Evidence for a Syncytial Origin of Eukaryotes from Ancestral State Reconstruction

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Accepted: 28 April 2021

Abstract

Modern accounts of eukaryogenesis entail an endosymbiotic encounter between an archaeal host and a proteobacterial endosymbiont, with subsequent evolution giving rise to a unicell possessing a single nucleus and mitochondria. The mononucleate state of the last eukaryotic common ancestor (LECA) is seldom, if ever, questioned, even though cells harboring multiple (syncytia, coenocytes, and polykaryons) are surprisingly common across eukaryotic supergroups. Here, we present a survey of multinucleated forms. Ancestral character state reconstruction for representatives of 106 eukaryotic taxa using 16 different possible roots and supergroup sister relationships, indicate that LECA, in addition to being mitochondriate, sexual, and meiotic, was multinucleate. LECA exhibited closed mitosis, which is the rule for modern syncytial forms, shedding light on the mechanics of its chromosome segregation. A simple mathematical model shows that within LECA's multinucleate cytosol, relationships among mitochondria and nuclei were neither one-to-one, nor one-to-many, but many-to-many, placing mitonuclear interactions and cytonuclear compatibility at the evolutionary base of eukaryotic cell origin. Within a syncytium, individual nuclei and individual mitochondria function as the initial lower-level evolutionary units of selection, as opposed to individual cells, during eukaryogenesis. Nuclei within a syncytium rescue each other's lethal mutations, thereby postponing selection for viable nuclei and cytonuclear compatibility to the generation of spores, buffering transitional bottlenecks at eukaryogenesis. The prokaryote-to-eukaryote transition is traditionally thought to have left no intermediates, yet if eukaryogenesis proceeded *via* a syncytial common ancestor, intermediate forms have persisted to the present throughout the eukaryotic tree as syncytia but have so far gone unrecognized.

Key words: syncytium, coenocyte, meiosis, mitosis, eukaryogenesis, endosymbiosis, units of selection.

Significance Statement

The transition of prokaryotes to eukaryotes involved endosymbiosis and a dramatic increase in intracellular cell complexity. While most theories on eukaryogenesis consider and illustrate the last eukaryotic common ancestor (LECA) as a mononucleated, sexual, flagellated population of cells, the origin of coordinated nuclear and organellar division coupled to the cell-cycle is rarely discussed. Using ancestral state reconstructions, we show that LECA most likely included a multinucleated stage which also allowed for conflict mediation between mitochondrial and nuclear genomes brought about by endosymbiotic gene transfer. The near-universal presence of the syncytial life stage across all major eukaryotic groups suggests that a multinucleated LECA is a viable intermediate that permitted intracellular experimentation and evolution of the complex eukaryotic processes we observe today.

Introduction

With more than 2 million described species, eukaryotes are morphologically the most diverse domain of life (Archibald et al. 2017; Adl et al. 2019), inhabiting a wide range of ecological habitats (López-García et al. 2007; Mora et al. 2011; Geisen et al. 2017). Eukaryotic cells are vastly more complex than prokaryotic cells as evident by their endomembrane system (Gould et al. 2016). They appear about 2 billion years later in the fossil record than prokaryotes do (Javaux et al. 2001; Javaux and Lepot 2018). There is a consensus among specialists that eukaryotes arose from prokaryotes, but the issue of how they arose from prokaryotes is intensely debated. All current theories for the origin of eukaryotes entail in some manner the concept of symbiogenesis (Mereschkowsky 1910; english translation in Kowallik and Martin 2021) because mitochondria trace to before the last eukaryote common ancestor LECA (Embley and Martin 2006; Tria et al. 2021) and there is no tenable way to explain the structure, DNA, and bioenergetic properties of mitochondria (and chloroplasts) without their endosymbiotic origin. The differences among current theories for eukaryote origin (reviewed in Martin et al. 2015; López-García and Moreira 2015; Dacks et al. 2016) mainly concern assumptions about the biological nature and cellular complexity of the host that acquired the mitochondrion.

In symbiogenic theories, the host is assumed to be a typical archaeon in terms of its cellular complexity, with the origin of mitochondria precipitating genetic, cell biological and bioenergetic changes within the host-symbiont consortium that ultimately led to LECA (Martin and Müller 1998; Lane and Martin 2012; Gould et al. 2016; Imachi et al. 2020). In gradualist theories, the host is assumed to be a descendant of the archaeal lineage, one that had however passed the threshold from prokaryotic to eukaryotic cell complexity by evolutionary mechanisms other than symbiosis, thereby bridging the gap between prokaryotic and eukaryotic complexity (Martijn and Ettema 2013; Spang et al. 2015) before the origin of mitochondria, which therefore had little impact on eukaryote complexity. In hybrid theories, the prokaryote to eukaryote transition involved one or more additional symbioses that preceded the origin of mitochondria, such as flagella (Sagan 1967), peroxisomes (de Duve 1969), the nucleus (López-García and Moreira 2020), or the ER (Gupta and Golding, 1996), or was precipitated by lateral gene transfer (LGT) to the host lineage, such that many hallmark traits of eukaryotes stem from genes that were invented in foreign lineages and donated to LECA via LGT (Pittis and Gabaldón 2016; Vosseberg et al. 2021) although the methods underpinning such claims have been called into question (Martin et al., 2017a; Tria et al. 2021; Nagies et al., 2020). Gradualist and hybrid theories typically posit an origin of phagotrophic feeding within the archaeal host lineage before the origin of mitochondria (Doolittle, 1998; Spang et al., 2015; Zaremba-Niedzwiedzka et al., 2017; Vosseberg et al. 2021), which is however a deeply problematic proposition from the physiological standpoint (Martin et al. 2017b) and at odds with evidence from the microfossil record indicating a late origin of phagocytosis (Mills, 2020). Eukaryotes are unquestionably genetic chimeras, with the majority of eukaryotic genes stemming from bacteria rather than archaea (Brueckner and Martin 2020), wherein the bacterial genes in eukaryotes trace to LECA, not to lineage-specific acquisitions during eukaryotic evolution (Nagies et al., 2020).

Despite their diversity and differing underlying premises, theories for eukaryote origin uniformly entail the assumption, usually implicit, that LECA was unicellular and mononucleate (Gould and Dring 1979; Cavalier-Smith 1987; Lake and Rivera 1994; Gupta and Golding 1996; Horiike et al., 2004; Imachi et al., 2020; Martijn and Ettema, 2013; Martin et al., 2015), an assumption that has almost never been called into question (Garg and Martin 2016). The uniformity of thought on the mononucleate nature of LECA is so pervasive that it is taken as a given, that is, it is rarely, if ever, even mentioned as an assumption. More tellingly, theories for eukaryote origin, if they are illustrated with a schematic diagram at all, invariably convey an image of LECA as a mononucleate cell. Such images are often symbolic in nature, depicting traits as opposed to living cells, but at the same time, they influence the way we conceptualize the problem of eukaryote origin. Models for eukaryogenesis that involve mitochondria in a mechanistic role usually entail one-to-one relationships or many-to-one relationships (Lane and Martin 2012) between mitochondria and the nucleus, whereby the nature of LECA's nuclear dynamics, heterogeneity among nuclei in LECA, its coordination of nuclear division with cell division, its cell cycle (meiotic vs. mitotic) and the evolutionary sequence linking organelle division, nuclear division, and cell division are seldom discussed (Cavalier-Smith 2010; Garg and Martin 2016).

Why is the possibility of a multinucleated state for LECA of interest? The main evolutionary benefit that a multinucleated state would confer upon LECA is evident: Gene mutations or even severe chromosome mutations, including aneuploidies that would otherwise be lethal in a mononucleated cell could be complemented by mRNA from other nuclei in the same cytosol, permitting the survival of the (multinucleated) individual as a collection of heterogeneous nuclei, a stable starting point from which the myriad differences between prokaryotic and eukaryotic chromosome segregation and handling across cell divisions could evolve (Garg and Martin 2016). In this way, the multinucleated state would buffer the transition from prokaryotic to eukaryotic chromosome division and furthermore decouple it from the evolutionary hurdle of surmounting the transition from prokaryotic to eukaryotic cell division as well as prokaryotic to eukaryotic chromatin organization during the cell cycle (Brunk and Martin 2019).

The occurrence of multinucleated taxa has been reported in members of all eukaryotic supergroups and in numerous

higher taxa, some ancient and some derived (Archibald et al. 2017; Adl et al. 2019; see supplementary table 3, Supplementary Material online). Well-known examples of multinucleated forms occur within the amoebozoan supergroup: the myxomycetes (myxogastrid amoebae), protosporangiids, dictyostelids, vampyrellids, and schizoplasmodids (fig. 1). Fungi are perhaps the most common coenocytes on Earth, wherein most of the classes and orders have multinucleated representatives, with unicellular forms being generally rare and often secondarily derived (Kiss et al. 2019). Besides fungi, within opisthokonts, nuclearid amoebae (Dirren and Posch 2016) and ichthyosporeans are also multinucleated, and syncytia are very well known among animals, for example, the body of hexactinellid sponges (Leys 2003), the muscles of all the other animals, and the larvae of holometabolous insects including *Drosophila*. Moreover, it has long been proposed that the common ancestor of Metazoa could have been multinucleated (Hadži 1953). Within Rhizaria, the deepest branch in SAR, there are numerous examples of multinucleated representatives (the most remarkable being Xenophyophorea). Furthermore, Opalinata and Apicomplexa have multinucleated forms as part of their life cycles as well (Archibald et al. 2017; Adl et al. 2019). Not only are syncytia found among heterotrophic eukaryotes but there are also numerous examples of multinucleate algae, both red (*Florideophyceae*) and green (*Ulvophyceae*), as well as various multinucleated tissues in land plants (Niklas et al. 2013). Multinucleated forms also occur among eukaryotes with secondary plastids such as in *Chlorarachniophyceae*, *Phaeophyceae* and *Xanthophyceae* (Niklas et al. 2013). The distribution and evolution of multinucleate tissues among eukaryotes with plastids reveal a great variety of form across 60 archaeplastid families and five diverse algal lineages (Niklas et al. 2013).

Some researchers distinguish between the terms syncytium and coenocyte based on the mechanism underlying the multinucleated state, with syncytia arising from cell fusions and coenocytes arising from chromosome segregation and nuclear divisions, without cytokinesis (Daubenmire 1936). Both lead to a multinucleated state and they are not mutually exclusive. We use the term multinucleated to describe the condition of having more than two (usually four or more) nuclei in the same cell without regard to the mechanism that gave rise to that state. Standard mitotic and meiotic intermediates are, obviously, not scored here as multinucleated states here, as this would trivialize the trait, making it as universal as the presence of nuclei themselves. The images in figure 1 convey an impression of a multinucleated state in the sense intended in this article.

The foregoing observations lead to the question of how far back in eukaryote evolution the syncytial state can be traced. Are multinucleated forms across all eukaryotic supergroups the result of convergence or do they reflect an ancestral state? Here, we explore the presence of multinucleated forms across the breadth of eukaryotic diversity, the likelihood of a multinucleated syncytial LECA using ancestral state reconstruction and the consequences for LECA's lifestyle.

Results

In order to capture the entire diversity of eukaryotes we generated an exhaustive list of 106 eukaryotic taxa (supplementary table 1, Supplementary Material online) including a wide array of organisms with sequenced relatives (see Materials and Methods). Among the 106 taxa chosen only 45 harbor sequenced relatives, highlighting the need for more sequencing of eukaryotic lineages. While there are recent concerted efforts to increase the diversity of sequenced genomes, they still fall short in capturing the immense phenotypic variation that sets the eukaryotes apart from the physiologically diverse prokaryotes. Nevertheless, a sufficient sample of taxa has been studied through microscopy to enable us to tabulate the presence of various eukaryotic traits from the literature, this substantial information is summarized in supplementary tables 2, 3 and 8, Supplementary Material online, including the presence of a multinucleated form in their life cycle. As mentioned in the Introduction section, the multinucleated state is usually reached by one of the two routes, namely through lack of cell division following nuclear division, leading to cells typically designated as coenocytes, and the fusion of mononucleated cells, leading to cells typically designated as syncytia. While the ontogenetic difference between the two states is distinct, in the absence of careful cell biological and cell cycle studies, which are lacking for many of the taxa examined here, it is not possible to accurately code the two form separately and hence are considered here together as being multinucleated. Note that our evolutionary investigation concerns the properties of multinucleated cells and the interactions of nuclei and mitochondria therein, irrespective of the process that generated the multinucleated state.

A cladogram for eukaryotes was generated based on extensive literature (Archibald et al. 2017; Cavalier-Smith 2018; Adl et al. 2019; Kiss et al. 2019, see supplementary tables 2 and 3, Supplementary Material online for complete list) and further refined by allowing for different configurations of polytomies and various accepted positions for the root (altogether 16), resulting in 30 different topologies for the eukaryotes (supplementary table 4, Supplementary Material online). While the tree shown in figure 2 is, among currently available alternatives, the least controversial and possibly most robust tree for those lineages of eukaryotes studied here, all three different topologies were used for ancestral state reconstructions (supplementary table 4, Supplementary Material online).

As control data sets for ancestral state reconstruction, we included several traits that are already annotated for many lineages across the eukaryotic domain. In addition to having mitochondria, the first eukaryote was sexual and had meiotic recombination (Speijer et al. 2015; Fu et al. 2019; Hofstatter

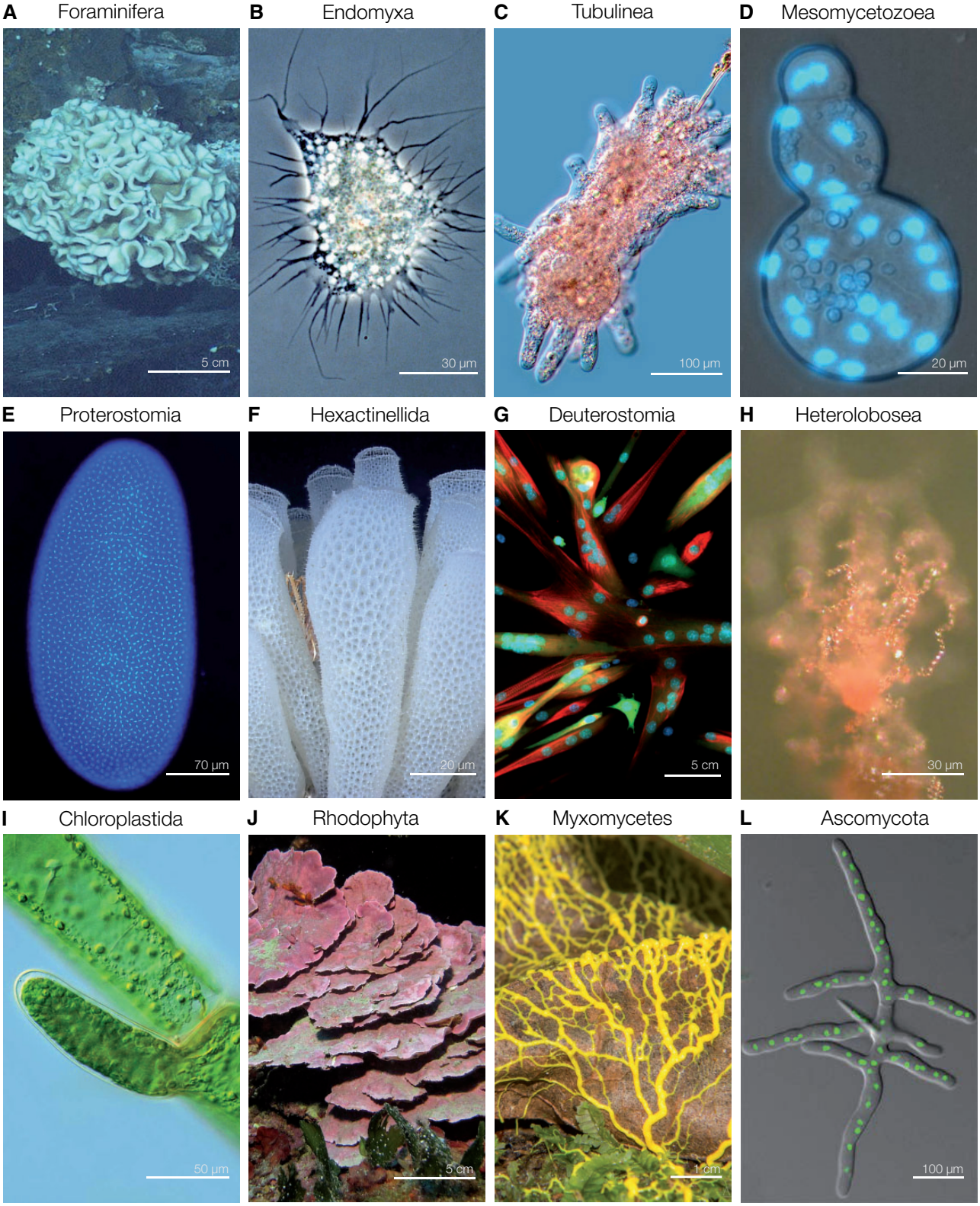

Fig. 1.—Representation of the diversity of the groups harboring multinucleated representatives. (A) Foraminifera: Filosa, a deep sea coenocytic xenophyophore; (B) Endomyxa: *Lateromyxa gallica*, multinucleated predatory amoeba; (C) Tubulinea: *Chaos* sp. multinucleated amoeba; (D) Mesomycetozoea: *Sphaeroforma arctica*, coenocyte with blue nuclei; (E) Protostomia: *Drosophila melanogaster*, multinucleated embryo; (F) Hexactinellida: *Euplectella aspergillum* coenocytic hexactinellid sponge; (G) Deuterostomia: multinucleated mouse muscle cells; (H) Heterolobosea: *Acrasis*

and Lahr 2019). It is known that hydrogenosomes and mitosomes arose from mitochondria via respiratory chain loss and ecological specialization in several independent lineages (Embley and Martin 2006; Müller et al. 2012; Maciszewski and Karnkowska 2019; Gould et al. 2019), that primary plastids arose once (Sánchez-Baracaldo et al. 2017) and that secondary plastids arose several times independently from eukaryotes containing a primary plastid (Maciszewski and Karnkowska 2019; Keeling 2004; Gould et al. 2008). Ancestral state reconstruction should map these traits accordingly.

A general outline of the relationship of prokaryotes to eukaryotes including symbiosis and depicting the number of described species in each group is given in figure 2. Using ancestral state reconstruction, we found that the last eukaryotic common ancestor (LECA) was a sexual, mitochondriate, and heterotrophic organism with closed nuclear division (mitosis) and likely harboring haploid nuclei (figure 3). The method and tree trace sexual reproduction and mitochondria back to the origin of eukaryotic complexity, in agreement with hitherto published studies (Speijer et al. 2015; Hofstatter and Lahr 2019). Lineages with hydrogenosomes, mitosomes and typical mitochondria (fig. 3*A*) represent ecological specializations from a common ancestral organelle (Müller et al. 2012). Consistent with previous reports, sex is recovered as being ubiquitous in the Eukarya domain and meiotic genes are present in all the supergroups in highly conserved manner (Ramesh et al. 2005; Speijer et al. 2015; Hofstatter and Lahr 2019).

The last common ancestor of archaeplastids was the first organism to have a primary plastid and is the first common ancestor of all the (secondary) plastids found in *Euglenida*, *Hacrobia*, and SAR. Despite being widely distributed across the eukaryotic tree, plastids did not trace to LECA with ancestral reconstruction, which serves as a form of internal control (fig. 3*C*). Since LECA did not have a plastid, it could not have been a photosynthetic, autotrophic eukaryote—it was a heterotroph. Primary plastids originated from a cyanobacterium in a symbiogenic event, which likely also involved a freshwater archaeplastid ancestor that was multinucleate in at least part of its life cycle (Sánchez-Baracaldo et al. 2017) (fig. 3*B*). Polyploidy ($>2n$) also originated several times independently. Though polyploid eukaryotes originated numerous times in evolutionarily well-separated groups, LECA most probably had haploid nuclei (fig. 3*A*). Polyploid trophobionts (feeding stages) are rare among eukaryotes. The only polyploid phases in most eukaryotes are the diploid zygote (especially its tetraploid phase before the first division) which ancestrally undergoes meiotic recombination, before the production of four different nuclei (trophic cells, spores, or gametes). In those lineages whose trophobionts are diploid, every somatic nucleus that has DNA replicated before segregation of chromosomes can be regarded as temporarily tetraploid although, as with zygote formation (see Materials and Methods), they were not scored as polyploid. Accordingly, LECA was not polyploid, but because it was meiotic, it harbored some form of karyogamic stage.

While the control traits were reconstructed as expected, the same analysis indicates that LECA was multinucleated and/or had a multinucleated stage during its life-cycle (fig. 3*A* and *B*). It was not a mononucleated protist-like flagellate eukaryote of the type salient to most theories, although it cannot be excluded that some phases of the life cycle might have been mononucleated, protist-like, and flagellated, for example, motile spores. The ancestral reconstruction indicates that the state of LECA might have been multinucleate with nuclei divided by closed mitosis, in which the nuclear envelope remained intact (fig. 3*A*). The ancestral presence of closed mitosis (closed chromosome segregation, not cytokinesis) is significant since chromosome segregation in syncytial forms demands an intact nuclear membrane consistent with an ancestral multinucleated stage. In our analyses, the probability that LECA was multinucleated is as high as the probability that it was sexual and possessed mitochondria (supplementary table 7, Supplementary Material online). For the full detailed results of ancestral character state reconstruction see supplementary table 6, Supplementary Material online.

No matter where we rooted the eukaryotic tree, nor how many unresolved branches we allowed, LECA was always reconstructed as multinucleate. Moreover, and crucially, not only was the ancestor of eukaryotes multinucleated, but the common ancestors of all eukaryote supergroups were also reconstructed as multinucleate as well, except the last common ancestor of Hacrobia (fig. 3*B*). Whether the Hacrobia ancestor was mononucleated or whether the information is missing that lead to inference of a mononucleated Hacrobia ancestor, while all other supergroup ancestors are reconstructed as multinucleated is unresolved. The ancestral reconstruction depicts LECA as multinucleated, a polykaryon whether syncytial or coenocytic, a population of interacting mitochondria and nuclei within the confines of a single cell membrane. Multiple nuclei in the same cytoplasm are not rare

rosea, fruiting body; (I) Chloroplastida: Ulvophyceae: *Cladophora* sp. syphonous thallus; (J)—Rhodophyta: Florideophyceae: *Lithophyllum* sp.; (K)—Myxomycetes: Multinucleated plasmodium of a *Physaraceae* member; (L) Ascomycota: *Eremothecium gossypii*, aseptate hyphae. Photo credits and Creative Commons (CC) sharing domain: A and F. NOAA, public domain; B. Norbert Hülsmann, BY-NC-SA 2.0; C. and I. Proyecto Agua, BY-NC-SA 2.0; D. Multicellgenome lab, BY 2.0; E. Billy Liar, BY-NC-SA 2.0; G. Kevin A. Murach, NIH Image Gallery, BY-CN 2.0; H. Shirley Chio, Biology of Fungi Lab, UC Berkeley, California, BY-SA 3.0; J. Christophe Quintin, BY-NC 2.0; K. André Amaral, distributed under CC BY-NC 4.0; L. Jaspersen Lab, public domain. Scale bar is approximate.

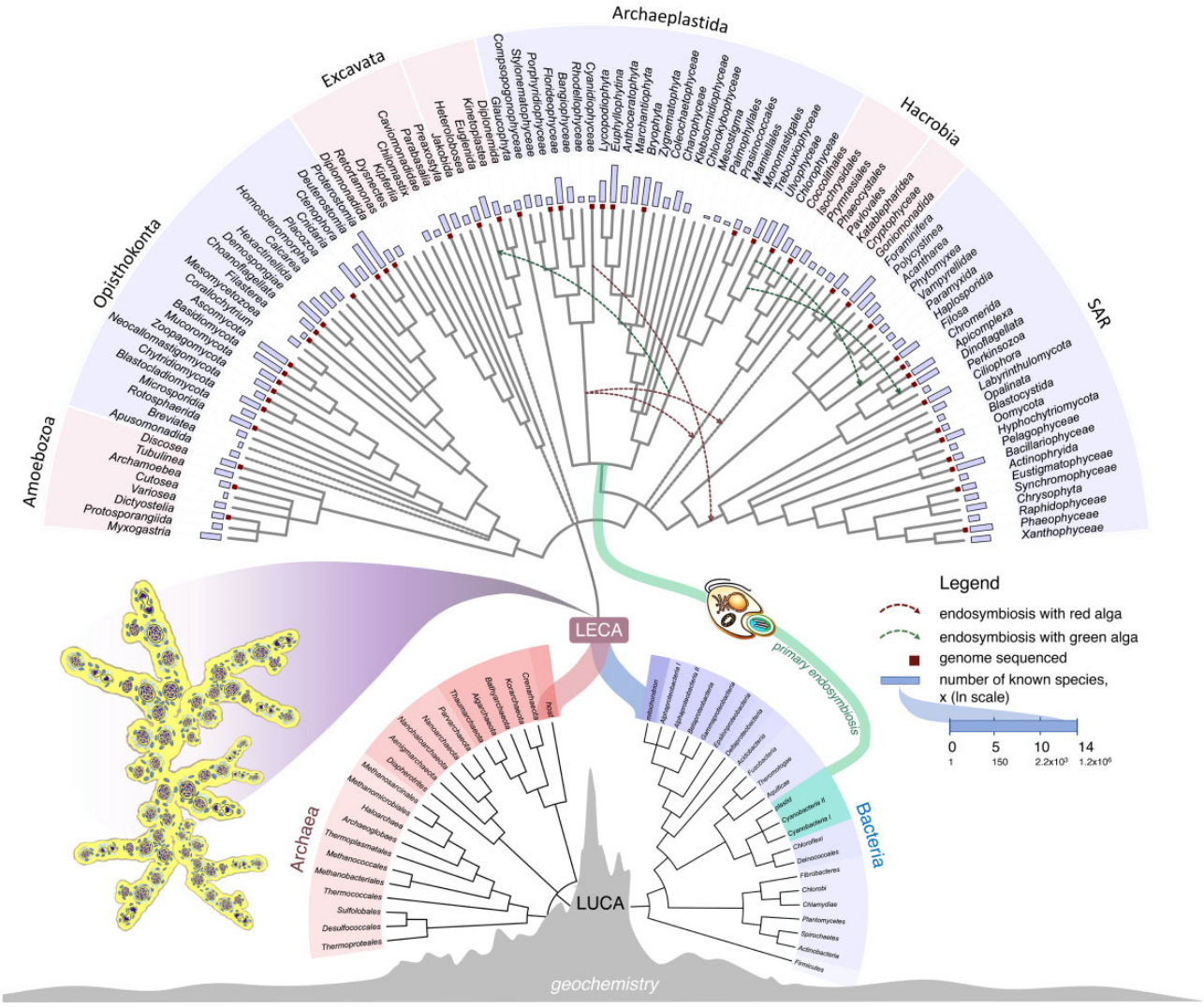

Fig. 2.—Schematic summary of cell evolution. The tree is rooted in physiology and geochemistry, with a nonfree-living Last Universal Common Ancestor (LUCA). Origin of Eukaryotes is depicted as a polyphyletic (symbiogenic) event, where two prokaryotic lineages, an archaeal lineage and an alphaproteobacterial lineage, gave rise to the eukaryotic lineage via LECA—the Last Eukaryotic Common Ancestor. Major prokaryotic groups (within archaea and bacteria) and eukaryotic supergroups are shown, altogether 106 taxa are included in this analysis. Comparison of the number of known species is shown in a logarithmic scale. Squares at the tip of certain branches denote in which groups genomes are sequenced. For reference tree of eukaryotes, see Materials and Methods section. Schematic prokaryotic tree of life was constructed based on literature. The tree was drawn using iTol. LUCA is depicted as arising at a hydrothermal vent, while LECA, which might also have arisen near hydrothermal vents as a geological source of H_2 (15, 23) is depicted as a multinucleate organism in which nuclei divide with their envelopes remaining intact. Primary endosymbiosis with cyanobacteria that gave rise to Archaeplastida is shown. Secondary endosymbiotic events, the multiple origins of secondary plastid, are shown as arrows. A 6-min animated video illustrating the origin of eukaryotes from symbiosis and the role of a syncytial state in the life cycle of LECA can be viewed at (https://www.youtube.com/watch?v=mmh_IpdgWvw&t=2s).

phenomena among eukaryotes. Syncytia and coenocytes are found across most of the higher eukaryotic groups (supplementary table 2, Supplementary Material online). Free-floating nuclei in the cytosol as opposed to being tethered to cell walls imply that in a syncytium they can only divide if nuclear division—and consequently chromosome segregation—is closed wherein the nuclear membrane remains intact throughout mitosis. Open mitosis in a coenocyte would potentially result in the spindle apparatus attaching to chromosomes from different nuclei and segregating them in an aberrant and likely lethal manner. The reconstruction (fig. 3*B*; table 1) suggests that open nuclear division (dissolution of the nuclear membrane at mitosis, as is well known in vertebrates) originated from closed mitosis via semi-open division, in which parts of the nuclear envelope dissolve, as the intermediate state (Boettcher and Barral 2013). Open nuclear division is typical for some mononucleate (both unicellular and multicellular), and most land-inhabiting eukaryotes (fig. 3*A*).

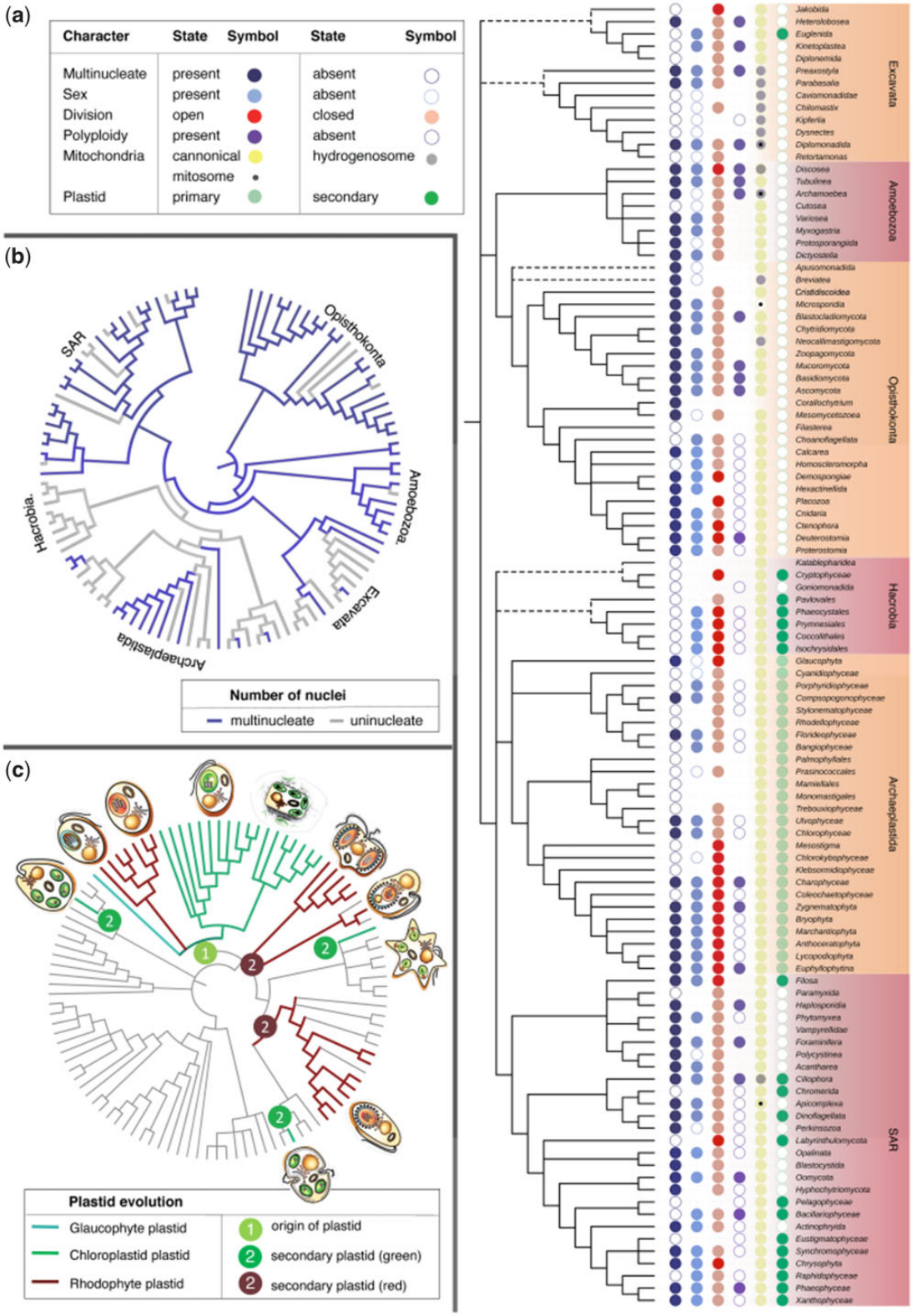

Fig. 3.—Overview of eukaryotic diversity with ancestral state reconstruction. (A) Traits are annotated on the reference eukaryote tree (1. presence of the multinucleated state, 2. sexual reproduction, 3. type of nuclear division, 4. polyploidy, 5. type of mitochondria and 6. type of plastids). (B) Ancestral state reconstruction of the multinucleate state is shown, as well as (C). the plastid evolution Representatives of main photosynthetic eukaryotes are depicted schematically. The data indicate that LECA was a multinucleate sexual heterotroph with closed mitosis.

Table 1
Summary of the results of the ancestral character state reconstruction by maximum parsimony, across 30 different topologies

	% Trees		
	Absent	Present	Ambiguous
Syncytium	0	0.8	0.2
Sex	0	0.867	0.133
Polyploidy	1	0	0
Closed division	0	1	0
Mitochondria	0	0.867	0.133
Plastid	1	0	0

Discussion

The origin of eukaryotes was a unique event from which all the complex life stems. The symbiosis that gave rise to eukaryotes occurred over 1.5 billion years ago (Knoll et al. 2006). While eukaryote origin cannot be forced to occur in the laboratory, endosymbiosis can (Mehta et al. 2018). The contours of eukaryogenesis, intermediate stages, and the sequence of events involved can be addressed via inference from the comparative investigation of modern lineages. The first eukaryote was the result of interactions between archaea and bacteria, two highly divergent cell lineages, that gave rise via interaction and cooperation to a new kind of organism, LECA, with new properties, novel bioenergetics, chimeric chromosomes, a cell cycle, novel genetics, reciprocal recombination, and cellular complexity. Descendants of these symbiotic partners are preserved as bacterial ribosomes in mitochondria and archaeal ribosomes in the eukaryotic cytosol.

LECA had sexual reproduction that included the fusion of haploid nuclei selected for the reproduction (gametes) and the recombination of their genetic material (meiosis). Mitochondria, sex, and multiple nuclei are signatures of LECA's state, with synergistic interactions. Unlike mitochondria, the nucleus has a large, complex genome with little size constraint. The genetic compatibility of nuclei and mitochondria inhabiting the same cytoplasm is crucial for the survival of eukaryotic cells. Internal competition or cytonuclear incompatibility can be lethal (Blackstone and Green 1999; Pesole et al. 2012; Rand and Mossman 2020) or render the organism dysfunctional. Inheritance of mitochondria is often uniparental. The inheritance of the nuclear genome is, however, bi-, tri-, or multi-parental. Uniparental inheritance of mitochondria indicates the existence of strict control on compatibility. Meiotic recombination, ancestrally during the zygote phase, is a compatibility checkpoint. At the onset of eukaryote evolution, the compatibility of mitochondria with newly arisen nuclei was essential. In mononucleate cells, only compatible combinations survived natural selection. In syncytia, many-to-many interactions among mitochondria and nuclei buffered compatibility within the environmental confines of a single cytoplasm. Spores spawned from a syncytial LECA presented a powerful bottleneck of selection for cytonuclear compatibility (Garg and Martin 2016).

An intriguing aspect of the multinucleated state for LECA concerns the transition from prokaryotic to eukaryotic chromosome segregation. In prokaryotes, chromosome segregation is linked to cell division via chromosome attachment to the cell wall. In eukaryotes, microtubule-dependent segregation of condensed chromosomes and cell division (cytokinesis) are neither physically nor mechanistically linked, though often temporally apposed. That is, chromosomes can, and often do, replicate and segregate in nondividing cells without the formation of spindles for the division of the nucleus itself (Geitler 1953), processes that were termed *Amitose* in the older literature (Strasburger 1908). If the origin of nuclear division (replication followed by segregation) preceded the origin of cell division at eukaryote origin (the converse could hardly be true), the resulting syncytium need not have possessed well-regulated chromosome segregation at the outset. It could have generated nuclei with aberrant chromosome numbers or aneuploid haploids. Such defective nuclei would be lethal for a mononucleate cell, but not in a syncytium, because even highly defective nuclei could complement each other freely via mRNA in the cytosol. The multinucleate state would thus buffer virtually all deleterious effects of nuclei arising as products of incorrect chromosome partitioning during a closed protomitosis at the origin of eukaryote chromosome segregation. This would have kept the syncytium as a unit of vegetative proliferation alive, while harboring nuclei with very different chromosome sets, nuclei that kept each other viable within the syncytium through complementation via mRNA in the cytosol. This involvement of ribosomes, whose synthesis requires massive rRNA gene expression, for complementation would explain why the nucleus: cytoplasm volume ratio (*Kern-Plasmarelation*) tends to approach a roughly constant value (Klieneberger 1917) of 1:10 even in syncytial cells (Sitte et al. 1991). As Strasburger (1908) put it: "In the Characeae, amitotic nuclear division in internodial cells is not a degenerate process, rather it is a means to amplify certain components of nuclear substance in relationship to the increase of cytoplasmic mass" (p. 40, translation by the authors).

Physical fusion of nuclei, a primitive and unregulated forerunner of karyogamy (present in LECA because LECA had sex), would generate new combinations of chromosomes at the same time as genes were being transferred from mitochondria to the nuclei (Lane and Martin 2012; Garg and Martin 2016). That generated a heterogeneous population of nuclei interreacting with a heterogeneous population of mitochondria, within the same syncytium. A syncytium could also become physically severed, generating segments or fragments that, provided means of sealing off ends, could have generated descendant progeny (as diaspores) without the requirement for regulated cell division. Syncytial fragments provided a mechanism for propagating populations of nuclei and mitochondria. But the main evolutionary hurdle to be crossed

was evolution of regulated, symmetric chromosome segregation that took into account the nutritional state of the cell (Brunk and Martin 2019) en route to a cell cycle—the backbone of eukaryotic cell biology.

Within a syncytium, both nuclei and mitochondria were units of selection and units of evolution. They were the intermediate state in the prokaryote to eukaryote transition. They coexisted within the same cytosol. Nuclei became heritable collections of genes able to influence their immediately surrounding cytosol, and able to interact with each other and with mitochondria via exported mRNA. Multinucleated cells are ubiquitous among the eukaryotes, both living (figs. 1 and 3A) and fossil, such as a recently reported 1-billion-year-old coenocytic green alga (Tang et al. 2020).

Conflict and Co-operation in a Syncytial LECA

Mitonuclear compatibility is important and is proportional to cell fitness (Rand and Mossman 2020). To compare the relative fitness of a mononucleated cell (monokaryon) and a multinucleated cell (polykaryon), one can consider the difference between the probability of survival for a population of unicellular mononucleate eukaryotes versus that for a single syncytium. For monokaryons, the probability of survival of the population is dependent on the individual survival probabilities which in turn depend on the fitness of the respective mitonuclear pair. However, in the case of a syncytium since the mitochondria and nuclei coexist in one cell the survival probability depends on the cumulative fitness of all possible combinations of mitonuclear pairs. This in turn allows the syncytium to behave similar to a population while allowing selection to resolve internal mito-nuclear conflicts independently. This is schematically shown in figure 4 and mathematically described in supplementary information 11, Supplementary Material online. A syncytium behaves as more like a population of nuclei and mitochondria than as an individual cell. Thus, the syncytium has a higher chance of survival than a population of monokaryons. Of course, there are ancient lineages of eukaryotes harboring mononucleate forms, including the excavates. However, a multinucleated LECA explains why modern eukaryote diversity is more readily derived from a syncytial ancestor than from a population of mononucleate unicellular ancestors (monokaryons). A population of monokaryons, especially that of haploid monokaryons, is not likely to accumulate genetic diversity. A syncytium on the other hand, easily accumulates genetic diversity within one cytosol, as nuclei with advantageous alleles complement deficiencies of other nuclei, and karyogamy, of which a meiotic LECA was capable, within a syncytium can generate novel chromosome combinations (fig. 4).

Evolutionary transitions in individuality involve cooperation and conflict (Buss 1987; Maynard Smith and Szathmáry 1996; Michod 1999). Without mechanisms for conflict mediation, cooperation cannot survive (Nowak 2006) and the higher-level unit cannot emerge (Radzvilavicius and Blackstone 2018). In evolution, the population structure has always been recognized as one of the most general mechanisms favoring cooperation. Even if selection favors non-cooperating defectors, as is typically the case, cooperation might still evolve in a structured population. Consider a population made up of individuals (the lower level) divided into groups (the higher level). While defectors are favored at the lower level, cooperators are favored at the higher. If a population was one large group, the selection at the higher level is weak, and defectors prevail. In a population with many small groups, however, the selection is potentiated at the higher. Groups of cooperators can form by chance and outcompete groups of defectors (Szathmáry and Demeter 1987). Thus, larger groups (e.g., a syncytial LECA) invite more conflict, while smaller groups (particularly sexually produced gametes) entail less. With larger cell sizes, stochastic processes may hence have been less important in mediating evolutionary conflict.

Origins of Flagellated Eukaryotes

In comparison to prokaryotes, the eukaryotic cell cycle is as unique as the processes behind mitosis and the physical separation of the newly emerging cell (cytokinesis). While a few homologous proteins are shared between archaeal binary fission and eukaryotic cytokinesis (Lindås et al. 2008), the mechanism of chromosome segregation through a centrosome-organized microtubular system and the subsequent actin-based cell constriction is not conserved across the prokaryote–eukaryote divide. The mechanism of eukaryotic chromosome segregation, like other eukaryote-specific traits, evolved de novo during the endosymbiotic integration of a bacterial partner within an archaeal cytosol en route to LECA. In a syncytium, chromosome segregation likely involves molecular selforganization of a chromosome separating machinery that requires no anchoring points at the plasma membrane.

Closed mitosis, in which the nuclear envelope remains largely intact, is considered ancestral to open mitosis (Cavalier-Smith 2010), consistent with our own results (fig. 3). All variants of mitosis share a microtubule-based network, which can be bundled or loose in a star-like manner, that reach out for the chromosomes and attach at the kinetochore which was present in LECA (Tromer et al. 2019). Centrosomes are however, not essential for chromosome separation (Heald et al. 1996). Crucially, eukaryotic chromosomes are separated largely by pushing forces along microtubules, in which the eukaryote-specific kinesin family of proteins play an essential role (Shimamoto et al. 2015). These mechanisms fit seamlessly with the biology of a syncytial cell, as mitosis of individual nuclei can occur independently of localized plasma membrane fixation points.

Consequently, the origin of mononucleated, flagellated protists can be viewed from a novel perspective. Images of the closest living relative of the archaeal host cell and a

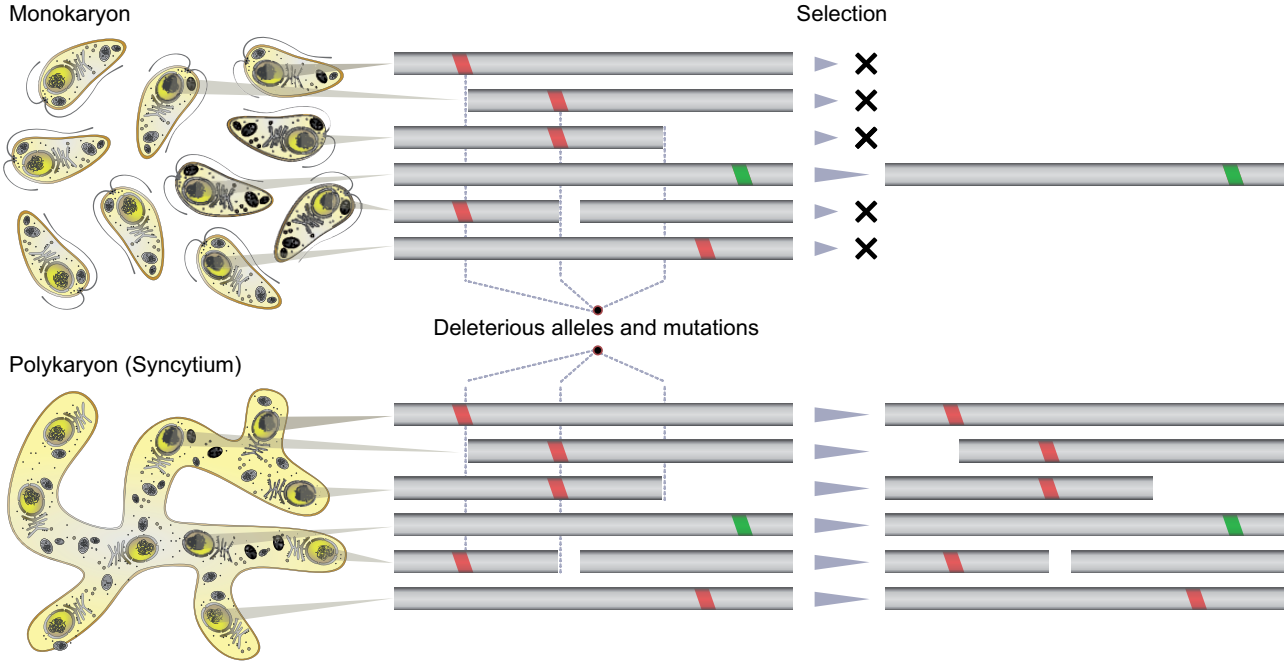

Fig. 4.—Syncytia buffer chromosome defects, unlike monokaryons. Schematic representation of a population of unicellular protists and a syncytial cell. Genomes of each nucleus are schematically shown as grey lines, deficient alleles as red rectangles whereas the beneficial allele is shown in green. If the same evolutionary constraints are applied, monokaryons' population is more likely to go extinct than a syncytium, as nuclei from different cells can neither cover each other's defects nor buffer mitonuclear incompatibilities, while in syncytium they can.

bacterial partner depict two sessile, nonmotile partners (Imachi et al. 2020), the syncytial LECA we propose was sessile, too. The microtubule organizing center (MTOC), or basal body, and the ability to form flagella was present in LECA. This trait diversified among eukaryotic supergroups and underwent recurrent loss (Yubuki and Leander 2013). Eukaryotic flagella are directly connected to basal bodies, or they form in a centriole-dependent manner de novo (Schrøder et al. 2011). The flagella pore complex shares a number of proteins with the nuclear pore complex (Dishinger et al. 2010; Kee et al. 2012; Gould et al. 2016). We suggest that the flagellum evolved on the basis of a (duplicated) centrosome-derived structure that subtended a region of the plasma membrane. Mononucleate, flagellated spores could have thus emerged from the syncytium with the actin cytoskeleton supporting final scission (Heidstra 2007).

Only spores containing viable mitonuclear interactions and capable of flagellar motion would have had the properties of motile gametes, provided that they were able to fuse with others of their kind, which is possible given the tendency of archaea themselves to fuse (Lange et al. 2011; Garg and Martin 2016; Shalev et al. 2017). Such spores would present motile units of selection. The nucleus of many flagellated protists is located in close proximity to the basal body, if not connected to it, as in numerous Archamoebea, Chytridiomycota, *Olpidium*, Pelagophyceae, Bacillariophyceae, Rhizaria and others (reviewed in ref. 2). It is possible that such gamete like cells became the founders of eukaryotic supergroups, all of which contain flagellated representatives that can generate syncytia (fig. 3*A*). We have no suggestion for the physical size of LECA as a syncytium, although we do suggest that it was a marine sediment dweller (Martin and Müller 1998), where anaerobic syntrophy is essential to symbiotic interactions (Imachi et al. 2020). The hyphae of modern fungal individuals can cover areas of square miles (Anderson et al. 2018). LECA could have been a large non-dividing multinucleate unicell that spawned supergroups through the extrusion of mitochondriate flagellated spores. A 6-min animated video illustrating the origin of eukaryotes from symbiosis and the role of a syncytial state in the life cycle of LECA can be viewed at (https://www.youtube.com/watch?v=mmh_IpdgWvw&t=2s)

Conclusion

Unlike prokaryotes, eukaryotes have complex systems of intracellular membrane flux and possess organelles. They are in terms of morphology the most diverse domain of life and originated via the origin of mitochondria. Eukaryote origin is usually depicted as a narrative of *two-cells-becoming-one*, a *one-on-one-model*, where an archaeon host engulfed a proteobacterial symbiont, with the units of selection being chimeric, mononucleate, free-living cells. Our results however suggest that at eukaryote origin, nuclei, and mitochondria were the units of selection and the units of evolution within

the confines of a syncytial LECA. Ancestral character state reconstruction based on taxon rich sampling spanning all supergroups suggest that LECA was 1) mitochondriate, 2) multinucleate (syncytial, coenocytic), 3) haploid, 4) with closed nuclear division, and 5) with sexual reproduction. It is often stated, also in many papers by the present authors, that the prokaryote to eukaryote transition left no intermediate forms. However, if our current thoughts are roughly on target, syncytia are in fact the intermediate state in the prokaryote to eukaryote transition, though hitherto unrecognized as such. In that light, the syncytia present throughout all eukaryote supergroups may harbor previously unrecognized forms of evidence about eukaryote origin and the prokaryote to eukaryote transition.

Materials and Methods

Selection of taxa

Based on an inspection of the literature (supplementary table 4, Supplementary Material online), a taxon-rich (Katz and Grant 2014) eukaryotic dataset comprising 106 higher taxa was constructed (supplementary table 1, Supplementary Material online). Representatives of six eukaryote supergroups are included. We employ the nomenclature of eukaryote supergroups as recently defined: *Amoebozoa, Archaeplastida, Excavata, Hacrobia, Opisthokonta*, and *SAR*, although for clarity, we have retained the more familiar term Opisthokonta instead of Obazoa here. The set consists mostly of higher categories, but in some cases, families and genera were included (table 2).

Table 2
Overview of eukaryote taxa considered in this analysis

	Phyla	Classes	Orders	Families	Genera	Per Supergroup
Amoebozoa	—	7	1	—	—	8
Archaeplastida	7	14	4	—	1	26
Excavata	1	4	3	1	4	13
Hacrobia	—	3	5	—	—	8
Opisthokonta	13	9	1	—	1	24
SAR	6	17	3	1	—	27
per rank	27	54	17	2	6	

Number of phyla, classes, orders, families, and genera are shown, corresponding to each supergroup (*per* supergroup).

Reference tree construction

Eukarya includes six supergroups—*Archaeplastida*, *Amoebozoa*, *Excavata*, *Hacrobia*, *Opisthokonta*, and *SAR*. Cladograms represented in this study are based on published relationships within each eukaryote supergroup (supplementary tables 3 and 4, Supplementary Material online). If we designate relationships as "resolved" it means that we incorporated the corresponding branching pattern for our 106-taxa tree. Branching patterns that were "unresolved" were translated to polytomies.

Tree topology and root

With or without a resolved species tree, the root branch is always informative and the output of phylogenetic analysis can vary depending on the position of the root (Tria et al. 2021). Within the supergroups, we deal mostly with resolved "species trees" (see Reference tree). However, relationships between the supergroups are not completely resolved, so we employed two models in ancestral state reconstruction—one that allows polytomy (unresolved branches), and the other which allows only dichotomies. Because there is no consensus on where the eukaryote root Eukarya lies, a set of reference trees was prepared with a collection of published proposals for the eukaryote root: *Excavata* or within excavates (Cavalier-Smith 2002; He et al. 2014; Tria et al. 2021), *Opisthokonta*, *Fungi* or within *Fungi* (e.g., *Microsporidia*) (Vossbrinck et al. 1987), *Amoebozoa* or within (Stechmann and Cavalier-Smith 2002; Katz and Grant 2015), *Amorphaea* (*Opisthokonta*+*Amoebozoa*) (Derelle et al. 2015). An unrooted set was also prepared. Detailed data underlying all parameters and trees are presented in supplementary table 3, Supplementary Material online.

Annotation of traits

To address LECA's traits, a data set comprising six characters was assembled: (I) the multinucleate state, (II) sexual, meiotic reproduction, (III) behavior of the nuclear envelope during division, (IV) polyploidy (>2*n*), (V) type of mitochondria, and (VI) presence and type of plastid. All the traits were numerically coded for ancestral state reconstruction (supplementary table 5, Supplementary Material online). For the multinucleated state, the trait was coded as 1 when there was an indication of the multinucleated state present in the whole group or part of the lifecycle of many (>2 genera) members, or if it was present within unresolved groups. The trait was considered ambiguous (0/1) for a group when either there is either a consensus that multinucleate state is present in a single derived species within the group, or when there is evidence for the presence of life cycle stages that closely resemble syncytia-like structures without a clear description in members of a group. Finally, the trait was coded as 0 when there is no indication at all that a multinucleate state exists within the known diversity of a certain taxon. The sources for the information are summarized in the supplementary tables 2 and 4, Supplementary Material online.

Ancestral character state reconstruction

Analyses of the ancestral state reconstruction were performed on the basis of the numerically coded character matrix

(supplementary table 5, Supplementary Material online) using maximum parsimony (ordered and unordered) (supplementary table 6, Supplementary Material online) as implemented in Mesquite 3.6 software (https://www.mesquiteproject.org/). Altogether 106 eukaryote taxa (from genus to phylum level) were selected (see Selection of taxa, supplementary table 1, Supplementary Material online). A set of 30 reference trees was then constructed, with different positions of root and different freedom towards polyphyly (see Setting tree topology and root, supplementary table 3, Supplementary Material online). The character matrix was prepared from literature data for six traits: the presence of multinucleated state, presence of polyploids, presence of sex/meiosis, behavior of nuclear envelope during division, type of mitochondria, and type of plastid (supplementary tables 2 and 5, Supplementary Material online). In some groups, certain members of the group exhibit one trait, while others exhibit the other. In cases like this, both traits were coded for that group (0/1 or 1/2).

Acknowledgments

This research was supported and funded by the Moore–Simons Project on the Origin of the Eukaryotic Cell GBMF9743 to W.F.M. and S.G.G. W.F.M. also would like to thank the ERC (666053) and the VW Foundation (93046 and 96742) for funding. S.B.G would like to thank VolkswagenStiftung (Life) for funding. We thank Prof. Klaus V. Kowallik for helpful discussions.

Author Contributions

All authors read the manuscript contributed to the final version of the manuscript. There are no conflicts of interests between the co-authors.

Data availability

All data associated with the manuscript is provided in the supplementary information.

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Associate editor: Martin Embley Dr.