First Cytogenetic Study of Puff-Faced Water Snake, *Homalopsis buccata* (Squamata, Colubridae) by Conventional Staining, Ag-NOR Banding and GTG-Banding Techniques

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Summary The first cytogenetics of the puff-faced water snake (*Homalopsis buccata*) from Khon Kaen Province, Thailand was obtained from the present study. Mitotic chromosome was prepared directly from the spleens of specimens after *in vivo* colchicines treatment. The metaphase spreads were performed on microscopic slides and air-dried. Conventional staining, Ag-NOR banding, and GTG-banding techniques were applied to stain the chromosome with Giemsa's solution. Results showed that the number of diploid chromosome was $2n=36$, while the fundamental number (NF) was 52 in both males and females. The types of macrochromosome were four large metacentric, two medium metacentric, six small metacentric, and two small acrocentric chromosome, and 20 microchromosomes. It was found that nucleolar organizer regions (NORs, the representative of chromosome marker) locate on the long arms near centromeres of a pair of microchromosomes, pair 15. The Z chromosome was a small metacentric chromosome, while the W chromosome was a small sub-metacentric chromosome. From the GTG-banding technique, it was found that each chromosome pairs could be clearly differentiated and the numbers of bands in the *H. buccata* was 109. The karyotype formula is as follows:

$$2n (36) = L_4^m + M_2^m + S_6^m + S_2^a + 20 \text{ microchromosomes} + \text{sex chromosomes (ZW)}$$

Key words Puff-faced water snake, *Homalopsis buccata*, Karyotype, Chromosome.

The snake fauna of Thailand is rich; there are more than 200 species. However, studies on snake karyotypes are very rare. Only a few species have been examined karyologically. The puff-faced water snake, *Homalopsis buccata* (Linnaeus 1758), is a member of class Reptilia, order Squamata, suborder Serpentes, family Colubridae, and subfamily Homalopsinae (Chan-ard *et al.* 1999). Colubridae is a family of snakes, and this broad classification of snakes includes about two-thirds of all snake species on earth. The earliest species of the snake family date back to the Oligocene epoch. With 304 genera and 1,938 species, Colubridae is the largest snake family. Colubrid species are found on every continent except Antarctica (Bauer *et al.* 1998). The Colubridae are not a natural group, as many are more closely related to other groups (Lawson *et al.* 2005).

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The genus *Homalopsis* can be distinguished from all other Southeast Asian snakes. Visual characteristics of *H. buccata* are: dark brown upper surface, with narrow, pale brown, black-edged transverse bands, which are yellow in young specimens; head pale with a triangular or V-shaped dark marking on the snout, an A-shaped spot on the occiput, and a dark band on each side passing through the eye and extending to before the eye. The lower surface is white or yellowish, with dark brown spots along each side; the tail has brown spots. The length of the head and body is 820 mm, the tail 230 mm. It eats fish and frogs (Murphy *et al.* 2012).

Karyological data act as a supplement to the morphological approach. Karyologically, snakes are highly conserved and variation is found only at family or subfamily level. They show a narrow range of chromosome numbers showing a preponderance of species with $2n=36$ including 16 macrochromosomes and 20 microchromosomes (Sharma and Kour 2005). Reptilian chromosomes have long been under discussion, particularly in relation to the sex determining mechanism, because in reptiles the situation is inconsistent showing female heterogamety in many species of snakes (Kobel 1967, Beçak *et al.* 1962, 1964, Singh 1972).

From a literature review about cytogenetic studies of snakes in the family Colubridae, covering reports by Nakamura (1927, 1928, 1935), Matthey (1931), Bhatnager (1959), Beçak (1965), Kobel (1967), Fischman *et al.* (1968), Beçak and Beçak (1969), Bury *et al.* (1970), Dutt (1970), Gorman and Gress (1970), Singh *et al.* (1970), Baker *et al.* (1972), Singh (1972), Gutiérrez *et al.* (1984), Moreno *et al.* (1987), Ota and Iwanaga (1996), Ota (1999), Beçak *et al.* (2003) and Aprea *et al.* (2003). The present cytogenetic study, the report on karyotype analysis and chromosomal characteristic of the nucleolar organizer regions (NORs) of *H. buccata*, is the first record. In the future, basic knowledge on and the cytogenetics of *H. buccata* could be applied to several areas of research and in particular for protection from extinction.

Materials and methods

Two adult males and females of *H. buccata* (family Colubridae) were captured in the field from Khon Kaen province, Thailand. Chromosome preparation was conducted by squash technique, from spleens. Conventional staining, Ag-NOR banding (Howell and Black 1980) and GTG-banding techniques (Rooney 2001) were applied to stain the chromosomes with Giemsa's solution. The lengths of short arm (Ls) and long arm (Ll) chromosomes were measured and calculated for the length of total arm chromosome (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 Chaiyasut (1989). All parameters were used in karyotyping and idiogramming.

Results and discussion

Karyotype analysis and morphometric measurements of the chromosomes of *H. buccata* have been conducted. An analysis of 50 well-spread metaphase plates from different somatic tissue of two male and two female specimens revealed a diploid chromosome count of $2n=36$ comprising 16 macrochromosomes and 20 microchromosomes (Fig. 1). The macrochromosomes include four large metacentric, two medium metacentric, six small metacentric, two small acrocentric, and two sex chromosomes (ZW system). The Z chromosome is the small metacentric chromosome, while the W chromosome is a small submetacentric chromosome.

There exists a shape distinction in size between macrochromosomes and microchromosomes; all the microchromosomes are very small, dot-like with a size ranging from 1.0 to 0.4 microns. The sex chromosomes could be distinguished in either sex. Only one nucleolar organizer region site was detected in the silver-stained cells (Ag-NOR banding technique) analyzed; this site locates only on the centromere of the long arm of the telocentric microchromosome pair 15 (Fig. 3). Very little is

Fig. 1. Metaphase chromosome plates and karyotypes of male (A) and female (B) puff-faced water snake (*Homalopsis buccata*), $2n=36$, by conventional staining technique. The arrows indicate sex-macrochromosomes pair 8 (scale bars $10\mu\text{m}$).

known about NORs in snakes. In some snakes, e.g., *Bothrops insularis*, *B. jararacussu* (Beçak *et al.* 1990), *Elephe taeniura* (Wang *et al.* 1991), *E. rofudorsata* (Guo *et al.* 1992), the NORs are distributed on the microchromosomes. But in others, e.g., *Macropisthodon rudis*, NORs are distributed on the macrochromosomes (Toriba 1990, Ruifang *et al.* 1996). This polymorphism may have been created by chromosomal rearrangement. Toriba (1990) and Wang *et al.* (1991) also proposed that in adults, the NORs are abrupt, but in juveniles, they are often clear. This phenomenon was interpreted as a higher synthesis rate in juveniles which is needed cytologically (Toriba 1990). In the present study, the NORs were detectable in adults.

The GTG-banding technique on *H. buccata* revealed that the number of bands on one set of haploid chromosomes, which includes 34 autosomes (14 macrochromosomes and 20 microchromosomes) and the Z and W chromosomes, is 109 bands (Fig. 5). The GTG-banding technique provides clearly identifiable chromosome bands which are represented by dark band (heterochromatin) and light band (euchromatin) regions on chromosomes. However, some chromosomes cannot be clearly identified because some bands have variation. As above, the chromosome band scoring is represented by the approximate bands that appear.

Fig. 2. Idiogram showing lengths and shapes of chromosomes of the puff-faced water snake (*Homalopsis buccata*), $2n=36$, by conventional staining technique. The arrow indicates sex-macrochromosomes pair 8.

For the family Colubridae, the $2n=36$ of *H. Buccata* which was found here is consistent with the report of Moreno *et al.* (1987), which showed $2n=36$ for *Philodryas chamissonis*. Baker *et al.* (1972) demonstrated a study on the family Colubridae of northern America, including *Arizona elegans*, *Coluber constrictor*, *Elaphe guttata*, *E. obsoleta*, *Heterodon nasicus*, *Lampropeltis calligaster*, *L. gelulus*, *L. mexicana*, *Masticophis flagellum*, *Ma. taeniatus*, *Natrix erythrogaster*, *N. harteri*, *N. rhombifera*, *Phyllorhynchus decurtatus*, *Pituophis metanoleucus*, *Salvadora grahamiae*, *S. lineate*, *Sloreria dekayi*, *Thamnophis radix*, *T. couchi*, *T. cyrolopsi*, *T. elegans*, *T. marcianus*, *T. proximas*, *T. rufipunctatus* and *T. sirlalis*. It showed that all above species have $2n=36$, apart from *E. suboc*, which has $2n=40$.

Moreover, the report of Ota and Iwanaga (1996) indicated that three species of the family Colubridae, namely *Amphiesma pryeri*, *A. conelarum* and *A. ishigakiense*, have $2n=36$. Singh (1972) demonstrated that *Ptyas mucosus* has $2n=34$ while other six species, namely *C. fasciolatus*, *N. stolata*, *Lycodon anulicus*, *Cerberus rhynchops*, *Boiga forsteni* and *B. trigonata*, have $2n=36$. In addition, the report of Beçak and Beçak (1969) demonstrated that *Hydrodynastes bicinctus* and *H. gigas* have diploid chromosome $2n=24$, and *Clelia occipitolutea* has $2n=50$. According to the comparative discussion described above, we suggest that members of the family Colubridae have a diploid chromosome number of 24 to 50.

A typical snake karyotype presents as follows: 1) Macrochromosomes and microchromosomes; 2) Diploid chromosome number is $2n=36$ in almost all of the families studied, normally of the 16 macrochromosomes and 20 microchromosomes type; 3) Ag-NORs and rDNA locate predominantly on microchromosomes; 4) Morphologically differentiated sex chromosomes are of the type ZZ/ZW or not morphologically differentiated in some groups; 5) Heterochromatin (visualized by C-banding technique) is predominantly centromeric or pericentromeric, and telomeric or sub-

Fig. 3. Metaphase chromosome plates and karyotypes of male (A) and female (B) puff-faced water snake (*Homalopsis buccata*), $2n=36$, by Ag-NOR banding technique. The arrows indicate satellite microchromosomes pair 15 (scale bars $10\mu\text{m}$).

telomeric. Interstitial C-bands were seldom reported (Oguiura *et al.* 2010). While, Beçak *et al.* (2003) reported that among Colubridae species there is wild variation of chromosome number and different levels of sex chromosome differentiation.

A common feature in Reptilia is the presence of macrochromosomes and microchromosomes, probably due to karyological ancestral derivation from forms that already presented this attribute. The C-banding technique showed that not all microchromosomes are heterochromatic. Moreover, even microchromosomes that are apparently totally heterochromatic may have some euchromatin in them. This condition may be or not advantageous, depending on environmental conditions (Olmo 2005, 2008, Beçak *et al.* 2003). *Hydrodynastes bicinctus bicinctus* has $2n=24$, a very small diploid chromosome number for snakes and no microchromosomes (Beçak and Beçak 1969), which may be interpreted as a formation of favorable linkage groups. This karyotype is clearly a derived one, when other species of the family are analyzed. In the other extreme, the *Apostolepis* genus presents the highest diploid chromosome number so far found in the suborder Serpentes and the highest number of microchromosomes, some of them very small (Beçak *et al.* 2003).

Fig. 4. Idiogram showing satellite microchromosome pair 15 of the puff-faced water snake (*Homalopsis buccata*), $2n=36$, by Ag-NOR banding technique. The arrow indicate nucleolar organizer region.

Besides most reptilian orders, microchromosomes are also found in some primitive amphibians, including the families Cryptobranchidae and Hynobiidae, at $2n=56-66$ with 14–19 pairs of microchromosomes and most avian (Morescalchi *et al.* 1977, 1979). Some reptiles and all *Crocodylia* lack microchromosomes (King *et al.* 1986); a puzzle given that the crocodilian lineage is, phylogenetically, the closest to birds. The genomes of amphibians, reptiles, fish and lower chordates do, however, differ from mammals and birds with little or no GC-rich isochore DNA (Holmquist 1989). Microchromosomes and a relatively small genome therefore appear to be characteristic of the common ancestor of terrestrial vertebrates (Burt 2002).

Morphologically differentiated sex chromosomes are spread all over the Metazoa. In vertebrates, they occur in all classes but not in all groups within a class. In non-avian reptiles, they occur in all orders but not homogeneously. In the Colubridae species, different stages of differentiation of the W chromosome occur: they may differ from the Z chromosome by the centromere position or by size or by both (Beçak and Beçak 1969, Beçak *et al.* 1990, Beçak *et al.* 2003).

The sex chromosomes in the family Colubridae exhibit various states of differentiation. In *Pseustes sulphureus sulphureus* (Beçak and Beçak 1969) and *P. mucosus* (Singh 1972), no heteromorphic pair of chromosomes have been observed in either sex. In *Coluber fasciolatus*, *Chironius bicarinatus*, *Dryadophis bifossatus bifossatus*, *Drymarchon corais corais*, *Spilotes pullatus anomalepis* and *S. p. maculatus*, there is a heteromorphic pair of sex chromosomes in the females. In these cases, both the Z and W chromosomes are almost identical in size but the Z chromosome has its centromere in the submedian region, whereas in the W chromosome it is in the subterminal region. The difference in the morphology between the Z and the W chromosomes can most simply be explained by the assumption of pericentric inversion. In *Elaphe quadrvirgata* and *E. climacophora*, the W chromosome is slightly smaller than the Z and it has its centromeres in the terminal and subterminal regions, respectively, whereas in Z it is in the submedian region. The Z chromosomes of

Fig. 5. Metaphase chromosome plates and karyotypes of male (A) and female (B) puff-faced water snake (*Homalopsis buccata*), $2n=36$, by GTG-banding technique. The arrows indicate sex-macrochromosomes pair 8 (scale bars $10\mu\text{m}$).

all the species in this group are more or less of similar size except in *C. fasciolatus*, *E. quadrivirgata* and *P. s. sulphureus* which are considerably larger. Similarly, the W chromosome in *C. fasciolatus* is the largest amongst all the species (Singh 1972).

The chromosome of mitotic metaphase cells and the karyotypes of *H. buccata* in male and female, as determined by conventional staining, GTG-banding, and Ag-NOR banding techniques, are shown in Fig. 1, 3, and 5, respectively. The chromosomes length of 20 cells (males and females) in mitotic metaphase was measured. The mean length of short arm chromosome (Ls), length of long arm chromosome (LI), total length of arm chromosome (LT), relative length (RL), centromeric index (CI), size and type of chromosome are presented in Table 1. The idiogram of *H. buccata* shows the gradually decreasing length of the autosomes (Fig. 2, 4, and 6). The *H. buccata* study revealed that the chromosome marker is the chromosome pair 1, which is the largest metacentric chromosome. The largest and smallest chromosomes show difference in size (approximately 7 folds). The karyotype formula is as follows:

$$2n (36) = L_4^m + M_2^m + S_6^m + S_2^s + 20 \text{ microchromosomes} + \text{sex chromosomes (ZW)}$$

Table 1. Mean length of the short arm chromosome (Ls), long arm chromosome (LI), and total arm chromosome (LT), relative length (RL), centromeric index (CI) and standard deviation (SD) of RL, CI from metaphase chromosomes in 20 cells of the puff-faced water snake (*Homalopsis buccata*) $2n=36$.

Chromosome pairs	Ls	LI	LT	RL±SD	CI±SD	Chromosome sizes	Chromosome types
1	158.05	181.66	339.71	0.209±0.020	0.535±0.031	Large	Metacentric
2	123.94	157.82	281.75	0.173±0.011	0.560±0.030	Large	Metacentric
3	100.71	121.81	222.53	0.137±0.013	0.547±0.037	Medium	Metacentric
4	72.73	94.20	166.93	0.103±0.013	0.564±0.043	Small	Metacentric
5	51.64	70.34	121.98	0.075±0.010	0.577±0.042	Small	Metacentric
6	34.16	83.56	117.72	0.072±0.011	0.710±0.025	Small	Acrocentric
7	46.20	64.77	110.97	0.068±0.008	0.584±0.046	Small	Metacentric
9	—	—	78.18	0.126±0.006	—	Microchromosome	
10	—	—	73.19	0.118±0.003	—	Microchromosome	
11	—	—	69.91	0.113±0.002	—	Microchromosome	
12	—	—	67.47	0.109±0.004	—	Microchromosome	
13	—	—	63.23	0.102±0.003	—	Microchromosome	
14	—	—	60.24	0.097±0.003	—	Microchromosome	
15*	—	—	57.37	0.092±0.004	—	Microchromosome	
16	—	—	53.98	0.087±0.003	—	Microchromosome	
17	—	—	50.64	0.082±0.005	—	Microchromosome	
18	—	—	46.38	0.075±0.004	—	Microchromosome	
Z	60.82	83.49	144.32	0.089±0.018	0.579±0.049	Small	Metacentric
W	42.30	79.70	122.00	0.075±0.006	0.653±0.055	Small	Submetacentric

Remark: *=satellite chromosome/NOR.

Fig. 6. Idiogram showing lengths and shapes of chromosomes of the puff-faced water snake (*Homalopsis buccata*), $2n=36$, by GTG-banding technique. The arrow indicates sex-macrochromosome pair 8.

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