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2 The origin of natural tetraploid loach *Misgurnus anguillicaudatus* (Teleostei: Cobitidae)
3 inferred from meiotic chromosome configurations

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12 Running title: Meiosis in tetraploid loach

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25 **Abstract**

26 In the loach, or Oriental weatherfish *Misgurnus anguillicaudatus* (Teleostei: Cobitidae),
27 diploid ($2n=50$) and tetraploid individuals ($4n=100$) are often sympatric in central China.
28 The evolutionary mechanism of this tetraploidization was analyzed with the observation
29 of meiotic behavior of chromosomes in both the germinal vesicles of mature oocytes
30 and the primary spermatocytes in diploid and tetraploid loaches. Whereas diploid
31 specimens usually showed 25 bivalents in meiotic cells, tetraploid loaches exhibited 0 to
32 6 quadrivalents and 38 to 50 bivalents in both sexes, with the modal number of
33 quadrivalents as three in females and four in males. In the diploid specimens, the two
34 largest metacentric chromosomes bearing nucleolar organizing regions (NORs)
35 identified by chromomycin A₃ (CMA₃) staining and fluorescence *in situ* hybridization
36 (FISH) with a 5.8S+28S rDNA probe formed one bivalent with terminal association. In
37 the tetraploids, four NOR-bearing chromosomes never formed a quadrivalent, but were
38 organized into two terminally-associated bivalents. These findings suggest an
39 autotetraploid origin of the natural tetraploid loach and subsequent rediploidization of
40 whole genome. The latter process, however, seems still in progress as inferred from the
41 concurrence of up-to several quadrivalents and the majority of bivalents.

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43

44 **Key words:** autotetraploid, bivalent, quadrivalent, rediploidization, teleost

45

46 **Introduction**

47 In Japanese populations of the loach or Oriental weatherfish *Misgurnus*
48 *anguillicaudatus* (Teleostei: Cobitidae), most individuals are diploid with $2n=50$
49 chromosomes, showing bisexual reproduction, but asexual diploid clones and natural
50 triploids ($3n=75$) also occur in certain localities (Zhang and Arai, 1999; Morishima et al.,
51 2002; Arai, 2003). Although the tetraploid individuals with 100 chromosomes have been
52 reported among specimens collected from fish markets (Ojima and Takai, 1979; Arai et
53 al., 1991), no wild tetraploid loach has been found yet in Japan despite intensive
54 screening with flow cytometry (Zhang and Arai, 1999; Morishima et al., 2002; Arai,
55 2003). In contrast, both diploid and tetraploid individuals are found in wild populations
56 along the Chang Jiang River system in central China (Li et al., 1983; 2008). Thus,
57 Chinese *M. anguillicaudatus* contain a diploid-tetraploid complex, which can be used as
58 a good model to elucidate the mechanisms of historical ploidy elevation as well as the
59 mechanisms of stable contemporary inheritance and reproduction.

60 There are two evolutionary hypotheses for tetraploidization: autotetraploidy,
61 caused by the doubling of the entire genome, and allotetraploidy or amphidiploidy
62 involving duplication of each non-homologous genome in a hybrid of different species.
63 In the autotetraploid amphibian species *Odontophrynus americanus*, many
64 quadrivalents were formed in testicular meiotic cells, owing to the high affinities among
65 four homologous chromosomes (Becak et al., 1966; Schmid et al., 1985). Such
66 quadrivalent configurations found in tetraploid animals suggest that these polyploid
67 animals are of autopolyploid origin. Dominance of quadrivalents has also been reported
68 in the *Datura* plant (Belling and Blakeslee 1924). On the other hand, typical
69 allotetraploid or amphidiploid plant species were reported to exhibit meiotic

70 configurations with bivalents, in that two sister chromosomes (duplicated from each
71 parent chromosome) probably behave as homologous chromosomes to form bivalent
72 pairs (Clausen and Goodspeed, 1925; Jenkins and Jimenez, 1995).

73 Meiotic configurations are useful to analyze the genome composition of polyploid
74 organisms, but generally considered difficult to observe in fish because of the large
75 number of small-sized chromosomes involved. Recently, we have successfully observed
76 meiotic chromosomes in the germinal vesicles (GVs) of mature oocytes of the
77 gynogenetic, clonal diploid loach (Itono et al., 2006) and in the meiotic hybridogenetic
78 clone-derived triploid loach using *in vitro* maturation techniques (Morishima et al.,
79 2008). In testicular tissues, on the other hand, meiotic chromosomes have been observed
80 in various cytotypes such as aneuploidies including extra supernumerary and micro
81 chromosomes (Zhang and Arai, 2003) and spontaneous polyploidies (Yoshikawa et al.,
82 2009).

83 In the present study, we observed meiotic configurations of chromosomes in both
84 the oocyte GVs and the spermatocytes of the diploid and tetraploid Chinese loaches,
85 with particular reference to the pairing behavior of homologous chromosomes
86 (univalents, bivalents or other unusual multivalents), to analyze the evolutionary
87 mechanism of the observed tetraploidization. The chromosomes bearing nucleolar
88 organizing regions (NORs) were used as a marker to detail the pairing profile of
89 homologous chromosomes, after differential fluorochrome staining and fluorescence *in*
90 *situ* hybridization (FISH) using a 5.8S+28S rDNA probe (Li et al., 2010).

91

92 **Materials and methods**

93

94 Diploid and tetraploid specimens of the loach *M. anguillicaudatus* were collected from
95 the Ching Jiang River and adjacent waters in Hubei Province, China, and samples were
96 transported to the laboratory of Dalian Ocean University, Dalian, Liaoning Province, for
97 analyses. Samples were sorted based on the ploidy status determined by chromosome
98 counts, DNA content estimated using flow cytometry, and erythrocytic measurements,
99 according to the methods described previously (Li et al., 2008).

100 Cytogenetic analyses of oocyte GVs were made on seven diploid females and six
101 tetraploid females following the procedures of Itono et al. (2006). Before incubation, all
102 the females were injected with 20 to 25IU human chorionic gonadotropin (Asuka
103 Pharmaceuticals, Tokyo) per gram of body weight and kept for 4 to 5 h in a 25°C
104 aquarium. Immediately after severing the medulla of each fish, mature oocytes were
105 then removed from the ovaries and incubated in goldfish saline (Kagawa et al., 1984)
106 containing 17 α -20 β dihydroxy-4-pregnene-3-one (Sigma) at room temperature. At
107 appropriate intervals, oocytes were fixed with 4% acetic acid to determine the initiation
108 of germinal vesicle migration (GVM). During GVM but before germinal vesicle break
109 down (GVBD), oocytes were fixed with chilled Carnoy's fixative (methylalcohol:acetic
110 acid=3:1) and the GVs were isolated by collecting as much yolk debris as possible with
111 fine forceps under a stereoscopic binocular microscope. The isolated GV was placed on
112 a clean slide glass and air-dried, and then the slide was stained with DAPI
113 (4',6-diamidino-2-phenylindole, Sigma) for 1 h prior to examination under a
114 fluorescence microscope.

115 Four diploid and four tetraploid males were injected with 6 μ g
116 phytohemagglutinin-A (Shanghai Ihua Medical, Co. Ltd.) per gram of body weight
117 and kept for 18 to 20 h in a 25°C aquarium, and then the same dose was again

118 administered and the fish were kept a further 4 to 6 h in the aquarium. Then, each fish
119 was injected with the appropriate volume of a 0.1% saline solution of colchicines (6 µg
120 per gram of body weight; Wako Pure Chemical Industries, Ltd.) and kept for a further 2
121 to 4 h in the aquarium. Immediately after severing the medulla of each fish, the testes
122 were removed and treated with a hypotonic solution, 0.8% trisodium citrate for 20 min.
123 Then, the testes were fixed with chilled Carnoy's fixative and kept in a - 20°C freezer. A
124 cell suspension was made from the testes of each male, and then one droplet was
125 pipetted onto a glass slide that had been cleaned with chilled 95% alcohol, and then air
126 dried. The slides were stained with Giemsa (Merck) diluted with phosphate buffer, pH
127 6.8..

128 The fluorochrome CMA₃ (chromomysin A₃, Wako) / DA (distamycin A, Sigma)
129 staining was performed following the protocol by Schweizer (1976, 1980). DA/DAPI
130 staining was performed according to Schweizer et al (1978). FISH with human
131 5.8S+28S rDNA as a probe (Fujiwara et al., 1998), after labeling with biotin-16-dUTP
132 by nick translation (Roche), was conducted following Li et al. (2010). After washing,
133 the slides were treated with avidin-FITC conjugate and counterstained with DAPI.
134 Hybridization signals were photographed under a Nikon Eclipse E800 fluorescence
135 microscope as previously (Li et al., 2010).

136

137 **Results**

138

139 **Meiotic chromosome configurations**

140

141 In diploid females, homologous chromosomes were paired during the first meiotic

142 division (MI), and 25 bivalents were observed in more than 90% of meiotic metaphases
143 (Fig. 1A, 2A), confirming the diploid chromosome number of 50 (Li et al., 2010). In a
144 total 32 oocytes of tetraploid loach, various pairing configurations were observed, with
145 the most frequent metaphases showing three ring-like quadrivalents (IV) and 44
146 bivalents (II) (Fig. 1B, 2B-D). Thus, all oocytes of tetraploid females had a total number
147 of 100 chromosomes, except for one oocyte with 102 chromosomes and 3IV+45II (Fig.
148 1B).

149 In the MI of diploid males, 41 out of 55 spermatocytes examined exhibited a
150 meiotic configuration in which 50 chromosomes were paired to form 25 bivalents (Fig.
151 1C, 3A). However, a few aneuploid cells with 23, 24 or 26 bivalents as well as
152 polyploid cells with 50 or 75 bivalents were also detected (Fig. 1C).

153 In the MI of tetraploid males (Fig. 1D), 38 MI metaphases observed were in
154 tetraploid and hypo-tetraploid ranges, 23 metaphases showed chromosome numbers in
155 hyper-tetraploid, hyper-hexaploid (6n), 8n, 12n, 16n and 24n ranges. Among the 33
156 tetraploid metaphases, metaphase with four quadrivalents (IV) and 42 bivalents (II) was
157 the most frequent (Fig.1D, 3E). The other meiotic configurations included 50II (Fig. 3B),
158 2IV+46II (Fig. 3C), 3IV+44II (Fig. 3D), 5IV+40II (Fig. 3F) and 6IV+38II (not shown).

159 Unusual metaphases with hypo- or hyper-tetraploid ranges showed various
160 configurations (Fig. 1D). In five eu-octoploid metaphases, the configurations 7IV+86II,
161 8IV+84II, and 9IV+82II were observed. Meiotic configurations showing hyper-8n, 12n,
162 16n and 24n-range spermatocytes were also observed.

163

164 **Differential fluorochrome staining and FISH profile**

165

166 In the diploid specimens, both terminals of one bivalent were always positive for CMA₃
167 staining (Fig. 4A), but no positive sites for DA/DAPI staining were observed (not
168 shown). In the tetraploid specimens, CMA₃-positive sites were never detected in the
169 quadrivalents, but occurred in two independent bivalents (Fig. 4C), in which both
170 terminals of each bivalent were CMA₃-positive as in the diploid specimens. This
171 suggests a tail-to-tail association of two homologous chromosomes because the
172 CMA₃-positive site is located at the telomeric region of the short arms of the largest
173 metacentric chromosomes (Li et al., 2010).

174 In each diploid metaphase, FISH signals of rDNA loci or NORs occurred at the
175 terminal CMA₃-positive sites in the bivalent, confirming the terminal associations of
176 two homologues (Fig. 4B). In tetraploid metaphases, FISH signals were observed in two
177 different bivalents (Fig. 4D), but no FISH signals were detected in any quadrivalents.

178

179 **Discussion**

180 In both ovarian and testicular cells of the present tetraploid loach, the occurrence
181 of several quadrivalents suggests an autotetraploid origin of tetraploidy. Chromosomes
182 of a putative ancestral diploid loach presumably doubled in the past and the resultant
183 four homologous chromosomes, with high affinities in each quartet, would have paired
184 to generate 25 quadrivalents during meiosis. However, meiotic cells always include
185 many bivalents in tetraploid loach. Such a large number of bivalents may have resulted
186 from subsequent pairwise differentiation among the chromosomes within each quartet
187 after autotetraploidization. In the present study, FISH signals and CMA₃-positive sites
188 were never detected in the quadrivalents, but two independent bivalents with NORs
189 gave two FISH signals and CMA₃-positive sites at both ends of each bivalent. These

190 results show that four NOR bearing chromosomes form two bivalents instead of a
191 quadrivalent in tetraploid loach. Presence of two bivalents within each quartet is
192 considered to be the evidence of rediploidization. Through the rediploidization,
193 contemporary tetraploid loach may be becoming stable in terms of genetics and
194 cytogenetics, because of the decrease of quadrivalents but the increase of bivalents. As a
195 corollary, quadrivalents may reduce reproductive capacity and survival due to an
196 unstable karyotype or irregular segregation of homologous chromosomes. In fact,
197 irregular meiosis often occur in tetraploid plants, causing the production of large
198 amounts of non-viable pollen (Davis 1933). Recovery of reproductive capacity by
199 rediploidization from autotetraploidy is a common phenomenon in plant breeding (Giles
200 and Randorph, 1951). Therefore, the concurrence of up to several quadrivalents and
201 many bivalents in the present tetraploid loach suggests that they are of autotetraploid
202 origin, whose rediploidization, however, is still in progress.

203 In the present study, both sexes exhibited up to several quadrivalents, but the
204 modal number was three in the female and four in the male. In the germinal vesicle of
205 oocytes from tetraploid loach, most metaphases showed 100 chromosomes and
206 numerical variation was small. Although, 33 out of 61 spermatocytes gave meiotic
207 configurations of 100 chromosomes, unusual aneuploidies (hypo- and hyper-tetraploids)
208 and higher polyploidies (with 6n, 8n, 12n, 16n and 24n ranges) were detected in other
209 spermatocytes. These unusual polyploid cells are considered to have resulted from
210 spontaneous endomitosis (or endoreduplication), i.e. duplication of chromosomes
211 without cytokinesis. Such premeiotic endomitosis has been observed in the unreduced
212 egg formation of triploid (Zhang et al., 1998) and clonal diploid (Itono et al., 2006), and
213 in unreduced sperm formation of sex-reversed clonal diploid (Yoshikawa et al., 2009) in

214 the loach. However, it remains to be examined the mechanism of the preferential
215 occurrence and the consequence of such unpredictable polyploid germ cells in the testes
216 of the present loach specimens. Further studies also are necessary to elucidate the
217 underlying mechanisms and the evolutionary consequence of genome duplication in the
218 loach.

219

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224

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311 **Figure legends**

312 Figure 1. Number of quadrivalents (IV) and bivalents (II), chromosome numbers and
313 ploidy status in 22 oocyte germinal vesicles (GVs) from 7 diploid females (A), 32
314 oocyte GVs from 6 tetraploid females (B), 55 spermatocytes from 4 diploid males (C)
315 and 61 spermatocytes from 4 tetraploid males (D) in the loach, *Misgurnus*
316 *anguillicaudatus*.

317

318 Figure 2. DAPI-stained meiotic configurations in oocyte germinal vesicles
319 comprising: 25 bivalents (II) from a normal diploid (A), 50II from a tetraploid (B), 3
320 quadrivalents (IV) and 44II from a tetraploid (C), 4IV and 42II from a tetraploid (D).
321 Arrows indicate ring-like quadrivalent configurations. Scales denote 20 μm .

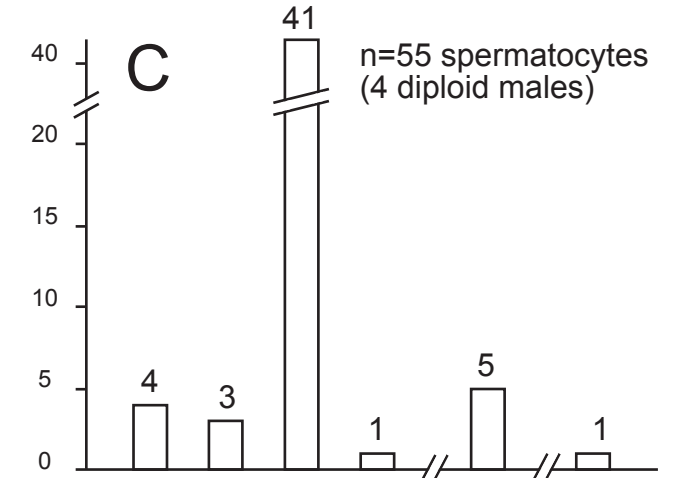
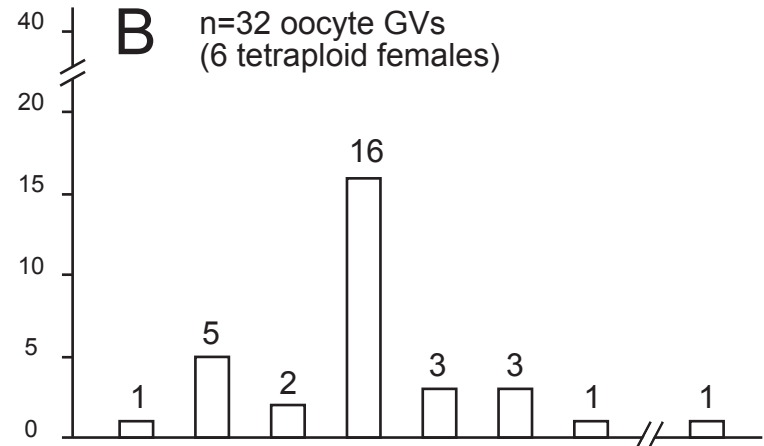
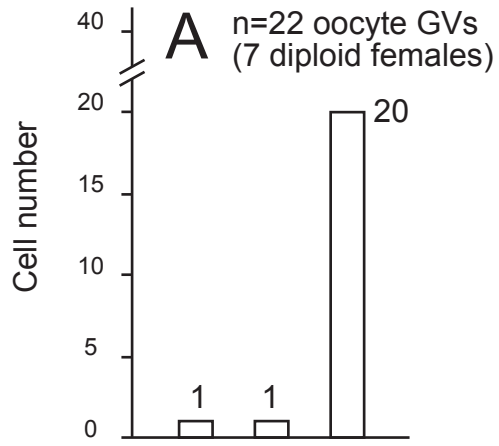
322

323 Figure 3. Meiotic metaphase with 25 bivalents (II) in a spermatocyte of a diploid male
324 (A), 50II in a spermatocyte of a tetraploid male (B), 2IV (quadrivalents) and 46II in a
325 spermatocyte of a tetraploid male (C), 3IV + 44II in a spermatocyte of a tetraploid male
326 (D), 4IV + 42II in a spermatocyte of a tetraploid male (E), 5IV + 40II in a spermatocyte
327 of a tetraploid male (F). Arrows indicate ring-like quadrivalent configurations. Scales
328 denote 10 μm .

329

330 Figure 4. CMA₃-staining meiotic chromosomes from diploid (A) and tetraploid cells (C)
331 and FISH, with human 5.8S+28S rDNA sequences as probe, in meiotic chromosomes
332 from diploid (B) and tetraploid cells (D). Arrows indicate CMA₃-positive sites.
333 Arrowheads indicate FISH signals. Note two CMA₃-positive sites and FISH signals in
334 one bivalent from a diploid cell and in each of two bivalents from a tetraploid cell.

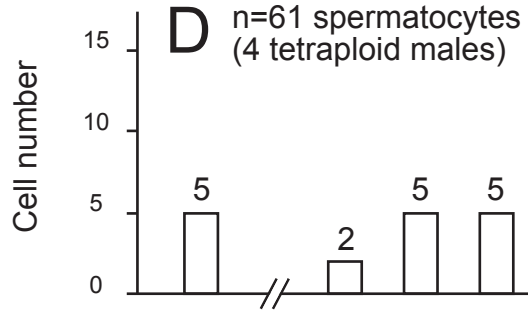
335 Scales denote 10 μm .



No. of IV	0	0	0
No. of II	23	24	25
Chrom. no.	50		
Ploidy	2n		

	0	1	2	3	4	5	6	3
	50	48	46	44	42	40	38	45
	100							102
	4n							hyper 4n

	0	0	0	0	0	0
	23	24	25	26	50	75
	50			52	100	150
	2n			hyper 2n	4n	6n



No. of IV	0-4	0	2	3	4	5	6	3-9	5-9	7	8	9	6,8	10,12	10,16	18
No. of II	40-48	50	46	44	42	40	38	45-69	78-84	86	84	82	92,101	128,130	264	158-172
Chrom. no.	94-96	100						108-160	186-192	200			216-226	300-304	376-400	600
Ploidy	hypo. 4n	4n						hyper 4n-6n	hypo. 8n	8n			hyper 8n	ca. 12n	ca. 16n	24n

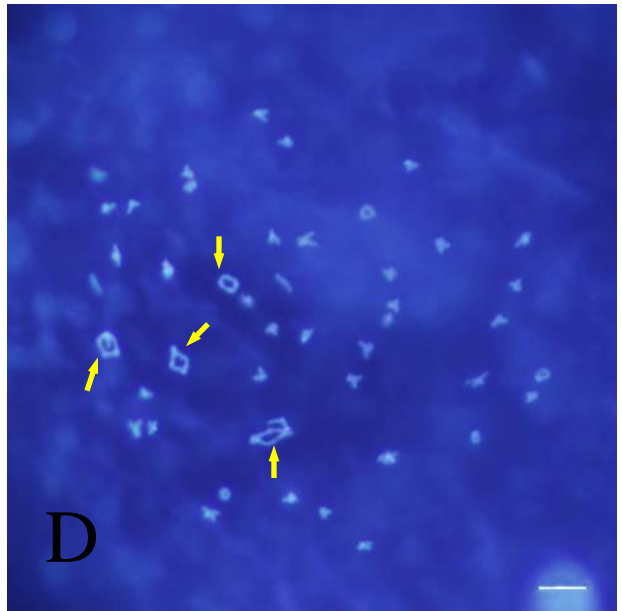
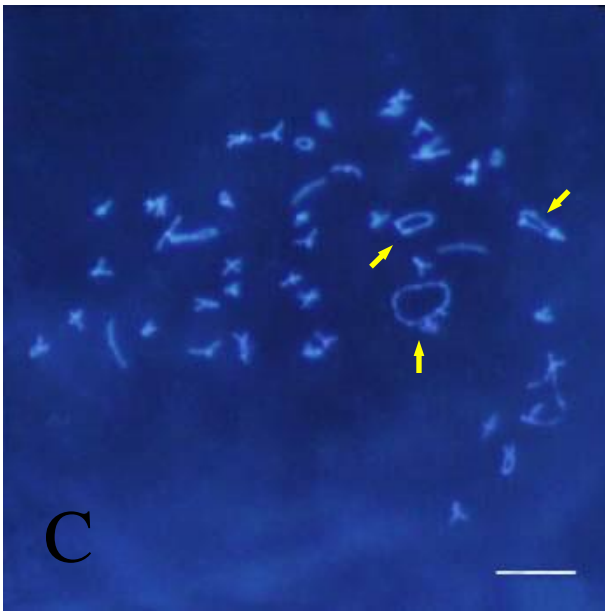
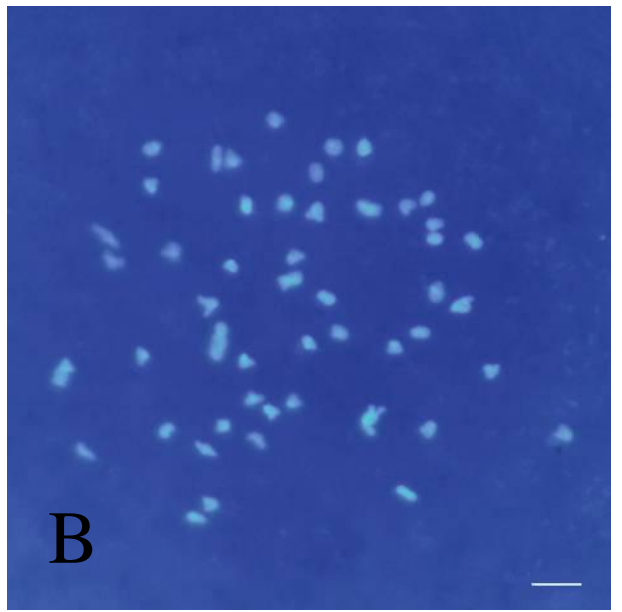
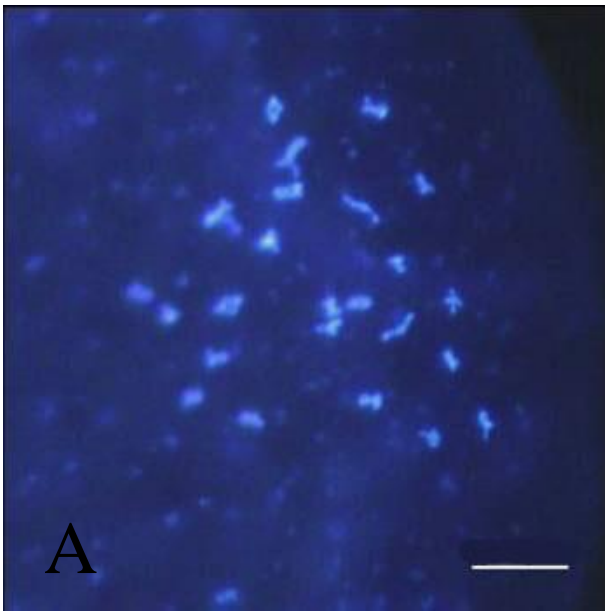


Fig .2

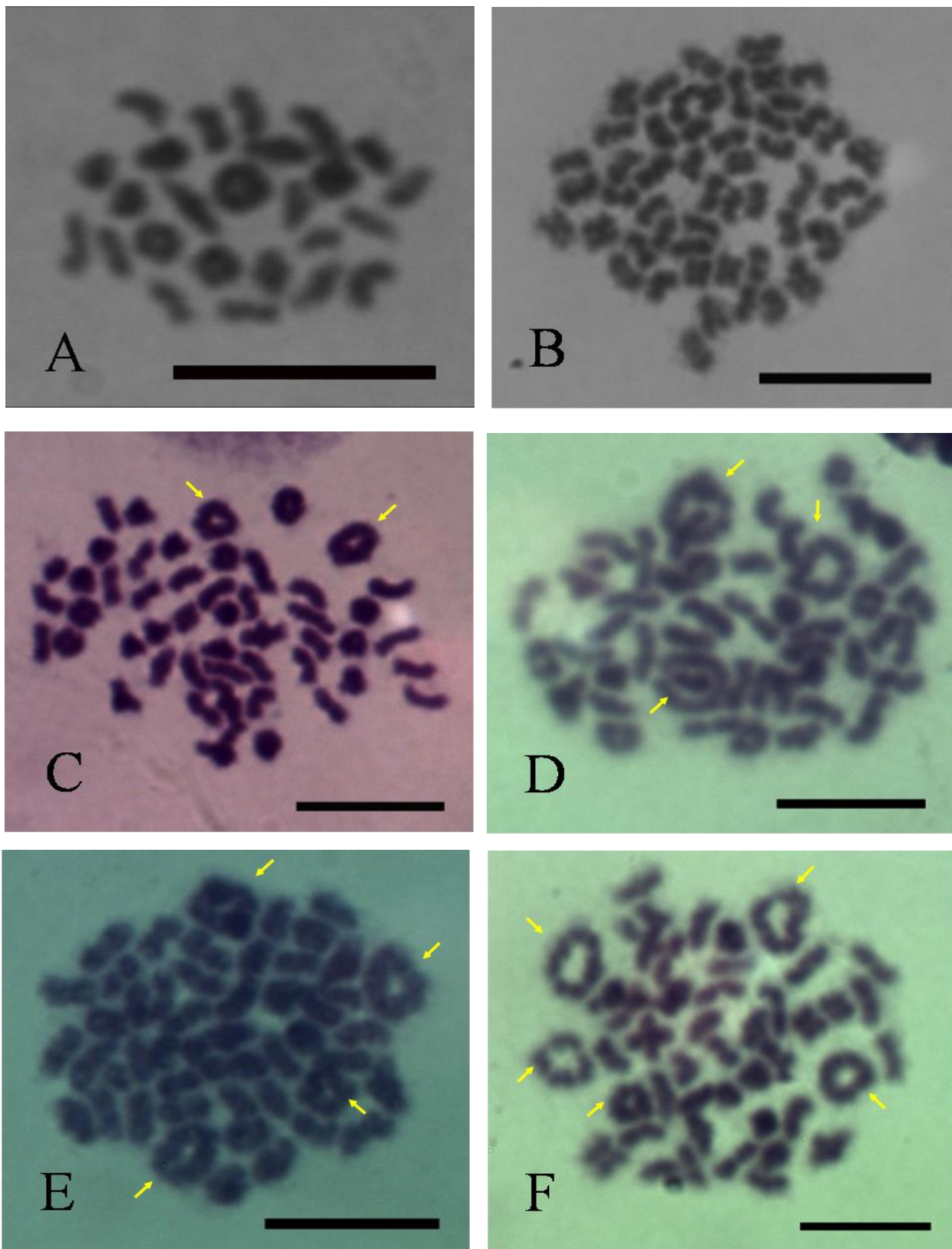


Fig.3

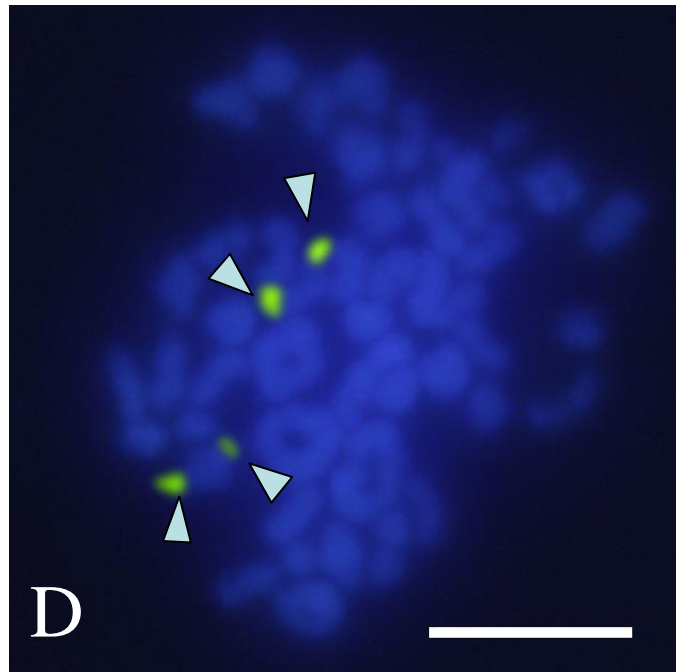
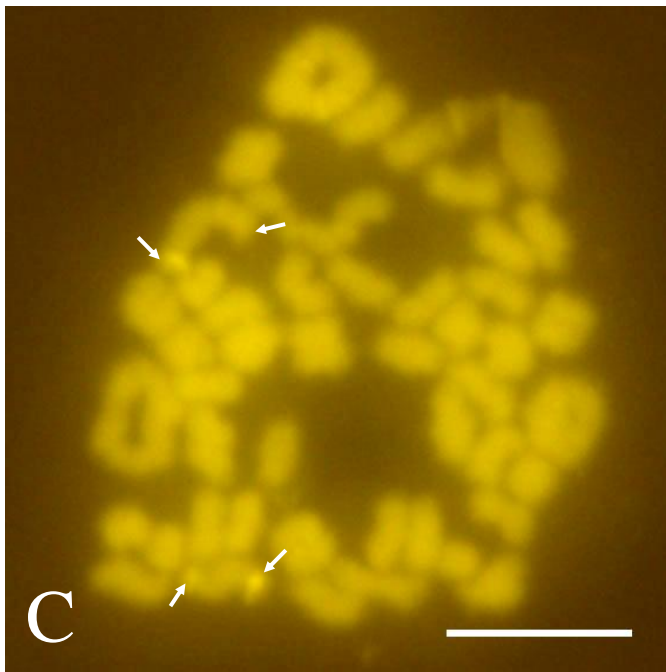
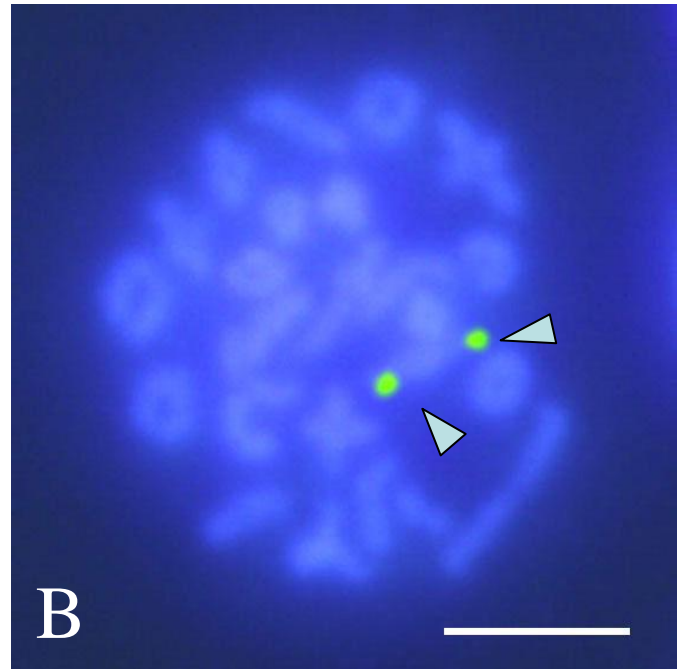
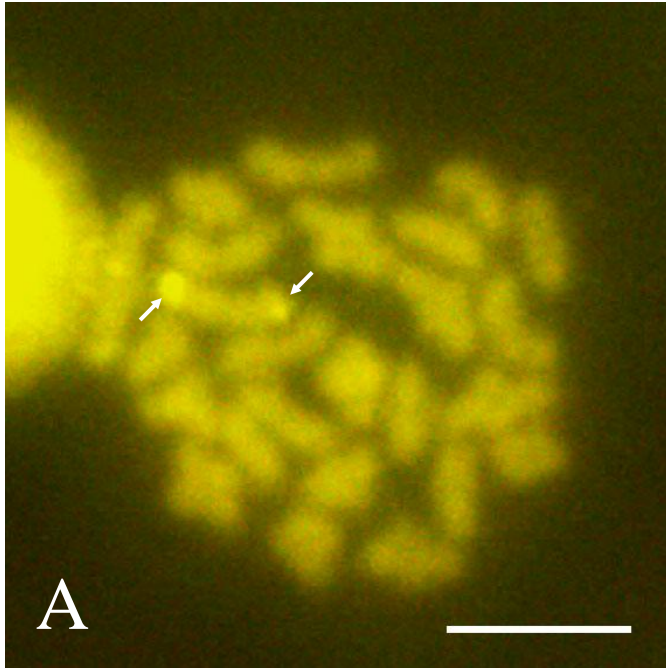


Fig .4