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Further insights into chromosomal evolution of the genus *Enyalius* with karyotype description of *Enyalius boulengeri* Etheridge, 1969 (Squamata, Leiosauridae)

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Abstract. The genus *Enyalius* is composed of 11 described species inhabiting forest areas in Amazônia, Cerrado and Atlantic forest biomes. Currently, eight species with high levels of chromosome variation have been karyotyped. The study aims to characterize the karyotype of *Enyalius boulengeri*, with classical and molecular techniques, and improve knowledge about the karyotype evolution of the lizard genus *Enyalius*. The species has *2n* = 36 chromosomes (8m + 4sm + 24mc), FN = 24; NORs and 18S rDNA were subtelomeric and located on chromosome pair 2. Repetitive DNA probes $(CAT)_{10}$ accumulated on centromeric and terminal regions of some macrochromosomes. $(GA)_{15}$ probe showed conspicuous accumulation on the pericentromeric region of chromosome pairs 1 and 6. Repetitive FISH patterns obtained with $(GC)_{15}$ probe marked the pericentromeric region of the first chromosome pair. All probes showed accumulation in the microchromosomes. The chromosomal formula found in *E. boulengeri* has been considered the ancestral karyotype for pleurodont Iguania. The genus *Enyalius* is characterized by two distinctive chromosomal groups; one with highly conserved karyotypes, whereas the other is karyotypically diverse. Our molecular cytogenetics data are promising and will increase knowledge about the genus *Enyalius* chromosome evolution.

Keywords: Ag-NOR banding; Cytogenetics; FISH; Lizards; rDNA 18S; Repetitive DNA probes.

INTRODUCTION

Cytogenetic studies on lizards of pleurodont Iguania infraorder suggest that there are two distinct trends on chromosome evolution in this taxon: some genera present chromosome variability, such as supernumerary chromosomes, sexual elements and large differences in chromosomal number and size (*e. g. Liolaemus*, *Norops*, and *Sceloporus*); on the other hand, many families show a conserved karyotype (Gorman & Atkins 1967; Pinna-Senn et al. 1987; Pellegrino et al. 1999; Bertolotto et al. 2002). Based on these results, the karyotype with $2n = 36$ chromosomes and distinction between macrochromosomes (M) and microchromosomes (mc) (12M + 24mc) has been proposed as the ancestral karyotype for Iguania (Paull et al. 1976). However, chromosome banding reveals that these conservative karyotypes present some polymorphisms, such as different positions of nucleolar organizing region (NOR) in some chromosome pairs, varying patterns of heterochromatin and different mc morphology (Bertolotto et al. 1996; Kasahara et al. 2004).

The advent of techniques banding heterochromatin regions in DNA is promising for the advance of the comprehension of the genome structure and evolution (Martins et al., 2011). Microsatellite regions apparently accumulate on regions with low levels of replication, such as telomeric and centromeric ones, and are easily detected by *Fluorescence in situ Hybridization* (FISH) techniques, as indicated in plants, anurans and fishes (Soares-Scott et al. 2005; Peixoto et al. 2015; Cunha et al. 2016). Although cytogenetic studies using molecular tools are still scarce on reptiles, they allow to understand relations between populations or/and species, and identify sexual elements at karyotypic components of species (Martins et al. 2011).

The genus *Enyalius* (Wied, 1821) is composed of 11 described species inhabiting forest areas in Amazônia, Cerrado and Atlantic forest biomes (Rodrigues et al. 2014; Costa & Bérnils 2018). Moreover, cryptic species are indicated to occur along the Atlantic forest (Rodrigues et al. 2014). Currently, eight species of the genus have been karyotyped (Bertolotto, 2006; Rodrigues et al., 2014), showing a certain degree of karyotypic variation. Some phylogenetic related species (clade A *sensu* Rodrigues et al. 2014) are proposed as bearers of the ancestral karyotype of Iguania. On the other hand, related species present variation in chromosome number and size and supernumerary elements (clade B *sensu* Rodrigues et al. 2014). However, two characters are highly conserved in *Enyalus*: the nucleolar organizing region is located on the chromosome pair number 2 and heterochromatic regions occur in the centromeric position, on M and on almost all mc (Bertolotto et al. 2002).

Phylogenetic relationships within this family are still unresolved, and studies employing banding techniques associated to molecular cytogenetics should reveal undetected synapomorphies. *Enyalius* is a widely distributed genus and a potential model for biogeographical analyses and chromosome evolution in Squamata. The study aims to characterize the karyotype of *Enyalius boulengeri*, with classical and molecular techniques and improve knowledge about the karyotype evolution of this genus.

MATERIAL AND METHODS

Specimens collection

Seven specimens of *Enyalius boulengeri* were analyzed: four specimens (two females - MZUFV 1358-1359- and two males – MZUFV 1353, 1362) from the APA Bom Jesus, Divino (20°35'52.85"S; 42°14'25.89"W) and three specimens (one female – MZUFV 1356 – and two males – MZUFV 1354-1355) from the Estação de Pesquisa, Treinamento e Educação Ambiental (EPTEA), Mata do Paraíso, Viçosa (20°48'0.40"S; 42°51'47.80"W), both in Minas Gerais State, Brazil. Proceedings were carried out according to the Animal Welfare Commission of the Universidade Federal de Viçosa and the current Brazilian laws (CONCEA 1153/95). All vouchers were deposited in the herpetological collection of the Museu de Zoologia João Moojen, at the Universidade Federal de Viçosa (MZUFV), Viçosa municipality, in Minas Gerais State, Brazil.

Conventional staining and molecular cytogenetic analyses

The specimens were fed 24 hours before being sacrificed. Each specimen was injected intraperitoneally with 0.1% solution of colchicine (0.1 ml per 10 g of body weight) 6 hours before euthanasia (carried out intraperitoneally with Hypnol solution 0.01 mL. mg^{-1}) to induce local anesthesia and pentobarbital (60 $mg.kg^{-1}$ – lethal dose). Mitotic chromosomes were obtained from gut epithelial cells, according to Schmid (1978) and stained using conventional protocols (5 % Giemsa diluted in Sorensen buffer). The best metaphases were photographed in digital Olympus BX53 light microscope with a DP73 Olympus camera, using the CellSens Dimensions® software system. Chromosome pairing and measurements were performed using Image Pro Plus® (IPP Version 4.5) to determine the modal value ($2n$) and the fundamental number (FN) for the species. Homologs were paired and grouped according to the centromere position, in decreasing size order, and classified in meta-

centrics (m), submetacentrics (sm), subtelocentrics (st) and telocentrics (t) (Green & Sessions 1991).

Active NORs in the preceding interphase were identified using silver nitrate precipitation (Ag-NORs) (Howell & Black 1980), whereas the heterochromatin-rich regions were detected using C-banding protocol (Sumner 1972). The FISH technique was performed according to Pinkel et al. (1986). The 18S rDNA probe was obtained from *E. boulengeri,* via polymerase chain reaction (PCR) with the following primers: 18Sf (5'-CCG CTT TGG TGA CTC TTG AT-3') and 18Sr (5'-CCG AGG ACC TCA CTA AAC CA-3') (Gross et al. 2010). The 18S probe was labeled by nick-translation with digoxigenina 11–dUTP, and the signal detection and amplification were performed using anti-digoxigenin-rhodamine (Roche Applied Science). The DNA repetitive probes were thynilated with cy3 at the 5' position (Sigma-Aldrich), using the following repetitive DNA probes: $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(GA)_{15}$, $(GC)_{15}$, $(TA)_{15}$, $(CAT)_{10}$, $(CAA)_{10}$, $(CAG)_{10}$, $(GAG)_{10}$, $(CGG)_{10}$, and the protocols followed Cioffi et al. (2011). FISH images were captured in a BX53 Olympus microscope with a XM10 camera. All procedures were carried out in the Laboratório de Sistemática Molecular BEAGLE, at the Universidade Federal de Viçosa, Viçosa municipality, Minas Gerais State, Brazil.

Cytogenetic tree

In order to comprehend the relationship between the phylogenetic hypothesis and cytogenetic data of the genus *Enyalius*, we overlapped our results on Rodrigues et al. (2014) hypothesis. The cytogenetic data were derived from the available data on literature (Gorman et al. 1967; Pellegrino et al. 1999; Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006; Rodrigues et al. 2014). The species tree was reconstructed in TNT 1.6 (Goloboff et al. 2008), and some rearrangements were made on Figtree software v1.3.1 (Rambaut 2009) and Illustrator v. CS3.

RESULTS

A karyotype with diploid number equal to $2n = 36$ chromosomes comprised of 12 macrochromosomes (M) and 24 microchromosomes (mc) (12M + 24mc) characterized the *E. boulengeri* populations (Figure 1). The M pairs 1, 3, 4, and 5 are metacentrics, and the pairs 2 and 6 are submetacentrics in all metaphases. The karyotype formula was 8m + 4sm + 24mc, with fundamental number (FN) equal to 24. A secondary constriction was observed in the distal end of the long arm of chromosome pair 2 (Figure 1). Heteromorphic sex chromosomes or supernumerary elements were not detected in any of these specimens.

The NORs were detected at the subtelomeric region of the long arm from both homologues on chromosome pair 2. NORs location corresponded to the conspicuous secondary constriction observed with Giemsa stain, and to the location of the 18S rDNA probe (Figure 1B). The C-banding results did not highlight heterochromatin regions from any chromosome pair. This result is probable related to the technique used in this study, once the presence of heterochromatin are reported to the other species of the genus (Bertolotto, 2006). Repetitive DNA probes $(GA)_{15}$ showed conspicuous accumulation on the pericentromeric region of chromosome pairs 1 and 6 and several mc (Figure 2A), whereas $(GC)_{15}$ probe marked the pericentromeric region of the first chromosome pair and a few mc (Figure 2B). Repetitive FISH patterns obtained with $(CAT)_{10}$ accumulated on the centromeric and terminal regions of some macrochromosomes and several microchromosomes (Figure 2C). The repetitive DNA probes $(A)_{30}$, $(C)_{30}$, $(CA)_{15}$, $(TA)_{15}$, $(CAA)_{10}$, $(CAG)_{10}$, $(CGG)_{10}$, and $(GAG)_{10}$ did not label any chromosome pair.

The two clades of *Enyalius* (Rodrigues et al. 2014) diverged on their cytogenetic patterns (Figure 3). The clade A (composed of five species with cytogenetic data available for three of them) presents the same chromosome formula (8m + 4sm + 24mc), and one species with cytogenetically differentiated sex chromosomes (*Enyalius perditus* 2). On the other hand, clade B (composed of seven species, six of them with cytogenetic data available), comprises species with high levels of caryological instability. The species possess different formulae (*i. e. E. pictus*: 8m + 4sm + 24mc and *E. bibronii*: 8m + 2sm + 2t + 24mc), as well as B chromosomes (*i. e. E. bilineatus*: 8m + 4sm+ 24mc + 1B/2B), sex chromosomes (*E. bilinetus* and *E. leechii*), besides some unusual telocentric chromosomes (*i. e. E. catenatus* 1: 6m + 2sm + 6t + 24mc and *E. erythroceneus*: 24t + 24mc).

DISCUSSION

Enyalius boulengeri showed a $2n = 36$ (12M + 24mc) karyotype that is ubiquitous among species of the genus and pleurodont Iguania (Gorman et al. 1967; Pellegrino et al. 1999; Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006; Rodrigues et al. 2013).

Furthermore, it was observed a similarity between the nucleolar organizing region (NOR) and the 18S rDNA labeling at the distal end of the long arm of both

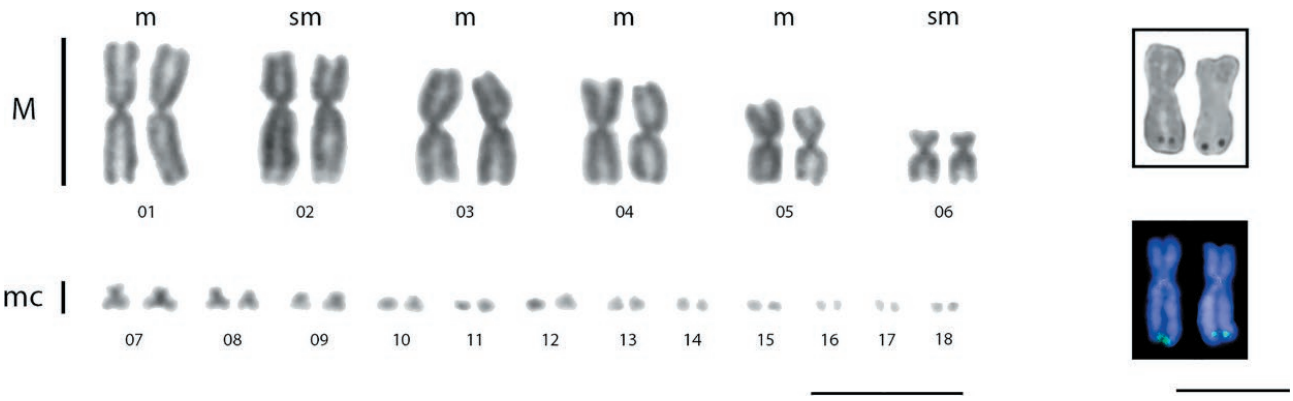

Figure 1. Giemsa-stained karyotypes of *Enyalius boulengeri*. Insets present chromosome pairs with Ag-NOR (above) and 18S rDNA (below) identified on chromosome pair number 2. Scale bars indicate 5 μm.

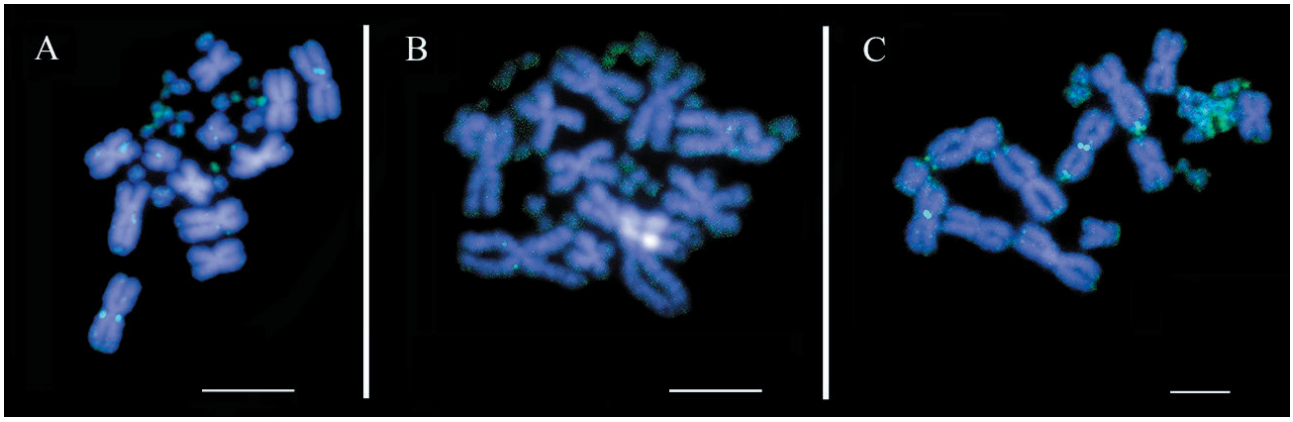

Figure 2. Mitotic chromosomes of *Enyalius boulengeri* labeled with the repetitive DNA probes: A: $(GA)_{15}$; B: $(GC)_{15}$; C: $(CAT)_{10}$. Scale bar indicated 10 μm.

homologues on chromosome pair 2. This same pattern (similarity between NOR and 18S rDNA probes and labeling in chromosome pair 2) is reported for all other species of the genus *Enyalius* and from Leiosauridae family (Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006). Here, the first *Enyalius* species was labelled with repetitive DNA probes, providing additional karyological data. This result will contributes to disentangling the phylogenetic relationships within the genus when similar studies are available to other species of the genus. The fundamental number of 24 is also shared with the other species from clade A of *Enyalius* (*E. perditus* and *E. iheringii*). The invariable number of mc (24 mc) in all species of the genus seems to be the rule in Squamata. Although clades A and B (Rodrigues et al. 2014) differ on macrochromosome constitution, mc are the same on all species of *Enyalius*. Thus, mc seem to be constituted by DNA sequences that represent a conserved part of the karyotypes of *Enyalius*. Patterns of repetitive DNA probes within this genus will be informative to test this hypothesis.

Two techniques corroborate that NORs are located on the subterminal region of the long arm of the second chromosome pair in *E. boulengeri*, with eight species of the genus presenting this same pattern (Bertolotto et al. 2002; Bertolotto 2006; Rodrigues et al. 2006). The conservation of chromosomal position of NORs also suggests the stability of this chromosome segment in Leiosauridae family (Bertolotto et al. 2002). The pattern of NOR banding should representing a phylogenetic sign for close related species. In addition, another pattern grabbing attention was the location of Ag-NOR and FISH 18S rDNA probe in the same chromosome region, which has been reported for several species from different families of clade Iguania (*i. e.* Agamidae (Patawang et al. 2015), Leiosauridae (Bertolotto et al. 2002), Liolaemidae (Bertolotto et al. 1996), Polychrotidae (Bertolotto et al. 2001), and Tropiduridae (Kasahara et al. 1987).

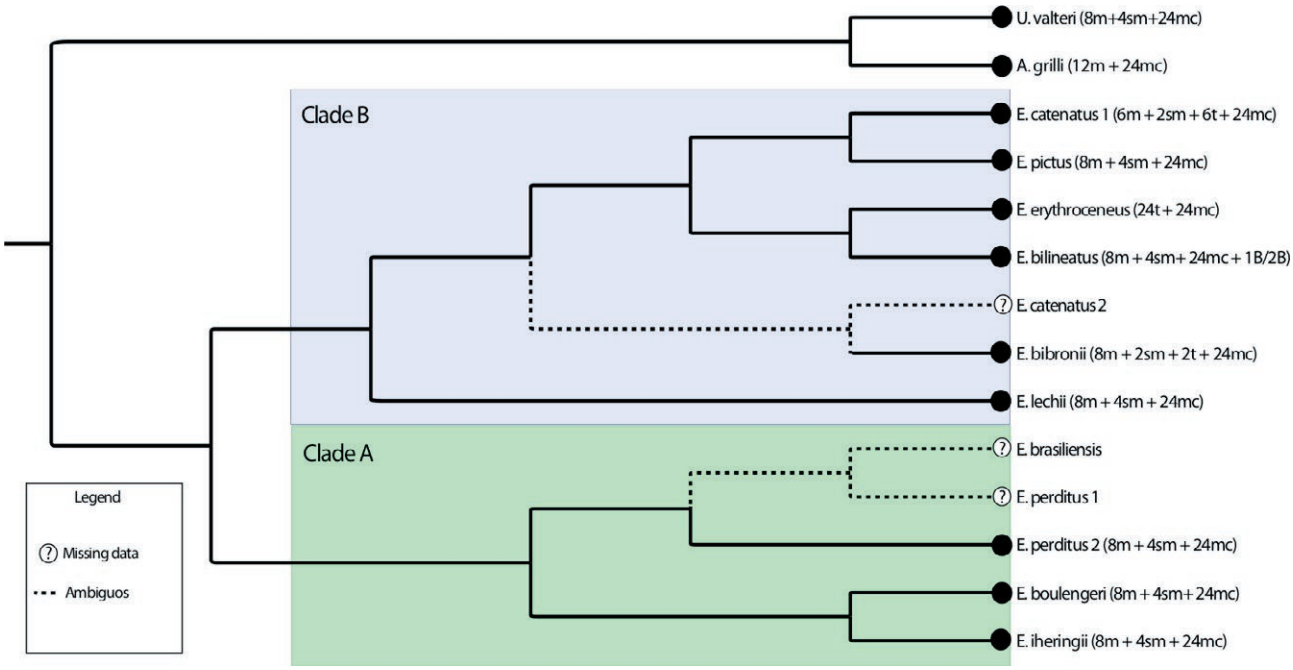

Figure 3. Compiled information about cytogenetic data in the genus *Enyalius* and some related species on a condensed phylogenetic hypothesis resulting from the analyses of Rodrigues et al. (2014).

E. boulengeri showed distribution of microsatellite repeats on pericentromeric and centromeric regions of the macrochromosome and many microchromossomes. No uneven accumulation of repetitive DNA probes was observed in the chromosome pairs, which corroborates the hypothesis this species have no system of sexual chromosomes. Studies using FISH to evaluate the genome distribution of microsatellite repeats on sex chromosomes was realized by Porkorna, 2011, this study showed certain microsatellite sequences are extensively accumulated over the whole length or parts of the W chromosome in *Eremias velox* (Pokorná et al. 2011). Giovannotti et al, (2018) isolated the repetitive element IMO-TaqI satDNA in several species of Lacertidae and found this element to be very abundant in the constitutive heterochromatin of the W-sex chromosome of the four Lacerta species investigated. On the other hand, repetitive probes also have evidenced chromosome stability among some species and populations of the *Scinax perpusillus* group and *Ololygon tripui* (Peixoto et al. 2015; Peixoto et al. 2016).

Our results highlighted that the chromosomal formula reported in *E. boulengeri* (8m + 4sm + 24mc) is shared with all species with described data in clade A. This pattern are possible result of the phylogenetic relation among the species that occurs in warmer climates in Southeastern and northeastern Brazil and once they belonged to the same clade inside the *Enyalius* genus. This character has been considered an ancestral karyotype for pleurodont Iguania, which includes the Leiosauridae family (Paull et al. 1976; Bertolotto et al. 2002) and might also be present in the two remaining species with unknown karyotypes (*E. brasiliensis* and *E. perditus* 1). Clade B members inhabit the cooler climates in Southeastern and Southern Brazil (Rodrigues et al. 2014), and presents a second distinct trend on chromosome evolution in Iguania, showing considerable karyotype variability. This trend is exemplified by *Anolis*, *Norops*, *Ctenonotus* also with relation to sex chromosomes (Castiglia et al. 2013; Giovannotti et al. 2017; Lisachov et al. 2019; Kichigin et al. 2019). For instance, some species present supernumerary chromosomes, different chromosomal formulae, besides some unusual telocentric chromosomes. In *E. erythroceneus* (24t + 24mc), the number of telocentric chromosomes seems to derive from fission events of chromosomes, assuming that the ancestral karyotype is 8m + 4sm. The karyotypes of two species in Clade B may also indicate fission events: *Enyalius catenatus* and *E. bibroni* are evidenced by the presence of telomeric elements (6m + 2sm + 6t + 24mc and 8m + 2sm + 2t + 24mc, respectively).

CONCLUSION

Our results support classical cytogenetics (diploid number, FN and NOR number and location) as an efficient tool to characterize lizard species in a family level, which corroborates the position of *E. boulengeri* within its genus. Repetitive DNA probes complement this conservative pattern and, if apply to other species of the genus, may allow to detect synapomorphies so as to improve knowledge about chromosome evolution and phylogenetic relationship within this genus.

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