

tance of 3.9 Å, resembles KBPAP in its two-metal-ion mechanism (19). Although the phosphate ester hydrolysis of alkaline phosphatase proceeds via a phosphoseryl intermediate with retention of the overall configuration at the phosphorus, the functional role of both metals is similar compared to that of the metals in the PAPs: One zinc ion primarily binds and preorients the phosphate group of the phosphoseryl intermediate for the attack of the water ligand that was activated by the other zinc ion. The weaker Lewis-acidity of zinc(II) compared to iron(III) leads to the shift of the phosphatase reaction to the alkaline side.

The first structure of a purple acid phosphatase presented here forms the structural basis for understanding the hydrolytic action of this enzyme class on phosphate esters. The results are consistent with the models for the dimetal center and with the proposed mechanism on the basis of several spectroscopic and kinetic studies, albeit the detailed structural and functional relation between the mammalian enzymes and the kidney bean PAP remains to be established.

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22. Two crystal forms were used for the structure determination: tetragonal crystals of space group $P4_32_12$ with unit cell dimensions $a = b = 104.4$ and $c = 309.9$ Å (6) and orthorhombic crystals of space group $C22_2$, with $a = 132.7$, $b = 347.3$, and $c = 128.7$ Å. The asymmetric unit contains two and four subunits for the $P4_32_12$ and the $C22_2$ space group, respectively. Because of the higher diffraction limit of 2.9 Å of the orthorhombic crystals compared with 3.5 Å of the tetragonal crystals, and because of the higher noncrystallographic symmetry, the diffraction data and improved phases of this space group were primarily used for model building. For brevity, only the data collection and phasing statistics of the orthorhombic crystals are included here and a detailed description of the structure determination procedure will be reported elsewhere. The orthorhombic crystals were grown from solutions of enzyme (12 mg/ml) and polyethylene glycol (PEG) 6000 (40 mg/ml), and 50 mM sodium acetate (pH 5.5). The native data set was collected on a MARRESEARCH imaging plate detector at the European Molecular Biology Laboratory, Heidelberg. The diffraction intensities of the derivatives were measured on a STOE imaging plate diffractometer in our laboratory in Münster. The data sets were scaled and evaluated with programs of the CCP4 program suite [Collaborative Computational Project, Number 4, *Acta Crystallogr. Sect. D* **50**, 760 (1994)]. The difference Patterson function of the first useful derivative, Na_2WO_4 , was deconvoluted and the heavy-atom binding sites of the second derivative, PIP, were located by a difference Fourier analysis. The MIR phases were improved and extended to 2.9 Å by solvent flattening and fourfold molecular averaging with the RAVE program [T. A. Jones, in *Molecular Replacement*, E. J. Dodson, Ed. (Science and Engineering Research Council, Daresbury, UK, 1992)]. The program O was used for all model building [T. A. Jones, J.-Y. Zou, S. W. Cowan, *Acta Crystallogr. Sect. A* **47**, 110 (1991)]. The high quality of the improved electron density, in conjunction with the amino acid sequence (4), allowed for an unambiguous tracing of the main chain. Structure refinement was performed with XPLOR, using the slow-cooling protocol (3000 K) [A. T. Brünger, XPLOR Version 3.1 Manual (Yale Univ. Press, New Haven, CT, 1993)]. The four monomers within the asymmetric unit have been refined with noncrystallographic symmetry (NCS) restraints. Residues involved in packing interactions were excluded from the NCS restraints. The R factor is currently 19.6% (free R factor = 23.3%) [A. T. Brünger, *Nature* **355**, 472 (1992)] for 51,523 unique reflections in the 8.0 to 2.9 Å shell ($F/\sigma_F > 2$, 82% complete). There was no interpretable density for the NH_2 -terminal residues 1 to 8 in any of the four monomers and they are assumed to be disordered. The root-mean-square (rms) deviations for bond distances and angles were 0.018 Å and 2.7°, respectively. The Luzzati plot suggests a coordinate error of 0.35 Å. Only one residue (His³²³) is in the disallowed regions of the Ramachandran plot.
23. We thank R. Fröhlich for help with data collection and W. N. Lipscomb and J. C. Sacchettini for reviewing the manuscript. Supported by the Deutsche Forschungsgemeinschaft and by the Fonds der Chemischen Industrie. This study is in partial fulfillment of the requirements of T. K. and N. S. for the degree of Dr rer. nat. at Westfälische Wilhelms-Universität, Münster.

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Multiple Origins of Lichen Symbioses in Fungi Suggested by SSU rDNA Phylogeny

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Phylogenetic hypotheses provide a context for examining the evolution of heterotrophic lifestyles. The lichen lifestyle, which is the symbiotic association of fungi with algae, is found in various representatives of *Dicaryomycotina*, both *Ascomycetes* and *Basidiomycetes*. A highly resolved parsimony analysis of small subunit ribosomal DNA (SSU rDNA) sequences suggests at least five independent origins of the lichen habit in disparate groups of *Ascomycetes* and *Basidiomycetes*. Because lichen associations arose from parasitic, mycorrhizal, or free-living saprobic fungi, neither mutualism nor parasitism should be construed as endpoints in symbiont evolution.

Lichens are a classic example of symbiosis ["Zusammenleben ungleichnamiger Organismen" (1)], with interactions ranging from mutualistic to parasitic (2). Hyphae of the fungal symbiont may lie within a matrix of algal cells, adhere to these cells as appressoria, invaginate these cells as haustoria, or occasionally penetrate cell walls and plasmalemmae (3). Long-term survival of the lichen association depends on balanced growth of the symbionts, yet this balance does not preclude killing or saprobic digestion of the algal symbiont (4). Lichen-forming fungi represent many diverse lineages of

Dicaryomycotina (5) that traditionally have been studied under the rubric of lichenology. These lineages are not descended from a single lichen-forming ancestor, yet it is not known how many times, and in which groups, the lichen habit originated. By examining the phylogenetic position of lichen-forming fungi relative to saprobic or pathogenic fungi, we can address a fundamental question of symbiont evolution: whether mutualistic symbioses are derived from more parasitic forms (6).

To determine the origins of the lichen habit, we included lichen-forming fungi within a phylogenetic analysis of *Amastigomycota*, members of *Eumycota* that lack motile stages. The major lineages of *Amastigomycota*—*Basidiomycetes*, *Ascomycetes*, and the paraphyletic *zygomycetous* fungi (7)—have few comparable morphological characters to serve as the basis for phylogenetic hypotheses. For example, the

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group Dikaryomycotina is distinguished by the single morphological feature of dikaryotic hyphae (5). Even the sexual structures used to define these groups do not allow for comparisons. Additionally, many fungi lack sexual structures and cannot be unambiguously classified.

Unlike morphological characters, molecular characters allow direct comparisons among extant Eumycota. Specifically, sequences of small subunit ribosomal DNA (SSU rDNA) have been used to propose phylogenetic hypotheses for fungi (8). The inclusion of SSU rDNA sequences from four lichen-forming representatives has produced a highly resolved phylogeny for Ascomycetes and has demonstrated that lichen-forming fungi are a key to understanding ascomycete relations (9). Our study used SSU rDNA sequences from 10 lichen-forming fungi and 65 other fungi in a cladistic analysis, which produced two equally parsimonious cladograms that differed only in sister taxa relations within one clade of three fungi (10) (Fig. 1). These phylogenetic hypotheses support Dikaryomycotina, Basidiomycetes, and Ascomycetes as monophyletic and resolve their relations (11). In the topologies, we identified the phylogenetic positions of lichen-forming fungi and nodes where discordance in lifestyles among sister taxa indicates an independent gain or loss of the lichen habit. In each case, it is most parsimonious to interpret the lichen habit as gained (12).

Within Basidiomycetes, our phylogenetic hypothesis supports three independent origins of the lichen habit, each corresponding to groups supported by morphological characters (13). The basal origin of the basidiolichen habit is within the coral fungi; a few species of *Multiclavula* (Fig. 2A) form loose lichen associations with the green alga *Coccomyxa* or occasionally with cyanobacteria. An independent lichen association with *Coccomyxa* is represented by *Omphalina umbellifera* (Fig. 2B), which produces typical gilled mushrooms and is allied with the button mushroom *Agaricus bisporus* and the oyster mushroom *Pleurotus ostreatus*. Another basidiolichen origin is indicated by *Dictyonema pavonia* (Fig. 2C), whose unique haustoria penetrate the cyanobacterium *Scytonema*. This tropical inhabitant is most closely related to the wood-rotting *Schizophyllum commune*. Two of the three basidiolichens, *Multiclavula mucida* and *O. umbellifera*, produce fruiting structures that are algae-free and closely resemble those of their non-lichen-forming relatives (13). The loose associations these basidiolichens form with algal colonies require few changes in overall morphology. These basidiolichen associations may have arisen multiple times, perhaps quite recently, within each closely related group.

Within Ascomycetes the lichen habit arose at least twice, according to our phylogenetic hypothesis; other independent origins may be detected with the inclusion of other taxa (14). The most basal origin is represented by *Lecanora dispersa* (Fig. 2D), *Porpidia crustulata*, and *Sphaerophorus globosus* (Fig. 2E), members of Lecanorales (9) that commonly form intimate haustorial connections to coccoid green algae such as *Trebouxia*. The nearly 6000 species of Lecanorales include familiar crustose, foli-

ose, and fruticose lichens with cup-shaped fruiting structures. Other lichen-forming groups such as Caliciales, as represented here by the saprobe *Mycocalicium albonigrum*, are sister taxa to Lecanorales. The calicialean lineage may have given rise to nonlichenized cleistothecial fungi, such as the laboratory model *Aspergillus*. A second origin of the ascolichen habit is in the lineage containing *Arthonia radiata* (Fig. 2F) and allied species of the order Arthoniales (15), whose hyphae may even penetrate

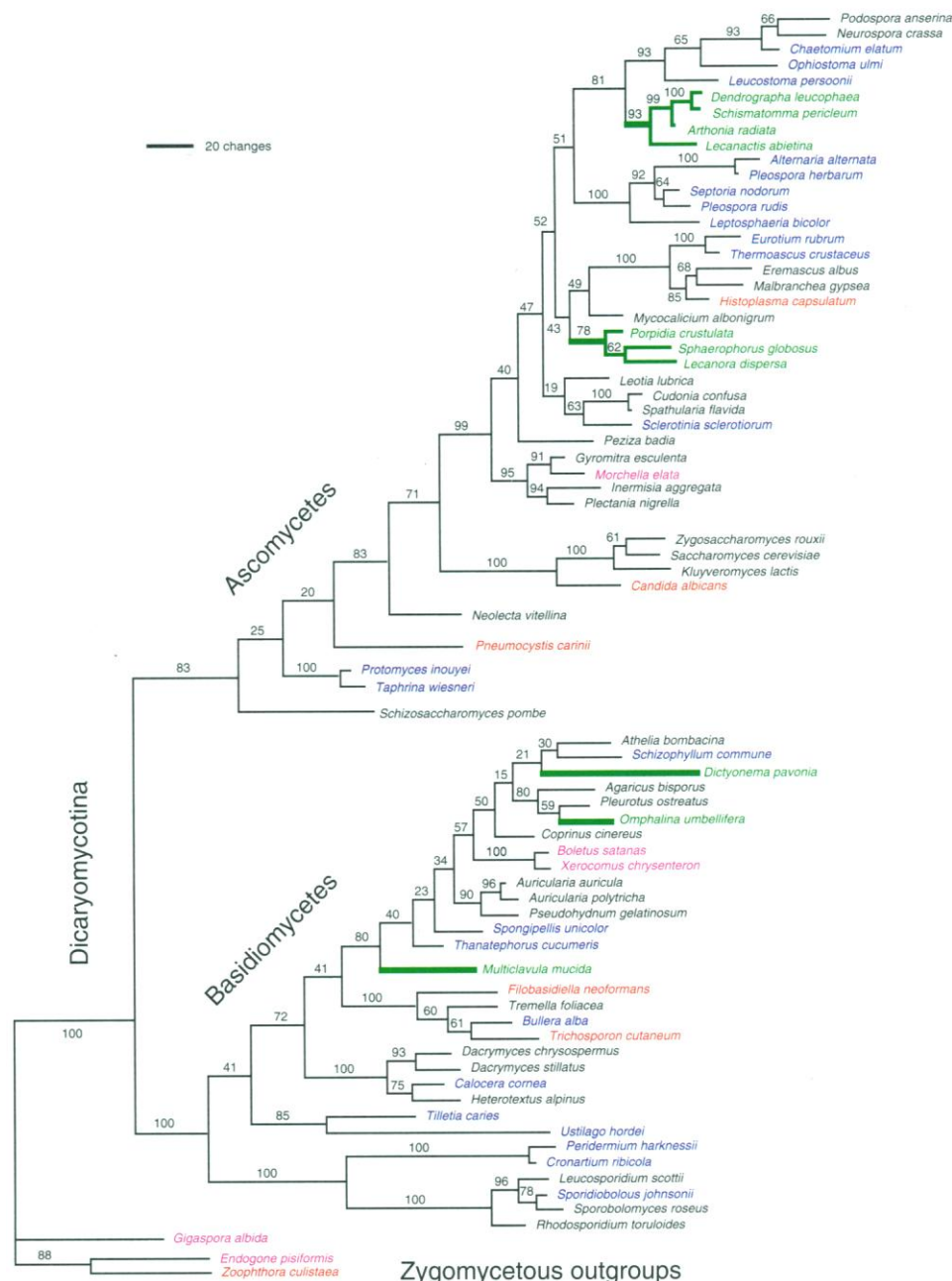
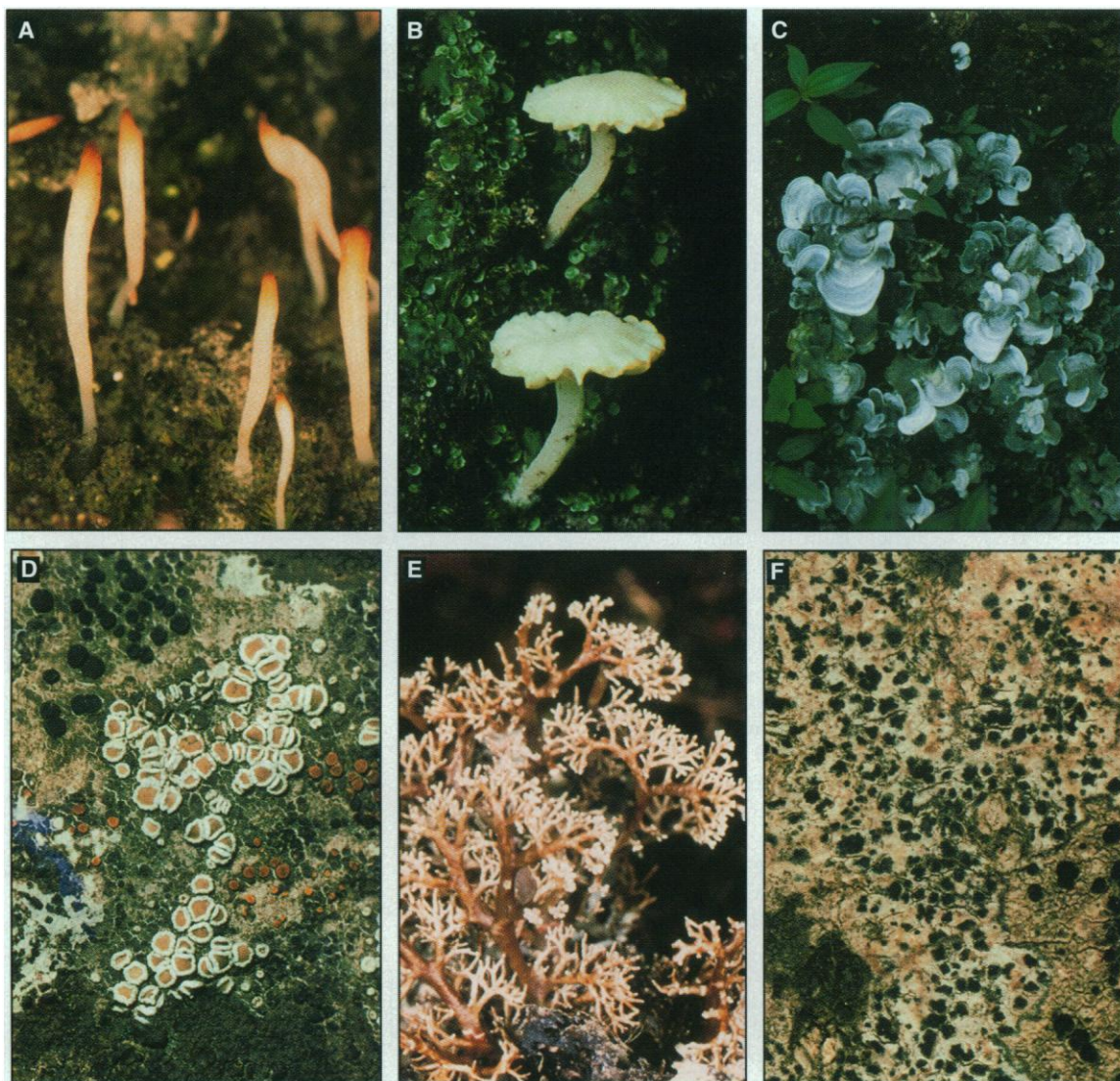


Fig. 1. Phylogenetic relations within Amastigomycota, as derived from parsimony analysis of 1927 nucleotides of SSU rDNA sequences from 75 representative fungi. One of two equally parsimonious cladograms of 3491 steps is shown; the cladograms differ only in sister taxa relations within the clade that includes *Agaricus bisporus*, *Omphalina umbellifera*, and *Pleurotus ostreatus*. Bootstrap percentages (21) from 200 replications are shown on each supported branch. The major fungal groups supported in this analysis are labeled adjacent to their nodes.

Fig. 2. Representatives of the five independent origins of lichen symbioses. **(A)** The coral fungus *Multi-clavula vernalis* (Schw.) R. H. Petersen, a close relative of *M. mucida*, on decaying wood. **(B)** The mushroom *Omphalina hudsoniana* (Jenn.) Bigelow, a close relative of *O. umbellifera*, on organic materials. **(C)** The foliose *Dictyonema pavonia* (Sw.) Parmasto on tropical soil. **(D)** The crustose *Lecanora dispersa* (Pers.) Sommerf. on rock. **(E)** The fruticose *Sphaerophorus globosus* (Hudson) Vainio on organic materials. **(F)** The crustose *Arthonia radiata* (Pers.) Ach. on bark. (A) through (C) are Basidiomycetes, (D) through (F) are Ascomycetes, and (D) and (E) are members of the order Lecanorales. Photographs (22) are by C. Scheidegger (A), V. Wirth (B, C, D, and F), and K. Rasbach (E).



their algal partner, typically the filamentous green alga *Trentepohlia*. This order of nearly 2000 species is closely related to perithecial fungi, including the model fungi *Neurospora crassa* and *Podospora anserina*. Separate origins of the lichen habit in Lecanorales and Arthoniales explain the notable differences in their morphology, haustoria, and algal symbionts. In contrast to the basidiolichens, ascolichens are intricate associations in which the lichen morphology differs from that produced by either of the symbionts cultured axenically. In Ascomycetes, lichen formation is a dominant lifestyle, with nearly half of the species associated with algae, and lichen origins are relatively ancient and evolutionarily successful.

Mycologists would never propose that all fungi pathogenic to plants, or all fungi that form mycorrhizae, are separate cohesive units. However, fungi that form lichens have been studied and classified in isolation from other fungi, even very recently (16).

Here, we show that lichen symbioses arose at least five times in phylogenetically distant groups; the concept of lichen is thus ecologically meaningful (17) but not phylogenetically meaningful. Within this phylogenetic context, the most parasitic lichen-forming fungi, Arthoniales (18), are closely related to groups that include virulent plant pathogens (*Leucostoma* and *Alternaria*), which suggests that lichen symbionts arose from parasitic fungi. Lichen formation is not a primitive lifestyle; other lichen symbionts arose from predominantly mycorrhizal fungi (basidiolichens) or from saprobic fungi (Lecanorales). Indeed, lecanoralean lichen symbionts may have given rise to fungi parasitic on lichens (19). Fungi are opportunists: Saprobes become symbionts; symbionts switch between mutualism and parasitism. On the basis of this phylogenetic analysis of fungi in symbiotic lichen associations, we find no support for the tenet that there is a general evolutionary progression from parasitism to mutualism.

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10. For this analysis, SSU rDNA sequences were obtained from nine fungi, including four Ascomycetes [*Arthonia radiata* (GenBank accession number U23537), *Dendrographa leucophaea* (U23538), *Lecanactis abietina* (U23539), and *Schismatomma pericleum* (U23540)] and five Basidiomycetes [*Agaricus bisporus* (U23724), *Dictyonema pavonia* (U23541), *Multiclavula mucida* (U23542), *Omphalina umbellifera* (U23543), and *Pleurotus ostreatus* (U23544)]. DNA was isolated and SSU rDNA was amplified by the polymerase chain reaction (PCR) from fungus-specific oligonucleotide primers as described in (9). Double-stranded PCR products were sequenced as described (9) or by the PRIZM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) with detection on a 373A automatic sequencing apparatus (Applied Biosystems). Sequence fragments were assembled manually. These DNA sequences were aligned to 8 SSU rDNA sequences from the first author's previous studies—*Lecanora dispersa* (L37535), *Leotia lubrica* (L37536), *Morchella elata* (L37537), *Mycocalicium albonigrum* (L37538), *Peziza badia* (L37539), *Porpidia crustulata* (L37540), *Sclerotinia sclerotiorum* (L37541), and *Sphaerophorus globosus* (L37532)—and to 58 sequences available from GenBank: *Alternaria alternata* (U05194), *Athelia bombacina* (M55638), *Auricularia auricula* (L22254), *Auricularia polytricha* (L22255), *Boletus satanas* (M94337), *Bullera alba* (X60179), *Calocera cornea* (L22256), *Candida albicans* (X53497), *Chaetomium elatum* (M83257), *Coprinus cinereus* (M92991), *Cronartium ribicola* (M94338), *Cudonia confusa* (Z30240), *Dacrymyces chrysospermus* (L22257), *Dacrymyces stillatus* (L22258), *Endogone pisiformis* (X58724), *Eremascus albus* (M83258), *Eurotium rubrum* (U00970), *Filobasidiella neofomans* (L05427), *Gigaspora albida* (Z14009), *Gyromitra esculenta* (Z30238), *Heterotextus alpinus* (L22259), *Histoplasma capsulatum* (X58572, S45469), *Inermisia aggregata* (Z30241), *Kluyveromyces lactis* (X51830), *Leptosphaeria bicolor* (U04202), *Leucosporidium scottii* (X53499), *Leucostoma personii* (M83259), *Malbranchea gypsea* (L28066), *Neolecta vitellina* (Z27393), *Neurospora crassa* (X04971), *Ophiostoma ulmi* (M83258), *Peridermium harknessii* (M94339), *Plectania nigrella* (Z27408), *Pleospora herbarum* (U05201), *Pleospora rudis* (U00975), *Pneumocystis carinii* (X12707), *Podospira anserina* (X54864), *Protomyces inouyei* (D11377), *Pseudohydnum gelatinosum* (L22260), *Rhodospiridium toruloides* (X60180), *Saccharomyces cerevisiae* (J01353, M27607), *Schizophyllum commune* (X54865), *Schizosaccharomyces pombe* (X54866), *Septoria nodorum* (U04236), *Spathularia flavida* (Z30239), *Spongipellis unicolor* (M59760), *Sporidobolus johnsonii* (L22261), *Sporobolomyces roseus* (X60181), *Taphrina wiesneri* (D12531, D01175), *Thanatephorus cucumeris* (M92990), *Thermoascus crustaceus* (M83263), *Tilletia caries* (U00972), *Tremella foliacea* (L22262), *Trichosporon cutaneum* (X60182), *Ustilago hordei* (U00973), *Xerocornus chrysenteron* (M94340), *Zoophthora culistaeae* (D29949), and *Zygosaccharomyces rouxii* (X58057). The 75 sequences were aligned with the program PileUp (Program Manual for the Wisconsin Package, Version 8, September 1994; Genetics Computer Group, Madison, WI); gaps were reduced by manual adjustment. An alignment of 1927 nucleotides from 75 taxa was used for parsimony analysis with the program PAUP 3.1 (20). No characters were excluded, invariant characters were ignored, all characters were equally weighted, and branch lengths equal to 0 were collapsed to polytomies. The analysis produced unrooted networks that were rooted with three zygomycetous fungi as outgroups. Two equally parsimonious trees were produced, with tree lengths of 3491, consistency indices of 0.3893, and retention indices of 0.6941; the trees differed only in sister taxa relations within one clade of three fungi (Fig. 1). Bootstrap percentages (21) to assess support for each branch were determined for 200 resamplings of the data set. Bootstrap values of
- >80% provided strong support for the monophyly of groups such as Dicaryomycotina, Ascomycetes, and Basidiomycetes.
11. This phylogenetic hypothesis supports the monophyly of most groups recognized in traditional classifications, and the addition of taxa may resolve finer relations. For example, in Basidiomycetes, *Auricularia* (represented by *Auricularia auricula*, *A. polytricha*, and *Pseudohydnum gelatinosum*) is recognized as monophyletic, yet its placement within the Homobasidiomycetes clade (defined by *Multiclavula mucida*, *Thanatephorus cucumeris*, *Spongipellis unicolor*, and *Athelia bombacina*) is problematic. In Ascomycetes, only the order Pezizales (represented by *Peziza badia*, *Gyromitra esculenta*, *Morchella elata*, *Inermisia aggregata*, and *Plectania nigrella*), here recognized as paraphyletic, differs from the first author's previous molecular phylogenies (9).
12. In Ascomycetes, the hypothesis of two independent gains of the lichen symbiosis is more parsimonious than that of a single origin with three subsequent losses. In Basidiomycetes, the hypothesis of three independent gains of the lichen symbiosis is more parsimonious, because a single origin would require one gain and eight losses. Although gain and loss can be variably weighted so that the loss of the lichen symbiosis is easier than its gain, this strategy ignores the necessary replacement of the lost lichen symbiosis with a gained saprobic, symbiotic, or parasitic habit.
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23. We thank V. Wirth, C. Scheidegger, and K. Rasbach for color photographs; the Laboratory of Molecular Systematics of the Smithsonian Institution for DNA sequencing facilities; D. L. Swofford and P. O. Lewis for support of phylogenetic analyses; and D. J. Futuyma, D. L. Hawksworth, F. Oberwinkler, R. H. Petersen, A. Y. Rossman, M. L. Sogin, A. R. Hornbuckle, and three anonymous reviewers for manuscript comments. A.G. and P.T.D. thank M. A. Faust, V. A. Funk, M. D. Kane, W. J. Kress, H. E. Robinson, and especially J. W. Taylor for support and encouragement. Supported by a Smithsonian Institution postdoctoral fellowship (A.G.), a Smithsonian Institution Scholarly Studies grant and a National Museum of Natural History Research Initiatives award (P.T.D. and A.G.), a Smithsonian Institution Visiting Scientist fellowship and Fonds zur Förderung der wissenschaftlichen Forschung (M.G.), and the Swedish National Research Council (A.T.).

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Muscle-Derived Neurotrophin-4 as an Activity-Dependent Trophic Signal for Adult Motor Neurons

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The production of neurotrophin-4 (NT-4) in rat skeletal muscle was found to depend on muscle activity. The amounts of NT-4 messenger RNA present decreased after blockade of neuromuscular transmission with α -bungarotoxin and increased during postnatal development and after electrical stimulation in a dose-dependent manner. NT-4 immunoreactivity was detected in slow, type I muscle fibers. Intramuscular administration of NT-4 induced sprouting of intact adult motor nerves. Thus, muscle-derived NT-4 acted as an activity-dependent neurotrophic signal for growth and remodeling of adult motor neuron innervation. NT-4 may thus be partly responsible for the effects of exercise and electrical stimulation on neuromuscular performance.

Intact adult motor neurons grow nerve processes or sprouts when muscles are partially denervated or in response to blockage of transmitter release and paralysis (1, 2). Candidate muscle-derived signaling factors whose expression is up-regulated by muscle inactivation include insulin-like growth factors (IGFs) (2) and some neurotrophic factors of the neurotrophin family (3, 4). However, motor nerve sprouting also occurs in normal vertebrate muscle under physio-

logical conditions (5). In the rat, qualitative changes in nerve terminal structure occur particularly during the first half year of life, when nerve terminal branches become organized in distinct groups with increased terminal length (6, 7). Muscle activity can directly influence the formation and maintenance of synaptic sites (5, 8, 9); in particular, exercise training has been shown to influence neuromuscular junction morphology and to induce axonal sprouting