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Meeting Report: Evolution of Protozoa and Other Protists, Linnean Society, London, September 13, 2004

Introduction

Over the last 10 years, the Linnean Society has acted as host to three international symposia on the evolution of Protozoa or Protists. At the first, in 1994, entitled "Kingdoms and Domains," discussion centred on the use of ribosomal RNA sequence comparisons in determining microbial phylogenetic relationships in the wake of Carl Woese's assertion that there are three, not two, primary phylogenetic groupings or Domains of organisms on this planet — Eubacteria, Archaea and Eukarya (Woese et al. 1990). A "Universal Tree of Life" was emerging that suggested that microbial diversity dwarfed that of the plant, fungal and animal kingdoms which had hitherto dominated the study of phylogeny. A major question was whether the Protista (or Protoctista) represent one Kingdom or several.

In 1996, a two-day symposium organised by the British Section of the Society of Protozoologists, the Systematics Association and the Linnean Society was held under the title of "Evolutionary Relationships among Protozoa" (Coombs et al. 1998). By then it had become recognised that the eukaryotic cell can be visualised as the result of a series of endosymbiotic events involving acquisition of new genetic material and cytoplasmic compartments, and the origin of mitochondria, plastids and hydrogenosomes had become of central importance in protozoan phylogenetics. But the Universal Tree was experiencing some rough weather as emphasised rather dramatically by the late André Adoutte at the meeting. Additional sequence data were beginning to reveal the extent to which lateral gene transfer had shaped the course of evolution over the long haul. In particular, there was uncertainty regarding the deep evolutionary branchings. It was odd that the deepest-branching protists were specialised parasites (*Giardia*, *Trichomonas*, microsporidians), though their lack of mitochondria suggested that they might be "Archezoa" — relicts of eukaryotes predating the endosymbiotic origin of mitochondria. Adoutte, however, pointed out to us that faster rates of molecular evolution in these branches may account for their displacement to the bottom of the tree. Indeed protein sequences had already suggested that the Microsporidia belonged with the fungi and could not be archezoans, moreover, like the Myxozoa, they were secondarily unicellular: the protozoa, as we knew them looked as though they were polyphyletic in origin and belonged to several Kingdoms.

In this third meeting, again supported by all three societies, it was hoped to resolve some of these doubts, and several interesting recent developments emerged from the talks and subsequent discussion, as indicated in the abstracts below. It now looks as though all amitochondriate eukaryotes are derived from mitochondriate ancestors and tenaciously hang on to their mitochondrial remnants (M. Embley, C. Rotte); if "Archezoa" exist they are yet to be found. Eukaryotes may share more genes with eubacteria than with their supposed sister group, the Archaea (C. Rotte), lending support to the view that prokaryote genome fusions leading up the eukaryotic condition are more realistically represented by a ring rather than a branching tree (Rivera and Lake 2004). Analytical artifacts still plague attempts to infer the

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deep phylogeny of eukaryotes from multiple gene data sets, but improved models of protein evolution may help to lay bare the deep structure of the eukaryote tree (A.J. Roger). In the meantime, derived gene fusions and gene insertions have been used to reconstruct branching order, providing an alternative to gene sequence trees (T. Cavalier-Smith). Phylogenetic analysis and ultrastructural evidence currently indicate that most of the Eukarya can be classified into six broad supergroups or Kingdoms (A.J. Roger), with a basic bifurcation into unikonts and bikonts shortly after emergence of the eukaryotic cell (T. Cavalier-Smith).

An aspect of protozoan evolution not evident in our previous meetings was consideration of protists that leave fossil remains in datable geological deposits, notably the foraminiferans, coccolithophorids and dinoflagellates. These organisms in comparison with extant forms, afford opportunities to explore other important aspects of protozoan evolution — the congruence of molecular and fossil data (J. Pawlowski), the link between morphology, habitat shifts and ecological success, speciation in the apparent absence of genetic isolation (M. Kucera), and the relationship between innovation and complex life cycles (J.R. Young). Lastly, the discovery of life-like preservation of soft-bodied protists in amber (W. Foissner), promises to illuminate whole microbiocoenoses of the past. As after previous meetings, participants left with a strong feeling that the study of protozoan evolution was in state of rapid flux, but, nevertheless, a fascinating and healthy one.

Evolution of Mitochondria, Hydrogeneosomes and their Relatives

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Over the past 10 years it has become apparent that parasitic protozoa collectively termed Archezoa, including *Entamoeba, Giardia, Trichomonas* and Microsporidia, that were once thought to have separated from other eukaryotes before the acquisition of mitochondria, contain genes from the mitochondrial endosymbiont (Embley et al. 2003). Antibodies to proteins encoded by these genes have now been used to show that representative species contain a double-membraned organelle to which the proteins are targeted. In *Trichomonas* this organelle is called a hydrogenosome because it makes molecular hydrogen. Hydrogenosomes also occur in anaerobic fungi and diverse anaerobic ciliates. The best-studied hydrogenosomes, those from *Trichomonas* and fungi, also import proteins using pathways that are otherwise typical of mitochondria (Embley et al. 2003). The recently discovered (Tovar et al. 2003) organelle in *Giardia* appears to play a role in the maturation of iron sulphur clusters, an essential biosynthetic function of yeast mitochondria. The organelles in *Entamoeba* and the microsporidian *Trachipleistophora* are called mitosomes and their function(s) are so far unknown. The simplest hypothesis to explain the shared similarities between hydrogenosomes, mitosomes and mitochondria is that they are homologues sensu Owen (1843) of the same organ (here organelle) in different animals under every variety of form and function. In the light of these data it appears reasonable to suggest that all eukaryotes will eventually be shown to contain a mitochondrial homologue, bearing testimony to the important role that the mitochondrial endosymbiosis has played in eukaryotic evolution. It remains to be seen if members of this family of organelles share a common function essential to the eukaryotic cell, that provides the underlying selection pressure for organelle retention under different living conditions.

Anaerobic Mitochondria — the Organelles that the Endosymbiont Hypothesis Forgot

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The current paradigm for the relatedness of eubacteria, archaebacteria and eukaryotes is the small subunit ribosomal RNA (rRNA) tree, also called the tree of life. In this rRNA tree, eukaryotes are depicted as sisters to the archaebacteria (Woese et al. 1990), yet the sister-group relationship between archaebacteria and eukaryotes implied in the rRNA tree is only reflected in some eukaryotic genes. Former studies already indicated that there are many more eubacterial genes in eukaryotic

genomes than would be expected on the basis of the rRNA paradigm (Brown 2003).

Pair-wise amino acid sequence identity in comparison of 6,214 nuclear-protein coding genes from baker's yeast *Saccharomyces cerevisiae* to 177,117 proteins encoded in sequenced genomes from 45 eubacteria and 15 archaebacteria were examined. The results show that, in yeast, eubacterial-derived genes outnumber the archaebacterial-derived genes by a factor of 3 (Esser et al. 2004). Carbon metabolism is the largest functional class among the eubacterial-specific genes, whereas the archaebacterial-specific genes are mostly involved in information processing. Further, the yeast genome shares more similarity with proteobacterial genomes among eubacteria and more similarity with methanogen genomes among archaebacteria than other prokaryotic genomes surveyed. Our findings indicate that at the level of overall amino acid sequence identity and gene content, yeast shares a sister-group relationship with eubacteria, not with archaebacteria, in contrast to the current phylogenetic paradigm based on ribosomal RNA.

The presence of eubacterial genes in eukaryotic genomes is now widely accepted to indicate some kind of chimaerism during eukaryotic evolution. It has spawned models in which additional endosymbiotic partners are invoked to explain the origins of these genes. According to the endosymbiont theory, mitochondria and plastids of eukaryotic cells were once free-living prokaryotes. Several models have been put forward to explain the origin of eukaryotes in a manner that could, in principle, account for the presence of too many eubacterial genes in eukaryotic genomes by the virtue of the intracellular relocation of genes from the endosymbiont to the host (Martin et al. 2001).

But in addition to such chimaerism, endosymbiotic models also need to account for the common origin of mitochondria and their anaerobic relatives, hydrogenosomes, as strongly suggested by newer findings. Mitochondria and hydrogenosomes are not only the powerhouses, but also the blacksmiths of eukaryotic cells. A basic metabolic function seems to be conserved in both types of organelles: the maturation of Fe/S clusters, which are required for biogenesis of Fe/S proteins that perform crucial functions within all types of cells. To date, Fe/S cluster maturation is the only biochemical process known to render yeast mitochondria essential (Mühlenhoff and Lill 2000). An intriguing question is, whether mitochondria (and their anaerobic relatives) are essential for maturation of Fe/S clusters in all eukaryotic cells.

Inferring the Deep Phylogeny of Eukaryotes with Multiple Gene Data Sets: Panacean or Panglossian?

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Over the last three decades, many phylogenetic questions concerning both ancient and recent divergences within bacteria and eukaryotes alike have been addressed by analyses of alignments of single genes encoding either structural RNA genes (e.g. rRNA) or proteins. Although much has been clarified by these analyses, the relationships amongst many of the earliest diverging lineages in the history of life remain difficult to resolve. Recent molecular phylogenetic analyses when considered in combination with current ultrastructural knowledge, indicate that most of the eukaryotic realm can be classified into 6 broad "super-groups" (Simpson and Roger 2004). Yet, single gene phylogenies fail to robustly recover the monophyly of many of these groups and the branching pattern amongst them is often either completely unresolved or strongly patterned by analytical artefacts (Gribaldo and Philippe 2002). Without a robust "deep" phylogeny of eukaryotes, progress in understanding the major evolutionary transitions in early evolution is stalled.

The difficulties in obtaining robust "deep" phylogenies of eukaryotes likely stem from: (i) saturation of sequence changes over deep evolutionary timescales, (ii) phylogenetic artefacts caused by model misspecification, (iii) rapid radiations of the taxa under study and (iv) lateral gene transfer amongst lineages. Saturation means that random error is increased while very few positions in the sequences will retain "deep" phylogenetic signal. Furthermore, if rapid radiations of the major eukaryote lineages occurred, then informative changes in sequences are expected to be extremely rare. One solution is to analyse data sets made up of

a large number of different genes. In principle this should provide much larger sample sizes that will reduce random error and potentially capture a sufficient number of positions with "deep" phylogenetic signal. Recent published analyses of multiple gene alignments have concatenated the sequences and analysed them as if they are one "super-gene". However, this approach can introduce systematic error since a single set of branch lengths and model parameters is assumed for all of the genes. Alternative approaches have been introduced that allow each gene to have its parameters separately optimised (Bapteste et al. 2002; Pupko et al. 2002) or allow the genes to have strictly proportional branch lengths (Pupko et al. 2002).

Some of these approaches for alignments of mixed protein/nucleotide data sets (actin, SSU rRNA, LSU rRNA) and multiple protein data sets (EF-1alpha, EF-2, alpha- & beta-tubulin, hsp70 and hsp90) were investigated. Using a bootstrapped Bayesian analysis with linked or unlinked parameter sets across the different genes, under several models of evolution, we demonstrate that choices in gene-by-gene parameter setup can strongly affect support for deep branches in the eukaryote tree. We have also developed a method based on iterated hierarchical likelihood ratio tests and clustering techniques that allows sets of mutually phylogenetically congruent/incongruent genes to be inferred. The software tool we have developed that implements this method, called CONCATERPILLAR, permits the identification of gene sets with similar histories and model parameters and analyses them separately from other sets. Despite these improvements, the complexity of the pattern of protein evolution over deep phylogenetic divergences continues to lead to pervasive artefactual signal in multigene datasets analysed under simple models. Improving these models of protein evolution is necessary if we hope to elucidate the deep structure of the eukaryote tree.

Early Evolution of Foraminifera

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The microfossils are widely used in biostratigraphy and paleoenvironmental reconstructions and provide a rich source of information on evolutionary history of many groups of protists. The origins of these groups and their early evolution, however, are obscured by a non-fossilised period, the importance of which is difficult to unravel from the fossil data. This hidden part of the evolutionary history of microfossil eukaryotes is essential to establish the phylogenetic relations and to infer the timescale of early eukaryote evolution.

The early evolution of foraminifera was examined by analysing the sequences of small subunit ribosomal RNA (Pawlowski et al. 2002) and actin-coding genes for a large number of extant non-fossilised species. These species included (i) the naked athalamiids that probably lost their tests secondarily, as an adaptation to the freshwater environment, (ii) the organic-walled unilocular allogromiids that are particularly abundant in deep-water and high latitude environments, and (iii) the agglutinated unilocular astrorhizids, the poorly preserved tests of which are rarely encountered in the fossil record. In traditional view of foraminiferal evolution, these three groups have been considered as successive steps in development of foraminiferal skeleton.

According to molecular data, the early evolution of foraminifera was not a gradual process of increasing the complexity of the test composition and structure, but rather a series of tentative experiments to develop a test by using various materials and construction methods. In molecular trees, the non-fossilised species form a large radiation, comprising numerous heterogeneous lineages, which often include species with both organic and agglutinated tests. Similar morphotypes developed independently in different lineages, throwing the present morphology-based classification of early foraminifers in total disarray. This radiation of non-fossilised unilocular foraminifers led to an independent divergence of two multilocular clades: the clade of Textulariida+Rotaliida and the clade of Spirillinida+Miliolida.

Based on fossil calibration using the multilocular species, we estimated that the beginning of foraminiferan radiation occurred in the Late Neoproterozoic, between 690 and 1150 million years ago (Pawlowski et al. 2003). Much younger dates (550—650 mya) were found using Bayesian methods for divergence time estimation, both in case of ribosomal RNA and actin genes. These dates are in agreement with interpretation of some Upper Vendian micro-

fossils as agglutinated foraminifers. Our study shows a relatively good congruence between molecular and fossil data, emphasising the importance of non-fossilised stages in early evolution of foraminifera.

Causes and Mechanisms of Speciation in Planktonic Foraminifera

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The colonization of the pelagic environment by foraminifera marks the last step in the ecological expansion of this extremely successful group of aquatic protists. The first planktonic foraminifera occurred in the Middle Jurassic, some 180 million years ago (Hart et al. 2003), but they did not become abundant until 50 million years later. Considering that several benthic foraminifer clades successfully crossed the ecological barrier to the pelagic realm, that biomineralisation in foraminifera evolved already in the Palaeozoic, and that planktonic radiolaria, a related group of marine amoebae with opal skeletons, are known from the earliest Palaeozoic, it remains a mystery why it took several hundred million years for foraminifera to mount the first sortie into the lucrative pelagic niche.

After their successful ascent into the pelagic realm, planktonic foraminifera have constituted an important element of oceanic microzooplankton. The prolific production and excellent preservation of their calcareous shells in oceanic sediments produced probably the best fossil record on earth, providing unparalleled archives of morphological change, abundance and habitat characteristics. Studies of the evolutionary record of planktonic foraminifera revealed enormous variation in rates of morphological evolution. Both sustained unidirectional trends lasting 10^6 years and rapid transitions of only 10^3 years have been documented (Norris 2001). Fossil data often indicate links between morphological evolution, ecological success and habitat shifts (Norris 2001), and molecular genetic studies show that even the finest skeletal morphological traits reflect genetic distinction (Darling et al. 2004). These observations suggest that the fossil morphological trends are genetically controlled and reflect adaptive processes.

The agents that trigger and guide evolution in marine plankton remain elusive. Schmidt et al. (2004) presented evidence for a link between morphological diversity among planktonic foraminifera and the strength of thermal gradients in the ocean. More evidence for abiotic forcing of speciation in planktonic foraminifera is emerging from studies combining fossil data with molecular genetics (Darling et al. 2004). In order to understand how marine plankton evolves, one needs to uncover the mechanisms causing genetic isolation. This is especially intriguing in the pelagic realm, where opportunities for geographical isolation are rare and many species show global dispersal potential. Thus, non-vicariant speciation models have been suggested as the main mechanism of plankton evolution (Norris 2001). In these models, isolation is mediated by divergence in depth and timing of reproduction, mate recognition systems or by disruptive selection along environmental gradients and in the presence of multiple adaptive optima. However, recent molecular genetic investigations have revealed that genetically distinct types with a greater degree of endemicity are common among morphologically defined species (e.g., Darling et al. 2004), lending support to the plausibility of allopatric speciation in the plankton.

The excellent fossil record of planktonic foraminifera combined with molecular genetic data allows investigations of gene flow through space and time, and its interactions with the physical environment. Such studies hold great promise for understanding of factors that promote and mediate evolution of marine microplankton.

Phytoplankton Life-Cycles and Biomineralization

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Life-cycles are a fundamental aspect of protist biology and their evolution is central to protist diversification, however for many groups they are still poorly characterised. Within the phytoplankton our knowledge of coccolithophore life-cycles has been greatly increased in recent years (e.g. Houdan et al.

2004; Young and Henriksen 2003) and we are currently investigating calcareous dinoflagellate life cycles. The results provide new insights to the linkages of evolution between life-cycle phases.

In both coccolithophores and non-calcifying haptophytes, it is now established that the basic life-cycle is haplo-diplontic, i.e. the haploid and the diploid phases are vegetative and can undergo indefinite mitotic division. Molecular genetic and stratophenetic data suggest that calcification evolved once only in the haptophytes and was initially expressed in the diploid phase, with heterococcolith production. Subsequently calcification appears to have been transferred to the haploid phase on a few occasions with different calcification modes occurring each time (holoccocoliths, Polycrater coccoliths and ceratoliths).

In dinoflagellates the basic life-cycle is haplontic, i.e. the haploid phase is dominant and undergoes asexual reproduction via mitosis whilst the diploid phase is subordinate, typically encysts, and does not reproduce mitotically. The calcareous dinoflagellates produce a calcareous exoskeleton or calcisphere in part of the life cycle. They form a rather well-defined clade within the dinoflagellates, as confirmed by molecular genetics but include two different functional groups; neritic species with calcispheres occurring as benthic resting cysts (e.g. *Scrippsiella trochoidea*) in the diploid stage, and planktonic species in which the calcispheres are apparently haploid vegetative phases (e.g. *Thoracosphaera heimii*) or possibly division cysts (e.g. *Leonella granifera*). As with coccolithophores this implies transfer of calcification from the diploid to the haploid phase.

These two examples highlight the fact that beneath the superficial variability of life-cycles within protist groups fundamental aspects related to the sexual life-cycle are highly conserved within groups, but also show that innovations in one ploidy phase of the life-cycle, specifically biomineralisation, can be transferred to the other phase.

Fossil Ciliates and Testate Amoebae: what do they Tell us?

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The earliest fossils of heterotrophic protists date back about 800 million years, that is, to the early Neoproterozoic to late Mesoproterozoic. However, diverse protist fossils are known only since the Cambrian 550 million years ago. Naturally, all fossils are from groups which have durable shells (tests), such as foraminifera, radiolarians, and dinoflagellates.

The oldest heterotrophic protists are the "vase-shaped microfossils" which were found worldwide in up to 800 million years old Neoproterozoic rocks from marine environments. Recent evidence, based on exceptionally well-preserved specimens from the Grand Canyon (USA), strongly suggested that the "vases" were testate amoebae (Porter and Knoll 2000). Many of these forms resembled extant testaceans, but were sufficiently different to be classified as new genera and species. Formerly, reliable records of testate amoebae were much younger, viz., from 100 million year old Cretaceous amber. Most of the young fossils resembled extant genera and species. This is emphasised by several excellently preserved specimens with richly structured siliceous scales, which were discovered in 15 million years old lacustrine sediments and could be investigated with the scanning electron microscope (Foissner and Schiller 2001). They were indistinguishable from the extant species *Euglypha crenulata* and *E. scutigera*.

Ciliates date back to at least the Ordovician (~500 million years ago), where loricas (Calpionellids) of tintinnids have been found; biochemical markers even suggested 750 million years. Tintinnids were diverse and abundant during several geological periods, and thus 76 fossil genera and hundreds of species have been described (Aescht 2001). Fossils from other shelled ciliates are extremely rare and known only from Triassic (~220 million years ago) peritrichs grown on ostracods and Cretaceous (~100 million years) folliculinid heterotrichs. Only recently, excellently preserved, about 100 million year old soft-bodied protists were discovered in amber from Bavaria (collected by U.C. Bauer). Indeed, these minute (<1 cm) amber pieces contained a complete microbiocoenosis composed of bacteria, fungi, algae, testate and naked amoebae, and various ciliates (Schönborn et al. 1999). Most, but not all, species found look similar to extant taxa, and several taxa to be expected, for instance, euglyphid testaceans and members of the ciliate genus *Colpoda*, were absent. Experi-

ments showed that living ciliates can be well preserved in resin from ancient plants (Cycas). Thus, the amber inclusions are not artifacts.

The paleontological data show that (1) diverse protist communities already existed 800—500 million years ago, suggesting that protists are much older than one milliard years; and (2) protist morphotypes can persist for long times. This, however, does not mean low extant protist species number because they could accumulate high diversity over time, although the Permian mass extinction also concerned protists.

Protist Diversification and the Tree of Life

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Protists — unicellular eukaryotes — are polyphyletic since they comprise not only those that were derived directly from the first eukaryote cell, but also those secondarily simplified from animal ancestors (Myxozoa) or fungal ancestors (e.g. microsporidia, yeasts). Eukaryotes and Archaebacteria together form the clade neomura, characterised by 20 novel characters, including highly modified DNA-handling enzymes, absent from eubacteria, which are probably far older and ancestrally had walls of the peptidoglycan murein — replaced by N-linked glycoproteins in the ancestral neomuran. The first eukaryote cell was almost certainly a phagotrophic heterotroph with a cilium (flagellum) but no chloroplast. It arose by the dramatic transformation of bacterial cell structure in association with the origin of phagotrophy, perhaps as recently as 800 million years ago; the concerted origin of the endomembrane system, internal cytoskeleton, nucleus, mitosis, sex, cilium, peroxisomes, and the enslavement of an ingested α-proteobacterium to form the first mitochondrion was the most extensive reordering of cell structure in the history of life. It probably took place in an early neomuran bacterium — a now missing link between Gram-positive eubacteria (specifically Actinobacteria) and Archaebacteria. Derived gene splits and molecular trees show that archaebacteria are sisters to eukaryotes, not their ancestors. Together with the dozen shared features of actinobacteria and neomura (e.g. proteasomes, phosphatidyl inositol), this refutes the hydrogen hypothesis for the origin of mitochondria and Martin's idea that eubacteria and neomura arose independently. Complementary gene fusions reveal a fundamental bifurcation among eukaryotes between two major clades: the ancestrally uniciliate (often unicentriolar) unikonts and the ancestrally biciliate bikonts, which undergo ciliary transformation by converting a younger anterior cilium into a dissimilar older posterior cilium. Unikonts comprise the ancestrally unikont protozoan phylum Amoebozoa and the opisthokonts (kingdom Animalia, phylum Choanozoa, their sisters or ancestors; and kingdom Fungi). They share certain derived gene fusions, absent from bikonts. Bikonts contrastingly share a derived gene fusion between dihydrofolate reductase and thymidylate synthase and include plants and all other protists, comprising the protozoan infrakingdoms Rhizaria [phyla Cercozoa, Foraminifera (probably sisters); Radiozoa] and Excavata (phyla Loukozoa, Metamonada, Euglenozoa, Percolozoa), plus the kingdom Plantae [Viridaeplantae, Rhodophyta (sisters); Glaucophyta] that arose by converting an enslaved ingested cyanobacterium into the first chloroplast, the chromalveolate clade, and the small protozoan phyla Apusozoa (Thecomonadea, Diphylleida) and Heliozoa (centrohelids only). Chromalveolates comprise kingdom Chromista (Cryptista, Heterokonta, Haptophyta) and the protozoan infrakingdom Alveolata [phyla Ciliophora, Myzozoa (Dinozoa, Apicomplexa)], which diverged from a common ancestor that enslaved an ingested red alga and evolved novel plastid protein-targeting machinery via host rough ER and the enslaved algal plasma membrane (periplastid membrane). The branching order of the five bikont groups is uncertain: Plantae may be sisters of or ancestral to chromalveolates (jointly designated corticates as they share cortical alveoli); if the formerly green algal plastid of euglenoids and chlorarachneans (Cercozoa) was enslaved in one event in their common ancestor, Rhizaria and Excavata (jointly cabozoa) are probably sisters. Apusozoa may be sisters of all other bikonts, or more likely just of Excavata; centrohelid heliozoa may be related to Rhizaria. Hydrogenosomes and mitosomes evolved polyphyletically from mitochondria as secondary anaerobic adaptations, the first eukaryote being a facultative aerobe. Our large-scale picture of diversification of the basal

eukaryote kingdom Protozoa (13 phyla) is probably now reasonably complete (Cavalier-Smith 2004). Important remaining phylogenetic uncertainties are the basal branching order in bikonts, and whether Amoebozoa are holophyletic or paraphyletic (Cavalier-Smith et al. 2004).

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