Genetic characterization of the progeny of a pair of the tetraploid silver crucian carp *Carassius auratus langsdorfii*

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Abstract

Silver crucian carp *Carassius auratus langsdorfii* comprises a diploid-polyploid complex in wild Japanese populations. Bisexually reproducing diploids are sympatrically distributed with gynogenetically developing triploids and tetraploids. Triploid and tetraploid males are very rare among Japanese silver crucian carp due to their gynogenetic reproduction. We examined the genetic characteristics of progeny that arose in a tank by natural spawning of tetraploid silver crucian carp pair. Ploidy status of 120 samples randomly collected from these progeny was determined to be tetraploid by DNA content flow cytometry. DNA fingerprints from a random amplified polymorphic DNA assay indicated that almost all the progeny examined had genotypes identical to the maternal tetraploid female with no paternally derived fragments. Selected specimens' cytogenetic analyses revealed that the progeny examined had tetraploid chromosome numbers, categorized into 40 metacentric (M), 80 submetacentric (SM), and 80 subtelocentric or telocentric (ST/T) chromosomes, which were arranged into quartets and six supernumerary microchromosomes (m). Fluorescence *in situ* hybridization signals were detected in four homologous chromosomes in all analyzed metaphases prepared from diploid goldfish specimens. Contrary, tetraploid silver crucian carp gave eight rDNA signals. These results suggest that gynogenetic development in eggs spawned by tetraploid females should be triggered by tetraploid males' homospecific sperm.

Keywords Silver crucian carp · Tetraploid · Gynogenesis · Flow cytometry · RAPD markers · FISH

Introduction

Silver crucian carp *C. auratus langsdorfii* comprises a diploid-polyploid complex in the wild populations of Japan. Bisexually reproducing diploids (2n = 100) are sympatrically distributed with gynogenetically developing triploids (3n = 156) and tetraploids (4n = 206) [1–3]. Frequencies of tetraploids are much lower than those of sympatric diploids and triploids [4, 5], and these tetraploids are considered to arise from triploids by accidental incorporation of a haploid sperm nucleus into a triploid egg [6–8]. The occurrence of relatively rare tetraploid from triploid and reproductive characteristics of such tetraploid progeny will provide good insights into mechanisms responsible for the origin, diversification and maintenance of such a diploid-polyploid complex in nature. Thus, the wild tetraploid Japanese silver crucian carp *C. auratus langsdorfii* has been considered excellent material to disclose the biological significance of polyploidy in fish reproduction and evolution.

Because unreduced eggs of both triploids and tetraploids develop by gynogenesis (sperm-dependent parthenogenesis) without any genetic contributions from males, the offspring are theoretically an all-female population due to no contribution by male-determinant Y chromosomes. Although a high male incidence has been observed among Chinese silver crucian carp *C. auratus gibelio*, because these triploid forms undergo a mixture of allogynogenesis (gynogenesis triggered by heterospecific sperm from other species) and bisexual gonochoristic reproduction [9], both triploid and tetraploid males are very rare in Japanese silver crucian carp *C. auratus langsdorfii* population [10, 11].

In 2003, we happened to discover a tetraploid male among samples from a rearing population in the Gunma Prefecture Fisheries Experiment Station. This male has been reared in the Nanae Fresh-Water Laboratory, Hokkaido University, Nanae, Hokkaido, since 2004. This male is kept in a tank along with a

66 conspecific tetraploid female whose sampling site and year were not recorded. They reproduced in this
67 tank by natural spawning, after which a large number of progeny appeared.
68 There are three possible genetic results for the progeny of a pair of tetraploids. (1) When some
69 reduced diploid eggs are laid by a tetraploid female and then fertilized with diploid sperm from a tetraploid
70 male, the resulting progeny are likely to be tetraploids with different genotypes. (2) Hexaploid progeny
71 with different genotypes may result when unreduced tetraploid eggs accidentally incorporate a diploid
72 sperm nucleus from a tetraploid male. (3) If unreduced tetraploid eggs of a tetraploid female initiate
73 gynogenetic development due to the intrusion of diploid sperm from a tetraploid male, all of these progeny
74 will grow to a second generation of gynogenetic tetraploids.
75 In the present study, 120 individuals were randomly selected from the large population of progeny
76 arising from a pair of tetraploids and were assessed for their ploidy status by flow cytometry analysis. Then,
77 using selected samples, the isogenic characteristics of these progeny were verified by random amplified
78 polymorphic (RAPD) DNA-PCR fingerprints. Chromosomes of these progeny were also prepared and
79 assessed by conventional karyotyping and rDNA detection using fluorescence *in situ* hybridization (FISH).
80
81 **Materials and methods**
82 *Ethics*
83 Research and handling of the animals were performed in lines with the Guide for the Care and Use of
84 Laboratory Animals at Hokkaido University.
85
86 *Source of samples*

A tetraploid male *C. a. langsdorfii* was found in a rearing population of the Gunma Prefectural Fisheries Station in 2003, and then transferred and kept at Azabu University. The rearing population of *C. a. langsdorfii* in the Gunma Prefectural Fisheries Station was originated from wild population of the Jounuma Lake, Gunma Prefecture. The tetraploid male was transferred to the Nanae Fresh-Water Laboratory, Hokkaido University, Nanae, Hokkaido in 2004. No sampling and rearing record of the female *C. a. langsdorfii* tetraploid individual was available, but it has been reared together with the tetraploid male in the same tank. The tetraploid pair reproduced firstly in the tank in May, 2009 and a large number of progeny appeared in the tank since this year.

A total of 120 individuals were randomly sampled from these progeny. Due to their small body sizes (body length, 36 to 54 mm), sex could not be determined for each individual by naked-eye observation. Fin clips were collected for somatic cellular DNA content determinations and stored in 100% ethanol at −30 °C for genomic DNA extraction. Samples of goldfish *Carassius auratus auratus* which were kept in the aquarium of the Environment Control Experiment Building, Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate City, Hokkaido, were also used as a source of diploid control samples for these analyses.

Flow Cytometry

Ploidy status of 120 individuals was determined by flow cytometry analysis using a previously described procedure [12]. Briefly, fin tissues were minced in DNA-extraction Buffer (Cystain DNA 2step, Partec GmbH, Münster, Germany) and then incubated at room temperature for 20 min. Samples were filtered through a 50-μm mesh filter (Cell TRICS Disposable filter units, Partec GmbH), after which five volumes of staining solution (Cystain DNA 2 step, Partec GmbH) containing DAPI (4′,6-diamidino-2-phenylindole

dihydrochloride) were added. DNA contents were then analyzed using a CyFlow® Ploidy Analyzer (Partec GmbH). Ploidy status was determined by comparing the relative DNA content against standard goldfish diploid DNA content (2C).

DNA Preparation and RAPD-PCR Reactions

Total genomic DNA was isolated from fin-clip samples of 100 individuals by the phenol–chloroform method. RAPD amplification was performed as described previously [13]. From a total of 40 commercially available decamer random primers (Kit A and B, Operon Technologies, Alameda, CA, USA), three primers, OPA-07 (Kit A-7: 5′-GAAACGGGTG-3′), OPB-05 (Kit B-5: 5′-TGCGCCCTTC-3′), and OPB-07 (Kit B-7: 5′-GGTGACGCAG-3′), were selected on the basis of a preliminary screening for stable amplification, reproducible fragment patterns, and the presence of polymorphisms between parents. The RAPD-PCR mixture contained 1.0 μl of a DNA template (100 ng/μl), 2.0 μl of 10 × PCR buffer (TaKaRa, Japan), 1.6 μl of a dNTP mixture (TaKaRa), 0.2 μl of rTaq polymerase (TaKaRa), 15.2 μl of DDW, and 1.0 μl of a random primer (20 μM). Amplification conditions were 3 min at 95 °C as an initial step, followed by 35 cycles of 95 °C for 30 s, 36 °C for 1 min for primer annealing, and 72 °C for 1 min for elongation. This was followed by a single final step of primer extension at 72 °C for 7 min. Approximately 9 μl of amplification products were separated by 1.5% agarose gel electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2.2 mM EDTA). Gels were stained with ethidium bromide and photographed on a UV trans-illuminator using a gel documentation system (Print-graph AE-6915H, Atto, Tokyo).

Chromosome preparation and karyotype analysis

Immature diploid goldfish and tetraploid silver crucian carp (total length: 40–65 mm; body weight: 1.0–3.0 g) were intraperitoneally injected with 50–150 μl of 0.001% colchicine 3 h prior to sacrifice. Gill tissues were removed and minced finely with forceps and suspended in goldfish Ringer's solution (150 mM NaCl, 3.0 mM KCl, 3.5 mM $MgCl_2$, 5.0 mM $CaCl_2$, 10 mM HEPES, pH 7.5). After centrifugation at 1,000 ×g for 12 min, the cells were resuspended in 0.075 M KCl hypotonic solution for 40–60 min at room temperature. Then, the cells were fixed with chilled Carnoy's solution (methanol:acetic acid = 3:1) and stored at −20 °C. One droplet of a cell suspension was air-dried on a slide, which had been cleaned in advance with chilled 50% ethanol. After Giemsa staining, karyotypes were analyzed on the basis of metaphase chromosomes, according to Levan et al [14].

Fluorescence in situ hybridization

FISH was performed using the 5.8S + 28S rDNA probe according to the protocol described by Fujiwara *et al*. [15] with minor modifications. Briefly, human 5.8S + 28S rDNA was labeled with biotin-16-dUTP using a nick translation kit (Roche, Germany). Chromosome metaphase spread slides (pretreated with RNase) were denatured in 70% formamide/2 × SSC (pH 7.0) at 70 °C for 2 min, dehydrated in cold 70% and 100% ethanol for 10 min each, and then air dried. Then, 150–200 ng of labeled rDNA probe was denatured at 75 °C for 10 min and quickly placed on ice before hybridization with each chromosome slide. After washing in 50% formamide/2 × SSC, 2 × SSC, 1 × SSC, and 4 × SSC, fluorescent signals were generated using an avidin–FITC (N-fluorescein isothiocyanate, Roche) conjugate. Finally, the slides were mounted in 100 μl of DABCO (1,4-Diazabicyclo-octane) antifade solution containing DAPI for counterstaining. The slides were observed under a Nikon ECLIPSE E800 microscope and images were captured with a Pixera Penguin 150CL-CU CCD camera (Pixera, San Jose, CA, USA).

152

153 **Results**

154 After testing all progenies (*n* =120), a prominent tetraploid-range DNA content peak (Fig. 1b) was detected
155 when compared with diploid goldfish as a control (Fig. 1a). RAPD-PCR was first performed for 100 (nos.
156 1–100) out of 120 tetraploid samples using OPA-07. No paternal contribution was detected by this primer,
157 and the fingerprint pattern of progeny was identical to that of the maternal tetraploid female. Furthermore,
158 no variations were detected among these progeny. Similar isogenic genotypes and all-female inheritance by
159 progeny were also verified in the same sample set (nos. 1–16) by RAPD-PCR using OPB-05 (Fig. 2).

160 Fingerprint pattern using OPB-07 primer exhibited no contribution of fragment specific to the father
161 in all the samples examined (*n*=16). Samples except for the sample (#3) demonstrated DNA fingerprints
162 identical to their mother, but a slight variation was found in electropherogram of one out of 16 progeny
163 examined: one maternally derived fragment was faint or lacking when compared with other progeny (Fig.
164 2). However, this variant individual did not show any polymorphism in fingerprints amplified by other
165 primers. These results indicated that almost all the second generation progeny of the tetraploid female were
166 genetically isogenic and thus likely a gynogenetically generated clone without any contribution of paternal
167 genome, and thus almost all the samples examined were likely genetically identical.

168 Table 1 shows the chromosome distributions determined for diploid goldfish and tetraploid silver
169 crucian carp samples. Tetraploid progeny (*n* = 3) had a modal number of chromosomes of 4n = 206 (Fig
170 3c), whereas control goldfish (*n* = 3) had a modal number of 2n = 100 (Fig 3a). In goldfish, chromosomes
171 were categorized into 20 metacentric (M), 40 submetacentric (SM), and 40 subtelocentric or telocentric
172 (ST/T) chromosomes that were arranged into 10 M, 20 SM, and 20 ST/T pairs in their karyotypes (Fig 3b).
173 In tetraploid progeny, the most informative spreads gave karyotypes that were categorized into 40 M, 80

174 SM, 80 ST/T, and six supernumerary microchromosomes (m). Regular-sized chromosomes were arranged
175 into 10 M, 20 SM, and 20 ST/T quartets in tetraploid karyotypes (Fig 3d).
176 FISH signals using the 5.8S + 28S rDNA probe were detected in four chromosomes in all
177 analyzed metaphases prepared from three goldfish samples (Fig 4a). Tetraploid progeny had rDNA signals
178 in eight chromosomes (Fig 4b). However, the exact morphologies of rDNA bearing chromosomes and the
179 exact locations of rDNA signals were difficult to identify.
180
181 **Discussion**
182 Tetraploid-range DNA contents and isogenic RAPD genotypes shown in these progeny clearly indicated
183 the occurrence of gynogenetic development in tetraploid eggs that were spawned by a tetraploid female
184 after fertilization by a tetraploid male sperm. These results indicated that sperm from the tetraploid male
185 had triggered gynogenesis in unreduced tetraploid eggs spawned by the tetraploid female. In the Chinese
186 silver crucian carp *C. auratus gibelio* both allogynogenetic and gonochoristic reproduction are involved
187 [16]. In this species, gynogenesis is initiated only by fertilization with heterospecific sperm from other
188 species (allogynogenesis), whereas normal bisexual fertilization occurs when eggs are fertilized with
189 homospecific sperm (gonochorism) [16]. As shown in the present study, eggs from a tetraploid female
190 initiated gynogenetic development even after fertilization with homospecific sperm from a tetralploid male.
191 Among 120 individuals examined, no hexaploid individuals arose due to the incorporation of a sperm
192 nucleus from the tetraploid male.
193 However, an exceptional progeny with a slight difference was detected by RAPD-PCR using
194 OPB-07 in 16 samples examined. This result suggests that the tetraploid female presumably produced large
195 number of unreduced clonal tetraploid eggs as well as very small number of exceptional tetraploid eggs

196 with genetic variation. Such variant eggs can be explained by a result of unusual meiotic recombination in
197 during oogenesis of the tetraploid female. Genetic and cytogenetic results showing the involvement of
198 meiotic recombination by the formation of synapsed bivalents and partially paired trivalents were reported
199 in gynogenetic triploid silver crucian carp [17, 18]. A few tetraploid eggs may undergo paring and
200 recombination between two homologous and/or homoeologous chromosomes, because they essentially
201 have four sets of chromosomes with relatively higher similarities or affinities. The other possibility may be
202 attributed to unstable reproducibility of RAPD-PCR itself in which purity of template DNA often affects
203 amplification of fragments [19]. Therefore, further study is required to confirm the presence or absence of
204 genetic variation among tetraploid eggs using more sensitive and reliable genetic markers such as a
205 microsatellite DNA.

206 The karyotypes of the goldfish examined here were very similar to those previously reported for
207 diploid *C. auratus* subsp. (2n = 100) and diploid Miyazaki race silver crucian carp *C. auratus langsdorfii*
208 (2n = 100) [1], which had 20 M + 40 SM + 40 ST/T chromosomes. The karyotype of a tetraploid silver
209 crucian carp *C. auratus langsdorfii* (4n = 206), obtained from the Kanto district was reported to be 44 M +
210 82 SM + 80 T [1], similar to our present results. They had 206 chromosomes, but six supernumerary
211 chromosomes were not distinguished from 200 regular chromosomes. The presence of six
212 microchromosomes found in the present study suggested that gynogenetic tetraploid may have arisen from
213 a triploid with 3n = 156 chromosomes [1] by incorporation of haploid chromosomes.

214 An rDNA locus with 5.8S + 18S + 28S genes and that with 5S genes are located on different
215 chromosomes in most taxa [20]. In triploid silver crucian carp *C. auratus gibelio*, 5S rDNA signals were
216 detected in three ST [21]. In the present study, rDNA signals were detected in four chromosomes of diploid
217 goldfish with 2n = 100, whereas eight signals were detected in chromosomes of tetraploid silver crucian

218 carp with 4n = 206. These FISH results indicated that the progeny of silver crucian carp must have a
219 tetraploid karyotype, including four sets of chromosomes. However, exact morphologies of rDNA bearing
220 chromosomes have not been determined and additional molecular cytogenetic studies will be required in
221 near future.

222 Gynogenesis usually occurs by preventing the decondensation of a sperm nucleus due to a failure
223 in the breakdown of the sperm nuclear envelope [22, 23] in fish species that spawn unreduced diploid or
224 triploid eggs [13, 24-26]. Unreduced diploid eggs are generated by the mechanism of premeiotic
225 endomitosis (chromosome doubling without cytokinesis) in the clonally reproducing diploid loach
226 *Misgurnus anguillicaudatus* [13], whereas clonal triploid eggs are formed by apomixis (i.e., ameiotic
227 division of oocytes by skipping the first meiotic division) in the silver crucian carp *C. auratus gibelio* [24,
228 25] and *C. auratus langsdorfii* [26] because the formation of a tripolar spindle may physically inhibit the
229 first meiotic division.

230 The occurrence of a tripolar spindle is presumably related to the presence of three sets of
231 chromosomes in gynogenetic triploid silver crucian carp. If so, then what would occur in unreduced
232 tetraploid eggs that includes four sets of chromosomes? This is an enigma that needs to be resolved. In
233 tetraploid eggs with an even number of chromosome sets, normal synapsis and subsequent meiosis can be
234 predicted, although tetraploid silver crucian carp produce unreduced tetraploid eggs that develop by
235 gynogenesis.

236 In tetraploid silver crucian carp, cytological mechanisms responsible for the formation of
237 unreduced tetraploid eggs and the initiation of gynogenesis remain to be determined. Our tetraploid
238 population is a reliable set of experimental animals for examining these problems. To identify these
239 mechanisms, the presence or absence of multipolar and other unusual spindles must be investigated in the

oocytes of tetraploid silver crucian carp, which can be induced to undergo final maturation by administering 17α-20β-dihydroxy-4-pregnene-3-one during *in vitro* culture [13, 26]. The configuration of meiotic chromosomes is also informative for determining the reproductive mode [13].

Furthermore, the second generation progeny of tetraploid silver crucian carp are reliable for generating sex-reversed tetraploid males, which are a genetic resource of diploid spermatozoa for further ploidy manipulations. A dimorphism of reproductive mode in the tetraploid silver crucian carp, showing gynogenetic unreduced oogenesis and reduced spermatogenesis, may provide an excellent model for reproductive biology in fishes.

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Figure captions:

Fig. 1 Flow cytometry results for relative DNA contents in fin cells from control diploid goldfish *Carassius auratus auratus* 2C (a) and a tetraploid silver crucian carp *Carassius auratus langsdorfii* progeny 4C (b)

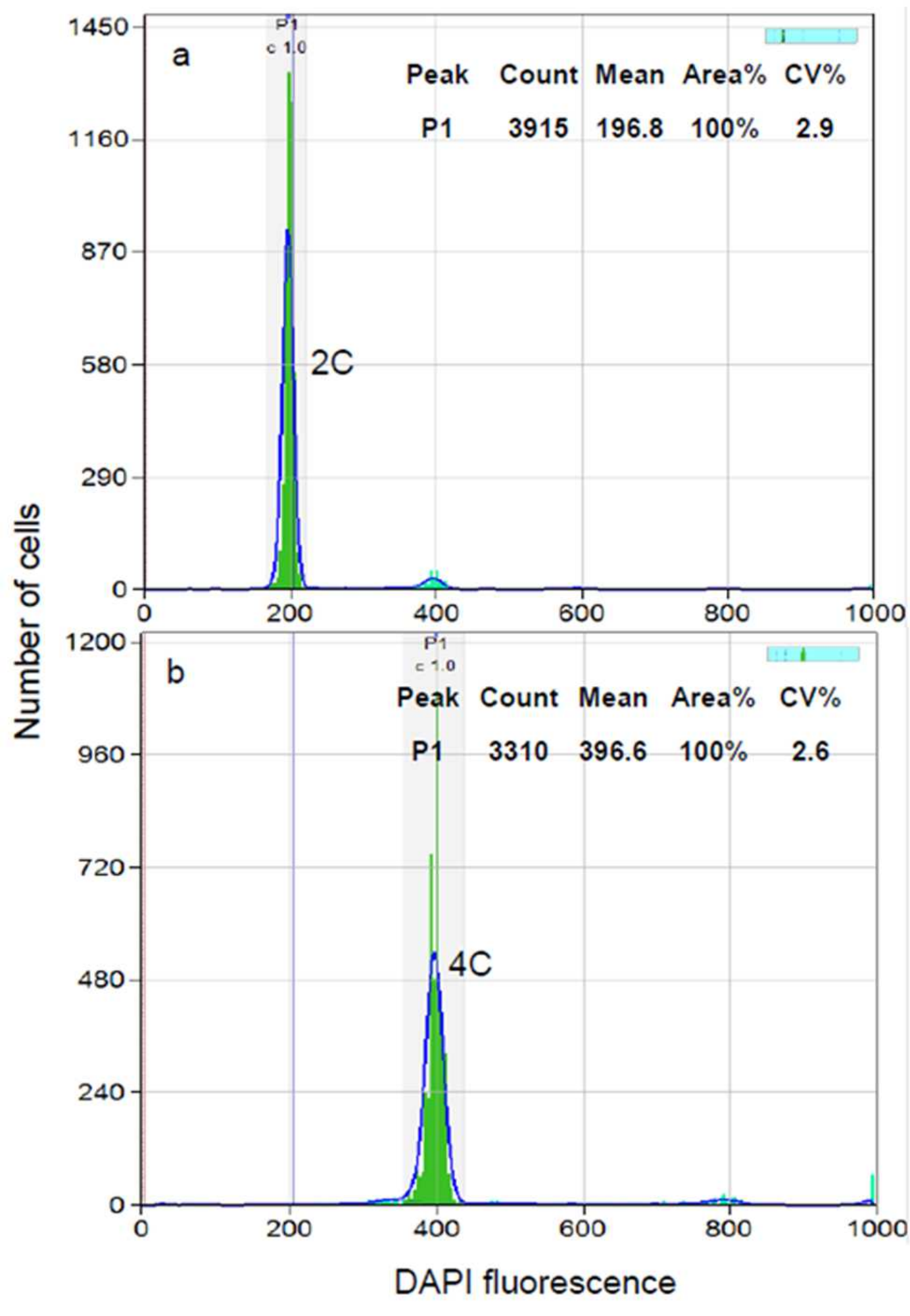
Fig. 2 Random amplified polymorphic DNA (RAPD)-PCR profiles using OPA-07 (top), OPB-05 (middle), and OPB-07 (bottom) primers for silver crucian carp *Carassius auratus langsdorfii*, tetraploid parents (♀ and ♂), and their progeny (nos. 1–10). M indicates molecular size markers

Fig. 3 Mitotic metaphase spread (a) and corresponding karyotype of goldfish *Carassius auratus auratus* with 100 chromosomes (b). Mitotic metaphase spread (c) and corresponding karyotype of silver crucian carp *Carassius auratus langsdorfii* (d) with 206 chromosomes. Arrows indicate microchromosomes. Scale bars = 10 μm

Fig. 4 Mitotic metaphase chromosomes identified after fluorescence *in situ* hybridization (FISH) probing with 5.8S + 28S rDNA sequences. Arrows indicate four fluorescent signals (green) in goldfish *Carassius auratus auratus* (a) and eight fluorescent signals (green) in tetraploid silver crucian carp *Carassius auratus langsdorfii* (b). All metaphase chromosomes were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Scale bars = 10 μm

Table 1 Chromosome distributions in somatic cells of diploid goldfish *Carassius auratus auratus* and tetraploid silver crucian carp *Carassius auratus langsdorfii*.

Fish	Sample no.	No. of cells with chromosomes							
		97	98	99	100	204	205	206	Total
Goldfish	1	0	0	0	12	-	-	-	12
	2	0	0	0	13	-	-	-	13
	3	1	0	0	11	-	-	-	12
Silver crucian carp	1	-	-	-	-	0	1	9	10
	2	-	-	-	-	1	0	10	11
	3	-	-	-	-	1	0	11	12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c 1.0
Peak Count Mean Area% CV%
P1 3310 396.6 100% 2.6
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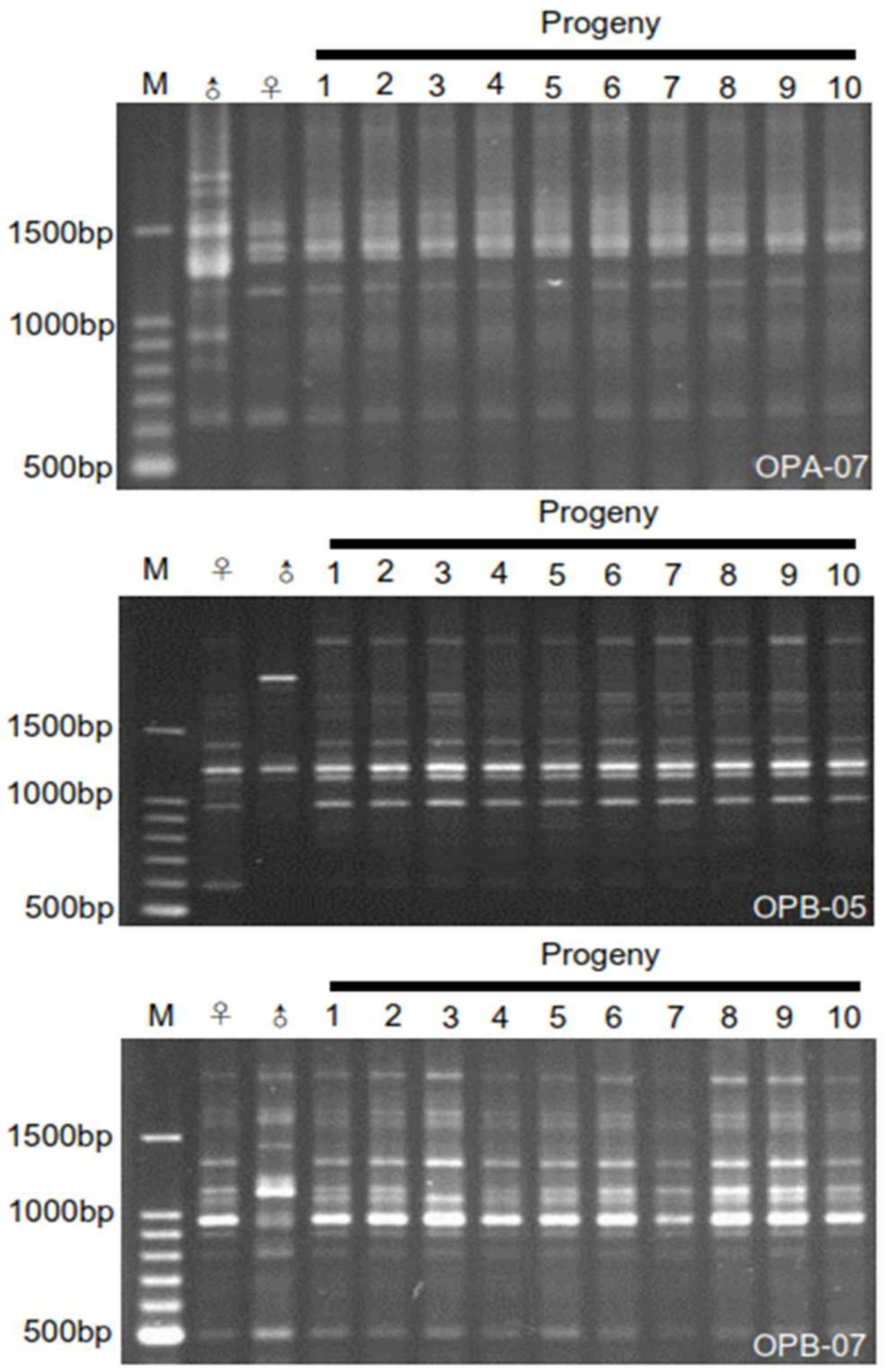
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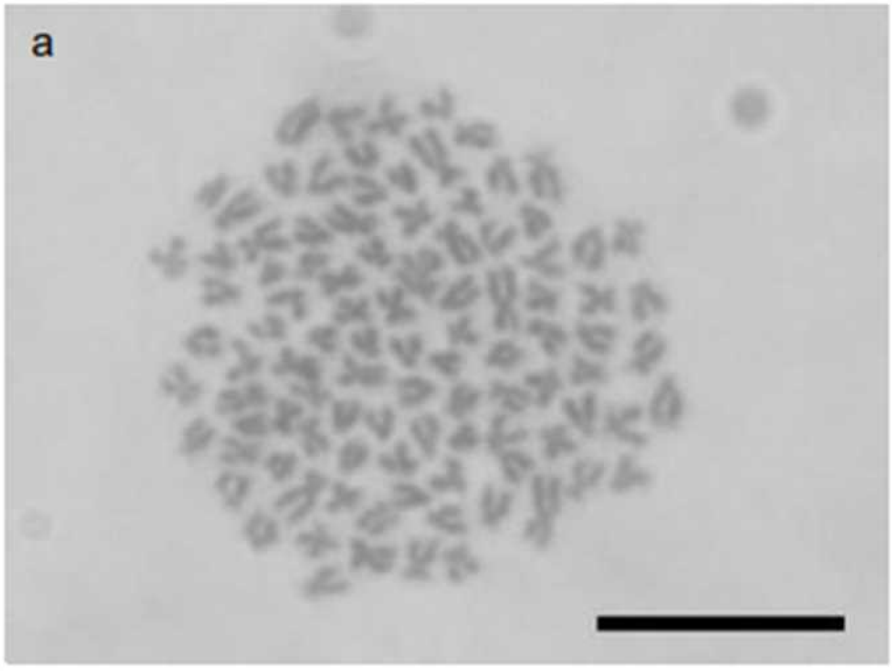
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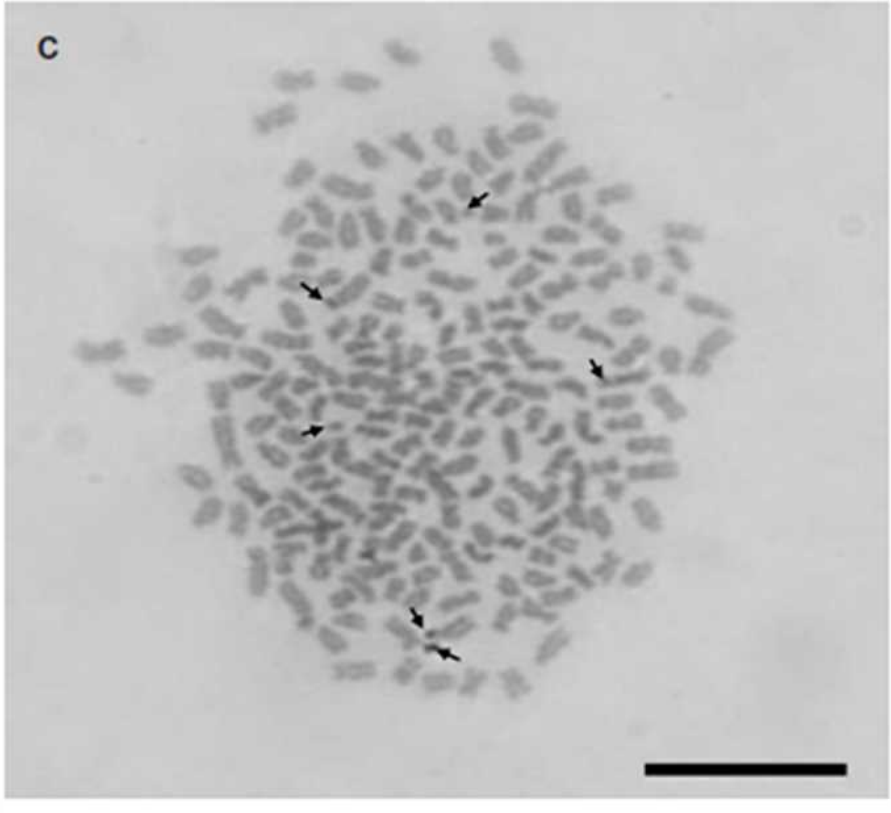
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(b)
M
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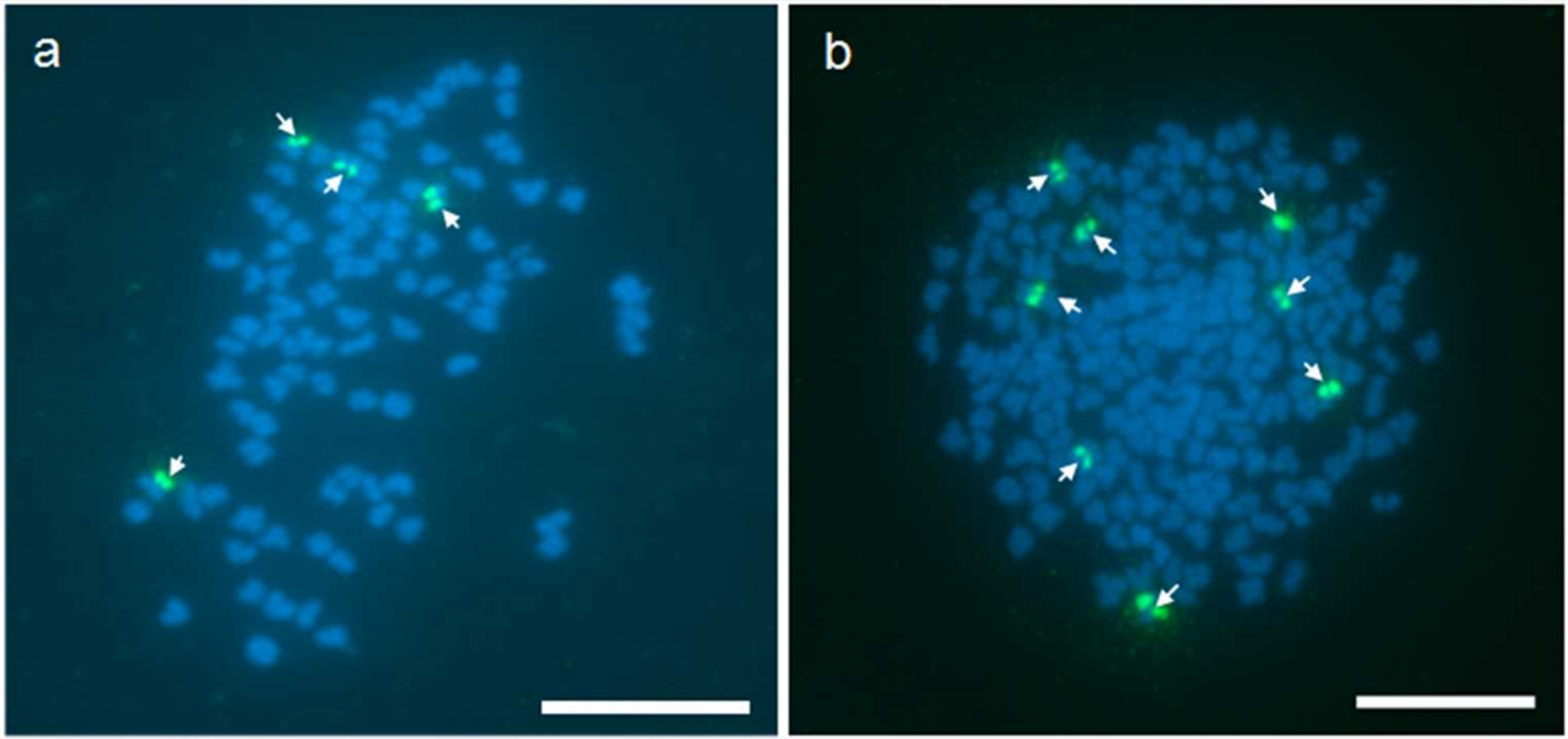
m


a
b