Opinion piece

Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree

I discuss eukaryotic deep phylogeny and reclassify the basal eukaryotic kingdom Protozoa and derived kingdom Chromista in the light of multigene trees. I transfer the formerly protozoan Heliozoa and infrakingdoms Alveolata and Rhizaria into Chromista, which is sister to kingdom Plantae and arguably originated by synergistic double internal enslavement of green algal and red algal cells. I establish new subkingdoms (Harosa; Hacrobia) for the expanded Chromista. The protozoan phylum Euglenozoa differs immensely from other eukaryotes in its nuclear genome organization (trans-spliced multicistronic transcripts), mitochondrial DNA organization, cytochrome *c*-type biogenesis, cell structure and arguably primitive mitochondrial protein-import and nuclear DNA prereplication machineries. The bacteria-like absence of mitochondrial outer-membrane channel Tom40 and DNA replication origin-recognition complexes from trypanosomatid Euglenozoa roots the eukaryotic tree between Euglenozoa and all other eukaryotes (neokaryotes), or within Euglenozoa. Given their unique properties, I segregate Euglenozoa from infrakingdom Excavata (now comprising only phyla Percolozoa, Loukozoa, Metamonada), grouping infrakingdoms Euglenozoa and Excavata as the ancestral protozoan subkingdom Eozoa. I place phylum Apusozoa within the derived protozoan subkingdom Sarcomastigota. Clarifying early eukaryote evolution requires intensive study of properties distinguishing Euglenozoa from neokaryotes and Eozoa from neozoa (eukaryotes except Eozoa; ancestrally defined by haem lyase).

1. INTRODUCTION

Darwin would be astounded by the recent reconstructions of the tree of life. In the very year he and Wallace published their natural selection ideas, Owen (1858) established the kingdom Protozoa for the most primitive unicellular organisms, which eventually undermined the two-kingdom animal-vegetable viewpoint dominating biological thinking since Linnaeus. Electron microscopic discoveries eventually led to Bacteria being separated as a distinct kingdom and a five-kingdom system for eukaryotes: basal Protozoa and four derived kingdoms: the ancestrally heterotrophic Animalia and

Fungi, and ancestrally phototrophic Plantae and Chromista (Cavalier-Smith 1981).

Recent phylogenetic advances reveal that several major protist groups formerly treated as Protozoa really belong in the kingdom Chromista, necessitating radical reinterpretation of chromist evolution and revision of higher classification of both kingdoms, effected here. Consequently, Chromista, sister to Plantae, with 10 phyla, now has a megadiversity second only to Animalia. The simpler picture for Protozoa, with only seven phyla of distinctive cellular body plan, makes it easier to solve longstanding problems of the position of the root of the eukaryotic tree (Roger & Simpson 2009) and nature of the first eukaryotes. These are illuminated here by the hypothesis that the eukaryotic root lies between the protozoan phylum Euglenozoa and all the remaining eukaryotes (neokaryotes).

2. ORIGIN AND EXPANSION OF KINGDOM CHROMISTA

I established Chromista as a kingdom distinct from Plantae and Protozoa because of the evidence that chromist chloroplasts were acquired secondarily by enslavement of a red alga, itself a member of kingdom Plantae, and their unique membrane topology (Cavalier-Smith 1981). Chromista originally included only three predominantly algal groups: Heterokonta, Haptophyta, Cryptomonada. Initially I defined chromists as organisms possessing one or both of two characters: (i) chlorophyll *c*-containing plastid(s) lying within an extra (periplastid) membrane inside the lumen of the rough endoplasmic reticulum (RER; typically within the perinuclear cisterna); (ii) tripartite or bipartite rigid tubular hairs on one or both cilia. I argued that both characters evolved simultaneously in the ancestral chromist and several descendants lost at least one. Parsimony in the evolution of protein targeting across these extra membranes was a key reason for proposing chromistan unity and origin by one symbiogenetic event.

In accord with my original intention that organisms demonstrated to have lost both characters should also be included in Chromista, I now place Alveolata, Rhizaria (phyla Cercozoa and Retaria) and centrohelid Heliozoa within Chromista (figure 1; electronic supplementary material, table S1), as multigene trees show that they belong to Chromista phylogenetically (Burki *et al.* 2007, 2008, 2009; Hackett *et al.* 2007). Like chromists, photosynthetic dinoflagellates have chlorophyll *c*, but were originally excluded from Chromista because their ciliary hairs and membrane topology are simpler; their closest evolutionary affinities are with Ciliophora and Apicomplexa, grouped with them as protozoan infrakingdom Alveolata. Later, extending parsimonious protein-targeting evolution arguments to embrace chromists and alveolates, I postulated that both (collectively 'chromalveolates') got their chloroplasts by the same secondary intracellular enslavement of a red alga (Cavalier-Smith 1999), which is now firmly established (Keeling 2009).

I propose that more than 400 non-streptophyte green algal genes suggested to have entered the ancestral chromist (Moustafa *et al.* 2009) perhaps did so by the same secondary symbiogenesis that implanted the nucleomorph and chloroplasts into cercozoan
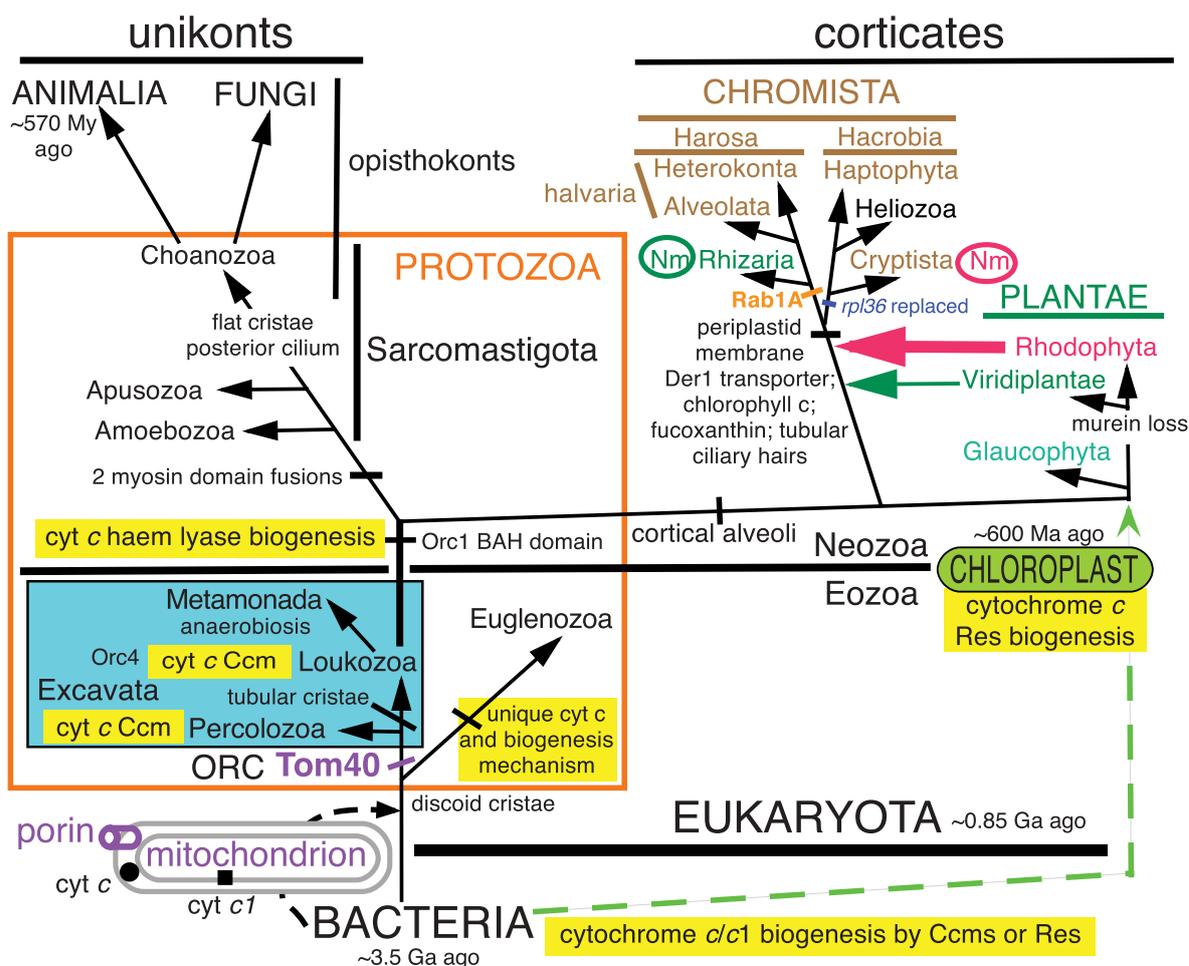
Figure 1. Evolutionary relationships of the six kingdoms. Chromist taxa with chlorophyll *c* are in brown (constituting the para-phyletic 'chromalveolates'); unlike previous classifications, Rhizaria and Heliozoa are within Chromista not Protozoa. Nm denotes retention as nucleomorphs by chlorarachnean Rhizaria of the green-algal and by Cryptophyceae of the red-algal nuclei from two ancestral secondary symbiogeneses that generated chromists. Cytochrome *c/c1* biogenesis mechanisms are highlighted in yellow. The ancestral bacterial cytochrome *c/c1* biogenesis mechanism (Ccm) is argued to have been inherited by excavate protozoa from the α-proteobacterial ancestor of mitochondria, and replaced by the novel haem lyase in the ancestor of neozoa. The complex pattern within corticates (ancestrally with both Ccms and haem lyase but later differentially lost) is omitted for clarity. The deepest branch having mitochondrial protein-import receptor Tom40 and origin recognition complex (ORC) is Percolozoa (electronic supplementary material, note 2). Taxa ranked as kingdoms or above are in upper case; clades not treated as taxa have lower-case initial. Diagnosis of new subkingdom Harosa: having Rab1A; typically with cortical alveoli or tripartite ciliary hairs or reticulose/filose pseudopods or ciliary gliding. The Alveolata/Heterokonta grouping (new clade halvaria) and likely eventual need to transfer Alveolata into Chromista were long foreseen (Cavalier-Smith 1995).

chlorarachnean algae (figure 1). On this double secondary symbiogenesis theory, chromists differentially lost green and red algal nuclei and chloroplasts several times, 'green' nucleomorphs persisting in the ancestral harosan (hence heterokonts retain more 'green genes' than haptophytes) and rhizarian, with only 'red' nucleomorphs persisting in the ancestral hacrobian and cryptist. Initial enslavement mechanisms of both plastids (evolving novel SNAREs targeting plastid-destined Golgi vesicles to the perialgal membrane; Cavalier-Smith 1999) were possibly shared, making one less independent evolutionarily onerous secondary symbiogenesis. The mechanistically much simpler later perialgal-nuclear-envelope membrane fusion, placing the enslaved red alga inside the RER, was evidently independent in Heterokonta (vesicle targeting and cytosolic location being retained by their alveolate sisters) and Hacrobia. Derived replacement of the chloroplast *rpl*36 gene by lateral gene transfer from a

bacterium in Hacrobia (Okamoto *et al*. 2009) followed their divergence from Harosa (figure 1). Electronic supplementary material, note 1 explains this further.

3. PRIMITIVE EOZOA VERSUS ADVANCED NEOZOA

Transfer of Alveolata, Rhizaria and Heliozoa to Chromista leaves seven phyla in the primitively heterotrophic Protozoa, in two contrasting subkingdoms: Eozoa (ancestrally rigid zooflagellates) and Sarcomastigota (ancestrally heterotrophic amoeboflagellates). The old idea that Eozoa are basal eukaryotes was set aside by arguments placing the root outside bikonts (Cavalier-Smith 2002). However, as Roger & Simpson (2009) and electronic supplementary material, note 3 explain, overlooked and new evidence demolished arguments against a bikont root. Recent evidence that eukaryotes have four different

mechanisms of biogenesis for cytochromes c and $c1$ (Allen *et al*. 2008), only two existing in bacteria, leads me to reinstate the idea that the root is within bikont Eozoa, not higher eukaryotes, which are here collectively called neozoa (figure 1).

Cytochromes c and $c1$ are key components of mitochondrial respiratory chains. Encoded in the nucleus and imported into mitochondria, they originated from the α-proteobacterium enslaved to make mitochondria, not its eukaryotic host. Their haem electron carrier is covalently attached via two cysteines (one only in Euglenozoa, uniquely in nature). Attachment is catalysed within the periplasm (intermembrane space) of mitochondria or bacteria by specific enzymes—in α-proteobacteria and excavate protozoa by the Ccm system of about eight inner membrane proteins named CcmA-H (Allen *et al*. 2008). Unikont eukaryotes have instead a simpler system: the enzyme haem lyase (sometimes one, sometimes two specialized for cytochrome c or $c1$, respectively), which evolved before their last common ancestor (figure 1). Bacteria lack homologous unimolecular haem lyases, a derived character unique to neozoa; the eukaryote root cannot be within neozoa (thus not between unikonts and bikonts) unless Eozoa secondarily lost haem lyase. Some plants and chromists have Ccms instead of haem lyase; this mosaic distribution of two mechanisms within Chromista and Plantae is most simply interpreted if haem lyase originated in the ancestor of neozoa, Ccms were lost quickly by the ancestral unikont, but coexisted with lyase relatively briefly in early corticates, one or the other being repeatedly lost during their early internal diversification; this is more parsimonious than hypothetical lateral gene transfer of haem lyase (Allen *et al*. 2008). Mosaic distribution of lyase and Ccms within corticates cannot be explained by placing the root within Plantae or Chromista, because the symbiogeneses creating each kingdom necessarily followed the origin of eukaryotes; a position between Plantae and Chromista would not explain it or why excavates have only the ancestral Ccms. Euglenozoa have neither Ccms nor haem lyase, but an unknown unique mechanism—probably derived, thus excluding the root from within Euglenozoa (if even the most divergent Euglenozoa possess it).

Previously I classified Euglenozoa within excavates, assuming that the eukaryotic root was outside bikonts and Euglenozoa secondarily lost excavate-specific characters (ciliary vanes; ventral feeding groove; characteristic cytoskeleton and ciliary roots; Cavalier-Smith 2002). But if the root is within Eozoa such dramatic loss need not be invoked: excavate ciliary vanes need never have become the euglenozoan-latticed rod or the ventral feeding groove be changed to the periciliary reservoir and distinctive mouthparts of Euglenozoa. Their contrasting cytoskeletal and ciliary structure would instead reflect independent divergence from a simpler common ancestor. As the rooting assumption behind earlier inclusion of Euglenozoa within Excavata is invalidated, I now exclude them from Excavata to emphasize their radical differences. The revised classification of kingdoms Chromista and Protozoa is shown in the electronic supplementary material, table S1.

4. WHERE IS THE EUKARYOTIC ROOT?

Cytochrome $c/c1$ biogenesis puts the eukaryotic root either within excavates (because of their ancestral Ccms) or between excavates and either neozoa or Euglenozoa, consistently with former reasons for placing the root outside bikonts now being invalid (Roger & Simpson 2009; see also electronic supplementary material, note 3). As Euglenozoa differ more profoundly from excavates and all other eukaryotes in genomic, mitochondrial and cytological organization than does any other phylum (electronic supplementary material, table S2), I now argue that the root is between Euglenozoa and all other eukaryotes (or possibly deeply within Euglenozoa). The most convincing new evidence is the absence of mitochondrial protein Translocator of the Outer Membrane (TOM) complex and origin recognition complex (ORC) genes from all three completely sequenced trypanosomatid genomes (Schneider *et al*. 2008; Godoy *et al*. 2009).

Tom40, a cylindrical β-barrel channel protein in the mitochondrial outer membrane (OM), is vital to almost all eukaryotes (even secondary anaerobes: Microsporidia; metamonads, e.g. *Giardia*, *Trimastix*) for importing nuclear-coded proteins. It ultimately evolved from a proteobacterial porin precursor like Usher (Cavalier-Smith 2006). Tom40 could never be lost without replacing its vital function with another protein. As TOM must interact with and recognize hundreds of mitochondrial protein presequences, changeover to radically different machinery is mechanistically almost inconceivable, making it highly unlikely that the trypanosomatid absence of Toms is secondary. Trypanosome mitochondrial protein-import machinery is radically simpler than in other eukaryotes: as postulated for the earliest mitochondria (Cavalier-Smith 2006), TIM translocase is one and not three proteins, and presequences are shorter (Schneider *et al*. 2008); but similar states in microsporidia must be simplifications, making this less compelling evidence for primitiveness than the absence of TOM. Unlike microsporidia, the aerobic trypanosomes lack obvious reasons for simplification; their Imp complex for presequence cleavage is also simpler. I suggest that after neokaryotes and Euglenozoa diverged, Tom40 evolved in the ancestral neokaryote by gene duplication and divergence of the ancestor of the sole trypanosomatid porin channel (VDAC). Originally, VDAC might have mediated both OM metabolite exchange and protein import, providing a remarkably simple way of originating mitochondria. Characterizing protein-import machinery in phylogenetically diverse Euglenozoa would test this and whether its greater simplicity in trypanosomes (and I suggest all Euglenozoa) is primitive. On VDAC/Tom40 mitochondrial porin trees, the root of the VDAC half is precisely between Euglenozoa and neokaryotes (Pusnik *et al*. 2009), as in my hypothesis.

Another clearly primitive trypanosome character is absence of the neozoan six-protein DNA replication ORC; they have only a Cdc6 single-protein replication initiator, like archaebacteria (Godoy *et al*. 2009). TOM complex and multicomponent ORC are arguably synapomorphies for neokaryotes (if no Euglenozoa have them) or

neokaryotes plus some Euglenozoa (if some Euglenozoa have them).

Other characters also substantially differ in trypanosomatids from neokaryotes. Some markedly simpler states in cytoskeleton and endomembranes than in neokaryotes (Berriman *et al.* 2005) might be consequences of parasitism; but most could, like Tom and ORC absence, be ancestral for all Euglenozoa, representing a simpler phase of eukaryote cell evolution before neokaryotes evolved. Irrespective of where within Eozoa the root is, the ancestral eozoan was non-amoeboid, with a rigid surface pellicle with cortical microtubules, not actomyosin, being cytoskeletally dominant (amoeboid surfaces evolved secondarily within Percolozoa and Metamonada). Such a rigid microtubule-supported cortex was previously thought to be essential for evolving mitosis and cytokinesis when cell surface-based DNA segregation machinery of bacteria became ineffective once phagotrophy originated and internalized DNA/membrane links (Cavalier-Smith 1987, 2002). Trypanosomatid emphasis on microtubules and relative deficiency in actomyosin-related machinery might be ancestral for Euglenozoa, possibly even eukaryotes, not parasitic reduction. Their mitotic kinetochore machinery is far simpler; they lack actin-severing and bundling machinery and activators of the actin-related protein complex Arp2/3, many proteins for microtubule ends and lateral decoration, and ciliary tektins (Berriman *et al.* 2005), all general for neozoa. Possibly, all evolved only in neokaryotes and not in the earliest eukaryotes. The hypothesis that the eukaryotic root is between Euglenozoa and neokaryotes neatly rationalizes this simplicity. Another 25 unusual genomic or cellular properties of Euglenozoa, trypanosomatids or euglenoids can be similarly interpreted (see electronic supplementary material, which highlights nine as further evidence for the root being between Euglenozoa and neokaryotes–and indicating the likely primitive state for the earliest eukaryotes).

Thomas Cavalier-Smith*
*Department of Zoology, University of Oxford,
South Parks Road, Oxford OX1 3PS, UK*
*tom.cavalier-smith@zoo.ox.ac.uk

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