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Ascospore Morphology Is a Poor Predictor of the Phylogenetic Relationships of *Neurospora* and *Gelasinospora*

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Dettman, J. R., Harbinski, F. M., and Taylor, J. W. 2001. Ascospore morphology is a poor predictor of the phylogenetic relationships of *Neurospora* and *Gelasinospora*. *Fungal Genetics and Biology* 34, 49–61. The genera *Neurospora* and *Gelasinospora* are conventionally distinguished by differences in ascospore ornamentation, with elevated longitudinal ridges (ribs) separated by depressed grooves (veins) in *Neurospora* and spherical or oval indentations (pits) in *Gelasinospora*. The phylogenetic relationships of representatives of 12 *Neurospora* and 4 *Gelasinospora* species were assessed with the DNA sequences of four nuclear genes. Within the genus *Neurospora*, the 5 outbreeding conidiating species form a monophyletic group with *N. discreta* as the most divergent, and 4 of the homothallic species form a monophyletic group. In combined analysis, each of the conventionally defined *Gelasinospora* species was more closely related to a *Neurospora* species than to another *Gelasinospora* species. Evidently, the *Neurospora* and *Gelasinospora* species included in this study do not represent two clearly resolved monophyletic sister genera, but instead represent a polyphyletic group of taxa with close phylogenetic relationships and significant morphological similarities. Ascospore morphology, the character that the distinction between the genera *Neurospora* and *Gelasinospora* is based upon, was not an accurate predictor of phylogenetic relationships.

Index Descriptors: Sordariaceae; *Neurospora*; *Gelasinospora*; phylogenetics; ascospore ornamentation; ascospore morphology.

INTRODUCTION

Members of the filamentous ascomycete family Sordariaceae are characterized by cylindrical unitunicate asci produced within darkly pigmented flask-shaped ascocarps (perithecia) with or without prominent ostioles. The genera placed within this family mainly are differentiated by ascospore morphology and ornamentation. For instance, the genera *Neurospora* Shear & Dodge (1927) and *Gelasinospora* Dowding (1933) are morphologically comparable except the former produces ascospores with elevated longitudinal ridges (ribs) separated by depressed grooves (veins), and the latter produces ascospores with spherical or oval indentations (pits). Within these two genera, the patterns of ascospore ornamentation can vary significantly, and significant overlap may occur in morphological traits that are used to distinguish species. For example, *N. sublineolata* and *N. discreta* produce ascospores with both pits and ribs (Furuya and Udagawa, 1976; Perkins and Raju, 1986).

Different members of these genera use one of three different mating strategies: heterothallism, homothallism, or pseudohomothallism. The mating-type locus has two alternate forms, *mat A* and *mat a*, which are so dissimilar

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that they have been termed "idiomorphs" rather than alleles (Metzenberg and Glass, 1990). Strains of heterothallic species possess a single mating-type idiomorph and are self-sterile obligate outbreeders that must mate with another strain that possesses the opposite idiomorph. Strains of homothallic species are self-fertile inbreeders which possess either the *mat A* or both mating-type idiomorphs (Glass *et al.*, 1990; Beatty *et al.*, 1994). Pseudohomothallic species produce four large dikaryotic ascospores instead of eight monokaryotic ascospores per ascus (Raju and Perkins, 1994). These dikaryotic ascospores have the *mat A* idiomorph in one nucleus and the *mat a* idiomorph in the other; so they are self-fertile and functionally homothallic. Due to abnormal ascospore formation, a small percentage of these ascospores are monokaryotic and give rise to functionally heterothallic strains which confer the possibility of outbreeding to these otherwise homothallic species. As a result, these species are termed "pseudohomothallic." In this study, both heterothallic and pseudohomothallic species are considered outbreeders.

The validity of the use of morphological characters (such as the size and shape of ascospores, asci, perithecia, and conidia) to delineate *Neurospora* species was questioned by previous authorities (Perkins *et al.*, 1976; Perkins and Raju, 1986; Perkins and Turner, 1988; Turner *et al.*, 2001), and a biological species concept has been applied to the five outbreeding species of this genus (*N. crassa*, *N. discreta*, *N. intermedia*, *N. sitophila*, and *N. tetrasperma*). Underscoring the incongruence between biological and morphological species concepts, all strains designated *N. discreta* are conspecific on the basis of fertility crosses, but strains sampled from different *N. discreta* populations may be morphologically distinct, even more so than strains from two other species (Perkins and Raju, 1986). Mating tests cannot be performed on the seven homothallic *Neurospora* species, so a purely morphological species concept has been retained for these taxa. Taxonomic placements within the genus *Gelasinospora* are also based upon a morphological species concept.

Most phylogenetic studies have focused on the relationships between the 1 pseudohomothallic and the 4 heterothallic *Neurospora* species (Natvig *et al.*, 1987; Taylor and Natvig, 1989; Randall and Metzenberg, 1995; Skupski *et al.*, 1997). Recently, Pöggeler (1999) included 6 homothallic *Neurospora* species in an analysis and reported that species with the same mating strategy were closely related. In the present study, we wanted to determine whether differences in ascospore morphology were consistent with genetic differences between *Neurospora* and *Gelasinospora* taxa. To address this question, 5 species of the pitted-spored genus *Gelasinospora* were included with the 12 described species of *Neurospora*, and their phylogenetic relationships were investigated with the nucleotide sequences of four nuclear genes. The results suggested that the *Neurospora* and *Gelasinospora* species included in this study do not represent two clearly resolved monophyletic sister genera, but instead represent a polyphyletic group of taxa with close phylogenetic relationships and significant morphological similarities. Species placed in a particular genus based upon ascospore morphology did not form well-supported clades to the exclusion of members of the other genus, and multiple origins of at least one of the ascospore morphologies are likely. Although the distinction between the genera *Neurospora* and *Gelasinospora* is based upon ornamentation of ascospores (pitted vs ribbed), this character was not an accurate predictor of the phylogenetic relationship as inferred from the sequence data analyzed in this study.

MATERIALS AND METHODS

Fungal Strains and Cultivation

The identification number and designated species names of the strains utilized in this study are listed in Table 1. Cultures were grown at 30°C on Vogel's minimal medium (0.5% yeast extract, 1× Vogel's salts, 1.5% agar; Vogel, 1964) amended with 1.5% sucrose.

DNA Extraction, Amplification, and Sequencing

DNA was extracted by the protocol of Lee *et al.* (1988). Gene fragments were PCR[2]-amplified from genomic DNA with an MJ Research PTC-100 Programmable Thermal Controller (MJ Research Inc., Watertown, MA). Oligonucleotide primers (Operon Technologies Inc., Alameda, CA) used for amplification were as follows: ITS5 and ITS4 (White *et al.*, 1990) for the ITS/5.8S rRNA region, N-gpd and C-gpd (Pöggeler, 1999) for the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene, Bal-5 and Bal-3 (Pöggeler, 1999) for the *mat A-1* gene of the *mat A*

[2] Abbreviations used: PCR, polymerase chain reaction; MP, maximum-parsimony; ML, maximum-likelihood; PHT, partition homogeneity test; KHT, Kishino–Hasegawa test; RFLP, restriction fragment length polymorphism.

TABLE 1
Strain Numbers and Mating Strategy for Taxa and Sources of Sequence Data for Four Genes

Taxa	Strain numbers[a]	Mating strategy[b]	Source of sequence data[c]			
			ITS/5.8S	*gpd*	*mat A-1*	*mat a-1*
Gelasinospora bonaerensis Stchigel & Guarro	—	Hom	AJ002029	—	—	—
Gelasinospora calospora (Mouton) Moreau & Moreau	FGSC 958	Hom	*AF388931*	*AF388933*	*AF388938*	*AF388942*
Gelasinospora cerealis Dowding	FGSC 959	Hom	*AF388932*	*AF388934*	*AF388939*	*AF388943*
*Gelasinospora*8239[d]	FGSC 8239	Het	*AF388912*	*AF388936*	Gene absent	*AF388945*
Gelasinospora tetrasperma Dowding	FGSC 7033	PsHom	*AF388911*	*AF388935*	*AF388940*	*AF388944*
Neurospora africana Huang & Backus	FGSC 1740	Hom	*AF388913*	AJ133012	AJ133139	Gene absent
Neurospora dodgei Nelson & Novak	FGSC 1692	Hom	*AF388920*	AJ133013	AJ133140	Gene absent
Neurospora galapagosensis Mahoney & Backus	FGSC 1739 (4628)	Hom	*AF388921*[e]	AJ133014	AJ133141	Gene absent
Neurospora lineolata Frederick & Uecker	FGSC 1910	Hom	*AF388924*	AJ133015	AJ133142	Gene absent
Neurospora pannonica Krug & Khan	FGSC 7221	Hom	*AF388925*	AJ133016	AJ133143	AJ133044
Neurospora sublineolata (Furuya & Udagawa) von Arx	FGSC 5508	Hom	*AF388927*	*AF388937*	*AF388941*	*AF388946*
Neurospora terricola Gochenaur & Backus	FGSC 1889	Hom	*AF388928*	AJ133017	AJ133144	AJ133045
Neurospora crassa Shear & Dodge	FGSC 987	Het	*AF388914*	U67457	M33876	M54787
Neurospora discreta Perkins & Raju	FGSC 3228 (3268, 6794, 8318, 8338)	Het	*AF388915*[f]	AJ133021	L42307	AJ133040
Neurospora intermedia Tai	FGSC 1762[g]	Het	*AF388923*	AJ133019	L42308	AJ133047
Neurospora sitophila Shear & Dodge	FGSC 1135	Het	*AF388926*	AJ133020	L42309	AJ133048
Neurospora tetrasperma Shear & Dodge	FGSC 7585	PsHom	*AF388929*	AJ133018	L42310	AJ133046
Sordaria fimicola (Roberge) Cesati & de Notaris	—	Hom	—	AJ133009	AJ133136	AJ133041
Sordaria macrospora Auerswald	—	Hom	—	AJ133007	Y10616	Y10616
Sordaria brevicollis Olive & Fantini	—	Het	—	AJ133010	AJ133137	AJ133042
Sordaria sclerogenia Fields & Grear	—	Het	—	AJ133011	AJ1330138	AJ133043
Podospora anserina (Cesati) Niessl	Mn/A 8A[h]	PsHom	*AF388930*	—	—	—

[a] If used in the present study. FGSC, Fungal Genetics Stock Center.
[b] Hom, homothallic; Het, heterothallic; PsHom, pseudohomothallic.
[c] Italicized GenBank accession numbers represent sequences produced during this study.
[d] An undescribed species reported by Glass *et al.* (1990).
[e] The sequence of the ITS/5.8S region of *N. galapagosensis* FGSC 4628 (*AF388922*) was identical to that of FGSC 1739.
[f] The sequence of the ITS/5.8S region of *N. discreta* FGSC 3268 and 6794 (*AF388916* and *AF388917*) and *N. discreta*-like FGSC 8318 and 8338 (*AF388918* and *AF388919*) differed from that of FGSC 3228 at a maximum of only two bases, and the consensus sequence was identical to that of FGSC 3228.
[g] This strain is of the orange rather than the rarer yellow ecotype.
[h] Genomic DNA donated by D. J. Cummings.

idiomorph, and Sal-5 and Sal-3 (Pöggeler, 1999) for the *mat a-1* gene of the *mat a* idiomorph. The regions of the genes amplified by the latter three primer sets have been previously described (Pögeller, 1999). Final PCR conditions were as follows: 200 μM dNTPs, 0.4 μM each primer (Operon Technologies Inc.), 1.0 unit of Ampli*Taq* DNA polymerase (Applied Biosystems, Foster City, CA), 1X PCR Buffer (supplied with enzyme). The thermal cycler protocol was as follows: initial denaturation at 94°C for 2 min; 31 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 1 min; 7-min extension at 72°C; and a final soak at 4°C. The PCR products were electrophoresed on 2% agarose gels to verify that a single fragment of appropriate length was produced. Amplified products were purified with the QIAquick PCR Purification Kit (QIAGEN Inc., Valencia, CA) and their nucleotide sequences were determined with the cyclic reaction termination method using fluorescently labeled dideoxyribonucleotide triphosphates. Dye Terminator (Amersham Pharmacia Biotech, Piscataway, NJ) or BigDye Terminator Cycle Sequencing Kits (Applied Biosystems) were utilized for sequencing reactions. Sequence data were collected from both strands with an ABI PRISM 377 DNA Sequencer and examined with the programs Sequencing Analysis and Sequence Navigator (version 3.4 and version 1.0.1, respectively; Applied Biosystems). The 36 nucleotide sequences produced during this study have been deposited in GenBank (National Center for Biotechnology Information). The GenBank accession numbers for these sequences and the other 42 DNA sequences utilized in this study are listed in Table 1. For the five outbreeding

Neurospora species, sequences may be from different strains because the *mat A-1* and *mat a-1* genes are not present in the same heterothallic strain.

Phylogenetic Analysis

DNA sequences were preliminarily aligned with ClustalX (version 1.8; Thompson *et al.*, 1997) with the multiple alignment parameters set to default, edited by visual inspection, and then verified by comparison to previously published amino acid sequences or alignments kindly provided by S. Pöggeler. Phylogenetic analysis was performed with the programs PAUP (version 4.0b4a; Swofford, 1998) and MacClade (version 3.08a; Maddison and Maddison, 1995). All gaps were treated as missing data. Maximum-parsimony (MP) analysis was performed with the heuristic search option with 10,000 replications of random addition searches. In this study, all MP trees produced by such a search were within a single tree island. Maximum-likelihood (ML) analyses was performed with the default settings, including Kishino–Hasegawa tests (KHTs; Kishino and Hasegawa, 1989). When multiple MP trees were produced, the tree that was the most likely as determined by ML (i.e., greatest −ln likelihood value) was chosen for display and is referred to as the "best tree." Transition/transversion ratios were determined by the importing of all MP trees into MacClade, the charting of unambiguous state changes, and the averaging of values across all trees. Parsimony bootstrapping was performed with default settings and 1000 replications. Partition homogeneity tests (PHTs; Farris *et al.*, 1995), also called incongruence length difference tests, were performed with the informative characters of the combined alignment and heuristic searches with 100 or 1000 replications.

RESULTS

Data Exploration

Nucleotide sequences were first aligned for the ITS1/5.8S/ITS2 ribosomal RNA region, glyceraldehyde-3-phosphate dehydrogenase (*gpd*), *mat A-1*, and *mat a-1* genes separately. The sequences of the four genes were also combined into a single alignment, excluding *G. bonaerensis* because fewer than three gene sequences were available for that taxon. Although the sequences for the *gpd*, *mat A-1*, and *mat a-1* genes were available for *Podospora anserina*, this taxon was eliminated from combined analysis (leaving 20 taxa) because the *Sordaria* genus was a closer outgroup; i.e., it had less sequence divergence from the ingroup. Sequence data from 18S ribosomal DNA had also indicated that *Neurospora* was more closely related to *Sordaria* than to *Podospora* (Lee and Hanlin, 1999). Members of *Sordaria* produce smooth ascospores surrounded by a clear gelatinous sheath, whereas members of *Podospora* produce two-celled ascospores with pedicels and gelatinous appendages. Table 2 summarizes the characteristics of the five sequence alignments. Analyses were conducted with *Sordaria* species as the outgroup taxa and *Gelasinospora* and *Neurospora* species as the ingroup taxa. The ITS/5.8S alignment lacked *Sordaria* species, so *P. anserina* was used as the outgroup taxon for rooting purposes. Including *P. anserina* in the analyses of the other three genes did not alter the topologies of the resulting trees. Unless individual genes are specified, discussion refers to the combined analysis of all four genes.

TABLE 2
Summary of Separate and Combined Analyses

Gene	Number of taxa	Mean ingroup seq. divergence (%)[a]	Number of nucleotides	Number of informative sites
ITS/5.8S[b]	18	1.06	569	13
gpd	20	3.78	433	48
mat A-1	19	5.40	481	141
mat a-1	16	5.37	372	78
Combined analysis	20	3.35	1855	278

[a] Uncorrected "p" distance. Ingroup = *Neurospora* and *Gelasinospora* taxa.
[b] Sequence data for the ITS/5.8S region extended 25 and 38 bp into the 18S and 28S rRNA genes respectively, but were retained to maximize the number of informative characters.

Base frequencies of entire antisense strands were not significantly different between taxa or genes, but the overall frequency of *C* (0.306) was significantly greater (χ^2, $P <$ 0.001) than any of the three other bases (0.230–0.234). Nucleotide composition bias was most obvious within the third codon positions, which were significantly A-deficient and C-rich (A = 0.045, C = 0.486, G = 0.262, T = 0.207; χ^2, $P < 0.001$). As inferred from the nine most parsimonious trees from combined analysis, the mean ratio of unambiguous transitions to transversions was 1.59, all types of transversions were equally common, and pyrimidine transitions (C ↔ T) were 2.19 times as common as purine transitions (A ↔ G). These values are consistent with the strong pyrimidine transition bias observed in

TABLE 3
Results Summary of Partition Homogeneity Tests

Partitions	*P* value[a]
ITS/5.8S:*gpd:mat A-1:mat a-1*	0.028
ITS/5.8S:*gpd:mat A-1*	0.15
ITS/5.8S:*gpd:mat a-1*	0.02
gpd:mat A-1:mat a-1	0.03
ITS/5.8S:*mat A-1:mat a-1*	0.22
ITS/5.8S:*gpd*	0.08
gpd:mat A-1	0.67
gpd:mat a-1	0.03
ITS/5.8S:*mat A-1*	0.80
ITS/5.8S:*mat a-1*	0.75
mat A-1:mat a-1	0.31
1st:2nd:3rd codon positions	0.218

[a] If 100 or 1000 replications were performed, two or three decimal places, respectively, are shown.

nuclear genes of Pyrenomycetes (Berbee and Taylor, 1992) and other fungi (Bruns and Szaro, 1992). As expected, the third codon position was the most variable and the second codon position the least variable. Sequence divergence (uncorrected distance) between ingroup taxa in combined analysis ranged from 0.07 to 6.15%, with a mean of 3.35%. The ITS/5.8S rRNA region was the least variable and the *mat A-1* gene was the most variable, with mean ingroup sequence divergences of 1.06 and 5.40%, respectively (Table 2). Although these are reasonable levels of sequence divergence, the possibility of mutational saturation was still investigated. First, uncorrected distances ("p") were plotted against corrected Kimura three-parameter distances (Kimura, 1981) for all pairwise ingroup sequence comparisons. Deviations from a linear relationship were not observed. When numbers of transitions were plotted against uncorrected distances for all pairwise ingroup sequence combinations, the results were similar. In general, there was no evidence of appreciable mutational saturation, so the nucleotide sequences of these genes were appropriate for phylogenetic analyses. A PHT was run with first, second, and third codon positions as separate partitions. No significant conflict between their phylogenetic signals was detected (Table 3; $P = 0.218$), so there was no reason to exclude or downweight certain classes of characters.

Separate Analysis

Three pairs of taxa had identical nucleotide sequences for the entire ITS/5.8S ribosomal RNA region: *N. dodgei* and *N. galapagosensis*, *N. terricola* and *Gelasinospora*8239, and *N. pannonica* and *G. tetrasperma*. Heuristic searches with the ITS/5.8S alignment produced 96 equally parsimonious trees (Length = 90, CI = 0.944, RI = 0.861), with the best tree displayed in Fig. 1A. The low variability and small number of informative sites in the ITS/5.8S rRNA region resulted in a poorly resolved gene tree with relatively short branch lengths. The topology of the ITS/5.8S tree did not support the monophyly of *Neurospora* or *Gelasinospora*, since branches that divided members of the same genus had high bootstrap support. The facts that two *Gelasinospora* strains had the same sequence as two *Neurospora* strains and that *G. calospora* grouped closely (70% bootstrap support) with *N. sublineolata* also suggested polyphyly of the two genera. Four of the homothallic *Neurospora* species (*N. africana, N. dodgei, N. galapagosensis*, and *N. lineolata*) were grouped together, as were the five outbreeding *Neurospora* species (*N. crassa, N. discreta, N. intermedia, N. sitophila*, and *N. tetrasperma*).

To briefly assess intraspecies variation, the ITS/5.8S rRNA region was sequenced from additional strains of heterothallic *N. discreta* and homothallic *N. galapagosensis. N. galapagosensis* FGSC 4628 had a sequence identical to that of *N. galapagosensis* FGSC 1739. The five strains from the *N. discreta* group (FGSC 3268, 3228, 6794, 8318, and 8338) displayed slight variation in this gene region, but any pair of strains differed by a maximum of only two bases. *N. discreta* FGSC 3228 and 6794 had identical sequences, as did *N. discreta* FGSC 8318 and 8338. Strains FGSC 8318 and 8338 have been referred to as *N. discreta*-like, which describes strains that do not mate well with any species tester strains, but mate least poorly with *N. discreta* (Turner *et al.,* 2001). Since the sequence of FGSC 3228 was the same as the consensus of the five strains, this sequence was used for phylogenetic analysis.

Three taxa had identical nucleotide sequences for the glyceraldehyde 3-phosphate dehydrogenase (*gpd*) gene segment: *N. africana, N. dodgei*, and *N. galapagosensis*. Heuristic searches with the *gpd* alignment produced two equally parsimonious trees (Length = 112, CI = 0.830, RI = 0.867), with the best tree displayed in Fig. 1B. The five outbreeding *Neurospora* species formed a well-supported clade (99%) in the *gpd* gene tree, as did the same four homothallic *Neurospora* species that grouped together in the ITS/5.8S tree (75%). *G. calospora, G. tetrasperma*, and *N. sublineolata* appeared closely related (96%), a result that was consistent with previously described morphological similarities of these two *Gelasinospora* species (Cailleux, 1971; von Arx, 1982).

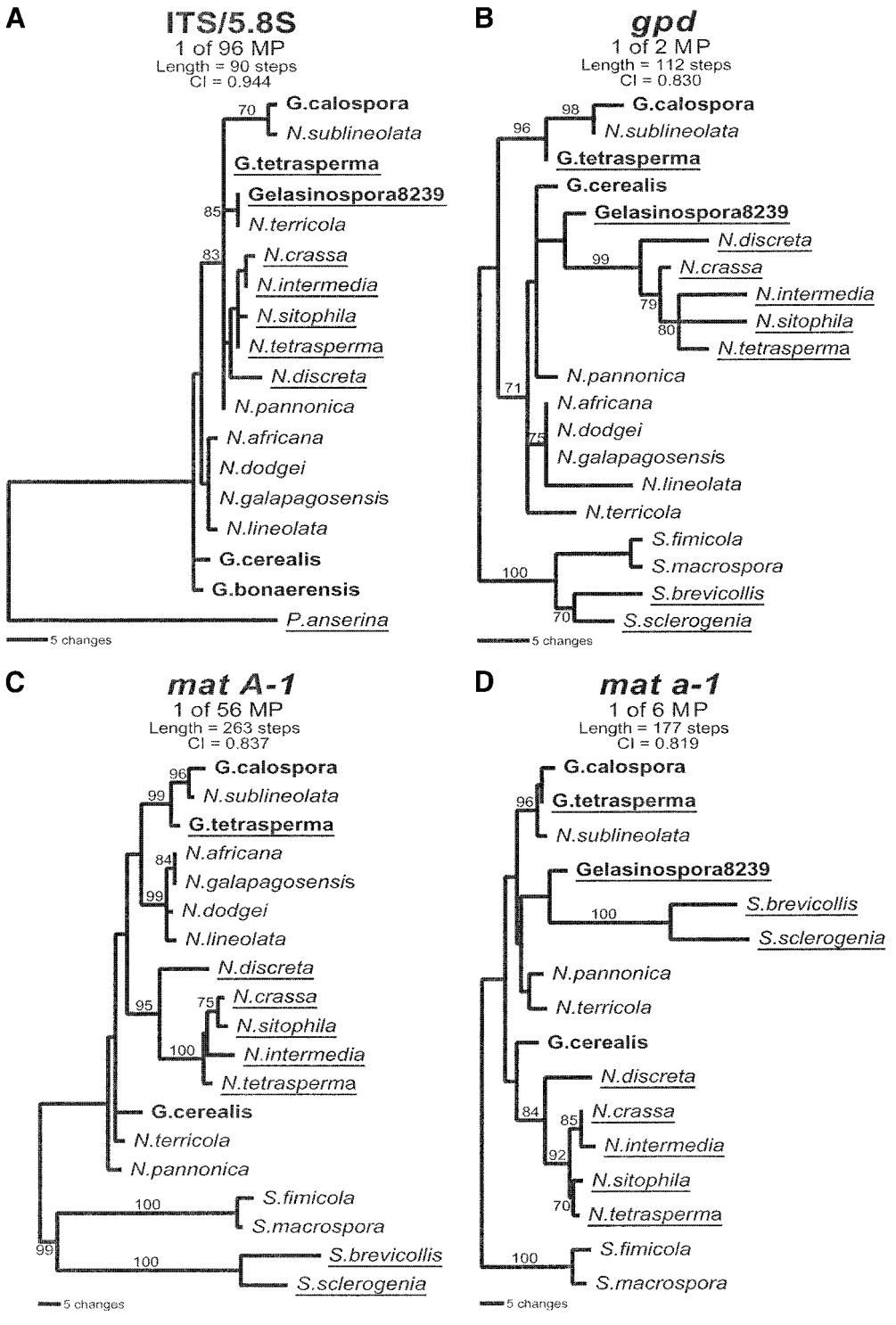

FIG. 1. (A–D) Maximum-parsimony phylograms produced from separate analysis of the ITS/5.8S (A), *gpd* (B), *mat A-1* (C), and *mat a-1* (D) sequence data sets. Outbreeding (heterothallic or pseudohomothallic) taxa are underlined, and taxa that produce ascospores with conspicuous pits are boldfaced and unitalicized. Numbers above or below branches represent the percentage (if ≥70%) of 1000 bootstrapped replicates that supported the branch.

Only one pair of taxa had identical nucleotide sequences for the *mat A-1* gene segment: *N. africana* and *N. galapagosensis*. Heuristic searches with the *mat A-1* alignment produced 56 equally parsimonious trees (Length = 263, CI = 0.837, RI = 0.866), with the best tree displayed in Fig. 1C. The topology of the *mat A-1* gene tree was quite similar to the topology of the *gpd* gene tree: the five outbreeding *Neurospora* species, the same four homothallic *Neurospora* species, and *G. calospora, G. tetrasperma*, and *N. sublineolata* formed well-supported clades (95, 99, and 99%, respectively).

Since four of the homothallic *Neurospora* species lack the *mat a-1* gene, the corresponding data set was limited to only 16 taxa, all of which had a unique sequence. Heuristic searches with the *mat a-1* alignment produced six equally parsimonious trees (Length = 177, CI = 0.819, RI = 0.811), with the best tree displayed in Fig. 1D. Surprisingly, the four *Sordaria* outgroup taxa did not form a monophyletic group. Although the *Sordaria* species were grouped together in the *mat a-1* gene tree produced by Pöggeler (1999), the MP bootstrap support was quite low (51%). Repetition of our analysis with *P. anserina* (GenBank X64195) as the outgroup did not change the topology of the gene tree. When the four *Sordaria* species were topologically constrained to monophyly, the best tree was only a single step longer (Length = 178, CI = 0.815, RI = 0.805) and was not significantly less likely than the unconstrained tree as determined by a KHT ($P > 0.48$; data not shown). Since most evidence suggests that *Sordaria* is a distinct sister group to *Neurospora* (e.g., Lee and Hanlin, 1999; Merrow and Dunlap, 1994), the constrained tree was preferred. Regardless, both trees support the clade of five outbreeding *Neurospora* species and the *G. calospora, G. tetrasperma*, and *N. sublineolata* clade.

Combined Analysis

The topologies of the four gene trees shown in Figs. 1A–1D were nearly congruent. There were no major conflicts between well-supported clades and such clades tended to be present in most gene trees. A conditional combination approach was taken and the degree of data conflict was assessed by PHTs with all possible combinations of two, three, or four gene partitions. Use of a *P* value of 0.05 may potentially result in false positives (Huelsenbeck *et al.*, 1996), so the *P* values suggested by Cunningham (1997) were used instead (i.e., the combining of data will cause phylogenetic accuracy to decrease if $P < 0.001$ and increase if $P > 0.01$). As shown in the PHT results summary in Table 3, the two most congruent partitions were ITS/5.8S and *mat A-1* ($P = 0.80$), whereas the two least congruent partitions were *gpd* and *mat a-1* ($P = 0.03$). For all possible combinations, significant conflict between partitions was not detected. When all four genes were compared, partitions did not have significant levels of incongruence and the combining of data was predicted to increase phylogenetic accuracy ($P = 0.028$; Table 3); thus, combined analysis was justified. Heuristic searches with the combined alignment produced six equally parsimonious trees (Length = 593, CI = 0.816, RI = 0.835), with the best tree displayed in Fig. 2. As expected, the clades that were well supported by separate analysis of each gene were also well supported by combined analysis. Various character and character state weightings were applied, but a tree with a different topology was produced only when transversions were weighted three times greater than transitions or when analysis was restricted to characters in the first and second codon positions only. Under both of these conditions, heterothallic *Gelasinospora*8239 was placed basal to the group of five outbreeding *Neurospora* species (trees not shown).

Assessment of Monophyly

The likelihood of monophyly of ascospore morphologies or taxa with similar mating strategies was assessed with Kishino–Hasegawa tests. Based upon the combined alignment, MP trees were constructed under specific topological constraints and their fit to the combined sequence data was compared with that of the best unconstrained tree (Fig. 2). Six of the different evolutionary hypotheses or topological constraints that were tested are listed in Table 4, along with the results. When all taxa that produce ribbed ascospores were forced to form a monophyletic clade (Fig. 3A), the best constrained tree was significantly less likely than the unconstrained tree ($P < 0.0001$). The hypothesis that ribbed ascospore morphology arose only once during the evolutionary history of these taxa could therefore be rejected. Similar results were obtained with pitted-spored taxa ($P < 0.0001$). Small inconspicuous dot-like pits occur within the veins of *N. sublineolata* (Furuya and Udagawa, 1976), and when *N. sublineolata* was included within the forced pitted-spored clade, monophyly could not be rejected ($P = 0.0872$).

Two of the well-supported clades in the combined gene tree were composed of all homothallic or all outbreeding *Neurospora* species, which suggested that mating strategy may be a good indicator of phylogenetic relationships (as discussed in Pöggeler, 1999) This statement holds true if *N. sublineolata* is omitted from analysis, but when *N.*

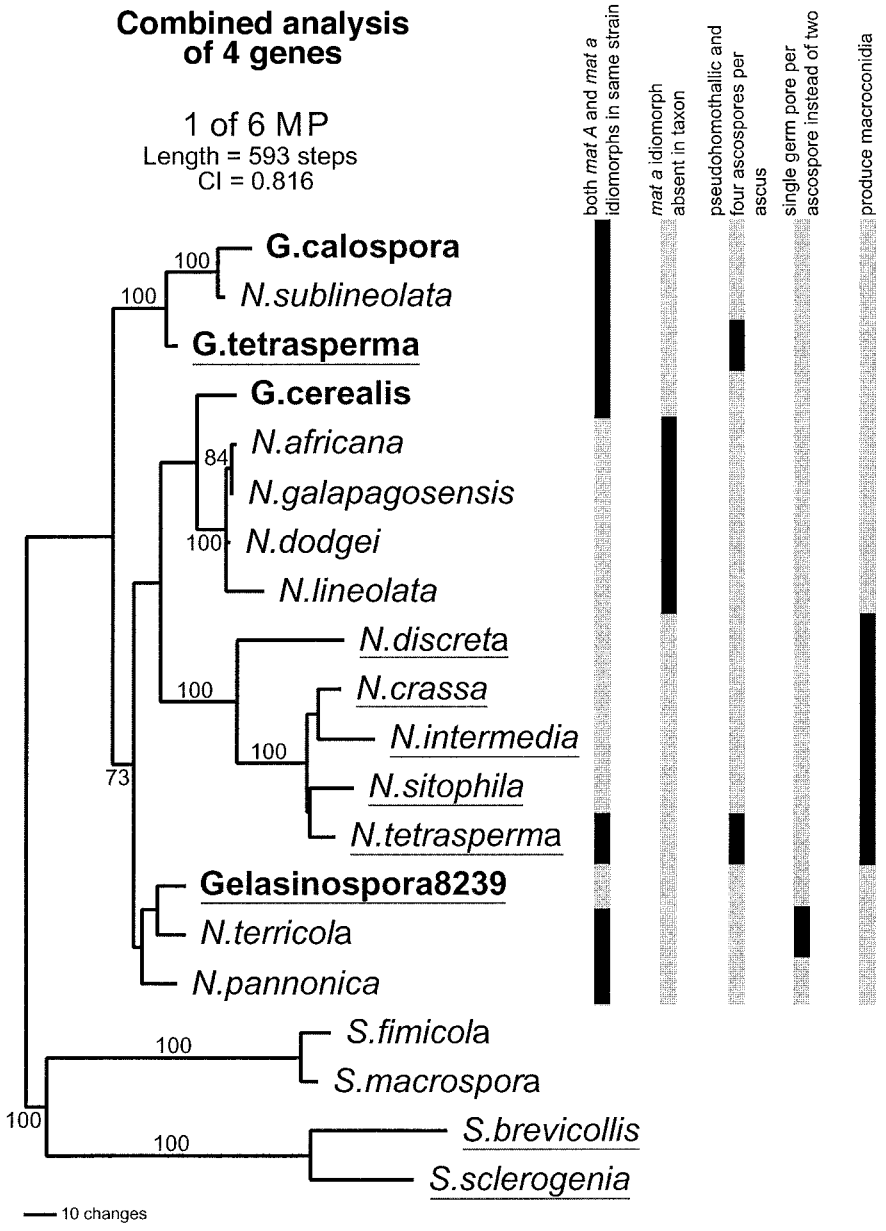

FIG. 2. Maximum-parsimony phylogram produced from combined analysis of the four gene sequence data sets. Some morphological/biological characters are mapped onto the phylogram, with black bars indicating character presence. Outbreeding (heterothallic or pseudohomothallic) taxa are underlined, and taxa that produce ascospores with conspicuous pits are boldfaced and unitalicized. Numbers above or below branches represent the percentage (if ≥70%) of 1000 bootstrapped replicates that supported the branch.

sublineolata is included, the monophyly of homothallism in *Neurospora* can be rejected ($P < 0.0001$; data not shown). Similarly, when all members of the ingroup taxa were included, the monophyly of an outbreeding, pseudohomothallic, or homothallic mating strategy (Fig. 3B) could be rejected in all cases ($P \leq 0.0003$; Table 4).

DISCUSSION

The phylogenetic relationships between various members of *Gelasinospora* and *Neurospora* were investigated with nucleotide sequence data from four nuclear genes: ITS/5.8S ribosomal RNA region, glyceraldehyde 3-phos-

TABLE 4
Results Summary of Kishino–Hasegawa Tests

Topologically constrained tree with monophyly of	Number of steps	Difference in −ln L values[a]	*t*	*P* value[b]
Ribbed ascospores (Fig. 3A)	628	168.479	5.492	<0.0001
Pitted ascospores	628	174.002	5.347	<0.0001
Pitted ascospores (including *N. sublineolata*)	600	26.660	1.711	0.0872
Outbreeding ingroup taxa	613	98.322	3.616	0.0003
Homothallic ingroup taxa (Fig. 3B)	615	101.548	3.732	0.0002
Pseudohomothallic ingroup taxa	658	299.751	9.202	<0.0001

[a] Between best unconstrained tree (593 steps, -In L = 5943.359) and constrained tree.
[b] Probability of obtaining a greater *t* value under the null hypothesis of no difference between trees.

phate dehydrogenase, mating-type *A-1*, and mating-type *a-1*. The most commonly used gene region for fungal phylogenetics or systematics at or below the genus level has been the ITS/5.8S rRNA region (White *et al.*, 1990), and *gpd* (Smith, 1989) and the well-characterized mating-type genes (*mat's*; Glass *et al.*, 1988) have also been used for similar purposes. For the taxa analyzed in this study, the mean sequence divergence and corresponding num-

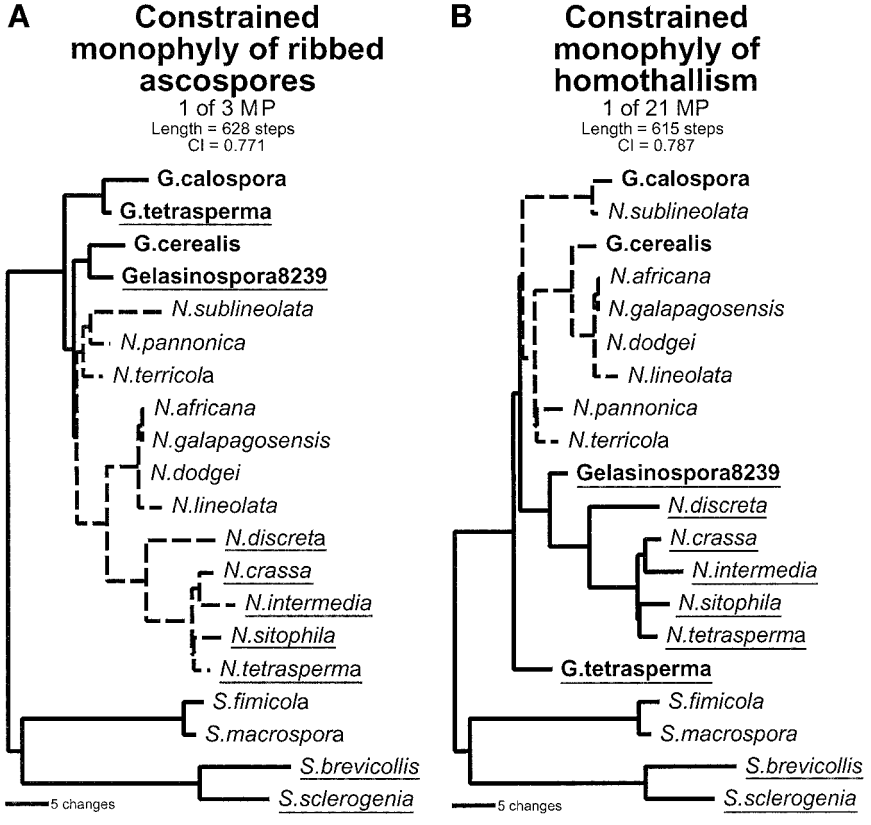

FIG. 3. Two examples of the maximum-parsimony phylograms produced from combined analysis with topological constraints applied to the ingroup. (A) All ingroup taxa that produce ribbed ascospores were constrained to monophyly. (B) All homothallic ingroup taxa were constrained to monophyly. Both trees were significantly less likely than the unconstrained tree ($P < 0.0001$ and $P = 0.0002$, respectively; see Table 4). Outbreeding (heterothallic or pseudohomothallic) taxa are underlined, taxa that produce ascospores with conspicuous pits are boldfaced and unitalicized, and constrained clades have dashed branches.

ber of parsimony-informative characters were relatively low for the ITS/5.8S rRNA region and high for the mating-type genes. This was consistent with previous reports of mating-type genes evolving at considerably higher rates than other genes (Ferris *et al.,* 1997; Turgeon, 1998; Pöggeler, 1999). Although the mating-type gene sequences were the most divergent between taxa, not even the highly variable third codon positions displayed signs of appreciable mutational saturation; thus, there was no *a priori* reason to downweight or exclude these characters from phylogenetic analyses. There were no significant conflicts between the phylogenetic signals from each gene, so sequence data for all four genes were combined for analyses.

Although the phylogenetic relationships of the five outbreeding species of *Neurospora* (heterothallic *N. crassa*, *N. discreta*, *N. intermedia*, and *N. sitophila*, and pseudohomothallic *N. tetrasperma*) have been studied in some detail, several issues remain unresolved. For instance, the placement of *N. intermedia* in relation to the other four species is unclear. In general, morphological characteristics of ascospores, asci, and perithecia are highly variable within species (Perkins *et al.*, 1976; Perkins and Turner, 1988; Turner *et al.,* 2001) and are not appropriate for the inferring of evolutionary relationships among *Neurospora* species. In the laboratory, *N. intermedia* can produce a small but significant number of viable ascospores when mated with either *N. crassa* or *N. sitophila* (Perkins *et al.,* 1976). Molecular-based studies by Natvig *et al.* (1987) using RFLPs of random nuclear DNA, Taylor and Natvig (1989) using RFLPs of mitochondrial DNA, and Skupksi *et al.* (1997) using upstream sequences of *al-1* and *frq* genes and RFLPs of random nuclear DNA suggested that *N. intermedia* was most closely related to *N. crassa*, whereas a study by Randall and Metzenberg (1995) of the *mat A* idiomorph and flanking sequence suggested otherwise. In Pöggeler's (1999) phylogenetic study using the *gpd, mat A-1,* and *mat a-1* genes, only the *mat a-1* gene supported a close relationship of *N. intermedia* and *N. crassa*. Although the present study suggested that *N. intermedia* and *N. crassa* were sister species, this grouping did not have high bootstrap support in the combined analysis tree. Also, a *N. intermedia–N. sitophila* relationship did not have a significantly worse fit to the data than a *N. intermedia–N. crassa* relationship (KHT, $P = 0.280$; data not shown). When all information was considered, there were only two well-supported conclusions: (1) the five outbreeding *Neurospora* species form a monophyletic group and (2) *N. discreta* is the most divergent of these five species and appears basal to the others. In the present study, all four genes support both of these conclusions.

The first phylogenetic study to investigate the relationships between outbreeding and homothallic *Neurospora* species included homothallic *N. africana, N. dodgei, N. galapagosensis, N. lineolata, N. pannonica,* and *N. terricola* in the analyses (Pöggeler, 1999). The bootstrap values and topologies of the gene trees presented here were similar to the consensus trees of Pöggeler (1999), since most of the published sequences were used. *N. africana, N. dodgei, N. galapagosensis,* and *N. lineolata* displayed low levels of sequence divergence and formed a well-supported monophyletic clade. Of 1483 aligned nucleotide sites, *N. africana, N. dodgei,* and *N. galapagosensis* differed at a maximum of only four sites, with *N. africana* and *N. galapagosensis* differing at only a single site. In addition to high sequence conservation, the ascospore ornamentation (see Austin *et al.* (1974) for scanning electron microscope images), meiotic nuclear behavior, and ascospore formation (Raju, 1978) are essentially identical in *N. africana, N. dodgei,* and *N. galapagosensis*. When *N. galapagosensis* and *N. africana* were first described by Mahoney *et al.* (1969), their "striking general resemblance to *N. dodgei* in both morphological and cultural features" was noted. The available morphological, cytological, and sequence data all suggest that these three taxa are in fact synonymous. Strains designated *N. africana, N. dodgei,* and *N. galapagosensis* may be individuals of the same species, and the minor morphological differences observed by previous authors might represent variation between populations from different geographic regions. Based upon similar hybridization patterns to cosmid and mating-type probes, Glass *et al.* (1990) suggested that all four of these homothallic taxa may represent a single species. Since *N. lineolata* is the most divergent of these four species in terms of gene sequence, and produces ascospores that are distinguishable from the others, it appears to represent a distinct lineage (*N. lineolata* ascospores have the least prominent topological features; see Frederick *et al.,* 1969; Austin *et al.,* 1974).

In contrast, three remaining homothallic *Neurospora* species, *N. pannonica, N. terricola,* and *N. sublineolata,* did not form a monophyletic group in any of the four gene trees. In the combined gene tree (Fig. 2), *N. pannonica* and *N. terricola* were grouped together with heterothallic *Gelasinospora*8239, and *N. sublineolata* was in a well-supported clade with two other *Gelasinospora* species. These *Neurospora* species are clearly distinct from the previously mentioned homothallic *Neurospora* species because *N. pannonica, N. sublineolata,* and *N. terricola* con-

tain both the *mat A* and the *mat a* idiomorphs in the same nucleus, whereas the other homothallics contain only the *mat A* idiomorph (Glass *et al.*, 1990; Beatty *et al.*, 1994). Interestingly, the *N. sublineolata* type strain (FGSC 5508) was originally placed in the genus *Anixiella* Saito and Minoura (Cain, 1961), the nonostiolate counterpart to the ostiolate genus *Gelasinospora*, because Furuya and Udagawa (1976) observed small inconspicuous pits along the faint veins of its ascospores. The specific epithet *sublineolata* was chosen to indicate the morphological ascospore similarities between this strain and the previously described strains of *N. lineolata* (Frederick *et al.*, 1969). This *Anixiella sublineolata* strain was later transferred to the genus *Neurospora* by von Arx (1981, p. 164). Based upon the gene sequence data presented here, *N. sublineolata* is clearly more closely related to *G. calospora* and *G. tetrasperma* than to *N. lineolata* or any of the other known *Neurospora* species.

Since this was the first study of the phylogenetic relationships between *Neurospora* and *Gelasinospora*, only five described species of *Gelasinospora* were included, one of which was omitted for the combined analysis. Even with such a small sample size, members of the genus *Gelasinospora* clearly did not form a monophyletic lineage that was distinct from the genus *Neurospora*. In the combined gene tree, each of the *Gelasinospora* species was more closely related to a *Neurospora* species than to another *Gelasinospora* species, and branches that divided members of the same genus had high bootstrap support. The two *Gelasinospora* species that appeared most closely related were *G. calospora* and *G. tetrasperma*, but *G. calospora* was also consistently placed in a well-supported clade that contained *N. sublineolata*. The phylogenetic affinity between these two *Gelasinospora* species is supported by remarkable morphological similarity. In taxonomic reviews of the genus by Cailleux (1971) and von Arx (1982), the only character that distinguished these two species was the number of ascospores per ascus (eight in *G. calospora*, four in *G. tetrasperma*).

In general, there was a correlation between mating strategy and phylogenetic affinity, in that well-supported ingroup clades tended to contain taxa with similar mating strategies (i.e., outbreeding *Neurospora* or homothallic *Neurospora*). In addition, the outgroup *Sordaria* taxa also formed two well-supported clades, one of homothallic species and the other of heterothallic species. When ingroup taxa with similar mating strategies were forced to form single clades regardless of ascospore morphology, the hypothesis of monophyly of outbreeding, heterothallic, homothallic, or pseudohomothallic taxa could be rejected in all cases ($P \leq 0.0003$).

Evidently, the *Neurospora* and *Gelasinospora* species included in this study do not represent two clearly resolved monophyletic sister genera, but instead represent a polyphyletic group of taxa with close phylogenetic relationships and significant morphological similarities. Species from both genera were interspersed among each other within the phylogenetic trees, and species placed in one genus based upon ascospore ornamentation did not form well-supported clades to the exclusion of members of the other genus. This indicated that pitted vs ribbed ornamentation of ascospores is a phylogenetically misleading character and, as such, is not appropriate for the prediction of evolutionary relationships of the Sordariaceae. The hypotheses of the single origin of either pitted or ribbed ascospores (i.e., monophyly of *Gelasinospora* or *Neurospora* species) could be rejected with KHTs ($P < 0.0001$), which suggested that multiple origins of at least one of the ascospore morphologies was likely. The monophyly of pitted-spored taxa could not be rejected (KHT, $P = 0.0872$) when *N. sublineolata* was included in the constrained pitted-spored clade. *N. sublineolata* produces small inconspicuous pits along the veins of its ascospores (Furuya and Udagawa, 1976), but similar small crater-like pits have also been observed along the ascospore veins of other *Neurospora* species (e.g., *N. dodgei* and *N. lineolata*; Austin *et al.*, 1974). Certain strains of *N. discreta* produce ascospores with both pits and ribs (Perkins and Raju, 1986), but the pits of *N. discreta* indent the ribs rather than the veins. Also, Cailleux (1971) identified four types of "pitted" ascospores within *Gelasinospora*, some with quite distinct structural features of the ascospore walls. Both of these findings support the possibility of multiple origins of pitted-spored morphology.

The fact that ascospore ornamentation can be a poor indicator of phylogenetic relationships in this group is an unsettling observation, considering that the distinction between the genera *Neurospora* and *Gelasinospora* is based solely upon this character, and the use of ascospore ornamentation to delineate genera is not restricted to the Sordariaceae. Similar examples can be found in other families of the Sordariales, such as Ceratostomataceae (*Melanospora* vs *Persiciospora* vs *Sphaerodes*; see Cannon and Hawksworth, 1982; Barr, 1990) and Triptosporaceae/Lasiosphaeriaceae (*Arnium* vs *Arniella*; see Barr, 1990). Another example with striking resemblance to the *Neurospora* vs *Gelasinospora* dichotomy can be found in Amphisphaeriaceae, Xylariales. The genera of Amphisphaeriaceae are classified by characters such as ascospore pig-

mentation, septation, and shape (Barr, 1994), but in some cases, only ascospore wall ornamentation is used to distinguish two genera. For instance, the genus *Lepteutypa* produces ascospores with thin circular pits, whereas the genus *Blogiascospora* produces smooth to slightly striate ascospores (Barr, 1975; Kang *et al.*, 1999). A characteristic more commonly used for distinction of genera is ascospore shape, which has also been shown to be an unreliable estimator of phylogenetic relationships in several cases. For example, while generic distinction of ascosporogenous yeasts is based primarily upon ascospore shape (and ornamentation), Kurtzman and Robnett (1994) demonstrated that these morphological differences were not consistent with phylogenetic relationships inferred from rRNA sequence similarity. If studies of the other groups of fungi demonstrate that similar ascospore shapes and ornamentation can arise through convergent evolution, what genetic, physiological, or environmental factors contributed to this process?

Before firm conclusions can be drawn in regard to the phylogenetic relationships of members of the genera *Neurospora* and *Gelasinospora*, more comprehensive studies must be performed. First of all, a wider taxonomic sampling of the Sordariaceae is necessary: related genera such as *Anixiella* and *Diplogelasinospora* have significant morphological similarities with *Gelasinospora* and *Neurospora*. A wider taxonomic sampling of *Gelasinospora* is also necessary: only 5 of over 20 described species were investigated in the present study. In addition, sampling multiple strains of each taxon from several geographic regions will indicate the levels of intraspecies variation and help to delineate the boundaries between populations and phylogenetic species. Particularly for *Neurospora* species, determination of population and intraspecies variation will be critical to understanding the relationships between *N. crassa* and the other 4 outbreeding species. Population genetic studies of closely related sympatric species will also determine whether interspecies gene flow is occurring and how it may affect the evolution and divergence of these taxa.

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