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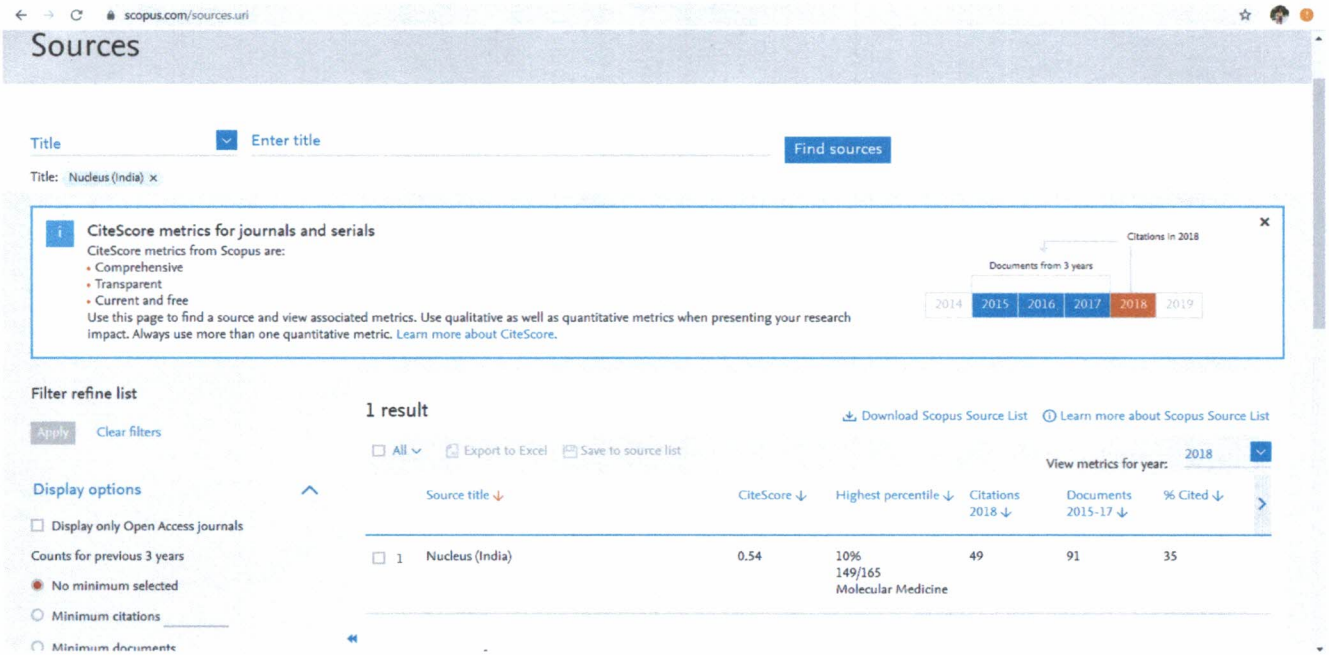

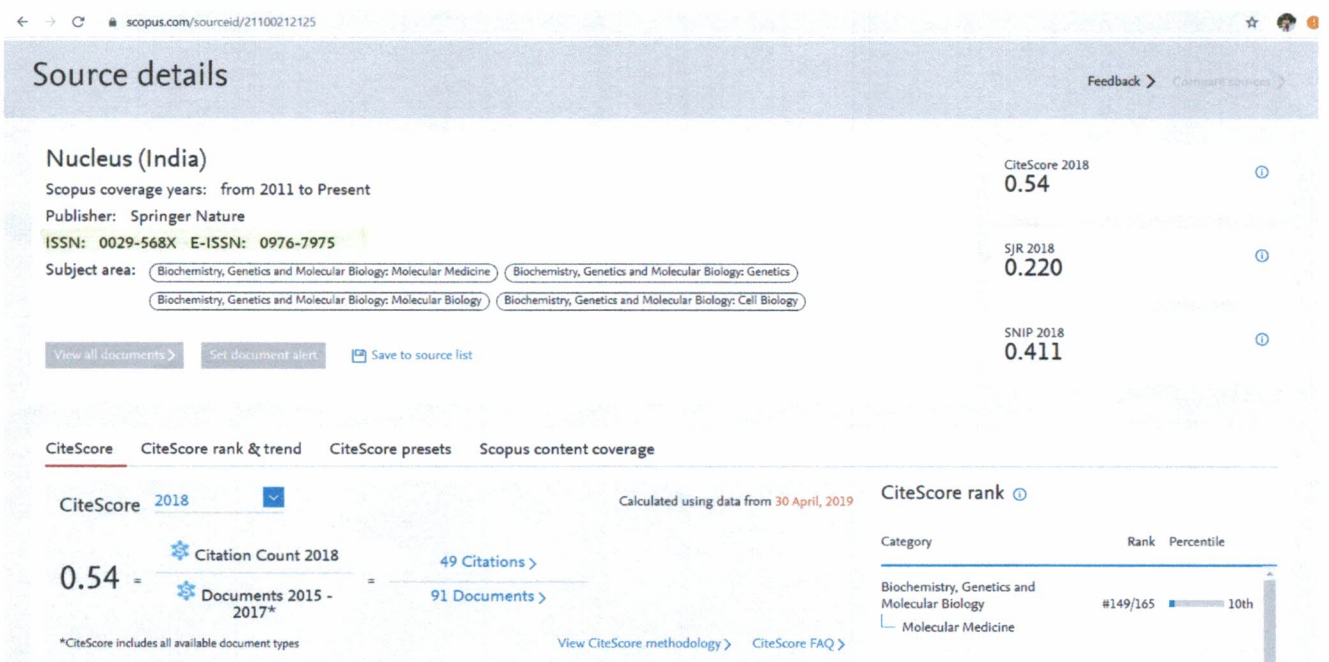

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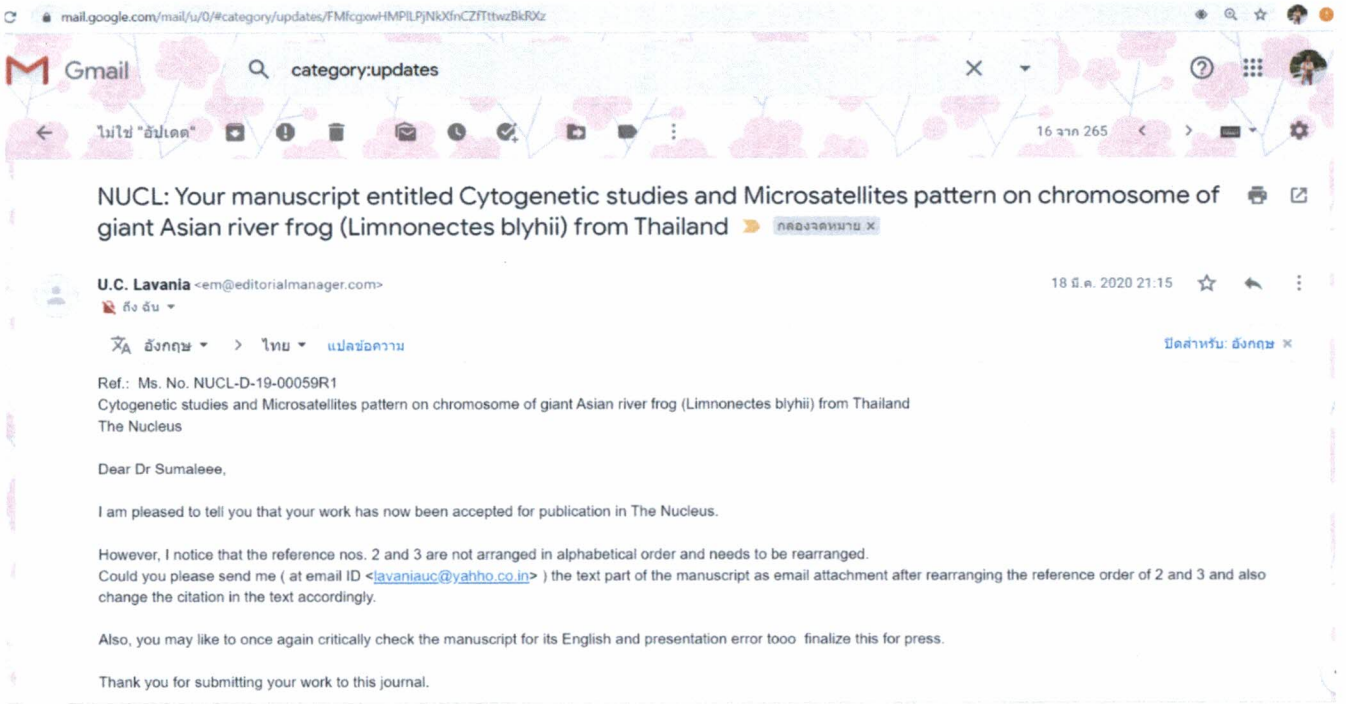

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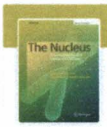

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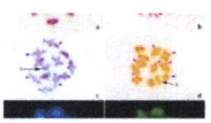

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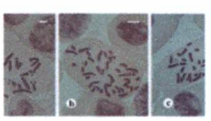

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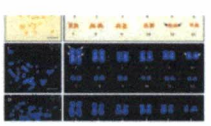

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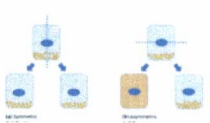

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REPORT

Karyomorphological delineation and linear differentiation of microsatellite patterns, and meiosis in giant Asian river frog (*Limnonectes blyhii*) from Thailand

Sumalee Phimphan[1] · S. Aiumsumang[1] · A. Tanomtong[2] · S. Jantarat[3]

Received: 11 September 2019 / Accepted: 15 April 2020

Abstract
The present investigation provides information on linear chromosomal differentiation and microsatellite patterns [$d(CA)_{15}$, $d(CGG)_{10}$, $d(TA)_{15}$] in the genome of giant Asian river frog (*Limnonectes blyhii*). Five male and female samples each collected from southern Thailand, were used for the study. The metaphase chromosome preparations were prepared from the bone marrow using the standard protocol. Results show that both male and female have the diploid chromosome number 2n = 24, and the fundamental number NF = 48. The karyotypes compose of 4 large metacentric, 4 large submetacentric, 2 medium metacentric, 8 small metacentric and 6 small submetacentric chromosomes. The Ag-NOR, FISH as well as hybridization with the microsatellite $d(CGG)_{10}$ confirm the location of NOR site in the subcentromeric region on chromosome pair 11. The in situ localization pattern of $d(CA)_{15}$ microsatellite was positive on chromosome pairs 1 and 6, while microsatellite $d(TA)_{15}$ show its location in the long arm of chromosome 5,8 and 10. The information provided has value as species specific marker for this species.

Keywords *Limnonectes blyhii* · Karyotype · Chromosomal microsatellite pattern

Introduction

The fanged frogs of Asia are a moderately species rich group of 53 described taxa distributed across Asia [3, 6]. The members of the genus *Limnonectes* (fam. Dicroglossidae) have a broad distribution in Asia from eastern and southern China, eastwards to Japan, throughout Indochina and southwards to Malaysia, Indonesia, Philippines and New Guinea [4]. *Limnonectes* is one of the most diverse group of amphibians with 69 species currently recognized, 15 of which have been described in the last 10 years [4]. The amphibian fauna in Thailand comprises of 137 species belonging to 8 families and 3 orders [7], of which 11 species reported are in the genus *Limnonectes* [1]. The giant Asian river frog (*Limnonectes blyhii*) grows up to a snout-vent length of 26 cm. Males of this species grow larger than the females. It has a robust body with a large head that is longer than wide. Its hind legs are long and muscular with the feet being extensively webbed. The skin on the back of adults is relatively smooth but warty in juveniles. It is brown or grey, sometimes with a broad yellowish stripe along its vertebrae. An enumerated data on chromosome numbers of ~ 1000 species is available [9].The list shows variation in chromosome number in most

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of the seven families of anuran amphibians classified within 33 genera.The typical karyotype of the family Dicroglossidae is diploid chromosome number, $2n=26$. Earlier reports on some species in the genus *Limnonectes* lists $2n=22$–26, NF = 44–52, including *L. kuhlii* and *L. blytthii* [16], *L. pileatus* [16, 17], *L. gruniens* and *L.modestus* [11], *L.blyhii* [2] and *L. taylori* [13]. All such studies further reveal vast variations with respect to chromosome number, type and size.

The molecular data providing sequence localization based on microsatellite probes to detect specific hybridization pattern in *L. blyhii* has not yet been reported. As such, the present study is the first report describing the molecular cytogenetic and karyotype study on chromosome size, standardized idiogram, karyotype formula and meiotic cell division of the species as well as information published earlier. The results obtained provide basic information on chromosomal details that has value in elucidating chromosome evolution and taxonomic affinities as well as conservation biology of the species and the genus.

Materials and methods

Field surveys were conducted in rainy season from southern (6° 51′ 21″ N/101° 16′ 3″ E position), Thailand. Five males and five females of mature *L. blyhii* were collected. The frogs were transferred to the laboratory and were kept under standard conditions for 3 days before the experimentation. The chromosomes were prepared in vivo [14] with slight modification. Colchicine was injected into the frog'sabdominal cavity. The frogs were left in a box for 8 h and then killed. 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. The tissues were cut into small pieces, and then transferred into 8 ml of cell sediments to a centrifuge tube and incubated for 30 min at 37 °C. After centrifugation at 1500 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 1500 rpm for 8 min. The fixation was repeated until the supernatant was clear, usually three times. Finally, the pellet was mixed with 1 ml fixative (depending on the amount of cell). The mixture was dropped onto a clean and cold slide by a micropipette, and then air-dried.

Conventional staining was done using 10% Giemsa's solution for 10 min [13]. 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 cells turned brownish. After that the slides were dipped in distilled water to remove the cover glass and air-dried. The microsatellites $(CA)_{15}$, $(TA)_{15}$ and $(CGG)_{10}$ were synthesized according to [8, 19]. These sequences were directly labeled with Cy_3 at the 5'terminus during synthesis by Sigma (St. Louis, MO, USA).

Chromosome counting was performed on mitotic metaphase cells under a light microscope. Twenty clearly observable and well-spread chromosomes of each male and female were selected and photographed. The length of the short chromosome arm (Ls) and the length of the long chromosome arm (Ll) were measured, and the length of the total chromosome (LT, LT = Ls + Ll) were 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 [10]. The fundamental number (number of chromosome arm, NF) was obtained by assigning a value of two to metacentric, submetacentric and acrocentric chromosomes and one to telocentric chromosome. All parameters were used in karyotyping and idiograming.

Results and discussion

The results showed that *L. blyhii* has diploid chromosome number of $2n=24$ and fundamental number (NF) = 48. The karyotype comprised of four large metacentric, four large submetacentric, two medium metacentric and 14 small metacentric chromosomes. The karyotype formula of *L. blyhii* is $2n=24=L_4^m+L_4^{sm}+M_2^m+S_{14}^m$ in both males and female, while sex chromosomes were cytologically indistinguishable (Fig. 1a). The average lengths of each chromosome including short and long arm length, total length, relative length, and centromeric index were calculated and are presented in Table 1. The previous relevant literatures have reported that the number of diploid chromosome and fundamental number in *Limnonectes* studied are $2n=22$–26 and NF = 44–52 including, *L. kuhlii* and *L. pileatus* (2n = 26, NF = 52) [16], *L. gruniens*, *L. modestus* and *L. blyhii* (2n = 24, NF = 48) [2, 11] and *L. taylori* (2n = 22, NF = 44) [13] (Table 2). Comparison to closely related species, *L. blyhii* has diploid chromosome number similar to *L. gruniens* and *L. modestus* ($2n=24$), but is higher than that in *L. taylori* ($2n=22$) and lower than *L. kuhlii* and *L. pileatus* ($2n=24$). This is in agreement to the diploid chromosome previous reported for *L.blyhii* [2]. Meanwhile, this species study herein has chromosomes with slight differences in morphological patterns.

After Ag-NOR staining, these regions produce numerous gene expressions and contain more non-histone protein than others regions on the chromosome. Accordingly, the dark band (NOR-positive) is induced by the reduction of organic silver by these proteins that change from silver to dark [15].

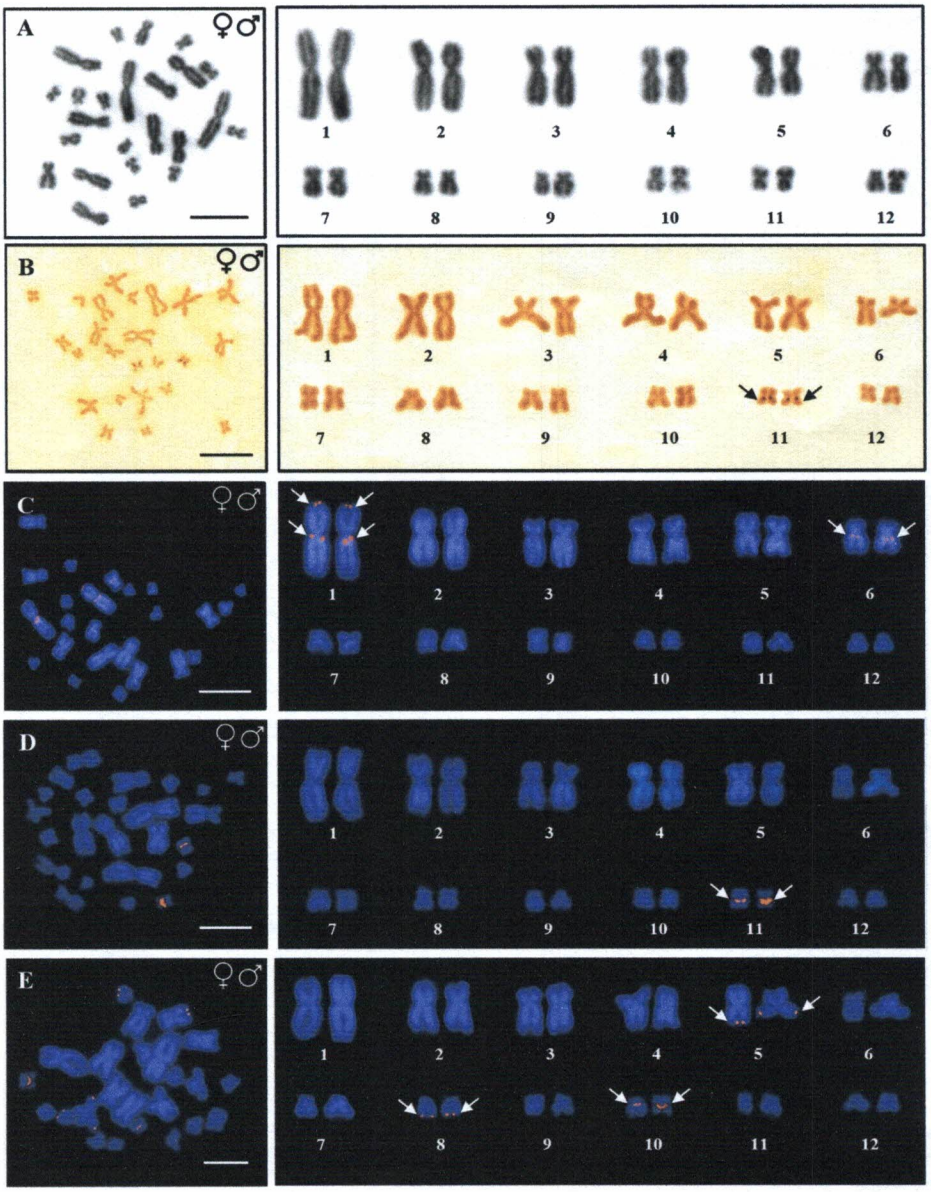

Fig. 1 Metaphase chromosome plates and karyotypes of giant Asian river frog (*Limnonectesblyhii*), $2n=24$ by: **a** conventional staining, **b** Ag-NOR banding, **c** $d(CA)_{15}$, **d** $d(CGG)_{10}$, **e** $d(TA)_{15}$ microsatellite pro. Scale bar = 10 μm

The NOR could be detected to subcentromeric region on long arm chromosome pair 11 (Fig. 1b). We found one pair of Ag-NOR sites in all of the samples examined. This is similar to the previous report on *L. kuhlii* [16], *L. blythii* [2, 16], *L. pileatus* [16, 17], *L. gruniens* and *L.modestus* [11], and *L. taylori* [13].

This is the first molecular cytogenetic study of metaphase chromosomes studied by FISH. The in situ hybridized localization of microsatellites $(CA)_{15}$, $d(CGG)_{10}$ and $d(TA)_{15}$. Microsatellites, also known as simple sequence repeats, consist of very short motifs (1–6 nucleotides in length) repeated in tandem arrays.Generally, they are located in the heterochromatic regions (telomeres, centromeres and in the sex chromosomes) of genomes, where a significant fraction of repetitive DNA is expected to be localized [18].The result of *L. blyhii* analyzed was being abundantly distributed on six chromosomes such as, the accumulation of $(CA)_{15}$ in chromosomal pair 1 and 6 (Fig. 1c), while $(CGG)_{10}$ detected subcentromeric region on chromosomal pair 1 (Fig. 1d) and $(TA)_{15}$ sequences are present in the chromosome pair 5, 8 and 10 (Fig. 1e).However, an intriguing feature exclusive for *L. blyhii* was the strong accumulation of all microsatellites at the regions of specific chromosomal pair, indicating that

Table 1 Karyomorphological details of the Asian river frog, *Limnonectesblyhii*, 2n = 24

Chromosome pairs	Ls (μm)	Ll (μm)	LT (μm)	CI ± SD (mean ± SD)	RL ± SD (mean ± SD)	Chromosome size	Chromosome type
1	9.165	11.728	20.893	0.561 ± 0.028	0.169 ± 0.007	Large	Metacentric
2	7.457	9.713	17.169	0.565 ± 0.025	0.139 ± 0.007	Large	Metacentric
3	5.788	8.876	14.664	0.607 ± 0.037	0.119 ± 0.005	Large	Submetacentric
4	5.531	7.789	13.320	0.603 ± 0.031	0.108 ± 0.004	Large	Submetacentric
5	5.425	6.814	12.239	0.555 ± 0.023	0.099 ± 0.003	Medium	Metacentric
6	3.839	5.860	9.699	0.590 ± 0.052	0.079 ± 0.003	Small	Metacentric
7	3.223	4.009	7.232	0.550 ± 0.043	0.059 ± 0.004	Small	Metacentric
8	2.575	3.772	6.346	0.590 ± 0.067	0.051 ± 0.003	Small	Metacentric
9	2.258	3.525	5.783	0.599 ± 0.048	0.047 ± 0.002	Small	Metacentric
10	2.514	3.272	5.786	0.565 ± 0.056	0.047 ± 0.004	Small	Metacentric
11*	2.505	2.952	5.457	0.541 ± 0.042	0.044 ± 0.003	Small	Metacentric
12	2.238	2.722	4.960	0.548 ± 0.037	0.040 ± 0.003	Small	Metacentric

Ls, short arm; Ll, long arm; LT, total chromosome length; CI, centromeric index; RL, relative length

*NORs bearing chromosomes (satellite chromosome)

Table 2 Review of the Karotypic data of other species the genus *Limnonectes*

Species	2*n*	Karyotype formula	NF	NORs	FISH	References
L. gruniens	24	24m	48	–	–	Nasaruddin et al. [11]
L. modestus	24	20m + 4t	44	–	–	Nasaruddin et al. [11]
L. kuhlii	26	8m + 14sm	52	2	–	Supaprom [16]
L. pileatus	26	16m + 10sm	52	2	–	Supaprom [16], Supaprom and Baimai [17]
L. taylori	22	16m + 6sm	44	2	–	Phimphan and Aiumsumang [13]
L. blyhii	24	10m + 12sm + 2a	48	–	–	Donsakul and Rangsiruji [2]
	24	20m + 4sm	48	2	+	Present study

these microsatellites may be used as chromosomal markers in this frog species.

The present study on the meiotic cell division of *L. blyhii* found that during interphase, nucleolus could be clearly seen, while chromatins were absent. In prophase, metaphase I (meiosis I) the homologous chromosomes showed synapsis, which can be defined as the 12 bivalent and 12 haploid chromosomes at metaphase II as diploid species. Thus it is confirmed that this species has 2n = 24 which is in agreement to previous reports. The largest metacentric chromosome pair 1 is the largest bivalent.We found that *L. blyhii* has the distinct character of the observable leptotene (initiation of chromosome shrinking), pachytene (completion of chromosome synapsis) and diakinesis (terminalization) according to Patawang et al. [12] (Fig. 2). In conclusion, this study provides the first molecular cytological details and Ag-NOR marker for *L. blyhii* from Thailand. The results support the karyotype of genus *Limnonectes* are conserved among

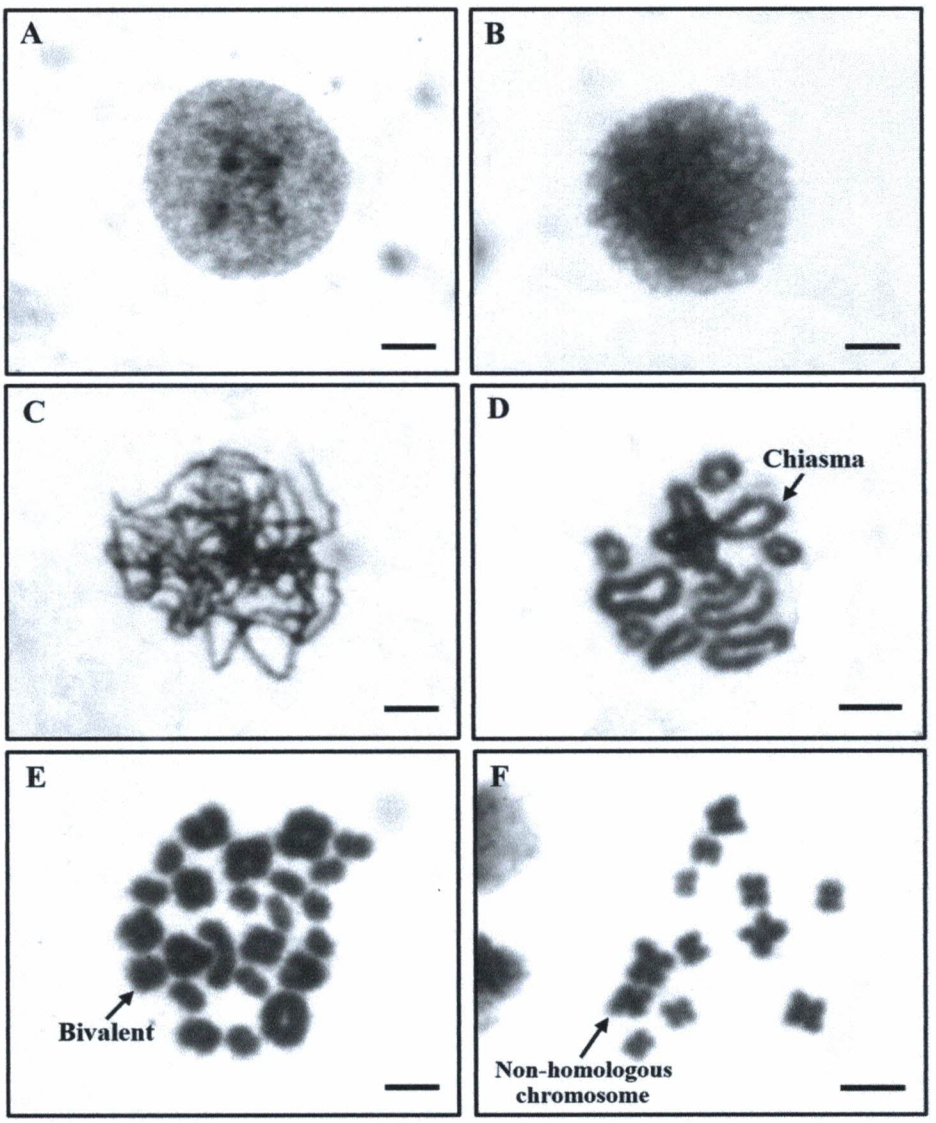

Fig. 2 Meiosis cell division ofgiant Asian river frog (*Limnonectesblyhii*), $2n = 24$: **a** interphase, **b** leptotene, **c** pachytene, **d** diakinesis, **e** metaphase I, **f** metaphase II. Scale bar = 10 μm

several other species. However, the chromosomal morphology may be slightly different depending on populations of *L. blyhii* present in different countries. Our results added new knowledge that can be used for comparative karyological analyses in *Limnonectes* species on the basis of classical and banding approach within this taxon.

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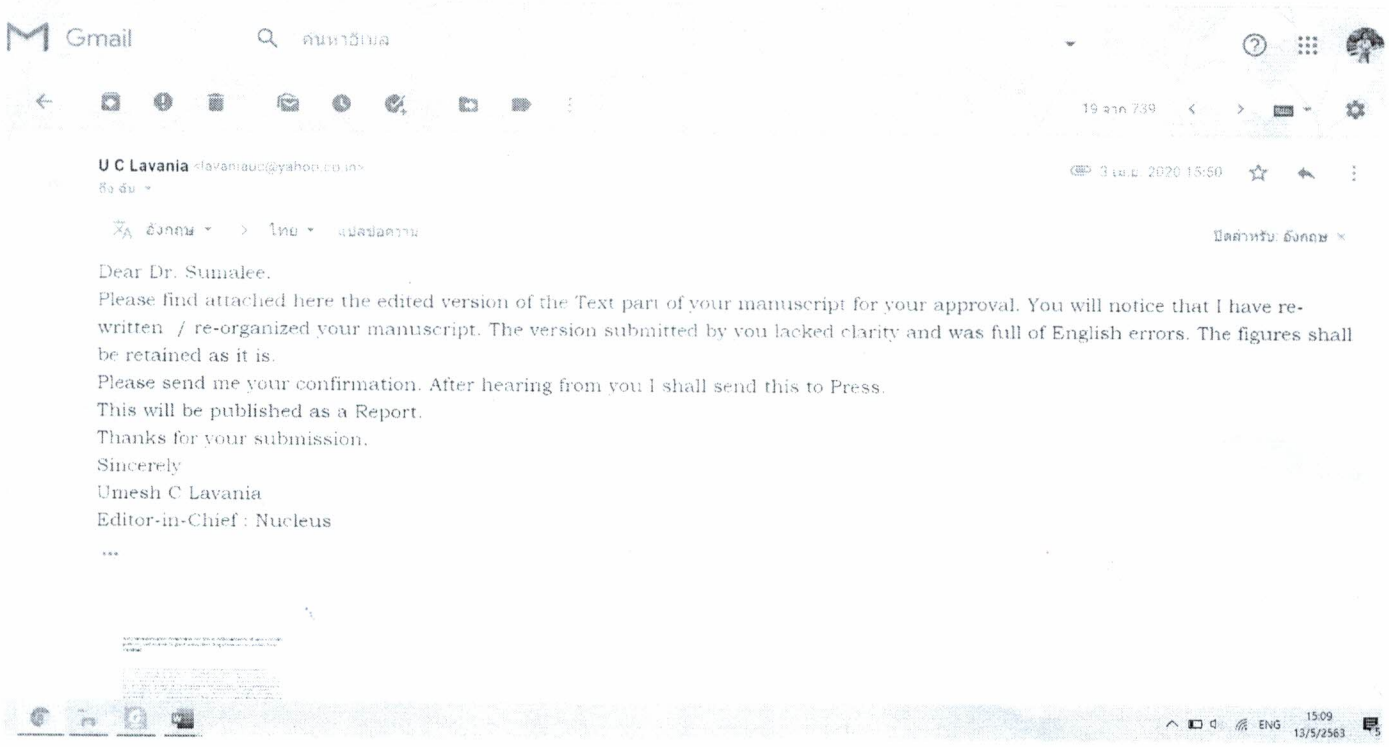
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