Standardized Karyotype and Idiogram of Bengal Monitor Lizard, *Varanus bengalensis* (Squamata, Varanidae)

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Received July 10, 2015; accepted November 10, 2015

Summary The standardized karyotype and idiogram of the Bengal monitor lizard (*Varanus bengalensis*) from the Kalasin Province of Thailand were studied. Blood samples were taken from male and female lizards. The standard T-lymphocyte cell was cultured at 26°C for 96 h. The mitotic chromosomes were harvested by colchicine-hypotonic-fixation-air drying technique. Conventional staining, Ag-NOR banding and C-banding techniques were applied to stain the chromosome with 20% Giemsa's solution. The results showed that the number of diploid chromosome was $2n=40$, while the fundamental number (NF) was 54 in males and 55 in females. The types of macrochromosomes were 4 large metacentric, 4 small metacentric, 6 small submetacentric, 2 small telocentric macrochromosomes and 24 microchromosomes. The Z chromosome is the small telocentric chromosome, while the W chromosome is the small metacentric chromosome. In addition, the short arm pericentromeric region of chromosome pair 1 showed clearly observable nucleolar organizer regions (NORs). This data is the first report on polymorphism of NORs in *V. bengalensis*. The result showed that the heteromorphic of one male had a different size of NORs of chromosome pair 1 (1a1b), while one female had an equal size of both chromosome pair 1 with a homomorphic (1a1a). The karyotype formula is as follows:

$$2n (40) = L_4^m + S_4^m + S_6^{sm} + S_2^t + Z(t)W(m) + 22 \text{ microchromosomes}$$

Key words Bengal monitor lizard, *Varanus bengalensis*, Karyotype, Chromosome.

The Bengal monitor lizard, *Varanus bengalensis* (Gray 1831) (Fig. 1), is a member of class Reptilia, order Squamata, suborder Lacertilia, superfamily Varanoidea and family Varanidae. All living monitor lizards are placed in a single genus *Varanus*, generally considered to be monophyletic. At this time, there are 46 species that are found in Africa, Asia and Australia. Four species of the genus *Varanus* have been recorded in Thailand: *V. bengalensis*, *V. salvator* (Laurenti 1768), *V. rudicollis* (Gray 1845) and *V. dumerilii* (Schlegel 1839) (Bennett 1998, Lauprasert and Thirakhupt 2001, Pianka 1995).

The Bengal monitor lizard is a member of the monitor or Varanid lizards group, native to Afghanistan, Pakistan, India, Sri Lanka, Bangladesh, Nepal, southern China, Myanmar, Laos, Vietnam, Cambodia and Thailand. The visual characteristics of *V. bengalensis* are a slender body, snout somewhat elongated, nostrils nearer

the eyes than the snout-tip, tail flattened, unicoloured or with a less distinct pattern, chin and throat with black spots, venter cream or yellow. The lizard's diet consists of insects, snails, frogs, small mammals, birds, lizards and snakes (Das 2010) (Fig. 1).

Lizards from the family Varanidae belong to the group of squamates for which the karyotypes appear highly conserved. Until now, there were only three previous cytogenetic studies of *V. bengalensis* according to the cytogenetic studies by Dutt (1968), Singh *et al.* (1970) and De Smet (1981). The present study is the first report on the chromosomal characteristics of *V. bengalensis* using Ag-NOR banding and C-banding techniques. The results of this study enhances cytogenetic information and enables future comprehensive studies to be conducted on taxonomy and evolutionary relationships.

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DOI: 10.1508/cytologia.82.75

Fig. 1. General characteristics of the Bengal monitor, *Varanus bengalensis* (Varanidae, Squamata) from Kalasin Province of Northeastern Thailand; scale bar indicates 10 cm.

Fig. 2. The metaphase chromosome plates and karyotypes of male (A) and female (B) Bengal monitors (*Varanus bengalensis*), $2n=40$, by conventional staining technique. The secondary constrictions appear on the subcentromeric on the short arm of the largest metacentric chromosome pair 1 (arrows). Scale bar indicates $5\mu\text{m}$.

Materials and methods

Blood samples and cell cultures

Blood samples from one male and one female *V. bengalensis* were maintained in the Kalasin Province of Thailand. The samples were collected from the blood vein using an aseptic technique, and kept on ice in 5 mL vacuum tubes coated with heparin to prevent blood clot-

ting. After cooling, 0.5 mL of whole blood were cultured in 5 mL of RPMI 1640 medium supplemented with 2% phytohemagglutinin (PHA) as a mitogen at 26°C , 5% CO_2 . The cultured bottle was loosely capped and regularly shaken twice a day, in the morning and evening. After a 96 h incubation, colchicine was introduced and mixed for further incubation for 30 min.

Fig. 3. The metaphase chromosome plates and karyotypes of male (A) and female (B) Bengal monitors (*Varanus bengalensis*), $2n=40$, by Ag-NOR banding technique. The result showed that a heteromorphic of one male had a different size of NORs of chromosome pair 1 (1a1b), while one male had an equal size of both chromosome pair 1 with a homomorphic (1a1a). Scale bar indicates $5\mu\text{m}$.

Fig. 4. The metaphase chromosome plates and karyotypes of male (A) and female (B) Bengal monitors (*Varanus bengalensis*), $2n=40$, by C-banding technique. Scale bar indicates $5\mu\text{m}$.

Table 1. Review of cytogenetic reports of lizards in family Varanidae (Reptilia, Squamata).

Species	2n	mac	mi	NORs	Reference
<i>Varanus acanthurus</i>	40 (ZW)	16 (14bi+2t)	24	1 st	King <i>et al.</i> (1982)
<i>V. albigularis</i>	40 (ZW)	16 (8bi+8t)	24	1 st	King and King (1975)
<i>V. bengalensis</i>	40	16 (8bi+8t)	24	—	Dutt (1968)
	40	16 (14bi+2t)	24	—	Singh <i>et al.</i> (1970)
	40	16 (16bi)	24	—	De Smet (1981)
	40 (ZW)	16 (14bi+2t)	24	1 st	Present study
<i>V. exanthematicus</i>	40	16 (8bi+8t)	24	—	De Smet (1981)
<i>V. flavescens</i>	40	16 (14bi+2t)	24	—	Singh <i>et al.</i> (1970)
<i>V. giganteus</i>	40	16 (10bi+6t)	24	1 st	King and King (1975)
<i>V. gilleni</i>	40	16 (14bi+2t)	24	1 st	King and King (1975)
<i>V. gouldii</i>	40	16 (8bi+8t)	24	—	Matthey (1931)
	40	16 (10bi+6t)	24	1 st	King and King (1975)
<i>V. griseus</i>	40	16 (16bi)	24	1 st	King and King (1975)
	40	16 (14bi+2t)	24	1 st	De Smet (1981)
<i>V. indicus</i>	40	16 (14bi+2t)	24	1 st	King and King (1975)
<i>V. mertensi</i>	40	16 (10bi+6t)	24	1 st	King and King (1975)
<i>V. nebulosus</i>	40	16 (16bi)	24	1 st	King and King (1975)
<i>V. niloticus</i>	40 (ZW)	16 (8bi+8t)	24	1 st	King and King (1975)
	40	16 (8bi+8t)	24	1 st	Porter <i>et al.</i> (1994)
<i>V. rosenbergi</i>	40	16 (10bi+6t)	24	1 st	King and King (1975)
<i>V. rudicollis</i>	40	16 (14bi+2t)	24	—	Gorman and Gress (1970)
<i>V. salvator</i>	40	16 (16bi)	24	1 st	King and King (1975)
	40	16 (14bi+2t)	24	—	De Smet (1981)
<i>V. scalaris</i>	40	16 (14bi+2t)	24	1 st	King and King (1975)
<i>V. semiremex</i>	40	16 (14bi+2t)	24	1 st	King and King (1975)
<i>V. spenceri</i>	40	16 (10bi+6t)	24	1 st	King and King (1975)
<i>V. storri</i>	40	16 (14bi+2t)	24	1 st	King and King (1975)
<i>V. tristis</i>	40	16 (14bi+2t)	24	1 st , 7 th	King and King (1975)
<i>V. varius</i>	40 (ZW)	16 (14bi+2t)	24	1 st	King and King (1975)

Remarks: 2n=diploid chromosome, mac=macrochromosome, mi=microchromosome, bi=bi-arms chromosome, t=telocentric chromosome and —=not available.

Table 2. Mean length (L) of the short arm chromosome (Ls), long arm chromosome (Ll), and total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes in 10 cells of male Bengal monitor (*Varanus bengalensis*), 2n=40.

Chro. pair	Ls	Ll	LT	CI±SD	RL±SD	Chro. type	Chro. size
1*	7.331	9.522	16.853	0.565±0.005	0.195±0.003	Metacentric	Large
2	7.022	8.431	15.453	0.546±0.004	0.179±0.003	Metacentric	Large
3	3.612	4.064	7.676	0.529±0.002	0.089±0.002	Metacentric	Small
4	0.000	5.752	5.752	1.000±0.000	0.067±0.005	Telocentric	Small
5	2.271	3.461	5.732	0.604±0.006	0.066±0.003	Submetacentric	Small
6	1.885	3.622	5.507	0.658±0.003	0.064±0.004	Submetacentric	Small
7	1.833	2.762	4.595	0.601±0.002	0.053±0.003	Submetacentric	Small
8	2.111	2.462	4.573	0.538±0.005	0.053±0.004	Metacentric	Small
9	—	—	2.201	—	0.025±0.002	Microchromosome	
10	—	—	2.002	—	0.023±0.002	Microchromosome	
11	—	—	1.701	—	0.020±0.001	Microchromosome	
12	—	—	1.653	—	0.019±0.003	Microchromosome	
13	—	—	1.634	—	0.019±0.003	Microchromosome	
14	—	—	1.603	—	0.019±0.002	Microchromosome	
15	—	—	1.556	—	0.018±0.002	Microchromosome	
16	—	—	1.537	—	0.018±0.002	Microchromosome	
17	—	—	1.482	—	0.017±0.003	Microchromosome	
18	—	—	1.431	—	0.017±0.003	Microchromosome	
19	—	—	1.300	—	0.015±0.001	Microchromosome	
Z	0.000	2.17	2.170	1.000±0.000	0.025±0.003	Telocentric	Small

Remarks: *=Nucleolar organizer region, (NOR) and chro.=chromosome

Cell harvest

After colchicine incubation, the blood mixture was centrifuged at 1200rpm for 10 min. After discarding the supernatant, the cells were treated with 10mL of hypo-

tonic solution (0.075 M KCl) and the cells were incubated at 26°C for 30min. The cells then were centrifuged and the supernatant was discarded. Fresh cool fixative (3 methanol:1 acetic acid) was used to fix the cells by

Table 3. Mean length (L) of the short arm chromosome (Ls), long arm chromosome (Ll), and total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes in 10 cells of female Bengal monitor (*Varanus bengalensis*), $2n=40$.

Chro. pair	Ls	Ll	LT	CI $\pm$ SD	RL $\pm$ SD	Chro. type	Chro. size
1*	8.302	10.761	19.063	0.564 $\pm$ 0.005	0.185 $\pm$ 0.003	Metacentric	Large
2	8.212	9.963	18.175	0.548 $\pm$ 0.003	0.176 $\pm$ 0.003	Metacentric	Large
3	4.031	4.864	8.895	0.547 $\pm$ 0.004	0.086 $\pm$ 0.004	Metacentric	Small
4	0.000	7.270	7.270	1.000 $\pm$ 0.000	0.070 $\pm$ 0.001	Telocentric	Small
5	2.385	3.911	6.296	0.621 $\pm$ 0.005	0.061 $\pm$ 0.002	Submetacentric	Small
6	2.077	4.205	6.282	0.669 $\pm$ 0.004	0.061 $\pm$ 0.001	Submetacentric	Small
7	1.891	3.683	5.574	0.661 $\pm$ 0.004	0.054 $\pm$ 0.003	Submetacentric	Small
8	2.593	2.792	5.385	0.518 $\pm$ 0.006	0.052 $\pm$ 0.003	Metacentric	Small
9	—	—	2.370	—	0.023 $\pm$ 0.003	Microchromosome	
10	—	—	2.301	—	0.022 $\pm$ 0.002	Microchromosome	
11	—	—	2.203	—	0.021 $\pm$ 0.002	Microchromosome	
12	—	—	2.054	—	0.020 $\pm$ 0.004	Microchromosome	
13	—	—	1.931	—	0.019 $\pm$ 0.002	Microchromosome	
14	—	—	1.866	—	0.018 $\pm$ 0.002	Microchromosome	
15	—	—	1.780	—	0.017 $\pm$ 0.002	Microchromosome	
16	—	—	1.707	—	0.017 $\pm$ 0.004	Microchromosome	
17	—	—	1.621	—	0.016 $\pm$ 0.003	Microchromosome	
18	—	—	1.563	—	0.015 $\pm$ 0.002	Microchromosome	
19	—	—	1.453	—	0.014 $\pm$ 0.001	Microchromosome	
Z	0.000	2.620	2.620	1.000 $\pm$ 0.000	0.025 $\pm$ 0.002	Telocentric	Small
W	1.161	1.720	2.881	0.597 $\pm$ 0.005	0.028 $\pm$ 0.004	Metacentric	Small

Remarks: *=Nucleolar organizer region, (NOR) and chro.=chromosome.

gradually adding it up to 8 mL. After centrifugation, the fixation was repeatedly done until the supernatant was clear. The cells were added to 1 mL fixative by dropping onto a clean cold slide and then dried by air-dry technique (Kasiroek *et al.* 2017). The slide was conventionally stained with 20% Giemsa's solution for 30 min (Choo-seangjaew *et al.* 2017).

C-banding method

The slides were heated at 60°C for two to three days, soaked in 0.2N HCl for 10–15 min, rinsed with distilled water, then soaked in 0.05N Ba(OH)₂ for 15 min at 37°C and rinsed with distilled water at 60°C. After that, the slide was soaked in 2X SSC at 60°C for one to two hours. The slide was then stained with 20% Giemsa's solution for 30 min (Rooney 2001, Patawang and Tanom-tong 2017).

Ag-NOR banding method

Two drops of 50% silver nitrate and 50% gelatin were added on to the slides, respectively. They were then sealed with cover glasses and incubated at 60°C for three hours. After that, the slides were soaked in distilled water until the cover glasses were separated. The slide was stained with 20% Giemsa's solution for one minute (Howell and Black 1980, Sangpakdee *et al.* 2017).

Results and discussion

Conventional staining of the karyotypes identified the diploid number of chromosomes for *V. bengalensis* to be $2n=40$ (Fig. 2). These karyotypes all consist of

16 macrochromosomes (14 bi-arms chromosomes and 2 mono-arm chromosomes) and 24 microchromosomes. We found the total chromosome number of macrochromosomes and microchromosomes and the morphologies of macrochromosomes of *V. bengalensis* in this study to be identical to those reported previously, confirming that at a gross morphological level, the karyotypes of *Varanus* species are conservative. Karyotypes have been reported for 22 *Varanus* species. All species examined so far have an identical chromosome number, $2n=40$, consisting of 16 macrochromosomes and 24 microchromosomes (Table 1), indicating that the karyotypes at this level are highly conserved in this family. This high conservation of karyotypes makes varanid lizards an excellent model to examine the fine scale molecular evolution of DNA sequences on some local chromosomal regions (Matsubara *et al.* 2014).

V. bengalensis has three types of macrochromosomes, which are 8 metacentric (four large and four small chromosomes), 6 submetacentric and 2 telocentric chromosomes (Table 2, 3). Different chromosomal features were reported by De Smet (1981), which consisted of 16 bi-arms and 24 uni-arms macrochromosomes. The microchromosomes in the *V. bengalensis* are 24 in number and all of them are nearly the same size. They behave like macrochromosomes but their small size inhibits critical analysis.

Current knowledge shows that the sex-chromosome system of the *V. bengalensis* is ZW, where the Z and W are classified as small telocentric and small metacentric chromosomes, respectively. The sex chromosomes are identified in four species (*V. acanthurus*, *V. albigularis*,

Fig. 5. The standardized idiogram of Bengal monitor (*Varanus bengalensis*), $2n=40$, by conventional staining (A) and Ag-NOR banding techniques (B); arrows indicate nucleolar organizer regions (NORs).

Fig. 6. The standardized idiogram of Bengal monitor (*Varanus bengalensis*), $2n=40$, by C-banding technique.

V. niloticus and *V. varius*). All four species have ZZ/ZW micro sex chromosomes in which the W is distinctively larger than other microchromosomes. The sex chromosomes of the many remaining species are unknown, and the molecular composition and constitution of the W chromosomes are yet to be investigated in any species (Matsubara *et al.* 2014).

The short arm pericentromeric region of chromosome pair 1 showed clearly observable nucleolar organizer regions (NORs) (Fig. 3). This is the first report on polymorphism of NORs in the *V. bengalensis*. The result showed that the heteromorphic of one male had a different size of NORs of chromosome pair 1 (1a1b), while one female had an equal size of both chromosome

Fig. 7. The comparison of secondary constriction/nucleolar organizer region on largest metacentric chromosome pair 1 between male (heteromorphic, 1a1b) and female (homomorphic, 1a1a) Bengal monitors (*Varanus bengalensis*) by conventional staining (A) and NOR-banding (B) techniques.

pair 1 with the homomorphic (1a1a) (Figs. 5, 7). Porter *et al.* (1994) found that the ribosomal genes (NORs) of the *V. niloticus* are found near the centromere of pair 1, which is a large metacentric chromosome. The region of hybridization corresponds with a distinct secondary constriction, which King and King (1975) correctly interpreted as the nucleolus organizer. The secondary constriction is found near the centromere of chromosome pair 1 in all karyotyped species of the genus (King and King 1975). Assuming that rDNA can be accurately located by the secondary constriction, it would appear that the ribosomal genes have remained in the same location throughout the evolution of genus *Varanus*. The proximal location of NORs in varanid chromosomes is unusual. Other squamate reptiles typically have the sites of NORs located near the chromosome ends.

The C-banding can provide a dark region on the chromosome, which represents the constitutive heterochromatin of the chromosome that can be found at all centromeres and some telomere of normal chromosomes. C-bands were detected at the centromeric regions of chromosome pairs 3, 5–8 (macrochromosomes), 9–10, 15, 17–19, and W (microchromosomes); on interstitial regions of chromosome pairs 1–6 (macrochromosomes),

14, and Z (microchromosomes); and on the telomeric regions of chromosome pairs 1, 4, 6–7 (macrochromosomes), 11, 13, and 16 (microchromosomes) (Fig. 4).

V. bengalensis demonstrated that the chromosome marker is the chromosome pair 1, which is the largest metacentric chromosome. The important karyotype feature of the *V. bengalensis* is the asymmetrical karyotype, which was found in three types of chromosomes (metacentric, submetacentric and telocentric chromosomes). The largest chromosome is eight times larger than the smallest chromosomes. Figures 5, 6 show the idiograms from conventional staining, Ag-NOR banding and C-banding techniques. The karyotype formula could be deduced as:

$$2n(40) = L_4^m + S_4^m + S_6^{sm} + S_2^t + Z(t)W(m) + 22 \text{ microchromosomes}$$

Acknowledgements

This work was financially supported by the Research Group on Toxic Substances in Livestock and Aquatic Animals, Khon Kaen University. We would like to thank the laboratory of animal cytogenetic group in the Department of Biology, Faculty of Science, Khon Kaen University and the laboratory of Department of Biology, Faculty of Science, Chiang Mai University for available help.

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