

Unusual chromosome heteromorphism in the Tsinling tree frog, *Hyla tsinlingensis*

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Abstract. Chromosome set is one of the most important characteristics of a species. Despite the fact that amphibian karyotypes are usually very stable, some species are characterized by various variants of chromosomal polymorphism. We have studied the karyotype of *Hyla tsinlingensis* from the Tsinling Mountains (China) and have compared it with the karyotypes of other four species of the *H. chinensis* group. In general it was very close to other representatives of the group and genera as a whole ($2n = 24$; $NF = 44$). The Ag-staining detected nucleolar organizer region (NOR) on long arms of the ninth pair of chromosomes. DAPI- and Q-bandings detected dark regions on the same part of chromosomes. The study of two males and a juvenile allowed us to reveal very unusual chromosome heteromorphism on this part of chromosomes. One chromosome was significantly smaller than the second in all the studied individuals. In a juvenile, both large and small chromosomes bear NORs and were characterized by nearly the same centromeric index. In males, NORs were lacking on a small chromosome that had different centromeric index. Assuming that the juvenile individual is a female, such heteromorphism could be classified as a case of dimorphic sex chromosomes. The homologues differ in size, centromeric indexes and presence/absence of NORs. In amphibians, the similar case (but without NOR dimorphism) of dimorphic sex chromosomes was described only once (in *Engystomops petersi*).

Key words: *Hyla tsinlingensis*, karyotype, sex chromosomes, Hylidae, Anura, Amphibia, Shaanxi, China.

Introduction

Chromosome set is one of most important characteristics of species. For example, study of karyotypes can help identify cryptic species, allows tracing phylogenetic relationships and revealing history of dispersal (Anderson 1991, Borkin et al. 2004, Schmid et al. 2010). Despite the fact that amphibian karyotypes are usually very stable, some of species are characterized by chromosomal polymorphism (Mahony 1991, Lourenço et al. 1998, 1999, Schmid et al. 2010, Skorinov et al. 2018, 2019).

The genus *Hyla* consists of two groups of species (Li et al. 2015). The West Palearctic *H. arborea* group includes ten species (Dufresnes et al. 2018, 2019, Frost 2020). The East Palearctic *H. chinensis* group consists of seven species, including *H. annectans*, *H. chinensis*, *H. hallowellii*, *H. sanchiangensis*, *H. simplex*, *H. tsinlingensis* and *H. zhaopingensis* (Li et al. 2015, Frost 2020). According to genetic data (Li et al. 2015), the Tsinling tree frog (*H. tsinlingensis*) is close related to the Jerdon's tree frog (*H. annectans*). *H. tsinlingensis* was described from the Tsinling (= Qinling) Mountains in the vicinities of Hou-tseng-tze (= Houzhenzicun) village, Couchih Hsien (= Zhouzhi) District, Shensi (= Shaanxi) Province, China (Hu et al. 1966). The species is an endemic of China. The main range is in the Tsinling Mountains in the south of Gansu and Shaanxi provinces, as well as in the north of Chongqing and Sichuan provinces (Fig. 1). An isolated part of the range is located about 800 km to the east in the Dabie Mountains in the northwestern part of Anhui Province (Zhao & Xu 1990, Guo & Dong 1992, Fei et al. 2012). The divergence between these allopatric populations took place in the Pliocene (Zhang et al. 2016).

Karyotypes of species of the *H. chinensis* group were analyzed several times (Kuramoto et al. 1973, Li et al. 1981, 1991, Gao 1989, Wei & Xu 1989, Guo et al. 1987, Anderson 1991,

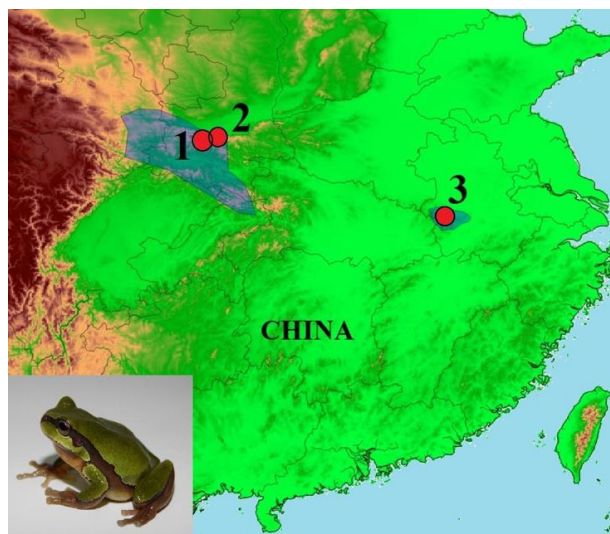


Figure 1. Male of *Hyla tsinlingensis* from Houzhenzicun (Zhouzhi, Shaanxi, China) and distributional range of *Hyla tsinlingensis* (purple areas; according to Li & Liang (2004) with corrections) with localities studied: 1. Houzhenzicun (our data); 2. Gujiapo (Wei & Xu 1989); 3. the Dabie Mountains (Guo & Dong 1992).

Guo & Dong 1992, Qian 2001, 2002, Yang 2003). Previously, karyotypes of *H. tsinlingensis* (Giemsa-, C- and Ag-bandings) were described two times for populations from the Tsinling and Dabie mountains (Wei & Xu 1989, Guo & Dong 1992). The aim of this paper was to first describe chromosome heteromorphism in the species and compare karyotypes of species of the *H. chinensis* group.

Material and Methods

We have studied 64 metaphase plates of three *H. tsinlingensis* indi-

Table 1. List of species of the *Hyla chinensis* group for which karyotypes were studied. ♂/♀/j is the number of males, females and juveniles (respectively).

Species	Locality	Coordinates	♂/♀/j	Reference
<i>Hyla tsinlingensis</i>	Houzhencun, Zhouzhi, Shaanxi, China	33.851°N 107.846°E	2/0/1	Present paper
<i>Hyla tsinlingensis</i>	Gujiapo, Lao Yu, Hu County, Shaanxi, China	~33.973°N 108.527°E	2/1/0	Wei & Xu (1989)
<i>Hyla tsinlingensis</i>	The Dabie Mountains, Anhui, China	~31.172°N 116.242°E	4/4/0	Guo & Dong (1992)
<i>Hyla annectans gongshanensis</i>	Dali, Yunnan, China	25.600°N 100.267°E	10/8/0	Qian (2002)
<i>Hyla annectans gongshanensis</i>	Kunming, Yunnan, China	~25.043°N 102.706°E	1/1/0	Li et al. (1981)
<i>Hyla chinensis</i>	Wenzhou County, Zhejiang, China	~28.000°N 120.700°E	17/9/0	Qian (2001)
<i>Hyla chinensis</i>	Fuzhou, Fujian, China	~26.043°N 119.409°E	3/7/0	Gao (1989)
<i>Hyla chinensis</i>	Huangshan, Anhui, China	~30.268°N 118.145°E	4/7/0	Yang (2003)
<i>Hyla sanchiangensis</i>	Huangshan, Anhui, China	~30.268°N 118.145°E	10/14/0	Guo et al. (1987)
<i>Hyla hallowellii</i>	Sumiyo, Oshima, Kagoshima, Japan	~28.295°N 129.437°E	10/0/0	Kuramoto et al. (1973)

viduals (two males and a juvenile) collected in 2014 from the type locality of the species in the Tsinling Mountains (Table 1). A day before tissue collection, specimens were injected with 0.5 ml of 0.4% colchicine solution. Each individual was anaesthetized by submersion in a 1% solution of 3-aminobenzoic acid ethyl ester (MS 222). After anesthesia, intestine and testes were taken. Before fixation, the tissue cell samples were kept in hypotonic 0.07 M KCl solution for 12 min. Then, tubes with samples were centrifuged at 755 g in hypotonical solution for 2 min. Supernatant liquid was poured out and samples were fixed in a mixture of methanol and glacial acetic acid (3:1). Fixative was changed three times each 30 min of incubation. For the production of metaphase plates, treated tissue fragments were kept in 60% acetic acid water solution, and then cell suspension was dropped on glass slides heated up to 60°C. The slides were Giemsa-, Ag-, DAPI/Distamycin A-, and Q-stained (Schmid 1978, Schmid et al. 1988).

Measurements of selected chromosome sets were performed on 13 metaphase plates obtained from all three individuals (9 and 1 plates from two males and 3 plates from a juvenile) using ImageJ (<https://imagej.nih.gov/ij/index.html>). We detected homologous chromosomes visually using similarities in their length, centromere position, Q-, DAPI/Distamycin A-, and Ag-stained regions. The relative length (RL) of a chromosome was calculated as a ratio of chromosome length to the total length of a haploid set of chromosomes (in percent). We have calculated the centromeric index (CI) as a ratio of length of short arm of chromosome to the total length of the chromosome. If CI was equal to 0.13 - 0.24, the chromosome was considered subtelocentric, if 0.25 - 0.37 as, submetacentric and if 0.38 - 0.50 as, metacentric (Green & Sessions 1991). We have used previously published data about relative lengths and centromeric indexes of chromosomes of *H. tsinlingensis* (Wei & Xu 1989) for ordering chromosomes of *H. tsinlingensis* from Houzhencun (type locality) and other species of the *H. chinensis* group (Table 1).

Yang (2003) described the karyotype of "*H. tsinlingensis*" from the vicinities of Huangshan City, Anhui, China. However, the species is absent in the region (Fei et al. 2012). Two other species of the group (*H. chinensis* and *H. sanchiangensis*) inhabit only the vicinities of Huangshan City. According to nucleolar organizer region (NOR) position on the chromosomes (10th or 11th pairs), the population should be assigned to *H. chinensis*. In addition, the data on chromosome measurements that were provided by the author were too rounded, and, therefore, we did not use them in our analyses of measurement data.

Additionally, the population of tree frogs from Dali City (Yunnan, China) was assigned by Qian (2002) to *H. chinensis* Günther, 1858. However, the species do not inhabit this region (Fei and Ye, 2016). Among species of the group, only *H. annectans* (= *H. gongshanensis*) is distributed here. Therefore, we guess that the karyotype described by Qian (2002) belongs to the last species (Table 1).

Besides, Li et al. (1991) have studied the chromosomes of tree frogs from Shitai County (Anhui, China). The authors attributed them to *H. chinensis*. However, the position (5th pair) of NORs does

not allow us to assign this population to the *H. chinensis* group, because all species of the group bear NORs on 9-10th pairs of chromosomes. Perhaps, the population belongs to other tree frog species, *Dryophytes immaculatus*, which looks quite similar and also inhabits the region (Borzée et al. 2018).

Results

The diploid karyotype of *H. tsinlingensis* consists of 24 biarmed chromosomes ($2n=24$; $NF=48$; Figs. 2-3). Six chromosomes were large (pairs 1-6; $RL > 8.1\%$; Table 2) and six were small (pairs 7-12; $RL < 6.2\%$). Two pairs of chromosomes (4th and 6th) were subtelocentric, three pairs were submetacentric (3rd, 5th and 7th), and six (1st, 2nd, 8th, 10th, 11th, and 12th) were metacentric (Table 3). The 9th pair of chromosomes was heteromorphic in all three individuals studied. The average relative length of the large chromosome (X1) from the 9th pair was 5.7% and small (presumed X2 in a juvenile and Y in males) was 4.3%. The mean differences between X1 and X2 (and/or Y) were equal 24.7%. The X1 chromosome was submetacentric ($CI=0.35$), and the X2 and Y chromosomes were metacentric ($CI=0.38$ and 0.45 respectively). The DAPI/Distamycin A- and Q-stained presumed X chromosomes (X1 and X2) had a darkened area in an interstitial region of long arms, and the region was positively Ag-stained (Fig. 4). Y chromosome was DAPI/Distamycin A-, Q- and Ag- negatively stained (Fig. 4). The chromosome formula was $2n=13m+7sm+4st$. The DAPI/Distamycin A- and Q- stainings of all other chromosomes did not revealed any banding patterns.

Discussion

Comparison of karyotypes in the *Hyla chinensis* group

Our study showed that *H. tsinlingensis* from Houzhencun (the Tsinling Mountains) were characterized by the same number of chromosomes ($2n=24$) as conspecific populations of the species and other species of the genus *Hyla* (Kuramoto et al. 1973, Anderson 1991, Guo & Dong 1992, Qian 2001). Six large and six small pairs of chromosomes were found in all populations of *H. tsinlingensis*, as well as in *H. chinensis*, *H. sanchiangensis*, and the population of *H. annectans* from Dali. However, five large and seven small pairs of chromosomes were found in *H. annectans* from Kunming and *H. hallowellii* (Table 2).

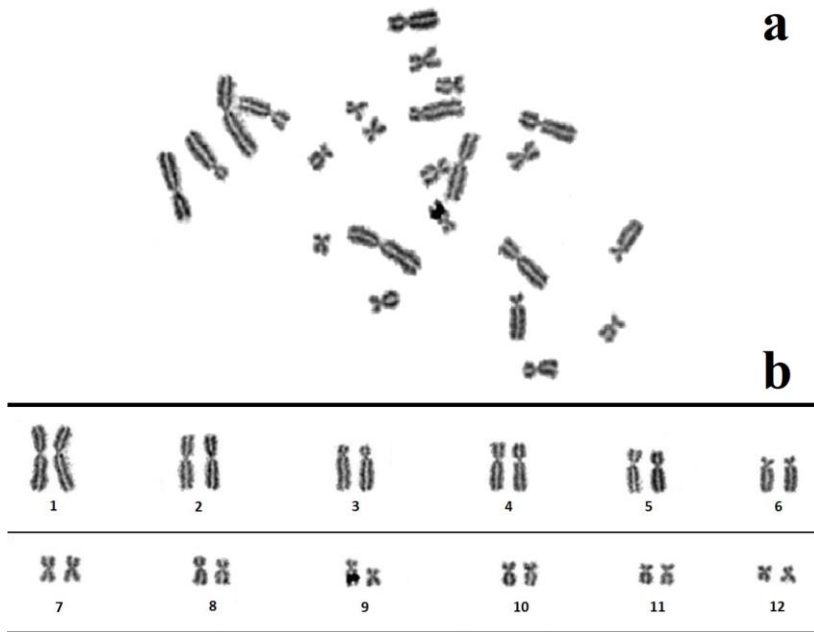


Figure 2. Diploid chromosome set of a male of *Hyla tsinlingensis* from Houzhenzicun (Zhouzhi, Shaanxi, China; Ag-stained). a - metaphase plate; b - karyotype. Chromosomes are arranged according to *H. tsinlingensis* from Gujiapo, Shaanxi, China (Wei & Xu 1989).

Table 2. Relative length of chromosomes (mean ± SD; in percents) in the *Hyla chinensis* group. CP is the number of chromosome pair. Chromosomes are arranged according to *H. tsinlingensis* from Gujiapo, Shaanxi, China (Wei & Xu 1989). *On the picture of karyotype in the paper of Qian (2001), the 9th pair of chromosomes was dimorphic, but measurements were given only for a chromosome (or in average).

CP	<i>tsinlingensis</i> (Houzhenzicun)	<i>tsinlingensis</i> (Gujiapo)	<i>tsinlingensis</i> (Dabie)	<i>annectans</i> (Dali)	<i>annectans</i> (Kunming)	<i>chinensis</i> (Wenzhou)	<i>chinensis</i> (Fuzhou)	<i>chinensis</i> (Huangshan)	<i>sanchiangensis</i> (Huangshan)	<i>hallowellii</i> (Sumiyo)
1	14.70±1.55	16.08±0.57	20.85±1.01	15.53±0.87	15.53	15.95±0.98	16.52±0.73	12.10±0.18	16.39±0.37	15.74±0.24
2	13.33±0.35	13.05±0.86	16.11±0.46	12.81±0.55	12.52	12.60±1.41	12.84±0.65	16.50±0.34	13.11±0.24	13.31±0.33
3	10.85±0.59	11.33±0.82	13.43±0.50	11.38±0.46	11.15	10.93±0.81	11.29±0.38	9.90±0.28	11.23±0.10	11.59±0.21
4	10.89±0.84	11.02±0.67	14.85±0.63	10.44±0.41	10.69	10.49±0.49	10.59±0.43	11.00±0.21	10.72±0.16	10.98±0.22
5	9.87±0.14	9.43±0.67	11.85±1.01	9.19±0.71	9.73	9.78±0.55	9.89±0.45	9.90±0.17	9.94±0.29	10.29±0.13
6	8.08±0.16	7.74±0.46	10.11±0.46	7.91±0.68	7.91	8.27±0.48	8.26±0.27	9.90±0.12	8.09±0.12	8.09±0.10
7	5.97±0.36	6.07±0.53	6.48±0.38	6.22±0.32	6.11	6.45±0.45	6.29±0.28	6.60±0.31	5.73±0.16	6.70±0.16
8	6.24±0.22	5.75±0.52	6.95±0.71	6.20±0.24	6.72	6.05±0.33	5.89±0.21	6.60±0.22	6.49±0.11	5.99±0.18
9X1	5.67±0.34	5.49±0.35	4.79±0.35	5.76±0.35	5.69	5.60±0.59*	5.04±0.24	4.40±0.19	5.46±0.31	5.17±0.12
9X2	4.26±0.05									
9Y	4.31±0.71									
10	5.37±0.28	5.13±0.52	6.16±0.31	5.34±0.42	5.18	5.43±0.41	5.38±0.29	4.40±0.23	5.06±0.11	4.54±0.14
11	4.60±0.37	4.42±0.62	3.11±0.27	5.04±0.40	4.79	4.70±0.32	4.45±0.33	3.30±0.34	4.23±0.14	4.17±0.18
12	4.43±0.63	3.42±0.55	4.74±0.30	4.15±0.43	3.97	3.79±0.55	3.55±0.35	5.50±0.40	3.42±0.10	3.35±0.17

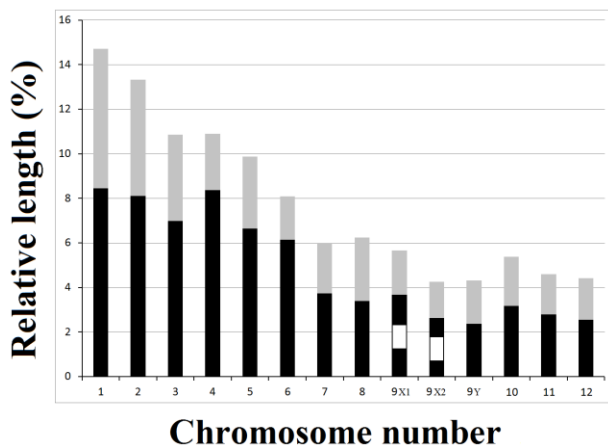


Figure 3. Idiogram of karyotype of *H. tsinlingensis*. Grey stands for short arms of chromosomes and black for long arms. White rectangle marks show position of nucleolar organizer regions (NORs).

Study of centromeric indexes revealed several regularities for the species of the *H. chinensis* group (Table 3). In all the studied species, 4th and 6th pairs of chromosomes (with the exception of *H. annectans* from Dali) were subtelocentric or submetacentric with average CI ranging from 0.16 to 0.28. The 5th pair of chromosomes was submetacentric in all the studied species (mean CI = 0.31-0.37). Four pairs (1st, 2nd, 10th, and 12th) were always metacentric (with the exception of *H. tsinlingensis* from Huangshan). Subtelocentrics were absent in the chromosome sets of *H. chinensis* from Wenzhou and both populations of *H. annectans*. Only several pairs of chromosomes in these populations were submetacentric (all other chromosomes were metacentric). Only *H. tsinlingensis* from Houzhenzicun and the Dabie Mountains, *H. chinensis* from Huangshan, and *H. hallowellii* were characterized by half or less than half of metacentric chromosomes.

It is important to note that not all pairs of homeological chromosomes from various species of the group can be cor-

Table 3. Centromeric indexes of chromosomes (mean \pm SD) in the *Hyla chinensis* group. m is metacentric chromosomes; sm is submetacentric; st is subtelocentric. CP is number of chromosome pair. *On the picture of karyotype in the paper of Qian (2001), the 9th pair of chromosomes was dimorphic, but measurements were given only for a chromosome (or in average).

CP	<i>tsinlingensis</i> (Houzhencicun)	<i>tsinlingensis</i> (Gujiapo)	<i>tsinlingensis</i> (Dabie)	<i>annectans</i> (Dali)	<i>annectans</i> (Kunming)	<i>chinensis</i> (Wenzhou)	<i>chinensis</i> (Fuzhou)	<i>chinensis</i> (Huangshan)	<i>sanchiangensis</i> (Huangshan)	<i>hallowellii</i> (Sumiyo)
1	0.42 \pm 0.00 (m)	0.41 (m)	0.43 (m)	0.44 (m)	0.43 (m)	0.43 (m)	0.42 (m)	0.45 (m)	0.42 (m)	0.40 (m)
2	0.39 \pm 0.01 (m)	0.40 (m)	0.39 (m)	0.41 (m)	0.42 (m)	0.42 (m)	0.43 (m)	0.40 (m)	0.38 (m)	0.40 (m)
3	0.36 \pm 0.01 (sm)	0.34 (sm)	0.37 (sm)	0.41 (m)	0.42 (m)	0.38 (m)	0.37 (sm)	0.33 (sm)	0.33 (sm)	0.36 (sm)
4	0.23 \pm 0.02 (st)	0.23 (st)	0.19 (st)	0.28 (sm)	0.28 (sm)	0.25 (sm)	0.22 (st)	0.20 (st)	0.26 (sm)	0.22 (st)
5	0.33 \pm 0.01 (sm)	0.34 (sm)	0.31 (sm)	0.37 (sm)	0.34 (sm)	0.35 (sm)	0.34 (sm)	0.33(sm)	0.35 (sm)	0.32 (sm)
6	0.24 \pm 0.00 (st)	0.22 (st)	0.16 (st)	0.32 (sm)	0.27 (sm)	0.25 (sm)	0.23 (st)	0.22 (st)	0.23 (st)	0.21 (st)
7	0.37 \pm 0.01 (sm)	0.38 (m)	0.29 (sm)	0.42 (m)	0.43 (m)	0.38 (m)	0.39 (m)	0.25 (sm)	0.38 (m)	0.33 (sm)
8	0.46 \pm 0.01 (m)	0.43 (m)	0.36 (sm)	0.45 (m)	0.43 (m)	0.44 (m)	0.47 (m)	0.50 (m)	0.42 (m)	0.44 (m)
9X1	0.35 \pm 0.02 (sm)	0.33 (sm)	0.27 (sm)	0.39 (m)	0.44 (m)	0.34 (sm)*	0.34 (sm)	0.25 (sm)	0.34 (sm)	0.36 (sm)
9X2	0.38 \pm 0.02 (m)									
9Y	0.45 \pm 0.02 (m)									
10	0.41 \pm 0.02 (m)	0.40 (m)	0.46 (m)	0.42 (m)	0.41 (m)	0.40 (m)	0.39 (m)	0.37 (sm)	0.38 (m)	0.41 (m)
11	0.39 \pm 0.03 (m)	0.43 (m)	0.39 (m)	0.42 (m)	0.43 (m)	0.39 (m)	0.41 (m)	0.33 (sm)	0.41 (m)	0.37 (sm)
12	0.42 \pm 0.01 (m)	0.45 (m)	0.43 (m)	0.48 (m)	0.49 (m)	0.44 (m)	0.46 (m)	0.40 (m)	0.43 (m)	0.43 (m)

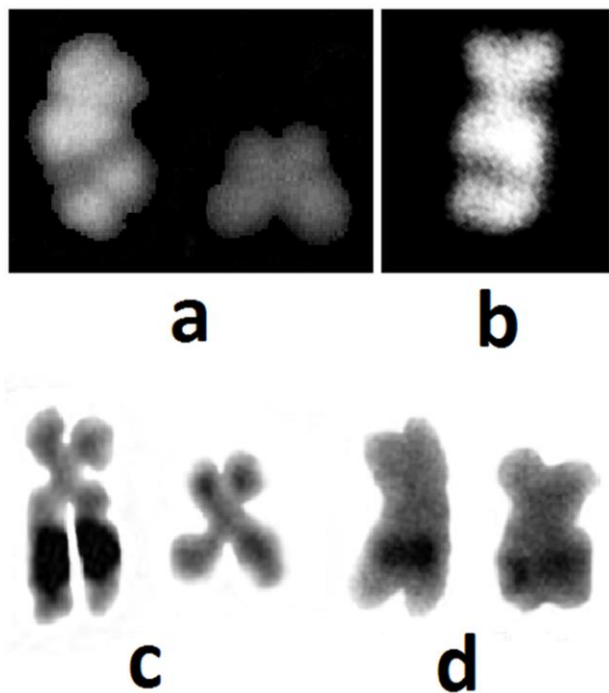


Figure 4. The 9th pair of chromosomes of *Hyla tsinlingensis*. DAPI/Distamycin A-stained presumed X1 and Y chromosomes (a); Q-stained X1 chromosome (b); Ag-stained X1 and Y chromosomes in a male (c); Ag-stained X1 and X2 chromosomes of the juvenile individual (d).

rectly determined only by using morphology. Small pairs of chromosomes of all studied species had quite similar relative length and centromeric indexes (Fig. 5). However, among them, the 9th pair of chromosomes could be easily identified by presence of NORs.

In *H. tsinlingensis* from Houzhencicun, which was studied by us, NORs were found in an interstitial region of the long arms of the 9th pair of chromosomes. NORs were in the same position in the Gujiapo's species population (Wei & Xu 1989), while in the Dabie Mountains species population, NORs were revealed in distal position on long arms of the 10th pair of chromosomes (Guo & Dong 1992). This can point

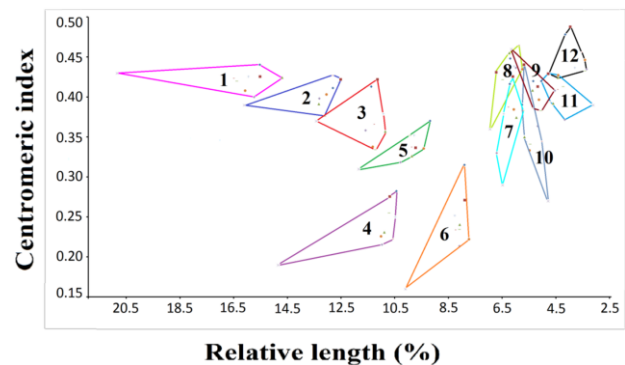


Figure 5. Relationship between relative length and centromeric indexes of chromosomes of the *Hyla chinensis* group. Various populations are designated by points with different color. Numbers indicate chromosome pairs (the 9th pair of *H. tsinlingensis* from Houzhencicun is represented by the largest X1 chromosome only).

out that allopatric populations from the Tsinling and Dabie mountains are genetically very different, which was also confirmed by molecular (mitochondrial DNA) data (Zhang et al. 2016).

Various authors found out that NORs are located on different pairs of chromosomes in species of the *H. chinensis* group. So Anderson (1991) noted that *H. chinensis* and *H. hallowellii* bear NORs in an interstitial region on long arms of the 10th pair of chromosomes (Table 4). In *H. sanchiangensis* NORs were found in an interstitial region on long arms of the 9th or 10th pairs of chromosomes (Guo et al. 1987, Guo & Dong 1992). According to Yang (2003), NORs are on the 11th pair of chromosomes of *H. chinensis* from Huangshan. However, according to our estimations, the 10th and 11th pairs of chromosomes in this population have no reliable differences. Perhaps, local tree frogs (as well as *H. chinensis* from Fuzhou) actually bear NORs on the 10th pair of chromosomes. Thus, we can conclude that *H. tsinlingensis* from the Tsinling Mountains, *H. annectans*, *H. sanchiangensis*, and *H. chinensis* from Wenzhou have NORs on the 9th pair of chromosomes. However, *H. hallowellii*, *H. tsinlingensis* from the Dabie Mountains and *H. chinensis* from Huangshan and Fuzhou bear NORs on the 10th pair of chromosomes (Li et al. 1981,

Table 4. Variation of karyological characters in species of the *Hyla chinensis* group.

Characters	<i>tsinlingensis</i> (Houzhencun)	<i>tsinlingensis</i> (Gujiapo)	<i>tsinlingensis</i> (Dabie Mountain)	<i>annectans</i> (Dali)	<i>annectans</i> (Kunming)
Number of large pairs of chromosomes	6	6	6	6	5
Position of NORs	Long arm of the 9 th pair, medially	Long arm of the 9 th pair, medially	Long arm of the 10 th pair, distally	Long arm of the 9 th pair	Long arm of the 9 th pair, medially
Number of metacentric pairs	6	7	5	9	9
Heteromorphic chromosomes	the 9 th pair	no	no	no	no

Characters	<i>chinensis</i> (Wenzhou)	<i>chinensis</i> (Fuzhou)	<i>chinensis</i> (Huangshan)	<i>sanchiangensis</i> (Huangshan)	<i>hallowellii</i> (Sumiyu)
Number of large pairs of chromosomes	6	6	6	6	5
Position of NORs	Long arm of the 9 th pair	Long arm of the 10 th pair	Long arm of the 11 th pair, medially	Long arm of the 9 th pair, medially	Long arm of the 10 th pair
Number of metacentric pairs	8	7	4	7	5
Heteromorphic chromosomes	no	no	no	no	no

Guo & Dong 1992, Qian 2002, Yang 2003). Moreover, we guess that NOR-bearing chromosomes in all species of the group are located on homeologous chromosomes. Therefore, in our Tables (2-3) we attributed them to the 9th pair (like in *H. tsinlingensis* from Houzhencun).

Chromosome heteromorphism of *Hyla tsinlingensis*

Chromosome heteromorphism can be related and not related to sex. In amphibians, cases of the not related to sex heteromorphism have been previously described two times. For example, in all species of the genus *Triturus* (Caudata) the 1th pair of chromosomes is heteromorphic (C-banding and lampbrush chromosomes) in both sexes (Macgregor & Horner 1980). In addition, the non-sex heteromorphism is known for the 13th pair of homologous chromosomes in *Discoglossus pictus* (Anura), identified by *in situ* hybridization (Amor et al. 2009). The 1st chromosome heteromorphism in newts of the genus *Triturus* is associated with the embryonic development violation of the individuals with homomorphic chromosomes, then the meaning of the 13th pair chromosome heteromorphism in *D. pictus* remains unclear.

In our case, assuming that the juvenile individual of *H. tsinlingensis* from Houzhencun is a female, the heteromorphism of the 9th pair of chromosomes could be classified as a case of dimorphic sex chromosomes. The homologues differ in size, centromeric indexes and presence/absence of NORs. In previous studies of *H. tsinlingensis*, the heteromorphism of chromosomes was not revealed (Wei & Xu 1989, Guo & Dong 1992). The presumed XY sex inheritance system in *H. tsinlingensis* is in a good agreement with data obtained from other species of the genus *Hyla* (Dufresnes et al. 2015).

In amphibians, the similar case of dimorphic sex chromosomes was described only once. Lourenço et al. (1999) have found two types of X chromosomes differing by relative length and centromeric indexes in the Peters' dwarf frog (*Engystomops petersi*) from Brazil. The single difference is that, in our case, heteromorphism was also associated with the presence/absence of NORs.

Sex chromosome heteromorphism is very uncommon among amphibians. At present, 7174 species of Anura are known (Frost 2020), but heteromorphic sex chromosomes are described in no more than 1.5% of them (about 100 species; Skorinov et al. 2019). Usually, heteromorphic sex chromosomes can be detected by size differences, centromeres and NORs location, as well as staining with various techniques. In the comprehensive review (Schmid et al. 2010), from all anurans, only ten species have NORs on sex chromosomes. Only three of them showed dimorphism associated with absence of NORs on one of sex chromosomes (W or Y). Additional cases in anurans heteromorphic sex chromosomes that are bearing NORs were noted in several other studies (Yosida 1957, Schmid 1978, Mahony 1991, Al-Shehri & Al-Saleh 2005, Skorinov et al. 2018, 2019). Further studies are needed to determine whether the discovered heteromorphism of the 9th pair of chromosomes in *H. tsinlingensis* is sex-related.

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