Systematics and Biodiversity

ISSN: 1477-2000 (Print) 1478-0933 (Online) Journal homepage: https://www.tandfonline.com/loi/tsab20

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To cite this article: Asa Kruys & Mats Wedin (2009) Phylogenetic relationships and an assessment of traditionally used taxonomic characters in the Sporormiaceae (Pleosporales, Dothideomycetes, Ascomycota), utilising multi-gene phylogenies, Systematics and Biodiversity, 7:4, 465-478, DOI: 10.1017/S1477200009990119

To link to this article: https://doi.org/10.1017/S1477200009990119

Published online: 11 Mar 2010.

Systematics and Biodiversity **7** (4): 465–478
doi:10.1017/S1477200009990119

Issued 1 December 2009

Phylogenetic relationships and an assessment of traditionally used taxonomic characters in the Sporormiaceae (Pleosporales, Dothideomycetes, Ascomycota), utilising multi-gene phylogenies

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submitted February 2009
accepted June 2009

Contents

Abstract The family Sporormiaceae (Pleosporales, Dothideomycetes, Ascomycota) occur worldwide and a majority of the species are coprophilous. The taxonomy and classification of the family are based on a small number of morphological and ecological characters. Several taxa are easily confused by their shared morphological features, and the relationships between genera are poorly known and in need of critical study. The aims of this study were to resolve the phylogenetic relationships within the Sporormiaceae, test the current generic classification, and study the utility of traditional characters for the taxonomy in the group. To resolve these questions, we analysed combined data sets of ITS-nLSU rDNA, mtSSU rDNA and ß-tubulin sequences with parsimony and Bayesian methods.

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Published online 11 Mar 2010

The results showed that many characters, which previously have been used in the taxonomy and classification of the Sporormiaceae, such as the substrate choice, presence or absence of an ostiole, and presence or absence of germ slits, were all homoplastic and not useful for circumscribing monophyletic groups. A number of characters may be useful for circumscribing smaller clades if used in combination with other features, such as the shape of the ascus with the shape of the ascospores. Our phylogenetic analyses show that *Preussia* and *Sporormiella* are non-monophyletic, and a constrained analysis forcing these genera into monophyly resulted in significantly worse trees. *Spororminula* is nested in *Preussia* s. lat., and *Eremodothis* and *Pycnidiophora* are nested within *Westerdykella*. Finally, we suggest a new generic classification for the family Sporormiaceae, including *Sporormia*, *Preussia* (including *Sporomiella* and *Spororminula)* and *Westerdykella* (including *Eremodothis* and *Pycnidiophora*). We also propose 14 new combinations: *Preussia alloiomera* (S.I. Ahmed & Cain) Kruys, *Preussia antarctica* (Speg.) Kruys, *Preussia bipartis* (Cain) Kruys, *Preussia borealis* (I.Egeland) Kruys, *Preussia dubia* (S.I. Ahmed & Cain) Kruys, *Preussia lignicola* (W. Phillips & Plowr.) Kruys, *Preussia longisporopsis* (S.I. Ahmed & Cain) Kruys, *Preussia minipascua* (S.I. Ahmed & Cain) Kruys, *Preussia octomera* (Auersw.) Kruys, *Preussia splendens* (Cain) Kruys, *Preussia tenerifae* (Arx & Aa) Kruys, *Preussia tetramera* (S.I. Ahmed & Cain) Kruys, *Westerdykella angulata* (A.C. Das) Kruys and *Westerdykella aurantiaca* (J.N. Rai & J.P. Tewari) Kruys.

Key words ß-tubulin, bitunicate, coprophilous, fungi, loculoascomycete, phylogeny

Introduction

The classification of many groups of Fungi has traditionally been based on a few easily observed characteristics, which often reflect aspects of their ecology and thus may be subject to parallel evolution or extreme specialisation in highly derived groups. There are numerous examples of non-monophyletic fungal groupings that have remained in classifications until very recently; like the well-known basidiomycetes in the Aphyllophorales that have been shown to comprise highly unrelated groups which all have the crust-like fruiting bodies in common (Larsson *et al.*, 2004; Binder *et al.*, 2005), and the mazaedia-producing Caliciales among the ascomycetes, which independently have acquired prototunicate, evanescent asci and passive spore dispersal (Tibell, 1984; Wedin & Tibell, 1997; Wedin *et al.*, 1998). With the progress of molecular phylogeny, the monophyly of these groups and the potentially synapomorphic traits defining them can be tested with independent means and classifications updated accordingly (e.g. Hibbet *et al.*, 2007; Lumbsch & Huhndorf, 2007). Still, potentially non-monophyletic groups defined on singular ecological or morphological traits prevail, particularly among taxa on lower systematic levels. One such example is the generic delimitation within the Sporormiaceae (Pleosporales, Dothidiomycetes, Ascomycota).

The Sporormiaceae occur worldwide and live as saprobes on various substrates including dung, plant debris, soil and wood. Recently, endophytic representatives have also been reported (Arenal *et al.*, 2007). Most taxa, however, are coprophilous and their ascospores are useful tools in paleoecological studies, when reconstructing animal abundances in the past (Burney *et al.*, 2003; van Geel *et al.*, 2003, 2007). The characteristic ascospores are thick-walled and dark brown, strongly constricted at septa and often fragmenting into part-spores at maturity (Barr, 2000). Germ slits in the spore walls are also common features (Fig. 1). The spores are developed in fissitunicate asci and pseudothecioid ascomata (Fig. 1). In the latest treatment (Barr, 2000), the Sporormiaceae comprises eight genera, and *c.* 100 species. One more genus, *Eremodothis*, has been included in the family since then (Kruys *et al.*, 2006; Lumbsch & Huhndorf, 2007). Also, the hyphomycete *Amorosia* should be transferred to the Sporormiaceae according to Mantle *et al.* (2006), but its closest relatives are unclear.

The type genus, *Sporormia*, is characterised by globose pseudothecia, which open with an ostiole. The ≥16-celled spores are typically joined together in a bundle with one common gelatinous sheath, when released from the ascus (Ahmed & Cain, 1972; Dissing, 1992). Germ slits in the spore walls, otherwise common and characteristic for the family, are absent in the type species *Sporormia fimetaria* (Ahmed & Cain, 1972). *Sporormia*, *Sporormiella* and *Preussia* are considered closely related and are easily confused by their shared morphological features (Munk, 1957; von Arx & Müller, 1975; Eriksson, 1981). *Sporormiella*, the most species-rich genus in the family, includes species with perithecioid ascomata and ≥4-celled spores with germ slits (Ahmed & Cain, 1972). *Preussia* differs morphologically from 4-celled *Sporormiella* species only by the ascomata being cleistothecioid (Cain, 1961; Barr, 2000). The taxonomic importance of the ostiole has, however, been questioned, since its presence in *Preussia* and *Sporormiella* seem to depend on growth conditions (von Arx, 1973; Guarro *et al.*, 1997a). According to experiments *in vitro*, different morphs can even be found within the same culture (Guarro *et al.*, 1997a).

Substrate choice has been another important diagnostic character for separating *Preussia* and *Sporormiella* utilised by von Arx and van der Aa (1987). *Preussia*, in their sense, includes species on plant debris, wood or soil, while *Sporormiella* is restricted to coprophilous species with perithecioid ascomata. Doveri (2004) also considered the preference for a certain substrate an important feature, together with the shape of the ascus where *Sporormiella* should have cylindric or cylindric-claviform asci and *Preussia* more or less clavate asci. He also believed that *Preussia* in general have more superficial ascomata. Typical species of *Sporormiella sensu* von Arx & van der Aa occur on substrates other than dung, while for instance

Figure 1 Ascomata (a) with ostiole (*Sporormiella* sp.) and (b) without ostiole (*Preussia funiculata*). (c) Spore with germ slits (*Sporormiella* sp.). Asci and spores of taxa representing major monophyletic groups in Sporormiaceae. (d) *Westerdykella* sp. (the *Westerdykella* clade). (e) *Sporormia fimetaria* (the *Sporormia* clade), (f) *Preussia funiculata* (the *P. funiculata* clade), (g) *P. vexans* (the *S. vexans* clade), (h) *P. intermedia* (the *S. intermedia* clade) and (i) *P. irregularis* (the *S. irregularis* clade). Scale bars correspond to 100 μm (a–b) or 25 μm (c–i).

P. funicalata often is coprophilous. *Preussia* and *Sporormiella* are therefore considered synonyms by several authors at present, and although this is not yet accepted in current classification (Lumbsch & Huhndorf, 2007), a large number of species has already been transferred (Valldosera & Guarro, 1990; Guarro *et al.*, 1997b; Abdullah *et al.*, 1999; Arenal *et al.*, 2004, 2005, 2007).

The monotypic genus *Sporormiella* is very similar to *Sporormiella* species with 8-celled spores, although the cells lack germ slits. Barr (2000) suggested, however, a close relationship between *Sporormiella* and *Sporormia*, as well as *Pleophragmia*, because of the presence of an ostiole, the lack of germ slits, and the spores, which should be triangular in transverse view. These ideas by Barr (2000) have not yet been tested in a phylogenetic context.

Westerdykella, *Pycnidophora* and *Chaetopreussia* form, like *Preussia*, cleistothecioid ascomata (Clum, 1955; Stolk, 1955; Locquin-Linard, 1977). Their spores lack germ slits and should be 4-celled, but separate into single cells at a very early stage in the ascus, according to Cain (1961), von Arx and van der Aa (1987) and Barr (2000). This last feature seems to be a long-lived misinterpretation concerning *Pycnidiophora* and *Westerdykella*, because both genera have been shown to develop 32 one-celled spores (Clum, 1955; Stolk, 1955; Thompson & Backus, 1966). *Pycnidiophora* has been treated as a synonym to *Westerdykella* by several authors (Cejp & Milko, 1964; von Arx & Storm, 1967; von Arx, 1981). However, both von Arx and van der Aa (1987) and Barr (2000) suggested that *Westerdykella* should be restricted to the type *W. ornata*, the only species with ornamented spores. None of these authors have formally transferred any species to *Pycnidiophora*, though.

It is clear that the phylogenetic relationships within the Sporormiaceae, and the utility of traditional characters for the taxonomy in the group, need a critical study. Here we intend to produce a phylogenetic hypothesis of the Sporormiaceae, to investigate if there are any well-supported monophyletic groups within the family, and to test the current generic classification. We will also investigate if the monophyletic groups found correlate with some of the morphological and ecological characters most commonly used for the taxonomy in the group; substrate choice, presence of an ostiole and presence of a germ slit. To resolve these questions, we will use sequences from both the nuclear and mitochondrial rDNA, as well as from the gene coding for the ß-tubulin protein.

Materials and methods

Taxon sampling

We have included 51 species from seven of the nine genera currently classified in the *Sporormiaceae*. *Chaetopreussia* and *Pleophragmia* had to be excluded due to lack of material. We compiled two combined data sets, one including 72 taxa with either the ITS or/and nLSU rDNA genes, and a second including 46 taxa that were complete for at least three of the four markers, nITS-LSU rDNA, mtSSU rDNA and ß-tubulin. Results of studies including the major families in the *Pleosporales* (Schoch *et al.*, 2006; Wang *et al.*, 2007) suggested that

Lepidosphaeria nicotiae is a suitable outgroup. Another seven taxa within the *Pleosporales*, closely related to the study group, were also included. Collection data for the sequenced specimens together with accession numbers for sequences retrieved from GenBank (http://www.ncbi.nlm.nih.gov) are listed in Table 1.

DNA extraction, amplifying and sequencing

DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Solna, Sweden), with a modification in the last step where DNA was eluted in sterile water. Double-stranded copies of the nITS-LSU rDNA, mtSSU rDNA and ß-tubulin were obtained by polymerase chain reaction (PCR) amplifications using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech Uppsala, Sweden). Sequences of ITS, nLSU and mtSSU rDNA were amplified with the following settings: initial denaturation at 94 °C for 5 min, 5 cycles of: 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, followed by 30 cycles of: 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. ß-tubulin was amplified with the settings: initial denaturation at 94 °C for 2 min, 40 cycles of 94 °C for 30 s, 50 °C for 15 s and 72 °C for 1 min, and final extension step at 72 °C for 10 min. The following primers were used for the PCR-amplifications: ITS1F in combination with LR1 (Vilgalys and Hester, 1990; Gardes & Bruns, 1993), ITS3 in combination with JS8 (White *et al.*, 1990; Landvik, 1996), MSU1 in combination with MSU7 (Zhou & Stanosz, 2001), and BT1819R in combination with BT2916 (Miller & Huhndorf, 2005). The PCR-products were then purified with the Qiagen PCR Purification Kit. The cycle sequencing reaction was performed with the DYE-namic ET terminator cycle sequencing kit (Amersham Pharmacia Biotech), with the following settings: 28 cycles (95 °C for 20 s, 50 °C for 15 s and 60 °C for 1 min). Additional primers used for the sequencing reactions were: ITS4 (White *et al.*, 1990), LR0R, LR5, LR3, LR3R (Vilgalys & Hester, 1990; Rehner & Samuels, 1995), nu-LSU-362–5′(Döring *et al.*, 2000), JS1 (Landvik, 1996), mrSSU1, mrSSU2, mrSSU3R (Zoller *et al.*, 1999), MS2 (White *et al.*, 1990), BT1283R (Miller & Huhndorf, 2005), and the new ß-tubulin primer BT1910R, which was designed for this study. The reverse primer BT1903R has the sequence TCT GGT CCT CRA CCT CCT TCA, and its position relative to the ß-tubulin sequence of *Neurospora crassa* (M13630) is 1903–1923. Post-reaction clean-up was carried out following the DYE-namic ET terminator cycle sequencing kit protocols (Amersham Pharmacia Biotech). The purified sequencing products were then loaded on a gel for automatic sequencing (ABI 377, Applied Biosystems, Stockholm, Sweden).

Sequence alignment

Sequence fragments were assembled and edited in Autoassembler 1.4.0 (Applied Biosystems Division, Perkin Elmer). The sequence alignments were done in the MegAlign 5.06 program of the LaserGene 1.67 package (DNASTAR Inc.) using the Clustal-W algorithm, followed by manual correction and optimisation. We excluded primer regions and ambiguously aligned parts. The ß-tubulin data set was compared with the amino acid translation of *Pleospora herbarum* (AY749032) as a control for editing and alignment mistakes.

Phylogenetic analyses

Before combining the individual data sets, we used a 70% reciprocal bootstrap criterion (de Queiroz, 1993; Mason-Garner & Kellogg, 1996) for detecting incongruence among the genes. The parsimony bootstrapping analyses for these comparisons were performed in PAUP version 4.0b10 (Swofford, 2002) with the following settings: 1000 replicates of bootstrapping, 50 random addition heuristic searches, TBR branch swapping, and 'MulTrees' off. Gaps were treated as missing data.

The combined '2-markers' (nITS+LSU rDNA) and '4-markers' data sets (nITS+nLSU+mtSSU rDNA+ß-tubulin) were both analysed with Bayesian Markov chain Monte Carlo (MCMC) analyses and parsimony bootstrapping analyses. Bayesian analyses were conducted in MrBayes version 3.2 (Ronquist & Huelsenbeck, 2003). The best-fit nucleotide substitution model for each partition was determined with the hierarchical likelihood ratio test, as implemented in Modeltest version 3.7 (Posada & Crandall, 1998). The selected evolutionary model for the ß-tubulin data set was GTR+I+G. The TrN+I+G was found to be optimal for the ITS rDNA partition, while TrNef+I+G was optimal for the nLSU rDNA dataset. The best-fit model for mtSSU rDNA was TVM+I+G. However, since the TrN, TrNef and TVM models are not implemented in MrBayes version 3.2, we instead analysed all data sets using the more complex GTR+I+G model (Huelsenbeck & Rannala, 2004). Partitions were run separately with unlinked shape parameter, proportion of invariable sites and state frequency. The partitions were also allowed to evolve at different rates by setting the rate prior to variable. The MCMC sampling was performed with two parallel runs, eight chains and every 100th tree saved. Sampling was halted when the critical value for the topological convergence diagnostic had reached 0.01. The burn-in fraction was set to 0.25, which corresponded to 5880 trees in the two-gene data set, and 1558 trees in the four-gene data set. The 2-markers data set was also analysed under the parsimony criterion in PAUP version 4.0b10, using heuristic search, 10 000 random sequence addition replicates, start from random trees, TBR branch swapping and save multiple trees.

Support for branching topologies of the 2-markers data set was evaluated with 1000 replicates of bootstrapping, 50 random addition heuristic searches, TBR branch swapping and 'MulTrees' off. The 4-markers data set was evaluated with 1000 replicates of bootstrapping, 10 random sequence addition heuristic searches, TBR branch swapping and multiple trees saved.

To test alternative topologies we performed a constrained analysis, where *Preussia sensu strictu* was forced to form one monophyletic group and *Sporormiella sensu strictu* another monophyletic group. The constrained analysis was performed with previous parsimony settings, except that the heuristic search was decreased to 1000 random addition replicates. Statistical support ($P < 0.05$) for the alternative tree topologies was investigated with the Shimodaira–Hasegawa test (Shimodaira

& Hasegawa 1999) implemented in PAUP, using RELL and 1000 bootstrap replicates.

Results

A total of 46 nITS rDNA, 36 nLSU rDNA, 26 mtSSU rDNA and 41 ß-tubulin sequences were produced (Table 1). These sequences, together with 62 GenBank sequences (Table 1) were distributed on the two different data sets (both submitted to TreeBASE). The 2-markers data set generated an alignment with 651 characters in the ITS rDNA dataset, 1451 characters in the nLSU rDNA and 2102 characters in the combined data set. In total, we included 1728 unambiguously aligned exon characters in the 2-markers combined data set. The number of parsimony informative sites versus variable sites of the ITS rDNA dataset was 171/222, 150/200 for the nLSU rDNA and 321/422 in total. The 4-markers data set generated an alignment with 612 characters in the ITS rDNA dataset, 1450 characters in the nLSU rDNA, 1308 characters in the mtSSU rDNA, 1006 characters in the ß-tubulin and 4376 characters in the combined data set. In total, we included 3549 unambiguously aligned exon characters in the 4-markers combined data set. The number of parsimony informative sites versus variable sites of the ITS rDNA dataset was 160/208, 142/189 for the nLSU rDNA, 99/170 for the mtSSU rDNA, 232/308 for the ß-tubulin and 633/875 in total.

Phylogenetic analyses of the 2-markers combined data set

The separate bootstrap analyses resulted in trees with different numbers of well supported clades. Significant support refers to bootstrap values (bs) ≥70% and posterior probability values (pp) ≥95% (Hillis & Bull, 1993; Alfaro *et al.*, 2003). The ITS rDNA tree had 20 well-supported clades and the nLSU rDNA trees had 18 well-supported clades. We found one significant incongruence between the two data sets, *Westerdykella cylindrica* was sister taxon to all other *Westerdykella* species (incl. *Pycnidiophora* and *Eremodothis*) in the ITS phylogeny (bs 100), but clustered with *W. nigra* and *W.* sp in the nLSU phylogeny (bs 78). We do not consider this within-group incongruence to be of any major importance for our purpose in this study and we therefore combined the two gene partitions. The Bayesian analysis of combined data resulted in a majority rule consensus tree of 35 282 sampled trees (Fig. 2). The parsimony analysis resulted in 21 910 equally parsimonious trees with the tree length 1585. The parsimony consensus tree was congruent with the Bayesian majority rule consensus tree, although less resolved.

Phylogenetic analyses of the 4-markers combined data set

The separate bootstrap analyses resulted in trees with different numbers of well supported groups, the ITS and nLSU rDNA trees had 14 well supported clades each, and the mtSSU and ß-tubulin trees had nine clades each. We revealed one significant incongruence when comparing the separate datasets. *Westerdykella cylindrica* was sister-taxon to *Westerdykella* spp. and *E. angulata* in the mtSSU analysis (bs 82), as well as in the ITS rDNA analysis (bs 100), while it clustered with *W. nigra* (bs 89) in the nLSU rDNA analysis. We did not consider this within-group incongruence to be of any major importance for our purpose in this study and we therefore combined the four gene partitions. The Bayesian analysis of combined data resulted in a majority rule consensus tree of 9348 sampled trees (Fig. 3).

Constrained analysis

The constrained topology analysis resulted in trees that were 90 steps longer than the most parsimonious solution, and significantly worse ($P < 0.001$) according to the S–H test.

Discussion

Sporormiaceae

The family forms a monophyletic group with close to significant support in the 2-markers phylogeny (bs 69, not shown in Fig. 2), and strong support in the 4-markers phylogeny (Fig. 3). The backbone structures of the trees had low or no support in our analyses, despite including sequence data from four loci of two different organellar genomes. Still, these analyses reveal much information that is useful for the interpretation and classification of this poorly known group of fungi. Within the Sporormiaceae, *Westerdykella* (incl. *Eremodothis* and *Pycnidiophora*), and several groups of *Preussia* and *Sporormiella* are significantly supported as monophyletic.

Sporormia

The type, *Sporormia fimetaria* is sister to the *Westerdykella* clade in the 2-markers phylogeny (supported by pp). In the 4-markers phylogeny, it clusters with *S. pulchella*, which is an unusual member of *Sporormiella* with its uniseriate spores in the asci. Neither of these taxa is an obvious closest relative to *Sporormia*. As the sister-group relationship is ambiguous and the genus is morphologically unique with its typical spores (Fig. 1e), we suggest that *Sporormia* should continue to be circumscribed in the sense of Ahmed and Cain (1972).

Preussia versus *Sporormiella*

Preussia, as circumscribed by Cain, is polyphyletic, as *P. isomera* and *P. mediterranea* are nested within the *S. intermedia* clade while *P. terricola* groups with *S. megalospora*, rather than with the monophyletic core-*Preussia*. The *Preussia terricola* 3 sequence originates from the isotype and we believe that it shows a more correct relationship of *P. terricola* than the AY544686 sequence, which groups with the unresolved *Preussia* clade in our 2-markers phylogeny. Our results confirm the findings of Guarro *et al.* (1997a); the presence or absence of an ostiole is not a useful character when delimiting *Preussia* and *Sporormiella*. Our results also do not support a distinction between *Preussia* and *Sporormiella* corresponding to substrate choice (Fig. 2), as suggested by von Arx and van der Aa (1987) and Doveri (2004). Several of the sequenced *Preussia* specimens were found on dung and intermix with soil-inhabiting

Species	Source[a]	Origin	GenBank accession no. ITS	nLSU	mtSSU	ß-tubulin
Amorosia littoralis Mantle & D. Hawksw.	GenBank	Bahamas, marine sediment	AM292047	AM292055	–	–
Curreya pityophila (J.C. Schmidt & Kunze) Petr.	CBS[b] 149.32	the Netherlands, root of *Picea* sp.	**GQ203756**	DQ384102	DQ384072	**GQ203679**
Eremodothis angulata (A.C. Das) Arx	1. CBS 610.74	India, rice-field soil	**GQ203757**	DQ384105	DQ384075	–
	2. IMI 090323	India, rice-field soil	**GQ203758**	**GQ203720**	–	**GQ203680**
Herpotrichia juniperi (Duby) Petr.	CBS 468.64	Switzerland, *Pinus mugo*	**GQ203759**	DQ384093	DQ384077	**GQ203681**
Lepidosphaeria nicotiae Parq-Leduc	CBS 559.71	Algeria, desert soil	**GQ203760**	DQ384106	DQ384078	**GQ203682**
Melanomma pulvis-pyrius (Pers.) Fuckel	Eriksson n. 901013–10 (UME)	Sweden, wood	–	DQ384095	DQ384079	**GQ203683**
Pleospora herbarum (Pers.) Rabenh.	GenBank	USA, onion leaf	AF229479	AF382386	AF229665	AY749032
Preussia aemulans (Rehm) Arx	GenBank	the Netherlands, soil	DQ468017	DQ468037	–	–
P. africana[c] Arenal, Platas & Peláez	GenBank	Spain, *Viburnum tinus* leaves	AY510418	AY510383	–	–
P. flanaganii Boylan	GenBank	Mexico, soil	AY943061	–	–	–
P. fleischhakii (Auersw.) Cain	CBS 565.63	Germany, soil	**GQ203761**	**GQ203721**	**GQ203653**	**GQ203684**
P. funiculata (Preuss) Fuckel	1. Huhndorf *et al.* 2577, (F)	USA, porcupine dung	**GQ203762**	**GQ203722**	**GQ203654**	**GQ203685**
	2. GenBank	Germany, soil	AY943059	–	–	–
P. isomera Cain	1. CBS 388.78	Venezuela, cow dung	**GQ203763**	**GQ203723**	**GQ203655**	**GQ203686**
	2. GenBank	USA, dung	AY943058	–	–	–
P. mediterranea Arenal, Platas & Peláez	GenBank	Spain, *Daphne gnidium* leaves	DQ468025	DQ468045	–	–
P. terricola Cain	1. GenBank	–	–	AY544686	-	–
	2. CBS 527.84	Tanzania, elephant dung	**GQ203764**	**GQ203724**	**GQ203656**	**GQ203687**
	3. CBS 317.65	Honduras, *Musa sapientum*	**GQ203765**	**GQ203725**	–	**GQ203688**
P. typharum (Sacc.) Cain	CBS 107.69	Japan, deer dung	**GQ203766**	**GQ203726**	**GQ203657**	**GQ203689**
P. vulgaris (Corda) Cain	Strid 18884 (S)	Sweden, hare dung	**GQ203767**	**GQ203727**	**GQ203658**	**GQ203690**
Pycnidiophora aurantiaca (J.N. Rai & J.P. Tewari) Mukerji & V.R. Rao	GenBank	India, mud	AY943057	–	–	–
P. dispersa Clum	1. CBS 297.56	USA, *Phlox drummondi*	**GQ203797**	**GQ203753**	DQ384085	**GQ203716**
	2. CBS 508.75	Armenia, salt-marsh soil	**GQ203798**	DQ384099	–	–
Sporormia fimetaria[c] De Not	1. Lundqvist 2302-c (UPS)	Sweden, cow dung	**GQ203768**	**GQ203728**	–	**GQ203691**
	2. Dissing Gr.81.194 (UPS)	Greenland, sheep dung	**GQ203769**	**GQ203729**	–	**GQ203692**
S. lignicola W. Phillips & Plowr.	CBS 363.69	the Netherlands, rabbit dung	**GQ203783**	DQ384098	DQ384087	**GQ203703**

Table 1 Sequences included in this study, with newly produced sequences in bold. [a]Herbarium acronyms follow Holmgren *et al.* (1990). [b] CBS, Centraalbureau voor Schimmelcultures, the Netherlands. [c]*Preussia africana* and *S. lignicola* would morphologically be included in *Sporormiella* sensu Barr.

Species	Source[a]	Origin	GenBank accession no. ITS	nLSU	mtSSU	ß-tubulin
Sporormiella affinis (Sacc., E. Bommer & M. Rousseau) S.I. Ahmed & Cain	Lundqvist 17739-j (S)	Denmark, rabbit dung	**GQ203770**	**GQ203730**	**GQ203659**	**GQ203693**
S. alloiomera S.I. Ahmed & Cain	Lundqvist 21345-p (S)	Norway, goat dung	**GQ203771**	**GQ203731**	**GQ203660**	**GQ203694**
S. antarctica (Speg.) S.I. Ahmed & Cain	Lundqvist 5279-c (UPS)	Sweden, hazelhen dung	**GQ203772**	–	–	–
S. australis (Speg.) S.I. Ahmed & Cain	1. GenBank	Namibia, gazelle dung	AY510412	AY510377	–	–
	2. Lundqvist 20884-a (S)	France, rabbit dung	**GQ203773**	**GQ203732**	–	**GQ203695**
S. bipartis (Cain) S.I. Ahmed & Cain	Lundqvist 17250-a (S)	Sweden, ptarmigan dung	**GQ203774**	**GQ203733**	**GQ203661**	–
S. borealis (I. Egeland) J.C. Krug	Lundqvist 16745-c (S)	Romania, horse dung	**GQ203775**	**GQ203734**	**GQ203662**	**GQ203696**
S. dakotensis (Griffiths) S.I. Ahmed & Cain	Thulin 2570-g (UPS)	Ethiopia, cow dung	**GQ203776**	**GQ203735**	–	–
S. dubia S.I. Ahmed & Cain	Strid 19562-G (S)	Iceland, horse dung	**GQ203777**	**GQ203736**	-	**GQ203697**
S. grandispora (Speg.) S.I. Ahmed & Cain	GenBank	–	DQ468032	DQ468052	–	–
S. heptamera (Auersw.) S.I. Ahmed & Cain	Lundqvist 3090b (UPS)	Sweden, horse dung	**GQ203778**	**GQ203737**	**GQ203663**	**GQ203698**
S. intermedia (Auersw.) Kobayasi	1. Kruys 304 (UPS)	Sweden, cow dung	**GQ203779**	**GQ203738**	**GQ203664**	**GQ203699**
	2. GenBank	USA, elk dung	AY510415	AY510380	–	–
S. irregularis (I. Egeland) S.I. Ahmed & Cain	Lundqvist 16568-f (S)	Hungary, cow dung	**GQ203780**	**GQ203739**	**GQ203665**	**GQ203700**
S. isomera S.I. Ahmed & Cain	GenBank	Kenya, elephant dung	AY943053	–	–	–
S. leporina (Niessl) S. I. Ahmed & Cain	1. Lundqvist 19873-a (S)	Sweden, hare dung	**GQ203781**	**GQ203740**	**GQ203666**	**GQ203701**
	2. Richardson MJR93/04, #217874 (E)	Canada, spruce grouse dung	**GQ203782**	**GQ203741**	–	**GQ203702**
S. longisporopsis S.I. Ahmed & Cain	Lundqvist 16551-g (S)	Hungary, rabbit dung	**GQ203784**	**GQ203742**	**GQ203667**	**GQ203704**
S. megalospora (Auersw.) S.I. Ahmed & Cain	Kruys 305 (UPS)	Sweden, cow dung	**GQ203785**	**GQ203743**	**GQ203668**	**GQ203705**
S. minima (Auersw.) S.I. Ahmed & Cain	1. Lundqvist 17212-a (S)	Sweden, cow dung	**GQ203786**	**GQ203744**	**GQ203669**	**GQ203706**
	2. GenBank	Spain, leaf litter	AY510427	AY510392		
S. minimoides S.I. Ahmed & Cain	GenBank	Argentina, pig dung	AY510423	AY510388	–	–
S. minipascua S.I. Ahmed & Cain	Kruys 306 (UPS)	Sweden, cow dung	**GQ203787**	**GQ203745**	**GQ203670**	**GQ203707**
S. octomera (Auersw.) S.I. Ahmed & Cain	Huhndorf *et al.* 2579 (F)	USA, porcupine dung	**GQ203788**	**GQ203746**	**GQ203671**	**GQ203708**
S. pilosella (Cain) S.I. Ahmed & Cain	GenBank	–	DQ468033	DQ468053	–	–
S. pulchella (E.C. Hansen) S.I. Ahmed & Cain	Richardson, MJR67/01, #216605 (E)	USA, rabbit? dung	**GQ203789**	**GQ203747**	–	**GQ203709**

Table 1 Continued

Species	Source[a]	Origin	GenBank accession no. ITS	nLSU	mtSSU	ß-tubulin
S. septenaria S.I. Ahmed & Cain	Espigores 00036 (S)	Argentina, sheep dung	**GQ203790**	**GQ203748**	**GQ203672**	**GQ203710**
S. similis R.S. Khan & Cain	GenBank	USA, dung	AY510419	AY510386	–	–
S. splendens (Cain) S.I. Ahmed & Cain	Lundqvist 11753-a (UPS)	Finland, hare dung	**GQ203791**	**GQ203749**	**GQ203673**	**GQ203711**
S. subticinensis (Mouton) Dugan & R.G. Roberts	GenBank	France, soil	AY943051	–	–	–
S. tetramera S.I. Ahmed & Cain	Lundqvist 13449 (UPS)	Sweden, moose dung	**GQ203792**	**GQ203750**	**GQ203674**	–
S. vexans (Auersw.) S.I. Ahmed & Cain	23.VIII.1995, Andersson (UME)	Sweden, moose dung	**GQ203793**	**GQ203751**	**GQ203675**	**GQ203712**
Sporormiella tenerifae Arx & Aa	1. CBS 354.86	Tenerife, rabbit dung	**GQ203794**	**GQ203752**	**GQ203676**	**GQ203713**
	2. GenBank	Tenerife, rabbit dung	AY943047	–	–	–
Trematosphaeria heterospora (De Not.) G. Winter	GenBank/ CBS 644.86	Switzerland, *Iris* sp.	**GQ203795**	AY016369	AF346429	**GQ203714**
Verruculina enalia (Kohlm.) Kohlm. & Volkm.-Kohlm.	GenBank/ CBS 304.66	Liberia, drift wood	**GQ203796**	AY016363	–	**GQ203715**
Westerdykella cylindrica (Malloch & Cain) Arx	GenBank	Kenya, cow dung	DQ491519	AY004343	AF346430	–
W. globosa (J. N. Rai & J. P. Tewari) Tad. Ito & Nakagiri	GenBank	India, soil	AY943046	–	–	–
W. multispora (Cain) Cejp & Milko	1. CBS 383.69	France, saline soil	**GQ203799**	**GQ203754**	**GQ203677**	**GQ203717**
	2. GenBank	Japan	AY943048	–	–	–
W. nigra (Routien) Arx	1. CBS 416.72	Pakistan, soil	**GQ203800**	**GQ203755**	**GQ203678**	**GQ203718**
	2. GenBank	–	AY943049	–	–	–
W. ornata Stolk	CBS 379. 55	Mozambique, mangrove mud	**GQ203801**	AY853401	AY853351	**GQ203719**
W. purpurea (Cain) Arx	GenBank	Togo, soil	AY943050	–	–	–
Westerdykella sp.	GenBank	Spain	DQ468029	DQ468049	–	–

Table 1 Continued

taxa. We therefore argue that *Preussia* and *Sporormiella* should be treated as synonyms, although we have no significant support for a joint monophyletic group in our phylogenies. To keep the two genera separated, as an ad hoc solution, is impractical when we do not yet know how to separate them morphologically, and a number of species have already been described in *Preussia sensu lato*.

Well-supported *Preussia/Sporormiella* clades

Sporormiella alloiomera grouped with a majority of the *Preussia* species, which form a well-supported monophyletic clade in both phylogenies. It is difficult to find unique morphological traits supporting this relationship, though. All the *Preussia* species have non-ostiolate ascomata, ± broadly clavate asci (Fig. 1f) and 4-celled spores that easily fall into part-spores and overlap in size, but this is not the case in *S. alloiomera*. There are, however, only small differences in branch lengths and morphology between several of the *Preussia* species in this clade, and further studies may show that some taxa should be treated as synonyms. In the 4-markers phylogeny, the *Preussia*-clade is sister to *Westerdykella*. Together, the two clades include a majority of the cleistothecioid species, although only supported by posterior probability.

The highly supported *Sporormiella vexans* clade, consisting of *S. affinis*, *S. heptamera*, *S. octamera* and *S. vexans*, is morphologically well characterised. All taxa in this clade have clavate asci with a short or a long stipe. The spores are 7- or 8-celled, fusiform and with an enlarged third cell (Fig. 1g). The central cells are broader than they are long. The highly supported sister-taxon to the *S. vexans* clade is *S. leporina*.

The *S. intermedia* clade, including slightly different taxa in the two phylogenies, is the largest monophyletic group in the *Sporormiella–Preussia* complex (supported only by pp). A majority of the members of this clade have cylindrical asci

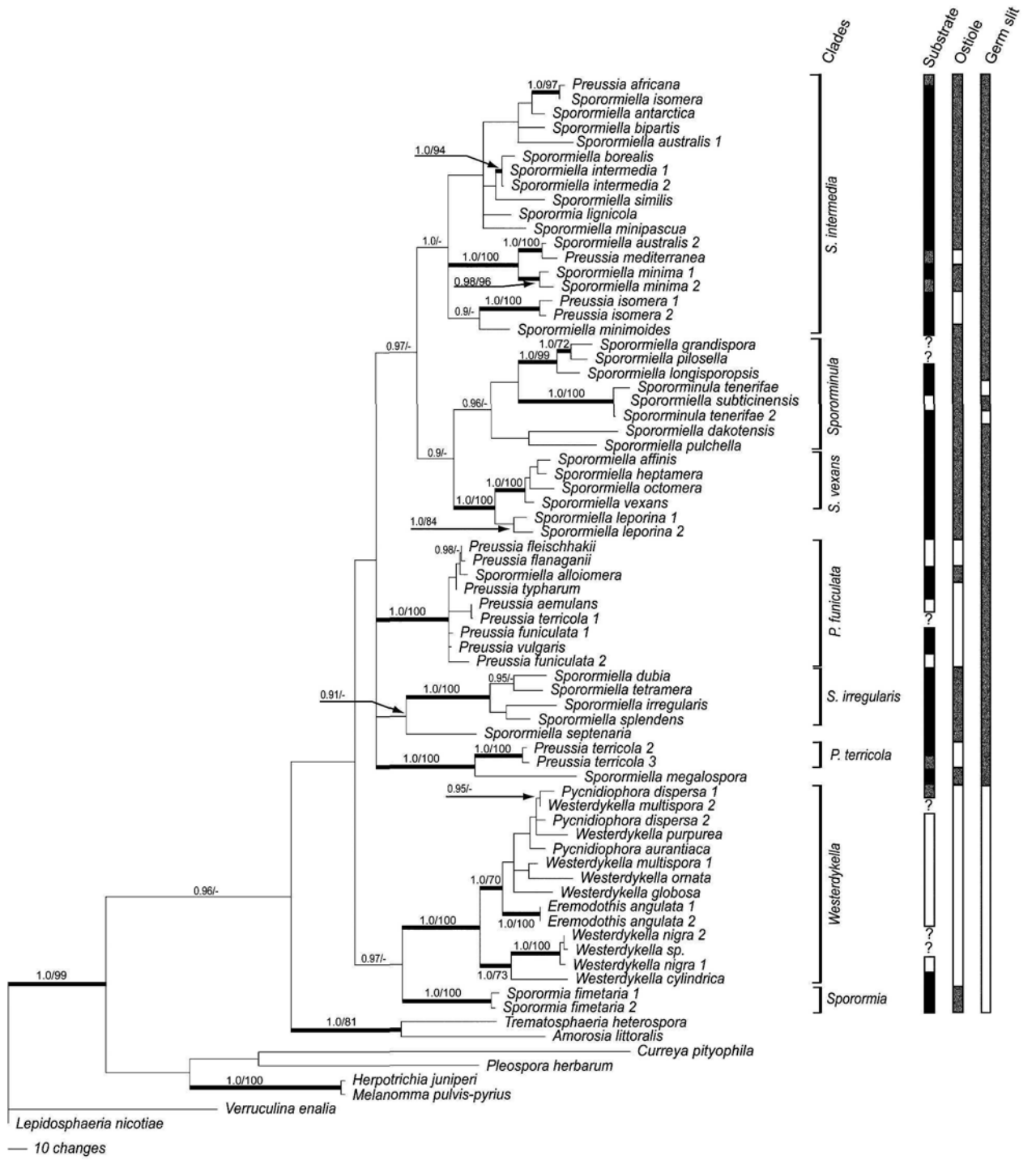

Figure 2 Majority rule consensus tree of the 35 282 sampled trees with best likelihood from a Bayesian analysis, based on ITS-nLSU rDNA sequences. Numbers on branches are posterior probability support followed by bootstrap support. Hyphen (-) represents support values below 0,9/70%. Bold lines represent significant support of both algorithms, i.e. bs ≥70% and pp ≥0.95. The different substrates are marked with different colours; soil (white), wood/plant material (grey) and dung (black). Question marks represent unknown substrates. The presence/absence of an ostiole and germ slits are marked with grey/white.

with a short stipe, and cylindrial spores with broadly rounded end-cells (Fig. 1h). Parallel-diagonal germ slits with a kink near the middle are only found in this group. One of these typical '*intermedia*-species' is *Sporormia lignicola*. It was never transferred to *Sporormiella*, since Ahmed and Cain (1972) only included coprophilous taxa in their revision.

Sporormiella dubia, *S. irregularis*, *S. splendens* and *S. tetramera* form a highly supported clade in both the 2-markers and the 4-markers phylogenies. These taxa have cylindrical-clavate asci with short ascus stipes. The spores are 4- or 8-celled and are tapered toward at least one end (Fig 1i). We also noticed that there are similarities in the structure of the

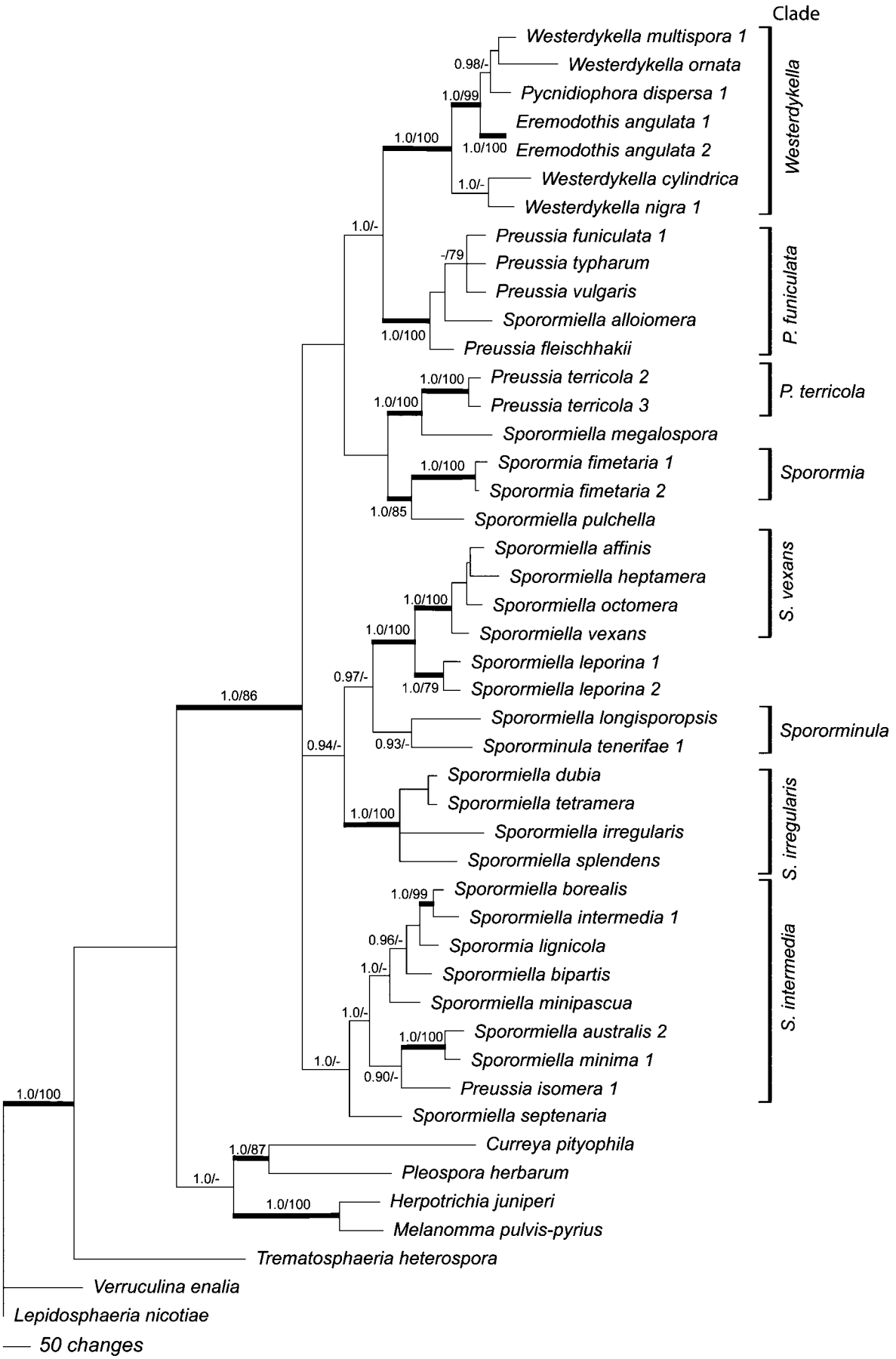

Figure 3 Majority rule consensus tree of the 9348 sampled trees with best likelihood from a Bayesian analysis, based on ITS-nLSU rDNA, mtSSU rDNA and ß–tublin sequences. Numbers on branches are posterior probability support followed by bootstrap support. Hyphen (-) represents support values below 0,9/70%. Bold lines represent significant support of both algorithms, i.e. bs ≥70% and pp ≥0.95.

peridium between the four taxa. The pseudothecium is comparatively dark and rough towards the upper part and especially at the neck, which also is scattered with small papillae, at least in *S. irregularis* and *S. splendens*.

Sporormiella

The triangular spore cells in transverse view and the absence of germ slits, claimed by Barr (2000) to be possible traits showing a close relationship between *Sporormiella* and *Sporormia*, are not homologous features uniting these species. *Sporormiella tenerifae* groups together with *S. longisporopsis*, with low support in the 4-markers phylogeny. There is, however, significant posterior probability support for a larger monophyletic group including *Sporormiella* and *S. longisporopsis*, with the *S. vexans* clade plus *S. leporina*. In the 2-markers phylogeny, *S. tenerifae* clusters with a diverse group of species including *S. subticinensis*, *S. longisporopsis*, *S. pilosella*, *S. grandispora*, *S. dakotensis* and *S. pulchella*. We noticed that the ITS sequences of *S. tenerifae* (AY943047, GQ203794) are identical to the sequence of *Sporormia subticinensis* (AY943051). We have studied the type material, but cannot decide from that material only, if the two should be synonyms. Additional collections should be studied to reach a conclusion.

Westerdykella

The *Westerdykella* clade is well distinguished from other genera in *Sporormiaceae* by the cleistothecioid ascomata, the small (<50 μm) asci with a short or almost absent ascus stipe, and the 1-celled spores, without germ slits (Fig. 1d). Our results do not support a separation between *Pycnidiophora* and *Westerdykella*, due to spore ornamentation, as suggested by von Arx and van der Aa (1987) and Barr (2000). The type of *Pycnidophora*, *P. dispersa* (= *Westerdykella dispersa*), is nested within the *Westerdykella* clade, close to the type of *Westerdykella*, *W. ornata* (Figs 2, 3). *Pycnidiopora* should, therefore, remain synonymous to *Westerdykella*. *Eremodothis* is also nested within *Westerdykella*, and should be treated as a synonym. It has the same morphological traits as the rest of this clade, apart from the eight pyramid-star-shaped spores.

New members of Sporormiaceae?

Mantle *et al.* (2006) included their new genus *Amorosia* in Sporormiaceae based on analyses of nLSU-SSU rDNA data. There is no clade including *Amorosia* within Sporormiaceae with significant support in their trees, though. The genus is a sister-taxon to Sporormiaceae, and whether it belongs within the family or not is then a question of interpretation. We included the available *A. littoralis* sequences in our ITS-nLSU rDNA analyses, where it grouped with *Trematosphaeria heterospora* with significant support outside Sporormiaceae. *Amorosia*'s position ought to be *incertae sedis*, until more knowledge is gained.

Morphological and ecological traits, their biological relevance and utility for taxonomy

A majority of the characters traditionally used as diagnostic features within the *Sporormiaceae* are poorly correlated with monophyletic groups in our phylogenies. This is most likely because these easily observed traits reflect the biology of these fungi, and not common ancestry. Substrate choice, an important feature when delimiting *Preussia* and *Sporormiella* by von Arx and van der Aa (1987), is clearly not characterising natural, monophyletic groups corresponding to genera in any suggested circumscription. A jump between substrates (e.g. dung, plant debris, wood) is apparently not a big step in evolutionary terms, in this group. The germ slit in the ascospores has apparently been lost or gained on several occasions during the evolution of the Sporormiaceae, and taxa with ostiolate and non-ostiolate ascomata do not form distinct groups in the tree, indicating that these features are widespread and not potentially synapomorphies, at least not for larger groups. However, these characters may be useful in combination with others. *Westerdykella* for instance, is well circumscribed by the non-ostiolate ascomata, as well as small asci with a short ascus stipe, and the 1-celled spores that lack germ slits. The small asci are an important feature also in *Sporormia*, together with the ≥16-celled spores that lack germ slits, and are typically joined together in a bundle. The number of cells in the spore is, to some extent, also a useful character circumscribing some of the *Preussia/Sporormiella* clades, considering the close relationship between the 7–8 celled species in the *S. vexans* clade. Our results shows, however, that a combination of ascus shape, stipe length and spore shape, which has been used for the identification of *Sporormiella* species in dichotomous keys (Ahmed & Cain 1972, Bell 2005, Doveri 2004), coincides best with molecular data for this group. Cylindrical asci with short stipes, and cylindrical spores circumscribe the *S. intermedia* clade, while cylindrical-clavate asci with short stipes, and spores that are tapered toward at least one end occur in the *S. irregularis* clade. Further, clavate asci with a short or a long stipe, and fusiform spores with an enlarged third cell, is typical for the *S. vexans* clade. As all groups are not fully resolved in our phylogenies we cannot see patterns for all taxa yet. A number of traits connected with asci and spores are clearly not homologues. Ascus length and width differ considerably in all *Preussia–Sporormiella* clades, Spore size is also a highly variable criterion, and for instance in the *S. irregularis* clade, the spore length has an extent of between 35 μm and 133 μm. Neither is the shape of the germ slit a useful character. Further studies of the ascomata may be worthwhile, though, considering the similarity in the cell structure of the peridium within the *S. irregularis* clade.

Conclusions and suggestions for the future

We suggest a new generic classification of the family Sporomiaceae, including *Sporormia*, *Preussia* (including *Sporormiella* and *Sporormiella*) and *Westerdykella* (including *Eremodothis* and *Pycnidiophora*). Future molecular studies of the Sporormiaceae should include more taxa, and explore additional molecular markers. The two genera *Chaetopreussia* and *Pleophragmia* remain to be investigated, and it is important for a final resolution of the *Preussia–Sporormiella* complex to

include the type species *Sporormiella nigropurpurea*, which we have failed to find good material of.

Identifying homologous/synapomorphic character states is clearly difficult in Sporormiaceae, although we have found a number of features that may be useful for circumscribing smaller clades. We believe an extended search for useful morphological characters is needed, though, preferably in combination with molecular studies. It is most important to facilitate continuing studies in this exciting group of fungi, by arriving at a well-supported and highly resolved phylogenetic hypothesis of the Sporormiaceae, in the near future.

Taxonomy

The following new combinations are proposed, based on our results from molecular data and morphological observations:

Preussia alloiomera (S.I. Ahmed & Cain) Kruys, comb. nov.
Basionym: *Sporormiella alloiomera* S.I. Ahmed & Cain, Ahmed & Cain (1972, p. 428)

Preussia antarctica (Speg.) Kruys, comb. nov.
Basionym: *Sporormia antarctica* Speg., Spegazzini (1888, p. 224)

Preussia bipartis (Cain) Kruys, comb. nov
Basionym: *Sporormia bipartis* Cain, Cain (1934, p. 106)

Preussia borealis (I. Egeland) Kruys, comb. nov
Basionym: *Sporormia borealis* I. Egeland, Egeland (1969, p. 217)

Preussia dubia (S.I. Ahmed & Cain) Kruys, comb. nov.
Basionym: *Sporormiella dubia* S.I. Ahmed & Cain, Ahmed & Cain (1972, p. 440)

Preussia lignicola (W. Phillips & Plowr.) Kruys, comb nov.
Basionym: *Sporormia lignicola* W. Phillips & Plowr., Phillips & Plowright (1877, p. 29)

Preussia longisporopsis (S.I. Ahmed & Cain) Kruys, comb. nov.
Basionym: *Sporormiella longisporopsis* S.I. Ahmed & Cain, Ahmed & Cain (1972, p. 448)

Preussia minipascua (S.I. Ahmed & Cain) Kruys, comb. nov.
Basionym: *Sporormiella minipascua* S.I. Ahmed & Cain, Ahmed & Cain (1972, p. 451)

Preussia octomera (Auersw.) Kruys, comb nov.
Basionym: *Sporormia octomera* Auersw., Auerswald (1868, p. 70)

Preussia splendens (Cain) Kruys, comb nov.
Basionym: *Sporormia splendens* Cain, Cain (1934, p. 107)

Preussia tenerifae (Arx & Aa) Kruys, comb nov.
Basionym: *Spororminula tenerifae* Arx & Aa, Arx & Aa (1987, p. 117)

Preussia tetramera (S.I. Ahmed & Cain) Kruys, comb. nov.
Basionym: *Sporormiella tetramera* S.I. Ahmed & Cain, Ahmed & Cain (1972, p. 464)

Westerdykella angulata (A.C. Das) Kruys, comb nov.
Basionym: *Thielavia angulata* A.C. Das, Das (1962, p. 545)

Westerdykella aurantiaca (J.N. Rai & J.P. Tewari) Kruys, comb nov.
Basionym: *Preussia aurantiaca* J.N. Rai & J.P. Tewari, Rai & Tewari (1963, p. 46)

Acknowledgements

This study was supported by The Swedish Taxonomy Initiative 34/07 1.4, The Swedish Research Council (NFR B5101–20005187, VR 629–2001-5756, VR 621–2002-349, VR 621–2003-3038), Kempestiftelserna and Stiftelsen Längmanska kulturfonden. We are grateful to the Directors and Curators of the cited herbaria and museums for the loan of specimens. Ove E. Eriksson contributed with valuable comments on the manuscript. Stefan Ekman (UPS) kindly lent out the photo-microscope used, and Mary Berbee is gratefully acknowledged for allowing us to use her extractions of three taxa.

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