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Phylogeny of frogs from the genus *Physalaemus* (Anura, Leptodactylidae) inferred from mitochondrial and nuclear gene sequences ☆

Luciana B. Lourenço [a,*], Cíntia P. Targueta [a], Diego Baldo [b], Juliana Nascimento [a], Paulo C.A. Garcia [c], Gilda V. Andrade [d], Célio F.B. Haddad [e], Shirlei M. Recco-Pimentel [a]

[a] Departamento de Biologia Estrutural e Funcional, Instituto de Biologia, Universidade Estadual de Campinas, 13083-863 Campinas, São Paulo, Brazil
[b] Laboratorio de Genética Evolutiva, Instituto de Biología Subtropical (CONICET-UNaM), Facultad de Ciencias Exactas Químicas y Naturales, Universidad Nacional de Misiones, Félix de Azara 1552, CPA N3300LQF, Posadas, Misiones, Argentina
[c] Laboratório de Herpetologia, Departamento de Zoologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, 31270-901 Belo Horizonte, Minas Gerais, Brazil
[d] Departamento de Biologia, Centro de Ciências Biológicas e da Saúde, Universidade Federal do Maranhão-UFMA, 65080-040 São Luís, Maranhão, Brazil
[e] Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista, C.P. 199, 13506-900 Rio Claro, São Paulo, Brazil

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ABSTRACT

Although some species groups have been recognized in the leiuperine genus *Physalaemus*, no phylogenetic analysis has previously been performed. Here, we provide a phylogenetic study based on mitochondrial and nuclear DNA sequences from 41 of the 46 species of *Physalaemus*. We employed the parsimony criterion using the software TNT and POY and the Bayesian criterion using the software MrBayes. Two major clades were recovered inside the monophyletic *Physalaemus*: (i) the highly supported *Physalaemus signifer* Clade, which included *P. nattereri* and the species previously placed in the *P. deimaticus* and *P. signifer* Groups; and (ii) the *Physalaemus cuvieri* Clade, which included the remaining species of *Physalaemus*. Five species groups were recognized in the *P. cuvieri* Clade: the *P. biligonigerus* Group, the *P. cuvieri* Group, the *P. henselii* Group, the *P. gracilis* Group and the *P. olfersii* Group. The *P. gracilis* Species Group was the same as that previously proposed by Nascimento et al. (2005). The *P. henselii* Group includes *P. fernandezae* and *P. henselii*, and was the sister group of a clade that comprised the remaining species of the *P. cuvieri* Clade. The *P. olfersii* Group included *P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi* and *P. lateristriga*. The *P. biligonigerus* Species Group was composed of *P. biligonigerus*, *P. marmoratus*, *P. santafecinus* and *P. riograndensis*. The *P. cuvieri* Group inferred here differed from that recognized by Nascimento et al. (2005) only by the inclusion of *P. albifrons* and the exclusion of *P. cicada*. The paraphyly of *P. cuvieri* with respect to *P. ephippifer* was inferred in all the analyses. Distinct genetic lineages were recognized among individuals currently identified as *P. cuvieri* and they were congruent with cytogenetic differences reported previously, supporting the hypothesis of occurrence of formally unnamed species.

1. Introduction

Physalaemus is one of the largest genera in Leptodactylidae, but its intergeneric and internal relationships remain unclear. Over the last decade, the taxonomy of leptodactylid anurans has changed as a result of a number of phylogenetic analyses (Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011; Faivovich et al., 2012; Fouquet et al., 2013; de Sá et al., 2014), and according to the latest studies *Physalaemus* is included in the subfamily Leiuperinae. Pyron and Wiens (2011) recognized the leptodactylid subfamily Leiuperinae to comprise the genera *Edalorhina*, *Engystomops*, *Eupemphix* [raised as a junior synonym of *Physalaemus* by Faivovich et al. (2012)], *Physalaemus*, *Pleurodema* (including *Somuncuria*) and *Pseudopaludicola*. Fouquet et al. (2013), in an extensive analysis of Leptodactylidae, also recovered a monophyletic Leiuperinae, whereas Faivovich et al. (2012), in a study designed to analyze the internal relationships in *Pleurodema*, did not recover *Pseudopaludicola* as belonging to the clade that includes all the remaining leiuperines. Although *Pleurodema* has been repeatedly recovered as the sister group of a clade composed of *Edalorhina*, *Engystomops* and *Physalaemus* (Frost et al., 2006; Grant et al., 2006; Lourenço et al., 2008; Pyron and Wiens, 2011;

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* Corresponding author.
E-mail address: bolsoni@unicamp.br (L.B. Lourenço).

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Faivovich et al., 2012; Fouquet et al., 2013), it is not clear which is the sister group of *Physalaemus*.

The internal phylogenetic relationships of *Physalaemus* are even less well studied than its intergeneric cladistic proximity. Currently, 46 named species of *Physalaemus* are known (listed in Frost, 2014), but even in the most comprehensive phylogenetic analyses of leiuperines conducted to date, only a few species of *Physalaemus* were included [in addition to *P. nattereri*, Pyron and Wiens (2011), Fouquet et al. (2013) and de Sá et al. (2014) analyzed eight species of *Physalaemus*, whereas Faivovich et al. (2012) analyzed only five].

Tárano and Ryan (2002), in a study of the advertisement call of *Physalaemus enesefae* (currently a junior synonym of *Physalaemus fischeri*), presented in the introduction of their paper a preliminary phylogenetic inference of *Physalaemus* relationships performed by D. Cannatella, M. Holder, D. Hillis, A. S. Rand and M. J. Ryan. This inference included ten species of *Physalaemus* in addition to "*P. enesefae*" and, according to Tárano and Ryan (2002), it was based on morphological characters, allozymes and mitochondrial DNA sequences, but no further information about the phylogenetic methods used was provided. Tárano and Ryan (2002) noted two monophyletic groups in that cladogram, one of them composed of the species currently assigned to *Engystomops* and the other composed of the species of *Physalaemus* (Fig. 1), but the authors did not discuss the internal relationships of those groups.

Despite the absence of a study specially designed to evaluate the phylogenetic relationships in *Physalaemus*, morphological groups of species have been recognized. Lynch (1970), who considered *Engystomops* a synonym of *Physalaemus*, recognized four species groups in *Physalaemus*: the *P. pustulosus* Group (including species currently in the genus *Engystomops*), the *P. cuvieri* Group, the *P. biligonigerus* Group and the *P. signifer* Group. Based on a phenetic analysis of morphometric data, external morphology, color patterns and osteological characters, Nascimento et al. (2005) reviewed the proposal of Lynch (1970) and in addition to resurrecting the genera *Engystomops* and *Eupemphix*, rearranged the *Physalaemus* species into seven groups (*P. albifrons*, *P. cuvieri*, *P. deimaticus*, *P. gracilis*, *P. henselii*, *P. olfersii* and *P. signifer* Species Group). However, the monophyly of each of these species groups has not been tested, and recent studies have noted the lack of known synapomorphies for two of them, *i.e.*, the *P. henselii* (Tomatis et al., 2009) and the *P. albifrons* (Vittorazzi et al., 2014) Groups.

The need for phylogenetic analysis of *Physalaemus* is evident not only from a taxonomic point of view but also because such analysis will enable accurate evolutionary studies of several character systems, including chromosomal data. Cytogenetic data are available for 29 of the 46 species of *Physalaemus* (Beçak, 1968; Beçak et al., 1970; Denaro, 1972; de Lucca et al., 1974; Silva et al., 1999, 2000; Amaral et al., 2000; Lourenço et al., 2006; Ananias et al., 2007; Quinderé et al., 2009; Tomatis et al., 2009; Milani et al., 2010; Nascimento et al., 2010; Provete et al., 2012; Vittorazzi et al., 2014), and this set of data has already been explored by Tomatis et al. (2009) and Vittorazzi et al. (2014) in an attempt to evaluate the arrangement of the *P. henselii* and *P. albifrons* Groups as proposed by Nascimento et al. (2005). Despite the notorious interspecific variation in the location of nucleolus organizer regions (NORs) in *Physalaemus*, the steps of karyotypic evolution in this genus and the recognition of chromosomal synapomorphies for species groups remain largely unknown (see comments in Tomatis et al., 2009 and Vittorazzi et al., 2014). One critical limitation to the cytogenetic studies of *Physalaemus* has been the scarcity

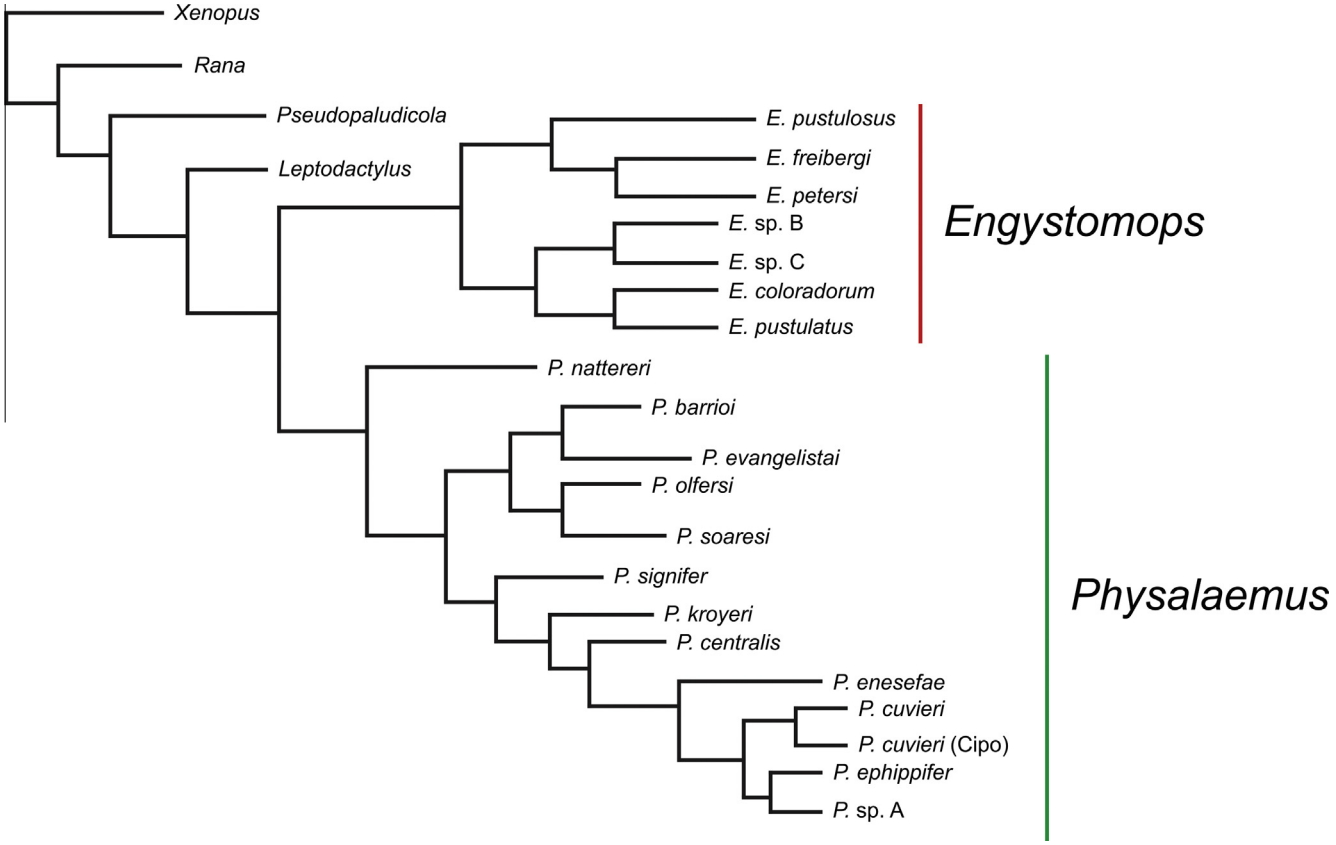

Fig. 1. Phylogenetic relationships of *Physalaemus* species presented by Tárano and Ryan (2002), based on unpublished analysis performed by D. Cannatella, M. Holder, D. Hillis, A.S. Rand and M.J. Ryan.

of chromosomal markers, which prevents a reliable recognition of interspecific chromosome homeology, especially with regards to the smallest of the 22 chromosomes of the diploid complement (*i.e.*, chromosome pairs 8–11) (see Vittorazzi et al., 2014). It is also noteworthy that the cytogenetic data, especially the NOR locations, have revealed cryptic diversity in *Physalaemus*, particularly with respect to *P. cuvieri*, which some have hypothesized indicates the presence of unnamed species (Quinderé et al., 2009). In this context, a phylogenetic hypothesis for the relationships of the species of *Physalaemus*, which should include a deeper analysis of the genetic diversity of *P. cuvieri*, would help elucidate the evolutionary variation of the chromosomal data.

In this study, we aim to investigate the interspecific relationships in *Physalaemus*, to test the monophyly of the recognized species groups and to provide a revision of the chromosomal data available for this genus in the light of a phylogenetic hypothesis.

2. Materials and methods

2.1. Taxon sampling

We analyzed 41 of the 46 currently known species of *Physalaemus* (Appendix A), including all the nominal species of the *P. albifrons*, *P. cuvieri*, *P. deimaticus*, *P. gracilis* and *P. henselii* Groups; all but one species of the *P. olfersii* Group; and all but three species of the *P. signifer* Group. For ten of the analyzed species, topotypical specimens or individuals from the vicinities of the type localities were sampled. Because high diversity in *P. cuvieri* was revealed by cytogenetic analyses (Quinderé et al., 2009), which raises the hypothesis of existence of unnamed species, we used a large sample of this taxon by analyzing specimens from 19 localities (Appendix A), which included most of the sites sampled by Quinderé et al. (2009).

We also included exemplars of all the other Leiuperinae genera (*i.e.*, *Edalorhina*, *Engystomops*, *Pleurodema* and *Pseudopaludicola*) and of the two other subfamilies of Leptodactylidae (*i.e.*, Leptodactylinae and Paratelmatobiinae) (Appendix A). Because the phylogenetic relationships of the family Leptodactylidae are still controversial (see Frost et al., 2006; Grant et al., 2006; Pyron and Wiens, 2011), we included representatives of the following families of Hyloidea: Alsodidae, Centrolenidae, Ceratophryidae, Cycloramphidae, Hylodidae, Odontophrynidae, Telmatobiidae and Hylidae, which was inferred by Frost et al. (2006) and Pyron and Wiens (2011) as the sister group of a clade that included Leptoctylidae and all the other aforementioned hyloid families, among others (Appendix A). The DNA sequences reported here and those recovered from GenBank are all indicated in Appendix A.

2.2. Mitochondrial and nuclear gene sequencing

Genomic DNA was extracted from tissue samples obtained from scientific collections (see Appendix A). Tissue samples were immersed in a TNES buffer solution (50 mM Tris, pH 7.5, 400 mM NaCl, 20 mM EDTA, 0.5% SDS). The solution was subsequently supplemented with proteinase K (to a final concentration of 100 μg/mL) and the samples were incubated for 5 h at 55 °C. Then, 1/3 volume of 5 M NaCl was added, and the samples were centrifuged. DNA was precipitated from the supernatant with isopropyl alcohol, washed with ethanol (70%), resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stored at −20 °C.

The mitochondrial 12S and 16S ribosomal genes and the intervening tRNA-val region were PCR amplified using the primer pairs MVZ59(L) (Graybeal, 1997)/TitusI(H) (Titus, 1992) and 12L13(L) (Feller and Hedges, 1998)/16Sbr(H) (Palumbi et al., 1991). For PCR amplification of a segment of the nuclear gene *RAG-1*, the primers RAG-1F and RAG-1R (Faivovich et al., 2005) were used. The PCR products were purified with GFX PCR and Gel Band DNA purification kits (GE Healthcare, England) and directly sequenced using BigDye Terminator kits (Applied Biosystems, Foster City, CA, USA) in an automatic DNA ABI/Prism sequencer (Applied Biosystems, Foster City, CA, USA). The mitochondrial genes were sequenced using the primers MVZ59(L) (Graybeal, 1997), MVZ50(H) (Graybeal, 1997), 12L13 (Feller and Hedges, 1998), TitusI(H) (Titus, 1992), Hedges16L2a (Hedges, 1994), Hedges16H10 (Hedges, 1994), 16Sar(L) (Palumbi et al., 1991) and 16Sbr(H) (Palumbi et al., 1991), whereas the *RAG-1* segment was sequenced with the same primers used for PCR amplification. DNA sequences were edited using Bioedit version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.3. Phylogenetic inferences

Homologous 165 segments with approximately 2.5 kb composed a mitochondrial DNA matrix, and these sequences together with 76 *RAG-1* sequences of 410 bp generated a concatenated matrix. Because missing data may compromise phylogenetic inferences (*e.g.*, Agnarsson and May-Collado, 2008; Simmons, 2012), we inferred phylogenetic relationships separately from the mitochondrial data matrix and from the concatenated data matrix. Each matrix was analyzed using both maximum-parsimony and Bayesian criteria. When using the maximum-parsimony criterion, we inferred the phylogenetic relationships based on both dynamic-homology and static-homology hypotheses.

The phylogenetic analysis based on direct optimization of unaligned characters was implemented with the software POY v.5.1.1 (Varón et al., 2010). The phylogenetic searches performed with POY included tree building (of at least 18 Wagner trees), tree bisection–reconnection (TBR) swapping, perturbation using a parsimony ratchet and tree fusing. The analyses were run with a maximum execution time of 5 days and cost 1 for gap opening, gap extension and nucleotide substitution. To obtain an implied alignment from the POY analysis, the characters were transformed into static characters and the generated matrix was exported using the command "phastwinclad." The exported matrix was loaded with TNT v.1.1 to calculate bootstrap support based on 1000 pseudoreplicates, using traditional search.

For the analysis using the static homology hypothesis, the mtDNA sequences were aligned with Muscle (Edgar, 2004). The alignment obtained under default parameters was improved twice with the "refine" command, rendering a matrix with 2528 characters. The alignment of the *RAG-1* sequences was unambiguous because this data matrix had no indel and the final concatenated data matrix included 2938 characters. The phylogenetic analyses of the aligned mitochondrial and concatenated data matrices under the maximum-parsimony criterion were performed using the software TNT v.1.1 (Goloboff et al., 2003). Gaps were considered a fifth character state. The most parsimonious trees were inferred through heuristic searches performed using the new technology search, including combined sectorial searches, the ratchet, tree drifting and tree fusing. The trees were obtained from driven searches, and the best length was hit 100 times. The bootstrap values of the branches inferred in these analyses were calculated with 1000 pseudoreplicates, using traditional search on TNT.

For the Bayesian analyses of the aligned data matrices, the GTR+I+G model of DNA evolution was inferred for the mitochondrial data, and the models JC+G, K80+G and SYM+G were inferred, respectively, for the first, second and third codon positions of the *RAG-1* sequences, using the software MrModeltest v.2.3 (Nylander, 2004). The phylogenetic analyses were implemented in the software MrBayes v.3.2 (Ronquist et al., 2011). Two

simultaneous analyses were run, each with four chains (three heated and one cold). In each analysis, 20 million generations were run. One tree was sampled every 100 generations. Consensus topology and posterior probabilities were produced after discarding the first 25% of the trees generated. The ASDSF (Average Standard Deviation of Split Frequencies) value was below 0.01 and the PSRF (Potential Scale Reduction Factor) values were approximately 1.000. The stabilization of posterior probabilities was checked using Tracer v. 1.5 (Rambaut and Drummond, 2007).

2.4. Genetic distances among lineages related to Physalaemus cuvieri, P. ephippifer and P. fischeri

The genetic distances among *Physalaemus ephippifer*, *P. fischeri* and the lineages of *P. cuvieri* recognized in the phylogenetic analyses were estimated by pairwise comparisons of ~500 bp sequences of the 16S mitochondrial rRNA gene using the software Mega v.6.0 (Tamura et al., 2013). Sequences of *P. cuvieri* specimens from Bolivia studied by Jansen et al. (2011) (JF789851-JF789858) were also included. Additionally, pairwise comparisons were provided for the other species of the *P. cuvieri* Species Group.

2.5. Cytogenetic analysis

Because the presence of a small telocentric chromosome becomes an important feature to be discussed in the present study and because no species of the *Physalaemus deimaticus* group had been karyotyped to date, we described the karyotype of the ZUEC 21193 specimen of *P. deimaticus* collected from Diamantina-MG, Brazil. Metaphase chromosome spreads were obtained from cell suspensions of intestine after an in vivo treatment with colchicine using a protocol adapted from King and Rofe (1976). Prior to the removal of the intestine, the animal was deeply anesthetized with a 2% lidocaine gel. Chromosomes were conventionally stained with 10% Giemsa and sequentially C-banded (King, 1980) and silver stained by the Ag-NOR method (Howell and Black, 1980).

3. Results

3.1. Phylogenetic inferences

In all our analyses, *Physalaemus* was monophyletic, *Pleurodema* was inferred as the sister group of a clade that included *Physalaemus*, *Edolorhina* and *Engystomops*, and *Pseudopaludicola* was the sister group of a clade composed of all the remaining leiuperines. In contrast, the relationships among *Physalaemus*, *Edalorhina* and *Engystomops* differed among the cladograms generated. Whereas *Edalorhina* was the sister group of the clade composed of *Physalaemus* and *Engystomops* in the Bayesian analyses (Appendices F and G), in all of the most parsimonious trees achieved by TNT and POY, *Edalorhina* was more closely related to *Engystomops* (Fig. 2; Appendices B–E).

The relationships inferred for the species of *Physalaemus* in all of the analyses were quite congruent (Figs. 3–5; Appendices B–G). *Physalaemus nattereri* (*Eupemphix nattereri* according to Nascimento et al., 2005) was nested within the *Physalaemus* clade as the sister taxon of a clade composed of all the representatives of the *P. signifer* and *P. deimaticus* Groups (sensu Nascimento et al., 2005) (Fig. 3; Appendices B–G). The monophyly of the *P. deimaticus* Group as defined by Nascimento et al. (2005) was inferred in all the analyses, with high statistical support (Fig. 3; Appendices B–G). A highly supported clade composed of the representatives of the *P. signifer* Group (sensu Nascimento et al., 2005), except *P. maculiventris*, was also recovered in all the analyses (Fig. 3; Appendices B–G). A clade with all the species of the *P. signifer* Group as recognized by Nascimento et al. (2005), including *P. maculiventris*, however, was not achieved in our analyses, since *P. maculiventris* was inferred as the sister taxon of a clade that included the remaining species of the *P. signifer* Group and all the species of the *P. deimaticus* Group (Fig. 3; Appendices B–G). The clade composed of *P. nattereri*,

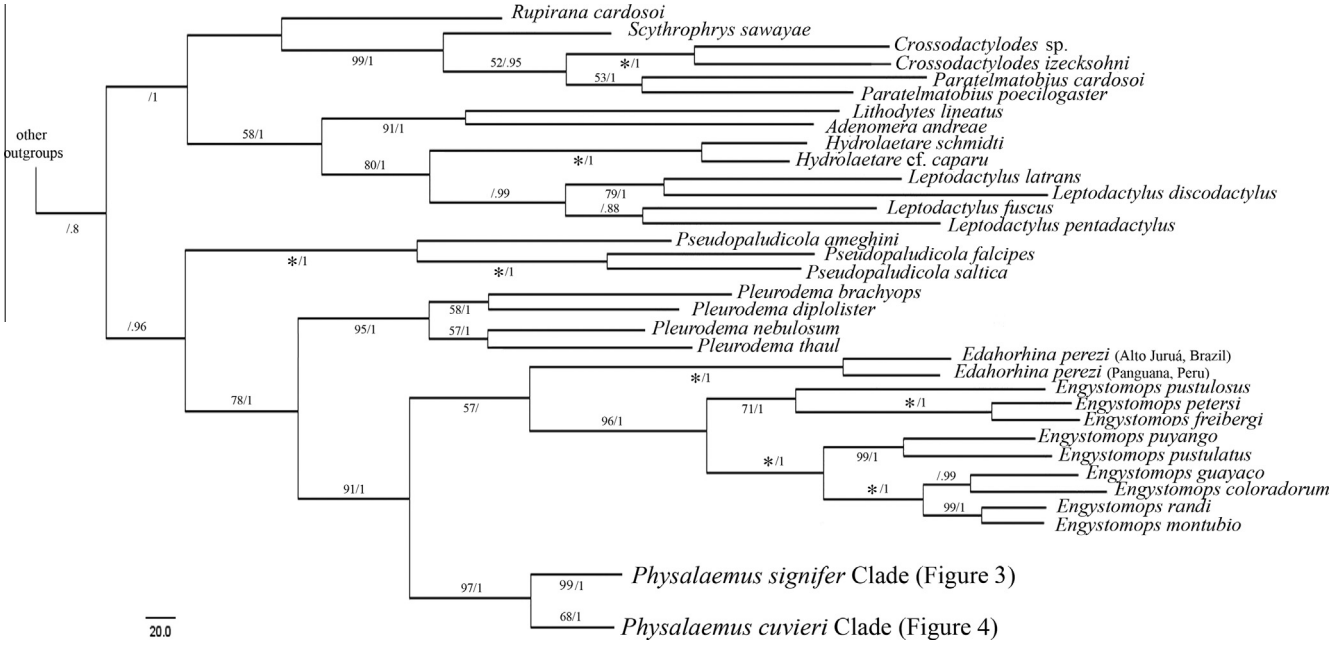

Fig. 2. Intergeneric relationships of *Physalaemus*. Overall relationships recovered in one of the 32 most parsimonious trees inferred in the TNT analysis of the concatenated mitochondrial and *Rag-1* sequences. Branch lengths are proportional to inferred amounts of sequence evolution. All of the nodes shown here were also present in the strict consensus tree. The interspecific relationships in the *Physalaemus signifer* Clade and *Physalaemus cuvieri* Clade are shown in Figs. 3 and 4. Numbers to the left of branches are bootstrap values (⩾ 50%). Asterisks indicate bootstrap value of 100%. Numbers to the right of branches are posterior probabilities achieved in the Bayesian analysis of the same data matrix (see Appendix F).

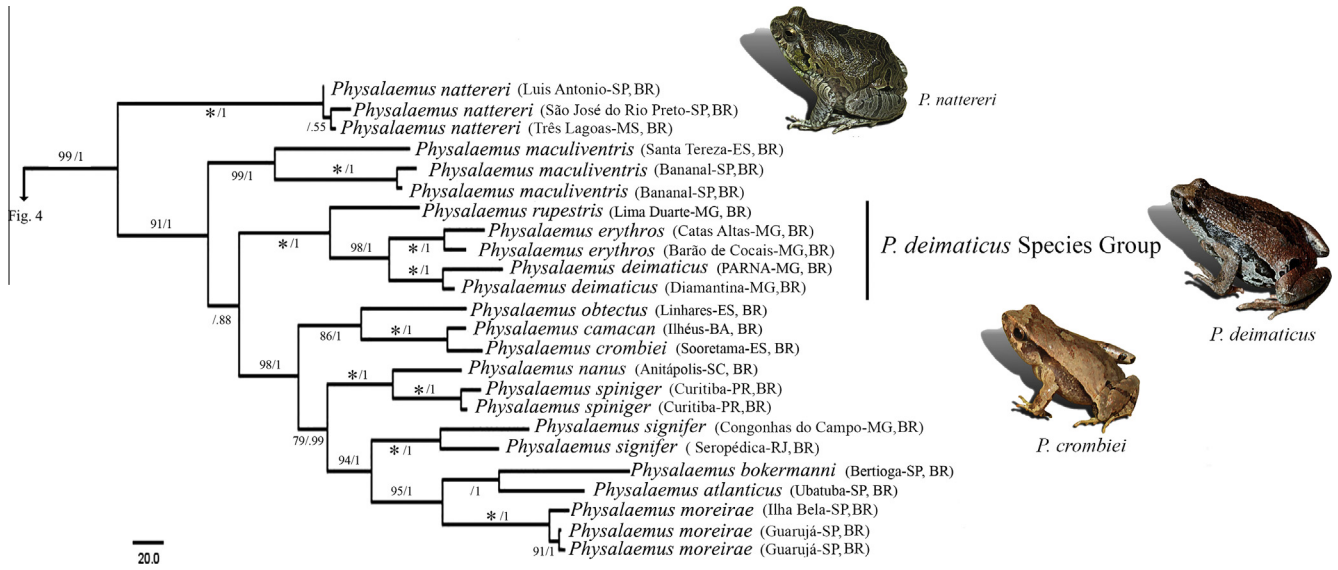

Fig. 3. Interspecific relationships in the *Physalaemus signifer* Clade. Partial view of one of the 32 most parsimonious trees inferred in the TNT analysis of the concatenated mitochondrial and *Rag-1* sequences. Branch lengths are proportional to inferred amounts of sequence evolution. All of the nodes were also present in the strict consensus tree. Numbers to the left of branches are bootstrap values (⩾ 50%). Asterisks indicate bootstrap value of 100%. Numbers to the right of branches are posterior probabilities achieved in the Bayesian analysis of the same data matrix (see Appendix F). Branches lacking posterior probability were not recovered in the Bayesian analysis.

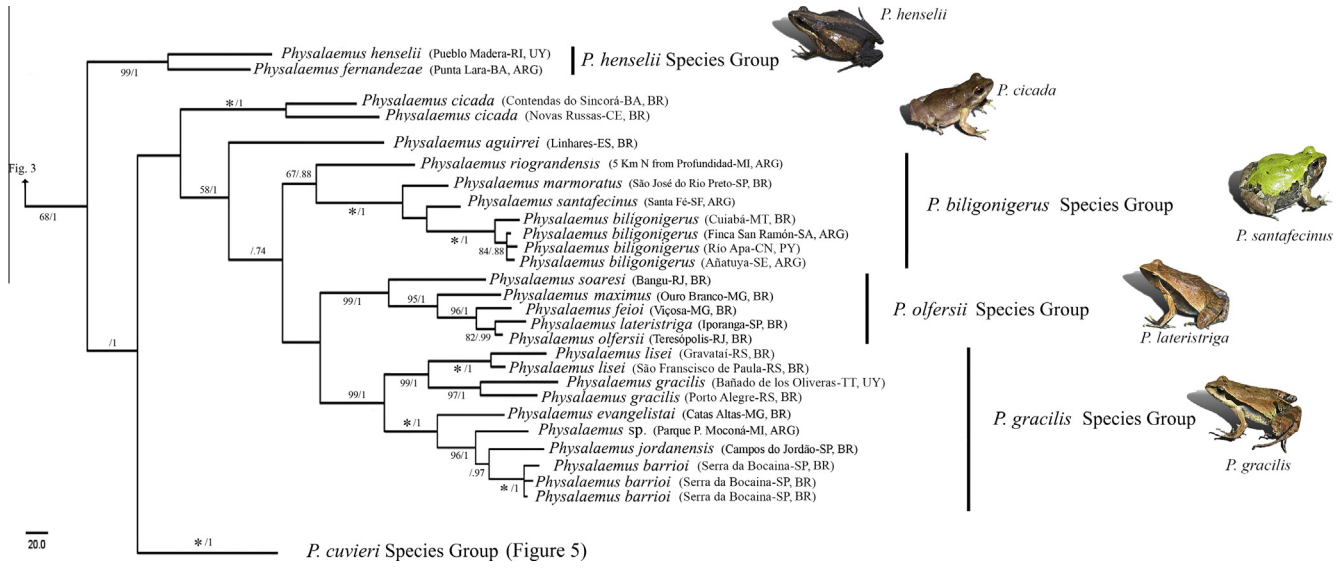

Fig. 4. Interspecific relationships in the *Physalaemus cuvieri* Clade. Partial view of one of the 32 most parsimonious trees inferred in the TNT analysis of the concatenated mitochondrial and *Rag-1* sequences. Branch lengths are proportional to inferred amounts of sequence evolution. All of the nodes shown here were also present in the strict consensus tree. The interspecific relationships in the *Physalaemus cuvieri* Species Group are shown in Fig. 5. Numbers to the left of branches are bootstrap values (⩾ 50%). Asterisks indicate bootstrap value of 100%. Numbers to the right of branches are posterior probabilities achieved in the Bayesian analysis of the same data matrix (see Appendix F). Branches lacking posterior probability were not recovered in the Bayesian analysis.

the *P. deimaticus* Group and all the species included in the *P. signifer* Group by Nascimento et al. (2005) was highly supported by bootstrap or posterior probability in our analyses (Fig. 3; Appendices B–G) and is hereafter called *P. signifer* Clade.

The species of *Physalaemus* not included in the *P. signifer* Clade were nested in a distinct group (Figs. 4 and 5), which we call the *P. cuvieri* Clade (Fig. 3; Appendices B–G). In this major clade, a monophyletic group with all the five species assigned to the *P. gracilis* Group (sensu Nascimento et al., 2005) was identified in all the inferences (Fig. 4; Appendices B–G). In contrast, we could not support the *P. albifrons*, *P. cuvieri*, *P. henselii* and *P. olfersii* phenetic groups, as they were recognized by Nascimento et al. (2005).

Because *Physalaemus albifrons* was nested within the *P. cuvieri* Group instead of being closely related to *P. biligonigerus*, *P. marmoratus* and *P. santafecinus*, the *P. albifrons* and *P. cuvieri* Groups proposed by Nascimento et al. (2005) were not corroborated. In addition, our analyses did not support inclusion of *P. cicada* in the *P. cuvieri* Group (Figs. 4 and 5; Appendices B–G).

The *Physalaemus henselii* Group of Nascimento et al. (2005) was not monophyletic because *P. riograndensis* was not closely related to *P. henselii* and *P. fernandezae*. In all our analyses, *P. riograndensis* was recovered as the sister species of a clade composed of *P. biligonigerus*, *P. marmoratus* and *P. santafecinus* (Fig. 4; Appendices B–G).

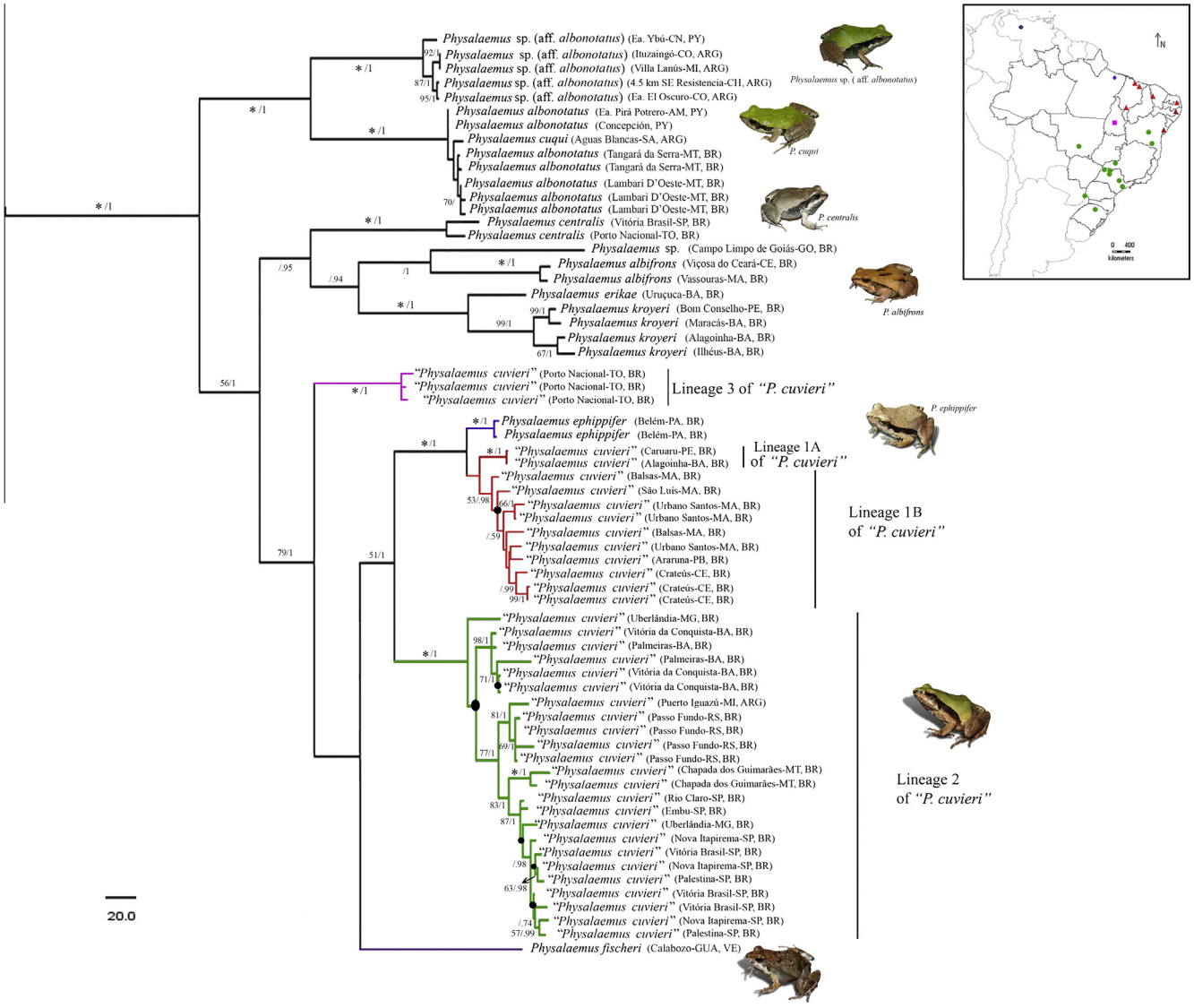

Fig. 5. Interspecific relationships in the *Physalaemus cuvieri* Species Group. Partial view of one of the 32 most parsimonious trees inferred in the TNT analysis of the concatenated mitochondrial and *Rag-1* sequences. Branch lengths are proportional to inferred amounts of sequence evolution. Circles indicate nodes that collapsed in the strict consensus tree (see Appendix B). Numbers to the left of branches are bootstrap values (⩾50%). Asterisks indicate bootstrap value of 100%. Numbers to the right of branches are posterior probabilities achieved in the Bayesian analysis of the same data matrix (see Appendix F). Branches lacking posterior probability were not recovered in the Bayesian analysis. The lineages of "*P. cuvieri*", *P. ephippifer* and *P. fischeri* are shown in different colors and their sampling localities are indicated in the inset map.

The *Physalaemus olfersii* Group of Nascimento et al. (2005) was polyphyletic because *P. aguirrei* did not form a clade with *P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi* and *P. lateristriga* (the two latter species were described after the proposal of the *P. olfersii* Group and were assigned to this group by Cassini et al., 2010) (Fig. 4; Appendices B–G). In all the analyses, *P. aguirrei* was the sister group of a clade composed of the *P. gracilis* Group, the clade (*P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi*, *P. lateristriga*) and the clade (*P. riograndensis*, *P. biligonigerus*, *P. marmoratus P. santafecinus*), but the relationships between these two latter clades and the *P. gracilis* Group varied among the analyses. In the TNT analyses, the clade (*P. riograndensis*, *P. biligonigerus*, *P. marmoratus*, *P. santafecinus*) was the sister group of a clade composed of the *P. gracilis* Group and the clade (*P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi*, *P. lateristriga*) (Fig. 4; Appendices B–C). In the POY analyses (Appendices D–E) and in the Bayesian analysis of the mitochondrial matrix (Appendix G), the clade (*P. riograndensis*, *P. biligonigerus*, *P. marmoratus*, *P. santafecinus*) was the sister group of the *P. gracilis* Group. In the Bayesian analysis of the concatenated matrix (Appendix F), the relationships between *P. gracilis* Group, the clade (*P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi*, *P. lateristriga*) and the clade (*P. riograndensis*, *P. biligonigerus*, *P. marmoratus*, *P. santafecinus*) remain unresolved.

3.1.1. Relationships of *Physalaemus cuqui* and *P. albonotatus*

We notice that *Physalaemus cuqui* composed with *P. albonotatus* a clade that was the sister group of a clade that included individuals collected from Paraguay and Argentina [here named *Physalaemus* sp. (aff. *albonotatus*)], which are morphologically similar to *P. albonotatus* but distinguished from it by the advertisement call (Fig. 5; Appendices B–G). In all the analyses, we also inferred paraphyly of *P. albonotatus* with regard to *P. cuqui* because the specimen of *P. cuqui* analyzed here was nested among the exemplars of *P. albonotatus* (Fig. 5; Appendices B–G). The genetic distance, estimated from 16S partial sequences, between *P. cuqui* and the group composed of these exemplars of *P. albonotatus* was only 0.3%. In contrast, a high uncorrected *p*-distance (7.3%) was observed between the clade (*P. albonotatus*, *P. cuqui*) and

Table 1
Uncorrected *p*-distances between 16S partial genes of individuals of species/lineages of the *Physalaemus cuvieri* Species Group. In gray, the uncorrected *p*-distances found within each lineage or species. –: data not estimated because only one sequence was available.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. Lineage 1A of "*P. cuvieri*"	0.000														
2. Lineage 1B of "*P. cuvieri*"	0.014	0.007													
3. *P. ephippifer*	0.015	0.012	0.000												
4. Lineage 2 of "*P. cuvieri*"	0.075	0.067	0.063	0.012											
5. Lineage 3 of "*P. cuvieri*"	0.049	0.041	0.040	0.060	0.004										
6. *P. fischeri*	0.087	0.081	0.078	0.081	0.079	–									
7. Bolivian Lineage A of *P. cuvieri*	0.055	0.051	0.053	0.071	0.034	0.094	0.001								
8. Bolivian Lineage B of *P. cuvieri*	0.053	0.049	0.051	0.071	0.036	0.101	0.031	0.000							
9. *P. kroyeri*	0.079	0.081	0.077	0.087	0.088	0.110	0.090	0.087	0.007						
10. *Physalaemus* sp. (Goiás. Brazil)	0.070	0.066	0.061	0.064	0.067	0.095	0.085	0.080	0.084	–					
11. *P. albifrons*	0.079	0.072	0.075	0.096	0.081	0.118	0.089	0.085	0.095	0.078	0.008				
12. *P. erikae*	0.072	0.074	0.070	0.077	0.073	0.106	0.081	0.076	0.036	0.078	0.084	–			
13. *P. centralis*	0.086	0.084	0.080	0.080	0.091	0.109	0.098	0.088	0.086	0.076	0.085	0.090	0.021		
14. (*P. albonotatus*, *P. cuqui*) clade	0.110	0.104	0.106	0.105	0.096	0.121	0.096	0.094	0.120	0.099	0.101	0.112	0.116	0.004	
15. *Physalaemus* sp. (aff. *albonotatus*)	0.100	0.100	0.096	0.087	0.084	0.110	0.092	0.096	0.108	0.086	0.117	0.096	0.123	0.073	0.004

its sister group [*Physalaemus* sp. (aff. *albonotatus*) clade] (Table 1).

3.1.2. Relationships of Physalaemus cuvieri, P. ephippifer and P. fischeri

The paraphyly of *Physalaemus cuvieri* with respect to *P. ephippifer* was inferred in all our analyses. The specimens first identified as *Physalaemus cuvieri*, collected from 19 localities within the wide geographical distribution of this species, clustered in distinct clades. One of the clades comprised the specimens distributed from Central to Southern Brazil and Argentina (Vitória da Conquista-BA, Palmeiras-BA, Chapada dos Guimarães-MT, Uberlândia-MG, Nova Itapirema-SP, Palestina-SP, Vitória Brasil-SP, Embu-SP, Rio Claro-SP, Passo Fundo-RG, Puerto Iguazú-MI,) (Fig. 5) and was highly supported in all our inferences (Fig. 5; Appendices B–G). Another highly supported clade clustered the specimens from Porto Nacional-TO (Fig. 5; Appendices B–G). The remaining specimens of *P. cuvieri*, which were distributed in Northern/Northeastern Brazil, were clustered into two clades, one of them composed of the specimens from Caruaru-PE and Alagoinha-BA, and the other composed of the individuals from Crateús–CE, Balsas–MA, São Luís–MA, Urbano Santos–MA and Araruna-PB (Fig. 5; Appendices B–G). In the maximum -parsimony analyses, both of these clades together composed the sister group of *P. ephippifer* (Fig. 5; Appendices B–E). In the Bayesian analysis of the concatenated matrix, the clade with the individuals from Caruaru-PE and Alagoinha-BA was recovered as the sister group of *P. ephippifer*, despite with very low support (0.54) (Appendix F). In the Bayesian analysis of the mitochondrial matrix, the clade that grouped the specimens from Caruaru-PE and Alagoinha-BA was recovered in a polytomy together with the *P. ephippifer* clade and the clade that clustered the remaining specimens from Northern/Northeastern Brazil (Appendix G). The clade composed of the two clades that comprised the exemplars of *P. cuvieri* from Northern/Northeastern Brazil and the *P. ephippifer* clade was highly supported in all the analyses; this clade was the sister group of the clade that comprised the specimens from Central to Southern Brazil and Argentina (Fig. 5; Appendices B–G).

Therefore, based on the phylogenetic inferences, four different groups, distributed over distinct geographic areas, may be recognized for *P. cuvieri*, diagnosing putatively independent evolutionary lineages, hereafter called Lineage 1A (Caruaru-PE, Alagoinha-BA), Lineage 1B (Crateús-CE, Urbano Santos-MA, Araruna-PB, Balsas-MA, São Luís-MA), Lineage 2 (Vitória da Conquista-BA, Palmeiras-BA, Chapada dos Guimarães-MT, Uberlândia-MG, Nova Itapirema-SP, Palestina-SP, Vitória Brasil-SP, Embu-SP, Rio Claro-SP, Passo Fundo-RG, Puerto Iguazú-MI) and Lineage 3 (Porto Nacional-TO) of "*P. cuvieri*".

The relationships of *Physalaemus fischeri* and the Lineage 3 of "*P. cuvieri*" with the clade that includes *P. ephippifer* and the remaining lineages recognized among the individuals first identified as *P. cuvieri* remain unclear. In the Bayesian inferences (Appendices F–G) and POY analyses (Appendices D–E) *P. fischeri* was the sister group of a clade that includes *P. ephippifer* and all the individuals first identified as *P. cuvieri*, whereas in the TNT analyses (Fig. 5; Appendices B–C) the Lineage 3 of "*P. cuvieri*" was the sister group of a clade composed of all the other lineages of "*P. cuvieri*", *P. ephippifer* and *P. fischeri*, rendering *P. cuvieri* paraphyletic also with respect to *P. fischeri*. Neither of these two arrangements was highly supported by bootstrap or posterior probability, whereas the clade that includes all the lineages of "*P. cuvieri*", *P. ephippifer* and *P. fischeri* was (Fig. 5; Appendices B–G).

3.2. Genetic comparisons among the lineages related to Physalaemus cuvieri, P. ephippifer and P. fischeri

Low genetic distance was observed between the Lineages 1A and 1B of "*P. cuvieri*" (1.4%) as well as between them and *P. ephippifer* (1.5% and 1.2%) (Table 1). In contrast, high values of uncorrected *p*-distance were observed when Lineages 2 and 3 of "*P. cuvieri*" were compared with each other (6.0%) or with *P. ephippifer* and the Lineages 1A and 1B of "*P. cuvieri*" (ranging from 4.0% to 7.5%) (Table 1). By comparing 16S gene fragments of specimens from two Bolivian lineages of *Physalaemus cuvieri* recognized by Jansen et al. (2011) with the equivalent gene fragments obtained from individuals nested in the four population-level lineages of *P. cuvieri* recognized here, high values of uncorrected *p*-distance were observed (3.4% to 7.1%), as was the distance between the Bolivian lineages (3.1%) (Table 1). Several of the aforementioned values were higher than that which emerged from the comparison of samples from *P. erikae* and *P. kroyeri* (3.6%), two valid species also assigned to the *P. cuvieri* Group (Table 1). The uncorrected *p*-distances estimated between *P. fischeri* and all of those *P. cuvieri* lineages were also high (ranging from 7.9% to 10.1%), as was that calculated between *P. fischeri* and *P. ephippifer* (7.8%) (Table 1).

3.3. The karyotype of Physalaemus deimaticus

All the 25 metaphases of the SMRP 497.2 specimen of *Physalaemus deimaticus* showed 22 chromosomes, including 10 pairs of metacentric or submetacentric chromosomes and one pair (chromosome pair 11) of telocentric chromosomes (Fig. 6A). Large amounts of C-banded heterochromatin were detected in the centromeric/pericentromeric regions of all the chromosomes (Fig. 6A). The nucleolus organizer region (NOR) was detected in

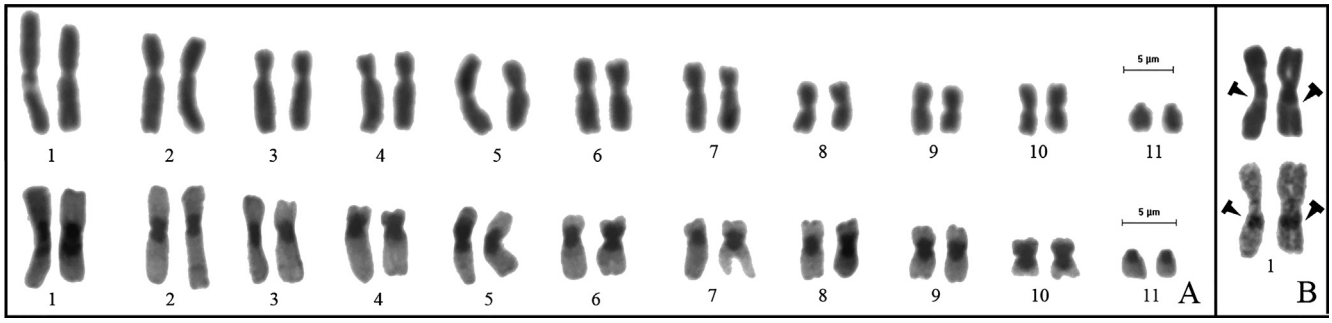

Fig. 6. A. Karyotype of *Physalaemus deimaticus* stained with Giemsa (top) and C-banded (bottom). Note the telocentric chromosome pair 11. In B, the same NOR-bearing chromosome 1 C-banded in A after staining with Giemsa (top) or submitted to the Ag-NOR method. The arrows in B indicate the secondary constrictions of the NORs in the Giemsa stained chromosome and the silver stained NORs.

the long arm of the large metacentric chromosomes classified as number 1, and it coincided with a C-band (Fig. 6B).

4. Discussion

4.1. Intergeneric relationships of Physalaemus

The close relationship of *Pleurodema* with a clade composed of *Physalaemus*, *Edalorhina* and *Engystomops*, previously inferred by Pyron and Wiens (2011), Faivovich et al. (2012) and Fouquet et al. (2013), was also recovered in all our phylogenetic inferences. Although with low statistical support, our inferences also provide additional evidence of the close relationship between *Pseudopaludicola* and the remaining Leiuperinae, corroborating Fouquet et al. (2013). In contrast, our analyses were not conclusive with regard to the closer intergeneric relationships of *Physalaemus*. Although the Bayesian inferences yielded *Physalaemus* as the sister genus of *Engystomops*, as also recovered by the Bayesian analyses of Pyron and Wiens (2011) and Fouquet et al. (2013), the maximum-parsimony analyses yielded a closer relationship between *Edalorhina* and *Engystomops*, as previously inferred by Frost et al. (2006), Grant et al. (2006), Lourenço et al. (2008) and Faivovich et al. (2012).

4.2. Interspecific relationships in Physalaemus

We increased from one to three the number of exemplars (from different localities) of *Physalaemus nattereri* and from eight to 41 the number of species of *Physalaemus* included in phylogenetic analyses, providing a reliable test of the monophyly of this genus. In all our phylogenetic inferences, *P. nattereri* was recovered inside the *Physalaemus* clade, corroborating that *Eupemphix* should be considered a junior synonym of *Physalaemus*, as previously stated by Faivovich et al. (2012).

The topologies recovered in all our phylogenetic inferences suggest two major clades in *Physalaemus*: (i) the *Physalaemus signifer* Clade, composed of *P. nattereri*, and the species previously placed in the *P. deimaticus* and *P. signifer* Groups; and (ii) the *Physalaemus cuvieri* Clade, with the remaining species of *Physalaemus*. This inference is congruent with the topologies obtained by previous authors (Pyron and Wiens, 2011; Faivovich et al., 2012).

Among leiuperines, two morphological characters have been used for supra-specific arrangements: the maxillary and premaxillary dentition (*e.g.*, Cochran, 1955; Bokermann, 1962, 1966; Cannatella et al., 1998); and the tarsal tubercle (the inner tarsal tubercle in some papers) (*e.g.*, Cochran, 1955; Cannatella and Duellman, 1984; Cannatella et al., 1998; Funk et al., 2008). Lynch (1970) considered this variation too discordant to reveal species relationships. However, more recently, Nascimento et al. (2005) noted the absence of premaxillary and maxillary dentition for *P. nattereri* (as *Eupemphix*) and for all species they assigned to the *P. signifer* and *P. deimaticus* Groups, which correspond to the *P. signifer* Clade inferred in our analyses. In contrast, they noted the presence of premaxillary and maxillary teeth in the remaining species groups (*i.e.*, the *P. albifrons*, *P. cuvieri*, *P. gracilis*, *P. henselii* and *P. olfersii* Groups). Nevertheless, as shown by several authors (Cardoso and Haddad, 1985; Heyer, 1985; Heyer and Wolf, 1989; Lobo, 1996; Pombal and Madureira, 1997; Haddad and Sazima, 2004; Pimenta et al., 2005; Weber et al., 2005; Cruz et al., 2007), most species of the *P. signifer* Clade have maxillary and premaxillary teeth (in some papers indicated as "teeth not visible but discernible by probe"). In addition, all species of *Edalorhina*, *Pleurodema* and *Pseudopaludicola*, and the species of *Engystomops* of the Duovox clade (*i.e.*, *E. coloradorum*, *E. guayaco*, *E. montubio*, *E. pustulatus*, *E. puyango* and E. *randi*) have maxillary teeth (Lynch, 1970; Lobo, 1995; Ron et al., 2006). The maxillary teeth are absent in species of *Engystomops* of the *E. edentulus* clade (*E. freibergi*, *E. petersi* and *E. pustulosus*), and this character state optimizes as a synapomorphy of this clade, as previously indicated (Cannatella et al., 1998; Ron et al., 2005; Ron et al., 2006; Ron et al., 2010). Therefore, the presence of premaxillary and maxillary teeth is plesiomorphic in *Physalaemus*, and the absence versus presence of premaxillary and maxillary teeth must be carefully considered in this genus.

In contrast, the tarsal tubercle clearly allows characterizing the species of *Physalaemus* as belonging to two major clades. All species of the *P. signifer* Clade lack tarsal tubercles, whereas all species of the *P. cuvieri* Clade have a tarsal tubercle with variable development (polymorphic in *P. lateristriga*, see Cassini et al., 2010). Analogously, this character was used for characterizing the two major groups in *Engystomops*, and the absence of the tarsal tubercle was suggested as a synapomorphy of the Duovox clade of *Engystomops* (Cannatella et al., 1998; Ron et al., 2005, 2006). In the remaining leiuperines, this character also shows variation; all species of *Pseudopaludicola* and most *Pleurodema* lack the tarsal tubercle (except for *P. allium* and *P. diplolister*, which do have tarsal tubercles; Maciel and Nunes, 2010), whereas in the two species of *Edalorhina*, tubercles are present (Heyer, 1975). Thus, although in both *Physalaemus* and *Engystomops* these characters discriminate major groups, their optimization in leiuperines is ambiguous.

4.2.1. The Physalaemus signifer Clade

A remarkable morphological characteristic of the species of the *Physalaemus signifer* Clade is the presence of a dark arrowhead-shaped mark on the dorsum (*e.g.*, Bokermann 1966; Cardoso and Haddad, 1985; Pimenta et al., 2005; Caramaschi

et al., 1991; Caramaschi et al., 2003). The presence of this trait in *P. nattereri* was noted by Ananias et al. (2007) and Kolenc et al. (2011), and it was previously proposed as a synapomorphy of the *P. signifer* Group (sensu Nascimento et al., 2005) shared with *P. nattereri* (Ananias et al., 2007). In our phylogenetic hypotheses, this character optimized as a synapomorphy of the *P. signifer* Clade.

Physalaemus angrensis, *P. caete* and *P. irroratus*, not included in our analyses, were originally included in the *P. signifer* Group (sensu Nascimento et al., 2005) by Weber et al. (2005), Nascimento et al. (2005) and Cruz et al. (2007), respectively. These species lack the tarsal tubercles and have a dark arrowhead-shaped mark on the dorsum. Therefore, we tentatively assign them to the *P. signifer* Clade.

Another synapomorphy for the *Physalaemus signifer* Clade emerges from the cytogenetic analysis. The five species of the *P. signifer* Group already karyotyped (*i.e.*, *P. atlanticus*, *P. crombiei*, *P. moreirae*, *P. spiniger* and *P. signifer*) have a telocentric chromosome pair as the smallest pair (chromosome pair 11) of the complement (de Lucca et al., 1974; Silva et al., 2000; Ananias et al., 2007), as do *P. nattereri* (Beçak, 1968; Lourenço et al., 2006; Ananias et al., 2007) and *P. deimaticus* (present work), which is the only species of the *P. deimaticus* Group karyotyped to date. In contrast, the remaining 23 species of *Physalaemus* studied cytogenetically have a biarmed chromosome pair 11 (Beçak et al., 1970; Denaro, 1972; de Lucca et al., 1974; Silva et al., 1999, 2000; Amaral et al., 2000; Quinderé et al., 2009; Tomatis et al., 2009; Milani et al., 2010; Nascimento et al., 2010; Provete et al., 2012; Vittorazzi et al., 2014), except for *P. fernandezae* (Tomatis et al., 2009). The phylogenetic relationships inferred herein provide strong evidence for the independent origin of the telocentric chromosome 11 of *P. fernandezae* and the chromosome 11 of species previously allocated to the *P. signifer* Group, corroborating the hypothesis discussed by Tomatis et al. (2009). Our analyses also strongly suggest that the telocentric chromosome 11 of *P. nattereri* is homeologous to the chromosome 11 found in the species of the *P. signifer* Group, constituting a synapomorphy for the *P. signifer* Clade (Fig. 5).

Inside the *Physalaemus signifer* Clade, a monophyletic group that includes all the species of the *P. deimaticus* Group previously proposed by Nascimento et al. (2005) could be recognized and was strongly supported by our analyses. *Physalaemus nattereri*, the *P. deimaticus* Species Group (*i.e.*, *P. deimaticus*, *P. erythros* and *P. rupestris*) and the remaining species of the *P. signifer* Clade (*i.e.*, *P. atlanticus*, *P. bokermanni*, *P. camacan*, *P. crombiei*, *P. nanus*, *P. obtectus*, *P. signifer*, *P. spiniger*, *P. maculiventris* and *P. moreirae*) occur in different biomes. *Physalaemus nattereri*, which is the sister taxon to a clade comprising the remainder of the *P. signifer* Clade, is widespread in open areas from east of Paraguay and Bolivia to central and southeastern Brazil (IUCN, 2013.2), whereas the species of the *P. deimaticus* Group are restricted to high elevations of the Espinhaço Mountain Range (Nascimento et al., 2005; Frost, 2014), localized in the Brazilian state of Minas Gerais between the Cerrado and Atlantic rainforest biomes, and the remaining species of the *P. signifer* Clade occur in the Atlantic rainforest (Nascimento et al., 2005). The Atlantic rainforest and the Espinhaço Mountain Range are areas of high endemism and biodiversity (Costa et al., 2000; Nogueira et al., 2011; Freitas et al., 2012).

4.2.2. The Physalaemus cuvieri Clade

This major clade, which comprised all the *Physalaemus* species not included in the *P. signifer* Clade, was not strongly supported in our analyses, and further studies are still necessary to confirm it. However, five species groups may be recognized in the *P. cuvieri* Clade (the *P. cuvieri* Group, the *P. biligonigerus* Group, the *P. gracilis* Group, the *P. henselii* Group and the *P. olfersii* Group) (discussed below) in addition to *P. cicada* and *P. aguirrei*. Despite the *P. henselii* Group being the sister taxon to the remainder of the *P. cuvieri* Clade and *P. aguirrei* being the sister taxon of a clade composed of the *P. biligonigerus*, *P. olfersii* and *P. gracilis* Groups in all our inferences, the relationships between these three latter species groups remain to be elucidated.

The larval oral disc in *Physalaemus* can have five different configurations based on the combination of three characters: ventral gap, ventrolateral gaps and number of lower tooth rows (see Vera Candioti et al., 2011). The common character that defines the oral disc configurations C4 and C5 (sensu Vera Candioti et al., 2011) is the presence of a ventral gap in the lower marginal papillae. This character state is unique among non-bufonid Leptodactyliformes and is shared by the *P. henselii* Group, *P. cicada* and all species of the *P. cuvieri* Group (as here defined). It is also present in a few *Pseudopaludicola* species (see Vera Candioti et al., 2011), but is not present in species of the *P. olfersii*, *P. biligonigerus* or *P. gracilis* Groups, or in *P. fischeri* (*P. cuvieri* Group) or in *P. aguirrei*. Optimization of this character on the phylogenetic hypotheses inferred here reveals that the presence of a ventral gap is a synapomorphy of the *P. cuvieri* Clade with reversions in *P. fischeri* and in the clade (*P. aguirrei*, *P. biligonigerus* Group, *P. gracilis* Group, *P. olfersii* Group).

4.2.2.1. The Physalaemus biligonigerus Species Group.

All our analyses recovered a highly supported clade consisting of *Physalaemus biligonigerus*, *P. marmoratus* and *P. santafecinus*, species that share some morphological similarities previously reported by Lynch (1970) and Nascimento et al. (2005). These species were not closely related to *P. nattereri* as proposed by Lynch (1970) or to *P. albifrons* as stated by Nascimento et al. (2005), leaving both the *P. nattereri* Group by Lynch and the *P. albifrons* Group as defined by Nascimento and colleagues polyphyletic.

Our phylogenetic inferences, therefore, corroborate that the large heterochromatic band present in the short arm of chromosome 3 of *Physalaemus biligonigerus*, *P. marmoratus* and *P. santafecinus* is a synapomorphy of this clade, and that this C-band had an independent origin from that found in the short arm of chromosome 3 of *P. nattereri*, as hypothesized by Vittorazzi et al. (2014).

In all our inferences, the clade composed of *Physalaemus biligonigerus*, *P. marmoratus* and *P. santafecinus* was the sister group of *P. riograndensis*, despite with low support. These four species share a similar tadpole oral disc morphogenetic pattern, characterized by two lower labial rows and marginal papillae developing without a ventral gap (C1 and C2 configurations sensu Vera Candioti et al., 2011). Whereas *P. riograndensis* maintains a configuration with ventrolateral gaps, tadpoles of the remaining three species have complete marginal papillae (Vera Candioti et al., 2011).

The presence of two lower labial tooth rows in *Physalaemus* is known only in these four species and, among other leiuperines, in three species of *Pleurodema* (*P. guayapae*, *P. nebulosum* and *P. tucumanum*; Cei, 1980), although the developmental processes involved differ (Vera Candioti et al., 2011). The presence of two lower labial tooth rows may represent, therefore, a synapomorphy of these species of *Physalaemus* and defines an oral disc that truncates its development with regard to the plesiomorphic larval labial tooth-row formula 2/3.

Based on this analysis of tadpole oral disc and on our phylogenetic inferences, we recognize a *Physalaemus biligonigerus* Species Group composed of *P. biligonigerus*, *P. marmoratus*, *P. santafecinus* and *P. riograndensis*.

4.2.2.2. The Physalaemus henselii Species Group. This group is composed of *Physalaemus henselii* and *P. fernandezae*, which were recovered as sister species in all our analyses. The species of the *P. henselii* Group have the most southerly distribution among the *Physalaemus* species, inhabiting open areas in the Pampa and Uruguayan Savanna Ecoregions, and their reproduction occurs in the winter (Barrio, 1964; Kolenc et al., 2006; Maneyro et al., 2008).

These species have been considered closely related based on similar adult and larval external morphology, ecology and geographic distribution (Barrio, 1964; Barrio, 1965). Lobo (1996) suggested that *Physalaemus fernandezae* and *P. henselii* are sister taxa based on the presence in both species of a nonbifurcated sternal style and an open frontoparietal fontanelle. The very incomplete osteological data for *Physalaemus* limits the interpretation of this character in the phylogenetic context.

Physalaemus henselii and *P. fernandezae* were formerly included in the *Physalaemus cuvieri* Group by Lynch (1970) or in the *P. henselii* Group (with *P. riograndensis*) by Nascimento et al. (2005). However, several works indicated that the larval configuration conflicts with the inclusion of *P. riograndensis* in these groups (Alcalde et al., 2006; Kolenc et al., 2006; Vera Candioti et al., 2011). *Physalaemus henselii* and *P. fernandezae* share with *P. cicada* the larval oral disc configuration that displays three lower labial tooth rows and marginal papillae with a ventral gap (C5 sensu Vera Candioti et al., 2011). This character combination of the oral disc is particular and, among the other leiuperines, was observed only in some specimens of *Pseudopaludicola falcipes* (revised by Vera Candioti et al., 2011). The presence of a third lower labial tooth row is a plesiomorphic character state observed in most leiuperines (except in *P. biligonigerus* Group and some species of *Pleurodema*, see Vera Candioti et al., 2011).

4.2.2.3. The Physalaemus gracilis Species Group. Our phylogenetic inferences corroborated the *Physalaemus gracilis* Group as previously recognized by Nascimento et al. (2005), which is composed of *P. barrioi*, *P. evangelistai*, *P. gracilis*, *P. jordanensis* and *P. lisei*. Additionally, an undescribed species from Argentina and Brazil (traditionally assigned to *P. gracilis*, Barrio, 1965) was recovered nested within this clade. No morphological synapomorphy supports the composition of this group, and the cytogenetic information is very fragmented, with chromosome data available only for *P. barrioi* (Provete et al., 2012) and *P. gracilis* (Brum-Zorrilla and Sáez, 1968). Most species of this clade are distributed in high regions of the Rain Atlantic Forest, although some species also inhabit the Uruguayan savannas ecoregion (*P. gracilis*), or transitional areas of Atlantic Forest, Cerrado and Campos Rupestres montane savannas in Serra do Cipó (*P. evangelistai*). Described tadpoles of this group share an oral disc configuration with three lower labial rows and complete marginal papillae (C3, sensu Vera Candioti et al., 2011). This condition is plesiomorphic and shared by the species included in *P. signifier* Clade, *P. olfersi* Species Group and several other Leiuperinaes.

4.2.2.4. The Physalaemus olfersii Species Group. The *Physalaemus olfersii* Group, as defined by Nascimento et al. (2005), was polyphyletic in all our analyses because *P. aguirrei* is not closely related to the clade composed of *P. olfersii*, *P. soaresi*, *P. maximus*, *P. feioi* and *P. lateristriga*; therefore, *P. aguirrei* should be excluded from the *P. olfersii* Species Group. Most of the species of the *P. olfersii* Group share a similar advertisement call, pulsed, without harmonic structure and without frequency modulation (see Cassini et al., 2010; Giaretta et al., 2009), whereas *P. aguirrei* have an unpulsed call with harmonic and frequency modulation like most of the species of the *P. cuvieri* and *P. gracilis* Groups (Bokermann, 1966).

Physalemus insperatus and *P. orophilus* were assigned to the *P. olfersii* Group (sensu Nascimento et al., 2005) in the original descriptions [Cruz et al. (2008) and Cassini et al. (2010), respectively]. Both species lack the dark arrowhead-shaped blotch in the dorsum, are morphologically very similar to other species of the group, and like all of them inhabit the Atlantic rainforest. In addition, *P. insperatus* have the tarsal tubercle weakly developed. In turn, *P. orophilus* lacks a tarsal tubercle, but adult males have an advertisement call consisting of only one pulsed note (with subpulses) like all the other members of *P. olfersii* Group (Giaretta et al., 2009; Cassini et al., 2010 and references therein). Therefore, we tentatively include both species in the *P. olfersii* Group.

4.2.2.5. The Physalaemus cuvieri Species Group. All our phylogenetic inferences show a clade that includes *Physalaemus albifrons*, *P. albonotatus*, *P. centralis*, *P. cuqui*, *P. cuvieri*, *P. ephippifer*, *P. erikae*, *P. fischeri* and *P. kroyeri*. This clade differs from the *P. cuvieri* Group recognized by Nascimento et al. (2005) only by the inclusion of *P. albifrons* and the exclusion of *P. cicada*. Our phylogenetic analyses conducted with POY and TNT did not group *P. cicada* with the species of the *P. cuvieri* Group, and in the Bayesian analyses (of the concatenated and mitochondrial matrices), the relationships of *P. cicada* in the *P. cuvieri* Clade remain unclear. Therefore, we avoided recognizing this species as a member of the *P. cuvieri* Group until further analyses are made.

According to Nascimento et al. (2005), *Physalaemus albifrons* was grouped together with *P. biligonigerus*, *P. marmoratus* and *P. santafecinus*. The close relationship of *P. albifrons* with *P. biligonigerus*, *P. marmoratus* and *P. santafecinus* was previously questioned by Vittorazzi et al. (2014) based on chromosomal data and tadpole morphology. Vittorazzi and colleagues noted that (i) the *P. albifrons* karyotype shows an interstitial heterochromatic band in the short arm of the metacentric chromosome 5, which is a characteristic shared by all the species of *P. cuvieri* group already karyotyped; and (ii) the *P. albifrons* karyotype does not have the large heterochromatic band in the short arm of chromosome 3 that is found in *P. biligonigerus*, *P. marmoratus* and *P. santafecinus* (discussed in section 4.2.2.1). The phylogenetic inferences shown herein allow us to interpret the interstitial heterochromatic band of the metacentric chromosome 5 as a synapomorphy of the *P. cuvieri* Group (including *P. albifrons*) as suggested by Vittorazzi et al. (2014).

With regard to tadpole morphological characters, the phylogenetic relationships recovered herein validate the hypothesis of Vittorazzi et al. (2014; based on data described by Vera Candioti et al., 2011), who proposed that the persistence of ventrolateral gaps in larval stages is a synapomorphy of the *Physalaemus cuvieri* Group (including *P. albifrons*).

Our phylogenetic analysis raised important taxonomic questions about some of the species included in the *Physalaemus cuvieri* Group. One question refers to *P. cuqui* and *P. albonotatus*. *Physalaemus cuqui* was described by Lobo (1993), who distinguished it from *P. albonotatus* by size, external morphology and osteological characters. Subsequently, Ferrari and Vaira (2001), based on exemplars from Parque Nacional Calilegua, Argentina, described the advertisement call of *P. cuqui*, which consisted of a long trilled whine substantially different from the unpulsed advertisement call of *P. albonotatus* described by Barrio (1965). In our study, the paraphyly of *P. albonotatus* with respect to the single specimen of *P. cuqui* was inferred, and a high level of genetic similarity between *P. cuqui* and *P. albonotatus* from Paraguay and Brazil was estimated, suggesting that a better study, including a morphological and bioacoustical revision and a better sampling of *P. cuqui*, is necessary for evaluating the possibility that *P. cuqui* Lobo, 1993 is a junior synonym of *Leiuperus albonotatus* Steindachner, 1864. In

contrast, a high value of genetic distance was found between the clade (*P. cuqui*, *P. albonotatus* from Paraguay and Brazil) and the clade composed of exemplars from Paraguay and Argentina that were not nested among the representatives of *P. albonotatus* (despite their morphological resemblance to this species). Because of these findings and because the exemplars from Paraguay and Argentina not nested inside the *P. albonotatus* clade differ greatly from *P. albonotatus* in advertisement call (Baldo et al., unpublished data), it is likely that these exemplars from Paraguay and Argentina represent a new species.

Other taxonomic questions arose from the inference of paraphyly of *Physalaemus cuvieri* with respect to *P. ephippifer* and possibly *P. fischeri*, which led to the recognition of distinct lineages among the Brazilian individuals first identified as *P. cuvieri*. The low values of genetic distance observed between the Brazilian Lineages 1A and 1B of "*P. cuvieri*" and *P. ephippifer*, which together constitute a monophyletic group, raise the hypothesis that these lineages may in fact represent *P. ephippifer* population-level groups. However, a significant karyotypic divergence was observed between *P. ephippifer* (Nascimento et al., 2010) and the specimens in Lineage B already studied cytogenetically (*i.e.*, specimens from Crateús-CE and Urbano Santos-MA) (Quinderé et al., 2009). The karyotype of the topotypes of *P. ephippifer* (including the two specimens used in the phylogenetic inferences presented herein) (Nascimento et al., 2010) differs from those found in the Lineage 1B of "*P. cuvieri*" (Quinderé et al., 2009) by presenting heteromorphic sex chromosomes Z and W. In the *P. ephippifer* karyotype, the NORs were restricted to the Z and W chromosomes (Nascimento et al., 2010), whereas the karyotypes of specimens in the Lineage 1B show the NORs in chromosomes 8 and 9, in a highly polymorphic condition (Quinderé et al., 2009). Therefore, further studies including the analysis of other genes and populations are needed to evaluate this taxonomic question as well as the role of the sex chromosome heteromorphism in the evolution of this group.

It is noticeable that the Brazilian Lineages 2 and 3 of "*P. cuvieri*", which showed high uncorrected *p*-distances in the 16S partial gene when compared with each other and with the Lineages 1A and 1B of "*P. cuvieri*" and *P. ephippifer*, correspond to distinct karyotypic groups that differ especially in the location of nucleolus organizer regions (NORs) (Silva et al., 1999; Quinderé et al., 2009). In the karyotypes of specimens in the Lineage 2 of "*P. cuvieri*", the principal NOR could be found in chromosome 8 or chromosome 11 [see Quinderé et al. (2009) for details], whereas in the karyotypes of the individuals from Porto Nacional (Lineage 3 of "*P. cuvieri*"), multiple NORs were present and could be found in chromosomes 1, 3, 4, 5 and 10 (Quinderé et al., 2009), in contrast to the karyotypes of the Lineages 1B and 2. Taking these chromosomal data together with the phylogenetic inferences and the *p*-distance data, we suggest that each of the Lineages 2 and 3 may represent a valid species. Based on the genetic divergences estimated from 16S partial gene, we also infer that these putative species do not correspond to either of those lineages of "*P. cuvieri*" found in Bolivian localities by Jansen et al. (2011). However, because of the wide geographical distribution of *Physalaemus cuvieri* and the uncertainty of its type locality, a deep review is still necessary to resolve the taxonomy of this putative species complex.

4.3. The nucleolus organizer regions of Physalaemus

The nucleolus organizer regions, in addition to the C-bands, are the most common traits used for the study of the karyotypes of anurans because techniques frequently employed for cytogenetic studies of other vertebrates, including G-banding, fail to provide good results in anuran chromosomes. In some of the anuran species karyotyped, a single pair of NORs was found in the diploid complement, and this character is apparently fixed in the sampled populations (*e.g.*, Schmid, 1978a,b; Lourenço et al., 2000; Veiga-Menoncello et al., 2003; Cardozo et al., 2011; Rodrigues et al., 2011). In contrast, multiple NORs as well as interpopulational and/or intrapopulational variations in the numbers or locations of NORs were reported for a number of species of anurans (*e.g.*, Wiley et al., 1989; Foote et al., 1991; Schmid et al., 1995; Kaiser et al., 1996; Bruschi et al., 2014). Such variation causes the inference of homology between interspecific NOR-bearing chromosomes as well as the evolutionary analysis of the NOR to be viewed with caution.

In the case of *Physalaemus*, some inferences may be made with regard to the character "location of fixed NORs" after the analysis of the phylogenetic relationships inferred here. The NOR-bearing chromosomes 8 found in *P. cuvieri*, *P. albonotatus* and *P. albifrons* (Silva et al., 2000; Quinderé et al., 2009; Vittorazzi et al., 2014) are very similar in morphology. The chromosomal morphology and the position of the NOR in the NOR-bearing chromosome 8 of *Pleurodema diplolister* (Lourenço et al., 2006) and, to a lesser extent, the chromosome 8 of *Edalorhina perezi* (Lourenço et al., 2000) indicate that these could be homeologous chromosomes. Therefore, the condition "presence of an interstitial NOR in 8q", as found in all of these species, may be plesiomorphic in relation to the other patterns of NOR occurrence found in species of *Physalaemus*, among which we note the following: the presence of a pericentromeric NOR in 9q (found in *P. centralis*; see Vittorazzi et al., 2014); the presence of NOR in 8q and in the metacentric chromosome 9 (found in *P. albonotatus*, see Vittorazzi et al., 2014); and the polymorphic condition found in exemplars from Porto Nacional identified here as Lineage 3 of "*P. cuvieri*") (Quinderé et al., 2009).

It is equally remarkable that the homoplastic telocentric chromosomes 11 of *Physalaemus fernandezae* and *P. nattereri* also show a terminal NOR, providing an illustrative example of the importance of considering this cytogenetic trait carefully.

At last, we note that the NOR location is a valuable trait in the cytogenetics of *Physalaemus* because this character may vary among species (*e.g.*, Vittorazzi et al., 2014), being helpful in detecting differences even among lineages morphologically undistinguishable as those of "*P. cuvieri*".

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Appendices A–G. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2015.06.011.

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