TITLE:

Development of nuclear DNA markers to characterize genetically diverse groups of *Misgurnus anguillicaudatus* and its closely related species

Takafumi Fujimoto[a]*, Aya Yamada[a], Yukihiro Kodo[b], Kohei Nakaya[b], Michiko Okubo-Murata[bc], Taiju Saito[ad], Kazuto Ninomiya[a], Michiko Inaba[a], Masamichi Kuroda[a], Katsutoshi Arai[a] and Masaru Murakami[b]

[a] Hokkaido University, Faculty and Graduate School of Fisheries Sciences, Hakodate, Hokkaido 041-8611, Japan.

[b] Azabu University, School of Veterinary Medicine, Sagamihara, Kanagawa 252-5201, Japan.

[c] (Present Address) Tokyo University of Agriculture, Faculty of Bioindustry, Abashiri, Hokkaido 099-2493, Japan.

[d] (Present Address) Ehime University, South Ehime Fisheries Research Center, Nishiura Station, Uchidomari, Ainan, Ehime 798-4206, Japan.

*Corresponding author: Takafumi Fujimoto

e-mail: fujimoto@fish.hokudai.ac.jp

Tel/Fax: +81-138-40-5536

Aya Yamada: shibakenmania@yahoo.co.jp

Yukihiro Kodo: yukihiro_1985@hotmail.com

Kohei Nakaya: sheepdog.skysail@gmail.com

Michiko Okubo-Murata: mo205725@bioindustry.nodai.ac.jp

Taiju Saito: taiju76@gmail.com

Kazuto Ninomiya: sa3b_u_rou6@yahoo.co.jp

Michiko Inaba: michiko178@eis.hokudai.ac.jp
Masamichi Kuroda:shindou19900120@yahoo.co.jp
Katsutoshi Arai: araikt@fish.hokudai.ac.jp
Masaru Murakami: murakami@azabu-u.ac.jp

Running title: Nuclear DNA markers of pond loach

Keywords: repetitive DNA sequence, RFLP, hybrid, species identification, Cobitidae

Abstract

Repetitive DNA sequences, ManDra and ManBgl, were isolated from the *Dra*I and *Bgl*II digests of the genomic DNA of the *Misgurnus anguillicaudatus*, respectively. A primer set of ManDra distinguished genetically different two groups (A and B) of *M. anguillicaudatus* by specific electrophoregrams. A primer set of ManBgl amplified the DNA of *M. anguillicaudatus* and *M. mizolepis*. The individuals of *M. anguillicaudatus* were divided into two groups depending on the fragment sizes, in which the group A and B (B-1 and B-2) showed 400 and 460 bp, respectively. *M. mizolepis* was distinguished by the different pattern (400, 460, and 510 bp fragments). PCR-RFLP analyses of *recombination activating gene 1* gave clear difference between A or B-2 (443 bp fragment) and B-1 groups (296 and 147 bp fragments). Clonal lineages and hybrids between B-1 and B-2 groups could be identified by appearance of three fragments (443, 296, and 147 bp). The combined analyses using the above three nuclear markers discriminated among nuclear genomes of genetic groups (A, B-1 and B-2) of *M. anguillicaudatus* and *M. mizolepis*. In several localities, natural hybridizations between the group B-1 and B-2 loaches and introgressions of clonal mitochondrial genomes into the group B-1 loaches were detected.

Introduction

Among wild populations of pond loach or Oriental weatherfish *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes) in Japan, bisexually reproducing gonochoristic wild-type diploid individuals (2n = 50) are most common, but clonal diploid lineages have been found in certain areas of Hokkaido and Ishikawa Prefectures [1, 2]. These clonal diploid loaches lay unreduced diploid eggs which are able to develop by gynogenesis after triggering with sperm of bisexual wild-type diploids. Bisexually reproducing gonochoristic tetraploids (4n = 100) occur together with sympatric wild-type diploids and infrequent triploids in China [3 – 5], but natural tetraploid loaches have not been so far detected in wild populations of Japan [6 – 8]. However, the occurrence of origin-unknown tetraploid loaches has been often reported in samples taken from fish markets of Japan [8 – 11]. Ploidy status and reproductive modes of Japanese pond loach *M. anguillicaudatus* are quite complicated and enigmatic.

Previous population genetic studies using allozymes (polymorphic enzymes) and sequences of the mitochondrial DNA control region (mtDNA-CR) revealed the presence of highly diversified groups A and B, in which the latter was further sub-divided to group B-1 and B-2, within wild populations of *M. anguillicaudatus* species in Japan [2, 12]. Similar conclusion was also obtained by the sequences of different region mtDNA, *cytochrome b* [13]. Genetic difference between A and B-1 groups was also indicated by microsatellite genotyping [14]. Recent genetic analyses using sequences of *recombination activating gene 1* (*RAG1*) strongly supported the presence of three groups A, B-1, and B-2 in Japanese *M. anguillicaudatus* populations and showed the simultaneous co-presence of *RAG1* sequences (alleles) from A and B-1 groups, i.e. heterozygosity, in the natural clonal loach [15]. The results based on sequences of *interphotoreceptor retinoid-binding protein 2* (*IRBP2*) also provided the same

84 conclusion [15]. These results strongly suggested the hybrid origin of clonal diploid
85 loach with atypical reproductive manner from the past hybridization event between
86 group A and B-1 as well as the existence of genetically diversified groups within *M.*
87 *anguillicaudatus* species.

88 In addition to the complex population structure of *M. anguillicaudatus* as
89 mentioned above, an exotic mud loach species *M. mizolepis* or *Paramisgurnus*
90 *dabryanus* has been often recorded in waters of Japan and such invasion makes a
91 situation much more complicated [16 – 20]. Moreover, mud loach *M. mizolepis* has
92 been considered as a synonym of large scale loach *P. dabryanus*, but it is premature to
93 conclude it due to the confusion of taxonomy and the shortage of other biological
94 information [18, 20, 21]. Thus, hereafter in the present study, we used *M. mizolepis* for
95 the exotic mud loach frequently appeared in Japan.

96 Here, firstly we tried to isolate repetitive sequences, which are generally known to
97 be useful for identifying a species and/or a population, from the *Dra*I and *Bgl*II digests
98 of the genomic DNA and then develop the genetic markers ManDra and ManBgl to
99 characterize three diverse groups (A, B-1 and B-2) of *M. anguillicaudatus*. Secondly,
100 we developed the genetic marker based on RFLP (restriction fragment length
101 polymorphism) of *RAG1* sequences to characterize different groups and clonal lineages
102 of *M. anguillicaudatus*. Finally, we have identified diverse groups of *M.*
103 *anguillicaudatus* originally defined by mtDNA haplotypes and by combining above
104 mentioned ManDra, ManBgl, and *RAG1* RFLP genetic markers in relatively large
105 numbers of Japanese loach samples (n =522) collected from 33 different sites. We also
106 tested availability of these molecular tools for species identification in specimens of
107 other cobitid species belonging to different genera and cyprinid fish.
108
109 **Materials and methods**
110

Fish specimens

A total of 522 pond loach *M. anguillicaudatus* (Cobitidae, Cypriniformes) were collected from 33 different localities from Hokkaido, Honshu, and Shikoku Islands, Japan (Table 1). A part of these samples were already analyzed for ploidy determination, mtDNA-CR haplotypes, DNA fingerprinting and sequencing of two nuclear genes *RAG1* and *IRBP2* [2, 15]. Preserved samples of the clonal lineage 1–4, which were genetically confirmed by Morishima et al. [1, 2] and Yamada et al. [15], were used as the standard reference controls in the present study. Mud loach *M. mizolepis* (n = 3) and European weatherfish *M. fossilis* (n = 5) were same to those analyzed in Morishima et al. [2]. Spinus loach *Cobitis taenia* (n = 2) were provided by the Polish scientist in 2004. Japanese spinous loach species *Cobitis biwae* (n = 3) were collected from Ashida River, Hiroshima Prefecture in 2012. Stone loach *Noemacheilus barbatulus toni* (n = 3) and Hokkaido eight barbell loach *Lefua nikkonis* (n = 3) were collected from waters in the Nanae town, Hokkaido Prefecture. Kuhlii loach *Pangio kuhlii* (n = 5) and eight barbel loach *L. echigonia* (n = 3) were commercially purchased from the local aquarium fish dealer. Goldfish *Carassius auratus* (n = 1) and common carp *Cyprinus carpio* (n = 1) samples were collected from the aquarium of the Faculty of Fisheries Sciences, Hokkaido University, Hakodate. Triploid silver crucian carp (ginbuna) *Carassius langsdorfii* (n = 1) was a rearing individual in the aquarium of the Faculty of Veterinary Medicine, Azabu University, Sagamihara.

Ploidy determination

Ploidy level of the samples was determined by nuclear DNA content measured by a flow cytometer PA or CyFlow (Partec GmbH, Münster, Germany) prior to molecular

genetic studies basically as described in Fujimoto et al.[22].

Grouping of specimens by mtDNA-CR haplotypes

DNA was extracted from tissue sample by the standard phenol/chloroform protocol [23]. The mtDNA-CR was amplified and then amplified product was analyzed to detect restriction fragment length polymorphism (RFLP), followed by the partial sequencing of the mtDNA-CR region (444-448 bp) according to the procedure of Morishima et al. [2]. Group identification of each specimen was carried out by RFLP pattern and/or 100 to 99.3% matching in the corresponding site between known 942-954 bp mtDNA-CR sequences (haplotypes) (AB306717-AB306793) and present partial 444-448 bp sequences. The genetic grouping based on mtDNA-CR was already made in parts of samples analyzed in Morishima et al. [2].

Development of the repetitive DNA sequences, ManDra marker

Preliminary screenings to digest the genomic DNA from a *M. anguillicaudatus*, which was purchased from a local aquarium shop, were conducted to find out the most appropriate endonuclease(s) among commercially available 75 restriction enzymes (data not shown) and *Dra*I was selected as a candidate. The *Dra*I digests were electrophoresed on a preparative 1.5 % agarose gel in TAE buffer and the separated bands were visualized with ethidium bromide on a UV trans-illuminator using a gel documentation system (UVP bioDoc-It^{TM} Imaging System, Cambridge, UK). The fragment of about 130 bp in size was excised and the DNA was purified by using Wizerd^{TM} SV Gel and PCR Clean-Up System (Promega), and then ligated into the *Sma*I-linealized plasmid vector pUC19. The ligated DNA was transformed into competent *E. coli* JM109. White colonies with recombinant plasmids were selected on

LB agar plates containing ampicillin, X-gal and IPTG. Selected plasmids were then purified by Wizerd™ Miniprep Resin (Promega) according to the manufacturer's instruction. The inserts were confirmed to have about 130 bp in size by agarose gel electrophoresis. Eight cloned plasmids purified with the resin were used as templates for sequencing. Both strands of each plasmid were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the automated sequencer, Genetic Analyzer 3100 (Applied Biosystems). The sequences were analyzed using GENETYX-MAC ver.15.0.4.

Procedures for the Southern blot hybridization were as follows. Genomic DNA of a *M. anguillicaudatus* sample was completely or partially cleaved with *Dra*I or *Apa*L1. The DNA fragments on agarose gels were depurinated, denatured and then neutralized. They were transferred to MagnaGraph nylon membrane (MSI) by capillary blotting and were immobilized by UV crosslinking. The DIG-labeled probe was prepared by PCR DIG Probe Synthesis Kit (Roche). Membranes were subsequently prewashed, prehybridized and hybridized with the DIG-labeled probe using Dig Easy Hyb (Roche) under conditions recommended by the manufacturer. Membranes were washed, blocked and antibody-conjugated by using DIG Wash and Block Buffer (Roche) and Anti-Digoxgenin-AP (Roche). Chemiluminescent detection of hybridized DNA was performed by CDP-*Star* Ready to Use (Roche) according to the manufacturer's protocol. The emitted light was recorded on X-ray film.

Based on sequences obtained (see the Result section), a primer set to amplify ManDra sequences was designed by using Oligo ver. 6.8 and AMPLIFY ver. 3.1, 4/3X. The PCRs were performed with 100 ng of genomic template DNA, 200 μM each of dNTPs, 0.021 U r*Taq* polymerase (TaKaRa) and 0.4 μM each of primers: ManDra-F 5'-TGTTTCATCCTTAGAATGCC-3' and ManDra-R 5'-CCAGCTCAGAAAAGCAGTTTAG-3' (Table 2). The cycling conditions were as follows: initial denaturation 3 min at 95 °C, 20 cycles of denaturation 30 s at 95 °C,

annealing 30 s at 50 °C, extension 30 s at 72 °C and then a 5 min extension at 72 °C. The products were electrophoresed and stained with ethidium bromide.

Development of the repetitive DNA sequences, ManBgl marker

The genomic DNA from a *M. anguillicaudatus* was completely digested with the selected *Bgl*II. The digests were fractionated on a preparative 1.5 % agarose gel in TAE buffer and were visualized with ethidium bromide. A fragment of 550 bp in length was excised and ligated into the *Bam* HI-linearized plasmid vector pUC19. The ligation and transformation were performed as described above for ManDra marker. Recombinant plasmids were verified by size and were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the automated sequencer, Genetic Analyzer 3100 (Applied Biosystems).

The Southern blot hybridization was performed as follows. Genomic DNA samples were separately cleaved to completion with *Bgl*II at 1 U /μg DNA, fractionated on 1.5% agarose gels and transferred to BIODYNE PLUS MEMBRANE (Pall BioSupport Division). DIG-labeled probe was made with PCR DIG Probe Synthesis Kit (Roche) and hybridization was performed at 42 °C for overnight. The labeled ManBgl-550-18 (see the Result section) was employed as a probe in this study. Chemiluminescent hybridization signals were detected with DIG Luminescent Detection Kit (Roche).

The PCRs were performed with 100 ng of genomic template DNA, 200 μM each of dNTPs, 0.021 U r*Taq* polymerase (TaKaRa) and 0.4 μM each of primers: 5' TCTKAKCATAGGCARCAATA 3' and 5' CTKTCAAAACWCAAAGACAC 3' was designed to amplify all ManBgl-550 sequences (Table 2). The cycling conditions were as follows: initial denaturation 3 min at 95 °C, 30 cycles of annealing 30 s at 51 °C, extension 45 s at 72 °C, denaturation 30 s at 95 °C and then a 5 min extension at 72 °C.

The products were electrophoresed and stained with ethidium bromide.

Development of the *RAG1*-RFLP marker

In the sequence (527bp) of *RAG1* gene determined in the clonal individual and the wild-type individuals of *M. anguillicaudatus* [15], restriction enzyme was selected to detect *M. anguillicaudatus* specimens belonging to genetically diverse groups and clonal lineages by using TaKaRa Cut-site Navigator (TaKaRa). Primer sets to amplify the *RAG1* gene region (443 bp) common to group A, B1 and B2 group of *M. anguillicaudates* were designed as RAG1-M.aF, 5'-GTTTGAATGGCAGCCAGCTCTG-3', and RAG1-M.aR, 5'-CCACAAACATGAGACACAGAGGTC-3' (Table 2). PCR was performed with a mixture containing 2.0 μl of the DNA sample, 12.3μl of DDW, 1.6μl of dNTP mixture (TaKaRa), 2.0 μl of 10 x PCR buffer (TaKaRa), 0.1μl of *rTaq* polymerase (TaKaRa), 1.5 μl of 10μM of RAG1-M.aF primer and 1.5μl of 10μM of RAG1-M.aR primer. The PCR conditions were as follows: initial denaturation 3 min at 93 °C, followed by 35 cycles of profile of 1 min at 93 °C, 1 min at 60 °C and 1 min at 72°C. The reaction was completed by a final extension at 72 °C for 7 min. Five μl of the PCR products was incubated in a mixture including 1 μl of *Pvu* II (TaKaRa), 1μl of 10 x M buffer and 3μl of DDW in a 0.2 ml micro tube at 37 °C for 4 h. About 4μl of digested sample mixed with 1μl of loading buffer was electrophoresed on 1.5% agarose gel for 40 min at 100 V and stained with ethidium bromide and photographed on a UV trans-illuminator using a gel documentation system (UVP bioDoc-It^{TM} Imaging System, Cambridge, UK).

Identification of genetically diverse groups and species of loach

Based on present results and previous analyses [15] of mtDNA-CR haplotype and

nuclear markers, samples of diploid wild-type loaches belonging to the group A (Ozora, Hokkaido, site no. 1, Table 1), B-1 (Notojima-Suso, Nanao, Ishikawa, site no. 21, Table 1) and B-2 (Ueda, Nagano, site no. 17, Table 1) were selected to demonstrate as the representative samples of each genetic group to examine the presence or absence of fragment(s) of above-mentioned nuclear DNA markers specific to the genetic groups. Preserved samples of clone 1 to 4 lineages including nuclear genomes of both A and B-1 loach [15] were also used as the reference standard of clonal loach to reveal the presence or absence of fragment(s) of the nuclear DNA markers characterizing the clonal lineages. In addition, samples of putative heterozygotes between B-1 and B-2 groups (Futtsu, Chiba, site no. 16, Table 1) were also used as representative samples.

Different species of the genus *Misgurnus* were also used to examine the presence of fragment(s) of the DNA markers specific to each species for developing species identification methods. Similar examinations were conducted in samples from other cobitid species belonging to the different genera such as *Cobitis*, *Noemacheilus*, *Lefua* and *Pangio* as well as cyprinid species such as goldfish, common carp and silver crucian carp.

In all the *M. anguillicaudatus* specimens (Table 1), ploidy status and haplotypes based on RFLP of mtDNA-CR or partial sequencing of mtDNA-CR (444--448 bp) were examined. Then, electrophoretic detections of the PCR products by ManDra and ManBgl sequences were carried out. RFLP analyses of PCR products of *RAG1* gene were followed. Combining all the results obtained by mtDNA-CR haplotyping as well as nuclear ManDra, ManBgl, and *RAG1*-RFLP genotyping, diversification of genetic groups was depicted in Japanese *M. anguillicaudatus*. Moreover, molecular methods to identify *M. anguillicaudatus* groups and other *Misgurnus* species were provided.

Results

ManDra marker

When the genomic DNA of the *M. anguillicaudatus* was digested with *Dra*I and the fragments electrophoresed, a discrete band of about 130 bp in size was observed (Fig. 1). The prominent fragment was cloned and the recombinants were identified by the size. Two types of nucleotide sequences, which appeared most frequently, were determined in eight colonies and then designated as ManDra (Fig.2). ManDra sequences were deposited in DDBJ/ GenBank/ EMBL under the accession numbers of LC149871. BLAST search of these sequences did not match any known sequences previously deposited in the NCBI DNA databases. The highest matching (about 72 %) was seen with Cal3nDr sequences from *Dra*I digests of triploid silver crucian carp [24]. Based on the ManDra sequences, we designed a primer set (ManDra-F and ManDra-R, amplified product 119 bp length, Table 2).

The ManDra sequences comprised the *Apa*LI cleaving site as shown in Fig. 2. When the genomic DNA sample of the *M. anguillicaudatus* was digested by *Apa*L1, a satellite band with approximately 130 bp in size was detected as in the above-mentioned case of *Dra*I digestion (Fig. 3a). Southern blot hybridization analysis in partially *Dra*I digested genomic DNA of a loach revealed ladder-like signals (Fig. 3b), thus suggesting the presence of tandem repetitive ManDra sequence in the genome. Even after complete digestion by *Dra*I, ladder-like signals appeared. The results indicated the presence of repetitive sequences which lacked the *Dra*I recognition site in ManDra sequences. No signals were detected in DNA samples of *Noemachelius barbatulus toni*, *Lefua echigonia, L. nikkonis,* goldfish, carp and spinous loach, *Cobitis biwae* by the present Southern blot hybridization (figure not shown).

When the PCRs were performed in samples from the group A of *M. anguillicaudatus*, a ladder-like electrophoretic pattern, which included fragments with 119bp then with an interval of approximately 130 bp, was clearly detected (Fig. 4).

While other representative samples of B-1 and B-2 groups always exhibited a smear-like pattern (Fig. 4). The sample of clone loach also gave the smear-like pattern (Fig. 4). There is no difference in ManDra profiles between clonal diploid individuals and clone-derived triploid individuals (figure not shown). On the other hand, similar fragment patterns were not seen in different loaches *M. mizolepis*, *M. fossilis*, *C. taenia*, *C. biwae*, and *L. nikkonis* under the present PCR conditions. However, an increase of PCR cycles to 25 - 30 often amplified weak ladder-like patterns. Weak fragments were seen in *N. b. toni*, but profile was different form that in *Misgurnus* (Fig. 4). No fragments appeared in goldfish, but weak bands appeared in common carp samples (Fig. 4). No PCR amplification was also confirmed in triploid silver crucian carp by a ManDra primer set (figure not shown).

ManBgl marker

When the genomic DNA of the *M. anguillicaudatus* was digested with *Bgl*II and the fragments were electrophoresed, a discrete band of about 550 bp in size was observed (Fig. 5). The prominent fragment of 550 bp was cloned and the recombinants were identified by the size. The complete nucleotide sequences, which we designated as ManBgl-550, of the inserts from a *M. anguillicaudatus* were determined (Fig. 6). ManBgl consensus sequences were deposited in DDBJ/ GenBank/ EMBL under the accession numbers of LC149872. BLAST search of these sequences did not match any known sequences previously deposited in the NCBI DNA databases.

To examine the genomic organization of ManBgl-550 repetitive DNA sequences, the genomic DNA of *M. anguillicaudatus* was digested with *Bgl*II and hybridized to a probe (Fig. 7, lane 1, 2 and 4). The digests exhibited two strongly hybridizing bands of about 490 and 550 bp, as well as several minor bands with larger sizes. A band of 550 bp was exhibited in the group B-1 and B-2 *M. anguillicaudatus* individuals, but a band of 490

bp was exhibited in the group A. All of the *M. mizolepis* showed the bands of both 490 and 550 bp together with minor bands with larger sizes (Fig. 7, lane 3). No bands were exhibited in other loaches examined: *C. biwae*, *P. kuhlii*, *N. b. toni*, *L. echigonia*, and *L. nikkonis* (figure not shown).

When the PCRs were performed to *M. anguillicaudatus*, *M. mizolepis*, *M. fossilis*, *C. taenia*, *C. biwae*, C. striata, *N. b. toni*, *L. nikkonis*, goldfish and common carp samples, the PCR bands were only detected in the individuals of *M. anguillicaudatus* and *M. mizolepis* according to our expectation (Fig. 8). No PCR products appeared in European weather fish *M. fossilis* and other cobitid and cyprinid species.

Two patterns were detected. The first pattern had prominent about 400 bp bands, while the second pattern had about 460 bp band (Fig. 8). The first and second patterns were shown in the group A and B (B-1 and B-2) samples of *M. anguillicaudatus*, respectively. The PCR band was 460 bp in size referred to ManBgl-550 sequences. Thus, the PCR band of 400 bp in size seemed to correspond to the southern blot hybridization band of 490 bp in size (Fig. 7). Thus, the group A of the *M. anguillicaudatus* could be easily distinguished by the presence of characteristic 400 bp band specific to the group A, while the group B of the *M. anguillicaudatus* by the absence of 400 bp band and the presence of 460 bp band specific to the group B (B-1 and B-2). Samples belonging to clonal lineages (1 to 4) gave intermediate electrophoretic patterns including both 400 bp and 460 bp bands, suggesting heterozygous state between two groups of the *M. anguillicaudatus*. On the other hand, the *M. mizolepis* samples showed characteristic pattern comprising about 400, 460, and 510 bp bands (Fig. 8). Thus, this exotic species could be identified by such specific electrophoretic pattern of ManBgl marker.

RAG1-RFLP marker

After the PCR amplification for *RAG1* gene region and its subsequent digestion with

Pvu II, samples of the group A and B-2 loach exhibited one fragment with 443 bp in size, while samples of the group B-1 loach showed two fragments with 147bp and 296 bp in size (Fig.9). Thus, group A or B-2 *M. anguillicaudatus* samples were clearly distinguishable from group B-1 samples by the present *RAG1*-RFLP analysis. Standard reference samples of clones exhibited the electrophoretic pattern comprising three fragments, 443 bp from the group A and 147 bp and 296 bp from the group B-1 loach (Fig. 9). By the occurrence of such three fragments, clonal individuals between group A and B-1 and inter-group hybrids between group B-1 and B-2 were discovered simply as well as clearly. Only one fragment with 443bp appeared in other *Misgurnus* loaches (*M. mizolepis* and *M. fossilis*). *Cobitis* samples exhibited one fragment with 443 bp length like *Misgurnus* loaches, but *Lefua* and *Noemacheilus* samples gave no amplification (Fig. 9). However, carp and goldfish amplified weak fragment with 443 bp and other minor fragments with larger sizes (Fig. 9).

Nuclear genomic constitutions in Japanese *M. anguilicaudatus* and molecular species identification

As mentioned in foregoing sections, nuclear genome group of each *M. anguillicaudatus* individual could be identified by genotyping of ManDra, ManBgl, and *RAG1*-RFLP markers and the electrophoretic patterns were summarized in Table 3. Samples with ladder-like electrophoretic pattern of ManDra, specific 400 bp ManBgl fragment, and specific 443 bp *RAG1*-RFLP band were identified as individuals with the group A nuclear genome. Samples with smear-like pattern of ManDra and specific 460 bp ManBgl band, but without 400 bp ManBgl band, were identified as individuals with the group B nuclear genome. Among such samples, group B-1 and B-2 genomes could be distinguished by two *RAG1* fragments (296 and 147 bp) and single *RAG1* fragment (443 bp) after RFLP, respectively. Natural clonal individuals were easily identified by sharing

both 400 and 460 bp ManBgl fragments and specific three-banded *RAG1*-RFLP pattern with 443, 296, and 147 bp fragments, which indicated heterozygous genomic state between group A and B-1. Smear-like pattern of ManDra was the intermediate result between ladder-like and smear-like patterns, in which higher numbers of repeats were presumably existed in smear-like patters of the group B. Heterozygous nuclear genomes including both group B-1 and B-2 could also be identified by the presence of group B specific 460bp ManBgl band (or the absence of group A specific 400bp ManBgl band) and the presence of three-banded pattern of *RAG1*-RFLP.

Exotic species *M. mizolepis* could be identified by the presence of species-specific ManBgl pattern comprising prominent 400, 460, and 510 bp bands. European weatherfish *M. fossilis* did not amplify ManBgl marker.

Population structure of Japanese *M. anguillicaudatus* based on nuclear genomes and mtDNA haplotypes

Nuclear genotypes and cytoplasmic mtDNA haplotypes of Japanese *M. anguillicaudatus* of each sampling site were individually shown in Table 4 (Fig. S1, Table S1). In most samples, nuclear genome and mtDNA haplotype were identified by ManDra, ManBGL, *RAG1*-RFLP and mtDNA-CR analyses, but nuclear genome was not precisely identified in a few samples collected from site nos. 3, 13, 18, 19, 20, 22 and 23. MtDNA-CR haplotype was also unknown in small number of samples from site nos. 13 and 24.

Samples which exhibited group A specific nucleus and mtDNA were only found in site nos. 1, 5 (Hokkaido Island), 11 and 16 (Honshu Island). However, individuals with group A specific nucleus and mtDNA were a few in the site no. 16. Among samples from site no. 1, about half had both nucleus and mtDNA specific to the group A, but others were identified as natural clones with heterozygous nuclear genomes from

both group A and B-1 and group A-specific mtDNA haplotype. Clonal individuals with the same genetic constitution also appeared in site no. 20 in Ishikawa Prefecture, Honshu Island, but the frequency of clonal diploids was much lower when compared with clones in site no. 1 in Hokkaido Prefecture. Triploid individuals in these sites also had the clone-origin nuclear (A/ B-1 heterozygous genome) and cytoplasmic (group A haplotype) composition (Table S1). In Ishikawa Prefecture (site nos. 19, 20, 23), individuals with nucleus specific to the group B-1 and mtDNA specific to the group A appeared. In these sites, minor numbers of heterozygous individuals with both B-1 and B-2 specific nucleus and the group A specific mtDNA also appeared.

In samples from Hokkaido and northern part of Honshu Island (site nos. 2, 3, 4, 6, 8, 9 and 10), both nuclear and cytoplasmic genomes were the group B-1 type. Similar situation was found in samples from western part of Honshu Island (site nos. 25, 27, 29, 30) and Shikoku Island (site no. 33). A small number of samples with group B-1 specific mtDNA from site nos 7, 18, 21, 22 and 28 had heterozygous B-1/ B-2 nucleus. In site no. 31, most samples had cytoplasmic mtDNA specific to the group B-1, but their nuclear genomes were changed to the group B-2 or B-1/ B-2 type. In site no. 32, more than half individuals with the group B-1 specifc mtDNA possessed heterozygous B-1/ B-2 nuclear genome. In site no. 20 of Ishikawa Prefecture, a very few number of samples with the clonal nucleus of heterozygous A/ B-1 and cytoplasmic B-1 specific mtDNA appeared.

In central part of Honshu Island (site nos. 12, 13, 14, 15, 16, 17), most samples had both nucleus and mtDNA specific to the group B-2, but small number of samples with mtDNA characteristic to the group B-2 showed B1/ B2 heterozygous nuclear genome. A small number of samples with heterozygous B1/ B2 nucleus and the group B2 specific mtDNA also appeared in site no. 24 in Ishikawa Prefecture. In site no. 26, samples with the group B-1 specific nucleus and the group B-2 specific mtDNA appeared in relatively high rate.

Discussion

The presence of genetically diverse three groups A, B-1 and B-2 was clearly recognized within Japanese pond loach *M. anguillicaudatus* populations by mtDNA-CR haplotypes [2], nuclear genotypes of *RAG1* [15] and other markers [12 - 14]. In the previous study, we strongly suggested the hybrid origin of gynogenetically reproducing clonal diploids by their heterozygosity due to past hybridization event between the group A and B-1 in the nuclear *RAG1* and *IRBP2* loci [15]. However, previous approaches required complicated procedures including cloning and sequencing when two or more doubled-peaks sites were detected in the sequences of the above two loci [15]. In addition, clonal individuals had to be genetically verified by microsatellite genotypes and RAPD-fingerprinting [15]. Therefore, here we reported easier and simpler molecular markers to distinguish genetic groups within *M. anguillicaudatus* as well as to identify clonal diploid individuals.

ManDra marker (PCR primer set) developed here gave different electrophoretic profile between the group A and B-1 (and B-2) loaches. The different profiles such as the ladder-like in the group A and the smear-like in the group B are presumably explained by the richness of repeats between them. The appearance of the smear-type electrophoregram of the clonal diploid samples was interpreted as intermediate heterozygous state between low number of repeats in the group A genome and higher number of repeats in the group B genome. The exact cause responsible for the difference in electrophoretic profile between genetic groups should be clarified by estimating number of repeats by comparative Southern blot hybridization and/or real-time PCR in near future.

Similar repetitive sequences of Cal3nDr was isolated from *Dra*I digests as polyploid-specific ones from the genomic DNA of a gynogenetically reproducing

triploid silver crucian carp [24]. ManDra sequences could not distinguish triploid individuals from other diploid *M. anguillicaudatus* like Cal3nDr in crucian carp. However, the isolation of *Dra*I digested repetitive sequences with approximately 130 bp monomer in size from both crucian carp and pond loach is very interesting from the viewpoints of similarity of reproductive manner, because both fish species include gynogenetically reproducing lineages and diploid-polyploid complexes. Further comparative molecular cytogenetic studies are required so as to clarify exact position and organization on their chromosomes by fluorescence *in situ* hybridization.

Considering the results from the Southern blot hybridization, the genomic organization of ManBgl-550 sequences was not a simple tandem array of monomer, because the hybridization band patterns were not like a ladder. The repetitive sequences were not detected in *M. fossilis* and other cobitid and cyprinid samples, but *M. anguillicaudatus* and *M. mizolepis*. Furthermore, nuclear genomes of two different genetic groups A and B could be identified by the presence or absence of 400 bp fragments in *M. anguillicaudatus*. Presence of both 400 bp and 460 bp frangments suggested clonal lineage with the hybrid origin between A and B groups. Exotic species *M. mizolepis* was also able to detect by specific electrophoregrams by this nuclear marker. These results indicated that ManBgl is the excellent marker not only to distinguish two diverse genetic groups of *M. anguillicaudatus*, but also to find candidates of clonal diploids. This ManBgl marker is also useful to identify exotic *M. mizolepis* from indigenous *M. anguillicaudatus* populations by the specific electrophoretic profile.

RAG1-RFLP marker was quite effective to find clonal individuals with both group A and B-1 specific nucleus and hybrid individuals with both group B-1 and B-2 specific nucleus. Although this marker cannot distinguish group A and B-2 specific nuclear genome, these two groups can be identified by using other markers such as ManBgl and ManDra.

Analyses using above mentioned ManDra, ManBgl and *RAG1*-RFLP markers on samples which were grouped by mtDNA haplotypes revealed general distribution of three groups loach as follows: (1) most individuals in eastern part of Hokkaido and one site in Tohoku area were members of the group A, (2) almost all individuals in central part of Hokkaido Island, Tohoku and western area of Honshu Island, and Shikoku Island were categorized to the group B-1, (3) most samples from Central area of Honshu Island had nucleus and mtDNA of the group B-2, (4) clonal diploids and clone-derived triploids with heterozygous A/ B-1 genomes and A group specific mtDNA exclusively appeared in eastern part of Hokkaido Island and Notojima-Hannoura, Nanao in Ishikawa Prefecture, Honshu Island, (5) different nucleo-cytoplasmic combinations between nucleus genome and mtDNA such as B-1 nucleus and A mtDNA, heterozygous B-1/ B-2 nucleus and A mtDNA, heterozygous B-1/ B-2 nucleus and B-1 mtDNA, B-1 nucleus and B-2 mtDNA, and B-2 nucleus and B-1 mtDNA were detected in different frequencies in several sites (Table 4, Fig. S1, Table S1).

Clonal lineages in eastern Hokkaido area are considered to be stably maintained, because sperm is provided to trigger gynogenetic development of clonal females from bisexually reproducing wild-type diploids with the group A-specific nucleus and mtDNA [1, 2, 25]. Triploids frequently appear by incorporation of sperm nucleus of diploid wild-type [25]. Resultant males are sterile, while resultant triploid females produce fertile haploid eggs only with the group-A specific nucleus by the atypical reproductive system, meiotic hybridogenesis [11, 26]. In contrast, clonal lineages in Ishikawa Prefecture were being unstably maintained and frequencies were lower than those in eastern area of Hokkaido Prefecture. This may be explained by sympatric distribution of the group B-1 wild-type. As already concluded in Yamada et al.[15], the individuals with group B-1 specific nucleus and clone specific A group mtDNA appeared and these loaches are presumably produced by the introgression of clonal mtDNA via meiotic hybridogenesis of clone-origin triploid individuals, which appeared

by incorporation of nucleus of the group B-1 of sympatric wild-type diploid. Such triploid females with clone-specific A type mtDNA included one set of the group A genome and two sets of the group B-1 genomes should produce fertile haploid gametes exclusively with the group B-1 nucleus by eliminating non-homologous A genome during oogenesis, i.e. meiotic hybridogenesis [26]. Such diploid loaches with the group B-1 nucleus and the group A mtDNA may produce progeny with heterozygous B-1/ B-2 nucleus by hybridization with wild-type diploid males with the group B-2 nucleus.

Other prominent nucleo-cytoplasmic combination was the loach with B-2 or B-1/ B-2 nucleus and B-1 mtDNA observed in Tottori and Shimane Prefecture. Such situation could be arisen by hybridization between females with the group B-1 nucleus and B-1 mtDNA and males with the group B-2 nucleus. Different types of hybrids between the group B-2 females and the group B-1 males were mainly seen in Gifu Prefecture. A small number of transient types including heterozygous B-1/ B-2 nucleus and B-2 mtDNA presumably from hybridization with the group B-1 males were observed in Niigata, Tochigi, Saitama, Chiba and Ishikawa Prefectures. The occurrence of these presumable inter-group hybrids may be caused by the invasion of *M. anguillicaudatus* loaches with B-2 nucleus and B-2 mtDNA, which may be genetically close to Continental strains [11], to the indigenous populations of the group B-1 loaches.

Acknowledgments

This work was supported in part by JSPS KAKENHI Grant Numbers 21380114 and 15H02457. We thank the staff of the Ishikawa Prefecture Fisheries Research Center for sampling loaches in the Ishikawa Prefecture. We also thank Dr. H Matsubara, Faculty of Bio-industry, Tokyo University of Agriculture, for sampling loaches in the eastern part of Hokkaido Prefecture.

References

[1] Morishima K, Horie S, Yamaha E, Arai K (2002) A cryptic clonal line of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) evidenced by induced gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus DNA fingerprinting. Zool Sci 19: 565-575.

[2] Morishima K, Nakamura-Shiokawa Y, Bando E, Li YJ, Boron A, Khan MMR, Arai K (2008) Cryptic clonal lineages and genetic diversity in the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) inferred from nuclear and mitochondrial DNA analyses. Genetica 132:159-171.

[3] Li YJ, Yin J, Wang JB, Yuan X, Wei J, Sun XW, Arai K (2008) A study on distribution of polyploid loaches in China. Nippon Suisan Gakkaishi 74: 177-182.

[4] Li YJ, Tian Y, Zhang MZ, Tian PP, Yu Z, Abe S, Arai K (2010) Chromosome banding and FISH with rDNA probe in the diploid and tetraploid loach *Misgurnus anguillicaudatus*. Ichthyol Res 57: 358-366.

[5] Li YJ, Zhang MS, Qin C, Gao M, Arai K (2012) Fertility and ploidy of gametes of diploid, triploid and tetraploid loaches, *Misgurnus anguillicaudatus*, in China. J Appl Ichthyol 28: 900-905.

[6] Zhang Q, Arai K (1999) Distribution and reproductive capacity of natural triploid individuals and occurrence of unreduced eggs as a cause of polyploidization in the loach, *Misgurnus anguillicaudatus*. Ichthyol Res 46: 153-161.

[7] Arai K (2003) Genetics of the loach *Misgurnus anguillicaudatus*: recent progress and perspectives. Folia Biol (Krakow) 51 Suppl: 107-117.

[8] Arai K, Fujimoto T (2013) Genome constitution and atypical reproduction in polyploid and unisexual lineages of the *Misgurnus* loach, a teleost fish. Cytogenet Genome Res 140: 226-240.

[9] Ojima Y, Takai A (1979) The occurrence of spontaneous polyploidy in the Japanese

570 common loach, *Misgurnus anguillicaudatus*. Proc Japan Acad 55B: 487-491.
571 [10] Arai K, Matsubara K, Suzuki R (1991) Karyotype and erythrocyte size of
572 spontaneous tetraploidy and triploidy in the loach *Misgurnus anguillicaudatus*.
573 Nippon Suisan Gakkaishi 57: 2167-2172.
574 [11] Zhao Y, Toda M, Hou J, Aso M, Arai K (2012) The occurrence of hyper tetraploid
575 and other unusual polyploid loaches *Misgurnus anguillicaudatus* among market
576 specimens in Japan. Fish Sci 78: 1219-1227.
577 [12] Khan MMR, Arai K. 2000. Allozyme variation and genetic differentiation in the
578 loach, *Misgurnus anguillicaudatus.* Fish Sci 66: 211-222.
579 [13] Koizumi N, Takemura T, Watabe K, Mori A (2009) Genetic variation and diversity
580 of Japanese loach inferred from mitochondri-al DNA—phylogenetic analysis using
581 the cytochrome *b* gene sequence. Trans. Jap. Soc. Irrig. Drain. Rural Engin. 77,
582 7-16.
583 [14] Arias-Rodriguez L, Morishima K, Arai K (2006) Genetically diversified
584 populations in the loach *Misgurnus anguillicaudatus* inferred from newly
585 developed microsatellite markers. Mol Ecol Notes 7: 82-85.
586 [15] Yamada A, Kodo Y, Murakami M, Kuroda M, Aoki T, Fujimoto T, Arai K (2015)
587 Hybrid origin of gynogenetic clones and the introgression of their mitochondrial
588 genome into sexual diploids through meiotic hybridization in the loach, *Misgurnus*
589 *anguillicaudatus*. J Exp Zool 323A: 593-606.
590 [16] Kanou K, Saito S, Fuchigami S, Imamura A, Imai H, Taki Y (2007) Occurrence
591 patterns and food habits of introduced alien loach *Paramisgurnus dabryanus* and
592 native loach *Misgurnus anguillicaudatus* at irrigation drainages around rice fields
593 in the Watarase river system, central Honshu, Japan. Aquaculture Sci 55: 109-114.
594 [17] Shimizu T, Takagi M (2010) Genetic structure of natural populations of *Misgurnus*
595 *anguillicaudatus* in Ehime Prefecture impacted by recent exotic introductions,
596 inferred from mitochondrial DNA analysis. Japan J Ichthyol 57: 13-26.

597 [18] Shimizu T, Takagi M (2010) Two genetic clades in populations of *Paramisgurnus*
598 *dabryanus*, an exotic invader in Ehime Prefecture. Japan J Ichthyol 57: 125-134.
599 [19] Takagi M, Ohyama A, Shimizu T (2010) Genetic variability and disturbance of the
600 Japanese common loach *Misgurnus anguillicaudatus* in Ehime Prefecture, Japan.
601 Aquaculture Sci 58: 113-120.
602 [20] Shimizu T (2014) Genetic status and consequences of commercial utilization of the
603 oriental weather loach *Misgurnus anguillicaudatus* in Japan. Japan J Ichthyol 61:
604 36-40.
605 [21] Fujita T (2007) Scientific name for the loach “Kara-Dojo” distributed in Japan.
606 Japan J Ichthyol 54: 243-244.
607 [22] Fujimoto T, Sakao S, Yamaha E, Arai K (2007) Evaluation of different doses of
608 UV irradiation to loach eggs for genetic inactivation of the maternal genome. J Exp
609 Zool 307A: 449-462.
610 [23] Asahida T, Kobayashi T, Saitoh K, Nakayama I (1996) Tissue preservation and
611 total DNA extraction from fish stored at ambient temperature using buffer
612 containing high concentration of urea. Fish Sci 62: 727-730.
613 [24] Murakami M, Fujitani H (1997) Polyploid-specific repetitive DNA sequences from
614 triploid ginbuna (Japanese silver crucian carp, *Carassius auratus langsdorfii*).
615 Genes Genet Syst 72: 107-113.
616 [25] Itono M, Okabayashi N, Morishima K, Fujimoto T, Yoshikawa H, Yamaha E, Arai
617 K (2007) Cytological mechanisms of gynogenesis and sperm incorporation in
618 unreduced diploid eggs of the clonal loach, *Misgurnus anguillicaudatus* (Teleostei:
619 Cobitidae). J Exp Zool 307A: 35-50.
620 [26] Morishima K, Yoshikawa H, Arai K (2008) Meiotic hybridogenesis in triploid
621 *Misgurnus* loach derived from a clonal lineage. Heredity 100: 581-586.
622
623

Figure Legends

Fig. 1. Detection of repetitive DNA sequences in *Sac*I, *Eco*RV, *Dra*I and *Kpn*I digests of the genomicDNA from the *M. anguillicaudatus* on a 1.5 % agarose gel. The arrow designates a satellite band of approximately 130 bp. The molecular markers show 100 bp ladder.

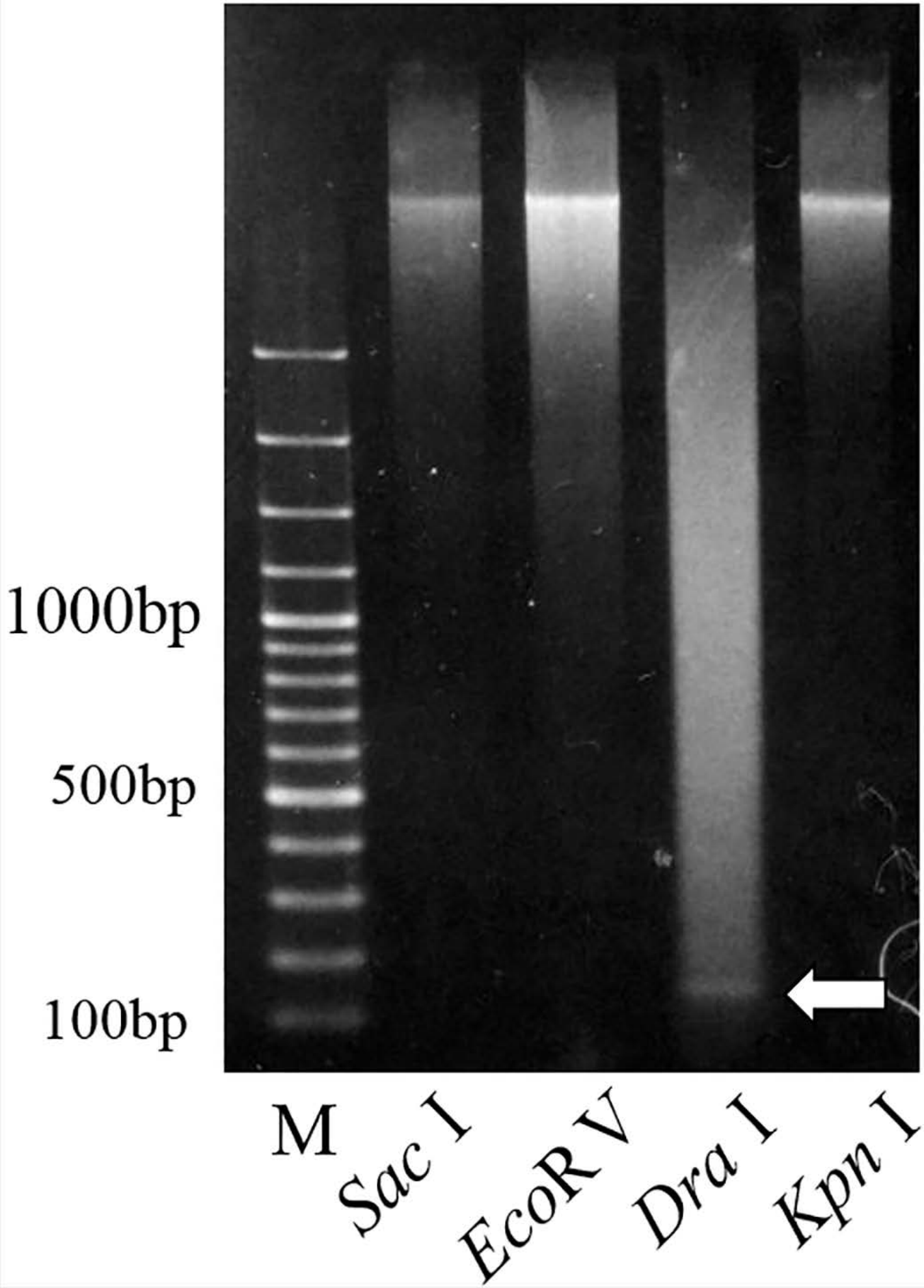
Fig. 2. Two types of ManDra sequences with 137 bp length which most frequently occurred in clones. Dots signify identical nucleotides. Substitutions are marked with the appropriate nucleotides. Arrows denote the sequences from which ManDra-F and ManDra-R primers were designed. Underline indicated recognition site (GTGCAC) of the restriction enzyme *Apa*LI.

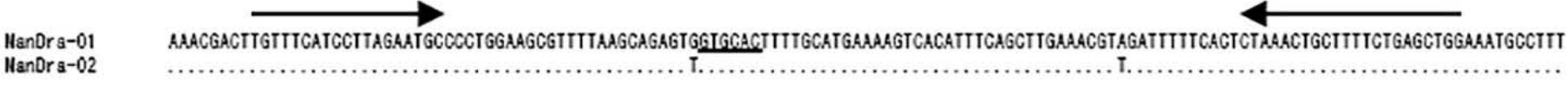
Fig. 3. (a) Detection of repetitive DNA sequences in *Apa*LI digests of the genomic DNA from the *M. anguillicaudatus* on a 1.5 % agarose gel. The arrow designates a satellite band of approximately 130 bp. The molecular markers show 100 bp ladder: (b) Southern hybridization of *M. anguillicaudatus* genomic DNA sample (10μg) with ManDra sequence as a probe after digestion with *Dra* I for 1 min, 5 min, 10 min, 20 min and over night (about 12 h). Note ladder like pattern after complete digestion.

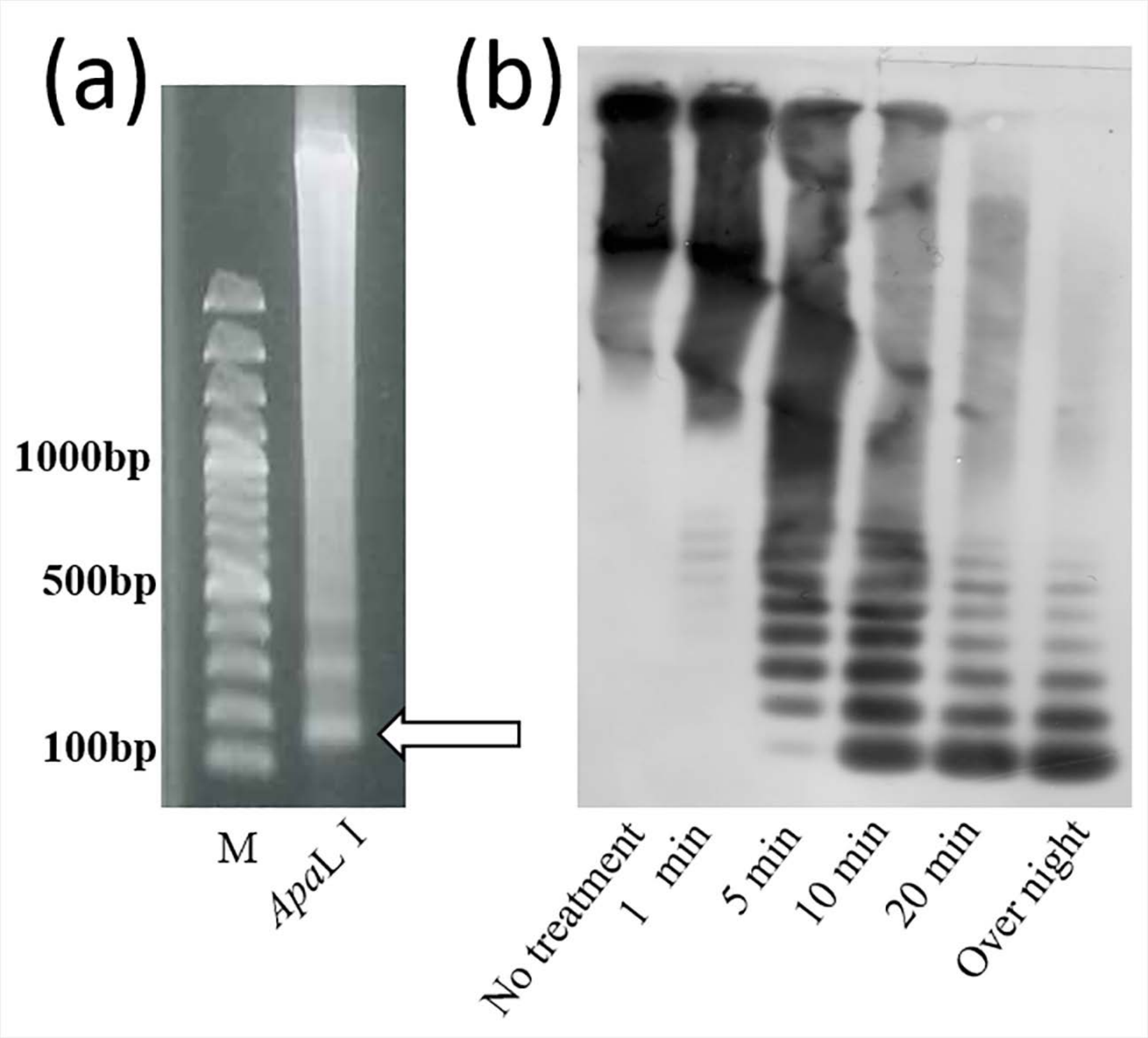
Fig. 4. Ladder-like pattern of ManDra in representative samples of group A (lane 2) and smear-like pattern in samples of group B-1 (lane 3), B-2 (lane 4) and clonal diploid (lane 5) within *Misgurnus anguillicaudatus* species. Weak ladder-like pattern also occurs in stone loach *Noemacheilus barbatulus toni* (lane 12). No detection of ManDra in other specimens under the present PCR conditions. Lane 1: molecular marker 100 bp ladder, 2: A group *M. anguillicaudatus*, 3: B-1 group *M. anguillicaudatus*, 4: B-2 group *M. anguillicaudatus*, 5: clonal lineage no.1 with A/ B-1 genome constitution *M. anguillicaudatus*, 6: B-1/ B-2 putative hybrid *M.*

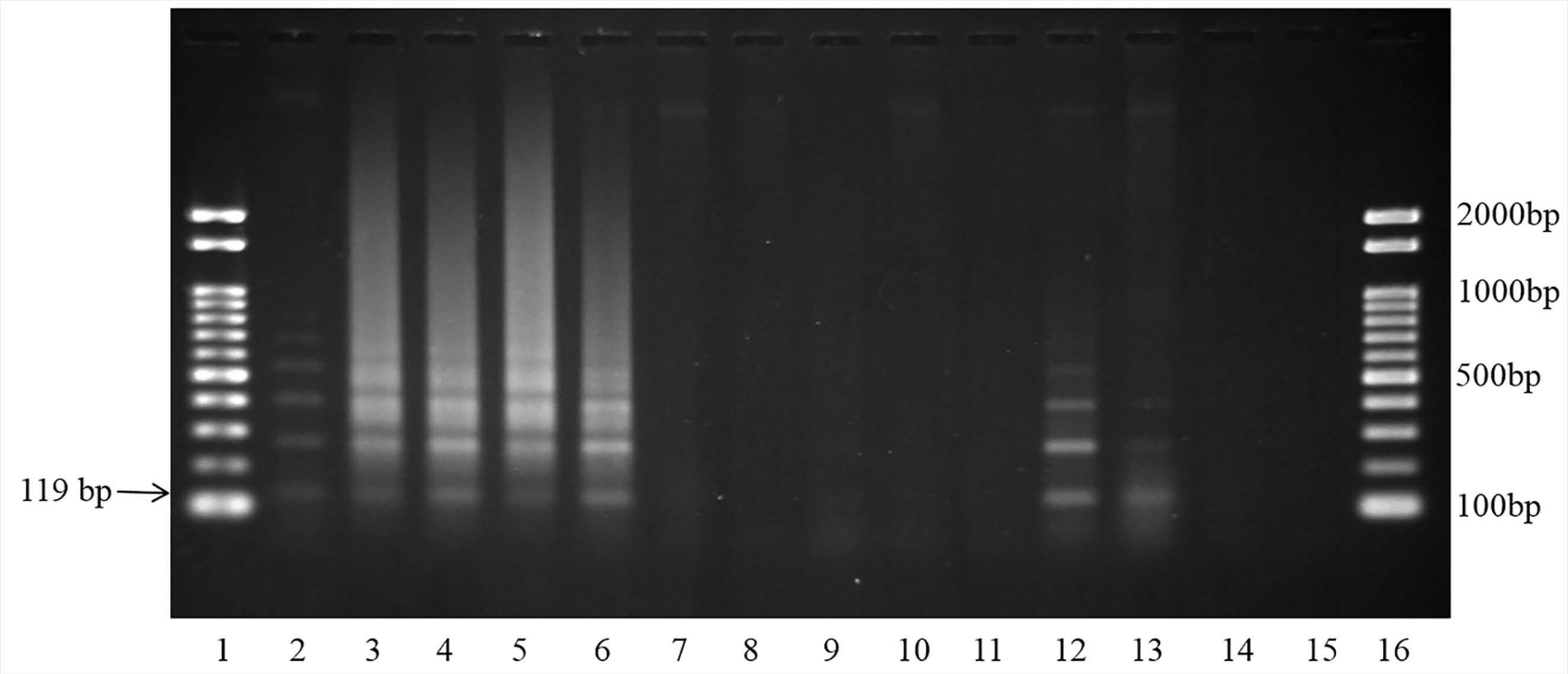
651 *anguillicaudatus*, 7: *M. mizolepis*, 8: *M. fossilis*, 9: *Cobitis taenia* from Poland, 10: *C.*
652 *biwae* from Hiroshima Pref., 11: *Lefua nikkonis*, 12: *N. barbatulus toni*, 13: *Cyprinus*
653 *carpio*, 14: *Carassius auratus*, 15: negative control, 16: molecular marker with 100
654 bp ladder.
655
656 Fig. 5. Detection of repetitive DNA sequences in *Bam*HI, *Sca*I, *Eco*RI, *Hin*dIII, *Nhe*I
657 and *Bgl*II digests of the genomic DNAs from the *M. anguillicaudatus* on a 1.5 %
658 agarose gel. The arrow designates a satellite band of approximately 550bp.
659
660 Fig. 6. Nucleotide sequences of ManBgl-550 monomers. Seventeen randomly selected
661 clones from a *M. anguillicaudatus* were sequenced and aligned using a Clustal W
662 program. Only complete sequence of ManBgl-550-c, which is the consensus
663 sequence determined by the majority rule, is shown and the sequence differences
664 relative to it are indicated. Dots and a dash signify identical nucleotides and a
665 sequence gap, respectively. Substitutions are marked with the appropriate nucleotides.
666 The pair of primers for the PCR experiment is indicated with arrows.
667
668 Fig. 7. Southern blots of restriction digests of the genomic DNAs from *M.*
669 *anguillicaudatus* (lanes 1, 2, 4) and *M. mizolepis* (lane 3) probed with ManBgl-550
670 sequences. Predominant bands (about 550 and 490 bp) are arrowed.
671
672 Fig. 8. Electrophoresis of the primed PCR products of ManBgl from the genetic DNA
673 of the *Misgurnus anguillicaudatus* (lanes 2 – 6) and *M. mizolepis* (lane 7). No
674 fragments appeared in other species. Predominant bands (about 400 bp and 460 bp)
675 are arrowed. Lane 1: molecular marker 100 bp ladder, 2: A group *M.*
676 *anguillicaudatus*, 3: B-1 group *M. anguillicaudatus*, 4: B-2 group *M.*
677 *anguillicaudatus*, 5: clonal lineage no.1 with A/ B-1 genome constitution *M.*

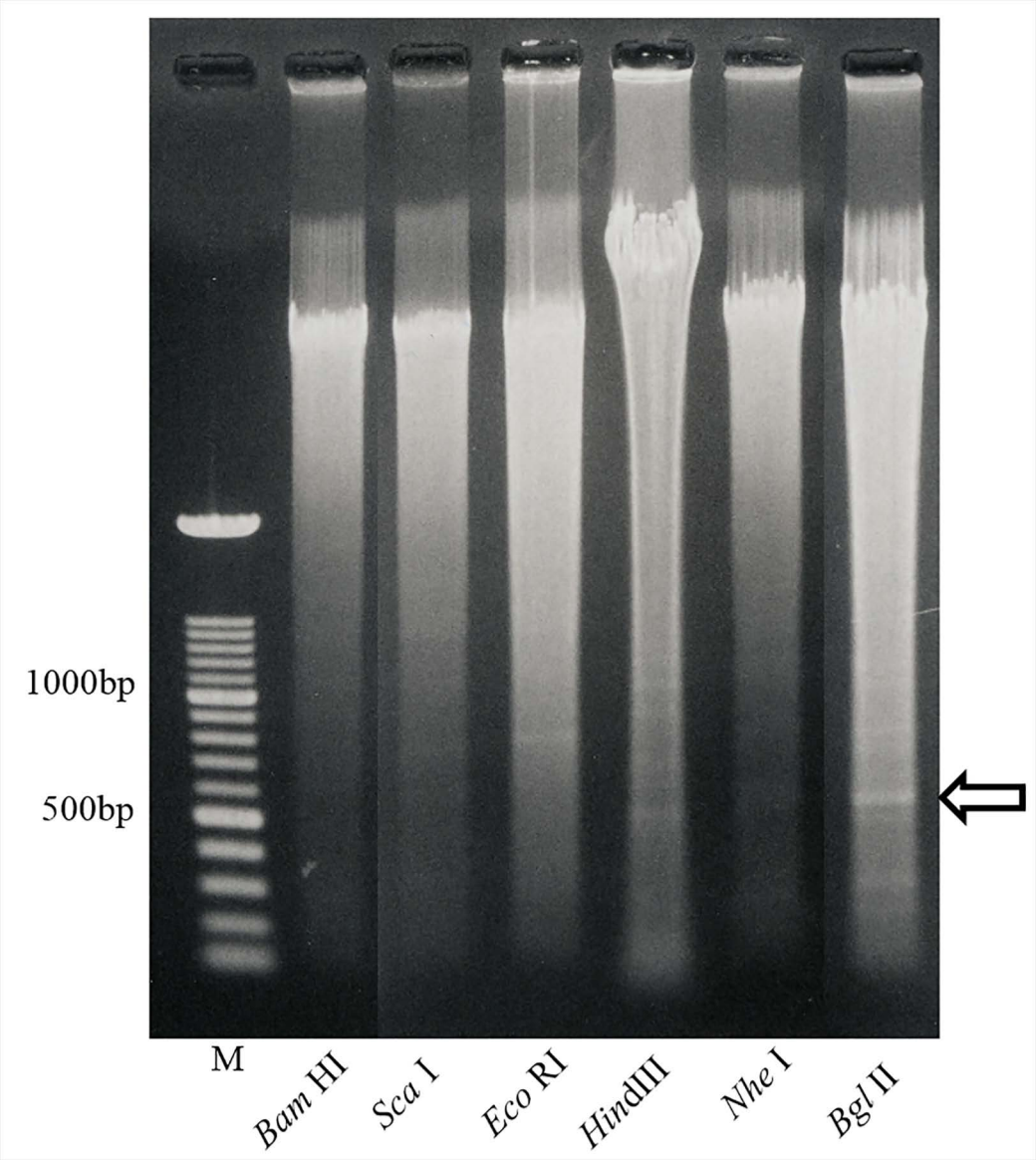
678 *anguillicaudatus,* 6: B-1/ B-2 putative hybrid *M. anguillicaudatus*, 7: *M. mizolepis*,
679 8: *M. fossilis*, 9: *Cobitis taenia* from Poland, 10: *C. biwae* from Hiroshima Pref., 11:
680 *Lefua nikkonis*, 12: *Noemacheilus barbatulus toni*, 13: *Cyprinus carpio*, 14:
681 *Carassius auratus*, 15: negative control, 16: molecular marker with 100 bp ladder.
682
683 Fig. 9. *RAG1*-RFLP analyses in *Misgurnus anguillicaudatus* and related species. A
684 group and B-2 group of *M. anguillicaudatus* (lanes 2 and 4) gave single fragment
685 with 443 bp size, while B-1 group (lane 3)gave two fragments with 226bp and 147
686 bp. Clonal lineage (lane 5) and B-1/ B-2 hybrid gave three fragments (443, 296 and
687 147 bp). Large fragment with 443 bp size also appeared in other cobitids (lanes 7
688 -10). Several fragments appeared in *Cyprinus carpio* (lane 13) and *Carassiun*
689 *auratus* (lane 14). Lane 1: molecular marker 100 bp ladder, 2: A group *M.*
690 *anguillicaudatus*, 3: B-1 group *M. anguillicaudatus*, 4: B-2 group *M.*
691 *anguillicaudatus,* 5: clonal lineage no.1 with A/ B-1 genome constitution of *M.*
692 *anguillicaudatus,* 6: B-1/ B-2 putative hybrid *M. anguillicaudatus*, 7: *M. mizolepis*,
693 8: *M. fossilis*, 9: *Cobitis taenia* from Poland, 10: *C. biwae* from Hiroshima Pref., 11:
694 *Lefua nikkonis*, 12: *Noemacheilus barbatulus toni*, 13: *Cyprinus carpio*, 14:
695 *Carassius auratus*, 15: negative control, 16: molecular marker with 100 bp ladder.
696

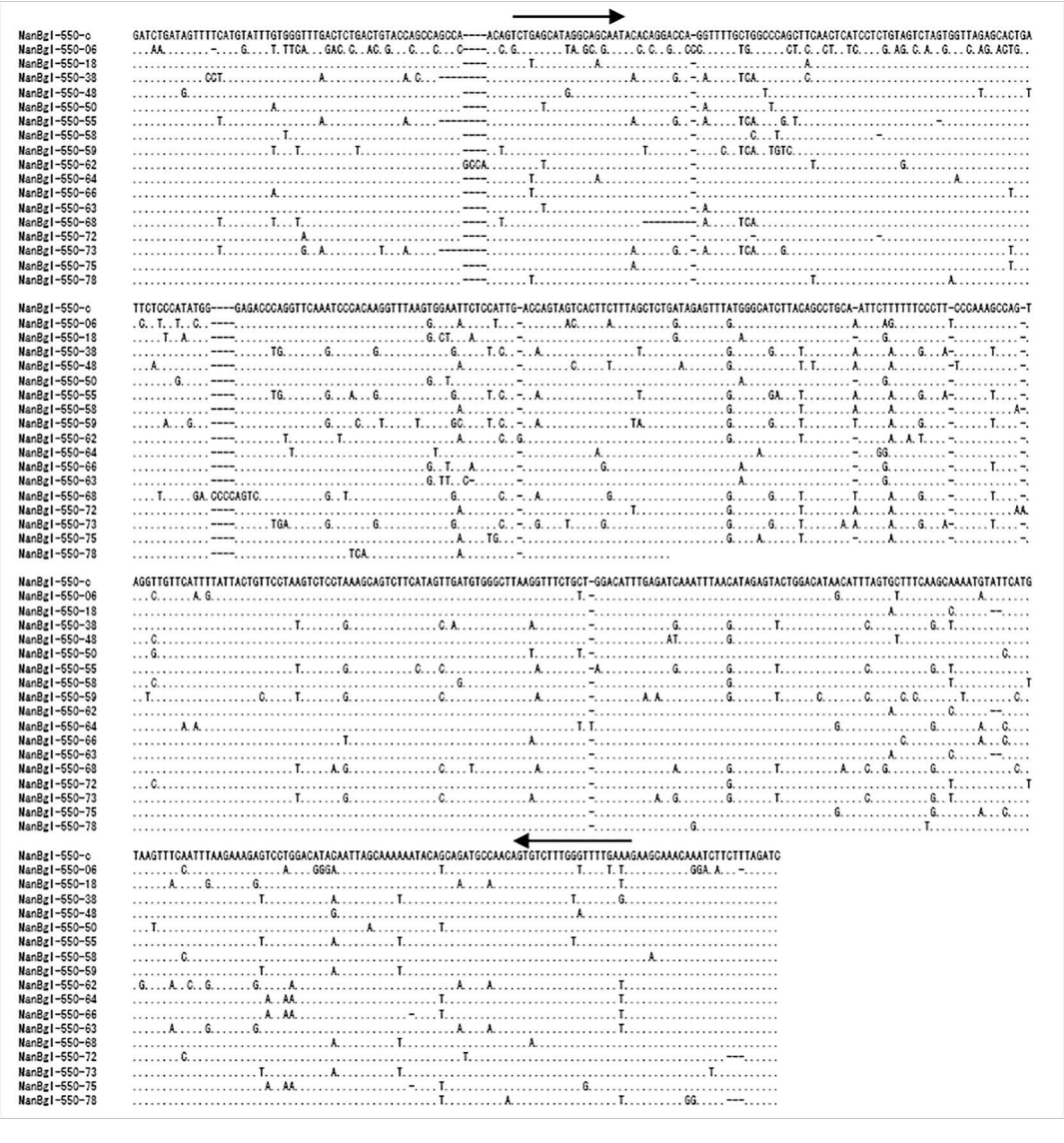

1000bp
500bp
100bp
M
Sac I
EcoR V
Dra I
Kpn I

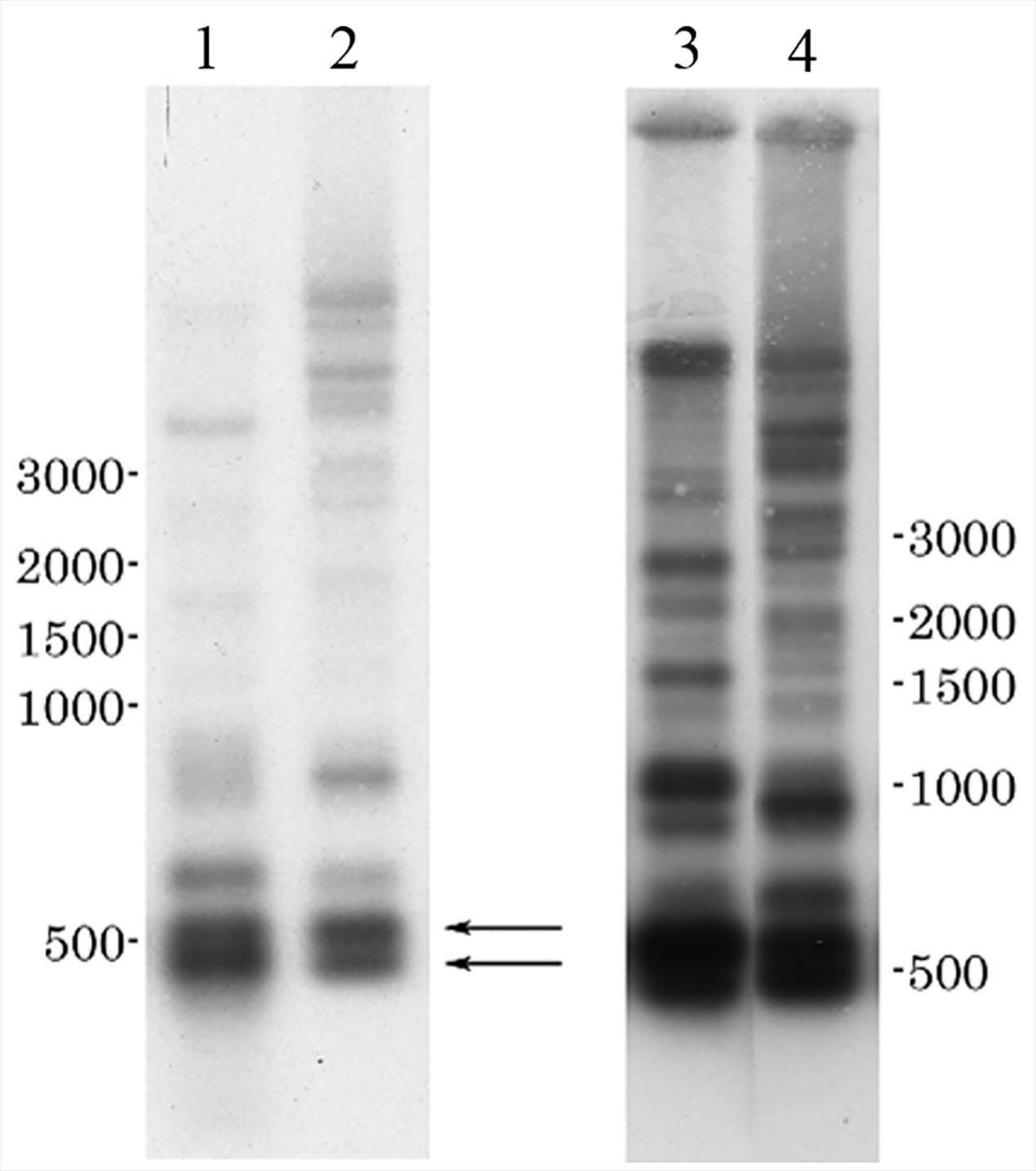

NanDrs-01
NanDrs-02
AAACGACTTGTTTCATCCTTAGAATGCCCCTGGAAGCGTTTTAAGCAGAGTGGTGCACTTTTGCATGAAAAGTCACATTTCAGCTTGAAACGTAGATTTTTCACTCTAAACTGCTTTTCTGAGCTGGAAATGCCTTT
T
T

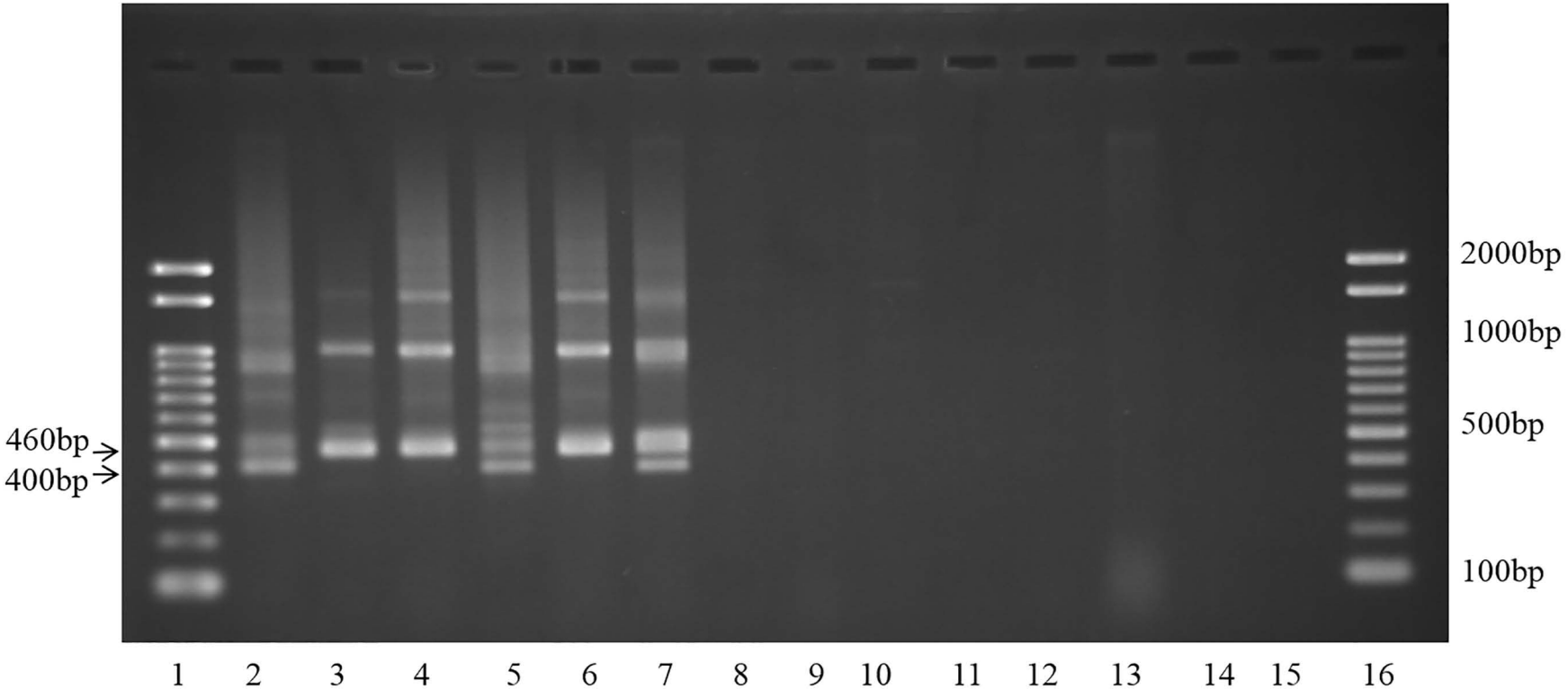

(a)
1000bp
500bp
100bp
M
ApaL I
(b)
No treatment
1 min
5 min
10 min
20 min
Over night

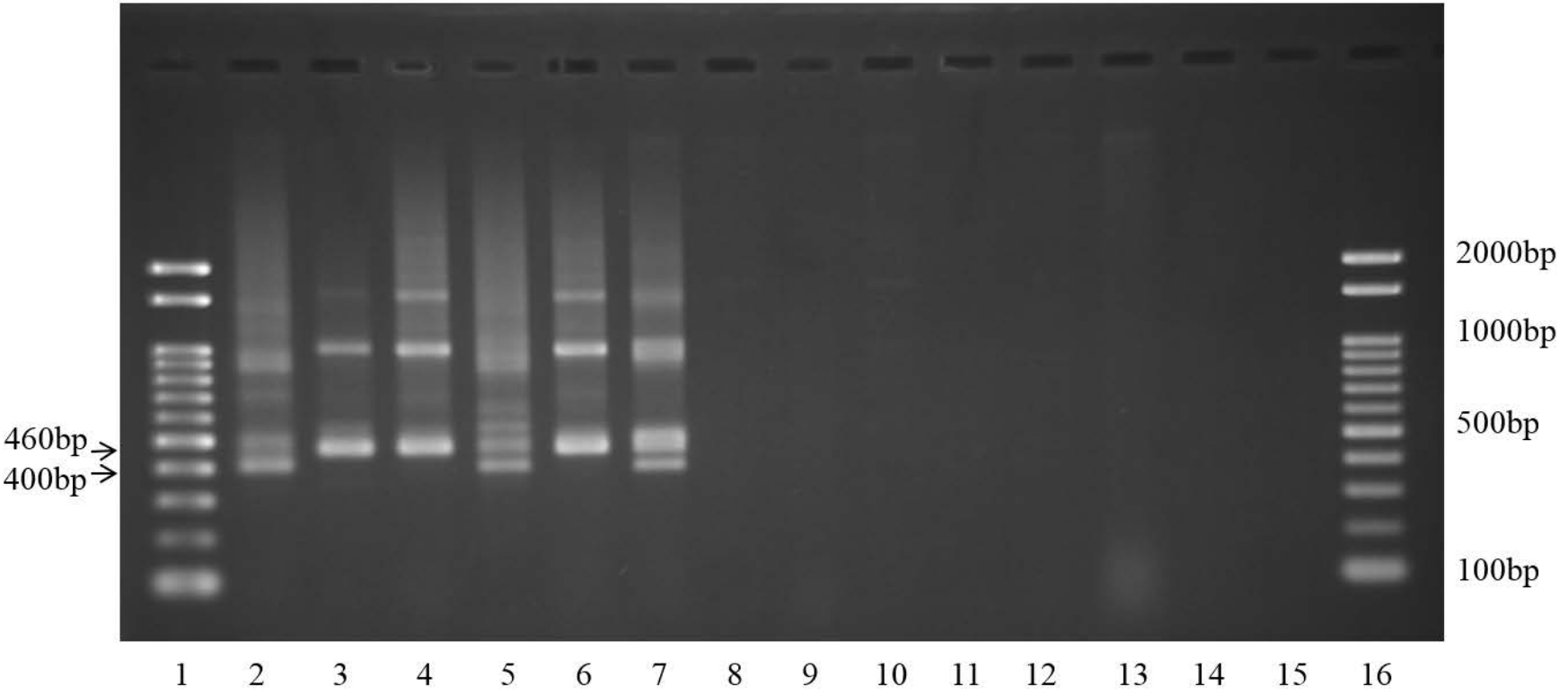

2000bp
1000bp
500bp
100bp
119 bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


1000bp
500bp
M
Bam HI
Sca I
Eco RI
HindIII
Nhe I
Bgl II


NanBgl-550-c
NanBgl-550-06
NanBgl-550-18
NanBgl-550-38
NanBgl-550-48
NanBgl-550-50
NanBgl-550-55
NanBgl-550-58
NanBgl-550-59
NanBgl-550-62
NanBgl-550-64
NanBgl-550-66
NanBgl-550-63
NanBgl-550-68
NanBgl-550-72
NanBgl-550-73
NanBgl-550-75
NanBgl-550-78


1
2
3
4
3000-
2000-
1500-
1000-
500-
-3000
-2000
-1500
-1000
-500


460bp
400bp
2000bp
1000bp
500bp
100bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


460bp
400bp
2000bp
1000bp
500bp
100bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

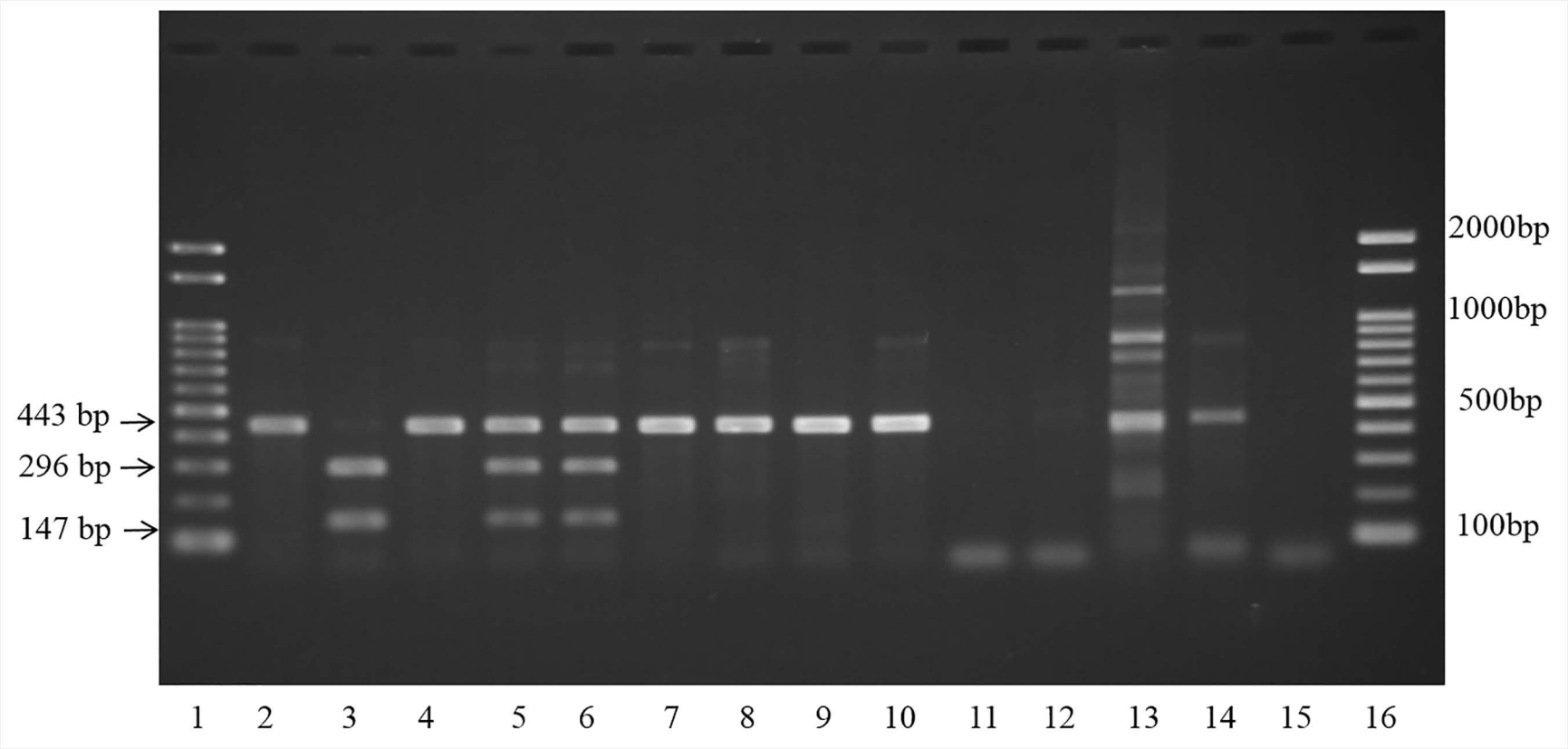

443 bp
296 bp
147 bp
2000bp
1000bp
500bp
100bp
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Table 1. Sampling sites, size and years of *Misgurnus anguillicaudatus* specimens for present genetic analysis

Site no.	Prefecture/Province	Localities (City, Town or Village)	Fish no.	Year of sampling
1	Hokkaido	Ozora (former, Memanbetsu)	58	2009
2		Higashikawa	8	1996
3		Iwamizawa	10	1996
4		Ebetsu	10	1998
5		Akkeshi	10	2001
6	Aomori	Yomogida	12	2009
7		Aomori	16	2009
8	Akita	Kakunodate	10	1998
9	Iwate	Hanamaki	20	2004
10	Yamagata	Tsuruoka	8	2004
11	Miyagi	Naruko	10	1998
12	Niigata	Hirokami	6	1996
13	Tochigi	Nasu	10	1997
14		Nikko	10	1998
15	Saitama	Hanyu	10	1999
16	Chiba	Futtsu	10	1998
17	Nagano	Ueda	10	1999
18	Ishikawa	Noto (Yanagida*)	30	2009
19		Noto (Kurikawashiri*)	20	2002
20		Nanao (Notojima-Hannoura*)	37	2009
21		Nanao (Notojima-Suso*)	20	2002
22		Nanao (Nakajima*)	10	2002
23		Nanao (Tadatsu*)	41	2009
24		Kaga (Oshiotsuji*)	44	2009
25	Aichi	Inazawa	3	2003
26	Gifu	Hashima	10	1999
27	Mie	Inabe	11	2004
28		Yokkaichi	20	2004
29	Shiga	Makino	8	1996
30	Fukui	Obama	10	1998
31	Tottori	Tomari	10	2000
32	Shimane	Izumo	10	1997
33	Tokushima	Hiwasa	10	1996
Total			522	

*Regional names in Noto Town, Nanao City or Kaga City.

Table 2. PCR primers for ManDra, ManBgl and *RAG1*

Primer name	Primer sequences
ManDra-1F	TGTTTCATCCTTAGAATGCC
ManDra-1R	CCAGCTCAGAAAAGCAGTTTAG
dbg13	TCTKAKCATAGGCARCAATA
dbg23	CTKTCAAAACWCAAAGACAC
RAG1-M.aF	GTTTGAATGGCAGCCAGCTCTG
RAG1-M.aR	CCACAAACATGAGACACAGAGGTC

Table 3. Genotypes of ManDra, ManBgl and *RAG1*-RFLP in each group of *Misgurnus anguillicaudatus* and *Misgurnus mizolepis* and *Misgurnus fossilis*.

	M. anguillicaudatus			*M. mizolepis*	*M. fossilis*
	A	B-1	B-2		
ManDra	Ladder	Smear	Smear	No amplification	No amplification
ManBgl	400 bp	460 bp	460 bp	400 bp, 460 bp, 510 bp	No amplification
RAG1-RFLP	443 bp	296 bp, 147 bp	443 bp	443 bp	443 bp

Table 4. Nuclear DNA and mitochondrial DNA genome constitution in individual *Misgurnus anguillicaudatus*

Site no.	Prefecture/ Province	Localities (City, Town or Village)	mtDNA genome	Nuclear genome	*N*
1	Hokkaido	Ozora (former, Memanbetsu)	A	A	29
			A	A/B-1	29
2		Higashikawa	B-1	B-1	8
3		Iwamizawa	B-1	B-1	9
			B-1	Unknown	1
4		Ebetsu	B-1	B-1	10
5		Akkeshi	A	A	10
6	Aomori	Yomogida	B-1	B-1	12
7		Aomori	B-1	B-1	15
			B-1	B-1/B-2	1
8	Akita	Kakunodate	B-1	B-1	10
9	Iwate	Hanamaki	B-1	B-1	20
10	Yamagata	Tsuruoka	B-1	B-1	8
11	Miyagi	Naruko	A	A	10
12	Niigata	Hirokami	B-2	B-1/B-2	2
			B-2	B-2	4
13	Tochigi	Nasu	A	Unknown	1
			B-2	Unknown	1
			B-2	B-1/B-2	1
			B-2	B-2	6
			Unknown	A	1
14		Nikko	B-1	B-2	1
			B-2	B-1/B-2	1
			B-2	B-2	8
15	Saitama	Hanyu	B-1	B-2	1
			B-2	B-2	9
16	Chiba	Futtsu	A	A	1
			B-1	B-1	1
			B-2	B-1/B-2	1
			B-2	B-2	7
17	Nagano	Ueda	B-1	B-2	1
			B-2	B-2	9
18	Ishikawa	Noto (Yanagida)	B-1	B-1	28
			B-1	B-1/B-2	2
19		Noto (Kurikawashiri)	A	B-1	2
			B-1	B-1	17
			B-1	Unknown	1
20		Nanao (Notojima-Hannoura)	A	A/B-1	4
			A	B-1	19
			A	B-1/B-2	2
			A	Unknown	1

			B-1	A/B-1	1
			B-1	B-1	10
21		Nanao (Notojima-Suso)	B-1	B-1	19
			B-1	B-1/B-2	1
22		Nanao (Nakajima)	B-1	B-1	9
			B-1	Unknown	1
23		Nanao (Tadatsu)	A	B-1	3
			A	B-1/B-2	1
			B-1	B-1	35
			B-1	Unknown	2
24		Kaga (Oshiotsuji)	B-1	B-1	36
			B-2	B-1/B-2	5
			B-2	B-2	1
			Unknown	B-1	2
25	Aichi	Inazawa	B-1	B-1	3
26	Gifu	Hashima	B-1	B-1	3
			B-2	B-1	6
			B-2	B-2	1
27	Mie	Inabe	B-1	B-1	11
28		Yokkaichi	B-1	B-1	19
			B-1	B-1/B-2	1
29	Shiga	Makino	B-1	B-1	8
30	Fukui	Obama	B-1	B-1	10
31	Tottori	Tomari	B-1	B-1/B-2	1
			B-1	B-2	9
32	Shimane	Izumo	B-1	B-1	4
			B-1	B-1/B-2	6
33	Tokushima	Hiwasa	B-1	B-1	10

Unknown: In mtDNA, the haplotypes have not been reported previously. In nuclear DNA, the genotypes were not categorized by our present markers. Detailed supplemental information is shown in a Table S1.

Supplemental Data:

Development of nuclear DNA markers to characterize genetically diverse groups of *Misgurnus anguillicaudatus* and its closely related species

Takafumi Fujimoto[a]*, Aya Yamada[a], Yukihiro Kodo[b], Kohei Nakaya[b], Michiko Okubo-Murata[bc], Taiju Saito[ad], Kazuto Ninomiya[a], Michiko Inaba[a], Masamichi Kuroda[a], Katsutoshi Arai[a] and Masaru Murakami[b]

[a] Hokkaido University, Faculty and Graduate School of Fisheries Sciences, Hakodate, Hokkaido 041-8611, Japan.

[b] Azabu University, School of Veterinary Medicine, Sagamihara, Kanagawa 252-5201, Japan.

[c] (Present Address) Tokyo University of Agriculture, Faculty of Bioindustry, Abashiri, Hokkaido 099-2493, Japan.

[d] (Present Address) Ehime University, South Ehime Fisheries Research Center, Nishiura Station, Uchidomari, Ainan, Ehime 798-4206, Japan.

*Corresponding author

Running title: Nuclear DNA markers of pond loach

Sapplemental figure:

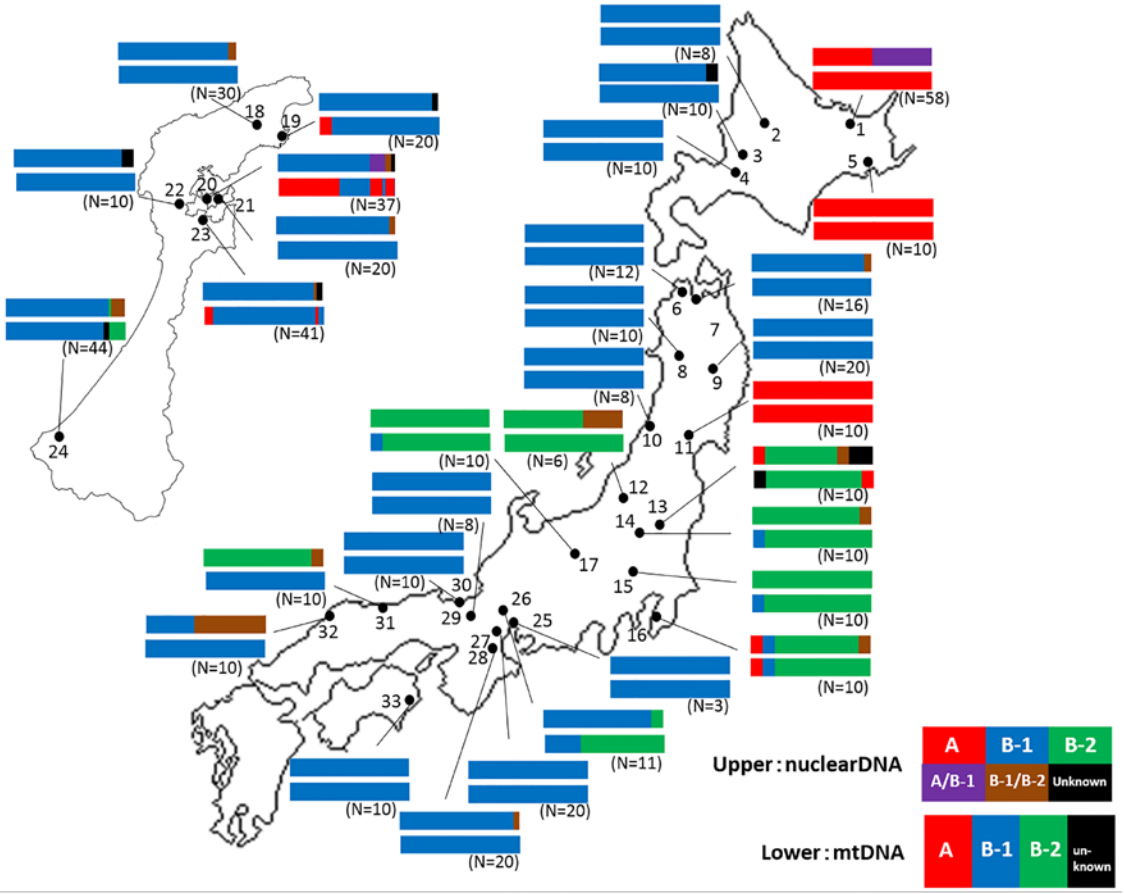


Fig. S1. Nuclear DNA (upper row) and mitochondrial DNA genome (lower row) constitution in individual *Misgurnus anguillicaudatus* specimens of each sampling site.

Table S1. Individual mtDNA and nuclear genome of *Misgurnus auguillicaudatus* specimens in the present genetic analyses by mtDNA control region (sequences/ RFLP), ManDra genotype, ManBgl genotype and *RAG1*-RFLP. Specimens described in Morishima et al [2] gave individual mtDNA genomes based on the previous results. But, individual samples of several sites were confirmed again by RFLP of mtDNA-CR.

Site no.	Individual no.	Ploidy	*Hae* Ⅲ	*Hin*f Ⅰ	RFLP	mtDNA haplotype	mtDNA genome	ManDra	ManBGL	*RAG1*	Nuclear genome
1	1	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	2	Diploid	A	A	Ⅰ	1-1	A	Ladder	400bp	443bp	A
	3	Diploid	A	D	Ⅳ	4-1	A	Ladder	400bp	443bp	A
	4	Diploid	A	C	Ⅱ	2-1, 2, 4	A	Ladder	400bp	443bp	A
	5	Diploid	A	C	Ⅱ	2-1	A	Ladder	400bp	443bp	A
	6	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	7	Diploid				1-1	A	Ladder	400bp	443bp	A
	8	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	9	Diploid	A	A	Ⅰ	1-7	A	Ladder	400bp	443bp	A
	10	Diploid	A	A	Ⅰ	1-3, 1-4, 1-5	A	Ladder	400bp	443bp	A
	11	Diploid	A	A	Ⅰ	1-1	A	Ladder	400bp	443bp	A
	12	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	13	Triploid				3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	14	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	15	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	16	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	17	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	18	Diploid	A	C	Ⅱ	1-7	A	Ladder	400bp	443bp	A
1	1	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	2	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	3	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	4	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	5	Diploid	A	E	Ⅸ	1-1	A	Ladder	400bp	443bp	A
	6	Diploid	A	C	Ⅱ	2-1	A	Ladder	400bp	443bp	A

7	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
8	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
9	Diploid	B	B	Ⅲ		A	Smear	400, 460bp	147, 296, 443bp	A/B-1
10	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
11	Diploid				1-7	A	Ladder	400bp	443bp	A
12	Diploid	A	C	Ⅱ		A	Ladder	400bp	443bp	A
13	Diploid	A	D	Ⅳ	4-1	A	Ladder	400bp	443bp	A
14	Diploid	A	D	Ⅳ		A	Ladder	400bp	443bp	A
15	Diploid	A	D	Ⅳ	4-1	A	Ladder	400bp	443bp	A
16	Diploid				3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
17	Diploid				2-1	A	Ladder	400bp	443bp	A
18	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
19	Diploid				3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
20	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
21	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
22	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
23	Diploid				3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
24	Diploid	A	E	Ⅸ	1-3, 1-4, 1-5	A	Ladder	400bp	443bp	A
25	Diploid	B	B	Ⅲ		A	Smear	400, 460bp	147, 296, 443bp	A/B-1
26	Diploid				1-1	A	Ladder	400bp	443bp	A
27	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
28	Diploid	A	C	Ⅱ	2-1	A	Ladder	400bp	443bp	A
29	Diploid				1-7	A	Ladder	400bp	443bp	A
30	Diploid				2-1	A	Ladder	400bp	443bp	A
31	Diploid	A	E	Ⅸ	1-1	A	Ladder	400bp	443bp	A
32	Diploid				1-3, 1-4, 1-5	A	Ladder	400bp	443bp	A
33	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
34	Triploid				3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
35	Diploid	A	C	Ⅱ	2-1	A	Ladder	400bp	443bp	A
36	Diploid	A	C	Ⅱ	2-1	A	Ladder	400bp	443bp	A
37	Diploid	A	C	Ⅱ		A	Ladder	400bp	443bp	A
38	Diploid	B	B	Ⅲ	3-1, 3-2	A	Ladder	400bp	443bp	A
39	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
40	Diploid	A	A	Ⅰ	1-7	A	Ladder	400bp	443bp	A

2 (6)*	1	B-1	Smear	460bp	147, 296bp	B-1
	2	B-1	Smear	460bp	147, 296bp	B-1
	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	460bp	147, 296bp	B-1
	5	B-1	Smear	460bp	147, 296bp	B-1
	6	B-1	Smear	460bp	147, 296bp	B-1
	7	B-1	Smear	460bp	147, 296bp	B-1
	8	B-1	Smear	460bp	147, 296bp	B-1
3 (8)	1	B-1	Smear	460bp	147, 296bp	B-1
	2	B-1	Smear	460bp	147, 296bp	B-1
	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	400bp	147, 296bp	unknown
	5	B-1	Smear	460bp	147, 296bp	B-1
	6	B-1	Smear	460bp	147, 296bp	B-1
	7	B-1	Smear	460bp	147, 296bp	B-1
	8	B-1	Smear	460bp	147, 296bp	B-1
	9	B-1	Smear	460bp	147, 296bp	B-1
	10	B-1	Smear	460bp	147, 296bp	B-1
4 (11)	1	B-1	Smear	460bp	147, 296bp	B-1
	2	B-1	Smear	460bp	147, 296bp	B-1
	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	460bp	147, 296bp	B-1
	5	B-1	Smear	460bp	147, 296bp	B-1
	6	B-1	Smear	460bp	147, 296bp	B-1
	7	B-1	Smear	460bp	147, 296bp	B-1
	8	B-1	Smear	460bp	147, 296bp	B-1
	9	B-1	Smear	460bp	147, 296bp	B-1
	10	B-1	Smear	460bp	147, 296bp	B-1
5 (10)	1	A	Ladder	400bp	443bp	A
	2	A	Ladder	400bp	443bp	A
	3	A	Ladder	400bp	443bp	A
	4	A	Ladder	400bp	443bp	A

	5						A	Ladder	400bp	443bp	A
	6						A	Ladder	400bp	443bp	A
	7						A	Ladder	400bp	443bp	A
	8						A	Ladder	400bp	443bp	A
	9						A	Ladder	400bp	443bp	A
	10						A	Ladder	400bp	443bp	A
6	1	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	E	V	5-20	B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid				5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	6	Diploid				5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	7	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid				5-20	B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	C	E	V	5-20	B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid				5-20	B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	C	E	V	5-20	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
7	1	Diploid				5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid	C	E	V	5-20	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	6	Diploid	C	E	V	5-20	B-1	Smear	460bp	147, 296bp	B-1
	7	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	E	V	5-25	B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid				5-20	B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid				5-20	B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	C	E	V	5-25	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
	14	Diploid				5-25	B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid				5-20	B-1	Smear	460bp	147, 296bp	B-1

	16	Diploid	C	E	V	5-1, 5-4	B-1	Smear	460bp	147, 296bp	B-1
8 (17)	1						B-1	Smear	460bp	147, 296bp	B-1
	2						B-1	Smear	460bp	147, 296bp	B-1
	3						B-1	Smear	460bp	147, 296bp	B-1
	4						B-1	Smear	460bp	147, 296bp	B-1
	5						B-1	Smear	460bp	147, 296bp	B-1
	6						B-1	Smear	460bp	147, 296bp	B-1
	7						B-1	Smear	460bp?	147, 296bp	B-1
	8						B-1	Smear	460bp	147, 296bp	B-1
	9						B-1	Smear	460bp	147, 296bp	B-1
	10						B-1	Smear	460bp	147, 296bp	B-1
9 (18)	1						B-1	Smear	460bp	147, 296bp	B-1
	2						B-1	Smear	460bp	147, 296bp	B-1
	3						B-1	Smear	460bp	147, 296bp	B-1
	4						B-1	Smear	460bp	147, 296bp	B-1
	5						B-1	Smear	460bp	147, 296bp	B-1
	6						B-1	Smear	460bp	147, 296bp	B-1
	7						B-1	Smear	460bp	147, 296bp	B-1
	8						B-1	Smear	460bp	147, 296bp	B-1
	9						B-1	Smear	460bp	147, 296bp	B-1
	10						B-1	Smear	460bp	147, 296bp	B-1
	11						B-1	Smear	460bp	147, 296bp	B-1
	12						B-1	Smear	460bp	147, 296bp	B-1
	13						B-1	Smear	460bp	147, 296bp	B-1
	14						B-1	Smear	460bp	147, 296bp	B-1
	15						B-1	Smear	460bp	147, 296bp	B-1
	16						B-1	Smear	460bp	147, 296bp	B-1
	17						B-1	Smear	460bp	147, 296bp	B-1
	18						B-1	Smear	460bp	147, 296bp	B-1
	19						B-1	Smear	460bp	147, 296bp	B-1
	20						B-1	Smear	460bp	147, 296bp	B-1
10 (20)	1						B-1	Smear	460bp	147, 296bp	B-1

	2				B-1	Smear	460bp	147, 296bp	B-1
	3				B-1	Smear	460bp	147, 296bp	B-1
	4				B-1	Smear	460bp	147, 296bp	B-1
	5				B-1	Smear	460bp	147, 296bp	B-1
	6				B-1	Smear	460bp	147, 296bp	B-1
	7				B-1	Smear	460bp	147, 296bp	B-1
	8				B-1	Smear	460bp	147, 296bp	B-1
11 (22)	1				A	Ladder	400bp	443bp	A
	2				A	Ladder	400bp	443bp	A
	3				A	Ladder	400bp	443bp	A
	4				A	Ladder	400bp	443bp	A
	5				A	Ladder	400bp	443bp	A
	6				A	Ladder	400bp	443bp	A
	7				A	Ladder	400bp	443bp	A
	8				A	Ladder	400bp	443bp	A
	9				A	Ladder	400bp	443bp	A
	10				A	Ladder	400bp	443bp	A
12 (31)	1				B-2	Smear	460bp	443bp	B-2
	2				B-2	Smear	460bp	443bp	B-2
	3				B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	4				B-2	Smear	460bp	443bp	B-2
	5				B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	6				B-2	Smear	460bp	443bp	B-2
13 (24)	1	C	F	Ⅶ	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	2	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	3	E	F	Ⅶ	B-2	Smear	400bp	443bp	unknown
	4	A			unknown	Ladder	400bp	443bp	A
	5	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	6	E	F	Ⅺ	A	Smear	400bp	443bp	unknown
	7	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	8	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	9	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2

	10	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
14 (26)	1	C	E	Ⅴ	B-1	Smear	460bp	443bp	B-2
	2	C	F	Ⅶ	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	3	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	4	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	5	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	6	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	7	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	8	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	9	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	10	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
15 (27)	1	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	2	C	E	Ⅴ	B-1	Smear	460bp	443bp	B-2
	3	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	4	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	5	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	6	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	7	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	8	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	9	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	10	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
16 (28)	1	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	2	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	3	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	4	C	E	Ⅴ	B-1	Smear	460bp	147, 296bp	B-1
	5	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	6	C	F	Ⅶ	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	7	E	F	Ⅺ	A	Ladder	400bp	443bp	A
	8	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	9	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2
	10	C	F	Ⅶ	B-2	Smear	460bp	443bp	B-2

17 (37)	1		C	F	VII		B-2	Smear	460bp	443bp	B-2
	2		C	F	VII		B-2	Smear	460bp	443bp	B-2
	3		C	F	VII		B-2	Smear	460bp	443bp	B-2
	4		C	F	VII		B-2	Smear	460bp	443bp	B-2
	5		C	F	VII		B-2	Smear	460bp	443bp	B-2
	6		C	F	VII		B-2	Smear	460bp	443bp	B-2
	7		C	E	V		B-1	Smear	460bp	443bp	B-2
	8		C	F	VII		B-2	Smear	460bp	443bp	B-2
	9		C	F	VII		B-2	Smear	460bp	443bp	B-2
	10		C	F	VII		B-2	Smear	460bp	443bp	B-2
18	1	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	6	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	7	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid				7-16	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	14	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	16	Diploid				7-16	B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	19	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	20	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	21	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	22	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1
	23	Diploid	C	F	VII	7-16	B-1	Smear	460bp	147, 296bp	B-1

	24	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	25	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	26	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	27	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	28	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	29	Diploid	C	E	Ⅴ	5-25	B-1	Smear	460bp	147, 296bp	B-1
	30	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
19	1	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid	B	B	Ⅲ		A	Smear	460bp	147, 296bp	B-1
	6	Diploid	C	E	Ⅴ		B-1	Smear	460bp		unknown
	7	Diploid	B	B	Ⅲ		A	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	10	Triploid	C	F	Ⅶ		B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	C	F	Ⅶ		B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	14	Diploid	C	F	Ⅶ		B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	16	Diploid	C	F	Ⅶ		B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	19	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	20	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
20	1	Diploid	C	E	Ⅴ	5-7, 7-5, 9-1	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	3	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	4	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	5	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	6	Diploid	B	B	Ⅲ	3-1, 3-2	A	Ladder	460bp	147, 296bp	unknown

	7	Diploid	C	F	Ⅶ	7-7	B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	E	Ⅴ	5-7, 7-5, 9-1	B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	C	E	Ⅴ	5-7, 7-5, 9-1	B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296, 443bp	B-1/B-2
	11	Diploid	C	F	Ⅶ	7-7	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	13	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	14	Diploid	C	E	Ⅴ	5-25	B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	16	Diploid	C	F	Ⅶ	7-7	B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	C	E	Ⅴ	5-25	B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	C	E	Ⅴ	5-25	B-1	Smear	460bp	147, 296bp	B-1
	19	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296, 443bp	B-1/B-2
	20	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	21	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	22	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	23	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	24	Diploid	B	B	Ⅲ	5-7, 7-5, 9-1	A	Smear	460bp	147, 296bp	B-1
	25	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	26	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	27	Diploid	C	E	Ⅴ	5-7, 7-5, 9-1	B-1	Smear	460bp	147, 296bp	B-1
	28	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	29	Diploid	C	F	Ⅶ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	30	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	31	Diploid	B	B	Ⅲ	7-7	A	Smear	460bp	147, 296bp	B-1
	32	Triploid	B	B	Ⅲ	5-7, 7-5, 9-1	B-1	Smear	400, 460bp	147, 296, 443bp	A/B-1
	33	Triploid	B	B	Ⅲ	3-1, 3-2	A	Smear	400, 460bp	147, 296, 443bp	A/B-1
	34	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	35	Diploid	C	E	Ⅴ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	36	Diploid	C	F	Ⅶ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
	37	Diploid	B	B	Ⅲ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
21 (34)	1	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1

	4	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	6	Diploid	A	E	IX		B-1	Smear	460bp	147, 296bp	B-1
	7	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	14	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	16	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	C	E	V		B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	19	Diploid	C	E	V		B-1	Smear	460bp	147, 296bp	B-1
	20	Diploid	C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
22 (35)	1		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	2		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	3		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	4		C	F	VII		B-1	Smear	460bp		
	5		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	6		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	7		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	8		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	9		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
	10		C	F	VII		B-1	Smear	460bp	147, 296bp	B-1
23	1	Diploid	C	E	V	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	F	VII	7-13	B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	F	VII	7-13	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	F	VII	7-13	B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid				3-1, 3-2	A	Smear	460bp	147, 296, 443bp	B-1/B-2
	6	Diploid	C	F	VII	7-13	B-1	Smear	460bp	147, 296bp	B-1

7	Triploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
8	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
9	Diploid	C	E	Ⅴ	7-13	B-1	Smear	460bp	147, 296bp	B-1
10	Diploid	C	E	Ⅴ	7-13	B-1	Smear	460bp	147, 296bp	B-1
11	Diploid	C	F	Ⅶ	5-2, 5-21	B-1	Smear	460bp	147, 296bp	B-1
12	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
13	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
14	Diploid	C	E	Ⅴ	7-13	B1	Smear	460bp	147, 296bp	B-1
15	Diploid	C	F	Ⅶ	5-7, 7-5, 9-1	B-1	Smear	460bp	147, 296bp	B-1
16	Diploid	C	F	Ⅶ	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
17	Diploid	C	F	Ⅶ	7-13	B-1	Smear	400bp	147, 296bp	unknown
18	Diploid	B	B	Ⅲ	7-13	B-1	Smear	460bp	147, 296bp	B-1
19	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
20	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
21	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
22	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
23	Diploid	C	F	Ⅶ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
24	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
25	Diploid	C	E	Ⅴ	7-13	B-1	Smear	460bp	147, 296bp	B-1
26	Diploid	C	F	Ⅶ	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
27	Diploid	C	E	Ⅴ	7-13	B-1	Smear	460bp	147, 296bp	B-1
28	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
29	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
30	Diploid	C	F	Ⅶ	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1
31	Diploid	C	E	Ⅴ	7-13	B-1	Smear	460bp	147, 296bp	B-1
32	Diploid	C	F	Ⅶ	5-2, 5-21	B-1	Smear	460bp	147, 296bp	B-1
33	Diploid	C	F	Ⅶ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
34	Diploid	B	B	Ⅲ	7-13	B-1	Smear	460bp	147, 296bp	B-1
35	Diploid	C	F	Ⅶ	5-2, 5-21	B-1	Smear	460bp	147, 296bp	B-1
36	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
37	Diploid	C	F	Ⅶ	7-13	B-1	Smear	460bp	147, 296bp	B-1
38	Diploid	C	E	Ⅴ	7-13	B-1	Smear	400bp	147, 296bp	unknown
39	Diploid	C	F	Ⅶ	3-1, 3-2	A	Smear	460bp	147, 296bp	B-1
40	Diploid				7-13	B-1	Smear	460bp	147, 296bp	B-1
41	Diploid	C	E	Ⅴ	5-5, 5-6, 5-24	B-1	Smear	460bp	147, 296bp	B-1

24	1	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	443bp	B-2
	5	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	6	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	7	Diploid	C	F	Ⅶ		unknown	Smear	460bp	147, 296bp	B-1
	8	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	9	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	10	Diploid	New	F	New	7-16	B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	C	F	Ⅶ	7-4, 7-9	B-2	Smear	460bp	147, 296, 443bp	B-1/B-2
	14	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	16	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	19	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	20	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	21	Diploid	New	F	New	7-16	B-1	Smear	460bp	147, 296bp	B-1
	22	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	23	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	24	Diploid	C	F	Ⅶ		unknown	Smear	460bp	147, 296bp	B-1
	25	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	26	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	27	Diploid	C	E	Ⅴ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	28	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	29	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	30	Diploid	New	F	New	7-16	B-1	Smear	460bp	147, 296bp	B-1
	31	Diploid	C	E	Ⅴ	5-20	B-1	Smear	460bp	147, 296bp	B-1
	32	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	33	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	34	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1

	35	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	36	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	37	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	38	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	39	Diploid	New	F	New	7-16	B-1	Smear	460bp	147, 296bp	B-1
	40	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	41	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	42	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	43	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
	44	Diploid	C	F	Ⅶ	7-16	B-1	Smear	460bp	147, 296bp	B-1
25 (40)	1						B-1	Smear	460bp	147, 296bp	B-1
	2						B-1	Smear	460bp	147, 296bp	B-1
	3						B-1	Smear	460bp	147, 296bp	B-1
26 (39)	1		C	F	Ⅶ		B-2	Smear	460bp	443bp	B-2
	2		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
	3		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
	4		C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	5		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
	6		C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	7		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
	8		C	E	Ⅴ		B-1	Smear	460bp	147, 296bp	B-1
	9		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
	10		C	F	Ⅶ		B-2	Smear	460bp	147, 296bp	B-1
27 (41)	1						B-1	Smear	460bp	147, 296bp	B-1
	2						B-1	Smear	460bp	147, 296bp	B-1
	3						B-1	Smear	460bp	147, 296bp	B-1
	4						B-1	Smear	460bp	147, 296bp	B-1
	5						B-1	Smear	460bp?	147, 296bp	B-1
	6						B-1	Smear	460bp	147, 296bp	B-1
	7						B-1	Smear	460bp?	147, 296bp	B-1
	8						B-1	Smear	460bp?	147, 296bp	B-1
	9						B-1	Smear	460bp	147, 296bp	B-1

	10		B-1	Smear	460bp	147, 296bp	B-1
	11		B-1	Smear	460bp?	147, 296bp	B-1
28 (42)	1	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	2	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	3	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	4	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	5	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	6	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	7	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	8	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	9	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	10	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	11	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	12	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	13	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	14	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	15	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	16	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	17	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	18	Diploid	B-1	Smear	460bp	147, 296bp	B-1
	19	Diploid	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	20	Diploid	B-1	Smear	460bp	147, 296bp	B-1
29 (46)	1		B-1	Smear	460bp	147, 296bp	B-1
	2		B-1	Smear	460bp	147, 296bp	B-1
	3		B-1	Smear	460bp	147, 296bp	B-1
	4		B-1	Smear	460bp	147, 296bp	B-1
	5		B-1	Smear	460bp	147, 296bp	B-1
	6		B-1	Smear	460bp	147, 296bp	B-1
	7		B-1	Smear	460bp	147, 296bp	B-1
	8		B-1	Smear	460bp	147, 296bp	B-1
30 (45)	1		B-1	Smear	460bp	147, 296bp	B-1
	2		B-1	Smear	460bp	147, 296bp	B-1

	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	460bp	147, 296bp	B-1
	5	B-1	Smear	460bp	147, 296bp	B-1
	6	B-1	Smear	460bp	147, 296bp	B-1
	7	B-1	Smear	460bp	147, 296bp	B-1
	8	B-1	Smear	460bp	147, 296bp	B-1
	9	B-1	Smear	460bp	147, 296bp	B-1
	10	B-1	Smear	460bp	147, 296bp	B-1
31 (48)	1	B-1	Smear	460bp	443bp	B-2
	2	B-1	Smear	460bp	443bp	B-2
	3	B-1	Smear	460bp	443bp	B-2
	4	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	5	B-1	Smear	460bp	443bp	B-2
	6	B-1	Smear	460bp	443bp	B-2
	7	B-1	Smear	460bp	443bp	B-2
	8	B-1	Smear	460bp	443bp	B-2
	9	B-1	Smear	460bp	443bp	B-2
	10	B-1	Smear	460bp	443bp	B-2
32 (49)	1	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	2	B-1	Smear	460bp	147, 296bp	B-1
	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	5	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	6	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	7	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
	8	B-1	Smear	460bp	147, 296bp	B-1
	9	B-1	Smear	460bp	147, 296bp	B-1
	10	B-1	Smear	460bp	147, 296, 443bp	B-1/B-2
33 (51)	1	B-1	Smear	460bp	147, 296bp	B-1
	2	B-1	Smear	460bp	147, 296bp	B-1
	3	B-1	Smear	460bp	147, 296bp	B-1
	4	B-1	Smear	460bp	147, 296bp	B-1

5	B-1	Smear	460bp	147, 296bp	B-1
6	B-1	Smear	460bp	147, 296bp	B-1
7	B-1	Smear	460bp	147, 296bp	B-1
8	B-1	Smear	460bp	147, 296bp	B-1
9	B-1	Smear	460bp	147, 296bp	B-1
10	B-1	Smear	460bp	147, 296bp	B-1

*Number in parenthesis indicates site number described in Morishima et al. [2]. Genetic grouping based on mtDNA-CR sequence or RFLPs was already done in [2].
Unknown: In mtDNA, the haplotypes have not been reported previously. In nuclear DNA, the genotypes were not categorized by our present markers.