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Comparative mitogenomic analyses reveal cryptic diversity of the bryozoan *Bugula neritina* Linnaeus, 1758, in the Yellow Sea

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Abstract. The bryozoan *Bugula neritina* Linnaeus, 1758, is known to be a complex of three cryptic species, namely Types S, D and N. In the present study, we determined the mitochondrial genomic features of *B. neritina* sampled from Qingdao (QD), China, and compared them with those of the genome reported for a specimen sampled from Taean Gun (TG), South Korea. The *B. neritina* QD mitochondrial genome has a duplication of *trn*L_2 and lacks *trn*V compared with *B. neritina* TG. Five tRNAs (*trn*L_1, *trn*A, *trn*E, *trn*Y and *trn*V) are encoded on the light-strand of *B. neritina* TG mitochondrial genome, but only one tRNA (*trn*A) is identified on the *B. neritina* QD mitochondrial light strand. In contrast to the *B. neritina* TG mitochondrial genome, deletion of *trn*V and duplication of *trn*L_2 are identified in the *B. neritina* QD mtDNA, and three tRNAs (*trn*E, *trn*L_1 and *trn*Y) exhibit translocation and inversion. The genetic distance in 12 protein-coding genes (PCGs) (amino acids) between the two *B. neritina* was 0.079, which is higher than interspecific values of 10 lophotrochozoan genera selected for comparison. All these results from comparison between the two *B. neritina* clearly indicate that they are genetically distinct species. Phylogenetic analysis based on *cox*1 and *lrRNA* sequences suggested that *B. neritina* TG belongs to the widely distributed Type S and *B. neritina* QD represents a new cryptic type closely related to Type N. This new type is designated as Type Y, for its occurrence in the Yellow Sea. The geographical range of the different types of *B. neritina* awaits further studies.

Additional keywords: genetic distance, gene rearrangement, mitogenome.

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Introduction

The bryozoan *Bugula neritina* Linnaeus, 1758, attracts significant interest as an important source of the cytotoxic chemicals bryostatins (Davidson *et al.* 2001; Lopanik *et al.* 2004; Manning *et al.* 2005; Sharp *et al.* 2007; Lei *et al.* 2010), which are potential anti-cancer agents now under clinical investigation. Previous studies of the mitochondrial gene *cox*1 have suggested that *B. neritina* is a complex of three cryptic species, namely Types S (shallow), D (deep) and N (North Atlantic) (McGovern and Hellberg 2003; Mackie *et al.* 2006). The three types have different distributional ranges, with Types N and D being geographically restricted, Type N occurring in the shallow waters of the north-western Atlantic (Delaware and Connecticut, USA), and Type D inhabiting the waters of the north-eastern Pacific (California, USA) at depths between 0 and 24 m (Davidson and Haygood 1999; Fehlauer-Ale *et al.* 2014). Type S is considered to be widespread in tropical, subtropical and temperate regions, occurring throughout Australia and in the Yellow Sea, the East and South China Seas, Curaçao, Hawaii, and England, for example (Fehlauer-Ale *et al.* 2014). All available data indicate that Type S is the only type of *B. neritina* known to occur in the Yellow Sea (Davidson and Haygood 1999; McGovern and Hellberg 2003; Mackie *et al.* 2006; Fehlauer-Ale *et al.* 2014).

The importance of recognising cryptic species is obvious. Researches on cryptic species have increased quickly over the past two decades, which has, in large part, resulted from the increasing availability of molecular data (Bickford *et al.* 2007). In recent years, detection of cryptic species on the basis of

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complete mitochondrial genomes has become increasingly popular (Iannelli *et al.* 2007; Krzywinski *et al.* 2011; Gasser *et al.* 2012; Meng *et al.* 2013; Burger *et al.* 2014; Griggio *et al.* 2014; Shen *et al.* 2014). The approach is much more informative than an individual gene sequence. Actually, mitogenomic analysis can provide sets of genome-level characteristics, such as gene content and relative gene arrangements. In the present study, we determined the mitochondrial genomic features of a *B. neritina* specimen sampled from Qingdao (QD), China, and compared them to those of a specimen from Taean Gun (TG), South Korea (Jang and Hwang 2009).

Materials and methods

Sample collection and genomic DNA extraction

A specimen of *B. neritina* was obtained from Luxun Park in Qingdao, Shandong Province, China. The specimen was identified by Prof. X. X. Liu according to his monograph on bryozoans of China (Liu *et al.* 2001). The fresh sample was washed with distilled water three times and then preserved in liquid nitrogen immediately. Genomic DNA was isolated using TIANamp Marine Animal DNA Kit (TIANGEN), following the manufacturer's protocol, and then stored at −20°C.

Sequencing using Illumina HiSeq 2000

The genomic DNA was fragmented by nebulisation, and then DNA was repaired with an 'A' ligated to the 3′ end. Adapters were ligated to the fragments, and the sample was size-selected aiming for an approximate 500-bp product. The size-selected product was amplified by polymerase chain reaction (PCR) and validated using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples were sequenced using the Illumina HiSeq 2000 platform (Illumina, San Diego, CA, USA), following the protocols supplied by Illumina. Reads were assembled using ABySS (kmer = 30; Simpson *et al.* 2009), and the contigs were then aligned with BLAST using the database of seven reported bryozoan mitochondrial genomes (Waeschenbach *et al.* 2006; Jang and Hwang 2009; Sun *et al.* 2009; Nesnidal *et al.* 2011; Sun *et al.* 2011; Shen *et al.* 2012; Waeschenbach *et al.* 2012). The largest matched contig is 15 101 bp in length. It lacks partial sequences of *cox*3 gene, when compared with *B. neritina* TG mitochondrial genome.

PCR amplification and sequencing

Based on the matched contig obtained above, four PCR primers (Bne-mt-F1: CTG GGC AGA AAC TGA CCT CCG ACC, Bne-mt-F2: CCA GAA ATC ACA AAG AAA GGA CAC, Bne-mt-R1: TTT ATG ATT TTG AGT GTG CCT TTT, Bne-mt-R2: ATC TAT ATG GCT GTT TTT GTG TTG) were designed to fill gaps of the mitochondrial genome. PCR amplifications were conducted in a Mastercycler gradient machine (Eppendorf, Hamburg, Germany) in a total volume of 25 μL, containing 18.7 μL of sterile distilled H_2O, 2.5 μL of 10 × PCR buffer (Mg^{2+} plus), 0.5 μL of dNTP (10 mM each), 1 μL of each primer (5 μM), 0.3 μL of *Taq* polymerase (5 U μL^{-1}), and 1 μL of DNA template (~50 ng). Cycle parameters included an initial denaturation step of 94°C for 2 min, 35 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 2 min, and terminated with a final extension cycle of 72°C for 10 min. The sequences of the PCR products (~300 bp in length) were obtained by conventional Sanger sequencing, which was conducted with ABI 3730x1 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Sequence assembly and basic analysis

Sequences were assembled and analysed using SeqMan 7.1.0 (LASERGENE). The locations of 13 protein-coding genes (PCGs) and two rRNA genes were determined with DOGMA (Wyman *et al.* 2004) and BLAST programs. The boundaries of tRNA genes were identified using tRNAscan-SE 1.21 (Schattner *et al.* 2005) and DOGMA. Gene map of the mitochondrial genome was drawn with OGDraw 1.2 (Lohse *et al.* 2013). The AT and GC skew values were calculated according to (A−T)/(A+T) and (G−C)/(G+C) respectively (Perna and Kocher 1995).

Genetic distance and phylogenetic analysis within Lophotrochozoa

With the mitochondrial genome sequence of *B. neritina* from China (QD), the currently available eight bryozoan mitochondrial genomes from GenBank were included in the phylogenetic analysis, including *B. neritina* from China (TG) (Jang and Hwang 2009), *Celleporella hyalina* (Waeschenbach *et al.* 2012), *Flustra foliacea* (Nesnidal *et al.* 2011), *Flustrellidra hispida* (Waeschenbach *et al.* 2006), *Membranipora grandicella* (Shen *et al.* 2012), *Tubulipora flabellaris* (Sun *et al.* 2011) and *Watersipora subtorquata* (Sun *et al.* 2009). In addition, 10 genera of *Lophotrochozoa* with at least two complete mitochondrial genomes reported, including *Mytilus*, *Crassostrea*, *Hyriopsis*, *Meretrix*, *Sepiella*, *Aplysia*, *Potamopyrgu*, *Conus*, *Haliotis* and *Sypharochiton*, were also incorporated in the analysis. The 24 species from the 10 genera were *Mytilus edulis* (Boore *et al.* 2004), *M. galloprovincialis* (Mizi *et al.* 2005), *M. trossulus* (Breton *et al.* 2006), *Crassostrea angulata*, *C. gigas*, *C. sikamea* (Ren *et al.* 2010), *Hyriopsis cumingii* (Wei *et al.* 2014), *H. schlegelii*, *Meretrix lusoria* (Wang *et al.* 2010), *M. meretrix* (He *et al.* 2011), *M. petechialis* (Ren *et al.* 2009), *Sepiella inermis* (Wang *et al.* 2013), *S. japonica*, *Aplysia californica* (Knudsen *et al.* 2006), *A. dactylomela* (Medina *et al.* 2011), *Potamopyrgus antipodarum*, *P. estuarinus* (Neiman *et al.* 2010), *Conus consors* (Brauer *et al.* 2012), *C. textile* (Bandyopadhyay *et al.* 2008), *Haliotis laevigata* (Robinson *et al.* 2014), *H. rubra* (Maynard *et al.* 2005), *H. tuberculata* (Van Wormhoudt *et al.* 2009), *Sypharochiton pelliserpentis* and *S. sinclairi* (Veale *et al.* 2014). Moreover, *Sepia officinalis* (Akasaki *et al.* 2006) from the same family of two *Sepiella* (*S. inermis* and *S. japonica*) was also included in the analysis so that a total of 33 mitochondrial genomes was used.

Amino acid sequences of 12 PCGs (*atp*8, ATPase subunit 8, is missing in many genomes) were obtained, with each separately aligned using Clustal X 1.83 with default settings (Larkin *et al.* 2007), and then concatenated as a single dataset for analysis (4242 sites). Model selection for the amino acid dataset was performed with ProtTest (Abascal *et al.* 2007). For maximum-likelihood analysis, the LG+I+G+F matrix was implemented in PhyML 3.0 (Guindon *et al.* 2010). The assessment of node reliability was undertaken using 100 bootstrap replicates. Pairwise genetic distances (p-distance) were calculated using MEGA 6.0 (Tamura *et al.* 2013). The average

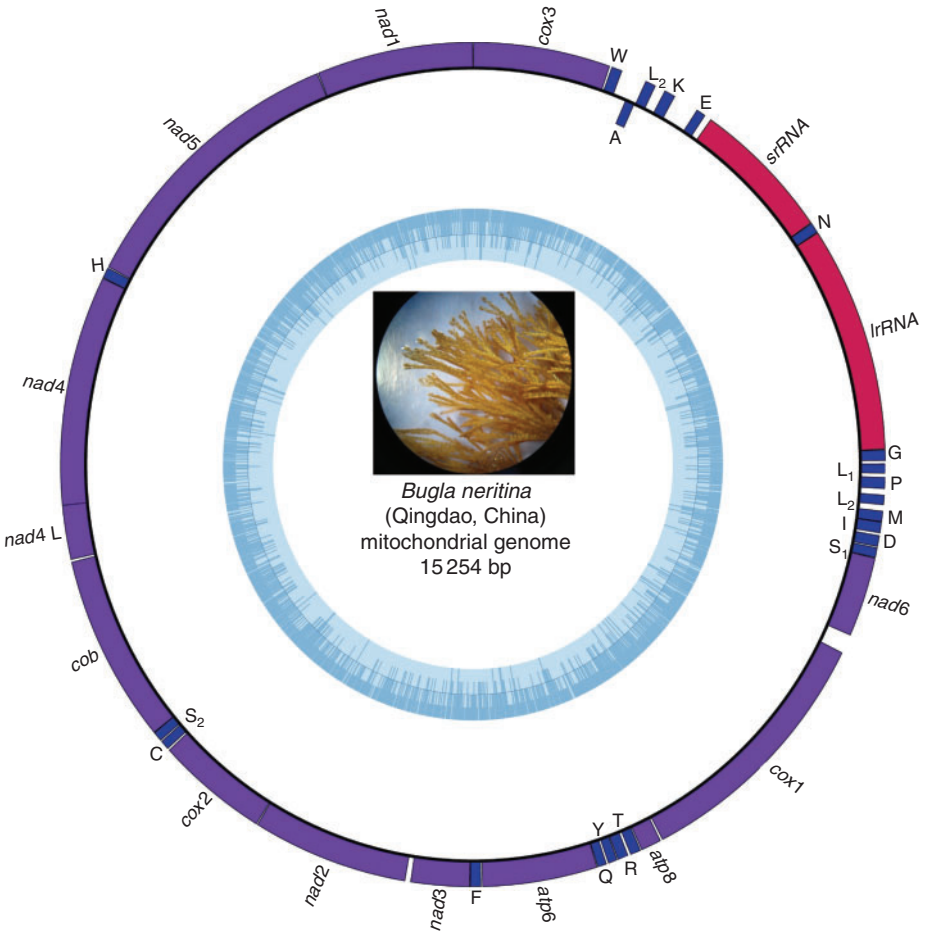

Fig. 1. The organisation of *Bugula neritina* (sampled from Qingdao, China) mitochondrial genome. Inner ring displays the GC content. Transfer RNA genes are designated by single-letter amino acid codes.

p-distance among three species is the average value of three pairs of pairwise distances.

Phylogenetic and p-distance analysis among B. neritina *types*

Besides the two mitochondrial genomes from QD and TG, currently available mitochondrial gene sequences (*cox*1 and *lrRNA*) of the three types of *B. neritina* were downloaded from GenBank (Table S1, available as Supplementary material for this paper). All of these sequences were aligned respectively using Clustal X 1.83 with default settings. Neighbour-joining trees were constructed using MEGA 6.0 (Tamura *et al.* 2013), with 1000 bootstrap replicates. The p-distances were also calculated using MEGA 6.0 (Tamura *et al.* 2013).

Results

Genomic characteristics of B. neritina *QD*

The mitochondrial genome of *B. neritina* QD (A: 0.378, C: 0.173, G: 0.122, T: 0.326) was 15 254 bp in length (Fig. 1), which was shorter than that of *B. neritina* TG (15 433 bp), with AT content of the former (70.5%) being slightly higher than that of the latter (70.0%). The AT and GC skew values of *B. neritina* QD mtDNA were 0.074 and −0.173 respectively. There were 27 intergenic regions in the *B. neritina* QD mitochondrial genome and the total length of non-coding DNA was 674 bp, which was shorter than that of *B. neritina* TG (902 bp). The largest non-coding region was found between *trn*K and *trn*E, which was 149 bp in length and shorter than that of *B. neritina* TG (271 bp). Because of the compactness of mitochondrial genome, gene overlaps on the same strand in the *B. neritina* QD mitochondrial genome could be found in three segments (Table 1). The *B. neritina* QD mitochondrial genome had a duplicated *trn*L_2 and lacked *trn*V as compared with *B. neritina* TG (Fig. 2). The entire *B. neritina* QD mitochondrial genome sequence was deposited in GenBank with Accession number KM983335.

Variation in protein-coding genes

Identical initiation codons were shared by each pair of the 12 mitochondrial PCGs in two *B. neritina* mitochondrial genomes, but their *nad*4 L had different start codons (Table 2), *viz*. ATG for *B. neritina* QD and ATT for *B. neritina* TG. Moreover, different termination codons were found in *cox*1 and *nad*6. The identities in 13 PCGs (nucleotide sequences) between the two *B. neritina* ranged from 78.5% (*nad*4 L) to 88.7% (*cox*1), and the

13 PCGs together had 1619 variable sites and 22 indels, with only 85.1% of invariant sites (Table 2). Because *atp*8 was missing in many mitochondrial genomes of *Lophotrochozoa* under comparison, the identities of the other 12 PCGs were compared (Table 3). The proportion of invariant sites of two *Aplysia* (*A. californica* and *A. dactylomela*) was 86.6%. The average identity (average value of pairwise identities) of the three *Crassostrea* (*C. angulata*, *C. gigas* and *C. sikamea*) was 88.6%, and the proportion of invariant sites between *C. angulata* and *C. gigas* was even as high as 95.0%. The identities of *Haliotis* (*H. laevigata* and *H. rubra*) and *Hyriopsis* (*H. cumingii* and *H. schlegelii*) were 89.6 and 90.3% respectively. Average identity of the three *Meretrix* (*M. meretrix*, *M. lusoria* and *M. petechialis*) and three *Mytilus* (*M. edulis*, *M. galloprovincialis* and *M. trossulus*) were 94 and 88.2%. The proportions of invariant sites in *Potamopyrgus* (*P. antipodarum* and *P. estuarinus*), *Sepiella* (*S. inermis* and *S. japonica*) and *Sypharochiton* (*S. pelliserpentis* and *S. sinclairi*) were 89.1, 93.2 and 88.4% respectively. However, the identity of the two *B. neritina* 12 PCGs was 85.1%, which was lower than most of the values for congeneric species (Table 3).

The p-distances of M2, M4 and C2 in 12 PCGs nucleic acid sequences were 0.005, 0.011 and 0.031 respectively (Fig. 3). In contrast, p-distances of five pairs of species ranged from 0.062 to 0.097, which included *Sepiella inermis* and *S. japonica* (0.062), *Meretrix petechialis* and *M. lusoria* (0.077), *M. meretrix* and *M. lusoria* (0.077), *Haliotis laevigata* and *H. rubra* (0.086), and *Hyriopsis cumingii* and *H. schlegelii* (0.097). In addition, the p-distances of the other five species pairs were larger than the above, yet they were less than 0.137, including for *Potamopyrgus antipodarum* and *P. estuarinus* (0.108), *Sypharochiton pelliserpentis* and *S. sinclairi* (0.115), *Aplysia californica* and *A. dactylomela* (0.123), *Crassostrea gigas* and *C. sikamea* (0.136), and *C. angulata* and *C. sikamea* (0.137).

Table 1. Mitochondrial genomic profile of *Bugula neritina* (sampled from Qingdao, China)
Negative intergenic-length numbers indicate overlapping nucleotides between adjacent genes

Gene	Strand	Position		Length (bp)	Codon		Anti-codon	Intergenic length (bp)
		Start	Stop		Start	Stop		
*cox*3	+	1	822	822	ATG	TAA		10
*trn*W	+	833	899	67			TCA	83
*trn*A	−	983	1044	62			TGC	4
trnL_2	+	1049	1114	66			CAA	59
*trn*K	+	1174	1243	70			TTT	149
*trn*E	+	1393	1452	60			TTC	37
srRNA	+	1490	2335	846				0
*trn*N	+	2336	2399	64			GTT	0
lrRNA	+	2400	3727	1328				0
*trn*G	+	3728	3792	65			TCC	15
trnL_1	+	3808	3865	58				20
*trn*P	+	3886	3954	69			TGG	33
trnL_2	+	3988	4047	60			TAA	33
*trn*M	+	4081	4144	64			CAT	−1
*trn*I	+	4144	4210	67			GAT	12
*trn*D	+	4223	4290	68			GTC	4
trnS_1	+	4295	4354	60			TGA	0
*nad*6	+	4355	4825	471	ATG	TAA		108
*cox*1	+	4934	6469	1536	ATA	TAG		10
*atp*8	+	6480	6605	126	ATG	TAA		4
*trn*T	+	6610	6675	66			TGT	13
*trn*R	+	6689	6755	67			TCG	2
*trn*Y	+	6758	6815	58			GTA	9
*trn*Q	+	6825	6881	57			TTG	1
*atp*6	+	6883	7572	690	ATG	TAA		5
*trn*F	+	7578	7643	66			GAA	0
*nad*3	+	7644	7997	354	ATG	TAA		30
*nad*2	+	8028	8963	936	ATG	TAA		3
*cox*2	+	8967	9638	672	ATG	TAA		9
*trn*C	+	9648	9705	58			GCA	5
trnS_2	+	9711	9770	60			TCT	−1
cob	+	9770	10 876	1107	ATG	TAA		11
*nad*4 L	+	10 888	11 208	321	ATG	TAA		−7
*nad*4	+	11 202	12 552	1351	ATT	T-		0
*trn*H	+	12 553	12 618	66			GTG	3
*nad*5	+	12 622	14 316	1695	ATG	TAA		2
*nad*1	+	14 319	15 254	936	ATG	TAA		0

However, the p-distance of two *B. neritina* 12 PCG sequences was 0.147, which was higher than values of the above pairs. Hence, the divergence of mitochondrial protein-coding genes between the two *B. neritina* generally exceeded the level of interspecies variation among *Lophotrochozoa* (Ren *et al.* 2009; He *et al.* 2011).

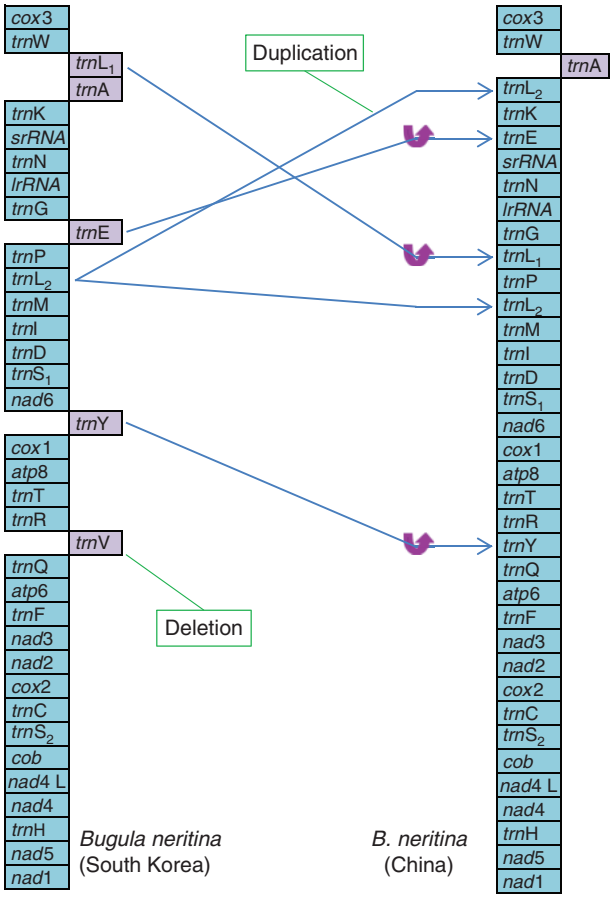

Fig. 2. Gene-order comparision of two *Bugula neritina* mitochondrial genomes.

Ribosomal RNA genes variation

Pairwise genetic distances of *srRNA* sequences between *Mytilus edulis* and *M. galloprovincialis* (M4) and between *Meretrix meretrix* and *M. petechialis* (M2) were 0.004 and 0.009 respectively (Fig. 3). Values between *Crassostrea angulata* and *C. gigas* (C2) and between *Aplysia californica* and *A. dactylomela* (A) were 0.012 and 0.024 respectively. Six pairs of *srRNA* p-distances ranged from 0.04 and 0.049, which included *Sepiella inermis* and *S. japonica* (0.04), *Potamopyrgus antipodarum* and *P. estuarinus* (0.041), *Haliotis laevigata* and *H. rubra* (0.041), *Meretrix petechialis* and *M. lusoria* (0.044), *Hyriopsis cumingii* and *H. schlegelii* (0.044), and *Meretrix meretrix* and *M. lusoria* (0.049). The p-distances of *srRNA* sequences between *Sypharochiton pelliserpentis* and *S. sinclairi* (S2) and between *Crassostrea angulata* and *C. sikamea* (C3) were 0.052 and 0.058 respectively. The value of 0.06 between the two *B. neritina* was higher than all of the above distances. The values for the other six congeneric species under comparison ranged from 0.073 to 0.127.

Similarly, the p-distances of M2 and M4 *lrRNA* sequences were 0.003 and 0.006 respectively (Fig. 3). Furthermore, those of C2, A and P were 0.011, 0.028 and 0.039 respectively. Moreover, seven pairs of *srRNA* p-distances ranged from 0.046 and 0.061, which included *Sepiella inermis* and *S. japonica* (0.046), *Haliotis laevigata* and *H. rubra* (0.052), *Meretrix petechialis* and *M. lusoria* (0.057), *Meretrix meretrix* and *M. lusoria* (0.057), *Hyriopsis cumingii* and *H. schlegelii* (0.057), *Crassostrea angulata* and *C. sikamea* (0.061), and *Crassostrea gigas* and *C. sikamea* (0.061). The p-distance of the two *B. neritina lrRNA* sequences was 0.067, which was higher than all the above values. The values for the other seven congeneric species under comparison ranged from 0.073 to 0.158. Therefore, p-distances of the two *B. neritina* rRNA genes were comparable to the values among congeneric species of *Lophotrochozoa*.

Table 2. Comparision of two *Bugula neritina* mitochondrial genomes

Bner-QD and Bner-TG mean *B. neritina* sampled from Qingdao (Shandong Province, China) and Taean Gun (Chungnam Province, South Korea) respectively

Gene	Bner-QD		Bner-TG		Site				
	Start	Stop	Start	Stop	All	Indel	Variable	Invariable	Identities (%)
atp6	ATG	TAA	ATG	TAA	690	0	114	576	83.48
atp8	ATG	TAA	ATG	TAA	126	0	20	106	84.13
cox1	ATA	TAG	ATA	TAA	1536	0	174	1362	88.67
cox2	ATG	TAA	ATG	TAA	672	0	92	580	86.31
cox3	ATG	TAA	ATG	TAA	822	0	111	711	86.50
cob	ATG	TAA	ATG	TAA	1107	0	148	959	86.63
nad1	ATG	TAA	ATG	TAA	936	0	149	787	84.08
nad2	ATG	TAA	ATG	TAA	936	0	160	776	82.91
nad3	ATG	TAA	ATG	TAA	354	0	51	303	85.59
nad4	ATT	T-	ATT	T-	1357	6	208	1143	84.23
nad4 L	ATG	TAA	ATT	TAA	321	15	54	252	78.50
nad5	ATG	TAA	ATG	TAA	1695	0	255	1440	84.96
nad6	ATG	TAA	ATG	TA-	471	1	83	387	82.17
Total	NA	NA	NA	NA	11 023	22	1619	9382	85.11

Table 3. Comparison of 12 mitochondrial protein-coding genes in *Lophotrochozoa*

Total sites do not include *atp8* because the gene is missing in many target mitogenomes. The abbreviations are as in Table 2

Genus	Species	Accession number	Species	Accession number	Total sites	Indel	Variable	Invariable	Identities (%)	Average (%)
Bugula	Bner-QD	KM983335	Bner-TG	NC_010197	10 897	22	1599	9276	85.12	85.12
Aplysia	*A. californica*	NC_005827	*A. dactylomela*	NC_015088	10 773	138	1307	9328	86.59	86.59
Conus	*C. consors*	NC_023460	*C. textile*	NC_008797	11 064	0	1833	9231	83.43	83.43
Crassostrea	*C. angulata*	NC_012648	*C. gigas*	NC_001276	11 163	231	334	10 598	94.94	88.58
	C. angulata	NC_012648	*C. sikamea*	NC_012649	11 154	6	1532	9616	86.21	
	C. gigas	NC_001276	*C. sikamea*	NC_012649	11 163	231	1488	9444	84.60	
Haliotis	*H. laevigata*	NC_024562	*H. rubra*	NC_005940	11 070	210	938	9922	89.63	84.61
	H. laevigata	NC_024562	*H. tuberculata*	NC_013708	11 067	198	1882	8987	81.21	
	H. rubra	NC_005940	*H. tuberculata*	NC_013708	11 062	20	1860	9182	83.00	
Hyriopsis	*H. cumingii*	NC_011763	*H. schlegelii*	NC_015110	10 911	6	1054	9851	90.29	90.29
Meretrix	*M. meretrix*	NC_013188	*M. lusoria*	NC_014809	12 157	140	920	11 097	91.28	94.00
	M. meretrix	NC_013188	*M. petechialis*	NC_012767	12 042	0	66	11 976	99.45	
	M. petechialis	NC_012767	*M. lusoria*	NC_014809	12 157	140	920	11 097	91.28	
Mytilus	*M. edulis*	NC_006161	*M. galloprovincialis*	NC_006886	11 169	148	117	10 904	97.63	88.17
	M. edulis	NC_006161	*M. trossulus*	NC_007687	11 160	145	1758	9257	82.95	
	M. galloprovincialis	NC_006886	*M. trossulus*	NC_007687	11 157	15	1776	9366	83.95	
Potamopyrgus	*P. antipodarum*	NC_020790	*P. estuarinus*	NC_021595	11 055	15	1188	9852	89.12	89.12
Sepiella	*S. inermis*	NC_022693	*S. japonica*	NC_017749	11 079	72	679	10 328	93.22	93.22
Sypharochiton	*S. pelliserpentis*	NC_024174	*S. sinclairi*	NC_024173	11 046	15	1272	9759	88.35	88.35
Urechis	*U. caupo*	NC_006379	*U. unicinctus*	NC_012768	10 962	0	1753	9209	84.01	84.01

Distances within and between different types of B. neritina

On the basis of 38 *cox*1-3P (segments of *cox*1 3′ end) sequences, the mean p-distances within the Type S was 0.0012, and those of Types D and N were 0 (Table 4). In contrast, the mean p-distances from *cox*1-5P (segments of *cox*1 5′ end) sequences were 0.0006, 0.0005 and 0.0009 for Types S, D and N respectively. Furthermore, within the Type S, the *lrRNA* sequences were identical. And the mean p-distances from *lrRNA* sequences were 0.0007 and 0.0006 for Types D and N respectively.

The mean p-distances between different types range from 0.0569 to 0.1328 on the basis of *cox*1-3P sequences. In contrast, the mean p-distances between different types from *cox*1-5P sequences were between 0.0457 and 0.1179. In addition, those based on *lrRNA* sequences ranged from 0.0239 to 0.0425.

Phylogenetic analysis

Phylogenetic tree constructed on the basis of 12 PCGs (amino acids) of the 33 mitochondrial genomes from lophotrochozoan genera showed that all genera with more than one genome constituted distinct monophyletic groups; in contrast, monophyly of Gymnolaemata and Cheilostomatida within Bryozoa was verified (Fig. 4). The average distances within *Crassostrea* (*C. angulata*, *C. gigas* and *C. sikamea*), *Mytilus* (*M. edulis*, *M. galloprovincialis* and *M. trossulus*), *Meretrix* (*M. lusoria*, *M. meretrix* and *M. petechialis*) and *Haliotis* (*H. laevigata*, *H. rubra* and *H. tuberculata*) were 0.049, 0.0393, 0.0193 and 0.026 respectively (Fig. 4). Pairwise genetic distances of other six genera ranged from 0.014 to 0.048, including *Hyriopsis* (0.044), *Aplysia* (0.036), *Potamopyrgus* (0.014), *Conus* (0.048), *Sepiella* (0.027) and *Sypharochiton* (0.038). The genetic distance between the two *B. neritina* 12 PCGs (amino acids) was 0.079, which was higher than those of all the above 10 genera. Most surprisingly, the distance of two *B. neritina* was even higher than the values between the genera *Sepia* (*Sepia officinalis*) and *Sepiella* (*S. inermis* and *S. japonica*), which were 0.073 and 0.076.

Discussion

Mitochondrial gene arrangements

Mitochondrial gene arrangement appeared to be highly variable among bryozoan species. Every newly sequenced mitochondrial genome contains a novel gene arrangement, none of which is identical to those of the other bryozoans (Waeschenbach *et al.* 2006; Jang and Hwang 2009; Sun *et al.* 2009, 2011; Nesnidal *et al.* 2011; Shen *et al.* 2012; Waeschenbach *et al.* 2012). In contrast, there is no variation in mitochondrial gene arrangements within each genus, including *Aplysia* (*A. californica* and *A. dactylomela*) (Knudsen *et al.* 2006; Medina *et al.* 2011), *Crassostrea* (*C. angulata*, *C. gigas* and *C. sikamea*) (Ren *et al.* 2010), *Haliotis* (*H. laevigata* and *H. rubra*) (Maynard *et al.* 2005; Robinson *et al.* 2014), *Hyriopsis* (*H. cumingii* and *H. schlegelii*) (Wei *et al.* 2014), *Mytilus* (*M. edulis* and *M. galloprovincialis*) (Boore *et al.* 2004; Mizi *et al.* 2005), *Potamopyrgus* (*P. antipodarum* and *P. estuarinus*) (Neiman *et al.* 2010), *Sepiella* (*S. inermis* and *S. japonica*) (Wang *et al.* 2013), *Sypharochiton* (*S. pelliserpentis* and *S. sinclairi*) (Veale

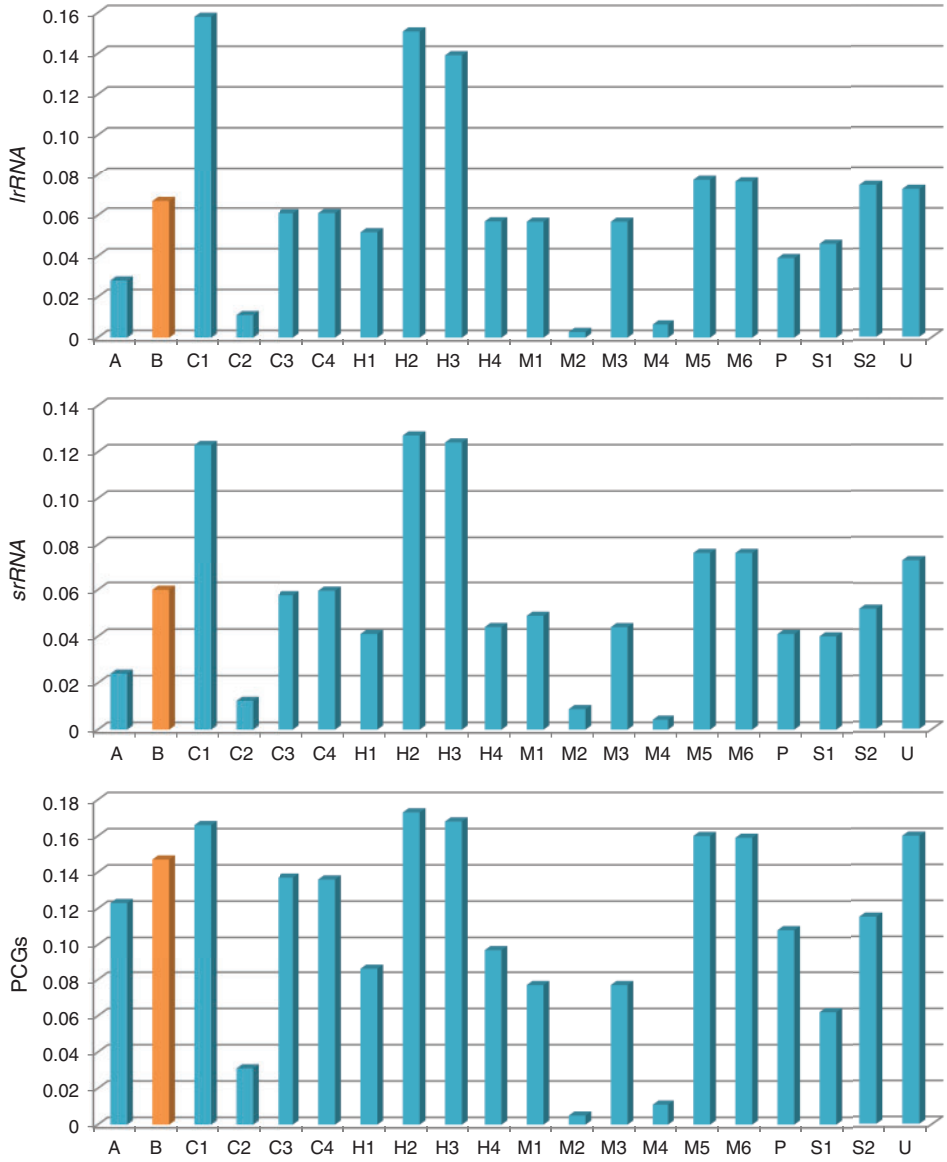

Fig. 3. Pairwise genetic distance (p-distance) of PCGs, *srRNA* and *lrRNA* nucleotide sequences. *Aplysia californica* v. *Aplysia dactylomela* (A), *Bugula neritina* QD v. *Bugula neritina* TG (B), *Conus consors* v. *Conus textile* (C1), *Crassostrea angulata* v. *Crassostrea gigas* (C2), *Crassostrea angulata* v. *Crassostrea sikamea* (C3), *Crassostrea gigas* v. *Crassostrea sikamea* (C4), *Haliotis laevigata* v. *Haliotis rubra* (H1), *Haliotis laevigata* v. *Haliotis tuberculata* (H2), *Haliotis rubra* v. *Haliotis tuberculata* (H3), *Hyriopsis cumingii* v. *Hyriopsis schlegelii* (H4), *Meretrix meretrix* v. *Meretrix lusoria* (M1), *Meretrix meretrix* v. *Meretrix petechialis* (M2), *Meretrix petechialis* v. *Meretrix lusoria* (M3), *Mytilus edulis* v. *Mytilus galloprovincialis* (M4), *Mytilus edulis* v. *Mytilus trossulus* (M5), *Mytilus galloprovincialis* v. *Mytilus trossulus* (M6), *Potamopyrgus antipodarum* v. *Potamopyrgus estuarinus* (P), *Sepiella inermis* v. *Sepiella japonica* (S1), *Sypharochiton pelliserpentis* v. *Sypharochiton sinclairi* (S2) and *Urechis caupo* v. *Urechis unicinctus* (U). Additionally, each *Crassostrea* mitochondrial genome has two fragments of *srRNA* and *lrRNA*, and the average distance values were used.

et al. 2014) and *Urechis* (*U. caupo* and *U. unicinctus*) (Boore 2004; Wu *et al.* 2009) (Fig. S1, available as Supplementary material for this paper).

In the present study, we sequenced the complete mitochondrial genome from *B. neritina* from Qingdao, China, and compared it to that from Taean Gun, South Korea (Jang and Hwang 2009). The *B. neritina* QD mitochondrial genome had a duplicated *trn*L_2 and lacked *trn*V as compared with *B. neritina* TG (Jang and Hwang 2009), although the gene numbers of the two *B. neritina* were identical. In the former, only *trn*A was encoded on the light strand, whereas all the other 36 genes (13 PCG, 2 rRNAs and 21 tRNAs) were encoded on the heavy strand (Fig. 2, Table 1). In contrast, five tRNAs (*trn*L_1, *trn*A, *trn*E, *trn*Y and *trn*V) were encoded on the light strand of

B. neritina TG mitochondrial genome. In addition, three tRNAs (*trn*E, *trn*L_1 and *trn*Y) exhibited translocation and inversion simultaneously. Gene content and arrangement differences of two specimens of *B. neritina* were rather distinct.

Table 4. Pairwise genetic distance (p-distance) within and between different types of *Bugula neritina*

	Type (s)	*cox*1-3P	*cox*1-5P	*lrRNA*
Distance within each type	S	0.0012	0.0006	0.0000
	D	0.0000	0.0005	0.0007
	N	0.0000	0.0009	0.0006
Distance between types	S *v.* D	0.0911	0.0867	0.0265
	S *v.* N	0.1328	0.1144	0.0422
	D *v.* N	0.1120	0.1139	0.0425
	Y *v.* S	0.1295	0.1056	0.0419
	Y *v.* D	0.1195	0.1179	0.0422
	Y *v.* N	0.0569	0.0457	0.0239

Two mechanisms of mitochondrial gene rearrangement are widely accepted, including 'duplication and random loss' and 'recombination'; however, the former cannot explain inversions (Boore and Brown 1998). Therefore, the gene inversions observed in *B. neritina* QD and *B. neritina* TG may be the result of recombination, which has been commonly found in many other metazoan mitochondrial genomes (Wang *et al.* 2011; Mao *et al.* 2014). At the same time, translocations in the same strand could be the result of duplication and random loss.

New cryptic type of the B. neritina

Phylogenetic trees constructed on the basis of *cox*1 and *lrRNA* sequences indicated clearly that *B. neritina* TG is nested within Type S, but *B. neritina* QD is clustered with Type N (Figs 5, S2, S3, with the latter two figures available as Supplementary material for this paper), which is believed to be geographically restricted to the north-western Atlantic (Delaware and Connecticut, USA; McGovern and Hellberg 2003; Fehlauer-Ale *et al.* 2014). Moreover, the genetic distance

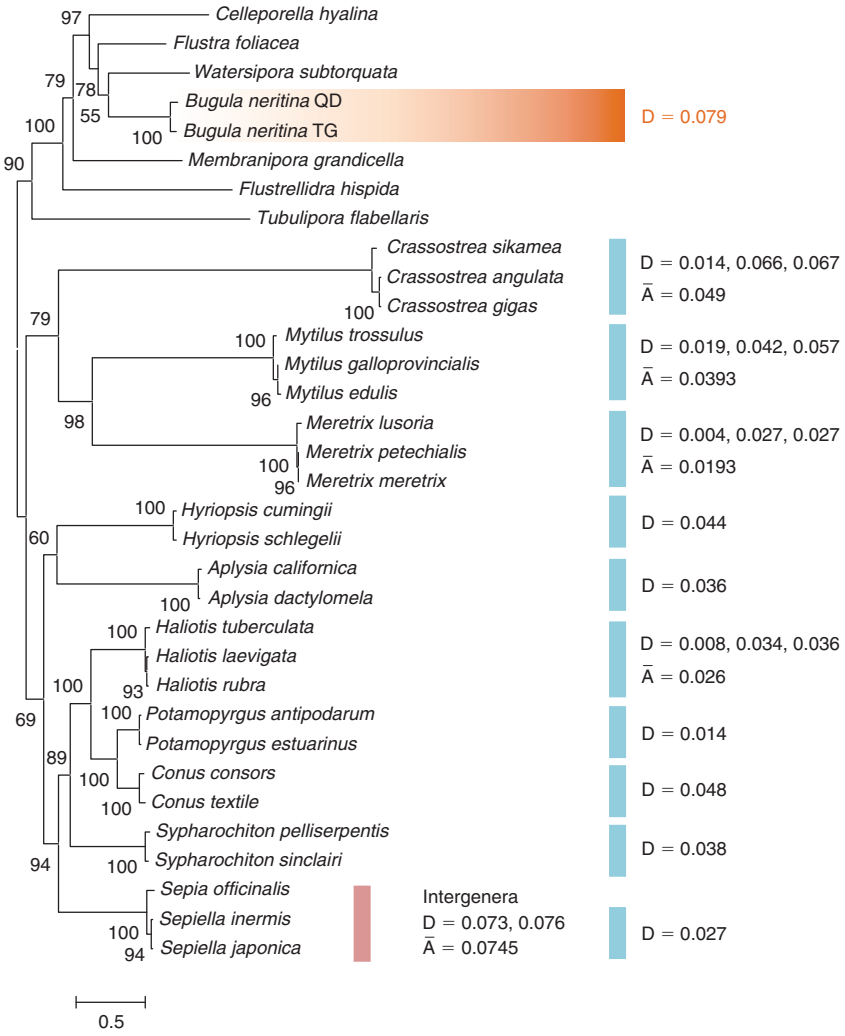

Fig. 4. Phylogenetic tree constructed from maximum-likelihood analyses of 12 mitochondrial protein-coding genes (amino acid sequences). The numbers at the nodes indicate the support values. D and Ā mean distance and average distance respectively.

between *B. neritina* QD and Type N is much greater than those among sequences of Type N from different localities (Coral Sea, north-eastern Pacific and north-western Atlantic; Fehlauer-Ale *et al.* 2014). The *B. neritina* sampled from Qingdao may represent a new cryptic type of the species. There are clearly two genetically distinct types of *B. neritina* occurring in the Yellow Sea.

Comparisons of gene contents, arrangements and divergence of major genes (rRNAs and PCGs) between two types of *B. neritina* occurring in the Yellow Sea have revealed that their differences are beyond intraspecific variation. Whereas *B. neritina* from Taean Gun (South Korea) belongs to the widely distributed Type S of the species, *B. neritina* from Qingdao represents a new type. We now designate this new type as Type Y, for its occurrence in the Yellow Sea. The cryptic diversity and distribution patterns of the different types of the bryozoan *B. neritina* should be further explored by molecular analysis of the species throughout its entire range.

There is the '10 × rule' that the mean interspecific genetic distance is 10 times higher than the mean distance within species (Hebert *et al.* 2004; Hickerson *et al.* 2006). On the basis of *cox*1-3P sequence, the minimum inter-type distance between Type Y and the other three types (0.0569) was 47.42 times the largest intra-type distance (Type S, 0.0012; Table 4). At the same time, the ratios were 50.78 and 34.14 from *cox*1-5P and *lrRNA* sequences respectively. Therefore, these ratios of inter-type and intra-type distances were much more than the threshold (10 times). Whereas the p-distance analysis clearly indicated that Types S, D, N and Y are each a separate species, the present knowledge of morphological characters fails to distinguish these species within the complex (McGovern and Hellberg 2003; Fehlauer-Ale *et al.* 2014).

Cryptic biodiversity is a common phenomenon in the phylum Bryozoa, which can be concluded from the current study and a lot of previous researches (McGovern and Hellberg 2003; Mackie *et al.* 2006; Nikulina *et al.* 2007; Schwaninger 2008;

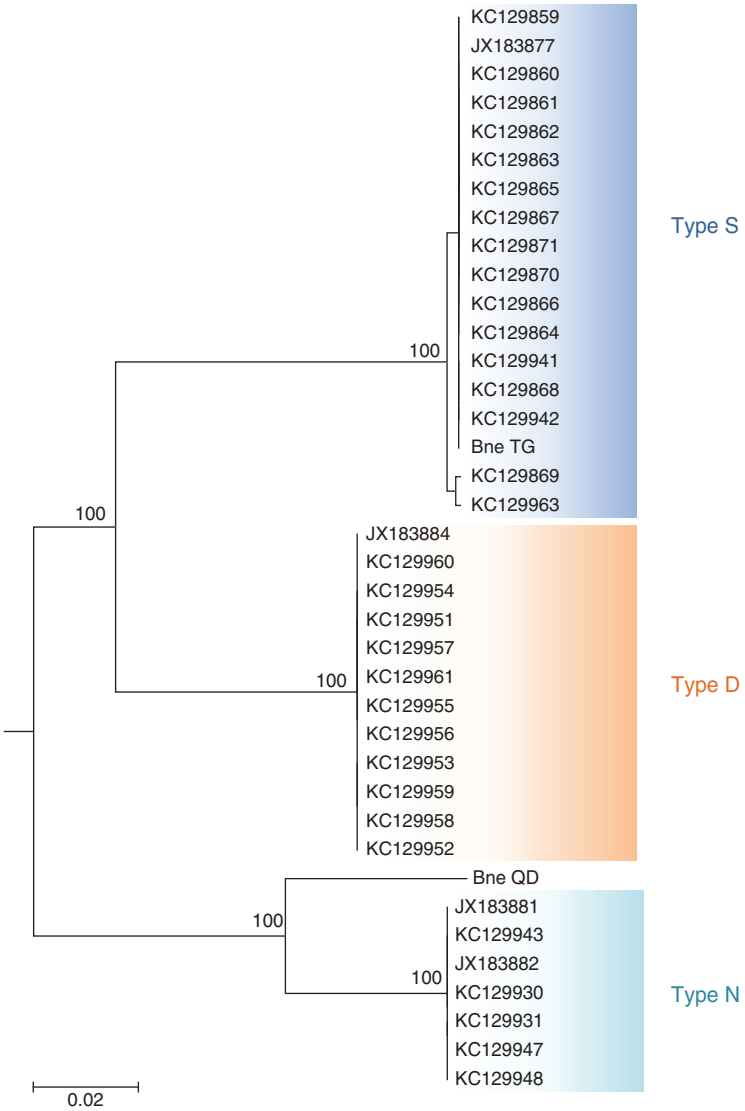

Fig. 5. Phylogenetic tree based on 38 *Bugula neritina cox*1-3P sequences.

Waeschenbach *et al.* 2012). In summary, the comparison of mitochondrial genomes revealed that the bryozoan *B. neritina* contains new cryptic diversity in the Yellow Sea. This finding challenges the previously assumed distribution pattern of the different types of *B. neritina*.

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