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Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes

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Mycorrhizae, the symbiotic associations of plant roots and fungal hyphae, are classic examples of mutualisms. In these ecologically important associations, the fungi derive photosynthetic sugars from their plant hosts, which in turn benefit from fungus-mediated uptake of mineral nutrients. Early views on the evolution of symbioses suggested that all long-term, intimate associations tend to evolve toward mutualism. Following this principle, it has been suggested that mycorrhizal symbioses are the stable derivatives of ancestral antagonistic interactions involving plant parasitic fungi[1]. Alternatively, mutualisms have been interpreted as inherently unstable reciprocal parasitisms, which can be disrupted by conflicts of interest among the partners[2–5]. To determine the number of origins of mycorrhizae, and to assess their evolutionary stability, it is necessary to understand the phylogenetic relationships of the taxa involved. Here we present a broad phylogenetic analysis of mycorrhizal and free-living homobasidiomycetes (mushroom-forming fungi). Our results indicate that mycorrhizal symbionts with diverse plant hosts have evolved repeatedly from saprotrophic precursors, but also that there have been multiple reversals to a free-living condition. These findings suggest that mycorrhizae are unstable, evolutionarily dynamic associations.

The evolution of mycorrhizae had a profound impact on terrestrial ecosystems. Fossils of arbuscular mycorrhizae from the Rhynie chert (400 million years before present) suggest that mycorrhizae facilitated the colonization of the land by plants, and today over 90% of plants form mycorrhizae of some kind[6,7]. The majority of mycorrhizae are arbuscular mycorrhizae, which involve the monophyletic Glomales and a broad range of herbaceous and woody plants[8]. The other major group of mycorrhizae are ectomycorrhizae, which are formed primarily by homobasidiomycetes, as well as a handful of ascomycetes and zygomycetes[8]. About thirty families of plants form ectomycorrhizae, including pines, oaks, dipterocarps and eucalypts[8]. Forests of these and other ectomycorrhizal trees dominate many cool temperate and some tropical ecosystems[7].

We inferred the evolutionary history of ectomycorrhizae among the homobasidiomycetes using a dataset of 161 species that includes 46 ectomycorrhizal species (29%), which is comparable to the proportion of known ectomycorrhizal taxa (about 35%)[9]. Characters for phylogenetic analyses[10] were obtained from overlapping datasets of nuclear small subunit (nuc-ssu) ribosomal DNA (rDNA), nuclear large subunit (nuc-lsu) rDNA and mitochondrial small subunit (mt-ssu) rDNA. Phylogenetic analysis yielded over 10,000 equally parsimonious trees (10,014 steps, consistency index, CI = 0.260; Fig. 1). Despite the large number of equally parsimonious trees, the strict consensus tree is highly resolved (Fig. 1).

Parsimony-based ancestral state reconstructions[11] on all equally parsimonious trees imply that there have been 15–16 transformations between ectomycorrhizal and free-living forms, including 7–13 gains and 3–9 losses (average: 9.0 gains, 6.3 losses). The ambiguity in the precise number of gains and losses is due to topological differences among the equally parsimonious trees, as well as the existence of several equally parsimonious reconstructions of ancestral states on all trees. Nevertheless, all reconstructions agree that the ancestor of the homobasidiomycetes was free-living, and that there have been many gains and losses of ectomycorrhizal symbioses within the homobasidiomycetes. This conclusion is also supported by maximum likelihood estimates of ancestral states[12] at 15 key nodes (Fig. 1). (Additional analyses are described in the Supplementary Information.)

Ectomycorrhizal homobasidiomycetes occur in six independent clades, which we refer to as groups 1–6 (Fig. 1). The plants that form ectomycorrhizae also make up a polyphyletic group, including conifers and multiple clades of angiosperms[8,13]. To evaluate broad patterns of host specificity, we examined the phylogenetic distribution of associations with six of the most ecologically important groups of ectomycorrhizal plants: Betulaceae, Dipterocarpaceae, Fagaceae, Myrtaceae, Pinaceae and Salicaceae. All six groups of ectomycorrhizal homobasidiomycetes form associations with Pinaceae, and four groups are reported to form associations with at least four of the angiosperm families that we investigated[14–19] (Fig. 1). At low taxonomic levels, there is considerable variation in the degree of host specificity exhibited by ectomycorrhizal fungi and plants, with both groups including generalist and specialist species[14,20]. It appears, however, that the majority of ectomycorrhizal fungi and plants are capable of entering into symbioses with multiple partners. For example, western hemlock, *Tsuga heterophylla*, forms associations with over 100 fungal species[21], which represent five of the six groups of ectomycorrhizal fungi that we recognize here, and the fly agaric, *Amanita muscaria*, forms associations with at least 23 species of Betulaceae, Fagaceae, Pinaceae and Salicaceae[14]. This broad host potential contributes to a dynamic community structure, in which individual plants form associations with suites of fungal species, whose composition may shift over time[7,8,22].

Adding to the dynamism of ectomycorrhizal assemblages is the occasional occurrence of reversals to a free-living, saprotrophic lifestyle within otherwise ectomycorrhizal clades of fungi. Our results suggest that as many as nine such reversals have occurred, although the precise number is not resolved with parsimony (Fig. 1). One unambiguous reversal occurs on the lineage leading to *Lentaria*, which is a small group of about 15 species of wood-decaying fungi that is nested in group 5 (Fig. 1). However, by far the largest concentration of secondarily saprotrophic taxa is in group 1, which is composed of three clades that have been termed the euagaric, bolete and thelephoroid clades[23] (Fig. 1). Collectively, it has been estimated that these clades include about 9,500 described species[23], which is 73% of the roughly 13,000 known species of homobasidiomycetes[9]. On the basis of current taxonomy, we estimate that there are about 8,500 described species of saprotrophic homobasidiomycetes, and that 4,700 of these species are nested in group 1. Thus, about half of all saprotrophic homobasidiomycetes may ultimately have been derived from ectomycorrhizal ancestors.

Saprotrophic homobasidiomycetes include two important (somewhat overlapping) guilds: wood-decay fungi, and soil and leaf-litter fungi[24]. Both play pivotal roles in nutrient cycling in terrestrial ecosystems. Ninety-four species in our data set, including

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both outgroup species, decay wood, which suggests that wood decay is the plesiomorphic condition in the homobasidiomycetes. Of the fourteen litter-decaying fungi in our data set, all but two are secondary saprotrophs in the euagarics clade (Fig. 1). In both ectomycorrhizal and litter-decaying fungi, the vegetative mycelia proliferate in the upper layers of soil, and fruiting bodies are produced directly on soil[25]. Therefore, derivation of litter decayers from ectomycorrhizal forms would require only a switch from a symbiotic to a free-living lifestyle, without a change of habitat.

Our inference that multiple lineages of homobasidiomycetes have 'escaped' from ectomycorrhizal symbioses is consistent with theoretical predictions that mutualisms are inherently unstable[2,3]. This view is also supported by empirical studies, which have demonstrated that symbioses can shift along a continuum of interactions ranging from mutualism to antagonism[26,27]. The situation in homobasidiomycetes is unusual, however, in that the transitions have not been between mutualists and parasites, but between mutualists and free-living forms[4]. Significantly, the eight timber pathogens in our data set do not appear to be closely related to ectomycorrhizal taxa (Fig. 1). The derivation of free-living forms from mutualists has received little attention, but might be common in groups where symbionts have some capacity for living independently and hosts have multiple potential symbionts. Besides ectomycorrhizae, such associations might include those involving corals and their photosynthetic zooxanthellae, or leaf-cutter ants and their cultivated fungi.

Several factors are thought to promote the stability of mutualisms, including compensating mechanisms that limit exploitation of one partner by another[5]; strictly vertical transmission of symbionts between host generations; asexual reproduction of symbionts; and one-to-one correspondence of host and symbiont species[3,4]. Based on these criteria, the apparent instability of ectomycorrhizae is not surprising. There are no known mechanisms that protect the fungi from exploitation by their plant hosts; fungal symbionts reproduce sexually and disperse spores independently of plant propagules; and most ectomycorrhizal plants draw partners from a diverse pool of potential symbionts. One factor that might be expected to promote the stability of ectomycorrhizal associations is the putative dependence of the fungus on the host for photosynthate. The view that ectomycorrhizal fungi are obligate symbionts is based on the slow growth of many of the fungi in pure culture, and their inability to derive adequate nutrition for growth from complex organic substrates (for example, cellulose)[25]. However, cultural studies have shown that certain ectomycorrhizal fungi can degrade cellulose, hemicellulose, pectin and lignin, albeit to a limited extent, and may act saprotrophically in the soil[25,28]. It appears that some ectomycorrhizal fungi have retained the genes for enzymes that can degrade plant cell walls, which may have facilitated repeated evolutionary reversals to saprotrophy. □

Methods

Sequence data were obtained by direct automated cycle sequencing of polymerase chain reaction products. Sequences generated for this study include 13 nuc-ssu rDNA, 10 mt-ssu rDNA and 50 nuc-lsu rDNA sequences, which were aligned with published sequences using Clustal X and adjusted by eye. Hypervariable regions that were deemed too divergent to align were excluded from analyses. The dataset included 157 nuc-ssu sequences, 158 mt-ssu sequences, and 60 nuc-lsu sequences, with 3,251 aligned positions, of which 1,111 are parsimony informative. Phylogenetic analyses using equally weighted parsimony were conducted using PAUP* 4.0 (ref. 11), with 1,000 heuristic searches with random taxon addition sequences, the maximum number of trees (MAXTREES) limited to 10,000,

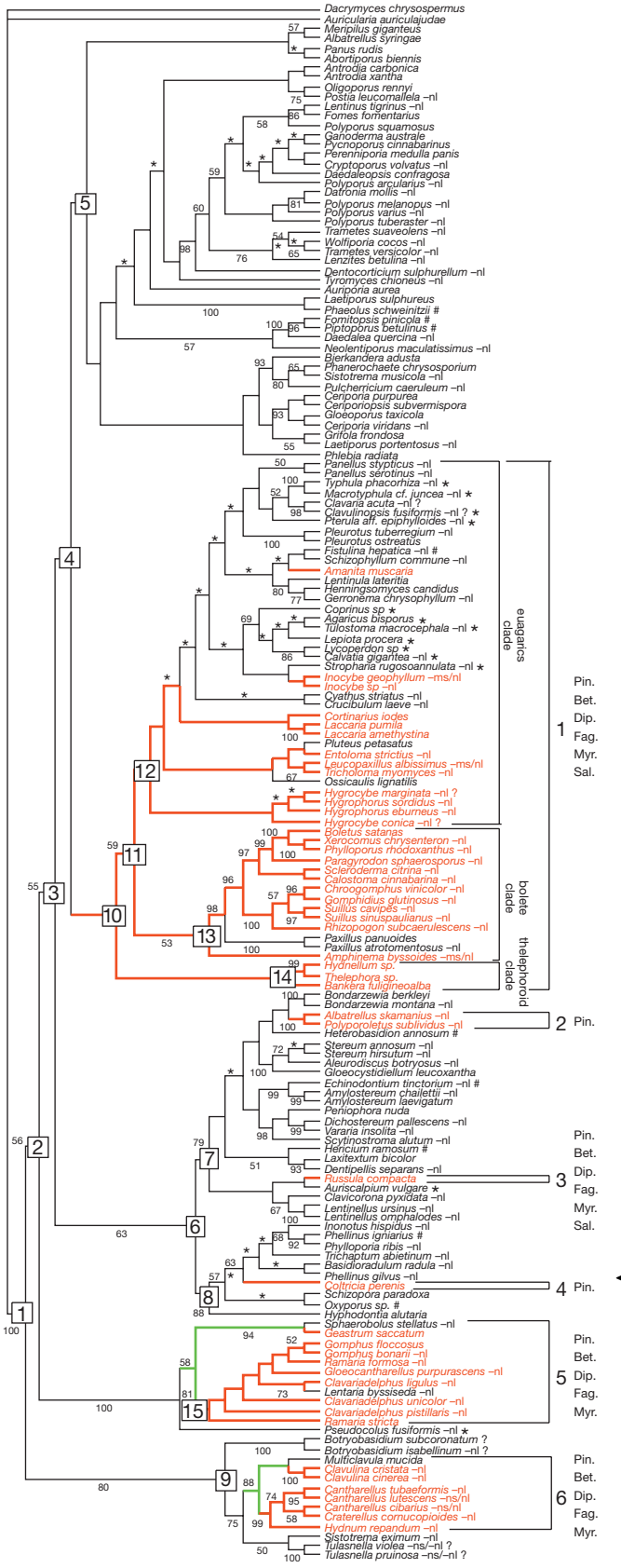

Figure 1 Phylogeny of homobasidiomycetes and evolution of ectomycorrhizal symbioses inferred from rDNA sequences. One of 10,000 equally parsimonious trees. Branches that collapse in the strict consensus tree are marked with asterisks. Bootstrap frequencies over 50% are shown. Ancestral states according to parsimony are indicated with branch shading: red is ectomycorrhizal, black is non-ectomycorrhizal, green is uncertain. Using maximum likelihood, nodes 1–9 are estimated to be non-ectomycorrhizal, and nodes 10–15 are estimated to be ectomycorrhizal ($\Delta \log L > 2$). Species are marked with symbols: coded as uncertain in parsimony optimization (questionmarks); litter-decaying saprotrophs (asterisks); tree pathogens (hashes); –ms means no mt-ssu rDNA sequence; –ns means no nuc-ssu rDNA sequence; –nl means no nuc-lsu rDNA sequence. Host plant codes: Pin., Pinaceae; Bet., Betulaceae; Dip., Dipterocarpaceae; Fag., Fagaceae; Myr., Myrtaceae; Sal., Salicaceae.

and tree bisection-reconnection (TBR) branch swapping. Bootstrap analyses used 100 heuristic searches, with one random taxon addition sequence per replicate, and with MAXTREES limited to 10. Based on previous analyses[29], the heterobasidiomycetes *Auricularia* and *Dacrymyces* were used for rooting purposes. Terminal taxa were coded as ectomycorrhizal, non-ectomycorrhizal or uncertain, and ancestral state reconstructions were performed on all trees with equally weighted parsimony using MacClade 3.0 (ref. 11). Maximum likelihood estimates of support for alternative states at selected nodes of one tree selected at random (with branch lengths estimated from molecular data using parsimony) were calculated using Discrete[12]. Because Discrete requires that all terminal taxa be scored for the character of interest, the estimated states from parsimony optimizations were assigned for the eight species that were coded as uncertain (Fig. 1). The 'local' method for estimating support for alternative ancestral states was used, with a difference of two units in log likelihood ($\Delta \log L > 2$)taken as an approximate criterion of significance[12].

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The genome sequence of the thermoacidophilic scavenger *Thermoplasma acidophilum*

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Thermoplasma acidophilum **is a thermoacidophilic archaeon that thrives at 59 °C and pH 2, which was isolated from self-heating coal refuse piles and solfatara fields[1,2]. Species of the genus *Thermoplasma* do not possess a rigid cell wall, but are only delimited by a plasma membrane. Many macromolecular assemblies from *Thermoplasma*, primarily proteases and chaperones, have been pivotal in elucidating the structure and function of their more complex eukaryotic homologues[3,4]. Our interest in protein folding and degradation led us to seek a more complete representation of the proteins involved in these pathways by determining the genome sequence of the organism. Here we have sequenced the 1,564,905-base-pair genome in just 7,855 sequencing reactions by using a new strategy. The 1,509 open reading frames identify *Thermoplasma* as a typical euryarchaeon with a substantial complement of bacteria-related genes; however, evidence indicates that there has been much lateral gene transfer between *Thermoplasma* and *Sulfolobus solfataricus*, a phylogenetically distant crenarchaeon inhabiting the same environment. At least 252 open reading frames, including a complete protein degradation pathway and various transport proteins, resemble *Sulfolobus* proteins most closely.**

Two basic approaches have been taken to genome sequencing. The statistical approach ('shotgun sequencing') relies on the determination of a highly redundant set of random genomic DNA sequences, which are assembled in the computer, the gaps remaining to be closed by other methods[5]. This approach rapidly yields 90–98% of the genomic information, but requires an extensive robotic infrastructure. The directed approach relies on a complete mapping of the genome, followed by the sequencing of large overlapping genomic fragments by shotgun sequencing or primer walking[6]. This approach reduces the infrastructure requirements but is slowed down by the need for a genetic map.

One of our aims was to establish a strategy for sequencing microbial genomes in reasonable time without extensive infrastructure. This strategy ('shotgun primer walking') combines features of the statistical and directed methods. After construction of several phagemid libraries and one cosmid library, inserts from randomly chosen clones were sequenced from the ends by primer walking (see Supplementary Information). Sequencing was stopped in regions that were redundant with already determined sequences. In total, 400 phagemids, covering 850 kilobase-pairs (kbp) (54%), and 469 cosmids, covering 1,533 kbp (98%), were partially or fully sequenced. With an average insert length of 40 kbp, the clones of the cosmid library statistically covered the genome 12 times. Because cosmids are susceptible to recombination events, the reverse strand of cosmid DNA was always sequenced with DNA templates from other cosmid clones covering the same region, or