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ORIGINAL ARTICLE

Molecular contributions to species boundaries in dicyemid parasites from eastern Pacific cephalopods

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Abstract
Dicyemids are enigmatic parasites found within the excretory systems of benthic cephalopods. The phylogenetic position and overall diversity of dicyemids remains poorly understood, in part because current species delimitation criteria are based solely on morphological traits. Understanding the diversity of parasite species is particularly problematic because they tend to be devoid of consistent (informative) morphological traits while simultaneously rich in morphological variation associated with developmental stages and environmental conditions. In this study, we tested the boundaries of currently described morphospecies of dicyemids using molecular phylogenetic data. Variation within sequences of the small subunit (18S) rRNA gene was explored because this marker (1) is known to be fast-evolving in parasitic eukaryotes, (2) is one of the few molecular markers to have been previously sequenced in some dicyemids, and (3) has been used successfully as a DNA barcode in other groups of parasites. Three species of cephalopods were collected, each hosting several different morphospecies of dicyemid parasites. Thirty-four individual dicyemids encompassing eight different morphospecies were isolated and their 18S rDNA sequenced. Molecular phylogenetic analyses of these data were incongruent with current morphology-based species descriptions. The 18S rDNA sequences suggest that each host species of cephalopod harbors one species of dicyemid encompassing a great deal of morphological variation. The addition of DNA sequences to understanding dicyemid diversity clarifies species boundaries in a lineage that is difficult to define in nearly every aspect.

Key words: *Coevolution, DNA barcode, dicyemid, Mesozoa, species*

Introduction

Dicyemids are obligate parasites that live within the kidneys of benthic cephalopods (Nouvel 1947). They ignite curiosity and bewilderment from all who encounter them, but surprisingly little is known about dicyemid biology. First discovered in 1839 by Filippo Calvolini, they were dubbed 'mesozoans' by Van Beneden (1876) because they hold both metazoan- and protozoan-like qualities. At the time, it was thought that they might form a bridge between microbial eukaryotes and animals. Scientists still debate if the 20–40 celled dicyemids are highly streamlined metazoans, complex 'protists', or even a chimera of the two (Kobayaski et al. 1999; Noto & Endoh 2004; Suzuki et al. 2010). Even with deeper insights afforded by molecular data, their phylogenetic position still remains uncertain (Ohama et al. 1984; Katayama et al. 1995; Pawlowski et al. 1996; Kobayaski et al. 1999; Suzuki et al. 2010).

Dicyemids were so named for their two-part life cycle consisting of both an asexual and a sexual phase. The asexual phase produces clones of the adults that live their lives inserted into the renal folds of a cephalopod host (Figure 1A). The sexual phase produces infusoriform larvae that are morphologically distinct from the adults and are capable of leaving the host with the excretory waste (Furuya & Tsuneki, 2003; Figure 1B). It is unknown where the larva travels once it leaves the host, whether there are intermediate hosts and how a new cephalopod is infected.

Dicyemid systematics

Slightly over 100 morphospecies of dicyemids have been described in 40 species of benthic cephalopods.

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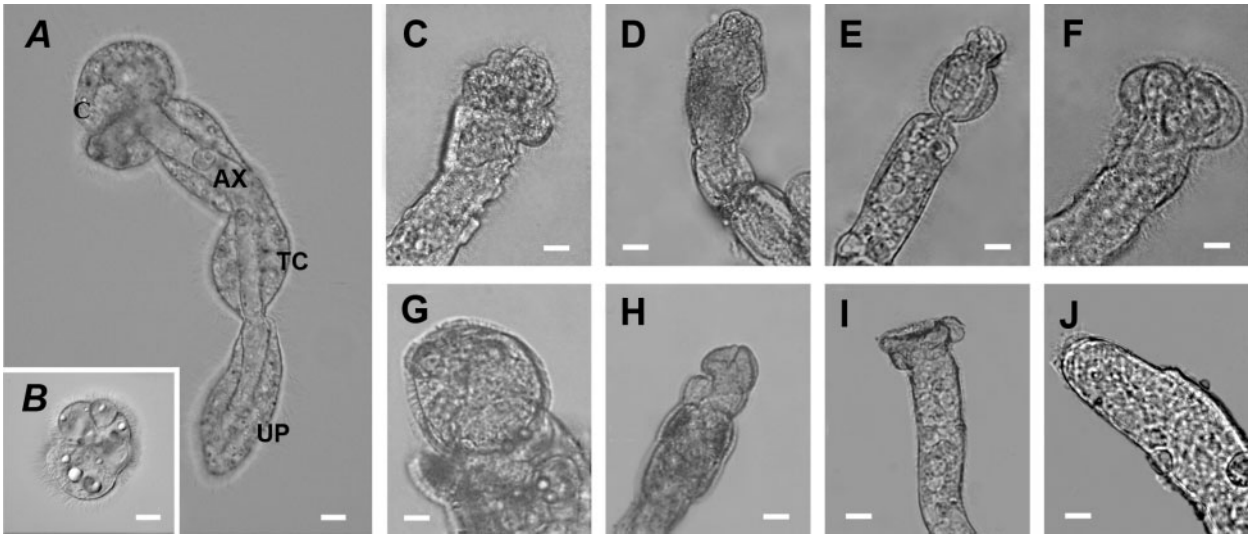

Figure 1. Light micrographs of the dicyemids characterized in this study with 18S rDNA sequences. (A) Vermiform adult representing *Dicyemennea brevicephala* found in the Pacific Red Octopus (*Octopus rubescens*) showing the disc-shaped calotte (C), the axial cell (AX), truck cells (TC) and uropolar cells (UP). Scale bar = 10 μm. (B) Infusoriform larva found in the Pacific Red Octopus (*O. rubescens*). Scale bar = 10 μm. (C) *Dicyemennea adscita* collected from *Octopus rubescens*. Scale bar = 10 μm. (D) *Dicyema apollyoni* collected from *O. rubescens*. Scale bar = 30 μm. (E) *Dicyemennea adminicula* collected from *O. rubescens*. Scale bar = 10 μm. (F) *Dicyemennea brevicephala* collected from *O. rubescens*. Scale bar = 10 μm. (G) *Dicyemodeca deca* collected from *Enteroctopus dofleini*. Scale bar = 10 μm. (H) *Dicyemennea abreida* collected from *E. dofleini*. Scale bar = 10 μm. (I) *Dicyemennea brevicephaloides* collected from *Rossia pacifica*. Scale bar = 40 μm. (J) *Dicyemennea rossiae* collected from *R. pacifica*. Scale bar = 10 μm.

Characterization of dicyemid genera is based on numbers of parapolar and metapolar cells found in the attachment organ or 'calotte' (Furuya 2006). There are four recognized shapes of calottes in dicyemids: conical, cap, disk, and irregular (Figures 1, 2). Differentiation at the species level has mainly been based on body size, cell number, calotte morphology, and host species (Furuya & Tsuneki 2003; Furuya et al. 2003). Up to four putative dicyemid morphospecies can be found in one cephalopod host species, and when multiple parasite species occur within a host, each of their co-habitants has a distinctive calotte shape. Never have two species of dicyemid with the same calotte shape been found in a single host species (Furuya et al. 2003).

Within the commonly found Northeast Pacific cephalopods, there are eight currently described species of dicyemids. *Enteroctopus dofleini* (Wülker, 1910) hosts *Dicyemennea abreida* McConnaughey, 1957 (Figures 1H, 2F) and *Dicyemodeca deca* (McConnaughey, 1957; Figures 1G, 2E). *Dicyemennea abreida* has 24–35 total cells, a conical-shaped calotte, and is about 1 mm in length. *Dicyemodeca deca* has 24–25 total cells, a disc-shaped calotte, and is also about 1 mm long (McConnaughey 1957). The biggest differences between the two species are the calotte shape and the extra metapolar cell that *Dicyemodeca* species have over *Dicyemennea* species.

Rossia pacifica S. S. Berry, 1911 hosts *Dicyemennea brevicephaloides* Bogolepova-Dobrokhotova, 1962 (Figures 1I, 2G) and *Dicyemennea rossiae* Bogolepova-Dobrokhotova, 1962 (Figures 1J, 2H) in their renal appendages. *Dicyemennea brevicephaloides* has 24 cells, a disc-shaped calotte and can grow up to 4 mm. *Dicyemennea rossiae* has 30–35 cells, a conical calotte and is about 2 mm in length (Furuya 2007). Both species have the same number of polar cells, and their differences lie mainly in body size and calotte shape.

Octopus rubescens Berry, 1953 hosts four morphospecies of dicyemids: *Dicyema apollyoni* Nouvel, 1947 (Figures 1D, 2B), *Dicyemennea adscita* McConnaughey, 1949 (Figures 1C, 2A), *Dicyemennea brevicephala* McConnaughey, 1941 (Figures 1F, 2D), and *Dicyemennea adminicula* McConnaughey, 1949 (Figures 1E, 2C). The calotte shapes are conical, cap-shaped, disc-shaped and irregular, respectively (Furuya et al. 2003). The boundaries between dicyemid species are based strongly on calotte shape. No molecular phylogenetic data have been used to validate existing species or determine new species, and the very little of the microscopy that has been performed has so far has centred on light microscopy, with a few instances of transmission electron microscopy (Ridley 1968, 1969; Czaker 2000).

DNA barcoding

DNA sequences offer a relatively unambiguous way to provide a 'barcode' capable of delimiting one species from another, the idea being that each barcoding gene has low intraspecific variation and

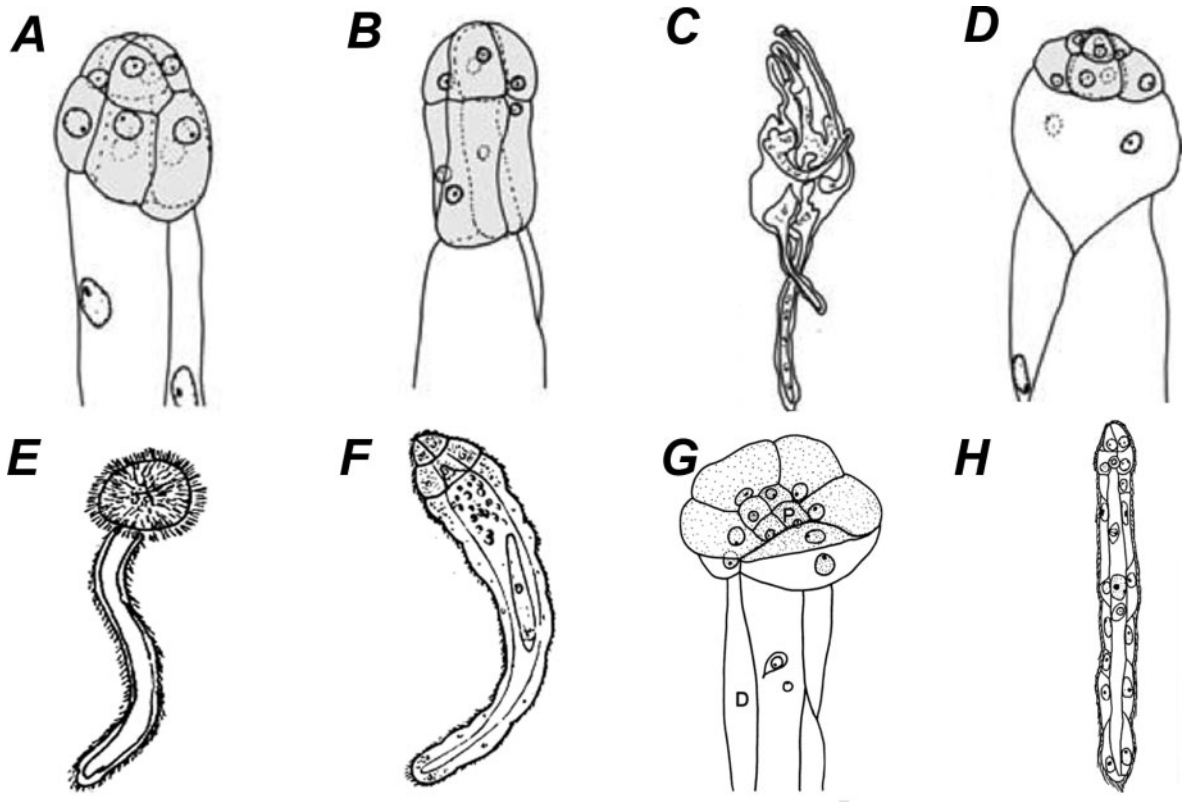

Figure 2. Illustrations of the dicyemid species characterized in this study with 18S rDNA sequences. (A) *Dicyemennea adscita* collected from *Octopus rubescens*. (B) *Dicyema apollyoni* collected from *O. rubescens*. (C) *Dicyemennea adminicula* collected from *O. rubescens*. (D) *Dicyemennea brevicephala* collected from *O. rubescens*. (E) *Dicyemodeca deca* collected from *Enteroctopus dofleini*. (F) *Dicyemennea abreida* collected from *E. dofleini*. (G) *Dicyemennea brevicephaloides* collected from *Rossia pacifica*. (H) *Dicyemennea rossiae* collected from *R. pacifica*. Figures reproduced with permission from: (A–D) H. Furuya, F. G. Hochberg & K. Tsuneki, 2003, Calotte morphology in the phylum Dicyemida: Niche separation and convergence, Journal of Zoology 259:361–73, Wiley, © The Zoological Society of London; (E,F) B. H. McConnaughey, 1957, Two new mesozoa from the Pacific Northwest, The Journal of Parasitology 43:358–64, Allen Press Publishing Services; (G,H) H. Furuya, 2007, Redescription of two *Dicyemennea* (phylum: Dicyemida) from *Rossia pacifica* (Mollusca: Cephalopoda: Decapoda), The Journal of Parasitology 93:841–49, Allen Press Publishing Services.

substantially more interspecific variation (the so-called 'barcoding gap'). DNA barcodes are particularly useful for determining species boundaries in organisms that have either limited morphological variation or high levels of intraspecific variation at the morphological level (Hebert et al. 2003; Moritz & Cicero 2004; Evans et al. 2007; Radulovici et al. 2010).

The most prevalent gene used to barcode eukaryotes is the cytochrome c oxidase subunit 1 (CO1) in the mitochondria (Hebert et al. 2003; Moritz & Cicero 2004; Evans et al. 2007). Mitochondrial genes, which are under less pressure to remain constant than nuclear genes, provide the relatively fast evolutionary rate necessary to distinguish and code organisms to the species level (Palumbi & Cipriano 1998). However, the mitochondria of endoparasites tend to be highly reduced because of the low oxygen environments they occupy within their hosts, so amplifying these genes can be very difficult if not impossible (Awata et al. 2005; Tsaousis et al. 2008).

The nucleus-encoded small subunit ribosomal gene (SSU rDNA or 18S rDNA) has mainly been used as a deep phylogenetic marker, but has been known to evolve rapidly in parasites, which facilitates DNA barcoding at the species level (Floyd et al. 2002; Powers 2004; Holterman et al. 2006, 2009; Crainey et al. 2009; Bucklin et al. 2011). Four sequences of the 18S rRNA gene have been amplified from a few species of dicyemids (Katayama et al. 1995; Pawlowski et al. 1996; Aruga et al. 2007). Therefore, amplifying additional sequences of this marker from more species is a logical starting point for deciphering inter- and intraspecific molecular variation in dicyemids.

So far, species of dicyemids have been established based only on comparative analysis of morphological traits (Furuya 2006). Molecular phylogenetic data have helped determine the position of the Dicyemida within the tree of eukaryotes, but these data have never been used to validate generic and species boundaries within the Dicyemida (Ohama et al. 1984; Katayama et al. 1995; Pawlowski et al. 1996; Kobayaski et al. 1999; Suzuki et al. 2010). Currently, a single cephalopod host can contain multiple

cosmopolitan genera based on comparative morphology. If true, then dicyemid genera would have had to remain stable throughout hundreds of thousands of years of cephalopod diversification. It is possible, however, that dicyemids are host-specific, but the morphological bases for current generic and species identification are misleading. If so, then one cephalopod species should be host to genetically similar species and more closely related cephalopods should host more closely related parasites. Molecular phylogenetic data can address this possibility and are expected to shed considerable light on whether current genera and species reflect phylogenetic relationships and whether they have coevolved with their hosts.

Material and methods

Specimen collection

Host cephalopods were collected in collaboration with fishermen of BC Spot Prawns. One *Enteroctopus dofleini*, three *Octopus rubescens*, and one *Rossia pacifica* were caught in prawn traps between 27 May and 4 June 2012 in the Jervis and Sechelt Inlets off the Sunshine Coast in British Columbia, Canada. Hosts were identified to species level using both morphological traits and CO1 sequences.

The kidneys were extracted from each sample and placed in 'Dicyemid Isolation Buffer' (DIB) (Lapan & Morowitz, 1975). Dicyemids were isolated under light microscopy via micropipette and placed in autoclaved seawater. From each host, 16 dicyemids representing 2–4 different morphospecies were photographed with differential interference contrast (DIC) using a Zeiss Axiovert 200 light microscope connected to a Pixelink-A662 digital camera and then deposited into a 0.2 ml PCR tube with 10 µl of autoclaved water. Micrographs were used to identify individual dicyemids to one of the currently recognized morphospecies found in the host species.

Dicyemid DNA extraction, PCR, cloning, and sequencing

DNA was extracted from the dicyemid isolates using the Biotechnologies Epicentre MasterPure™ Complete DNA & RNA Purification Kit and stored in 35 µl of TE buffer. 18S rDNA sequences were PCR amplified using Illustra™ PuReTaq™ Ready-To-Go™ PCR beads, 23 µl autoclaved distilled water, 1 µl of extracted template DNA and 0.5 µl of each of the following primers: F3 (5′-CGG CTCATTAAATCGGACATAC-3′) and R2 (5′-CC AACAACCTCACCAAATCATTC-3′) compiled from dicyemid 18S sequences on GenBank (Benson et al. 2005). The PCR protocol involved an initial denaturation period (94°C for 2 min), 40 cycles of denaturing (94°C for 45 s), annealing (50°C for 45 s), and elongation (72°C for 2 min), and a final elongation period (72°C for 5 min).

These PCR products were then diluted to 1 in 10 parts water and used as the template for two different semi-nested PCR amplifications. The first reaction used primers F3 and R3 (5′-CACTG TGTTCGGCCCGGGTGAG-3′); the second reaction used primers F2 (5′-GTGGATTAGATCTCG TCGTAG-3′) and R2 compiled from the same GenBank sequences as above. The PCR programme for these reactions was the same as described above except the 40 cycles were reduced to 25 cycles. Purified PCR products were sequenced using ABI Big-Dye™ reaction mixed with the amplification primers. The new DNA sequences from the dicyemid samples were identified using BLAST and confirmed with molecular phylogenetic analyses. The 34 new sequences were deposited into GenBank (accession numbers KJ786919–KJ786928).

DNA was extracted from *Octopus rubescens* and *Enteroctopus dofleini* kidneys using Quiagen DNeasy® Blood & Tissue Kit. CO1 sequences were amplified with the forward primer 1490 (5′-GGTCAACA AATCATAAAGATATTGG-3′) and reverse primer 2198 (5′-TAAACTTGAGCCTGACGAAAAAAAT C-3′) (Folmer et al. 1994). PCR samples were prepared with 12.5 µl EconoTaq® DNA Polymerase, 10.5 µl autoclaved distilled water, 0.5 µl forward primer, 0.5 µl reverse primer and 1 µl of extracted template DNA. The PCR samples were held at an initial denaturation period (94°C for 5 min), then 40 cycles of denaturation (92°C for 1 min), annealing (40°C for 1 min), elongation (72°C for 1 min) and a final elongation period (72°C for 5 min). PCR products were then cloned using the Agilent Technologies StrataClone PCR Cloning Kit. Purified DNA was sequenced in both directions from eight clones per individual host using ABI Big-Dye™ reaction mix and the cloning primers. The new DNA sequences from the host samples were identified using BLAST (accession numbers KJ786929 and KJ786930).

Sequence alignments and molecular phylogenetic analyses

Thirty-four new DNA sequences from dicyemids were analysed and edited using Sequencher® before being aligned with the web-based MUSCLE: multiple sequence alignment with high accuracy and throughput (Edgar 2004). In addition, three available 18S rDNA sequences from dicyemids were pulled from GenBank: *Dicyema acuticephalum* Nouvel, 1947, a parasite of *Octopus vulgaris* Cuvier, 1797 collected from Japan; *Dicyema orientale* Nouvel & Nakao, 1938, a parasite of *Sepioteuthis lessoniana*

Lesson, 1830 collected from Japan; and *Dicyema* sp. collected from a *Sepia officinalis* Linnaeus, 1758 in the Mediterranean Sea (Katayama et al. 1995; Pawlowski et al. 1996). These three sequences plus the new ones generated in this study formed a 37-taxon alignment that was edited by eye using MacClade (Maddison & Maddison 2005). The ends of the alignment were trimmed to equalize different lengths of recovered sequences. Two indel sections (one three-character section at position 507 and one four-character section at position 518) were excluded from the alignment, resulting in 1245 unambiguously aligned sites. The NEXUS file was submitted to RAxML to infer a Maximum Likelihood (ML) tree and 100 bootstrap replicates (invariable sites = 0.8) (Stamatakis 2006). A genetic distance matrix was constructed using PAUP version 4 (Swofford 2002).

Results

Dicyemid morphospecies

Dicyemids representing all eight morphospecies previously recognized from *Octopus rubescens*, *Enteroctopus dofleini*, and *Rossia pacifica* were found. Two *Dicyema apollyoni*, two *Dicyemennea adminicula*, four *Dicyemennea brevicephala*, and six *Dicyemennea adscita* sequences were collected from three Pacific Red Octopus (*O. rubescens*) individuals. Six *Dicyemodeca deca* and five *Dicyemennea abreida* sequences came from one Giant Pacific Octopus (*E. dolfleini*), while five *Dicyemennea brevicephaloides* and four *Dicyemennea rossiae* sequences were collected from one Stubby Squid (*R. pacifica*) (Table I).

Molecular phylogeny analyses

The 14 dicyemids collected from three different individuals of *Octopus rubescens* had identical 18S rDNA sequences; these dicyemids represented the morphotypes of *Dicyemennea adscita*, *Dicyemennea adminicula*, *Dicyemennea brevicephala*, and *Dicyema apollyoni*. The 18S rDNA sequences derived from 11 dicyemids collected from *Eneteroctopus dofleini*, which represented the morphotypes of *Dicyemodeca deca* and *Dicyemennea abreida*, were identical to each other and 0.32% different from the dicyemid sequences collected from *O. rubscens*. The nine dicyemids collected from *Rossia pacifica*, which represented the morphotypes of *Dicyemennea brevicephaloides* and *Dicyemennea rossiae*, were identical and 4.4% different from the dicyemid sequences collected from *E. dofleini* and *O. rubescens* (Figure 3).

The molecular phylogenetic analyses of 18S rDNA sequences demonstrated a polyphyletic distribution of isolates representing the current genera of dicyemids. Representatives of *Dicyema*, for instance, were nested within three different clades (Figure 3). Of the three dicyemid sequences found in GenBank, *Dicyema orientale* grouped near the *Dicyemennea brevicephaloides*/*Dicyemennea rossiae* clade while *Dicyema acuticephalum* and an undescribed *Dicyema* species diverged from the same lineage as the *E. dofleini* and *O. rubescens* clades.

The two clades of dicyemid species from octopods, namely (1) *Dicyemennea adscita*, *Dicyemennea brevicephala*, *Dicyemennea adminicula*, and *Dicyema apollyoni* from *O. rubescens*, and (2) *Dicyemodeca deca* and *Dicyemennea abreida* from *E. dofleini* formed a monophyletic group to the exclusion of the clade of dicyemid species from teuthoids, with the exception of the unnamed *Dicyema* sp. from *S. officinalis* (Figure 3).

Discussion

The 18S rDNA sequence data suggest that (1) *Dicyemennea brevicephala*, *Dicyemennea adscita*, *Dicyemennea adminicula*, and *Dicyema apollyoni* are all one species; (2) *Dicyemodeca deca* and *Dicyemennea abreida* represent one species, and (3) *Dicyemennea rossiae* and *Dicyemennea brevicephaloides* represent one species. Therefore, all three dicyemid genera investigated here are polyphyletic from a molecular phylogenetic point of view, indicating that the morphological differences between the previously

Table I. Dicyemid morphospecies sequenced in this study and the host cephalopods from which they were collected.

Host species	Number collected	Dicyemid morphospecies	Number of individuals sequenced
Octopus rubescens	3	*Dicyema apollyoni*	3
		Dicyemennea brevicephala	4
		Dicyemennea adminicula	3
		Dicyemennea adscita	9
Enteroctopus dofleini	1	*Dicyemodeca deca*	6
		Dicyemennea abreida	5
Rossia pacifica	1	*Dicyemennea brevicephaloides*	5
		Dicyemennea rossiae	4

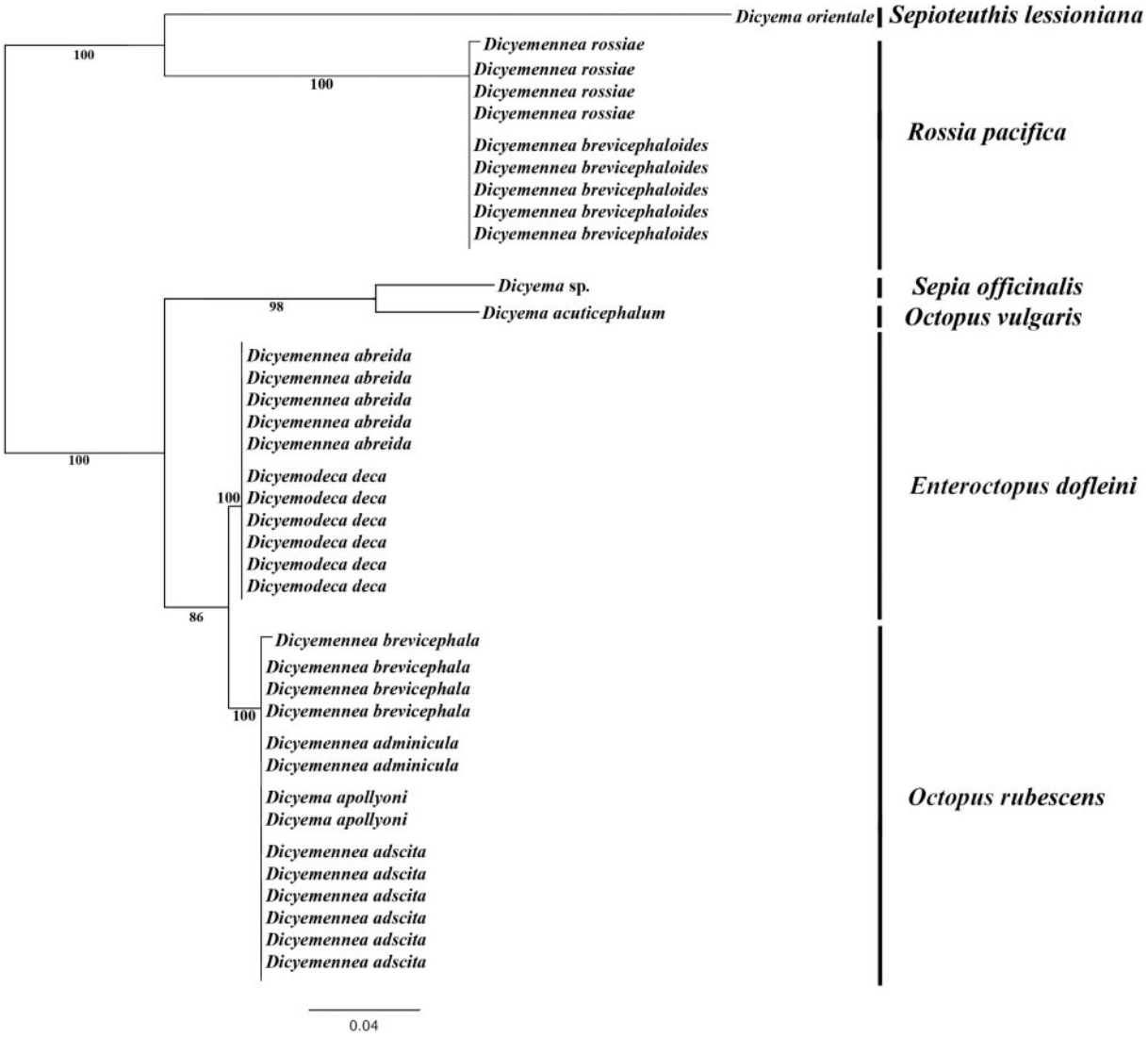

Figure 3. Maximum Likelihood tree of all dicyemid 18S rDNA sequences used in this study representing eight different morphospecies: 34 new dicyemid sequences and three dicyemid sequences from GenBank. The host species for each dicyemid species is labelled to the right of each group of dicyemid species. Dicyemids representing the morphology-based genera *Dicyema*, *Dicyemennea*, and *Dicyemodeca* are polyphyletic. Numbers above or below the branches represent bootstrap values; branch lengths represent the mean number of nucleotide substitutions per site.

recognized species reflect intraspecific variation. The 18S rDNA sequences of dicyemids therefore show higher similarity between different morphotypes within a host than the morphotypes indicative of currently recognized genera, suggesting that coevolutionary pressures between host and parasite are more significant than previously recognized. This molecular phylogenetic context is therefore critical for understanding species diversity and the convergent evolution of morphological traits in dicyemids.

Parasites tend to occupy a predictable environment in their specific hosts. As such, they can synchronize with their host so tightly that any small change made in the host selects for changes in the parasite and vice versa, so large evolutionary patterns can be mirrored between host and symbiont (Brooks 1979; Kuris et al. 1980; Poulin et al. 2011). The intraspecific morphological variation in dicyemids, especially the four different calotte shapes, might be attributable to different contours present in the host's renal folds. The 18S rDNA sequence data suggest that the four recognized calotte shapes are transient traits that change in each dicyemid species as they conform to variations in the host's renal anatomy. In other words, dicyemid individuals are probably capable of adopting any suitable calotte shape during development, a level of morphological plasticity that appears to be common in parasites (Nolte et al. 2010; Beldade et al. 2011; Rueckert et al. 2011).

DNA barcodes can overcome many of the challenges associated with species that are rich in intraspecific morphological variation and have complex life cycles. The fate of infusoriform larvae in dicyemids, for instance, remains a mystery. These larvae contain magnesium inositol hexaphosphate, a heavy metal, which potentially causes the larvae to sink into the marine sediments (Furuya & Tsuneki 2003). If the infusoriform larvae are present in sediments, then they could be more easily detected and identified using environmental DNA sequencing surveys of organismal diversity. The life cycle of dicyemids might also contain currently unknown stages with diverse morphological traits in intermediate hosts. DNA barcodes from dicyemids, like those reported here, provide powerful data to detect this possibility in non-cephalopod hosts (e.g. prey animals), potentially leading to the discovery of important components of the dicyemid life cycle. Regardless of whether dicyemids infect intermediate hosts, infusoriform larvae are capable of surviving in seawater much longer and can swim much faster than their vermiform counterparts (McConnaughey 1951). They could be travelling longer distances than expected and be living in unexpected habitats. Environmental DNA surveys could prove to be a powerful tool for extracting information on where these organisms reside outside of their cephalopod hosts.

Although rapidly evolving mitochondrial genes, such as 16S rRNA and COI, are also useful markers for species delimitation in several other lineages of organisms (Hebert et al. 2003; Ratnasingham & Hebert 2007; Mitani et al. 2009; Bucklin et al. 2011; Schoch et al. 2012), these genes are of limited use in organisms with reduced mitochondria, such as endoparasites and free-living organisms living in low-oxygen environments. The mitochondria of adult dicyemids, for instance, are highly reduced and are only present in a few somatic cells (Awata et al. 2005). Moreover, markers that evolve too rapidly can obscure species boundaries because the DNA sequences may be so variable that they are impossible to align (Schloss & Eisen 2010). However, the addition of different genetic markers, such as mitochondrial genes, will test and improve our understanding of dicyemid evolution. These data will not only help evaluate the use of 18S rDNA as a barcode for dicyemids, but also provide another way to reveal the boundaries between closely related species of dicyemids.

Variation within sequences of the 18S rDNA gene was explored in this study because this maker (1) is known to be fast-evolving in parasitic eukaryotes (e.g. nematodes, gregarine apicomplexans, diplomonads, and parabasalids), (2) is one of the few molecular markers to have been previously sequenced in a few dicyemid species, and (3) has been used successfully as a DNA barcode in other groups of parasites (Hopkins et al. 1997; Powers 2004; Holterman et al. 2006; Leander 2008; Holterman et al. 2009; Rueckert et al. 2011; Stensvold 2013; Tai et al. 2013). Including the data presented here, there are currently only nine distinct 18S rDNA sequences that reflect the diversity of dicyemid species.

Over 100 species of dicyemids have been described in about 40 species of cephalopods (Castellanos-Martinez et al. 2011). Our molecular data suggest that the total number of different dicyemid species described so far is probably much smaller and that very few of the approximately 600 species of benthic cephalopods harbour more than one species of dicyemid. Nonetheless, by using molecular tags as species identifiers, researchers will be able to identify new dicyemid species in unexplored hosts, collapse misidentified morphospecies into one phylogenetic species, elucidate the complete life cycle of these parasites, and reconstruct the co-evolutionary history of these parasites with their cephalopod hosts.

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