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Phylogeny of Discomycetes and Early Radiations of the Apothecial Ascomycotina Inferred from SSU rDNA Sequence Data[1]

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GARGAS, A., AND TAYLOR, J. W. 1995. Phylogeny of discomycetes and early radiations of the apothecial Ascomycotina inferred from SSU rDNA sequence data. *Experimental Mycology* **19**, 7–15. We used nucleotide sequences of the small subunit ribosomal genes (SSU rDNA) to examine evolutionary relationships of apothecial ascomycetes (division Ascomycota; class Discomycetes *sensu*), commonly known as the cup fungi. The apothecial ascomycetes include both lichen-forming and free-living fungi. We sequenced the SSU rDNA from representatives of 10 fungal genera from four orders: Pezizales (*Ascobolus lineolatus, Morchella elata* agg., *Peziza badia*); Leotiales (*Leotia lubrica, Sclerotinia sclerotiorum*); Caliciales (*Calicium tricolor, Mycocalicium albonigrum, Sphaerophorus globosus*); and Lecanorales (*Lecanora dispersa, Porpidia crustulata*). Of these, *C. tricolor, S. globosus, L. dispersa,* and *P. crustulata* are lichen-forming fungi. Based on parsimony analyses of approximately 1750 aligned nucleotides of their SSU rDNA, we determined a most parsimonious tree (MPT). This hypothesis suggests that the apothecial ascomycetes are a paraphyletic assemblage, basal to other groups of filamentous ascomycetes including representatives of the perithecial fungi and cleistothecial fungi. The most parsimonious tree produced using this dataset supported the monophyly of the orders Pezizales, Leotiales, and Lecanorales. However, there was no support for monophyly of the representative Caliciales; *S. globosus* had affinities with members of the Lecanorales. This phylogenetic hypothesis recognizes Pezizales as basal and supports Nannfeldt's hypothesis (1932) of a primitive apothecial ascomata with subsequent evolution of perithecial and cleistothecial forms. This MPT provides a foundation for understanding evolution of the ascomycetous fungi.

INDEX DESCRIPTORS: fungi; Ascomycetes; discomycetes; lichen-forming; apothecia; 18S rDNA; SSU rDNA; small subunit ribosomal DNA; molecular evolution; phylogeny.

Fungi that produce cup-shaped ascomata (apothecial ascomycetes) are commonly known as the cup fungi or Discomycetes. The placement of this group within the filamentous ascomycetes is important since the apothecial fungi represent almost 9000 of the 28,000 described ascomycete species (Hawksworth *et al.*, 1983). There is no historical consensus on the evolutionary relationships within the apothecial ascomycetes, nor on their relationships to other fungi. Most of the apothecial ascomycetes (around 6000 spp.) are lichen-forming fungi in symbiotic relationships with green algae or cyanobacteria. The 13,000 species of lichen-forming ascomycetes, including members of the apothecial ascomycetes as well as members of other classes, constitute nearly half of the described species of ascomycetes. These fungi have been treated separately from pathogenic and free-living fungi, but must be considered to produce a comprehensive phylogeny of the ascomycetes. To address the relationships of the apothecial ascomycetes, we sequenced

[1] Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. L35732–L35741.

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the small subunit rDNA gene (SSU rDNA)[3] from representatives of 10 species and used these nucleotide sequences to produce a phylogenetic hypothesis.

Classification schemes for apothecial ascomycetes have been reviewed by Kimbrough (1970) and for all ascomycetes by Hawksworth (1985). Based on reproductive structures of open, simple cups in contrast to the closed structures of the cleistothecial ascomycetes or the flask-shaped structures of the perithecial ascomycetes, mycologists have variously proposed that the cup fungi constitute a monophyletic group of recent origin, an ancestral group, or several independent groups. Korf and Eriksson have differed as to whether or not the Discomycetes are monophyletic. Korf (1973) placed the apothecial ascomycetes in the single class "Discomycetes." Eriksson (1981), in an outline of the ascomycetes, classified the apothecial ascomycetes into groups of one to several families. Eriksson concluded that without a comprehensive investigation of their morphology and ontogeny, the ascomycetes could not be arranged into classes. Even when morphology and ontogeny were well known, interpretation was difficult. Eriksson (1981) focused attention on the orders of ascomycetes and discouraged the use of supraordinal taxa; the Dictionary of Fungi (Hawksworth *et al.*, 1983) and Outline of Ascomycetes (Eriksson and Hawksworth, 1993) present the ascomycete fungi without class distinctions.

To examine monophyly of the morphologically defined class discomycetes, as well as orders within this group, we have sought a source of characters independent from their morphology. An independent test of phylogenetic hypotheses based on morphological characters can be provided by molecular data. For example, recent analyses using SSU rDNA sequence data (Berbee and Taylor, 1992) from ascomycetes with closed ascomata (cleistothecia) and flask-shaped ascomata (perithecia) have suggested that these are useful phylogenetic characters, and that the classes based on these characters, Plectomycetes and Pyrenomycetes, respectively, are supported as monophyletic lineages. Landvik *et al.* (1993) included members of the Leotiales and Pezizales, but found no clear monophyly for either group. Saenz *et al.* (1994) showed the apothecial ascomycetes to be a basal assemblage which included the powdery mildew *Blumeria graminis* (Erysiphales). The first analysis to include SSU rDNA from lichen-forming fungi (Gargas, 1992) also found the Caliciales, Lecanorales, Leotiales, and Pezizales to be a basal assemblage within the filamentous ascomycetes, although monophyly of each of these groups was not rejected by maximum likelihood analysis of the SSU rDNA dataset.

In the present study we have used SSU rDNA sequences to test the monophyly of the class Discomycetes, those fungi which possess apothecia. We selected taxa of apothecial ascomycetes that would address the following questions: (1) within all ascomycetes, are the filamentous forms supported as a single lineage? (2) which type of ascoma should be considered ancestral within the ascomycetes? (3) within the nonyeast ascomycetes, are the apothecial ascomycetes supported as monophyletic and separate from the cleistothecial and perithecial ascomycetes? and (4) within the apothecial ascomycetes, are representatives of the four orders Pezizales, Caliciales, Leotiales, and Lecanorales each supported as monophyletic?

MATERIALS AND METHODS

Isolates

We sequenced the SSU rDNA from 10 fungal species representing four orders of

[3] Abbreviations used: PCR, polymerase chain reaction; MPT, most parsimonious tree; SSU, small subunit.

ascomycete apothecial fungi. One species from a representative genus for each order was sequenced, as well as one or two other species from genera considered to be within the order. The species sequenced from each order were: Pezizales—*Ascobolus lineolatus* Brumm., *Morchella elata* agg., *Peziza badia* Pers.; Leotiales—*Leotia lubrica* Pers.:Fr., *Sclerotinia sclerotiorum* (lib. DeBary); Caliciales—*Calicium tricolor* F. Wilson, *Mycocalicium albonigrum* (Nyl.) Tibell, *Sphaerophorus globosus* (Huds.) Vain.; and Lecanorales—*Lecanora dispersa* (Pers.) Sommerf., *Porpidia crustulata* (Ach.) Hertel and Knoph (syn: *Lecidea crustulata*).

DNA Extraction and Amplification

We used standard fungal miniprep protocols (Lee and Taylor, 1990; White *et al.*, 1990) to extract the total DNA from fungal cultures, freshly collected material, or herbarium specimens. From dilutions (10^{-1}, 10^{-2}) of this total DNA we specifically amplified the fungal nuclear SSU rDNA using polymerase chain reaction (PCR) primers designed to favor the amplification of fungal over green-algal DNA (NS17UCB-NS24UCB; Gargas and Taylor, 1992). The initial PCR amplification ran for 30 cycles (each cycle was for 2 min at 97°C, 1 min at 48°C, 45 s at 72°C with a 4 s/cycle extension at 72°C). A second PCR amplification of 30 cycles was used to obtain either single-stranded (Gyllensten and Erlich, 1988) or double-stranded DNA products (Kusukawa *et al.*, 1990) for sequencing by the dideoxy-labeling method (TAQuence kit; U.S. Biochemical Corp., Cleveland, OH).

Sequencing Reactions

We used the PCR primers NS2-NS7 (White *et al.*, 1990), UCBNS17-UCBNS24 (Gargas and Taylor, 1992), MB2 (the complement of UCBNS23; M. Berbee, personal communication), and CNS26 (TCGAA AGTTG ATAGG GCAG; gift of B. Bowman) to sequence both the coding and the noncoding strands.

To confirm the identity of the fungal DNA being sequenced, sequence data for a diagnostic region was obtained from at least one related species, or the fungal sequence was compared to that obtained by another researcher (S. Landvik, personal communication). The SSU rDNA sequences from the 10 fungi have been archived in GenBank.

Sequence Alignment and Construction of Phylogenetic Trees

The sequences were aligned with the following fungi (with GenBank accession Nos.): *Saccharomyces cerevisiae* Meyen *ex* Hansen (J01353, M27607) (Rubtsov *et al.*, 1980); *Neurospora crassa* Shear and Dodge (X04971) (Chambers *et al.*, 1986); *Ascosphaera apis* (Maasen *ex* Claussen) Olive *ex* Spiltoir (M83264), *Byssochlamys nivea* Westling (M83256), *Chaetomium elatum* Kunze (M83257), *Eremascus albus* Eidam (M83258), *Leucostoma persoonii* Höhn (M83259), *Ophiostoma ulmi* (Buism.) Nannf. (M83258), and *Thremoascus crustaceus* (Apinis and Chesters) A. C. Stolk (M83263) (Berbee and Taylor, 1992); *Eurotium rubrum* König, Spieckermann, and W. Bremer (U00970), *Pleospora rudis* Rabenh. *ex* Ces. and de Not. (U00975) (Berbee and Taylor, 1993); *Cudonia confusa* Bres. (Z30240), *Gryromitra esculenta* (Pers.) Fr. (Z30238), *Inermisia aggregata* Berk. and Broome (Z30241), *Neolecta vitellina* (Bres.) Korf and J. K. Rogers (Z27393), *Plectania nigrella* (Pers.:Fr.) P. Karst (Z27408), and *Spathularia flavida* Pers. (Z30239) (Landvik *et al.*, 1993); *Blumeria graminis* (DC.) Speer f. sp. *hordei* (L26253) (Saenz *et al.*, 1994).

We aligned the sequences with the PileUp computer program (Genetics Computer Group, Madison, WI) and hand-corrected this alignment. *S. cerevisiae* and *N. vitellina* were chosen as the outgroups

based on the results of Landvik *et al.*, 1993. We used maximum-parsimony analysis (Camin and Sokal, 1965; PAUP 3.1, Swofford, 1991) to determine a single most parsimonious tree (MPT) from this dataset. We resampled the alignment 500 times using bootstrap (Felsenstein, 1985) to derive 500 replicate datasets. Each of these datasets was subjected to maximum-parsimony analysis to determine a MPT. From the MPTs of these replicate datasets, we calculated the percentage of MPTs which had a particular branch as an assessment of support for each internal branch.

RESULTS

We sequenced over 1600 nucleotides, or over 90%, of SSU rDNA from each of 10 taxa currently recognized as members of the four orders Caliciales, Lecanorales, Leotiales, and Pezizales. These four orders reflect the diversity of morphologies and nutritional modes (saprobic, parasitic, and lichen-forming) found within the apothecial ascomycetes. The nucleotide sequences were obtained by direct sequencing of PCR products from specific amplifications of the SSU rDNA. To assure the accuracy of this data, both strands of the SSU rDNA were sequenced (except for a short region near the 5′ and 3′ ends, close to primers UCBNS17 and UCBNS24). These nucleotide sequences were comparable to those of the SSU rDNA reported for other fungi from the division Ascomycota. Although most eukaryotic SSU rDNA sequences have been approximately 1800 nucleotides long, the PCR products from the isolates *M. albonigrum, L. dispersa* and *P. crustulata* were longer, with *L. dispersa* nearly double the expected length. Upon sequencing, we found that the increase in length was due to discrete insertions of 78–388 nucleotides. A total of 15 insertions were found with between 1 and 8 insertions per isolate (Gargas *et al.*, 1995), several of these insertions being group I introns (Gargas and Damberger, manuscript in preparation).

Parsimony Analysis Supports the Orders Pezizales, Leotiales, and Lecanorales

To test for monophyly of various groups within the ascomycetes, our alignment included the apothecial ascomycetes with other representative ascomycetes. This alignment of 1750 nucleotides had a total of 496 variable sites and 307 informative sites. Initial analyses showed that the sequence of *C. tricolor* had many apomorphic changes in its SSU rDNA sequence which caused "long-branch-attraction" problems (leading to spurious convergences); for this reason *C. tricolor* was excluded from the determination of the MPT. The MPT (Fig. 1) required 1095 steps; adding one step produced 9 equally parsimonious trees; adding two steps added 28 equally parsimonious trees. Each branch supported by bootstrap at greater than 50% (derived from 500 bootstrap replications) are indicated in Fig. 1. This analysis suggests that the apothecial ascomycetes radiated early in the evolution of the filamentous ascomycetes and that cleistothecial and perithecial forms were derived from within this lineage. The group which includes apothecial ascomycetes is separated by 35 synapomorphies from the outgroups *N. vitellina* and the yeast *S. cerevisiae*. The order Pezizales is supported by 17 synapomorphies, the order Leotiales is supported by 10 synapomorphies, including the Erysiphales (powdery mildew) representative *B. graminis*. The cleistothecial and perithecial ascomycetes are each supported by 41 and 43 synapomorphies, respectively, a result consistent with the conclusions of Berbee and Taylor (1992).

DISCUSSION

Analysis for Monophyly of Each of the Orders

Phylogenetic analysis of the SSU rDNA sequences from representative asco-

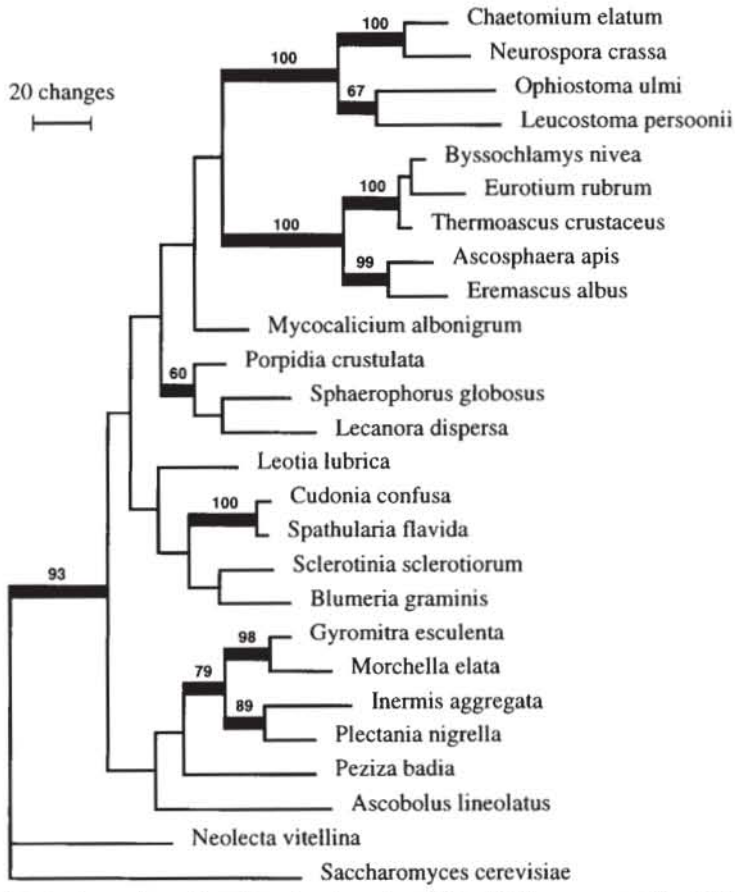

FIG. 1. The single most parsimonious tree based on SSU rDNA sequence data (1750 aligned nucleotides, 1097 steps, consistency index = 0.604). Bootstrap percentages >50% based on 500 replications are shown over thickened branches. Distance is relative to line length (scale on figure).

mycetes suggests that the class "Discomycetes" is paraphyletic and basal to the cleistothecial and perithecial fungi. However, the same analysis supported most of the traditional orders, recognized by characteristic ascomata and ascal tip structures, as monophyletic. The order Lecanorales is monophyletic if the taxon *S. globosus* (formerly Caliciales) is included; the order Leotiales is supported as paraphyletic, including the powdery mildew *B. graminis* (formerly Erysiphales). Taxon sampling is critical to the resolution of monophyly of each order in parsimony analyses; analyses which sampled fewer taxa, or a less diverse range of taxa within each order, did not consistently recognize each order as monophyletic. The basal taxa within each order (i.e., *A. lineolatus* or *Le. lubrica*) having many apomorphic changes, were subject to long branch attraction to taxa in other orders. The inclusion of several diverse taxa within each order reduced these long branches to a series of hierarchical internodes and resolved the clades. With proper taxon sampling we may be able to include problem taxa such as the long-branched *C. tricolor*.

The order Pezizales or true cup fungi includes species with minute, simple cups as in *A. lineolatus*, larger cups such as those in *Pe. badia*, as well as species with large,

convoluted, ascocarps such as *Mo. elata* and *G. esculenta*. Their asci are usually operculate, though this is not evident in all members. Typically the Pezizales are saprobic, though some form mycorrhizal associations. The MPT shows support for the Pezizales representatives as a monophyletic group. Bootstrapping supports the sister taxa relationships of *G. esculenta* and *Mo. elata*, of *I. aggregata* and *Pl. nigrella*, as well as a group of all four of those taxa; relationships predicted by ultrastructural characters (J. Kimbrough, personal communication). The MPT places *A. lineolatus* basal to the other Pezizales, its branch includes 63 apomorphic changes. As the most basal of the Pezizales, *A. lineolatus* should be examined for characters it may share with the basal members of other apothecial orders, such as simple ascomata. The branch leading to the Pezizales including *A. lineolatus* was not supported by bootstrapping, as is typical for clades including taxa with many apomorphic changes.

The Leotiales produce small, fleshy apothecia whose asci typically open with a simple pore. This group includes saprobic fungi and also many important plant pathogens such as species of *Sclerotinia*. The branch leading to this assemblage of Leotialean fungi was supported in the MPT, yet bootstrapping supported only the branch leading to *Cu. confusa* and *Sp. flavida*, consistent with the results of Landvik *et al.* (1993). Interestingly, the powdery mildew (formerly Erysiphales) *B. graminis* grouped with *Sc. sclerotiorum*, a result consistent with Saenz *et al.* (1994). Further research should address whether other Erysiphales have their closest relatives within the Leotiales. The most basal taxon in Leotiales was the type-genus *Leotia*. Previous analyses, including fewer Leotialean taxa, have had difficulties resolving *Leotia* as monophyletic with other members of this order (Gargas, 1992; Landvik *et al.*, 1993; Saenz *et al.*, 1994). Probably the large number of apomorphic changes resulted in long branch attraction between *Le. lubrica* and other nonLeotialean taxa.

The Caliciales are a group of less than 400 species of mostly lichen-forming fungi characterized by stalked ascomata or mazaedia. Their asci are simple, or prototunicate, and the ascospores are released passively in a powdery mass. We included these fungi to test whether mazaedia are a derived character that can delineate this group. Representatives of the lichen-forming *S. globosus*, *C. tricolor*, and the saprobic *M. albonigrum* were sequenced from this group. The MPT was determined without *C. tricolor*, as long branch attraction made the position of *C. tricolor* ambiguous. The MPT placed *S. globosus* as a sister taxon to *L. dispersa* within the Lecanorales; *M. albonigrum* was placed on a lineage between the Lecanorales and cleistothecial and perithecial ascomycetes. The MPT does not support recognition of the Caliciales including *S. globosus*. When we used the MPT as a backbone constraint (PAUP 3.1.1) to determine the most parsimonious placement of *C. tricolor*, *C. tricolor* was a sister taxon to *Le. lubrica* with a long branch of 87 autapomorphies. We consider the placement of *C. tricolor* to be ambiguous as the result of its many autapomorphies. Careful taxon sampling of more representatives of the Caliciales is critical to further testing of these hypotheses. The MPT suggests that mazaedia presence is not sufficient to delimit this group, and may have arisen multiple times within the ascomycetes. Kimbrough (1970) classified the caliciaceous fungi as the family Caliciaceae within the Helotiales (here considered to be Leotiales), placement of *C. tricolor* in the Leotiales could not be excluded based on its SSU rDNA sequence.

The Lecanorales is a large and diverse order containing most of the lichen-forming fungi. Lichen thalli from this group may be crustose, foliose, or fruticose. Lecanorales has the greatest diversity of ascal types of

the four orders examined; some elaborate asci have been described as "nearly tritunicate" (Hawksworth, 1985). The MPT shows that *L. dispersa* and *P. crustulata* are monophyletic, if one includes the caliciaceous *S. globosus*. Although *S. globosus* produces mazaedia, it has asexual characters similar to those found in the Lecanorales (J. Hafellner and L. Tibell, personal communication). Other members of the Sphaerophoraceae should be examined to see if they should be recognized as members of the Lecanorales.

Evolution of Ascomata within the Filamentous Ascomycetes

The MPT suggests that the apothecial ascomycetes diverged after the outgroups represented by *N. vitellina* and the yeast *Sa. cerevisiae*. The distinction of representatives of *N. vitellina* as separate from the other ascomycetes is supported by their lack of paraphyses and the unusual staining of their asci. This analysis suggests that paraphyses evolved within the ascomycetes before divergence of the apothecial forms and brings into question whether the ancestral ascomycetes were yeast-like or filamentous. The position of the apothecial ascomycetes as paraphyletic and ancestral to the cleistothecial and the perithecial fungi supports the hypothesis that fungi with relatively undifferentiated apothecia gave rise to derived cleistothecia and perithecia as proposed by Nannfeldt (1932). Nannfeldt suggested a gradual transition between apothecia and perithecia, and transitory forms may be identified when more taxa of intermediate positions are included in molecular studies. The Eurotiales are a derived group within the filamentous ascomycetes even though they have many reductions in morphology such as naked ascogonia and simple asci. The derived state of perithecia from apothecia differs from the hypothesis that cleistothecia were basal to perithecia and that the open apothecia of the discomycetes arose from within perithecial forms (Luttrell, 1955), but has been supported by other recent molecular analyses (G. Haase, personal communication).

The MPT suggests that within the apothecial ascomycetes the Pezizales are basal, and this group may have retained traits which are primitive for the ascomycetes. The Pezizales are distinguished by operculate asci and ascospores, which are single-celled and often highly ornamented. Developmentally, the Pezizales lack spermatia and the vegetative primordia within which ascogonia develop, further supporting a separation of he Pezizales from the inoperculate discomycetes and from the perithecial ascomycetes (Kimbrough, 1981). Within this phylogenetic context evolution of lifestyle may be considered. It is interesting that the Pezizales contain few plant parasites. If the habit of parasitism arose, perhaps many times, in other lineages, it is reasonable that basal taxa would retain an ancestral saprobic lifestyle.

Based on the MPT the lichen habit arose in the ascomycetes after the divergence of Pezizales and Leotiales. It is likely that the lichen habit was acquired separately in other groups such as the Arthoniales (A. Tehler, personal communication). Lichenologists have recently used phylogenetic methods to examine relationships of the lichen-forming fungi. Hafellner's (1988) classification incorporated both free-living and lichen-forming fungi. Using ascal characters Hafellner grouped the Leotiales (as Helotiales), Caliciales, and Pezizales (with functionally unitunicate asci), as separate from the Lecanorales (which have ascal tips with internal apical beaks). The MPT is consistent with Hafellner's grouping. Both Hafellner (1988) and Tehler (1988, 1990) treated the lichen-forming fungi along with saprobic and parasitic fungi and concurred that the lichen-forming habit was widespread within the ascomycetes and could

not be used to separate these fungi into a monophyletic group.

CONCLUSION

The most parsimonious tree based on SSU rDNA gene sequences implies an early radiation of apothecial fungi within the filamentous ascomycetes (Fig. 2). The cleistothecial and perithecial ascomycetes have undergone more recent radiations. *N. vitellina* represents a basal lineage which is distinct from the apothecial ascomycetes. The MPT supports monophyly for the orders Pezizales and Leotiales and the Lecanorales (including *S. globosus*). The Caliciales as currently recognized (Tibell, 1984) could not be supported as monophyletic; we propose that this group may need redefinition. With this view of the apothecial fungi as an unnatural assemblage, we are free to form new hypotheses of ascomycete evolution. Building on this stable phylogeny for the ascomycetes, which recognizes morphologically supported orders, we can extend our analyses to include problematic taxa such as those with highly derived and reduced morphology or those that lack teleomorphic states. The inclusion of more taxa from other diverse groups of ascomycetes will reveal other details of fungal evolution.

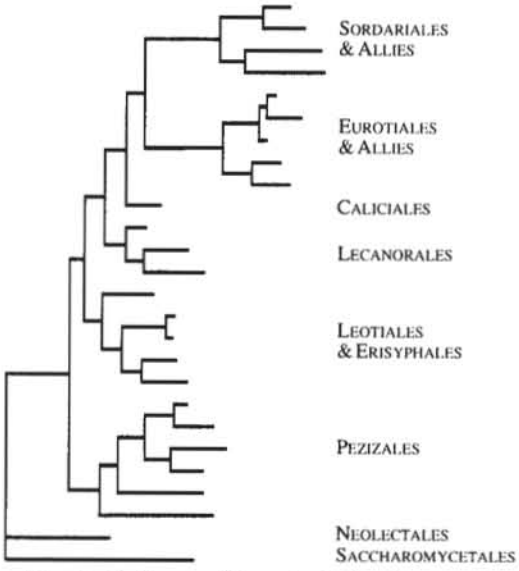

FIG. 2. Phylogeny of the subdivision Ascomycotina suggested by the most parsimonious tree from SSU rDNA sequences. This phylogenetic hypothesis supports the traditional orders Pezizales, Leotiales, and Lecanorales (including *S. globosus*) and suggests that Erysiphales is within the Leotiales. The apothecial ascomycetes are paraphyletic and basal to the perithecial and cleistothecial ascomycetes which are of comparatively recent divergence.

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