Two Ascomycete Classes Based on Fruiting-Body Characters and Ribosomal DNA Sequence[1]

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Traditional fruiting body–based classification of ascomycetes has been under attack for 2 decades. Fruiting-body types can converge, and few researchers now assume that either the closed fruiting bodies (cleistothecia) characterizing the class Plectomycetes or the flask-shaped fruiting bodies (perithecia) characterizing the class Pyrenomycetes are stable, unifying characters. Unless we identify characters uniting major ascomycete groups, orders of ascomycetes remain narrowly defined, and supraordinal classification is impossible. We sequenced both strands of 18s rDNA from nine ascomycete fungi, adding three sequences from GenBank into our analysis. The phylogeny, inferred from 162 informative sites in 1,700 bp of DNA sequence data and using yeast as an outgroup, divided the fungi into two groups correlating well both with fruiting-body type and with the traditional classes Plectomycetes and Pyrenomycetes. Each group received strong statistical support. Genera producing cleistothecia, such as *Talaromyces* (with a *Penicillium* asexual state) and the human pathogen *Ajellomyces capsulatus* (causing histoplasmosis), fall within the plectomycete group. Plectomycetes also includes *Eremascus albus* and the bee pathogen *Ascosphaera apis,* although both lack typical fruiting bodies. The Dutch elm disease fungus groups with pyrenomycetes such as *Neurospora,* in spite of its confusing mixture of class-level characters.

Introduction

The Ascomycotina, including almost 40% of known fungus species, is the largest subdivision of fungi (Hawksworth et al. 1983). Ascomycetes produce spores in an ascus, or sac, and traditional ascomycete classes have been defined both by the form of the fruiting bodies bearing the asci and by the type and arrangement of the asci. Members of the class Plectomycetes have cleistothecia, closed fruiting bodies characterized by an irregular distribution of asci and ascospore release following disintegration or deliquescence of the ascus wall (fig. 1) (Fennell 1973). In the past, the class Plectomycetes included the organisms with *Penicillium* or *Aspergillus* asexual states, as well as the fungi causing ringworm and histoplasmosis. Pyrenomycetes included *Neurospora* and other ascomycetes forming flask-shaped perithecia with a single layer of asci and forcible discharge of ascospores from the ascus (fig. 1) (Müller and von Arx 1973).

The problem with traditional classification is that fruiting-body characters can converge (Cain 1972; Malloch 1981). A species normally producing flasklike fruiting bodies can be induced to form closed fruiting bodies under certain environmental

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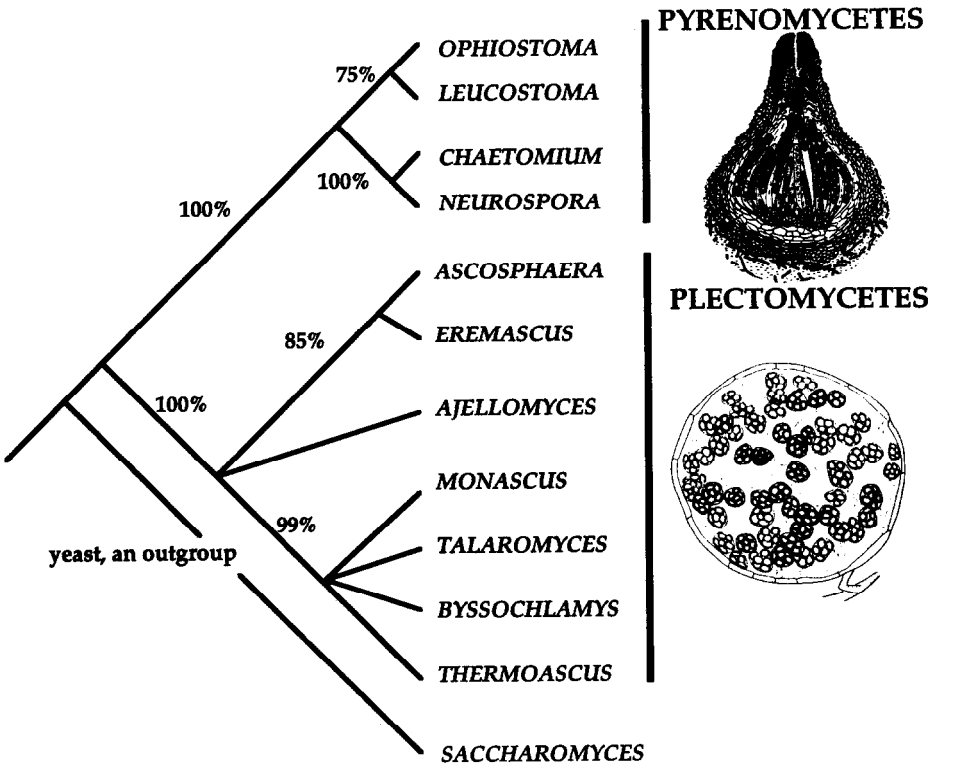

FIG. 1.—Two classes of ascomycetes corresponding to phylogenetic groups inferred from rDNA sequence data. The percentages are the frequencies with which a given branch appeared in 500 bootstrap replications. Branches that appeared in ≥95% of the replications are strongly supported by the data set. Branches with frequencies <75% have been reduced to polychotomies. The two most parsimonious trees generated by the Branch and Bound option in PAUP require 462 changes. The above tree is identical to one generated from a consensus of the 25 most parsimonious Branch and Bound trees with lengths under 464. (The graphic depicting Pyrenomycetes is reproduced, with permission from the publisher, from *Fungal Spores: Their Liberation and Dispersal,* by C. T. Ingold. Oxford: Oxford University Press, 1971. The graphic depicting Plectomycetes is reproduced, with permission from publisher, from *Introduction to Fungi,* by J. Webster. New York: Cambridge University Press and Academic Press, 1980.)

conditions (von Arx 1973). Some genera of fungi with flasklike fruiting bodies are closely related to genera of fungi with closed fruiting bodies (von Arx 1973). Some species, such as *Ophiostoma ulmi* (the dutch elm disease fungus), have both the fruiting-body type characteristic of the Pyrenomycetes and the ascus arrangement characteristic of the Plectomycetes. Without characters uniting major ascomycete groups, orders of ascomycetes must remain narrowly defined, and supraordinal classification is impossible (Erikkson 1982; Hawksworth et al. 1983). At present, Plectomycetes and Pyrenomycetes and other classes are falling into disuse (Hawksworth et al. 1983) (fig. 2). Classless taxonomic systems, while prudent, offer no help to the molecular biologist wanting to know whether the human pathogenic fungi are (1) more closely related to *Aspergillus* than to *Neurospora* and therefore (2) more likely to undergo homologous than nonhomologous transformation (Fincham 1989).

If fruiting-body characters indicate common descent, we would expect that the genera we studied would be divided into two groups corresponding to traditional

1971 6 classes 25 orders	1983 0 classes 37 orders
HEMIASCOMYCETES	Arthoniales
Endomycetales [1,7]	Ascosphaerales [8]
Protomycetales	Caliciales
Taphrinales	Clavicipitales
	Coryneliales
PLECTOMYCETES	Cyttariales
Ascosphaerales [8]	Diaporthales [11]
Eurotiales [2,3,4,5,6]	Diatrypales
Microascales [12]	Dothidiales
Erysiphales	Elaphomycetales
Meliolales	Endomycetales [1]
	Erysiphales
PYRENOMYCETES	Eurotiales [2,3,4]
Hypocreales	Graphidiales
Sphaeriales [9,10,11]	Gyalectales
Clavicipitales	Gymnoascales [6]
Coryneliales	Helotiales
Coronophorales	Hypocreales
	Laboulbeniales
DISCOMYCETES	Lecanidiales
Phacidiales	Lecanorales
Helotiales	Microascales
Ostropales	Opegraphales
Pezizales	Ophiostomatales [12]
Tuberales	Ostropales
	Peltigerales
LABOULBENIOMYCETES	Pertusariales
Laboulbeniales	Pezizales [?5,?7]
	Polystigmatales
LOCULOASCOMYCETES	Pyrenulales
Myriangiales	Rhytismatales
Pleosporales	Sordariales [9,10]
Hysteriales	Sphathulosporales
Dothideales	Sphaeriales
Capnodiales	Taphrinales
Microthyriales	Teloschistales
	Verrucariales

FIG. 2.—*The Dictionary of the Fungi,* a standard reference for fungal terminology, placed ascomycete orders into classes in 1973 but dropped the classes from the 1983 edition. The number of orders within the Ascomycotina increased from 25 in the 1973 edition to 37 in the 1983 edition. By eliminating classes and defining orders narrowly, the dictionary minimizes the chances of uniting unrelated, morphologically convergent organisms but provides little information about higher-level relationships. Superscript numbers show the classification of fungal genera included in the present study: 1 = *Saccharomyces;* 2 = *Thermoascus;* 3 = *Byssochlamys;* 4 = *Talaromyces;* 5 = *Monascus;* 6 = *Ajellomyces;* 7 = *Eremascus;* 8 = *Ascosphaera;* 9 = *Neurospora;* 10 = *Chaetomium;* 11 = *Leucostoma;* and 12 = *Ophiostoma.* [Columns are reproduced, with permission from the publisher, from Hawksworth et al. (1983).]

ascomycete classes and correlating with fruiting-body characters. If fruiting-body characters arose convergently, we would anticipate that phylogeny inferred from sequence might correlate with other morphological characters. Either way, sequence characters

had the potential to suggest which morphological characters usually indicate phylogenetic relationships leading to a more cohesive system of ascomycete taxonomy.

Material and Methods

We sequenced the nuclear 18S rDNA of nine ascomycetes in six orders (Hawksworth et al. 1983) and added sequence from *Neurospora crassa* (GenBank NEURRNAS), *Ajellomyces capsulatus* (GenBank X58572), and *Saccharomyces cerevisiae* (GenBank YSCRGEA) into our analysis (fig. 1). We amplified the rDNA subunit from miniprepped DNA by using primers NS1 and NS8 and 30 cycles (each cycle = 2 min at 97°C, 1 min at 48°C, and 45 s at 72°C, with a 4 s/cycle extension at 72°C) of the polymerase chain reaction (PCR) (Lee and Taylor 1990; White et al. 1990). We sequenced single-stranded template from asymmetric amplification of double-stranded PCR template by using primer pairs including NS1-8 (White et al. 1990) and NS19-22 (A. Gargas, personal communication), with the primer in excess at 0.5 μm and the limiting primer at 0.025 μm (White et al. 1990). Only one strand was sequenced near primers NS 1 and NS 8, and in an ~100-nucleotide-long region near NS 5. Otherwise, both strands were sequenced. We aligned the sequences visually and excluded ambiguously aligned sites from our analysis. Phylogenetic trees with identical topologies were generated using either the maximum-parsimony method, PAUP 3.01 (Camin and Sokal 1965; Swofford 1989), or the distance neighbor-joining (Saitou and Nei 1987) method. Sequences have been deposited in GenBank, and fungal strains followed by GenBank accession codes are *Ascosphaera apis* UCB 78-018 (M83264), *Byssochlamys nivea* FRR 2205 (M83256), *Chaetomium elatum* UCB 81-063 (M83257), *Eremascus albus* UCB 50-026 (M83258), *Leucostoma persoonii* LP8 Gerry Adams personal collection (M83259), *Monascus purpureus* ATCC 16365 (M83260), *Ophiostoma ulmi* ATCC 32437 (M83261), *Talaromyces flavus* var. *macrospora* FRR 2386 (M83262), and *Thermoascus crustaceus* FRR 1328 (M83263) (UCB = University of California, Berkeley collection; FRR = Food Research Laboratory, North Ryde, New South Wales; and ATCC = American Type Culture Collection). Alignment is available on request.

Results and Discussion

From ~1,700 bp of sequence per fungus, 1,628 sites were well aligned for all 12 fungi. Out of the 302 variable sites, the 162 phylogenetically informative sites were the basis for inferring phylogenetic relationships by using maximum-parsimony methods (Camin and Sokal 1965; Swofford 1989). Eleven ascomycete genera in seven orders (Hawksworth et al. 1983) fall into two groups, with *Saccharomyces cerevisiae,* a 12th fungus, as an outgroup in a parsimony-based phylogenetic tree from sequence of 18S nuclear ribosomal RNA (rDNA). The groups correspond to traditional ascomycete classes Plectomycetes and Pyrenomycetes. The first group, the Plectomycetes, includes organisms with cleistothecial fruiting bodies, as well as both *Ascosphaera apis* (causing chalk brood disease of bees), which has a vesicle rather than a hyphal cleistothecial wall, and *Eremascus albus,* which lacks a fruiting body. The second group, the Pyrenomycetes, includes *Neurospora crassa,* with typical pyrenomycete characters, and the Dutch elm disease fungus *Ophiostoma ulmi,* with a pyrenomycete-like fruiting body containing a plectomycete-like distribution of asci. We did a bootstrap analysis and found that branches leading to Plectomycetes and Pyrenomycetes were supported at the 100% level. Levels >95% indicate strong statistical support for branches

(Felsenstein 1985). At least 42 nucleotide changes showing no homoplasy occurred on the branches leading either to Plectomycetes or to Pyrenomycetes, and 18 of these were unambiguously assignable to each branch. The yeast *S. cerevisiae* was used as an outgroup, on the basis of (1) its distance from the other taxa included in the present study, (2) morphological considerations, and (3) the results of preliminary study comparing the sequence of the small nuclear subunit of rDNA of chytridiomycetes, basidiomycetes, and ascomycetes (Bowman et al. 1992).

The plectomycetes, with morphologically simple reproductive structures, have been viewed either as primitive or as a heterogeneous assemblage of reduced, unrelated ascomycetes. We found that morphologically diverse representatives of Plectomycetes together form a distinct monophyletic group (fig. 1). Within the Plectomycetes, saprobes with *Penicillium* or *Penicillium*-like asexual states, forming dry chains of asexual spores from specialized cells (phialids), grouped together (family Trichochomaceae: *Talaromyces, Thermoascus,* and *Byssochlamys*) (Malloch and Cain 1972) along with, unexpectedly, *Monascus purpureus* (a fungus exuding red-purple pigment used in coloring some Asian foods). *Monascus purpureus* had been placed in its own family both because of its unusual sex organs (gametangia and cleistothecia) and because it lacks the phialidic asexual spores characterizing the Trichochomaceae (Cole and Kendrick 1968).

Ajellomyces capsulatus (the human pathogen causing histoplasmosis) and the three human pathogens most closely related to it (B. Bowman, personal communication) are also plectomycetes, making typical cleistothecia if reproducing sexually.

Ascosphaera apis, causing chalk brood disease of bees, and *Eremascus albus* have been taxonomic puzzles. They have been classified with yeasts, because they lack a hyphal fruiting body (Harrold 1950; Fennell 1973; Kreger-von Rij 1973), or in Plectomycetes, because they are mycelial, form hyphal gametangia, and have eight ascospores in each ascus (Spiltoir and Olive 1955; von Arx 1981, pp. 88 and 138). On the basis of rDNA sequence, the two are plectomycetes, demonstrating the strength of rDNA sequence data in placing organisms when diagnostic cleistothecial morphology is lacking.

The pyrenomycetes that we examined varied both in ascus structure and in presence of sterile tissue surrounding the perithecia, but all are similar in form, and, with the exception of the Dutch elm disease fungus, all are generally considered to be related (von Arx 1981, pp. 19 and 150–178; Barr 1990). The Dutch elm disease fungus *Ophiostoma ulmi* was originally considered to be a member of Pyrenomycetes because of its dark-colored, flask-shaped fruiting bodies (Müller and von Arx 1973; von Arx 1981, p. 150). However, like plectomycetes, its asci are not organized into a single layer in the fruiting body, and ascospores are released when the ascus wall deliquesces, rather than through a forcible discharge mechanism (Nannfeldt 1932; Benny and Kimbrough 1980). When sequences are compared, the Dutch elm disease fungus groups with other pyrenomycetes rather than with plectomycetes or with the yeast *Saccharomyces cerevisiae.*

We have found that sequence data support a simple but controversial taxonomic hypothesis—i.e., that ascomycetes from different orders but with similar fruiting-body characters belong in the same class. Not all fungi in a class have all characters of the class, and sequence data can indicate relationships when some morphological characters are atypical. We demonstrated that the fruiting-body type, rather than ascus type and arrangement, of the Dutch elm disease fungus was most consistent with its class-level relationships. As the nucleic acid sequences of more ascomycetes become available,

the boundaries of the Plectomycetes, Pyrenomycetes, and other classes will be defined. With equal certainty, support for the Pyrenomycetes and Plectomycetes will remain strong, judging from the robust statistical support for branches grouping the diverse organisms included in the present study.

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