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The complete mitochondrial genome of *Pallisentis celatus* (Acanthocephala) with phylogenetic analysis of acanthocephalans and rotifers

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Abstract: Acanthocephalans are a small group of obligate endoparasites. They and rotifers are recently placed in a group called Syndermata. However, phylogenetic relationships within classes of acanthocephalans, and between them and rotifers, have not been well resolved, possibly due to the lack of molecular data suitable for such analysis. In this study, the mitochondrial (mt) genome was sequenced from *Pallisentis celatus* (Van Cleave, 1928), an acanthocephalan in the class Eoacanthocephala, an intestinal parasite of rice-field eel, *Monopterus albus* (Zuiew, 1793), in China. The complete mt genome sequence of *P. celatus* is 13 855 bp long, containing 36 genes including 12 protein-coding genes, 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs) as reported for other acanthocephalan species. All genes are encoded on the same strand and in the same direction. Phylogenetic analysis indicated that acanthocephalans are closely related with a clade containing bdelloids, which then correlates with the clade containing monogononts. The class Eoacanthocephala, containing *P. celatus* and *Paratenuisentis ambiguus* (Van Cleave, 1921) was closely related to the Palaeacanthocephala. It is thus indicated that acanthocephalans may be just clustered among groups of rotifers. However, the resolving of phylogenetic relationship among all classes of acanthocephalans and between them and rotifers may require further sampling and more molecular data.

Keywords: Eoacanthocephala, mitochondrion, mt genome, rice-field eel, China, phylogeny, Syndermata

Acanthocephalans, which use vertebrates as definitive hosts and arthropods as intermediate hosts, are a small group of obligate endoparasites with a total of about 1 200 species in the world (Schmidt 1985). They were historically classified in the phylum Nematoda (Dougherty 1951), but were more recently recognized as a group within the phylum Rotifera in either traditional morphological (Melone et al. 1998, Sørensen and Giribet 2006) or molecular phylogenetic analyses (García-Varela and Nadler 2006). Acanthocephalans and rotifers are now grouped in the taxon Syndermata (see Ahlrichs 1997). However, the phylogenetic relationship of acanthocephalans with three classes of rotifers, i.e. Bdelloidea, Monogononta and Seisonidea, has not been resolved (Garey et al. 1998, García-Varela and Nadler 2006, Witek et al. 2008, Fontaneto and Jondelius 2011).

However, parasitologists considered acanthocephalans or spiny-headed worms to represent the phylum Acanthocephala, which contains four classes, Archiacanthocephala, Eoacanthocephala, Palaeacanthocephala, and Polyacanthocephala (Amin 1987, Monks 2001). Several authors have attempted to examine the phylogenetic relationship of these classes (Near et al. 1998, García-Varela et al. 2000, Monks 2001, Near 2002, García-Varela and Nadler 2006), but their phylogenetic relationships remain to be resolved (García-Varela and Nadler 2005). Nevertheless, it seems possible that the Eoacanthocephala and Polyacanthocephala are closely related, representing a sister group to the Palaeacanthocephala, with the Archiacanthocephala as a separate clade (García-Varela et al. 2002, García-Varela and Nadler 2006).

These phylogenetic studies have been based on the analysis of one or two genes, such as ribosomal RNA genes (García-Varela et al. 2002, García-Varela and Nadler 2005, 2006, Mark Welch 2005, Passamaneck and Halanych 2006, Sørensen and Giribet 2006), ITS (Kráľová-Hromadová et al. 2003, Perrot-Minnot 2004), or *cox1* (Perrot-Minnot 2004), which may not be phylogenetically informative as mt genomes used much more commonly in phylogenetic studies (Stach et al. 2010, Zhao et al. 2010, Fontaneto and Jondelius 2011, Park et al. 2011, Abascal et al. 2012). It is only recently that the mt genome, which may be much more informative for phylogenetic analysis, has been sequenced in acanthocephalans,

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Table 1. PCR primers used for cloning of the mitochondrial genome of *Pallisentis celatus*.

Primer	DNA sequence (5'–3')	Estimated size of PCR products	Reference
cox1F	GGTCAACAAATCATAAAGATATTGG	680 bp	Folmer et al. (1994)
cox1R	TAAACTTCAGGGTGACCAAAAAATCA		
cox2F	GGWCAYCARTGATATTGA	340 bp	This study
cox2R	CAATKACAATYGGTATAAA		
rrnLF	GACYGTRCTWAGGTAGCRTRATC	600 bp	Gazi et al. (2012)
rrnLR	AWRDRATRATCCAACATCGAGGTA		
cobF	CTTTTTTAGGGTATGTTTTACC	600 bp	Gazi et al. (2012)
cobR	TCWACARYAYAWCCTCC		
cox1-16sF	CTTTGGTGGTTACTGCTTTCTTAG	1.9 kb	This study
cox1-16sR	ATCCCCAGAGTAACTACACCCTTG		
16S-cobF	TTCAAGGGTGTAGTTACTCTGGGG	7.5 kb	This study
16S-cobR	GACACCAATGCCTCACCAACTACA		
cob-cox2F	AATCCACTGGGGGTGTTTTCTTCT	2.8 kb	This study
cob-cox2R	GTCAAATACACGCCTACCCAAGAA		
cox2-cox1F	AGGTTTTTGGGGGTAGGGTCAGAT	2.3 kb	This study
cox2-cox1R	AAACCTCCCATAAAAGCAGGCATT		

namely *Leptorhynchoides thecatus* (Linton, 1891) of the Palaeacanthocephala, *Oncicola luehei* (Travassos, 1917) and *Macracanthorhynchus hirudinaceus* (Pallas, 1781) of the Archiacanthocephala, and *Paratenuisentis ambiguus* of the Eoacanthocephala (see Steinauer et al. 2005, Gazi et al. 2012, Weber et al. 2013). More mitochondrial genome sequences are apparently needed to resolve the phylogenetic relationship of acanthocephalans at various taxonomical levels, as well as to understand their relationship with other animals.

Pallisentis celatus of the class Eoacanthocephala is an endoparasite in intestines of rice-field eel, *Monopterus albus* (Zuiew). Its wide distribution in China and in some Southeast Asian countries, such as Vietnam, and its abundant occurrence in the eel provide opportunities for sampling the parasite easily (Amin et al. 2004, Wang et al. 2004). Previous studies on this parasite have been carried out with focus on its morphology, epidemiology and population dynamics, and even infection control (Amin et al. 2004, Wang et al. 2004, Zeng and Wang 2007).

In the present study, the complete mt genome of *P. celatus* obtained from *M. albus* was sequenced. The gene arrangement, nucleotide composition and codon usage were further analyzed, and the phylogenetic relationship of *P. celatus* was examined with the combination of mt genomes from other acanthocephalans and rotifers in order to assess phylogenetic relationships within the Syndermata.

MATERIALS AND METHODS

Parasite samples and DNA extraction

Pallisentis celatus was dissected out under a microscope from intestines of *Monopterus albus*, which were captured in rice field around Wuhan, Hubei Province of China. Parasite specimens were then washed in 0.6% sodium chloride, before being kept in 70% ethanol at -20 °C for further use. The total genomic DNA was extracted using the Promega Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's protocol.

PCR amplification and sequencing

Initially, four partial gene fragments of *cox1*, *cox2*, *cob* and *rrnL* were amplified using reported primer sets (cox1F/cox1R, cobF/cobR, and rrnLF/rrnLR) (Folmer et al. 1994, Gazi et al. 2012) (Table 1), and primer set (cox2F/cox2R) designed from conserved regions of published sequences of acanthocephalans (*Leptorhynchoides thecatus* and *Oncicola luehei* with their GenBank accession numbers listed in Table 2) and two rotifer species (*Rotaria rotatoria* and *Brachionus plicatilis*; Table 2). Polymerase chain reaction (PCR) was carried out with the following amplification conditions: 94 °C for 3 min; 35 cycles of 94 °C for 30 s, 45 °C for 30 s and 72 °C for 1 min, and incubation at 72 °C for 10 min.

The amplified PCR products were cloned into pMD18-T vector (Takara), and transformed into competent *Escherichia coli* strain DH5α. The nucleotide sequences obtained from these partial gene fragments were then used to design specific primers for long PCR amplification for cloning the remaining sequence of mt genome (Table 1). PCRs were carried out with the primers (Table 1) and the LA Taq (Takara) at the following conditions: 94 °C for 3 min; 33 cycles of 94 °C for 30 s, 60 °C for 30 s and 68 °C for 10 min, and extension at 72 °C for 10 min. The amplified DNA fragments were approximately 1.9, 7.0, 2.8 and 2.3 kb long, respectively, which extended from downstream of *cox1* to upstream of *rrnL*, from downstream of *rrnL* to upstream of *cob*, from downstream of *cob* to upstream of *cox2*, and from downstream of *cox2* to upstream of *cox1*.

The amplified long PCR products were gel-isolated and extracted using the Omega Gel Extraction Kit (Omega). After gel purification, each of the long PCR products was ligated into pMD18-T cloning vector (Takara) and then transformed into competent *E. coli* DH5α. The recombinant clones were selected and sequenced using a Big Dye Terminators Cycle-Sequencing Kit (ABI) in both directions by the primer walking method. Sequence data were analyzed using the SeqMan program from DNASTAR (http://www.DNASTAR.com/). The complete nucleotide sequence was submitted to the GenBank database with accession number JQ943583.

Table 2. Species and their mitochondrial genomes used in the phylogenetic analysis. Newly sequenced mt genome in bold.

Species	Taxonomic group	GenBank accession no.
Lophotrochozoa		
Mytilus galloprovincialis	Mollusca	NC_006886
Musculista senhousiavoucher	Mollusca	GU001954
Terebellides stroemi	Annelida	EU236701
Lumbricus terrestris	Annelida	NC_001673
Loxosomella aloxiata	Entoprocta	AB264800
Loxocorone allax	Entoprocta	NC_010431
Urechis caupo	Echiura	AY619711
Urechis unicinctus	Echiura	NC_012768
Sipunculus nudus	Sipunculida	NC_011826
Laqueus rubellus	Brachiopoda	NC_002322
Terebratalia transversa	Brachiopoda	AF331161
Phoronis psammophila	Phoronida	AY368231
Flustra foliacea	Bryozoa	JQ061319
Watersipora subtorquata	Bryozoa	NC_011820
Platyzoa		
Pallisentis celatus	**Acanthocephala; Eoacanthocephala**	**JQ943583**
Paratenuisentis ambiguus	Acanthocephala; Eoacanthocephala	FR856885
Leptorhynchoides thecatus	Acanthocephala; Palaeacanthocephala	NC_006892
Oncicola luehei	Acanthocephala; Archiacanthocephala	NC_016754
Macracanthorhynchus hirudinaceus	Acanthocephala; Archiacanthocephala	FR856886
Rotaria rotatoria	Rotifera; Bdelloidea	NC_013568
Philodina citrina	Rotifera; Bdelloidea	FR856884
Adineta vaga	Rotifera; Bdelloidea	1
Habrotrocha constricta	Rotifera; Bdelloidea	2
Macrotrachela quadricornifera	Rotifera; Bdelloidea	3
Adineta ricciae	Rotifera; Bdelloidea	4
Philodina roseola	Rotifera; Bdelloidea	5
Brachionus plicatilis	Rotifera; Monogononta	NC_010472 part-I NC_010484 part-II
Brachionus calyciflorus	Rotifera; Monogononta	6
Brachionus manjavacas	Rotifera; Monogononta	7
Paragonimus westermani	Platyhelminthes	NC_002354
Taenia solium	Platyhelminthes	NC_004022
Taenia crassiceps	Platyhelminthes	NC_002547
Echinococcus multilocularis	Platyhelminthes	NC_000928
Ecdysozoa		
Homarus americanus	Arthropoda	HQ402925
Scutigera coleoptrata	Arthropoda	NC_005870
Opisthopatus cinctipes	Onychophora	NC_014273
Epiperipatus biolleyi	Onychophora	NC_009082
Priapulus caudatus	Priapulida	NC_008557
Deuterostomia		
Branchiostoma floridae	Cephalochordata	NC_000834
Acanthaster planci	Echinodermata	NC_007788
Sarotherodon melanotheron	Chordata	NC_015611
Rhabdopleura compacta	Hemichordata	NC_015649
Radiata (outgroup)		
Montastraea annularis	Cnidaria	NC_007224

Abbreviations: 1 – indicates some fragments of the mt genome, i.e. JX183993, JX184001, JX184009, JX184017, JX184025, JX184033, JX184056, JX184064, JX184072; 2 – indicates some fragments of the mt genome, i.e. JX183994, JX184002, JX184010, JX184018, JX184026, JX184034, JX184057, JX184065, JX184073; 3 – indicates some fragments of the mt genome, i.e. JX183995, JX184003, JX184011, JX184019, JX184027, JX184035, JX184059, JX184067, JX184075; 4 – indicates some fragments of the mt genome, i.e. JW861109, JW861111, JW861112; 5 – indicates some fragments of the mt genome, i.e. JW861079, JW861081, JW868082, JW861084, JW861085; 6 – indicates some fragments of the mt genome, i.e. JX463644, JX463643, JX463645, JX463646, JX433647, JX463648, JX433649, JX463650, JX463651; 7 – indicates some fragments of the mt genome, i.e. JW861101, JW861103, JW861104, JW861106.

Sequence analyses and genome drawing

Nucleotide composition, amino acid composition and codon usage were initially analyzed using MEGA version 5 (Tamura et al. 2011). Protein-coding genes were analyzed through the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) using invertebrate mt code. Two rRNAs were identified in their similarity to those published mt genomes of acanthocephalans by BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The 5' and 3' ends of the large ribosomal RNA (*rrnL*) and small ribosomal RNA (*rrnS*) were assumed to be adjacent to the flank-

Table 3. Mitochondrial genome organization of *Pallisentis celatus*.

Nucleotide	Length (bp)	A (%)	C (%)	T (%)	G (%)	A+T (%)	G+C (%)
Entire sequence	13 855	20.7	9.7	40.8	28.8	61.5	38.5
Protein coding sequence	10 041	18.4	9.4	42.8	29.4	61.2	38.8
Codon position*							
1st	3 347	20.9	8.3	36.5	34.3	57.4	42.6
2nd	3 347	13.2	12.7	51.8	22.3	65.0	35.0
3rd	3 347	21.2	7.3	40.1	31.4	61.3	38.7
Ribosomal RNA genes sequence	1 457	28.2	10.2	35.4	26.2	63.6	36.4
Transfer RNA genes sequence	1 242	23.8	10.1	38.1	28.0	61.9	38.1
Non-coding region 1	424	28.1	10.6	25.4	36.8	52.6	47.4
Non-coding region 2	317	29.0	11.7	37.5	21.8	66.6	33.4

*Termination codons were excluded.

ing genes. The 22 tRNAs were identified using the tRNAscan-SE program (Lowe and Eddy 1997), DOGMA (Wyman et al. 2004) or manually through comparing specific anticodon sequences and secondary structures with those found in *L. thecatus* and *O. luehei*, when tRNA genes were not recognized by these two programs. The circular mt genome map of *P. celatus* was drawn by using GenomeVx (Conant and Wolfe 2008).

Phylogenetic analysis

In order to include as many rotifer species as possible, phylogenetic analysis was performed using 9 of the 12 protein-coding genes, as *nad2*, *nad3* and *nad6* were absent from sequence data of some species of rotifers (Lasek-Nesselquist 2012). The metazoan groups represented in the analysis included 14 lophotrochozoans, 19 platyzoans with *P. celatus* and other four acanthocephalans, 5 ecdysozoans and 4 deuterostomes. The mt genome sequence data from one cnidarian, *Montastraea annularis* (Ellis et Solander, 1786), was included in the analysis as outgroup. A list of species and GenBank accession numbers of their mt genomes are given in Table 2. Nine concatenated protein-coding genes of *P. celatus* were translated into amino acids using invertebrate mt genetic code and then aligned using ClustalX with default options (Thompson et al. 1997). The most conserved sequence regions (1 125 amino acid) were selected for phylogenetic analysis from 9 concatenated genes using Gblocks program with default options (Castresana 2000).

Phylogenetic analysis of conserved amino acid sequences was performed using Bayesian Inference (BI) by MrBayes version 3.2 (Huelsenbeck and Ronquist 2001). For BI analysis, the best fit model was estimated by using Akaike Information Criterion (AIC) and ProTest version 2.0 (Abascal et al. 2005). ProTest selected MtArt with a gamma distribution (+G), a portion of invariable sites (+I), and empirical base frequencies (+F) as the best-fit model for amino acid substitution, followed by MtArt+G+F, MtArt+I+G, MtArt+G, and RtREV+I+G+F. Since none of the MtArt model is implemented in MrBayes, RtREV as the next best available model was used for the matrix (again applying options +I, +G and +F). Bayesian analysis was run for 1 000 000 generations and sampled every 100 generations with four Markov Chain Monte Carlo (MCMC) chains. Bayesian posterior probability (BPP) values were determined after discarding the initial 2 000 saved trees (the first 2×10^5 generations) as burn-in.

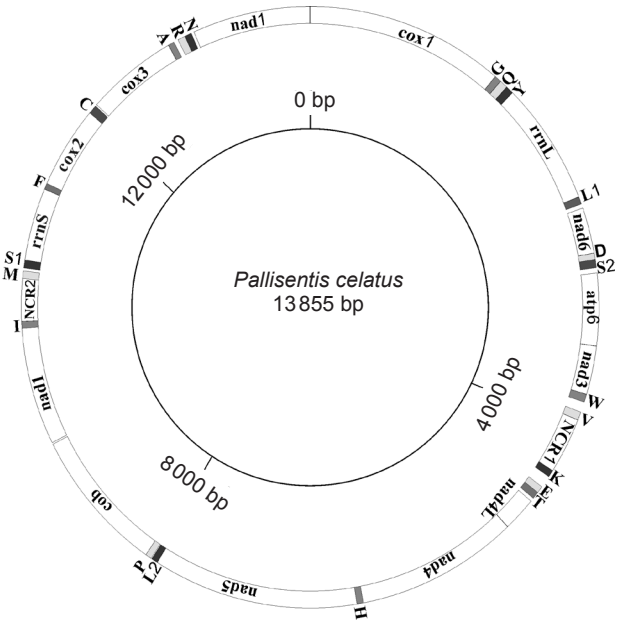

Fig. 1. Circular presentation of the complete mitochondrial genome of *Pallisentis celatus*. All genes are encoded in the same direction and 22 tRNA genes are designated by a single-letter abbreviation. The two leucine and serine tRNA genes are labeled, according to their anticodon sequences, as L1 (*trnL*-uag), L2 (*trnL*-uaa), S1 (*trnS*-ucu) and S2 (*trnS*-uga), respectively.

RESULTS

Genes and organization of the mitochondrial genome

The complete mt genome of *Pallisentis celatus* is a circular, double-stranded DNA molecule, with a total length of 13 855 bp. The nucleotide composition of the entire genome sequence is 40.8% for T, 28.8% G, 20.7% A and 9.7% C (Table 3). The genome contains 36 genes including 12 protein-coding genes, 22 tRNAs and 2 rRNAs (*rrnL* and *rrnS*). All genes are encoded on the same strand (Fig. 1). A list of gene order, gene length, and intergenic spacer regions is given in Table 4.

Codon usage and sequence features of protein-coding genes

The entire mt genome of *P. celatus* excessively favours AT. The overall AT composition of protein-coding genes in the genome is 61.2%, which is a little more than that of *Oncicola luehei* (58.8%), and less than that of *Leptorhynchoides thecatus* (71.6%), *Paratenuisentis ambiguus* (67.1%) and *Macracanthorhynchus hirudinaceus* (63.9%). The codon usage of *P. celatus* protein-coding genes is shown in Table 5.

The protein-coding genes of *P. celatus* mt genome are composed of T-rich codons. The total length of 12 protein-coding genes in the genome is 10 041 bp, which consist of 3 347 codons, excluding termination codons. Analysis of the codon usage of 12 protein-coding genes revealed that three codons are used frequently, with TTT

Table 4. Nucleotide composition of the mitochondrial genome of *Pallisentis celatus*.

Gene	Position		Size		Codons		Intergenic sequence
	Start	Finish	No. of nt	No. of aa	Initiation	Termination	
cox1	1	1534	1534	511*	GTG	T	0
trnG	1535	1587	53				0
trnQ	1589	1650	62				1
trnY	1651	1711	61				0
rrnL	1712	2634	923				0
trnL1	2635	2690	56				0
nad6	2722	3063	342	114*	ATG TAA	TAA	31
trnD	3064	3112	49				0
trnS2	3110	3172	63				-3
atp6	3211	3748	538	179*	ATG	T	38
nad3	3749	4111	363	121*	ATA	TAG	0
trnW	4112	4171	60				0
trnV	4249	4310	62				77
NCR1	4311	4731	421				0
trnK	4732	4783	52				0
trnE	4874	4926	53				-10
trnT	4926	4981	56				-1
nad4L	5008	5266	259	86*	ATT	T	26
nad4	5268	6519	1252	417*	ATG	T	1
trnH	6518	6568	51				-2
nad5	6568	8157	1590	530*	GTG	TAG	-1
trnL2	8158	8207	50				0
trnP	8208	8262	55				0
cob	8261	9349	1089	363*	ATG	TAG	-2
nad1	9362	10238	877	292*	GTG	T	12
trnI	10238	10287	50				-1
NCR2	10288	10602	317				0
trnM	10603	10659	57				-2
trnS1	10681	10739	59				21
rrnS	10740	11273	534				0
trnF	11274	11328	55				0
cox2	11327	11941	615	205*	GTG	TAG	-2
trnC	11941	12009	69				-1
cox3	12021	12714	694	231*	GTG	T	11
trnA	12721	12777	57				6
trnR	12808	12865	58				30
trnN	12865	12919	55				-1
nad2	12942	13853	912	304*	TTG	TAA	22

*Stop codons were not included; nt – nucleotide; aa – amino acid.

codon used most frequently (8.98%), followed by TTG (7.40%) and GTT (6.92%), whereas CGC, TGC, GCC and CCG codons are used only at 0.06%, 0.24%, 0.27%, and 0.27%, respectively (Table 5). The most frequently encoded amino acids include leucin (Leu – 16.24%), valim (Val – 15.84%) and glycin (Gly – 10.82%), accounting for 42.90% of total amino acid components (Fig. 2).

Four protein-coding genes, *nad6*, *atp6*, *nad4*, *cob*, start with ATG. Five other protein-coding genes, *cox1*, *nad5*, *nad1*, *cox2*, *cox3*, start with GTG, and *nad3* starts with ATA, and *nad4L* with ATT, and *nad2* with TTG. Six genes use complete stop codons, including four genes with TAG (*nad3*, *nad5*, *cob* and *cox2*) and two with TAA (*nad6* and *nad2*), whereas other six genes appear to end in incomplete stop codons with T (*cox1*, *cox3*, *atp6*, *nad4*, *nad4L* and *nad1*). Details of initiation and termination codons of 12 protein-coding genes are shown in Table 4.

Table 5. Genetic code and codon usage for the 12 mitochondrial protein-coding genes of *Pallisentis celatus*.

Codon	aa	N.	%	Codon	aa	N	%
TTT	Phe	301	8.98	TAT	Tyr	93	2.77
TTC	Phe	36	1.07	TAC	Tyr	21	0.63
TTA	Leu	203	6.05	TAA	*	2	0.06
TTG	Leu	248	7.40	TAG	*	4	0.12
CTT	Leu	46	1.37	CAT	His	28	0.84
CTC	Leu	11	0.33	CAC	His	10	0.30
CTA	Leu	16	0.48	CAA	Gln	16	0.48
CTG	Leu	19	0.57	CAG	Gln	19	0.57
ATT	Ile	121	3.61	AAT	Asn	33	0.98
ATC	Ile	23	0.69	AAC	Asn	12	0.36
ATA	Met	68	2.03	AAA	Lys	32	0.95
ATG	Met	110	3.28	AAG	Lys	33	0.98
GTT	Val	232	6.92	GAT	Asp	54	1.61
GTC	Val	21	0.63	GAC	Asp	8	0.24
GTA	Val	79	2.36	GAA	Glu	35	1.04
GTG	Val	198	5.91	GAG	Glu	49	1.46
TCT	Ser	84	2.51	TGT	Cys	33	0.98
TCC	Ser	22	0.66	TGC	Cys	8	0.24
TCA	Ser	38	1.13	TGA	Trp	38	1.13
TCG	Ser	22	0.66	TGG	Trp	71	2.12
CCT	Pro	24	0.72	CGT	Arg	21	0.63
CCC	Pro	11	0.33	CGC	Arg	2	0.06
CCA	Pro	23	0.69	CGA	Arg	13	0.39
CCG	Pro	9	0.27	CGG	Arg	11	0.33
ACT	Thr	23	0.69	AGT	Ser	57	1.70
ACC	Thr	10	0.30	AGC	Ser	16	0.48
ACA	Thr	30	0.89	AGA	Ser	41	1.22
ACG	Thr	17	0.51	AGG	Ser	75	2.24
GCT	Ala	51	1.52	GGT	Gly	144	4.29
GCC	Ala	9	0.27	GGC	Gly	24	0.72
GCA	Ala	31	0.92	GGA	Gly	45	1.34
GCG	Ala	20	0.60	GGG	Gly	149	4.44

*Stop (termination) codon; aa – amino acid; N – number of copies.

Transfer RNA and rRNA genes

Twenty-two tRNAs encoded in the mt genome of *P. celatus* are identified on the basis of their respective anticodons and secondary structures (Fig. 3); they vary in length from 49 (*trnD*) to 69 (*trnC*) nucleotides, with the inclusion of two *trnL* and two *trnS*. The *trnR*, *trnC*, *trnF*, and the two *trn*S have dihydrouridine (DHU) arm and pseudouridine (TΨC) arm. The *trnA*, *trnN*, *trnE*, *trnT*, *trnY* and *trnV* lack a DHU arm, and the remaining 12 tRNAs lack a TΨC arm (Fig. 3).

The two rRNAs, *rrnL* and *rrnS,* of *P. celatus* mt genome were identified by sequence comparison with those of *L. thecatus*, *O. luehei*, *Paratenuisentis ambiguus*, and *M. hirudinaceus*, and the entire flanking regions between the gene boundaries of their respective adjoining genes were designated as *rrnL* and *rrnS*. The length of the *rrnL* and *rrnS* genes are 923 and 534 bp, respectively, and the AT contents are 62.8% and 64.8%, respectively. The *rrnL* lies between *trnL1* and *trnY*, a same position as observed in mt genomes of *L. thecatus*, *O. luehei* and *P. ambiguus*, but different from the genome in *M. hirudinaceus*, in which lies between *trnY* and *trnL2*, whereas the *rrnS* is situated between *trnS1* and *trnF*, same as in *L. theca-*

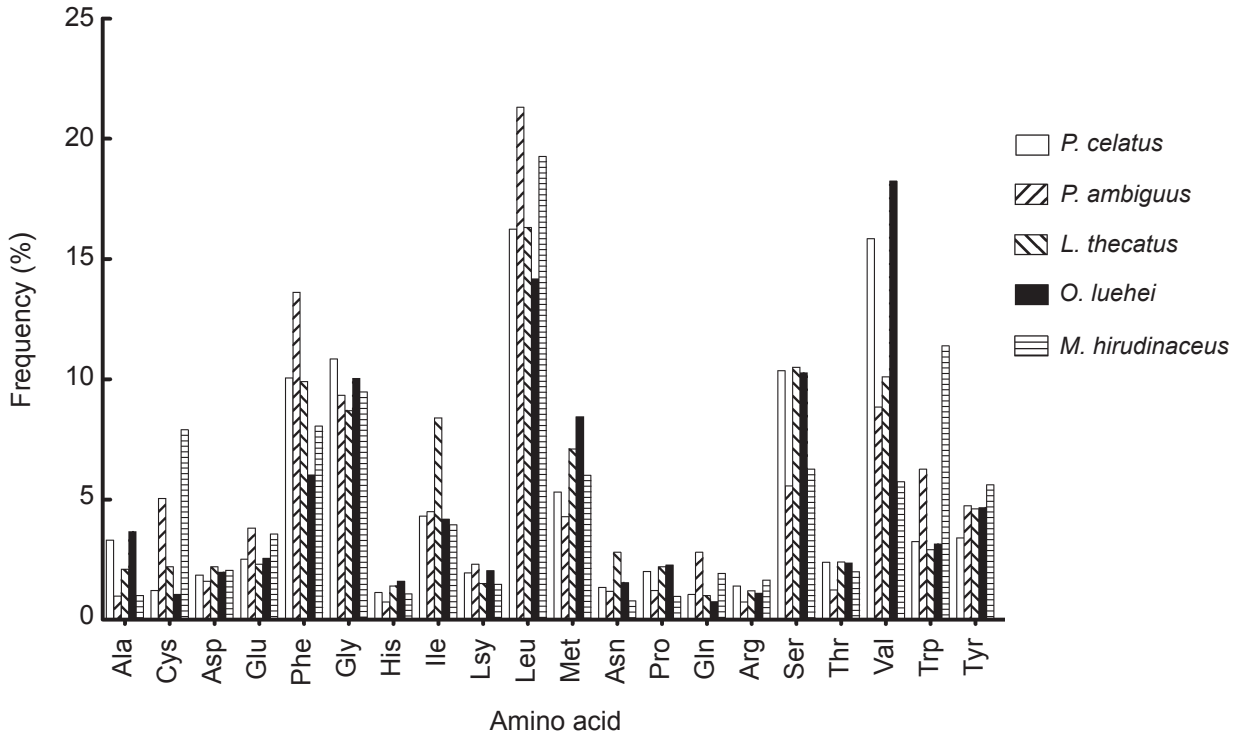

Fig. 2. Comparison in amino acid composition of 12 protein-coding genes in mitochondrial genomes of five acanthocephalan species, *Pallisentis celatus*, *Paratenuisentis ambiguus*, *Macracanthorhynchus hirudinaceus*, *Oncicola luehei* and *Leptorhynchoides thecatus*.

tus, but different in *O. luehei*, *P. ambiguus* and *M. hirudinaceus*, in which it lies between *trnM* and *trnF* (Fig. 4).

Non-coding region

The total length of the non-coding region (NCR) for *P. celatus* is 1 104 bp, which is composed of 16 intergenic spacer sequences, ranging from 1 to 421 bp. Among these, two regions (NCR1 and NCR2) are most prominent in their length. The NCR1 located between *trnV* and *trnK* is 421 bp long, whereas NCR2 between *trnI* and *trnM* is 315 bp long. The A + T contents of NCR1 and NCR2 for the *P. celatus* mt genome are 52.6% and 66.6%, respectively (Table 3).

Phylogenetic analysis

Phylogenetic analysis was performed with complete mt genomes of 43 metazoan species (Fig. 5), with cnidarian *Montastraea annularis* as the outgroup. Five species of acanthocephalans form a clade with high nodal support (1.00 BPP in BI analysis, Fig. 5), among which *Pallisentis celatus* and *Paratenuisentis ambiguus* form a clade of Eoacanthocephala (1.00 BPP), which then correlates with *L. thecatus* (1.00 BPP). *Macracanthorhynchus hirudinaceus* and *O. luehei* form a separate clade within the acanthocephalan clade. Seven species of Bdelloidea are clustered together with a high support value (1.00 BPP), and three species of Monogononta clustered together with a high support (1.00 BPP). The clade of acanthocephalans is more closely related with the clade containing species in the Bdelloidea, and then with species in the Monogononta. It is shown that acanthocephalans together with Bdelloidea and Monogononta form a clade of Syndermata (Fig. 5).

DISCUSSION

The mt genome of *Pallisentis celatus*, an acanthocephalan of the class Eoacanthocephala, was sequenced in the present study, which represents the second mt genome of a species from the same class and one of the five mt genomes for acanthocephalans. The genome shows characters common for other mt genomes in acanthocephalans, particularly in gene content and gene order of protein-coding genes. The genome of *P. celatus*, as well as those of other species of acanthocephalans, *Paratenuisentis ambiguus*, *Leptorhynchoides thecatus*, *Macracanthorhynchus hirudinaceus* and *Oncicola luehei,* are encoded on the same strand and in the same direction, although most metazoan species are encoded on two strands and in two directions (Gissi et al. 2008), and they all contain a total of 36 genes and 12 protein-coding genes, including 22 transfer RNAs (tRNAs) and 2 ribosomal RNAs (rRNAs). The complete mt genome in *P. celatus* (13 855 bp) falls into the range of genome sizes in acanthocephalans (ranging between 13 574 and 14 281 bp) and its overall A + T content (61.5%) is also comparable with those in other acanthocephalans, ranging from 60.2% in *O. luehei* to 71.46% in *L. thecatus* (Steinauer et al. 2005, Gazi et al. 2012, Weber et al. 2013).

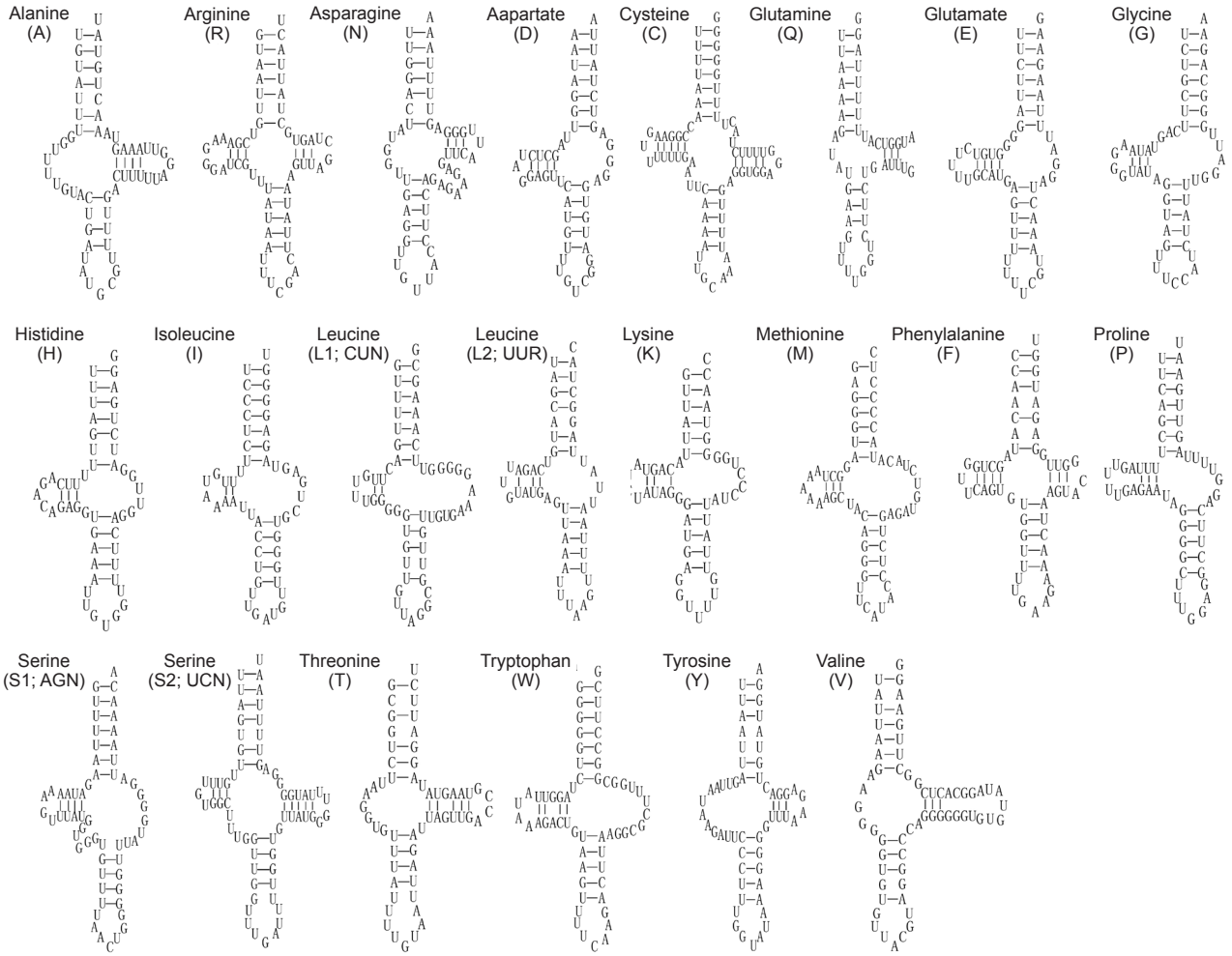

Fig. 3. The predicted secondary structure of 22 tRNAs in mitochondrial genome of *Pallisentis celatus*.

The 12 protein-coding genes in the mt genome of *P. celatus* share features in start and stop codons with those in other acanthocephalans. The start codon, ATG, is observed for *nad6*, *atp6*, *nad4* and *cob*, GTG observed for *cox1*, *nad5*, *nad1*, *cox2* and *cox3*, and ATA, ATT and TTG for *nad3*, *nad4L* and *nad2*, respectively. Six genes use complete stop codons, including four genes with TAG (*nad3*, *nad5*, *cob* and *cox2*) and two genes with TAA (*nad6* and *nad2*), whereas other six genes appear to end in incomplete stop codons with T (*cox1*, *cox3*, *atp6*, *nad4*, *nad4L* and *nad1*). The incomplete stop codon (T) has also been found in *cox1*, *nad1*, *nad4*, *nad5* and *cob* genes for *L. thecatus* (Steinauer et al. 2005), and T for *cox1*, *cox3* and *nad5*, and TA for *nad6* and *cob* for *O. luehei* (Gazi et al. 2012).

Moreover, the character of T-rich codons in protein-coding genes of the *P. celatus* mt genome is also observed in some mt genomes of other invertebrates (Kurabayashi and Ueshima 2000, Lessinger et al. 2000, Steinauer et al. 2005, Gazi et al. 2012). As observed in *Pallisentis celatus,* mt genomes in other acanthocephalans, i.e. *L. thecatus*, *M. hirudinaceus* and *P. ambiguus*, also contain leucin as the most abundant amino acid in mt genomes of acanthocephalans, being 16.38%, 19.27%, 21.31%, respectively, with the exception in *O. luehei* which has valin (18.24%) being the most abundant amino acid and leucin (14.17%) the second most abundant (Fig. 2). However, it would be interesting to analyze if this is a character typical of mt genomes of other acanthocephalans.

Comparison in mt gene orders has often been used as a tool for assessing phylogenetic affinity in metazoans, as gene order may show degree of conservation (Boore and Brown 2000, Lavrov and Lang 2005, Park et al. 2011). However, gene rearrangement was discovered also in acanthocephalans, as found in some other metazoans (Vallès and Boore 2006, Tang and Hyman 2007, Stach et al. 2010). The gene arrangement shows a certain degree of variation in the five mt genomes of acanthocephalans, despite the conserved location of 12 protein-coding genes and the rrnL and rrnS genes. It seems much more likely that *trnK* and *trnV* may have a reciprocal position and same is likely true for *trnS1* and *trnM*. *trnS2*, *trnL1* and *trnL2* may differ in their positions in the genome for different species of acanthocephalans. Nevertheless, the

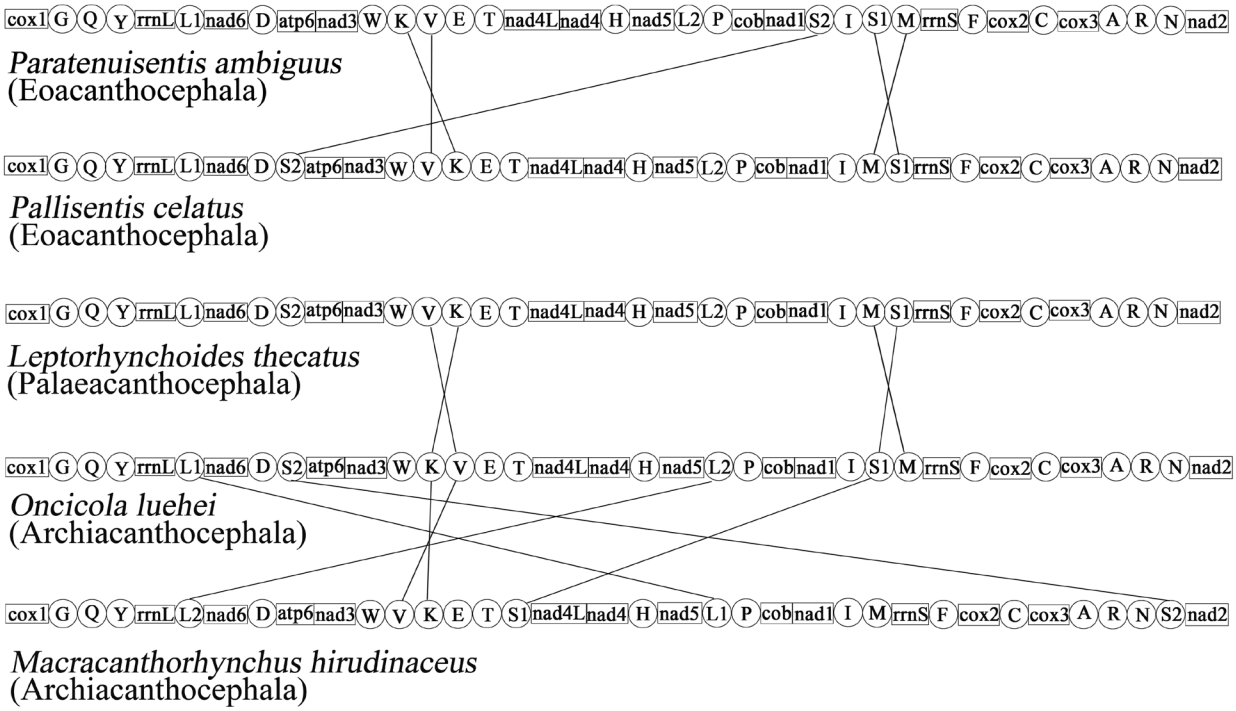

Fig. 4. Linearized comparison in the arrangement of mitochondrial genes in five acanthocephalan species, *Pallisentis celatus*, *Paratenuisentis ambiguus*, *Macracanthorhynchus hirudinaceus*, *Oncicola luehei* and *Leptorhynchoides thecatus*. Gene and genome size are not shown in scale. All genes are transcribed in the same direction (from left to right). The tRNAs are labeled by single-letter abbreviations.

gene order in the mt genome of *P. celatus* is exactly the same as in that of *L. thecatus* (Fig. 4). It is thus indicated that mt genomes in acanthocephalans show certain degree of conservation at least in the gene content and gene order.

Importantly, with the release of mt genome information from a few species of rotifers (Suga et al. 2008, Min and Park 2009, Lasek-Nesselquist 2012, Weber et al. 2013), it becomes possible to analyze the relationship between acanthocephalans and rotifers. Due to the unavailability of some genes in the mt genome of rotifers (Lasek-Nesselquist 2012), 9 protein-coding genes were used in the present phylogenetic analysis. It appears certain that acanthocephalans are closely related with bdelloids and that they together form a clade with high support value, which then correlates with another group, monogononts in rotifers. This relationship has been supported by analyses using other molecular markers, such as 16S rRNA, 18S rRNA and 28S rRNA (Garey et al. 1998, García-Varela and Nadler 2006, Witek et al. 2008), and using mt genes and/or genomes (Steinauer et al. 2005, Min and Park 2009, Fontaneto and Jondelius 2011, Gazi et al. 2012, Weber et al. 2013). Using also mt genomes, Podsiadlowski et al. (2009) included only one species of acanthocephalan (listed as the genus *Leptorhynchoides*) and a monogonont (*Brachionus*) in the phylogenetic analysis of lophotrochozoans, but phylogenetic relationship in the Syndermata was not even dealt with in their research.

With protein-coding genes, the clade containing acanthocephalans and bdelloids is well revealed with high supporting value in the phylogenetic tree, and it can then be concluded, at least tentatively, that acanthocephalans are closely related to bdelloids and are clustered phylogenetically among rotifers. As three taxa, Bdelloidea, Monogononta and Seisonidea, are reported in the Rotifera (Ahlrichs 1997, Melone et al. 1998), more rotifer samples, especially those in the Seisonidea, which contains only three species (Sørensen et al. 2005), should be included in further phylogenetic research on the Syndermata in order to resolve the phylogeny of these taxa.

It is clearly indicated that the classes Eoacanthocephala and Palaeacanthocephala are closely related, forming a clade, with the Archiacanthocephala as a sister group. This phylogenetic relationship is supported by other studies, with mt genome as molecular marker (Gazi et al. 2012, Weber et al. 2013). Other studies also confirmed that the Palaeacanthocephala and Eoacanthocephala form a sister group. Using 18S rRNA, Near et al. (1998) found that the Palaeacanthocephala and Eoacanthocephala are sister taxa. Morphological phylogeny also supported such relationship (Monks 2001). However, other authors (García-Varela et al. 2002, García-Varela and Nadler 2006), using SSU and LSU rDNA and *cox1* sequences, found that the Polyacanthocephala are closely related with the Eoacanthocephala. As mt genome information on any species of the Polyacanthocephala is absent, phylogenetic relation-

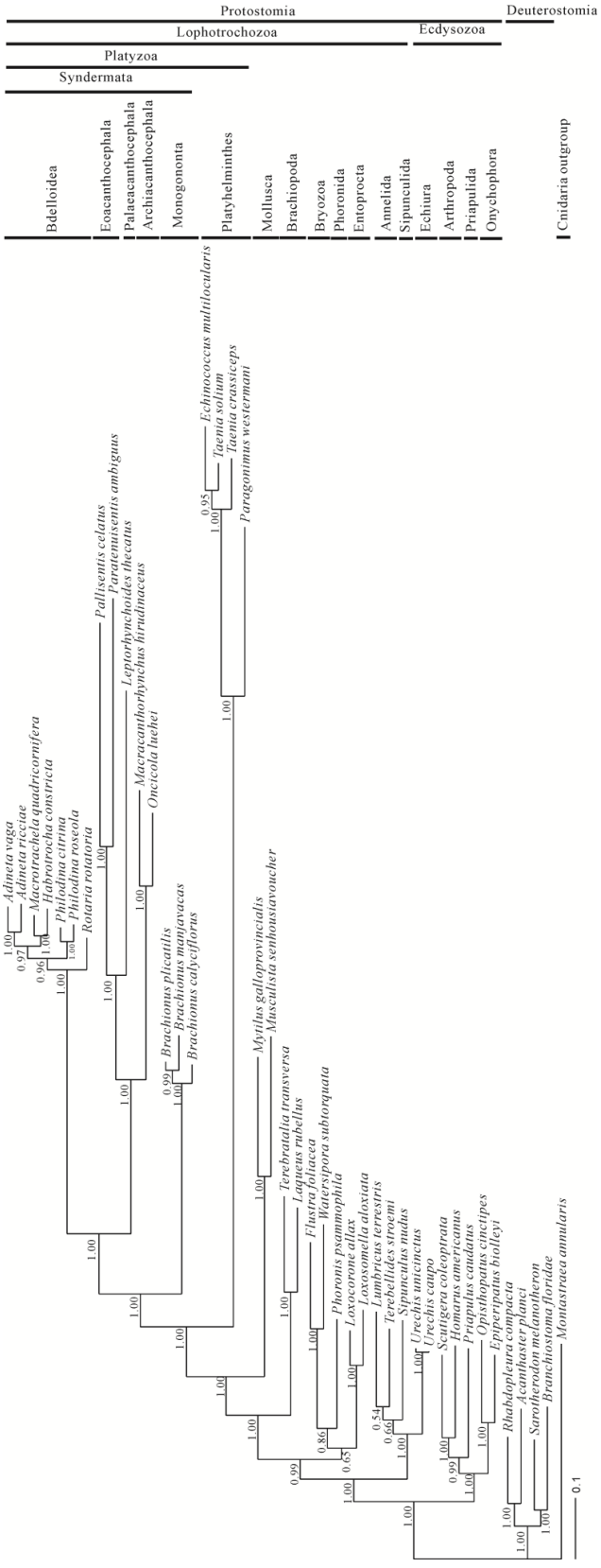

Fig. 5. Bayesian phylogenetic tree inferred from amino acid sequence dataset of 9 protein-coding genes for 43 metazoan mitochondrial genomes. The tree shows the topology based on concatenated data of nine mitochondrial encoded protein sequences (*cox1*, *atp6*, *nad4L*, *nad4*, *nad5*, *cob*, *nad1*, *cox2*, *cox3*). Reconstruction was performed by MrBayes version 3.2 with the protein model of RtRev. The numerical values near internal nodes represent Bayesian posterior probability (BPP) values.

ship among the classes of acanthocephalans awaits to be further resolved.

In addition, the monophyly of Ecdysozoa was supported (1.00 BPP), which is a sister clade to Lophotrochozoa with high supporting value (1.00 BPP), as reported by other authors (Dunn et al. 2008, Min and Park 2009). However, further phylogenetic research should include more taxa in order to resolve phylogenetic relationship among acanthocephalans and rotifers and in-between.

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