could be formulated corresponding to the 15 unrooted tree topologies with *P. herbarum* and *P. gaudefroyi* as sister taxa. Subsequently, the number of *a priori* hypotheses was reduced to three, since we considered *Cochliobolus sativus* and *Setosphaeria rostrata* to be sister taxa. This assumption was based on a study by Berbee et al. (1999) where the phylogenetic relationships of species of *Alternaria*, *Cochliobolus*, *Pleospora*, *Pyrenophora*, and *Setosphaeria*, were investigated using a dataset with ITS and *GPD* sequences. The results showed members of *Cochliobolus* and *Setosphaeria* to be sister groups with 72% bootstrap support. This agreed with results from RAPD data, where representatives of *Cochliobolus* and *Setosphaeria* were more similar to one another than either one of them to *Pyrenophora* (Bakonyi *et al.*, 1995). *Pyrenophora* also differed ecologically from both *Cochliobolus* and *Setosphaeria*: Members of *Pyrenophora* were pre-

dominantly found on grasses of the Pooideae, whereas *Cochliobolus* and *Setosphaeria* occurred generally on members of the Chloridoideae (Alcorn, 1983; Watson & Dallwitz, 1992). Thus, using the program SHTests v1.0 (Rambaut, 2000), the three *a priori* hypotheses were compared to the ML tree obtained in PAUP*. All three were significantly worse than the ML tree ($P < 0.02$).

4.3.4 Phylogenetic analysis of the ITS rDNA dataset

The ITS rDNA dataset consisting of eight taxa was subjected to a parsimony analysis with *Leptosphaeria maculans* (M96384) as outgroup. Out of 625 characters, 198 were variable (32%), of which 113 were parsimony informative (18%). One most parsimonious (MP) tree was obtained, measuring 410 steps (CI = 0.759, RI = 0.434). *Pleospora gaudefroyi* grouped with 100% bootstrap support with *Alternaria alternata*, *Cochliobolus sativus, Pleospora herbarum*, *Pyrenophora tritici-repentis*, and *Setosphaeria rostrata* (data not shown). The latter five taxa formed a monophyletic group with 61% bootstrap support. The remaining bootstrap support percentages were below 50% (data not shown). The ITS alignment contained many ambiguously aligned sites, so that we chose to emphasize results based on easily alignable SSU rDNA data. However, the placement of *P. gaudefroyi* in ITS rDNA analyses was consistent with results from SSU rDNA data.

4.4. Discussion

4.4.1 Pleospora gaudefroyi transferred to new genus *Decorospora*

Phylogenetic analyses of SSU and ITS rDNA data, as well as test results of SSU rDNA datasets supported morphological and ecological data suggesting that *P. gaudefroyi* and *P. herbarum* were distinct species. The molecular analyses further revealed that *P. gaudefroyi* should be transferred to another genus. In both parsimony and Neighbor joining analyses of the SSU rDNA dataset, representatives of the Pleosporaceae without *P. gaudefroyi* clustered together with 100% bootstrap support (Figure 4-5). In an ITS rDNA analysis, *P. gaudefroyi* clustered with 100% bootstrap support with members of the Pleosporaceae as well (data not shown). However, constraining the genus *Pleospora* to be monophyletic resulted in significantly worse trees as evaluated by the Kishino-Hasagewa and Shimodaira-Hasegawa tests using SSU rDNA datasets. Thus, *P. gaudefroyi* was excluded from *Pleospora*, and transferred to the new genus *Decorospora*. The establishment of a new genus was necessary due to the lack of any existing genus in the Dothideomycetes characterized by a *Pleospora*-like morphology combined with ornamented ascospores.

4.4.2 *Decorospora*, a new genus in the Pleosporaceae

Phylogenetic analyses indicated that of all included taxa, *D. gaudefroyi* was closest related to members of the Pleosporaceae sensu Eriksson (1999): In both parsimony and Neighbor joining analyses, *D. gaudefroyi* and the remainder of the Pleosporaceae formed a monophyletic group with 100% bootstrap support (Figure 4-5). Thus, *Decorospora* is placed in the Pleosporaceae.

4.5. Taxonomy

4.5.1 The new genus *Decorospora*

Decorospora Inderbitzin, Kohlm. et Volkm.-Kohlm. gen. nov.
Genus Pleosporacearum. Ascomata subglobosa ad ellipsoidea, immersa, ostiolata, epapillata vel breve papillata, carbonacea, nigra. Peridium cellulis pachydermis luminibus grandis, in sectione longitudinali texturam angularem formantibus. Hamathecium pseudoparaphysibus septatis, ramosis. Asci octospori, clavati, breve pedunculati, pachydermi, fissitunicati, sine apparatu apicale. Ascosporae biseriatae, ellipsoideae, muriformes, brunneae, tunica gelatinosa tectae; tunica extensa ad apices ambos in 2 vel 3 protuberationes subconicas.

A genus of Pleosporaceae. *Ascomata* subglobose to ellipsoidal, immersed, ostiolate, epapillate or short papillate, carbonaceous, black (Figure 4-1). *Peridium* composed of thick-walled cells with large lumina, forming a *textura angularis* in longitudinal section. *Hamathecium* composed of septate, ramose pseudoparaphyses (Figure 4-4). *Asci* eight-spored, clavate, short pedunculate, thick-walled, fissitunicate, without apical apparatuses (Figure 4-3, Figure 4-4). *Ascospores* biseriate, ellipsoidal, muriform, brown, covered by a gelatinous sheath that is slightly constricted around the center and drawn out at each apex into 2 or rarely 3 subconical extensions (Figure 4-2).

Type species. Decorospora gaudefroyi (Pat.) Inderbitzin, Kohlm. & Volkm.-Kohlm.

Etymology. From the Latin *decorus*: beautiful, and *sporus*: spore, in reference to the ornate ascospores.

4.5.2 Transfer of *Pleospora gaudefroyi* to *Decorospora*

Decorospora gaudefroyi (Pat.) Inderbitzin, Kohlm. et Volkm.-Kohlm., comb. nov., Figure 4-1 - Figure 4-4.

Basionym: *Pleospora gaudefroyi* Pat., Tabulae Analyticae Fungorum, Paris, Deuxième Sér., p. 40, No. 602, 1886.
≡ *Pleospora salsolae* Fuckel var. *schoberiae* Sacc., Michelia 2, 69. 1880.
≡ *Pleospora schoberiae* (Sacc.) Berl., Icon. Fung. 2, 23. 1895.
= *Pleospora lignicola* J. Webster & M. T. Lucas, Trans. Brit. Mycol. Soc. 44, 431. 1961.

= *Pleospora salicorniae* Jaap, Verh. Bot.Ver. Prov. Brandenburg 49, 16. 1907 (non *Pleospora salicorniae* P. A. Dang. 1888).
≡ *Pleospora herbarum* (Fr.) Rabenh. var. *salicorniae* (Jaap) Jaap, Ann. Mycol. 14, 17. 1916 (non *Pleospora herbarum* f. *salicorniae* Auersw. *in* Rabenhorst, Fungi Europaei Exsiccati, Cent. 2, No. 145. 1860, invalid name).

4.5.3 Specimens examined

FRANCE. PAS DE CALAIS: Marais de la Pointe de Touquet, near Etaples, 56°12'30"N, 0°48'40"W, on *Suaeda maritima*, 15 Aug. 1879, *O. Hariot* (HOLOTYPE PC); sub *Pleospora salsolae* f. *schoberiae*, from Herb. E. Gaudefroy. CROATIA: Island of Rab, Lopar, 44°49'N, 14°45'E, on *Halimione portulacoides*, 16 Oct. 1971, *J. & E. Kohlmeyer* J.K. 2903 (IMS); same location and date, on *Salicornia* sp., *J. & E. Kohlmeyer* J.K. 2904 (IMS). ARGENTINA. BUENOS AIRES: Near Villa del Mar, SE of Bahia Blanca, 38°49'S, 62°19'W, on *Salicornia* sp., 23 Oct. 1973, *J. & E. Kohlmeyer* J.K. 3520 & 3522 (IMS); same location and date, on *Salicornia ambigua*, *J. & E. Kohlmeyer* J.K. 3521 (IMS). CANADA. BRITISH COLUMBIA: On small island in Malaspina Inlet near Lund, ca. 50°03'N, 124°47'W, on *Salicornia virginica*, *P. Inderbitzin* P162 (UBC F14076). Data on other collections has already been reported in the literature (see paragraph on Geographic Distribution).

4.5.4 Commentary

Decorospora gaudefroyi has been fully described and illustrated by Kohlmeyer & Kohlmeyer (Kohlmeyer & Kohlmeyer, 1964; 1979). Yusoff et al. (1993) described the ultrastructure of *D. gaudefroyi* ascospores with surrounding sheath while still in the ascus (i.e., without the unfolded sheath extensions), and Hyde et al. (1986) depicted an ascospore with extended sheath in SEM. Ascospore ornamentations, found among many marine ascomycetes (Kohlmeyer & Volkmann-Kohlmeyer, 1991) are considered adaptations to the marine habitat, enhancing the attachment of spores to submerged substrates (Hyde *et al.*, 1986). Because of its characteristically ornamented ascospores (Figure 4-2), *D. gaudefroyi* cannot be confused with any other marine ascomycete. A superficially similar species is *Nimbospora octonae* Kohlm. (Halosphaeriales) which, however, has ascospores with a gelatinous sheath, enclosing a number of subulate appendages (Kohlmeyer, 1985). Other marine species with somewhat extended ascospore sheaths are *Frondicola tunitricuspis* K.D. Hyde and *Carinispora nypae* K.D. Hyde (1992), whereas the sheath in *Massarina armatispora* K.D. Hyde, Vrijmoed, Chinnaraj & E. B. G. Jones appears simply drawn out at the poles (Hyde *et al.*, 1992). Ascospore sheaths without extensions occur frequently also in terrestrial ascomycetes, e.g. in *Phaeosphaeria* and *Massariosphaeria* (Eriksson, 1967; Leuchtmann, 1984).

4.5.5 Substrates

Decorospora gaudefroyi is an obligate marine fungus, growing at or above the high water mark. It is not host-specific, as it occurs on a variety of cellulosic substrates, such as dead marsh plants, driftwood and pilings. Among the host plants found so far are *Halimione portulacoides* (L.) Aellen, *Salicornia ambigua* Michx., *Salicornia virginica* L., *Salicornia* spp., and *Suaeda maritima* (L.) Dum. The species is able to grow under conditions of high salinity, as it was found in a salina in southern France with a salinity of 60‰, and formed ascomata even on the salt-encrusted top of a piling (Kohlmeyer, 1962). In pure culture *D. gaudefroyi* grows well, decomposes balsa wood, and even dissolves cellulose of a tunicate mantle (Kohlmeyer, 1962).

4.5.6 Geographic distribution

Decorospora gaudefroyi appears to be restricted to temperate waters. In Europe it was collected at the northern coast of France (Patouillard, 1886), at the Mediterranean coast of France (Kohlmeyer, 1962), at the North Sea coast of England (Webster & Lucas, 1961), at the German coast of the North Sea (Jaap, 1907), and in Croatia (this paper). In North America the species was found in Massachusetts, USA (Gessner & Lamore, 1978) and in Canada (British Columbia, this paper). The only collections of *D. gaudefroyi* from the southern hemisphere are from Argentina (this paper). *Decorospora gaudefroyi* can be compared in its habitat and geographical distribution with *Passeriniella obiones* (P. Crouan & H. Crouan) K. D. Hyde & Mouzouras. The latter grows also on decaying marsh plants and wood, and occurs throughout Europe, on the United States east coast, in British Columbia and Argentina (Kohlmeyer & Kohlmeyer, 1979).

4.6. Acknowledgements

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Table 4-1. GenBank accession numbers and classification of the species used in this study. New sequences are in bold.

Taxon	Accession No. SSU rDNA	Accession No. ITS rDNA	Order / Family [a]
Alternaria alternata (Fr.:Fr.) Keissler	U05194	AF071346	Pleosporales / Pleosporaceae [b]
Cochliobolus sativus (Ito & Kurib.) Drechsler ex Dasturin	U42479	AF071325	Pleosporales / Pleosporaceae
Decorospora gaudefroyi (Pat.) Inderbitzin, Kohlm. & Volkm.-Kohlm.	AF394542	AF394541	Pleosporales / Pleosporaceae [c]
Delitschia winteri W. Phillips & Plowr.	AF164354	-	Pleosporales / Delitschiaceae
Didymella exigua (Niessl) Sacc.	AF164355	-	incertae sedis
Didymosphaerella opulenta (De Not.) Checa & M. E. Barr	AF164370	-	incertae sedis / Montagnulaceae
Herpotrichia diffusa (Schw.) Ellis & Everh.	U42484	-	Pleosporales / Lophiostomataceae
Kirschsteiniothelia elaterascus Shearer	AF053727	-	Pleosporales / Pleosporaceae
Leptosphaeria maculans (Desm.) Ces. & De Not.	U04233	M96383 / M96384	Pleosporales / Leptosphaeriaceae
Leptospora rubella (Pers.:Fr.) Rabenh.	AF164361	-	incertae sedis
Lophiostoma crenatum (Pers.:Fr.) Fuckel	U42485	-	Pleosporales / Lophiostomataceae
Massaria platani Ces.	AF164363	-	Pyrenulales [b] / Massariaceae [b]
Massarina australiensis K. D. Hyde	AF164364	-	Pleosporales / Lophiostomataceae
Massariosphaeria phaeospora (Müll.) Criv.	AF164368	-	Pleosporales / Lophiostomataceae
Melanomma pulvis-pyrius (Pers.:Fr.) Fuckel	AF164369	-	Pleosporales / Melanommataceae
Mycosphaerella citrullina (C. O. Sm.) Grossenb.	U79487	-	incertae sedis / Mycosphaerellaceae
Phaeodothis winteri (Niessl) Aptroot	AF164371	-	incertae sedis / Phaeosphaeriaceae [b]
Phaeosphaeria nodorum (E. Müll.) Hedj.	U04236	-	incertae sedis / Phaeosphaeriaceae
Pleomassaria siparia (Berk. & Broome) Sacc.	AF164373	-	incertae sedis / Pleomassariaceae
Pleospora herbarum (Pers.:Fr.) Rabenh. ex Ces. & De Not.	U05201	AF071344	Pleosporales / Pleosporaceae

Pseudotrichia aurata (Rehm) Wehm.	AF164374	-	Pleosporales / Melanommataceae
Pyrenophora tritici-repentis (Died.) Drechsler	U42486	AF071348	Pleosporales / Pleosporaceae
Rhytidhysteron rufulum (Spreng.) Speg.	AF201452	-	Patellariales / Patellariaceae
Setosphaeria rostrata K. J. Leonard	U42487	AF071342	Pleosporales / Pleosporaceae
Sporormia lignicola W. Phillips & Plowr.	U42478	-	incertae sedis / Sporormiaceae
Sporormiella australis (Speg.) Ahmed & Cain	U79483	-	incertae sedis / Sporormiaceae
Trematosphaeria hydrela (Rehm) Sacc.	AF164376	-	Pleosporales / Melanommataceae
Westerdykella dispersa (Clum) Cejp & Milko	U42488	-	incertae sedis / Sporormiaceae

[a] According to Eriksson et al. (2001).
[b] According to GenBank.
[c] This paper.

Figure 4-1. ***Decorospora gaudefroyi*** **from** ***Salicornia*** **sp., Argentina. Longitudinal section (20 µm) through ascoma (J.K. 3521). From Kohlmeyer, BioScience 25:89, 1975, reprinted with permission. Bar = 50 µm.**

Figure 4-2. ***Decorospora gaudefroyi*** **from** ***Salicornia*** **sp., Argentina. Ascospores enclosed in gelatinous sheaths with apical extensions (J.K. 3520). From Kohlmeyer, McIlvainea 6:46, 1984, reprinted with permission. Both in Nomarski interference contrast. Bar = 20 µm.**

Figure 4-3. ***Decorospora gaudefroyi*** **from** ***Salicornia*** **sp., Croatia. Immature ascus, ascospores enclosed in gelatinous sheaths (J.K. 2904). From Kohlmeyer, McIlvainea 6:46, 1984, reprinted with permission. Bar = 20 µm.**

Figure 4-4. 4. ***Decorospora gaudefroyi*** **from** ***Suaeda maritima*****, France. Mature asci and pseudoparaphyses (HOLOTYPE). Both in Nomarski interference contrast. Bar = 25 µm.**

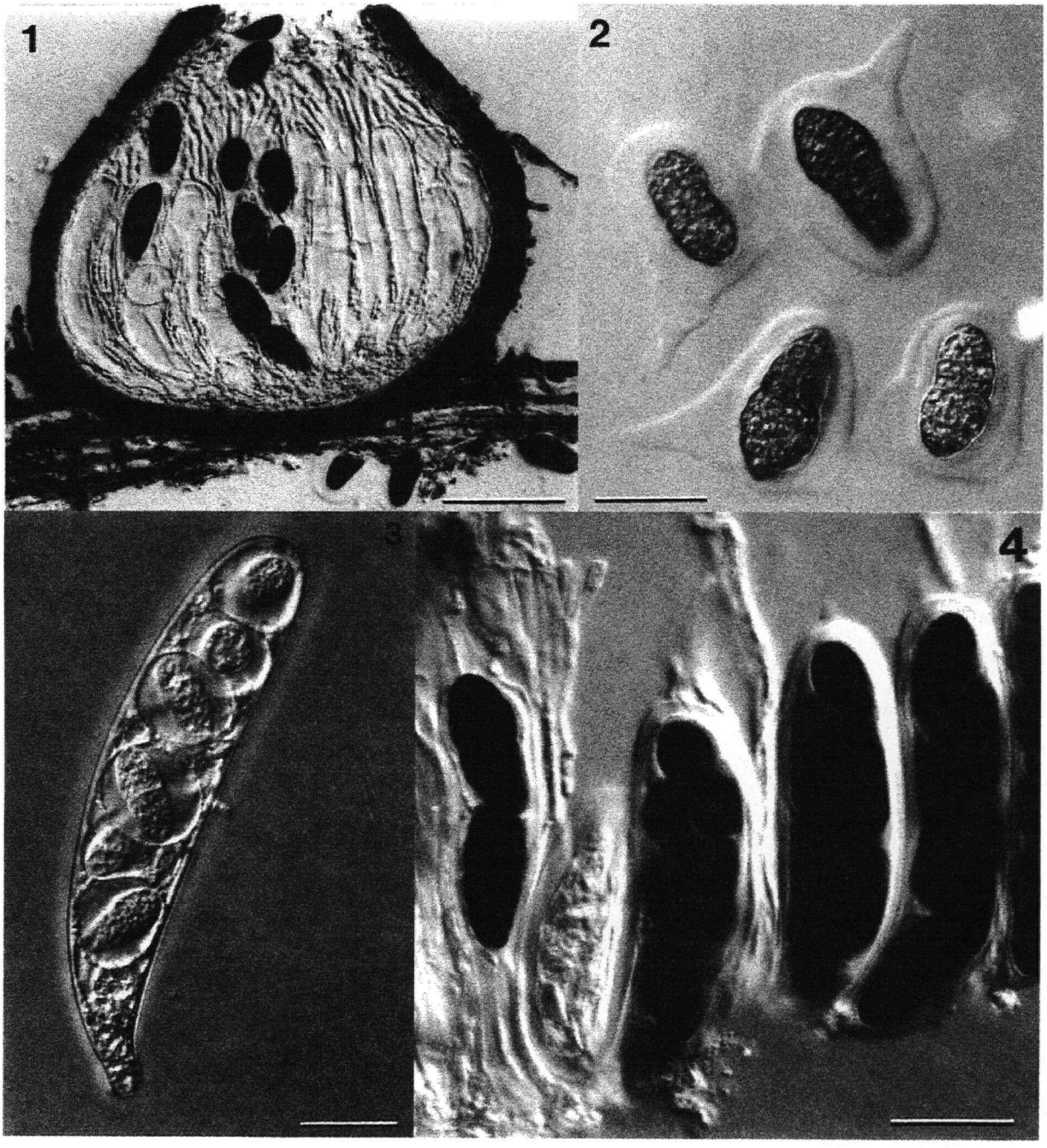
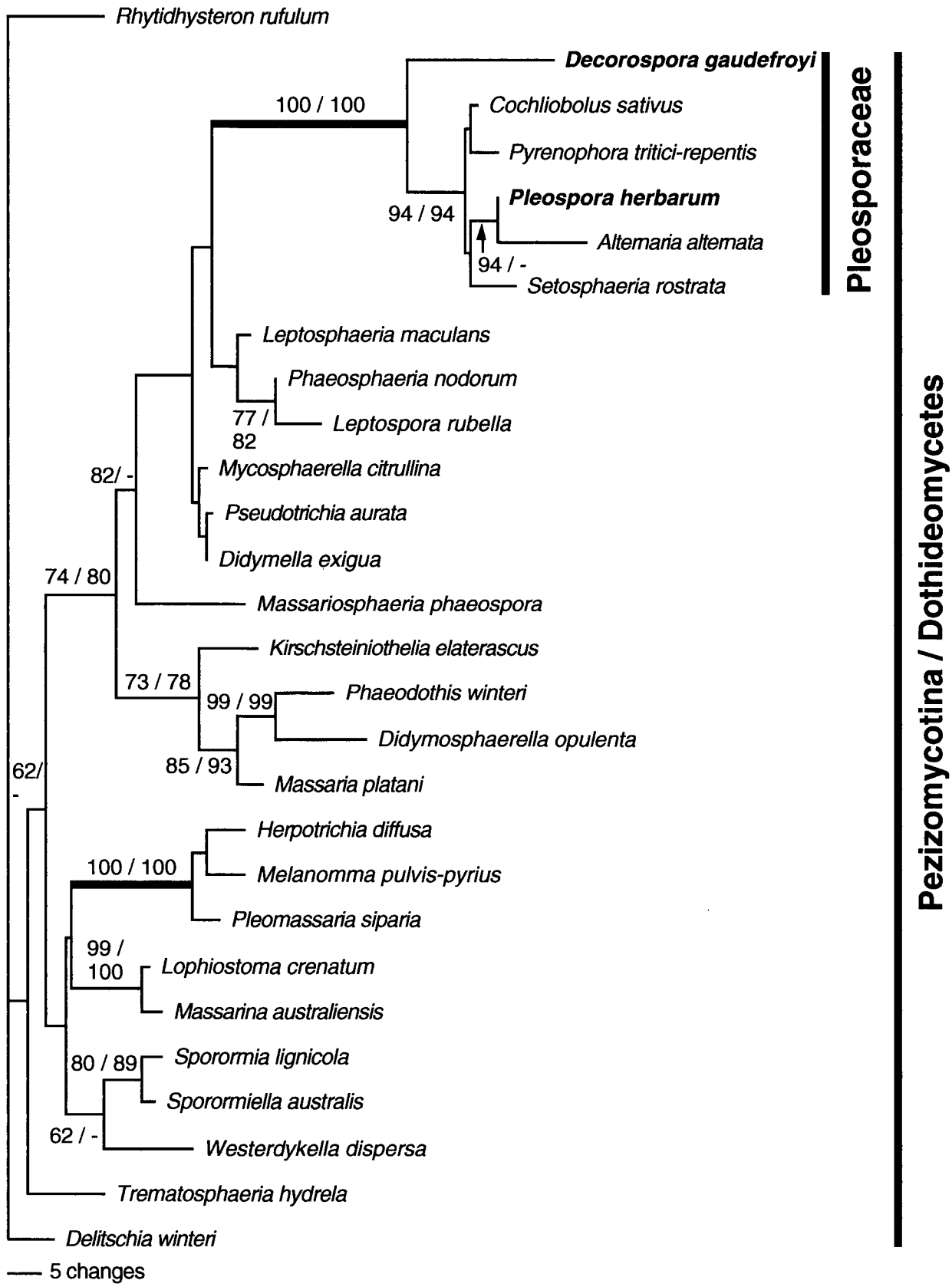

1
2
3
4

Figure 4-5. Single most parsimonious tree obtained from a SSU rDNA dataset containing 28 taxa and 1720 characters, using *Rhytidhysteron rufulum* as outgroup (tree length = 348 steps; CI = 0.690; RI = 0.794). Higher taxonomic levels are given on the right, and follow Eriksson et al. (2001) for the most part (see Table 4-1 for details). Numbers by the branches are bootstrap support percentages in parsimony and Neighbor joining analyses. Branches with 100% bootstrap support in both analyses are in bold. *Decorospora gaudefroyi* and *Pleospora herbarum* were the focus of this study and are therefore in bold. *Decorospora gaudefroyi* grouped with 100% bootstrap support in both analyses with taxa in the Pleosporaceae. Note that *D. gaudefroyi* and *P. herbarum* were not closest relatives: The Pleosporaceae without *D. gauderoyi* were supported by 94% of the bootstrap replicates in both analyses. Exclusion of *D. gaudefroyi* from *Pleospora* was also suggested by results from both Kishino-Hasegawa and Shimodaira-Hasegawa tests which showed that constraining the genus *Pleospora* to be monophyletic yielded significantly worse trees (see text for details).


Rhytidhysteron rufulum
Decorospora gaudefroyi
100 / 100
Cochliobolus sativus
Pyrenophora tritici-repentis
94 / 94
Pleospora herbarum
Alternaria alternata
94 / -
Setosphaeria rostrata
Pleosporaceae
Leptosphaeria maculans
Phaeosphaeria nodorum
77 / 82
Leptospora rubella
82/ -
Mycosphaerella citrullina
Pseudotrichia aurata
Didymella exigua
74 / 80
Massariosphaeria phaeospora
Kirschsteiniothelia elaterascus
73 / 78
99 / 99
Phaeodothis winteri
Didymosphaerella opulenta
85 / 93
Massaria platani
62/ -
Herpotrichia diffusa
100 / 100
Melanomma pulvis-pyrius
Pleomassaria siparia
99 / 100
Lophiostoma crenatum
Massarina australiensis
80 / 89
Sporormia lignicola
Sporormiella australis
62 / -
Westerdykella dispersa
Trematosphaeria hydrela
Delitschia winteri
5 changes
Pezizomycotina / Dothideomycetes

4.7. Bibliography

Alcorn, J. L. (1983). Generic concepts in *Drechslera, Bipolaris* and *Exserohilum. Mycotaxon* **17**, 1-86.

Bakonyi, J., Pomázi, A., Fischl, G. & Hornok, L. (1995). Comparison of selected species of *Bipolaris, Drechslera* and *Exserohilum* by random amplification of polymorphic DNA. *Acta Microbiologica et Immunologica Hungarica* **42**, 355-366.

Berbee, M. L., Pirseyedi, M. & Hubbard, S. (1999). *Cochliobolus* phylogenetics and the origin of known, highly virulent pathogens, inferred from ITS and *glyceraldehyde-3-phosphate dehydrogenase* gene sequences. *Mycologia* **91**, 964-977.

Eriksson, O. (1967). On graminicolous pyrenomycetes from Fennoscandia. 2. Phragmosporous and scolecosporous species. *Arkiv for Botanik* **6**, 381-441.

Eriksson, O. E. (1999). Outline of Ascomycota - 1999. *Myconet* **3**, 1-88.

Eriksson, O. E., Baral, H.-O., Currah, R. S., Hansen, K., Kurtzman, C. P., Rambold, G. & Laessøe, T. (2001). Outline of Ascomycota - 2001. *Myconet* (published on the internet).

Gargas, A. & Taylor, J. W. (1992). Polymerase Chain Reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. *Mycologia* **84**, 589-592.

Gessner, R. V. & Lamore, B. J. (1978). Fungi from Nantucket salt marshes and beaches. *Rhodora* **80**, 581-586.

Goldman, N. J., Anderson, P. & Rodrigo, A. G. (2000). Likelihood-based tests of topologies in phylogenetics. *Systematic Biology* **49**, 652-670.

Hyde, K. D. (1992). Fungi from decaying intertidal fronds of *Nypa fruticans*, including three new genera and four new species. *Botanical Journal of the Linnean Society* **110**, 95-110.

Hyde, K. D., Jones, E. B. G. & Moss, S. T. (1986). How do fungal spores attach to surfaces? In *Biodeterioration 6*, pp. 584-589. Edited by H. D. Barry S, Llewelyn GC, O'rea CE. London: Commonwealth Agricultural Bureau and the Biodeterioration Society.

Hyde, K. D., Vrijmoed, L. L. P., Chinnaraj, S. & Jones, E. B. G. (1992). *Massarina armatispora* sp. nov., a new intertidal ascomycete from mangroves. *Botanica Marina* **35**, 325-328.

Inderbitzin, P., Landvik, S., Abdel-Wahab, M. A. & Berbee, M. L. (2001). Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. *American Journal of Botany* **88**, 52-61.

Inderbitzin, P., Volkmann-Kohlmeyer, B., Kohlmeyer, J. & Berbee, M. L. (2002). *Decorospora*, a new genus for the marine ascomycete *Pleospora gaudefroyi. Mycologia* **94**, 651-659.

Jaap, O. (1907). Zweites Verzeichnis zu meinem Exsiccatenwerk "Fungi selecti exsiccati", Serien V-VIII (Nummern 101-200), nebst Beschreibungen neuer Arten und Bemerkungen. *Verh Bot Ver Prov Brandenburg* **49**, 7-29.

Kohlmeyer, J. (1962). Über *Pleospora gaudefroyi* Patouillard. *Willdenowia* **3**, 315-324.

Kohlmeyer, J. (1985). *Nimbospora octonae*, a new marine ascomycete from Hawaii. *Canadian Journal of Botany* **63**, 1122--1125.

Kohlmeyer, J. & Kohlmeyer, E. (1964). *Icones Fungorum Maris. Fasc I.* Weinheim: J. Cramer.

Kohlmeyer, J. & Kohlmeyer, E. (1979). *Marine mycology: the higher fungi.* New York: Academic Press.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. (1991). Illustrated key to the filamentous higher marine fungi. *Botanica Marina* **34**, 1-61.

Kumar, S., Tamura, K. & Nei, M. (1993). *MEGA: Molecular Evolutionary Genetics Analysis, version 1.01*: The Pennsylvania State University, University Park, PA 16802.

Lee, S. B. & Taylor, J. W. (1990). Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols*, pp. 282-287. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Leuchtmann, A. (1984). Über *Phaeosphaeria* Miyake und andere bitunicate Ascomyceten mit mehrfach querseptierten Ascosporen. *Sydowia* **37**, 75-194.

Liew, E. C. Y., Aptroot, A. & Hyde, K. D. (2000). Phylogenetic significance of the pseudoparaphyses in Loculoascomycete taxonomy. *Molecular Phylogenetics and Evolution* **16**, 392-402.

Patouillard, N. (1886). *Tabulae Analyticae Fungorum.* Paris: Klincksieck.

Rambaut, A. (1995). *Se-Al v1.d1*, Version 1.0 alpha edn. Oxford, U.K: Department of Zoology, University of Oxford.

Rambaut, A. (2000). Shimodaira and Hasegawa Tests of Phylogenetic Hypotheses SHTests v1.0.

Swofford, D. L. (2001). *PAUP*. Phylogenetic analysis using parsimony (*and other methods). Version 4.0b5.* Sunderland, Massachusetts: Sinauer Associates.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**, 4673-4680.

Watson, L. & Dallwitz, M. J. (1992). *The grass genera of the world.* Wallingford, Oxon, UK: CAB International.

Webster, J. & Lucas, M. T. (1961). Observations on British species of *Pleospora*. II. *Transactions of the British Mycological Society* **44**, 417-436.

Wehmeyer, L. E. (1961). *A world monograph of the genus Pleospora and its segregates.* Ann Arbor: The University of Michigan Press.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols*, pp. 315-322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Winka, K. & Eriksson, O. E. (1998). Molecular evidence for recognizing the Chaetothyriales. *Mycologia* **90**, 822-830.

Yusoff, M., Moss, S. T. & Jones, E. B. G. (1993). Ascospore ultrastructure of *Pleospora gaudefroyi*. *Canadian Journal of Botany* **72**, 1-6.

CHAPTER 5. Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata[2]

[2] This chapter is a slightly modified version of the article: **Inderbitzin, P., Landvik, S., Abdel-Wahab, M. A., & Berbee, M. L. (2001).** Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. *American Journal of Botany* **88**: 52-61. It was a collaboration with Sara Landvik, a postdoc in our lab, and Mohamed A. Abdel-Wahab from the Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong Special Administrative Region, People's Republic of China. I would like to thank the collaborators for their contributions.

5.1. Introduction

Microfungi are rarely collected and poorly known. The majority of known microfungi belong to the Ascomycota, the largest phylum of the fungi with more than 30,000 described species (Hawksworth *et al.*, 1995). Hawksworth et al. (1997) estimated that the total number of known Fungi constitute only 5% of the existing mycota. For the Ascomycota alone, that would leave 570,000 species to be discovered. This lack of knowledge of microfungal diversity greatly hampers our capability of correctly inferring and understanding many aspects of fungal biology and phylogeny.

Not surprisingly, taxonomic studies of microfungi from little explored areas regularly yield high numbers of undescribed species. For instance, in a monograph of the Coronophorales (Ascomycetes) from India, Subramanian and Sekar (1990) found that ten out of 23 fungi (43%) collected from the Western Ghats were new to science (Hawksworth, 1991).

On two short surveys of ascomycetous, fruitbody-forming microfungi on decaying wood in Thailand and southern China, we found a total of six species. Only one fungus could be named with certainty, and five seemed to be undescribed. The time invested in collecting decaying wood in the field and the amount of material returned to the laboratory for microscopic examination were minimal: less than 1 h was spent collecting and approximately half the volume of a large backpack of decaying wood was returned to the laboratory.

The number of existing Fungi worldwide has been estimated to 1.5 million species, based on the 1:6 ratio of vascular plants to fungi on the British Isles (Hawksworth, 1991). For a country like China, with ~ 27,000 species of vascular plants (Eriksson & Yue, 1988), and ~ 7000 species of Fungi (Tai, 1979), it follows that 155,000 Fungi or more than 90% of the mycota present in China would yet have to be found. For Thailand, no comparable reliable data were available (R. Bandoni, personal communication, Ladner, British Columbia, Canada).

Two of the new fungi that we found, one from Thailand and one from China, were particularly interesting. They are described here as members of the new genus *Aliquandostipite*, placed in the new family Aliquandostipitaceae.

Initial examination of hyphae and ascomata (organs of sexual sporogenesis in ascomycetes) suggested the two new fungi were strikingly different from known ascomycetes. Both species of *Aliquandostipite* were characterized by the presence of hyphae that were five times wider than the widest hyphae known in ascomycetes. In ascomycetes in general, ascomata form superficially or immersed in the substrate, becoming superficial at maturity. In species of *Aliquandostipite*, however, ascomata seemed to be either borne by thick hyphae, which function as stalks, or were unstalked and erumpent from the substratum at maturity. The conventional, sessile ascomata and the stalked as-

comata, resembling a tiny moss sporophyte more than a fungus, were present side by side on the substratum. By comparing the morphology of ascomata from nature and observing ascomata in culture, we investigated whether stalked and sessile forms represented dimorphisms within a species.

Initial observation suggested that species of *Aliquandostipite* belong to the Dothideomycetes (Eriksson & Winka, 1998), a group of ascomycetes formerly referred to as Loculoascomycetes. Members of this group are characterized by the presence of a functionally bilayered ascus wall developing in a lysogenic cavity, the centrum, within a compact hyphal body, called the ascoma. Fungi of the Dothideomycetes have traditionally been separated into orders based on the morphology of their centrum tissue (Barr, 1987). The centrum tissue consists of asci containing sexual spores and sterile filaments, which sometimes are present intermingled among the asci. The presence or absence of the sterile filaments is taxonomically important.

Among the orders with bilayered ascus wall, the Dothideales lack sterile filaments in their centrum, while filaments are present in the Pleosporales and Patellariales (Barr, 1987). Ascomata of most orders of the Dothideomycetes open by a pore at maturity, through which the ascospores are released. In the Patellariales, however, the ascomata open by an apical cleft, and the outer wall recurves, detaches from the hamathecium, and reveals the centrum.

In the latest system of classification, the Pleosporales and the Dothideales are united in the Dothideomycetes, but the affinity of the Patellariales is unclear (Eriksson & Winka, 1998). Morphology suggests that species of *Aliquandostipite* belong in the Pleosporales, but molecular evidence contradicts this placement.

In this paper, we describe two new species in the new genus *Aliquandostipite*, show that they are closely related based on morphological and molecular characters, and infer their phylogenetic relationship to the three orders of Dothideomycetes outlined above.

5.2. Materials and methods

5.2.1 Collection, examination and isolation of fungi

Decaying branches were collected and stored in plastic bags until return to the laboratory. Fungi were located with an Olympus ZH10 or a Leica Wild M3Z stereomicroscope. Semi-thin cryosections of material embedded in Jung tissue-freezing medium were cut with a Jung CM 1500 cryostat. Cryosections and squash mounts of fungal material in water or glycerol were examined with an Olympus BH-2 or a Leica Leitz DMRB microscope. Photographs were taken with an Olympus C-35AD-4 or a Leica Wild MPS 48/52 photoautomat using Kodak TMAX 100 film.

To obtain cultures derived from single ascospores, ascomata were cut open with a razor blade, and the centrum tissue containing ascospores was removed with sterile forceps and placed in sterile tap water. Small drops of this ascospore suspension were placed on Corn Meal Agar (Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) petri dishes and incubated at 25°C in the dark. Germinated ascospores were transferred to new Potato Dextrose Agar (Difco Laboratories, Detroit, Michigan, USA) petri dishes with sterile forceps and incubated at 25°C in the dark or at room temperature on a laboratory bench subject to artificial and day light and darkness at night.

5.2.2 Molecular work--Species of *Aliquandostipite*

DNA was extracted from the mycelium of *Aliquandostipite khaoyaiensis* scraped off the surface of a PDA petri dish, and from the centrum tissue of a single ascoma of *A. sunyatsenii* stored in DNA lysis buffer (Lee and Taylor, 1990) for ~ 4 mo. A QIAamp Tissue Kit (Qiagen Inc., Mississauga, Ontario, Canada) was used for DNA extractions.

From *A. khaoyaiensis*, the small subunit (SSU) ribosomal DNA (rDNA) gene was amplified by polymerase chain reaction (PCR) with the primers SL1 (Landvik, 1996) and NS8 (White *et al.*, 1990). The internal transcribed spacer (ITS) 1 and ITS2 regions, including 5.8 S rDNA, were amplified by ITS5 and ITS4 (White *et al.*, 1990). Ready-To-Go PCR beads (Amersham Pharmacia Biotech, Uppsala, Sweden) with a reaction volume of 25 µL were used. The PCR protocol consisted of 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1 min at 56°C, and 1 min with a time extension of 4 s per cycle at 72°C, and a final extension at 72°C for 7 min PCR products were purified by QIAquick PCR Purification Kit (Qiagen Inc., Mississauga, Ontario, Canada). Sequencing was performed with the AmpliTaq DNA Polymerase FS Dye Terminator Cycle Sequencing kit (Perkin Elmer Corp., Norwalk, Connecticut, USA) in a reaction volume of 20 µL containing ~ 90 ng of purified PCR product and 3.2 pmol of the sequencing primer according to the following PCR protocol: rapid thermal ramp to 96°C, followed by 25 cycles of 30 s at 96°C, 15 s at 50°C, and 4 min at 60°C. Primers used for sequencing were NS2, NS3, NS4 (White *et al.*, 1990), SL 1, SL344 and SL887 (Landvik, 1996) for the SSU rDNA region, and primers ITS5 and ITS3 for the ITS region (White *et al.*, 1990).

For *A. sunyatsenii*, parts of the SSU region and the ITS2 region of the rDNA gene were amplified with the primer pairs SL1 / NS4 and ITS3 / ITS4, respectively, and purified. PCR products were cloned following the instructions of the TOPO TA Cloning kit (Invitrogen, Carlsbad, California, USA). Ten bacterial colonies containing the respective PCR product insertions were transferred into 1.5-mL centrifuge tubes aliquoted with 1 mL of LB medium and 50 µg/mL ampicillin prepared according to the manufacturer's instructions. The tubes were incubated at room temperature overnight and then centrifuged at 3000

rpm for 5 min The supernatants were removed, and the bacterial pellets were resuspended in 200 µl TE buffer (White *et al.*, 1990) and incubated at 95°C for 10 min Two microlitres of each bacterial suspension were used for a PCR reaction either with the primer pair SL1 / NS2 or ITS3 / ITS4. The PCR products were purified and sequenced, using the primers SL1 and ITS3 for the SSU and ITS products, respectively. All sequenced products were purified by ethanol precipitation (95% EtOH, 7.5 mol/L NaOAc) prior to processing by an ABI 377XL Automatic Sequencer (Perkin Elmer Corp., Norwalk, Connecticut, USA).

5.2.3 Additional sequences obtained

Tubeufia helicoma (Phill. & Plowr.) Pirozynski was collected 7 February 1999 by A. & R. Bandoni, S. Landvik, and P. Inderbitzin in South Arm Marshes Nature Reserve, Ladner, British Columbia, Canada, on a decaying decorticated log on the ground. A dried culture was deposited at the herbarium of the University of British Columbia, Vancouver, Canada (UBC F13877).

Rhytidhysteron rufulum (Spreng.: Fr.) Petrak was collected 6 June 1999 by M. A. Abdel-Wahab, at Pat Heung, New Territories, Hong Kong, on a decaying branch on the ground. The material and a dried culture were deposited at the herbarium of the University of British Columbia, Vancouver, Canada (UBC F13903). Molecular work was carried out as described above. DNA was isolated from a culture derived from a single ascospore, and the SSU rDNA was sequenced in both directions with the following primers: NS1, NS2, and cITS 5, NS19 (Gargas & Taylor, 1992), SL344 and SL887, MB20 (Winka & Eriksson, 1998), MB1 (5'-GGA GTA TGG TCG CAA GGC TG-3'), MB2 (5'-GTG AGT TTC CCC GTG TTG AG-3'), Bas3 (5'-AGA GTG TTC AAA GCA GGC-3'), and cBas3.

5.2.4 Data analysis

New sequences were assembled using AutoAssembler Version 1.4 (Applied Biosystems, Perkin Elmer Corp., Norwalk, Connecticut, USA). Thirty SSU rDNA sequences were retrieved from GenBank (Table 5-1). Most of them were longer than 1700 bp, with the exceptions of *Sphaerophorus globosus* and *Botryosphaeria rhodina* (around 1650 bp), *Peltigera neopolydactyla* and *Solorina crocea* (1567 bp), and *Tubeufia helicomyces* (357 bp). 45 bp at the 3'-end of *Lecanora dispersa* were removed due to ambiguous alignment. The first 20 positions at the 5'-end of *Aliquandostipite khaoyaiensis*, the introns in *T. helicoma* and *Monodyctis castaneae* were excluded from analysis. Sequences were manually aligned using Se-Al Version 1.0 alpha 1 (Rambaut, 1995). Datasets were analyzed using PAUP Version 4.0b3 (Swofford, 2000) on a Power Macintosh G3 (Apple Computer, Inc., Cupertino, California, USA). Unless otherwise noted, default settings were used.

Most parsimonious trees were generated in PAUP with 30 replicated heuristic searches and random taxon addition. Support for the branches was based

on 500 bootstrap replicates. Most likely trees were found with 30 replicated heuristic searches with random addition of taxa; or with a heuristic search using the most likely of the parsimony trees as the starting tree. Support for the branches was based on 100 bootstrap replicates. The Kishino-Hasegawa test was used as implemented by PAUP. Neighbor joining analysis generated a distance-based tree. Neighbor joining branch support was based on 500 bootstrap-replicates.

5.3. Results

We first formally describe the following new taxa of ascomycetes: the new family Aliquandostipitaceae, based on the new genus *Aliquandostipite*, which contains the two new species *A. khaoyaiensis* (holotype species), and *A. sunyatsenii*. The short Latin diagnoses are followed by extensive descriptions in English. Then we present the data from molecular work, including new sequences obtained and results from phylogenetic analyses.

5.3.1 Taxonomy

5.3.1.1 Establishment and definition of the new family Aliquandostipitaceae fam. nov.

Aliquandostipitaceae Inderbitzin fam. nov. Characteribus genere typico *Aliquandostipite* Inderbitzin gen. nov.

Characters like the holotype genus *Aliquandostipite* Inderbitzin gen. nov.

5.3.1.2 Establishment and definition of the new genus *Aliquandostipite* gen. nov.

Aliquandostipite Inderbitzin gen. nov. Etymology: from Latin *aliquando*, sometimes, and *stipite*, with a stalk.

Ascomata immersa-erumpentia vel superficialia. Hamathecium pseudoparaphyses. Asci bitunicati, fissitunicati. Hyphae usque ad 50 µm latas, aliquando ascoma ferentem. Generitypus *Aliquandostipite khaoyaiensis* Inderbitzin sp. nov.

Ascomata immersed-erumpent or superficial. Hamathecium comprising pseudoparaphyses. Asci bitunicate, fissitunicate. Mycelium visible on the substratum, comprising up to 50 µm wide hyphae, which may bear ascomata. Holotype species *Aliquandostipite khaoyaiensis* Inderbitzin sp. nov.

5.3.1.3 Establishment and description of the new species *A. khaoyaiensis* sp. nov.

Aliquandostipite khaoyaiensis Inderbitzin sp. nov. (Figure 5-1 - Figure 5-13). Etymology: from the type locality, Khao Yai National Park in Thailand.

Ascomata globosa vel subglobosa, ~ 250 µm diameter, papillata. Pseudoparaphyses septatae, ramosae, persistentes. Asci clavati, ~ 167 X 46 µm. Ascosporae uniseptatae, ovales, ~ 62 X 16 µm, appendiculatae. Mycelium

praesens in pagina substrato. Hyphae usque ad 42 µm latas, aliquando ascoma ferentem. Holotypus: in ligno emortuo, Khao Yai, KY3.4 (UBC F13875).

Sessile ascomata singly immersed to erumpent or superficial on old, decorticated branch lying on the ground in a tropical rain forest, globose to broadly ellipsoidal, 216-290 µm high, 220-344 µm wide, papillate, appearing pale brown when young or dark brown with age beneath stereomicroscope (Figure 5-8, Figure 5-9). Ascomal wall membranous, one-layered, in surface view pallid brown, forming a textura angularis-globulosa, in vertical section cells rounded to elongate (Figure 5-8 - Figure 5-10). Ascomal wall in basal part 6-16 µm thick, 1-2 cells wide, cells 3-15 µm in diameter, in apical part 13-31 µm thick, 2-4 cells wide, cells up to 22.5 µm in diameter (Figure 5-8, Figure 5-9). Cell walls of outermost cells up to 3.5 µm thick and refractive and the largest cells may protrude up to 8 µm (Figure 5-9). Papilla ~ 50 µm high, 70 µm wide (Figure 5-9). Hamathecium pseudoparaphyses, septate, sparsely branched, up to 3.5 µm wide (Figure 5-8, Figure 5-11). Asci 136-194 X 36-58 µm (166.67 X 45.57 µm on average, N = 30), eight-spored, clavate, bitunicate, fissitunicate, with thickened apical region, spores variably arranged, small pedoncle observed at times (Figure 5-11). Ascospores oval in outline, 49.6-70 X 12.8-20 µm (61.80 X 16.27 µm on average, N = 50), one-septate, constricted at the septum and there 11.2-16.8 µm wide (15.59 µm on average, N = 50), upper cell slightly longer and narrower than lower cell, smooth, pale brown, guttulate or not, sheathed (Figure 5-11 - Figure 5-13). Sheath first appressed to the wall, gradually expanding and detaching from the polar regions towards the septum, then balloon-like at the poles, finally surrounding the entire ascospore, ~ 150 X 50 µm (Figure 5-12 - Figure 5-13).

Superficial mycelium: consisting of light to dark brown, up to 40 µm wide hyphae, septate every 40-100 µm, wall refractive in vertical section, 4-6 µm thick (Figure 5-2, Figure 5-7). In substrate repeatedly branching into narrower, finally ~ 2 µm wide hyphae. Single hyphae may bear ascomata (Figure 5-1, Figure 5-3).

Stalked ascomata: stalk up to 1.6 mm long and 42 µm wide, wall up to 15 µm thick, arising singly from superficial hypha (Figure 5-7), or singly or gregariously from substrate (Figure 5-1, Figure 5-3). Apical segment of stalk broadening to up to 3 times the width of the one beneath, and comprising several rounded cells, ~ 25 µm in diameter in basal part, diminishing in size and merging with the peridium in the upper part (Figure 5-4, Figure 5-5). Ascomata globose to oval and then tapering towards the stalk, 140-320 µm high, 100-320 µm wide (Figure 5-4, Figure 5-5). Papilla up to 40 µm high, 110 µm wide (Figure 5-6). Ascomal wall a textura angularis-globulosa in surface view. In vertical section 1-3 cells wide, in basal part ~ 10 µm thick consisting of hyaline, thin-walled cells, 15-25 µm thick in apical part, cell walls 1-4 µm thick and refractive (Figure 5-4, Figure 5-5). Asci 116-180 X 30-46 µm (146.60 X

39.17 µm on average, N = 30). Ascospores 54.4-66.4 X 12.8-20.8 µm (61.46 X 16.73 µm on average, N = 50), at the septum 12.4-17.6 µm wide (14.67 µm on average, N = 50).

Ascospores from sessile ascomata germinated on CMA petri dishes overnight in the dark at 25°C. Germination hyphae were up to 16 µm wide, constricted at the septa and there up to 10 µm wide. Germinated ascospores were transferred to PDA petri dishes and incubated on a laboratory bench exposed to artificial and daylight, and darkness at night. After 5 wk, colonies measured 2-4 µm in diameter, and the mycelium was immersed in the agar and dark olive-brown to black. After 10 mo, colonies measured up to 4 cm in diameter. At the margin, the mycelium was immersed in the agar and comprised up to 20 µm wide hyphae. Towards the center, the mycelium was erumpent from the agar, forming a dark-brown, prosenchymatous stroma intermixed with agar. Stalked ascomata formed on the stroma. The stalks were up to ~ 500 µm long and 30 µm wide, bearing globose ascomata up to ~ 400 µm in diameter. Ascomata contained pseudoparaphyses and sterile asci. A culture was deposited at CBS.

Habitat and distribution: on decaying branch lying on the ground in tropical rain forest in Khao Yai National Park, Thailand.

Specimen examined: KY3.4, holotype (UBC F13875), on decaying branch, Khao Yai National Park, Thailand, July 1998, leg. A. M. Abdel-Wahab.

5.3.1.4 Establishment and description of the new species *A. sunyatsenii* sp. nov.

Aliquandostipite sunyatsenii Inderbitzin sp. nov. (Figure 5-14 - Figure 5-17). Etymology: after Dr. Sun Yat-Sen, a native of Zhongshan.

Ascomata globosa, ~ 350 µm diameter, papillata. Pseudoparaphyses septatae, ramosae, persistentes. Asci elongati, ~ 145 X 52 µm. Ascosporae uniseptatae, ellipsoideae, ~ 49 X 20 µm, appendiculatae. Mycelium praesens in pagina substrato. Hyphae usque ad 50 µm latas, aliquando ascoma ferentem. Holotypus: in ligno emortuo, Wu Gui Shan, Z1.2 (UBC F13876).

Sessile ascomata singly erumpent from decorticated branch immersed in small stream, rounded, 300-400 µm in diameter, papillate, ostiolate, light to dark brown, membranous (Figure 5-14). Ascomal wall one-layered, 25-40 µm thick, 2-5 cells wide, forming a textura globulosa-angularis in surface view. Outermost cells rounded to elongate, up to 30 µm in diameter, some protruding up to 13 µm above surrounding cells (Figure 5-14), inner cells elongate and laterally compressed, cell walls 1-5 µm thick, refractive (Figure 5-14). Cells at the base and towards papilla dark pigmented at times. Ostiole apically lined by elongate cells, ~ 10 X 5 µm (Figure 5-14). Pseudoparaphyses persistent, septate, branched, ~ 2.5 µm wide. Asci originating from a cushion-shaped ascogenous tissue at the base of the ascomata, 128-193 X 45-57.5 µm (145 X 51.5 µm on average, N = 20). When young, saccate with thick-walled apex, ocular

chamber and short stalk, completely filled by ascospores and ovoid to elongate when mature, bitunicate, fissitunicate, eight-spored. Ascospores straight or slightly curved (39-) 46-52 X 16-23 µm (49 X 19.5 µm on average, N = 30), one-septate up to 5 µm above or 4 µm below the middle (0.3 µm above on average, N = 30), upper hemispore up to 3 µm wider than lower hemispore (1 µm wider on average, N = 30), constricted at the septum, light brown, heavily guttulate (Figure 5-16, Figure 5-17). Two helmet-shaped appendages are present on either side of both upper and lower poles, tending to unite over the respective pole (Figure 5-17).

Superficial mycelium dark brown, up to 35 µm wide, septate at intervals of 35-45 µm, carrying single presumptive ascoma primordia at times (Figure 5-15). Connections between sessile ascomata and superficial hyphae seen.

Stalked ascoma: one stalked ascoma was found, ~ 350 µm in diameter, originating from the apex of a concolorous stalk (Figure 5-15). Stalk septate at intervals of 30-40 µm, thick-walled (up to 7.5 µm), 50 µm wide and 0.5 mm long, at the base branching into 15 µm wide hyphae. Asci contained were 137.5-142.5 X 45-62.4 µm, and ascospores 50-52.5 X 17.5-20 µm (both N = 4) (Figure 5-15).

Ascospores failed to germinate in culture.

Habitat and distribution: on decaying branches immersed in a small stream at Wu Gui Shan, near Zhongshan, Guangdong Province, People's Republic of China.

Specimen examined: Z1.2, holotype (UBC F13876), on decaying branch at Wu Gui Shan, 15 km south of Zhongshan, Guangdong Province, China, 11 November 1998, leg. Eduardo M. Leaño and P. Inderbitzin.

5.3.2 Molecular data

5.3.2.1 New sequences obtained

From the following species, new SSU rDNA sequences were obtained and submitted to GenBank: *Aliquandostipite khaoyaiensis* (AF201453), 1739 bp corresponding to positions 17-1716 of *Saccharomyces cerevisiae* Meyen ex E. C. Hansen from GenBank (V01335), *A. sunyatsenii* (AF201454), 440 bp corresponding to positions 130-571 of *S. cerevisiae*, *Tubeufia helicoma* (AF201455), 2110 bp corresponding to positions 65-1690 of *S. cerevisiae*, and *Rhytidhysteron rufulum* (AF201452), 1616 bp corresponding to positions 51-1665 of *S. cerevisiae*. In *T. helicoma*, introns of 81 and 402 bp were present at positions 467 and 565, respectively. From the following taxa, sequences from the ITS rDNA-region were obtained and submitted to GenBank: 548 bp of ITS1, 5.8S rDNA, and ITS2-region of *A. khaoyaiensis* (AF201728), and 395 bp of the 5.8 rDNA and ITS2-region for *A. sunyatsenii* (AF201727). The latter two sequences were too divergent to be aligned.

SSU rDNA sequences of *A. khaoyaiensis* and *A. sunyatsenii* were more similar to one another than to any other sequence. Their overlapping region of 438 unambiguous sites differed in 1.8% of the sites. Among the other taxa included in the analyses, the homologous region in *Botryosphaeria ribis* was most similar to taxa of *Aliquandostipite*, differing from *A. khaoyaiensis* in 4.5% of the sites. *Aliquandostipite khaoyaiensis* and *A. sunyatsenii* clustered together with high bootstrap support (Figure 5-18).

Since a complete SSU rDNA sequence of a member of the Tubeufiaceae was not available from GenBank, we sequenced the SSU rDNA region of *T. helicoma*. This new sequence differed in 0.8% of the sites from the homologous partial sequence of *T. helicomyces* retrieved from GenBank (Table 5-1). This was comparable to the degree of variability in homologous regions of other closely related taxa of the Dothideomycetes included in this study, such as *Setosphaeria rostrata* and *Pleospora herbarum*, which differed in 0.8% of the sites. On the other hand, the homologous regions of the most divergent taxa in the Dothideomycetes in this study, *P. herbarum* and *Aureobasidium pullulans*, differed by 7.2%. *Tubeufia helicoma* clustered with the 357 bp sequence of *T. helicomyces* with bootstrap support (Figure 5-18).

Similarly, a complete sequence of the SSU rDNA region of a member of the Patellariales was not available, and so we sequenced the gene from *R. rufulum*. The phylogenetic placement of *R. rufulum* inferred in this study agreed with results of previous studies (Winka & Eriksson, 1998) and confirmed the identity of our new sequence. A fragment of *R. rufulum* retrieved from GenBank (U20506) differed from ours in the overlapping region of 1046 bp in five (0.48%) sites, and another sequence (AF164375) differed from ours in 11 (1.03%) of the 1063 overlapping sites. The latter two fragments differed in 12 (1.15%) of the 1046 overlapping sites. As a comparison, *Pleospora herbarum* and *Setosphaeria rostrata* (Figure 5-18) differed in 0.36% of 1046 sites.

5.3.2.2 Phylogenetic analyses

The new SSU rDNA sequences of *A. khaoyaiensis*, *A. sunyatsenii*, *T. helicoma* and *R. rufulum* were aligned with 30 sequences retrieved from GenBank, using *Boletus satanas* as an outgroup (Table 5-1). Hence, the data matrix contained 34 taxa and 1799 characters. The alignment was submitted to TreeBase. Ambiguous sites were excluded from analyses. These included 72 characters in the following positions: 35-39, 99-105, 141-160, 452-458, 1336-1361, 1484-1490. Of the remaining 1727 characters 1255 were constant, 211 of the variable characters were parsimony uninformative, and 261 were parsimony informative. Characters were weighted equally, gaps were ignored.

Parsimony and likelihood trees inferred from a data matrix with ambiguous sites included were not significantly different from the analyses with am-

biguous sites excluded, based on a Kishino-Hasegawa test ($P > 0.05$). However, 88 MPTs were found (data not shown). For this reason, ambiguous sites were excluded in all the following analyses.

In 30 heuristic searches using parsimony, two MPTs, each requiring 964 steps, were found (Consistency Index = 0.623, Retention Index = 0.661). The two MPTs differed in the arrangement of taxa within a clade of the Pleosporales: *Leptosphaeria maculans* appeared as sister taxon to either *Septoria nodorum* or *Cucurbitaria elongata*. The overall tree topology agrees with results from other authors (Winka & Eriksson, 1998). The most likely MPT (-ln likelihood = 8204) differed from the most likely tree in Figure 5-18 by the rearrangement of branches receiving less than 50% bootstrap support: *Chaetomium elatum* was sister taxon to the Chaetothyriales, the Lecanorales formed a sister group to the Peltigerales, and *Aureobasidium pullulans*, *Dothidea insculpta*, *Coccodinium bartschii*, and *Aliquandostipite* were sister group to the remainder of the Dothideomycetes.

The most likely tree of the two MPTs was used as the starting tree in a likelihood analysis, which yielded the same tree topology as 30 likelihood heuristic searches with taxa added by random stepwise addition. The most likely tree (-ln likelihood = 8198) was 965 steps long, one step longer than the MPTs, and not significantly different from either MPT ($P > 0.6$). Clades supported by at least 50% of the bootstrap replicates in either most likely tree or MPTs were present in both trees.

Based on a Kishino-Hasegawa test, the Neighbor joining tree was significantly worse than the most likely tree ($P < 0.05$), and thus is not discussed here in detail.

Clades with relevance in this study and high likelihood, parsimony, and Neighbor joining bootstrap support include: Pleosporales, with 99, 95, and 87% support in the respective analyses, Pleosporales and *R. rufulum* with 95, 89, and 58% support, species of *Aliquandostipite* with 100, 92, and 100% support, and species of *Tubeufia* with 91, 73, and 83% bootstrap support (Figure 5-18). In neither analysis did the Dothideales, i.e. *Dothidea insculpta*, *Coccodinium bartschii*, *Aureobasidium pullulans*, and species of *Botryosphaeria* receive support as a monophyletic group. The Dothideomycetes consisting of Pleosporales and Dothideales formed a monophyletic group in both parsimony and likelihood analyses, receiving the highest support in the likelihood analysis with 63% of the bootstrap replicates. The morphologically related taxa in Pleosporales, Patellariales, Tubeufiaceae, and species of *Botryosphaeria* formed a monophyletic group in all analyses, with a maximum bootstrap support of 58% in the likelihood analysis.

Constraining species of *Aliquandostipite* to the Pleosporales, yielded four trees that were 11 steps longer than the MPTs and significantly worse than either one of them ($P < 0.05$), as evaluated by the Kishino-Hasegawa test.

5.4. Discussion

In the previous section, we gave evidence that the new species of *Aliquandostipite* have morphologically dimorphic ascomata within one species, that they are closely related based on morphological and molecular data, and distant from their presumptive closest relatives based on morphology.

In the following, we justify the inclusion of both new species in one genus, the establishment of the new genus *Aliquandostipite* and the new family Aliquandostipitaceae. Finally, we show that the stalked and sessile ascomata present side by side on the substratum belong to the same species and discuss another distinguishing feature of both new species, the unusually wide hyphae.

5.4.1.1 Two new congeneric species

Molecular evidence for a close relationship of species of *Aliquandostipite* included the high support that their clade received in phylogenetic analyses of the SSU rDNA sequences using different methods. Both Neighbor joining and maximum likelihood clustered *A. khaoyaiensis* and *A. sunyatsenii* together with 100% bootstrap support (Figure 5-18). Bootstrap values obtained with parsimony support the *Aliquandostipite* clade with 92% (Figure 5-18). Morphological characters common to both species of *Aliquandostipite* were a light-colored, one-layered ascomal wall, downward-growing sterile filaments, functionally two-layered asci completely filled by ascospores at maturity, and one-septate, sheathed ascospores. The habitats and ecology of both species of *Aliquandostipite* were similar. They were found on old, decorticated branches in very humid and warm habitats: *Aliquandostipite khaoyaiensis* on branches lying on the ground of a tropical rain forest in Thailand, *A. sunyatsenii* on a branch immersed in a stream in subtropical southern China.

Hence, morphological and ecological characters supported SSU rDNA data and indicated a close relationship of *A. khaoyaiensis* and *A. sunyatsenii*. Even though the ITS rDNA sequences of the species of *Aliquandostipite* were too different to be aligned, the inclusion of both species into one genus seemed most appropriate at present.

5.4.1.2 The new genus *Aliquandostipite* and new family Aliquandostipitaceae

Species of *Aliquandostipite* did not group with any significant support with other taxa included in the phylogenetic analyses. In the most likely tree, the genus *Aliquandostipite* was sister group to the Dothideomycetes (Figure 5-18). The Dothideomycetes comprise fungi traditionally placed in the Dothideales and Pleosporales. The morphological characters of species of *Aliquandostipite*, the presence of bitunicate asci, and the presence of sterile filaments, both developing in a stroma, are consistent with a placement in the Dothideomycetes.

Except for the stalked ascomata, all morphological features of species of *Aliquandostipite* are encountered in the Pleosporales, the light-colored ascomal wall suggesting a possible affinity with the family Tubeufiaceae (M. E. Barr, personal communication, Sidney, British Columbia, Canada). In phylogenetic analyses, however, species of *Aliquandostipite* did not cluster within the Pleosporales. *Rhytidhysteron rufulum* in the Patellariales, appeared as sister taxon to the Pleosporales, excluding both *Tubeufia* and *Aliquandostipite.* The genera *Tubeufia* and *Aliquandostipite* were as similar to other filamentous ascomycetes as to one another, and did not form a monophyletic group (Figure 5-18). Constraining the genus *Aliquandostipite* to be within the Pleosporales, yielded significantly worse trees than the most parsimonious tree ($P < 0.05$).

The Dothideales are defined morphologically by the absence of sterile filaments (Barr, 1987), and the presence of sterile filaments excludes *Aliquandostipite* from this group. In the likelihood analysis, the Dothideales did not cluster together (Figure 5-18). In the parsimony analysis, *Aureobasidium pullulans*, *Dothidea insculpta*, and *Coccodinium bartschii* of the Dothideales, and *Aliquandostipite* formed a monophyletic group without significant support. However, molecular data provided little support for membership of *Aliquandostipite* in the Dothideales, but poor resolution of branching order made a monophyletic relationship of *Aliquandostipite* and Dothideales impossible to exclude.

Hence, the genus *Aliquandostipite* could neither be included in the Dothideales nor in the Pleosporales. The lack of morphological and molecular affinity to taxa known to us justified the establishment of the new family Aliquandostipitaceae and the new genus *Aliquandostipite* for the two new species of *A. khaoyaiensis* and *A. sunyatsenii.*

5.4.1.3 Dimorphic ascomata and the widest hyphae in ascomycetes

Besides the characters mentioned above, the new family Aliquandostipitaceae is supported by the presence in both species of two unique features, distinguishing them from all other Euascomycetes. These are the widest hyphae reported in the ascomycetes and the formation of both sessile and stalked ascomata side by side on the substratum.

Stalked ascomata are atypical among ascomycetes. In species of *Aliquandostipite*, stalked and sessile ascomata are present side by side on the substratum. Stalked ascomata are rounded to elongate and lack the flattened base of the sessile, dome-shaped ascomata. The stalks originate either directly from the substratum (Figure 5-3) or from a superficial hypha (Figure 5-7). In *A. sunyatsenii*, superficial hyphae were observed to be connected to sessile ascomata as well. Hence, both stalked and sessile ascomata may have issued from the same mycelium. This situation is identical to what is encountered in

culture: Single ascospore isolates from sessile ascomata of *A. khaoyaiensis* produced both sessile and stalked ascomata.

Microscopic features of the centrum tissue and ascomal wall in stalked and sessile ascomata vary only to a degree to be expected within one species. In *A. khaoyaiensis*, the ascospores of both stalked and sessile ascomata are nearly identical in size and the dimensions of the asci clearly overlap, being on average 12% longer and 14% narrower in the sessile than in the stalked ascomata. In *A. sunyatsenii*, dimensions of asci and ascospores overlap as well, and their means are very close. A more detailed comparison is not possible, because of the fact that only few asci and ascospores from one stalked ascomata could be measured. Vertical sections of stalked and sessile ascomata in *A. khaoyaiensis* show the same type of sterile filaments, which are apically attached and seem to have grown downwards (Figure 5-4 - Figure 5-6, Figure 5-8). The ascomal wall is one-layered and light colored, and the constituting cells are largest at the exterior of the ascoma, and diminish in size towards the inside. Some of the outermost cells in sessile ascomata of *A. khaoyaiensis* and *A. sunyatsenii* were observed to protrude up to 13 µm above the surrounding cells (Figure 5-9, Figure 5-14). This was not observed in stalked ascomata of *A. khaoyaiensis*. In *A. sunyatsenii*, stalked ascomata were not sectioned.

Species of *Aliquandostipite* produce the widest hyphae of any known ascomycete. The ascomal stalks, which are single hyphae, are up to 50 µm wide and 1.6 mm long. This is five times wider than the widest hyphae previously reported in the ascomycetes. So far, the widest hyphae in lignicolous ascomycetes were known from species in the genus *Botryosphaeria*, reaching a width of 10 µm (M. E. Barr, personal communication, Sidney, British Columbia, Canada). In the lignicolous genus *Wolfiporia* of the basidiomycetes, hyphae may in rare cases reach a width of 20 µm (L. Ryvarden, personal communication, University of Oslo, Norway). In lamellae of certain Basidiomycetes, 30 µm wide hyphae are possible (H. Clémençon, personal communication, University of Lausanne, Switzerland).

The results of this study are surprising, in that two short surveys in geographically distant localities yielded two new, closely related species that cannot be placed in a known family. We hope these results will encourage further study of fungal diversity in little explored areas of the world.

Table 5-1. Fungal species and GenBank accession numbers for SSU rDNA sequences from GenBank used in phylogenetic analyses. Ordinal classification used in this paper following Barr (1987), and Eriksson and Winka (1998). Supraordinal classification follows Eriksson and Winka (1998).

Species	Accession	Order	Class
Aspergillus fumigatus Fresen.	M60300	Eurotiales	Eurotiomycetes
Aureobasidium pullulans (de Bary) G. Arnaud	M55639	Dothideales	Dothideomycetes
Boletus satanas Lenz	M94337	Boletales	-
Botryosphaeria rhodina (Berk. & Curtis) Arx	U42476	Dothideales	Dothideomycetes
Botryosphaeria ribis Grossenb. & Duggar	U42477	Dothideales	Dothideomycetes
Bulgaria inquinans Fries	AJ224362	Leotiales	Leotiomycetes
Capronia mansonii (Schol-Schwarz) Mueller & al.	X79318	Chaetothyriales	Chaetothyriomycetes
Ceramothyrium linnaeae (Dearness) Hughes	AF022715	Chaetothyriales	Chaetothyriomycetes
Chaetomium elatum Kunze	M83257	Sordariales	Sordariomycetes
Coccodinium bartschii A. Massal.	U77668	Dothideales	Dothideomycetes
Cucurbidothis pityophila (Schmidt & Kunze) Petrak	U42480	Pleosporales	Dothideomycetes
Cucurbitaria elongata (Fr.) Grev.	U42482	Pleosporales	Dothideomycetes
Dothidea insculpta Wallr.	U42474	Dothideales	Dothideomycetes
Eurotium rubrum Koenig & al.	U00970	Eurotiales	Eurotiomycetes
Lecanora dispersa (Pers.) Sommerf.	L37535	Lecanorales	Lecanoromycetes
Leptosphaeria maculans (Des.) Ces. & de Not.	U04233	Pleosporales	Dothideomycetes
Lophiostoma crenatum (Pers.) Fuckel	U42485	Pleosporales	Dothideomycetes
Malbranchea filamentosa Sigler & Carmichael	L28065	Onygenales	Eurotiomycetes
Monodictys castaneae (Wallr.) Hughes	Y11715	Pleosporales	Dothideomycetes
Morchella elata Fries	U42641	Pezizales	Pezizomycetes
Pleospora herbarum (Pers.) Rabenh.	U05201	Pleosporales	Dothideomycetes
Pleospora rudis Berl.	U00975	Pleosporales	Dothideomycetes
Peltigera neopolydactyla (Gyeln.) Gyeln.	X89218	Peltigerales	Lecanoromycetes
Septoria nodorum E. Müller	U04236	Pleosporales	Dothideomycetes
Setosphaeria rostrata Leonard	U42487	Pleosporales	Dothideomycetes
Sphaerophorus globosus (Huds.) Vain.	L37532	Lecanorales	Lecanoromycetes
Solorina crocea (L.) Ach.	X89220	Peltigerales	Lecanoromycetes
Tubeufia helicomyces von Höhnel	L35296	Pleosporales	Dothideomycetes
Uncinocarpus reesii Sigler & Orr	L27991	Onygenales	Eurotiomycetes
Westerdykella dispersa (Clum) Cejp & Milko	U42488	Pleosporales	Dothideomycetes

Stalked ascomata and hyphae of *Aliquandostipite khaoyaiensis*. Features of stalked ascomata and wide hyphae.

Figure 5-1. Stalked ascomata on substrate.

Figure 5-2. Mycelium on the substrate. Note thick, branching hyphae.

Figure 5-3. Cluster of stalks bearing ascomata.

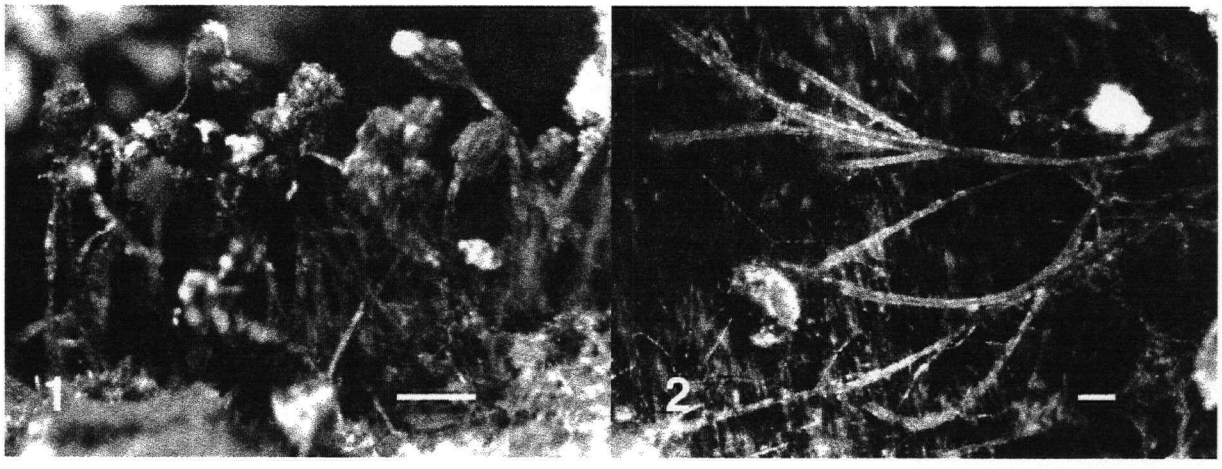
Figure 5-4. Vertical cryosection of stalked ascomata mounted in glycerol.

Figure 5-5. Vertical cryosection of basal section of stalked ascomata mounted in glycerol. Note the stalk formed by a single hypha with thick, refractive walls, and the widening apical segment of the stalk merging with the ascomal wall.

Figure 5-6. Vertical cryosection of apical section of stalked ascomata mounted in glycerol. Note papilla and apically attached, branched and septate sterile filaments.

Figure 5-7. Squash mount in glycerol of superficial hypha bearing an ascoma on a lateral branch.

Scale bars: Figure 5-1, Figure 5-3 = 0.5 mm, Figure 5-2, Figure 5-4, Figure 5-7 = 100 µm, Figure 5-5, Figure 5-6 = 50 µm. Figure 5-1 - Figure 5-3 in brightfield with stereomicroscope, Figure 5-4 - Figure 5-6 in Nomarski inference contrast, Figure 5-7 in phase contrast.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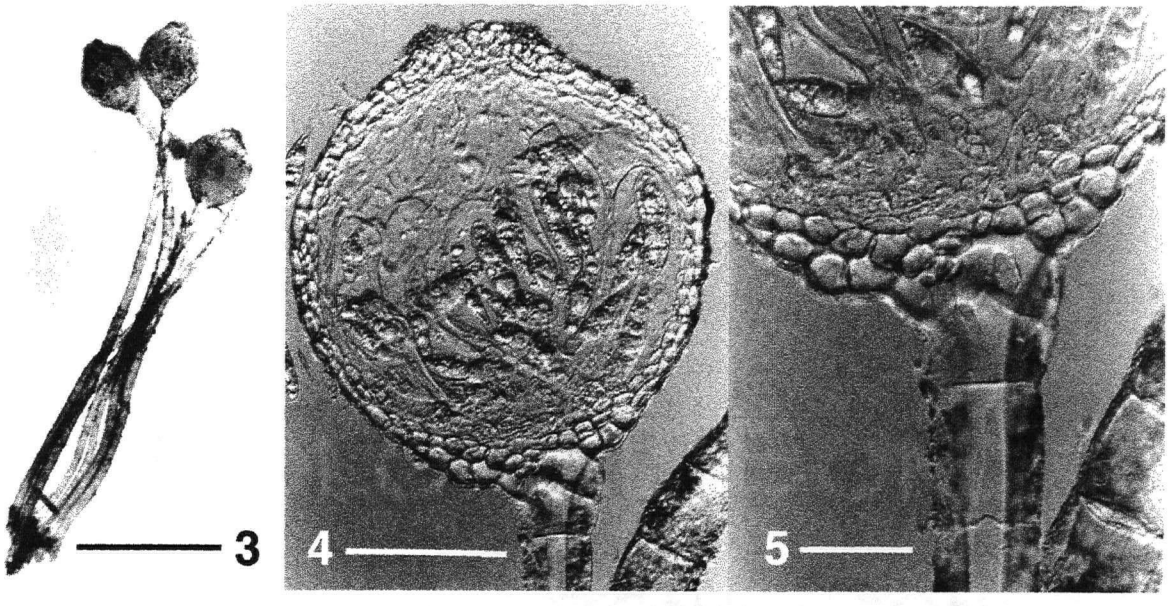
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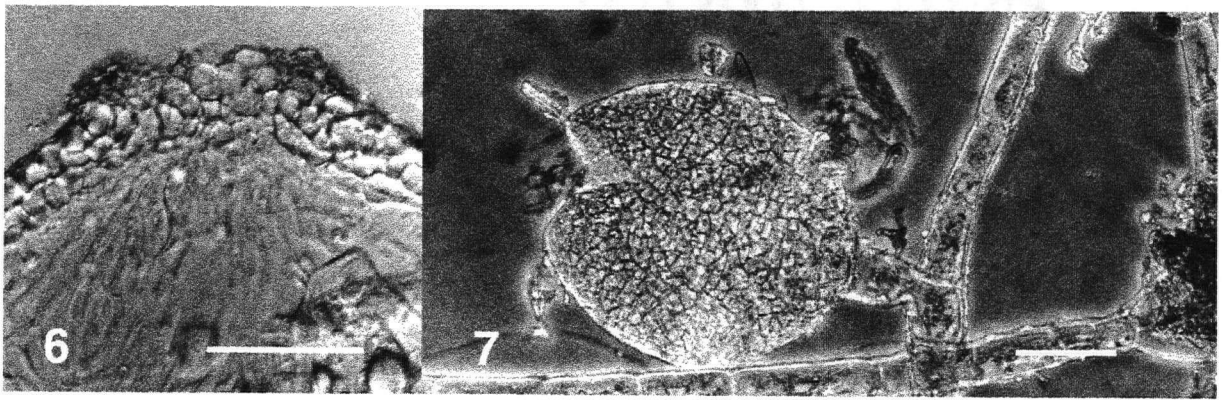
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Sessile ascomata, asci and ascospores of *Aliquandostipite khaoyaiensis*.

Figure 5-8. Vertical cryosections of sessile dome-shaped sessile ascoma with flattened base, mounted in glycerol.

Figure 5-9. Vertical cryosections of apical section of sessile ascomata with papilla and protruding ascomal wall cell (arrow), mounted in glycerol.

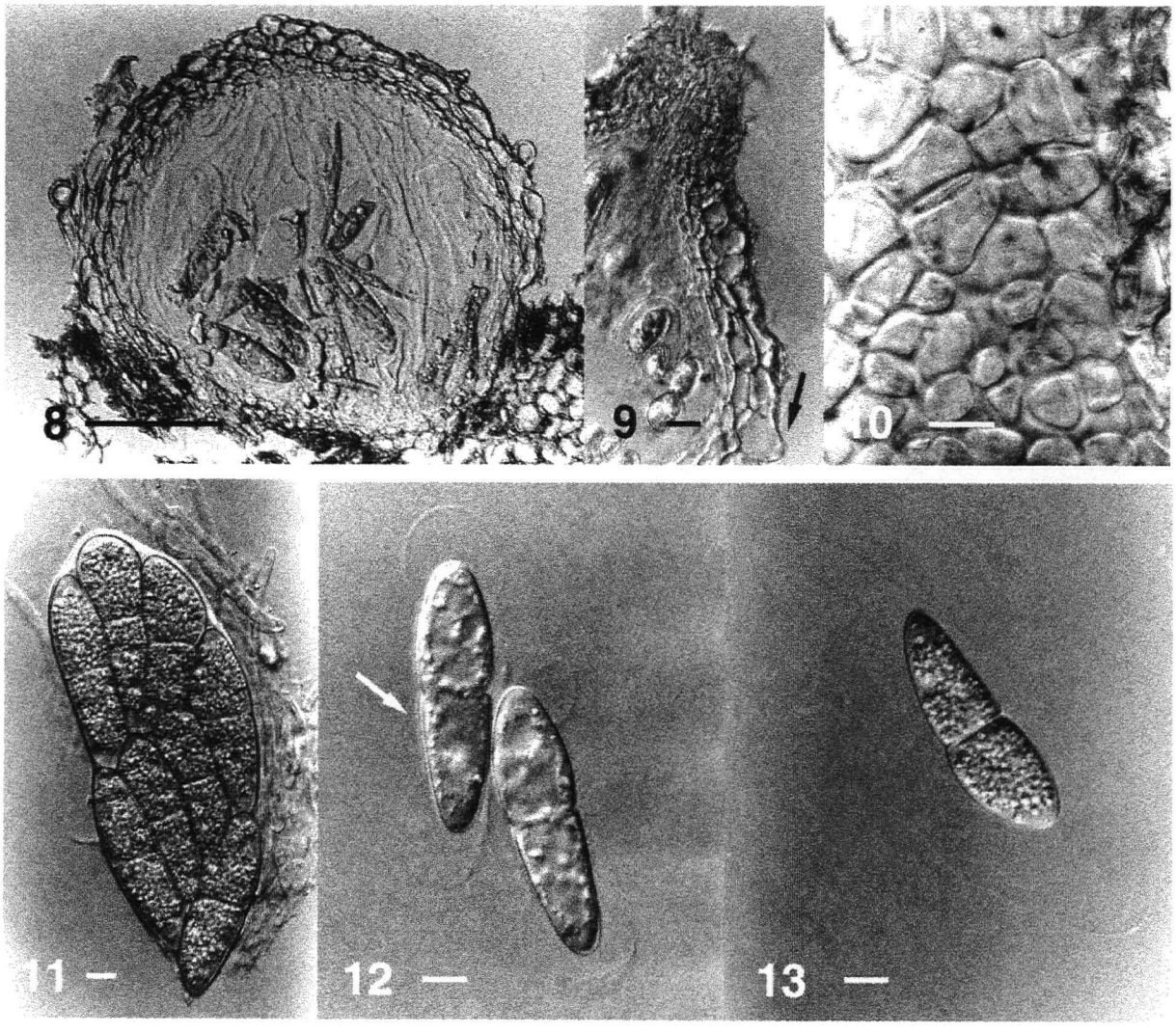
Figure 5-10. Superficial view of ascomal wall. Note the light-colored, thick-walled cells, mounted in glycerol.

Figure 5-11. Ascus and sterile filaments. Squash mount in glycerol.

Figure 5-12. Ascospores with detaching sheath. Note the appressed sheath (arrow) in the equatorial region, detaching towards the poles. Squash mount in glycerol.

Figure 5-13. Ascospore in water with completely detached sheath.

Scale bars: Figure 5-8 = 100 µm, Figure 5-9 - Figure 5-13 = 10 µm. Figure 5-8 - Figure 5-13 in Nomarski inference contrast.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Ascomata, asci and ascospores of *Aliquandostipite sunyatsenii* mounted in glycerol.

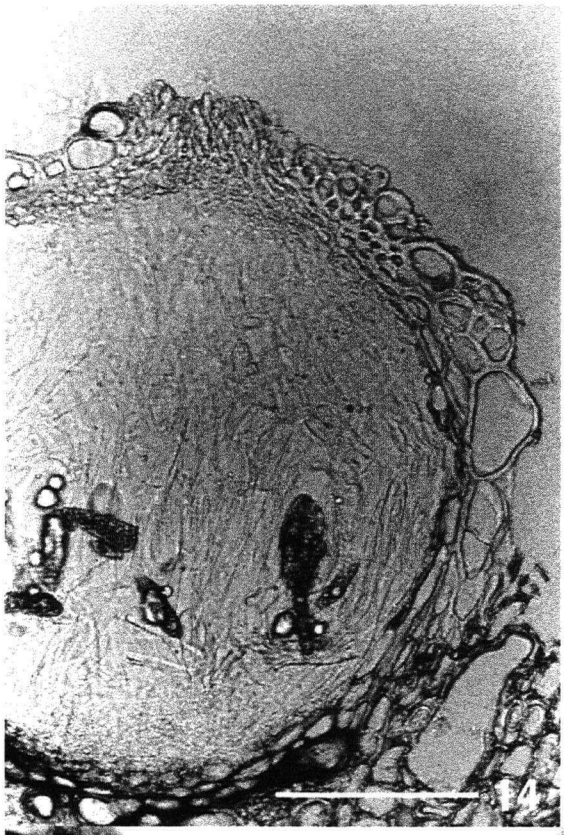
Figure 5-14. Vertical cryosection of ascoma. Note rounded ascoma, large protruding cells of ascomal wall, and dark cells at basal ascomal wall.

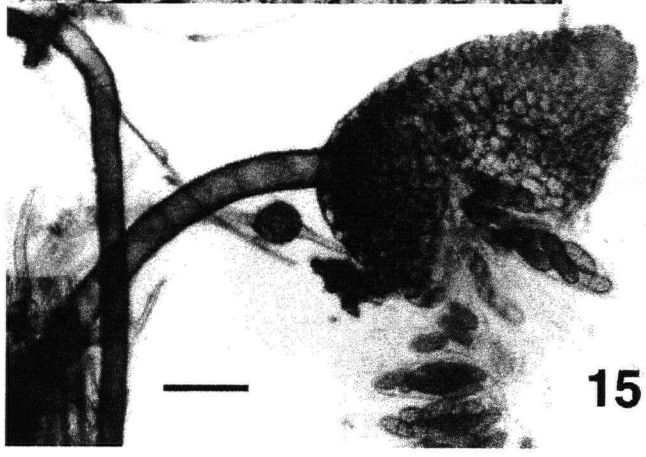
Figure 5-15. Squash mount of superficial hypae with stalked ascoma. Stalked, elongate ascoma liberating asci and ascospores, superficial hyphae of different sizes, presumptive ascoma-primordia borne by a thin hypha.

Figure 5-16. Ascus completely filled by ascospores.

Figure 5-17. Ascospores. Note polar, none-detaching appendages.

Scale bars: Figure 5-14, Figure 5-15 = 100μm, Figure 5-16, Figure 5-17 = 10 μm. Figure 5-14 - Figure 5-17 in Nomarski inference contrast.





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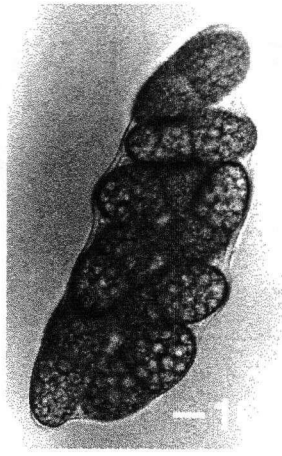


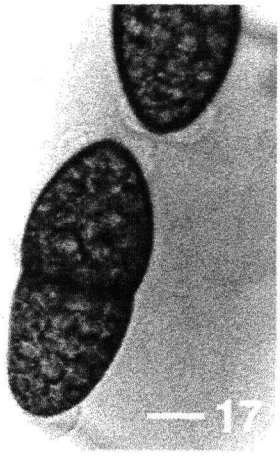
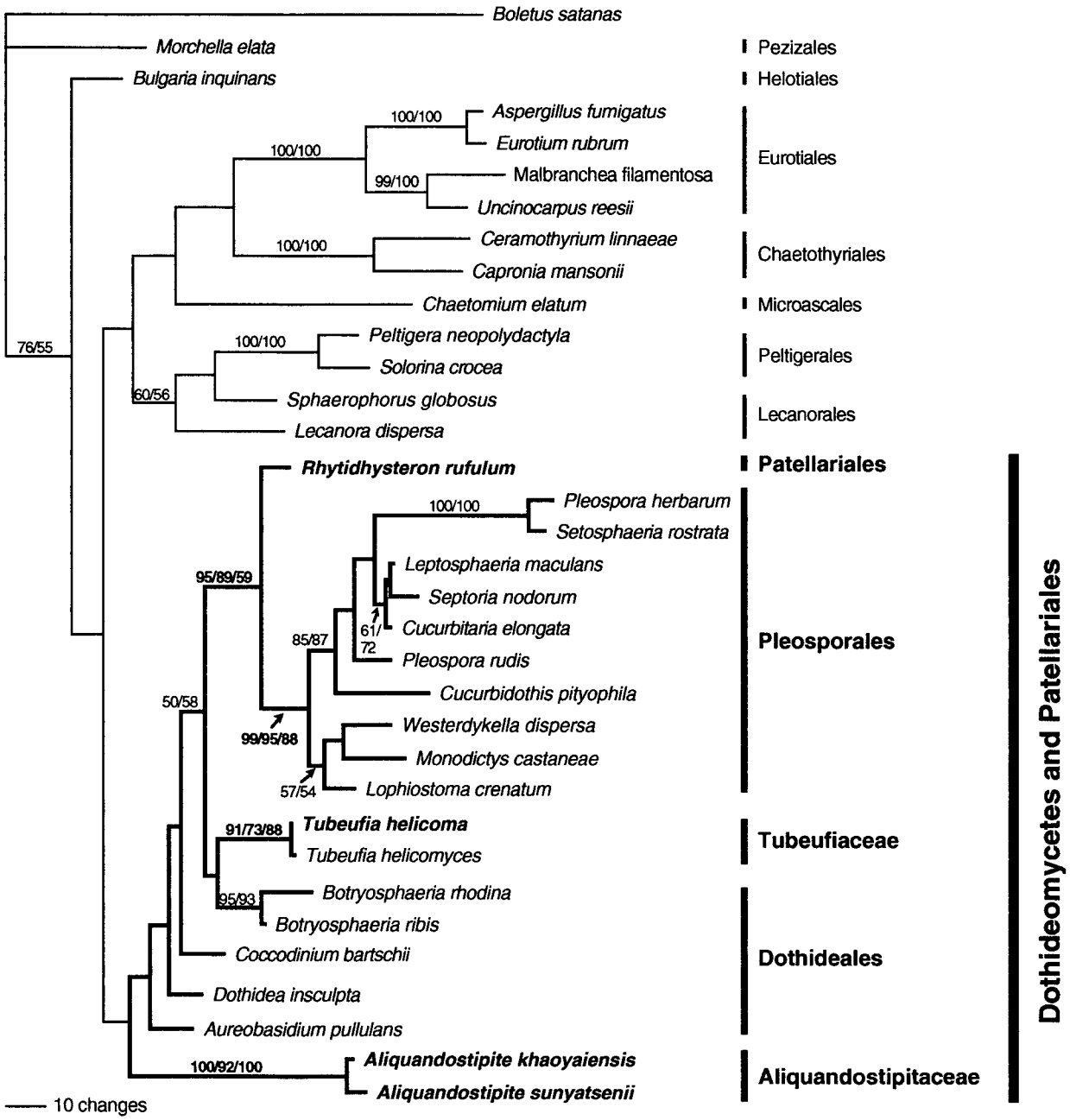


Figure 5-18. Most likely tree (-ln likelihood = 8198). Bootstrap support percentages are shown above the branches. The first numbers are likelihood bootstrap percentages based on 100 replicates, and the second numbers are parsimony bootstrap percentages based on 500 replicates. Only bootstrap percentages higher than 50% in both analyses were included. For groups relevant to this study, bootstrap percentages are given in boldface and Neighbor joining bootstrap percentages (based on 500 replicates) are given last in the series of numbers. Branches of Dothideomycetes and Patellariales are in boldface. Species for which new sequences were obtained are also in boldface. Note the well-supported clades of *Aliquandostipite* spp. and *Tubeufia spp.*, clustering outside the clade comprising *Rhytidhysteron rufulum* and Pleosporales.


Boletus satanas
Morchella elata
Bulgaria inquinans
Aspergillus fumigatus
Eurotium rubrum
Malbranchea filamentosa
Uncinocarpus reesii
Ceramothyrium linnaeae
Capronia mansonii
Chaetomium elatum
Peltigera neopolydactyla
Solorina crocea
Sphaerophorus globosus
Lecanora dispersa
Rhytidhysteron rufulum
Pleospora herbarum
Setosphaeria rostrata
Leptosphaeria maculans
Septoria nodorum
Cucurbitaria elongata
Pleospora rudis
Cucurbidothis pityophila
Westerdykella dispersa
Monodictys castaneae
Lophiostoma crenatum
Tubeufia helicoma
Tubeufia helicomyces
Botryosphaeria rhodina
Botryosphaeria ribis
Coccodinium bartschii
Dothidea insculpta
Aureobasidium pullulans
Aliquandostipite khaoyaiensis
Aliquandostipite sunyatsenii
100/100
100/100
99/100
100/100
76/55
100/100
60/56
95/89/59
85/87
61/72
50/58
99/95/88
57/54
91/73/88
95/93
100/92/100
10 changes
Pezizales
Helotiales
Eurotiales
Chaetothyriales
Microascales
Peltigerales
Lecanorales
Patellariales
Pleosporales
Tubeufiaceae
Dothideales
Aliquandostipitaceae
Dothideomycetes and Patellariales

5.5. Bibliography

Barr, M. E. (1987). *Prodromus to class Loculoascomycetes.* Amherst, Massachusetts: Published by the author.

Eriksson, O. E. & Yue, J.-z. (1988). *The Pyrenomycetes of China, an annotated checklist.* Umeå: University of Umeå.

Eriksson, O. E. & Winka, K. (1998). Families and higher taxa of Ascomycota. *Myconet* **1**, 17-24.

Gargas, A. & Taylor, J. W. (1992). Polymerase Chain Reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. *Mycologia* **84**, 589-592.

Hawksworth, D. L. (1991). The fungal dimension of biodiversity: Magnitude, significance, and conservation. *Mycological Research* **95**, 641-655.

Hawksworth, D. L., Kirk, P. M., Sutton, B. C. & Pegler, D. N. (1995). *Dictionary of the Fungi*, 8 edn. Wallingford, Oxon, UK: CAB International.

Hawksworth, D. L., Minter, D. W., Kinsey, G. C. & Cannon, P. F. (1997). Inventorying a tropical fungal biota: intensive and extensive approaches. In *Tropical Mycology*, pp. 29-50. Edited by K. K. Janardhanan, C. Rajendran, K. Natarajan & D. L. Hawksworth. Enfield, New Hamphshire: Science Publishers, Inc.

Landvik, S. (1996). Phylogenetic rDNA studies of Discomycetes (Ascomycota): Department of Ecological Botany, Umeå University.

Rambaut, A. (1995). *Se-Al v1.d1*, Version 1.0 alpha edn. Oxford, U.K: Department of Zoology, University of Oxford.

Subramanian, C. V. & Sekar, G. (1990). Coronophorales from India - A Monograph. *Kavaka* **18**, 19-90.

Swofford, D. L. (2000).PAUP*. Phylogenetic analysis using parsimony (*and other methods). Sunderland, Massachusetts: Sinauer Associates.

Tai, F. L. (1979). *Sylloge Fungorum Sinicorum.* Beijing: Science Press, Academia Sinica.

White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols*, pp. 315-322. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Winka, K. & Eriksson, O. E. (1998). Molecular evidence for recognizing the Chaetothyriales. *Mycologia* **90**, 822-830.

CHAPTER 6. Conclusion

The research presented in this thesis contributes to the knowledge of ascomycete diversity in the genus *Pleospora* and the new family Aliquandostipitaceae, both in the class Dothideomycetes. In the concluding statement, the thesis results are discussed in relation to each other and to the field of study, and further research is suggested.

6.1. *Pleospora*: Generic delimitation, phylogenetics and mating system evolution

The main part of this thesis was centered on the investigation of mating system evolution in *Pleospora*. This required a well-supported phylogeny, restriction of the genus *Pleospora* to isolates with the *Stemphylium* asexual state, and the delimitation of 22 phylogenetic species. Subsequently, the sex regulating master locus MAT that contains the mating type idiomorphs was cloned from *Pleospora* and used for phylogenetic analyses as well as structural analyses. The combined evidence from all data led to the conclusion that homothallism in *Pleospora* is polyphyletic with three independent evolutionary origins.

6.1.1 Generic delimitation and phylogenetics of *Pleospora* sensu stricto

Pleospora is a vast and polyphyletic genus (Crivelli, 1983; Holm, 1962). Câmara et al. (2002) showed that the type species of *Pleospora, P. herbarum*, formed a monophyletic group with other *Pleospora* species that have a *Stemphylium* asexual state, together constituting *Pleospora* sensu stricto. These results were confirmed in chapter 3 with MAT protein analyses. In chapter 4 I contributed to the monophyly of *Pleospora* by removing the marine species *P. gaudefroyi* that lacks a sexual state, to the new genus *Decorospora*.

Chapters 2 and 3 dealt with the phylogenetic relationships within *Pleospora* sensu stricto. My results confirmed the phylogenetic findings by Câmara et al. (2002), generally increased the statistical support of the tree nodes, and revealed the relationships of additional species that were not included in the Câmara et al. (2002) study.

The sampling of *Pleospora* strains used in this thesis was biased towards examination of the delimitation of the type species of *Pleospora*, *P. herbarum*. Câmara et al. (2002) found that *P. herbarum* could not be distinguished from closely related morphological species based on phylogenetic analyses of two loci. The studies in chapter 2 with four loci confirmed their results.

6.1.1.1 Further research

A considerable amount of work remains necessary for a complete phylogenetic and taxonomic treatment of *Pleospora*. The hypothesis of the delimitation of *Pleospora* sensu stricto as a monophyletic group characterized by the *Stemphylium* asexual state has to be confirmed through study of additional species,

and all *Pleospora* species that do not fall into this group have to be placed in appropriate alternative genera. Wehmeyer's (1961) slide collection from his monographic work on *Pleospora* could serve as backbone and starting point for further morphological study. Fresh collections of additional *Stemphylium* species are needed for molecular investigations.

The phylogenetic species of *Pleospora* sensu stricto obtained in chapter 2 have to be reexamined. All but the phylogenetic species *P. herbarum* contained fewer than ten isolates. Of the 22 recognized phylogenetic species, 18 contained fewer than five isolates. Thus, to prove a history of genetic isolation for all phylogenetic species, more research with a larger sample size is necessary, as for example in Kroken & Taylor (2001).

Gene flow within the phylogenetic species *P. herbarum* remains an issue. *Pleospora herbarum* is the type species of *Pleospora,* and its phylogenetic species contains several described morphological species. Whether these all fall within one phylogenetic species, sharing a very recent history of sexual reproduction and gene flow could be tested with a population genetics approach, for example using RFLP markers. This was done by Zhan et al. (2003) where a regularly recombining population structure was detected in the outcrosser *Mycosphaerella graminicola* (Fuckel) Schroeter based on a sample size of 1673 strains.

6.1.2 The divergent protein-encoding intron in *EF-1 alpha*

The gene *EF-1 alpha* of which intron and exon DNA sequences were used for phylogenetic analyses, contained an unusual intron in the phylogenetic species *S. lancipes*, *S. trifolii* and *Stemphylium sp.* strain P246. The intron in these three phylogenetic species was distinct from all other introns found in this study. It was up to 1678 bp long, more than 1400 bp longer than in other *Pleospora* isolates, encoded a protein and was delimited at the 5'-end by the non-canonical splice site GGT instead of GT. The ORF encoded by the introns of all three species was most similar to a hypothetical zinc-finger protein from the filamentous ascomycete *Gibberella zeae.* Non-canonical, 5'-GGT splice sites have not been reported in fungi before.

6.1.2.1 Further research

In case of experimental verification, the long, *EF-1 alpha* introns in the phylogenetic species *S. lancipes*, *S. trifolii* and *Stemphylium sp.* strain P246 could be the first 'parasitic' splice sites in fungi (Burset *et al.*, 2000). Similar 'parasitic' splice sites were also found in *EF-1 alpha* of the divergent *Pleospora* species *P. papaveracea*, but were not confirmed experimentally (Inderbitzin, O'Neill, Shoemaker and Berbee, unpublished). To prove involvement of these putative fungal 'parasitic' intron splice sites in mRNA maturation, absence of the re-

spective introns in *EF-1 alpha* mRNA could be assayed by PCR amplification of a cDNA library using specific primers.

6.1.3 Mating system evolution in *Pleospora* sensu stricto, in the following referred to as *Pleospora*

Combined evidence from the species phylogeny in chapters 2 and mating type evidence from chapter 3, led to the conclusion that the three groups of homothallics in *Pleospora* evolved in three different ways. One homothallic lineage evolved by fusion of the *MAT1-1* and *MAT1-2* loci, a second homothallic clade originated by horizontal transfer of the fused MAT locus, and a third origin resulted in a clade of homothallics with only a single detectable forward-pointing *MAT1-1* idiomorph. The evolution of homothallism by horizontal transfer as in *Pleospora* has never been reported before.

Throughout chapter 3, the mating system in *Pleospora* was compared to the one in *Cochliobolus* that is in the same family as *Pleospora.* Unlike *Pleospora*, *Cochliobolus* homothallics evolved repeatedly and independently from heterothallics, by the inclusion of both MAT loci within one genome (Yun *et al.*, 1999). A recent study of the B trichothecene-producing clade of *Fusarium* found that, unlike *Cochliobolus* and *Pleospora*, all homothallics of this group evolved once from heterothallics (O'Donnell *et al.*, 2004). This was supported by the fact that all homothallics shared the same fused MAT locus arrangement.

6.1.3.1 Further research

Mating system evolution should be investigated in other ascomycete groups. The *Nectria haematococca-Fusarium solani* species complex and the two intermingled genera *Neurospora* and *Gelasinospora* would be appropriate subjects for studies of mating type gene evolution because MAT locus sequences are available for some species in each group.

The phylogenetic distribution of homothallics in the *Nectria haematococca-Fusarium solani* species complex is discontinuous (O'Donnell, 2000), as in *Pleospora* or *Cochliobolus*. Examination of the MAT locus and phylogenetic analyses would be necessary in this case to decide if the discontinuous distribution is due to vertical or lateral transfer.

The two intermixed genera *Neurospora* and *Gelasinospora* also contain both heterothallics and homothallics (Dettman *et al.*, 2001). A broader taxon sampling, clarification of generic limits, and additional structural information about the MAT loci would be needed to determine mating system evolution in this case.

Pleospora represents an ideal genus in which to investigate the effects of different mating systems on genetic structure of populations and species.

Pleospora contains three homothallic mating systems, each of which evolved independently from a heterothallic system. Using population genetics approaches, some of the questions that can be addressed are as follows. The homothallic *Gibberella zeae* (Schwein.) Petch can outcross (Bowden & Leslie, 1999). Do homothallics of *Pleospora* outcross, and how does the outcrossing rate compare to closely related heterothallics? In *Pleospora*, *Cochliobolus* and the B trichothecene-producing clade of *Fusarium*, homothallics are derived from heterothallics. Does the genetic structure of homothallic fungal populations differ from the ones of heterothallic close relatives?

The homothallic phylogenetic species *P. herbarum* has a global distribution, including the campus of the University of British Columbia (*Stemphylium sp.* strain P301, see Table 2-2). This distribution would allow for an assessment of the genetic structure at different levels, from populations to continents. Genetic structures of heterothallic *Pleospora* species could also be determined, and compared to homothallic genetic structures for different levels. To assess the importance of outcrossing and other evolutionary forces to the population structure of heterothallics and homothallics, mark-release-recapture experiments can be done in conjunction with computer simulations (Zhan & McDonald, 2004).

6.2. The new family Aliquandostipitaceae

Chapter 5 of my thesis is a morphological and phylogenetic study of two new species from Thailand and China (Inderbitzin *et al.*, 2001). This chapter is linked to the previous ones in that it investigated questions of diversity and evolution, but in a more fundamental sense. Whereas the genus *Pleospora* was described and well-known prior to the beginning of my studies, morphological features present in *Aliquandostipite* had never been reported in the ascomycetes. Characters reported for the first time for *Aliquandostipite* included the dimorphic fruitbodies with both morphological types side by side on the substrate, and the widest hyphae ever reported in ascomycetes. The overall morphological features were of the order Pleosporales. However, phylogenetic analyses showed that species of *Aliquandostipite* did not belong to the Pleosporales, but represented a previously unknown in the class Dothideomycetes. Because of the phylogenetic placement and the novel morphological features, the two new species were placed in the new family Aliquandostipitaceae.

After the publication of the manuscript reprinted in chapter 5, Pang et al. (2002) showed that *Aliquandostipite* was related to fungi in the genus *Jahnula*. Reinterpretation of the *Jahnula* morphology showed that it also contained the fruitbody dimorphism and the wide hyphae of the Aliquandostipitaceae.

Pang et al. (2002) showed in phylogenetic analyses that one species of *Aliquandostipite*, *A. sunyatsenii*, was more closely related to species with mor-

phological similarity to the type of *Jahnula,* than to the type of *Aliquandostipite.* Thus, they transferred *A. sunyatsenii* to *Jahnula.*

6.2.1 Further research

Jahnula and *Aliquandostipite* are ideal genera for a study of phylogenetic significance of morphological characters. Unlike *Pleospora* for example, they contain a considerable amount of morphological diversity that can be used for taxonomic purposes. Morphological variation occurs in the shape of the asci, presence of an apical ring and an ocular chamber in the ascus apex, ascospore shape and color, ascospore appendage type, presence of both stalked and sessile ascomata and several apomorphic morphological characters (Hyde, 1999; Inderbitzin *et al.*, 2001; Pang *et al.*, 2002).

A comprehensive study addressing the taxonomic confusion in the two genera would be fruitful at this point. The study should include morphological and phylogenetic analyses of at least the type specimens of both genera, together with additional species to represent the morphological diversity of the entire group. Protein coding genes, in addition to ribosomal genes, could provide additional phylogenetic resolution.

6.3. Bibliography

Bowden, R. L. & Leslie, J. F. (1999). Sexual recombination in *Gibberella zeae. Phytopathology* **89**, 182-188.

Burset, M., Seledtsov, I. A. & Solovyev, V. V. (2000). Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Research* **28**, 4364-4375.

Câmara, M. P. S., O'Neill, N. R. & Berkum, v., P (2002). Phylogeny of *Stemphylium* spp. based on ITS and glyceraldehyde-3-phosphate dehydrogenase gene sequences. *Mycologia* **94**, 660-672.

Crivelli, P. G. (1983). Über die heterogene Ascomycetengattung *Pleospora* Rabenh.; Vorschlag für eine Aufteilung. Ph.D. thesis. Eidgenössische Technische Hochschule, Zürich, Switzerland.

Dettman, J. R., Harbinski, F. M. & Taylor, J. W. (2001). Ascospore morphology is a poor predictor of the phylogenetic relationships of *Neurospora* and *Gelasinospora. Fungal Genetics and Biology* **34**, 49-61.

Holm, L. (1962). Lewis E. Wehmeyer, a world monograph of the genus *Pleospora* and its segregates. *Svensk Botanisk Tidskrift* **56**, 377-381.

Hyde, K. D. (1999). Tropical Australian Freshwater Fungi. XV. The ascomycete genus *Jahnula*, with five new species and one new combination. *Nova Hedwigia* **68**, 489-509.

Inderbitzin, P., Landvik, S., Abdel-Wahab, M. A. & Berbee, M. L. (2001). Aliquandostipitaceae, a new family for two new tropical ascomycetes with unusually wide hyphae and dimorphic ascomata. *American Journal of Botany* **88**, 52-61.

Kroken, S. & Taylor, J. W. (2001). A gene genealogical approach to recognize phylogenetic species boundaries in the lichenized fungus *Letharia. Mycologia* **93**, 38-53.

O'Donnell, K. (2000). Molecular phylogeny of the *Nectria haematococca-Fusarium solani* species complex. *Mycologia* **92**, 919-938.

O'Donnell, K., Ward, T. J., Geiser, D. M., Kistler, H. C. & Aoki, T. (2004). Genealogical concordance between the mating type locus and seven other nuclear genes supports formal recognition of nine phylogenetically distinct species within the *Fusarium graminearum* clade. *Fungal Genetics and Biology* **41**, 600-623.

Pang, K.-L., Abdel-Wahab, M. A., Sivichai, S., El-Sharouney, H. M. & Jones, E. B. G. (2002). Jahnulales (Dothideomycetes, Ascomycota): a new order of lignicolous freshwater ascomycetes. *Mycological Research* **106**, 1031-1042.

Wehmeyer, L. E. (1961). *A world monograph of the genus Pleospora and its segregates.* Ann Arbor: The University of Michigan Press.

Yun, S. H., Berbee, M. L., Yoder, O. C. & Turgeon, G. (1999). Evolution of fungal self-fertile reproductive life style from self-sterile ancestors. *Proceedings of the National Academy of Sciences of the USA* **96**, 5592-5597.

Zhan, J. & McDonald, B. A. (2004). The interaction among evolutionary forces in the pathogenic fungus *Mycosphaerella graminicola. Fungal Genetics and Biology* **41**, 590-599.

Zhan, J., Pettway, R. E. & McDonald, B. A. (2003). The global genetic structure of the wheat pathogen *Mycosphaerella graminicola* is characterized by high nuclear diversity, low mitochondrial diversity, regular recombination, and gene flow. *Fungal Genetics and Biology* **38**, 286-297.

CHAPTER 7. Appendices

7.1. DNA, PCR and sequencing

7.1.1 DNA extraction, purification and quantification

The DNA extraction, purification and quantification protocols given below were used in this thesis. Only for chromosome walking, DNA was purified by removal of RNA, and the DNA concentration was measured. Otherwise, the unpurified DNA extract was used directly for PCR, diluted ten or 100 times.

DNA extraction was based on Lee & Taylor (1990) with modifications suggested by M. Berbee and S. Landvik (personal communications). DNA purification and measurement was based on a Qiagen Inc. handbook (Anonymous, 2000), and a Spectral Genomics Inc. protocol (Anonymous, 2002a).

7.1.1.1 Reagents for DNA extraction

- DNA extraction buffer: 50 mM Tris-HCl (pH 7.2), 50 mM EDTA, 3% SDS, with 1% beta-mercaptoethanol added just before extraction.
- Phenol : Chloroform = 1 : 1.
- Chloroform.
- Sodium acetate 3 M, pH 8.0.
- Isopropanol.
- Ethanol 70%.
- TE buffer with low EDTA concentration: 10 mM Tris-HCl, 0.1 mM EDTA pH 8.0.

Liquid nitrogen if needed.

7.1.1.2 Additional reagent for DNA purification

RNase A stock solution (100 mg/ml) from Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada).

7.1.1.3 Preparation of fungal material for DNA extraction

The DNA extraction protocol consisted of two main parts. Preparation of the material and extraction of the nucleic acids, DNA plus RNA. Preparation for extraction depended on the kind of material and the desired quantity of DNA required.

7.1.1.3.1 Fresh mycelium and small amounts of herbarium material

Step 1. Mycelium was scraped off a Petri dish with a spatula, deposited into 30 µl lysis buffer in a 1.5 ml-Eppendorf tube and ground with a few turns of a micropestle treated with HCl.

Step 2. 670 µl lysis buffer were added and the Eppendorf tube incubated at 65°C for one hour.

Step 3. Tube was stored in freezer or directly used in DNA extraction in step 4, see below.

7.1.1.3.2 Lyophilized fungal material for high DNA yield

Step 1. Fungi were grown in liquid culture on a shaker. The harvested mycelium was filtered, rinsed with distilled water, frozen in liquid nitrogen and lyophilized without delay. Mycelial pellets of ca. 1 cm^3 required two days for completion of lyophilization. Lyophilized material was stored in airtight containers.

Step 2. Lyophilized mycelium was ground in liquid nitrogen with pre-chilled mortar and pestle. Ground mycelium was added to a 1.5 ml-Eppendorf tube, up to one third of the conical portion (20 to 60 mg). 700 µl of lysis buffer was added, homogenized by vortexing and incubated at 65°C for one hour.

Step 3. Tube was stored in the freezer or directly used in DNA extraction in step 4, see below. Remaining powdered mycelium was stored in airtight container.

7.1.1.4 Extraction of nucleic acids

Step 4. To Eppendorf tube, 700 µl of phenol : chloroform = 1 : 1 was added, homogenized by vortexing, and spun in a microcentrifuge for 15 minutes.

Step 5. A maximal volume of supernatant was transferred to a new tube containing an equal volume of chloroform, homogenized by vortexing and spun in a microcentrifuge for 15 minutes.

Step 6. A maximal volume of supernatant (y µl) was transferred to a new tube, preferably a tube with a non-sticky inner surface (e. g. Eppendorf Flex-Tubes 1.5 mL, distributed by Brinkmann Instruments Ltd., Mississauga, ON, Canada). 10 µl of 3 M sodium acetate (pH 8.0) was added, followed by y µl times 0.54 isopropanol (see below) and gently homogenized by inversion of the tube. For herbarium material, tube was placed in a freezer over night for precipitation (Ristaino *et al.*, 2001). For non-problematic materials, this step was omitted, and tubes were directly spun in a microcentrifuge for 2 minutes. The isopropanol was carefully removed by pipetting without disturbing the pellet. For y µl of supernatant, the following amounts of isopropanol were used:

y = 300 µl isopropanol = 162 µl
y = 350 µl isopropanol = 189 µl
y = 400 µl isopropanol = 216 µl
y = 450 µl isopropanol = 243 µl
y = 500 µl isopropanol = 270 µl
y = 550 µl isopropanol = 297 µl
y = 600 µl isopropanol = 324 µl

Step 7. 700 µl 70% ethanol was added, gently inverted to mix and spun in a microcentrifuge for 2 minutes.

Step 8. As much liquid as possible was removed by slowly pipetting it out using a small pipette tip. The pellet was dried at room temperature for 5-10 minutes, overdrying was avoided.

Step 9. If DNA was intended for PCR amplification, 200 µl of TE buffer was added and incubated at 65°C for one hour to resuspend DNA. If DNA was to be purified, 700 µl of TE buffer was added.

7.1.1.5 Removal of RNA

RNA was only removed when the DNA was used for chromosome walking, in order to measure the concentration of DNA with a spectrophotometer.

Step 10. Per 100 µl of nucleic acid extract, 1 µl of RNase A stock solution (100 mg/ml) from Qiagen DNeasy Plant Mini Kit (Qiagen Inc., Mississauga, ON, Canada) was added, aiming for a final RNase concentration of 1 mg/ml. The tube was then slowly turned end-over-end by hand for a while to homogenize, and incubated 30 minutes at 37°C. Then steps 4-9 were followed, except that the solutions were mixed gently by hand, DNA was left to precipitate for 15 minutes at room temperature after addition of sodium acetate and DNA was suspended in 100 µl of TE buffer by gentle pipetting prior to measuring the DNA concentration.

7.1.1.6 Measuring of extracted DNA concentration

Step 11. Serial dilutions from RNase treated DNA stock were made. Spectrophotometer readings had to fall between 0.1 and 1.0 to be accurate. An absorbency of 1.0 at 260 nm corresponded to 50 µg of DNA/ml. Pure DNA had a A260/A280 ratio of 1.7-1.9. After measurement, two to 10 µl of DNA were electrophoresed on an agarose gel. Non-degraded, ethydium bromide stained DNA was visible under UV light as a wide band in the upper part of the DNA ladder. If the DNA was degraded, the band was less distinct, and there was a smear towards the bottom of the DNA ladder. Contaminating RNA was visible as a smear with highest intensity in the bottom part of the DNA ladder (ca. 100-500 bp).

7.1.2 Preparation of PCR reactions

PCR amplifications were done in 25 µl final reaction volumes, including 12.5 µl of DNA extract using ReadyToGo PCR Beads, or puReTaq Ready-To-Go PCR Beads (Amersham Biosciences, Piscataway, NJ, USA), following the manufacturer's instructions, or self-prepared master mix with GIBCO BRL Taq DNA polymerase (Life Technologies, Inc., Gaithersburg, MD) (dNTP 0.2 mM each, primer 0.5 uM each, MgCl 0.75 mM, Taq 0.625 units per reaction, enzyme buffer).

7.1.3 Electrophoresis of PCR products

4 µl PCR product was separated by electrophoresis in TAE running buffer (0.1 M Tris, 12.5 mM sodium acetate, 1 mM EDTA, pH8.1) on a 1% agarose gels (SeaKem GTG agarose, Cambrex Bio Science Walkersville, Inc, Walkersville, MD, USA) and stained in an ethydium bromide solution for 15 minutes (from 10 mg/ml ethydium bromide stock, 10 µl were added to distilled water tray holding one gel).

For small PCR products below 400 bp in length, 3% agarose gels formulated to resolve small bands were used. The 3% gels containing one third SeaKem GTG agarose (Cambrex Bio Science Walkersville, Inc, Walkersville, MD, USA) and two thirds NuSieve GTP agarose (FMC Bioproducts, Rockland, ME, USA), a low-melting agarose.

7.1.4 Reamplification of weak PCR bands

Weak PCR bands were cut out of the gel with a razor blade under UV illumination, melted in 50 µl TE buffer (2 min at 95°C), diluted 10 and 100 times, and reamplified with the initial PCR primers or internal primers, with ca. 3°C higher annealing temperature.

7.1.5 Precipitation of PCR products

For DNA sequencing, PCR products were cleaned by sodium acetate - ethanol precipitation. To a 1.5 ml-Eppendorf centrifuge tube with non-stick inner surface, 2 µl of sodium acetate (3 M, pH 4.5) were added, followed by the remaining 21 µl of PCR product and 57.5 µl 95% ethanol, and mixed by vortexing. The tube was placed directly on ice in the freezer for 20 minutes, followed by a 10-minute spin in the microcentrifuge. The liquid was removed by pipetting using a tip with small opening, without touching the pellet. To remove excess sodium acetate, 700 µl of 75% ethanol were added, the tube gently inverted to mix and spun in the centrifuge for 5 minutes. All ethanol was removed by pipetting, and the pellet dried under vacuum in a speed vac for 5 minutes. The pellet was resuspended in 12 µl of PCR quality water at room temperature.

To concentrate PCR products, several PCR reactions were pooled for precipitation, with sodium acetate and 95% ethanol adjusted accordingly.

7.1.6 Measuring of PCR product concentrations

To give accurate measurements of DNA concentration, the spectrophotometer reading had to fall within 0.1 and 1.0. By addition of 5 µl purified PCR product to 95 µl of water, a spec reading of at least 0.150 could be expected. This corresponded to 150 ng/µl of DNA (DNA concentration = spec reading x dilution x 50).

7.1.7 Preparation of DNA sequencing reactions

Sequencing reactions had a final volume of 20 µl, consisting of 4 µl Big Dye Terminator Cycle Sequencing Kit v2.0 or 3.0 (Applied Biosystems, Foster City, CA, USA), 3 µl primer (1 uM) and 13 µl PCR product adjusted to 10 ng/µl DNA. A quick centrifuge spin was used for homogenization.

7.1.8 Precipitation of DNA sequencing products and DNA sequence determination

To each sequencing reaction, 2 µl sodium acetate (3 M, pH 4.5) and 50 µl 95% ethanol were added, and precipitated at room temperature for 15 minutes. The remainder was as for PCR products. The dried pellet was stored in the freezer until DNA sequence determination by the Nucleic Acids Protein Services (NAPS) Unit of the University of British Columbia.

7.2. Chromosome walking

For chromosome walking, DNA extracted from lyophilized mycelium and purified by RNase treatment was used. However, Sara Landvik was successful with DNA extracted from wet mycelium, without RNase treatment (S. Landvik, personal communication). The DNA was digested, and adapters for primer sites were then ligated to the digested DNA. The following protocols are slightly modified after the original Vectorette chromosome walking protocol (Anonymous, 2002b).

7.2.1 Restriction enzyme digest of DNA

To a 0.5 or 1.5 ml-Eppendorf tube, 33 µl PCR quality water and 5 µl restriction enzyme buffer were added, as well as 10 µl DNA visible on a gel as a bright band. Finger vortexing was used to mix, followed by a quick centrifuge spin. Then, 2 µl or ca. 20 units restriction enzyme were added, finger vortexed and spun down, followed by incubation at 37°C for 2h. To check the digest, 10 µl were run on an agarose gel.

7.2.2 Adapter ligation to digested DNA

To a 0.5 or 1.5 ml-Eppendorf tube, 15 µl DNA digest were added as well as 1.5 µl Vectorette adapter units (0.9 pmol), 0.5 µl 100 mM ATP, and 0.5 µl 100 mM DTT, finger vortexed to mix, and placed in the centrifuge for a quick spin. Then 0.5 µl DNA ligase was added (0.5 units), finger vortexed to mix, and quickly spun in a microcentrifuge. Three cycles of incubation at 20°C for one hour, followed by 37°C for half an hour were used for effective ligation of digested DNA to adapters. To stop enzymatic reactions, 60 µl of PCR quality water was added after completion of the three cycles.

7.2.3 Chromosome walking with touchdown and hot start PCR protocols

For higher yield and specificity, decreasing annealing temperature and addition of the Taq enzyme to heated PCR mixture were used. In the first set of PCR reactions, the ligated DNA was diluted 50 times, and 12.5 µl was added to each PCR reaction containing only 12.0 µl PCR cocktail, overlayed with a drop of mineral oil. The PCR program was started with a heating step to 94°C where 0.5 µl Expand High Fidelity PCR System Taq was added (Roche Diagnostics GmbH, Mannheim, Germany). The products of the first PCR reactions were diluted 50 times in PCR quality water, and 12.5 µl was used per reaction in a second round of PCR with touchdown conditions and hot start. For details on PCR programs, see section 3.2.3.2.4 on page 74.

7.3. Bibliography

Anonymous (2000). *DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit Handbook*, August 2000 edn. Mississauga, Ontario, Canada: Qiagen Inc.

Anonymous (2002a). DNA Clean up protocol, pp. 1-2: Spectral Genomics Inc.

Anonymous (2002b). The Vectorette System, pp. 1-28. The Woodlands, Texas, USA: Sigma-Genosys.

Lee, S. B. & Taylor, J. W. (1990). Isolation of DNA from fungal mycelia and single spores. In *PCR Protocols*, pp. 282-287. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego: Academic Press, Inc.

Ristaino, J. B., Groves, C. T. & Parra, G. R. (2001). PCR amplification of the Irish potato famine pathogen from historic specimens. *Nature* **411**, 695-697.