Phylogenetic investigations of Sordariaceae based on multiple gene sequences and morphology

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A B S T R A C T

The family Sordariaceae incorporates a number of fungi that are excellent model organisms for various biological, biochemical, ecological, genetic and evolutionary studies. To determine the evolutionary relationships within this group and their respective phylogenetic placements, multiple-gene sequences (partial nuclear 28S ribosomal DNA, nuclear ITS ribosomal DNA and partial nuclear β-tubulin) were analysed using maximum parsimony and Bayesian analyses. Analyses of different gene datasets were performed individually and then combined to generate phylogenies. We report that Sordariaceae, with the exclusion Apodus and Diplogelasinospora, is a monophyletic group. Apodus and Diplogelasinospora are related to Lasiosphaeriaceae. Multiple gene analyses suggest that the spore sheath is not a phylogenetically significant character to segregate Asordaria from Sordaria. Smooth-spored Sordaria species (including so-called Asordaria species) constitute a natural group. Asordaria is therefore congeneric with Sordaria. Anixiella species nested among Gelasinospora species, providing further evidence that non-ostiolate ascomata have evolved from ostiolate ascomata on several independent occasions. This study agrees with previous studies that show heterothallic Neurospora species to be monophyletic, but that homothallic ones may have a multiple origins. Although Gelasinospora and Neurospora are closely related and not resolved as monophyletic groups, there is insufficient evidence to place currently accepted Gelasinospora and Neurospora species into the same genus.

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Introduction

The family Sordariaceae (Sordariales, Ascomycetes) comprises taxa characterised by dark, usually ostiolate ascomata, and unitunicate, cylindrical asci, usually with a small J- apical ring. Ascospores are brown to black, often with a gelatinous sheath or with wall ornamentations, but lack gelatinous appendages (Kirk et al. 2001). Morphologically, Sordariaceae is closely related to Lasiosphaeriaceae, another family in Sordariales (Lundqvist 1972; Huhndorf et al. 2004). Anamorphs of sordariaceous species are mostly hyphomycetes, such as Chrysonilia (Arx 1981). Sordariaceous species have been used extensively as model organisms in various biological, biochemical, ecological, genetic and evolutionary studies (e.g. Randall & Metzenberg 1995; Nelson 1996; Coppin et al. 1997; Dettman et al. 2003a, b; Jacobson et al. 2004).

Sordariaceae is represented by well-known and important genera such as Gelasinospora, Neurospora and Sordaria. These fungi, although closely related, occupy different natural habitats. Most species of Neurospora have been reported from soil and none occur on dung (Frederick et al. 1969), while Gelasinospora species are predominantly terricolous, with only a few
species being coprophilous (Lundqvist 1972). Most Sordaria species however, are strictly coprophilous (Arx et al. 1987; Guarro & Arx 1987). The distribution of sordariaceous taxa, especially Neurospora species have been well investigated, they are found to be ubiquitous in humid tropical and subtropical regions (e.g. Turner et al. 2001). Neurospora species are also common primary colonizers of trees and shrubs killed by forest fires in cold and dry temperate regions (Jacobson et al. 2001). Gelasinospora species, on the other hand, are more frequently collected from tropical and subtropical regions (Krug et al. 1994).

Sordariaceae presently comprises 7–10 genera (Kirk et al. 2001; Eriksson et al. 2004). The intimate relationships between Gelasinospora, Neurospora and Sordaria have been discussed by various authors based on biological, morphological and molecular data (Carr & Olive 1958; Lu 1967; Lundqvist 1972; Raju 1980; Beauty et al. 1994; Dettman et al. 2001). These fungi have primarily been differentiated on ascospore morphology and ornamentation (Lundqvist 1972). Their intergeneric relationships are however, unclear.

Sordaria species have ascospores that are smooth-walled with a basal germ pore and gelatinous sheath (Guarro & Arx 1987). In Gelasinospora and Neurospora, however, ascospores have ornamented walls and usually have two germ pores (Dowding 1933; Mahoney et al. 1969). Gelasinospora and Neurospora are morphologically distinguished by differences in ascospore ornamentation. The former possesses ascospores which are spherical or oval, with pitted or reticulate cell wall ornamentation, while in Neurospora, ascospores are broadly fusiform, with longitudinal ribs and intercostal veins. These characteristic pits or ribs are most easily observed in young ascospores. The fully pigmented ascospores of Gelasinospora and Neurospora may be mistaken as Sordaria (Dowding 1933; Arx 1982). The phylogenetic significance of spore ornamentation is at present obscure. A recent phylogenetic study by Dettman et al. (2001) has shown that ascospore ornamentation previously used to segregate this group of fungi is a poor predictor of phylogenetic relationships. The latest taxonomic studies on Gelasinospora and Neurospora are those of García et al. (2004). Based on ultrastructural morphologies and neighbour-joining analyses of partial 28S rDNA, they synonymised Gelasinospora with Neurospora.

The life-cycle of species in Sordariaceae can be heterothallic, homothallic or pseudo-homothallic. Most phylogenetic studies of Sordariaceae have focused on heterothallic and pseudohomothallic Neurospora species (e.g. Natvig et al. 1987; Taylor & Natvig 1989; Randall & Metzenberg 1995; Skupski et al. 1997). Pöggeler (1999) reported that species with the same mating strategy were closely related based on mating-type gene and gpd gene.

There are some other genera which are presently included in Sordariaceae, but their evolutionary relationships and respective phylogenetic placements remain uncertain. Apodus and Diplogelasinospora are currently in Sordariaceae (Kirk et al. 2001; Eriksson et al. 2004). Several authors, however, have pointed out that they may have close phylogenetic relationships with some lasiosphaeriaceous species (Maniottis 1965; Malloch & Cain 1971; Udagawa & Horie 1972). The name Anixiella has been used for non-ostiolate forms of Gelasinospora (Cain 1961; Horie & Udagawa 1974; Udagawa 1980). This concept however, has not been accepted by other authors. Both ostiolate and non-ostiolate forms of the G. fallaciosa have been recognised and both ascomatal types occur together in the type strains of G. seminuda and G. novoguineensis (Arx 1973, 1982). On the other hand, developmental and cytological studies inferred that the two genera are related but sufficiently distinct to warrant segregation (Uecker 1979). Anixiella has been treated as a synonym of Gelasinospora (Kirk et al. 2001), but this is still questionable as morphological characters are inadequate to clarify their phylogenetic relationship. Arx et al. (1987) established Asordaria for species with smooth ascospores without a gelatinous sheath. The lack of gelatinous sheath, has been given much taxonomic weight when separating Asordaria from Sordaria (Arx et al. 1987). However, the phylogenetic significance of this morphological character has been widely debated (Arx 1973, 1982; Eriksson & Hawksworth 1988; Khan & Krug 1989a, b; Uecker 1979) and whether Asordaria and Sordaria are distinct or congeneric has been a matter of personal opinion.

The intergeneric relationships and phylogenetic affinities of this group of fungi are still obscure. Based on phylogenetic analyses of multi-gene sequences (partial nuclear 28S rDNA, nuclear ITS rDNA and partial β-tubulin sequences), together with the re-evaluation of morphological features, we aimed to: (1) examine the monophyly of the Sordariaceae and clarify the phylogenetic affinities of Apodus and Diplogelasinospora; (2) assess the phylogenetic relationships between Gelasinospora, Neurospora, and Sordaria; (3) evaluate the phylogenetic significance of non-ostiolate ascomata and gelatinous spore sheaths, on which Anixiella and Asordaria were established.

Materials and methods

**Fungal isolates and DNA extraction**

Cultures were obtained from different collections: CBS (Utrecht), IFO (Usaka) and ICMP (Auckland; Table 1). Isolates were grown on potato dextrose agar (PDA) for 2–4 wk and total genomic DNA was extracted from fresh mycelium using a modified protocol of Doyle & Doyle (1987) as outlined by Lacap et al. (2003).

**DNA amplification and sequencing**

DNA amplification was performed by PCR. Partial 28S rDNA, complete ITS rDNA and partial β-tubulin were amplified using fungal specific primers LROR and LR5 (Vilgalys & Hester 1990), ITS4 and ITS5 (White et al. 1990) and Bt2A and Bt2B (Glass & Donaldson 1995) respectively. The amplification reaction was performed in a 50 μl reaction volume as outlined by Jeewon et al. (2004). The PCR thermal cycle for all of the three regions were similar, consisting of 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 50 s and elongation at 72 °C for 1 min, with a final extension step of 72 °C for 10 min. PCR products were checked on 1% agarose electrophoresis gels stained with ethidium bromide. PCR products were then purified using minicolumns, purification resin and buffer according to the manufacturer’s protocols (Amersham product code: 27-9602-01). DNA
sequencing was performed using the primers mentioned above in an Applied Biosystem 3730 DNA Analyser at the Genome Research Centre, The University of Hong Kong.

**Sequence alignment and phylogenetic analyses**

For each fungal strain, sequences obtained from paired primers were aligned to obtain an assembled sequence using Bioedit (Hall 1999). In total, five datasets were analysed. To investigate the relationships of *Sordariaceae* and related families and resolve the phylogenetic affinities of *Apodus* and *Diplogelasinospora*, a 28S rDNA dataset containing newly generated sequences and reference sequences obtained from GenBank was analysed (Dataset I). Together with taxa from *Sordariales*, other reference taxa included in this dataset were members from *Boliniales*, *Chaetosphaeriales*, *Coniochaetales*, *Diaporthales*, *Halosphaeriales*, *Hypocreales*, *Ophiostomatales*, and *Xylariales*. Four additional datasets based on different genes and combined genes were analysed to reveal the intergeneric relationships among *Sordariaceae* members. They are datasets based on 28S rRNA (Dataset II), ITS rRNA (Dataset III), β-tubulin (Dataset IV) and combined 28S rDNA, ITS rDNA and β-tubulin sequences (Dataset V). The statistical congruence of the sequence datasets was tested for Dataset V using the partition homogeneity test (Farris et al. 1995; Huelsenbeck et al. 1996) as implemented in PAUP* 4.0b10 (Swofford 2002). In all, 102 novel sequences generated from this study were submitted to GenBank (Table 1). Sequences for each strain, together with reference sequences obtained from GenBank (Table 2), were aligned using Clustal X (Thomson et al. 1997).

### Table 1 – Newly generated sequences in this study: taxon, isolate code, and GenBank accession number

<table>
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<th>Species</th>
<th>Isolate code</th>
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<th>ITS rDNA</th>
<th>β-tubulin</th>
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*a* Abbreviations: CBS, Centraalbureau voor Schimmelcultures (Utrecht); IFO, Institute for Fermentation (Osaka); ICMP, International Collection of Microorganisms from Plants, Landcare Research (Auckland).
The 28S rDNA dataset-I consisted of 12 newly sequenced taxa and 30 taxa from GenBank. The final dataset comprised 869 characters after alignment, of which four ambiguous regions of 33 characters were excluded in the analyses. The best-fit evolutionary model selected by Modeltest 3.06 was TrNef+Γ+G. Unweighted parsimony (UP) resulted in six trees, while weighted parsimony (WP) yielded only one tree. Based on K-H test, these seven trees were not significantly different (details not shown). Treating gaps as fifth state under both criteria resulted in trees with similar topologies. The single parsimonious tree (TL = 988, CI = 0.461, RI = 0.621, RC = 0.286, HI = 0.539, –ln L = 6587.91107) generated from WP and treating gaps as missing data is shown in Fig 1.

This 28S rDNA Dataset II consisted of 28 newly sequenced taxa and 3 taxa from GenBank. The final alignment used in the analyses comprised 841 characters with no ambiguous regions. Likelihood-ratio test in Modeltest 3.06 suggested that the best-fit model of evolution for this dataset is TrN+Γ+G. Four trees were generated from UP, while only one

<table>
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<th>Species</th>
<th>GenBank Accession Nos.</th>
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<td>Xyliora hypoxylon</td>
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</table>

This analysis was repeated five times starting from different random trees to ensure trees from different tree space were being sampled (Miller & Huhndorf 2004).

To assess the likelihood that taxa sharing similar morphologies and mating type strategies are monophyletic, constrained analyses were performed using the combined dataset (Dataset V) by using PAUP* 4.0b10 (Swofford 2002). Unconstrained analyses were performed in a same way as constrained analyses. Constrained trees were searched using heuristic search option (1000 random sequence addition, TBR and Maxtrees unlimited). Tree with the best –ln L score resulting from each constrained analysis was evaluated against the best unconstrained tree, using KHT and Shimodaira-Hasegawa test (SHT) (Shimodaira & Hasegawa 1999). Eight different hypotheses or topological constraints that are tested are shown in Table 3.

### Results

<table>
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<tr>
<th>Topologically constrained tree with monophyly of</th>
<th>Length</th>
<th>–ln L</th>
<th>P (KHT)</th>
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<td>Unconstrained</td>
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<td>(1) Asordaria (smooth spore without sheath)</td>
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<td>0.0000&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(2) Anixiella (pitted spore and non-ostiolate ascoma)</td>
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<td>7018.74089</td>
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<td>(3) Galasinospora (pitted spore)</td>
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<td>0.0000&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>(5) Galasinospora and Neurospora (ornamented spore)</td>
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<td>(6) Homothallic Neurospora</td>
<td>733</td>
<td>6838.46572</td>
<td>0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.001&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(7) Heterothallic Neurospora</td>
<td>716</td>
<td>6748.74780</td>
<td>1.0000&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.0000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(8) Sordaria and Asordaria (smooth spore)</td>
<td>721</td>
<td>6764.06821</td>
<td>0.3180&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.2200&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

- Only the tree with best –ln L score was tested.
- Indicates significant as P < 0.05 under the null hypothesis.
Fig 1 – Phylogram depicting the relationships of Apodus and Diplogelasinospora species with respect to other members of Sordariales and reference taxa. The single tree was generated from parsimony analysis based on 28S rDNA sequences (\( TL = 988, CI = 0.461, RI = 0.621, RC = 0.286, HI = 0.539, -\ln L = 6587.91107 \)). Data were analysed with random addition sequence, weighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap (equal or above 50 %). Thickened branches represent significant Bayesian posterior probabilities (equal or above 95 %). The tree is rooted with Dothidea sambuci.
comprised 632 characters with no ambiguous regions. The best-fit model of evolution determined by Modeltest 3.06 was TrNef+G. For this dataset, treating gaps as fifth state resulted in better resolved trees, and clades received better bootstrap support. Under above gap mode, UP generated 105 trees while WP gave 60 trees. K-H test showed these trees were not significantly different. One of the 60 parsimonious trees (TL = 272, CI = 0.875, RI = 0.815, RC = 0.713, HI = 0.125, \(-\ln L = 1788.08538\)) generated from WP and treating gaps as fifth state is shown in Fig 3.

Fig 2 – Phylogram of single tree generated from parsimony analysis based on 28S rDNA sequences (TL = 136, CI = 0.853 RI = 0.906, RC = 0.772, HI = 0.147, \(-\ln L = 1931.65148\)). Data were analysed with random addition sequence, weighted parsimony and treating gaps as missing data. Values above the branches are parsimony bootstrap (equal or above 50 %). Thickened branches represent significant Bayesian posterior probabilities (equal or above 95 %). The tree is rooted with Diaporthe pustulata.
Thirty newly sequenced taxa were included in Dataset IV. The final alignment comprised 525 characters, of which 6 ambiguous regions of 28 characters were excluded in the analyses. \( \text{TrN} + \text{G} \) was selected by Modeltest 3.06 as the best-fit model of evolution for this dataset. Both UP and WP analyses treating gaps as missing data resulted in only one tree with identical tree topology. Treating gaps as fifth state did not result in significantly different trees. The single maximum parsimonious tree (TL = 421, CI = 0.710, RI = 0.707, RC = 0.502, HI = 0.290, \(-\ln L = 2804.33026\))

Fig 3 – Phylogram of one of 60 trees generated from parsimony analysis based on ITS rDNA sequences (TL = 272, CI = 0.875, RI = 0.815, RC = 0.713, HI = 0.125, \(-\ln L = 1788.08538\)). Data were analysed with random addition sequence, weighted parsimony and treating gaps as newstate. Values above the branches are parsimony bootstrap (equal or above 50 %). Thickened branches represent significant Bayesian posterior probabilities (equal or above 95 %). The tree is rooted with Chaetomium globosum and Lasiosphaeris hispida.
generated from UP and treating gaps as missing data is shown in Fig 4.

The combined dataset (dataset-V) consisted of 30 newly generated sequences. The final dataset comprised 1944 characters after alignment, of which seven ambiguous regions of 39 characters were excluded in the analyses. Homogeneity partition tests indicated that the three datasets were congruent and combinable (P = 0.055) (Cunningham 1997; Sullivan 1996). The best-fit model of selection estimated by Modeltest 3.06 was TrNef+I+G. UP and WP resulted in four trees and two trees respectively, which were not significantly different. Treating gaps as fifth state generated tree topologies which were less resolved. One of the two equally maximum parsimonious trees (TL = 716, CI = 0.756, RI = 0.752, RC = 0.569,

Fig 4 – Phylogram of the single tree generated from parsimony analysis based on β-tubulin sequences (TL = 421, CI = 0.710, RI = 0.707, RC = 0.502, HI = 0.290, −ln L = 2804.33026). Data were analysed with random addition sequence, unweighted parsimony and treating gaps as missing data. Values above or below the branches are parsimony bootstrap (equal or above 50 %). Thickened branches represent significant Bayesian posterior probabilities (equal or above 95 %). The tree is rooted with Achaetomium strumarium and Lasiosphaeris hispida.
HI = 0.244, $-\ln L = 6748.74780$) obtained from WP and treating gaps as missing data was used to represent relationships among members of Sordariaceae (Fig 5).

Analyses revealed associations of *Apodus oryzae* with *Triangularia mangenotii* and *Cercophora mirabilis*, and *A. deciduus* with *Cercophora neufeldiana*, taxa of Lasiosphaeriaceae (Fig 1). Both subclades received high bootstrap support (91 % and 100 % respectively). On the other hand, *Diplogelasinospora inaequalis* and *D. grovesii* clustered with each other as sister group of other members of the Lasiosphaeriaceae (Fig 1). Sordariaceae members represented by Gelasinospora, Neurospora, and Sordaria, formed a highly supported monophyletic clade (100 % BT
and 100 % PP, Fig 1). In Figs 2–5, all investigated Asordaria species were interspersed among Sordaria species and clades uniting them received high statistical support (Figs 2–5). Pitted spored Gelasinospora species formed two different groups in the combined gene tree (Fig 5), and the phylogenies resulting from individual datasets are also similar (Figs 2–4). In Fig 5, clade G1 contains species of G. bonaerensis, G. brevispora, G. ripopopotama, G. reticulata and G. udagawae, while the second group comprises G. calospora, G. cerealis, G. cratophora, G. dictyophora, G. endodonta, G. saitoi, G. seminuda and G. tetrasperma (clade G2). These two clades were supported by a bootstrap of 99 % and 71 % respectively. In addition, the non-ostiolate Gelasinospora species (Gelasinospora endodonta, G. reticulata and G. saitoi, so-called Anixiella species) did not cluster together as would be expected. Instead, they interspersed with other Gelasinospora species possessing ostiolate ascomata (Figs 2–5). G. reticulata grouped in clade G1, while G. endodonta and G. saitoi grouped in clade G2 (Fig 5). Heterothallic Neurospora species constituted a monophyletic group in all generated phylogenies as previously reported (Dettman et al. 2001; García et al. 2004). In the ITS tree where more Neurospora members were added, all heterothallic and pseudohomothallic Neurospora species constituted a monophyletic clade N1 which is well supported by bootstrap (83 %) and PP (95 %) (Fig 3). Homothallic Neurospora species however, failed to cluster together (Figs 3–5) and grouped with other Sordaria species or Gelasinospora species.

The results of the KHT and SHT of the comparison of constrained trees with unconstrained tree are given in Table 3. As shown, constrained analyses failed to reject two hypotheses: (1) taxa having smooth-walled ascospores (Asordaria and Sordaria) are monophyletic (KHT P = 0.318, SHT P = 0.220; the constrained tree is five steps longer than the unconstrained tree, with only a few nodes re-arranged); and (2) heterothallic (including pseudohomothallic) Neurospora species are monophyletic (KHT P = 1.000, SHT P = 1.000; best constrained tree identical to the best unconstrained tree). Analyses based on other hypotheses as mentioned in Table 3 generated constrained trees which were significantly less likely than Fig 5 (with 17–50 steps longer than the unconstrained tree).

Discussion

Phylogenetic affinities of Apodus and Diplogelasinospora

The phylogenetic placement of Apodus and Diplogelasinospora, based on molecular characters, is not congruent with established morphological classifications (Kirk et al. 2001; Eriksson et al. 2004). Apodus is characterised by dark, non-ostiolate ascomata, and clavate to cylindrical asci with an indistinct apical ring. The ascospores are mostly brown, ellipsoid, one-celled (occasionally two-celled) with a single germ pore, resembling species in Sordaria (Arx 1975). However, Malloch & Cain (1971) pointed out that Apodus is closer to Lasiospheariaceae than Sordariaceae based on cultural characters and ascomatal morphology (non-ostiolate). Another clear morphological difference between Apodus and Sordaria is that ascospores of Apodus species occasionally have a transverse septum and a paler basal cell. Many lasiospheariaceous species produce ascospores with a paler basal cell, e.g. Cercophora, Podospora, Strattonia, and Triangularia species, and this is what possibly links Apodus to the Lasiospheariaceae (Lundqvist 1972). Huhndorf et al. (2004) recently redefined Lasiospheariaceae and pointed out that lasiospheariaceae species are characterised by possessing ascospores with a brown, ellipsoid cell and different degrees of a hyaline cell. The ascospore morphology of Apodus (brown, ellipsoid, sometimes with a paler end cell) fits well with the current concept of Lasiospheariaceae as proposed by Huhndorf et al. (2004). Our molecular data are also in agreement with the taxonomic concept as postulated by Malloch & Cain (1971) and Lundqvist (1972). Apodus should therefore be transferred to the Lasiospheariaceae.

The phylogenetic relationship of Diplogelasinospora is not completely resolved. Diplogelasinospora species have pitted ascospores as those found in Gelasinospora (Cain 1961; Arx 1982), but the former differs in having two-celled ascospores, with one cell being hyaline and another being black and opaque. Cain (1961) stated that morphological characters in Diplogelasinospora, which have evolved from Gelasinospora, may be an adaptation to fruiting in unexposed locations and for delayed dispersal of the ascospores. Our molecular data however, do not support this evolutionary hypothesis. In Fig 1, Diplogelasinospora is phylogenetically unrelated to Gelasinospora and nested between members of Chaetomiaceae and Lasiospheariaceae. Constrained analysis forcing Diplogelasinospora and Gelasinospora into monophyletic clade resulted in trees which were significantly less resolved than the unconstrained tree (details not shown). Our result is congruent with Maniots (1965), who stated that it is inappropriate to link Diplogelasinospora to Gelasinospora by their pitted ascospores. The pitted ascospores have possibly evolved independently within different lineages. In Diplogelasinospora, the presence of a spore septum, and the basal cell, which is hyaline and often collapses, are more typical for Lasiospheariaceae (Udagawa & Horie 1972; Guarro et al. 1991). Even though results show that Diplogelasinospora is more related to Lasiosphaeraceae, further investigation with more taxa may help to conclusively resolve its phylogenies.

Phylogeny of Sordaria

Arx et al. (1987) argued that Asordaria might be more closely related to Gelasinospora and Neurospora because of their fast growing colonies with broad expanding hyphae and unsheathed ascospores. Our cladistic analysis does not support this statement as in all phylogenies, Asordaria species are more related to Sordaria than Gelasinospora and Neurospora (Figs 2–5). In addition, constrained analysis forcing Asordaria species into a monophyletic clade resulted in trees which are significantly less resolved than the best unconstrained tree (Table 3). However, the hypothesis that species having smooth-walled ascospores (Asordaria and Sordaria species) are monophyletic could not be rejected (KHT, P = 0.362, SHT, P = 0.237). Both Asordaria and Sordaria are characterised by some common morphologies. These include smooth-walled ascospores and a single germ pore. Both genera have previously been treated as congeneric (Khan & Krug 1989b; Eriksson & Hawksworth 1988; Kirk et al. 2001). Our molecular data
corroborates with established classification and provides evidence on the congeneric status of Asordaria and Sordaria. S. alcina appears to be distantly related to other Sordaria species (Figs 2–5). This is consistent with S. alcina having ascosporores which are ellipsoidal to cylindrical (Lundqvist, 1972), rather than the generally broad ellipsoidal to subglobose spores in other Sordaria species. Previous studies have shown that ascospore shape is useful in delimiting species within a genus (e.g. Câmara et al. 2002; Jeewon et al. 2003a). Also, note worthy is that A. conoidea, A. prolifica and S. tomento-alba constitute a small clade in the β-tubulin, ITS and combined gene trees (Figs 3–5). Morphologically, these species have narrower ascospores (widths less than 12 µm), while ascospore widths of other Sordaria /Asordaria species investigated are greater than 12 µm. The only ambiguity is S. fimicola, which has ascospores 11–13 µm wide and lies in the main clade. It is also worth mentioning that spore length is a criterion that should be given less taxonomic weight as compared to spore width as exemplified in this study (e.g. A. conoidea and A. tenerifae have the same ascospores length, but fall in different clades) and previous studies (e.g. Jeewon et al. 2003a).

**Phylogeny of Gelasinospora and Neurospora and their relationships**

Phylogenies generated in this study showed that taxa possessing similar ascomatal structures may not necessarily be phylogenetically related. In all analyses, non-ostiolate Gelasinospora species failed to constitute a monophyletic clade (Figs 2–5). KH and SH tests (Table 3) also showed that when species having non-ostiolate ascomata were constrained to be monophyletic, resulting trees were significantly worse than the unconstrained tree (Fig 5). The phenomenon that both types of ascomata occur in some strains (Arx 1973, 1982; Khan & Krug 1989a) strongly suggests that ostiole is not a reliable character in delimiting this group of fungi. As suggested in previous studies, non-ostiolate ascoma may have evolved independently from ostiolate ascomata on different occasions (e.g. Berbee & Taylor 1992; Rehner & Samuels 1995). Modern classifications have tended to place non-ostiolate ascomycetes in primarily ostiole groups (Rehner & Samuels 1995; Suh & Blackwell 1999). At familial and higher levels, the use of this morphological character has also caused confusion (e.g. Cephalotheceae and Pseudenurotaceae) (Malloch & Cain 1970; Suh & Blackwell 1999). In this study, the clades comprising taxa with ostiole and nonostiolate ascomata (Figs 2–5) also imply that evolutionary changes have occurred within different lineages. The distinction between non-ostiole and ostiolate ascomata is therefore not phylogenetically significant in delimiting genera.

The heterothallic (including pseudohomothallic) Neurospora species are found to be monophyletic (Figs 2–5, Table 3) and they may share a common ancestor. (Garcia et al. 2004) Those homothallic ones, however, did not group together (Figs 2–5, Table 3). Constrained analysis is congruent with above tree topologies, in which when homothallic Neurospora species were constrained to be monophyletic, the resulting tree was significantly less resolved than the unconstrained tree (Table 3). Similar findings were reported in previous studies of Pöggeler (1999) and Dettman et al. (2001). The question as to whether homothallic fungi arose from heterothallic ancestors or vice versa has been widely debated, but is not fully resolved based on current knowledge (Pöggeler 1999). That homothallic and heterothallic species are widely dispersed amongst different ascomycete genera and families shows that at least one of these strategies must have numerous independent origins. Further studies on the characterisation of mating-type loci may help to determine the origins of different mating strategies.

Neurospora terricola is distantly related to other Neurospora species and appears to be closely related to Sordaria species (Figs 2–5). Mahoney et al. (1969) stated that N. terricola was the most divergent Neurospora species because of its small ascomata and small ovoid ascospores with a single germ pore. These characters, particularly the single germ pore, are however, mostly restricted to Sordaria (Lundqvist 1972). In his review of Sordariaceae, Lundqvist (1972) suggested that an organism may be primitive in one respect and advanced in another. In N. terricola, the ascospores possess a single germ pore (a character of Sordaria) and ribbed wall ornamentation (a character of Neurospora). This species possibly represents an intermediate stage in evolution between Neurospora and Sordaria. Cain (1961) placed species with ornamented spores (Gelasinospora and Neurospora) in Neurosporaceae. This concept is not accepted in the present study. In the constrained analysis; the hypothesis that Gelasinospora species and Neurospora species are monophyletic could not be accepted (Table 3). Intergeneric relationships between these genera have been detailed by Dettman et al. (2001) based on phylogenies derived from four nuclear genes (ITS, mat A-1, mat a-1 and gpd gene). Gelasinospora and Neurospora species included in their study do not represent two clearly resolved monophyletic lineages and they suggested multiple origins for ornamented-sporo morphology. Similar phylogenetic inferences are derived in this study. In the combined gene tree (Fig 5), N. africana grouped together with Gelasinospora species (clade G1) in a well-supported clade, while N. terricola grouped together with Sordaria species.

In a recent morphological study coupled with phylogenies based on neighbour-joining analysis of partial 28S rDNA sequences, Garcia et al. (2004) demonstrated that epispore morphology was useful in delimiting Gelasinospora and Neurospora species. There appears, however, to be little justification to support this. For instance, their analysis revealed a close association between N. terricola and N. nigeriensis, characterised by smooth and ornate epispores respectively (Garcia et al. 2004). With a different taxonomic sampling regime, and analyses from three different genes, this study presents a different perspective on the utility of epispore layer morphology. It appears that epispore layer morphology may not be necessarily as phylogenetically informative as previously suggested. For example, G. bomaensis, G. reticulata, G. udagawae, and Neurospora africana, which have smooth epispore, grouped in clade G1. However, clade G1 also includes G. hippopomata and G. brevispora, which have an inwardly projecting epispore (Khan & Krug 1989a; Krug et al. 1994). The similarity of the inwardly projecting pits between G. brevispora (in clade G1, Fig 5) and G. calospora (in clade G2, Fig 5) was pointed out by Khan & Krug (1989a). Although phylogenies generated in this study depict essentially similar species groupings, there does not
seem to be enough molecular evidence to synonymize Gelasinospora and Neurospora as postulated by García et al. (2004). Their dataset, with only 6% of parsimony informative characters, did not include any related Sordaria species and was based only on neighbour-joining analyses of partial 28S rDNA. It might be that inclusion of more sequences from different genes and maximum parsimony and Bayesian analyses will reflect and clarify more evolutionary and taxonomic issues. In our combined gene tree (Fig 5), a Sordaria clade is nested between clades G1 and G2. Furthermore, there is statistical support (PP 98%) uniting the clade containing the Sordaria clade, the Gelasinospora clade G1, and the heterothallic Neurospora clade (Fig 5). This may indicate that species in clade G1 might be more closely related to some Sordaria species than other species in clade G2. Constrained analysis forcing the Neurospora and Gelasinospora species into a monophyletic clade also resulted in significantly worse trees (Table 3). Thus, it would be inappropriate, at this stage, to accept the synonymization of Gelasinospora with Neurospora.

Another ambiguity observed is the phylogenetic affinity of G. cerealis, characterised by smooth epispore. We report that this species (AY681154) is more related to other Gelasinospora species with ornate epispores (G. cratophora and G. dicytophora), while García et al. (2004) found that G. cerealis (AJ579560) was related to G. reticulata, G. bonaenesis, and G. microperutua, characterised by a smooth epispore. Despite the same species being investigated, we noted 26 nucleotide differences between the two 28S rDNA sequences, including six single base pair insertions in AJ579560. It seems less likely that different strains of the same species can be so genetically different given that 28S rDNA is quite conserved. We double checked our sequences and realigned available allied sequences from GenBank and point out that sequence AJ579560 deposited by García et al. (2004) needs to be updated, as it was the only ambiguous one.

Other possible members of Sordariaceae

The 28S rDNA sequence of a Copromyces sp. obtained from GenBank was included in this study (Huhndorf et al. 2004). Copromyces appears to be phylogenetically related to other members of the Sordaria. Copromyces species have non-ostiolate ascomata, 2-spored asci, and verrucose ascospores with a slight apical pore and a single germ pore (Lundqvist 1967), which fits well with the taxonomic concept of the Sordariaceae. Again, possibly the presence of single germ pore links Copromyces to Sordaria species (Fig 2), despite its verrucose ascospore ornamentation. As the species used here (CBS 386.78) is not validly described, the phylogenetic relationship of Copromyces may require further investigation.

Boothiella is another genus which has been referred to Sordariaceae (Kirk et al. 2001; Eriksson et al. 2004). It is characterised by light-coloured, non-ostiolate ascomata, cylindrical, 4-spored asci with a small apical ring, and smooth or slightly verrucose, one-celled ascospores with a single germ pore. Presently there are no molecular data for Boothiella. From a morphological perspective, Boothiella is similar to Copromyces and Sordaria. Some authors have argued that Boothiella resembles Thielavia (Chaetomiaceae) in some aspects, such as the non-ostiolate ascomata and ascospores with a germ pore (Arx & Mahmood 1968; Arx 1975). However, it has been shown in various studies that non-ostiolate ascomata have evolved independently and may not be informative in understanding phylogenetic relationships (Rehner & Samuels 1995; Suh & Blackwell 1999). The ascospore with germ pore is, however, not an exclusive character of Chaetomiaceae. On the other hand, as suggested by Udagawa & Furuya (1977), the cylindrical asci and ascospore morphology found in Boothiella are strongly suggestive of Sordariaceae.

Summary

The current study does not support the familial boundary of Sordariaceae adopted by Kirk et al. (2001) and Eriksson et al. (2004). The 28S rDNA analyses and morphological characters reveal that Apodus and Diplogelasinospora do not belong to Sordariaceae, but bear phylogenetic affinities to lasiosphaericeous genera. With the exclusion of Apodus and Diplogelasinospora, Sordariaceae appears to be a natural group, in which the ascospores are one-celled, smooth-walled or ornamented, and with one to two or occasionally multiple germ pores. Gelasinospora and Neurospora, together with Sordaria, are shown to have an intimate relationship, and to be representatives of Sordariaceae. This study, together with existing morphological data, provide insights to the understanding of the phylogenetic relationships within Sordariaceae. The gelatinous sheath surrounding the ascospores is shown to be an unreliable morphological character to segregate Asordaria from Sordaria. Anixiella, the name used for non-ostiolate Gelasinospora species, is artificial based on molecular data and previous cultural studies. N. terricola is more related to Sordaria species than to other Neurospora species, and this is consistent with the single germ pore in the ascospores of N. terricola. In addition, it is highly unlikely that Gelasinospora should be treated as congeneric to Neurospora, as discussed above.

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References


Multigene phylogenetics of Sordariaceae


