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PHYLOGENY OF LICHEN- AND NON-LICHEN-FORMING OMPHALINOID MUSHROOMS AND THE UTILITY OF TESTING FOR COMBINABILITY AMONG MULTIPLE DATA SETS

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Abstract.—As an initial step toward developing a model system to study requirements for and consequences of transitions to mutualism, the phylogeny of a group of closely related lichenized and nonlichenized basidiomycetes (*Omphalina*) was reconstructed. The phylogenetic analyses are based on four data sets representing different regions of the nuclear ribosomal repeat unit (ITS1, 5.8S, ITS2, and 25S) obtained from 30 species of *Omphalina* and related genera. The resulting phylogenetic trees from each of these four data sets, when analyzed separately, were not identical. Testing for the combinability of these four data sets suggested that they could not be combined in their entirety. The removal of ambiguous alignments and saturated sites was sufficient, after reapplying the combinability test on the pruned data sets, to explain the topological discrepancies. In this process, the first of two complementary tests developed by Rodrigo et al. (1993, N.Z. J. Bot. 31:257–268) to assess whether two data sets are the result of the same phylogenetic history was found to be biased, rejecting the combinability of two data sets even when they are samples of the same phylogenetic history. Combining the four pruned data sets yielded phylogenies that suggest the five lichen-forming species of *Omphalina* form a monophyletic group. The sister group to this symbiotic clade consists mostly of dark brown *Omphalina* species intermixed with species from the genera *Arrhenia* and *Phaeothellus*. The genera *Omphalina* and *Gerronema* are shown to be polyphyletic. The lichen-forming species *O. ericetorum* and the nonmutualistic species *O. velutipes*, *O. epichysium*, and *O. sphagnicola* are the best candidates for experimental work designed to gain a better understanding of mechanisms involved in symbiotic interactions and the role symbiosis has played in the evolution of fungi. [Basidiomycetes; data set combinability; data set homogeneity; fungi; lichen symbiosis; model system; molecular phylogenies; mutualism; nuclear ribosomal DNA; *Omphalina*; phylogenetic signal.]

The high frequency of mycobiont–photobiont symbiotic associations in nature and the concentration of mutualistic species in specific taxonomic groups strongly suggest that mutualism is an important evolutionary mechanism in the evolution of fungi, cyanobacteria, algae, and land plants (Pirozynski and Malloch, 1975; Cooke, 1977; Malloch et al., 1980; Law and Lewis, 1983; Jansen, 1985; Ahmadjian and Paracer, 1986; Pirozynski and Hawksworth, 1988; Margulis and Fester, 1991; Simon et al., 1993; Lutzoni and Vilgalys, 1995b). Not only have mutualistic interactions been understudied in proportion to their importance (Risch and Boucher, 1978; Boucher, 1985; Hammerstein and Hoekstra, 1995), but most available studies have been large-

ly descriptive (Bronstein, 1991). One of the major reasons for slowed progress in the study of mutualism as an evolutionary mechanism has been the lack of a model system allowing direct empirical investigation of a transitional process from a non-mutualistic to a mutualistic state. Experimental approaches for elucidating mechanisms involved in the origin and evolution of lichenization will require appropriate groups of symbiotic organisms that are amenable to laboratory study and whose phylogenetic relationships are well known. Such a group of fungi, the genus *Omphalina*, was recently proposed as a model system for study of evolutionary mechanisms associated with lichenization (Lutzoni and Vilgalys, 1995b). Because symbiotic interactions (including parasitism, commensalism, and mutualism) are part of a continuum (Lewis, 1985; Templeton and Gilbert, 1985), a better understanding of the evolution of mutualistic in-

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teractions in this system may also provide insight into other symbiotic interactions.

The use of a group of closely related species within which lichenization occurred recently is essential to minimize extinction events and the number of incidental differences between organisms with different nutritional modes. In such a system, the potential for the observed differences to be associated with the transition from a nonmutualistic state to an obligatory mutualistic state would be maximized. *Omphalina* includes several closely related nonmutualistic and mutualistic (lichen forming) species that are very similar phenotypically.

The use of multiple data sets in systematics has raised many unresolved questions and controversies regarding how to best integrate this information in our search to find the true organismal phylogeny (e.g., Kluge, 1989; Swofford, 1991; Bull et al., 1993; de Queiroz et al., 1995; Huelsenbeck et al., 1996). Significant topological differences derived from different data sets can be artifactual or real. Sampling error or the use of an inappropriate evolutionary model for a given data set are examples of artifactual differences; in such cases, the independent data sets can be combined once the source of the discrepancies among data sets has been identified and corrected. When topological differences among independent data sets reflect different phylogenetic histories, e.g., due to lineage sorting, recombination, or gene duplication followed by extinction, these data sets cannot be combined without removing at least one taxon responsible for the conflicting phylogenies (Rodrigo et al., 1993). On the basis of this information, the debate should not be about partitioning versus combining multiple independent data sets but rather about finding which tests are best to use when deciding whether two phylogenetic data sets are combinable or not (Swofford, 1991; Bull et al., 1993; Rodrigo et al., 1993; Hillis, 1995; Cunningham, 1997).

An empirical comparison of three different combinability tests showed that these tests have different properties (Lutzoni

and Vilgalys, 1995a). In that study, the method developed by Rodrigo et al. (1993) was superior to an adapted version of Faith's (1991) T-PTP test and to Kishino and Hasegawa's (1989) likelihood test. The protocol of Rodrigo et al. includes a series of three interdependent tests. The first test is to determine whether trees from different data sets are more similar than are pairs of random trees. The second and third tests are complementary and address whether two different sets of data result from the same phylogenetic history. The second test explores the variability of the estimated phylogenies by bootstrapping the two different data sets and then comparing the trees recovered from the bootstrapped data sets. According to Rodrigo et al., if the spectra of possible topologies from both data sets do not overlap, then the null hypothesis can be rejected that both topologies estimate the same phylogeny. However, if there is some degree of overlap (Rodrigo et al. were not specific about how much overlap is needed; however, it seems that one common topology would be sufficient according to them), the third test is implemented. This third test determines whether the symmetric differences (Penny and Hendy, 1985; Swofford, 1993) between topologies derived from two separate data sets are significantly different from symmetric differences among trees derived from random (bootstrapped) subsets of a single data set. Lutzoni and Vilgalys (1995a) reported that the second test of Rodrigo et al. is the weakest part of the protocol, the number of shared trees being dependent on the number of bootstrap replicates when dealing with small numbers of replicates. Properties of this second test were further investigated in this study.

Taxonomic delimitation of the genus *Omphalina* has not been satisfactory since the genus was first described by Quélet in 1886 (Singer, 1962, 1964, 1975, 1986; Bigelow, 1974; Moser, 1983; Kühner, 1980; Lange, 1981; Clémenton, 1982; Kuyper, 1986; Redhead and Kuyper, 1987). Uncertainty about the limits of this genus is due mainly to the presence of three more or

less distinct groups that have been included and excluded at different times and to the presence of many satellite groups of species with uncertain affinities. The largest of these peripheral groups of species is the genus *Gerronema*. This genus is in itself very heterogeneous and often is used as a default entity for the classification of omphalinoid agarics of unknown affiliation. The present taxonomic status of *Omphalina* seems worse than ever because it also has been entangled in a complex, yet unsettled, nomenclatural riddle for more than 10 years (Redhead and Weresub, 1978; Lange, 1981; Redhead, 1984, 1986; Redhead and Kuyper, 1987, 1988; Jørgensen and Ryman, 1989a, 1989b; Norvell et al., 1994). The genus *Omphalina* is mostly arctic-alpine circumpolar, with some species having distributions extending into the Boreal and Temperate zones. The lichenized species of *Omphalina* are restricted to arctic-alpine environments (Gulden et al., 1985; Gulden and Jenssen, 1988) except for *O. ericetorum*, which is also found in boreal forests (Redhead, 1989). *Omphalina ericetorum* is also the lichenized species with the broadest ecological amplitude.

The first objective of this study was to reconstruct the phylogenetic relationships among a group of closely related mutualistic and nonmutualistic species of *Omphalina* as a first step toward developing a model system for evolutionary studies of mycobiont-photobiont symbioses. The second objective was to demonstrate the usefulness (and weaknesses) of the method developed by Rodrigo et al. (1993) to test for combinability. The underlying goals were to determine the number of independent transitions to mutualism within the genus *Omphalina* and to determine which two most similar species (genotypically), one being lichenized, flank the transitional event. In an attempt to provide additional independent transitions to mutualism for future comparative studies on the causes and consequences of a transition to mutualism (Lutzoni and Pagel, 1997), two species of the lichenized coral fungus *Multiclavula*, one mutualistic species of the omphalinoid genus *Gerronema*, and one mutualistic spe-

cies of the genus *Rickenella* were included. All lichenized *Omphalina* and *Multiclavula* are associated with the unicellular green alga *Coccomyxa*. The species *G. marchantiae* and *R. pseudogrissella* are associated with the thallose liverworts *Marchantia polymorpha* and *Blasia pusilla*, respectively.

MATERIALS AND METHODS

Taxon Sampling

For this study, *Omphalina* was considered to consist of approximately 40 species that can be grouped into three main, and additional minor *incertae sedis* groups (Lutzoni and Vilgalys, 1995b). A total of 17 species were selected to represent the whole spectrum of variation within *Omphalina* based on previous systematic work. Five of these species are obligate mutualists (lichenized species): *O. ericetorum* (Fr.) M. Lange [Lutzoni 930817-2, DUKE; U66445], *O. grisella* (Weinm.) Moser [Lutzoni 930822-6, DUKE; U66443], *O. hudsoniana* (Jennings) Bigel. [Lutzoni 920728-4a, DUKE; U66446], *O. luteovitellina* (Pilát & Nannf.) M. Lange [Lutzoni 930816-8, DUKE; U66447], and *O. velutina* (Quél.) Quél. [Lutzoni 930812-1, DUKE; U66454]. The 12 other species of *Omphalina* included in this study are nonmutualists: *O. brevisidiata* (Sing.) Sing. [Lutzoni 930826-1, DUKE; U66441], *O. epichysium* (Pers.:Fr.) Quél. [Redhead 5223 for ITS1, 5.8S, and ITS2; Redhead 3140 for large subunit nuclear ribosomal RNA (nrRNA) gene; DAOM; U66442], *O. grossula* (Pers.) Sing. [Gulden 417/75, O; U66444], *O. obscurata* Reid [Lamoure L73-101, polyspore culture; U66448], *O. philonotis* (Lasch) Quél. [Lutzoni 930804-5, DUKE and O; U66449], *O. pyxidata* (Pers.:Fr.) Quél. [Lamoure L66-118h14, culture; U66450], *O. rivulicola* (Favre) Lam. [Lamoure L64-41h3, culture; U66451], *O. rosella* (M. Lange) Moser [Redhead 7501, DAOM; U66452], *O. sphagnicola* (Berk.) Moser [Lutzoni 930810-1, DUKE; U66453], *O. velutipes* Orton [Lamoure L77-166h11Xh4, culture; U66455], *O. viridis* (Hornem.) Kuyper [Norvell 930506-4, DUKE; U66456], and *O. wynniae* (Berk. & Br.) Ito [A. H. Smith 82899, MICH; U66457].

To produce a stable delimitation of the genus *Omphalina*, 11 additional species were selected from seven putatively related genera: *Arrhenia auriscalpium* (Fr.) Fr. [Lutzoni 930731-3, DUKE; U66428], *Arrhenia lobata* (Pers.:Fr.) Redhead [Lutzoni & Lamoure 910825 for ITS1, 5.8S, and ITS2; Lutzoni & Lamoure 910824-1 for large subunit nrRNA gene; DUKE; U66429], *Chrysomphalina chrysophylla* (Fr.) Clément. [A. H. Smith 76299 for ITS1, 5.8S, and ITS2, MICH; S. A. Redhead 7700 for large subunit nrRNA gene, DUKE; U66430], *Clitocybe lateritia* Favre [Lutzoni 930803-1, DUKE; U66431], *Gerronema marchantiae* Sing. & Clément. [Lutzoni 910826-1, DUKE; U66432], *G. strobodes* (Berk. & Mont.) Sing. [Kuyper 2984, WBS; U66433], *G. subclavatum* (Peck) Redhead [Redhead 5175, DAOM; U66434], *Hygrocybe citrinopallida* (Smith & Hesler) Kobay [Lutzoni 930731-1, DUKE; U66435], *Phaeotellus griseopallidus* (Desm.) Kühner & Lam. [Lutzoni & Lamoure 910824-4, DUKE; U66436], *Rickenella mellea* (Sing. & Clément.) Lam. [Lamoure 74-20h 1/9.91, poly-spore culture; U66438], and *R. pseudogriseella* (A. H. Smith) Gulden [Lutzoni 930728-3, DUKE; U66437]. Two clavarioid species (Aphylophorales), *Multiclavula vernalis* (Schw.) Petersen [Lutzoni 930806-1, DUKE; U66439] and *M. corynoides* (Pk.) Petersen [Lutzoni 930804-2, DUKE; U66440], were included to provide an additional independent transitional event to mutualism. The nucleotide sequence from the large subunit of *Lentinus lepideus* (Fr.:Fr.) Fr. (Hibbett and Vilgalys, 1993; M98644, M98583, M98618), a nonlichenized species, was used to root the tree.

DNA Isolation, Amplification, Sequencing, and Sequence Alignment

DNA was isolated from herbarium materials or cultures using the DTAB-CTAB method developed by Armaleo and Clerc (1995). A region of about 1.4 kb starting at the 5' end of the large subunit of the nuclear ribosomal DNA (nrDNA) repeat unit was amplified by polymerase chain reaction (PCR) using primers LR0R and LR7 (Moncalvo et al., 1993). The nrDNA inter-

nal transcribed spacer (ITS) region was amplified using primers ITS5 and ITS4 (White et al., 1990). The amplified products were cleaned using low-binding regenerated cellulose 30,000 NMWL filter units (Millipore).

Both strands of the nrDNA large subunit were sequenced using the following primers: LR0R, LR7, LR16, LR5, LR6, LR17R, LR21, LR3R, P2, P2R, and LR3 (Guadet et al., 1989; Moncalvo et al., 1993; Hopple and Vilgalys, 1994; Rehner and Samuels, 1994; Vilgalys and Sun, 1994). Both strands of the nrDNA ITS region (including ITS1, 5.8S, and ITS2) were sequenced using primers ITS5, ITS2, 5.8SR, and ITS4 (Vilgalys and Hester, 1990; White et al., 1990). Sequencing was performed using ABI 373A and ABI 373 stretch automated sequencers.

Sequence fragments were assembled using Sequencher 2.1 (Gene Codes). The nrRNA large subunit sequences were aligned using the same program, and the alignment was optimized by eye and exported as a Nexus file for phylogenetic analyses. Because of major length variation in the ITS region, it was not possible to use the GCG program PILEUP (Devereux et al., 1984) for alignment. Instead, the same concepts used in PILEUP and CLUSTAL (Higgins and Sharp, 1988), i.e., using distance matrices to determine the order in which the sequences are aligned, were applied manually. A distance matrix generated using PAUP 3.1.1 (Swofford, 1993) with the large subunit alignment was used to select the putatively most similar ITS sequence pairs. Sequences and groups of aligned sequences were added in decreasing order of similarity until all 30 sequences from the ITS region were integrated within one alignment. A second distance matrix was generated from this initial ITS alignment and was used to optimize the first ITS alignment.

Combinability and Phylogenetic Structure Tests

The combinability of the different data sets was tested using the part of the Rodrigo et al. (1993) protocol that determines whether or not the observed symmetric

differences measured between all the best trees derived from the different molecular data sets are due to sampling error. This test was applied as described by Lutzoni and Vilgalys (1995a). The complete data sets were found to be incompatible, which was interpreted as indicating that hyper-variable regions of the large subunit and parts of ITS1 and ITS2 were saturated or that the alignments were ambiguous because of large numbers of indels. In either case, these regions were removed. These pruned data sets were then reanalyzed with the same combinability test.

The potential problem with the second test of the Rodrigo et al. (1993) protocol was explored by comparing the pruned 25S data set with a subset of the same data set obtained by randomly deleting half of all characters. This operation was performed using the jackknife resampling option of the program SEQBOOT in PHYLIP 3.53c (Felsenstein, 1993).

Because there is no algorithm designed specifically to delimit hypervariable regions in a given alignment (i.e., regions of an alignment that are ambiguously aligned and that have a high proportion of indels), the alignment was segmented into indel-rich and indel-poor regions by eye by focusing on regions with high numbers of gaps and then delimiting them by identifying flanking regions, ideally without any gaps and without alignment ambiguity. Phylogenetic structure in indel-rich regions of the nrRNA large subunit alignment were tested using the g_1 statistic (Hillis and Huelsenbeck, 1992). Källersjö et al. (1992) reported that this method can give misleading results because it is too sensitive to character state frequencies and not sensitive enough to the number of characters and because it relies on counts of arbitrarily resolved bifurcating trees. For these reasons, the PC test (version 1.3.2; Alroy, 1994) was used as a second test. Because Källersjö et al. argued that the sensitivity of the PTP test (Archie, 1989; Faith and Cranston, 1991) was not restricted to hierarchical phylogenetic patterns but also included patterns of association in general, the PC test, which was designed to correct

this problem, was chosen over the PTP test to determine if the structure in these regions is significantly different from structure resulting from a stochastic process. Alroy's PH test was not used because in the present version it cannot handle multistate characters (Alroy, pers. comm.). The decision to exclude a given gap-rich region from the nrDNA large subunit for subsequent phylogenetic analyses was based on the results of the g_1 statistic, the PC test, and the degree of alignment ambiguity associated with the insertion of gaps in the alignment for this region. When the alignment was very ambiguous for a given gap-rich region, this region was excluded even if it was not significantly noisy, as indicated by the PC test or g_1 statistic. The rationale in these cases was that the structure detected by the PC test or g_1 statistic was an artifact resulting from the large number of gaps needed to align this indel-rich region. Use of the method developed by Gatesy et al. (1993) to describe the amount of alignment ambiguity was not considered necessary because the removal of ambiguously aligned segments was done after the phylogeny was reconstructed using all the data. A combinability test was then implemented with the trees derived from the pruned data sets to confirm that the ambiguous regions removed for the second round of phylogenetic analyses were indeed responsible for the incongruence among different data sets.

All indel-rich regions of both ITS data sets were pruned out, even if they contained a significant amount of signal, because the alignment of these regions was highly ambiguous. The presence of significant signal in these indel-rich regions of ITS1 and ITS2, as revealed by the PC test and g_1 values (results not shown), was interpreted as artifactual. The structure is very likely the result of inserting a large number of gaps in the alignment. Again, such clear cases of ambiguous alignment did not necessitate application of the Gatesy et al. (1993) method for the exclusion of alignment-ambiguous nucleotide sites. In such extreme cases, the sequence align-

ment and the result of phylogenetic analyses probably would vary when sequences are aligned in a different order (Lake, 1991). The PC test and g_1 statistic were only applied to the gap-poor regions of ITS1 and ITS2 to screen out noisy regions from subsequent phylogenetic analyses. As for the gap-rich regions of the large subunit and of both ITS data sets, a high degree of ambiguity in the alignment of the gap-poor regions of ITS1 and ITS2 was sufficient to exclude these regions from subsequent analyses, even if the PC test or g_1 statistic detected significant amounts of signal. The g_1 statistics for each data set and for sections of each data set were estimated by generating tree length distributions with 100,000 random trees using PAUP 3.1.1. For the PC test, gaps were considered a fifth character state and all character states were unordered. For this test, 100 replicates were performed except for borderline cases for which the P values were based on 1,000 replicates.

The complete alignments along with delimitation of the gap-rich/gap-poor regions, g_1 statistics, PC tests, and exclusion/inclusion decisions for each region of the large subunit, 5.8S, and ITS1 and ITS2 are provided in the Appendix.

Phylogenetic Analyses

Phylogenetic analyses were performed using maximum parsimony as implemented in PAUP 3.1.1 (Swofford, 1993) and maximum likelihood as implemented in fastDNAml 1.1.1a (Felsenstein, 1981a; Olsen et al., 1994). All parsimony analyses were done using gaps as a fifth character state. Both equally and unequally weighted parsimony analyses were applied to the combined data set. Using MacClade 3.05 (Maddison and Maddison, 1992), a priori weights for the transformational changes were calculated using the Character Steps option of the Chart menu, restricting the changes from one nucleotide or gap to another nucleotide or gap (e.g., from A to T and from T to A). To avoid circularity, because this procedure necessitates an a priori tree building operation, the tree structure dependence of this procedure

was factored out by using MacClade to calculate the average frequency of restricted changes for 100 random equiprobable trees. This procedure produces estimates comparable to the average frequency of changes calculated from pairwise comparisons of sequences, as generated with the program package MEGA 1.01 (Kumar et al., 1993). The absolute average frequency for all 10 pairs of possible transformational changes (without polarity) was converted to percentages. These probabilities of reciprocal change were converted to costs of changes using the negative natural logarithm of the probability (Felsenstein, 1981b; Wheeler, 1990; Maddison and Maddison, 1992). The costs were rounded to integers, which were used in a symmetric step matrix. The triangle inequality was tested for each step matrix using MacClade 3.05. When the triangle inequality was violated, the values in the step matrix were modified using MacClade's Type Edit dialog box by setting the cost of going between any two states to be the minimum cost along any path between two states. The unequally weighted parsimony analyses were performed by implementing these four step matrices simultaneously on each of the four respective data sets that formed the combined data set. The parsimony heuristic searches were always performed with 1,000 random-addition sequence replicates, TBR branch swapping, MULPARS option on, collapse zero-length branches off, and interpreting multiple character states as partial uncertainties. (A Nexus file of the combined data set is available on the *Systematic Biology* Home Page at <http://www.utexas.edu/depts/systbiol/>.)

The fastDNAml searches on the combined data set were performed with four different categories of evolutionary rates for each of the four molecular data sets (5.8S = 0.60, 25S = 0.92, ITS1 = 1.68, ITS2 = 1.96). These category rates were obtained by calculating the average frequency of nucleotide changes per site using the absolute frequencies of nucleotide substitutions. Gaps were treated as unknowns. The average transition/transversion ratio

TABLE 1. Statistical significance of structure present in four data sets from the nuclear ribosomal DNA (nrDNA) repeat unit for 30 taxa of *Omphalina* and related genera.

Data set	No. nucleotide sites	No. parsimony-informative sites	g_1^a	PC test (P)
Large subunit	1,400	367	-1.048*	<0.001
5.8S	161	19	-0.540*	<0.001
ITS1	474	413	-0.806*	<0.001
ITS2	532	409	-0.756*	<0.001
Pruned				
Large subunit	1,264	249	-1.122*	<0.001
ITS1	40	29	-0.433*	0.230
ITS2	85	62	-0.687*	<0.001
Combined ^b	1,550	359	-1.085*	<0.001

^a All data sets are significantly more structured ($P = 0.01$) than random data as extrapolated from critical values estimated by Hillis and Huelsenbeck (1992).

^b nrDNA 5.8S and all pruned data sets fused to form the combined data set.

estimate (2.14) for the entire combined data set was based on the same absolute values of frequency of nucleotide substitutions. One hundred independent searches were performed using different random number seeds, global rearrangements off, and empirical frequencies on. All the different topologies generated by these 100 searches were used as starting points for global rearrangements using the user tree option and the global option of fastDNAML. All best topologies from the maximum parsimony and maximum likelihood analyses were compared using the Kishino and Hasegawa test (Kishino and Hasegawa, 1989) as implemented in fastDNAML 1.1.1a.

The support for the internodes of the most-parsimonious trees was estimated by 1,000 bootstrap replicates (Felsenstein, 1985) with a heuristic search but with two random-addition sequences for each bootstrap replicate. The support for the internodes of the maximum likelihood tree was estimated by 350 bootstrap replicates, with bootstrapped data sets generated using the SEQBOOT program in PHYLIP. One global search was done on each bootstrapped data set using fastDNAML with four rate categories, the jumble option, and a transition/transversion ratio of 2.14. The 350 searches were performed using the "P4" code, which distributed the computational task to 63 processors in parallel on an Intel Paragon supercomputer. The bootstrap

values were computed for the resulting 350 bootstrapped trees using the majority rule consensus option of PAUP 3.1.1.

RESULTS

Sequence Alignment, Combinability, and Phylogenetic Structure

The alignment of the 30 sequences from the 5' end of the nrDNA large subunit was 1,400 nucleotide positions in length. Of these, 367 positions were parsimony informative. The 5.8S alignment resulted in 161 nucleotide positions, only 19 of which were parsimony informative. The alignments for ITS1 and ITS2 contain a large number of indels (Appendix). The alignment of ITS1 resulted in 474 sites with 413 informative sites, and that of ITS2 resulted in 532 nucleotide positions, of which 409 were informative. All four data sets, when tested in their entirety with both the g_1 statistic and the PC test, contained significant amounts of structure (Table 1). According to the g_1 statistic, the large subunit was the data set with the most structure, followed by ITS1, ITS2, and 5.8S.

To determine if these data sets could be combined in their entirety, the Rodrigo et al. (1993) test was applied to the unpruned nrDNA large subunit, ITS1, and ITS2 data sets as described by Lutzoni and Vilgalys (1995a). This test was not applicable to the 5.8S data because this data set has very little resolving power. Because its incongru-

TABLE 2. Synopsis of results from equally weighted parsimony analyses when implemented with each of the complete and pruned data sets and with the combined data set from the nuclear ribosomal (nrDNA) repeat unit.

Data set	Tree length	No. most-parsimonious trees	Indices ^a			Trees
			CI	RI	RC	
Large subunit	1,206	1	0.458	0.637	0.291	Fig. 1
5.8S	NA ^b	NA	NA	NA	NA	NA
ITS1	1,804	2	0.361	0.574	0.207	Fig. 2a
ITS2	1,887	4	0.355	0.513	0.182	Fig. 2b
Pruned						
Large subunit	705	8	0.499	0.672	0.335	Fig. 4a
ITS1	NA	NA	NA	NA	NA	NA
ITS2	261	14	0.448	0.604	0.271	Fig. 4b
Combined ^c	1,182	3	0.464	0.628	0.291	Fig. 5

^a CI = consistency index; RI = retention index; RC = rescaled consistency index.

^b NA = not applicable. These data sets alone have very little resolving power.

^c nrDNA 5.8S and all pruned data sets fused to form the combined data set.

ence with other data sets is very likely to be weak and not significantly different than expected from sampling error, the 5.8S data set was considered combinable with the other data sets. One, two, and four most-parsimonious trees were recovered when equally weighted parsimony analyses were applied to the entire large subunit, ITS1, and ITS2, respectively (Table 2; Figs. 1, 2). The symmetric difference among these topologies was 26 between the large subunit and ITS1 trees, 28 between the large subunit and ITS2 trees, and 22 between the ITS1 and ITS2 trees (Table 3; Fig. 3).

The first test of the protocol by Rodrigo et al. (1993) was designed to determine whether trees from two data sets are more similar than pairs of random trees. This problem of similarity among trees due to chance is a problem only for data sets with few taxa; there was no need to apply this test in the present study.

The second test of the protocol by Rodrigo et al. (1993) determines whether the differences between topologies obtained from two different data sets can be explained by the variation associated with each data set. The spectrum of possible topologies for each data set is explored by separately bootstrapping the data sets and recovering the most-parsimonious trees, forming two bootstrap tree files, one for each data set. In analyzing those two data

sets, if no bootstrap trees are common to both bootstrap tree files, Rodrigo et al. suggested that the null hypothesis—that both topologies are estimates of the same phylogeny—must be rejected and, therefore, there is no need for their third test, i.e., to determine whether the differences among topologies associated with different data sets are due to sampling error.

When the second test was applied to all data sets (pruned and unpruned), only the pairwise comparison of the tree files from the unpruned and pruned nrDNA large subunit had bootstrap trees in common, with 200 shared bootstrap trees representing 54 different topologies. For example, none of the 1,897 bootstrap trees recovered from 1,000 bootstrap replicates from the entire ITS2 were identical to any of the 219,074 bootstrap trees recovered from the pruned version of ITS2. Because these pruned data sets probably share the same phylogenetic history (topology) with their respective complete data sets, this result suggested that the second test of the protocol by Rodrigo et al. (1993) might be biased toward rejecting data set combinability.

To explore this hypothesis, this second test by Rodrigo et al. (1993) was applied to the pruned 255 data set (1,264 characters) and a subset where half of the characters were randomly deleted. Tree files generated by 100 bootstrap replicates and

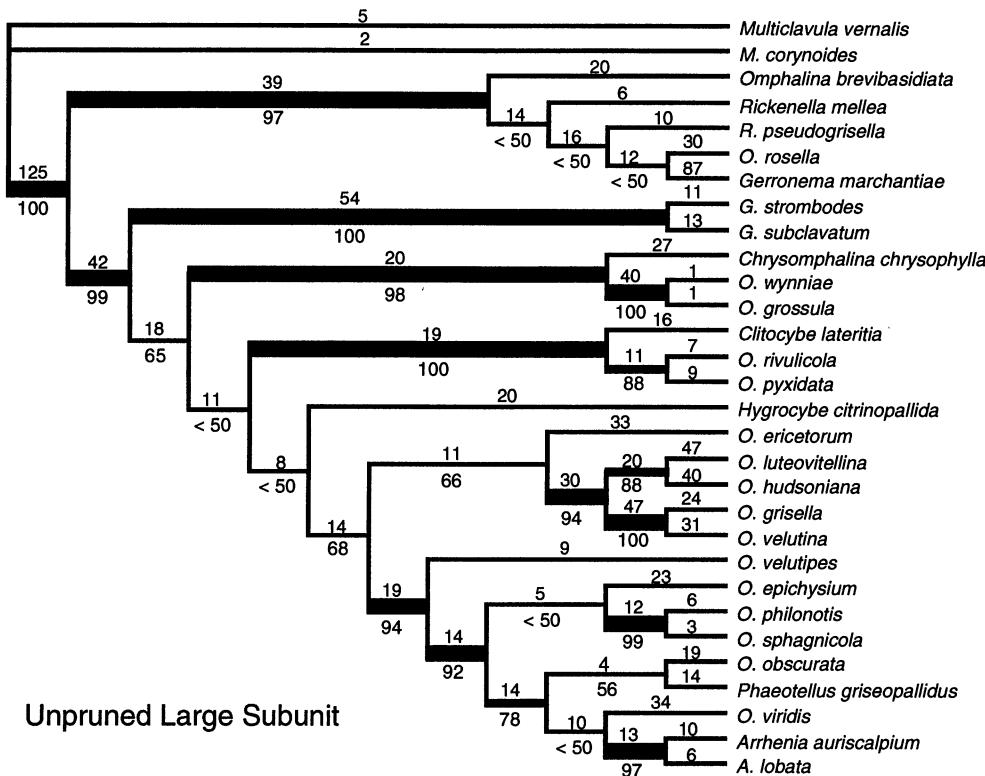


FIGURE 1. Single most-parsimonious unrooted tree from an equally weighted analysis of the nrDNA large subunit of lichenized and nonlichenized omphalinoid mushrooms. In this analysis, the ambiguous and saturated regions of the alignment were not removed. The number above each branch is the number of unambiguous character state changes, and the number below is the percentage of 1,000 bootstrap replicates supporting the same binary partition shown. The thick internodes have bootstrap values of 90–100% inclusively, the intermediate internodes have values of 70–89% inclusively, and the narrow internodes have values ≤69%.

500 bootstrap replicates were compared (Table 4). This investigation with two data sets known to sample the same phylogenetic history (topology) confirms that this second step in the protocol of Rodrigo et al. is biased toward rejecting the combinability of two data sets when comparing a data set with high resolving power (1,264 characters, Table 4) with a data set with low resolving power (632 characters, Table 4). For all 10 comparisons with 100 bootstrap replicates, no trees were ever found in common even when comparing a tree file of >50,000 bootstrap trees with a tree file of >1,000 bootstrap trees (Table 4). However, trees were found in common for this same case when 500 bootstrap replicates were done (Table 4). Of the four data sets in this study, some have high and oth-

ers have low resolving power; therefore, this part of the Rodrigo et al. protocol was not used to decide whether two data sets could be combined.

The combinability criterion used in this study corresponds to the third part of the protocol of Rodrigo et al. (1993), which determines whether the symmetric differences (Penny and Hendy, 1985; Swofford, 1993) between topologies derived from two separate data sets are significantly different from symmetric differences among trees derived from random (bootstrap) subsets of one of the two data sets. This test is applied reciprocally to both data sets compared. The first step is to determine the symmetric differences among the most-parsimonious trees of two different data sets (Table 3). These observed sym-

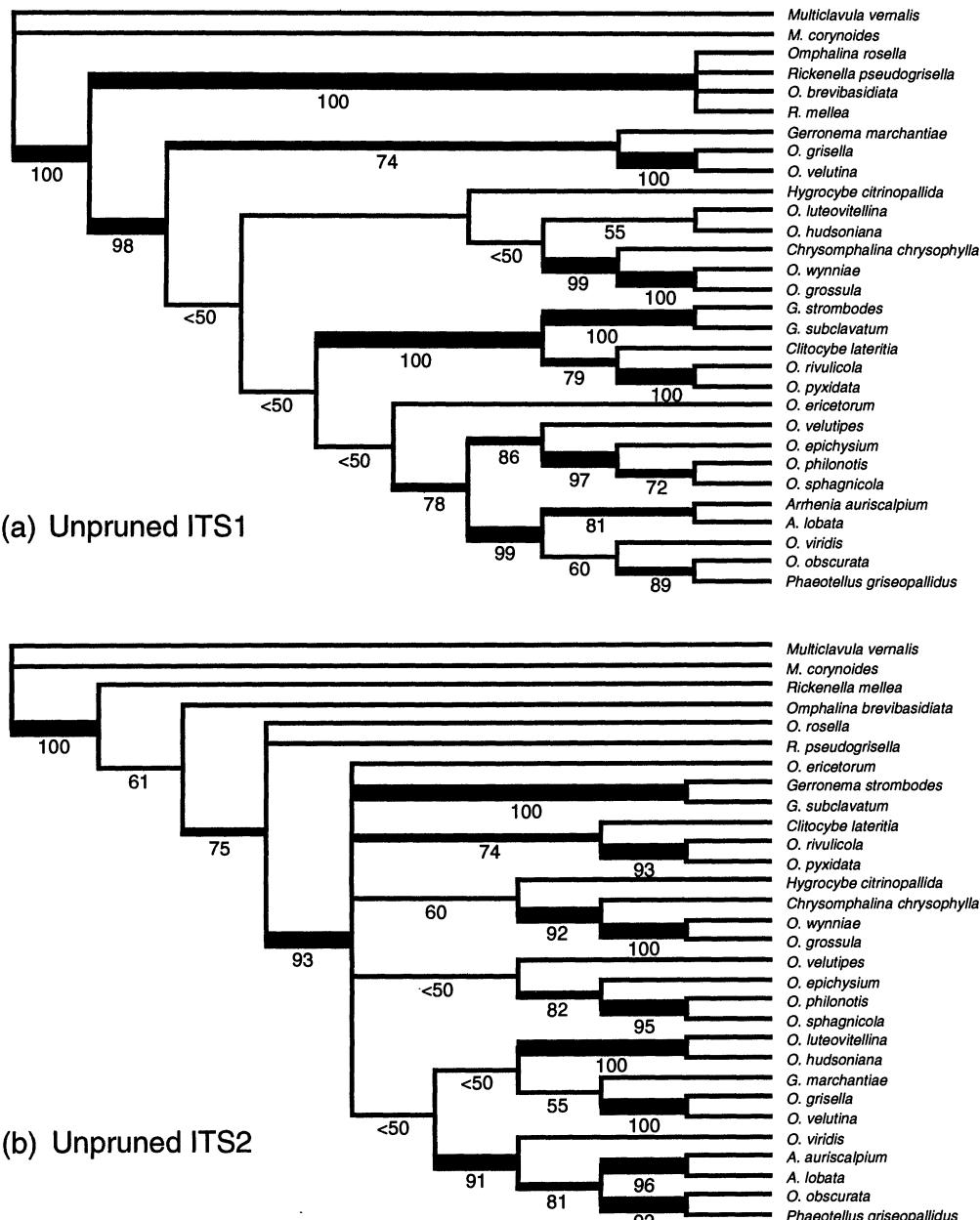


FIGURE 2. Strict consensus of unrooted most-parsimonious trees for lichenized and nonlichenized omphaloid mushrooms. In both analyses, the ambiguous and saturated regions of the alignment were not removed. The number below each internode is the percentage of 1,000 bootstrap replicates supporting the same binary partition shown. The thick internodes have bootstrap values of 90–100% inclusively, the intermediate internodes have values of 70–89% inclusively, and the narrow internodes have values $\leq 69\%$. (a) Unrooted strict consensus tree of the two most-parsimonious trees generated from an equally weighted analysis of the entire ITS1 region. (b) Unrooted strict consensus tree of the four most-parsimonious trees generated from an equally weighted analysis of the entire ITS2 region.

TABLE 3. Matrix of observed symmetric differences when comparing equally weighted most-parsimonious trees derived from data sets from the nrDNA repeat unit. The number after the colon represents the observed symmetric difference; the number before the colon indicates its frequency. The parts of Figure 3 (e.g., 3a, 3b) listed indicate the respective histograms showing the frequency distribution of expected symmetric differences when two data sets are samples of the same phylogenetic history.

	Large subunit	5.8S	ITS1	ITS2	Pruned		
					Large subunit	ITS1	ITS2
Large subunit	—						
5.8S	NA ^a	—					
ITS1	2:26 3a, 3c	NA	—				
ITS2	4:28 3a, 3d	NA	8:22 3c, 3d	—			
Pruned large subunit	1:6 2:8 1:10 3:12 1:16 3a, 3b	NA	2:28 6:30 6:32 2:34 3b, 3c	4:28 4:30 20:32 4:34 3b, 3d	—		
Pruned ITS1	NA	NA	NA	NA	NA	—	
Pruned ITS2	1:28 3:30 6:32 4:34 3a, 3e	NA	4:32 8:34 16:36 3c, 3e	8:32 16:34 32:36 3d, 3e	1:28 5:30 45:32 29:34 40:36 20:38 3b, 3e	NA	—

^a NA = not applicable because at least one of the two data sets compared has very little resolving power.

metric differences were then compared with the null distribution of expected symmetric differences between trees obtained from two series of 100 independent bootstrap data sets sampled from one of the two data sets compared (Fig. 3). Because the two series of bootstrap replicates are from the same data set, this null distribution represents the differences expected among trees derived from data sets sampling the same phylogenetic history (topology). The results of these pairwise tests are summarized in Table 5. The only unpruned data sets that were consistently combinable in both reciprocal tests were ITS1 and ITS2.

The first possible reason for this significant incongruence between the large subunit and the two ITS regions was the presence of regions with ambiguous alignments and/or noise. For the large subunit, 14 indel-rich regions were delimited (Appendix). Each indel-rich region was tested using the PC test and g_1 statistic to determine

whether the region was significantly different from noise. Only 4 of these 14 regions were unambiguously aligned and significantly different from noisy data. The 10 other indel-rich regions were therefore excluded to form the pruned nrDNA large subunit data set. A total of 136 positions were removed from the large subunit, none from the 5.8S, 434 from ITS1, and 447 from ITS2 (Table 1).

ITS1 and ITS2 were subdivided into a total of 30 intermittent indel-poor and indel-rich regions (Appendix). The results of the PC test and the g_1 statistic and the degree of alignment ambiguity for the 16 indel-poor regions of the two ITS data sets were used to further prune these two data sets. As a result, five and three of these gap-poor regions were excluded from the ITS1 and ITS2, respectively.

The same phylogenetic and combinability tests were carried out on the pruned large subunit and pruned ITS2 data sets. The pruned ITS1 was considered combin-

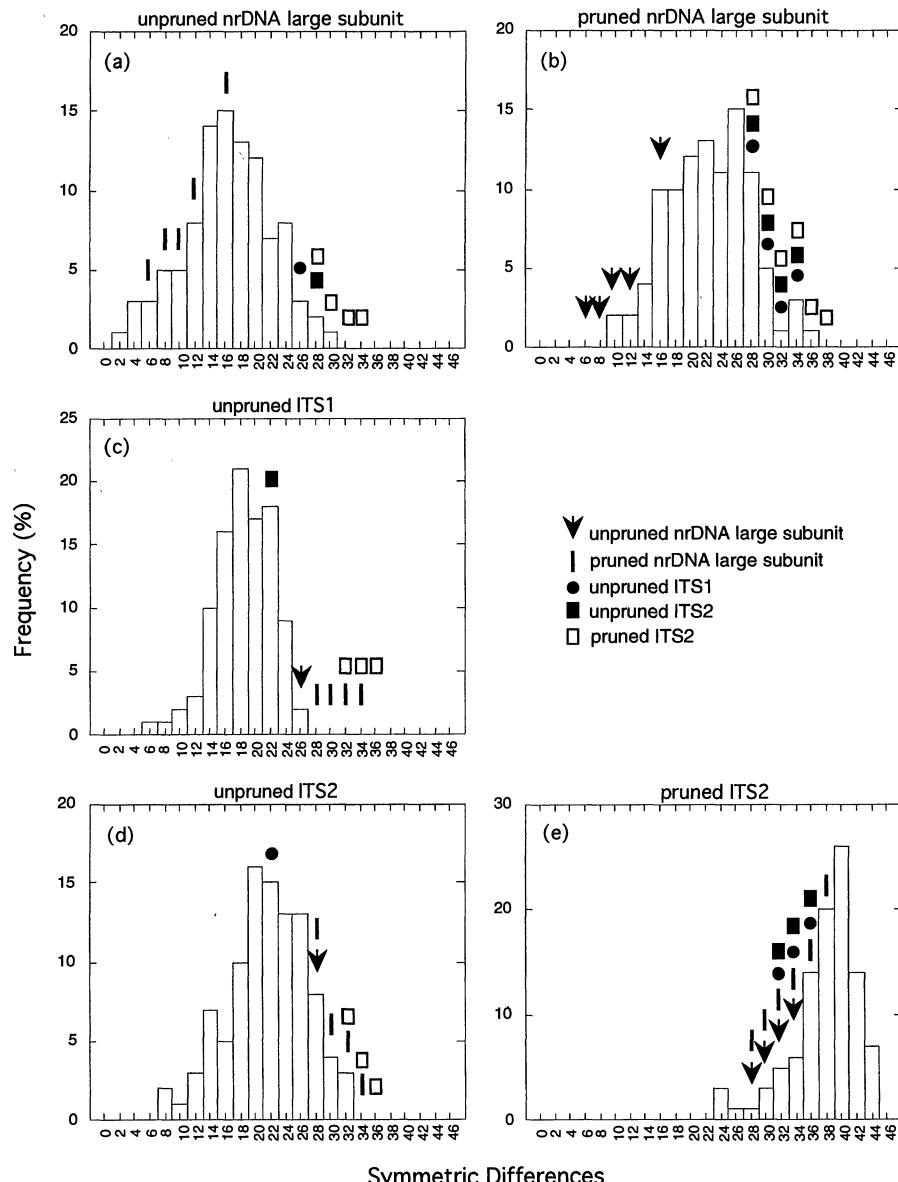


FIGURE 3. Null distribution of expected symmetric differences between most-parsimonious trees obtained from two sets of 100 bootstrapped data sets sampled from the same original data set (data set indicated above each null distribution). This procedure was applied to five of the seven data sets in Tables 3 and 4. The symbols represent the observed symmetric differences between most-parsimonious trees from two different data sets for omphaloid mushrooms. For example, in (a) ● = the observed symmetric difference between the most-parsimonious trees from the unpruned nrDNA large subunit and those from the unpruned ITS1 (see also Tables 3, 5). The smallest observed symmetric difference was used for the test when comparisons involving equally parsimonious trees generated multiple observed symmetric differences. For example, in (a) the observed symmetric difference of 28 was used to test whether the pruned ITS2 tree could be combined with the unpruned nrDNA large subunit tree.

TABLE 4. Properties of the second test of the Rodrigo et al. (1993) protocol, when two data sets are known to be sampling the same phylogenetic history. All comparisons are between bootstrap tree files obtained from the pruned 25S data set. In the first comparison, both data sets (1 and 2) compared have high resolving power. In the second series of comparisons, one data set has more resolving power than the other data set with which it is compared. In the third series of comparisons, both data sets compared have low resolving power. These three series of comparisons were done with 100 bootstrap replicates and with 500 bootstrap replicates. The third column shows the number of trees shared between the two tree files.

Comparison	No. bootstrap replicates	No. bootstrap trees		
		Set 1	Set 2	Shared
High ^a and high resolutions	100	1,162	1,038	10
		1,162	1,211	33
		1,038	911	14
		1,038	949	24
		911	949	41
		1,162	911	47
		1,162	949	35
		1,038	1,211	14
		911	1,211	35
		1,211	949	26
High and low ^b resolutions	500	5,713	5,615	3,790
		1,162	50,771	0
		1,038	17,215	0
		911	63,304	0
		1,211	26,092	0
		949	47,908	0
		1,038	50,771	0
		1,162	17,215	0
		911	47,908	0
		949	26,092	0
Low and low resolutions	500	1,211	63,304	0
		5,615	205,290	6
		5,713	146,829	14
		50,771	17,215	6
		50,771	63,304	72
		50,771	26,092	206
		50,771	47,908	24
		17,215	63,304	68
		17,215	26,092	40
		17,215	47,908	6
500	205,290	63,304	26,092	24
		63,304	47,908	122
		26,092	47,908	102
		205,290	146,829	4,184

^a High = complete data set (1,264 nucleotide sites).

^b Low = half of the data set (632 nucleotide sites), obtained by jackknifing.

able for the same reasons stipulated for the complete 5.8S data set, i.e., because of its low resolving power, incongruences with other data sets are very likely to be not significantly different from what is expect-

ed by chance alone. More than 100 informative sites for the large subunit were lost in this pruning process. The effect of data pruning was very drastic for the two ITS regions, especially ITS1 where only 29 of 413 parsimony-informative sites were considered reliable (Table 1). The g_1 values increased for the large subunit but decreased for both ITS regions as a result of this pruning. However, the three pruned data sets still contained a significant amount of signal according to the g_1 statistic; only the pruned ITS1 data set contained a significant amount of noise according to the PC test (Table 1). This decrease in signal is mostly due to the removal of artifactual structure and not to the removal of phylogenetic signal.

The equally weighted parsimony analysis revealed 8 most-parsimonious trees for the pruned large subunit versus 1 from the unpruned data set and 14 most-parsimonious trees for the pruned ITS2 versus 4 from the unpruned ITS2 data set (Table 2; Fig. 4). The consistency index (CI), retention index (RI), and rescaled consistency index (RC) values increased in both cases for the pruned data sets, suggesting that this approach was successful in removing noise from each of these data sets. The pruning of the large subunit did not cause a significant loss of resolution or overall bootstrap support. Some internodes even gained support as a result of this procedure (Figs. 1, 4a). As expected for ITS2, the pruning of ambiguously aligned and noisy regions drastically reduced the resolution of phylogenetic relationships because of the loss of 347 putative informative sites (Table 1; Figs. 2b, 4b). Despite this massive exclusion of sites, the g_1 did not change much, and the CI, RI, and RC values increased substantially.

The pruning of putative noisy regions from the data sets had two effects on the null distribution of expected pairwise symmetric differences of the combinability test. For both the large subunit and ITS2, the distribution became narrower and was shifted to the right (Figs. 3a, 3b, 3d, 3e). This shift was caused by the greater probability of generating bootstrap trees with

TABLE 5. Summary matrix of the results from the combinability test (applying the third part of the protocol described by Rodrigo et al., 1993) as shown in Figure 3. Numbers shown are *P* values.

	Large subunit	5.8S	ITS1	ITS2	Pruned		
					Large subunit	ITS1	ITS2
Large subunit	—	NA ^a	C ^b 0.06	NC ^c 0.03	C 0.94	NA	NC 0.03
5.8S	NA	—	NA	NA	NA	NA	NA
ITS1	NC 0.02	NA	—	C 0.29	NC 0.00	NA	NC 0.00
ITS2	C 0.15	NA	C 0.56	—	C 0.15	NA	NC 0.03
Pruned large subunit	C 1.00	NA	C 0.21	C 0.21	—	NA	C 0.21
Pruned ITS1	NA	NA	NA	NA	NA	—	NA
Pruned ITS2	C 0.96	NA	C 0.92	C 0.92	C 0.96	NA	—

^a NA = not applicable because at least one of the two data sets compared has very little resolving power.

^b C = combinable.

^c NC = not combinable.

larger differences because of the loss of resolving power resulting from pruning informative (but noisy) characters. This pruning also had the beneficial effect of eliminating most of the incongruence among data sets ($P = 0.15$ and 0.03 when testing the combinability of the unpruned ITS2 and nrDNA large subunit vs. $P = 0.96$ and 0.21 when testing the pruned versions of these data sets). The result of filtering out noisy data was that data sets that could not be combined previously were combinable after pruning one or both data sets (Figs. 3b, 3e; Table 5). Only the pairwise tests restricted to pruned data sets (Table 5) consistently showed combinability. Based on these results, the pruned large subunit, the 5.8S, the pruned ITS1, and the pruned ITS2 were combined into one data set to estimate the phylogenetic relationships among *Omphalina* species and related genera. This combined data set included 1,550 sites, 359 of which were parsimony informative (Table 1). The g_1 value for this combined data set was -1.085 . Only the pruned large subunit data set had more structure ($g_1 = -1.122$) according to this statistic.

Phylogenetic Relationships

The equally weighted parsimony analysis of the combined data set yielded three

most-parsimonious trees (Table 2; Fig. 5a). The CI, RI, and RC values for these topologies were intermediate between the values for the pruned large subunit and the values for the pruned ITS2. These three topologies are most similar, but not identical, to the most-parsimonious trees produced with the pruned data set from the large subunit, followed by the most-parsimonious tree from the unpruned large subunit.

The statistical analyses of the four individual data sets, which constituted the combined data set, revealed that each data set has unique characteristics. The average transition/transversion ratio for each data set was 3.80 for 5.8S, 2.05 for 25S, 1.05 for ITS2, and 0.91 for ITS1. The two most similar data sets, in terms of cost of changes among character states, were the pruned ITS1 and pruned ITS2 (Fig. 6). The pruned large subunit and the 5.8S data sets were the next most similar in this regard. The unequally weighted parsimony analysis of the combined data set generated two most-parsimonious topologies (Fig. 5b). The results from this analysis were very similar to those from the equally weighted analysis. One of these two topologies was identical to one of the three most-parsimonious topologies obtained from the equally

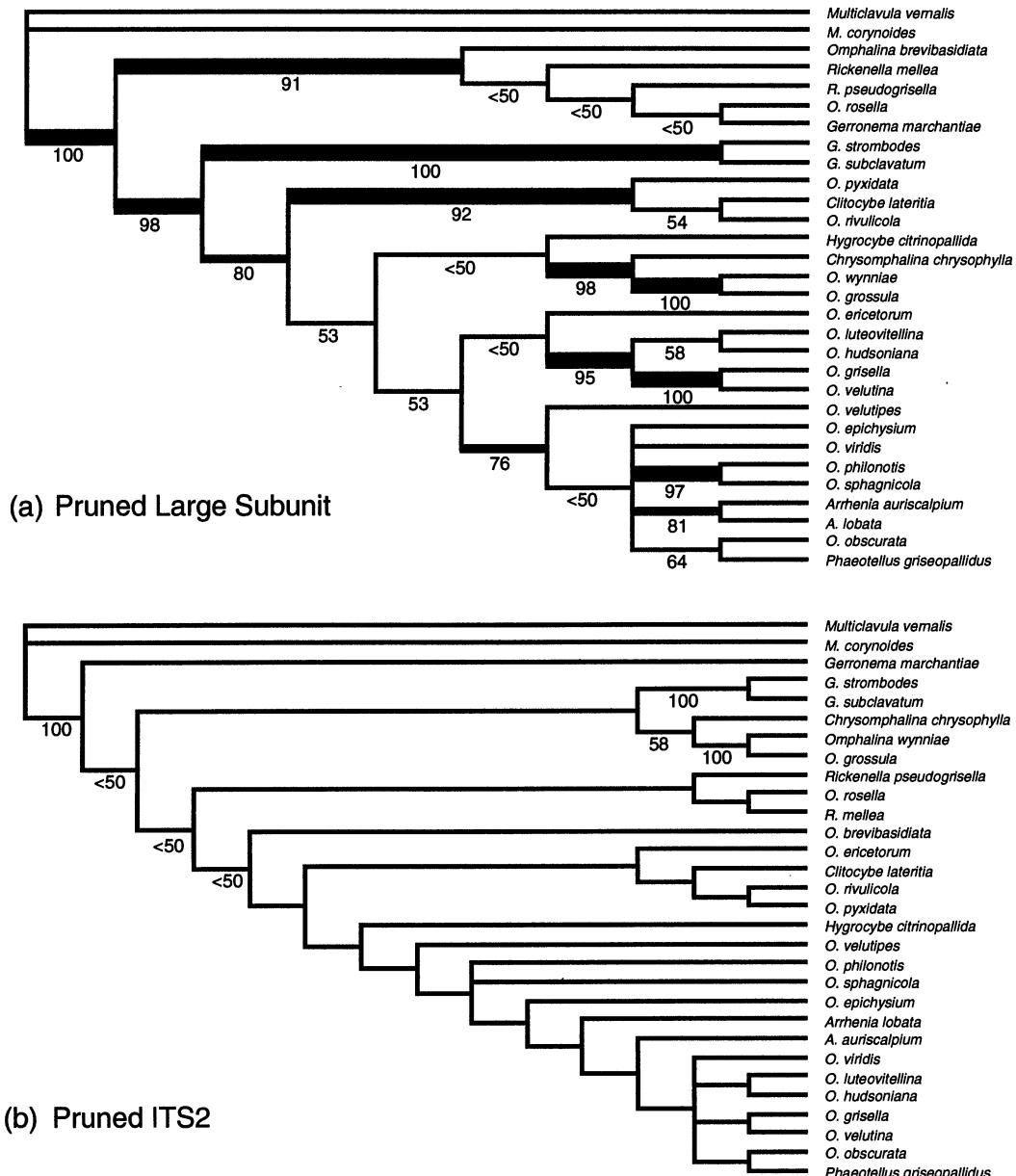


FIGURE 4. Majority-rule consensus of unrooted most-parsimonious trees for lichenized and nonlichenized omphalind mushrooms. In both analyses, the ambiguous and saturated regions of the alignment were removed. The number below each internode is the percentage of 1,000 bootstrap replicates (100 bootstrap replicates for pruned ITS2) supporting the same binary partition shown. The thick internodes have bootstrap values of 90–100% inclusively, the intermediate internodes have values of 70–89% inclusively, and the narrow internodes have values ≤69%. (a) Consensus of the eight most-parsimonious trees for the pruned nrDNA large subunit. (b) Consensus of the 14 most-parsimonious trees for the pruned ITS2.

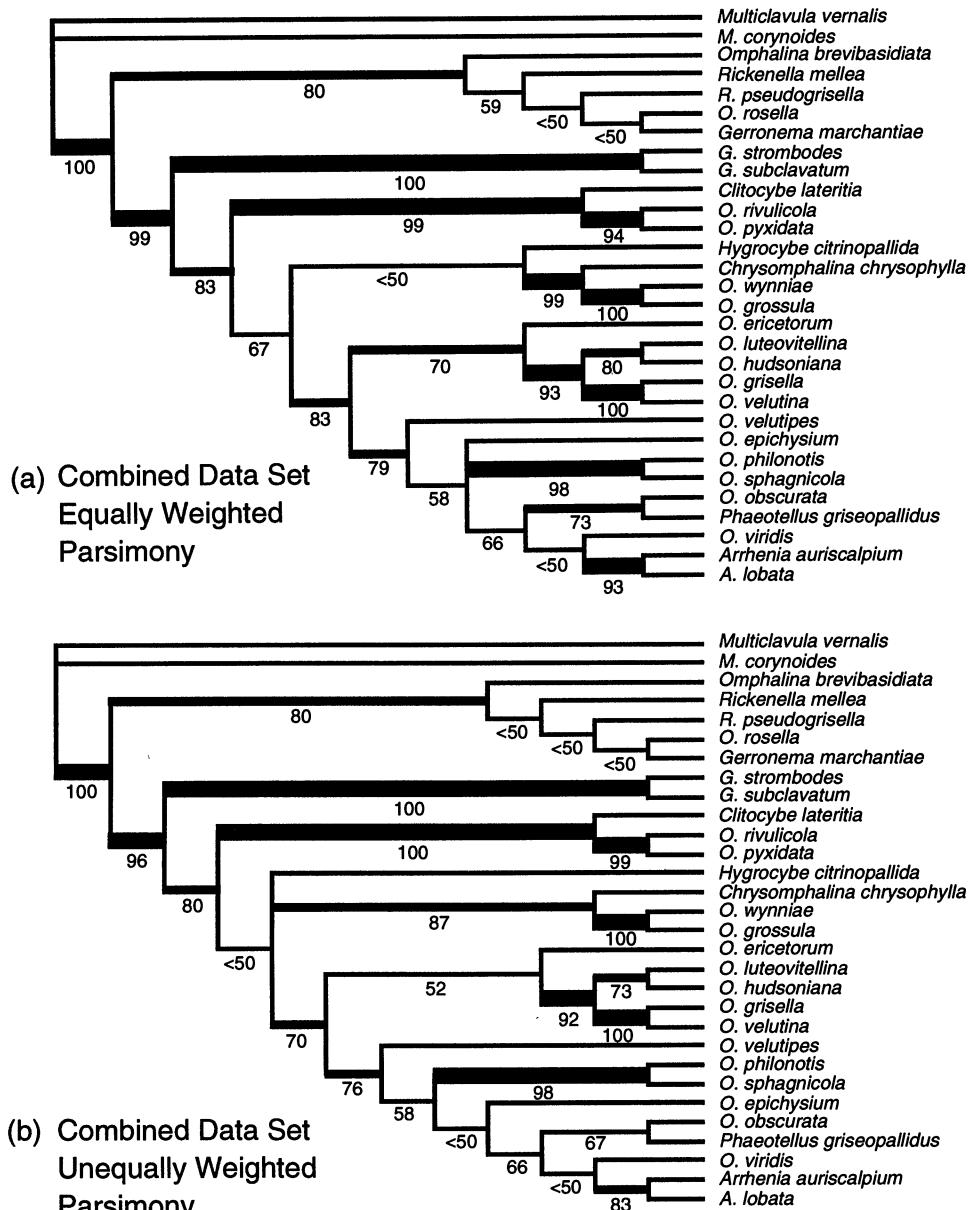


FIGURE 5. Unrooted majority-rule consensus trees from parsimony analyses of combined omphaloid mushroom data set consisting of the fusion of the pruned large subunit nrDNA, 5.8S, pruned ITS1, and pruned ITS2 data sets. In both analyses, the ambiguous and saturated regions of the alignment were removed. The number below each internode is the percentage of 1,000 bootstrap replicates supporting the same binary partition shown. The thick internodes have bootstrap values of 90–100% inclusively, the intermediate internodes have values of 70–89% inclusively, and the narrow internodes have values ≤69%. (a) Consensus of three most-parsimonious trees generated from equally weighted analysis. (b) Consensus of two most-parsimonious trees generated from unequally weighted analysis.

pruned LSU					5.8S						
A	C	G	T	-	A	C	G	T	-		
[A]	.	3	1	2	3	[A]	.	3	1	3	3
[C]	3	.	3	1	4	[C]	3	.	3	1	4
[G]	1	3	.	3	3	[G]	1	3	.	3	4
[T]	2	1	3	.	3	[T]	3	1	3	.	5
[-]	3	4	3	3	.	[-]	3	4	4	5	.

pruned ITS1					pruned ITS2						
A	C	G	T	-	A	C	G	T	-		
[A]	.	3	4	3	2	[A]	.	3	3	3	2
[C]	3	.	4	2	2	[C]	3	.	3	2	2
[G]	4	4	.	3	2	[G]	3	3	.	3	2
[T]	3	2	3	.	1	[T]	3	2	3	.	1
[-]	2	2	2	1	.	[-]	2	2	2	1	.

FIGURE 6. Individual step matrices indicating costs of changes among character states calculated for the four omphalinoid mushroom data sets included in the combined analysis. These step matrices were simultaneously implemented for the unequally weighted analysis of the combined data set. LSU = nrDNA large subunit; - = gaps.

weighted parsimony analysis of the combined data set. Bootstrap values were lower when using step matrices (Fig. 5).

Ten different topologies were recovered from the 100 local rearrangement searches with random addition sequences using fastDNAML. Global rearrangement searches with each of these 10 topologies used as a starting point for branch swapping converged on the three most likely topologies obtained from the local rearrangement procedure (Table 6). The most likely tree recovered using the maximum likelihood optimization criterion (\ln likelihood = -7370.71124) was by far the topology most frequently recovered (60%) during the search process with the fastDNAML program (Fig. 7; Table 6).

The tree length and log likelihood were calculated for each of the five most-parsimonious trees from the maximum parsimony (equally and unequally weighted) analyses of the combined data set and from the three best likelihood trees. None of the best trees recovered by maximum parsimony were more likely than the best trees recovered using maximum likelihood, and vice versa (Fig. 8). This result suggests that the tree space was thoroughly explored using the maximum likelihood and maximum parsimony approaches. De-

TABLE 6. Maximum likelihood phylogenetic analyses. Results from 100 random addition sequences with local rearrangements, followed by global rearrangements on all different topologies obtained with local rearrangements.

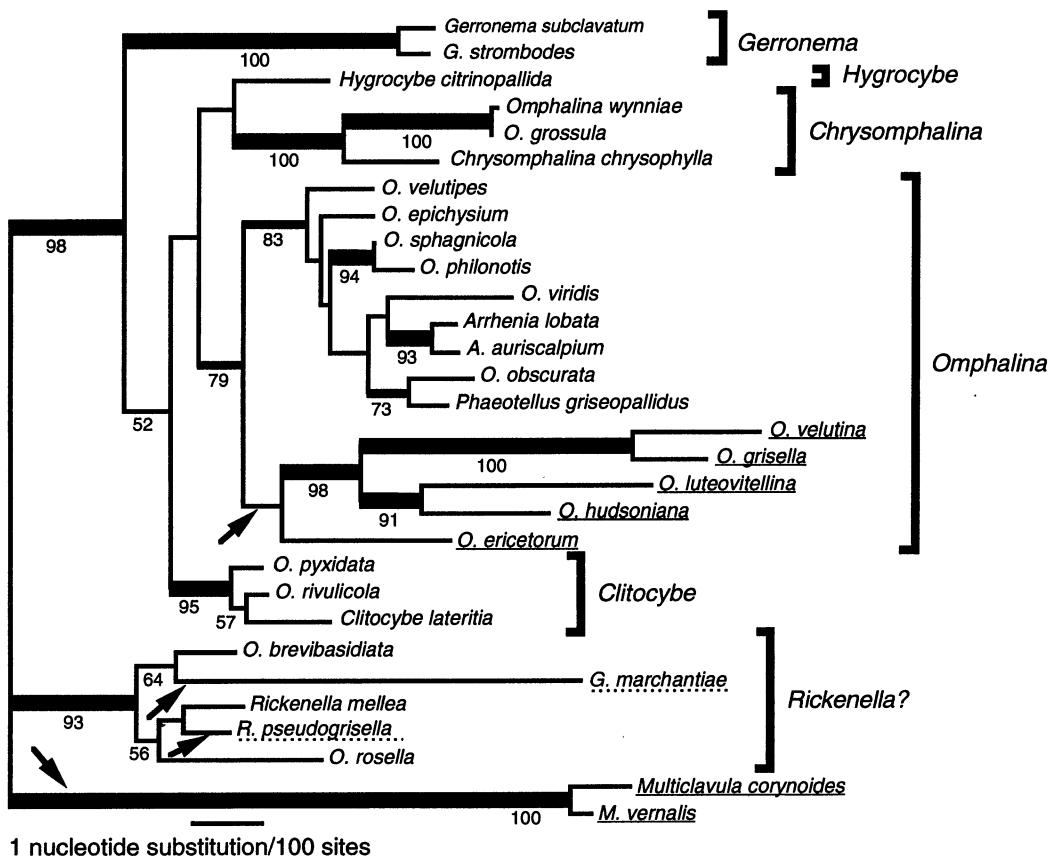
Log likelihood	Frequency (%)	Local rearrangements		Log likelihood	Global rearrangements:
-7370.71124	36			-7370.71124	
-7371.26639	9			-7371.26639	
-7371.62575	19			-7371.62575	
-7372.44002	8			-7371.26639	
-7372.62855	23			-7370.71124	
-7373.71281	1			-7370.71124	
-7377.68027	1			-7371.26639	
-7380.01859	1			-7371.62575	
-7380.58516	1			-7371.26639	
-7393.69493	1			-7371.62575	

spite the apparent large differences (in terms of log likelihoods and tree lengths) between the likelihood trees and the parsimony trees (Fig. 8), none of these topologies were significantly worse than any other tree for explaining the data (Table 7).

DISCUSSION

Best Estimate of Phylogenetic Relationships among Omphalinoid Mushrooms

All best topologies based on the combined data set, generated with maximum parsimony and maximum likelihood analyses, were very similar (Figs. 5, 7). However, among the eight best phylogenetic estimates using the maximum parsimony and maximum likelihood optimization criteria, the most likely topology (\ln likelihood = -7370.71124) recovered using maximum likelihood was preferred as the best estimate of the relationships among omphalinoid mushrooms (Fig. 7, ML1 of Fig. 8). This choice was based on the following observations. The five most-parsimonious trees obtained from the equally and unequally weighted parsimony analyses (Figs. 5, 8) consistently show *Omphalina rosella* as sister species to *Gerronema marchantiae*, with the two *Rickenella* species and *O. brevibasidiata* forming a grade. *Omphalina rosella* and *G. marchantiae* have the longest terminal branches in this clade of five species with short internodes (Figs. 7,



Combined Data Set

Maximum Likelihood

FIGURE 7. Unrooted, most likely topology ($\ln \text{likelihood} = -7370.71124$) resulting from maximum likelihood analyses of combined omphalinoid mushroom data set consisting of the fusion of the pruned nrDNA large subunit, 5.8S, pruned ITS1, and pruned ITS2 data sets. The dotted underline indicates taxa forming an obligatory mutualistic association with a thallose liverwort. Underlined taxa are lichen-forming species. Arrows indicate branches where the four independent transitions to mutualism took place, using parsimony as the optimization criterion (MacClade 3.0). Generic names associated with square brackets delimiting monophyletic groups are guidelines derived from this study for the future reclassification of omphalinoid mushrooms. The number below each internode is the percentage of 350 bootstrap replicates supporting the same binary partition shown. Bootstrap values $\leq 50\%$ are not shown. The thick internodes have bootstrap values of 90–100% inclusively, the intermediate internodes have values of 70–89% inclusively, and the narrow internodes have values $\leq 69\%$.

9). The three most likely trees obtained with maximum likelihood suggest that *G. marchantiae* is a sister species to *O. brevispiciata* and that the two *Rickenella* species form a monophyletic group. The genus *Rickenella* (Raithelhuber, 1973) is one of the most homogeneous genera of all those with an omphalinoid habit (Lamoure, 1979). Moreover, based on simulation stud-

ies by Kuhner and Felsenstein (1994), parsimony is expected to perform poorly when compared with maximum likelihood for recovering the correct topology when dealing with unequal substitution rates on different branches. The morphological data, the topological characteristics, and simulation studies strongly suggest that the topology selected using the maximum

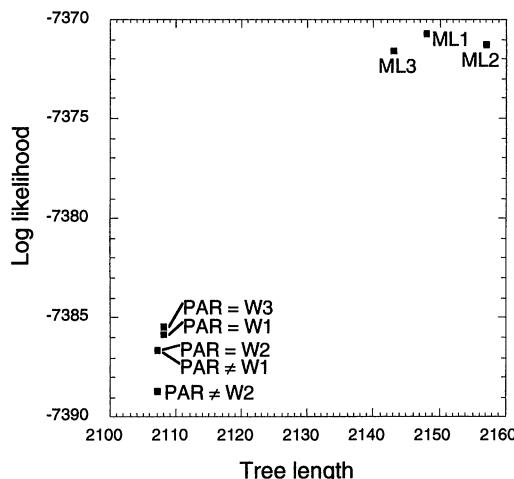


FIGURE 8. Topology comparison on tree length and log-likelihood axes of the most-parsimonious trees recovered by the parsimony analyses and the most likely trees recovered by the likelihood analyses of the omphalinoïd mushroom data. ML1–ML3 represent the three most likely trees. PAR = W1–W3 represent the three most-parsimonious trees recovered from the equally weighted parsimony analyses; PAR ≠ W1 and W2 represent the two most-parsimonious trees recovered by the unequally weighted parsimony analyses.

likelihood criterion is more likely to be correct than that based on parsimony for that portion of the tree.

Mapping unambiguous transformational changes and their genomic source on the most likely tree demonstrates the advantage of using multiple data sets (Fig. 9). One of the major contributions of the combined data set was to provide complemen-

tary support at critical internodes. The best example is internode 1 (Fig. 9), where most of the support is provided by the pruned ITS2 data set. This result demonstrates that even rapidly evolving molecules such as ITS1 and ITS2 can be very helpful in resolving deep internodes once the ambiguous portions of the alignment and the saturated sites have been removed. Conversely, transformational changes in conserved regions such as the 5.8S are distributed toward the tips of the tree (Fig. 9). These results support the idea that it is preferable to obtain more data from different molecules than to gather more from the same molecule (Cummings et al., 1995). For internodes 2–8, half or more of the support came from the pruned ITS2, pruned ITS1, and/or the 5.8S data sets. Internode 3 had 70% bootstrap support in the equally weighted parsimony analysis. This internode is the most critical area of the tree because it supports the monophyly of the lichenized *Omphalina* species. More than half the support at this internode was provided by the 5.8S and pruned ITS1.

The Utility of Testing for Combinability

Combinability testing can indicate whether data sets should or should not be combined in their entirety; it is also a tool for exploring the specific characteristics of each data set that cause phylogenetic incongruence. Hypothetical factors that cause phylogenetic discrepancies can be

TABLE 7. Kishino and Hasegawa (1989) likelihood test as implemented in fastDNAMl 1.1 (Olsen et al., 1994) on most likely and most-parsimonious trees generated from the combined data set. None of these trees were significantly worse than any other for explaining the data.

Tree	Log likelihood	Difference of log likelihood	SD
Most likely tree*	-7370.71124		
Second most likely tree	-7371.26639	-0.55515	10.1992
Third most likely tree	-7371.62575	-0.91451	13.3747
Most-parsimonious equal-weight tree 3	-7385.42607	-14.71483	14.7666
Most-parsimonious equal-weight tree 1	-7385.83291	-15.12167	15.4106
Most-parsimonious equal-weight tree 2	-7386.60575	-15.89451	15.3619
Most-parsimonious unequal-weight tree 1	-7386.60575	-15.89451	15.3619
Most-parsimonious unequal-weight tree 2	-7388.73590	-18.02466	16.5177

* This tree is the best tree when using the maximum likelihood criterion. A given tree is considered significantly worse than the most likely (best) tree in explaining the observed nucleotide sequences, when the log likelihood difference is more than twice the standard deviation.

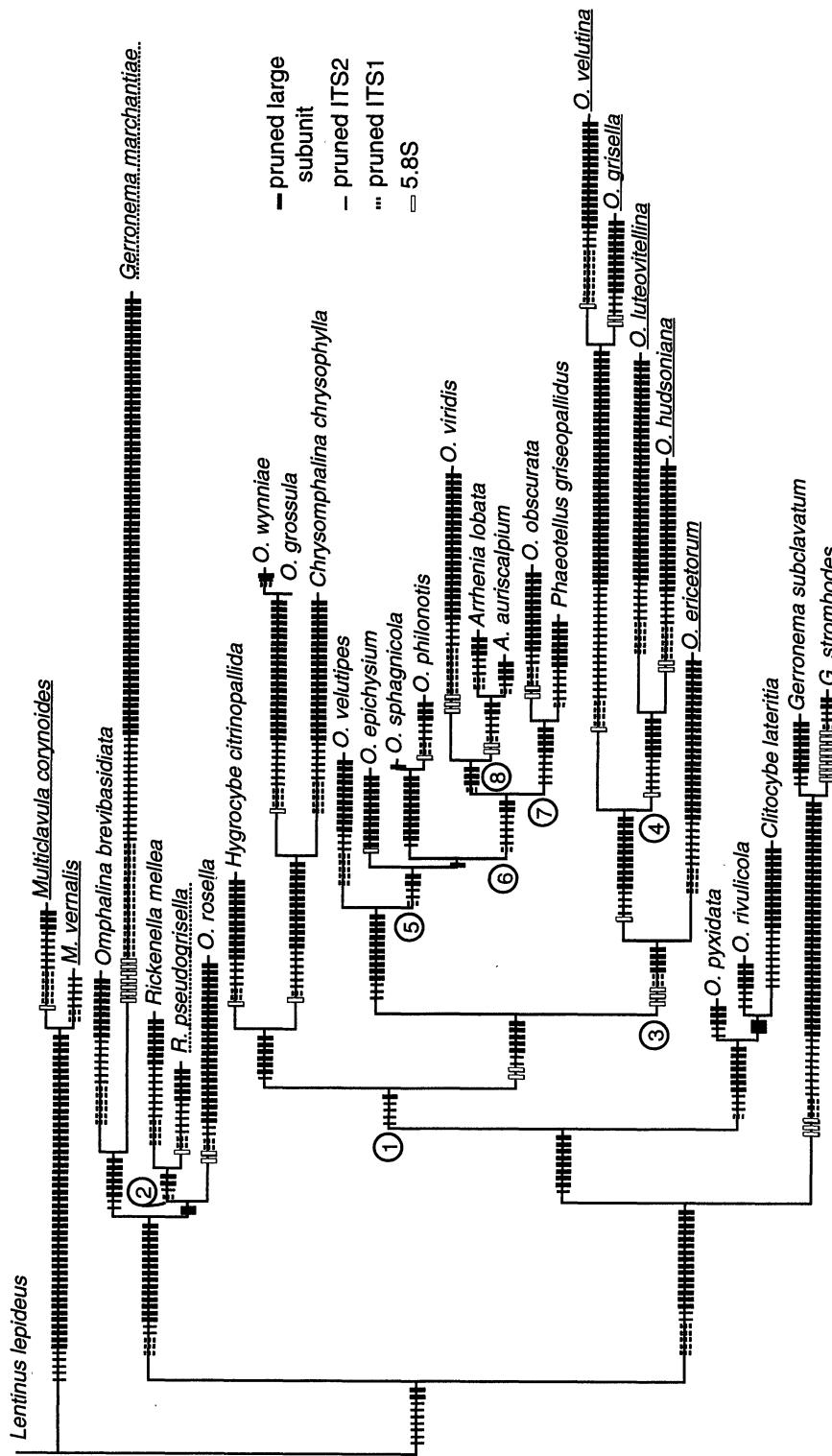


FIGURE 9. Distribution of observed unambiguous transformational changes for each of the four data sets on the most likely ($\ln \text{likelihood} = -7370.71124$) tree depicting phylogenetic relationships among *Omphalina* species and related genera. Circled numbers indicate internodes where $\geq 50\%$ of the synapomorphies are not from the nrDNA large subunit. Underlined taxa are lichenized. Dotted underlines indicate taxa forming an obligatory mutualistic association with thallose liverworts.

investigated by removing or correcting them one at a time and reapplying the test. In this study, testing for combinability confirmed that the significant incongruences between the nrDNA large subunit and the two ITS regions were mainly due to the large number of gaps and saturated sites present in ITS1 and ITS2. The strong support for the best estimates of the omphalinoid phylogeny provided in the present study is due to (1) using multiple data sets, (2) testing for combinability, and (3) removing noise from the data sets. Each of these three components contributed toward this strong support, but the combinability test was pivotal in this analytical process.

Bias in the Second Step of Rodrigo et al.'s Homogeneity Test

The second of the three steps proposed by Rodrigo et al. (1993) determines whether two suites of data are the result of the same phylogenetic process by exploring the variation associated with each data set. Our results indicate that this test is biased, especially when comparing one data set with high resolving power with another data set with low resolving power. This bias was demonstrated by comparing bootstrap tree files from two data sets known to sample the same phylogenetic history, i.e., one data set was a random subset of the original data set. For such cases, the null hypothesis that the two data sets are samples of the same phylogenetic history was consistently rejected when only 100 bootstrap replicates were used (Table 3). With 500 bootstrap replicates, very few trees were found in common, even though this is an ideal case where we know that both data sets are indeed sampling the same phylogenetic history (Table 3). In a real situation, independent data sets are likely to be more incongruent even if they still are derived from the same phylogenetic process. In such situations, it is very likely that no bootstrap trees would be found in common, even with 500 bootstrap replicates.

Using a high number of bootstrap replicates to perform this part of the Rodrigo

et al. (1993) test involves generating large tree files that are awkward to manipulate and require considerable CPU time to compare. Moreover, as the number of taxa increases, more bootstrap replicates will be required to recover common trees when comparing data sets with unequal resolving power. Unfortunately, this situation is common in phylogenetic studies. For example, morphological data sets with many operational taxonomic units often have low resolving power, but they are still critical in our search for the true organismal phylogeny (Lutzoni and Vilgalys, 1995a; Pryer et al., 1995). The situation is the same for multiple molecular data sets; in this study, the resolving power of the pruned ITS2 data set was not as high as that of the pruned 25S data set, but the ITS2 data were essential for resolving and increasing the level of confidence at several internodes (Fig. 9). For these reasons, it is preferable to skip this second part of the Rodrigo et al. method and proceed to their third test.

Testing for Phylogenetic Structure

Although the idea of filtering out saturated regions from molecular data sets that are subjected to phylogenetic analyses is very appealing, there is as yet no satisfactory way of implementing this idea. At least two aspects need to be considered when assessing phylogenetic signal for non-protein-coding sequences: (1) alignment ambiguity due to the insertion of gaps and (2) saturation of nucleotide sites. As the proportion of gaps to nucleotides increases, often so do the number of ways to reach the same overall optimal similarity or likelihood for a given portion of the alignment. The favored arrangement of these gaps can be assigned based on unambiguous regions of the alignment or on putative models for insertion and deletion processes (see Gatesy et al., 1993). Depending on the order in which the sequences are aligned (Lake, 1991), this practice can introduce structure where there might be very little or none. In this study, the gap-rich regions of ITS1 and ITS2 almost always had a significant amount of structure

according to both the PC test and the g_1 statistic ($P < 0.01$, data not shown) despite the extreme amount of alignment ambiguity in these regions. Gap-rich regions are associated with the most rapidly evolving regions of the nrDNA unit and are expected to be prone to saturation. Nevertheless, these regions have a significant amount of structure according to current tests. Therefore, the strict use of these tests for detecting structure in non-protein-coding sequences is not advisable, especially in gap-rich regions, and they should be used only after removing regions that are ambiguously aligned.

In gap-poor regions with no, or very little, ambiguity in the alignment, there were many discrepancies between the different tests for structure. The g_1 statistic using Hillis and Huelsenbeck's (1992) critical values was a very weak test. None of the regions tested in this study (including all gap-rich regions and the entire ITS1 and ITS2 data sets) were noisy according to this test (Appendix). The PC test was more stringent; 14 of the 30 regions tested were not significantly different from randomly permuted data ($P = 0.05$). These two tests also behaved in a contradictory manner. For instance, the GP.4 region of ITS1 (Appendix) had the second lowest g_1 value (-0.341) for ITS1, suggesting that it is noisier than other regions, but the signal was highly significant with the PC test. Within the same molecule, GP.2 and GP.7 (Appendix) have the most skewed distributions ($g_1 = -3.978$ and -3.370 , respectively), suggesting very strong structure, but have the highest P values with the PC test (0.718 and 0.995, respectively), strongly suggesting that these regions were not different from randomly permuted data. Similar patterns can be observed in the ITS2 and 25S data.

Perhaps a more appropriate method for testing for phylogenetic structure would be the use of regression analyses for the observed pairwise distances versus expected (corrected for multiple hits) distances (Moritz et al., 1992). Unfortunately, this method cannot account for the presence of gaps, which is essential when at-

tempting to detect phylogenetic structure in rapidly evolving regions of nrDNA. This area of phylogenetics needs to be explored further, and new methods proposed must be compared using both simulations and empirical studies.

Classification Guidelines for the Genus Omphalina

None of the classifications proposed in the past completely match the monophyletic groupings revealed by the present phylogenetic study (Fig. 7). In this study, the lichen-forming *Omphalina* species represent a monophyletic group (the *O. velutina*–*O. ericetorum* clade). This grouping agrees with the taxonomic conclusions of Redhead and Kuyper (1987) and Norvell et al. (1994) that the lichenized *Omphalina* species form a natural group. Their proposals to recognize this lichen clade at the generic level is not in contradiction with the results presented here. However, the bootstrap support for the lichen clade is only 70% in the equally weighted parsimony analysis, 52% in the unequally weighted parsimony analysis, and <50% in the maximum likelihood analysis (Figs. 5, 7). More extensive studies might show that *O. ericetorum* is basal to the sister group of the lichen clade or to the genus *Omphalina* as delimited in Figure 7. With the present knowledge of relationships among *Omphalina* species and for nomenclatural stability, I recommend that the lichenized clade and its sister group be maintained in the same genus.

Model Species for Studies on Mycobiont–Photobiont Symbiotic Interactions

One of the goals of this phylogenetic study was to identify transition(s) to a lichen state that occurred during the evolution of omphalinoid mushrooms and to determine which two species (one lichenized and the other nonlichenized) are most similar, genetically and phenotypically, to the ancestors flanking the transition. All best phylogenetic estimates from this study of the relationships among *Omphalina* species (Figs. 7–9) suggest that there was a single transition to a mutualistic state during

the evolution of *Omphalina* (internode 3, Fig. 9). If molecular rates of evolution are any indication of the overall rate of species evolution, *O. ericetorum*, with the slowest evolutionary rate among the lichen-forming *Omphalina* species, is likely to be the most similar extant species to the lichenized ancestor of this clade (Fig. 7). *Omphalina velutipes*, *O. sphagnicola*, and *O. epichysium*, three basal species of the sister group to this lichen clade that have the slowest rates of nucleotide substitutions, are probably most similar to the ancestral lineage in which the transition to a lichen state occurred. Redhead and Kuyper (1987) reported that *O. epichysium*, a lignicolous species, can also be found associated with bryophytes and algae. They further suggested that these facultative fungus–alga and fungus–bryophyte associations are precursors to the development of lichenization. Future experimental molecular and developmental work on these species should show the advantages of using a phylogenetic approach in molecular genetic studies of symbiotic associations and in building stronger links between the two fields.

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APPENDIX

Alignment of the entire ITS1, 5.8S, ITS2, and the 5' end portion of the nrDNA large subunit sequenced from 28 omphalinoid mushroom species and two coral fungus species of the genus *Multiclavula*. N = unknown; – = gap; • = variable but parsimony-uninformative site; + = parsimony-informative site. For ITS1 and ITS2, the underlined parts of the alignment (single continuous line) are indel-rich regions that were excluded from phylogenetic analyses because of high alignment uncertainties, regardless of the g_1 statistic and PC test result. Parts of the alignment underlined with double dashed lines represent more conserved regions. For ITS1 and ITS2, variable sites are shown only for these conserved regions and were the only ones tested for phylogenetic signal. For the nrDNA large subunit, underlined (hypervariable) regions were tested for the presence of phylogenetic signal. The g_1 statistic and PC test result are shown under each conserved region of ITS1 and ITS2 and for the hypervariable regions of the large subunit, with the decision to include or exclude these regions from phylogenetic analyses. The alignment is also posted on the *Systematic Biology* Home Page at <http://www.utexas.edu/depts/systbiol/>.

ITS2 (check bracketed) ->	
A-----GTCGTATTCAGTTCCTATCCTATGACCG	GCT-----GAGATACCCGCGTGTATACGGCTGT
A-----ATTGTGTTATGCAATT-A	-AATTCAGTGGCTTGAACTTACGAGCACTAGTC
O. hudsoniana	
O. grisea	
O. weberi	
O. virens	
O. virilicola	
O. viridis	
O. obscurata	
A. arisicabium	
H. citrinopallidus	
Citocybe lateritia	
O. rivulicola	
O. pseudagrissella	
Chrysomphalina chrysophylla	
O. grossula	
O. myrriae	
Germonia strobmodes	
G. sublavatum	
O. brevisetigera	
R. kennedia melaea	
A. pseudagrissella	
O. tessula	
G. merchantiae	
M. corynoides vermalis	
M. corynoides	

#331

<----ITS2	
A-----CCCTAACGCTPAC-TTTC-T-O-TGA-C-AAC	
A-----TT-G-TTG-A-TCA-C-AAC	
O. hudsoniana	
O. grisea	
O. weberi	
O. virens	
O. exickerum	
O. velutipes	
O. epithysium	
O. philonotis	
O. sphagnicola	
O. viridis	
H. citrinopallidus	
O. obscurata	
A. arisicabium	
H. citrinopallidus	
Citocybe lateritia	
O. rivulicola	
O. pseudagrissella	
Chrysomphalina chrysophylla	
O. grossula	
O. myrriae	
Germonia strobmodes	
G. sublavatum	
O. brevisetigera	
R. kennedia melaea	
A. pseudagrissella	
O. tessula	
G. merchantiae	
M. corynoides vermalis	
M. corynoides	

GP 6
PC Least P = 0.265
S1 = 0.647
Included

<----ITS2	
O. hudsoniana	
O. grisea	
O. weberi	
O. virens	
O. exickerum	
O. velutipes	
O. epithysium	
O. philonotis	
O. sphagnicola	
O. viridis	
H. citrinopallidus	
O. obscurata	
A. arisicabium	
H. citrinopallidus	
Citocybe lateritia	
O. rivulicola	
O. pseudagrissella	
Chrysomphalina chrysophylla	
O. grossula	
O. myrriae	
Germonia strobmodes	
G. sublavatum	
O. brevisetigera	
R. kennedia melaea	
O. roseola	
G. merchantiae	
M. corynoides vermalis	
M. corynoides	

#496

<i>Emphalina luteovitelina</i>	CTAAAGGATTCCTTAATGCGGAACTGCAACTTGCGGGTCAACCTTTGCGGCCGT-CCCTTGCGGCGCGGGTG-GT-CCTT-TGGGGCTTCGCGGTATTGA-TTGGGGCGTCTGGA-CCTTGTCAAGGATCCTTTGCGA-GCTAACTAAATTAACTCGGATGTT-
<i>O. hudsoniana</i>	
<i>O. grisea</i>	
<i>O. velutina</i>	
<i>O. elaeocerorum</i>	
<i>O. velutipes</i>	
<i>O. epichrysium</i>	
<i>O. philonotis</i>	
<i>O. sparsa</i>	
<i>O. pyriformis</i>	
<i>Pheuctellus griseopallidus</i>	
<i>O. obscurata</i>	
<i>A. lobata</i>	
<i>A. arizicalium</i>	
<i>Brycore B. citrinopallidus</i>	
<i>Citocoris lateritiae</i>	
<i>O. rufincola</i>	
<i>O. pseudosella</i>	
<i>O. rosea</i>	
<i>Chrysomphalina chrysophylla</i>	
<i>O. grossula</i>	
<i>O. wynniae</i>	
<i>Gerronema stroboides</i>	
<i>G. subulatum</i>	
<i>O. brevisubulata</i>	
<i>Rickeia R. meliae</i>	
<i>O. pseudopseuda</i>	
<i>O. rosea</i>	
<i>G. merchaniae</i>	
<i>Mutillia vernalis</i>	
<i>M. cyathoides</i>	#1

GR. 1
PC test: P = 0.000
N = 0.309
EXCLUDED

<i>Emphalina luteovitelina</i>	CCCTCTTCAGGCCTCA-TGGCGGCTTGCCTTGATGTTGGCAGGCGAACTGCGGTAACTGCGGGTCAACCTTTGCGGCCGT-CCCTTGCGGCGCGGGTG-GT-CCTT-TGGGGCTTCGCGGTATTGA-TTGGGGCGTCTGGA-CCTTGTCAAGGATCCTTTGCGA-GCTAACTAAATTAACTCGGATGTT-
<i>O. Hudsoniana</i>	
<i>O. grisea</i>	
<i>O. velutina</i>	
<i>O. elaeocerorum</i>	
<i>O. velutipes</i>	
<i>O. epichrysium</i>	
<i>O. philonotis</i>	
<i>O. sparsa</i>	
<i>Pheuctellus griseopallidus</i>	
<i>O. obscurata</i>	
<i>A. lobata</i>	
<i>A. arizicalium</i>	
<i>Brycore B. citrinopallidus</i>	
<i>Citocoris lateritiae</i>	
<i>O. rufincola</i>	
<i>O. pseudosella</i>	
<i>O. rosea</i>	
<i>Chrysomphalina chrysophylla</i>	
<i>O. grossula</i>	
<i>O. wynniae</i>	
<i>Gerronema stroboides</i>	
<i>G. subulatum</i>	
<i>O. brevisubulata</i>	
<i>Rickeia R. meliae</i>	
<i>O. pseudopseuda</i>	
<i>O. rosea</i>	
<i>G. merchaniae</i>	
<i>Mutillia vernalis</i>	
<i>M. cyathoides</i>	#166

GR. 2
PC test: P = 0.000
N = 0.465
EXCLUDED

