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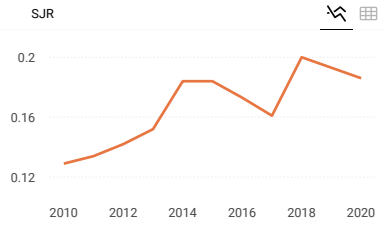

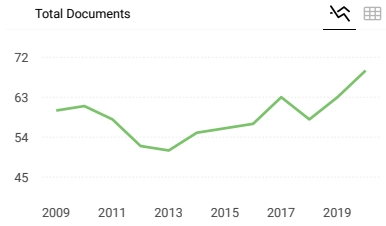

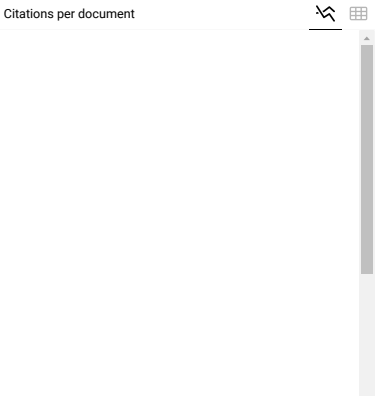

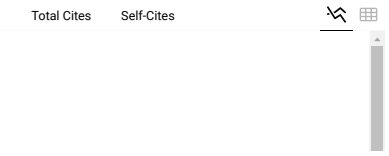

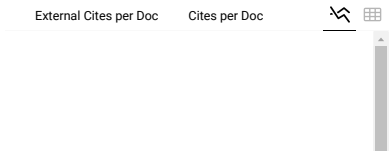

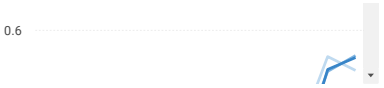

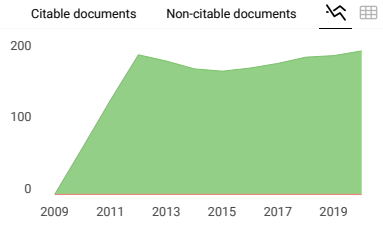

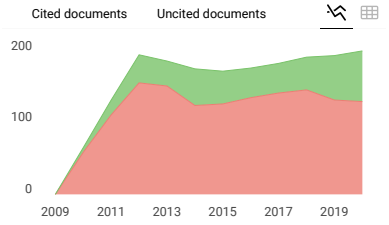

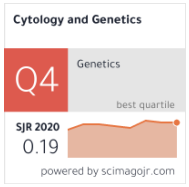

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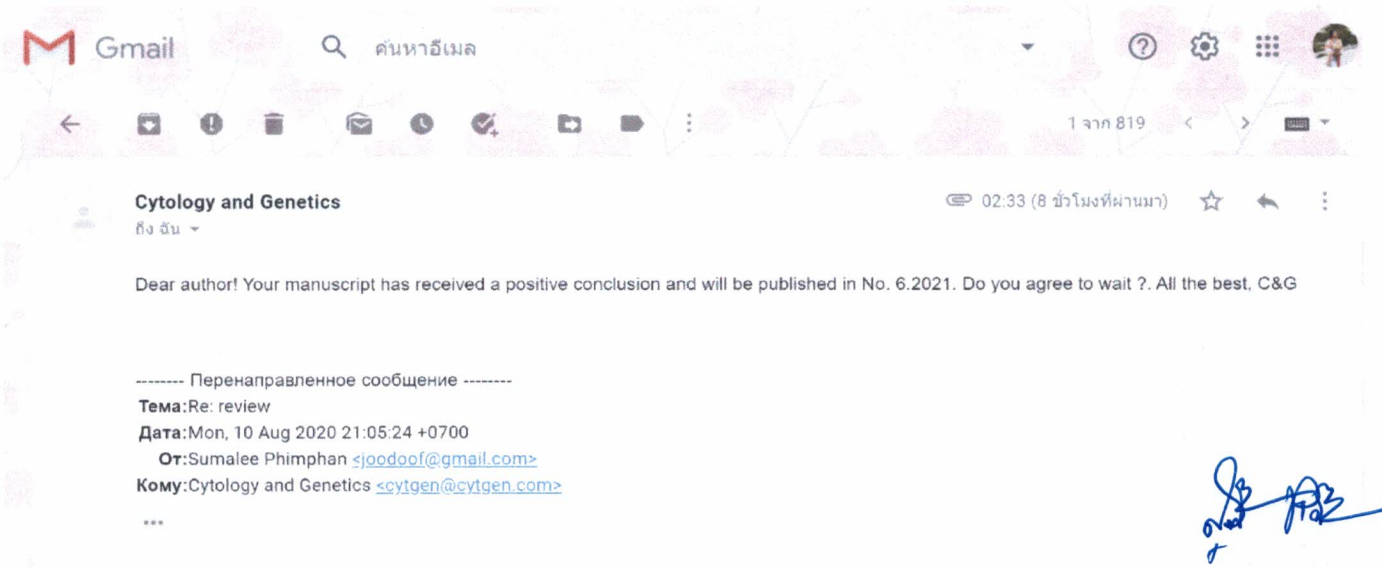

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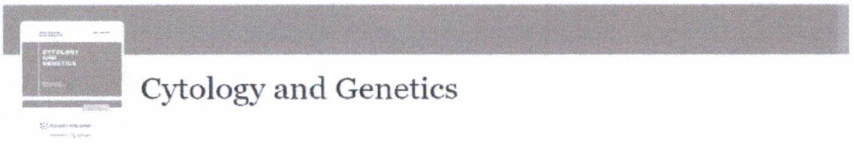

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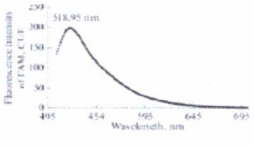

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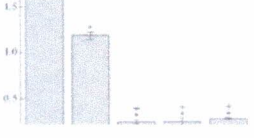

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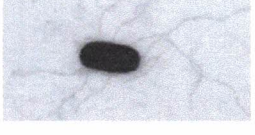

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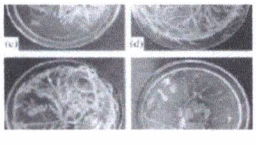

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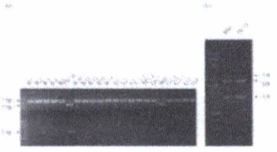

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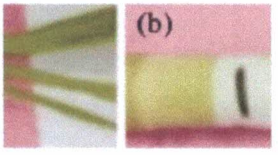

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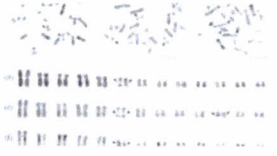

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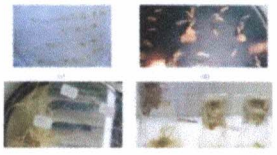

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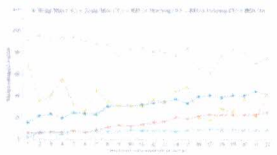

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Characterization of Chromosomal and Repetitive Elements in the Genome of "*Rana nigrovittata*" (Anura, Ranidae): Revealed by Classical and Molecular Techniques

Sumalee Phimphan[a], *, Surachest Aiumsumang[a], and Alongklod Tanomtong[b]

[a] *Biology program, Faculty of Science and Technology, Phetchabun Rajabhat University, Phetchabun, 67000 Thailand*

[b] *Department of Biology, Faculty of Science, Khon Kaen University, Muang, Khon Kaen, 40002 Thailand*

**e-mail: sumalee.phi@pcru.ac.th*

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Abstract—Karyotype study and microsatellites pattern in the genome of *Rana nigrovittata* were studied, with the aim to provide a standard karyotype, chromosome marker and the distribution of repetitive DNA elements, informative knowledge of cytogenetics and evolutionary events. Here, we analyzed the karyotype structure and the distribution of repetitive DNA sequence in this species using conventional banding and Fluorescence in situ hybridization techniques. The ten specimens (five males and five females) were collected from Phitsanulok province, Thailand. Mitotic metaphases were prepared from the bone marrows by the standard protocol. The result showed that *R. nigrovittata* had the diploid chromosome number of $2n = 26$ and the fundamental number (NF) were 52 in both males and females. The karyotypes compose of six large metacentric, four large submetacentric, two medium metacentric, two medium submetacentric and 12 small submetacentric chromosomes. No sex related chromosome heteromorphism was observed in male (XY) or female (ZW) of this species. The NOR was observed in subcentromeric region on chromosome no. 11. The C-positive heterochromatin blocks are mainly distributed in the centromere of most chromosomes, while some additionally in paracentromeric and telomeric regions. The large heterochromatic blocks were found on chromosome no 6. Some of repetitive elements were scattered while some were specific in the karyotype. The combine of conventional banding and molecular cytogenetics provide information for a cytogenetic determination of the examined species.

Keywords: *Rana nigrovittata*, karyotype, microsatellites, chromosome

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INTRODUCTION

Cytogenetics of anura has been of immense significance in unraveling the taxonomy and systematics of the thousands of these species worldwide over the past century. The beginning of cytogenetic study of anuran was originally observed with the widespread toads, Bufo species. It was 1920's when the work was done to describe the basic chromosome number and morphology by the conventional staining methods (Beccari, 1926; Stohler, 1927). Ranid frogs are quite abundant in Thailand. At least 52 species are widely distributed throughout the country (Khonsue et al., 2001). Many species in Thailand have been revised and reclassified by Chan-Ard, 1999. *Rana nigrovittata*, a wild frog species which is occupied a wide range in continental Southeast Asia, including Assam (India) to Yunnan (China), Vietnam, south to Malaya (Frost, 1985) as well as throughout Thailand (Matsui et al., 2001).

The Karyotypic analysis of anura belonging to the family Ranidae has been carried out predominantly among member species of the genus *Rana* (Seto, 1965; Guillemin, 1967; Schmid, 1978; Blommers-Schlosser, 1978; Kuramoto, 1980). Some of previous literatures evidenced that the diploid chromosome number of most *Rana* species was uniformly $2n = 26$ chromosomes, except for a few species that had $2n = 24$ and 22 chromosomes (Kuramoto, 1990; Supaprom and Baimai, 2004). Most of these are found in species from southeast Asia. Some of these frogs, such as the 22 chromosomes *R. kuhlii* and *R. namiyei* (Kuramoto, 1972; Kuramoto, 1979), the 24 chromosomes *R. magna*, *R. macrodon* and *R. blythii* (Schmid, 1980; Kuramoto, 1989; 1992). According to such karyotypically conservative groups of genus *Rana*, the classical and molecular cytogenetic approaches based on the differential staining of conventional, constitutive heterochromatin, NORs and FISH technique are particularly helpful.

The molecular organization and cytogenetic mapping of repetitive DNA elements, including satellites, multigene families and microsatellite repeats, have been analyzed in a large number of species, demonstrating their enormous potential for expanding the

knowledge of the karyotype differentiation, as reviewed in fish species (Cioffi, 2012). Microsatellites, which are abundant repeated sequences that are present in all eukaryotic genomes studied thus far, are found either between the coding regions of structural genes or between other repetitive sequences (Tautz and Renz, 1984). The in situ investigation of repetitive DNA elements provided useful characteristics for comparative genomics at the chromosomal level, offering new insights into the karyotype evolution of *Rana* species.

The present paper, we analyzed the karyotype structure in *R. nigrovittata* using conventional, NOR-, C-band and molecular cytogenetic techniques using microsatellite probes. We aimed to provide a characterization on standard karyotype, chromosome marker and the distribution of repetitive DNA elements which considerably improved upon the knowledge of cytogenetics and evolutionary events of this wildly distributed frogs.

METERIALS AND METHODS

Sample Collection

Ten samples of mature *R. nigrovittata* (five males and five females) were collected in rainy season from Phitsanulok province, Thailand. The samples were confirmed for the correct species using the methods of (Chan-Ard et al., 1999). The frogs were brought back to the laboratory at Khon Kaen University and were kept under standard conditions for 3 days before the experimentation.

Chromosome Preparation

The Mitotic chromosomes were prepared directly from bone marrow cells after in vivo colchicine treatment (Sangpakdee et al., 2017) with minor modification as follows. The wild caught specimens were injected with 0.1% colchicine into the abdominal cavity and left for 8 hours before being paralyzed with diethylether. The head and end of the long bone e.g., femur, tibiofibular and humerus were then cut off. The mitotic cells were taken by the injection of 0.075 M KCl to drive out from the bone marrow. Some of large morrow tissues were gently cut to small pieces as possible. The cells sediment was treated with 0.075 M KCl solution and incubated for 30 min at 37°C. Then the cell suspension was centrifuged at 1500 rpm and fixed with 8 mL of fresh-cold Carnoy's solution. The first 1–2 mL of fixative was added drop-wise while constantly shaking the suspension. The fixed materials were washed twice with freshly prepared fixative. The cells were then resuspended in 1 ml of fixative (depend on the cells density) and 2 drops of this suspension were dropped onto cleaned slides and then air-dried.

Conventional Band and FISH Experiment

Conventional staining was done using 20% Giemsa's solution for 10 min (Sangpakdee et al., 2017). Ag-NOR banding was performed Howell and Black, 1980 and C-banding was prepare by standard protocol (Sumner, 1972).

Detection of Repetitive DNA Sequences by FISH

The microsatellites $(A)_{20}$, $(C)_{30}$, $(CA)_{15}$, $(GC)_{15}$, $(TA)_{15}$, $(CAA)_{10}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(CGG)_{10}$, $(GAA)_{10}$ and $(GAG)_10$ were synthesized by standard protocol. These sequences were directly labeled with Cy_3 at the 5'terminus during synthesis by Sigma (St. Louis, MO, USA). Fluorescence in situ hybridization was performed under high stringency conditions on mitotic chromosome spreads according to Pinkel et al., 1986.

Microscopic Analysis and Image Processing

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 0.90–1.0 were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively (Chaiyasut, 1989). 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 chromosomal complements of *R. nigrovittata* had diploid number of $2n = 26$ and the fundamental number (NF) were 52 in both males and females. Their karyotype can be grouped into 3 distinct sizes, 6 large metacentric (pairs 1, 4 and 5), 4 large submetacentric (pairs 2 and 3), 2 medium submetacentric (pair 6), 2 medium metacentric (pair 7) and 12 small submetacentric chromosomes (pairs 8–13). No sex chromosome heteromorphism was evident. All chromosome parameters obtained from the average of 20 mitotic metaphases consequently the karyotype formula as follow: $2n\ (26) = L_6^m + L_4^{sm} + M_2^m + M_2^{sm} + S_{12}^{sm}$, with mean relative length ranging from 0.139 ± 0.005 to 0.057 ± 0.004 (Table 1). The metaphase plate and karyotype performed by conventional staining were

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 standard deviation (SD) from 20 metaphases of male and female sapgreen stream frog (*Rana nigrovittata*) $2n = 26$

Chromosome pairs	Ls, μm	Ll, μm	LT, μm	CI ± *SD*	RL ± SD	Chromosome size	Chromosome type
1	8.295	9.604	17.900	0.535 ± 012	0.139 ± 0.005	Large	Metacentric
2	5.613	9.409	15.022	0.605 ± 018	0.100 ± 0.003	Large	Submetacentric
3	5.085	8.672	13.757	0.628 ± 022	0.092 ± 0.005	Large	Submetacentric
4	5.586	7.336	12.922	0.586 ± 025	0.061 ± 0.002	Large	Metacentric
5	5.271	6.498	11.769	0.561 ± 017	0.052 ± 0.004	Large	Metacentric
6	3.285	6.596	9.881	0.613 ± 019	0.049 ± 0.002	Medium	Submetacentric
7	3.338	4.515	7.853	0.592 ± 031	0.046 ± 0.003	Medium	Metacentric
8	2.687	4.868	7.555	0.616 ± 031	0.043 ± 0.003	Small	Submetacentric
9	2.644	4.695	7.339	0.643 ± 039	0.117 ± 0.005	Small	Submetacentric
10	2.745	3.950	6.696	0.608 ± 037	0.107 ± 0.004	Small	Submetacentric
11*	2.525	3.677	6.203	0.604 ± 030	0.078 ± 0.004	Small	Submetacentric
12	2.388	3.470	5.858	0.607 ± 038	0.059 ± 0.004	Small	Submetacentric
13	2.300	3.159	5.458	0.617 ± 028	0.057 ± 0.004	Small	Submetacentric

*NOR bearing chromosome.

illustrated in Figs. 1A and 1C, and the idiogram was shown in Fig. 3.

As expected, the karyotype of *R. nigrovittata* was similar to those of *Rana* species that showed the same in diploid number and all of chromosomes were biarmed consequently NF = 52 (Table 2), however some species are exhibited a difference of diploid number and NF such as *R. kuhlii*, *R. namiyei*, *R. magna*, *R. macrodon* and *R. blythii* (Kuramoto, 1972; 1979; 1989; 1992; Schmid, 1980). Interestingly, a special karyotype was observed in *Rana phrynoides* distributed in Hengduan Mountains. In this species, $2n = 64$ consisting of all telocentric chromosomes (Liu and Zan, 1984).

Among amphibian, Cytogenetic and genetic approaches have clarified the presence of various types

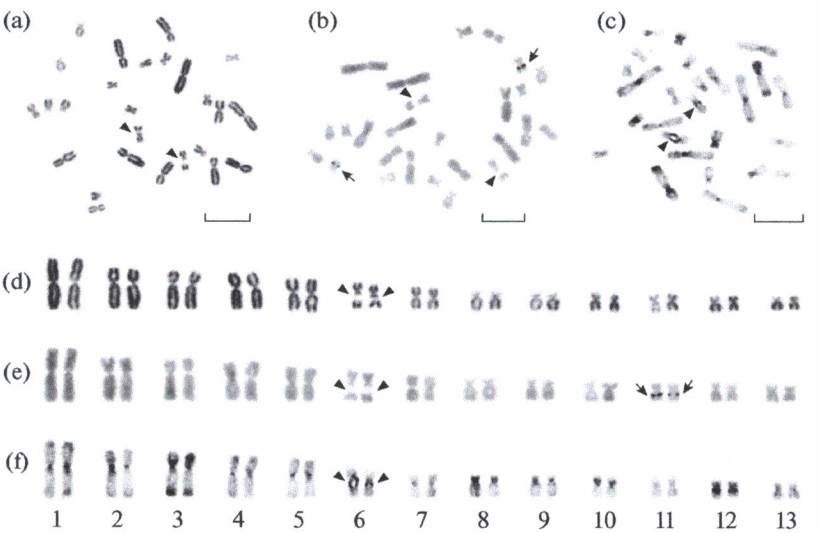

Fig. 1. Metaphase chromosome plates and karyotypes of *Rana nigrovittata* by conventional staining (a and d), Ag-NOR banding (b and e) and C-banding (c and f). *Arrows* indicate nucleolus organizer regions. *Arrowheads* show heterochromatin block-NOR like region (scale bars = 10 μm).

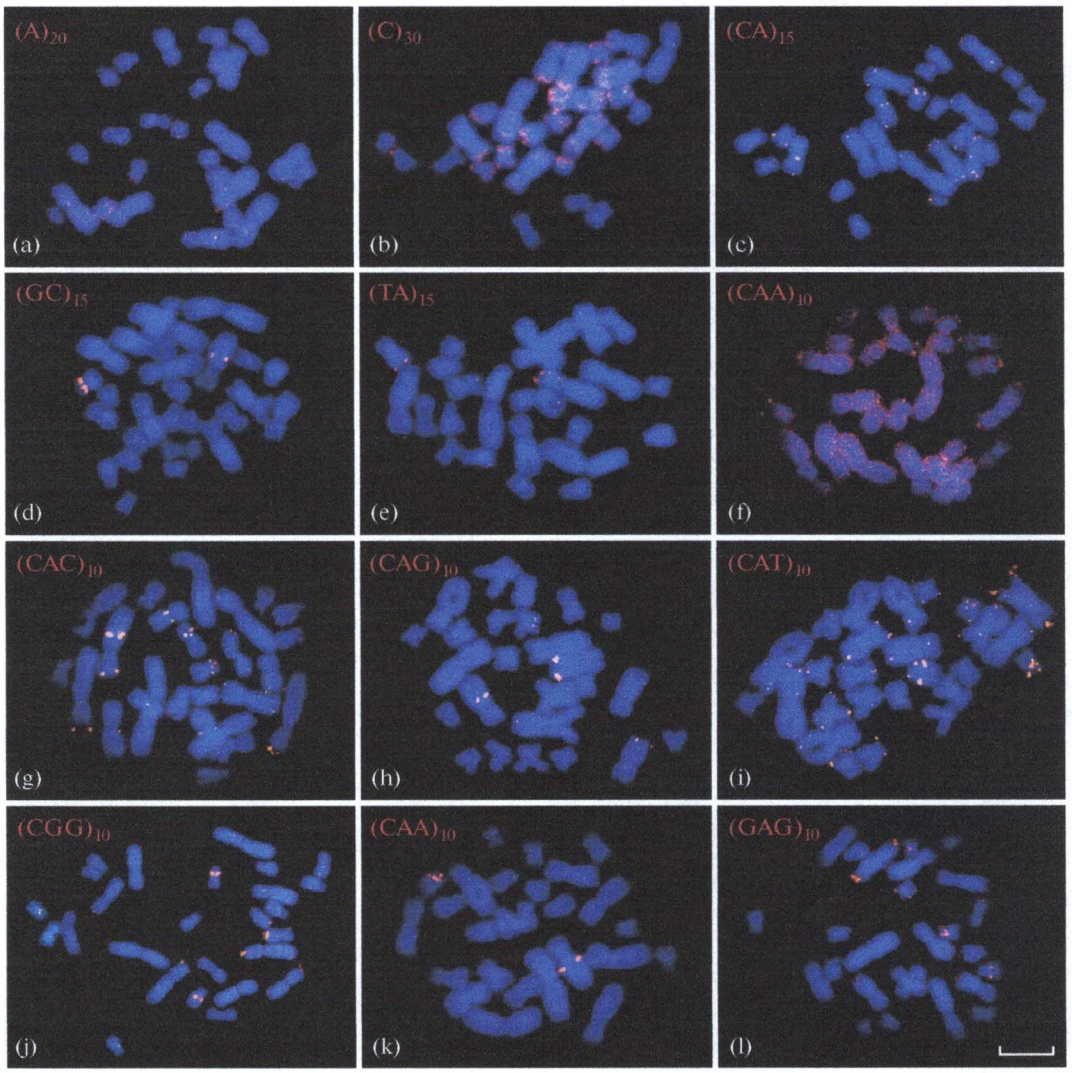

Fig. 2. Metaphase chromosome plate of *R. nigrovitata* mapped with different repeated DNAs. Microsatellite probes used are indicated on upper-left of each image (scale bar = 10 μm).

of chromosomal sex differentiation types. So far, eleven species with cytologically detected sex chromosomes, including XY and ZW systems, even an OW/OO system of sex determination and multiple sex chromosomes in one genome (Schmid et al., 1990) were discovered. Few sex-specific chromosome pairs in heterogametic individuals are heteromorphic and most of them are homomorphic. Here, we proposed that *R. nigrovittata* had homogametic sex chromosomes. This means that the heterogametic sex chromosomes, X and Y, or Z and W, have similar morphology and are not identifiable by microscopy. Sex chromosomes have been revealed to differ considerably even in the same species or population of amphibians as described in reviews (Schmid et al., 1990; Hills and Green, 1990).

The morphology of the chromosome complements and the location of chromosome markers for this species, were obviously similar to that reported by (Supaprom and Baimai, 2004), i.e. (i) chromosome form is also highly conserved with most of the karyotypes having symmetrically arranged biarmed chromosomes of metacentric, submetacentric and in few cases acrocentric chromosomes and (ii) a secondary constriction was located at the long arm of chromosome nearby to centromere position which is designated as no. 11 (11q) (Figs. 1b and 1e). The presence and position of NOR served as markers for cytogenetically

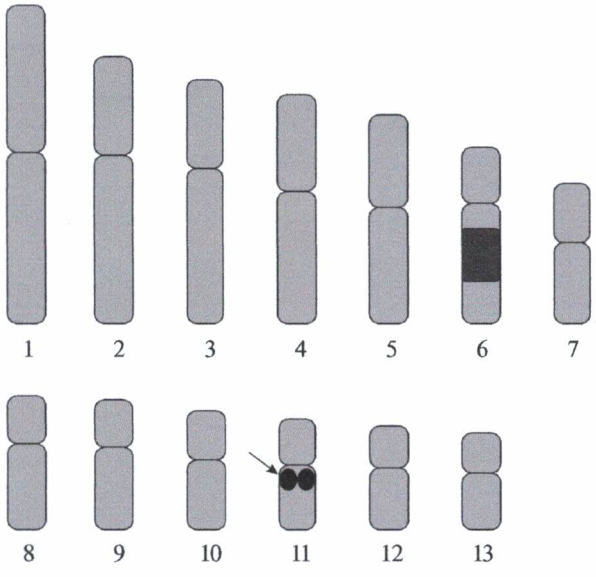

Fig. 3. Idiogram in haploid set (*n*) of *Rana nigrovittata* created from average parameters of length of each chromosome from 20 metaphases. Arrow indicates nucleolus organizer region (NOR). Black rectangle on chromosome no. 6 represents heterochromatic block-NOR like region.

identifying the species. With regards to amphibian karyotypes, especially those of *Rana* species as reviewed in Birstein, 1984.

In many ranid frogs, cytogenetically analysis by mean of C-banding has been accomplished (Heppich, 1978; Heppich and Tunner, 1979; Iizuka, 1989; Miura, 1994; Odierna, 2001). It has been shown that the C-heterochromatin is confined to the centromere (Schmid, 1978). The present investigation agrees with the earlier observations. C-positive heterochromatin blocks in karyotype of *R. nigrovittata* are mainly distributed in the centromere of most chromosomes, of those the C-positive paracentromeric region are exhibited on the short arm of chromosome nos. 3, 8, 9, 10 and extended to both arm of no. 12. The telomeric ends of the short and long arms of no. 1 were distinctly C-positive; these are also found in nos. 2 and 3 but only in the long arm. Surprisingly, every mitotic metaphase analyzed, the chromosome no. 6 of examined species being similar to secondary constriction chromosome was also positive for C-banding. The morphology of no. 6 in the present study was no observed in the previous reported (Supaprom and Baimai, 2004). The molecular study of this species had suggested that genetic differentiation of *R. nigrovittata* seem to have been strongly affected by the geohistory of

Table 2. Review of some cytogenetic publications of family Ranidae (genus *Rana*)

Species	2*n*	Karyotype formula	NF	NORs	Reference
R. exilispinosa	26	16m + 10sm	52	2	(Matsui et al., 1995)
R. chalconota	26	–	–	–	(Singh and Banerjee, 2004)
R. livida	26	16m + 10sm	52	2	(Matsui et al., 1995)
	26	–	–	–	(Singh and Banerjee, 2004)
	26	10m + 14sm + 2a	52	2	(Supaprom, 2003)
R. camerani	26	12m + 8sm + 6a	52	–	Popov and Dimitrov, 1999
R. narina	26	2m + 18sm + 6a	52	2	(Kuramoto, 1979)
R. ishikawae	26	2m + 18sm + 6a	52	2	(Kuramoto, 1979)
R. subaspera	26	2m + 18sm + 6a	52	4	(Kuramoto, 1979)
R. holsti	26	2m + 18sm + 6a	52	2	(Kuramoto, 1979)
R. okinavana	26	2m + 18sm + 6a	52	2	(Kuramoto, 1979)
R. namiyei	22	2m + 20sm	44	2	(Kuramoto, 1979)
	26	10m + 12sm + 4a	52	2	(Supaprom, 1999)
R. nigrovittata	26	10m + 16sm	52	2	(Supaprom, 2003)
	26	10m + 16sm	52	2	(Supaprom and Baimai 2004)
	26	**16m +10sm**	**52**	**2**	**Present study**
R. nigromaculata	26	–	–	2	(Shi et al., 2006)
R. curtipes	26	18m + 8sm	52	2	(Joshy et al., 2006)
R. temporalis	26	22m + 4sm	52	2	(Joshy et al., 2006)
R. malabarica	26	14m + 12sm	52	2	(Joshy et al., 2006)

2*n* = diploid chromosome number, NF = fundamental number, m = metacentric, sm = submetacentric, a = acrocentric, NORs = Ag-NOR banding.

their distributional ranges (Matsui et al., 2001). We therefore preliminarily characterized this remarkable chromosome as heterochromatin block-NOR like region which is unique for this species. The localization and distribution of C-band was evident in Figs. 1C and 1F.

Theoretically, as mentioned in Spasic-Boskovic et al., 1997 that most of the chromosomal rearrangements took place in the centromeric regions of chromosome, i.e. in regions of constitutive heterochromatin. We estimated that no. 6 of *R. nigrovittata* were chromosome that has possibility to break apart in aforementioned event.

The FISH technique carried out in *R. nigrovittata* using microsatellite probes revealed the distribution patterns of repetitive elements which scattered and specific signals on chromosome complements (Fig. 2). The intensity of signals were observed less intensity for $(A)_{20}$ in comparison to the other repeats. The microsatellites $(CAA)_{10}$ were dispersed and accumulated in telomeric region of several chromosomes, whereas less of intensity accumulated detecting for $(C)_{30}$. Regarding the microsatellites with specific signals some differences of Interstitial, terminal and proximal blocks were evidenced as follows: (i) in several chromosomes for $(CA)_{15}$, $(CAC)_{10}$, and $(CAT)_{10}$; (ii) in some chromosomes for $(TA)_{15}$, $(CAG)_{10}$, $(CGG)_{10}$, and $(GAG)_{10}$; and finally (iii) only two chromosomes for $(GC)_{15}$ and $(CAA)_{10}$. This study provides the first chromosomal mapping of repetitive elements in the genome of *R. nigrovittata*. The in situ investigation of repetitive DNA elements provided useful characteristics for comparative genomics at the chromosomal level, offering new insights into the karyotype evolution of *Rana* species in the latter.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflict of interests.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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