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First record of the natural occurrence of pentaploid loach, *Misgurnus anguillicaudatus* in Hubei Province, China

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Abstract. Natural occurrence of pentaploid loach, *Misgurnus anguillicaudatus* discovered from Liangzi Lake area, Hubei Province, central China, is reported here for the first time. The evidences from karyotyping, DNA content analysis and nuclear volume measurements were described to confirm the pentaploid nature of the identified individual. This individual was phenotypically indistinguishable from its sympatric diploid and tetraploid counterparts. The chromosome number was 5n = 125, the mean erythrocyte nuclear DNA content was 2.62 and 1.25 times of their diploid and tetraploid counterparts, respectively. An origin of such polyploidy form either from genome addition of normal sperm nuclei to unreduced egg and the mating of tetraploid individual and hexaploid individual, respectively, is hypothesized.

Key words: chromosomes, ploidy identification, unusual cytotype, weather loach

Introduction

The cobitid loach, *Misgurnus anguillicaudatus*, a small freshwater species is widely distributed in Japan, Korea, Taiwan and eastern coasts of Asian continent from the River Amur to North Vietnam (Saitoh 1989). This fish is commercially important in China as both traditional Chinese medicine and food (Gao et al. 2007).

The most interesting aspect of *M. anguillicaudatus* is the occurrence of polyploid and unisexual biotypes in nature besides bisexually reproducing diploid individuals (Arai 2003). This fish appears to tolerate genomic changes from diploidy to polyploidy even aneuploidy in nature or by means of artificial chromosome manipulation. Ojima & Takai (1979) first identified the natural occurrence of triploid and tetraploid individuals among fishes obtained from the fish market as well as from nature using chromosome counting and DNA content determination. Since then, a number of issues pertaining to the polyploidy and cytogenetics of various *M. anguillicaudatus* populations have been investigated in Japan, Korea and China. The polyploidy in Japanese *M. anguillicaudatus* populations was comprehensively reviewed by Arai (2003), who reported that besides the most common bisexual diploid individuals (2n = 50), a relatively high frequency of triploids and asexually reproducing clonal diploid *M. anguillicaudatus* could also be found in some localities. In China, the diploid *M. anguillicaudatus* with 50 chromosomes is also the most bisexually reproducing cytotype in natural populations. However, a large number of tetraploid individuals have also been recorded along the Yangtze River basin (Yu et al. 1989, Wang et al. 1993, Ma 1996, Lou 1997, Chang et al. 2000, Yin et al. 2005, Li et al. 2008). Furthermore, a few triploid individuals has been discovered in several places out of 29 localities examined for ploidy status by measurement of erythrocyte nucleus and DNA content determination using flow cytometry (Li et al. 2008). A recent study has even detected fewer hexaploid specimens near the Yangtze River basin (Abbas et al. 2009). Owing to such natural ploidy diversity, this loach is a suitable animal model not

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only for studying genome differentiation due to ploidy alterations but also for genetic and breeding studies in aquaculture (Arai 2003). However, the screening of ploidy levels among populations of the Chinese *M. anguillicaudatus* is restricted, partly due to lack of detailed cytogenetic information on this loach (Abbas et al. 2009).

During the past several years, a series of studies of our laboratory investigated to the biogeographic distribution of various polyploid forms of *M. anguillicaudatus* in China. Besides the diploid, triploid, tetraploid and hexaploid cytotypes of *M. anguillicaudatus* reported previously (Abbas et al. 2009), we unexpectedly discovered the natural occurrence of pentaploid specimen from Liangzi Lake population, Hubei province, central China in August, 2011. The aim of this report was to identify this phenomenon, using karyotyping, DNA content analysis, and nuclear volume measurement.

Material and Methods

The *M. anguillicaudatus* individuals were collected from 62 locations in China during the years 2005-2011. As many as 30 individuals were randomly selected from each population for confirmation of their ploidy level by flow cytometric DNA content analysis. While examining the ploidy status of samples from Liangzi Lake area (30°12′55″ N 114°30′7″ E) population near the Yangtze River basin, the occurrence of a pentaploid *M. anguillicaudatus* specimen was recorded. This specimen was subjected to morphometric measurement. Subsequently, cytogenetic examination including karyotyping, DNA content analysis and nuclear volume measurements was carried out after anaesthetizing the fish with MS-222 (200 mg/l). All the methods were conducted according to Zhou et al. (2008). Briefly, the fish were injected intraperitoneally with PHA and colchicine with a final concentration of 8-10μg g^{-1} and 2-4μg g^{-1} body weight, respectively. After four hours of treatment, the peripheral blood was collected by caudal vein puncturing and preserved for the flow cytometric analysis and blood smears preparation. The specimens were sacrificed to collect head kidneys for karyotype analysis. Using flow cytometer (Becton Dickinson FACS Calibur, USA), the DNA content was evaluated. For this, the blood cells were suspended in 1 ml of staining buffer consisting of 0.1 % sodium citrate, 0.1 % triton × 100 and 50μg ml^{-1} propidium iodide and analyzed within five minutes. Erythrocytes of karyologically identified *M. anguillicaudatus* with 2n = 50 gave a relative DNA content of 2n as the diploid standard while 4n = 100 give a relative DNA content of 4n as the tetraploid standard, both of which was used as internal control (Hardie et al. 2002). Absolute DNA contents were measured on the basis of chicken erythrocytic DNA content (2.5 pg $nukleus^{-1}$) (Tiersch & Chandler 1989). For volumetric analysis of erythrocyte nuclei, the major axis (a) and minor axis (b) of the nuclei of 100 randomly selected erythrocytes were measured and recorded with computer image analyses software (Motic Images Advanced 3.2, USA). The volume (V) of the nuclei was computed using the following formula: $V = 4/3 \times \pi\ (a/2) \times (b/2)^2$.

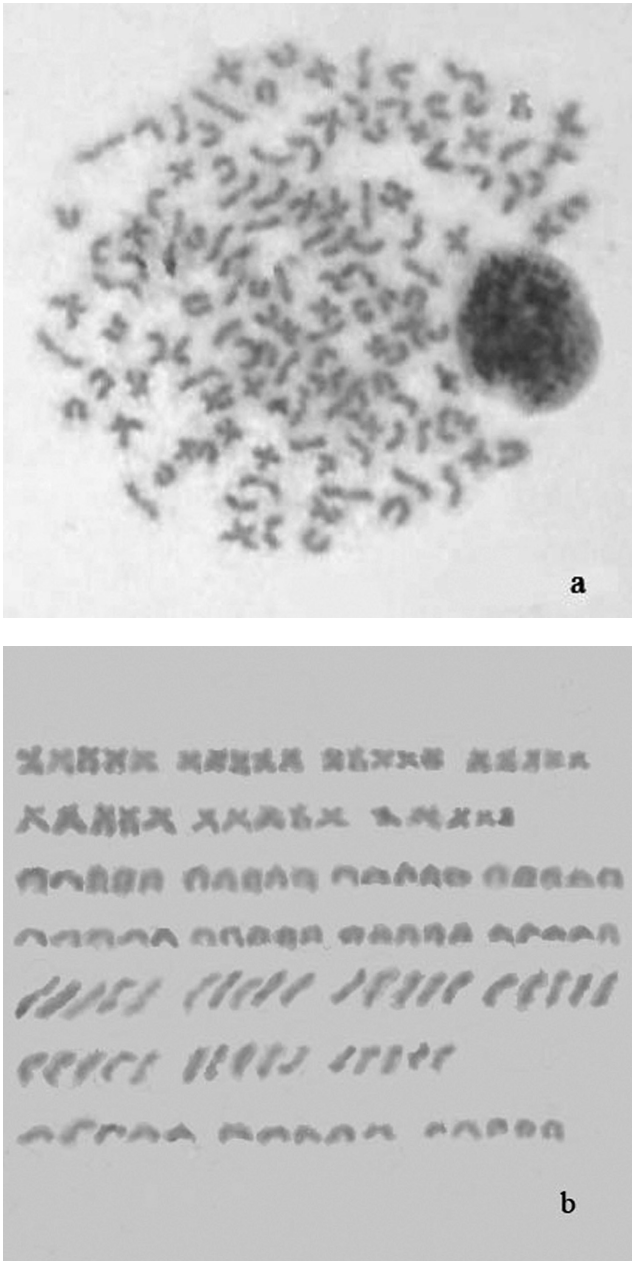

Fig. 1. A representative metaphase (a) and corresponding karyotype of the pentaploid *M. anguillicaudatus* (b).

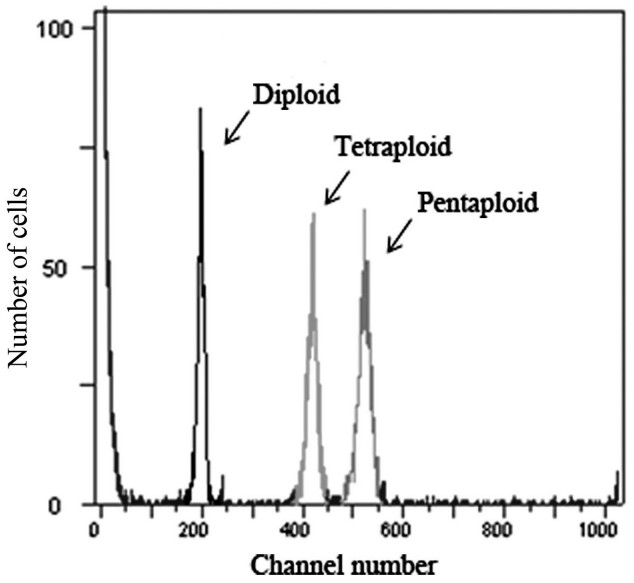

Fig. 2. Flow cytometry profiles of cellular DNA contents of the pentaploid individual with the sympatric diploid and tetraploid *M. anguillicaudatus* as control.

Results

The flow cytometric results revealed that the tetraploid *M. anguillicaudatus* was dominant cytotype in Liangzi Lake. Of the 30 specimens examined, 26 (86.67 %) were exclusively tetraploid, three (10 %) exclusively diploid, and only one (3.33 %) of the sample was identified as pentaploid.

The pentaploid individual had the external appearance, i.e. shape and colour, the same as its sympatric diploid and tetraploid counterparts. A representative metaphase chromosome spread of the identified individual is shown in Fig. 1a and the corresponding karyotype in Fig. 1b. The chromosome number was 125 and complement consisted of 20 metacentric (m), 15 submetacentric (sm) and 90 telocentric (t) chromosomes. No well-defined sex chromosomes were detected. The diploid chromosome complement of sympatric diploid inviduals was 2n = 50 and composed of 8 m, 6 sm and 36 t while symptaric tetraploids had 4n = 100 and complement composed of 16 m, 12 sm and 72 t.

The flow cytometric profiles of erythrocyte nuclear DNA contents of the pentaploid specimen, as well as its sympatric diploids and tetraploids are presented in Fig. 2. The pentaploid specimen had a mean channel number of 521.7, while the diploids, tetraploids and chicken erythrocyte control had a mean channel number of 198.8, 418.5 and 200.3, respectively. Thus, it can be inferred that DNA content of the pentaploid loach is 6.5pg and about 2.62 and 1.25 times higher than its sister diploid and tetraploid counterparts,

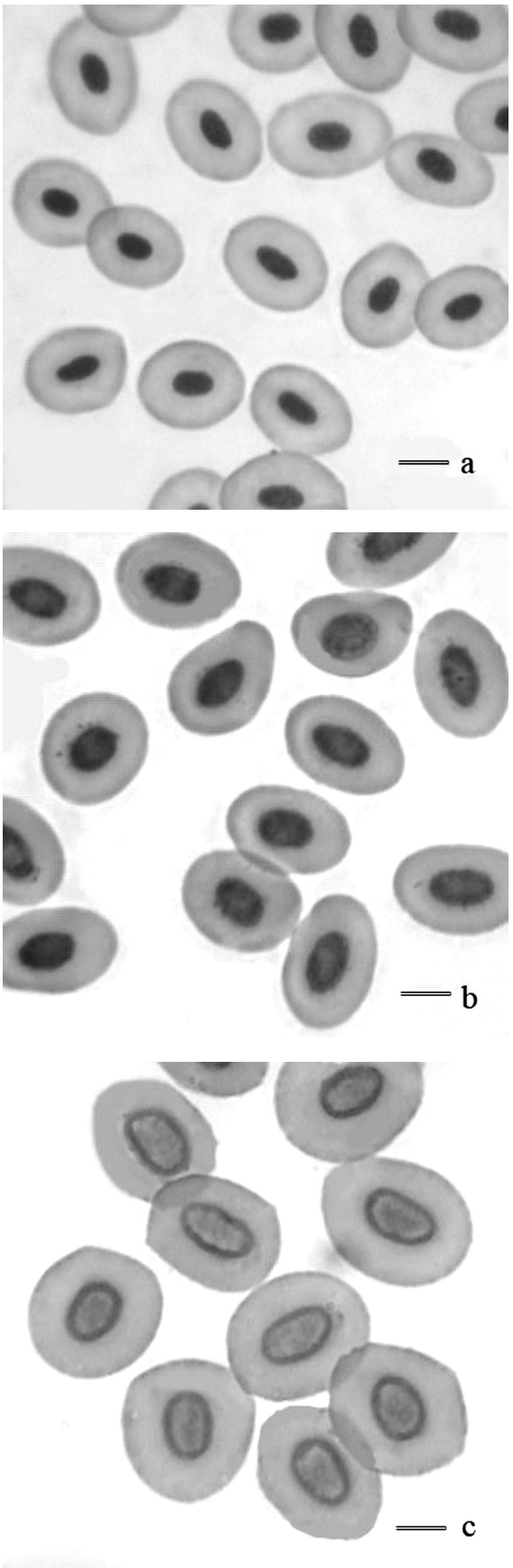

Fig. 3. Erythrocytes of diploid (a), tetraploid (b) and pentaploid (c) individuals of *M. anguillicaudatus*. Magnification of 1000×. Scale bar = 5μm.

Table 1. Comparison of cytogenetic characteristics of the natural pentaploid with the sympatric diploid individual and tetraploid individuals of *M. anguillicaudatus.*

Ploidy type	Number of samples	Chromosome number	RBCs nuclear Volume ± S.D. (μm^3)*1	Flow cytometric results of DNA content	
				Average channel number ± S.D.	DNA content ± S.D. (pg $nukleus^{-1}$)*2
Diploid	3	50	71.19 ± 9.49	198.8	2.48 ± 0.02
Tetraploid	26	100	145.32 ± 15.95	418.5	5.25 ± 0.06
Pentaploid	1	125	179.11 ± 17.84	521.7	6.50

*1 RBCs: red blood cells;
*2The DNA contents were calculated by using chicken erythrocytes DNA content (2.50 pg $nucleus^{-1}$) as standard.

respectively. Similar profiles were obtained from the measurements of erythrocyte nuclear volume (Fig. 3 and Table 1).

Discussion

The natural occurrence of pentaploid individual completed known ploidy cytotypes among natural *M. anguillicaudatus*, populations after previous discoveries of diploid, triploid, tetraploid, and hexaploid *M. anguillicaudatus* individuals (Abbas et al. 2009). To the best of our knowledge, no report is as yet available for natural occurrence of pentaploid in any fish species (Arai 2011), although some publications reported on the induction of viable pentaploidy in fishes (Chourrout & Nakayama 1987, Arai et al. 1991, 1993, Liu et al. 2007).

Artificial pentaploids had been successfully produced by inhibition of the second polar body extrusion of eggs laid by natural tetraploid female after fertilization with haploid sperm of normal diploid (Arai et al. 1991, 1993, Arai 2003). These results could be considered as a plausible explanation for the observed natural occurrence of pentaploid *M. anguillicaudatus* – the spontaneous failure in releasing second polar body in a normal egg produced by a tetraploid female and then fertilized by a haploid sperm of a diploid *M. anguillicaudatus* results in rise of pentaploid individual. It happends so because the spontaneous failure of second polar body extrusion is considered relatively common in wild *M. anguillicaudatus* (Zhang & Arai 1999, Arai 2003) and other species, such as *Crussostrea gigus* (Guo et al. 1992) and artificial diploid hybrids between common carp, *Cyprinus carpio*, and crucian carp, *Carassius auratus* (Liu et al. 2001, Sun et al. 2007). However, we cannot rule out the possibility of other pathways. The pentaploid fish also might have been originated by spontaneous failure in releasing second polar body in a normal diploid egg of diploid female and then fertilized by triploid sperm of hexaploid *M. anguillicaudatus*. The other possibility is fusion of normal gametes of tetraploid and hexaploid *M. anguillicaudatus*, to produce pentaploid level as has been hypothesized in our previous studies (Abbas et al. 2009).

Unfortunately, nothing is known about occurrence of more pentaploid *M. anguillicaudatus* individuals in natural populations of China at present. Moreover, most of the interpretations attributed to the origin of pentaploid fish are hypothetical unless further studies will investigate a number of genetic parameters of progeny originated from experimental crossings of various ploidy biotypes (Arai 2003).

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