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Report

Colponemids Represent Multiple Ancient Alveolate Lineages

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Summary

The alveolates comprise three well-studied protist lineages of significant environmental, medical, and economical importance: apicomplexans (e.g., *Plasmodium*), dinoflagellates (e.g., *Symbiodinium*), and ciliates (e.g., *Tetrahymena*). These major lineages have evolved distinct and unusual characteristics, the origins of which have proved to be difficult evolutionary puzzles. Mitochondrial genomes are a prime example: all three groups depart from canonical form and content, but in different ways. Reconstructing such ancient transitions is difficult without deep-branching lineages that retain ancestral characteristics. Here we describe two such lineages and how they illuminate the ancestral state of alveolate mitochondrial genomes. We established five clonal cultures of colponemids, predatory alveolates without cultured representatives and molecular data. Colponemids represent at least two independent lineages at the phylum level in multilocus phylogenetic analysis; one sister to apicomplexans and dinoflagellates, and the other at a deeper position. A genome survey from one strain showed that ancestral state of the mitochondrial genomes in the three major alveolate lineages consisted of an unusual linear chromosome with telomeres and a substantially larger gene set than known alveolates. Colponemid sequences also identified several environmental lineages as colponemids, altogether suggesting an untapped potential for understanding the origin and evolution of apicomplexans, dinoflagellates, and ciliates.

Results and Discussion

Colponemids Represent Multiple Early-Diverging Alveolate Lineages

Alveolates are a large and well-studied group of microbial eukaryotes of significant environmental and ecological diversity [1–5]. The three main alveolate lineages are each diverse and important in their own right, and each includes a number of significant model organisms: the apicomplexans include the causative agent of malaria (*Plasmodium*), the dinoflagellates include harmful algal species and important endosymbionts of corals (*Alexandrium* and *Symbiodinium)*, and the ciliates include model systems for cell biology (*Tetrahymena* and *Paramecium*). These and related species have played a key role in our discovery and understanding of many characteristics that either are unique to eukaryotes or provide a basis for eukaryote-wide concepts, including the structure and function of telomeres [6], catalytic RNAs [7], the apical complex and cell invasion [8], permanently condensed chromosomes [9], nuclear dualism and DNA rearrangements [10], and mRNA processing [11]. Alveolate plastids and mitochondria have been particularly well studied, because of their functional importance and unique evolutionary histories [12–15], and have thus come to play a central role in our understanding of endosymbiosis and organellar evolution in general [16].

Reconstructing the evolutionary histories of these characters has proved challenging because of the great evolutionary distance between apicomplexans, dinoflagellates, and ciliates, but one solution has come from identifying early-diverging alveolates. The discovery of *Chromera velia* [17] most obviously demonstrates how such lineages can reveal much about these major evolutionary transitions [16, 18, 19]. However, the potential of *Chromera* and other similarly early-diverging lineages (e.g., perkinsids and colpodellids) is limited by the fact that they are specifically related to a particular alveolate lineage, either dinoflagellates or apicomplexans, and cannot address many of the earliest events in alveolate evolution.

Colponemids are an alveolate lineage whose morphology suggests a possible early-diverging position within the group; however, they are not available in culture, and no molecular and little ultrastructural data are available for this lineage [20–22]. To examine their potential to resolve some of the major transitions in alveolate evolution, we isolated five colponemid strains from saline lake, soil, and freshwater samples collected from diverse locations worldwide (Peru, Vietnam, Chukotka, and Caucasus; Experimental Procedures). Clonal cultures from all strains were established from single cells using the prey cultures of *Spumella* sp. OF-40, *Parabodo caudatus*, and *Procryptobia sorokini* grown on a *Pseudomonas fluorescens* suspension. Light microscopy suggested that the five isolates represent at least three morphologically distinguishable species, one corresponding to the previously described *Colponema edaphicum* [20] and two undescribed variants, which we provisionally refer to here as "*Colponema* sp. Vietnam" and "colponemid-like sp. Peru" (Figure S1 available online). The 18S ribosomal RNA gene (18S rDNA) was sequenced to determine their relationship to one another. Phylogenetic analyses supported the interpretation that they represent three distinct species and, more surprisingly, suggested that they form not one, but two distinct lineages within alveolates (Figure S2A). To further address this relationship, we sequenced five additional genes (28S rDNA, *hsp90*, *actin*, *alpha-tubulin*, and *beta-tubulin*) from strains representing all three species, as well as the *Spumella* prey (see the Experimental Procedures). 28S rDNA and Hsp90 trees were

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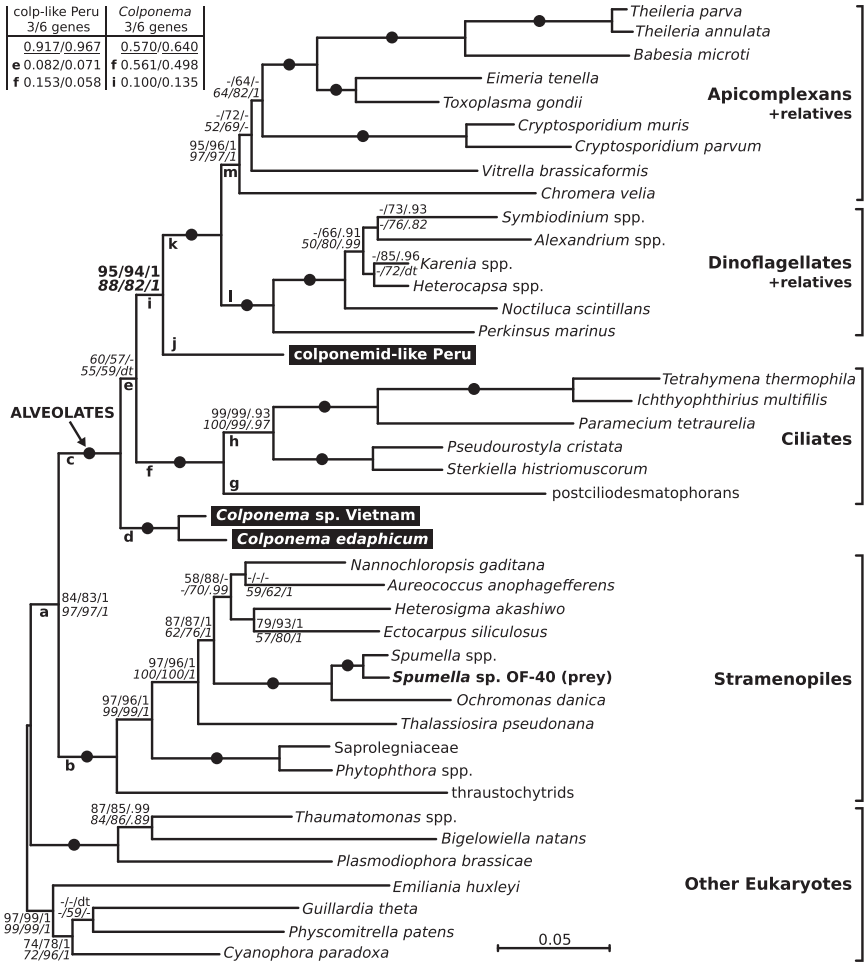

Figure 1. Colponemids Represent Two Deep-Branching Lineages of Alveolates

Maximum-likelihood phylogenies (RAxML) of mixed rDNA and protein matrices of three loci (18S + 28S rDNA + Hsp90; upper values) and six loci (addition of actin + alpha tubulin + beta tubulin; lower values in italics). RAxML rapid bootstrap/Treefinder LR-ELW/MrBayes posterior probability branch supports (>50/>80/>0.90) are shown (dt, different topology). Black dots indicate complete support (100/100/1). Significance values (AU test) for alternative placements of both colponemid lineages (either group at all positions labeled a–m on the tree) were calculated. Tests that were not rejected at the significance level of 0.01 are shown in the upper-left corner (values for the unmodified topology are underlined). Those that were rejected include the sister grouping of both colponemid lineages, a specific relationship of either lineage to apicomplexans or dinoflagellates, and their branching within the ciliates or outgroup. See also Figures S1 and S2.

congruent with the 18S rDNA tree topology (Figures S2A–S2C), whereas Alpha- and Beta-tubulins and Actin separated the strains into the same two lineages, but were inconclusive as to how they related to other alveolates (Figures S2D–S2F). Distinct paralogs of *C. edaphicum* and *Colponema* Vietnam Hsp90 and colponemid-like Peru Alpha-tubulin were found (Figures S2C and S2E). Phylogenies inferred from the combined data set of either all six genes or, alternatively, the three most robust markers (18S rDNA, 28S rDNA, and Hsp90) were consistently well resolved using all algorithms (Figure 1) and strongly support the monophyly of alveolates, ciliates, dinoflagellates, and myzozoans (apicomplexans + dinoflagellates). Colponemid sequences consistently formed two deep and independent alveolate branches: colponemid-like Peru is the closest sister to myzozoans with strong support, whereas *C. edaphicum* and *Colponema* Vietnam branch together at a deeper position near the base of alveolates, or sister to ciliates (Figure 1). Some alternative positions for either colponemid lineage were not rejected in topology tests (Figure 1 and the Experimental Procedures), but the monophyly of colponemids was rejected, as was any specific relationship to either apicomplexans or dinoflagellates, or branching within either of them or ciliates. Colponemids therefore represent two previously unrecognized alveolate groups of great potential utility in reconstructing deep early evolution of apicomplexans, dinoflagellates, and ciliates, at the both morphological and molecular levels.

Colponemid morphology is easily reconcilable with this phylogeny. Colponemids contain typical alveolate features (cortical alveoli and tubular mitochondrial cristae), but feed by phagocytosis (engulfment), presumably as did the ancestor of ciliates, but not the ancestor of myzozoans, which probably fed by myzocytosis (sucking) [23]. Previously, there was a debate about whether colponemids ancestrally also fed by myzocytosis [23, 24], but the phylogeny shows they diverged prior to myzocytotic lineages. Myzocytosis is therefore unlikely to be an ancestral feature of colponemids, although the exact timing of its appearance is limited by the little data currently available. The general morphology of both colponemid lineages is very similar (Figure S1), which suggests that most of their defining characteristics are likely ancestral to all alveolates and that this ancestor was a free-living predator with two heterodynamic flagella, a posterior digestive vacuole, and extrusive organelles for active prey capture (toxicysts or trichocysts) interspersed between the submembrane alveolar vesicles.

The Mitochondrial Genome of Colponemid-like Peru Retains an Ancestral Structure and Gene Content

From the molecular perspective, colponemids have even greater potential to elucidate the origin of a number of unique characteristics in alveolate evolution, and here we investigate their unusual mitochondrial genomes. Myzozoans contain the smallest and some of the most unusually organized mitochondrial genomes, which encode only three protein-coding genes and fragments of the ribosomal RNA genes. The evolution of this extreme reduction has been studied in some detail, but its early evolution is unclear. We conducted a genomic survey from colponemid-like Peru and assembled its mitochondrial genome. The assembled mitochondrial genome mapped as a single 50.4 kbp contig and consisted of a central single-copy region flanked by 17.6 kbp terminal inverted repeats (TIRs) (Figure 2A). The TIRs themselves terminate with a

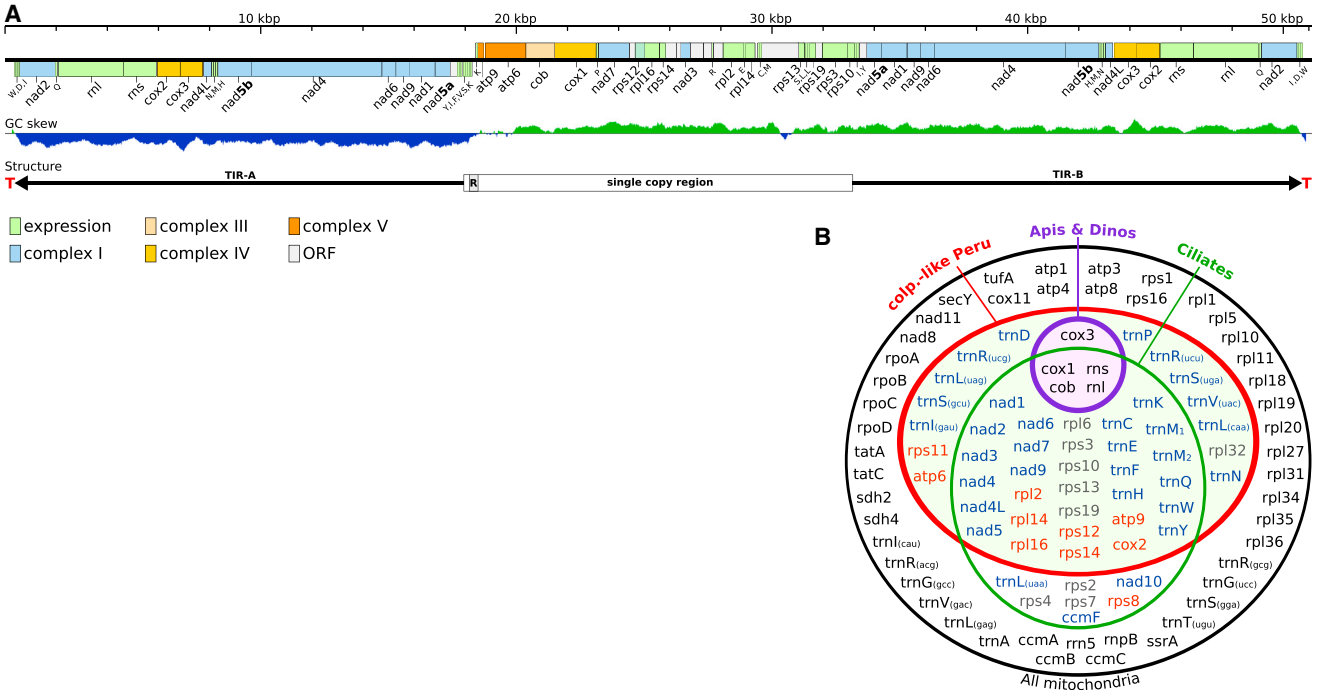

Figure 2. Mitochondrial Genome Structure and Gene Content

(A) Mitochondrial genome map of colponemid-like Peru (top) with genes represented by boxes and color coded according to the key. Genes above the line are transcribed to the right, and genes below are transcribed to the left. GC skew (middle) reveals a putative replication origin in the single-copy region (R). The mitochondrial genome structure (bottom) is linear with two large TIRs terminated by telomeres (T in red; lengths of the telomeres are not known).

(B) Venn diagram of mitochondrial gene content. The sums of all mitochondrial genes in a given lineage are shown in colored circles. Genes acquired secondarily and tRNA genes of rare occurrence were removed, including *trnI(aat)* in colponemid-like Peru. Genes known to have been transferred to the nucleus in myzozoans are in red, those that were lost are in blue, and those that cannot be assigned to either are in gray. Note that alveolate tRNA-Met genes could not be unambiguously distinguished.

See also Figure S3.

conserved 38 bp tandem telomeric repeat. The presence of TIRs, telomeres, and a putative replication origin in the single-copy region (Figure 2A, and see below) suggests that the mitochondrial DNA of colponemid-like Peru is linear monomeric in structure. Telomeres are rarely found in mitochondrial genomes and are best known from fungi and, most interestingly, ciliates [25–27]. Ciliate mitochondrial DNA structure shares much in common with that of colponemid-like Peru: both contain TIRs (or subterminal IRs) and telomeric repeats of a similar length (31–64 bp). Telomeres are widespread in ciliates [28], although they may be absent in *Paramecium* [29]. Colponemid-like Peru and *Tetrahymena* also both share *rnl* genes located in the TIRs and a rapid shift in GC skew (imbalance between G and C content on a given DNA strand) within the single-copy region, indicative of a putative DNA replication origin and bidirectional replication (Figure 2A) [30]. This structure was once thought of as being highly derived, but its distribution in ciliates and colponemid-like Peru (which branches at the base of apicomplexans and dinoflagellates) suggests that this actually represents the ancestral state of mitochondria in all the three major alveolate lineages and colponemid-like Peru. Myzozoan mitochondrial DNAs are also linear, but they lack telomeres, and only those in piroplasmid apicomplexans (e.g., *Theileria* and *Babesia*) are known to be monomeric molecules with TIRs [31]. Because concatemers are more prevalent in apicomplexans, the genomes of piroplasms were considered to be secondarily derived [32], but the colponemid-like Peru genome suggests the opposite: that piroplasms retain the ancestral state. Indeed, in retrospect, it is easier to imagine how either concatemers (e.g., in *Plasmodium* and *Eimeria*) or highly fragmented genomes (e.g., in dinoflagellates) evolved from linear monomers than than vice versa.

Comparing the gene content of the colponemid-like Peru mitochondrion with that of other eukaryotes reveals that it has retained the largest mitochondrial gene set among all alveolates, including 14 genes previously unknown in the group (Figure 2B). One gene, *rpl32*, is only otherwise found in the gene-rich genomes of jakobids. The direct sister relationship of colponemid-like Peru to myzozoans (Figure 1) provides a unique opportunity to examine their mitochondrial genome reduction. In total, 45 mitochondrion-encoded genes must have been lost after the divergence of colponemid-like Peru from the myzozoans (Figure 2B), which represents the largest known genome reduction in any aerobic mitochondria. The main force behind this reduction can be traced to two processes: import of aminoacylated tRNAs from the cytosol [33] and use of alternative NADH dehydrogenase [34]. Together, these processes account for two-thirds of the gene loss from ancestral mitochondrial gene set (30 genes). Of the remaining 15 genes, nine are encoded in the nuclear genome in *Plasmodium*, and the fate of the remaining six is uncertain; all of them encode ribosomal proteins and may have been lost, or they may be too divergent to be detected in the nuclear genome. In either case, this suggests that accelerated relocation of mitochondrial genes to the nucleus played an important supplementary role in the myzozoan mitochondrial genome

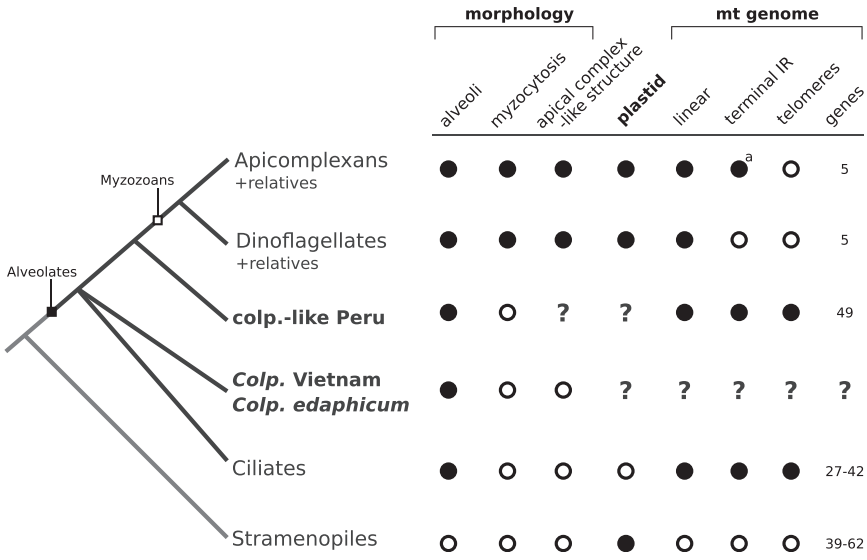

Figure 3. Distribution of Selected Characteristics among Alveolates

Presence or absence of each character is recorded based on the putative ancestral state in the lineage.

reduction. The mechanism of tRNA import is unknown, but it was previously interpreted to have originated very early in alveolate evolution, because ciliate mitochondrial genomes are also somewhat tRNA gene poor (three to nine). However, colponemid-like Peru has retained a large set of 21 mitochondrial-encoded tRNA genes (Figure 2B) and requires import of only three tRNA species, *trnA*, *trnG*, and *trnR*(ncg), in addition to *trnT*, which is absent in most mitochondrial genomes. The ancestral alveolate mitochondrial genome was therefore tRNA rich, and reduction in ciliates and myzozoans occurred independently. An unusual *trnI*(uau) gene with a conserved fold is present in colponemid-like Peru, which is presumably a modified *trnI*(cau). Colponemid-like Peru has also retained a canonical mitochondrial genetic code in contrast to ciliate mitochondrial genomes, where UGA encodes for tryptophan [29].

The colponemid Peru mitochondrial genome also sheds light on a number of unusual modifications in alveolate mitochondrial ribosomal RNAs and proteins. The large and small subunits of the myzozoan ribosomal RNAs are fragmented into multiple pieces [35, 36], whereas those in ciliates are split into two fragments each [37]. The ciliate *nad1* is likewise split into two fragments that are expressed independently, suggestive of a split at the protein level [38]. The homologous genes in colponemid-like Peru are all intact, showing these splits to be independently derived in other alveolate lineages. The myzozoan cytochrome oxidase subunit 2 (Cox2; complex IV) is also split and transferred to the nucleus, the evolutionary significance of which has been a major point of debate [39–41]. The colponemid-like Peru *cox2* is intact and encoded in the mitochondrial genome, supporting the conclusion that the myzozoan *cox2* split occurred independently from other eukaryotes [40]. The cytochrome oxidase subunits in ciliates are even more unusual: both Cox1 and Cox2 contain long insertions, and Cox3 is absent altogether [42]. A number of smaller modifications are also found in ciliates, including the highly divergent C terminus of Cob (complex III) and multiple insertions in complex I subunits (Nad2, Nad4, Nad6, and Nad9). In all cases, colponemid-like Peru encodes significantly less derived genes without insertions or modifications. Interestingly, the colponemid-like Peru *nad5* has been split into two fragments that are widely separated in the genome.

Alveolate mitochondrion-encoded proteins are also notoriously fast evolving [36], so we analyzed a combined matrix of 17 mitochondrion-encoded proteins using the PhyloBayes CAT inference, which provides a more realistic estimate of branch length than standard likelihood approaches [43]. The resulting phylogeny (Figure S3) shows that mitochondrial proteins in both ciliates and myzozoans are substantially faster evolving than those in other eukaryotes, including colponemid-like Peru. Altogether, therefore, the mitochondrial genome of colponemid-like Peru has retained a number of ancestral characteristics that help resolve the course of unusual evolutionary transitions in its stranger but better-studied relatives (see Figure 3 for a summary).

Colponemid Diversity Has a Potential to Illuminate Major Evolutionary Transitions

Previously, no validated colponemid data existed to compare with environmental sequences, so we searched environmental sequence databases using the colponemid 18S rDNA sequences generated in this study. Several matches were identified from diverse habitats and geographical locations, but, remarkably, only two were closely related to any of the five isolates sequenced here (Figure 4). The remaining 21 sequences formed five colponemid-related lineages (CRLs). All CRLs branched either independently of other alveolates or together with the *Colponema edaphicum* clade, albeit with no support (Figure 4). This suggests that the majority of colponemid diversity is yet to be characterized, and each of these deep-branching alveolate lineages may help resolve the many evolutionary transitions that took place during the course of alveolate evolution.

For example, no plastid sequences were identified in our sequence survey. It is possible that plastid genes were simply not sampled, or that plastids were present ancestrally [44], but they or their genomes and all related genes were lost secondarily [45, 46]. If, on the other hand, colponemids ancestrally lacked plastids, then myzozoans must have acquired theirs independently of other eukaryotes. Distinguishing between these scenarios will be critical to reconstructing the evolution of photosynthesis in a major proportion of eukaryotic diversity and for understanding general principles of plastid acquisition and loss.

Phagocytosis in colponemids is another noteworthy process, since it provides the closest ancestral state to myzocytosis, which is associated with the apical complex-like structures used for infection in medically important apicomplexans (Figure 3). Preliminary evidence now suggests that the apical complex may have evolved from the flagellar apparatus [47], which makes colponemid-like Peru a promising candidate in understanding the apical complex origin. Importantly, colponemid-like Peru lacks the longitudinal groove

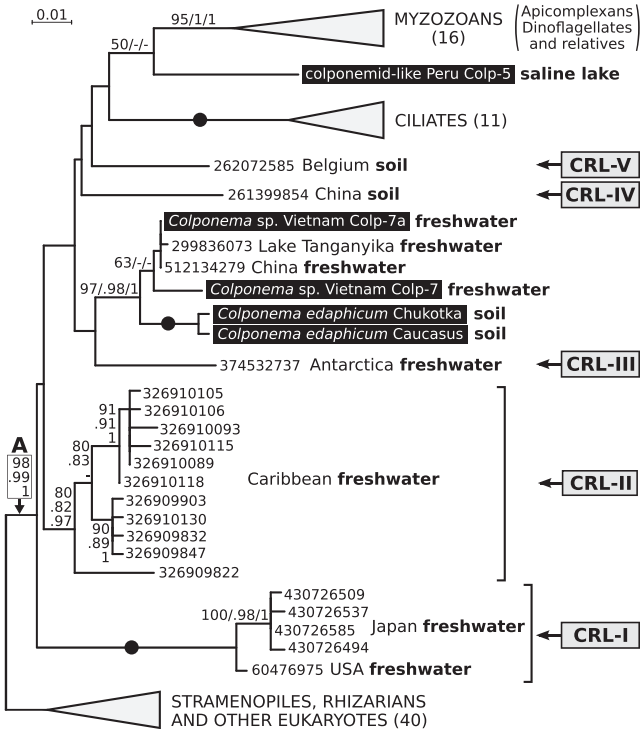

Figure 4. Environmental Diversity of Colponemid-Related Lineages

Maximum-likelihood 18S rDNA phylogeny (RAxML) reveals environmental sequence evidence for further unexplored diversity of colponemids, including additional novel deep-branching clades (A, branch uniting all putative alveolates). RAxML rapid bootstrap/PhyML aLRT/MrBayes posterior probability branch supports (>50/>0.80/>0.90) are shown. Black dots indicate complete support (100/1/1). The number of species in collapsed clades are indicated in brackets.

used for phagocytosis in other colponemids [20–22], and its feeding apparatus remains unknown. It is possible that colponemid-like Peru diverged during the transition between phagocytosis and myzocytosis, so characterizing its flagellar apparatus and excretory organelles (e.g., resembling apicomplexan/myzozoan rhoptries and micronemes) could potentially provide insights into the origin and evolution of the apical complex.

Predatory protists are generally poorly studied, which is unfortunate because many represent lineages diverged around the time of major evolutionary transitions, and characterizing them can resolve a variety of evolutionary problems. Molecular data now show that colponemids represent at least two lineages of deep-branching alveolates that diverged when major changes were taking place in alveolate evolution. Their position in the tree suggests that their general morphology was dominant throughout early alveolate history and that the major lineages, apicomplexans, dinoflagellates, and ciliates, are divergent variants. Their mitochondrial genomes tell a similar story, being composed of relatively gene-rich linear chromosomes with TIRs and telomeres, probably representing the ancestral organization of alveolate mitochondrial genomes, from which the better studied variants arose by massive gene loss, architectural reorganization, and the accumulation of processes like RNA editing. Overall, the colponemids described here, together with other recently described lineages (e.g., *Chromera*), have breathed new life into old debates by offering new ways to reconstruct ancient evolutionary events that are difficult or impossible to resolve by focusing too much attention on mainstream lineages and model organisms.

Experimental Procedures

Colponema sp. Vietnam Colp-7 and Colp-7a were isolated from the sediment of the freshwater Dau Tron Lake (107°20′50″ E, 11°28′47N″), Vietnam, and a shallow pool near the forest road of Cát Tiên National Park (107° 25′55.6″ E, 11°26′38.1″ N), Vietnam, respectively. Colponemid-like sp. Peru was isolated from the sediments of saline lake Supay (76°14′44.38″ W, 14°0′5.18 N″), Pisco Province, Peru. Clonal cultures of *Colponema* sp. Vietnam Colp-7 and Colp-7a and colponemid-like Peru were established from single cells and cultivated using *Spumella* sp. OF-40, *Parabodo caudatus* BAS-1, and *Procryptobia sorokini* B-69 prey, respectively. *Colponema edaphicum* was isolated as described previously [20]. Sampling and culturing are detailed in the Supplemental Experimental Procedures. Genomic DNA was extracted from fresh cells (Colp-7a and colponemid-like Peru) or cells preserved in 96% ethanol (Colp-7). Colponemid ribosomal RNA and protein-coding genes (57 unique clones; only subset of genes was sequenced from closely related isolates; see Figure S2) were amplified using universal eukaryotic primers. All six loci from *Spumella* sp. prey were sequenced; bodonid contaminants and error-containing reads were excluded. Total genomic DNA of colponemid-like Peru was sequenced using Illumina 100 bp paired-end HiSeq technology and assembled in Ray 2.0 using kmer = 21. The mitochondrial genome of colponemid-like Peru (50393 bp) was identified using Blastp searches on two overlapping contigs, the larger of which could be connected to each end of the smaller forming a TIR (17,584 bp each). A conserved tandem telomeric repeat 5′-CCTCTGAGTGAGATTATCTTAATATTCAAAACAAACCC-3′ was found on the outward-oriented side of the TIR (no additional contigs carrying alveolate mitochondrial genes or telomeric sequence were identified). Contamination by *Procryptobia* mitochondrial DNA reads was easily recognizable due to their divergence. Mitochondrial genes were annotated based on Blastp homology to their closest orthologs. All intervening open reading frames longer than 50 amino acids were annotated. Protein-coding genes with low homology were identified using HHpred [48] and Phyre [49]. tRNA genes were predicted in tRNAscan-SE [50] using Mito/Chloroplast search: all had high Cove scores (^{3}57) except for one of the two *trnM* genes (33.56). Mitochondrial genome maps in Figure 2A were drawn with the aid of GView. So that the Venn diagram could be drawn (Figure 3), the identity of all tRNA and protein-coding and genes in alveolate and stramenopile mitochondrial genomes was verified using tRNAscan-SE and BLAST searches, respectively. The identity of the ciliate *trnC*(gca) gene is questionable: it is only found in *Sterkiella* (*Oxytricha*) and has a very low Cove score (21.05). Gene transfers to the nucleus in myzozoans (Figure 2B) were evaluated using BlastP searches in apicomplexan and *Perkinsus* genomes using multiple queries. Preparation of phylogenetic data sets of nuclear and mitochondrial loci, alignment (MAFFT), alignment processing (Trimal, Gblocks), phylogenetic analyses (RAxML, PhyML, TreeFinder, MrBayes, and Phylobayes), and tree topology testing (AU test in Consel) are detailed in the Supplemental Experimental Procedures.

Accession Numbers

The GenBank accession numbers for the sequence data reported in this paper are KF651062–KF651132 and KF651061.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.062.

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