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Changes in heterochromatin content and ancient chromosome fusion in the endemic Malagasy boid snakes *Sanzinia* and *Acrantophis* (Squamata: Serpentes)

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Boid snakes are represented in Madagascar by the genera *Sanzinia* Gray, 1849 (Malagasy tree boas) and *Acrantophis* Jan, 1860 (Malagasy ground boas), which form a monophyletic group (Vences et al. 2001). They are considered to be phylogenetically related to African mainland boas of the genus *Calabaria* Gray, 1858 (Noonan & Chippendale 2006, Reynolds et al. 2014) and classified either in the boid subfamily Sanziniinae (Pyron et al. 2013) or even in their own family, Sanziniidae (Reynolds & Henderson 2018). Malagasy tree boas comprise two species (Reynolds et al. 2014, Reynolds & Henderson 2018), *S. madagascariensis* (Duméril & Bibron, 1844) and *S. volontany* Vences & Glaw, 2004, with the former occurring in the east and *S. volontany* in the west, south and north of Madagascar (Vences & Glaw 2003, Orozco-Terwengel et al. 2008). Molecular phylogenetic analysis has revealed that the two *Sanzinia* species have highly divergent haplotypes (Orozco-Terwengel et al. 2008) and led to the hypothesis that the two former subspecies (*S. m. madagascariensis* and *S. m. volontany*) might actually represent different species. The Malagasy ground boas are also regarded as two species, *A. madagascariensis* (Duméril & Bibron, 1844), distributed in the northern half of the island, and *A. dumerili* Jan, 1860, which is widespread in the south (Glaw & Vences 2007, Orozco-Terwengel et al. 2008).

Only 37 of 175 boa and python species have as yet been karyotyped, and a recent review of the cytogenetic literature revealed that there are no published reports of cytogenetically identifiable sex chromosomes in any boa or python species, except for the finding of a heteromorphic pair of chromosomes in a single *A. dumerili* sample (Mengden & Stock, 1980). However, the heteromorphic chromosomes in this species could represent either a ZW or a XY system (Gamble et al. 2017). In general, chromosome changes may precede or follow molecular differentiation, they may cause cladogenesis, or be a result of the processes of lineage diversification (see King 1993). In either case, they can be useful to detect plesio- and apomorphic states, different evolutionary lineages of taxonomic relevance and to reconstruct evolutionary trends in the studied species (Mezzasalma et al. 2014, 2016, 2017).

In order to identify and evaluate the evolutionary significance of possible karyological differences between the Malagasy tree boas as well as between *Sanzinia* and the closely related Malagasy ground boas of the genus *Acrantophis*, we conducted a comparative chromosomal analysis, using standard and C-banding methods. Experimental procedures were performed on six Malagasy specimens, collected in 2003–2004 (collection permits of the Malagasy Ministère de l'Environnement, des Eaux et des Forêts, 156-MEF/SG/DGEF/DADF/SCB dated 12 December 2002 and 238-MINENVEF/SG/DGEF/DPB/SCBLF dated 14 November 2003; export permits 063C-EA02/MG03 dated 26 February 2003). The studied samples include a male of *A. dumerili* from Analalava Forest, Isalo (ZSM 949/2003, field number FGMV 2002-1580); two juveniles, two females and a male of *Sanzinia* spp., respectively, from Ranomafana (UADBA-R 24494, ZCMV 610), probably Isalo (UADBA-R, FGMV 2002-2249), near Ifanadiana (ZSM 794/2003, FGMV 2002-646, FGMV 2002-3278), and Analalava Forest, Isalo (ZSM 950/2003, FGMV 2002-1584). These specimens were deposited in the collec-

tion of the Université d'Antananarivo, Mention Zoologie et Biodiversité Animale, Madagascar (UADBA) and in the Zoologische Staatssammlung München, Germany (ZSM), respectively. The taxonomic affinities of the collected specimens were established by means of molecular analyses, using a fragment of the 16S rRNA mitochondrial gene. The 16S rRNA (16S) was chosen considering the available number of sequences for several populations of Malagasy boas (Orozco-Terwengel et al. 2008). DNA was extracted from chromosome suspensions using the standard method by Sambrook et al. (1989). The primers used to amplify the 16S fragment were 16Sar-L and 16Srb-H (Palumbi et al. 1991) with PCR parameters set as detailed by Vences & Glaw (2003). From these analyses we identified the following taxa: *A. dumerili* (ZSM 949/2003), *S. volontany* (UADBA-R [FGMV 2249], ZSM 950/2003), and *S. madagascarensis* (ZSM 794/2003, FGMV 2002-3278). GenBank accession numbers are LR535674-LR535678. The sample UADBA-R 24494, ZCMV 610, was attributed to *S. madagascariensis* based only on its morphological characteristics and the sampling locality. The phylogenetic analysis was performed with Maximum Likelihood (ML) in MEGA6 (Tamura et al. 2013), using our newly generated sequences and homologous sequences taken from GenBank. We produced a tree congruent with the two main haplotype groups in *Sanzinia* (corresponding to *S. madagascariensis* and *S. volontany*) as reported by Vences & Glaw (2003) and Orozco-Terwengel et al. (2008) (not shown).

Concerning the 16S, the maximum intraclade genetic diversity within both *S. volontany* and *S. madagascariensis* was < 2% (uncorrected p-distance), while interclade genetic distance between the two taxa was about 3–4%. Similarly, the only sample of the genus *Acrantophis* analysed here (ZSM 949/2003, FGMV 2002-1580) showed a genetic identity of 99.8% with homologous sequences of *A. dumerili* from GenBank. Chromosomes were obtained from intestine and testis of the studied samples as described in Mezzasalma et al. (2014). Giemsa standard staining and sequential C-banding + Giemsa + Chromomicyn A_3 (CMA)+DAPI were performed as described in Mezzasalma et al. (2018).

The karyotype of the studied sample of *A. dumerili* resembled the one already described by Mengden & Stock (1980) from a sample of unknown provenance, namely with 2n = 34 elements of which 16 were macrochromosomes (six biarmed and two uniarmed pairs) and 18 were microchromosomes (Fig. 1). Sequential C-banding + Giemsa + CMA + DAPI staining evidenced a very scarce presence of heterochromatin on biarmed macrochromosomes, while centromeric C-bands, almost all of them negative to CMA and DAPI, were present in the centromeric regions of three telocentric pairs and four pairs of microchromosomes (Fig. 1). Mengden & Stock (1980) found in a sample of this species a heteromorphic 4th chromosome pair, identified as a ZW sex system. However, Gamble et al. (2017) questioned this hypothesis, suggesting that the heteromorphic pair could represent either a XY or ZW sex chromosome system, because an ambiguous determination of the sex of some samples used by Mengden & Stock (1980). However, the male sample of *A. dumerili* studied here did not have any heteromorphic pair, thus supporting the ZW sex chromosome system suggested by Mengden & Stock (1980). Using a Restriction Site-Associated DNA (RADseq) method, Gamble et al. (2017) demonstrated that a XY sex chromosome system was present in two species *Boa imperator* and *Python bivittatus*, highlighting its independent origin. Our results confirm that in snakes, and in particu-

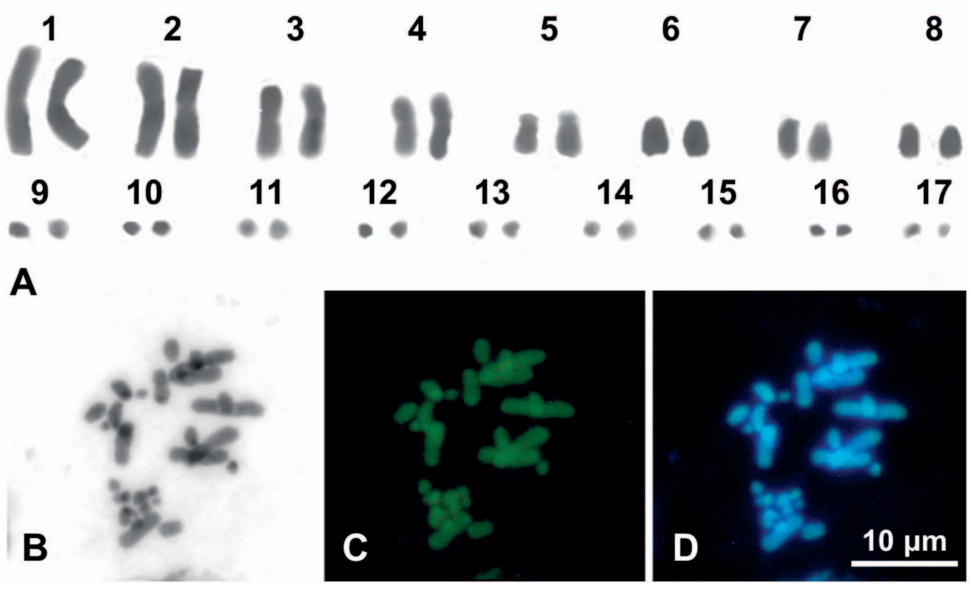

Figure 1. Giemsa-stained karyotype (A) and a metaphase plate of *Acrantophis dumerili* sequentially stained with C-banding + Giemsa, (B) + CMA(C) + DAPI (D).

lar in Boidae, different genetic sex-determination systems evolved multiple times independently, following a pathway leading to either male or female heterogamety in different evolutionary lineages.

The studied male and female of *S. madagascariensis* and *S. volontany* exhibited no sex-linked heteromorphism, at least not with the methods here used. In fact, the samples of *S. volontany* and *S. madagascariensis* both had similar karyotypes (2n = 34 chromosomes, with 12 biarmed and six uniarmed macrochromosomes plus 16 microchromosomes) (Fig. 2). Furthermore, the two species also exhibited similar patterns of heterochromatin distribution after C-banding, namely: four pairs of microchromosomes that were almost completely heterochromatic; the short arms of macrochromosome pairs five and six and the long arm of macrochromosome pair nine were also completely heterochromatic (Fig. 2). The chromosome morphologies and C-banding patterns of *S. madagascariensis* and *S. volontany* are like those described by Mengden & Stock (1980) for a male *Sanzinia* sp. sample from an unknown locality. The chromosome morphologies and C-banding patterns of *A. dumerili* and two *Sanzinia* have been schematically summarized in Fig. 3.

Karyologically, *A. dumerili* differs from *S. madagascariensis* and *S. volontany* in the number of macrochromosomes (16 vs 18, respectively) and microchromosomes (18 vs 16, respectively). After C-banding, the long arms of the last macrochromosome pair (9) of both *Sanzinia* species were found to be completely heterochromatic (see Fig. 3). This allows to hypothesise, as has been suggested already by Mengden & Stock (1980), that the karyotypes of *S. madagascariensis* and *S. volontany* could be derived from a karyotype like that of *A. dumerili* by means of addition of heterochromatin to a proto-9th microchromosome pair (see Fig. 3). However, the opposite process heterochromatin deletion, cannot be ruled out considering the available data. In fact, the lack of karyological data from the closest relative of the Malagasy boids, the African mainland Calabar ground boa, *Calabaria reinhardtii* (Schlegel, 1851), prevents a direct inference on the polarity of this chromatin rearrangement.

Considering the entire clade Ophidia, it is interesting to note that the karyotype of *A. dumerili* differs from the supposed ancestral snake karyotype, which is composed by 2n = 36 chromosomes with 16 macro- and 20 microchromosomes (Gorman & Gress 1970, Olmo 1986, Oguiura et al. 2009), in lacking a pair of microchromosomes. During the species diversification of Squamata, and more in general of vertebrates, there is no evidence of a loss of microchromosomes, but rather that they have been translocated to macro- and/or other microchromosomes (Olmo 2005, Oguiura et al. 2009, Gamble & Zarkower 2012, Uno et al. 2012). Following this evidence, the karyotype of *A. dumerili* may have evolved from the ancestral snake karyotype by means of a translocation of a microchromosome pair to a macrochromosome one. In turn, the karyotype of *Sanzinia* may have derived from a karyotype like that of *A. dumerili* by means of an addition of heterochromatin to a microchromosome pair. Again supposing an original karyotype similar to that of *A. dumerili*, the heterochromatic short arms of the macrochromosome pairs five and six of *Sanzinia* may have evolved by means of an euchromatin transformation into heterochromatin, a rearrangement that is believed to have been occurred in various taxa (King 1980, Galetti et al. 1991).

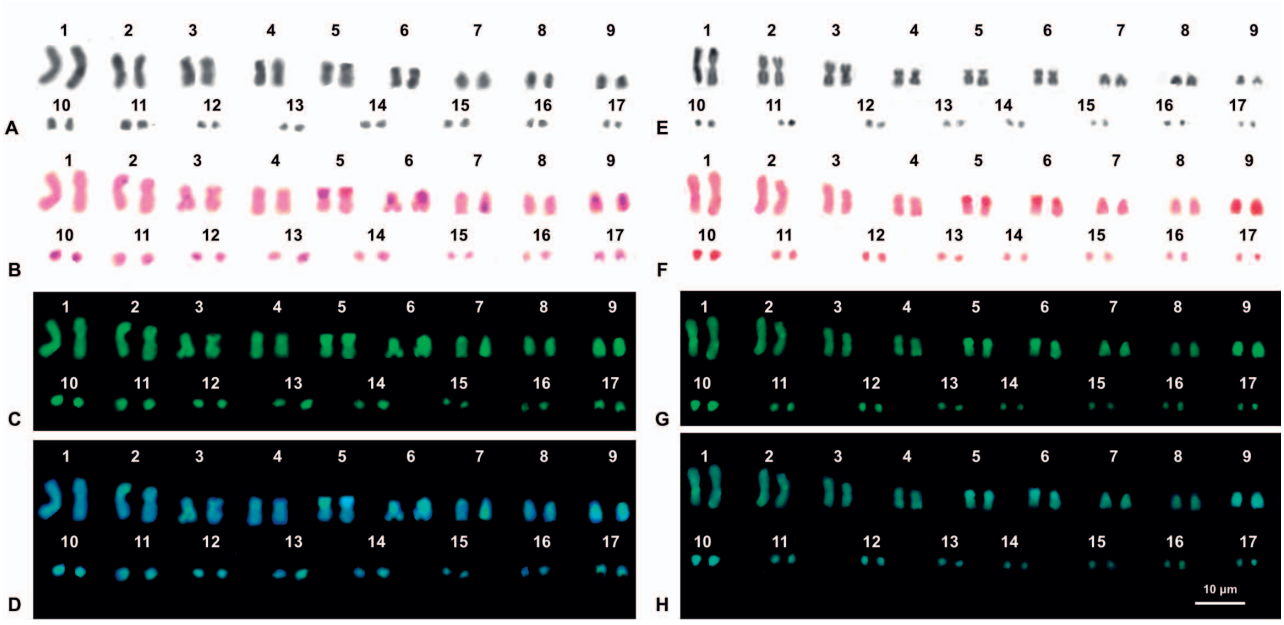

Figure 2. Giemsa (A, E) and sequentially C-banding + Giemsa (B, F) + CMA (C, G) + DAPI (D, H) -stained karyotypes of *Sanzinia madagascariensis* (A, B, C, D) and *S. volontany* (E, F, G, H) ().

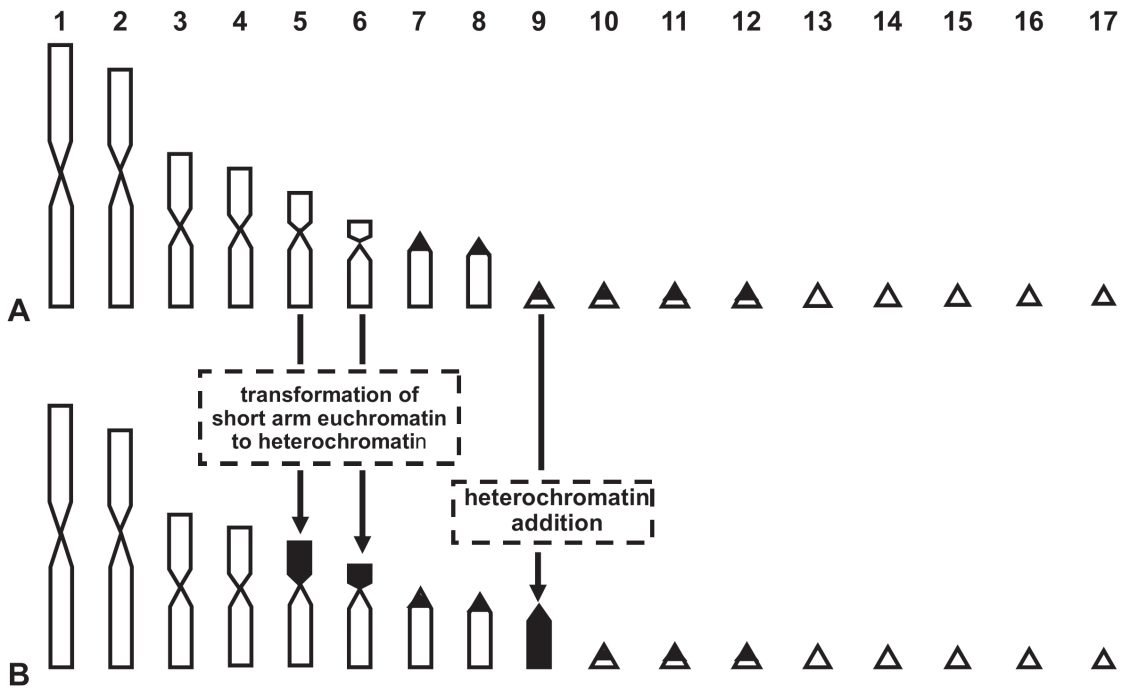

Figure 3. Schematic karyograms of *Acrantophis dumerili* (A), and *Sanzinia madagascariensis* and *S. volontany* (B), with the distribution of their C-banding-positive heterochromatin (solid black blocks). The supposed directional evolutionary heterochromatin changes (arrows) are indicated in the stippled frames.

Furthermore, the transformation of euchromatin into heterochromatin has been recognised as a relevant factor in speciation processes, acting as a post-zygotic barrier to hybridisation by preventing correct chromosome pairing and the formation of chiasms (King 1993). Conversely, the role of heterochromatin addition in speciation processes has been largely debated (Miklos et al. 1980, King 1993), but recent evidence suggests the likely occurrence of similar post-zygotic barriers (see Hughes & Hawley 2009, Kawakami et al. 2011, Sawamura 2012, Fukagawa 2013, Mezzasalma et al. 2017). In addition, heterochromatin is a rapidly evolving genomic material (Hughes & Hawley 2009) and differences in its content and genomic distribution can often precede those observed at molecular level (in den Bosch et al. 2003, Jang et al. 2013, Gutiérrez-Flores et al. 2018).

In conclusion, the results presented here suggest that the 2n = 34 chromosomes of the karyotypes of *A. dumerili* and *Sanzinia* spp. may have derived from the supposed ancestral snake karyotype of 2n = 36 elements by means an ancient translocation of a microchromosome pair to a macrochromosome one. Furthermore, our results also suggest that the evident differences in the heterochromatin content may have played a relevant role in the diversification between *Sanzinia* and *Acrantophis*. Similar evidences were not observed between the two species of *Sanzinia*, suggesting that the diversification observed at molecular level (Orozco-Terwengel et al. 2008) occurred without any evident karyological modifications.

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