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

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Running title: Meiosis in tetraploid loach

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**Abstract**

In the loach, or Oriental weatherfish *Misgurnus anguillicaudatus* (Teleostei: Cobitidae), diploid (2n=50) and tetraploid individuals (4n=100) are often sympatric in central China. The evolutionary mechanism of this tetraploidization was analyzed with the observation of meiotic behavior of chromosomes in both the germinal vesicles of mature oocytes and the primary spermatocytes in diploid and tetraploid loaches. Whereas diploid specimens usually showed 25 bivalents in meiotic cells, tetraploid loaches exhibited 0 to 6 quadrivalents and 38 to 50 bivalents in both sexes, with the modal number of quadrivalents as three in females and four in males. In the diploid specimens, the two largest metacentric chromosomes bearing nucleolar organizing regions (NORs) identified by chromomycin $A_3$ ($CMA_3$) staining and fluorescence *in situ* hybridization (FISH) with a 5.8S+28S rDNA probe formed one bivalent with terminal association. In the tetraploids, four NOR-bearing chromosomes never formed a quadrivalent, but were organized into two terminally-associated bivalents. These findings suggest an autotetraploid origin of the natural tetraploid loach and subsequent rediploidization of whole genome. The latter process, however, seems still in progress as inferred from the concurrence of up-to several quadrivalents and the majority of bivalents.

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

## Introduction

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

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

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

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

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

## Materials and methods

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

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

Four diploid and four tetraploid males were injected with 6 μg phytohemagglutinin-A (Shanghai Ihua Medical, Co. Ltd.) per gram of body weight 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 $CMA_3$ (chromomysin $A_3$, 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

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

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

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

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

**Differential fluorochrome staining and FISH profile**

In the diploid specimens, both terminals of one bivalent were always positive for $CMA_3$ staining (Fig. 4A), but no positive sites for DA/DAPI staining were observed (not shown). In the tetraploid specimens, $CMA_3$-positive sites were never detected in the quadrivalents, but occurred in two independent bivalents (Fig. 4C), in which both terminals of each bivalent were $CMA_3$-positive as in the diploid specimens. This suggests a tail-to-tail association of two homologous chromosomes because the $CMA_3$-positive site is located at the telomeric region of the short arms of the largest metacentric chromosomes (Li et al., 2010).

In each diploid metaphase, FISH signals of rDNA loci or NORs occurred at the terminal $CMA_3$-positive sites in the bivalent, confirming the terminal associations of two homologues (Fig. 4B). In tetraploid metaphases, FISH signals were observed in two different bivalents (Fig. 4D), but no FISH signals were detected in any quadrivalents.

**Discussion**

In both ovarian and testicular cells of the present tetraploid loach, the occurrence of several quadrivalents suggests an autotetraploid origin of tetraploidy. Chromosomes of a putative ancestral diploid loach presumably doubled in the past and the resultant four homologous chromosomes, with high affinities in each quartet, would have paired to generate 25 quadrivalents during meiosis. However, meiotic cells always include many bivalents in tetraploid loach. Such a large number of bivalents may have resulted from subsequent pairwise differentiation among the chromosomes within each quartet after autotetraploidization. In the present study, FISH signals and $CMA_3$-positive sites were never detected in the quadrivalents, but two independent bivalents with NORs gave two FISH signals and $CMA_3$-positive sites at both ends of each bivalent. These

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

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

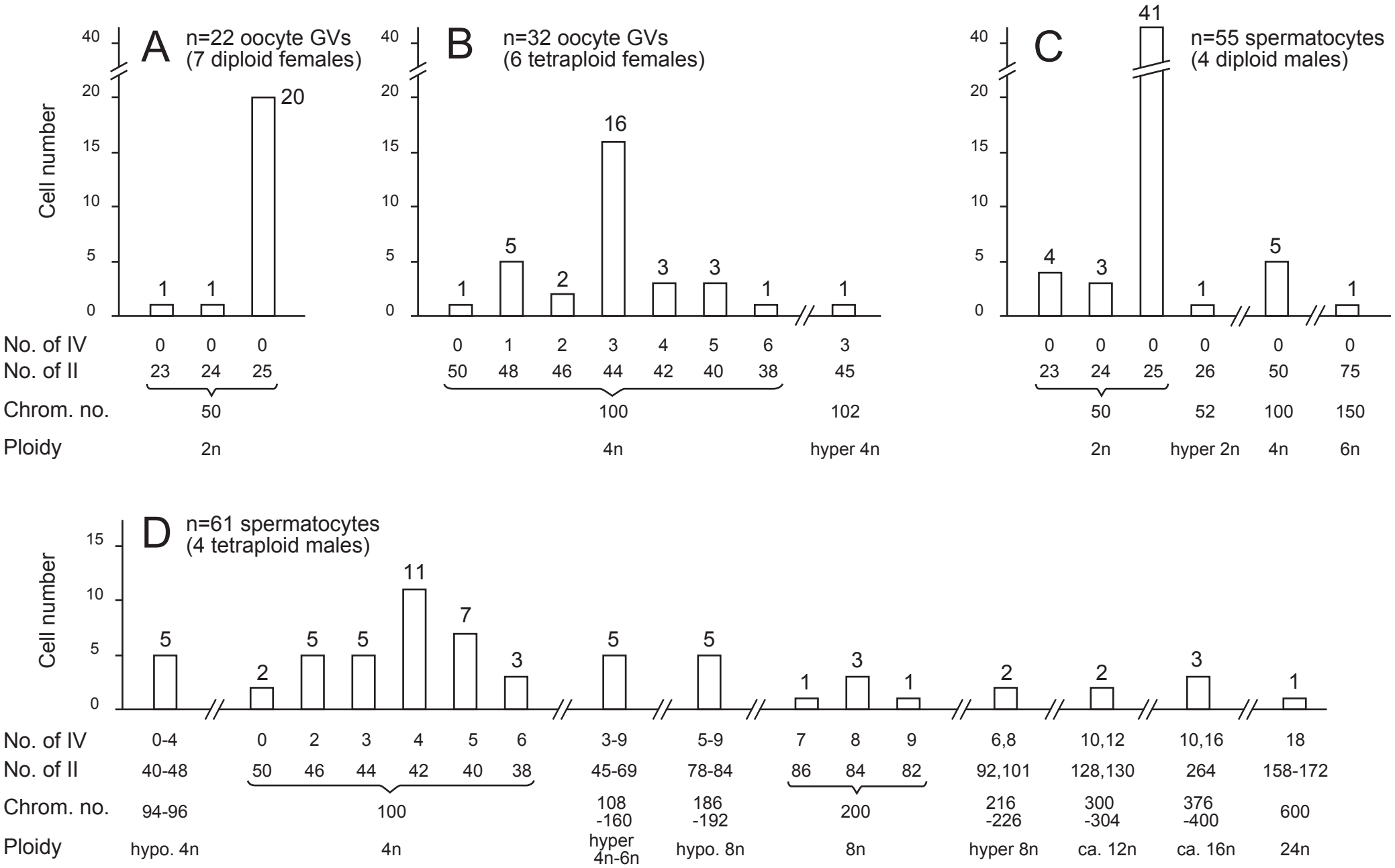
Figure 1. Number of quadrivalents (IV) and bivalents (II), chromosome numbers and ploidy status in 22 oocyte germinal vesicles (GVs) from 7 diploid females (A), 32 oocyte GVs from 6 tetraploid females (B), 55 spermatocytes from 4 diploid males (C) and 61 spermatocytes from 4 tetraploid males (D) in the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

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

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

Figure 4. $CMA_3$-staining meiotic chromosomes from diploid (A) and tetraploid cells (C) and FISH, with human 5.8S+28S rDNA sequences as probe, in meiotic chromosomes from diploid (B) and tetraploid cells (D). Arrows indicate $CMA_3$-positive sites. Arrowheads indicate FISH signals. Note two $CMA_3$-positive sites and FISH signals in one bivalent from a diploid cell and in each of two bivalents from a tetraploid cell.

335 Scales denote 10 μm.


A
n=22 oocyte GVs
(7 diploid females)
B
n=32 oocyte GVs
(6 tetraploid females)
C
n=55 spermatocytes
(4 diploid males)
D
n=61 spermatocytes
(4 tetraploid males)
Cell number
No. of IV
No. of II
Chrom. no.
Ploidy
2n
4n
hyper 4n
hyper 2n
6n
hypo. 4n
hyper 4n-6n
hypo. 8n
8n
hyper 8n
ca. 12n
ca. 16n
24n

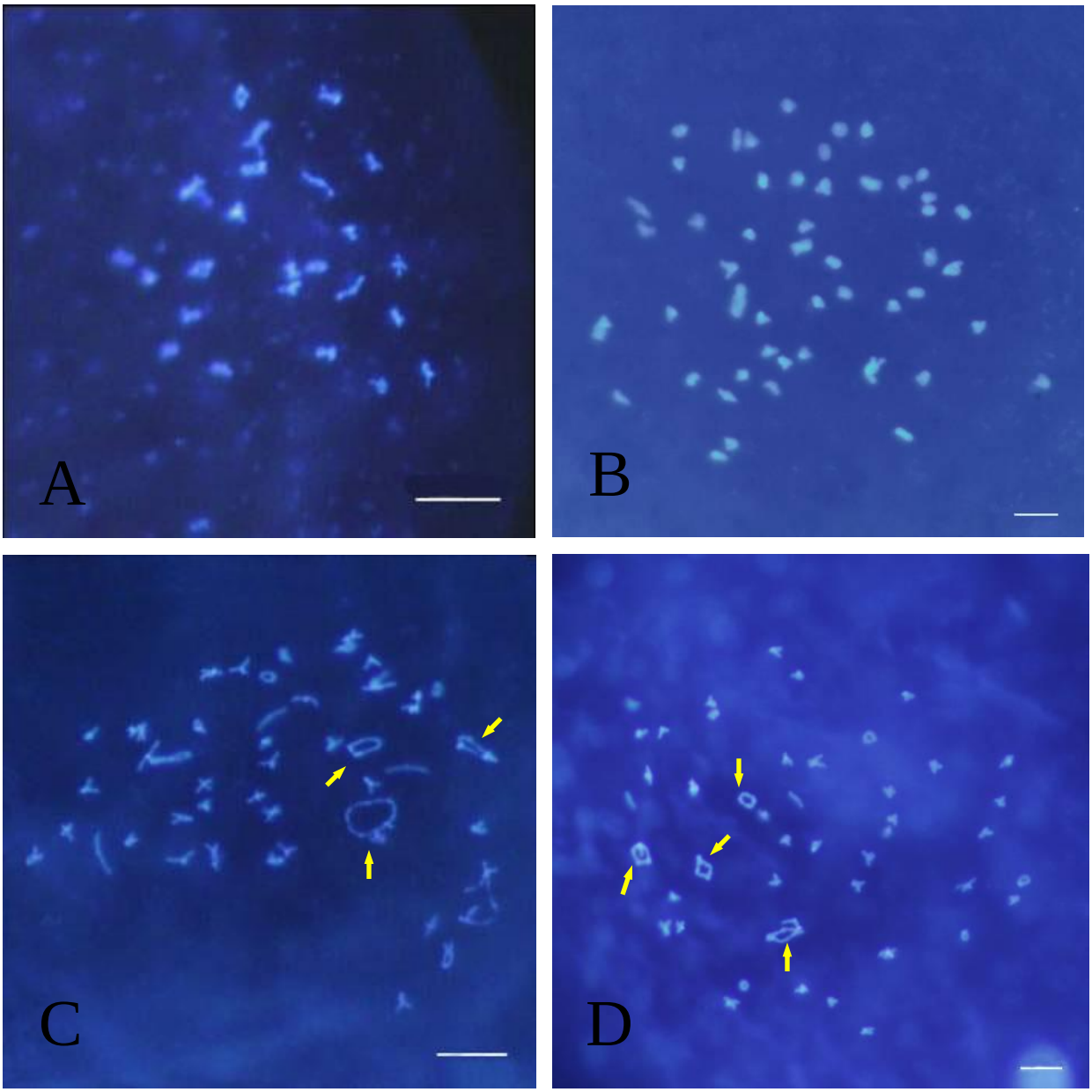



**Fig .2**

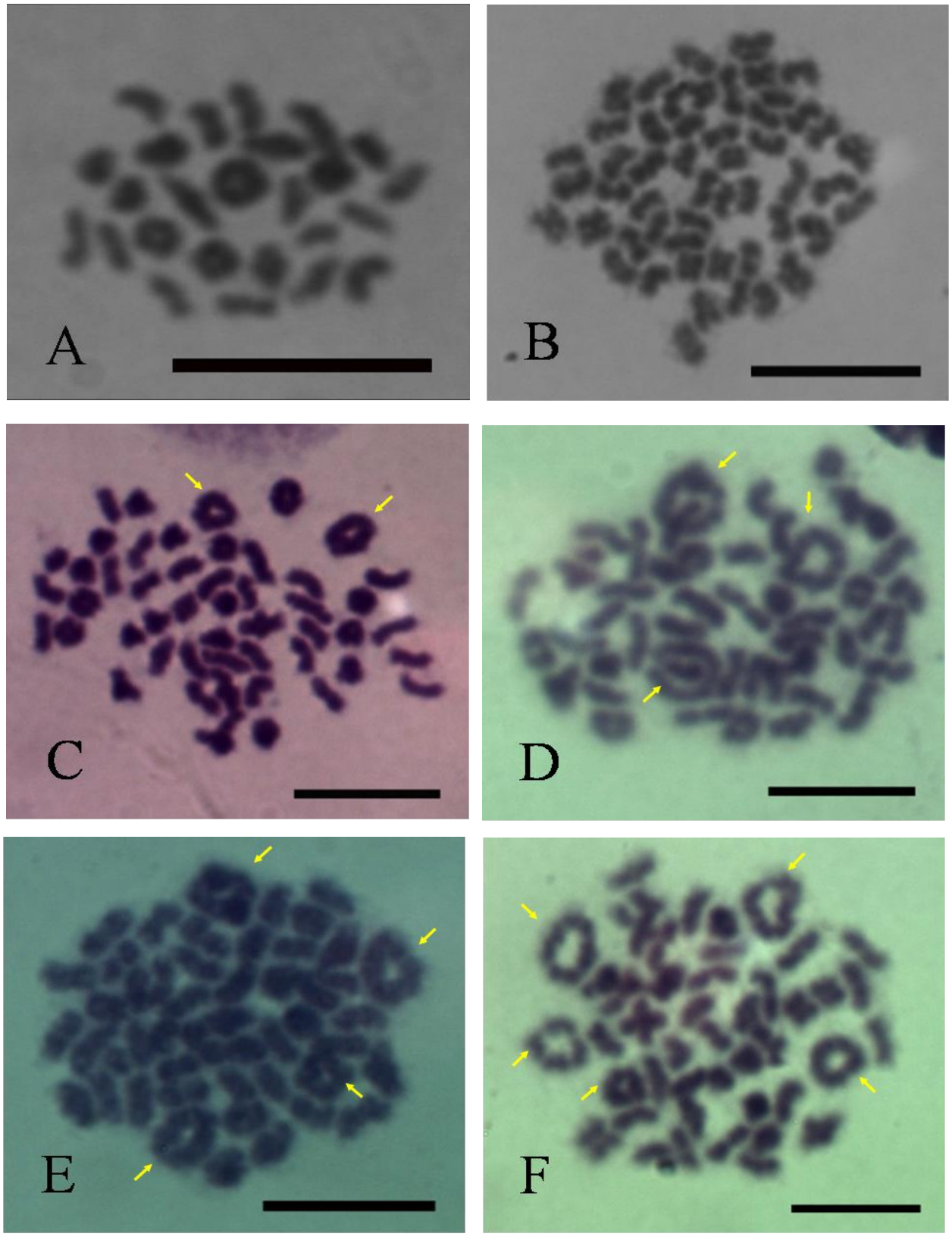



Fig.3

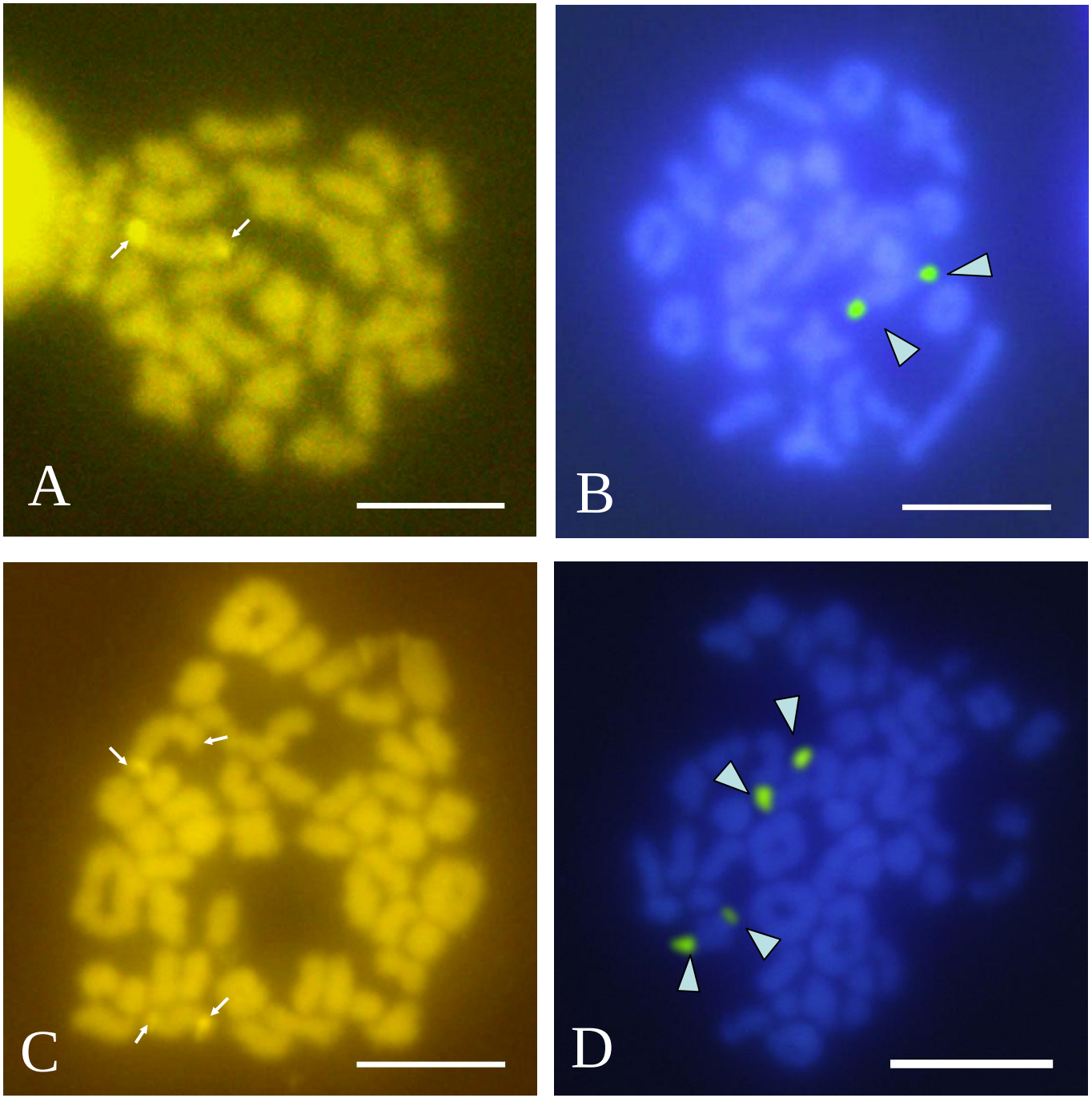



Fig .4