Title

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

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Abstract

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

Keywords

Clone, Hybrid, PCR-RFLP, Unisexuality

Introduction

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

Different nuclear DNA markers, ManDra (hereafter designated as ManDra-B in this paper), ManDra-A, and ManBgl, were developed from repetitive sequences isolated by digestion of genomic DNA with the restriction enzymes *Dra*I and *Bgl*II (Fujimoto et al. 2017; Kuroda et al. 2021). The DNA markers ManDra-B and ManDra-A were designed to amplify isolated repetitive sequences by PCR and were used for grouping based on the electrophoretic patterns of the PCR products. Specifically, ManDra-B yields ladder-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 (*RAG1*-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 *RAG1*-RFLP marker using two restriction enzymes, *Pvu*II and *Stu*I. 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).

Materials and Methods

Experimental animals

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

Group identification by mtDNA-CR RFLP haplotypes

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

Group identification by nuclear DNA markers ManDra-A and ManDra-B

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

5.0 μL 2×Quick Taq HS DyeMix, and 0.2 μL of each primer (10 μM). The PCR cycling conditions were as follows: initial denaturation for 3 min at 95°C, followed by 20 cycles of denaturation for 30 s at 95°C, 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 al. 2017; Kuroda et al. 2021). Group identification based on ManDra-A and ManDra-B was performed by comparing the electrophoretic patterns of the two markers (Fujimoto et al. 2017; Kuroda et al. 2021).

Improvement of *RAG1*-RFLP marker analysis

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

Results and Discussion

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

Declarations

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Conflicts of interest

The authors declare that they have no conflict of interest.

Availability of data and material

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

Code availability

Not applicable.

Author contribution statement

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

Ethics approval

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

Consent to participate

Not applicable.

Consent for publication

Not applicable.

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Table

Table 1 Sampling sites, numbers of individuals, year of sampling, and presence or absence of previous

DNA data sets of individuals for this study

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

Figure Legends

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

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

Electronic Supplementary Material

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 *Stu*I restriction site (indicated by a solid line). Sequences of group B1 (AB698049) contain a *Stu*I restriction site and a *Pvu*II restriction site (indicated by a dotted line). Sequences of group B2 (AB698061) have no *Stu*I and *Pvu*II restriction sites. Thick arrows indicate the primer sequences to amplify *RAG1* sequences (443 bp) by PCR.

Table 1 Sampling sites, number of individuals, year of sampling, and presence or absence of previous DNA data sets† 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
Total			105		

†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[†]

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

Table 2 continued

			1-8	C	F	VII	**B1 or B2**	-	smear	443	**B2**
9	Saitama	Hanyu	1-6	C	F	VII	**B1 or B2**	faint smear	smear	443	**B2**
			7	C	E	V	**B1**	faint smear	smear	443	**B2**
			8	C	G	VI	**B2**	faint smear	smear	443	**B2**
			1-6, 8	C	F	VII	**B1 or B2**	-	smear	443	**B2**
			7	C	E	V	**B1**	-	smear	443	**B2**
10	Chiba	Futtsu	1-4	C	F	VII	**B1 or B2**	faint smear	smear	443	**B2**
			5	C	E	V	**B1**	faint smear	smear	198/147/98	**B1**
			6	C	F	VII	**B1 or B2**	faint smear	smear	443/198/147/98	**B1×B2**
			7	E	F	XI	**A**	smear	ladder	245/198	**A**
			8	C	F	VII	**B1 or B2**	smear	ladder	443/245/198	**A×B2**
			1-4, 8	C	F	VII	**B1 or B2**	-	smear	443	**B2**
			5	C	E	V	**B1**	-	smear	296/147	**B1**
			6	C	F	VII	**B1 or B2**	-	smear	443/296/147	**B1xB2**
			7	E	F	XI	**A**	-	ladder	443	**A**
11	Nagano	Ueda	1-6	C	F	VII	**B1 or B2**	faint smear	smear	443	**B2**
			7	C	E	V	**B1**	faint smear	smear	443	**B2**
			8	C	F	VII	**B1 or B2**	faint smear	ladder	443	**B2**
			1-6, 8	C	F	VII	**B1 or B2**	-	smear	443	**B2**
			7	C	E	V	**B1**	-	smear	443	**B2**
12	Tokushima	Hiwasa	1-2	C	E	V	**B1**	ladder	smear	198/147/98	**B1**
			3-7	C	E	V	**B1**	faint smear	smear	198/147/98	**B1**
			8	C	E	V	**B1**	faint smear	faint smear	245/198	**A**
			1-8	-	-	-	**B1**	-	smear	296/147	**B1**

† 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).

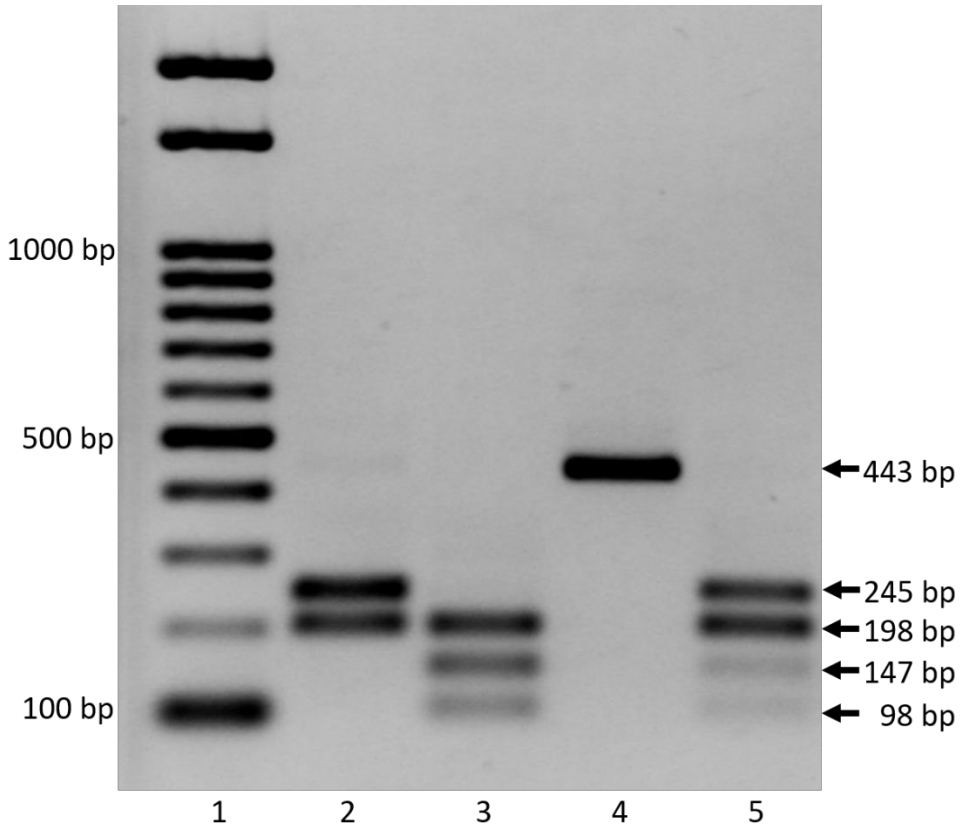
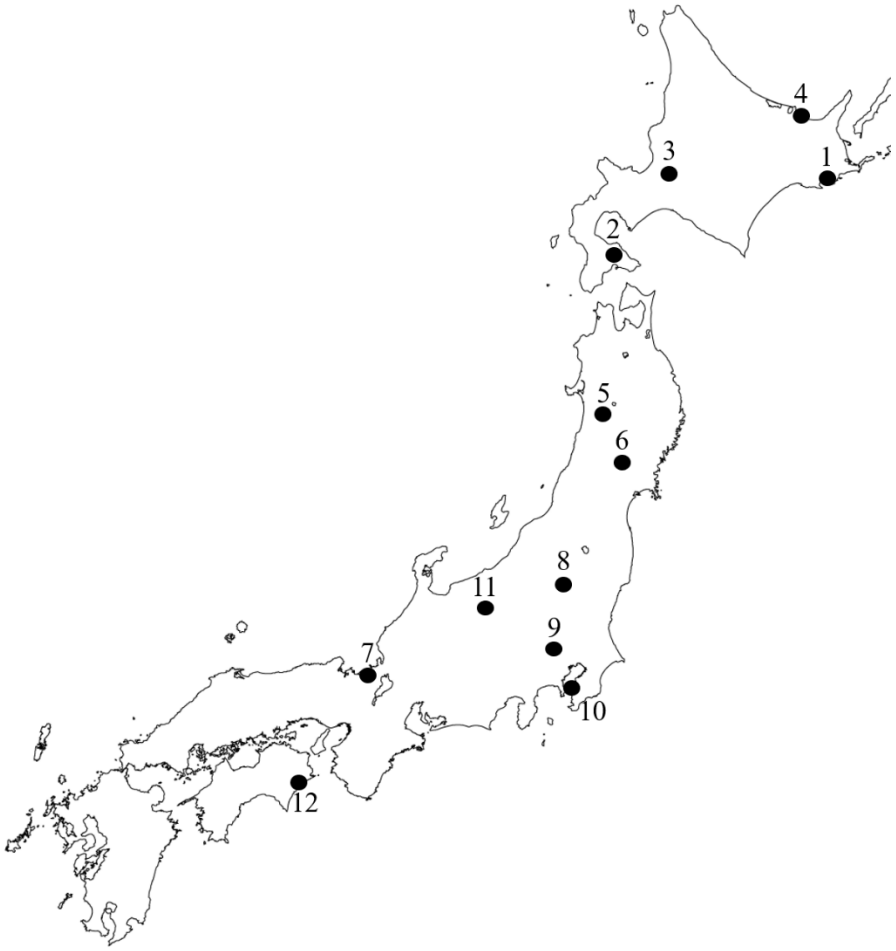
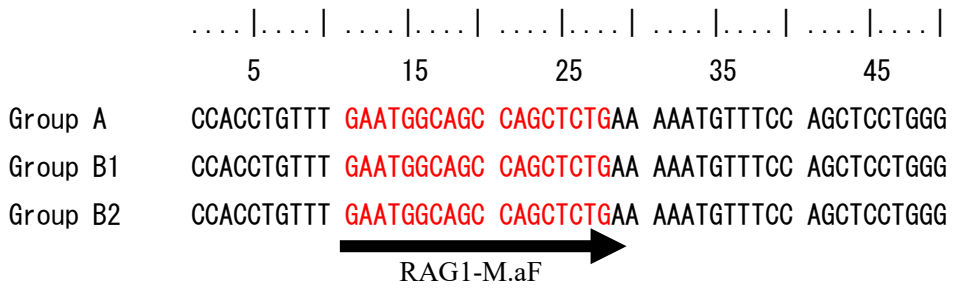


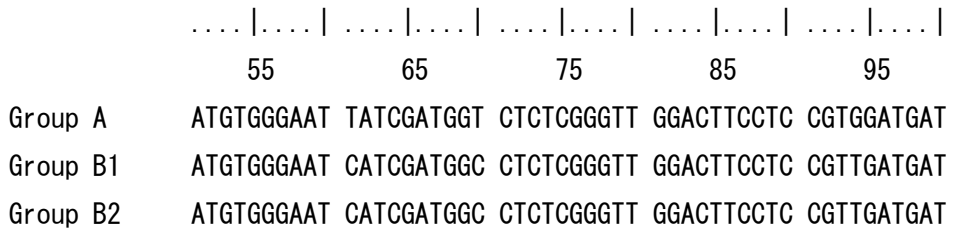
Fig. 1

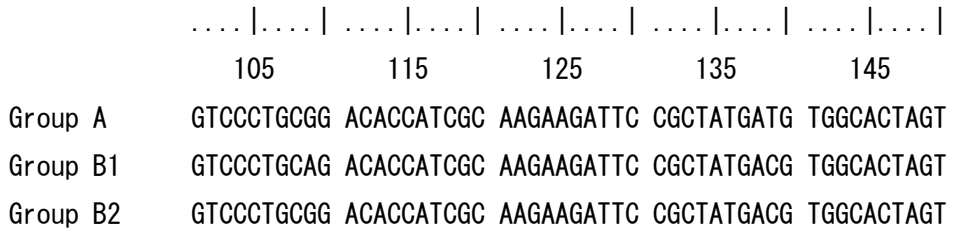


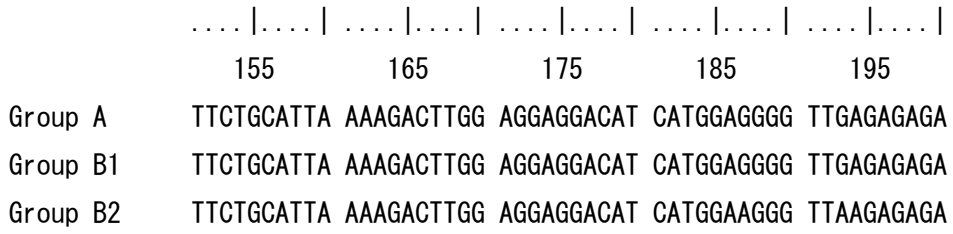
Supplementary Fig. S1 Sampling locations in this study

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

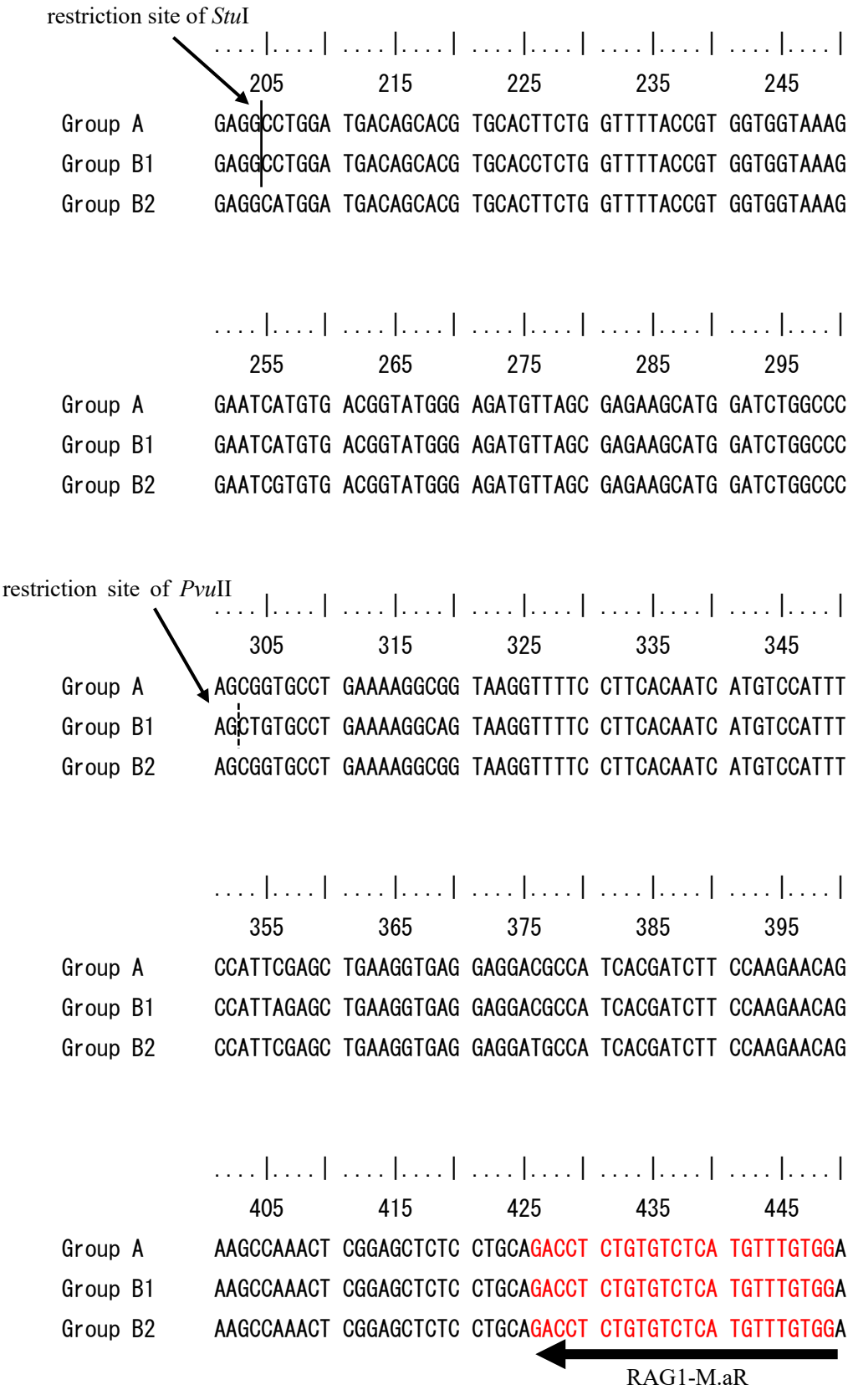








Supplementary Fig. S2 Representative *RAG1* sequences (527 bp) of group A, B1, and B2 loaches Sequences of group A (AB698051) contain a *Stu*I restriction site (indicated by a solid line). Sequences of group B1 (AB698049) contain a *Stu*I restriction site and a *Pvu*II restriction site (indicated by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



Supplementary Fig. S2 Continued

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                ....|....| ....|....| ....|....| ....|....| ....|....|
                   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        AGCGGAAAGC CATGCTGGAA AGTCGAC
Group B2        AGCGGAAAGC CATGCTGGAA AGTCGGC
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Supplementary Fig. S2 Continued