Phylogenetic analysis of nuclear and mitochondrial rDNA sequences supports the view that loculoascomycetes (Ascomycota) are not monophyletic

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The loculoascomycetes are defined by the initiation of ascoma development prior to dikaryotisation and functionally bitunicate asci. Previous molecular studies suggested non-monophyly of loculoascomycetes and consequently, Chaetothyriomycetes and Dothideomycetes were distinguished. We have used sequences of nu SSU rDNA, nu LSU rDNA, and mt SSU rDNA to re-evaluate the monophyly of loculoascomycetes. 13 new sequences of these regions from 10 species, including two representatives of Pezizomycetes used as outgroup, were aligned with sequences obtained from GenBank. A combined data set was analysed phylogenetically using maximum parsimony. The Chaetothyriomycetes and Eurotiomycetes form a sister-group, supported by a bootstrap value of 97%, suggesting that loculoascomycetes are not monophyletic. A topology constrained to loculoascomycete monophyly can be rejected as being significantly worse using parametric bootstrapping. Our results also indicate that mt SSU rDNA sequence data are useful as additional characters to elucidate the phylogeny of ascomycetes at the rank of different classes. Additional data from more representatives of these groups and other ascomycetes are required to clarify whether the loculoascomycetes are a paraphyletic or polyphyletic assemblage.

INTRODUCTION

The loculoascomycetes are a group of fungi in which the ascoma development starts before dikaryotisation. A locule is formed in which ascogonia develop, while the aschyomennial fungi start their ascoma formation after the beginning of the production of ascogonia. The peridium of mature ascomata in loculoascomycetes is derived from the wall of the primordial locule, while the margin of ascomata in aschyomennial fungi develops during ascoma formation. Hence, the ascomata of the loculoascomycetes are often referred to as pseudothecia. In addition to their distinct ascoma development, loculoascomycetes are characterized by the predominant presence of functionally bitunicate asci and usually multiseptate, often pigmented, ascospores. Given these derived morphological features, loculoascomycetes were generally believed to form a monophyletic group and were recognized in several classifications as a supraordinal taxon, such as Ascoloculares (Nannfeldt 1932), Bitunicatae (Luttrell 1951), Loculoascomycetes (Luttrell 1955), or Loculoascomycetidae (Luttrell 1981). Traditionally the Chaetothyriales were included in the Loculoascomycetes (Barr 1987, Barr & Huhndorf 2000). Surprisingly, nuclear 18S rDNA sequence data did not support the monophyly of the loculoascomycetes (Berbee 1996, Haase et al. 1995, Silva-Hanlin & Hanlin 1999, Spatafora, Mitchell & Vilgalys 1995, Winka, Eriksson & Bång 1998), but suggested a closer relationship of the Chaetothyriales with the Eurotiomycetes or Lecanoromycetes. Subsequently, the Chaetothyriales were separated from the Dothideomycetes as a separate class Chaetothyriomycetes (Eriksson & Winka 1997). Phylogenetic analyses based on sequence data of the RPB2 gene (Liu, Whelen & Hall 1999) and the nuclear LSU rRNA gene (Lumbsch, Lindemuth & Schmitt 2000) supported the non-monophyly of the loculoascomycetes and also placed the Chaetothyriomycetes as sister-group to the Eurotiomycetes. However, such a sister-group relationship had only poor bootstrap support in all analyses and an alternative topology constrained to loculoascomycete monophyly could not be rejected using the Kishino-Hasegawa test (1989) in the latter two data sets (Liu et al. 1999, Lumbsch et al. 2000).

In connection with an ongoing study on the phylogeny of loculoascomycetes (cfr Lumbsch & Lindemuth 2001), we started to assess in the relationships of the Chaetothyriomycetes and Dothideomycetes. We have now employed an additional molecular data set, the mitochondrial SSU rDNA, to further evaluate the monophyly of loculoascomycetes and added this data set in a combined analysis together with nuclear SSU rDNA and LSU rDNA sequence data.
MATERIALS AND METHODS

Specimens and DNA extraction

New sequences were obtained from ten species as listed in Table 1. Total DNA was extracted from fresh culture material using a modified CTAB method (Armaleo & Clerc 1995, Cubero et al. 1999).

PCR Amplification

Dilutions (10⁻¹ up to 10⁻⁴) of the total DNA or undiluted DNA were used for PCR amplifications of the genes coding for the nuclear SSU and LSU rRNA, and the mitochondrial SSU rRNA. Primers (nu rDNA primer nomenclature follows Gargas & DePriest 1996) for amplification were: (1) for the nuclear SSU rDNA: nu-SSU-0021-5' (Gargas & DePriest 1996), nu-SSU-0819-5', nu-SSU-1293-3', nu-SSU-1750-3' (Gargas & Taylor 1992); (2) for the nuclear LSU rDNA: nu-LSU-0155-5' (= AL1R) (Döring et al. 2000), nu-LSU-0042-5' (= LR0R), nu-LSU-0654-5' (= LR3R), nu-LSU-1050-5' (= LR17R), nu-LSU-0635-3' (= LR3), nu-LSU-1125-3' (= LR6), nu-LSU-1432-3' (= LR7) (Vilgalys homepage), and the newly designed primer nu-LSU-973-5' (AGGTAA-AGCGAAATGATTAG); (3) for the mt SSU rDNA gene: mr SSU1 (Zoller et al. 1999) and MSU 7 (Zhou et al. pers. comm.). Cycle sequencing was executed with the following program: 25 cycles of 95 °C for 30 s, 48 °C for 15 s, 72 °C for 4 min. Sequencing products were precipitated and dried before they were loaded on an ABI Prism 377 (Perkin Elmer) automatic DNA sequencer. Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually adjusted.

Sequencing alignment

Sequences of 12 species (Table 1) were aligned. Preliminary multiple alignments were generated using Clustal W (Thompson, Higgins & Gibson 1994) and manually optimized. Missing data at the 5'- and 3'-end of partial sequences were coded by '?'. Major insertions were excluded. Portions of the alignment with ambiguous positions that may not be homologous were eliminated using the software program Gblocks (Castresana 2000). The three data sets were combined and their congruence was examined, employing the partition homogeneity test (Farris et al. 1994) as implemented in PAUP*. Invariant characters were excluded before applying the test as recommended by Cunningham (1997).

Phylogenetic analysis

The alignment was analysed using the PAUP* 4.0 software package (Swofford 1998). The polarity of characters was assessed using two representatives of Pezizomycetes as following sequencing primers were used: a) for the SSU rRNA gene: nu-SSU-0021-5' (Gargas & DePriest 1996), nu-SSU-0402-5', nu-SSU-0819-5', nu-SSU-1203-5', nu-SSU-0852-3', nu-SSU-1293-3', nu-SSU-1750-3' (Gargas & Taylor 1992), nu-SSU-1184-3' (Gargas, DePriest & Taylor 1995), and nu-SSU-0553-3' (White, Bruns & Taylor 1990); (2) for the LSU rRNA gene: nu-LSU-0155-5' (= AL1R) (Döring et al. 2000), nu-LSU-0042-5' (= LR0R), nu-LSU-0654-5' (= LR3R), nu-LSU-1050-5' (= LR17R), nu-LSU-0635-3' (= LR3), nu-LSU-1125-3' (= LR6), nu-LSU-1432-3' (= LR7) (Vilgalys homepage), and the newly designed primer nu-LSU-973-5' (AGGTAA-AGCGAAATGATTAG); (3) for the mt SSU rDNA gene: mr SSU1 (Zoller et al. 1999) and MSU 7 (Zhou et al. pers. comm.). Cycle sequencing was executed with the following program: 25 cycles of 95 °C for 30 s, 48 °C for 15 s, 72 °C for 4 min. Sequencing products were precipitated and dried before they were loaded on an ABI Prism 377 (Perkin Elmer) automatic DNA sequencer. Sequence fragments obtained were assembled with SeqMan 4.03 (DNAStar) and manually adjusted.

Table 1. Specimens of ascomycetes used for phylogenetic analysis with Genbank accession number (newly obtained sequences in bold).

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection</th>
<th>Class according to Eriksson (2000)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
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<tr>
<td>Aspergillus nigerus</td>
<td>CBS 451.66</td>
<td>Eurotiomycetes</td>
<td>AB008404 U28095 U29227</td>
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<td>Dothideomycetes</td>
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<tr>
<td>Eurotium javanicum</td>
<td>CBS 20437</td>
<td>Eurotiomycetes</td>
<td>AF346419 AF346420 AF346425</td>
</tr>
<tr>
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<td>Eurotiomycetes</td>
<td>AF346419 AF346420 AF346425</td>
</tr>
<tr>
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<td>incertae sedis</td>
<td>AF346419 AF346420 AF346425</td>
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<tr>
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<td>CBS 20437</td>
<td>Pezizomycetes</td>
<td>AF346426 U42642 U29227</td>
</tr>
<tr>
<td>Peziza succina</td>
<td>CBS 193.58</td>
<td>Pezizomycetes</td>
<td>AF346427</td>
</tr>
<tr>
<td>Sydnothidiolus puccinoides</td>
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<td>Pezizomycetes</td>
<td>AF346427</td>
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<td>Termatophyllum heterospora</td>
<td>CBS 244.86</td>
<td>Dothideomycetes</td>
<td>AF346428</td>
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<td>Westradylla cylindrica</td>
<td>CBS 454.72</td>
<td>Dothideomycetes</td>
<td>AF346429</td>
</tr>
</tbody>
</table>
Multi-gene analysis of loculoascomycetes

outgroup, which appear as a basal group to other filamentous ascomycetes in recent molecular studies (Gargas & Taylor 1995, Liu et al. 1999, Lumbsch et al. 2000). A maximum parsimony (MP) tree was inferred with PAUP* using the branch and bound search option. Nonparametric bootstrap support (Felsenstein 1985) for each clade was tested based on 5000 replications, using the branch and bound search option. Phylogenetic trees were drawn using TREEVIEW (Page 1996). The consistency index (CI; Kluge & Farris 1969), retention index (RI; Farris 1989), and rescaled consistency index (RC; Farris 1989) were obtained from PAUP*.

Parametric bootstrapping was employed to check whether an alternative topology constrained to loculoascomyecete monophyly could be rejected as being significantly worse than the MP tree. The constrained MP tree was parameterized using maximum likelihood (ML), assuming the Tamura & Nei (1993) model with rate heterogeneity among sites (TrN + Γ).

We obtained sequences of mt SSU rDNA varying in length between 787 bp in Capronia mansonii and 1003 bp in Trematosphaeria heterospora, nu SSU rDNA of 1751 bp in Glyphium elatum and 1801 bp in Ceramothyrium carniolicum, and nu LSU rDNA of 891 bp in Peziza succosa and 1409 bp in Glyphium elatum (including insertions). Approximately 98% of

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**RESULTS**

We obtained sequences of mt SSU rDNA varying in length between 787 bp in Capronia mansonii and 1003 bp in Trematosphaeria heterospora, nu SSU rDNA of 1751 bp in Glyphium elatum and 1801 bp in Ceramothyrium carniolicum, and nu LSU rDNA of 891 bp in Peziza succosa and 1409 bp in Glyphium elatum (including insertions). Approximately 98% of

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![Fig. 1](image-url) One of two most parsimonious trees obtained from a branch-and-bound search using PAUP*. Bootstrap support values are shown at nodes. The branch with an asterisk collapses in the strict consensus tree. Classes according to Eriksson & Winka (1997) are indicated at the margin.
the nu SSU and LSU sequence lengths were sequenced in both directions in all species, except *Ceramothyrium carniolicum*—nu SSU with 88%. For mt SSU sequences at least 57% were obtained in both directions. Major insertions were present in the nu SSU gene of *C. carniolicum* and the mt SSU genes of *Trematosphaeria heterospora* and *Westerdykella cylindrica*; these were excluded from the analyses.

Sequences of the 12 taxa were aligned to produce a matrix of 992 characters for the mt SSU, 1709 for the nu SSU, and 1404 nucleotide-position characters for the nu LSU data set. Gblocks selected conservative blocks for phylogenetic analysis from the combined data set with 91% of the original positions. The alignment is available in TreeBASE under accession no. S615 (http://herbaria.harvard.edu/treebase/). The partition homogeneity test revealed that the three data sets are congruent and can be analysed in combined analyses (P = 0.22). The data set examined included 700 parsimony-informative sites. A MP search revealed two most parsimonious trees (Fig. 1) of 1937 steps length, with CI = 0.66, RI = 0.66, and RC = 0.48. The two trees differed only in the phylogeny within the *Chaetothyriomycetes* which appears as a non-resolved polytomy in the strict consensus tree.

The three representatives of the *Eurotiomycetes* cluster together with 100% bootstrap support, and also the *Chaetothyriomycetes* form a group supported with 100% bootstrap support, including *Glyphium*. These two clades appear as sister-groups in the MP tree with 97% bootstrap support. The *Chaetothyriomycales/Eurotiomycetes* clade is a sister-group of the *Dothideomycetes*. The four taxa of *D. elatum* form a clade with 87% bootstrap support. Two of the included *Dithidioomycetes* are aporaphysate (*Capnodiun* and *Stylolothis*) and two have pseudoparaphyses (*Trematosphaeria* and *Westerdykella*). These two groups form well-supported sister-groups (98%, resp. 100% bootstrap support) within the *Dithidioomycetes*.

The single MP tree enforced to loculoascomycete monophyly had a length of 1960 steps, thus being 23 steps longer than the unconstrained MP tree. A parametric bootstrap analysis revealed that the monophyly of the loculoascomycetes could be rejected as being significantly worse (P = 0.022) than the unconstrained tree that places the *Chaetothyriomycetes* as sister-group to *Eurotiomycetes*.

To evaluate the potential presence of long-branch attraction in our analysis we performed a χ²-test and a relative-rate test. All sequences included in the study passed the χ²-test (P = 0.83–0.96 for *Chaetothyriomycetes*, P = 0.56–0.94 for *Eurotiomycetes*, and P = 0.70–0.99 for *Dithidioomycetes*) including the outgroup (P = 0.10 for *Marchella* and P = 0.76 for *Peziza*), indicating that none of the sequences had a significantly deviating nucleotide composition. The results of the relative-rate tests showed that the three ingroups included in this study (*Chaetothyriomycetes*, *Dithidioomycetes*, *Eurotiomycetes*) do not differ significantly in their substitution rate. The results were not significant in all three cases examined (P = 0.0553 for *Chaetothyriomycetes* vs. *Dithidioomycetes*, P = 0.0583 for *Chaetothyriomycetes* vs. *Eurotiomycetes*, P = 0.283 for *Dithidioomycetes* vs. *Eurotiomycetes*).

The genus *Glyphium* was listed in ‘*Chaetothyriales*/*D. incertae sedis*’ by Eriksson (2000), indicating that its placement was uncertain. Previous authors have placed the genus into orders which are currently classified in the *Dothideomycetes*, e.g. *Dothiorales* (Zogg 1962), *Hysteriales* (Luttrell 1973), or *Melanommatiales* (Barr 1987). In our molecular analysis, *G. elatum* clustered with two *Chaetothyriomycetes*.

**DISCUSSION**

The results of our combined analysis of nuclear SSU and LSU rDNA and mitochondrial SSU rDNA sequence data show that the loculoascomycetes are not monophyletic, as already suggested by molecular studies using single-gene approaches (Berbee, 1996, Haase et al. 1995, Liu et al. 1999, Lumbsch et al. 1999, Silva-Hanlin & Hanlin 1999, Spatafora et al. 1995, Winka et al. 1998). But additionally in this multi-gene approach such a relationship is well supported and the monophyly of the loculoascomycetes can be rejected based on parametric bootstrapping. However, it should be noted that the taxon sampling in our study is restricted to a few species and certainly a much broader sampling is required to evaluate the variation within the different classes. Especially the *Dithidioomycetes* is a species-rich group and therefore it remains uncertain whether the *Dithidioomycetes* is a sister-group to *Chaetothyriomycetes/Eurotiomycetes* as in our study or whether the *Dithidioomycetes* itself is paraphyletic at a basal position to the *Chaetothyriomycetes/Eurotiomycetes* clade as in the MP tree by Berbee (1996). The distinction of taxa lacking pseudoparaphyses and the pseudoparaphyseate *Pleosporales* within the *Dithidioomycetes* agrees with previously published results (Berbee 1996, Liew, Aptroot & Hyde 2000, Lumbsch & Lindemuth 2001), but again the sampling is too small to permit any conclusions drawn from this study.

Our results suggest that the ascomromatic development of loculoascomycetes cannot be used as a synapomorphy for a group of fungi. It remains uncertain, however, whether the loculoascomycetes is a paraphyletic or polyphyletic assemblage. Given the special morphological characters, such as the ascoma development, bitunicate ascus-type, or predominance of septate and pigmented ascospores, it may be more plausible to suppose a paraphyly of loculoascomycetes. However, as long as the branching order of classes within filamentous ascomycetes is unclear, this question will remain unknown. We hope that additional sequence data from less conservative genes, such as mitochondrial SSU rDNA, of additional classes of *Pezizomycotina* will help to answer this open question.

The non-monophyly of loculoascomycetes raises the question of how to distinguish members of the *Chaetothyriomycetes* and *Dithidioomycetes* morphologically. The *Chaetothyriales* were resurrected by Barr (1976, 1979, 1987) and the main characters for the distinction of this group included the presence of periphysoids, sometimes amyloid reaction of the hymenial gel, and often asymmetric ascospores. The only consistent key character for the recognition of *C. simoni*us (Barr 1987) was the presence of periphysoids. However, this character was shown to be of limited use in loculoascomycetes, since periphysoids in...
different members of that group do not appear to be homologous (Reynolds 1998, Winka et al. 1998). At the moment taxa with uncertain relationship in the loculoascomycetes can only be placed unequivocally in one of the two classes using molecular markers. Morphological and chemical characters are poorly known and additional investigations together with molecular studies are urgently needed to further evaluate the distinction of the two classes of ascolocular fungi.

The placement of *Glyciphium elatum* in the Chaetothyriomycetes with 100% bootstrap support remains uncertain as long as the identity of the culture is not confirmed. Notes on the original culture and the referring specimen are given in Lohman (1933) and indicate a correct determination. However, it is uncertain whether the culture today still represents that species.

Sequence data of mt SSU rDNA have been mainly used in ascomycete systematics at the level of species complexes and for intraspecific problems (Hong et al. 2000, Li, Tam & Hartman 2000, Nikoh & Fukatsu 2000, Zhou et al. pers. comm.). However, our results show that data from this mitochondrial gene may also be helpful in phylogenetic questions at higher ranks in ascomycetes, even between different classes, if used in combination with more conservative genes, such as the nuclear ribosomal gene. This is in agreement with results from phylogenetic studies on basidiomycetes (Hibbett et al. 1997, Pine, Hibbett & Donoghue 1999).

The well-supported (97% bootstrap support) clade including Chaetothyriomycetes and Eurotiomycetes in our multi-gene study is remarkable, since most single-gene studies fail to resolve any relationships above the rank of classes (as circumscribed by Eriksson & Winka 1997) in the Pezizomycotina. Separate analysis of the three data sets (and combinations of two of these) all supported a sister-group relationship of Chaetothyriomycetes and Eurotiomycetes (results not shown), but single-gene approaches failed to reject loculoascomycete monophyly statisitcally.

Although a sister-group relationship of Chaetothyriomycetes and Eurotiomycetes has been documented repeatedly in molecular studies (Tehler et al. 2000, Winka 2000), such a close relationship has never been proposed based on morphological studies and obvious morphological synapomorphies are unknown. Therefore Winka (2000) suggested that this relationship might be due to long-branch attraction (Felsenstein 1978). If two unrelated lineages have had an accelerated substitution rate compared to other groups included in a study, they will have accumulated characters that will distance them from other taxa in an analysis, resulting in a false clustering based on convergencies (Swoford et al. 1996). However, the results of the $\chi^2$-test and the relative-rate tests reject such an assumption. The Eurotiomycetes and Chaetothyriomycetes do not differ significantly in their nucleotide composition and substitution rate from the Dothideomycetes. Therefore, long-branch attraction cannot be considered as being of major importance in this case. The lack of morphological support for the close relationship of Chaetothyriomycetes and Eurotiomycetes, however, is a major concern and should stimulate additional morphological research in these two groups of ascomycetes.

In our study, only the combination of the three data sets yielded the resolution power to reject an alternative topology (monophyly of loculoascomycetes) on a statistical basis. This makes us confident that the lack of resolution in single-gene studies (cf. Gargas & Taylor 1995, Liu et al. 1999, Lumbsch et al. 2000) concerning the relationships of the different classes currently recognized in the Pezizomycotina may not be due to a rapid and ancient radiation of filamentous ascomycetes, but to a lack of resolution attributable to the analysis of individual genes. If this were true, multi-gene approaches could overcome these difficulties.

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REFERENCES


