The Phylogenetic Position of the Enigmatic, *Polypodium hydriforme* (Cnidaria, Polypodiozoa): Insights from Mitochondrial Genomes

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Abstract

*Polypodium hydriforme* is an enigmatic parasite that belongs to the phylum Cnidaria. Its taxonomic position has been debated: whereas it was previously suggested to be part of Medusozoa, recent phylogenomic analyses based on nuclear genes support the view that *P. hydriforme* and *Myxozoa* form a clade called Endocnidozoa. Medusozoans have linear mitochondrial (mt) chromosomes, whereas myxozoans, as most metazoan species, have circular chromosomes. In this work, we determined the structure of the mt genome of *P. hydriforme*, using Illumina and Oxford Nanopore Technologies reads, and showed that it is circular. This suggests that *P. hydriforme* is not nested within Medusozoa, as this would entail linearization followed by recirculation. Instead, our results support the view that *P. hydriforme* is a sister clade to *Myxozoa*, and mt linearization in the lineage leading to medusozoans occurred after the divergence of *Myxozoa+P. hydriforme*. Detailed analyses of the assembled *P. hydriforme* mt genome show that: (1) it is encoded on a single circular chromosome with an estimated size of ∼93,000 base pairs, making it one of the largest metazoan mt genomes; (2) around 78% of the genome encompasses a noncoding region composed of several repeat types; (3) similar to *Myxozoa*, no mt tRNAs were identified; (4) the codon TGA is a stop codon and does not encode for tryptophan as in other cnidarians; (5) similar to myxozoan mt genomes, it is extremely fast evolving.

Key words: Cnidaria, Myxozoa, mtDNA, Oxford Nanopore Technologies, Endocnidozoa, tRNA loss.

Significance

Resolving the phylogenetic position of morphologically derived and fast-evolving organisms is often difficult. *Polypodium hydriforme* is a tiny cnidarian parasite of sturgeon eggs that has been previously classified as a member of Medusozoa (e.g., jellies and hydras), a lineage characterized by linear mitochondrial (mt) genome. We determined the mt genome sequence of this organism. Our results indicate that *P. hydriforme* encodes a circular mt genome, supporting previous molecular studies that grouped *P. hydriforme* with parasitic myxozoans rather than with medusozoans. Our study demonstrates that analyzing variations in mt genome structures can help decipher the evolutionary relationships among enigmatic and fast-evolving organisms.
Introduction

*Polypodium hydriforme* is an intriguing organism that develops inside the oocytes of Acipenseridae (i.e., sturgeons and paddlefish) into an elongated inside-out stolon (the endoderm layer is outside, whereas the ectoderm is inside), which upon host spawning everts to reveal tentacles (Raikova 1994). Individuals subsequently emerge from the host oocytes, transitioning into medusoid-like forms, which develop reproductive organs. The gametophores then infect the larva of the host fish to restart the life cycle (Raikova 1980, 1994, 2002).

*Polypodium hydriforme* is a member of Cnidaria. It was historically classified based on morphology as a member of the Narcomedusae (subphylum Medusozoa, class Hydrozoa), which includes parasitic members (Berrill 1950; Bouillon 1987). Nonetheless, *P. hydriforme* has several distinct characteristics that differentiate it from all hydrozoans. The latter convinced Raikova (1994) to erect a distinct cnidian class, Polypodiozoa, to host *P. hydriforme*. The first molecular studies to include a *P. hydriforme* sequence in their analyses did not support a relationship of *P. hydriforme* with Hydrozoa (Siddall et al. 1995; Siddall and Whiting 1999). Instead, these studies, which were based on rRNA sequences, supported a close relationship between *P. hydriforme* and Myxozoa. Myxozoa (Grassé 1970) is a large group of ~2,600 species. They are microscopic organisms with a very simple structural organization but with a complex life cycle, which involves two hosts, usually a fish and an annelid. They are the causative agents of significant fish diseases, such as whirling disease and proliferative kidney disease (reviewed in Okamura et al. 2015).

The putative sister-group relationship between *P. hydriforme* and Myxozoa prompted the suggestion of a new clade: Endocnidozoa (Zrzavý and Hypša 2003). The presence of “cell-in-a-cell” early developmental stages, where a large primary cell contains secondary and tertiary cells, has been suggested as a unifying synapomorphy for this clade (Zrzavý and Hypša 2003; Morris 2012).

The phylogenetic position of the *P. hydriforme*+Myxozoa clade was, however, unstable in subsequent rDNA studies even with a large taxonomic sampling representative of the cnidian diversity (Evans et al. 2008). A larger-scale phylogenetic study based on hundreds of protein-coding genes provided strong support to the sister-clade relationship of *P. hydriforme* and Myxozoa (Chang et al. 2015). Chang et al. (2015) also placed the *P. hydriforme*+Myxozoa clade as sister to Medusozoa with high support. Holzer et al. (2018) reanalyzed the data of Chang et al. (2015) and obtained the same phylogenetic results. However, they suggested instead that the grouping of *P. hydriforme* and Myxozoa could stem from a long-branch attraction artifact and that parasitism evolved convergently in these two clades. Thus, the phylogenetic position of *P. hydriforme* is still debated (fig. 1).

Analysis of the mitochondrial (mt) genome structure may help resolve the phylogenetic position of *P. hydriforme* within Cnidaria. Typical animal mt genomes are composed of a single circular and compact chromosome, which encodes 13 protein-coding genes and 22 tRNAs (Bernt et al. 2013; Lavrov and Pett 2016). Second, medusozoans have a unique mt genome structure. In contrast to a single circular mt genome found in other animals including anthozoan cnidarians, medusozoans possess linear mt chromosomes (fig. 1), and their mt genome can be encoded on one or several chromosomes (Bridge et al. 1992; Kayal et al. 2012, 2015). Recently, it was reported that the anthozoans belonging to Ceriantharia (tube anemones) may possess fragmented linear mt genomes (Stampar et al. 2019), suggesting that, in Cnidaria, mt-genome structures are more complex than previously understood. This finding is, however, debated (Smith 2020). Mitochondrial genomes have been sequenced for five myxozoan species from three genera: *Kudoa* (three species), *Enteromyxum* (one species), and *Myxobolus* (one species) (Takeuchi et al. 2015; Yahalomi et al. 2017, 2020). Two genera have unusual partitioned mt genomes, ranging from two circular chromosomes in *Kudoa* to eight mega-circular chromosomes in *Enteromyxum* (Yahalomi et al. 2017). The circular structure reported for Myxozoa (Takeuchi et al. 2015; Yahalomi et al. 2017, 2020) suggests that their position is outside of Medusozoa, which is in agreement with phylogenomic studies (Nesnidal et al. 2013; Feng et al. 2014; Chang et al. 2015).

The determination of the mt genome structure of *P. hydriforme* could help resolve the phylogenetic position of this species within Cnidaria (fig. 1). In this study, we resolve the sequence and structure of the *P. hydriforme* mt genome using various sequencing methods.

Results and Discussion

The Use of Long Oxford Nanopore Technology Reads

The groups of D.H., I.F., and P.C. have failed to determine the *P. hydriforme* mt genome using long-range PCR and Illumina sequencing for over a decade. It is now clear that sequences that contain large regions composed of repeated elements, or large duplicated regions cannot be assembled using Illumina sequencing alone, due to the short-read lengths (100–300 bp) (Treangen and Salzberg 2011). These problematic regions are at best not useful for assembly but can be captured by a more direct sequencing method, in this case, Membrane-Integrated NanoFilterer (MIF) OXN. After sequencing with MIF, the resulting reads were filtered out and aligned to the mitochondrial genome of *Enteromyxum stichomi* (accession number: LM663978) to determine the correct orientation and boundaries of the reads. By using a combination of MIF and Nanopore sequencing, we were able to generate a complete mtDNA sequence of *P. hydriforme*, which is 17,988 bp long and contains 13 protein-coding genes, 22 tRNA genes, and two rRNA genes (fig. 2).

A phylogenetic analysis was conducted using RAxML, and the results are shown in figure 3. The mt genome of *P. hydriforme* was found to be a circular structure, similar to that of medusozoans. This supports the idea that parasitism evolved convergently in these two clades, as suggested by Chang et al. (2015). However, further research is needed to determine the exact position of *P. hydriforme* within Cnidaria.
The mitochondrial genome of the enigmatic Polypodium hydriforme

assembled, or assembled into chimeric sequences leading to a misrepresentation of the sequence of origin (e.g., Treangen and Salzberg 2011; Tørresen et al. 2017; Gan, Grandjean, et al. 2019). In our specific case, we discovered a long noncoding region of over 70 kb, composed mainly of repeated elements. We could not PCR amplify this region because long PCR amplifications are limited to fragments below 40 kb, whereas our noncoding region is significantly longer. Moreover, the presence of numerous repeats excluded the design of specific primers needed to perform primer-walking sequencing. In this work, we were able to determine the mt sequence using a hybrid approach combining Oxford Nanopore Technology (ONT) long-read sequencing in conjunction with short-read next-generation sequencing (supplementary materials and methods, Supplementary Material online). This approach was previously used in other studies to successfully determine sequences harboring: (1) long-regions with multiple repeats (e.g., Tørresen et al. 2017; Ring et al. 2018; Gan, Grandjean, et al. 2019); (2) complicated structural variations (Wang et al. 2018); (3) large duplications (Gan, Grandjean, et al. 2019); (4) AT-rich regions (Gan, Linton, et al. 2019). However, in our case, we cannot exclude the possibility that our results underestimate the size of the large noncoding region. The number of repeated elements (fig. 2A) has been determined based on the assembly performed by the Canu (Koren et al. 2017) and Necat (Chen et al. 2021) assemblers (supplementary fig. S1 and materials and methods, Supplementary Material online). We found reads supporting the number of repeated elements present in all repeated regions except repeat region 4 (dark red color, fig. 2B). Consequently, some uncertainty remains regarding the exact number of repeats in this region.

Genome Architecture

The P. hydriforme mt genome is encoded on a single circular chromosome whose estimated size is ∼90 kb (fig. 2A). It is thus inferred to be the largest circular mt genome sequenced hitherto in animals, and the second largest mt genome after the partitioned genome of the myxozoan Enteromyxum leei ∼165,843 bp (Yahalomi et al. 2017). By comparison, the next largest animal mt genomes sequenced to this point are from cerianthids (tube anemone) ∼80 kb (Stampar et al. 2019); calcarean sponges ∼50 kb (Lavrov et al. 2016); bivalves ∼48 kb (Hou et al. 2016; Williams et al. 2017), and placozoans ∼43 kb (Dellaporta et al. 2006). Interestingly, unlike Placozoa, the size increase in P. hydriforme does not originate from the presence of additional genes and introns, but from the presence of a large noncoding region of ∼72 kb composed of six main repetitive regions (four of them composed from tandem repeats), ranging from ∼2.5 to ∼23 kb, with different repeated elements. Most of the P. hydriforme mt genome is thus noncoding. Moreover, unlike Ceriantharia (Stampar et al. 2019) and Placozoa (Dellaporta et al. 2006), the noncoding part of the genome is not spread between genes (i.e., divided into several noncoding regions). Rather, most of the repeats are encoded in a single noncoding region. Polypodium hydriforme shares with the myxozoan genus Kudoa a genome divided into two regions: a compact coding region, and a large noncoding region (Takeuchi et al. 2015; Yahalomi et al. 2017). Thus, such a large noncoding region within the mt-genome is a likely synapomorphy of Endocnidozoa.

Additional support for the correctness of the above mt structure is provided by mapping the longest ONT reads
Fig. 2.—Deciphering the circular structure of the Polypodium hydridorme genome. (A) Organization of the mt genome. Dark blue—canonical mt genes (ATP6, COX1-3, CYTB, ND1-6); red—rRNA (rns, rnl); orange—unidentified putative proteins (ORF_126, ORF_146, ORF_281, ORF 510); gray rectangle—noncoding region. All other arrows indicate repeated elements. Repeated elements with the same sequence have the same color. (B) Mapping of ONT reads longer than 25,000 bp to the mt genome. Genes and repeated elements were annotated for each read before mapping. The exact number of repeats could not be determined for the repeat region 4 (located between 39,947 to 63,093 bp) as indicated by the black box.
onto the assembled genome (fig. 2B). This mapping shows the presence of reads that include each main repetitive region and its flanking regions, except for the repeat region 4 as indicated above. It also shows the presence of reads harboring multiple repeats. In addition, support for the repetitive elements being of mt origin rather than of nuclear origin is provided by coverage analysis (supplementary fig. S2, Supplementary Material online). The noncoding region (e.g., the region around 70 kb) includes both repetitive and nonrepetitive regions, and the later were found to have the same level of coverage as the nonrepetitive mt region, which includes the coding genes (supplementary fig. S2A, Supplementary Material online). This coverage is higher than the nuclear genome coverage, suggesting that these regions are genuine mt regions. Moreover, it is worth noting that the *P. hydriforme* sequence does not contain inverted repeats, which could hint at the presence of telomeres. In contrast, all medusozoans studied thus far present inverted repeats as telomeric structures (Kayal et al. 2012). Finally, southern blot hybridizations failed to determine the size of the mt genome of *P. hydriforme*, whereas a control plasmid containing the *cytb* sequence of *P. hydriforme* successfully hybridized with our probe (supplementary materials and methods and fig. S3, Supplementary Material online). The large size of the mt genome could explain these results because the agarose gel would only separate DNA molecules up to 30 kb (supplementary fig. S3, Supplementary Material online).

The coding region includes 17 ORFs longer than 100 aa, all with transmembrane domains, and rnl and rns genes. No tRNA or self-splicing introns were detected. Among the 17 ORFs, we identified 11 of the 13 canonical mt protein-coding genes (*atp6*, *cox1-3*, *nad1-6*, and *cytb*; fig. 2A). Neither of the two additional medusozoan-specific genes was identified (i.e., *dnaB* and *orf324*). Because of the fast rate of sequence evolution of the *atp8*, *nad4L*, *dnaB*, and *orf324*, these genes may still be present but not identifiable, and indeed six unknown ORFs could be detected in the mt genome of *P. hydriforme*.

The organization of the genes is markedly different from other cnidarians, including myxozoans. The only similarity is the location of *cox1* gene at the end of the coding region, which is similar to its location in Hydrozoa. Both canonical and unknown genes are oriented on the same strand. Interestingly, mt tRNA genes could not be identified. The absence of tRNA genes is in agreement with previous studies on myxozoan species (Lavrov and Pett 2016; Yahalomi et al. 2017). This suggests that the loss of tRNA<sub>Met</sub><sup>CAC</sup> and tRNA<sub>Trp</sub><sup>UCG</sup> is a characteristic of Endocnidozoa. Further, all mt aminoacyl-tRNA synthetase genes have been lost in *P. hydriforme* (supplementary table S1, Supplementary Material online), including the mt tryptophanyl-tRNA synthetase gene, which is present in most cnidarians (Pett and Lavrov 2015). Interestingly, the loss of tRNA<sub>Trp</sub><sup>UCG</sup> is also accompanied by the transition of the TGA codon into a stop codon in *P. hydriforme* (see below).
**Fig. 4.**—Phylogenetic analyses of cnidarian mt genomes. (A) Phylogenetic tree inferred from the concatenation of five mt proteins (cox1, cox2, cytb, nad1, nad5; 96 taxa and 1,700 amino-acid sites). Maximum-likelihood (ML) tree reconstructed under the LG + C60 + F + G mixture model. ML bootstrap support and Bayesian posterior probabilities computed under the CAT+F mixture model are only indicated for Endocnidozoa branches (for the other branches see supplementary fig. S5, Supplementary Material online). The branch lengths exemplify the fast evolutionary rate of *P. hydriforme* and Myxozoa. (B) Evolution of the mt genome structure in cnidarians (based on Yahalomi et al. 2017); the topology follows Kayal et al. (2018). Red and black branches indicate linear and circular mt genomes, respectively (as illustrated by the diagrams at the end of each branch).
Polypodium Reverted to the Standard Genetic Code

In addition to the complete mt genome of a *P. hydriforme* individual from the US (described above), we have also obtained, using Sanger sequencing, a 6,675 bp mt genome fragment from a Russian *P. hydriforme* individual (supplementary materials and methods, Supplementary Material online). Protein-coding gene alignments between these two individuals reveal that stop codons in one individual are often aligned with a TGA codon in the other, which in Cnidaria encodes tryptophan. In addition, the first appearance of a TGA codon is always aligned to a stop codon in the other individual (fig. 3). Together, this suggests that TGA is a stop codon and that the *P. hydriforme* mt genome does not use the “The Mold, Protozoan, and Coelenterate” mt code (i.e., Translation Table 4) typically utilized by other cnidarians. *Polypodium hydriforme* has instead reverted to the standard genetic code because the only difference between both genetic codes is the UGA codon assignment.

Putative Cryptic Polypodium Species

The complete US and partial Russian mt sequences present the same gene order over the region in which they overlap. The genetic distance between the nucleotide sequences of the six shared protein-coding genes of the US and partial Russian mt sequences was relatively large: the *p*-distances ranged from 10.5% to 14.0%, and the K2P distance for the barcoding region (i.e., between the HCO 1490 and the LCO primers) is 11.7%. This distance exceeds the generally accepted 2% nucleotide sequence difference cutoff in COI barcoding region for being different species (Hajibabaei et al. 2006). However, given the fast evolutionary rate in Endocnidozoa, the threshold of 2% is likely irrelevant for detecting cryptic species within this group. Nevertheless, this cutoff threshold seems to be below 10% even in fast-evolving myxozoans. As a case in point, the distance between two *Kudoa* sequences, which belong to different species, can be as low as 3–5% (Sakai et al. 2018; Sakai et al. 2019). However, it should be noted that in this case, the comparison involves a different COI region than the barcoding one. This suggests that the Russian and US populations of *P. hydriforme* could represent cryptic species. Sampling additional *Polypodium* specimens would help resolve this issue.

Phylogenetic Reconstruction

Myxozoa and *P. hydriforme* mt DNA show an extremely fast rate of evolution in agreement with previous studies (Lavrov and Pett 2016; Yahalomi et al. 2017). Based on branch lengths and the low number of mt genes that we were able to identify (five genes previously identified in Myxozoa and eleven in *P. hydriforme* in this study), it appears that myxozoans evolve faster than *P. hydriforme*. This high evolutionary rate resulted in a lack of similarity between the endocnidozoan sequences and those of the other cnidarians analyzed. Thus, the phylogenetic reconstructions based on mt sequences did not resolve the phylogenetic position of Endocnidozoa among cnidarians (fig. 4A and supplementary figs. S4 and S5, Supplementary Material online). Even when myxozoans are excluded, the long *P. hydriforme* branch precludes accurate phylogenetic inferences and the phylogenetic position of *P. hydriforme* within Cnidaria is uncertain (supplementary fig. S6, Supplementary Material online). Nevertheless, Endocnidozoa were recovered as a monophyletic group, because *P. hydriforme* mt sequences formed a well-supported clade with Myxozoa (bootstrap percentage; BP = 100, Bayesian posterior probabilities PP = 0.98). The reconstructions also supported the monophyly of Myxozoa (BP = 80/PP = 0.95).

Implications for Classification Within Cnidaria

The circular structure of the mt chromosome of *P. hydriforme* supports the view that the ancestor of Endocnidozoa harbored a circular mt genome and that linearization of the medusozoan mt genome occurred after the divergence of Endocnidozoa from Medusozoa (figs. 1B and 4B). These results are in agreement with previous studies that showed a circular structure of Myxozoa mt genome (Takeuchi et al. 2015; Yahalomi et al. 2017, 2020). Furthermore, among cnidarians, myxozoans and *P. hydriforme* have lost all mt tRNA genes and share a unique organization (in having compact coding region and a large noncoding region). These discrete molecular characters, defining the mt genome architecture, provide strong evidence in support of the positioning of the Myxozoa and *P. hydriforme* as sister taxa outside Medusozoa (Siddall et al. 1995; Chang et al. 2015). The monophyly of Endocnidozoa is also supported by: (1) these two taxa being endoparasites; (2) the presence of cell within a cell early developmental stages (Morris 2012); (3) shared distinct minicollagens gene structures (Shpirer et al. 2014; Kyslik et al. 2021).

Conclusions

Our study suggests that the evolution of mt genomes in cnidarians is more complex than previously thought. The circular structure of the mt DNA, the loss of mt tRNAs, and the unexpected insertion of a large, noncoding region within the mt genome of *P. hydriforme* provide additional evidence uniting *P. hydriforme* with Myxozoa.

Supplementary Material

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

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Data Availability

The ONT reads and the annotated mt genome sequence of US *P. hydriforme* were deposited at the National Center for Biotechnology Information (NCBI) under accession numbers PRJNA558922 and MN794187, respectively. The partial mt sequence from the Russian *P. hydriforme* was submitted under accession MN714000. Gene annotations based on hidden Markov models were not accepted by NCBI. ATP6, COX3, and ND6 are hence annotated as ORFs in NCBI.

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