The Ancestral Mitotic State: Closed Orthomitosis With Intranuclear Spindles in the Syncytial Last Eukaryotic Common Ancestor

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Abstract

All eukaryotes have linear chromosomes that are distributed to daughter nuclei during mitotic division, but the ancestral state of nuclear division in the last eukaryotic common ancestor (LECA) is so far unresolved. To address this issue, we have employed ancestral state reconstructions for mitotic states that can be found across the eukaryotic tree concerning the intactness of the nuclear envelope during mitosis (open or closed), the position of spindles (intranuclear or extranuclear), and the symmetry of spindles being either axial (orthomitosis) or bilateral (pleuromitosis). The data indicate that the LECA possessed closed orthomitosis with intranuclear spindles. Our reconstruction is compatible with recent findings indicating a syncytial state of the LECA, because it decouples three main processes: chromosome division, chromosome partitioning, and cell division (cytokinesis). The possession of closed mitosis using intranuclear spindles adds to the number of cellular traits that can now be attributed to LECA, providing insights into the lifestyle of this otherwise elusive biological entity at the origin of eukaryotic cells. Closed mitosis in a syncytial eukaryotic common ancestor would buffer mutations arising at the origin of mitotic division by allowing nuclei with viable chromosome sets to complement defective nuclei via mRNA in the cytosol.

Key words: last eukaryotic common ancestor, ancestral state reconstruction, mitosis, syncytium, eukaryogenesis.

Significance

Knowledge about the ancestral state of mitosis (nucleus, chromosome, and cell division) in eukaryotes would shed light on the biology of the last eukaryotic common ancestor (LECA). To address that question, we used methods of ancestral state reconstruction to ascertain the type of mitosis present in the LECA. We found that LECA did not disintegrate its nuclear membrane at chromosome division, but instead kept the nuclear membrane intact so that it divided by a process similar to constriction. The chromosomes were pushed apart by microtubules that formed within the mother nucleus. The data indicate that nuclear division took place without cell division in LECA, giving its cells a filamentous multinucleated state. This reconstructed state sheds light on an important aspect of the prokaryote to eukaryote transition.

Introduction

The origin of eukaryotes is a classical topic of debate. There was a time, not too long ago, when the prospect was discussed that prokaryotes arose from eukaryotes (Forterre and Phillippe 1999; Poole et al. 1999). Today it is now generally agreed that eukaryotes arose from prokaryotes, that the endosymbiotic event that led to mitochondria played a role in their origin, that eukaryotes and mitochondria share a single common origin, and that eukaryotes therefore share a last eukaryotic common ancestor (LECA). Based upon the universal distribution of the traits among major eukaryotic groups, it is furthermore agreed that LECA possessed, in addition to mitochondria (Lane and Martin

2010), a nucleus (Mans et al. 2004; Bapteste et al. 2005; Neumann et al. 2010), an endoplasmic reticulum (Kontou et al. 2022), linear chromosomes with centromeres (Ishikawa and Naito 1999; van Hooff et al. 2017), flagellae (Carvalho-Santos et al. 2011; Lindemann 2022), microtubule organizing centers (Yubuki and Leander 2013), nucleoli (Gardner et al. 2010; Hoeppner and Poole 2012), meiosis, and sex (Villeneuve and Hillers 2001; Loidl 2016). Those traits are easily traced to LECA because they are present in all eukaryotes. Yet eukaryotes exhibit almost boundless cytological and morphological diversity, leaving the biological nature of the LECA far less clearly resolved than one might tend to think (Katz 2012; Koumandou et al. 2013; Booth and Doolittle 2015; López-García and Moreira 2015; Porter 2020; Gabaldón 2021; Roger et al. 2021; Mills et al. 2022).

The traditional method of inferring information about the nature of any last common ancestor is to construct an evolutionary tree for the group and assign a root, in hope that traits that are variable across the tree might map to the rooted tree in such a manner as to reveal the state of the trait at the root, ideally, without conflicting data (Jermann et al. 1995; Kohn et al. 1996; Gold et al. 2015; Klim et al. 2018). The traditional approach is very difficult for eukaryotes, however, because there is little agreement among experts (Williams 2014; Keeling and Burki 2019; Burki et al. 2020) and little agreement across molecular data sets (Stechmann and Cavalier-Smith 2003; Richards and Cavalier-Smith 2005; Kim et al. 2006; Rodríguez-Ezpeleta et al. 2007; Cavalier-Smith 2009; Roger and Simpson 2009; Rogozin et al. 2009; Derelle and Lang 2012; Katz et al. 2012; He et al. 2014; Cerón-Romero et al. 2022) as to the position of the root in the eukaryotic tree. Two main causes are discussed for the differing pictures concerning the position of the eukaryotic root: (1) the time between the divergence of major eukaryotic supergroups may have been very short in the sense of a "radiation" rendering resolution difficult (Philippe et al. 2000; Eme et al. 2014), and (2) hundreds of gene duplications that took place in the genome that led to LECA, generating vast amounts of hidden paralogy in gene trees hence discordant placements of roots (Tria et al. 2021), or both.

For traits that are universal among eukaryotes, reconstruction to LECA is trivial. For traits that are not universal, reconstruction of the trait in LECA requires more work. One example is phagocytosis, the ability to eat and digest other cells as food. Many lineages of eukaryotes possess phagocytosis but many do not; reconstruction of the trait indicates that LECA was not phagocytotic (Bremer et al. 2022). Many lineages of eukaryotes possess multinucleated states that are distinct from those generated during meiosis, whereas many eukaryotes lack such multinucleated (syncytial) states; reconstruction of the trait indicates that LECA had a syncytial (multinucleated) habit (Skejo et al. 2021; Bremer et al. 2022). An ancestrally multinucleated state for LECA bears upon the nature of mitosis in LECA because there exist a variety of mitotic types in eukaryotes which differ in their compatibility with the syncytial habit. In the present study, we are interested in reconstructing the ancestral state of mitosis in LECA.

Though LECA possessed the molecular machinery required for mitotic chromosome division (Tromer et al. 2019), there is also no doubt that LECA possessed meiotic sex (Speijer et al. 2015), leaving open the question of whether mitosis preceded meiosis on the path to LECA or vice versa (Garg and Martin 2016). The state of mitosis in LECA is the focus of our present study. Mitotic types across the eukaryotic tree are diverse. The greatest differences are the state of the nuclear envelope and the position and symmetry of the spindles (fig. 1). Different combinations of those traits can be found across the eukaryotic tree. The nuclear envelope can either remain intact during mitosis (closed mitosis) or it can be partly or completely dispersed (semi-open or open mitosis). When the nuclear envelope remains intact, the position of the central spindle can either be intranuclear or extranuclear. The symmetry of the spindles can either be axial (orthomitosis) or bilateral (pleuromitosis). Almost all combinations of those three traits can be found in eukaryotes, with the exception of open pleuromitosis and closed extranuclear orthomitosis that are topologically self-exclusive (Raikov 1994). During open orthomitosis, the nuclear envelope dissolves completely and the spindles have an axial symmetry. This form of mitosis can be found for example in the algal species *Chilomonas paramecium* (Heywood 1988) and *Isochrysis galbana* (Hori and Green 1985). If the nuclear envelope disperses only partly and the symmetry of spindles is axial, the mitotic type is called semi-open orthomitosis. This process is not universal and can be found in several variants (reviewed in Raikov 1994). Examples for this type of mitosis have been found in *Amoeba proteus* (Gromov 1985) and the algal flagellates *Pavlova lutheri* and *Pavlova salina* (Green and Hori 1988). During semi-open pleuromitosis, the nuclear envelope disperses partly and the spindles have a bilateral symmetry. This type of mitosis is typical for species of the phylum Apicomplexa and was found for example in *Aggregata eberthi* (Bělař 1926). Species that perform closed intranuclear pleuromitosis have an intact nuclear envelope with bilaterally symmetrical intranuclear spindles. This constellation can be found in a variety of species across the eukaryotic tree, including for example fungi, Kinetoplastida, and Haplosporidia (Heath 1980). Closed intranuclear orthomitosis is characterized by an intact nuclear envelope with intranuclear spindles that have an axial symmetry. Multiple variants of this type can be found in eukaryotes (reviewed in Raikov 1994). One variant for example has been found in the testate amoebae *Arcella vulgaris* (Raikov and Mignot 1991). The remaining combination of states is a closed extranuclear pleuromitosis. The interesting

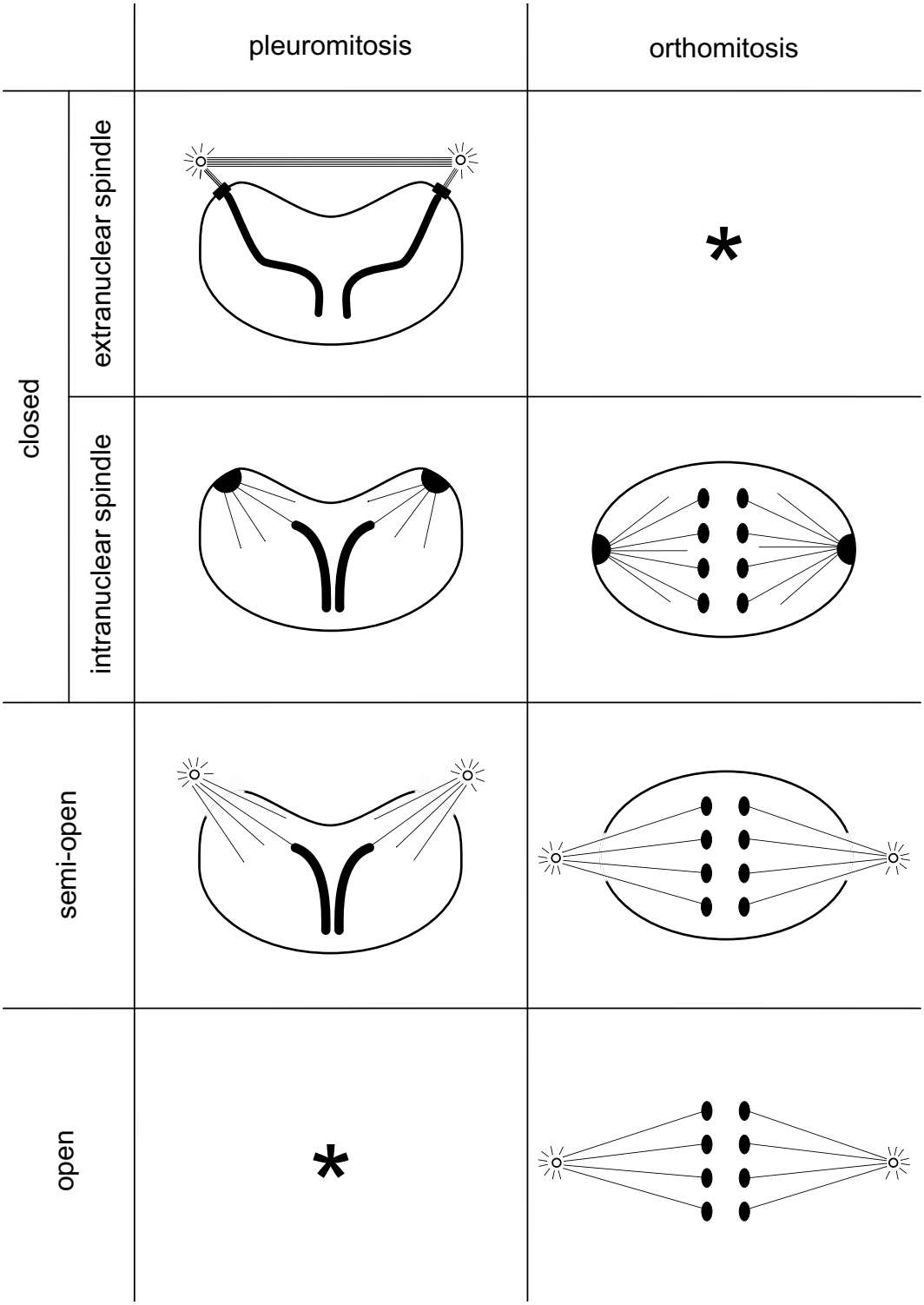

Fig. 1.—Mitotic traits and combinations studied in this manuscript (using symbolism of Raikov 1994). During mitosis, the nuclear envelope can remain intact (closed), disperse partially (semi-open), or disperse completely (open). If the nuclear envelope stays intact during mitosis, the spindles can be either intranuclear or extranuclear. The symmetry of spindles is divided into axial symmetry (orthomitosis) and bilateral symmetry (pleuromitosis). An asterisk (*) indicates that the combination is not possible according to Raikov (1994).

part of this type of mitosis is that the spindles are extranuclear with a bilateral symmetry although the nuclear envelope stays intact. One prominent species with this type of mitosis is *Trichomonas vaginalis* (Ribeiro et al. 2005).

Different types of mitosis have been observed within eukaryotes, but there is no consensus concerning the type of mitosis within LECA. This is mainly due to open and closed mitosis being widespread across various groups of eukaryotes (Sazer et al. 2014). It has been suggested that closed mitosis must have been the ancestral state as it occurs among suspectedly primitive or simple eukaryotic organisms (Pickett-Heaps 1969; Leedale 1970; Pickett-Heaps 1974). Though it has been suggested that mitosis is never completely open mitosis nor completely closed (Dey and Baum 2021), the terms have standard meaning and eukaryotes studied can be classified along that spectrum. Open mitosis of animals and streptophytes has been interpreted as convergent secondary adaptions (Cavalier-Smith 2010). Another correlation concerns the fate of the nuclear envelope and the size of the eukaryotic cell. A larger cell results in a larger nucleus due to the classical "Kernplasmarelation" or karyoplasmic ratio (Hertwig 1903; Jorgensen et al. 2007; Neumann and Nurse 2007). As a consequence of this, a larger nucleus has a larger change in surface during mitosis. The amount of additional membrane that has to be produced in order to perform closed mitosis could force large cells to change their mitosis to an open mitosis (Boettcher and Barral 2013).

Some species can exhibit more than one mitotic type depending on the life cycle stage. For example, the slime mold *Physarum polycephalum* can form a syncytial plasmodium with multiple nuclei but can also exist as a uninucleate amoeba. Depending on the phase of its life cycle, its mitotic type changes. In its syncytial (multinucleated) state, it undergoes closed mitosis and switches to open mitosis during its uninucleate phase (Solnica-Krezel et al. 1991; Tanaka 1973). This mitotic polymorphism during different phases of the life cycle is also seen in *Physarum flavicomum*. Throughout its myxamoebal form the nuclear envelope disperses during prometaphase and remains absent until telophase, whereas during its plasmodial form, the nuclear envelope remains intact and slightly discontinuous at the poles in late stages (Aldrich 1969). Closed nuclear division with intranuclear spindles is typical for cells with a syncytial (multinucleated) habit. This is because open mitosis or extranuclear spindles in a syncytium would lead to microtubule attachment to chromosomes from different nuclei, hence missegregation of chromosomes and therefore a failing mitosis (De Souza and Osmani 2007). Skejo et al. (2021) recently published ancestral state reconstructions (ASRs) indicating that LECA possessed a syncytial morphology, in contrast with standard depictions of LECA as a mononucleated cell, but consistent with earlier suggestions (Garg and Martin 2016) that the syncytial habit of LECA would dramatically ease the transition from prokaryotic to eukaryotic cell division. Here we investigate the ancestral state of mitosis in LECA.

Results and Discussion

Framework and Data

We used a data set of 4 eukaryotic traits (3 mitotic traits)—nuclear envelope during mitosis, symmetry of the spindle apparatus, the position of the central spindle in the presence of an intact nuclear envelope, and sexual reproduction, as well as the distribution of these traits across 150 eukaryotic species spanning a total of 6 lineages: Opisthokonta, Archaeplastida, Hacrobia, Excavata, SAR, and Mycetozoa (fig. 2; see Methods for details). We performed ASR in order to time the origin of these traits relative to the LECA. We clustered 1,848,936 protein-coding genes from the 150 eukaryotic genomes using MCL (Enright et al. 2002) and obtained a total of 239,012 gene families as previously described (Bremer et al. 2022). Since the reconstruction of a reliable eukaryotic species tree remains challenging and the position of the root in the eukaryotic tree is still debated (Williams 2014; Keeling and Burki 2019; Burki et al. 2020), we used a total of 1,789 gene families with at least one representative species of each of the six supergroups. The reconstruction of a reliable species tree is furthermore complicated by the paucity of "universal" orthologs in the data set. The causes for this are frequent gene duplications and gene losses.

Our approach of analyzing 1,789 rooted gene trees—instead of one or a few published rooted species trees—covers a wider range of phylogenetic history recorded in genes. Each eukaryotic gene tree has its own history and therefore the root position will vary across different trees. This is important because eukaryotic evolution (and evolution in general) is, obviously, not recorded or reconstructed the same for each gene. If all eukaryotic genes tended to generate exactly the same tree, the eukaryotic tree would have been inferred, and rooted, decades ago. One could also argue that our method has some similarities with conventional methods. The analysis of widely distributed genes, in our case distributed in each supergroup, is similar to the summation of signals across a sample of gene trees in the case of building a consensus tree. In our method, we have the benefit that the individual phylogenetic signal of each gene is recorded because the analyzed gene trees were reconstructed from independent phylogenetic markers.

LECA Reconstructs With Closed Intranuclear Orthomitosis and Sexual Reproduction

We labeled the species at the tips of each tree according to their trait-state annotations and performed maximum-likelihood ASR. A gene tree was informative for ASR of a given trait if it contained representative species for both possible trait states. Trees with only one trait state across all annotated tips were not considered for ASR as they

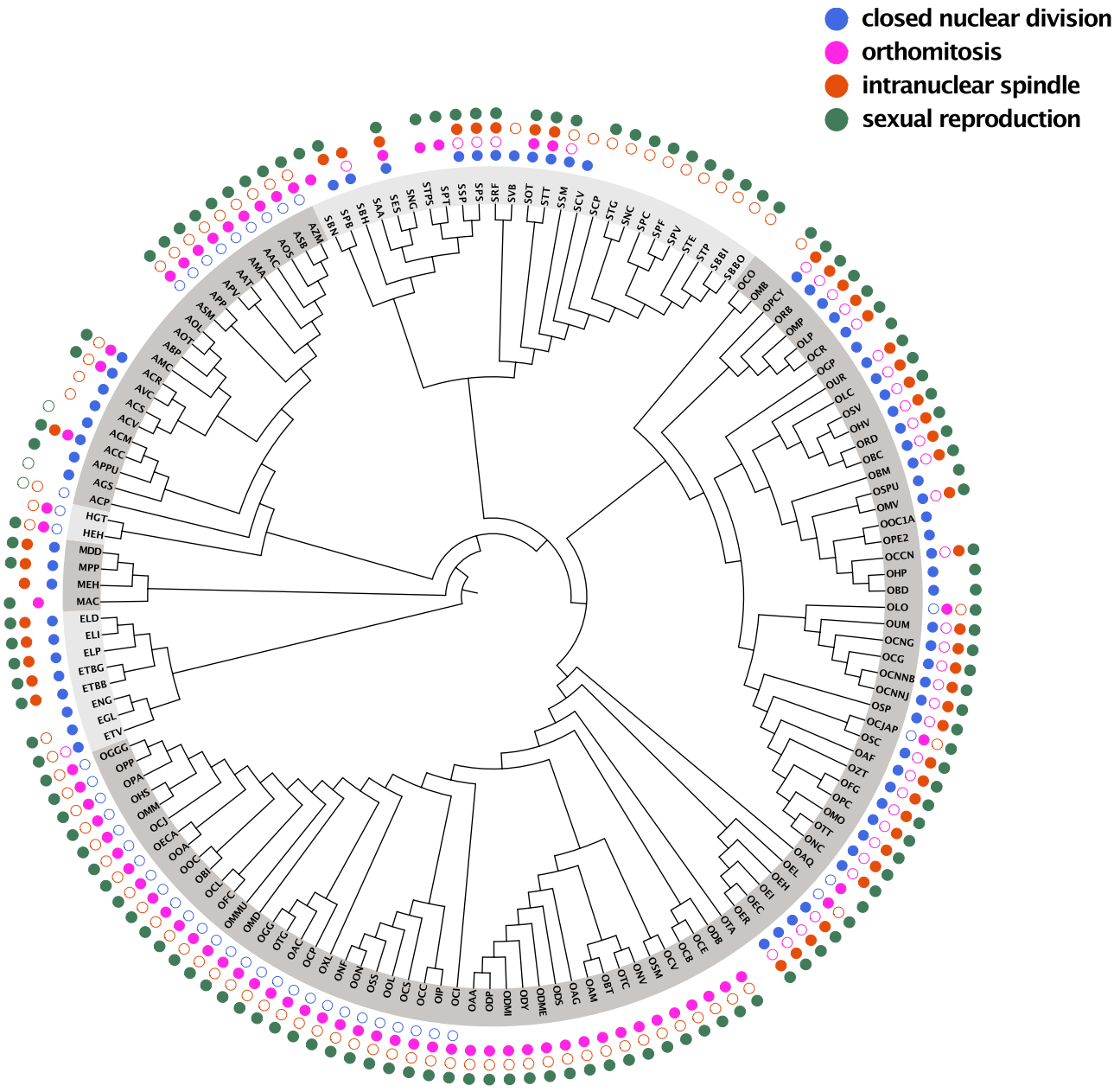

Fig. 2.—Presence (filled circle) absence (empty circle) distribution of 4 traits in 150 eukaryotic species. Species with no circle for a given trait indicate missing annotation. The reference tree was inferred from the alignment of 18*S* RNA sequences, rooted on the Excavates branch, with the sole purpose of data display (see Methods). Tip labels are species codes (see supplementary table S1, Supplementary Material online, for complete species names and detailed trait annotations). The first character of the species codes indicates supergroup affiliation of the species: Excavates (*E*), Mycetozoa (*M*), Hacrobia (*H*), Archaeplastida (*A*), SAR (*S*), and Opisthokonta (*O*). The shades of gray show the clades of the six eukaryotic supergroups.

were uninformative. Due to the fact that each tree has at least one representative from each of the six eukaryotic supergroups, the root corresponds to LECA. In a first step, we summarized the ASR across all trees by counting the frequency of each trait-state appearance in LECA (table 1). High frequencies indicate the most likely state of a trait in LECA, whereas low frequencies indicate lineage specific origins of the trait or errors.

With this majority rule, we found that 90% of the trees reconstruct a closed nuclear envelope at the root node and therefore in LECA. An absence of this trait state in LECA was only found in 2% of the trees. The remaining 8% had ambiguous results at the root node. Our analysis also shows that 65% of the trees recover orthomitosis as the ancestral state, whereas only 9% result in pleuromitosis in LECA. The remaining 26% of the trees have unresolved

Table 1
Maximum-likelihood Ancestral Reconstruction of 4 Traits From 150 Eukaryotic Species, Across a Broad Sample of Gene Trees as Estimates of the Underlying Phylogeny

Trait	Presence	Absence	Ambiguous	Total (*N*)
Single-copy gene trees				
Closed nuclear division	13 (62%)	0	8 (38%)	21
Orthomitosis	8 (40%)	0	12 (60%)	20
Intranuclear spindle	3 (15%)	3 (15%)	14 (70%)	20
Sexual reproduction	10 (91%)	0	1 (9%)	11
Multicopy gene trees				
Closed nuclear division	1,595 (90%)	29 (2%)	144 (8%)	1768
Orthomitosis	1,152 (66%)	154 (9%)	448 (25%)	1754
Intranuclear spindle	782 (44%)	491 (28%)	494 (28%)	1767
Sexual reproduction	1,480 (98%)	2 (0.1%)	23 (1.5%)	1505

Note.—Absolute values indicate the number of trees with a trait state (presence/absence) tracing to LECA. The total number of trees used (*N*) as well as the number of trees with ambiguous reconstructions in LECA are indicated. All analyzed traits were modeled as binary traits.

ASR for that trait. For the third trait, we reconstructed intranuclear spindle in 44% of the analyzed trees. The absence of this trait, and therefore the presence of extranuclear spindles, was recovered only in 28% of the trees and the remaining 28% of the trees had ambiguous results. The majority rule indicates that LECA had a closed nuclear envelope with axial symmetry of the spindle apparatus and intranuclear spindles.

The reconstruction of the sexual reproduction resulted in 98% of the trees with this trait being present in LECA and <2% with ambiguous results. The absence of this trait was only recovered in 0.1% of all analyzed trees. The presence of sexual reproduction in LECA is in accordance with the conservation of meiosis across all major eukaryotic groups despite a remarkable variability of this process across the eukaryotic tree (Egel and Penny 2007; Loidl 2016).

Whereas the reconstructions of a sexual reproduction and a closed nuclear envelope were robust, the analyses for orthomitosis and intranuclear spindles had a higher proportion of unresolved reconstructions, with both however still favoring presence of the trait in LECA. To further analyze these ambiguities, we tested the statistical significance of our results by matching marginal probabilities of all possible trait states for each tree (fig. 3). This was done regardless of the reconstruction results in LECA (trait-presence, trait-absence, or unresolved). The differences in distributions were assessed using the Wilcoxon-signed-rank test. This test is a refinement relative to the majority rule as it not only uses the reconstruction result at LECA, but also takes the magnitude of probabilities into account. The advantage of this analysis is that we can also look at trees with unresolved ASR in LECA. The two-tailed Wilcoxon tests indicate that closed nuclear envelope, orthomitosis, intranuclear spindle, and sexual reproduction were present in LECA at $P < 0.01$.

The 200 Best Trees for Tree Quality, Sampling, and Conflicting Evidence Recover the Same Reconstructions

Ancestral state reconstructions depend on the quality of the underlying gene trees. This quality can be influenced by the sequence alignment, the rooting or species sampling. In order to show that our reconstructions with 1,789 trees are not the result of low-quality trees, we examined the tree quality for eight independent criteria by analyzing only the top 200 trees for each criteria individually. Quality of sequence alignments was tested by performing heads or tails (HoT) analyses (Landan and Graur, 2007). For this, we compared the original alignment (heads) against the alignment that was obtained using the same sequences, but in reverse amino acid order (tails). Our analysis showed that tree quality in the majority of our 1,789 trees is high. Most trees have a mean column score above 0.6 and a mean residue pair score above 0.9. Additionally, ASRs of only the best 200 trees according to both scores individually uncover the same reconstructions (supplementary fig. S1, Supplementary Material online). The Wilcoxon tests are significant for almost all traits, except for the intranuclear spindle, yet the reconstruction of closed orthomitosis as the ancestral state inevitably leads to intranuclear spindle as this is the only possible combination of these three trait states (see fig. 1). Therefore, the alignment quality does not impact our results obtained with 1,789 gene trees.

Tree rooting can have an influence on the results of ASRs. The rooting method we used for our trees is the minimal ancestor deviation (MAD) approach (Tria et al. 2017). It has been shown in independent studies that this approach outperforms other current rooting methods (Wade et al. 2020; Lamarca et al. 2022). Additionally, MAD does not require an outgroup for rooting a gene tree. Here, we analyzed two root scores: the ancestor deviation (AD) statistic and the root ambiguity index (AI). AD scores measure the degree of deviation from the molecular clock corresponding to the

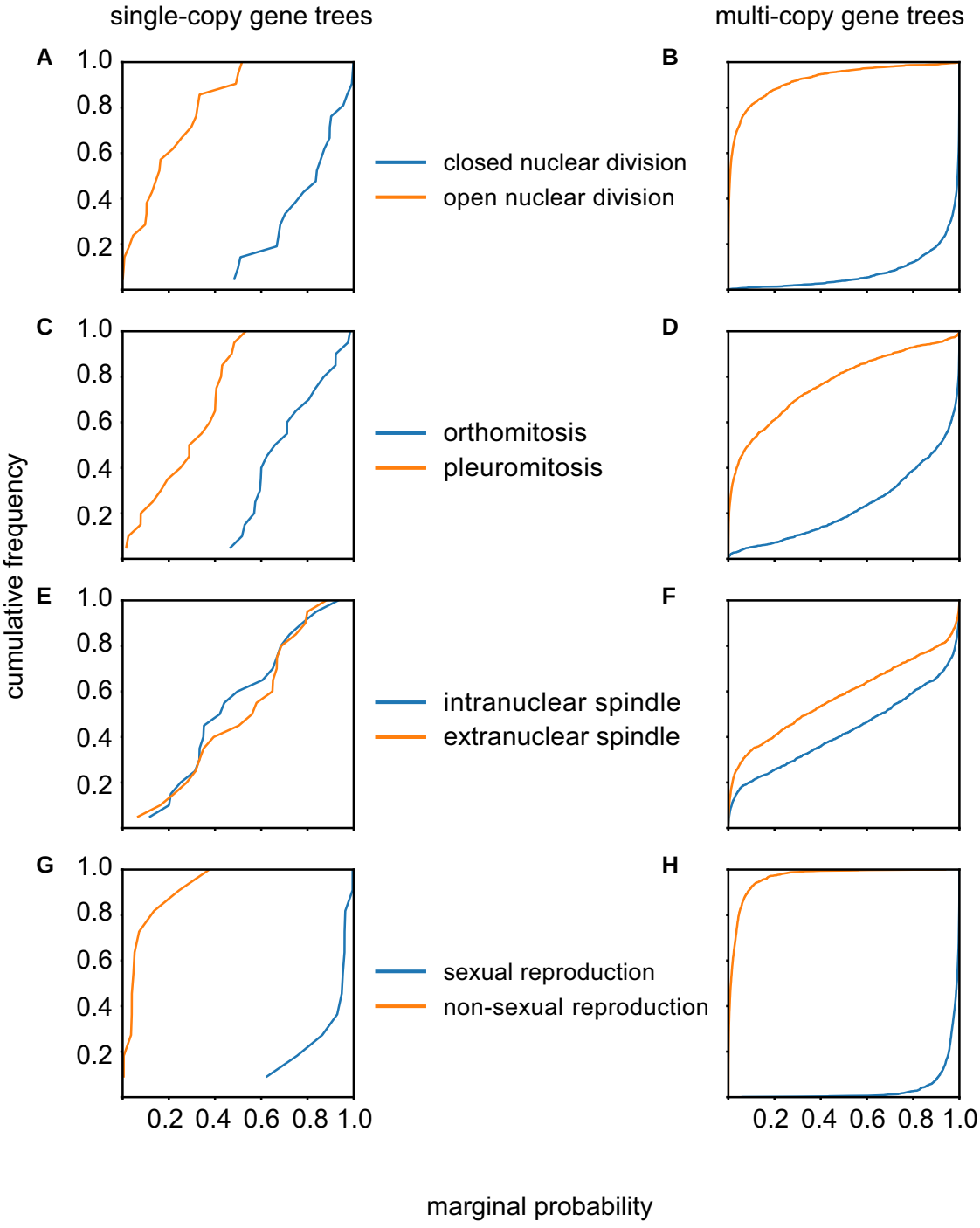

Fig. 3.—Distribution of marginal probabilities for alternative trait states in LECA across a maximum of 21 single-copy gene trees (A,C,E,G; without paralogs) and a maximum of 1,768 multicopy gene trees (B,D,F,H; with paralogs). All traits were treated as binary traits. The number of trees used in the analyses are show in table 1. Trait states with high probabilities of reconstructing to LECA in the trees have distributions (colored lines) that are right shifted in the plots, for example, sexual reproduction (*G*, *H*).

inferred root. AI scores are the ratio of AD scores for the inferred root over the second-best root. Both scores were noticeably similar for trees uncovering different trait states at the root node. This suggests that our rooting method did not cause significant bias during reconstructions. The Wilcoxon tests for the top 200 trees judged by AD and AI recovered the same reconstruction results, with the exception of intranuclear spindle being not

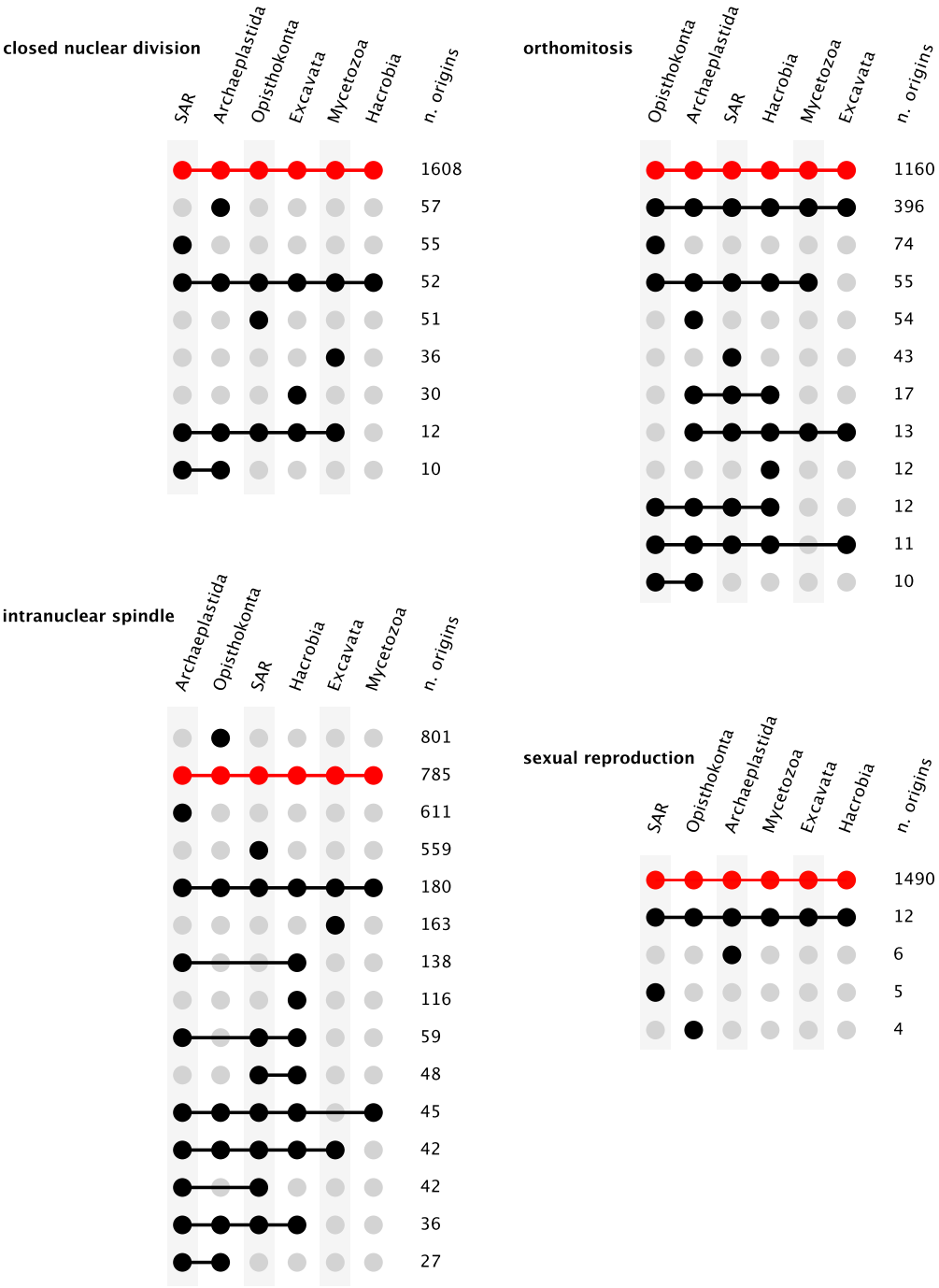

Fig. 4.—Distribution of supergroups descending from origin nodes across 1,789 trees. For each internal node reconstructed as a trait origin, all the species (tips) descending from it were used to score an origin to the combination of supergroups (filled circles) to which the descending species belong. Origins at the root node (LECA) are shown in red (number of origins: 1608; 1160; 785; 1490).

significant (supplementary fig. S1, Supplementary Material online). As explained above, the only possible combination having a closed mitosis and axial spindle (orthomitosis) requires intranuclear spindle (see fig. 1).

Another aspect that can influence the results of ASRs are the sampled species within the analysis. For the construction of gene clusters, we avoided metagenomic and transcriptomic sequences. It has been shown recently that

there is more contamination, base calling and assembly errors in those sequences (Garg et al. 2021). We relied on genomic sequences mainly from RefSeq (O'Leary et al. 2016; see Supplementary file 1, Supplementary Material online). It is inevitable due to our tree selection (at least one species from each eukaryotic supergroup) that gene families are not uniformly distributed across the sampled genomes. We therefore tested four different criteria to investigate the effect of differential sampling: (1) the total number of operational taxonomic units; (2) the total number of species; (3) the fraction of basal lineages (Excavates and Mycetozoa) relative to Opisthokonts; and (4) the fraction of the least frequent trait state occurring at the tips of our trees. The Wilcoxon tests for the top 200 trees independent for all four sampling criteria significantly recovered the same reconstructions we found while using 1,789 trees. The only exception is, once again, the intranuclear spindle being only significantly recovered in two out of the four cases: total number of species and fraction of the least frequent trait state (supplementary fig. S1, Supplementary Material online). It is still the only possible state for this trait due to the other significant reconstructions (see fig. 1).

Timing Trait Origins Relative to the Emergence of Eukaryotic Supergroups

We investigated eukaryotic species that descended from internal trait origin nodes in order to time the origin of those traits relative to the divergence of the six eukaryotic supergroups that were considered here: Opisthokonta, Archaeplastida, SAR, Hacrobia, Excavata, and Mycetozoa. For this, we recorded the combination of descending species of each internal origin node for each trait across all trees. The distribution of those combinations of supergroups was plotted (fig. 4). Internal origin nodes are defined as internal nodes with a newly acquired trait state that is not present in its parent node. We distinguish between a descending combination of all six supergroups that has its trait origin at the root node (six red circles) from trait origins with descendants from each supergroup that were found at other internal nodes (six black circles). The former case identifies the presence of the trait at the root (LECA). The latter case identifies the presence of a node in the tree that subtends descendants of all six supergroups but is not the root (LECA), which would be compatible with trait origin in LECA but could also be the consequence of phylogenetic error or duplications (Bremer et al. 2022).

Combinations with a high frequency (fig. 4) likely represent a true origin node in the underlying supergroup phylogeny. Low-frequency supergroup combinations can be interpreted as likely conflicting results. In three out of the four analyzed eukaryotic traits the results are very clear. For the trait "closed nuclear division," we see that the majority of origin nodes is found in the root node of the tree ($n = 1{,}608$). The same was observed for the trait "sexual reproduction." Almost all origins of the trait were found in LECA ($n = 1{,}490$). "Orthomitosis" also has the majority of origins in the root node ($n = 1{,}160$) followed by additional origins with descendants of all six supergroups that were not at the root node ($n = 396$). Origin nodes for "intranuclear spindles" were found with a wider spectrum of combinations of descending supergroups. The highest number of origins was found in Opisthokonta ($n = 801$) followed closely by origins within LECA ($n = 785$), Archaeplastida ($n = 611$) and SAR ($n = 559$), indicating that for the present sample, intranuclear spindles are more common in opisthokonts than in other supergroups.

Number of Origins of Different Traits Across Eukaryotic Trees

When looking at the origin of a trait in the tree, it is not only of interest to investigate the ancestral state, but also how

Table 2

Summary Statistics for the Number of Trait Origins Across Trees

Trait	Number of origin[a]		
	Terminal nodes	Internal nodes	All nodes
Single-copy gene trees			
Closed nuclear division	2, 1, 2.97	1.23, 1, 0.88	3.23, 2, 3.59
Orthomitosis	1.45, 1, 1.88	1, 1, 0.56	2.45, 2, 1.90
Intranuclear spindle	1.8, 2, 1.40	0.85, 1, 0.93	2.65, 2.5, 1.27
Sexual reproduction	0.64, 0, 1.57	1.18, 1, 0.75	1.82, 1, 1.60
Multi-copy gene trees			
Closed nuclear division	0.69, 0, 1.83	1.64, 1, 1.58	2.33, 1, 3.17
Orthomitosis	0.43, 0, 2.69	1.22, 1, 0.68	1.6, 1, 2.89
Intranuclear spindle	1.94, 2, 1.90	2.35, 2, 1.38	4.29, 4, 2.88
Sexual reproduction	0.75, 0, 1.26	1.38, 1, 1.07	2.13, 1, 2.04

NOTE.—Trait origin in internal and terminal nodes are distinguished. Single-copy trees (without paralogs) were distinguished from multicopy trees (with paralogs).
[a]Numbers indicate mean, median, and standard deviation across trees.

many times a trait arose within eukaryotes. In order to analyze this, we counted the number of origins for each analyzed gene tree. The average number of origins of all analyzed traits are shown in table 2. Despite no trait having a clear single origin, the number of average origins is still comparatively low. This makes sense as all analyzed traits have been reconstructed to being ancestral in LECA. The additional origins within the trees can be the results of turnovers of these traits. As we already highlighted above, the syncytial state of a cell favors a closed mitosis and therefore a change in the lifestyle could have also changed the traits analyzed here. We have recently shown that LECA was multinucleated, but the trait itself had a high turnover rate ranging on average from three to seven origins per tree (Bremer et al. 2022). The present data indicate that mitotic traits evolved more stable than the multinucleated state, whereby the presence of an intracellular spindle and closed mitosis are required for the syncytial state to become manifest.

Conclusions

Despite the reconstruction of LECA being syncytial with closed orthomitosis using intranuclear spindles, is it still possible that open mitosis was somehow present in LECA but escaped identification? The key difference between open and closed mitosis concerns continuity of the nuclear envelope. A complete breakdown and reassembly of the nuclear envelope at every cell division requires more in the way of membrane fragmentation and reassembly processes (Heath 1980) than closed mitosis, which is mechanistically simpler, entailing enlargement and median constriction of the nuclear envelope (reviewed in Ungricht and Kutay 2017). Eukaryotes arose from simple prokaryotic ancestors having prokaryotic chromosome and cell division processes. The presence of closed mitosis with intranuclear spindles in a syncytial LECA eases the prokaryote to eukaryote transition, because it decouples the processes of chromosome division, chromosome partitioning, and cell division (cytokinesis), allowing them to evolve in sequence as independent traits rather than simultaneously, while also buffering for the existence of defective chromosome combinations through intracellular complementation from nuclei with viable chromosome combinations via mRNA in the cytosol. Therefore, it is unlikely that open mitosis was present in LECA and escaped identification. In summary, the present findings indicate that LECA had, in addition to a nucleus (Mans et al. 2004; Bapteste et al. 2005; Neumann et al. 2010), an endoplasmic reticulum (Kontou et al. 2022), linear chromosomes with centromeres (Ishikawa and Naito 1999; van Hooff et al. 2017), flagellae (Carvalho-Santos et al. 2011; Lindemann 2022), microtubule organizing centers (Yubuki and Leander 2013), nucleoli (Gardner et al. 2010; Hoeppner and Poole 2012), meiosis and sex (Villeneuve and Hillers 2001; Loidl 2016), facultatively anaerobic mitochondria (Müller et al. 2012; Mills et al. 2022), and a syncytial habit (Skejo et al. 2021) that lacked phagocytosis (Bremer et al., 2022), closed orthomitosis with intranuclear spindles. In terms of overall physiology and lifestyle, LECA is beginning to look like a filamentous fungus (Martin et al. 2003) able to survive in anaerobic environments.

Materials and Methods

Phylogenetic Trees

We clustered protein sequences from 150 eukaryotic genomes by firstly performing an all-versus-all BLAST (Altschul et al. 1990) and selecting the best reciprocal BLAST hits with an expectation value (*e*-value) $\leq 10^{-10}$. Those hits were then globally aligned using the Needleman–Wunsch algorithm implemented in the EMBOSS needle program (Rice et al. 2000). Protein pairs with a global identity <25% were discarded before clustering with the MCL algorithm (Enright et al. 2002), version 12-068 using default parameters. A total of 1,789 protein clusters that possessed at least one species of each eukaryotic supergroup were found and selected for further analyses. Alignments of those protein clusters were generated using MAFFT (Katoh et al. 2002), using the iterative refinement method that assimilates local pairwise alignment information (L-INS-i). The alignments were not trimmed and maximum-likelihood trees were reconstructed with IQ-Tree (Nguyen et al. 2015), using the best-fit model and the following parameters: "-bb 1000" and "-alrt 1000." We differentiated between trees without paralogs (single-copy trees) and trees with paralogs (multicopy trees) for further analyses. The trees were rooted with MAD (Tria et al. 2017). All 1,789 analyzed trees showed no ambiguous root inferences.

Trait Annotation, Coding, and Definition

All four analyzed traits were coded as binary traits (presence "1" or absence "0"). While the sexual reproduction may be either present or absence, the mitotic traits have to be seen a little different. An absence for the closed nuclear division means that the nuclear envelope is open or semi-open during mitosis. For the intranuclear spindles, an absence corresponds to extranuclear spindles and the absence of orthomitosis (axial symmetry) stands for pleuromitosis (bilateral symmetry). The annotation of traits is based on literature (supplementary table S1, Supplementary Material online). Not every species in our data set is annotated for every trait in literature. We therefore applied a majority rule for each group in question. If there is data on the exact ancestral state of a trait, we annotated it to be present in the whole group. In cases where only one representative of a group is annotated in literature, this annotation was

suspected to be present in the whole group. Groups with different annotations for different members were annotated by majority rule. No cases with a 50:50 distribution were found in our data set. Two species in our data set are annotated with incompatible mitotic combinations (closed orthomitosis with extranuclear spindle): *Chlamydomonas reinhardtii* and *Volvox carteri*, both members of the taxon Chlorophyceae. Although the combination of traits itself is incompatible, the majority rule resulted in this combination for the group.

Ancestral State Reconstruction

The reconstruction of ancestral states was performed using PastML version 1.9.20 (Ishikawa et al. 2019). The chosen parameters were a maximum-likelihood approach based on marginal posterior probabilities approximation and the F81 model of character evolution (Felsenstein 1981). We annotated the tips of the trees based on a trait matrix for the 150 eukaryotic species (supplementary table S1, Supplementary Material online), with the inclusion of missing data (unknown tip state). Trees with the same state of a trait at each tip of the tree were discarded from the analysis for this specific trait. Analyses of phylogenetic trees and trait origins were performed using the python toolkit Environment for Tree Exploration ETE v3 (Huerta-Cepas et al. 2016).

18S RNA Reference Tree

The reconstruction of a reference tree was performed with 18S RNA sequences for all 150 eukaryotes in our data set. Sequences were primarily searched for in the SILVA rRNA database (release 138.1 from November 2020; Quast et al. 2013). As not all eukaryotic species from our data set were found within this database, sequences were secondarily searched for within the PR^2 sequence database (version 4.12.0 from August 2019; Guillou et al. 2013). For a total of eight species, we were not able to find 18S RNA sequences in both databases and therefore used alternatives from the same genus. We generated an alignment using MAFFT (Katoh et al. 2002), using the iterative refinement method that assimilates local pairwise alignment information (L-INS-i), reconstructed a maximum-likelihood tree with IQ-tree (Nguyen et al. 2015) and rooted the resulting tree on the branch leading to Excavates. The sole purpose of the reconstruction and rooting of this tree was to display our data.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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Data Availability

Sequence alignments, phylogenetic trees and ASR are available as Supplemental Data under https://doi.org/10.6084/m9.figshare.20591172.

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