Animals and Their Unicellular Ancestors

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Advanced article

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Animals belong to the Opisthokonta, one of the major divisions of the eukaryotic Tree of Life. This supergroup also includes other well-known groups such as fungi and choanoflagellates, in addition to some newly discovered unicellular taxa as the Ichthyosporea or the Filasterea. To unveil the origin of animal multicellularity, it is vital to understand the evolution of their single-celled relatives, as they might hold key genetic clues that might help us understand how the unicellular ancestors of animals became animals. Our current knowledge of unicellular animal relatives, their specific phylogenetic relationships and the role they might play in future research is being improved, thanks to molecular data.

Introduction

Among all living creatures, animals (or metazoans) have drawn, and continue to draw, many researchers' attention. This is partially because of our persistent anthropocentric bias, that is, we are animals, therefore we think they are interesting, and also because many metazoans are relatively large and therefore highly conspicuous compared to microbes. Furthermore, until recently, researchers were short of tools – such as modern microscopy and molecular biology techniques – to explore the biodiversity and biology of microscopic organisms. Hence, it is not surprising that one of the most widespread classifications of living beings, the Five Kingdoms of Whittaker (1959), placed the most conspicuous multicellular groups (i.e. animals, fungi and plants) at the same biological level as Protista (a clade comprising all single-celled eukaryotes) and the so-called Monera (which comprises the prokaryotic bacteria and archeae).

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Thanks to many advances in molecular techniques, this rather simplistic view is being largely improved. First came Carl Woese's proposal (Woese and Fox, 1977) to divide the Tree of Life into three domains: two prokaryotic domains, Archaea and Bacteria, and an eukaryotic domain (Eukaryota). From then on, a myriad of molecular phylogenies improved that scheme, dividing the Eukaryota into a few major groups. Cavalier-Smith (2002) grouped those eukaryotic supergroups into two major clades: the unikonts and the bikonts. The name 'Unikonta' (from Greek, *uni* meaning 'single' and *kontos* meaning 'pole', referring to the flagellum) refers to the structure of kinetids, that is, the flagellar apparatus composed of one or two basal bodies (centrioles plus their related microtubules) in conjunction with their associated cilia/flagella. Cavalier-Smith has claimed (2002) that the last common ancestor of Unikonta had only one centriole and one cilium per kinetid. Unikonts include Amoebozoa and Opisthokonta, the clade that includes animals and fungi. The unikont–bikont division of eukaryotes is still highly contentious, because, even if considered derived states, many current unikonts have two centrioles or two cilia. In any case, molecular phylogenies (Ruiz-Trillo *et al.*, 2008), different molecular synapomorphies (Richards and Cavalier-Smith, 2005) and recent analyses on rare genomic changes (Rogozin *et al.*, 2009) seem to corroborate the alliance of opisthokonts with amoebozoans. **See also**: Protozoan Evolution and Phylogeny

Embedded among the unikonts, 'Opisthokonta' (from Greek, opisthō means 'posterior'; Cavalier-Smith, 1987) are unikonts with (1) a single posterior flagellum (in at least one vital phase sometimes secondarily lost) with a pair of centrioles and (2) exhibiting mitochondria with flat cristae (compartments outlined by the mitochondrion inner membrane). Opisthokonta comprises animals, plus fungi and several unicellular groups, such as the choanoflagellates (**Figure 1**). This is why opisthokonts are sometimes informally named as the 'Metazoa/Fungi clade'. The monophyly of opisthokonts has been confirmed by molecular phylogenies (Lang *et al.*, 2002; Ruiz-Trillo *et al.*, 2004, 2008), an approximately 12-amino acid insertion present in the translation elongation factor 1-alpha (EF1-α; Baldauf and Palmer, 1993) and the presence of a

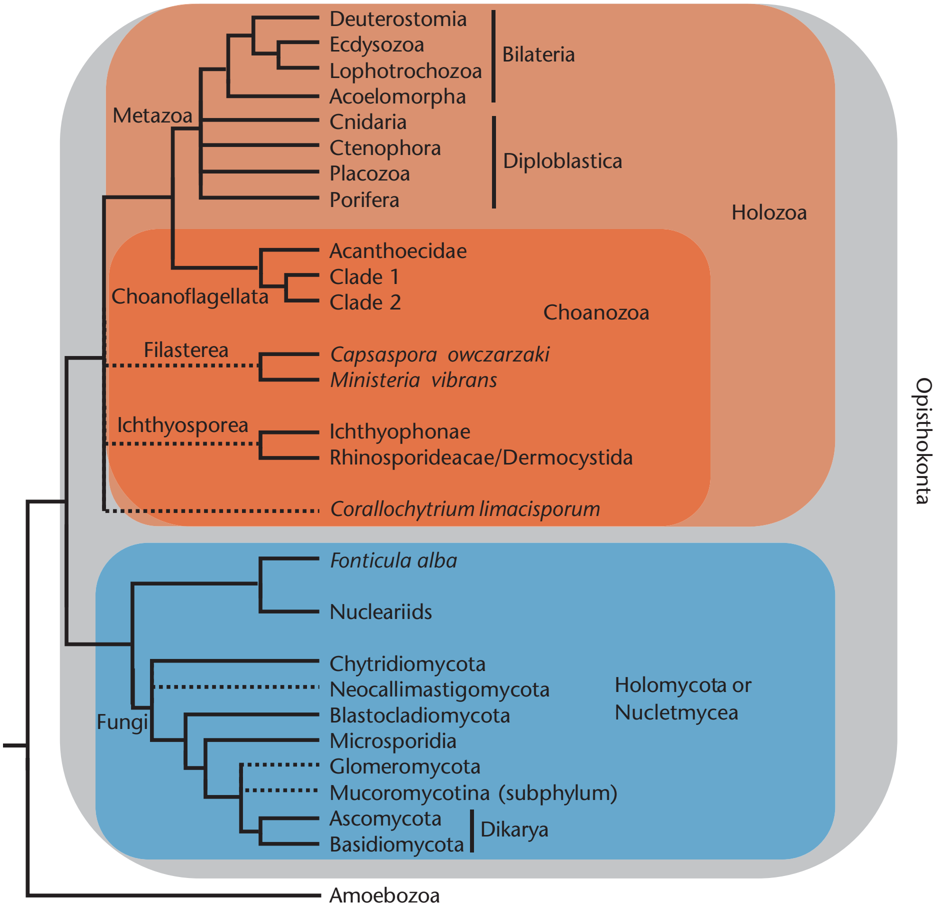

Figure 1 Phylogeny of the Opisthokonta. Summary of the phylogenetic relationships of the opisthokonts, based on recent molecular phylogenies. Amoebozoans are sister group to Opisthokonta, together forming the Unikonta. Shaded boxes remark widely accepted supergroups. See text for further discussion.

haloarchaeal type tyrosyl-transfer ribonucleic acid (tRNA) synthetase (Huang *et al.*, 2005).

First Animals

'Animals' were defined by Cavalier-Smith (1998) as phagotrophic multicellular eukaryotes with a connective tissue, located between two dissimilar epithelia (except in Mesozoa and Placozoa) and composed of collagen (**Figure 2a**). Later, Adl *et al.* (2005) extended this definition by adding traits such as osmotrophic nutrition, absence of cell wall, presence of intercellular junctions holding cells and sexual reproduction with production of an egg cell that is fertilised by a smaller, often monociliated, sperm cell. There are a million and half described animal species, grouped in around 35 phyla. However, some estimations suggest that the real number is probably greater than 11 million species. **See also**: Diversity of Life

Animal relationships have been explored since Haeckel coined the term *phylogeny*, one century and a half ago, and modern molecular phylogenetics has presented a widely accepted 'new animal phylogeny' (Aguinaldo *et al.*, 1997; Halanych, 2004; Halanych *et al.*, 1995). This novel scheme distributes the animals into two big groups, Diploblastica (animals with radial symmetry) and Bilateria (animals with bilaterian symmetry). The new rearrangement made by molecular systematics mostly affected the relationships within bilaterians, defining novel groups such as Lophotrochozoa or Ecdysozoa (Aguinaldo *et al.*, 1997; Halanych *et al.*, 1995). It is widely accepted that diploblasts are the earliest-branching Metazoa, embracing phyla such as Porifera (sponges), Cnidaria (sea anemones and jellyfishes), Ctenophora (comb jellies) and the puzzling Placozoa (with only a single species described, *Trichoplax adhaerens*). Whereas the position of Cnidaria seems to be settled as a sister group to bilaterians (Halanych, 2004), the remaining deep animal relationships are still problematic and questions about the monophyly of sponges or the position of sponges, ctenophores and placozoans have not been cleared yet.

Early phylogenetic studies favoured the paraphyletic status of 'Porifera', with Hexactinellida (glass sponges), then Demospongiae (spongin fibres) and then Calcarea (calcareous sponges) splitting off in order from the metazoan last common ancestor (Borchiellini *et al.*, 2001). Recent studies have failed to provide a definitive answer on to the Porifera status, because some trees show Porifera as a monophyletic assemblage (Pick *et al.*, 2010) whereas others support their paraphyly (Hejnol *et al.*, 2009; Sperling *et al.*, 2009). However, most of these phylogenomic studies agree in the sister group relationship between hexactinellids and demosponges.

The morphologically simple 'Placozoa' have also been candidates to be direct descendants from the metazoan last common ancestor (Schierwater *et al.*, 2009). However, molecular phylogenies have shown placozoans in every possible position among the diploblasts, from sister group to Cnidaria (Bridge *et al.*, 1995), to basal diploblasts (Dellaporta *et al.*, 2006; Schierwater *et al.*, 2009), to an intermediate position between sponges and cnidarians (Srivastava *et al.*, 2008). Finally, the diploblast phylum 'Ctenophora' has recently been shown to be the earliest-branching metazoans (Dunn *et al.*, 2008; Hejnol *et al.*, 2009). This position is highly controversial and whether is real or a result of an artefact caused by low taxon sampling or long-branch attraction (Pick *et al.*, 2010) awaits additional data.

Widely Known Relatives of Animals: Choanoflagellata and Fungi

The other major group of opisthokonts is the Fungi. 'Fungi' are defined as heterotrophic organisms, with unicellular or multicellular species, with vegetative and spore cells, often with cell walls of chitin and beta-glucan (at least in spores) and without phagocytosis (Cavalier-Smith, 1998). They display multinucleate hyphae, an alpha-aminoadipate (AAA) lysine biosynthesis pathway (although this pathway may present in other eukaryotes, see Torruella *et al.*, 2009), mitochondria and peroxisomes but lack other plastids (Adl *et al.*, 2005). The Fungi share some traits with metazoans as the presence of chitin, storage of glycogen and mitochondrial codon UGA encoding tryptophan; both have spores or gametes with a single posterior flagellum, but only the fungi chytrids have kept this character (Cavalier-Smith, 1987). Around 75 000 species of fungi have been described, but the estimates suggest there are above one million species. Their diversity range from the simple yeast or molds (i.e. *Saccharomyces* or *Penicillium*) to the more eye-catching mushrooms (**Figure 2b**), including a huge array of plant pathogens (such as the rice blast *Magnaporthe grisea*) or organisms of medical interest such as *Candida*, *Aspergillus* or *Cryptococcus*. A recent thorough revision, heavily based in molecular data, has reorganised all the fungi groups into seven phyla (Hibbett *et al.*, 2007; see **Figure 1** for phyla names). The most remarkable modifications affect the first splitting fungi, the two traditional phyla Chytridiomycota and Zygomycota, which currently are recognised as polyphyletic groups (James *et al.*, 2006). Recent multigenic (James *et al.*, 2006) and phylogenomics studies (Liu *et al.*, 2009) show Chytridiomycota (*sensu* Hibbett *et al.*, 2007) as the most basal fungi, together or followed by Neocallimastigomycota (**Figure 1**). **See also**: Diversity of Life

The other major, and well-known, opisthokont lineage besides fungi and animals is the 'Choanoflagellata'. Choanoflagellates comprise around 140 species of mostly marine heterotrophic, flagellate single-celled organisms, although some of them are able to form simple colonies (i.e. Proterospongia, see King, 2005). They present an egg-shaped cell 3–10 μm in diameter and bear a single posterior flagellum enclosed by a collar of thin microvilli. The idea that choanoflagellates are close relatives of animals was long ago posited on the basis of the striking resemblance between choanoflagellates and the collared cells (choanocytes) of sponges (James-Clark, 1866). In fact, recent molecular phylogenies have confirmed that choanoflagellates are indeed the sister group to Metazoa (Lang *et al.*, 2002; Carr *et al.*, 2008; Ruiz-Trillo *et al.*, 2008). Choanoflagellates external morphology is remarkably diverse, with outer coverings ranging from very simple ones to incredibly intricate coats. Actually, their external diversity is the basis of their traditional classification into three families: Codosigidae, Salpingoecidae and Acanthoecidae. The Codosigidae are the 'naked' choanoflagellates, they only have the cell membrane as an outer covering. The Salpingoecidae have a flask-shaped membrane or 'theca', whereas the Acanthoecidae have beautiful silica baskets or 'lorica'. A recent molecular study analysing four genes for 17 choanoflagellates species (Carr *et al.*, 2008) confirmed the existence of acanthoecids, while rejecting the monophyly of the other two families. Owing to their pivotal position as the closest unicellular relative to animals, our knowledge on them is relatively good compared with other no-parasite protists, with a genome sequence already published (King *et al.*, 2008), another in progress (Ruiz-Trillo *et al.*, 2007) and some expressed sequence tags (ESTs) projects done (King *et al.*, 2003; Ruiz-Trillo *et al.*, 2008).

Enigmatic Cousins of Animals

In addition to the major opisthokonts groups explored earlier (animals, fungi and choanoflagellates), recent molecular data have shown that Opisthokonta includes other poorly known single-celled lineages, such as ichthyosporeans, filastereans, nucleariids, *Corallochytrium limacisporum*, and the most recent addition, the slime mold *Fonticula alba*. Later, we describe each of those groups.

'Ichthyosporea' (Cavalier-Smith, 1998) was originally defined as the DRIP group (by the acronym of *Dermocystidium*, rossete agent, *Ichthyophonus* and *Psorospermium*), a node-based clade closely related to animals and choanoflagellates (Herr *et al.*, 1999). Later their name was amended to Mesomycetozoea (Mendoza *et al.*, 2002), based on the clade name 'mesomycetozoa' (Herr *et al.*,

Figure 2 Pictures representatives of different Opisthokonta. (a) Picture of the frog *Phyllomedusa vaillantii*. Courtesy of Carlos Palacín and Alejandro de Mendoza. (b) Picture of the fungi *Amanita muscaria*. (c) Scanning microscopy picture of the filasterean *Ministeria vibrans*. (d) Scanning microscopy picture of the ichthyosporean *Creolimax fragrantissima* in colonial stage. Courtesy of Arnau Sebé-Pedrós.

1999) that refers to their evolutionary position between fungi and animals (*meso* means 'middle', *myceto* stands for fungi and *zoa* for animals). However, probably to avoid confusion with Mycetozoa, Ichthyosporea seems to be nowadays the most accepted name for this clade. Ichthyosporea are single-celled organisms, with flat cristae like other opisthokonts, and some show colonial forms (**Figure 2d**). Most are animal parasites or endosymbionts, although there are some free-living and saprotrophic species (Adl *et al*., 2005). Ichthyosporeans can be divided into two groups, the human parasitic Rhinosporideacae and the Ichthyophonae. Some Rhinosporideacae (Mendoza *et al*., 2002; Dermocystida in Cavalier-Smith, 1998) show a posterior flagellum, and when inside their hosts, they exhibit sphere-shaped cells with several endospores that are eventually released. Ichthyophonae (Mendoza *et al*., 2002; Ichthyophonida Cavalier-Smith, 1998), on the contrary, includes parasites of fish or aquatic arthropods. Several of them show amoeboid cells typical of the group, although in others, amoeboid cells are absent or have not been detected yet. None of them shows flagellum, albeit a contentious monoflagellated phase is reported in *Pseudoperkinsus tapetis*. Interestingly, some ichthyosporeans have the capacity to form microcolonies as part of the cell cycle (Ruiz-Trillo *et al*., 2007).

Early molecular trees related ichthyosporeans to choanoflagellates (Herr *et al*., 1999; Mendoza *et al*., 2002), but later when filastereans (see discussion later) started to be added to phylogenetic or phylogenomic analyses, Ichthyosporea was shown as sister group to filastereans or to animals, choanoflagellates and filastereans (Carr *et al.*, 2008; Ruiz-Trillo *et al.*, 2008; Shalchian-Tabrizi *et al.*, 2008; Steenkamp *et al.*, 2006). Aphelidea (Gromov, 2000) are intracellular parasites of algae of undetermined phylogenetic position with an intricate life cycle that starts with an amoeboid cell invading the algae and finishes when cell division releases flagellate or amoeboid cells. Although aphelids have been proposed as members of Ichthyosporea (Shalchian-Tabrizi *et al.*, 2008), the lack of molecular data for them keeps their position unsettled.

'Filasterea' is a newly defined group (Shalchian-Tabrizi *et al.*, 2008), mainly based on molecular data, which currently comprises two species, *Capsaspora owczarzaki* (ATCC 30864) and *Ministeria vibrans*. Both organisms are unicellular, lack flagella and display flat cristae and long filopodia emerging from their membranes. *Capsaspora* amoeboid-like cells (3.0–7.0 μm) were isolated from the pulmonate snail *Biomphalaria glabrata* (Stibbs *et al.*, 1979), where they seem to feed from the larvae of the trematode *Schistosoma mansoni* that parasite the snail. *Capsaspora* is, thus, a symbiont of *B. glabrata* and was first named as *Nuclearia* sp. and considered a member of the nucleariids (Amaral-Zettler *et al.*, 2001). Further morphological and phylogenetic analyses showed that *C. owczarzaki* is not a nucleariid but an independent opisthokont lineage, closely related to ichthyosporeans (Ruiz-Trillo *et al.*, 2004). *Ministeria* (Tong, 1997) are free-living marine microorganisms, around 5 μm in diameter (**Figure 2c**). Some single-gene or multi-gene phylogenies related it to animals (Cavalier-Smith and Chao, 2003). Later analyses including data from both *Capsaspora* and *Ministeria* showed their sister group relationship and placed them closer to animals and choanoflagellates, forming the group Filozoa (Brown *et al.*, 2009; Shalchian-Tabrizi *et al.*, 2008).

'Corallochytrea' comprises a single species, *C. limacisporum* (Raghu-Kumar, 1987), a marine saprotrophic organism, 4.5–20 μm diameter, found in coral reefs in the Indian Ocean. During their life cycle, they undergo binary fissions and produce several amoeboid cells. The presence of flagellum has been reported. They were first classified as a kind of Fungi (as a Thraustochytrid, Raghu-Kumar, 1987), but early molecular phylogenies placed it close to choanoflagellates (Cavalier-Smith and Chao, 2003; Mendoza *et al.*, 2002) and later multigenic analyses pointed to a relationship with ichthyosporeans (Carr *et al.*, 2008; Ruiz-Trillo *et al.*, 2004; Steenkamp *et al.*, 2006). Unfortunately, there is no living culture available to researchers; therefore, its specific phylogenetic position within the opisthokonts may be unclear for some time more.

'Nucleariidae' (Cavalier-Smith, 1993) comprise several free-living, heterotrophic amoeboids. Besides their extending hyaline filopodia, nucleariids have either discoidal-shaped or flattened mitochondrial cristae. They typically have a spherical floating form and an amoeboid form when adhered to a substrate. Molecular phylogenies have strongly shown that nucleariids are the sister group to Fungi (Liu *et al.*, 2009; Ruiz-Trillo *et al.*, 2004; Steenkamp *et al.*, 2006).

Finally, the most recent addition to the opisthokonts is the cellular slime mold '*F. alba*' (Brown *et al.*, 2009). This species, the only on its genus, is an amoeboid protist that forms a multicellular fruiting body by aggregation of several individuals (Worley *et al.*, 1979). Molecular phylogenies have shown that *F. alba* is the sister group to nucleariids.

Opisthokonts, an Evolutionary Window to Understanding the Unicellular–Multicellular Transition

Our current phylogenetic framework of the opisthokonts splits them into two major branches: one containing Fungi and their single-celled relatives, named as Nucletmycea (Brown *et al.*, 2009) or Holomycota (Liu *et al.*, 2009), and another one containing Metazoa and their unicellular relatives, known as Holozoa (see **Figure 1**; Lang *et al.*, 2002). Moreover, the single-celled holozoans, that is, the closest animal relatives, are also known as choanozoans.

Interestingly, within the opisthokonts, there are currently five types of multicellular organisation: animal, fungal, aggregative colonial, 'syncitial type' colonial and aggregative fruiting bodies. Animal multicellularity is the product of embryogenesis, where a single cell develops into various differentiated cells and tissues, whereas Fungi have mycelia, which are interconnected, walled filamentous cells, which may display syncitial features. Choanoflagellates and *F. alba* can form colonies or fruiting bodies, respectively, by aggregation of several individuals, whereas some ichthyosporeans develop 'syncitial' type microcolonies.

Thus, the opisthokonts offer a unique evolutionary window among eukaryotic supergroups to understand multicellular origins. In this regard, the single-celled members of Holomycota, nucleariids and *F. alba*, are crucial to understanding the origin of fungal multicellularity, whereas the single-celled members of Holozoa, that is filastereans, ichthyosporeans, choanoflagellates and *Corallochytrium*, appear to be critical to understanding the origin of animal multicellularity. The importance of animal relatives to understanding the origin of animals has often been neglected, with most research efforts focusing on the study of basal metazoans, such as cnidarians, poriferans and placozoans. Studies of those basal animals have already unravelled important insights into the origin of Metazoa, and now researchers have complete genome sequence of two cnidarians (*Hydra magnipapillata* and *Nematostella vectensis*), one poriferan (*Amphimedon queenslandica*) and the placozoan *Trichoplax adhaerens* (**Table 1**). However, only a single genome of an animal relative (that of the choanoflagellate *Monosiga brevicollis*) has, so far, been published (King *et al.*, 2008). That genome showed us that genomic comparisons between basal

Table 1 Current available genomic data from Opisthokonta in NCBI

Organisms	Genomes completed	Genomes in progress	ESTs reads
Metazoa			
Bilaterian invertebrates	24	58	20×10^6
Cnidaria	2	0	640×10^3
Ctenophora	0	0	51×10^3
Placozoa	1	0	11×10^3
Porifera	0	1	95×10^3
Choanoflagellata	1	1	106×10^3
Filasterea			
Capsaspora owczarzaki	0	1	9×10^3
Ministeria vibrans	0	1	68
Ichthyosporea			
Sphaeroforma arctica	0	1	8×10^3
Creolimax fragrantissima	0	1	–
Amoebidium parasiticum	0	1	4×10^3
Fungi	200	50	2.5×10^6
Nucleariids	0	0	3×10^6
Fonticula alba	0	1	–

Source: Extracted from NCBI web page (http://www.ncbi.nlm.nih.gov/).

animals and their current unicellular relatives will provide significant insights into the emergence of animals from their protistan ancestors.

However, if one looks at the opisthokont phylogenetic framework (see **Figure 1**), it is clear that only by incorporating additional genomic data from the other animal relatives, such as ichthyosporean, filastereans and *Corallochytrium*, in the comparative analyses, we will be able to fully disentangle the 'metazoan genetic starter kit'. This is actually the goal of the UNICORN initiative, recently funded by the National Human Genome Research Institute (NHGRI) and being performed at the Broad Institute at MIT and Harvard (Ruiz-Trillo *et al*., 2007). UNICORN is obtaining the genome sequence of several holozoans and holomycots, with the aim to better understand the origins of both fungi and animals. A first look at what this data can show us is provided by the analysis of the integrin-mediated signalling and adhesion machinery that conclusively shows that choanoflagellates (and fungi) have suffered some lineage-specific gene losses of this important machinery (Sebé-Pedrós *et al*., 2010). This should be taken as a cautionary tale, because gene loss is undoubtedly an important, and sometimes neglected, player in evolutionary history and that broad taxonomic sampling is critical in comparative genomic analyses. Thus, we envision, and hope, that animal unicellular relatives, such as *Capsaspora*, *Ministeria* or ichthyosporeans, will not only offer us important insights into animal origins but also no longer be neglected by researchers.

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