Research Article

Title

Fluorescence *in situ* hybridization identifies chromosome differentiation between contemporary genomes of wild types and the ancestral genome of unisexual clones of dojo loach, *Misgurnus anguillicaudatus*

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Short Title

Chromosome differentiation between contemporary and ancestral genomes in fish

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Number of Tables: 0

Number of Figures: 5

Word count: 4766

Keywords

Chromosome differentiation, Hybrid, Polyploidy, Unisexual fish, Unreduced gametes

Abstract

In dojo loach (*Misgurnus anguillicaudatus*), although most wild types are gonochoristic diploids that are genetically differentiated into two groups, A and B, clonal lineages appear in certain localities. Clonal loaches have been considered to have hybrid origins between the two groups by a series of genetic studies. In this study, using fluorescence *in situ* hybridization (FISH) with a newly developed probe (ManDra-A), we identified 26 (1 pair of metacentric and 12 pairs of telocentric chromosomes) of 50 diploid chromosomes in contemporary wild type group A loach. In contrast, ManDra-A signals were not detected on metacentric chromosomes derived from the ancestral group A of clonal loach. The FISH results clearly showed the presence of certain differentiations in metacentric chromosomes between ancestral and contemporary group A loach. Two-color FISH with ManDra-A and group B specific ManDra (renamed ManDra-B) probes reconfirmed the hybrid origin of clones by identifying chromosomes from both groups A and B in metaphases. Our results showed the hybrid origin of clonally reproducing fish and the possibility that chromosomal differentiation between ancestral and contemporary fish can affect gametogenesis. In meiotic spermatocytes of sex-reversed clones, ManDra-A, and not ManDra-B, signals were detected in 12 out of 50 bivalents. Thus, the results further support the previous conclusion that clonal gametogenesis was assured by pairing between sister chromosomes duplicated from each ancestral chromosome from group A or B. Our study deepens knowledge about the association between clonality and hybridity in unisexual vertebrates.

Introduction

Wild type dojo loach, *Misgurnus anguillicaudatus* (Cobitidae; Teleostei), have gonochoristic diploidy (2n = 50 chromosomes). It reproduces bisexually in most Japanese populations, whereas unisexual clonal lineages inhabit certain regions of Hokkaido and Ishikawa Prefectures, Japan [Morishima et al., 2002, 2008a; Arai and Fujimoto, 2013]. Clonal loach lay unreduced diploid eggs that develop gynogenetically without any genetic contribution of the sperm genome [Itono et al., 2006, 2007; Arai and Fujimoto, 2013]. Such clonal lineages may have arisen from past hybridization events between genetically diversified groups A (genomic constitution of AA) and B (BB), as shown by the heterozygosity of the *RAG1* and *IRBP2* sequences [Yamada et al., 2015]. Repetitive sequences were previously isolated from the genomic DNA of group B loach using the restriction enzyme *Dra*I. The repetitive sequences named ManDra ("Man" comes from *M. anguillicaudatus* and "Dra" comes from a restriction enzyme *Dra*I) were used as a nuclear DNA marker (hereafter designated as ManDra-B in this paper) [Fujimoto et al., 2017]. Karyological differences were not morphologically detected among gonochoristic diploid wild type group A, group B, and clonal dojo loaches, since they have the same (2n = 50) chromosomes, categorized into 10 metacentric, 4 submetacentric, and 36 telocentric chromosomes [Itono et al., 2006]. When we performed fluorescence *in situ* hybridization (FISH) using the ManDra-B sequence as a probe in somatic cells of clonal diploids (2n = 50), a haploid set of chromosomes (n = 25) from the group B loach, clear ManDra-B signals specific to the centromeric region were detected; however, another haploid set of chromosomes from group A loach was not identified [Kuroda et al., 2018]. Thus, the hybrid origin of the clonal loach was cytogenetically proven, and the genomic constitution of clonal loach has been designated as the heterozygous genotype AB [Yamada et al., 2015; Fujimoto et al., 2017; Kuroda et al., 2018].

In contrast, hybrids from laboratory crosses between the two extant groups A and B (AB) show different reproductive features compared to clonal loach (AB). Hybrid females were induced to confirm whether they could produce gynogenetically developing diploid eggs, such as clonal loaches [Arias-Rodriguez et al., 2009]. Some hybrid females mainly laid diploid eggs, while others laid haploid, aneuploid, and polyploid eggs together with diploid eggs [Arias-Rodriguez et al., 2009]. Moreover, the diploid eggs never developed by gynogenesis but generated triploid progeny after incorporating a sperm nucleus at the time of fertilization [Arias-Rodriguez et al., 2009]. Artificially sex-reversed clonal males were fertile and produced isogenic diploid spermatozoa [Yoshikawa et al., 2007, 2009; Kuroda et al., 2018]. However, inter-group hybrid males showed post-zygotic sterility because they produced non-motile spermatozoa or

spermatozoon-like cells with morphological abnormalities, and the cell populations of testis and semen were composed of various ploidy levels (haploid, diploid, and tetraploid) [Arias-Rodriguez et al., 2010]. In European spined loach (genus *Cobitis*), clonal lineages of hybrid origin between different species are present [Janko et al., 2007a]. The clonal diploids produce unreduced eggs, which develop by gynogenesis [Janko et al., 2007a, b]. Artificially induced hybrid females also produce isogenic diploid eggs [Choleva et al., 2012]. Although most of these unreduced eggs developed into triploid fish by fertilization with haploid sperm, a very small portion of fishes from only a single backcross family were genetically identical to their mother since they developed by gynogenesis [Choleva et al., 2012].

In Poeciliidae, two genera (*Poecilia* and *Poeciliopsis*) contain unisexuals. The clonal fish Amazon molly, *Poecilia formosa*, was discovered to be the first unisexual vertebrate that reproduces by gynogenesis [Hubbs and Hubbs, 1932]. Amazon molly arose from hybridization between two sexual species, Atlantic molly, *P. mexicana* and sailfin molly, *P. latipinna* [Avise et al., 1991; Schartl et al., 1995b]. Despite a large number of crossing experiments, gynogenetic hybrids could never be synthesized from laboratory crosses between extant *P. mexicana* and *P. latipinna* [Stöck et al., 2010]. Hemi-clonal *Poeciliopsis monacha-lucida* is an all-female fish originating from past hybridization between *P. monacha* and *P. lucida* [Schultz, 1969; Cimino, 1972]. However, artificial hybrids from laboratory crosses between *P. monacha* and *P. lucida* showed a low survival rate [Schultz, 1973]. Natural hybrids of hemi-clonal greenling (*Hexagrammos* species) produce haploid eggs containing only the maternal genome after elimination of the paternal genome by hybridogenesis [Kimura-Kawaguchi et al., 2014]. However, artificial hybrids between extant parental species produce recombinant gametes by regular meiosis [Kimura-Kawaguchi et al., 2014]. Moreover, hybridogenetic natural *Hexagrammos* hybrids have several large metacentric chromosomes and microchromosomes specific to hemi-clones, which were not seen in extant parental species and their induced hybrids [Suzuki et al., 2017, 2020].

These results show the difficulty of artificial synthesis of clonal or hemi-clonal fish by hybridization of extant parental species. The appearance of metacentric chromosomes and microchromosomes specific to natural hybridogenetic *Hexagrammos* hybrids indicated the occurrence of structural differentiation between ancestral chromosomes maintained in hemi-clonal hybrids and contemporary chromosomes in extant gonochoristic wild types. In dojo loach, however, such a chromosomal differentiation was not detected between the ancestral genomes of group B in the clone lineage and contemporary genomes of extant wild type group B, when analyzed by FISH with ManDra-B and 5.8S + 28S rDNA probes [Kuroda et al., 2018].

On the other hand, there is no cytogenetic evidence of chromosomal differentiation between ancestral and contemporary A genomes because a molecular-cytogenetic tool to identify chromosomes derived from the group A loach has not yet been developed.

In this study, we developed a new FISH probe named ManDra-A from repetitive sequences of group A dojo loach to clarify chromosomal differentiation between the ancestral and contemporary A genomes. Two-color FISH with the newly developed ManDra-A and previously reported ManDra-B probes was performed in somatic cells of clonal loaches to verify the hybrid origin of the clonal loach. Meiotic configurations analyzed by FISH with the ManDra-B probe indicated a pairing between sister chromosomes duplicated from each chromosome of group B as the mechanism for clonal gametogenesis [Kuroda et al., 2018]. In this system, any genetic variation does not arise because crossing over or recombination occurs between identical elements of sister chromosomes, which are duplicated from the original same chromosome by premeiotic endomitosis [Itono et al., 2006; Yoshikawa et al., 2009; Kuroda et al., 2018]. However, the lack of a probe to detect chromosomes from group A has weakened the conclusion on the mechanisms to produce isogenic clonal gametes. Thus, we determined the occurrence of sister chromosome pairing by the presence or absence of ManDra-A and ManDra-B FISH signals in the spermatocytes of artificially sex-reversed clonal males. Our study clearly shows the hybrid origin of clonally reproducing fish by FISH techniques; it also suggests the possibility that chromosomal differentiation between ancestral and contemporary fish can affect gametogenesis even in the same origin. These results help deepen our knowledge about the association between clonality and hybridity in unisexual vertebrates that have been often discussed [Dawley, 1989; Vrijenhoek, 1994; Beukeboom and Vrijenhoek, 1998; Lamatsch and Stöck, 2009; Arai and Fujimoto, 2013].

Materials and methods

Experimental animals

In total, 39 dojo loach (*M. anguillicaudatus*) individuals were used in this study (Supplementary Table S1). For chromosome analyses, wild type loach belonging to group A (n = 11), wild type loach belonging to group B (n = 6), artificial inter-group hybrids between groups A and B (n = 6), clonal loach females (n = 12), sex-reversed clonal loach males (n = 2), and clone-origin triploids (n = 2) were used. Group A, group B, and clonal loaches were identified by mitochondrial DNA-control region haplotype [Morishima et al., 2008a], restriction fragment length polymorphism (RFLP) analysis of the *RAG1* gene [Fujimoto et al., 2017], and electrophoretic pattern of ManDra-B sequences [Fujimoto et al., 2017]. Ploidy was determined by flow cytometry, as described in a previous study [Morishima et al., 2002]. Inter-group hybrids were induced by artificial fertilization of group B wild type eggs from Nanae, Hokkaido Prefecture, or Ishikawa Prefecture with group A wild type sperm from Abashiri, Hokkaido Prefecture [Kuroda et al., 2018]. Although unreduced diploid eggs of clonal loach normally develop by natural gynogenesis, clonal diploid embryos were produced by fertilization of diploid eggs laid by clonal loach with genetically inactivated UV-irradiated goldfish sperm so as not to produce clone-origin triploids by accidental incorporation of the sperm nucleus [Morishima et al., 2002]. Sex-reversed clonal males were induced as previously described with the administration of 17-α methyltestosterone [Yoshikawa et al., 2007].

Isolation of repetitive sequences from genomic DNA

Group A-specific repetitive DNA was isolated by genomic DNA digestion using restriction enzymes according to Fujimoto et al. [2017]. Briefly, genomic DNA (3 μg) extracted from group A loach was digested with 29 different restriction enzymes (Supplementary Table S2). The digested fragments were electrophoresed on a 1.5% agarose gel and extracted using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Dueren, Germany). The isolated repetitive DNA was inserted and cloned into a plasmid vector using the Zero Blunt TOPO PCR cloning kit for sequencing, without competent cells (Thermo Fisher Scientific, Massachusetts, USA) and One Shot TOP10 chemically competent *E. coli* (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. Plasmid sequence data from independent five colonies were obtained using DNA sequence service (FASMAC, Kanagawa, Japan) and analyzed by BioEdit (version 7.0.5.3).

Primer design

Based on the identified repetitive sequences named ManDra-A (see the Results section), a primer set (ManDra-AF 5′-TCATCATAAGAATGCTCCTGTAAGC-3′ and ManDra-AR 5′-GCATTTTAGTATGAGAATTCAACTT-3′) was designed using Primer-BLAST (NCBI) to amplify the ManDra-A region. PCR analyses using the ManDra-A primer set were performed with 1 μL of genomic template DNA (100 ng/μL), 1.2 μL water, 5 μL 2X PCR Buffer for KOD FX Neo (TOYOBO, Osaka, Japan), 2.0 μL dNTPs (2 mM), 0.2 μL KOD FX Neo (TOYOBO, Osaka, Japan), and 0.3 μL each of the ManDra-A primer set (10 μM). The PCR cycling conditions were as follows: initial denaturation for 3 min at 95 °C, 20 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 50°C, extension for 30 s at 72°C, and a final extension for 5 min at 72 °C. The PCR products were electrophoresed in a 1.5% agarose gel to confirm whether each group showed a different banding pattern.

Sequence analysis of PCR products

In group A, an amplicon of approximately 650 bp was extracted after electrophoresis using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Dueren, Germany). The extracted amplicon was inserted into a plasmid vector and cloned to confirm the sequences using the methods described above.

Chromosome preparation

To prepare chromosome slides from embryos at the optic vesicle stage, yolks were mechanically removed under a binocular microscope. The embryos were incubated in 0.0025% colchicine (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) dissolved in physiological saline (7.5 g NaCl, 0.2 g KCl, 0.264 g/L $CaCl_2 \cdot 2H_2O$) for 30 min. The embryos were then placed in a hypotonic solution (0.075 M KCl) for 20 min and fixed with Carnoy's solution (3:1 methanol/acetic acid). Fixed embryos were stored at -30°C until FISH analyses. For chromosome preparations from the kidney and testis, goat serum (100 μL/gm body weight) was individually injected 1 and 5 days before sacrifice. Subsequently, 0.01% colchicine in physiological saline was injected 2.5 h before sacrifice. Kidney and testis tissues were collected from the individuals and cut into small pieces using forceps. The pieces were treated with the hypotonic solution for 1 h and fixed with Carnoy's solution. Fixed pieces were stored at -30°C until FISH analyses. Cell suspensions from embryos, kidney, and testis were dropped onto glass slides and air-dried. The slides were incubated at 65°C for 24 h for hardening.

192

193 **Two-color FISH**

194 Plasmids containing a single ManDra-A region or five tandemly repeating units of the ManDra-A region
195 (see the Results section) were used as ManDra-A and ManDra-A 5 repeat probes. To identify chromosomes
196 derived from group B, the ManDra-B probe was used [Kuroda et al., 2018, 2019]. The ManDra-A probe
197 was labeled with biotin-16-dUTP using the Biotin-Nick translation mix (Roche, Basel, Switzerland) or
198 digoxigenin-11-dUTP using the Dig-Nick translation mix (Roche, Basel, Switzerland). The ManDra-A 5
199 repeat probe was labeled with biotin-16-dUTP by Biotin-Nick translation mix. The ManDra-B probe was
200 labeled with digoxigenin-11-dUTP using Dig-Nick translation mix.

201 Two-color FISH was performed according to Kuroda et al. [2018]. Biotin-labeled ManDra-A or
202 ManDra-A 5 repeat probes were detected with streptavidin and Alexa Fluor 488 conjugate (Thermo Fisher
203 Scientific, Massachusetts, USA). The signals were amplified using biotinylated anti-avidin antibody
204 (Vector Laboratories, California, USA). Digoxigenin-labeled ManDra-B probe was detected with anti-
205 digoxigenin-rhodamine, Fab fragments (Roche Basel, Switzerland). The slides were counterstained with
206 ProLong Gold Antifade Mountant with 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Thermo
207 Fisher Scientific, Massachusetts, USA).

208 Metaphases were observed using a fluorescence microscope, DM5500B (Leica, Wetzlar, Germany).
209 Images of metaphase were recorded with a DFC 365FX camera (Leica, Wetzlar, Germany). Image
210 processing was performed using PhotoShop Elements11 (Adobe, California, USA).

211

Results

Isolation of repetitive sequence and the sequence analysis

When genomic DNA from group A loach was digested with the restriction enzyme *Dra*I, satellite DNA fragments (approximately 130 bp) were observed after electrophoresis (Supplementary Fig. S1). The fragments were isolated and cloned. Sequence analysis showed that all five colonies had the same sequence (136 bp) (Fig. 1). The sequence was named ManDra-A.

Primer set and comparison of PCR products

Based on the determined ManDra-A sequence, a primer set (ManDra-AF, ManDra-AR) was designed to amplify the internal region of ManDra-A (Fig. 1). A smear-like electrophoretic pattern was detected in group A and clonal loaches (Fig. 2). In contrast, a ladder-like electrophoretic pattern with fragments of 110 bp and an interval of approximately 130 bp was detected in group B (Fig. 2).

Sequence analysis of the PCR product

In group A, a PCR amplicon (approximately 650 bp) was extracted following electrophoresis. Sequence analysis showed that the amplicon contained five tandemly repeating units of the ManDra-A region. Specifically, compared to the ManDra-A sequence (Fig. 1), unit 1 was a partial ManDra-A region (121 bp) because of the ManDra-AF primer attached (Supplementary Fig. S2). Substitutions (2 bases) and deletions (3 bases) were found in unit 1 (Supplementary Fig. S2). Units 2, 3, and 4 contained the whole ManDra-A region (136 bp) (Supplementary Fig. S2), and substitutions were identified in 7 bases, 6 bases, and 4 bases, respectively (Supplementary Fig. S2). Unit 5 was a partial ManDra-A region (113 bp) because the ManDra-AR primer attached (Supplementary Fig. S2), and contained a substitution in 1 base (Supplementary Fig. S2). The sequences were named ManDra-A 5 repeat and used for subsequent FISH studies.

FISH with ManDra-A probe in wild type dojo loach

In 44 somatic cells from group A wild type loach ($n = 6$), 26 (2 metacentric and 24 telocentric chromosomes) out of 50 chromosomes had ManDra-A signals at centromeric regions in most of the cells examined (Fig. 3a, Supplementary Table S3). However, in most metaphases, ManDra-A signals were weak, and it was difficult to detect stable signals. In contrast, 57 cells from group B wild type loach ($n = 6$) had no ManDra-A signals (Fig. 3b, Supplementary Table S3).

FISH with ManDra-A 5 repeat probe

In 19 somatic cells of wild type loach from group A (n = 5), 26 (two metacentric and 24 telocentric chromosomes) out of 50 chromosomes showed ManDra-A 5 repeat signals at centromeric regions in most metaphases examined (Supplementary Table S4, Supplementary Fig. S3a). In contrast, 54 cells from group B wild type loach (n = 5) had no ManDra-A 5 repeat signals (Supplementary Table S4, Supplementary Fig. S3b).

Comparison of FISH signals between ManDra-A and ManDra-A 5 repeat probes

To confirm whether ManDra-A and ManDra-A 5 repeat signals were detected at the same number and regions of chromosomes, two-color FISH with ManDra-A and ManDra-A 5 repeat probes were performed. In an inter-group hybrid between wild type group A and B loach, both ManDra-A and ManDra-A 5 repeat signals were detected in the centromeric regions of the same 13 (1 metacentric and 12 telocentric chromosomes) out of 50 chromosomes (Supplementary Fig. S4). ManDra-A 5 repeat signals were more stable than ManDra-A signals.

Identification of parental chromosomes in inter-group hybrid by two-color FISH

Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed. In 31 somatic cells of inter-group hybrids between wild type dojo loach groups A and B (n = 5), 25 out of 50 chromosomes had ManDra-B signals at centromeric regions, suggesting that the chromosomes were derived from group B (Fig. 4a, Supplementary Table S5). Moreover, 13 (1 metacentric and 12 telocentric chromosomes) out of 25 chromosomes without ManDra-B signals showed ManDra-A 5 repeat signals at centromeric regions suggesting the chromosomes were derived from group A (Fig. 4a, Supplementary Table S5).

Further evidence of hybrid origin in clonal loach

Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed on 117 clonal diploid cells (n = 12). Twenty-five out of 50 chromosomes had ManDra-B signals at centromeric regions (Fig. 4b, Supplementary Table S6). In contrast, in the other 25 chromosomes without ManDra-B signals, ManDra-A 5 repeat signals were detected in 12 telocentric chromosomes, in the centromeric region (Fig. 4b,

Supplementary Table S6). No metacentric chromosomes exhibited ManDra-A 5 repeat signals in the somatic cells of clonal loach (Fig. 4b).

Two-color FISH in clone-origin triploid loach

Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed in 29 somatic cells of clone-origin triploids (n = 2). Twenty-five of 75 chromosomes had only ManDra-B signals at centromeric regions (Fig. 4c, Supplementary Table S7). In the other 50 chromosomes without any ManDra-B signals, ManDra-A 5 repeat signals were detected in 25 (one metacentric and 24 telocentric chromosomes) chromosomes at the centromeric region (Fig. 4c, Supplementary Table S7). Thus, 12 telocentric chromosomes out of 25 ManDra-A 5 repeat positive chromosomes were derived from the ancestral A genome. The other metacentric and 12 telocentric chromosomes with ManDra-A 5 repeat signals were derived from the contemporary A genome. Moreover, the genome composition of the triploids could be designated as AAB, with AB (derived from a diploid egg of clonal loach) and A (derived from sperm of wild type group A loach) genomes.

Chromosome pairing in meiosis of clonal loach

Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed in sex-reversed clonal males (n = 2). On spermatocytes with 50 bivalents, indicating the occurrence of premeiotic genome doubling, two ManDra-B signals were detected in 25 out of 50 bivalents (Fig. 5). Moreover, two ManDra-A 5 repeat signals were detected in 12 out of 25 bivalents without ManDra-B signals (Fig. 5). Most of the FISH signals were detected on both sides of bivalents (tail-to-tail association), although some signals were detected around the center (head-to-head association) (Fig. 5).

Discussion

Previous FISH analyses using the ManDra-B probe revealed that clonal loaches contained one set of genomes (i.e., haploid chromosomes) derived from group B dojo loach [Kuroda et al., 2018]. Although cytogenetic evidence was not provided, another set of genomes presumably originated from group A. Thus, the hybrid origin of clonal loach was strongly suggested [Kuroda et al., 2018]. Moreover, our previous two-color FISH with ManDra-B and 5.8S + 28S rDNA probes provided evidence of pairing between sister chromosomes that were duplicated from each chromosome derived from group B loach by premeiotic endomitosis to form bivalents in the course of gametogenesis. Such a pairing should assure the formation of isogenic unreduced gametes because crossing over or recombination did not give rise to any genetic variation due to the exchange of identical elements of sister chromosomes [Kuroda et al., 2018]. In a previous study, however, the pairing of sister chromosomes originating from group A loach was not completely proven due to the lack of a FISH probe to detect chromosomes from group A loach.

In this study, we proved the hybrid origin between groups A and B in clonal loach using newly developed group A-specific FISH probes, ManDra-A and ManDra-A 5 repeat. In clonal diploids, 25 chromosomes had ManDra-B signals, whereas ManDra-A 5 repeat signals were detected in 12 chromosomes that did not show any ManDra-B signals, clearly indicating that clonal loaches should contain both A and B genomes. In wild type diploid loach from group A, however, ManDra-A 5 repeat signals were detected in the centromeric region of two metacentric and 24 telocentric chromosomes. No signals were detected in the other chromosomes. Thus, one metacentric chromosome detected by the ManDra-A 5 repeat probe was transmitted from the group A wild type to the artificial hybrid between groups A and B. In contrast, the ManDra-A 5 repeat signal was never detected in any metacentric chromosome of the clonal diploids, although 12 telocentric chromosomes from group A exhibited ManDra-A 5 repeat signals. The results suggest that metacentric chromosomes maintained in the ancestral group A genome in clonal loach should be structurally different from those in the contemporary group A genome of the extant group A wild type loach. FISH analyses clarified that our clone-origin triploid samples were generated by the incorporation of haploid sperm nuclei from extant wild type group A loach into clonal diploid eggs. Thus, the clone-origin triploids contained three kinds of genomes: one contemporary A genome derived from extant group A wild type sperm, one ancestral group A genome derived from clonal loach, and one ancestral but indifferent genome from the extant group B derived from clonal loach. The result that only one metacentric chromosome exhibited a ManDra-A 5 repeat signal indicated that the metacentric chromosome

should be derived from the group A wild type. Metacentric chromosomes from clonal fish did not show any ManDra-A 5 repeat FISH signals. Thus, metacentric chromosomes should already be differentiated between the ancestral clonal and contemporary wild type genomes.

Chromosomal differentiation between asexual and wild type genomes has also been reported in hybridogenetic hemi-clonal fishes [Suzuki et al., 2017]. Natural hybrids of greenling between *Hexagrammos octogrammus* and *H. otakii* or *H. agrammus* produce hemi-clonal haploid eggs exclusively, including non-recombinant maternally derived *H. octogrammus* genomes by the reproductive system of hybridogenesis [Kimura-Kawaguchi et al., 2014]. However, fertile artificial interspecific hybrids between extant parental species *H. octogrammus* and *H. otakii* or *H. agrammus* produced recombinant gametes by regular meiosis [Kimura-Kawaguchi et al., 2014]. Further karyological studies clarified that karyotypes and chromosome numbers of the artificial hybrids were intermediate between the two parental species [Suzuki et al., 2017], but hemi-clonal natural hybrids differed from the artificial hybrids because natural hybrids had several large metacentric chromosomes and microchromosomes derived from the hemi-clonal *H. octogrammus* genome [Suzuki et al., 2017, 2020]. Maternal backcrosses (natural hybrid female × *H. octogrammus* male) had one hemi-clonal *H. octogrammus* genome containing several large metacentric chromosomes and one extant *H. octogrammus* genome [Suzuki et al., 2017, 2020]. The backcrosses produced recombinant gametes by regular meiosis and large metacentric chromosomes fissured to form two separate chromosomes during meiosis [Suzuki et al., 2017]. Thus, there are no karyological differences between two *H. octogrammus* genomes in offspring from a crossing between the maternal backcross and *H. octogrammus*, implying that genetic factors tightly associated with hybridogenesis may be located on the large metacentric chromosomes of hemi-clonal hybrids [Kimura-Kawaguchi et al., 2014; Suzuki et al., 2017, 2020].

Chromosomal differentiation between gonochoristic parental species and unisexual biotypes has also been reported in Amazon molly (*Poecilia formosa*), in which gynogenetic fish stably inherited microchromosomes derived from the paternal genome. These microchromosomes are thought to extend genetic diversity in asexual lineages [Schartl et al., 1995a; Nanda et al., 2007]. Similarly, microchromosomes derived from paternal blunt snout bream (*Megalobrama amblycephala*) were observed in gynogenetic gibel carp (*Carassius gibelio*), and some parts of the sperm-derived DNA fragment were incorporated into the gibel carp genome [Yi et al., 2003; Chen et al., 2020]. The incorporated DNA fragments are believed to increase genetic diversity and introduce new traits into unisexual animals [Chen

et al., 2020]. Thus, chromosomal differentiation is an important factor not only to determine the asexual reproductive mode but also to extend genetic diversity through gene leakage by the paternal genome.

Clone-origin triploid (genomic constitution: AAB) females produce haploid eggs containing only the A genome by quasi-normal meiosis after eliminating the unmatched group B genome, i.e., meiotic hybridogenesis [Morishima et al., 2008b]. Twenty-five bivalents were observed in germinal vesicles of oocytes from the triploid female [Morishima et al., 2008b], indicating that chromosome pairing correctly occurred between ancestral and contemporary A genomes. Thus, although chromosomal differentiation is present at least in the centromeric region of ManDra-A 5 repeat positive metacentric chromosomes, the degree of difference was not enough to prevent homologous chromosome pairing between asexual and wild type genomes at the moment. In this case, recombinant haploid eggs should be generated by crossing over and random segregation of ancestral and contemporary A chromosomes. If the haploid egg fertilizes with spermatozoa from group A males in the natural population, the genomic constitution of the progeny will be AA and will behave as contemporary group A loach, even though some genetic factors are derived from ancestral group A loach.

Most ManDra-A 5 repeat-positive chromosomes were telocentric chromosomes. This suggests that mutations around centromeric sequences should occur independently in each chromosomal category: metacentric, submetacentric, and telocentric chromosomes. In medaka fish, sequence mutations around the centromeric region have been reported to occur more frequently in chromosomes containing centromeres near the center than in those containing centromeres near the terminal [Ichikawa et al., 2017]. In our study, FISH analyses using ManDra-A and ManDra-A 5 repeat probes detected certain differentiations of the centromeric region in metacentric chromosomes between ancestral and contemporary group A loach. However, whether chromosomal differentiation occurs in other chromosomes is unknown. Thus, the next approach to confirm the occurrence of chromosomal differentiation is FISH using various repetitive satellite DNA sequences as probes based on whole-genome sequencing data, such as European spined loach (genus *Cobitis*) [Marta et al., 2020].

FISH analyses using the new ManDra-A 5 repeat probe together with a previous ManDra-B probe in the spermatocytes of sex-reversed clones indicated the pairing between sister chromosomes derived from the same ancestral group of the loach. Twenty-five out of 50 bivalents had two ManDra-B signals, indicating pairing between chromosomes exclusively derived from group B. While 12 out of 50 bivalents contained two ManDra-A 5 repeat signals, indicating the pairing between chromosomes exclusively derived from

group A. Thus, our present results verify the previous conclusion that isogenicity of unreduced diploid gametes of the clone was assured by sister chromosome pairing [Kuroda et al., 2018]. In this study, although meiosis in oocytes of clonal females has not been cytogenetically confirmed, the same mechanisms should be involved in both oogenesis and spermatogenesis of clonal dojo loach because both clonal females and males produce unreduced isogenic gametes.

Statements

Acknowledgement

We would like to thank the members of Laboratory of Aquaculture Genetics and Genomics at Hokkaido University, Nanae Freshwater Station at Hokkaido University, and Laboratory of Aquaculture Science at Tokyo University of Agriculture.

Statement of Ethics

This study was performed according to the Guide for the Care and Use of Laboratory Animals at Hokkaido University. All animal experiments were approved by the animal study ethical committee of Hokkaido University (Approval number 29-3).

Conflict of interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This study was supported by Grants-in-Aid from JSPS (Japan Society for the Promotion of Science) KAKENHI Grant Number 15H02457 and JSPS Research Fellow Number 17J01971.

Author Contributions

MK, TF, MM, EY, and KA conceived and designed the study. MK conducted the experiments. KS contributed to the new analytical tools. MK analyzed the data. MK and KA wrote the manuscript. All authors read and approved the manuscript.

414 **References**

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506

Figure Legends

Fig. 1. ManDra-A sequences (136 bp). Arrows indicate the primer sequences to amplify ManDra-A sequences by PCR.

Fig. 2. Representative electrophoregram pattern after PCR using ManDra-A primers. Group A (lane 2) and clonal diploids (lane 5) show a smear-like pattern. Group B (lane 3, 4) shows a ladder-like pattern. The 100 bp ladder molecular marker is shown in lane 1.

Fig. 3. FISH with the ManDra-A probe in somatic cells from wild type dojo loach. Representative metaphase (left) and karyotype (right) of somatic cells from wild type diploid dojo loach of group A (a) and group B (b) after FISH with the ManDra-A probe. ManDra-A probe was labeled with biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). All chromosomes were counterstained with DAPI (blue). Scale bars denote 10 μm. M, metacentric chromosome; SM, submetacentric chromosome; T, telocentric chromosome. Asterisks indicate metacentric chromosomes with ManDra-A signals.

Fig. 4. Two-color FISH with ManDra-A 5 repeat probe and ManDra-B probe in somatic cells of inter-group hybrid, clonal diploid, and clone-origin triploid dojo loach. Representative metaphase (left) and karyotype (right) of a somatic cell from an inter-group hybrid between wild type dojo loach groups A and B (a), clonal diploid dojo loach (b), and clone-origin triploid dojo loach (c) after two-color FISH with ManDra-A 5 repeat probe and ManDra-B probe. The ManDra-A 5 repeat probe was labeled with biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). ManDra-B probe was labeled with digoxigenin-11-dUTP and detected by anti-digoxigenin-rhodamine, Fab fragments (red). All chromosomes were counterstained with DAPI (blue). Scale bars denote 10 μm. M, metacentric chromosome; SM, submetacentric chromosome; T, telocentric chromosome. Asterisks indicate metacentric chromosomes with ManDra-A 5 repeat signals.

Fig. 5. Two-color FISH with ManDra-A 5 repeat and ManDra-B probes in spermatocytes of sex-reversed clonal diploid male dojo loach. Representative meiotic metaphase (left) and karyotype (right) in spermatocytes from sex-reversed clonal diploid males dojo loach after two-color FISH with ManDra-A 5 repeat and ManDra-B probes. Asterisks indicate bivalents with ManDra-B signals around the center.

ManDra-A 5 repeat probe was labeled with biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). ManDra-B probe was labeled with digoxigenin-11-dUTP and detected by anti-digoxigenin-rhodamine, Fab fragments (red). All bivalents were counterstained by DAPI (blue). Scale bar denotes 10 μm.

Online Supplementary Material

Supplementary Fig. S1. Isolation of repetitive DNA sequences with restriction enzymes *Afa* I and *Dra* I in the genomic DNA of group A dojo loach. Arrow indicates a satellite band of approximately 130 bp.

Supplementary Fig. S2. Sequences of ManDra-A and each repeat unit in the ManDra-A 5 repeat region. The upper sequence indicates ManDra-A, and the lower sequence indicates each repeat unit of ManDra-A 5 repeat. Units 1 and 2 contain partial ManDra-A sequences. Red characters indicate different bases compared to the ManDra-A reference sequence. Hyphens (-) indicate base deletions.

Supplementary Fig. S3. FISH with ManDra-A 5 repeat probe in somatic cells from wild type dojo loach. Representative metaphase of somatic cells from wild type diploid dojo loach of group A (a) and group B (b) after FISH with ManDra-A 5 repeat probe. The ManDra-A 5 repeat probe was labeled with biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). All chromosomes were counterstained with DAPI (blue). Scale bars denote 10 μm.

Supplementary Fig. S4. Two-color FISH with ManDra-A and ManDra-A 5 repeat probes in somatic cells of inter-group hybrids between wild type dojo loach groups A and B. Representative metaphase in somatic cells from inter-group hybrids between wild type dojo loach groups A and B after two-color FISH with ManDra-A signals (a) and ManDra-A 5 repeat signals (b). ManDra-A 5 repeat probe was labeled with biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). ManDra-A probe was labeled with digoxigenin-11-dUTP and detected by anti-digoxigenin-rhodamine, Fab fragments (red). All chromosomes were counterstained with DAPI (blue). Scale bars denote 10 μm.

Supplementary Table S1. Individuals used for chromosome preparations in this study.

Supplementary Table S2. Restriction enzymes used for satellite DNA isolation and the presence of satellite DNA bands.

Supplementary Table S3. Number of chromosomes and ManDra-A FISH signals detected in the somatic cells of dojo loach.

Supplementary Table S4. Number of chromosomes and ManDra-A 5 repeat FISH signals detected in the somatic cells of dojo loach.

Supplementary Table S5. Number of chromosomes and ManDra-A 5 repeat and ManDra-B FISH signals detected in the somatic cells of the inter-group hybrids between groups A and B dojo loach.

Supplementary Table S6. Number of chromosomes and ManDra-A 5 repeat and ManDra-B FISH signals detected in the somatic cells of clonal dojo loach.

Supplementary Table S7. Number of chromosomes and ManDra-A 5 repeat and ManDra-B FISH signals detected in the somatic chromosomes of two clone-origin triploid dojo loaches.