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Comparative cytogenetic analysis in *Erythrolamprus* snakes (Serpentes: Dipsadidae) from Argentina

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ABSTRACT

We described the karyotypes of five snake taxa from Argentina: *Erythrolamprus almadensis, E. ceii*, *E. poecilogyrus caesius, E. p. schotti* and *E. p. sublineatus*, and also intergrading individuals between the last two subspecies by conventional staining, chromosome bandings and fluorescent *in situ* hybridization (FISH) with 28S ribosomal DNA probes. *Erythrolamprus ceii* and *E. almadensis* share a diploid chromosome number of 2n= 28, whereas in *E. poecilogyrus* intraspecific variations were observed: *E. p. caesius* has 2n= 28, *E. p. schotti* and *E. p. sublineatus* as well as in the intergrading individuals have 2n= 32. In *E. almadensis* and *E. p. caesius*, the 2nd and 6th chromosome pairs respectively are heteromorphic by size, morphology and C-banding pattern. These results allow us to suggest that these chromosome pairs might be considered as the ZW sex chromosomes in these species. The present comparative cytogenetic analyzes contributes to the already remarkable karyotypic variability in *Erythrolamprus* genus and propose a hypothesis about potential mechanisms involved in the chromosome evolution among taxa analyzed. Furthermore, the karyotypic differences observed between *E. p. caesius* (2n= 28) and *E. p. schotti* and *E. p. sublineatus* (2n= 32) might play a causal role in speciation.

Key words: Ag-NOR, C-banding, Xenodontini, fluorescent banding, rDNA-FISH, sex chromosomes.

INTRODUCTION

The cytogenetic analysis has been useful for taxonomic studies in Serpentes with inter- and intraspecific chromosome variability as *Micrurus* (Gutiérrez and Bolaños 1981) and *Vipera* (Aprea et al. 2006). Moreover, the differences in the karyotype through the redistribution of the chromosomes have been identified as a key mechanism in the evolutionary process of speciation (White 1968, 1969, Olmo 2005).

The Xenodontini comprises roughly 70 species of South American Dipsadidae snakes, currently grouped in three genera: *Erythrolamprus* Boie 1826, *Lygophis* Fitzinger 1843 and *Xenodon* Boie 1826 (Vidal et al. 2010, Grazziotin et al. 2012, Pyron et al. 2013, Uetz and Hošek 2013). *Erythrolamprus* includes 48 species distributed in Central and South

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America (Vidal et al. 2000, Zaher et al. 2009, see debate in Curcio et al. 2009, Uetz and Hošek 2013). Cytogenetic studies were carried out in only six species of *Erythrolamprus* by conventional staining and the number and location of nucleolar organizer regions (NORs) were determined in *E. poecilogyrus schotti* by fluorescent *in situ* hybridization (FISH) (Beçak et al. 1966, 1971, 1975, Beçak and Beçak 1969, Gutiérrez et al. 1984, Trajtengertz et al. 1995). Despite the scarce cytogenetic information available, karyotype variability has been reported among the species studied, i.e., karyotypes with 28 chromosomes that differ in number of macro- and micro-chromosomes and in morphology of macro-chromosomes, and one karyotype with 32 chromosomes (Beçak et al. 1966, 1971, 1975, Beçak and Beçak 1969, Gutiérrez et al. 1984). On the other hand, in *E. almadensis*, *E. miliaris*, *E. aesculapii venustissimun* and *E. bizona* the 4th chromosome pair was recognized as ZW sex pair (Beçak et al. 1966, 1975, Beçak and Beçak 1969, Gutierrez et al. 1984).

In this cytogenetic study, we describe the karyotype of Argentinean snakes included in *poecilogyrus* morphological group (Cei 1993): *Erythrolamprus almadensis* (Wagler 1824), *E. ceii* (Dixon 1991), *E. poecilogyrus caesius* (Cope 1862), *E. p. schotti* (Schlegel 1837) and *E. p. sublineatus* (Cope 1860) and the intergrading individuals between the last two subspecies through conventional staining, different chromosome banding (C, DAPI/ CMA_3 and Ag-NOR) and fluorescent *in situ* hybridization (FISH) of ribosomal DNA. *Erythrolamprus poecilogyrus* (Wied-Neuwied 1825) is considered one of the most noteworthy examples of polymorphism due to the geographic variation in color and design patterns and natural mosaic of morphometric characters and lepidosis (Dixon and Markezich 1992). The cytogenetic characters described for each analyzed taxon were taxonomically informative and allowed us to provide a hypothesis about potential mechanisms involved in the chromosome evolution into the *poecilogyrus* group.

MATERIALS AND METHODS

Cytogenetic analyses were performed on females and males of *Erythrolamprus ceii*, *E. almadensis*, *E. poecilogyrus caesius*, *E. p. schotti* and *E. p. sublineatus* and intergrading individuals (see details in Appendix). Voucher specimens are deposited in the Herpetological Collection of the National University of the Northeast (UNNEC), Corrientes, República Argentina.

Erythrolamprus poecilogyrus subspecies were identified by the different color patterns according to Fernandes da Silva (2006). Individuals with intermediate patterns of coloration between *E. p. schotti* and *E. p. sublineatus* were considered as intergrading individuals.

The specimens were injected intraperitoneally with 0.1% colchicine (1ml/100g body weight) for 3 hours prior to animal dissection. We practiced euthanasia according to the method described by Beaupre et al. (2004). The intestine was dissected and swollen for 50 minutes at RT in a hypotonic solution (0.075 M KCl), cut into small fragments, and then fixed in freshly prepared fixative solution (methanol:glacial acetic acid, 3: 1) and stored at -20ºC until further use.

For mitotic chromosome preparations, two-three intestine fragments were transferred to a tube with a few drops of 60% acetic acid for a few minutes until the epithelial cells shed, and then the gut fragments were removed. Some drops of cell suspension were carefully placed onto slides, which had been previously pre-heated in a thermal bath at 45º C for maintaining a thin film of water at the time when the drops fall on the slide. Cells were spread on the slide using a heating plate at 45°C as described in Traut (1976). Then, the preparations were dehydrated in an ethanol series (70, 80, and 96%, 30 s each) and stored at -20°C until further

use. For conventional staining, preparations were stained with 10% Giemsa solution (pH 6.8) for 7 min at RT.

C-and sequential fluorescent bandings were performed according to Papeschi (1988) and Poggio et al. (2011). Detection of the nucleolus organizer regions (NORs) on mitotic chromosomes was done following the silver staining method of Howell and Black (1980). Fluorescent *in situ* hybridization (FISH) with biotinylated 28S rDNA probe of *Xenopus laevis* was performed following the procedure in Bressa et al. (2009). The 28S rDNA fragment was labeled by nick translation with biotin 14-dUTP (Bionick Labeling System, GIBCO BRL, USA) (Bressa et al. 2009). Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA).

Chromosome preparations were observed in a epifluorescence microscope Leica DMLB equipped with a Leica DFC350 FX CCD camera and Leica IM50 software, version 4.0 (Leica Microsystems Imaging Solutions). Black-and-white images of chromosomes were recorded separately for each fluorescent dye. Images were pseudocolored (light blue for DAPI, green for CMA_3, and red for Cy3) and processed with an appropriate software.

Chromosome sizes were measured using the computer application MicroMeasure version 3.3 (available at http://www.colostate.edu/Depts/Biology/Micromeasure) (Reeves and Tear 2000). Measurements were performed on ten metaphase plates of each specimen. Relative chromosomal lengths (RL) and centromeric index (CI) were calculated and expressed as percentage of the haploid set. These data were used to describe the karyotype of each Argentinean snake according to Levan et al. (1964). The chromosomal formula was determined following to Peccinini-Seale (1981) (2n = I+II+III) being I = metacentric or submetacentric macrochromosomes, II = telocentric or subtelocentric macrochromosomes and III = microchromosomes. Based on RL values three chromosome groups could be recognized: small (1 - 2.5% of haploid set), medium (2.51 - 5%) and large (> 5%).

RESULTS

CHROMOSOME COMPLEMENTS

Erythrolamprus ceii, E. almadensis and *E. poecilogyrus caesius* share a diploid chromosome number 2n = 28 (Fig. 1a-d). Based on the RL values the chromosome pairs 1 to 3 are larger, 4 to 7 pairs have medium size and 8 to 14 pairs are smaller (Fig. 1a-d; Table I).

The karyotype formula is 28 = 28+0+0, NF = 56 in *E. ceii* and *E. almadensis* (Fig. 1a-c; Table I). In females of *E. almadensis*, the chromosome pair 2 is heteromorphic in size, being one homologous chromosome smaller (RL = 6.77%) than the other (RL = 8.66%), whereas in males both homologous have the same relative size (RL = 7.35%) (Table I). In males of *E. poecilogyrus caesius*, the karyotype formula is 28 = 26+2+0, NF = 54 with a pair 6 formed by two subtelocentric chromosomes, whereas females have 28 = 27+1+0, in which the pair 6 comprises one metacentric chromosome and the other subtelocentric (Fig. 1d; Table I).

The karyotype formula of both sexes of *E. poecilogyrus schotti*, *E. p. sublineatus* and intergrading individuals is 32 = 24+8+0, NF = 56 (Fig. 1e-g). The chromosome pairs 1, 4 - 6, and 9 - 16 are metacentric and the pairs 2, 3, 7, and 8 are telocentric (Fig. 1; Table I). According to the RL values, the pair 1 belongs to the large group, the pairs 2 to 9 to the medium one, and pairs 10 to 16 to the smaller (Table I). In these taxa neither a heteromorphic pair and no secondary constriction is observed on the *poecilogyrus* group chromosome complements.

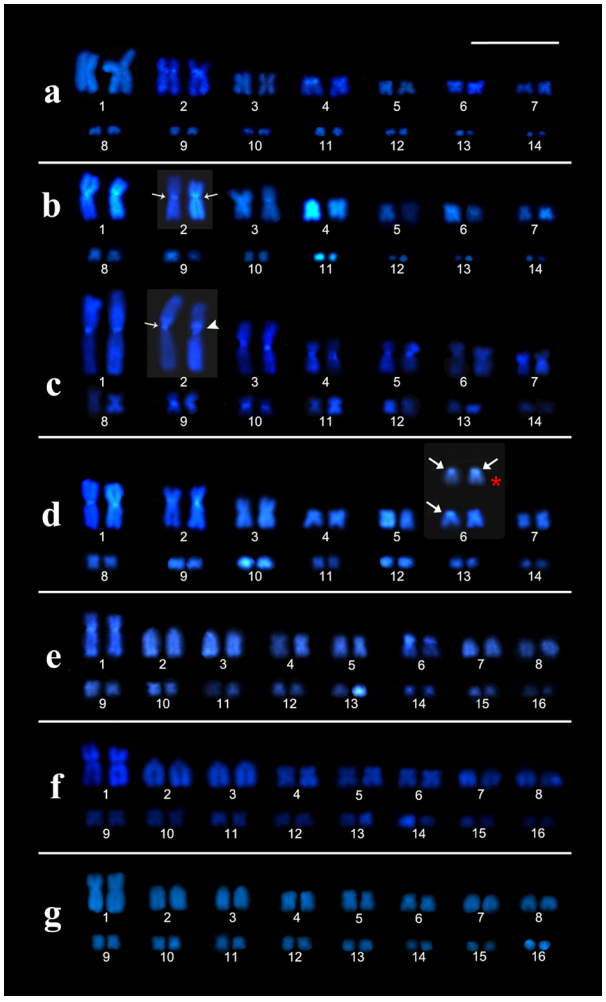

Figure 1 - C-banding patterns staining with DAPI on mitotic chromosomes of *Erythrolamprus poecilogyrus* group species. **a**) *E. ceii*, **b**) *E. almadensis* (male), **c**) *E. almadensis* (female), **d**) *E. poecilogyrus caesius*, **e**) *E. p. schotti*, **f**) *E. p. sublineatus* and **g**) Intergrading individuals. Arrowhead: pericentromeric C-positive band. Arrows: centromeric C-positive bands in the heteromorphic chromosome pairs of *E. almadensis* and *E. poecilogyrus caesius*. Red asterisk: pair 6 in males in *E. poecilogyrus caesius*. The gray boxes indicate the heteromorphic pairs. Scale bar= 10 µm.

C- AND FLUORESCENT BANDINGS

In *Erythrolamprus ceii*, three chromosome pairs have heterochromatic C- positive bands at centromeric regions, i.e., the chromosome pair 2, 4 and 6 (Fig. 1a). In males of *E. almadensis*, the chromosome pairs 1, 2, 3, 6, and 9. In female mitotic preparations, C-positive bands are present at centromeric regions of the chromosome pairs 1, 3-6, 9, and 10 (Fig. 1b, c). In the heteromorphic chromosome pair 2, the large homologous chromosome shows a C-positive band at the centromeric region, whereas the small one has a pericentromeric C-positive band in the p arm (Fig. 1c). In *E. p. caesius*, C-positive centromeric bands are revealed on each chromosome of pairs 1 and 2 (Fig. 1d). Besides, both sexes show differences in the C-banding pattern of the chromosome pair 6. In males, each subtelocentric homologous chromosome has C-positive centromeric bands. In females, no C-bands are detected on the metacentric chromosome, whereas a C-positive band is observed in its subtelocentric homologous (Fig. 1d). In *Erythrolamprus p. schotti*, the chromosome pair 1, 6 and 14 show heterochromatic C-positive bands placed on centromeric regions (Fig. 1e). No C-positive bands were detected in both *E. p. sublineatus* and the intergrading specimens (Fig. 1f, g).

All mitotic chromosomes of *E. ceii* are stained homogenously without any DAPI- or CMA_3-bright bands. A small chromosome pair of *E. almadensis*, shows a DAPI-negative/CMA_3-bright band at the centromeric region (Fig. 2a-c). In mitotic chromosomes of *E. p. caesius*, DAPI-dark/CMA_3-bright bands are observed at centromeric regions of the chromosome pairs 3, 5, 10, 11, 12, 13, 14 and in one homologous chromosome of the pairs 1 and 6, DAPI-bright/CMA_3-bright centromeric bands in chromosome pair 2, and DAPI-bright/CMA_3-dark bands at centromeric regions of pairs 7, 8 and 9 (Fig. 2d-f). The fluorescent banding in *E. p. schotti* reveals DAPI-dark/CMA_3-bright bands placed on centromeric regions of two small chromosome pairs (Fig. 2g-i). No DAPI/CMA_3 bands are detected in mitotic chromosomes of *E. p. sublineatus*. Lastly different fluorescent banding patterns are observed in the intergrading individuals: i) all chromosomes are stained homogenously without any DAPI- or CMA_3-bright bands, ii) centromeric DAPI-dark/CMA_3-bright bands on the chromosome pair 1, a medium-sized and small pairs (Fig. 2j-l), iii) DAPI-dark/CMA_3-bright bands at telomeric regions on

TABLE I

Relative length (RL), centromeric index (CI) and standard deviation (SD) of *Erythrolamprus* species analyzed in this study.

Species	*Erythrolamprus ceii*			*E. almadensis*			*E. poecilogyrus caesius*			*E. p. schotti*			*E. p. sublineatus*			Intergrading individuals		
CP	RL %	CI ± SD	Type	RL %	CI ± SD	Type	RL %	CI ± SD	Type	RL %	CI ± SD	Type	RL %	CI ± SD	Type	RL %	CI ± SD	Type
1	9.87	46.89 ± 0.58	M	9.17	48.16 ± 0.24	M	9.24	47.08 ± 1.86	M	8.11	40.03 ± 0.83	M	7.24	44.73 ± 0.58	M	8.26	41.88 ± 0.29	M
2*	7.96	40.49 ± 0.59	M	**8.66**	**44.96 ± 1.84**	**M**	8.37	39.10 ± 0.17	M	4.96	4.38 ± 0.30	T	4.90	9.04 ± 1.38	T	4.96	4.39 ± 0.35	T
				6.77	**41.12 ± 1.15**	**M**				4.75	5.66 ± 0.76	T	4.91	8.70 ± 1.19	T	4.65	6.06 ± 0.82	T
3	5.83	48.37 ± 1.18	M	5.28	46.55 ± 1.65	M	5.90	45.90 ± 0.27	M	3.51	41.30 ± 0.47	M	3.64	49.30 ± 0.09	M	4.00	45.20 ± 0.51	M
4	3.64	47.48 ± 1.50	M	3.94	45.31 ± 0.14	M	4.25	46.74 ± 0.77	M	3.09	41.62 ± 0.47	M	3.37	43.49 ± 0.13	M	3.58	44.11 ± 0.68	M
5	3.57	43.94 ± 1.04	M	3.30	40.78 ± 0.76	M	3.43	46.57 ± 1.77	M	3.11	46.40 ± 1.32	M	2.86	47.65 ± 1.63	M	3.25	43.34 ± 0.55	M
6*	2.96	45.78 ± 1.01	M	3.18	40.08 ± 1.66	M	**3.24**	**44.86 ± 1.23**	**M**	3.14	7.68 ± 0.13	T	3.35	5.18 ± 1.35	T	3.31	8.07 ± 0.60	T
							3.05	**19.52 ± 0.59**	**ST**	3.10	6.11 ± 0.17	T	3.00	8.80 ± 0.48	T	3.02	9.16 ± 1.25	T
7	2.69	43.30 ± 0.11	M	3.09	41.20 ± 0.83	M	2.82	43.71 ± 1.59	M	2.64	45.16 ± 1.04	M	2.60	39.55 ± 0.26	M	2.79	43.76 ± 1.17	M
8	2.50	46.51 ± 0.54	M	2.50	44.29 ± 1.04	M	2.49	46.92 ± 0.07	M	2.38	42.94 ± 0.03	M	2.44	46.75 ± 0.78	M	2.39	47.02 ± 0.36	M
9	2.32	46.78 ± 1.13	M	2.49	45.72 ± 2.31	M	2.26	47.25 ± 1.54	M	2.26	46.29 ± 0.20	M	2.40	40.54 ± 0.16	M	1.94	46.85 ± 0.10	M
10	2.06	45.37 ± 0.08	M	2.27	45.90 ± 0.09	M	2.00	48.72 ± 0.21	M	2.10	43.70 ± 1.07	M	2.33	47.26 ± 1.33	M	1.83	46.71 ± 0.76	M
11	1.91	44.54 ± 0.63	M	2.13	45.47 ± 1.14	M	1.82	46.54 ± 0.07	M	1.96	44.89 ±1.17	M	2.13	38.99 ± 0.91	M	1.70	46.79 ± 0.89	M
12	1.70	45.32 ± 0.47	M	1.87	46.38 ± 1.53	M	1.60	46.50 ± 1.28	M	1.79	44.49 ±1.38	M	1.74	48.14 ± 0.11	M	1.59	47.57 ± 0.12	M
13	1.58	46.87 ± 1.19	M	1.63	46.13 ± 0.62	M	1.41	47.07 ± 1.69	M	1.75	47.48 ±0.87	M	1.64	40.45 ± 1.31	M	1.43	47.01 ± 1.60	M
14	1.41	44.19 ± 1.11	M	1.35	46.31 ± 0.33	M	1.27	43.72 ± 0.76	M	1.60	45.56 ±0.56	M	1.38	43.86 ± 0.40	M	1.29	44.97 ± 1.09	M

2*, 6* heteromophic pair in females of *E. almadensis* and *E. p. caesius.* respectively. CP: chromosome pairs. M: metacentric, ST: subtelocentric and T: telocentric.

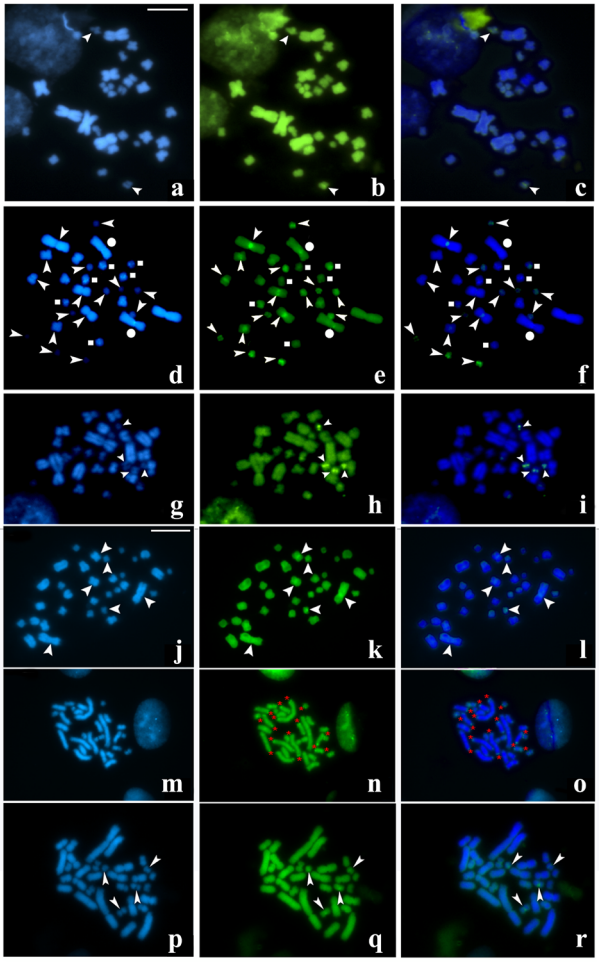

Figure 2 - Sequential DAPI/ CMA_3 banding patterns of the *Erythrolamprus poecilogyrus* group species. **a-c**, *E. almadensis*. **d-f**, *E. p. caesius*. **g-i**, *E. p. schotti*. **j-r**, intergrading individuals. **a**, **d**, **g**, **j**, **m**, **p** DAPI banding; **b**, **e**, **h**, **k**, **n**, **q** CMA_3 banding and **c**, **f**, **i**, **l**, **o**, **r** combination of figures **a**+**b**, **d**+**e**, **g**+**h**, **j**+**k**, **m**+**n** and **p**+**q** respectively. Arrowheads: centromeric DAPI-dark/CMA_3-bright bands. Dots: centromeric DAPI-bright/CMA_3-bright bands. Squares: centromeric DAPI-bright/CMA_3-dark bands. Red asterisks: telomeric DAPI-dark/CMA_3-bright bands. Scale bar= 10 µm.

most chromosome pairs (Fig. 2m-o), and iiii) centromeric DAPI-dark/CMA_3-bright bands on two small chromosome pairs (Fig. 2p-r).

LOCATION OF rDNA BY FISH AND Ag-NOR BANDING

At mitotic metaphases, positive Ag-NORs are observed in one of the small chromosome pairs in all species of the *Erythrolamprus* analyzed, i.e. *Erythrolamprus ceii* (Fig. 3a), *E. almadensis* (Fig. 3b), *E. p. caesius* (Fig. 3c), *E. p. schotti* (Fig. 3d), and *E. p. sublineatus* (Fig. 3e), and also in intergrading individuals (Fig. 3f). In mitotic metaphases of males of *E. p. sublineatus* and females of *E. p. schotti*, FISH experiments with the 28S rDNA probe showed a single cluster of rDNA genes located in each homologous chromosomes of one of the small pair (Fig. 3g-l).

DISCUSSION

The present comparative study provides new evidences that strengthen the remarkable karyotype variability in Xenodontini tribe and also in *Erythrolamprus* genus (Falcione et al. 2016) (Table II). To the present, two diploid chromosome numbers are described for the genus: 2n= 28 in *E. aesculapii venustissimun*, *E. epinephelus*, *E. bizona*, *E. miliaris* and *E. almadensis*, but showing variations in the number of uni- and biarmed macro- and micro-chromosomes (Table II) (Beçak et al. 1966, 1975, Beçak and Beçak 1969, Gutierrez et al. 1984), and 2n = 32 in *E. poecilogyrus* and *E. p. schotti* (Beçak et al. 1971, Trajtengertz et al. 1995).

In relation to the number of biarmed and uni-armed macro-chromosomes (M) pairs, it deserves attention that all the species and subspecies of the genus *Erythrolamprus* with 2n = 28 exhibit a low number of uni-armed M pairs: two in *E. aesculapii venustissimun* (9 and 10) and *E. epinephelus* (6 and 9), one in *E. bizona* (pair 6), and without any of them in *E. miliaris*, *E. ceii* and *E. almadensis* (Table II). Particularly, into the *poecilogyrus* group inter- and intra-specifics chromosomes variations are revealed about to the number of uni- and bi-armed: *Erythrolamprus ceii* and *E. almadensis* have karyotypes with 2n = 28 = 28+0+0 (♂/♀) whereas in *E. poecilogyrus* intraspecifics variations were observed: *E. p. sublineatus* and *E. p. schotti* and the intergrading individuals have 2n = 32= 24+8+0

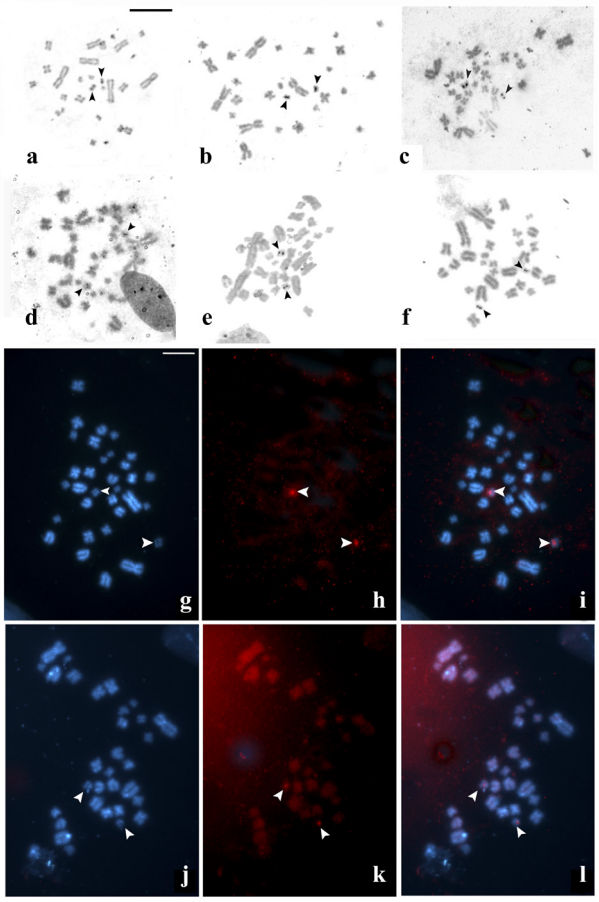

Figure 3 - Location of rDNA by FISH and Ag-NOR banding of *Erythrolamprus poecilogyrus* group species. Ag-NOR-stained metaphases of **a**) *Erythrolamprus ceii*, **b**) *E. almadensis*, **c**) *E. p. caesius*, **d**) *E. p. schotti*, **e**) *E. p. sublineatus* and **f**) Intergrading individuals. The black arrowheads indicate the chromosomes bearing NORs. FISH with DNAr 28S probe in male mitotic metaphase of *E. p. sublineatus* (**g-i**) and female of *E. p. schotti* (**j-l**). Chromosomes counterstained with DAPI (blue) (**g**, **j**), hybridization signals (red) (**h**, **k**) and combination of figures **g**, **j** with **h** and **k**, respectively (**i-l**). The white arrowheads indicate the hybridization signals and the chromosomes carriers. Scale bar= 10 µm.

(♂/♀) whereas *E. p. caesius* has 2n = 28 = 26+2+0 ♂/ 27+1+0 ♀.

The comparison of cytogenetic characteristics within *E. poecilogyrus* allow distinguishing two karyotypes: one with both higher diploid chromosome number and fundamental number (2n = 32, NF = 56), consisting of less biarmed M and more uni-armed M (24+8+0) (*E. poecilogyrus schotti*, *E. p. sublineatus* and intergrading individuals), and the other with both lower diploid number and fundamental number (2n = 28, NF = 54), formed by more biarmed M and less uni-armed M (26+2+0) (*E. p. caesius*).

In snakes, the karyotype 2n = 36 (16 macro-chromosomes+20 micro-chromosomes) is proposed to be an ancestral character shared throughout most families (Oguiura et al. 2009). Taking into account this hypothesis, both chromosome changes must have occurred throughout the karyotype evolution of *Erythrolamprus poecilogyrus* group and the different karyotypes should be considered as derivatives. The lowest diploid numbers could derive through fusion between macro and micro-chromosomes and/or between micro-chromosomes. Moreover, differences in chromosome morphology could be due to pericentric inversions and heterochromatin addition. In Squamata, there is a trend to reduce the number of micro-chromosome because of translocation onto macro-chromosomes, or fusion among micro-chromosomes (Olmo 2008, Uno et al. 2012).

Concerning the content, distribution and location of constitutive heterochromatin, different C-banding patterns have been previously described in snakes, i.e., large blocks at centromeric regions, and also at terminal and interstitial position in macro-chromosomes, whole heterochromatic arms (Mengden and Stock 1980, Moreno et al. 1987), only placed on microchromosomes (Singh and Majumdar 1994) and scarce heterochromatin in autosomes (Mezzasalma et al. 2014). The results obtained here revealed a low content of constitutive heterochromatin among the species of the *poecilogyrus* group, intra- and intraspecific differences and the enrichment of heterochromatin mainly of CG base pairs.

The karyotypes of *Erythrolamprus ceii*, *E. almadensis* and *E. p. caesius* (2n = 28) are similar after conventional staining, although we detected differences from the comparison of their DAPI/ CMA_3 banding patterns. Our cytogenetic

TABLE II
Diploid number, chromosome formula and sex chromosome morphology and position into *Erythrolamprus* genus.

Species	2n	Chromosome formula	Sex chromosomemorphology		ZW position	References
			Z	W		
Erythrolamprus aesculapii venustissimun	28	16+4+8 (♂,♀)	Metacentric	Submetacentric	4th pair	Beçak et al. 1966, Beçak and Beçak 1969
E. epinephelus	28	16+4+8 (♂,♀)	–		–	Gutiérrez et al. 1984
E. miliaris	28	19+1+8 (♀)	Metacentric	Acrocentric	4th pair	Beçak and Beçak 1969
		20+0+8 (♂)	–		–	
E. bizona	28	18+2+8 (♀)	Submetacentric	Submetacentric	4th pair	Gutiérrez et al. 1984
E. almadensis	28	27+1+0 (♀)	Metacentric	Acrocentric	4th pair	Beçak et al. 1975
		28+0+0 (♂)	–		–	
		28+0+0 (♂,♀)	Metacentric	Metacentric	2nd pair	This study
E. ceii	28	28+0+0 (♂,♀)	–		–	This study
E. poecilogyrus caesius	28	27+1+0 (♀)	Subtelocentric	Metacentric	6th pair	This study
		26+2+0 (♂)	–		–	
E. poecilogyrus	32	–	–		–	Beçak et al. 1971
E. p. schotti	32	–	–		–	Trajtengertz et al. 1995
	32	24+8+0 (♂,♀)	–		–	This study
E. p. sublineatus	32	24+8+0 (♂,♀)	–		–	This study

findings provide a deeper knowledge of chromatin organization and constitution of the chromosomes among related species. It should be mentioned that the fluorescence bands patterns of the intergroup individuals are noteworthy since they some share with *E. p. schotti* DAPI-dark/CMA3-bright bands on the two pairs of small chromosomes and others with *E. p. sublineatus* without evident DAPI/CMA3 bands. The distribution and content of constitutive heterochromatin are considered as variable characters for the karyotypes of some reptiles (Olmo et al. 1986). Furthermore, the intra- and interspecific variation observed in the C-band patterns would appear to be unrelated to phylogeny (Odierna et al. 1985). Thus, the diversity observed in the C-banding pattern of the species and subspecies studied here constitutes evidence that supports the evolutionary trend for reptiles.

The number and location of the NORs in a pair of micro-chromosomes is frequent in Serpentes and also in Squamata (Camper and Hanks 1995, Aprea et al. 2006, Mezzasalma et al. 2014). Within Xenodontini, the location in micro-chromosomes has been reported in *E. poecilogyrus schotti* and three *Lygophis* species (Trajtengertz et al. 1995, Falcione et al. 2016). In all taxa studied here, a single NOR has been observed in one pair of small chromosomes.

In most organisms, the repeating unit of the ribosomal genes (rDNA) is often G+C-rich (Miller 1981, Sumner 2003). The presence of a CMA_3-bright band is generally associated to NORs (Sumner 2003, Aprea et al. 2006). From the results of the analysis of fluorescent bandings in *E. almadensis*, the CMA_3-bright band placed on one small chromosome pairs could represent an NOR.

The presence of a heteromorphic sex chromosome pair ZZ/ZW has been already previously described in four species of *Erythrolamprus*: *E. miliaris*, *E. bizona*, *E. aesculapii venustissimun* and *E. almadensis* (Beçak et al. 1966, 1975, Beçak and Beçak 1969, Gutiérrez et al. 1984). These species share the position of the ZZ/ZW on the 4th chromosome pair of the karyotype, but sex chromosomes Z and W shows a variable morphology and size (Beçak et al. 1966, 1975, Beçak and Beçak 1969, Gutiérrez et al. 1984). The ZW heteromorphism is considered a putative synapomorphy of Colubroidea (Oguiura et al. 2009), and their position on the 4th pair is reported in colubrids and crotalids species (Baker et al. 1972, Beçak and Beçak 1969, 1981, Singh 1972, Gutiérrez et al. 1979, Mengden and Stock 1980, Ota 1999, Aprea et al. 2003, 2006, Falcione et al. 2016). However, based on partial gene content, part of the sex chromosomes is homologous in all families of caenophidian snakes (Rovatsos et al. 2015).

In the present study, the 2nd and 6th chromosome pairs are assigned as sex chromosome pair (ZZ/ZW) in both *E. almadensis* and *E. p. caesius*, respectively. In females of *E. almadensis*, both homologous are metacentric but differ in size. However, in males the two chromosomes are of equal size. Furthermore, the smallest metacentric chromosome of pair 2 exhibits a pericentromeric C-positive band on the p arm in females. Therefore, the presence of this heteromorphic pair in females allows us to propose that chromosomal pair 2 would be the ZW sex pair, because the smallest metacentric chromosome would be resting to females, being W chromosome.

In the female diploid chromosome complement of *E. poecilogyrus caesius*, the pair 6 is heteromorphic by morphology, since one of the chromosomes is metacentric and the other sub-telocentric. In contrast, male karyotype the same pair is homomorphic, both homologues being subtelocentric. Similarly, in females only the subtelocentric chromosome of this pair has a C-positive band in the p-arm, whereas in males C-positive bands are distinguished in the p-arm of both subtelocentric chromosomes. Considering to these result, we suggest that the metacentric chromosome of the pair 6 would be the W chromosome, whereas the subtelocentric chromosome the Z, because it is only present in the female complement. Pericentric inversions, amplification of pre-existent heterochromatin or the addition of new heterochromatin, and deletions of euchromatic regions would have been the mechanisms involved in the evolution of sex chromosomes of *Erythrolamprus almadensis* and *E. p. caesius* as there were proposed in Serpentes (Ohno 1967, Ray-Chaudhuri et al. 1971, Singh et al. 1976, Beçak 1983, Matsubara et al. 2006).

Cytotaxonomy plays a key role in elucidating the taxonomy and chromosomal evolution of snakes when morphological characteristics are insufficient for the resolution of taxonomic problems (Gutiérrez and Bolaños 1981). From this point of view, we provide an overview of the current cytogenetic *Erythrolamprus* genus. Moreover, our results show that *E. p. caesius* (2n = 28) has karyological specific characteristics that differentiate it from *E. p. schotti* and *E. p. sublineatus* (2n = 32). This karyotype might constitute a mechanism of reproductive isolation making it a different species: *Erythrolamprus caesius*. Complementarily morphological and molecular analysis should provide further information.

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APPENDIX

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

Erythrolamprus ceii (n = 3): UNNEC- 12690 (♂), UNNEC- 12691 (♂) and UNNEC- 12692 (♂), Isla de Cañas (22º53′55″S, 64º39′59″W), Salta province, Argentina.

Erythrolamprus almadensis (n = 8): UNNEC-10621 (♂) Isla Apipé (27º34′20″S, 56º48′05″W), Corrientes province, Argentina; UNNEC-10939 (♂) Santa Ana (27º27′18″S, 58º39′12″W), Corrientes province, Argentina; UNNEC-11093 (♀), UNNEC-11243 (♂), UNNEC-11826 (♀) and UNNEC-11827 (♂) San Cayetano (27º33′09″S, 58º40′47″W), Corrientes province, Argentina; UNNEC-11012 (♀) Chavarria (28º53′47″S, 58º30′11″W), Corrientes province, Argentina; UNNEC-11048 (♂) Concepción (28º17′24″S, 58º02′08″W), Corrientes province, Argentina.

Erythrolamprus poecilogyrus caesius (n = 11): UNNEC-11092 (♀), UNNEC-10897 (♀), UNNEC-11898 (♀) and UNNEC-11107 (♀) Taco Pozo (25º37′02″S, 63º16′07″W), Chaco province, Argentina; UNNEC-12751 (♀) and UNNEC-12750 (♂) Campo Largo (26º48′01″S, 60º50′21″W), Chaco province, Argentina; UNNEC-12693 (♂) Pampa Del Índio (26º01′07″S, 59º58′01″W), Chaco province, Argentina; UNNEC-11094 (♂) San Roque (28º34′28″S, 58º42′32″W), Corrientes province, Argentina; UNNEC-11828 (♀), UNNEC-11829 (♀) and UNNEC-11830 (♂) Calchaqui (30º01′06″S, 60º19′36″W), Santa Fé province, Argentina.

Erythrolamprus poecilogyrus schotti (n = 19): UNNEC-9930 (♂) and UNNEC-9934 (♀) Galarza (28º04′20″S, 56º38′44″W), Corrientes province, Argentina; UNNEC-10073 (♀) Loreto (27º45′21″S, 57º18′42″W), Corrientes province, Argentina; UNNEC-10205 (♀) and UNNEC-10207 (♂) Isla Apipé (27º31′12″S, 56º44′32″W), Corrientes province, Argentina; UNNEC-12696 (♂) and UNNEC-12697 (♀) Tres Cerros (29º06′47″S, 56º55′08″W), Corrientes province, Argentina; UNNEC-12330 (♀) Ituzaingó (27º35′24″S, 56º41′22″W), Corrientes province, Argentina; UNNEC-11262 (♂) Yapeyú (29º27′03″S, 56º49′06″W), Corrientes province, Argentina; UNNEC-11095 (♂) and UNNEC-12699 (♀) San Luis del Palmar (27º34′41″S, 58º30′37″W), Corrientes province, Argentina; UNNEC-11245 (♂) and UNNEC-11246 (♀) Capitá Miní (28º56′00″S, 58º22′10″W), Corrientes province, Argentina; UNNEC-11247 (♀) San Cayetano (27º33′09″S, 58º40′47″W), Corrientes province, Argentina; UNNEC-12752 (♀) Santa Ana (27º27′18″S, 58º39′12″W), Corrientes province, Argentina; UNNEC-12698 (♂) San Miguel (27º59′35″S, 57º35′19″W), Corrientes province, Argentina; UNNEC-12700 (♀) and UNNEC-12701 (♂) Empedrado (27º47′52″S, 58º45′40″W), Corrientes province, Argentina; UNNEC-11249 (♀) Capital (27º28′16″S, 58º50′22″W), Corrientes province, Argentina.

Erythrolamprus poecilogyrus sublineatus (n = 9): UNNEC-10626, 10630 (♂) and UNNEC-10572, 10628 (♀) Concepción (28º25′44″S, 57º57′54″W), Corrientes province, Argentina; UNNEC-12703 (♂) and UNNEC-12702 (♀) Tres Cerros (29º06′47″S, 56º55′08″W), Corrientes province, Argentina; UNNEC-9823 (♂) Galarza (28º05′48″S, 56º41′07″W), Corrientes province, Argentina; UNNEC-12704 (♂) Bonpland (29º49′03″S, 57º25′41″W), Corrientes province, Argentina; UNNEC-10940 (♂) Guardamonte (32º05′19″S, 59º15′39″W), Entre Rios province, Argentina.

Intergrading Individuals (n = 8): UNNEC-10583 (♂) and UNNEC-10572 10629 (♀) Concepción (27º55′01′′S, 57º27′29′′W), Corrientes province, Argentina; UNNEC-11481 (♀) Monte Caseros (30º27′47′′S, 58º01′20′′W), Corrientes province, Argentina; UNNEC-11480 (♂) Sauce (30º10′08′′S, 59º01′55′′W), Corrientes province, Argentina; UNNEC-11248 (♂) Mercedes (28º55′24′′S, 58º17′50′′W), Corrientes province, Argentina; UNNEC-11246 (♀) Capitá Miní (28º56′00′′S, 58º22′10′′W), Corrientes province, Argentina; UNNEC-12755 (♂) and UNNEC-2753 (♀) Santa Ana (27º27′18′′S, 58º39′12′′W), Corrientes province, Argentina.