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1 **Research Article**

2 **Title**

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

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17

18 **Short Title**

19 Chromosome differentiation between contemporary and ancestral genomes in fish

20

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31 **Abstract**

32 In dojo loach (*Misgurnus anguillicaudatus*), although most wild types are gonochoristic diploids that are  
33 genetically differentiated into two groups, A and B, clonal lineages appear in certain localities. Clonal  
34 loaches have been considered to have hybrid origins between the two groups by a series of genetic studies.  
35 In this study, using fluorescence *in situ* hybridization (FISH) with a newly developed probe (ManDra-A),  
36 we identified 26 (1 pair of metacentric and 12 pairs of telocentric chromosomes) of 50 diploid chromosomes  
37 in contemporary wild type group A loach. In contrast, ManDra-A signals were not detected on metacentric  
38 chromosomes derived from the ancestral group A of clonal loach. The FISH results clearly showed the  
39 presence of certain differentiations in metacentric chromosomes between ancestral and contemporary group  
40 A loach. Two-color FISH with ManDra-A and group B specific ManDra (renamed ManDra-B) probes  
41 reconfirmed the hybrid origin of clones by identifying chromosomes from both groups A and B in  
42 metaphases. Our results showed the hybrid origin of clonally reproducing fish and the possibility that  
43 chromosomal differentiation between ancestral and contemporary fish can affect gametogenesis. In meiotic  
44 spermatocytes of sex-reversed clones, ManDra-A, and not ManDra-B, signals were detected in 12 out of  
45 50 bivalents. Thus, the results further support the previous conclusion that clonal gametogenesis was  
46 assured by pairing between sister chromosomes duplicated from each ancestral chromosome from group A  
47 or B. Our study deepens knowledge about the association between clonality and hybridity in unisexual  
48 vertebrates.  
49

50 **Introduction**

51 Wild type dojo loach, *Misgurnus anguillicaudatus* (Cobitidae; Teleostei), have gonochoristic diploidy  
52 ( $2n = 50$  chromosomes). It reproduces bisexually in most Japanese populations, whereas unisexual clonal  
53 lineages inhabit certain regions of Hokkaido and Ishikawa Prefectures, Japan [Morishima et al., 2002,  
54 2008a; Arai and Fujimoto, 2013]. Clonal loach lay unreduced diploid eggs that develop gynogenetically  
55 without any genetic contribution of the spermgenome [Itono et al., 2006, 2007; Arai and Fujimoto, 2013].  
56 Such clonal lineages may have arisen from past hybridization events between genetically diversified groups  
57 A (genomic constitution of AA) and B (BB), as shown by the heterozygosity of the *RAG1* and *IRBP2*  
58 sequences [Yamada et al., 2015]. Repetitive sequences were previously isolated from the genomic DNA of  
59 group B loach using the restriction enzyme *DraI*. The repetitive sequences named ManDra (“Man” comes  
60 from *M. anguillicaudatus* and “Dra” comes from a restriction enzyme *DraI*) were used as a nuclear DNA  
61 marker (hereafter designated as ManDra-B in this paper) [Fujimoto et al., 2017]. Karyological differences  
62 were not morphologically detected among gonochoristic diploid wild type group A, group B, and clonal  
63 dojo loaches, since they have the same ( $2n = 50$ ) chromosomes, categorized into 10 metacentric, 4  
64 submetacentric, and 36 telocentric chromosomes [Itono et al., 2006]. When we performed fluorescence *in*  
65 *situ* hybridization (FISH) using the ManDra-B sequence as a probe in somatic cells of clonal diploids ( $2n$   
66  $= 50$ ), a haploid set of chromosomes ( $n = 25$ ) from the group B loach, clear ManDra-B signals specific to  
67 the centromeric region were detected; however, another haploid set of chromosomes from group A loach  
68 was not identified [Kuroda et al., 2018]. Thus, the hybrid origin of the clonal loach was cytogenetically  
69 proven, and the genomic constitution of clonal loach has been designated as the heterozygous genotype AB  
70 [Yamada et al., 2015; Fujimoto et al., 2017; Kuroda et al., 2018].

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

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

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

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

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

113 In this study, we developed a new FISH probe named ManDra-A from repetitive sequences of group A  
114 dojo loach to clarify chromosomal differentiation between the ancestral and contemporary A genomes.  
115 Two-color FISH with the newly developed ManDra-A and previously reported ManDra-B probes was  
116 performed in somatic cells of clonal loaches to verify the hybrid origin of the clonal loach. Meiotic  
117 configurations analyzed by FISH with the ManDra-B probe indicated a pairing between sister chromosomes  
118 duplicated from each chromosome of group B as the mechanism for clonal gametogenesis [Kuroda et al.,  
119 2018]. In this system, any genetic variation does not arise because crossing over or recombination occurs  
120 between identical elements of sister chromosomes, which are duplicated from the original same  
121 chromosome by premeiotic endomitosis [Itono et al., 2006; Yoshikawa et al., 2009; Kuroda et al., 2018].  
122 However, the lack of a probe to detect chromosomes from group A has weakened the conclusion on the  
123 mechanisms to produce isogenic clonal gametes. Thus, we determined the occurrence of sister chromosome  
124 pairing by the presence or absence of ManDra-A and ManDra-B FISH signals in the spermatocytes of  
125 artificially sex-reversed clonal males. Our study clearly shows the hybrid origin of clonally reproducing  
126 fish by FISH techniques; it also suggests the possibility that chromosomal differentiation between ancestral  
127 and contemporary fish can affect gametogenesis even in the same origin. These results help deepen our  
128 knowledge about the association between clonality and hybridity in unisexual vertebrates that have been  
129 often discussed [Dawley, 1989; Vrijenhoek, 1994; Beukeboom and Vrijenhoek, 1998; Lamatsch and Stöck,  
130 2009; Arai and Fujimoto, 2013].

131

132 **Materials and methods**

133 **Experimental animals**

134 In total, 39 dojo loach (*M. anguillicaudatus*) individuals were used in this study (Supplementary Table S1).  
135 For chromosome analyses, wild type loach belonging to group A ( $n = 11$ ), wild type loach belonging to  
136 group B ( $n = 6$ ), artificial inter-group hybrids between groups A and B ( $n = 6$ ), clonal loach females ( $n =$   
137 12), sex-reversed clonal loach males ( $n = 2$ ), and clone-origin triploids ( $n = 2$ ) were used. Group A, group  
138 B, and clonal loaches were identified by mitochondrial DNA-control region haplotype [Morishima et al.,  
139 2008a], restriction fragment length polymorphism (RFLP) analysis of the *RAG1* gene [Fujimoto et al.,  
140 2017], and electrophoretic pattern of ManDra-B sequences [Fujimoto et al., 2017]. Ploidy was determined  
141 by flow cytometry, as described in a previous study [Morishima et al., 2002]. Inter-group hybrids were  
142 induced by artificial fertilization of group B wild type eggs from Nanae, Hokkaido Prefecture, or Ishikawa  
143 Prefecture with group A wild type sperm from Abashiri, Hokkaido Prefecture [Kuroda et al., 2018].  
144 Although unreduced diploid eggs of clonal loach normally develop by natural gynogenesis, clonal diploid  
145 embryos were produced by fertilization of diploid eggs laid by clonal loach with genetically inactivated  
146 UV-irradiated goldfish sperm so as not to produce clone-origin triploids by accidental incorporation of the  
147 sperm nucleus [Morishima et al., 2002]. Sex-reversed clonal males were induced as previously described  
148 with the administration of 17- $\alpha$  methyltestosterone [Yoshikawa et al., 2007].

149

150 **Isolation of repetitive sequences from genomic DNA**

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

161

162 **Primer design**

163 Based on the identified repetitive sequences named ManDra-A (see the Results section), a primer set  
164 (ManDra-AF 5'-TCATCATAAGAATGCTCCTGTAAGC-3' and ManDra-AR 5'-  
165 GCATTTTAGTATGAGAATTCAACTT-3') was designed using Primer-BLAST (NCBI) to amplify the  
166 ManDra-A region. PCR analyses using the ManDra-A primer set were performed with 1 µL of genomic  
167 template DNA (100 ng/µL), 1.2 µL water, 5 µL 2X PCR Buffer for KOD FX Neo (TOYOBO, Osaka, Japan),  
168 2.0 µL dNTPs (2 mM), 0.2 µL KOD FX Neo (TOYOBO, Osaka, Japan), and 0.3 µL each of the ManDra-  
169 A primer set (10 µM). The PCR cycling conditions were as follows: initial denaturation for 3 min at 95 °C,  
170 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  
171 final extension for 5 min at 72 °C. The PCR products were electrophoresed in a 1.5% agarose gel to confirm  
172 whether each group showed a different banding pattern.

173

174 **Sequence analysis of PCR products**

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

178

179 **Chromosome preparation**

180 To prepare chromosome slides from embryos at the optic vesicle stage, yolks were mechanically removed  
181 under a binocular microscope. The embryos were incubated in 0.0025% colchicine (FUJIFILM Wako Pure  
182 Chemical Corporation, Osaka, Japan) dissolved in physiological saline (7.5 g NaCl, 0.2 g KCl, 0.264 g/L  
183 CaCl<sub>2</sub>·2H<sub>2</sub>O) for 30 min. The embryos were then placed in a hypotonic solution (0.075 M KCl) for 20 min  
184 and fixed with Carnoy's solution (3:1 methanol/acetic acid). Fixed embryos were stored at -30°C until FISH  
185 analyses. For chromosome preparations from the kidney and testis, goat serum (100 µL/gm body weight)  
186 was individually injected 1 and 5 days before sacrifice. Subsequently, 0.01% colchicine in physiological  
187 saline was injected 2.5 h before sacrifice. Kidney and testis tissues were collected from the individuals and  
188 cut into small pieces using forceps. The pieces were treated with the hypotonic solution for 1 h and fixed  
189 with Carnoy's solution. Fixed pieces were stored at -30°C until FISH analyses. Cell suspensions from  
190 embryos, kidney, and testis were dropped onto glass slides and air-dried. The slides were incubated at 65°C  
191 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 Elements 11 (Adobe, California, USA).

211

212 **Results**

213 **Isolation of repetitive sequence and the sequence analysis**

214 When genomic DNA from group A loach was digested with the restriction enzyme *DraI*, satellite DNA  
215 fragments (approximately 130 bp) were observed after electrophoresis (Supplementary Fig. S1). The  
216 fragments were isolated and cloned. Sequence analysis showed that all five colonies had the same sequence  
217 (136 bp) (Fig. 1). The sequence was named ManDra-A.

218

219 **Primer set and comparison of PCR products**

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

224

225 **Sequence analysis of the PCR product**

226 In group A, a PCR amplicon (approximately 650 bp) was extracted following electrophoresis. Sequence  
227 analysis showed that the amplicon contained five tandemly repeating units of the ManDra-A region.  
228 Specifically, compared to the ManDra-A sequence (Fig. 1), unit 1 was a partial ManDra-A region (121 bp)  
229 because of the ManDra-AF primer attached (Supplementary Fig. S2). Substitutions (2 bases) and deletions  
230 (3 bases) were found in unit 1 (Supplementary Fig. S2). Units 2, 3, and 4 contained the whole ManDra-A  
231 region (136 bp) (Supplementary Fig. S2), and substitutions were identified in 7 bases, 6 bases, and 4 bases,  
232 respectively (Supplementary Fig. S2). Unit 5 was a partial ManDra-A region (113 bp) because the ManDra-  
233 AR primer attached (Supplementary Fig. S2), and contained a substitution in 1 base (Supplementary Fig.  
234 S2). The sequences were named ManDra-A 5 repeat and used for subsequent FISH studies.

235

236 **FISH with ManDra-A probe in wild type dojo loach**

237 In 44 somatic cells from group A wild type loach ( $n = 6$ ), 26 (2 metacentric and 24 telocentric chromosomes)  
238 out of 50 chromosomes had ManDra-A signals at centromeric regions in most of the cells examined (Fig.  
239 3a, Supplementary Table S3). However, in most metaphases, ManDra-A signals were weak, and it was  
240 difficult to detect stable signals. In contrast, 57 cells from group B wild type loach ( $n = 6$ ) had no ManDra-  
241 A signals (Fig. 3b, Supplementary Table S3).

242

243 **FISH with ManDra-A 5 repeat probe**

244 In 19 somatic cells of wild type loach from group A ( $n = 5$ ), 26 (two metacentric and 24 telocentric  
245 chromosomes) out of 50 chromosomes showed ManDra-A5 repeat signals at centromeric regions in most  
246 metaphases examined (Supplementary Table S4, Supplementary Fig. S3a). In contrast, 54 cells from group  
247 B wild type loach ( $n = 5$ ) had no ManDra-A 5 repeat signals (Supplementary Table S4, Supplementary Fig.  
248 S3b).

249

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

251 To confirm whether ManDra-A and ManDra-A 5 repeat signals were detected at the same number and  
252 regions of chromosomes, two-color FISH with ManDra-A and ManDra-A5 repeat probes were performed.  
253 In an inter-group hybrid between wild type group A and B loach, both ManDra-A and ManDra-A5 repeat  
254 signals were detected in the centromeric regions of the same 13 (1 metacentric and 12 telocentric  
255 chromosomes) out of 50 chromosomes (Supplementary Fig. S4). ManDra-A 5 repeat signals were more  
256 stable than ManDra-A signals.

257

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

259 Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed. In 31 somatic cells of  
260 inter-group hybrids between wild type dojo loach groups A and B ( $n = 5$ ), 25 out of 50 chromosomes had  
261 ManDra-B signals at centromeric regions, suggesting that the chromosomes were derived from group B  
262 (Fig. 4a, Supplementary Table S5). Moreover, 13 (1 metacentric and 12 telocentric chromosomes) out of  
263 25 chromosomes without ManDra-B signals showed ManDra-A 5 repeat signals at centromeric regions  
264 suggesting the chromosomes were derived from group A (Fig. 4a, Supplementary Table S5).

265

266 **Further evidence of hybrid origin in clonal loach**

267 Two-color FISH with ManDra-A 5 repeat and ManDra-B probes was performed on 117 clonal diploid cells  
268 ( $n = 12$ ). Twenty-five out of 50 chromosomes had ManDra-B signals at centromeric regions (Fig. 4b,  
269 Supplementary Table S6). In contrast, in the other 25 chromosomes without ManDra-B signals, ManDra-A  
270 5 repeat signals were detected in 12 telocentric chromosomes, in the centromeric region (Fig. 4b,

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

273

#### 274 **Two-color FISH in clone-origin triploid loach**

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

285

#### 286 **Chromosome pairing in meiosis of clonal loach**

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

293

294 **Discussion**

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

306 In this study, we proved the hybrid origin between groups A and B in clonal loach using newly  
307 developed group A-specific FISH probes, ManDra-A and ManDra-A 5 repeat. In clonal diploids, 25  
308 chromosomes had ManDra-B signals, whereas ManDra-A 5 repeat signals were detected in 12  
309 chromosomes that did not show any ManDra-B signals, clearly indicating that clonal loaches should contain  
310 both A and B genomes. In wild type diploid loach from group A, however, ManDra-A 5 repeat signals were  
311 detected in the centromeric region of two metacentric and 24 telocentric chromosomes. No signals were  
312 detected in the other chromosomes. Thus, one metacentric chromosome detected by the ManDra-A5 repeat  
313 probe was transmitted from the group A wild type to the artificial hybrid between groups A and B. In  
314 contrast, the ManDra-A 5 repeat signal was never detected in any metacentric chromosome of the clonal  
315 diploids, although 12 telocentric chromosomes from group A exhibited ManDra-A 5 repeat signals. The  
316 results suggest that metacentric chromosomes maintained in the ancestral group A genome in clonal loach  
317 should be structurally different from those in the contemporary group A genome of the extant group A wild  
318 type loach. FISH analyses clarified that our clone-origin triploid samples were generated by the  
319 incorporation of haploid sperm nuclei from extant wild type group A loach into clonal diploid eggs. Thus,  
320 the clone-origin triploids contained three kinds of genomes: one contemporary A genome derived from  
321 extant group A wild type sperm, one ancestral group A genome derived from clonal loach, and one ancestral  
322 but indifferent genome from the extant group B derived from clonal loach. The result that only one  
323 metacentric chromosome exhibited a ManDra-A5 repeat signal indicated that the metacentric chromosome

324 should be derived from the group A wild type. Metacentric chromosomes from clonal fish did not show any  
325 ManDra-A5 repeat FISH signals. Thus, metacentric chromosomes should already be differentiated between  
326 the ancestral clonal and contemporary wild type genomes.

327 Chromosomal differentiation between asexual and wild type genomes has also been reported in  
328 hybridogenetic hemi-clonal fishes [Suzuki et al., 2017]. Natural hybrids of greenling between  
329 *Hexagrammos octogrammus* and *H. otakii* or *H. agrammus* produce hemi-clonal haploid eggs exclusively,  
330 including non-recombinant maternally derived *H. octogrammus* genomes by the reproductive system of  
331 hybridogenesis [Kimura-Kawaguchi et al., 2014]. However, fertile artificial interspecific hybrids between  
332 extant parental species *H. octogrammus* and *H. otakii* or *H. agrammus* produced recombinant gametes by  
333 regular meiosis [Kimura-Kawaguchi et al., 2014]. Further karyological studies clarified that karyotypes and  
334 chromosome numbers of the artificial hybrids were intermediate between the two parental species [Suzuki  
335 et al., 2017], but hemi-clonal natural hybrids differed from the artificial hybrids because natural hybrids  
336 had several large metacentric chromosomes and microchromosomes derived from the hemi-clonal *H.*  
337 *octogrammus* genome [Suzuki et al., 2017, 2020]. Maternal backcrosses (natural hybrid female  $\times$  *H.*  
338 *octogrammus* male) had one hemi-clonal *H. octogrammus* genome containing several large metacentric  
339 chromosomes and one extant *H. octogrammus* genome [Suzuki et al., 2017, 2020]. The backcrosses  
340 produced recombinant gametes by regular meiosis and large metacentric chromosomes fissured to form  
341 two separate chromosomes during meiosis [Suzuki et al., 2017]. Thus, there are no karyological differences  
342 between two *H. octogrammus* genomes in offspring from a crossing between the maternal backcross and  
343 *H. octogrammus*, implying that genetic factors tightly associated with hybridogenesis may be located on  
344 the large metacentric chromosomes of hemi-clonal hybrids [Kimura-Kawaguchi et al., 2014; Suzuki et al.,  
345 2017, 2020].

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

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

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

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

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

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

389

#### 390 **Statements**

#### 391 **Acknowledgement**

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394 Tokyo University of Agriculture.

395

#### 396 **Statement of Ethics**

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

400

#### 401 **Conflict of interest Statement**

402 The authors have no conflicts of interest to declare.

403

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407

#### 408 **Author Contributions**

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

412

413

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506

507 **Figure Legends**

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

510

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

514

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

521

522 **Fig. 4.** Two-color FISH with ManDra-A 5 repeat probe and ManDra-B probe in somatic cells of inter-group  
523 hybrid, clonal diploid, and clone-origin triploid dojo loach. Representative metaphase (left) and karyotype  
524 (right) of a somatic cell from an inter-group hybrid between wild type dojo loach groups A and B (a), clonal  
525 diploid dojo loach (b), and clone-origin triploid dojo loach (c) after two-color FISH with ManDra-A 5  
526 repeat probe and ManDra-B probe. The ManDra-A 5 repeat probe was labeled with biotin-16-dUTP and  
527 detected by streptavidin Alexa Fluor 488 conjugate (green). ManDra-B probe was labeled with digoxigenin-  
528 11-dUTP and detected by anti-digoxigenin-rhodamine, Fab fragments (red). All chromosomes were  
529 counterstained with DAPI (blue). Scale bars denote 10  $\mu$ m. M, metacentric chromosome; SM,  
530 submetacentric chromosome; T, telocentric chromosome. Asterisks indicate metacentric chromosomes  
531 with ManDra-A 5 repeat signals.

532

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

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

541

#### 542 **Online Supplementary Material**

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

545 **Supplementary Fig. S2.** Sequences of ManDra-A and each repeat unit in the ManDra-A 5 repeat region.  
546 The upper sequence indicates ManDra-A, and the lower sequence indicates each repeat unit of ManDra-A  
547 5 repeat. Units 1 and 2 contain partial ManDra-A sequences. Red characters indicate different bases  
548 compared to the ManDra-A reference sequence. Hyphens (-) indicate base deletions.

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

554 **Supplementary Fig. S4.** Two-color FISH with ManDra-A and ManDra-A5 repeat probes in somatic cells  
555 of inter-group hybrids between wild type dojo loach groups A and B. Representative metaphase in somatic  
556 cells from inter-group hybrids between wild type dojo loach groups A and B after two-color FISH with  
557 ManDra-A signals (a) and ManDra-A 5 repeat signals (b). ManDra-A 5 repeat probe was labeled with  
558 biotin-16-dUTP and detected by streptavidin Alexa Fluor 488 conjugate (green). ManDra-A probe was  
559 labeled with digoxigenin-11-dUTP and detected by anti-digoxigenin-rhodamine, Fab fragments (red). All  
560 chromosomes were counterstained with DAPI (blue). Scale bars denote 10  $\mu$ m.

561

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

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

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

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

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

571 **Supplementary Table S6.** Number of chromosomes and ManDra-A5 repeat and ManDra-B FISH signals  
572 detected in the somatic cells of clonal dojo loach.

573 **Supplementary Table S7.** Number of chromosomes and ManDra-A5 repeat and ManDra-B FISH signals  
574 detected in the somatic chromosomes of two clone-origin triploid dojo loaches.

575