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GEOGRAPHICAL DISTRIBUTION OF PLOIDY LEVEL VARIATION OF LOACH *MISGURNUS ANGUILLICAUDATUS* IN CHINA

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A screening of ploidy level in 2146 individuals from 52 populations of the loach *Misgurnus anguillicaudatus* in China was performed. Ploidy level variation and its geographical distribution in this study was combined with previously reported data from another 28 populations. Two major (2n = 50, 4n = 100) and three minor (3n = 75, 5n = 125, 6n = 150) ploidy levels were found. Diploids were the dominant ploidy level showing broad ecological amplitude. Tetraploid populations were dominantly, especially concentrated in the central Yangtze River basin. Diploid and tetraploid individuals were observed to coexist in seven of the 80 examined populations. In four of those seven populations, no triploids were detected, indicating reproductive isolation between the two sympatric ploidy levels. In the Liangzi Lake area, the sympatric occurrence of diploids, triploids, tetraploids, and pentaploids was observed, although the triploids and pentaploids were rare (0.1%). In the Diaochahu area, Yangtze River basin, two male hexaploids were found. No clearly defined geographical ranges or boundaries could be identified between the diploid and polyploid *M. anguillicaudatus*.

Keywords: Cobitid loaches, DNA content, ploidy variation, reproduction isolation

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INTRODUCTION

The cobitid loach *Misgurnus anguillicaudatus* (Cobitidae, Cypriniformes) is a small freshwater teleost that inhabits the muddy bottoms of creeks, ponds, wetlands and paddy fields (Kitagawa *et al*., 2011). This species is widespread from China to Japan and other Southeast Asian countries, such as Indonesia and India (Zeng *et al*., 2012). The loach can be used as traditional Chinese medicine or folk remedies for treatment of hepatitis, osteomyeitis, carbuncles, inflammations and cancers, as well as for patient's recovery from debilities caused by various pathogens and aging (Wang *et al*., 2008a, 2010). In the past two decades, commercial farming for *M. anguillicaudatus* occupies a significant position in freshwater fish aquaculture production in Asia (Wang *et al*., 2008b, 2009).

The *M. anguillicaudatus* is full of interest to science in part because of the extensive ploidy variability in nature. Since the first report of the natural occurrence of triploid and tetraploid individuals in Japan (Ojima and Takai, 1979), a number of issues pertaining to the polyploidy and cytogenetics of *M. anguillicaudatus* populations have been investigated in Japan, Korea, and China. Arai (2003) described the ploidy status of Japanese loach populations and reported that, in addition to the most common bisexual diploid individuals (2n = 50), a relatively high frequency of triploid loaches (3n = 75) could be found in some localities. Experimental crosses, including induced gynogenesis and inter-specific hybridization with goldfish, shows that these triploid individuals resulted from fertilization of unreduced eggs produced by sympatric diploids (Morishima *et al*., 1999; Zhang and Arai 1999; Arai, 2003). In China, populations of both diploid and tetraploid (4n = 100) loaches have been frequently recorded (Yu *et al*., 1989; Wang *et al*., 1993; Ma, 1996; Lou, 1997; Chang *et al*., 2000; Yin *et al*., 2005). A few triploid individuals have been detected in several locations in China though measurement of the erythrocyte nucleus and determination of DNA content using flow cytometry (Li *et al*., 2008). A recent study has detected rare hexaploid individuals (6n = 150) in the Yangtze River basin (Abbas *et al*., 2009). Due to such natural variability in ploidy levels, *M. anguillicaudatus* can be promising model for genetic as well as breeding studies related to aquaculture (Arai, 2003). However, the data about ploidy level variation and its geographical distribution in *M. anguillicaudatus* is limited, especially due to the lack of detailed cytogenetic screening of its populations across China.

The present study examined ploidy level variation and its geographic distribution of several polyploid forms of *M. anguillicaudatus* in China to address the following questions: (1) What is the ploidy level structure of *M. anguillicaudatus* populations in China? (2) How common are mixed-ploidy populations, and which ploidy levels are present in the studied populations? (3) What minor ploidy level can be found and under what conditions? (4) What is the small-scale distribution pattern in mixed-ploidy populations?

MATERIALS AND METHODS

Altogether, 2146 individuals of *M. anguillicaudatus* were collected from 52 locations in China during years 2005-2012. The sampling locations, time of collection, sample size, and the population data included from previous studies of are given in Table 1. As various ploidy levels have previously been reported in the Yangtze River basin (Yu *et al*., 1989; Yin *et al*., 2005; Li *et al*., 2008; Abbas *et al*., 2009), intensive sampling was conducted in this area. Collected individuals were transported to Huazhong Agricultural University for ploidy level determination.

Before handling, fish were anaesthetized using 200 mg l^{-1} MS-222. The ploidy level of each individual was determined using flow cytometry (Becton Dickinson FACS Calibur, USA): Peripheral blood was collected by caudal puncture and kept at 4℃. Blood cells were suspended in 1 ml of staining buffer consisting of 0.1% sodium citrate, 0.1% triton×100, and 50 µg ml^{-1} propidium iodide and analyzed within 5 min. Erythrocytes of karyologically identified *M. anguillicaudatus* with 2n = 50 gave a relative DNA content of 2n as the diploid standard, which was used as internal control (Hardie *et al*., 2002). Absolute DNA content was measured on the basis of chicken erythrocyte DNA content (2.5 pg $nucleus^{-1}$) (Tiersch and Chandler, 1989).

After the ploidy level was determined, representative individuals for further cytological examination, including karyotyping and nuclear volume, were selected according to the following methods: For the populations with only diploids or only tetraploids, three individuals were randomly selected. In the populations exhibiting coexistence of individuals with different ploidy levels, three individuals with major ploidy level were randomly selected while all individuals with minor ploidy level were included for further analysis. Individuals were karyotyped using direct chromosome preparation from the head kidney as described by Zhou *et al.* (2008). Briefly, fish were injected intraperitoneally with PHA and colchicine at a final concentration of 8-10 µg g^{-1} and 2-4 µg g^{-1} body mass, respectively. After four hours, the peripheral blood was collected and the fishes were killed and the head kidneys collected for karyotype analysis. Blood smears we prepared and stained with Wright-Giemsa (WG) fluid. The major axis (a) and minor axis (b) of the nuclei of 100 randomly selected erythrocytes were measured using a Motic BA400 microscope and Motic Image Advanced 3.2 software (Motic Co., Ltd. http://www.motic.com/). The volume (V) of the nuclei was computed using the following formula: $V=4/3\times\pi(a/2)\times(b/2)^2$. Five fin formulae (anal-fin, dorsal-fin, pectoral-fin, pelvic-fin, caudal-fin) of the representative individuals were also recorded for comparison.

Table 1. The frequency and distribution of different ploidy level *Misgurnus anguillicaudatus* in China

Loc. no.	River basin	Tributary（ locality）	Ploidy (%)					*N*	Sampling time or reference
			2n	3n	4n	5n	6n		
1	Yangtze River	Tuo River (Luzhou）	34(100)	0(0)	0(0)	0(0)	0(0)	34	Aug. 2007
2		Minjiang River (Chengdu)	31(100)	0(0)	0(0)	0(0)	0(0)	31	Aug. 2007
3		Jialing River (Chongqing)	40(100)	0(0)	0(0)	0(0)	0(0)	40	Sep. 2007
4		Wujiang River (Guiyang）	30(100)	0(0)	0(0)	0(0)	0(0)	30	Aug. 2007
5		Qingjiang River (Enshi)	38(100)	0(0)	0(0)	0(0)	0(0)	38	June 2006
6		Xiangxi River (Zigui)	39(100)	0(0)	0(0)	0(0)	0(0)	39	July 2006
7		Malao River (Zhijiang)	38(100)	0(0)	0(0)	0(0)	0(0)	38	Mar. 2006
8		Songzi River (Shashi)	5(11.4)	1(2.3)	38(86.4)	0(0)	0(0)	44	Aug. 2010
9		Honghu Lake	25(69.4)	3(8.3)	8(22.2)	0(0)	0(0)	36	May 2010
10		Juzhanghe River (Jinmen)	42(100)	0(0)	0(0)	0(0)	0(0)	42	Apr. 2006
11		Hanjiang River (Hanzhong)	31(100)	0(0)	0(0)	0(0)	0(0)	31	Sep. 2012
12		Hanjiang River (Ankang)	4(14.3)	0(0)	24(85.7)	0(0)	0(0)	28	Sep. 2012
13		Hanjiang River (Shiyan)	37(100)	0(0)	0(0)	0(0)	0(0)	37	Sept. 2006
14		Hanjiang River (Fangxian)	0(0)	0(0)	40(100)	0(0)	0(0)	40	Aug. 2007
15		Hanjiang River (Songbai, Shenlongjia)	0(0)	0(0)	43(100)	0(0)	0(0)	43	Aug. 2007
16		Hanjiang River (Dajiuhu Lake, Shenlongjia)	42(100)	0(0)	0(0)	0(0)	0(0)	42	Aug. 2007
17		Hangjiang River (Xiangyang)	39(100)	0(0)	0(0)	0(0)	0(0)	39	July 2006
18		Hanjiang River (Yicheng)	41(100)	0(0)	0(0)	0(0)	0(0)	41	Apr. 2006
19		Hanjiang River (Qianjiang)	39(100)	0(0)	0(0)	0(0)	0(0)	39	June 2006

20		Hanjiang River (Tianmen)	22(61.1)	0(0)	14(38.9)	0(0)	0(0)	36	June 2006
21		Yunshui River (Suizhou)	18(41.9)	0(0)	25(58.1)	0(0)	0(0)	43	Apr. 2006
22		Yunshui River (Xiaogan)	23(52.3)	0(0)	21(47.7)	0(0)	0(0)	44	Mar. 2006
23		Hanjiang River (Xiantao)	0(0)	0(0)	39(100)	0(0)	0(0)	39	July 2006
24		Yangtze River (Dongxihu)	0(0)	0(0)	40(100)	0(0)	0(0)	40	Apr. 2006
25		Diaochahu Lake	0(0)	0(0)	44(95.7)	0(0)	2(4.3)	46	May 2008
26		Yangtze River (Caidian)	0(0)	0(0)	42(100)	0(0)	0(0)	42	Apr. 2006
27		Junshui River (Chibi)	5(11.4)	2(4.5)	37(84.1)	0(0)	0(0)	44	Sept. 2009
28		Yangtze River (Jiangxia)	0(0)	0(0)	40(100)	0(0)	0(0)	40	May 2006
29		Yangtze River (Xinzhou)	0(0)	0(0)	41(100)	0(0)	0(0)	41	May 2006
30		Liangzi Lake	32 (11.1)	4(1.4)	249 (86.5)	3(1)	0(0)	288	Aug. 2011/ June/Aug.2012
31		Daoshui River (Hongan)	39(100)	0(0)	0(0)	0(0)	0(0)	39	May 2006
32		Yangtze River (Huanggang)	0(0)	0(0)	41(100)	0(0)	0(0)	41	May 2006
33		Yangtze River (Ezhou)	0(0)	0(0)	39(100)	0(0)	0(0)	39	Aug. 2006
34		Yangtze River (Huangshi)	41(100)	0(0)	0(0)	0(0)	0(0)	41	Apr. 2006
35		Fushui River (Yangxin)	40(100)	0(0)	0(0)	0(0)	0(0)	40	Apr. 2006
36		Yangtze River (Wuxue)	0(0)	0(0)	40(100)	0(0)	0(0)	40	Aug. 2006
37		Dongting Lake	36(100)	0(0)	0(0)	0(0)	0(0)	36	July 2007
38		Yuanjiang (Changde)	28(100)	0(0)	0(0)	0(0)	0(0)	28	Aug. 2012
39		Xiangjiang (Changsha)	32(100)	0(0)	0(0)	0(0)	0(0)	32	Aug. 2012
40		Poyang Lake	(100)	0(0)	0(0)	0(0)	0(0)	Not given	Mao 2005
41		Xinjiang (Shangrao)	(100)	0(0)	0(0)	0(0)	0(0)	Not given	
42		Taihu Lake	(100)	0(0)	0(0)	0(0)	0(0)	Not given	Shen et al.1984
43		Shuiyangjiang (Ningguo)	30(100)	0(0)	0(0)	0(0)	0(0)	30	Li et al. 2008
44		Yangtze River (Nanjing)	25(100)	0(0)	0(0)	0(0)	0(0)	25	
45		Qiantang River (Hangzhou)	29(96.7)	1(3.3)	0(0)	0(0)	0(0)	30	
46		Huangpu River (Shanghai)	20(100)	0(0)	0(0)	0(0)	0(0)	20	
47		Huangpu River (Chongming)	Major	0(0)	Minor	0(0)	0(0)	Not given	Guo et al. 2009
48		Huangpu River (Songjiang)	Major	0(0)	Minor	0(0)	0(0)	Not given	
49		Huangpu River (Nanhui)	Major	0(0)	Minor	0(0)	0(0)	Not given	
50		Huangpu River (Jinshan)	Major	0(0)	Minor	0(0)	0(0)	Not given	
51	Yellow River	Daheihe River (Hohhot)	29(96.7)	1(3.3)	0(0)	0(0)	0(0)	30	Li et al. 2008
52		Weihe River (Xian)	30(100)	0(0)	0(0)	0(0)	0(0)	30	Sep. 2012
53	Hwai River	Jinshui River (Zhengzhou)	26(100)	0(0)	0(0)	0(0)	0(0)	26	Aug. 2012
54		Wei River (Xinxiang)	(100)	0(0)	0(0)	0(0)	0(0)	Not given	Kang et al. 2008
55		Shihe River (Xinyang)	21(100)	0(0)	0(0)	0(0)	0(0)	21	Aug. 2012
56		Yihe River (Linyi)）	26(100)	0(0)	0(0)	0(0)	0(0)	26	Aug. 2006
57		Wohe River (Bengbu)	Major	0(0)	Minor	0(0)	0(0)	Not given	Guo et al. 2009
58		Feihe River (Hefei)	30(100)	0(0)	0(0)	0(0)	0(0)	30	Aug. 2012
59	Heilongjiang	Nenjiang River (Heihe)	30(100)	0(0)	0(0)	0(0)	0(0)	30	Li et al. 2008
60		Nenjiang River (Wudalianchi)	26 (100)	0(0)	0(0)	0(0)	0(0)	26	
61		Nenjiang River (Keshan)	29(100)	0(0)	0(0)	0(0)	0(0)	29	
62		Songhuajiang River (Jiamusi)	30(100)	0(0)	0(0)	0(0)	0(0)	30	
63		Songhuajiang River (Hulan)	15(100)	0(0)	0(0)	0(0)	0(0)	15	
64		Songhuajiang River (Harbin)	30(100)	0(0)	0(0)	0(0)	0(0)	30	
65		Songhuajiang River (Binxian)	19(100)	0(0)	0(0)	0(0)	0(0)	19	
66	Pearl River	Xijiang River (Nanning)	(100)	0(0)	0(0)	0(0)	0(0)	Not given	Shen et al.

67		Beijiang River (Shaoguan)	0(0)	0(0)	(100)	0(0)	0(0)	Not given	1984
68		Xiangjiang River (Guilin)	36(100)	0(0)	0(0)	0(0)	0(0)	36	Sep. 2011
69		Pearl River Delta (Zhujiang)	26(100)	0(0)	0(0)	0(0)	0(0)	26	Aug. 2012
70	Liaohe River	Hunhe River (Shenyang)	48(100)	0(0)	0(0)	0(0)	0(0)	48	Aug. 2012
71		Taizi River (Liaoyang)	20(100)	0(0)	0(0)	0(0)	0(0)	20	Li et al. 2008
72		Linghe River (Huludao)	30(100)	0(0)	0(0)	0(0)	0(0)	30	
73	Yalu River	Yalu River (Dandong)	28(100)	0(0)	0(0)	0(0)	0(0)	28	
74		Zhuanghe River (Zhuanghe)	30(100)	0(0)	0(0)	0(0)	0(0)	30	
75	Haihe River	Daqing River (Yixian)	28(100)	0(0)	0(0)	0(0)	0(0)	28	Sep. 2009
76		Daqing River (Baodingshi)	31(100)	0(0)	0(0)	0(0)	0(0)	31	Sep. 2009
77		Yongding River (Tianjin)	28(94.7)	1(5.3)	0(0)	0(0)	0(0)	29	Aug. 2012
78	Jiaodong coastal	Dagu River (Yantai)	29(100)	0(0)	0(0)	0(0)	0(0)	29	Aug. 2008
79		Dagu River (Qindao)	20(100)	0(0)	0(0)	0(0)	0(0)	20	Li et al. 2008
80	Southeast coastal	Lincheng River (Zhoushan)	35(100)	0(0)	0(0)	0(0)	0(0)	35	Aug. 2012
∑			1705(65.88)	13(0.5)	865(33.42)	3(0.12)	2(0.08)	2588	

RESULTS

***Ploidy level variation of M. anguillicaudatus*:** A total of 2146 *M. anguillicaudatus* individuals from 52 populations were examined for ploidy level, and 196 individuals were karyotyped. Five ploidy levels were detected: (1) diploid, the DNA content was 2.49 ± 0.02 pg nucleus^{-1}, and the chromosome number was 50, consisting of eight metacentrics (m), six submetacentrics (sm), and 36 acrocentrics (a), *i.e.* karyotype 2n = 50 (8m+6sm+36a); (2) triploid, 3.75 ± 0.04 pg nucleus^{-1} and 3n = 75 (12m+9sm+54a); (3) tetraploid, 4.96 ± 0.05 pg nucleus^{-1} and 4n = 100 (16m+12sm+72a); (4) pentaploid, 6.12 ± 0.07 pg nucleus^{-1} and 5n = 125 (20m+15sm+90a); (5) hexaploid, 7.43 ± 0.09 pg nucleus^{-1} and 6n = 150 (24m+18sm+108a). The representative metaphase cells, and the corresponding karyotype of each ploidy level, are shown in Figure 1.

As expected, the major and minor axes of the erythrocytes and their nuclei in different ploidy *M. anguillicaudatus* increased along with the increasing ploidy level. The cytological parameters of each ploidy level *M. anguillicaudatus* were summarized in Table 2.

Note: The DNA content was calculated using chicken erythrocytes DNA content (2.50 pg nucleus^{-1}) as standard.

The polyploid *M. anguillicaudatus* shares the external appearance, i.e. shape and colour, with its sympatric diploid counterparts. The five fin formula was: dorsal fin 2,6; pectoral fin 1,9; pelvic fin 1,5; anal fin 1,6; caudal fin 2,12, no difference among the ploidy levels could be detected.

***Geographical distribution of different ploidy levels*:** As shown in Figure 2, diploid *M. anguillicaudatus* was the dominant ploidy level found in the present study, comprising 65.9% of all indivuduals. Of the 80 populations (52 populations currently sampled and 28 previously reported), 68 included diploids or were exclusively diploids. The second most abundant were tetraploids, accounting for 33.4% of the total. In contrast to the widely distributed diploids, the tetraploids were primarily found in the central Yangtze River basin, especially near the mainstream, including locations 24, 26, 29, 32, 33, and 36. Along the Hanshui tributary in the Yangtze River basin, diploids and tetraploids were observed to coexist in some populations (locations 20, 21, and 22), but interestingly, no triploids were detected. In other populations (locations 8, 9, and 27), a small number of triploids was discovered along with diploids and tetraploids. At each of the locations 45, 51, and 77, only a single triploid co-occurred with diploid individuals.

In Shenlongjia area of the Yangtze River basin, ploidy levels varied depending on altitude. At 2500 m (Dajiuhu Lake, location 16), all individuals screened were found to be diploid, while samples from 1200 m (Songbai, location 15) and 600 m (Fangxian, location 14) were only tetraploids. No sympatric population of different ploidy levels were detected in these localities.

In Liangzi Lake area, Yangtze River basin (location 30), a single pentaploid individual was found in August, 2011. To verify this phenomenon, 288 additional individuals were collected in two further samplings. Flow cytometric analysis revealed that the tetraploid *M. anguillicaudatus* was the dominant in the area. Of the 288 individuals, 249 (86.46%) were tetraploid, 32 (11.11%) diploid, four (1.39%) triploid, and three individuals (1.04%) were identified as pentaploid, demonstrating the sympatric occurrence of diploid, triploid, tetraploid, and pentaploid loach. Typical distribution of DNA content in red blood cells (RBC) of polyploid loach from the populations is shown in Figure 3.

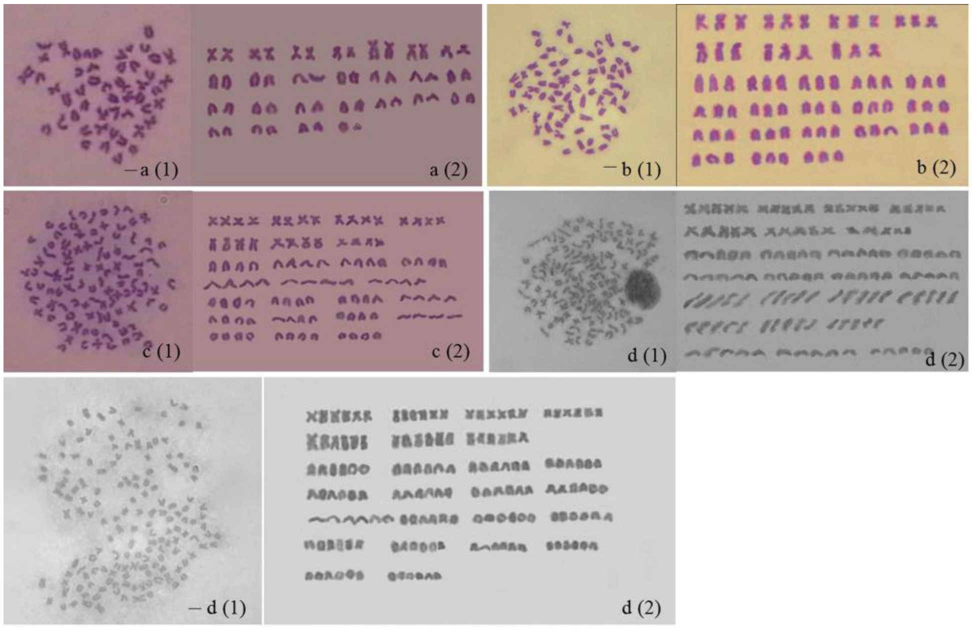

Figure 1. The representative metaphase chromosome (1) and the corresponding karyotype (2) of diploid (a), triploid (b), tetraploid (c), pentaploid (d), and hexaploid (e) loach *Misgurnus anguillicaudatus* from China.

Table 2. Comparison of cytogenetic characteristics of natural diploid and polyploid loach *Misgurnus anguillicaudatus*

Ploidy level	**No. of samples**	**Chromosome number**	**Erythrocyte nuclear Volume ± S.D. (μm^3)**	**Flow cytometric results of DNA content**	
				Average channel number ± S.D.	**DNA content ± S.D. (pg nucleus^{-1})**
Diploid	32	50	71.19 ± 9.49	203.4±3.19	2.49±0.02
Triploid	4	75	106.13±11.26	306.3±2.75	3.75±0.04
Tetraploid	249	100	142.32 ± 12.95	405.5±2.08	4.96±0.05
Pentaploid	3	125	176.11±15.84	499.6±1.99	6.12±0.07
Hexaploid	2	150	213.69±16.38	607.2±2.11	7.43±0.09

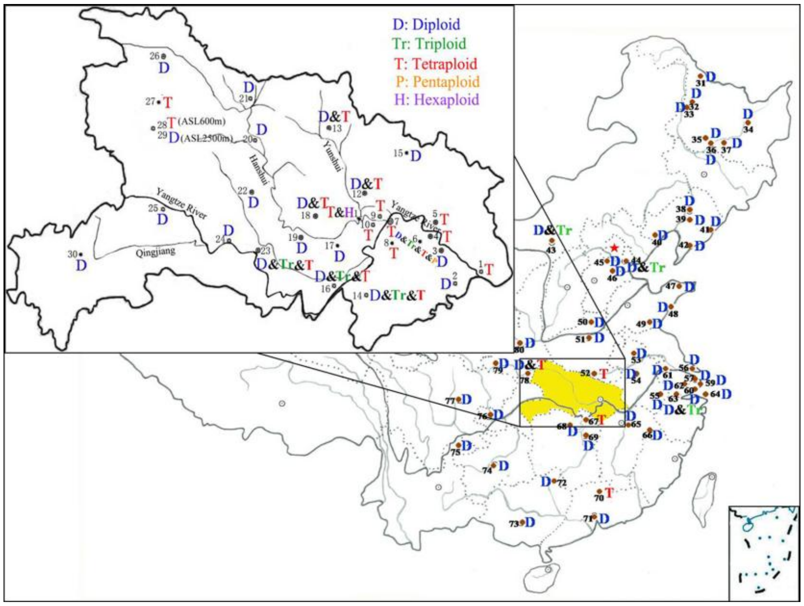

Figure 2. Geographical distribution of different ploidy level loach *Misgurnus anguillicaudatus* in China

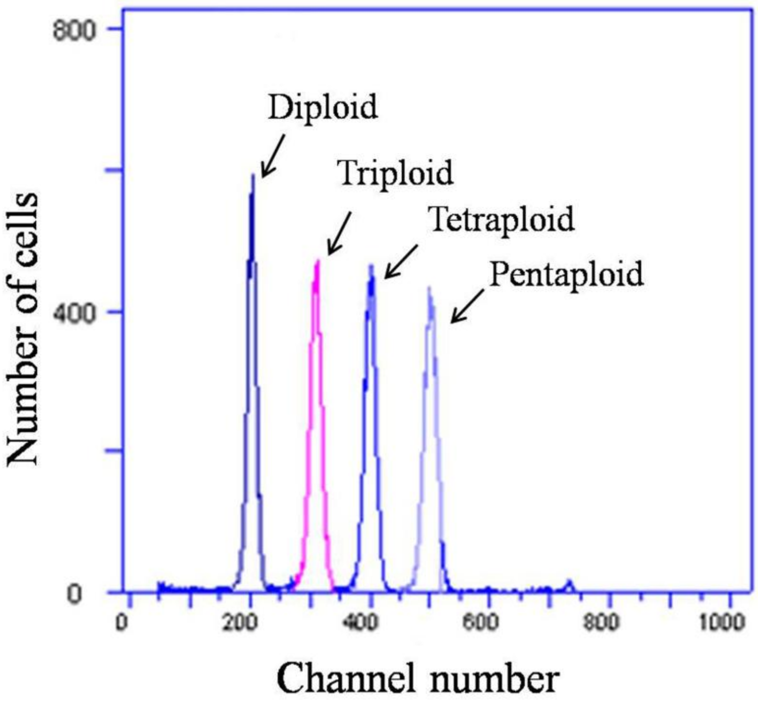

Figure 3. Flow cytometry profiles of cellular DNA content of the diploid, triploid, tetraploid, and pentaploid loach *Misgurnus anguillicaudatus* from Liangzi Lake area, Yangtze River Basin, China.

In the Diaochahu Lake area (location 25), Yangtze River basin, two male hexaploids with 150 chromosomes were detected during May, 2008. No additional hexaploid individual was found, despite repeated screening. No clearly defined geographical ranges or boundaries of the distribution of ploidy levels were found both for diploids and tetraploids.

DISCUSSION

Diploidy is the dominant ploidy level of *M. anguillicaudatus* in the majority of the Chinese populations assayed in the present and previous studies, as is the case in Japan (Arai, 2003). The tetraploid *M. anguillicaudatus* is most likely endemic to China, since no tetraploid individual has been identified in wild populations in other countries of eastern Asia (Zhang and Arai, 1999). For a long time, the evolutionary origin of the tetraploid *M. anguillicaudatus* distributed in the Yangtze River basin, China remained unknown. The recent molecular phylogenetic study of Cobitidae species using nuclear (Slechtova *et al*., 2008; Saitoh *et al*., 2010) as well as mitochondrial genome sequences (Morishima *et al*., 2008; Slechtova *et al*., 2008; Koizumi *et al*., 2009; Saitoh *et al*., 2010; Kitagawa *et al*., 2011) indicate that an hybridization event occurred between an ascestral species of *Cobitis* and ascestral species of *Misgurnus* early in the evolutionary history of *Misgurnus*. The interspecific hybridization tends to trigger the development of clonality and gynogenesis, and subsequently, polyploidisation, as well recognized in the spined loaches (Choleva *et al*., 2012; Janko *et al*., 2012). Therefore, the natural tetraploid *M. anguillicaudatus* can be regarded as a consequence of this ancient hybridization event.

Seven of the 80 studied populations exhibited coexistence of diploids and tetraploids, but a small number of triploids were detected in only three of seven populations, even after repeated sampling. As reciprocal hybridization between natural diploid and tetraploid *M. anguillicaudatus* can easily produce a large number of triploid progeny (Arai, 2003; *Zhou et al*., 2010), the sympatric diploids and tetraploids probably subjected to strong selection pressure to mate with their co-mates, resulted in reproductive isolation of the two-level ploidies. Further research on reproductive isolation between diploid and tetraploid *M. anguillicaudatus* has not been accomplished yet, but in the striated spined loach (the *striata* type of the *Cobitis taenia* complex), researchers found that spawning site segregation is an important factor in reproductive isolation of two sympatric populations- the small and mid-sized forms (Saitoh, 1990). In anuran species, its was reported that females prefer larger males (Sullivan *et al.*, 1995), or males of a particular size (Robertson, 1990), and researchers have domenstrated the positive relationship between the size of males and females during amplexus in the sunset frog *Spicospina flammocaerulea* (Smith *et al*., 2003). Researchers concerning spawning behaviour of the loach species argued that, the significantly smaller sized males than females is common characteristic in most genera of Cobitidae, which is a required condition to from a complete ring around the female to activate spawning (Bohlen, 2008). During the studies, we observed that the tetraploid *M. anguillicaudatus* exhibit obviously larger body size than sympatric diploids, male and female, respectively. Thus, the female-male size ratio may be a factor affecting the reproductive success in this species, either through male-male competition or female mate choice.

It is often assumed that polyploids spread because they can better tolerate and invade a wider range of environments than their diploid counterparts, owing to increased genetic buffering provided by additional genome copies (Otto and Whitton, 2000). Greater ecological amplitude has been demonstrated in some polyploid plants compared to their diploid progenitors. Polyploid plants are reported to inhabit regions of higher altitude and tolerate extreme conditions, whereas the lowlands are differentially inhabited by diploid populations. However, this trend may not be the rule in fishes, at least in *M. anguillicaudatus.* In our results, the diploid *M. anguillicaudatus* was the dominant ploidy level in most of the sampled localities, showing a broader range than their tetraploid derivatives. Loach from a higher altitude (location 16, Dajiuhu Lake, Shenlongjia area, 2500 m a.s.l.) were exclusively diploid, whereas those from lower altitude (location 15, Songbai, Shenlongjia, 1200 m a.s.l. and location 14, Fangxian, ASL 600 m a.s.l.) were tetraploid. In addition, the tetraploid *M. anguillicaudatus* has a conspicuously bigger adult body size than the diploid form (Zhou *et al*., 2010), and, as a consequence, the fecundity of tetraploids is higher than that of diploids. However, the occurrence of tetraploids in natural populations of *M. anguillicaudatus* is relatively low, possibly indicating that the increased cell surface of tetraploids limits cellular metabolism and, thus, poor survival in adverse environmental conditions (Cassani *et al*., 1990; Mable, 2003; Zhou *et al*., 2010).

The discovery of naturally occurring pentaploid and hexaploid *M. anguillicaudatus* expands our knowledge on ploidy variability of wild *M. anguillicaudatus*. Hexaploidy of *M. anguillicaudatus* has been prevously reported (Abbas *et al*., 2009). However, data on natural occurrence of pentaploid fish is scarce (Arai, 2011), although a few publications have reported the artificial induction of pentaploidy in fish (Arai *et al*., 1991, 1993). Pentaploids have been produced by inhibition of the second polar body extrusion of eggs of natural tetraploid females after fertilization with haploid sperm of normal diploid males (Arai *et al*., 1991, 1993; Arai, 2003). These results could be considered as an heuristic explanation for the observed natural occurrence of pentaploid *M. anguillicaudatus*: the spontaneous failure in releasing a second polar body in a normal tetraploid egg produced by a tetraploid female and subsequent fertilization by the 1n sperm of a diploid *M. anguillicaudatus* leading to production of a pentaploid individual. The failure of extrusion of a second polar body is considered relatively common in wild *M. anguillicaudatus* (Zhang and Arai, 1999; Arai, 2003).

Unlike amphibians (Smith *et al*., 2003) and plants (Travnicek *et al*., 2011), where majority of polyploids exhibit a regular biogeographical pattern, no clearly defined geographical ranges or boundaries for the distribution of diploids and tetraploids were observed in the present study, making it difficult to draw conclusion regarding the processes contributing to the origin and divergence of polyploidy *M. anguillicaudatus*.

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