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The complete mitochondrial genome of *Watersipora subtorquata* (Bryozoa, Gymnolaemata, Ctenostomata) with phylogenetic consideration of Bryozoa

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ABSTRACT

The phylogenetic position of the Bryozoa has long been controversial. In this paper, we have determined the complete mitochondrial genome of the *Watersipora subtorquata* (Bryozoa, Gymnolaemata, Ctenostomata). It is a circular molecule of 14,144 bp, relatively small compared with most other metazoan mitochondrial genomes, and bears some unusual features. All genes in the *W. subtorquata* mtDNA, unlike those in two bryozoan mtDNAs and most other metazoan mtDNAs published previously, are transcribed from the same strand. It has a unique gene order which differs radically from that of other metazoans. Drastic gene rearrangements were also found among bryozoan mtDNAs. To investigate the phylogenetic position of Bryozoa, analyses based on amino acid sequences of 11 protein-coding genes (excluding *atp6* and *atp8*) from 25 metazoan mtDNAs were made utilizing ML and Bayesian methods. Lophotrochozoa was recovered as monophyletic with strong support in our analyses. Lophophorate was undoubted within Lophotrochozoa, but appears as polyphyletic, which indicates that the lophophores of this group may be of different origin. The existence of Phoronozoa was rejected. Our analyses indicated that Phoronida is more closely related to Annelid instead of Brachiopod. Chaetognatha appeared as the sister group of Bryozoa and they formed a clade together with strong support. More evidence is needed to clarify the relationship of these two phyla.

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1. Introduction

Bryozoans (= Ectoprocta, Polyzoa) are small, sessile, aquatic, colonial animals, and commonly known as moss animals, with approximately 5000 living species (Ruppert et al., 2004). Traditionally, Bryozoa has been divided into three classes: the Stenolaemata all inhabit the marine environment, the Gymnolaemata are mostly marine but have a few freshwater members in the order Ctenostomata, and the Phylactolaemata which are exclusively freshwater (Francis, 2001).

Traditionally, Bryozoa along with Brachiopoda and Phoronida are grouped together as "lophophorates", because they share a special ciliated tentacular feeding apparatus called the lophophore (Hyman, 1959; Brusca and Brusca, 2002; Willmer, 1990). Lophophorates have embryological and morphological characters of both protostomes and deuterostomes (Willmer, 1990; Halanych, 1996), resulting in the ambiguity of the phylogenetic position of this group. They have been considered as protostomes, deuterostomes or intermediate group in the past. Based on 18S rDNA data, Halanych et al. (1995) proposed that lophophorates are derived protostomes, and along with mollusks, annelids and other phyla form a node-based clade Lophotrochozoa. Lophotrochozoa is well supported by multiple sets of data, e.g., rDNA (Halanych et al., 1995; Mackey et al., 1996; Conway-Morris et al., 1996; Mallatt and Winchell, 2002), *Hox* gene (de Rosa et al., 1999; Passamaneck and Halanych, 2004), ATPase α-subunit gene (Anderson et al., 2004), mitochondrial genome (Stechmann and Schlegel, 1999; Waeschenbach et al., 2006) and multigenes (Helmkampf et al., 2007). However, the branching order of phyla within Lophotrochozoa still remains unclear (Adoutte et al., 2000; Halanych, 2004).

Bryozoa is one of the most puzzling phyla in phylogenetic studies. Analyses based on different types of evidence have suggested

Abbreviations: *atp6* and *8*, ATPase subunits 6 and 8; bp, base pair; *cox1–3*, cytochrome *c* oxidase subunits I–III; PCGs, protein-coding genes; nCR, non-coding region; *cob*, cytochrome *b*; mtDNA, mitochondrial DNA; *nad1–6* and *4L*, NADH dehydrogenase subunits 1–6 and 4L; *srRNA* and *lrRNA*, small and large subunits ribosomal RNA; *tRNA*, transfer RNA; L_1, $tRNA^{Leu(CUN)}$; L_2, $tRNA^{Leu(UUR)}$; S_1, $tRNA^{Ser(AGN)}$; S_2, $tRNA^{Ser(UCN)}$; BPP, Bayesian posterior probability; BP, bootstrap probability of Maximum Likelihood.

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bryozoans are basal protostomes (Giribet et al., 2000; Giribet and Ribera, 2000), basal lophotrochozoans (Peterson and Eernisse, 2001; Passamaneck and Halanych, 2004) or members of lophotrochozoans (Helmkampf et al., 2007; Waeschenbach et al., 2006). Moreover, though traditionally grouped with Brachiopoda and Phoronida as lophophorates, this classification has been questioned by morphological and molecular analyses (Nielsen, 2002). Nielsen (1985, 1987) proposed based on morphological characters that Lophophorata is polyphyletic, brachiopods and phoronids tend to form a clade (Phoronozoa), while bryozoans are excluded from them. Analyses of 18S rDNA (Halanych et al., 1995; Mackey et al., 1996; Eernisse, 1997), morphology and 18S rDNA (Zrzavy et al., 1998, Giribet et al., 2000; Peterson and Eernisse, 2001) and morphological characters (Nielsen et al., 1996; Nielsen, 1985, 1987, 2001) supported this viewpoint. Cohen (2000) even proposed that the phylum Phoronida should be reduced to a subphylum within the Brachiopoda based on 18S rDNA data. However, the existence of Phoronozoa was rejected by analyses based on mitochondrial protein-coding genes (Waeschenbach et al., 2006) and multigene data (Helmkampf et al., 2007). The relationship of these three phyla remains controversial.

The comparison of complete mitochondrial genomes has become a powerful tool for inferring metazoan phylogenetic relationships, not only because they are more informative than single genes, but also they have some genome level characters, such as gene order (Boore et al., 1995, 2005). There are over one thousand complete mtDNAs available; however, the taxonomic sampling has been highly biased. Bryozoan species are poorly sampled, and until now, complete mitochondrial genomes have been determined only for two bryozoans, *Flustrellidra hispida* (Waeschenbach et al., 2006) and *Bugula neritina* (Jang, unpublished).

In this paper, we reported the complete mitochondrial genome of the cheilostome bryozoan *Watersipora subtorquata* (Bryozoa, Gymnolaemata, Ctenostomata). To investigate the phylogenetic position of Bryozoa, phylogenetic analyses based on amino acid sequences of 11 protein-coding genes (excluding *atp6* and *atp8*) from 25 metazoan mitochondrial genomes were made utilizing ML and Bayesian methods.

2. Materials and methods

2.1. Sample collection and DNA extraction

Colonies of *W. subtorquata* attached on seaweed surfaces were collected from Qingdao Huiquan Beach (Qingdao, China), and stored in 100% ethanol immediately after washing with distilled water for three times. Total genomic DNA was isolated using a DNeasy tissue kit (Qiagen) following the manufacturer's protocol.

Table 1
Primers used in amplifying and sequencing of *W. subtorquata* mitochondrial genome.

Name	Sequences	References
Universal		
LCO	GGTCAACAAATCATAAAGATATTGG	Folmer et al., 1994
HCO	TAAACTTCAGGGTGACCAAAAAATCA	
cox2F	AAGCWAATWGGNCATCARTGRTATTG	Burger et al., 2007
cox2R	CTCCRCATATTTCNGARCATTGNCC	
cox3F	TGGTGGCGAGATGTKKTNCGNGA	Burger et al., 2007
cox3R	ACWACGTCKACGAAGTGTCARTATCA	
16SarF	CGCCTGTTTATCAAAAACAT	Palumbi et al., 1991
16SbrR	CCGGTCTGAACTCAGATCACGT	
Long PCR		
WScox1LF	TCCCATTATTTGTTTGAGCCGTA	This study
WScox1LR	AGTTCATCCAGTCCCTGCCCCTC	
WScox2LF	TGCTGTTCCTGGACGACTAAATC	
WScox2LR	GATTTAGTCGTCCAGGAACAGCA	
WScox3LF	ATCAAGAACACACAAAGACACCC	
WScox3LR	CTCCTACAGGTGGTCAAGTCGCC	
WS16sLF	CTAATTGAAGGTAAGGATTGCGAC	
WS16sLR	AGATTACGCTGTTATCCCTAAGG	

Table 2
Mitochondrial genome profiles of *W. subtorquata*.

Gene	Strand	Position	Size(bp)	Start codon	Stop codon	Intergenic nucleotides[a]
cox1	+	1–1548	1548	ATC	TAA	
ATP8	+	1559–1654	100	ATG	TAA	10
tRNA$^{Leu(CUN)}$	+	1652–1708	57			−3
cox3	+	1717–2502	786	ATA	TAG	8
tRNA^{Ile}	+	2501–2560	60			−2
nad1	+	2561–3483	923	ATG	TA[b]	0
tRNA^{Gly}	+	3483–3544	62			−1
lrRNA	+	3545–4676	1132			0
tRNA$^{Ser(AGN)}$	+	4679–4734	56			2
tRNA^{Trp}	+	4735–4801	67			0
tRNA^{Asp}	+	4801–4863	63			−1
nad5	+	4867–6546	1680	ATG	TAG	3
tRNA^{Ala}	+	6544–6598	55			−3
tRNA^{Lys}	+	6597–6659	63			−2
tRNA^{Asn}	+	6664–6730	67			4
nad2	+	6731–7693	963	ATG	TAA	0
tRNA^{His}	+	7694–7754	61			0
cob	+	7755–8849	1095	ATG	TAA	0
tRNA^{Arg}	+	8848–8914	67			−2
srRNA	+	8915–9684	770			0
tRNA$^{Ser(UCN)}$	+	9696–9748	53			11
tRNA$^{Leu(UUR)}$	+	9748–9803	56			−1
cox2	+	9803–10478	676	ATG	T[b]	−1
tRNA^{Pro}	+	10479–10541	63			0
tRNA^{Glu}	+	10541–10602	62			−1
tRNA^{Phe}	+	10602–10666	65			−1
nad3	+	10667–11018	352	ATG	T[b]	0
tRNA^{Tyr}	+	11019–11069	51			0
tRNA^{Thr}	+	11070–11133	64			0
tRNA^{Cys}	+	11134–11193	60			0
nad4L	+	11197–11490	294	ATG	TAA	3
nad4	+	11511–12824	1314	ATA	TAA	20
atp6	+	12830–13508	679	ATG	T[b]	5
tRNA^{Gln}	+	13505–13564	60			−4
nad6	+	13567–14028	462	ATA	TAA	2
tRNA^{Met}	+	14027–14089	63			−2
tRNA^{Val}	+	14093–14144	52			3

[a] Numbers correspond to the nucleotides separating different genes. Negative numbers indicate overlapping nucleotides between adjacent genes.
[b] Termination codons completed via polyadenylation.

2.2. PCR amplification and sequence determination

Initially, partial sequences for the *cox1*, *cox2*, *cox3* and *srRNA* genes were amplified via PCR using universal primers (Table 1). Amplification employed an initial denaturation step of 95 °C for 2 min, 38 cycles of 94 °C for 20 s, 50 °C for 50 s and 72 °C for 2 min, and a final extension cycle of 72 °C for 5 min. PCR products were purified using the Montage PCR Cleanup Kit (Millipore) and directly sequenced with ABI 3730x1 DNA Analyzer.

Long PCR primers were designed according to sequences obtained above (Table 1), and the complete mitochondrial genome were amplified in four fragments: WS*cox1*LF-WS*cox3*LR, WScox3LF-WS16sLR, WS*cox2*LF-WS*cox1*LR, and WS16sLF-WS*cox2*LR, with the PCR products of 1.5 kb, 2.3 kb, 4.4 kb and 5.8 kb in length, respectively. The amplification included an initial denaturation step of 95 °C for 2 min, 36 cycles of 94 °C for 20 s, 50 °C for 50 s and 65 °C for 1 min/kb, and a final extension cycle of 72 °C for 10 min.

The 1.5 kb, 2.3 kb and 4.4 kb fragments were purified and sequenced directly using primer walking strategy. The 5.8 kb fragment was sheared into fragments of 1–3 kb and ligased to pUC19 vector (Fermentas) after blunt ended with T4 DNA polymerase (NEB). Plasmids were extracted and sequenced with M13 primers using ABI 3730x1 DNA Analyzer. Sequences were assembled and analyzed using phredPhrap (Ewing and Green, 1998; Ewing et al., 1998). All

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