CompCytogen 14(1): 61–74 (2020)
doi: 10.3897/CompCytogen.v14i1.46852
http://compcytogen.pensoft.net

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative cytogenetics of the ground frogs *Eupsophus emiliopugini* Formas, 1989 and *E. vertebralis* Grandison, 1961 (Alsodidae) with comments on their inter- and intraspecific chromosome differentiation

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Academic editor: *N. Golub* | Received 27 September 2019 | Accepted 10 December 2019 | Published 27 January 2020

http://zoobank.org/EB3B23D9-CD35-49F6-A518-0848CC9E8B78

Citation: Quercia CA, Suárez-Villota EY, Foresti F, Nuñez JJ (2020) Comparative cytogenetics of the ground frogs *Eupsophus emiliopugini* Formas, 1989 and *E. vertebralis* Grandison, 1961 (Alsodidae) with comments on their inter- and intraspecific chromosome differentiation. Comparative Cytogenetics 14(1): 61–74. https://doi.org/10.3897/CompCytogen.v14i1.46852

Abstract

South American frogs of the genus *Eupsophus* Fitzinger, 1843 comprise 10 species. Two of them, *Eupsophus vertebralis* Grandison, 1961 and *E. emiliopugini* Formas, 1989 belong to the *Eupsophus vertebralis* group, exhibiting 2n = 28. Fundamental number differences between these species have been described using conventional chromosome staining of few specimens from only two localities. Here, classical techniques (Giemsa, C-banding, CMA_3/DAPI banding, and Ag-NOR staining), and fluorescence *in situ* hybridization (FISH, with telomeric and 28S ribosomal probes), were applied on individuals of both species collected from 15 localities. We corroborate differences in fundamental numbers (FN) between *E. vertebralis* and *E. emiliopugini* through Giemsa staining and C-banding (FN = 54 and 56, respectively). No interstitial fluorescent signals, but clearly stained telomeric regions were detected by FISH using telomeric probe over spreads from both species. FISH with 28S rDNA probes and Ag-NOR staining confirmed the active nucleolus organizer regions signal on pair 5 for both species. Nevertheless, one *E. emiliopugini* individual from the Puyehue locality exhibited 28S ribosomal signals on pairs 4 and 5. Interestingly, only one chromosome of each pair showed Ag-NOR positive signals, showing a nucleolar dominance pattern. Chromosomal rearrangements, rRNA gene dosage control, mobile NORs elements, and/or species hybridization process could be involved in this interpopulation chromosomal variation.

Keywords
Karyotype variations, FISH, Patagonian frogs, ribosomal probe, NOR polymorphism

Introduction

Eupsophus Fitzinger, 1843 is a South American genus of frogs that currently comprises 10 species (Frost 2019, Suárez-Villota et al. 2018a), endemic from the temperate *Nothofagus* forests from Chile and Argentina (Formas 1978, Ibarra-Vidal et al. 2004). Based on ethologic (advertisement calls; Formas and Brieva 1994), morphometrics (Nuñez 2003), molecular (allozymes and DNA sequences; Formas et al. 1992, Blotto et al. 2013), and cytogenetic (Formas 1991, Veloso et al. 2005) analyses, this genus is divided into the *Eupsophus roseus* and the *Eupsophus vertebralis* groups.

The *E. roseus* group is composed of eight species: *E. calcaratus* (Günther, 1881), *E. contulmoensis* Ortiz, Ibarra-Vidal & Formas, 1989, *E. septentrionalis* Ibarra-Vidal, Ortiz, & Torres-Pérez, 2004, *E. nahuelbutensis* Ortiz & Ibarra-Vidal, 1992, *E. insularis* (Philippi, 1902), *E. migueli* Formas, 1978, *E. roseus* (Duméril & Bibron, 1841), and *E. altor* Nuñez, Rabanal & Formas, 2012 (Suárez-Villota et al. 2018a) exhibiting the same diploid number 2n = 30 with some species specific characteristics (*e.g.* fundamental number, sex chromosomes, secondary constriction location; Iturra and Veloso 1986, Veloso et al. 2005, Nuñez et al. 2012). On the other hand, the *E. vertebralis* group, composed of *E. vertebralis* Grandison, 1961 and *E. emiliopugini* Formas, 1989, exhibit 2n = 28, do not have sex chromosomes, and present a secondary constriction in pair 5 (Formas 1991). Moreover, the pair 13 is metacentric in *E. emiliopugini* and telocentric in *E. vertebralis*, differing in their fundamental number (FN = 56 and FN = 54, respectively).

Having in mind the hypothetical ancestrality of telocentric chromosomes in amphibians (Morescalchi 1980), Formas (1991) proposed two alternative hypotheses to explain the origin of the differences on the pair 13 in the *E. vertebralis* group. The first one is a pericentric inversion in a telocentric pair of *E. vertebralis*, which shifted the centromere to the metacentric position in *E. emiliopugini*. The second hypothesis is the addition of heterochromatic segments in the centromeric region of the telocentric pair in *E. vertebralis*, which leads to a metacentric pair in *E. emiliopugini*. Formas (1991) considered the first alternative as a reasonable hypothesis because telocentric and metacentric pairs 13 are the same size.

Although the hypothesis of Formas (1991) is well argued from the data, it should be considered with caution since the conclusions are obtained using only conventional stain and specimens from only two locations, preventing the findings from being extrapolated, and increasing the chance of assuming as true a false premise. Here we combined classical and molecular cytogenetic techniques to characterize the karyotypes of these species using samples from several localities. Thus, we analyzed at population level the nucleolus organizer regions (NORs) position using Ag-NOR banding and

fluorescent *in situ* hybridization (FISH) with 28S rDNA probe. Using FISH with telomeric probe and CMA_3/DAPI banding, we sought interstitial signals, which could suggest chromosomal rearrangements in both species. Our comparative cytogenetic analyses provide a detailed description of the *E. vertebralis* group karyotypes and their inter- and intraspecific chromosome differentiation.

Methods

Sample collection and cytological preparations

Cytological preparations were obtained from 14 and nine individuals of *Eupsophus vertebralis* and *E. emiliopugini*, respectively (See Suppl. material 1: Table S1). These individuals were collected according to permit of Servicio Agrícola y Ganadero (No. 9244/2015) from 15 locations in Southern Chile (Fig. 1). Mitotic plates were obtained from intestine cell suspension. For this purpose, we injected 30 µl/g of 0.1% colchicine (Sigma-Aldrich) into the abdominal cavity of each individual. After 12 hours, the individuals were euthanized with oversaturated benzocaine, according to the recommendations of the Bioethics and Biosecurity Committee of the Universidad Austral de Chile (UACh, resolution No. 236/2015 and 61/15). Immediately after euthanasia, the gut cells were extracted and prepared according to Schmid et al. (1978) protocol. Then, the specimens were included in the herpetological collection of Instituto de Ciencias Marinas y Limnológicas, UACh (voucher numbers in Suppl. material 1: Table S1).

Classical cytogenetic techniques

Mitotic plates were stained with 10% Giemsa for karyotype determination. Active NORs were detected using silver nitrate staining (Ag-NOR) according to (Howell and Black 1980). This chromosomal material was analyzed in Siedentopf trinocular microscope (AmScope T340B-DK-LED) and photographed with AmScope camera using IS capture software. Karyotypes were arranged according to Formas (1991).

To identify constitutive heterochromatic regions, we carried out a C-banding protocol using formamide for DNA denaturation, according to Fernández et al. (2002) and staining with DAPI (1 µg/ml). CG-rich and AT-rich regions were detected using CMA_3/DAPI stains, respectively follow to Schweizer (1976). In this technique, we used pretreated metaphases with formamide according to Pieczarka et al. (2006) as well as FISH pretreated plates (Suárez et al. 2013). For both C-banding and CMA3/DAPI stains, mitotic plates were mounted with Vectashield antifade. Subsequently, metaphases were visualized through a BX61 Olympus microscope, and captured with adequate filter using a DP70 Olympus digital camera with PRO MC Image software. All images were overlaid and contrast enhanced using Adobe Photoshop CS6.

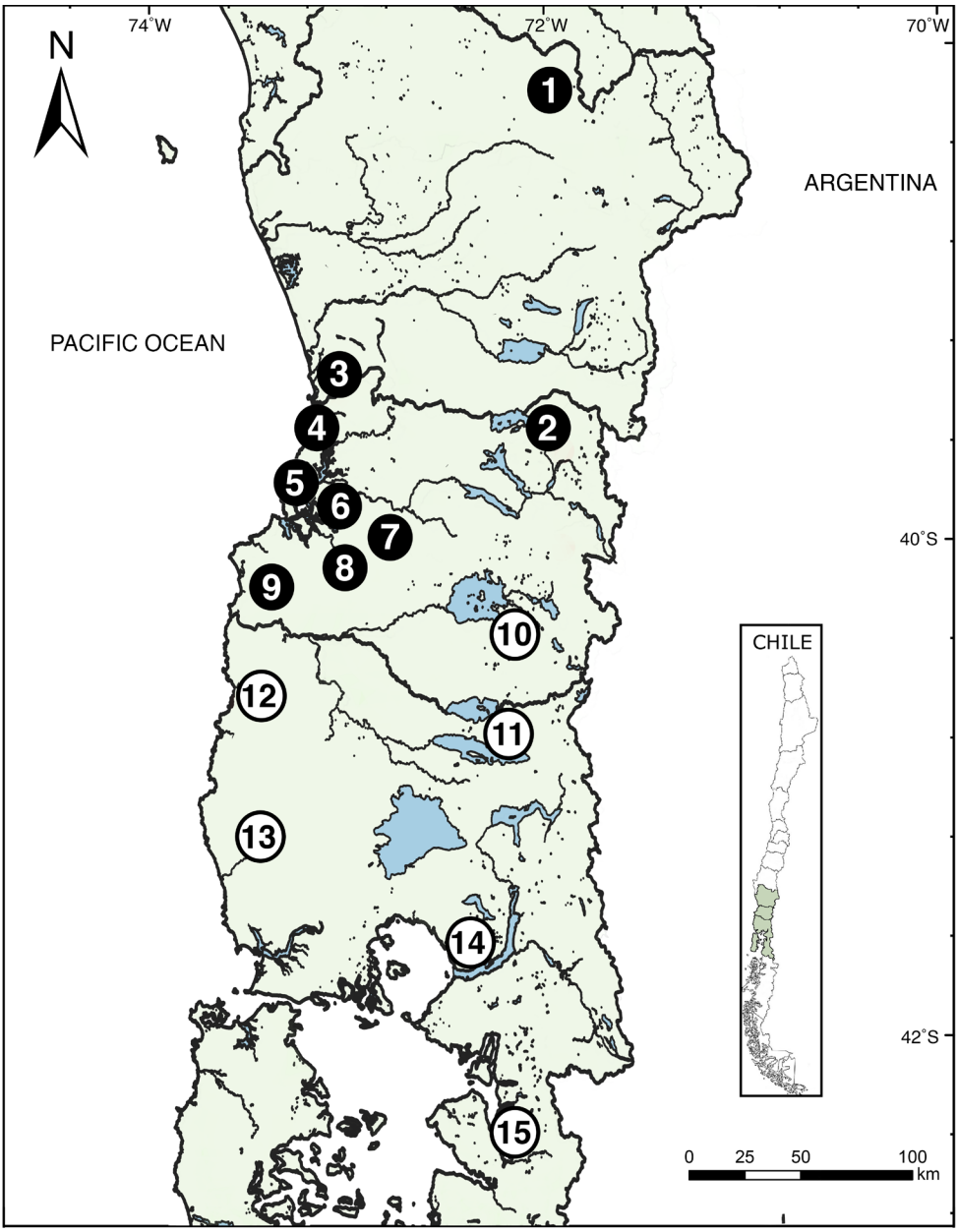

Figure 1. Map depicting 15 collection localities of the *Eupsophus vertebralis* group specimens in Southern Chile. *E. vertebralis* locations are represented by black circles, and *E. emiliopugini* locations are shown with white circles. The numbers inside the circles corresponds with the follow localities: **1**) Tolhuaca, **2**) Lago Pellaifa, **3**) Colegual Alto, **4**) Chanchan, **5**) Oncol, **6**) Llancahue, **7**) Reumén, **8**) Chamil, **9**) Cordillera Pelada, **10**) Los Mañios, **11**) Puyehue, **12**) Pucatrihue, **13**) Cordillera del Sarao, **14**) Parque Alerce Andino, and **15**) Huinay.

Molecular cytogenetic techniques

The physical map of the rDNA genes was detected by FISH on mitotic plates from *E. vertebralis* (from Colegual Alto and Reumén), and *E. emiliopugini* (from Puyehue, Cordillera del Sarao, and Parque Alerce Andino) specimens. For this purpose, 28S rDNA fragment from *E. vertebralis* DNA was amplified using 28SV (5′-AAGGTAGCCAAATGCCTCGTCATC-3′) and 28SJJ (5′-AGTAGGGTAAAACTAACCT-3′) primers (Hillis and Dixon 1991). PCR was carried out according to the manufacturer's instructions for *Taq* Platinum DNA Polymerase (Cat. No. 10966, Invitrogen), at 55 °C of annealing temperature. The 28S probe was PCR-labeled with 11-digoxigenin dUTP (Cat. No. 11093088910, Sigma-Aldrich), hybridized according to Ferreira et al. (2011), and detected with Anti-Digoxigenin-Rhodamine, Fab fragments (Cat. No. 11207750910, Roche).

Telomere detection by FISH was carried out on metaphase chromosomes from *E. vertebralis* (from Tolhuaca, Reumén, and Colegual Alto), and *E. emiliopugini* (from Puyehue, Parque Alerce Andino, and Cordillera del Sarao) specimens. Universal telomeric probes $(TTAGGG)_n$ were PCR-generated and labeled with fluorescein-12-dUTP (Cat. No 11373242910, Roche) (Ijdo et al. 1991). Fluorescent *in situ* hybridization followed to Ferreira et al. (2011) without final immunodetection protocol.

Slide mounting and image capture for both 28S rDNA and telomeric FISH assays were carried out as described previously for C-banding protocol.

Results

Classical cytogenetic techniques

We analyzed 88 mitotic plates showing 2n = 28 for each species, without evidence of sexual chromosomes (Fig. 2). All the *E. emiliopugini* plates showed only chromosomes bi-armed with a FN = 56. The pairs 1, 3, 8–14 were metacentric, pair 7 was submetacentric, and pairs 2, 4–6 were subtelocentric (Fig. 2, top) following the descriptions by Formas (1991).

Mitotic plates of *E. vertebralis* exhibited a telocentric pair 13 presenting FN = 54, while the other karyotypic features were similar to *E. emiliopugini* (Fig. 2, middle). Although it is not clear for all plates, secondary constriction was observed in the short arms of pair 5 from both species (Fig. 2, top and middle, black arrows). In one *E. emiliopugini* specimen collected at Puyehue (hereafter, the Puyehue specimen) was difficult to establish morphological homology among chromosomes of pairs 5 and 4 (Fig. 2, bottom, black arrows).

C-banding and DAPI staining detected predominantly centromeric regions in chromosomes of *E. emiliopugini* and *E. vertebralis* (Fig. 3a, top and middle, respectively). Interstitial heterochromatic signals were detected on the long arms of chromosomes of pair 5 (Fig. 3a, white arrows). When applying C-banding over mitotic plates from Puyehue specimen, secondary constrictions were detected in one chromosome of the pair 4, and in one chromosome of the pair 5 (Fig. 3a, bottom, red arrows). This final arrangement

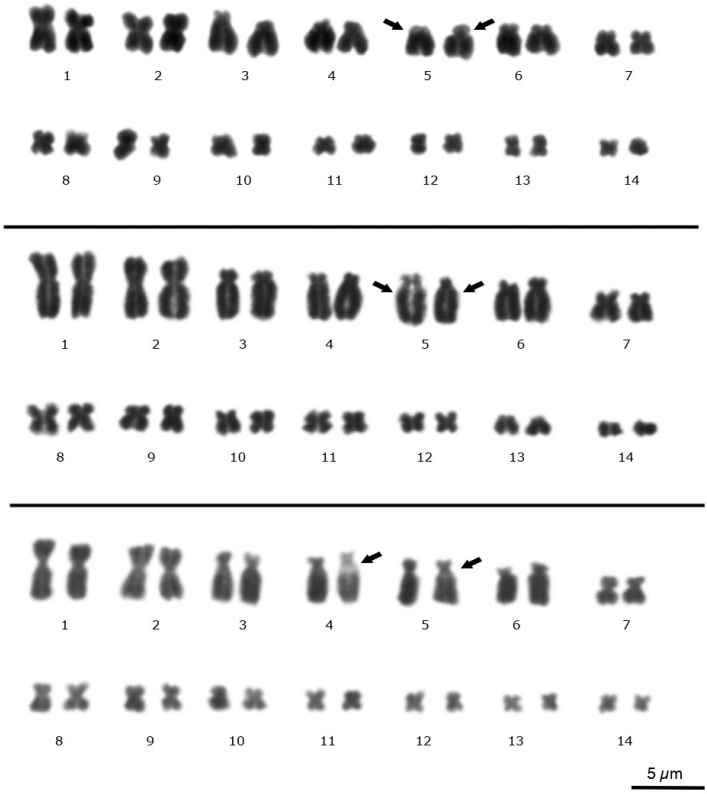

Figure 2. Conventional Giemsa banding on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). Note metacentric (top and bottom) or telocentric (middle) pair 13. Secondary constrictions are indicated with black arrows on pairs 4 or 5 (see text for details).

among chromosomes of pairs 4 and 5 was based on Ag-NOR technique as described below. CMA_3 positive signals were detected on pair 5 of both karyotypes (Fig. 3b, top and middle, white arrows), but in that of the Puyehue specimen, these signals were detected in both chromosomes on pairs 4 and 5 (Fig. 3b, bottom, white arrows).

Ag-NOR staining detected active NORs on short arms of chromosomes of pair 5 in both *E. emiliopugini* and *E. vertebralis* karyotypes (Fig. 4a, top and middle, respectively). This technique detected active NORs, corresponding to secondary constriction, on long arm from one chromosome of the pair 4, and on short arm from one chromosome of pair 5 (Fig. 4a, bottom) in the Puyehue specimen.

Molecular cytogenetic techniques

Coincident with Ag-NOR staining results, signals on short arms of chromosomes of pair 5 in both *E. emiliopugini* and *E. vertebralis*, were detected through FISH using 28S rDNA

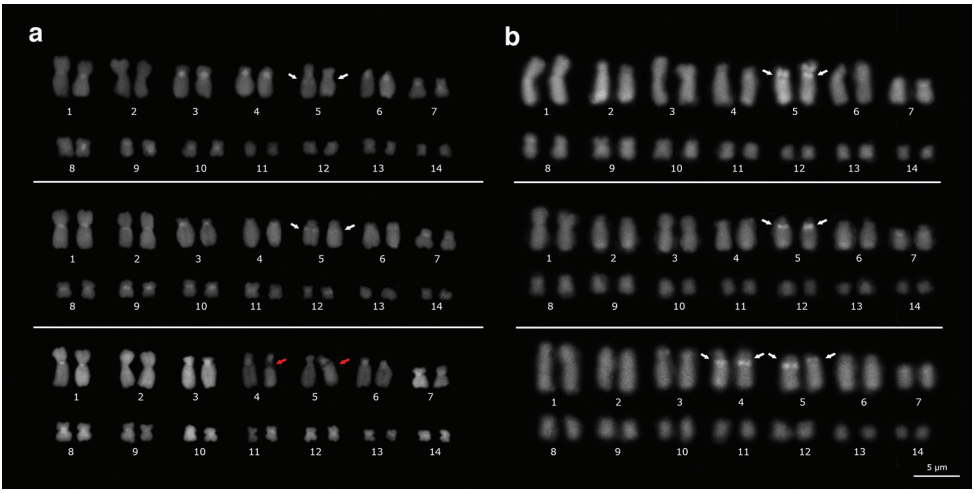

Figure 3. DAPI staining (**a**) and CMA_3 (**b**) on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). White arrows indicated heterochromatic interstitial bands in **(a)** and CMA_3 positive signals in (**b**). Red arrows indicated secondary constriction in *E. emiliopugini* Puyehue specimen.

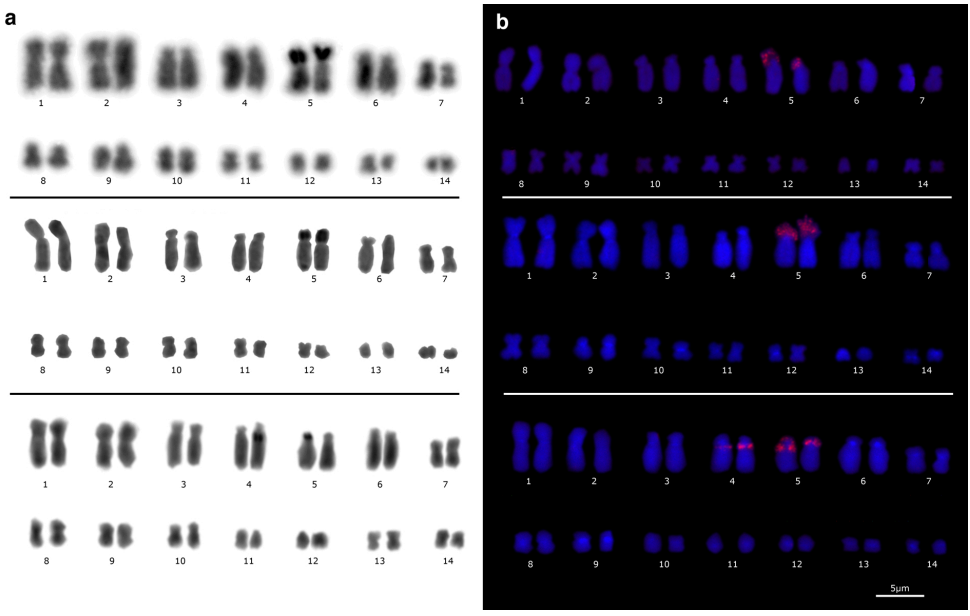

Figure 4. Ag-NOR staining (**a**), and FISH using 28S rDNA probe (**b**) on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). Note colocalization of AgNOR and FISH signals on pair 5 (top and middle). FISH signals on four chromosomes, two of them AgNOR stained are observed in *E. emiliopugini* from Puyehue (bottom, see text for details).

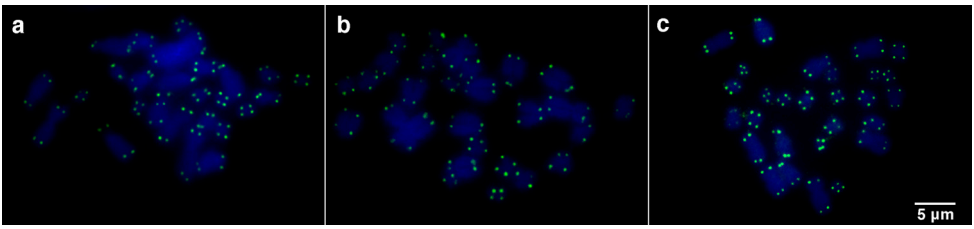

Figure 5. Fluorescent *in situ* hybridization over mitotic plates from the *Eupsophus vertebralis* group, using the telomeric probe. *Eupsophus emiliopugini* (**a**), *E. vertebralis* (**b**), and *E. emiliopugini* from Puyehue locality (**c**) mitotic plates are shown. Note the absence of interstitial signals in all chromosomes.

probe (Fig. 4b, top and middle, respectively). In the Puyehue specimen, this probe detected a long arm region of chromosomes in pair 4 and short arm regions of chromosomes in pair 5 (Fig. 4b, bottom).

Telomeric, but no centromeric or interstitial signals were detected on all chromosomes in both species through FISH using universal telomeric probe (Fig. 5a, b, respectively). This pattern was also observed on mitotic plates from the Puyehue specimen (Fig. 5c).

Discussion

Karyotypic patterns of *E. emiliopugini* and *E. vertebralis*

We present the first comparative cytogenetic study using classical and molecular cytogenetic techniques among specimens from different localities of *E. emiliopugini* and *E. vertebralis*. According with previous works (Formas 1989, 1991), *E. emiliopugini* and *E. vertebralis* exhibit 2n = 28, and FN = 56 and 54, respectively, derived of polymorphisms in pair 13 (Fig. 2). We did not detect sex chromosomes in the *E. vertebralis* group as it was observed by Formas (1991) (Fig. 2). Since, the lineage that gave origin to *E. vertebralis* and *E. emiliopugini* diverged early in the evolutionary history of *Eupsophus* (Suárez-Villota et al. 2018a), and sex chromosomes are detected in some species of the *E. roseus* group (*E. roseus*, *E. migueli*, *E. insularis*, and *E. septentrionalis*; Iturra and Veloso 1986, Cuevas and Formas 1996, Veloso et al. 2005), we agree with the notion that sex chromosomes correspond to an apomorphic condition in *Eupsophus* (Iturra and Veloso 1986, King 1991, Cuevas and Formas 1996, Veloso et al. 2005).

C-banding has been largely used in amphibians to compare karyotypes and to distinguish species with the same diploid number (Bogart 1970, Cuevas and Formas 2003, Nogueira et al. 2015, Sangpakdee et al. 2017, Targueta et al. 2018). Moreover, homogeneous C-banding patterns among related species has been associated with low genetic differentiation (Pellegrino et al. 1997, Lourenço et al. 1998, Bruschi et al. 2012) and enrichment of repetitive elements, characteristic of amphibian chromosomes (Schmid et al. 1978, Bruschi et al. 2012, Zlotina et al. 2017). Therefore, the absence of interspecific variations in heterochromatin banding reported in this

study (Fig. 3), could be associated with the recent and low differentiation between *E. vertebralis* and *E. emiliopugini* as has been reported in divergence times estimates and mitogenomic analyses (Suárez-Villota et al. 2018a, b).

Nucleolus organizer regions (NORs)

Ag-NOR banding combined with FISH using rDNA probes allow us to characterize the NORs in *E. emiliopugini* and *E. vertebralis* (Fig. 4). NORs locus correspond to rDNA coding for 18S rRNA, 5.8S rRNA and, 28S rRNA (Preuss and Pikaard 2007, McStay 2016). Thus, while Ag-NOR staining detects active NORs, FISH checks the total number of loci rDNA (Zaleśna et al. 2017). For both species of the *E. vertebralis* group, excluding the Puyehue specimen, we detected Ag-NOR signals on the short arms of pair 5 (Fig. 4a, top and middle), colocalized with the secondary constriction, and with 28S rDNA FISH signal (Fig. 4b, top and middle, red signal). Therefore, rDNA locus was transcriptionally active in both homologues of pair 5 for *E. emiliopugini* and *E. vertebralis*. Thus, it was not possible to determine a species-specific pattern relative to numbers and locations of NORs between both species. Consequently, NORs polymorphism is not a well indicative of species differentiation in this group as occur in some species of *Alsodes* Bell, 1843 [*A. pehuenche* Cei, 1976, *A. vanzolinii* (Donoso-Barros, 1974) and *A. verrucosus* (Philippi, 1902); Cuevas and Formas 2003]. However, different situation occurs in some species of the *E. roseus* group. For example, *E. contulmoensis* and *E. migueli* show specific Ag-NOR banding patterns (Veloso et al. 2005).

Intraspecific polymorphism in NORs was detected in the Puyehue specimen (Fig. 4a, b, bottom). We observed CMA_3 positive banding and 28S FISH signals on pairs 4 and 5 (four NOR loci, Figs 3b, 4b, bottom), of which only one chromosome of each pair showed secondary constriction (Fig. 2, bottom, black arrows) and Ag-NOR positive signal (Fig. 4a, bottom). The absence of secondary constriction in one chromosome from one pair is a cytologic phenomenon known as differential amphiplasty (Navashin 1928, Pikaard 2000). This phenomenon could be a manifestation of rRNA gene dosage control, regulating the number of active rRNA genes according to the cellular demand, or an epigenetic phenomenon from interspecific hybrids where the expression of rRNA genes inherited from one progenitor are silenced (Pikaard 2000, Tucker et al. 2010). Thus, the four rRNA loci with nucleolar dominance detected in Puyehue specimen could be related with chromosomal rearrangements (Schweizer and Loidl 1987), mobiles NORs (Schmid et al. 2017) or hybrid origin (Pereyra et al. 2009), as it has been also associated to polymorphic NORs in other species.

Hypothesis about the evolution of pair 13

C-banding and CMA_3/DAPI stains results did not show a heterochromatic region in the short arms of metacentric pair 13 of *E. emiliopugini* (Fig. 3a, b, top). Moreover, telomeric

probe hybridized over *E. emiliopugini* and *E. vertebralis* mitotic plates detected telomeric/subtelomeric signals but not interstitial signals (Fig. 5). Therefore, our data did not support the addition of heterochromatic segments in the telocentric pair of *E. vertebralis* and not show insights of inversions in the pair 13 of the *E. vertebralis* group. Since, these phenomena could be expected under hypothesis to explain the differentiation of pair 13 in this group (Formas 1991), we cannot refuse the proposed explanations. In this regard, telomeric sequences at telomeric/subtelomeric region are conserved in vertebrates (Meyne et al. 1989) whereas interstitial telomeric sequences could result from chromosomal rearrangements in animals (Ruiz-Herrera et al. 2002, Vitturi et al. 2002, Castiglia et al. 2006). Therefore, the pericentric inversion proposed by Formas (1991) to explain the differences in pair 13 between *E. emiliopugini* and *E. vertebralis* could be unlikely or it did not include the telomeric regions. Additionally, interstitial telomeric sequences could also be lost, as it has been reported in mammals (Rogatcheva et al. 2002, Castiglia et al. 2006). Thus, we cannot falsify the inversion hypothesis in pair 13 of the *E. vertebralis* group.

In conclusion, our analyses corroborate species-specific cytogenetic pattern differences between *E. emiliopugini* and *E. vertebralis* by detecting metacentric or telocentric pair 13 in populations of these species, respectively. Although, our results do not allow rejecting hypotheses of chromosomal rearrangements or heterochromatin addition in the origin of chromosomes of pair 13, a euchromatic pattern without interstitial telomeric sequences characterized these chromosomes. We reported an intraspecific polymorphism related to number, location, and activation of NORs for one specimen of *E. emiliopugini* from Puyehue locality. Chromosome rearrangements, hybridization event and transposition could be involved in the origin of this polymorphism. Future studies using probes from chromosome 13, more samples of *E. emiliopugini* from Puyehue locality, and molecular sequences analyses will allow a better understanding of the chromosomal evolution in the *E. vertebralis* group.

Acknowledgments

We are grateful to Dr. Cristian Araya-Jaime and Dr. Duílio M. Z. de A. Silva for his laboratory assistance. We thank to the reviewers Dr. Vladimir Kryloy and Dr. Pablo Suárez and to the editor Natalia Golub, whose comments significantly improve the manuscript. We appreciate the great fieldwork assistance of Engr. Nicolás González. Fondecyt 3160328 to EYS-V. and CONICYT grant 22180766 to CAQ funded this research.

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Supplementary material 1

Table S1. *Eupsophus* specimens analyzed in the present study

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Data type: speciemens data

Explanation note: Map number (Fig. 1), species, localities, coordinates, numbers of samples, and vouchers from herpetological collection of Instituto de Ciencias Marinas y Limnológicas (UACh), are shown.

Link: https://doi.org/10.3897/CompCytogen.v14i1.46852.suppl1