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Comparative mapping of a new repetitive DNA sequence and chromosome region-specific probes unveiling rearrangements in an Amazonian frog complex

Kaleb Pretto Gatto, Lucas H.B. Souza, Juliana Nascimento, Pablo Suárez, and Luciana Bolsoni Lourenço

Abstract: The frog species *Physalaemus ephippifer* exists in the Amazonian region and harbors heteromorphic Z and W chromosomes. A genetic lineage closely related to this species was recognized based on its mitochondrial DNA and RADseq-style markers, but its taxonomic status is still unclear and has been referred to as Lineage 1 of "*P. cuvieri*". The heteromorphic sex chromosomes found in *P. ephippifer* are not present in this lineage and which of its chromosome pairs is homologous to the sex chromosomes of *P. ephippifer* remain to be elucidated as well as the role of such a karyotypic divergence in the evolution of these frogs. Here, we described a new family of repetitive DNA and used its chromosomal sites along with the markers detected by a probe constructed from the microdissected segment of the Z chromosome of *P. ephippifer* to infer chromosomal homology. We also analyzed an unnamed species that is considered to be the sister group of the clade composed of Lineage 1 of "*P. cuvieri*" and *P. ephippifer*. Our results suggest that complex rearrangements involving the chromosomes that were inferred to be homeologous to the sex chromosomes of *P. ephippifer* have occurred during the divergence of this group of frogs.

Key words: *Physalaemus cuvieri*, *Physalaemus ephippifer*, sex chromosome, chromosome evolution.

Résumé : La grenouille *Physalaemus ephippifer* est présente en Amazonie et elle comporte des chromosomes hétéromorphes Z et W. Une lignée génétiquement proche a été identifiée sur la base de son ADN mitochondrial et de marqueurs de type RADseq, mais sa taxonomie demeure incertaine tout en étant nommée Lignée 1 du « *P. cuvieri* ». Les chromosomes sexuels hétéromorphes présents chez le *P. ephippifer* ne le sont pas au sein de cette lignée et il demeure inconnu laquelle des paires de chromosomes est homologue aux chromosomes sexuels du *P. ephippifer* de même que le rôle d'une telle divergence caryotypique dans l'évolution de ces grenouilles. Dans ce travail les auteurs décrivent une nouvelle famille d'ADN répété et ils emploient les sites chromosomiques de ces séquences ainsi que des marqueurs détectés au moyen d'une sonde dérivée d'un segment (obtenu par microdissection) du chromosome Z du *P. ephippifer* pour déterminer l'homologie chromosomique. Les auteurs ont également analysé une espèce sans nom qui est considérée comme un groupe proche du clade composé de la Lignée 1 du « *P. cuvieri* » et du *P. ephippifer*. Les résultats suggèrent que des réarrangements complexes, impliquant les chromosomes déterminés comme étant homéologues des chromosomes sexuels du *P. ephippifer*, seraient survenus au cours de la divergence au sein de ce groupe de grenouilles. [Traduit par la Rédaction]

Mots-clés : *Physalaemus cuvieri*, *Physalaemus ephippifer*, chromosome sexuel, évolution des chromosomes.

Introduction

Frogs are a very interesting vertebrate group for studies of sex chromosome evolution and its role in speciation, as they present distinct sex chromosome systems and different levels of sex chromosome heteromorphism (for a review, see Schmid et al. 2012). Although some studies have previously provided important interspecific comparisons regarding sex chromosomes (see Miura and Ogata 2013; Jeffries et al. 2018; Cauret et al. 2020), the recognition of interspecific chromosome homeologies remains a relevant challenge in the study of sex chromosome evolution in anurans, either due to

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technical limitations in chromosome characterization or the low coverage of studied species. Here, we infer chromosomal homeologies in a species complex of frogs that shows both homomorphic and heteromorphic sex chromosomes, the *Physalaemus cuvieri* – *Physalaemus ephippifer* complex.

The frog species *Physalaemus ephippifer* (Steindachner 1864) is found in the Amazonian region, but its geographical range is still unclear (Nascimento et al. 2019). Phylogenetically, *P. ephippifer* is closely related to *P. cuvieri*, and the delimitation of these species remains to be elucidated (Nascimento et al. 2019). Based on its mitochondrial DNA sequences, RADseq-style markers, and cytogenetic data, Nascimento et al. (2019) suggested the existence of undescribed species related to *P. ephippifer* and *P. cuvieri*, and argued that a taxonomic revision of this group is required.

Physalaemus ephippifer is the sister group of a clade that includes specimens from northeastern Brazil, known as Lineage 1 of "*P. cuvieri*" (Lourenço et al. 2015; Nascimento et al. 2019). The low level of mitochondrial DNA divergence observed between this lineage and the *P. ephippifer* clade supports the hypothesis that the former is indeed *P. ephippifer* (Lourenço et al. 2015; Nascimento et al. 2019). The sequence-based species delimitation analyses, however, were not conclusive with respect to the number of species present in this group (Nascimento et al. 2019). In addition, an undescribed species, which includes specimens from the Pará and Roraima states of Brazil, was recognized as the sister group of the clade composed of the Lineage 1 of "*P. cuvieri*" and *P. ephippifer* (Nascimento et al. 2019). A high level of divergence was found between this *Physalaemus* sp. clade and that composed of Lineage 1 of "*P. cuvieri*" and *P. ephippifer*, in contrast to the low level of mitochondrial DNA divergence observed between these latter two groups (Nascimento et al. 2019).

Notorious cytogenetic differences were noticed among *P. ephippifer* (Nascimento et al. 2010), Lineage 1 of "*P. cuvieri*" (specimens from Urbano Santos and Crateús in Quinderé et al. 2009), and *Physalaemus* sp. from western Pará and Viruá (Nascimento et al. 2019), especially with respect to the nucleolus organizer region (NOR)-bearing chromosomes. In *P. ephippifer*, the NORs are found in the Z and W chromosomes, which are heteromorphic in morphology, C-banding pattern, and in the number of NORs (Nascimento et al. 2010). In Lineage 1 of "*P. cuvieri*", otherwise, no chromosome heteromorphism related to sex was detected, although notorious variation concerning the NOR-bearing chromosomes 8 and 9 was found (see karyotypes of specimens from Urbano Santos and Crateús in Quinderé et al. 2009). Both chromosome 8 and chromosome 9 were highly polymorphic with respect to NOR size, and chromosome 9 also varied in the number of NORs, but no chromosomal variant was exclusively found in all females or males (see Quinderé et al. 2009). It is also noteworthy that chromosomes 8 and 9 of Lineage 1 of "*P. cuvieri*" are very similar in morphology but can be easily distinguished from each other by C-banding, as the NORs in chromosome 9 coincide with C-bands, while the NOR in chromosome 8 is flanked by small C-bands (Quinderé et al. 2009).

Based on the available cytogenetic data, some similarities can be observed between the Z chromosome of *P. ephippifer* and the NOR-bearing chromosome pairs of the specimens from the Lineage 1 of "*P. cuvieri*", but a proper inference of chromosomal homeology between any of them is still not possible. Because the Lineage 1 of "*P. cuvieri*" and *P. ephippifer* do not share the same heteromorphic sex chromosomes, Nascimento et al. (2019) suspected that such a karyotypic divergence may generate a reproductive barrier, but this hypothesis has not been tested yet.

In the case of *Physalaemus* sp., it is still not possible to evaluate whether sex chromosome heteromorphism is present because only male specimens have been analyzed to date (Nascimento et al. 2019). Despite that, it is noteworthy that the NOR-bearing chromosome found in males of *Physalaemus* sp. greatly differ from those observed in the Lineage 1 of "*P. cuvieri*" or *P. ephippifer* (Nascimento et al. 2019).

In this paper, we used probes for a repetitive DNA sequence and for a segment of the long arm of the Z chromosome of *P. ephippifer* to provide a better comparison between the sex chromosomes of *P. ephippifer* and the NOR-bearing chromosomes of Lineage 1 of "*P. cuvieri*" and *Physalaemus* sp., aiming to assess the hypothesis of the homology of these chromosomes.

Materials and methods

We isolated a repetitive DNA sequence from genomic restriction fragments of *P. ephippifer*, and we used microdissection to isolate a segment of the long arm of the Z chromosome of this species. We constructed probes from the isolated materials and mapped them to the karyotypes of *P. ephippifer* and its closely related lineages (Lineage 1 of "*P. cuvieri*" and *Physalaemus* sp. from western Pará and Viruá) to deepen their comparison. We also constructed a probe from the chromosomal segment of *Physalaemus* sp. (from western Pará) that was previously detected by the probe constructed from the Z chromosome of *P. ephippifer*. To combine the data, we obtained using all these probes with those previously generated for these frogs, the karyotypes in comparison also had their C-band and NOR patterns assessed. In the next sections, we describe in detail all the procedures we used.

Specimens

Seven individuals (four females and three males) of *P. ephippifer*, two males of Lineage 1 of "*P. cuvieri*", and eight males of *Physalaemus* sp. were analyzed. The specimens were collected under a permit issued by the Instituto Chico Mendes de Conservação da Biodiversidade/Sistema de Autorização e Informação em Biodiversidade (ICMBio/SISBIO) (permit number 32483) and were deposited in the amphibian collection of the Museu de Zoologia "Prof. Adão José Cardoso" at the Institute of

Table 1. Sampling locality, sex, and museum vouchers of the individuals of *Physalaemus ephippifer*, Lineage 1 of "*P. cuvieri*", and *Physalaemus* sp. used in the present study.

Species/lineage	Locality	Sex	Museum vouchers
Physalaemus ephippifer	Belém, Pará state, Brazil	Female	ZUEC 13706, 13735, 13736, and 13741
	Belém, Pará state, Brazil	Male	ZUEC 13733
	Santa Bárbara, Pará state, Brazil	Male	ZUEC 24907–24908
Lineage 1 of "*P. cuvieri*"	Urbano Santos, Maranhão state, Brazil	Male	ZUEC 13360 and 13363
Physalaemus sp. (western Pará clade)	Prainha, Pará state, Brazil	Male	ZUEC 17594 and 17599
	Alenquer, Pará state, Brazil	Male	ZUEC 18191 and 18198
	Óbidos, Pará state, Brazil	Male	ZUEC 18188 and 22693
Physalaemus sp. (Viruá clade)	Viruá National Park, Roraima state, Brazil	Male	ZUEC 17602 and 17606

Note: ZUEC, Museu de Zoologia "Prof. Adão José Cardoso" at the Institute of Biology – University of Campinas.

Biology – University of Campinas (ZUEC). The sampling locality and museum voucher number of the analyzed specimens are shown in Table 1.

Chromosome preparation, NOR detection, and C-banding

Chromosome preparations were made from intestine cell suspensions deposited at the cytogenetic collection Shirlei Maria Recco Pimentel (SMRP) at the Institute of Biology – University of Campinas. Although most of the cell suspensions were previously obtained by Nascimento et al. (2010, 2019), some of them were prepared following King and Rofe (1976), after an in vivo treatment of specimens with 2% colchicine for 4 h. Before the removal of the intestine, the specimens were anesthetized with 2% lidocaine. This protocol was approved by the Committee for Ethics in Animal Use of the University of Campinas (CEUA/UNICAMP) (permit number 4802-1).

For a proper identification of each chromosome pair of all the analyzed karyotypes, the chromosome preparations were sequentially subjected to Giemsa-staining (with a 10% Giemsa solution), C-banding (following King 1980) for the detection of constitutive heterochromatin, and silver-impregnation (following the Ag-NOR method; Howell and Black 1980) for the detection of NORs. For the detection of NORs, metaphase chromosomes were also hybridized to the nucleolar rDNA probe HM 123 (Meunier-Rotival et al. 1979) or a 28S rDNA fragment (Bruschi et al. 2012) labeled by nick translation (HM 123 probe) or PCR (28S probe) with digoxigenin-dUTP (Roche). The hybridization step followed the method of Viegas-Péquignot (1992); the probes were detected by anti-digoxigenin conjugated to rhodamine (Roche) (0.06 μg/mL), and the chromosomes were stained with DAPI (4′,6-diamidino-2-phenylindole; 0.5 μg/mL). Because the Ag-NOR method may detect some heterochromatic regions in addition to the NORs, the in situ hybridization of nucleolar rDNA is an important approach to unequivocally identify the NORs.

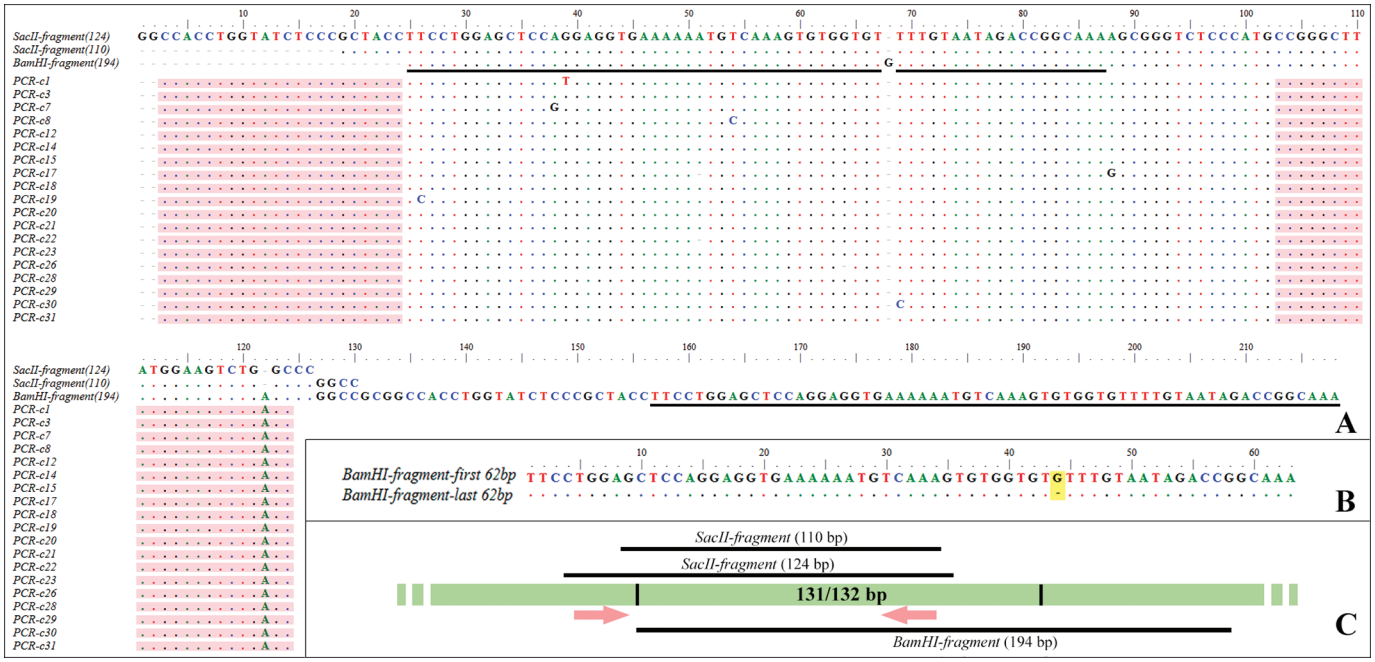
Isolation and characterization of a new repetitive DNA — the PepBS sequence

We used genomic DNA digestion to isolate the repetitive DNA sequence of *P. ephippifer* we used in this work. For that, approximately 3 μg of genomic DNA extracted from liver samples of the females ZUEC 13735 and ZUEC 13736 were digested for 16 h with *Bam*HI (Fermentas) and *Sac*II (Fermentas), respectively. The restriction fragments were precipitated by using NaCl and ethanol, resuspended in sterile milli-Q water, and separated by electrophoresis in 1.2% agarose gel. In both cases, bands containing fragments of 100–200 bp were observed after ethidium bromide staining, and they were cut out of the gel using a sterile blade. The restriction fragments were isolated from the agarose gel using GFX PCR and Gel Band DNA Purification kits (GE Healthcare) according to the manufacturer's instructions. Complementation of the cohesive ends of the restriction fragments was achieved using *Taq* polymerase at 70 °C for 2 h, as described by Sánchez et al. (1996). The resulting fragments were inserted into the pGEM-T Easy Vector (Promega) and cloned into *Escherichia coli* JM109 using a TransformAid Bacterial Transformation kit (Fermentas) following the manufacturers' instructions. Cloned fragments were sequenced using the BigDye Terminator kit (Applied Biosystems) and the primers T7 and SP6. The isolated sequence was named PepBS, which makes a reference to ***P****hysalaemus* ***ep****hippifer* and the restriction enzymes used for its isolation, ***B****am*H1 and ***S****ac*II.

Based on the nucleotide sequence of the cloned fragments, the primers PepBS-F (5′–CCACCTGGTATCTCCCGC TACC–3′) and PepBS-R (5′–GCTCAGACTTCCATAAGCCC GG–3') were designed and used for the PCR amplification of specific fragments from the genomic DNA of the *P. ephippifer* specimen ZUEC 13733. The PCR-amplified fragments were cloned and sequenced as mentioned above. All of the nucleotide sequences were edited and compared to each other using BioEdit Sequence Alignment Editor software v. 7.2.5 (Hall 1999).

Then, we searched for the PepBS sequence among the 3RAD reads available in the Sequence Read Archive (SRA) for *P. ephippifer* (SRX5561028 and SRX5561025), Lineage 1 of "*P. cuvieri*" (SRX5561014, SRX5561026, SRX5561029, SRX5561036, and SRX5561033), Lineage 2 of "*P. cuvieri*" (SRX5561034 and SRX5561031), and *Physalaemus* sp. from western Pará and Viruá (SRX5561011-5561013, SRX5561015-5561025, and SRX5561027). For this purpose, the reads

Fig. 1. PepBS sequence of *Physalaemus ephippifer*. (A) Nucleotide sequence alignment of the fragments resulting from *Sac*II and *Bam*HI digestion of genomic DNA (three first sequences) and PCR with the primers PepBS-F and PepBS-R (19 last sequences). The equivalent segments found in the *Bam*HI fragment are underlined and their alignment is shown in (B). Highlighted (yellow) in B, the only site that differs between the repeated segments of the *Bam*HI fragment. (C) Spatial representation of the *Sac*II and *Bam*HI restriction fragments as well as the annealing sites of the primers PepBS-F and PepBS-R in relation to the inferred tandemly repeated PepBS array.



were mapped to a PepBS sequence using BWA v.0.7.17 (Li and Durbin 2009) with the mem option under the default parameters. Samtools v.1.9 (Li et al. 2009) was employed for alignment sorting, statistics recovery, and to generate *.bam files. Alignments from the *.bam files were inspected in Geneious (Biomatters. New Zealand).

Chromosome mapping of PepBS sequences

For in situ hybridization, the cloned PepBS fragments were labeled with digoxigenin-dUTP (Roche) or biotin-dUTP (Roche) by PCR with the primers PepBS-F and PepBS-R. The hybridization step and the detection of the biotinylated probes followed the method of Viegas-Péquignot (1992). The probes labeled with digoxigenin were detected by anti-digoxigenin conjugated to rhodamine (Roche) (0.06 μg/mL). The chromosomes were stained with DAPI (0.5 μg/mL).

For a proper identification of the chromosomes, the same chromosome preparations hybridized with the PepBS probe were sequentially subjected to hybridization with a rDNA probe, C-banded, and silver-impregnation, following the procedures cited before.

Construction and in situ hybridization of the Zqper probe and the 8p probe

To better compare the sex chromosomes of *P. ephippifer* with chromosomes of the specimens of Lineage 1 of "*P. cuvieri*" and *Physalaemus* sp. from western Pará and Viruá, we constructed a probe for a pericentromeric (per) region of the long arm (q) of the Z chromosome (Zqper probe). We avoid using the short arm of the Z chromosome when constructing a probe because it was shown to be polymorphic with respect to an interstitial C-band (see Nascimento et al. 2010; Fig. S1[1]). Also, the region we chose for probing does not include the NOR present in the long arm of the Z chromosome (Fig. S1[1]).

For the microdissection of the segment of interest, cell suspensions from a male *P. ephippifer* were dropped onto coverslips covered with a polyethylene membrane (PEN). For better visualization of the secondary constrictions of the NORs, the chromosomes were stained with 10% Giemsa. The segment localized between the NOR (visualized as a secondary constriction; see Fig. S1[1]) and the centromere of the Z chromosome long arm was dissected using a P.A.L.M. MicroBeam system 4.1 (Zeiss) coupled with an inverted microscope (Olympus). A total of 22 segments were captured, and the collected material was amplified using a GenomePlex Single Cell Whole Genome Amplification Kit WGA4 (Sigma–Aldrich) and purified with a Fermentas Genomic DNA Purification Kit. For probe construction, samples of the purified DNA were amplified with the GenomePlex Single Cell Whole Genome Reamplification Kit WGA3 (Sigma–Aldrich), using a

[1]Supplementary data are available with the article at https://doi.org/10.1139/gen-2020-0199.

dNTP mix containing 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 6.5 mM dTTP, and 3.5 mM dUTP-digoxigenin. The resulting probe (Zqper probe) was purified and hybridized to karyotypes of *P. ephippifer*, *Physalaemus* sp. from western Pará and Viruá clades, and Lineage 1 of "*P. cuvieri*", following the protocol for fluorescent in situ hybridization (FISH) suggested by Krylov et al. (2010).

We also constructed a probe by microdissecting the region from the chromosome 8 short arm (8p) of *Physalaemus* sp. (from the western Pará clade; ZUEC 22693) that was detected by the Zqper probe (Fig. S2[1]). In this case, 15 chromosomes were microdissected following the same protocol presented above, and the resulting probe (8p probe) was hybridized to karyotypes of *P. ephippifer*, *Physalaemus* sp. from western Pará and the Viruá clades, and Lineage 1 of "*P. cuvieri*".

Results

Repetitive DNA PepBS

One *Bam*HI and two *Sac*II restriction fragments (GenBank accession numbers MW314576–MW314578) resulting from the digestion of genomic DNA of *P. ephippifer* showed the same type of sequence, named the PepBS sequence. The *Bam*HI fragment was 194 bp (GenBank accession number MW314578), being comprised of an entire unit of the PepBS sequence (the first 132 bp) juxtaposed to a partial one (the last 62 bp) (Fig. 1). The tandemly repeated segments observed in the *Bam*HI fragment differed from each other only by an additional G present in the complete PepBS unit (Fig. 1B). The *Sac*II fragments were 124 and 110 bp, differing from each other by their ends (Fig. 1).

Nineteen PepBS sequences were obtained by PCR from the genomic DNA of *P. ephippifer* (Fig. 1; GenBank accession numbers MW314579–MW314597), which were 99.24% similar in nucleotide sequence. No significant similarity was found between the PepBS sequences and the sequences available in GenBank or Repbase.

The PepBS sequences were identified in the 3RAD libraries available for *P. ephippifer*, Lineages 1 and 2 of "*P. cuvieri*", and the *Physalaemus* sp. from western Pará and Viruá clades (Table 2). All of the mapped reads showed a high similarity with the segment of PepBS used as reference, with only a few mismatches.

Chromosome mapping of the PepBS sequences

In the *P. ephippifer* karyotype, the PepBS sequences were mapped to a distal site in the long arm of chromosomes Z and W as well as in a distal region of the W chromosome short arm. In all of these chromosomal sites, the PepBS probe signals overlapped the hybridization signals of a nucleolar rDNA probe (Fig. 2A). In one of the *P. ephippifer* specimens from Santa Bárbara (ZUEC 24907), an additional NOR was found interstitially in one of the homologs of pair 10, and this NOR was also detected by the PepBS probe (Fig. 2B).

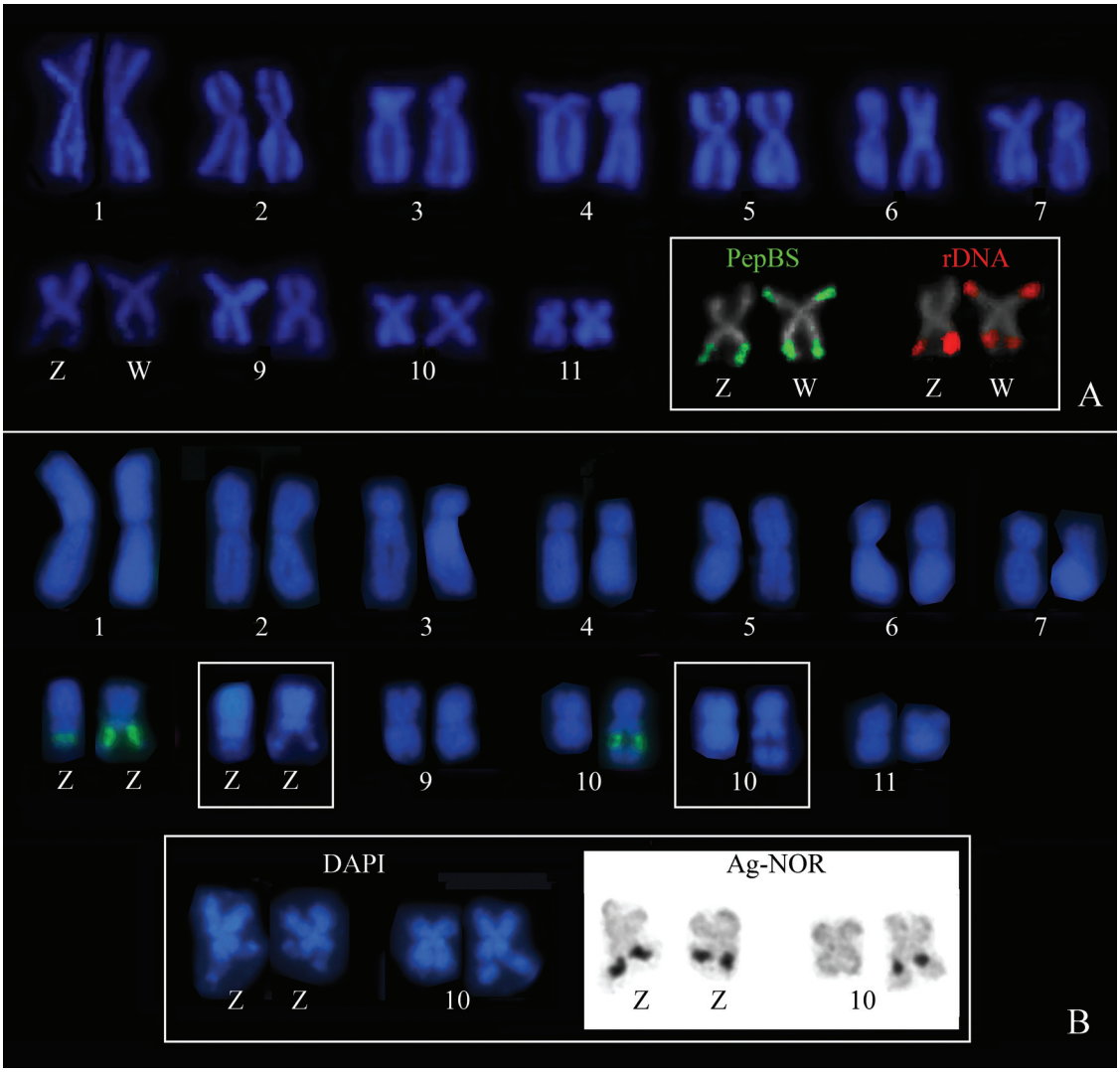
Table 2. Summary of the mapping of reads from 3RAD libraries of *Physalaemus ephippifer* – *P. cuvieri* available in the SRA against the PepBS sequence.

Species/3RAD library	No. of mapped reads	Mean similarity to PepBS (%)
P. ephippifer		
L9	23	99.20
L13	10	99.70
"*P. cuvieri*" L1		
L12	49	94.00
L43	218	97.67
"*P. cuvieri*" L2		
L45	55	96.91
L46	17	96.22
Physalaemus sp. (western Pará clade)		
L14	16	96.30
L15	15	95.47
L16	16	95.80
L17	8	94.87
L21	11	94.23
L22	23	94.92
L23	14	94.92
L24	18	96.83
L25	20	97.84
L26	9	96.81
L27	4	93.43
Physalaemus sp. (Viruá clade)		
L28	3	93.46
L29	2	94.20

In the specimen of the Lineage 1 of "*P. cuvieri*", the PepBS probe strongly detected the sites of chromosome 9 also evidenced as NORs and C-bands (Fig. 3A). In some metaphases, a very faint signal of this probe could be seen in the NORs of chromosome 8 (inset in Fig. 3A), which were flanked by weak C-bands instead of being heterochromatic as were the NORs of chromosome 9 (Figs. 3B, 3E). Both chromosome pairs 8 and 9 of this male specimen were heteromorphic in relation to the NOR sites, as evidenced by the Ag-NOR method (Fig. 3C). In chromosome 9, the hybridization signals of the PepBS probe were much brighter than those shown by the hybridization of the 28S probe, although they colocalized (Fig. 3D). Such a difference was not observed in relation to the sites detected by these probes in chromosome 8. It is also noteworthy that the NORs in chromosome 8 were visualized as conspicuous secondary constrictions in DAPI-stained metaphases, while the secondary constrictions of the NORs in chromosome 9 were less evident (Fig. 3F).

In karyotypes of *Physalaemus* sp. specimens from Alenquer and Óbidos (referred to as cytotype I of *Physalaemus* sp. in Nascimento et al. 2019), as well as in karyotypes of specimens from the Viruá National Park (cytotype II in Nascimento et al. 2019), the PepBS probe detected the NOR site located in the short arm of chromosome 8. In contrast, no hybridization signal of this probe was seen in the NOR found in the long arm of chromosome 8 of the specimens from Alenquer and Óbidos (Fig. 4).

Fig. 2. Mapping of PepBS sequences to the *Physalaemus ephippifer* karyotype. (A) A female karyotype stained with DAPI and hybridized with PepBS (green) and HM123 (red) probe. The PepBS probe detected the distal NOR in chromosome Z and both NORs of chromosome W. (B) Karyotype of the male ZUEC 24907 stained with DAPI and hybridized with PepBS (green). DAPI-stained images of the sex chromosome pair and chromosome pair 10 were shown in insets. In the lower inset in B, the sex chromosome pair and chromosome pair 10 from another metaphase of the same male stained with DAPI and subsequently impregnated by the Ag-NOR method. Note that this male has three NORs, and all of them are visualized as secondary constrictions in DAPI-stained metaphases and hybridize to the PepBS probe.



FISH with the Zqper probe and the 8p probe

The Zqper probe, which was constructed from the microdissection of the pericentromeric region of the long arm of the Z chromosome of *P. ephippifer* (Fig. S1[1]), detected a site adjacent to the centromere in the short arm of chromosome 9 (9p per) of specimens assigned to the Lineage 1 of "*P. cuvieri*" (Fig. 5A). In *Physalaemus* sp. specimens from the western Pará and Viruá clades, the Zqper probe detected the site adjacent to the NOR in the short arm of chromosome 8 (8p) (Figs. 5B and 5C).

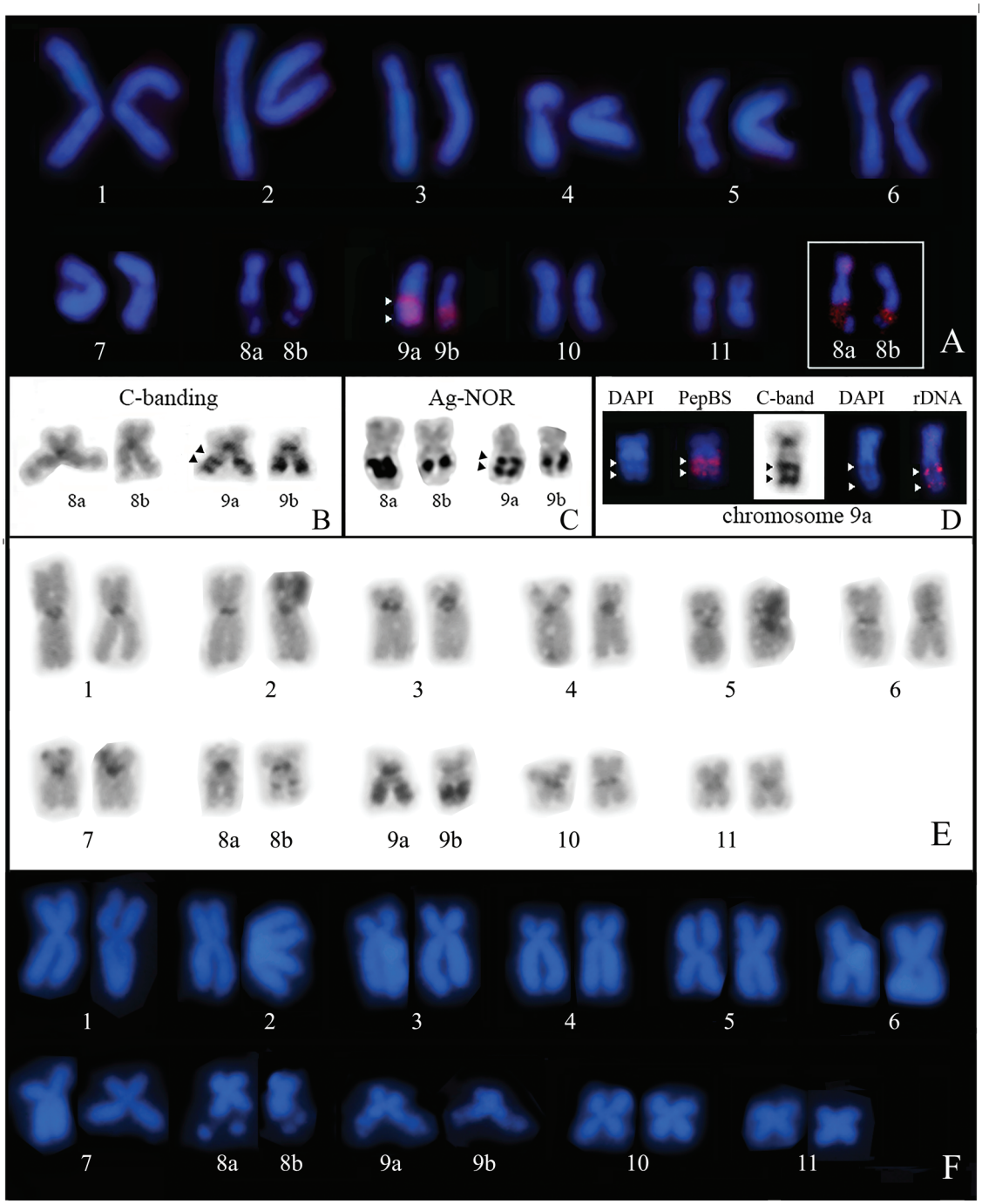
The 8p probe (derived from the microdissection of the site revealed by the Zqper probe in the *Physalaemus* sp. karyotype) detected a pericentromeric region in the long arm and an interstitial site in the short arm of the Z chromosome and a pericentromeric region in the W chromosome of *P. ephippifer* (Fig. 6) as well as the 9p per region in the karyotype of Lineage 1 of "*P. cuvieri*". The interstitial site detected by the 8p probe in the Z chromosome of *P. ephippifer* varied greatly in size in both analyzed males (see the ZZ pairs shown in Figs. 6A and 6B) and among the two analyzed females, being barely visible in some cases. The Zqper/8p site detected interstitially in the short arm of the Z chromosome coincides with the site detected by C-banding (Fig. 6C).

Discussion

The PepBS repetitive DNA

The PepBS sequences isolated from restriction fragments of *P. ephippifer* refer to a family of repetitive DNA whose

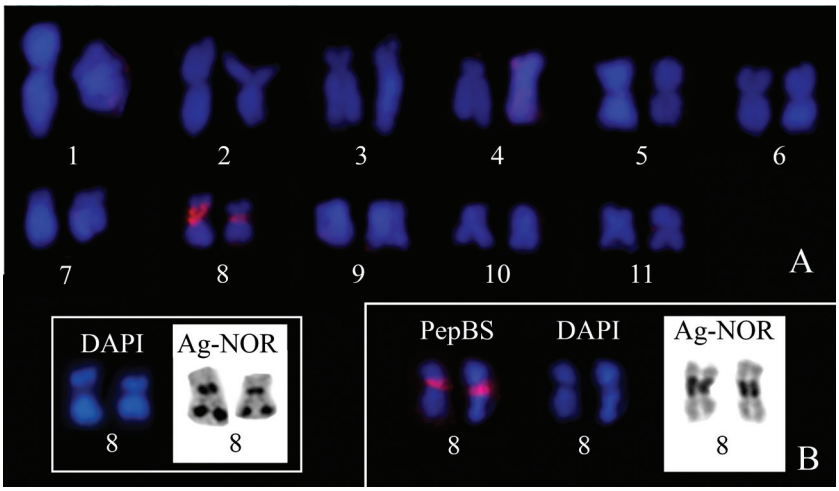
Fig. 3. Mapping of PepBS sequences to the karyotype of Lineage 1 of "*Physalaemus cuvieri*". (A, E–F) Karyotype of the specimen ZUEC 13360 hybridized with the PepBS probe (A), C-banded (E), and DAPI-stained (F). In the inset in A, there is an overly contrasted image of the same chromosome pair 8 shown in the karyogram, in which the probe signal can be visualized. (B–C) C-banded (B) and silver-impregnated (C) chromosome pairs 8 and 9. Note the NOR/C-band heteromorphism in pairs 8 and 9. (D) Chromosome 9a hybridized with the PepBS probe (left panel) and to the 28S rDNA probe (right panel), and C-banded (middle). Arrowheads point to the two PepBS sites of chromosome 9a, which are coincident to C-bands and NORs.



presence in Lineage 1 of "*P. cuvieri*" and *Physalaemus* sp. (from western Pará and Viruá, Brazil) could be confirmed by in situ hybridization and an analysis of the 3RAD libraries. All 3RAD reads that were mapped to the PepBS sequence showed high similarity to this sequence (above 90%), although few reads were recovered in this analysis, which could be a result of the underrepresentation of the genome in these libraries.

Although the molecular organization of PepBS remains to be elucidated, the finding of a fragment (the *Bam*HI

Fig. 4. Hybridization of the PepBS probe to chromosomes of *Physalaemus* sp. from western Pará (A) and Viruá (B). DAPI-stained and a silver-impregnated chromosome pairs 8 are also shown. For a Giemsa-stained karyotype of *Physalaemus* sp. from western Pará, see Fig. S2.[1]



fragment) comprised of more than one unit of the PepBS sequence allowed us to infer that a repetitive unit of approximately 130 bp may be tandemly repeated in the genome of *P. ephippifer*. Another interesting characteristic presented by the PepBS sequences is their colocalization with NORs in all of the karyotypes analyzed, although not every NOR colocalized with a PepBS site. Because no similarity between the PepBS nucleotide sequence and the 18S, 5.8S, or 28S rRNA genes was observed, we can hypothesize that the PepBS sequences are contained in the intergenic spacer located between the clusters of rRNA genes or that they compose heterochromatic blocks interspersed with nucleolar ribosomal DNA.

The presence of repetitive DNA in ribosomal intergenic spacers has been reported from several organisms (e.g., De Lucchini et al. 1997; Macas et al. 2003; Uno et al. 2013; Ishijima et al. 2017), and in some cases (De Lucchini et al. 1997; Mandrioli et al. 1999), such an association was argued to be related to the variation observed with respect to the NOR sites. It is worth noting that, in our analysis, one of the NOR-bearing chromosomes in which the PepBS sequences colocalized with rDNA sites was chromosome 9 of the specimen of the Lineage 1 of "*P. cuvieri*", which is particularly polymorphic in the number and size of NOR sites, as reported by Quinderé et al. (2009). The higher brightness of the PepBS hybridization signals when compared with the 28S probe signals, a condition observed exclusively in these sites on chromosome 9, may suggest that the ratio of PepBS sequences/rRNA genes is larger in these sites than in the other PepBS/rDNA sites analyzed. Accordingly, this could be the reason why these sites of chromosome 9 were also revealed as heterochromatic bands by C-banding. Taken together, these data raise questions about a possible involvement of the PepBS sequences with a high level of variation related to chromosome 9 in the Lineage 1 of "*P. cuvieri*".

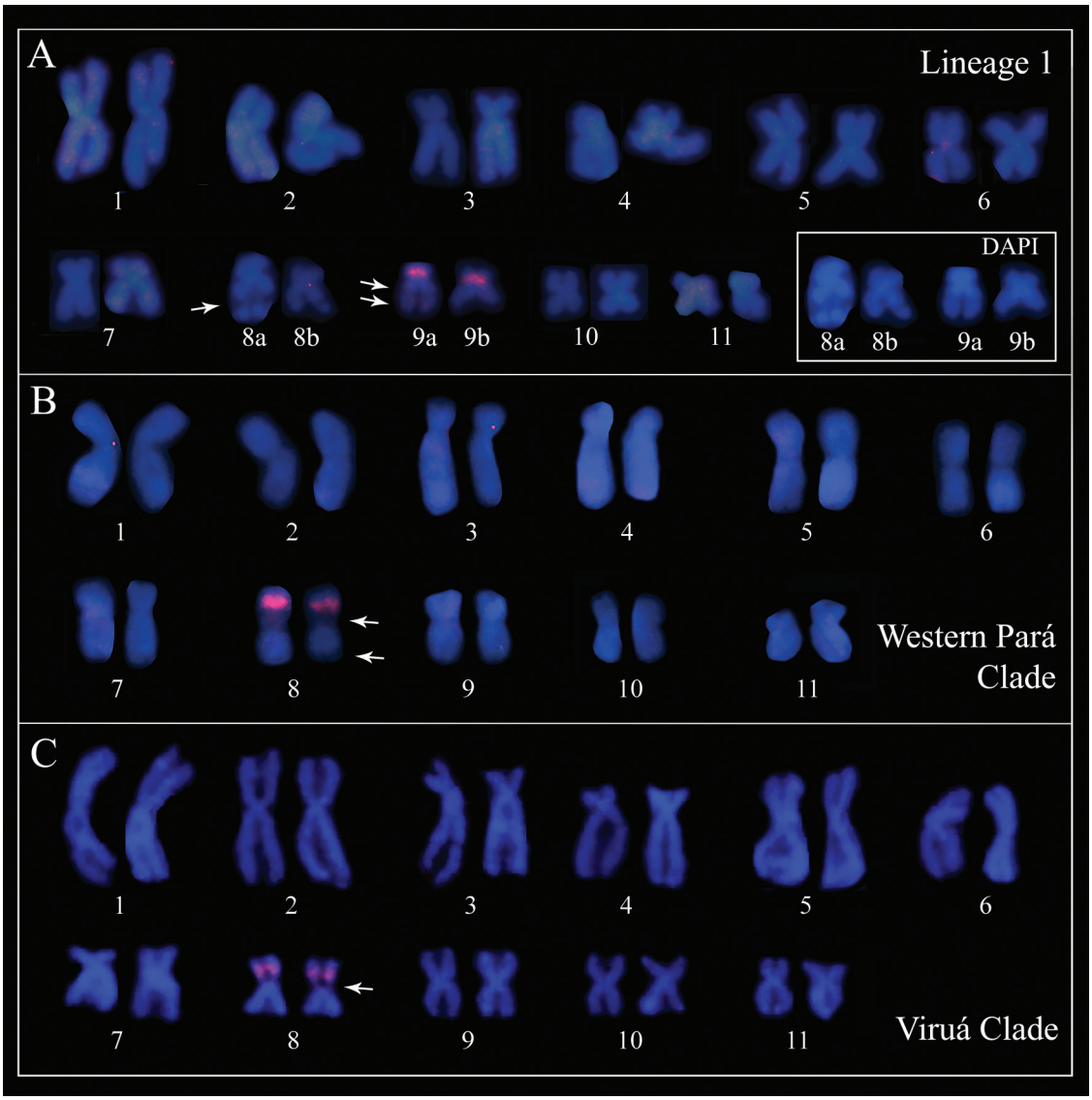
It is also remarkable that the expressive richness in PepBS sequences observed for the NORs of chromosome 9 of Lineage 1 of "*P. cuvieri*" specimen was not observed in the NORs of chromosome 8 of the same specimen, which barely presented signals of hybridization of the PepBS probe. Similarly, the distal NOR of chromosome 8 found in the cytotype I of *Physalaemus* sp. (from western Pará) was not revealed by the PepBS probe, while the pericentromeric NOR localized in the short arm of the same chromosome was. Although repetitive DNA is expected to evolve in concert, the evolutionary dynamics of these DNA fractions is a complex process, which may also involve the creation of a chromosome-specific set of sequences (Dover 1986; reviews by Ugarković and Plohl 2002; Plohl et al. 2008). The differential profiles presented by those NORs of cytotype I of *Physalaemus* sp. (from western Pará) and by the NORs of Lineage 1 of "*P. cuvieri*" showed that independent evolutionary forces operated in these sites. On the other hand, the two NORs found in *P. ephippifer* were also detected as PepBS sites. This result may indicate that homogenization forces are more effective in the NORs of *P. ephippifer* than in the others but, alternatively, may be explained by a recent origin of one of the NORs from a pre-existing one in *P. ephippifer*.

When a repetitive DNA is chromosome-specific, it may lead to the recognition of interspecific chromosomal homeologies, such as reported by Archidiacono et al. (1995) in a study with the alphoid sequence of the human X chromosome, whose probe also detected the X chromosome of chimpanzee, pygmy chimpanzee, and gorilla (Archidiacono et al. 1995). In the case of PepBS repetitive DNA, it helped us to infer some interspecific homeologies, as discussed in the next section.

Sex chromosome heteromorphism in *P. ephippifer*

Previous studies have shown that the Z and W chromosomes of *P. ephippifer* differ from each other, particularly

Fig. 5. Hybridization of the Zqper probe to karyotypes of Lineage 1 of "*Physalaemus cuvieri*" (A) and *Physalaemus* sp. from western Pará (B) and *Physalaemus* sp. from Viruá (C). The arrows point to secondary constrictions coincident to NORs. Note that in DAPI-stained image (inset in A), chromosomes 8 can be easily distinguished from chromosome 9 because the NORs in chromosomes 8 are conspicuous secondary constrictions (for more details about these chromosomes, see Fig. 3).



in the short arm, which bears an additional segment composed of an NOR and a heterochromatic block in the W chromosome (Nascimento et al. 2010; Vittorazzi et al. 2014). The Z and W long arms, although very similar in size and having a distal NOR (Nascimento et al. 2010), varied in the size/intensity of a pericentromeric cluster of PcP190 satellite DNA (Vittorazzi et al. 2014). Here, we provide additional evidence of the similarity between Zq and Wq, as a pericentromeric segment was detected in both chromosome arms by the Zqper/8p probes.

Additionally, the Zqper/8p probes revealed the existence of different types of the Z chromosome in *P. ephippifer*, as the conspicuous interstitial band revealed by these probes in the short arm of some of the Z chromosomes was barely seen in others. As expected, the two types of Z chromosomes were found in both males and females. This interstitial Zqper/8p band was not seen in Wp in any of the analyzed females.

Inference of chromosome homeology in the species complex *P. ephippifer* – *P. cuvieri*

The Zqper/8p probes detected exclusively specific regions of the sex chromosomes of *P. ephippifer*, chromosome pair 8 of *Physalaemus* sp. from western Pará and Viruá, and chromosome pair 9 of the Lineage 1 of "*P. cuvieri*", suggesting these chromosomes are homeologous. The long arm of the Z and W chromosomes of *P. ephippifer* and the short arm of chromosome 8 of *Physalaemus* sp. from western Pará and Viruá bear a Zqper/8p segment and a PepBS/rDNA site. These chromosome arms, however, differ from each other by the relative location of the Zqper/8p and PepBS/rDNA

Fig. 6. Hybridization of the 8p probe to *Physalaemus ephippifer* chromosomes. (A) Karyotype of the male ZUEC 24907 hybridized to the 8p probe. Note that the size of the hybridization signals varied between the two Z homologous chromosomes. In the inset in A, the DAPI image of the same ZZ pair shown in the karyogram hybridized to the probe. The ZZ pair from another metaphase of the same male is shown in B, in which the small 8p probe site is better visualized (arrowhead). (C) Z and W chromosomes of the female ZUEC 13706 submitted to C-banding (left) and in situ hybridization with the 8p probe (DAPI image in the middle and merged DAPI and probe images in the right). Note >that the short arm of the Z chromosome of this female shows a conspicuous interstitial hybridization site of the 8p probe and an interstitial C-band.

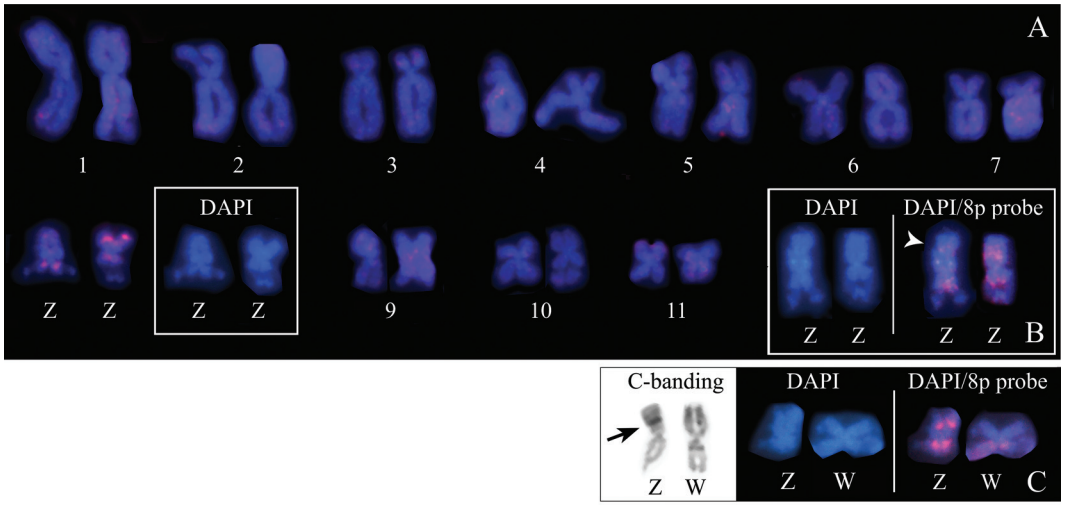
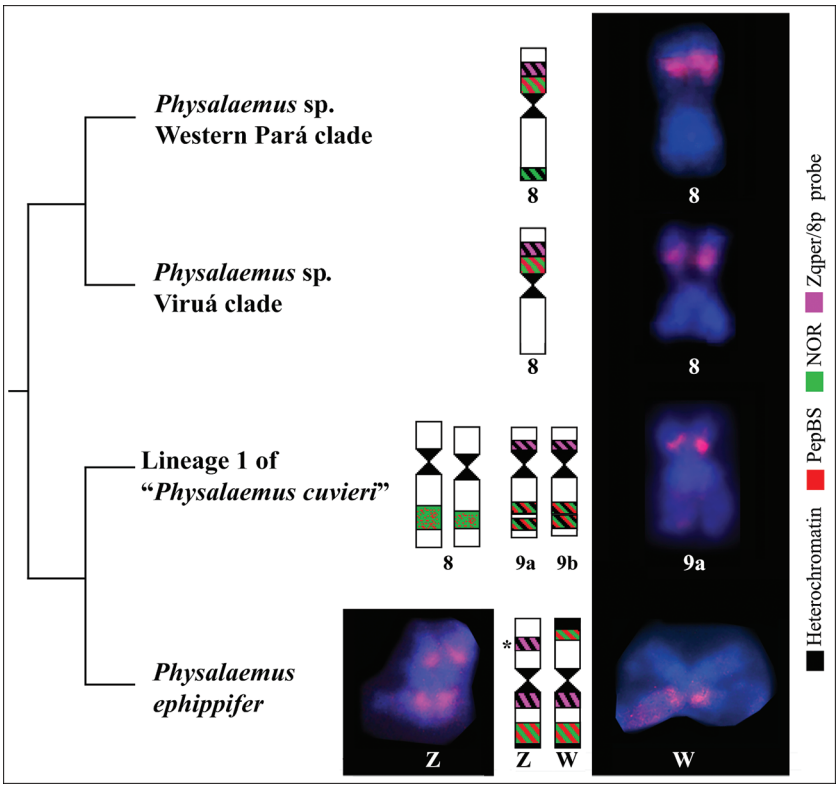


Fig. 7. Schematic representation of the NOR-bearing chromosomes of *Physalaemus ephippifer*, L1 of "*P. cuvieri*", and *Physalaemus* sp. (western Pará and Viruá clades) along with the phylogenetic relationships of these genetic lineages as inferred by Nascimento et al. (2019) and the chromosomes hybridized with the Zqper/8p probes. * denotes the band detected by the Zqper/8p probes in the short arm of the Z chromosome of *P. ephippifer* varies in size (see text for details) and only the larger band is schematized here. The ideograms of chromosomes 8 and 9 of L1 of "*P. cuvieri*" shown here refer to the specimen ZUEC 13360 (which we used to map the PepBS sequence and Zqper/8p probes), but other variants of chromosome 9, including that with a pericentromeric NOR in the long arm, are present in this lineage and may be seen in Quinderé et al. (2009).



markers. In Zq and Wq of *P. ephippifer*, the Zqper/8p region is pericentromeric and the PepBS/rDNA site is distal, whereas in 8p of *Physalaemus* sp., the pericentromeric PepBS/rDNA region is adjacent to the PepBS/rDNA site, which occupies an interstitial region in this chromosome arm. Thereby, during the evolutionary divergence of these species, chromosome rearrangements such as inversions must have occurred in the chromosomes that bear the Zqper-PepBS/rDNA region.

In contrast to the condition observed in *P. ephippifer* and *Physalaemus* sp., the pericentromeric segment detected by the Zqper/8p probes in chromosome 9 of the specimen of the Lineage 1 of "*P. cuvieri*" is not in the same arm that bears the PepBS/rDNA site. Whereas the Zqper/8p segment is in 9p, the PepBS/rDNA site is in 9q. Therefore, although the presence of a conspicuous PepBS/rDNA site and a segment detected by the Zqper/8p probes in this chromosome 9 suggests its homology to the sex chromosomes of *P. ephippifer* and chromosome 8 of *Physalaemus* sp., the distinct relative positions occupied by those markers clearly differentiate chromosome 9 of the Lineage 1 of "*P. cuvieri*" (Fig. 7).

Such a difference between the sex chromosomes of *P. ephippifer* and the homologous chromosome 9 of the Lineage 1 of "*P. cuvieri*" reinforces the need for further investigation about the role (if any) of this cytogenetic divergence on reproductive barriers between these genetic lineages. Because hybrids between individuals with different sex chromosomes may suffer from genetic incompatibilities, sex chromosomes may play an important role in speciation (Qvarnström and Bailey 2009; Graves 2016). In frogs, which harbor a high diversity of sex chromosomes and different levels of sex chromosome heteromorphism, some interesting evidence about the role of sex chromosomes in species evolution (e.g., Dufresnes et al. 2016, 2020) and even in the origin of new sex chromosomes (Miura and Ogata 2013; Ogata et al. 2018) has been reported. Thereby, the chromosome markers generated here would be very helpful in further studies on the evolution of the species complex *P. cuvieri* – *P. ephippifer*, which could even have relevant taxonomic implications.

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