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Phylogenomics reveals Adeleorina are an ancient and distinct subgroup of Apicomplexa

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ABSTRACT

Apicomplexans are a diverse phylum of unicellular eukaryotes that share obligate relationships with terrestrial and aquatic animal hosts. Many well-studied apicomplexans are responsible for several deadly zoonotic and human diseases, most notably malaria caused by *Plasmodium*. Interest in the evolutionary origin of apicomplexans has also spurred recent work on other more deeply-branching lineages, especially gregarines and sister groups like squirmids and chrompodellids. But a full picture of apicomplexan evolution is still lacking several lineages, and one major, diverse lineage that is notably absent is the adeleorinids. Adeleorina apicomplexans comprises hundreds of described species that infect invertebrate and vertebrate hosts across the globe. Although historically considered coccidians, phylogenetic trees based on limited data have shown conflicting branch positions for this subgroup, leaving this question unresolved. Phylogenomic trees and large-scale analyses comparing cellular functions and metabolism between major subgroups of apicomplexans have not incorporated Adeleorina because only a handful of molecular markers and a couple organellar genomes are available, ultimately excluding this group from contributing to our understanding of apicomplexan evolution and biology. To address this gap, we have generated complete genomes from mitochondria and plastids, as well as multiple deep-coverage single-cell transcriptomes of nuclear genes from two Adeleorina species, *Klossia helicina* and *Legerella nova*, and inferred a 206-protein phylogenomic tree of Apicomplexa. We observed distinct structures reported in species descriptions as remnant host structures surrounding adeleorinid oocysts. *Klossia helicina* and *L. nova* branched, as expected, with monoxenous adeleorinids within the Adeleorina and their mitochondrial and plastid genomes exhibited similarity to published organellar adeleorinid genomes. We show with a phylogeneomic tree and subsequent phylogenomic analyses that Adeleorina are not closely related to any of the currently sampled apicomplexan subgroups, and instead fall as a sister to a large clade encompassing Coccidia, Protococcidia, Hematozoa, and Nephromycida, collectively. This resolves Adeleorina as a key independently-branching group, separate from coccidians, on the tree of Apicomplexa, which now has all known major lineages sampled.

1. Introduction

Apicomplexans are widespread obligate parasites and symbionts infecting diverse animal hosts around the world. Over 6,000 species have been described among the millions estimated to infect aquatic and terrestrial vertebrates and invertebrates (Votýpka et al., 2017; del Campo et al., 2019). As obligate parasites and symbionts, all apicomplexans must pass through at least one host to complete their life cycle, however their propensity to influence host development and health exists on a spectrum, from the highly virulent and deadly, such as the malaria-causing *Plasmodium* spp. (Sato, 2021), to less well-studied commensal and potentially mutulistic members such as *Nephromyces* (Saffo et al., 2010; Paight et al., 2019). Similar forms of obligate symbiosis with animal hosts have arisen at least three times in the apicomplexans and the closely-related squirmids and chrompodellids, all of which arose from a free-living photosynthetic ancestor. Within these groups, convergent evolution has been identified as a recurring theme, affecting their red-algae derived plastids, mitochondria, and apical structure used for feeding and infection (Mathur et al., 2019).

Major subgroups within the Apicomplexa are the Coccidia, Protococcidia, Hematozoa, Nephromycida, Marosporida, Gregarinasina, and Cryptosporidia. Among these subgroups, the coccidians and

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hematozoans have been best-studied because they include model systems and human pathogens like *Toxoplasma* and *Plasmodium* (Kim and Weiss, 2004). More recently, there has been progress on the genomics of early-branching gregarines because they have provided insights into the origin and early evolution of apicomplexans (Mathur et al., 2019; Boisard and Florent, 2020). This latter work was powered by significant advances in culture-free genomics and transcriptomics, which have enabled previously less well-studied major apicomplexan subgroups to be assessed with phylogenomic methods that generate more robust phylogeny by using hundreds of proteins instead of just one gene, like the SSU rDNA.

Culture-free genomics and phylogenomics have also recently been used to pin-point the phylogenetic position of other apicomplexans whose relationships have been historically enigmatic. In particular, the recent demonstration that several disparate groups that were not thought to be related actually collectively form a new class, Marosporida (Mathur et al., 2021). These advances have led to a very robust tree of apicomplexans, but a few lineages remain to be added, and perhaps the largest and most diverse of these are the adeleorinids. Adeleorina is a suborder of apicomplexans comprising over 500 described intracellular species infecting blood and tissue cells in invertebrate and vertebrate hosts across the world (Barta, 2000; Netherlands et al., 2019). The group consists of monoxenous lineages that infect one host (Adeleidae, Legerellidae, and Klossiellidae), known as the adelines, and a monophyletic heteroxenous lineage, known as the haemogregarines, where members infect two or more hosts (Dactylosomatidae, Haemogregarinidae, Hepatozoidae, and Karyolysidae) (Barta, 2000; Votýpka et al., 2017; Barta et al., 2012). The families Adeleidae and Klossiellidae diverge near the root of this group in phylogenetic analyses but the position of Legerellidae is unresolved as this is the only known group within Adeleorina with no genetic data reported (Barta et al., 2012; Lévellé et al., 2019a). Indeed, molecular data for the group are scarce in general, in contrast to other long-established major apicomplexan clades. The majority of reported molecular data is restricted to SSU rDNA markers representing a small portion of all described species. Mitochondrial and apicoplast genomes have been reported from a few adeleorinids (Léveillé et al., 2014; Léveillé et al., 2020; Zeldenrust and Barta., 2021; Léveillé et al., 2019a,b; Hrazdilová et al., 2021). No genome or transcriptome data has been reported from any member of Adeleorina.

Adeleorina apicomplexans have traditionally been considered coccidians (Votýpka et al., 2017), but their position in the tree of Apicomplexa is essentially unresolved (Barta et al., 2012; Maia et al., 2016). Phylogenies using the SSU rDNA from a wide range of adeleorinid and other apicomplexan taxa have generated trees using three different phylogenetic analysis methods, but these could only conclude that Adeleorina formed a monophyletic clade in various possible positions related to Coccidia and Hematozoa (Barta et al., 2012). Subsequent SSU rDNA analyses have shown adeleorinids in different positions: within the Hematozoa sister to Piroplasmida (Léveillé et al., 2019b), sister to Coccidia (del Campo et al., 2019; Kopecná et al., 2006; Chagas et al., 2021; Zeldenrust and Barta, 2021), sister to Hematoza and Coccidia collectively (Cavalier-Smith, 2014; Vázquez et al., 2022; Pales Espinosa et al., 2023), or, when combined with LSU rRNA, branching with Nephromycida and collectively sister to Coccidia (Kwong et al., 2020). The only other molecular data are from the organelles: an apicoplast protein phylogeny showed Hematozoa and Marosporida forming a clade branching sister to Adeleorina (Pales Espinosa et al., 2023), whereas a mitochondrial protein-based tree showed Adeleorina branching sister to Coccidia (Kwong et al., 2020).

To provide a more comprehensive molecular dataset to compare adelorinids with other major apicomplexan groups, we have generated genomic and transcriptomic data from two species, *Klossia helicina* and *Legerella nova*. These are the first nuclear transcriptomes from any adelorinid, and *L. nova* also represents the only remaining family within Adeleorina without any molecular data to date. We also sequenced complete plastid and mitochondrial genomes from both species. Using phylogenomics we resolve the placement of Adeleorina in the phylogeny of Apicomplexa, revealing a strongly supported clade that is not closely related to any other major subgroup. Instead, the adeleorinds branch after Marosporidia and sister to Coccidia, Protococcidia, Hematozoa, and Nephromycida collectively.

2. Materials and methods

2.1. Sample collection

The definitive host of *Klossia helicina*, the grove snail *Cepaea nemoralis*, were obtained from residential areas from the municipalities of Richmond, Delta, and Vancouver in British Columbia, Canada. *Cepaea nemoralis* were euthanized prior to dissection using a 2-step method involving anesthetization followed by euthanasia (Gilbertson and Wyatt, 2016). To ensure complete anesthetization of *C. nemoralis*, which are of a different species and size than the snails from the original method (Gilbertson and Wyatt, 2016), the ethanol concentration was increased to 10 % and 80 % for the anesthetization and euthanasia steps, respectively, and an incubation period of 1 h was used for each step. Euthanized snails were rinsed in water to remove ethanol. After removing the shell, the snail body was resubmerged in water to rinse off residual ethanol prior to dissection. Kidney tissue was transferred to a new dish with 1 × phosphate-buffered saline (PBS), teased apart using fine forceps, then observed under a Leica DM IL LED inverted microscope.

Cells of *K. helicina* were collected using hand-drawn glass micropipettes and rinsed twice in 1 × PBS to wash off host cell debris. Images were taken at 63 × magnification with a Sony alpha 7RIII digital camera attached to the microscope. Cells were sorted by life cycle stage based on previous descriptions (Woodcock, 1911; Barta, 2000) and 4–20 cells were pooled into individual 20 μl thin-walled PCR tubes containing 2 μl of Smart-Seq2 cell lysis buffer (2 U/μl RNaseOUT Recombinant Ribonuclease Inhibitor [Invitrogen] in 0.2 % [vol/vol] Triton X-100) for single-cell transcriptome preparation, and 2 μl of ultrapure water for single cell whole genome amplification (WGA) (Barta, 2000; Picelli et al., 2014). Previous studies used liquid nitrogen and 37 °C water bath freeze–thaw cycles to break open oocysts of *K. helicina* for the extraction of genetic material (Barta et al., 2012). Our attempt at breaking open mixed cyst stages including oocysts using this method with the freezing temperature at −80 °C was unsuccessful for RNA extraction. Instead, a 1000 μl pipette tip was melted at the tip over a Bunsen burner to form a closed ball approximately the size of the end of the 20 μl collection tube, at the same time sterilizing it. Once the pipette tip has cooled down, it was used as a pestle to manually break open cells post freeze–thaw to release cell material for downstream steps.

The host of *Legerella nova*, the pill millipede *Glomeris marginata*, were collected in Bonn, Germany in April 2022. Specimens were kept at 8 °C until dissection. *Glomeris marginata* were euthanized in a kill jar with acetone vapor, after which their Malpighian tubules were removed into 1 × PBS and inspected for the presence of oocysts under 63 × magnification with a Leica DM IL LED inverted microscope and a Fujifilm X-S10 camera. In total, 23 pill millipedes were dissected (3 males, 20 females, 2 undetermined sex), of which 4 (1 male, 2 females, 1 undetermined) had *L. nova* oocysts. Cells picked with glass micropipettes, washed with 1 × PBS, and combined into pools of up to 6 cells in 2 μl of cell lysis buffer (2 U/μl RNaseOUT Recombinant Ribonuclease Inhibitor [Invitrogen] in 0.2 % [vol/vol] Triton X-100) and frozen until Smart-Seq2 library preparation. A piece of Malpighian tubule with a high density of *L. nova* was collected in 40 μl of 1 × PBS for genomic DNA extraction.

2.2. Single cell genome and transcriptome extraction, sequencing, and assembly

Transcriptomes were generated from *K. helicina* and *L. nova* cells

using the Smart-Seq2 (Illumina Inc.) protocol, with PCR amplification adjusted to 23 cycles due to low quantity of starting cell material (Picelli et al., 2014). Genomic DNA was extracted from *K. helicina* single cells using the 4BB TruePrime Single Cell WGA Kit (4basebio SLU). To obtain *L. nova* genomic DNA from Malpighian tubule tissue, we used the DNeasy PowerSoil Pro Kit (Qiagen) according to the manufacturer's instructions and eluted the DNA in 50 μl of 1 mM Tris solution. DNA was quantified with the Qubit dsDNA HS Assay Kit on a Qubit 2.0 Fluorometer. For *K. helicina* and *L. nova* cDNA and *K. helicina* genomic DNA, samples above 2 ng/μl concentration were sequenced. For *L. nova* genomic DNA, a single sample of 1.5 ng/μl concentration was sequenced.

Sequencing libraries for genomic DNA and cDNA products were generated using the Nextera XT DNA Library Preparation Kit (Illumina Inc.). Sequencing was performed on the Illumina NextSeq platform using 150 bp paired-end reads at the University of British Columbia Sequencing Centre (cDNA libraries and *K. helicina* genomic DNA) and at the Instituto Gulbenkian de Ciência Genomics Facility (*L. nova* genomic DNA).

Raw reads from multiple samples and sequencing runs were merged to generate final transcriptome datasets. Recovered reads for *K. helicina* samples ranged from 8,342,353 (1,259,695,303 bp) to 16,617,794 (2,509,286,894 bp). Sequencing runs were repeated for two samples recovering 61,700,273 (9,316,741,233 bp) and 86,078,183 (12,997,805,633 bp) from each repeat. The original runs, repeated runs, and 6 additional *K. helicina* samples were merged to assemble the final *K. helicina* transcriptome dataset. The final *L. nova* transcriptome dataset was assembled by merging 13 samples with reads ranging from 7,254,148 (1,080,868,052 bp) to 14,663,520 (2,184,864,480 bp). Raw read quality was assessed using FastQC. TrimGalore v0.6.6 was used to remove adaptor sequences and low-quality bases. Transcriptome reads were assembled using SPAdes v3.15.1 with the -rna option. *Klossia helicina* genomic reads were assembled with SPAdes v3.15.1 and the -sc option for genome assembly. *Legerella nova* genomic reads were assembled with MegaHit v1.2.9 (Li et al., 2015) with default options. To confirm that the sequenced samples indeed contained *K. helicina* and *L. nova*, SSU rRNA sequences from the assemblies were extracted and queried by BLASTN against the NCBI GenBank nr database. Transcriptome samples that passed this identification were reassembled together with SPAdes v3.15.1 to maximize completeness. Animal host contamination was filtered out from genomes and transcriptomes by processing assembled reads through megaBLAST searches against the NCBI nr nucleotide database followed by diamond BLASTX against the UniProt reference proteome database (Altschul et al., 1990; UniProt Consortium, 2021). Visualization of contig contaminants was achieved using BlobTools and contaminants belonging to bacterial, archaeal, and metazoan groups were removed (Laetsch and Blaxter, 2017). TransDecoder v5.1.0 was used to predict open reading frames (ORFs) and coding genes and longest ORFs were searched against the Uniprot database using BLASTP to generate annotations based on similarity (Haas, 2015; UniProt Consortium, 2021). Estimations of assembly completeness were made using BUSCO v5.4.3, using the alveolate database (Simão et al., 2015).

2.3. SSU rRNA phylogenetic analyses

Nuclear SSU rRNA genes of *K. helicina* and *L. nova* were extracted from assembled transcriptomes. Published sequences were retrieved from NCBI GenBank. To maximize phylogenetic resolution, sequences < 1000 bp in length were excluded, except for *Haemogregarina bigemina* which was previously thought to be an adeleorinid but instead branches with other unidentified marine fish-infecting apicomplexans sister to corallicolids on phylogenetic trees and is the only described member of this clade (Hayes and Smit, 2019). Sequences were aligned with MAFFT v7.481 (Katoh et al., 2002), then unaligned 5′ and 3′ ends were manually trimmed before trimming with trimAl v1.4 using the automated1 method (Capella-Gutiérrez et al., 2009). A maximum likelihood tree was built using IQ-TREE v1.6.12 with 1000 non-parametric bootstrap replicates and a GTR + F + R5 substitution model chosen by ModelFinder (Nguyen et al., 2014; Kalyaanamoorthy et al., 2017).

2.4. Apicoplast and mitochondrial genome assembly and annotation

Klossia helicina and *L. nova* genome assemblies were used to search for apicoplast contigs using BLAST (Altschul et al., 1990) with published Adeleorina apicoplast sequences as queries. Contigs identified as belonging to the apicoplast were then used as templates which all trimmed raw reads were mapped against them with Bowtie2 v2.4.2 (Langmead and Salzberg, 2012). Reads that successfully mapped were extracted with SAMtools (Danecek et al., 2021), and manually reassembled and visualized using Tablet v1.12.02.08 (Milne et al., 2010). This procedure was repeated to assemble and close (circularize) the genome. Apicoplast genome annotation was completed manually on Geneious Prime 2022.0.1 (Biomatters Ltd.) using tRNA predictions from tRNAscan-SE 2.0 (Chan and Lowe, 2019) and rRNA, ribosomal protein genes, and other protein encoding gene predictions from a combination of RNAmmer v1.2 (Lagesen et al., 2007), MFannot (https://megasun.bch.umontreal.ca/apps/mfannot/), and Geneious Prime's find open reading frame and annotation prediction function with apicomplexan apicoplast reference genomes.

Similarly, mitochondrial contigs were recovered from the *K. helicina* and *L. nova* genome assemblies by selecting high-coverage low GC content contigs and verifying against related apicomplexan mitochondrial sequences using BLAST. Multiple *K. helicina* mitochondrial contigs were identified but could not be circularized using the technique described to close the apicoplast genome. The single *L. nova* mitochondrial contig we identified was circularizable, suggesting it represents a complete mitochondrial genome. The *L. nova* mitochondrial genome was annotated manually on Geneious Prime 2022.0.1 (Biomatters Ltd.) using predictions of protein coding genes from MFannot with translation table 4 (Mold, Protozoan, and Coelenterate Mitochondrial) (https://megasun.bch.umontreal.ca/apps/mfannot/) and predictions of small and large rDNA fragments made using Geneious Prime's annotation prediction function with *K. helicina* (MT084563), *K. razorbacki* (NC_058856), and *Klossiella equi* (MH203050) mitochondrial reference genomes. The putative fragments were checked for sequence similarity using BLAST and predictions recovering 97.5–100 percent identity to apicomplexan mitochondrial genomes retained.

2.5. Phylogenomic tree construction and analyses

Newly generated transcriptomic data from *K. helicina* and *L. nova* and additional taxa with publicly available genomic and transcriptomic data were added to an existing set of 263 genes used previously for apicomplexan phylogenomic analyses containing representatives from most eukaryotic supergroups (Mathur et al., 2023). BLASTP was used to search coding sequences predicted by TransDecoder v5.1.0 during assembly for 263 genes used in the set with an e-value threshold of 1e-20 and query coverage threshold of 50 % (Haas, 2015). To identify poorly aligned sequences to extract from newly identified genes, BLAST outputs were searched against the UniProt database using BLASTP (UniProt Consortium, 2021). After poorly aligned sequences were removed the final 263 gene set was aligned using MAFFT L-INS-i v7.481 (Katoh et al., 2002). The alignments were used to construct single gene trees with IQ-TREE v1.6.12 and viewed with FigTree v1.4.4 to manually screen for contaminants and paralogs (Nguyen et al., 2015; Rambaut, 2014). Cleaned tree sets that had a maximum of 40 % missing genes were trimmed with the automated1 method in trimAl. The initial dataset included all taxa, including long-branching taxa. To account for the effects of extremely divergent members within Apicomplexa like long branch attraction or other artefacts, long-branching taxa were removed from the main dataset used to generate the main figures. The final

dataset was concatenated in SCaFoS v4.55 resulting in a concatenated alignment consisting of 67 taxa, 206 proteins, and 51,460 amino acid sites (Roure et al., 2007). A phylogenomic maximum likelihood tree was built using IQ-TREE v1.6.12 under the LG + C60 + F + G4 mixture model with 1,000 ultrafast bootstrap replicates (UFB) (Nguyen et al., 2015; Minh et al., 2013).

Several subsequent analyses were performed to explore the support and robustness of the position where Adeleorina emerged. The former dataset that still contained long-branching taxa was analyzed in parallel to the main dataset using the same parameters in all additional analyses including the generation of a phylogenomic maximum likelihood tree with 74 taxa, 203 proteins, and 48,966 amino acid sites. The initial maximum likelihood tree was used as the input guide tree to run the Posterior Mean-Site Frequency (PMSF) model under the LG + C60 + F + G4 model using 200 non-parametric bootstrap replicates (BS) in IQ-TREE v1.6.12 for rapid approximation of the profile mixture model to evaluate node support (Wang et al., 2017). Bayesian analyses was performed with PhyloBayes-MPI 20,201,026 using the CAT + GTR model with 4 parallel chains allowed to run simultaneously until a cutoff point of minimum 10,000 cycles each was reached (Lartillot et al., 2009). The first 10 % of trees generated in each chain were discarded as burn-in before calculating differences in bipartition frequencies with -bpcomp

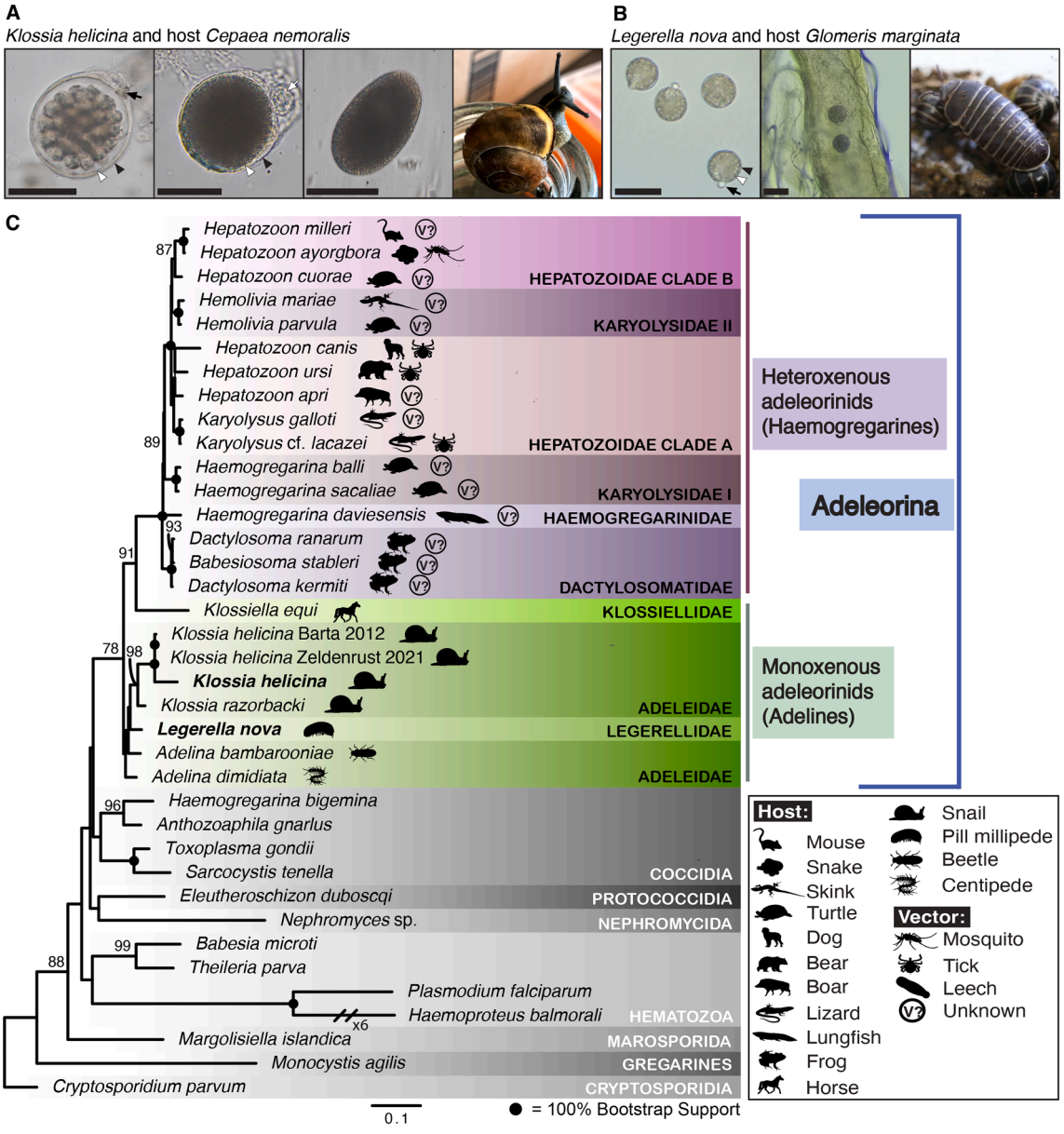

Fig. 1. Light micrographs of *Klossia helicina* and *Legerella nova* cells, pictures of hosts, and phylogeny of the Adeleorina **A.** From left to right the micrographs show a *Klossia helicina* sporulated oocyst, unsporulated oocyst or macrogamete associated with a microgamete, unsporulated oocyst or macrogamete, and the grove snail host *Cepaea nemoralis*. **B.** Light micrographs of *Legerella nova* from left to right show oocysts with naked sporozoites, oocysts in Malpighian tubules of the pill millipede host, and the pill millipede host *Glomeris marginata.* Scale bar = 50 μm. Black arrow = host nucleus; White arrow = host nucleus or microgamete; White triangle = inner membrane originating from parasite; Black triangle = outer membrane originating from host. **C.** Maximum-likelihood SSU rDNA tree of 37 apicomplexan taxa, with a focus on Adeleorina subgroups, generated under the GTR + F + R5 model with 1000 non-parametric bootstrap replicates. Heteroxenous lineages of Adeleorina (haemogregarines) are shaded in purple. Monoxenous Adeleorina (adelines) are shaded in green. All other major apicomplexan subgroups are shaded in grey. Newly added adeleorinids *Klossia helicina* and *Legerella nova* are indicated in bold. The branch supporting *Haemoproteus balmorali* has been reduced to 1/6 of its original length. The tree is rooted on *Cryptosporidium parvum.* Bootstrap supports under 70 % are omitted, nodes with full bootstrap support are denoted by a black circle. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to assess convergence of the four chains and manually inspecting consensus trees from each chain (maxdiff = 1). We removed fast-evolving sites in 5 % incremental steps until a maximum 95 % was removed (Irwin, 2021). The site-specific substitution rates were generated using the -wsr option in IQ-TREE v1.6.12. A maximum likelihood phylogenomic tree was made for each increment with IQ-TREE v1.6.12 using the LG + C60 + F + G4 model and 1,000 ultrafast bootstrap replicates. To assess topology robustness the approximately unbiased (AU) test (Shimodaira, 2002) was performed on 12 trees representing a range of possible topologies possible, some based on the original maximum likelihood tree, maximum likelihood tree including long-branching apicomplexans, positions in published phylogenetic trees (Léveillé et al., 2014; Léveillé et al., 2020; Zeldenrust and Barta, 2021; Léveillé et al., 2019a; Léveillé et al., 2019b; Hrazdilová et al., 2021).

3. Results and discussion

3.1. Identification and isolation of Klossia helicina and Legerella nova

Klossia helicina and *L. nova* were manually isolated from dissected kidney tissue of grove snails and the Malpighian tubules of pill millipedes (Fig. 1A, B), respectively, and the material was used to generate transcriptomes and organellar genomes. This study is the first report of *K. helicina* in grove snails in Canada. Previous attempts at finding the adeleorinid in Eastern Canada were unsuccessful and until now reports of *K. helicina* were limited to grove snails originating from Europe (Barta et al., 2012). Sporulated oocysts and cells that may be unsporulated oocysts or macrogametes of *K. helicina* were spherical and ranged from 70 to 80 μm in length (Fig. 1A). Sporozoites within sporulated oocysts measured 12.5 μm in diameter (Fig. 1A). A distinctively elongated cell that was either an unsporulated oocyst or macrogamete measured 90 μm in length and 51 μm in width (Fig. 1A). *Legerella nova* oocysts with naked sporozoites measured up to 30 μm (Fig. 1B). This was consistent with previous reports of comparatively large adeleorinid oocysts and macrogametes, relative to invertebrate host cells (Barta, 2000; Volkmann, 1967; Vincent, 1927).

Both adeleorinids had distinct inner and outer membranes with a structure situated in between (Fig. 1A, B). Previous descriptions identify the inner membrane originating from the adeleorinid while the outer membrane is what remains of the host membrane highly distended well beyond its original size (Vincent, 1927; Volkmann, 1967). The structure situated between the membranes in *K. helicina* and *L. nova* sporulated oocysts is likely to be the remaining hypertrophied host nucleus (Fig. 1A, B) (Volkmann, 1967; Vincent, 1927). However, distinguishing this structure as a hypertrophied host nucleus beside an unsporulated oocyst or, alternatively, a microgamete attached to a macrogamete in syzygy in *K. helicina* is difficult under light microscopy as both a microgamete and a hypertrophied host nucleus would appear as small structures directly adjacent to a larger pigmented cell (Fig. 1A) (Volkmann, 1967; Schulte, 1971, Scholtyseck et al., 1971). The mechanism behind the manipulation, maintenance, and function of these host structures and the adeleorinid parasite requires further investigation to be understood.

3.2. Phylogenetic placement based on SSU rDNA

Small subunit rRNA sequences were extracted from *K. helicina* and *L. nova* transcriptomes and used to infer a Maximum-likelihood (ML) phylogenetic tree with 37 taxa representing all 7 known sub-groups of Adeleorina (Fig. 1C, Table S1) (Barta, 2000). *Legerella nova* is the only taxon representing Legerellidae since it is the first member of the group with available molecular data. The genera *Cyrillia* and *Disseria* are considered to be members of the Haemogregarinidae, but could not be included in the adeleorinid tree as presently no molecular data have been obtained from any members of these genera. Adeleorinid taxa from a variety of definitive and intermediate hosts as well as host geographical location were selected to represent adeleorinid prevalence and distribution, as they have been found on every continent except Antarctica so far (Fig. 1C, Table S1).

Klossia helicina from grove snails from Western Canada branched with *K. helicina* obtained from grove snails in Europe with strong support (Fig. 1C, 98 % BS), forming a *Klossia* clade with *K. razorbacki* from the North American magnolia three-toothed snail *Triodopsis hopetomensis* (Fig. 1C, 65 % BS) (Zeldenrust and Barta, 2021). *Legerella nova* branched within Adeleorina, but without significant support and within the Adeleidae clade sister to the *Klossia* clade (Fig. 1C, 65 % BS). Availability of published molecular data from monoxenous adeleorinids is scarce compared to heteroxenous adeleorinids although many species have been described from the former group, and from Legerellidae more specifically (Barta et al., 2000; Vincent et al., 1927; Tuzet and Manier, 1957). Obtaining molecular data from additional monoxenous adeleorinids, especially members of the Legerellidae, in combination with phylogenetic analysis using more data than just the SSU rRNA (e.g., phylogenomics or at least the mitochondrial COI protein) may improve the resolution of the position of Legerellidae within Adeleorina.

Interestingly, a greater sampling of taxa from Adeleorina not only has the potential of illuminating phylogenetic relationships within Adeleorina, but also across Apicomplexa as a whole. Phylogenetic analysis of the marine fish haemogregarine, *Haemogregarina bigemina,* revealed it is not really a haemogregarine at all: instead, it was found to branch outside of Adeleorina as sister to the marine, coral-infecting corallicolids (Fig. 1C) (Hayes and Smit, 2019; Kwong et al., 2021). Only a limited number of arthropods and gastropods have been inspected for presence of adeleorinids, and marine sampling of invertebrate-infecting apicomplexans is very poor (Rueckert et al., 2019), altogether suggesting that these abundant and species-rich animal groups probably harbour a greater diversity of new lineages.

3.3. Apicoplast and mitochondrial genomes

Apicoplast genomes extracted from *K. helicina* and *L. nova* are extremely similar to each other, and to the only other known apicoplast genome from Adeleorina. The apicoplast genomes of *K. helicina* and *L. nova* were circular-mapping like the apicoplast genome of the adeleorinid *Hepatozoon canis* (Léveillé et al., 2019b). The *K. helicina* and *L. nova* apicoplast genomes were 33,281 bp (21.9 % GC content) and 32,955 bp (22.6 % GC content), respectively. Both genomes retained inverted repeat regions that included the LSU (23S) and SSU (16S) rRNA (Fig. 2A), like the apicoplast genome of *H. canis* (Léveillé et al., 2019b). No genes were found that are involved in photosynthesis or chlorophyll biosynthesis (chlL, chlN, chlB, and acsF) (Kwong et al., 2019).

A mitochondrial genome sequence was obtained from *L. nova,* and a fragmented genome was also found in *K. helicina*. The *L. nova* mitochondrial genome was 6,750 bp long (36.3 % GC content), circular-mapping, and contained large and small ribosomal RNA (rns and rnl) fragments and protein coding genes: cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit III (COIII), and cytochrome *b* (COB) (Fig. 2B). Four contigs that had high percent identity to a previously published *K. helicina* mitochondrial genome were also identified (Fig. 2B). These contigs included the COI, COB, and COIII genes, rnl and rns fragments, and shared 87.93–94.63 % identity to the previous *K. helicina* mitochondrial genome (MT084563). Unlike the previous *K. helicina* and *K. razorbacki* mitochondrial genomes, we could not fully circularize our *K. helicina* mitochondrial genome (Zeldenrust and Barta, 2021).

3.4. Phylogenomic analyses

Phylogenomics was used to place Adeleorina on a multigene tree of Apicomplexa. Transcriptomes of *K. helicina* and *L. nova* generated in this study, and genomes and transcriptomes from *Besnoitia besnoiti, Cystoisospora suis, Eimeria necatrix, Eimeria brunetti, Cyclospora caytanensis, Goussia janae,* and *Cardiosporidium cionae* extracted from online

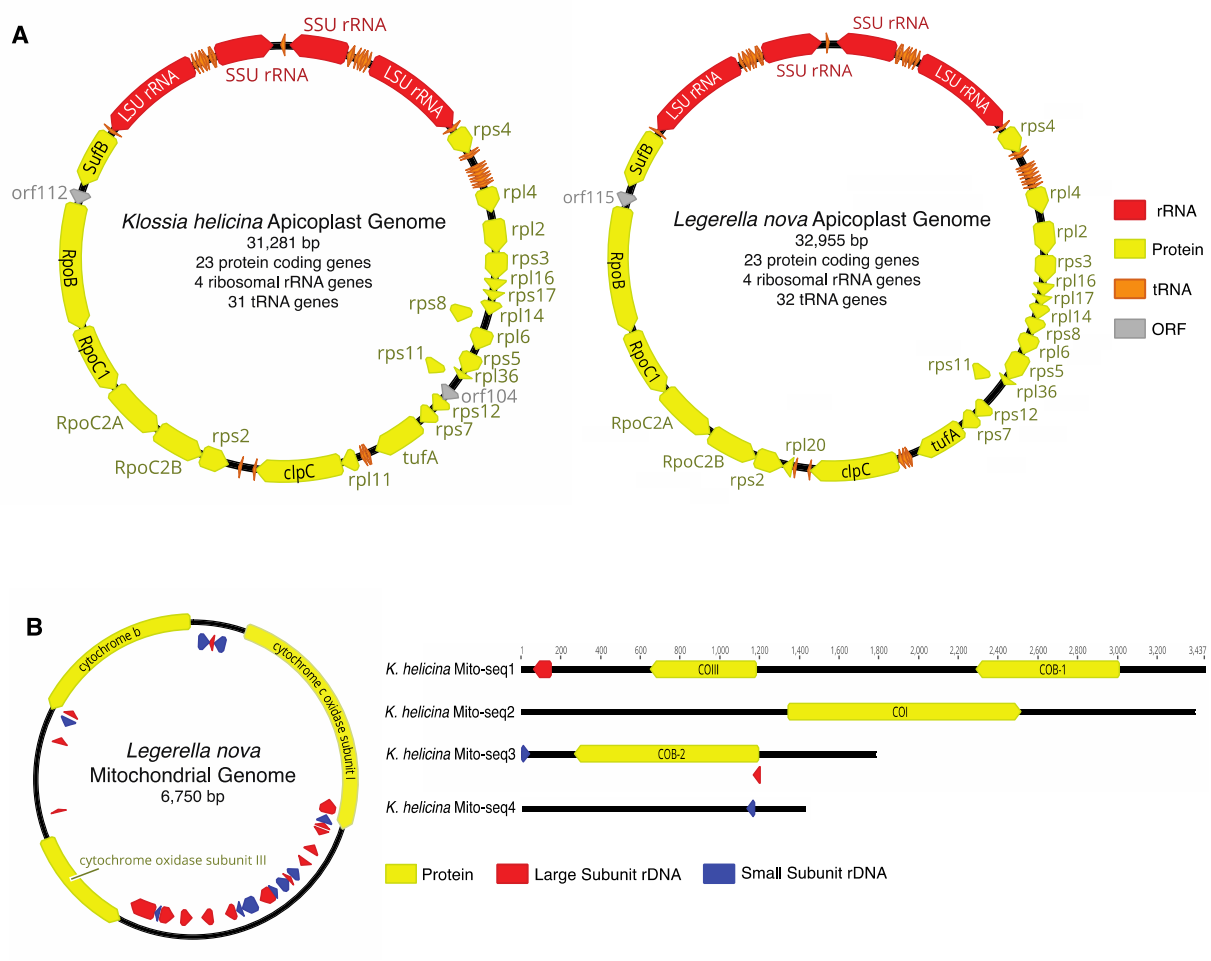

Fig. 2. Organellar genomes and associated sequences from *Klossia helicina* and *Legerella nova*. **A.** Complete apicoplast genomes of *Klossia helicina* and *Legerella nova* generated in this study. rRNAs are shown in red, ribosomal and other proteins are shown in yellow, tRNAs are shown in orange, and ORFs are shown in grey. **B.** Complete mitochondrial genome of *Legerella nova* and mitochondria-associated sequences of *Klossia helicina*. Large and small subunit rDNA are in red and blue, respectively. Other proteins are in yellow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

databases, were added to an existing dataset (Mathur et al., 2023) (Table S2). In a 67-taxa 206-protein ML phylogenomic tree, the two Adeleorina species formed a monophyletic clade that was strongly supported as sister to a large group consisting of Coccidia, Protococcidia, Hematozoa, and Nephromycida (Fig. 3, 98 % UFB; 100 % PMSF-BS; Fig. S2A, 1 pp). Marosporida, another less well-studied group, branched sister to this clade and Adeleorina (Fig. 3,100 % UFB; 100 % PMSF-BS; Fig. S2A, 1 pp). This position of adeleorinds was also observed in some previous analyses of SSU rDNA (Cavalier-Smith, 2014; Vazquez et al., 2022; Espinosa et al., 2023), but with poor support and with members of the Marosporida in different positions. The position of Adeleorina remained the same in our 74-taxa 203-protein ML tree that included long-branching apicomplexans in its dataset (Fig. S1, 92 % UFB; 100 % PMSF-BS; Fig. S2B 1 pp).

In Bayesian analysis none of the four chains for each dataset converged, but none of the differences between chains were related to the position of Adeleorina which was fully supported in all cases (Fig. S2, 1 pp). In all four chains generated using the main dataset (Fig. S2A) and main dataset plus long branching taxa (Fig. S2B) Adeleorina remained sister to Coccidia, Protococcidia, Hematozoa, and Nephromycida collectively and Marosporida remained sister to Adeleorina, Coccidia, Protococcidia, Hematozoa, and Nephromycida collectively. Bootstrap support values for Adeleorina normalized to 100 % UFB after the first 5 % incremental removal step of fast-evolving sites in trees made from both datasets and remained at 100 % UFB until 40 % of fast-evolving sites were removed (Fig. S3). The support value for Adeleorina in the original dataset lowered to 99 % UFB when 40–50 % fast-evolving sites were removed, then rose to 100 % UFB again when 55–65 % fast-evolving sites were removed. The dataset that included long-branching taxa lowered to 99 % at 65 % fast-evolving site removal, and, along with the original dataset, lowered to 98 % once 70 % fast-evolving sites were removed. Tree topology significantly diverged from published literature when 75 % or greater fast-evolving sites were removed and were thus not included as part of the analysis. The position of Adeleorina was also strongly supported in the AU tests where 11 out of 12 tested topologies were rejected: only the topology matching the maximum-likelihood tree where Adeleorina branches sister to Coccidia, Protococcidia, Hematozoa, and Nephromyces was not rejected at a 95 % confidence interval (Fig. S4, Table S3).

Phylogenomic trees made using multiple slow-evolving proteins can more robustly represent phylogenetic relationships and have been used to resolve conflicting branch patterns in Apicomplexa as well as inform evolutionary history (Mathur et al., 2021; Salomaki et al., 2021). The phylogenomic positions of Marosporida, Gregarines and now Adeleorina were resolved within Apicomplexa using multigene phylogenomic methods, just as the demonstration that squirmids were not apicomplexans was also made with multigene phylogenies, altogether changing our interpretation of apicomplexan evolution by demonstrating how parasitism, plastid loss, and loss of photosynthesis occurred multiple times (Mathur et al., 2021; Salomaki et al., 2021). The importance of

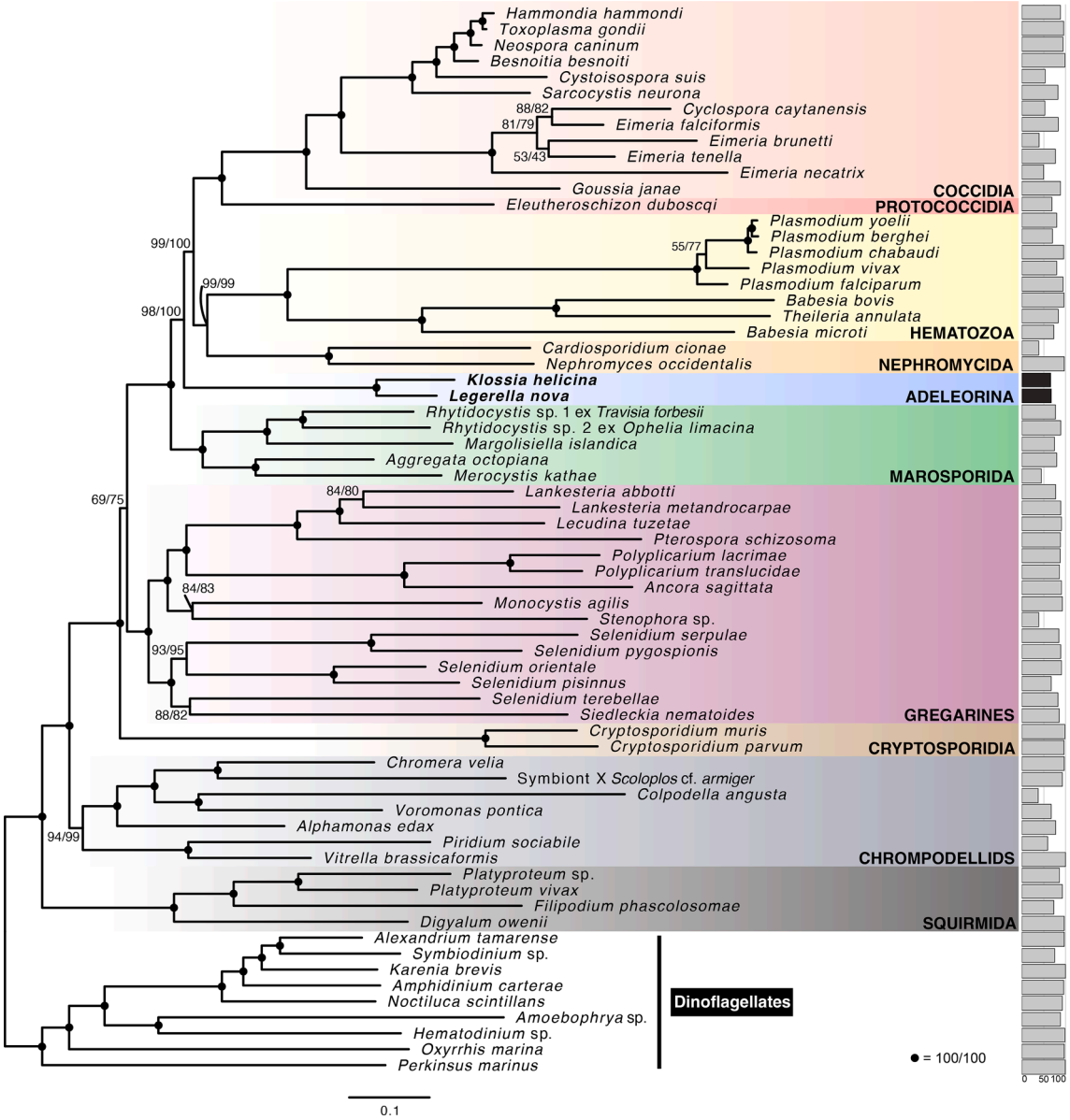

Fig. 3. Maximum likelihood phylogeny of 67 taxa inferred from 206 genes (51,460 amino acid sites) under the LG + C60 + F + G4 mixture model with 1000 UFBs. Major apicomplexan subgroups are separated by colour. The tree is rooted on Dinoflagellates. Adeleorinids *Klossia helicina* and *Legerella nova* from this work are in bold. Black dots indicate nodes with 100 % ultrafast bootstrap support, and 100 % posterior mean site frequency (PMSF) bootstrap support from 200 non-parametric bootstrap replicates. Values otherwise are indicated in this order from left to right. The bar graph on the right shows the percentage of genes represented in the phylogeny for each taxon with black bars representing the newly added adeleorinids from this work.

parallel evolutionary changes is also underscored by our analyses, as the phylogenomic tree shows the transition from a monoxenous parasitic lifestyle to a heteroxenous lifestyle, the emergence of blood parasitism, and the transmission by invertebrate blood vectors have also all emerged multiple times independently within Apicomplexa. Both monoxenous and heteroxenous taxa are represented in Coccidia, Adeleorina, Marosporida, and Gregarines, but a pattern of strictly monoxenous taxa close to the root within these subgroups to heteroxenous taxa at the tips of these subgroups appear in the Coccidia, Adeleorina, and Gregarines. Among apicomplexans, intracellular invasion of blood cells appears in two major subgroups that branch in different positions of the phylogenomic tree: the Hematozoa and the Adeleorina (and also seemingly in the lineage of marine fish-infecting apicomplexans previously mis-identified as Haemogregarines). Blood-infecting apicomplexans in these two major subgroups are also transmitted by blood vectors such as mosquitoes and ticks (and gnathiid isopods and leeches in the case of the marine fish-infecting group (Hayes and Smit, 2019)).

Phylogenomics can help us understand deeper relationships among groups within the Apicomplexa, while mitochondrial and apicoplastidal data appear to be more effective in investigating more closely related taxa. Other drawbacks to the organelle genomes are that mitochondrial and/or apicoplast genomes have been lost in cryptosporidians and multiple clades of gregarines (Putignani et al., 2004; Zhu et al., 2000; Toso and Omoto, 2007; Mathur et al., 2019; Janouškovec et al., 2019). Further, using apicoplast data alone has been shown to generate less robust phylogenies of Apicomplexa (Muñoz-Gómez et al., 2019; Mathur et al., 2021). Sampling for more transcriptome and genome data from members of the Adeleorina can contribute to a better understanding of their phylogenetic relationships and biological diversity as well as investigate species of interest that infect endangered, domesticated, or economically important animals. Compared to heteroxenous members of the Adeleorina, monoxenous adeleorinids are more easily isolated and

can be cultured (Moltmann, 1980). This enables the use of techniques requiring manipulation of live cells to be completed with these taxa, making adeleorinids—particularly *K. helicina*—a potential candidate model organism for future research. Strong phylogenomic support for Adeleorina forming an independent subgroup sister to Coccidia, Protococcidia, Hematozoa, and Nephromycida puts the adeleorinids in an interesting position, diverging around the time when apicomplexans appear to have been transitioning from mostly-extracellular and benign symbionts to more intracellular and more likely to be pathogenic, making them interesting cases in the study of the origin of parasitism that are largely overlooked.

4. Conclusions

Adeleorina, a major apicomplexan subclade infecting a large variety of vertebrate and invertebrate animals across the world, has previously been excluded from phylogenomic analyses due to unavailable molecular data. Here, we obtained transcriptomes from two members of the Adeleorina to use in a phylogenomic analysis, resolving its position as a major group branching in a position sister to a clade formed by Coccidia, Protococcidia, Hematozoa, and Nephromycida. This demonstrates that the Adeleorina are not Coccidia, as in classical taxonomy, but instead, like Marosporida, diverged from other apicomplexans early in the transition from a mostly-extracellular to a mostly-intracellular infection strategy was emerging. From this updated tree of Apicomplexa multiple independent instances of a transition from monoxenous to heteroxenous parasitic lifestyle, blood parasitism, and transmission through an animal blood-feeding vector appear to have arisen within multiple subgroups. *Legerella nova* is the only member of the *Legerellidae* in the Adeleorina thus far to have genetic material sequenced, demonstrating the need for further sampling from monoxenous members of Adeleorina to better resolve this area of the Adeleorina tree. *Klossia helicina* was previously believed to be endemic to *C. nemoralis* and *C. hortensis* in Europe, but were revealed to be present in hosts that have been transferred to North America and therefore dispersed more widely than was previously known. *Klossia helicina* and *L. nova* retain apicoplasts closely resembling other Adeleorina with circular-mapping apicoplast genomes. A circular-mapping mitochondrial genome could be extracted from *L. nova,* but the mitochondrial contigs extracted from *K. helicina* could not be circularized. The tree of Apicomplexa continues to be refined by using phylogenomics and adding previously unsampled taxa, largely through culture-free genomics and transcriptomics methods. Resolving the position of the historically established, widespread, and extensively described Adeleorina on the tree of Apicomplexa as an independent subgroup changes how we must interpret characteristics attributed to adeleorinids, and point to the need to further develop these methods and datasets to really understand apicomplexan evolution and diversity.

CRediT authorship contribution statement

Ina Na: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Claudia Campos:** Investigation, Writing – review & editing. **Gordon Lax:** Methodology, Writing – review & editing. **Waldan K. Kwong:** Conceptualization, Formal analysis, Investigation, Resources, Writing – review & editing. **Patrick J. Keeling:** Conceptualization, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Transcriptome and genome raw reads are available on NCBI SRA under BioProject PRJNA1032149. SSU rDNA sequences are available at the NCBI GenBank Nucleotide Database under accession numbers OR680781 (*Klossia helicina)* and OR689432 (*Legerella nova)*. The plastid and mitochondrial data generated in this study are available at the NCBI GenBank Nucleotide Database under the following accession numbers: OR693319, OR693320, OR693321, PP329795, PP329796, PP329797, PP329798. The alignments used to generate our phylogenomic trees are available on Mendeley Data under the DOI: 10.17632/nmjjgbckb4.1.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2024.108060.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410.

Barta, J.R., Ogedengbe, J.D., Martin, D.S., Smith, T.G., 2012. Phylogenetic position of the adeleorinid coccidia (myzozoa, apicomplexa, coccidia, eucoccidiorida, adeleorina) inferred using 18S rDNA sequences. J Eukaryot Microbiol. 59 (2), 171–180. https://doi.org/10.1111/j.1550-7408.2011.00607.x. PMID: 22313415.

Barta, J. R. (2000). Adeleorina. In: Lee, J. J., Leedale, G. F. & Bradbury, P. (eds) An Illustrated Guide to the Protozoa, 2nd ed. Allen Press Inc., KS 66044 USA. p. 305– 318.

Boisard, J., Florent, I., 2020. Why the -omic future of apicomplexa should include gregarines. Biol Cell. 112 (6), 173–185. https://doi.org/10.1111/boc.202000006. PMID: 32176937.

Capella-Gutiérrez, S., Silla-Martínez, J.M., Gabaldón, T., 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. Bioinformatics. 25 (15), 1972–1973. https://doi.org/10.1093/bioinformatics/btp348. PMID: 19505945; PMCID: PMC2712344.

Cavalier-Smith, T., 2014. Gregarine site-heterogeneous 18S rDNA trees, revision of gregarine higher classification, and the evolutionary diversification of sporozoa. Eur J Protistol. 50 (5), 472–495. https://doi.org/10.1016/j.ejop.2014.07.002. PMID: 25238406.

Chagas, C.R.F., Harl, J., Preikša, V., Bukauskaitė, D., Ilgūnas, M., Weissenböck, H., Valkiūnas, G., 2021. Lankesterella (apicomplexa, Lankesterellidae) blood Parasites of passeriform birds: prevalence, Molecular and morphological Characterization, with notes on sporozoite persistence in vivo and development in vitro. Animals (basel). 11 (5), 1451. https://doi.org/10.3390/ani11051451. PMID: 34070187; PMCID: PMC8158525.

Chan, P.P., Lowe, T.M., 2019. tRNAscan-SE: Searching for tRNA genes in genomic sequences. Methods Mol Biol. 1962, 1–14. https://doi.org/10.1007/978-1-4939-9173-0_1. PMID: 31020551; PMCID: PMC6768409.

Danecek, P., Bonfield, J.K., Liddle, J., Marshall, J., Ohan, V., Pollard, M.O., Whitwham, A., Keane, T., McCarthy, S.A., Davies, R.M., Li, H., 2021. Twelve years of SAMtools and BCFtools. Gigascience. 10(2):giab008. doi: 10.1093/gigascience/giab008. PMID: 33590861; PMCID: PMC7931819.

Del Campo, J., Heger, T.J., Rodríguez-Martínez, R., Worden, A.Z., Richards, T.A., Massana, R., Keeling, P.J., 2019. Assessing the diversity and distribution of apicomplexans in host and free-living environments using high-throughput amplicon data and a phylogenetically informed reference framework. Front Microbiol. 10, 2373. https://doi.org/10.3389/fmicb.2019.02373. Erratum. In: Front Microbiol. (2020) 11:576322. PMID: 31708883; PMCID: PMC6819320.

Gilbertson, C.R., Wyatt, J.D., 2016. Evaluation of euthanasia techniques for an invertebrate species, land snails (Succinea putris). J Am Assoc Lab Anim Sci. 55 (5), 577–581. PMID: 27657713; PMCID: PMC5029829.
Haas, B., 2015. TransDecoder (find coding regions within transcripts) [WWW document]. GitHub. https://github.com/TransDecoder/TransDecoder.
Hayes, P.M., Smit, N.J., 2019. Molecular insights into the identification and phylogenetics of the cosmopolitan marine fish blood parasite, Haemogregarina bigemina (adeleorina: Haemogregarinidae). Int J Parasitol Parasites Wildl. 8, 216–220. https://doi.org/10.1016/j.ijppaw.2019.01.006. PMID: 30891401; PMCID: PMC6404647.
Hrazdilová, K., Červená, B., Blanvillain, C., Foronda, P., Modrý, D., 2021. Quest for the type species of the genus *hepatozoon* – phylogenetic position of hemogregarines of rats and consequences for taxonomy. Systematics and Biodiversity 19 (6), 622–631. https://doi.org/10.1080/14772000.2021.1903616.
Irwin, N.A.T., 2021. Phylogenomic-analysis [WWW document]. accessed 7.6.21 GitHub. https://github.com/nickatirwin/Phylogenomic-analysis.
Janouškovec, J., Paskerova, G.G., Miroliubova, T.S., Mikhailov, K.V., Birley, T., Aleoshin, V.V., Simdyanov, T.G., 2019. Apicomplexan-like parasites are polyphyletic and widely but selectively dependent on cryptic plastid organelles. Elife. 8, e49662.
Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., Jermiin, L.S., 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat. Methods 14 (6), 587–589.
Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. Nucleic Acids Res. 30 (14), 3059–3066.
Kim, K., Weiss, L.M., 2004. Toxoplasma gondii: the model apicomplexan. Int J Parasitol. 34 (3), 423–432. https://doi.org/10.1016/j.ijpara.2003.12.009. PMID: 15003501; PMCID: PMC3086386.
Kopecná, J., Jirků, M., Oborník, M., Tokarev, Y.S., Lukes, J., Modrý, D., 2006. Phylogenetic analysis of coccidian parasites from invertebrates: search for missing links. Protist. 157 (2), 173–183. https://doi.org/10.1016/j.protis.2006.02.005. PMID: 16621694.
Kwong, W.K., del Campo, J., Mathur, V., Vermeij, M.J.A., Keeling, P.J., 2019. A widespread coral-infecting apicomplexan with chlorophyll biosynthesis genes. Nature. 568, 103–107.
Kwong, W.K., Irwin, N.A.T., Mathur, V., Na, I., Okamoto, N., Vermeij, M.J.A., Keeling, P. J., 2021. Taxonomy of the apicomplexan symbionts of coral, including corallicolida ord. nov., reassignment of the genus gemmocystis, and description of new species corallicola aquarius gen. J. Eukaryot. Microbiol. 68, e12852 nov. sp. nov. and Anthozoaphila gnarlus gen. nov. sp. nov.
Laetsch, D.R., Blaxter, M.L., 2017. BlobTools: interrogation of genome assemblies. [version 1; peer review: 2 approved with reservations]. F1000Research 6, 1287. https://doi.org/10.12688/f1000research.12232.1.
Lagesen, K., Hallin, P., Rødland, E.A., Staerfeldt, H.H., Rognes, T., Ussery, D.W., 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res. 35 (9), 3100–3108. https://doi.org/10.1093/nar/gkm160. PMID: 17452365; PMCID: PMC1888812.
Langmead, B., Salzberg, S.L., 2012. Fast gapped-read alignment with bowtie 2. Nat Methods. 9 (4), 357–359. https://doi.org/10.1038/nmeth.1923. PMID: 22388286; PMCID: PMC3322381.
Lartillot, N., Lepage, T., Blanquart, S., 2009. PhyloBayes 3: a bayesian software package for phylogenetic reconstruction and molecular dating. Bioinformatics 25, 2286–2288. https://doi.org/10.1093/bioinformatics/btp368.
Léveillé, A.N., Ogedengbe, M.E., Hafeez, M.A., Tu, H.H., Barta, J.R., 2014. The complete mitochondrial genome sequence of hepatozoon catesbianae (apicomplexa: coccidia: adeleorina), a blood parasite of the green frog, lithobates (formerly Rana) clamitans. J Parasitol. 100, 651–656. https://doi.org/10.1645/13-449.1.
Léveillé, A.N., Bland, S.K., Carlton, K., Larouche, C.B., Kenney, D.G., Brouwer, E.R., Lillie, B.N., Barta, J.R., 2019. Klossiella equi infecting kidneys of Ontario horses: life cycle features and multilocus sequence-based genotyping confirm the genus klossiella belongs in the adeleorina (apicomplexa: coccidia). J Parasitol. 105 (1), 29–40. PMID: 30807709.
Léveillé, A.N., Baneth, G., Barta, J.R., 2019. Next generation sequencing from hepatozoon canis (apicomplexa: coccidia: adeleorina): complete apicoplast genome and multiple mitochondrion-associated sequences. Int J Parasitol. 49, 375–387. https://doi.org/10.1016/j.ijpara.2018.12.001.
Léveillé, A.N., El Skhawy, N., Barta, J.R., 2020. Multilocus sequencing of hepatozoon cf. griseisciuri infections in Ontario eastern gray squirrels (Sciurus carolinensis) uncovers two genotypically distinct sympatric parasite species. Parasitol Res. 119, 713–724. https://doi.org/10.1007/s00436-019-06583-5.
Li, D., Liu, C.M., Luo, R., Sadakane, K., Lam, T.W., 2015. MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de bruijn graph. Bioinformatics 31 (10), 1674–1676.
Maia, J.P., Carranza, S., Harris, D.J., 2016. Comments on the systematic revision of adeleid Haemogregarines: are more data needed? J Parasitol. 102 (5), 549–552. https://doi.org/10.1645/15-930. PMID: 27385057.
Mathur, V., Kolísko, M., Hehenberger, E., Irwin, N.A.T., Leander, B.S., Kristmundsson, Á., Freeman, M.A., Keeling, P.J., 2019. Multiple independent origins of apicomplexan-like Parasites. Curr Biol. 29 (17), 2936–2941.e5. https://doi.org/10.1016/j.cub.2019.07.019. PMID: 31422883.
Mathur, V., Kwong, W.K., Husnik, F., Irwin, N.A.T., Kristmundsson, Á., Gestal, C., Freeman, M., Keeling, P.J., 2021. Phylogenomics identifies a new major subgroup of apicomplexans, Marosporida class nov., with extreme apicoplast genome reduction. Genome Biol Evol. 13(2):evaa244 https://doi.org/10.1093/gbe/evaa244. PMID: 33566096; PMCID: PMC7875001.
Milne, I., Bayer, M., Cardle, L., Shaw, P., Stephen, G., Wright, F., Marshall, D., 2010. Tablet–next generation sequence assembly visualization. Bioinformatics. 26 (3), 401–402. https://doi.org/10.1093/bioinformatics/btp666. PMID: 19965881; PMCID: PMC2815658.
Minh, B.Q., Nguyen, M.A., von Haeseler, A., 2013. Ultrafast approximation for phylogenetic bootstrap. Mol Biol Evol. 30 (5), 1188–1195. https://doi.org/10.1093/molbev/mst024. PMID: 23418397; PMCID: PMC3670741.
Moltmann, U.G., 1980. Light and electron microscopic studies on the merogony of Klossia helicina (coccidia; adeleidea) in snail kidney tissue cultures. Z. Parasitenkd. 62, 165–178. https://doi.org/10.1007/BF00927862.
Muñoz-Gómez, S.A., Durnin, K., Eme, L., Paight, C., Lane, C.E., Saffo, M.B., Slamovits, C. H., 2019 Oct 1. Nephromyces represents a diverse and novel lineage of the apicomplexa that has retained apicoplasts. Genome Biol Evol. 11 (10), 2727–2740. https://doi.org/10.1093/gbe/evz155. PMID: 31328784; PMCID: PMC6777426.
Netherlands, E.C., Cook, C.A., Du Preez, L.H., Vanhove, M.P.M., Brendonck, L., Smit, N. J., 2019. An overview of the dactylosomatidae (apicomplexa: adeleorina: dactylosomatidae), with the description of dactylosoma kermiti n. sp. parasitising Ptychadena anchietae and sclerophrys gutturalis from South Africa. Int J Parasitol Parasites Wildl. 11, 246–260. https://doi.org/10.1016/j.ijppaw.2019.12.006. PMID: 32195110; PMCID: PMC7078462.
Nguyen, L.T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 32 (1), 268–274. https://doi.org/10.1093/molbev/msu300. PMID: 25371430; PMCID: PMC4271533.
Paight, C., Slamovits, C.H., Saffo, M.B., Lane, C.E., 2019. Nephromyces encodes a urate metabolism pathway and predicted Peroxisomes, demonstrating that these are not ancient losses of apicomplexans. Genome Biol Evol. 11 (1), 41–53. https://doi.org/10.1093/gbe/evy251. PMID: 30500900; PMCID: PMC6320678.
Pales Espinosa, E., Bouallegui, Y., Grouzdev, D., et al., 2023. An apicomplexan parasite drives the collapse of the bay scallop population in New York. Sci Rep. 13, 6655. https://doi.org/10.1038/s41598-023-33514-3.
Picelli, S., Faridani, O.R., Björklund, Å.K., Winberg, G., Sagasser, S., Sandberg, R., 2014. Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. 9 (1), 171–181. https://doi.org/10.1038/nprot.2014.006.
Putignani, L., Tait, A., Smith, H.V., Horner, D., Tovar, J., Tetley, L., Wastling, J.M., 2004. Characterization of a mitochondrion-like organelle in *Cryptosporidium parvum*. Parasitology. 129 (Pt 1), 1–18. https://doi.org/10.1017/s003118200400527x.
Mathur, V., Salomaki, E.D., Wakeman, K.C., Na, I., Kwong, W.K., Kolisko, M., Keeling, P. J., 2023. Reconstruction of Plastid Proteomes of Apicomplexans and Close Relatives Reveals the Major Evolutionary Outcomes of Cryptic Plastids. Mol Biol Evol. 40(1): msad002. doi: 10.1093/molbev/msad002. PMID: 36610734; PMCID: PMC9847631.
Rambaut, A. (2014). FigTree v1.4.2, A Graphical Viewer of Phylogenetic Trees. Available from hhttp//tree.bio.ed.ac.uk/software/figtree/i [Internet] 1.4.2. Available from: http//tree.bio.ed.ac.uk/software/ figtree.
Roure, B., Rodriguez-Ezpeleta, N., Philippe, H., 2007. SCaFoS: a tool for selection, concatenation and fusion of sequences for phylogenomics, in. BMC Evolutionary Biology. 7 (S2) https://doi.org/10.1186/1471-2148-7-S1-S2.
Rueckert, S., Pipaliya, S.V., Dacks, J.B., 2019. Evolution: Parallel paths to Parasitism in the apicomplexa. Curr Biol. 29 (17), R836–R839. https://doi.org/10.1016/j.cub.2019.07.047. PMID: 31505182.
Saffo, M.B., McCoy, A.M., Rieken, C., Slamovits, C.H., 2010. Nephromyces, a beneficial apicomplexan symbiont in marine animals. Proc Natl Acad Sci U S a. 107 (37), 16190–16195. https://doi.org/10.1073/pnas.1002335107. PMID: 20736348; PMCID: PMC2941302.
Salomaki, E.D., Terpis, K.X., Rueckert, S., Kotyk, M., Varadínová, Z.K., Čepička, I., Lane, C.E., Kolisko, M., 2021. Gregarine single-cell transcriptomics reveals differential mitochondrial remodeling and adaptation in apicomplexans. BMC Biol. 19 (1), 77. https://doi.org/10.1186/s12915-021-01007-2. PMID: 33863338; PMCID: PMC8051059.
Sato, S., 2021. Plasmodium-a brief introduction to the parasites causing human malaria and their basic biology. J Physiol Anthropol. 40 (1), 1. https://doi.org/10.1186/s40101-020-00251-9. Erratum. In: J Physiol Anthropol. (2021) 40(1):3. PMID: 33413683; PMCID: PMC7792015.
Scholtyseck, E., Mehlhorn, H., Hammond, D.M., 1971. Fine structure of macrogametes and oocysts of coccidia and related organisms. Z Parasitenkd. 37 (1), 1–43. https://doi.org/10.1007/BF00259543. PMID: 4327670.
Schulte, E., 1971. Cytochemische untersuchungen an den feinstrukturen von Klossia helicina (coccidia, adeleidea). Z. f. Parasitenkunde. 36, 140–157. https://doi.org/10.1007/BF00259585.
Shimodaira, H., 2002. An approximately unbiased test of phylogenetic tree selection. Syst Biol. 51 (3), 492–508. https://doi.org/10.1080/10635150290069913. PMID: 12079646.
Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., Zdobnov, E.M., 2015. BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31 (19), 3210–3212.
Toso, M.A., Omoto, C.K., 2007. *Gregarina niphandrodes* may lack both a plastid genome and organelle. J Eukaryot Microbiol. 54 (1), 66–72.
Tuzet, O., Manier, J.-F., 1957. Écologie parasitaire chez *Glomeris marginata* villers. Vie Et Milieu. 8, 58–71.
UniProt Consortium. UniProt: the universal protein knowledgebase in 2021. (2021). Nucleic Acids Res. 49(D1):D480-D489. doi: 10.1093/nar/gkaa1100. PMID: 33237286; PMCID: PMC7778908.
Vázquez, N., Freeman, M.A., Cremonte, F., Gilardoni, C., Kristmundsson, Á., 2022. A phylogenetically unresolved apicomplexan (APXSc) causing swirl lesions in the tehuelche scallop, Aequipecten tehuelchus, from the Southwest Atlantic coast. Int J

Parasitol Parasites Wildl. 17, 295–307. https://doi.org/10.1016/j.ijppaw.2022.03.009. PMID: 35342711; PMCID: PMC8943341.

Vincent, M., 1927. On Legerella hydropori n.sp., a coccidian Parasite of the malpighian tubules of Hydroporus palustris L. (coleoptera). Parasitology 19 (4), 394–400. https://doi.org/10.1017/S0031182000005862.

Volkmann, B., 1967. Vergleichend elektronenmikroskopische und lichtmikroskopische untersuchungen an verschiedenen entwicklungsstadien von Klossia helicina (coccidia, adeleidea). Zeitschrift Für Parasitenkunde 29, 159–208.

Votýpka, J., Modrý, D., Oborník, M., Šlapeta, J., Lukeš, J., 2017. Apicomplexa. In: Archibald, J., Simpson, A., Slamovits, C. (Eds.), Handbook of the Protists. Springer, Cham. https://doi.org/10.1007/978-3-319-28149-0_20.

Wang, H.-C., Minh, B.Q., Susko, E., Roger, A.J., 2017. Modeling site heterogeneity with posterior mean site frequency profiles accelerates accurate phylogenomic estimation. Syst. Biol. 67, 216–235.

Woodcock, H. M. (1911). "Coccidia" Encyclopaedia Britannica (11th ed.). Vol 6. Cambridge University Press.

Zeldenrust, E.G., Barta, J.R., 2021. Description of the first Klossia species (apicomplexa: eucoccidiorida: adeleorina: adeleidae) infecting a pulmonate land snail, Triodopsis hopetonensis (mollusca: polygyridae). North America. J Parasitol. 107 (3), 421–429. https://doi.org/10.1645/21-3. PMID: 34048570.

Zhu, G., Marchewka, M.J., Keithly, J.S., 2000. *Cryptosporidium parvum* appears to lack a plastid genome. Microbiology. 146 (Pt 2), 315–321.