Phylogenetic relationships of cantharelloid and clavarioid Homobasidiomycetes based on mitochondrial and nuclear rDNA sequences

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Abstract: Sequence data from mitochondrial and nuclear small subunit rDNA were used to estimate phylogenetic relationships of cantharelloid and clavarioid Homobasidiomycetes. Sixty-five diverse Homobasidiomycete species were investigated, including 23 cantharelloid and clavarioid species. Although nodes deep in the tree could not be resolved, four lineages containing cantharelloid and clavarioid fungi were identified. (i) Cantharellaceae (Cantharellus, Cruciferellus) is closely related to Hydnaceae, which is toothed, Stichocarpaceae, which is a simple club, and Clavulinaceae, which is coralloid. These taxa all have stichic nuclear division, which is a synapomorphy supporting this clade. (ii) Clavariadellaceae is closely related to Gomphaceae and Ramariaceae. This relationship is supported by green reactions of sporocarps treated with iron salts, which is reflective of the presence of the compound pistillar. The nearest relatives of these cantharelloid and clavarioid fungi are gastromycetes, including the earth star Geastrum, the stinkhorn Pseudocolus, and the "cannon-ball fungus" Sphaerobolus. (iii) The clavarioid fungi Clavaria, Clavulinopsis, Pleurotus, and Typhula appear to be derived from the lineage that contains most of the gilled fungi. (iv) Clavicorona is closely related to Auriscalpium, which is toothed, and Lentinellus, which is gilled. This lineage is united by amylolytic spor ornamentation. Although these results suggest that there has been extensive convergence in fruiting body morphology, certain anatomical and biochemical features appear to be phylogenetically informative, notably stichic nuclear division, presence of pistilar, and cyanophily or amyloidy of spor ornamentation.

Key Words: Cantharellaceae, Clavariaceae, evolution, fungi, Gomphaceae, phylogeny, ribosomal DNA, systematics

INTRODUCTION

Fruiting bodies of cantharelloid and clavarioid Homobasidiomycetes include funnel-shaped or pileate sporocarps with smooth, wrinkled, or lamellate hymenophores, and unbranched club or branched coralloid sporocarps with smooth or folded hymenophores. Ecological habits range from saprophytism and parasitism to ectomycorrhizal and lichenized mutualisms. Anatomical and biochemical diversity is found in characters such as spore ornamentation and reactivity, hypoval structure, patterns of meiotic division, secondary compounds, and chemical structure of pigments (Table I).

Although all modern authors agree that the cantharelloid and clavarioid fungi are polyphyletic (e.g., 2, 4, 10, 11, 12, 14, 19, 21, 24, 43, 58, 72, 74, 83), evolutionary relationships of monophyletic taxa have not been resolved. Relatively few morphological characters have been identified that can be compared across genera, and many of these support incongruent relationships. Various authors have emphasized different suites of characters and consequently have proposed conflicting evolutionary histories (e.g., 10, 12, 14 vs 19 vs 72). A preliminary phylogenetic analysis using published morphological characters failed to resolve relationships among genera of cantharelloid and clavarioid fungi (EM Pine unpubl). Results presented here use DNA sequence data as an independent and abundant source of characters for comparisons across diverse lineages.

This discussion treats only taxa and characters relevant to results of this study. Corner (10, 12, 14), Donk (19), and Petersen (74) provide broad taxonomic reviews of cantharelloid and clavarioid fungi. Selected authors' taxonomic treatments of key genera are summarized (see Table II).

Cantharelloid and clavarioid fungi figure prominently in hypotheses about the origin of the fleshy basidiomycetes (12, 15, 31, 32, 43, 58, 72, 83, 84). Their fruiting forms can be arranged in a transformation series, with simple clubs at one extreme, cantharelloid forms intermediate, and agaric forms at the other extreme. Corner (15) proposed the "Cla-
varia theory’ of basidiomycete evolution, which treats the cantharellloid and clavarioid fungi as a basal paraphyletic group from which all other Homobasidiomycetes have been derived. Corner suggested that the simple club with a smooth hymenophore (e.g., Clavaria) is the ancestral state of the fleshy fungi, from which have been derived first club-shaped and cantharellloid intermediates with folded hymenophores (e.g., Clavariadelphus and Cantharellus, whose hymenial configurations differ from true lamellae in the orientation of hyphae in the trama), and eventually gilled mushrooms. Corner’s model has had a strong influence on subsequent evolutionary hypotheses. For example, Jülich (43) suggested that Clavariaceae was derived from the Auriculariales (jelly fungi) or their ancestors, and that Cantharellales is the basal group of Homobasidiomycetes. Miller and Watling (58 p 439) state that “the logical extension from the clavarioid condition among epigean taxa is the cantharellloid basidiome,” and suggest that agarics have been derived multiple times from cantharellloid ancestors. Other authors agree that there must have been transformations among coraloid, cantharellloid, and agaric forms, but propose the opposite polarity, suggesting that lineages containing cantharellloid, coral, and club fungi have been derived from agaric ancestors (2, 30, 72, 83).

The agarics Hygrocybe and Gerronema have been suggested as close relatives of Cantharellaceae. Hygrocybe is similar to Cantharellus in having thick, waxy, decurrent gills, bright orange and yellow pigments, long and narrow basidia, and hyaline, unornamented, non-reactive spores (34). Gerronema (= Omphalinus) chrysophyllum resembles members of Cantharellaceae in spore color, hynenial anatomy, basidiole color, general aspect, and molecular structure of carotenoid pigments (2, 29, 48, 83). Yet chante-trelies depart from true mushrooms in several important characters (Table 1), including anatomical differences between cantharellloid gill-folds and true agaric gills (12 p 19), leading several authors to ascribe similarities between Cantharellaceae and Hygrocybe or Gerronema to convergent evolution (12, 19 p 245, 53).

Singer (83 p 126) suggested that “A further ‘bridge’ between Aphyllophorales and Agaricales might be seen in Linderomyces,” a genus with a bilateral trama (true gills) and unusual “coccoid” (sieve-like) hyphae (82), but with microscopic features and chemical reactions characteristic of Gomphaceae (13, 69, 71). Singer originally placed Linderomyces in Paxillaceae (82); he later concluded that the genus represented an independent origin of gills within Gomphaceae, but thought it might be “a starting point for an evolution which would lead from the Gomphaceae to the Paxillaceae” (83 p 126). Petersen (69) concluded that Linderomyces was a synonym for Gloeocantharellus, a gomphaceous genus whose morphology has been described as intermediate between Cantharellus and Gomphus (81). Corner (12, 13) and Petersen (71, 72) agreed with Singer that Gloeocantharellus/Linderomyces belongs in Gomphaceae, but thought it could represent an evolutionary link with Paxillaceae and some Boletaceae.

Anatomical features suggest that cantharellloid and clavarioid fungi comprise several independent lineages (Table 1). Spore morphology can be used to delineate three groups. Hyaline, unornamented spores that do not react to Melzer’s reagent or Cotton Blue are characteristic of most of the known cantharellloid and clavarioid fungi. Spores with distinctive amyloid ornamentation are found in the coraloid genus Clavicipitcula; Donk (19) used this feature, along with presence of gloeocystidia, to transfer Clavicipitcula from Clavariaceae to Hericaceae. The remaining spore type, ochraceous with cyanophilic ornamentation, is found in genera with a variety of fruiting body forms: Gomphus (cantharellloid), Ramaria (coraloid), Bresia (hydnoid), Kavinia and Ramicaricia (resupinate), and Gloeocantharellus (= Linderomyces) (agaric). Despite their macromorphological diversity, these taxa have been grouped in Gomphaceae (18, 19, 21, 44, 46, 52, 71, 83), a placement that is supported by shared green reactivity of fruiting bodies treated with FeSO4. The club-shaped genus Clavariadelphus also reacts with ferric salts, but has smooth, hyaline, unornamented spores (56). If macrochemical reactions and mode of nuclear division are emphasized, Clavariadelphus is placed with Gomphaceae (2, 19, 30, 32, 56, 72, 90), but emphasis on spore morphology supports a relationship with Cantharellaceae or Clavaria (10 p 25, 11, 12, 14, 58, 65, 68, 72).

The position and orientation of the first nuclear division of meiosis has been proposed as a taxonomically important character (19, 41, 42, 55). In most Homobasidiomycetes that have been examined, division takes place near the apex of the basidium with the meiotic spindle transverse to the long axis of the basidium (6 p 267, 39, 42, 55, 63). This pattern is called chiasmatic division (see Fig. 3). In contrast, in Cantharellus (39, 42, 55), Craterellus (42, 55), Clavulina (42, 55, 63), Sticholowaria (= Multiclavula) (39, 42), Clavulicium (6 p 267), Sistroverum (6 p 267), and Hydnium s. s. (55, 63, 80), division is near the middle of the basidium, with the spindle axis more or less parallel to the basidial axis. This pattern is called stichic division (see Fig. 3). Meiotic division can be observed only in fresh, mature fruiting bodies, and has not been examined in many taxa.
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*a* Scoring is for the entire taxon listed, with rare states indicated in parentheses.

*b* Fruiting body morphology: cl = clavarioid (simple club), ca = coralloid (branching cylinder), ca = cantharelloid (tube-shaped), pi = pileate/agaricoid.

*c* Discontinuous hymenium: − = sterile area continuous across top of basidiocarp, + = apex of fruiting body sterile (assumed true for "pi" fruiting bodies).

*d* Hymenial configuration: sm = smooth (assumed true for clavarioid and coralloid taxa unless otherwise reported), wr = wrinkled/ridged, la = lamellate/appearing gilled, to = toothed/hydnoid.

*e* Thickening hymenium (sensu 10): − = hymenial layer constant thickness throughout development, + = developing basidiomata push past mature basidiomata.

*f* Habit/ecological strategy (substrate reported only if specific ecological data not available): em = ectomycorrhizal, li = lichenized, pa = parasitic, sa = saprobic, te = terrestrial, wo = found on dead plant matter.

*g* Spore ornamentation: − = none (spores smooth), ro = roughened, wa = warty, ri = ridged or with anastomosed warts, sp = spiny/echinulate.

*h* Spore pigmentation: hy = hyaline (unpigmented), pi = pink, ye = yellow, or = orange, bu = light buff, oc = ochraceous.

*i* Reactivity of spore wall ornamentation (not including cytoplasm): − = not staining blue in Melzer's reagent or Cotton Blue, am = amyloid, cy = cyanophilic, blank = reactivity not reported for either reagent.

*j* Number of spores (or sterigmata) per basidium.

*k* Presence/type of cystidia in hymenium: − = no cystidia of any type, le = leptocystidia/undifferentiated cystidia, gi = gloeocystidia (also known as coccocystidia).
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<td>Ramaria</td>
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<td>Stichoclavaria</td>
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1 Skeletal hyphae in basidiocarp: - = absent (monomitic), + = present (dimitic).
2 Gloeocleroplerous hyphae in basidiocarp: - = absent (or not reported), + = present, lu = laticiferous hyphae (superficially similar to gloeocleroplerous hyphae, but not staining in Cotton Blue).
3 Clamp connections throughout basidiocarp: - = absent (or a single clamp at the base of the basidium, as seen in Clavaria), + = present.
4 Orientation of first meiotic nuclear division (original reports only): st = stichic (spindle oblique or parallel to long axis of basidium), ch = chiasitic (spindle transverse), blank = not reported, original classification of examined specimen is listed if it was later transferred to a different genus.
5 Reaction of fruiting body with iron salts (i.e., FeSO₄ or FeCl₃): - = no reaction, lg = light green, re = red, gr = dark green, vi = violet, +pi = compound pistillaris demonstrated to be present, -pi = pistillaris assayed but not present, blank = reactivity not reported.
6 Carotenoid pigments in fruiting body extractions: - = absent, + = present.
7 Characteristic of Clavaria helicoides, whose generic position is uncertain (see 14: p. 34-35).
8 C. fusiformis has been placed variously in Clavulinopsis, Ramariopsis, and Clavaria, so scoring is limited to the single species.
9 Lentaria s.s. refers to taxa remaining after the segregation of Multiclavula Petersen.
10 Macroturphula scoring is based largely on Corner's (10) Clavariadelphus subg. Typhulopsis, Methven (56) equates the two.
11 Stichoclavaria Ulbrich is used for Multiclavula Petersen, see text.
Shared possession of stictic division suggests that Cantharellaceae is closely related to Hydnum (17, 19, 72, 74) and Clavulinaceae (17, 19). If stichobasidia are deemphasized as a taxonomic character, other features suggest different relationships (Table 1). For example, Corner (11) used hymenophore configuration, fruiting body development, clamp connections, and presence/absence of a sterile apex or pileus to split Cantharellaceae, suggesting placement of Cantharellus with Clavariadelphus, Craterellus with Stereum, and Clavulina with Clavaria and Clavulinopsis. Petersen (66) agreed that stichic Clavulina was related to chistic Clavulinopsis, but placed Clavariadelphus with this complex rather than with Cantharellus. Reijnders and Staplers (79) found a different pattern of hymenophore trama development in Hydnum repandum than in Cantharellaceae, which, combined with the absence of carotenoid pigments in Hydnum, led them to reject a close relationship between Cantharellaceae and Hydnum.

Circumscription of genera within Cantharellaceae has been controversial (74). Craterellus has been distinguished mainly by absence of clamp connections (4, 11, 12), but Petersen (74) noted that some species that lack clamps have been included in Cantharellus. Corner (11) proposed the genus Pseudocraterellus to contain unclamped, secondarily septate chanterelles otherwise similar to Cantharellus; Corner also emphasized patterns of fruiting body development, but this feature is difficult to examine and has been largely ignored by subsequent workers. Petersen (70, 74) and Bigelow (4) criticized secondary septation as a taxonomic character since it is variable among individual fruiting bodies, especially those of different ages, and is difficult to ascertain in herbarium material. Furthermore, many authors have noted cantharelloid species that exhibit combinations of features used to define different genera (e.g., Craterellus carolinensis) or whose placements by Corner’s criteria conflict with those supported by other well-accepted characters (4, 11, 13, 70, 75). Corner himself (13) pointed out that Cantharellus inanitus and C. subcubarius can be clamped and secondarily septate; his description of C. cuticulatus, which is “so very obviously a Cantharellus” (p 786) led him to conclude that “secondarily septate hyphae without clamps, such as characterize Pseudocraterellus, occur in this species of Cantharellus” (p 785). Despite examination of pigmentation (30), spore wall anatomy (45), secondary septation, fruiting body ontogeny, and hyphal anatomy (11, 12, 13), no synapomorphies have been recognized that unambiguously distinguish Craterellus, Cantharellus, and Pseudocraterellus. Although these difficulties have led some authors to collapse all the species of Cantharellaceae into one genus (e.g., 50), or to segregate Craterellus into its own family (e.g., 43), such changes in taxonomic rank have not clarified relationships among cantharelloid lineages.

Clavarioid basidiomycetes are a heterogeneous group whose phylogenetic relationships have also proved extremely difficult to resolve. A few genera, such as Clavicipitaceae and Ramaria, share distinctive features with other lineages of Homobasidiomycetes and have been removed from Clavariaceae (18, 19). Other species have autapomorphic features that have allowed segregation of the umbrella Clavaria into distinct genera. For example, Clavulinaceae is characterized by secondarily-septate basidia with two strongly incurved stigmata, Pterula by a dimitic hyphal system, and Typhula by the formation of sclerotia (10, 14). But such characters do not suggest higher-level relationships, and although some authors have promoted these genera to segregate families (19, 43), their nearest relatives have not been identified. Stichic division (found in Stichocladus and Clavulina) and carotenoid pigmentation (found in Clavaria subg. Clavulina sensu Petersen, 77), link some genera to other lineages of Homobasidiomycetes (Table 1), but these characters have not been widely accepted as synapomorphies. Thus Clavariaceae is still a polyphyletic group that is defined largely by the absence of distinguishing features.

MATERIALS AND METHODS

Twenty-five cantharelloid and clavarioid exemplars were selected to represent 25 species in 12 genera and 8 families sensu Corner (12, 14). Taxa were chosen to emphasize taxonomically controversial traits (e.g., stichic nuclear division, spore ornamentation, FeSO4 reactivity), with an effort to include multiple species of each genus of chanterelles and several clavarioid genera (Table II). Sequences for Clavicorona ferox were published previously (35).

Because higher-level evolutionary relationships of cantharelloid and clavarioid fungi are controversial, a broad sampling of other Homobasidiomycetes was imperative. Four taxa were chosen to represent proposed relatives of Cantharellaceae: Hydnum repandum, Gerronema chrysophyllum, and two species of Hygrophoraceae. Gloeocantharellus purpurascens, with true gills, was the sole representative of noncantharelloid or clavarioid Gomphaceae. Sequences for additional taxa were available from published and ongoing studies of Homobasidiomycete relationships (35, 36, 37). Thirty-six exemplars were selected to represent traditional families of basidiomycetes as well as unclassified lineages identified by previous phylogenetic analyses. In total, 21 families sensu Donk (19) and Singer (83) were represented.

DNA was isolated from dried or fresh fruiting bodies or mycelia. Some taxa proved extremely difficult to extract, particularly those with dark pigments (e.g., Craterellus falax), and protocols were modified to reduce the concentr-
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* Accessions are dried fruiting bodies, except those marked with *, which are mycelial cultures.

*b Accession is a yellow mutant of the species (1, 64).

c Also known as Stichoclavaria mucida.
tions of these pigments. Fragments of fruiting bodies were first soaked in a buffer of 20% DMSO, 250 mM EDTA, and saturated NaCl (S. Rehner pers. comm.) 1–3 d, then rinsed with 1× TE pH 8.0 (10 mM Tris-HCl, 1 mM EDTA) 10 min. Extraction protocol was as follows: a small fragment (0.25 cm² or less) of fungal tissue was placed in 1.5 mL microcentrifuge tube with 400 µL hot (60 °C) 1% SDS extraction buffer and sterile sand. Tube contents were homogenized with a plastic pestle fitted into a hand drill (recalcitrant tissue was ground in a mortar under liquid nitrogen, then added to hot buffer). Tubes were incubated at 60 °C 10–30 min, then extracted once with 25:24:1 phenol: chloroform: isoamyl alcohol and once with 24:1 chloroform: isoamyl alcohol. DNA was removed from solution using GeneClean II (Bio 101, La Jolla, California) and eluted into 50 µL 1× TE pH 8.0 (10 mM Tris-HCl, 1 mM EDTA). Serial dilutions of genomic DNA (1:10–1:1000) were used as template for the polymerase chain reaction.

Two unlinked genes were examined: mitochondrial small subunit rDNA (mt-sus-rDNA) and nuclear small subunit rDNA (nuc-sus-rDNA). Amplification and sequencing used the primers MS1 and MS2 (91) for mt-sus-rDNA, and SR1c and NS41 (35) for nuc-sus-rDNA. Double-stranded PCR products were purified using GeneClean II (Bio 101, La Jolla, California) or QIAquick spin columns (QIAGEN, Inc., Chatsworth, California). PCR and sequencing protocols were as described by Hibbett and Donoghue (36). Sequences were edited and assembled using SeqEd v. 3.0.1 (Applied Biosystems, Inc., Foster City, Calif.) or Sequencher v. 3.9 (Gene Codes Corp., Ann Arbor, Mich.).

Sequences were aligned manually in SeqApp v. 1.9a169 and PAUP v. 3.1.1 (86) and test version 4.0d54 of PAUP* (written by David L. Swofford) on a Power Macintosh 8500/220 and Sun workstation. Heuristic searches were performed, with 100 random stepwise addition replicates with MULPARS on, steepest descent off, and TBR branch swapping. A two-step search was performed: first, no more than two trees were saved from each replicate, then exhaustive swapping was performed on all of the most parsimonious trees discovered. The resulting trees were rooted with Tremella, as suggested by the results of Swann and Taylor (85). One thousand bootstrap replicates were performed with the following settings: MULPARS option off, simple addition sequence, heuristic search, and TBR branch swapping. Analyses of the two subset alignments (gompheid-phaoid and stichic) used the branch-and-bound search algorithm, which guarantees discovery of all most parsimonious trees.

RESULTS

The number of included, variable, and parsimony-informative characters for each data set is shown in Table III, along with the number and length of optimal trees found in each analysis. Independent analyses of mt-sus-rDNA and nuc-sus-rDNA suggest that there is evolutionary rate heterogeneity among lineages in both genes. In the mt-rDNA tree (Fig. 1), there are long branches leading to Clavaria zollingeri (33 steps), Sparassis spathulata (49 steps), the branch linking these three isolates (53 steps), and the branch linking these taxa to Stichodactylaria (34 steps). These are four of the five longest branches in the tree; the fifth consists of the 45 autapomorphic characters leading to Boletus satanas. In the nuc-rDNA tree (Fig. 2), 63 autapomorphic changes lead to Cantharellus cibarius, and there is a long branch of 38 steps supporting monophyly of Cantharellaceae. The next longest branch is 35 steps, leading to Daedycybe chrysospermus, at the three base of the tree; no other branch is more than 25 steps long. The most obvious conflict
Table III. Description of the various data sets analyzed and the most parsimonious trees found

<table>
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<tr>
<th>Data set</th>
<th>Size of Matrix</th>
<th>Characters Included</th>
<th>Variables</th>
<th>Informative</th>
<th>Shortest Trees Number</th>
<th>Length</th>
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*Single alignment including all 65 taxa.
*b Clavulinula ornata was excluded.
*c New alignment including only the 12 taxa in the gomphoid-phalloid clade.
*d New alignment including only the 11 taxa in the stichic clade.

Between the two gene phylogenies concerns relationships of taxa on these long branches. The mt-rDNA tree (Fig. 1) depicts monophyly of Clavaria zollingeri and Sparassis spathulata and places these taxa as the sister group of Stichocladaria, although with less than 70% bootstrap support. The nuc-rDNA tree (Fig. 2), in which these taxa are not associated with unusually long branches, supports monophyly of all Clavaria species and Clavulinopsis, and places Sparassis as the sister group of Laetiporus. The mt-rDNA tree (Fig. 1) gives strong support (99% bootstrap) for the monophyly of Cantharellaceae and Hydnaceae, but the nuc-rDNA tree (Fig. 2) places Cantharellus and Craterellus near the base of the phylogeny, and leaves Hydnaceae with the remainder of the stichic clade. Other nodes that differ between the two gene trees either collapse in the strict consensus of equally parsimonious trees or receive less than 60% bootstrap support from one or both genes.

Results of analyses of the three exclusion sets of the combined data (datasets 1–3) differed slightly in bootstrap values and degree of resolution of the strict consensus tree, but no conflicting nodes received even moderate (>50%) bootstrap support. Because the major conclusions of this study are congruent with all three sets of analyses, only results of dataset 2 will be presented. Combined analyses (Figs. 3, 4) place Clavaria zollingeri and Sparassis together and support monophyly of Cantharellaceae and Hydnaceae, reflecting the mt-rDNA results (Fig. 1). The branch leading to Clavaria zollingeri and Sparassis is the longest in the tree (68 steps). Furthermore, two of the three next longest branches lead to Sparassis itself (60 steps) and Clavaria zollingeri (63 steps). The remaining unusually long branch leads to the divergent Cantharellus cibarius (64 steps). The strict consensus tree (Fig. 4) does not resolve relationships of stichic taxa, but 71% of the bootstrap replicates support monophyly of stichic taxa. Lack of resolution in the strict consensus tree is due to conflicting placements of Clavaria zollingeri and Sparassis; alternate equally parsimonious positions are marked with dashed branches in Figs. 3, 4. When Clavaria zollingeri and Sparassis were excluded from analyses, monophyly of stichic taxa was supported by all most parsimonious trees and 100% of bootstrap replicates.

Cantharelloid and clavarioid fungi appear in four groups (Fig. 5). Gomphus, Ramaria, Clavocantharellus, Lentaria, and Clavariadelphus form a clade including Pseudocolus, Geastrum, and Sphaerobolus (henceforth referred to as the gomphoid-phalloid clade), with 100% bootstrap support. The stichic genera Cantharellus, Craterellus, Hydnaceae, Clavulinopsis, and Stichocladaria are monophyletic, including Sparassis and Clavaria zollingeri in some of the most parsimonious trees. Clavicornospora is the sister group of Auriscalpinum and Lentinellus. The remaining clavarioid fungi are nested within the clade including most of the gilled fungi and the polypore Fistulina hepatica (henceforth termed the euagaric clade after Hibbett et al. 37).

Restricting attention to members of each of the first two clades (gomphoid-phalloid and stichic) allowed unambiguous alignment of more of the sequence data. Compared to the alignment including all 64 taxa, fewer gaps were required, reducing the matrix length, and reduced homoplasy provided fewer, shorter most parsimonious trees with better-resolved fine-scale relationships (Table III). The relationships supported by the single most parsimonious tree for the gomphoid-phalloid clade realignment (Fig. 5) are congruent with those supported by some of the most parsimonious trees for analyses including all taxa (e.g., Fig. 3). Gomphus is monophyletic (99% bootstrap), and closely related to Ramaria formosa. Although Ramaria stricta is the sister group of this...
Fig. 1. Phylogram of mt-ssu rDNA gene tree. One of 372 equally parsimonious trees. L = 1234, CI = 0.332, RI eq 0.583, RC = 0.194. The following conventions are used in all figures: cantharelloid and clavarioid taxa are in boldface, bootstrap values (greater than 50% in Figs. 1, 2, 4–6) are indicated next to the appropriate branch, branches receiving >70% bootstrap support are thickened, * indicates branches that collapse in the strict consensus of all most parsimonious trees.
Fig. 2. Phylogram of nuc-su rDNA gene tree. One of >4800 equally parsimonious trees. L = 682, CI = 0.526, RI = 0.732, RC = 0.385. See Fig. 1 for explanation of conventions.
FIG. 3. Phylogram of combined data for mit-ssu rDNA and rnc-ssu rDNA. One of 4458 equally parsimonious trees, see Methods for analysis parameters. L (length) = 2022, CI (consistency index) = 0.380, RI (retention index) = 0.606, RC (rescaled consistency index) = 0.291. See Fig. 1 for explanation of conventions. In this figure, dashed branches indicate alternate placements of the branch leading to Sparassis spathulata and Clavaria zollingeri (arrow), which are reflected in Figs. 1, 2. Line drawings (after 41) depict stichic vs chastic meiotic division in immature basidia.
Fig. 4. Strict consensus tree for combined data. Consensus of 4458 equally parsimonious trees, L eq 2022, normalized CFI (consistency fork index) = 0.422. See Fig. 1 for explanation of conventions. Dashed branches indicate alternate placements of the branch leading to Sparassis spathulata and Clavaria zollingeri (arrow).
clade in the most parsimonious tree, bootstrapping does not support monophyly of 
Gomphus and Ramaria. The genus Ramaria appears to be paraphyletic. Clavaria delphus
pisillaris and C. unicolor are sister taxa, as are C. ligulatus and Lentaria byssiseda. Clava
riadelphus is monophyletic if L. byssiseda is included. The Clavariadelphus lineage is nested 
within Gomphaceae, although bootstrap support is weak. Gloeocantharelus appears to be the 
basal lineage within Gomphaceae, but its position is not supported by bootstrapping. Pseudoculus, Geastrum, and Sphaerobolus are weakly supported as the monophyletic sister 
group of Gomphaceae.

Among stichic taxa, Cantharellus lutescens and
C. tubaeformis form a clade which is the sister group 
of Craterellus fallax and C. cornucopioides (Fig. 6). There is strong support (100%) for the monophyly of these taxa, to the exclusion of Cantharellus cibarius. Hydnum repandum is the sister group of Cantharellaceae. Unfortunately, attempts to amplify DNA isolated from Pseudocraterellus were unsuccessful. Clavulina is monophyletic and is the sister group of Stichocla
vara.

**DISCUSSION**

In many cases, inclusion of additional characters in phylogenetic analysis increases the probability of correctly estimating the underlying tree topology (87). But inclusion of ambiguously-aligned regions introduces characters whose homology is questionable. Furthermore, inclusion of phylogenetically informative characters for which multiple taxa are scored as missing sometimes can result in spurious resolution of artificial clades during parsimony analysis (53). Thus, there is a dilemma in phylogeny reconstruction of omitting large numbers of characters vs including characters that may add noise or be positively misinformative. However, results from analyses of the three exclusions sets suggest that these factors did not affect conclusions of this study, since all phylogenetic resolution receiving even moderate bootstrap support from any of the three exclusion sets tested is compatible with results from all three.

Possible causes for incongruence of the underlying phylogeny of two genes from the same taxon include: incomplete lineage sorting, hybridization, and other modes of horizontal transfer (54). The first two phenomena most likely occur among closely related species; this study focuses on relationships among genera and families that presumably diverged long ago. Horizontal transfer of the genomic regions used in this study has never been reported. Thus, we expected the mt-ssu-rDNA and nuc-ssu-rDNA sequences of the taxa in this study to represent the same underlying phylogeny. This expectation was supported by comparison of bootstrap values for the two gene phylogenies (Figs. 1, 2)—all positive conflict between the two trees receives less than 70% bootstrap support in at least one gene phylogeny.

The most distinctive topological conflict between the two gene trees concerns the placement of Clavaria zollingeri and Sparassis spathulata. Mitochondrial data depict these taxa as monophyletic, and place them within a clade that is otherwise stichic (Fig. 1). This differs from their placement based on nuc-ssu-rDNA (Fig. 2) or morphological characters, but strong support for any of these placements is lacking. Previous analyses of 1.2 kb of nuc-ssu-rDNA and the MS1/MS2 fragment of mt-ssu-rDNA support monophyly of Sparassis, Phaeolus schweinitzii, and Laetiporus sulphureus, but only with moderate (50%) bootstrap support (35). However, when additional taxa are sampled and complete nuc-ssu-rDNA se-

**Fig. 6.** One of the two most parsimonious trees for realigned combined data of stichic taxa. L = 419, CI = 0.924, RI = 0.921, RC = 0.850. See Fig. 1 for explanation of conventions. Topology is congruent with some of the most parsimonious trees for all taxa (e.g., Fig. 3). Rooted after Fig. 5 and Hibbett et al (37).
quences (1.8 kb) are included, bootstrap support for this clade rises to 98% (37). *Sparassis, Laetiporus,* and *Phaeolus* all have ellipsoid-ovoid, smooth, mammary spores, produce a brown rot, and can cause root and butt rot of living trees, although host ranges differ. Taken together, the ecological and anatomical characters and nuc-ssu-rDNA evidence suggest that the correct placement of *Sparassis* is with *Laetiporus.* The lengths of the branches leading to *Clavaria zollingeri,* *Sparassis,* and their putative sister taxa (see Results) in both the combined tree (Fig. 3) and the mt-rDNA gene tree (Fig. 1) suggest that long branch attraction could be responsible for their placement. In certain cases of grossly unequal branch lengths, parsimony analysis has been demonstrated to artificially connect extremely long branches that are unrelated in the true underlying phylogeny (27, 40). The branch leading to *Stichocladus* is the longest in the mt-ssu-rDNA tree (Fig. 1) if *C. zollingeri* and *Sparassis* are deleted (55 steps), suggesting that it is a likely candidate for this analytical artifact. Furthermore, monophyly of stichic taxa, *C. zollingeri,* and *Sparassis* receives only marginal (62%) bootstrap support, while monophyly of stichic taxa receives 92% bootstrap support in analyses of mt-ssu-rDNA that exclude the problematic *C. zollingeri* and *Sparassis.* Thus the mitochondrial data alone do not unambiguously support the placement of *C. zollingeri* and *Sparassis,* and furthermore, underlying phylogenetic signal supports monophyly of stichic taxa. Although branch lengths are more evenly distributed in combined analyses (Fig. 3), *Sparassis* and *C. zollingeri* are still extremely divergent and are grouped together. There is no support for their placement in the tree, resulting in three alternative placements (Figs. 3, 4) and lack of resolution in the strict consensus of equally parsimonious trees (Fig. 4). In the most conservative estimate, data presented in this study are insufficient to resolve relationships of *Clavaria zollingeri* and *Sparassis.* However, if evidence from anatomical and ecological characters and nuc-ssu-rDNA are given precedence over the dubious mt-rDNA results, *Clavaria zollingeri* belongs with *Clavaria acuta* and *Clavulinopsis,* *Sparassis* is the sister group of *Laetiporus,* and neither are nested within the stichic clade.

A similar argument can be used to explain the polyphyly of stichic taxa found in the nuc-ssu-rDNA analyses (Fig. 2). The longest branches in the tree are found within the Cantharelaceae, and the only other branch that is nearly as long is at the base, leading to *Dacrymyces.* The rest of the stichic clade exhibits extremely short branches. We conclude that Cantharelaceae is probably drawn to a basal position in analyses based on nuc-ssu-rDNA because of its high degree of divergence. Its position as the sister group of *Hydnium repandum* receives unequivocal support in analyses with more evenly distributed branch lengths (Figs. 1, 3).

Overview—Higher level relationships of Homobasidiomycetes are not resolved by these analyses (Fig. 4). Nevertheless, in no analyses do cantharelloid and clavarioid fungi appear to form a basal, paraphyletic group from which the rest of the Hymenomycetes have been derived. Thus, this study provides no support for Corner’s *Clavaria* theory of Homobasidiomycete evolution. Additionally, there is no evidence for a relationship between Cantharelaceae and the agarics *Gerronema* or *Hygrocybe,* supporting Donk’s (19) and Heinemann’s (33) conclusions that similarities of these genera to Cantharelaceae are due to convergence. Instead, it appears that many clavarioid fungi, traditionally placed in Clavariaceae, are derived from a lineage (designated euagaric) that also gave rise to the gilled mushrooms *Lenitula, Pleurotus,* *Hygrophorus,* *Hygrocybe,* and *Gerronema,* and to the polyzore *Fistulina.*

Coral- and club-shaped fungi have been derived in four lineages, two of which have also given rise to cantharelloid fungi. The fruiting bodies of the nearest extant relatives of the different lineages represent a wide range of forms: gilled mushrooms, toothed fungi, puffballs, sinkhorns, and the cannon-ball fungus. Similar rapid evolution of fruiting-body macro-morphology has been documented in diverse lineages of Homobasidiomycetes (7, 37, 38, 47, 59). The agarics *Neolentinus lepideus* and *Lentinellus* can produce clavarioid fruiting bodies under appropriate environmental conditions (9, 57, 61 p 184), and Donk (19 p 207–208) discusses several taxa whose fruiting bodies can be either corticioid or clavarioid. It appears that superficial similarity of form is not a good predictor of evolutionary proximity in the cantharelloid and clavarioid fungi. Instead, our results suggest that certain anatomical features are conserved within lineages that are otherwise morphologically diverse. For example, the coralloid *Clavicornis,* the toothed *Auriscalpium,* and the gilled *Lenitella* form a monophyletic group characterized by amyloid spore ornamentation. One of the goals of this study was to evaluate putative synapomorphies for other lineages with cantharelloid and clavarioid members.

**Gomphoid-phalloid clade.—** *Gomphus,* *Ramaria,* and *Gloeocantharelus* are united by cyanophilic, warty spore ornamentation and by green reactivity to iron salts (Table 1). *Gloeocantharelus* has a cantharelloid aspect, but has true gills and contains abundant gloeoplerous hyphae. Our results strongly support the accepted placement of *Gloeocantharelus* with *Gomphus* and *Ramaria,* indicating that *Gloeocantha-
rellus represents an independent derivation of gills within the gomphoid-phalloid lineage, and is unrelated to any other agaric or boletoid fungi examined thus far.

Other fungi reported with spores and macrochemical reactivity similar to Gomphaceae include hydnoid Beemdia (44, 52, 60), and the resupinates Knavia, which is toothed, and Ramaricium, which has a smooth hymenophore (19, 24, 52). Although these taxa are not represented in this study, sequences from the mitochondrial large subunit rDNA support placement of Knavia with Gomphus and Ramaria (8), suggesting that spore morphology and iron salt reactivity may be a synapomorphy of this group. Another relative of Gomphaceae seems to be Gattaria, a false-truffle with striate, brown-pigmented spores that are also reported to be cyanophilous (46). Sequence data from mitochondrial large subunit rDNA (8) and nuclear large subunit rDNA (J. Spatafora pers comm) support placement of Gattaria with Gomphaceae. Petersen (71 p 15) reported that “the staining reaction and general construction of the spore wall” of Gymnopilus and the boletoid taxa, Porphyrellus subflavidus, Strobilomyces confusus, and S. floccopus are very similar to that of Gomphus, but relationships among these taxa and Gomphaceae have not been examined further.

Members of the club-shaped genus Clavariadelphus react green on contact with iron salts (Table 1), reflecting the presence of pistillarin (56). These analyses provide 100% bootstrap support for the placement of Clavariadelphus within Gomphaceae, rejecting a relationship with Cantharellaceae and with Clavariaceae. Although Clavariadelphus spores are smooth, hyaline, and unreactive, like those of Cantharellaceae and Clavariaceae, this state appears to be plesiomorphic conservation of ancestral features.

Lentaria in the restricted sense is a homogenous group of branched, lignicolous clavarioid fungi characterized by white, smooth spores and thick-walled generative hyphae that give the fruiting body a leathery texture (67, 72). Corner (10, 14) included in the genus phycophilous, stichic species that Petersen (67) segregated into Multiclavula (= Stichoclavaria). Although Corner (10 p 24) left Lentaria in Clavariaceae, he noted a resemblance between L. byssidea and the Stricta group of Ramaria; shared green reactions with iron salts and thick-walled skeletal hyphae led Petersen (65, 72 Fig 10) to conclude that Lentaria s. s. was derived within Ramaria. Corner (14) moved Lentaria from Clavariaceae into a new family, Ramariaceae. Our results support Petersen’s separation of Multiclavula (= Stichoclavaria) and Lentaria s. s., as well as the placement of the latter genus in Gomphaceae (Fig. 3), although Lentaria byssidea appears to be nested within Clavariadelphus rather than Ramaria (Fig. 5).

Several other taxa have been reported to stain green on contact with iron salts. Petersen (68, 72) described green or gray-green reactions of Cantharellus cibarius and some species of Clavulinopsis (which he redefined as Ramario specif in 1978), but later (78) reported that pistillarin, the compound responsible for the green reaction in Gomphaceae and Clavariadelphus, was not present in Clavulinopsis. Our results suggest that neither Cantharellus nor Clavulinopsis are related to Gomphaceae, indicating that green iron salt reactions in the absence of pistillarin are not phylogenetically informative. Welden (90) suggested that Stereum radicans (= Stereopsis Reid) was related to Gomphaceae and Clavariadelphus, since it also stains green on contact with iron salts, but its pistillarin content has not been examined and it was not represented in this study.

The remaining taxa in the gomphoid-phalloid clade are Pseudocoles, Geastrum, and Sphaerobolus. Bootstrap support for the placement of these gasteromycetes with Gomphaceae is unequivocal (100%), and inclusion of the rest of the nuclear 18S rDNA and additional taxa does not alter this result (37). Furthermore, sequences from nuclear large subunit rDNA (28S) support a relationship between Gomphales and Phallales (J. Spatafora pers comm). Relationships among stinkhorns, earth-stars, the canan-ball fungus, and cantharelloid and clavarioid fungi have never been proposed in the taxonomic literature, and no morphological synapomorphy has yet been identified for this diverse clade. Although Pellegrini and Patrignani’s (62) examination of septal pore apparatuses let them to suggest that “the genus Clavariadelphus could be placed closer to Phallales owing to the perforate parentheses with small irregular holes,” they observed intact dolipore septa in all Ramaria species examined. Fungi in the gomphoid-phalloid clade are remarkably ecologically and morphologically diverse, and have traditionally been examined by different groups of mycologists. Comparative studies of the anatomy and biochemistry of these taxa might elucidate morphological features that unite the lineage, and should be pursued. For example, iron salt reactions and pistillarin content of Pseudocoles, Sphaerobolus, and Geastrum should be investigated.

Stichic clade.—Although monophyly of stichic taxa is not supported by all analyses, placement of Sparassis and Clavaria zollingeri in the midst of otherwise stichic clade is difficult to accept. Because such a relationship is contradicted by all evidence except mtssu-rDNA sequences, which may be susceptible to
long branch attraction of these taxa and do not provide strong bootstrap support, we reject the mt-su
rDNA results for Sparassis and C. soliingeri in favor of the placements supported by nuc-su-rDNA. Similarly, the removal of Cantharelloides from the stichic clade to a basal position in the tree, seen only in the nuc-rDNA analyses (Fig. 2), can be explained by long branch attraction. Thus we conclude that stichic taxa form a monophyletic group, Sparassis and Laetiporus, both brown rot fungi, are sister taxa, and the genus Clavaria is most likely monophyletic and nested within the euagaric clade.

Our results provide strong (100% bootstrap) support for the monophyly of Cantharelloides, but Cantharellus as previously defined appears to be paraphyletic (Fig. 6). Cantharellus cibarius is the sister taxon of a clade consisting of C. tubaeformis, C. lutescens (= xanthopus, see 20), Craterellus fallax, and Cx. cornucopioides. These results confirm earlier findings by Feibelman et al. (26) and Dahlman et al. (pers. comm). Feibelman et al. (26) recently proposed a new circumscription of genera within Cantharelloides based on results from phylogenetic analyses of nuclear large subunit (28S) rDNA sequences. Feibelman et al included only three of the species in our study, but their conclusion that a clade containing C. cibarius can be separated from a clade including Cx. fallax and C. tubaeformis is concordant with our results (Fig. 6). They revised Cantharellus to contain C. cibarius and its relatives, and suggested that the genus Craterellus be expanded to include C. tubaeformis and Pseudocraterellus sinuosus, in addition to traditional members of Craterellus (e.g., Cx. fallax, Cx. odoratus). If our results are fitted to their generic circumscription, C. lutescens must also be transferred to Craterellus. Feibelman et al. also evaluated some of the morphological features discussed in the Introduction, and concluded that ‘‘shape and texture seem to be more important [characters] than clamps, secondary septa, development, or hymenial configuration’’ in evaluating relationships of Cantharelloides.

Analyses of carotenoid pigments of Cantharelloides provide some support for the circumscription suggested by Feibelman et al. (26). Cantharellus lutescens, C. tubaeformis, and other members of Cantharellus subg. Phaeo: cantharellus sensu Corner (12) accumulate carotenoids with alliphatic structure exclusively, while C. cibarius and other members of Corner’s subgenus Cantharellus (roughly corresponding to genus Cantharellus sensu 26) accumulate predominantly bicyclic carotenoids (2, 28). However, published reports of pigment analyses provide conflicting results in some cases. For example, Arpin and Flaslon (2 p 84) state that ‘‘Citr. cornucopioides links closely to the group C. lutescens-C. tubaeformis, from which it differs only in having a weaker carotenogenesis, with correspondingly relatively strong development of dark pigments of another sort.’’ In contrast, Fiasson et al. (30) found that Cx. cornucopioides was totally devoid of carotenoids, while Cx. fallax, which is otherwise very similar to Cx. cornucopioides, possessed the same carotenoids as C. cibarius. It is intriguing that carotenoid pigment structure seems to correlate with relationships supported by other characters, but until more taxa are examined and conflicting reports are resolved it is impossible to determine the pattern of pigment evolution within Cantharelloides. Our data do support multiple derivation of bicyclic carotenoids in diverse lineages, since neither Gomphus nor Clavulinopsis are closely related to any members of Cantharelloides.

Hydnum repandum is the sister group of Cantharelloides, supporting Donk’s (17, 19) and Petersen’s (72) conclusions based on morphological similarity. Note that we are using Donk’s (19) restricted definition of Hydnum, typified by H. repandum; the name Dentinum, which has been used for this group, is invalid (76). Although nuc-su-rDNA data taken alone remove Cantharelloides from the stichic clade (Fig. 2), the extreme divergence of the nuclear rDNA of Cantharelloides (18S: note the long branches in Fig. 2; ITS: ref. 25; 28S: J. Spatafora pers. comm) makes it very likely that long branch attraction is responsible for the placement of Cantharelloides near the base of the tree and for the absence of support from bootstrapping when the nuc-su-rDNA is taken alone. The branch length disparity of Cantharelloides is much less severe in combined analyses, which provide unequivocal (100% bootstrap) support for the monophyly of Cantharelloides and Hydnum (Fig. 3). Although Hydnum has a toothed hymenophore, it is similar to Cantharellus in color, aspect, anatomy, and flavor (Table 1), and also has stichic nuclear division (80).

Stichic nuclear division (see Fig. 3) was first described by Juel (41), soon after which Maire (55) proposed a classification scheme for the fleshy basidiomycetes based on the distinction between stichic and chistiac basidia. Ulbrich (88) erected new genera and families for stichic taxa, but his classification was largely ignored by subsequent literature. Many authors since have criticized the use of this character for taxonomy (10 p 27, 12 p 11, 43, 66, 83). Although Donk’s early work (17) gave strong weight to stichic nuclear division, placing Clavulinopsis and Hydnum s. s. within Cantharelloides, he later (18, 19) revised his opinion, removing Clavulinopsis to its own family on the grounds that it was so evolutionarily divergent that its nearest relative could not be determined. Our results support Maire’s (55), Ulbrich’s (88), and Donk’s...
original (17) concepts of close relationships among all stichic taxa.

Petersen (67) segregated small, lichenized, unbranched clavarioid fungi into the genus Multiclava, but suggested that Multiclava belonged in a generic complex with Clavaria (72). Hubbard and Petersen (39) concluded that Juel (42) was likely examining a Multiclava when he described the nuclear state of Clavaria falcata. In 1928, Ulbrich erected the family Stichoclavariaceae, including two genera—Stichoclava, typified by C. falcata, and Stichoramaria, including S. rugosa, S. cristata, S. cinerea, and S. grisea—for stichic clavarioid fungi. Although Ulbrich’s Stichoramaria is a synonym for the older Clavulina, we concur with Hubbard and Petersen’s suggestion that “Stichoclava should be reconsidered as the correct name for the Multiclava complex.”

Our results support Petersen’s segregation of Stichoclava from other clavarioid fungi, but suggest that similarities between Stichoclava and Clavaria are due to convergence; the nearest relatives of Stichoclava are taxa with the same mode of meiotic nuclear division.

The only reportedly stichic genera not represented in this study are the resupinate fungi Clavulicium and Sistotrema. Clavulicium is anatomically very similar to Clavulina (5), while Sistotrema possesses unique urniform basidia that make its relationship to other basidiomycetes difficult to ascertain; no known data contradict a relationship with the stichic clad revealed by our analyses. Because such a wide range of chaotic genera were sampled, it is likely that stichobasidia are indeed uniquely derived and have never been reversed. Still, nuclear behavior during meiosis has yet to be examined in many groups of basidiomycetes. Attempts to identify correlated characters, such as narrow, elongate basidia, have been strongly criticized (19 p 220). For example, Hygrocybe is anatomically very similar to stichic fungi, notably in basidial shape (34), but is reported to be chaotic (55). If stichobasidia are as phylogenetically informative as these results suggest, examination of more taxa may identify other relatives of Cantharellaceae, Hydnaceae, Stichoclavariaceae, and Clavulina.

Euagaric clade.—Although the evolutionary relationships of the remaining clavarioid genera are not definitively resolved by these data, they appear to be nested within the lineage containing the major radiation of gilled mushrooms (Figs. 3, 4). The monophyly of Macrotypula juncea and Typhula phaenorrhiza is well supported (100% bootstrap), suggesting that earlier placements of Macrotypula with Clavariadelphus (14, 43) were erroneous. Clavaria acuta and Clavulinopsis (= Ramaripopsis) fusiformis are monophyletic in most analyses, although without strong bootstrap support. A clade including these clavarioid fungi and Pierula, the mushrooms Hygrocybe, Hygrophorus, Pleurotus, Gerronema, Lentinula, and the polypleurine Fistulinula appears in all of the most parsimonious trees from the combined data (Fig. 4). Although this clade does not receive strong bootstrap support (97%), analyses including more non-cantharellioid or clavarioid taxa and the rest of the nucl-ssu-rDNA provide 97% bootstrap support for the placement of Typhula and Fistulinula with Pleurotus, Lentinula, and other members of the euagaric clade (37). Future mycological studies cannot assume that mushrooms and coral and club fungi represent distinct lineages. Furthermore, it is now clear that coral and club fungi have been derived multiple times from diverse lineages, and do not represent an ancestral group that gave rise to the more complex fruiting forms found in the Basidiomycetes.

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