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Cytogenetics of four foam-nesting frog species of the *Physalaemus gracilis* group (Anura, Leptodactylidae)

JUAN M. FERRO, ALBERTO TAFFAREL, CRISTIAN TOMATIS, CLAUDIO BORTEIRO, FRANCISCO KOLENC, KALEB P. GATTO, LUCIANA B. LOURENÇO & DIEGO BALDO

Abstract: Intending to increase the knowledge about cytogenetics of *Physalaemus* and the sparsely studied *P. gracilis* group, we analyzed the karyotypes of *P. carrizorum*, *P. gracilis*, *P. lisei*, and *P.* sp. aff. *gracilis*. We studied chromosome morphology, heterochromatin patterns, Ag-NORs location and mapped the repetitive DNA sequence PcP190. All species showed diploid karyotypes composed of 22 bi-armed chromosomes and similar C- bands and Ag-NOR patterns. C-bands were mainly centromeric and pericentromeric; non-centromeric C-bands were detected on the telomeres of pair 1 in *P. lisei*, although polymorphic, and interstitially on pair 10 of *P. gracilis*. This last character is useful to distinguish *P. gracilis* from its sibling species *P.* sp. aff. *gracilis*. The Ag-NOR sites were detected on the long arms of chromosome pair 8 but with a variable position among species. Clusters of PcP190 showed centromeric and pericentromeric positions coincident with conspicuous C-bands, on pairs 2 and 9 in *P. gracilis* and *P.* sp. aff. *gracilis*, pair 3 in *P. carrizorum*, and pair 7 in *P. lisei*. These results significantly increase the knowledge about *Physalaemus* cytogenetics and encourage further studies on the satellite PcP190 in other genera of Leiuperinae to better understand its taxonomic distribution and the evolutionary dynamics.

Key words: Leiuperinae, Ag-NORs, C-Bands, satDNA, PcP190.

INTRODUCTION

The foam-nesting frog genus *Physalaemus* is a monophyletic group of Neotropical leptodactylids belonging to the sub-family Leiuperinae that inhabits several South American ecoregions, from the southern part of the Guianas and Venezuela to central Argentina, including a large portion of Brazil, southeastern Colombia, eastern Bolivia, Paraguay and Uruguay (Frost 2019). Current knowledge about the phylogenetic relationships of these frogs arranges them into two major clades. The *P. signifer* clade comprises the *P. deimaticus* and *P. signifer* species groups, plus *P. maculiventris* and *P. nattereri*. Additionally, the *P. cuvieri* clade consists of five species groups: *P. biligonigerus*, *P. cuvieri*, *P. henselii*, *P. olfersii*, and *P. gracilis*, and the species *P. cicada* unassigned to any group (Lourenço et al. 2015).

The cytogenetics of *Physalaemus* has provided valuable information to elucidate the taxonomic relations and chromosome evolution of these frogs. Only 27 of the 48 currently recognized species have been karyotyped (Quinderé et al. 2009, Tomatis et al. 2009, Vittorazzi et al. 2014a, 2016, Lourenço et al. 2015 and references therein). As a rule, *Physalaemus* species have diploid karyotypes with 2n = 22, although two different fundamental numbers (FN) can be observed (42 and 44). The

karyotypes with FN = 42 have a small telocentric chromosome and are found in all species of the *P. signifer* clade and *P. fernandezae*, whereas a FN = 44 is present in all the remaining species of the *P. cuvieri* clade.

Characteristic of many *Physalaemus* species is the location of the Nucleolar Organizer Regions (NORs) on small-sized chromosomes (i.e., pairs 8 to 11). However, the homology of this character among species should be interpreted cautiously, since it showed broad intraspecific variability (Ananias et al. 2007a, Quinderé et al. 2009, Vittorazzi et al. 2014a, Nascimento et al. 2019) or could be homoplastic (Lourenço et al. 2015). Within the *P. cuvieri* clade, most species show NORs on pair 8, which is a plesiomorphic condition of the genus (Lourenço et al. 2015). Similarly, C-bands are other interesting characters that may be phylogenetically informative. For instance, all karyotyped species of the *P. cuvieri* group share an interstitial C-band on the metacentric pair 5, and almost all species of the *P. biligonigerus* group share a pericentromeric C- band on the short arm of pair 3 (Vittorazzi et al. 2014a, and references therein). Besides, the satellite DNA (satDNA) sequence PcP190 that was mapped in several species of *Engystomops*, *Physalaemus*, and *Pseudis* is a promissory marker for the establishment of chromosomal homologies and its variation can reveal phylogenetically informative patterns in anurans (Vittorazzi et al. 2011, 2014b, 2016, Targueta et al. 2018, Gatto et al. 2016, 2018, 2019).

The *Physalaemus gracilis* group is composed of six species (*P. barrioi*, *P. carrizorum*, *P. evangelistai*, *P. gracilis*, *P. jordanensis*, and *P. lisei*) that inhabit southeastern Brazil, northeastern Argentina, and Uruguay (Nascimento et al. 2005, Lourenço et al. 2015, Cardozo & Pereyra 2018). Cytogenetic data for this group is scarce and restricted to *P. barrioi* (Provete et al. 2012) and *P. gracilis* (Brum-Zorrilla & Sáez 1968, de Lucca et al. 1974), which have karyotypes of 22 bi-armed chromosomes (FN = 44). In *P. barrioi*, there is additional information regarding the position of NORs terminal on pair 10 and C-banding pattern that is mostly centromeric and pericentromeric, except for an interstitial band present on the long arm of pair 4 (Provete et al. 2012).

In this paper, we cytogenetically studied four species of the *Physalaemus gracilis* group: *P. carrizorum*, *P. gracilis*, *P. lisei*, and a different species from Uruguay referred herein as *P.* sp. aff. *gracilis*. We provide information concerning Ag-NORs, C-banding patterns, and the chromosomal location of the repetitive sequence PcP190, and discuss our findings under the view of current taxonomy and phylogenetic hypotheses.

MATERIALS AND METHODS

We studied 13 specimens of *Physalaemus carrizorum*, 4 of *P. gracilis,* 7 of *P.* sp. aff. *gracilis*, and 7 of *P. lisei*. Mitotic metaphases were obtained from intestines and bone marrow tissue following Schmid et al. (2010). The chromosome number and morphology were studied on preparations stained with buffered Giemsa solution (10%). C-bands and Ag-NOR were obtained according to Sumner (1972) and Howell & Black (1980), respectively. The satDNA sequence PcP190 (Vittorazzi et al. 2011) was mapped to mitotic preparations by *in situ* hybridizations after Pinkel et al. (1986). Probes consisted of PcP190 sequences amplified from a cloned type 1-PcP190 fragment (KX170909) using a PCR dig Probe Synthesis Kit (Roche) and were detected with Rhodamine conjugated with anti-digoxigenin (Roche). Chromosomes were measured using the software DRAWID (Kirov et al. 2017), excluding secondary constrictions in measurements, and classified either as metacentric (m), submetacentric (sm), and

subtelocentric following Green & Sessions (1991). In the karyograms, the chromosome pairs were ordered to reflect our hypotheses of chromosomal homeologies (Lourenço et al. 2015, Targueta et al. 2018), regardless of chromosome size.

Collected specimens are housed in the herpetological collections of the Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical, Posadas, Misiones, Argentina (LGE), and Museo Nacional de Historia Natural, Montevideo, Uruguay (MNHN), and the Collection of tissue and chromosome preparation "Shirlei Maria Recco Pimentel", Department of Structural and Functional Biology at the Biology Institute of the University of Campinas, São Paulo, Brazil. The collections of specimens were approved by the Ministerio de Ecología y Recursos Naturales Renovables (Argentina, MEyRNR 061/2015, 073/2016, 035/2017 and 047/2018), Instituto Chico Mendes de Conservação da Biodiversidade and Sistema de Autorização e Informação em Biodiversidade (Brazil, ICMBio/SISBIO 32483-3), and División Fauna (Uruguay, MGAP Res 195/06). Protocols of euthanasia and preservation of specimens were performed with the approval of the Ethics Committee in Animal Use (CEUA UNICAMP 4802-1 and MNHN 1/2019). The sex and collection information of studied specimens are detailed below.

Physalaemus sp. aff. *gracilis* — URUGUAY: San José: Estancia Maitea, Sierra de Mahoma, MNHN 9984 (♀), 9985 (♂), 9986 (♂); Maldonado: Route 109 2 km southern Aiguá, MNHN 9982 (♂), 9984 (♂), LGE 15682 (♀); Treinta y Tres: Bañado de Los Oliveras, MNHN 9511 (♀).

Physalaemus gracilis — BRAZIL: Rio Grande do Sul: Gravataí, SMRP 37.16 (♂), 37.17 (♂), 37.18 (♂), 37.19 (♂)

Physalaemus carrizorum — ARGENTINA: Misiones: Moconá Provincial Park, LGE 15317(♂), LGE 15318, 15319 (♂), 15320 (♂); Arroyo Los Muertos, near Provincial route N°2, LGE 24602 (♂), LGE 15325 (♀); Piñalito Provincial Park, LGE 24602 (♂), 20450 (♂), 20453 (undetermined); Provincial route N°18, 25 km west Bernardo de Irigoyen, LGE 20433 (♂), 20434 (♂); Provincial route N° 18, 45 km northwest Bernardo de Irigoyen, LGE 3383 (♂); National route N° 14 km northwest Tobuna, LGE 21828 (♂).

Physalaemus lisei — BRAZIL: Rio Grande do Sul: Gravataí, SMRP 38.9 (♀), 38.10 (♀), 38.11 (♀), 38.12 (♀), 38.13 (♀), 38.14 (♂), 38.15 (♂).

RESULTS

The four species studied herein presented similar karyotypes with 11 bi-armed chromosome pairs (2n = 2x = 22; FN = 44), containing seven large to medium-sized and four small-sized ones. Pairs 1, 2, 5, 6, and 9–11 were metacentric, while pairs 3, 4, and 7 were submetacentric. Pair 8 invariably showed secondary constrictions, being metacentric in *Physalaemus gracilis*, *P.* sp. aff. *gracilis*, and *P. lisei* but submetacentric in *P. carrizorum* (Figure 1 a,d, Table I).

The Ag-NORs were detected only on the long arms of chromosome pair 8, coinciding with secondary constrictions in Giemsa stained metaphases. They were terminal in *Physalaemus* sp. aff. *gracilis* and *P. gracilis*, but interstitial in *P. carrizorum* and *P. lisei* (Figure 1 a,d). Besides, one female of *P.* sp. aff. *gracilis* (MNHN 9511) and one male of *P. gracilis* (SMRP 37.16) showed different sizes for the Ag-NORs between homologues (Figure 1 a,d).

Heterochromatic bands were observed in the centromeric regions and stained more intensely on (peri)centromeres of pair 3 in the four species and pair 7 in *Physalaemus lisei* (Figure 2a, c, e, g). In *Physalaemus* sp. aff. *gracilis* and *P. gracilis*, the NORs were also associated with C-bands (Figure 2c, e). In *P.*

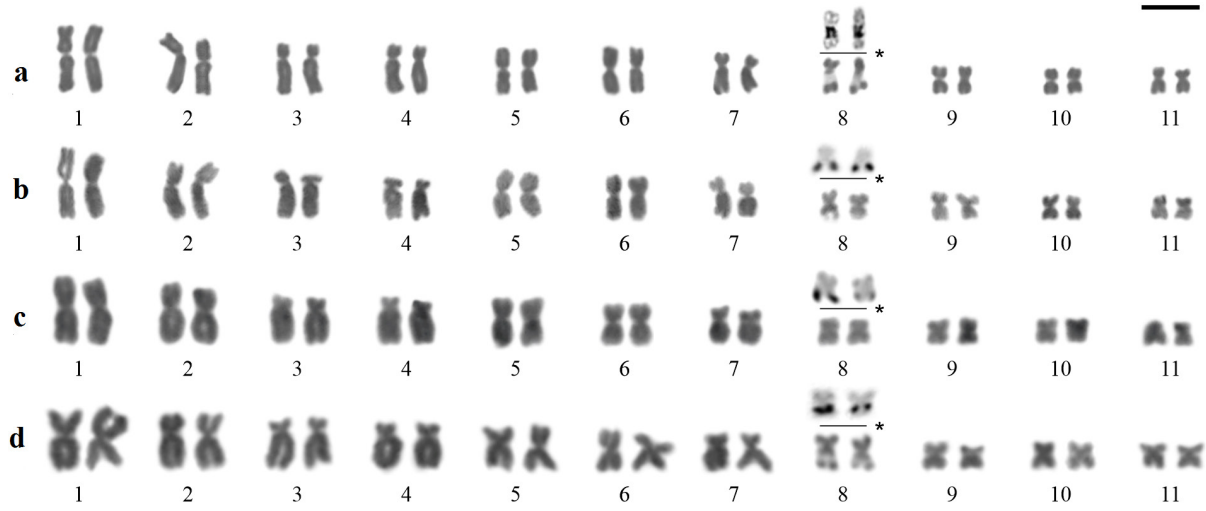

Figure 1. Giemsa stained karyotypes of four species of the *Physalaemus gracilis* group. (a) *P. carrizorum*, (b) *P. gracilis*, (c) *P.* sp. aff. *gracilis*, (d) *P. lisei*. The insets (*) show chromosomes with Ag-NORs. Bar = 10 µm.

gracilis, an additional interstitial C-band was observed in pair 10. Moreover, in *P. lisei*, we observed polymorphisms for terminal C-bands associated with chromosomes of pair 1. Of the four specimens analyzed by this technique (3♀, 1♂), the three females showed positive C-bands on both homologues in 1q while the male was heterozygous for such band, being present in only one chromosome. On the other hand, two of these females showed additional C-bands in both homologues in 1p (Figure 2i).

FISH with PcP190 probe showed fluorescent centromeric/pericentromeric marks in one chromosome pair of *Physalaemus carrizorum* and *P. lisei*, and in two chromosome pairs in *P.* sp. aff. *gracilis* and *P. gracilis*, always associated with heterochromatin but with a different location between species: pairs 2 and 9 in *P. gracilis* and *P.* sp. aff. *gracilis*, pair 3 in *P. carrizorum*, and pair 7 in *P. lisei* (Figure 2b, d, f, h).

DISCUSSION

Previous reports on cytogenetics of the *Physalaemus gracilis* group only referred to the chromosome number of specimens from Uruguay (presumably *P.* sp. aff. *gracilis*, as *P. gracilis*, Brum-Zorrilla & Sáez 1968), karyotype descriptions of *P. gracilis* from an uncertain locality in Brazil (de Lucca et al. 1974), and of *P. barrioi* from the type locality of the species (Serra de Bocaina-Brazil, Provete et al. 2012). To date, there is no cytogenetic information about *P. evangelistai* and *P. jordanensis*.

The basic chromosome number (x = 11) observed in the *Physalaemus gracilis* group is shared by all currently analyzed species of the genus (Lourenço et al. 2015, and references therein; present study). Within Leptodactylidae, this character is widespread in the remaining four genera of Leiuperinae, *Engystomops*, *Edalorhina*, *Pleurodema*, and *Pseudopaludicola* (Barrio & Rinaldi de Chieri 1970, Lourenço et al. 2000, 2006, Targueta et al. 2011, 2018, Cardozo et al. 2016, 2018, and also in *Leptodactylus* (Leptodactylinae, Coelho et al. 2016, and references therein). In contrast, different numbers are observed in Paratelmatobiinae (x = 12 in *Paratelmatobius* and *Scythrophrys*, Lourenço et al. 2008), and in other genera of Leptodactylinae (x = 9 in *Lithodytes* and x = 12 and 13 in *Adenomera*, Bogart 1970, Zaracho & Hernando 2011, Coelho et al. 2016).

Table I. Chromosome measurements of species of the *Physalaemus gracilis* group. The chromosome percentage is relative to the haploid set. Centromeric index ± Standard Deviation. Chromosome morphology: metacentric (m); submetacentric (sm); subtelocentric (st).

	P. carrizorum	*P.* sp. aff. *gracilis*	*P. gracilis*	*P. lisei*
1	14.37 **(m)** 0.44 ± 0.01	15.31 **(m)** 0.45 ± 0.01	14.65 **(m)** 0.46 ± 0.02	15.15 **(m)** 0.43 ± 0.02
2	12.78 **(m)** 0.39 ± 0.01	13.04 **(sm)** 0.34 ± 0.03	12.81 **(sm)** 0.36 ± 0.03	11.74 **(m)** 0.38 ± 0.01
3	10.94 **(sm)** 0.30 ± 0.00	10.99 **(st)** 0.23 ± 0.07	11.11 **(sm)** 0.25 ± 0.02	10.61 **(sm)** 0.29 ± 0.03
4	10.47 **(sm)** 0.26 ± 0.01	10.19 **(st)** 0.25 ± 0.02	10.34 **(st)** 0.23 ± 0.02	10.55 **(sm)** 0.28 ± 0.03
5	9.86 **(m)** 0.46 ± 0.03	10.17 **(m)** 0.42 ± 0.01	10.43 **(m)** 0.43 ± 0.03	10.22 **(m)** 0.41 ± 0.03
6	9.77 **(m)** 0.45 ± 0.03	9.65 **(m)** 0.43 ± 0.02	9.20 **(m)** 0.41 ± 0.02	9.58 **(m)** 0.39 ± 0.04
7	8.67 **(sm)** 0.32 ± 0.01	8.66 **(sm)** 0.36 ± 0.02	8.39 **(sm)** 0.34 ± 0.05	8.37 **(sm)** 0.34 ± 0.02
8	5.15 **(m)** 0.45 ± 0.01	5.76 **(m)** 0.42 ± 0.04	5.48 **(m)** 0.46 ± 0.01	5.96 **(m)** 0.47 ± 0.02
9	6.48 **(m)** 0.45 ± 0.01	5.50 **(m)** 0.42 ± 0.07	6.43 **(m)** 0.44 ± 0.03	6.23 **(m)** 0.45 ± 0.01
10	5.83 **(m)** 0.45 ± 0.03	5.57 **(m)** 0.45 ± 0.04	5.79 **(m)** 0.45 ± 0.02	6.15 **(m)** 0.44 ± 0.03
11	5.68 **(m)** 0.41 ± 0.01	5.16 **(m)** 0.44 ± 0.03	5.37 **(m)** 0.44 ± 0.04	5.44 **(m)** 0.39 ± 0.04

Moreover, within the higher taxon Hyloidea, this character state is shared by the families Brachycephalidae, Craugastoridae (Schmid et al. 2010, and references therein), Bufonidae (Baldo et al. 2012, and references therein), and Odontophrynidae (Ananias et al. 2007b, Rocha et al. 2017, and references therein). It is remarkable that in some recent phylogenomic analyzes, all these taxa were recovered within the major clade Commutabirana that includes the superfamily Brachycephaloidea (Brachycephalidae, Craugastoridae, Eleutherodactylidae) and the families Allophrynidae, Bufonidae, Centrolenidae, Dendrobatidae, Leptodactylidae, and Odontophrynidae (Feng et al. 2017, Streicher et al. 2018), and in which the state x = 11 optimizes as a synapomorphy. Alternatively, in a more inclusive phylogenetic hypothesis recently proposed by Jetz & Pyron (2018), x = 11 optimizes as a synapomorphy for a minor clade that excludes Brachycephaloidea.

As previously stated, among the *Physalaemus* karyotypes, are observed two distinct FN, FN = 44 and FN = 42 (Vittorazzi et al. 2016, and references therein). Karyotypes composed of only bi-armed chromosomes (FN = 44) are present in species of the *P. cuvieri* clade but also among the other genera of leiuperines *Edalorhina*, *Engystomops*, *Pleurodema*, and *Pseudopaludicola* (Barrio & Rinaldi de Chieri 1970, Lourenço et al. 2000, 2006, Targueta et al. 2011, 2018, Cardozo et al. 2016, 2018. Conversely, karyotypes with one small pair of telocentric chromosomes (FN = 42) were reported for species of the *P. signifer* clade and *P. fernandezae* (*P. cuvieri* clade), and with one small or medium pair in some species of *Engystomops* (Tomatis et al. 2009, Lourenço et al. 2015, Targueta et al. 2018). The similar FN observed in these three taxa likely corresponds to homoplasy, and the character state FN = 42 could be considered a synapomorphy of the *P. signifier* clade (Lourenço et al. 2015). It should be stressed that the relation between *P. fernandezae* and *P. henselii*, and the remaining species of the *P. cuvieri* clade is poorly supported (Lourenço et al. 2015) and, in this regard, future

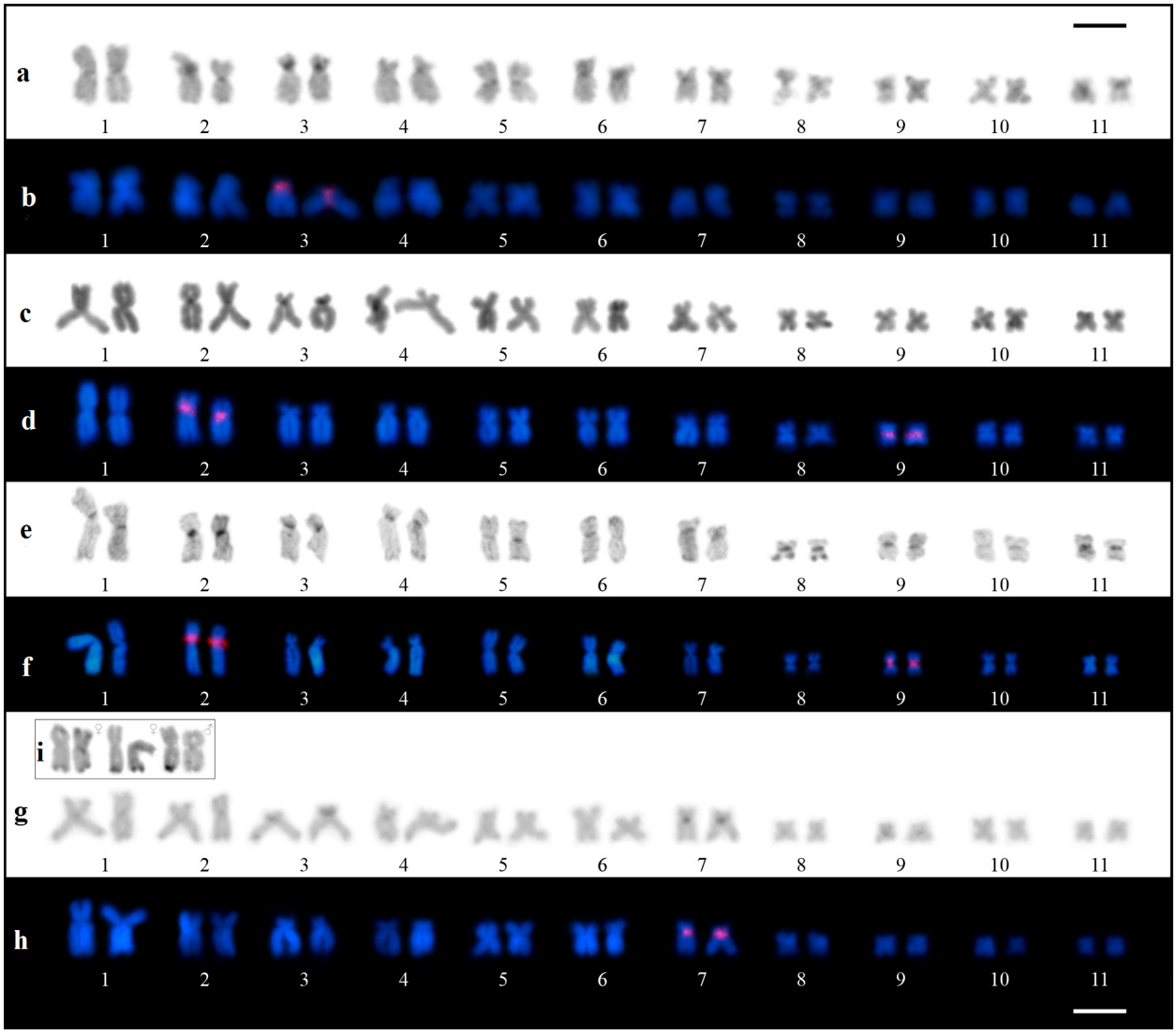

Figure 2. Mitotic chromosomes of four species of the *Physalaemus gracilis* group after C-banding (a, c, e, g, i) and FISH with PcP190 satDNA (b, d, f, h). (a, b) *P. carrizorum*, (c, d) *P. gracilis*, (e, f) *P.* sp. aff. *gracilis*, (g,i) *P. lisei.* The polymorphic variation for C-bands of pair 1 in *P. lisei* are shown in (i). Bar = 10 µm.

interpretations of the transformation observed in *P. fernandezae* would change as more information would become available.

Almost all karyotyped species of the *Physalaemus gracilis* group show the NORs in chromosome pair 8, excepting *P. barrioi* in which this marker is present in pair 10 (Provete et al. 2012). In the *P. olfersii* group, a clade phylogenetically closely related to the *P. gracilis* group, the Ag-NORs are present in pairs 3 and 7 in *P. feioi* (as *P. olfersii* from the locality of Viçosa, state of Minas Gerais, Brazil, Milani et al. 2011), 3 and 4 in *P. olfersii* (in specimens from Teresópolis, state of Rio de Janeiro, Brazil, Milani et al. 2011), and possibly in pair 11 in *P. soaresi* inferred by the presence of secondary constrictions (de Lucca et al. 1974). Moreover, in the *P. biligonigerus* group, which is phylogenetically closely related to the *P. gracilis* and *P. olfersii* groups, all species show distal Ag-NORs in pairs 8 or 9 (Amaral et al. 2000, Silva et al. 1999, Tomatis et al. 2009, Vittorazzi et al. 2014a). In this sense, NORs in pair

3 would be an apomorphy of a less inclusive clade within the *P. olfersii* group, pending the study of this marker on *P. lateristriga* and *P. soaresi* to confirm this assumption. Besides, the similar size and morphology of chromosome pairs 8–10 in species of the *P. biligonigerus* and *P. gracilis* groups allow us to assume that NOR-bearing chromosomes of these species are homeologous, with the caveat that this hypothesis should be tested with the aid of other chromosome markers. Moreover, Lourenço et al. (2015) stated that the NORs on pair 8 would represent a plesiomorphic condition for *Physalaemus*, as it is shared by other genera of Leiuperinae and Leptodactylinae that also present 2n = 22 karyotypes (Bogart 1974, Lourenço et al. 2000, 2008, 2015, Coelho et al. 2016).

Two specimens, one of *Physalaemus gracilis* and one of *P.* sp. aff. *gracilis* showed heteromorphisms for Ag-NOR size. This sort of variation is frequently reported as a source of polymorphisms in anuran cytogenetics and may be the consequence of different mechanisms such as unequal meiotic exchanges or tandem duplication of rDNA (Schmid 1982, Schmid et al. 2010).

All species in the *Physalaemus gracilis* group have similar patterns of (peri)centromeric C-bands. Besides, *P.* sp. aff. *gracilis* and *P. gracilis* show heterochromatin associated with the NOR sites, and *P. barrioi* and *P. gracilis* have interstitial C-bands on pairs 4 and 10, respectively (Provete et al. 2012, this study). In *P. gracilis*, the C-band on chromosome pair 10 has an additional taxonomic value as it allows us to distinguish *P. gracilis* from its sibling species *P.* sp. aff. *gracilis*. Finally, in *Physalaemus lisei*, the detection of conspicuous telomeric C-bands varies among specimens for both arms (1p and 1q) and, although the only male studied by this technique had a particular pattern not observed in females, with a single C-band on one homologue of 1q, it is still necessary to study more males in order to exclude that this last polymorphism is sex-biased.

CONCLUSION

Some studies have demonstrated an extensive taxonomic distribution regarding the PcP190 sequences, a satDNA probably derived from 5S rDNA (Vittorazzi et al. 2011). Its presence was confirmed for the families Leptodactylidae (*Engystomops*, *Leptodactylus*, and *Physalaemus*), Hylodidae (*Crossodactylus*), and Hylidae (*Pseudis*). This satDNA marker was previously mapped on the karyotypes of 8 species of *Engystomops*, 7 species of *Physalaemus*, and 7 species of *Pseudis* (Vittorazzi et al. 2011, 2014b, 2016, Gatto et al. 2016, Targueta et al. 2018, and references therein; Gatto et al. 2019). As a generality, the chromosome position of PcP190 is biased towards the centromeres or proximally associated with C- bands (Vittorazzi et al. 2014b). Exceptions are the sex chromosomes of *Physalaemus ephippifer* and species of *Pseudis*, in which PcP190 clusters were detected in interstitial heterochromatic bands, suggesting a possible role in the differentiation of sex chromosomes (Vittorazzi et al. 2014b, Gatto et al. 2016, 2019). In a similar way to what is observed in other species of *Physalaemus*, all four species in the *P. gracilis* group analyzed in this work have variable PcP190 location. It is worth noting that its presence on pair 3 is a recurrent feature shown by 7 of 11 studied species in the genus: *P. albifrons*, *P. albonotatus*, *P. carrizorum*, *P. centralis* (in addition to several pairs), *P. cuvieri* (in addition to several pairs), *P. kroyeri* (in addition to pair 1), and *P. ephippifer* (in addition to the W chromosome) (Vittorazzi et al. 2011, 2014b, 2016, this study). This feature was also reported in almost all studied species

of *Engystomops* (6 of 8 species), excepting *E. coloradorum* and *E.* "magnus" (see Targueta et al. 2018). The recurring occurrence of PcP190 in pair 3 of both *Engystomops* and *Physalaemus* is a promising informative marker to establish homeology. However, in order to obtain a better picture of this character distribution within Leiuperinae, a more considerable amount of data has to be collected in the remaining groups of the *P. cuvieri* clade (i.e., *P. biligonigerus*, *P. henselii*, and *P. olfersii* groups), the *P. signifer* clade, *Edalorhina*, *Pseudopaludicola*, and *Pleurodema* (for details about the phylogenetic relationships of these taxa, see Lourenço et al. 2015). Moreover, given the similarity between PcP190 and 5S nucleotide sequences, further data should also include information about the chromosomal mapping of 5S rDNA.

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JUAN M. FERRO[1]
https://orcid.org/0000-0003-0287-5567

ALBERTO TAFFAREL[1]
https://orcid.org/0000-0002-9479-5152

CRISTIAN TOMATIS[2]
https://orcid.org/0000-0001-7860-7951

CLAUDIO BORTEIRO[3]
https://orcid.org/0000-0002-8722-0334

FRANCISCO KOLENC[3]
https://orcid.org/0000-0002-9985-3630

KALEB P. GATTO[4,5]
https://orcid.org/0000-0002-2261-229X

LUCIANA B. LOURENÇO[4]
https://orcid.org/0000-0001-6602-6850

DIEGO BALDO[1]
https://orcid.org/0000-0003-2382-0872

[1] Universidad Nacional de Misiones, Facultad de Ciencias Exactas Químicas y Naturales, Instituto de Biología Subtropical (CONICET-UNaM), Laboratorio de Genética Evolutiva, Félix de Azara 1552, N3300LQH Posadas, Misiones, Argentina

[2]Universidad Nacional de Misiones, Facultad de Ciencias Exactas Químicas y Naturales, Félix de Azara 1552, N3300LQH Posadas, Misiones, Argentina

[3]Museo Nacional de Historia Natural, Sección Herpetología, Miguelete 1825, 11800 Montevideo, Uruguay

[4]Universidade Estadual de Campinas, Laboratório de Estudos Cromossômicos, Departamento de Biologia Estrutural e Funcional, Bertrand Russell, s/n, 13083-865 Campinas, SP, Brazil

[5]Universidade Estadual Paulista, Instituto de Biociências, Centro de Aquicultura e Laboratório de Herpetologia, Departamento de Zoologia, Avenida 24A, Bela Vista, 13506-900 Rio Claro, SP, Brazil

Correspondence to: **Diego Baldo**
E-mail: diegobaldo@gmail.com

Author contributions

Juan M. Ferro: collected specimens, performed cytogenetic studies, analyzed the data and wrote the manuscript. Alberto Taffarel: performed cytogenetic studies, contributed to analyze the data, revised the manuscript. Cristian Tomatis: collected specimens, performed cytogenetic studies, contributed to analyze the data, revised the manuscript. Claudio Borteiro: collected specimens, revised the manuscript. Francisco Kolenc: collected specimens, revised the manuscript Kaleb P. Gatto: performed cytogenetic studies, revised the manuscript. Luciana B. Lourenço: contribute to analyze data, revised the manuscript. Diego Baldo: acquired the financial resources for this research, designed the study, collected specimens, analyzed the data, revised the manuscript. All authors discussed the results and approved the final version of the manuscript.