Songklanakarin J. Sci. Technol.
35 (4), 383-387, Jul. - Aug. 2013

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Chromosome banding of two *Litoria* species (Anura, Hylidae)

Wanpen Kakampuy[1]*, Thavorn Supaprom[1], Claus Steinlein[2], and Michael Schmid[2]

[1] *Department of Biological Sciences, Faculty of Science, Ubon Ratchathani University, Warin Chamrab, Ubon Ratchathani, 34190 Thailand.*

[2] *Department of Human Genetics, Biocenter, University of Würzburg, D-97074 Würzburg, Germany.*

Received 26 April 2012; Accepted 2 May 2013

Abstract

This research was the first cytogenetic studies of green-eyed tree frog (*Litoria genimaculata*) and Nyakala frog (*L. nyakalensis*). The mitotic chromosomes were prepared directly from bone marrow after *in vivo* colchicine treatment and analysed following conventional, silver staining and C-banding techniques. These species showed similar karyotypes with 2*n*=26, the fundamental number (NF) are 52 chromosomes and Ag-NOR positions located in the short arm near centromeres of chromosome pair 7 in *L. genimaculata* and in the long arm near telomeres of chromosome pair 9 in *L. nyakalensis*. The C-banded karyotypes showed heterochromatin bands at the centromeres and telomeres of all chromosomes. Both species were characterized by the number and position of constitutive heterochromatin in the karyotypes. The mitotic karyotypes of *L. genimaculata* exhibited greater amounts of C-band positive heterochromatin than *L. nyakalensis*.

Keywords: standard karyotype, *Litoria*, Hylidae, banding patterns

1. Introduction

Members of the family Hylidae are commonly called tree frogs or hylids. The family is particularly species rich in the New World tropics; however, their distribution extends to Europe, North Africa, Asia, North, South and Central America, the West India, Australia and New Guinea (King, 1990 and Frost, 2010). Currently, the family Hylidae has been found to consist of five subfamilies including Hylinae, Phyllomedusinae, Hemiphractinae, Amphignathodotinae, and Pelodryadinae.

The genus *Litoria* includes in Pelodryadinae comprises 181 species (Frost, 2010). A small number of *Litoria* species have been analyzed chromosomally at varying levels of resolution, ranging from chromosome number to relatively sophisticate banding techniques and the *in situ* hybridization of 18S+28S cRNA probes. All species have 2*n*=26, NF=52 chromosomes, except for giant tree frog *L. infrafrenata*, which has 2*n*=24, NF=48. The chromosomes of *Litoria* species are all very similar in their morphology and are typically hylid in format (King *et al*., 1979; 1990).

There are several cytogenetic reports on tree frogs in the genus *Litoria* (Menzies and Tippett, 1976; King, 1980; Schmid *et al*., 2003). However, there is no report on *L. genimaculata* (green-eyed tree frog) and *L. nyakalensis* (Nyakala frog). Both species are important to the ecosystem because they are considered excellent bioindicators and these animals are an Australian endemic. Populations of both species have declined; especially *L. nyakalensis* has been listed as critically endangered because its population size is estimated to number fewer than 50 mature individuals (IUCN, 2012). In this work, the karyotypes of these species were studied using conventional staining, silver staining and C-banding techniques. This is the first report describing the banding patterns of *L. genimaculata* and *L. nyakalensis*. This study provides important data for breeding, genetic conservation, and evolutionary studies in the *Litoria* genus.

* Corresponding author.
Email address: kakampuy@hotmail.com

2. Materials and Methods

2.1 Chromosome preparation and banding analyses

The localities from where the specimens of *L. genimaculata* and *L. nyakalensis* karyotyped in this study were obtained from are shown in Table 1. Nineteen males of *L. genimaculata* and four males of *L. nyakalensis* were collected in the North-eastern part of Australia. Mitotic metaphase cells were prepared directly from bone marrow of all animals after *in vivo* colchicine treatment. The preparation of cell suspensions, hypotonic treatment, and fixation of the cells were conducted as described previously (Schmid, 1978; Schmid *et al*., 1983). Labeling with Giemsa staining, silver staining and C-banding were performed according to the method of Schmid *et al*. (1983, 1988).

Giemsa staining. The slides were stained for six minutes in 5% Giemsa solution. Then, the slides were rinsed with running tap water to remove excess stain. Afterwards, the slides were allowed to air dry at room temperature.

Silver staining. The slides were flood with 50% $AgNO_3$ solution, covered with a cover glass, incubated for 2 hours at 60°C, rinsed in distilled water and air-dried. The slides were stained in 2% Giemsa solution for 30 seconds, rinsed in distilled water and air-dried.

C-banding. The air dried slides were placed in 0.2 N HCl for 30 minutes, rinsed in distilled water and air dried. The slides were placed in saturated $Ba(OH)_2$ solution at 30°C for 5 to 10 minutes, dipped briefly in 0.2 N HCl and rinsed in distilled water. The slides were then placed in 2XSSC at 60°C for 1.30 hour, rinsed in distilled water and stained in 10% Giemsa solution for 5 to 15 minutes. Slides were then rinsed in distilled water, dried and mounted.

2.2 Photography and analysis of banding patterns

At least 25 well spread metaphase cells, which exhibited the greatest banding clarity, size uniformity, and straightness for each species, were observed for analysis of the chromosome number and the demonstration of secondary constriction as chromosome marker. At least 10 metaphase cells of them were selected for photomicrography. All microscopic analyses were conducted with Zeiss photomicroscope III and Zeiss Axiophot microscope equipped with incident HBO 50 W mercury lamp illumination. All photographs were taken with Agfaortho 25 ASA film.

2.3 Idiogram construction and karyotyping

The high-quality C-banded mitoses cells from *L. genimaculata* and *L. nyakalensis* were selected for chromosome measurements. These cells were in a middle stage of metaphase and had no overlapping chromosomes. Chromosome lengths were determined by comparing chromosome photographs with a Zeiss 10 mm objective micrometer photographs at the same magnification. Photomicrographs of at least 10 well-spread metaphase chromosome sets, which had the best banding clarity, size uniformity, and straightness, were selected for each species of amphibians for ideogram construction and karyotyping. The percent of relative length of chromosome is RL (%) = chromosome length × 100 / total chromosome length, and the arm ratio or centromeric ratio is CR = length of long arm / length of short arm (Green and Sessions, 1991). The position of the centromere was determined by the arm ratio as metacentric = 1.00-1.67, submetacentric = 1.68-3.00, subtelocentric = 3.01-7.00 and telocentric = 7.00-µ (Green and Session, 1991). The chromosome pairs from photomicrography prints were cut and arranged according to size in parallel rows and in order of decreasing mean length.

3. Results

3.1 Giemsa staining

Karyotypes of *L. genimaculata* and *L. nyakalensis* were shown a diploid number (2*n*) of 26 chromosomes, all bi-armed, so the fundamental number (NF, number of chromosome arms) is 52. The first six pairs (pairs 1 to 6, RL % of *L. genimaculata* = 14.20 to 7.94, *L. nyakalensis* = 14.26 to 7.80) were distinctly larger than the rest of the chromosomes (pairs 7 to 13, RL % of *L. genimaculata* = 6.34 to 3.11, *L. nyakalensis* = 6.96 to 3.72). In the *L. genimaculata*, pairs 1 and 4 were large metacentric chromosomes, pair 2 was large submetacentric chromosome, pairs 3, 5 and 6 were large subtelocentric chromosomes; pairs 7, 8, 9, 10 and 11 were small submetacentric chromosomes and pairs 12 and 13 were small metacentric chromosomes. A secondary constriction was present on the short arm near telomere of pair 7 (Table 2, Figure 1a). In the *L. nyakalensis*, chromosome pairs 1 and 4 were large metacentric chromosomes, pair 3 was large subtelocentric chromosome, pairs 2, 5 and 6 were large submetacentric chromosomes; pair 7 was small subtelocentric

Table 1. Localities of the two species of *Litoria* referred to in this study.

Species	2*n*	Collection locality	Sex	Number of specimens
L. genimaculata (green-eyed tree frog)	26	Mt. Lewis, Qld.	m	19
L. nyakalensis (Nyakala frog)	26	Paluma, Qld.	m	4

Table 2. Relative length (RL, %), centromeric ratio (CR) and nomenclature for centromeric position (CP) on mitotic chromosomes according to Green and Session (1991).

Species		Chromosome No.												
		1	2	3	4	5	6	7	8	9	10	11	12	13
L. genimaculata	RL%	14.20	13.85	12.38	11.55	9.48	7.94	6.34	5.43	4.76	4.34	3.32	3.29	3.11
	CR	1.40	2.50	3.06	1.07	3.15	3.36	2.50	1.97	1.83	1.98	1.93	1.31	1.58
	CP[a]	m	sm	st	m	st	st	sm	sm	sm	sm	sm	m	m
L. nyakalensis	RL%	14.26	12.10	10.67	10.44	8.70	7.80	6.96	6.17	5.28	5.14	4.91	4.15	3.72
	CR	1.45	1.99	3.05	1.53	2.07	2.05	3.03	2.00	1.68	1.43	1.41	1.64	1.47
	CP[a]	m	sm	st	m	sm	sm	st	sm	sm	m	m	m	m

Remarks: m = metacentric, sm = submetacentric, st = subtelocentric chromosome.

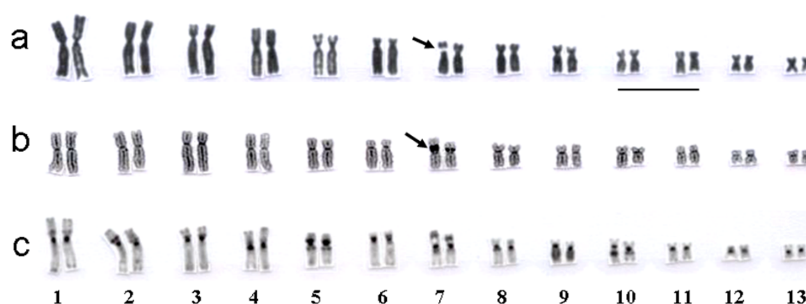


Figure 1. Karyotypes of male green-eyed tree frog (*Litoria genimaculata*) showing conventional staining (a), silver staining of the nucleolar organizer regions (b), and C-banding of the constitutive heterochromatin (c). Arrows indicate secondary constrictions. In this and all other figures the bar scale is equal to 10 mm.

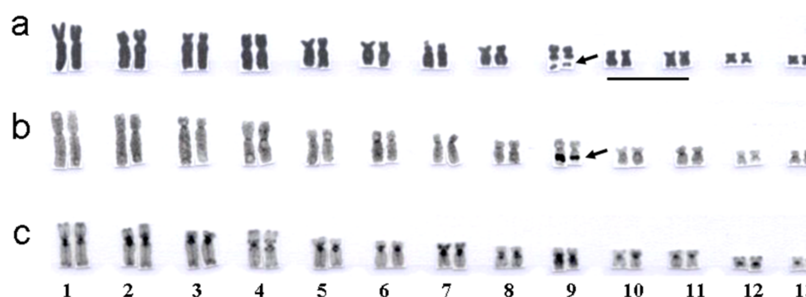


Figure 2. Karyotypes of male Nyakala frog (*Litoria nyakalensis*) showing conventional staining (a), silver staining of the nucleolar organizer regions (b), and C-banding of the constitutive heterochromatin (c). Arrows indicate secondary constrictions.

chromosome, pairs 8 and 9 were small submetacentric chromosomes and pairs 10, 11, 12 and 13 were small metacentric chromosomes. A secondary constriction was present on the long arm near telomere of pair 9 (Table 2, Figure 2a).

3.2 Silver staining

Silver staining showed that the nucleolar organizer regions (NORs) of *L. genimaculata* was located in the short arm near centromere of chromosome pair 7 (Figure 1b), whereas in *L. nyakalensis* it was positioned in the long arm near telomere of chromosome pair 9 (Figure 2b), which corresponded to the conspicuous secondary constriction detected in conventional staining (Figure 1a and 2a). All metaphase cells showed heteromorphic NORs.

3.3 C-banding

Constitutive heterochromatins were located in the centromeric and telomeric regions of all chromosomes of *L. genimaculata* and *L. nyakalensis*. In *L. genimaculata*; interstitial C-bands were visible in the pericentromeric regions of the short arms of chromosome pairs 1 and 2, long arms of chromosome pairs 5 and 10, large centromeric band of chromosome pair 5, large telomeric band on the short arm of chromosome pair 7 and the whole arm C-band on the long arm

of chromosome pair 9 (Figure 1c). In *L. nyakalensis*; large centromeric and pericentromeric bands of chromosome pairs 2, 3 and 9 and the short arm of chromosome pair 7 was completely heterochromatin (Figure 2c). Figure 3(b) and 4(b) show the idiogram of *L. genimaculata* and *L. nyakalensis* from C-banding. The karyotype formula of *L. genimaculata* and *L. nyakalensis* were as follows:

L. genimaculata $2n=26=8m+12sm+6st$
L. nyakalensis $2n=26=12m+10sm+4st$

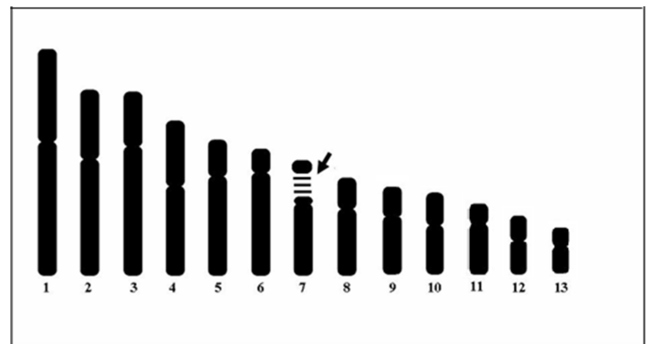
4. Discussion

This study is the first cytogenetic report of two rare *Litoria* species from Australia, including *L. genimaculata* and *L. nyakalensis*. The two *Litoria* species are $2n$=26. Similarly, 45 species of *Litoria* were reported by Menzies and Tippett (1976), King (1980) and Schmid *et al*. (2003), all have been found to have $2n$=26 but the exception is *L. infrafrenata* which have $2n$=24. Menzies and Tippett (1976) and King (1980) suggested a variation in chromosome numbers was due to the centric fusion between a pair of small and medium sized chromosomes which reduced the chromosome number from $2n$=26 to $2n$=24.

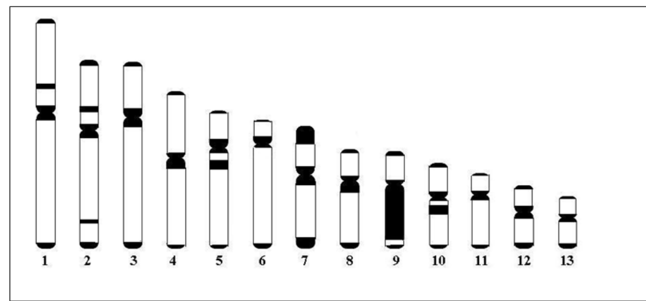
This examination also revealed that the fundamental numbers of the *L. genimaculata* and *L. nyakalensis* were 52; a diploid number of 26 chromosomes, all chromosomes are biarm. This is the same NF of other *Litoria* species (Menzies and Tippette, 1976; King, 1980 and Schmid *et al*., 2003).

King (1980) demonstrated that in terms of arm ratios and centromere positions the chromosome morphology of *Litoria* species is very characteristic. The *Litoria* species chromosome pairs 1 and 4 are metacentric chromosomes, pairs 2 and 6 are submetacentric chromosomes and pairs 3 and 5 are subtelocentric chromosomes. In terms of the overall size the members of the karyotype fall naturally into two clusters: pairs 1 to 6 and pairs 7 to 13. In the present study, the overall sizes of the karyotype of the two species are same as in King's report (1980). We found that *L. genimaculata* and *L. nyakalensis* were similar in the shapes of chromosomes as follows: metacentric, submetacentric and subtelocentric, but differed in a number of chromosomes; metacentric chromosome pairs 1, 4, 12 and 13 in *L. genimaculata* and pairs 1, 4, 10, 11, 12 and 13 in *L. nyakalensis*, submetacentric chromosome pairs 2, 7 to 11 in *L. genimaculata* and pairs 2, 5, 6, 8 and 9 in *L. nyakalensis*, subtelocentric chromosome, pairs 3, 5 and 6 in *L. genimaculata* and pairs 3 and 7 in *L. nyakalensis*. Regarding the secondary constriction, *L. genimaculata* has a secondary constriction on the short arm of chromosome pair 7 but on the long arm of chromosome pair 9 in *L. nyakalensis*.

In this investigation, the two nucleolar organizer regions (NORs 1 pair satellite chromosomes) of the *L. genimaculata* are located on the short arm near the centromere of chromosome pair 7 (2 positions) and the *L. nyakalensis* are located on the long arm near the telomere of chromosome pair 9 (2 positions). It is similar to the reports of King (1980) and Schmid *et al*. (2003) that indicated that NORs of the *L. moorei, L. chloris, L. lesueurii, L. peronii* and *L. meiriana* appear on the long arm near the telomere 13, 11, 10, 11 and 11 (2 positions), respectively and *L. infrafrenata*

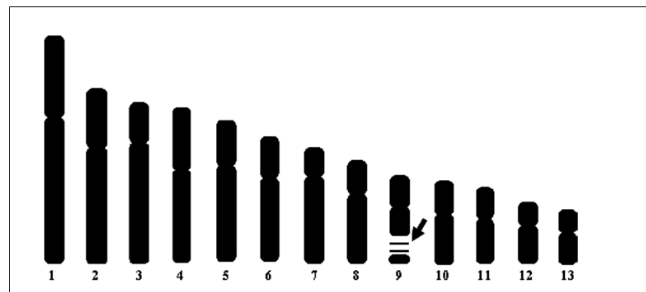


(a)

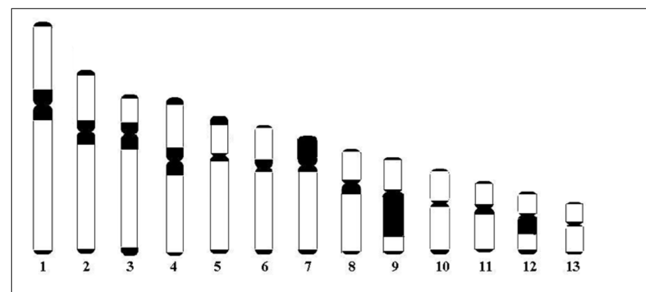


(b)

Figure 3. Idiogram of *L. genimaculata*, by (a) conventional staining, (b) C-banding. Arrows indicate secondary constrictions.



(a)



(b)

Figure 4. Idiogram of *L. nyakalensis*, by (a) conventional staining, (b) C-banding. Arrows indicate secondary constrictions.

appear on the short arm of pair 5 (2 positions).

Heteromorphic NORs have been described for a large number of species in anurans. Schmid (1982) detected 67% of heteromorphic NORs in 260 individuals and related them to tandem duplication or triplication in one of the NOR-bearing homologue chromosomes. King *et al*. (1990) also observed this sort of heteromorphism after conducting *in situ* hybridization in Australian hylids. They suggested amplifications in some parts of the ribosomal sequences of the duplications/ triplications of the rDNA cistrons. The NORs are the chromosomal sites of genes and are transcribed for 18S and 28S ribosomal RNA. These were presumably transcribed at a preceding interphase and are important in the relation to their intimate relationship with protein synthesis (Howell and Black, 1980).

Centromeric C-bands were observed to be commonly present in each chromosome of each species, but with regards to the interstitial and telomeric bands, differences could be clearly observed. The chromosomes of the two species of *Litoria* reported here showed patterns of centromeric, interstitial and telomeric bands. The mitotic karyotypes of *L. nyakalensis* exhibited greater amounts of C-band positive heterochromatin than *L. genimaculata*. We found that the two species of *Litoria* were similar in the C-banding patterns. *L. genimaculata* has four interstitial C-bands on chromosome pairs 1, 2, 5 and 10 which is absent in *L. nyakalensis.* It is clear from the C-banding studies on *Litoria* (King, 1980) that chromosomal reorganization has indeed been preceded by internal adjustments in the amount and location of the heterochromatin without any accompanying major structural rearrangements. King (1980) concluded that the mode of heterochromatin evolution in *Litoria* might have been through two primary processes, i.e. addition of heterochromatin by tandem duplication and transformation of euchromatin into heterochromatin. The findings of this study for the two species seem to be in accordance with King (1980).

Studies using banding patterns are quite uncommon in amphibian groups. However, cytogenetic data represents an important tool in order to contribute to systematic approaches and to understand the evolutionary and phylogenetic relationships in this group. The results of this study obtained will be hopefully useful for amphibian categorization and can also be applied in future evolutionary studies.

Acknowledgment

We are very grateful to G. Hesse for her dedicated and expert photographic assistance.

References

Bogart, J.P. 1991. The influence of life history on karyotypic evolution in frogs. In Amphibians Cytogenetics and Evolution. D.M. Green and S.T. Sessions, Editors. Academic Press, New York, U.S.A., pp. 33-258.

Frost, D.R. 2010. Amphibian Species of the World, *Litoria* species, http://research.anh.org/herpetology/amphibia [September 12, 2010]

Green, D.M. and Session, S.T. 1991. Nomenclature for chromosome. In Amphibian Cytogenetics and Evolution. D.M. Green and S.T. Session, Editors. Academic Press, San Diego-New York, U.S.A., pp. 431-433.

http://research.amnh.org/herpetology/amphibia/names.php [March 03, 2012]

http://www.iucnredlist.org/details/full/12149/0/Litoria [February 03, 2013]

Howell, W.M. and Black, D.A. 1980. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia. 36, 1014-1015.

King, M., Tyler, M.J., Davies, M. and King, D. 1979. Karyotypic studies on *Cyclorana* and associated genera of Australian frogs. Australian Journal Zoology. 27, 373-393.

King, M. 1980. C-banding studies on Australian hylid frogs: secondary constriction structure and the concept of euchromatin transformation. Chromosoma. 80, 191-217.

King, M. 1990. Animal Cytogenetics, Vol 4/2, Gebrüder Borntraeger, Berlin, Germany. pp. 23-202.

King, M., Contreras, N., Honeycutt, R.L. 1990. Variation within and between nuclelolar organizer regions in Australian hylid frogs (Anura) shown by 18S+28S *in-situ* hybridization. Genetica. 80, 17-29.

Menzies, J.I. and Tippett, J. 1976. Chromosome numbers of Papuan hybrid frogs and the karyotype of *Litoria infrafrenata* (Amphidia, Anura, Hylidae). Journal of Herpetology. 10(3), 167-173.

Schmid, M. 1978. Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma. 66, 361-388.

Schmid, M. 1982. Chromosome banding in Amphibia. VII. Analysis of the structure and variability of NORs in Anura. Chromosoma. 87, 327-344.

Schmid, M., Haff, T., Geile, B. and Sims, B. 1983. Chromosome banding in Amphibia. VIII. An unusual XY/XX sex chromosome system in *Gastrotheca riobambae* (Anura, Hylidae). Chromosoma. 88, 69-82.

Schmid, M., Steinlein, C., Feichtinger, W., Almeida, C.G. and Duellman, W.E. 1988. Chromosome banding in Amphibia. XIII. Sex chromosomes, heterochromatin and meiosis in marsupial frogs (Anura, Hylidae). Chromosoma. 97, 33-42.

Schmid, M., Steinlein, C. and Haaf, T. 2003. Chromosome banding in Amphibia XXVII. DNA replication banding patterns in three anuran species with greatly differing genome sizes. Cytogenetic Genome Research. 101, 54-61.