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Diploid-dominant life cycles characterize the early evolution of Fungi

Kevin R. Amses[a,1,2], D. Rabern Simmons[a,1], Joyce E. Longcore[b], Stephen J. Mondo[c], Kensuke Seto[a], Gustavo H. Jerônimo[a,d], Anne E. Bonds[a], C. Alisha Quandt[a,e], William J. Davis[a,3], Ying Chang[f,g], Brian A. Federici[h], Alan Kuo[c], Kurt LaButti[c], Jasmyn Pangilinan[c], William Andreopoulos[c], Andrew Tritt[c], Robert Riley[c], Hope Hundley[c], Jenifer Johnson[c], Anna Lipzen[c], Kerrie Barry[c], B. Franz Lang[i], Christina A. Cuomo[j], Nicolas E. Buchler[k], Igor V. Grigoriev[c,l], Joseph W. Spatafora[f], Jason E. Stajich[m,n], and Timothy Y. James[a,4]

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Most of the described species in kingdom Fungi are contained in two phyla, the Ascomycota and the Basidiomycota (subkingdom Dikarya). As a result, our understanding of the biology of the kingdom is heavily influenced by traits observed in Dikarya, such as aerial spore dispersal and life cycles dominated by mitosis of haploid nuclei. We now appreciate that Fungi comprises numerous phylum-level lineages in addition to those of Dikarya, but the phylogeny and genetic characteristics of most of these lineages are poorly understood due to limited genome sampling. Here, we addressed major evolutionary trends in the non-Dikarya fungi by phylogenomic analysis of 69 newly generated draft genome sequences of the zoosporic (flagellated) lineages of true fungi. Our phylogeny indicated five lineages of zoosporic fungi and placed Blastocladiomycota, which has an alternation of haploid and diploid generations, as branching closer to the Dikarya than to the Chytridiomyceta. Our estimates of heterozygosity based on genome sequence data indicate that the zoosporic lineages plus the Zoopagomycota are frequently characterized by diploid-dominant life cycles. We mapped additional traits, such as ancestral cell-cycle regulators, cell-membrane– and cell-wall–associated genes, and the use of the amino acid selenocysteine on the phylogeny and found that these ancestral traits that are shared with Metazoa have been subject to extensive parallel loss across zoosporic lineages. Together, our results indicate a gradual transition in the genetics and cell biology of fungi from their ancestor and caution against assuming that traits measured in Dikarya are typical of other fungal lineages.

life cycle evolution | phylogenomics | aquatic fungi | plesiomorphy

Significance

It has been assumed that fungi are characterized by a haploid-dominant life cycle with a general absence of mitosis in the diploid stage (haplontic life cycles). However, this characterization is based largely on information for Dikarya, a group of fungi that contains mushrooms, lichens, molds, yeasts, and most described fungi. We now appreciate that most early-diverging lineages of fungi are not Dikarya and share traits with protists, such as flagellated life stages. Here, we generated an improved phylogeny of the fungi by generating genome sequences of 69 zoosporic fungi. We show, using the estimated heterozygosity of these genomes, that many fungal lineages have diploid-dominant life cycles (diplontic). This finding forces us to rethink the early evolution of the fungal cell.

Fungi and Metazoa evolved from a common protist-like ancestor, yet the two kingdoms have diverged in ways that make their kinship as Opisthokonts barely recognizable. Fungi grow on or within their food and feed by external digestion (osmotrophy), while animals mostly eat things smaller than themselves via ingestion. This difference is the basis for massive changes in morphology, including loss of motility during feeding and polarized cell growth in fungi (1, 2). The two kingdoms are also considered intrinsically different in life cycles, because fungi are characterized as being haplontic (haploid-dominant life cycle) while animals are diplontic (diploid-dominant). However, this textbook difference is inaccurate in two ways. First, the subkingdom Dikarya, with the majority of fungal species diversity, comprises lineages that spend some or most of their life cycles in a dikaryotic phase wherein two haploid nuclei undergo conjugate division, a cell type genetically analogous to a diploid (3). Further, life cycles have not been carefully investigated in most early-diverging fungal lineages (EDF), which include many phyla outside of Dikarya (e.g., non-Dikarya fungi). EDF have retained ancestral traits also retained in Metazoa, such as flagellation, actin structures used for crawling, presence of cholesterol in cell membranes, vitamin dependencies, and cell-cycle genes (4–8). However, life-cycle transitions between the Opisthokont ancestor and the extant Fungi are shrouded due to a lack of information on the genetic characteristics of EDF and the undersampling of their genomic diversity (9–11). The goal of this paper is to provide a robust and comprehensive phylogeny of the Fungi, emphasizing zoosporic taxa, to reassess the evolution of life-cycle and cellular characters during early fungal diversification using genomic data.

Although fungi are often considered to have haploid-dominant life cycles, there are many variations observed (Fig. 1). In a haplontic life cycle, mitosis is restricted to the haploid phase, and meiosis ensues immediately following sex and nuclear fusion (Fig. 1*A*). In contrast, in the diplontic life cycle that generally characterizes Metazoa, mitosis is restricted to diploid cells (Fig. 1*B*). The alternation between haploid and diploid mitotic cycles, which generally characterizes plants, is documented, albeit rarely, in fungi, such as baker's yeast and the water mold *Allomyces* (Fig. 1*C*). Despite this general

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[1]K.R.A. and D.R.S. contributed equally to this work.

[2]Present address: Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331.

[3]Present address: Department of Biological Sciences, Kent State University, Kent, OH 44243.

[4]To whom correspondence may be addressed. Email: tyjames@umich.edu.

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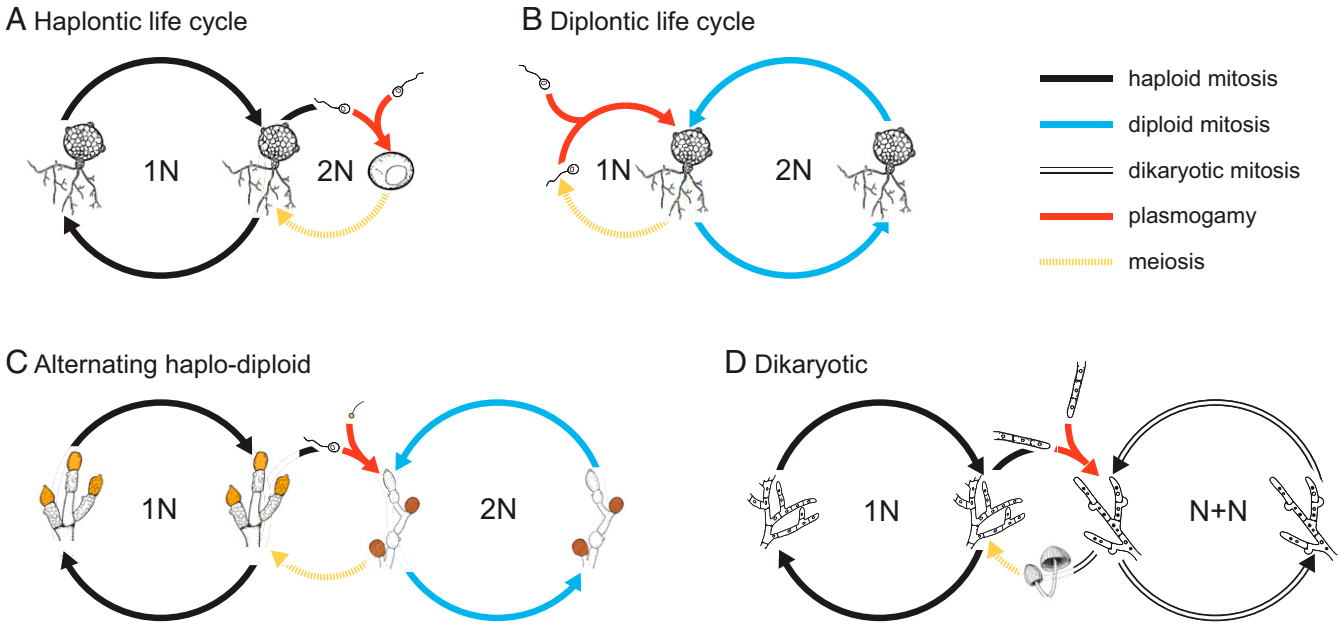

Fig. 1. Illustrated life cycles observed in fungi. (*A*) In haplontic life cycles mitosis is limited to the haploid phase, with plasmogamy of gametes followed by meiosis. (*B*) In diplontic life cycles, mitosis only occurs in the diploid phase with haploid cells only functioning as gametes. (*C*) Life cycles may alternative between haploid and diploid mitotic phases and may show morphological differences between ploidies as in *Allomyces*. (*D*) The dikaryotic life cycle is an alternative to alternation of haploid and diploid generations which lacks diploid mitosis and instead has a phase with two nuclear genotypes undergoing synchronous division.

avoidance of diploid mitosis in fungi, many Dikarya show a distinctive dikaryotic life cycle wherein, following mating, haploid nuclei of the two partners remain paired and undergo synchronous mitoses (Fig. 1*D*). This life cycle is analogous to diploidy with respect to genetic dominance (12) and would provide some of the proposed advantages of diploidy, such as buffering against somatic mutation (13). Overall, although we appreciate that fungal life cycles have great potential to vary, we have a poor understanding of life cycles of the EDF which represent the majority of the phylogenetic diversity of Fungi.

We consider EDF to comprise 11 phyla, including 8 zoosporic phyla that reproduce with swimming spores and form a contentious paraphyletic grade along the backbone of the fungal tree (9, 10, 14–16). The deeply diverging phyla, Rozellomycota/Cryptomycota and Aphelidiomycota, are endoparasites that have the ability to phagocytize, which enables them to ingest host cytoplasm, a trait presumably retained from the most recent common ancestor (MRCA) of Opisthokonta (17, 18). The remaining free-living zoosporic phyla have microscopic vegetative thalli that may be unicellular or mycelial (*SI Appendix*, Fig. S1), and the greatest species diversity is found in the Chytridiomycota, which has an estimated 14 orders (9). Chytridiomycota is united with the phyla Monoblepharidomycota + Neocallimastigomycota in subkingdom Chytridiomyceta, though the branching order of the three phyla is uncertain (14, 15, 19).

Blastocladiomycota is an enigmatic group with a life cycle alternating between morphologically distinctive haploid and diploid thalli (Fig. 1*C*) (20, 21). Members include the water mold, *Allomyces*, that has been used as a model system for genetics and physiology (22) and a genus of obligate fatal parasites, *Coelomomyces*, that has a haploid phase in copepods and a diploid phase in mosquitoes (23). The precise phylogenetic placement of the Blastocladiomycota has been controversial (10, 15, 19, 24), with nearly equal support for the Blastocladiomycota diverging before the Chytridiomyceta or after the Chytridiomyceta. Several traits of Blastocladiomycota ally them with the terrestrial fungi (here defined as the nonzoosporic phyla Mucoromycota and Zoopagomycota and subkingdom Dikarya): closed mitosis, the presence of a Spitzenkörper, beta 1,3 glucans in the cell wall, and true mycelial growth in some members (22, 25). The detection of mating types in *Coelomomyces* (26), which have only been otherwise documented in terrestrial fungi, may be indicative that Blastocladiomycota is more closely related to Dikarya than Chytridiomyceta.

Mating and sexuality are poorly described in zoosporic fungi beyond the well-characterized water mold model *Allomyces*. According to mycological textbooks, life cycles of Chytridiomycota are characterized as being haplontic with zygotic meiosis (27–29), but the majority of assumptions of meiotic stages are unconfirmed by cytology. Moreover, the requisite genetic studies using molecular markers to confirm ploidy cycling have not been accomplished for these presumably sexual species. Importantly, the best-studied chytrid fungus, *Batrachochytrium dendrobatidis*, has a life cycle that appears to be dominated by asexually reproducing diploid, or aneuploid, thalli (30). More recently, additional studies have indicated that non-Dikarya phyla have heterozygosity indicative of diploidy or higher ploidy (31–34), suggesting that the assumption of haplontic life cycles for the Chytridiomyceta and other EDF may be false.

Current sequencing technologies now create the potential for leveraging genomic sequencing to broadly sample fungal genomes for estimating ploidy and other cellular traits in a robust phylogenomic framework. Here, we sampled 69 zoosporic fungal genomes using both culture and single-cell approaches. Our genome analyses provide a strongly supported phylogeny for understanding taxonomy and the evolution of ploidy and other traits which had previously been held to be distinctive between Fungi and Metazoa. These data bolster the growing picture that many traits including motility, feeding modes, and life cycles changed gradually during the early diversification of fungi. The high levels of heterozygosity estimated from genomes analyzed in this study reveal that somatic diploidy is much more common in Fungi than previously appreciated.

Results

Phylogenomic Analyses Reveal a Robustly Supported Paraphyly of Zoosporic Fungi. We generated draft genome assemblies for 69 zoosporic fungi to address the controversial phylogenetic relationships among the EDF and the evolutionary history of key genetic traits. Assembly sizes of the newly sequenced fungi ranged from 11.70 to 81.19 Mb, with gene numbers of 5,512 to 16,599, and genome completeness values of 34.30 to 94.99% (*SI Appendix*, Table S1). One single cell (PSC023) of the alga *Micrasterias* cf *truncata* parasitized by a chytrid (*SI Appendix*, Fig. S1*D*) revealed two fungal genomes (with >60% genome completeness), one of which grouped with Rhizophydiales and the other with Rozellomycota. We used the BUSCO odb10 fungi ortholog set of 758 markers to search all fungal and outgroup genomes for a gene set enriched in single-copy orthologs. After filtering out genes with missing data from >25% of taxa or issues with paralogy, we limited our data set to 487 markers. The average of occupancies following filtering and subsequent alignment trimming was 82.02% with a range of: 69.34 to 96.35% (*SI Appendix*, Fig. S2).

The branching order of the zoosporic fungi along the backbone of the fungal tree is controversial. Our phylogenomic reconstructions based on concatenation covering 197,423 amino acid positions over 133 fungal taxa and four outgroups generated a robustly supported tree by maximum likelihood (ML) analysis using IQTree with 100% bootstrap support for all nodes (Fig. 2 and *SI Appendix*, Fig. S3). We recovered a paraphyletic grade of the five zoosporic lineages: Rozellomycota+Microsporidia, Aphelidiomycota, Chytridiomyceta, Blastocladiomycota, and Olpidiomycota, in this order of branching from the root. These relationships are largely consistent with other phylogenomic analyses of zoosporic fungi, although many studies place Blastocladiomycota closer to the fungal root than Chytridiomyceta (14, 15, 35), whereas others place Chytridiomyceta more basal (10, 32, 36). Within Chytridiomyceta, the Monoblepharidomycota is sister to the Neocallimastigomycota. The zoosporic plant pathogen *Olpidium bornovanus* was placed as the sister to the terrestrial fungi. Among the terrestrial fungi, Zoopagomycota was recovered as diverging first, with Mucoromycota supported as the sister clade to Dikarya. The relationships among classes and orders of Chytridiomycota had poor support with one exception. A clade comprising Rhizophydiales+Spizellomycetales+Rhizophlyctidales +*Blyttiomyces helicus* was strongly supported.

Although our concatenated phylogeny provides robust support for all branches by bootstrapping, concatenation can often produce an inflated perspective of phylogenetic support (37). Therefore, we also assessed support for the concatenated topology by generating a coalescence-based species tree using individual gene trees with the program ASTRAL (*SI Appendix*, Fig. S4). The ASTRAL tree was largely congruent with the concatenated tree, with only nine nodes differing. For example, in the concatenated tree Neocallimastigomycota groups with Monoblepharidomycota, whereas in the ASTRAL tree Neocallimastigomycota groups with Chytridiomycota. We also estimated support from individual genes via gene concordance factors and internode certainty, which are highly conservative, less-biased metrics based on splits or quartets in underlying gene trees (*SI Appendix*, Fig. S5). These metrics provided support for relationships within a taxonomic order; however, interordinal relationships were rarely supported by these measures, as expected due to their conservative nature (38). We then addressed whether individual gene phylogenies provide consistent support for alternative resolutions of controversial placements of zoosporic lineages in the fungal tree (*SI Appendix*, Fig. S6). This was done by examining the relative likelihood of alternative arrangements of quartets involving these contentious relationships (four taxon trees) using constrained searches (see *SI Appendix*, *Supplemental Results* for more details). These results showed that individual genes generally supported the relationships present in the concatenated tree over the ASTRAL tree (*SI Appendix*, Fig. S6). For example, the majority of the genes (*SI Appendix*, Fig. S6*A*) provide support for Monoblepharidomycota to group with Neocallimastigomycota (50%) versus the alternative placement of Neocallimastigomycota with Chytridiomycota (20%). For the controversial placement of the Blastocladiomycota, 44% of gene trees support Blastocladiomycota as sister to terrestrial fungi and *Olpidium*, and 32% of trees favor Blastocladiomycota as branching before Chytridiomycota (*SI Appendix*, Fig. S6*C*). An approximately unbiased test of these two placements of Blastocladiomycota was nonsignificant ($P = 0.413$), showing that this genetic dataset is equivocal for resolving this relationship (*SI Appendix*, Fig. S7).

Most Major Lineages in the Fungi Are Diplontic. Our genome sequence data provided an unprecedented opportunity to evaluate the ploidy and life cycles of EDF using heterozygosity extracted from the underlying sequencing reads. This approach relies on the representation of *k*-mers or specific sequences of a given length *k* in short-read data (Fig. 3*A*) or the ratios of reads mapped to heterozygous bases in a reference genome supporting reference or alternate alleles (Fig. 3*B*). In the case of a truly heterozygous position in a genome, half of the reads are expected to match one allele and the remaining half should match the alternative allele. We used these approaches to infer ploidy for the 112 taxa included in our phylogenomic analyses for which short-read data were available. The ploidy of another 14 taxa was inferred from long-read data (*SI Appendix*, *Supplemental Materials and Methods*) or existing literature (*SI Appendix*, Table S1). Ploidy inferences for 11 taxa are missing.

The resulting *k*-mer and allele frequency (AF) histograms (*SI Appendix*, Fig. S8) were systematically binned by ploidy based on their similarity to canonical examples of *k*-mer (Fig. 3*A*) and AF (Fig. 3*B*) histograms, in addition to measured density of heterozygous positions (i.e., single-nucleotide polymorphisms [SNPs]) postfiltering. Using all evidence available, we characterized 62 species as diploid, 59 as haploid, 14 as uncertain, and 1 as triploid. Although a portion of our histograms displayed canonical distributions (as shown in Fig. 3 *A* and *B*), many others had noncanonical distributions (e.g., *SI Appendix*, Fig. S8*J*). These noncanonical distributions appear to result from low sequencing depth or whole-genome amplification, among other unknown factors. In general, *k*-mer histograms were less reliable at low sequencing coverage, and we therefore relied more heavily on AF histograms, and their associated SNP densities, to make ploidy calls in marginal cases.

The density of SNPs per base pair appeared to separate noise from sequencing or mapping error from actual heterozygosity. When the density of SNPs of each genome was plotted against the proportion of SNPs falling within one SD of depth-scaled binomial distributions assuming diploidy ("expected range"), we observed clear separation of genomes assigned to either haploid and diploid/triploid ploidy into two groups (Fig. 3*C*). Haploid-annotated genomes formed a tight cluster at low SNP densities (mean = $5.70{\cdot}10^{-5}$; SD = $8.75{\cdot}10^{-5}$) and low numbers of SNPs within the expected range (mean = 16.12%; SD = 16.65%) (Fig. 3*C*). In contrast, diploid genomes formed a broad cluster at high SNP densities (mean = $2.04{\cdot}10^{-3}$;

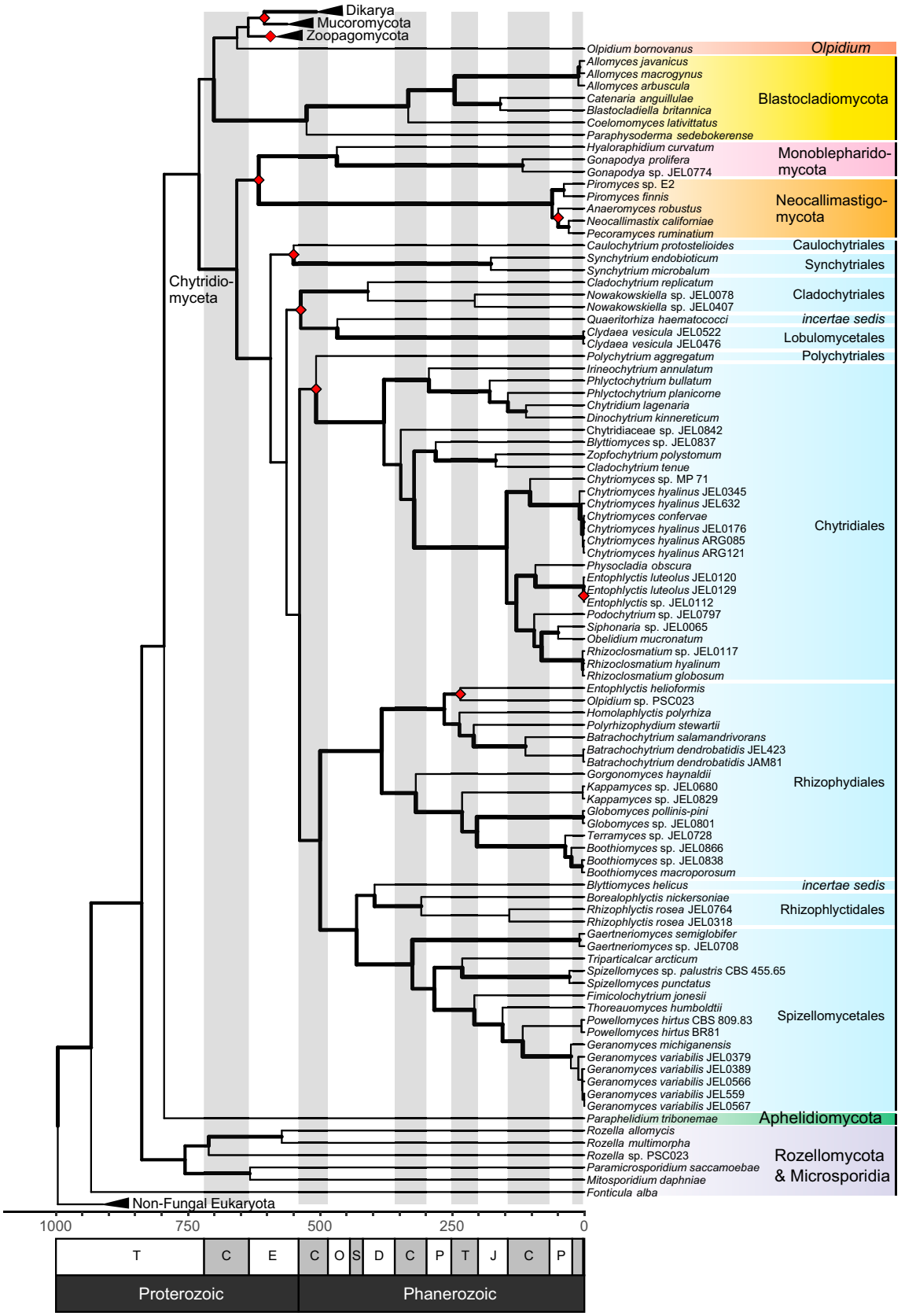

Fig. 2. Annotated, time-calibrated concatenated ML tree of kingdom Fungi, including 68 newly sequenced genomes of zoosporic fungi, based on 197,423 amino acid positions. All bootstrap support values are 100%; edge thickness indicates gCF support, and red diamonds indicate clades that were not present in ASTRAL tree. Blue shading are all taxa in the most species diverse phylum Chytridiomycota. Fossil-based calibration points were used to constrain minimum ages of the MRCA of several clades following Chang et al. (75): Blastocladiomycota = 407 Ma, Chytridiomycota = 407 Ma, Ascomycota = 407 Ma, Basidiomycota = 330 Ma, Mucorales = 315 Ma. A range of dates were used to constrain ages of the MRCA of Dikarya (500 to 650 Ma). The time scale in the chronogram is in millions of years before present with epochs abbreviated in the following order: T: Tonian, C: Cryogenian, E: Ediacaran, C: Cambrian, O: Ordovician, S: Silurian, D: Devonian, C: Carboniferous, P: Permian, T: Triassic, J: Jurassic, C: Cretaceous, P: Paleogene.

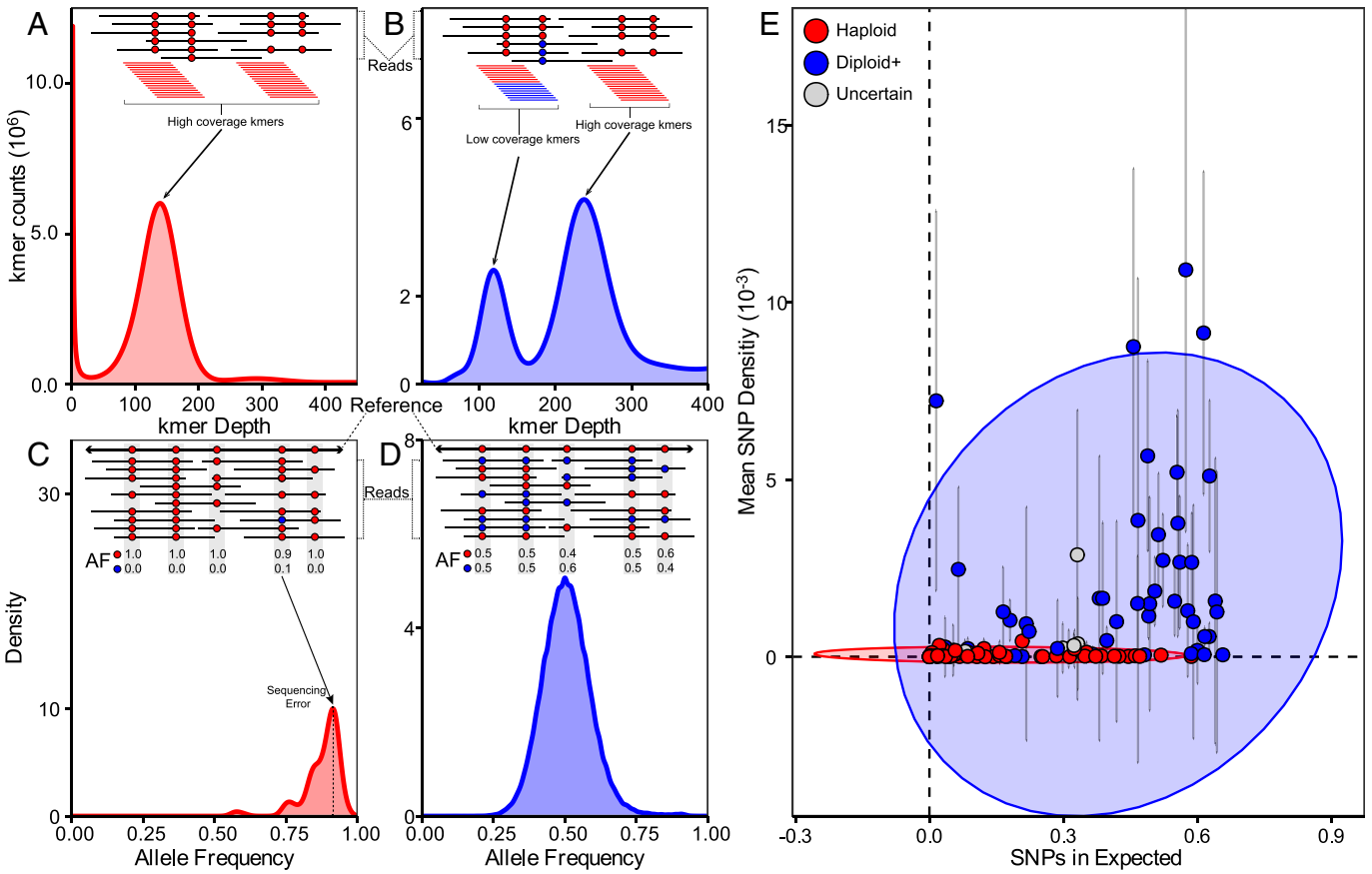

Fig. 3. Summary of ploidy inference for 112 assemblies and their underlying short reads. Curves (*A*–*D*) or points (*E*) are colored by ploidy state (diploid+: blue; haploid: red, uncertain: gray). (*A*) Histogram of *k*-mer counts ($k = 23$) generated from short-read data of *Lobosporangium transversale* CBS455.65 showing the unimodal distribution typical of read libraries derived from haploid genomes. Single peak corresponds to relatively high coverage *k*-mers alone and is approximately centered at mean sequencing depth. (*B*) Histogram of *k*-mer counts ($k = 23$) generated from short-read data of *A. javanicus* California 12 showing the bimodal distribution typical of read libraries derived from diploid genomes. Peaks respectively correspond to relatively high coverage *k*-mers that cover only homozygous positions and low-coverage *k*-mers that also cover heterozygous positions. Peaks are approximately centered at mean sequencing depth and 1/2 mean sequencing depth, respectively. (*C*) Canonical haploid AF histogram (from *L. transversale*) showing right-skewed unimodal distribution corresponding to SNPs introduced by sequencing error. (*D*) Canonical diploid AF histogram (from *A. javanicus*) showing unimodal distribution centered at 0.50 corresponding to SNPs introduced by heterozygous positions on homologous chromosomes. (*E*) Scatter plot of genomes by weighted mean of filtered SNP density across L50 contig set (*y* axis) and proportion of filtered SNPs from L50 contig set falling within 1 SD of the mean of each genome's theoretical binomial distribution (*x* axis). Ellipses are normal ellipses around diploid+- or haploid-annotated points. Error bars represent SD. Dashed lines indicate the origin.

SD = $2.59{\cdot}10^{-3}$) and proportions of SNPs occurring within the expected range (mean = 42.27%, SD = 19.85%) (Fig. 3*C*).

Given the wide range of diversity of ploidy observed across the EDF, we then traced the evolutionary history of ploidy across Fungi by performing an ancestral state reconstruction. We found that most of the ancestral nodes in the tree were reconstructed with diploidy as the more likely dominant phase of the life cycle (Fig. 4 and *SI Appendix*, Fig. S9), but the relative likelihoods of diploidy versus haploidy were mostly similar. We inferred the probability of a diploid or higher ploidy MRCA of all phyla diverging prior to the Mucoromycota, except for the Neocallimastigomycota, to be >50%, with the MRCA of all Fungi having a 59% relative likelihood of being diploid (Fig. 4). Our marginal ancestral state reconstructions were corroborated with a Bayesian Markov chain Monte Carlo stochastic ancestral state reconstruction based on 1,000 simulations (Fig. 4). However, these analyses point to ambiguity in ancestral states at deeper nodes, consistent with frequent transitions between diploidy and haploidy in early fungal evolution. Using a maximum parsimony ancestral state reconstruction, we found that a minimum of 27 transitions between haploidy and diploidy were required to explain the phylogenetic distribution of ploidy we observed (*SI Appendix*, Fig. S10).

Diverse EDF Lineages Lost Ancestral Traits Independently. Discovery of widespread diploidy in zoosporic fungi parallels recent studies that show interpretation of fungal traits based only on analysis of Dikarya may be misleading (4, 5, 7, 8, 39). Many of these misinterpreted traits were those inherited from the Opisthokont MRCA, i.e., are plesiomorphic. We show by mapping these traits onto our comprehensive phylogenies a striking pattern of parallel loss of these plesiomorphic traits throughout fungi (Fig. 5). The best-studied example of parallel loss is the flagellum that has been lost multiple times in the fungi, rendering the zoosporic fungi paraphyletic (Fig. 5). Our genomic data allow us to test for similar trends in genetic traits absent in Dikarya which presumably were traits of the Opisthokont MRCA. The elongation factor-like (EFL) protein that presumably cooccurred with the canonical elongation factor 1-alpha protein in the Opisthokont MRCA (40, 41) showed a punctate pattern with EFL proteins present in *Basidiobolus*, *Olpidium*, *Rozella allomycis*, most members of the Blastocladiomycota, and all examined taxa of the Spizellomycetales (Fig. 5). Selenoproteins, or proteins containing the 21st amino acid selenocysteine, and cobalamin-associated enzymes are examples of genetic traits that were assumed to be absent from fungi but have been recently detected in EDF (7, 39). Both traits are

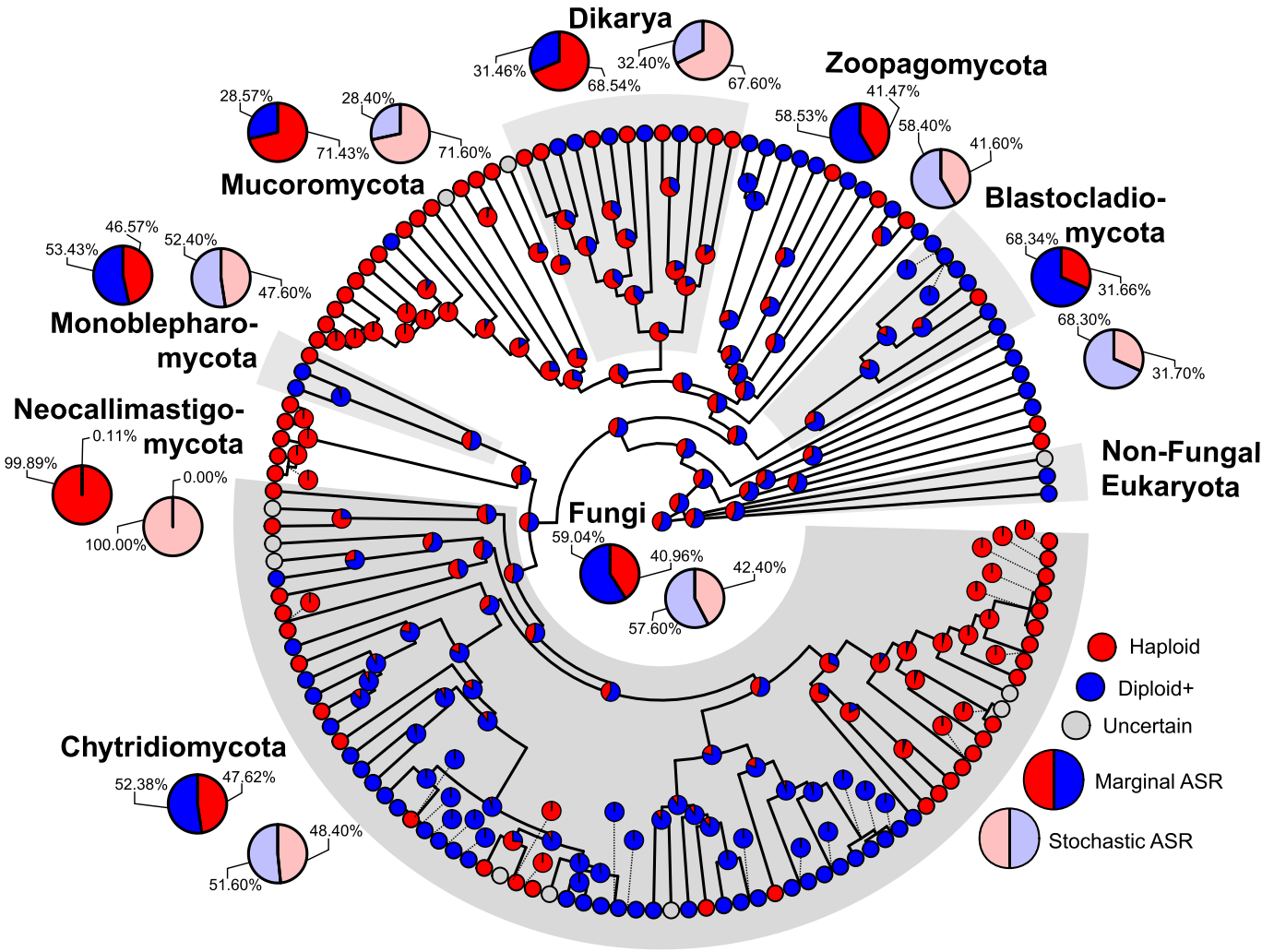

Fig. 4. Best concatenated ML tree annotated with inferred ploidy. Tips are colored according to the ploidy of each genome they represent (red: haploid, blue: diploid, gray: uncertain). Pie charts on internal nodes represent ancestral state probabilities inferred from marginal ancestral state reconstruction of ploidy status across Fungi. Major clades are labeled with bold text and alternating gray–white insets. Major clades are further annotated with the enlarged pie charts that show the ancestral state probabilities of their ploidy status inferred via marginal ancestral state reconstruction (solid, and present on tree edge) or stochastic ancestral state reconstruction (transparent, and absent on tree edge).

widely distributed in EDF but show different phylogenetic distribution patterns. For example, selenocysteine is only encoded in 11 genomes of EDF; however, it is present in all phyla except Ascomycota, Basidiomycota, Aphelidiomycota, and Neocallimastigomycota (Fig. 5). The eight enzymes involved in cobalamin usage are widespread across EDF but absent in Dikarya, with certain lineages containing subsets of enzymes or being completely devoid of detectable enzymes (Fig. 5).

The fungal cell wall and membrane are often considered defining of the kingdom and are frequently used as drug targets (42). Although chitin is a near-universal component of fungal cell walls, the polysaccharides and sterols present are variable (8, 25, 43). Despite the lack of evidence that zoosporic fungi encoded homologous proteins for alpha 1,3 glucan synthesis, we detected AGS1 homologs in diverse Chytridiomycota (Fig. 5). FKS1, the protein responsible for synthesis of beta 1,3 glucans, was only known from Blastocladiomycota among zoosporic EDF (25); however, we detected it in both Aphelidiomycota and Chytridiomycota, consistent with its recent biochemical detection in a chytrid (44). EDF are known to produce a wider diversity of sterols than Dikarya (8), which are reflected in variable presence of protein homologs of the C22 sterol desaturase ERG5, the sterol 14-demethylase ERG11, and DHCR7, the 7-dehydrocholesterol reductase (Fig. 5). DHCR7, associated with cholesterol production, was absent in all Dikarya but detected in all EDF phyla except *Olpidium* and Neocallimastigomycota.

Dikarya, such as budding yeast, have served as premier models for understanding the regulation of cell cycle. Although Dikarya and Metazoa have a similar cell cycle network topology, the actual regulatory proteins are nonhomologous (5). This divergence in regulatory evolution was shown to be driven by the horizontal transfer of the transcription factor SBF from a virus which rewired the ancestral E2F–Rb cell cycle pathway at some point in the early evolution of the Fungi (5). Strikingly, many EDF were recently shown to have different combinations of the ancestral E2F–Rb regulators as well as the derived SBF-Whi5 regulators, whereas Dikarya lost the ancestral pathway and only have the SBF-Whi5 pathway (5). We searched for E2F, Rb, SBF, and Whi5 orthologs across the diverse EDF lineages (Fig. 5). All components of the hybrid network are largely present in the Spizellomycetales and Rhizophlyctidales, whereas differential loss of E2F, Rb, and/or Whi5 characterize most other chytrid groups, such as the Chytridiales and Rhizophydiales. Interestingly, we find some EDF species (e.g., *Quaeritorhiza*, *Hyaloraphidium*, and *Paraphysoderma*) that have all the components of the hybrid network, even though the

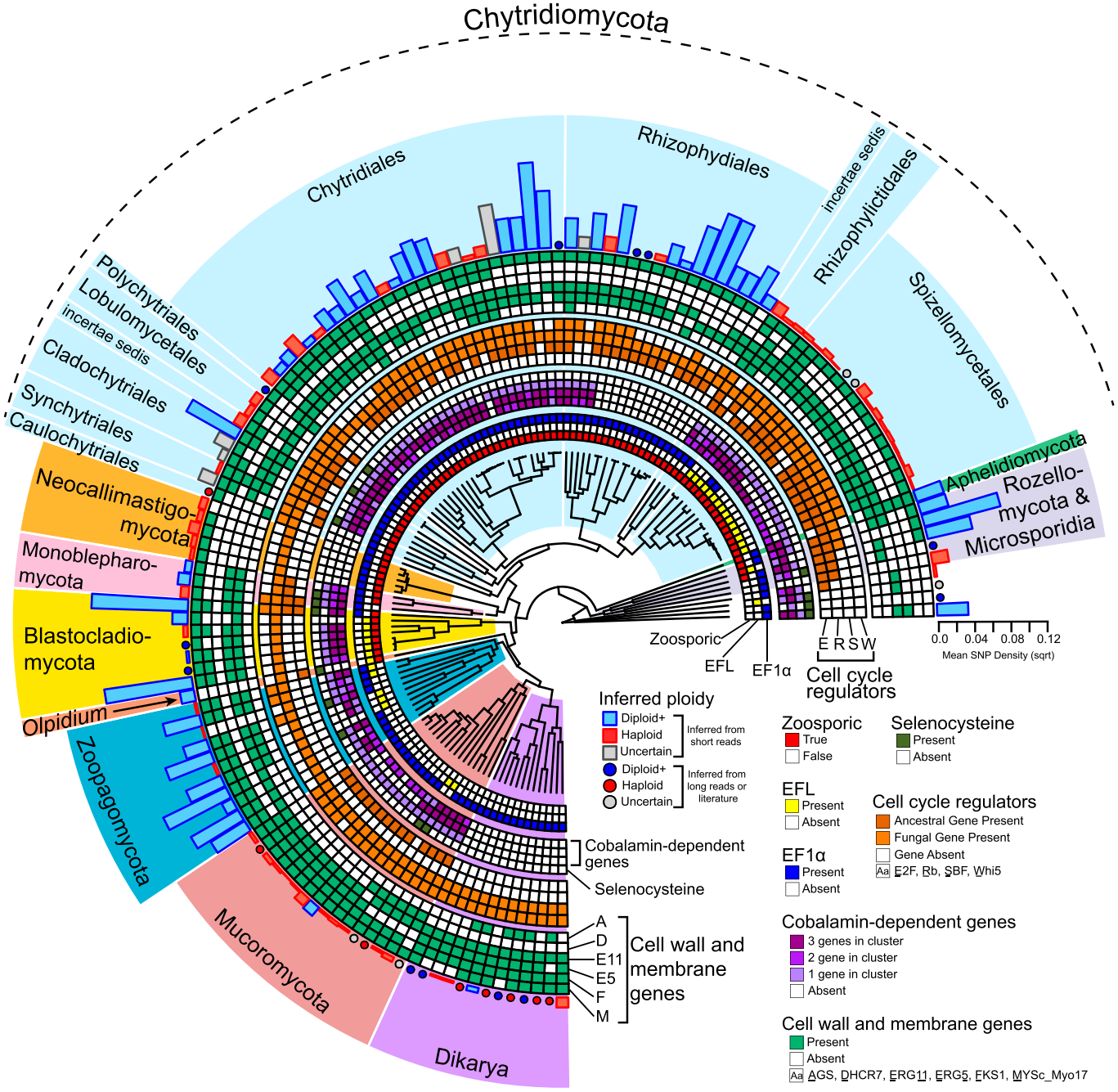

Fig. 5. Best concatenated ML tree showing the distribution of life history or genetic character states across zoosporic fungi. Tracks are colored or abbreviated according to the legend (*Bottom Right*). Genetic traits were characterized across the 137-taxon dataset through searches with gene-specific HMMs. SNP densities (square root-scaled) across draft genomes for which short reads were available are displayed on the outermost track (bars) and colored according to inferred ploidy. Inferred or presumed ploidy in cases where short reads were not available, and SNP density was not calculated are also indicated (points).

other species within their groups (e.g., Lobulomycetales, Monoblepharidomycota, and Blastocladiomycota) are all missing components. Mapping of the SBF-Whi5 origin across the earliest divergences suggests the presence of the transcriptional regulator SBF but not the inhibitor Whi5 in the MRCA of Fungi.

Discussion

The most unexpected result from this research is the finding of widespread vegetative diploidy throughout the non-Dikarya fungal phyla. Although population genomics and single-cell genome sequencing (30, 31, 34) have indicated that nondikaryotic fungi can be diploid, our results extend diploidy to additional lineages, such as Aphelidiomycota, Entomophthoromycotina, Monoblepharidomycota, and Olpidiomycota. Our results are based primarily on heterozygosity of cultivated strains, which presumably represent the dominant life-cycle stage of these organisms. This finding demands a reconsideration of the canonical life cycle of fungi as being primarily haplontic and lacking mitosis at the diploid stage. Instead, the MRCAs of Fungi and most phyla of Fungi may have been diplontic and the haplontic life cycles that characterize some of the major groups (e.g., Mucoromycota, Neocallimastigomycota, and Spizellomycetales) are likely derived.

One caveat to interpreting our ploidy estimates is that because they mostly rely on genome-wide heterozygosity, homozygous genomes will appear haploid. Therefore, our estimation of diploidy is likely an underestimate. Conversely, heterozygosity could

be overestimated by sequencing or genome amplification errors or from mismapping of reads, for example in areas of the genome assembly where there have been recent gene duplications. However, our SNP filtering approach drastically reduced the size of the final SNP set, likely favoring the removal of true heterozygosity over including falsely heterozygous positions. Our pipeline robustly inferred high levels of heterozygosity in some taxa that are well-documented as being diploid, such as *Batrachochytrium* spp. (30, 45) and *Allomyces javanicus* (46) and confirmed haploidy in some taxa that were expected to be, such as the gametophyte stage of *Coelomomyces* (23) and Mucorales (47). In contrast, the life cycles of some zoosporic fungi, including *Chytriomyces hyalinus* (48), *Catenaria anguillulae* (49), and *Paraphysoderma sedebokerense* (50), were suspected of being haplontic; however, all three of these species were observed to have highly heterozygous genomes. In the Blastocladiomycota taxa *Paraphysoderma sedebokerensis* and *C. anguillulae*, ultrastructural views of synaptonemal complexes (49, 50) led to the conclusion that meiosis occurs at the zygote stage leading to a haploid-dominant life cycle.

How can these cytological observations that seem to suggest haplontic life cycles be reconciled with heterozygosity data that suggest that diploidy is the dominant vegetative phase? One possible explanation is that the life cycles of these fungi cycle between diploid and tetraploid. Although this is conceivable, it is also unlikely given the errors that are likely to occur in autotetraploid meiosis (51). Despite the finding that at least *R. allomycis* is triploid, no cases of tetraploidy were uncovered using *k*-mer or allele frequencies as might be expected if this were the case. Another possibility is that many taxa have undergone recent whole-genome duplication, creating a scenario of two similar genomes wherein the heterozygosity is actually divergence between paralogs. If this were the case one might expect to see more divergent alleles and patchy heterozygosity as duplicated regions of the genome either diverge or are lost over time. Both the low level of heterozygosity and evenness of heterozygosity across the genome indicate that this model is unlikely to be accurate. However, whole-genome duplication appears to have occurred in at least one example. *Cladochytrium replicatum* was identified to have a substantial amount of segmental duplication (~70% of assembly duplicated) but with low amino acid identity between copies (~83%). Unlike with other assemblies, mapping reads onto this genome, perhaps unsurprisingly, did not show a typical binomial distribution of allele frequencies. Another possible explanation for the discrepancy between expected and observed rates of diploidy is the possibility that the ultrastructural studies are misinterpreted. There is precedent of synapsis without meiosis in some somatic cells, such as *Drosophila* (52), but the presence of tripartite synaptonemal complexes is considered a meiosis-specific hallmark. Both enhanced studies relating DNA replication and pairing to formation of synaptonemal complexes and studies tracing genetic segregation in appropriate zoosporic fungal models are needed to resolve these discrepancies.

These results show that vegetative haploidy should not be assumed for Fungi, and instead vegetative diploidy is common. However, ploidy is not homogeneously distributed across the nondikaryotic lineages. For example, the Neocallimastigomycota and Spizellomycetales were all estimated as haplontic. For Blastocladiomycota, we identified both haploid and diploid genomes, consistent with the alternation of haploid–diploid generations known for the group. Ploidy differences were observed between the phyla of former Zygomycota (Mucoromycota+ Zoopagomycota), which corroborates their phylogenetic distinctiveness (53). Although diploid mitosis is generally avoided in the Dikarya, some species, including yeasts (54) and the *Armillaria* mushrooms (55), are vegetatively diploid. Furthermore, the dikaryotic life stage that forms the dominant portion of the life cycle in many of the Basidiomycota, and is present in the sexual development of most Ascomycota (56), is genetically analogous to diploidy. The widespread phylogenetic distribution of diploidy and dikaryosis emphasizes the nonhaploid nature of the fungal individual.

Beyond a better basic understanding of fungal life cycles, what are the implications of diploidy among the EDF? The advantages and disadvantages of alternative ploidies have been much discussed (57). One group of explanations focuses on evolutionary advantages. Diploidy is associated with larger organisms, because it buffers against somatic mutations, whereas haploidy is associated with parasitism, which naturally is associated with smaller organisms (58). Over short evolutionary time periods, adaptive evolution may proceed faster in haploids relative to diploids depending on the dominance of beneficial mutations (59). None of these mechanisms is likely to fully explain diploidy of EDF, however. Multicellular and pathogenic taxa exist in many of the EDF phyla, and the haploid lineages we identified are not distinguished by any obvious additional characteristics. Further confounding these issues, the two zoosporic lineages that are apparently haplontic, Neocallimastigomycota and Spizellomycetales, are ones which have never been observed to undergo sexual reproduction and might be expected to suffer from mutation accumulation.

A second group of explanations focuses on the differences in cell biology and metabolism of the different ploidies. It has been suggested that the relationship between ploidy and evolution could be explained by differences in cell or nucleus size (60, 61), given that cells with higher ploidy typically are larger and have a lower surface area/volume ratio. However, it is hard to relate this hypothesis to all fungi due to the incredible differences in size and shape of fungal cells. Yet, two aspects stand out as differentiating Dikarya cells from EDF: increased compartmentalization of cells and lack of motility. First, compartmentalization may have facilitated developmental complexity in Dikarya, and dikaryons may be viewed as diploids with additional cellular complexity. Dikaryotic fungi, particularly the mushroom fungi with dikaryotic phases dominating the life cycle, may have both the advantages of diploidy and the advantages of haploidy, the latter of which includes the increased promiscuity and mating potential demonstrated by haploid nuclei (62). We propose that one overlooked aspect underpinning trends in ploidy in fungi is the motility (or the lack of it) of individuals and gametes as relates to mating. Specifically, a trend toward haplontic life cycles in terrestrial fungi could reflect the loss of motility, because the difficulty of finding a mate is increased in fungi that are immotile. If mating opportunities are limiting, haploids or dikaryons could wield an advantage over diploids in that they need not go through meiosis upon encountering a compatible mating partner. The observation that Oomycetes, the other major group of mycelium-like organisms, have largely retained motility and are generally considered diploid further fuels this speculation (63).

A renewed focus on the EDF of Fungi in recent years has taken place, in part, because phylogenetic studies have revealed an underappreciated diversity in gene content (4, 5, 64). Here, we extend this genetic diversity to life cycle differences and highlight characteristics of cell structure and biochemistry that are much more diverse in Fungi when considering all EDF. Beyond vegetative diploidy, we found that numerous

characteristics of the fungal MRCA cell that are now absent in the Dikarya were maintained during the separation of the major fungal lineages in the Pre-Cambrian. For example, the common ancestor of Chytridiomycota and Blastocladiomycota (~750 Ma) had a complete hybrid cell-cycle network, used the amino acid selenocysteine, had cobalamin-dependent enzymes, and was motile. Since then, the genes encoding these traits were independently lost in the different EDF lineages ("extinction at the tips"). Together, these data indicate a previous fungal dependence on molecules, such as selenium and cobalamin, for many hundreds of millions of years in the Proterozoic during which these phyla separated into major lineages. By the end of the Cambrian, we estimate that most of the EDF phyla and orders of Chytridiomycota were already diversifying, consistent with the fossil record of a wide diversity of chytrid-like fossils by the Devonian (65) and consistent with diversification of algae, which were the dominant photosynthetic life in the Pre-Cambrian (19). In order to expand our understanding of the early branches Fungal tree of life and its full phylogenetic and biochemical diversity, we must turn to the numerous lineages that cannot be cultured and incorporate their genomes using careful consideration of each step of phylogenetic analysis, including homolog retrieval, alignment, model selection, and tree construction. These results should also prompt detailed studies of basic Mendelian genetics and cytology in the overlooked EDF.

Materials and Methods

Strain Selection, Nucleic Acid Preparation, Sequencing, Assembly, and Annotation. We generated genome sequences for 69 chytrid fungi at high coverage, low coverage, or using low-input approaches (*SI Appendix*, Table S1). Cultured strains have been deposited in the CZEUM collection (66). Details on growth and extraction of DNA from cultured strains for sequencing are described in Simmons et al. (66). RNA was extracted according to a modified TRIzol reagent (Invitrogen) protocol. Nucleic acids were either sequenced on PacBio SMRT, Illumina, Sanger, or a combination of PacBio and Illumina platforms. Resulting sequence data were assembled into draft genomes using a variety of assembly, polishing, or filtering methods, mostly dependent on depth of coverage (i.e., high or low coverage), sequencing platform used, and prior knowledge of underlying genome ploidy. Special considerations were made for read libraries prepared via low-input methods [e.g., single-cell (67)]. High-coverage genomes were mostly annotated with the Joint Genome Institute (JGI) annotation pipeline (68) or the Broad Institute annotation pipeline for *Allomyces macrogynus* (69), while both low-coverage and low-input genomes were annotated with funannotate v1.7.4 (70). See *SI Appendix, Supplemental Materials and Methods* for a full description of the above analyses.

Phylogenomic Methods and Related Analyses. To conduct genome-scale phylogenomic analyses that excluded paralogous sequences we generated a filtered set of 487 genes derived from the 758 conserved markers comprising the BUSCO fungi_odb10 database (71). Our filtered gene set was selected through iterative rounds of automatic (e.g., score- or topology-based) or manual filtering of marker gene trees. Gene trees were computed from hidden Markov models (HMMs) and predicted proteomes using a standard approach, with the important modification that multiple sequences for each species were allowed. At the conclusion of filtering, each species was represented by, at most, one sequence per marker (*SI Appendix, Supplemental Materials and Methods*). Our set of 487 markers had a mean representation of 82.02% of species across our 137-taxon set. An ML phylogenomic tree and associated support values (i.e., 100 nonparametric bootstraps, gene concordance factors, and quartet internode certainty) were computed based on the 192,423 amino acid alignment under the LG+R6 substitution model with ModelFinder and IQTree2 (72), or QuartetScores (38). Marginal and stochastic ancestral state reconstructions of ploidy were conducted with phytools (73) in R. We generated a time-calibrated phylogeny from our best concatenated ML tree with r8s v1.81 (74) using fossil-based calibration points for most major clades and a range of allowable dates for Dikarya (75, 76). A coalescent tree was computed based on the 487 individual gene trees in ASTRAL 5.7.3 (77). Separately, using newly compiled or established sets of HMMs, we searched our 137-taxon set for genes linked to dynamic traits of EDF (e.g., use of the 21st amino acid selenocysteine and cell cycle proteins). See *SI Appendix, Supplemental Materials and Methods* for a full description of the above analyses.

Ploidy Estimation. To estimate the ploidy of assemblies across as many of our 137-taxon set as possible, we employed a bipartite *k*-mer ($k = 23$) and AF counting approach (i.e., for taxa with available short reads) or a combined BLAT–BLAST approach (i.e., for taxa with only available long reads). For short reads, *k*-mer counting was conducted on raw short reads using kmercountexact in bbtools (https://sourceforge.net/projects/bbmap/) and allele frequencies were calculated from haploid or haploidized assemblies via a standard SNP calling approach using bwa mem v0.7.15 (78), samtools v1.5 (79), and GATK HaplotypeCaller v4.1.0.0 (80). *K*-mer and AF data were used to construct histograms that were then characterized as supporting haploid (N) or diploid or higher (2N+) heterozygosity. For long reads, BLAT was used to identify syntenic regions of each assembly. These potential allelic regions were investigated for shared gene content and presumed 2N+ when >20% of all gene models were found in allelic regions. Other types of duplicated content (e.g., segmental duplication) were also explored. See *SI Appendix, Supplemental Materials and Methods* for a full description of the above analyses.

Data, Materials, and Software Availability. The sequencing data, assembly, and annotations are available from JGI Fungal Genome portal MycoCosm (68) (https://mycocosm.jgi.doe.gov/mycocosm/home) and have been deposited in GenBank under the Bioprojects listed in *SI Appendix*, Table S1. Additional data and code are available at https://github.com/Michigan-Mycology/Chytrid-Phylogenomics (81).

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Author affiliations: [a]Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI 48109; [b]School of Biology and Ecology, University of Maine, Orono, ME 04473; [c]US Department of Energy Joint Genome Institute, Lawrence Berkeley National Laboratory, Berkeley, CA 94720; [d]Secretaria de Infraestrutura e Meio Ambiente, Instituto de Pesquisas Ambientais, São Paulo 04301-902, Brazil; [e]Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309; [f]Department of Botany and Plant Pathology, Oregon State University, Corvallis, OR 97331; [g]Life Sciences, Yale-NUS College, 138527 Singapore; [h]Department of Entomology, University of California, Riverside, CA 92521; [i]Department of Biochemistry, Robert Cedergren Centre, Université de Montréal, Montréal, QC H3C 3J7, Canada; [j]Infectious Disease and Microbiome Program, Broad Institute of MIT and Harvard, Cambridge, MA 02142; [k]Department of Molecular Biomedical Sciences, North Carolina State University, Raleigh, NC 27606; [l]Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720; [m]Department of Plant Pathology & Microbiology, University of California, Riverside, CA 92521; and [n]Institute for Integrative Genome Biology, University of California, Riverside, CA 92521

Author contributions: K.R.A., D.R.S., I.V.G., J.W.S., J.E.S., and T.Y.J. designed research; K.R.A., D.R.S., J.E.L., S.J.M., K.S., G.H.J., A.E.B., C.A.Q., W.J.D., Y.C., B.A.F., A.K., K.L., J.P., W.A., A.T., R.R., H.H., J.J., A.L., K.B., B.F.L., C.A.C., and N.E.B. performed research; J.E.L. contributed new reagents/analytic tools; K.R.A., D.R.S., S.J.M., K.S., G.H.J., C.A.Q., W.J.D., Y.C., B.A.F., A.K., K.L., J.P., W.A., A.T., R.R., H.H., J.J., A.L., K.B., B.F.L., C.A.C., N.E.B., and T.Y.J. analyzed data; and K.R.A., D.R.S., J.E.L., S.J.M., K.S., N.E.B., I.B.G., J.W.S., J.E.S., and T.Y.J. wrote the paper.

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