Title

Aberrant meiotic configurations cause sterility in clone-origin triploid and inter-group hybrid males of the dojo loach, *Misgurnus anguillicaudatus*

Author Affiliations

Masamichi Kuroda[a,d*] · Takafumi Fujimoto[a] · Masaru Murakami[b] · Etsuro Yamaha[c] · Katsutoshi Arai[a]

[a]Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan

[b]School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa, Japan

[c]Nanae Freshwater Station, Field Science Center for Northern Biosphere, Hokkaido University, Nanae, Hokkaido, Japan

[d]Japan Society for the Promotion of Science, Tokyo, Japan

Short Title

Hybrid fish sterility caused by aberrant meiotic configurations

***Corresponding Author**

Masamichi Kuroda

Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan. +81-0138-40-5536. shindou19900120@eis.hokudai.ac.jp

Keywords

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Abstract

Gonochoristic wild-type dojo loaches (*Misgurnus anguillicaudatus*) are diploid (2n = 50) and reproduce bisexually. However, sympatric clonal diploids generate unreduced diploid isogenic eggs, which develop gynogenetically. Clone-origin triploidy arises following the incorporation of a haploid wild-type sperm nucleus into the diploid egg. Triploid females produce fertile haploid eggs by meiotic hybridogenesis, while triploid males are sterile. Clonal loaches arose from past hybridization event(s) between genetically diverse groups, A and B. Artificial hybrid females between the two groups produce unreduced and/or aneuploid eggs, but the hybrid males are sterile. In this study, using fluorescence *in situ* hybridization (FISH), we analyzed chromosome pairing in the meiotic cells of clone-origin triploid and inter-group hybrid males to clarify the cytogenetic mechanisms underlying the male-specific sterility. We used a repetitive sequence probe to identify group B-derived chromosomes and a 5.8S + 28S rDNA probe to identify pairs of homologous chromosomes. We found that asynapsis and irregular synapsis occur in triploid and hybrid males containing two different genomes and that this may cause the

35 formation of sterile germ cells. These results will help us understand hybrid sterility from

36 the viewpoint of synapsis behavior.

Introduction

Diploid wild-type dojo loaches, *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes, Teleostei), are gonochoristic and reproduce bisexually in most Japanese populations [Arai and Fujimoto 2013]. However, clonal individuals appear in several populations in Hokkaido and Ishikawa Prefectures, Japan [Morishima et al., 2002, 2008a]. Clonal dojo loaches are essentially all female because this species has a male heterogametic (XX female, XY male) sex determination system [Suzuki et al., 1985], and thus no contribution of Y chromosome is predicted in clonal progeny. The clonal females generate unreduced isogenic diploid eggs, which develop by gynogenesis triggered by sperm from sympatric wild-type diploid males [Morishima et al., 2002; Itono et al., 2006, 2007]. Our genetic studies revealed that clonal loaches were heterozygotes that originated from past hybridization event(s) presumably between genetically diverse group A females and group B males [Khan and Arai 2000; Arias-Rodriguez et al., 2007; Morishima et al., 2008a; Yamada et al., 2015], as previously reported in other unisexual vertebrates [Dawley 1989; Vrijenhoek 1994; Lutes et al., 2010]. Recently, nuclear DNA markers based on repetitive sequences were developed to distinguish the genomes of group A and B loaches [Fujimoto et al., 2017]. One of these, ManDra, exclusively detects 25 group B

54 loach chromosomes (haploid number) in the diploid metaphases of clonal loaches with
55 2n = 50 karyotype by FISH [Kuroda et al., 2018]. Both in meiosis of oocytes from clonal
56 females and spermatocytes from artificially sex-reversed clonal males, 50 bivalents were
57 easily counted. Thus, pairing should occur between sister chromosomes doubled from
58 each ancestral chromosome from two different groups by a mechanism of premeiotic
59 endomitosis [Itono et al., 2006; Yoshikawa et al., 2007, 2009; Kuroda et al., 2018].
60 Therefore, clonal loaches are of hybrid origin with heterozygous genotype (AB),
61 including group A and B chromosome sets, and clonally reproduce by gynogenesis.
62 However, isogenic diploid eggs sometimes incorporate the haploid sperm nucleus of
63 sympatric wild-type diploids to become clone-origin triploids with two chromosome sets
64 (genomes) of group A and one chromosome set of group B, i.e., trigenomic type AAB
65 [Itono et al., 2007; Morishima et al., 2008b]. Such triploid females produce haploid eggs
66 including chromosomes exclusively from group A by quasi-normal meiosis after
67 eliminating unmatched group B chromosomes through meiotic hybridogenesis during
68 oogenesis [Morishima et al., 2008b]. In contrast, triploid males produce few haploid,
69 triploid, and hexaploid spermatozoa or spermatozoon-like cells, most of which have no
70 motility after the addition of ambient water and exhibit morphological abnormalities such

71 as larger head sizes and short, or no, flagellum [Oshima et al., 2005]. None or very few
72 progeny appear after the fertilization of normal eggs with sperm of clone-origin triploid
73 males, indicating that clone-origin triploid males are post-zygotic sterile [Oshima et al.,
74 2005].

75 While the clonal loach is of hybrid origin, artificial hybrids between extant genetic
76 groups A and B produce different reproductive consequences. Although such hybrid
77 females produce fertile diploid and/or aneuploid eggs, the resultant unreduced diploid
78 eggs never develop gynogenetically and become triploid progeny by incorporating a
79 sperm nucleus at the time of fertilization [Arias-Rodriguez et al., 2009]. In contrast,
80 hybrid males produce haploid, diploid, and tetraploid cell populations in testis and semen,
81 most of which exhibit abnormal morphology [Arias-Rodriguez et al., 2010]. Very few
82 diploid and triploid progeny appear after fertilization of normal eggs with such sperm,
83 suggesting that inter-group hybrid males are sterile [Arias-Rodriguez et al., 2010].

84 Both females and artificially sex-reversed males of clonal diploid loaches are fertile
85 [Morishima et al., 2002; Itono et al., 2006, 2007; Yoshikawa et al., 2007, 2009], but clone-
86 origin triploids and inter-group hybrids males are sterile [Oshima et al., 2005; Arias-
87 Rodriguez et al., 2010]. All of these biotypes are essentially of hybrid origin and include

88 chromosome sets (genomes) from genetically different groups A and B [Khan and Arai
89 2000; Arias-Rodriguez et al., 2007; Morishima et al., 2008a, 2008b; Yamada et al., 2015;
90 Kuroda et al., 2018]. The genomic constitution of clonal diploid, clone-origin triploid,
91 and artificial hybrids can be designated as AB, AAB, and AB, respectively. Why clone-
92 origin triploids and artificial hybrids exhibit male sterility and clonal diploids of both
93 sexes are fertile, in spite of their similar heterozygous genomic constitutions, remains
94 unknown. There are relatively few cytogenetic studies on the mechanisms of hybrid
95 sterility in fish, and other vertebrates, even though there are numerous reports of hybrid
96 formation [Schwenk et al., 2008]. Unbalanced meiotic configurations, including
97 univalent and multivalent chromosomes, are frequently observed in interspecific hybrids
98 of mammals [Bhattacharyya et al., 2013; Torgasheva et al., 2016], birds [Islam et al.,
99 2013], amphibians [Müller 1977], and fish [Shimizu et al., 1997] indicating failure of
100 synapsis as the cause of postzygotic sterility. To the best of our knowledge, cytogenetic
101 analyses using FISH with species-specific probes have not been performed in fish hybrids.
102 Here, we examined the presence or absence of regular pairing of homologous and/or
103 homoeologous chromosomes in testicular cells of sterile clone-origin triploid and inter-
104 group hybrid males to gain insight into the cytogenetic mechanisms underlying the

observed male-specific sterility. We used FISH techniques to distinguish chromosomes derived from group B and to identify nucleolar organizing region (NOR) bearing chromosomes.

Materials and methods

Group identification and ploidy determination of experimental fish

Two dojo loaches were used in this study: a clone-origin triploid male and an inter-group hybrid male between groups A and B wild-type loach. Group A wild-type, group B wild-type, clone-origin triploid, and inter-group hybrid were distinguished by mitochondrial DNA-control region haplotype [Morishima et al., 2008a], nuclear DNA-*RAG1* gene sequence [Yamada et al., 2015] and RFLP [Fujimoto et al., 2017], and electrophoretic pattern of repetitive sequences ManDra and ManBgl [Fujimoto et al., 2017]. Sample ploidy was determined by flow cytometry as described previously [Morishima et al., 2002]. The triploid loach was caught in Abashiri city, Hokkaido Prefecture. To improve the accuracy of ploidy determination in the triploid, we confirmed chromosome number in somatic cells and used flow cytometry. The inter-group hybrid was induced by artificial fertilization with group B wild-type eggs from Nanae town, Hokkaido Prefecture and group A wild-type sperm from Abashiri city, Hokkaido Prefecture.

Chromosome preparation

To prepare chromosome slides from kidneys and testis, goat serum (100 μl/g body weight) was individually injected five and one day prior to sacrifice. Subsequently 0.01%

125 colchicine in physiological saline (NaCl 7.5 g, KCl 0.2 g, $CaCl_2 \cdot 2H_2O$ 0.264 g DW/L)
126 was injected 2.5 h prior to sacrifice. After kidney and testis tissues were removed from
127 the body, the tissues were cut into small pieces. These pieces were treated with a
128 hypotonic solution (0.075 M KCl) for 20 min and fixed in Carnoy's solution (3:1
129 methanol/acetic acid) until use. Cell suspensions from the tissues were dropped onto glass
130 slides and air-dried. Before being used for FISH, the slides were incubated at 65 °C for
131 24 h for hardening.

132 **Two-color FISH**

133 Repetitive sequences (approximately 130 bp), designated as ManDra, were isolated from
134 group B genomic DNA by *Dra*I restriction enzyme digestion described in Fujimoto et al.
135 [2017]. Plasmids containing inserted ManDra repetitive sequences were used as ManDra
136 probes to distinguish group B-derived chromosomes [Kuroda et al., 2018]. The ManDra
137 probe was labeled with biotin-16-dUTP by Biotin-Nick Translation Mix (Roche).

138 Human 5.8S + 28S rDNA sequences were used as probes [Fujiwara et al., 1998] to
139 determine the largest (first) metacentric homologous chromosomes in the dojo loach [Li
140 et al., 2010, 2011, 2012, 2015, 2016; Kuroda et al., 2018]. Two rDNA signals can be
141 detected in diploid loaches and three rDNA signals can be detected in triploid loaches.

142 The rDNA probe was labeled with digoxigenin-11-dUTP by Dig-Nick Translation Mix
143 (Roche).

144 Two-color FISH was performed according to Kuroda et al. [2018]. The biotin-labeled
145 ManDra probe was detected with streptavidin, Alexa Fluor 488 conjugate (Thermo Fisher
146 Scientific). The signals were amplified using a biotinylated anti-avidin antibody (Vector
147 Laboratories). The digoxigenin-labeled 5.8S + 28S rDNA probe was detected with Anti-
148 Digoxigenin-Rhodamine, Fab fragments (Roche). The slide was counterstained with 4,
149 6-diamidino-2-phenylindole, dihydrochloride (DAPI).

150 **Statistics**

151 Data are shown as mean ± standard deviation (SD). Statistical significance in the number
152 of bivalent chromosomes (II-AA, II-AB) was confirmed using Student's *t*-test. *P*-values <
153 0.05 were considered statistically significant.

Results

Genomic composition analysis in clone-origin triploid male using two-color FISH

In a clone-origin triploid sample, somatic metaphases comprised 75 chromosomes: four out of five metaphases showed eutriploidy with 75 chromosomes, while one was from a broken cell with 68 chromosomes. ManDra signals were detected at the centromeric regions of 25 out of 75 chromosomes (Fig. 1a). Therefore, these 25 chromosomes were derived from group B, and the other 50 chromosomes were derived from group A. 5.8S + 28S rDNA signals were clearly observed in NORs at the short arms of the three largest (first) metacentric homologous chromosomes (Fig. 1 (a, b) arrow and dotted arrows). ManDra signal was detected in one of the three rDNA bearing homologous chromosomes (Fig. 1b). Therefore, the genomic composition of the triploid was cytogenetically confirmed to be AAB, with AB (egg-derived) and A (sperm-derived) genomes.

Meiosis in clone-origin triploid male

Meiotic metaphases of clone-origin triploid males frequently comprised both bivalent and univalent chromosomes, but trivalent chromosomes were scarce. There were two types of NOR-bearing chromosomes with rDNA signals among the bivalent chromosomes: bivalent chromosomes paired between homologues from group A, without ManDra signal

171 (II-AA, Fig. 2 (a, b)); and bivalent chromosomes paired between a chromosome without
172 ManDra signal from group A and a chromosome with ManDra signal from group B (II-
173 AB, Fig. 2 (c, d)). Failure of pairing generated one ManDra-positive univalent
174 chromosome (I-B) and two ManDra-negative univalent chromosomes (I-A) (Fig. 2 (e, f)).
175 Infrequent trivalent chromosomes (III-AAB) showed one ManDra-positive and two
176 ManDra-negative elements among the three elements with rDNA signals (Fig. 2 (g, h)).
177 Using the presence or absence of ManDra signal as the defining criteria, bivalent
178 chromosomes were categorized as II-AA (pairing between chromosomes from group A)
179 and II-AB (pairing between chromosomes from two groups A and B) (Fig. 2). Univalent
180 chromosomes could also be categorized as I-A and I-B and trivalent chromosomes as III-
181 AAB (Fig. 2). The numbers of uni-, bi-, and trivalent chromosomes largely varied from
182 metaphase to metaphase, but the chromosome number at each metaphase was consistently
183 75, equivalent to triploidy (Table 1). Homogenomic bivalent chromosomes (II-AA) were
184 predominant in triploid metaphases, but their number varied between 10 (20
185 chromosomes) and 24 (48 chromosomes), while the number of heterogenomic bivalent
186 chromosomes (II-AB) varied between one (two chromosomes) and nine (18
187 chromosomes) (Table 1). Bivalent chromosome number per cell was mean 17.4 ± SD 3.0

188 for II-AA, and was significantly larger (t = 19.1, $p = 1.96 \times 10^{-19} < 0.05$) than the 3.8 ±
189 1.9 observed for II-AB (Table 1). Accordingly, the mean number of univalent
190 chromosomes per cell was 21.2 ± SD 1.8 and 11.3 ± 5.4 for I-B, and I-A, respectively
191 (Table 1). Formation of a trivalent chromosome was difficult, even in a triploid metaphase,
192 and only one cell with trivalent chromosomes was observed in a total of 34 metaphases
193 (Table 1).
194
195 **Confirmation of genome origin in the hybrid loach**
196 Twenty-five ManDra signals were detected in the inter-group hybrid. Therefore, the
197 hybrid comprised 25 chromosomes derived from group A and 25 chromosomes derived
198 from group B (Fig. 3).
199 **Meiosis in inter-group hybrid male**
200 Spermatocytes of the inter-group hybrid contained different numbers of bivalent and
201 univalent chromosomes (Fig. 4, Table 2). Twenty-five ManDra probe signals were
202 detected in all 43 meiotic cells examined. Two-color FISH indicated the presence of
203 heterogenomic bivalent chromosomes (II-AB) formed by pairing between a group A-
204 derived (ManDra-negative) and group B-derived (ManDra-positive) chromosomes in

NOR bearing largest (first) metacentric bivalent chromosomes with rDNA signal (Fig. 4 (a, b)). The mean number of bivalent chromosomes per cell was 21.8 ± SD 1.6 for II-AB (Table 2). The other metaphases showed two univalent chromosomes from NOR bearing chromosomes with rDNA signal, one also had the ManDra signal from group B (I-B) and another had no signal from group A (I-A) (Fig. 4 (c, d)). The mean number of univalent chromosomes per cell was 3.2 ± SD 1.6 for I-A and I-B (Table 2). The frequency of univalent chromosomes was reduced due to the increased number of bivalent chromosomes. Most metaphases (36/43 cells) included 21 to 23 bivalent chromosomes and four to eight univalent chromosomes.

Discussion

In the clone-origin triploid male, bivalent chromosomes preferentially synapsed between chromosomes derived from the same group, group A. Consequently, univalent chromosomes of group A chromosomes (mean 11.3 ± SD 5.4) were observed less frequently than those of group B (21.2 ± 1.8), because they tended to pair with their counterpart from the same origin. The numbers of bivalent and univalent chromosomes largely varied among spermatocytes, and no clear modal configuration was observed. A trivalent chromosome was identified in only one spermatocyte. Taken together, these results show that synapsis occurs between homologous chromosomes derived from the same origin with higher affinity, but that synapsis between chromosomes derived from different origins is also possible, but occurs infrequently. Consequently, in triploid meiosis, 10 to 24 II-AA, 1 to 9 II-AB, 1 to 23 I-A, and 16 to 24 I-B appeared. Bivalent chromosomes should segregate to each pole, while univalent chromosomes might segregate randomly. Therefore, various kinds of aneuploid spermatozoa were predicted in the clone-origin triploid, but no or very few functional sperm were produced. Natural clone-origin triploid males exhibit triploid and hexaploid cell populations in testis, but functional haploid spermatozoa are very rarely observed and most spermatozoa and

232 spermatozoon-like cells are morphologically abnormal and non-motile [Oshima et al.,
233 2005]. Therefore, in the clone-origin triploid male, replication would proceed to form
234 hexaploid cells with 6C DNA content. These cells may then accumulate in testis without
235 completing the first meiotic division, due to their aberrant chromosome configurations
236 including irregular univalent chromosomes and trivalent chromosomes. Spermiogenesis
237 without completion of meiosis was reported in the diploid interspecific medaka hybrid,
238 in which non-motile spermatozoon-like cells with tetraploidy (4C DNA content) were
239 formed [Shimizu et al., 1997]. Similar phenomenon may occur in clone-origin triploid
240 testis with germ cells able to replicate but unable to complete regular meiosis, leading to
241 the appearance of abnormal spermatozoon-like cells. This reproductive feature differs
242 greatly from those observed in the clone-origin triploid female [Morishima et al., 2008b].
243 In the triploid female, 25 bivalent chromosomes were observed in germinal vesicles of
244 mature oocytes, and fertile haploid eggs were produced, presumably by eliminating
245 unmatched genomes and subsequent quasi-normal bivalent chromosome formation
246 during meiosis (meiotic hybridogenesis) [Morishima et al., 2008b]. Here, meiotic cells
247 composed of 25 bivalent chromosomes, without any univalent and trivalent chromosomes,

248 were never observed, suggesting genome elimination occurs in later stages or that there
249 is no genome elimination.

250 The inter-group hybrid male, between groups A and B, showed post-zygotic sterility
251 because it produced non-motile spermatozoa or spermatozoon-cells [Arias-Rodriguez et
252 al., 2010]. Here, we observed that most chromosomes (34 to 48 of 50) paired to form
253 bivalent chromosomes (17 to 24 of 25) between heterogenomic chromosomes derived
254 from groups A and B, but two to 16 chromosomes, which could not find a counterpart
255 with which to pair, formed two to 16 univalent chromosomes in the hybrid male. Although
256 univalent chromosomes were observed in all meiotic cells, the number of bivalent and
257 univalent chromosomes differed in each cell. As observed in other sterile interspecific
258 hybrids of vertebrates [Müller 1977; Shimizu et al., 1997; Bhattacharyya et al., 2013;
259 Islam et al., 2013; Torgasheva et al., 2016], failure of synapsis was observed in the inter-
260 group hybrid loach male. Coexistence of bivalent and univalent chromosomes may cause
261 the formation of aneuploid gametes due to random univalent segregation, but hybrid
262 males produced haploid, diploid, and tetraploid spermatozoa or spermatozoon-like cells
263 with low ability to generate next generation zygotes [Arias-Rodriguez et al., 2010].

264 Similar to the triploid males, tetraploid cells with 4C content were observed in the
265 sperm and testis of inter-group hybrid males and they were considered to be
266 spermatocytes that underwent replication but could not complete meiosis [Arias-
267 Rodriguez et al., 2010]. Thus, tetraploid spermatocytes presumably accumulated in the
268 testes, and differentiated into spermatozoon-like cells without completion of meiosis.
269 Such a situation was reported in interspecific medaka hybrids [Shimizu et al., 1997].

270 In a clonal diploid loach, unreduced diploid gametes are produced by pairing between
271 sister chromosomes doubled from each ancestral chromosome before meiosis (premeiotic
272 endomitosis) [Kuroda et al., 2018]. If the same mechanism acted in clone-origin triploid
273 males and inter-group hybrid males, 50 ManDra signals should be detected. In this study,
274 however, 25 ManDra signals were detected in all meiotic cells observed, suggesting no
275 occurrence of premeiotic endomitosis. It is unclear why premeiotic endomitosis did not
276 occur in the inter-group hybrid male (AB), which had a genomic constitution similar to
277 the clonal male (AB).

278 Analysis of mitochondrial DNA-control regions indicated that the genetic distance
279 between the two groups, A and B, is larger than that between different species, *M.*
280 *mizolepis* and *M. fossilis* [Morishima et al., 2008a]. Similar results were obtained by

281 analyses of allozyme variation and nuclear DNA-*RAG1* gene [Khan and Arai 2000;
282 Yamada et al., 2015]. The presence of asynapsis observed in our study provides
283 cytogenetic evidence that distant genetic differentiation occurred between the two groups
284 of dojo loach.

285 The reproductive system in hybrids of European spined loach (genus *Cobitis*) is
286 similar to dojo loach. The spined loach hybrid complex is composed of diploid, triploid,
287 and tetraploid [Janko et al., 2007a]. Moreover, clonal lineages originating from
288 hybridization between different *Cobitis* species are present [Janko et al., 2007a]. Clonally
289 reproducing diploid females are present in both the dojo and spined loach [Janko et al.,
290 2007a, 2007b; Choleva et al., 2012]. However, in triploid females, the clone-origin
291 triploid dojo loach produces haploid eggs by a mechanism of meiotic hybridogenesis
292 [Morishima et al., 2008b], but the triploid spined loach lays unreduced triploid eggs which
293 develop by gynogenesis [Janko et al., 2007b]. On the other hand, the males showed
294 common characteristics. Spermatozoa from a triploid male of spined loach did not move
295 after adding ambient water [Vasil'ev et al., 2003]. Moreover, diploid hybrid males were
296 sterile [Choleva et al., 2012; Juchno and Boroń 2018]. Thus, sterility of the male spined
297 loach may be caused by aberrant meiotic configurations, as observed in dojo loach.

Our results show that asynapsis and irregular synapsis occurred in triploid and hybrid males containing two different genomes and that this may lead to the formation of sterile spermatozoa. These results will help us to understand hybrid sterility from the viewpoint of synapsis behavior.

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Statement of Ethics

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

Disclosure Statement

315 The authors have no conflicts of interest to declare.

316

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423

424 **Table**

425

426 **Table 1.** Number of univalent, bivalent, and trivalent chromosomes in meiotic
427 metaphases of clone-origin triploid male. I: Univalent chromosome, II: Bivalent
428 chromosome, III: Trivalent chromosome.

429

Univalent (I)		Bivalent (II)		Trivalent (III)	Chromo.	No. of
A	B	AA	AB	AAB	no.	cell
1	24	24	1	0	75	1
2	21	22	4	0	75	1
5	24	22	1	0	75	1
4	19	20	6	0	75	2
5	20	20	5	0	75	1
9	24	20	1	0	75	1
6	21	20	4	0	75	1
8	21	19	4	0	75	1
9	22	19	3	0	75	2
10	23	19	2	0	75	1
9	20	18	5	0	75	3
11	22	18	3	0	75	3
10	19	17	6	0	75	1
11	20	17	5	0	75	1
13	22	17	3	0	75	2
12	22	17	2	1	75	1
14	21	16	4	0	75	1
16	23	16	2	0	75	3
17	22	15	3	0	75	1
17	20	14	5	0	75	1

18	21	14	4	0	75	1
20	23	14	2	0	75	1
17	16	12	9	0	75	1
20	19	12	6	0	75	1
23	18	10	7	0	75	1
11.3* (5.4)**	21.2 (1.8)	17.4 (3.0)	3.8 (1.9)			Total 34 cells

430 *: Mean, **: Standard deviation (SD)

431

Table 2. Number of univalent and bivalent chromosomes in meiotic metaphases of inter-group hybrid male between groups A and B. I: Univalent chromosome, II: Bivalent chromosome.

Univalent (I)		Bivalent (II)	Chromo.	No. of
A	B	AB	no.	cell
8	8	17	50	1
7	7	18	50	2
6	6	19	50	1
5	5	20	50	1
4	4	21	50	10
3	3	22	50	11
2	2	23	50	15
1	1	24	50	2
3.2* (1.6)**	3.2 (1.6)	21.8 (1.6)		Total 43 cells

*: Mean, **: Standard deviation (SD)

Figure Legends

Figure 1. Two-color FISH using ManDra and 5.8S + 28S rDNA probes in the somatic cells of the clone-origin triploid male. Representative somatic metaphase (**a**) and the partial karyotype of the largest homologous chromosomes (**b**) in the clone-origin triploid

443 after two-color FISH with ManDra and 5.8S + 28S rDNA probes. The arrow indicates the
444 largest homologous metacentric chromosome with ManDra and rDNA signals. Dotted
445 arrows indicate the largest homologous metacentric chromosomes with rDNA signal but
446 not ManDra signal. The ManDra probe was labeled with biotin-16-dUTP and detected
447 using streptavidin Alexa Fluor 488 conjugate (Green). The 5.8S + 28S rDNA probe was
448 labeled with digoxigenin-11-dUTP and detected using Anti-Digoxigenin-Rhodamine,
449 Fab fragments (Red). All chromosomes were counterstained with DAPI (Blue). Scale bars
450 are 10 μm.
451
452 **Figure 2.** Two-color FISH with ManDra and 5.8S + 28S rDNA probes in spermatocytes
453 of clone-origin triploid male. Representative meiotic metaphases (**a**, **c**, **e**, **g**), partial
454 karyotypes and schematic diagrams (**b**, **d**, **f**, **h**). Bivalent chromosome that originated
455 from group A and a univalent chromosome that originated from group B (**b**), bivalent
456 chromosome originating from groups A and B and a univalent chromosome that
457 originated from group A (**d**), three univalent chromosomes that originated from groups A
458 and B (**f**), and one trivalent chromosome that originated from groups A and B (**h**) in
459 spermatocytes of a clone-origin triploid after two-color FISH with ManDra and 5.8S +

460 28S rDNA probes. Arrows indicate the bivalent chromosome with two rDNA signals.
461 Dotted arrows indicate the univalent chromosomes. The arrowhead indicates the trivalent
462 chromosome with three rDNA signals. The ManDra probe was labeled with biotin-16-
463 dUTP and detected using streptavidin Alexa Fluor 488 conjugate (Green). The 5.8S + 28S
464 rDNA probe was labeled with digoxigenin-11-dUTP and detected using Anti-
465 Digoxigenin-Rhodamine, Fab fragments (Red). All chromosomes were counterstained
466 with DAPI (Blue). I: Univalent chromosome, II: Bivalent chromosome, and III: Trivalent
467 chromosome. Scale bars are 10 μm.
468
469 **Figure 3.** Two-color FISH with ManDra and 5.8S + 28S rDNA probes in somatic cells of
470 the inter-group hybrid. Representative metaphase (**a**) and partial karyotype of a pair of
471 the largest homologous metacentric chromosomes (**b**) in somatic cells of hybrid between
472 wild-type loach groups A and B after two-color FISH with ManDra and 5.8S + 28S rDNA
473 probes. The arrow indicates one of a pair of the largest homologous metacentric
474 chromosomes with ManDra signals. The dotted arrow indicates the other of a pair of the
475 largest homologous metacentric chromosomes without ManDra signals. The ManDra
476 probe was labeled with biotin-16-dUTP and detected using streptavidin Alexa Fluor 488

conjugate (Green). A pair of the largest homologous metacentric chromosomes with nucleolar organizing regions (NORs) were determined using the 5.8S + 28S rDNA probe labeled with digoxigenin-11-dUTP and detected with Anti-Digoxigenin-Rhodamine, Fab fragments (Red). All chromosomes were counterstained with DAPI (Blue). Scale bars are 10 μm.

Figure 4. Two-color FISH with ManDra and 5.8S + 28S rDNA probes in spermatocytes of the inter-group hybrid. Representative meiotic metaphases (**a**, **c**) and partial karyotypes and schematic diagrams (**b**, **d**) of the bivalent chromosome (**b**) and two univalent chromosomes (**d**) in spermatocytes of the inter-group hybrid (between groups A and B) after two-color FISH with ManDra and 5.8S + 28S rDNA probes. The arrow indicates the bivalent chromosome with a ManDra signal and two rDNA signals. The dotted arrows indicate the two univalent chromosomes with rDNA signals. One of two univalent chromosomes with rDNA signals had a ManDra signal. ManDra probe was labeled with biotin-16-dUTP and detected using streptavidin Alexa Fluor 488 conjugate (Green). The rDNA sequences of the bivalents were visualized by labeling the 5.8S + 28S rDNA probe with digoxigenin-11-dUTP and detection using Anti-Digoxigenin-Rhodamine, Fab

494 fragments (Red). All chromosomes were counterstained with DAPI (Blue). I: Univalent

495 chromosome. II: Bivalent chromosome. Scale bars are 10 μm.

Figure Legends

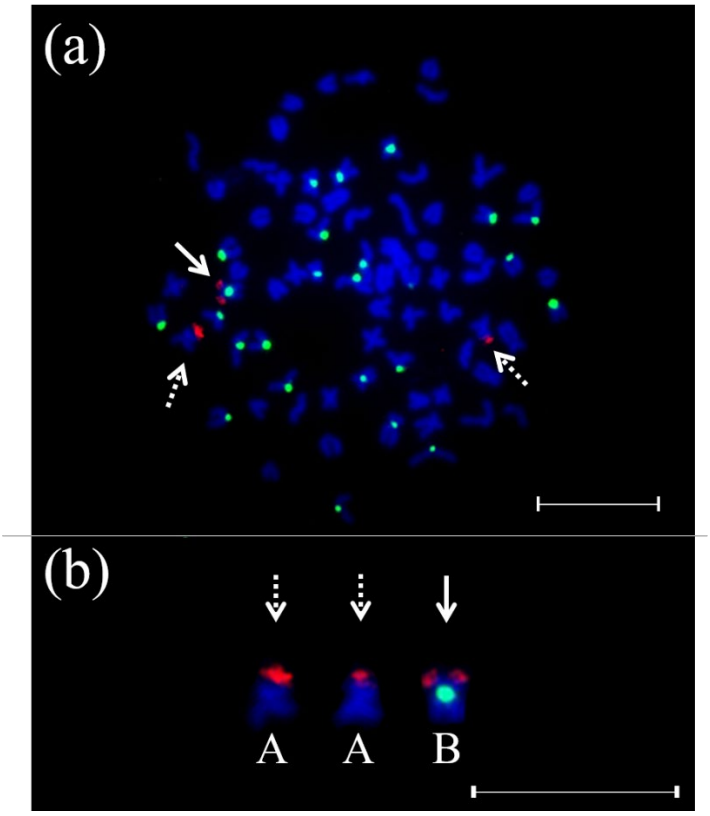

Figure 1.

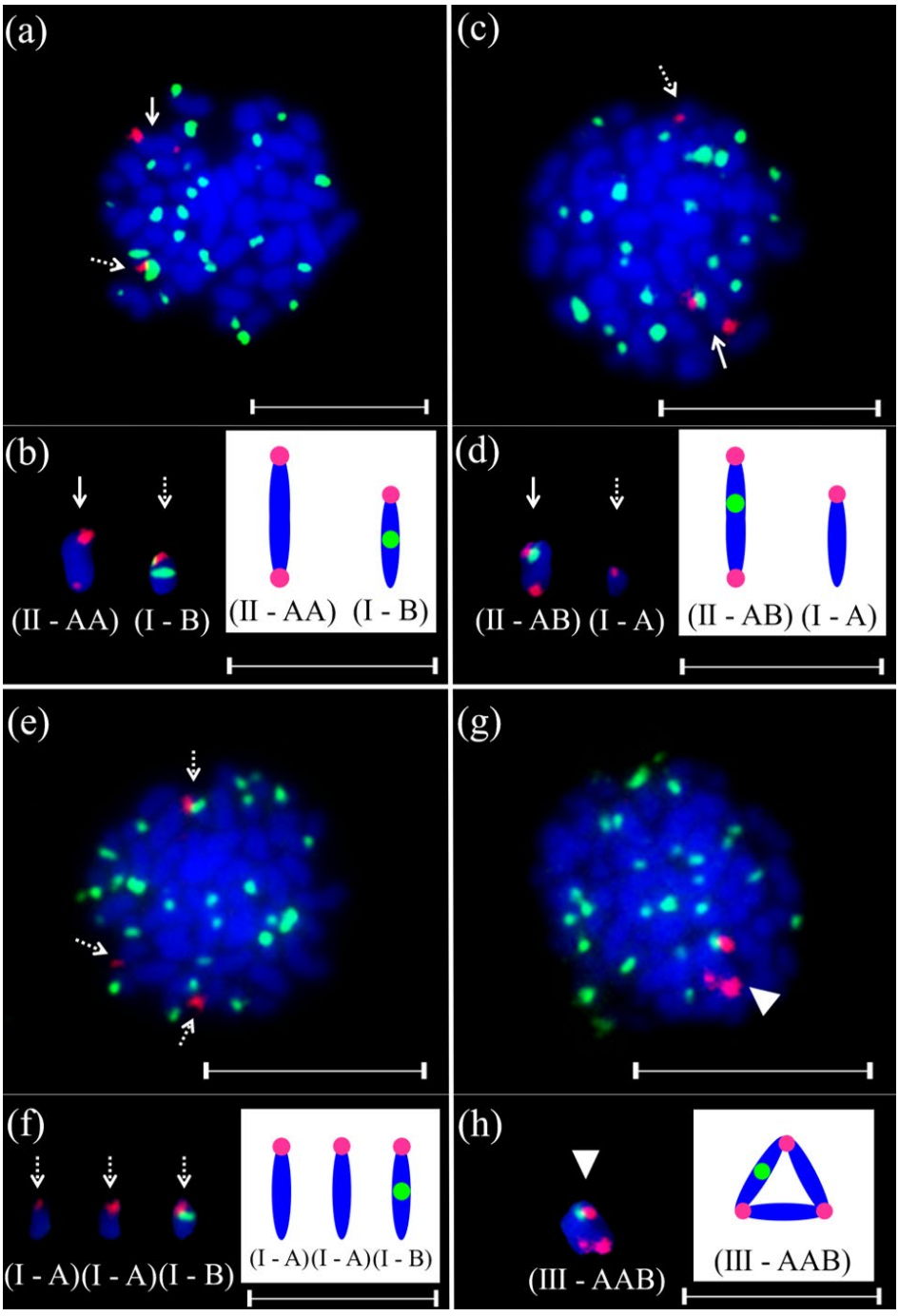
(a)
(c)
(b)
(II - AA) (I - B)
(II - AA) (I - B)
(d)
(II - AB) (I - A)
(II - AB) (I - A)
(e)
(g)
(f)
(I - A)(I - A)(I - B)
(I - A)(I - A)(I - B)
(h)
(III - AAB)
(III - AAB)

Figure 2.

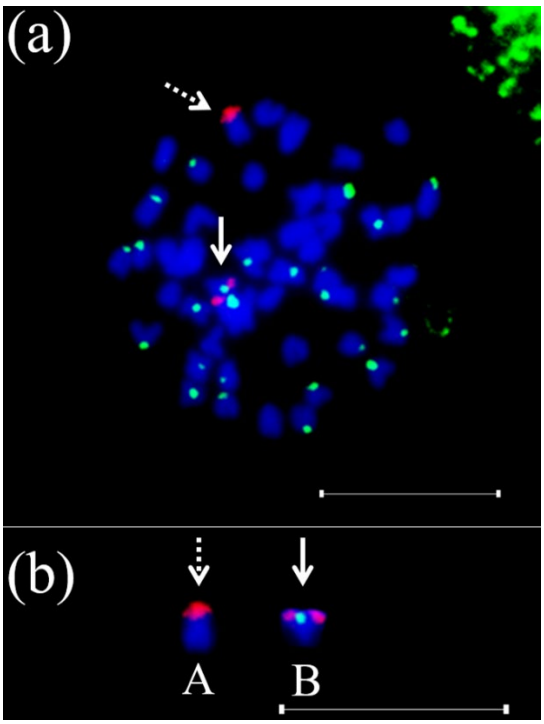

Figure 3.

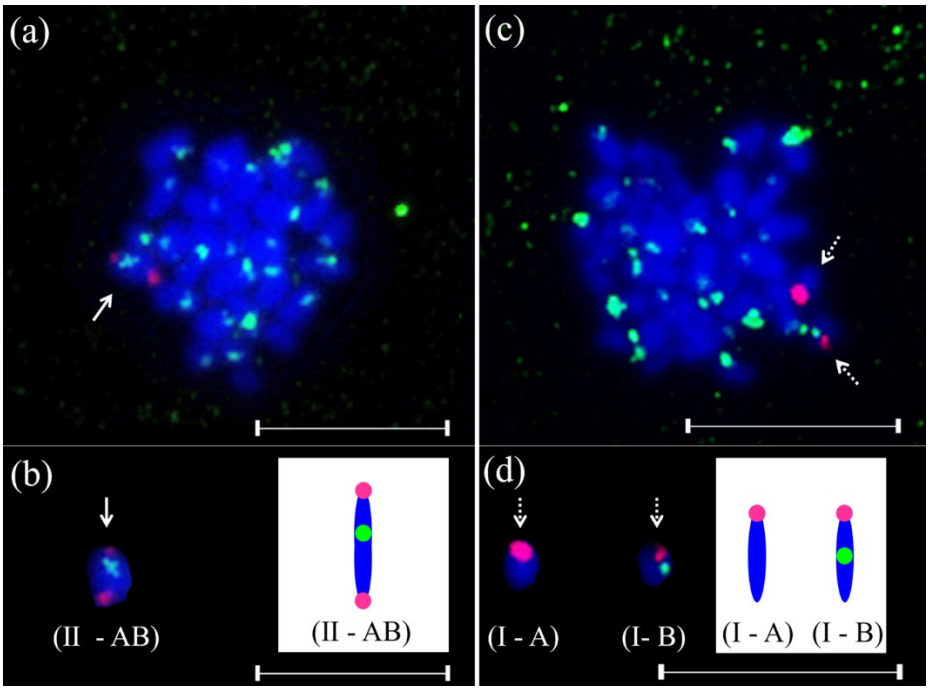

Figure 4.