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Chromosomes of *Hynobius chinensis* Günther and *Hynobius amjiensis* Gu from China, and Comparison with Those of 19 Other *Hynobius* Species

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ABSTRACT—We report for the first time the chromosome numbers, karyotypes and C-banding patterns of *Hynobius chinensis* and *Hynobius amjiensis* from the middle-eastern region of mainland China. Both species had a diploid set of 56 chromosomes, with similar karyotypes. Among *Hynobius* species, the C-banding patterns of *H. chinensis* and *H. amjiensis* were the most similar to those of *H. leechii* from the northern region of South Korea. The chromosome number of *H. chinensis* and *H. amjiensis* was identical to that of 11 *Hynobius* species distributed throughout Korea and Japan. This identity indicates close phylogenetic relationships among the 13 species.

INTRODUCTION

Salamanders in the genus *Hynobius* are distributed throughout mainland China, Taiwan, Korea and Japan. Among 20 *Hynobius* species recognized in Taiwan, Korea and Japan, the chromosome numbers of 19 have been examined (Azumi and Sasaki, 1971; Ikebe and Kohno, 1979a, b; Morescalchi *et al*., 1979; Seto *et al*., 1983, 1986, 1987, 1988; Seto and Matsui, 1984a, b; Matsui *et al*., 1985; Ikebe *et al*., 1986, 1987; Kohno *et al*., 1987; Seto and Utsunomiya, 1987; Yamamoto *et al*., 1988; Iizuka *et al*., 1989). *Hynobius leechii*, *H. chinensis*, *H. amjiensis* and *H. mantchuricus* have been recognized in mainland China (Zhao and Adler, 1993). Among these four species, *H. leechii* is distributed across northeast China and Korea, and its karyotype and C- and R-banding patterns have been examined in Korean populations (Seto *et al*., 1986; Kohno *et al*., 1987; Seto and Iizuka, 1993). On the other hand, *H. chinensis*, *H. amjiensis* and *H. mantchuricus* have not been cytogenetically studied.

Of these three species, *H. chinensis* and *H. amjienesis* are found in the middle-eastern region of mainland China, and the history of taxonomic studies on these two species proceeded as follows. Günther (1889) described *H. chinensis* as a new species from specimens collected in Ichang, Hubei Province. Cai (1985) described *H. yiwuensis* as a new species, based on specimens collected in Dachen, Zhejiang Province. Adler and Zhao (1990) examined Günther's specimens, which are preserved in the British Museum, and relegated *H. yiwuensis* to the synonymy of *H. chinensis*. Gu (1991) described *H. amjiensis* as a new species from specimens collected in Mt. Longwang, Zhejiang Province. *Hynobius amjiensis* is endemic in Mt. Longwang at an altitude of 1300 m, whereas *H. chinensis* is found in the lowlands of the middle-eastern region of China.

In the present paper, we describe the chromosome numbers, karyotypes and C-banding patterns of *H. chinensis* and *H. amjiensis*. We compared these results with cytogenetic data from 19 other *Hynobius* species, and discuss their phylogenetic relationships.

MATERIALS AND METHODS

Hynobius chinensis and *H. amjiensis* were collected from Dinghai (Dinghai County) and Mt. Longwang (Amji County), respectively, in Zhejiang Province (Fig. 1).

The investigated materials are presented in Table 1. Chromosomes from embryos were prepared for Giemsa staining and C-banding as described by Kohno *et al*. (1987). Chromosomes were prepared from adult male intestines and testes and from female intestines. Chromosome preparations from intestines and testes were Giemsa stained and those from intestines were C-banded. Testicular chromosomes were prepared by the method of Imai *et al*. (1981) with some modifications. Details of the procedure have been described

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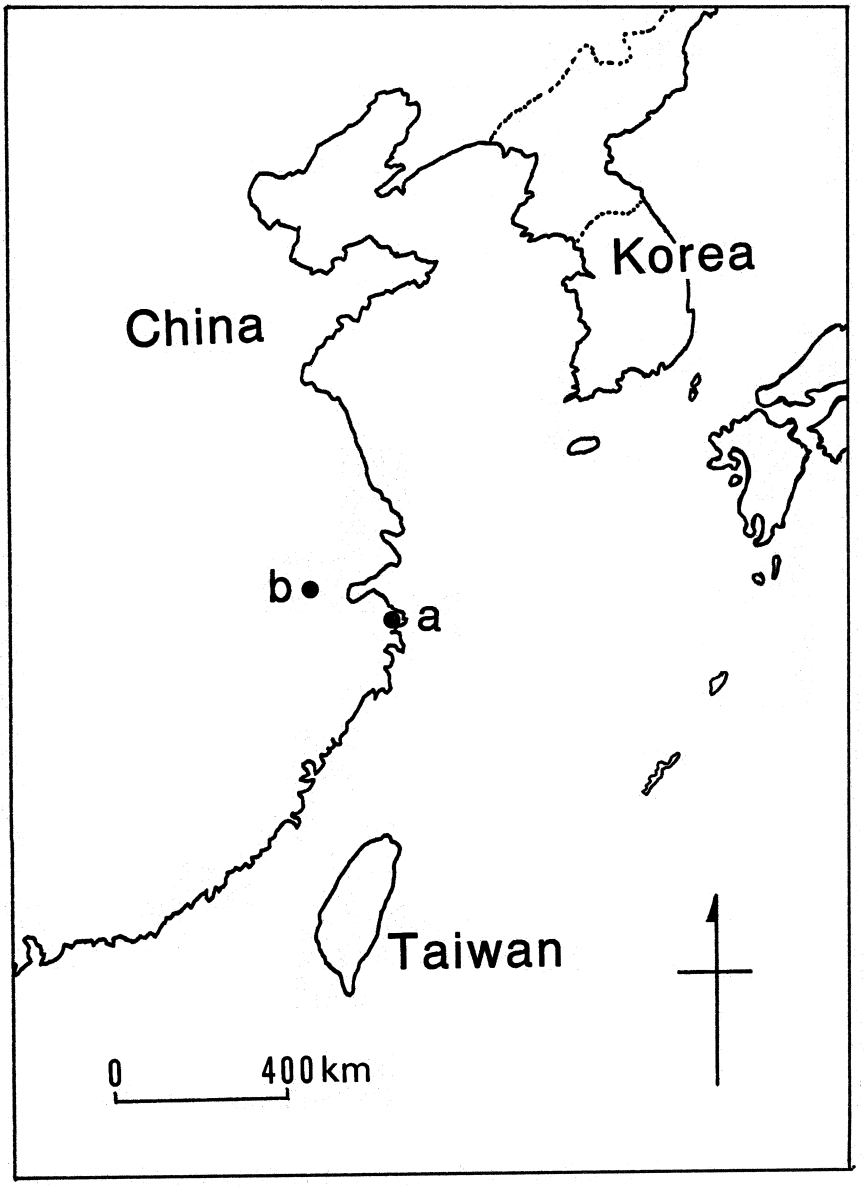

Fig. 1. Map of parts of mainland China and Japan, Korea and Taiwan. (a) Site of *Hynobius chinensis* collection (Dinghai). (b) Site of *Hynobius amjiensis* collection (Mt. Longwang).

Table 1. Number of adult specimens, egg sacs and embryos of *Hynobius chinensis* (*H. c.*) and *H. amjiensis* (*H. a.*) used in this study

Species	No. of males			No. of females			No. of egg sacs	No. of embryos		
	G*	C**	total	G*	C**	total		G*	C**	total
H. c.	1 (5)	1 (7)	1	1 (5)	1 (6)	1	2	8 (22) 3 (4)	7 (25) 1 (4)	8 3
H. a.	2 (10)	2 (10)	2	0	0	0	2	7 (19) 1 (3)	6 (20) 1 (1)	8 2

Numbers of metaphases in perentheses.
*: Giemsa staining.
**: C-banding.

by Kuro-o *et al*. (1998). Chromosomes were prepared from intestines as described by Kezer and Sessions (1979). Chromosomes were C-banded using the CBG technique described by Sumner (1972).

The centromere position in each chromosome was classified following the nomenclature of Levan *et al*. (1964).

RESULTS

A diploid set of 56 chromosomes was identified from 36 clear metaphases in *H. chinensis*. The diploid chromosome number of *H. amjiensis* was also determined as 56 from 32 clear metaphases. The relative length and the arm ratio of *H. chinensis* and *H. amjiensis* chromosomes were calculated from each of five metaphases from embryos (Table 2). We identified nine large (nos. 1-9), four medium (nos. 10-13) and 15 small pairs of chromosomes (nos. 14-28) in both species. The latter consisted of five biarmed (meta- or submetacentric) and 10 uniarmed (acrocentric) chromosome pairs.

The karyotypes of these two species were very similar, except for a near-terminal secondary constriction in the short arm of chromosome 10 in *H. amjiensis* (Fig. 2). This secondary constriction was observed in one chromosome 10 of eight embryos in one egg-sac, but was never found in that of embryos in other egg-sacs or male specimens of this species. Since we examined the chromosomes of only two *H. amjiensis* males, we have no information regarding sex chromosomes. We found no differences between karyotypes of male and female *H. chinensis*.

We analyzed 42 C-banded metaphases from *H. chinensis* and 31 C-banded metaphases from *H. amjiensis*. C-banding identified 15 of 28 chromosome pairs from both species (Fig. 3). Centromeric C-bands and multiple bands on the arms of chromosomes were evident. The near-terminal secondary constriction in the short arm of chromosome 10 of *H. amjiensis* was weakly stained by C-banding. Except for this secondary constriction, C-banding patterns between *H. chinensis* and *H. amjiensis* did not differ. We used the length of the terminal C-band of chromosome 2 as a feature for comparing C-banding patterns of *Hynobius* species. The terminal bands of chromosome 2 in *H. chinensis* and *H. amjiensis* were 12.45 ± 1.61% (N = 12) and 11.35 ± 1.00% (N = 12) in length, respectively. This value represents the length of the terminal band divided by the length of the chromosome from which the terminal band was subtracted.

Table 2. Characteristics of chromosomes from *Hynobius chinensis* and *H. amjiensis*

No.	*Hynobius chinensis*			*Hynobius amjiensis*		
	Relative length ± S.D.*	Arm ratio ± S.D.**	Centromere position***	Relative length ± S.D.*	Arm ratio ± S.D.**	Centromere position***
1	11.13 ± 0.57	1.21 ± 0.17	m	11.75 ± 1.20	1.26 ± 0.18	m
2	10.16 ± 0.47	1.68 ± 0.16	m or sm	10.78 ± 1.39	1.65 ± 0.13	m or sm
3	7.37 ± 0.47	1.78 ± 0.24	m or sm	7.00 ± 0.37	1.65 ± 0.15	m or sm
4	7.41 ± 0.43	3.35 ± 0.47	st	7.02 ± 0.60	3.47 ± 0.44	st
5	6.90 ± 0.54	2.28 ± 0.23	sm	7.00 ± 0.26	2.47 ± 0.42	sm
6	7.51 ± 0.59	1.24 ± 0.12	m	7.74 ± 0.33	1.30 ± 0.17	m
7	6.77 ± 0.63	1.41 ± 0.08	m	7.06 ± 0.56	1.24 ± 0.11	m
8	5.61 ± 0.55	1.93 ± 0.26	sm	5.86 ± 0.39	1.98 ± 0.18	sm
9	4.94 ± 0.41	1.21 ± 0.13	m	5.10 ± 0.43	1.29 ± 0.09	m
10	3.78 ± 0.32	2.20 ± 0.16	sm	3.99 ± 0.22#	2.11 ± 0.27#	sm
11	3.18 ± 0.22	2.82 ± 0.36	sm or st	3.29 ± 0.35	2.53 ± 0.19	sm
12	3.01 ± 0.22	2.49 ± 0.22	sm	2.91 ± 0.30	2.31 ± 0.18	sm
13	3.59 ± 0.29	1.13 ± 0.09	m	3.33 ± 0.22	1.17 ± 0.14	m
14	2.16 ± 0.19	1.37 ± 0.26	m	2.15 ± 0.23	1.55 ± 0.23	m or sm
15	2.04 ± 0.13	1.21 ± 0.17	m	1.86 ± 0.11	1.42 ± 0.34	m or sm
16	1.75 ± 0.15	1.21 ± 0.28	m	1.75 ± 0.15	1.35 ± 0.19	m
17	1.39 ± 0.14	1.04 ± 0.05	m	1.36 ± 0.17	1.09 ± 0.11	m
18	1.21 ± 0.24	1.09 ± 0.16	m	1.12 ± 0.17	1.08 ± 0.10	m
19	1.62 ± 0.08	–	a	1.50 ± 0.21	–	a
20	1.51 ± 0.08	–	a	1.39 ± 0.14	–	a
21	1.33 ± 0.18	–	a	1.22 ± 0.12	–	a
22	1.03 ± 0.19	–	a	1.00 ± 0.19	–	a
23	0.95 ± 0.16	–	a	0.91 ± 0.22	–	a
24	0.83 ± 0.17	–	a	0.68 ± 0.20	–	a
25	0.74 ± 0.14	–	a	0.67 ± 0.21	–	a
26	0.74 ± 0.12	–	a	0.58 ± 0.19	–	a
27	0.70 ± 0.12	–	a	0.50 ± 0.23	–	a
28	0.60 ± 0.13	–	a	0.47 ± 0.22	–	a

* Length of one chromosome × 100 / total length of chromosomes ± standard deviation.
** Length of long arm / length of short arm ± standard deviation.
*** According to Levan *et al*. (1964).
\# The near-terminal secondary constriction was not calculated.

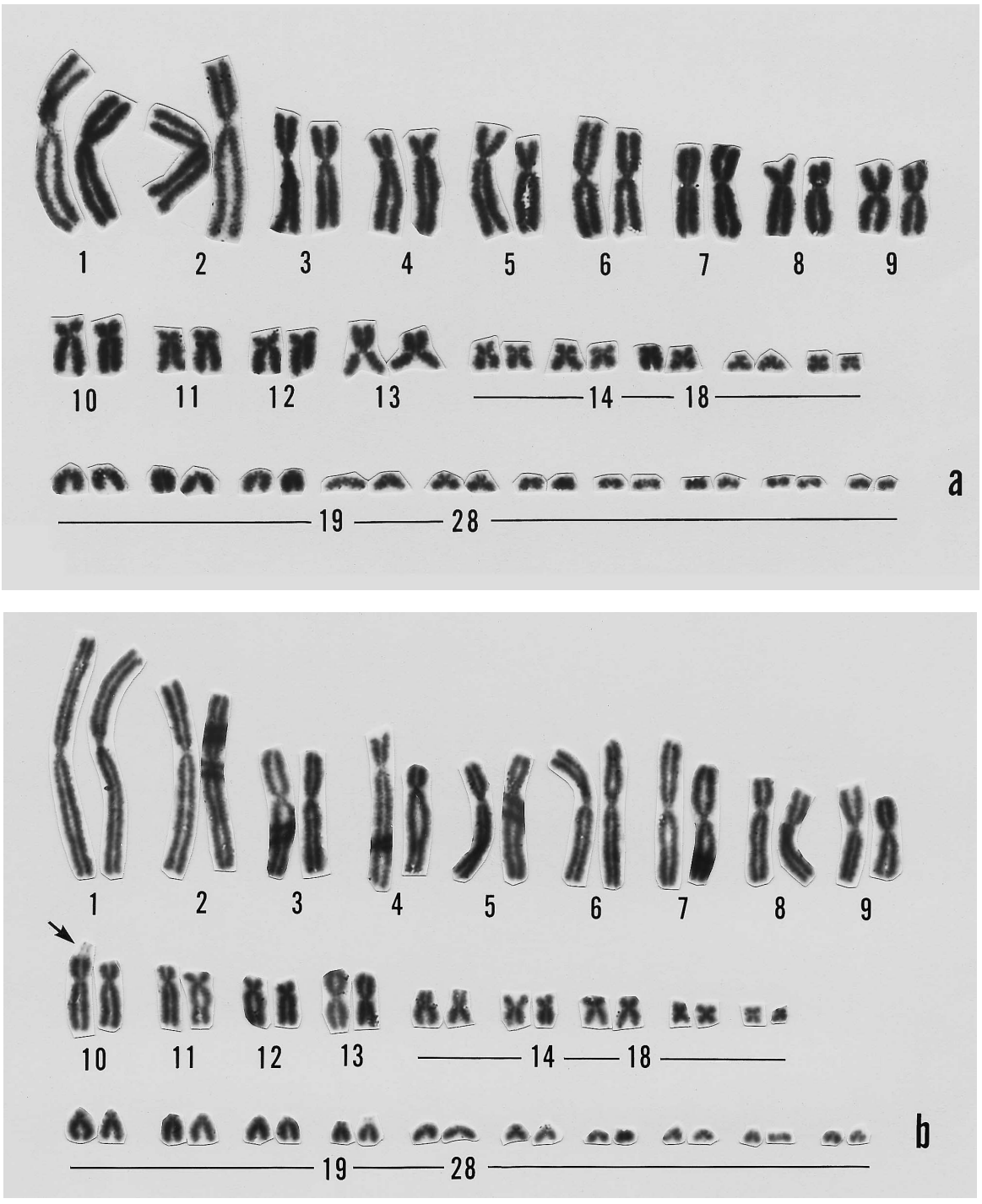

Fig. 2. (**a**) Karyotype of *Hynobius chinensis* from an embryo. (**b**) Karyotype of *Hynobius amjiensis* from an embryo. Arrow indicates near-terminal secondary constriction in chromosome 10.

DISCUSSION

We showed that both *H. chinensis* and *H. amjiensis* have a diploid set of 56 chromosomes, and that their Giemsa-stained and C-banded karyotypes are very similar. Recent chromosome analyses have identified sex-chromosomes (zz/zw type) in *H. tokyoensis*, *H. nebulosus*, *H. leechii* and *H. lichenatus* (Saso *et al*., 1995; Seto, 1995; Ikebe *et al*., 1996; Irie *et al*., 1996). However, we could not identify morphological or C-banding differences between male and female karyotypes of *H. chinensis*. The short arm of chromosome 10 had a secondary constriction in eight embryos from one egg-sac in *H. amjiensis*. We considered that this was due to autosomal heteromorphism, because only embryos from one egg-sac had

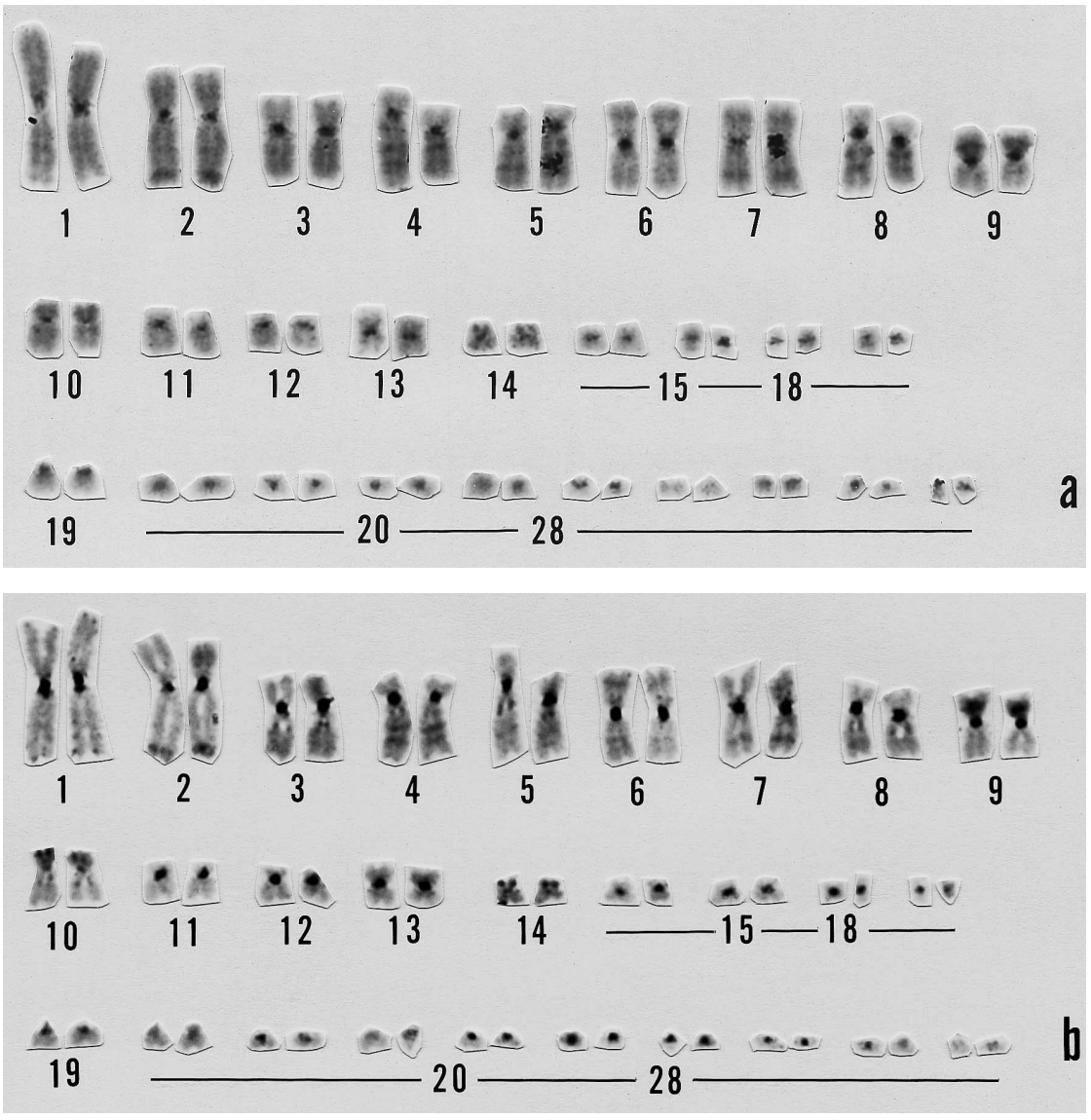

Fig. 3. (**a**) C-banded karyotype of *Hynobius chinensis* from an embryo. (**b**) C-banded karyotype of *Hynobius amjiensis* from an adult male.

this type of variation.

C- and/or R-banding analyses of 11 *Hynobius* species (*H. abei*, *H. dunni*, *H. kimurae*, *H. leechii*, *H. lichenatus*, *H. nebulosus*, *H. nigrescens*, *H. retardatus*, *H. takedai*, *H. tokyoensis*, and *H. tsuensis*: Kohno *et al*., 1983, 1987; Seto *et al*., 1986; Kuro-o *et al*., 1987; Ikebe *et al*., 1987, 1990; Izumisawa *et al*., 1990; Ikebe and Kohno 1991) have indicated inter- and intraspecific size variations in the terminal band of the long arm of chromosome 2 and in the short arm of chromosome 10. In the present study, we observed relatively large terminal bands in chromosome 2 in *H. chinensis* and *H. amjiensis* (12.45 ± 1.61% and 11.35 ± 1.00%, respectively) and these value are similar to those of *H. leechii* and *H. nebulosus* (12.29 ± 1.28% and 11.38 ± 1.35%, respectively: Izumisawa *et al*., 1990). The arm ratio of chromosome 10 (2.20 ± 0.16 in *H. chinensis* and 2.11 ± 0.27 in *H. amjiensis*) (Table 2) is the most similar to those of *H. leechii* collected from the norhern regions of South Korea (2.14 ± 0.21: Ikebe *et al*., 1990). Therefore, the C-banding patterns of *H. chinensis* and *H. amjiensis* are the most similar to those of *H. leechii* from the northern region of South Korea (Fig. 4).

Members of the genus *Hynobius* distributed in Japan, Korea and Taiwan can be divided into three groups according to chromosome number (Kohno *et al*., 1991). One of these groups has a diploid set of 56 chromosomes (11 species), one has 58 chromosomes (seven species), and one species has 40 chromosomes. According to molecular surveys of some members of *Hynobius* using repetitive DNA (Kuro-o *et al*., 1992) and protein electrophoresis (Matsui *et al*., 1992), these three groups have evidently differentiated from each other. Therefore, the two Chinese species with 56 chromosomes, *H. chinensis* and *H. amjiensis*, are probably closer relatives to the 11 species with the same number of chromosomes (*H. abei*, *H. dunni*, *H. hidamontanus*, *H. leechii*, *H. lichenatus*, *H.*

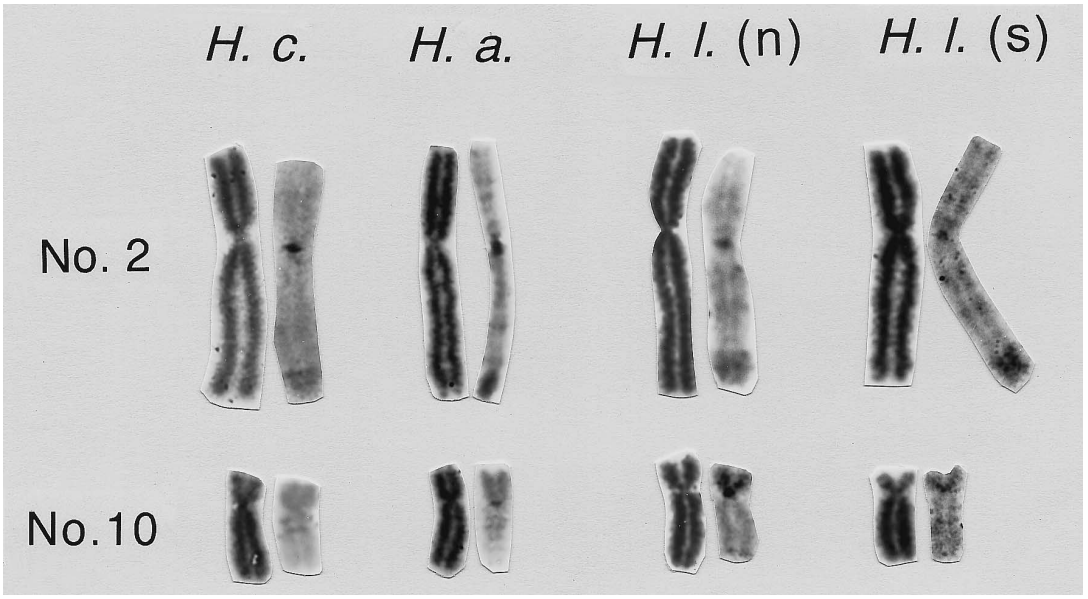

Fig. 4. Giemsa-stained and C-banded chromosomes 2 and 10 of *Hynobius chinensis* (*H. c.*), *H. amjiensis* (*H. a.*), *H. leechii* from Yangju (northern region of South Korea) (*H. l.* (n)) and *H. leechii* from Kyongju (southern region of South Korea) (*H. l.* (s)).

nebulosus, *H. nigrescens*, *H. okiensis*, *H. takedai*, *H. tokyoensis* and *H. tsuensis*) than other species with 58 or 40 chromosomes. According to ecological and morphological characteristics, Japanese, Korean and Taiwanese *Hynobius* species are divided into pond and mountain-brook types, and all species with 56 chromosomes except for *H. okiensis* belong to the pond type (lentic breeder) (Sato, 1943; Matsui and Miyazaki, 1984; Matsui, 1987). Since both *H. chinensis* and *H. amjiensis* are of this type, according to their lentic breeding habitat (Cai, 1985; Gu,1991), the ecological features of these species are also consistent with those of the ten species with 56 chromosomes.

Of the *Hynobius* species with 56 chromosomes, two species are distributed in the middle-eastern region of China, one in Korea and 10 are found in Japan. Seven species with 58 chromosomes are distributed in Taiwan (three species) and Japan (four species), and one species with 40 chromosomes is found in Hokkaido, Japan. The phylogenetic differentiation of the *Hynobius* species seems to be related to the geological history of the Asian continent, Taiwan and Japan. The phylogenetic relationships among *Hynobius* species should be investigated more precisely using various approaches.

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