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A karyological study of three typhlopid species with some inferences on chromosome evolution in blindsnakes (Scolecophidia)

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ABSTRACT

Blindsnakes are poorly studied reptiles from the karyological point of view since chromosomal data are only known for eight out of more than 420 species. The present paper shows the results of a karyological study conducted by means of standard and banding staining (Ag-NOR-banding and C-banding plus CMA and DAPI fluorochromes) on three typhlopid species, *Madatyphlops arenarius, Xerotyphlops vermicularis* and the parthenogenetic blindsnake, *Indotyphlops braminus*. *Madatyphlops arenarius* has conserved the primitive snake karyotype of 2n = 36 with 16 biarmed macro- and 20 microchromosomes and NORs on a microchromosome pair. The karyotype of *X. vermicularis* differed in showing a reduced number of microchromosomes (16) and NORs on the long arms of the third macrochromosome pair. The parthenogenetic *I. braminus* exhibited a triploid karyotype of 3n = 42 with seven macro- and seven microtriplets and NORs on a microtriplet. Comparison with chromosome data from the available literature suggests that the primitive snake karyotype of 2n = 36 occurred in the common ancestor of Leptotyphlopidae + Typhlopidae and in the common ancestor of Typhlopidae. In the latter family chromosome evolution may well have proceeded towards a decrease in the chromosome number from 2n = 36, 34, 32 until it reached 2n = 28, mainly involving micro- to microchromosome and/or macrochromosome translocations. Furthermore, these chromosome rearrangements occurred independently and repeatedly in the different evolutionary lineages of this family and are partially concordant with phylogenetic relationships. Cytologically identifiable sex chromosomes were not revealed by either standard or C-banding staining.

1. Introduction

With a total body length of about 10–100 cm, the infraorder Scolecophidia comprises the smallest snakes and is characterized by extreme morphological and ecological adaptations as well as a peculiar evolutionary history and systematic position (Hedges, 2008; Nagy et al., 2015; Hsiang et al., 2015). In total, the Scolecophidia radiation includes more than 420 living species, distributed on all continents except Antarctica and subdivided into five distinct families: Anomalepididae, Gerrhopilidae, Leptotyphlopidae, Xenotyphlopidae and Typhlopidae, the latter family comprising about 263 described species alone (see e.g. Uetz and Hošek, 2016). However, despite the large number of species and very wide distribution, scolecophidians are among the least studied snakes, mostly because of their highly specialized fossorial lifestyle and very elusive habits (Hedges, 2008).

Insights into the evolutionary history of these burrowing snakes have been gained by several recent molecular studies (e.g., Adalsteinsson et al., 2009; Vidal et al., 2010; Kornilios et al., 2012, 2013; Marin et al., 2013a, 2013b; Hedges et al., 2014; Pyron and Wallach, 2014; Nagy et al., 2015), which consider the Scolecophidia as an ancestral sister group to all other crown group snakes, collectively known as the Alethinophidia. However, the most recent phylogenetic studies have shown that the systematic classification of the Scolecophidia is still problematic: the uncertain position of the family Anomalepididae would render the Scolecophidia paraphyletic if only genetic data are considered (see Wiens et al., 2012; Pyron et al., 2013); but monophyletic if genetic and phenotypic data are combined (Hsiang et al., 2015).

All recent phylogenetic analyses of this group are concordant in placing all the remaining scolecophidian families in a monophyletic group, with the Leptotyphlopidae in a basal position, as the sister

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group of a clade composed by Gerrhopilidae, Xenotyphlopidae and Typhlopidae (see e.g. Wiens et al., 2012; Pyron et al., 2013; Nagy et al., 2015).

In contrast, karyological data of worm-like snakes are still very scarce and refer to only one species of Leptotyphlopidae, namely *Myriopholis macrorhyncha* (Jan, 1860) (Werner, 1959), and seven species of Typhlopidae, including two representatives of *Afrotyphlops* and *Typhlops,* and one species of *Amerotyphlops*, *Indotyphlops*, and *Letheobia* (see Table 1 for karyotype formulas and references). Moreover, all these species were investigated only by morphological staining methods, except *Amerotyphlops brongersmianus* and *Indotyphlops braminus* (Daudin, 1802 [1802–1803]) (Vanzolini, 1976; Patawang et al., 2016) for which the Ag-NOR banding method was also applied (see Table 1 for details).

The present paper reports on the results of a karyological study conducted by means of standard and banding methods (Ag-NOR and C-banding) on two blindsnake species with an unknown karyotype, namely *Madatyphlops arenarius* (Grandidier, 1872) and *Xerotyphlops vermicularis* (Merrem, 1820), and on the parthenogenetic *Indotyphlops braminus*. The results were compared with the karyological data available from the literature, and coupled with the current systematic classification of the Scolecophidia, allowing us to propose a hypothesis on the chromosome evolution of blindsnakes.

2. Materials and methods

We studied three Malagasy samples of *M. arenarius*, comprising one female (from Ranohira), one juvenile (from Ilakaka) (field number Franco Andreone Zoological Collection, FAZC 11844 = Museo Regionale di Scienze Naturali, Torino, Italy, MRSN R2368, and FAZC 11865 = MRSN R2362) and one male (from Belalanda) (field number Gennaro Aprea, GA 474; deposited in the Department of Biology of Naples, code Typhare1); one female of the Malagasy blindsnake *I. braminus* from Maroantsetra (FAZC 11720 = MRSN R2360), and one male and one female of the European blindsnake *X. vermicularis* from Petrina (Greece) (specimens deposited in the Department of Biology of Naples – code Typhver1 and Typhver2, male and female respectively).

All samples were collected during fieldwork in 2003–2006.

Chromosomes were obtained using the air-drying method. In brief, after capture, animals were injected with 0.5 mg/ml colchicine solution (0.1 ml/10 g body weight). One hour later, animals were euthanized by deep exposition to ethyl ether vapor, and intestines, lungs, spleen and gonads were removed, incubated for 30 min in a solution of sodium citrate (0.5%), fixed in a mix of methanol and acetic acid (3:1) and dissected on a sieve. Aliquots of 20 μl of chromosome suspensions were dropped on cleaned slides and stained with both standard methods (5% Giemsa solution at pH 7 for 10 min) and chromosome banding techniques, namely C-banding (Sumner, 1972), sequential C-banding + CMA + DAPI staining (Mezzasalma et al., 2014) and Ag-NOR banding (Howell and Black, 1980). For karyotype determination we used at least five metaphase plates per studied sample, following the chromosome classification proposed by Levan et al. (1964).

3. Results

The examined male, female and juvenile specimens of *Madatyphlops arenarius* showed karyotypes of 2n = 36 chromosomes, with eight macrochromosome pairs, and 20 microchromosomes. Among the macrochromosomes, the first three pairs were metacentric and distinctly larger than the other five pairs, which included two metacentric (pairs 4 and 7) one submeta/metacentric (pair 6) and two submeta/subtelocentric pairs (pairs 5 and 8), as illustrated in Fig. 1A (see Table S1 for relative lengths and centromeric indices). The Ag-NOR banding showed NORs on a microchromosome pair, tentatively the 2nd micro-pair (Fig. 1A), while C-banding staining evidenced a scarce presence of heterochromatin, C-banding positive and negative to both fluorochromes, on the centromeric region of various macrochromosomes and on several microchromosomes (Fig. 2A).

The examined male and female specimens of *Xerotyphlops vermicularis* showed a karyotype composed by 2n = 32 chromosomes, with 16 macro- and 16 microchromosomes. Macrochromosomes were very similar in morphology and length to those of *M. arenarius* (Fig. 1B; Table S1). Loci of NORs were located on the long arms of the third pair, in a peritelomeric position (Fig. 2B). Faint centromeric C-bands, CMA-positive, were observed on almost all macrochromosomes (Fig. 2B).

The examined female of *Indotyphlops braminus* had a karyotype composed by 3n = 42 chromosomes, settling in seven triplets of macrochromosomes and seven triplets of microchromosomes. All the macrotriplets were metacentric with the exception of the last, which was submeta/subtelocentric. Furthermore, the first four macrotriplets were distinctly bigger than the three others (Fig. 1C) (Table S1). Loci of NORs were located on a microtriplet, tentatively indicated as the 2nd microtriplet (Fig. 1C). Heterochromatin was scarce and particularly evident only on the microtriplet, probably the NOR-associated one (Fig. 2C).

4. Discussion

Combining our newly obtained data with previously published findings, at least four different chromosome formulas occur in the blindsnake radiation, namely with a chromosome number of 2n = 32, 34 and 36 and a peculiar triploid condition of 3n = 42 of the parthenogenetic *I. braminus* (see Table 1 and Results).

Furthermore, in all the examined blindsnake species the number of homologous macrochromosomes is highly conserved (seven in *I. braminus* and eight in all the other species hitherto studied), while the number of microchromosomes varies among different evolutionary lineages (see Figs. 1, 3 and 4).

These data provide interesting insights into the chromosome evolution of blindsnakes and their phylogenetic position in the suborder Serpentes. As previously shown in different taxa by several karyological studies (see e.g. Odierna et al., 2002; Aprea et al., 2013; Mezzasalma et al., 2014, 2015), adding karyological to phylogenetic data can be useful to detect evolutionary trends and plesiomorphic and apomorphic states, allowing to propose hypotheses on the karyotype diversification in the studied species (see Figs. 3 and 4).

Concerning the Scolecophidia, various molecular phylogenetic trees are currently available (e.g., Vidal et al., 2010; Kornilios et al., 2012, 2013; Marin et al., 2013a, 2013b; Hedges et al., 2014; Pyron and Wallach, 2014; Nagy et al., 2015). In the present paper, we coupled the available karyological data to phylogenetic relationships proposed by Nagy et al. (2015) since they largely resolved the systematic position of all the involved blindsnake taxa.

Interestingly, the hypothetical primitive karyotype of snakes, composed by 2n = 36 chromosomes and with 16 macro- and 20 microchromosomes (see e.g. Olmo, 2005; Oguiura et al., 2009; Gamble and Zarkower, 2012; Uno et al., 2012; Mezzasalma et al., 2014), was shown by both Leptotyphlopidae, represented by the slender blindsnake *Leptotyphlops phillipsi*, now a synonym of *Myriopholis macrorhyncha* (Werner, 1959) and Typhlopidae, represented by the Malagasy blindsnake *M. arenarius* (see Results).

These data further support a 2n = 36 karyotype as the ancestral condition of the common ancestor of Scole-