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PHYLOGENETIC ANALYSIS OF DICYEMID MESOZOANS (PHYLUM DICYEMIDA) FROM INNEXIN AMINO ACID SEQUENCES: DICYEMIDS ARE NOT RELATED TO PLATYHELMINTHES

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ABSTRACT: Dicyemid mesozoans are endoparasites, or endosymbionts, found only in the renal sac of benthic cephalopod molluscs. The body organization of dicyemids is very simple, consisting of usually 10 to 40 cells, with neither body cavities nor differentiated organs. Dicyemids were considered as primitive animals, and the out-group of all metazoans, or as occupying a basal position of lophotrochozoans close to flatworms. We cloned cDNAs encoding for the gap junction component proteins, innexin, from the dicyemids. Its expression pattern was observed by whole-mount in situ hybridization. In adult individuals, the innexin was expressed in calottes, infusorigens, and infusoriform embryos. The unique temporal pattern was observed in the developing infusoriform embryos. Innexin amino acid sequences had taxon-specific indels which enabled identification of the 3 major protostome lineages, i.e., 2 ecdysozoans (arthropods and nematodes) and the lophotrochozoans. The dicyemids show typical, lophotrochozoan-type indels. In addition, the Bayesian and maximum likelihood trees based on the innexin amino acid sequences suggested dicyemids to be more closely related to the higher lophotrochozoans than to the flatworms. Flatworms were the sister group, or consistently basal, to the other lophotrochozoan clade that included dicyemids, annelids, molluscs, and brachiopods.

Dicyemid mesozoans (phylum Dicyemida) are endoparasites, or endosymbionts, found only in the renal sac of benthic cephalopod molluscs. The body organization of dicyemids is very simple, consisting of usually 10 to 40 cells, with neither body cavities nor differentiated organs. The vermiform body is organized in a 2-layer structure consisting of a single, central, cylindrical axial cell surrounded by a sheet of ciliated peripheral cells. They have the distinct anterior region termed a ''calotte'' and insert into folds or crypts of the renal appendage of the host (Ridley, 1968; Furuya et al., 1997). The calotte is distinct from the other regions in having stiffer, shorter, and thicker cilia (Nouvel, 1947; McConnaughey, 1951; Hochberg, 1990; Furuya et al., 2007). Infusoriform embryos are more differentiated than vermiform stages (Ridley, 1968; Matsubara and Dudley, 1976; Furuya et al., 1997, 2004, 2007; Furuya, 1999). Internally, infusoriform embryos have 4 large cells, called urn cells, each containing a germinal cell.

The dicyemids have been considered as primitive animals and the sister taxon of all metazoans (Van Beneden, 1876; Hyman, 1940, 1956; Lapan and Morowitz, 1975), or as secondary reduced animals, referring the simple body organization to the result of specialization for parasitism (Nouvel, 1947; Stunkard, 1954, 1972; Ginetsinskaya, 1988). Molecular phylogenetic studies have suggested that the dicyemids are not primitive animals, as considered previously, but are members of the metazoans (Katayama et al., 1995; Pawlowski et al., 1996; Siddall and Whiting 1999), probably a member of the lophotrochozoans (Kobayashi et al., 1999). Katayama et al. (1995) suggested platyhelminth affinities, as did Stunkard (1954), Nouvel (1947), and Ginetsinskaya (1988), who proposed the same relationship based on the morphology and life cycles. Telford et al. (2000) analyzed the mitochondrial genetic code of concatenated Cox I, II, and III sequences from the dicyemids and showed that the dicyemids have the canonical invertebrate mitochondrial genetic code, suggesting that the dicyemids are at least triploblastic. Zrzavý (2001) reviewed the interrelationships of metazoan parasites, based on recent morphological and molecular phylogenetic analyses, and suggested that the Mesozoa are closely related to the Acoelomorpha. These recent studies have revealed that dicyemids might not be truly primitive animals deserving the name of mesozoans, whereas some authors still consider dicyemids to be a primitive animal because dicyemids have several protozoan-like features, i.e., the double-stranded ciliary necklace common to opalinids and ciliates, tubular mitochondrial cristae, the endocytotic uptake of particulate materials from the outer surface of peripheral cells, and the absence of a common extracellular matrix (Cavalier-Smith, 1993; Czaker, 2000; Noto et al., 2003; Noto and Endoh, 2004; Awata et al., 2005, 2006; Czaker, 2006). More recently, a genomic cDNA sequence analysis on the *Pax6* and *Zic* intron positions supported the assertion that dicyemids are reduced bilaterians (Aruga et al., 2007). Thus, the phylogenetic position of dicyemids is still uncertain, and they remain one of the most enigmatic groups of lower invertebrates.

The most significant synapomorphy of multicellular animals is their multicellularity, which is characterized by cell interactions that are mediated by intercellular junctions and a variety of adhesion molecules. As a consequence, cells are connected and can communicate with one another. By contrast, such features are never seen in colonial organisms such as colonial choanoflagellates. Cell adhesion was obviously crucial, not only for evolution of the multicellular state, but also for construction of tissues and organs. One approach to understanding the origin of multicellularity is to study the intercellular adhesion systems of living primitive organisms. Using a freeze-fracture method, Revel (1988) observed the gap junction in dicyemids. Subsequently, Furuya et al. (1997) described some intercellular junctions, and their distribution, in the body of dicyemids. These suggest dicyemids may be the most primitive multicellular animals to have several basic cell junctions such as adherens junctions, septate junctions, and innexin–pannexin gap junctions.

In addition to cell-to-cell attachment, the basic requirement for the multicellular level of organization is cell-to-cell communication between 2 interacting cells. Gap junctions play a role in such communication. Gap junctions are seen in all eumetazoans, with the exception of anthozoans and scyphozoans (Mackie et al., 1984; Shestopalov and Panchin, 2008). Primitive multicellular

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animals with monociliary cells (sponges and placozoans) do not have gap junctions. In sponges, however, Loewenstein (1967) and Green and Bergquist (1979) suggested the presence of intercellular communicating channels. Grell and Ruthmann (1991) demonstrated several patterns of organized behavior in placozoans, which might suggest the presence of some communication system. In this context, innexin whose proteins form the gap junction may be regarded as "toolkit genes" like the genes encoding *Hox*, *parahox*, and other developmentally critical transcription factors.

We conducted an analysis of cDNA sequences, by random sequencing, to examine various aspects of dicyemid biology. We cloned cDNAs encoding for innexin from the Dicyemidae, i.e., *Dicyema japonicum* Furuya and Tsuneki, 1992 and *Dicyema koshidai* Furuya and Tsuneki, 2005. In amino acid sequences of innexin, we found some distinct indels which identified 3 major protostome lineages, 2 groups of ecdysozoans (arthropods and nematodes) and the lophotrochozoans. These indels indicated clear differences between Platyhelminthes and lophotrochozoans, suggesting the possible clade "Platyzoa." Innexin might be a useful toolkit gene for phylogenetic studies. Using these advantages of innexin, we determined whether or not the dicyemids are related to Platyhelminthes. The dicyemids were apparently more closely related to the higher lophotrochozoans, such as molluscs and annelids, than to the Platyhelminthes.

MATERIALS AND METHODS

Animals

Most known innexin genes are from the model animals. We sampled several animals to classify relationships among dicyemids, parasitic flatworms, and other lophotrochozoans.

Two dicyemids, *D. japonicum* and *D. koshidai*, were obtained from renal sacs of the Octopodidae, *Octopus vulgaris* Cuvier, 1797 and the Loliginidae, *Sepioteuthis lessoniana* Lesson, 1830, respectively. *Octopus vulgaris* were obtained from a fisherman who collected them in Osaka Bay (Akashi, Hyogo, Japan). *Sepioteuthis lessoniana* were obtained from a fisherman who collected them in Wakasa Bay (Obama, Fukui, Japan). The Lingulidae, *Lingula anatina* Lamarck, 1801 were obtained from a fisherman who collected them in the Ariake Sea (Yanagawa, Fukuoka, Japan). The Oligobrachiidae, *Oligobrachia mashikoi* Imajima, 1973 were obtained in Tsukumo Bay (Marine Biological Laboratory of Kanazawa University at Noto, Ishikawa, Japan). The Urechidae, *Urechis unicinctus* von Drasche, 1881 was obtained from a fisherman who caught it off the coastline of South Korea. The Heterophyidae, *Metagonimus yokogawai* Katsurada, 1912 was obtained from the pectoral and caudal fins of the Osmeridae, *Plecoglossus altivelis*, Temminck and Schlegel, 1846 (Chikusa River, Okayama, Japan).

Cloning of cDNAs and RACE PCR

We used the dicyemid species, *D. japonicum* and *D. koshidai*, living in the renal sac of *O. vulgaris* and *S. lessoniana*, respectively, for obtaining RNA (see Furuya et al., 1992a; Furuya and Tsuneki, 2005). The life cycle and the morphology are shown in Figure 1. Dicyemids were isolated from the kidney of the host using a pipette. Host cells in the isolated dicyemids suspension were carefully removed with a pipette using a stereoscopic microscope. Collected dicyemids were washed several times with artificial seawater.

Total RNA was extracted using TRIZOL reagent (Invitrogen, Carlsbad, California). Subsequently, cDNA was synthesized from 1 μg of total RNA using the BD SMART^{TM} RACE cDNA amplification kit (BD Biosciences, Franklin Lakes, New Jersey). In this reaction, we used BD SMART II^{TM} (BD Biosciences) A oligonucleotide and oligo dT adapter primer (5′-AACTGGAAGAATTCGCGGCT$_{18}$VN-3′) instead of packed oligo dT primer. From this reaction, we obtained cDNAs with the adapter sequence at both the 5′ and 3′ ends. These adapter sequences were used as the priming site for cDNA amplification with PCR.

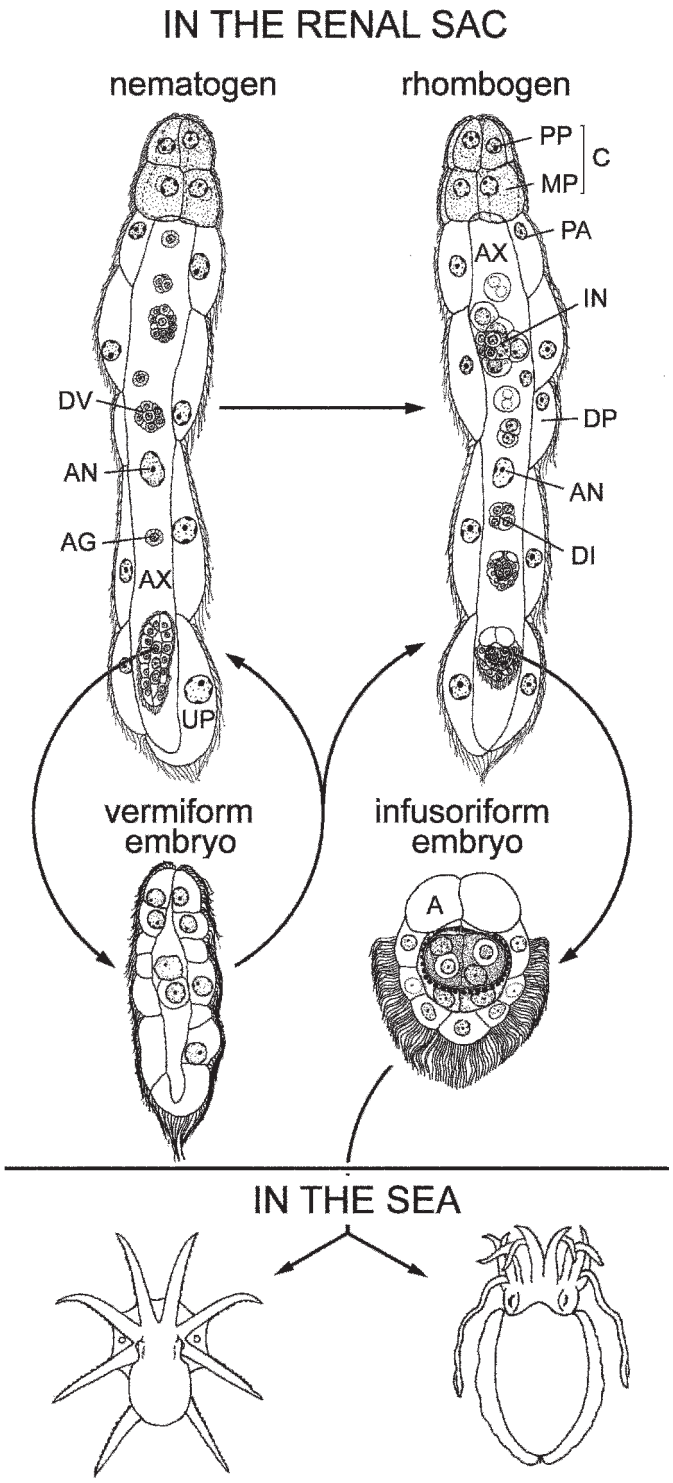

FIGURE 1. Life cycle of the dicyemids (modified from Furuya et al., 2003). The development of infusorigens, gametegenesis around the infusorigen, and development of 2 types of embryos all proceed within the axial cell cytoplasm. Abbreviations: apical cell (A), agamete (AG), axial cell nucleus (AN), axial cell (AX), calotte (C), developing infusoriform embryo (DI), diapolar cell (DP), developing vermiform embryo (DV), infusorigen (IN), metapolar cell (MP), parapolar cell (PA), propolar cell (PP), uropolar cell (UP).

TABLE I. Combination of primers used for obtaining sequences from genome.

Organisms	Primer
Octopus vulgaris	inx-A, inx-D
Sepioteuthis lessoniana	inx-C, inx-F
Metagonimus yokogawai	inx-B, inx-E
Urechis unicinctus	inx-A, inx-F
Oligobrachia mashikoi	inx-C, inx-F
Lingula anatina	inx-C, inx-E

It was difficult to obtain the dicyemid first-strand cDNA in enough quantity to construct the cDNA library, so we amplified the first-strand cDNA by PCR using BD Advantage 2 polymerase mix (BD Biosciences, San Jose, California). The PCR product was ligated into a pGEM-T vector (Promega, Madison, Wisconsin), which was introduced to a JM109-competent cell by the heat shock method. We randomly picked up a transformed cell and extracted a plasmid containing dicyemid cDNA. A cloned cDNA was sequenced with a BigDye® terminator v. 3.1 and an ABI 3700 auto sequencer (Applied Biosystems, Foster City, California). The sequence data of cDNA were analyzed by the BlastX program on the DNA Data Bank of Japan (DDBJ) server.

To obtain a full length of dicyemid innexin sequences, we conducted 5′ and 3′ RACE PCR with Universal Primer A mix (UPM) in the BD Advantage 2 polymerase mix and gene-specific primer. In *D. japonicum*, gene-specific primers were designed based on innexin sequences cloned from cDNA library by random cloning. The 5′ RACE PCR was conducted with UPM and gene-specific primer (5′-GGTATGGATGG-CAATGATTAGG-3′). The 3′ RACE PCR was conducted with UPM and gene-specific primer (5′-CCTACGTTGGCAGTTCTGG-3′). In the *D. koshidai* innexin sequence, gene-specific primers (5′-CGTCCCCGT-GATTGTATGG-3′ and 5′-GCAGTAAGTCACGCGCGG-3′) were designed based on the innexin sequence cloned from a partial sequence of *D. koshidai* innexin. Partial sequence was obtained from genomic PCR by degenerate primers (5′-GGSGAMCCRATYCACTGCTGG-3′ and 5′-AGCCARAACCARATGAAKATRWARAT-3′).

We deduced the full length of *D. japonicum* and *D. koshidai* innexin sequences by combining the resultant sequence data of 5′ and 3′ RACE PCR.

Genome extraction

Genomes of several metazoan taxa, i.e., *O. vulgaris*, *S. lessoniana*, *D. koshidai*, *D. japonicum*, *U. unicincus*, *Ol. mashikoi*, *L. anatina*, and *M. yokogawai*, to be included in the phylogenetic analysis, were extracted by QIAGEN DNeasy Blood & Tissue kit (QIAGEN, Germanton, Maryland). Genomes of *P. altivelis*, *O. vulgaris*, *S. lessoniana*, *U. unicinctus*, and

TABLE II. Animals used for phylogenetic analysis. Bolded portions are newly determined sequences.

Phylum	Organisms	Sequence	Source	Accession numbers
Dicyemida	***Dicyema japonicum***	**innexin**	**cDNA**	**AB517609**
	Dicyema koshidai	**innexin**	**cDNA**	**AB517610**
Platyhelminthes	*Dugesia japonica*	Innexin-4*	database	BAE78813
		Innexin-5	database	BAE78814
		Innexin-7	database	BAE78815
		Innexin-8	database	BAE78816
		Innexin-9	database	BAE78817
		Innexin-10*	database	BAE78818
		Innexin-11*	database	BAE78819
		Innexin-12*	database	BAD83778
		Innexin-13	database	BAE78820
	Metagonimus yokogawai	**innexin**	**genome**	**AB517613**
Brachiopoda	***Lingula anatina***	**innexin**	**genome**	**AB517614**
Annelida	*Hirudo medicinalis*	Innexin-9	database	ABB16290
		Innexin-11	database	ABB16292
		Innexin-12	database	ABB16293
Annelids (Pogonophora)	***Oligobrachia mashikoi***	**innexin**	**genome**	**AB517615**
Annelids (Echiura)	***Urechis unicinctus***	**innexin**	**genome**	**AB517616**
Mollusca	*Aplysia californica*	Pannexin-1	database	AAV33848
		Pannexin-2	database	AAV33850
		Pannexin-3	database	AAV33851
	Clione limacina	Pannexin-1	database	AAF75839
	Octopus vulgaris	**innexin**	**genome**	**AB517611**
	Sepioteuthis lessoniana	**innexin**	**genome**	**AB517612**
Arthropoda	*Homarus gammarus*	Innexin-1	database	CAJ58681
	Schistocerca americana	Innexin-1	database	AAD29305
	Tribolium castaneum	Innexin-1	database	XP_968503
	Bombyx mori	Innexin-2	database	AAS77384
	Drosophila melanogaster	Innexin-1	database	P27716
		Innexin-2	database	Q9V427
Vertebrata†	*Mus musculus*	Pannexin-1	database	NP_062355
		Pannexin-2	database	NP_001002005
	Danio rerio	Pannexin-1	database	NP_957210
		Pannexin-2	database	XP_686064

* Used only in alignment comparison.
† Used only in phylogenetic tree.

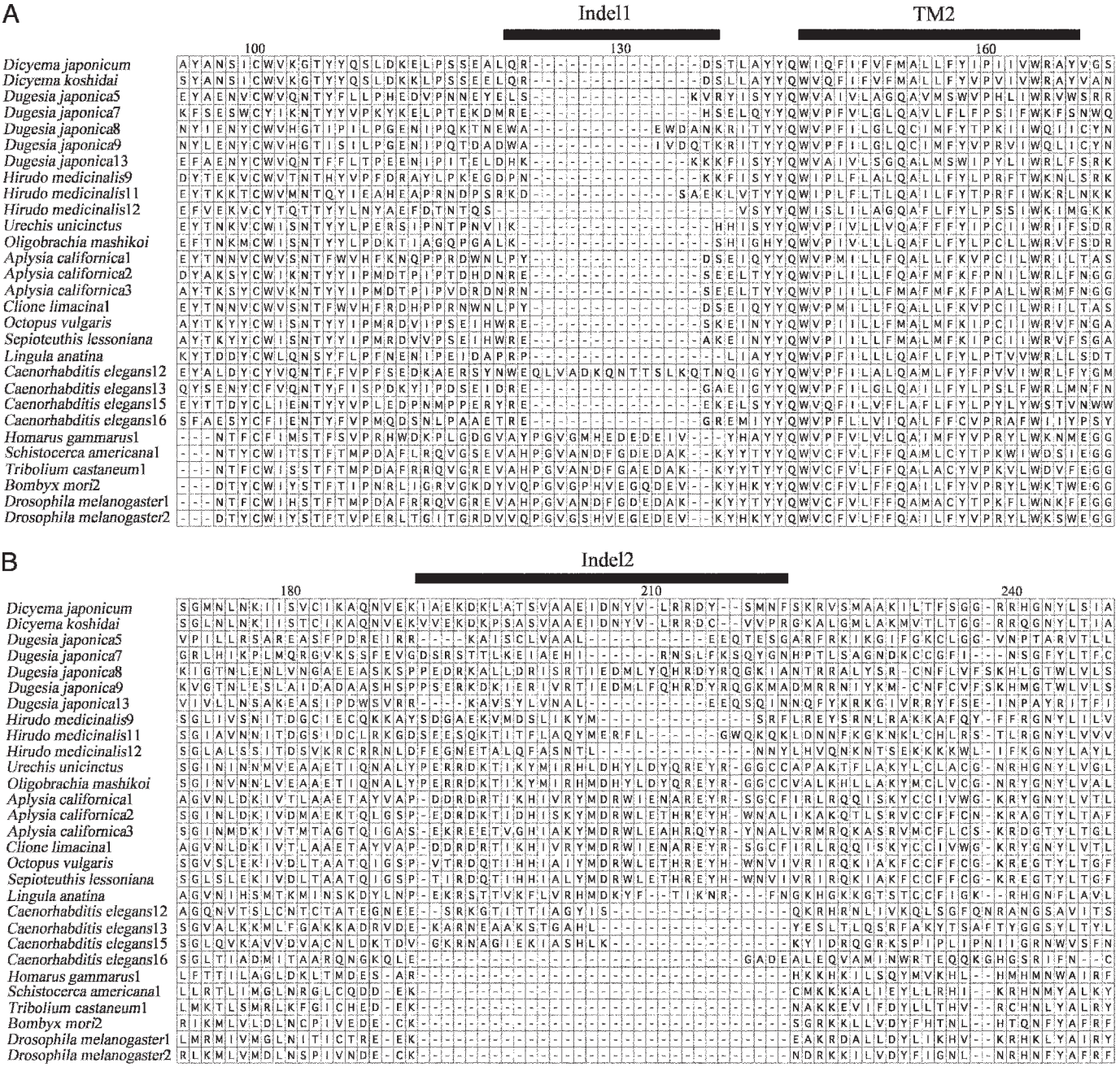

FIGURE 2. Indels in innexin amino acid sequence in 19 lophotrochozoan innexins of molluscs, annelids, brachiopods, platyhelminths, dicyemids, and 10 ecdysozoan innexin sequence of arthropods and nematodes. TM2 indicates location of the second trans-membrane domain deduced by Dykes et al. (2004). Indel 1 and indel 2 indicate locations of specific indels.

L. anatina were obtained from their muscles. Genomes of *O. mashikoi*, *M. yokogawai*, and dicyemids were obtained from the whole body after fixation in 70% ethanol.

PCR amplifications and sequencing

Six degenerate primers were used to obtain innexin sequence. Each primer for amplifications of gene sequences of innexin was designed based on conserved sequences of innexin in flatworms, annelids, and dicyemids (*D. japonicum*), as follows: inx-A: 5′-CARTACGTCGGAGACCCRATYCA-3′; inx-B: 5′-TYCCYWKCATTMTYTGGMG-3′; inx-C: 5′-GGSGAMCCRATYCACTGCTGG-3′; inx-D: 5′-ARRTTVATMGGMARRACRCAYTG-3′; inx-E: 5′-CASWWSGTGACYCKSGGGAA-3′; inx-F: 5′-AGCCARAACCARATGAAKATRWARAT-3′ (R = A + G, Y = T + C, M = C + A, W = T + A, K = G + T, S = C + G). Combinations of primers are shown in Table I. The polymerase chain reaction was performed in 20 µl reaction volumes containing 100 ng extracted genomic DNA, 2 µl 10× PCR buffer, 1.6 µl 10mM dNTPs, 1 µl 10µM primer each, and 0.1 µl 0.5 U Takara Ex Taq polymerase (TaKaRa Bio, Shiga, Japan). The cycling conditions were as follows: initial denaturation for 3 min at 94 C, followed by 35 cycles of 1 min at 94 C, 1 min at gradient 50 C to 60 C, and 1 min 15 sec at 72 C, and completed by 5 min at 72 C.

Amplified fragments were separated by agarose gel electrophoresis and extracted by Quantum Prep® Freeze 'N Squeeze DNA Spin Columns (Bio Rad, Hercules, California). Extracted fragments were ligated into a

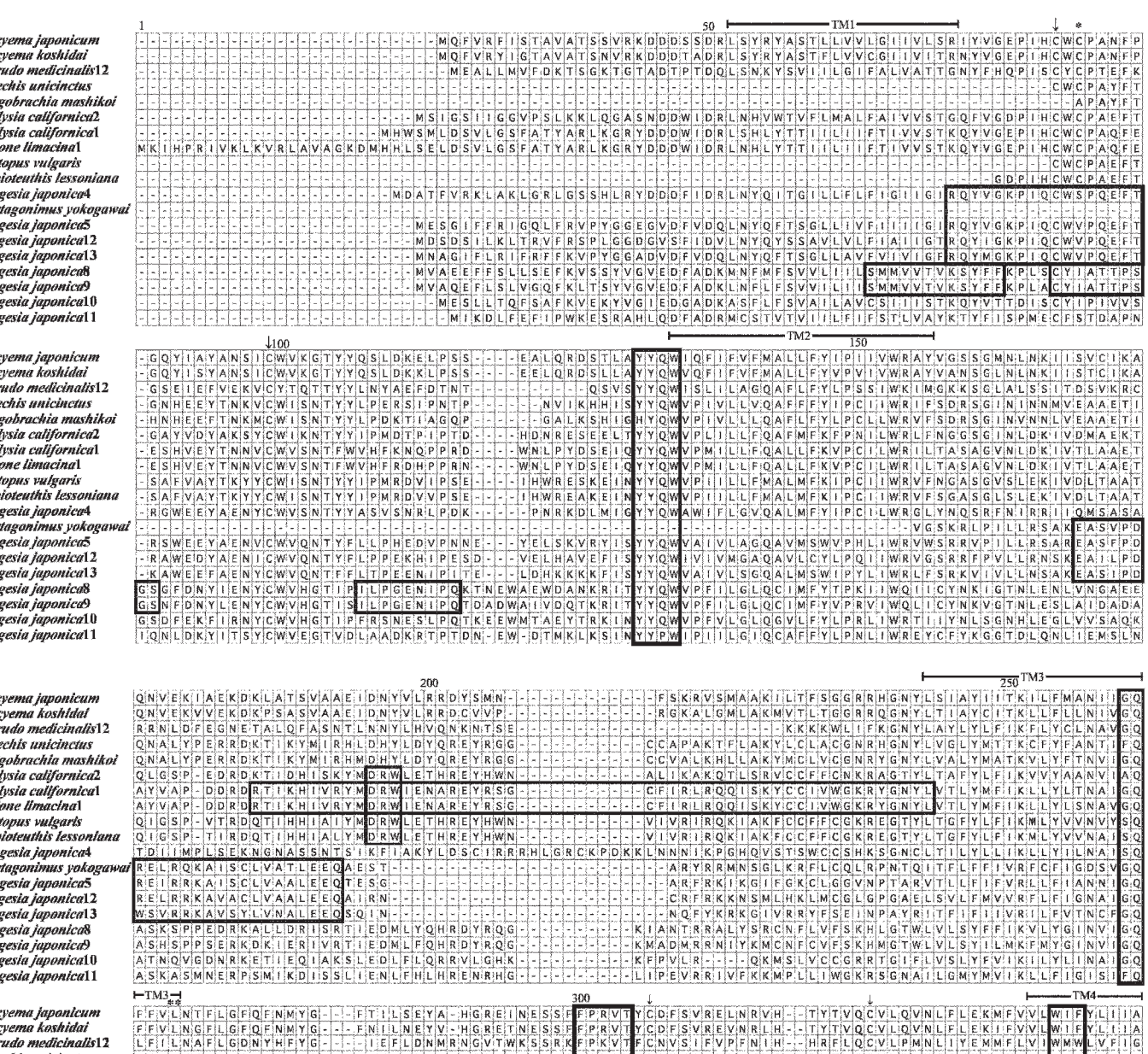

FIGURE 3. Specific conserved regions in 19 innexin sequences of molluscs, annelids, platyhelminths, and dicyemids. Deduced amino acid sequences of the dicyemid innexin show their full lengths. TM indicates the locations of trans-membrane domains deduced by Dykes et al. (2004). Boxes indicate conserved sequences in each taxa. Conserved cysteine except for platyhelminths (*), conserved leucine in annelid, molluscan and dicyemid lineage (**), conserved cysteine in all taxa (↓).

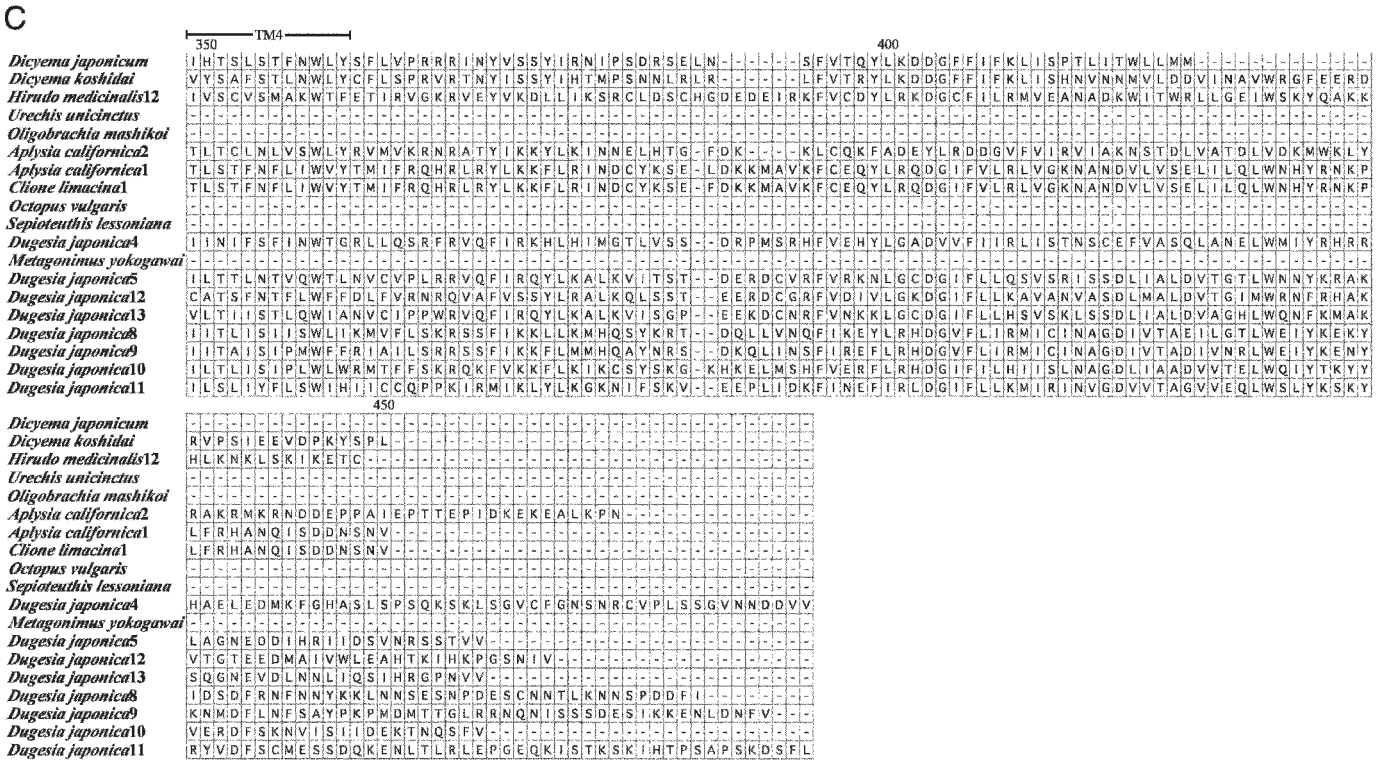

FIGURE 3. Continued.

pGEM®-T vector (Promega), which was introduced to the JM109-competent cell by heat-shock methods. We randomly picked up a transformed cell and extracted a plasmid containing innexin sequence. A cloned fragment was sequenced by BigDye terminator version 3.1 and an ABI 3700 auto sequencer (Applied Biosystems). Sequence data of fragments were analyzed by the BlastX program on the DDBJ server. The accession numbers of sequences used in this study are shown in Table II.

Phylogenetic analysis

Phylogenetic trees were constructed based on the deduced amino acid sequences by Sequence Analysis v. 1.6.0 (Gilbert, 2002) and on already known sequences collected from the NCBI database (Table II). Sequences were collected from four kinds of taxa: Vertebrata, Ecdysozoa, Platyhelminthes, and higher Lophotrochozoa. Sequences contained both innexin and pannexin sequences because these sequences are regarded as orthologous (Panchin et al., 2000; Yen and Saier, 2007). Sequences were aligned by ClustalX (Thompson et al., 1997) and then adjusted by eye. The construction of a phylogenetic tree was performed using Phyml (Guindon and Gascuel, 2003) for the maximum likelihood (ML) analysis and with MrBayes, v. 3.12 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) for the Bayesian analysis. In the ML analysis, we used the WAG (Whelan and Goldman, 2001) + I + G + F model. Gamma distribution was 2.46 and the proportion of invariable sites was 0.02. The starting tree was generated by BIONJ (Gascuel, 1997). Bootstrap values were generated through 1,000 replicates. The settings of the ML analysis were based on the results from the Akaike Information Criterion (AIC) by model generator v. 0.81 (Keane et al., 2004). In the Bayesian analyses, we used the WAG + I + G model and set rates = invgamma and outgroup = mouse PNX-1. The settings of the Bayesian analysis were based on the result from the Bayes information criterion (BIC) by model generator v. 0.81. For the Bayesian analyses, we ran 2,000,000 generations, with 1 cold and 3 incrementally heated chains, ran random starting trees for each chain, and sampled trees every 100 generations. After running 500,000 generations, the average standard deviation of split frequencies dropped to below 0.01. The constructed trees were viewed with TreeView 1.66 (Page, 1996).

Whole-mount in situ hybridization

We amplified the DNA fragment from cloned *D. japonicum* cDNA, by PCR, using primers 5′-TCAGAAAGGACGACGATT-3′ and 5′-TGGGCATATTCACTGAGA-3′. The fragment was ligated to the plasmid, and we used this plasmid for a synthesis of DIG-labeled RNA probe. DIG-labeled RNA probes were made by in vitro transcription from the linearized plasmid with a DIG RNA labeling kit (Roche, Indianapolis, Indiana). We used *Nco* 1 and *Not* 1 to linearize the plasmid. T7 or SP6 RNA polymerase (Roche) was used in the in vitro transcription to synthesize an antisense RNA probe, and a sense RNA probe was used as a negative control for hybridization. Whole-mount in situ hybridization was conducted by previously described methods (Ogino et al., 2007a, 2007b).

RESULTS

Isolation of innexin genes

We cloned the innexin gene by the random cloning of *D. japonicum* cDNA and obtained a full length of the gene (1,246 bp) by RACE PCR. We also cloned the innexin gene by RACE PCR amplification of *D. koshidai* cDNA and obtained a full length of the gene (1,263 bp). Partial sequences of the innexin gene of *O. vulgaris*, *S. lessoniana*, *U. unicinctus*, *Ol. mashikoi*, *L. anatina*, and *M. yokogawai* were obtained from the genome by distinct primer sets (Table I). In *D. japonicum* and *D. koshidai*, and in the trematode *M. yokogawai*, we confirmed that the same sequences were not obtained from their host.

Alignment

Alignments of amino acid sequences of innexins are shown in Figures 2 and 3. The alignment was conducted based on deduced amino acid sequences by ClustalX. Deduced amino acid sequences of dicyemids had 367 aa in *D. japonicum* and 394 aa in *D. koshidai*. Specific indels appeared in 29 innexin sequences from 17 species of 7 phyla containing arthropods, molluscs, annelids, brachiopods, flatworms, nematodes, and dicyemids (Fig. 2). Specific and conserved indels appeared up- and downstream of the TM2 (see Fig. 2A, indel 1, and Fig. 2B, indel 2).

There were 2 typical indels; the arthropod-type and the lophotrochozoan-type. In the number of amino acid sequences around the TM2 region, the arthropods had 7–18 aa longer upstream sequences than the lophotrochozoans, but 15–29 aa shorter downstream sequences than the lophotrochozoans. Nematode innexins share both the lophotrochozoan- and the arthropod-type. Thus, Innexin-12 of *Caenorhabditis elegans* (Rhabditidae) is unique in containing arthropod-type indel 1 and lophotrochozoan-type indel 2, and Innexin-16 is unique in containing lophotrochozoan-type indel 1 and arthropod-type indel 2 (Fig. 2). The other types of *C. elegans* innexins are similar to the lophotrochozoan type, although the downstream sequence of the TM2 is 4–14 aa shorter than a typical lophotrochozoan-type. Dicyemids have the lophotrochozoan type sequence both up- and downstream of TM2.

Conserved sequences are shown in the deduced innexin amino acid sequences of the lophotrochozoans containing 19 innexin sequences from 11 species of 4 phyla; molluscs, annelids, flatworms, and dicyemids (Fig. 3). Well-conserved amino acid sequences are located around 4 trans-membrane domains, such as cysteines between TM1 and TM2 or TM3 and TM4 (see the arrows in Fig. 3); the amino acid alignment sequences "YYQW" upstream of TM2; "GQ" in TM3; "FPRVT" at the upper site of the TM4; and "WFW" in TM4, although the dicyemid innexin has "WIF" at the upper site of the TM4. Molluscs, annelids, and dicyemids share some amino acids, but the cysteine located in the 11th amino acid from the end of the TM1 was not found in any platyhelminth innexins, and the leucine located in the 4th amino acid from well-conserved "GQ" within the TM3 was not conserved in platyhelminths (see the asterisks in Fig. 3).

Molluscan innexin sequences have the amino acid alignment sequence "DRW" around the 195 aa region. Additionally, 45 aas containing the up- and downstream of "DRW" were well conserved in *Aplysia californica* (Aplysiidae) innexin 1 and *Clione limacina* (Clionidae) innexin 1. Sequences of 2 cephalopod species, *O. vulgaris* and *S. lessoniana*, were almost the same (210 of 222 aa). In annelids, sequences between TM2 and TM3 regions were conserved between *O. mashikoi* and *U. unicincutus*, but not in *Hirudo medicinalis* (Hirudinidae). These molluscan- and annelid-specific amino acid sequences were not found in the dicyemid innexins.

There are 3 types of platyhelminth innexin: the neuronal type of *Dugesia japonica* (Planariidae) (Innexin-2, Innexin-3, Innexin-4, Innexin-5, Innexin-12, and Innexin-13), the mesenchyme type of *D. japonica* (Innexin-8, Innexin-9, Innexin-10, and Innexin-11), and the intestine type of *D. japonica* (Innexin-1) and *Girardia tigrina* (Planariidae) (Pannexin-1) (Nogi and Levin, 2005). The neuronal type shows some specific amino acid sequences at the downward side of TM1 region, "RQY (V or I or M) GKPIQCW (I or S or V), and PQEFT." Other neuronal type of innexins, such as *D. japonica* Innexin-5, Innexin-12, and Innexin-13, share sequences; "EA (S or I) (F or L or I) PD (R or W) (E or S) (I or L or V) RRKA (I or V) (A or S) (C or Y) LV (A or N) ALEEQ" between the TM2 and TM3 region. The trematode, *M. yokogawai*, also has a similar sequence (EASVPDRELRQ-KAISCLVATLEEQ).

In the mesenchyme type, Innexin-8 and Innexin-9 have some conserved sequences, such as "SMMVVTVKSYFF," "CY-IATTPSGS," and "ILOGENIPQ" between the TM1 and TM2 region, but the Innexin-11 does not. Although the Innexin-10 is less homologous to the Innexin-8 and Innexin-9, it has a similar

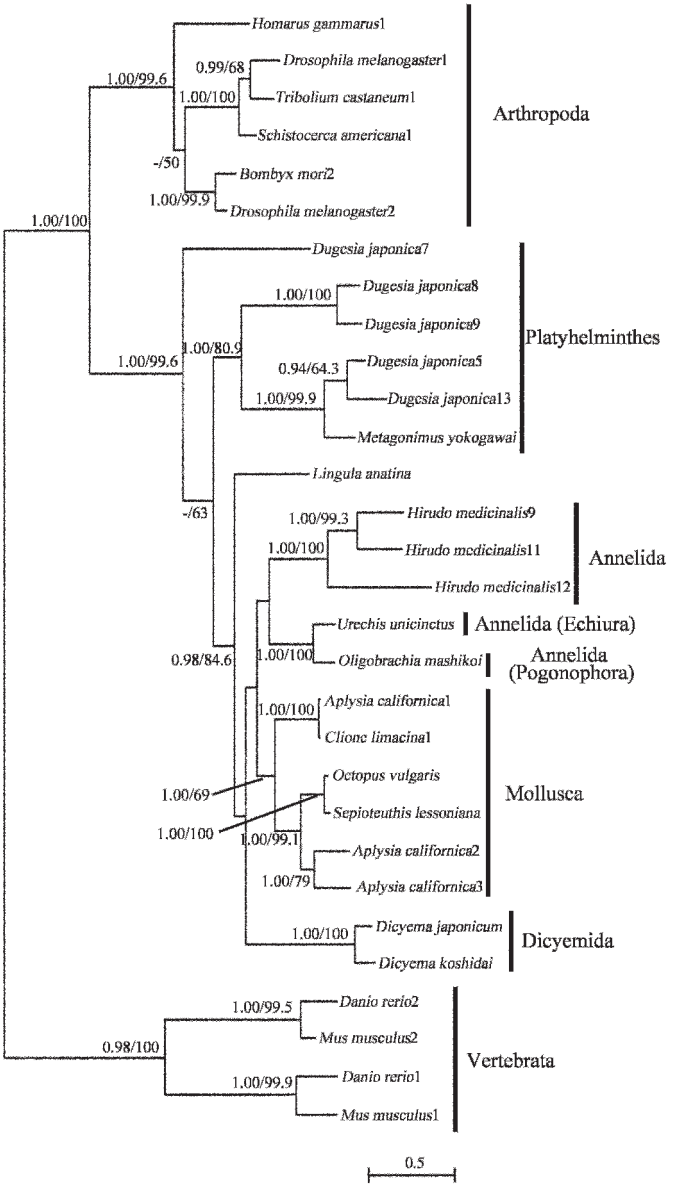

FIGURE 4. Phylogenetic tree based on innexin amino acid sequences by the Bayesian method. Number after species name indicates the number of innexin or pannexin. Support values: posterior probability by Baysian method/bootstrap values by maximum likekihood method. Values lower than 0.95 in posterior probability and 50% in maximum likelihood method are shown as "–". Bar represents the branch length.

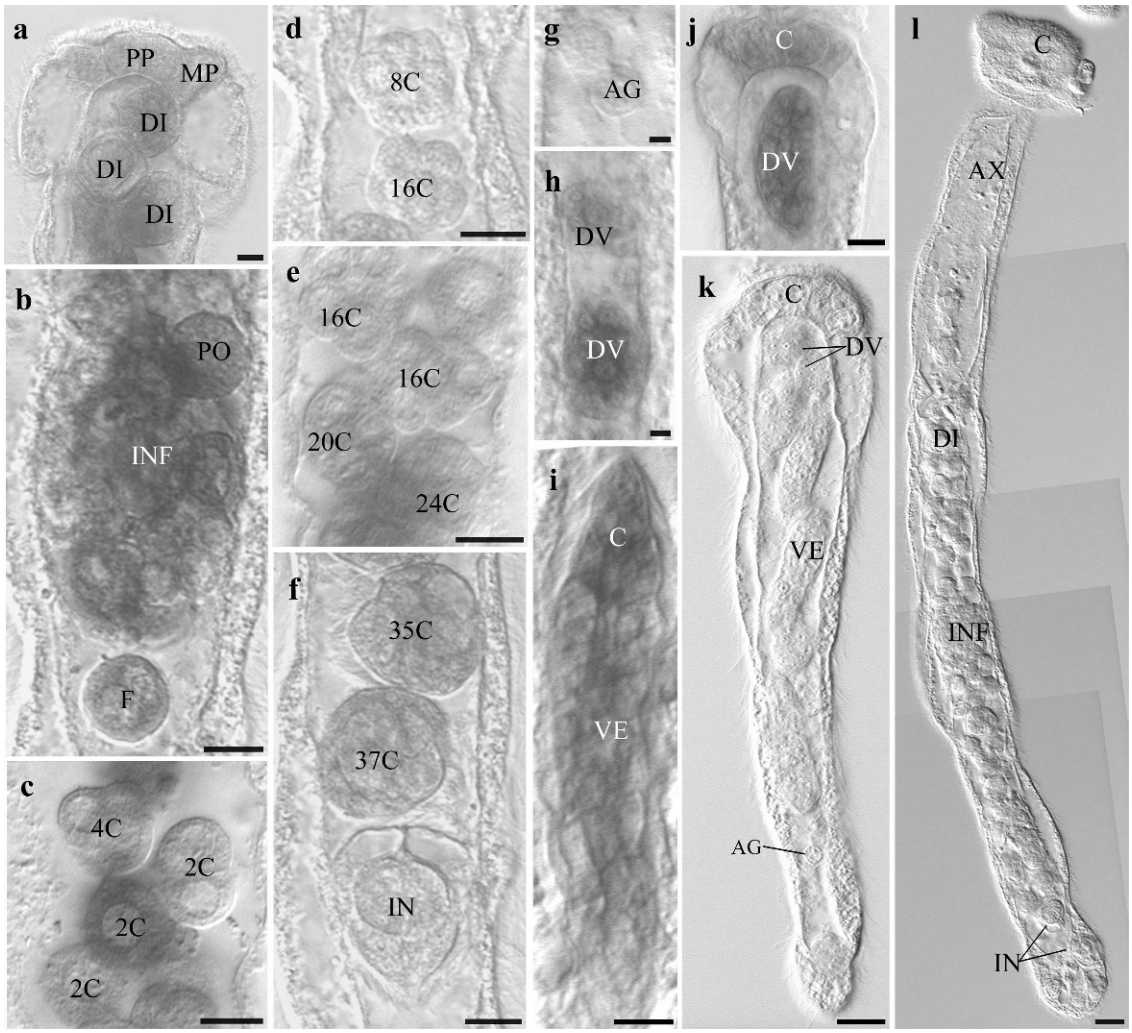

FIGURE 5. Expression patterns of innexin in *Dicyema japonicum*. (**a–f, l**) Rhombogen. (**g–k**) Nematogen. (**a**) Calotte of rhombogen and developing infusoriform embryo (DI), metapolar cell (MP), and propolar cell (PP). (**b**) Infusorigen (INF), primary oocyte (PO), and fertilized egg (F). (**c**) 2-cell stage (2C) and 4-cell stage (4C). (**d**) 8-cell stage (8C) and 16-cell stage (16C). (**e**) 16-cell stage (16C), 20-cell stage (20C), and 24-cell stage (24C). (**f**) 35-cell stage (35C), 37-cell stage (37C), and infusoriform embryo (IN). (**g**) Agamete (AG) in axial cell of nematogen. (**h**) Developing vermiform embryo (DV). (**i**) Calotte (C), veriform embryo (VE). (**j**) Calotte of nematogen (C) and developing vermiform embryo (DV). (**k**) Negative control of nematogen; agamete (AG), Calotte (C), developing veriform embryo (DV), veriform embryo (VE). (**l**) Negative control of rhombogen; axial cell (AX), Calotte (C), developing infusoriform embryo (DI) infusoriform embryo (IN), infusorigen (INF). Bars = 20 μm (**k, l**), 10 μm (**a–f, i, j**), 2 μm (**g, h**).

sequence, "CYIPIVVSGS," corresponding to the sequence "CYIATTPSGS" found in both Innexin-8 and Innexin-9.

Molecular phylogenetic analysis

We studied the relationship of major metazoan groups based on the deduced aligned amino acid sequences. Phylogenetic trees showed that the resulting clades consist of 3 groups, i.e., vertebrates, arthropods, and lophotrochozoans (Fig. 4). These clades were supported by high posterior probability and bootstrap values; 0.98 and 100% in vertebrate clade and 1.00 and 99.6% in both arthropod and lophotrochozoan clades. Flatworms were consistently basal to the other lophotrochozoans, which included dicyemids, annelids, molluscs, and brachiopods. The lophotrochozoan clade, without the flatworms, was supported with high values (0.98 posterior probability and 84.6% bootstrap value). However, the hypothesized relationship among the dicyemids, annelids, molluscs, and brachiopods obtained from ML analyses was not strictly congruent with that obtained with the Bayesian approach. In ML analyses, annelids were basal to dicyemids and molluscs, and dicyemids were clustered with higher lophotrochozoans.

Expression patterns of dicyemid innexin

Expression patterns of the innexin gene in *D. japonicum* were visualized by performing whole-mount in situ hybridization. Temporal expression patterns were observed in the antisense probes (Fig. 5). In adult individuals, the innexins were expressed in calottes, infusorigens, and infusoriform embryos. A unique temporal pattern was observed in the developing infusoriform embryos. The innexin first appeared in fertilized eggs, disappeared from 2-cell to early 24-cell stage, and was expressed from late 24-cell stage to the nearly formed embryo (Fig. 6). Subsequently, when embryos were fully formed, innexin expressions disappeared. In the vermiform embryo, innexin was expressed in all

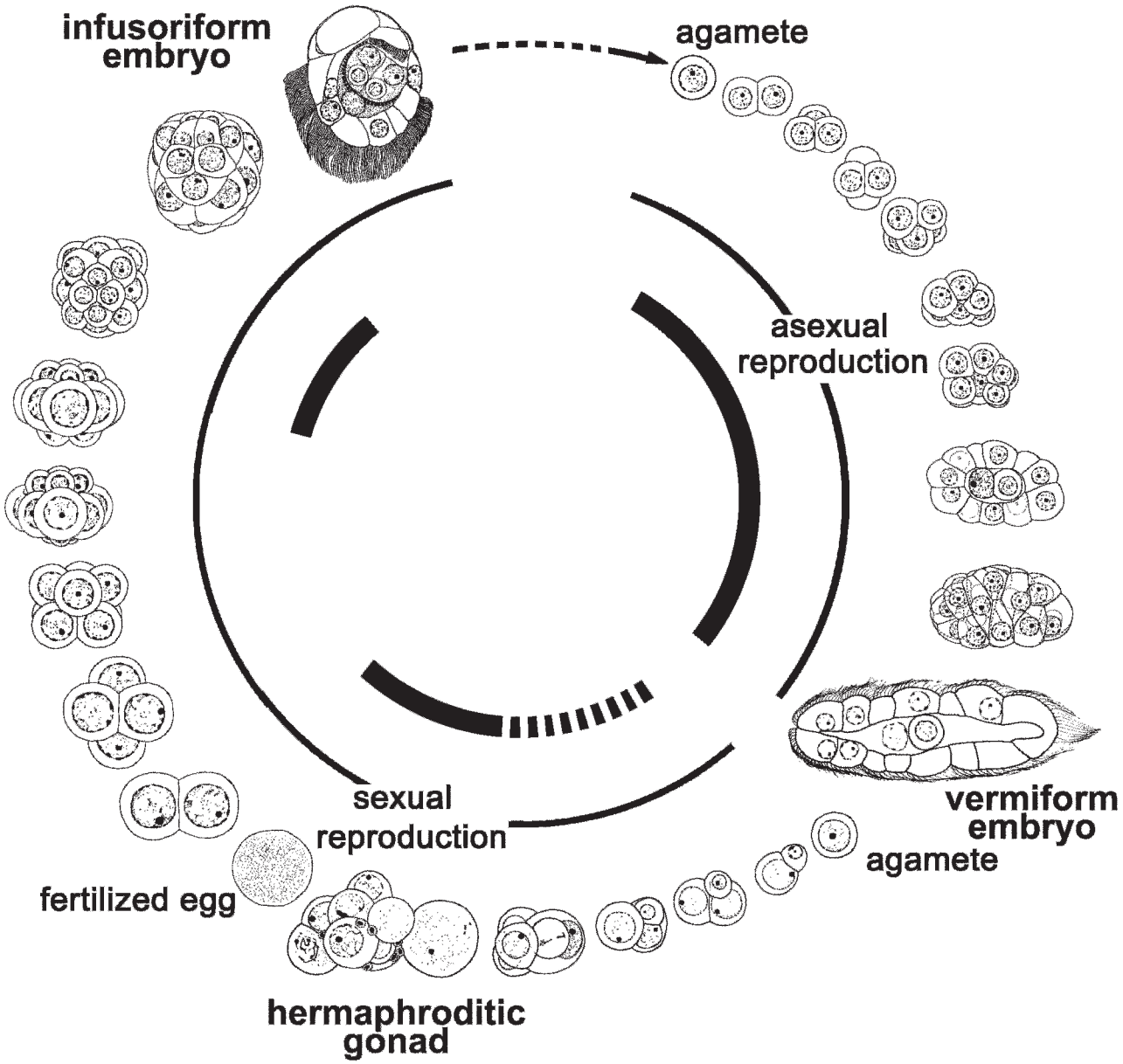

FIGURE 6. Innexin expression patterns in 2 types of embryo. The dashed arrow indicates an unknown process involved in the infection into a new cephalopod and development into adult forms. Inner lines represent the timings at which expression of the innexin gene was observed. The sketches of embryos were modified from Furuya et al., (1993, 1994, 2001) and Furuya and Tsuneki (2003).

somatic cells of developing embryos. When embryos were fully developed, expression of innexin remained only in calottes. No signal was detected in the negative-control experiment.

DISCUSSION

The taxon-specific large sequence changes, such as signature sequences in the *Hox* gene, are known as ''rare genomic changes'' (Rokas and Holland, 2000). These changes are useful for phylogenetic analyses. In fact, specific sequence changes in the innexin amino acid sequences revealed taxon-specific indels which distinguished 3 major protostome lineages, 2 ecdysozoans (arthropods and nematodes) and a lophotrochozoan. Innexin may be regarded as a ''toolkit gene'' similar to the genes encoding *Hox*, *parahox*, and other developmentally critical transcription factors. The innexin amino acid sequences can also be used to evaluate sister taxon relationships among major metazoan groups. The present study revealed the lophotrochozoans apparently consist of 2 groups, the Platyzoa (Platyhelminthes) and the Trochozoa (Annelida, Mollusca, Pogonophora, Echiura, Brachiopoda) (Giribet et al., 2000). The nematode *C. elegans* interestingly possesses the arthropod-type, and additional lophotrochozoan-type, sequences in its innexin amino acid sequences (Fig. 2). A recent report has suggested the nematode to be a primitive ecdysozoan (Dunn et al., 2008). Thus, lophotrochozoan-type sequences might have been lost during evolution of the ecdysozoan lineage.

Several studies have argued that the simple body plan of dicyemids is likely secondarily derived from higher lophotrochozoan animals as a result of their endoparasitic, or endosymbiotic, lifestyle in the cephalopod kidney (Katayama et al., 1995; Pawlowski et al., 1996; Kobayashi et al., 1999; Telford et al., 2000). In the present study, alignment of innexin amino acid sequences, as well as the phylogenetic trees based on the innexin amino acid sequences, showed that dicyemids are lophotrochozoans. This is supported by the evidence of the spiral cleavage in early infusoriform embryos (Furuya et al., 1992b). Thus, dicyemids are certainly spiralians.

Some previous analyses maintained that dicyemids are related to Platyhelminthes. Analysis using 18S rRNA suggested an affinity to Platyhelminthes (Katayama et al., 1995). Some parasitologists considered the dicyemids to be derived from the parasitic Platyhelminthes, such as trematodes, because dicyemids show a complex life cycle (Nouvel, 1947; Stunkard, 1954; Ginestinskaya, 1988). However, the alignment of innexin amino acid sequences of dicyemids contains no Platyhelminthes-specific amino acid sequence and suggests an affinity to annelids or molluscs rather than to Platyhelminthes, including the turbellarian, *D. japonica*, and the trematode, *M. yokogawai*. Phylogenetic trees using the innexin amino acid sequences also showed the dicyemids are not included in the platyhelminth clade. Sparse taxon sampling analysis has a limited utility for classification (May-Collado and Agnarsson, 2006). Although our taxon sampling is limited, topology of our tree is congruent with the broad taxon sampling analysis across the invertebrates (Dunn et al., 2008). This gives credibility to our findings that dicyemids are possibly higher lophotrochozoans.

Although infusoriform larvae of dicyemids are free-swimming organisms, the body organization is not regarded as achieving the grade of the tissue level (Furuya et al., 1996). A germ layer is absent in the infusoriform embryos, and groups of cells are characterized only as being external or internal in their location (Furuya et al., 2004). It is unclear whether or not infusoriform embryos without tube-shaped guts and ciliary bands represent the true level of organization. Molecular clues to trace the evolutionary history of degeneration in the dicyemid embryogenesis and life cycle, from those of ancestral lophotrochozoan animals, were provided using the developmental expression patterns of regulatory genes; the central type *Hox* gene, *otx*, and the *brachyury* homologs (Kobayashi et al., 2009). *Do-otx* was expressed in the vegetal pole cells of the developing infusoriform embryos, suggesting that the invagination in infusoriform embryos is homologous to the gastrulation of other metazoans. *Do-bra* is expressed in the presumptive ventral cells, which are ventral to the opening of the urn cavity. The expression of *Do-bra* suggests that the urn cavity opening of the infusoriform embryo is comparable to the stomodeum of trochophore larvae. Dicyemid larvae are likely to change the body plan with some molecular traces of trochophore larvae. Thus, dicyemids might be specialized from a higher triploblast animal rather than from the Platyhelminthes.

Several, organ-specific innexin genes were reported in a leech, *H. medicinalis* (Dykes and Macagno, 2006), in *Drosophila melanogaster* (Drosophilidae) (Stebbings et al., 2002), and in *C. elegans* (Starich et al., 2003; Chuang et al., 2007; Whitten and Miller, 2007). In embryonic stages, they were exclusively expressed in germ layers, but the dicyemid innexin was expressed in all blastomeres of developing embryos after the 24-cell stage. In adult stages of dicyemids, expression was limited to the calotte and the hermaphroditic gonad, the infusorigen. No gap junctions were observed in infusorigens (Ridley, 1968; Matsubara and Dudley, 1976; Furuya et al., 1997). However, expressions appeared in the primary oocytes and fertilized eggs on the surface of infusorigens, suggesting this innexin mRNA was maternal, for use in early embryonic stages. In *C. elegans* embryos, the zygotic innexin was expressed after the 28-cell stage (Starich et al., 2003). In dicyemids, innexin appears again after the 24-cell stage, suggesting the zygotic expression. In dicyemids, the innexin expression also appears in the calotte cells of vermiform stages. The calotte consists of 8–9 cells, each of which adheres to 4–6 neighboring calotte cells and an internal axial cell. There may be more gap junctions and channels in calotte cells than in other peripheral cells.

By comparing the alignment of amino acid sequences of innexins, major invertebrate taxa were distinguishable at the phylum or superphylum level. The basal ecdysozoan, *C. elegans*, shared both arthropod and lophotrochozoan features. In conclusion, dicyemids appear to be higher lophotrochozoans and not related to the Platyhelminthes.

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