Evolution of Marine Mushrooms

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Fungi make up one of the most diverse, ecologically important groups of eukaryotes. The vast majority of fungi are terrestrial, but the chytridiomycetes, a basal group of fungi, includes flagellated, unicellular, aquatic forms, and it is likely that this was the ancestral condition of the group (1). The more derived groups of fungi—zygomycetes, ascomycetes, and basidiomycetes—are all predominantly filamentous and terrestrial, and lack flagellated cells at any stage of the life cycle. Within the basidiomycetes, the most conspicuous group is the homobasidiomycetes, which includes about 13,000 described species of mushrooms and related forms. Eleven species of homobasidiomycetes (in eight genera) occur in marine or freshwater habitats. To resolve the relationships among terrestrial and aquatic homobasidiomycetes, we assembled a data set of ribosomal DNA (rDNA) sequences that includes 5 aquatic species and 40 terrestrial species. Phylogenetic trees obtained using parsimony and maximum likelihood (ML) methods suggest that there have been three or four independent transitions from terrestrial to aquatic habitats within the homobasidiomycetes. Three of the marine taxa in our data set are associated with mangroves, suggesting that these ecosystems provide a common evolutionary stepping-stone by which homobasidiomycetes have reinvaded aquatic habitats.

One of the major themes in the evolution of eukaryotes is the repeated transition from aquatic to terrestrial habitats that has occurred in several major clades, including fungi, plants, and animals. In a classic paper, Pirozynski and Malloch (2) suggested that fungi and plants were the first eukaryotes to colonize the land, and that this ecological shift was made possible by the establishment of mycorrhizal symbioses (associations involving fungal hyphae and plant roots). This hypothesis has been bolstered by the recent discovery of spores of putatively mycorrhizal fungi from the Ordovician (3). Fungi have radiated extensively in terrestrial habitats, where they play pivotal ecological roles, as decayers, pathogens, and symbionts of plants and animals.

One group of fungi that is elegantly adapted to life on the land is the homobasidiomycetes. Adaptations to terrestrial habitats displayed by some homobasidiomycetes include rootlike rhizomorphs that allow the fungi to forage along the forest floor, drought-resistant sclerotia, and tough, perennial fruiting bodies. Aerial spore dispersal in homobasidiomycetes is accomplished by a forcible discharge mechanism termed ballistospory. However, several lineages of terrestrial homobasidiomycetes have lost ballistospory, including puffballs and other forms with enclosed spore-bearing structures.

Aquatic homobasidiomycetes include four species that have retained ballistospory and seven species that have lost ballistospory. The ballistosporic forms can be tentatively assigned to certain terrestrial groups on the basis of the morphology of spores and fruiting bodies (4–12). However, the aquatic homobasidiomycetes that have lost ballistospory have no obvious close relatives among terrestrial groups. This taxonomically enigmatic assemblage includes several marine genera that have elongate or appendaged spores, which superficially resemble the spores of many aquatic ascomycetes (4, 5; Fig. 1).

To resolve the relationships among terrestrial and aquatic homobasidiomycetes, we assembled a data set that includes 4 marine species, 1 freshwater species, and 40 diverse terrestrial species (Fig. 2). The heterobasidiomycete "jelly fungus" *Auricularia auricula-judae* was included for rooting purposes. The data set contains sequences of four rDNA regions, including nuclear and mitochondrial small and large subunit rDNA (3.8 kb total). Four species in the data set lack the mitochondrial large subunit rDNA sequence (Fig. 2). Sequences from 38 terrestrial species and one marine species, *Nia vibrissa*, were drawn from earlier studies (13, 14).
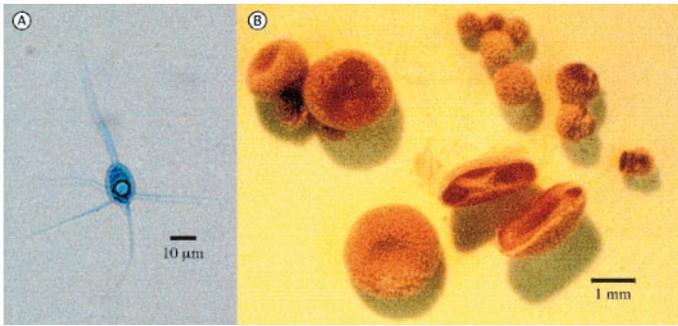
Figure 1. Appendaged spore (A) and enclosed fruiting bodies (B) of the marine homobasidiomycete *Nia vibrissa.*

Parsimony analysis (15) resulted in two shortest trees (5175 steps, consistency index (CI) = 0.372, retention index (RI) = 0.410), and ML analysis resulted in one optimal tree (−lnL = 29962.65066; Fig. 2). In all trees, the aquatic species occur in four separate lineages (Fig. 2). There are two equally parsimonious reconstructions of shifts between terrestrial and aquatic habitats (on all three trees). One reconstruction suggests that there have been four independent transitions from terrestrial to aquatic habitats (Fig. 2A), whereas the other reconstruction suggests that there have been three shifts to aquatic habitats and one reversal from aquatic to terrestrial habitats (Fig. 2B). Under the latter scenario, the terrestrial species *Cyphellopsis anomala* would be derived from marine ancestors.

All of the aquatic species in our data set are nested in a strongly supported group (parsimony bootstrap = 90%/ML bootstrap = 99%) called the euagarics clade, which has been estimated to contain roughly 7400 species (57%) of homobasidiomycetes (Fig. 2; 16). Most members of the euagarics clade are typical mushrooms, with a cap, gills, and (often) a stalk. Familiar taxa in our data set include the cultivated button mushroom *Agaricus bisporus* and the mycorrhizal "fly agaric" *Amanita muscaria.* The ancestor of the euagarics clade was probably a gilled mushroom (14), but contemporary aquatic fungi bear scant resemblance to such forms, as described below.

Three marine species in our data set, *Halocyphina villosa, Calathella mangrovei,* and *Physalacria maipoensis* are ballistosporic, have exposed spore-bearing surfaces, and occur in intertidal mangrove communities. *Halocyphina villosa* and *Calathella mangrovei* produce "cyphelloid" fruiting bodies, which are minute (*ca.* 0.3–1.0 mm diameter), cup-shaped structures, whereas *Physalacria maipoensis* produces a "capitate" fruiting body, which has a globose head on a short stalk (*ca.* 0.5–2.5 mm high; 8, 9, 11). The genera *Calathella* and *Physalacria* each include terrestrial species, as well as the marine species sampled here (9, 11). *Halocyphina* contains only one species, but Ginns and Malloch

(8) suggested that it could be closely related to the terrestrial cyphelloid genera *Henningsomyces* or *Rectipilus.* Consistent with this prediction, our results suggest that the terrestrial cyphelloid genera *Henningsomyces* and *Cyphellopsis* are closely related to marine homobasidiomycetes (Fig. 2).

The remaining aquatic species in our dataset, *Nia vibrissa* (marine) and *Limnoperdon incarnatum* (freshwater), have lost ballistospory and produce spores inside minute (*ca.* 0.3–1.2 mm diameter), enclosed, puffball-like fruiting bodies (5, 7, 17, 18; Fig. 1). *Nia vibrissa* is further distinguished by having appendaged basidiospores (Fig. 1). *Nia vibrissa* and *Limnoperdon incarnatum* bear a superficial resemblance to terrestrial puffballs, but their phylogenetic relationships have been obscure. Our results indicate that *Nia vibrissa* is strongly supported (bootstrap = 99%/100%) as the sister group of *Halocyphina villosa* (Fig. 2). The precise placement of *Limnoperdon incarnatum* is not resolved with confidence, although it is strongly supported as a member of the euagarics clade (bootstrap = 90%/99%; Fig. 2).

The close relationship of *Nia vibrissa* and *Halocyphina villosa* could not have been predicted based on morphology. Aside from their small size and marine habit they have no distinguishing features in common. Moreover, *Halocyphina villosa* occurs in mangroves, whereas *Nia vibrissa* and the related species *N. epidermoidea* and *N. globospora* have been collected on fully submerged substrates, including driftwood and the wreckage of a sunken ship, and have been isolated by baiting with submerged wooden test panels, *Spartina* culms, horsehair, and feathers (17, 19–22). Nevertheless, the *Nia-Halocyphina* clade is strongly supported and is nested in another strongly supported clade (bootstrap = 100%/100%) that includes the mangrove-inhabiting species *Calathella mangrovei* and two terrestrial species, *Cyphellopsis anomala* and *Favolaschia intermedia* (Fig. 2). With its appendaged spores, enclosed fruiting body, and habit on submerged substrates, *Nia vibrissa* is the most derived member of this clade. Transformations leading to the evolution of this unusual basidiomycete probably involved a shift from terrestrial to periodically immersed to fully submerged substrates, loss of ballistospory, and evolution of appendaged spores and an enclosed fruiting body. Significantly, the cyphelloid fruiting body of *Halocyphina villosa* is enclosed during parts of its ontogeny, and at maturity the opening of the fruiting body is partially covered by interwoven hyphae (8, 18). Thus, the mangrove-inhabiting *Halocyphina villosa* appears to be morphologically and ecologically intermediate between *Nia vibrissa* and terrestrial cyphelloid forms, such as *Cyphellopsis anomala.*

In the mangroves where they occur, *Calathella mangrovei, Halocyphina villosa,* and *Physalacria maipoensis* are all periodically submerged in seawater (4, 5, 8, 11). *Physalacria maipoensis,* however, has also been found in adjacent upland forests that are not inundated (9).
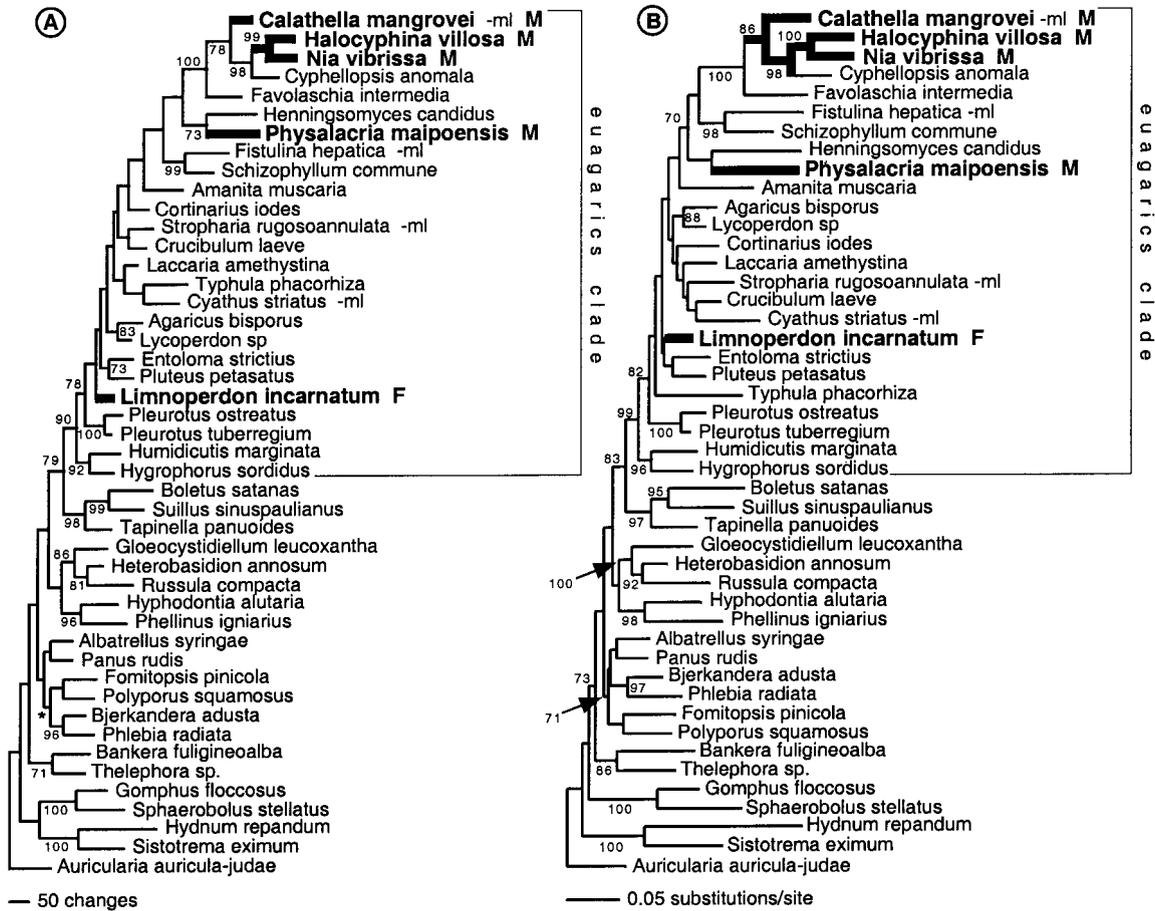
Figure 2. Phylogenetic relationships of terrestrial, marine, and freshwater homobasidiomycetes inferred from nuclear and mitochondrial ribosomal DNA (rDNA) sequences, and alternative reconstructions of the history of shifts between terrestrial and aquatic habitats. (A) One of two phylogenetic trees inferred using parsimony (asterisk indicates the one node that collapses in the strict consensus tree). (B) Phylogenetic tree inferred using maximum likelihood (ML). Names of aquatic taxa are in bold type; M = marine, F = freshwater. Taxa marked −ml lack mitochondrial large subunit rDNA sequences. Bootstrap values are indicated next to branches (only values above 70% are shown). Branch shading indicates reconstruction of ancestral habitats; thin lines = terrestrial, thick lines = aquatic. The parsimony tree (A) shows a reconstruction of habitat shifts that involves four independent transitions from terrestrial to aquatic habitats. The ML tree (B) shows an equally parsimonious reconstruction of ancestral states that involves three transitions from terrestrial to aquatic habitats, and one reversal. Methods: DNA was isolated from cultured mycelium, and nuclear and mitochondrial rDNA regions were amplified and sequenced using protocols and primers that have been reported elsewhere (13, 14). Sequences were aligned by eye in the PAUP* (15) data editor. After excluding 185 positions that were deemed to be ambiguously aligned, the data set included 3574 aligned positions, of which 1267 were variable and 827 were parsimony-informative. Parsimony analysis used 1000 heuristic searches with random taxon addition sequences, tree bisection-reconnection (TBR) branch-swapping, and MAXTREES set to autoincrease, with all characters and transformations equally weighted. Bootstrapped parsimony analysis used 1000 replicates with one heuristic search per replicate, with other settings as in the baseline analysis. ML analysis used the HKY85 model of sequence evolution, with empirical base frequencies, transition-transversion bias set to 2, and among-site rate heterogeneity modeled on a discrete gamma distribution, with four rate classes and shape parameter α set to 0.5. The ML analysis used a heuristic search, with the trees obtained in the parsimony analysis used as starting trees for branch swapping with TBR. Bootstrapped ML analyses used 100 replicates, with one heuristic search per replicate, using a starting tree generated with neighbor-joining (Kimura two-parameter distances), and TBR branch swapping. A time limit of 1 hour per bootstrap replicate was enforced. Sequences have been deposited in GenBank (accession numbers AF426948-AF426970, which should be consulted for strain data) and the data set has been deposited in TreeBASE (accession number S666).

Inderbitzin and Desjardin (9) regarded *Physalacria maipoensis* as "halotolerant," and suggested that it is closely related to certain terrestrial species of *Physalacria*. It is tempting to speculate that *Physalacria maipoensis* represents an early stage in the transition from terrestrial to marine environments in homobasidiomycetes.

Acknowledgments

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