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Karyomorphology and microsatellites characterization of *Limnonectes gyldenstolpei*: first report from Thailand

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Abstract. In the present investigation, karyotype and microsatellites pattern in the chromosome of Gyldenstolpe's Frog (*Limnonectes gyldenstolpei*) have been analyzed. The aspect of chromosome numbers, morphology, nucleolus organizer region (NOR) locations and microsatellites pattern [$d(CA)_{15}$, $d(CGG)_{10}$, $d(GC)_{15}$, $d(TA)_{15}$]. We provided the karyotype and idiogram of this species by conventional staining, Ag-NOR banding and Fluorescence *in situ* hybridization techniques. For the study, five male and female samples collected from northern Thailand, were used. The metaphase chromosome preparations were prepared from the bone marrows by the standard protocol. The result shows that *L. gyldenstolpei* had the diploid chromosome number ($2n$) was 26 and the fundamental number (NF) were 56 in both males and females. The karyotype is composed of 4 large metacentric, 4 large submetacentric, 2 medium metacentric, 14 small metacentric and 2 small submetacentric chromosomes. The NORs bearing chromosome were in close to the telomere region on chromosome pair 1. In addition, the microsatellite $d(CGG)_{10}$ and $(GC)_{15}$ hybridization results confirmed the NOR region. The *in situ* localization pattern of $d(CA)_{15}$ microsatellites was positive on all telomere chromosome, while microsatellites $d(TA)_{15}$ have no signal on chromosome. Here we provide a classical and some molecular genetics information for *L. gyldenstolpei* useful as a species specific marker.

Keywords: *Limnonectes gyldenstolpei*, karyotype, chromosome, microsatellites.

INTRODUCTION

Limnonectes gyldenstolpei is a species of frog in the Dicroglossidae family. It has been recorded throughout much of Thailand, northeastern Lao,

southwestern Cambodia, and central Vietnam. It has recently also been recorded from the Phong Nha-Kẻ Bàng National Park in central Vietnam (Luu et al., 2013). The members of the genus *Limnonectes* 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 (Frost, 2016). *Limnonectes* is one of the most diverse groups amphibians with 69 currently species recognized and 15 of which have been described in the last ten years (Frost, 2016).

The gross chromosome numbers of 1,000 amphibian species were reported by Kuramoto (1990). But only 837 of the 3,521 anuran species have been analyzed chromosomally (King, 1991). The amphibian fauna in Thailand comprises of 176 species in 8 families and 3 orders (Khonsue and Thirakhupt, 2001). In the genus *Limnonectes* reported 11 species (Niyomwan, et al., 2019). The list shows chromosome number variation occurs in most of the seven families of anuran amphibians classified with 33 genera. The typical karyotype of the family Dicroglossidae is diploid chromosome number (2*n*)=22, 24 and 26. For the genus *Limnonectes*, there were some cytogenetic studies reported the diploid number was 2*n*=22-26, NF=44-52, including *L. kuhlii* and *L. blytthii* (Supaprom, 2003), *L. pileatus* (Supaprom, 2003; Supaprom and Baimai, 2004), *L. gruniens* and *L. modestus* (Nasaruddin, 2009), *L. blytthii* (Donsakul and Rangsiruji, 2005; Phimphan et al., 2020) and *L. taylori* (Phimphan and Aiumsumang, 2019). All previous knowledge demonstrated that there are several patterns of chromosomes (number, type, size).

This is the first report describing the molecular cytogenetic and karyotype study of chromosome size, standardized idiogram, karyotype formula and meiotic cell division of the *L. gyldenstolpei* species. The molecular data, microsatellite probes are used to detect if there is some specific hybridization pattern in *L. gyldenstolpei* has not been studied yet. The results obtain can be fulfilled to the basic knowledge. In addition, our knowledge advances cytogenetic information for further study on taxonomy relationship. Moreover, we provide useful basic information for the conservation and chromosome evolution study of this frog.

MATHERIAL AND METHODS

Field surveys were conducted in rainy season from northern (16.42°N 101.16°E), Thailand. Five males and five females of *L. gyldenstolpei* were mature obtained during. The frogs were transferred to the laboratory and were kept under standard conditions for 3 days before the experimentation. Experiments were performed in accordance with ethical protocols (Ref No. U1-04498-2559). The chromosomes were prepared *in vivo* with slight adaptations as follows (Sangpakdee et al., 2016). The colchicine was injected into the frogs' abdominal cavity. Then, the frogs were left in a box for eight hours 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. 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 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 the air-dry technique was applied.

Conventional staining was done using 10% Giemsa's solution for 10 min (Phimphan and Aiumsumang, 2019). Ag-NOR banding was performed (Howell and Black, 1980) 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. The microsatellites $(CA)_{15}$, $(CGG)_{10,}$ $(GC)_{15}$, and $(TA)_{15}$ were synthesized according to (Kubat etal., 2008; Supiwong et al., 2014). 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 arm chromosome (Ls) and the length of the long arm chromosome (Ll) were measured, and the length of the total arm chromosome (LT, LT = Ls+Ll) 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 (Levan, 1964). 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 *L. gyldenstolpei* had diploid chromosome number of 2*n*=26 and fundamental number (NF)=52, the karyotype comprised four large metacentric, four large submetacentric, two medium metacentric, 14 small metacentric and two small submetacentric chromosomes. The karyotype formula of *L. gyldenstolpei* is 2*n*(26)= L^m_4+L^{sm}_4+M^m_2+S^m_{14}+S^{sm}_2 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 presented in Table 1. The previous relevant literatures have been reported that the numbers of diploid chromosome and fundamental number in *Limnonectes* studied herein are 2*n*=22-26 and NF=44-52 including, *L. kuhlii* and *L. blytthii* (Supaprom, 2003), *L. pileatus* (Supaprom, 2003; Supaprom and Baimai, 2004), *L. gruniens* and *L. modestus* (Nasaruddin, 2009), *L. blytthii* (Donsakul and Rangsiruji, 2005; Phimphan et al., 2020) and *L. taylori* (Phimphan and Aiumsumang, 2019). (Table 2). Comparison to closely related species, *L. gyldenstolpei* had diploid chromosome number similar to *L. gruniens* and *L. modestus* (2*n*=24), but is higher than that in *L. taylori* (2*n*=22) and lower than *L. kuhlii* and *L. pileatus* (2*n*=24). This result was the first report on *L. gyldenstolpei*. These characteristics are consistent with the theory that reorganization from the original karyotype resulted from Robertsonian fissions, fusions, or pericentric inversions (Gorman 1973; King 1978). Our results confirmed 2n for *L. gyldenstolpei* species but with differences in the diploid chromosome number. This incongruence reflects probably the number of the *Limnonectes* chromosomes, especially those of polyploids.

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 (Sharma et al., 2002). If these regions were active during the interphase prior to mitosis, they can be detected by silver nitrate staining (Howell and Black 1980). The NOR could be detected to near telomeric region on long arm chromosome pairs 1 (Fig. 1B). We found one pair of Ag-NOR sites in all of the samples examined. However, the results were similar to the previous report on *L. kuhlii* and *L. blytthii* (Supaprom, 2003), *L. pileatus* (Supaprom, 2003; Supaprom and Baimai, 2004), *L. gruniens* and *L. modestus* (Nasaruddin, 2009), *L. blytthii* (Donsakul and Rangsiruji, 2005; Phimphan et al., 2020) and *L. taylori* (Phimphan and Aiumsumang, 2019). The most striking variation is seen in the morphology of the secondary constrictions. Generally, one major nucleolar organizer region is present per genome (n), which may vary in its position between species. However, closely related and often morphologically very similar species share the same type and location of their nucleolar organizer regions, which can therefore provide an effective taxonomic.

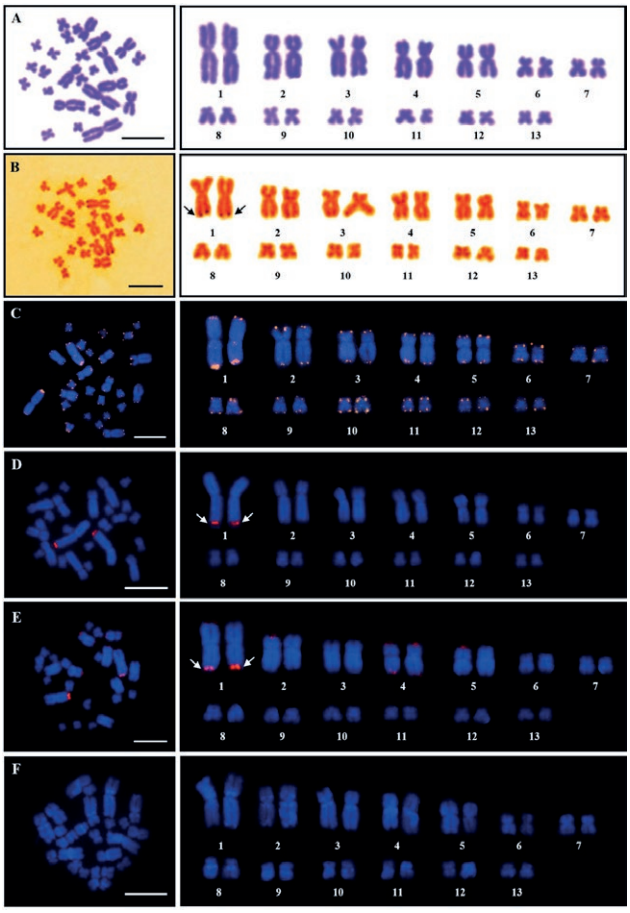

Figure 1. Metaphase chromosome plates and karyotypes of *Limnonectes gyldenstolpei*, 2*n*=26 by conventional staining [A.], Ag-NOR banding [B.], $d(CA)_{15}$ [C.], $d(CGG)_{10}$ [D.] $d(GC)_{15}$ [E.] and $d(TA)_{15}$ microsatellite probe [F.]. Note scale bars indicate 10 micrometers.

Here the first molecular cytogenetic study in metaphase chromosomes stained by FISH. The *in situ* hybridized localization of microsatellites $d(CA)_{15}$, $d(CGG)_{10,}$ $d(GC)_{15}$, 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 (Supiwong et al., 2013). The

Table 1. 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 male and female *Limnonects gyldenstolpei*, 2*n* (diploid)=26.

Chromosome pairs	Ls	Ll	LT	CI±SD	RL±SD	Chromosome size	Chromosome type
1*	6.162	7.628	13.790	0.552±0.012	0.161±0.005	Large	metacentric
2	4.166	6.849	11.015	0.622±0.014	0.128±0.004	Large	submetacentric
3	3.469	5.991	9.460	0.633±0.019	0.110±0.004	Large	submetacentric
4	3.786	5.168	8.954	0.580±0.022	0.105±0.002	Large	metacentric
5	3.700	4.503	8.203	0.550±0.018	0.096±0.003	Medium	metacentric
6	2.179	2.966	5.145	0.577±0.014	0.060±0.002	Small	metacentric
7	1.866	2.892	4.758	0.606±0.032	0.056±0.002	Small	submetacentric
8	1.876	2.799	4.675	0.597±0.037	0.054±0.002	Small	metacentric
9	1.766	2.590	4.356	0.595±0.017	0.051±0.002	Small	metacentric
10	1.799	2.475	4.274	0.577±0.021	0.050±0.002	Small	metacentric
11	1.609	2.387	3.996	0.596±0.015	0.047±0.001	Small	metacentric
12	1.505	2.260	3.765	0.598±0.018	0.044±0.001	Small	metacentric
13	1.486	1.967	3.452	0.572±0.024	0.040±0.002	Small	metacentric

* NORs bearing chromosomes (satellite chromosome).

Table 2. Review of cytogenetic publications of family Dicroglossidae (genus *Limnonectes*).

Species	2*n*	Karyotype formula	NF	NORs	FISH	Reference
L. gruniens	24	24m	48	-	-	Nasaruddin et al. 2009
L. modestus	24	20m+4t	44	-	-	Nasaruddin et al. 2009
L. kuhlii	26	8m+14sm	52	2	-	Supaprom 2003
L. pileatus	26	16m+10sm	52	2	-	Supaprom 2003
	26	16m+10sm	52	2	-	Supaprom and Baimai 2004
L. taylori	22	16m+6sm	44	2	-	Phimphan and Aiumsumang 2019
	24	10m+12sm+2a	48	-	-	Donsakul and Rangsiruji 2005
L. blythii	24	20m+4sm	48	2	+	Phimphan et al. 2021a
L. gyldenstolpei	26	20m+6sm	52	2	+	Present study

2*n* diploid chromosome number, *NF*=fundamental number (number of chromosome arms), *m* metacentric, *sm* submetacentric, *a* acrocentric, *t* telocentric chromosome, *NORs* Ag-NOR banding, *FISH* Fluorescence in situ hybridization, + positive and - not available.

result of *L. gyldenstolpei* analyzed was being abundantly distributed on all telomere chromosomes such as, the accumulation of $(CA)_{15}$ in long arm chromosomal pair 1 (Fig 1C), while $(CGG)_{10}$ and $(GC)_{15}$ detected subtelomeric region on long arm chromosomal pair 1 (Fig. 1D, 1E) and $(TA)_{15}$ sequences are not present in the all chromosome (Fig. 1F). However, an intriguing feature exclusive for *L. gyldenstolpei* was the strong accumulation of all microsatellites at the regions of specific chromosomal pair, indicating that these microsatellites may be used as chromosomal markers in this frog species. In the frog genomes, microsatellites are usually abundant in the telomeric and centromeric regions, Otherwise, the dinucleotides $(CA)_{15}$, $(GC)_{15}$ and $(CGG)_{10}$ accumulated exclusively in telomeric and subcentromeric chromosomal regions, corroborating findings from other frog groups studied to date (Phimphan, et al. 2021a; 2021b). These molecular cytogenetics data could also be a substantial prerequisite for future frog genome projects. This study discovered that the cytogenetic maps of *L. gyldenstolpei* allowed us to map out the steps involved in this species' chromosomal rearrangement. This is the first report on the Fluorescence *in situ* hybridization (FISH) study of this species in Thailand.

The present study on the meiotic cell division of *L. gyldenstolpei* found that during interphase, nucleolus

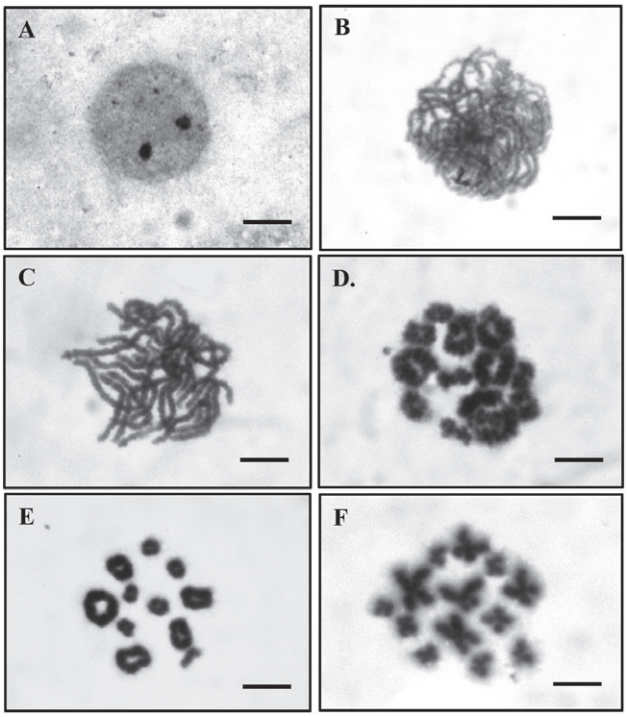

Figure 2. Meiosis cell division of the *Limnonectes gyldenstolpei*, 2*n*=26 on interphase (A.), leptotene (B.), pachytene (C.), diakinesis (D.), metaphase I (E.) and metaphase II (F.) by conventional staining technique. Scale bars indicate 10 micrometers.

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 13 bivalent and 13 haploid chromosomes at metaphase II as diploid species. It is confirmed for this species had 2n=26 in similar to previous reports. The largest metacentric chromosome pair 1 is the largest bivalent. We found that *L. gyldenstolpei* had the distinct character of the observable leptotene (initiation of chromosome shrinking), pachytene (completion of chromosome synapsis) and diakinesis (terminalization) according to Patawang (Patawang et al., 2013) (Fig. 2). In conclusion, this study provides the first chromosome, molecular cytological details and Ag-NOR marker for *L. gyldenstolpei* from Thailand. The results support the karyotype of genus *Limnonectes* are conserved among several other species. However, the chromosomal morphology may be slightly different depending on populations of *L. gyldenstolpei* present in different countries. Our results added new knowledge that can be used for karyological comparative analyses in *Limnonectes* species, on the basis of classical and banding approach within this taxon.

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