〈Original Article〉

Comparative genomics of mitochondrial DNA in *Branchiostoma* species

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Summary A comparative genomic analysis of mitochondrial DNAs (mtDNAs) of *Branchiostoma* was performed using 10 types of mtDNA data, including those for mtDNAs of *Branchiostoma belcheri* (*B. belcheri*; a Pacific lancelet), *Branchiostoma lanceolatum* (*B. lanceolatum*; an Atlantic lancelet), and *Branchiostoma floridae* (*B. floridae*; an Atlantic lancelet), as well as 6 more types of mtDNA data obtained from databases. The *Branchiostoma* species corresponding to the 10 types of mtDNA data were classified into the following 4 clusters: 2 clusters of *B. belcheri*, 1 cluster of *B. lanceolatum*, and 1 cluster of *B. floridae*. Spruyt *et al*. have reported that *B. lanceolatum* and *B. floridae* have an identical mtDNA, which has therefore been considered by some investigators to be attributable to a misidentification of *B. lanceolatum* used as a research subject. The results of the present analysis suggest that the *B. lanceolatum* examined by Spruyt *et al*. can be classified as a species or group closely related to *B. floridae*. We also analyzed polymorphisms among *Branchiostoma* species (thus establishing a basis for their classification by molecular biological approaches), and we clarified their geographical distribution. Our findings should prove very useful for future studies in the fields of phylogenetics, evolutionary biology, and phylogenetic systematics.

Key words: *Branchiostoma belcheri*, *Branchiostoma lanceolatum*, *Branchiostoma floridae*, Mitochondrial DNA, Polymorphism

1. Introduction

The anatomical features of *Branchiostoma* that distinguish its various species include relative location of the notochord, structure of the feeding organ, entire length of the body, shapes of fins, locations of the peribranchial cavity and anus, numbers of sarcomeres, branchial chambers, and reproductive glands[1)]. Since these features may be greatly affected by habitat, comparisons among the various *Branchiostoma* species by DNA sequencing is considered preferable. In a series of evolutionary studies of *Branchiostoma*, we have found[2, 3)] that the entire sequence[4)] of the mitochondrial DNA (mtDNA) of *Branchiostoma*

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lanceolatum (*B. lanceolatum*)[5] is quite similar to that of *Branchiostoma floridae* (*B. floridae*)[6]. On the other hand, Cañestro et al.[7] found a marked difference between the alcohol dehydrogenase (*Adh*) gene (a nuclear gene), of *B. lanceolatum* and that of *B. floridae*. Furthermore, Nohara *et al.*[8,9] noted a discrepancy in DNA sequencing among the results of previous molecular studies. In the present study, with the aim of addressing this discrepancy, we performed a comparative genomic analysis of *Branchiostoma* using 10 types of mtDNA data, including new data for mtDNAs of *Branchiostoma belcheri* (*B. belcheri*), *B. lanceolatum*, *B. floridae*[10], and 6 other types of mtDNA data obtained from databases. We also searched for polymorphisms among *B. belcheri*, *B. lanceolatum*, and *B. floridae* using mtDNAs obtained from multiple individuals of these species, and we discuss differences in habitat, in order to objectively discriminate among *Branchiostoma* species.

2. Materials and methods

Ten individuals of *B. belcheri* (material abbreviated here as *Bb*-T), a Pacific species, were collected in waters close to Japan. Ten individuals of *B. lanceolatum* (*Bl*-M), an Atlantic species, collected in Argeles-sur-Mer on the Mediterranean coast of France were kindly provided by Dr. Michael Schubert from the Institut de Génomique Fonctionnelle de Lyon (IGFL) (*Bl*-M). Twenty individuals of *B. floridae* (*Bf*) collected in Tampa Bay, Florida, in the USA were kindly provided by Dr. Michael Schubert from IGFL (*Bf*-M) and Dr. Linda Holland from the Scripps Institution of Oceanography University of California-San Diego (*Bf*-H).

Sequences of mtDNAs were determined by PCR-direct sequencing[10]. Nucleotide sequences were translated into amino acid sequences using EMBOOS

Table 1 Localization of mitochondrial DNA genes and non-coding regions of *Branchiostoma*

Gene	*Bb*-T		*Bl*-M		*Bf*-M		*Bf*-H		*Bb*-G		*Bj*-W		*Bb*-W		*Bl*-N		*Bl*-S		*Bf*-B	
	Size		Size		Size		Size		Size		Size		Size		Size		Size		Size	
	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)	(bp)	(aa)
cytb	1,141	380	1,143	380	1,143	380	1,143	380	1,141	380	1,141	380	1,142	380	1,143	380	1,143	380	1,143	380
tRNA-Thr	65		65		64		64		65		65		68		65		66		64	
tRNA-Pro	65		67		66		66		65		65		68		67		68		66	
12S rRNA	847		843		846		846		847		847		876		843		844		846	
tRNA-Phe	63		66		66		67		63		63		66		66		67		66	
tRNA-Val	72		67		67		67		72		72		67		67		67		67	
16S rRNA	1,383		1,376		1,367		1,367		1,384		1,382		1,381		1,379		1,367		1,371	
tRNA-Leu (TTR)	70		71		71		71		70		70		71		71		71		71	
NADH1	945	314	940	313	940	314	940	314	945	314	945	313	944	314	943	313	942	313	940	313
tRNA-Ile	63		64		63		63		63		63		65		64		63		63	
tRAN-Met	67		67		67		67		67		67		67		67		67		67	
tRNA-Gln	68		69		69		69		68		68		69		69		69		69	
NADH2	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346	1,041	346
tRNA-Asn	72		67		70		70		72		72		69		67		70		70	
tRNA-Trp	67		66		69		69		67		67		65		66		69		69	
tRAN-Ala	63		62		63		63		63		63		63		63		63		63	
tRNA-Cys	59		59		58		58		59		59		57		59		58		58	
tRNA-Tyr	65		66		66		66		65		65		66		66		66		66	
COI	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515	1,548	515
tRNA-Ser (TCN)	71		71		70		70		71		71		71		71		71		71	
tRNA-Asp	63		66		65		65		63		63		66		66		65		65	
COII	691	230	691	230	691	230	691	230	691	230	691	230	691	230	691	230	720	239	691	230
tRNA-Lys	65		66		66		66		65		65		66		66		66		66	
ATP8	165	54	165	54	165	54	165	54	165	54	165	54	165	54	165	54	165	54	165	54
ATP6	683	227	683	227	683	227	683	227	684	227	684	227	683	227	683	227	684	227	683	227
COIII	789	262	789	262	788	262	788	262	789	262	789	262	789	262	792	262	789	262	788	262
NADH3	354	117	354	117	352	117	352	117	354	117	354	117	352	117	354	117	354	117	352	117
tRNA-Arg	62		63		67		67		62		62		62		63		69		67	
NADH4L	276	91	275	91	275	91	275	91	276	91	276	91	275	91	275	91	276	91	275	91
NADH4	1,359	452	1,358	452	1,358	452	1,358	452	1,359	452	1,359	452	1,358	452	1,358	452	1,359	452	1,358	452
tRNA-His	67		65		66		66		67		67		66		65		66		66	
tRNA-Ser (AGY)	67		67		66		66		67		67		67		67		66		66	
tRNA-Leu (CTN)	66		68		68		68		66		66		68		68		68		68	
NADH5	1,797	598	1,797	598	1,797	598	1,797	598	1,797	598	1,797	598	1,794	598	1,797	598	1,797	598	1,800	599
NC sequence	110		190		129		129		110		110		184		191		129(57)		129	
tRNA-Gly	67		67		68		68		67		67		67		67		68		68	
NADH6	504	167	503	167	504	167	504	167	504	167	504	167	504	167	504	167	504	167	504	167
tRNA-Glu	68		66		65		65		68		68		67		66		65		65	
mtDNA	15,075		15,139		15,076		15,077		15,076		15,075		15,182		15,146		15,076		15,083	

Data from: *Bb*-T (*Branchiostoma belcheri* T01, AB478554), *Bb*-G (*Branchiostoma belcheri*, NC_004537), *Bj*-W (*Branchiostoma japonicum*, DQ407722), *Bb*-W (*Branchiostoma belcheri*, AY932825), *Bl*-M (*Branchiostoma lanceolatum* M01, AB478564), *Bl*-N (*Branchiostoma lanceolatum*, AB194383), *Bl*-S (*Branchiostoma lanceolatum*, Y16474), *Bf*-M (*Branchiostoma floridae* M01, AB478574), *Bf*-H (*Branchiostoma floridae* H01, AB478581), *Bf*-B (*Branchiostoma floridae*, AF098298).

Numbers in parentheses indicate corrected gene size.

Table 2 Percentage base composition of mitochondrial DNA in *Branchiostoma* species

Base	*Bb*-T	*Bl*-M	*Bf*-M	*Bf*-H	*Bb*-G	*Bj*-W	*Bb*-W	*Bl*-N	*Bl*-S	*Bf*-B	Mean
Adenine (A)	27.6	26.0	27.0	26.9	27.5	27.5	25.8	26.1	26.9	26.9	26.8
Cytosine (C)	13.9	16.4	15.5	15.9	13.9	14.0	13.5	16.5	15.9	15.9	15.1
Guanine (G)	21.4	21.8	21.4	21.5	21.3	21.4	22.8	21.8	21.4	21.4	21.6
Thymine (T)	37.1	35.7	36.1	35.7	37.3	37.1	37.9	35.7	35.8	35.8	36.4
A+T	64.7	61.7	62.7	62.7	64.8	64.6	63.7	61.8	62.7	62.7	63.2
C+G	35.3	38.3	37.3	37.3	35.2	35.4	36.3	38.2	37.3	37.3	36.8

Abbreviations are the same as in Table 1.

Transeq (http://www.ebi.ac.uk/Tools/emboss/transeq/index.html) provided by EMB-EBI. Structures of transfer RNA (tRNA) genes were analyzed using tRNAscan-SE 1.21 (http://lowelab.ucsc.edu/tRNAscan-SE/) provided by Todd M. Lowe[11]. Multiple sequence alignment analysis was performed using a CLUSTAL-W program (http://www.ddbj.nig.ac.jp/Welcome-j.html) provided by the DNA Data Bank of Japan (DDBJ)[12-14]. Molecular evolutionary trees were created using the NJplot (http://pbil.univ-lyon1.fr/software/njplot. html)[15], with the previously-reported mtDNA sequences of *B. belcheri* (*Bb*-G: NC_004537)[2], *B. japonicum* (*Bj*-W: DQ407722), *B. belcheri* (*Bb*-W: AY932825), *B. lanceolatum* (*Bl*-N: AB194383)[9], *B. lanceolatum* (*Bl*-S: Y16474)[4], and *B. floridae* (*Bf*-B: AF098298)[6] used as references and that of *Lampetra fluviatilis* (*Lam*: Y18683)[16] used as an outgroup.

3. Results

1. Mitochondrial DNA size

The sizes of mtDNAs analyzed were as follows: 15,075 base pairs (*bp*) for *Bb*-T, 15,139-15,141 bp for *Bl*-M, 15,076-15,077 bp for *Bf*-M, 15,075-15,077 bp for *Bf*-H, 15,076 bp for *Bb*-G, 15,075 bp for *Bj*-W, 15,182 bp for *Bb*-W, 15,146 bp for *Bl*-N, 15,076 bp for *Bl*-S, and 15,083 bp for *Bf*-B (Table 1).

The mean frequencies of each base in mtDNAs were as follows: 26.8% for adenine (A), 15.1% for cytosine (C), 21.6% for guanine (G), and 36.4% for thymine (T). Similar patterns of base distribution were observed among *Bb*-T, *Bb*-G, and *Bj*-W, between *Bl*-M and *Bl*-N, and between *Bf* and *Bf*-B. *Bb*-W exhibited the highest frequencies of G (22.8%) and T (37.9%) and the lowest frequency of C (13.5%) among all *Branchiostoma* species tested. Base frequencies differed 0.5-2.6% among species. The base frequencies in *Bl*-S differed from that of *Bl*-M, but was the same as that of *Bf*. The mean GC content was 36.8%, with no intra-species difference and a maximum of 3.0% inter-species difference (Table 2).

2. Protein-coding genes

With regard to protein-coding genes, the size of the *NADH1* was the same among *Bb*-T, *Bb*-G, *Bj*-W, and *Bb*-W, and among *Bl*-M, *Bl*-N, and *Bl*-S, while that of the *NADH5* differed between *Bf* and *Bf*-B. The *COII* of *Bl*-S coded for 239 amino acids (aa), which was longer by 9 aa than the corresponding gene of the other *Branchiostoma* species (230 aa). The sizes of other genes were the same among all *Branchiostoma* species tested (Table 1).

Eleven of protein-coding genes of *Bb*-T, *Bb*-G, *Bj*-W, *Bb*-W, *Bf*, *Bl*-S, and *Bf*-B had an ATG initiation codon, while the *NADH1* and *NADH4L* of *Bb*-T, *Bb*-G, and *Bj*-W, and the *NADH1* and *COI* of *Bf*, *Bb*-W, *Bl*-S, and *Bf*-B had a different initiation codon. The *NADH1* of *Bl*-S and *Bf*-B had an ATA initiation codon. In *Bl*-M and *Bl*-N, 12 of 13 genes excluding the *COI* had an ATG initiation codon.

Termination codons can be divided into complete codons consisting of TAG or TAA and an incomplete codon ending with T or TA. A complete termination codon of TAG or TAA was found in the following genes: all protein-coding genes examined, except the *cytb*, *COII*, and *ATP6*, in *Bb*-T and *Bb*-G; the *cytb*, *COI*, *COIII*, *NADH2*, *NADH3*, *NADH5*,

Table 3 Initiation and termination codons of protein-coding genes in *Branchiostoma* species

Gene	*Bb*-T		*Bl*-M		*Bf*-M		*Bf*-H		*Bb*-G		*Bj*-W		*Bb*-W		*Bl*-N		*Bl*-S		*Bf*-B	
	Codon		Codon		Codon		Codon		Codon		Codon		Codon		Codon		Codon		Codon	
	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination	Initiation	Termination
cytb	ATG	T*	ATG	TAG	ATG	TAG	ATG	TAG	ATG	T*	ATG	TAG	ATG	TA*	ATG	TAG	ATG	TAG	ATG	TAG
NADH1	GTG	TAG	ATG	T*	GTG	T*	GTG	T*	GTG	TAG	GTG	TAG	GTG	TA*	ATG	T*	ATA	T*	ATA	T*
NADH2	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG
COI	ATG	TAG	GTG	TAG	GTG	TAG	GTG	TAG	ATG	TAG	ATG	TAG	GTG	TAG	GTG	TAG	GTG	TAG	GTG	TAG
COII	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*	ATG	T*
ATP8	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG
ATP6	ATG	TA*	ATG	TA*	ATG	TA*	ATG	TA*	ATG	TAA	ATG	TAA	ATG	TA*	ATG	TA*	ATG	TAA	ATG	TA*
COIII	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAG	ATG	TAG	ATG	TAA	ATG	TAA
NADH3	ATG	TAG	ATG	T*	ATG	T*	ATG	T*	ATG	TAA	ATG	TAA	ATG	T*	ATG	T*	ATG	TAA	ATG	T*
NADH4L	GTG	TAA	ATG	TA*	ATG	TA*	ATG	TA*	GTG	TAA	GTG	TAA	ATG	TA*	ATG	TA*	ATG	TAA	ATG	TA*
NADH4	ATG	TAG	ATG	TA*	ATG	TA*	ATG	TA*	ATG	TAG	ATG	TAG	ATG	TA*	ATG	TA*	ATG	TAG	ATG	TA*
NADH5	ATG	TAA	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAA	ATG	TAG	ATG	TAG
NADH6	ATG	TAG	ATG	TA*	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TAG	ATG	TA*	ATG	TAG	ATG	TAG

Abbreviations are the same as in Table 1.
Asterisks indicate no such potential reasonably exists and that the termination codon is incomplete.
Corrected codons are shown in parentheses.

and *ATP8* in *Bl*-M and *Bl*-N; the *cytb*, *COI*, *COIII*, *NADH2*, *NADH3*, *NADH5*, *NADH6*, and *ATP8* in *Bf* and *Bf*-B; all protein-coding genes examined, except the *COII* gene, in *Bj*-W and *Bl*-S; and the *COI*, *COIII*, *NADH2*, *NADH3*, *NADH5*, *NADH6*, and *ATP8* in *Bb*-W (Table 3).

The mean frequencies of amino acids in protein-coding genes were as follows: 7.8% for glycine (G), 7.3% for alanine (A), 9.2% for valine (V), 16.5% for leucine (L), 6.1% for isoleucine (I), 5.3% for methionine (M), 6.9% for phenylalanine (F), 2.9% for tryptophan (W), 4.2% for proline (P), 7.7% for serine (S), 4.9% for threonine (T), 3.1% for asparagine (N), 2.3% for glutamine (Q), 1.0% for cysteine (C), 2.1% for aspartic acid (D), 2.7% for glutamic acid (E), 1.9% for lysine (K), 2.4% for histidine (H), 2.0% for arginine (R), and 4.0% for tyrosine (Y), with hydrophobic amino acids accounting for 66.0%, neutral amino acids 18.9% and hydrophilic amino acids 15.1% (Table 4). No differences were observed in the frequencies of C, D, and H among species, while 0.1-0.7% differences were observed in the frequencies of other amino acids among species. Similar patterns of amino acid distribution were observed among *Bb*-T, *Bb*-G, and *Bj*-W, between *Bl*-M and *Bl*-N, and between *Bf* and *Bf*-B. *Bb*-W exhibited the highest frequency for V (9.9%) among all *Branchiostoma* species tested, with a 1.1% difference compared to the frequency of V in *Bb*-T. The profile of amino-acid frequency in *Bl*-S was closer to that in *Bf* than to that in *Bl*-M. A total of 804 aa were found to differ among *Bb*-T, *Bl*-M, and *Bf*; among them, 197 aa differed between any two of the three species (Table 5). The two groups composing *Bf*, *Bf*-M, and *Bf*-H differed from each other with respect to 7 aa (polymorphism; over 15%) (Table 6).

3. RNAs

The mean length of tRNA genes among the Branchiostoma species tested was 66.3 nucleotides (nt). The mean length of tRNA genes varied among species: 66.1 nt for *Bb*-T, 66.1 nt for *Bl*-M, 66.4 nt for *Bf*, 66.1 nt for *Bb*-G, 66.1 nt for *Bj*-W, 66.4 nt for *Bb*-W, 66.2 nt for *Bl*-N, 66.7 nt for *Bl*-S, and 66.4 nt for *Bf*-B. Among tRNA genes, only the $tRNA^M$ exhibited

Table 4 Percentage amino-acid composition of protein-coding genes in *Branchiostoma* species

	Amino acid	*Bb*-T	*Bl*-L	*Bf*-L	*Bf*-H	*Bb*-G	*Bj*-W	*Bb*-W	*Bl*-N	*Bl*-S	*Bf*-B	Mean
hydrophobic residues	G	7.8	7.6	7.8	7.8	7.9	7.8	7.8	7.6	7.8	7.8	7.8
	A	7.4	7.4	7.2	7.2	7.4	7.5	7.1	7.3	7.1	7.1	7.3
	V	8.8	9.5	9.2	9.1	8.8	8.8	9.9	9.4	9.1	9.1	9.2
	L	16.1	16.6	16.5	16.6	16.2	16.0	16.6	16.8	16.5	16.6	16.5
	I	6.5	5.7	6.2	6.2	6.4	6.5	5.9	5.6	6.2	6.2	6.1
	M	5.5	4.8	5.3	5.4	5.6	5.6	5.1	4.8	5.4	5.4	5.3
	F	6.9	7.0	6.8	6.8	6.9	6.9	6.8	7.0	6.8	6.7	6.9
	W	2.9	2.9	2.8	2.8	2.9	2.9	2.9	2.9	2.8	2.8	2.9
	P	4.1	4.2	4.2	4.2	4.1	4.1	4.1	4.2	4.2	4.2	4.2
	Total	66.0	65.7	66.0	66.1	66.2	66.1	66.2	65.6	65.9	65.9	66.0
neutral residues	S	7.4	7.9	7.8	7.8	7.3	7.4	7.8	7.8	7.9	7.9	7.7
	T	4.9	5.1	4.7	4.7	5.0	4.9	4.6	5.2	4.7	4.7	4.9
	N	3.2	3.1	2.9	3.0	3.2	3.2	3.0	3.1	3.0	3.0	3.1
	Q	2.3	2.2	2.3	2.3	2.3	2.3	2.2	2.2	2.3	2.3	2.3
	C	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	Total	18.8	19.3	18.7	18.8	18.8	18.8	18.6	19.3	18.9	18.9	18.9
hydrophilic residues	D	2.1	2.1	2.1	2.1	2.1	2.2	2.1	2.1	2.1	2.1	2.1
	E	2.6	2.7	2.7	2.8	2.6	2.7	2.8	2.7	2.7	2.7	2.7
	K	2.0	1.8	2.0	1.9	2.0	1.9	1.9	1.8	2.0	1.9	1.9
	H	2.4	2.4	2.4	2.4	2.4	2.3	2.4	2.4	2.4	2.4	2.4
	R	1.9	1.9	2.0	2.0	1.9	1.9	1.9	2.0	2.0	2.0	2.0
	Y	4.1	4.1	3.9	3.9	4.1	4.1	4.2	4.1	4.0	3.9	4.0
	Total	15.1	15.0	15.1	15.1	15.1	15.1	15.3	15.1	15.2	15.0	15.1

Abbreviations are the same as in Table 1 and text.

Table 5 Numbers of amino-acid substitutions in mitochondrial-encoded protein in which *Branchiostoma* species are distinguished

Gene	aa	*Bb*-T	*Bl*-M	*Bf*	All
ATP6	227	12	7	9	12
ATP8	54	5	3	8	9
COI	515	9	5	5	0
COII	230	8	4	2	2
COIII	262	8	0	11	3
cytb	380	24	6	9	8
NADH1	313	16	16	10	9
NADH2	346	32	12	34	43
NADH3	117	7	11	3	9
NADH4	452	48	65	34	35
NADH4L	91	5	7	4	4
NADH5	598	58	22	43	47
NADH6	167	15	11	19	16
Total	3,752	247	169	191	197

Abbreviations are the same as in Table 1.

Table 6 Numbers of amino-acid substitutions in protein-coding genes identified within each *Branchiostoma* species

Gene	*Bb*-T	*Bl*-M	*Bf*	*Bf*-M	*Bf*-H
ATP6	2	1	3	0	1
ATP8	0	0	0	0	1
COI	0	0	0	0	0
COII	0	0	0	0	0
COIII	1	2	0	0	1
cytb	0	1	0	0	0
NADH1	1	1	0	0	0
NADH2	1	1	2	0	0
NADH3	0	0	0	0	0
NADH4	6	2	3	0	2
NADH4L	0	0	0	0	0
NADH5	3	1	0	1	0
NADH6	0	2	0	1	0
Total	14	11	8	2	5

Abbreviations are the same as in Table 1.

Table 7 Numbers of base substitutions in tRNA genes in which *Branchiostoma* species are distinguished

Gene	*Bb*-T	*Bl*-M	*Bf*	All
tRNA-Gly	8	2	4	0
tRAN-Ala	2	1	4	0
tRNA-Val	1	0	1	1
tRNA-Leu (TTR)	1	4	2	0
tRNA-Leu (CTN)	8	6	3	4
tRNA-Ile	4	3	4	2
tRAN-Met	0	2	0	0
tRNA-Phe	15	3	10	3
tRNA-Trp	8	6	2	3
tRNA-Pro	4	1	1	1
tRNA-Ser (TCN)	2	4	4	0
tRNA-Ser (AGY)	5	7	5	1
tRNA-Thr	2	3	2	1
tRNA-Asn	10	4	4	1
tRNA-Gln	3	2	3	1
tRNA-Cys	5	7	1	4
tRNA-Asp	10	5	5	6
tRNA-Glu	6	5	6	2
tRNA-Lys	7	4	8	3
tRNA-His	8	7	7	3
tRNA-Arg	10	3	4	3
tRNA-Tyr	13	6	3	1
Total	132	85	83	40

Abbreviations are the same as in Table 1.

Table 8 Numbers of base substitutions in tRNA genes identified within each *Branchiostoma* species

Gene	*Bb*-T	*Bl*-M	*Bf*	*Bf*-M	*Bf*-H
tRNA-Gly	0	0	0	0	0
tRAN-Ala	0	0	0	0	0
tRNA-Val	0	0	0	0	0
tRNA-Leu (TTR)	1	3	0	0	0
tRNA-Leu (CTN)	0	0	0	0	0
tRNA-Ile	0	0	0	0	0
tRAN-Met	0	0	0	0	0
tRNA-Phe	1	1	0	1	3
tRNA-Trp	0	0	0	0	0
tRNA-Pro	0	0	0	0	0
tRNA-Ser (TCN)	0	0	0	0	0
tRNA-Ser (AGY)	0	0	0	0	0
tRNA-Thr	0	0	0	0	0
tRNA-Asn	0	0	0	0	1
tRNA-Gln	0	0	0	0	0
tRNA-Cys	0	0	0	0	0
tRNA-Asp	0	0	0	1	0
tRNA-Glu	0	0	0	0	0
tRNA-Lys	0	0	1	0	0
tRNA-His	0	0	0	0	1
tRNA-Arg	0	0	0	2	1
tRNA-Tyr	0	0	0	0	0
Total	2	4	1	4	6

Abbreviations are the same as in Table 1.

the same size in all species, while other genes showed 1-7 nt differences among species (Table 1). A total of 327 bases differed among species, 40 of which differed among *Bb*-T, *Bl*-M, and *Bf* (Table 7). In addition, 10 base substitutions were identified between *Bf*-M and *Bf*-H, the two groups comprising *Bf* (Table 8).

With regard to ribosomal RNA (rRNA) genes, the size of the *12S rRNA* was 847 bp for *Bb*-T, 843 bp for *Bl*-M, 846 bp for *Bf*, 847 bp for *Bb*-G, 847 bp for *Bj*-W, 876 bp for *Bb*-W, 843 bp for *Bl*-N, 844 bp for *Bl*-S, and 846 bp for *Bf*-B, with a mean of 849 bp. The size of the *12S rRNA* of *Bb*-W differed (by about 30 bp) from that of the other *Branchiostoma* species. The *12S rRNA* of *Bl*-S was intermediate in size between those of *Bl*-M and *Bf*. The size of the *16S rRNA* was 1,383 bp for *Bb*-T, 1,376 bp for *Bl*-M, 1,367 bp for *Bf*, 1,384 bp for *Bb*-G, 1,382 bp for *Bj*-W, 1,381 bp for *Bb*-W, 1,379 bp for *Bl*-N, 1,367 bp for *Bl*-S, and 1,371 bp for *Bf*-B, with a mean of 1,375 bp. The size of the *16S rRNA* was similar among *Bb*-T, *Bb*-G, *Bj*-W, and *Bb*-W, between *Bl*-M and *Bl*-N, and between *Bf* and *Bf*-B, while that for *Bl*-S was distinct from those for *Bl*-M and *Bl*-N but similar to those for *Bf* and *Bf*-B (Table 1).

4. Unassigned DNA

The size of unassigned DNA sequences was as small as 137-221 nt. In the mtDNAs of *Bb*-T and *Bb*-G, 110 nt of unassigned DNA were present in a single region between the *NADH5* and $tRNA^{G}$, 8 nt between the $tRNA^{S(TCN)}$ and $tRNA^{D}$, 4 nt between the $tRNA^{Y}$ and *COI*, 3 nt each between the $tRNA^{F}$ and $tRNA^{V}$, the $tRNA^{D}$ and *COII*, and the $tRNA^{R}$ and *NADH4L*, and 1 nt each in 5 other regions. In the mtDNAs of *Bl*-M and *Bl*-N, 190 nt were found in a single region between the *NADH5* and $tRNA^{G}$, 10 nt between the $tRNA^{Y}$ and *COI*, 7 nt between the $tRNA^{S(TCN)}$ and $tRNA^{D}$, 3 nt between the $tRNA^{L(TTR)}$ and *NADH1*, 2 nt each between the $tRNA^{F}$ and $tRNA^{V}$ and the $tRNA^{R}$ and *NADH4L*, and 1 nt each in 3 other regions. In the mtDNA of *Bf*, 129 nt were observed in a single region between the *NADH5* and $tRNA^{G}$, 10 nt between the $tRNA^{Y}$ and *COI*, 8 nt between the $tRNA^{S(TCN)}$ and $tRNA^{D}$, 2 nt each between the $tRNA^{F}$ and $tRNA^{V}$ and the $tRNA^{R}$ and *NADH4L*,

and 1 nt each in 3 other regions. In the mtDNA of *Bj*-W, 110 nt were found in a single region between the *NADH5* and $tRNA^{G}$, 8 nt between the $tRNA^{S(TCN)}$ and $tRNA^{D}$, 4 nt between the $tRNA^{Y}$ and *COI*, 3 nt each between the $tRNA^{F}$ and $tRNA^{V}$, the $tRNA^{D}$ and *COII*, and the $tRNA^{R}$ and *NADH4L*, and 1 nt each in 6 other regions. In the mtDNA of *Bb*-W, 184 nt were in a single region between the *NADH5* and $tRNA^{G}$, 11 nt

Table 9 Lengths and sequences of unassigned DNA in *Branchiostoma* species

Distributed block			Bb-T Size (nt)	Bb-T Sequence	Bf-M Size (nt)	Bf-M Sequence	Bj-M Size (nt)	Bj-M Sequence	Bf-H Size (nt)	Bf-H Sequence	Bb-G Size (nt)	Bb-G Sequence	Bj-W Size (nt)	Bj-W Sequence	Bb-W Size (nt)	Bb-W Sequence	Bf-N Size (nt)	Bf-N Sequence	Bf-S Size (nt)	Bf-S Sequence	Bf-B Size (nt)	Bf-B Sequence
cyt b	–	*tRNA-Thr*	1	G							1	G	1	G								
tRNA-Thr	–	*tRNA-Pro*																				
tRNA-Pro	–	*12S rRNA*																				
12S rRNA	–	*tRNA-Phe*																				
tRNA-Phe	–	*tRNA-Val*	3	CTG	2	GA	2	GA	2	GA	3	CTG	3	CTG	11	AGGTAATTTAT	2	GA	2	GA	2	GA
tRNA-Val	–	*16S rRNA*											1	A								
16S rRNA	–	*tRNA-Leu (TTR)*																				
tRNA-Leu (TTR)	–	*NADH1*			3	GTG											3	GTG	3	GTG	3	GTG
NADH1	–	*tRNA-Ile*																				
tRNA-Ile	–	*tRAN-Met*																				
tRAN-Met	–	*tRNA-Gln*																				
tRNA-Gln	–	*NADH2*	1	T	1	T	1	T	1	T	1	T	1	T	1	T	1	T	1	T	1	T
NADH2	–	*tRNA-Asn*																				
tRNA-Asn	–	*tRNA-Trp*																				
tRNA-Trp	–	*tRAN-Ala*																				
tRAN-Ala	–	*tRNA-Cys*																				
tRNA-Cys	–	*tRNA-Tyr*																				
tRNA-Tyr	–	*COI*	4	AATT	10	TGAAGAATTT	10	AGGATAGCTT	10	AGGATAGCTT	4	AATT	4	AATT	10	GGAATAAATT	10	TGAAGAATTT	10	AGGATAGCTT	10	AGGATAGCTT
COI	–	*tRNA-Ser (TCN)*																				
tRNA-Ser (TCN)	–	*tRNA-Asp*	8	TTTGTTTA	7	TAGATTA	8	TTTAAAYA	8	TTTAAAYA	8	TTTGTTTA	8	TTTGTTTA	8	CTTAGTTA	7	TAGATTA	8	TTTAAACA	8	TTTAAACA
tRNA-Asp	–	*COII*	3	TCG							3	TCG	3	TCG								
COII	–	*tRNA-Lys*																				
tRNA-Lys	–	*ATP8*																				
ATP8	–	*ATP6*																				
ATP6	–	*COIII*																				
COIII	–	*NADH3*																				
NADH3	–	*tRNA-Arg*																				
tRNA-Arg	–	*NADH4L*	3	GTT	2	GT	2	GT	2	GT	3	GTT	3	GTT	3	TAT	2	GT	1	T	2	GT
NADH4L	–	*NADH4*	1	A							1	A	1	A								
NADH4	–	*tRNA-His*																				
tRNA-His	–	*tRNA-Ser (AGY)*																				
tRNA-Ser (AGY)	–	*tRNA-Leu (CTN)*																				
tRNA-Leu (CTN)	–	*NADH5*																				
NADH5	–	*tRNA-Gly*	110	NC sequence	190	NC sequence	129	NC sequence	129	NC sequence	110	NC sequence	110	NC sequence	184	NC sequence	191	NC sequence	129	NC sequence	129	NC sequence
tRNA-Gly	–	*NADH6*																				
NADH6	–	*tRNA-Glu*	1	T	1	A	1	A	1	A	1	T	1	T			1	A	1	A	1	A
tRNA-Glu	–	*cyt b*	1	T	1	T	1	T	1	T	1	T	1	T	3	ATA	1	T	1	T	1	T

Abbreviations are the same as in Table 1.

Asterisks indicate unassigned DNA sequences that disappear after correction of the initiation codon for the *NADH1*.

Table 10 Percentage of identical base and amino-acid sequences for protein-coding genes and rRNA genes in *Branchiostoma* species

ATP6	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	98-100	85-86	84-87	84-86	98-99	98-99	89-90	85-86	85	85
Bf-M		99-100	86-88	86-88	85	85	86-87	99-100	87	87-88
Bj-M			98-100	97-100	84-85	84-85	86-87	87-88	98-99	99
Bf-H				97-100	84-85	84-85	86-88	86-88	98-99	99-100
Bb-G					100	98	89	85	85	85
Bj-W						100	89	85	84	85
Bb-W							100	87	86	86
Bf-N								100	87	88
Bf-S									100	99
Bf-B										100

cyt b	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	89-90	88-89	87-89	99-100	99-100	91	90	88	89
Bf-M		99-100	93	92-93	89-90	89-90	92-93	99	93	93
Bj-M			98-100	98-100	88-89	88-89	92	93-94	99	97-99
Bf-H				97-100	87-89	87-89	91-92	93-94	97-99	97-99
Bb-G					100	100	91	90	88	89
Bj-W						100	91	90	88	89
Bb-W							100	93	92	92
Bf-N								100	93	94
Bf-S									100	99
Bf-B										100

NADH4L	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	100	82	85	84-85	98	82	87	82	85	85
Bf-M		100	83	83-84	83	82	89	100	83	83
Bj-M			100	98-100	86	86	84	83	100	100
Bf-H				98-100	85-86	85-86	83-84	83-84	98-100	98-100
Bb-G					100	100	89	83	86	86
Bj-W						100	89	83	86	86
Bb-W							100	89	84	84
Bf-N								100	83	83
Bf-S									100	100
Bf-B										100

ATP8	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	94-100	66-68	55-59	55-59	98-100	98-100	68-70	66-68	57-59	55-59
Bf-M		100	61-62	61-62	68	68	74	100	62	62
Bj-M			100	96-100	57-59	57-59	61-62	61-62	98-100	98
Bf-H				96-100	57-59	57-59	61-62	61-62	94-100	98
Bb-G					100	100	70	68	59	57
Bj-W						100	70	68	59	57
Bb-W							100	74	62	61
Bf-N								100	62	62
Bf-S									100	98
Bf-B										100

NADH1	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	85-87	88-89	88-89	98-99	99	90	85-86	88-89	88-89
Bf-M		99-100	87-88	88	85-86	86	90	97	88	88
Bj-M			99-100	99-100	88	88	89	96-97	99-100	99-100
Bf-H				99-100	88	88	87	99-100	99-100	
Bb-G					100	99	90	85	88	88
Bj-W						100	90	85	88	88
Bb-W							100	88	88	88
Bf-N								100	87	87
Bf-S									100	100
Bf-B										100

NADH5	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	77-78	74	74	99-100	99	78	78	74	74
Bf-M		99-100	80	80	77-78	77-78	82	99-100	80	80
Bj-M			98-100	98-100	74	74	76-77	80	98-99	99
Bf-H				99-100	74	74	76-77	80	99	99
Bb-G					100	99	78	78	74	74
Bj-W						100	78	78	74	74
Bb-W							100	82	76	76
Bf-N								100	80	80
Bf-S									100	99
Bf-B										100

COI	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	96-97	95-97	96-97	99-100	99	97	97	97	97
Bf-M		99-100	97-98	97-98	97	96-97	97-98	99	97	97
Bj-M			98-100	98-100	96-97	95-97	97-98	97	98-99	99
Bf-H				99-100	97	96-97	98	97	99	99
Bb-G					100	99	97	97	97	97
Bj-W						100	97	97	97	97
Bb-W							100	98	98	98
Bf-N								100	98	98
Bf-S									100	99
Bf-B										100

NADH2	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	98-100	72-74	67-68	67-68	98-99	97-98	72-77	73	67	67
Bf-M		99-100	72-73	73	74	73-74	76	98-99	73	73
Bj-M			97-100	97-100	66-67	66-67	69-70	71-73	98-99	99-100
Bf-H				98-100	66-67	66-67	69-70	72-73	98-99	99-100
Bb-G					100	98	73	73	67	67
Bj-W						100	73	73	67	67
Bb-W							100	75	70	70
Bf-N								100	72	73
Bf-S									100	99
Bf-B										100

NADH6	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	97-100	72-74	67-70	68-70	98-100	98-99	73	73	67-69	67-69
Bf-M		98-100	71-73	71-73	73-74	72-73	74-76	98-100	71-73	71-73
Bj-M			98-100	97-100	68-69	67-68	66-67	72-73	98-100	99-100
Bf-H				98-100	68-69	68	67	73	98-100	98-100
Bb-G					100	99	73	73	68	68
Bj-W						100	73	73	68	68
Bb-W							100	75	67	67
Bf-N								100	73	73
Bf-S									100	100
Bf-B										100

COII	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	93	93-94	93-94	99-100	99-100	91-92	93	94	94
Bf-M		99-100	95-96	95-96	93	93	94-95	99-100	95-96	95-96
Bj-M			99-100	99-100	94	94	92-93	95-96	99-100	99-100
Bf-H				99-100	94	94	92-93	95-96	99-100	99-100
Bb-G					100	100	92	93	94	94
Bj-W						100	92	93	94	94
Bb-W							100	95	93	93
Bf-N								100	96	96
Bf-S									100	100
Bf-B										100

NADH3	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	76-77	82-83	83-84	99-100	99-100	79	76-77	83	83
Bf-M		99-100	79-81	76	76	82	99-100	79-80	79-80	
Bj-M			98-100	98-100	82-83	82-83	79-80	80-81	99-100	99-100
Bf-H				98-100	83-84	83-84	79-80	79-81	99-100	99-100
Bb-G					100	100	79	76	83	83
Bj-W						100	79	76	83	83
Bb-W							100	82	79	79
Bf-N								100	80	80
Bf-S									100	100
Bf-B										100

12S rRNA	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	78-79	74-76	74-75	99-100	99	79-79	78-79	75	74-75
Bf-M		98-100	77-78	77-79	78-79	78-79	79-80	99-100	77-78	77
Bj-M			98-99	98-100	75-76	75-76	76-77	77-78	97-99	97-99
Bf-H				98-99	75	75-76	76-77	77-79	98-99	97-98
Bb-G					100	99	79	79	75	75
Bj-W						100	78	76	76	76
Bb-W							100	80	77	76
Bf-N								100	78	77
Bf-S									100	98
Bf-B										100

COIII	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99-100	95	90-91	90-91	99-100	99	94-95	95	91	91
Bf-M		99-100	93-94	93-94	95	95	96-97	99-100	94	94
Bj-M			99-100	99-100	91	90-91	95	94	99-100	99-100
Bf-H				99-100	91	90-91	95	94	99-100	99-100
Bb-G					100	99	95	95	91	91
Bj-W						100	94	95	91	91
Bb-W							100	95-97	95	95
Bf-N								100	94	94
Bf-S									100	100
Bf-B										100

NADH4	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	98-100	66-67	72-74	72-74	99	99	78	75-76	72-74	73-74
Bf-M		99-100	69-70	69-70	66-67	66-67	71	98	70	69
Bj-M			98-100	98-100	72-73	72-73	78-79	78-79	99-100	98-99
Bf-H				98-100	72-73	72-73	78-79	78-79	98-100	98-99
Bb-G					100	99	78	75	73	73
Bj-W						100	78	75	73	73
Bb-W							100	80	78	78
Bf-N								100	79	78
Bf-S									100	99
Bf-B										100

16S rRNA	Bb-T	Bf-M	Bj-M	Bf-H	Bb-G	Bj-W	Bb-W	Bf-N	Bf-S	Bf-B
Bb-T	99	79-80	77-78	77-78	99	99	78	79	77	77-78
Bf-M		99-100	75-76	76	79	79-80	77-78	99	76-77	76
Bj-M			98-99	98-99	77-78	77	76-77	75-76	98-99	98-99
Bf-H				98-100	77-78	77	76-77	75-76	98-99	98-99
Bb-G					100	99	79	79	77	77
Bj-W						100	79	79	77	77
Bb-W							100	78	76	76
Bf-N								100	76	76
Bf-S									100	98
Bf-B										100

Abbreviations are the same as in Table 1.

between the *tRNA*^{F} and *tRNA*^{V}, 10 nt between the *tRNA*^{Y} and *COI*, 8 nt between the *tRNA*$^{S(TCN)}$ and *tRNA*^{D}, 3 nt each between the *tRNA*^{R} and *NADH4L* and the *tRNA*^{E} and *cytb*, and 1 nt each in 2 other regions. In the mtDNA of *Bl*-S, 129 nt were identified in a single region between the *NADH5* and *tRNA*^{G}, 10 nt between the *tRNA*^{Y} and *COI*, 8 nt between the *tRNA*$^{S(TCN)}$ and *tRNA*^{D}, 3 nt between the *tRNA*$^{L(TTR)}$ and *NADH1*, 2 nt between the *tRNA*^{F} and *tRNA*^{V}, and 1 nt each in 4 other regions. In the mtDNA of *Bf*-B, 190 nt were found in a single region between the *NADH5* and *tRNA*^{G}, 10 nt between the *tRNA*^{Y} and *COI*, 8 nt between the *tRNA*$^{S(TCN)}$ and *tRNA*^{D}, 3 nt between the *tRNA*$^{L(TTR)}$ and *NADH1*, 2 nt each between the *tRNA*^{F} and *tRNA*^{V} and the *tRNA*^{R} and *NADH4L*, and 1 nt each in 3 other regions (Table 9).

5. Homology

A comparison of the homology in protein-coding genes revealed 94-100% homology within each species of *Bb*-T, *Bl*-M, and *Bf*, and 55-96% homology between species. Among protein-coding genes, the *COI*, *COII*, and *COIII* exhibited high degrees of homology (≧90%) both within and between species. The *ATP8* exhibited high intra-species homology (94-100%) but the lowest inter-species homology (55-68%). High degrees of homology in protein-coding genes were observed among *Bb*-T, *Bb*-G, and *Bj*-W (97-100%), between *Bl*-M and *Bl*-N (90-100%), and between *Bf* and *Bf*-B (98-100%). Protein-coding genes of *Bb*-W exhibited low degrees of homology with those of *Bb*-T (68-97%), *Bl*-M (71-98%), and *Bf* (61-97%), as did most of those of *Bl*-S with those of *Bb*-T (57-97%) and *Bl*-M (62-97%), while exhibiting high degrees of homology with those of *Bf* (94-100%) (Table 10).

Molecular phylogenetic trees created by the NJplot revealed the following: *Bb*-T, *Bb*-G, and *Bj*-W were classified into the same cluster as the one for all genes tested; *Bb*-W was classified into a different cluster

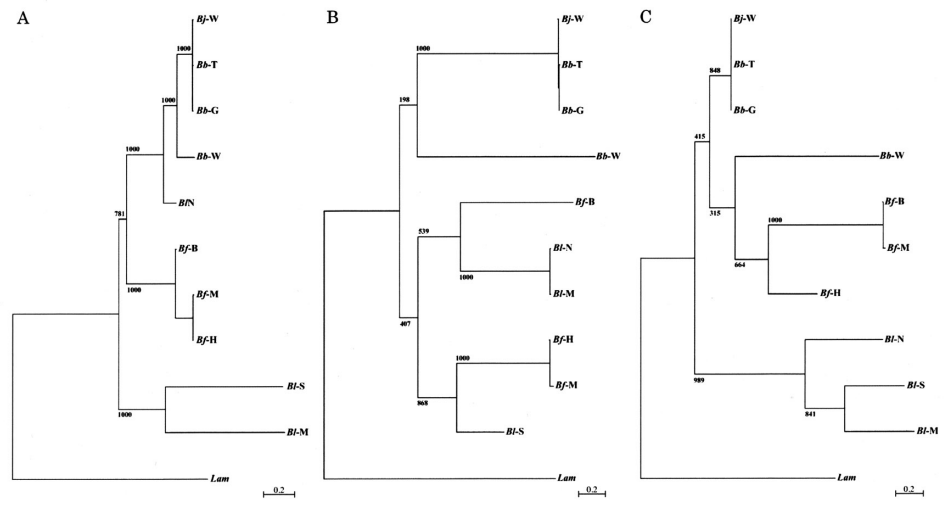

Fig. 1 Neighbor-joining analysis of the relationships between representative mitochondrial genes
An analysis based on 10 genotypes was conducted using CLUSTAL-W program (DDBJ). Bootstrap testing was performed (1,000 replicas). Numbers at forks represent bootstrap percentages. Tree is unrooted.
A) Amino acid sequence analysis of 13 protein-coding genes; B) Base sequence analysis of rRNA genes; C) Base sequence analysis of tRNA genes.
Abbreviations are same as in Table 1.
Lam (*Lampetra fluviatilis*: Y18683) was included as an outgroup.

than that for *Bb*-T, *Bb*-G, and *Bj*-W; *Bl*-M and *Bl*-S were classified into the same cluster for protein-coding genes and tRNA genes and different clusters for rRNA genes; *Bl*-N and *Bl*-M were classified into the same cluster for rRNA and tRNA genes, but into a cluster similar to *Bb*-T, *Bb*-G, *Bj*-W, and *Bb*-W for protein-coding genes; *Bf*-M and *Bf*-H were classified into the same cluster for all genes tested; and *Bf*-B was classified into the same cluster as that for *Bf*-M and *Bf*-H for protein-coding genes and tRNA genes, but into a cluster similar to *Bl*-N and *Bl*-M for rRNA genes (Fig. 1).

4. Discussion

1. Base frequency

Base frequency exhibited no significant intra-species differences but did exhibit significant inter-species differences ($P<0.001$)[17]. No significant differences were observed in base frequency among *Bb*-T, *Bb*-G, and *Bj*-W, between *Bl*-M and *Bl*-N, or between *Bf* and *Bf*-B. The base frequency in the mtDNA of *Bl*-S differed from that for *Bl*-M, but did not significantly differ from that for *Bf*. *Bb*-W exhibited a profile of frequencies of C, G, and T different from those for the other *Branchiostoma* species. A comparison of GC content, while revealing no intra-species differences, did show inter-species differences. Similar levels of GC content were observed among *Bb*-T, *Bb*-G, and *Bj*-W, between *Bl*-M and *Bl*-N, and between *Bf* and *Bf*-B. The GC content in the mtDNA of *Bl*-S differed from that for *Bl*-M, but was the same as that for *Bf*. *Bb*-W exhibited a level of GC content different from those for the other *Branchiostoma* species, and intermediate between those for *Bb*-T and *Bf*. Differences in basic structural factors, such as base frequency and GC content, are in general thought to be attributable to differences in mutation pressure[18]. Possession of sufficient levels of these basic structural factors is a requirement for the production of a functional protein[19]. The above findings thus suggest the following: *Bb*-T, *Bb*-G, and *Bj*-W, *Bl*-M and *Bl*-N, and *Bf* and *Bf*-B are identical species; *Bb*-W differs from the other *Branchiostoma* species, while *Bl*-S is identical to *Bf*. Such differences in the basic structural factors observed thus suggest that *Branchiostoma* species have long been under selective pressure due to differences in habitat.

2. Protein-coding genes

The sizes and sequences of protein-coding genes of Branchiostoma were found to be very similar to those for other metazoans. While the numbers of amino acids were the same within each species, the mtDNA of *Bl*-M was 1 aa shorter than those of *Bb*-T and *Bf*. This inter-species difference in the number of amino acids was attributable to the *NADH1* of *Bl*-M being 1 aa shorter than that of the other two species. The *COII* of *Bl*-S was 9 aa longer than that of all the other species, making a total gene size of 3,761 amino acids, the largest among all Branchiostoma species tested. This suggests that, if a reduction of mtDNA size is required for the rapid replication of mitochondria, *Bl*-S is under less selective pressure to reduce its size than are the other *Branchiostoma* species.

The *ATP8* of *Bj*-W exhibited 68-70% homology with those of *Bb*-T, *Bb*-G, and *Bb*-W. This value was comparable to the degree of homology found in the *ATP8* between *Bb*-T and *Bl*-M (66-68%). The *ATP8* of *Bl*-S showed 62% homology with that of *Bl*-M, but 94-100% homology with that of *Bf*. This indicates that *Bj*-W is a species located between *Bb*-T and *Bl*-M, and that *Bl*-S is distinct from *Bl*-M but identical to *Bf*. The *ATP8*, which exhibited the greatest variation among *Branchiostoma* species, may thus be a useful marker for discriminating among *Branchiostoma* species.

3. Initiation and Termination codons

The initiation codons for *Bb*-G, *Bl*-N, and *Bf*-B were the same as those for *Bb*-T, *Bl*-M, and *Bf*, respectively. The initiation codon for the *NADH1* of *Bl*-S was found to be ATA, which was different from that for *Bl*-M but the same as that for *Bf*-B. Since we have already presented evidence that the initiation codon for *Bf* is GTG, it is also reasonable to infer that the initiation codon for the *NADH1* of *Bl*-S and *Bf*-B is GTG. On this basis, we have determined the initiation codon for the *NADH1* to be GTG in *Bb*-T, *Bb*-G, *Bj*-W, *Bb*-W, *Bl*-S, *Bf*, and *Bf*-B, and ATG in *Bl*-M

and *Bl*-N. The initiation codon for the *COI* differs among *Bb*-T, *Bl*-M, and *Bf*: being ATG in *Bb*-T and *Bl*-M, and GTG in *Bf*. Conversely, the initiation codon for the *NADH4L* was GTG in *Bb*-T and *Bl*-M and ATG in *Bf*. These findings suggest that the difference in initiation codons is reflected by inter-species differences. It also appears that *Bl*-S and *Bf* are identical species.

The termination codons for *Bb*-G, *Bl*-N, and *Bf*-B were the same as those for *Bb*-T, *Bl*-M, and *Bf*, respectively. *Bj*-W has a complete termination codon of TAG in the *COI*, *cytb*, *NADH1*, *NADH2*, *NADH4*, *NADH6*, and *ATP8*, a complete termination codon TAA in the *COIII*, *NADH3*, *NADH4L*, *NADH5*, and *ATP6*, and an incomplete termination codon ending with T in the *COII*. *Bb*-W shows a complete termination codon of TAG in the *COI*, *COIII*, *NADH2*, *NADH6*, and *ATP8*, a complete termination codon TAA in the *NADH5*, and incomplete termination codons ending with T or TA in the other genes. *Bl*-S has a complete termination codon of TAG in the *COI*, cytb, *NADH1*, *NADH2*, *NADH4*, *NADH5*, *NADH6*, and *ATP8*, a complete termination codon TAA in the *COIII*, *NADH3*, *NADH4L*, and *ATP6*, and an incomplete termination codon ending with T in the *COII*. These three species (*Bj*-W, *Bb*-W, and *Bl*-S) exhibited patterns of termination codon usage different from those of the other *Branchiostoma* species.

These findings suggest that the initiation and termination codons vary among *Branchiostoma* species, and that *Bb*-T, *Bb*-G, and *Bj*-W, *Bl*-M, and *Bl*-N, and *Bf* and *Bf*-B are identical species, respectively, while *Bb*-W and *Bl*-S are distinct from the other *Branchiostoma* species. *Bl*-S thus appears to be a species closely related to *Bf*.

4. Unassigned DNA

The insertion sites, sequences, and sizes of unassigned DNA sequences were the same in *Bb*-T and *Bb*-G as in *Bl*-M and *Bl*-N. The unassigned DNA makeup of *Bf*-B is the same as that of *Bf*, provided that the initiation codon for the *NADH1* of *Bf*-B is corrected. The number of insertion sites, of 1 nt unassigned DNA was larger by 1 site in *Bj*-W than in *Bb*-T and *Bb*-G, although the number of insertion sites, of other unassigned DNA sequences was the same among the three species. *Bb*-W differed from the other *Branchiostoma* species with respect its insertion sites, sequences, and sizes of unassigned DNA sequences. The unassigned DNA sequences located between the $tRNA^F$ and $tRNA^V$ are characteristic of this species, with the size of the unassigned DNA sequences of *Bb*-W being 11 nt, making it considerably larger than the 2-3 nt in the other *Branchiostoma* species. *Bl*-S differed from *Bl*-M and *Bl*-N with respect to its sequences and the sizes of its unassigned DNA in 3 regions, and differed from *Bf*-B with respect only to its sequence and the size of an unassigned DNA in 1 region. As in the case of *Bf*-B, the unassigned DNA makeup of *Bl*-S is the same as that of *Bf*, provided that the initiation codon for the *NADH1* of *Bl*-S is corrected. Although the sizes of unassigned DNA sequences (not including non-coding (NC) sequences) in the *Branchiostoma* species tested ranged from 25-37 nt, those in the *Branchiostoma* species (excluding *Bb*-W) ranged from only 25-28 nt, with no significant difference among species. The difference in the size of NC regions is thus reflected by the size difference in unassigned DNA sequences. A characteristic finding for the unassigned DNA is that its insertion sites, sequences, and sizes vary among species. In particular, the unassigned DNA sequences located between the $tRNA^F$ and $tRNA^V$, the $tRNA^Y$ and *COI*, the $tRNA^{S(TCN)}$ and $tRNA^D$, and the $tRNA^R$ and *NADH4L* were found in all *Branchiostoma* species tested, and their sequences and sizes were found to be species-specific. The differences in unassigned DNA makeup thus appear to constitute inter-species differences. It also appears that, if a reduction of the mtDNA size is required for rapid replication of mitochondria, the inter-species differences observed have been generated by selective pressure.

5. Transfer RNAs

A comparison of tRNA genes from multiple individuals of *Bb*-T, *Bl*-M, and *Bf* has revealed species-specific base substitutions (polymorphism) in almost all tRNA genes. Among polymorphisms that distinguish one species from the other two, those that distinguish Bb-T from the other two were most

common (132 polymorphisms). *Bl*-M was distinguished from the other two by 85 polymorphisms, and *Bf* from the other two by 83 polymorphisms. tRNA genes that frequently exhibited polymorphisms included the genes for $tRNA^{F}$, $tRNA^{D}$, $tRNA^{H}$, and $tRNA^{Y}$, with more tRNA genes related to hydrophilic amino acids than to hydrophobic amino acids. In terms of inter-species difference, polymorphisms were commonly identified in the $tRNA^{F}$, $tRNA^{Y}$, $tRNA^{N}$, $tRNA^{D}$ and $tRNA^{R}$ in *Bb*-T, the $tRNA^{S(AGY)}$, $tRNA^{C}$, and $tRNA^{H}$, in *Bl*-M, and the $tRNA^{F}$, $tRNA^{K}$, and $tRNA^{H}$ in *Bf*.

Intra-species polymorphisms were identified in the genes for $tRNA^{F}$, $tRNA^{L(TTR)}$, $tRNA^{N}$, $tRNA^{D}$, $tRNA^{K}$, $tRNA^{R}$, and $tRNA^{H}$. One particularly noteworthy finding is the presence of polymorphisms between the two groups comprising *Bf*. The conventional classification of the *Branchiostoma* is based on the findings reported in previous classical biological studies[5, 20]. The presence of 10 polymorphisms identified in the $tRNA^{F}$, $tRNA^{N}$, $tRNA^{D}$, $tRNA^{R}$, and $tRNA^{H}$ suggests that the species of *Branchiostoma* exhibiting the same morphological characteristics may be further divided into different groups. On the other hand, the genes for $tRNA^{M}$, $tRNA^{V}$, and $tRNA^{L(TTR)}$ have fewer base substitutions than do the other tRNA genes, and thus appear to be better conserved in their structure. The initiation codons used in the mitochondria of *Branchiostoma* have been shown to be ATG, GTG, and ATA. However, the results of our present analyses of tRNA genes and the *NADH1* have suggested that the initiation codon for the *NADH1* of *Bl*-S and *Bf* is GTG. This indicates that only ATG and GTG need be considered as candidates for initiation codons used by the *Branchiostoma*. The $tRNA^{M}$ in particular, exhibits the least inter-species variation. The $tRNA^{M}$ as well as the $tRNA^{V}$, carries an anticodon for the translation initiation codon, thus playing an important role in protein synthesis. In the mtDNA of vertebrates, transcription is initiated in an inverted promoter on the D-loop, followed by the production of the primary transcript. The transcription level of mitochondrial rRNA genes is usually regulated by a transcription termination factor that specifically binds to the nucleotide sequences of tRNA genes. In humans, a transcription termination factor-binding site consisting of a sequence of TGGCAGAGCCCGG is present on the $tRNA^{L(TTR)}$ [21], and the binding of a 34-kDa transcription termination factor to that site terminates transcription, resulting in the production of rRNAs. If no transcription termination factor binds to the site, transcription resumes further downstream, resulting in the production of mRNAs and tRNAs[22, 23]. This transcription termination factor-binding site carries a point mutation (3243A-G) responsible for mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)[24, 25]. The reduced ability of the transcription termination factor to bind to DNA that carries this point mutation has also been demonstrated[23]. Although the transcription termination factor-binding site in the $tRNA^{L(TTR)}$ of the *Branchiostoma* has not yet been identified, it may be involved in regulating of the production of rRNAs and mRNAs in *Branchiostoma*, as it does in humans. It is thus clear that the three types of tRNA genes are more universally required across species than are the other types of tRNA genes.

6. Polymorphism

While the mtDNAs of *Bb*-T, *Bl*-M, and *Bf* exhibited intra-species identity with respect to base and amino-acid composition, size, initiation/termination codon usage, and unassigned DNA makeup, several features varied among the three species, indicating the presence of distinctive genetic differences among them. Within-species variations in amino-acid frequency and base/amino-acid polymorphisms were less frequently observed in *Bl*-M than in *Bb*-T and *Bf*.

Bb-G was identical to *Bb*-T with respect to all features examined. *Bb*-W was similar to *Bl*-M in gene size and to *Bf* in unassigned DNA makeup, but was distinct from *Bb*-T, *Bl*-M, and *Bf* in initiation/termination codon usage and base/amino-acid composition. *Bj*-W was identical to *Bb*-T in gene size, base/amino-acid composition, initiation codon usage, and NC sequence makeup, but was identical to *Bf* in termination codon usage. *Bl*-M and *Bl*-N were

identical in all features. *Bl*-S was closer to, or identical with, *Bf* than to *Bl*-M in all features, but distinct from *Bb*-T, *Bl*-M, and *Bf* with respect to base/amino-acid composition and termination codon usage. *Bf* and *Bf*-B were identical in all features. These findings indicate the following: 1) *Bb*-T and *Bb*-G, *Bl*-M and *Bl*-N, and *Bf* and *Bf*-B are identical species, respectively; 2) *Bj*-W is close to *Bb*-T but distinct from *Bb*-T; 3) *Bb*-W is distinct from *Bb*-T, *Bl*-M, and *Bf*; and 4) *Bl*-S is close to but distinct from *Bf*. Analyses of various types of genes have been conducted to address these inter-species differences and to elucidate the evolution of *Branchiostoma*[26-31]. In particular, rRNA-based phylogenetic analyses have often been performed and have yielded valuable findings[8, 32]. Samples are now usually frozen with liquid nitrogen or treated with an RNA-preserving compound and are thus kept in a condition relatively close to that of live samples. However, the extrapolations of genetic characteristics to ancient organisms from those of currently existing organism are still speculative Since fossils of cephalochordata were found in the Chengjiang fauna of the Early Cambrian (around 540 million years ago) and fossils of an agnathan fish termed conodont were discovered among the same fauna, it is believed that urochordata, cephalochordata (e.g., *Branchiostoma*), and vertebrates appeared simultaneously between 6 and 10 million years during the Early Cambrian[33]. If this is so, it will be important to examine the genomic information obtained from these fossils as a means of enabling practical genetic analyses using comparative genomic approaches. For this purpose, inter-species polymorphisms need to be clarified to provide a basis for comparative genomic approaches.

With regard to protein-coding genes, a total of 804 amino-acid polymorphisms were identified among *Bb*-T, *Bl*-M, and *Bf*, and in 197 polymorphisms among them the three species were distinguished. tRNA genes carry a total of 340 polymorphisms, 40 of which distinguish *Bb*-T, *Bl*-M, and *Bf*, which is further divided into *Bf*-M and *Bf*-H. We examined whether it is possible to distinguish these two subgroups, and identified 15 polymorphisms in protein-coding genes and 10 in tRNA genes. Five characteristic amino acids translated from protein-coding genes and 7 characteristic bases in tRNA genes distinguished *Bf*-M and *Bf*-M subgroups from other individuals, respectively. To distinguish highly similar groups, tRNA genes, which carry 6 polymorphisms out of 1,461 nt (0.4%), are slightly advantageous compared with the *16S rRNA*, which carries 4 polymorphisms out of 1,367 nt (0.3%). Within the species of *Bb*-T, *Bl*-M and *Bf*, the frequencies of polymorphisms in protein-coding genes and tRNA genes were lower in *Bl*-M than in *Bb*-T and *Bf*, suggesting the presence of a bottle-neck effect[34, 35]. Within the *Bf* species, the frequency of polymorphisms is lower in *Bf*-M than in *Bf*-H, representing an inter-group difference. These findings suggest that a geographical classification of *Branchiostoma* species can be achieved by molecular biological approaches.

Many of the findings obtained in the present study are consistent with those derived from the analysis of nuclear genes by Cañstro *et al.*[7] and those obtained from the analysis of mtDNAs by Nohara *et al.*[9]. This may be due to the fact that the research material identified as *B. lanceolatum* in the study conducted by Spruyt *et al*[4]. was actually *B. floridae*, or because they misinterpreted their findings. However, several findings have revealed differences between *Bl*-S and *Bf*, the size of the *COII* of *Bl*-S (239 aa) was 9 aa longer than that of *Bf*; the *NADH1*, *NADH4*, *NADH4L*, and *ATP6* of *Bl*-S had complete termination codons; and the molecular phylogenetic trees for protein-coding genes and tRNA genes showed that *Bl*-S was close to *Bl*-M. It is thus reasonable to conclude that *Bl*-S is a sub species that is close to *Bf*.

5. Conclusion

The results of the present analysis of mtDNAs have demonstrated that *B. belcheri*, *B. lanceolatum*, and *B. floridae* are distinct species. We have also identified species-specific polymorphisms and have clarified the phylogenetic classification and geographical distribution of *Branchiostoma* by molecular biological approaches. These results indicate that the *Branchiostoma* in early developmental stages exhibited a region-specific distribution pattern and have subse-

quently undergone independent evolutions. The distinctiveness of *B. lanceolatum* from *B. belcheri* and *B. floridae* suggests the presence of a bottleneck effect. We have also provided a reason for reconsidering the research material used by Spruyt *et al.* as *B. lanceolatum* to be a species or group that is much closer to *B. floridae* than to *B. lanceolatum*. These findings were derived from a comparative genomic analysis of new data for *B. belcheri*, *B. lanceolatum*, and *B. floridae*, as well as from other existing data. *Branchiostoma* have been used in a number of studies, and their molecular biological findings are frequently cited. The findings obtained in the present study should prove very useful for future studies in the fields of phylogenetics, evolutionary biology, and phylogenetic systematics.

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