

1 The Protistan Origins of Animals and Fungi

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I. Introduction

Fungi and Metazoa constitute two of the major multicellular eukaryotic lineages and a large body of robust data confirms that they are close relatives (Baldauf and Palmer 1993; Burki et al. 2007; Hackett et al. 2007; Hampl et al. 2009; Wainright et al. 1993). Along with three major groups of protists – nucleariids, ichthyosporeans and choanoflagellates – and four enigmatic *incertae sedis*

groups – Aphetidea, *Capsaspora*, Corallochytreia and *Ministeria* – Fungi and Metazoa make up the phylogenetic supergrouping Opisthokonta (Adl et al. 2005; Cavalier-Smith 1987). The ichthyosporeans, nucleariids and *incertae sedis* groups were placed together in the taxon Mesomycetozoa by Adl et al. (2005). Whilst the Opisthokonta is a well established grouping, Mesomycetozoa remains controversial as it is paraphyletic (Fig. 1.1) and not universally recognised. Therefore each of the mesomycetozoan lineages will be discussed separately.

The relationships among the opisthokont groups, and those of the opisthokonts with other eukaryotic supergroups, are slowly becoming clearer (Fig. 1.1). We discuss here the major taxonomic groups within Opisthokonta and their relationships with each other. It now seems clear that the deepest bifurcation within the opisthokonts resulted in two major lineages, now referred to as Holozoa and Holomycota. The Holomycota is composed of the Fungi and nucleariids (Lara et al. 2010). Metazoa, the choanoflagellates, ichthyosporeans and the four *incertae sedis* protists are collectively known as Holozoa (Lang et al. 2002; Ruiz-Trillo et al. 2004; Steenkamp et al. 2006).

Taxon-rich phylogenies of the opisthokonts are frequently ribosomal DNA (rDNA) studies. This is because these genes are multi-copy in eukaryotes and possess highly conserved regions that facilitate the binding of universal PCR primers – two factors which allow relatively rapid and easy DNA sequencing. The same, or similar, protocols can therefore be employed to produce orthologous sequences across a broad variety of taxa.

Whilst there are many advantages in producing large-scale phylogenies based on rDNA sequences, it is often necessary to use sequences from multiple genes when attempting to resolve deep branches. Combining small- and large-subunit rDNA sequences (Medina et al. 2003; Moreira et al. 2007) is one simple approach to

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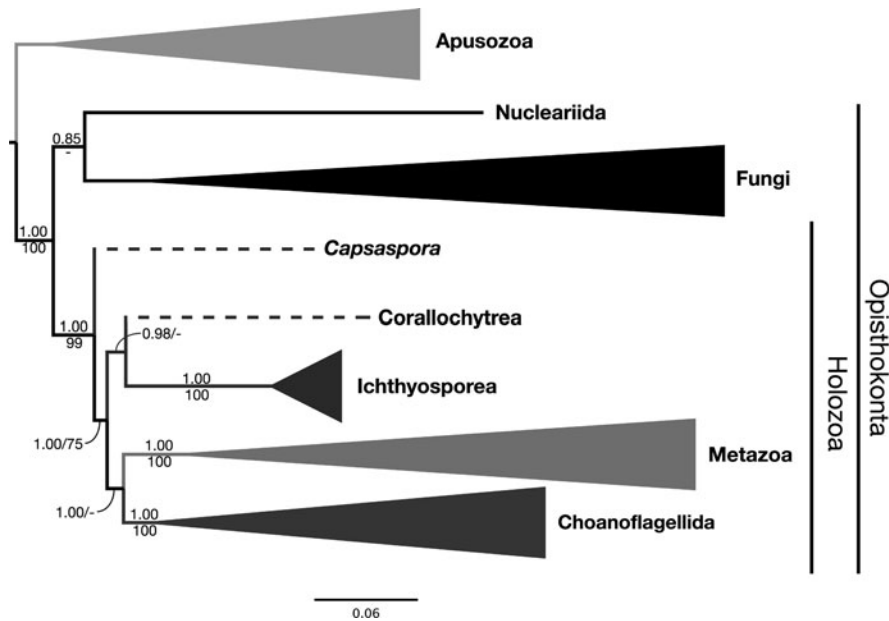


Fig. 1.1. Simplified four-gene phylogeny of the opisthokonts. The tree shown was derived by Bayesian inference, using MrBayes 3.1.1 (Ronquist and Huelsenbeck 2003), based on a combined rDNA (SSU, LSU), α -tubulin (*tubA*), 90-kDa heat-shock protein (*hsp90*) dataset. The 6415 aligned nucleotide positions were analysed using the GTR+I+ Γ substitution model, with separate partitions for rRNA, first + second codon positions and third positions. Support values are also shown from a maximum

likelihood tree derived using RAxML 7.0.3 (Stamatakis et al. 2005) utilizing the GTRCAT substitution model. Bayesian inference posterior probabilities (biPP) and maximum likelihood bootstrap percentages (mlBP) values are given above and below branches respectively. Branches are drawn proportional to the number of nucleotide substitutions per site as indicated by the scale bar at the lower left. Dotted lines indicate taxa of uncertain phylogenetic position (modified from Carr et al. 2008a)

creating phylogenies from more than one gene. However, the two genes are together in the genome, transcribed as a single unit (Perry et al. 1970) and function in the same biosynthetic pathway. It is therefore likely that both subunits will be affected by similar evolutionary forces (natural selection, local mutation bias, etc.) and may, in effect, act as a single locus. A preferential method for producing robust opisthokont phylogenies is to use multiple ribosomal and protein-coding genes in conjunction; such sequences can be produced using targeted PCR with degenerate primers (Carr et al. 2008a; Ruiz-Trillo et al. 2004; Steenkamp et al. 2006) or expressed sequence tag (EST) libraries (Patron et al. 2007; Philippe et al. 2004; Shalchian-Tabrizi et al. 2008). The sequencing of whole genomes and EST libraries allows the construction of datasets composed of many thousands of aligned amino acid positions. This field, termed phylogenomics, allows the powerful analyses of deep phylogenetic relationships (Burki et al. 2008; Minge et al. 2009; Philippe et al. 2009). At present, the sequencing of either whole

genomes or EST libraries is still a relatively expensive procedure and as a result genomic data are publicly available for less than ten opisthokont protists.

Sparse taxon sampling is a difficult issue in producing accurate phylogenies. Whilst a wealth of molecular data exists for many of the major lineages of Fungi and Metazoa, there are data for far fewer opisthokont protists. Nonetheless multiple protein-coding gene sequences are available for 15 species of choanoflagellates, two species of ichthyosporeans, a single nucleariid and three of the *incertae sedis* holozoan taxa (*Capsaspora owczarzaki*, *Corallochytrium limacisporum*, *Ministeria vibrans*).

Poorly sampled taxa can lead to species being present on isolated long branches; this, in turn, may lead to problems when reconstructing phylogenies due to the phenomenon of long-branch attraction (Hendy and Penny 1989). When distantly related sequences share a relatively high number of characters due to convergence rather than ancestry the true phylogenetic signal may

be overwhelmed. Long-branch effects can also be produced by unequal rates of evolution; therefore, when possible, it is advisable to screen taxa and pick those most suitable for phylogenetic reconstruction. This however is not always possible, particularly in the case of the opisthokont protists where some lineages are only represented by a single known species.

II. Opisthokonta

The Opisthokonta was originally postulated by Cavalier-Smith, partly based on the presence of a posteriorly directed flagellum common to both Fungi and Metazoa. However as the depth of diversity in opisthokont lineages has been uncovered, it has become apparent that there are no recognised universal morphological characters unique to this group. The posterior flagellum is not present in all opisthokont groups; loss of the flagellum must have occurred on multiple occasions within Fungi (James et al. 2006a), as well within the nucleariids, ichthyosporeans, *Capsaspora*, Corallochytreia and possibly ministeriids. Throughout the opisthokonts, the morphology of mitochondrial cristae is predominantly flat (Adl et al. 2005), but this appears to be a plastic trait, with lamellar, tubular and discoidal cristae also present (Amaral-Zettler et al. 2001; Ragan et al. 1998).

Nonetheless, these characteristics are widespread across the opisthokonts and may point to the ancestral state of the group. The abilities to produce amoeboid cells and also to engulf particles by phagocytosis are present in all of the major lineages. Moreover, Metazoa is the only major opisthokont lineage that does not contain species with cell walls and it has been suggested that the last common ancestor of the opisthokonts also possessed the potential to produce a cell wall (Mendoza et al. 2002).

Nearly all described eukaryotes can be assigned to one of six super-groupings (for a review, see Adl et al. 2005), namely Opisthokonta, Amoebozoa, Archaeplastida, Chromalveolata, Excavata and Rhizaria. Of these, Amoebozoa appear to form a super-assemblage with the opisthokonts (Burki et al. 2007; Stechmann and Cavalier-Smith 2003). Opisthokonta and Amoebozoa both contain species possessing a single flagellum, giving rise

to the name Unikonta for the super-assemblage (Stechmann and Cavalier-Smith 2003). Under this hypothesis, the other eukaryotic groups, termed bikonts, may have ancestrally possessed two flagella (Cavalier-Smith 2009).

The enigmatic Apusozoa are also proposed to be the closest group to the opisthokonts (Kim et al. 2006), however if the biflagellate Apusozoa are the true sister-group to the opisthokonts, then the unikonts must be a polyphyletic grouping. Competing hypotheses however suggest that the apusozoans may alternatively be an early branching lineage within either the amoebozoans or bikonts (Minge et al. 2009).

The uncertainty over the sister-group of the opisthokonts may only be resolved by increasing the number of apusozoan and amoebozoan taxa for which multiple gene sequences are available.

III. Fungi

Fungi are a large, diverse group of heterotrophs, which predominantly employ an absorptive mode of nutrition. Species often produce multinucleate hyphae and cell walls that comprise both β -glucan and chitin (Cavalier-Smith 1998, 2001; Kirk et al. 2001; Tehler 1988). When present, mitochondrial cristae are flat.

Fungal classification and phylogeny is currently in a state of flux, with many traditional taxa (e.g., Zygomycota) now considered redundant. At its simplest level, Fungi can be divided into the subkingdom Dikarya (formerly Neomycota; Cavalier-Smith 1998) and nine basal groups (Blastocladiomycota, Chytridiomycota, Entomophthoromycotina, Glomeromycota, Kickxellomycotina, Microsporidia, Mucoromycotina, Neocallimastigomycota, Zoopagomycotina; Hibbett et al. 2007). Dikarya is characterised by species possessing pairs of unfused haploid nuclei (dikaryons). The taxon is composed of the phyla Ascomycota and Basidiomycota and comprises the majority (~98%) of described fungal species (James et al. 2006a). The two phyla form a well supported monophyletic group within Fungi (Fig. 1.2; James et al. 2006a; Liu et al. 2006).

In contrast to Dikarya, the relationships between the basal lineages of Fungi are not, at present, well resolved. Several genera (e.g., *Basidiobolus*, *Olpidium*, *Rozella*) are not currently associated

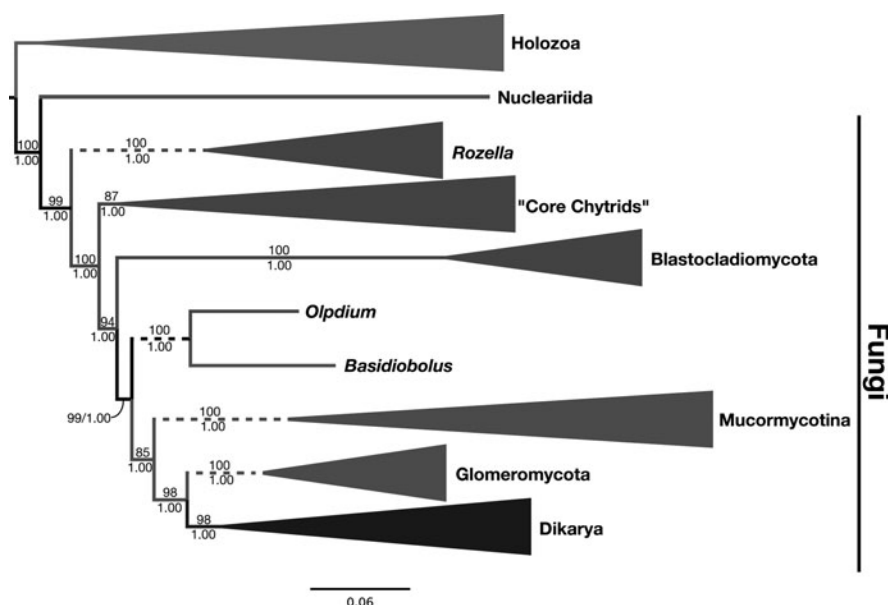


Fig. 1.2. Simplified four-gene phylogeny of the Fungi and Nucleariida. The tree was derived by a maximum likelihood method, using RAXML 7.0.3, from partial sequences of rRNA (SSU and LSU) *tubA* and *hsp90*. The 6411 aligned nucleotide positions were analysed using the GTRCAT substitution model, with separate partitions for rRNA, first + second codon positions and third positions. Support values are also shown from a maximum likelihood tree derived using MrBayes 3.1.1 utilizing the GTR+I+Γ

substitution model; mlBP and biPP values are given above and below branches respectively. The long-branch and phylogenetically unstable subphyla Entomophthoromycotina, Kickxellomycotina and Zoopagomycotina have been omitted. Branches are drawn proportional to the number of nucleotide substitutions per site as indicated by the scale bar at the lower left. Dotted lines indicate taxa of uncertain phylogenetic position

with any higher taxa (Hibbett et al. 2007). Chytridiomycota was long considered the most basal lineage within Fungi, with the Zygomycota viewed as the sister-group to Dikarya. Molecular phylogenies have confirmed that the former chytridiomycetes do indeed fall at the base of the fungal tree (Fig. 1.2; James et al. 2006a; Tehler et al. 2000), but show that the traditional taxon is polyphyletic comprising at least four major lineages: *Rozella* spp., the “core chytrids”, Blastocladiomycota and *Olpidium* spp. (Fig. 1.2; James et al. 2006a, b). Of these, it appears that *Rozella* forms the earliest branching clade within Fungi (Lara et al. 2010). In fact, recent evidence indicate that it is a member of a large clade, the “cryptomycota”, which is known almost exclusively from metagenomic data (Jones et al. 2011). The former Chytridiomycota is now formally separated into the Chytridiomycota, Neocallimastigomycota and Blastocladiomycota (Hibbett et al. 2007; James et al. 2006b).

Zygomycota also appears to be a polyphyletic assemblage (Fig. 1.2; James et al. 2006a; Keeling 2003; Voigt and Wöstemeyer 2001). A recent

revision of fungal classification considered the Zygomycota to be a redundant taxon (Hibbett et al. 2007) and divided the former group into one phylum (Glomeromycota) and four subphyla *incertae sedis* (Entomophthoromycotina, Mucormycotina, Kickxellomycotina and Zoopagomycotina). Within the former zygomycete groups, the glomeromycetes have been tentatively placed as the closest lineage to Dikarya (James et al. 2006a; Voigt and Wöstemeyer 2001). This result however may be heavily biased by a reliance on rDNA sequences, since phylogenies based only on protein-coding genes place the Mucormycotina as the sister-group to Dikarya (Lee and Young 2009; Liu et al. 2009).

The position of Microsporidia within Fungi remains unclear (see Chapter 2 in this volume). Liu et al. (2006) considered Microsporidia as a sister-group to all other fungi; however, using a larger dataset, James et al. (2006a) placed these highly reduced obligate parasites as an early branching lineage and the sister-group to *Rozella* spp (see Chapter 3 in this volume). A more derived

position, within the former zygomycetes, was proposed on the basis of protein-coding genes (Gill and Fast 2006; Keeling 2003) and genome-wide conserved gene order (synteny; Lee et al. 2008).

A robust phylogeny of Fungi is vital in order to understand how the group evolved. Due to the widespread presence of a posteriorly positioned flagellum in the basal fungal lineages (James et al. 2006a), parsimony argues that it was present in the last common ancestor of Fungi and that flagella loss has subsequently occurred. However, due to competing phylogenies the number of losses that have occurred is uncertain. Liu et al. (2006) presented a phylogeny that only required a single loss of the flagellum, whereas other phylogenies (James et al. 2006a; Tanabe et al. 2005) highlight multiple flagella losses.

Although considered one of the major multicellular kingdoms, unicellular species are found across the fungal tree. Only unicellular forms are known for the early branching Microsporidia and *Rozella*. In addition, secondary reversions to unicellularity (exemplified by the ascomycete yeasts) appear to have evolved on multiple occasions within the kingdom (James et al. 2006a). Only a minority of described fungal species are marine and it is unclear from phylogenetic reconstructions whether the last common ancestor of Fungi was a marine or freshwater organism (James et al. 2006a). It does appear however that the radiation of the major extant fungal lineages occurred in the terrestrial environment and, in fact, it has been proposed that the presence of glomeromycetes on land was essential for terrestrial colonisation by green plants (James et al. 2006a; Pirozynski and Malloch 1975).

Despite the recent, and continuing, advances using multigene phylogenies, a complete robust tree of Fungi remains elusive. At the time of writing, the Deep Hypha initiative (<http://ocid.nacse.org/research/deephyphae/>) is in the process of producing a large-scale phylogeny by sequencing seven loci from 300 basal fungal taxa and 1200 Dikarya species.

IV. Nucleariida

Nucleariids are a small group of mainly free-living amoeboid protists. The protoplast (cell body) appears as a spherical or flattened elongated amoeba with radiating filopodia (Amaral-Zettler

et al. 2001). Flagellated cells have not been observed in any of the stages in the life cycles of any nucleariid. All described species thus far have been isolated from freshwater (Maldonado 2004).

Most described species are algivorous or bacterivorous (Patterson 1984). One potential exception to this is *Nuclearia pattersoni*, which was discovered living in the gills of freshwater fish (Dykov et al. 2003). However the authors did not ascertain whether the association between the amoebae and host is parasitic or commensal.

There is considerable variation within the ultrastructure of species in the group (for a review, see Yoshida et al. 2009).

Two types of mitochondrial cristae have been reported; most species have discoidal cristae (Amaral-Zettler et al. 2001) but *Nuclearia pattersoni* exhibits flattened cristae (Dykov et al. 2003). The majority of known species have small (15–25 µm in diameter), uninucleate cells; in contrast *Nuclearia delicatula* has larger (50 µm) cells with 2–12 nuclei (Yoshida et al. 2009). Cyst formation has been observed in *Nuclearia pattersoni* and *Nuclearia simplex*, but both *Nuclearia delicatula* and *Nuclearia moebiusi* appear incapable of producing cysts. Additionally, a mucoidal extracellular matrix, absent from *Nuclearia moebiusi*, is present in *Nuclearia delicatula*, *Nuclearia pattersoni* and *Nuclearia simplex* (Dykov et al. 2003; Patterson 1984).

The taxonomic classification of the nucleariids has been reappraised over recent years through the use of molecular phylogenetics. Due to the morphology of their mitochondrial cristae, they were initially placed within the Filosea, a subgroup of Amoebozoa (Cavalier-Smith 1993; Page 1987). The monophyly of Filosea was questioned by Patterson (1999) on the basis that filopodia are likely to have evolved on multiple occasions within eukaryotes, as well as the unexpected presence of three mitochondrial cristae morphologies in the group. Amaral-Zettler et al. (2001) confirmed the polyphyly of Filosea using small-subunit rDNA sequences, which strongly supported the inclusion of nucleariids within Opisthokonta and not Amoebozoa. This study also highlighted polyphyly within the sequenced nucleariids, showing that one species (*Nuclearia* sp. ATCC 30864) did not group with the other taxa. This species was later reclassified as *Capsaspora owczarzaki* and moved to Mesomycetozoa (Hertel et al. 2002; see below). Early small-subunit rDNA studies did not robustly resolve the position of the nucleariids within the opisthokonts

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