



Title	Improvement in group identification of dojo loach, <i>Misgurnus anguillicaudatus</i> , using PCR-restriction fragment length polymorphism
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1 **Title**

2 Improvement in group identification of dojo loach, *Misgurnus anguillicaudatus*, using PCR-restriction  
3 fragment length polymorphism

4

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26 Tokyo University of Agriculture.

27

28 **Abstract**

29 In most Japanese populations of dojo loach (*Misgurnus anguillicaudatus*), gonochoristic diploids of  
30 genetically diversified groups (A and B, further subdivided into B1 and B2) are present, whereas unisexual  
31 clonal lineages inhabit certain localities in the Hokkaido and Ishikawa Prefectures in Japan. Through a  
32 series of genetic studies including DNA markers, the clonal loaches were deemed to originate from a  
33 hybridization event(s) between the A and B1 groups. However, combined analyses with other DNA markers  
34 are needed to identify each genetic group. In this study, we improved the PCR-restriction fragment length  
35 polymorphism (RFLP) analysis of the *recombination activating gene 1* (*RAG1*) gene using digestion with  
36 two restriction enzymes, *PvuII* and *StuI*. The improved *RAG1*-RFLP analysis showed different fragment  
37 patterns for each group: two fragments (245 and 198 bp) for group A, three fragments (198, 147, and 98  
38 bp) for group B1, and a single fragment (443 bp) for group B2. The clonal loaches exhibited four fragments  
39 (245, 198, 147, and 98 bp) derived from both groups A and B1. Moreover, the DNA markers were able to  
40 detect two different hybrid genotypes (A × B2 and B1 × B2). Thus, the improved *RAG1*-RFLP markers  
41 allowed for quick and accurate group identification of the dojo loaches.

42

43 **Keywords**

44 Clone, Hybrid, PCR-RFLP, Unisexuality

45

46

47 **Introduction**

48 In most Japanese populations of dojo loach, *Misgurnus anguillicaudatus* (Cobitidae; Teleostei), bisexually  
49 reproducing gonochoristic diploids ( $2n = 50$ ) are present, whereas unisexual clonal lineages inhabit certain  
50 localities in the Hokkaido and Ishikawa Prefectures in Japan (Morishima et al. 2002, 2008; Arai and  
51 Fujimoto 2013). The clonal diploids generate unreduced diploid eggs that develop by gynogenesis without  
52 any genetic contribution of sperm from sympatric bisexual wild types (Itono et al. 2006, 2007; Arai and  
53 Fujimoto 2013). Previous population genetic studies using allozymes (Khan and Arai 2000), microsatellites  
54 (Arias-Rodriguez et al. 2007), and sequences of the control region in mitochondrial DNA (mtDNA-CR)  
55 (Morishima et al. 2008) clarified that there are two highly diversified groups, A and B (the latter further  
56 subdivided into B1 and B2) in the Japanese wild populations. In Nakaikemi Wetland, Fukui Prefecture,  
57 although groups A and B loaches are sympatric, reproductive isolates between the two groups were  
58 confirmed and suggested Japanese dojo loach should be recognized as two distinct biological species  
59 (Okada et al. 2017). Sequence analyses of *recombination activating gene 1* (*RAG1*) and *interphotoreceptor*  
60 *retinoid-binding protein 2* (*IRBP2*) genes also supported the presence of diverse groups (Yamada et al.  
61 2015). A hybrid origin between groups A and B1 was strongly suggested in clonal loaches because of the  
62 heterozygosity of *RAG1* and *IRBP2* sequences (Yamada et al. 2015). Restriction fragment length  
63 polymorphism (RFLP) analyses of *RAG1* sequences with the restriction enzyme *PvuII* provided different  
64 fragment patterns among the groups. Specifically, groups A and B2 showed a single fragment (443 bp),  
65 while group B1 showed two fragments (296 and 147 bp) (Fujimoto et al. 2017). Clonal loaches had three  
66 fragments (443, 296, and 147 bp) derived from both groups A and B1 (Fujimoto et al. 2017). Although the  
67 *RAG1*-RFLP marker is a useful tool for identifying genetic groups in dojo loaches, discrimination of groups  
68 A and B2, and the hybrids between groups A and B2 is impossible because all of the individuals show a  
69 single fragment (443 bp). Similarly, it is impossible to distinguish clonal loaches from hybrids between  
70 groups B1 and B2 because the three fragments that are detected are the same sizes (443, 296, and 147 bp).

71 Different nuclear DNA markers, ManDra (hereafter designated as ManDra-B in this paper), ManDra-A,  
72 and ManBgl, were developed from repetitive sequences isolated by digestion of genomic DNA with the  
73 restriction enzymes *DraI* and *BglII* (Fujimoto et al. 2017; Kuroda et al. 2021). The DNA markers ManDra-  
74 B and ManDra-A were designed to amplify isolated repetitive sequences by PCR and were used for  
75 grouping based on the electrophoretic patterns of the PCR products. Specifically, ManDra-B yields ladder-  
76 like electrophoretic patterns in group A, but smear-like patterns in groups B1 and B2 (Fujimoto et al. 2017).

77 In contrast, ManDra-A shows smear-like patterns in group A, but ladder-like patterns in group B1 (Kuroda  
78 et al. 2021). Thus, both ManDra-B and ManDra-A show smear-like patterns in clonal loaches (Fujimoto et  
79 al. 2017; Kuroda et al. 2021). Similarly, for the ManBgl marker, a 400 bp fragment has been amplified by  
80 PCR in group A, while a 460 bp fragment without the 400 bp fragment has been shown in groups B1 and  
81 B2 (Fujimoto et al. 2017). Clonal loaches show both the 400 and 460 bp fragments because of the  
82 heterozygous nuclear genomes (Fujimoto et al. 2017).

83 Therefore, combined genetic analyses using the abovementioned DNA markers (*RAGI*-RFLP, ManDra-  
84 B, ManDra-A, and ManBgl) are needed to completely distinguish the genetic groups of dojo loaches. Here,  
85 we have improved the *RAGI*-RFLP marker using two restriction enzymes, *PvuII* and *StuI*. The improved  
86 marker allowed quick and accurate identification of each group (A, B1, and B2), clonal lineage, and even  
87 hybrid genotypes (A × B2 or B1 × B2).

## 88 **Materials and Methods**

### 89 **Experimental animals**

90 In total, 105 dojo loach (*M. anguillicaudatus*) individuals were collected from 12 localities in Japan (Table  
91 1; Fig. S1). Although most individuals had been grouped (except those from Nanae and Abashiri) by genetic  
92 analyses of mtDNA-CR RFLP haplotypes, ManDra-B, ManBgl, and *RAG1*-RFLP genotypes in previous  
93 studies (Morishima et al. 2008; Yamada et al. 2015; Fujimoto et al. 2017), mtDNA-CR RFLP haplotypes,  
94 ManDra-A, ManDra-B, and the improved *RAG1*-RFLP marker were analyzed for all samples in this study  
95 (Table 2). Table 2 shows the previous DNA datasets (Morishima et al. 2008; Fujimoto et al. 2017), as well  
96 as the results of the new DNA analyses in this study.

### 97 **Group identification by mtDNA-CR RFLP haplotypes**

98 Genomic DNA was extracted from tissue samples using a standard phenol/chloroform protocol (Asahida et  
99 al. 1996). The mtDNA-CR was amplified by PCR using a previously published primer set (0F 5'-  
100 CTGACATTCCGACCAATCAC-3' and 1565R 5'-CTCTCGTATAACCGCGGT-3') (Morishima et al.  
101 2008). PCR analyses were performed with 1.0 µL of genomic template DNA (100 ng/µL), 3.6 µL double  
102 distilled water, 5.0 µL 2×Quick Taq HS DyeMix (TOYOBO, Osaka, Japan), and 0.2 µL of each primer (10  
103 µM). The PCR cycling conditions were as follows: initial denaturation for 3 min at 93°C, followed by 30  
104 cycles of denaturation for 1 min at 93°C, annealing for 1 min at 58°C, and extension for 1 min at 72°C. The  
105 PCR products were digested using the restriction enzymes *HaeIII* and *HinI* (Takara Bio, Shiga, Japan)  
106 (Morishima et al. 2008). Five microliters of each PCR product was mixed with 1.0 µL restriction enzyme,  
107 1.0 µL 10×M Buffer (for *HaeIII*) or 10×H Buffer (for *HinI*) (Takara Bio), and 3.0 µL double distilled water  
108 in a 0.2 mL microcentrifuge tube. After incubation at 37°C for 9 h, 5.0 µL of the digested sample was  
109 electrophoresed on a 1.5% agarose gel for 40 min at 100 V and visualized with ethidium bromide.  
110 According to the method described by Morishima et al. (2008), the genetic group of each sample was  
111 determined by the RFLP haplotype of the mtDNA-CR.

### 112 **Group identification by nuclear DNA markers ManDra-A and ManDra-B**

113 The repetitive sequences, ManDra-A and ManDra-B, were amplified by PCR using previously reported  
114 primer sets (ManDra-A: ManDra-AF 5'-TCATCATAAGAATGCTCCTGTAAGC-3' and ManDra-AR 5'-  
115 GCATTTTAGTATGAGAATTCAACTT-3'; ManDra-B: ManDra-F 5'-TGTTTCATCCTTAGAATGCC-3'  
116 and ManDra-R 5'-CCAGCTCAGAAAAGCAGTTTAG-3') (Fujimoto et al. 2017; Kuroda et al. 2021). PCR  
117 analyses were performed with 1.0 µL of genomic template DNA (100 ng/µL), 3.6 µL double distilled water,

118 5.0  $\mu$ L 2 $\times$ Quick Taq HS DyeMix, and 0.2  $\mu$ L of each primer (10  $\mu$ M). The PCR cycling conditions were as  
119 follows: initial denaturation for 3 min at 95°C, followed by 20 cycles of denaturation for 30 s at 95°C,  
120 annealing for 30 s at 50°C, extension for 30 s at 72°C, and a final extension for 5 min at 72°C (Fujimoto et  
121 al. 2017; Kuroda et al. 2021). Group identification based on ManDra-A and ManDra-B was performed by  
122 comparing the electrophoretic patterns of the two markers (Fujimoto et al. 2017; Kuroda et al. 2021).

### 123 **Improvement of *RAG1*-RFLP marker analysis**

124 *RAG1*-RFLP marker (Fujimoto et al. 2017) was developed using *RAG1* gene sequences (527 bp) of groups  
125 A (AB698051-AB698056), B1 (AB698049-AB698050, AB698057-AB698060), and B2 (AB698061-  
126 AB698064) determined by Yamada et al. (2015) (Fig. S2). To clarify improved *RAG1*-RFLP marker can  
127 identify each group accurately compared to *RAG1*-RFLP marker, same *RAG1* gene sequence datasets  
128 (Yamada et al. 2015) were used as representative sequence of each group in this study. Optimal restriction  
129 enzymes that allow identification of each group (A, B1, B2, and clonal loaches) from the sizes and numbers  
130 of the digested fragments were selected using CLC Genomics Workbench (ver. 9.5.3) (QIAGEN, Venlo,  
131 Netherlands). Primer set RAG1-M.aF (5'-GTTTGAATGGCAGCCAGCTCTG-3') and RAG1-M.aR (5'-  
132 CCACAAACATGAGACACAGAGGTC-3') was designed to amplify 443 bp of the *RAG1* gene region (Fig.  
133 S2) (Fujimoto et al. 2017). PCR analyses were performed with 1.0  $\mu$ L of genomic template DNA (100  
134 ng/ $\mu$ L), 3.6  $\mu$ L double distilled water, 5.0  $\mu$ L 2 $\times$ Quick Taq HS DyeMix, and 0.2  $\mu$ L of each primer (10  $\mu$ M).  
135 The PCR cycling conditions were as follows: initial denaturation for 2 min at 94°C, followed by 35 cycles  
136 of denaturation for 1 min at 94°C, annealing for 1 min at 60°C, extension for 1 min at 68°C, and a final  
137 extension for 7 min at 68°C. Five microliters of each PCR product was mixed with 1.0  $\mu$ L each of restriction  
138 enzymes *PvuII* and *StuI* (New England Biolabs, Massachusetts, USA), 1.0  $\mu$ L CutSmart Buffer (New  
139 England Biolabs), and 2.0  $\mu$ L double distilled water in a 0.2 mL microcentrifuge tube. After incubation at  
140 37°C for 9 h, 5.0  $\mu$ L of the digested sample was electrophoresed on a 1.5% agarose gel for 40 min at 100  
141 V and visualized with ethidium bromide.

142 **Results and Discussion**

143 The mtDNA haplotypes and nuclear genotypes from the 12 localities are shown in Table 2. The combined  
144 use of two restriction enzymes (*PvuII* and *StuI*) allowed the identification of each group (A, B1, and B2)  
145 from the sizes and numbers of the digested fragments of the amplified *RAG1* gene region (443 bp) (Fig.  
146 S2). The sequences of group A (AB698051-AB698056) contained a restriction site for *StuI* (AB698051  
147 was shown in Fig. S2). The sequences of group B1 (AB698049-AB698050 and AB698057-AB698060)  
148 contained a restriction site for *StuI* and a restriction site for *PvuII* (AB698049 was shown in Fig. S2). There  
149 were no restriction sites for *StuI* and *PvuII* in the group B2 sequences (AB698061-AB698064) (AB698061  
150 was shown in Fig. S2). Thus, three diversified groups (A, B1, and B2) and the clonal loaches showed  
151 different electrophoretic fragment patterns using the improved *RAG1*-RFLP (Fig. 1). Specifically, two  
152 fragments (245 and 198 bp), three fragments (198, 147, and 98 bp), and a single fragment (443 bp) were  
153 detected in groups A, B1, and B2, respectively (Fig. 1). The clonal loaches exhibited four fragments (245,  
154 198, 147, and 98 bp) derived from both groups A and B1 (Fig. 1). Thus, the improved *RAG1*-RFLP markers  
155 clearly distinguished the genetic groups in the dojo loaches. Group B2 is known as non-native loaches  
156 artificially introduced from Chinese continent (Koizumi et al. 2009, Shimizu and Takagi 2010, Matsui and  
157 Nakajima 2020). Thus, the improved *RAG1*-RFLP marker should be a strong tool for discriminating  
158 Japanese native populations and invasive populations. Moreover, the DNA markers allowed the detection  
159 of various natural hybrid genotypes. For example, A × B1 hybrid genotype was found sympatrically with  
160 group B1 loaches in Obama in Fukui Prefecture (Table 2). Two different hybrid genotypes (A × B2 and B1  
161 × B2) were found sympatrically, as well as groups A, B1, and B2 loaches in Futtsu in Chiba Prefecture  
162 (Table 2). The clonal loaches had specific mtDNA-CR haplotype III, which has been classified into four  
163 lineages by random amplified polymorphic DNA (RAPD)-PCR and DNA fingerprints (Morishima et al.  
164 2008). Individuals with hybrid genotypes in Obama and Futtsu may carry out clonal reproduction, although  
165 their mtDNA-CR haplotypes V and VII differs from that of the clonal loaches. This occurs because clonal  
166 reproduction is closely associated with hybridization in many species and is observed in clonal loaches that  
167 are supposed to be of hybrid origin between groups A and B1 (Dawley, 1989; Vrijenhoek, 1994; Beukeboom  
168 and Vrijenhoek, 1998; Lamatsch and Stöck, 2009; Arai and Fujimoto, 2013). Thus, experiments using  
169 artificial fertilization should be performed in the future to confirm whether unreduced diploid gametes are  
170 produced.

171



172 **Declarations**

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177

178 **Conflicts of interest**

179 The authors declare that they have no conflict of interest.

180 **Availability of data and material**

181 All data and material sets generated during the current study are available from the corresponding author  
182 upon reasonable request.

183 **Code availability**

184 Not applicable.

185 **Author contribution statement**

186 MK, TF, EY, and KA conceived and designed the study. MK conducted the experiments. MK analyzed the  
187 data. MK and TF wrote the manuscript. All authors read and approved the manuscript.

188 **Ethics approval**

189 This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of  
190 Hokkaido University. All animal experiments were approved by the Animal Study Ethical Committee of  
191 Hokkaido University (approval number 29-3).

192 **Consent to participate**

193 Not applicable.

194 **Consent for publication**

195 Not applicable.

196

197 **References**

198 Arai K, Fujimoto T (2013) Genomic constitution and atypical reproduction in polyploid and unisexual  
199 lineages of the *Misgurnus* loach, a teleost fish. *Cytogenet Genome Res* 140(2-4):226-240.  
200 <https://doi.org/10.1159/000353301>

201 Arias-Rodriguez L, Morishima K, Arai K (2007) Genetically diversified populations in the loach *Misgurnus*  
202 *anguillicaudatus* inferred from newly developed microsatellite markers. Mol Ecol Notes 7(1):82-  
203 85. <https://doi.org/10.1111/j.1471-8286.2006.01536.x>

204 Asahida T, Kobayashi T, Saitoh K, Nakayama I (1996) Tissue preservation and total DNA extraction from  
205 fish stored at ambient temperature using buffer containing high concentration of urea. Fish Sci  
206 62:727-730. <https://doi.org/10.2331/fishsci.62.727>

207 Beukeboom LW, Vrijenhoek RC (1998) Evolutionary genetics and ecology of sperm-dependent  
208 parthenogenesis. J Evol Biol 11(6):755-782. <https://doi.org/10.1046/j.1420-9101.1998.11060755.x>

209 Dawley RM (1989) An introduction to unisexual vertebrates. In: Dawley RM, Bogart JP (ed) Evolution and  
210 Ecology of Unisexual Vertebrates. New York State Museum, Albany, New York, pp 1-18

211 Fujimoto T, Yamada A, Kodo Y et al (2017) Development of nuclear DNA markers to characterize  
212 genetically diverse groups of *Misgurnus anguillicaudatus* and its closely related species. Fish Sci  
213 83(5):743-756. <https://doi.org/10.1007/s12562-017-1108-y>

214 Itono M, Morishima K, Fujimoto T, Bando E, Yamaha E, Arai K (2006) Premeiotic endomitosis produces  
215 diploid eggs in the natural clone loach, *Misgurnus anguillicaudatus* (Teleostei: Cobitidae). J Exp  
216 Zool A Comp Exp Biol 305(6):513-523. <https://doi.org/10.1002/jez.a.283>

217 Itono M, Okabayashi N, Morishima K et al (2007) Cytological mechanisms of gynogenesis and sperm  
218 incorporation in unreduced diploid eggs of the clonal loach, *Misgurnus anguillicaudatus* (Teleostei:  
219 Cobitidae). J Exp Zool A Comp Exp Biol 307(1):35-50. <https://doi.org/10.1002/jez.a.344>

220 Khan MR, Arai K (2000) Allozyme variation and genetic differentiation in the loach *Misgurnus*  
221 *anguillicaudatus*. Fish Sci 66(2):211-222. <https://doi.org/10.1046/j.1444-2906.2000.00037.x>

222 Koizumi N, Takemura T, Watabe K, Mori A (2009) Genetic variation and diversity of Japanese loach  
223 inferred from mitochondrial DNA-phylogenetic analysis using the cytochrome *b* gene sequence.  
224 Trans JSIDRE 259:7-16. <https://doi.org/10.11408/jsidre.77.7>

225 Kuroda M, Shibata K, Fujimoto T, Murakami M, Yamaha E, Arai K (2021) Fluorescence *in situ*  
226 hybridization identifies chromosome differentiation between contemporary genomes of wild types  
227 and the ancestral genome of unisexual clones of dojo loach, *Misgurnus anguillicaudatus*. Cytogenet  
228 Genome Res. Accepted. doi: 10.1159/000515107

- 229 Lamatsch DK, Stöck M (2009) Sperm-dependent parthenogenesis and hybridogenesis in teleost fishes. In:  
 230 Schön I, Martens K, Dijk P (eds) Lost sex: the evolutionary biology of parthenogenesis. Springer,  
 231 Dordrecht, pp 399-432
- 232 Matsui S, Nakajima J (2020) Distribution of native and non-native lineages of *Misgurnus anguillicaudatus*  
 233 in Osaka Prefecture, Japan, and development of morphological identification for each lineage.  
 234 Bulletin of the Osaka Museum of Natural History 74:1-15. <http://doi.org/10.20643/00001424>
- 235 Morishima K, Horie S, Yamaha E, Arai K (2002) A cryptic clonal line of the loach *Misgurnus*  
 236 *anguillicaudatus* (Teleostei: Cobitidae) evidenced by induced gynogenesis, interspecific  
 237 hybridization, microsatellite genotyping and multilocus DNA fingerprinting. *Zoolog Sci* 19(5):565-  
 238 575. <https://doi.org/10.2108/zsj.19.565>
- 239 Morishima K, Nakamura-Shiokawa Y, Bando E et al (2008) Cryptic clonal lineages and genetic diversity  
 240 in the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) inferred from nuclear and  
 241 mitochondrial DNA analyses. *Genetica* 132(2):159-171. [https://doi.org/10.1007/s10709-007-9158-](https://doi.org/10.1007/s10709-007-9158-1)  
 242 1
- 243 Okada R, Inui T, Iguchi Y, Kitagawa T, Takata K, Kitagawa T (2017) Molecular and morphological analyses  
 244 revealed a cryptic species of dojo loach *Misgurnus anguillicaudatus* (Cypriniformes: Cobitidae) in  
 245 Japan. *J Fish Biol* 91(3):989-996. <https://doi.org/10.1111/jfb.13393>
- 246 Shimizu T, Takagi M (2010) Genetic structure of natural populations of *Misgurnus anguillicaudatus* in  
 247 Ehime Prefecture impacted by recent exotic introduction, inferred from mitochondrial DNA  
 248 analysis. *Japanese Journal of Ichthyology* 57:13-26. <https://doi.org/10.11369/jji.57.13>
- 249 Yamada A, Kodo Y, Murakami M et al (2015) Hybrid origin of gynogenetic clones and the introgression of  
 250 their mitochondrial genome into sexual diploids through meiotic hybridogenesis in the loach,  
 251 *Misgurnus anguillicaudatus*. *J Exp Zool A Ecol Genet Physiol* 323(9):593-606.  
 252 <https://doi.org/10.1002/jez.1950>
- 253 Vrijenhoek RC (1994) Unisexual fish: model systems for studying ecology and evolution. *Annu Rev Ecol*  
 254 *Syst* 25(1):71-96
- 255
- 256
- 257 **Table**
- 258 **Table 1** Sampling sites, numbers of individuals, year of sampling, and presence or absence of previous

259 DNA data sets of individuals for this study

260 **Table 2** MtDNA and nuclear genomes of all samples used in this study

261 **Figure Legends**

262 **Fig. 1** Representative fragment patterns of improved *RAG1*-RFLP analysis in dojo loaches

263 Group A (lane 2) produced two fragments of 245 and 198 bp. Group B1 (lane 3) produced three fragments  
264 of 198, 147, and 98 bp. Group B2 (lane 4) produced a single fragment of 443 bp. The clonal loach (lane  
265 5) produced four fragments of 245, 198, 147, and 98 bp. The 100 bp ladder molecular marker is shown in  
266 lane 1.

267

268 **Electronic Supplementary Material**

269 **Supplementary Fig. S1** Sampling locations in this study

270 Each number (1-12) corresponds to a site no. in Table 1.

271

272 **Supplementary Fig. S2** Representative *RAG1* sequences (527 bp) of group A, B1, and B2 loaches

273 Sequences of group A (AB698051) contain a *StuI* restriction site (indicated by a solid line). Sequences of  
274 group B1 (AB698049) contain a *StuI* restriction site and a *PvuII* restriction site (indicated by a dotted line).  
275 Sequences of group B2 (AB698061) have no *StuI* and *PvuII* restriction sites. Thick arrows indicate the  
276 primer sequences to amplify *RAG1* sequences (443 bp) by PCR.

277

278

279

**Table 1** Sampling sites, number of individuals, year of sampling, and presence or absence of previous DNA data sets<sup>†</sup> of individuals for this study

Site no.	Prefecture	Localities (city, town, or village)	Total no. individuals	Year of sampling	Previous DNA data
1	Hokkaido	Akkeshi	8	2001	present
2		Nanae	4	2018	absent
3		Ebetsu	8	1998	present
4		Abashiri	21	2018	absent
5	Akita	Kakunodate	8	1998	present
6	Miyagi	Naruko	8	1998	present
7	Fukui	Obama	8	1998	present
8	Tochigi	Nikko	8	1998	present
9	Saitama	Hanyu	8	1999	present
10	Chiba	Futtsu	8	1998	present
11	Nagano	Ueda	8	1999	present
12	Tokushima	Hiwasa	8	1996	present
<b>Total</b>			105		

<sup>†</sup>Previous DNA data sets (Morishima et al. 2008; Fujimoto et al. 2017) include mtDNA control region RFLP haplotypes, nuclear DNA ManDra-B, and nuclear *RAG1*-RFLP markers (standard methodology, not improved)

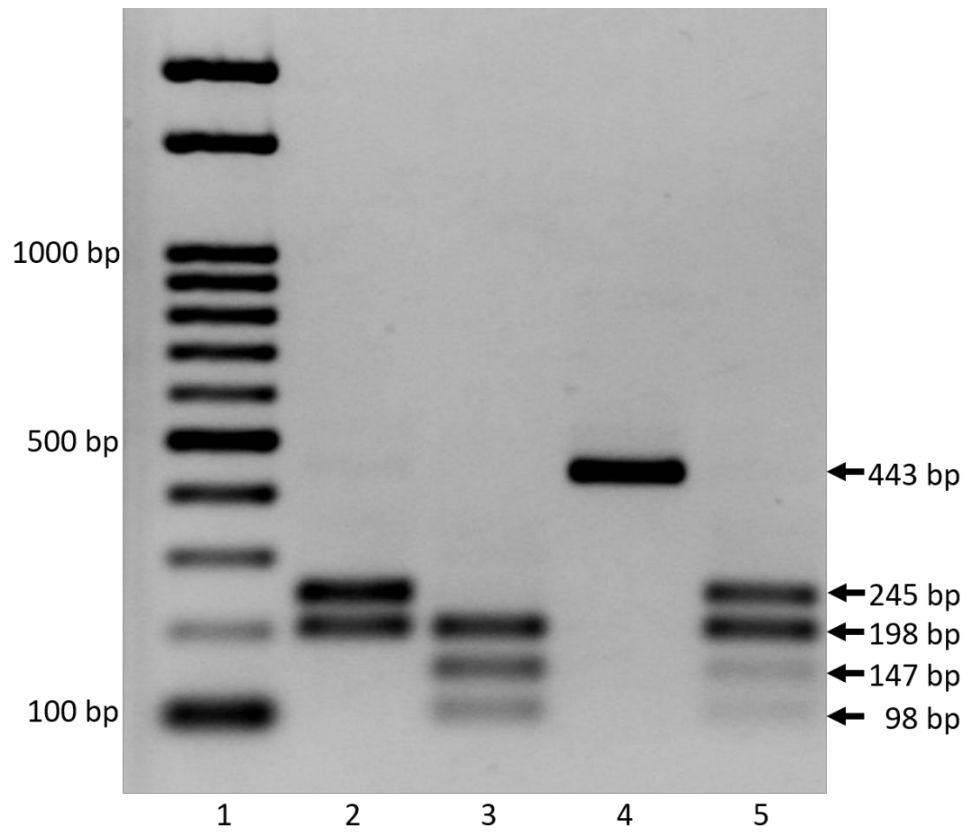
**Table 2** MtDNA and nuclear genomes of all samples used in this study<sup>†</sup>

Site no.	Prefecture	Localities (city, town, or village)	Individual no.	<i>Hae</i> III	<i>Hin</i> I	CR-RFLP	MtDNA genome	ManDra-A	ManDra-B	<i>RAG1</i> -RFLP	Nuclear genome
1	Hokkaido	Akkeshi	1-8	A	A	I	<b>A</b>	smear	ladder	245/198	<b>A</b>
			1-8	-	-	-	<b>A</b>	-	ladder	443	<b>A</b>
2		Nanae	1-3	C	E	V	<b>B1</b>	ladder	smear	198/147/98	<b>B1</b>
			4	C	E	V	<b>B1</b>	unknown	smear	198/147/98	<b>B1</b>
3		Ebetsu	1-2	C	E	V	<b>B1</b>	ladder	smear	198/147/98	<b>B1</b>
			3-7	C	E	V	<b>B1</b>	faint smear	smear	198/147/98	<b>B1</b>
			8	C	E	V	<b>B1</b>	faint smear	smear	245/198	<b>A</b>
1-8	-	-	-	<b>B1</b>	-	smear	296/147	<b>B1</b>			
4		Abashiri	1-2	A	D	IV	<b>A</b>	smear	ladder	245/198	<b>A</b>
			3-6	A	A	I	<b>A</b>	smear	ladder	245/198	<b>A</b>
			7-8	A	C	II	<b>A</b>	smear	ladder	245/198	<b>A</b>
			9-21	B	B	III	<b>A</b>	smear	smear	245/198/147/98	<b>Clone</b>
5	Akita	Kakunodate	1-8	C	E	V	<b>B1</b>	faint smear	smear	198/147/98	<b>B1</b>
			1-8	-	-	-	<b>B1</b>	-	smear	296/147	<b>B1</b>
6	Miyagi	Naruko	1-8	C	I	X	<b>A</b>	smear	ladder	245/198	<b>A</b>
			1-8	-	-	-	<b>A</b>	-	ladder	443	<b>A</b>
7	Fukui	Obama	1	C	E	V	<b>B1</b>	faint smear	unknown	245/198/147/98	<b>A×B1</b>
			2-3	C	E	V	<b>B1</b>	faint smear	smear	245/198/147/98	<b>A×B1</b>
			4-8	C	E	V	<b>B1</b>	faint smear	smear	198/147/98	<b>B1</b>
1-8	-	-	-	<b>B1</b>	-	smear	296/147	<b>B1</b>			
8	Tochigi	Nikko	1-8	C	F	VII	<b>B1 or B2</b>	faint smear	smear	443	<b>B2</b>

Table 2 continued

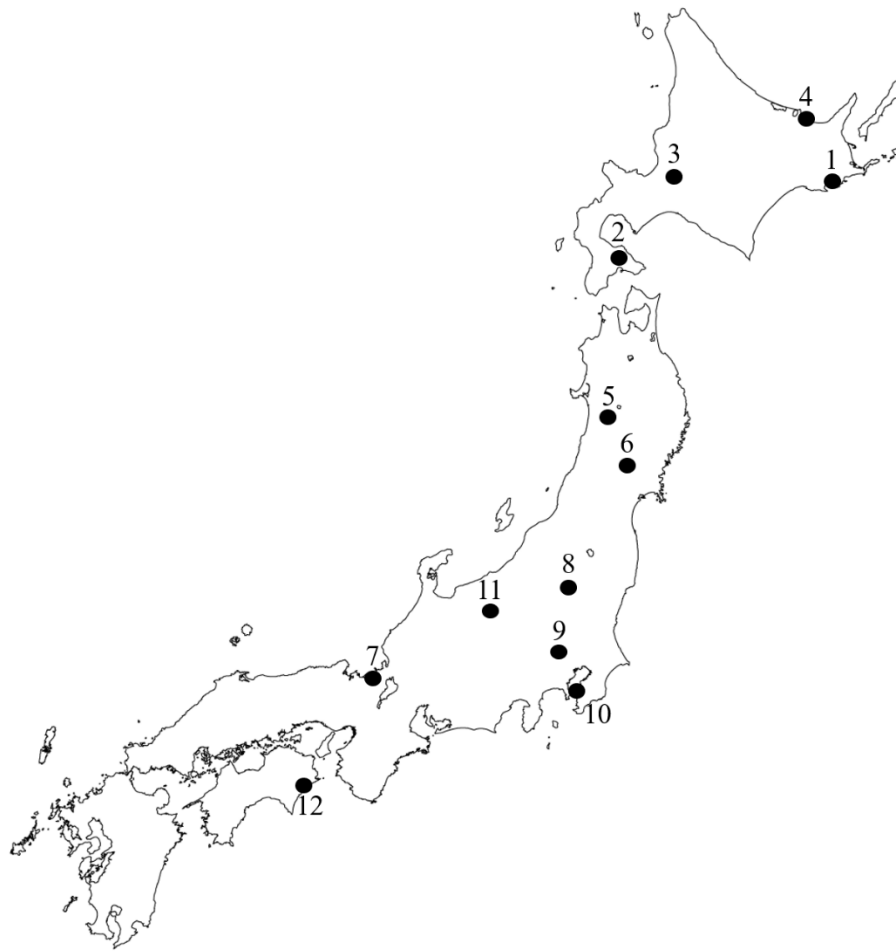
			1-8	C	F	VII	<b>B1 or B2</b>	-	smear	443	<b>B2</b>
9	Saitama	Hanyu	1-6	C	F	VII	<b>B1 or B2</b>	faint smear	smear	443	<b>B2</b>
			7	C	E	V	<b>B1</b>	faint smear	smear	443	<b>B2</b>
			8	C	G	VI	<b>B2</b>	faint smear	smear	443	<b>B2</b>
			1-6, 8	C	F	VII	<b>B1 or B2</b>	-	smear	443	<b>B2</b>
			7	C	E	V	<b>B1</b>	-	smear	443	<b>B2</b>
10	Chiba	Futtsu	1-4	C	F	VII	<b>B1 or B2</b>	faint smear	smear	443	<b>B2</b>
			5	C	E	V	<b>B1</b>	faint smear	smear	198/147/98	<b>B1</b>
			6	C	F	VII	<b>B1 or B2</b>	faint smear	smear	443/198/147/98	<b>B1×B2</b>
			7	E	F	XI	<b>A</b>	smear	ladder	245/198	<b>A</b>
			8	C	F	VII	<b>B1 or B2</b>	smear	ladder	443/245/198	<b>A×B2</b>
			1-4, 8	C	F	VII	<b>B1 or B2</b>	-	smear	443	<b>B2</b>
			5	C	E	V	<b>B1</b>	-	smear	296/147	<b>B1</b>
			6	C	F	VII	<b>B1 or B2</b>	-	smear	443/296/147	<b>B1×B2</b>
			7	E	F	XI	<b>A</b>	-	ladder	443	<b>A</b>
11	Nagano	Ueda	1-6	C	F	VII	<b>B1 or B2</b>	faint smear	smear	443	<b>B2</b>
			7	C	E	V	<b>B1</b>	faint smear	smear	443	<b>B2</b>
			8	C	F	VII	<b>B1 or B2</b>	faint smear	ladder	443	<b>B2</b>
			1-6, 8	C	F	VII	<b>B1 or B2</b>	-	smear	443	<b>B2</b>
			7	C	E	V	<b>B1</b>	-	smear	443	<b>B2</b>
12	Tokushima	Hiwasa	1-2	C	E	V	<b>B1</b>	ladder	smear	198/147/98	<b>B1</b>
			3-7	C	E	V	<b>B1</b>	faint smear	smear	198/147/98	<b>B1</b>
			8	C	E	V	<b>B1</b>	faint smear	faint smear	245/198	<b>A</b>
			1-8	-	-	-	<b>B1</b>	-	smear	296/147	<b>B1</b>

† MtDNA genome was determined using control region RFLP haplotypes (CR-RFLP). Nuclear genome was comprehensively determined from results of ManDra-A genotype, ManDra-B genotype, and improved *RAG1*-RFLP. Dashed boxes indicate previous results obtained from the same individuals including the *RAG1*-RFLP markers (standard methodology, not improved) described in Morishima et al. (2008) and Fujimoto et al. (2017).



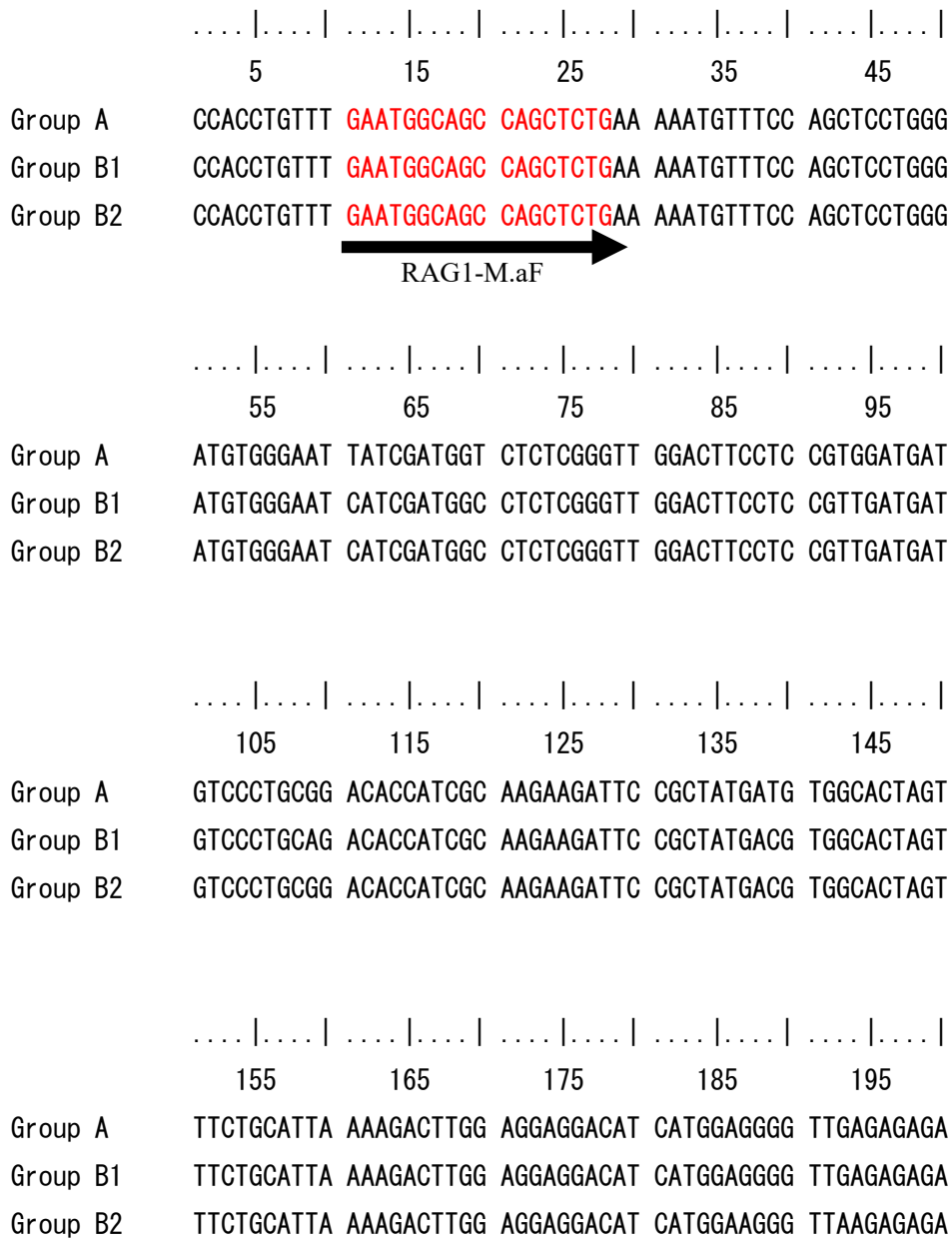
**Fig. 1**





**Supplementary Fig. S1** Sampling locations in this study

Each number (1-12) corresponds to a site no. in Table 1.



**Supplementary Fig. S2** Representative *RAG1* sequences (527 bp) of group A, B1, and B2 loaches. Sequences of group A (AB698051) contain a *StuI* restriction site (indicated by a solid line). Sequences of group B1 (AB698049) contain a *StuI* restriction site and a *PvuII* restriction site (indicated by a dotted line). Sequences of group B2 (AB698061) have no *StuI* and *PvuII* restriction sites. Thick arrows indicate the primer sequences to amplify *RAG1* sequences (443 bp) by PCR.

restriction site of *StuI*

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

205 215 225 235 245

Group A GAGGCCTGGA TGACAGCACG TGCACTTCTG GTTTTACCGT GGTGGTAAAG

Group B1 GAGGCCTGGA TGACAGCACG TGCACCTCTG GTTTTACCGT GGTGGTAAAG

Group B2 GAGGCATGGA TGACAGCACG TGCACTTCTG GTTTTACCGT GGTGGTAAAG

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

255 265 275 285 295

Group A GAATCATGTG ACGGTATGGG AGATGTTAGC GAGAAGCATG GATCTGGCCC

Group B1 GAATCATGTG ACGGTATGGG AGATGTTAGC GAGAAGCATG GATCTGGCCC

Group B2 GAATCGTGTG ACGGTATGGG AGATGTTAGC GAGAAGCATG GATCTGGCCC

restriction site of *PvuII*

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

305 315 325 335 345

Group A AGCGGTGCCT GAAAAGGCGG TAAGGTTTTC CTTACAATC ATGTCCATTT

Group B1 AGCTGTGCCT GAAAAGGCAG TAAGGTTTTC CTTACAATC ATGTCCATTT

Group B2 AGCGGTGCCT GAAAAGGCGG TAAGGTTTTC CTTACAATC ATGTCCATTT

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

355 365 375 385 395

Group A CCATTCGAGC TGAAGGTGAG GAGGACGCCA TCACGATCTT CCAAGAACAG

Group B1 CCATTAGAGC TGAAGGTGAG GAGGACGCCA TCACGATCTT CCAAGAACAG

Group B2 CCATTCGAGC TGAAGGTGAG GAGGATGCCA TCACGATCTT CCAAGAACAG

.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|.....|

405 415 425 435 445

Group A AAGCCAAACT CGGAGCTCTC CTGCAGACCT CTGTGTCTCA TGTGGTGGG

Group B1 AAGCCAAACT CGGAGCTCTC CTGCAGACCT CTGTGTCTCA TGTGGTGGG

Group B2 AAGCCAAACT CGGAGCTCTC CTGCAGACCT CTGTGTCTCA TGTGGTGGG

← RAG1-M.aR

Supplementary Fig. S2 Continued

	..... .....	..... .....	..... .....	..... .....	..... .....
	455	465	475	485	495
Group A	CGAGTCAGAC	CACGAAACCC	TCACGGCCAT	TTTGGGACCA	GTGGTGGCAG
Group B1	CGAATCAGAC	CACGAAACAC	TCACGGCCAT	CTTGGGACCA	GTGGTGGCAG
Group B2	CGAATCAGAC	CACGAAACAC	TCACGGCCAT	CTTGGGACCA	GTGGTGGCAG

	..... .....	..... .....	..... ..
	505	515	525
Group A	AGCGAAAAGC	CATGCTGGAA	AGTCGGC
Group B1	AGCGAAAAGC	CATGCTGGAA	AGTCGAC
Group B2	AGCGAAAAGC	CATGCTGGAA	AGTCGGC

**Supplementary Fig. S2** Continued