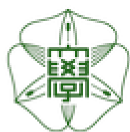
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Author(s)	Yamada, Aya; Kodo, Yukihiro; Murakami, Masaru; Kuroda, Masamichi; Aoki, Takao; Fujimoto, Takafumi; Arai, Katsutoshi
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Hybrid Origin of Gynogenetic Clones and the Introgression of Their Mitochondrial Genome into Sexual Diploids through Meiotic Hybridogenesis in the Loach, *Misgurnus anguillicuadatus*

Aya Yamada[1], Yukihiro Kodo[2], Masaru Murakami[2], Masamichi Kuroda[1], Takao Aoki[1], Takafumi Fujimoto[1], Katsutoshi Arai [1]*

[1]Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, 041-8611, Japan
[2]Faculty of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, 252-5201, Japan

18 pages text including this title page, five figures, three tables

Abbreviated title: Hybrid origin of clonal diploid loach (38 letters and spaces)

*** Correspondence to:** Faculty and Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1, Minato, Hakodate, Hokkaido 041-8611, Japan
Telephone: +81-138-40-5535
Fax: +81-138-43-5015
Email: araikt@fish.hokudai.ac.jp

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ABSTRACT

In a few Japanese populations of the loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae), clonal diploid lineages produce unreduced diploid eggs that normally undergo gynogenetic reproduction; however the origin of these clones remains elusive. Here, we show the presence of two diverse clades, A and B, within this loach species from sequence analyses of two nuclear genes *RAG1* (recombination activating gene 1) and *IRBP2* (interphotoreceptor retinoid-binding protein 2), and then demonstrate heterozygous genotypes fixed at the two loci as the evidence of the hybrid nature of clonal lineages. All the clonal individuals were identified by clone-specific mitochondrial DNA haplotypes, microsatellite genotypes and random amplified polymorphic DNA fingerprints; they commonly showed two alleles, one from clade A and another from clade B, whereas other wild-type diploids possessed alleles from either clade A or B. However, we also found wild-type diploids with clone-specific mitochondrial DNA and nuclear genes from clade B. One possible explanation is an introgression of a clone-specific mitochondrial genome from clonal to these wild-type loaches. These individuals likely arose by a cross between haploid sperm from bisexual B clade males and haploid eggs with clone-specific mtDNA and clade B nuclear genome, produced by meiotic hybridogenesis (elimination of unmatched A genome followed by meiosis after preferential pairing between two matched B genomes) in clone-origin triploid individual (ABB).

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2 The loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) is a gonochoristic diploid
3 with 2n = 50 chromosomes and it reproduces sexually, but clonal diploid lineages have
4 been found in the northern area of Hokkaido Prefecture and the Notojima Island of
5 Ishikawa Prefecture, Japan (Morishima et al., 2002, 2008a). These clonal diploid
6 loaches are essentially all-female and generate unreduced diploid eggs by premeiotic
7 endomitosis, i.e., chromosome doubling without cytokinesis (Itono et al. 2006). Most
8 clonal diploid eggs initiate gynogenetic development after fertilization by sperm of
9 sympatric sexually reproducing wild-type males, whereas some incorporate the sperm
10 nucleus into the egg to develop as triploid animal (Itono et al., 2007). Genetics and
11 atypical reproduction of clonal diploid and other polyploid *Misgurnus* loaches have been
12 reviewed by Arai and Fujimoto (2013).
13 Interspecific hybridization is considered to be involved in the origin of unisexual
14 vertebrates, which atypically reproduce by parthenogenesis, gynogenesis
15 (sperm-dependent parthenogenesis), or hybridogenesis, and this is often accompanied
16 by polyploidy (reviewed in Dawley,'89; Vrijenhoek et al.,'89; Vrijenhoek, '94), although
17 nonhybrid origins were recently reported in parthenogenetically reproducing lizard
18 *Lepidophyma* genus (Sinclair et al., 2010). Hybridization often causes a disruption of
19 regular meiosis because of unsuccessful pairing between non-homologous chromosomes
20 derived from different species, and it often results in sterility, but it seldom induces
21 atypical gametogenesis and unusual modes of reproduction in fish species (Dawley et
22 al., '85; Fujimoto et al., 2008). Examples of unisexual fish with an apparent hybrid
23 origin are included in the genera *Poecilia*, *Poeciliopsis*, *Menidia*, *Phoxinus*, *Squalius,*
24 *Carassius,* and *Cobitis* (reviewed in Dawley, '89; Vrijenhoek et al., '89; Gui and Zhou,
25 2010; Collares-Pereira et al., 2013). Recent large-scale multi-locus analyses revealed the
26 hybrid origin of asexuality, establishment of clones in different ploidy levels, and effect
27 of asexual complexes to the initiation of clonality and polyploidy in *Cobitis* loach,
28 different members of the family Cobitidae (Janko et al., 2012). In the *Misgurnus* loach,
29 however, a relationship between clonal reproduction in certain diploids and
30 hybridization is ambiguous, because *M. anguillicaudatus* has been identified as a single
31 species entity (Saitoh, '89).
32 There are two major groups of Japanese *M. anguillicaudatus*, namely, A and B,
33 which are further clustered into two sub-groups, B-1 and B-2, based on highly
34 differentiated sequences (average sequence divergence, 13%) of the mitochondrial DNA
35 control region (mtDNA-CR) (Morishima et al., 2008a). Similar inter-generic equivalent
36 diversity has also been observed in *M. anguillicaudatus* (Koizumi et al., 2009), in which

different mtDNA regions, particularly cytochrome b sequences, were analyzed, and a large genetic divergence was observed (average sequence divergence, 15 - 18%). However, recent molecular phylogenetic studies strongly suggest that the entire mitochondrial genome of group B *Misgurnus* must have originated from the ancestral loach genus *Cobitis* through introgression; thus, the presence of two different lineages diverging early in the speciation process within *M. anguillicaudatus* is highly spurious (Slechtova et al., 2008; Kitagawa et al., 2011). The hypotheses about the mitochondrial introgression are supported by the recent analyses of the complete mitochondrial genomes of different polyploid *M. anguillicaudatus* (Zhou et al., 2014). Although these results raise the question of genealogy based on previous mtDNA analyses (Morishima et al., 2008a; Koizumi et al., 2009), the presence of two different groups in the Japanese wild populations have also been demonstrated by using allozymes (Khan and Arai, 2000) and microsatellite analyses (Arias-Rodriguez et al., 2006). Recent molecular phylogenetic studies on *Misgurnus* from the Far Eastern region of Russia also suggested the presence of different species within *M. anguillicaudatus* (Perdices et al., 2012). Disruption of normal meiosis and subsequent gametogenesis was also reported in inter-populational *M. anguillicaudatus* hybrids between group A females and group B males as in atypical reproduction occurred in inter-specific hybrids: most hybrids generated unreduced diploid and other types of unusual gametes (Arias-Rodriguez et al., 2009, 2010). All these studies support the hybrid-origin theory for the occurrence of gynogenetically reproducing clones in nature.

In natural triploid loaches arising from the spontaneous incorporation of a haploid sperm nucleus from a wild-type diploid into an unreduced diploid egg of clonal lineage, haploid eggs are formed by meiotic hybridogenesis: one of the two different chromosome-sets (haploid genome) of the clone preferentially pairs with the genetically similar haploid set from a sperm donor to form bivalents and then they produce haploid gametes by regular meiosis, but another haploid set of the clone is eliminated before meiosis because of the low pairing affinity between the two haploid genomes of the clone (Morishima et al., 2008b). This unusual pattern of meiotic hybridogenesis also suggests that the genomic constitution of the clonal loach might be heterozygous because of its presumptive hybrid origin.

All the above-mentioned results support the involvement of hybridization in the origin of clonal diploid loaches in nature, but direct genetic evidence has not been obtained so far. In the present study, we analyzed sequences of the nuclear genes, *RAG1* (recombination activating gene 1) and *IRBP2* (interphotoreceptor retinoid-binding protein 2), which have been utilized for molecular phylogenetic studies in Cypriniformes

1 fishes, including loach species (Slechtova et al., 2008; Saitoh et al., 2010; Perdices et al., 2 2012), in putative clonal diploid loaches and wild-type diploid loaches from different 3 localities. Here, we demonstrate that the sequences of the *RAG1* and *IRBP2* genes can 4 be classified into two different clades, which closely correspond to two groups that were 5 previously observed in mtDNA-CR sequences. We then show that clonal diploid 6 specimens with clone-specific mtDNA-CR sequences and microsatellite genotypes are 7 heterozygotes possessing two different alleles from diverse clades at both *RAG1* and 8 *IRBP2* loci, indicating the hybrid origin of clonal lineage.

9

10 **MATERIALS AND METHODS**

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12 Ethics

13 All experiments procedures were performed in accordance with Institutional guidelines 14 on animal experimentation and care, and were approved by the Animal Research 15 Committee of Hokkaido University.

16

17 Fish specimens

18 From 2000 to 2010, *M. anguillicaudatus* (*n*=54) were collected from 12 Japanese 19 localities (Table 1). The ploidy status of each individual was examined by flow 20 cytometry according to Morishima et al. (2002) and one triploid individual was detected 21 among the specimens collected from site no. 1. Clonal individuals (#3 and #6) were 22 previously identified by reproduction experiments including artificial fertilization with 23 normal sperm, hybridization with goldfish sperm, and induced gynogenesis with 24 UV-irradiated sperm as well as genetically identical genotypes in several microsatellite 25 loci and multi-locus DNA fingerprinting (Morishima et al., 2002). Clone 1 strain was 26 established by reproduction of the identified female (#3: Morishima et al., 2002) and 27 then maintained in the Aquarium room of the Environment Control Experiment 28 Building of the Faculty of Fisheries Sciences, Hokkaido University, Hakodate. Clone 1 29 strain was used as reference for clone 1. The other clonal strains (2 to 4) were later 30 identified by the presence of mtDNA-CR haplotype III as well as absolutely identical 31 profiles in random amplified polymorphic DNA (RAPD)-PCR analyses using several 32 random primers and multi-locus DNA fingerprinting using a tetranucleotide probe 33 (Morishima et al., 2008a). Preserved specimens of clones 2 to 4 were also used as 34 reference control samples to identify clonal strains. The mud loach *M. mizolepis* 35 specimen (*n* = 1), obtained from a commercial aquarium shop, was also used in the 36 analysis.

Mitochondrial DNA analysis

DNA was extracted from fin clips or muscle tissue by using the standard phenol/chloroform protocol (Asahida et al., '93). The mtDNA-CR was amplified and subjected to restriction fragment length polymorphism (RFLP) analysis, followed by determination of the partial sequences of the mtDNA-CR region (444 - 448 bp) according to the procedures described in Morishima et al. (2008a). Precise group identification of each specimen was performed by 100 to 99.3 % matching in the corresponding site between the deposited 942 - 954 bp mtDNA-CR sequences (AB306717-AB306793) clustered into different genetic groups (A, B-1, and B-2; Morishima *et al.,* 2008a) and present partial 444 - 448 bp sequences by using Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Identification of clonal diploid individuals by DNA marker analyses

Since all the previously identified clonal individuals and clone-origin triploid individuals had mtDNA-CR RFLP haplotype III, i.e., sequences III-1 or III-2 (Morishima et al., 2008a), candidates of clones were predicted to have such a clone-specific haplotype. Thus, samples taken from localities (site no, 1, 7 and 11), where previously known clones were found, were firstly screened to separate candidates of clones among diploid loaches by presence or absence of the clone-specific mtDNA-CR haplotype.

Next, selected candidates of clones were analyzed by independent microsatellite loci (*Mac* 3, *Mac37, Mac* 404, *Mac458* and *Mac477*; Morishima et al., 2008b, c, 2012) in order to find out clonal individuals with genotypes identical to those of the reference control samples of clones 1, 2, 3, and 4. These five loci were used to genotype progeny of clone 1 in Morishima et al. (2008b, 2012)

Candidates of clones were further examined by RAPD (random amplified polymorphic DNA) fingerprinting using three different random primers, Wako-01 (Arbitorary Primer set A-01, Wako, Osaka, Japan), OPA-11 (Kit A-11, Operon Technologies, Alameda, CA, USA) and OPA-12 (Kit A-12) according to the procedures described by Itono et al. (2006, 2007) and Morishima et al. (2008a). The other specimens without clone-specific mtDNA-CR haplotype, clonal microsatellite genotypes and clonal RAPD fingerprints were conveniently categorized to wild-type diploids, although the bisexual reproductive mode was not individually confirmed by reproduction experiments.

For microsatellite genotyping, PCR was performed with a 10 μL mixture containing

1 1.0 µL of the DNA sample, 6.4 5µL of DDW, 0.8 µL of dNTP mixture (TaKaRa, Otsu,
2 Shiga, Japan), 1.0 µL of 10xPCR buffer (TaKaRa), 0.05 µL of *r Taq* DNA Polymerase
3 (TaKaRa), 0.37 µL of the primer mix (Forward:Reverse=1:10), and 0.33 µL of the
4 M13-tailed forward primer (with fluorescence-labeled 5' ends). The PCR conditions were
5 as follows: initial denaturation at 94° for 1 min, 35 cycles of 94° for 15 s, 56° for 15 s and
6 72° for 1 min 30 s. The reaction was completed by a final extension at 72° for 60 min.
7 Electrophoresis was carried out using ABI PRISM 3130xl (Applied Biosystems, Foster
8 City, CA, USA). The alleles were distinguished by differences in the molecular size
9 using the Gene Scan 500LIZ (Applied Biosystems). Genotyping was performed using the
10 Gene Mapper Software v.3.7 (Applied Biosystems).
11 The amplification reactions were conducted for RAPD fingerprinting in a mixture
12 comprising DNA template (100ng/µL), DDW 16.0 µL, dNTP mixture (TaKaRa) 1.6 µL,
13 10x PCR buffer (TaKaRa) 2.0 µL, *rTaq* polymerase (TaKaRa) 0.2 µL and random primer
14 0.2 µL in 0.2 mL microtube. Amplification profiles were follows: initial step of 3 min at
15 95°, subsequent 30 cycles of 30s at 95°, 1 min at 36° and 1 min at 72°, followed by final
16 primer extension for 7 min at 72°. About 5 µL of PCR products were electrophoresed on
17 1.5% agalose gel and stained with ethidium bromide and photographed on a UV
18 transilluminator using a gel documentation system (UVP BioDoc-It™ Imagining
19 System, Cambridge, UK).
20
21 Sequencing of the *RAG1* gene
22 *RAG1* was amplified using the primer RAG-1F
23 (5'-AGCTGTAGTCAGTAYCACAARATG-3': Quenouille et al., 2004) and RAG-RV1
24 (5'-TCCTGRAAGATYTTGTAGAA-3': Slechtova et al., 2007). PCR was performed with a
25 20 µL mixture containing 1.0µL of the DNA sample, 13.3 µL of DDW, 1.6 µL of a dNTP
26 mixture (TaKaRa), 2.0 µL of 10x*Ex Taq* Buffer (TaKaRa), 0.1µL of *Ex Taq* DNA
27 Polymerase (TaKaRa), 1.0 µL of 10 µM RAG-1F primer, and 1.0 µL of 10 µM RAG-RV1
28 primer. The PCR conditions were as follows: initial denaturation at 95° for 5 min,
29 touch-down profile of 1 min at 94°, 1min 30 s at 60 - 55° (1° /cycle), and 2 min at 72°
30 followed by 30 cycles with annealing temperature held at 54°. The reaction was
31 completed by a final extension at 72° for 7 min. The PCR products were purified using
32 AMPure Kits (Beckman Coulter, Brea, CA, USA) and the purified products were
33 sequenced with PCR primers using BigDye Terminator v3.1 Cycle Sequencing Kit
34 (Applied Biosystems). The sequence products were purified using Clean SEQ (Beckman
35 Coulter) and resolved on an ABI PRISM 3130xl (Applied Biosystems). The sequences
36 were aligned using ClustalW in the program BioEdit (http://www.mbio.-

1 ncsu.edu/BioEdit/bioedit.html), and the alleles were identified when no or only one
2 double-peaked site was observed. When two or more double-peaked sites were detected,
3 the PCR product was cloned again using the TA Cloning Kit (Invitrogen, Paisley, UK),
4 and each allele per clone was identified.
5
6 Sequencing of the *IRBP-2* gene
7 *IRBP-2* was amplified using the primer IRBP 76F
8 (5'-CTTRTTGTGGATATGGCAAAAAT-3') and IRBP 1162R
9 (5'-TGGTGGWCTTYAGGCACTTGT-3') (Chen et al., 2008). PCR was performed with a
10 20μL mixture containing 1.0 μL of the DNA sample, 13.3 μL of DDW, 1.6 μL of dNTP
11 mixture (TaKaRa), 2.0 μL of 10x *Ex* Taq Buffer (TaKaRa), 0.1 μL of *Ex* Taq DNA
12 polymerase (TaKaRa), 1.0 μL of 10 μM IRBP 76F primer, and 1.0 μL of 10 μM IRBP
13 1162R primer. The PCR conditions were as follows: initial denaturation at 95° for 4 min,
14 30 cycles of 95° for 40 s, 58° for 40 s, and 72° for 1min 30 s. The reaction was completed
15 by a final extension at 72° for 7 min. The PCR products were purified using AMPure
16 Kits (Beckman Coulter), and the purified products were sequenced using PCR primers
17 and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The
18 sequence products were purified using Clean SEQ (Beckman Coulter) and resolved on
19 ABI PRISM 3130xl (Applied Biosystems). The sequences were aligned using ClustalW
20 in the program BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and alleles
21 were identified when no or only one double-peaked site was observed. When two or more
22 double-peaked sites were detected, the PCR product was cloned using a TA Cloning Kit
23 (Invitrogen), and alleles were identified in the clone.
24
25 Data analysis
26 We conducted maximum likelihood (ML) phylogenetic analysis by using MEGA 5
27 (Tamura et al., 2011). The best-fit substitution models indicated that the Kimura
28 2-parameter + discrete gamma model (Kimura, 1980; Yang, 1994) was the best fit
29 implemented in MEGA 5. Bootstrap analysis was performed on 1,000 replications in the
30 phylogenetic tree. We obtained *Cobitis biwae* (RAG-1:AB531299, IRBP-2: AB531238),
31 *Cobitis striata* (RAG-1: AB531286, IRBP-2: AB531223) and *Acantopsis choirorhynchos*
32 (RAG-1: AB531313, IRBP-2: 531254) as the outgroup from DDBJ/GenBank/EMBL. The
33 genetic distance between the groups was also calculated using MEGA5 against the
34 *p*-distance.
35
36 **RESULTS**

1 Table 2 shows the genetic groups inferred from mtDNA-CR RFLP haplotypes and the
2 partial sequences of all the loach specimens examined. Clone-specific mtDNA-CR
3 haplotypes (III-1 or III-2) were detected in six out of 12 samples from site no. 1, one out
4 of 9 samples from site no. 7, and six out of nine samples from site no. 11. No
5 clone-specific mtDNA-CR haplotypes were detected in specimens from other sampling
6 sites.

7 When microsatellite genotyping was performed at five different loci in
8 above-mentioned samples, five candidates from site no. 1 were identified as clone 1,
9 because they exhibited microsatellite genotypes identical to the reference control of
10 clone 1 and other samples gave polymorphic variations (Table 3). On the other hand,
11 clone 2 was found in site no. 7, and clones 3 and 4 were detected in site no. 11 (Table 3).
12 Clonal individual was not detected by microsatellite genotyping in other candidates
13 from site no. 11, although they had clone-specific mtDNA-CR III-1 or III-2 haplotype
14 (Table 2, 3). A naturally triploid individual (fish no.7 from site no. 1) harbored two
15 microsatellite alleles specific to clone 2, which may presumably be a spontaneous
16 triploid progeny of clone 2 (Table 3).

17 RAPD-fingerprinting with three different random primers (Wako-01, OPA-11 and
18 OPA-12) verified genetically identical characteristics of five candidates from site no.1
19 (clone 1), one candidate from site no.7 (clone 2) and two candidates from site no. 11
20 (clone 3 and 4), because they gave fingerprints identical to that of reference control
21 samples of clone 1-4 (Fig. 1). Triploid sample (no.7 from site no. 1; lane 12 in left gels)
22 gave DNA fingerprints comprising all fragments of clone 2 and additional fragments
23 (Fig. 1). Genetic variation was found by RAPD fingerprinting in other diploids from site
24 no. 1, 7 and 11 (Fig. 1). Several samples that had clone-specific mtDNA-CR III-1 or III-2
25 haplotype also gave polymorphic fingerprints (Fig. 1).

26 The 527-bp *RAG1* gene sequence was determined in 52 individuals of *M.*
27 *anguillicaudatus* and one individual of *M. mizolepis*. Consequently, 16 different
28 sequences were distinguished among *M. anguillicaudatus* specimens examined and all
29 these sequences were deposited in DDBJ/GenBank/EMBL as accession numbers
30 AB698049 to AB698064, with allele names serially designated as *RAG1-01* to *RAG1-16*
31 in the present study. Two sequences (AB704298, 704299) from a *M.mizolepis* specimen
32 were also deposited in DDBJ/GenBank/EMBL and designated as *RAG1-a* and *RAG1-b*,
33 respectively. ML phylogenetic analysis resulted in the construction of a tree (Fig. 2),
34 which clustered in two major clades, A and B, with clade B further divided in two
35 sub-clades, B-1 and B-2. *RAG1* sequences of *M. anguillicaudatus* were clearly
36 distinguished from those of *M. mizolepis* and this difference indicated the presence of

1 genetically diversified groups within the species. The genetic distance of *RAG1* by mean
2 uncorrected *p*-distance was 4.3 ± 0.8% between *Cobitis* and *Misgurnus*, 3.1 ± 0.6% between
3 *M. anguillicaudatus* and *M. mizolepis*, 2.6 ± 0.6% between clade A and B, and 2.4 ± 0.6%
4 between sub-clades B-1 and B-2.
5 Among *M. anguillicaudatus* and *M. mizolepis* specimens examined, 20 genotypes
6 were detected in the *RAG1* gene locus (Table 2). In wild-type diploids of *M.*
7 *anguillicaudatus* from site nos. 2 to 5, 7, 9, 10, and 12, the individuals were either
8 homozygous (*Rag1-01/Rag1-01, Rag1-02/Rag1-02*) or heterozygous (*Rag1-01/Rag1-02,*
9 *Rag1-01/Rag1-11, Rag1-01/Rag1-12*) for the *RAG1* alleles of the sub-clade B-1. All these
10 genotypes had mtDNA-CR sequences classified in the B-1 group. Loaches from site no.8
11 were either homozygous (*Rag1-13/Rag1-13*) or heterozygous (*Rag1-13/Rag1-14,*
12 *Rag1-13/Rag1-16, Rag1-14/Rag1-15*) for the *RAG1* alleles of the sub-clade B-2. All these
13 genotypes contained an mtDNA-CR classified as B-2. In 13 loaches from site no. 1, five
14 wild-type diploids were either homozygous (*Rag1-05/Rag1-05*) or heterozygous
15 (*Rag1-03/Rag1-04, Rag1-04/Rag1-05, Rag1-05/Rag1-06*) for the *RAG1* alleles of clade A.
16 The wild-type diploids with *RAG1* alleles of the clades A, B-1, and B-2 were categorized
17 into genetic groups A, B-1, and B-2, as inferred from mtDNA-CR, respectively (Table 2).
18 On the other hand, clonal diploid loaches from site nos. 1, 7, and 11 showed the
19 characteristic *RAG1* genotype: all clonal individuals were heterozygous for clade A and
20 clade B-1 (*Rag1-01/Rag1-07, Rag1-07/Rag1-10*), i.e., hybrids between genetically
21 different clades. In site no. 11, however, wild-type diploid loaches with homozygous
22 (*Rag1-10/Rag1-10*) or heterozygous genotypes (*Rag1-01/Rag1-09, Rag1-01/Rag1-10,*
23 *Rag1-01/Rag1-11*) within the same B-1 clade showed clone-specific mtDNA-CR.
24 The 575-bp *IRBP2* gene sequence was determined in 35 individuals of *M.*
25 *anguillicaudatus* and one individual of *M. mizolepis*. Consequently, 15 different alleles
26 were detected in *M. anguillicaudatus* specimens and all these sequences were deposited
27 in DDBJ/GenBank/EMBL under accession numbers AB702651 to AB702665, with allele
28 names serially designated as*IRBP2-01* to *IRBP2-15*. Two sequences (AB704300 and
29 704301) from a *M. mizolepis* specimen were also deposited in DDBJ/GenBank/EMBL
30 and designated as *IRBP2-a* and *IRBP2-b*, respectively. ML phylogenetic analysis
31 resulted in the construction of a tree (Fig. 3), which clustered at two major clades, A and
32 B. The *IRBP2* sequences of *M. anguillicaudatus* were clearly distinguished from those
33 of *M. mizolepis* and indicated the presence of two genetically differentiated groups in *M.*
34 *anguillicaudatus*. Further sub-clade analysis was not possible using the phylogenetic
35 tree. The genetic distance of *IRBP2* by mean uncorrected *p*-distance was 5.3 ± 0.7%
36 between *Cobitis* and *Misgurnus*, 3.8 ± 0.6% between *M. anguillicaudatus* and *M.*

mizolepis, and 2.9 ± 0.6% between clades A and B.

A total 15 genotypes were detected in the *IRBP2* gene locus (Table 2). Wild-type diploid samples from site nos. 2 to 5, 7, and 8 were either homozygous (*IRBP2-01/ IRBP2-01, IRBP2-02/ IRBP2-02, IRBP2-06/ IRBP2-06*) or heterozygous (*IRBP2-01/ IRBP2-02, IRBP2-02/ IRBP2-04, IRBP2-05/ IRBP2-06, IRBP2-14/ IRBP2-15*) for *IRBP2* sequences of the clade B. All these genotypes harbored an mtDNA-CR that was classified as belonging to either the B-1 or B-2 subgroup. In loaches from site no. 1, five diploid included only the *IRBP2* alleles of clade A (*IRBP2-11/ IRBP2-12, IRBP2-12/ IRBP2-13, IRBP2-10/ IRBP2-12*). The wild-type loach individuals with *IRBP2* alleles of the clades A and B were categorized into genetic groups A and B, as inferred from mtDNA-CR, respectively.

On the other hand, all the clonal diploid individuals from site nos. 1, 7, and 11 exhibited heterozygosity between alleles from clades A and B of the *IRBP2* gene (*IRBP2-05/ IRBP2-08, IRBP2-06/ IRBP2-09*). However, in wild-type diploid loaches from site no. 11, four individuals showed *IRBP2* gene sequences exclusively from clade B (*IRBP2-06/ IRBP2-06*), although these showed an mtDNA-CR belonging to group A (Table 2).

DISCUSSION

Although recent molecular phylogenetic studies using both nuclear genes and mitochondrial DNA markers suggest that the entire mitochondrial genome of one of the two major groups in Japanese *M. anguillicaudatus* should have been replaced by that of the genus *Cobitis* due to introgression after a hybridization event that occurred approximately 7 to 10 million years ago (Slechtova et al., 2008; Kitagawa et al., 2011; Pardices et al., 2012), both *RAG1* and *IRBP2* sequences examined in this study were clustered into two clades of the same species. Among wild-type diploid *M. anguillicaudatus* specimens within clade A of both *RAG1* and *IRBP2* gene sequences were categorized as group A, as inferred from its mtDNA-CR, whereas those within clade B of both gene sequences as that of group B. Specimens within the subclade B-1 of *RAG1* and those within the subclade B-2 also correspond to the B-1 and B-2 groups, as inferred from the mtDNA-CR, respectively. Thus, genetic results from mtDNA and nuclear gene sequence analyses strongly suggest the presence of different groups within *M. anguillicaudatus.*

The inter-clade genetic distance for *RAG1* (2.6 ± 0.6%) estimated in the present study was slightly higher than the inter-specific genetic distance reported for several

Misgurnus species (2.1 ± 0.8%; Perdices et al., 2012) and similar to that for the *Cobitis* species (2.5 ± 0.5%; Perdices et al., 2012). A similar inter-clade distance was also estimated in the present study for *IRBP2* (2.9 ± 0.6%). Molecular systematics of Asian and Far Eastern *Misgurnus* are poorly understood (Perdices et al., 2012), but present *RAG1* and *IRBP2* genetic distances between clades of the Japanese *M. anguillicaudatus* suggest the presence of genetically diverse groups or cryptic species in loaches.

Wild-type loaches were homozygous or heterozygous for *RAG1* and *IRBP2* gene alleles within the same clade. In contrast, all the clonal diploid individuals that were identified by mtDNA-CR haplotype, microsatellite genotype analyses and RAPD fingerpinting showed heterozygous genotypes between clades A and B alleles in both *RAG1* and *IRBP2* genes. These results show that natural clonal diploid loaches may have occurred from an earlier hybridization event between the loaches from different genetic groups (Fig. 4). Hybridization presumably caused a disruption of normal meiosis and subsequent irregular gametogenesis because of the failure of pairing between chromosomes, with each set originating from a different species or genetic group. However, in the inter-populational (or inter-clade) *Misgurnus* hybrids, females might have generated genetically identical unreduced eggs to recover fertility, and very few such eggs might have acquired the developing mode of gynogenesis through mutation for clonal reproduction. The unreduced diploid egg formation was reported in inter-populational *M. anguillicaudatus* hybrids between group A and B-1, as inferred from mtDNA-CR sequences (Arias-Rodriguez et al., 2009). Recently in the genus *Cobitis* spinous loaches, clonality and polyploidy were artificially synthesized by hybridization: F_1 hybrids between two bisexual species produced unreduced eggs, a few of which initiated gynogenesis in nature (Choleva et al., 2012). The formation of unreduced diploid eggs has also been reported in inter-specific hybridizations in *Salmo* (Johnson and Wright, '86), *Oryzias* (Shimizu et al., 2000), *Lepomis* (Dawley et al., '85) and in the inter-generic hybridization between *Carassius* and *Cyprinus* (Liu et al., 2001). As a cytological mechanism responsible for unreduced oogenesis, premeiotic endomitosis has been proposed because natural clonal diploid loaches were shown to form unreduced diploid eggs through this mechanism (Itono et al., 2006), probably occurring at an early germ cell stage (Yoshikawa et al., 2009 ; Morishima et al., 2012).

In specimens from site no. 11 (Hannoura, Notojima, Nanao City, Ishikawa Prefecture, site no. 33 in Morishima et al. 2008a), wild-type diploid loaches with clone-specific mtDNA-CR haplotypes occurred sympatrically with clonal diploid individuals, although these harbored *RAG1* and *IRBP2* gene sequences exclusively from clade B. The mitochondrial genome of clade B loaches may have thus been replaced by

1 that from the natural clonal lineage. One possible explanation to this occurrence is an
2 introgression of clone-specific mitochondrial genomes from clones to sympatric
3 wild-type diploid loaches. However, introgression of mitochondrial genome by
4 back-crossing of paternal species (group) to hybrids is not realistic, because most
5 hybrids from intergeneric crosses are often sterile or sometimes nonviable (reviewed in
6 Chevassus, '86). Inter-generic hybrids between *M. anguillicaudatus* and *Cobitis* species
7 have been reported to produce viable progeny (Minamori, '53; Kusunoki et al., '94),
8 although sterility in most hybrids has also been reported by Minamori ('57). It is also
9 been shown that inter-populational *Misgurnus* hybrids derived from genetically
10 different groups are generally sterile or undergo unusual reproduction. Experimental
11 *Misgurnus* hybrids from groups A and B-1 produce a small number of haploid eggs, a
12 large number of unreduced diploid eggs, and other genetically abnormal eggs, including
13 aneuploids and mosaicisms (Arias-Rodriguez et al., 2009). The inter-populational male
14 hybrids generated a very small number of fertile haploid or/and diploid spermatozoa
15 (Arias-Rodriguez et al., 2010). Similar results were also obtained in hybrids within the
16 genus *Cobitis* (Choleva et al., 2012). Production of functional gametes by hybrids is a
17 prerequisite for the introgression theory by subsequent backcrossing of fertile hybrids
18 with its original species or population.
19 Wild-type diploid genotypes with clone-specific mtDNA-CR are likely to occur
20 through meiotic hybridogenesis (Morishima et al., 2008b). Unreduced diploid eggs with
21 a heterozygous genotype (AB) are formed by a clone from a crossing between a clade A
22 female with clone-specific mtDNA and a clade B male (Fig. 4) and these diploid eggs
23 infrequently develop into triploid fish with genotype ABB: a haploid sperm from a
24 wild-type male from clade B was thus accidentally incorporated into an unreduced
25 diploid egg (genotype AB) of the clonal diploid (Fig. 5). These clone-derived triploid
26 loaches (genotype ABB) are known to produce haploid eggs by meiotic hybridogenesis
27 (Morishima et al., 2008b), i.e., elimination of an unmatched chromosome set from clade
28 A and pairing between two chromosome sets from clade B, followed by quasinormal
29 meiosis (Fig. 5). Thus, the occurrence of wild-type diploid loaches with chromosome sets
30 of clade B and a mtDNA-CR of group A in site no. 11 is well explained by this meiotic
31 hybridogenetic mechanism. Consequently, the formation of triploids from clonal
32 lineages and the subsequent reproduction through meiotic hybridogenesis presumably
33 produce bisexually-reproducing diploid loaches with clone-specific mtDNA. Therefore,
34 introgression of clonal mtDNA through meiotic hybridogenesis of clone-origin triploids
35 produces bisexually reproducing loaches with clone-derived group A mitochondrial
36 genomes

The present study concluded that the *RAG1* and *IRBP2* genotypes in natural clonal loaches, that generate unreduced diploid eggs with identical genotypes and develop through gynogenesis, arose through the hybridization of two genetically different groups of *M. anguillicaudatus*.

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LITERATURE CITED

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.

Arias-Rodriguez L, Morishima K, Arai K. 2006. Genetically diversified populations in the loach *Misgurnus anguillicaudatus* inferred from newly developed microsatellite markers. Mol Ecol Notes 7: 82-85.

Arias-Rodriguez L, Yasui GS, Arai K. 2009. Disruption of normal meiosis in artificial inter-populational hybrid female *Misgurnus* loach. Genetica 136: 49-56.

Arias-Rodriguez L, Yasui GS, Kusuda, S., Arai, K., 2010. Reproductive and genetic capacity of spermatozoa of inter-populational hybrid males in the loach, *Misgurnus anguillicaudatus.* J Appl Ichthyol 26: 653-658.

Asahida T, Kobayashi T, Saitoh K, Nakayama I. 1996. Tissue preservation and total DNA extraction from fish stored at ambient temperature using buffer containing high concentration of urea. Fish Sci 62: 727-730.

Chen WJ, Miya M, Saitoh K, Mayden RL. 2008. Phylogenetic utility of two existing and four novel nuclear gene loci in reconstructing tree of life of ray-finned fishes: the order Cypriniformes (Ostariophysi) as a case study. Gene 425:125-134.

Chevassus B. 1983. Hybridization in fish. Aquaculture 33: 245-262.

Choleva L, Janko K, De Gelas, K, Bohlen J, Šlechtová V, Rábová M, Ráb P. 2012. Synthesis of clonality and polyploidy in vertebrate animals by hybridization between two sexual species. Evolution 66-7: 2191-2203.

Collares-Pereira MJ, Matos I, Morgado-Santos M, Coelho MM. 2013. Natural pathways towards polyploidy in animals: the *Squalius alburnoides* fish complex as a model system to study genome size and genome reorganization in polyploids. Cytogenet Genome Res 140: 97-116.

Dawley RM. 1989. An introduction to unisexual vertebrates. In: Dawley RM, Bogart JP,

1 editors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum,
2 Albany. Bulletin 466, p. 1-18.
3 Dawley RM, Graham JH, Schultz RJ. 1985. Triploid progeny of pumpkinseed x green
4 sunfish hybrids. J Hered 76: 251-257.
5 Fujimoto T, Yasui GS, Yoshikawa H, Yamaha E, Arai K. 2008. Genetic and reproductive
6 potential of spermatozoa of diploid and triploid males obtained from interspecific
7 hybridization of *Misgurnus anguillicaudatus* female with *M. mizolepis* male. J Appl
8 Ichthyol 24: 430-437.
9 Gui JF, Zhou L, 2010. Genetic basis and breeding application of clonal diversity and
10 dual reproduction modes in polyploidy *Carassius auratus gibelio*. Sci China, Life
11 Science 53: 409-415.
12 Itono M, Morishima K, Fujimoto T, Bando E, Yamaha E, Arai, K. 2006. Premeiotic
13 endomitosis produces diploid eggs in the natural clone loach, *Misgurnus*
14 *anguillicaudatus* (Teleostei: Cobitidae) J Exp Zool 305A: 513-523.
15 Itono M, Okabayashi N, Morishima K, Fujimoto T, Yoshikawa H, Yamaha E, Arai K.
16 2007. Cytological mechanisms of gynogenesis and sperm incorporation in
17 unreduced diploid eggs of the clonal loach, *Misgurnus anguillicaudatus* (Teleostei:
18 Cobitidae). J Exp Zool 307A: 35-50.
19 Janko K, Kotusz J, De Gelas K, Šlechtová V, Opoldusová Z, Drozd P, Choleva L,
20 Popiołek M, Baláž M. 2012. Dynamic formation of asexual diploid and polyploidy
21 lineages: multilocus analysis of *Cobitis* reveals the mechanisms maintaining the
22 diversity of clones. PLoS One 7(9): e45384.
23 Johnson KR, Wright JE. 1986. Female brown trout x Atlantic salmon hybrids produce
24 gynogens and triploids when backcrossed to male Atlantic salmon. Aquaculture 57:
25 345-358.
26 Khan MMR, Arai K. 2000. Allozyme variation and genetic differentiation in the loach,
27 *Misgurnus anguillicaudatus.* Fish Sci 66: 211-222.
28 Kimura M. 1980. A simple method for estimating evolutionary rates of base
29 substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:
30 111-120.
31 Kitagawa T, Fujii Y, Koizumi N. 2011. Origin of the two major distinct mtDNA clades of
32 the Japanese population of the oriental weather loach *Misgurnus anguillicaudatus*
33 (Teleostei: Cobitidae). Folia Zool 60: 340-346.
34 Koizumi N, Takemura T, Watabe K, Mori A. 2009. Genetic variation and diversity of
35 Japanese loach inferred from mitochondrial DNA—phylogenetic analysis using the
36 cytochrome *b* gene sequence. Trans. Jap. Soc. Irrig. Drain. Rural Engin. 77, 7-16.

1 Kusunoki T, Arai K, Suzuki R. 1994. Viability and karyotypes of interracial and
2 intergeneric hybrids in loach species. Fish Sci 60:415-422.
3 Liu S, Liu Y, Zhou G, Zhang X, Luo C, Feng H, He X, Zhu G, Yang H. 2001. The
4 formation of tetraploid stocks of red crucian carp x common carp hybrids as an
5 effect of interspecific hybridization. Aquaculture 192: 171-186.
6 Minamori S. 1953. Physiological i-solation in cobitidae II. Inviability of hybrids between
7 the mud loach and some local races of spinous loaches. J. Sci. Hiroshima Univ.,
8 Series B, Div. 1, 14: 125-149.
9 Minamori S. 1957. Physiological isolation in cobitidae V. Sterility of the hybrids between
10 the mud loach and six races of spinous loaches. J. Sci. Hiroshima Univ., Series B,
11 Div. 1, 17: 55-65.
12 Morishima K, Horie S, Yamaha E, Arai K. 2002. A cryptic clonal line of the loach
13 *Misgurnus anguillicaudatus* (Teleostei: Cobitidae) evidenced by induced
14 gynogenesis, interspecific hybridization, microsatellite genotyping and multilocus
15 DNA fingerprinting. Zool Sci 19: 565-575.
16 Morishima K, Nakamura-Shiokawa Y, Bando E, Li YJ, Boron A, Khan MMR, Arai K.
17 2008a. Cryptic clonal lineages and genetic diversity in the loach *Misgurnus*
18 *anguillicaudatus* (Teleostei: Cobitidae) inferred from nuclear and mitochondrial
19 DNA analyses. Genetica 132:159-171.
20 Morishima K, Yoshikawa H, Arai K, 2008b. Meiotic hybridogenesis in triploid
21 *Misgurnus* loach derived from a clonal lineage. Heredity 100: 581-586.
22 Morishima K, Nakayama I, Arai K. 2008c. Genetic linkage map of the loach *Misgurnus*
23 *anguillicaudatus* (Teleostei: Cobitidae). Genetica 132: 227-241.
24 Morishima K, Yoshikawa H, Arai K. 2012. Diploid clone produces unreduced diploid
25 gametes but tetraploid clone generates reduced diploid gametes in the *Misgurnus*
26 loach. Biol Reprod 86(2): 33, 1-7.
27 Perdices A, Vasil'ev V, Vasil'eva E. 2012. Molecular phylogeny and intraspecific
28 structure of loaches (genera *Cobitis* and *Misgurnus*) from the Far East region of
29 Russia and some conclusion on their systematic. Ichthyol Res 59:113-123.
30 Quenouille B, Bermingham E, Planes S. 2004. Molecular systematic of the damselfishes
31 (Teleostei: Pomacentridae): Bayesian phylogenetic analyses of mitochondrial and
32 nuclear DNA sequences. Mol Phylogenet Evol 31, 66-68.
33 Saitoh K. 1989. Asian Pond loach in: Kawanabe H, Mizuno N, editors. Freshwater
34 Fishes of Japan. Yamakei Pub, Tokyo. p. 382-385.
35 Saitoh K, Chen WJ, Mayden RL. 2010. Extensive hybridization and tetraploidy in
36 spined loach fish. Mol Phylogenet Evol. 56: 1001-1010.

Shimizu Y, Shibata N, Sakaizumi M, Yamashita M. 2000. Production of diploid eggs through premeiotic endomitosis in the hybrid medaka between *Oryzias latipes* and *O. curvinotus*. Zool Sci 17, 951-958.

Sinclair EA, Pramuk JB, Bezy RL, Crandall KA, Sites JW Jr. 2010. DNA evidence for nonhybrid origins of parthenogenesis in natural populations of vertebrates. Evolution 64: 1346-1357.

Šlechtová V, Bohlen J, Perdices A. 2008. Molecular phylogeny of the freshwater fish family Cobitidae (Cypriniformes: Teleostei): Delimination of genera, mitochondrial introgression and evolution of sexual dimorphism. Mol Phylogenet Evol 47: 812-831.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony method. Mol Biol Evol 28: 2731-2739.

Vrijenhoek RC. 1994. Unisexual fish: Model systems for studying ecology and evolution. Annu Rev Ecol Syst 25: 71-96.

Vijenhoek RC, Dawley RM, Cole CJ, Bogart JP. 1989. A list of the known unisexual vertebrates. In: Dawley RM, Bogart JP,ediors. Evolution and Ecology of Unisexual Vertebrates. New York State Museum, Albany. Bulletin 466, p. 19-23.

Yang Z. 1994. Maximum likelihood phylogenetic estimation from DNA sequences with variable rates over: approximate methods. J Mol Evol 39: 306-314.

Yoshikawa H, Morishima K, Fujimoto T, Saito T, Kobayashi E, Yamaha E, Arai K. 2009. Chromosome doubling in early spermatogonia produces diploid spermatozoa in a natural clone fish. Biol Reprod 80: 973-979.

Zhou X, Yu Y, Li Y, Wu J, Zhang X, Guo X, Wang W. 2014. Comparative analysis of mitochondrial genomes in distinct nuclear ploidy loach *Misgurnus anguillicaudatus* and its implications for polyploidy evolution. PLoS ONE 9(3): e92033.

Figure legends

Fig. 1. RAPD-PCR fingerprints using random primers WAKO-01(A), OPA-11(B) and OPA-12(C). Left gels: Lanes 1-4 denote reference control samples of clone 1, 2, 3 and 4, respectively. Lane 5, blank. Lanes 6 -13 denote fish no. 1 - 8 from site no.1 (see Table 1 and 2). Central gels: Lanes 1 - 3 denote fish no. 7, 8 and 9 from site no. 7 (see Table 1 and 2). Right gels: Lanes 1-4 denote reference control samples of clone 1, 2, 3 and 4, respectively. Lane 5, blank. Lanes 6 -13 denote fish no. 1 - 9 from site no. 11 (see Table 1 and 2). M in a gel indicates size marker. Left bars also indicate molecular size marker of 3.0, 1.5, 1.0 and 0.5 kbp.

Fig. 2. ML phylogenetic tree using *RAG1* sequences. The numbers on the branches are bootstrap values.

Fig. 3. ML phylogenetic tree using *IRBP2* sequences. The numbers on the branches are bootstrap values.

Fig. 4. A scenario for the formation of natural clonal diploids through the hybridization of a group A female and a group B male in *Misgurnus* loaches. III in figure means clone-specific mitochondrial DNA control region haplotype transmitted from A group female.

Fig. 5. A scenario of introgression of clone-specific mtDNA from clonal diploid loaches to sexual diploid loaches through meiotic hybridogenesis of triploid progeny with one group A genome and two group B genomes, accidentally formed by incorporation of a group B sperm nucleus into a diploid unreduced egg from a clonal diploid loach. III in figure means clone-specific mitochondrial DNA control region haplotype transmitted from A group female.

Table 1. Sampling sites, sample size and year of sampling of specimens used in the present

the present study

Site no.	Prefecture	Locality City/Town/Village (Former name) [Site no. in Morishima *et al.*(2008a)]	N	Year of sampling
1	Hokkaido	Ozora (Memanbetsu) [5]	12 (1)[1]	1998/2009
2	Hokkaido	Iwamizawa [8]	8	1996
3	Aomori	Yomogita	2	2009
4	Iwate	Hanamaki [18]	2	2004
5	Yamagata	Tsuruoka [20]	2	2004
6	Miyagi	Osaki (Naruko) [22]	1	1998
7	Niigata	Niigata (Maki) [30]	9	1996
8	Saitama	Hanyu [27]	4	1998
9	Aichi	Inazawa [40]	2	2003
10	Mie	Yokkaichi [42]	2	2004
11	Ishikawa	Nanao[2] [33]	9	2009
12	Shimane	Izumo [49]	1	1997
Total			56	

[1] Number of triploid individual is indicated in parentheses

[2] Hannoura region, Notojima island.

Table 2. Clones and wild-type diploids inferred from mtDNA-CR haplotypes, microsatellite genotypes and RAPD fingerprinting and clade A and B alleles of *RAG1* and *IRBP2* loci

Site no.	Fish no.	mtDNA-CR				Identification of Clone[3]	*RAG1* Genotype[5]		*IRBP2* Genotype[5]	
		RFLP	Haplotype (444-448 bp)	Group[1]	Clone-specific Haplotype[2]		Clade A allele	Clade B allele	Clade A allele	Clade B allele
1	1	III	III-1, III-2	A	+	**Clone 1**	7	1	9	6
	2	I	I-3, I-4, I-5	A	-	-	4 5	-	11 12	-
	3	III	III-1, III-2	A	+	**Clone 1**	7	1	9	6
	4	III	III-1, III-2	A	+	**Clone 1**	7	1	9	6
	5	III	III-1, III-2	A	+	**Clone 1**	7	1	9	6
	6	IV	IV-1	A	-	-	5 5	-	11 12	-
	7	III	III-1, III-2	A	+	**Triploid** [4]	7	1	.n.d.[6]	
	8	III	III-1, III-2	A	+	**Clone 1**	7	1	9	6
	9	I	I-3, I-4, I-5	A	-	-	3 4	-	n.d.	
	10	I	I-7	A	-	-	5 5	-	12 13	-
	11	I	I-7	A	-	-	5 6	-	10 12	-
	12	I	I-7	A	-	-	.n.d.		10 12	-
2	1	V	V-1, V-4	B-1	-	-	-	1 1	-	1 1
	2	V	V-3	B-1	-	-	-	1 2	-	1 2
	3	V	V-1, V-4	B-1	-	-	-	2 2	-	2 2
	4	V	V-1, V-4	B-1	-	-	-	1 2	-	1 2
	5	V	V-1, V-4	B-1	-	-	-	2 2	n.d.	
	6	V	V-1, V-4	B-1	-	-	-	1 1	-	1 2
	7	V	V-1, V-4	B-1	-	-	-	2 2	n.d.	
	8	V	V-1, V-4	B-1	-	-	-	2 2	-	2 3
3	1	V	V-5, V-6, V-24	B-1	-	-	-	1 1	-	1 2
	2	V	V-5, V-6, V-24	B-1	-	-	-	1 12	-	1 2
4	1	V	V-2, V-21	B-1	-	-	-	1 12	-	2 4
	2	V	V-27	B-1	-	-	-	1 1	-	2 4

5	1	V	V-5, V-6, V-24	B-1	-	-	-	1 1	n.d.	
	2	V	V-5, V-6, V-24	B-1	-	-	-	1 1	-	2 2
6	1	X	n.d.	A	-	-	8 8		7 7	
7	1	V	V-23	B-1	-	-	-	1 1	-	6 6
	2	VII	VII-8, VII-15	B-1	-	-	-	1 1	n.d.	
	3	V	V-23	B-1	-	-	-	1 1	n.d.	
	4	VII	VII-8, VII-15	B-1	-	-	-	1 1	n.d.	
	5	V	V-5, V-6, V-24	B-1	-	-	-	1 1	n.d	
	6	V	V-23	B-1	-	-	-	1 1	n.d.	
	7	IX	IX-1	B-1	-	-	-	1 1	n.d.	-
	8	V	V-23	B-1	-	-	n.d.		-	6 6
	9	III	III-1, III-2	A	+	**Clone 2**	7	1	9	6
8	1	VII	VII-1, VII-12	B-2	-	-	-	14 15	n.d.	
	2	VII	VII-4, VII-9	B-2	-	-	-	13 13	n.d.	
	3	VII	VII-14	B-2	-	-	-	13 14	n.d.	
	4	VII	VII-1, VII-12	B-2	-	-	-	13 16	-	14 15
9	1	V	V-19, V-26	B-1	-	-	-	1 1	n.d.	
	2	V	V-23	B-1	-	-	-	1 1	n.d.	-
10	1	V	V-20	B-1	-	-	-	1 11	d.d.	-
	2	V	V-20	B-1	-	-	-	1 11	n.d	-
11	1	III	III-1, III-2	A	+	**Clone 4**	7	10	8	5
	2	III	III-1, III-2	A	+	-	-	1 9	-	6 6
	3	III	III-1, III-2	A	+	-	-	10 10	-	6 6
	4	III	III-1, III-2	A	+	-	-	1 9	-	5 6
	5	V	V-7	B-1	-	-	-	1 10	-	5 6
	6	III	III-1, III-2	A	+	-	-	1 10	-	6 6
	7	VII	VII-7	B-1	-	-	-	1 9	-	5 6
	8	V	V-7	B-1	-	-	-	1 10	-	6 6
	9	III	III-1, III-2	A	+	**Clone 3**	7	1	8	5
12	1	VII	VII-11	B-1	-	-	-	1 1	n.d.	

[1] Group inferred from mtDNA-CR haplotypes (RFLP, sequences) by Morishima *et al.* (2008a).

[2] Presence (+) or absence (-) of clone-specific mtDNA haplotypes based on Morishima *et al.* (2008a).

[3] Clone-candidates were first screened for presence of clone-specific mtDNA haplotypes and then identified as clone 1 to 4 based

on identical microsatellite genotypes as shown in Table 3 and identical RAPD fingerprints as shown Figure 1. Symbol "-" means non-clonal wild-type.

[4] Triploid probably derived from clone 1 diploid by accidental sperm nucleus incorporation into diploid egg.

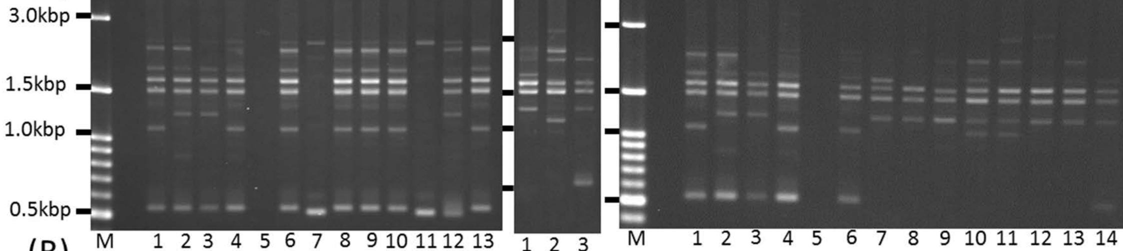
[5]Symbol "-" means absence of allele categorized to clade A or B.

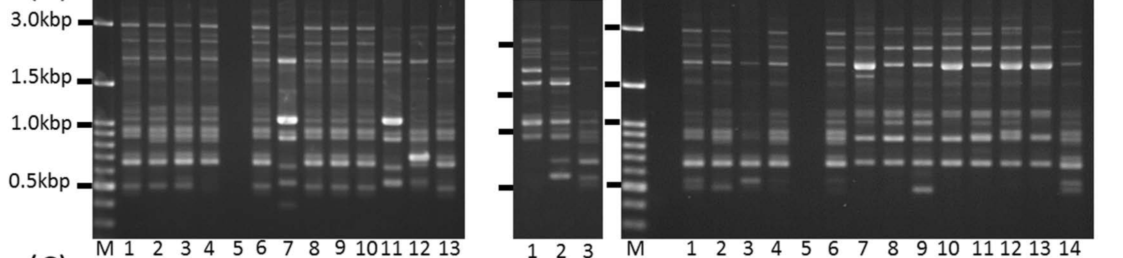
[6]Not determined

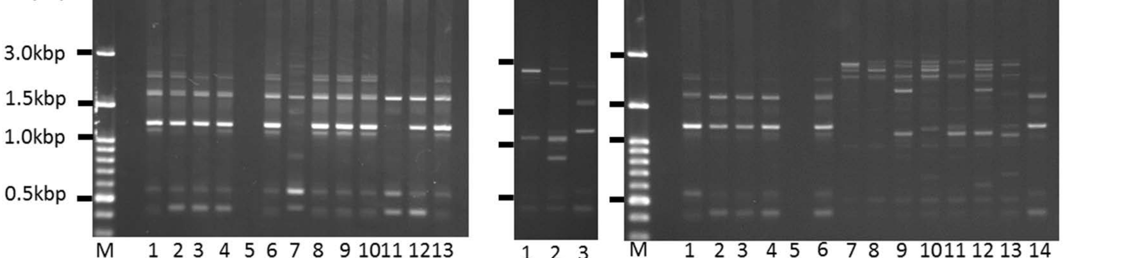
Table 3. Identification of clonal individuals by matching of genotypes of five independent microsatellite loci with those of reference control samples identified as clones 1 - 4 by Morishima et al. (2002, 2008ab, 2012)

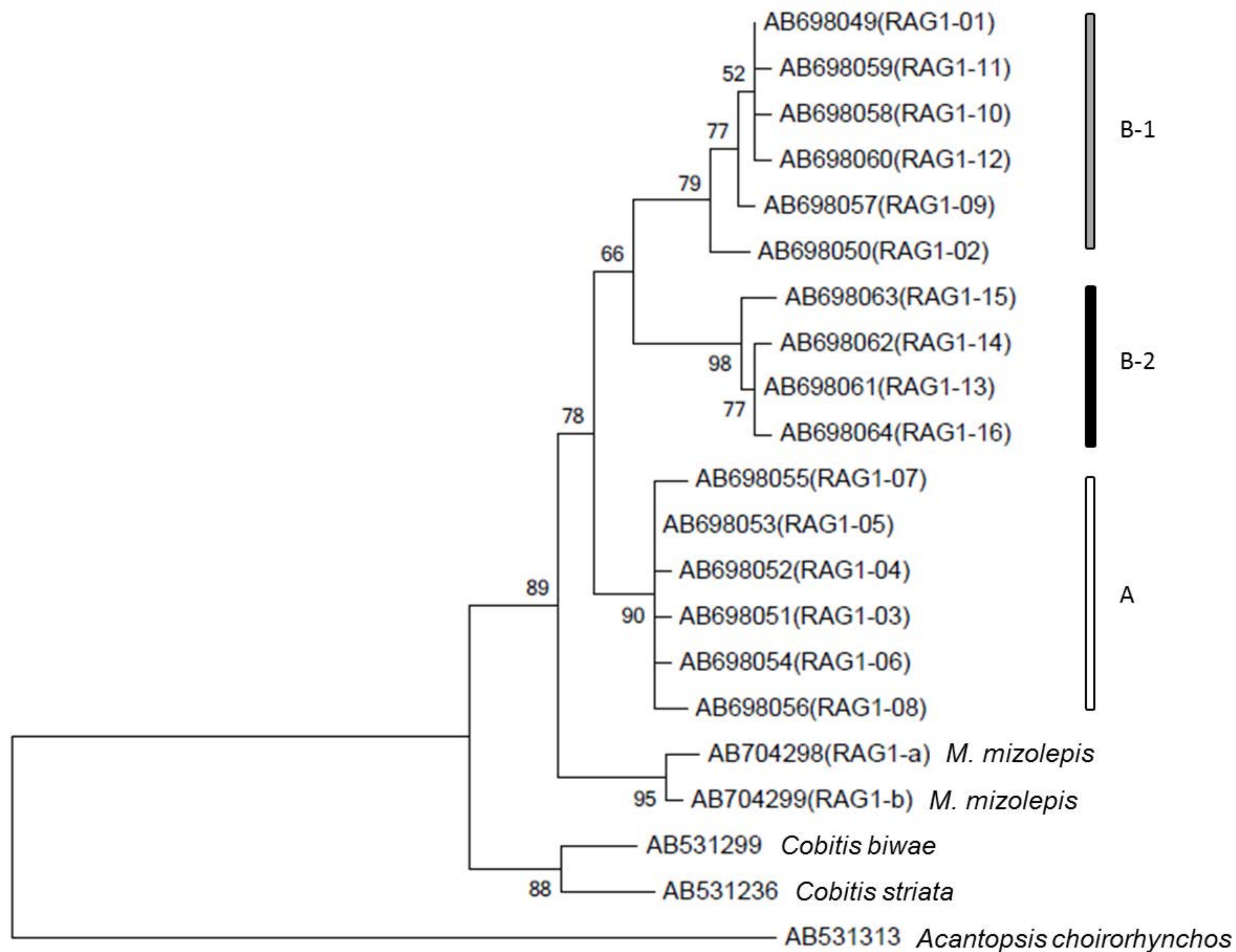
Site No.	Fish No.	Microsatellite locus (Linkage group)					Identification of clone
		Mac 3(12)	Mac37 (10)	Mac404 (4)	Mac458 (16)	Mac477 (11)	
Reference control	clone 1	103/145	101/107	207/211	224/232	127/192	clone 1
	clone 2	103/145	101/105	213/223	232/232	124/202	clone 2
	clone 3	103/161	101/107	203/211	228/234	124/196	clone 3
	clone 4	103/161	101/107	203/211	216/238	124/196	clone 4
1	1*	103/145	101/107	207/211	224/232	124/192	clone 1
	2	113/113	99/105	195/199	228/244	126/126	
	3*	103/145	101/107	207/211	224/232	124/192	clone 1
	4*	103/145	101/107	207/211	224/232	124/192	clone 1
	5*	103/145	101/107	207/211	224/232	124/192	clone 1
	6	117/117	79/85	181/189	250/264	126/126	
	7*	103/113/145	93/101/105	201/213/223	232/260	124/126/202	clone 2 origin 3n
	8*	103/145	101/107	207/211	224/232	124/192	clone 1
7	7	123/151	105/109	193/243	234/264	136/140	
	8	109/163	107/107	193/201	212/260	138/202	
	9*	103/145	101/105	213/223	232/232	124/202	clone 2
11	1*	103/161	101/107	203/211	216/238	124/196	clone 4
	2*	137/143	105/131	203/219	200/200	178/194	
	3*	135/135	105/107	219/219	218/236	196/216	
	4*	135/151	105/107	203/219	202/216	178/214	
	5	103/145	105/107	203/229	218/234	178/178	
	6*	105/135	105/131	203/205	200/200	178/196	
	7	137/151	115/117	203/219	202/228	194/212	
	8	103/143	105/107	203/219	202/230	178/216	
	9*	103/161	101/107	203/211	228/234	124/196	clone 3

* Samples with clone-specific mtDNA haplotypes

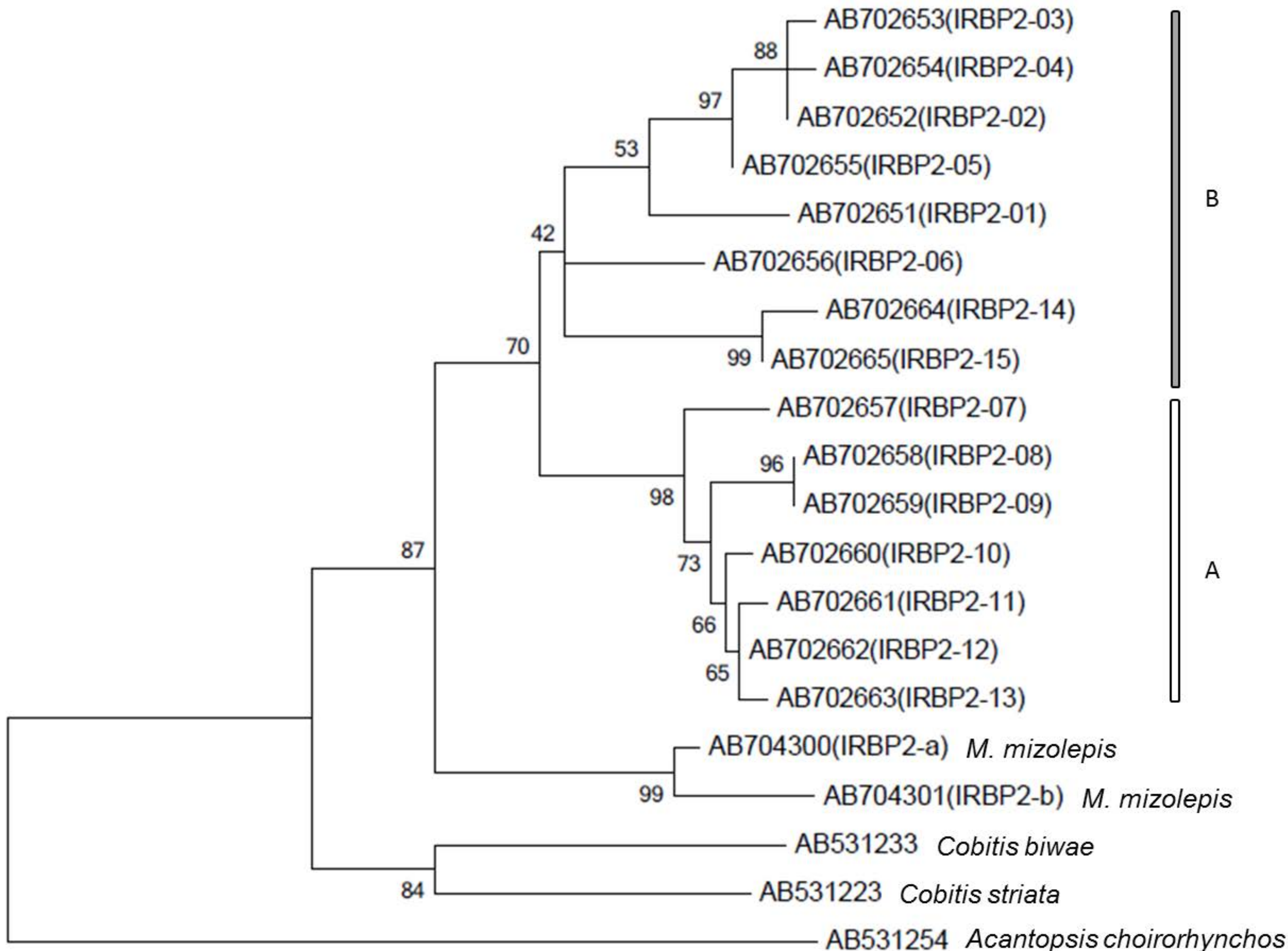

(A)
3.0kbp
1.5kbp
1.0kbp
0.5kbp
M 1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

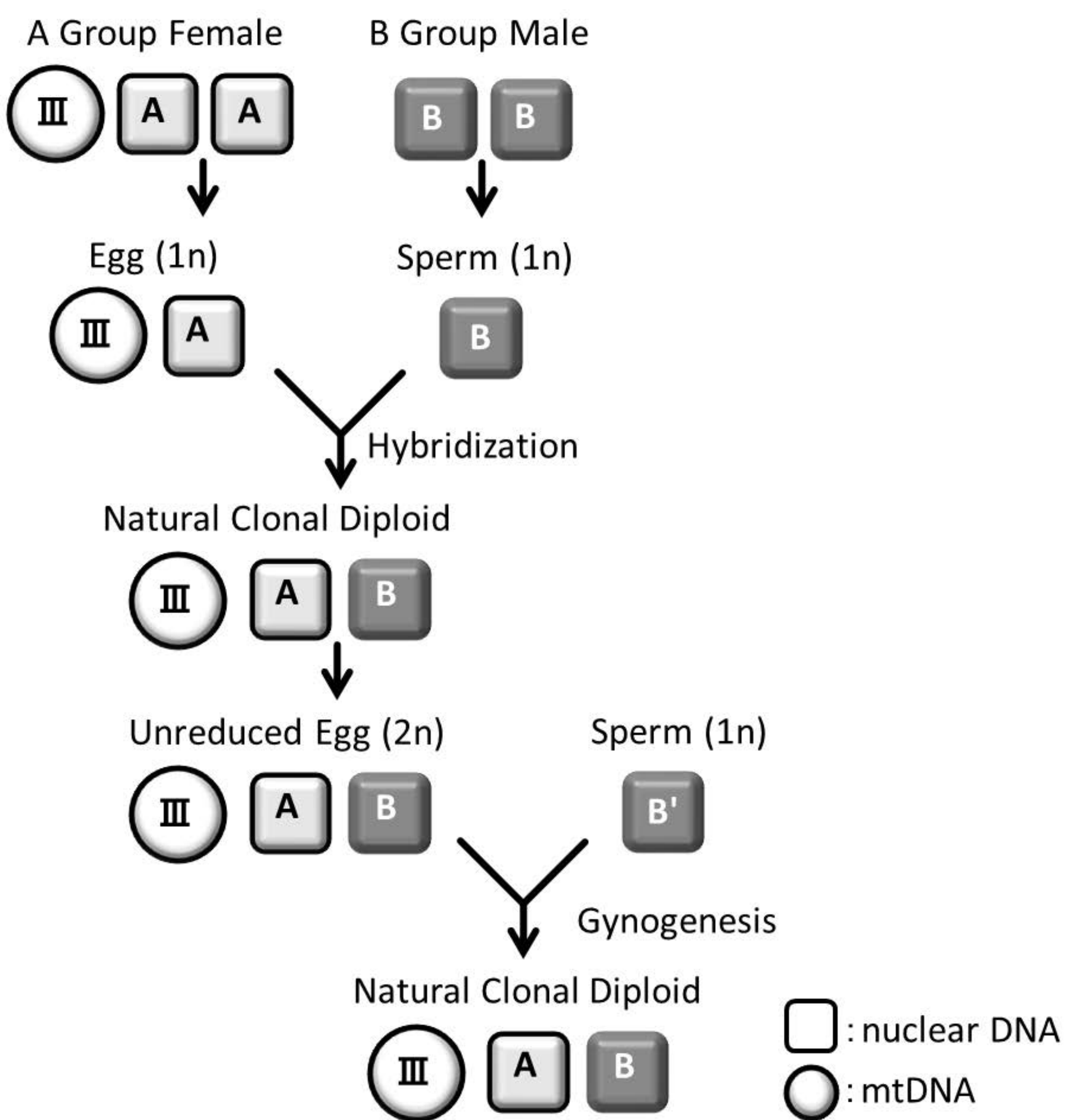

(B)
3.0kbp
1.5kbp
1.0kbp
0.5kbp
M 1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

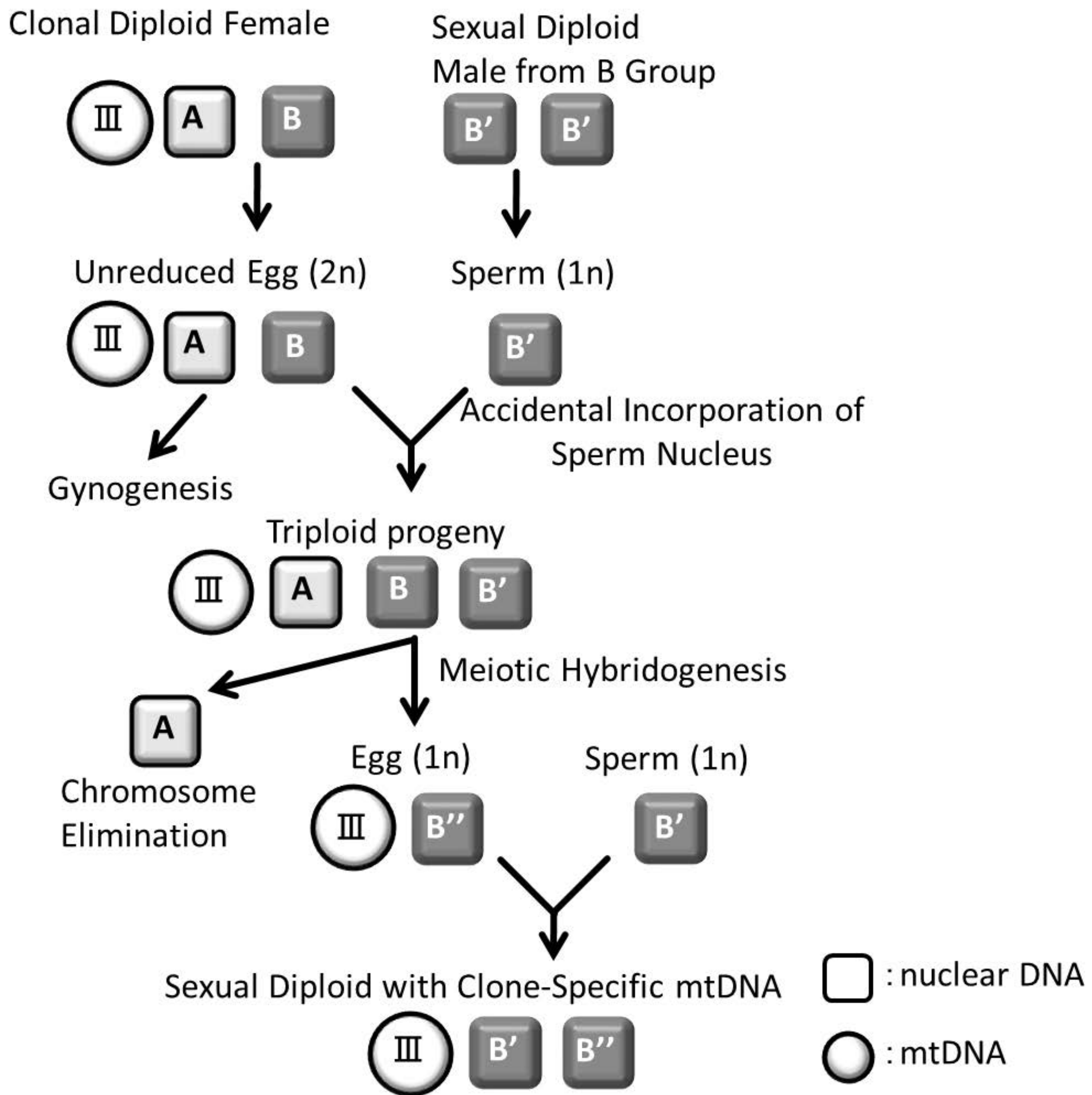

(C)
3.0kbp
1.5kbp
1.0kbp
0.5kbp
M 1 2 3 4 5 6 7 8 9 10 11 12 13
1 2 3
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14



AB698049(RAG1-01)
52
AB698059(RAG1-11)
AB698058(RAG1-10)
77
AB698060(RAG1-12)
79
AB698057(RAG1-09)
66
AB698050(RAG1-02)
AB698063(RAG1-15)
AB698062(RAG1-14)
98
AB698061(RAG1-13)
77
78
AB698064(RAG1-16)
AB698055(RAG1-07)
AB698053(RAG1-05)
AB698052(RAG1-04)
89
90
AB698051(RAG1-03)
AB698054(RAG1-06)
AB698056(RAG1-08)
AB704298(RAG1-a) M. mizolepis
95
AB704299(RAG1-b) M. mizolepis
AB531299 Cobitis biwae
88
AB531236 Cobitis striata
AB531313 Acantopsis choirorhynchos
0.02
B-1
B-2
A


AB702653(IRBP2-03)
AB702654(IRBP2-04)
AB702652(IRBP2-02)
AB702655(IRBP2-05)
AB702651(IRBP2-01)
AB702656(IRBP2-06)
AB702664(IRBP2-14)
AB702665(IRBP2-15)
AB702657(IRBP2-07)
AB702658(IRBP2-08)
AB702659(IRBP2-09)
AB702660(IRBP2-10)
AB702661(IRBP2-11)
AB702662(IRBP2-12)
AB702663(IRBP2-13)
AB704300(IRBP2-a) M. mizolepis
AB704301(IRBP2-b) M. mizolepis
AB531233 Cobitis biwae
AB531223 Cobitis striata
AB531254 Acantopsis choirorhynchos
88
97
53
42
99
70
98
96
73
66
65
87
99
84
B
A
0.01


A Group Female
Ⅲ
A
A
B Group Male
B
B
Egg (1n)
Ⅲ
A
Sperm (1n)
B
Hybridization
Natural Clonal Diploid
Ⅲ
A
B
Unreduced Egg (2n)
Ⅲ
A
B
Sperm (1n)
B'
Gynogenesis
Natural Clonal Diploid
Ⅲ
A
B
: nuclear DNA
: mtDNA


Clonal Diploid Female
Ⅲ
A
B
Sexual Diploid
Male from B Group
B′
B′
Unreduced Egg (2n)
Ⅲ
A
B
Sperm (1n)
B′
Accidental Incorporation of
Sperm Nucleus
Gynogenesis
Triploid progeny
Ⅲ
A
B
B′
Meiotic Hybridogenesis
A
Chromosome
Elimination
Egg (1n)
Ⅲ
B′′
Sperm (1n)
B′
Sexual Diploid with Clone-Specific mtDNA
Ⅲ
B′
B′′
: nuclear DNA
: mtDNA