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

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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 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

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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,

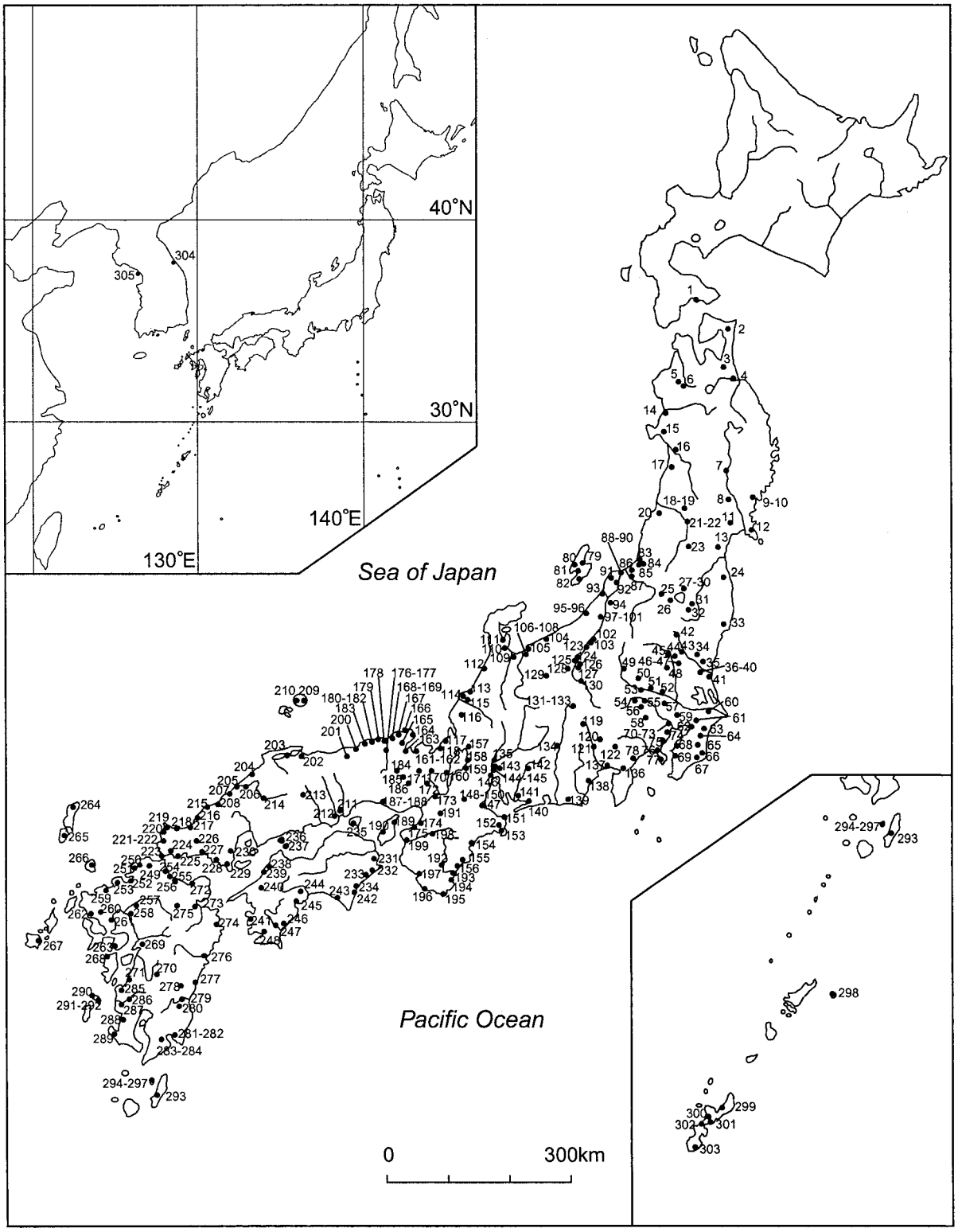


**Fig. 1.** Collection sites of *O. latipes*. The numbers refer to the locations listed in Table 1, except for two Korean sites mentioned in the text.

**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 |
|---|---|---|---|
| 1. | Hakodate, Hokkaido Pref. | 5 | B11(5) |
| 2. | Higashidori, Aomori Pref. | 4 | A1(1), A13*(3) |
| 3. | Kamikita, Aomori Pref. | 1 | A1(1) |
| 4. | Momoishi, Aomori Pref. | 4 | A1(4) |
| 5. | Iwaki, Aomori Pref. | 3 | A1(2), A4(1) |
| 6. | Hirosaki, Aomori Pref. | 1 | A1(1) |
| 7. | Hanamaki, Iwate Pref. | 5 | B11(2), B27(3) |
| 8. | Ichinoseki, Iwate Pref. | 5 | B11(5) |
| 9. | Kesennuma1, Miyagi Pref. | 3 | B11(3) |
| 10. | Kesennuma2, Miyagi Pref. | 2 | B11(2) |
| 11. | Tajiri, Miyagi Pref. | 5 | B11(5) |
| 12. | Onagawa, Miyagi Pref. | 5 | B11(5) |
| 13. | Sendai, Miyagi Pref. | 5 | B11(5) |
| 14. | Noshiro, Akita Pref. | 5 | A1(3), A2*(2) |
| 15. | Hachirogata, Akita Pref. | 5 | A15(5) |
| 16. | Yuwa, Akita Pref. | 4 | A16*(4) |
| 17. | Honjo, Akita Pref. | 4 | A1(4) |
| 18. | Sinjo1, Yamagata Pref. | 5 | A1*(5) |
| 19. | Shinjo2, Yamagata Pref. | 5 | A1(5) |
| 20. | Tsuruoka, Yamagata Pref. | 5 | A1(3), A4*(2) |
| 21. | Obanazawa1, Yamagata Pref. | 5 | A5(5) |
| 22. | Obanazawa2, Yamagata Pref. | 5 | A1(5) |
| 23. | Yamagata, Yamagata Pref. | 1 | A5*(1) |
| 24. | Soma, Fukushima Pref. | 4 | B11(4) |
| 25. | Aizubange, Fukushima Pref. | 4 | A1(2), A19(2) |
| 26. | Aizuwakamatsu, Fukushima Pref. | 5 | A1(5) |
| 27. | Inawashiro1, Fukushima Pref. | 4 | A1(1), A8*(3) |
| 28. | Inawashiro2, Fukushima Pref. | 3 | A19(3) |
| 29. | Inawashiro3, Fukushima Pref. | 5 | A1(5) |
| 30. | Inawashiro4, Fukushima Pref. | 5 | A1(4), A8(1) |
| 31. | Koriyama, Fukushima Pref. | 6 | A1*(3), A19(1), B1a(1), B27(1) |
| 32. | Sukagawa, Fukushima Pref. | 5 | B1a(5) |
| 33. | Iwaki, Fukushima Pref. | 8 | B1a(3), B11(5) |
| 34. | Urizura, Ibaraki Pref. | 5 | B11(5) |
| 35. | Naka, Ibaraki Pref. | 1 | B11(1) |
| 36. | Mito1, Ibaraki Pref. | 1 | B11*(1) |
| 37. | Mito2, Ibaraki Pref. | 1 | B11(1) |
| 38. | Mito3, Ibaraki Pref. | 4 | B11(2), C1(2) |
| 39. | Mito4, Ibaraki Pref. | 5 | B1a(4), B27(1) |
| 40. | Mito5, Ibaraki Pref. | 5 | B11(5) |
| 41. | Oarai, Ibaraki Pref. | 5 | B11(5) |
| 42. | Otawara, Tochigi Pref. | 5 | B11(5) |
| 43. | Karasuyama, Tochigi Pref. | 5 | B11(2), B15(3) |
| 44. | Takanezawa, Tochigi Pref. | 5 | B15(4), B27(1) |
| 45. | Kawachi, Tochigi Pref. | 5 | B11(5) |
| 46. | Mooka1, Tochigi Pref. | 6 | B11(3), B15*(2), C1*(1) |
| 47. | Mooka2, Tochigi Pref. | 5 | B15(5) |
| 48. | Mibu, Tochigi Pref. | 5 | B11(4), B15(1) |
| 49. | Maebashi, Gunma Pref. | 5 | B1a*(5) |
| 50. | Nitta, Gunma Pref. | 5 | B1a(3), B15(2) |
| 51. | Tatebayashi, Gunma Pref. | 6 | B1a(3), B15(3) |
| 52. | Itakura, Gunma Pref. | 5 | B1a(3), B11(1), B15(1) |
| 53. | Menuma, Saitama Pref. | 6 | B1b*(3), B15(3) |
| 54. | Ogawa, Saitama Pref. | 5 | B11(5) |
| 55. | Yoshimi, Saitama Pref. | 7 | B1a*(3), B11(2), B15(1), B36(1) |
| 56. | Hatoyama, Saitama Pref. | 6 | B1a(1), B11(5) |
| 57. | Hasuda, Saitama Pref. | 2 | B11(1), B15(1) |
| 58. | Iruma, Saitama Pref. | 6 | B11(3), B27(3) |
| 59. | Nagareyama, Chiba Pref. | 9 | B1a(3), B11(5), B35*(1) |
| 60. | Sawara, Chiba Pref. | 6 | B1a(3), B11(1), B15(2) |
| 61. | Sakura, Chiba Pref. | 5 | B11(5) |
| 62. | Chiba, Chiba Pref. | 5 | B11(5) |
| 63. | Naruto, Chiba Pref. | 7 | B1a(1), B11(6) |
| 64. | Oamishirasato, Chiba Pref. | 5 | B11(5) |
| 65. | Mobara, Chiba Pref. | 6 | B1a(3), B27(1), C1(2) |
| 66. | Ohara, Chiba Pref. | 5 | B11(5) |
| 67. | Onjuku, Chiba Pref. | 5 | B11(5) |
| 68. | Kisarazu, Chiba Pref. | 2 | B11(1), B15(1) |
| 69. | Futtsu, Chiba Pref. | 9 | B1a(3), B11(4), B34*(1), C2*(1) |
| 70. | Kitaku, Tokyo Pref. | 5 | B15*(5) |
| 71. | Chiyodaku, Tokyo Pref. | 5 | B11(5) |
| 72. | Setagayaku1, Tokyo Pref. | 10 | B1a(2), B11(4), B27(3), B34(1) |
| 73. | Setagayaku2, Tokyo Pref. | 5 | B36*(5) |
| 74. | Kawasaki, Kanagawa Pref. | 4 | B11(4) |
| 75. | Yokohama, Kanagawa Pref. | 4 | B11(4) |
| 76. | Kamakura, Kanagawa Pref. | 3 | B27(3) |
| 77. | Miura, Kanagawa Pref. | 3 | B11(3) |
| 78. | Odawara, Kanagawa Pref. | 7 | B11(2), B27*(3), C1(2) |
| 79. | Ryotsu, Niigata Pref. | 2 | A1(2) |
| 80. | Aikawa, Niigata Pref. | 3 | A1(3) |
| 81. | Sawata, Niigata Pref. | 1 | A1(1) |
| 82. | Hamochi, Niigata Pref. | 5 | A1(5) |
| 83. | Arakawa, Niigata Pref. | 5 | A1*(5) |
| 84. | Sekikawa, Niigata Pref. | 5 | B27(5) |
| 85. | Kurokawa, Niigata Pref. | 2 | A2(2) |
| 86. | Shibata, Niigata Pref. | 4 | A1(3), A20(1) |
| 87. | Toyosaka, Niigata Pref. | 5 | A1(5) |
| 88. | Niigata1, Niigata Pref. | 5 | A1(3), A6(1), A7*(1) |
| 89. | Niigata2, Niigata Pref. | 4 | A1(1), A3(1), A6*(2) |
| 90. | Niigata3, Niigata Pref. | 5 | A1(2), A3*(1), A19(2) |
| 91. | Nishikawa, Niigata Pref. | 5 | A1(5) |
| 92. | Shirone, Niigata Pref. | 5 | A1(2), A19(3) |
| 93. | Teradomari, Niigata Pref. | 3 | A1(2), A3(1) |
| 94. | Mitsuke, Niigata Pref. | 4 | A1(1), A19(3) |
| 95. | Kashiwazaki1, Niigata Pref. | 2 | A1(2) |
| 96. | Kashiwazaki2, Niigata Pref. | 4 | A1(4) |
| 97. | Ojiya1, Niigata Pref. | 2 | A19*(2) |
| 98. | Ojiya2, Niigata Pref. | 2 | A19(2) |
| 99. | Ojiya3, Niigata Pref. | 2 | A19(2) |
| 100. | Ojiya4, Niigata Pref. | 2 | A19(2) |
| 101. | Ojiya5, Niigata Pref. | 5 | A19(3), B27(2) |
| 102. | Nakasato, Niigata Pref. | 5 | A3(2), B1a(3) |
| 103. | Tsunan, Niigata Pref. | 4 | A1(4) |
| 104. | Itoigawa, Niigata Pref. | 4 | A1(4) |
| 105. | Kurobe, Toyama Pref. | 4 | A1(4) |
| 106. | Kamiichi1, Toyama Pref. | 10 | A1(6), A20*(4) |
| 107. | Kamiichi2, Toyama Pref. | 4 | A1(4) |
| 108. | Kamiichi3, Toyama Pref. | 2 | A1(2) |
| 109. | Oshima, Toyama Pref. | 1 | A1(1) |
| 110. | Himi, Toyama Pref. | 1 | A1(1) |
| 111. | Nanao, Ishikawa Pref. | 5 | A11(3), A9*(2) |
| 112. | Kanazawa, Ishikawa Pref. | 5 | A11*(5) |
| 113. | Kaga, Ishikawa Pref. | 1 | A11(1) |
| 114. | Awara, Fukui Pref. | 5 | A10*(1), A11(3), A12*(1) |
| 115. | Kanazu, Fukui Pref. | 5 | A1(5) |
| 116. | Sabae, Fukui Pref. | 1 | A1(1) |
| 117. | Mihama, Fukui Pref. | 1 | A1(1) |
| 118. | Obama, Fukui Pref. | 4 | A1*(4) |
| 119. | Kobuchizawa, Yamanashi Pref. | 1 | B11(1) |
| 120. | Tatomi, Yamanashi Pref. | 5 | B11(5) |
| 121. | Rokugo, Yamanashi Pref. | 2 | B11(2) |
| 122. | Fujiyoshida, Yamanashi Pref. | 5 | B11(5) |
| 123. | Sakae, Nagano Pref. | 5 | A19(5) |
| 124. | Iiyama, Nagano Pref. | 5 | B11(5) |
| 125. | Toyota, Nagano Pref. | 5 | B11(5) |
| 126. | Nakano, Nagano Pref. | 3 | B27(3) |
| 127. | Obuse, Nagano Pref. | 5 | B11(5) |
| 128. | Nagano, Nagano Pref. | 5 | B11(5) |
| 129. | Omachi, Nagano Pref. | 5 | B11(5) |
| 130. | Tobu, Nagano Pref. | 3 | B11(3) |
| 131. | Suwa1, Nagano Pref. | 5 | B11(5) |
| 132. | Suwa2, Nagano Pref. | 2 | B11(2) |
| 133. | Suwa3, Nagano Pref. | 2 | B11(2) |
| 134. | Iida, Nagano Pref. | 5 | B11(5) |
| 135. | Anpachi, Gifu Pref. | 5 | B40*(5) |
| 136. | Mishima, Shizuoka Pref. | 5 | B18*(5) |
| 137. | Fuji, Shizuoka Pref. | 5 | B18(4), B19*(1) |
| 138. | Shizuoka, Shizuoka Pref. | 4 | B21*(4) |
| 139. | Iwata, Shizuoka Pref. | 4 | B5*(3), B27(1) |
| 140. | Kozakai, Aichi Pref. | 1 | B27(1) |
| 141. | Nishio, Aichi Pref. | 5 | B27(5) |
| 142. | Seto, Aichi Pref. | 4 | B40(4) |
| 143. | Iwakura, Aichi Pref. | 7 | B12(2), B27(1), B40(4) |
| 144. | Saori, Aichi Pref. | 4 | B12*(3), B40(1) |
| 145. | Tatsuta, Aichi Pref. | 3 | B12(1), B40(2) |
| 146. | Nagashima, Mie Pref. | 4 | B1c(3), B12(1) |
| 147. | Tsu, Mie Pref. | 5 | B1c*(5) |
| 148. | Ueno1, Mie Pref. | 1 | B1a(1) |
| 149. | Ueno2, Mie Pref. | 5 | B1a(5) |
| 150. | Ueno3, Mie Pref. | 5 | B1a(5) |
| 151. | Toba, Mie Pref. | 5 | B1c(4), B27(1) |
| 152. | Isobe, Mie Pref. | 5 | B1c(1), B5(3), B11(1) |

**Table 1.** Continued

| | Collection site | N | Mitotype |
|---|---|---|---|
| 153. | Ago, Mie Pref. | 5 | B1c(5) |
| 154. | Miyama, Mie Pref. | 5 | B5(1), B33(4) |
| 155. | Kumano, Mie Pref. | 5 | B33*(5) |
| 156. | Mihama, Mie Pref. | 4 | B33(4) |
| 157. | Kinomoto, Shiga Pref. | 5 | B22(5) |
| 158. | Hikone, Shiga Pref. | 5 | B22*(5) |
| 159. | Notogawa, Shiga Pref. | 5 | B22(5) |
| 160. | Katata, Shiga Pref. | 3 | B1a(1), B22*(2) |
| 161. | Maizuru1, Kyoto Pref. | 6 | A15*(2), A17*(1), A18*(3) |
| 162. | Maizuru2, Kyoto Pref. | 3 | A15(3) |
| 163. | Miyazu, Kyoto Pref. | 3 | A1(3) |
| 164. | Ine, Kyoto Pref. | 5 | A1(5) |
| 165. | Omiya, Kyoto Pref. | 2 | B30*(2) |
| 166. | Tango, Kyoto Pref. | 3 | B28*(3) |
| 167. | Amino, Kyoto Pref. | 2 | B29(2) |
| 168. | Kumihama1, Kyoto Pref. | 5 | B5*(1), B14*(4) |
| 169. | Kumihama2, Kyoto Pref. | 3 | B7*(3) |
| 170. | Keihoku, Kyoto Pref. | 5 | B22(5) |
| 171. | Tanba, Kyoto Pref. | 4 | B38*(4) |
| 172. | Kameoka, Kyoto Pref. | 4 | B22(3), B38(1) |
| 173. | Hirakata, Osaka Pref. | 2 | B1a(1), B22(1) |
| 174. | Izumi, Osaka Pref. | 5 | B6*(5) |
| 175. | Kaizuka, Osaka Pref. | 3 | B1a(3) |
| 176. | Toyooka1, Hyogo Pref. | 3 | A14*(3) |
| 177. | Toyooka2, Hyogo Pref. | 6 | A14(3), B14(3) |
| 178. | Kinosaki, Hyogo Pref. | 5 | A14(5) |
| 179. | Takeno, Hyogo Pref. | 2 | B31*(2) |
| 180. | Kasumi1, Hyogo Pref. | 2 | B29*(2) |
| 181. | Kasumi2, Hyogo Pref. | 1 | B29(1) |
| 182. | Kasumi3, Hyogo Pref. | 3 | B29(3) |
| 183. | Hamasaka, Hyogo Pref. | 7 | B15(3), B37*(4) |
| 184. | Hikami, Hyogo Pref. | 5 | B22(5) |
| 185. | Kaibara, Hyogo Pref. | 7 | B9(1), B22(4), B27(2) |
| 186. | Tannan, Hyogo Pref. | 4 | B39*(4) |
| 187. | Himeji1, Hyogo Pref. | 5 | B9*(5) |
| 188. | Himeji2, Hyogo Pref. | 2 | B1a(1), B9(1) |
| 189. | Higashiura, Hyogo Pref. | 5 | B1a(4), B22(1) |
| 190. | Seidan, Hyogo Pref. | 4 | B1a(4) |
| 191. | Kawai, Nara Pref. | 1 | B1a(1) |
| 192. | Hongu, Wakayama Pref. | 6 | B11*(5), B32(1) |
| 193. | Shingu, Wakayama Pref. | 8 | B1a(1), B22(1), B32*(3), B33*(1), B41*(2) |
| 194. | Nachikatsuura, Wakayama Pref. | 7 | B11(4), B33(3) |
| 195. | Kushimoto, Wakayama Pref. | 5 | B1a(4), B27(1) |
| 196. | Hikigawa, Wakayama Pref. | 3 | B1a(1), B10*(2) |
| 197. | Tanabe, Wakayama Pref. | 1 | B10(1) |
| 198. | Hashimoto, Wakayama Pref. | 4 | B1a(2), B22(2) |
| 199. | Wakayama, Wakayama Pref. | 4 | B1a(3), B1b(1) |
| 200. | Iwami, Tottori Pref. | 3 | B20*(3) |
| 201. | Tottori, Tottori Pref. | 2 | B31(2) |
| 202. | Yonago, Tottori Pref. | 5 | B4*(3), B29(2) |
| 203. | Matsue, Shimane Pref. | 1 | B29(1) |
| 204. | Oda, Shimane Pref. | 3 | B29(2), B31(1) |
| 205. | Gotsu, Shimane Pref. | 3 | B15(1), B29(2) |
| 206. | Sakurae, Shimane Pref. | 2 | B29(2) |
| 207. | Hamada, Shimane Pref. | 3 | B29(3) |
| 208. | Masuda, Shimane Pref. | 5 | B15(5) |
| 209. | Saigo, Shimane Pref. | 3 | B29(3) |
| 210. | Tsuma, Shimane Pref. | 2 | B3(1), B29(1) |
| 211. | Seto, Okayama Pref. | 5 | B1a*(5) |
| 212. | Okayama, Okayama Pref. | 4 | B1a(4) |
| 213. | Tessei, Okayama Pref. | 1 | B1a(1) |
| 214. | Miyoshi, Hiroshima Pref. | 4 | B1a(4) |
| 215. | Tamagawa, Yamaguchi Pref. | 5 | B3*(2), B15(3) |
| 216. | Abu, Yamaguchi Pref. | 5 | B15(5) |
| 217. | Hagi, Yamaguchi Pref. | 1 | B15(1) |
| 218. | Nagato, Yamaguchi Pref. | 4 | B15(4) |
| 219. | Yuya, Yamaguchi Pref. | 4 | B15(1), B17(3) |
| 220. | Hohoku, Yamaguchi Pref. | 2 | B15(2) |
| 221. | Toyoura1, Yamaguchi Pref. | 1 | B1a(1) |
| 222. | Toyoura2, Yamaguchi Pref. | 4 | B15(4) |
| 223. | Shimonoseki, Yamaguchi Pref. | 6 | B15*(6) |
| 224. | Sanyo, Yamaguchi Pref. | 2 | B15(2) |
| 225. | Ube, Yamaguchi Pref. | 5 | B4(3), B15(2) |
| 226. | Yamaguchi, Yamaguchi Pref. | 1 | B1a(1) |
| 227. | Hofu, Yamaguchi Pref. | 5 | B13*(5) |
| 228. | Kudamatsu, Yamaguchi Pref. | 3 | B13(3) |
| 229. | Hirao, Yamaguchi Pref. | 4 | B1a(4) |
| 230. | Iwakuni, Yamaguchi Pref. | 4 | B1a(4) |
| 231. | Anan, Tokushima Pref. | 4 | B1a(4) |
| 232. | Yuki, Tokushima Pref. | 4 | B1a(4) |
| 233. | Hiwasa, Tokushima Pref. | 5 | B1a(5) |
| 234. | Kainan, Tokushima Pref. | 4 | B2*(4) |
| 235. | Ikeda, Kagawa Pref. | 2 | B1a(2) |
| 236. | Kamiura, Ehime Pref. | 5 | B1a(5) |
| 237. | Yoshiumi, Ehime Pref. | 4 | B1a(4) |
| 238. | Matsuyama, Ehime Pref. | 6 | B1a(6) |
| 239. | Masaki, Ehime Pref. | 5 | B1a(5) |
| 240. | Uchiko, Ehime Pref. | 1 | B1a(1) |
| 241. | Tsushima, Ehime Pref. | 4 | B1d(4) |
| 242. | Toyo, Kochi Pref. | 5 | B1a(5) |
| 243. | Aki, Kochi Pref. | 5 | B1d(5) |
| 244. | Tosa, Kochi Pref. | 4 | B1d(4) |
| 245. | Susaki, Kochi Pref. | 5 | B1d*(5) |
| 246. | Saga, Kochi Pref. | 5 | B1d(5) |
| 247. | Nakamura, Kochi Pref. | 5 | B1d(4), B42*(1) |
| 248. | Sukumo, Kochi Pref. | 3 | B1d(3) |
| 249. | Kurate, Fukuoka Pref. | 4 | B15(4) |
| 250. | Munakata, Fukuoka Pref. | 5 | B15(4), B16*(1) |
| 251. | Fukuma, Fukuoka Pref. | 3 | B15(2), B24(1) |
| 252. | Hisayama, Fukuoka Pref. | 4 | B1a(1), B15(3) |
| 253. | Shima, Fukuoka Pref. | 4 | B15(2), B17(2) |
| 254. | Yukuhashi, Fukuoka Pref. | 5 | B15(5) |
| 255. | Shiida, Fukuoka Pref. | 5 | B15(5) |
| 256. | Buzen, Fukuoka Pref. | 5 | B15*(5) |
| 257. | Mihashi, Fukuoka Pref. | 5 | B24(5) |
| 258. | Yamato, Fukuoka Pref. | 5 | B24*(5) |
| 259. | Karatsu, Saga Pref. | 5 | B17(4), B24(1) |
| 260. | Arita, Saga Pref. | 4 | B26(4) |
| 261. | Kashima, Saga Pref. | 1 | B24(1) |
| 262. | Sasebo, Nagasaki Pref. | 5 | B26*(5) |
| 263. | Kazusa, Nagasaki Pref. | 3 | B23*(3) |
| 264. | Kamiagata, Nagasaki Pref. | 1 | B17(1) |
| 265. | Izuhara, Nagasaki Pref. | 1 | B17(1) |
| 266. | Ashibe, Nagasaki Pref. | 5 | B17*(5) |
| 267. | Fukue, Nagasaki Pref. | 4 | B26(4) |
| 268. | Reihoku, Kumamoto Pref. | 1 | B23(1) |
| 269. | Uto, Kumamoto Pref. | 5 | B23(3), B24(2) |
| 270. | Taragi, Kumamoto Pref. | 5 | B24(5) |
| 271. | Minamata, Kumamoto Pref. | 4 | B24(4) |
| 272. | Bungotakada, Oita Pref. | 5 | B15(5) |
| 273. | Beppu, Oita Pref. | 1 | B1a(1) |
| 274. | Saiki, Oita Pref. | 4 | B1a(4) |
| 275. | Kusu, Oita Pref. | 2 | B15(2) |
| 276. | Nobeoka, Miyazaki Pref. | 2 | B13(2) |
| 277. | Takanabe, Miyazaki Pref. | 4 | B13(4) |
| 278. | Saito, Miyazaki Pref. | 5 | B4(5) |
| 279. | Miyazaki, Miyazaki Pref. | 5 | B4*(5) |
| 280. | Kiyotake, Miyazaki Pref. | 1 | B4(1) |
| 281. | Kushima1, Miyazaki Pref. | 5 | B4(5) |
| 282. | Kushima2, Miyazaki Pref. | 5 | B4(5) |
| 283. | Higashikushira, Kagoshima Pref. | 5 | B4(5) |
| 284. | Kushira, Kagoshima Pref. | 5 | B4(5) |
| 285. | Takaono, Kagoshima Pref. | 3 | B24(3) |
| 286. | Hiwaki, Kagoshima Pref. | 3 | B24(3) |
| 287. | Sendai, Kagoshima Pref. | 5 | B24*(5) |
| 288. | Hiyoshi, Kagoshima Pref. | 4 | B24(4) |
| 289. | Oura, Kagoshima Pref. | 5 | B24(5) |
| 290. | Kamikoshiki, Kagoshima Pref. | 5 | B25*(5) |
| 291. | Sato1, Kagoshima Pref. | 3 | B24(3) |
| 292. | Sato2, Kagoshima Pref. | 2 | B24(2) |
| 293. | Nakatane, Kagoshima Pref. | 5 | B8*(5) |
| 294. | Mageshima1, Kagoshima Pref. | 3 | B24*(3) |
| 295. | Mageshima2, Kagoshima Pref. | 3 | B24(3) |
| 296. | Mageshima3, Kagoshima Pref. | 3 | B8(3) |
| 297. | Mageshima4, Kagoshima Pref. | 3 | B8(2), B24(1) |
| 298. | Kikai, Kagoshima Pref. | 2 | B24*(2) |
| 299. | Ogimi, Okinawa Pref. | 5 | B24(5) |
| 300. | Nago, Okinawa Pref. | 2 | B24*(2) |
| 301. | Ginoza, Okinawa Pref. | 5 | B24(5) |
| 302. | Ishikawa, Okinawa Pref. | 4 | B24(4) |
| 303. | Gushikami, Okinawa Pref. | 1 | B24*(1) |
| Inbred strains | | | |
| | HNI* | 2 | A1(2) |
| | 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 μM of each primer, 1 μl of template DNA (below 100 ng), and 0.6 units of *Ex Taq* polymerase and *Ex Taq* 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 1min 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, *Mbo*I, *Msp*I, *Rsa*I and *Taq*I), in accordance with the instructions provided by the suppliers. Enzyme *Taq*I 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*.,

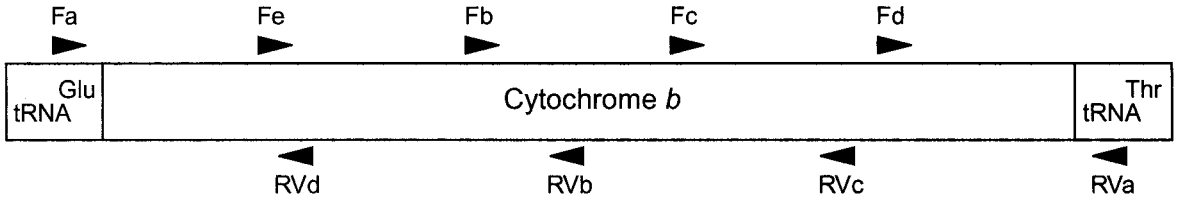


**Fig. 2.** Positions (arrowheads) of primers used for amplification and/or sequencing of cytochrome *b* gene. The nucleotide sequences of the primers are described in the text.

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 *Hae*III (A–Z), 14 for *Mbo*I (A–N), eight for *Msp*I and *Rsa*I (A–H), and four for *Taq*I (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 (*Hae*III, *Mbo*I, *Msp*I, and *Rsa*I). A special analysis with another restriction endonuclease, *Taq*I, 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 | *Hae*III | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fragment pattern | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T | U | V | W | X | Z |
| 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 | | |

| Endonuclease | *Mbo*I | | | | | | | | | | | | | | *Msp*I | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fragment pattern | A | B | C | D | E | F | G | H | I | J | K | L | M | N | A | B | C | D | E | F | G | H |
| 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 |
| | | | | | | | | | | | | | | 91 | 37 | 11 | 13 | | | 11 | | 11 | 13 |
| | | | | | | | | | | | | | | 37 | | | 11 | | | | | | 11 |

| Endonuclease | *Rsa*I | | | | | | | | | *Taq*I | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fragment pattern | A | B | C | D | E | F | G | H | | A | B | C | D |
| Molecular size (bp) | 1241 | 867* | 885* | 734 | 507 | 574* | 592* | 436 | 592 | 948 | 895 | 472 | 878 | 895 |
| | | 374* | 356* | 507 | 414 | 356 | 356 | 356 | 356 | 293 | 237 | 423 | 237 | 237 |
| | | | | | 320 | 293 | 293 | 293 | 197 | | 61 | 237 | 61 | 105 |
| | | | | | | 18* | | 156 | 96 | | 44 | 61 | 61 | 4 |
| | | | | | | | | | | | 4 | 44 | 4 | |
| | | | | | | | | | | | | 4 | | |

**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.

| Mitotype | Composite | *Taq*I | No. of localities | N |
|---|---|---|---|---|
| A1 | AAAA | | 47 | 145 |
| A2 | AAAB | | 2 | 4 |
| 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 | 4 |
| A17 | FAAA | | 1 | 1 |
| A18 | GAAA | | 1 | 3 |
| A19 | HAAA | | 12 | 30 |
| A20 | IAAA | | 2 | 5 |
| 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 | 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 | MFEE | | 2 | 9 |
| B19 | MFEG | | 1 | 1 |
| B20 | MFGB | | 1 | 3 |
| B21 | MFHE | | 1 | 4 |
| B22 | NFEE | | 12 | 39 |
| B23 | OFCA | | 3 | 7 |
| B24 | OFDA | | 24 | 75 |
| B25 | OFDB | | 1 | 5 |
| B26 | OLDA | | 3 | 13 |
| B27 | PFEE | | 19 | 41 |
| B28 | PFDA | | 1 | 3 |
| B29 | PFDB | | 12 | 24 |
| B30 | PGDA | | 1 | 2 |
| B31 | PHDB | | 3 | 5 |
| B32 | QFEB | | 2 | 4 |
| B33 | QFEE | | 5 | 17 |
| B34 | RFEE | | 2 | 2 |
| B35 | SFEE | | 1 | 1 |
| B36 | TJEE | | 2 | 6 |
| B37 | UFFE | | 1 | 4 |
| B38 | VFEE | | 2 | 5 |
| B39 | VFDE | | 1 | 4 |
| B40 | WFEE | | 5 | 16 |
| B41 | XFEE | | 1 | 2 |
| B42 | HFEE | | 1 | 1 |
| C1 | ZMCH | | 4 | 7 |
| C2 | ZNCH | | 1 | 1 |
| | | | 413 | 1225 |

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

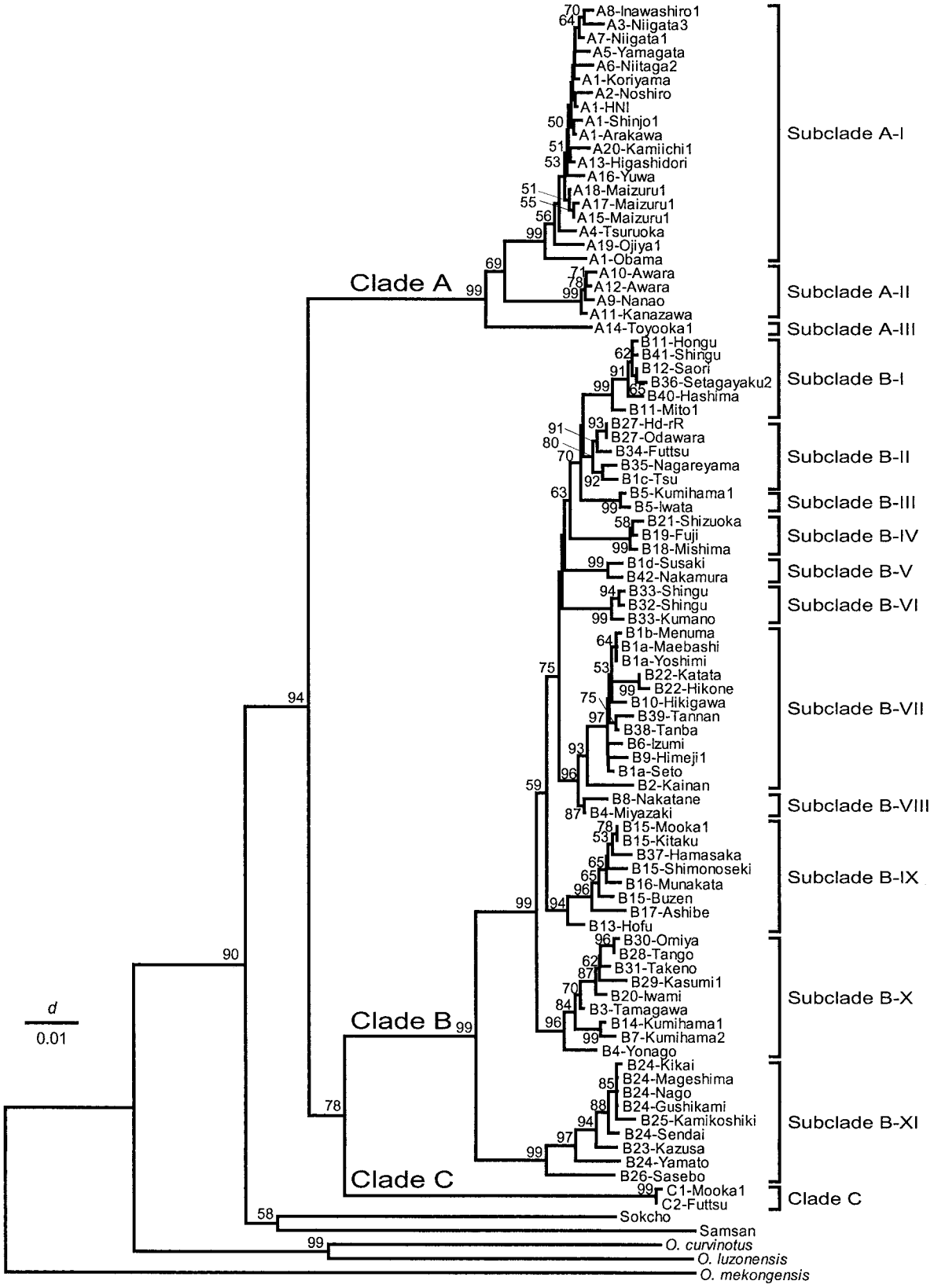


**Fig. 3.** Neighbor-joining tree of the entire cytochrome *b* gene (1141-bp). The sequence divergences were calculated with Kimura's (1980) two-parameter method. BP values with 1000 replicates associated with each node are shown. Branches without BP values were supported by <50%.

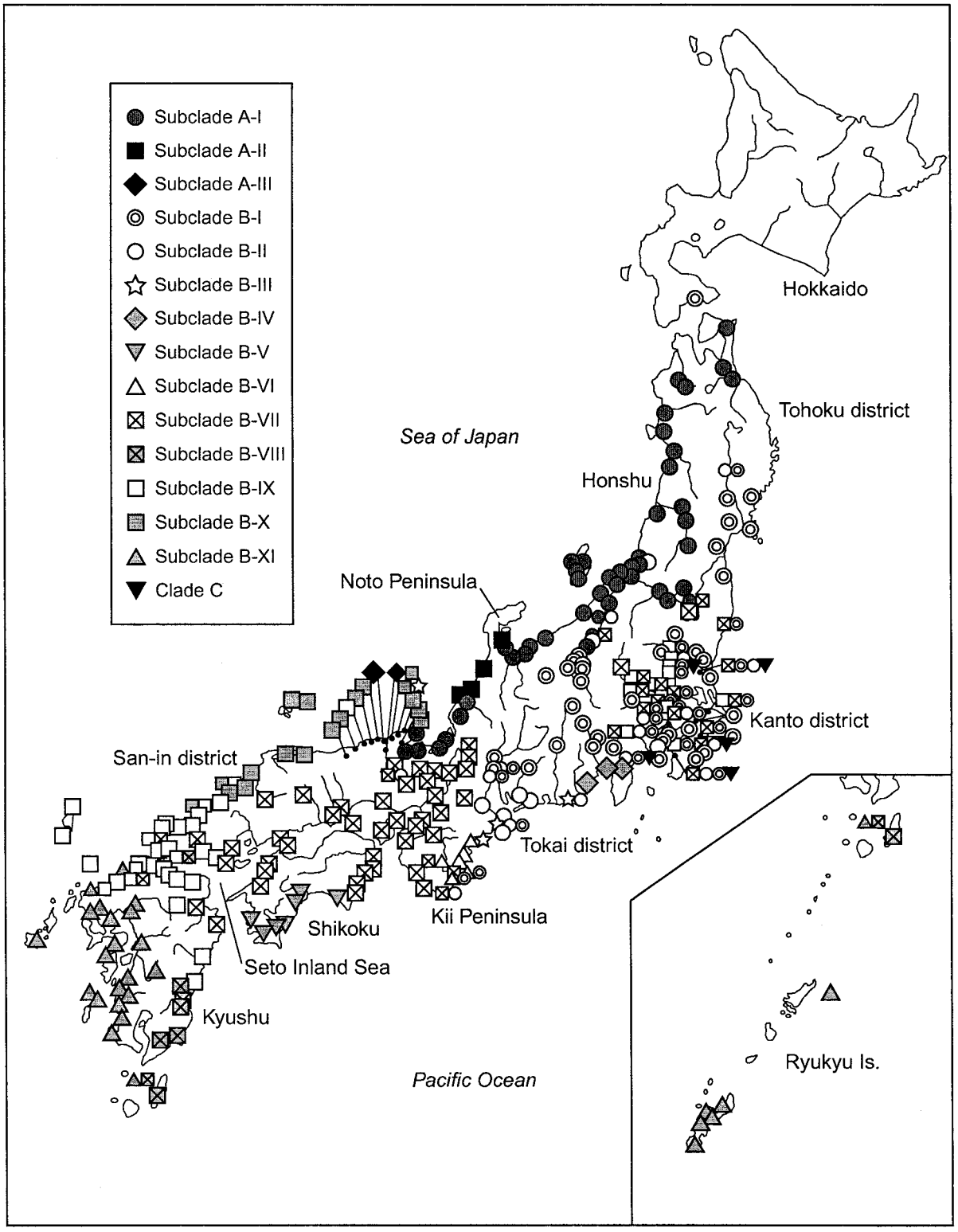


**Fig. 4.** Geographic distribution of mitotypes. Clustering of mitotypes was inferred from the NJ tee (Fig. 3). Intra-populational polymorphism is indicated by the combination of small symbols.

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 San-in 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.

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