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Karyomorphological delineation, and the NOR loci on the sex chromosome in three species of Chrysopeleinid (Chrysopeleinae: Colubridae) from Thailand

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Abstract. *Donbundit N, Bausriyod P, Tanomtong A, Srisamoot N, Sumontha M, Thongnetr W, Patawang I, Supiwong W, Ditcharoen S, Muanglen N, Kaewmad P. 2022. Karyomorphological delineation, and the NOR loci on the sex chromosome in three species of Chrysopeleinid (Chrysopeleinae: Colubridae) from Thailand. Biodiversitas 23: 3813-3819.* The description of chromosomal characteristics of three snakes from Thailand namely, *Ahaetulla fusca*, *A. prasina*, and *Chrysopelea ornata* was by using conventional staining and Ag-NOR banding techniques. The results showed that the same diploid chromosome number (2*n*) = 36 (16 macrochromosomes + 20 microchromosomes) and the fundamental number (NF) was 54 while the karyotype formulae in these three species are as follows: *A. fusca*; $2n$ (36) = $L^{m}_{6} + S^{m}_{2} + S^{sm}_{4} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes, *A. prasina*; $2n$ (36) = $L^{m}_{6} + S^{m}_{6} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes and *C. ornata*; $2n$ (36) = $L^{m}_{4} + L^{sm}_{2} + S^{m}_{2} + S^{sm}_{4} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes. Besides, the ZZ/ZW sex chromosome was observed in all species, whereas *C. ornata* was discovered the heteromorphic sex chromosome for the first time. Nucleolar organizer regions (NORs) are located on telomeric region of one pair in all species, while located on microchromosomes in *A. fusca* and *A. prasina*, whereas *C. ornata* was detected on sex chromosomes. Therefore, the Z chromosome revealed the NOR on the telomeric region of the long arm, while NOR of the W chromosome was located on the telomeric region of the short arm.

Keywords: Cytogenetics, karyotype, nucleolar organizer regions, sex chromosomes, snake

INTRODUCTION

Subfamily Chrysopeleinae is a member of the family Colubridae with 63 currently recognized species belonging to five genera containing *Ahaetulla*, *Chrysopelea*, *Dendrelaphis*, *Dryophiops*, and *Proahaetulla* which are widespread throughout South and Southeast Asia (Mallik et al. 2019). The Chrysopeleinae in Thailand consists of 17 species belonging to four genera, i.e.: *Ahaetulla*, *Chrysopelea*, *Dendrelaphis*, and *Dryophiops* (Pyron et al. 2013; Chan-ard et al. 2015; Uetz et al. 2021).

The largest genus *Dendrelaphis* comprises 47 species but Thailand found only 11 species, i.e.: *D. caudolineatus* (Gray, 1834), *D. cyanochloris* (Wall, 1921), *D. formosus* (Boie, 1827), *D. haasi* Van Rooijen and Vogel 2008, *D. pictus* (Gmelin, 1789), *D. kopsteini* Vogel and Van Rooijen 2007, *D. striatus* (Cohn, 1905), *D. subocularis* (Boulenger, 1888), *D. ngansonensis* (Bourret, 1935), *D. nigroserratus* (Vogel, Van Rooijen and Hauser 2012), and *D. vogeli* Jiang, Guo, Ren and Li, 2020 (Vogel and Rooijen 2011; Chan-ard et al. 2015; Hauser et al. 2021; Jiang et al. 2020; Pawangkhanant et al. 2021). Members of the genus *Dendrelaphis* are slender, diurnal species that are predominantly arboreal (Rooijen and Vogel. 2012; Jiang et al. 2020). The genus *Dryophiops* has 2 species but found *Dryophiops rubescens* (Gray, 1834) only in Thailand (Wallach et al. 2014; Holden and Poyarkov 2021).

The vine snake genus *Ahaetulla* comprises 19 species but in Thailand only four species are found: *Ahaetulla fasciolata* (Fischer, 1885), *A. mycterizans* (Linnaeus, 1758), *A. nasuta* (Lacépède, 1789) and *A. prasina* (Boie, 1827). The *A. nasuta* was changed to the *A. fusca* (Duméril, Bibron and Duméril, 1854) by David et al. (2022), recently. Morphologically, *Ahaetulla* is split into two groups, that presents a dermal appendage and another without a dermal appendage (Mohapatra et al. 2017). Only *A. fusca* has features with dermal appendages presenting all *Ahaetulla* members in Thailand.

Three species of the flying snake genus *Chrysopelea* occur in Thailand namely, *Chrysopelea ornata* (Shaw, 1802), *C. paradisi* Boie, 1827, and *C. pelias* (Linnaeus, 1758) from total of five species (i.e., *C. rhodopleuron* Boie, 1827, and *C. taprobanica* Smith, 1943) (Silva 2013). Features of this group include a long and slender body with an elongated hand, neck distinct, snout much depressed, broadly truncated, large eyes with round pupils, and 13-17 smooth to weakly-keeled mid-body dorsal scale rows (Figueroa et al. 2016).

Morphological characteristics and molecular data were used to understand the evolutionary success of snake phylogenies (Pyron et al. 2013, 2014; Figueroa et al. 2016; Mohapatra et al. 2017; Zaher et al. 2019), and with species-level sampling (Pyron et al. 2013; Figueroa et al. 2016; Zheng and Wiens 2016). Moreover, the cytogenetic study has become valuable data for taxonomic studies in Serpentes (Falcione et al. 2016). The standard karyotype of snake is very stable with $2n$ = 36 chromosomes (16 bi-armed macrochromosomes and 20 microchromosomes), which is conserved in almost all species of non-caenophidian and caenophidian snakes and widely accepted as the ancestral trait (Gamble and Zarkower 2012). However, the heteromorphic ZZ/ZW sex chromosomes were performed only on caenophidian snakes (Acrochordidae, Colubridae, Elapidae, Homalopsidae, Lamprophiidae, Pareatidae, Viperidae, Xenodermatidae) (Rovatsos et al. 2015a).

In addition, the subfamily Chrysopeleinae still has problems of nomenclatural confusion based on morphological for complete systematics (Figueroa 2022), and the cytogenetics of *Ahaetulla* and *Chrysopelea* both genera are poorly known while described at least four species (Singh 1974; Sharma and Nakhasi 1980; Mengden 1982; Supanuam et al. 2020). Moreover, the karyological information has only one previously reported of *Dendrelaphis pictus* (Supanuam et al. 2020) in Thailand. For this reason, we definitely analyze cytogenetics and compare it with previous reports of three snakes in the subfamily Chrysopeleinae. Besides, we described the karyotype of three species namely, *Ahaetulla fusca*, *A. prasina*, and *Chrysopelea ornata* by conventional and Ag-NOR staining techniques location of nucleolar organizer regions (NORs) with the marker chromosome to determine aimed to identify characters between interspecies. Therefore, this cytogenetic data provides useful knowledge and basic information for comprehensively examining taxonomy and evolutionary relationship with other independent characters.

MATERIALS AND METHODS

Sample collection

Mature snakes were collected from three provinces and unknown exact localities in Thailand including two females and one male of *A. fusca*, two females and males of *A. prasina* and three females and two males of *C. ornata*. The snakes were transferred to the laboratory and were kept under standard conditions for 7 days or until eaten before the experiment. The procedures followed the ethical protocols; anesthesia was conducted by keeping in a freeze before anesthesia as approved by the Institutional Animal Care and Use Committee of Khon Kaen University, based on the Ethics of Animal Experimentation of National Research Council of Thailand (Record No. IACUC-KKU-41/64 and Reference No. 660201.2.11/177 (29)).

Chromosome preparation

Chromosomes were directly prepared in vivo with slight adaptations as follows Donbundit et al. (2020). Colchicine was injected into the snake's abdominal cavity (1 mL/100 g body weight) and left in a box for 12 hours after that sacrifice. The bone marrows were cut into small pieces then squashed and mixed with hypotonic solution (0.075 M KCl). Next, then transferred 7 mL of cell sediments to a centrifuge tube and incubated for 30 min at room temperature. After that centrifuged at 3,200 rpm for 10 min, then KCl was discarded. Cells were fixed with fresh cool fixative (3 methanol: 1 glacial acetic acid) added to 7 mL before being centrifuged again at 3,200 rpm for 10 min (Pinthong et al. 2013; Patawang et al. 2016).

Chromosome staining

Conventional staining was done using 20% Giemsa's stock solution for 30 min (Patawang et al. 2014). 50% $AgNO_3$ and 2% gelatin were used for Ag-NOR banding technique (Patawanget al. 2017; Sangpakdee et al. 2017; Phimphan et al. 2020; Thongnetr et al. 2021).

Chromosome checks

The chromosome length of 20 cells (male and female) was measured and calculated by the length of short arms (Ls) and long arms (Ll) of chromosomes for the length of total arm chromosomes (LT, LT = Ls + Ll) and centromeric index (CI) was also computed to classify the types of chromosomes. The CI (q/p+q) between 0.50-0.59, 0.60-0.69, 0.70-0.89, and 0.90-0.99 according to Supiwong et al. (2017) were described as metacentric, submetacentric, acrocentric and telocentric chromosomes, respectively. The fundamental number (NF) was obtained by assigning a value of 1 to the telocentric chromosome and 2 to the metacentric, submetacentric and acrocentric chromosomes. All data were used in karyotyping and diagramming (Tanomtong et al. 2014; Chooseangjaew et al. 2017). The microchromosomes are classified between the size of large chromosome groups and smaller chromosome groups. All of the microchromosomes are very small and the total length is often less than 0.5 micrometers (Waters 2021).

RESULTS AND DISCUSSION

Results

Mitotic chromosome features from Giemsa staining

This research presented the karyomorphology of the *A. fusca*, *A. prasina* and *C. ornata* sharing the same diploid chromosome number ($2n$) was 36 (16 macrochromosomes and 20 microchromosomes) and the fundamental number (NF) was 54 of all species analyzed. The three *A. fusca* had 16 macrochromosomes: six large metacentrics (pairs 1, 2, and 3), three small metacentrics (pair 4 and Z-chromosome), five small submetacentrics (pairs 7, 8, and W-chromosome) and two small acrocentrics (pair 6), as well as the microchromosomes, were composed of one pair of metacentrics and nine pairs incapable to define on account of their very small (Figures 1A and 1B). Karyotype formula could be deduced as 2*n* (36) = $L^{m}_{6} + S^{m}_{2} + S^{sm}_{4} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes. Four individuals of *A. prasina* had macrochromosomes consisting of six large metacentrics (pairs 1, 2, and 3), seven small metacentrics (pairs 4, 7, 8, and Z-chromosome) and three small acrocentrics (pair 6 and W-chromosome). Moreover, the morphology of microchromosomes had 10 pairs consisting of two metacentrics, and those of 18 microchromosomes are unable to determine (Figure 1C and 1D). Its karyotype formula was 2*n* (36) = $L^{m}_{6} + S^{m}_{6} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes. Chromosome pair 5 of these two species showed a small-sized heteromorphic sex chromosome in females consisting of the Z as metacentric chromosome and the W as submetacentric chromosome in *A. fusca*, while *A. prasina* showing the Z as metacentric chromosome and the W as acrocentric chromosome. Additionally, five individuals of *C. ornata* had macrochromosomes including four large metacentrics (pairs 1 and 3), three large submetacentric (pair 2 and W-chromosome), one medium metacentric (Z-chromosome), two small metacentrics (pair 5), four small submetacentrics (pairs 7 and 8) and two small acrocentrics (pair 6). Furthermore, microchromosomes revealed the only metacentric in pair 1 that differ from others and is unable to be determined. Karyotype formula could be deduced as 2*n* (36) = $L^{m}_{4} + L^{sm}_{2} + S^{m}_{2} + S^{sm}_{4} + S^{a}_{2}$ + ZZ/ZW + 20 microchromosomes. Consequently, the *C. ornata* is the first cytogenetically described heteromorphic sex chromosome on pair 4 in females showing that the Z as metacentric chromosome and the W as submetacentric chromosome that is medium and large size, respectively (Figure 1E and 1F).

Nucleolar organizer region from Ag-NOR banding

The NOR position was performed on one pair in all these arboreal snakes. Mitotic metaphases of *A. fusca* and *A. prasina* were observed in the centromeric regions of microchromosome pairs 11 and 10, respectively (Figure 2G-2J). Besides, we found the clearly located NORs on the telomeric region of sex chromosome pair 4 on the long arm of the Z chromosome and the short arm of the W chromosome of *C. ornata* (Figure 2K and 2L).

Discussion

On cytogenetic focus, the diploid chromosome number (2*n*) in the subfamily Chrysopeleinae was obtained from three species namely, *A. fusca*, *A. prasina* and *C. ornata* analyzed herein for the first time in Thailand. The result of all species showed that 2*n* = 36 (16 macrochromosomes+20microchromosomes) similar to those previous reports in subfamily Chrysopeleinae (Singh 1974; Sharma and Nakhasi 1980; Mengden 1982; Supanuam et al. 2020) (Table 1) very conserved and shared an ancestral karyological character almost all species of family Colubridae, Viperidae, and Boidae (Uno et al. 2012). In addition, our study showed a fundamental number of 54, but one pair of microchromosomes revealed that a metacentric could derive through the pericentric inversion.

Table 1. Review of cytogenetic reports of snakes in the subfamily Chrysopeleinae (Colubridae)

Species	2*n*	Karyotype formulae	Sex chromosome morphology		ZW	Ag-NORs	References
			Z	W			
Ahaetulla fusca	36	16M+20mi	Metacentric	Submetacentric	5th pair	11th pair	*This study*
(*Dryophis nasutus*)	36	16M+20mi	Not explain	Not explain	-	-	Singh (1974)
A. parsina	36	16M+20mi	Metacentric	Acrocentric	5th pair	10th pair	*This study*
Chrysopelae ornata	36	16M+20mi	-	-	-	-	Singh (1974)
	36	16M+20mi	Metacentric	Submetacentric	4th pair	4th pair	*This study*
Dendrelaphis punctulatus (*D. punctulata*)	36	16M+20mi	Submetacentric	Submetacentric	-	-	Mengden (1982)
D. pictus	36	16M+20mi	-	-	-	-	Sharma and Nakhasi (1980)
(*D. ahaetulla*)	36	16M+20mi	Submetacentric	Telocentric	8th pair	-	Supanuam et al. (2020)

Note: 2*n*: diploid chromosome number, M: macrochromosome and mi: microchromosome

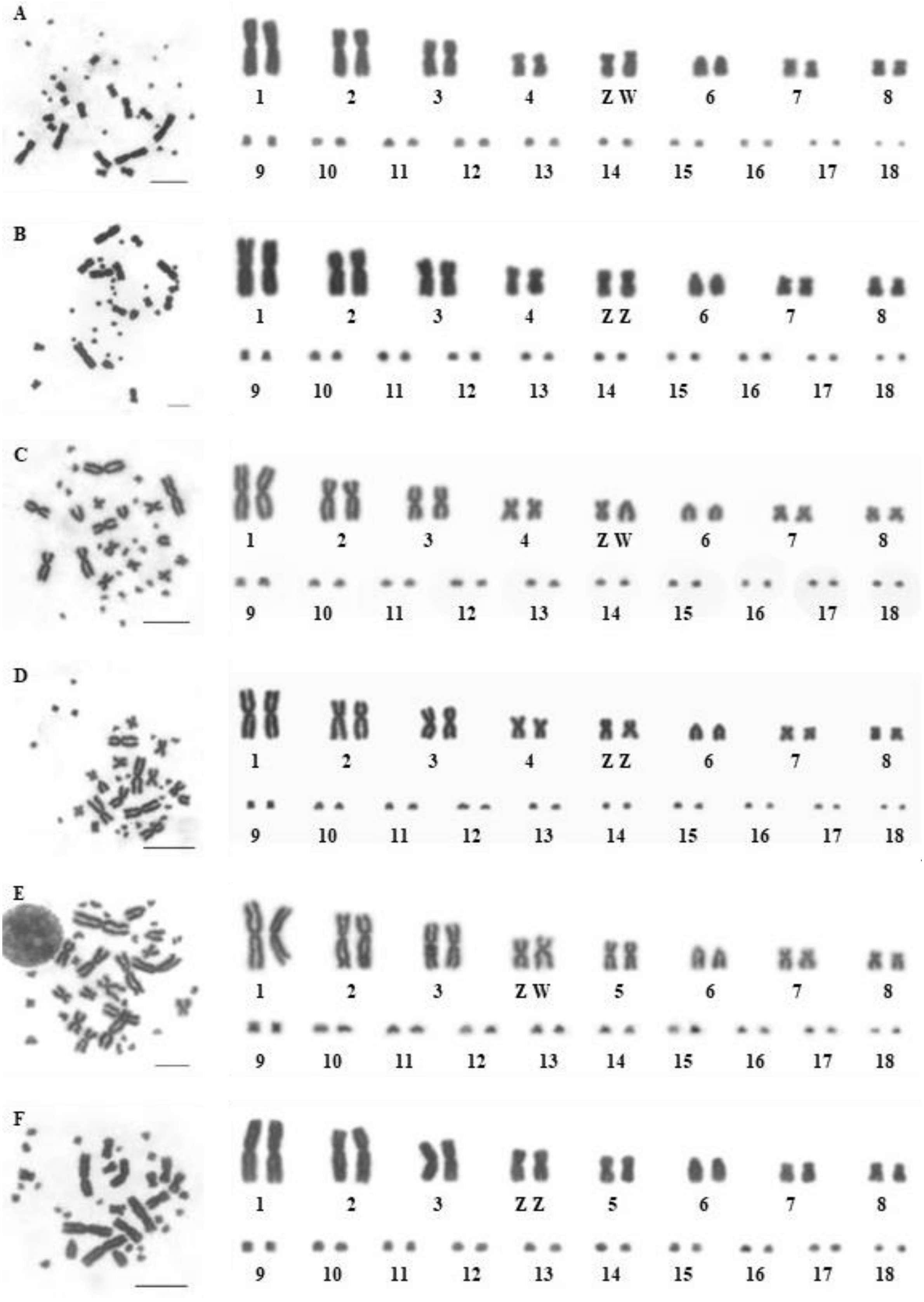

Figure 1. Metaphase chromosomes and karyotypes by conventional staining. A. *A. fusca* female, B. *A. fusca* male, C. *A. parsina* female, D. *A. parsina* male, E. *C. ornata* female, F. *C. ornata* male. Scale bars: 10 µm

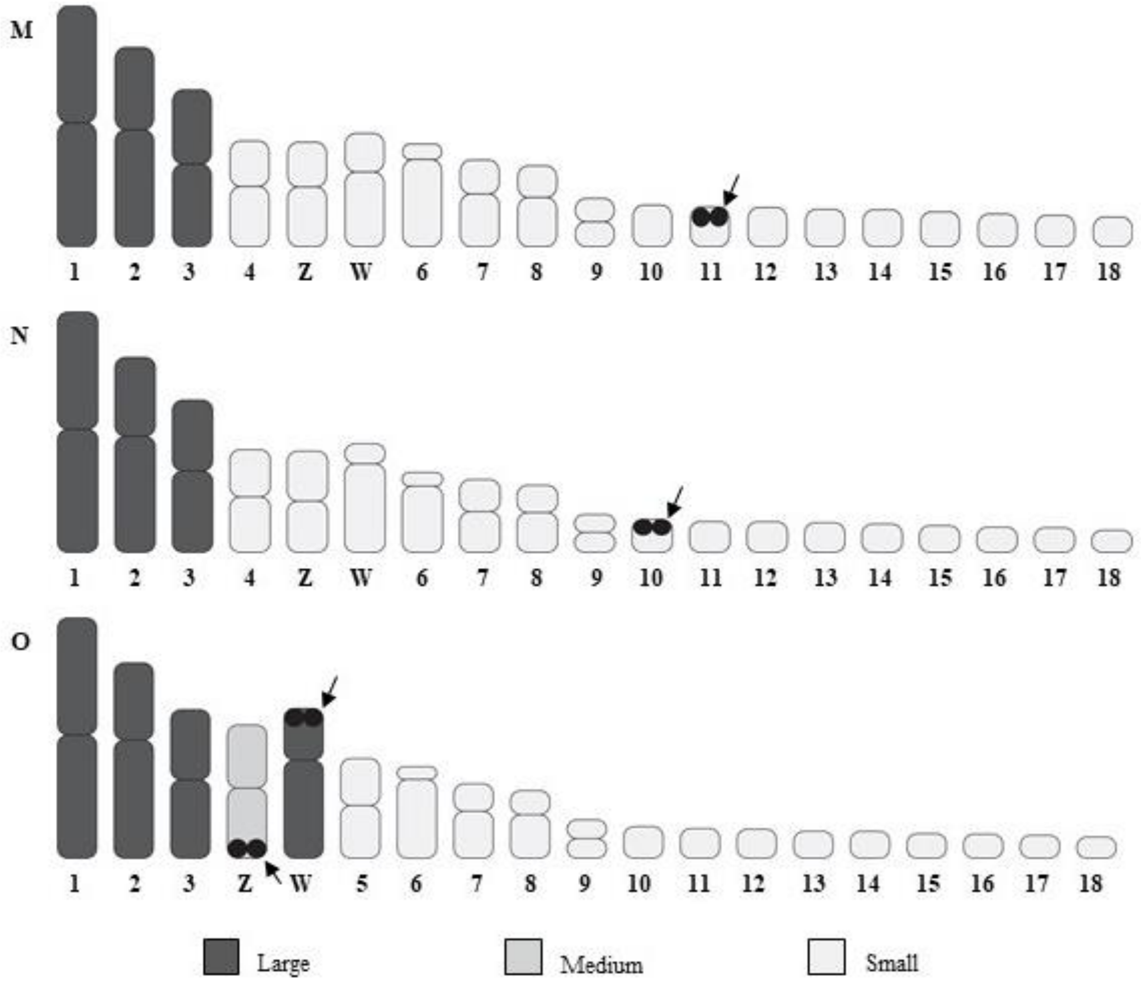

Figure 3. Idiogram showing length and shape by conventional staining and Ag-NOR staining techniques. M. *A. fusca*, N. *A. parsina*, O. *C. ornata*. Arrows indicate NORs position

The females of this study were characterized by heteromorphic sex chromosomes as the ZZ/ZW system had been previously reported in *A. fusca* and *A. prasina* (Singh 1974; Mengden 1982). The heteromorphism revealed the size and morphology of the chromosome pair 5 of these species. The ZW chromosomes of the *A. fusca* consisted of metacentric and submetacentric chromosome dissimilar to the *A. prasina* revealed that metacentric and acrocentric chromosomes, respectively (Figure 3M and 3N). Similar to chromosome pair 4 of *C. ornata* appeared the submetacentric chromosome would be the W chromosome and the Z chromosome was the metacentric chromosome (Figure 3O). However, the previous study was not observed the different morphology of macrochromosome (Singh 1974). For our study, ZW heteromorphism performed the W larger than the Z chromosomes. This situation is normal in an early stage of ZZ/ZW sex chromosomes and differs in Colubroidea snakes (Matsubara et al. 2006; Oguiura et al. 2009). The position of the Z chromosome was usually presented on the fourth pair in almost all of the advanced snakes and is often found in pair 5 (Mengden and Stock 1980; Rao et al. 2009; Rovatsos et al. 2015b). Falcione et al. (2016) proposed the degree of heteromorphy of the sex chromosome (ZW) is caused by the heterochromatin distribution that leads to similar or differs morphologically in shape and/or size.

The localization of NOR loci in all taxa studied was performed for the first time. A single NOR was observed on microchromosomes in *A. fusca* and *A. prasina* on pair 11 and 10, respectively. The number of NOR loci on one microchromosome pair is frequent in Serpentes (Mezzasalma et al. 2014; Falcione et al. 2018), while the NOR-bearing may be detected either on macrochromosomes, a microchromosome pair or on macro- and microchromosomes (Mezzasalma et al. 2016). NOR on macrochromosome was found in *C. ornata* interestingly, this species revealed that NORs on the sex chromosome. Actually, NORs are located on different regions of the Z and W chromosomes. They were detected on the telomeric region of the long arm (q) as observed for the Z chromosome, while the W chromosome was revealed on the telomeric region of the short arm (p). However, reptile species whose observed NORs located on sex chromosome has been few reported in taxa differed from agamids and turtles (Matsubara et al. 2019), and are similar to the previous reports of the occurrence of NORs on sex chromosomes in other vertebrates such as fish, amphibians, several marsupials and eutherian mammals species (Scacchetti et al. 2015; Proskuryakova et al. 2018; Paim et al. 2020). Previous studies have hypothesized that the NOR-bearing on sex chromosomes may play a key role in the evolution of sex chromosomes by recombination between proto-sex chromosomes. Differentiated sex chromosomes then structural rearrangement were loss of active genes or segmental insertions and deletions, while the location of the NOR and the accumulation of heterochromatin are often associated with it (Singchat et al. 2018).

In conclusion, our report on the three species of subfamily Chrysopeleinae are *A. fusca*, *A. prasina*, and *C. ornata* represented the same karyotype structures herein chromosome numbers (2*n*=36) and fundamental numbers (NF=54). The karyotype of genus *Ahaetulla* appeared to have similar chromosome morphology except for sex chromosome and NOR regions. The ZW chromosomes of the *A. fusca* consisted of metacentric and submetacentric chromosome dissimilar the karyotype of *A. prasina* revealed that metacentric and acrocentric chromosome, respectively. In addition, the NOR regions are located at the same centromeric position but differ to pair 11 between pair 10, respectively. Furthermore, we revealed the strongly heteromorphic sex chromosome in *C. ornata* and the first detect location of the NORs bearing on sex-chromosome in this species. We proposed this different both characterizations can be the chromosome marker. Our study of an association between the NOR and sex chromosomes also provides support for the hypothesis that recombination between proto sex chromosomes. Overall, the cytogenetic study must be still ongoing in this group to clearly play a key role in cytotaxonomy and the karyotypic diversity of snakes.

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