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## Phylogenetic investigations of *Sordariaceae* based on multiple gene sequences and morphology

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### ABSTRACT

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  $\beta$ -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 *sordariaceae* species are mostly hyphomycetes, such as

*Chrysonilia* (Arx 1981). *Sordariaceae* species have been used extensively as model organisms in various biological, biochemical, ecological, genetic and evolutionary studies (e.g. Randall & Metzberg 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

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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. 2004). *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; Beatty 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 ornamentations 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 (Maniotis 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. fallaciosus* 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  $\beta$ -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  $\beta$ -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  $\mu$ l reaction volume as outlined by Jee-won 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

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

Species	Isolate code <sup>a</sup>	GenBank nos.		
		28S rDNA	ITS rDNA	β-tubulin
<i>Apodus deciduus</i>	CBS 506.70	AY681165	AY681199	AY681233
<i>A. oryzae</i>	CBS 376.74	AY681166	AY681200	AY681234
<i>Asordaria arctica</i>	CBS 143.68	AY681141	AY681175	AY681209
<i>A. conoidea</i>	CBS 563.72	AY681145	AY681179	AY681213
<i>A. prolifica</i>	CBS 567.72	AY681140	AY681174	AY681208
<i>A. sicutii</i>	CBS 768.96	AY681146	AY681180	AY681214
<i>A. tenerifae</i>	CBS 264.86	AY681138	AY681172	AY681206
<i>Diplogelasinospora inaequalis</i>	CBS 436.74	AY681167	AY681201	AY681235
<i>D. grovesii</i>	CBS 340.73	AY681168	AY681202	AY681236
<i>Gelasinospora calospora</i>	IFO 32008	AY681155	AY681190	AY681223
<i>G. cerealis</i>	IFO 6759	AY681154	AY681187	AY681222
<i>G. reticulata</i>	IFO 32837	AY681156	AY681189	AY681224
<i>G. seminuda</i>	IFO 32891	AY681153	AY681186	AY681221
<i>G. endodonta</i>	IFO 30835	AY681157	AY681191	AY681225
<i>G. bonaerensis</i>	CBS 102191	AY681143	AY681177	AY681211
<i>G. brevispora</i>	CBS 548.94	AY681162	AY681196	AY681230
<i>G. cratophora</i>	CBS 245.89	AY681163	AY681197	AY681231
<i>G. dictyophora</i>	CBS 529.95	AY681147	AY681181	AY681215
<i>G. hippopotama</i>	CBS 561.94	AY681148	AY681182	AY681216
<i>G. tetrasperma</i>	CBS 178.33	AY681144	AY681178	AY681212
<i>G. udagawae</i>	CBS 309.91	AY681150	AY681183	AY681218
<i>G. saitoi</i>	CBS 435.74	AY681151	AY681184	AY681219
<i>Neurospora africana</i>	IFO 32896	AY681152	AY681185	AY681220
<i>N. crassa</i>	ICMP 6360	AY681158	AY681193	AY681226
<i>N. intermedia</i>	CBS 131.92	AY681149	AY681192	AY681217
<i>N. terricola</i>	CBS 298.63	AY681142	AY681176	AY681210
<i>N. tetrasperma</i>	IFO 32011	AY681159	AY681194	AY681227
<i>Sordaria alcina</i>	CBS 109460	AY681164	AY681198	AY681232
<i>S. lappae</i>	CBS 154.97	AY681137	AY681171	AY681205
<i>S. firmicola</i>	CBS 508.50	AY681160	AY681188	AY681228
<i>S. superba</i>	CBS 784.96	AY681139	AY681173	AY681207
<i>S. tomento-alba</i>	CBS 260.78	AY681161	AY681195	AY681229
<i>Lasiosphaeria hispida</i>	CBS 955.72	AY681169	AY681203	AY681237
<i>Achaetomium strumarium</i>	CBS 333.67	AY681170	AY681204	AY681238

a Abbreviations: CBS, Centraalbureau voor Schimmelcultures (Utrecht); IFO, Institute for Fermentation (Osaka); ICMP, International Collection of Microorganisms from Plants, Landcare Research (Auckland).

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 *Boliales*, *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 rDNA (Dataset II), ITS rDNA (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<sup>†</sup> 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). Alignment was manually adjusted to allow maximum alignment and minimize gaps.

Phylogenetic analyses were performed by using PAUP<sup>†</sup> 4.0b10 (Swofford 2002). Ambiguously aligned regions were excluded from all analyses. Unweighted parsimony (UP) and weighted parsimony (WP) analyses were performed. Gaps were treated as missing data or fifth character under both UP and WP to find better resolved trees (Jeewon *et al.* 2003b). WP analyses were performed using a symmetric step matrix generated with the program SMatrix version 2.2 (François Lutzoni & Stefan Zoller, Department of Biology, Duke University, Durham, NC), by which the relative frequencies of nucleotide substitutions were calculated and converted into costs of changes. Trees were inferred using the heuristic search option with

**Table 2 – Additional sequences used in the analyses obtained from GenBank**

Species	GenBank Accession Nos.
<i>Ambrosiella macrospora</i>	AF282873
<i>Amphisphaeria umbrina</i>	AF452029
<i>Aporoethelavia leptoderma</i>	AF096186
<i>Barrina polyspora</i>	AY346261
<i>Bombardia bombardia</i>	AY346263
<i>Bombardioidea anartia</i>	AY346264
<i>Camarops microspora</i>	AY083821
<i>C. tubulina</i>	AY346266
<i>Cercophora mirabilis</i>	AY346271
<i>C. newfieldiana</i>	AF064642
<i>C. globosum</i>	AY545729
<i>Chaetomium cupreum</i>	AF286400
<i>C. globosum</i>	AY429056
<i>Chaetosphaeria innumera</i>	AY017375
<i>Coniochaetidium savoryi</i>	AY346276
<i>Copromyces</i> sp.	AY346277
<i>Diaporthe pustulata</i>	AF408358
<i>Dothidea sambuci</i>	AF382387
<i>Farrowia longicollea</i>	AF286408
<i>F. seminuda</i>	AF286410
<i>Halosphaeria appendiculata</i>	U46885
<i>Hypomyces sibirinae</i>	AJ583486
<i>Lasiosphaeria ovina</i>	AY436413
<i>L. sorbina</i>	AY436415
<i>Lignincola laevis</i>	U46890
<i>Melanochaeta hemipsila</i>	AY346292
<i>Nectria grammicospora</i>	AF193238
<i>Neurospora discreta</i>	AF388917
<i>N. dodgei</i>	AF388920
<i>N. galapagosensis</i>	AF388921
<i>N. lineolata</i>	AF388924
<i>N. sitophila</i>	AF388926
<i>Ophiostoma piliferum</i>	AF221625
<i>Sordaria macrospora</i>	AY346301
<i>S. macrospora</i>	AF246293
<i>Thielavia cephalothecoides</i>	AF286413
<i>Triangularia mangelotii</i>	AY346303
<i>Valsella salicis</i>	AF408389
<i>Xylaria hypoxylon</i>	U47841

TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited. Branches of zero length were collapsed and all parsimonious trees were saved. TL, CI, RI, RC, HI, and log likelihood  $[-\ln L]$  (HKY model) were calculated for trees generated under different optimality criteria. Clade stability was assessed in a bootstrap analysis with 1000 replicates, each with ten replicates of random stepwise addition of taxa. Kishino-Hasegawa tests (KHT) (Kishino & Hasegawa 1989) were performed in order to determine whether trees were significantly different. Trees were viewed in Treeview (Page 1996).

Model of evolution was estimated by using Modeltest 3.06 (Posada & Crandall 1998). Bayesian posterior probabilities (PP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001), using above estimated model of evolution. Six simultaneous Markov chains were run for 1 m generations and trees were sampled every 100th generations (resulting 10 000 total trees). First 2,000 trees that represented the burn-in phase of the analyses were discarded. The remaining 8,000 (post-burning) trees were used to generate a majority rule consensus tree.

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<sup>v</sup> 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

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 evolution model selected by Modeltest 3.06 was TrNef+I+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.

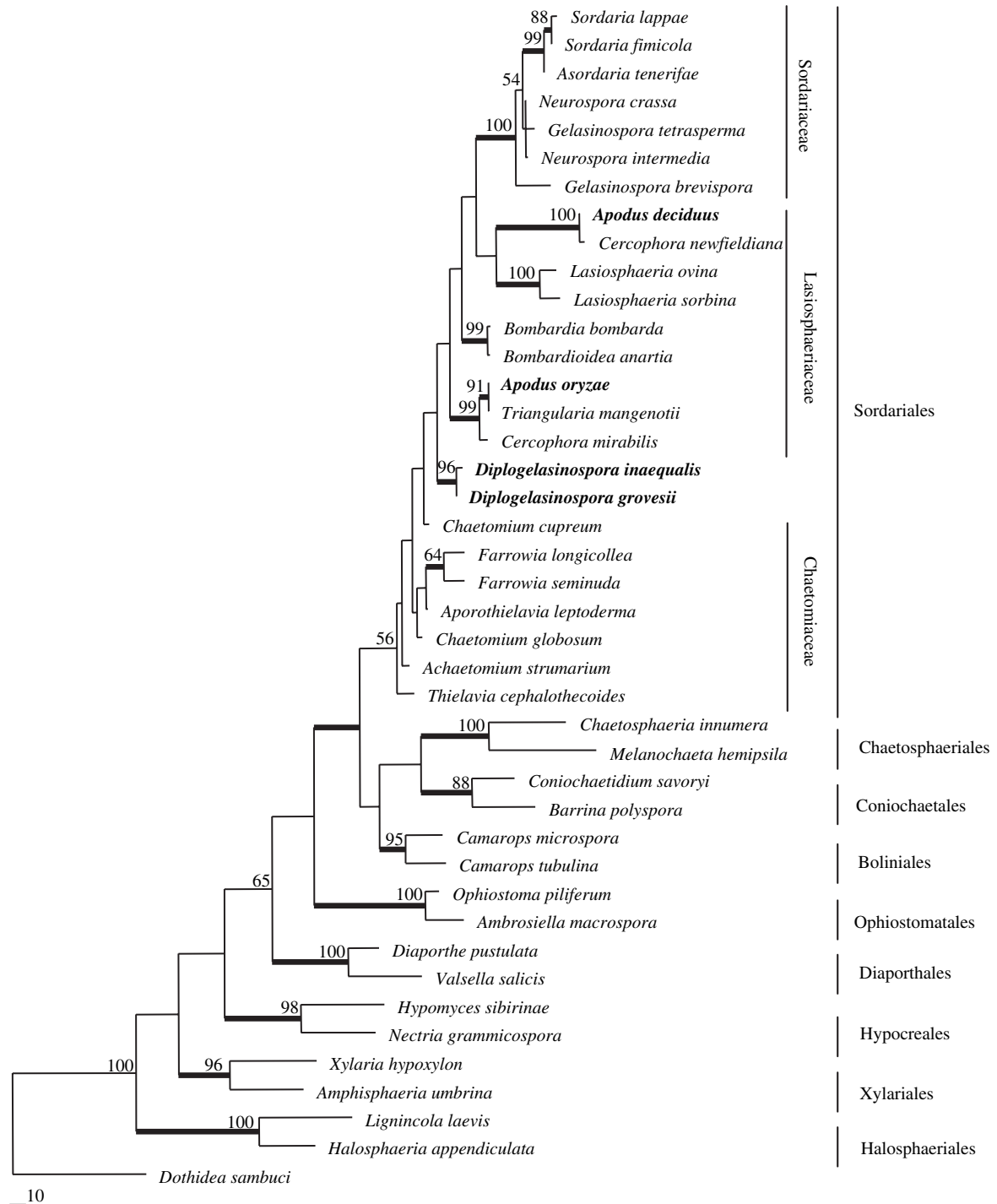
This 28S rDNA Dataset II consisted of 28 newly sequenced taxa and 3 taxa from GenBank. The final alignment used in the analyses comprised 841 characters with no ambiguous regions. Likelihood-ratio test in Modeltest 3.06 suggested that the best-fit model of evolution for this dataset is TrN+I+G. Four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

**Table 3 – Results of KHT and SHT on the comparison of constrained trees with unconstrained tree<sup>a</sup>**

Topologically constrained tree with monophyly of	Length	$-\ln L$	P (KHT)	P (SHT)
Unconstrained	716	6748.74780	-	-
(1) <i>Asordaria</i> (smooth spore without sheath)	756	6958.81836	0.000 <sup>b</sup>	0.000 <sup>b</sup>
(2) <i>Anixiella</i> (pitted spore and non-ostiolate ascoma)	766	7018.74809	0.000 <sup>b</sup>	0.000 <sup>b</sup>
(3) <i>Gelasinospora</i> (pitted spore)	737	6858.30365	0.000 <sup>b</sup>	0.000 <sup>b</sup>
(4) <i>Neurospora</i> (ribbed spore)	737	6851.23041	0.000 <sup>b</sup>	0.000 <sup>b</sup>
(5) <i>Gelasinospora</i> and <i>Neurospora</i> (ornamented spore)	740	6868.78778	0.000 <sup>b</sup>	0.000 <sup>b</sup>
(6) Homothallic <i>Neurospora</i>	733	6838.46572	0.001 <sup>b</sup>	0.001 <sup>b</sup>
(7) Heterothallic <i>Neurospora</i>	716	6748.74780	1.000	1.000
(8) <i>Sordaria</i> and <i>Asordaria</i> (smooth spore)	721	6764.06821	0.318	0.220

a Only the tree with best  $-\ln L$  score was tested.

b Indicates significant as  $P < 0.05$  under the null hypothesis.

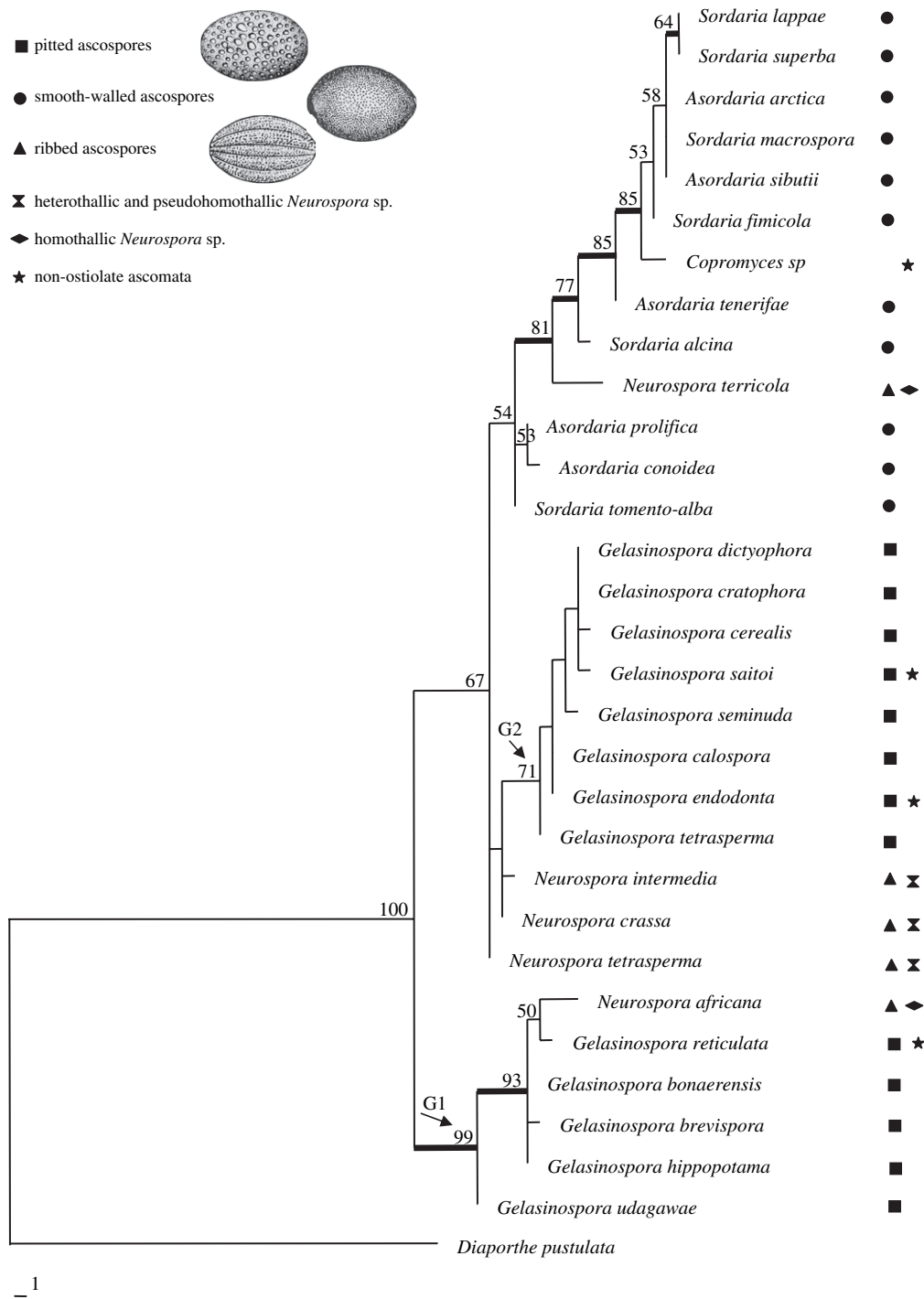


**Fig 1 - Phylogram depicting the relationships of *Apodus* and *Diplogelasinospora* species with respect to other members of Sordariales and reference taxa. The single tree was generated from parsimony analysis based on 28S rDNA sequences (TL = 988, CI = 0.461, RI = 0.621, RC = 0.286, HI = 0.539,  $-\ln L = 6587.91107$ ). 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*.**

tree was generated from WP. K-H test showed that these 5 trees were statistically not different. Treating gaps as fifth state resulted in trees with identical phylogeny. The single parsimonious tree (TL = 136, CI = 0.853, RI = 0.906, RC = 0.772,

HI = 0.147,  $-\ln L = 1931.65148$ ) generated from WP and treating gaps as missing data is shown in Fig 2.

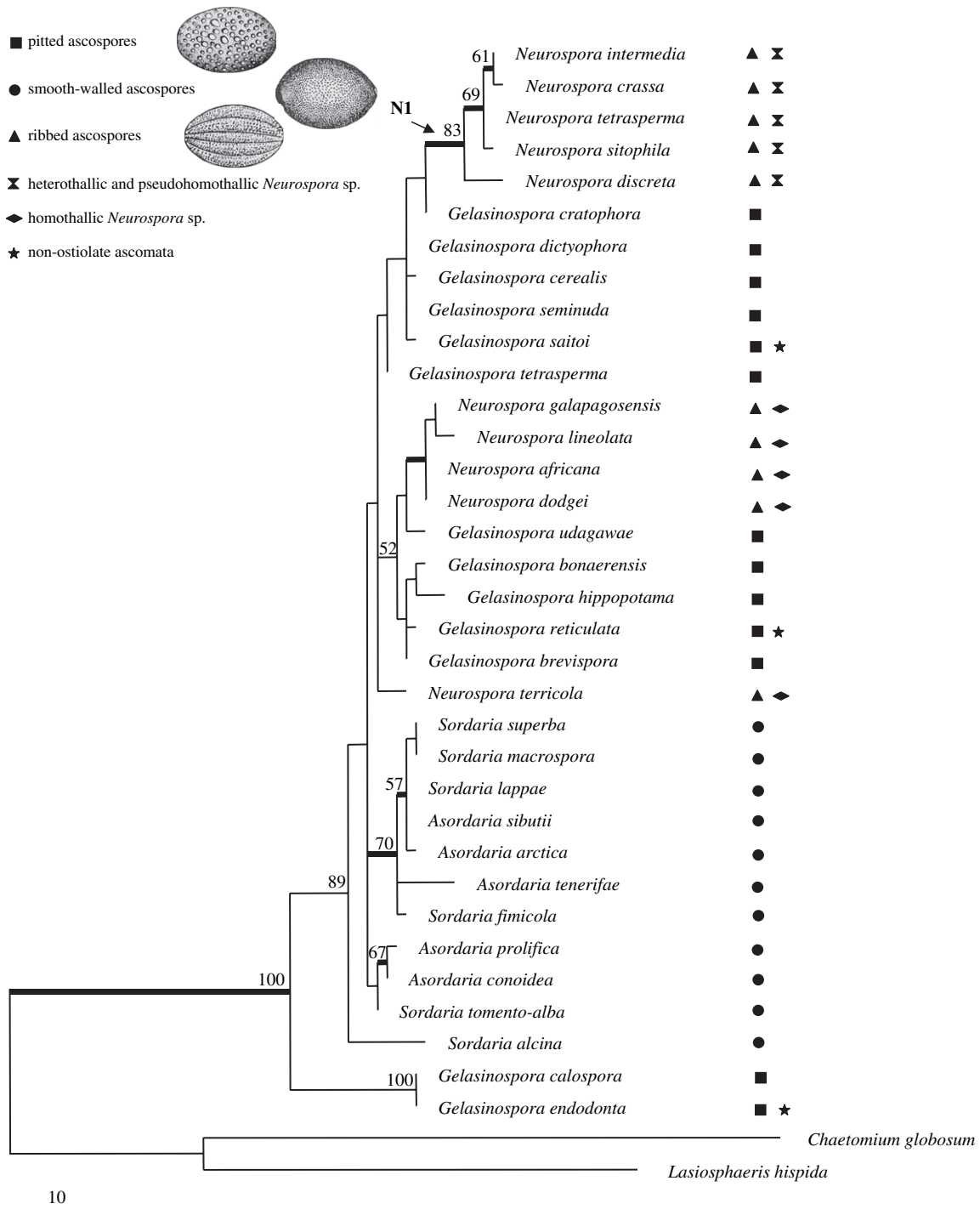
ITS sequences of 29 newly sequenced taxa and 7 taxa from GenBank were aligned (Dataset III). The final alignment



**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*.**

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

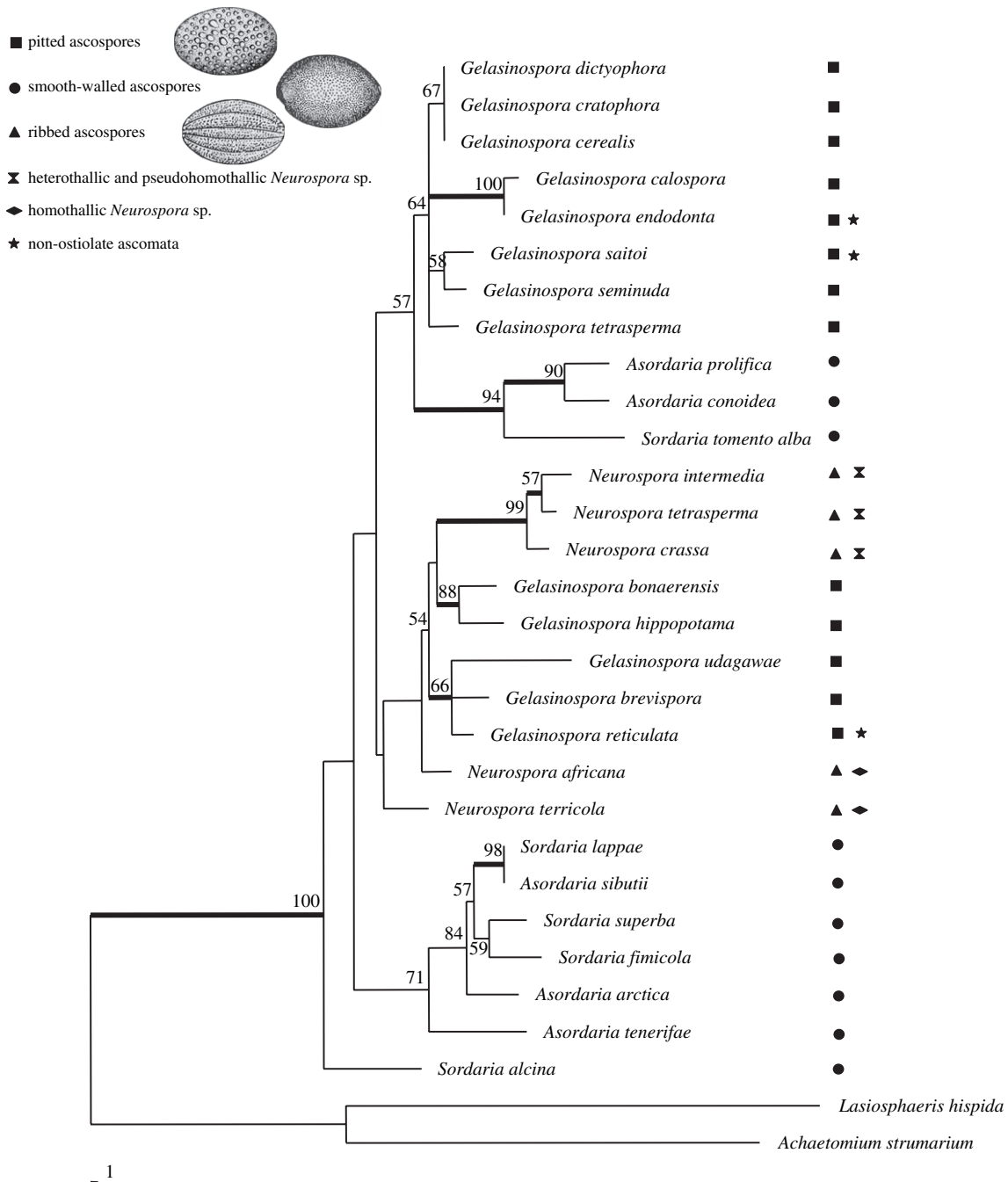
trees while WP gave 60 trees. K-H test showed these trees were not significantly different. One of the 60 parsimonious trees (TL = 272, CI = 0.875, RI = 0.815, RC = 0.713, HI = 0.125,  $-\ln L = 1788.08538$ ) generated from WP and treating gaps as fifth state is shown in Fig 3.



**Fig 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 *Lasiochaeris hispida*.**

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. TrN+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)

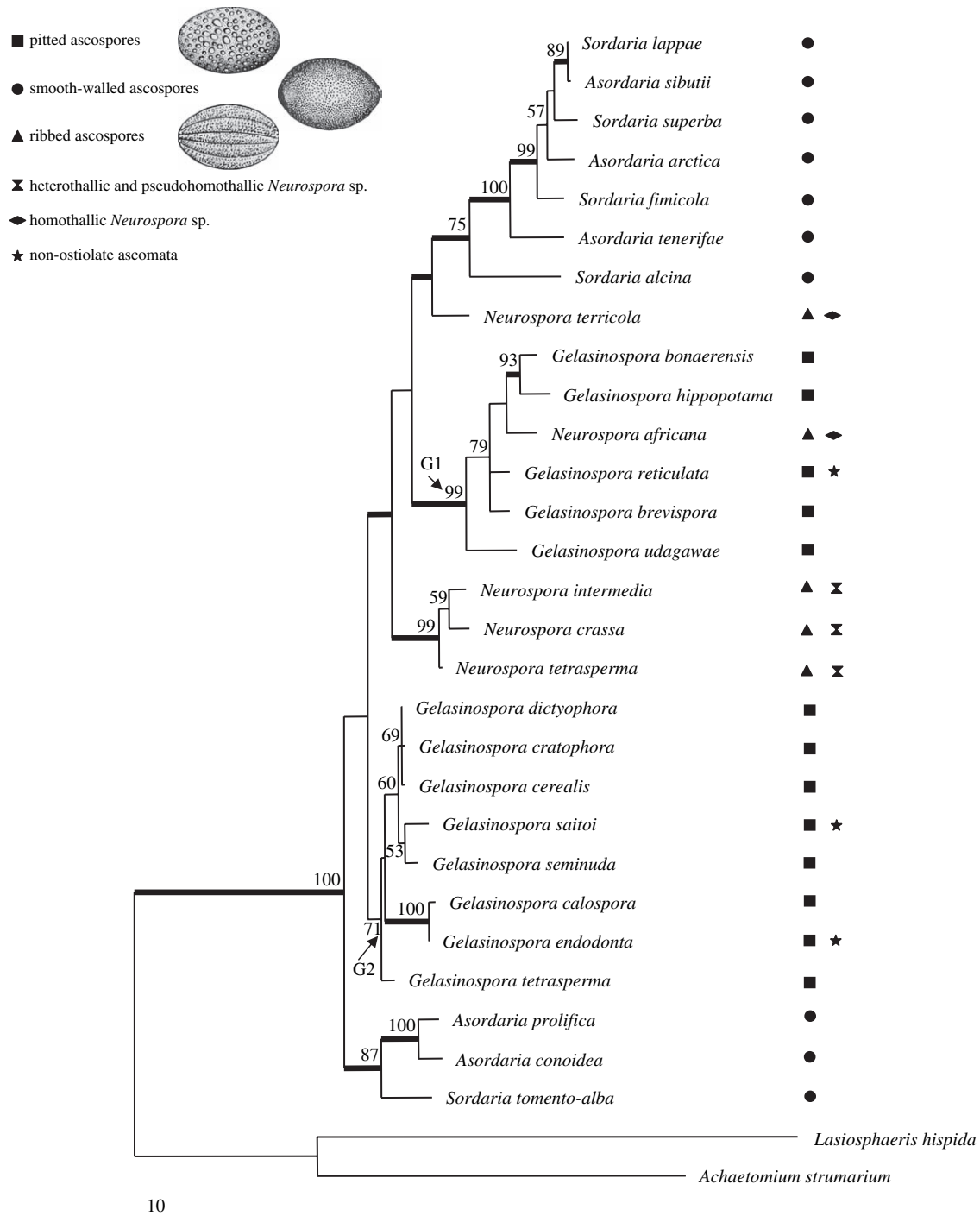


**Fig 4 – Phylogram of the single tree generated from parsimony analysis based on  $\beta$ -tubulin sequences (TL = 421, CI = 0.710, RI = 0.707, RC = 0.502, HI = 0.290,  $-\ln L = 2804.33026$ ). Data were analysed with random addition sequence, unweighted parsimony and treating gaps as missing data. 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 *Lasio-sphaeria 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 evolution selected 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 5 – Phylogram of one of the two trees generated from parsimony analysis based on combined  $\beta$ -tubulin, ITS rDNA and 28S rDNA sequences (TL = 716, CI = 0.756, RI = 0.752, RC = 0.569, HI = 0.244,  $-\ln L = 6748.74780$ ). Data were analysed with random addition sequence, weighted 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 *Lasio-sphaeris 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 mangentii* and *Cercophora mirabilis*, and *A. deciduus* with *Cercophora newfieldiana*, 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. hippopotama*, *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 *Lasiosphaeriaceae* 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 *lasiosphaeriaceae*

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 *Lasiosphaeriaceae* (Lundqvist 1972). Huhndorf et al. (2004) recently redefined *Lasiosphaeriaceae* and pointed out that *lasiosphaeriaceae* 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 *Lasiosphaeriaceae* 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 *Lasiosphaeriaceae*.

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 *Lasiosphaeriaceae*. 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 Maniotis (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 *Lasiosphaeriaceae* (Udagawa & Horie 1972; Guarro et al. 1991). Even though results show that *Diplogelasinospora* is more related to *Lasiosphaeriaceae*, further investigation with more taxa may help to conclusively resolve its phylogenies.

### Phylogeny of *Sordaria*

Arx et al. (1987) augured 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 ascospores 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  $\beta$ -tubulin, ITS and combined gene trees (Figs 3–5). Morphologically, these species have narrower ascospores (widths less than 12  $\mu\text{m}$ ), while ascospore widths of other *Sordaria* /*Asordaria* species investigated are greater than 12  $\mu\text{m}$ . The only ambiguity is *S. fimicola*, which has ascospores 11–13  $\mu\text{m}$  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 ascoma on different occasions (e.g. Berbee & Taylor 1992; Rehner & Samuels 1995). Modern classifications have tended to place non-ostiolate ascomycetes in primarily ostiolate groups (Rehner & Samuels 1995; Suh & Blackwell 1999). At familial and higher levels, the use of this morphological character has also caused confusion (e.g. *Cephalothecaceae* and *Pseudeurotiaceae*) (Malloch & Cain 1970; Suh & Blackwell 1999). In this study, the clades comprising taxa with ostiolate and nonostiolate ascomata (Figs 2–5) also imply that evolutionary changes have occurred within different lineages. The distinction between non-ostiolate 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. (García *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-spore 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, García *et al.* (2004) demonstrated that episporic 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 episporic respectively (García *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 episporic layer morphology. It appears that episporic layer morphology may not be necessarily as phylogenetically informative as previously suggested. For example, *G. bonaerensis*, *G. reticulata*, *G. udagawae*, and *Neurospora africana*, which have smooth episporic, grouped in clade G1. However, clade G1 also includes *G. hippopotama* and *G. brevispora*, which have an inwardly projecting episporic (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. dictyophora*), while García *et al.* (2004) found that *G. cerealis* (AJ579560) was related to *G. reticulata*, *G. bonaerensis*, and *G. micropertusa*, 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 pairs 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 ascospores, 2-spored asci, and verrucose ascospores with a slight apiculus 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.

*Boothiaella* 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 ascospores, 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 *Boothiaella*. From a morphological perspective, *Boothiaella* is similar to *Copromyces* and *Sordaria*. Some authors have argued that *Boothiaella* resembles *Thielavia* (*Chaetomiaceae*) in some aspects, such as the non-ostiolate ascospores and ascospores with a germ pore

(Arx & Mahmood 1968; Arx 1975). However, it has been shown in various studies that non-ostiolate ascospores 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 *Boothiaella* 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 lasiosphaeriacean 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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