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Complete mitochondrial genome of *Membranipora grandicella* (Bryozoa: Cheilostomatida) determined with next-generation sequencing: The first representative of the suborder Malacostegina

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ABSTRACT

Next-generation sequencing (NGS) has proven a valuable platform for fast and easy obtaining of large numbers of sequences at relatively low cost. In this study we use shot-gun sequencing method on Illumina HiSeq 2000, to obtain enough sequences for the assembly of the bryozoan *Membranipora grandicella* (Bryozoa: Cheilostomatida) mitochondrial genome, which is the first representative of the suborder Malacostegina. The complete mitochondrial genome is 15,861 bp in length, which is relatively larger than other studied bryozoans. The mitochondrial genome contains 13 protein-coding genes, 2 ribosomal RNAs and 20 transfer RNAs. To investigate the phylogenetic position and the inner relationships of the phylum Bryozoa, phylogenetic trees were constructed with amino acid sequences of 11 PCGs from 30 metazoans. Two superclades of protostomes, namely Lophotrochozoa and Ecdysozoa, are recovered as monophyletic with strong support in both ML and Bayesian analyses. Somewhat to surprise, Bryozoa appears as the sister group of Chaetognatha with moderate or high support. The relationship among five bryozoans is *Tubulipora flabellaris* + (*M. grandicella* + (*Flustrellidra hispida* + (*Bugula neritina* + *Watersipora subtorquata*))), which supports for the view that Cheilostomatida is not a natural, monophyletic clade. NGS proved to be a quick and easy method for sequencing a complete mitochondrial genome.

1. Introduction

The phylum Bryozoa (Ectoprocta, moss animals) contains more than 6000 described species, globally distributed, and common inhabitants in both marine and fresh water environments. Though the phylum Bryozoa itself is regarded as a monophyletic group, the phylogenetic position and the inner relationships of the phylum are still controversial (Fuchs et al., 2009; Hausdorf et al., 2010). Traditionally, Bryozoa along with Brachiopoda and Phoronida are grouped together as "lophophorates", because they share a special ciliated tentacular feeding apparatus called the lophophore. However, this classification has been questioned by morphological and molecular analyses (Giribet et al., 2000; Helmkampf et al., 2008). Over the past decades, inference of a deeper phylogenetic relationship of metazoans with complete mitochondrial genomes has gained popularity, which not only because mitochondrial genomes are more informative than single genes, but also because they provide some genome level characters, such as gene rearrangement (He et al., 2011; Shen et al., 2011). Despite the importance and biodiversity of the phylum Bryozoa, only 4 bryozoans complete mitochondrial genomes have been determined, including *Tubulipora flabellaris* (Stenolaemata: Tubuliporida), *Watersipora subtorquata* (Gymnolaemata: Cheilostomatida), *Bugula neritina* (Gymnolaemata: Cheilostomatida) and *Flustrellidra hispida* (Gymnolaemata: Ctenostomata) (Waeschenbach et al., 2006; Jang and Hwang, 2009; Sun et al., 2009).

DNA sequencing is now gradually moving from Sanger-based technology to next-generation sequencing (NGS) approaches (Metzker, 2010). NGS has proven a valuable platform for quick and easy obtaining of large numbers of sequences at relatively low cost. NGS is having a significant impact on fields related to genomics and has been presented as a valuable method to obtain animal mitochondrial genome sequences (Feldmeyer et al., 2010; Jex et al., 2010; Nabholz et al., 2010; Webb and Rosenthal, 2011).

Abbreviations: *atp6* and *8*, ATPase subunits 6 and 8; *cox1–3*, cytochrome *c* oxidase subunits I–III; PCGs, protein coding genes; *cob*, cytochrome *b*; mtDNA, mitochondrial DNA; *nad1–6* and *4 L*, NADH dehydrogenase subunits 1–6 and 4 L; *srRNA* and *lrRNA*, small and large subunits ribosomal RNA; tRNA, transfer RNA; L_1, $tRNA^{Leu(CUN)}$; L_2, $tRNA^{Leu(UUR)}$; S_1, $RNA^{Ser(AGN)}$; S_2, $tRNA^{Ser(UCN)}$.

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In this study we make use of shotgun sequencing method on Illumina Genome Analyzer IIx, to obtain enough mitochondrial sequences for the assembly of the bryozoan *Membranipora grandicella* (Bryozoa: Gymnolaemata: Cheilostomatida) mitochondrial genome, which is the first representative from the suborder Malacostegina. Furthermore, we described the gene organization, codon usage and gene order of *M. grandicella* mitochondrial genome. In addition, the phylogenetic position and the inner relationships of the phylum Bryozoa were analyzed based on 30 complete mitochondrial genomes.

2. Materials and methods

2.1. Sample collection and genomic DNA extraction

Colonies of *M. grandicella* were collected from Gaogong Island (Lianyungang, China). After washed with distilled water for three times, the samples were stored in 100% ethanol immediately. Total genomic DNA was isolated using a DNeasy tissue kit (Qiagen) following the manufacturer's protocol.

2.2. Sequencing using Illumina Hiseq2000 and assembled using Abyss

The sequenced sample was prepared according to the Illumina protocols. Briefly, genomic DNA was fragmented by nebulization, and the fragmented DNA was repaired with an 'A' ligated to the 3′ end. Illumina adapters were then ligated to the fragments, and the sample was size selected aiming for approximate 500 bp product. The size-selected product was PCR amplified and validated using the Agilent Bioanalyzer. Samples were sequenced using the Illumina HiSeq 2000, following the Illumina supplied protocols. The short reads were assembled (kmer = 31) using Abyss (Simpson et al., 2009).

2.3. PCR amplification and sequencing

PCR reactions were conducted in a Mastercycler gradient machine (Eppendorf AG Inc.) in a total volume of 25.0 μL, containing 17.3 μL sterile distilled H_2O, 2.5 μL 10 × LA PCR buffer II (Mg^{2+} plus, Takara), 2.0 μL dNTP (2.5 mM each), 1.0 μL each primer (10 μM), 0.2 μL *Taq* polymerase (5 U/μL, Takara), and 1.0 μL DNA template. PCR condition was as follows: 94 °C for 5 min, 35 cycles of 94 °C for 45 s, 60 °C for 45 s and 72 °C for 1 min, and 72 °C for 10 min. PCR products were purified using the Montage PCR Cleanup Kit (Millipore) and directly sequenced with ABI 3730 × 1 DNA Analyzer. Sequences were assembled and analyzed using the software DNAMAN.

2.4. Gene annotation and sequence analysis

The boundaries of protein-coding genes and rRNA genes were initially identified via DOGMA (Wyman et al., 2004), and then refined by alignment with mitochondrial genomes of studied bryozoans. Most tRNA genes were identified using tRNAscan-SE 1.21 (Lowe and Eddy, 1997) with the invertebrate mitochondrial genetic code and 'mito/chloroplast' source. Remaining tRNA genes were identified by inspecting sequences for tRNA-like secondary structures and anticodons manually. Gene map of mitochondrial genome of *M. grandicella* was drawn by OGDRAW (Lohse et al., 2007). Codon usage in 13 PCGs of the mitochondrial genome was estimated with DnaSP 4.10.7 (Rozas et al., 2003).

2.5. Phylogenetic analyses

A broad representation of taxa was chosen for phylogenetic analyses in order to investigate the phylogenetic position and the inner relationships of the phylum Bryozoa. Amino acid sequences from 11 PCGs (excluding *atp6* and *atp8*) were individually aligned using Clustal X (Thompson et al., 1997) under default settings. The amino acid dataset was analyzed using the Bayesian inference method by MrBayes (Ronquist and Huelsenbeck, 2003; Altekar et al., 2004) and the Maximum Likelihood method by PhyML (Guindon and Gascuel, 2003), respectively.

Both analyses employed the mtREV + G + I amino acid substitution model. For the Bayesian analysis, four chains of 1,000,000 generations were run sampling every 1000 generations. The first 250 trees were excluded from the analysis as "burn-in", while the remaining 750 trees were used to estimate the Bayesian posterior probabilities (BPP). For the ML analysis, 1000 bootstraps were used to estimate the nodal reliability (BPM).

3. Results and discussion

3.1. Sequences alignment and PCR primers

DNA samples were sequenced using the Illumina HiSeq 2000 and the short reads were assembled using Abyss. Then BLAST searches to known mitochondrial genomes and partial *lrRNA* gene sequence obtained previously, two contigs (12,540 bp and 2983 bp in length, respectively) were determined as partial sequences of *M. grandicella* mitochondrial genome. To close the gaps between two contigs, two pairs of primers (Mgr-cg1-F12415: 5′-CTT TGA CTT AGC AGA GGG AGA ATC-3′/Mgr-cg2-R101: 5′-GTT GAT CAT ATC GTA GGC GAG GGT-3′; Mgr-cg2-F2838: 5′-AAC CAA TAA CCG CAC AAC TTA CAT-3′/Mgr-cg1-R136: 5′-TTA GTA GGG ATG GAA GGG AAA ATA-3′) near the gap regions were designed.

3.2. General features and differences of bryozoan mitochondrial genomes

The length of *M. grandicella* mitochondrial genome is 15,861 bp, which is larger than the other four studied bryozoan mitochondrial genomes (Table 1). It contains 35 genes, which is lack of 2 transfer RNA genes ($tRNA^{Asn}$ and $tRNA^{Tyr}$) in contrast to typical metazoan mitochondrial genomes (Fig. 1). A total of 1771 bp of non-coding sequences were observed at 27 intergenic regions. The largest one is 1057 bp in length between *nad4* and $tRNA^{Gln}$, which is suggested to be a putative control region due to its high A + T content and potential secondary structure. The entire *M. grandicella* mitochondrial genome sequence was deposited in GenBank with accession number JF957859.

The overall A + T content of *M. grandicella* (70.8%), which is similar to other studied bryozoans (Table 1). The lengths of five studied bryozoans mitochondrial genomes are ranging from 13,026 bp (*F. hispida*) to 15,861 bp (*M. grandicella*), which result from length differences of *lrRNA*, *srRNA* and protein-coding genes. Genomic characteristics of five studied bryozoans mitochondrial genomes are shown in Table 1.

3.3. Protein-coding genes and codon usage

Protein-coding genes were identified with DOGMA and subsequently aligned with the mitochondrial genomes of other studied bryozoans. All 13 PCGs encoding on the α strand. Mitochondrial genes commonly use several alternatives to ATN as start codons. However, all PCGs in the mitochondrial genome of *M. grandicella* initiate with the ATD start codon (ATG, ATT or ATA). All open reading frames are terminated with TAA, which is different from other studied bryozoans mitochondrial PCGs.

The pattern of codon usage in the *M. grandicella* mitochondrial genome was also studied. There are 3577 codons for 13 PCGs in total. The most frequently used amino acids are Leu (14.65%), followed by Ser (9.73%), Phe (8.16%) and Met (7.63%) (Table 2). The overall A + T content of PCGs is 68.9%, and at the third positions it elevates to 77.6%, which are within the range observed in other four bryozoans (Table 1).

Table 1
Genomic characteristics of mitochondrial genomes of five bryozoans.

Species	GenBank accession no.	α-strand		Protein-coding genes			*lrRNA* gene		*srRNA* gene		tRNA genes	
		Length (bp)	A+T (%)	Number of amino acid	A+T (%)		Length (bp)	A+T (%)	Length (bp)	A+T (%)	Length (bp)	A+T (%)
					All positions	Third codon positions						
M. grandicella	JF957859	15,861	70.8	3577	68.9	77.6	1273	74.9	871	71.2	1279	74.0
T. flabellaris	NC_015646	13,763	73.4	3310	72.6	85.0	1127	75.3	732	71.7	1265	77.3
W. subtorquata	NC_011820	14,144	70.6	3581	69.8	78.5	1132	74.5	770	68.8	1327	73.5
B. neritina	NC_010197	15,433	70.0	3657	68.7	74.8	1327	71.9	840	69.0	1318	73.2
F. hispida	NC_008192	13,026	58.9	3350	58.5	61.8	670	63.1	653	53.3	1163	62.9

3.4. Ribosomal and transfer RNA genes

Both rRNA genes are encoded on the α strand. The *lrRNA* gene lies between $tRNA^{Ser(UCN)}$ and $tRNA^{Met}$, while the *srRNA* gene lies between $tRNA^{Met}$ and $tRNA^{Phe}$. The lengths of *srRNA* and *lrRNA* are 871 and 1273 bp, respectively. The A+T contents are 71.2% and 74.9% for *srRNA* and *lrRNA*, respectively, which are within the range observed in other studied bryozoans (Table 1). Most tRNA genes were identified using tRNAscan-SE 1.21, and the remaining were identified by inspecting sequences for tRNA-like secondary structures and anticodons. The *M. grandicella* mtDNA encodes 20 tRNA genes, ranging in size from 55 ($tRNA^{Cys}$) to 71 ($tRNA^{Phe}$) nucleotides.

3.5. Gene arrangement

Comparison of gene arrangement may be a useful approach for phylogenetic researches, especially when some ancestral relationships were focused (Boore and Brown, 1998). Comparison of mitochondrial gene arrangement among five bryozoans and other metazoans showed that bryozoan mitochondrial genomes have experienced drastic rearrangements (Fig. 2) (Waeschenbach et al., 2006; Jang and Hwang, 2009; Sun et al., 2009). In this study, we identified that the mitochondrial genome of *M. grandicella* has a distinct gene order when compared with other metazoans. Gene order will be useful for inferring the phylogenetic position and inner

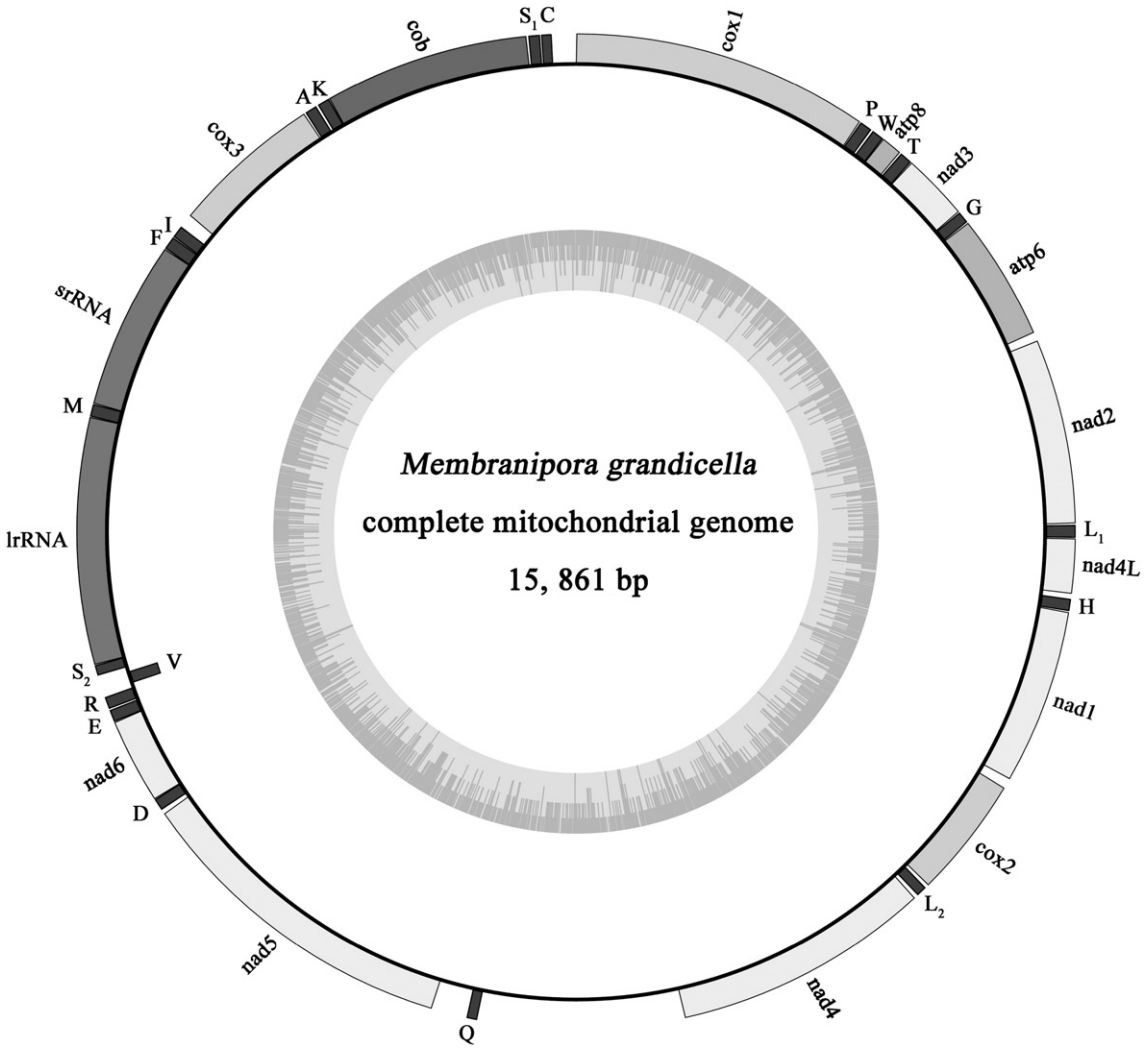

Fig. 1. Gene map of mitochondrial genome of *M. grandicella* (Bryozoa: Cheilostomatida).All genes are encoded on the α-strand, except for one transfer RNA gene ($tRNA^{Val}$). Inner ring shows GC content graph.

Table 2
Codon usage in 13 protein-coding genes of *M. grandicella* (Bryozoa: Cheilostomatida).

Phe	UUU	215	6.01	Ser	UCU	106	2.96	Tyr	UAU	96	2.68	Cys	UGU	24	0.67
	UUC	77	2.15		UCC	45	1.26		UAC	65	1.82		UGC	19	0.53
Leu	UUA	229	6.40		UCA	52	1.45	End	UAA	13	0.36	Trp	UGA	69	1.93
	UUG	38	1.06		UCG	3	0.08		UAG	0	0.00		UGG	16	0.45
Leu	CUU	93	2.60	Pro	CCU	67	1.87	His	CAU	42	1.17	Arg	CGU	8	0.22
	CUC	36	1.01		CCC	26	0.73		CAC	25	0.70		CGC	1	0.03
	CUA	112	3.13		CCA	35	0.98	Gln	CAA	51	1.43		CGA	34	0.95
	CUG	16	0.45		CCG	6	0.17		CAG	5	0.14		CGG	2	0.06
Ile	AUU	250	6.99	Thr	ACU	104	2.91	Asn	AAU	97	2.71	Ser	AGU	22	0.62
	AUC	69	1.93		ACC	46	1.29		AAC	58	1.62		AGC	5	0.14
Met	AUA	248	6.93		ACA	68	1.90	Lys	AAA	105	2.94		AGA	97	2.71
	AUG	25	0.70		ACG	5	0.14		AAG	16	0.45		AGG	18	0.50
Val	GUU	57	1.59	Ala	GCU	72	2.01	Asp	GAU	37	1.03	Gly	GGU	29	0.81
	GUC	18	0.50		GCC	60	1.68		GAC	27	0.75		GGC	20	0.56
	GUA	108	3.02		GCA	56	1.57	Glu	GAA	73	2.04		GGA	111	3.10
	GUG	11	0.31		GCG	7	0.20		GAG	12	0.34		GGG	20	0.56

relationships of the phylum Bryozoa when more mitochondrial genome data from Bryozoa are available.

3.6. Phylogenetic analysis

To investigate the phylogenetic position and the inner relationships of the phylum Bryozoa, phylogenetic trees were constructed with amino acid sequences of 11 PCGs from 30 metazoans. In this study, both ML and Bayesian analyses lead to very similar phylogenies (Fig. 3). Two superclades of protostomes, namely Lophotrochozoa and Ecdysozoa were recovered as monophyletic with strong support in our analyses (BPP = 100, BPM = 100).

Analyses based on molecular data have confirmed the protostome affinity of Bryozoa, but failed to resolve its phylogenetic position within Protostome. Analyses based on different types of data have suggested bryozoans are basal protostomes (Giribet et al., 2000), basal lophotrochozoans (Passamaneck and Halanych, 2004) or members of lophotrochozoans (Waeschenbach et al., 2006; Helmkampf et al., 2008; Sun et al., 2009). In current analyses, Bryozoa grouped within the Lophotrochozoa, which is consistent with analyses based on multigenes (Helmkampf et al., 2008) and mitochondrial genomes (Waeschenbach et al., 2006; Jang and Hwang, 2009; Sun et al., 2009). Chaetognatha (arrow worms) is another phylum with enigmatic phylogenetic position but which has been treated traditionally as a deuterostome (Zrzavý et al., 1998) and later grouped with the protostomes, most times to the Ecdysozoa based on molecular analyses (Helmkampf et al., 2008). In our analyses Chaetognatha groups within Lophotrochozoa instead of Ecdysozoa, which is similar to previous study (Sun et al., 2009). Somewhat to surprise, it appears as sister group of Bryozoa with moderate or high support in both analyses (BPP = 99, BPM = 51). However, more evidence is needed to clarify the relationship between two groups.

Five bryozoans cluster a monophyletic clade, which support the phylum Bryozoa is a natural group. The Gymnolaemata is the most diverse class within Bryozoa and is usually subdivided into two orders, the softbodied Ctenostomata and the calcified Cheilostomatida. The monophyly of these two orders has been questioned by a number of investigators (Fuchs et al., 2009). Based on mitochondrial genomic evidence, the relationship among five bryozoans is *T. flabellaris* + (*M. grandicella* + (*F. hispida* + (*B. neritina* + *W. subtorquata*))), which supports for the view that Cheilostomatida is not natural, monophyletic clade. Meanwhile, the bootstrap value is very high (Fig. 3), so further analyses with more mitochondrial genomes from Cheilostomatida and related groups are needed. Given many questions in the phylogenetic relationships of the phylum Bryozoa remain unanswered, it is desirable

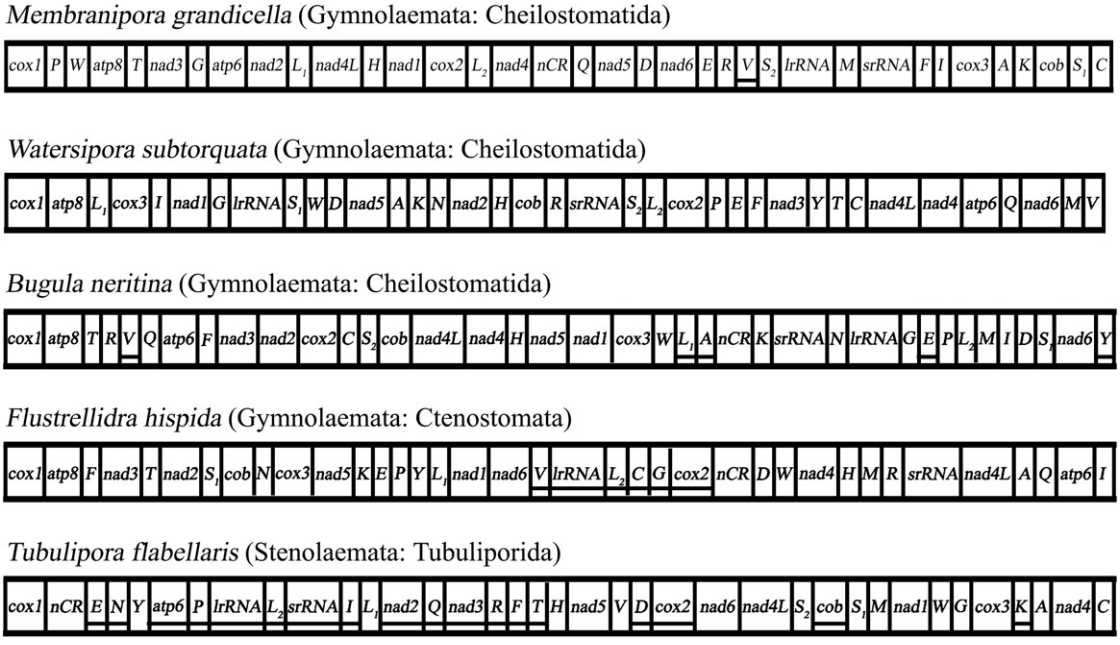

Fig. 2. Linearized representation of mitochondrial gene arrangement for five studied bryozoans. All genes are encoded on α-strand except those indicated by underlining, which are encoded on β-strand.

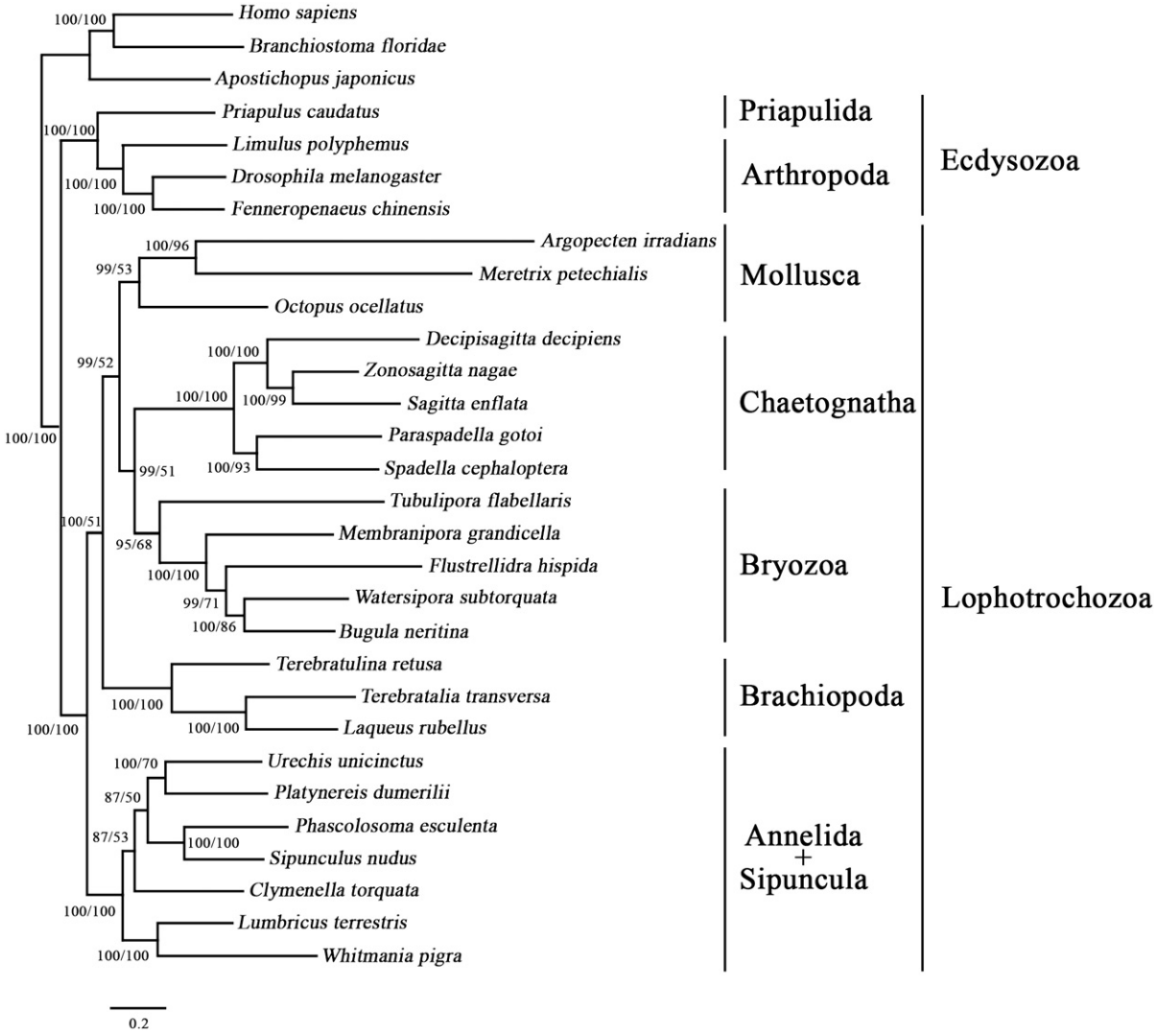

Fig. 3. Phylogenetic tree based on 11 concatenated mitochondrial PCGs. Tree topologies produced by the two methods were very similar. Nodal support indicated by Bayesian posterior probabilities (BPP) and Bootstrap value (BPM), respectively.

to increase the resolution by adding more mitochondrial genome data from the phylum and related groups.

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