The karyotype of *Hynobius maoershanensis* (Urodela: Hynobiidae), a newly described species with rare banding patterns

Qing Li-yan[1,2], Wang Yi-ding[2], Zeng Xiao-mao[1], Chen Qin[1] & Hou Mian[3]

[1]*Chengdu Institute of Biology, The Chinese Academy of Sciences, Chengdu, China*
[2]*College of Life Sciences, Sichuan Normal University, Chengdu, China*
[3]*Continuing Education College of Sichuan Normal University, Chengdu, China*

The karyotype of *Hynobius maoershanensis*, a newly described salamander species from mainland China, is described for the first time using Giemsa conventional stain, C-banding and Ag-NORs techniques. All individuals have a diploid number of 56 chromosomes, which is consistent with karyotypes of the pond-type *Hynobius* group that lives in the lowlands and spawns in small ponds. Several differences distinguish *H. maoershanensis* from other *Hynobius* karyotypes: chromosome no. 13 is subtelocentric while in others it is metacentric; there is a prominent and unique dark C-banding pattern encompassing the whole short arm of chromosome no. 13, which can be used as a marker to distinguish this species from other species of *Hynobius*; multiple C-bands are found only in chromosomes 4, 9 and 13 in this species, much fewer than in other species; NORs are rarely located at the terminal position on the short arm of a large biarmed chromosome (no. 8) in *H. maoershanensis*, but are commonly observed on the small telocentric microchromosomes (nos. 20, 21 or 23) in other species with 2n=56. All five species from six populations of *Hynobius* from mainland China can be ecologically attributed to the pond-type group and have 2n=56. Karyologically, they form two subgroups. One subgroup has ten while the other has only nine pairs of telocentric microchromosomes.

Key words: Ag-NORs, C-banding, Chinese hynobiid salamander

INTRODUCTION

The genus *Hynobius* is endemic to East Asia, occurring in mainland China, Taiwan, Korea and Japan. There are 31 species known in the genus, of which nine occur in mainland China, and three in Taiwan (Zhou et al., 2006; Frost, 2008). The karyotypes of 24 species have been examined by Giemsa conventional stain. C-banding or R-banding techniques have been applied to 17 of these species, but the Ag-NORs technique has only been applied to the karyotypes of seven species (Iizuka & Kakegawa, 1989; Kohno et al., 1991; Cha & Lee, 1995; Zeng et al., 1997; Ikebe et al., 1998, 2000, 2005; Kuro-o et al., 2002; Nishikawa et al., 2005). Most of these investigations primarily deal with species distributed in Japan, Korea and Taiwan. Only four species from five localities have been cytogenetically studied in mainland China: one population for each of *H. amjiensis*, *H. leechii* and *H. guabangshanensis* and two populations of *H. yiwuensis* were studied using conventional Giemsa stain. *H. amjiensis* from Mt Longwang and *H. yiwuensis* from Zhenhai County have also been studied by C-banding (Zeng et al., 1997; Ikebe et al., 1998; Fu et al., 2003; Chen et al., 2008; Xiong et al., 2008). So far, Ag-NORs of salamanders from mainland China have not been reported. Recently, with more *Hynobius* species described from mainland China (Shen et al., 2004; Zhou et al., 2006), additional cytogenetic studies can provide more information for this genus.

In this paper, we describe the karyotype of *Hynobius maoershanensis*, which was recently described as a new species in the genus *Hynobius* (Zhou et al., 2006). We used not only Giemsa conventional stain, but also C-banding and Ag-NORs techniques. In addition, we discuss the karyotypes of Chinese *Hynobius* salamanders.

MATERIALS AND METHODS

Thirteen egg masses of *Hynobius maoershanensis* were collected from swamps in Guangxi Maoershan Natural Reserve, Guangxi Province, China (25º53'N, 110º25'E, altitude 1986 m) on 25 January 2006. Late tail-bud stage embryos were used for cytogenetic studies.

Chromosome preparation and Giemsa conventional stain

Embryos were incubated in their jelly capsules at 20 °C for more than three days. Tail-bud stage embryos were carefully removed from the capsule using forceps, scissors, and needles, and were kept alive at 20 °C in a 0.5% colchicine solution for 96 h. After the yolk was removed, the embryos were thoroughly disrupted with Pasteur pipettes. Cells were treated with hypotonic solution (1 part amphibian saline: 15 parts distilled water) for 20–30 min and fixed in (3:1) methanol:acetic acid. Suspended cells were dropped on glass slides, and the slides were air dried and stained with 4% Giemsa solution in phosphate-buff-

Correspondence: Zeng Xiao-mao, Chengdu Institute of Biology, The Chinese Academy of Sciences, Chengdu 610041, Sichuan, China. *E-mail:* zengxm@cib.ac.cn

Table 1. Chromosome characteristics of *H. maoershanensis*. Abbreviations: m = metacentric; sm = submetacentric; st = subtelocentric; t = telocentric.

No.	Relative length	Arm ratio	Centromere position
1	12.96±0.99	1.37±0.19	m
2	10.81±0.65	1.40±0.18	m
3	8.69±0.58	1.29±0.16	m
4	7.78±0.53	3.66±0.76	sm or st
5	7.57±0.55	1.44±0.30	m or sm
6	6.98±0.41	1.46±0.41	m or sm
7	6.60±0.56	2.52±0.30	sm
8	5.78±0.54	2.42±0.43	sm
9	4.95±0.46	1.23±0.20	m
10	4.16±0.40	2.35±0.47	sm
11	3.34±0.44	1.25±0.22	m
12	2.99±0.34	4.27±0.50	st
13	2.69±0.27	4.30±0.80	st
14	2.22±0.39	1.20±0.19	m
15	1.73±0.33	2.89±0.83	sm or st
16	1.38±0.35	1.14±0.12	m
17	1.02±0.28	1.36±0.47	m
18	0.75±0.31	1.12±0.18	m
19	1.69±0.46	—	t
20	1.24±0.38	—	t
21	0.96±0.29	—	t
22	0.99±0.56	—	t
23	0.82±0.25	—	t
24	0.62±0.17	—	t
25	0.49±0.12	—	t
26	0.41±0.09	—	t
27	0.32±0.09	—	t
28	0.22±0.06	—	t

ered saline (pH 6.8) for 15–20 min, then the slides were rinsed in distilled water and dried at room temperature.

Fifty metaphase plates were used to count the chromosome numbers, and karyotypes were assembled from ten good metaphases. The chromosomes were described following the nomenclature for centromeric position on chromosomes by Leven et al. (1964).

C-banding

Staining of constitutive heterochromatin (C-bands) was done using the BSG method described by Sumner (1972) with slight modification. The air-dried slides were placed in 0.2 N HCl at room temperature for 30–45 min and rinsed with distilled water before being placed in saturated $Ba(OH)_2$ solution at 50 °C for 7–10 min. Subsequently, they were incubated in 2xSSC at 60 °C for 1 h and rinsed with distilled water. Finally, the slides were stained with 4% Giemsa (pH 6.8) for 30–45 min, rinsed again in distilled water, and dried at room temperature. Five metaphase cells were examined and photographed. Chromosome pairs were cut out from photographic prints and arranged according to size in parallel rows, so that the conformity of the heterochromatin banding patterns could be observed and analysed.

Ag-NORs

The nucleolus organizer regions (NORs) were detected using the Ag-NOR banding method described by Tan et al. (1986) with slight modification. Air-dried slides were kept at 54–55 °C for a few seconds, then 2% gelatine solution and 50% Ag-NO_3 solution were added onto the slides and intermixed gently. The slides were incubated for about 5 min, rinsed in distilled water and dried at room temperature. Five metaphase cells were examined and photographed.

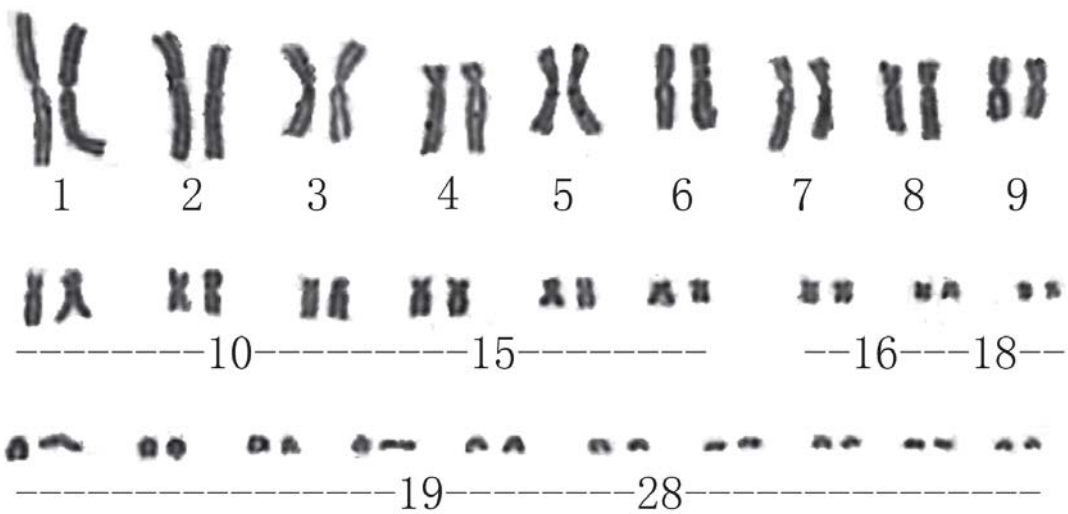

Fig. 1. Karyotype of *H. maoershanensis*.

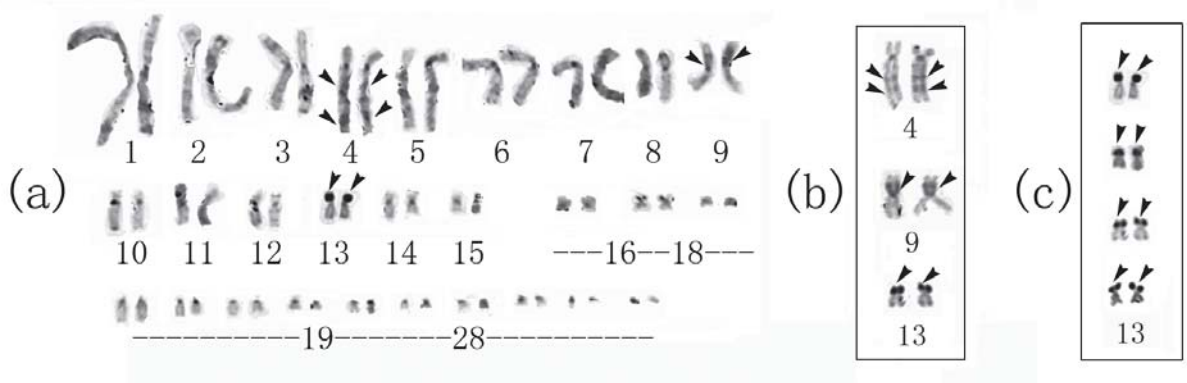

Fig. 2. C-banded karyotype of *H. maoershanensis*. (a) All the chromosomes have centromeric C-bands; (b) multiple C-banding patterns on chromosomes 4, 9 and 13; (c) prominent, dark C-banding pattern on chromosome 13 from four metaphase plates. Arrows point to the interstitial C-bands on chromosomes 4 and 9, and the prominent dark C-banding on chromosome 13.

RESULTS

The diploid complement of *H. maoershanensis* consisted of 56 chromosomes that could be divided into four groups by size and centromeric position (Table 1; Fig. 1). The first group contained nine pairs of large chromosomes (nos. 1–9); no. 4 was submetacentric or subtelocentric, nos. 5 and 6 were metacentric or submetacentric, nos. 7 and 8 were submetacentric, and the remaining four pairs were metacentric. Chromosomes 10–15 comprised the second group, which were medium-sized chromosomes. Chromosome 10 was submetacentric, 11 and 14 were metacentric, 12 and 13 were subtelocentric, and 15 was submetacentric or subtelocentric. The third group contained three metacentric chromosomes (nos. 16–18) while the fourth group consisted of ten acrocentric microchromosomes (nos. 19–28). No conspicuous secondary constriction was observed in any of the metaphase plates.

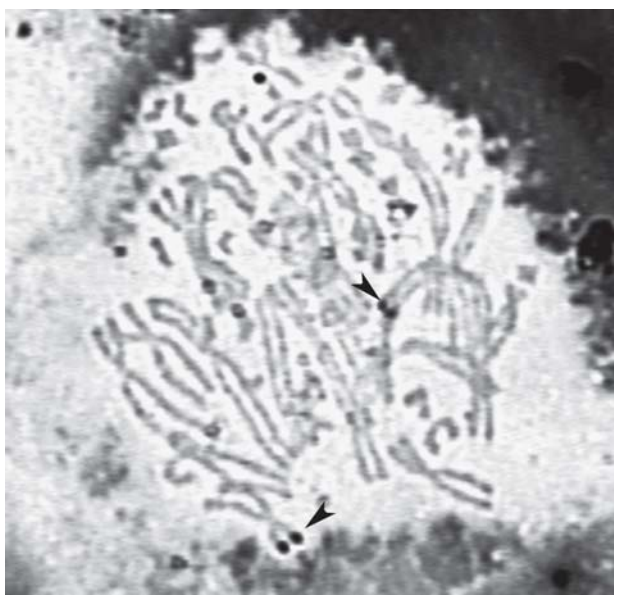

Fig. 3. Silver-stained karyotype of *H. maoershanensis*. Arrows point to the active NORs in the telomeric regions on the short arms of chromosome 8.

C-bands were detected in the centromeric region in all chromosome pairs (Fig. 2a). Two interstitial C-bands were found in the long arms of chromosome 4 and there was a major C-positive block at the centromeric region of the short arms of chromosome 9 (Fig. 2b). In addition, the whole short arm of chromosome 13 displayed a prominent, dark C-band (Fig. 2c). We did not detect any C-banding patterns on the small telocentric chromosomes. NORs were observed in the telomeric region of the short arms of chromosome pair 8 (Fig. 3)

DISCUSSION

Hynobius karyotypes

Members of the genus *Hynobius* distributed in Japan, Korea and Taiwan can be divided into three groups according to the number of diploid chromosomes (Kohno et al., 1991). The first group has a diploid number of 56 chromosomes, the second has 58 chromosomes, and a third group, with only one species (*H. retardatus*), has 40 chromosomes. *H. maoershanensis* has 2n=56 and the karyotype is similar to the other 16 species with 56 chromosomes (Morescalchi, 1973; Ikebe & Kohno, 1979a,b; Kohno et al., 1983, 1987, 1991; Seto et al., 1983, 1986, 1988; Seto & Matsui, 1984b; Ikebe et al., 1986, 1987, 1990, 1998, 2005; Matsui et al., 1985; Kuro-o et al., 1987a,b, 2002; Yamamoto et al., 1988; Izumisawa et al., 1989; Cha & Lee 1995; Irie et al., 1996; Zeng et al., 1997; Xiong et al,. 2008). In all of the 56-chromosome species of *Hynobius*, the chromosomes can be divided into four groups by size, and the first group consists of nine pairs of large-sized chromosomes. Despite these similarities, *H. maoershanensis* is easily distinguished from the other 16 species of 56-chromosome *Hynobius* by having a subtelocentric, and not a metacentric chromosome 13 (Table 1; Fig.1). The unique morphology of chromosome 13 can be considered a distinguishing marker in the karyotype of *H. maoershanensis*.

C-bands

Of 24 species in the genus *Hynobius* analysed chromosomally, 17 have been investigated for C-banding and only two of those species were from mainland China

Table 2. The grouping of *Hynobius* salamanders based on the criteria of Sato (1943) and Matsui et al. (1985). P: pond type; M: mountain-brook type.

Species	Locality	Ecological group	2n	Pairs of medium-sized uniarmed chromosomes	Pairs of telocentric micro-chromosomes	References
Species from mainland China						
H. leechii	Huanren Co., Liaoning	P	56	0	9	Zeng et al., 1997
H. yiwuensis	Wuyi Mt., Fujian	P	56	0	9	Zeng et al., 1997
H. amjiensis	Anji Co., Zhejiang	P	56	0	10	Ikebe et al., 1998; Zeng et al., 1997
H. yiwuensis	Zhenhai Co., Zhejiang	P	56	0	10	Ikebe et al., 1998
H. guabangshanensis	Guabangshan Nature Reserve, Hunan	P	56	0	10	Xiong et al., 2008
H. maoershanensis	Maoershan Nature Reserve, Guangxi	P	56	0	10	This study
Species beyond mainland China						
	Japan	P	56*	1	8, 9, 10	Ikebe & Kohno, 1979a; Ikebe et al., 1987, 2000; Seto & Matsui, 1984a,b; Seto et al., 1983, 1988
	Japan, Korea	P	56*	0	8, 9, 10	Ikebe & Kohno, 1979a,b; Ikebe et al., 1998; Seto & Iizuka, 1993; Seto et al., 1983, 1986; Zeng et al., 1997
	Japan	M	58	1**	8	Iizuka & Kakegawa, 1989
	Japan, Taiwan	M	58	0	7–12	Iizuka et al., 1989; Iizuka & Kakegawa, 1989; Ikebe & Kohno, 1991; Ikebe et al., 1986; Seto et al., 1987

*Only *H. okiensis*, *H. tsuensis* with 56 chromosomes are mountain-brook type (Seto et al., 1986, 1987).
**Only the population of *H. kimurae* from Hakuba, Nagano Pref., Japan has a pair of medium-sized uniarmed chromosomes.

(Kohno et al., 1983, 1987, 1991; Seto et al., 1986; Ikebe et al., 1987, 1990, 1998, 2000, 2005; Iizuka & Kakegawa 1989; Izumisawa et al., 1989; Ikebe & Kohno, 1991; Kuro-o et al., 2002). In keeping with the other 17 species, all the chromosomes exhibited centromeric C-bands in *H. maoershanensis* (Fig. 2a). Telomeric, pericentric and interstitial C-bands are only found on chromosomes 4, 9 and 13 (Fig. 2a,b) in *H. maoershanensis*, while C-bands have been detected on chromosomes 1–15, and even on telocentric chromosomes 20–23 in other 56-chromosome species. These observations suggest that *H. maoershanensis* has a different total amount of constitutive heterochromatin (C-positive regions) than other *Hynobius* species.

Ikebe et al. (1990) concluded that, in pond-type *Hynobius* species, the main evolutionary changes of chromosome no. 10 were decreases in the C-positive region and a transformation of the biarmed type of this chromosome to the uniarmed type by a pericentric inversion that involved the C-positive region. *H. maoershanensis* has a similar submetacentric chromosome no. 10 to the other five pond-type species described by Ikebe et al. (1990). However, there is no interstitial C-banding pattern on the short arm of chromosome no. 10 of *H. maoershanensis*, unlike other pond-type species. But two Chinese species (*H. yiwuensis* and *H. amjiensis*) possess a submetacentric chromosome 10 and have dark C-bands on the short arm like *H. leechii*, one of five pond species described by Ikebe et al. (1990, 1998). Relatively, the C-bands of chromosome in *H. maoershanensis* are distinctively different.

Significantly, the distinctive dark C-banding pattern on the whole short arm of chromosome 13 is unique in *H. maoershanensis*. Combined with its special

subtelocentric centromere, it can be considered as a marker to distinguish this species from the other species' karyotypes.

Silver-stained karyotype

Silver staining is a method of detecting NORs, and has been applied to seven *Hynobius* species from Japan, Korea and Taiwan (Iizuka & Kakegawa, 1989; Cha & Lee, 1995; Ikebe et al., 2000, 2005; Kuro-o et al., 2002). The Ag-NORs of species with 2n=56 chromosomes, treated by Howell & Blacks' (1980) method, are reported to be located mainly on the chromosomes of the small telocentric group: on chromosome 23 in *H. leechii*; on chromosomes 20 and 21 in *H. tokyoensis*; on chromosomes 21 and 23 in *H. quelpaertensis* and on chromsomes 20 and 21 in *H. tenuis*. The Ag-NORs of three species with 2n=58 chromosomes, detected by the method of Macgregor & Varley (1983), commonly occurred at the centromeric regions, i.e. on chromosomes 3, 6, 19 and 23 in *H. formosanus*, on some large-sized chromosomes in *H. kimurae* and on all chromosomes in *H. naevius*. Although the absence or presence of centromeric NORs may vary with different methods of staining, Ag-NORs have generally been reported on microchromosome pairs. Only in *H. formosanus* are the NORs observed on the long arm of a medium-sized pair of chromosomes (no. 10). *H. maoershanensis* was not particularly different from either the 56- or the 58-chromosome group, except for the location of the Ag-NORs, which are telocentrically situated on the large-sized submetacentric chromosome no. 8 (Fig. 3). A similar phenomenon was observed in the Anura (Li & Hu, 1995, 1996a,b; Dong et al., 2004). For example, the Ag-NORs observed in *Paa* species are generally found on the short arms of chromosome no. 6. In *P. shini* and *P. quadrana*, however, the Ag-NORs were found on the short arm of chromosomes 1 and 10 or 12 respectively. A reciprocal translocation involving the Ag-NORs was inferred to explain these various position of Ag-NORs by Li & Hu (1995). The differing locations of Ag-NORs between *H. maoershanensis* and other *Hynobius* species can probably be explained by a reciprocal translocation involving Ag-NORs. However, more evidence is needed. *H. maoershanensis* is the only species of Chinese *Hynobius* that has been studied using the Ag-NORs technique, and variation in the number and position of NORs remains unknown.

Karyotypes of Chinese *Hynobius* salamanders (Table 2)

Members of the genus *Hynobius*, most of which are endemic to Japan, can be divided into two groups according to their ecological features: the pond type, which inhabits the lowlands and spawns in small ponds, and the mountain-brook type that inhabits mountain regions and spawns primarily in cool streams (Sato, 1943). Most members of the former type have 2n=56 chromosomes (except for *H. retardatus* with 2n=40), while those of the latter have 2n=58 chromosomes (except for *H. okiensis* and *H. tsuensis* with 2n=56) (Seto & Matsui, 1984a; Seto et al., 1986, 1987; Iizuka & Kakegawa, 1989; Iizuka et al., 1989; Kohno et al., 1991; Nishikawa et al., 2005).

With the present data from *Hynobius maoershanensis*, the karyotypes of five species including six populations in mainland China have been investigated. From the chromosome number and breeding habits, these six populations can be viewed as the typical "pond-type" *Hynobius*. Just like some other "pond-type" species mentioned by Iizuka et al. (1989) from outside mainland China, they all have a similar karyotype of 2n=56, with the large-sized group of chromosomes 1–9.

Karyologically, similar *Hynobius* salamanders can also be partitioned into two main groups based primarily on the presence or absence of a medium-sized uniarmed chromosome, and secondarily by the number of microchromosomes that are telocentric (Matsui et al., 1985). All five Chinese species are characterized by the absence of a medium-sized uniarmed chromosome, and belong to "group II" of Matsui et al. (1985). For the second criterion, the Chinese populations can be divided into two clear subgroups: *H. amjiensis*, *H. yiwuensis* from Zhejiang, *H. maoershanensis* and *H. guabangshanensis* have ten telocentric microchromosome pairs, while *H. leechii* and a Fujian population of *H. yiwuensis* have only nine pairs. These two subgroups are not geographically separated. Of the species with nine pairs of telocentric microchromosomes, *H. leechii* is found in the northeastern part of mainland China, while Fujian populations of *H. yiwuensis* occur with species that have ten pairs in the central and eastern parts of China.

With additional sampling of Chinese salamanders, such as the rediscovery of *H. chinensis* from the type locality after 116 years (Wang et al., 2007), further cytogenetic studies at the population level are becoming increasingly important and urgent, because of the rapid destruction and deterioration of isolated habitats.

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