The highly repetitive genome of *Myxobolus rasmusseni* n. sp, a myxozoan parasite of fathead minnows

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Abstract

Background

The Myxozoa is a group of at least 2,400 endoparasites within the phylum Cnidaria. All myxozoans have greatly reduced in size and morphology compared to free-living members of the phylum. They are best known for causing disease in economically important fish across the world; for example, *Myxobolus cerebralis* causes whirling disease, which can kill 90% of infected juvenile salmonid fish. In 2017, a new myxozoan species was identified in Alberta. *Myxobolus rasmusseni* n. sp. causes distinct lesions in fathead minnows, which are ultimately fatal. Here, we sequenced, assembled and analyzed the genome of *M. rasmusseni* to understand how the parasite interacts with its fish host and identify potential strategies to counter this emerging threat.

Results

At 185 Mb, the *M. rasmusseni* genome is the largest myxozoan genome sequenced so far. This large genome size is, in part, due to the high repetitive content; 68% of the genome was interspersed repeats, with the MULE-MuDR transposon covering 18% of the *M. rasmusseni* genome. Similar to myxozoan genomes, the *M. rasmusseni* genome has lost many genes well conserved in other eukaryotes. However, we also identified multiple expansions in gene families—serine proteases, hexokinases, and FLYWCH-domain containing proteins—which suggests their functional importance in the parasite. The mitochondrial genome of *M. rasmusseni* encodes only five of the thirteen protein-coding genes typically found in animals. We found that

the mitochondrial gene *atp6* was transferred to the nucleus and acquired a mitochondria-targeting signal in *M. rasmusseni*.

Conclusions

Our study provides valuable insights into myxozoan biology and identify promising avenues for future research. We also propose that *M. rasmusseni* is promising myxozoan model to explore host-parasite interactions in these parasites.

1. Introduction

Myxozoa (phylum Cnidaria) are an enigmatic group of approximately 2,400 species of intracellular parasites, which have drastically simplified their morphology, compared to their free-living relatives: jellyfish, corals, and sea anemones (Alama-Bermejo & Holzer, 2021) (Figure 1A). Myxozoans are found in both freshwater and marine environments, and cycle between invertebrate (definitive) and vertebrate (intermediary) hosts (reviewed in (Eszterbauer et al., 2015)). While myxozoans have been identified in birds (Bartholomew et al., 2008), amphibians (Eiras, 2005), reptiles (Eiras, 2005), and even mammals (Friedrich et al., 2000), they are infamous for causing disease in fish, and pose significant ecological, conservation, and economic threats globally (Alama-Bermejo & Holzer, 2021; Hutchins et al., 2021; James et al., 2021; Koel et al., 2006; Sarker et al., 2015).

Myxobolus (Bütschli 1882) is the most species-rich genus in Myxozoa, with approximately 850 species described so far (Liu et al., 2019). Arguably the most well-known member of the genus is *Myxobolus cerebralis* (Hofer 1903), the causative agent of whirling disease in salmonid fish (Bartholomew & Reno, 2002). *M. cerebralis* attacks the cartilage of juvenile rainbow trout (*Oncorhynchus mykiss*), leading to skeletal deformities. Its definitive host is the oligochaete *Tubifex tubifex* (sludge worm). In 2016, whirling disease was first detected in Canada, in Johnson Lake in Banff National Park, Alberta (Canadian Food Inspection Agency, 2019), and *M. cerebralis* has now been found in four major Albertan water sheds, with approximately 85% of wild rainbow trout fingerlings in the Crowsnest River showing pathological signs of disease (James et al., 2021). This poses severe social, economic, and conservation concerns. The Crowsnest River rainbow trout are sympatric with the endangered westslope cutthroat trout, *Oncorhynchus clarkii lewisi*, which are also susceptible to *M. cerebralis*, raising concerns for their conservation (Koel et al., 2006). Further, rainbow trout is the third most valuable recreational fish species in Alberta, a sport that generates more than $1.1 billion/year for Alberta (Veillard & Clayton, 2020). Multiple other members of the genus cause disease in fish, for example *Myxobolus honghuensis* which infects the pharynx of the economically important gibel

carp (*Carassius auratus gibelio*) and *Myxobolus holzerae,* which causes severe gill disease in an Indian major carp (*Labeo rohita*) (Liu et al., 2012; Gupta & Kaur, 2017).

In 2017, a new member of the genus, *Myxobolus rasmusseni*, was discovered in fathead minnows (*Pimephales promelas*) in southern Alberta (Tilley *et al*. submitted, 2024). As with *M. cerebralis, M. rasmusseni* uses *T. tubifex* as a definitive host (Figure 1B). Fathead minnows infected with *M. rasmusseni* have large lesions in the head, ocular, oral, and sinus cavities, leading to reduced olfactory acuity, and increased fatigue, hyperactivity and death compared to uninfected minnows. It is too soon to know the population-level effect on fathead minnows. A concern is that *M. rasmusseni* could—or already has—spread to other sympatric cyprinid species, including the western silvery minnow (*Hybognathus argyritis*), which is already designated as threatened in Canada (COSEWIC Assessment and Status Report, 2017). Further, fathead minnows have their own important place in the aquatic ecosystem, as a food source for larger fish, and feeding on algae thereby limiting algal overgrowth.

Despite their environmental and economic importance, myxozoans remain under studied. The diagnoses, monitoring, and treatment of parasitic diseases has greatly benefited from knowledge of the genome—and other omics data—for the causative organisms. Myxozoans have been relatively slow to enter the genomic age. To date, genomes have been published for only eight myxozoan species, with transcriptomes available for a further eight species (reviewed in (Alama-Bermejo & Holzer, 2021)). These resources are assisting myxozoan researchers answer questions related to virulence and diversity. Further, of the ~2400 myxozoan species, only five have a laboratory-confirmed life cycles with just another fifty having partially describe life cycles (Eszterbauer et al., 2015). This knowledge gap has limited efforts to explore myxozoan-host interactions and develop strategies for myxozoan control.

In this study, we set out to sequence, assemble, and annotate the genome of *M. rasmusseni*. This is only the second time that long-read sequencing technology has been used for the myxozoans, the first being *Myxobolus honghuensis* (Guo et al., 2022). The assembled genome is the largest of any myxozoan sequenced to date. The use of long-read sequencing allowed us to explore the repeat landscape of the parasite genome which showed that *M. rasmusseni* has one of the most repetitive animal genomes. We compared the genome with other myxozoan and free-living cnidarians and identified multiple gene families that have undergone expansion in *M. rasmusseni*, like serpins, peroxiredoxins, and hexokinases. In addition, we also present the mitochondrial genome of *M. rasmusseni* and show that the parasite possesses a highly reduced mitochondrial genome and mitochondrial proteome.

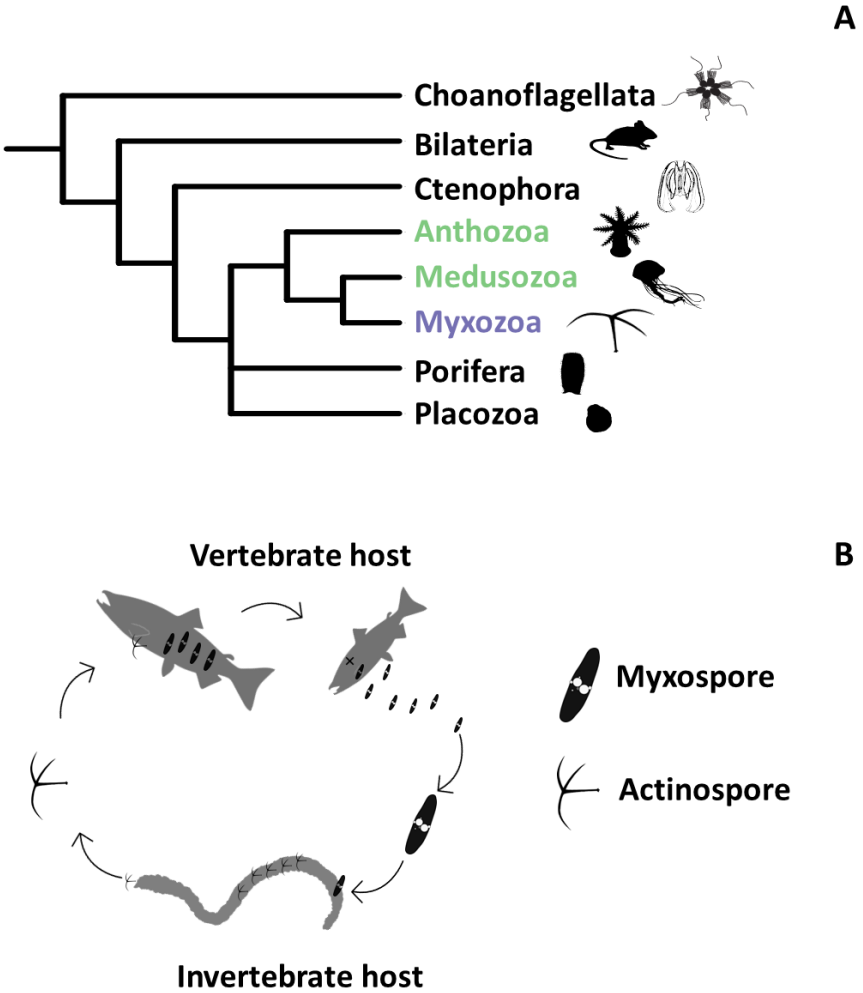

Figure 1. A) Phylogenetic relationships between the five metazoan groups (Porifera, Cnidaria, Ctenophora, Placozoa, and Bilateria) and the outgroup Choanoflagellata, where the free-living classes of phylum Cnidaria are colored in green and Myxozoa is colored in purple. Silhouettes for the animals are taken from PhyloPic (www.phylopic.org). B) A simplified life cycle of *M. rasmusseni.*

2. Methods and Materials

2.1. Sample Collection, DNA extraction, and Genome Sequencing

Myxobolus rasmusseni myxospores were isolated from lesions in fathead minnows following the protocols established by Dr. Cameron Goater and Molly Tilley (Tilley *et al.* 2024 *submitted*). High molecular weight was extracted using the MagAttract® HMW DNA Qiagen kit (Ref.67563). Clean-up used a combination of two kits: the beads from the Select-a-size Magbead kit (Zymo Cat# D4084-3-10) and the buffers from Genomic DNA Clean & Concentrator -25 (Zymo Cat# D4064). A detailed protocol is attached as Supplementary File S1. For sequencing, the SQK-LSK112 Ligation Sequencing Kit (Q20+) from Oxford Nanopore Technologies (ONT) was used to generate the libraries. No changes were made to the steps outlined in the manual for the SQK-LSK112 Ligation Sequencing Kit. The Mk1C MinION sequencer from ONT was used to sequence the genome and Guppy v2.1.7 was used for base-calling with default options.

2.2. Genome assembly and contamination removal

To remove the host (fathead minnow) reads, all the base-called reads were mapped to the fathead minnow draft genome (GCF_016745375.1) using minimap2 v2.24 (default options) (Li, 2018; Martinson et al., 2022). Three assemblies for *M. rasmusseni* from the unmapped reads using different assembly software:

1. FLYE v2.9 (Kolmogorov et al., 2019) ("flye –nano-hq <READSFILE> -o flye -t 48 -g 50m"),
2. wtdbg2 (Ruan & Li, 2020) ("wtdbg2 -x ont -g 50m -t 14 -i <READSFILE> -fo mras; wtpoa-cns -t 14 -i mras.ctg.lay.gz -fo mras.ctg.fa"),
3. Shasta (Shafin et al., 2020) ("shasta-Linux-0.10.0 –input <READSFILE> --threads 14 –config Nanopore-May2022 –Reads.minReadLength 5000").

BUSCO v5.4.3 (Eukaryota lineage) and N50 was used to select the preferred genome assembly for subsequent annotation and downstream analysis (Simão et al., 2015). The selected assembly was polished with MEDAKA v1.6.0 (https://www.github.com/nanoporetech/medaka). A further round of decontamination was performed using BlobTools v1.1 (Laetsch & Blaxter, 2017), Uniprot Reference Proteomes (downloaded 01 May 2023) (The UniProt Consortium, 2019), and DIAMOND BLASTX (Buchfink et al., 2021). All assembly contigs assigned as chordate, prokaryote, or viral origin were discarded.

148 ## 2.3. RNA extraction and *de novo* transcriptome assembly

149 Samples of fish muscles containing myxospores were lysed by adding 200 µl of Trizol followed
150 by manual homogenization in dry ice, for a total of three freeze-thaw cycles in a final volume of
151 600 µl. The resulting mixture was centrifuged, and the supernatant was collected into a new tube
152 where RNA was extracted using the Direct-zol RNA Miniprep kit (Zymo cat. no. R2050)
153 following the manufacturer's instructions. The extracted RNA was then cleaned using the RNA
154 Clean and Concentrator-5 kit (Zymo cat. no. R1015) following the manufacturer's instructions.
155 The quantity and quality of the total RNA were assessed on an Agilent 2200 TapeStation RNA
156 ScreenTape. Libraries for sequencing were prepared using the NEBNext Ultra II Directional
157 RNA Library Prep Kit with PolyA capture following the manufacturer's instructions. Libraries
158 were multiplexed and paired-end sequenced on Illumina NextSeq 500 for 300 cycles (2 × 150 bp)
159 using a mid-output cell, following the manufacturer's instructions. FastQC v0.11.9 was used to
160 assess the quality of the RNAseq reads before and after filtering reads (Andrews & others, 2010).
161 Adapter sequences and low-quality reads were identified with Trimmomatic v0.39 and removed
162 (Bolger et al., 2014). A *de novo* transcriptome assembly was generated with SPAdes v3.15.4
163 using default parameters (Bushmanova et al., 2019).
164

165 ## 2.4. Identification of repeat content and genome annotation

166 Interspersed repeats and low complexity DNA sequences in the *M. rasmusseni* genome were
167 masked using RepeatModeller v2.0.1 (Flynn et al., 2020) and RepeatMasker v4.1.4
168 (www.repeatmasker.org) with default parameters. This soft-masked genome was used as input
169 for BRAKER v2.1.6 to predict protein-coding genes with protein evidence (Brůna et al., 2021).
170 The OrthoDB v10 metazoa protein database was used as the protein evidence for the genome
171 annotation software BRAKER (Kriventseva et al., 2019). BRAKER was also used to annotate
172 the four publicly available myxozoan genome without genome annotations and to re-annotate the
173 four myxozoan genomes which had publicly-available genome annotations. For this, BRAKER
174 was run using protein evidence (OrthoDB v10 metazoa protein database) (Table S2). BUSCO
175 v5.4.3 was used to analyze the completeness of the myxozoan genomes. The lineage used was
176 'eukaryota_odb10' (Creation date: 2020-09-10, number of genomes: 70, number of BUSCO
177 genes: 255). For myxozoan species for whom BRAKER did not improve existing genome
178 annotations based on BUSCO analysis, previously published genome annotations were used for
179 downstream analysis.
180

181 ## 2.5. Analysis of the mitochondrial genome and proteome

182 A database of mitochondrial genomes from four myxozoan species, *Polypodium hydriforme,* two
183 cnidarian species, human, *Caenorhabditis elegans*, *Drosophila melanogaster*, and yeast were
184 used to identify whether *M. rasmusseni* has a mitochondrial genome (Table 1). BLASTN was

185 used to align these genomes to the *M. rasmusseni* genome (e-value ≤ $1e^{-5}$) (Camacho et al.,
186 2009). Two methods were used to identify genes in the mitochondrial genome. First, MITOS2
187 was used to annotate the contig containing the mitochondrial genome with parameters listed in
188 Table 2 (Bernt et al., 2013). MITOS2 was also used to annotate the five publicly available
189 myxozoan mitochondrial genomes. Second, an HMM model approach was used to identify
190 protein-coding genes in the mitochondrial genome. HMM models for the thirteen protein coding
191 genes found in the majority of animal mitochondrial genomes were downloaded from the
192 eggNOG protein database V5.0.0 (Cantalapiedra et al., 2021). HMMer (hmmer.org) was used to
193 search the mitochondrial genome of *M. rasmusseni* and the publicly-available myxozoan
194 mitochondrial genomes. PROKSEE was used to visualize the mitochondrial genome (Grant et
195 al., 2023).
196
197 Most proteins that function in the mitochondria are encoded in the nuclear genome and then
198 imported into the mitochondria (Meisinger et al., 2008). To identify these proteins, human
199 mitochondrial proteins catalogued in MitoCarta v3 (Rath et al., 2021) were downloaded.
200 BLASTP was used to align these human mitochondrial proteins to the proteins from all
201 myxozoan and free-living cnidarian nuclear genomes (e-value ≤ $1e^{-5}$).
202
203 **Table 1** Publicly available mitochondrial genomes used in this analysis to identify the
204 mitochondrial genome in *Myxobolus rasmusseni*

Species	**GenBank ID**
Polypodium hydriforme	MN794187.1 (Novosolov et al., 2022)
Myxobolus squamalis	MK087050.1 (Yahalomi et al., 2020)
Kudoa hexapunctata	LC009437.1 (Takeuchi et al., 2015)
Kudoa septempunctata	AB731753.1 (Takeuchi et al., 2015)
Kudoa iwatai	LC009438.1 (Takeuchi et al., 2015)
Nematostella vectensis	OW052000.1 (Fletcher & Pereira da Conceicoa, Lyndell, n.d.)
Dendronephthya gigantea	NC_013573.1 (Park Eun-Ji et al., 2010)
Homo sapiens	NC_012920.1 (Anderson et al., 1981)
Caenorhabditis elegans	NC_001328.1 (Wolstenholme et al., 1994)
Drosophila melanogaster	NC_024511.2 (Hoskins et al., 2015)
Saccharomyces cerevisiae	NC_027264.1 (Wolters et al., 2015)

205
206 **Table 2** The parameters used in MITOS2 to annotate the mitochondria genome of *M. rasmusseni*

Property	**Value**
Reference	RefSeq 63 Metazoa
Genetic Code	4
Proteins	TRUE
tRNAs	TRUE
rRNAs	TRUE
OH	TRUE
OL	TRUE

Circular	TRUE
Use Al Arab et al.	FALSE
E-value Exponent	2
Final Maximum Overlap	50nt
Fragment Quality Factor	100
Standard Code	FALSE
Cutoff	50.00%
Clipping Factor	10
Fragment Overlap	20.00%
Local only	TRUE
Sensitive only	FALSE
ncRNA overlap:	50 nt

2.6. Genome Analysis

2.6.1. Identifying the function of protein-coding genes

If a gene had multiple transcripts, the longest transcript and associated protein per gene was selected. eggNOG-mapper v2.1.9 (Cantalapiedra et al., 2021) to annotate the predicted proteins using default parameters, except two (percentage identity was changed to 30% and all queries were realigned to the PFAM database instead of importing PFAM annotations from orthologs). The output of eggNOG-mapper was parsed to extract 1) PFAM domains, 2) Gene Ontology (GO) terms, and 3) KEGG pathways mapped to each protein.

2.6.2. Gene family analysis

OrthoFinder v2.5.4 was used, with default parameters, to identify groups of orthologous proteins (OGs) between the predicted protein sets of *M. rasmusseni*, six publicly-available myxozoan species, and seven cnidarian species (listed in Table 3) (Emms & Kelly, 2019). The sets of predicted proteins for the free-living cnidarian species were downloaded from UniProt (one protein per gene). We omitted two myxozoan species (*K. iwatai* and *S. zaharoni*) as they had a high rate of duplication and therefore OrthoFinder identified considerably lower number of single-copy orthologs.

The single-copy orthologs identified by OrthoFinder were used to analyze phylogenetic relationships between these 14 species. First, all single-copy orthologs were aligned using MAFFT ("--auto" option) (Katoh et al., 2002) and concatenated into a single alignment using the script "catfasta2phyml.pl". Next, the alignment was trimmed using trimAI (Capella-Gutiérrez et al., 2009). The resulting alignment was used as input for IQ-TREE v2.2.5 (Nguyen et al., 2015). The appropriate substitution model was identified using ModelFinderPlus ("-MFP" option)

232 (Kalyaanamoorthy et al., 2017) based on the Bayesian information criterion (BIC). Branch
233 supports were assessed with 1,000 standard ultrafast bootstrap approximation (UFBoot)
234 replicates (Hoang et al., 2018; Minh et al., 2013). All phylogenetic trees in this study were
235 visualized using iTOL (Letunic & Bork, 2021). CAFE5 (Computational Analysis of gene Family
236 Evolution) was used to identify rapidly expanding and contracting gene families (p-values <
237 0.05) using the OrthoFinder results and the IQ-TREE-generated phylogenetic tree (Mendes et al.,
238 2021).
239
240 Further, 98 proteomes from the class Actinopterygii were downloaded from UniProt. These were
241 used to identify *M. rasmusseni* proteins that lack sequence similarity to proteins from
242 Actinopterygii. For this, *M. rasmusseni* proteins were aligned to a database of proteins from
243 these 98 species (e-value ≤ $1e^{-3}$).
244
245 **Table 3** Details about the seven publicly-available myxozoan genomes used in this study

Species	Source	Reference
Sphaeromyxa zaharoni	GCA_001455285.1	(Chang et al., 2015)
Kudoa iwatai	GCA_001407235.2	(Chang et al., 2015)
Enteromyxum leei	GCA_001455295.2	(Chang et al., 2015; Yahalomi et al., 2017)
Myxobolus squamalis	GCA_010108815.1	(Yahalomi et al., 2020)
Henneguya salminicola	GCA_009887335.1	(Yahalomi et al., 2020)
Thelohanellus kitauei	GCA_000827895.1	(Yang et al., 2014)
Myxobolus honghuensis	Harvard Dataverse	(Guo, 2021; Guo et al., 2022)
Ceratonova shasta	GCA_918559645.1	

246

247 2.6.3. Search for minicollagens and nematogalectins

248 Protein sequences for minicollagens and nematogalectins from the study done by (Shpirer et al.,
249 2014) were downloaded and BLASTP was used to align them to the myxozoan genomes (e-value
250 ≤ $1e^{-5}$).
251

252 2.6.4. Identification of proteases/inhibitors

253 The MEROPS Scan Sequences database was downloaded, which is a non-redundant subset of
254 the MEROPS database that is used for the MEROPS batch BLAST searches (Rawlings et al.,
255 2010). BLASTP was used to align the proteins from all species in this study to the MEROPS
256 Scan database (e-value ≤ $1e^{-5}$).
257

258 2.6.5. Searching for homologs of drug target proteins from ChEMBL

259 All 9,072 single protein drug targets from ChEMBL 33 (www.ebi.ac.uk/chembl) were
260 downloaded. BLASTP was used to align *M .rasmusseni* proteins to the database of these
261 ChEMBL targets (e-value $\leq 1e^{-10}$).

262

3. Results

3.1. Genome assembly and repeat content analysis

3.1.1. Genome assembly statistics

We extracted DNA from *M. rasmusseni* myxospores that were isolated from lesions on the heads of fathead minnows. Approximately 3.2 million sequence reads (11.38 Gb) were generated from a single Oxford Nanopore run and passed base-calling filters. Of these, ~2.1 million reads (65%) could be mapped to the available genome assembly of the fathead minnow, and so were flagged as contamination and removed. The remaining ~1.1 million reads were used to assemble the *M. rasmusseni* genome. To achieve the best possible assembly, we generated assemblies from three algorithms: FLYE, wtdbg2, and Shasta. By reviewing assembly metrics—the most complete BUSCO complement and assembly continuity—we selected the FLYE assembly for the subsequent analysis (Table 4). We used BlobTools to remove further contamination by discarding all contigs that were either mapped to bacteria, eukaryote, or viruses (92 contigs). The final *M. rasmusseni* genome was comprised of 1,503 contigs (185 Mb with N50 of 407.91 Kb). At 185 Mbp, the *M. rasmusseni* genome assembly is the largest of any myxozoan species sequenced so far (Table 5).

Table 4 Comparison of the *M. rasmusseni* genome assembled using three genome assemblers (FLYE, wtdbg2, and Shasta) based on their BUSCO scores (Eukaryota lineage in the genome mode) and N50 values.

Assembler	Genome size (nt)	# Contigs	BUSCO (Eukaryota) % where n=225	N50
FLYE	190,986,082	1,595	C:32.2 [S:31. 4, D:0.8], F:19.2, M:48.6	383 kb
wtdbg2	178,055,605	1,282	C:28.2 [S:27.8, D:0.4], F:18.8, M:53.0	331 kb
Shasta	165,736,712	3,742	C:27.5 [S:27.1, D:0.4], F:17.3, M:55.2	80 kb

3.1.2. Repeat content analysis

Approximately 68% of the genome is comprised of interspersed repeats (Table 6). To confirm that the large repeat content in *M. rasmusseni* was not an artefact of the genome assembler, we also analyzed the repeat content of the genomes assembled using two other tools- wtdbg2 and Shasta. We found that these two genome assemblies were highly repetitive as well (67.2% for the wtdbg2 assembly and 68.2% for the Shasta assembly). In addition, we compared the types and prevalence of different types of repeats with *M. rasmusseni*. At the time of this analysis, the *M. honghuensis* assembly was the only other myxozoan built from long-reads, giving us

292 confidence in the assembly of repetitive loci. Using the same tools, we found that just over half
293 the *M. honghuensis* genome (51.0%) was comprised of repetitive regions.
294
295 The *M. rasmusseni* transposable element (TE) landscape was dominated by DNA transposons,
296 which occupied 43.8 % of the genome and retrotransposons occupied a further 9.3 % of the
297 genome (Table 6). In contrast, the *M. honghuensis* assembly is comprised of 14.7 % DNA
298 transposons and 1% retrotransposons. Of the *M. rasmusseni* DNA transposons, MULE-MuDR
299 transposons were the most abundant and were identified in 18.1% of the total genomic sequence.
300 Compared to *M. rasmusseni,* we found that just 2.5% of the genome was occupied by MULE-
301 MuDR elements. It is important to note that more repeats in *M. honghuensis* were unclassified
302 (35.4%) compared to *M.* rasmusseni (15.1%).
303
304 **Table 5** Genome statistics of all publicly-available myxozoan genomes and *M. rasmusseni*

Species	Platform	# Contigs	Size (Mb)	Contig N50 (Kb)	BUSCO (genome mode, eukaryote dataset) % where n=225
Myxobolus rasmusseni	Oxford Nanopore	1503	184.9	407.9	C:31.4 [S:31.0, D:0.4], F:19.2, M:49.4
Myxobolus honghuensis	PacBio + Illumina HiSeq 2500	1118	161.1	1273.5	C:32.6 [S:31.8, D:0.8], F:20.8, M:46.6
Myxobolus squamalis	Illumina HiSeq 3000	37921	43.7	1.3	C:26.3 [S:26.3, D:0.0], F:17.3, M:56.4
Sphaeromyxa zaharoni	Illumina HiSeq 2000	70935	173.6	4.5	C:30.6 [S:11.0, D:19.6], F:23.1, M:46.3
Kudoa iwatai	Illumina HiSeq 2000 / Illumina Genome Analyzer IIx	1646	31.2	39.5	C:31.8 [S:16.1, D:15.7], F:17.3, M:50.9
Enteromyxum leei	Illumina HiSeq 2000	69178	68.2	1.0	C:22.8 [S:20.4, D:2.4], F:21.6, M:55.6
Henneguya salminicola	Illumina HiSeq 3000	18332	61.4	1.2	C:32.9 [S:32.9, D:0.0], F:20.8, M:46.3
Thelohanellus kitauei	454 GS FLX + Illumina Genome Analyzer II	15632	150.3	13.0	C:32.2 [S:31.4, D:0.8], F:18.0, M:49.8
Ceratonova shasta	Illumina HiSeq 2000	24903	69.8	9.3	C:27.1 [S:25.1, D:2.0], F:8.2, M:64.7

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312 **Table 6** Results of RepeatModeller and RepeatMasker analysis for genomes of *Myxobolus*
313 *rasmusseni and Myxobolus honghuensis*

Repeat Elements		*Myxobolus rasmusseni*			*Myxobolus honghuensis*		
		# elements	**length occupied**	**% of sequence**	**# elements**	**length occupied**	**% of sequence**
Retroelements		14495	17182784	9.29	1121	1469706	0.91
	SINEs	0	0	0	0	0	0
	Penelope	0	0	0	0	0	0
	LINEs	532	258231	0.14	0	0	0
	LTR elements	13963	16924553	9.15	1121	1469706	0.91
DNA transposons		112916	80978766	43.79	43041	23662456	14.69
	hobo-Activator	6092	6575235	3.56	18240	11145247	6.92
	Tc1-IS630-Pogo	37716	22841177	12.35	12193	4972777	3.09
	En-Spm	0	0	0	0	0	0.00
	MULE-MuDR	39574	33412948	18.07	6488	4008242	2.49
	PiggyBac	41	30174	0.02	733	508393	0.32
	Tourist/Harbinger	0	0	0	0	0	0.00
	Other_(Mirage,P-element, Transib)	0	0	0	0	0	0.00
Rolling-circles		2705	949873	0.51	0	0	0
Unclassified		116134	27904502	15.09	166939	57023818	35.4
Total interspersed repeats			126066052	68.17		82155980	51.00
Small RNA		0	0	0	0	0	0
						`	
Satellite		0	0	0	0	0	0
Simple repeats		18636	870644	0.47	36080	1727836	1.07
Low complexity		8293	422235	0.23	12949	668608	0.42

314

315 ## 3.2. Genome annotation and analysis

316 We identified 10,106 protein-coding genes in the *M. rasmusseni* genome, which is around 65%
317 of the number of genes in the *M. honghuensis* genome. The average intron length, exon length
318 and gene lengths in *M. rasmusseni* and *M. honghuensis* were similar (Table 7). We reannotated

319 all eight publicly-available myxozoan genomes (with protein evidence) (Table 8). Four of these
320 eight genomes lacked publicly-available annotations and a further two species— *Myxobolus*
321 *squamalis* and *Thelohanellus kitauei*—received improved annotations, based on their BUSCO
322 scores (Table 8).
323
324 **Table 7** Distribution of intron lengths, exon lengths, and gene lengths for *M. rasmusseni* and *M.*
325 *honghuensis,* from BRAKER results

	Intron lengths		Exon length		Gene length	
	M. rasmusseni	*M. honghuensis*	*M. rasmusseni*	*M. honghuensis*	*M. rasmusseni*	*M. honghuensis*
values	32,234	52,165	43,272	69,808	10,106	16,867
min	5	4	3	3	196	108
max	59,944	24,000	12,697	12,542	89,608	113,913
range	59,939	23,996	12,694	12,539	89,412	113,805
sum	24,066,630	36,410,620	11,571,580	17,172,630	33,931,130	51,515,720
median	22	28	155	150	1,007	1,208
mean	746.62	697.99	267.41	246.00	3,357.52	3,054.23
std.dev	3,262.03	1,701.45	397.77	345.06	6,684.27	4,959.44

326
327 **Table 8** BUSCO analysis of predicted proteins from myxozoan genomes using the eukaryote
328 database. For myxozoans with publicly-available annotations, the predicted protein sets from
329 both the original annotation and from our BRAKER annotation were assessed using BUSCO.
330 N.A. not available. Note 1: We could not find the annotations file from the publication.

Species	**Original annotations**		**BRAKER2 annotations**	
	Protein-coding genes	**BUSCO (Eukaryota) % where n=225**	**Protein-coding genes**	**BUSCO (Eukaryota) % where n=225**
Sphaeromyxa zaharoni	N.A.	N.A.	18,437	C:35.7 [S:13.7, D:22.0], F:23.5, M:40.8
Kudoa iwatai	5,533	N.A.[1]	6,921	C:67.1 [S:31.0, D:36.1], F:7.5, M:25.4
Enteromyxum leei	N.A.	N.A.	20,185	C:22.8 [S:21.6, D:1.2], F:24.7, M:52.5
Myxobolus squamalis	5,710	C:22.0 [S:22.0, D:0.0], F:19.2, M:58.8	4,538	C:29.8 [S:29.8, D:0.0], F:16.9, M:53.3
Henneguya salminicola	8,187	C:35.3 [S:35.3, D:0.0], F:18.8, M:45.9	4,163	C:32.6 [S:31.0, D:1.6], F:17.3, M:50.1
Thelohanellus kitauei	15,020	C:29.4 [S:28.6,D:0.8], F:15.7, M:54.9	9,498	C:37.3 [S:34.9, D:2.4], F:14.9, M:47.8
Ceratonova shasta	N.A.	N.A.	15,032	C:27.1 [S:25.1, D:2.0], F:8.2, M:64.7
Myxobolus honghuensis	15,433	C:51.0 [S:49.4, D:1.6], F:14.1, M:34.9	16,867	C:44.4 [S:42.4, D:2.0], F:16.9, M:38.7
Myxobolus rasmusseni	-	-	10,106	C:44.3 [S:41.6, D:2.7], F:16.5, M:39.2

Next, we assigned functional annotations to 57% (5,757 of 10,106) *M. rasmusseni* proteins with: GO terms to 2,611 proteins; KEGG pathways to 1,873 proteins; and PFAM domains to 4,671 proteins. Nearly all KEGG pathways assigned to at least one *M. rasmusseni* protein (357/361 KEGG pathways) were common between *M. rasmusseni* and *M. honghuensis.* Further, 340 pathways contained at least one protein from all myxozoan species. The number of pathways with at least one protein was similar across free-living and parasitic cnidarians, ranging from 349 in *C. shasta* to 382 in *T. kitauei* in myxozoans and from 390 in *C. hemisphaerica* to 405 in *S. pistillata.* When we looked at the 339 KEGG pathways with at least one protein from all myxozoan and free-living cnidarian species, we observed that, on average, free-living cnidarians had more proteins assigned to the pathway compared to myxozoans.

We assigned 1,729 PFAM domains to at least one *M. rasmusseni* protein, of which 1,522 domains (88%) were also found in the genome of *M. honghuensis.* Of these 1,729 domains, nine were not identified in any of the free-living cnidarian species (Table 9). Ten proteins had the "Arg_repressor" (Arginine repressor, DNA binding domain) protein domain in *M. rasmusseni* and *C. shasta,* two in *T. kitauei* and none in any free-living cnidarians analyzed in this study.

Table 9 Number of myxozoan proteins containing the nine domains that were identified in at least one *M. rasmusseni* protein but none of the free-living cnidarians used in the study

Pfam domain identifier	*M. rasmusseni*	*M. honghuensis*	*M. squamalis*	*C. shasta*	*E. leei*	*H. salminicola*	*K. iwatai*	*S. zaharoni*	*T. kitauei*
Arg_repressor	10	0	0	10	0	0	0	0	2
Terminase_5	4	0	0	0	0	0	0	0	0
DUF1398	1	0	0	0	0	0	0	0	0
Lambda_Bor	1	0	0	0	0	0	0	0	0
Mononeg_RNA_pol	1	0	0	0	0	0	0	3	0
Phage_holin_3_1	1	0	0	0	0	0	0	0	0
Phage_lysis	1	0	0	0	0	0	0	0	0
RdRP_2	1	0	0	0	0	0	0	0	0
Saw1	1	0	0	0	0	0	0	0	0

A further 22 PFAM domains were present in more *M. rasmusseni* proteins compared to free-living cnidarians (the number of proteins with a domain in *M. rasmusseni* was at least three times the average number of proteins containing the domain in free-living cnidarian species). From these 22 domains, "RVP_2", "DDE_Tnp_IS1595", and "FLYWCH" were the top three most abundant protein domains. Proteins with the FLYWCH domain are a part of a superfamily of

DNA transposon-derived conserved families of transcription factors. The number of FLYWCH domains ranged from none in three myxozoans to 55 in *M. rasmusseni* in myxozoans, and none in two free-living cnidarians to three in *S. pistillata* in free-living cnidarians. In *M. rasmusseni,* the majority of proteins with the FLYWHC domain also contained the MULE transposase protein domain.

We found that both *M. rasmusseni* and *M. honghuensis* had more proteins containing the "Hexokinase_1" and "Hexokinase_2" protein domains when compared to free-living cnidarians. *M. rasmusseni* had six proteins containing both domains. Phylogenetic analysis of all proteins that contained the two domains showed that the family underwent duplication in these two species (Figure 2).

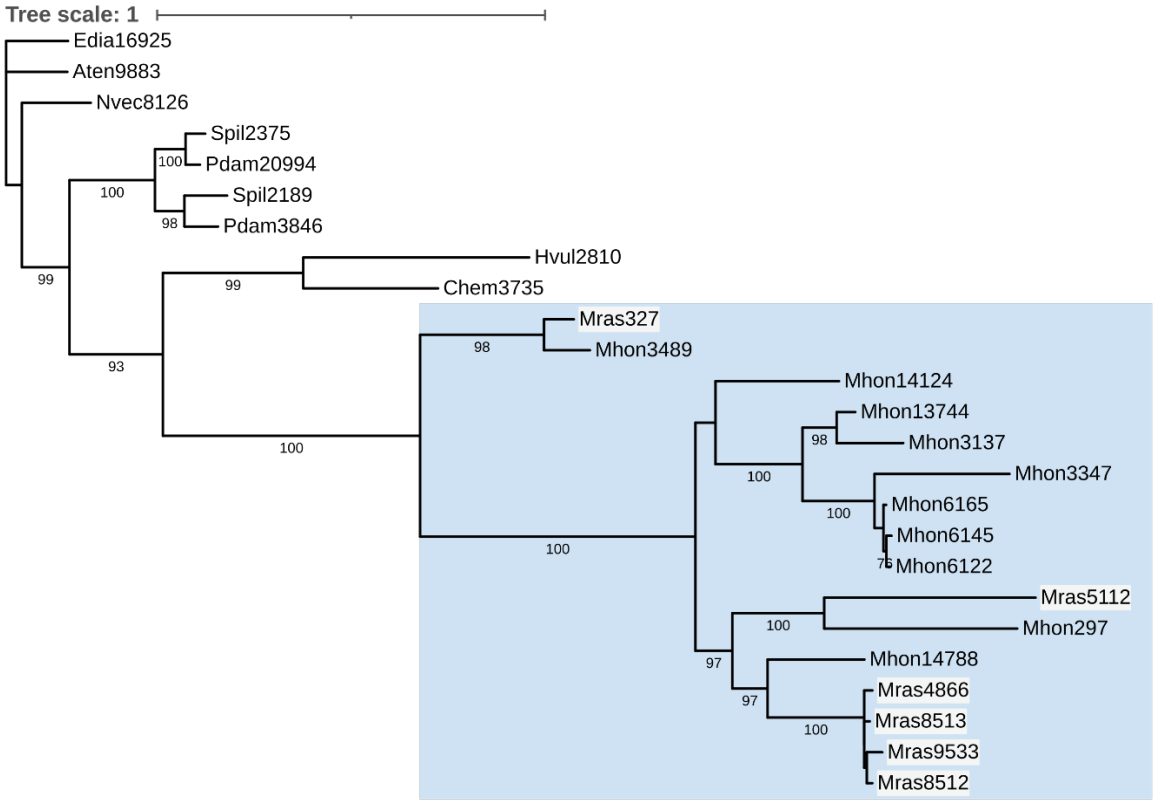


Figure 2 Phylogenetic tree of proteins containing the "Hexokinase_1" and "Hexokinase_2" protein domains in free living cnidarians and *M. rasmusseni* and *M. honghuensis*, in which a blue box represents the clade containing all myxozoan proteins and *M .rasmusseni* proteins have a white background. Bootstrap support values of 75% and above are annotated on the branches of the tree.

3.3. Gene family analysis

Using OrthoFinder, we identified 9,843 groups of orthologous proteins (OGs) that contained at least one myxozoan protein, of which 3,972 OGs contained at least one *M. rasmusseni* protein. Of these, 3,095 OGs also contained protein from *M. honghuensis*. There were 877 OGs with at least one *M. rasmusseni* protein but not *M. honghuensis,* and 1070 OGs vice versa. Less than one-third of all 9,843 OGs (2,727 OGs) contained a protein from the majority of myxozoan species used in the analysis (four of the seven). These 2,727 OGs represent a set of core conserved proteins in myxozoans.

3.3.1. Phylogenetic relationships between the myxozoan species

Using the 57 single-copy orthologs, we reconstructed a ML-based phylogeny of both myxozoans and free-living cnidarians and found that *M. honghuensis* is the sister species to *M. rasmusseni* with the other relationships agreeing with previous studies (Figure 3).

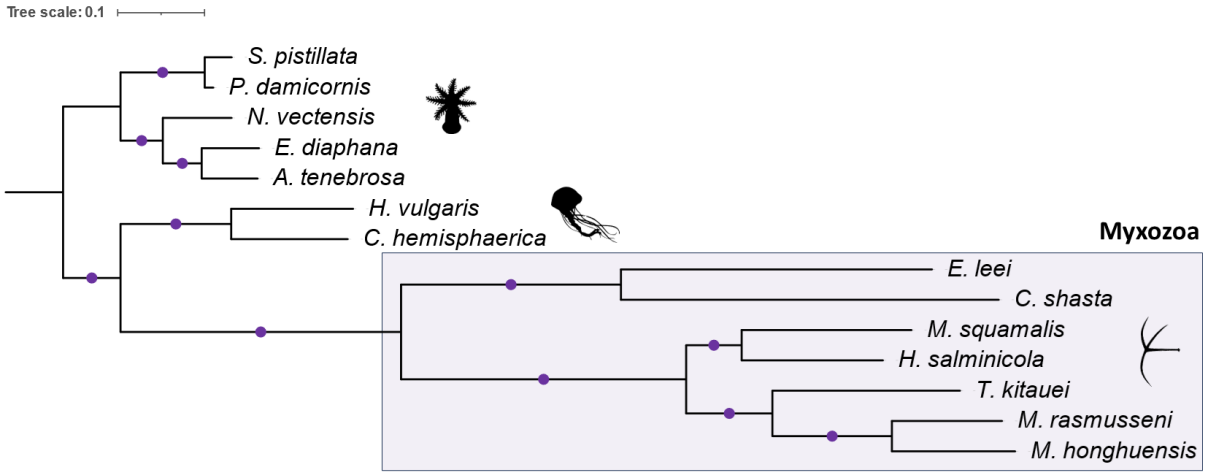


Figure 3 Phylogenetic relationships between free-living and parasitic cnidarians generated using IQTREE. Single-copy orthologs identified by OrthoFinder were used as input. All nodes with boot-strap values greater than 90% are annotated with purple circles. The silhouettes for Anthozoa, Medusozoa, and Myxozoa are taken from PhyloPic (www.phylopic.org).

3.3.2. Gene family expansions in *M. rasmusseni*

Using the species tree and full set of orthology clusters results, we identified 10 gene families that were significantly expanded in myxozoans. We identified a further 19 gene families that were significantly expanded in only *M. rasmusseni*, the majority of which were involved in repeat-related processes, and hence were not analyzed further. Here, we describe on three gene families of interest.

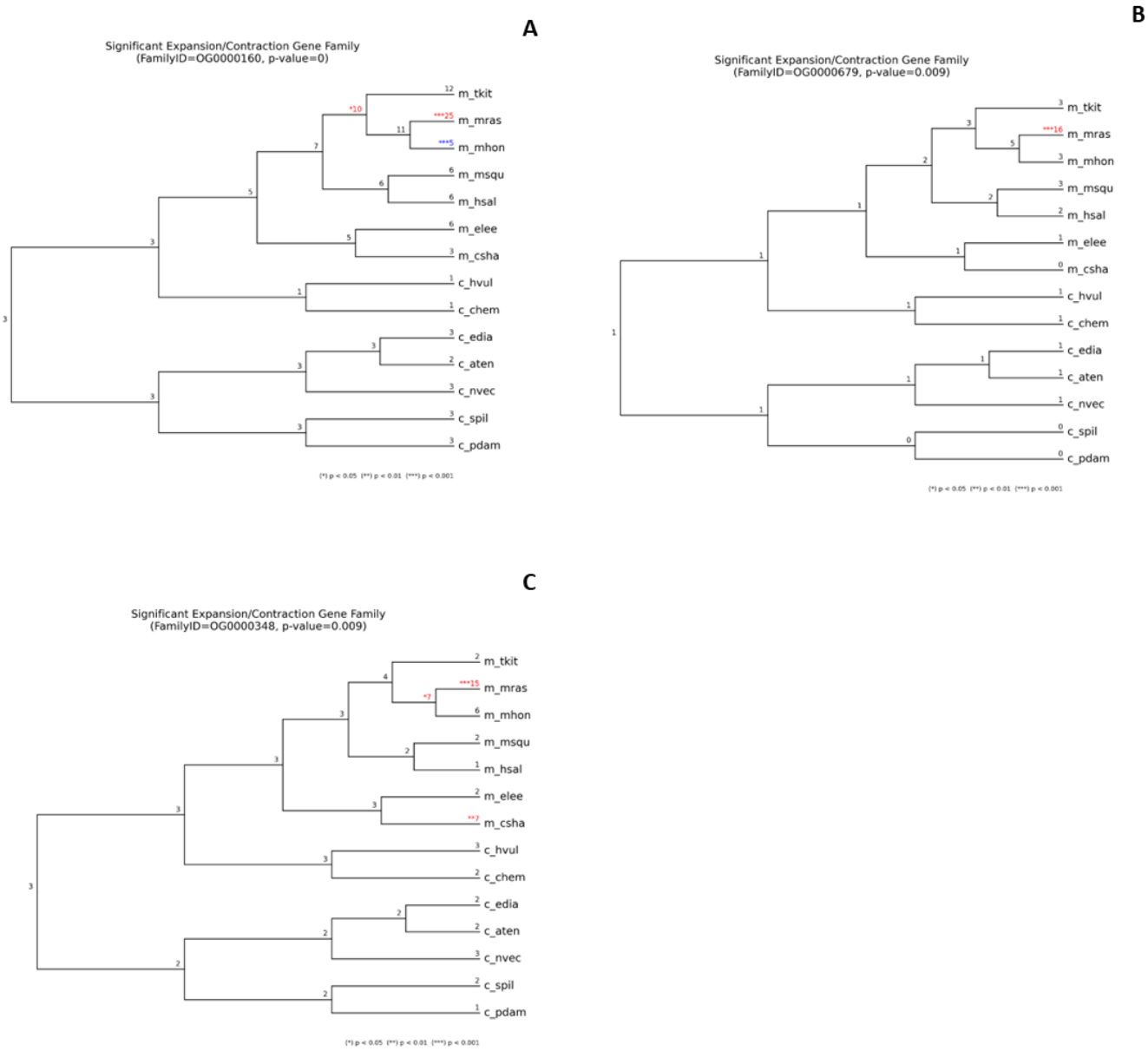
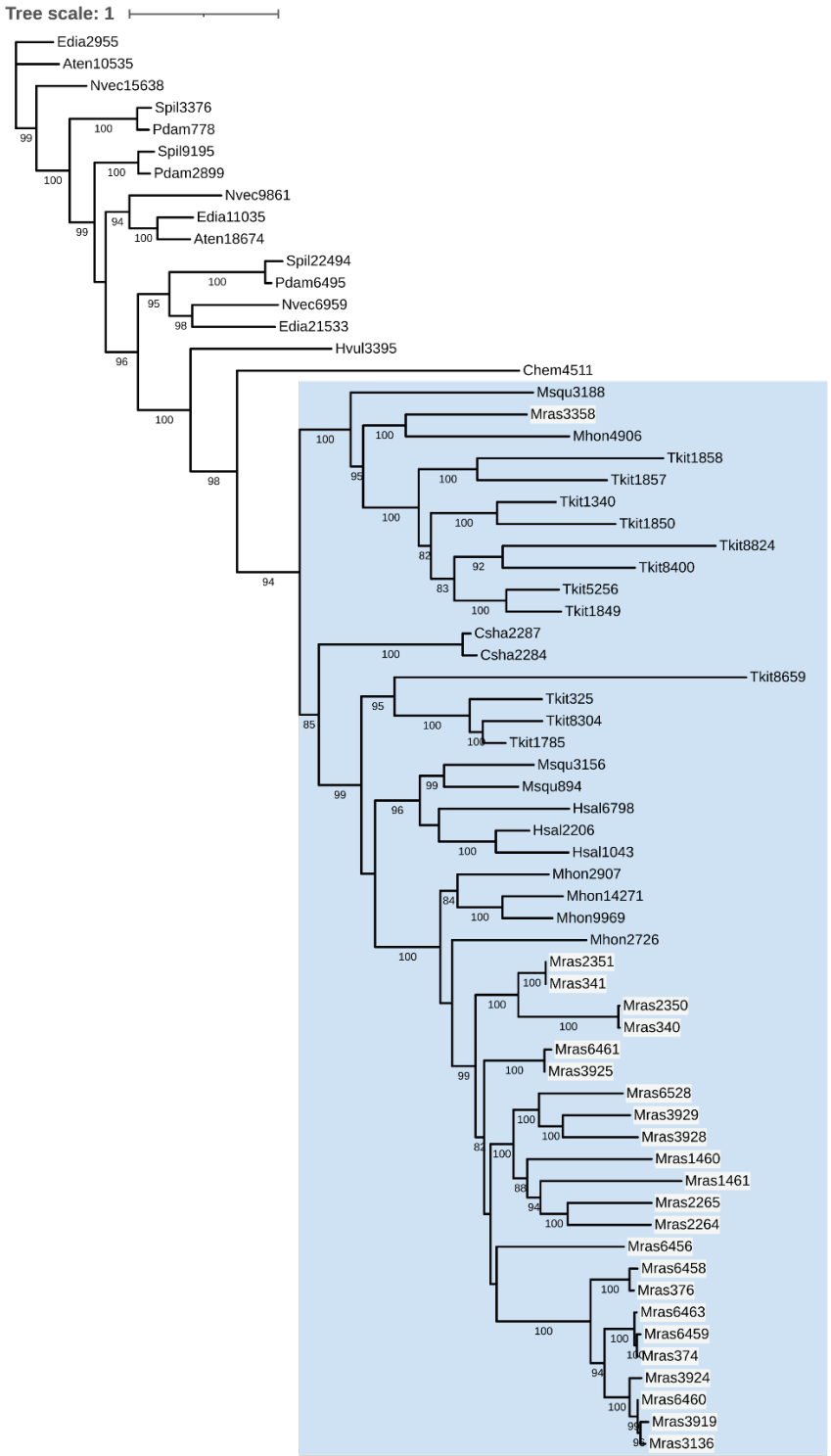


Figure 4 CAFE5 analysis of A) OG0000160, B) OG0000679, and C) OG0000348 with significant expansions and contractions colored in red and blue, respectively. Significant changes on the branch are labelled with asterisks, where red color indicates gene family expansion and blue color indicates gene family contraction. (*) indicates a p-value < 0.05, (**) indicates a p-value < 0.01, and (***) indicates a p-value < 0.001.

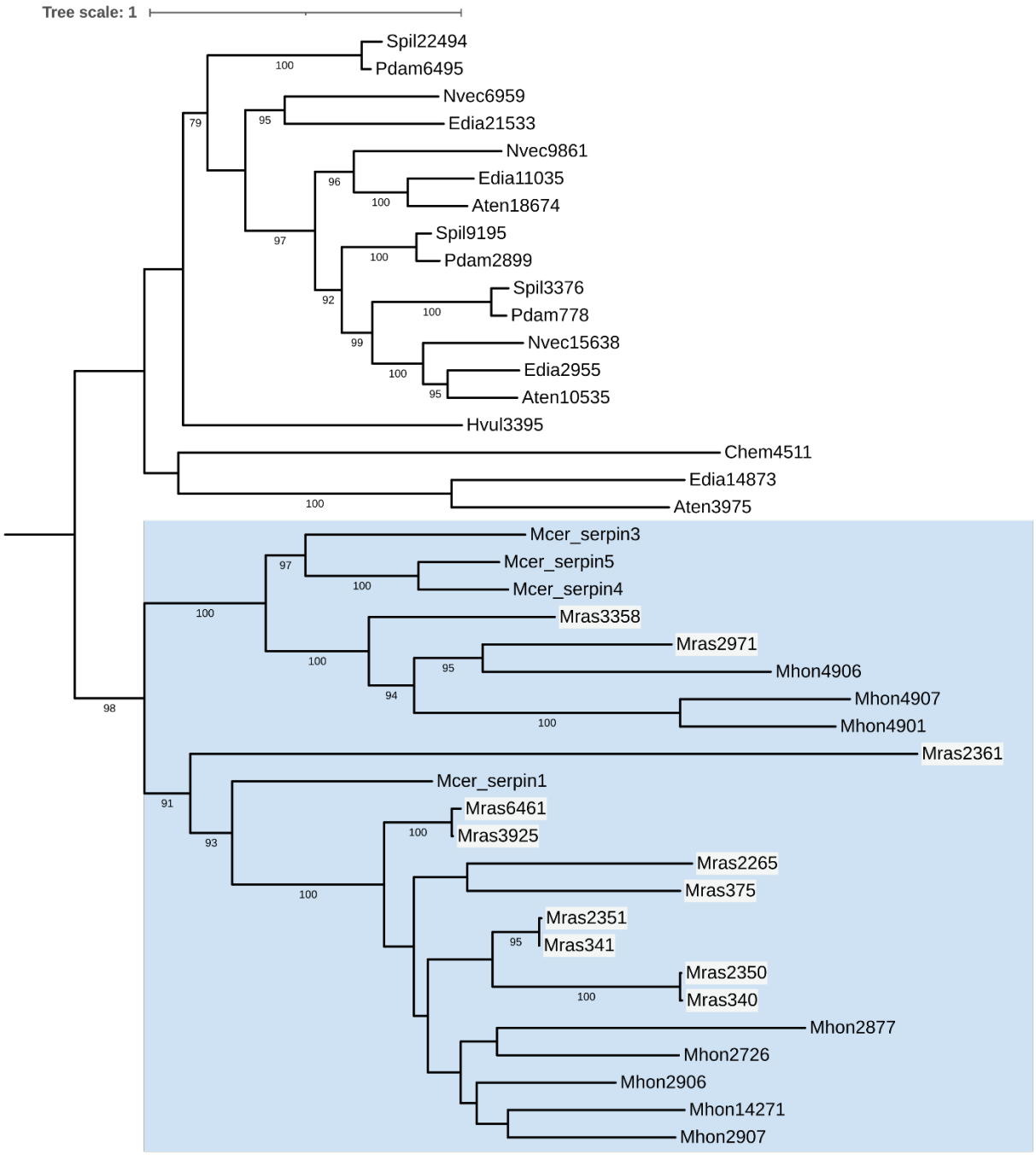
The first gene family of interest (OG0000160) was comprised of the orthologs of the *H. vulgaris* SERPINB6 (serine protease inhibitor B6). This family underwent expansion on the branch leading to *M. rasmusseni, M. honghuensis,* and *T. kitauei,* which was followed by a contraction

412 on the branch leading to *M. honghuensis* (Figure 4A). All myxozoan proteins in this OG
413 clustered together in the maximum likelihood (ML) phylogenetic tree (Figure 5). Since serpins
414 are considered as potential targets for myxozoan control and have been extensively studied in *M.*
415 *cerebralis*, we inspected this gene family further. We observed that only 11 of the 25 *M.*
416 *rasmusseni* proteins from this OG contained the "Serpin" domain. Additionally, two more OGs
417 contained *M. rasmusseni* proteins with the "Serpin" domain. Of these, one OG (OG0022393) had
418 proteins only from *M. rasmusseni* and *M. honghuensis.* We analyzed the phylogenetic
419 relationships between all proteins containing serpin domains from *M. rasmusseni*, *M.*
420 *honghuensis, M. cerebralis,* and free-living cnidarians. We observed that all the myxozoan
421 serpins clustered together (Figure 6). Further, myxozoan homologs of *M. cerebralis* serpin 1
422 underwent expansion in *M. rasmusseni* and *M. honghuensis*.
423
424 The second gene family of interest (OG0000679) underwent expansion in *M. rasmusseni* and
425 was comprised of orthologs of the *H. vulgaris* protein "Uncharacterized protein LOC100211173
426 isoform X1". Proteins in this OG harbor the "Lupus La" protein domain. This gene family
427 experienced an expansion on the branch leading to both *M. rasmusseni* and *M. honghuensis*
428 (Figure 4B, 7). Finally, the third gene family of interest that experienced expansions in *M.*
429 *rasmusseni* consisted of orthologs of three peroxiredoxin proteins in *H. vulgaris*. This gene
430 family experienced an expansion on the branch leading to *M. rasmusseni, M. honghuensis,* and
431 *T. kitauei,* and also in *C. shasta* (Figure 4C, 8).
432


Tree scale: 1
Edia2955
Aten10535
Nvec15638
Spil3376
Pdam778
Spil9195
Pdam2899
Nvec9861
Edia11035
Aten18674
Spil22494
Pdam6495
Nvec6959
Edia21533
Hvul3395
Chem4511
Msqu3188
Mras3358
Mhon4906
Tkit1858
Tkit1857
Tkit1340
Tkit1850
Tkit8824
Tkit8400
Tkit5256
Tkit1849
Csha2287
Csha2284
Tkit8659
Tkit325
Tkit8304
Tkit1785
Msqu3156
Msqu894
Hsal6798
Hsal2206
Hsal1043
Mhon2907
Mhon14271
Mhon9969
Mhon2726
Mras2351
Mras341
Mras2350
Mras340
Mras6461
Mras3925
Mras6528
Mras3929
Mras3928
Mras1460
Mras1461
Mras2265
Mras2264
Mras6456
Mras6458
Mras376
Mras6463
Mras6459
Mras374
Mras3924
Mras6460
Mras3919
Mras3136

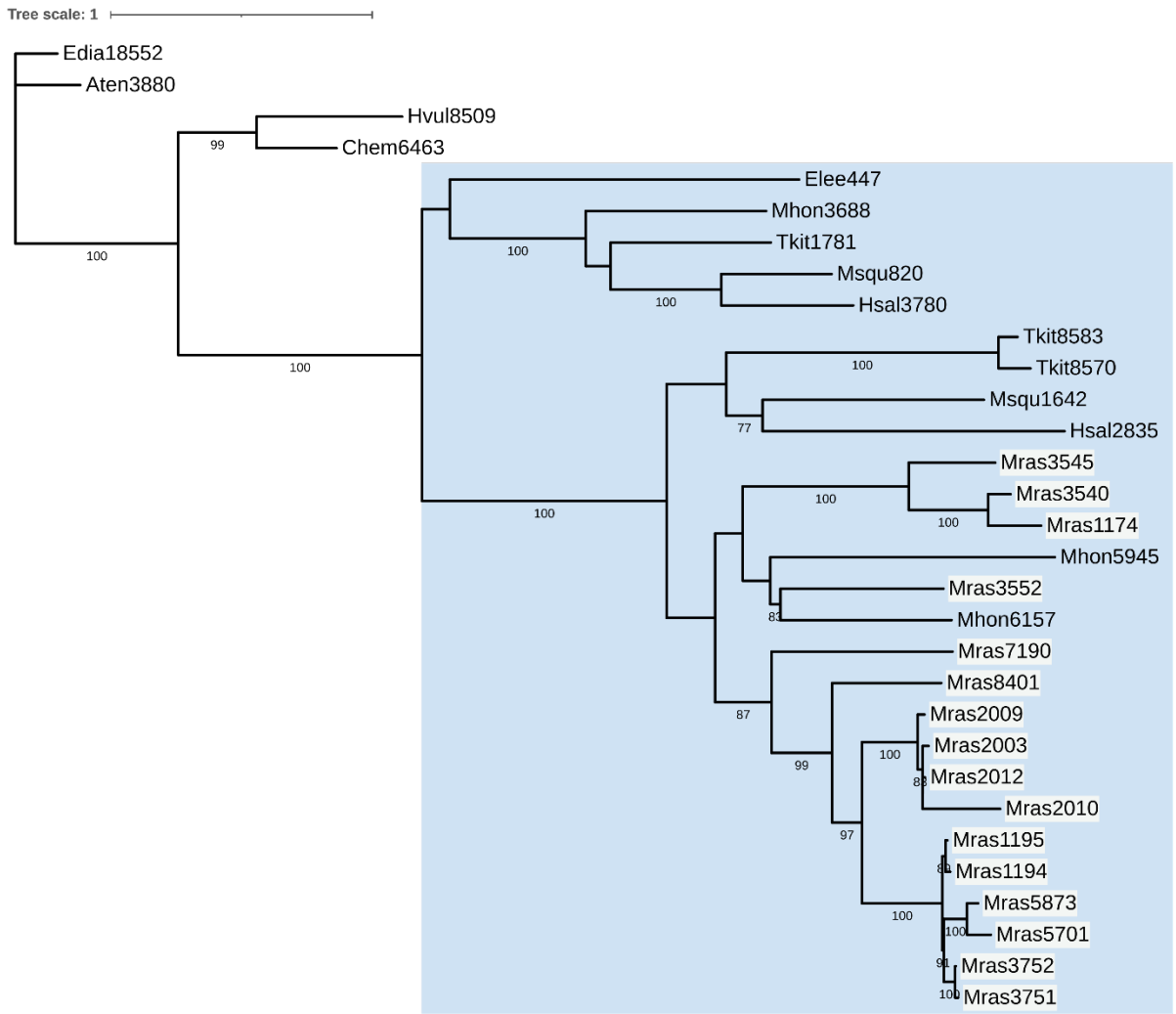
433

434 **Figure 5** Phylogenetic tree of the gene family OG000160, in which the *M. rasmusseni* proteins
435 have a white background and a blue box represents the clade containing all myxozoan proteins.
436 Bootstrap support values of 75% and above are annotated on the branches of the tree.
437



438
439 **Figure 6** Phylogenetic tree of proteins containing the "Serpin" domain in free-living cnidarians,
440 *M. rasmusseni*, *M. honghuensis*, and *M. cerebralis* in which the *M. rasmusseni* proteins have a

441 white background and a blue box represents the clade containing all myxozoan proteins.
442 Bootstrap support values of 75% and above are annotated on the branches of the tree.
443



444
445 **Figure 7** Phylogenetic tree of the gene family OG000679 in which the *M. rasmusseni* proteins
446 have a white background and a blue box represents the clade containing all myxozoan proteins.
447 Bootstrap support values of 75% and above are annotated on the branches of the tree.
448
449

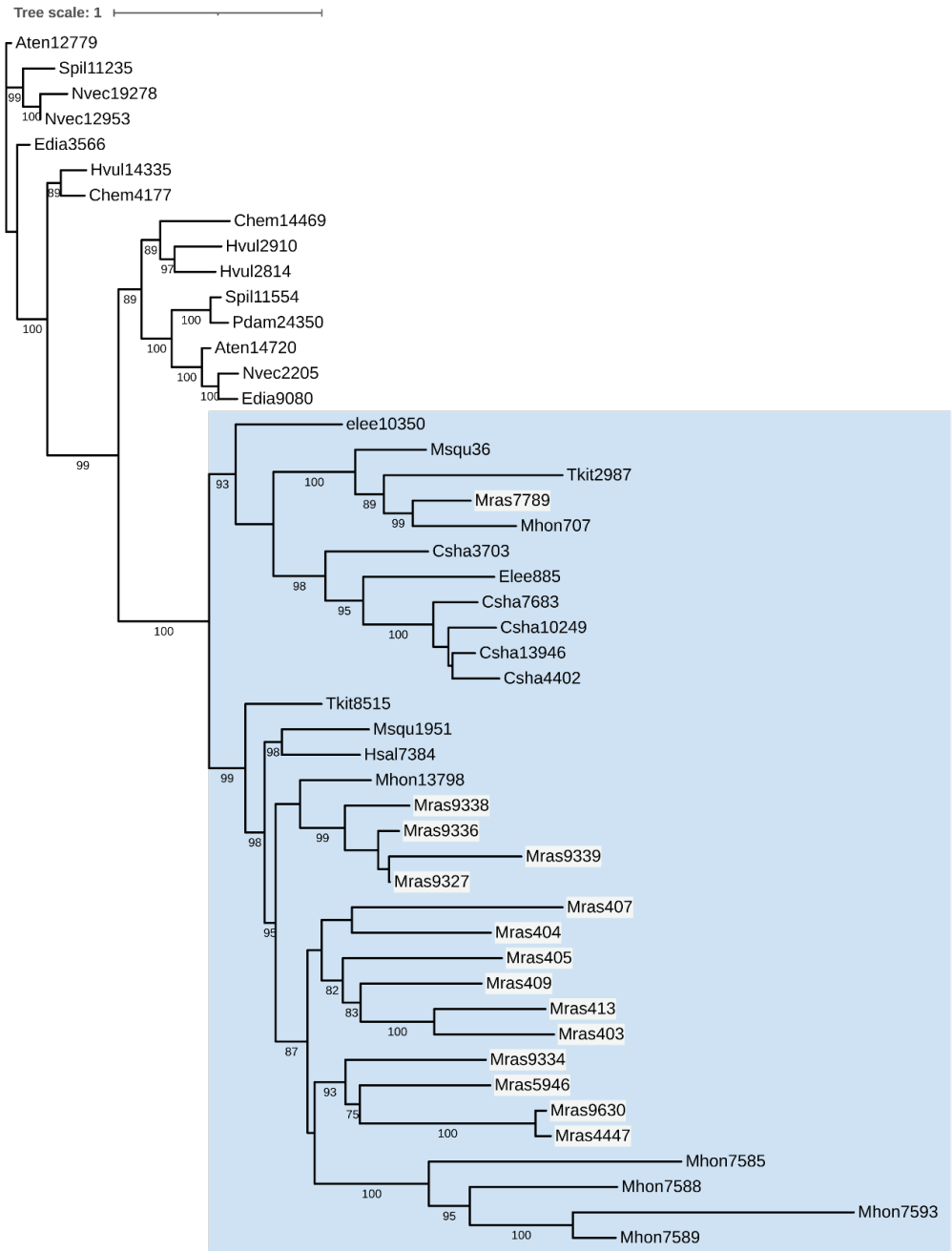


Figure 8 Phylogenetic tree of the gene family OG000348 (peroxiredoxin) in which the *M. rasmusseni* proteins have a white background and a blue box represents the clade containing all myxozoan proteins. Bootstrap support values of 75% and above are annotated on the branches of the tree.

3.3.3. Gene families of interest in *M. rasmusseni*

In addition to these three expanded gene families, we explored the evolution of two gene families that have been of interest in myxozoan biology. First, we analyzed the complement of proteases found in *M. rasmusseni* since proteases have been proposed to be promising targets for myxozoan control. We observed that 377 *M. rasmusseni* proteins had a top hit to 155 proteases from the MEROPS database: 39 metalloproteases, 38 cysteine proteases, 22 serine proteases, 19 threonine proteases, 17 aspartic proteases, and 20 protease inhibitors. The most abundant category of proteases was A28B (the aspartic peptidase "skin SASPase"), followed by S01A (serine peptidase "chymotrypsin A"), and C01A (cysteine peptidase "papain"). We also identified 31 proteins from the I04 family of serine and cysteine endopeptidase inhibitors.

We aligned all but 16 of these 377 *M. rasmusseni* proteases to proteins from the fathead minnow and 13 of the remaining 16 proteins could be aligned to Actinopterygii (ray-finned fish) proteins from the NR database (e-value $\leq 1e^{-3}$). We inspected the function annotation remaining three proteins (mras5248, mras6483, and mras9733): protein mras5248 belonged to the same homologous superfamily as the mouse retroviral-like aspartic protease 1 (A28B subfamily) and also has the same PFAM protein domain ("gag-asp_protease" gag-polyprotein putative aspartyl protease); protein mras6483 was assigned to the same homologous superfamily as the mouse retroviral-like aspartic protease 1 (A28B subfamily) but did not have any predicted PFAM protein domains; protein mras9733 could not be assigned any family membership or PFAM domains.

Next, we analyzed the repertoire of minicollagen and nematogalectin proteins in *M. rasmusseni*. We searched for significant sequence similarity with minicollagen sequences from myxozoans and free-living cnidarians, and analyzed phylogenetic relationships between them (Figure 9). Previously, minicollagens were shown to belong to three different clades: I, II, and III. We found one *M. rasmusseni* protein in each of clades II and III, and none from clade I. In addition, we identified one *M. rasmusseni* and one *M. honghuensis* protein that grouped with free-living cnidarian minicollagens from clade III. There were two *M. rasmusseni* proteins and two *M. honghuensis* proteins that clustered together but did not have sufficient phylogenetic support to assign to a minicollagen clade. For the nematogalectins, we found three proteins in *M. rasmusseni*: one closely related to *M. bejeranoi* nematogalectin A; one to *M. bejeranoi* nematogalectin C; and the third to a putative nematogalectin in *S. zaharoni* (Figure 10).

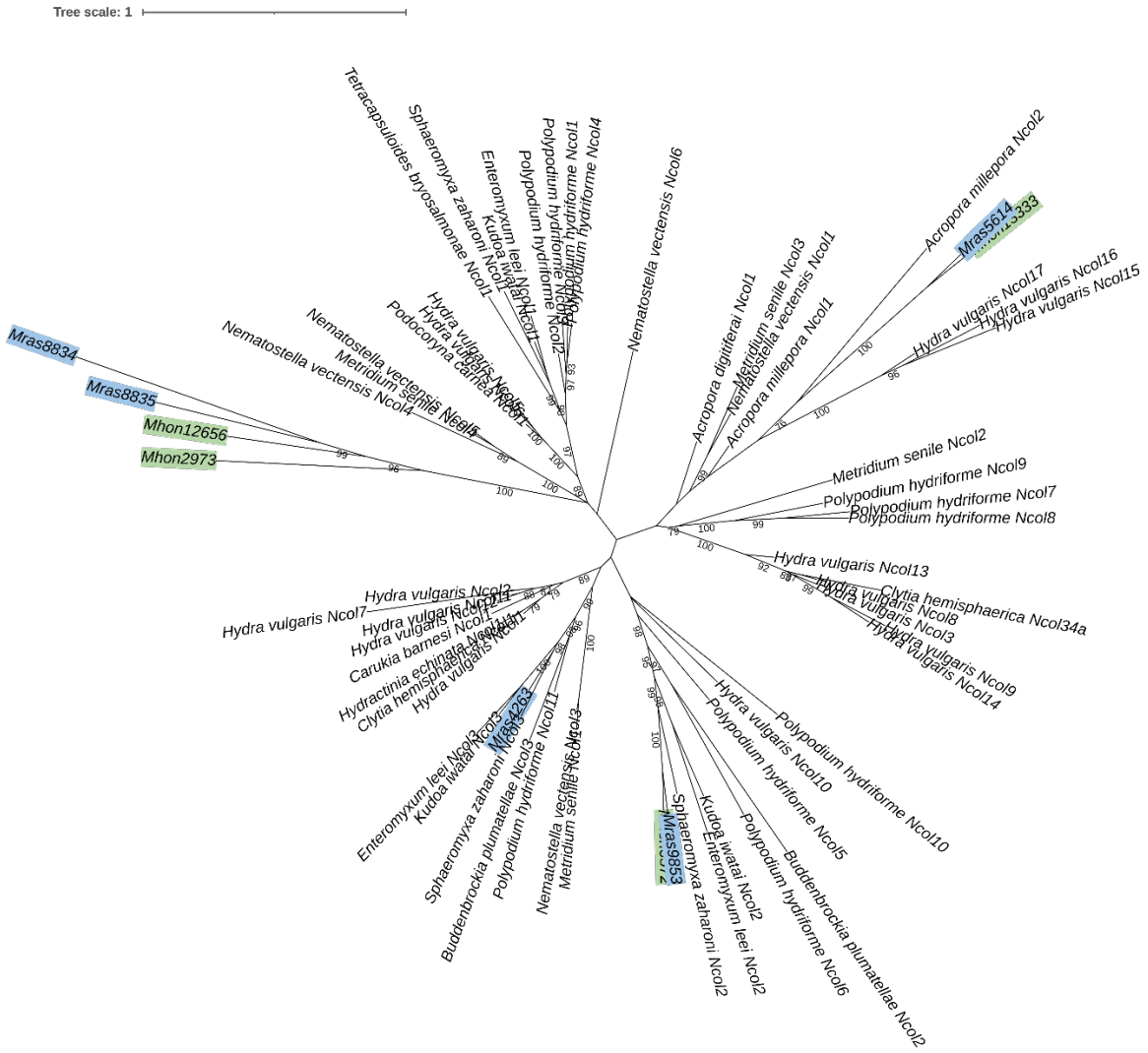


Figure 9 Phylogenetic tree of the minicollagens, in which *M. rasmusseni* and *M. honghuensis* sequences are labelled with blue and green color respectively. Bootstrap support values of 75% and above are annotated on the branches of the tree.

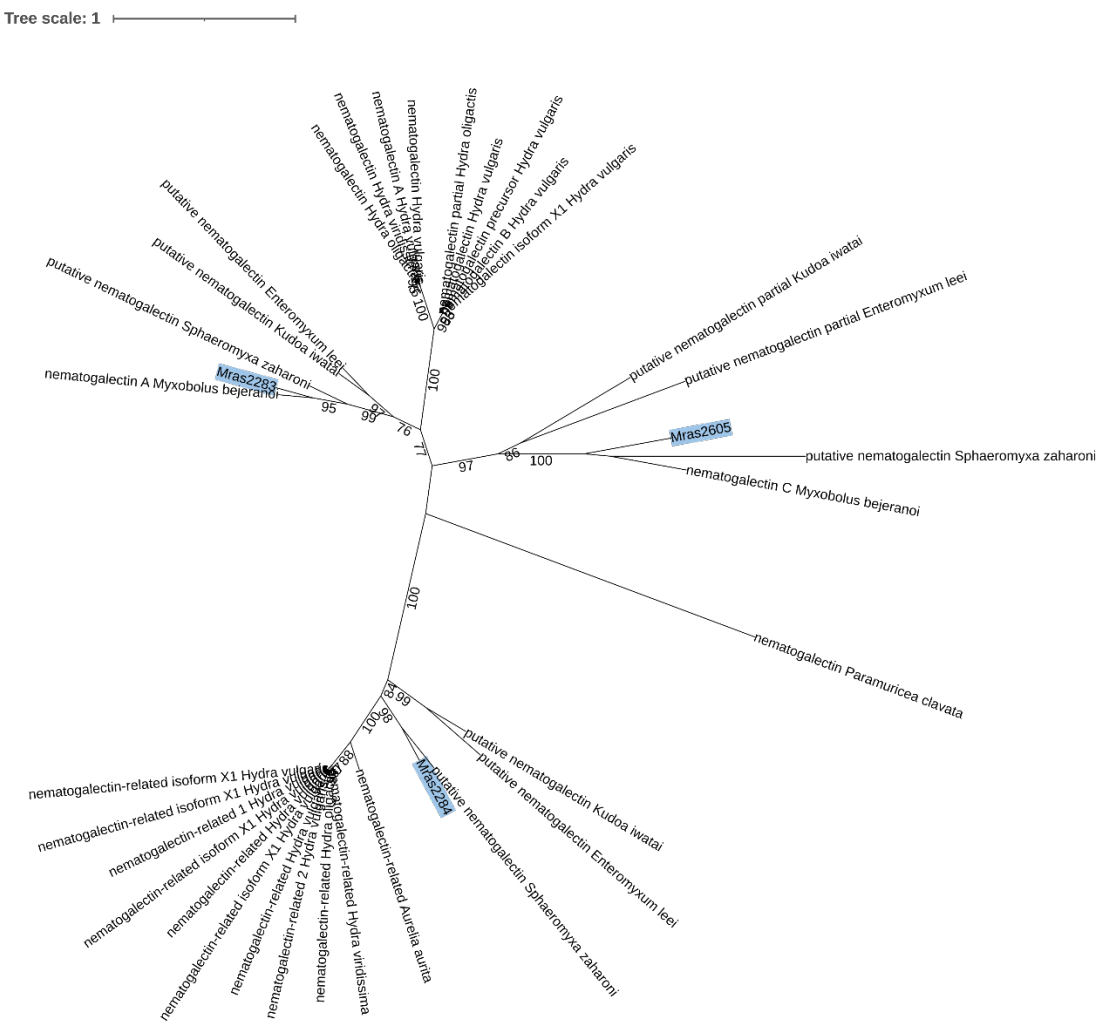


Figure 10 Phylogenetic tree of the nematogalectins in which *M. rasmusseni* sequences are labelled with blue. Bootstrap support values of 75% and above are annotated on the branches of the tree.

3.3.4. Gene-loss in *M. rasmusseni*

We identified 111 OGs with proteins from *M. honghuensis* and the majority of the remaining five myxozoan species, but none from *M. rasmusseni.* We extracted the 178 *M. honghuensis* proteins from these 111 OGs and examined the protein domains and KEGG pathways associated with these proteins as determined by EggNOG-mapper. The top three most abundant protein domains were “Dimer_Tnp_hAT” (hAT family C-terminal dimerisation region), “Pkinase” (Protein kinase domain), and “UAA” (UAA transporter family). The top three most abundant

507 KEGG pathways were “Metabolic pathways” (map01100), “Cellular senescence” (map04218),
508 and “cGMP-PKG signaling pathway” (map04022).
509

510 ### *3.3.5. M. rasmusseni* proteins with no sequence similarity to proteins from the fathead
511 minnow

512 We detected 3,444 *M. rasmusseni* proteins that did not have sequence similarity to any fathead
513 minnow protein, as identified using BLASTP (e-value ≤ $1e^{-3}$), of which 639 proteins also had
514 BLASTP hits to proteins from Actinopterygii species (e-value ≤ $1e^{-3}$). The remaining 2,805 *M.*
515 *rasmusseni* proteins were queried against the ChEMBL database to search for potential drug
516 targets. To this end, we identified eight *M. rasmusseni* proteins which had a BLASTP hit to a
517 protein from the ChEMBL database (e-value ≤ $1e^{-5}$). For a more stringent selection criteria, we
518 only looked at *M. rasmusseni* proteins that did not have a BLASTP hit to proteins from the
519 fathead minnow (e-value ≤ 0.05). This yielded three proteins which we analyzed in detail.:
520 “mras18” was aligned to inositol phosphorylceramide synthase (CHEMBL1641343),
521 “mras2094” to gag-pol polyprotein (CHEMBL3638331), and “mras2163” to heat shock protein
522 hsp-16.2 (CHEMBL2146313).
523

524 ## 3.4. Characterization of the mitochondrial genome and mitochondrial
525 proteome

526 We identified a single 31,057 bp contig as the mitochondrial genome (mt-genome) of *M.*
527 *rasmusseni* (Figure 11A). The *M. rasmusseni* mt-genome was predicted to encode only five of
528 the 13 protein-coding genes that are typically found in most animal mitochondrial genomes—
529 *cox1*, *cox2*, *nad1*, *nad5*, and *cob*—as well as two ribosomal RNA genes (*rrnS* and *rrnL*). The
530 *rrnL* gene was split into two fragments. We aligned both the fragments of the 16S RNA gene
531 (*rrnL*) to the NT database and found that the top hit to both fragments was from the myxozoan
532 *Myxobolus squamalis*. When we searched the nuclear genome of *M. rasmusseni* with the same
533 HMM profiles of protein-coding genes encoded in the mt-genome, we found that *atp6* was
534 transferred to the nuclear genome and had acquired introns and an mitochondria-targeting signal
535 (MTS). In fact, we found that *atp6* was transferred to the nuclear genome and acquired an MTS
536 in *M. honghuensis*, *S. zaharoni*, *T. kitauei, K. iwatai*, and *M. squamalis*. None of the other
537 protein-coding genes in the mt-genome were transferred to the nuclear genome in myxozoans.
538

539 Next, we analyzed the mitochondrial proteome—the set of proteins encoded in the nuclear
540 genome and imported into the mitochondrion—of *M. rasmusseni*. First, we identified homologs
541 of nuclear-encoded mitochondrial proteins from humans in the cnidarian genomes. For this, we
542 aligned the set of human MitoCarta v3 proteins to the proteomes from myxozoans and free-living
543 cnidarians. *M. rasmusseni* had homologs of 542 of the 1,136 human MitoCarta genes (Figure
544 11B). On average, myxozoans had homologs of 526 MitoCarta genes, compared to 951 for the

545 free-living cnidarians. We identified 741 MitoCarta genes that had homologs in at least one
546 myxozoan species, of which 241 were present in all. We used ConsensusPathDB to find out the
547 functions of these 241 mitochondrial protein-coding genes. The top five enriched KEGG
548 pathways included “Valine, leucine and isoleucine degradation”, “TCA cycle”, “Fatty acid
549 degradation”, and “Aminoacyl-tRNA biosynthesis”. On the contrary, 395 mitochondrial protein-
550 coding genes did not have a significant sequence similarity hit to any myxozoan protein in this
551 study, representing a potential loss of mitochondrial functions in myxozoans. ConsensusPathDB
552 found that the top five enriched pathways in this missing set included “Oxidative
553 phosphorylation”, “Thermogenesis”, and “Apoptosis”.
554
555 Next, we identified potential mitochondria-targeted proteins in *M. rasmusseni* which do not have
556 homologs to known mitochondrial proteins in humans. For this, we identified *M. rasmusseni*
557 proteins that are predicted to possess a mitochondrial targeting signal (MTS) at their N-terminus
558 end using MitoFates. We observed that only 271 *M. rasmusseni* proteins were predicted to
559 harbor an MTS, of which 36 were predicted to possess an MTS with a high probability (at least
560 90%). Of these, 15 had a hit to a human protein, for which the top human hit was also predicted
561 to possess a MTS. A further seven *M. rasmusseni* proteins had a hit to a human protein that did
562 not have MTS. These proteins represent potential instances of neolocalization in the parasite (an
563 instance where the homolog of a non-mitochondrial protein in human is targeted to the
564 mitochondria in *M. rasmusseni*). These included the endogenous retrovirus group K member 113
565 Pol protein (P63132), NYNRIN (Q9P2P1), alpha-1,3-mannosyl-glycoprotein 2-beta-N-
566 acetylglucosaminyltransferase (P26572), adenosine 5'-monophosphoramidase HINT1 (P49773),
567 and cytoplasmic asparagine--tRNA ligase (O43776). The remaining 14/36 proteins did not have
568 BLASTP hit to any human protein, and we used BLASTP to search for proteins with sequence
569 similarity in the NR database. Of these, five did not have a BLASTP hit to any non-myxozoan
570 protein in the NR database.
571

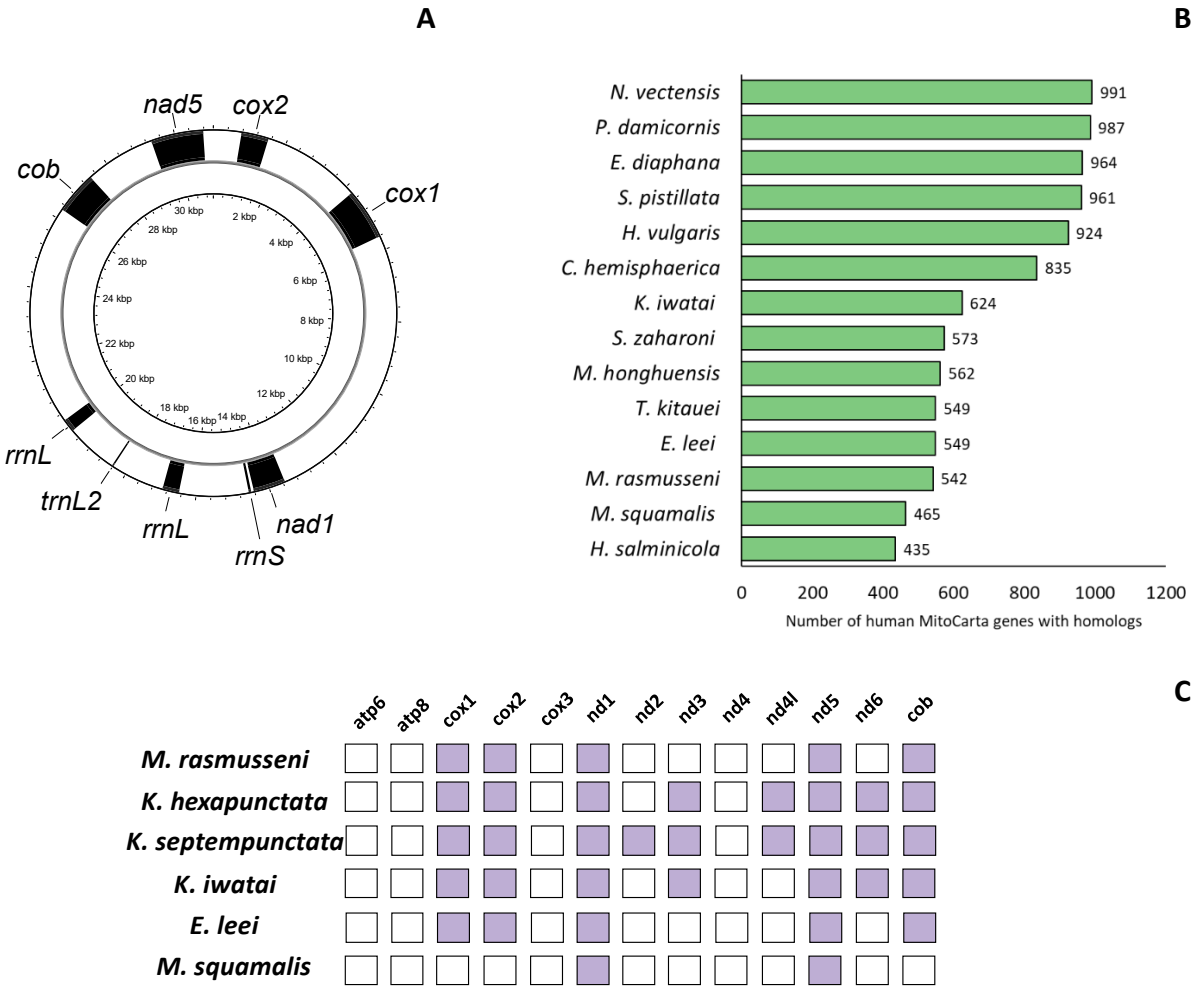


Figure 11 A) The mitochondrial genome of *M. rasmusseni* visualized using PROKSEE, B) The number of human MitoCarta genes with Blast hits to myxozoan (purple) genomes and cnidarian (green) genomes, C) The presence/absence of protein-coding genes in the mitochondrial genomes of myxozoans.

4. Discussion

4.1. The high repeat content of the *Myxobolus rasmusseni* genome contributes to genome expansion and necessitates the use of long-read sequencing of myxozoan genomes

In this study, we sequenced, annotated, and analyzed the genome of *Myxobolus rasmusseni*, a recently discovered myxozoan parasite of fathead minnows in southern Alberta. We used the Oxford Nanopore long-read sequencing technology, and thus were able to provide the second

584 long-read genome in this species-rich group of parasites. While relatively short for an animal
585 genome, it is one of the most repetitive. The only other long-read-based myxozoan genome, *M.*
586 *honghuensis*, is also highly repetitive. Such repetitive sequences can negatively affect the
587 assembly of a genome, especially in genomes sequenced using short-read platforms where
588 repetitive loci are more likely to be misassembled, which likely explains the relatively high
589 number of scaffolds in both the *M. honghuensis* and our *M. rasmusseni* assemblies (Tørresen et
590 al., 2019). Since both long-read genomes belong to a single genus in myxozoans, we cannot say
591 with confidence whether high repeat content is a feature of all myxozoan genomes.
592
593 The high repeat content in the *M. rasmusseni* genome is a significant factor explaining the large
594 genome size in this species. We detected a proliferation of the MULE-MuDR elements in the
595 genome. A recent analysis of the diversity of these Mutator-like elements (MULEs) across
596 eukaryotes found that, outside of plants, many genomes containing these elements were from
597 pathogens (Dupeyron et al., 2019). Indeed, in fungal pathogens, such TE-expansions have been
598 proposed to account for genome expansion (Raffaele & Kamoun, 2012). Furthermore, the
599 increase in repeat content in parasites could result in new functions and genome arrangements
600 which benefit the parasite's efforts to escape host defenses (Dong et al., 2015). It is possible that
601 the small sizes of myxozoan genomes that were sequenced using short-read sequencing
602 platforms, and the smaller repeat content predicted in them, are an underestimation of the true
603 genome size of these parasites.
604
605 The increase in repeat content of the *M. rasmusseni* genome can also further our understanding
606 of myxozoan biology. A good example of this is the expansion in proteins with the FLYWCH
607 domains identified in *M. rasmusseni*. A recent analysis of DNA transposon-derived transcription
608 factors in animals found that FLYWCH-domain containing transcription factors are rare in
609 animals but have undergone expansion in one phylum of non-bilaterian animals – Ctenophora
610 (Mukherjee & Moroz, 2023). This study did not include myxozoans in their comparisons. We
611 show that *M. rasmusseni* could contain the highest repertoire of these FLYWCH proteins in
612 animals.

613 4.2. Gene family expansions

614 A prominent feature of myxozoan genome evolution is the loss of genes that are otherwise well-
615 conserved in free-living eukaryotes. Hence, gene families that defy this trend and instead
616 undergo expansion are interesting as they represent potential adaptations to the parasitic
617 myxozoan lifestyle.
618
619 In one such example of gene family expansion, serpins (serine proteases) play an important role
620 in parasitic organisms, like defense against host proteases in *Schistosoma mansoni* and acting as
621 immunomodulators and inhibit host immune responses in nematodes (Maizels et al., 2001;
622 Valdivieso et al., 2015). Recently, a study analyzed the expression-profiles of *M. cerebralis*

serpins and proposed that they represent good targets for inactivating the parasite at an early stage in the fish host (Eszterbauer et al., 2021). We found a group of *M. rasmusseni* proteins that clustered together with serpins 3, 4, and 5 from *M. cerebralis.* Researchers have shown that the expression of these three serpins is upregulated during fish invasion and early development, and their functional predictions suggest that these three are caspase inhibitors. Thus, the *M. rasmusseni* proteins that cluster together with these three serpins represent promising targets for parasite control. Additionally, serpin 1, predicted to be a putative chymotrypsin-like inhibitor, has undergone extensive duplication in *M. rasmusseni.* While this suggests that the homologs of serpin 1 are probably involved in important functions in *M. rasmusseni,* developing targets against them would be challenging.

Further, we also identified an expansion in a family of peroxiredoxins in *M. rasmusseni* and other myxozoans. Peroxiredoxins are ubiquitous enzymes with roles in antioxidation and signaling systems (Gretes et al., 2012). These enzymes play important roles in parasitic defenses against reactive oxygen and nitrogen species generated by the host immune system. These enzymes have been proposed to be drug targets several parasites like trypanosomes and as vaccine antigens in *L. major* (Bayih et al., 2014).

We also observe that both *M. rasmusseni* and *M. honghuensis* have a higher number of hexokinase domain containing proteins. Hexokinases catalyze the first step of glycolysis. The increase in the hexokinase repertoire in the parasite could reflect the reliance of the parasite on glycolysis to compensate for a reduced and inefficient oxidative phosphorylation system. Indeed, we noticed that several human OXPHOS proteins did not have homologs in *M. rasmusseni*. These could represent a lead for future antiparasitic strategy.

4.3. *Myxobolus rasmusseni* has a reduced mitochondrial genome and proteome

Most animal mitochondrial genomes are single circular compact molecules that encode 37 genes (13 protein coding genes, 2 rRNA genes, and 22 tRNA genes) (Lavrov & Pett, 2016). Myxozoan mitochondrial genomes differ from this archetypal animal mitochondrial genome in several aspects. For instance, all myxozoan genomes have a reduced set of genes (reviewed in (Lavrov & Pett, 2016)). *Enteromyxum leei* has a multipartite mitochondrial genome, comprised of eight megacircles (Yahalomi et al., 2017). The mitochondrial genome of *M. rasmusseni* is no exception. It represents just the second mitochondrial genome from the species-rich *Myxobolus* genus. The only other mitochondrial genome from the genus belongs to *M. squamalis,* and only a single protein coding gene can be identified in the genome. Further, while researchers produced a high-quality nuclear genome of *M. honghuensis*, they were unable to identify a contig containing the mitochondrial genome. Thus, here we present the most complete mitochondrial genome from the genus *Myxobolus*. It is worth noting that while the mitochondrial genome of *M. rasmusseni* lacks several protein-coding genes, it is larger than the human mitochondrial genome, due to

large intergenic regions, instead of one large non-coding region in most animal mitochondrial genomes. Further, myxozoan mitochondrial genomes have some of the fastest rates of sequence evolution in animals (Lavrov & Pett, 2016), which could make identifying some mitochondrial genes difficult. In fact, some myxozoan mitochondrial genomes from the genus *Kudoa* have some unidentified open reading frames (ORFs) (Takeuchi et al., 2015), which could be standard animal mitochondrial genes that have evolved beyond recognition by sequence-based approaches.

We find that the mitochondrial gene *atp6* is transferred to the nuclear genome in multiple myxozoans and has acquired a mitochondrial targeting signal. This suggests that the protein encoded by this gene is imported into the organelle, however proteomic analyses need to be done to confirm this. Such a transfer of *atp6* to the nuclear genome and the acquisition of a mitochondrial targeting signal has been reported before, like in the ctenophore *Mnemiopsis leidyi* (Pett et al., 2011) and in the non-animal eukaryote *Chlamydomonas reinhardtii* (Funes et al., 2002). This transfer of *atp6* to the nuclear genome and its potential for its subsequent import back into the organelle make myxozoans potential models to study mitochondrial protein import for aiding efforts in mitochondrial gene therapy (Figueroa-Martínez et al., 2011; Manfredi et al., 2002), as was suggested for *M. leidyi* (Pett et al., 2011).

All proteins encoded by genes in the mitochondrial genome function in complexes that require proteins that are encoded in the nuclear genome and imported into the organelle. The majority of mitochondrial processes rely on just the import of nuclear-encoded proteins. This underscores the need to analyze the mitochondrial proteome for analyzing the evolution of mitochondrial processes in myxozoans. The analysis of mitochondrial proteomes outside of model animals is limited. Only a few studies have looked at the evolution of the mitochondrial proteome in myxozoan species, both showing that myxozoans lack several mitochondrial proteins found in mammals (Muthye & Lavrov, 2018; Yahalomi et al., 2020). Our analysis of the mitochondria proteomes from myxozoans align with these results. On average, just less than half the mitochondrial proteins from mammals had homologs in myxozoans, compared to ~85% in the three non-parasitic cnidarians. We found that around 35% of the human mitochondrial proteins were missing in all the myxozoan species analyzed, representing a set of core lost mitochondrial functions. These missing proteins were enriched in pathways such as oxidative phosphorylation and apoptosis. These findings are consistent with a recent study that found that several apoptosis proteins are lost in myxozoan genomes (Neverov et al., 2023).

4.4. *Myxobolus rasmusseni* represents a potential model system to study host-parasite interactions in the genus *Myxobolus*

The lack of experimentally confirmed life cycles from myxozoans impedes efforts to analyze how these parasites infect the hosts, transmit between them, and interact with their hosts post

infection. Within the Myxozoa, only six life cycles have been established in the laboratory, of which three are from the genus *Myxobolus*: *M. cerebralis* (El-Matbouli et al., 1999; Wolf & Markiw, 1984), *Myxobolus pseudodispar* (Kallert et al., 2007; Marton & Eszterbauer, 2012; Székely et al., 1999, 2001), and *Myxobolus parviformis* (Kallert et al., 2005). However, these three species currently lack genomic resources; only a transcriptome is available only for *M. cerebralis*. Thus, *M. rasmusseni* represents a promising model to study infection and host-parasite interactions within *Myxobolus* – since both hosts have been molecularly confirmed, a good quality long-read genome has been sequenced, and a transcriptome is available. Further, the maintenance of this life cycle involving fathead minnows in a laboratory setting is much easier and cost-effective than doing the same with salmonid fish.

5. Conclusion

We sequenced, assembled, and annotated the genome of *M. rasmusseni*, an emerging parasite of fathead minnows in Alberta, using the long read Oxford Nanopore sequencing technology. The *M. rasmusseni* genome is the largest genome among myxozoan species sequenced till date, and one of the most repetitive genomes among animals. We compare the *M. rasmusseni* genome to genomes from other myxozoan and free-living cnidarian species, and show that, like other myxozoan species, *M. rasmusseni* has lost several well-conserved eukaryotic genes, and at the same time, has also experienced expansion in several gene families. Our analysis demonstrated the value of long read sequencing platforms for all myxozoan parasites. We also propose that *M. rasmusseni* presents a promising potential model to study host-parasite interactions in myxozoans.

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