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Dicyema sphyrocephalum (Phylum Dicyemida: Dicyemidae) isolated from Korean common octopus *Callistoctopus minor* in Korea

Ilson Whang [1], **Beomseok Lee** [2], **Rahul Krishnan** [2], **Hiroaki Nakajima** [3], **Hidetaka Furuya** [3,*], **Sang Phil Shin** [4,*]

[1]Department of Genetic Resources Research, National Marine Biodiversity Institute of Korea (MABIK), Seocheon 33662, Korea
[2]Department of Aqualife Medicine, Chonnam National University, Yeosu 59626, Korea
[3]Department of Biology, Graduate School of Science, Osaka University, Toyonaka 560-0043, Japan
[4]Department of Marine Life Science & Marine Science Institute, Jeju National University, Jeju 63243, Korea

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***Corresponding authors:**
Hidetaka Furuya
Department of Biology, Graduate School of Science, Osaka University, 1 Machikaneyamacho, Toyonaka 560-0043, Japan.
E-mail: hfuruya@bio.sci.osaka-u.ac.jp

Sang Phil Shin
Department of Marine Life Science & Fish Vaccine Research Center, Jeju National University, 102 Jejudaehak-ro, Jeju 63243, Korea.
E-mail: ssp1201@jejunu.ac.kr

ORCID iDs
Ilson Whang
https://orcid.org/0000-0002-3431-3895
Beomseok Lee
https://orcid.org/0000-0002-0721-5472
Rahul Krishnan
https://orcid.org/0000-0002-2492-094X

ABSTRACT

Background: Dicyemids are parasites found in the renal sac of cephalopods. The first species of dicyemid was found from kidneys of the Korean common octopus *Callistoctopus minor*.
Objectives: This study aimed to identify the dicyemid and investigate the effect on renal sac of host.
Methods: In this study, we compared the morphological characteristics of isolate to dicyemids (*Dicyema sphyrocephalum*, *Dicyema clavatum*, and *Dicyema dolichocephalum*) reported from *C. minor* in Japan. We compared the 18S ribosomal RNA (rDNA) and cytochrome c oxidase subunit I (COI) sequences of isolate to the sequences of *D. shyrocephalum* and *D. clavatum*. The infected octopuses renal tissues were histologically compared with the tissues of uninfected individuals.
Results: The morphological characteristic of this isolated species corresponds to *D. sphyrocephalum*. The sequences similarities of 18S rDNA and COI gene of isolate are 99.7% and 98.1% with *D. sphyrocephalum*. We observed morphological changes in the epithelia folds of kidney at the dicyemids attached areas.
Conclusions: The present study identified the isolate as *D. sphyrocephalum* and this is the first report of dicyemid species from Republic of Korea. Further studies on the effects of dicyemids on growth and health status of cephalopods will be needed.

Keywords: *Dicyema sphyrocephalum*; Korean common octopus; *Callistoctopus minor*; kidney

INTRODUCTION

Dicyemids are parasites found in the renal sac of cephalopods. The dicyemid bodies consist of only 8 to 40 cells and they have neither body cavities nor differentiated organs [1]. The life-cycle of dicyemids consists of 2 morphologically distinct stages: the vermiform stage (nematogen or rhombogen) and the infusoriform embryo [1]. Dicyemids were regarded as an intermediate between the Protozoa and the Metazoa [2]. However, developmental and genomic studies have identified as the parasite as degenerate triploblastic, and a member of the Spiralia [3,4]. To date, no report exists on dicyemids in Korea, although 124 species

Hiroaki Nakajima
https://orcid.org/0000-0002-4865-1625
Hidetaka Furuya
https://orcid.org/0000-0002-7086-3122
Sang Phil Shin
https://orcid.org/0000-0001-9677-4526

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Shin SP, Whang I, Furuya H; Data curation: Shin SP, Whang I, Furuya H, Nakajima H; Formal analysis: Shin SP, Whang I, Furuya H, Nakajima H; Funding acquisition: Shin SP, Whang I; Investigation: Lee B, Krishnan R, Furuya H, Nakajima H; Methodology: Shin SP, Lee B, Krishnan R, Furuya H, Nakajima H; Project administration: Whang I; Resources: Whang I; Software: Shin SP; Supervision: Whang I; Validation: Whang I; Visualization: Shin SP, Whang I, Furuya H; Writing - original draft: Shin SP; Writing - review & editing: Whang I, Furuya H.

have been reported worldwide [5]. The present study reports a species of dicyemid, *Dicyema sphyrocephalum* Furuya, 1999, found in the Korean common octopus *Callistoctopus minor* (Sasaki, 1920) (syn. *Octopus minor*) and the parasitic effect on renal organs. This study is valuable for future diagnostics and identification of dicyemid species.

MATERIALS AND METHODS

Animals and morphological identification

C. minor (n = 12, body weight = 126.8 ± 5.0 g) was obtained from Shinan Mudflat located in the far Southwest part of Republic of Korea on June 6, 2018. Wet mount samples of the octopus renal tissue were examined for dicyemids under light microscope and the small pieces of renal tissues were smeared on a slide glass. Slides were promptly fixed in Bouin's fluid, later stained with Ehrlich's acid hematoxylin. After staining, the preparations were dehydrated and mounted in Entellan New (Merck) for observation of dicyemids. The kidney, gonad, branchial heart, digestive gland, and gills were procured for histological processing. Tissue portions were fixed in 10% neutral buffered formalin and embedded in paraffin prior to being sectioned. Sections of approximately 4 μm thickness were stained using the hematoxylin and eosin stain.

Molecular identification

The vermiform stages (rhombogen) were isolated from 2 of octopus samples for molecular phylogenetic analyses. DNA was extracted from the parasite using AccuPrep Genomic DNA Extraction Kit (BIONEER, Korea) following the manufacturer's instructions. Portions of 18S ribosomal RNA (rDNA) were amplified by PCR using eukaryote universal primers (18e: 5′-CTGGTTGATCCTGCCAGT-3′ and ERIB10: 5′-CTTCCGCAGGTTCACCTA-3′) [6,7]. In addition, 3 primer sets (cytochrome c oxidase subunit I [COI]_9F: 5′-ATYATYGCWGTYCCBACWGG-3′, COI_6R: 5′-CCRAAGAAYCARAANARRTG-3′, COI_1-1-4F: 5′-TTRYTRATTCCKTATCATTG-3′, COI_1-1-4R: 5′-ACAAGRATVCCRTAHACRAGWGA-3′, COI_1-1-5F: 5′-TTYCCNCGDWTDAATGC-3′, and COI_1-1-5R: 5′-CCWGADGTWCCNCCRAT-3′) were designed newly to amplify COI of the dicyemid. PCR products were treated with AccuPrep Genomic PCR Purification Kit (BIONEER) to remove excess primers and dNTPs, and directly sequenced with BigDyeTM Terminator v3.1 in an ABI 3730xl Sequencer. The 18S rDNA sequence showed a high degree of similarity in the GenBank database using the Basic Local Alignment Search Tool search engine. In addition, the sequence was compared with *Dicyema clavatum* and *D. sphyrocephalum* which had been isolated from *C. minor* in Japan. Multiple alignments of 18S rDNA sequence were made by Clustal X 2.0 [8] with the homologous 23 sequences of dicyemids and 1 sequence of *Rhopalura ophiocomae* (Orthonectida; outgroup) (**Table 1**). Ambiguously aligned regions in 18S rDNA datasets were removed using Gblocks v0.91b [9] under default parameters, which resulted in half the taxa having gaps. For Bayesian inference analysis, nucleotide substitution models were selected using the Akaike information criterion and the Bayesian information criterion implemented in jModeltest 2.1.7 [10,11], GTR + I + G and TIM2 + G were chosen as the best-fit nucleotide substitution models for the 18S rDNA data sets, respectively. The metropolis-coupled Markov chain Monte Carlo algorithm implemented in MrBayes 3.2.4 [12] was performed for a sufficient number of generations until the average standard deviation of the split frequencies was < 0.05. The sampling frequency was set at every 100 generations for 1,000,000 generations. The first 100,000 generations from each run were discarded as burn-in, and the remaining were analyzed using the “Sumt” command in MrBayes software. Gaps were treated as missing data. A consensus tree was created using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 1. Phylogenetic trait and 18S ribosomal RNA accession number of dicyemids used for phylogenetic analysis in this study

Parasite	Accession No.	Locality	Host		
			Order	Family	Genus species
Dicyema sphyrocephalum	MK271740	Republic of Korea	Octopoda	Octopodidae	*Octopus minor*
Dicyema sphyrocephalum	LC571906	Japan	Octopoda	Octopodidae	*Octopus minor*
Dicyema clavatum	LC571905	Japan	Octopoda	Octopodidae	*Octopus minor*
Dicyemidae sp. DS-2016	LT669909	France	Sepiida	Sepiidae	*Sepia orbignyana*
Dicyema sp. E2 DS-2016	LT669863	Tunisia	Octopoda	Eledonidae	*Eledone moschata*
Pseudicyema truncatum	LT669919	Tunisia	Sepiida	Sepiidae	*Sepia officinalis*
Dicyemidae sp. DS-2016-S03S04CN2011	LT669841	France	Sepiida	Sepiidae	*Sepia officinalis*
Dicyema acuticephalum	D26530	Japan	Octopoda	Octopodidae	*Octopus vulgaris*
Dicyemidae sp. DS-2016-S15DS18CN2012	LT669856	France	Sepiida	Sepiidae	*Sepia officinalis*
Dicyemidae sp. DS-2016-S53CN2013	LT669895	France	Sepiida	Sepiidae	*Sepia orbignyana*
Dicyema sp. O16 DS-2016	LT669916	Tunisia	Octopoda	Octopodidae	*Octopus vulgaris*
Dicyema sp. O11H2 DS-2016	LT669879	Tunisia	Octopoda	Octopodidae	*Octopus vulgaris*
Dicyemennea sp. L8 DS-2016	LT669882	Tunisia	Octopoda	Eledonidae	*Eledone cirrhosa*
Dicyemidae sp. DS-2016-S30H1TN2013	LT669864	Tunisia	Sepiida	Sepiidae	*Sepia officinalis*
Dicyemodeca deca	KJ786922	Canada	Octopoda	Enteroctopodidae	*Enteroctopus dofleini*
Dicyemennea brevicephaloides	KJ786924	Canada	Octopoda	Octopodidae	*Octopus rubescens*
Dicyemennea eledones	LT669890	France	Octopoda	Eledonidae	*Eledone cirrhosa*
Dicyemidae sp. DS-2016-S23CN2012	LT669845	France	Sepiida	Sepiidae	*Sepia officinalis*
Dicyemennea rossiae	KJ786921	Canada	Sepiida	Sepiolidae	*Rossia pacifica*
Dicyema orientale	D26529	Japan	Myopsida	Loliginidae	*Sepioteuthis lessoniana*
Dicyemidae sp. DS-2016-S30H2TN2013	LT669876	Tunisia	Sepiida	Sepiidae	*Sepia officinalis*
Dicyema sp. O12 DS-2016	LT669877	Tunisia	Octopoda	Octopodidae	*Octopus vulgaris*
Dicyema sp. O11H1 DS-2016	LT669878	Tunisia	Octopoda	Octopodidae	*Octopus vulgaris*
Rhopalura ophiocomae	U58369	USA	Ophiurida	Amphiuridae	*Amphipholis squamata*

RESULTS

In this study, we found only one dicyemid species from *C. minor* in Shinan, the southwest part of Republic of Korea. In the wet mount preparation, the dicyemid was observed in 5 out of 12 octopuses; it was found in the folds and surfaces of the renal epithelia. Other tissues such as the gonad, branchial heart, digestive gland, and gills were normal.

The dicyemid has the following characteristics. Bodies of adult stages, nematogens and rhombogens, are hammer-like (**Fig. 1A**), 500–600 μm long; 50–70 μm wide. The peripheral cell number is 22 (4 propolars, 4 metapolars, 2 parapolars, 10 diapolars, and 2 uropolars). This species is regarded as genus *Dicyema* by the cell number in the calotte (4 propolar cells + 4 metapolar cells) and the orientation of cells (opposite) (**Fig. 1C**). The calotte is disc-shaped in large individuals (**Fig. 1B and C**). Cilia on calotte is about 5 μm long, oriented forward. Uropolar cells form verruciform and cilia on these cells longer than on other trunk cells. The axial cell is cylindrical and rounded anteriorly, extending forward to base of propolar cells. About 20 vermiform embryos typically are present in axial cell of large individuals. Accessory nuclei few, sometimes are present in uropolar cells. Rhombogens have one, rarely 2 infusorigens in axial cell. About 20 infusoriform embryos typically are found in axial cells of large individuals of rhombogens.

Full-grown vermiform embryos are 50–70 μm long, 12–15 μm wide (**Fig. 1D**). The peripheral cell number is 22. The anterior end of calotte is rounded. Trunk cells are arranged in opposed pairs. Axial cell is rounded anteriorly, extending forward to base of propolar cells. Axial cell nucleus usually is in the center or occasionally in anterior half of axial cell. Axial cells of full-grown embryos consistently contain 1–2 agametes. Infusorigens are medium-sized (**Fig. 1E**). Axial cells of infusorigens usually are rounded, 13–18 μm in diameter. In mature infusorigens

the number of external cells (oogonia and primary oocytes) are 5–12, number of internal cells (spermatogonia, primary spermatocytes, and secondary spermatocytes) 2–5, and number of sperms 4–10. Fertilized eggs are 12.1 μm in diameter, that of sperm 2.0 μm in diameter (**Fig. 1E**).

Infusoriform embryos are ovoid, bluntly rounded to pointed posteriorly (**Fig. 1F and G**). In full-grown embryos, length (excluding cilia) 18–23 μm. Cilia at posterior end are 7 μm long. Refringent bodies present, solid, relatively small, about same size as single urn cell, occupying about 30% of anterior part of embryo length in the lateral view (**Fig. 1F**). Cilia project from ventral internal cells into urn cavity. Capsule cells contain many large granules.

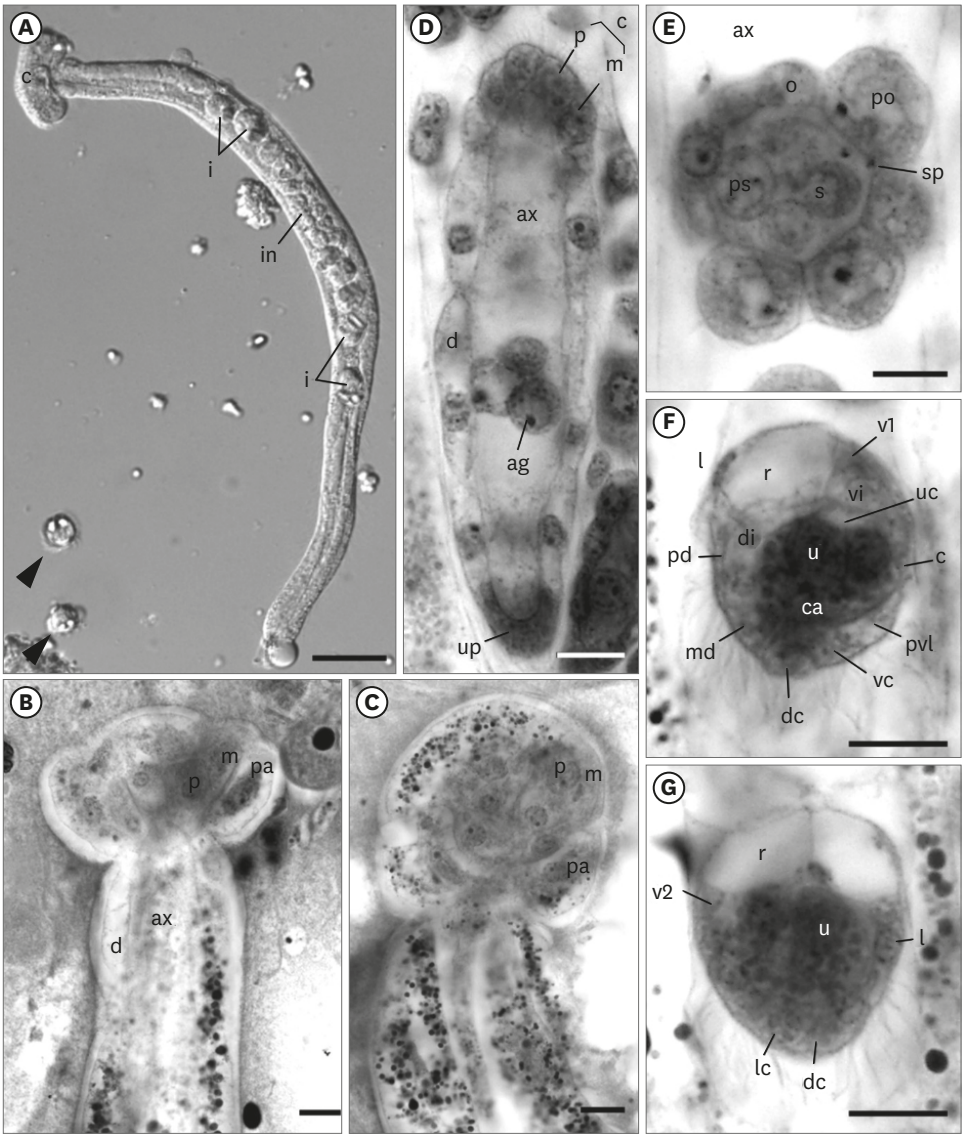

Fig. 1. Dicyemid species in the kidney of *Callistoctopus minor*. (A) rhombogen containing a hermaphroditic gonad, infusorigen, developing infusoriform embryos, and infusoriform larvae escaped from the rhombogen (arrows). (B) Anterior region of nematogen. (C) Anterior region of rhombogen. (D) Vermiform embryo. (E) Infusorigen. (F) Sagittal section of infusoriform embryo. (G) Horizontal section of infusoriform embryo. Scale bar = 50 μm (A), 20 μm (B, C), 10 μm (D, G). ag, agamete; ax, axial cell; c, calotte; ca, capsule cell; d, diapolar cell; dc, dorsal caudal cell; di, dorsal internal cell; i, infusoriform embryo; in, infusorigen; l, lateral cell; lc, lateral caudal cell; m, metapolar cell; md, median dorsal cell; o, oogonium; p, propolar cell; pa, parapolar cell; pd, paired dorsal cell; po, primary oocyte; ps, primary spermatocyte; pvl, posterolateral cell; r, refringent body; s, secondary spermatocyte; sp, sperm; u, urn cell; uc, urn cavity; up, uropolar cell; vc, ventral caudal cell; vi, ventral internal cell; v1, first internal cell; v2; second ventral cell.

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Full-grown infusoriform embryos consist of 37 cells: 33 somatic and 4 germinal cells. Somatic cells comprise external cells covering large part of anterior and lateral surfaces of embryo (2 enveloping cells), external cells with cilia on external surfaces (2 paired dorsal cells, 1 median dorsal cell, 2 dorsal caudal cells, 2 lateral caudal cells, 1 ventral caudal cell, 2 lateral cells, and 2 posteroventral lateral cells), external cells with refringent bodies (2 apical cells), external cells without cilia (2 first ventral cells, 2 second ventral cells, 2 third ventral cells, and 1 couvercle cell), internal cells with cilia (2 ventral internal cells), internal cells without cilia (2 dorsal internal cells, 2 capsule cells, and 4 urn cells). Each urn cell contains 1 germinal cell plus 1 nucleus. Nuclei of second ventral cells are pycnotic. All somatic nuclei become pycnotic as infusoriform embryos mature.

We obtained 1,561 base pairs of 18S rDNA and 1,687 base pairs of COI gene of this dicyemid species and deposited with GenBank (accession number MK271740 and LC575089). The sequences similarities of 18S rDNA and COI gene are as follows; 99.7% (1,436/1,440) and 98.1% (1,655/1,687) with *D. sphyrocephalum* (LC571906 and LC571909) whereas 95.9% (1,385/1,445) and 72.2% (811/1,124) with *D. clavatum* (LC571905 and LC571907) from *C. minor* in Japan, respectively.

The phylogenetic tree divided into 6 groups and the isolate clustered with 5 species of dicyemids (**Fig. 2**). However, no specific relationship was observed in host specificity and geographic location among the species of the cluster (group I). The species isolated from Octopoda or Sepiida (*Eledone moschata*, *C. minor*, and *Sepia officinalis*), and the dicyemids have been reportedly found France, Tunisia, Japan, and Republic of Korea (**Fig. 2**, **Table 1**).

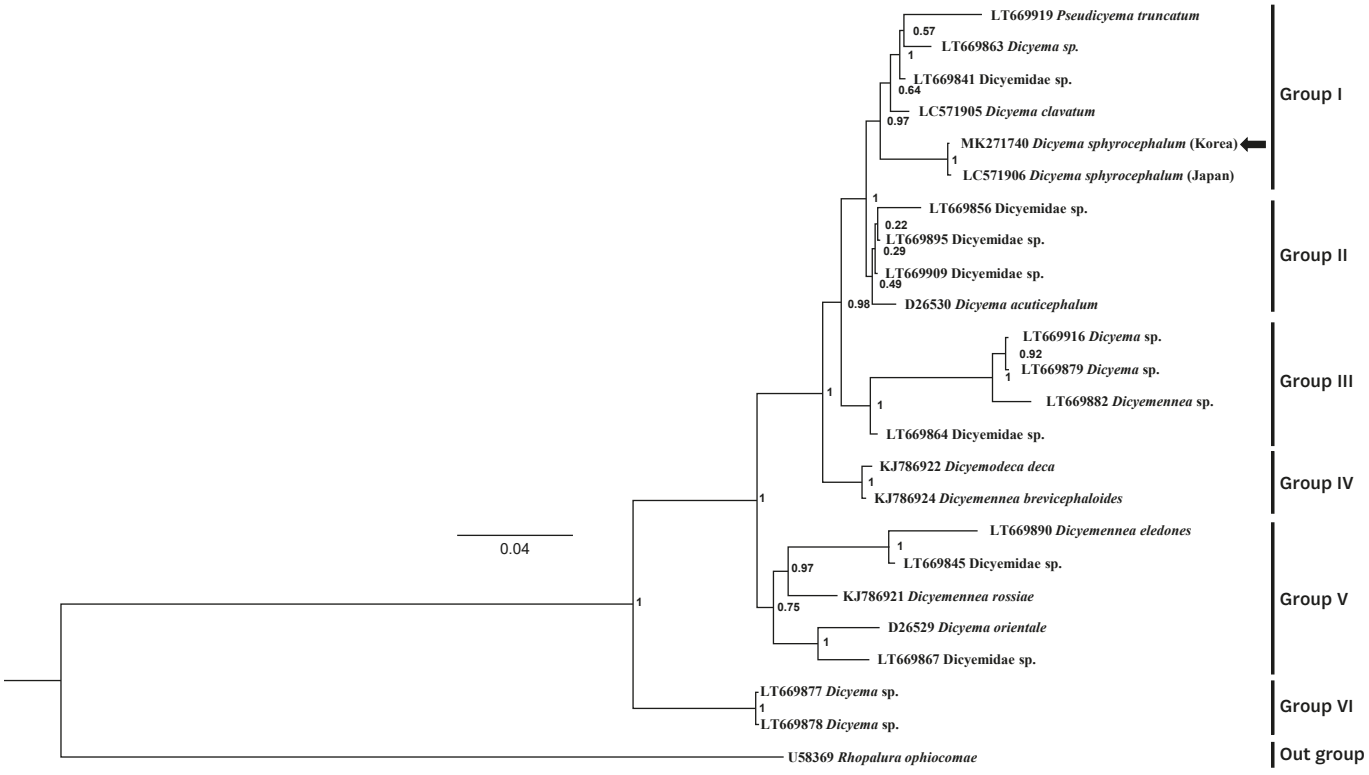

Fig. 2. Phylogenetic tree generated by Bayesian analysis of the aligned partial 18S ribosomal RNA sequences of *D. sphyrocephalum* obtained during the present study (arrow) and other dicyemid species. *R. ophiocomae* was used as the outgroup, and the posterior probabilities are shown on the branches.

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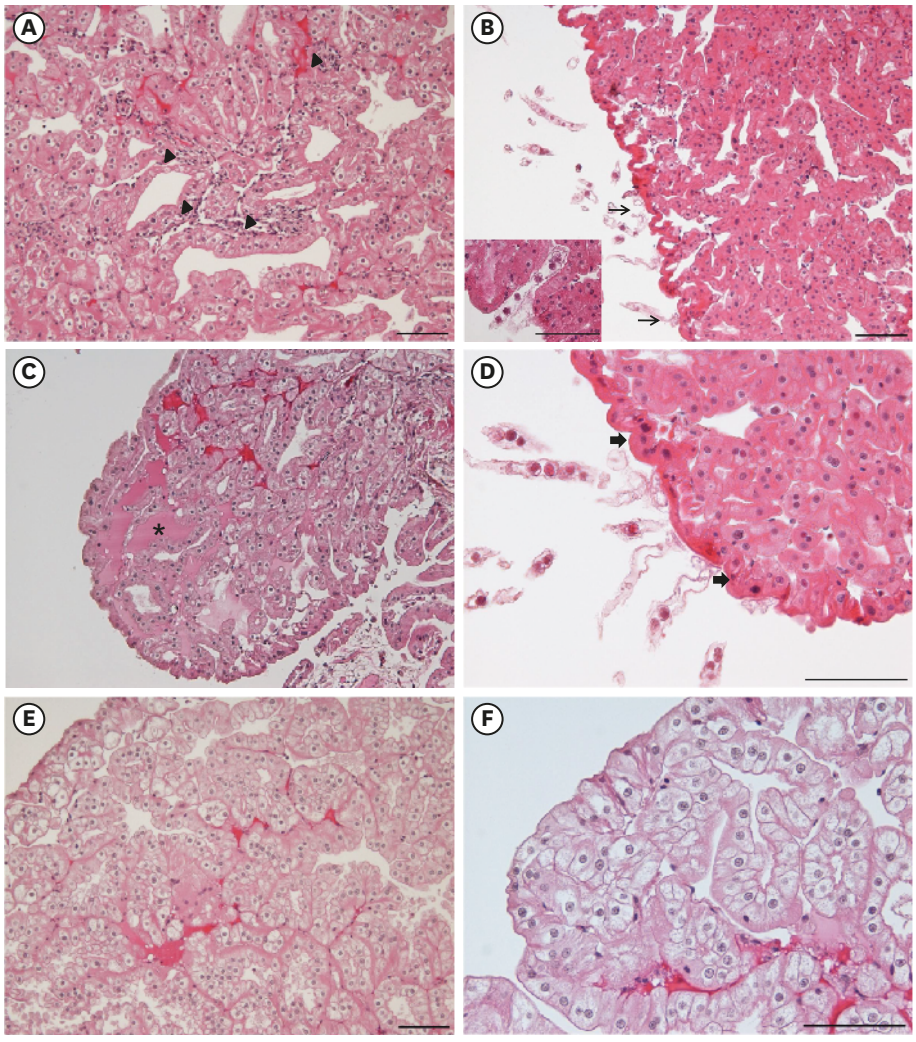

Fig. 3. Sections of renal organ of *Callistoctopus minor*. *Dicyema sphyrocephalum* infected (A-D) and uninfected (E, F) by H&E stain. (A) The lumen of vena cava branches had hemocytic infiltration (arrow heads). (B) The parasite attached (arrows) and invaded to the renal appendage (insight square). (C) The lumen of the renal sac shows fluid accumulation (asterisk). (D) There is increased epithelial cytoplasmic density and edema over epithelial folds (arrows). (E, F) There are no pathological and morphological changes in uninfected renal sacs. H&E stain. Scale bar = 50 μm.
H&E, hematoxylin and eosin stain.

Five infected octopuses tissues were histologically compared with the tissues of uninfected 7 individuals. **Fig. 3** shows the histopathology of the parasite-infected and -uninfected renal organs of the octopus. The lumen of vena cava branches showed a marked inflammation characterized by hemocytic infiltration (**Fig. 3A**). The parasite invaded the organ capsule and firmly attached to the folds/crypts of the renal appendage using its anterior calotte (**Fig. 3B**). Marked hypertrophy of renal vena cava branches with varying stages of fluid accumulation was also observed (**Fig. 3C**). Further analysis showed increased epithelial cytoplasmic density over the infected epithelial folds (**Fig. 3D**) compared to the uninfected sample (**Fig. 3E and F**).

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DISCUSSION

C. minor is widely distributed in the Korean Peninsula, upper continental shelf to littoral zone of Eastern China, and Japan. This species is known as the Korean common octopus (syn. long arm octopus), one of the important economic species of cephalopods in Asia. In the Japanese water *C. minor* harbors 3 dicyemid species, D. sphyrocephalum [13], D. dolichocephalum [13], and D. clavatum [14]. They are distinguishable one another by their distinct calotte shapes as the name suggests. *D. sphyrocephalum* has a disc-shaped calotte, *D. clavatum*, and *D. dolichocephalum* have a cap-shaped calotte and an elongated calotte, respectively. The morphological characteristic of this isolated species corresponds to *D. sphyrocephalum*. In addition, the isolated species showed the highest similarities of 18S rDNA and COI gene e with *D. sphyrocephalum* Based on the morphological and molecular comparison, we identified that the present isolate is *D. sphyrocephalum*.

Furuya et al. [14] has suggested the relationship between the species of dicyemids, and host specificity or geographical location. According to the study, the degree of host specificity differs among different species of dicyemids, and ecological specificity of the host may account for the distribution of parasite. Unfortunately, we were unable to uncover the relationship in this study. Nakajima and Furuya [15] have suggested the host specificity and host-switching in dicyemids by molecular phylogenetic analyses using 35 dicyemid species in the Japanese water. We need to obtain as many species data as possible to clear relationships between dicyemids and cephalopods. In addition, further studies that investigate the ecology of cephalopods and use other gene markers will be required.

Although histopathological analysis in this study revealed no severe cellular abnormalities such as necrosis in the kidney, morphological changes in the epithelia folds of kidney were evident at the attached areas. Further, the accumulation of bodily fluids was observed. However, this study did not reveal whether the observed change (and phenomenon) related to functional disturbance and health status of host. In addition, the previous study reported the similar phenomenon (the accumulation of bodily fluids) from octopus and cuttlefish that dicyemids not infected [16]. Thus, this study has a limit to confirm the pathogenicity of dicyemid and further studies on the effects of dicyemids on growth and health status of cephalopods will be needed.

To the best of our knowledge, this is the first report of dicyemid species from Republic of Korea. Furthermore, the present study identifies the isolate as the same species as *D. sphyrocephalum* based on morphological and molecular comparison. Morphological and phylogenetic information obtained from this study will contribute to identification and classification of dicyemids in future work. In addition, the histopathological information will be utilized to reveal the pathogenicity of the parasite in the host.

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