Food Agricultural Sciences and Technology (FAST)
2026; *12*(1). https://doi.org/10.14456/fast.2026.5

Research Article

Karyotypic analysis of the blunt-headed burrowing frog (*Glyphoglossus molossus*) using conventional staining and NOR-banding techniques

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Abstract – This study investigated the karyotype of the blunt-headed burrowing frog (*Glyphoglossus molossus*) using ten individuals (five males and five females) collected from Chombueng district, Ratchaburi province. Chromosome preparations were made from bone marrow cells, and mitotic chromosomes were obtained using a colchicine-hypotonic-fixation-air drying technique. Conventional staining was performed with 20% Giemsa's solution to assess the diploid number, shape, and size of the chromosomes. The results revealed that the diploid chromosome number (2n) for the blunt-headed burrowing frog is 26, with the fundamental number (NF) of 52 in both males and females. The karyotype consists of 6 large metacentric chromosomes, 2 medium metacentric, 2 medium submetacentric, 12 small metacentric, and 4 small submetacentric chromosomes. NOR staining revealed a pair of NOR loci located at the subtelomeric region of the short arm of a submetacentric chromosome. Notably, no distinguishable sex chromosomes were observed. The karyotype formula for the species is 2n (26) = L^{m}_{6}+ M^{m}_{2}+M^{sm}_{2}+ S^{m}_{12}+ S^{sm}_{4}.

Keywords: *Glyphoglossus molossus*, chromosome, karyotype, Thailand

Received: 31st March 2025,
Revised: 23rd June 2025,
Accepted: 4th July 2025

Citation: Sribenja, K., & Getlekha, N. (2025). Karyotypic analysis of the blunt-headed burrowing frog *(Glyphoglossus molossus)* using conventional staining and NOR-banding techniques. *Food Agricultural Sciences and Technology (FAST)*, *12*(1), 50-58. https://doi.org/10.14456/fast.2026.5

1. Introduction

Family Microhylidae consists of 10 subfamilies, approximately 64 genera, and around 436 species. In Thailand, the Asian painted frog (*Kaloula pulchra*) and Inornate Froglet (*Micryletta inornata*) are the most common, while certain species, like the blunt-headed burrowing frog (*Glyphoglossus molossus*), are also consumed (Lauhachinda, 2009). The blunt-headed burrowing frog is the sole living species in the *Glyphoglossus* genus. It features a stout body, a very short face, a narrow, blunt mouth, small eyes, and a body that is typically brown or gray-black with a white belly; some individuals may also have yellow spots scattered across their body. All four feet are webbed for swimming, and there is a ridge under the hind feet for digging. In Thailand, this species is found exclusively in areas north of Prachuap Khiri Khan Province. Due to over-harvesting for consumption and habitat changes, it is currently at risk of extinction. The Department of Fisheries has been supporting efforts to farm it as an economic species (Siripiyasing et al., 2018; Nonsrirach, 2019).

The Ratchaburi region lies at the intersection of the Sundaic and Indochinese sub-zoological zones. Species inhabiting this transitional zone often exhibit low population densities and are closely related to sibling species occupying similar ecological niches. Consequently, while this area harbors a rich diversity of species, the close morphological and ecological similarities among them pose challenges in distinguishing and studying each species.

Cytogenetics involves the study of the components within a cell's nucleus, focusing on the number, shape, and size of chromosomes to better understand their distinct features and characteristics (Chaiyasut, 1989). Since chromosomes are key features that define each organism, the study of cellular genetics plays a crucial role in applying this knowledge to various fields, such as classification, breeding, genetic conservation, and understanding evolutionary relationships. There are several methods to study cellular genetics, including directly preparing chromosomes on slides (direct chromosome preparation), culturing cells or tissues in vitro, photographing chromosomes under a microscope, measuring the size and length of chromosomes, and conducting karyotype analysis (Supaprom et al., 1992).

Previous investigations into the cytogenetics of the blunt-headed burrowing frog (*G. molossus*) have revealed studies conducted in the northeastern regions, including Nakhon Ratchasima, Kalasin, and Ubon Ratchathani prov inces (Donsakul & Rangsiruji, 2005; Phapakdee et al., 2019; Supaprom et al., 1992). However, these studies are limited compared to the broader distribution of *G. molossus*, with no reports found in certain regions. Basic biological and ecological data on the *G. molossus* remain scarce in Thailand, including cellular genetic research. Such data is crucial not only for conservation management but also for understanding the evolutionary history of the species, conserving its genetic resources, providing foundational information for future studies, and supporting taxonomic classification.

This study aims to investigate the fundamental cytogenetic characteristics of the blunt-headed burrowing frog (*G. molossus*) populations from Chom Bueng District, Ratchaburi Province, Thailand. By comparing these findings with data from northeastern Thailand populations, the research seeks to identify the presence of sex chromosome heteromorphism and the localization of nucleolus organizer regions (NORs) within the species. The outcomes will provide essential chromosomal data to inform future genetic conservation strategies.

2. Materials and methods

2.1 Sampling collection

Ten male and female of the blunt-headed burrowing frogs (*G. molossus*) were collected from various water sources in Chom Bueng District, Ratchaburi Province. In order to the trials minimize pain during the slaughter process, the animals were immersed in ice-cold water to induce anesthesia prior to euthanasia. This procedure was reviewed and approved by the Ethics Committee of Muban Chombueng Rajabhat University and the Institute of Animal for Scientific Purposes Development (IAD) Committee (Approval No. U1-04484-2559). They were classified and identified by comparing their morphology with the descriptions provided in the study by Niyomwan et al. (2019) before proceeding to the next phase of the analysis.

Figure 1. Characteristics of the blunt-headed burrowing frog (*G. molossus*).

2.2 Cytogenetic investigation

Chromosome preparations were made using the direct method, based on Sumner (1990) technique. Frog samples were injected with a 0.02% colchicine solution (1 ml/100 g body weight) into the abdomen and left for 12 hours. The bone marrow from the arms and legs was surgically extracted and then finely chopped in a hypotonic solution (0.075 M KCl) using scissors. The chopped cell sediment was transferred into a 15 ml centrifuge tube containing the hypotonic solution and left for 30 minutes. Afterward, it was centrifuged at 2,500 rpm for 8 minutes, and the supernatant was discarded, leaving only the sediment. The first fixation step involved slowly adding a fixative solution (a 3:1 ratio of methanol and concentrated acetic acid) at 5°C to the tube, mixing the sediment with the fixative. The volume of the fixative was adjusted to 7 ml, and the mixture was centrifuged again, with the supernatant removed. This process was repeated until a cloudy white cell precipitate formed. The remaining fixative was removed, leaving about 3-4 ml, and the precipitate was mixed thoroughly with the remaining solution. A small portion (about 0.2 ml) of the cell precipitate was dropped onto a clean, dry slide placed on a heating plate at 80°C, with two drops per slide. The slide was left to dry, stained with a 20% Giemsa stain solution for 20 minutes and NORs were detected using the silver staining technique described by Howell and Black (1980), with minor modifications. Finally, the slide was examined and photographed using a light microscope with a 1,000X magnification.

2.3 Chromosomal analysis

The diploid chromosome number and the fundamental number (NF: metacentric, submetacentric, and acrocentric chromosomes were assigned a value of 2, while telocentric chromosomes were assigned a value of 1) were classified. The karyotype and idiogram preparation followed the method outlined by Chaiyasut (1989). Metaphase chromosome images were selected based on criteria such as well-distributed chromosomes, appropriate length, complete chromosome count, and clear visibility of shapes. The lengths of chromosomes from 20 cells (both male and female) were measured, including the short arm length (Ls), long arm length (Ll), total length (LT), centromeric index (CI: Ll/LT), and relative length (RL: LT/ΣLT), to determine the type and size of the chromosomes. A standard idiogram was then constructed using a computer program, which classified the chromosomes

according to their shape and size based on the measured lengths.

3. Results

The blunt-headed burrowing frog (*G. molossus*) from Chom Bueng District, Ratchaburi Province has a diploid chromosome number of 26 and the fundamental number of 52. Chromosome size was measured through metaphase analysis, with the short arm length (Ls), long arm length (Ll), total chromosome length (LT), centromeric index (CI), and relative length (RL) being recorded (Table 1) to categorize the type and size of the chromosomes. This data was then used to construct a karyotype and standard idiogram. The analysis revealed that the chromosomes of *G. molossus* can be grouped into three size categories: large chromosomes (> 4.180 μm) totaling 6 chromosomes (pairs 1-3); medium-sized chromosomes (3.206 to 4.180 μm), totaling totaling 4 chromosomes (pairs 4-5) and small chromosomes (< 3.206 μm) totaling 16 chromosomes (pairs 6-13) (Figures 2 and 3). The karyotype of the *G. molossus* consists of 6 large metacentric chromosomes, 2 medium metacentric chromosomes, 2 medium sub metacentric chromosomes, 12 small metacentric chromosomes, and 4 small sub metacentric chromosomes (Figures 2 and 3). The Ag-NOR sites are located in the subtelomeric region of the short arm of a sub metacentric chromosome (pair 12). No karyotypic differences were observed between male and female frogs. The karyotype formula formula for thespecies is 2n (26) = L^m_6+ M^m_2+M^{sm}_2+ S^m_{12}+S^{sm}_4

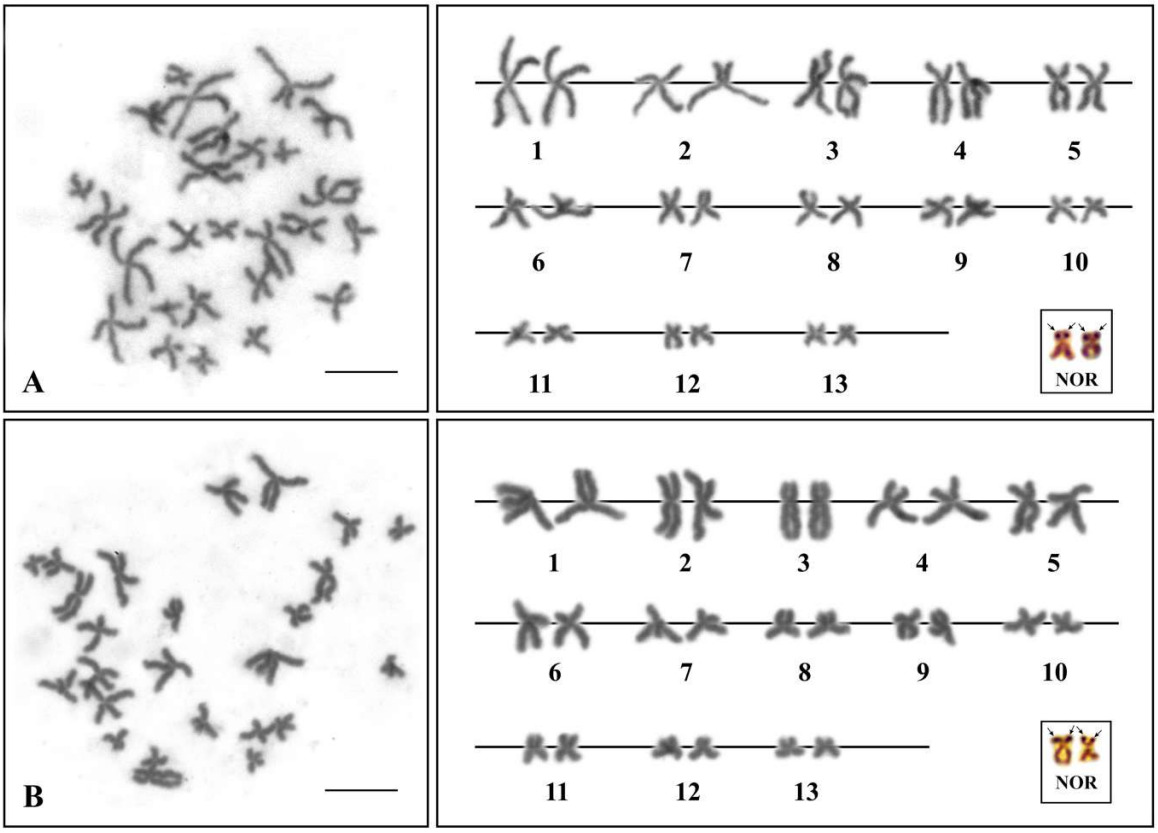

Figure 2. Metaphase cells and karyotypes of both male (A) and female (B) of the blunt-headed burrowing frogs (*G. molossus*, 2n=26) were analyzed using the conventional staining method. The boxes indicate the NORs, with a scale bar of 5 μm.

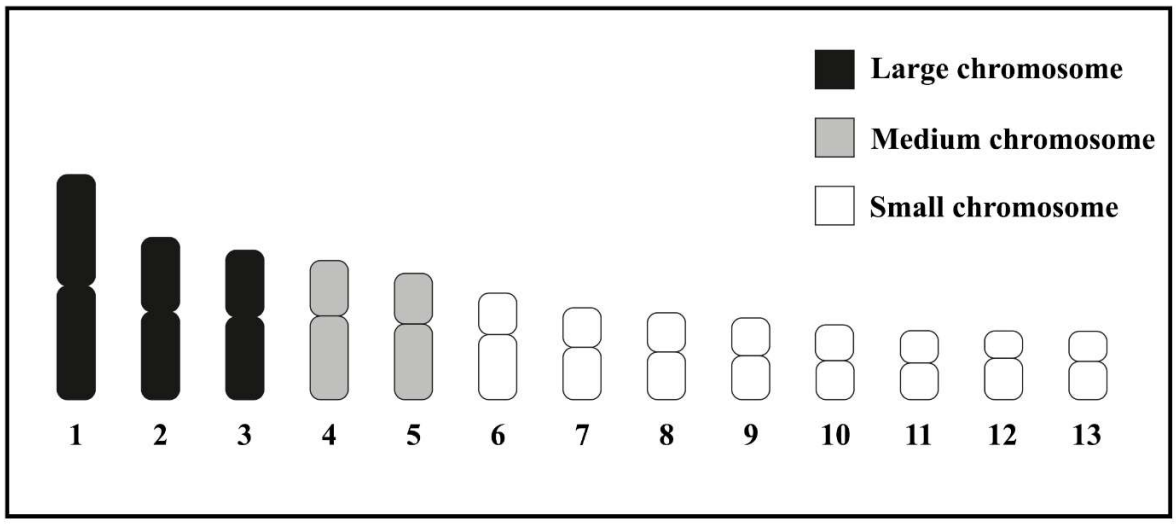

Figure 3. Standard idiogram of the blunt-headed burrowing frog (*G. molossus*, 2n=26) using the conventional staining technique.

Table 1. Mean length of short chromosome arm (Ls), long chromosome arm (Ll), length of each chromosome pair (LT) in micrometers, centromeric index (CI), relative length (RL), and standard deviation (SD) of CI and RL of the blunt-headed burrowing frog in both male and female (*G. molossus*, 2n=26, NF=52).

Pairs	Ls	Ll	LT	CI ± SD	RL ± SD	Size	Type
1	3.170	3.243	6.413	0.500±0.042	0.155±0.011	Large	Metacentric
2	2.119	2.504	4.623	0.543±0.037	0.112±0.007	Large	Metacentric
3	1.900	2.358	4.257	0.554±0.026	0.103±0.006	Large	Metacentric
4	1.578	2.386	3.964	0.602±0.010	0.096±0.005	Medium	Submetacentric
5	1.440	2.158	3.598	0.599±0.020	0.088±0.007	Medium	Metacentric
6	1.181	1.855	3.036	0.610±0.029	0.074±0.006	Small	Submetacentric
7	1.125	1.492	2.617	0.568±0.046	0.064±0.005	Small	Metacentric
8	1.117	1.360	2.477	0.547±0.029	0.060±0.002	Small	Metacentric
9	1.076	1.253	2.330	0.537±0.029	0.056±0.003	Small	Metacentric
10	1.021	1.111	2.133	0.517±0.040	0.052±0.002	Small	Metacentric
11	0.920	1.045	1.965	0.526±0.076	0.047±0.005	Small	Metacentric
12*	0.776	1.181	1.957	0.602±0.028	0.047±0.002	Small	Submetacentric
13	0.858	1.090	1.948	0.557±0.057	0.047±0.003	Small	Metacentric

Note: Chromosomes size categories: large chromosomes (> 4.180 μm); medium-sized chromosomes (3.206 to 4.180 μm), and small chromosomes (< 3.206 μm) and * = NOR-bearing chromosomes.

4. Discussion

This study prepared chromosomes from actively dividing bone marrow cells of *G. molossus* and arrested cell division at the metaphase stage using a colchicine solution. The chromosomes were then stained with conventional Giemsa stain to examine their number, size, and shape, followed by karyotyping, a widely recognized method known for providing accurate and reliable results. The karyotype analysis of *G. molossus* revealed a diploid chromosome count of 2n = 26, which aligns with previous studies conducted in the northeastern regions of Nakhon Ratchasima, Kalasin, and Ubon Ratchathani provinces (Supaprom et al., 1992; Donsakul & Rangsiruji, 2005; Supaprom & Udomsirichakorn, 2005; Phapakdee et al., 2019), all of which also reported 2n = 26 chromosomes for *G. molossus* (Table 2) this suggests that *G. molossus* might share a common ancestor. Furthermore, Aiumsumang et al. (2023) reported a diploid chromosome count of 2n = 26 for *Polypedates leucomystax* and *Fejervarya limnocharis* from Phetchabun Province. This consistency provides valuable foundational information for further studies, including species identification, taxonomic relationships, and the evolutionary analysis of *G. molossus* across different populations, as well as in other anuran species.

The chromosomes of the *G. molossus* consist of three sizes: 6 large chromosomes, 4 medium chromosomes, and 16 small chromosomes. All chromosomes are bi-armed, and the fundamental number is 52 in both males and females. In amphibian evolution, chromosome number reduction often occurs through the fusion of smaller chromosomes into larger ones, primarily via centric or tandem fusions. Initially, small chromosomes may fuse end-to-end (tandem fusion), forming telocentric chromosomes. Subsequently, these telocentric chromosomes can undergo centric fusion, resulting in biarmed (metacentric or submetacentric) chromosomes. This progressive fusion process has led to the predominance of biarmed chromosomes over telocentric ones in many amphibian lineages (Supanuam, 2018).

This study aims to determine whether sex chromosome heteromorphism exists within the species. However, no morphological differences were observed between the sex chromosomes. In most amphibians, sex chromosomes are homomorphic (undifferentiated) in both sexes and exhibit frequent turnover. This contrasts with the sex chromosomes of mammals and birds, where they are heteromorphic in one sex and remain highly conserved. The mechanisms of sex determination in anuran amphibians, especially concerning the turnover of sex-determining genes and chromosomes, are outlined, and their evolution is explored (Miura, 2018).

In this study, we identified a pair of Ag-NOR sites located in the subtelomeric region of the short arm of a submetacentric chromosome. This conserved NOR location aligns with the observations of Schmid et al. (1990), who reported that in Anura species, NORs are typically situated in the same chromosomal region among closely related species. However, there are several exceptions, such as in dendrobatid species of the genus *Epipedobates* (Aguiar et al., 2002). Although no other species of *Glyphoglossus* have been studied using Ag–NOR labeling, it is probable that the NORs are located in the same chromosomal region in other species as well. The NOR location is a conserved trait, supported by the presence of NOR-bearing secondary constrictions observed in all karyotypically analyzed species of *Hylodes* and *Crossodactylus*. This finding aligns with Schmid et al. (1990), who asserted that in closely related species, NORs are typically located in the same chromosomal region. In the case of *Megaelosia* (Rosa et al., 2003), although the NOR is positioned on different chromosome pairs, the chromosomal regions appear to be homeologous across the three karyotypes described. This indicates

that the NOR's position may have shifted due to a series of structural rearrangements in the chromosomes (Aguiar et al., 2004).

However, comparing the karyotypes of *G. molossus* from previous studies, differences were found in the types of chromosomes, but the basic chromosome number remained consistent (2n=26). All chromosomes were bi-armed in all reports, with most being metacentric or submetacentric (Table 2). These findings suggest that the chromosomes of *G. molossus* are highly conserved.

Table 2. Comparative of karyotype studies of the blunt-headed burrowing frog (*G. molossus*).

Species	2n	NF	Karyotype	Refereces
Glyphoglossus molossus	26	52	18m +8sm	Donsakul & Rangsiruji (2005)
	26	52	20m +6m	Phapakdee et al. (2019)
	26	52	6 m+18sm +2a	Supaprom et al. (1992)
	26	52	14m+12sm	Supaprom & Udomsirichakorn (2005)
	26	52	20m+6sm	**Present study**

Note: m=metacentric, sm=submetacentric and a=acrocentric chromosome.

Previous research has indicated that amphibians exhibit a limited range of karyotypic variation. Karyotype analysis is a valuable tool for studying comparative differences between genera and species, and it plays an important role in taxonomic classification (Stace, 2000). Variation in karyotypes is often observed between males and females, as well as in somatic cells and gametes, particularly in populations sampled from different regions.

In the order Anura, the diploid chromosome number in Thai amphibians varies between 2n = 22 to 28, which is divided into two suborders: Mesobratachia and Neobratachia. The suborder Mesobratachia retains a relatively primitive karyotype, characterized by a chromosome count of 2n = 24-26, comprising both bi-armed and mono-armed chromosomes. In contrast, the suborder Neobratachia exhibits more advanced karyotypes, with a chromosome count of 2n = 22-28, consisting solely of bi-armed chromosomes (Supanuam, 2018). No instances of polyploid chromosome numbers were observed in any Thai amphibians, most species possess bi-armed chromosomes, which are categorized as symmetrical karyotypes, meaning the chromosomes are primarily metacentric and/or submetacentric. These species do not exhibit bimodal karyotypes, which would involve distinct sets of large and small chromosomes. This suggests a high level of chromosomal conservation within amphibians of the order Anura (Chaithiangtham & Patawang, 2020).

In Thailand, the evolution of Microhylidae chromosomes has been characterized by 10 distinct karyotypes, exhibiting varying diploid numbers of 2n = 22, 24, 26, and 28. Correspondingly, the fundamental numbers differ among these karyotypes, encompassing 44, 48, 52, and 56. Chromosomal morphology predominantly includes metacentric and submetacentric types, with the potential presence of subtelocentric or acrocentric chromosomes. These karyotypes are characterized by symmetrical arrangements and unimodal

distribution patterns. The family exhibits a range of karyotypic diversity, from moderately to highly developed structures, characterized by relatively low diploid chromosome numbers. Their karyotypes are symmetrical and unimodal, reflecting a gradual decrease in chromosome size without distinct macro- and microchromosome differentiation (Supanuam, 2018).

5. Conclusion

This report provides essential information on the number of chromosomes, NOR positions, and karyotype patterns of *G. molossus*, serving as a foundational knowledge base. This data lays the groundwork for more detailed and expansive studies in the future. It can also serve as a model or reference for similar research on other amphibians or in different regions of the country.

Thailand is home to a rich diversity of amphibians; however, studies focused on these species, particularly in the field of cellular genetics, remain limited in comparison to the number of species identified. This research adds valuable biological data to the existing body of knowledge on amphibians. Cellular genetic knowledge, especially chromosomal markers, is increasingly being utilized to explore the evolutionary relationships of amphibians and is important for cell taxonomy (Donsakul & Rangsiruji, 2005). Moreover, the stability of karyotypes in the face of low genetic diversity underscores the necessity for urgent conservation measures. Genetic information is pivotal for breeding programs, aiding in the enhancement of amphibian population quality and resilience.

Acknowledgement

This research was supported by the Plant Genetic Conservation Project Under the Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn (RSPG), Muban Chombueng Rajabhat University, for their financial support. We would also like to express our gratitude to the Department of Biology, Faculty of Science and Technology, Muban Chombueng Rajabhat University, for providing facilities, encouragement, and valuable assistance in verifying the accuracy of the reports.

References

Aguiar, O., Jr., de Carvalho, K. A., & Giaretta, A. A. (2004). Cytogenetics of *Hylodes* and *Crossodactylus* species (Anura, Leptodactylidae) with comments on Hylodinae/Dendrobatidae relationships. *Genetica, 121*(1), 43–53. https://doi.org/10.1023/B:GENE.0000019926.50310.26

Aguiar, O., Lima, A. P., Giaretta, A. A., & Recco-Pimentel, S. M. (2002). Cytogenetic analysis of four poison frogs of the *Epipedobates* genus (Anura: Dendrobatidae). *Herpetologica*, *58*(3), 293–303. https://doi.org/10.1655/0018 -0831(2002)058[0293:CAOFPF]2.0.CO;2

Aiumsumang, S., Khoomsab, K., Tangsuwan, I., & Phimphan, S. (2023). Standardized chromosome pattern of some amphibians in Phetchabun Province. *KKU Science Journal*, *51*(3), 306–313. https://doi.org/10.14456/kkuscij.2023.25

Chaithiangtham, P., & Patawang, I. (2020). Karyotypic study of Taylor's stream frog (*Limnonectes taylori*) in Chiang Mai, Thailand. *Journal of Science and Technology, Mahasarakham University*, *39*(4), 355–360.

Chaiyasut, K. (1989). *Cell genetics and phylogenetic cell classification of the Zephyranthes genus* [Doctoral dissertation, Chulalongkorn University]. Thai-Lis.

Donsakul, T., & Rangsiruji, A. (2005). Liver karyotypes in *Limnonectes blythii, Rana erythraea, Rana leptoglossa, Occidozyga martensii* and *Glyphoglossus molossus*

(Amphibia, Anura). In *Proceedings of the 43rd Kasetsart University Annual Conference: Veterinary Science and Science Fields* (pp. 544–551). Kasetsart University.

Howell, W. M., & Black, D. A. (1980). Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: A 1-step method. *Experientia*, *36*(8), 1014–1015. https://doi.org/10.1007/BF01953855

Lauhachinda, W. (2009). *Herpetology of reptiles and amphibians* [in Thai]. Kasetsart University.

Miura, I. (2018). Sex determination and sex chromosomes in Amphibia. *Sexual Development*, *11*(5–6), 298–306. https://doi.org/10.1159/ 000485270

Niyomwan, P., Srisom, P., & Pawangkhanant, P. (2019). *Amphibians of Thailand* [in Thai]. Pappim.

Nonsrirach, T. (2019). *Genetic diversity, genetic structure, and morphological variation of the truncated-snouted burrowing frog (Glyphoglossus molossus) in northeastern Thailand* [Unpublished master's thesis]. Mahasarakham University.

Phapakdee, J., Pakdeenarong, N., & Lauprasert, K. (2019). Cell genetics of the frogs consumed in Kalasin Province. In *Proceedings of the 14th academic conference of Mahasarakham University: "50 years of Mahasarakham University, wisdom for the people"* (pp. 702–710). Faculty of Humanities and Social Sciences, Mahasarakham University.

Rosa, C., Aguiar, O., Giaretta, A. A., &Recco-Pimentel, S. M. (2003). Karyotypic variation in the genus *Megaelosia* (Anura, Hylodinae) with the first description of a B-chromo some in a leptodactylid frog. *Copeia*, *2003*(1), 166–174. https://doi.org/10.1643/0045-8511(2003) 003[0166:KVITGM]2.0.CO;2

Schmid, M., Steinlein, C., Nanda, I., & Epplen, J. T. (1990). Chromosome banding in Amphibia. In E. Olmo (Ed.), *Cytogenetics of amphibians and reptiles* (pp. 21–45). Birkhäuser Verlag.

Siripiyasing, P., Srihabut, J., Aunboon, C., & Yomsawat, C. (2018). *Research report on cell genetics of some amphibians in the area of Mahasarakham Rajabhat University*. Mahasarakham Rajabhat University.

Stace, C. A. (2000). Cytology and cytogenetics as a fundamental taxonomic resource for the 20th and 21st centuries. *Taxon*, *49*(3), 451–477. https://doi.org/10.2307/1224344

Sumner, A. T. (1990). *Chromosome banding*. Unwin Hyman.

Supanuam, P. (2018). Chromosome evolution of amphibians in Thailand. *KKU Science Journal*, *46*(4), 654–663. retrieved from https://ph01.tci-thaijo.org/index.php/KKUSciJ/article/view/249939

Supaprom, T., & Udomsirichakorn, K. (2005). *Cytogenetics and biodiversityof amphibians in Patam National Park Ubon Ratchathani Province: Report on the implementation of the Cultural Conservation Project*. Ubon Ratchathani University.

Supaprom, T., Arunmongkhonphon, W., & Udomsirichakorn, K. (1992). Studies on the chromosome complements and karyotypes of *Glypholossus molossus* Gunther and *Rhacophorus leucomystax* Kuhl. In *Proceedings of the 30th Kasetsart University Annual Conference* (pp. 717–724). Kasetsart University.