

Genomic insights into the evolutionary origin of Myxozoa within Cnidaria

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The Myxozoa comprise over 2,000 species of microscopic obligate parasites that use both invertebrate and vertebrate hosts as part of their life cycle. Although the evolutionary origin of myxozoans has been elusive, a close relationship with cnidarians, a group that includes corals, sea anemones, jellyfish, and hydroids, is supported by some phylogenetic studies and the observation that the distinctive myxozoan structure, the polar capsule, is remarkably similar to the stinging structures (nematocysts) in cnidarians. To gain insight into the extreme evolutionary transition from a free-living cnidarian to a microscopic endoparasite, we analyzed genomic and transcriptomic assemblies from two distantly related myxozoan species, *Kudoa iwatai* and *Myxobolus cerebralis*, and compared these to the transcriptome and genome of the less reduced cnidarian parasite, *Polypodium hydriforme*. A phylogenomic analysis, using for the first time to our knowledge, a taxonomic sampling that represents the breadth of myxozoan diversity, including four newly generated myxozoan assemblies, confirms that myxozoans are cnidarians and are a sister taxon to *P. hydriforme*. Estimations of genome size reveal that myxozoans have one of the smallest reported animal genomes. Gene enrichment analyses show depletion of expressed genes in categories related to development, cell differentiation, and cell-cell communication. In addition, a search for candidate genes indicates that myxozoans lack key elements of signaling pathways and transcriptional factors important for multicellular development. Our results suggest that the degeneration of the myxozoan body plan from a free-living cnidarian to a microscopic parasitic cnidarian was accompanied by extreme reduction in genome size and gene content.

Myxozoa | Cnidaria | *Polypodium* | parasite | genome evolution

Obligate parasitism can lead to dramatic reduction of body plans and associated morphological structures (1, 2). One of the most spectacular examples is the microscopic Myxozoa, which spend most of their parasitic life cycle as just a few cells in size (3). The most conspicuous myxozoan cell type houses a polar capsule, which is a complex structure with an eversible tube (or filament) that is thought to facilitate attachment to the host. The polar capsule bears remarkable similarity to the stinging structures (nematocysts) of cnidarians (corals, sea anemones, jellyfish, and hydroids), suggesting that nematocysts and polar capsules are homologous and that myxozoans are related to cnidarians (4, 5).

Myxozoa are a diverse group of obligate endoparasites that comprise over 2,180 species (6). The vast majority of myxozoan species alternate between a fish and annelid host. In *Myxobolus cerebralis* (7), the causative agent for whirling disease in rainbow trout (8), the annelid host *Tubifex tubifex* releases infective actinospores (Fig. 1A), which subsequently anchor onto the fish, injecting the sporoplasm into the host tissue (9). Infective myxospores develop within the fish and are eventually ingested by the annelid where they develop into an actinospore, thereby completing the parasitic life cycle (Fig. 1A). Although the vast majority of myxozoan species are composed of just a few cells, some malacosporean myxozoans, such as *Buddenbrockia plumatellae*, have a complex

vermiform life cycle stage (10), which is a derived trait that has been lost and regained several times within this particular lineage (11).

Another obligate cnidarian endoparasite, *Polypodium hydriforme*, in contrast to myxozoans, does not have a degenerate body form and instead displays conventional cnidarian-like features, including tentacles, a gut, and a mouth (Fig. 1B). *P. hydriforme* lies dormant as a binucleate cell within the oocytes of female acipenseriform fishes (paddlefish and sturgeon) (Fig. 1B), eventually developing into an elongated stolon, which emerges from the host's oocyte upon spawning. Once freely living, the stolon begins to fragment into multiple individuals, each developing a mouth with which to feed (Fig. 1B). Adult *P. hydriforme* infect juvenile female fish to repeat the life cycle (12).

Classification of Myxozoa and *P. hydriforme* has been controversial (13–15). From the first descriptions in the 1880s until relatively recently myxozoans were considered to be protists, largely due to their highly reduced, microscopic construction (16). Unlike Myxozoa, the placement of the monotypic species *P. hydriforme* as a cnidarian has long been proposed based on morphology (17, 18). With the advent of molecular phylogenetics, it was discovered that myxozoans are not protists, but instead are metazoans (19, 20). The first studies, based mainly on analyses of 18S rDNA, usually recovered Myxozoa as the sister taxon to *P. hydriforme*. However, the

Significance

Myxozoans are a diverse group of microscopic parasites that infect invertebrate and vertebrate hosts. The assertion that myxozoans are highly reduced cnidarians is supported by the presence of polar capsules, which resemble cnidarian stinging structures called "nematocysts." Our study characterizes the genomes and transcriptomes of two distantly related myxozoan species, *Kudoa iwatai* and *Myxobolus cerebralis*, and another cnidarian parasite, *Polypodium hydriforme*. Phylogenomic analyses that use a broad sampling of myxozoan taxa confirm the position of myxozoans within Cnidaria with *P. hydriforme* as the sister taxon to Myxozoa. Analyses of myxozoan genomes indicate that the transition to the highly reduced body plan was accompanied by massive reduction in genome size, including depletion of genes considered hallmarks of animal multicellularity.

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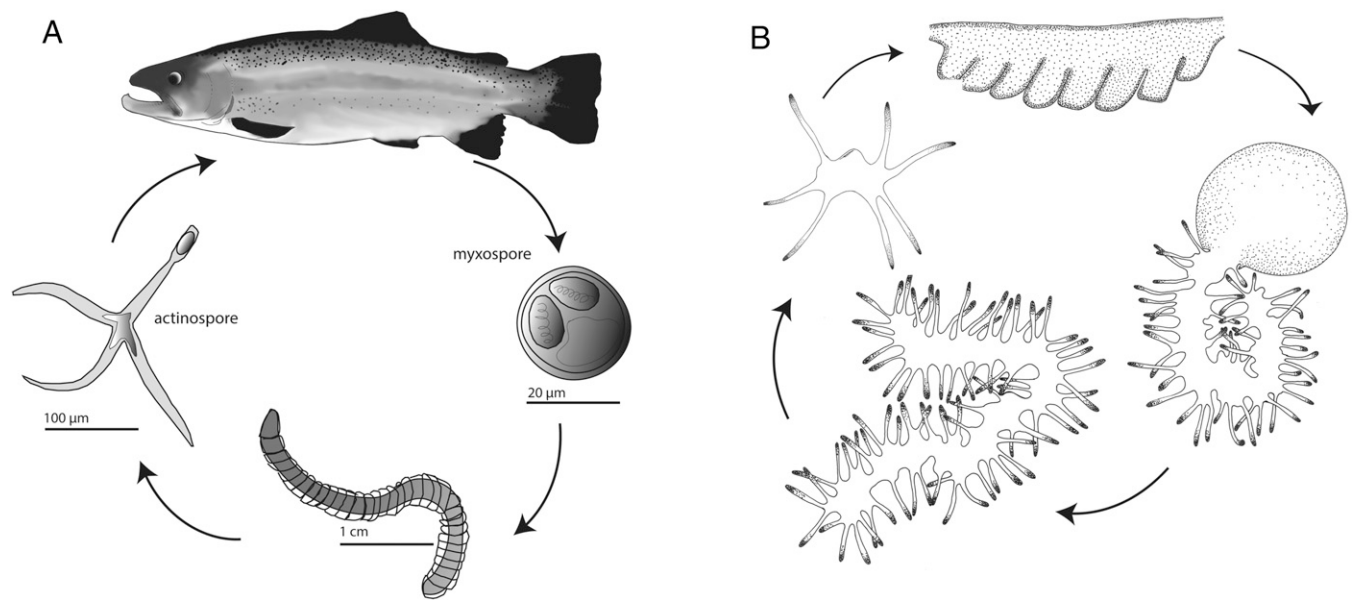


Fig. 1. Life cycles of *M. cerebralis* and *P. hydriforme*. (A) *M. cerebralis* alternates its development between a fish (salmonid) host and an annelid (*T. tubifex*) host. The myxospore is produced in the fish (Right), and the actinospore is produced in the annelid (Left). Both stages consist of just a few cells, including those housing polar capsules. (B) In *P. hydriforme*, the stolon stage (Top) develops inside the ovaries of its host (acipenseriform fish). Upon host spawning, *P. hydriforme* emerges from the host's oocyte (Right), fragments, and lives as a free-living stage with a mouth (Left) before infecting its host.

position of this clade was unstable. It was either placed as the sister clade to Bilateria or nested within Cnidaria, depending on taxon sampling, alignment, optimization method, and the characters considered (13, 19–23). Recent phylogenomic studies support a position of Myxozoa within Cnidaria, as the sister clade to Medusozoa (24–26). However, in these studies *P. hydriforme* and representatives of major lineages of myxozoan and nonmyxozoan cnidarians were notably absent. Thus, the precise phylogenetic position within Cnidaria remains uncertain.

In recent studies, myxozoans were found to possess the cnidarian-specific minicollagen and nematogalactin genes (24, 27, 28), each of which has been shown to play important roles in the nematocyst structure in *Hydra* (29, 30). These studies support previous morphology-based assertions that myxozoan polar capsules are homologous to cnidarian nematocysts (4, 5, 31, 32) and thus indirectly suggest a close evolutionary relationship between these two groups.

For this study, we analyzed genomic and transcriptomic assemblies from two distantly related myxosporean myxozoans, *Kudoa iwatai* and *M. cerebralis*, as well as the cnidarian parasite *P. hydriforme*, to gain insight into the evolutionary transition to parasitism and extreme reduction of body plans from a free-living cnidarian. First, we used these newly generated data, in conjunction with publicly available data, to determine the phylogenetic relationships between all major lineages of myxozoans (33), *P. hydriforme*, and other cnidarians to reconstruct the evolutionary history of endoparasitism in Cnidaria. Second, we compared genome size, gene number, gene content, and enrichment of expressed genes between myxozoans, *P. hydriforme*, and published cnidarian genomes to determine if the degeneration of the cnidarian body plan displayed in Myxozoa (but not in *P. hydriforme*) was accompanied by genome reduction and gene loss. Our findings reaffirm a cnidarian origin for myxozoans and recover them as the sister group to *P. hydriforme*. Analysis of genome and transcriptome assemblies reveal that the highly degenerate body plan of myxozoans coincided with extreme reduction in genome size and gene loss while retaining some genes necessary to function as an obligate parasite. By contrast, *P. hydriforme*, which displays many cnidarian-like morphological features, has a genome size and gene content similar to that of published cnidarian genomes.

Results

Phylogenetic Position. A phylogenomic analysis was performed using the newly generated transcriptome assemblies from *K. iwatai*, *M. cerebralis*, and *P. hydriforme* as well as genomic data of *Enteromyxum leei* and *Sphaeromyxa zaharoni* in conjunction with published sequences from three additional myxozoans (*Buddenbrockia plumatellae*, *Tetracapsuloides bryosalmonae*, and *Thelohanellus kitauei*), altogether encompassing 22 cnidarians, 38 representatives of the Metazoan diversity, and 9 unicellular opisthokont taxa. Both Bayesian analyses using the CAT model (34) and a maximum-likelihood (ML) analysis using the GTR model recovered *P. hydriforme* as sister to a monophyletic Myxozoa with maximal support [Bayesian posterior probability (PP) of 1.0, ML bootstrap percentage (BP) of 100]. Within a monophyletic Cnidaria (PP = 1.0, BP = 100), the Myxozoa + *P. hydriforme* clade was recovered as sister to the medusozoan clade with maximal support (PP = 1.0, BP = 100) (Fig. 2). The Bayesian and ML topologies differed only in the position of two taxa (*Porites* and *Strigamia*). Several analyses were conducted to evaluate the robustness of the position of the Myxozoa + *P. hydriforme* clade within Cnidaria. Because it has been claimed that ribosomal genes can contain a different signal from nonribosomal genes (35), phylogenetic analyses were conducted on a dataset of 41,237 amino acids, excluding ribosomal genes (Fig. S1). Additionally, because taxon sampling can affect phylogenetic inferences, phylogenetic reconstructions were performed either with only cnidarian taxa (Fig. S2) or after removing either Myxozoa or *P. hydriforme*. None of these analyses affected the position of Myxozoa and *P. hydriforme* or its position as the sister clade to Medusozoa.

Estimation of the Completeness of Genome and Transcriptome Assemblies. RNA libraries from *M. cerebralis*, *K. iwatai*, and *P. hydriforme* and DNA libraries from the latter two were sequenced using a short-read Illumina platform. Data were deposited in the National Center for Biotechnology Information (NCBI) archives (Table S1). Previously generated *M. cerebralis* genomic data (26) were downloaded from the NCBI (SRX208206). Assembly statistics are shown in Table 1, and size distribution of the transcriptome sequences is shown in Fig. S3. Completeness of the genome and transcriptome assemblies was estimated by determining the presence of the 248 ultra-conserved core eukaryotic

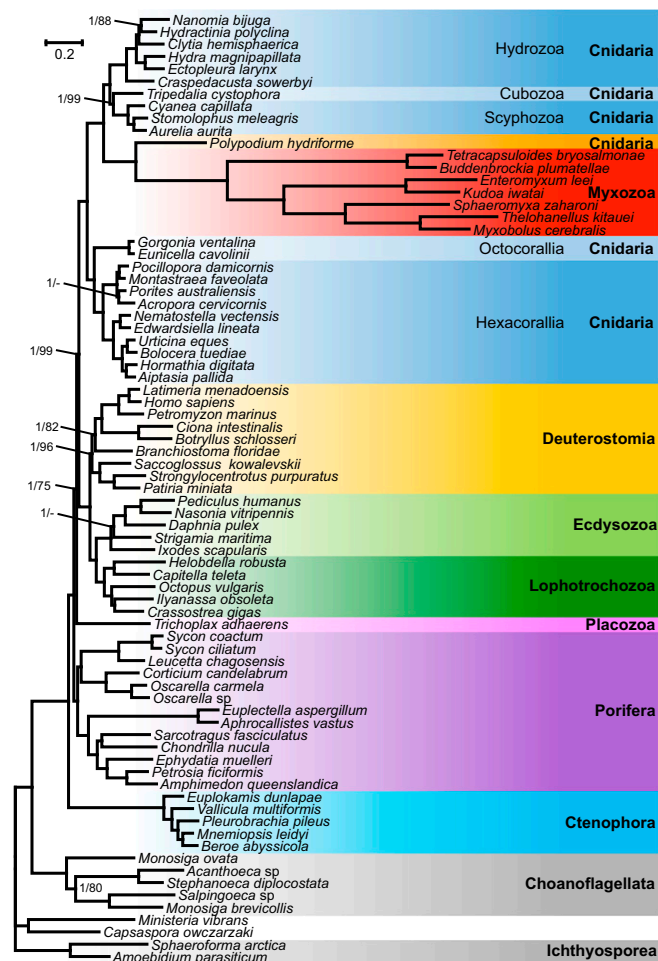


Fig. 2. Phylogenetic tree generated from a matrix of 51,940 amino acid positions and 77 taxa using Bayesian inference under the CAT model. Support values are indicated only for nodes that did not received maximal support. Bayesian posterior probabilities/ML bootstrap supports under the PROTGAMMAGTR model are given near the corresponding node. A minus sign (“-”) indicates that the corresponding node is absent from the ML bootstrap consensus tree.

genes (CEGs), obtained from the Core Eukaryotic Genes Mapping Approach (CEGMA) database (36) (Table 1). The *K. iwatai* genome and transcriptome assemblies recovered over 70% of the CEGs with over 1,000x estimated mean base-pair coverage. The *M. cerebralis* transcriptome was less complete, recovering only 39% of the CEGs. We were unable to recover any CEGs for *M. cerebralis* from its published genomic data, most likely due to their low coverage. Although the *P. hydriforme* genome assembly

recovered very few CEGs due to low coverage, its transcriptome assembly recovered 90% of complete CEGs.

General Characteristics of Genomes. Genome size estimates based on overall coverage of known individual genes are shown in Table 2. These estimates suggest that the myxozoan genome of *K. iwatai* (22.5 Mb) is one of the smallest reported animal genomes, comparable to the genome of the recently reported parasitic nematode (~20 Mb) (37). The *K. iwatai* genome is more than 20-fold smaller than the estimated size of the *P. hydriforme* genome (561 Mb) and the published genome of the cnidarian *Nematostella vectensis* (450 Mb) (38) and more than 40-fold smaller than the published estimated genome size of the cnidarian *Hydra magnipapillata* (1,005 Mb) (39). Although the published estimated genome size of the myxozoan *Thelohanellus kitauei* (188.5 Mb), which was based on K-mer distribution (40), is eightfold larger than our estimated *K. iwatai* genome size, it is still significantly smaller than the nonmyxozoan cnidarian genomes. As an independent test of the accuracy of genome-size estimation, we compared genome size based on the overall assembly coverage of the *K. iwatai* genome from two independent sequencing runs (*SI Materials and Methods*). This revealed a very similar genome-size estimate (23.5 Mb). Due to the low coverage of the published *M. cerebralis* genomic read data, it was not possible to estimate its genome size.

The number of protein-coding genes and average intron and exon sizes were estimated from the genome assemblies using the Maker2 (*SI Materials and Methods*). These analyses revealed that the number of protein-coding genes in the *K. iwatai* genome (5,533) is less than 30% of those estimated in *P. hydriforme* (17,440), *H. magnipapillata* (16,839), and *N. vectensis* (18,000) (Table 2). In addition, the myxozoan genome appears to be much more compact, with a mean intron size of 82 bp in *K. iwatai*, compared with 1,163 bp in *P. hydriforme*, 2,673 bp in *H. magnipapillata*, and 799 bp in *N. vectensis* (Table 2).

Characteristics of Transcriptomes: Comparisons of Gene Ontology and Gene Orthology. To identify the biological pathways that have gained or lost a significant fraction of expressed genes in the myxozoans *M. cerebralis*, *K. iwatai*, and *P. hydriforme* compared with the cnidarian model species *H. magnipapillata* and *N. vectensis*, Fisher’s exact tests were used to infer enrichment and depletion in the proportion of genes present in 112 Gene Ontology (GO) categories as defined by the GOSlim list of CateGORizer (41) (Table 3 and Dataset S1). Because the GO terms of *P. hydriforme* were more similar to *N. vectensis* and *H. magnipapillata* than to the myxozoans *M. cerebralis* and *K. iwatai*, the most informative comparison was between *M. cerebralis* and *K. iwatai* versus *P. hydriforme*, *H. magnipapillata*, and *N. vectensis* (Dataset S1, Tab 2). Of the top 20 GO categories with the highest occurrences of GO terms (Fig. 3), the expressed myxozoan genes appear to be significantly depleted (by comparison with other cnidarians) in categories that are related to development, cell differentiation, and cell-to-cell communication (Table 3), consistent with lack of a complex multicellular body in myxosporean myxozoans. By contrast, myxozoan-expressed genes have an abundance of categories such as cellular function, for which the number of genes does not differ significantly

Table 1. Assembly statistics for sequenced genomes and transcriptomes

	<i>K. iwatai</i>		<i>M. cerebralis</i>		<i>P. hydriforme</i>	
	Genome	Transcriptome	Genome*	Transcriptome	Genome	Transcriptome
Raw reads	167,917,062	154,253,215	NA	312,202,378	229,917,588	467,431,688
Contigs	1,637	6,528	NA	52,972	83,415	24,523
N50	40,195	1,662	NA	994	3,865	1,475
CEGs (c), % [†]	179/72	190/77	0/0	97/39	14/6	223/90
CEGs (p), % [†]	188/76	208/84	0/0	164/66	56/23	232/94

*Published ESTs.

[†]Number/percentage of 248 ultra-conserved CEGs. c, complete; p, partial.

Table 2. Estimated genome characteristics

	<i>Kudoa</i>	<i>Polypodium</i>	<i>Hydra</i> (39)	<i>Nematostella</i> (38)
Genome size (Mb)	22.5	561	1,005	450
No. protein genes	5,533	17,440	16,839	18,000
GC content, %	28	47	29	41
Mean intron size, bp	82	1,163	2,673	799
Mean exon size, bp	102	216	218	208

from the number observed in cnidarians (e.g., a similar number of nucleoplasm genes was found in both) (Dataset S1, Tab 2). Although these analyses were from transcriptomes, the general patterns likely reflect overall genome content as multiple life cycle stages are represented in the combined transcriptome of *K. iwatai* and *M. cerebralis* (SI Materials and Methods). To confirm this, we also performed a GO comparison analysis of the genes predicted based on genomic sequences, which revealed the same general patterns for *K. iwatai*, but not for *P. hydriforme* whose transcriptome assembly was of better quality than its genome assembly (Dataset S1 and Fig. S4).

Using the OrthoMCL database (42), we determined the number of orthologous groups (OG) that could be identified from our transcriptome assemblies of *P. hydriforme* and *K. iwatai*, compared with published predicted proteins from *H. magnipapillata* (39) and *N. vectensis* (38) (Fig. S5). A total of 8,021 unique OGs were recovered from *H. magnipapillata*, 11,162 from *N. vectensis*, 5,451 from *P. hydriforme*, and 2,735 from *K. iwatai*. Although there was more overlap in the number of OGs between *H. magnipapillata*, *N. vectensis*, and *P. hydriforme* than between *K. iwatai* and the other three, this is consistent with the significantly lower number of total OGs in *K. iwatai* (Fig. S5).

Analyses of Gene Pathways and Candidate Genes in the Transcriptome and Genome Assemblies. We searched for several candidate genes and gene pathways that have been previously characterized as important for cnidarian cell signaling and development in the transcriptome and genome assemblies of *M. cerebralis*, *K. iwatai*, and *P. hydriforme*. Using BLAST searches and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses we determined presence/absence of representatives within a gene family, but not precise orthology, within the particular families. Myxozoan genomes appear to lack key genes and signaling pathways that are present in *P. hydriforme* and other cnidarians (Table 4). Specifically, the conserved transcriptional factors belonging to the Hox and Runx gene families, which have been shown to be important for cnidarian patterning and cellular differentiation, respectively (43, 44), were found in neither the genomes nor transcriptomes of myxozoans, but were nevertheless present in *P. hydriforme* (Table 4 and Dataset S2). In addition, myxozoans appear to have lost the ligands, receptors, and most downstream elements of the Wnt- and Hedgehog-signaling pathways, which have been shown to be important for axial patterning (45) and cell signaling (46), respectively, whereas nearly all components of these pathways were recovered in *P. hydriforme* (Table 4 and Dataset S3). By contrast, myxozoans and *P. hydriforme* possess orthologs to the stem-cell markers FoxO (47) and Piwi (48), and *P. hydriforme* and *K. iwatai* appear to have the gene Hap2, which was shown to be involved in gamete fusion in *Hydra* (49) (Table 4 and Dataset S2). The two myxozoans and *P. hydriforme* were also found to have key elements of the Notch-signaling pathway, and *M. cerebralis* possessed some elements of the TGF β pathway (Table 4 and Dataset S3). Notch is reported to have an important role in differentiation of stem-cell lineages (50), whereas TGF β appears to play a more general role in cell signaling (51).

Discussion

Our analyses of transcriptomic and genomic assemblies of myxozoans have yielded significant insight into the evolution of these microscopic parasites from free-living cnidarians. We report, for the first time to our knowledge, a broad phylogenomic sampling of myxozoans, including representatives from the malacosporean clade and the freshwater and marine myxosporean clades (33), as well as the only phylogenomic study to date to include *P. hydriforme*. In addition, we have a more comprehensive sampling of cnidarians than previous phylogenomic studies, addressing the placement of myxozoans (24–26). We recover *P. hydriforme* as the sister taxon to Myxozoa and can confirm, with an increased sampling and thus a higher degree of confidence, the placement of this clade as the sister taxon to medusozoan cnidarians. These results are consistent with those of other molecular phylogenetic studies (19, 23), although these have been criticized as possible artifacts of long-branch attraction (21). The monophyly of Myxozoa + *P. hydriforme* is also supported by endoparasitism in fish, a unique cell-within-cell developmental stage, possession of a single similar type of nematocyst (19, 23), and similarity in minicollagen sequences (28). This phylogenomic pattern suggests that endoparasitism in Cnidaria was a single event that occurred at the base of Myxozoa + *P. hydriforme*, but that the dramatic reduction in body plan occurred following the divergence of *P. hydriforme* from myxozoans, as *P. hydriforme* retains many cnidarian features.

The Myxozoa represent an extreme example of degeneration of body plans due to parasitism. Genome and transcriptome analyses reveal that this degeneration was accompanied by massive genome reduction, with myxozoans having one of the smallest reported animal genomes. Genome size reduction included loss of many genes considered hallmarks of metazoan development, yet retention of genes necessary to function as obligate parasites, such as nematocyst-specific genes. In contrast to myxozoans, *P. hydriforme* has a genome similar in size, gene number, and gene content to the model system *Hydra*. This finding is not surprising given that, although *P. hydriforme* is an obligate parasite, it has maintained its cnidarian-like body plan, including epithelia, mouth, gut, and tentacles. Our study provides a robust phylogenetic hypothesis for myxozoan placement within Cnidaria, as the sister taxon to *P. hydriforme*, and a framework for comparative genomic studies, which should be valuable for future phylogenetic and genomic investigations of Cnidaria sensu lato.

Materials and Methods

Phylogenetic Reconstruction. Curated sequence alignments of 200 protein markers (52) were augmented with sequences from the four myxozoan species generated for this study, from *P. hydriforme*, and from the NCBI databases (www.ncbi.nlm.nih.gov) [GenBank and Sequence Read Archive (SRA)] as described (52). After removing positions with unreliable alignment, the final dataset included 51,940 sites and 12% missing data (Dataset S4). Bayesian tree reconstructions were conducted under the Bayesian CAT model (34) as implemented in Phylobayes MPI vs.1.5 (53) (SI Materials and Methods). The ML analysis was conducted under the PROTGAMMAGTR model, as implemented in RAxML 8.1.3 (54). Alignments and Bayesian tree

Table 3. Transcriptome-based Gene Ontology categories showing depletion in myxozoans compared with other cnidarians

Category	Myxozoa, %*	Cnidaria, %†
Cell differentiation	548/0.0234	1763/0.0302
Development	1052/0.0450	3892/0.0667
Morphogenesis	456/0.0195	1693/0.0290
Receptor activity	46/0.00197	310/0.00531
Signal transducer activity	49/0.0002	328/0.0056

Myxozoan total number = 23,388. Cnidarian total number = 53,354. All categories had a *P* value ≤ 0.0001 .

*Myxozoa = *K. iwatai* + *M. cerebralis*.

†Cnidaria = *H. magnipapillata* + *N. vectensis* + *P. hydriforme*.

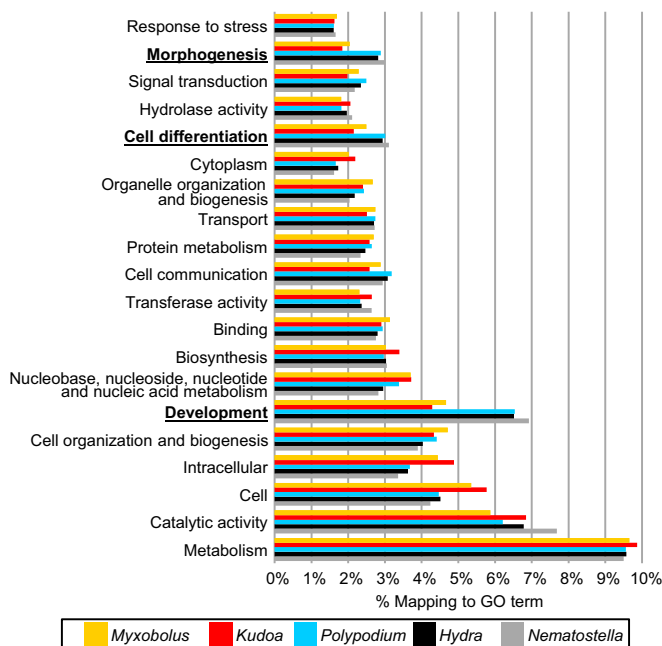


Fig. 3. GO annotation of unigenes in transcriptomes. The top 20 GO categories are shown as a percentage of total GO terms from the transcriptome assemblies of *M. cerebralis*, *K. iwatai*, *P. hydriforme*, and the published protein sequences of *H. magnipapillata* and *N. vectensis*. Categories for which myxozoans present significantly fewer GO terms than other cnidarians are indicated by boldface type.

have been deposited in the TreeBASE repository (55) (purl.org/phylo/treebase/phyloids/study/TB2:S17743?format=html).

Specimen Collection. Collection of specimens of *P. hydriforme*, *K. iwatai* plasmodia, *E. leei* trophozoites, and *S. zaharoni* plasmodia for both genomic and transcriptomic purposes was carried out as described in ref. 28 and *SI Materials and Methods*. Flash-frozen *M. cerebralis* actinospores were kindly provided by Ron Hedrick (University of California at Davis).

Illumina Sequencing. For the *M. cerebralis* transcriptome assembly, RNA extraction, library preparation, and sequencing was performed for the *P. hydriforme* transcriptome as described (28). Library preparations and genome sequencing of *P. hydriforme* was carried out at the Genome Sequence Facility at the University of Kansas Medical School. *P. hydriforme* gDNA was sheared to a size of 350 bp, and 100 bp paired-end (PE) sequencing was performed on an Illumina HiSeq 2000. Library preparation and Illumina 100-bp PE HiSeq 2000 sequencing of *K. iwatai* (transcriptome and genome), *S. zaharoni* (genome), and *E. leei* (genome) was described (28). In addition to the Illumina HiSeq sequencing, the *K. iwatai* genomic library was also independently sequenced with an Illumina Genome Analyzer IIx platform, which produced 95,434,687 paired reads (76 bp).

Genome and Transcriptome Assemblies. The *M. cerebralis* transcriptome was filtered for read quality and assembled following protocols described for the *P. hydriforme* transcriptome (28). Genome de novo assemblies for *P. hydriforme*, *E. leei*, *S. zaharoni*, and *K. iwatai* were performed with ABySS v. 1.3.6 (56), and the transcriptome de novo assembly of *K. iwatai* was performed with Trinity (57). Contigs shorter than 500 and 300 bp were removed from the genomic and transcriptomic assemblies, respectively. Filtering methods for host contaminants are described in *SI Materials and Methods*. The accession of the different assemblies and short read data are indicated in Table S1.

Analysis of Assembly Completeness. For each transcriptome and genome assembly, relative completeness was assessed using CEGMA that searches for the presence of 248 ultra-conserved CEGs (58). *P. hydriforme* assemblies were run using default settings. Because our evidence indicates that myxozoans have unusually small intron sizes (see below), the `-max_intron_size` parameter for *M. cerebralis* and *K. iwatai* genomic CEGMA runs was adjusted to match the maximum intron size of the *M. cerebralis* intron size distribution (2,630 bp).

Table 4. Presence (+) or absence (–) of genes and KEGG pathways that have been characterized in other cnidarians

	<i>K. iwatai</i>	<i>M. cerebralis</i>	<i>Polypodium</i>
Genes			
Hox-like	–	–	+
Runx	–	–	+
Piwi	+	+	+
FoxO	+	+	+
Hap2	+	–	+
KEGG pathways			
Wnt	–	–	+
Hedge	–	–	+
TGF β	–	+	+
Notch	+	+	+

Estimation of Genome Size and Content. Output from the CEGMA runs was used for coverage-based estimates of the genome size for *P. hydriforme* and *K. iwatai*. We used the “dna” output files from the genomic CEGMA runs, which include the raw sequence for each region identified by CEGMA as a partial or complete core gene, as well as the 2,000 bp of sequence on each side. Because CEGs were chosen to minimize in-paralogy and therefore should be largely single-copy, mapping reads to these regions provide a simple unbiased estimate of genome coverage. For each of the genomes, the complete set of raw reads was mapped to the CEGMA output files using the Burrows-Wheeler Aligner, BWA-MEM (v.0.7.8), with default settings (59). Coverage was calculated using QualiMap 2 (60). Under the assumption that the coverage estimated from the conserved CEGMA-identified regions is representative of genome-wide coverage, the number of base pairs used in the whole read set for each assembly was divided by the calculated coverage for that species, thus providing an estimate of the genome size for each species (Table 2). In addition, an independent approach based on coverage of all contigs was used to estimate the genome size of *K. iwatai* (*SI Materials and Methods*). Genome annotation was conducted using MAKER2 (*SI Materials and Methods*). The OrthoMCL database (42) was used to determine the number of orthologous groups identified in the transcriptome assemblies of *K. iwatai* and *P. hydriforme*, compared with published predicted proteins in *H. magnipapillata* (*SI Materials and Methods*).

Gene Enrichment Analysis of Transcriptomes. Before conducting the gene enrichment analysis, duplicate sequences and alternative transcripts were removed from the transcriptomes of *P. hydriforme* and *M. cerebralis* using methods described in *SI Materials and Methods*. No redundant contigs were found in the transcriptome of *K. iwatai*. We used the Trinotate pipeline (*SI Materials and Methods*) to annotate the *K. iwatai*, *M. cerebralis*, and *P. hydriforme* unigenes. In particular, Trinotate searched the GO database (61) and recovered for each transcript its relevant GO terms. The GO terms of each transcriptome were reduced using the GOSlim list in CateGORizer (41). The database of *H. magnipapillata* and *N. vectensis* protein sequences was downloaded from the Metazome website (metazome.net). GO terms were also assigned to *H. magnipapillata* and *N. vectensis* using Trinotate (*SI Materials and Methods*). The GO terms categories of Myxozoa and nonmyxozoan Cnidaria were compared for depletion or enrichment using Fisher’s exact tests. The significance level was corrected for multiple testing using a Bonferroni correction (specifically, $\alpha = 0.05$ was corrected to $\alpha = 0.000446$). Gene enrichment analysis of genomes is described in *SI Materials and Methods*.

Analysis of Gene Pathways and Candidate Genes. We searched assembled genomes and transcriptomes for genes and gene pathways that are considered important for cnidarian development. A multistep BLAST-based approach (62) was used for identifying candidate genes (*SI Materials and Methods*). All BLAST-search results are found in Dataset S2. We also assessed the completeness of candidate signaling pathways in our assemblies using KEGG (63) (*SI Materials and Methods*).

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