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Karyotypes of four species of Xenodontini snakes (Serpentes) and implications for taxonomy

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Abstract

The karyotypes of four Xenodontini snake species, *Lygophis dilepis*, *L. meridionalis*, *L. flavifrenatus* and *L. anomalus*, are here described for the first time. We studied specimens from northeastern Argentina using conventional and silver (Ag-NOR) staining. While the typical ophidian karyotype is 2n = 36, we found that the karyotype of the studied species is 2n = 34, with metacentric and submetacentric chromosome pairs. The Ag-NOR staining revealed that nucleolar organizer regions (NORs) are located on one pair of microchromosomes. In *L. dilepis* and *L. anomalus* the 4th chromosome pair is heteromorphic, and we suggest that it might be considered as the ZW sex chromosome pair. The optimization of available karyological data on a molecular phylogenetic tree of the tribe Xenodontini shows that the diploid numbers 2n = 28, 30 and 34 represent putative synapomorphy for *Erythrolamprus*, *Xenodon* and *Lygophis*, respectively. Our results provide new insights which fill gaps in our knowledge on the cytology in the genus *Lygophis* and identified a possible diagnostic character for the genus.

Contents

Introduction

In recent years, phylogenetic studies based on morphological and molecular data have resulted in modifications in the systematic and phylogenetic relationships of Serpentes (Zaher, 1999; Vidal *et al*., 2000, 2010; Zaher *et al*., 2009; Grazziotin *et al*., 2012; Pyron *et al*., 2013). The monophyletic tribe Xenodontini (Vidal *et al*., 2000, 2010; Grazziotin *et al*., 2012) is one of the South American snake radiations comprising about 70 species (Uetz and Jirí, 2013). According to the classification considered the clade is included in the subfamily Xenodontinae into Dipsadidae (Zaher *et al*., 2009) or in the subfamily Dipsadinae into Colubridae (Pyron *et al*., 2013).

The Xenodontini (*sensu* Vidal *et al*., 2010 and Grazziotin *et al*., 2012) is composed by the genera *Lygophis* Fitzinger 1843, *Erythrolamprus* Boie 1826 and *Xenodon* Boie 1826, clade also recover in other phylogenetic analysis (Zaher *et al*., 2009; Pyron *et al*., 2013; see nomenclatural discussion in Curcio *et al*., 2009). The genus *Lygophis* was resurrected by Zaher *et al*. (2009), and confirmed by other studies (Vidal *et al*., 2010; Grazziotin *et al*., 2012; Pyron *et al*., 2013). *Lygophis* comprises eight species grouped in the *'anomalus'* and the *'lineatus'* morphological groups; with three and five species, respectively (Dixon, 1985, Michaud and Dixon, 1987).

The cytogenetics of the Xenodontini is poorly known. Chromosomal data are restricted to six *Erythrolamprus* species and four *Xenodon* species (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1971, 1975; Gutiérrez *et al*., 1984). Karyological information has been obtained using conventional cytological staining protocols. Localization of the nucleolar organizer regions (NORs) was only carried out on *E. poecilogyrus schotti* (Trajtengertz *et al*., 1995).

Although information is scarce, the Xenodontini appear to be a karyologically diverse tribe. In fact, four diploid numbers (2n = 28, 30, 32, 34), eight karyotype

Table 1. Centromeric index (CI) and chromosome type of macrochromosome pairs (1-8) of *Lygophis* snakes analyzed in this study. a, the heteromorphic pair. Abbreviations: M = metacentric, SM = submetacentric.

Chromosome pair number	Sex		1	2	3	4[a]		5	6	7	8
L. flavifrenatus	♂	CI	47.62 ±1.08	46.37 ±2.23	45.56 ±2.98	46.27 ±1.99		46.68 ±1.56	46.99 ±1.90	44.90 ±1.92	45.69 ±1.69
		Type	M	M	M	M		M	M	M	M
L. dilepis	♀	CI	47.19 ±3.51	35.71 ±2.44	47.76 ±0.97	47.10 ±1.49	34.04 ±2.11	47.15 ±1.13	47.51 ±1.21	45.35 ±2.22	46.61 ±1.53
		Type	M	SM/M	M	M	SM	M	M	M	M
	♂	CI	47.30 ±2.63	35.95 ±2.15	47.77 ±1.09	45.33 ±2.27		45.74 ±2.49	47.01 ±2.23	46.94 ±1.99	46.33 ±1.46
		Type	M	SM/M	M	M		M	M	M	M
L. meridionalis	♀	CI	49.16 ±0.45	43.52 ±2.23	46.09 ±0.77	31.73 ±3.22	48.27 ±1.42	47.47 ±1.55	45.88 ±2.31	45.01 ±1.08	44.64 ±2.40
		Type	M	M	M	SM	M	M	M	M	M
L. anomalus	♀	CI	48.94 ±1.31	44.05 ±0.67	45.49 ±1.47	42.59 ±0.49	33.10 ±1.17	43.16 ±0.33	42.26 ±1.54	40.73 ±1.86	47.64 ±1.53
		Type	M	M	M	M	SM	M	M	M	M
	♂	CI	47.76 ±0.69	39.40 ±1.09	46.90 ±1.13	46.43 ±1.41		45.05 ±1.91	31.85 ±1.56	41.37 ±1.40	38.43 ±1.58
		Type	M	M	M	M		M	SM	M	SM/M

Table 2. Intra and intergeneric variation in the diploid number, chromosome formula, and sex chromosome morphology in species of tribe Xenodontini. Abbreviations: M = macrochromosome, m = microchromosome.

Species	2n	Chromosome formula	Sex chromosome morphology Z	W	Reference
Erythrolamprus aesculapii venustissimun	28	16+4+8 (♂,♀)	Metacentric	Submetacentric	Beçak *et al*., 1966; Beçak and Beçak, 1969
E. epinephelus	28	16+4+8 (♂,♀)	-		Gutiérrez *et al*., 1984
E. almadensis	28	27+1+0 (♀) 28+0+0 (♂)	Metacentric	Acrocentric	Beçak *et al*., 1975
E. bizona	28	18+2+8 (♀)	Submetacentric	Submetacentric	Gutiérrez *et al*., 1984
E. miliaris	28	19+1+8 (♀) 20+0+8 (♂)	Metacentric	Acrocentric	Beçak and Beçak, 1969
E. poecilogyrus schotti	32	-	-		Beçak *et al*., 1971; Trajtengertz *et al*., 1995
Xenodon merremi	30	16+0+14 (♂,♀)	Metacentric	Metacentric	Beçak, 1968
X. neuwedii	30	14+2+14 (♂,♀)	Metacentric	Metacentric	Beçak and Beçak, 1969
X. severus	30	14M+16m	-		Beçak *et al*., 1971
X. rabdocephalus	34	14+8+12 (♂,♀)	Submetacentric	Metacentric	Gutiérrez *et al*., 1984
Lygophis anomalus	34	16+0+18 (♂,♀)	Metacentric	Submetacentric	This study
L. dilepis	34	16+0+18 (♂,♀)	Metacentric	Submetacentric	This study
L. meridionalis	34	16+0+18 (♀)	-		This study
L. flavifrenatus	34	16+0+18 (♂)	-		This study

formulas, the ZW sex determination system, and a remarkable intra- and inter-generic karyotypic variability were described by previous studies (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1971, 1975; Gutiérrez *et al*., 1984). In *Erythrolamprus*, one karyotype 2n = 32 and five karyotypes 2n = 28 which differ in micro- and macro-chromosome number and macrochromosomes morphology have been found (Beçak and Beçak, 1969; Beçak *et al*., 1971, 1975; Gutiérrez *et al*., 1984). Three species of *Xenodon* have the same diploid number of 2n = 30 including 14 or 16 macrochromosomes and microchromosomes, while *X. rab-*

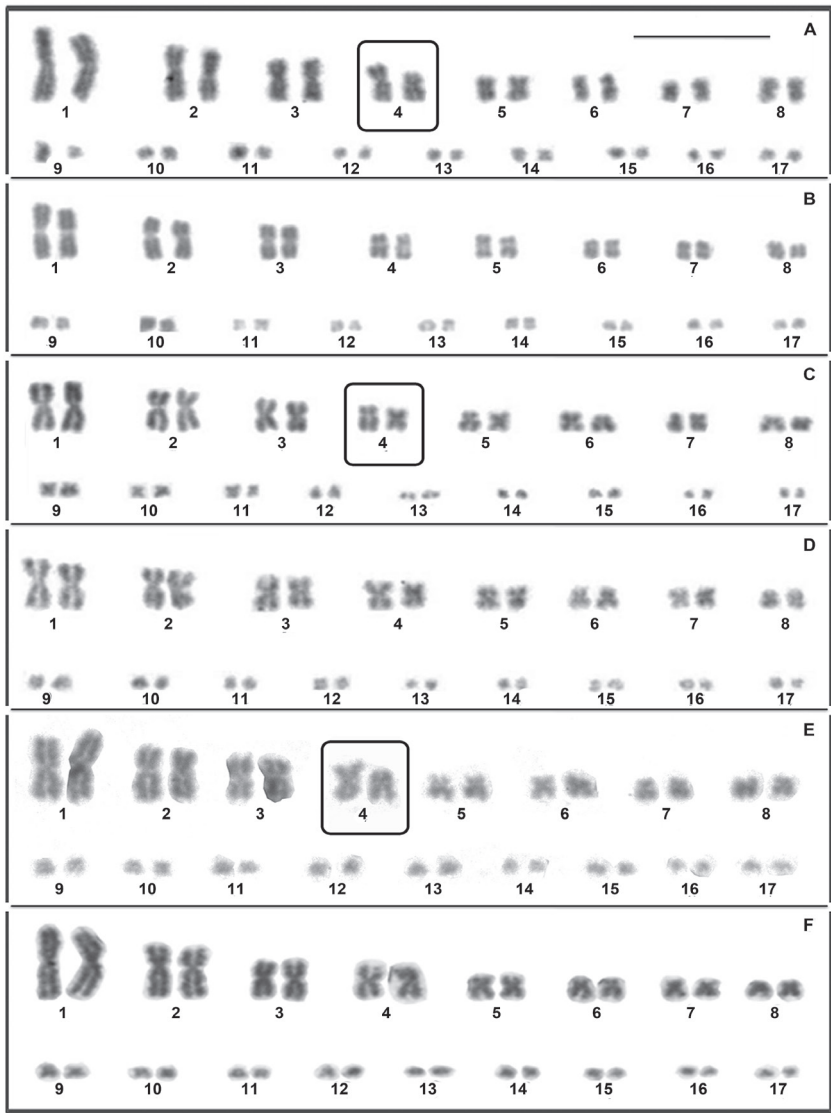

Fig. 1. Mitotic karyotypes of four *Lygophis* species. A) *Lygophis dilepis* (female), B) *L. dilepis* (male), C) *L. meridionalis* (female), D) *L. flavifrenatus* (male), E) *L. anomalus* (female) and F) *L. anomalus* (male). Rounded boxes illustrate the heteromorphic pair. Scale bar = 10 μm.

docephalus (2n = 34) exhibits 22 macro-chromosomes and a reduced micro-chromosome complement of 12 (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1971; Gutiérrez *et al*., 1984).

Cytogenetic characters can be used to infer evolutionary relationships if they are analysed together with other independent characters (morphological, molecular, immunological, isozyme) (Sites and Reed, 1994). Indeed, such an approach has been demonstrated to be important to understand the diversity and evolution in snakes (Oguiura *et al*., 2009; Mezzasalma *et al*., 2014).

Hitherto no cytological characters were known for the genus *Lygophis*. To fill this information vacuum, we documented the karyotype and the location of Ag-NORs in four species: *L. dilepis* Cope, 1862, *L. meridionalis* (Schenkel, 1902) and *L. flavifrenatus* Cope, 1862 (all belonging to the '*lineatus*' group *sensu* Michaud and Dixon, 1987) and *L. anomalus* (Günther, 1858) (belonging to the '*anomalus*' group *sensu* Dixon, 1985).

Material and methods

Chromosome analyses were carried out in males and females of *L. flavifrenatus*, *L. meridionalis*, *L. dilepis* and *L. anomalus* (see details in the Appendix). Voucher specimens are deposited in the Colección Herpetológi-

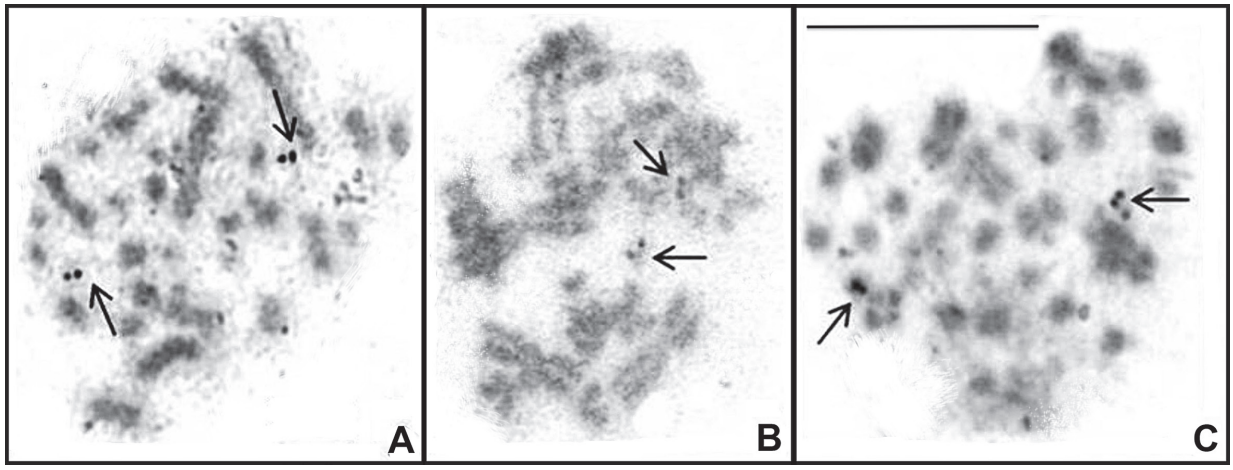

Fig. 2. Metaphases after silver staining (Ag-NORs). Arrows indicate the NORs on a pair of microchromosomes. A) *Lygophis dilepis*, B) *L. meridionalis*, C) *L. flavifrenatus*. Scale bar = 10 µm.

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Four hours prior to animal dissection, specimens were injected intraperitoneally with 0.1% colchicine (1 ml/100 g body weight). The euthanasia method proposed by Beaupre *et al*. (2004) was used. Chromosomes were obtained from intestinal epithelium by dispersion of cells on the hot stage. Chromosome preparations were stained conventionally using a 10% Giemsa solution at pH 6.8. The NORs were detected using the silver staining (Ag-NOR) technique applied by Howell and Black (1980). The Ag-NORs banding staining was performed only for species which provided a sufficient number and quality of metaphase plate.

To calculate the centromeric index (CI), the arms of macrochromosomes were measured on ten metaphase plates of each specimen using the software MicroMeasure version 3.3 (Reeves and Tear, 2000). The chromosomal formula was determined following Peccinini-Seale (1981): (2n = I + II + III), with I = metacentric or submetacentric macrochromosomes, II = telocentric or subtelocentric macrochromosomes and III = microchromosomes. Chromosomes that measured around 1 micron (μm) were classified as microchromosomes.

To reconstruct karyotype evolution within the Xenodontini clade, the diploid number (from our results and from the literature) of Xenodontini species was optimized on the most recent phylogenetic hypothesis of Squamata by Pyron *et al*. (2013) using the parsimony criterion with TNT software (Goloboff *et al*., 2008).

Results

The diploid chromosome complements of *L. dilepis*, *L. meridionalis*, *L. flavifrenatus* and *L. anomalus* were similar (Fig. 1A-F). The karyotype was 2n = 34 (16 + 0 + 18) and consisted of eight pairs of metacentric or submetacentric macrochromosomes, gradually decreasing in size (Table 1) and nine pairs of microchromosomes. No secondary constrictions were observed.

In females of *L. dilepis*, *L. meridionalis* and *L. anomalus* the 4th chromosomal pair was heteromorphic. In *L. dilepis* and *L. anomalus*, the larger element was metacentric and the smaller submetacentric (Fig. 1A, E). In *L. meridionalis*, the submetacentric corresponded to the larger chromosome and the metacentric to the smaller (Fig. 1C). In males of *L. dilepis*, *L. flavifrenatus* and *L. anomalus*, the homologous of pair 4 were metacentric and of similar size (Fig. 1B, D, F).

The NORs were detected on one pair of microchromosomes in *L. dilepis*, *L. flavifrenatus*, *L. meridionalis* (Fig. 2A-C).

Discussion

In snakes, chromosome numbers range from 2n = 24 to 2n = 52, with 2n = 36 being the most frequent number (16 macrochromosomes and 20 microchromo-

somes) (Beçak and Beçak, 1969; Oguiura *et al*., 2009). Karyotypes differing from this formula and with the chromosome number varying from 2n = 28 to 2n = 34 have been documented in 14 Xenodontini species belonging to the genera *Erythrolamprus* and *Xenodon*, as reported by several studies (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1971; 1975; Gutiérrez *et al*., 1984), and to the genus *Lygophis*, as here studied (Table 2).

Five *Erythrolamprus* species share the diploid number 2n = 28 but with variations in the number of bi- and uniarmed macro- and microchromosomes. *Erythrolamprus aesculapii venustissimun*, *E. epinephelus*, *E. bizona* and *E. miliaris* have karyotypes with 20 macrochromosomes and eight microchromosomes whereas 28 macrochromosomes and no microchromosomes were described in *E. almadensis* (Beçak and Beçak, 1969; Beçak *et al*., 1975; Gutiérrez *et al*., 1984). When considering all currently known chromosomal data for *Erythrolamprus* (Table 2), two out of the 10 pairs of macrochromosome are uniarmed in *E. aesculapii venustissimun* (pairs 9 and 10) and *E. epinephelus* (pairs 6 and 9) and one in *E. bizona* (pair 6). In *E. miliaris* and *E. almadensis* all macrochromosomes are biarmed. In *E. poecilogyrus schotti*, 2n = 32, and there is no clear distinction between macro- and microchromosomes (Beçak *et al*., 1971; Trajtengertz *et al*., 1995). In the genus *Xenodon*, *X. merremi* and *X. newiedii* have a similar 2n = 30 karyotype with eight macrochromosome pairs and seven microchromosome pairs (Beçak, 1968; Beçak and Beçak, 1969). Although *X. severus* also exhibits 2n = 30, it has seven macro- and eight microchromosomes (Beçak *et al*., 1971). According to Beçak *et al*. (1971), in the karyotype of *X. severus*, a translocation that occurred between macrochromosomes can explain the numeric differences in the karyotypes of *Xenodon* species. Moreover, *X. rabdocephalus* exhibits the highest diploid number known in Xenodontini (2n = 34). Its karyotype includes 22 macrochromosome pairs of which 14 are biarmed and 8 uniarmed, coupled to 12 microchromosomes (Gutiérrez *et al*., 1984). The chromosome complement of *X. rabdocephalus* has more uniarmed macrochromosome pairs but one or two fewer microchromosome pairs than the karyotype of 2n = 30 *Xenodon* species (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1971).

Lygophis dilepis, *L. meridionalis*, *L. flavifrenatus* and *L. anomalus* have a similar karyotype consisting of 34 chromosomes (16 biarmed macrochromosome pairs and 18 microchromosomes). Although 2n = 34 has also been reported for *X. rabdocephalus*, differences can be noted between these two taxa, since the *Lygophis* karyotype consists of eight pairs of biarmed macrochromosomes and three additional microchromosomes pairs when compared with the karyotype of *X. rabdocephalus*.

Considering that the karyotype 2n = 36 (16 macrochromosomes and 20 microchromosomes) occurred in the common ancestor of snakes (Oguiura *et al*., 2009), several mechanisms have been proposed to explain chromosomal evolution of Xenodontini. In *Erythrolamprus* (2n = 28 and 32) and *Xenodon* (2n = 30 and 34) the reduction in the diploid number resulted from unequal translocations between macro- and microchromosomes (Gutiérrez *et al*., 1984) and Robertsonian fusions of microchromosomes (Beçak and Beçak, 1969). Additional centric fissions of macrochromosomes probably played an important role in the differentiation of *Xenodon* karyotypes (Gutiérrez *et al*., 1984). On the other hand, we suggest that chromosomal inversion and reduction in the number of microchromosomes were involved in the karyotype evolution of *Lygophis* (2n = 34). Lower microchromosomes number also have observed in others Dipsadidae snakes (*Hydrodynastes*: 2n = 24, *Philodryas serra*: 2n = 28, *Thamnodynastes strigatus*: 2n = 32 and *T. hypoconia*: 2n = 34) (Beçak and Beçak, 1969).

The Boidae genera *Eryx*, *Acrantophis* and *Sanzinia* (2n = 34) and *Micrurus* species (Elapidae) from Central America (2n = 26 to 2n = 34) tend to reduce the chromosome number probably due to fusion microchromosomes processes (Beçak and Beçak, 1969; Gutiérrez and Bolaños, 1979; Mengden and Stock, 1980; Oguiura *et al*., 2009). From an ancestral karyotype 2n = 48 in Pseudoxyrhophiinae (Lamprophiidae), the chromosomal diversification and reduction of the chromosome number may have occurred as a result of translocations of microchromosomes to macrochromosomes, tandem fusions and centric fissions and fusions (Mezzasalma *et al*., 2014). Recently, it has been proposed that during diversification of Squamata the decrease in number of microchromosomes may have occurred by repeated fusions between macro- and/or other micro-chromosomes (Uno *et al*., 2012).

The presence of ZW sex chromosomes in the 4th position was reported in many colubrids, elapids, and viperids (Beçak and Beçak, 1969; Singh, 1972; Gutiérrez *et al*., 1979; Mengden and Stock, 1980; Beçak and Beçak, 1981; Ota, 1999; Aprea *et al*., 2003, 2006) and has been considered a putative synapomorphy of the superfamily Colubroidea (Oguiura *et al*., 2009). The

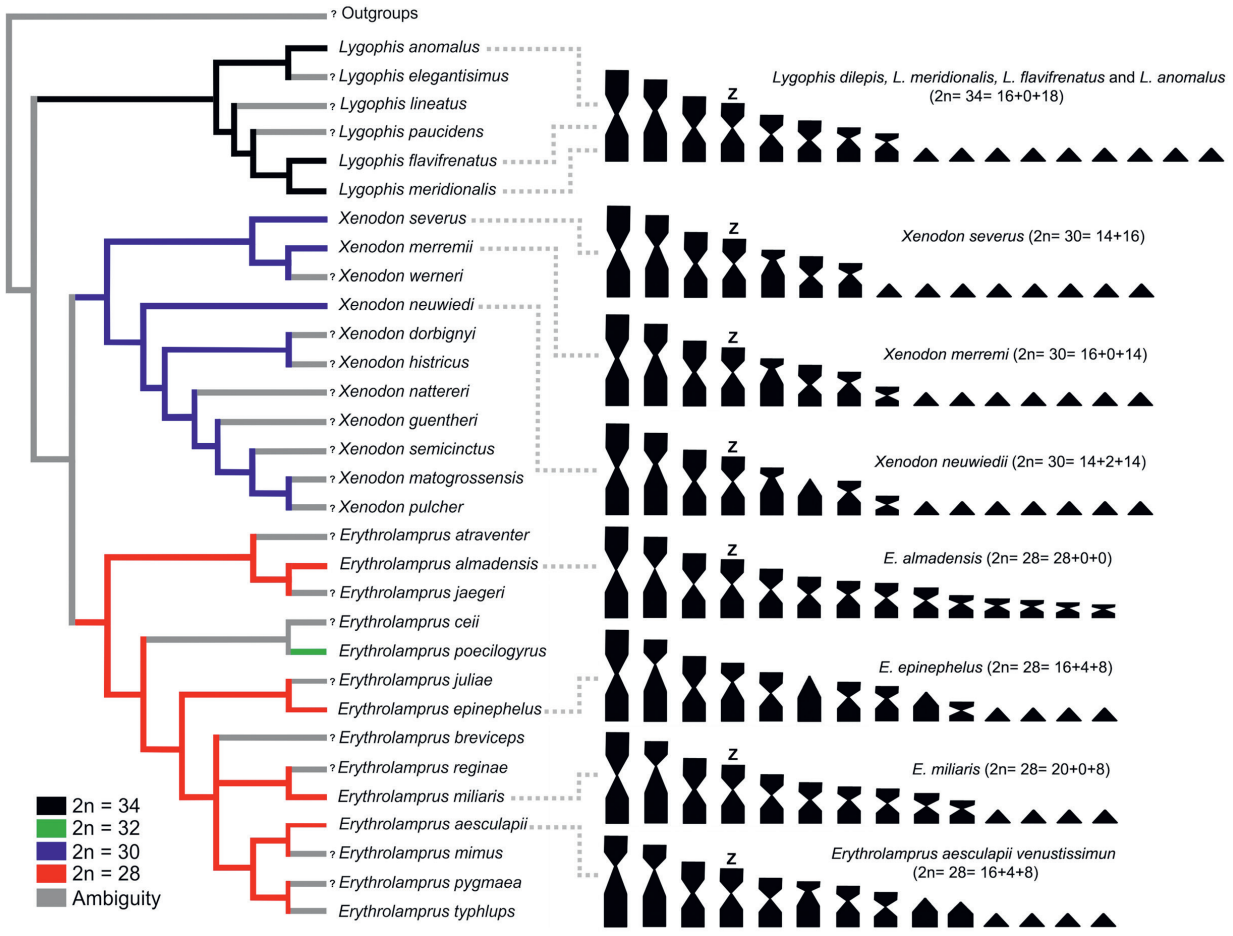

Fig. 3. Diploid number optimization within Xenodontini clade based on the molecular phylogenetic hypothesis presented by Pyron *et al*. (2013). The outgroup consisted of the sister clade (*Uromacer catesbyi* + *U. frenatus* + *U. oxyrhynchus*). The gray branches represent ambiguity and the question symbols (?) the missing entries. On the right are the karyograms of the included species for which chromosomal information is available.

degree of heteromorphy of the ZW chromosomes is variable; they can be similar morphologically or differ in shape and/or size and in heterochromatin distribution (Beçak and Beçak, 1969; Singh, 1972; Mengden and Stock, 1980; Olmo, 1986; Oguiura *et al*., 2009). A ZZ:ZW system among Xenodontini was also reported for *X. merremi*, *X. neuwedii*, *X. rabdocephalus*, *E. almadensis*, *E. miliaris* and *E. bizona* (Table 2) (Beçak, 1968; Beçak and Beçak, 1969; Beçak *et al*., 1975; Gutiérrez *et al*., 1984).

In *L. dilepis* and *L. anomalus* females metaphases exhibited a metacentric/submetacentric heteromorphic 4th pair, and the respective males possessed two homomorphic metacentric chromosomes on the same position. Although based on the analysis of Giemsa-stained metaphases, these observations lead us to suggest that the 4th pair could be related to the ZW system of sex determination. Beçak and Beçak (1969) and Matsubara *et al*. (2006) suggested that, in snakes, heteromorphic sex chromosomes are the result of pericentric inversions, heterochromatinization and deletion of euchromatic regions.

Lygophis dilepis, *L. flavifrenatus* and *L. meridionalis* have a similar NORs location (one pair of microchromosomes). The same position has been reported for Xenodontini in *E. poecilogyrus schotti* (Trajtengertz *et al*., 1995) and in Old and New World snakes (Camper and Hanks, 1995; Aprea *et al*., 2006). As such,

this number and location of NORs appears be prevalent in Serpentes (Camper and Hanks, 1995; Aprea *et al*., 2006). However, in Xenodontini, further studies are necessary to understand the distribution of this character within this group.

Of the thirteen species of the Xenodontini tribe cytogenetically described to date eleven are included in the phylogeny proposed by Pyron *et al*. (2013) and the available data only allow evaluation of the chromosome number and morphology. Our optimization show that diploid numbers represent putative synapomorphies for each genus (Fig. 3): *Lygophis* 2n = 34, *Xenodon* 2n = 30 and *Erythrolamprus* 2n = 28 (with an increase in *E. poecilogyrus* to 2n = 32). Unfortunately, we have no karyological data for the sister clade (*Uromacer catesbyi* + *U. frenatus* + *U. oxyrhynchus*) and for this reason it was not possible to evaluate the ancestral diploid number of the study group.

This study provides new data on the karyology of the Xenodontini. Yet, more studies on the karyology as well as on phylogenetic relationships in the Xenodontini species are necessary. Moreover, karyological information is available for three species that have not yet been included in molecular phylogenetic studies: *E. bizona* (2n = 28), *L. dilepis* (2n = 34), and *X. rabdocephalus* (2n = 34). Consequently, this information could not be optimized.

Although based on only four species, our study reinforce that there is remarkable variation in the karyotypes of Xenodontini. The karyotype 2n = 34 = 16 + 0 + 18 is shared among the studied *Lygophis* species and could thus be a diagnostic character for the genus. Increasing the taxonomic sampling in combination with the analysis of the banding patterns will allow us to further elucidate chromosomal evolution in Xenodontini.

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Appendix

Species names, sex, collection numbers, and localities of specimens of *Lygophis* sampled in this study. UNNEC = Colección Herpetológica de la Universidad Nacional del Nordeste, Corrientes, Argentina.

Lygophis anomalus (n = 5): UNNEC-13001 (♀) Paso de los Libres (29°34'04''S, 57°25'45''W), Corrientes province, Argentina; UNNEC-13002 (♀) La Cruz (29°8'21''S, 56°52'35''W), Corrientes province, Argentina; UNNEC-11047 (♂) Mercedes (28°41'22''S, 57°28'32''W), Corrientes province, Argentina; UNNEC-10843 (♂) Mercedes (28°42'12''S, 57°28'26''W), Corrientes province, Argentina; UNNEC-11046 (♀) Medanos (33°25'54''S, 59°04'17''W), Entre Rios province, Argentina.

Lygophis dilepis (n = 6): UNNEC-09736 (♂) Fontana (25°20'18''S, 59°41'17''W), Formosa province, Argentina; UNNEC-10102 (♂) Corrientes Capital (27°28' 09''S, 58°46'56''W), Corrientes province, Argentina; UNNEC-10204 (♀), UNNEC-10211 (♀), and UNNEC-10212 (♀) Paraje Perichón (27°25'45''S, 58°44' 45''W), Corrientes province, Argentina; UNNEC-11831 (♂) Calchaquí (29°57'55''S, 60°19'49''W), Santa Fé province, Argentina.

Lygophis flavifrenatus (n = 2): UNNEC-11013 (♂) San Roque (28°52'37''S, 58°28'09''W), Corrientes province, Argentina; UNNEC-11263 (♂) Corrientes Capital (27°28'09''S, 58°46'56''W), Corrientes province, Argentina.

Lygophis meridionalis (n = 2): UNNEC-10209 (♀) Isla Apipé (27°31'12''S, 56°44'32''W), Corrientes province, Argentina; UNNEC-11263 (♀) Concepción (28°23'01''S, 57°51'57''W), Corrientes province, Argentina.