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The fungal mitochondrial genome project: evolution of fungal mitochondrial genomes and their gene expression

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Abstract The goal of the fungal mitochondrial genome project (FMGP) is to sequence complete mitochondrial genomes for a representative sample of the major fungal lineages; to analyze the genome structure, gene content, and conserved sequence elements of these sequences; and to study the evolution of gene expression in fungal mitochondria. By using our new sequence data for evolutionary studies, we were able to construct phylogenetic trees that provide further solid evidence that animals and fungi share a common ancestor to the exclusion of chlorophytes and protists. With a database comprising multiple mitochondrial gene sequences, the level of support for our mitochondrial phylogenies is unprecedented, in comparison to trees inferred with nuclear ribosomal RNA sequences. We also found several new molecular features in the mitochondrial genomes of lower fungi, including: (1) tRNA editing, which is the same type as that found in the mitochondria of the amoeboid protozoan *Acanthamoeba castellanii*; (2) two novel types of putative mobile DNA elements, one encoding a site-specific endonuclease that confers mobility on the element, and the other constituting a class of highly compact, structured elements; and (3) a large number of introns, which provide insights into intron origins and evolution. Here, we present an overview of these results, and discuss examples of the diversity of structures found in the fungal mitochondrial genome.

Key words Chytridiomycetes · Mitochondria · Comparative genomics · Gene expression

Dedicated to Professor Fritz Kaudewitz, Founding Editor of *Current Genetics*, in recognition of his long service to the Journal

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Introduction

Current molecular research in fungi is largely restricted to the "higher fungi", i.e., Ascomycota and Basidiomycota, which include the majority of the approximately 70 000 described fungal species. The less well known "lower fungi", the Chytridiomycota and Zygomycota, have been relatively neglected. Although the latter two groups of organisms probably represent most of the genetic diversity of fungi, their evolutionary relationships to each other and to the "higher fungi", as well as their modes of gene expression and their ecological importance, are not well understood. In order to overcome these limitations, the fungal mitochondrial genome project (FMGP) has started to investigate selected members of all major fungal lineages, with an emphasis on lower fungi, by the sequencing of complete mtDNAs, and by analyzing their evolution and molecular expression.

Historically, the FMGP has its roots in the very active phase of mitochondrial genetics research in yeast and fission yeast initiated in the early 1970s. Major contributions in this field came from the "Institut für Genetik und Mikrobiologie der Universität München", which was directed by Dr. F. Kaudewitz, who established a strong program in mitochondrial biochemistry, genetics and gene expression. Although most scientists involved in the early mitochondrial research of the Institute have now moved to other research institutions, they have continued to contribute to mitochondrial research.

A definition of the kingdom fungi

Historically, the concept of a fungal lineage has been controversial, because the evolutionary relationships of "zoosporic fungi" and slime molds to the Zygomycota, Ascomycota and Basidiomycota were difficult to assess when only morphological characters were considered. Zoosporic

fungi were either classified in the kingdom Fungi (Whittaker 1969; Sparrow 1973; Barr 1992) or, alternatively, included in the Protoctista (Protista) (Corliss 1984; Margulis and Schwartz 1988), an ill-defined eukaryotic kingdom uniting the most ancient and diverse lineages of unicellular eukaryotes. Zoosporic fungi are now understood to be a polyphyletic group. Evidence for this view includes differences in ultrastructural features such as mitochondrial cristae (Olive 1975) and the architecture of the flagellar apparatus (Barr 1980, 1981, 1983, 1992), together with differences in biochemical features, such as pathways of lysine biosynthesis (Vogel 1965) and the composition of cell walls (Bartnicki-Garcia 1970). More recently, hypotheses about fungal evolution that are based on ultrastructural and biochemical evidence have been supported by molecular phylogenies inferred from nuclear small-subunit ribosomal RNA (*rns*) sequences. According to molecular phylogenies, zoosporic fungi that are classified in the Oomycota are related to the golden-brown algal lineage (Stramenopila), and only zoospore-producing fungi in the Chytridiomycota are related to the higher fungi (Gunderson et al. 1987; Förster et al. 1990). Because in this article it is important to distinguish between organisms belonging to the kingdom Fungi and organisms that are fungal by virtue of their life style, we restrict the term "fungi" to organisms in the Chytridiomycota, Zygomycota, Basidiomycota and Ascomycota. We will use "oomycetous fungi" or "pseudo-fungi" to refer to organisms in the Oomycota.

An important portion of our research focuses on members of the Chytridiomycota because their diversity has been defined only by cellular morphology and ultrastructure, and not in molecular terms. Further, until now it has been unclear how the chytridiomycetes are related in an evolutionary sense to the higher fungi, and whether they are a monophyletic group of organisms.

The diversity of chytridiomycete fungi

The Chytridiomycota contains a single class, five orders, and about 112 genera. Chytrids are microscopic and cannot be isolated by standard microbiological methods; consequently, they have not been seen by many biologists. These "lower fungi" are neglected in many general biology text books and are sometimes even misclassified (see review in Powell 1993).

(1) Morphology and ecology of the chytrids

The morphology of Chytridiomycetes ranges from small (usually between 10–100 μm diameter), sack-like thalli, which may have absorptive root-like rhizoids, to indeterminant mycelial forms. The thalli of many of the simpler forms are uniquely decorated with teeth, spines, ridges, etc. Asexual reproduction is by the production of swimming spores (zoospores; 2–10 μm diameter), which are formed within zoosporangia. A variety of sexual mechanisms exists within the fungal phylum (reviewed in Sparrow 1960) including fusion of motile isogametes or anisogametes, gametangial conjugation (Doggett and Porter 1996), fusion of thalli, and fertilization of eggs with motile sperm. Sexual reproduction, however, has not been reported for the majority of chytrid species. The thalli of chytrids are haploid, except for *Allomyces*, which has an isomorphic alternation of generations, and some other members of the Blastocladiales that have a diploid stage.

Chytrids are saprobes and parasites in aquatic and semi-aquatic habitats and can be found either by directly examining organic substrates with the compound microscope or by enriching water cultures with organic baits that later can be examined microscopically (Barr 1987). Although they are commonly known as aquatic fungi, chytrids are also widespread in soils and a few are marine. Some species are of agricultural importance. *Synchytrium endobioticum* causes black wart disease of potato; *Olpidium brassicae* parasitizes the roots of plants in the cabbage family and is a vector of viruses; and members of the order Neocallimastigales are obligate anaerobes that, along with other microorganisms, degrade cellulose in the guts of mammalian herbivores. *Coelomomyces* species are parasitic in mosquito larvae and have received attention as possible bio-control agents (Lucarotti et al. 1985). Chytrid species that parasitize algae can be present in sufficient numbers to affect host populations (Bruning et al. 1992). The ecological importance of most chytrids, however, is unknown. It is probable that many saprobic chytrids have a key function as primary invaders of organic matter in aquatic situations, including wet soils.

Phylogeny of the chytrids, based on ultrastructure

Many chytridiomycete thalli are so simple that few light microscopic characters are available on which to base phylogenetic hypotheses. The same body plan evolved in several phylogenetically distant lines, which has made phylogenetic hypotheses based on light microscopy suspect. For example, *Olpidium* with posteriorly uniflagellate zoospores and *Olpidiopsis* with biflagellate zoospores have simple sac-like thalli and at one time were both placed in the same family (Fischer 1892). It was not until the taxonomic importance of the zoospore flagellum was realized that these genera were placed in different orders (Sparrow 1973), and more recently in different major groups of the eukaryotes (Fungi and Stramenopila; Sogin 1994).

Sparrow (1958) began using the class name Chytridiomycetes for the orders of zoosporic fungi whose members possessed posteriorly uniflagellate zoospores, separating them from orders that sometimes had similar morphology but biflagellate or anteriorly uniflagellate spores. In so doing Sparrow followed Scherffel's (1925) ideas and placed primary taxonomic emphasis on the zoospore structure. Unfortunately, taxonomy, which is based on the asexual reproductive spore, has probably excluded taxa that are phylogenetically allied with the Chytridiomycetes (Nagahama et al. 1995).

The ability to study the fine structure of zoospores using transmission electron microscopy added taxonomic

characters to the few previously available. Beginning in the 60s and especially in the 70s (see reviews in Barr 1978; Powell 1978; Lange and Olson 1979) representatives of many taxa were studied and by the 1980s ultrastructural features characteristic for the class and orders were delineated (Barr 1980, 1981). Members of the Chytridiomycetes have a posteriorly directed flagellum attached to a kinetosome (basal body) that is associated with a secondary centriole (non-functional centriole, C2, dormant centriole). Also associated with the kinetosome are nine props, each of which originates at the distal end of the kinetosome and is attached to the plasma membrane. These features seemed nearly universal until studies of the Neocallimastigales determined that these anaerobic gut fungi belong in the Chytridiomycetes (Bowman et al. 1992; Li and Heath 1992). Zoospores of the gut fungi lack the second centriole and props, possess a kinetosome-related skirt and collar not seen in any other Chytridiomycetes, and zoospores of species in some genera are polyflagellate. A chytrid-like morphology and the directionality of their zoospore flagellum suggested that the gut fungi belonged in the Chytridiomycetes but similarities in thallus morphology are frequently convergent, and it was sequencing evidence that confirmed their placement. Orders are also classified on the basis of zoospore ultrastructure (Barr 1980, 1990) and we selected representatives of four of these orders (Neocallimastigales are not included because they lack mitochondria) for the fungal mitochondrial genome project (FMGP).

The fungal mitochondrial genome project

Published molecular phylogenies still include only a few chytridiomycete fungi, and tree topologies are not robust (e.g., Bruns et al. 1992). This problem is not only due to a lack of species in the currently available data sets, but reflects the general difficulty in resolving fungal phylogeny with sequences from single genes. In order to overcome these limitations, we are currently sequencing complete mtDNAs from selected members of all major fungal lineages, and we use concatenated protein sequences for the phylogenetic analyses. In addition to providing rich material for phylogenetic reconstructions, the comparative analysis of the genomic sequences permits us to detect conserved intergenic sequence motifs that might pay a role in transcription, translation, or gene regulation. Further, the genome comparison also allows us to address the mechanisms by which the especially rapid changes in fungal mitochondrial DNAs can be explained, and to determine how gene expression correlates with these changes. In the course of this research program, more than 600 kbp of new mitochondrial sequence from selected members of the major fungal lineages will become available.

The results of our research are available on the World Wide Web (http://megasun.bch.umontreal.ca/People/lang/FMGP/), usually much in advance of journal publication. Web pages contain information on methods, strain descriptions, organismal information and images, genome maps, sequences, etc.

Materials and methods

Strains, cultures, purification and cloning of mtDNA. The strains *Spizellomyces punctatus* BR11, *Rhizophlyctis rosea* BR186 and *Rhizophus stolonifer* (DAOM14842) were kindly provided by Dr. D. J. S. Barr (Agriculture Canada, Ottawa), *Allomyces macrogynus* (strain Burma 3-35 (35°C) his1; ATCC 46923) by Dr. L.W. Olson (Copenhagen, Denmark) and *Schizophyllum commune* (homokaryotic strain UVM 4-40) by Dr. C. P. Novotny (University of Vermont, US). *Harpochytrium* strains, other Monoblepharidales and *Rhizophydium* sp. are from the personal collection of Marilyn Mollicone and J. E. Longcore, University of Maine. *Phytophthora infestans* (ATCC 16981) was purchased from ATCC. *S. punctatus* was grown on a medium containing 0.5% yeast extract, 3% dextrose or glycerol (pH 5.8). *R. rosea* was grown on the same medium, supplemented with 1.7% yeast nitrogen base without amino acids and ammonium sulphate (Difco). *Rhizophydium* sp., *Harpochytrium* sp. and *Monoblepharella* sp. were grown on a medium containing 0.1% tryptone, 0.1% peptone and 0.5% dextrose. *P. infestans* was grown on the media formulation provided by ATCC. More information on cell-culture methods are available on the World Wide Web at URL: http//megasun/People/lang/FMGP/methods.html. Cell culture, purification of mtDNA by cesium chloride/bisbenzimide density gradient centrifugation of total cellular DNA, and cloning procedures were all performed according to previously published protocols (Paquin et al. 1995 b). Some of our protocols are also available on the World Wide Web at URL: http://megasun.bch.umontreal.ca/ogmp/mbmethods.html.

Sequencing of mtDNA. The mtDNAs were sequenced using random clone libraries (nebulized mtDNA cloned in a modified Bluescript KS+ vector; http://megasun.bch.umontreal.ca/ogmp/bank.html). Sequencing of single-stranded DNA templates was performed by the dideoxy chain-termination method (Sanger et al. 1977). To permit extended reading of sequences, high-resolution polyacrylamide-gel electrophoresis was performed (Lang and Burger 1990). Both DNA strands were sequenced.

Phylogenetic analysis. The COX1, COX2, COX3 and COB sequences that were used for phylogenetic analysis were either obtained from Genbank or were translated from the mtDNA sequences determined by us. A collection of all protein sequences used in this study can be obtained via the WWW at http://megasun.bch.umontreal.ca/People/lang/FMGP/proteins.html. Multiple protein sequences were aligned with CLUSTAL V or W (Higgins et al. 1992; Thompson et al. 1994), which was executed from GDE (genetic data environment, Smith et al. 1994). Only amino-acid positions that could be aligned without ambiguities were used for the analysis. Phylogenetic trees were inferred using distance approaches. First, a distance matrix was calculated with PROTDIST (Felsenstein 1993), this matrix was then used as an input in tree-building algorithms, either FITCH (Fitch and Margoliash 1967) or NEIGHBOR (Saitou and Nei 1987). The robustness of the tree topology was tested by bootstrap analysis (Felsenstein 1985). These programs were used as installed in the PHYLIP package (Felsenstein 1993). Alternative trees (data not shown) were constructed with a maximum-likelihood algorithm (PROTML, Kishino et al. 1990), using an exhaustive tree-optimization procedure with the -jf parameters. No difference was observed between the trees constructed either by the two distance approaches (PROTDIST with FITCH or NEIGHBOR) or by the maximum-likelihood algorithm.

Results and discussion

Description of selected mitochondrial genomes

In this section we describe the mitochondrial genomes from three representative fungi: the chytridiomycete *A. macro-*

gynus, the ascomycete *Schizosaccharomyces pombe* and the basidiomycete *S. commune*. We compare the mtDNAs of these taxa with that of *P. infestans* to demonstrate that this oomycete should definitely not be included in the eufungal kingdom.

A. macrogynus

Organismal description. A. macrogynus is a member of the Blastocladiales, one of the five orders of the Chytridiomycota. The filamentous cells of *Allomyces* grow essentially without limitation in length, and divide in a characteristic dichotomous pattern that results in a perfectly geometric mycelium, on solid media. Hyphae (filament segments) are separated by pseudo septa that allow free circulation of intracellular materials along the filaments. The reproductive structures, which are located at the end of the hyphae, are isolated by complete septa. *Allomyces* is most commonly found in damp soil in the tropics, although it has been identified in many different regions of the world. Its life cycle can alternate between haploid gametophytic and diploid sporophytic stages, reflecting its capacity for both sexual and asexual reproduction. For a more detailed description of *Allomyces* see the review of Olson (1984), which describes many details of its life cycle, light microscopic and ultrastructural views, genetics, basic experiments in teaching, etc.

Mitochondrial DNA. A. macrogynus has a circular mitochondrial DNA of 57 473 bp that has been completely sequenced (Fig. 1; Paquin and Lang 1996). The mitochondrial genome of *A. macrogynus* resembles that of many other fungal mtDNAs in that it contains the usual set of genes (Table 1) interrupted by numerous introns and separated by relatively long intergenic spacers (up to 747 bp in length). The alliance of *Allomyces* to the higher fungi is corroborated by phylogenetic studies inferred from a comparison of gene contents and genome complexities of mtDNAs (Sankoff et al. 1992) and mitochondrial protein sequences (Paquin et al. 1995 a, b; see also the section on phylogeny).

Although the *A. macrogynus* mtDNA is typically "fungal", sequencing of the whole genome revealed some unusual and unique features: (1) the overall A+T content is exceptionally low (60% as compared to 70–80% in most other fungi); (2) the universal genetic code is used, which contrasts with a modified code employed in nearly all ascomycetes (with the exception of *S. pombe*). In mitochondria of *Neurospora* and *Aspergillus*, for instance, the 'stop' codon UGA specifies tryptophan; (3) for the first time, a ribosomal protein gene (*rps3*) was identified (Fig. 1), which is clearly similar to bacterial homologues (Paquin and Lang 1996); (4) four unique ORFs were identified, one of which, *orf360*, codes for an endonuclease that is part of a mobile element (Paquin et al. 1994; see below); and finally, (5) we have identified 80 highly structured and conserved DNA elements of 26–79 bp that are inserted in intergenic spacers, introns, and variable regions of the *rnl* and *rns* genes (see below).

For a more detailed description of this mitochondrial genome, see Paquin and Lang (1996).

S. pombe

Organismal description. S. pombe ("fission yeast") is a member of the class Archiascomycetes, Ascomycota. As the prefix "archi" indicates, members of this group have been assumed to be an ancestral assembly of the ascomycetes. The common name "fission yeast" is misleading, because *S. pombe* does not have any more features in common with budding yeasts (e.g., *Saccharomyces* or *Pichia*) than with non-yeast ascomycetes (e.g., *Neurospora* or *Penicillium*). *S. pombe* is almost as easily cultured and manipulated as yeast, and is well characterized with respect

Table 1 Number of different types of genes in mtDNAs. For a discussion of the gene content of animal, plant and fungal mtDNAs, see also the review by Gray (1992). Further mtDNA data used to compile this table: *Phytophthora infestans*, (this paper); the amoeboid protist *Acanthamoeba castellanii* (Burger et al 1995); *Marchantia polymorpha* (Oda et al. 1992). The difference in gene repertoire of animals *versus* fungi consists in the additional occurrence of *atp9* in many fungi, the presence of a pseudogene coding for a ribosomal protein (*rps3*) in *A. macrogynus*, and the *var1* gene in several budding yeasts. The VAR1 protein is associated with mitochondrial ribosomes but no homolog is recognized by sequence comparison with bacterial or eukaryotic ribosomal proteins (Butow et al. 1985). Fungal mtDNAs tend to code for several further unidentified protein genes. Animal mitochondrial genes do not normally contain introns. The only documented exception is in the sea anemone *Metridium senile* (Beagley et al. 1996). The major difference in gene repertoire between fungal and protist (including *P. infestans*) or plant mtDNAs is the presence of ribosomal protein genes in the latter.

Type of gene	Animals	Fungi	Phytophthora	Acanthamoeba	Plants
Respiratory chain					
NADH dehydrogenase (*nad*)	7	0–7	10	10	11–12
apocytochrome *b* (*cob*)	1	1	1	1	1
cytochrome oxidase (*cox*)	3	3	3	3	3
ATP synthesis (*atp*)	2	2–3	4	3	4
Ribosomal RNAs (*rnl*, *rns*, *rrn5*)	2	2	2	2	2–3
Ribosomal proteins (*rps*, *rpl*)	–	(1)	16	16	16
Introns	(+)	+++	–	3	++

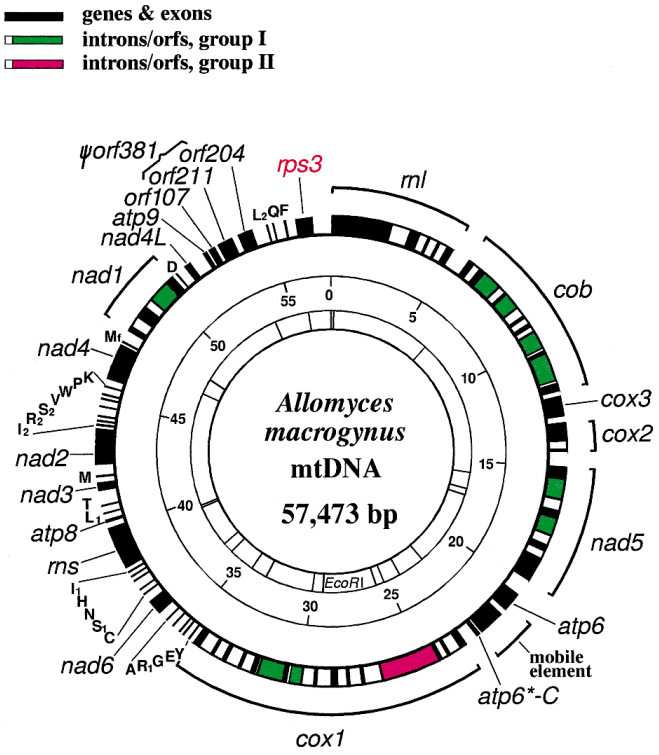

Fig. 1 Map of *A. macrogynus* mtDNA. *Numbers* in the inner circle are in kbp and the start position has been set at the start site of the *rnl* gene. An *Eco*RI restriction map is also shown. All genes are transcribed clockwise and split genes are indicated by *brackets*. The endonuclease-containing, mobile element (Paquin et al. 1994) is also *bracketed*. The gene *rps3* (*in red*) is the only known example of a ribosomal protein-coding gene-homolog in fungal mitochondria (see text and Table 1). tRNA genes are designated using the one-letter-code of the amino acid they specify. In the following cases, two isoacceptors are found: I_1, $tRNA^{Ile}$ (GAU); I_2, $tRNA^{Ile}$ (CAU); L_1, $tRNA^{Leu}$ (AAG); L_2, $tRNA^{Leu}$ (UAA); R_1, $tRNA^{Arg}$ (UCU); R_2, $tRNA^{Arg}$ (UCG), S_1, $tRNA^{Ser}$ (UGA); S_2, $tRNA^{Ser}$ (GCU)

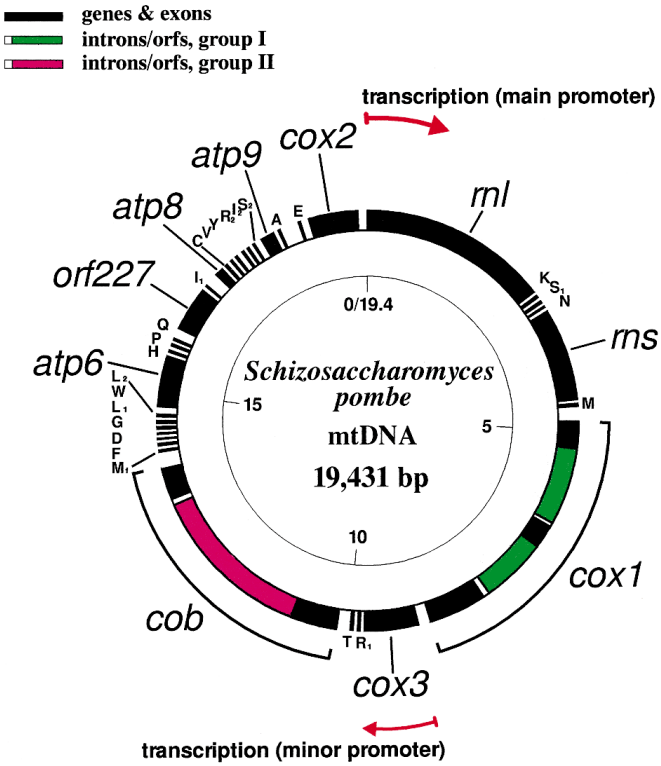

Fig. 2 Map of *S. pombe* mtDNA. *Numbers* in the inner circle are in kbp and position 0/19.4 is at the start of the *rnl* gene. Boundaries of intron-containing genes are indicated by *brackets*. Genes are transcribed clockwise from two promoters as shown by *arrows*. Designation of tRNAs when two isoacceptors are used: S_1, $tRNA^{Ser}$ (GCU); S_2, $tRNA^{Ser}$ (UGA); R_1, $tRNA^{Arg}$ (UCU); R_2, $tRNA^{Arg}$ (UCG); L_1, $tRNA^{Leu}$ (UAA); L_2, $tRNA^{Leu}$ (UAG); I_1, $tRNA^{Ile}$ (GAU); I_2, $tRNA^{Ile}$ (CAU)

to both classical and molecular genetics. Its nuclear genome is being sequenced and this species is likely to become an alternative fungal model system, comparable to the yeast *Saccharomyces cerevisiae*.

Mitochondrial DNA. The 19 430-bp-long circular mtDNA of *S. pombe* has been completely sequenced (Swiss Prot accession number MISPCG; Lang et al. 1983; Lang 1993; Fig. 2). This mtDNA is tightly packed with genes that are separated by short, A+T-rich, 'non-coding' sequences, which contrasts with the structure of the mtDNA of *A. macrogynus* described above, in which genes are separated by relatively long, highly G+C-rich spacers. Nonetheless, the mitochondrial gene content of *A. macrogynus* and *S. pombe* is quite similar, with the exception that *S. pombe* lacks *nad* genes (coding for NADH dehydrogenase subunits).

The universal translation code is used in mitochondrial protein-coding genes of *S. pombe*, contrasting with code deviations found in the mitochondria of all other ascomycetes. Despite the small genome size, three introns are present in the *S. pombe* mtDNA (see Table 2; Lang 1984; Lang et al. 1985). The genome is transcribed in two units (indicated by arrows in Fig. 2), starting from the two promoters situated opposite one another on the circular DNA molecule, one upstream of *rnl* and the other upstream of *cox3*. Transfer RNA genes that are interspersed between

Table 2 Distribution of introns and intron ORFs in some fungal mtDNAs. Group-I intron ORFs were classified in three categories, LAGLI-DADG (LAGLI) (Waring et al. 1982; Hensgens et al. 1983), Omega (Dujon 1980; Lazowska et al. 1980) and GIY-YIG (Burger and Werner 1985; Michel and Cummings 1985). Group-II intron ORFs usually encode proteins showing significant similarities to reverse transcriptases (RT) (Michel and Lang 1985).

mtDNA	Group-I introns				Group-II introns	
	Total	LAGLI	Omega	GIY-YIG	Total	RT-like
A. macrogynus	26	2	6	1	2	1
S. punctatus	10	1	3	2	0	
Harpochytrium	0				0	
R. stonolifer	9	3	1	1	0	
S. commune	0				0	
S. pombe	2	2			1	1

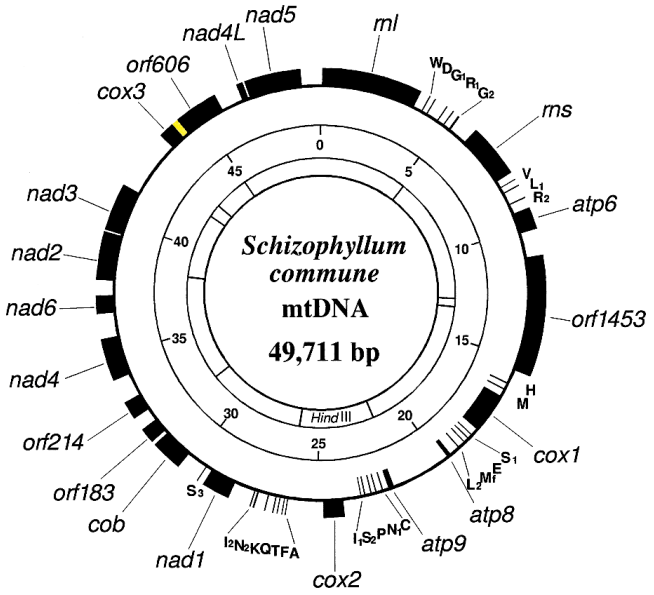

Fig. 3 Map of *S. commune* mtDNA. Genes are represented by *filled boxes* and those located on the outer ring are transcribed clockwise, whereas those on the inner ring are transcribed anti-clockwise. The *yellow region* indicates the 242-nucleotide overlap between *cox3* and *orf606*. The innermost circle is a *Hin*dIII restriction map. Isoacceptor tRNAs: G_1 *and* G_2, $tRNA^{Gly}$ (UCU); R_1, $tRNA^{Arg}$ (UCU); R_2, $tRNA^{Arg}$ (UCG); L_1, $tRNA^{Leu}$ (UAG); L_2, $tRNA^{Leu}$ (UAA); S_1, $tRNA^{Ser}$ (GCU); S_2 *and* S_3, $tRNA^{Ser}$ (UGA); N_1, $tRNA^{Asn}$ (GUU); N_2, $tRNA^{Asn}$ (AUU); I_1, $tRNA^{Ile}$ (CAU); I_2, $tRNA^{Ile}$ (GAU)

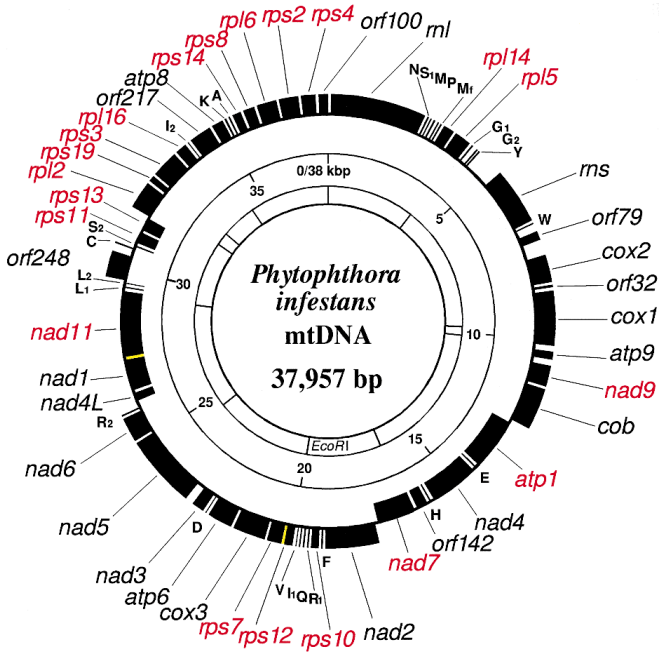

Fig. 4 Map of *P. infestans* mtDNA. An *Eco*RI restriction map is displayed in the inner circle. Genes are shown as *filled boxes* and their position on either the outward or inward circle relate to their direction of transcription (clockwise or counterclockwise, respectively). *Yellow regions* indicate overlaps between genes and genes in *red* are those that are not usually found in fungal mtDNAs. Instances where two isoacceptor tRNAs are used: S_1, $tRNA^{Ser}$ (GCU); S_2, $tRNA^{Ser}$ (UGA); G_1, $tRNA^{Gly}$ (GCC); G_2, $tRNA^{Gly}$ (UCC); R_1, $tRNA^{Arg}$ (GCG); R_2, $tRNA^{Arg}$ (UCU); I_1, $tRNA^{Ile}$ (GAU); I_2, $tRNA^{Ile}$ (CAU); L_1, $tRNA^{Leu}$ (UAG); L_2, $tRNA^{Leu}$ (UAA)

most other genes may be used for processing the large RNA precursors containing them. A similar mechanism has been proposed in the case of animal mitochondria (Bibb et al. 1981).

S. commune

Organismal description. S. commune is a basidiomycete fungus with world-wide distribution. Its taxonomic classification in the order Aphyllophorales (Alexopoulos et al. 1996; Nakasone 1996) remains problematic. Although its fruiting body has the appearance of a "mushroom", the lamellae on the fruiting body of *Schizophyllum* are most likely not homologous with the gills of typical mushrooms (Agaricales). *S. commune* grows on dead branches and twigs of hardwoods and lumber, is cultivated very easily in the laboratory, and is used as an experimental organism in studies dealing with the function of mating-type loci, genetics, physiology, morphogenesis and many other topics.

Mitochondrial DNA. The very A+T-rich (78%) mtDNA of *S. commune* is circular and has a length of 49 711 bp (Fig. 3). Its gene content is characteristic of fungal mitochondria, and includes the *nad* genes. In addition to the usual genes (see Table 1), *S. commune* mtDNA contains four long ORFs. The longest ORF potentially codes for a peptide of 1453 amino acids, the sequence of which is distantly related to RNA polymerase, although the significance of the similarity remains to be determined. The three other ORFs have no obvious similarity to any known protein sequence in the databases. The *nad3* gene includes an unusual carboxy terminal extension of 473 amino acids of unknown function. This may have evolved from an in-phase overlap with a downstream ORF. As in many other fungi, the genes are often separated by long, A+T-rich spacers. The genes are coded on both DNA strands although most of them are transcribed from one strand; only 11 tRNA and three protein-coding genes, located within a single 7.5-kb segment, are inverted with respect to the other genes. Based on the sequence of the *cox3* gene, others (Phelps et al. 1988) have postulated that the universal translation code might be used in *S. commune* mitochondria. Our analysis of the complete sequence, however, shows that more than 20% of the tryptophan codons occurring in conserved mitochondrial protein genes (such as *cob* or *nad5*) are specified by UGA. Despite a high bias towards codons with A or T in the third position in *S. commune*, UGA (Trp) codons are not preferentially used, as they are in other species (e.g., yeast). This leads us to believe that the introduction of this codon occurred only re-

cently in the evolution of the Basidiomycota. Also, the anticodon of the $tRNA^{Trp}$ is CCA, as in mitochondria using the universal translation code. The *S. commune* mitochondrial $tRNA^{Trp}$ (CCA) must be able to recognize not only the preferred UGG codons but also, to some degree, UGA codons. It is conceivable that the C in the wobble position of the anticodon has to be partially modified, to permit the binding to and the recognition of UGA (Trp) codons.

Some aspects of the *S. commune* mtDNA are clearly uncharacteristic of fungal mitochondria. Most surprisingly, we have not detected any introns, which is a rare exception to the otherwise intron-rich fungal mtDNAs. The *nad* genes are clustered and arranged in a conspicuous fashion: the stop codon of the *nad2* gene is directly adjacent to the initiation codon of the *nad3* gene, and the *nad4L* and *nad5* coding regions are separated by only two nucleotides. Also, *cox3* and *orf606* overlap by 242 nucleotides. Finally, the tRNA genes are unusual in two respects. First, 2 of the 27 tRNA genes in *S. commune* mtDNA, $tRNA^{Gly}$ and $tRNA^{Ser}$ (UGA), originate from gene duplications; and secondly, two different tRNAs read the asparagine codons AAY (Y stands for pyrimidine). Generally, a single $tRNA^{Asn}$ (GUU) is sufficient to read both codons because the G in the wobble position is able to pair with either C or U. But in *S. commune*, the two asparagine codons may be read by two different tRNAs, $tRNA^{Asn}$ (GUU) and $tRNA^{Asn}$ (AUU). Because an A in the wobble position of an anticodon is normally de-aminated to I, it would result in a most unusual tRNA (IUU) recognizing both asparagine and lysine codons. We currently have no alternative explanation for the function of this particular tRNA gene. No $tRNA^{Tyr}$ has been detected despite the presence of numerous UAU and UAC codons in mitochondrial genes. We assume that this tRNA is imported from the cytoplasm. Although *S. commune* mtDNA has the highest number of tRNA genes yet found in fungi, its set is apparently not sufficient to read all codons.

P. infestans

Organismal description. P. infestans is an oomyceteous fungus (pseudo-fungus) that causes the devastating "late blight disease" of potatoes. Other species in the genus are also economically important pathogens of higher plants and, consequently, much is known about the biology of the genus (e.g., Lucas et al. 1991). As in the Chytridiomycota, which are true fungi, asexual reproduction is by the production of motile spores, and thus the oomycetes have also been termed "zoosporic fungi". In contrast to fungi, zoospores of *Phytophthora* have an apically directed flagellum, which has flagellar hairs, and a posteriorly directed whiplash flagellum, as do other taxa in the kingdom Stramenopila (Alexopoulos et al. 1996). Therefore, *Phytophthora* should correctly be termed a stramenopile. The indeterminate mycelium of *Phytophthora* is diploid, and meiosis takes place during the formation of many male and female gametangia per thallus. In *P. infestans* two mating types are required for sexual reproduction. A male gametangium, or antheridium, attaches to a female gametangium, or oogonium, and forms a connection through which a haploid nucleus migrates and fuses with a haploid nucleus within the oogonium. After nuclear fusion a single thick-walled oospore is formed, which can germinate either directly by forming a mycelium from a germ tube or by forming zoospores inside a zoosporangium.

Mitochondrial DNA. The circular 37 914 kbp mtDNA of *P. infestans* has been completely sequenced (Fig. 4; Lang and Forget 1993; our unpublished results). It is A+T-rich (76%) and the genes, which are coded on both DNA strands, are separated by short spacers, resulting in an extremely tightly packed genome. In two instances, adjacent genes (*rps12-rps7* and *nad1-nad11*) actually overlap. In concordance with such a compact organization, *P. infestans* mtDNA has no introns; in fact, more than 95% of the genome is coding. The gene map of *P. infestans* presented here agrees with a partial gene map of *Phytophora megasperma* obtained by others through Southern hybridization (Förster et al. 1987). The mtDNA sequences of these two *Phytophthora* species deviate by only a few percent (Sachay et al. 1993; our unpublished sequence data).

As expected from the unrelatedness of fungi and oomycetes, the gene content of the *P. infestans* mtDNA is quite different from that of fungal mitochondria. Indeed, many *P. infestans* genes do not occur in the mtDNAs of animals and fungi, including three subunits of the NADH dehydrogenase complex (*nad7*, *nad9* and *nad11*), 11 small-subunit and five large-subunit ribosomal protein genes (*rps* and *rpl*), the gene encoding the ATPase-alpha subunit (*atp1*), *orf248*, which corresponds to a conserved mitochondrial orf (ymf16) found in various plants (e.g., Oda et al. 1992) and protists (Wolff et al. 1994; Leblanc et al. 1996), and at least six unique ORFs. *Orf32* was detected by comparative sequence analysis of *P. infestans* and *P. megasperma* mtDNAs, showing that, although very short (32 amino acids), this ORF is probably expressed and functionally important.

In many respects, the set of *Phytophtora* genes parallels that of protist and plant mitochondria (see Table 1). Indeed, after excluding unassigned ORFs, the same genes are found in *P. infestans* and *Acanthamoeba castellanii* mtDNAs, except for two ribosomal protein genes (*rps10* is absent in *A. castellanii*, and *rpl11* is absent in *P. infestans*). Interestingly, the organization of some ribosomal protein gene clusters in *P. infestans*, *A. castellanii* (Burger et al. 1995) and *Marchantia* (Oda et al. 1992) resembles that of bacteria and chloroplasts.

All mitochondrial protein genes of *Phytophthora* can be translated using the universal code, as in the mitochondria of plants, some lower fungi and many protists. However, the 25 tRNAs encoded in the *P. infestans* mtDNA are not sufficient to read all codons. There are two tRNAs decoding the GGN glycine-codon family, one carrying the expected UCC anticodon, and one with a GCC anticodon. According to the extended wobble hypothesis in mitochondria (reviewed by Dirheimer and Martin 1990; Osawa et al. 1992), an unmodified U at the first position of the antico-

don allows reading of all four bases, whereas a modified U restricts the reading to codons ending with a purine. It is conceivable that the $tRNA^{Gly}$ (UCC) has a modified U at the wobble position, so that both $tRNAs^{Gly}$ are necessary to read the GGN codon family.

The only tRNA capable of reading the CGN codon family (R_1 in Fig. 4) has a GCG anticodon (which should decode only CGC and CGU codons), rather than the usual UCG anticodon, which would recognize all four CGN codons. CGA and CGG codons are frequently used in protein-coding genes of *P. infestans* mtDNA. Therefore, we hypothesize that the corresponding arginine tRNA is imported into mitochondria. Likewise, no mitochondrial $tRNA^{Thr}$ gene was detected and so presumably has to be supplied via a nuclear gene. Interestingly, $tRNA^{Thr}$ is also absent in the mtDNAs of related species (e.g., *Cafeteria roenbergensis* and *Ochromonas danica*; G. Burger and B. F. Lang, unpublished results). Import of $tRNA^{Thr}$ from the cytoplasm may be a common feature in the stramenopile lineage.

The tRNA genes are interspersed with many genes, suggesting their general role as processing signals in large RNA precursors. Several promoters may transcribe the gene clusters encoded on both DNA strands, but we have not yet been able to define promoter motifs.

Mobile elements, introns and tRNA editing in lower fungal mitochondria

Mobile elements in Allomyces *mitochondria*

We have identified two novel types of putative mobile sequence elements in the mitochondria of *A. macrogynus*. The first involves an endonuclease (*orf360*) and the second consists of a class of short, conserved, sequence elements (DHE). Both types of elements have also been detected in other chytridiomycete and zygomycete mtDNAs.

A new type of a mobile endonuclease. Recently, we have described a novel type of mobile element in the *A. macrogynus* mtDNA, including of the C-terminus of *atp6* plus an open reading frame (*orf360*) that codes for an endonuclease (Paquin et al. 1994). According to our interpretation, this element has been inserted into the resident *atp6* gene of *A. macrogynus* such that a functional hybrid *atp6* was created, while the original C-terminal *atp6* region was displaced (Fig. 5). Similar to the group-I intron-homing mechanism (for a review see Dujon 1989), the ORF360 endonuclease cuts an insert-less *atp6* gene version at a specific site, which permits the subsequent transposition of the mobile element via gene conversion. Interestingly, the similarity between the acquired and the original *atp6* sequences is lower at the nucleotide level than at the protein level, especially at the recognition site of the endonuclease. Consequently, although the protein underwent only minor changes, the nucleotide sequence of the hybrid genes is different enough to prevent further endonucleolytic cleavage and deletion of the inserted element.

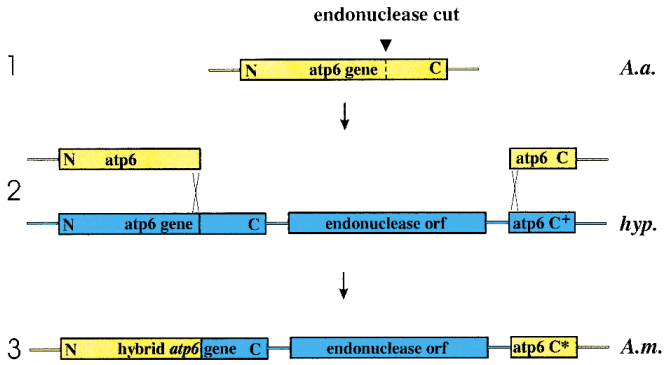

Fig. 5 Creation of a functional hybrid gene by insertion of a mobile element. (*1*) The original un-interrupted *atp6* gene as found in *A. arbusculus* (*A.a.*; *yellow box*) is cleaved by a specific endonuclease. (*2*) Gene conversion occurs between the ends of the cut *atp6* gene, and a hypothetical *atp6* gene of another species containing the mobile element (*hyp.*; *blue boxes*). (*3*) The conversion results in a topology as found in *A. macrogynus* mtDNA: a hybrid *atp6* gene, followed by an endonuclease orf and the displaced C-terminal portion of the original *atp6* gene. Note that co-conversion of a variable number of flanking nucleotides adjacent to the position of the cleavage site is likely to occur (not indicated by the colouring scheme)

We predicted that this type of mobile element may be present in mitochondrial protein genes of a variety of species, and may have been even overlooked because such an integration event would only be revealed by comparative sequence analysis (Paquin et al. 1994). In fact, we now have evidence that two further, quite similar, mobile elements are inserted in the *atp9* genes of the chytridiomycete *Rhizophydium sp.* and the zygomycete *R. stolonifer*.

A new class of short, conserved sequence elements (DHE). The intergenic regions in *A. macrogynus* mtDNA are unusually G+C-rich (49%). This bias reflects the presence of numerous G+C-rich sequence elements of 26–79 bases that can be folded into a distinctive structure, consisting of two adjacent hairpins with few mismatches and small loops of 3–6 bases (Fig. 6; Paquin and Lang 1996). Although the sequence similarity among most of these double-hairpin elements (DHE) is limited, the overall secondary structure is highly similar. Most sequence variations occur in the loops, and the conserved sequences are G+C-rich motifs. The hairpins also contain compensatory base changes in the paired regions. Presumably, such a bias reflects an evolutionary constraint to conserve secondary structure that has an important, but yet unidentified, function. These elements are scattered throughout the genome and located mostly in intergenic regions (39) and in introns (35), but they also occur in rRNA genes (6). The DHE insertion sites in introns are either in peripheral regions outside the conserved catalytic core or, in five instances, in intronic ORFs, resulting in frame shifts that potentially inhibit the complete translation of the intronic ORFs. Similarly, the DHEs inserted in rRNAs are located in variable regions. Apparently, DHEs are tolerated only in non-essential regions of the mitochondrial genome.

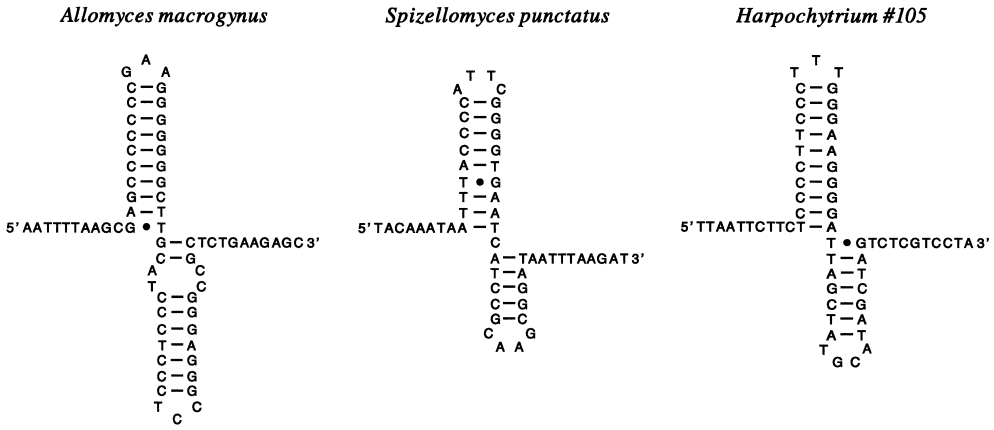

Fig. 6 Structured elements in the mtDNAs of three chytrids. A double hairpin element (DHE) from *A. macrogynus* mtDNA and two analogous structured elements from *S. punctatus* and *Harpochytrium* (strain 105) mtDNAs are illustrated. *Dots* indicate non-canonical base pairs, and *bars* stand for Watson-Crick interactions. Note the similar, compact structures of these elements from members of three orders of the Chytridiomycota. The *A. macrogynus* element is DHE20 and its sequence corresponds to nucleotide positions 23 539 to 23 610 (accession number U41288)

Some DHEs of *A. macrogynus* are identical, or nearly identical, in primary sequence to DHEs that occur in non-homologous locations of the *A. arbusculus* mtDNA. This distribution pattern suggests that DHEs are mobile elements and that deletions and transpositions are frequent events. Interestingly, similar DHEs have been identified in other chytridiomycete mtDNAs, e.g., in members of the Monoblepharidales and Spizellomycetales (Fig. 6), and also in the zygomycete *R. stolonifer* (our unpublished results). It is not clear if the presence of DHEs in different lower fungal mtDNAs reflects their inheritance from a common ancestor (and loss in the higher fungal clade after the divergence of *Rhizopus*), or, rather, lateral transfer. Currently, we also have no information on whether or not they occur in the nuclear genomes of fungi.

Distribution and evolution of introns in fungal mitochondrial genomes

Organellar introns have been classified into group I and group II on the basis of conserved secondary structures and the occurrence of short, primary sequence motifs (Michel et al. 1982). Since then, the secondary structure models for both types of intron have been refined (Michel et al. 1989; Michel and Westhof 1990) and a tertiary structure model has been proposed for the catalytic core of group-I introns (Michel and Westhof 1990; Cate et al. 1996). Organellar types of introns also occur in eukaryotic nuclear genomes (group-I introns only) and in eubacteria (groups I and II).

Ascomycete mitochondria often contain large numbers of introns. The most extreme example is *P. anserina* where 36 introns occupy about 60% of the mitochondrial genome (Cummings et al. 1990). The majority of the fungal mitochondrial introns belong to group I. In *P. anserina*, for example, only 3 of the 36 introns are group II (Cummings et al. 1990). Conversely, group-I introns are absent in land-plant mitochondria (reviewed by Schuster and Brennicke 1994), with only few exceptions (Oda et al. 1992; Vaughn et al. 1995).

Our sequence analysis revealed a large number of group-I introns, but only two group-II introns (both in *A. macrogynus*), in chytridiomycete and zygomycete mtDNAs, and many of these introns encode ORFs (see Table 2). Interestingly, although the presence of group-I and -II introns and their respective ORFs is a common feature of fungal mitochondria, there are two notable exceptions. The mtDNAs of two closely related species of *Harpochytrium* (http://megasun.bch.umontreal.ca/People/lang/species/harpo/harpo.html) and *S. commune* (Fig. 4) completely lack introns. This absence of mitochondrial introns is not a specific feature of Monoblepharidales and basidiomycetes, because partial sequences from the mtDNA of the basidiomycete *Trimorphomyces papilionaceus* contain several introns (Hong et al. 1993; accession number X73821), and a relative of *Harpochytrium, Monoblepharella sp.*, contains at least six introns (Lang et al., unpublished).

In fungi, introns are inserted in many different mitochondrial genes, with a strong preference for protein-coding genes, most frequently *cox1* and *cob*. For example, of the 28 introns in *A. macrogynus* mtDNA 12 are inserted in *cox1* and 6 in *cob*. No introns have been found in mitochondrial tRNA genes of fungi and relatively few are located in rRNA genes. Genes such as *nad6*, *cox2* or *atp9* rarely contain introns. This bias indicates that introns prefer to reside in sequence regions that are highly conserved among species (e.g., Lang 1984). In fact, the *cox1* and *cob* genes are the most conserved mitochondrial genes.

Mitochondrial introns of different species are often located in identical gene positions. One of the most striking examples is an intron inserted near amino-acid position 240 of the *cox1* genes of the fungi *S. punctatus (cox1*i1), *R. stolonifer (cox1*i3), *S. cerevisiae (cox1*i4), and *P. anserina* (*cox1*i9), the choanoflagellate *Monosiga brevicollis* (*cox1*i2), the chlorophyte alga *Prototheca wickerhamii* (*cox1*i3), the liverwort *Marchantia polymorpha* (*cox1*i7), and the angiosperm *Peperomia polybotrya* (Vaughn et al.

1995). It was suggested that these introns, most of which are also closely related at the nucleotide sequence and intron structure level, were inherited from a common ancestor shared by chlorophytes, fungi and animals (Ohta et al. 1993; Wolff et al. 1993). However, the presence of this intron in an angiosperm is better explained by horizontal transfer (Vaughn et al. 1995), as is the presence of one of two highly similar *cox1* introns in *S. pombe* and *Aspergillus nidulans* (Lang 1984; Waring et al. 1984). A group-I intron inserted at an identical position of the *rnl* gene of *P. wickerhamii* (*rnl*i1) and that of the fungi *A. nidulans* (*rnl*i1), *Neurospora crassa* (*rnl*i1), *P. anserina* (*rnl*i2), *S. cerevisiae* (*rnl*i1) and *A. macrogynus* (*rnl*i2) was also proposed to be "ancient" because phylogenetic trees inferred with the intron sequences and the *rns* gene sequences were congruent (Wolff et al. 1993).

Further instances of unusual sequence similarity between two introns inserted in different locations of the same genome (e.g., Wolff et al. 1993 and B.F.L. unpublished), as well as between unrelated genomes (e.g., Michel and Dujon 1986), suggest that introns are not only capable of homing into homologous sites, but also of transposing. As outlined above, introns were presumably present during early mitochondrial evolution, most probably before the radiation of fungi and before the divergence of the chlorophyte lineage. However, there is no evidence that introns were present in the eubacterial progenitor of mitochondria before the endosymbiotic event. The only intron found in the alpha-proteobacteria, a group-I intron in the $tRNA^{Arg}$ (CCU) of *Agrobacterium tumefaciens* (Reinhold-Hurek and Shub 1992), has little sequence similarity to mitochondrial introns, and group-I introns in mitochondrial tRNA genes have never been observed. Although our data point to a relatively ancient origin of most fungal mitochondrial introns, their phylogenetic distribution indicates frequent exchanges, even among distant species (for a review of intron mobility see Lambowitz and Belfort 1993; Belfort and Perlman 1995).

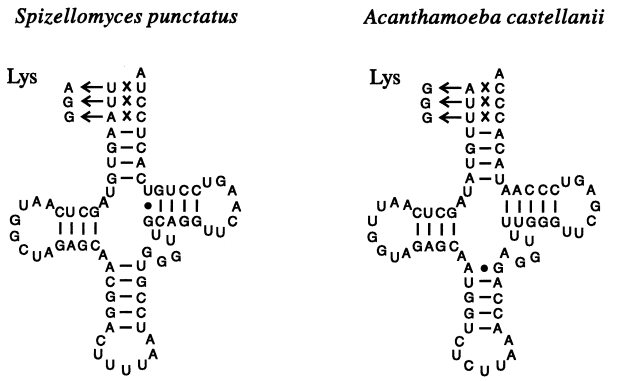

Fig. 7 Mitochondrial tRNA editing in a fungus and a protist. Comparison of editing patterns for $tRNA^{Lys}$ (UUU) in the chytridiomycete *S. punctatus* and $tRNA^{Lys}$ (CUU) in the amoeboid protozoan *A. castellanii*. tRNA structures as predicted from the mtDNA sequence of both species contain mismatches in their acceptor stems (indicated by *X*). As revealed by RNA sequencing, canonical base pairing is restored post-transcriptionally in *S. punctatus* (*arrows* pointing to the corrected bases). The editing of the *Acanthamoeba* $tRNA^{Lys}$ has not yet been shown experimentally

tRNA editing in chytridiomycete fungi

A few examples of tRNA editing have been found in the mitochondria of the protist *A. castellanii* (Lonergan and Gray 1993 a, b), land snails (Yokobori and Pääbo 1995) and marsupials (Börner and Pääbo 1996), and in mitochondria and chloroplasts of plants (Marchfelder et al. 1996). In this section we review our results on tRNA editing in the mitochondria of Chytridiomycota, the first example of such editing found in the fungi (Laforest et al. 1996).

The mtDNA of *S. punctatus* encodes a set of only eight tRNA genes and modelling of their secondary structures shows that all of them have one-to-three mis-pairings at the first three base pairs of their acceptor stems. These tRNAs must be edited to correct the mis-pairing that would prevent tRNA processing by RNAse P and by the 3′-end processing enzymes, and would also prevent amino-acylation. Moreover, the pattern of mis-pairing and the changes necessary to restore the conserved secondary structure follow the rules for a type of editing first described in *A. castellanii* mitochondrial tRNA (Lonergan and Gray 1993 a; see also Fig. 7). We have demonstrated by cDNA sequencing that the *S. punctatus* mitochondrial tRNAs are indeed edited, that all changes occur exclusively at the mispaired sites at the first three positions of the 5′ end, and that editing restores standard base pairs. The editing involves the replacement of ribonucleotides by purines, preferentially from A to G, followed by U to G, U to A and C to A. Because of the striking similarity of the editing patterns in *A. castellanii* and *S. punctatus*, it is likely that the process is mediated by similar enzymes in these two organisms (Laforest et al. 1996).

This editing mechanism can be considered to be a form of "directed mismatch repair", in which the 3′ half of the tRNA acceptor stem defines the position and the nature of the edited nucleotides. It cannot be explained by a base-modification mechanism such as the C to U or U to C editing in plant organelles (Gray and Covello 1993; Yu and Schuster 1995); rather, it must involve either a nucleotide replacement or a base replacement. A nucleotide replacement would involve the sequential, or total, removal of the first three nucleotides followed by their re-synthesis. In principle, any nucleotide exchange should be possible with this mechanism. However, an analysis of all editing sites in *A. castellanii* and *S. punctatus* shows that the nucleotide that restores the secondary structure is always a purine (the 3′ partner is always a pyrimidine, predominantly a C). Such nucleotide-biased editing could be explained by a base-replacement mechanism analogous to the post-transcriptional trans-glycosylation reaction that catalyzes tRNA modifications, where the hyper-modified base queenine (Q base) is incorporated at the wobble position of anticodons by a tRNA-guanine transglycosylase. A similar enzyme could drive the type of tRNA editing described here;

however, a transglycosylase with a different specificity would need to be postulated.

The origin of virtually identical editing mechanisms in *S. punctatus* and *A. castellanii* cannot be easily explained by common ancestory. *S. punctatus* is a member of the Chytridiomycota, which are unquestionably fungi. *A. castellanii*, in contrast, is an amoeboid protist, in a group that branches basally to plants, animals and fungi (Sogin et al. 1986; Wainright et al. 1993). To postulate a common origin for this type of tRNA editing, one would need to assume that this feature has been lost independently in plants, animals and fungi. Alternatively, tRNA editing could have evolved independently in fungi and protists or has been transferred laterally.

We are currently examining the mtDNA of representatives of the orders Monoblepharidales and Chytridiales to determine the distribution of mitochondrial tRNA editing in the Chytridiomycota. So far, we have found that some mitochondrial tRNA gene sequences in the Monoblepharidales *Monoblepharella* sp. and *Harpochytrium* species #94 and #105 (URL: http://megasun.bch.umontreal.ca/People/lang/FMGP/seqprojects.html), have mis-pairings in their acceptor stems and are probably edited. No tRNA editing, however, was found in the chytridiomycetes *A. macrogynus* (Paquin and Lang 1996) and *Rhizophydium* sp. (see our web site). In molecular phylogenies, *Rhizophydium* sp. is more closely related to *S. punctatus* than to the Monoblepharidales (see also the next section). This distribution of tRNA editing in two separate phylogenetic lineages of the Chytridiomycota does not support the hypothesis of a single origin of tRNA editing within the fungi.

Molecular phylogeny of the fungi and evolution of their mtDNAs

The value of mitochondrial sequences for phylogenetic analysis

The reconstruction of phylogenies is most reliably achieved with several different data sets, such as morphological and ultrastructural characters, gene sequences from different genomes, as well as gene content and genome order. We have contributed to the knowledge of fungal phylogeny by generating and analyzing complete mitochondrial sequences. We based our selection of candidate taxa on morphological and ultrastructural features, so as to include the widest possible divergence of species. A similar approach has been undertaken for protists by the organelle genome megasequencing program (OGMP; http://megasun.bch.umontreal.ca/ogmpproj.html).

The rationale for sequencing complete mitochondrial genomes rather than single genes is that: (1) single genes do not necessarily reflect the historical record of the entire genome, and (2) eight or more homologous, highly conserved mitochondrial genes are found in almost all mtDNAs, and these can be used for comparative phylogenetic analysis. The reason for using mitochondrial data is that most eukaryotic lineages contain mitochondria, and secondary endosymbioses involving the transfer of mitochondria between distant eukaryotic lineages does not seem to occur. Consequently, mitochondrial sequence data can be used to analyze a period of approximately 1.5 billion years (Doolittle et al. 1996), the approximate time span since the primary endosymbiosis that gave rise to mitochondria.

Protein sequences have overcome many drawbacks in phylogenetic analyses previously encountered with mitochondrial rRNA sequences (Paquin et al 1995 a, b). Mitochondrial phylogenies based on multiple, concatenated protein sequences yield reliable trees of unprecedented resolution. For the first time, animals and fungi are grouped together with high support (bootstrap values exceeding 95% at most nodes; see below), to the exclusion of a sister lineage that combines red algae, green algae, and cryptomonads. Phylogenetic analyses based on nuclear sequences or ultrastructural data are essentially congruent with those produced with mitochondrial protein data.

Phylogenies using single and multiple, concatenated protein sequences

A phylogenetic tree was inferred using the concatenated COX1, COX2, COX3 and COB protein sequences. The topology of the resulting tree is robust in bootstrap analysis (Fig. 8), with the fungi clustering in a clade that is highly supported (99%). The chytridiomycetes are the earliest diverging fungi, which is consistent with their taxonomic classification as “lower fungi”; however, they constitute a paraphyletic group. *Allomyces* (Blastocladiales) branches early in the higher fungal lineage, whereas *Spizellomyces* (Spizellomycetales) and *R. rosea* are on a separate branch. Up to now, the taxonomic position of *Rhizophlyctis* has not been unambiguously defined (Barr and Désaulniers 1984), but our data suggest that it may be considered a member of the Spizellomycetales. The paraphyly of the chytridiomycetes is even more accentuated when Monoblepharidales and Chytridiales are included in the analysis (for details, see our Web pages). When this is done, all chytridiomycetes except the Blastocladiales cluster together with the Spizellomycetales. Interestingly, the paraphyly of the Chytridiomycetes is not seen consistently in molecular phylogenies based on nuclear SSU rRNA sequences (Li and Heath 1992; Paquin et al. 1995 b); moreover, when topologies similar to our protein-based trees are obtained, they are not supported by bootstrap analysis (Bruns et al. 1992; Li and Heath 1992).

Some phylogenetic positions, including those of *S. commune* and *S. pombe*, remain unresolved even with the large data set used to infer the tree shown in Fig. 8. These nodes are separated by very short distances (indicated by dashed lines in Fig. 8), and are poorly supported by bootstrap analysis. The inclusion of more species and the use of additional protein sequences alleviates this problem (unpublished results). Except for the re-grouping of *S. pombe* with the budding yeasts, the branching order of the fungal clades in Fig. 8 is in agreement with classical taxonomy (Spar-

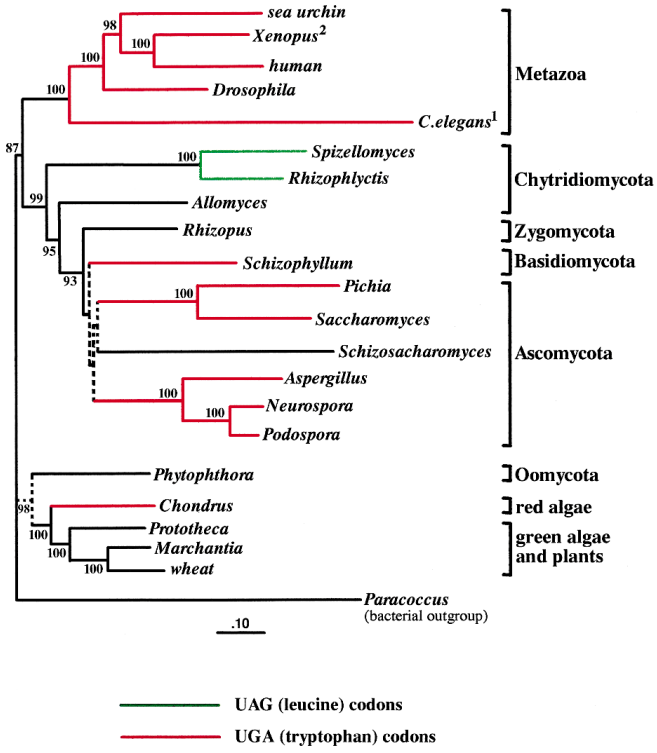

Fig. 8 Phylogenetic tree of mitochondrial protein sequences. The displayed phylogenetic tree was inferred from the concatenated protein sequences COX1, COX2, COX3 and COB using a combination of PROTDIST and FITCH as described in Materials and methods. Bootstrap support (calculated from 500 replicates) is shown at each node (in%, rounded numbers), except for the unsupported branches (*dashed regions*, values below 50%). The same tree topology was obtained when a maximum-likelihood approach (PROTML) was used. Modifications of the universal translation code are exemplified by coloured branches: *red* for the use of UGA tryptophan codons and *green* for the use of UAG leucine codons. Note that the appearance of UGA tryptophans codons has occurred several times independently in mitochondrial evolution. Source of the sequences: *C. elegans* (Okimoto et al. 1992); *Drosophila* (Clary and Wolstenholme 1985); human (Anderson et al. 1981); *Xenopus* (Roe et al. 1985); sea urchin (Jacobs et al. 1988); *Spizellomyces*, *Rhizophlyctis*, *Rhizopus*, *Schizophyllum* and *Phytopthora* (this paper and Laforest et al. 1996); *Allomyces* (Paquin and Lang 1996); *Pichia* (*Hansenula*) (Sekito et al. 1995); *Saccharomyces* (de Zamaroczy and Bernardi 1986 and references therein), *Aspergillus* (Brown 1993 and references therein); *Neurospora* (Collins 1993 and references therein); *Podospora* (Cummings et al. 1990); *Chondrus* (Leblanc et al. 1996); *Prototheca* (Wolff et al. 1994); *Marchantia* (Oda et al. 1992); Wheat (Pereira de Souza et al. 1991); *Paracoccus* (Kurowski and Ludwig 1987; Raitio et al. 1987)

row et al. 1973; Cavalier-Smith 1987), as well as with molecular phylogenies based on nuclear SSU rRNA (Förster et al. 1990; Illingworth et al. 1991; Bruns et al. 1992; Li and Heath 1992; Paquin et al. 1995 b). Mitochondrial phylogenies based on single protein sequences (Paquin et al. 1995 a, b), or on gene order and genomic content (Sankoff et al. 1992), also have yielded topologies consistent with that shown in Fig. 8.

[1] Nematode worm, *Caenorhabditis elegans* [2] Clawed toad, *Xenopus laevis*

Fungi and animals share a common ancestor

Based on nuclear ribosomal and protein sequences, it was suggested that animals and fungi share a common ancestor to the exclusion of the chlorophytes and protists (Baldauf and Palmer 1993; Wainright et al. 1993). The mitochondrial data set used in our study provides further solid evidence for this association (bootstrap values of 87%; Fig. 8). A common ancestor of fungi and animals was also predicted from a phylogenetic analysis using the mitochondrially encoded NAD5 protein sequences, although with weaker support (76.5%, Paquin et al. 1995 a).

One method to test the stability of phylogenetic trees is the stepwise elimination of species, and a comparison of the resulting tree topologies. We have found greater than 80% bootstrap support for a common ancestor of fungi and animals, after certain manipulations involving: (1) the elimination of one or all ascomycete, basidiomycete, zygomycete and *R. rosea* sequences from the data set; (2) further reduction of the fungal lineage to contain only ascomycetes but not including *S. pombe*, while at the same time eliminating *Caenorhabditis elegans* from the animal lineage.

The elimination of taxa from phylogenetic analyses can be justified when individual species in an otherwise homogenous data set are highly derived. Such a case can be made for many species of our data set, including *C. elegans*, *S. pombe*, *P. canadensis* and *S. cerevisiae* (note the long branches for these species, indicating that they are highly derived; Fig. 8). Other taxa that have long branches and are difficult to place in phylogenetic trees with mitochondrial data include: *Chlamydomonas* species, *A. castellanii*, *Paramecium caudatum*, *Tetrahymena pyriformis*, *Plasmodium* species and *Dictyostelium discoideum*. Although the red and green algae are sister clades in our analysis, supported by an impressive 100% bootstrap support (Fig. 8), the green alga *Chlamydomonas* (when included) does not group with the green alga *P. wickerhamii* as expected, and the further addition of the highly derived protist sequences mentioned above makes an assessment of the origin of the red and green algae and their possible common ancestry impossible. Only the addition of numerous additional taxa will be likely to resolve this problem. However, the fungal plus animal clade as represented in our analysis, retains its integrity and topology with or without the addition of highly derived taxa, which we interpret as solid support for this portion of the tree.

Phylogenetic position of P. infestans

The dissimilarity between the *P. infestans* and fungal mtDNAs (see the section describing the *Phytophthora* mtDNA) is corroborated by our molecular phylogenies, which show that *P. infestans* is not included in the fungal clade (Fig. 8). However, its association with the plant lineage in this analysis, although supported by a high bootstrap value, is clearly a phylogenetic artifact (dotted branch in Fig. 8). *P. infestans* is the only species of the Stramen-

opila included in our data set. Because its mitochondrial gene sequences are little derived (Lang and Forget 1993), like those of plants, they artificially group together with the plants. In fact, molecular phylogenies based on nuclear small subunit rRNA sequences (Gunderson et al. 1987; Förster et al. 1990), or alternatively on the mitochondrial NAD4L protein sequences (Chesnick et al. 1996), clearly show that the Oomycota are members of the Stramenopila, and not of fungi or plants. In agreement with this view, mtDNA sequences from two members of the Stramenopila (*Cafeteria roenbergensis* and *Ochromonas danic*a; G. Burger and B.F. Lang, unpublished results) have a gene content similar to that of *P. infestans*. The strong support for a common ancestry of an oomycete with plants as shown in Fig. 8 should serve as a warning that when very deep phylogenies include only a few species of one lineage, incorrect conclusions may be drawn.

A relatively constant gene content in fungal mitochondria

The usual set of fungal mitochondrial genes includes 11 genes coding for subunits of the respiratory chain (*cox1-3*, *cob*, *nad1-6* and *nad4L*), three genes coding for subunits of the ATP-synthetase complex (*atp6*, *atp8* and *atp9*, although the latter is either absent or a pseudo-gene in some ascomycete mtDNAs), two genes coding for ribosomal RNAs (*rns* and *rnl*) and a number of tRNA genes (Table 1). Members of the Zygomycota and Ascomycota encode a complete set of mitochondrial tRNAs, which is sufficient to de-code all codons used, but the basidiomycete *S. commune* (see above, the chapter on *S. commune*) lacks a tRNA recognizing tyrosine codons. Also, *Allomyces* has a complete tRNA set, but members of other chytridiomycete lineages encode only eight tRNA genes in their mtDNAs. The most parsimonious explanation of these findings is that most of the tRNA genes were lost in an ancestor of the Monoblepharidales-Spizellomycetales-Chytridiales clade. Besides this variation in the number of tRNA genes, the major difference in the gene content of fungal mtDNAs is the absence of *nad* genes in some ascomycetes, such as *S. pombe* and *S. cerevisiae*, and the occurrence of a variable number of unique ORFs in almost all fungi.

Evolution of the fungal mitochondrial genetic code

The zygomycete *R. stolonifer*, the chytridiomycetes *Allomyces*, and members of the Monoblepharidales use the standard translation code in their mitochondria. Protein-coding genes from members of the Spizellomycetales and Chytridiales, however, contain many in-frame UAG "stop" codons. On the basis of protein sequence alignments, we conclude that these UAG codons are most probably translated as leucine. In fact a tRNA with the anticodon CUA (recognizing UAG codons) has been found in *S. punctatus* mtDNA; this tRNA sequence is strikingly similar to $tRNA^{Leu}$ sequences from other species, including the presence of a long extra arm.

Most likely, the universal translation code is an ancestral trait in fungal mitochondria, one shared with plants and many protists, whereas the switch of UAG codons to spe-

Table 3 Sequence information on fungal, choanoflagellate and oomycete mitochondrial DNAs, resulting from the FMGP program. "(PCR)" indicates that only the partial, PCR-amplified *cox1* sequence was determined. The known or estimated sizes of mtDNAs are indicated in kbp, and the percentage of the known sequence reflects the status as of November, 1996. For updates on the status and for more detailed information, consult the pages of the WWW site at http://megasun.bch.umontreal.ca/People/lang/FMGP/progress.html

Species	Taxonomic group	Size of mtDNA	Completion
Schizosaccharomyces pombe	Ascomycota	19.432	100%
Schizophyllum commune	Basidiomycota	49.711	100%
Rhizopus stolonifer	Zygomycota	54.191	100%
Phytophthora infestans	Ooomycota	37.957	100%
Monosiga brevicollis	Choanozoa	76.568	100%
	Chytridiomycota		
Allomyces macrogynus	Blastocladiales	57.473	100%
Allomyces arbusculus[a]		54	15%
Catenaria anguillulae		~50	4% (PCR)
Spizellomyces punctatus[b]	Spizellomycetales	60.218	100%
Rhizophlyctis rosea		~50	5% (PCR)
Rhizophydium #136	Chytridiales	~100	95%
Harpochytrium #94[c]	Monoblepharidales	19.473	100%
Harpochytrium #105		24.162	100%
Monoblepharella #15		55	90%
Monoblepharis #20		~40	5% (PCR)

[a] Two 4-kbp long *Hin*d III fragments were completely sequenced.
[b] The mitochondrial genome of *Spizellomyces punctatus* consists of three circular 'chromosomes', of 58.7, 1.4 and 1.1 kb.
[c] *Harpochytrium* and the Harpochytriaceae were placed in the Chytridiales (Barr 1990) because the zoospore of *Harpochytrium* was reported to be similar to those of the Chytridiales (Gauriloff et al 1980). Our analyses show, however, that *Harpochytrium* belongs in the Monoblepharidales (Lang et al. unpublished)

cify leucine most probably occurred in the ancestor of the Spizellomycetales-Chytridiales clade, after the divergence of the Monoblepharidales. Accordingly, the introduction of UGA tryptophan codons occurred after the divergence of the zygomycete *R. stolonifer* (red branches in Fig. 8). It is likely that UGA tryptophan codons were introduced at least twice during fungal evolution, once in the basidiomycete *S. commune* and independently in the ascomycetes, after the divergence of *S. pombe.* The same modification of the genetic code also occurred, independently, at least once in animals and once in red algae.

Ribosomal protein genes in mtDNAs of the fungal ancestor ?

When we first identified a gene coding for a ribosomal protein (rp) in *A. macrogynus* mtDNA, we hypothesized that this was an ancestral trait and that *rp* genes were likely to be present in mitochondria of other lower fungi. Analysis of available sequences (including complete or nearly complete mtDNAs from three species from different orders of the chytridiomycetes, see Table 3) has not revealed any other *rp* genes. The presence of such a gene in the genus *Allomyces* (*A. arbusculus* mtDNA also encodes *rps3*; our unpublished results) is therefore an exception. Interestingly, the mtDNA of the choanoflagellate *Monosiga* contains many *rp* genes (see our web site for a map of this mtDNA). According to our preliminary mitochondrial phylogeny (see our web site), *Monosiga* diverges in a trichotomy with the animal and fungal clades and, consequently, branches in close proximity to a putative common ancestor of animals and fungi. It is therefore possible that the mitochondria of the ancestral fungus did encode some *rp* genes that were lost during evolution in every fungal lineage shortly after the divergence of fungi and animals. The *rps3* pseudo-gene in *Allomyces* (Paquin and Lang 1996) appears to be the last vestige of their presence. However, we cannot exclude a scenario in which these genes were quickly lost before the emergence of the first fungi and that *rps3* has been horizontally acquired more recently in *Allomyces*.

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