UNIVERSITY OF CALIFORNIA SAN DIEGO

Using DNA sequencing to better understand the diversity of larval trematode and cestode parasites in food webs

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Biology

by

Cassandra Nicole Bernas

Committee in charge:

Professor Ryan F. Hechinger, Chair
Professor Andrew Barton, Co-Chair
Professor Stephanie Mel

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The thesis of Cassandra Nicole Bernas is approved,
and it is acceptable in quality and form for publication
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Co-Chair

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Chair

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2020

DEDICATION

To my family. Thank you for always motivating me. A wise man once told me…

“whatever”.

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ABSTRACT OF THE THESIS

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Cassandra Nicole Bernas

Master of Science in Biology

University of California San Diego, 2020

Professor Ryan F. Hechinger, Chair
Professor Andrew Barton, Co-Chair

While parasites can alter community structure, comprise a substantial proportion of the diversity in food webs, and also help us resolve trophic links, few studies have incorporated parasites into food webs. One of the main challenges of adequately adding parasites in food webs is accurate identification of parasites to the

species level. In addition to having a large, undescribed diversity of species, many parasites have multiple-host complex life cycles, with poorly documented larval stages, infecting a wide range of vertebrate and invertebrate hosts. It is difficult to connect the larval stages to the adult stages, even when adult stages are described. DNA sequencing can inform efforts to document the parasite diversity in food webs and to connect different parasite life stages. This thesis seeks to inform a large effort to put parasites into the food webs for 13 estuaries along the California and Baja California coast. I obtained DNA sequences of the cytochrome oxidase 1 (CO1) gene and/or 18S rDNA for 123 samples of parasitic platyhelminthes (larval cestodes and trematodes), from 15 species of invertebrate hosts (including: bivalves, decapods, arthropods, and gastropods), that were only previously crudely identified based on morphology. Three different phylogenies were created to provide an improved degree of taxonomic resolution compared to the previous working names. I worked to test and develop new degenerate COI primers that had great promise in terms of sequencing efficiency (percentage of samples sequenceable). However, much testing and trouble-shooting revealed the inadequacy of the new primers, and other primers were used to obtain additional sample sequences. On the whole, this work provided sequences for 31 parasite samples, 26 species, corrected 3 misidentifications, and increased the taxonomic resolution for 22 samples. This work sets the stage for continuing efforts to link the larval stages to other parasite life stages, and permits a more accurate depiction of parasite diversity in food webs.

INTRODUCTION

Parasites affect host populations (Hechinger 2010, Lafferty 1993), ecological communities (Wood et al. 2007, Dobson and Hudson 1986), and food web robustness (Lafferty and Kurtis 2009). Parasites can indirectly alter ecosystems by influencing host growth, mortality, and behavior (Mouritsen and Poulin 2005, Lafferty et al. 2006a, Wood et al. 2007, Lefèvre et al. 2009, Preston et al. 2010). While parasites were thought to contribute little biomass to ecosystems (Loreau et al. 2005, Polis and Strong 1996), despite their small size, they make up a large proportion of the ecosystem's biomass (Kuris et al. 2008, Lambden and Johnson 2013) and significantly alter food web structure (Lafferty et al. 2006b). For example, parasite biomass exceeds that of top predators within California and Baja California estuaries (Kuris et al. 2008). While parasites alter community structure and help determine trophic links, few studies have incorporated these parasites into food webs (Marcogliese and Cone 1997, Sukhdeo and Hernandez 2004). The goal of this work is to help incorporate parasites into food webs along the California and Baja California coasts. 31 different parasitic platyhelminthes (larval cestodes and trematodes) were identified, from 15 species of invertebrate hosts (including: bivalves, decapods, arthropods, and gastropods), that were previously identified solely by morphology.

Digeneans are the most geographically diverse and numerically abundant group of parasitic metazoans, with ~18,000 currently known species (Pérez-Ponce de León and Hernández-Mena 2019, Littlewood et al. 2015, Olson et al. 2003, Cribb et al. 2001). Cestodes (tapeworms) and trematodes (fukes) have complex life cycles that involve

multiple host species. Some of the transmission steps for trematodes, and all of those for cestodes, typically involve a form of trophic transmission, whereby the host for the trophically transmitted larval stage must be eaten by the final host predator (e.g., a shark, ray, bony fish, or bird). To facilitate transmission to the final host, some parasites manipulate host behavior to increase the probability of predation (e.g., Lafferty and Morris 1996). In their life cycles, these parasites can infect more than one intermediate host including mollusks, arthropods, annelids, ctenophores, echinoderms, hexapods and vertebrates, before reaching sexual maturity in the final host (Littlewood et al. 2015, Caira et al. 2013, Aznar et al. 2007, Levy 2002, Cribb et al. 2001, Campbell and Beveridge 1994, Martin 1950).

Studies elucidating marine tapeworm life cycles can provide guidance to identify cryptic trophic links (Randhawa 2011a, Isbert et al. 2015, Caira et al. 2014). For example, Randhawa and Brickle (2011b) revealed a trophic link between the squid *Doryteuthis gahi* and porbeagle sharks that leads to the transmission of tetraphyllidean tapeworms. They connected larvae recovered from *Doryteuthis gahi* to the adult cestodes *Clistobothrium cf. montaukensis* and *Dinobothrium sp.* in the shark. Connecting life stages of this parasite helped draw a trophic association between squid and porbeagle sharks that was never seen before (Randhawa and Brickle 2011b). Thus, as shown in this research, bridging these helminth life stages could expand our knowledge about host biology.

Many helminth life cycles are unknown, including the platyhelminth species. This is because taxonomy is based on adult morphology and the larvae tend to lack the

taxonomically key features of the adult stages. The exception is elasmobranch cestodes, of the order Trypanorhyncha, due to their distinctive hooked tentacles that are actually present in larval stages (Campbell and Beveridge 1994). However, for most other parasites, it is very difficult to connect life stages by simply observing morphological features. Laboratory experimental infections is the classic way of studying these life cycles (Hoffman 1999, Martin 1950). While this is a promising approach to characterize a parasite, it limits the study to a single species. Also, the research is confined to a limited number of hosts, where some do not reflect the natural system. For example, parasites will be set up to infect a single host species, for each life stage. However, in a natural environment, they infect a larger array of intermediate and final hosts.

Nonetheless, DNA sequencing and analytical techniques make it possible to overcome these difficulties (Olson & Tkach 2005). For instance, Jensen and Bullard (2010) noted that it is challenging to confidently assign species or even genera to larval tapeworms based on morphology alone. Thus, they amplified a region of the 28S rDNA gene to align with adult rhinebothriidean and tetraphyllidean sequences (Healy et al. 2009, Olson et al. 2001, Waeschenbach et al. 2007). Organisms that played a role in trophic transmission were successfully identified using these techniques. However, the minimal amount of adult tapeworm sequences available is a large limitation of this approach (Olson & Tkach 2005, Brickle et al. 2001, Agusti et al. 2005, Aznar et al. 2007, Jensen & Bullard 2010). That is, even with good DNA sequences of a larval

stage, the adult stage may have never been sequenced; there is nothing to which to match the larval stages sequences.

Another issue with identifying platyhelminthes solely by morphology is the occurrence of cryptic species. Cryptic species are sibling species that are morphologically indistinguishable (with current knowledge). Luckily, molecular techniques such as barcoding have been proven to be effective when identifying parasites (McManus and Bowles 1996). The cytochrome oxidase 1 (COI) gene is frequently used for species identification of larval stages and can define cryptic species (Moszczynska et al. 2009). However, only a small number of flatworms have previously been barcoded (Kvist 2013) and the high level of sequence divergence makes it difficult to target all platyhelminthes (Moszczynska et al. 2009). Hence, the 18S region was also used to create phylogenies in this study.

Ribosomal DNA (rDNA), especially the 18S (small subunit), contains highly conserved regions and is well used for species identification (Hillis & Dixon 1991, Littlewood et al. 2015, Caira et al. 2013, Foronda et al. 2004, Olson et al. 2001, Fernandez et al. 1998). 18S rDNA has been proven to be a strong ally for creating and analyzing platyhelminth phylogenies (Littlewood and Olson 2001). For example, Cribb et al. (2001) did a comparison across multiple taxa of a morphological tree to a phylogeny based on rDNA. They found that the phylogeny displayed a more accurate depiction of species identification, compared to the morphological data.

To begin mapping life stages of helminth parasites, in this study, invertebrate hosts were collected from California and Baja California wetlands. From these hosts, 31

platyhelminth species were identified using both morphology and sequencing data. The 18S region was amplified for species identification. The CO1 region was also used for DNA barcoding, to decipher possible cryptic species, using degenerate primers and Dice primers (Steenkiste et al. 2014). During this research, we tested new degenerate CO1 primers, trying to amplify a wide range of taxa, at a higher rate than traditional universal primers. After sequencing, samples were placed within phylogenetic trees for identification and to display species diversity. Finally, connections were drawn to their invertebrate, intermediate hosts. This work will directly (1) establish the taxonomic identity of the difficult to identify larval stages, (2) inform efforts to link those larval stages to other life stages in the same ecosystems, and (3) permit a more accurate depiction of parasite diversity in food webs.

MATERIALS AND METHODS

General Overview

This project is part of a larger study examining each parasite's flow through the coastal food webs and ecosystem. In order to understand the specific impact each parasite has throughout its lifecycle and to tackle the most challenging stages to deal with morphologically, we began connecting each species to their intermediate host. A wide range of invertebrates from California and Baja California wetlands were collected and dissected. Various cestodes and trematodes were extracted from these hosts and amplified using three different primer sets, targeting two different loci. After sequencing, samples were placed within their distinct phylogenies for analysis. Below, is the detailed experimental design.

Collection of Specimens and Extraction of DNA

Intermediate hosts were collected, as part of a large expedition in 2014 from 13 estuaries in California and Baja California. I obtained sequences from material originating from the following subset of those estuaries (ordered North to South): Drakes Estero, Bolinas Lagoon, southeast San Francisco Bay, Morro Bay, Ballona Lagoon, Mission Bay, or Guerrero Negro. All hosts were kept live or frozen until dissection. Platyhelminth samples were extracted from each host, identified based on morphological features, and placed in 70% ethanol, in dram vials, for genetic analysis. Prior to DNA extraction, each sample was rinsed with 500µL of DI water 3 times, to remove ethanol. The HotSHOT method was used to extract DNA (Truett et al. 2000).

After the addition of the lysis buffer, each sample was flick vortexed every 20 minutes, while sitting in a 95°C bath for an hour. Then, they were transferred to an ice bath for 3 minutes, flick vortexed again, and the 75 uL of neutralization buffer was added. Entire specimens were placed into the lysis buffer, sometimes containing host tissue. A total of 18 cestoda samples and 111 trematoda samples were extracted.

Polymerase Chain Reaction

Three different PCR reaction conditions were performed, all with final volumes of 20µL. For amplification of the 18S rDNA, primers Worm-A (5'-GCGAATGGCTCATTAAATCAG - 3') and Worm-B (5'-CTTGTTACGACTTTTACTTCC - 3') were used (Littlewood and Olson 2001). These primers were tagged with universal sequencing tails T7 Term and T3 respectively. Each reaction contained 1.5U of Taq DNA Polymerase (Qiagen, CA; Mat # 1007837; lot # 124125007), 2µL of 10X PCR Buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$), 0.2 mM of dNTPs, 0.25µM of each PCR primer, and 2µL of extracted genomic DNA. Thermal protocol was based off of Olson et al. (2003).

The first 570-580bp of the Folmer region, within the mitochondrial gene cytochrome c oxidase 1 (CO1), was amplified using Dice1F (5' - TTWCNTTRGATCATAAG - 3') and Dice 11R (5' - GCWGWACHAAATTTHCGATC - 3'). The reaction conditions and thermal protocol were followed according to the touchdown protocol listed within Steenkiste et al. (2014).

A 600bp region within the CO1 gene was also amplified using newly designed primers, CestCOIdgenF1 (5' - CAYATGTTTTGRTTYTTTGGNCAYC - 3') and CestCOIdgenR1 (5' - CCAAARTAATGCATVGGRAAA - 3') (Metz, unpublished data). Universal sequencing tails T7 term and T3 were added to the end of each primer respectively. Two different reactions were used to troubleshoot sequencing inconsistencies. The first reaction comprised 0.6U of Taq DNA Polymerase (Qiagen), 2µL of 10X PCR Buffer (Tris-Cl, KCl, $(NH_4)_2SO_4$, 15mM $MgCl_2$), 0.2mM of dNTPs, 0.25µM of each PCR primer, and 2µL of extracted genomic DNA. The thermal conditions were 94℃ for 2 min for initiation. Then, 37 cycles of 94℃ for 45 sec, 50℃ for 45 sec, and 72℃ for 1 min, with a final extension at 72℃ for 5 min. The second reaction contained the same reagent volumes, however, there was a final concentration of 3.5mM $MgCl_2$. A touchdown thermal protocol was used containing the following: 94℃ for 2 min, 3 cycles with 94℃ for 40 sec, 50℃ for 40 sec, and 72℃ for 1 min, 5 cycles with 94℃ for 40 sec, 49℃ for 40 sec (decreasing a degree each cycle), and 72℃ for 1 min, 35 cycles with 94℃ with 40 sec, 41℃ with 40 sec, and 72℃ for 1 min, and an extension at 72℃ for 5 min.

Sequence Processing

All PCR products were purified and sequenced, in the forward direction, using sanger sequencing (Eton Biosciences). The chromatograms were analyzed using 4Peaks v 1.8. (4Peaks, RRID:SCR_000015). Ambiguous or incorrect base calls were manually edited and trimmed to erase low quality sequence data. Each samples' most

compatible reference sequences, with the highest alignment rates, from Genbank were collected and compared (www.ncbi.nlm.nih.gov). A list of these outgroup and reference sequences are in Table 1. To align sequences, multiple sequence comparison by log-expectation (MUSCLE) analysis within MEGA X v. 7 was used (Kumar et al. 2018). The length for each alignment, including gaps, was as follows: 18S = 676bp, Dice = 491bp, and Degenerate = 624bp.

Phylogenetic Analysis

Maximum likelihood analysis was conducted using PAUP* v. 4b10 (Swofford 2002) and MEGA X (Kumar et al. 2018). Models of nucleotide substitution were evaluated for each data partition independently using MrModelTest v. 2.4 (Nylader 2004). For each data set, the general time reversible model including estimates of invariant sites and gamma distributed among site rate variation (GTR+I+G) was proven best fit according to Akaike's Information Criterion (AIC). Each data set was run with the parameters shown in Table 2. These phylogenies were analyzed to connect parasite species to their hosts. Turbellaria species were used as an outgroup for each tree.

Table 1: Reference sequences and their accession numbers used for phylogenetic analysis. Each accession number is categorized with the appropriate primers used.

Species Name	18S	Dice	Degenerate
Acanthoparyphium sp.			KJ956253
Acanthoparyphium spinulosum isolate		KM880011	
Acanthotaenia shipleyi isolate		MK328931	
Acanthotaenia sp.		MK328926	
Allocreadium gotoi			LC215273
Anonchocephalus chilensis isolate			KR780782
Ascocotyle sp.		JQ241161	
Brachycladium goliath	KR703279		
Calicophoron daubneyi isolate			KP979656
Calyptrobothrium sp.	KF685848		
Caulobothrium opisthorchis voucher	FJ177066		
Cladotaenia vulturi			KU559932
Clistobothrium montaukensis	AF286996		
Clistobothrium sp.		KU987913	
Cloacitrema narrabeenensis	AY222134		
Collyriclum faba	JQ231122		KJ434372
Degeneria halosauri	AJ287497		
Dicrocoeliidae sp.	MG822661		
Digenea sp.	KY417091		
Dilepididae sp.		KM538088	
Dipylidium caninum			NC021145
Echinochasmidae sp.		MH532417	
Echinococcus canadensis			AB813185
Echinococcus felidis			AB732958
Echinococcus granulosus			KU601616
Echinococcus multilocularis			MN251848
Echinococcus oligarthrus			AB208545
Echinostoma caproni	L06567		
Echinostoma miyagawai		MN116740	
Echinostoma paraensei	FJ380226		
Echinostoma revolutum	AY222132		
Encyclometra colubrimurorum	AY222142		
Euparyphium capitaneum isolate		KY636236	

Table 1: Reference sequences and their accession numbers used for phylogenetic analysis. Each accession number is categorized with the appropriate primers used.

Species Name	18S	Dice	Degenerate
Fasciola gigantica			MF287791
Fasciola hepatica			AP017707
Fasciola jacksoni	MF077356	MN131131	
Fascioloides magna	EF051080		
Fasciolopsis buski	AY311386		KX169163
Halysiorhynchus macrocephalus isolate	DQ642940		
Haplorchis taichui isolate	KX815126		
Heronimus mollis	AY222118		
Himasthla elongata	KU886143		
Hurleytrematoides sp.		JN969509	
Hymenolepis microstoma			LR215992
Kirstenella gordoni isolate			KR780796
Kotorella pronosoma			EF103923
Maritrema novaezealandensis isolate			FJ765475
Maritrema oocysta	AJ287534		
Metagonimus yokogawai isolate	HQ832630		
Microphallidae sp.	AB974359		FJ765510
Microphallus fusiformis	AJ287531		
Microphallus primas	AJ287541		
Microphallus sp.			FJ765480
Milanella familiaris isolate			KR780783
Nagmia floridensis	AY222145		
Nasitrema sp.	KM258666		
Neobothriocephalus aspinosus isolate			KR780805
Opecoelidae sp.			FJ765503
Oschmarinella macrorchis	LC269094		
Pachybothrium hutsoni		JQ268551	
Parachristianella sp.	FJ572902		
Paraechinophallus japonicus isolate			KR780804
Paragonimus heterotremus isolate			KY952166
Paragonimus mexicanus isolate		KC562288	
Parorchis sp.		LC438938	KJ868195
Penetrocephalus ganapattii isolate			KR780799
Plilophthalimid sp.	AJ287560		
Phoreiobothrium lewinense isolate	KF685830		

Table 1: Reference sequences and their accession numbers used for phylogenetic analysis. Each accession number is categorized with the appropriate primers used.

Species Name	18S	Dice	Degenerate
Plagiorchis sp.		MG964028	
Pleorchis uku	DQ248203		
Probothriocephalus alaini isolate			KR780784
Pseudopsilostoma varium		JX468064	
Raillietina sp.			EU665478
Rhinebothroides sp.		JF803679	
Rhopalias macracanthus isolate		MK982785	
Saccocoelioides orosiensis isolate		MK749598	
Schistosoma japonicum isolate			KU196375
Spirometra erinaceieuropaei			KJ599680
Stephanostomum baccatum	AJ287577		
Steringophorus margolisi	AJ287578		
Taenia crocutae		AB905201	
Taenia laticollis		AB731727	
Taenia ovis		AB731675	
Taenia sp.		AB905202	
Trichobilharzia anseri isolate			KP901385
Trimacracanthus aetobatidis isolate	DQ642942		
Troglotrema acutum		KJ722062	
Turbellaria sp.	U45961	KT383430	KT383430
Uvitellina sp.		NC042722	
Versteria mustelae		AB732960	MK681866

Table 2: Parameters for each phylogeny.

Phylogeny	Model	Base	Nst	Rmat	Rates	Shape	Pinvar
18S	GTR	0.2387 0.2115 0.2893	6	1.480631232 3.389058352 1.929102421 0.798881531 5.479024410	Gamma	0.3833	0.1054
CO1 Dice	GTR	0.2171 0.1187 0.2263	6	0.092590421 4.318057060 1.510504246 1.670088768 4.199796200	Gamma	0.6321	0.2676
CO1 Degenerate	GTR	0.2334 0.0754 0.2240	6	2.297921658 5.658803940 1.544232607 3.891034842 7.756382465	Gamma	0.4118	0.0961

RESULTS

General Overview

Out of 129 platyhelminthes samples, 8 cestodes and 23 trematodes were amplified and sequenced. They were placed within 3 maximum likelihood phylogenies (18S, COI Dice, and CO1 Degenerate). These samples span across 5 orders (Tetraphyllidea, Trypanorhyncha, Bucephalata, Echinostomata, and Xiphidiata), infecting 15 different hosts (bivalves, decapods, arthropods, and gastropods). Most trematodes fell within the families Himasthlidae and Microphallidae. Each phylogeny's subtrees were assembled according to Tkach (2015), Olson et al. (2003), Olson et al. (2009), and Jensen and Bullard (2010). On average, sequencing improved taxonomic resolution of morphologically identified species by at least one taxonomic level (displayed in Table 3). Inaccurate taxonomic identifications were also revealed. These newly identified species were mapped to each individual phylogeny, with their hosts indicated. Nineteen samples were placed into the 18S phylogeny, 8 into the CO1 Dice phylogeny, and 10 into the CO1 Degenerate phylogeny. Six platyhelminth samples were displayed in more than one phylogeny (Table 3). They were identified as the same species in CO1 phylogenies, if there was a <3% alignment difference. Samples with a larger alignment difference within the same family, were identified as cryptic species. Overall, 2 different Himasthla quissitensis parthenitae cryptic species were identified and 22 samples had improved taxonomic resolution.

Table 3: Summary data for the 31 samples that were successfully sequenced. Updated parasite identification, parasite identification based off of morphology alone, host, and primer pair used are listed

New Parasite Identification	Initial Parasite Identification (Morphology)	Host Species	Primer*
Platyhelminthes:Neodermata:Cestoda:Eucestoda			
Tetraphyllidea			
Tetraphyllidean sp.2	Tetraphyllidean sp.	*Saxidomus nuttalli*	Dgn CO1
Tetraphyllidea incertae sedis			
Tetraphyllidean sp.1	Tetraphyllidean sp.	*Tresus sp.*	Dgn CO1, Worm A/B
Tetraphyllidean sp.1 10 loculi	Tetraphyllidean sp. 10 loculi	*Macoma nasuta*	Dice, Worm A/B
Tetraphyllidean sp.1	Tetraphyllidean sp.	*Macoma nasuta*	Dice
Trypanorhyncha			
Trypanobatoida			
Trypanobatoidan sp.1.1	Trypanorhynchid sp.	*Neotrypaea gigas*	Dgn CO1
Trypanobatoidan sp.1.2	Trypanorhynchid sp.	*Neotrypaea gigas*	Dgn CO1
Trypanobatoidan sp.2	Trypanorhynchid sp.	*Neotrypaea gigas*	Dgn CO1
Trypanobatoidan sp.3 plerocercoid	Trypanorhynchid sp.	*Pachygrapsus crassipes*	Worm A/B
Platyhelminthes:Neodermata:Trematoda:Digenea:Plagiorchiida			
Bucephalata			
Gymnophalloidea			
Gymnophallidae			
Gymnophallid sp.1	Bucephalid sp.	*Cryptomya californica*	Worm A/B
Gymnophallid sp.2	Bucephalid sp.	*Cryptomya californica*	Worm A/B
Gymnophallid sp.3 parth	Gymnophallid sp. parth	*Nutricola tantilla*	Worm A/B
Echinostomata			
Echinostomatiodea			
Himasthlidae			
Himasthlid sp.1 parth	Echinostome sp.	*Littirina plena*	Dgn CO1, Worm A/B
Himasthlid sp.2 parth	Echinostome sp.	*Littirina plena*	Dice
Himasthlid sp.2 mc	Unknown mc	*Tagelus subteres*	Dice

Table 3: Summary data for the 31 samples that were successfully sequenced. Updated parasite identification, parasite identification based off of morphology alone, host, and primer pair used are listed

New Parasite Identification	Initial Parasite Identification (Morphology)	Host Species	Primer*
Himasthla			
Himasthla sp.1 quissitensis parth	*Himasthla* sp. quissitensis parth	*Nassarius tiarula*	Dice, Worm A/B
Himasthla sp.2 quissitensis parth	*Himasthla* sp. quissitensis parth	*Nassarius tiarula*	Worm A/B
Himasthla sp.3 quissitensis parth	*Himasthla* sp. quissitensis parth	*Nassarius tiarula*	Dice
Xiphidiata			
Allocreadioidea			
Allocreadioidean sp. mc	mc oval	*Uca crenulata*	Worm A/B
Allocreadioidean sp. parth	*Zoogonus rubellus* sp. parth	*Illyanassa obsoleta*	Dgn CO1
Acanthocolpidae			
Stephanostomum			
Stephanostomum sp. cf S. tenue	*Stephanostomum* sp.	*Ilyanassa obsoleta*	Worm A/B
Gorgoderoidea			
Gorgoderidae			
Gorgoderid sp.1	mc	Tresus sp.	Dice, Worm A/B
Gorgoderid sp.1	mc	*Pectinaria californiensis*	Dice
Microphalloidea			
Microphallidae			
Microphallinae			
Microphalline sp.1	*Maritrema* sp.	*Pachygrapsus crassipes*	Worm A/B
Microphalline sp.2	*Maritrema* sp.	*Pachygrapsus crassipes*	Worm A/B
Microphalline sp.3 mc large	mc large	*Hemigrapsus oregonensis*	Worm A/B
Microphallus			
Microphallus sp.1 mc small	*Microphallus* sp. mc small	*Traskorchestia traskiana*	Dgn CO1, Worm A/B
Microphallus sp.2 mc rnd large	mc round large	*Hemigrapsus oregonensis*	Worm A/B
Microphallus sp.3 mc large	mc large	*Traskorchestia traskiana*	Worm A/B
Microphallus sp.4 mc oval	mc oval	*Traskorchestia traskiana*	Worm A/B

Table 3: Summary data for the 31 samples that were successfully sequenced. Updated parasite identification, parasite identification based off of morphology alone, host, and primer pair used are listed

New Parasite Identification	Initial Parasite Identification (Morphology)	Host Species	Primer*
Zoogonidae			
Zoogonus			
Zoogonus rubellus sp. parth	*Zoogonus rubellus* sp. parth	*Illyanassa obsoleta*	Dgn CO1
Unknown sp. mc huge	mc huge	*Traskorchestia traskiana*	Dgn CO1

* Worm A/B targets the 18S region (Littlewood and Olson 2001), Dice targets the CO1 region (Van Steenkiste et al. 2014), and Dgn CO1 are the new degenerate primers that target the CO1.

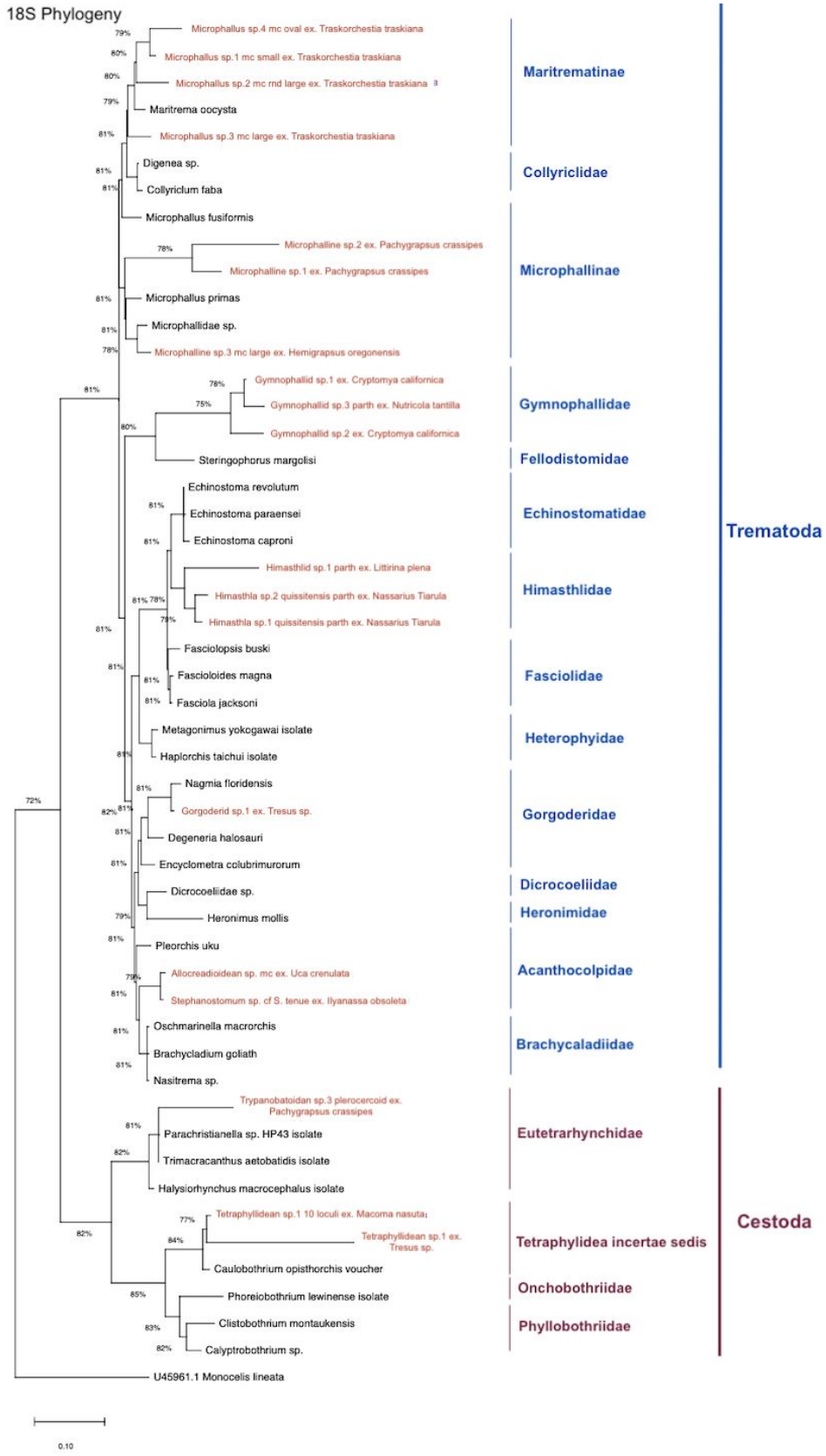
18S rDNA Phylogeny

Nineteen platyhelminthes were successfully sequenced using rDNA. Thirty six additional reference sequences were added to the maximum likelihood tree, including a turbellarid as an outgroup. The bootstrap range was from 75% to 85%. One sample was identified down to the sub order, one to the super family, 11 to the family, and 6 down to the genus (Figure 1). Most samples fell within the Microphalloidea. 3 Gymnophallid and 2 Himasthla species were closely related (Figure 1).

The Microphallidae split into two clades, Maritrematinae and Microphallinae. The Maritrematinae branch hosted 4 newly identified species and the Microphallinae had 3. *Steringophorus margolisi* is a part of Gymnophalloidea, which also embeds Gymnophallidae. Within Gymnophallidae, 3 different species were identified. Another large clade consisting of newly identified species was the Himasthlidea, which was displayed, within Echinostomata, according to Tkach et al. (2016).

For the cestodes, there were two main clades, Tetraphyllidea and Trypanorhyncha. The two tetraphyllidean samples are grouped within “Tetraphilidea incertae sedis”. The one trypanorhynchid fell within Trypanobatiodia.

Figure 1: Maximum Likelihood Phylogeny based off of 18S rDNA with bootstrapping percentages shown. Red indicates new species. For accession numbers see Table 1.


18S Phylogeny
Microphallus sp.4 mc oval ex. Traskorchestia traskiana
Microphallus sp.1 mc small ex. Traskorchestia traskiana
Microphallus sp.2 mc rnd large ex. Traskorchestia traskiana
Maritrema oocysta
Microphallus sp.3 mc large ex. Traskorchestia traskiana
Digenea sp.
Collyriclum faba
Microphallus fusiformis
Microphalline sp.2 ex. Pachygrapsus crassipes
Microphalline sp.1 ex. Pachygrapsus crassipes
Microphallus primas
Microphallidae sp.
Microphalline sp.3 mc large ex. Hemigrapsus oregonensis
Gymnophallid sp.1 ex. Cryptomya californica
Gymnophallid sp.3 parth ex. Nutricola tantilla
Gymnophallid sp.2 ex. Cryptomya californica
Steringophorus margolisi
Echinostoma revolutum
Echinostoma paraensei
Echinostoma caproni
Himasthlid sp.1 parth ex. Littirina plena
Himasthla sp.2 quissitensis parth ex. Nassarius Tiarula
Himasthla sp.1 quissitensis parth ex. Nassarius Tiarula
Fasciolopsis buski
Fascioloides magna
Fasciola jacksoni
Metagonimus yokogawai isolate
Haplorchis taichui isolate
Nagmia floridensis
Gorgoderid sp.1 ex. Tresus sp.
Degeneria halosauri
Encyclometra colubrimurorum
Dicrocoeliidae sp.
Heronimus mollis
Pleorchis uku
Allocreadioidean sp. mc ex. Uca crenulata
Stephanostomum sp. cf S. tenue ex. Ilyanassa obsoleta
Oschmarinella macrorchis
Brachycladium goliath
Nasitrema sp.
Trypanobatoidan sp.3 plerocercoid ex. Pachygrapsus crassipes
Parachristianella sp. HP43 isolate
Trimacracanthus aetobatidis isolate
Halysiorhynchus macrocephalus isolate
Tetraphyllidean sp.1 10 loculi ex. Macoma nasuta
Tetraphyllidean sp.1 ex. Tresus sp.
Caulobothrium opisthorchis voucher
Phoreiobothrium lewinense isolate
Clistobothrium montaukensis
Calyptrobothrium sp.
U45961.1 Monocelis lineata
0.10
Maritrematinae
Collyriclidae
Microphallinae
Gymnophallidae
Fellodistomidae
Echinostomatidae
Himasthlidae
Fasciolidae
Heterophyidae
Gorgoderidae
Dicrocoeliidae
Heronimidae
Acanthocolpidae
Brachycaladiidae
Eutetrarhynchidae
Tetraphylidea incertae sedis
Onchobothriidae
Phyllobothriidae
Trematoda
Cestoda

COI Dice Phylogeny

8 platyhelminth samples were successfully sequenced with Dice primers and placed into a phylogeny, along with 27 other species collected from Genbank (Table 1) (www.ncbi.nlm.nih.gov). The trematodes grouped together within Himasthlidae and Gorgoderidae and the 2 cestodes grouped within the Tetraphillidea order (Figure 3). The bootstrap percentage was 97% for trematodes and 98% for cestodes. 3 pairs of helminth samples (Himasthlid, Gorgoderid, and Tetraphyllidean) were identified as the same species. “Tetraphyllidean sp. 10 loculi ex. Macoma nasuta”, “Himasthlid sp.1 quissitensis parth ex. Nassarius tegula”, and “Gorgoderid sp.1 ex. Tresus drakes” were incorporated into both the 18S tree and CO1 Dice tree.

4 samples grouped into Himasthlidae. Acanthoparyphium spinulosum appeared to be closely related to two of these new species. Thus, we identified two species as “Himasthlid” and two species as “Himasthla”. “Gorgoderid sp.1 ex. Tresus drakes” was first identified within the 18S phylogeny (see Figure 1). When incorporated into the CO1 Dice tree, it aligned, having a >3% difference, with another Gorgoderid from pectinaria morro. Thus, they were identified as the same species. The same trend occurred with “Tetraphyllidean sp.1 10 loculi ex. Macoma nasulta” (also in the 18S phylogeny) and “Tetraphyllidean sp.1 ex. Macoma nasulta”.

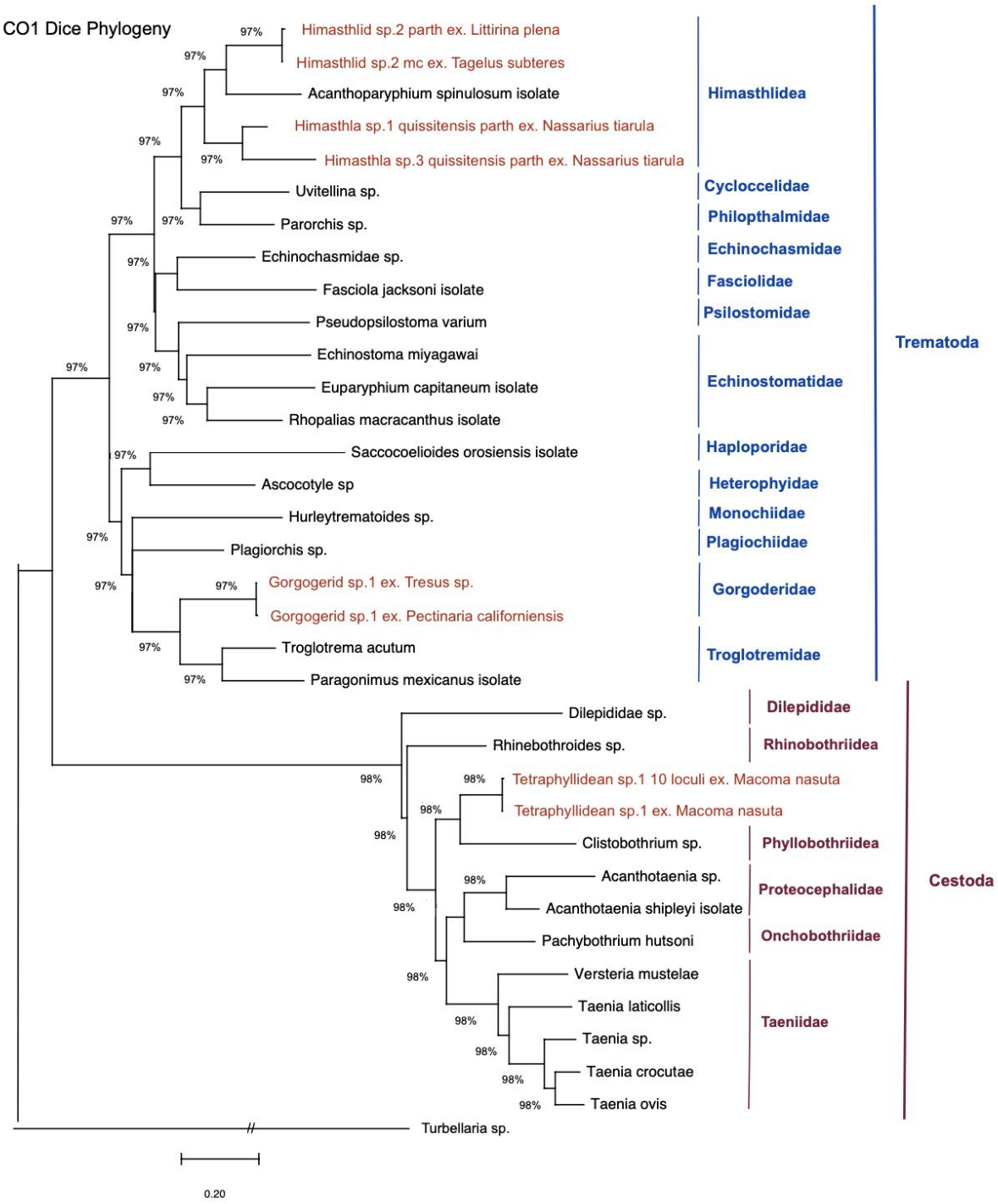


Figure 2: Maximum Likelihood Phylogeny based off of COI data from Dice primers with bootstrapping percentages shown. Red indicates new species. For accession numbers see Table 1.

COI Degenerate Phylogeny

The new degenerate primers, which targeted the COI region, had a ~55% success rate for amplification during gel electrophoresis. However, sequencing had a success rate of ~15%, of those that amplified. During experimentation, over seven dilution series of individual reagents were run, the thermal conditions were altered six times, and the primers were modified eight times, which still resulted in mostly low quality sequence data. 10 out of 123 platyhelminth samples were successfully sequenced and placed into a maximum likelihood tree (Figure 3). The phylogeny had a bootstrapping range from 97% to 98%. Half of the platyhelminthes were trematodes and the other half were cestodes, with most samples falling into the Trypanorhynch order.

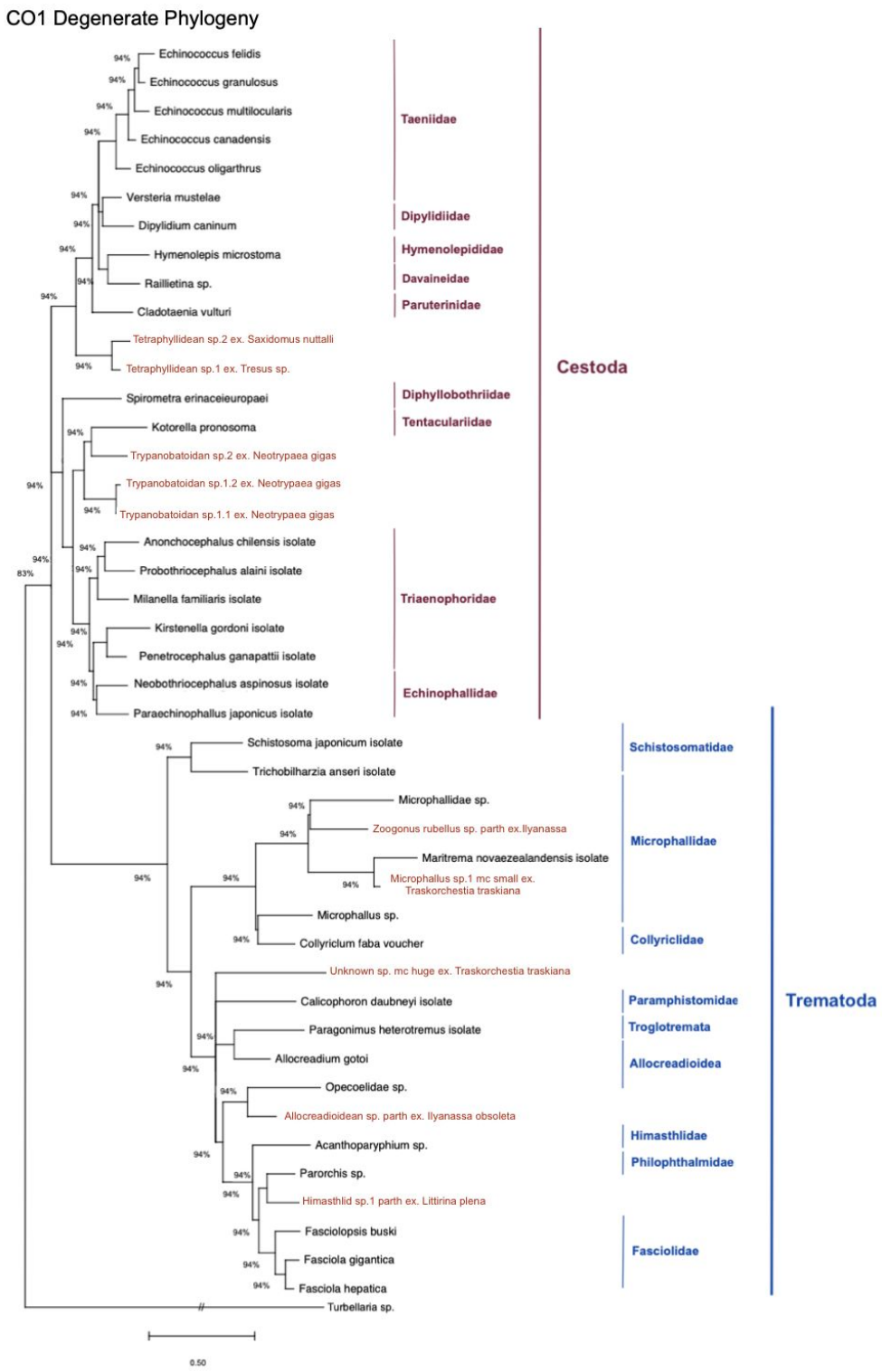


Figure 3: Phylogeny based off of COI data from new degenerate primers with bootstrapping percentages shown. Red indicates new species. For accession numbers see Table 1

DISCUSSION

Primer Troubleshooting

Due to the low success rate of the new COI degenerate primers, we were led to believe that the sequencing error was potentially a result of (1) contamination, (2) nonspecific amplification with a very similar sized base pair region, (3) primer dimerization, and/or (4) an issue with PCR purification. Each of these possibilities were evaluated, modified, and tested. Plus, the use of 18S primers further verified that there wasn't an issue with DNA quality. However, we found no major correlations in what was causing sequencing issues. Over ~80% of sequences produced noisey chromatograms. Even successful samples returned with illegible sequences for the first and last 100 base pairs.

Many studies have shown the benefit of lower taxon specific primers compared to broader ones (Dzikowski et al. 2004, Levy 2011, Moszynska et al. 2009). This method is limiting because the samples have to be partially identified by morphological features to determine what primers to use. Helminth larvae can be particularly tedious to classify (Jensen & Bullard 2010), making it advantageous to use primers that amplify at a phylum level. However, the higher the taxon the less successful the primers may be. For example, universal primers, such as the Folmer primers (Folmer et al. 1994), tend to have a low percentage of success and occasionally target the host tissue over the parasite. This trend is perhaps what we are seeing with the new COI degenerate primers and Dice primers (Steenkiste et al. 2014).

Moszynska et al. (2009) also found the COI region to be difficult to sequence with phylum level primers. They suggest that the high level of sequence divergence across platyhelminthes makes it unlikely that we will ever be able to use 'universal primers' to amplify species throughout such a large taxonomic level. Rather, we should use primers that target lower level taxonomic groups (Moszynska et al. 2009). However, the high success of amplification during gel electrophoresis implies that these degenerate primers may still be useful for barcoding, after the sequencing issue is resolved. They were especially fruitful within the Himasthlidae and Microphallidae families and provided higher species identification compared to previous classification based solely on morphology.

Phylogenetic Analysis

Some new helminth samples were named to larger taxonomic groups due to low resolution of the phylogenies because of the limited amount of reference sequences available. Particularly, the CO1 trees had the lowest resolution. For example, most samples were identified down to family level, not genus, due to the limited amount of cestode sequences accessible.

Within the 18S tree, there should be 2 main branches of microphallidae (Microphallinae and Maritrematinae), according to Tkach et al. (2003). Collyriclidae should be outside Microphallidae, but is within the Microphalloidea. Also, for the "Allocreadioidean sp. mc" (Table 2, Figure 1), we were unable to identify it as part of the *Stephanostomum* genus or the Acanthocolpidae family, even though it aligns closely to

our newly identified "*Stephanostomum sp.".* This is because acanthocolpids are not known to be found within decapods. Usually, they use mollusks or fish as their intermediate hosts (Bartoli and Bray 2004, Grano-Maldonado et al. 2019). Thus, we went up a taxa to Allocreadioidea, which does consist of a family, Opecoelidae, that infects crustaceans (Levia et al. 2017).

Moving Forward

Although we set out to sequence a greater number of parasites, these findings represent a large diversity of platyhelminth larvae. Also, 31 species were identified and their taxonomic resolution improved due to this sequencing data. An additional aspect of this work is that it will permit downstream connecting of other life stages to the stages sequenced here. For instance, when adult stage trematodes and cestodes are sequenced from estuarine birds, some of their larval stages will undoubtedly be found in the material sequenced for this project. This will shed further light on this understudied aspect of diversity, including the nature of these parasites' complex life cycles and the way they fit into food webs.

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