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Species and sex comparisons of karyotype and genome size in two *Kurixalus* tree frogs (Anura, Rhacophoridae)

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Abstract. *Kurixalus* is a rhacophorid genus of tree frogs that are similar in morphology but vary in reproductive behavior. We investigated the cytogenetic features and genome size using conventional G-banding, C-banding and silver-staining techniques, fluorescence in situ hybridization (FISH), and flow cytometry in two representatives of *Kurixalus* (*K. eiffingeri* Boettger, 1895 and *K. idiootocus* Kuramoto and Wang, 1987) and compared the data between species and sex. The two *Kurixalus* species share a diploid chromosome number 2n = 26 and fundamental number FN = 52. Prominent differences between species were noted in the distribution of secondary constriction (SC)/nucleolus organizer region (NOR) and dense heterochromatin. Other interspecies differences including variations in the number of metacentric and submetacentric chromosomes and staining intensity of heterochromatin were also found. The cytogenetic results are consistent with the observed differences in their genome sizes. FISH with telomeric motif (TTAGGG)n for both species detected signals in the terminal regions. Intersex comparisons revealed no differences in terms of cytogenetic features and genome size in the two species. Despite the apparent highly conserved diploid chromosome number, data on the karyotype microstructure characterize the cytogenetic profile of the two *Kurixalus* species that contribute to clarification of the chromosomal homologies and the rearrangement mechanisms occurring during the karyotype evolution of *Kurixalus*. No heteromorphic chromosome pair in both species is consistent with the view that homomorphic sex chromosome is common in amphibians.

Keywords. *Kurixalus eiffingeri, K. idiootocus,* karyotype, homomorphic chromosome, sex determination.

INTRODUCTION

Kurixalus is a rhacophorid genus of small tree frogs that occurs across East to Southeast Asia, including the Ryukyu Islands, Taiwan, China, India, Myanmar, Cambodia, Vietnam, Thailand, Malays, Sumatra, Borneo and the Philippines (Frost, 2017). Phylogenetic data support the

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monophyletic origin of *Kurixalus* despite its broad distribution (Li et al., 2013; Biju et al., 2016; Jiang et al., 2016). However, since these frogs are similar in morphology and overlap in body size, taxonomy is difficult and mainly based on molecular analyses (Wilkinson et al., 2002; Li et al., 2008; Li et al., 2009; Hertwig et al., 2013; Yu et al., 2013; Nguyen et al., 2014a; Nguyen et al., 2014b; Wu et al., 2016). Currently, 14 species are recognized in *Kurixalus* (Frost, 2017).

Despite the taxonomy of *Kurixalus* species continues to update, considerable differences in the reproductive behavior are noted among some known species. For example, *K. eiffingeri* Boettger, 1895, a species found in Taiwan and two southern islands of Japan (Maeda and Matsui, 1989), breeds in water-filled tree holes and bamboo stumps, and displays a complex parental care including paternal care for eggs and subsequent maternal care for larvae. The tadpoles are oophagous, feeding exclusively on unfertilized eggs laid by the female frogs which return to nests (Kam et al., 1997; Kam et al., 2001). On the contrary, *K. idiootocus* Kuramoto and Wang, 1987, a species endemic to Taiwan, breeds in soils and crevices that are frequently covered with dead leaves. Eggs hatch when heavy rainfall occurs and the hollow fills up, and the tadpoles get washed into nearby waterways or pools. The tadpoles are herbivorous, subsisting on algae and plants. While much attention has been put on subjects of the reproductive behavior in *Kurixalus* species (Lin and Kam, 2008; Cheng WC et al., 2013; Tung et al., 2015), little information is available on their genetic complements in terms of species difference and sex differentiation. It is thus interesting to investigate the cytogenetic and genomic features in members of this genus.

Variations in chromosome number and structure among species and/or populations can be used to estimate phylogenetic relationships (Yates et al., 1979) and has been applied by various kinds of cytogenetic techniques for classifying species with similar morphology (Medeiros et al., 2003; Rosa et al., 2003; Veiga-Menoncello et al., 2003; Carvalho et al., 2009). Cytogenetic analyses can also reveal sex determination of targeted species. If heteromorphic sex chromosomes are present in species, genetic sex determination is inferred (Hanada, 2002). Even though heteromorphic chromosome pairs are not always recognized in species, different chromosome staining techniques, such as G-banding, C-banding and Ag-NOR staining are still widely accepted in detecting cryptic structural alterations of chromosomes (Hanada, 2002; Wu et al., 2007). Additionally, sophisticated painting techniques, such as fluorescence *in situ* hybridization (FISH) and comparative genomic hybridization (CGH), are also useful tools in the studies of comparative cytogenetics. This can be instanced by the use of FISH with a highly conserved vertebrate telomeric motif (TTAGGG)n to identify chromosome rearrangements (e.g., fusion, fission and translocation events) if the interstitial telomeric sequences (ITSs) were detected. The important role of the telomeric sequences in karyotypic diversity, chromosome evolution, and chromosome instability has been highlighted (Bruschi et al., 2014). The CGH, a FISH-based method using total DNA from two taxa as probes, can differentiate chromosomes from different genomes and has been used in studies of genome-wide comparison (Yu et al., 2012).

Karyotypes in the genus *Kurixalus* are poorly understood. Available cytogenetic data are restricted to the common features, such as diploid chromosome number, in two species (Kuramoto, 1977; Kuramoto and Wang, 1987). In this study, with the aim of providing cytogenetic information of *Kurixalus*, the karyotypes of two representatives of *Kurixalus* (*K. eiffingeri* Boettger, 1895 and *K. idiootocus* Kuramoto and Wang, 1987) were characterized using G-banding, C-banding, and Ag-NOR staining. Because karyotypic variations may involve rearrangements that are not detected by conventional karyotyping, we also included here the telomeric FISH and CGH analyses in the karyotypes of both species. Moreover, the genomic sizes were also examined to yield a natural link to the cytogenetic data that provide a genetic basis for comparative studies of interspecies as well as intersex differentiation in *Kurixalus*.

MATERIALS AND METHODS

Sampling

A total of 40 specimens of *Kurixalus eiffingeri* (male: n = 10, female: n = 10) and *K. idiootocus* (male: n = 10, female: n = 10) were collected from Xitou (elevation 1,092 m a.s.l.; 23°40'35.2"N, 120°47'46"E) and Yuchi (elevation 634 m; 23°53'47"N, 120°54'13"E), respectively, in Nantou County, Taiwan (Fig. 1). Both of the two *Kurixalus* species examined have been taxonomically identified, in addition to phenotypic classification, by phylogenetic analysis using the mitochondrial DNA cytochrome c oxidase subunit 1 (CO1) and the 16S rDNA sequencing (Wu et al., 2016). The reference CO1 and 16S rDNA sequences have been uploaded to GenBank with accession numbers of DQ468678 and DQ468670 for *K. eiffingeri*, and DQ468682 and DQ468674 for *K. idiootocus*. Tissues of gonad (from both sexes) and kidney were obtained and cultured according to Chinchar and Sinclair (1978).

Cytogenetic analyses

For chromosome preparation, mitotic metaphase arrest was induced by adding 0.1 μg/ml colcemid 4 h before cell harvest-

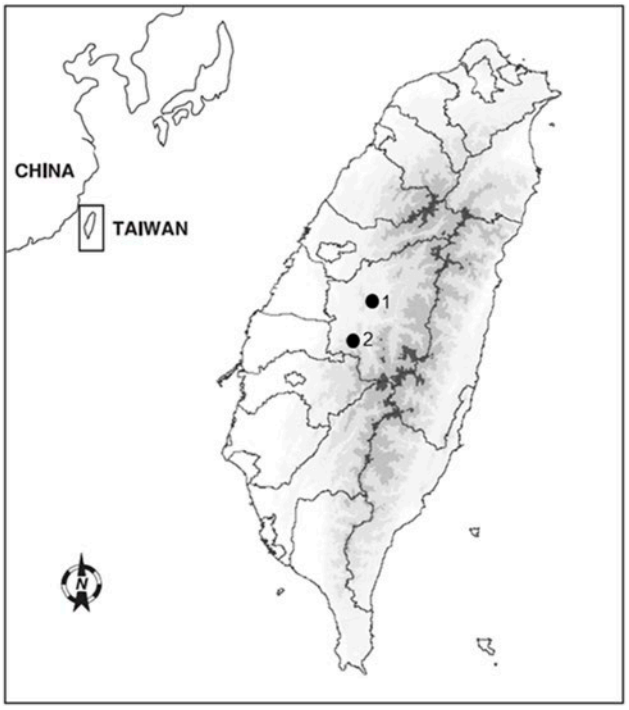

Fig. 1. Map showing sampling locations of the two *Kurixalus* species in Nantou County, Taiwan. —1. Yuchi (23°53'47"N, 120°54'13"E) for *K. idiootocus*; —2. Xitou (23°40'35.2"N, 120°47'46"E) for *K. eiffingeri*.

ing. Hypotonic KCl solution (0.075 M) was applied for 20 min, followed by fixation with fresh Carnoy's fixative (3:1 of methanol to glacial acetic acid) at 4 °C for 1 h. G-banding, C-banding and Ag-NOR staining were carried out according to published protocols (Wang et al., 2004; Wu et al., 2007). In short, for G-banding, the metaphase chromosomes were digested with trypsin at room temperature (RT) for 15 sec and subsequently stained with Wright's stain at RT for 2 min; for C-banding, the metaphase chromosomes were treated with an alkali barium hydroxide 5% $Ba(OH)_2$ at 50 °C for 15 sec, incubated in 2 x SSC solution at 60 °C for 1 h, and afterward stained with Wright's dye; for Ag-NOR staining, the working solution (mixture of 100 µl of the 2% gelatin colloid solution and 200 µl of the silver nitrate solution) was mixed immediately before use and pipetted onto the slides with the metaphase chromosomes at 70 °C for 2 min. After rinsing off with deionized water, the metaphase chromosomes were stained with Wright's dye at RT for 2 min.

Telomeric FISH

The physical map of telomeric sequences was detected by fluorescence *in situ* hybridization (FISH) using All Human Telomeres Probe (TTAGGG)n (Qbiogene/MP Biomedicals LLC, Tucson, AZ, USA) and followed manufacturer's manual. Briefly, 10 µl of the fixed, pre-treated and homogenized samples were applied on a microscope slide and dried at 50 °C for 15 min, immersed the slide in 2x SSC for 2 min at RT without agitation, followed by a successive dehydration step with 70%, 85% and 100% ethanol respectively for 2 min and allowed to dry. The sample and probe were co-denatured by heating the slide at 75 °C for 2 min, and then hybridizations were performed at 37 °C for 12 h. The slide were immersed in 0.4 x SSC (pH 7.0) at 72 °C for 2 min and then were immersed in 2 x SSC, 0.05% Tween-20 at RT (pH 7.0) for 1 min. Finally, the slides were drained and applied 10µl of 0.125 µg/ml DAPI antifade onto hybridization areas, and were viewed with a fluorescence microscope.

Comparative genomic hybridization (CGH)

Comparative genomic hybridization is often used for the evaluation of the differences between the chromosomal complements of different sexes (Badenhorst et al., 2013; Matsubara et al., 2013; Rovatsos et al., 2015). The CGH between sexes was performed as previously described (Yu et al., 2012), with some modifications. Briefly, the genomic DNA from male and female were labeled with fluorescein isothiocyanate (FITC)-12-dUTP and Texas Red (TxRed)-5-dUTP (Perkin Elmer Life Sciences, Boston, MA, USA) respectively by Nick Translation Kit (Roche Diagnostics, Basel, Switzerland) and co-precipitated with a 200-fold excess of Cot-1 DNA. Genomic probes were re-dissolved and kept in hybridization buffer (50% formamide, 10% dextran sulphate, and 2 x SSC). The hybridization mixture was denatured at 80 °C for 10 min, followed by preannealing at 37 °C for 60 min. The slides were incubated in a moist chamber at 37 °C for 72 h. After standard washing procedure, chromosomes were counterstained with 0.125 µg/ml DAPI added to antifade reagent (Abbott, Illinois, USA).

Image capture and analysis

Karyotype images, FISH and CGH results were analyzed for at least 10 well-spread metaphases in each sample by CytoVision system (Applied Imaging, Carlsbad, CA, USA). For karyotyping, chromosome types were classified according to the nomenclature of Levan et al. (1964).

Genome size estimation

The genome sizes were estimated by flow cytometry based on the description of Matsuba and Merilä (2006). Blood samples from each sex of both species were collected in phosphate-buffered saline without divalent cations and stored at 4 °C. As internal references, male blood cells from *Lithobates catesbeianus* (haploid genome size: C value = 7.37 pg) and *Homo sapiens* (C value = 3.5 pg) were analyzed simultaneously in 1:10 mixed with surveyed cells. Cells were suspended in 0.5 ml of a solution containing 25 µg propidium iodide (PI), 0.1% sodium citrate, 25 µg RNAase, and 0.1% Triton X-100. Samples were filtered through 30 µm mesh and kept at 4 °C in the dark for over 2 h. Mean fluorescence of co-stained nuclei was quantified

on a Beckman-Coulter EPICS XL-MCL flow cytometer with an argon laser (emission at 488 nm/15 mW power), and analyzed with WinMDI 2.9 software. The PI fluorescence and genome size of *L. catesbeiana* and *H. sapiens* were used as standards to calculate the unknown genome sizes in samples (Vinogradov, 1998). Student T-test was used to compare the difference of genome sizes between species/sexes by SPSS software (IBM Corp., Armonk, NY, USA). The Bonferroni correction was applied to set the significance cut-off at α/n, with α = 0.05 and n = number of samples.

RESULTS

A total of 138 and 125 metaphase spreads from *K. eiffingeri* (individual number = 10) and *K. idiootocus* (individual number = 10) respectively were subjected to chromosome analyses. Both species had the same haploid and diploid chromosome numbers (n = 13 and 2n = 26), and chromosomes are readily classified into two distinct size groups, large pairs of chromosomes (no. 1-5) and small pairs of chromosomes (no. 6-13). All chromosome pairs were either metacentric or submetacentric, giving a fundamental number (FN) of 52 (Table 1). Though karyotypes of the two species were similar, differences were noted in detailed chromosome morphology and banding pattern (Fig. 2). No chromosomal polymorphism (e.g., inversions, translocations, deletions and duplications) was noted among individuals examined in each of the two species. In *K. eiffingeri* the chromosome pairs 2, 3, and 9 were submetacentrics and the others metacentrics while in *K. idiootocus* chromosome pairs 2 and 4 were submetacentrics and the others metacentrics. The karyotype formulas of *K. eiffingeri* and *K. idiootocus* were thus 10 metacentric + 3 submetacentric and 11 metacentric + 2 submetacentric, respectively. An achromatic secondary constriction (SC) was found in the long arm near the centromere of chromosome pair 8 and pair 12 in *K. eiffingeri* and *K. idiootocus* respectively (Fig. 2). Additionally, significant differences (ANOVA test, $P < 0.0001$) in relative chromosome length were found in chromosome pairs 1-3 (longer in *K. eiffingeri*) and in chromosome pairs 6-9 and 11-13 (longer in *K. idiootocus*) (Table 2). No heteromorphic chromosome pair (sex chromosomes) could be identified in each species. C-banding analysis showed that constitutive heterochromatin was located at the centromeres of all chromosomes of *K. eiffingeri* and *K. idiootocus*, but heterochromatins differed in banding intensity corresponding with blocks of constitutive heterochromatin. An additional dense heterochromatin was only found on proximal short arm of chromosome pair 8 of *K. eiffingeri* (Fig. 3). The nucleolus organizer region (NOR) was detected by silver staining on the long arm near the centromere of chromosome pair 8 in *K. eiffingeri*, but on the long arm near the centromere of chromosome pair 12 in *K. idiootocus* (Fig. 2). The active NORs are within the corresponding regions of SCs in both species (Fig. 2 and Table 1).

FISH with telomeric probe (TTAGGG)n detected strong signals on the chromosome ends of all chromosomes of *K. eiffingeri* and *K. idiootocus* (Fig. 4). No (TTAGGG)n signal was found at interstitial region in either species. The CGH for *K. eiffingeri* and *K. idiootocus* revealed no notable chromosome difference between sexes. Most of stronger painting signals were observed in the centromeres with blocks of tandemly repeated satellite sequences, which are embedded in heterochromatic regions. A strong hybridization signal was noted in the telomeric region of the long arm of chromosome pair 3 of *K. eiffingeri* using either male or female probe (Fig. 5).

The DNA C value of was estimated as 5.06 ± 0.13 pg (male: 5.02 ± 0.08 pg, female: 5.11 ± 0.16 pg) in *K. eiffingeri* (n = 20), and 4.18 ± 0.21 pg (male: 4.23 ± 0.22 pg, female: 4.13 ± 0.20 pg) in *K. idiootocus* (n = 17) (Table 1). Significant difference in genome size between species was evidenced (Student t-test, $t_{35} = 15.29$, $P < 0.0001$). In contrast, no sexual difference in genome size was found in both species (Student t-test, $t_{18} = -1.597$, $P = 0.128$ in *K. eiffingeri*, 10 male vs 10 female; $t_{15} = 0.968$, $P = 0.349$ in *K. idiootocus*, 9 male vs 8 female).

DISCUSSION

The chromosomes of frogs in the family Rhacophoridae is considered to be conservative since almost all species studied so far show invariable 2n = 26 karyotypes (Li, 2007). Distinct diploid chromosome number in this family is extremely rare and only reported in few species (e.g., *Chiromantis doriae* 2n = 16, in Rao and Yang, 1996, but 2n = 16 or 26 in Tan, 1987). Rhacophorid karyotypes also share other characteristics including composition of two size groups of chromosomes (i.e., large and small chromosome pairs) and predominant presence of metacentric and submetacentric chromosomes (Kuramoto, 1977; Blommers-Schlösser, 1978; Kuramoto, 1985; Kuramoto and Wang, 1987; Tan, 1987; Rao and Yang, 1996; Joshy and Kuramoto, 2011). These features, together with the fact that very few acrocentric and telocentric chromosomes were found, have led to the suggestion that the karyotype of Rhacophoridae represent a derived condition in Anura (Rao and Yang, 1996) because the primary direction of Anuran karyotype evolution has been advocated to reduce chromosome number by fusions of acrocentrics to metacentrics (Morescalchi, 1980; Suarez

Table 1. Summary of karyotype and chromosomes features of the two species: 2n, diploid chromosome number; M, metacentric chromosomes; SM, submetacentric chromosomes; FN, fundamental number; SC, secondary constriction; NOR, nucleolus organizer region; NSF, no specific findings.

	2n	Karyotype formula	FN	Size group of chromosomes (large + small)	SC	NOR	Heterochromatins (by C-banding)	Telomeric motif (TTAGGG)n (by telomeric FISH)	CGH	C value (pg; mean ± SD)
K. eiffingeri										
Male	26	10 M + 3 SM	52	5 + 8	8q	8q	1. Centromere of all chromosomes 2. Proximal 8p (dense)	Telomeres of all chromosomes	Stronger painting signal on 3q	5.02 ± 0.08
Female	26	10 M + 3 SM	52	5 + 8	8q	8q	1. Centromere of all chromosomes 2. Proximal 8p (dense)	Telomeres of all chromosomes	Stronger painting signal on 3q	5.11 ± 0.16
Overall	26	10 M + 3 SM	52	5 + 8	8q	8q	1. Centromere of all chromosomes 2. Proximal 8p (dense)	Telomeres of all chromosomes	Stronger painting signal on 3q	5.06 ± 0.13
K. idiootocus										
Male	26	11 M + 2 SM	52	5 + 8	12q	12q	Centromere of all chromosomes	Telomeres of all chromosomes	NSF	4.23 ± 0.22
Female	26	11 M + 2 SM	52	5 + 8	12q	12q	Centromere of all chromosomes	Telomeres of all chromosomes	NSF	4.13 ± 0.20
Overall	26	11 M + 2 SM	52	5 + 8	12q	12q	Centromere of all chromosomes	Telomeres of all chromosomes	NSF	4.18 ± 0.21

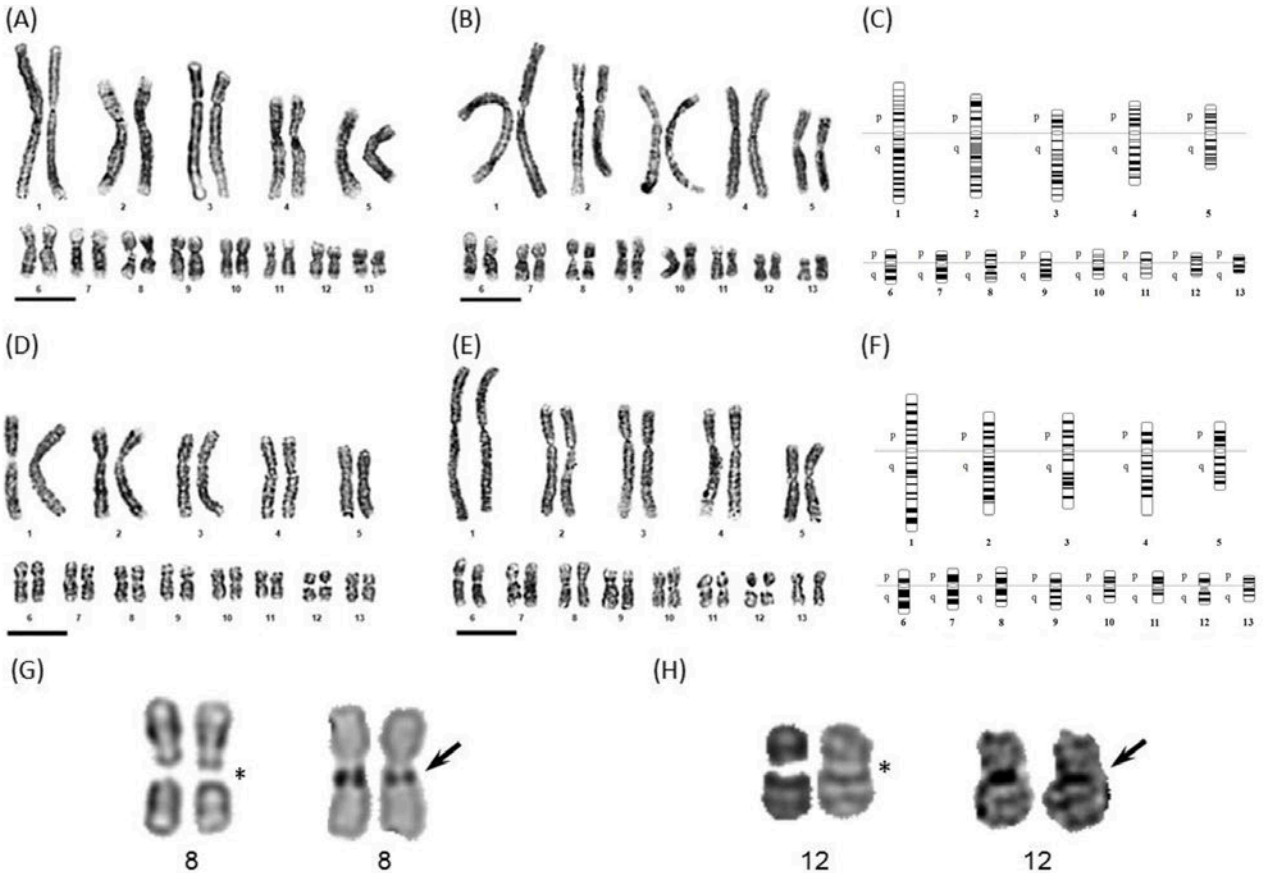

Fig. 2. G-banding karyotypes of *K. eiffingeri* for male (A), female (B); and proposed idiogram (C), and *K. idiootocus* for male (D), female (E) and proposed idiogram (F). The G-banding karyotypes of female and male are similar in each of the two species. Bars = 10 μm. An achromatic secondary constriction (SC; indicated by stars) was found in the long arm near the centromere of chromosome pair 8 of *K. eiffingeri* (G) and in the long arm near the centromere of chromosome pair 12 of *K. idiootocus* (H). The SCs were accompanied by positive Ag-NOR staining (indicated by arrows) in both species (G and H).

et al., 2013). The two *Kurixalus* species analyzed here revealed conservative karyological features of Rhacophoridae, including the modal diploid number, 5 larger and 8 smaller chromosomal pairs, but no acrocentric (either metacentric or submetacentric) chromosomes.

Although the diploid states of the two *Kurixalus* species are similar, evidence of karyotype divergence through chromosomal rearrangements was found. The two *Kurixalus* species are different in karyotype microstructure. A highly dense heterochromatin was only found in the proximal short arm of the chromosome pair 8 in *K. eiffingeri*, which could be a chromosome marker in karyotype of this species. Besides, the long arm of chromosome pair 3 of *K. eiffingeri* was painted with strong fluorescence signals in karyotypes of both sexes, suggesting the region is rich in repeated sequences in this species. In both species, NORs were only found in one pair of chromosomes and the NOR localizations were coincided with regions of SC. However, the NOR-bearing chromosome pairs differ between *K. eiffingeri* and *K. idiootocus*: one in pair 8 and the other in pair 12. Extensive studies have shown that NORs can differ in extent, localization and number among chromosomes from different species that make NORs a useful chromosome marker in comparative cytogenetic studies (Morescalchi, 1980; Mahony and Robinson, 1986; Bruschi et al., 2014). For instance, the NOR on chromosome pair 7 has been recognized as a structural distinction between Z and W chromosomes in *Buergeria buergeri*, which is the only known rhacophorid species with heteromorphic sex chromosomes (Schmid et al., 1993). Recently, the possible role of NORs as rearrangement hotspots during evolution is also discussed (Cazaux et al., 2011; Britton-Davidian et al., 2012).

Chromosomal rearrangements can be traced by chromosomal mapping of telomeric sequences, which located at the ends of eukaryotic chromosomes with an

Table 2. Arm ratio (length of long arm/short arm) and relative length (percentage of total haploid chromosome length) of chromosomes in *K. eiffingeri* and *K. idiootocus* (mean ± SD). Abbreviation M and SM indicate metacentric and submetacentric respectively, and n is the number of metaphase spreads analyzed

Pair no.	*K. eiffingeri*					*K. idiootocus*					ANONA test	
	Arm ratio		Attribute†	Relative length (%)		Arm ratio		Attribute†	Relative length (%)			
	Male (n = 19)	Female (n = 17)		Male (n = 19)	Female (n = 17)	Male (n = 21)	Female (n = 19)		Male (n = 21)	Female (n = 19)	F	*P*
1	1.26 ± 0.09	1.23 ± 0.10	M	17.00 ± 0.33	16.87 ± 0.36	1.34 ± 0.07	1.35 ± 0.11	M	15.92 ± 0.62	16.06 ± 0.48	80.3	<0.0001‡
2	1.84 ± 0.08	1.88 ± 0.11	SM	13.34 ± 0.63	13.43 ± 0.61	1.73 ± 0.09	1.76 ± 0.11	SM	12.65 ± 0.26	12.33 ± 0.46	57.4	<0.0001‡
3	2.64 ± 0.33	2.55 ± 0.35	SM	13.22 ± 0.54	13.13 ± 0.47	1.53 ± 0.17	1.48 ± 0.09	M	11.82 ± 0.26	12.02 ± 0.37	171.7	<0.0001‡
4	1.50 ± 0.12	1.47 ± 0.10	M	11.46 ± 0.29	11.55 ± 0.41	1.81 ± 0.24	1.84 ± 0.17	SM	11.22 ± 0.28	11.35 ± 0.41	7.3	0.0086
5	1.38 ± 0.07	1.41 ± 0.11	M	9.61 ± 0.45	9.69 ± 0.58	1.29 ± 0.04	1.31 ± 0.11	M	9.68 ± 0.25	10.04 ± 0.47	3.9	0.0040
6	1.52 ± 0.18	1.55 ± 0.15	M	5.48 ± 0.28	5.53 ± 0.41	1.56 ± 0.13	1.54 ± 0.15	M	5.79 ± 0.22	5.71 ± 0.19	15.7	<0.0001‡
7	1.51 ± 0.17	1.53 ± 0.14	M	5.06 ± 0.12	4.98 ± 0.21	1.41 ± 0.09	1.36 ± 0.07	M	5.46 ± 0.14	5.27 ± 0.25	61.2	<0.0001‡
8	1.59 ± 0.17	1.56 ± 0.13	M	4.85 ± 0.22	4.97 ± 0.21	1.27 ± 0.09	1.23 ± 0.08	M	5.18 ± 0.16	5.11 ± 0.22	27.4	<0.0001‡
9	1.82 ± 0.19	1.78 ± 0.21	SM	4.67 ± 0.11	4.74 ± 0.11	1.70 ± 0.18	1.62 ± 0.13	M	5.04 ± 0.19	5.01 ± 0.21	74.3	<0.0001‡
10	1.40 ± 0.18	1.42 ± 0.13	M	4.53 ± 0.13	4.57 ± 0.14	1.11 ± 0.07	1.08 ± 0.06	M	4.45 ± 0.16	4.44 ± 0.22	6.9	0.0106
11	1.21 ± 0.12	1.20 ± 0.11	M	3.90 ± 0.17	3.83 ± 0.17	1.13 ± 0.11	1.09 ± 0.09	M	4.40 ± 0.16	4.37 ± 0.21	154.2	<0.0001‡
12	1.37 ± 0.16	1.41 ± 0.13	M	3.77 ± 0.07	3.65 ± 0.14	1.41 ± 0.15	1.36 ± 0.11	M	4.30 ± 0.18	4.26 ± 0.19	234.4	<0.0001‡
13	1.24 ± 0.11	1.22 ± 0.08	M	3.11 ± 0.24	3.06 ± 0.23	1.36 ± 0.09	1.33 ± 0.09	M	4.09 ± 0.22	4.03 ± 0.17	376.5	<0.0001‡

†Levan et al. (1964).

‡Statistical significance (α = 0.05/76, after Bonferroni correction).

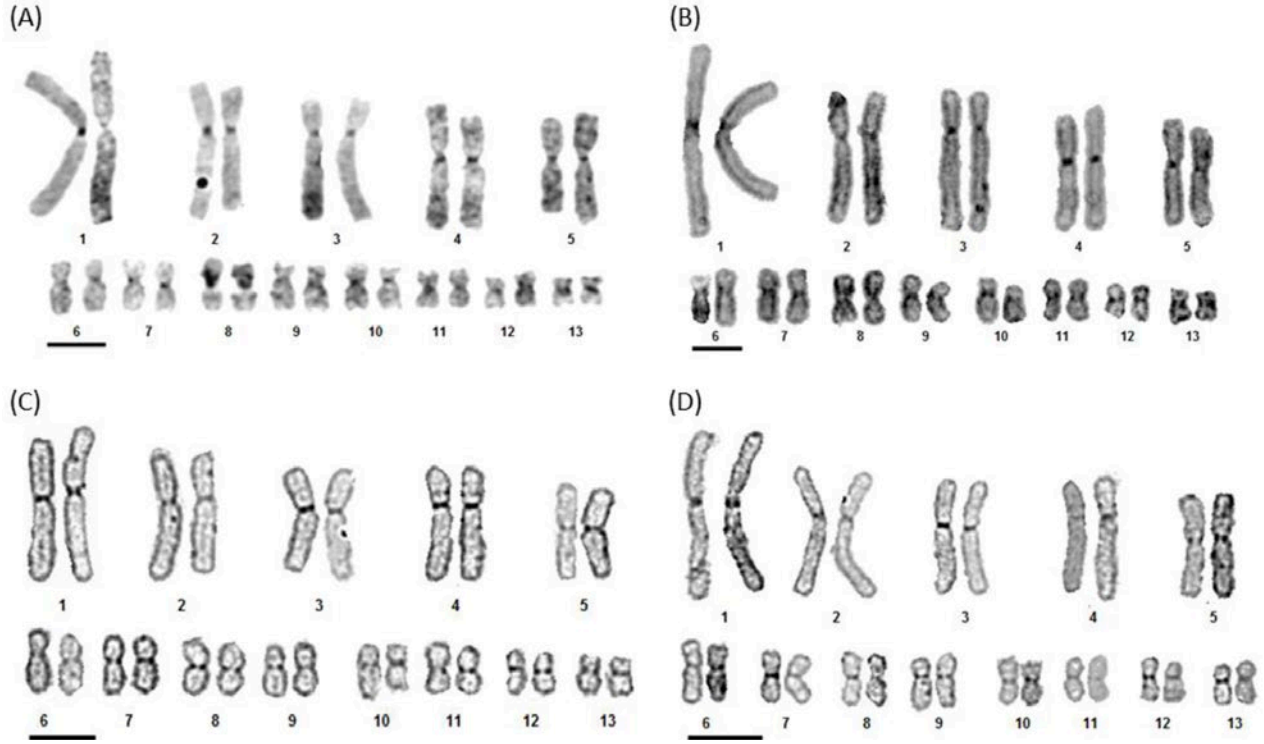

Fig. 3. C-banding karyotypes of *K. eiffingeri* for male (A) and female (B), and *K. idiootocus* for male (C) and female (D). Constitutive heterochromatins were regularly detected on the centromeres of all chromosomes. Particularly, a dense heterochromatin was also noted on the proximal region of short arm of chromosome pair 8 in *K. eiffingeri*. The C-banding karyotypes of female and male are similar in each of the two species. Bars = 10 μm.

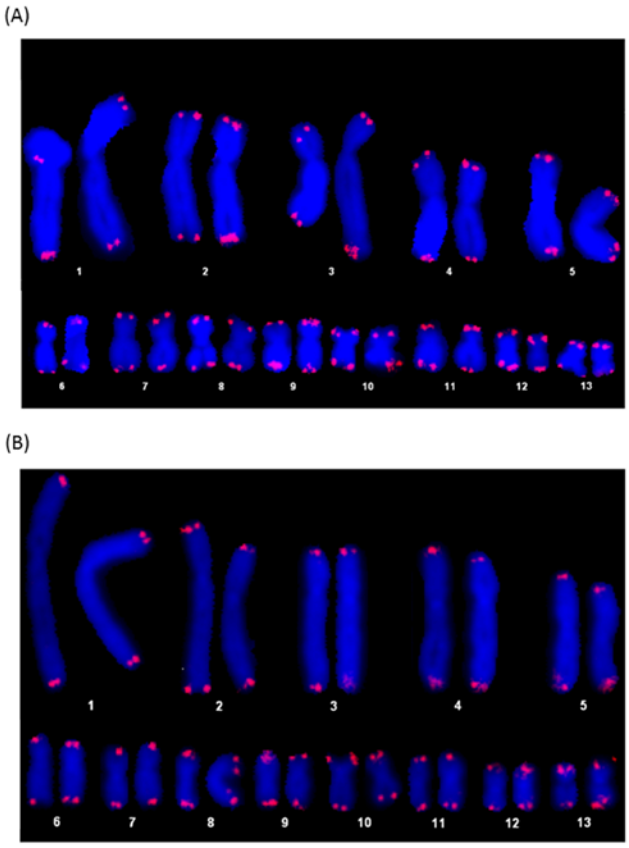

Fig. 4. Fluorescence in situ hybridization (FISH) with telomeric probe exclusively mapped (TTAGGG)n repeats to terminal regions of all chromosomes of *K. eiffingeri* (a) and *K. idiootocus* (b). No interstitial telomeric sequence (ITS) was detected in both species.

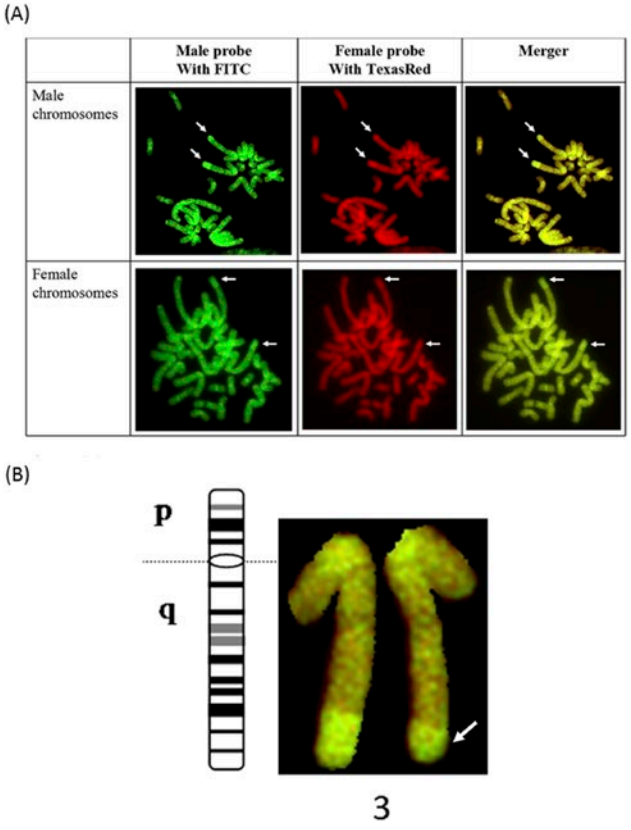

Fig. 5. Comparative genomic hybridization (CGH) using male and female genomic probes (labeled by FITC and TxRed, respectively) for chromosomes from male and female of *K. eiffingeri* showed similar hybridization patterns between sexes (A). A large chromosomal fragment with stronger painting signal (indicated by an arrow) were observed on the terminal regions of long arm of chromosome pair 3, presumably rich in repeated sequences (B). Similar patterns between sexes without any stronger painting signal in chromosome pairs were observed in *K. idiootocus* (data not showed). p, short arm; q, long arm.

important function of chromosomal stability by protecting them from end-to-end fusion, degradation, and recombination (Fagundes and Yonenaga-Yassuda, 1998; Bruschi et al., 2014). The presence of interstitial telomeric sequences (ITSs) in chromosomes has been considered as evidence of chromosome rearrangements that occurred during the evolution of karyotypes. Telomeric FISH for the karyotypes of the two *Kurixalus* species showed that the (TTAGGG)n motifs are restricted to the terminal regions of chromosomes. The absence of ITSs cannot be explained by the absence of chromosome rearrangements because evidence of karyotypic differences between *K. eiffingeri* and *K. idiootocus* were noted. We postulate that subtle chromosome changes irrelevant to ITSs (e.g., intrachromosomal rearrangements) may play a role in karyotype evolution. Recent genomic studies have shown that amphibians have a slow rate of interchromosomal rearrangements but intrachromosomal rearrangements of loci are not uncommon (Smith and Voss, 2006; Sun et al., 2015). Thus, the undetected chromosome changes in *Kurixalus* may be uncovered by advanced molecular analyses. The present data demonstrated that the karyotypes of the two *Kurixalus* species seem to be conserved, despite minor differences in chromosome morphology were observed. As a result, the interspecies differences at chromosomal level are unsatisfying to link directly with their behavioral difference in reproductive mode.

In addition to cytogenetic characteristics, genome sizes of the two *Kurixalus* species are different. The estimated DNA C value of *K. eiffingeri* is about 1.21 times greater than that of *K. idiootocus*. Currently, no information is available about the C values of other rhacophorid species and the few data collected in Genome Size Database (http://www.genomesize.com/) actually belonged species in the Mantellidae. In amphibians, the C values ranged from

0.95 to 120 pg. The more than 130-fold variation in C value has rendered amphibian a taxon with the most variable genome size among vertebrates (Gregory, 2003). Genome size can be affected by various genetic events, such as duplication, insertion, recombination, deletion and polyploidization (Gregory, 2005). Difference in genome sizes thus reflects dissimilarity in genomic compositions.

Identification of heteromorphic chromosome pairs has been generally acknowledged as the first step in recognition of sex determination mechanism (Valenzuela et al., 2003). Some heteromorphic chromosome pairs differ in chromosomal appearance and are easily observable, whereas others require advanced high-resolution tools to clarify. In this study, cytogenetic analyses for the two *Kurixalus* species revealed all chromosome pairs are homomorphism. Even the CGH was executed for both sexes in each species, no recognizable sex difference in karyotypes. This is not surprising because in amphibians most species lack morphologically distinguishable sex chromosomes despite exhibiting gonochorism (distinct male and female). So far, only a few of amphibian species with heteromorphic sex chromosomes were reported (Hayes, 1998; Berset-Brandli et al., 2006; Ezaz et al., 2006).

It is a little strange that, despite the lack of heteromorphism in chromosome pairs of most amphibian species, genetic sex determination is generally considered the rule for this group (Nakamura, 2009). It was proposed that since sex chromosomes evolved from homomorphic autosomes through the acquisition of a sex determining gene (Ohno, 1967), the reason that heteromorphic sex chromosomes could not be highlighted in amphibians was not due to their nonexistence but because the genes involved in sex determination were located on poorly discriminated regions of the chromosomes (Hayes, 1998). For example, the gene for ADP/ATP translocase was described as a sex-linked marker in *Rana rugosa* (Miura et al., 1998) of XX/XY system and sex-specific loci in *Xenopus laevis* (Mawaribuchi et al., 2017) of ZZ/ZW system, but none of the species exhibited notable differences in karyotypes between sexes. However, in the two *Kurixalus* species analyzed the sexual differences in genomic size are too small to be detected, leading to inconclusive evidence if genetic differentiation exists between sexes.

Traditionally, a good strategy to understand the possible sex determination mode in species is to examine the sex ratio in natural populations (Janzen and Paukstis, 1991). Balanced sex ratio is the most general explanation for species with genetic determination. The sex ratio of *K. idiootocus* in nature was not known, but *K. eiffingeri* had balanced adult sex ratio (51.2% proportion of males) (Kam et al., 2000). Although this provides a possible link to the genetic sex determination in *K. eiffingeri*, segregation distortion, such as temperature-induced differential mortality, differential fertilization, and embryo abortion, can result in biased primary (fertilization) or secondary (births) sex ratio (Valenzuela et al., 2003). Besides, in a few frog species the sex can be affected by temperature despite they are considered as genetic sex determination (Witschi, 1914; Piquet, 1930; Yoshikura, 1963). Basically, all above arguments must move ahead from a well-established base of sufficient cytogenetic information just like our efforts in the two *Kurixalus* species.

We considered that the loss of cytogenetic differences between sexes of the two *Kurixalus* species does not refute the idea that these species possess genetic sex determination. However, the present data did not provide evidence to support the existence of sex-specific genetic markers in either species. It is possible that the sex chromosomes might be homomorphic, with the extensive pseudoautosomal region (Roco et al., 2015), and small sex specific regions that harbor key genes from the sex-determination pathway, such as the cases with sex specific *Dmrt1* gene in *Rana temporaria* (Ma et al. 2016; Rodriguez et al 2017), which would need more advanced molecular approaches (e.g., genotyping by length-polymorphic markers) to exploring the genetic sex-determination. At the moment, it remains unclear if all amphibian species are of genetic sex determination, but species with diverse reproductive behaviors like *Kurixalus* provides an interesting object to study this question.

We demonstrated that the two *Kurixalus* tree frogs have distinct karyotypes and genome sizes. Cytogenetic characteristics, such as the distribution of SC/NOR and staining intensity of heterochromatins that distinguished the karyotypes of the two species, were not previously reported. In either species, the finding of no differences in cytogenetic and genomic features between sexes is in line with previous reports that showed no heteromorphic chromosome pair in most rhacophorid species. Moreover, our telomeric FISH demonstrated no ITS in chromosomes of both species, suggesting subtle chromosome changes irrelevant to ITSs (e.g., intrachromosomal rearrangements) may play a role in their karyotype evolution. The cytogenetic data and the genomic information described in this work contribute to our understanding of the genetic features in *Kurixalus* species and are potentially useful as taxonomic and reproductive traits for additional studies in this genus as well as in the family Rhacophoridae.

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