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Comparison of the Genetic Relationships and Osteological Aspects in Six Branchiostegid Fish Species (Perciformes)

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Abstract: We analyzed partial sequences of cytochrome *b* (cyt-*b*), a mitochondrial DNA (mtDNA) gene, to determine the genetic relationships between six horsehead fish species: *Branchiostegus japonicus, Branchiostegus albus, Branchiostegus auratus, Branchiostegus argentatus, Branchiostegus wardi,* and an unidentified *Branchiostegus* species. The specimens were collected in Korea, China, Japan, and Vietnam. We compared their molecular phylogenetic relationships inferred from mtDNA cyt-*b* sequences with an osteological analysis. The unidentified species, *B.* sp., was similar to *B. albus* in terms of the lack of triangular silver-white dot at the posterior region of eyes (vs. large one present in *B. japonicus*), but was also similar to *B. japonicus* in terms of the presence of a straight-shaped first hemal spine (vs. a curve-shaped hemal spine in *B. albus*). Analysis of the mtDNA cyt-*b* sequences indicated that the smallest estimated sequence divergence was between the *B. japonicus* and *B.* sp. (0.70-0.94%), whereas the largest difference was between *B. auratus* and *B. argentatus* (23.06-23.36%). Both the maximum parsimony and maximum likelihood trees showed that the *B.* sp. was closely clustered with *B. japonicus*, and that *B. auratus* was most distant from the other species. When comparing the osteological characters, UPGMA tree showed that the *B. japonicus* and *B.* sp. were the most closely clustered species, and *B. auratus* was the most distantly clustered fish relative to the other species. The shape of the nasal, otolith and first hemal spine was informative for distinguishing *B. auratus* from the other species. These osteological differences were consistent with the differences in mtDNA.

Key words: *Branchiostegus*, cytochrome *b*, osteological analysis, molecular phylogeny

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INTRODUCTION

The genus *Branchiostegus* is an attractive taxon in the field of phylogeny because of its morphological similarity and the lingering controversy regarding its higher taxonomic classification (Kishinouye, 1907; Ochiai, 1953; Johnson, 1984; Kim and Ryu, 1998). The *Branchiostegus* species have usually been classified based on body color, and not on meristic or morphometric characteristics, because of the extent of overlap of the latter (Irie, 1953; Dooley, 1978; Dooley and Kailola, 1988; Nakabo, 2002). There are four species in the genus *Branchiostegus* in Korea (i.e., *B. japonicus*, *B. albus*, *B. auratus*, and *B. argentatus*) (Kim et al., 2005b), six species in Japan (Nakabo, 2002), and 16 species throughout the world (Nelson, 2006). Several taxonomic studies have examined morphology, osteology, or myology to clarify the taxonomic position of the Branchiostegidae (or Malacanthidae) and to identify appropriate taxonomic characteristics, even after formalin-fixation (Irie, 1953; Ochiai, 1953; Marino and Dooley, 1982; Johnson, 1984; Kim and Ryu, 1998; Imamura, 2000). For example, some researchers consider *B. japonicus* and *B. auratus* to be the same species, which should be subdivided into different subspecies (Ochiai, 1953; Chyung, 1977), while others consider them to be different species (Dooley, 1978).

In the phylogenetic study of the family Branchiostegidae (or Malacanthidae), Dooley (1978) proposed that this family be separated into the two families, Branchiostegidae and Malacanthidae, based on 13 characteristics (e.g. external features, osteological features, and habitat). The monophyly of the family Branchiostegidae was subsequently supported by Marino and Dooley (1982), based on a comparison of the adductor mandibulae of 21 branchiostegid fishes with those of other malacanthid fishes (outgroup);

however, Johnson (1984) insisted that the family Malacanthidae was valid, based on a common characteristic (i.e. nasal fusion) that existed during the early stages of life in both families (Branchiostegidae and Malacanthidae). Later, Imamura (2000) suggested that the branchiostegid fishes should be included in the family Dactylopteridae, based on a common derived character (i.e. synapomorphy) present in both families (Dactylopteridae and Malacanthidae). The controversy over the classification of the *Branchiostegus* species into higher taxa was due to either morphological similarities or polyphyly of Percoidei (Nelson, 2006); therefore, new molecular phylogeny approaches are required to classify the branchiostegid fishes.

Although morphological characteristics remain widely used in phylogenetic analyses (Moser et al., 1984; Nelson, 1994; Stiassny et al., 1996; Minaka, 1997; Wiens, 2000; Springer and Johnson, 2004), the development of DNA analysis methods has resulted in an abrupt increase in the number of reports on molecular phylogeny and phylogeography in the past decade (Hwang and Kim, 1999; Avise, 2000; Inoue et al., 2001; Whelan et al., 2001; Nelson, 2006). In particular, maternal inheritance of mitochondria DNA (mtDNA), especially the cyt-*b* gene which contains both conserved and variable sites, is now widely used in molecular phylogeny (Zhu et al., 1994; Grant and Bowen, 1998; Kartavtsev and Hanzawa, 2007; Zhang et al., 2007; Lee et al., 2008).

The goal of the present study was to estimate the molecular phylogenetic relationships among six *Branchiostegus* species (*B. japonicu, B. albus, B. auratus, B. argentatus, B. wardi*, and *B.* sp.) based on mtDNA cyt-*b* gene sequences, and to further compare them with the results obtained using osteological analyses.

MATERIALS AND METHODS

Sampling

Six *Branchiostegus* species (i.e., *B. japonicus, B. albus, B. auratus, B. argentatus, B. wardi*, and *B.* sp.) were collected in Korea, China, Japan, and Vietnam (Table 1). For outgroup comparison (Watrous and Wheeler, 1981), the partial cytochrome *b* sequences of *Salanx ariakensis* and *Salangichthys microdon* were obtained from GenBank (AB196848, AB196906; Kim et al., 2006), and the osteological analysis of the two species, *Epinephelus septemfasciatus* and *Scombrops boops* were conducted.

DNA isolation, PCR and sequencing

Total genomic DNA was extracted from muscle tissues preserved at –30°C, using the method described by Asahida et al. (1996). The mtDNA cytochrome *b* gene was amplified using a universal primer set (Kocher et al., 1989), a light-strand Cyt-L primer (5'-CGAAGCTTGATATGAAAAAC CATCGTT-3'), and a heavy-strand Cyt-H primer (5'-AA ACTGCAGCCCCTGCTCAGAATGATATTTGTCCTCA-3'). The PCR reaction mixture contained 4 μL of genomic DNA (50 ng/μL), 4 μL of 10×buffer, 3.2 μL of 0.2 mM dNTP, 2 μL of primers (10 pmole), and 1.25 units of *Taq* polymerase (Takara, Japan), to a final mixture of 40 μL. PCR reactions were carried out using the following conditions: an initial denaturation step at 94°C for 5 min; 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 2 min; and a final extension at 72°C for 5 min. PCR products were purified using a PCR purification kit (Qiagen, Germany), and were then used in direct cycle sequencing reactions with the Big Dye terminator system (Applied Biosystems, USA). DNA

Table 1. List of *Branchiostegus* species and outgroups

Abbreviation	Species	Sampling location/date	Accession No.
AU1	*Branchiostegus auratus*	Nagasaki, Japan/Jul., '98	EU821585
JA1	*Branchiostegus japonicus*	Jeju, Korea/Apr., '97	EU821586
JA2	*Branchiostegus japonicus*	Jeju, Korea/Apr., '97	EU821587
WA1	*Branchiostegus wardi*	Hainan, China/Jul., '99	EU821588
WA2	*Branchiostegus wardi*	Hainan, China/Jul., '99	EU821589
HA1	*Branchiostegus* sp.	Hainan, China/Apr., '98	EU821590
AR1	*Branchiostegus argentatus*	Vietnam /Apr., '99	EU821591
AR2	*Branchiostegus argentatus*	Hainan, China/Jul., '99	EU821592
AL1	*Branchiostegus albus*	Tongyong, Korea/May, '96	EU821593
AL2	*Branchiostegus albus*	Jeju, Korea/Apr., '97	EU821594
AL3	*Branchiostegus albus*	Jeju, Korea/Apr., '97	EU821595
SA1	*Salanx ariakensis*	Jindo, Korea/May, '04	AB196848
SA2	*Salangichthys microdon*	Mikata, Japan/Apr., '04	AB196906

sequences were obtained using the ABI 377 automated DNA sequencer (Applied Biosystems, USA) and were subsequently arrayed using the DNASIS ver. 2.5 software (Hitachi, Japan). Nucleotide sequence data reported here have been supplied to the DDBJ/EMBL/GenBank nucleotide sequence databases (accession numbers EU821585-EU821595).

DNA analysis

Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed using PAUP 4.0b10 (Swofford, 2002). Heuristic MP analyses were conducted with tree bisection-reconnection (TBR) branch swapping and 100 random addition sequences. The Hasegawa-Kishino-Yano model with variable sites assumed to follow a discrete gamma distribution (HKY+Γ; Hasegawa et al., 1985) was selected as the best fit model of nucleotide substitution (ModelTest ver. 3.06; Posada and Crandall, 1998). All ML heuristic searches were carried out using TBR branch-swapping.

Osteological analysis

Osteological characteristics assessed by alizarin red-S staining were visualized and sketched using an Olympus SZH10 stereomicroscope equipped with a Lucida camera. Clearing and staining were performed according to Kawamura and Hosoya (1991). We analyzed and compared the morphology of cranium, visceral skeleton, vertebrae, caudal skeleton, shoulder girdle and jaw bones. Of which, a total of six osteological characters, being informative, were scored and presented in Table 3. The osteological terminology was the same as in Ochiai (1953), Rojo (1991), and Kim (1989). Cluster analysis was performed with the unweighted pair-group method, arithmetic average (UPGMA), using the NTSYS version 1.50 software (Rohlf, 1988).

RESULTS

Molecular relationship

Analysis of 430 bp of the mtDNA cyt-*b* gene sequence revealed variations at a total of 85 nucleotide positions, which were primarily base substitutions. *B. auratus* was clearly differentiated from the other species (Fig. 1) and transversions were found at 39 nucleotide positions, at a very high frequency (46%). Most variations were transitions, with transversions found at only seven nucleotide positions, which suggests a higher frequency of base substitution in transitions than in transversions. *B. auratus* had a specific sequence at 54 nucleotide positions, including position numbers 13, 40, 50, and 58. In addition, *B. argentatus* had a species-specific sequence at three nucleotide positions (i.e. positions number 91, 294, and 412), of which the transversion at nucleotide 91 was clearly different from the other species, and was considered a species-specific genetic

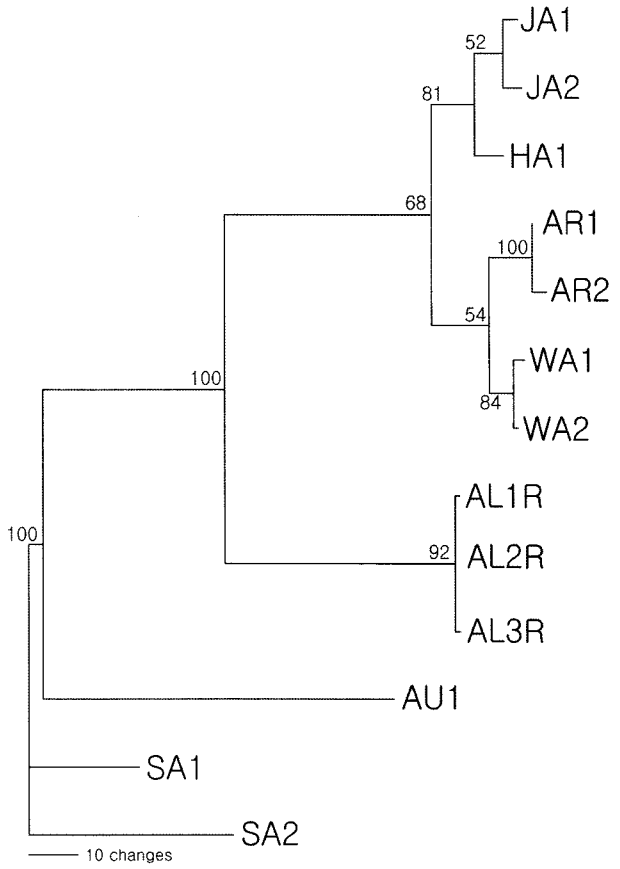

Fig. 1. Maximum parsimony tree inferred from the first 430 bp of mtDNA cyt-*b* gene sequences among the six *Branchiostegus* species with two outgroups (SA1 and SA2). Abbreviation of fish taxon corresponds to Table 1. Tree length, 237, consistency index, 0.8144; retention index, 0.8402; and rescaled consistency index, 0.7303. The numbers in the branches indicate bootstrap probabilities in 1,000 bootstrap replications using the heuristic search option in PAUP 4.0b 10 (Swofford, 2002).

marker. The genetic divergence within each species was very low: 0.62%±0.37 (mean±standard error) in *B. albus*, 0.70%±0.41 in *B. argentatus*, and 1.17%±0.53 in *B. japonicus*. The estimated genetic divergence among the six branchiostegid fishes was slightly higher, 0.70-23.36%. This increase in genetic divergence reflects the fact that the genetic divergence between *B. auratus* and the other species was significantly higher, ranging from 19.56% to 23.36%. Moreover, the genetic divergence between *B. albus* and *B.* sp. was found to range from 4.32% to 4.82%, suggesting that these two fish may be considered different species. In contrast, the genetic divergence between *B. japonicus* and *B.* sp. was 0.70-0.94%; therefore, using this criterion, these two fish belong to the same species (Table 2).

In both the MP and ML trees, the *B.* sp. was closely clustered with *B. japonicus*, which was supported by the 81% bootstrap value determined using MP analysis, whereas the location of *B. auratus* on the tree was distant

Table 2. Genetic divergence among the six *Branchiostegus* species with two outgroups (SA1 and SA2), based on mtDNA cytochrome *b* gene sequences

	JA1	JA2	HA1	AL1	AL2	AL3	AR1	AR2	WA1	WA2	AU1	SA1
JA1												
JA2	0.0117											
HA1	0.0070	0.0094										
AL1	0.0507	0.0582	0.0482									
AL2	0.0483	0.0531	0.0432	0.0070								
AL3	0.0508	0.0557	0.0457	0.0094	0.0023							
AR1	0.0434	0.0482	0.0409	0.0560	0.0535	0.0561						
AR2	0.0508	0.0507	0.0483	0.0635	0.0610	0.0636	0.0070					
WA1	0.0409	0.0408	0.0334	0.0482	0.0432	0.0457	0.0434	0.0508				
WA2	0.0384	0.0407	0.0310	0.0508	0.0458	0.0483	0.0359	0.0433	0.0117			
AU1	0.2019	0.1989	0.1988	0.2016	0.1956	0.1986	0.2306	0.2336	0.2209	0.2272		
SA1	0.2641	0.2633	0.2606	0.2780	0.2682	0.2717	0.2892	0.2996	0.2849	0.2852	0.2636	
SA2	0.3208	0.3197	0.3169	0.3060	0.2996	0.2958	0.3246	0.3355	0.3169	0.3135	0.2879	0.1448

Abbreviation of fish taxon corresponds to the Table 1. Genetic distances were corrected using Kimura's (1980) two-parameter model.

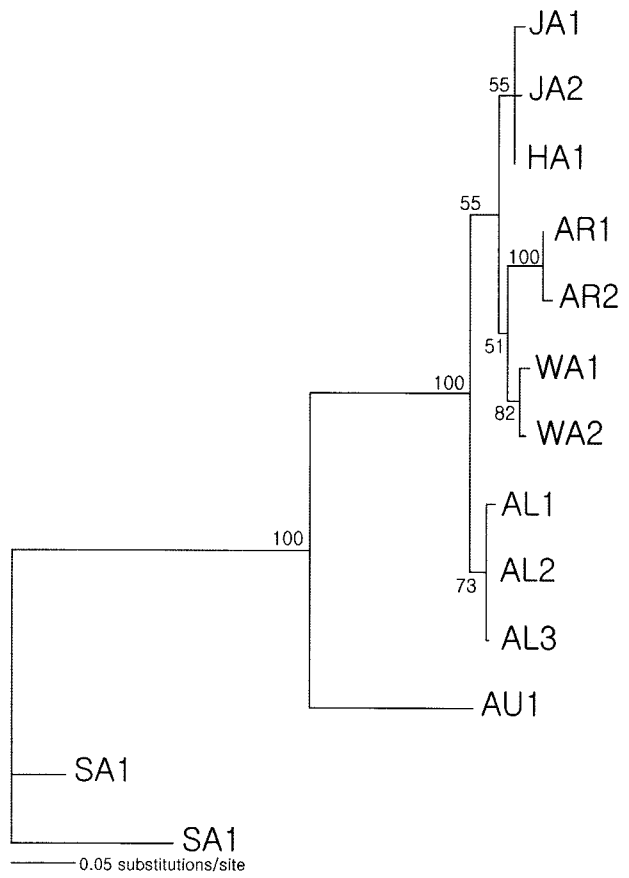

Fig. 2. Maximum likelihood tree inferred from the first 430 bp of mtDNA cyt-*b* gene sequences among the six *Branchiostegus* species with two outgroups (SA1 and SA2). Abbreviation of fish taxon corresponds to Table 1. The HKY+Γ model of sequence evolution (Hasegawa et al., 1985) was used. The numbers in the branches indicate bootstrap probabilities in 500 bootstrap replications using the heuristic search option in PAUP 4.0b 10 (Swofford, 2002).

from the remaining species. Among the six branchiostegids, three individuals of *B. albus* (AL1, 2, 3) were also located considerably far from the three species (*japonicus*, *argentatus* and *wardi*) (Figs. 1, 2).

Osteological relationship

Analyses of the osteological characteristics of the *Branchiostegus* species revealed interspecies differences in the shape of the nasal, preorbital, first hemal spine, basisphenoid, otolith and scale. The anterior process of the nasal was absent or rudimentary in *B. japonicus*, *B. albus*, *B. argentatus*, *B. wardi*, and *B.* sp. (Fig. 3A, B, D, E, and F), while it was highly pronounced in *B. auratus* (Fig. 3C). The external shape of the preorbital was quadrilateral in *B. albus*, *B. auratus*, and *B. argentatus* (Fig. 3B', C', and D') and asymmetric in *B. japonicus*, *B. wardi*, and *B.* sp. (Fig. 3A', E', and F'). The ratio of the length of the first and second hemal spines showed interspecies differences. In *B. auratus* (Fig. 3C"), the length of the first hemal spine was about half that of the second hemal spine; however, the length between the first and second hemal spines was similar in all other species (Fig. 3A", B", D", E", and F"). The shape of the first hemal spine was curved in *B.* sp. (Fig. 3B"), but was straight in the other species (Fig. 3A", D", E", and F"). The width of the otolith was very thick in *B. auratus*, but was very thin in the other species. In the shape of scale, well developed ctenoid scales were found in *B. argentatus*, but both cycloid and ctenoid scales (usually cycloid scale) were found in the other species.

A character matrix-based UPGMA tree was constructed (Table 3) and showed that the *B. japonicus* and *B.* sp. were the most closely clustered species, whereas *B. auratus* was

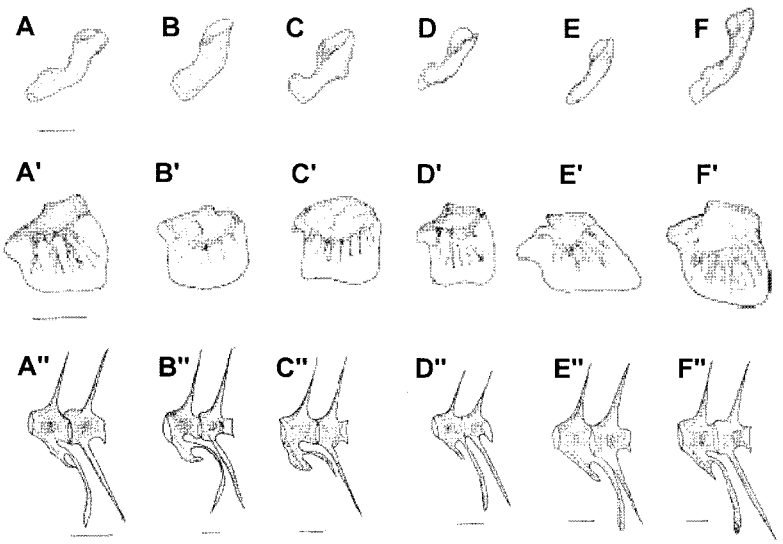

Fig. 3. Comparison of nasal (A–F), preorbital bones (A'–F'), and the first-second caudal vertebrae (A"-F") of the six *Branchiostegus* species. A-A": *B. japonicus*; B-B": *B. albus*; C-C": *B. auratus*; D-D": *B. argentatus*; E-E": *B. wardi*; F-F": *B.* sp. Scale bars=2 mm.

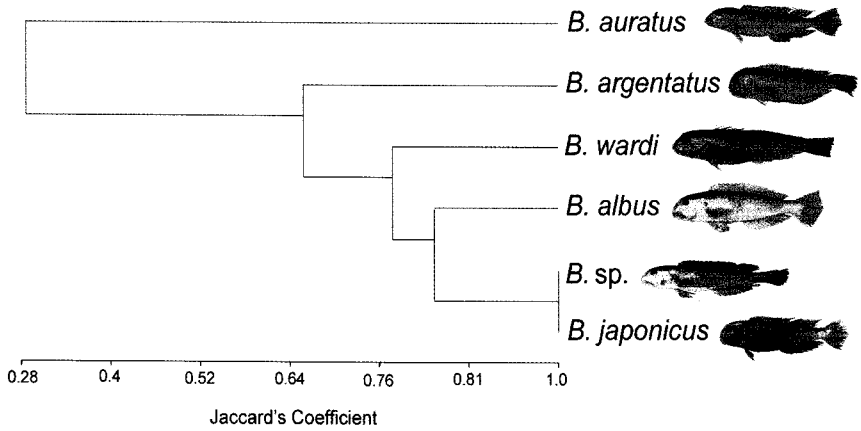

Fig. 4. UPGMA tree inferred from six osteological characters among the six *Branchiostegus* species. The tree was derived from Jaccard's coefficient.

Table 3. Matrix of osteological characteristics of the six *Branchiostegus* species

Character/region	JA	AL	AU	AR	WA	HA
Basisphenoid/cranium	1	1	1	1	0	1
Otolith/cranium	1	1	0	1	1	1
Nasal/cranium	1	1	0	1	1	1
Hemal spine/vertebrae	1	1	0	1	1	1
Scale/surface	1	1	1	0	1	1
Preorbital/orbital region	1	0	0	0	1	1

Abbreviation of fish taxon corresponds to the Table 1.

the most distantly clustered fish relative to the other species (Fig. 4).

DISCUSSION

Comparison of the mtDNA cyt-*b* gene sequence in six *Branchiostegus* species revealed that *B. auratus* was clearly distinct from the other species. A similar result was obtained using a osteological comparison analysis. Interestingly, in the comparative sequence analysis, *B. auratus* may have differed from the other species at the genus level. The divergence in the mtDNA cyt-*b* gene sequence was 0.32-4.0% (1.55±0.56) at the population level, 3.5-12.0% (5.52 ±1.34) at the sibling species level, 2.3-26.2% (10.96±1.34) at the species level, and 6.6-32.8% (18.51±2.09) at the genus level (Kartavtsev and Lee, 2006). The genetic distance between *B. auratus* and the other species was so high (18.7-21.8%) that this species could potentially belong to a different genus. Further studies of the demographic history of *B. auratus* and its relationship with other genera are needed to clarify the taxonomic position of this fish.

Kishinouye (1907) was the first to report differences among the three *Branchiostegus* species (*B. japonicus, B. auratus*, and *B. albus*), namely in the shape of the first

caudal vertebra and of the preopercle; however, Ochiai (1953) suggested that the first two species were the same, because the shape of the first caudal vertebra (straight) was the same in both species, which was in contrast to the shape found in *B. albus* (curved shape). Unlike Ochiai (1953), we found that the shape of the first caudal vertebra of *B. japonicus* was clearly different from that of *B. auratus* (Fig. 3A" vs. C"). In *B. japonicus*, the length of the first hemal spine was identical to that of the second, but in *B. auratus*, the first hemal spine was about half the length of the second; these results contradict those of Kishinouye (1907) and Ochiai (1953). In addition, Irie (1953) considered *B. japonicus* and *B. auratus* to be distinct species, based on color pattern (i.e. the presence of a silver-white stripe under the eyes and round-shaped blotches in the caudal fin in *B. auratus*). This hypothesis was subsequently accepted by many ichthyologists (Dooley, 1978, 1988; Nakabo, 2002; Kim et al., 2005a, b). In the present study, the shape of the nasal, otolith and first hemal spine was used as characteristics to distinguish *B. auratus* from other species.

Although some morphological differences (e.g., triangular silver-white dot at the posterior region of eyes) were found between *B. japonicus* and *B.* sp., only a few differences in the mtDNA sequence between the two species were observed, suggesting that the latter is a color variation of the former. Furthermore, the UPGMA tree (Fig. 4) supports the hypothesis that the *B.* sp. could be a color variation of *B. japonicus*, based on a comparison of six osteological characteristics. However, because body color is considered one of the most important taxonomic characteristics in the Branchiostegidae (or Malancanthidae) (Dooley, 1978; Dooley and Kailola, 1988), investigations into additional characteristics (e.g., muscle) will be necessary to further clarify the taxonomic position of *B.* sp.

Marino and Dooley (1982) classified *Branchiostegus* into four groups, according to the shape and insertion of the adductor mandibulae. *B. argentatus*, *B. japonicus*, and *B. albus* were included in the third group, and *B. wardi* was placed in the fourth; however, our results reveal that *B. wardi* was closely clustered with *B. argentatus*, whereas *B. albus* was distantly clustered relative to the other species (Figs. 1 and 4). These results contradict the findings of Marino and Dooley (1982). Conflicting evidence regarding the phylogenetic relationships between mtDNA and nuclear DNA has arisen in recent years (Shaw, 2002; Holcroft, 2005). Furthermore, mtDNA may not reflect the true evolutionary history of a species because of its maternal inheritance (Seehausen et al., 2003; William et al., 2004). Holcroft (2005) demonstrated that a nuclear DNA tree explains the morphological relationship between species better than mtDNA; therefore, additional study including nuclear DNA sequence analysis is necessary to determine the true phylogenic relationships among the *Branchiostegus* species.

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