Phylogenomics Identifies a New Major Subgroup of Apicomplexans, Marosporida \textit{class nov.}, with Extreme Apicoplast Genome Reduction

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Abstract

The phylum Apicomplexa consists largely of obligate animal parasites that include the causative agents of human diseases such as malaria. Apicomplexans have also emerged as models to study the evolution of nonphotosynthetic plastids, as they contain a relict chloroplast known as the apicoplast. The apicoplast offers important clues into how apicomplexan parasites evolved from free-living ancestors and can provide insights into reductive organelle evolution. Here, we sequenced the transcriptomes and apicoplast genomes of three deep-branching apicomplexans, \textit{Margolisiella islandica}, \textit{Aggregata octopiana}, and \textit{Merocystis kathae}. Phylogenomic analyses show that these taxa, together with \textit{Rhytidocystis}, form a new lineage of apicomplexans that is sister to the Coccidia and Hematozoa (the lineages including most medically significant taxa). Members of this clade retain plastid genomes and the canonical apicomplexan plastid metabolism. However, the apicoplast genomes of \textit{Margolisiella} and \textit{Rhytidocystis} are the most reduced of any apicoplast, are extremely GC-poor, and have even lost genes for the canonical plastidial RNA polymerase. This new lineage of apicomplexans, for which we propose the class Marosporida \textit{class nov.}, occupies a key intermediate position in the apicomplexan phylogeny, and adds a new complexity to the models of stepwise reductive evolution of genome structure and organelle function in these parasites.

Key words: organelle evolution, plastids, apicomplexans, phylogenomics.

Significance

Apicomplexans are obligate parasites of animals, however, they evolved from algae and retain a nonphotosynthetic plastid (the apicoplast). Using phylogenomics, we resolve the branching order of major apicomplexan lineages, and identify a new class of apicomplexans, the Marosporida. We also show marosporidians have the most reduced apicoplast genomes sequenced to date, which lack canonical plastidial RNA polymerase and provide new insights into reductive organelle evolution.
Introduction

The Apiclopedia is a phylum of obligate animal parasites including agents of significant human disease such as malaria (*Plasmodium* spp.) and toxoplasmosis (*Toxoplasma gondii*), and core symbionts of corals (Seeber et al. 2013; Kwong et al. 2019). They are abundant parasites in nature, with over 6,000 species described and thousands more likely yet to be discovered (Votýpka et al. 2017). Apicomplexan-like parasitism has arisen at least four times in parallel from a free-living plastid-bearing ancestor (Janouškovec et al. 2019; Mathur et al. 2019). In each case, the parasite morphology has converged around the use of an ancestral “apical complex” structure, which was originally used for feeding but was coopted for infection (Dos Santos Pacheco et al. 2020). Likewise, during each transition to parasitism, the chloroplast underwent convergent reduction, giving rise to a reduced, nonphotosynthetic chloroplast, known as the apicoplast. Since its surprising discovery (McFadden et al. 1996; Wilson et al. 1996), the apicoplast has been thoroughly investigated as a potential drug target for apicomplexan diseases, and for clues into the evolutionary origins of apicomplexans (Ralph et al. 2001). This reduced organelle is responsible for the essential biosynthesis of isoprenoids, fatty acids, and iron–sulfur clusters (Lim and McFadden 2010). Although the evolutionary origin of the apicoplast was previously contentious (Keeling 2010), it is now known to be a secondary, red-algal derived plastid that shares a common ancestor with the peridinin-containing plastids found in the sister group to apicomplexans, the dinoflagellates (Janouškovec et al. 2010).

The apicoplast genome is highly reduced, and has become a model for genome evolution in cryptic organelles. Across apicomplexan lineages, the gene content of apicoplasts has proven to be remarkably conserved: they have lost all genes encoding proteins that function directly in photosynthetic electron transfer (i.e., photosynthesis-related genes), they retain genes of nonphotosynthetic function, and their genomes are thought to be maintained due to the retention of a small number of nonhousekeeping genes (Janouškovec et al. 2010). One major exception is the recently described corallicolids (coral symbionts) whose plastids contain four genes involved in chlorophyll biosynthesis in addition to the traditional gene repertoire (Kwong et al. 2019). Phylogenies of plastid-encoded proteins place the corallicolids at the base of the Apicomplexa, which suggests that this may be an ancestral state that was simply lost early in other apicomplexan lineages. In contrast, the nuclear small subunit rRNA gene and mitochondrial phylogenies place the corallicolids closer to the Coccidia, suggesting a more complex history of apicoplast gene loss. A similar incongruence between plastid and nuclear phylogenies was recently observed in the mutualistic apicomplexan, *Nephromyces* (Muñoz-Gómez et al. 2019), and both studies suggested that the currently poor sampling of plastid data from deep-branching and diverse apicomplexan lineages may be a reason for conflicting phylogenetic signals.

To gain further insights into the evolutionary history of the apicoplast, and plastid evolution more generally, we performed whole-genome shotgun (WGS) and transcriptome sequencing surveys on three understudied, deep-branching apicomplexan species, *Aggregata octopiana*, *Merocystis kathae*, and *Margoli optima islandica*. Using phylogenomics, we present a robust multigene apicomplexan phylogeny incorporating all published apicomplexan taxa to date (Janouškovec et al. 2019; Mathur et al. 2019; Muñoz-Gómez et al. 2019). We show that species of the genera *Aggregata*, *Merocystis*, *Margoli optima*, together with *Rh tydotaxis*, are part of a previously unrecognized, monophyletic group of marine invertebrate-infecting apicomplexans that is sister to the Haemosporidia, Piroplasmida, and Coccidia. We also reconstruct the complete apicoplast genomes and plastid metabolism of all three species, in addition to that of four other deep-branching apicomplexan species, *Siedleckia nematoideas*, *Eleutherorhichion diboschii*, *Rh tydotaxis* sp. 1, and *Rhydotaxis* sp. 2. We find that the apicoplasts of *Aggregata*, *Merocystis*, *Siedleckia*, and *Eleutherorhichion* closely resemble other known apicomplexans in genome content and structure. However, the apicoplast genomes of *Margoli optima* and *Rhydotaxis* spp. differ from all known apicoplasts, in that they are more severely reduced, divergent, and have lost the highly conserved plastid-encoded RNA polymerase (rpoB) operon.

Results and Discussion

A Resolved Multiprotein Phylogeny of the Apicomplexa

We generated new transcriptomes and WGS sequencing data for *M. kathae* and *M. islandica*, and WGS data from *A. octopiana*, after isolating the parasites from their marine mollusc hosts (SRA PRJNA645464). *Margoli optima islandica* is known to infect Icelandic scallops (*Chlamys islandica*) where it causes an intracellular infection in the heart auricle (Kristmundsson et al. 2011), *A. octopiana* primarily infects the gastrointestinal tract of the common octopus (*Octopus vulgaris*) with various intermediate crustacean hosts (Gestal et al. 1999; Castellanos-Martínez et al. 2013, 2019), and *M. kathae* infects the renal tissues of the common whelk (*Buccinum undatum*) with intermediate life stages in scallops (Krstmundsson and Freeman 2018) (fig. 1Aa–c). Host tissue infected with oocysts were dissected and washed to isolate parasite sporocysts and sporozoites from which RNA and DNA were extracted and sequenced for transcriptome and WGS analysis.

To place these species within a phylogenomic context, we added them to a data set of slow-evolving nuclear genes previously used to resolve deep phylogenetic relationships within the Apicomplexa (Mathur et al. 2019). This data set
was also expanded to incorporate 11 other recently published transcriptomes (Janouškové et al. 2019; Muñoz-Gómez et al. 2019). The final phylogenetic matrix included 55 taxa, 194 conserved, nucleus-encoded genes, and 58,611 amino acid sites (supplementary table S1, Supplementary Material online). Maximum-likelihood phylogenies using an empirical profile mixture model (LG+GTR+CAT) and Bayesian analyses using the CAT-GTR model (chain bipartition discrepancies: max diff. = 0.017) (Lartillot et al. 2009; Stamatakis 2014) produced congruent topologies that were well resolved and statistically supported at most internal nodes (fig. 1B). The resulting phylogenomic tree confirms the polyphyletic distribution of apicomplexan parasitism, with at least four origins. *Digyalum* is robustly sister to *Platyproteum*, together forming the “Squirmida” (Cavalier-Smith 2014), a group sister to all apicomplexans and chrompodellids (chromerids and colpodellids). *Nephromyces* is sister to the hematozoa (Muñoz-Gómez et al. 2019) and the gregarines (eugregarines and archigregarines) form a fully-supported monophyletic group. The position of *Cryptosporidium* remains problematic: in these analyses, it is recovered as sister to the gregarines, but with somewhat weaker support.

**A New Apicomplexan Class, Marosporida, That Infects Marine Invertebrates**

*Aggregata*, *Merozystis*, and *Margolisiella* all branch with *Rhytidocystis* in a robustly supported monophyletic group (fig. 1B). The recovery of *Aggregata* and *Merozystis* as sister taxa is congruent with traditional taxonomic studies and 18S rDNA small subunit gene phylogenies, which have led to their placement in the family, Aggregatidae (Patten 1935; Kristmundsson and Freeman 2018) (supplementary fig. S1, Supplementary Material online). However, the Aggregatidae has been placed within the Coccidia, which is not consistent with the phylogenomic tree (fig. 1B). Similarly, the placement

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**Fig. 1.**—Phylogeny of the Apicomplexa. (A) Light micrographs of oocysts of the species sequenced, left to right, *Merocystis kathae*, *Margolisiella islandica*, and *Aggregata octopiana*. Scale bars are indicated on the figure. (B) A maximum-likelihood tree of apicomplexans and their relatives based on 195 nucleus-encoded protein markers and 58,611 amino acid sites. Newly sequenced species from this study are shown in bold. Circles at the nodes correspond to nonparametric bootstrap support (1,000 replicates, LG+GTR model) and Bayesian posterior probabilities (PP) (PhyloBayes, consensus of two independent chains, GTR+CAT model). All nodes shown have a PP of 1 unless otherwise indicated. The list of proteins used in the phylogenetic matrix, missing data proportions, and the BUSCO completeness of the newly sequenced species can be found in supplementary table S1, Supplementary Material online. * denotes that Nephromyces is a chimeric OTU assembled from several most closely related lineages from inside the renal sac of a single host. (C) A maximum-likelihood tree of apicomplexans based on 22 plastid-encoded genes and 5,759 amino acid sites. Branch support values are inferred from 500 nonparametric bootstrap replicates (IQ-TREE model LG+F+G4) and are indicated by shaded circles on the nodes. Nodes with support <70% have been collapsed into polytomies. The shading corresponds to the groupings colored in figure 1A. * denotes taxa that only have plastid genome data available.
of Margolisiella as the sister taxon to Rhytidocystis has also been observed previously in rRNA phylogenies, although with variable statistical support (Kristmundsson et al. 2011; Miroljiubova et al. 2020) (supplementary fig. S1, Supplementary Material online). Historically, however, Rhytidocystis and Margolisiella have typically been classified into separate coccidian families, Agamococcidiida and Eimeriidae, respectively (Levine 1979; Desser and Bower 1997; Leander and Ramey 2006), and very recently proposed to be a new subgroup, Eococcidia, based on concatenated multiprotein phylogenomics that these taxa are sisters, 

Merocystis and Eococcidia. The Eococcidia (Miroliubova et al. 2020) is also sister to all apicomplexans other than corallicolids, as the sister taxon to Rhytidocystis and Margolisiella, based on concatenated rRNA operon phylogeny, which did not include Aggregata or Merocystis (Miroljiubova et al. 2020). Here, we show with robust multiprotein phylogenomics that these taxa are sisters, but are not coccidians (fig. 18).

The past taxonomic treatments of all these organisms are complex and contradictory. Indeed, the entire apicomplexan lineage is in need of a major revision to better reflect conclusions from molecular and phylogenomic analyses. To best represent their relationships and avoid the confusion of names representing contradictory taxonomic proposals, we propose a new apicomplexan Class, Marosporida, named to reflect the marine nature of the currently recognized members. Within this group, we propose existing subgroups that do not contradict the phylogenetic relationships can be retained: the Aggregatidae is therefore transferred from the Coccidia to the Marosporida to reflect the sister relationship of Aggregata and Merocystis, and similarly the Rhytidocystidae, which was erected for the genus Rhytidocystis (Levine 1979; Leander and Ramey 2006; Rueckert and Leander 2009), can also be transferred to the Marosporida. The Eococcidia (Miroljiubova et al. 2020) is also consistent with current phylogenomics, and could be transferred to the Marosporida, although it carries with it the potentially misleading reference to Coccidia. The Agamococcidiida is an extremely problematic group that will need to be revisited and perhaps abandoned. This group originally contained Rhytidocystidae and Gemmocystidae, which included one member: Gemmocystis cylindrus, a coral-infecting species that was suggested from histology to be closely related to Rhytidocystis (Upton and Peters 1986). Gemmocystis is now often hypothesized to be related to a broader group of coral-infecting apicomplexans, the corallicolids (Kwong et al. 2019). This cannot be tested with molecular data since none was produced for Gemmocystis, but we can conclude that corallicolids are not related to Rhytidocystidae (Kwong et al. 2019; Miroljiubova et al. 2020). Whether the corallicolids are also members of Marosporida is still an open question. Corallicold transcriptomic data remain unavailable, however, 18S rRNA and mitochondrial gene phylogenies do not support this grouping (supplementary figs. S1 and S2, Supplementary Material online; Miroljiubova et al. 2020), which, together with the lack of data from other key taxa like Pseudoklossia and Adelina, highlight the need for comparable sequencing data from additional apicomplexan lineages.

**Taxon Sampling Does Not Improve Congruence between Apicoplast and Nuclear Phylogenies**

Strongly conflicting signals between apicoplast-encoded and nuclear gene phylogenies have been observed in recent publications (Kwong et al. 2019; Muñoz-Gómez et al. 2019), which is unexpected given their shared evolutionary history. One explanation for the incongruence is that deep-branching apicomplexan genomes are poorly sampled, making phylogenetic reconstructions less reliable. To fill this gap, we reanalyzed the apicoplast phylogeny with significantly greater taxonomic diversity. To obtain apicoplast genomes from Aggregata, Merocystis, and Margolisiella, we conducted WGS sequencing using DNA from parasite sporocysts isolated directly from host tissue. In addition, we also assembled a number of complete or near-complete apicoplasts from other previously reported transcriptome data that contained plastid genes, including those of Rhytidocystis sp. 1, Rhytidocystis sp. 2, Eleutheroschizon, Siedleckia, Selendium, and Dicyalum (Januškovec et al. 2019). We used hidden Markov models (HMMs) to comprehensively search these transcriptomes for 40 apicoplast-encoded proteins using alignments curated for plastid phylogenomic analyses (Mathur et al. 2019). Apicoplast-encoded protein sequences were filtered and concatenated resulting in a phylogenetic matrix consisting of 58 taxa, 22 proteins, and 5,759 amino acid sites (supplementary table S2, Supplementary Material online). Using this matrix in combination with ML and Bayesian phylogenetic analyses, we recovered a poorly resolved phylogeny (fig. 1C).

The plastid phylogeny, even with the addition of 10 new deep-branching apicomplexans, remains poorly supported and incongruent with the nuclear phylogeny, specifically with respect to the branching order of the major groups (fig. 1C). Both phylogenies fully support the sister relationships between Merocystis and Aggregata, and Margolisiella and Rhytidocystis. The plastid phylogeny also recovers a monophyletic grouping of the Hematozoa, Pirolplasmodia, and Coccidia. However, the positions of the gregarines, Selendium and Siedleckia, as well as Nepromyces, Hepatozoon, and Eleutheroschizon are not resolved. Interestingly, the position of corallicolids as the sister to all other apicomplexans in the plastid phylogeny is fully supported in agreement with previous analyses with less diversity (Kwong et al. 2019). The phylogeny was repeated excluding plastid genes extracted from transcriptome data (supplementary fig. S3, Supplementary Material online), which did not improve the support. We also progressively removed fast-evolving sites from the phylogenomic matrix, and tested the stability of the poorly supported nodes (supplementary fig. S4, Supplementary Material online). The node placing Aggregata and Merocystis sister to all apicomplexans other than corallicolids,
remains stable, as does support for *Eleutheroschizon* branching basal to the Coccidia (which is also consistent with its position in the nuclear topology). All other deep nodes with poor support have low and fluctuating bootstrap support with the progressive removal of fast-evolving sites indicative of phylogenetic artifacts. Overall, the significant augmentation of apicoplast diversity does little to resolve the incongruence between plastid and nuclear gene trees. Given the fast-evolving nature of the extremely AT%-rich apicoplast genomes, together with the fact that far fewer genes are available in the plastid genome for phylogenomic analyses (5,759 sites in the plastid tree compared with 58,611 sites in the nuclear tree), we conclude, in agreement with the findings of Muñoz-Gómez et al. (2019), that the apicoplast-based analyses are less robust in resolving phylogenetic relationships within the Apicomplexa.

**Apicoplast Genomes in Margoliisiella and Rhytidocystis Are Highly Reduced and Lack RNA Polymerase Genes**

The *Aggregata* and *Merocystis* apicoplast genomes are extremely similar in gene content, synteny, and size (fig. 2A).
They contain compact (~38 kb) circular-mapping genomes with an inverted repeat including the 55, 16S, and 23S rRNAs and the ribosomal protein gene rps4, like apicoplasts of Coccidia and coralloidid. They lack all genes involved in photosynthesis, including the four chlorophyll biosynthesis genes found in the coralloidids (chlor, chlN, chlB, and acsF). The only significant differences between the two genomes are the presence of the ribosomal protein gene rpl11 and the RNA polymerase (RNAP) subunit rpoC2A being split in two open reading frames in Merocystis but not Aggregata. Therefore, their apicoplast genomes are overall extremely similar to each other in both structure and gene content, and do not differ substantially from the apicoplast genomes found in the Coccidia and Haemosporidia (fig. 2B).

Unlike Merocystis and Aggregata, the plastid genome of Margolisiella is strikingly reduced compared with all other apicoplasts sequenced to date (fig. 2A and B). The genome is very small (18 kb), with a strong AT% bias (13.3% GC). The genome is circular, extremely compact, and contains a single copy of the 16S and 23S rRNA genes, and a reduced complement of 18 tRNA genes and 13 ribosomal proteins, along with a single copy of the tufA, clpC, and sufB proteins, three ribosomal protein pseudogenes, and two hypothetical proteins (fig. 2A and B). Margolisiella also uses an alternate genetic code where UGA (the “opal” stop codon in the standard genetic code) encodes tryptophan, which is also used in the coralloidids, Nephromyces, and the two chromodellids, Piridium and Chromera (Janouškovce et al. 2010, Kwong et al. 2019, Mathur et al. 2019, Muñoz-Gómez et al. 2019). Unlike all other known apicoplasts, the Margolisiella apicoplast genome has lost all four of its plastid-encoded RNAP genes, which are presumed to be solely responsible for the transcription of the apicoplast genome and therefore are functionally indispensable (Nisbet and McKenzie 2016).

A similarly AT-rich fraction of sequence reads was also observed by Janouškovce et al. (2019) in two species of Rhytidocystis, where the authors found apicoplast proteins in their transcriptome data. Organellar genomes with their high copy number and elevated expression levels can be highly represented not only in genome sequences but also in transcriptomes if their AT-rich transcripts are enriched by the poly-A selection step (Smith 2013). To determine whether the rhytidocystid apicoplast genomes resembled that of Margolisiella, we mined the publicly available transcriptomes from Rhytidocystis sp. 1 (which infects Travisia forbesii) and Rhytidocystis sp. 2 (which infects Ophelia limacina), for plastid sequences and were able to assemble complete apicoplast genomes. These searches also revealed numerous plastid contigs that allowed for the assembly of complete circular genomes from S. nematoides and E. duboscqui (supplementary fig. S5, Supplementary Material online) and fragmented genomic contigs that included most of the expected genes from Selenidium and Digyalum (see Materials and Methods) (supplementary table S2, Supplementary Material online).

The Rhytidocystis apicoplast genomes are even more reduced than Margolisiella (13 and 14 kb, in Rhytidocystis sp. 1 and sp. 2, respectively). They are extraordinarily AT-rich, with a GC content of 11.6% in Rhytidocystis sp. 1, and 9% in Rhytidocystis sp. 2. These are the most AT-rich plastid genomes sequenced to date, and Rhytidocystis sp. 2 even surpasses the AT-richness of the holoparasitic plant, Balanophora (Su et al. 2019). Although the two species are in the same genus, their apicoplast genomes show considerable divergence. Rhytidocystis sp. 1 is more reduced, and encodes only six ribosomal proteins, the 16S and 23S rRNAs, 4 tRNAs, clpC and sufB, whereas Rhytidocystis sp. 2 encodes seven ribosomal proteins (in addition to three that are pseudogenized), the 16S and 23S rRNAs, nine tRNAs, clpC, and sufB (fig. 2B). Strangely, Rhytidocystis sp. 1 uses an alternate genetic code (UGA encodes tryptophan), but Rhytidocystis sp. 2 uses the standard genetic code (fig. 2B).

Both genomes lack all genes for RNAP, but interestingly have also lost the translation elongation factor, tufA, which is present in all other apicoplast genomes sequenced to date. The extreme compaction of the Rhytidocystis and Margolisiella apicoplast genomes demonstrates that genome reduction has not reached an “end point” in the majority of apicoplasts, despite the appearance of little variation from the best-studied groups, and further emphasizes the likely importance of only two genes, sufB and clpC, as a barrier to outright loss of the apicoplast genome (Janouškovce et al. 2015).

The Enigmatic Transcription of Margolisiella and Rhytidocystis Plastid Genes

The lack of plastid-encoded RNAPs in Margolisiella and Rhytidocystis raises the question of how their apicoplast genes are transcribed. We first explored the possibility that the RNAP genes had been transferred to the nucleus and that the plastid-derived polymerase proteins are imported back to the organelle, as many other plastid proteins are, and dinoflagellate plastid RNAPs are (Mungpakdee et al. 2014). We used a combination of BLAST and HMMs (Altschul et al. 1990; Finn et al. 2011) to comprehensively search for RNAP proteins based on domain structure and sequence composition in Margolisiella transcriptome and WGS data, and Rhytidocystis transcriptomes, but found no homologs, despite identifying plastid-encoded polymerase proteins (rpoB and rpoC) in all other apicoplast-bearing apicomplexans (fig. 3A). By comparison, we also searched for the “missing” tufA protein in Rhytidocystis, and found tufA transcripts from both species with canonical plastid targeting leaders, indicating that tufA has been transferred to the nucleus and that its protein product is targeted back to the apicoplast.

Another possible explanation, for which there is a precedent, is that the ancestral plastid-derived RNAP has been lost entirely, and apicoplast transcription relies on a separate and distinct nuclear-encoded polymerase derived from some other
proteins were found. (A) The presence of eukaryotic RNAs I, II, III and other RNAs with uncertain phylogenetic association, plastid-encoded RNA polymerases (PEP), and viral RNA polymerases are represented as portions of the circles. Empty circles indicate that no proteins were found. (B) The two domains of the bacteriophage-derived T7 polymerase (mitochondrial) are represented by circles. A line joining the circles signifies a complete T7 polymerase where the N-terminal domain is attached to the rest of the protein, and no line indicates fragmented proteins. Numbers within the circles denote the number of unique proteins identified.

source. Land plant plastids, for example, use two different RNAs: a nuclear-encoded polymerase related to homologs from T7 bacteriophage that transcribes nonphotosynthesis-related genes, and the ancestral plastid polymerase that transcribes photosynthesis-related genes (Liere et al. 2011). Intriguingly, some holoparasitic plants that have lost many or all photosynthesis-related genes have also lost their functional plastid-encoded RNAs and in the genus Cuscuta, it has been demonstrated that all transcription is now carried out by the phage-derived polymerase (Krause et al. 2003; Krause 2008). To see if such an alternative polymerase exists in Margolisella and Rhytidocystis, we searched their transcripts as well as the predicted proteins of other plastid-bearing apicomplexans with Pfam HMMs and identified all proteins containing domains associated with the two largest RNAP subunits, phage-type RNAPs, and other viral RNAPs (El-Gebali et al. 2018) (fig. 3A). We identified subunits of the eukaryotic RNA polymerases (RNAPI, II, and III) in all the apicomplexans searched, as well as a few phylogenetically ambiguous proteins that were not associated distinctly with a certain polymerase (labeled as “uncertain”) (fig. 3A).

These searches also found T7 bacteriophage derived RNAPs in all taxa, except for Merocystis (fig. 3A), which may be due to the incompleteness of the Merocystis transcriptome (refer to supplementary table S1, Supplementary Material online, for BUSCO completeness scores). Most eukaryotic mitochondria use a T7 phage polymerase for transcription of mitochondrial genes, and such polymerases have been found in the genomes of both dinoflagellates and apicomplexans (Li et al. 2001; Teng et al. 2013). All T7 polymerases that we recovered were found to be homologous to these mitochondrially targeted polymerases. In Selenidium, Siedleckia, and Eleutheroschizon we retrieved truncated proteins that lack the complete N-terminal domain, whereas in Toxoplasma, Piridium, and Digyalum, we recovered the complete protein (fig. 3B). Intriguingly, we found additional T7 polymerases in several apicomplexans, including Rhytidocystis. Two N-terminal domain fragments were found in Rhytidocystis sp. 1, whereas in Rhytidocystis sp. 2, we found two truncated T7 polymerase transcripts that are missing the N-terminal domain (fig. 3B). Margolisella contained two nonoverlapping T7 polymerase fragments, but it was not clear if they are part of one protein or two different proteins. It is possible that a T7 phage-derived polymerase might be targeted to the apicoplast, or even that the mitochondrial T7 polymerase is dually targeted in these nonmodel species. A precedent comes from land plants, where a dually targeted RNAP with an ambiguous targeting sequence allows a mitochondrial T7 polymerase to be imported into both the chloroplast and the mitochondria (Hedtke et al. 2000). Another possibility is that mitochondrial T7 polymerase may contain a “twin” targeting sequence, represented by a mitochondrial and a chloroplast targeting sequence in tandem. This is seen in the protoporphyrinogen oxidase II enzyme in spinach, which has two in-frame initiation codons, and thus two different proteins are made by alternative translation where the longer protein is targeted to the chloroplasts and the shorter one to the mitochondria (Watanabe et al. 2001). Based on present sequencing data, we cannot convincingly identify the protein responsible for transcription of the Margolisella and Rhytidocystis apicoplast-encoded genes, but we hypothesize that an unrecognized but ancient redundancy in apicomplexan RNAPs exists and that some single-subunit polymerase, such as a T7 phage polymerase, is targeted to these apicoplasts.

The Canonical Apicoplast Biosynthetic Function Is Conserved in Marosporida

Given the variability in apicoplast genomes in Marosporida, we explored the diversity of organelle function in Aggregata, Merocystis, and Margolisella. Generally, apicoplasts are involved in biosynthesis of isoprenoids (MEP), fatty acids (FASII), iron–sulfur (Fe–S) clusters, and part of the tetrapyrrole (heme) biosynthesis pathway (Sheiner et al. 2013). Most apicomplexans retain genes for all four pathways, however, the pirophosphas and “Symbioint-X” only carry out isoprenoid biosynthesis (Janouškové et al. 2015, 2019), and the marine gregarine clade that includes Pterospora, Lankesteria, and...
**Lecudina** only retain fatty acid biosynthesis (Mathur et al. 2019).

All plastid homologs for enzymes in these pathways were identified by HMM searches with previously curated alignments (Mathur et al. 2019; see Materials and Methods). We found evidence for the presence of all four pathways in the Marosporida (fig. 2C and supplementary table S3, Supplementary Material online). In Margolisiella, we found the complete fatty acid biosynthesis pathway, a near-complete Fe–S cluster assembly pathway, and homologs for several enzymes involved in both isoprenoid (ispC, ispE, ispG) and heme biosynthesis (hemE and hemH). In Aggregata, we found the complete isoprenoid biosynthesis and near-complete fatty acid pathway, as well as most enzymes involved in the Fe–S and heme pathways. We also found homologs from all pathways except the MEP pathway in Merocystis, however, none was complete, probably because the Merocystis transcriptomic data were not sequenced as deeply (refer to supplementary table S1, Supplementary Material online and fig. 2C for BUSCO completeness scores). We then searched for putative targeting leaders indicated by signal and transit peptides at the N-terminus of these plastid-targeted proteins using predictions from SignalP v5.0 and ChloroP v1.1 (Emanuelsson et al. 1999; Dyrlev Bendtsen et al. 2004). We found plastids targeting N-terminal signatures in 3 of these proteins (supplementary table S3, Supplementary Material online), including components of the Fe-S and FASII pathways. Interestingly, we also found evidence for the cytosolic type I fatty acid synthase in Margolisiella and Aggregata. This pathway has been lost in many apicomplexans, but is also retained in the Coccidia, suggesting that both Coccidia and Marosporida retain both type I (cytosolic) and type II (plastidic) fatty acid biosynthesis pathways (Mazumdar and Striepen 2007).

**Summary and Future Directions**

Here, we present a well-resolved phylogenetic framework of the Apicomplexa that includes nearly all recognized apicomplexan groups. This facilitated the identification of a new clade of diverse apicomplexans previously classified into several distinct subgroups, the Marosporida. Whole-genome shotgun and transcriptome sequencing shows that plastid metabolisms of this new group are conserved, but the apicoplast genome structure and content are highly variable. Apicoplasts from Margolisiella and Rhytidocystis have the smallest, most reduced, and most AT-biased genomes known to date, and have distinctively lost plastid-encoded RNAP genes. Taken together with the recent discovery of chlorophyll biosynthesis genes in corallicolids and the intragenus variation of the Rhytidocystis plastids, the patterns of gene retention and loss in deep-branching apicomplexans is more complex than previously thought, as are other relatively stable characters, such as noncanonical genetic codes. This variability will likely continue to increase with greater taxon sampling, given that we only have a handful of representatives from most lineages, and entirely lack complete apicoplast genomes from several key taxa, such as the archigregarines and squirmeids. We also still lack nuclear genomic resources for several important groups, such as corallicolids and adeleids. Although our overall understanding of reductive plastid evolution and the emergence of parasitism in the Apicomplexa is still challenged by both gaps in taxon and subcellular compartment sampling, the unexpected genetic diversity and complex evolutionary patterns that have been revealed here and in other recent studies bring us closer to a comprehensive understanding of apicomplexan biology and evolution.

**Taxa**

Marosporida Mathur, Kristmundsson, Gestal, Freeman, and Keeling 2020

Definition: The phylogenetic clade containing *A. octopiana* Frenzel 1885 (Aggregatidae), *M. kathae* Dakin, 1911 (Aggregatidae), *Ma. islandica* (Kristmundsson et al. 2011), *Rhytidocystis* sp. 1 and *Rhytidocystis* sp. 2 (Rhytidocystidae) (Janouškovec et al. 2019).

Etymology: “Maro” refers to the marine environment that the hosts of these parasites inhabit.

Reference phylogeny: Figure 1, this paper. *Aggregata octopiana* is closely related to *M. kathae*, and *Ma. islandica* to *Rhytidocystis* sp. 1 and 2.

Comments: This clade is inferred from molecular phylogenies. This is a zoological name above level of order and as such falls outside the zoological (and botanical) codes of nomenclature.

**Materials and Methods**

Sample Collection and DNA/RNA Extraction

*Merocystis kathae* was isolated from a common whelk (*B. undatum*) and *Ma. islandica* was isolated from an Icelandic scallop (*C. islandica*) that were collected by dredging in Breiðafjörður Bay, Iceland (65°7.576’N; 22°44.738’W). Prior to sampling, both the whelks and the scallops were sedated using 0.1% MgSO4 in seawater for 1–2 h. The renal organ of the whelks was examined under a dissecting microscope for the presence of *Merocystis* infections. Subsequently, the relatively large gametogenic stages of *Merocystis* were retrieved by gently squeezing the renal organ with pointed forceps until the parasites were released. The resulting exudate was collected into concave glass spot plates containing filtered seawater and rinsed with autoclaved seawater three times to remove as much host tissues and mucous as possible. The auricles of the scallops were excised under a dissecting microscope, and infections of *Margolisiella* (all life stages present) examined from a small drop of hemolymph from the
contigs of interest and extracted using Bowtie v2.2.6 and BlobTools bamfilter (Laetsch and Blaxter 2017; Langmead et al. 2009). These extracted reads were used for the final apicoplast genome assemblies with Spades v3.11.1 (Bankevich et al. 2012). NOVOPlasty v2.6.3 was used to assemble the inverted repeat regions and close (circularize) the apicoplast genomes (Dierckxsens et al. 2016). The plastid genomes of *S. nematoides, Eleutherocochron duboscpi, and Rhytidocystis* sp. 1 and sp. 2 were assembled by data mining publicly available RNA-Seq data. *Siedleckia* was assembled using NOVOPlasty v2.6.3, and *Eleutherocochron* and *Rhytidocystis* were assembled based on contig overlaps from assemblies of raw transcript reads using maSPAdes 3.13.2 (Bushmanova et al. 2019). The apicoplast genomes were annotated manually in Geneious Prime (www.geneious.com/prime/) and ORFs larger than 100 amino acids were predicted, followed by BLAST searches against the NCBI GenBank non-redundant databases (Agarwala et al. 2018). rRNA genes were annotated based on predictions made by RNaMer v1.2 using the “Bacteria” setting and tRNAs were annotated using tRNAscan-SE 2.0 (Lagesen et al. 2007; Chan and Lowe 2019).

Plastid Phylogenomic Analyses
A data set comprising 40 plastid-encoded proteins was compiled based on a previously published data set (Mathur et al. 2019) and enriched with all apicomplexan plastids that have been sequenced as of January 2020 (supplementary table S2, Supplementary Material online) as well as the plastid-encoded proteins of *A. octopiana, M. kathae*, and *Ma. islandica*. Profile HMMs searches with the above-mentioned protein alignments were also used to identify plastid-encoded proteins from transcriptomic data published by Janouškové et al. (2019). Hits from the HMM search were aligned with MAFFT v7.222 and poorly aligned regions were removed using trimAl v1.2 (-gt 0.8) (Capella-Gutierrez et al. 2009; Katoh and Standley 2013). Maximum-likelihood trees were made with FastTree v2.1.7 using the default options (Price et al. 2010). The resulting phylogenies were inspected manually to remove contaminants and paralogs. The selected proteins were then added to the final protein alignments for phylogenomic analyses (see supplementary table S2, Supplementary Material online, for the list of taxa and proteins). The final protein alignments were aligned with MAFFT v7.222 using the –auto option and trimmed with trimAl v1.2 (-gt 0.6) (Capella-Gutierrez et al. 2009; Katoh and Standley 2013). These alignments were then filtered so that they contained only a maximum of 26% missing OTUs and concatenated using SCaFoS v1.2.5 (Roure et al. 2007). The resulting concatenated alignment consists of 22 genes spanning 5,759 amino acid positions from 58 taxa (available at Mendeley data doi:10.17632/rndc4xsk2h.1). The phylogenomic maximum-likelihood analyses were done in IQ-TREE.
v.1.5.4 with the model LG+F+I+R7 and 500 nonparametric bootstraps. This model best fits the data according to the Akaike information criterion and the corrected Akaike information criterion as determined by ModelFinder (Kalyaanamoorthy et al. 2017). Fast evolving site removal was done using site rates generated in IQTREE v.1.5.4 (-wsr option) (Nguyen et al. 2015).

Transcriptome Sequencing and Assembly

Reverse transcription of RNA samples from *M. kathae* and *Ma. islandica* was carried out using the Smart-Seq2 protocol (Picelli et al. 2014). The cDNA concentration was quantified on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.). Prior to high-throughput sequencing, 1 μl of the final cDNA product was used as a template for a PCR amplification of the V4 region of the 18S rRNA gene using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Thermo Scientific) and the general eukaryotic primer pair TAReuk454FWD1 and TAReukREV3 (Stoeck et al. 2010). The PCR product was then sequenced by Sanger sequencing. The SSU rRNA gene sequences were used to confirm species specificity and avoid animal host contamination using BlastN to look for similar sequences in the nonredundant NCBI database (Johnson et al. 2008). Sequencing libraries were then prepared using the Nextera XT protocol, and sequenced on the Illumina MiSeq sequencer using 250-bp paired-end reads. All raw reads have been deposited under SRA PRJNA645464. The raw Illumina sequencing reads were merged using PEAR v0.9.6, and FastQC was used to assess the quality of the paired reads (Zhang et al. 2014; Andrews et al. 2015). The adapter and primer sequences were trimmed using Trimmomatic v0.36 and the transcripts were assembled with Trinity v2.4.0 (Grabherr et al. 2011; Bolger et al. 2014). The contigs were then filtered for animal host contamination using BlastN to look for similar sequences in the nonredundant NCBI database (Johnson et al. 2008). Sequencing libraries were then prepared using the Nextera XT protocol, and sequenced on the Illumina MiSeq sequencer using 250-bp paired-end reads. All raw reads have been deposited under SRA PRJNA645464. The raw Illumina sequencing reads were merged using PEAR v0.9.6, and FastQC was used to assess the quality of the paired reads (Zhang et al. 2014; Andrews et al. 2015). The adapter and primer sequences were trimmed using Trimmomatic v0.36 and the transcripts were assembled with Trinity v2.4.0 (Grabherr et al. 2011; Bolger et al. 2014). The contigs were then filtered for animal host contamination using BlobTools in addition to BlastN and BlastX searches against the NCBI nt database and the SWISS-PROT database, respectively (Agarwala et al. 2018; Bateman 2019). Coding sequences were predicted using a combination of TransDecoder v3.0.1 and similarity searches against the SWISS-PROT database, respectively (Agarwala et al. 2018; Bateman 2019). The completeness of the transcriptomes were assessed with BUSCO v4.0.6 using the alveolate marker gene set (Simão et al. 2015) (supplementary table S1, Supplementary Material online).

Nuclear Phylogenomics Analyses

Transcriptome data from *M. kathae*, *Ma. islandica*, and recently published apicomplexan transcriptomes by Janouškovec et al. (SRA PRJNA557242) and Muñoz-Gómez et al. (SRR8618777) were added to our data set. The transcriptomes were searched using BlastP for a set of 263 genes that have been previously used for apicomplexan phylogenomic analyses and that represent a wide range of eukaryotes (Altschul et al. 1990; Burki et al. 2016; Mathur et al. 2019). The hits were filtered using an e-value threshold of 1e-20 and a query coverage of 50%. In addition to this transcriptomic data set, the genomic reads of *A. octopiana* and *M. kathae* were also searched for the 263 genes using TBlastN with an e-value threshold of 1e-20. The complete regions of the contigs that contained hits were extracted and coding regions were predicted using Exonerate v2.2.0 and TransDecoder-v5.1.0 (Slater and Birney 2005; Haas et al. 2013). The final 263 gene alignments were then aligned using MAFFT L-INS-i v7.722 and trimmed using trimAl v1.2 (-gt 0.8) (Capella-Gutiérrez et al. 2009; Katoh and Standley 2013). Single gene trees were then constructed to identify paralogs and contaminants using RAxML v8.2.12 (PROT-GAMMA-LG model) with support from 1,000 bootstraps (Stamatakis 2014) The resulting trees were manually viewed in FigTree v1.4.3 and contaminants and paralogous sequences were identified and removed (Rambaut 2014). The final cleaned gene-sets were filtered so that they contained only a maximum of 40% missing OTUs and then concatenated in SCaFoS v1.2.5 (Roure et al. 2007). The resulting concatenated alignment consisted of 194 genes spanning 58,611 amino acid positions from 54 taxa (available at Mendeleev data doi:10.17632/rrdc4xsk2h.1). The phylogenomic maximum-likelihood tree was constructed with the heterogeneous mixture LG+C40+F+G4 model as implemented in IQ-TREE (Quang et al. 2008; Nguyen et al. 2015). Statistical support was inferred using 1,000 bootstrap replicates using the LG+F+I+G4 model in RAxML (Stamatakis 2014). The Bayesian tree was computed in PhyloBayes v4.1 using the GTR-CAT model with constant sites removed from the analyses (Lartillot et al. 2009). Four independent chains were run for 10,000 generations and two chains converged with max. diff. = 0.017, whereas two chains got stuck at local maxima. However, all four chains recovered the same topology in regards to the support of the Marosporida clade with posterior probability of 1. Furthermore, the chains that recovered the same topology as the best tree had higher log likelihoods.

RNA Polymerase Analysis

To assess the presence and absence of RNAPs in Margoliussiella and *Rhytidocystis* sp. 1 and sp. 2, we searched genomic and transcriptomic protein predictions from plastid bearing apicomplexans using PFAM HMMs (E < 10\(^{-5}\), incE < 10\(^{-5}\), domE < 10\(^{-5}\)) to identify proteins containing domains associated with the two largest RNAP subunits, Rpa1/Rpb1/Rpc1/RpoC (PF00623, PF04983, PF04990, PF04992, PF04997–PF05001), and Rpa2/Rpb2/Rpc2/RpoB (PF00562, PF04560, PF04561, PF04563, PF04565–PF04567, PF10385), as well as phage-type/mitochondrial RNAPs (PF00940, PF10385), and other viral RNAPs (PF00680, PF00978, PF00998, PF02123, PF07925, PF17501) (for a total of 24 PFAM domains) using PFAM HMMs (Kalyaanamoorthy et al. 2017). The cDNA concentration was quantified using the Smart-Seq2 protocol (Picelli et al. 2014). The cDNA concentration was quantified on a Qubit 2.0 Fluorometer (Thermo Fisher Scientific Inc.). Prior to high-throughput sequencing, 1 μl of the final cDNA product was used as a template for a PCR amplification of the V4 region of the 18S rRNA gene using Phusion High-Fidelity DNA Polymerase (New England Biolabs, Thermo Scientific) and the general eukaryotic primer pair TAReuk454FWD1 and TAReukREV3 (Stoeck et al. 2010). The PCR product was then sequenced by Sanger sequencing. The SSU rRNA gene sequences were used to confirm species specificity and avoid animal host contamination using BlastN to look for similar sequences in the nonredundant NCBI database (Johnson et al. 2008). Sequencing libraries were then prepared using the Nextera XT protocol, and sequenced on the Illumina MiSeq sequencer using 250-bp paired-end reads. All raw reads have been deposited under SRA PRJNA645464. The raw Illumina sequencing reads were merged using PEAR v0.9.6, and FastQC was used to assess the quality of the paired reads (Zhang et al. 2014; Andrews et al. 2015). The adapter and primer sequences were trimmed using Trimmomatic v0.36 and the transcripts were assembled with Trinity v2.4.0 (Grabherr et al. 2011; Bolger et al. 2014). The contigs were then filtered for animal host contamination using BlobTools in addition to BlastN and BlastX searches against the NCBI nt database and the SWISS-PROT database, respectively (Agarwala et al. 2018; Bateman 2019). Coding sequences were predicted using a combination of TransDecoder v3.0.1 and similarity searches against the SWISS-PROT database (Haas et al. 2013; Bateman 2019). The completeness of the transcriptomes were assessed with BUSCO v4.0.6 using the alveolate marker gene set (Simão et al. 2015) (supplementary table S1, Supplementary Material online).
searches were conducted against the SWISS-PROT database to identify nonapicomplexan outgroups (Bateman 2019). Identified SWISS-PROT and apicomplexan proteins were aligned using MAFFT v.7.222 using the PFAM seed alignments as references (Katoh and Standley 2013). The resulting alignments were then trimmed using trimAl v1.2 (-gt 0.3) before being used to generate maximum-likelihood phylogenies using FastTree v2.1.3 (Capella-Gutiérrez et al. 2009; Price et al. 2010). To identify which polymersome complexes these proteins corresponded to (e.g., eukaryotic RNAPI, RNAPII, RNAPIII, or prokaryotic RNAP), proteins were annotated using BlastP searches against the SWISS-PROT database (max_target_seqs 1, E < 1e-5) and their phylogenetic context was interpreted in FigTree. Phylogenetically ambiguous proteins that were not clearly associated with a certain polymersome were labeled as “uncertain.”

Search for Plastid-Derived Biosynthetic Proteins
Profile HMMs were used to identify plastid metabolic proteins in our transcriptomes based on previously curated alignments (Mathur et al. 2019). Profile HMMs were generated using these alignments and HMM searches were conducted on all transcriptomes and genomes using HMMP v3.1 and an e-value threshold of 1e-5 (Finn et al. 2011). In addition to this transcriptomic data set, the WGS contigs of Aggregata, Merocystis, and Margoliisa were also searched for the plastid-targeted proteins using TBlastN with an e-value threshold of 1e-20. The complete regions of the contigs that contained hits were extracted and coding regions were predicted using Exonerate v.2.2.0 and TransDecoder-v5.1.0 (Slater and Birney 2005; Haas et al. 2013). All resulting hits were then extracted and incorporated into the original alignments and realigned using MAFFT v7.222 (–auto option). The alignments were then used to generate phylogenies in FastTree v2.1.3 (Price et al. 2010). The phylogenies were manually scanned in FigTree v1.4.2 and contaminants, paralogs, mitochondrial sequences, and long-branching divergent sequences were identified and removed (Rambaut 2014). The remaining sequences were realigned and used to generate maximum-likelihood phylogenies in IQ-TREE v1.6.9 (Nguyen et al. 2015). Phylogenetic models were selected for each tree individually based on Bayesian Information Criteria using ModelFinder as implemented in IQ-TREE, and statistical support was assessed using 1,000 ultrafast bootstrap replicates (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017). Phylogenetic models were selected for each tree individually based on Bayesian Information Criteria using ModelFinder as implemented in IQ-TREE, and statistical support was assessed using 1,000 ultrafast bootstrap replicates (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017).

Mitochondrial and 18S Ribosomal Small Subunit Gene Phylogenies
The three mitochondria-encoded proteins, cox1, cox3, and cob, were extracted using BLAST searches against the transcriptomes and WGS assemblies (Altschul et al. 1990). Single protein alignments were generated using MAFFT v7.222 (–auto option) and trimmed using trimAl v1.2 (-gt 0.6) (Capella-Gutiérrez et al. 2009; Katoh and Standley 2013). Proteins were concatenated in Geneious Prime v 2020.1.1. The phylogeny was constructed in IQ-TREE with the LG+F+I+G4 model and 1,000 ultrafast bootstraps. The best-fit model was chosen according to the Bayesian information criterion (BIC) using Model Finder (Kalyaanamoorthy et al. 2017). Nuclear 18S rRNA genes were extracted using BLAST searches against the transcriptomes and genomes (Altschul et al. 1990). Genes were aligned with MAFFT v7.222 (–auto option) and trimmed using trimAl v1.2 (-gt 0.6) (Capella-Gutierrez et al. 2009; Katoh and Standley 2013). Phylogenies were constructed in IQ-TREE with the GTR+G+I model and 1,000 ultrafast bootstraps.

Supplementary Material
Supplementary data are available at Genome Biology and Evolution online.

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Author Contributions
V.M. and P.J.K. designed the study. A.K., M.F., and C.G. obtained samples. V.M. and F.H. performed transcriptomics and WGS preparation. V.M., F.H., and W.K.K. assembled and annotated the plastids. V.M. and N.A.T.I. carried out the RNAP analysis. V.M. analyzed the rest of the data. V.M. and P.J.K. wrote the paper with input from all authors.
Data Availability
The plastid genomes generated here are available at the NCBI GenBank Nucleotide Database (www.ncbi.nlm.nih.gov/nucleotide/) and can be accessed with the following accession numbers: MW088710, MW088711, and MW088712. The raw sequencing reads are available on the Sequence Read Archive (www.ncbi.nlm.nih.gov/sra) and can be accessed with the accession PRJNA645464. The alignments for the phylogenomics analyses and transcriptome-mined plastid genomes are available at Mendeley Data (www.mendeley.com/data-sets) and can be accessed with the doi:10.17632/rdc4xsk2h.3.

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