Nucleus (2021) 64:129–133
https://doi.org/10.1007/s13237-019-00291-2

REPORT

Chromosomal characteristics of Taolor's stream frog (*Limnonectes taylori*) (Amphibia, Anura) from Thailand

Sumalee Phimphan[1] · **S. Aiumsumang**[1]

Received: 16 December 2018 / Accepted: 8 August 2019 / Published online: 14 August 2019

Abstract
The somatic chromosomal architecture of the Taolor's stream frog (*Limnonectes taylori*) is described in relation with the location of nucleolus organizer region (NOR) and C-banding pattern. The 10 male and 10 female samples collected from Phitsanulok and Phayao province, Northern Thailand, were used for the study. Chromosome preparations were prepared from bone marrow following standard protocol. The results show that both male and female constitute the diploid chromosome number $2n = 22$, and fundamental numbers = 44. No cytologically distinguishable sex chromosomes could be identified. *L. taylori* had the NORs adjacent to the subcentromeric on the long arm of chromosome pair 11. The C-positive heterochromatin blocks are distributed in the centromere of most chromosomes. The large heterochromatic blocks were found on short arm chromosome pair 7. The karyotype formula of *L. taylori* was comprised of $2n$(diploid)$22 = L^{m}_{4} + L^{sm}_{4} + M^{m}_{4} + M^{sm}_{2} + S^{m}_{8}$.

Keywords *Limnonectes taylori* · Karyotype · Chromosome

Introduction

The total amphibian species of Thailand consist of 137 species that have been categorized, all of which have been found and reported in Thailand, including 5 species that are vulnerable, 33 species that are near threatened, 64 species that are of least concern, 35 species that are deficient in data, and 7 species are endemic [8]. *Limnonectes taylori* is a species of frog in the family Dicroglossidae, first described from Doi Inthanon, Thailand. It occurs in northwestern Thailand and into northern Laos and extreme east-central Myanmar, possibly into adjacent Vietnam. Very little study has been conducted on chromosome, karyotype, and genetic organization of amphibian fauna in Thailand [15], although advancement in cytogenetic technique achieved during the last two decades have made it possible to perform very precise analyses of the fine structure of chromosome [13].

The karyotypic analysis of anurans belonging to the genus *Limnonectes* show $2n = 24–26$, NF = 48–52, including *L. kuhlii* and *L. blytthii* [13], *L. pileatus* [13, 14], *L. gruniens* and *L. modestus* [14], *L. blyhii* [2] (Table 1). Nowadays, chromosomal homologies and chromosomal rearrangements among difference species can be recognized by chromosome banding techniques. However basic cytogenetic studies can be used for further informative study, and support the classification of the living amphibians.

The present study provides first report on the chromosomal characteristics of *L. taylori* based on conventional staining, Ag-NOR banding and C-banding techniques. The results provide karyomorphological details of the target species to supplement taxonomy and evolutionary studies.

Materials and methods

Sample collection

Mature frogs, 10 male and 10 female were collected during rainy season from Phitsanulok province (Latitude 16.8390578, Longitude 100.381629) and Phayao province (Latitude 19.030821, Longitude 99.925868), Northern Thailand. The frogs were transferred to the laboratory and

✉ Sumalee Phimphan
joodoof@gmail.com

S. Aiumsumang
topthesun1978@gmail.com

[1] Biology Program, Faculty of Science and Technology, Phetchabun Rajabhat University, Phetchabun 67000, Thailand

Table 1 Review of cytogenetic publications of genus *Limnonectes*

Genus/species	2n	Karyotype formula	NF	NOR banded	References
Limnonectes					
L. kuhlii	26	8m + 14sm	52	2	Supaprom [8]
L. pileatus	26	16m + 10sm	52	2	Supaprom [8]
	26	16m + 10sm	52	2	Supaprom and Baimai [9]
L. blytthii	26	6m + 16sm + 2a	52	2	Supaprom [8]
	24	10m + 12sm + 2a	48	–	Donsakul and Rangsiruji [2]
L. gruniens	24	24m	48	–	Nasaruddin et al. [5]
L. modestus	24	20m + 4t	44	–	Nasaruddin et al. [5]
L. taylori	22	16m + 6sm	44	2	Present study

2n diploid chromosome number, *NF* fundamental number, *m* metacentric chromosome, *sm* submetacentric chromosome, *a* acrocentric chromosome, *t* telocentric chromosome, – not available

were kept under standard conditions for 3 days before the experimentation.

Chromosome preparation

Chromosomes were directly prepared in vivo with slight adaptations as follows [7]. The colchicine was injected into the frog's abdominal cavity. Then, the frogs were left in a box for eight hours and then sacrificed. The bone marrow was collected by cutting the head and the end of femurs and tibias, and then a syringe was used to inject 0.075 M KCl into the marrow to drive out the bone marrow tissue or cells into the plate. We gently cut the tissue to pieces as small as possible. We transferred 8 mL of cell sediments to a centrifuge tube and incubated it for 30 min at 37 °C. After centrifugation at 2000 rpm for 8 min, the KCl was discarded. Cells were fixed in fresh cool fixative up to 8 mL by gradually adding it before being centrifuged again at 2000 rpm for 8 min. Finally, the pellet was mixed with 1 mL fixative. The mixture was dropped onto a clean and cold slide by a micropipette, and then the air-dry technique was applied.

Chromosome staining

Conventional staining was done using 20% Giemsa's solution for 10 min [7]. Ag-NOR banding was performed [5] by applying two drops of 2% gelatin on the slides, followed with four drops of 50% silver nitrate. The slides were then covered with a cover slip and incubated at 60 °C for 5 min or until the slide changed brownish. After that the slides were dipped in distilled water to remove the cover glass and air-dried on the slide. C-banding was performed according to the standard protocol [12].

Microscopic analysis and image processing

The metaphase chromosomes were examined under microscope Nikon ECLIPSE. Twenty clear metaphase spreads were photographed with digital CCD camera (Nikon DS-Fi1). The length of the short arm chromosome (Ls) and the long arm chromosome (Ll) were measured and the length of the total arm chromosome (LT, LT = Ls + Ll) was calculated. The relative length (RL), the centromeric index (CI), and standard deviation (SD) of RL and CI were estimated. The CI (q/p + q) between 0.50–0.59, 0.60–0.69, 0.70–0.89, and 0.90–0.99 were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively [1, 11].

Results and discussion

The karyotypic analysis revealed that the chromosome complement of *L. taylori* consists of two large metacentric (pair 1 and 2), two large submetacentric (pair 3 and 4), two medium metacentric (pair 5 and 6), one medium submetacentric (pair 7) and four small metacentric chromosome (pair 8, 9, 10 and 11) (Fig. 1). The chromosome lengths in micrometers of 20 cells (males and females) in mitotic metaphase were measured. The length of short arm chromosome, length of long arm chromosome, length of total arm chromosomes, relative length, centromeric index, standard deviation of RL and CI, size and type of chromosome are presented in Table 2 and showed that the mean value of relative length ranged from 0.077 ± 0.004 to 0.021 ± 0.002. The proposed karyotype of this species was $2n(22) = L^{m}_{4} + L^{sm}_{4} + M^{m}_{4} + M^{sm}_{2} + S^{m}_{8}$.

The numbers of diploid chromosome and NF in this species studied herein are coincided with previous literature which reported diploid number of $2n = 24$ (NF = 44) in *L. modestus* while *L. gruniens* (2n = 24, NF = 48) [14] and 2n = 24, NF = 48 in *L. kuhlii*, *L. blytthii* [2, 13], *L. pileatus* [13, 14]. The most *Limnonectes* spp., except *L. blytthii* [2], *L. gruniens* and *L. modestus* reveal the diploid number of 2n = 22–24 and NF = 44–48. Majority, Anuran species have bi-armed metacentric or submetacentirc chromosomes and acrocentric chromosomes occur less frequently.

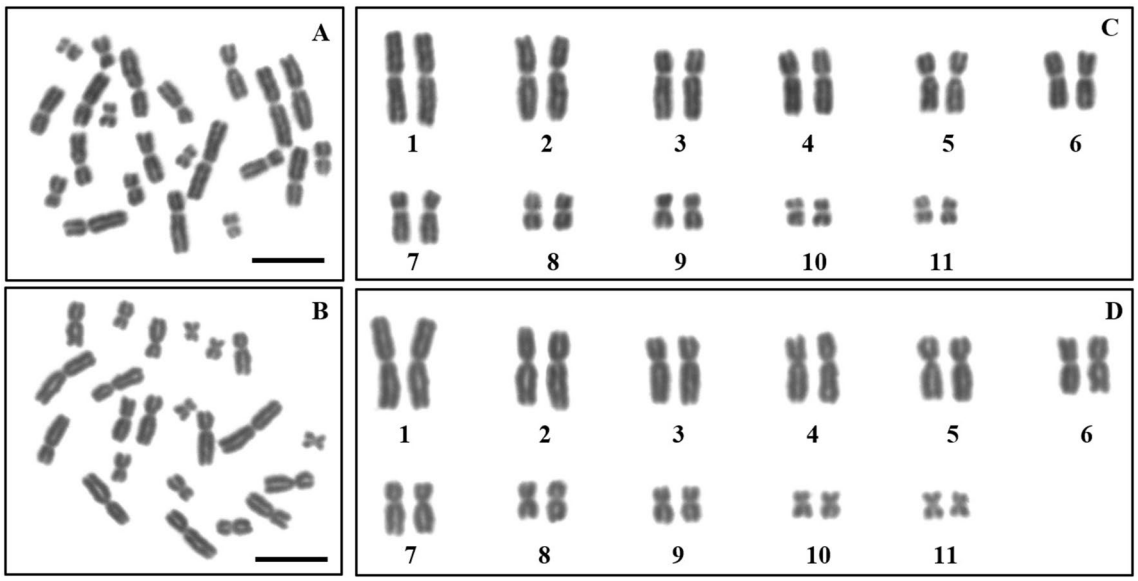

Fig. 1 Metaphase chromosome plate male (**a**) female (**b**) and karyotypes male (**c**) female (**d**) of *Limnonectes taylori*, 2*n* = 22 by conventional staining technique. Scale bar = 10 µm

Table 2 Mean length of short arm chromosome (Ls), length of long arm chromosome (Ll), length of total chromosomes (LT), relative length (RL), centromeric index (CI) and standart deviation (SD) from 20 metaphases of *Limnonectes taylori*, 2*n* (diploid) = 22

Chromosome pairs	Ls (µm)	Ll (µm)	LT (µm)	CI ± SD (mean ± SD)	RL ± SD (mean ± SD)	Chromosome size	Chromosome type
1	4.743	5.169	9.912	0.522 ± 0.012	0.077 ± 0.004	Large	Metacentric
2	3.676	4.925	8.601	0.573 ± 0.019	0.066 ± 0.002	Large	Metacentric
3	2.831	4.643	7.474	0.621 ± 0.019	0.058 ± 0.002	Large	Submetacentric
4	2.696	4.247	6.943	0.612 ± 0.017	0.054 ± 0.001	Large	Submetacentric
5	2.650	3.661	6.310	0.580 ± 0.013	0.049 ± 0.002	Medium	Metacentric
6	2.541	3.498	6.038	0.579 ± 0.027	0.047 ± 0.002	Medium	Metacentric
7	2.070	3.202	5.272	0.607 ± 0.026	0.041 ± 0.002	Medium	Submetacentric
8	1.820	2.260	4.080	0.554 ± 0.019	0.031 ± 0.002	Small	Metacentric
9	1.641	2.162	3.803	0.569 ± 0.016	0.029 ± 0.002	Small	Metacentric
10	1.327	1.704	3.031	0.562 ± 0.015	0.023 ± 0.002	Small	Metacentric
11[a]	1.192	1.590	2.782	0.572 ± 0.024	0.021 ± 0.002	Small	Metacentric

[a]NORs bearing chromosomes (satellite chromosome)

In the present study acrocentric chromosomes were absent. We suggest here that no cytologically distinguishable sex-chromosome was observed which is consistent to *L. kuhlii* and *L. blytthii* [13], *L. pileatus* [13, 14], *L. gruniens* and *L. modestus* [14], *L. blyhii* [2] and other anuran in the subfamily Dicroglossidae. It may be possible that the frog's sex-chromosomes are at the initiation of differentiation and hence these chromosomes which contain the sex determination gene cannot be detected by cytogenetic analyses.

The development of silver staining technique [3] to detect metaphase chromosome sites of NORs has greatly facilitated comparative studies of NORs variation. Silver staining of NORs is considered as one of the standard banding methods and has assumed considerable importance in the characterization of a species karyotype. The results of NORs in *L. taylori* could be observed in one pair of chromosomes in both male and female at the near top (subcentomeric region) of the long arm of metacentric chromosome pair 11 (Fig. 2). From C-banding technique has been shown that the C-heterochromatin is confined to the centromere (Fig. 3) [9]. The present investigation agrees with the earlier observations [3, 4, 6, 7, 10]. C-positive heterochromatin blocks in karyotype of *L. taylori*are mainly distributed in the centromere of most

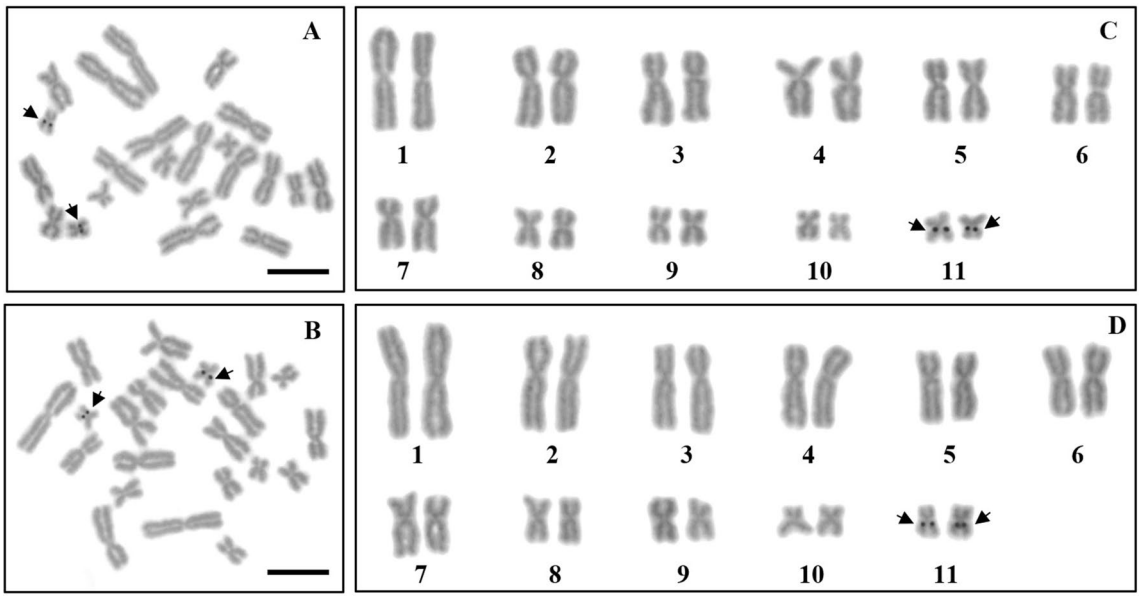

Fig. 2 Metaphase chromosome plate male (**a**) female (**b**) and karyotypes male (**c**) female (**d**) of *L. taylori*, $2n = 22$ by Ag-NOR banding technique. Scale bar indicate 10 µm. Arrows indicate satellite chromosome/NORs pair 11

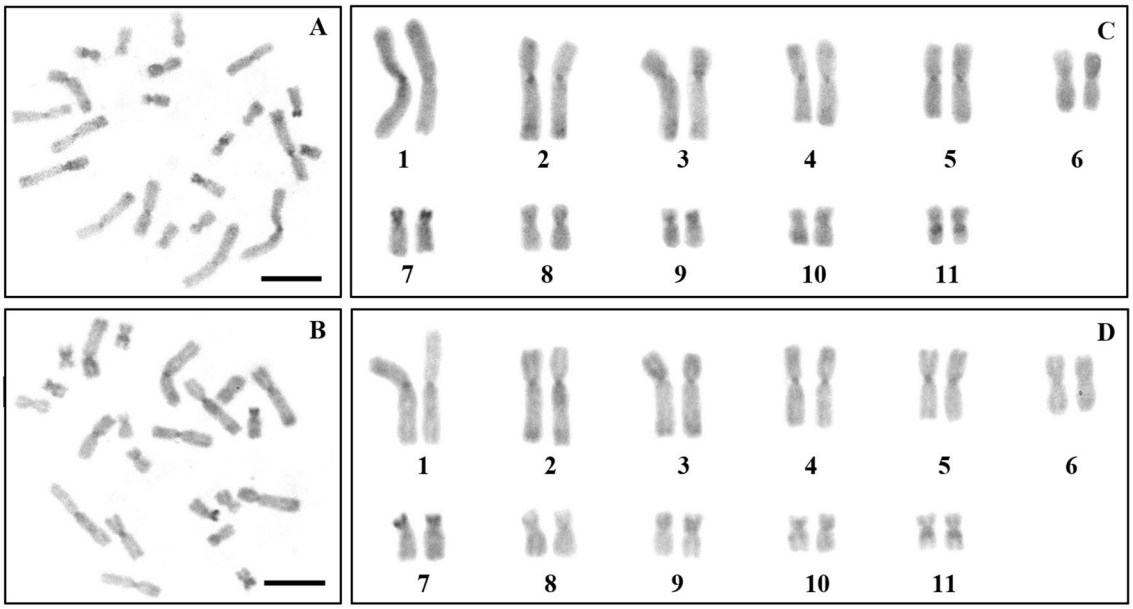

Fig. 3 Metaphase chromosome plate male (**a**) female (**b**) and karyotypes male (**c**) female (**d**) of *L. taylori*, $2n = 22$ by C-banding technique. Scale bar = 10 µm

chromosomes, of those the C-positive are exhibited on the short arm of chromosome pair 7. The telomeric ends of the long arms of pairs 2, 3 were distinctly C-positive and these are found in paracentromeric region on long arm chromosome pair 11. Their respective idiograms of the *L. taylori*from conventional staining and Ag-NORs banding techniques are shown in Fig. 4. Such studies are useful in resolving taxonomic ambiguities among closely related frog species and can also throw light on karyoevolution and speciation of the anuran species.

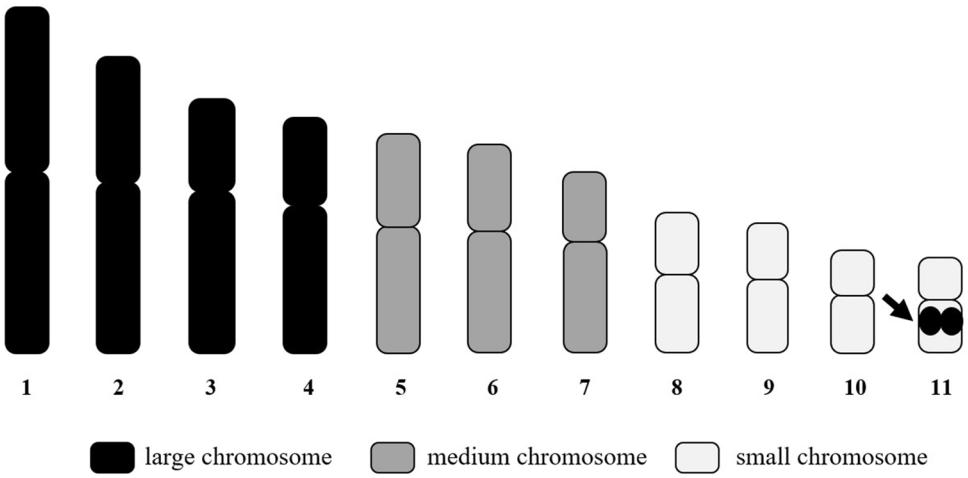

Fig. 4 Idiogram showing lengths and shape of chromosomes of the *L. taylori*, $2n = 22$ by conventional staining and Ag-NOR banding technique (the arrows indicate satellite chromosome/NORs pair 11)

Acknowledgements The authors are grateful to Phetchabun Rajabhat University and Prof. Dr. Alongklod Tanomtong for his valuable suggestions.

References

1. Chaiyasut K. Cytogenetics and cytotaxonomy of the family Zephyranthes. Bangkok: Chulalongkorn University Press; 1989 (in Thai).
2. Donsakul T, Rangsiruji A. Liver karyotypes in *Limnonectes blythii*, *Rana erythraea*, *Rana leptoglossa*, *Occidozyga martensii* and *Glyphoglossus molossus* (Amphibia, Anura). In: Proceedings of 43rd Kasetsart University annual conference, Thammasart University, Bangkok; 2005.
3. Heppich S. Hybridogenesis in *Rana esculenta*: C-band karyotypes of *Rana ridibunda*, *Rana lessonae* and *Rana esculenta*. Z Zool Syst Evolut Forsch. 1978;16:27–39.
4. Heppich S, Tunner HG. Chromosomal constitution and C-banding in homotypic *Rana esculenta* crosses. Mitt Zool Mus Berlin. 1979;55:111–4.
5. Howell WM, Black DA. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia. 1980;36:1014–5.
6. Iizuka K. Constitutive heterochromatin and nucleolus organizer regions in Japanese brown frogs, *Rana japonica* and *Rana ornativentris*. Jpn J Herpetol. 1989;13:15–20.
7. Miura I. Sex chromosome differentiation in the Japanese brown frog, *Rana japonica*. Sex-related heteromorphism of the distribution pattern of constitutive heterochromatin in chromosome no. 4 of the Wakuya population. Zool Sci. 1994;11:797–806.
8. Nabhitabhata J, Chan-ard T. Thailand red data: mammals, reptiles and amphibians. Bangkok: Office of Natural Resources and Environmental Policy and Planning (ONEP); 2005.
9. Nasaruddin, Suriana, Adi DA, Salamansyah. The karyotype of seven species of amphibians (Anuran order) from South-east Sulawesi. Veteriner. 2009;10:77–86.
10. Odierna G, Vences M, Aprea G, Lotters S, Andreone F. Chromosome data for Malagasy poison frogs (Amphibia: Ranidae: Mantella) and their bearing on the taxonomy and phylogeny. Zool Sci. 2001;18:505–14.
11. Phimphan S, Tanomtong A, Saengphan N, Sangpakdee W. The first karyological study in freshwater Prawn, *Macrobrachium lanchesteri* (Decapoda, Palaemonidae) from Thailand. The Nucleus. 2018;62:77–82.
12. Sumner AT. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res. 1972;75:304–6.
13. Supaprom T. Cytogenetics of amphibians in Thailand. Ph.D. dissertation, Mahidol university; 2003.
14. Supaprom T, Baimai V. Karyotypes of ten species of ranid frogs (Anura: Ranidae) from Thailand. Amphib Reptil. 2004;25:104–11.
15. Taylor EH. The amphibian fauna of Thailand. Univ Kansas Sci Bull. 1962;43:265–599.