9. Cronin, T. W. & Forward, R. B., Jr *J. comp. Physiol.* A**162,** 463–478 (1988).
10. Lipetz, L. E. & Cronin, T. W. *Vision Res.* **28,** 1083–1093 (1988).
11. Bruno, M. S., Barnes, S. N. & Goldsmith, T. H. *J. comp. Physiol.* **120,** 123–142 (1977).
12. Hiller-Adams, P., Widder, E. A. & Case, J. F. *J. comp. Physiol.* A**163,** 63–72 (1988).
13. Avery, J. A., Bowmaker, J. K., Djamgoz, M. B. A. & Downing, J. E. G. *J. Physiol., Lond.* **334,** 23P-24P (1983).
14. Hårosi, F. I. & Hashimoto, Y. *Science* **222,** 1021–1023 (1983).
15. Ohtsuka, T. *J. comp. Neurol.* **237,** 145–154 (1985).
16. Ohtsuka, T. *Science* **229,** 874–877 (1985).
17. Jane, S. D. & Bowmaker, J. K. *J. comp. Physiol.* A**162,** 225–235 (1988).
18. Cummins, D. R. & Goldsmith, T. H. *J. comp. Physiol.* **142,** 199–202 (1981).
19. Hardie, R. C. in *Progress in Sensory Physiology* Vol. 5 (ed. Ottoson, D.) 1–79 (Springer, Berlin, 1985).
20. Arikawa, K., Inokuma, K. & Eguchi, E. *Naturwissenschaften* **74,** 297–298 (1987).
21. Caldwell, R. L. & Dingle, H. *Naturwissenschaften* **62,** 214–222 (1975).
22. Barlow, H. B. *Vision Res.* **22,** 635–643 (1982).
23. Bowmaker, J. K. *Trends neurosci.* **6(2),** 41–43 (1983).
24. Dingle, H. *Crustaceana* **7,** 236–240 (1964).
25. Bernard, G. D. *J. opt. Soc. Am.* **A4,** 123 (1987).

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Accelerated evolution of a false-truffle from a mushroom ancestor

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THE false-truffles (Hymenogastrales) are a group of basidiomycetous fungi that produce underground truffle-like basidiocarps. They are generally believed to be independently derived from several mushroom lineages[1–4], but extensive morphological divergence often obscures recognition of these phylogenetic connections. Comparisons of mitochondrial DNA now demonstrate a surprisingly close relationship between species of false-truffles in the genus *Rhizopogon* (Hymenogastraceae) and the mushroom genus *Suillus* (Boletaceae). The striking morphological differences separating all *Suillus* species from *Rhizopogon* imply an acceleration in the rate of morphological change relative to molecular change during the evolution of these false-truffles from their mushroom ancestors. This acceleration can best be explained by rapid morphological divergence resulting from selective pressures which may have acted on a small number of developmental genes.

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Two types of molecular evidence demonstrate that *Rhizopogon* is closely related to *Suillus*. First, the structure of the mitochondrial genome of *Rhizopogon subcaerulescens* is identical to that of 14 species of *Suillus* with respect to the order of 15 regions (Fig. 1). The common order of these fragments in *Suillus* and *R. subcaerulescens* is highly significant because mitochondrial gene order is known to be extremely variable among distantly related species of fungi[5–7], and because differences in the order of these 15 regions exist both within the genus *Suillus* and among related genera of the Boletaceae[8]. Second, nucleotide sequences from a portion of the mitochondrial large subunit ribosomal RNA gene from two species *Rhizopogon*, three species of *Suillus* and three species from other genera in the Boletaceae, demonstrate a high level of similarity between *Rhizopogon* and *Suillus* and significant divergence of both of these genera from the other members of Boletaceae sampled (Fig. 2). Cladistic analyses of these sequences demonstrate that *Rhizopogon* and *Suillus* are a monophyletic group that has diverged significantly from the three members of the Boletaceae sampled (Fig. 3).

The extreme morphological divergence between *Rhizopogon* and *Suillus* provides a striking contrast to their molecular similarity. *Suillus* shares many obvious morphological similarities with *Paragyrodon sphaerosporus* such as the presence of tubes, annulate stipes and large pilei. Microscopic features of their basidia and spore attachment are also similar, and the latter species has previously been placed within *Suillus*[9]. *Rhizopogon* differs from both taxa with respect to all of these features and has traditionally been placed in a different family or order[10–12], but molecular criteria (Figs 1 and 2) demonstrate that it is more closely related to *Suillus* than either *Suillus* or *Rhizopogon* is to *P. sphaerosporus*. These results demonstrate an accelerated rate of morphological change relative to molecular change during the evolution of *Rhizopogon*.

This acceleration can be caused by either an increase in the rate of morphological evolution or a decrease in the rate of molecular divergence in *Rhizopogon*, relative to the other taxa studied. The latter explanation would require a major departure from the molecular clock hypothesis, and would not explain the structural similarity of the mitochondrial genomes (Fig. 1). The rate of sequence divergence in *Rhizopogon* would have had to have a fourfold decrease just to set its time of divergence from its closest relatives within *Suillus* to that of its nearest mushroom relative outside the genus (that is, *P. sphaerosporus*), and even this adjustment would be insufficient to match the morphological divergence between *Rhizopogon* and *Suillus* to an outside point of reference. Furthermore, this fourfold rate difference clearly represents an underestimation, because four regions of the gene that align well in comparisons of *Suillus* and *Rhizopogon* were excluded from comparisons with the highly divergent sequences of the other three members of the Boletaceae (Fig. 2). Deviations

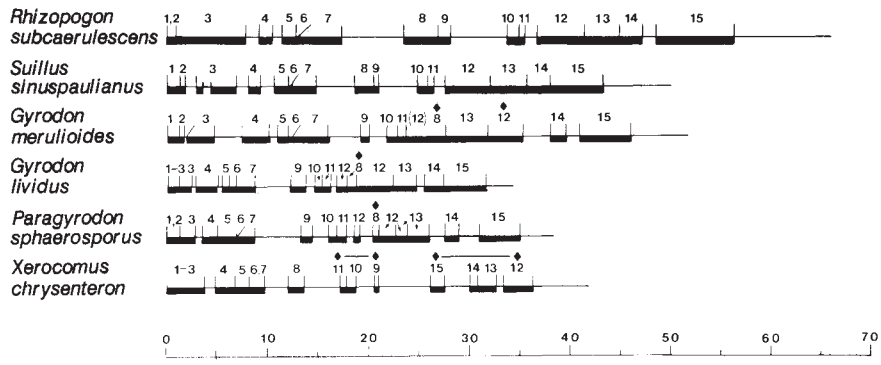

FIG. 1 Fragment order of *R. subcaerulescens* and five taxa from the Boletaceae. The circular mitochondrial genomes of the six taxa are shown linearized in approximate alignment. Regions that hybridize to 14 cloned fragments of *Suillus* mitochondrial DNA (mtDNA) and to portions of the ATPase 9 gene of *Saccharomyces cerevisiae* (region 8) were determined by Southern blot hydridizations. The fragment order shown in *S. sinuspaulianus* is shared by 14 of the 15 species of *Suillus* previously examined[8]. Rearrangements relative to this predominate *Suillus* order were not found in *R. subcaerulescens* but do exist (diamonds) in all other members of the Boletaceae sampled previously[8]. Approximately 75% of the *S. sinuspaulianus* mitochondrial genome and at least eight mitochondrial genes are included in these fragments[8]. MtDNA size varies threefold within *Suillus*[7] and is a poor indicator of phylogenetic affinity[8]. Size scale at the bottom is in kilobase pairs. Light hybridization of clone 12 to a portion of the *G. merulioides* genome is shown with parentheses. The clones, hydridization conditions, and restriction enzymes mapped are described elsewhere[8].

FIG. 2 Aligned sequences of *Rhizopogon subcaerulescens* (Rs), *R. ochraceorubens* (Ro), *Suillus cavipes* (Sc), *S. luteus* (Sl), *S. sinuspaulianus* (Ss), *Boletus satanas* (Bs), *Xerocomus chrysenteron* (Xc) and *Paragyrodon sphaerosporus* (Ps) from a 351 base-pair (bp) portion of the mitochondrial large subunit rRNA gene. Gaps introduced into the sequence to facilitate the alignments are indicated by stops. Bases that differ from the consensus are underlined. The four large underlined regions highlight areas where the aligned sequences of *Suillus* and *Rhizopogon* species cannot be unambiguously aligned with those of the three other taxa. The sequences start at ~122 nucleotides from primer 1 and end 20 nucleotides from primer 2 (see below). Initial alignments were made with GENALIGN and visually adjusted.

METHODS. A fragment containing the mitochondrial large subunit rRNA gene of *S. sinuspaulianus* was cloned into a bluescript phagemid vector[8], and subcloned by *Exo*III deletions[22]. Single strands of individual clones were rescued[23] with the helper phage M13K07 and sequenced by standard dideoxy-termination method with modified T7 DNA polymerase (Sequenase). Conserved regions of the *Suillus* gene were identified by comparison with published sequences of *Saccharomyces cerevisiae* available in GENBANK. The following two oligonucleotides primers, which are complementary to opposite strands of two conserved regions, were synthesized on a Biosearch 8700 DNA synthesizer: ACCCTATGCAGCTTCTACTG, and TTATCCCTAGCGTAACTTTTATC. In the *S. sinuspaulianus* gene these regions are separated by 480 bp (data not shown). *Hin*dIII fragments containing the large subunit rRNA gene from *S. luteus*, *S. cavipes* and *R. subcaerulescens* were cloned into pUC19 by methods described previously[8]. The two primers were used to sequence the region between them in each of these clones by using CsCl-purified double-stranded plasmids as templates[24]. The same primers were used in conjunction with the polymerase chain reaction to amplify and sequence the homologous region in the three other members of the Boletaceae and in *R. ochraceorubens*. Double-stranded fragments were amplified in 100-μl reaction with the following components: 50 pmol each primer, 2.5 units *Taq* polymerase, 0.1–10 ng fungal DNA in a solution of 50 mM KCl, 10 mM Tris, pH 8.4, 2.5 mM $MgCl_2$, 0.1 mg ml^{-1} gelatin and 200 μM each of the four deoxynucleotide triphosphates. These reactions were subjected to 25 cycles in a Perkin-Elmer-Cetus DNA Thermal Cycler under the following temperature regime: 25 s at 95 °C, 55 s at 53 °C, 2 min at 72 °C. Amplified fragments were isolated from a 5-μl aliquot by gel electrophoresis in 1.5% low-melting agarose. Gel slices containing the fragments were melted into 1 ml of 10 mM Tris, pH 8.0, 1 mM EDTA and diluted 1,000-fold in water. Single-stranded templates of both strands were amplified separately and sequenced directly from 1 μl the diluted fragment by the asymmetric primer ratio method[25]. Conditions for these second amplifications were as described above, except that 40 cycles were run with the annealing temperature set at 50 °C and the primer ratios of 1:50 pmol and 50:3 pmol were used to produce strands complementary to the first and second primer respectively.

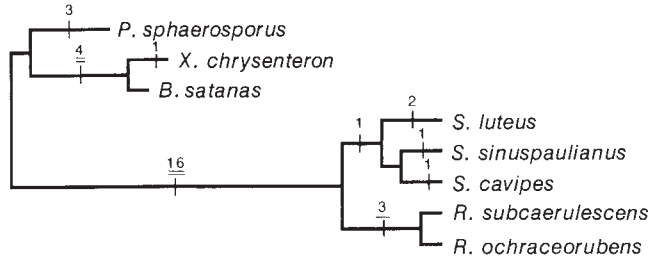

FIG. 3 Phylogenetic relationships between *Rhizopogon* and members of the Boletaceae based on cladistic analyses of nucleotide sequence differences. The tree is based on parsimony analysis, which minimizes the number of mutational events necessary to account for the sequence differences. It was constructed using the branch and bounds algorithm of the DNAPENNY program[26]. All site differences analysed were consistent. Thus, this tree postulates no convergence or reversals. Branch lengths correspond to changes within the well-aligned portion of the large subunit rRNA sequence (that is, excluding the four underlined regions; Fig. 2). Branching patterns depicted within the *Suillus–Rhizopogon* lineage are based on differences within the entire sequenced region, as these five sequences are aligned throughout. We estimated the confidence in all putatively monophyletic groups larger than one with a bootstrap analysis of sample-size 100 using the DNABOOT program[26]. Those groups that occurred in 97% or 100% of the trees are indicated by branch lengths that are underlined once or twice respectively. The groupings of all *Suillus* species, and of *S. sinuspaulianus* with *S. cavipes* were found in 90 and 69 per cent of trees, respectively.

in the molecular clock have been documented[13–16], but differences of this magnitude are generally seen only in comparisons of distantly related lineages[15]. No obvious mechanism is available to explain such a drastic rate reduction in this case.

Rapid morphological divergence could be accomplished if the number of underlying genetic changes is small and if selection is intense. A model for the evolution of gastromycetes proposed by Thiers[2] invokes both of these factors and is applied to *Rhizopogon* in Fig. 4. Unopened, distorted mushrooms, known as secotioid forms, are known in many unrelated lineages and are thought to be caused by developmental arrest during mushroom maturation[2]. In one such case, genetic studies suggest that this arrest may involve only a single recessive gene[17]. The presence of secotioid or partially secotioid derivatives of several *Suillus* species shows that a similar process occurs within the genus[18–20]. Transition between the secotioid and underground false-truffle forms presumably requires numerous additional mutations. But, many of the genes responsible probably regulate processes related to mushroom development (for example, expansion of the stipe, organization of the hymenium) and changes in such genes might be expected to have pleiotropic effects on the morphology. Furthermore, most morphological differences are losses of ancestral structures; given that there are many ways in which gene function can be lost, this process might be expected to be more rapid than one that requires extensive acquisition of novel morphological structures. The enclosed nature of spore-producing tissues in secotioid mushrooms might also be expected to initially increase the frequency of inbreeding, because large clumps of spores would generally be dispersed from a single basidiocarp. A high frequency of

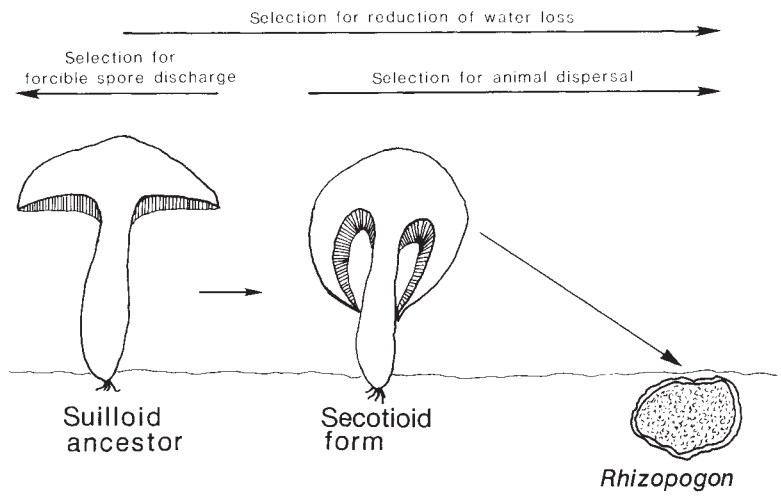

FIG. 4 Model for the evolution of *Rhizopodon* adapted from Thiers[2]. Fruit bodies of a suilloid ancestor, a secotioid form, and *Rhizopogon* are shown in cross-section. Spore-bearing regions are shown with lines (that is, tubes in the suilloid and secotioid forms) and stippling (in *Rhizopogon*). The initial transition between suilloid mushroom and a secotioid form is thought to involve the arrest of normal pileus expansion. Further modification, by loss or reduction of ancestral features, results from continued selection for reduction of water loss and selection for rodent dispersal of fruitbodies[27]. The establishment of the initial secotioid form also removes selection for the maintenance of forcible spore discharge, which in turn permits the loss of precisely aligned tubes, loss of spore asymmetry, and the formation of aberrant basidia. Other changes which occur during the transition to the *Rhizopogon* form are the reduction and loss of the stipe, size reduction of the basidiocarp, loss of cystidia, loss of sterigmata, and completion of development underground.

inbreeding would enable a rapid increase in the frequency of recessive alleles in the progeny.

The sparsity of intermediates between *Rhizopogon* and *Suillus* provides indirect evidence of intense selective pressure. Putatively intermediate taxa are limited to three secotioid forms of *Suillus* and two to three species of *Truncocolumella*. Collectively, these fungi do not approach a gradual set of intermediates. With the exception of *Truncocolumella citrina*, all are rare and limited in distribution. In contrast, both *Suillus* and *Rhizopogon* have over 80 described species, many of which are common and widely distributed in north temperate regions. This pattern is consistent with the concept of a selective gradient in which intermediates, ill-adapted to either air or animal dispersal and lacking the derived modifications necessary for seasonally xeric conditions, would be eliminated at a high frequency.

The dramatic change in morphology and lack of intermediates seen in these extant organisms is analogous to the patterns seen in the fossil records of many other taxa[21]. The case of *Suillus* and *Rhizopogon* thus provides a modern example of rapid morphological evolution; one in which a detailed proposed mechanism exists, and where the underlying molecular genetic basis can be examined in more detail. □

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1. Heim, R. in *Evolution in the Higher Basidiomycetes* (ed. Petersen, R. H.) 505–534 (Univ. Tennessee Press, Knoxville, 1971).
2. Thiers, H. D. *Mycologia* **76,** 1–8 (1984).
3. Savile, D. B. O. in *The Fungi Vol.* 3 (eds Ainsworth, G. C. & Sussman, A. S.) 649–675 (Academic, New York, 1965).
4. Shaffer, R. L. *Mycologia* **67,** 1–18 (1974).
5. Grossman, L. I. & Hudspeth, M. E. S. in *Gene Manipulations in Fungi* (eds Bennet J. W. & Linda, L.) 56–104 (Academic, New York, 1985).
6. Hoeben, P. & Clark-Walker, G. D. *Curr. Genet.* **10,** 371–379 (1986).
7. Bruns, T. D., Palmer, J. D., Shumard, D. S., Grossman, L. I. & Hudspeth, M. E. S. *Curr. Genet.* **13,** 49–56 (1988).
8. Bruns, T. D. & Palmer, J. D. *J. molec. Evol.* **28,** 349–362 (1989).
9. Smith, A. H. & Thiers, H. D. *The Boletes of Michigan* (Univ. Michigan Press, Ann Arbor, 1971).
10. Ainsworth, C. W. *Dictionary of the Fungi* 6th Edn (Commonwealth Mycological Institute, Kew, 1971).
11. Pegler, D. N. & Young, T. W. K. *Trans. Br. mycol. Soc.* **76,** 103–146 (1981).
12. Julich, W. *Higher Taxa of Basidiomycetes* (Cramer, Vaduz, 1981).
13. Templeton, A. R. *Evolution* **37,** 221–224 (1983).
14. Wu, C. I. & Li, W. H. *Proc. natn. Acad. Sci. U.S.A.* **82,** 1741–1745 (1985).
15. Britten, R. J. *Science* **231,** 1391–1398 (1986).
16. Li, W.-H. & Tanimura, M. *Nature* **326,** 93–95 (1987).
17. Rosinski, M. A. & Robinson, A. D. *Am. J. Bot.* **55,** 242–246 (1968).
18. Thiers, H. D. & Trappe, J. M. *Brittonia* **21,** 244–254 (1969).
19. Singer, R. & Both, E. E. *Mycologia* **69,** 59–72 (1977).
20. Singer, R. *The Agaricales in Modern Taxonomy* 4th Edn (Koeltz, Koenigstein, 1986).
21. Eldridge, N. & Gould, S. J. in *Models in Paleobiology* (ed. Schof, T. J. M.) 82–115 (Freeman, San Francisco, 1972).
22. Henikoff, S. *Gene* **28,** 351–359 (1984).
23. Dente, L., Cesareni, G. & Cortese, R. *Nucleic Acids Res.* **11,** 1645–1655 (1983).
24. Lim, H. M. & Pène, *Gene anal. Techn.* **5,** 32–39 (1988).
25. Gyllensten, U. B. & Erlich, H. A. *Proc. natn. Acad. Sci. U.S.A.* **85,** 7652–7655 (1988).
26. Felsenstein, J. *PHYLIP 3.1.* (Users Manual, Univ. Washington, Seattle, 1988).
27. Maser, C., Trappe, J. M. & Nussbaum, R. A. *Ecology* **59,** 799–809 (1978).

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Origin of the algae

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EUKARYOTIC algae are traditionally separated into three broad divisions: the rhodophytes, the chromophytes and the chlorophytes. The evolutionary relationships between these groups, their links with other eukaryotes and with other photosynthetic groups, such as euglenophytes and cryptophytes, have been the subject of much debate and speculation[1–8]. Here we analyse partial sequences of the large (28S) cytoplasmic ribosomal RNA from ten new species of protists belonging to various groups of unicellular algae. By combining them with the homologous sequences from 14 other unicellular and multicellular eukaryotes, we show that rhodophytes, chromophytes and chlorophytes emerge as three distinct groups late among eukaryotes, that is, close to the metazoa–metaphytes radiation. This implies a relatively late occurrence of eukaryotic photosynthetic symbiosis. We also provide details of intra- and inter-phyla relationships.

The ten new sequences, as shown in Fig. 1, correspond to the ~450 nucleotides of the 5′ end of the cytoplasmic large ribosomal RNA (28S). This molecule can be used as a tracer of the history of the nucleo-cytoplasmic compartment independently of the chloroplast compartment, which is now widely considered to result from a single or from several endosymbiotic events[9–12]. We have shown that the specific domain sequenced provides a good phylogenetic index over very broad evolutionary distances[13,14]. The phylogenetic relationships of algae with the eukaryotic kingdoms (metazoa, metaphytes, fungi and protists) show that all the algal groups emerge later than all other unicellular eukaryotes (Fig. 2). Both Sogin's group[15] and ours[13] have previously reported that a set of non-photosynthetic flagellated protists emerge very early from the eukaryotic tree. It now appears that the three main classes of photosynthetic protists as well as the cryptophytes diverged at a later stage (as predicted by Cavalier-Smith[16] on the basis of several ultrastructural features), during a period of intense diversification that is relatively close to the metazoa–metaphytes radiation and which comprises the fungi and other groups of protists such as ciliates. This rapid diversification makes it difficult to resolve branching orders in distance trees. However, several alternative procedures for treating the data, including parsimony approaches