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Full Length Research Paper

Phylogenetic relationships of the lancelets of the genus *Branchiostoma* in China inferred from mitochondrial genome analysis

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It is commonly accepted that the *Branchiostoma* lancelets in South and North China belong to *Branchiostoma belcheri* (Gray, 1847) and *Branchiostoma belcheri tsingtauense* (Tchang and Koo, 1934), respectively. Three partial mitochondrial DNA (mtDNA) fragments of cytochrome oxidase *c* subunit I (COI), cytochrome *b* (Cyt*b*), and 16S ribosomal RNA (16S rRNA) genes were sequenced to analyze phylogenetic relationships of the *Branchiostoma* lancelets from South (Xiamen) and North (Qingdao and Rizhao) China, and phylogenetic trees constructed also included the existing data from Japanese waters. The genetic distances of the lancelets between South and North China averaged 0.19, 0.21, and 0.17 based on partial sequences analysis of COI, Cyt*b*, and 16S rRNA genes, respectively, which were much higher than those observed in other intraspecific variations. However, the value between North China and Japanese waters was only 0.01 based on partial sequences analysis of three mtDNA genes, which indicated low intraspecific genetic divergence existed in the two areas. The results also clearly indicated two monophyletic clades (clade A (North China and Japanese waters), clade B (South China)) existed in the specimens, corresponding to the South and North China, respectively. Above all, our results indicate that the *Branchiostoma* lancelets in South and North China should belong to different species, and the original subspecies *B. belcheri tsingtauense* together with the lancelets in most Japanese waters is an independent species. According to the rule of priority and present studies, the *B.* lancelets in North China and most Japanese waters should be revised to *Branchiostoma japonicus*. The divergence time between *B. belcheri* and *B. japonicus* was estimated at about 39.90 - 43.24 million years ago.

Key words: Lancelets, *Branchiostoma*, mitochondrial DNA genes, *Branchiostoma japonicus.*

INTRODUCTION

The lancelets (subphylum Cephalochordata), a benthic marine invertebrate taxon, are believed to be the sister group of vertebrates and widely used to study for clues to the origin of vertebrates (Gee, 1996; Hall, 1998). The lancelets of the genus *Branchiostoma* in China Sea including two species (*Branchiostoma belcheri* and *Branchiostoma belcheri* var. *tsingtauense*) widely distribute along the east coast of China from Xiamen waters to the Gulf of Po-Hai (Zhou, 1958; Wang et al., 2004). Gray (1847) first named the lancelets from Borneo (now Sarawak state, Malaysia) *Amphioxus belcheri* and then renamed it as *B. belcheri*. To the present studies, the species is thought to be widely distributed throughout the Indo-West Pacific regions (Poss and Boschung, 1996). Xiamen (China) used to be a famous lancelets fishery for its surprising yield (Light, 1923) and the species distributing there was identified as *B. belcheri* (Boring and Li, 1932). Tchang and Koo (1936) reported their finding of new distribution of lancelets in the Kiaochow Bay (south side of Shandong Peninsula) and named them as a new variety *B. belcheri* var. *tsingtauense* based on the morphological observations. To date, researchers have noticed the morphological differentiation of the lancelets along the

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Table 1. Sample size and gene information of *Branchiostoma* lancelets including sequence variability and nucleotide substitution model for each data partition.

Gene	Qingdao (n)	Rizhao (n)	Japan (n)	Xiamen (n)	Aligned sites	Gapped sites	Parsimony informative sites	Variable sites	Best model	% invariable sites	Gamma values (Γ)
COI	5	3	5	4	403 bp	0	65	75	HKY + Γ	0	0.16
Cyt*b*	3	7	4	4	472 bp	0	86	97	HKY + Γ	0	0.10
16S rRNA	5	8	5	8	574 bp	10	91	109	K81uf + Γ	0	0.19

coast of China from south to north (Zhou, 1958; Zhang, 1988; Cao et al., 2001). Nishikawa (1981) examined specimens from different localities based on the morphological divergences of lancelets and concluded that the *Branchiostoma* lancelets in Japanese waters, except those from Ariake Sea and Amakusa on the west coast of Kyusyu Island, belonged to the subspecies *B. belcheri* var. *tsingtauense* recorded from Kiachow Bay, North China. Currently, differentiation of the lancelets is so obscure that the taxonomic status of *B. belcheri* var. *tsingtauense* remains controversial (Lin, 2001).

The recent progress in molecular biological techniques like isoenzyme technique, DNA sequencing and DNA molecular marker has made the exploration of genetic diversities of organisms more straight forward, and the genetic analyses aided by these techniques have been effective particularly in evolutionary studies of morphologically similar organisms. Indeed, comparative genetic studies of congeneric animals using molecular sequencing method (e.g. mtDNA Cyt*b*) have revealed large genetic differentiation (Glenn and Avise, 1998), suggesting that their general body organization such as the lancelets has remained stable for a long time following phylogenetic splitting. Lancelets have persisted for a long period of time as indicated by fossil records (e.g. *Pikaia* and *Cathaymyrus* from the Lower Cambrian) referred to the cephalochordates (Shu et al., 1996). Therefore, although the lancelets have shown few morphological changes, it is possible that they have accumulated significant genetic changes at molecular level and the diversification among the extant species have been examined by genetic survey based on the mitochondrial DNA genes (Nohara et al., 2004).

In the present study, three partial fragments of mitochondrial DNA (mtDNA) genes (COI, Cyt*b*, 16S rRNA) were sequenced to examine the extent of the genetic differentiation of *Branchiostoma* lancelets between South and North China, and the taxonomic status of the lancelets in North China and Japanese waters were also discussed.

MATERIALS AND METHODS

Sample collection

61 sequences including the existing data for the *Branchiostoma.* lancelets in Japanese waters (COI: AB078191 (Sea of Genkai), AB083383 (Sea of Genkai), AB083384 (Akashi), AB083385 (Akashi), AB105136 (Awajishima Island); Cyt*b*: AB078191, AB083383, AB083384, AB083385; 16S rRNA: AB105142 (Awajishima Island), AB078191, AB083383, AB083384, AB083385) and Xiamen (Cyt*b*: AY700108, AY700110) were analyzed in the present study. 13 specimens were collected from Qingdao in 2004, 18 specimens were collected from Rizhao in 2007, and 14 specimens were collected from Xiamen in 2005 (Figure 1 and Table 1). All specimens were fixed soon after collection and preserved in 95% ethanol until DNA extraction. *Epigonichthys lucayanum* (AB105140 and AB110092) and *Branchiostoma lancelantum* (Y16474) were used as out-groups for COI, Cyt*b*, and 16S rRNA in the present phylogenetic analysis, respectively. *Branchiostoma malayanum* (AB248229 and AB105138) was used to estimate divergence time based on the COI gene.

DNA extraction, amplification and sequencing

Genomic DNA was isolated from muscle of lancelets. Muscle tissues were digested with proteinase K in a buffer of 10 mM Tris-HCl (pH 7.5) with 125 mM NaCl, 50 mM EDTA, 8 M urea, and 1% SDS by at 50℃ temperature and purified by standard phenol-chloroform extraction (Sambrook and Russell, 2001). The mitochondrial DNA sequences of lancelets were amplified with the following primers: COI_R: (5`-TAA TAG TAG TAA ATA AGC AGT-3`) and COI_F: (5`-CTA CTA ATC ATA AAG ATA TTG G-3`); Cyt*b*_R: (5`-TGT TGC ATT ATC AAC AGA AA-3`) and Cyt*b*_F: (5`-AGA ATT TAA GCA TGA AAA GC-3`), 16S rRNA_R: (5`-CGC CTG TTT AAC AAA AAC A -3`) and 16S rRNA_F: (5`-TCC GGT CTG AAC TCA GAT CAC GTC -3`) (Nohara et al., 2004; Wang et al., 2004). The PCR amplification was carried out in a thermal cycler 9700 (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial 3 min denaturation at 94℃, and 39 alternating cycles of 1 min at 94℃ for denaturation, 1 min at 50℃ for annealing, and 1 min at 72℃ for extension, and a final extension at 72℃ for 10 min. A total of 2 µL of each PCR product was used for 1% agarose gel electrophoresis for verifying the amplified fragment length with a standard size marker (TaKaRa, Dalian). PCR products were purified with Gel Extraction Mini Kit (Watson BioTechnologies, Shanghai). Both strands were sequenced using the BigDye Terminator Cycle Sequencing Kit (ver.2.0, PE Biosystems, Foster City, California) and run on an ABI Prism 3700 (Applied Biosystems) automatic sequencer according to the manufacturer's recommendations. The primers used for sequencing were the same as those for PCR amplification.

Sequence analysis

All raw sequences data of lancelets COI, Cyt*b*, and 16S rRNA genes were reevaluated using DNASTAR package (DNASTAR Inc., Madison, USA). Then the obtained sequence alignments were initiated with Clustal X (Thompson et al., 1997), subsequent inspection and correction being made manually. Genetic variation indices such as the number of haplotypes, polymorphic sites, transitions,

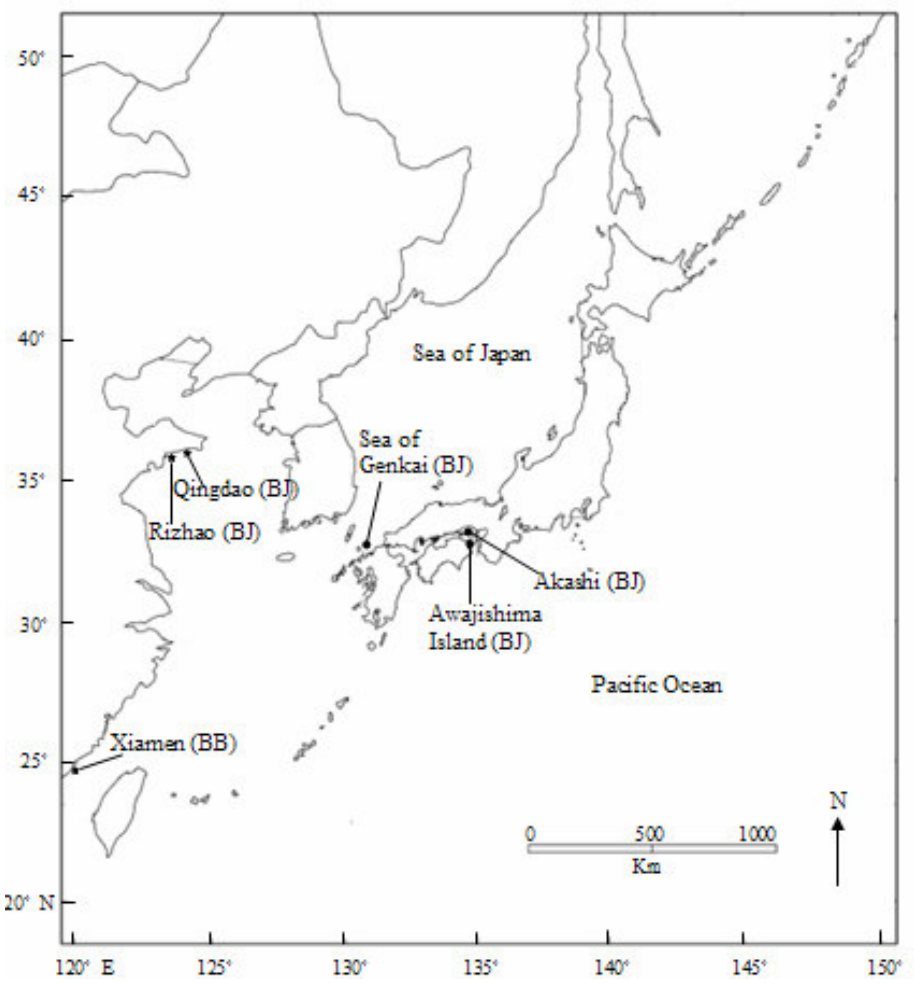

Figure 1. Map showed sample locations of *Branchiostoma* lancelets including the existed data for Japanese lancelets. BB: *Branchiostoma belcheri*, BJ: *B. japonicus*.

and transversions were obtained using the program ARLEQUIN version 2.000 (Schneider et al., 2000). The gamma distribution with shape parameter (Γ) for the rate heterogeneity among sites and nucleotide sequence evolution models were evaluated using likelihood-ratio tests implemented by the program Modeltest version 3.06 (Posada and Crandall, 1998). Genetic relationships among haplotypes of the lancelets for the three mitochondrial DNA genes sequence data were reconstructed using the neighbor-joining method implemented in PAUP* and the robustness of each internal branch of the phylogenetic trees was evaluated with 5 000 bootstrap replicates (Saitou and Nei, 1987). Genetic distances were generated of phylogenetic reconstruction using the best fit evolution models corrected with the shape parameter of a gamma distribution (Γ) substitution suggested by Model test.

RESULTS

Sequence variability and divergence among the lancelets

61 sequences of the genus *Branchiostoma* lancelets in South and North China together with Japanese waters were anal- yzed. After unreadable sites close to the primers at either end were removed, the Clustal X was used to analyze these sequences getting 403, 472 and 574 bp DNA fragments for COI, Cyt*b*, and 16S rRNA genes, respectively (Table 1). Genetic variation indices for three partial fragments of mtDNA genes are shown in Table 1. The frequency of variable sites in Cyt*b* (20.55%) gene was higher than those in COI (18.61%) and 16S rRNA (18.99%) genes, and 10 base-pair insertion/deletion events were observed in the 16S rRNA gene fragment (Table 1, Figure 2). No insertion/deletions were found in the partial fragments of COI and Cyt*b* genes (Figure 2a, b). 49 haplotypes (m_{COI} = 13; $m_{Cyt\,b}$ = 15; $m_{16S\ rRNA}$ = 21) were defined by 281 variable sites of the three mtDNA genes fragments in the 61 sequences (Table 2). All haplotypes of *Branchiostoma* lancelets from Qingdao, Rizhao, Japan, and Xiamen were coded as BJQ, BJR, BJ, and BBX, respectively. BJQ2 and BBX1 were occupied by 4 (Qingdao and Rizhao) and 2 (Xiamen) individuals, respectively, and the left haplotypes were unique based on the COI gene (Table 2a). The similar result was also

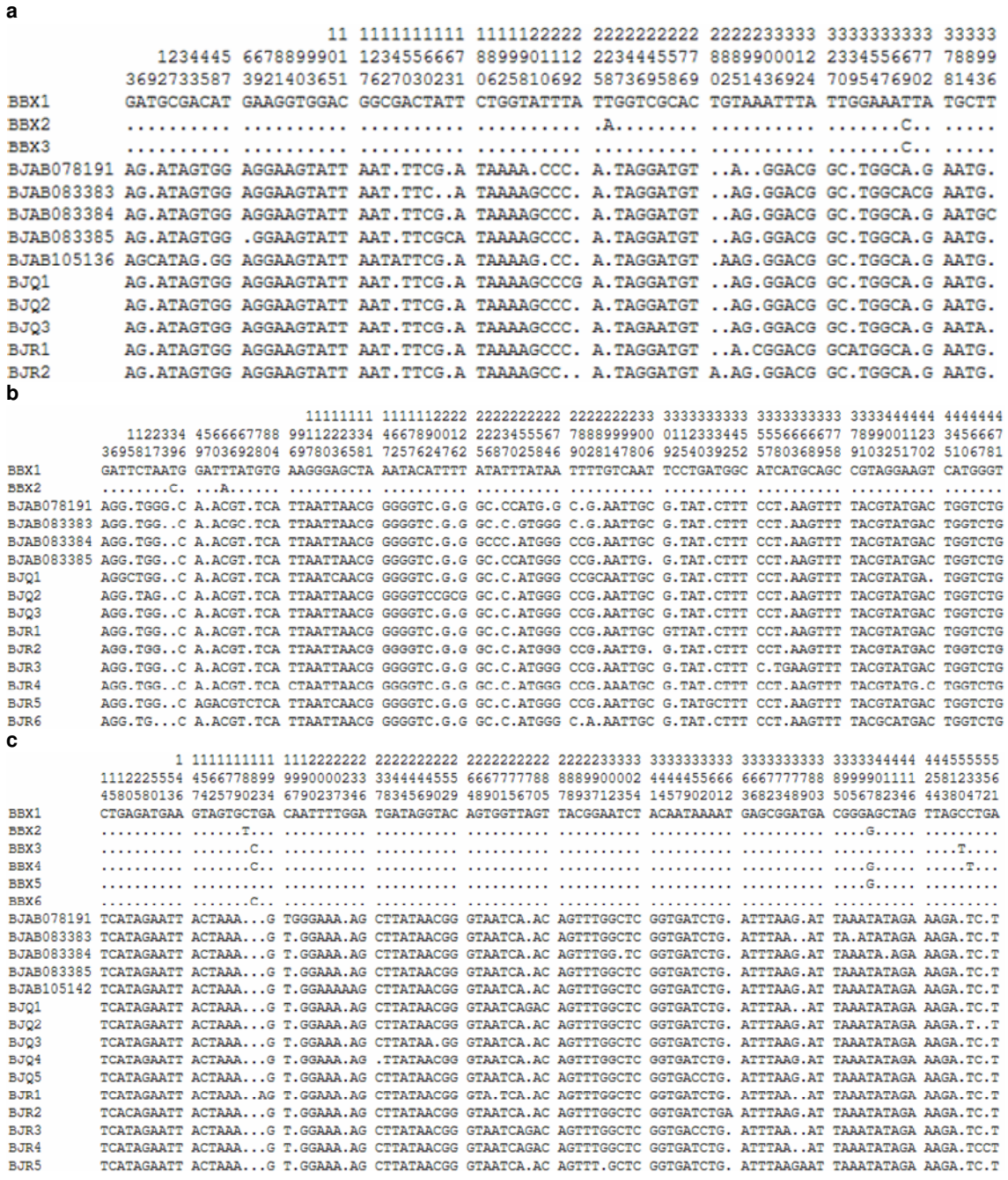

Figure 2. Nucleotide variations of COI, Cyt*b* and 16S rRNA genes haplotypes in *Branchiostoma* lancelets, ".", identical bases. (a): COI gene, (b): Cyt*b* gene, (c): 16S rRNA gene. BBX: Xiamen lancelets; BJQ: Qingdao lancelets; BJR: Rizhao lancelets; BJ: Japan lancelets.

found in the Cyt*b* gene as shown in Table 2b. In the partial fragment of 16S rRNA gene, most of the haplotypes were unique except for BJQ1, BJAB083385, and BBX6 which shared 2 (Qingdao and Rizhao), 3 (Rizhao and Japan), and 3 (Xiamen) individuals, respectively (Table 2c). Low values (α_{COI} = 0.16; $\alpha_{Cyt\,b}$ = 0.10; $\alpha_{16S\ rRNA}$ = 0.19) of the gamma distribution shape parameter were calculated for COI, Cyt*b*, and 16S rRNA genes, respectively, which indicated extant heterogeneities existed among sites within the three mtDNA genes fragments of the genus *Branchiostoma* (Table 1).

The nucleotide compositions of the three mitochondrial DNA genes fragments of *Branchiostoma* lancelets were all AT-rich (A + T > 60%), and variations consisted predominantly of transition substations (Table 3). The average of nucleotide compositions of Qingdao (T = 38.0%, C

Table 2a. Frequencies of haplotypes for COI, Cyt*b* and 16S rRNA in *Branchiostoma* lancelets. (a): COI gene, (b): Cyt*b* gene, (c): 16S rRNA gene.

Haplotype	Qingdao	Rizhiao	Japan	Xiamen
BJQ1	1			
BJQ2	3	1		
BJQ3	1			
BJR1		1		
BJR2		1		
BJAB078191			1	
BJAB083383			1	
BJAB083384			1	
BJAB083385			1	
BJAB105136			1	
BBX1				2
BBX2				1
BBX3				1

Table 2b.

Haplotype	Qingdao	Rizhiao	Japan	Xiamen
BJQ1	1			
BJQ2	1			
BJQ3	1	1		
BJR1		1		
BJR2		1		
BJR3		1		
BJR4		1		
BJR5		1		
BJR6		1		
BJAB078191			1	
BJAB083383			1	
BJANB083384			1	
BJANB083385			1	
BBX1				2
BBX2				2

Table 2c.

Haplotype	Qingdao	Rizhiao	Japan	Xiamen
BJQ1	1	1		
BJQ2	1			
BJQ3	1			
BJQ4	1			
BJQ5	1			
BJR1		1		
BJR2		1		
BJR3		1		
BJR4		1		
BJR5		1		
BJAB105142			1	
BJAB078191			1	
BJAB083383			1	
BJAB083384			1	
BJAB083385		2	1	
BBX1				1
BBX2				1
BBX3				1
BBX4				1
BBX5				1
BBX6				3

= 15.6%, A = 22.9%, G = 23.5%) and Rizhao (T = 38.0%, C = 15.6%, A = 23.0%, G = 23.4%) lancelets in North China were similar with that of Japanese lancelets (T = 37.8%, C = 15.8%, A = 23.0%, G = 23.4%), but had significantly differences with that of Xiamen lancelets (T = 39.2%, C = 16.5%, A = 21.7%, G = 22.6%) in South China based on COI gene. The similar results of nucleotide compositions were also checked in the partial fragments of Cyt*b* and 16S rRNA genes. The average numbers of transitiongs/transversions (Ti_{COI} : Tv_{COI} = 24 / 12, $Ti_{Cyt\,b}$: $Tv_{Cyt\,b}$ = 18 / 13, $Ti_{16S\ rRNA}$: $Tv_{16S\ rRNA}$= 28 / 18) of the lancelets between South and North China were larger than those (Ti_{COI} : Tv_{COI} = 4 / 0, $Ti_{Cyt\,b}$: $Tv_{Cyt\,b}$ = 4 / 0, $Ti_{16S\ rRNA}$: $Tv_{16S\ rRNA}$ = 3 / 0) between North China and Japanese waters based on the COI, Cyt*b*, and 16S rRNA genes, respectively (Table 3). The average numbers of transitiongs/transversions were 24/16 between South China lancelets and *B. malayanum*. Genetic distances among *Branchiostoma* lancelets from Qingdao, Rizhao, Japanese and Xiamen waters were generated on the basis of the best fit evolution models corrected with the shape parameter of a gamma distribution (Tables 1 and 3). The percentages of pair wise differences of lancelets within North China, Japanese waters, and South China based on COI gene averaged 0.80, 1.21 and 0.33%, respectively (Figure 2a). And those within North China, Japanese waters, and South China based on Cyt*b* and 16S rRNA genes averaged 1.04, 0.42, 0.93%, and 0.37, 0.40, 0.55%, respectively (Figure 2b, c). The genetic distances of lancelets between North and South China based on the COI, Cyt*b*, and 16S rRNA genes averaged 0.19, 0.21, and 0.17, respectively, but those between North China and Japanese waters based on the COI, Cyt*b*, and 16S rRNA genes averaged only 0.01, 0.01, and 0.01, respectively (Table 3). The genetic distance between Qingdao and Rizhao lancelets is very similar to that between Qingdao and Japanese waters lancelets based on the three mitochondrial DNA genes fragments (Table 3).

Phylogenetic relationships

Three phylogenetic trees constructed using the neighbor-joining method under the best fit substitution models showed the same results that North and South

Table 3. Pair wise genetic distances (PGD) and average sequence differences (transitions: transversions, Tr : Tv) of *Branchiostoma* lancelets in COI, Cyt*b*, and 16sRNA genes from Xiamen, Qingdao, Rizhao and Japan.

Sample	COI		Cyt*b*		16S rRNA	
	PGD	Tr : Tv	PGD	Tr : Tv	PGD	Tr : Tv
BBX vs. BJQ	0.18	24/12	0.21	27/23	0.17	28/18
BBX vs. BJR	0.18	25/13	0.21	24/20	0.17	30/20
BBX vs. BJ	0.18	25/12	0.21	27/23	0.17	28/18
BJQ vs. BJR	0.01	2/0	0.01	4/0	0.01	2/0
BJQ vs. BJ	0.01	3/0	0.01	4/0	0.01	2/0
BJR vs. BJ	0.01	4/0	0.01	4/0	0.01	2/0
South vs. North China	0.19	24/12	0.21	18/13	0.17	28/18
North China vs. Japan	0.01	4/0	0.01	4/0	0.01	3/0

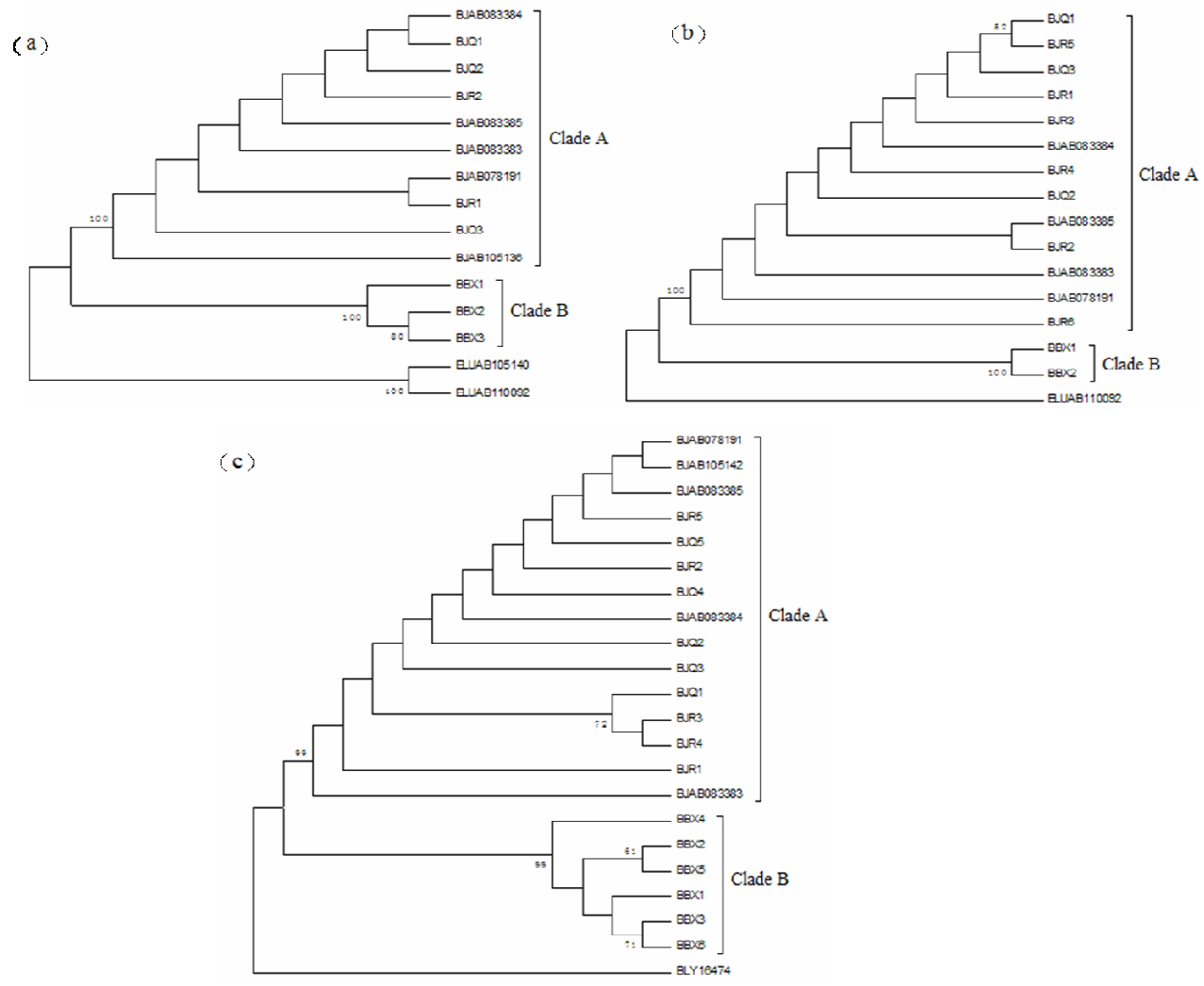

Figure 3. Three neighbor-joining trees of haplotypes for COI, Cyt*b*, and 16S rRNA genes based on their own best fit models. Bootstrap supports of > 50% in 5000 replicates are shown. (a): COI gene, (b): Cyt*b* gene, (c): 16S rRNA gene.

China lancelets of the genus *Branchiostoma* were reciprocally clustered into two monophyletic clades (clade A and clade B), supported by high bootstrap probabilities (Figure 3a, b, c). Within monophyletic clade A was recognized on the basis of COI, Cyt*b*, and 16S rRNA genes fragments, including lancelets from North China

(Qingdao and Rizhao) and Japanese waters with high confidence values. And monophyletic clade B only included South China (Xiamen) lancelets (Figure 3).

DISCUSSION

In previous studies, Tchang and Koo (1936) firstly reported the new distribution of Chinese lancelets in Kiaochow Bay and named them as a new variety *B. belcheri tsingtauense* on the basis of their morphological differentiations. From that time on, a commonly accepted opinion is that the lancelets along the east coast of China from South China Sea to Po-Hai Sea and Japanese waters are *B. belcheri* (Wang et al., 2004). But the surveys using the morphological methods appeared to show that the differentiation had occurred among the *B.* lancelets along the coast of China (Cao et al., 2001). The results of the present surveys from mtDNA genes analysis clearly indicated that *B. belcheri tsingtauense* in North China was markedly distinguishable from *B. belcheri* in South China, as concluded from the previous morphological analysis by Yan et al. (2005). The data also showed that *B. belcheri tsingtauense* in North China and Japanese waters lancelets shared a very close genetic relationship (Figure 2). The genetic distances of the lancelets between North and South China averaged more than 0.17 based on the three partial mtDNA genes fragments, which were similar with those observed in other interspecific variations (Nohara et al., 2004). However, the values between North China and Japanese waters *Branchiostoma* lancelets were less than 0.01 based on the three mtDNA genes, which indicated low intraspecific genetic divergence existed in the two areas (Table 3). All genetic information strongly supported that the *Branchiostoma* lancelets from North and South China should belong to different species and the lancelets from North China and most Japanese waters should belong to the same species.

According to Nishikawa's (1981) research, the Japanese lancelets except those from Ariake Sea and Amakusa belonged to the subspecies *B. belcheri* var. *tsingtauense* as first named by Tchang and Koo (1936). But Willey (1897) first named the Japanese lancelets as a variety *B. belcheri* var. *japonicus* on the basis of the morphological observation. Based on the rule of priority and recent taxonomic studies coupled with the present genetic results on the *Branchiostoma* lancelets, the subspecies name, *B. belcheri* var. *tsingtauense*, is invalid nomenclature for lancelets in North China and most Japanese waters. Comparison with *B. belcheri* in South China based on the present results and the rule of priority, the original subspecies *B. belcheri tsingtauense* together with the *B.* lancelets in most Japanese waters is an independent species and its name should be revised to *B. japonicus*. Consequently, there are at least two species of the genus *Branchiostoma* existed in China Sea.

Divergence time for the two *Branchiostoma* species also has been estimated from the Cyt*b* and COI genes sequences data. The rate of mitochondrial DNA (mtDNA) evolution (7.1×10^{-10} tranversions/sites· year) determined from the COI and Cyt*b* genes of sharks was used as the molecular clock for mtDNA genes of lancelets (Andrew et al., 1992). Sharks are regarded as the closest relatives of lancelets among all the animals that have been studied for evolutionary rates of mtDNA, so the molecular clock from shark mtDNA may supply a reasonable estimate of divergence time for the two *Branchiostoma* species. According to Andrew et al. (1992) estimated method, the divergence time between *B. belcheri* and *B. japonicus* was reckoned at about 39.90 - 43.24 million years ago (Mya), middle Eocene, and that between *B. belcheri* and *B. malayanum* was at 58.26 Mya. The divergence time between *B. japonicus* and *B. malayanum* was at 97.75 Mya estimated by Nohara et al. (2004). The divergence-time estimates among the three species indicated that dispersal had occurred for *B.* lancelets from south to north in ancient Western Pacific Ocean. Geographic events (e.g. glacial period) and benthic habits may have played some roles in bringing about such genetic differentiation for the *Branchiostoma* species.

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