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...
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-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

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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

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Strain numbers</th>
<th>Mating strategy</th>
<th>Source of sequence data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>ITS/5.8S</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gpd</td>
</tr>
</tbody>
</table>

| Gelatinospora bonaensis    | —              | H om          | AJ00209                 |
| Gelatinospora calospora   | F GSC 958      | H om          | AF388931 AF388933 AF388938 AF388942 |
| Gelatinospora cerealis    | F GSC 959      | H om          | AF388932 AF388934 AF388939 AF388943 |
| Gelatinospora tetrasperma | F GSC 8239     | H et          | AF388912 AF388936 Gene absent AF388945 |
| Gelatinospora tetrasperma | F GSC 7033     | Psh om        | AF388911 AF388935 AF388940 AF388944 |
| Neurospora africana       | F GSC 1740     | H om          | AF388913 AJ133012 AJ133139 Gene absent |
| Neurospora dodgei         | F GSC 1692     | H om          | AF388920 AJ133013 AJ133140 Gene absent |
| Neurospora galapagosensis | F GSC 1739 (4628) | H om        | AF388921 AJ133014 AJ133141 Gene absent |
| Neurospora lineolata      | F GSC 1910     | H om          | AF388924 AJ133015 AJ133142 Gene absent |
| Neurospora pannonica      | F GSC 7221     | H om          | AF388925 AJ133016 AJ133143 AJ133044 |
| Neurospora sublineolata   | F GSC 5508     | H om          | AF388927 AF388937 AF388941 AF388946 |
| Neurospora terricola      | F GSC 1889     | H om          | AF388928 AJ133017 AJ133144 AJ133045 |
| Neurospora crassa         | F GSC 987      | H et          | AF388914 U67457 M38376 M54787 |
| Neurospora discreta       | F GSC 3228 (3268, 6794, 8318, 8338) | H et | AF388915 AJ133021 L42307 AJ133040 |
| Neurospora intermedia Tai | F GSC 1762     | H et          | AF388923 AJ133019 L42308 AJ133047 |
| Neurospora sitophilia     | F GSC 1135     | H et          | AF388926 AJ133020 L42309 AJ133048 |
| Neurospora tetrasperma    | F GSC 7585     | Psh om        | AF388929 AJ133018 L42310 AJ133046 |
| Sordaria fimbicola        | F GSC 1899     | H et          | — AJ133009 AJ133136 AJ133041 |
| Sordaria macrospora       | F GSC 7821     | H om          | — AJ133007 Y10616 Y10616 |
| Sordaria brevicollis      | F GSC 9501     | H et          | — AJ133010 AJ133137 AJ133042 |
| Podospora anserina        | F GSC 3226     | H et          | — AJ133011 AJ133038 AJ133043 |

a If used in the present study. FGSC, Fungal Genetics Stock Center.
b Hom, homothallic; H et, heterothallic; Psh om, pseudohomothallic.
c Italized 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 F GSC 4628 (AF388922) was identical to that of F GSC 1739.
f The sequence of the ITS/5.8S region of N. discreta F GSC 3268 and 6794 (AF388916 and AF388917) and N. discreta-like F GSC 8318 and 8338 (AF388918 and AF388919) differed from that of F GSC 3228 at a maximum of only two bases, and the consensus sequence was identical to that of F GSC 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 (Pöggeler, 1999). Final PCR conditions were as follows: 200 μM dNTPs, 0.4 μM each primer (Operon Technologies Inc.), 1.0 unit of AmpliTaq 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 deoxyribonucleotide 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 characters of the combined alignment and heuristic searches, 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 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-phos- phate 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. bonaeren- sis 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.

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 ($\chi^2$, 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; $\chi^2$, 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>
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<tr>
<th>Gene</th>
<th>Number of taxa</th>
<th>Mean ingroup seq. divergence (%)</th>
<th>Number of nucleotides</th>
<th>Number of informative sites</th>
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<td>18</td>
<td>1.06</td>
<td>569</td>
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<tr>
<td>gpd</td>
<td>20</td>
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<td>433</td>
<td>48</td>
</tr>
<tr>
<td>mat A-1</td>
<td>19</td>
<td>5.40</td>
<td>481</td>
<td>141</td>
</tr>
<tr>
<td>mat a-1</td>
<td>16</td>
<td>5.37</td>
<td>372</td>
<td>78</td>
</tr>
<tr>
<td>Combined analysis</td>
<td>20</td>
<td>3.35</td>
<td>1855</td>
<td>278</td>
</tr>
</tbody>
</table>

* Uncorrected “p” distance. Ingroup = Neurospora and Gelasinospora taxa.

* 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.
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 divergent (uncorrected distance) between ingroup taxa and the mean of 3.35%. The ITS/5.8S rRNA region was sequenced from additional strains of Neurospora and Gelasinospora. 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 region, 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).

### TABLE 3

Results Summary of Partition Homogeneity Tests

<table>
<thead>
<tr>
<th>Partitions</th>
<th>P value*</th>
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<tbody>
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<td>ITS/5.8S:gpd:mat A-1:mat a-1</td>
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</tr>
<tr>
<td>ITS/5.8S:gpd:mat A-1</td>
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<td>gpd:mat A-1:mat a-1</td>
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<tr>
<td>ITS/5.8S:mat A-1:mat a-1</td>
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</tr>
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<td>ITS/5.8S:gpd</td>
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</tr>
<tr>
<td>gpd:mat A-1</td>
<td>0.67</td>
</tr>
<tr>
<td>gpd:mat a-1</td>
<td>0.03</td>
</tr>
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<td>mat A-1:mat a-1</td>
<td>0.31</td>
</tr>
<tr>
<td>1st:2nd:3rd codon positions</td>
<td>0.218</td>
</tr>
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</table>

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

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 Gelasinospora8239, 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).

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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 (Hulsenbeck 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 Gelasinospora8239 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.
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 Gelatinispora and Neurospora were investigated with nucleotide sequence data from four nuclear genes: ITS/5.8S ribosomal RNA region, glyceraldehyde 3-phos-
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-

<table>
<thead>
<tr>
<th>Topologically constrained tree with monophyly of</th>
<th>Number of steps</th>
<th>Difference in $-\ln L$ values$^a$</th>
<th>t</th>
<th>P value$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribbed ascospores (Fig. 3A)</td>
<td>628</td>
<td>168.479</td>
<td>5.492</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pitted ascospores</td>
<td>628</td>
<td>174.002</td>
<td>5.347</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pitted ascospores (including N. sublineolata)</td>
<td>600</td>
<td>26.660</td>
<td>1.711</td>
<td>0.0872</td>
</tr>
<tr>
<td>Outbreeding ingroup taxa</td>
<td>613</td>
<td>98.322</td>
<td>3.616</td>
<td>0.0003</td>
</tr>
<tr>
<td>Homothallic ingroup taxa (Fig. 3B)</td>
<td>615</td>
<td>101.548</td>
<td>3.732</td>
<td>0.0002</td>
</tr>
<tr>
<td>Pseudohomothallic ingroup taxa</td>
<td>658</td>
<td>299.751</td>
<td>9.202</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

$^a$ Between best unconstrained tree (593 steps, $-\ln L = 5943.359$) and constrained tree.

$^b$ Probability of obtaining a greater t value under the null hypothesis of no difference between trees.

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.
umber 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 Skupski 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 Geisleromyces arbuscorum² and N. sublineolata was in a well-supported clade with two other Geisleromyces 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 M inoura (Cain, 1961), the nonostiolate counterpart to the ostiolate genus Gelasinospora, because Furuya and U dagawa (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 < 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 Sordariaeae. 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 Sordariaeae. Similar examples can be found in other families of the Sordariales, such as Ceratostomataceae (Melanospora vs Persiciospora vs Sphaerodas; see Cannon and Hawksworth, 1982; Barr, 1990) and Triptosporaceae/Lasiothecaeeae (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-

Phylogenetic Relationships of Neurospora and Gelasinospora

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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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