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First Study on Chromosomal Feature and NORs Localization of the Yellow-spotted Keelback Snake, *Fowlea flavipunctatus* (Squamata, Natricinae) in Thailand

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Abstract-The first report on chromosome of the yellow-spotted keelback snake (*Fowlea flavipunctatus*), from Khon Kaen Province, Thailand, was investigated using classical cytogenetic approach. The mitotic chromosomes were directly prepared from the bone marrow cells of four adult male and female specimens after *in vivo* colchicine treatment. The results shown that the diploid chromosome number was 2*n*=42, while the fundamental number (NF) was 58 in both male and female. The types of macrochromosome were six large metacentric (m), two large acrocentric (a), four small metacentric and four small telocentric (t) chromosomes, and 24 microchromosomes ($mi^m_2 + mi^t_{22}$). Nucleolar organizer regions (NORs) located on subtelomeric position of short arm of macrochromosomes pair 4. The sex chromosome system was ZW. The karyotype formula of *F. flavipunctatus* is as follows: 2*n* (42) = $L^m_6 + L^a_2 + S^m_4 + S^t_4 + mi^m_2 + mi^t_{22}$ + 2 sex chromosomes (ZW).

Keywords: *Fowlea flavipunctatus,* Yellow-spotted keelback, Cytogenetic, Karyotype

1. Introduction

The yellow-spotted keelback snake, *Fowlea flavipunctatus* (Hallowell, 1860) belongs to the subfamily Natricinae of Colubridae. This subfamily consists of 245 species in 37 genera (Dubey *et al*., 2012; Zaher *et al*., 2019). The genus *Fowlea* comprises eight species namely, *Fowlea asperrimus* (BOULENGER, 1891), *F. flavipunctatus* (HALLOWELL, 1860), *F. melanzostus* (GRAVENHORST, 1807), *F. piscator* (SCHNEIDER, 1799), *F. punctulatus* (GÜNTHER, 1858), *F. sanctijohannis* (BOULENGER, 1890), *F. schnurrenbergeri* (KRAMER, 1977) and *F. tytleri* (BLYTH, 1863).

Visual characteristics of *F. flavipunctatus* are maximum recorded total length 991 mm in adult, body cylindrical, head distinct from the neck and eye large. This species has body-coloured as: olive, brown, dark grey or pale grey. The neck is often marked with conspicuous Y-shaped; venter pattern has darker shades only on the outermost edges. This species is distributed throughout Southeast Asia (Vogel and David, 2006).

Phylogenetic relationships within this subfamily and the systematic position of various species and genera were recently analyzed using molecular and morphological data (e.g. Boulenger, 1986; Cox *et al*., 1998; Dubey *et al*., 2012; Kramer, 1977; Smith, 1943; Taylor, 1965; Vogel and David, 2006; Zaher, 1999). In contrast, there are poorly known karyological data of any species of this genus. The previous reports were restricted to other genera of Natricinae (Table. 1). Karyological information has been obtained using standard staining protocols. From literature review about cytogenetic studies of snakes in the subfamily Natricinae, covering reports by Eberle, (1971); Rossman and Eberle (1977); Trinco and Smith (1971) and Itoh *et al*. (1970) reported a range of karyotypes from $2n$=17 to 21 pairs of chromosomes.

The cytogenetic analysis has been useful for taxonomic studies in Serpentes with inferring evolutionary relationships if they are analyzed together with other independent characters e.g morphological, molecular, immunological and isozyme (Falcione, 2016; Sites and Reed, 1994). In the present study, a report on karyotype analysis and chromosomal characteristics of the nucleolar organizer regions (NORs) of *F. flavipunctatus* is the first cytogenetic record in Thailand. This advanced cytogenetic technique provides useful basic information for genetic research on the taxonomy and evolution of the species.

2. Materials and methods

2.1 Sample collection

Four adult males and females of *F. flavipunctatus* were collected from Khon Kaen Province, Thailand.They were transferred to the laboratory and kept under standard conditions for 1 day prior to the experiments.

2.2 Chromosome preparation

Chromosomes were directly prepared *in vivo* using the following method (Ota, 1999). The 0.01% colchicine was injected into the snake's abdominal (1 mL/100 g body weight) and left for 12 hours. Bone marrows were cut into small pieces, then squashed and treated with 0.075 M

hypotonic KCl for 30 min. Cells were fixed in a cool fixative solution (3 absolute methanol: 1 glacial acetic acid).

Table 1. Review of cytogenetic reports of subfamily Natricinae

Species	2*n*	Mac	Mi	Reference
Fowlea flavipunctatus (*Xenochrophis flavipunctatus*)	42	18	24	Present study
Natrix piscator (*Xenochrophis piscator*)	40	10	30	(Singh *et al*., 1968 ; Rao *et al*., 2010)
Xenochrophis piscator	36 and 38	16 and 10	20 and 28	(Dutt, 1970)
Xenochrophis vittattus	34	26	8	(Hanifa and Nurhandayani, 2015)

As stated above, 2n=diploid chromosome, Mac=macrochromosome, Mi=microchromosome

2.3 Chromosome staining

Conventional staining was done using 20% Giemsa's stock solution for 30 min. 50% $AgNO_3$ and 2% gelatin were used for Ag-NOR banding technique (Howell and Black, 1980).

2.4 Chromosome checks

The length of short arms (Ls) and long arms (Ll) of chromosomes were measured and calculated for the length of total arm chromosomes (LT, LT = Ls + Ll). Relative length (RL) and centromeric index (CI) were estimated. CI was also computed to classify the types of chromosomes according to Turpin and Lejeune (1965). Classification of microchromosomes is based on the presence of a size between groups of large chromosomes and smaller chromosomes, all the microchromosomes are very small are often less than 1.5 micrometeres (Ezaz and Young, 2013). All parameters were used in karyotyping and idiograming.

3. Results and discussion

The diploid chromosome number of *F. flavipunctatus* was 2*n*=42 (16 macrochromosomes + 24 microchromosomes) and consisted of six large metacentric, two large acrocentric, four small metacentric and four small telocentric chromosomes, and 24 microchromosomes. The types of microchromosomes series were two metacentrics and 22 telocentric microchromosomes (Fig.1). The fundamental number (NF, chromosome arm number) was 58. The idiogram of *F. flavipunctatus* revealed that the gradually decreasing length of the autosomes (Fig. 2). Only one nucleolar organizer region was detected on the subtelomeric of the long arm of the large acrocentric macrochromosome pair 4 (Fig.1, 2). The sex chromosome showed a heteromorphic ZZ/ZW sex chromosome system, both the Z and W chromosomes are small submetacentric chromosomes.

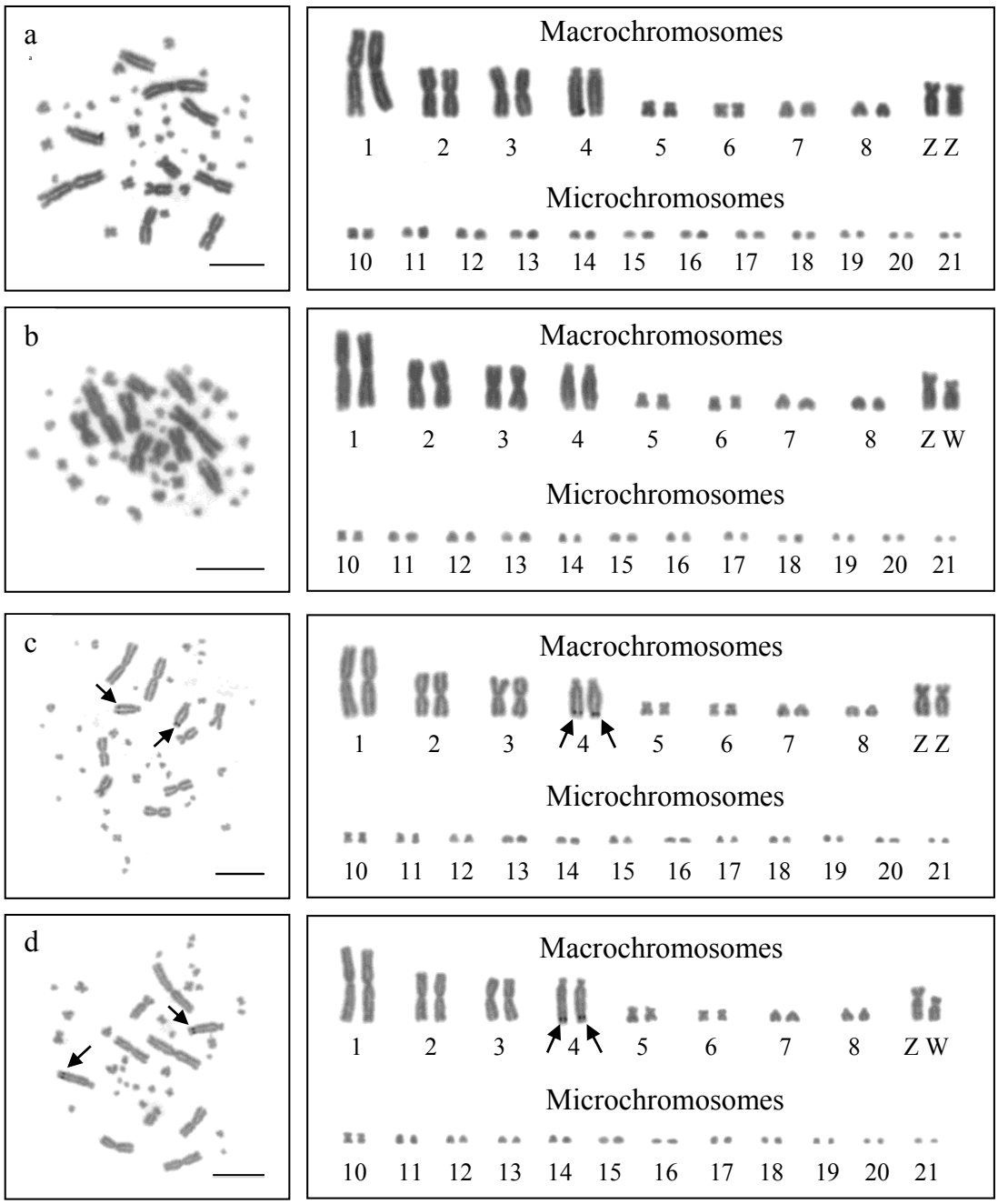

Figure. 1 Metaphase chromosome plates and standardized karyotypes of *Fowlea flavipunctatus*, 2*n*=42 by conventional staining of male (a) and female (b) and Ag-NOR banding technique of male (c) and female (d). The arrows indicate subtelomeric NOR on macrochromosome pair 4 (scale bar=5 μm).

The *F. flavipunctatus* is member of Natricinae. The letter being reported the generic name *Fowlea* was used member for subspecies under the *Natrix piscator* (Smith, 1943), which was later changed to the genus *Xenochrophis* (Wallach, *et al*., 2014). This study was different from previous research, the 2*n* of genus *Xenochrophis* ranges from 34 to 40 (Table 1). The natricine snakes mostly have a karyotype of 2*n*=36 chromosomes, with 16 macrochromosomes and 20 microchromosomes (Beçak and Beçak, 1969; Oguiura *et al*., 2009).

Snake chromosome complements, like avian chromosome complements are comprised of both macrochromosomes and microchromosomes. The karyotype 2*n*=36 (16 bi-armedmacrochromosomes + 20 microchromosomes) is widely Accepted: as the ancestral character shared throughout the large family Colubridae, most of Viperidae and Boidae (Oguiura *et al*., 2009).

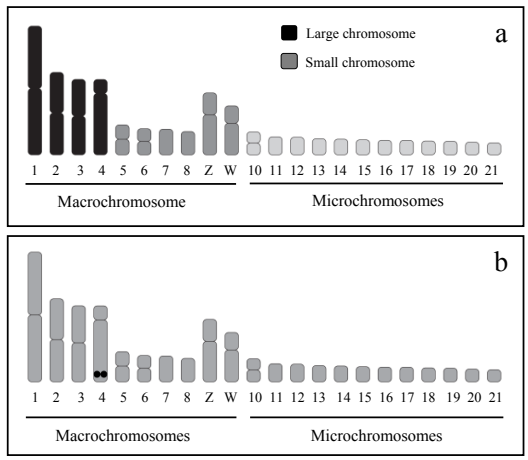

Figure. 2 Standardized idiogram of Yellow-spotted keelback snake (*Fowlea flavipunctatus*), 2*n*=42 by conventional staining (a) and Ag-NOR banding (b) techniques. The black dot is indicate subtelomeric NOR macrochromosome

Table. 2 Mean length of the short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI, RL from metaphase chromosomes in 20 cells of the Yellow-spotted keelback snake (*Fowlea flavipunctatus*), 2*n*=42

Chro.	Ls	Ll	LT	RL±SD.	CI±SD.	Size	Type
1	2.475	2.637	5.113	0.181±0.010	0.516±0.015	L	m
2	1.610	1.666	3.276	0.181±0.011	0.508±0.008	L	m
3	1.461	1.537	2.998	0.181±0.012	0.513±0.020	L	m
4*	0.554	2.440	2.994	0.181±0.013	0.813±0.023	L	a
5	0.569	0.622	1.192	0.181±0.014	0.522±0.022	S	m
6	0.508	0.532	1.041	0.181±0.015	0.512±0.013	S	m
7	0.000	1.020	1.020	0.181±0.016	1.000±0.000	S	t
8	0.000	0.927	0.927	0.181±0.017	1.000±0.000	S	t
9	0.445	0.468	0.914	0.181±0.018	0.513±0.012		mi (m)
10	0.000	0.717	0.717	0.025±0.003	1.000±0.000		mi (t)
11	0.000	0.700	0.700	0.025±0.003	1.000±0.000		mi (t)
12	0.000	0.644	0.644	0.023±0.002	1.000±0.000		mi (t)

Table. 2 Mean length of the short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), centromeric index (CI), relative length (RL) and standard deviation (SD) of CI, RL from metaphase chromosomes in 20 cells of the Yellow-spotted keelback snake (*Fowlea flavipunctatus*), 2*n*=42 (continue)

Chro.	Ls	Ll	LT	RL±SD.	CI±SD.	Size	Type
13	0.000	0.626	0.626	0.022±0.003	1.000±0.000		mi (t)
14	0.000	0.599	0.599	0.021±0.002	1.000±0.000		mi (t)
15	0.000	0.580	0.580	0.021±0.002	1.000±0.000		mi (t)
16	0.000	0.578	0.578	0.020±0.002	1.000±0.000		mi (t)
17	0.000	0.551	0.551	0.020±0.002	1.000±0.000		mi (t)
18	0.000	0.539	0.539	0.019±0.002	1.000±0.000		mi (t)
19	0.000	0.505	0.505	0.018±0.002	1.000±0.000		mi (t)
20	0.000	0.479	0.479	0.017±0.003	1.000±0.000		mi (t)
Z	0.866	1.596	2.463	0.044±0.003	0.647±0.026	S	sm
W	0.690	1.257	1.947	0.035±0.003	0.649±0.030	S	sm

As stated above, Chro.=chromosome pair, m=metacentric, sm=submetacentric, a=acrocentric, t=telocentric, mi=microchromosome, L=large, S=small, * subtelomeric macrochromosome/NOR

However, the hypothesis of transition from the plesiomorphic snake karyotype (2*n*=36) to the karyotype of *F. flavipunctatus* (2*n*=42) may have occurred as a two-step of six centric fissions from bi-armed macrochromosome to eight mono-armed macrochromosome and four microchromosomes. Then four pericentric inversions from four mono-armed macrochromosomes to four bi-armed macrochromosomes. Moreover, the metacentric of microchromosome is the result of pericentric inversion.

In suborder Serpentes, presence of ZZ/ZW sex chromosome system was found in all female heterogamety (Gamble and Zarkower, 2012; Matsubara *et al*., 2006; Oguiura *et al*., 2009; Olmo, 2005). The variable morphology of heteromorphy of the ZW chromosomes are remarkable; they can be similar morphologically or differ in their shape and/or size, centromeric position and in heterochromatin distribution (Beçak and Beçak, 1969; Mengden and Stock, 1980; Oguiura *et al*., 2009; Olmo, 1986; Singh, 1972).

The localization of NOR loci is poorly known and also provides interesting data. The presence of NOR loci on a single microchromome pair is Accepted: as a plesiomorphic condition in Squamata, while a derived condition is with loci of NORs either on macrochromosomes or two chromosome pairs (Porter *et al*., 1991). Therefore, *F. flavipunctatus* show NORs on a macrochromosomes would indicate as the apomorphic state of NORs. Similarly, the *Macropisthodon rudis* presence the NORs location on the macrochromosomes (Ruifang *et al*, 1996; Toriba, 1990).

Cytotaxonomy is a key role in explaining the taxonomy and chromosomal evolution of snake when morphological characteristics are scarce for the determination of taxonomic problems (Dubey *et al*.,

2012; Wallach *et al*., 2014). In this investigation, we provide new data for the current karyology in the *F. flavipunctatus*, as determined by conventional staining and Ag-NOR banding techniques. The number of diploid chromosome was 2*n*=42 (18 macrochromosomes + 24 microchromosomes), while the fundamental number was 58 in both sex. The sex chromosome presence of ZZ/ZW system. The NORs appeared on the long arms subtelomeric region of the 4th acrocentric of macrochromosomes. Moreover, increasing the taxonomic sampling will allow us for further study on taxonomy and evolutionary relationships in genus *Fowlea***.**

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