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Intraspecific Differentiation in the Japanese Brown Frog *Rana japonica* Inferred from Mitochondrial DNA Sequences of the Cytochrome *b* Gene

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ABSTRACT—Intraspecific differentiation of the Japanese brown frog *Rana japonica* was investigated by analyzing nucleotide sequences of the mitochondrial cytochrome *b* (*cyt b*) gene in order to clarify phylogenetic relationships among three population groups known to exist in this species. The nucleotide sequences of 447 base pair (bp) segments were determined by the PCR-direct sequencing method on 31 individuals from 14 populations of *R. japonica* from Honshu, and phylogenetic trees were constructed by the UPGMA and NJ methods using *Rana catesbeiana* as an outgroup. A sequence alignment provided 92 variable sites (15 corresponded to the first codon position, three to the second, and 74 to the third), and 19 haplotypes were identified from 31 frogs. The sequence divergences were 0.22~2.50% ($\bar{x}$ = 0.65%) within populations, 0.22~12.02% ($\bar{x}$ = 7.34%) between populations, and 23.59~27.89% ($\bar{x}$ = 26.19%) between *R. japonica* and *R. catesbeiana*. Although many nucleotide substitutions were silent mutations, 12 amino acid replacements were found to occur within *R. japonica*. A high frequency of transitions relative to transversions was observed within *R. japonica*. The present nucleotide sequence data showed that the eastern and western groups of *R. japonica* was considerably differentiated to each other, and that the Akita population of the northwestern group was evidently derived from the eastern group, but the Nakajo and Izumozaki populations of the northwestern group diverged considerably from each of the eastern and the western groups.

INTRODUCTION

The Japanese brown frog *Rana japonica* is widely distributed through Japan, from Honshu (except for the northern end) to Kyushu (Maeda and Matsui, 1989). In this species, the Ichinoseki population (eastern Honshu) is reproductively isolated from the Hiroshima population (western Honshu) by the incomplete sterility of male hybrids caused by the failure of homologous chromosome paring (Sumida, 1981, 1994). Although the two populations are not morphologically distinct, they are distinguishable both karyologically and biochemically. Allozyme analysis of 25 populations from Honshu and Kyushu distinguished the eastern and western groups of *R. japonica*, and introgressions of the eastern alleles were noted at several loci in the northwestern populations of Honshu (Sumida and Nishioka, 1994a). These two groups are geographically isolated from each other by the high mountains in the Izu-Hakone area and the mountain ridges in the Tohoku area. The locus linked with the sex-determining gene varied among local populations (Sumida and Nishioka, 1994b). Extensive intraspecific crossing experiments among 20 populations from Honshu and Kyushu revealed that three groups of populations, the eastern, western, and northwestern groups, were reproductively isolated from each other by incomplete male hybrid sterility (Sumida, 1996). By the mtDNA RFLP analysis of 16 populations, Sumida (1997b) showed a distinct divergence between the eastern and western groups of *R. japonica*. On the basis of the results of allozyme analysis, crossing experiments and mtDNA RFLP analysis, he suggested that the eastern and western groups of *R. japonica* have experienced secondary contact, and that introgression has occurred in the northwestern populations of Honshu.

Mitochondrial DNA evolves more rapidly than nuclear DNA, perhaps 5~10 times faster than typical single-copy nuclear DNA (Brown *et al*., 1979; Vawter and Brown, 1986), and is maternally inherited (Kaneda *et al*., 1995). Unlike nuclear DNA, mtDNA mutations that arise in different individuals do not recombine during sexual reproduction. Thus the mtDNA of higher animals is an ideal molecular system for matriarchal phylogenetic analysis among closely related taxa (Avise *et al*., 1987). The mitochondrial cytochrome *b* gene or D-loop region sequences have proven useful to resolve the phylogenetic relationships among populations of the same species and between closely related species. With the development of PCR, it has recently become routine to use gene sequences from DNA fragments in evolutionary studies (Hedges *et al*., 1993; Hillis *et al*., 1996).

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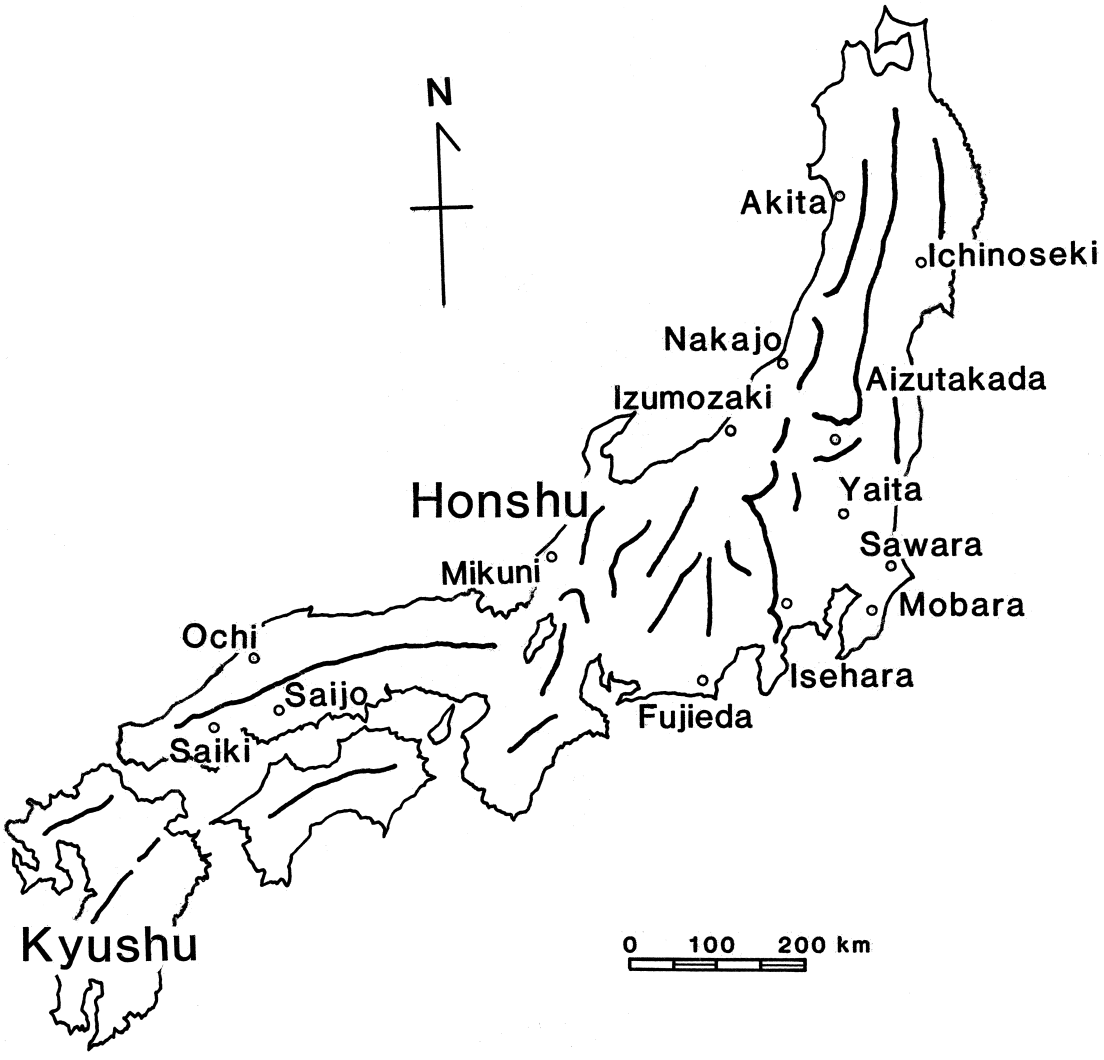

Fig. 1. Map showing the collecting stations of the Japanese brown frog *Rana japonica* used in the present study. Solid lines in the map are ranges of mountains. Geographic distribution of *R. japonica* in Honshu was conveniently divided into three groups, the eastern, western and northwestern groups, which included three to six populations (Table 1).

Table 1. Specimens of *Rana japonica* used and haplotypes identified in the present study

Group	Prefecture	Locality	No. of specimens	Population	Haplotype
Eastern	Iwate	Ichinoseki-shi, Sannoseki	6	Ichinoseki	Ichi 1~4
	Fukushima	Onuma-gun, Aizutakada-machi	2	Aizutakada	Aizu 1, 2
	Tochigi	Yaita-shi	1	Yaita	Yait
	Chiba	Sawara-shi	1	Sawara	Sawa
	Chiba	Mobara-shi	3	Mobara	Moba,Yait,Sawa
	Kanagawa	Isehara-shi, Sannomiya	2	Isehara	Yait,Sawa
Northwestern	Akita	Akita-shi, Toyoiwaishidazaka	3	Akita	Akit 1, 2
	Niigata	Kitakanbara-gun, Nakajo-machi	2	Nakajo	Naka
	Niigata	Santo-gun, Izumozaki-machi	1	Izumozaki	Izum
Western	Fukui	Sakai-gun, Mikuni-cho	2	Mikuni	Miku
	Shizuoka	Fujieda-shi	1	Fujieda	Fuji
	Shimane	Ochi-gun, Ochi-cho	4	Ochi	Ochi 1, 2
	Hiroshima	Higashihiroshima-shi, Saijo-cho	2	Saijo	Saij
	Hiroshima	Saiki-gun, Saiki-cho, Iinoyama	1	Saiki	Saik
		Total	31	14	19

In the present study, the mtDNA sequences of the *cyt b* gene were investigated for the Japanese brown frog *Rana japonica* in order to clarify phylogenetic relationships among three population groups at the nucleotide sequence level, and to investigate the introgression in the northwestern group postulated by the present senior author.

MATERIALS AND METHODS

MtDNA sources

A total of 31 frogs from 14 populations of Honshu were used, and these populations represented three geographic groups, the eastern, western and northwestern groups (see Table 1, Fig. 1). These frogs were screened from among 78, of which the mtDNA was analyzed by the RFLP method (Sumida, 1997b), and covered all mtDNA RFLP haplotypes. MtDNA was extracted from the livers or ovaries of each frog according to the method reported by Sumida (1997a), which was modified from that of Yonekawa *et al*. (1980). The nucleotide sequence of *Rana catesbeiana* reported by Yoneyama (1987) was used for outgroup comparisons, but the additional 5'-upstream 102 bp were determined by the present study.

PCR primers

Primers for amplification and sequencing were designed to cover a 447-bp segment of the *cyt b* gene corresponding to sites 16942-17388 in *Xenopus laevis* sequences (Roe *et al*., 1985) based on the conserved regions between nucleotide sequences of *Rana catesbeiana* (Yoneyama, 1987; Okazaki, unpublished) and *Xenopus laevis* (Roe *et al*., 1985). The primer sequences were F17 (5'-CCA TAC TTC TCC TAC AAA GAC-3'), RS03I (5'-TCC TGC AGG GAC TGC CAA C-3'), RS03F (5'-GTT CTG CAG GGA TTG CCA A-3') and RS03H (5'-GTT CTG CAG GGA TCG CTA A-3').

PCR and product detection

PCR mixtures were prepared with a TaKaRa Taq™ Kit as recommended by the manufacturer (TaKaRa) in a final volume of 50 μl. The *cyt b* gene was amplified by 30 cycles, each cycle consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 1 min at 72°C. To detect PCR products, 3 μl of the reaction mixture was applied to a gel of 4% SeaKem ME agarose (FMC BioProduct) and electrophoresed in the standard 1 × TAE buffer. The gel was stained with 0.1 μg/ml ethidium bromide for 20 min.

Direct sequencing

The PCR products were purified by MicroSpin™ S-300 HR Columns (Pharmacia Biotech). Purified DNA was sequenced by the DyeDeoxy™ Terminator Cycle Sequencing method using 373A DNA Sequencing System Ver. 1.2 (ABI). Amino acid sequences were deduced from the nucleotide sequences by DNASIS (Ver. 3.2, Hitachi Software Engineering Co. Ltd.).

Phylogenetic analysis

Genetic relationships among haplotypes were estimated based on the pairwise matrix of sequence divergence calculated by Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were reconstructed by the neighbor-joining (NJ) method (Saitou and Nei, 1987) and the unweighted pair-group arithmetic average (UPGMA) clustering method (Sneath and Sokal, 1973; Nei, 1975), using the programs included in version 3.5c of PHYLIP (Felsenstein, 1993), to compare these trees with those obtained from the allozyme and RFLP analyses reported previously. Using PHYLIP, bootstraps were tested with 1000 replications for both the NJ tree and the UPGMA dendrogram to obtain the approximate confidence of the trees.

Table 2. Frequencies of haplotypes of *cyt b* gene sequences in 14 populations of the Japanese brown frog *Rana japonica*

Population	No.of frogs	Haplotypes (Frequencies)																		
		Ichi 1	Ichi 2	Ichi 3	Ichi 4	Aizu 1	Aizu 2	Yait	Sawa	Moba	Akit 1	Akit 2	Naka	Izum	Miku	Fuji	Ochi 1	Ochi 2	Saij	Saik
Ichinoseki	6	2 (0.33)	1 (0.17)	2 (0.33)	1 (0.17)															
Aizutakada	2					1 (0.50)	1 (0.50)													
Yaita	1							1 (1.00)												
Sawara	1								1 (1.00)											
Mobara	3							1 (0.33)	1 (0.33)	1 (0.33)										
Isehara	2							1 (0.50)	1 (0.50)											
Akita	3										1 (0.33)	2 (0.67)								
Nakajo	2												2 (1.00)							
Izumozaki	1													1 (1.00)						
Mikuni	2														2 (1.00)					
Fujieda	1															1 (1.00)				
Ochi	4																3 (0.75)	1 (0.25)		
Saijo	2																		2 (1.00)	
Saiki	1																			1 (1.00)

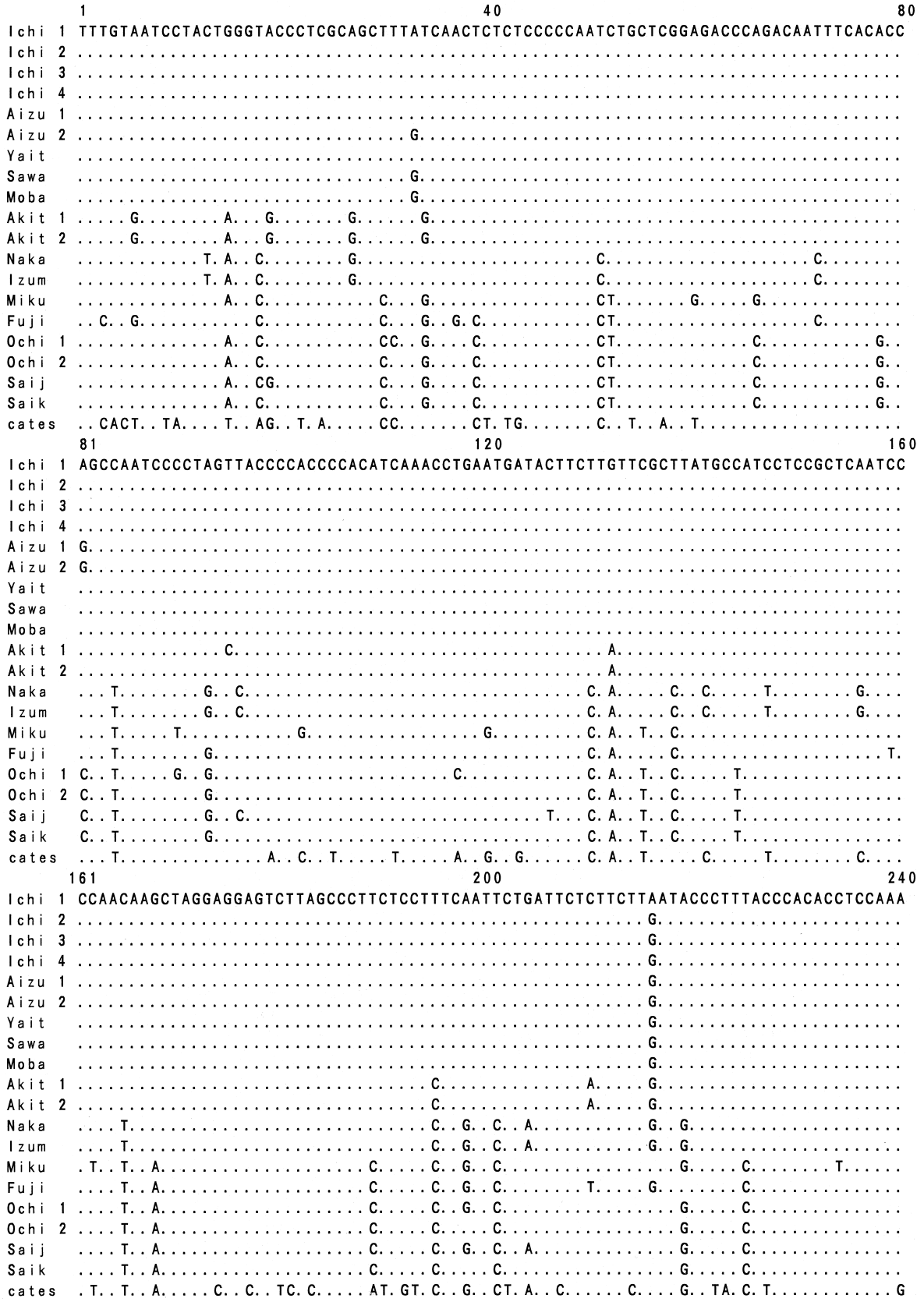

Fig. 2. Aligned sequences of a 447-bp segment of the mitochondrial cytochrome *b* gene from 19 haplotypes of the Japanese brown frog *Rana japonica* and outgroup *R. catesbeiana* (cates). The abbreviations of 19 haplotypes are shown in Table 1. Dots indicate identity to the sequence of Ichi 1.

```
          241                                       280                                       320
Ichi  1 CTTCGCTCTCTTATGTTCCGCCCTGTCGCTAAAATCTTCTTTTGATCTCTAATCGCTAACACAGCCATTCTGACATGAAT
Ichi  2 .......................A.....................................................................
Ichi  3 ..........................................................A.....................
Ichi  4 ................................................................................
Aizu  1 ..........................................................A.....................
Aizu  2 ..........................................................A.....................
Yait    ..............................................................C.....................
Sawa    ................................................................................
Moba    ..............................................................A.....................
Akit  1 ..................................................T.............................T..G...
Akit  2 ..................................................T.............................T..G...
Naka    ..C..T........A.........A..A.C.................A.........C.....................G...
Izum    ..C..T.................A..A...................A.........C.....................G...
Miku    ..C..T..C..............A.....................A.......T.........T..C..A.........
Fuji    ..C..T..C..............A.....................A.......T.........T..............
Ochi  1 ..C..T..C.............CA.....................T......A.C.....................A..G......
Ochi  2 ..C..T..C...........T..CA.T...................T......A.C.....................A..G......
Saij    ..C..T..C...........T..CA.....................T......A.C.....................A..G......
Saik    ..C..T..C.............CA.T...................T......A.C.....................A..G......
cates   ..C..T..A..C..A........AA.T...............C...A.C.....T.........T.....T.........
          321                                       360                                       400
Ichi  1 CGGAGGCCAACCCGTAGAAGACCCATTTATCATAATCGGCCAAATCGCCTCAGGCCTTTATTTCTTAATCTTTGTCCTCC
Ichi  2 ..............................................................C.....................
Ichi  3 ..............................................................C.....................
Ichi  4 ..............................................................C.....................
Aizu  1 ..............................................................C.....................
Aizu  2 ..............................................................C.....................
Yait    ..............................................................C.....................
Sawa    ..............................................................C.....................
Moba    ..............................................................C.....................
Akit  1 ...................................T..........................C.....................
Akit  2 ..............................................................C.....................
Naka    ......T.....T..T........G.....................G.............C.....................T.
Izum    ......T.....T..G........G.....................G.............C.....................T.
Miku    ........................G...........T..T........T........C.....................T.
Fuji    ......G..G..T...........G...........T..T........T........C.....................T.
Ochi  1 ...G..A.................G.....................T........C.....................T.
Ochi  2 ......A.................G..C..................T........C......G..............T.
Saij    ......A.................G.....................T........C.....................T.
Saik    ......A.................G..C..................T........C......G..............T.
cates   ...G..A.....A...........T.....T..C........G...A.......A..A..C........T.....T..T.
          401                                       440
Ichi  1 TTGTTCCCACTATAGGCCTCTTAGAAAATAAACTCCTTAAAATCTAA
Ichi  2 ...........................................
Ichi  3 ...........................................
Ichi  4 ...........................................
Aizu  1 ...........................................
Aizu  2 ...........................................
Yait    ...........................................
Sawa    ...........................................
Moba    ...........................................
Akit  1 ...........................................
Akit  2 ...........................................
Naka    ..................C........C........C.........
Izum    ..................C........C........C.........
Miku    ...C..............C........C........C.........
Fuji    ..................C........C........C...G.....
Ochi  1 ...C..............C........C........C.........
Ochi  2 ...C..............C........C........C.........
Saij    ...C..............C........C........C.........
Saik    ...C..............C........C........C.........
cates   ..A.C..A..AT.......T..G.....C..G..T......G.....
```

Table 3. Percent similarities of nucleotide sequences (above diagonal) with number of nucleotide substitutions in parenthesis and percent two-parameter method (below diagonal) among haplotypes of *cyt b* gene sequences in the Japanese brown frog *Rana japonica* and outgroup

Haplo-type	Ichi 1	Ichi 2	Ichi 3	Ichi 4	Aizu 1	Aizu 2	Yait	Sawa	Moba	Akit 1	Akit 2	Naka	Izum	Miku	Fuji
Ichi 1	–	99.3 (3)	99.3 (3)	99.6 (2)	99.1 (4)	98.9 (5)	99.3 (3)	99.3 (3)	99.1 (4)	96.6 (15)	97.1 (13)	90.8 (41)	91.3 (39)	89.9 (45)	89.9 (45)
Ichi 2	0.67	–	99.6 (2)	99.8 (1)	99.3 (3)	99.1 (4)	99.6 (2)	99.6 (2)	99.3 (3)	96.9 (14)	97.3 (12)	91.5 (38)	91.9 (36)	90.2 (44)	90.6 (42)
Ichi 3	0.67	0.45	–	99.8 (1)	99.8 (1)	99.6 (2)	99.6 (2)	99.6 (2)	99.3 (3)	96.9 (14)	97.3 (12)	91.1 (40)	91.5 (38)	89.7 (46)	90.2 (44)
Ichi 4	0.45	0.22	0.22	–	99.6 (2)	99.3 (3)	99.8 (1)	99.8 (1)	99.6 (2)	97.1 (13)	97.5 (11)	91.3 (39)	91.7 (37)	89.9 (45)	90.6 (42)
Aizu 1	0.90	0.67	0.22	0.45	–	99.8 (1)	99.3 (3)	99.3 (3)	99.1 (4)	96.6 (15)	97.1 (13)	90.8 (41)	91.3 (39)	89.5 (47)	89.9 (45)
Aizu 2	1.13	0.90	0.45	0.67	0.22	–	99.1 (4)	99.6 (2)	99.3 (3)	96.4 (16)	96.9 (14)	90.6 (42)	91.1 (40)	89.3 (48)	89.7 (46)
Yait	0.67	0.45	0.45	0.22	0.67	0.90	–	99.6 (2)	99.6 (2)	96.9 (14)	97.3 (12)	91.1 (40)	91.5 (38)	89.9 (45)	90.2 (44)
Sawa	0.67	0.45	0.45	0.22	0.67	0.45	0.45	–	99.8 (1)	96.9 (14)	97.3 (12)	91.1 (40)	91.5 (38)	89.7 (46)	90.2 (44)
Moba	0.90	0.67	0.67	0.45	0.90	0.67	0.45	0.22	–	96.6 (15)	97.1 (13)	90.8 (41)	91.3 (39)	89.9 (45)	89.9 (45)
Akit 1	3.43	3.20	3.20	2.97	3.43	3.67	3.20	3.20	3.43	–	99.6 (2)	90.6 (42)	91.1 (40)	89.3 (48)	89.9 (45)
Akit 2	2.97	2.74	2.74	2.50	2.97	3.20	2.74	2.74	2.97	0.45	–	91.1 (40)	91.5 (38)	89.3 (48)	89.9 (45)
Naka	9.68	8.94	9.43	9.19	9.68	9.93	9.45	9.43	9.68	9.98	9.48	–	99.6 (2)	90.6 (42)	91.7 (37)
Izum	9.17	8.44	8.93	8.68	9.17	9.42	8.94	8.93	9.17	9.46	8.97	0.67	–	91.1 (40)	92.2 (35)
Miku	10.68	10.43	10.93	10.68	11.19	11.44	10.70	10.93	10.68	11.46	11.46	9.93	9.42	–	94.2 (26)
Fuji	10.70	9.95	10.45	10.20	10.70	10.95	10.46	10.45	10.70	10.72	10.72	8.71	8.20	6.02	–
Ochi 1	11.51	11.26	11.76	11.51	11.76	12.02	11.53	11.76	11.51	12.55	12.04	9.99	9.48	5.80	7.48
Ochi 2	10.75	10.50	11.00	10.75	11.00	11.26	11.02	11.00	11.26	12.04	11.53	9.74	9.23	6.28	7.24
Saij	11.24	10.98	11.49	11.24	11.49	11.75	11.51	11.49	11.75	12.02	11.51	8.73	8.23	6.27	7.23
Saik	10.50	10.24	10.75	10.50	10.75	11.00	10.76	10.75	11.00	11.78	11.27	9.49	8.98	6.04	7.00
cates	26.64	26.64	27.26	26.95	27.57	27.89	26.90	27.26	27.26	28.67	28.04	25.41	25.36	23.59	24.45

RESULTS

Haplotypes of nucleotide sequences

Of the 19 haplotypes (haplotype names abbreviated to the first four letters of the population name) identified (Table 1), most were found in only one population, but two (Sawa and Yait) were found in three populations (Table 2). The 447-bp segment provided 92 variable sites (15 corresponding to the first codon position, three to the second, and 74 to the third) within *R. japonica* (Fig. 2), and 141 variable sites (29 corresponding to the first codon position, five to the second, and 107 to the third) between *R. japonica* and *R. catesbeiana* (Fig. 2).

Sequence divergences among haplotypes

The intrapopulational sequence divergences were small, 0.22~0.67%, within the Ichinoseki, Aizutakada, and Akita populations, but were as large as 2.50% within the Ochi population (Table 3). There were one to three nucleotide substitutions within the former three populations, and 11 nucleotide substitutions within the Ochi population (Table 3). The interpopulational sequence divergences were 0.22~1.13% ($\bar{x}$ = 0.59%) among the six eastern populations with four substitutions, 0.22~7.48% ($\bar{x}$ = 4.87%) among the five western populations with 1~32 substitutions, and 8.97~9.98% ($\bar{x}$ = 9.47%) between the northwestern Akita population and the northwestern Nakajo and Izumozaki populations with 38~42 substitutions (Table 3). The interpopulational sequence divergences were not so large, 2.50~3.67% ($\bar{x}$ = 3.07%), between the Akita population and the six eastern populations with 11~16 substitutions, whereas they were very large, 10.72~12.55% ($\bar{x}$ = 11.59%), between the former population and the five western populations with 45~52 substitutions (Table 3). On the other hand, the interpopulational sequence divergences were large,

sequence divergences calculated by Kimura's
R. catesbeiana (cates)

Ochi 1	Ochi 2	Saij	Saik	cates
89.3	89.9	89.5	90.2	77.9
(48)	(45)	(47)	(44)	(99)
89.5	90.2	89.7	90.4	77.9
(47)	(44)	(46)	(43)	(99)
89.0	89.7	89.3	90.2	77.4
(49)	(46)	(48)	(44)	(101)
89.3	90.2	89.5	90.2	77.6
(48)	(44)	(47)	(44)	(100)
89.0	89.7	89.3	89.9	77.2
(49)	(46)	(48)	(45)	(102)
89.0	89.5	89.0	89.7	77.0
(49)	(47)	(49)	(46)	(103)
89.3	89.7	89.3	89.9	77.6
(48)	(46)	(48)	(45)	(100)
89.0	89.7	89.3	89.9	77.4
(49)	(46)	(48)	(45)	(101)
89.3	89.5	89.0	89.7	77.4
(48)	(47)	(49)	(46)	(101)
88.4	88.8	88.8	89.0	76.5
(52)	(50)	(50)	(49)	(105)
88.8	89.3	89.3	89.5	77.0
(50)	(48)	(48)	(47)	(103)
90.6	91.1	91.7	91.1	78.7
(42)	(40)	(37)	(40)	(95)
91.1	91.3	92.2	91.5	78.7
(40)	(39)	(35)	(38)	(95)
94.4	94.0	94.0	94.2	80.1
(25)	(27)	(27)	(26)	(89)
92.8	93.1	93.1	93.3	79.4
(32)	(31)	(31)	(30)	(92)
–	97.5	97.5	97.8	79.9
	(11)	(11)	(10)	(90)
2.50	–	98.2	99.9	79.2
		(8)	(1)	(93)
2.50	1.81	–	98.0	79.6
			(9)	(91)
2.27	0.22	2.04	–	79.4
				(92)
23.98	24.89	24.24	24.59	–

8.44~9.93% ($\bar{x}$ = 9.24%), between the northwestern Nakajo and Izumozaki populations and the six eastern populations with 36~42 substitutions, and 8.20~9.99% ($\bar{x}$ = 9.18%) between the former two populations and the five western populations with 35~42 substitutions (Table 3). The interpopulational sequence divergences were very large, 9.95~12.02% ($\bar{x}$ = 11.01%), between the five western populations and the six eastern populations with 42~49 substitutions (Table 3). The interspecific sequence divergences were extremely large, 23.59~28.67% ($\bar{x}$ = 26.19%), between *R. japonica* and *R. catesbeiana* with 89~105 substitutions (Table 3).

Within *R. japonica* many nucleotide substitutions were transitions, and transversions were either rare or nonexistent, whereas between *R. japonica* and *R. catesbeiana* the number of transversions increased relative to that of transitions (Table 4). The percentage of transitions was 73~100% within *R. japonica* except the Yaita population, whereas it was 62~70% between *R. japonica* and *R. catesbeiana* (Table 4).

Amino acid sequences

Amino acid sequences were deduced from the nucleotide sequences of the 447-bp segment of the *cyt b* gene by DNASIS (Fig. 3). Although many nucleotide substitutions were situated at the third codon position and were silent mutations, 12 amino acid replacements occurred within *R. japonica* (Fig. 3). Four amino acid replacements, the 12th, 89th, 96th and 135th, occurred between the eastern and western populations (Fig. 3). The 12th amino acid was Ser (TCA) in the six eastern and the northwestern Nakajo and Izumozaki populations, whereas it was Ala (GCA) in the Akita and the five western populations (Fig. 3). The 89th amino acid was Val (GTC) in the six eastern and the Akita populations, except one haplotype of the Ichinoseki population, whereas it was Ile (ATC or ATT) in the Nakajo and Izumozaki and the five western populations (Fig. 3). The 96th amino acid was Ser (TCT) in the six eastern populations, Phe (TTT) in the Akita population, and Thr (ACT or ACC) in the two northwestern and the five western populations (Fig. 3). The 135th amino acid was Ala (GCT) in the four western populations, whereas it was Val (GTT) in the other 11 populations (Fig. 3). The other eight amino acid replacements occurred in only one to three haplotypes of one or two populations (Fig. 3). Twenty two amino acid replacements were found between *R. japonica* and *R. catesbeiana* (Fig. 3).

Phylogenetic relationships among haplotypes

As shown in the UPGMA dendrogram (Fig. 4), the outgroup *R. catesbeiana* was clearly separated from the ingroup *R. japonica*. The ingroup, which was strongly supported in 100% of 1000 bootstrap iterations, was largely divided into three clusters. The first cluster contained two subclusters (100% of 1000 bootstrap iterations), one of which consisted of nine haplotypes of the six eastern populations, and the other two haplotypes of the northwestern Akita population. The second cluster contained two haplotypes of the northwestern Nakajo and Izumozaki populations. The third cluster contained three subclusters which consisted of six haplotypes of the five western populations.

The topology of the NJ tree was slightly different from that of the UPGMA dendrogram (Fig. 5). The ingroup was largely divided into four clusters. In the first cluster, the sister relationship between the two haplotypes of the northwestern Nakajo and Izumozaki populations and the 11 haplotypes of the six eastern and the northwestern Akita populations was supported in 90% of 1000 bootstrap iterations. The other three clusters which contained six haplotypes of the five western populations showed a polytomous relationship with the first cluster (Fig. 5).

DISCUSSION

Many researchers have recently reported on amphibians using mtDNA sequences to reconstruct intra- and interspecific phylogenies (Moritz *et al*., 1992; Hedges *et al*., 1993;

Table 4. Numbers of transition sites (above diagonal) with percentage in parenthesis and transversion sites (below diagonal) among haplotypes brown frog *Rana japonica* and outgroup *R. catesbeiana* (cates)

Haplo-type	Ichi 1	Ichi 2	Ichi 3	Ichi 4	Aizu 1	Aizu 2	Yait	Sawa	Moba	Akit 1	Akit 2	Naka	Izum	Miku	Fuji
Ichi 1	–	3 (100)	3 (100)	2 (100)	4 (100)	5 (100)	2 (67)	3 (100)	4 (100)	12 (80)	10 (77)	39 (95)	38 (97)	43 (96)	42 (93)
Ichi 2	0	–	2 (100)	1 (100)	3 (100)	4 (100)	1 (50)	2 (100)	3 (100)	11 (79)	9 (75)	36 (95)	35 (97)	42 (95)	39 (93)
Ichi 3	0	0	–	1 (100)	1 (100)	2 (100)	1 (50)	2 (100)	3 (100)	12 (86)	9 (75)	38 (95)	37 (97)	44 (96)	41 (93)
Ichi 4	0	0	0	–	2 (100)	3 (100)	0 (0)	1 (100)	2 (100)	10 (77)	8 (73)	37 (95)	36 (97)	43 (96)	39 (93)
Aizu 1	0	0	0	0	–	1 (100)	2 (67)	3 (100)	4 (100)	12 (80)	10 (77)	39 (95)	38 (97)	45 (96)	42 (93)
Aizu 2	0	0	0	0	0	–	3 (75)	2 (100)	3 (100)	13 (81)	11 (79)	40 (95)	39 (98)	46 (96)	43 (93)
Yait	1	1	1	1	1	1	–	1 (50)	1 (50)	10 (71)	8 (67)	37 (93)	36 (95)	42 (93)	40 (91)
Sawa	0	0	0	0	0	0	1	–	1 (100)	11 (79)	10 (83)	38 (95)	37 (97)	44 (96)	41 (93)
Moba	0	0	0	0	0	0	1	0	–	12 (80)	10 (77)	39 (95)	38 (97)	43 (96)	42 (93)
Akit 1	3	3	2	3	3	3	4	3	3	–	2 (100)	37 (88)	36 (90)	45 (94)	41 (91)
Akit 2	3	3	3	3	3	3	4	2	3	0	–	35 (88)	34 (89)	45 (94)	41 (91)
Naka	2	2	2	2	2	2	3	2	2	5	5	–	2 (100)	40 (95)	34 (92)
Izum	1	1	1	1	1	1	2	1	1	4	4	0	–	39 (98)	33 (94)
Miku	2	2	2	2	2	2	3	2	2	3	3	2	1	–	25 (96)
Fuji	3	3	3	3	3	3	4	3	3	4	4	3	2	1	–
Ochi 1	6	6	6	6	6	6	7	6	6	7	7	6	5	4	3
Ochi 2	6	6	6	6	6	6	7	6	6	7	7	5	5	4	3
Saij	5	5	5	5	5	5	6	5	5	6	6	5	4	3	2
Saik	6	6	6	6	6	6	7	6	6	7	7	6	5	4	3
cates	32	32	32	32	32	31	31	32	32	35	35	32	31	32	31

Tanaka *et al*., 1994, 1996; Shaffer and McKnight, 1996; García-París *et al*., 1998; Sumida *et al*., 1998), and the mitochondrial *cyt b* gene or the D-loop region sequences have proven useful and adequate in resolving relationships among closely related taxa.

The present study revealed that the nucleotide divergences within a population ranged from 0.22 to 2.50% with a mean of 0.65%. There is very little data on the level of variability within a population derived from a single location in amphibians. Shaffer and McKnight (1996) revealed that inner locality variation was 0.1% in *Ambystoma velasci* and *A. tigrinum nebulosum*. Sumida *et al*. (1998) reported that the intrapopulational sequence divergences of *R. nigromaculata* and *R. porosa* were 0.22~0.45% ($\bar{x}$ = 0.34%). Tanaka *et al*. (1994) reported that the intrapopulational sequence divergences were 0.8% in *R. japonica*, *R. ornativentris* and *R. tagoi*. The values for *R. japonica* in the present study were not very different from those previously reported for other amphibians.

The present study also showed that the sequence divergences between populations were 0.22~12.02% ($\bar{x}$ = 7.34%). The sequence divergences between the six eastern populations and the five western populations were 9.95~12.02% ($\bar{x}$ = 11.01%). These values were similar to those between *R. japonica* and *R. ornativentris* (11.58%) and between *R. ornativentris* and *R. pirica* (11.49%) reported by Tanaka *et al*. (1994), and those between *R. nigromaculata* and *R. porosa* (10.38~11.92%, $\bar{x}$ = 11.39%) reported by Sumida *et al*. (1998). The present study also showed that there was a considerable number of nucleotide substitutions in the *cyt b* gene within *R. japonica*, and 12 amino acid changes occurred among 148 amino acids of 19 haplotypes. Of these 12 amino acid changes, four occurred between the eastern and western populations, and the other eight occurred in only one or two populations (Fig. 3). Between *R. nigromaculata* and *R. porosa*, only one

of *cyt b* gene sequences in the Japanese

Ochi 1	Ochi 2	Saij	Saik	cates
42	39	42	38	67
(88)	(87)	(89)	(86)	(68)
41	38	41	37	67
(87)	(86)	(89)	(86)	(68)
43	40	43	38	69
(88)	(87)	(90)	(86)	(68)
42	38	42	38	68
(88)	(86)	(89)	(86)	(68)
43	40	43	39	70
(88)	(87)	(90)	(87)	(69)
43	41	44	40	72
(88)	(87)	(90)	(87)	(70)
41	39	42	38	69
(85)	(85)	(88)	(84)	(69)
43	40	43	39	69
(88)	(87)	(90)	(87)	(68)
42	41	44	40	69
(88)	(87)	(90)	(87)	(68)
45	43	44	42	70
(87)	(86)	(88)	(86)	(67)
43	41	42	40	68
(86)	(85)	(88)	(85)	(66)
36	35	32	34	63
(86)	(88)	(86)	(85)	(66)
35	34	31	33	64
(88)	(87)	(89)	(87)	(67)
21	23	24	22	57
(84)	(85)	(89)	(85)	(64)
29	28	29	27	61
(91)	(90)	(96)	(90)	(66)
–	9	10	8	56
	(82)	(91)	(80)	(62)
2	–	7	1	58
		(88)	(100)	(62)
1	1	–	8	58
			(89)	(64)
2	0	1	–	58
				(63)
34	35	33	34	–

amino acid change was found to occur out of 147 amino acid residues of cytochrome *b* (Sumida *et al*., 1998). The occurrence of four amino acid changes might suggest a considerable divergence between the eastern and western groups of *R. japonica*.

The fact that the sequence divergences of the northwestern Akita population from the six eastern populations averaged 3.07%, but 11.59% from the five western populations, supports the idea that the mtDNA of the Akita population was derived from that of the eastern populations, as was already suggested by mtDNA RFLP analysis (Sumida, 1997b). On the other hand, the sequence divergences were 8.44~9.93% ($\bar{x}$ = 9.24%) between the northwestern Nakajo and Izumozaki populations and the six eastern populations, 8.20~9.99% ($\bar{x}$ = 9.18%) between the former and the five western populations, and 8.97~9.98% ($\bar{x}$ = 9.47%) between the former and the northwestern Akita population. These results suggest that the mtDNA gene of the Nakajo and Izumozaki populations diverged considerably from the mtDNA genes of the Akita, eastern, and western populations. Judging from aligned sequences of a 447-bp segment of the *cyt b* gene (Fig. 2), it is likely that the mtDNAs of the Nakajo and Izumozaki populations were derived from the western group but diverged uniquely in these northwestern populations. It is noticeable that the mtDNAs of the western Mikuni and Fujieda populations also diverged considerably from those of the other three western populations.

From an electrophoretic analysis of enzymes and blood proteins of 25 populations, Sumida and Nishioka (1994a) observed distinct differentiation between the eastern and western groups of *R. japonica*. At the *Hb-II* locus, the eastern and western groups were characterized by a fixed allelic difference, whereas at the *PEP-A* and *Alb* loci, introgressions of the "eastern" alleles were noted in the northwestern populations. A steep cline in allelic frequencies was observed in the northwestern region from Akita to Toyama at the *PEP-A* locus. In the Akita population, the *Hb-II* locus was fixed for the "western" allele, whereas the *Alb* and *PEP-A* loci were fixed for the "eastern" allele. Unique alleles at the *LDH-B* and *MDH-B* loci were found in the Akita population. These results suggest that the Akita population was formed by an introgression between the eastern and western groups followed by random drift at each locus. Sumida (1996) showed that although there was neither gametic isolation nor hybrid inviability among populations of *Rana japonica*, a remarkable preponderance of male reciprocal hybrids was observed among three groups of populations, the eastern, western, and northwestern groups, that were reproductively isolated from one another by incomplete male hybrid sterility. The degree of male hybrid sterility was largest in reciprocal hybrids between the western and eastern groups, and smallest in reciprocal hybrids between the northwestern and eastern groups. By conducting mtDNA RFLP analysis, Sumida (1997b) showed that *Rana japonica* in Honshu was first divided into the eastern and western groups. The eastern group thereafter diverged into the Akita population and a subgroup containing six eastern populations, and the western group differentiated into several subgroups. He suggested that the mtDNA of the Akita population was derived from that of the eastern group.

Based on the present data on mitochondrial cytochrome *b* gene sequence divergences as well as those on allozyme analysis, crossing experiments and mtDNA RFLP analysis stated above, it is considered that there exist two geographic groups (probably races), which are genetically differentiated to each other, and are reproductively isolated to some extent, and that the northwestern group contains several populations which are derived from the hybridization between the eastern and western groups, and have various genetic traits. After *R. japonica* diverged into the eastern and western groups by geographical isolation of the mountain ridges between them, they might have come into secondary contact with each other, and introgressive hybridization occurred in the northwestern populations of Honshu, leading to the substantial introgres-

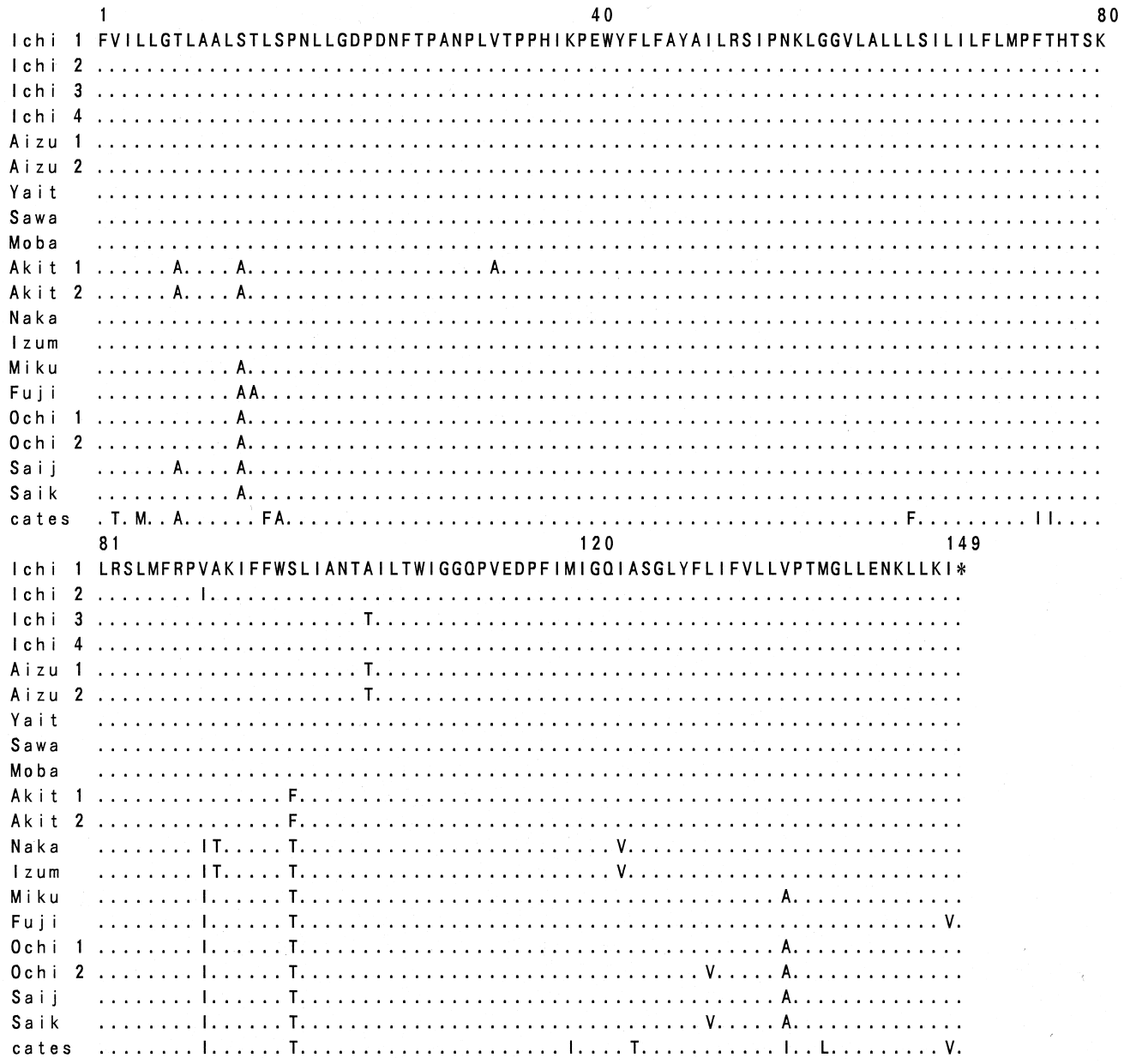

Fig. 3. Deduced amino acid sequences of the mitochondrial cytochrome *b* gene from 19 haplotypes of the Japanese brown frog *Rana japonica* and outgroup *R. catesbeiana* (cates). The abbreviations of 19 haplotypes are shown in Table 1. Dots indicate identity to the sequence of Ichi 1. Asterisk indicates the stop codon.

sion of mtDNA and nuclear genes. The degree of introgressions depended on the populations in this region. They were probably isolated geographically thereafter by marine transgression or other biogeographical events during the interglacial period or during the last glacial period (Minato *et al.*, 1965). It is thus not unreasonable that the eastern and western groups might be named the Ichinoseki and Hiroshima races, respectively, as proposed by Sumida (1996), and the northwestern populations are considered to be derived from the introgressive hybridization between the eastern and western groups.

Conditions suitable for the interspecific transfer of mtDNA may occasionally occur either when sympatric but ecologically separated species interbreed locally or when species with parapatric distributions hybridize along their zone of contact (Szymura *et al.*, 1985). Several studies have provided evidence for interspecific mtDNA transfer in the absence of nuclear gene introgression (Ferris *et al.*, 1983; Powell, 1983; Spolsky and Uzzell, 1984). On the other hand, nuclear and mitochondrial genes were found to change concordantly within hybrid populations or in a transect across hybrid zones in some animals (Avise *et al.*, 1984; Szymura *et al.*, 1985; Harrison *et al.*, 1987; Wallis and Arntzen, 1987; Rand and Harrison, 1989; Sumida and Ishihara, 1997). Based on the UPGMA dendrograms, the patterns for mtDNA gene divergence of *R. japonica* were mostly concordant with allozyme data, in which the phylogenetic analysis was carried out only by the UPGMA method

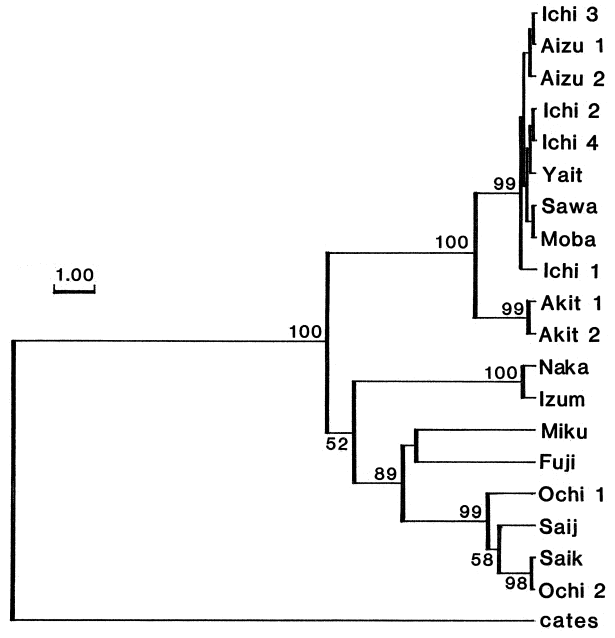

Fig. 4. The UPGMA dendrogram based on the nucleotide sequence of 447-bp segment of the mitochondrial cytochrome *b* gene from 19 haplotypes of the Japanese brown frog *Rana japonica* and outgroup *R. catesbeiana* (cates). Numbers on the dendrogram are bootstrap values (above 50%) based on 1000 replications. A scale bar represents branch length in terms of percent divergences (%).

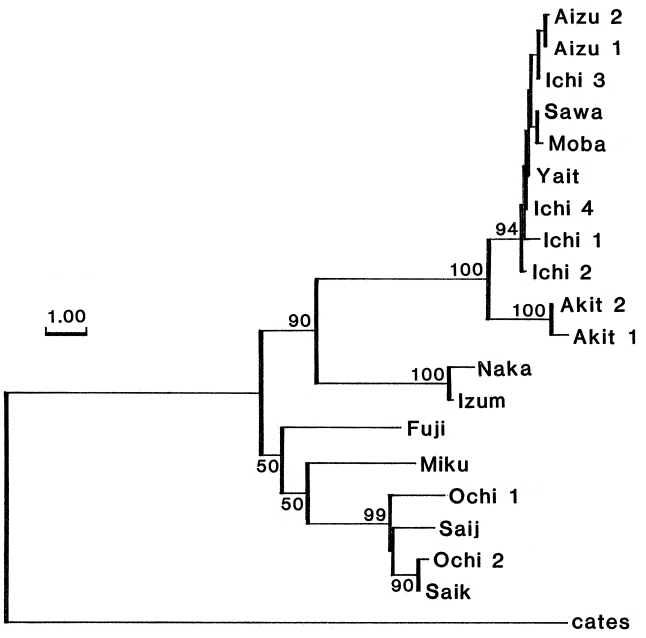

Fig. 5. The neighbor-joining (NJ) tree based on the nucleotide sequence of 447-bp segment of the mitochondrial cytochrome *b* gene from 19 haplotypes of the Japanese brown frog *Rana japonica* and outgroup *R. catesbeiana* (cates). Numbers on the tree are bootstrap values (above 50%) based on 1000 replications. A scale bar represents branch length in terms of percent divergences (%).

(Sumida and Nishioka, 1994a).

The nucleotide substitutions in the mitochondrial *cyt b* gene of the Japanese brown frog *R. japonica* showed a high frequency of transitions relative to transversions (Table 4). The similar phenomena were observed in the mitochondrial 12S and 16S rRNA genes of caecilians (Hedges *et al*., 1993), in the mitochondrial *cyt b* gene of Japanese brown frogs (Tanaka *et al*., 1994), and in the mitochondrial 12S rRNA and *cyt b* genes of Japanese pond frogs (Sumida *et al*., 1998). These phenomena are interpreted as evidence that the nucleotide substitutions are not saturated with respect to multiple substitutions, because the percentages of transitions usually reach a plateau around 40~50%, where multiple substitutions begin occurring at the same site (Brown *et al*., 1982; Hedges *et al*., 1991, 1993). Based on these lines of evidence, it seems probable that the substitutions are not saturated among *R. japonica*, whereas they are almost saturated between *R. japonica* and *R. catesbeiana*.

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