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Source: Zoological Science, 20(10) : 1279-1291

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.1279

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Geographic Variation and Diversity of the Cytochrome *b* Gene in Japanese Wild Populations of Medaka, *Oryzias latipes*

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ABSTRACT—We conducted a polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) analysis of the mitochondrial cytochrome b gene to elucidate the detailed genetic population structure of Japanese wild populations of medaka, Oryzias latipes. The analysis of 1,225 specimens collected from 303 sites identified 67 mitotypes. Subsequently we determined the nucleotide sequences of the complete cytochrome b gene (1141-bp) to clarify the phylogenetic relationships among mitotypes. The phylogenetic tree based on nucleotide sequences indicated three major clades (A, B and C) that differed by 11.3-11.8%, corresponding to three clusters previously identified by RFLP analysis of entire mitochondrial DNAs. The geographic distribution of mitotypes in clades A and B was fully concordant with the Northern and Southern Populations defined by allozymes. Clade A could be subdivided into three subclades and clade B into eleven, with sequence divergences among subclades of 1.3-5.8%. Each distribution of mitotypes in subclades roughly corresponded to that of mtDNA haplotypes in subclusters previously identified. Mitotypes in clade C were found only in the Kanto district. The phylogenetic relationships and the estimated divergence times suggest that three Japanese clades originated from a common ancestor and were separated during the Pliocene, and that the regional differentiation of subclades was closely connected with the geological history of the Quaternary. This study has also demonstrated the possibility of artificial disturbance of natural distribution especially in the Kanto district and the superior efficacy of PCR-RFLP analysis as a simple method for detecting genetic variation and artificial gene flow of medaka.

Key words: phylogeography, conservation, mitochondrial DNA, PCR-RFLP, Oryzias latipes

INTRODUCTION

Medaka, *Oryzias latipes*, is an egg-laying freshwater fish native to Japan, Korea and China. It inhabits marshes, ponds and irrigation canals amid rice fields in flat alluvial lowlands. Recently the Japanese wild population of medaka has been decreasing rapidly, mainly because of destruction of their habitat (Hosoya, 2000), and this fish has been listed within the rank of 'Threatened II' in the 1999 Red List of Threatened Animals of Japan (Ministry of the Environment Japan, 1999).

Allozyme studies have shown that wild populations of medaka consist of four genetically different groups: the Northern Population from the Sea of Japan coast of eastern Japan, the Southern Population from the Pacific coast of

* Corresponding author: Tel. +81-25-262-6368; Fax. +81-25-262-6368. E-mail: yusuke@env.sc.niigata-u.ac.jp eastern Japan and from western Japan, the East Korean Population from eastern and southern Korea, and the China-West Korean Population from China and western Korea (Sakaizumi *et al.*, 1983; Sakaizumi, 1986; Sakaizumi and Joen, 1987). The boundaries separating the geographic distributions of these four groups are clear, and major divergences among groups have been observed. However, it is evident that male and female progeny from hybrids among the four groups are fully fertile (Sakaizumi *et al.*, 1992).

Studies of restriction fragment length polymorphism (RFLP) of the entire mitochondrial DNA (mtDNA) of medaka has confirmed the existence of four groups on the basis of the results of allozymic analyses (Matsuda *et al.*, 1997a; Matsuda *et al.*, 1997b). RFLP analysis of the Japanese wild population, revealed a total of 63 mtDNA haplotypes that formed three distinct clusters (A, B and C). The geographic distribution of mtDNA haplotypes in clusters A and B fully corresponds to the Northern Population and the Southern

Population. Cluster C was found only in the Kanto district. Furthermore, distributions of mtDNA haplotypes in the subclusters, show strong geographical correlations. However, the genetic variability within populations and the origin of two Japanese groups remain to be clarified because of the small sample sizes (one individual per site), the limited number of polymorphic sites, and the lack of informative outgroup data.

Mitochondrial DNA can be a powerful molecular marker for reconstructing evolutionary lineages in animals (Avise,



Fig. 1. Collection sites of O. latipes. The numbers refer to the locations listed in Table 1, except for two Korean sites mentioned in the text.

Table 1. Collection sites, sample size (N) and observed mitotypes of *Oryzias latipes*. Numbers in parentheses indicate number of individuals representing each mitotype. Asterisks (*) show samples that we sequenced. HNI and Hd-rR are inbred stains derived from the Northern and Southern Populaions, respectively.

	Collection site	N	Mitotype		Collection site	N	Mitotype
1.	Hakodate, Hokkaido Pref.	5	B11(5)	77.	Miura, Kanagawa Pref.	3	B11(3)
2.	Higashidori, Aomori Pref.	4	A1(1), A13*(3)	78.	Odawara, Kanagawa Pref.	7	B11(2), B27*(3), C1(2)
3.	Kamikita, Aomori Pref.	1	A1(1)	79.	Ryotsu, Niigata Pref.	2	A1(2)
4.	Momoishi, Aomori Pref.	4	A1(4)	80.	Aikawa, Niigata Pref.	3	A1(3)
5.	Iwaki, Aomori Pref.	3	A1(2), A4(1)	81.	Sawata, Niigata Pref.	1	A1(1)
6.	Hirosaki, Aomori Pref.	1	A1(1)	82.	Hamochi, Niigata Pref.	5	A1(5)
7.	Hanamaki, Iwate Pret.	5	B11(2), B27(3)	83.	Arakawa, Niigata Pref.	5	A1^(5)
8. 0	ICHINOSEKI, IWate Pret.	5	B11(5) B11(2)	84.	Sekikawa, Niigata Pref.	5	B27(5)
10	Kesennuma2 Miyagi Pref	2	B11(2)	86	Shibata Nijgata Pref	2	$\Delta_{1}(2)$ $\Delta_{20}(1)$
11	Tajiri Miyagi Pref	5	B11(5)	87	Tovosaka Niigata Pref	5	A1(5)
12.	Onagawa, Miyagi Pref.	5	B11(5)	88.	Niigata1, Niigata Pref.	5	A1(3), A6(1), A7*(1)
13.	Sendai, Miyagi Pref.	5	B11(5)	89.	Niigata2, Niigata Pref.	4	A1(1), A3(1), A6*(2)
14.	Noshiro, Akita Pref.	5	A1(3), A2*(2)	90.	Niigata3, Niigata Pref.	5	A1(2), A3*(1), A19(2)
15.	Hachirogata, Akita Pref.	5	A15(5)	91.	Nishikawa, Niigata Pref.	5	A1(5)
16.	Yuwa, Akita Pref.	4	A16*(4)	92.	Shirone, Niigata Pref.	5	A1(2), A19(3)
17.	Honjo, Akita Pref.	4	A1(4)	93.	Teradomari, Niigata Pref.	3	A1(2), A3(1)
18.	Sinjo1, Yamagata Pret.	5	A1 ⁽⁵⁾	94.	Mitsuke, Nilgata Pret.	4	A1(1), A19(3)
19.	Silinjo2, faillagala Piel.	5	$A1(3)$ $A4^*(2)$	95.	Kashiwazaki2, Niigata Pref.	2	A1(2)
20.	Obanazawa1 Yamagata Pref	5	A5(5)	97	Oiiva1 Niigata Pref	2	A19*(2)
22.	Obanazawa2, Yamagata Pref.	5	A1(5)	98.	Ojiva2. Nijgata Pref.	2	A19(2)
23.	Yamagata, Yamagata Pref.	1	A5*(1)	99.	Ojiya3, Niigata Pref.	2	A19(2)
24.	Soma, Fukushima Pref.	4	B11(4)	100.	Ojiya4, Niigata Pref.	2	A19(2)
25.	Aizubange, Fukushima Pref.	4	A1(2), A19(2)	101.	Ojiya5, Niigata Pref.	5	A19(3), B27(2)
26.	Aizuwakamatsu, Fukushima Pref.	5	A1(5)	102.	Nakasato, Niigata Pref.	5	A3(2), B1a(3)
27.	Inawashiro1, Fukushima Pref.	4	A1(1), A8*(3)	103.	Tsunan, Niigata Pref.	4	A1(4)
28.	Inawashiro2, Fukushima Pref.	3	A19(3)	104.	Itoigawa, Niigata Pref.	4	A1(4)
29.	Inawashiro3, Fukushima Pref.	5	A1(5) A1(4) A8(1)	105.	Kurobe, Toyama Pref.	4	A1(4) A1(6) A20*(4)
30.	Koriyama, Eukushima Pref	6	A1(4), A0(1) $A1^{*}(3) A19(1) B1_{2}(1) B27(1)$	100.	Kamiichi? Toyama Pref	10	$\Delta 1(4)$
32.	Sukagawa, Fukushima Pref.	5	B1a(5)	107.	Kamijchi3, Toyama Pref.	2	A1(2)
33.	Iwaki, Fukushima Pref.	8	B1a(3), B11(5)	109.	Oshima, Toyama Pref.	1	A1(1)
34.	Urizura, Ibaraki Pref.	5	B11(5)	110.	Himi, Toyama Pref.	1	A1(1)
35.	Naka, Ibaraki Pref.	1	B11(1)	111.	Nanao, Ishikawa Pref.	5	A11(3), A9*(2)
36.	Mito1, Ibaraki Pref.	1	B11*(1)	112.	Kanazawa, Ishikawa Pref.	5	A11*(5)
37.	Mito2, Ibaraki Pref.	1	B11(1)	113.	Kaga, Ishikawa Pref.	1	A11(1)
38.	Mito3, Ibaraki Pref.	4	B11(2), C1(2)	114.	Awara, Fukui Pref.	5	A10*(1), A11(3), A12*(1)
39.	Mito4, Ibaraki Pref.	5	B11(5)	115.	Kanazu, Fukui Pref.	5	A1(5)
40. 41	Millos, Ibaraki Pref. Oaraj Ibaraki Pref	5	B11(5)	110.	Sabae, Fukui Piel. Mihama, Eukui Pref	1	Δ1(1)
42	Otawara Tochigi Pref	5	B11(5)	118	Obama Fukui Pref	4	A1*(4)
43.	Karasuyama, Tochigi Pref.	5	B11(2), B15(3)	119.	Kobuchizawa, Yamanashi Pref.	1	B11(1)
44.	Takanezawa, Tochigi Pref.	5	B15(4), B27(1)	120.	Tatomi, Yamanashi Pref.	5	B11(5)
45.	Kawachi, Tochigi Pref.	5	B11(5)	121.	Rokugo, Yamanashi Pref.	2	B11(2)
46.	Mooka1, Tochigi Pref.	6	B11(3), B15*(2), C1*(1)	122.	Fujiyoshida, Yamanashi Pref.	5	B11(5)
47.	Mooka2, Tochigi Pref.	5	B15(5)	123.	Sakae, Nagano Pref.	5	A19(5)
48.	Mibu, Tochigi Pret.	5	B11(4), B15(1)	124.	liyama, Nagano Pret.	5	B11(5)
49. 50	Nitto, Gunma Prof.	5	B1a'(5) B1a(2) $B15(2)$	125.	Toyota, Nagano Pret.	5	B11(5) B27(2)
50.	Tatebayashi Gunma Pref	6	B1a(3), B15(2) B1a(3) B15(3)	120.	Obuse Nagano Pref	5	B27(3) B11(5)
52.	Itakura, Gunma Pref.	5	B1a(3), B11(1), B15(1)	128.	Nagano, Nagano Pref.	5	B11(5)
53.	Menuma, Saitama Pref.	6	B1b*(3), B15(3)	129.	Omachi, Nagano Pref.	5	B11(5)
54.	Ogawa, Saitama Pref.	5	B11(5)	130.	Tobu, Nagano Pref.	3	B11(3)
55.	Yoshimi, Saitama Pref.	7	B1a*(3), B11(2), B15(1), B36(1)	131.	Suwa1, Nagano Pref.	5	B11(5)
56.	Hatoyama, Saitama Pref.	6	B1a(1), B11(5)	132.	Suwa2, Nagano Pref.	2	B11(2)
57.	Hasuda, Saitama Pref.	2	B11(1), B15(1)	133.	Suwa3, Nagano Pref.	2	B11(2)
58.	Iruma, Saltama Pret.	6	B11(3), B27(3) B12(2), B11(5), B25*(1)	134.	lida, Nagano Pret.	5	B11(5)
59. 60	Nagareyama, Chiba Pref. Sawara, Chiba Pref	9	B1a(3), B11(3), B35(1) B1a(3), B11(1), B15(2)	135.	Mishima Shizuoka Pref	5	B40 (5) B18*(5)
61	Sakura, Chiba Pref	5	B11(5)	137	Fuji Shizuoka Pref	5	B18(4) B19*(1)
62.	Chiba, Chiba Pref.	5	B11(5)	138.	Shizuoka, Shizuoka Pref.	4	B21*(4)
63.	Naruto, Chiba Pref.	7	B1a(1), B11(6)	139.	lwata, Shizuoka Pref.	4	B5*(3), B27(1)
64.	Oamishirasato, Chiba Pref.	5	B11(5)	140.	Kozakai, Aichi Pref.	1	B27(1)
65.	Mobara, Chiba Pref.	6	B1a(3), B27(1), C1(2)	141.	Nishio, Aichi Pref.	5	B27(5)
66.	Ohara, Chiba Pref.	5	B11(5)	142.	Seto, Aichi Pref.	4	B40(4)
67.	Onjuku, Chiba Pref.	5	B11(5)	143.	Iwakura, Aichi Pref.	7	B12(2), B27(1), B40(4)
68.	Kisarazu, Uniba Pref.	2	BII(1), B15(1) B12(2) B11(4) B24*(1) C2*(1)	144.	Saori, Alchi Pret. Tateuta Aichi Pref	4	B12(1), B40(2)
09. 70	r uttsu, Utilba Frei. Kitaku Tokyo Prof	9	B15*/5)	145.	Taisula, Alciii F181. Nagashima Mic Prof	3	B1c(3) B12(1)
71.	Chivodaku, Tokvo Pref.	5	B11(5)	147.	Tsu. Mie Pref.	5	B1c*(5)
72.	Setagayaku1, Tokyo Pref.	10	B1a(2), B11(4), B27(3), B34(1)	148.	Ueno1, Mie Pref.	1	B1a(1)
73.	Setagayaku2, Tokyo Pref.	5	B36*(5)	149.	Ueno2, Mie Pref.	5	B1a(5)
74.	Kawasaki, Kanagawa Pref.	4	B11(4)	150.	Ueno3, Mie Pref.	5	B1a(5)
75.	Yokohama, Kanagawa Pref.	4	B11(4)	151.	Toba, Mie Pref.	5	B1c(4), B27(1)
76.	Kamakura, Kanagawa Pref.	3	B27(3)	152.	Isobe, Mie Pref.	5	B1c(1), B5(3), B11(1)

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Table 1. Continued

	Collection site	N	Mitotype		Collection site	N	Mitotype
152	Age Mic Brof	5	Plo(5)	220	Iwakupi Vamaguchi Brof	1	Blo(4)
155.	Ago, Mie Frei. Miyama, Mia Brof	5	DIC(3) DE(1) = D22(4)	230.	Apap Tokushima Prof	4	$B_{1a}(4)$
154.	Kumano Mie Pref	5	B33*(5)	231.	Vuki Tokushima Prof	4	$B_{12}(4)$
155.	Mihama Mia Prof	1	B33 (3)	202.	Hiwaca, Takuchima Prof	-4 5	D1a(4) P1a(5)
157	Kinomoto Shiga Pref	5	B22(5)	234	Kainan Tokushima Pref	4	$B2^{*}(4)$
158	Hikone Shiga Pref	5	B22*(5)	235	Ikeda Kagawa Pref	2	B1a(2)
159	Notogawa Shiga Pref	5	B22(5)	236	Kamiura Ehime Pref	5	B1a(5)
160.	Katata, Shiga Pref.	3	B1a(1), B22*(2)	237.	Yoshiumi, Ehime Pref.	4	B1a(4)
161.	Maizuru1, Kvoto Pref.	6	A15*(2), A17*(1), A18*(3)	238.	Matsuvama, Ehime Pref.	6	B1a(6)
162.	Maizuru2, Kyoto Pref.	3	A15(3)	239.	Masaki, Ehime Pref.	5	B1a(5)
163.	Miyazu, Kvoto Pref.	3	A1(3)	240.	Uchiko, Ehime Pref.	1	B1a(1)
164.	Ine. Kvoto Pref.	5	A1(5)	241.	Tsushima. Ehime Pref.	4	B1d(4)
165.	Omiya, Kyoto Pref.	2	B30*(2)	242.	Toyo, Kochi Pref.	5	B1a(5)
166.	Tango, Kyoto Pref.	3	B28*(3)	243.	Aki, Kochi Pref.	5	B1d(5)
167.	Amino, Kyoto Pref.	2	B29(2)	244.	Tosa, Kochi Pref.	4	B1d(4)
168.	Kumihama1, Kyoto Pref.	5	B5*(1), B14*(4)	245.	Susaki, Kochi Pref.	5	B1d*(5)
169.	Kumihama2, Kyoto Pref.	3	B7*(3)	246.	Saga, Kochi Pref.	5	B1d(5)
170.	Keihoku, Kyoto Pref.	5	B22(5)	247.	Nakamura, Kochi Pref.	5	B1d(4), B42*(1)
171.	Tanba, Kyoto Pref.	4	B38*(4)	248.	Sukumo, Kochi Pref.	3	B1d(3)
172.	Kameoka, Kyoto Pref.	4	B22(3), B38(1)	249.	Kurate, Fukuoka Pref.	4	B15(4)
173.	Hirakata, Osaka Pref.	2	B1a(1), B22(1)	250.	Munakata, Fukuoka Pref.	5	B15(4), B16*(1)
174.	Izumi, Osaka Pref.	5	B6*(5)	251.	Fukuma, Fukuoka Pref.	3	B15(2), B24(1)
175.	Kaizuka, Osaka Pref.	3	B1a(3)	252.	Hisayama, Fukuoka Pref.	4	B1a(1), B15(3)
176.	Toyooka1, Hyogo Pref.	3	A14*(3)	253.	Shima, Fukuoka Pref.	4	B15(2), B17(2)
177.	Toyooka2, Hyogo Pref.	6	A14(3), B14(3)	254.	Yukuhashi, Fukuoka Pref.	5	B15(5)
178.	Kinosaki, Hyogo Pref.	5	A14(5)	255.	Shiida, Fukuoka Pref.	5	B15(5)
179.	Takeno, Hyogo Pref.	2	B31*(2)	256.	Buzen, Fukuoka Pref.	5	B15*(5)
180.	Kasumi1, Hyogo Pref.	2	B29*(2)	257.	Mihashi, Fukuoka Pref.	5	B24(5)
181.	Kasumi2, Hyogo Pref.	1	B29(1)	258.	Yamato, Fukuoka Pref.	5	B24*(5)
182.	Kasumi3, Hyogo Pref.	3	B29(3)	259.	Karatsu, Saga Pref.	5	B17(4), B24(1)
183.	Hamasaka, Hyogo Pref.	7	B15(3), B37*(4)	260.	Arita, Saga Pref.	4	B26(4)
184.	Hikami, Hyogo Pref.	5	B22(5)	261.	Kashima, Saga Pref.	1	B24(1)
185.	Kaibara, Hyogo Pref.	7	B9(1), B22(4), B27(2)	262.	Sasebo, Nagasaki Pref.	5	B26*(5)
186.	Tannan, Hyogo Pref.	4	B39*(4)	263.	Kazusa, Nagasaki Pref.	3	B23*(3)
187.	Himeji1, Hyogo Pref.	5	B9*(5)	264.	Kamiagata, Nagasaki Pref.	1	B17(1)
188.	Himeji2, Hyogo Pref.	2	B1a(1), B9(1)	265.	Izuhara, Nagasaki Pref.	1	B17(1)
189.	Higashiura, Hyogo Pref.	5	B1a(4), B22(1)	266.	Ashibe, Nagasaki Pref.	5	B17*(5)
190.	Seidan, Hyogo Pref.	4	B1a(4)	267.	Fukue, Nagasaki Pref.	4	B26(4)
191.	Kawai, Nara Pref.	1	B1a(1)	268.	Reihoku, Kumamoto Pref.	1	B23(1)
192.	Hongu, Wakayama Pref.	6	B11*(5), B32(1)	269.	Uto, Kumamoto Pref.	5	B23(3), B24(2)
193.	Shingu, Wakayama Pref.	8	B1a(1), B22(1), B32*(3),	270.	Taragi, Kumamoto Pref.	5	B24(5)
101	Na dillata una Malana Dati	-	B33^(1), B41^(2)	2/1.	Minamata, Kumamoto Pref.	4	B24(4)
194.	Nachikatsuura, Wakayama Pret.		B11(4), B33(3)	2/2.	Bungotakada, Oita Pref.	5	B15(5)
195.	Kushimoto, Wakayama Pret.	5	B1a(4), B27(1)	273.	Beppu, Olta Pref.	1	B1a(1)
196.	Hikigawa, wakayama Pref.	3	B1a(1), B10 ⁻ (2)	274.	Salki, Olta Pref.	4	B1a(4)
197.	Tanabe, Wakayama Pref.	1	B10(1) B12(2) B22(2)	275.	Nusu, Olta Pref.	2	B15(2)
190.	Makayama Makayama Pref.	4	D1a(2), D22(2) D1a(2), D1b(1)	270.	Nobeoka, Miyazaki Fiel.	2	B13(2)
200	Iwami, Tottori Prof	4	B20*(3)	277.	Saita Miyazaki Pref	4	B13(4) B4(5)
200.	Tottori, Tottori Prof	3	B20 (3) B21(2)	270.	Miyazaki Miyazaki Pref.	5	B4(3) B4*(5)
201.	Vonago Tottori Pref	5	$B_{1}(2)$ $B_{1}(2)$ $B_{2}(2)$	279.	Kiyotaka Miyazaki Pref	1	B4 (5) B4(1)
202.	Mateue Shimane Pref	1	B20(1)	200.	Kuchimat Miyazaki Prof	5	B4(1) B4(5)
203.	Oda Shimane Pref	3	$B_{20}(2) = B_{21}(1)$	201.	Kushima2 Miyazaki Prof	5	B4(5)
205	Gotsu Shimane Pref	3	B15(1) B29(2)	283	Higashikushira Kagoshima Pref	5	B4(5)
206	Sakurae Shimane Pref	2	B29(2)	284	Kushira Kagoshima Pref	5	B4(5)
207	Hamada Shimane Pref	3	B29(3)	285	Takaono Kagoshima Pref	3	B24(3)
208	Masuda Shimane Pref	5	B15(5)	286	Hiwaki Kagoshima Pref	3	B24(3)
209	Saigo Shimane Pref	3	B29(3)	287	Sendai Kagoshima Pref	5	B24*(5)
210.	Tsuma, Shimane Pref.	2	B3(1), B29(1)	288.	Hivoshi, Kagoshima Pref.	4	B24(4)
211.	Seto, Okavama Pref.	5	B1a*(5)	289.	Oura, Kagoshima Pref.	5	B24(5)
212.	Okavama, Okavama Pref.	4	B1a(4)	290.	Kamikoshiki, Kaqoshima Pref.	5	B25*(5)
213.	Tessei. Okavama Pref.	1	B1a(1)	291.	Sato1. Kagoshima Pref.	3	B24(3)
214.	Mivoshi, Hiroshima Pref.	4	B1a(4)	292.	Sato2, Kagoshima Pref.	2	B24(2)
215.	Tamagawa, Yamaguchi Pref.	5	B3*(2), B15(3)	293.	Nakatane, Kagoshima Pref.	5	B8*(5)
216.	Abu, Yamaguchi Pref.	5	B15(5)	294.	Mageshima1, Kagoshima Pref	3	B24*(3)
217.	Hagi, Yamaguchi Pref.	1	B15(1)	295.	Mageshima2, Kagoshima Pref.	3	B24(3)
218.	Nagato, Yamaguchi Pref.	4	B15(4)	296.	Mageshima3, Kagoshima Pref.	3	B8(3)
219.	Yuya, Yamaguchi Pref.	4	B15(1), B17(3)	297.	Mageshima4, Kagoshima Pref.	3	B8(2), B24(1)
220.	Hohoku, Yamaguchi Pref.	2	B15(2)	298.	Kikai, Kagoshima Pref.	2	B24*(2)
221.	Toyoura1, Yamaguchi Pref.	1	B1a(1)	299.	Ogimi, Okinawa Pref.	5	B24(5)
222.	Toyoura2, Yamaguchi Pref.	4	B15(4)	300.	Nago, Okinawa Pref.	2	B24*(2)
223.	Shimonoseki, Yamaguchi Pref.	6	B15*(6)	301.	Ginoza, Okinawa Pref.	5	B24(5)
224.	Sanyo, Yamaguchi Pref.	2	B15(2)	302.	Ishikawa, Okinawa Pref.	4	B24(4)
225.	Ube, Yamaguchi Pref.	5	B4(3), B15(2)	303.	Gushikami, Okinawa Pref.	1	B24*(1)
226.	Yamaguchi, Yamaguchi Pref.	1	B1a(1)				
227.	Hofu, Yamaguchi Pref.	5	B13*(5)	Inbred	strains		
228.	Kudamatsu, Yamaguchi Pref.	3	B13(3)		HNI*	2	A1(2)
229.	Hirao, Yamaguchi Pref.	4	B1a(4)		Hd-rR*	3	B27(3)

1994; Kocher and Stepien, 1997). The cytochrome b gene is found in the mitochondrial genome of nearly all eukaryotic organisms and in many diverse prokaryotes, indicating a very ancient origin (Esposti et al., 1993). This gene has been one of the most frequently utilized segments of mtDNA because it is easy to align and it has been characterized in many vertebrates, including several fish species (Kocher and Stepien, 1997; Kocher et al., 1989; Zadoya and Doadrio, 1999; Orti et al., 1994; Brito et al., 1997). In the case of fish, the cytochrome b gene has been used to address many phylogenetic questions, from relationships among closely related cichlids (Meyer et al., 1990) to deep phylogenetic questions such as the relationships among sarcopterygian fishes and tetrapods (Meyer and Wilson, 1990; Meyer and Dolven, 1992; Hedges et al., 1993; but see Meyer, 1994).

In this study, we surveyed the detailed genetic population structure in Japanese wild populations of medaka by PCR-RFLP analysis of the cytochrome *b* gene in order to clarify the genetic variability within populations. This procedure resulted in the identification of a large number of mitotypes. In addition, we obtained nucleotide sequences of all mitotypes of the cytochrome *b* gene discriminated by PCR-RFLP analysis. This report concerns the phylogenetic relationships among mitotypes, and discusses the origin of the Northern and Southern Populations in medaka and their evolutionary history.

MATERIALS AND METHODS

Materials

During the period 1979–2001, we collected wild individuals of *O. latipes* from 303 different sites in Japan (Fig. 1). The collection sites are listed in Table 1. We also examined two inbred strains, HNI derived from the Northern Population and Hd-rR from the Southern Population (Hyodo-Taguchi and Sakaizumi, 1993).

Two individuals captured from Sokcho (site #304 in Fig. 1) and Samsan (site #305) in Korea were included for intraspecific phylogenetic analysis. The former belongs to the East Korean Population and the latter to the China-West Korean Population. In addition, three species of the genus *Oryzias, O. curvinotus, O. luzonensis* and *O. mekongensis*, were analyzed for outgroup comparison. They have been classified into the bi-armed chromosome group with *O. latipes* (Uwa, 1986).

DNA extraction and amplification of cytochrome b gene

Total DNA was extracted from the caudal fin by proteinase K digestion, phenol:chloroform extraction and isopropanol precipitation (Shinomiya *et al.*, 1999), and the DNA samples were dissolved

in TE buffer (1 mM EDTA, 10 mM Tris pH 8.0).

For the design of the PCR primers, two complete mitochondrial genomic libraries were first constructed from two wild strains, Maizuru from the Northern Population and Himeji from the Southern Population, and nucleotide sequences were obtained from around the cytochrome *b* gene. We then used these sequences to design a pair of PCR primers to amplify a 1241-bp segment including the complete cytochrome b gene. The nucleotide sequences of the primers were Cytb Fa (5'-AGG ACC TGT GGC TTG AAA AAC CAC-3') and Cytb RVa (5'-TYC GAC YYC CGR WTT ACA AGA CCG-3'). The reaction mixture for amplification by PCR contained 0.2 mM of dNTPs, 0.25 μ M of each primer, 1 μ l of template DNA (below 100 ng), and 0.6 units of Ex Tag polymerase and Ex Tag Buffer (TaKaRa shuzo Co., Kyoto, Japan). PCR amplifications with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) were performed under the following conditions: 94°C for 2min for denaturation, 30 cycles of amplification (94°C for 1.5 min, 55°C for 2 min, 72°C for 2 min) and 1 min for the final extension at 72°C, using.

PCR-RFLP analysis

RFLP analysis of the cytochrome b gene was conducted for 1,225 wild individuals from 303 sites in Japan and the two inbred strains.

Amplified segments were digested with five restriction endonucleases (*Hae*III, *Mbol*, *Mspl*, *Rsal* and *Taql*), in accordance with the instructions provided by the suppliers. Enzyme *Taql* was used only for specimens representing mitotype B1 identified by the other four enzymes, because of the wide distribution of mitotype B1. The fragments thus obtained were separated by electrophoresis on 6% polyacrylamide gels. RFLP patterns were visualized and photographed under UV light after ethidium bromide staining.

Sequencing for phylogenetic analysis

The entire cytochrome *b* gene was subjected to sequence analysis. We sequenced eighty-seven Japanese individuals of *O. latipes*, including all mitotypes identified by PCR-RFLP analysis, two Korean individuals and three closely-related species. The sequenced Japanese samples are shown with an asterisk (*) in Table 1.

Nucleotide sequences were determined directly from PCR products. DNA sequencing used an Applied Biosystems 310 Genetic Analyzer with ABI BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems) in accordance with the manufacturer's instructions. The following primers were used for sequencing: CytbFa, CytbFb (5'-CAA ATA TCA TTT TGA GGG GCC ACT GT-3'), CytbFc (5'-CGA CAA AGT ATC CTT CCA CCC TTA CTT-3'), CytbFd (5'-CCC TAT TCT ACA CAC CTC TAA ACA ACG-3'), CytbFe (5'-CTC GTC AGT TGC ACA CAT CTG CCG-3'), CytbRVa, CytbRVb (5'-ACT GAA AAT CCC CCT CAA ATT CAT TG-3'), CytbRVc (5'-CCT CCA AGT TTG TTT GGA ATT GAT CGT AG-3'), and CytbRVd (5'-GCA TGT ATA TTC CGG ATT AGT CAG CCG TA-3'). The positions of these primers are shown in Fig. 2.

DNA sequences were aligned by using the multiple-sequence alignment program CLUSTAL X, version 1.81 (Thompson *et al.*,



Fig. 2. Positions (arrowheads) of primers used for amplification and/or sequencing of cytochrome *b* gene. The nucleotide sequences of the primers are described in the text.

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1994). Phylogenetic analyses were performed with MEGA2, version 2.1 (Kumar et al., 2001). Pairwise sequence divergences were calculated with Kimura's two-parameter method (Kimura, 1980), and phylogenetic trees were reconstructed with the neighbor-joining (NJ) method (Saitou and Nei, 1987). The reliability of the tree was evaluated with the aid of 1000 bootstrap replicates (Felsenstein, 1985), although the literature on the interpretation of bootstrap proportions (BPs) has not reached a consensus yet (Felsenstein and Kishino, 1993; Hillis and Bull, 1993). We followed the interpretation by Shaffer et al. (1997), which considers BPs >90% to be highly significant, 70-89% as marginally significant, and <70% as constituting limited evidence of monophyly. The sequences reported in this paper have been deposited in the DDBJ/EMBL/GenBank under accession numbers AB084670-AB084756 and AB094503-AB094507.

RESULTS

PCR-RFLP analysis

Multiple fragment patterns of the segments (1241-bp) including the entire cytochrome b gene could be identified with the five restriction endonucleases. The fragment patterns detected by each of the endonucleases are shown in Table 2. Twenty-five different patterns were found for HaeIII (A-Z), 14 for Mbol (A-N), eight for Mspl and Rsal (A-H), and four for Taql (A-D). However, we treated several different fragments as practically the same, since the detection of small differences (below 20-bp) between fragments was not possible with this method.

A total of 1,225 individuals were surveyed and 67 mitotypes were observed (Table 3). The composite mitotypes were identified from four restriction endonucleases (HaeIII, Mbol, Mspl, and Rsal). A special analysis with another restriction endonuclease, Tagl, was used for Mitotype B1, yielding four submitotypes (B1a-B1d). Twenty-four mitotypes were found at only one site, 15 shared two sites, and seven three sites. Eleven mitotypes were observed at four to seven sites, and ten at more than nine sites.

Sequencing and phylogenetic analysis

The 1141-bp region of the complete cytochrome b gene was successfully sequenced for all individuals without any insertions or deletions. In the 1141-bp sequences from Japanese populations, 326 sites were variable, and 311 transitions and 60 transversions were observed (45 sites show both transition and transversion). Most substitutions (270; 82.8%) were in the third codon position, with only 44 (13.5%) in the first and 12 (3.7%) in the second positions, and total of resulting in 37 (9.7%) amino acid changes. A nonsense mutation observed at nucleotide position 1136 in the sample from Mishima (site #136) eliminated two amino

Table 2. Fragment patterns and molecular size (bp) generated by five endnucleases of an amplified segment (1241-bp) comprising the complete cytochrome b gene. Asterisks (*) show fragments including two digestion patterns that could not be distinguished by electrophoresis, because their molecular size was very similar.

Endonuclease	Haelli																									
Fragment pattern	Α		В	С	D	Е	F	G	н	I	J	К	L	М	Ν	0	Ρ	Q	R	S	Т	U	٧	W	Х	Ζ
Molecular size (bp)	563	312*	306*	480	284	765	1049	765	563	563	563	423	563	563	563	306	563	486	284	765	625	282	563	284	423	734
	284	284	284	284	251	284	173	284	284	486	284	284	236	486	284	284	486	480	282	284	284	281	202	248	423	175
	202	251*	257*	202	229	173	19	120	202	173	202	202	202	192	202	257	139	139	281	139	140	236	159	202	202	140
	173	202	202	173	202	19		53	192	19	139	140	139		120	202	53	83	202	53	139	202	139	175	140	139
	19	173	173	83	173			19			53	139			53	139		53	139		53	139	125	140	53	53
		19	19	19	83							53			19	53			53			53	53	139		
					19																	48		53		
		Mbol										Msp														
Fragment pattern	A	В	С	D	E	F	G	н	I	J	K		_	М	N	A	В	С	D	E	F	G	н			
Molecular size (bp)	659	730	537	659	659	531	531	893	531	531	531	410*	403*	348	356	542	473	675	542	542	640	473	411			
	348	348	348	211	511	362	362	348	348	348	348	362	362	265	348	318	318	542	416	389	318	416	389			
	163	163	163	163	71	348	211		182	282	260	348	348	256	256	259	259	13	259	259	259	259	259			
	71		122	137			137		180	80	102	121*	128*	138	138	98	98	11	13	27	13	69	131			
			71	71										106	106	13	69		11	13	11	13	27			
															37	11	13			11		11	13			
														37	0.		11						11			
														57												
Endonuclease					R	sal					Taql															
Fragment pattern	Α	l	В	С	D		E	F	G	н	Α	В	С	D												
Molecular size (bp)	1241	867*	885*	734	507	574*	592*	436	592	948	895	472	878	895												

423 237 237

4

105

61 4

237

61 237 61

44 61 44 4

4

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374* 356* 507 414 356 356

320 293 293

18*

356 356

293

156

197

96

293

Table 3. Mitotypes, composite fragment patterns, number of localities in which the mitotype was found, and sample size (N) of *Oryzias latipes.* The order of the enzymes is *Hae*III, *Mbo*I, *Msp*I, and *Rsa*I.

A1 AAAA 47 145 A2 AAAB 2 4 A3 AAABA 2 3 A4 ABAA 2 3 A5 ACAA 2 6 A6 ADAA 2 3 A7 AEAA 1 1 A8 BABA 2 4 A9 BBAA 1 1 A10 BBCA 1 1 A11 BBDA 4 12 A12 BBDC 1 1 A13 CAAA 3 10 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 3 A19 HAAA 12 30 A20 IAAA 1 3 A19 HAAA 12 30 B10 JFEE B 2 4 B10 JFEE B 2 3 B11 KFEE 1	Mitotype	Composite	Taql	No. of localities	Ν
A2 AAB 2 4 A3 AABA 4 5 A4 ABAA 2 3 A5 ACAA 2 3 A6 ADAA 2 3 A7 AEAA 1 1 A8 BBAA 2 4 A9 BBAA 1 2 A10 BBCA 1 1 A11 BBDC 1 1 A12 BDC 1 1 A13 CAAA 3 10 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 2 5 B10 JFEE B 2 4 B10 JEE 3 7 B10 JEE 3 7 B10 JEE 3 7	A1	AAAA		47	145
A3 AABA 4 5 A4 ABAA 2 3 A5 ACAA 2 6 A6 ADAA 2 3 A7 AEAA 1 1 A8 BABA 2 4 A9 BBAA 1 2 A10 BBCA 1 1 A11 BBDA 4 12 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 1 A17 FAAA 1 1 A18 GAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B10 JFEE D 7 30 B2 JFEF 1 4 8 B6 JHEE 1 3 10 B9 <td< td=""><td>A2</td><td>AAAB</td><td></td><td>2</td><td>4</td></td<>	A2	AAAB		2	4
A4 ABAA 2 3 A5 ACAA 2 3 A6 ADAA 2 3 A7 AEAA 1 1 A8 BABA 2 4 A9 BBAA 1 1 A10 BBCA 1 1 A11 BBDA 4 12 A12 BDC 1 1 A13 CAAA 3 10 A14 DADD 3 11 A15 EAAA 1 3 A14 DADD 3 10 A16 EBAA 1 4 A17 FAAA 1 3 A18 GAAA 1 3 A19 HAAA 2 5 B16 JFEE D 7 30 B2 JEE D 7 30 B2 JGEE 4 8 8 B10 JFEE 0 7 30 B3	A3	AABA		4	5
A6 ADAA 2 3 A7 AEAA 1 1 A8 BBAA 1 2 A40 BBCA 1 1 A9 BBCA 1 1 A10 BBCA 1 1 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 111 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE D 7 30 B2 JEE 3 10 3 B3 JFDB 2 3 3 B4 JFDE 9 37 10 B4 JFDE 3 10 3 </td <td>A4</td> <td>ABAA</td> <td></td> <td>2</td> <td>3</td>	A4	ABAA		2	3
A0 ADAA 2 3 A7 AEAA 1 1 A8 BABA 2 4 A9 BBAA 1 2 A10 BECA 1 1 A11 BBDA 4 12 A11 BBDA 4 12 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 1 A16 EAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B1c JFEE D 7 30 B2 JFEF 1 4 8 B6 JHDE 3 10 3 B14 JFEE 6 1 2 B15 JGEE 4 7 7 <td>A5</td> <td>ACAA</td> <td></td> <td>2</td> <td>6</td>	A5	ACAA		2	6
A8 BABA 1 1 1 A8 BABA 1 2 A10 BBCA 1 1 A11 BBDA 4 12 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 1 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B11 KJEE 4 7 <tr< td=""><td>A0 A7</td><td></td><td></td><td>2</td><td>3</td></tr<>	A0 A7			2	3
A9 BBAA 1 2 A10 BBCA 1 1 A11 BBDA 4 12 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B10 JFEE D 7 30 B2 JFFF 1 4 4 B3 JFDE 2 3 3 B4 JFDE 9 37 37 B5 JGEE 4 8 8 B6 JHEE 1 1 3 B7 JHDB 1 3 3 B8 JHDE	A8	BABA		2	4
A10 BBCA 1 1 A11 BBDC 1 1 A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 3 B1a JFEE A 53 157 B1b JFEE B 2 4 B10 JFEE D 7 30 B2 JFEF 1 4 8 B6 JHEE 2 3 10 B2 JGEE 4 8 8 B6 JHEE 3 10 3 B3 JDE 3 10 3 B4 JFEE 3 10 3 B9 <td< td=""><td>A9</td><td>BBAA</td><td></td><td>1</td><td>2</td></td<>	A9	BBAA		1	2
A11 BBDA 4 12 A12 BDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B10 JFEE C 5 18 B11 JFEE D 7 30 B2 JFFF 1 4 8 B10 JFEE Q 3 1 B3 JFDB 2 3 3 B4 JFDE 9 37 3 B5 JGEE 4 8 8 B6 JHEE 1 3 3 B7 JHDB 1 3 3 B8 JHEE 2 7 3 <tr< td=""><td>A10</td><td>BBCA</td><td></td><td>1</td><td>1</td></tr<>	A10	BBCA		1	1
A12 BBDC 1 1 A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE D 7 30 B2 JFFF 1 4 8 B1d JFEE D 7 30 B2 JFFF 1 4 8 B3 JFDE 2 3 3 B4 JFDE 9 37 3 3 B5 JGEE 4 8 8 3 10 B9 JIEE 3 7 3 3 3 B10 JJEE 2 7 3 1 3	A11	BBDA		4	12
A13 CAAA 1 3 A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 3 A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE D 7 30 B2 JEFF 1 4 4 B3 JFDB 2 3 3 B4 JFDE 9 37 10 B2 JEFF 1 4 8 B6 JHEE 1 3 10 B7 JHDB 1 3 10 B9 JEE 2 3 7 B10 JEE 2 7 11 B14 LFDE 4 14 14	A12	BBDC		1	1
A14 DADD 3 11 A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 2 30 A20 IAAA 2 4 B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE D 7 30 B2 JFEF 1 4 8 B3 JFDB 2 3 3 B4 JFEE 9 37 7 B5 JGEE 4 8 8 B6 JHEE 1 5 7 B7 JHDB 1 3 10 B9 JIEE 3 7 10 B10 JZEE 2 7 7 B11 KJEE 4 7 11 B14 LFDE 3 10<	A13	CAAA		1	3
A15 EAAA 3 10 A16 EBAA 1 4 A17 FAAA 1 1 A18 GAAA 1 3 A19 HAAA 2 5 B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE C 5 18 B1d JFEE D 7 30 B2 JEE D 7 30 B2 JFEF 1 4 83 B1d JFEE D 7 30 B2 JEE 2 3 3 B1 JFDB 2 3 3 B4 JEE 3 10 3 B5 JGEE 4 8 6 B7 JHDB 1 3 3 B10 JEE 2 3 3 B11 KFEE 61 226 7 B13 LFDE <td>A14</td> <td>DADD</td> <td></td> <td>3</td> <td>11</td>	A14	DADD		3	11
A10 EDAA 1 4 A17 FAAA 1 1 A18 GAAA 12 30 A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE D 7 30 B2 JEFF 1 4 B3 JFDB 2 3 B4 JFEE D 7 30 B2 JEFF 1 4 B3 JFDB 2 3 B4 JFEE A 8 B6 JHEE 1 3 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 3 7 B10 JEE 2 7 B11 KJEE 4 7 B12 KJEE 4 7 B13 LFDE 37 110 B16	A15			3	10
A18 GAAA 1 3 A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE D 7 30 B2 JEF 1 4 B3 JFDB 2 3 B4 JFEE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JEE 2 3 B10 JJEE 2 7 B13 LFDE 4 14 B14 LFDE 1 1 B15 LFFE 37 110 B16 LKFE 1 1 B17 LIFE 6 16 B18 MFEG 1 3 B20 MFGB	Δ17	ΕΔΑΑ ΕΔΔΔ		1	4
A19 HAAA 12 30 A20 IAAA 2 5 B1a JFEE B 2 4 B1b JFEE C 5 18 B1d JFEE D 7 30 B2 JFEF 1 4 B3 JDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 2 3 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 14 B14 LFDE 6 16 B15 LFFE 37 110 B16 LKFE 1 1 B20 MF6B 1 3 B21 MFHE 1 4 B22 NFEE <td>A18</td> <td>GAAA</td> <td></td> <td>1</td> <td>3</td>	A18	GAAA		1	3
A20 IAAA 2 5 B1a JFEE A 53 157 B1b JFEE C 5 18 B1d JFEE D 7 30 B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 3 7 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 7 B13 LFDE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEG 1 1 B20 MFGB 1 3 B21 MFHE 19 41 B22 NFEE<	A19	HAAA		12	30
B1a JFEE A 53 157 B1b JFEE B 2 4 B1c JFEE C 5 18 B1d JFEE D 7 30 B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JEE 3 7 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 7 B13 LFDE 37 110 B16 LFFE 1 1 B17 LLFE 6 16 B18 MFEG 1 1 B20 MFGB 1 3 B21 MFHE 1 4 B22	A20	IAAA		2	5
Bib JFEE B 2 4 Bic JFEE C 5 18 Bid JFEE D 7 30 B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 2 3 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 14 B14 LFDE 4 14 B14 LFDE 6 16 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEE 2 9 B19 MFEG 1 3 B20 MFBB <td>B1a</td> <td>JEEE</td> <td>Δ</td> <td>53</td> <td>157</td>	B1a	JEEE	Δ	53	157
Bit JFEE C 5 18 B1d JFEE D 7 30 B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 2 3 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 7 B13 LFDE 4 14 B14 LFDB 2 7 B15 LFFE 37 110 B16 LKFE 1 1 B20 MFGB 1 3 B21 MFHE 1 4 B22 OFCA 3 13	B1b	JFEE	В	2	4
B1d JFEE D 7 30 B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 2 3 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 7 B13 LFDE 4 14 B14 LFDB 2 7 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEE 2 9 B19 MFGB 1 3 B21 MFHE 1 4 B22 NFEE 19 41 B28 PFDA 1 3 <td>B1c</td> <td>JFEE</td> <td>c</td> <td>5</td> <td>18</td>	B1c	JFEE	c	5	18
B2 JFEF 1 4 B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 2 3 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 14 B14 LFDB 2 7 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEE 2 9 B19 MFEG 1 1 B20 MFGB 1 3 B21 MFHE 1 4 B22 OFCA 3 7 B24 OFDA 24 75 B25 OFDB 1 2 <t< td=""><td>B1d</td><td>JFEE</td><td>D</td><td>7</td><td>30</td></t<>	B1d	JFEE	D	7	30
B3 JFDB 2 3 B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 3 7 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 14 B14 LFDB 2 7 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEE 2 9 B19 MFEG 1 1 B20 MFGB 1 3 B21 MFHE 1 4 B22 NFEE 12 39 B23 OFCA 3 75 B26 OLDA 3 13 3 B29 PFDB 12 24	B2	JFEF		1	4
B4 JFDE 9 37 B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 3 7 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 7 B13 LFDE 4 14 B14 LFDB 2 7 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEG 1 3 B20 MFGB 1 3 B21 MFHE 1 4 B22 NFEE 12 39 B23 OFCA 3 13 B27 PFEE 19 41 B28 PFDA 1 3 B30 PGDA 1 2 B31 PHDB 3 5 B32 QFEB 2 4 B33 QFEE 5 B34 RFEE	B3	JFDB		2	3
B5 JGEE 4 8 B6 JHEE 1 5 B7 JHDB 1 3 B8 JHDE 3 10 B9 JIEE 3 7 B10 JJEE 2 3 B11 KFEE 61 226 B12 KJEE 4 14 B14 LFDE 4 14 B15 LFFE 37 110 B16 LKFE 1 1 B17 LLFE 6 16 B18 MFEG 1 3 B20 MFGB 1 3 B21 MFEE 12 39 B23 OFCA 3 7 B26 OLDA 3 13 B27 PFEE 19 41 B28 PFDA 1 2 B30 PGDA 1 2 B31 PHDB 3 5 B32 OFEB 2 4	B4	JFDE		9	37
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C1 ZMCH 4 7 C2 ZNCH 1 1	042				-
02 ZNUH 1 1	C1	ZMCH		4	7
	62	ZNGH		1	1

acids in the C-terminal, although this mutation did not seem to have affected viability because the fish looked normal.

The NJ tree based on Kimura's two-parameter model is shown in Fig. 3. This tree demonstrates that the 67 mitotypes found among Japanese wild population could be divided into three major clades (A, B and C) accompanied by highly significant BPs (99% each). Clade A comprised 20 mitotypes (A1-A20), clade B 42 (B1-B42) and clade C only two, C1 and C2. Clades A and B were subdivided into, respectively, three and 11 subclades. We designated the subclades of clade A A-I to A-III and those of clade B B-I to B-XI. The BP values of these subclades were never less than 80%. We analyzed nucleotide sequences of 2-6 individuals for 10 mitotypes that had large distribution ranges. As a result, while the same mitotype was usually clustered in one subclade, the exception was mitotype B4, shared between two subclades B-VIII and B-X. The sample from Yonago (site #202) was included in subclade B-X and the specimen from Miyazaki (site #279) in subclade B-VIII.

The average sequence divergence was 11.3% (SE=0.9) between clades A and B, 11.8% (SE=1.1) between clades A and C and 11.4% (SE=1.0) between clades B and C. The respective sequence divergences within clade A and clade B averaged 1.6% (SE=0.2) and 3.2% (SE=0.3). The average divergence values were 3.0-4.1% among subcaldes A-I to A-III and 1.3–5.8% among subclades B-I to XI.

The tree also showed intraspecific relationships and the branching order of clades and subclades. Monophyly of the ingroup (*O. latipes* lineage) was supported by a BP value of 90%. The ingroup was divided into two lineages, one consisting of three Japanese clades and supported by a BP value of 94%, and the other contained Sokcho and Samsan samples with limited BP support (58%). In the former lineage, clade A divided at the basal portion, and clade B-C, supported by a BP value of 78%, subsequently branched into clades B and C. In clade A, subclade A-III split first, and the remainder (BP=69%) was divided into subclades A-I and A-II. Clade B first divided into subclade B-XI and the remainder consisting of 10 subclades, which split in descending order according to their subclade designation, although several nodes were not supported by substantial BPs (<50%).

Geographic distribution of mitotypes

Fig. 4 shows the geographic distribution of the mitotypes of the three clades and 14 subclades making up the NJ tree. The distribution patterns demonstrated strong geographical associations.

The mitotypes of clade A were found along the Sea of Japan northward from Hyogo Prefecture, while those of clade B were distributed along the Pacific coast southward from Iwate Prefecture and along the Sea of Japan westward from Kyoto Prefecture. The mitotypes of clade C were found at five sites in the Kanto district.

The mitotypes of subclade A-I were widely distributed covering the entire distribution range of clade A, but those of subclade A-II were observed at only four sites westward



Fig. 3. Neighbor-joining tree of the entire cytochrome *b* gene (1141-bp). The sequence divergences were calculated with Kimura's (1980) two-parameter method. BP values with 1000 replicates associated with each node are shown. Branches without BP values were supported by <50%.

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Fig. 4. Geographic distribution of mitotypes. Clustering of mitotypes was inferred from the NJ tee (Fig. 3). Intra-populational polymorphism is indicated by the combination of small symbols.

from the Noto Peninsula. Subclade A-III contained only one mitotype, A14. This mitotype was found at three sites along the northern coast of Hyogo Prefecture (sites #176, 177, 178), and this area corresponded to the distribution region

of the Boundary Population (Sakaizumi, 1984).

The mitotypes of subclade B-I are widely distributed from the eastern part of Kii Peninsula to the Tohoku district. The mitotypes of subclade B-II were found in the same region as those of subclade B-I (except for Sekikawa; site #84), and the mitotypes of subclade B-III mainly in the Tokai district. The mitotypes of subclades B-IV, B-V and B-VI were found exclusively and respectively in Shizuoka Prefecture, along the Pacific coast in Shikoku, and in the eastern part of Kii Peninsula, and the mitotypes of subclade B-VII mainly around the Seto Inland Sea as well as in the Kanto district. Although subclade B-VII consists of nine mitotypes, all specimens from the Kanto district represented the same mitotype, B1a. The mitotypes of subclade B-VIII were found in eastern Kyushu, Tanegashima Island and Mageshima Island. The mitotypes of subclade B-IX were mainly distributed along the western edge of Honshu, in the northern part of Kyushu, and also in the Kanto district. Although subclade B-IX contains five mitotypes, all individuals from Kanto district showed only one mitotype, B15. The mitotypes of subclade B-X were found in the San-in district, and the mitotypes of subclade B-XI in the western region of Kyushu and in the Ryukyu Islands.

DISCUSSION

Phylogenetic analysis of the cytochrome b gene sequence revealed the differentiation of three major clades (A, B and C) within the Japanese wild populations of medaka. Matsuda et al. (1997b) also identified three major clusters among Japanese medaka on the basis of their RFLP analysis of the entire mtDNA. The distributions of the three clades identified in cytochrome b sequences completely agreed with those of the three clusters detected in RFLP analysis. That is, clades A, B and C correspond to, respectively, mtDNA clusters A, B and C. Moreover, the geographic distributions of the mitotypes in clades A and B appear to be identical to the previously described ranges of the Northern and Southern Populations as determined by allozymic analysis (Sakaizumi et al., 1983). This agreement among the mitotypes of cytochrome b, mtDNA haplotypes and allozyme genotypes supports the concept that the two Japanese populations evolved in complete isolation in the distant past.

Matsuda *et al.* (1997b) conducted RFLP analysis of mtDNA for one individual per site, and identified subclusters that tended to reflect the region-specific geographic distribution. The distribution of mitotypes in each subclade revealed in this study roughly corresponded to that of mtDNA haplotypes in the subclusters. This consistency can be explained by the low genetic variability within the population. Most of populations had monotypic mitotype or mitotypes belonging to the same subclade. This result shows only small nucleotide changes and close phylogenetic relationships among mitotypes in single populations. Consequently, it is likely that the mtDNA haplotype of each individual substantially reflected that of each population.

Our analysis also showed phylogenetic relationships among clades and subclades. The NJ tree shows that in *O. latipes*, the initial divergence occurred between the continental clade and the Japanese clade. After the three Japanese clades had separated off, each of the subclades diverged further. Several authors have proposed different molecular clocks for the cytochrome b gene: about 0.81% per million years (myr) from elasmobranchs (Cantatore et al., 1994), about 0.92%/myr from Sebastes fishes (Rocha-Olivares et al., 1999), about 2.5%/myr from mammals (Irwin et al., 1991) and about 2.8%/myr from sticklebacks (Orti et al., 1994). Recently, Harada et al. (2001) estimated evolutionary rates of 2.5-2.8%/myr from floating gobies, Gymnogobius species, based on the paleogeographic data. We followed these rates, and estimated the divergence times: 5.4-6.0 million years ago (mya) between the continental clade and the Japanese clade, 4.0-4.7 mya among clades A, B and C, 1.1-1.6 mya among subclades A-I to A-III, and 0.5-2.3 mya among subclades B-I to B-XI.

On the basis of these findings, we suggest that the original stock of medaka, like many cyprinid fishes, dispersed from the Eurasian continent into the western part of Japan (Nishimura, 1980; Tomoda, 1989), and that the ancestor of the Japanese populations became separated from the continental populations during the late Miocene. Subsequently, this ancestor divided into three groups (clades A, B and C) during the Pliocene. The era of colonization by the three clades corresponds to the period of the establishment of the protoform of Japan (Fujita, 1990). It is supposed that the dispersal of freshwater fish is restricted by geographic features such as mountains and sea, so that fish populations are inevitably confined to their own watershed or island, resulting in regional differentiation. The boundary separating the Northern and Southern Populations corresponds to the backbone mountains of Honshu, which are surmised to have created a barrier between the two populations (Sakaizumi et al., 1983). It is therefore very likely that the three Japanese groups emerged during this period as a result of geographic isolation. After colonization by the three groups, populations within the groups underwent genetic interaction for a long time until later separation.

Mitotypes in clade C are rare variants of the Japanese wild population and found only at five sites in the Kanto district. That this clade is more closely related to clade B than to clade A is supported by a BP of 78%. The fish representing the haplotype in cluster C has an allozymic genotype similar to that of the fish with haplotypes in cluster B in spite of major differences in the sequence estimated in the mtDNA (Matsuda et al., 1997b). These results suggest that the mitotypes in clade C are 'relics', which diverged from the ancestor of the Japanese medaka and remained in a limited region (the Kanto district) for a long time. The distribution pattern of mitotypes in clade C is similar to that of cyprinid fish, Tanakia tanago. This species is also restricted to a few sites in the Kanto district (Hosoya, 1993). These similar distribution patterns between clade C in O. latipes and T. tanago may suggest the same colonization history.

In clade A, which corresponds to the Northern Population, three subclades (A-I to A-III) were recognized. The NJ tree indicates that subclade A-III (distributed along the northern coast of Hyogo Prefecture) was the first to separate from the common ancestor of clade A, followed by subclades A-II (distributed in Ishikawa Prefecture) and A-I. It is likely that the dispersal of the Northern Population throughout its distribution area, took place from South to North. Subclades A-II and A-III originated independently during the Quaternary in the regions where they now occur, because the boundaries between the subclades are very distinct. Subclade A-I, with the largest distribution range in clade A, is characterized by limited differentiation, as evidenced by the short branch on the NJ tree. It is surmised that this decline in genetic variation can be attributed to a bottleneck effect, and that subclade A-I recently expanded its distribution area (mya between subclades A-I and A-II <1.2).

Clade B, which corresponds to the Southern Population, can be divided into 11 subclades, B-I to B-XI. The mitotypes of subclade B-XI are found in the western region of Kyushu and the Ryukyu Islands, and this subclade may have been the first to separate in the late Pliocene from a common ancestor of clade B, followed by the remaining subclades after a period of genetic interaction with each other. Subclade B-X and B-IX were next to become isolated from the other subclades. Mitotypes of B-X are found in the Sanin district, and those of B-IX along the western edge of Honshu and in the northern part of Kyushu. The remaining subclades, on the other hand, diverged in almost the same period, followed by subclades B-I, B-II and B-III in eastern Japan, which have diverged most recently. The boundaries of the distribution of the mitotypes of each subclade are relatively clear and correlate with geographical features such as mountains. These results suggest that the dispersal of the Southern Population occurred from south-west to north-east in its distribution area, except for the Ryukyu Islands. Furthermore, the regional differentiation of subclades is mainly due to isolation during the Quaternary, as a result of geographic isolation and thus has been maintained until now.

As for subclade B-XI that were distributed from western Kyushu to the Ryukyu Islands, the populations from western Kyushu had four mitotypes (B23-B26), while those from Mageshima Island (sites #294 and 295) and the Ryukyu Islands (sites #298-303) had only one mitotype B24. In contrast to the sequence divergences among samples from Kyushu (sites #262, 263, 287 and 290) in subclade B-XI (0.71-3.1%), those among specimens from the Ryukyu Islands (sites #298, 300 and 303) and Mageshima Island (site #294) were extremely low (0–0.09%). Furthermore, the NJ tree showed a close relationship between samples from the Ryukyu Islands and western Kyushu in subclade B-XI, with a minimum divergence between them of 0.35% (Sendai vs. Mageshima/Nago/Gushikami, and Kamikoshiki vs. Mageshima/Nago/Gushikami). These results indicate that medaka in western Kyushu recently expanded their range into the Ryukyu Islands (mya<0.14). Based on analysis of stratigraphic, tectonostructural and geomorphic data for the Ryukyu Cordillera and adjacent regions, Kimura (1996) proposed a paleogeographic hypothesis that a large land bridge may have formed from the Chinese continent through Taiwan and the Ryukyu Islands to the Kyushu main island during the middle to the late Pleistocene (0.2–0.02 mya). It is thus possible that dispersal of medaka occurred via the land bridge formed from Kyushu to the Ryukyu Islands during the last Ice Age.

Although distribution patterns of the mitotypes in each of subclades show strong geographical associations, mitotypes in subclade B-VII and B-IX show an exceptionally scattered distribution in western Japan and the Kanto district. This disjunct distribution between western Japan and the Kanto district has also been observed at the ACP locus (Sakaizumi et al., 1983). Our results showed that the populations from western Japan had seven mitotypes in subclade B-VII and five mitotypes in subclade B-IX. On the other hand, those from the Kanto district had only two mitotypes, B1a and B1b, and one mitotype B15 in respective subclades. The sequence divergences were 0-0.09% between samples from Maebashi (B1a; site #49), Yoshimi (B1a; site #55), and Menuma (B1b; site #53), and 0% between samples from Mooka (B15; site #46) and Kita-ku (B15; site #70). These results indicate little genetic variability of mitotypes in each of the subclades, which were distributed in the Kanto district. Furthermore, it is notable that a large genetic variability within populations from the Kanto district, was observed. Our study clearly shows that distinct mitotypes, differentiated in various regions, exist even within populations in this district. These findings strongly suggest that this distribution pattern was a result of gene flow, and could have been caused by human action such as the release of fish. It is possible that medaka from western Japan may have been released in the Kanto district.

Furthermore, populations from natural parks such as Mito4 (site #39, Ibaraki Pref., B1a and B27), Sekikawa (site #84, Niigata Pref., B27), and Nakasato (site #102, Niigata Pref., A3 and B1a) surely represent different mitotypes from those originally distributed in adjacent regions. These unusual distribution patterns may also have been caused by artificial transplantation.

Our study also demonstrated the high efficacy of PCR-RFLP analysis of mitochondrial cytochrome b gene as a simple method for detecting genetic variation among Japanese wild populations of medaka. The mitotypes identified by PCR-RFLP, reflect variations in cytochrome b sequences and make the detailed detection of endemism possible. As stated earlier, Japanese wild populations of medaka are characterized by high intraspecific variation and this genetic variation has been maintained for a long time by geographical isolation. Our results demonstrate that transportation of medaka between rivers or between regions strongly affects the genetic features of local populations and the natural distribution of intraspecific variation. To conserve the genetic diversity of medaka, including geographic variations, it is important to preserve local populations and to prevent further artificial disturbance. We hope that our PCR-RFLP

method can be applied to genetic monitoring and thus contribute to the conservation of wild populations of medaka. Furthermore it is likely that this method can be applied to other species.

ACKNOWLEDGEMENT

We are grateful to Dr. Satoshi Hamaguchi (Niigata University) for his valuable advice.

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(Received November 22, 2002 / Accepted May 30, 2003)