Heredity (2021) 126:396–409
https://doi.org/10.1038/s41437-020-00385-7

the geneticssociety

ARTICLE

Sex chromosome evolution in frogs—helpful insights from chromosome painting in the genus *Engystomops*

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Received: 16 September 2020 / Revised: 27 October 2020 / Accepted: 28 October 2020 / Published online: 12 November 2020

Abstract

The differentiation of sex chromosomes is thought to be interrupted by relatively frequent sex chromosome turnover and/or occasional recombination between sex chromosomes (fountain-of-youth model) in some vertebrate groups as fishes, amphibians, and lizards. As a result, we observe the prevalence of homomorphic sex chromosomes in these groups. Here, we provide evidence for the loss of sex chromosome heteromorphism in the Amazonian frogs of the genus *Engystomops*, which harbors an intriguing history of sex chromosome evolution. In this species complex composed of two named species, two confirmed unnamed species, and up to three unconfirmed species, highly divergent karyotypes are present, and heteromorphic X and Y chromosomes were previously found in two species. We describe the karyotype of a lineage estimated to be the sister of all remaining Amazonian *Engystomops* (named *Engystomops* sp.) and perform chromosome painting techniques using one probe for the Y chromosome and one probe for the non-centromeric heterochromatic bands of the X chromosome of *E. freibergi* to compare three *Engystomops* karyotypes. The Y probe detected the Y chromosomes of *E. freibergi* and *E. petersi* and one homolog of chromosome pair 11 of *Engystomops* sp., suggesting their common evolutionary origin. The X probe showed no interspecific hybridization, revealing that X chromosome heterochromatin is strongly divergent among the studied species. In the light of the phylogenetic relationships, our data suggest that sex chromosome heteromorphism may have occurred early in the evolution of the Amazonian *Engystomops* and have been lost in two unnamed but confirmed candidate species.

Introduction

In contrast to most mammals and birds, which show highly differentiated sex chromosomes, in some vertebrates, including anurans, sex chromosome heteromorphism is an unusual trait (Schartl 2004; Graves 2008; Schmid et al. 2012; Targueta et al. 2018). Although variable sex determination systems, including XX/XY, ZZ/ZW, and multiple chromosome systems, have been described for anuran species (e.g., Schmid et al. 1990; 2003; Nishioka et al. 1993; Busin et al. 2008; Nascimento et al. 2010), sex chromosomes in this group are frequently detected only by advanced cytogenetic techniques, such as chromosome banding (Schmid et al. 1993, 2012; Cuevas and Formas 1996; Odierna et al. 2007). Sex chromosome differentiation is thought to have occurred several times during the divergence of anuran species (Hillis and Green 1990; Schmid and Steinlein 2003), but

Associate editor Ben Evans

Supplementary information The online version of this article (https://doi.org/10.1038/s41437-020-00385-7) contains supplementary material, which is available to authorized users.

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the mechanisms that triggered these processes in anurans are poorly understood.

Two nonexclusive hypotheses proposed to explain the prevalence of homomorphic sex chromosomes in frogs are the turnover of sex chromosome systems (see discussion in Volff et al. 2007 and Evans et al. 2012) and the fountain-of-youth model (Perrin 2009). Sex chromosome turnover is achieved by the translocation of a sex-determining gene to a different chromosome or by the replacement of a sex-determining gene by a new one (Evans et al. 2012). The first strong evidence of sex chromosome turnover came from studies of the Japanese frog *Glandirana rugosa* (Miura and Ogata 2013), which showed different populations with XY and ZW sex chromosome systems, respectively. More recently, Jeffries et al. (2018) used RADseq analysis to identify sex-linked markers and compared them with the genome assembly of *Xenopus tropicalis*, and the results suggested at least 13 events of sex chromosome turnover in the family Ranidae. The second hypothesis (fountain-of-youth model) proposed by Perrin (2009) points out that occasional recombination between X and Y chromosomes precludes Y evolutionary decay and, therefore, prevents sex chromosome differentiation. Recent evidence supporting this model was found in *Rana temporaria* (Rodrigues et al. 2018) and *Litoria aurea* (Sopniewski et al. 2019), where recombination between X and Y chromosomes was found in sex-reversed XY females.

Considering that sex chromosome turnover and/or occasional XY recombination are frequent phenomena among anurans, it would be expected that the loss of sex chromosome heteromorphism would be easily inferred from the evaluation of chromosomal evolution in a phylogenetic context. However, experimental data testing this expectation are still scarce. Here, we provide evidence of the loss of sex chromosome heteromorphism in the Amazonian frogs of the genus *Engystomops*, which constitutes an intriguing group with a complex evolutionary history and incompletely resolved taxonomy (Targueta et al. 2010; Funk et al. 2012 and references therein).

The tropical genus *Engystomops* (Leptodactylidae) encompasses two major clades, namely, the Edentulus Clade and Duovox Clade (Ron et al. 2006). At least three out of nine named species of *Engystomops* exhibit heteromorphic sex chromosomes. Two species of the Edentulus Clade, *Engystomps freibergi* and *E. petersi*, have heteromorphic X and Y chromosomes, whereas one representative of the Duovox Clade, *E. coloradorum*, has heteromorphic Z and W chromosomes (Targueta et al. 2010, 2012).

Engystomops freibergi and *E. petersi* are the only two named species of an Amazonian species complex composed of five to seven species (Funk et al. 2012). To date, besides *E. freibergi* and *E. petersi*, two unnamed but confirmed candidate species (CCS) closely related to *E. petersi* have been cytogenetically analyzed. These CCS were defined by Funk et al. (2012) and referred to as *E. "magnus"* and *E. "selva"* by Trillo et al. (2017), names we used hereafter although they are *nomina nuda* because the species still need to be formally described. The karyotypes of *E. "magnus"* and *E. "selva"* were distinct from that of *E. petersi* from Puyo-Ecuador, particularly with respect to the sex chromosomes (Targueta et al. 2010). *Engystomops petersi* males from Puyo-Ecuador have a heteromorphic sex chromosome pair that is not present in males of *E. "magnus"* from Yasuní-Ecuador (Targueta et al. 2010). Based on their similarity, the sex chromosome pair of *E. petersi* and chromosome pair 11 of *E. "magnus"* were presumed to be homologous (Targueta et al. 2010). The karyotype of *E. "selva"* from La Selva-Ecuador differs greatly from the two above mentioned karyotypes. In this species, the chromosomes are not similar to the sex chromosomes of *E. petersi* or to chromosome pair 11 of the *E. "magnus"* karyotype (Targueta et al. 2010).

In contrast, the X chromosome and, especially, the Y chromosome of *Engystomops petersi* are morphologically similar to the *E. freibergi* X and Y chromosomes (Targueta et al. 2010). However, the X chromosome of *E. petersi* shows only one terminal heterochromatic band in the long arm, whereas in *E. freibergi*, there is an additional interstitial heterochromatic band (Targueta et al. 2010).

Combining chromosome data with interspecific phylogenetic relationships, Targueta et al. (2010) proposed the following two alternative hypotheses: (i) differentiation between X and Y chromosomes occurred in a common ancestor of the Edentulus Clade of *Engystomops*, with sex chromosome heteromorphism lost at least in the lineage including the Yasuní population (*E. "magnus"*), and (ii) independent processes led to the sex chromosome differentiation in *E. petersi* and *E. freibergi* (Fig. 1). Differences noted between the sex chromosomes of *E. petersi* and *E. freibergi* may be explained by both hypotheses; thus, further studies are required to decide which is correct. Such studies should include a deeper characterization of *E. freibergi* and *E. petersi* sex chromosomes and the cytogenetic study of the Amazonian *Engystomops* candidate species that have not been karyotyped to date (namely, the French Guiana Lineage, the Pará Lineage, and the lineage referred to as Clade B by Funk and colleagues; Fig. 1).

In this context, we (i) described the karyotype of specimens of *Engystomops* sp. from a Brazilian site in the state of Amapá, which is close to French Guiana (Fig. 1), and (ii) analyzed the sex chromosomes of the Amazonian *Engystomops* using specific probes for the Y chromosome and the heterochromatic bands of the X chromosome of *Engystomops freibergi*. The results were interpreted in the light of a phylogenetic analysis of the genus, which enabled a character-state reconstruction of sex chromosome traits and

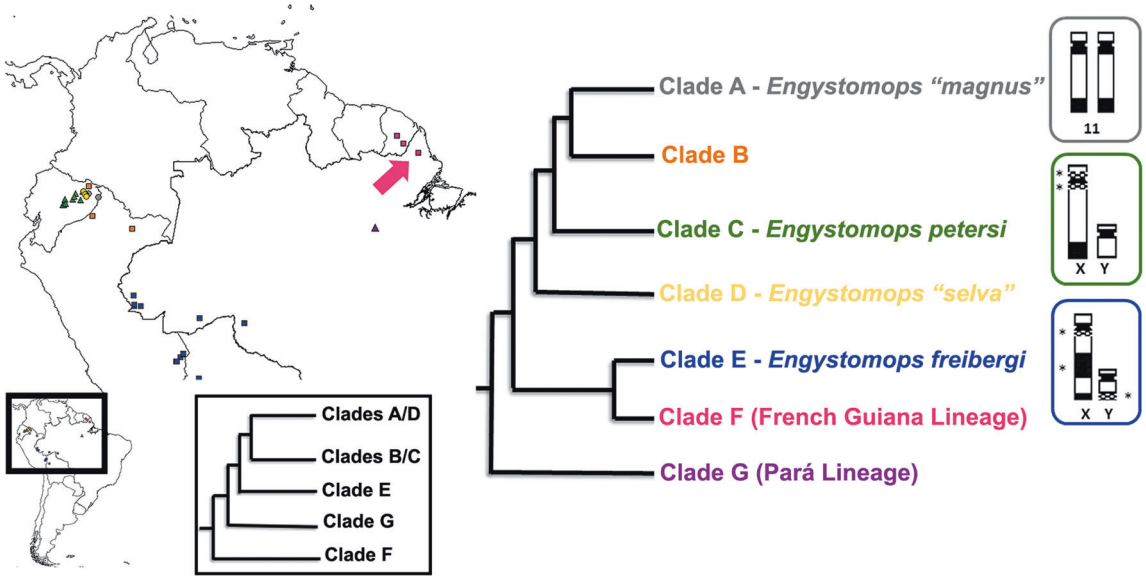

Fig. 1 Phylogenetic relationships of the Amazonian *Engystomops* inferred from mitochondrial (main image) and nuclear genes (inset) by Funk et al. (2012). Note the incongruence between both inferences. In the map, the geographical distribution of the clades is shown (identified by different colors). The arrow in the map indicates *Engystomops* sp. from Amapá, which was studied herein. Sex chromosomes are presented as ideograms based on Targueta et al. (2010). Black regions represent heterochromatic C-bands, and circles represent NORs. *Intra-populational variation is reported with respect to the size of non-centromeric heterochromatic bands and to the presence of NORs (Targueta et al. 2010).

the proposal of evolutionary hypotheses regarding chromosome evolution in *Engystomops*.

Materials and methods

Karyotype description of *Engystomops* sp. from Amapá

For classical cytogenetic analysis, we sampled five *Engystomops* sp. males from the Lourenço district (Calçoene municipality) in the state of Amapá, Brazil under a permit issued by the Chico Mendes Institute for Biodiversity Conservation/Biodiversity Information and Authorization System (ICMBio/SISBIO) (permit number 32483), which also included authorization for extracting tissue samples. The animal vouchers were deposited in the amphibian collection of the "Prof. Adão José Cardoso" Museum of Zoology at the Institute of Biology—University of Campinas (ZUEC) (accession numbers ZUEC 22680-22684). These males were processed for cytogenetic analysis following King and Rofe (1976) with modifications from Gatto et al. (2018). This protocol was approved by the Committee for Ethics in Animal Use of the University of Campinas (CEUA/UNICAMP) (protocol # 4363-1).

Chromosome preparations were sequentially stained with 10% Giemsa, C-banded following King (1980) and stained with 4′-6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml) and mithramycin (MM, 0.5 mg/ml). Nucleolar organizer regions (NOR) were detected by silver staining through the Ag-NOR method (Howell and Black 1980).

Sex chromosome analysis using chromosome painting

Chromosome preparations

In addition to chromosome preparations from the five *Engystomops* sp. males from Amapá mentioned in the previous section, we used chromosome preparations from seven male *E. freibergi*, one male and three female *E. petersi* and one male *E. "magnus"*, which were previously generated by Targueta et al. (2010) and made available at the cell suspension bank housed at the Chromosome Studies Laboratory (LabEsC), State University of Campinas, Campinas, Brazil. The specimens of *Engystomops freibergi* were collected from the Zoobotanical Park of the Federal University of Acre, Acre State, Brazil (ZUEC 14440, ZUEC 14443, ZUEC 14450, ZUEC 14454-14456, and ZUEC 14465). The individuals of *E. petersi* were collected from Puyo, Provincia de Pastaza, Ecuador; the specimen of *E. "magnus"* was collected from the Estación Científica Yasuní, Provincia de Orellana, Ecuador; and all were deposited at Museo de Zoologia da Pontifícia Universidad Católica del Ecuador (QCAZ) (accession numbers QCAZ 34935, QCAZ 34937, QCAZ 34940, QCAZ 34942, and QCAZ 34947).

Laser microdissection of X and Y sex chromosomes of Engystomops freibergi and probe preparation

Intestinal cell suspensions from male *Engystomops freibergi* specimens from Acre, Brazil (ZUEC 14454 and ZUEC 14465) were dropped onto coverslips, covered with a polyethylene membrane (PEN) and stained with 10% Giemsa. Only the X and Y chromosomes that did not contain NORs were used in this step. The whole Y chromosome and the long arm of the X chromosome were dissected from 15 mitotic metaphases and collected separately using a P.A.L.M. MicroBeam system (Zeiss, Jena, Germany) coupled with an inverted microscope (Olympus, Tokyo, Japan). Laser microdissection, DNA amplification, and labeling were performed as described previously by Krylov et al. (2010). The resulting probes were purified using a GeneJet PCR Purification Kit (Fermentas) and named pY and pXh, respectively.

For in situ hybridization experiments, the probes were diluted to 20 ng/µl in a final volume of 22 µl containing formamide (50%), dextran sulfate (10%), 2X saline-sodium citrate (SSC), and competitor DNA (~10–78 ng/µl). The competitor DNA consisted of 75- to 500-bp fragments, which were obtained after autoclaving a sample of the genomic DNA of *Engystomops freibergi* in 0.3-M NaCl at 1.4 atm/120 °C for 30 min. In some experiments with the pXh probes, sonicated salmon sperm DNA (10 ng/µl) was used as the competitor DNA and, in others, no competitor DNA was added.

Fluorescence in situ hybridization (FISH) with probes obtained from microdissected material

Engystomops freibergi cell suspensions were dropped onto slides and stored at −20 °C for at least one night. The chromosomes were denatured in formamide (70%) diluted in 2X SSC for 2 min at 72 °C. Then, the chromosomes were dehydrated in a methanol series (70, 90, and 100%) at room temperature and dried.

The probes were denatured at 72 °C for 10 min and renatured at 37 °C for 80 min. Approximately 22 µl of probe was dropped onto the slides with denatured chromosomes and hybridized at 37 °C for 16 h in a wet chamber.

The chromosome preparations with the pY probe were washed in two consecutive 50% formamide/2X SSC baths at 42 °C (5 min each) and in 0.4X SSC at room temperature (3 baths, 5 min each). Alternatively, the specimens with the pXh probe were washed in 50% formamide/2X SSC at 37 °C for 5 min and then in 2X SSC. All chromosome preparations were then placed in PBT [PBS pH 7.4 (130-mM NaCl, 7-mM Na_2HPO_4, 3-mM NaH_2PO_4), 8.33% BSA, and 0.1% Tween 20] for 5 min. For probe detection, the chromosome preparations were exposed to a PBT solution containing anti-digoxigenin Ab coupled with rhodamine (Roche) (1:200) for 60 min. After washing in PBT (3 baths, 5 min each), the slides were mounted in 0.5-µg/ml DAPI diluted in Vectashield (Vector, California, U.S.A.), and the probe signals were visualized and documented under an Olympus fluorescence microscope coupled with a camera. The images were processed with Adobe Photoshop version CS6.

The probes obtained from the X (pXh) and Y (pY) chromosomes of *Engystomops freibergi* were also hybridized with the karyotypes of *Engystomops* sp. from Amapá, *E. petersi* from Puyo, and *E. "magnus"* from Yasuní. For this purpose, we prolonged the hybridization time to 72 h. The two alternative conditions used for washing probes in the *E. freibergi* chromosome preparations were also employed in the cross-species FISH procedure.

Phylogenetic analysis and genetic distances

We used DNA sequences to obtain a phylogenetic tree to infer the ancestral states of sex chromosomes of *Engystomops* (see next section). In addition, considering the taxonomic uncertainties regarding the Amazonian *Engystomops*, a DNA-based analysis was fundamental to properly identify to which evolutionary lineage the specimens from Amapá belong.

For this analysis, genomic DNA was extracted from liver samples of *Engystomops* sp. from Amapá, Brazil following Medeiros et al. (2013) (SM1, for samples discrimination). A region of ~2300 bp containing the mitochondrial ribosomal genes 12S and 16S and the RNAt-Val gene (the H1 fragment) was isolated by PCR using the primer pairs MVZ 59 (Graybeal 1997)—Titus I (Titus 1992) and 12L13 (Feller and Hedges 1998)—16Sbr (Palumbi et al. 2002). The products of these PCR reactions were purified using the ReliaPrep™, Wizard and Maxwell gDNA purification kits (Promega, Wisconsin, U.S.A.). The samples were sequenced using the BigDye Terminator kit (Applied Biosystems, California, U.S.A.), with the primers mentioned above, in addition to MVZ50 (Graybeal 1997), 16SL2a (Hedges 1994), 16H10 (Hedges 1994), and 16Sar (Palumbi et al. 2002), in an ABI 3730xL DNA Analyzer automatic sequencer (Applied Biosystems, California, U.S.A.).

Nucleotide sequences available in GenBank for the Amazonian *Engystomops* species were downloaded and are listed in the Supplementary Material (SM1). We did not include sequences of *Engystomops* from Ecuador that were previously recognized by Funk et al. (2012) as mitochondrial DNA that had introgressed from one clade to another in the analysis (QCAZ 25790, QCAZ 28169, QCAZ 28178, QCAZ 25790, QCAZ 44073, QCAZ 24029, QCAZ 28578, QCAZ 28577, QCAZ 46032, QCAZ 15164, QCAZ 46033, QCAZ 23975, QCAZ 28576, QCAZ 38133, QCAZ 38134).

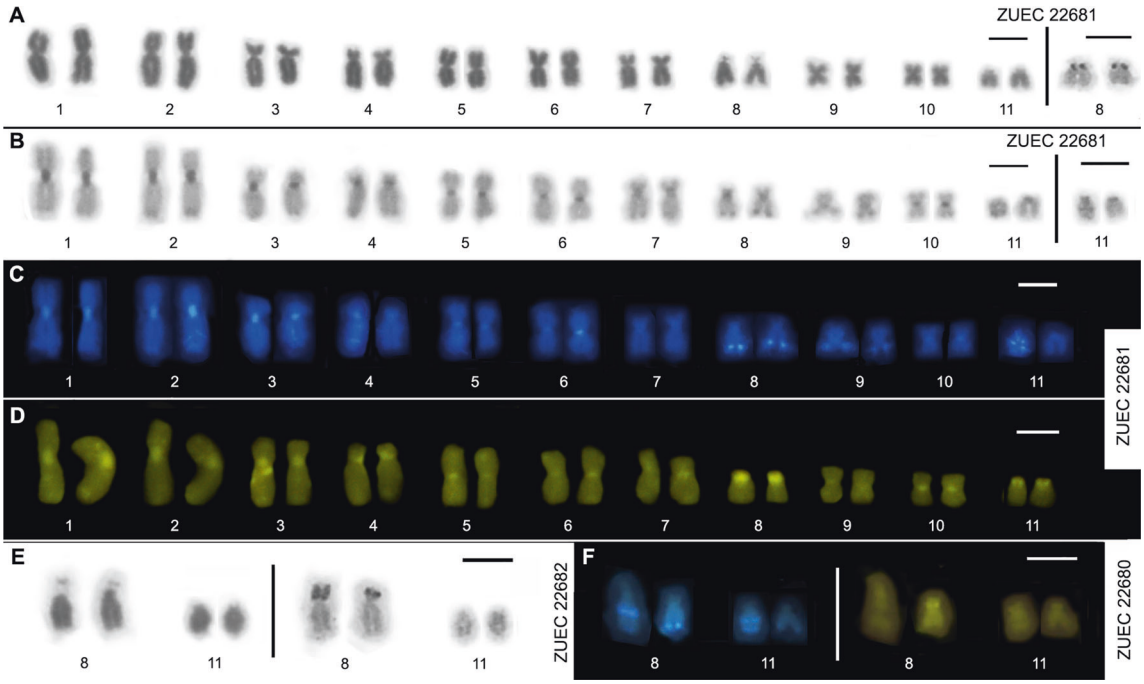

Fig. 2 Karyotype of *Engystomops* sp. from Amapá, Brazil. Chromosomes stained with Giemsa (**A**), submitted to C-banding (**B**), and sequentially stained with DAPI (**C**) and MM (**D**). In inset in **A**, a silver-stained chromosome pair 8 bearing a homomorphic pair of NORs is shown. In inset **B**, note the heteromorphism in a C-banding pattern in chromosome pair 11. In **E**, chromosome pairs 8 and 11 of the specimen ZUEC 22682 are stained with Giemsa and silver-stained by the Ag-NOR method. Note the heteromorphic pair 8 with a homolog (on the left) bearing a larger NOR. In **F**, chromosome pairs 8 and 11 of specimen ZUEC 22680 were sequentially submitted to C-banding and staining with DAPI and MM. Note that pair 8 is heteromorphic with respect to the location of a DAPI-positive C-band on the long arm.

As an outgroup, we included at least one sequence of each of the seven remaining species of *Engystomops* (SM1). We also included representative sequences of the two major clades of the genus *Physalaemus* (*P. signifer* Clade and *P. cuvieri* Clade) and one species of *Edalorhina* (SM1), as both genera were inferred as closely related to *Engystomops* (see Lourenço et al. 2015).

The sequences were edited using BioEdit version 7.2.5 (Hall 1999) and aligned with MAFFT Online (https://mafft.cbrc.jp) using the G-INS-I strategy. Phylogenetic analysis under the Bayesian approach was performed using MrBayes version 3.2 (Ronquist et al. 2011). The GTR + I + G evolutionary model was used as inferred by jModelTest (Posada 2008). Bayesian analyses were run for 30 million generations. Tracer version 1.5 (Rambaut and Drummond 2007) was used to check for the stabilization of posterior probabilities. The consensus tree was edited in Figtree software.

Genetic distances (p-distance) between the principal clades of the Amazonian *Engystomops* inferred from phylogenetic analysis were calculated using MEGA 7.0 (Kumar et al. 2016), not considering gaps in pairwise comparisons. The genetic distances were estimated based on the H1 fragment used in our phylogenetic analysis and the 16Sar-16Sbr fragment, which has been largely used for evaluating genetic diversity in anurans (Fouquet et al. 2007; Lyra et al. 2017).

Character-state reconstruction

We used the 50% majority-rule consensus tree recovered from MrBayes to reconstruct the ancestral states of sex chromosomes in the software BayesTraits version 3 (Meade and Pagel 2019). The sex chromosome states were set as homomorphic sex chromosomes, heteromorphic XY, and heteromorphic ZW. The unknown state was assigned to the species and confirmed candidate species not karyotyped to date. We ran the MultiState model and MCMC approach for 10^6 iterations with a burn-in of 10,000. Posterior probabilities of ancestral states were estimated and plotted in pie charts.

FISH with telomeric probes

Telomeric sequences presented in *E. freibergi* karyotypes were detected by FISH using the PNA probe $(CCTAA)_3$ following the manufacturer's instructions (PNABio, California, U.S.A.).

Results

Karyotype of *Engystomops* sp. from Amapá, Brazil

The males of *Engystomops* sp. from Amapá, Brazil presented a $2n = 22$ karyotype, with chromosome pairs 1, 5, 6, 9, and 10 being metacentric, chromosome pairs 2–4 and 7 being submetacentric and pairs 8 and 11 being subtelocentric (Fig. 2).

The centromeric region of all chromosomes was revealed by C-banding (Fig. 2B) and stained with both DAPI (Fig. 2C) and MM (Fig. 2D) fluorochromes. Heterochromatic C-bands were also found terminally in the long arm of some chromosome pairs, especially pairs 8 and 11, and terminally in the short arm of chromosome pair 11 (Fig. 2B). The terminal C-band in the long arm of pair 8 was strongly stained by DAPI but not by MM, whereas the C-band in the short arm of chromosome 11 was stained by MM and not by DAPI (Fig. 2C, D). None of the C-bands, not even the MM-positive C-band on the short arm of chromosome 11, were recognized as NORs by the Ag-NOR method (Fig. 2D).

Chromosome pair 11 was heteromorphic in all analyzed males due to the presence of a conspicuous interstitial DAPI-positive/MM-negative heterochromatic band in only one homolog (Fig. 2B–D). In addition, chromosome 11 that carries this interstitial C-band also bears a conspicuous terminal C-band in the long arm, whereas its homolog has a faint terminal C-band in the long arm (see Fig. 2F), which is hardly seen in some metaphases.

In one individual (ZUEC 22680), pair 8 was also heteromorphic, since homologs differed with respect to the position of a DAPI-positive/MM-negative C-band in the long arm. While in one chromosome 8, this band was terminal, its counterpart bore an interstitial band (Fig. 2F).

The NOR was adjacent to the centromere in the short arm of chromosome 8 and MM-positive/DAPI-negative in all analyzed animals (Fig. 2A-inset and 2D). In three individuals (ZUEC 22680, ZUEC 22682, ZUEC22683), chromosome pair 8 was notably heteromorphic with respect to the NOR size (Fig. 2E).

Chromosome painting of *Engystomops* sex chromosomes

In *Engystomops freibergi* from Acre, the pY probe detected the entire Y chromosome, as expected, and stained a small region around the centromere of the X chromosome (Fig. 3). In this population, the Y chromosome differs from the X chromosome in its size and heterochromatin amount. The Y chromosome is smaller and bears only one heterochromatic band, which is located in the centromere region, whereas the X chromosome shows interstitial and terminal heterochromatic bands in the long arm in addition to the centromeric C-band (Fig. 3). The pXh probe strongly detected these non-centromeric heterochromatin regions in the long arm of the X chromosome (Fig. 3B). It is worth noting that besides these heterochromatic regions, no additional X chromosome segment was detected by the pXh probe, although this probe was derived from the microdissection of the entire X chromosome long arm. In addition, the pXh probe produced a small but consistent signal at the terminal region of the long arm of chromosome 11 (Fig. 3D), whereas the interstitial C-band located on this chromosome arm did not show any positive hybridization signal (Fig. 3D). When hybridized to a telomeric probe (SM2), only the terminal regions of all chromosomes of *E. freibergi* were detected, with no signal being present in the interstitial heterochromatic band of the long arm of the X chromosome.

In meiotic XY bivalents of *Engystomops freibergi*, the region at the X chromosome detected by the pY probe was near the single chiasma between the short arm of the X chromosome and the Y chromosome, while the segment detected by the pXh probe was more distant from this chiasma (Fig. 3F).

In the karyotype of *E. petersi* from Puyo, the pY probe detected the whole Y chromosome and a proximal segment in the long arm of the X chromosome, which was barely seen in some metaphases (Fig. 4A, B). The hybridization of the pY probe with the karyotype of *Engystomops* sp. from Amapá painted the whole homolog of pair 11 that avoids the interstitial DAPI-positive heterochromatic band (Fig. 4C, D). In addition, only a small hybridization signal was seen pericentromerically in the long arm of chromosome 11 that bears conspicuous interstitial and terminal heterochromatic bands (Fig. 4C, D).

When the pXh probe was hybridized with chromosome preparations of *Engystomops* sp. from Amapá, *Engystomops petersi* from Puyo, and *E. "magnus"* from Yasuní, no signal was detected, despite the low stringent conditions used in the FISH procedure (data not shown).

Sex chromosome evolution

Our phylogenetic analysis clustered the specimens from Amapá, Brazil within the highly supported Amazonian clade of *Engystomops* and recovered all the major monophyletic groups recognized previously by Funk et al. (2012) in the Edentulus Clade (SM3). The Amapá specimens were placed, with strong support, in the same clade as specimens of *Engystomops* from French Guiana (SM3). Low genetic divergence was found between the specimens from Amapá and those from French Guiana in both the H1 fragment (0.1%) and the 16Sar-16Sbr fragment (0.1%). In contrast, high genetic distances were

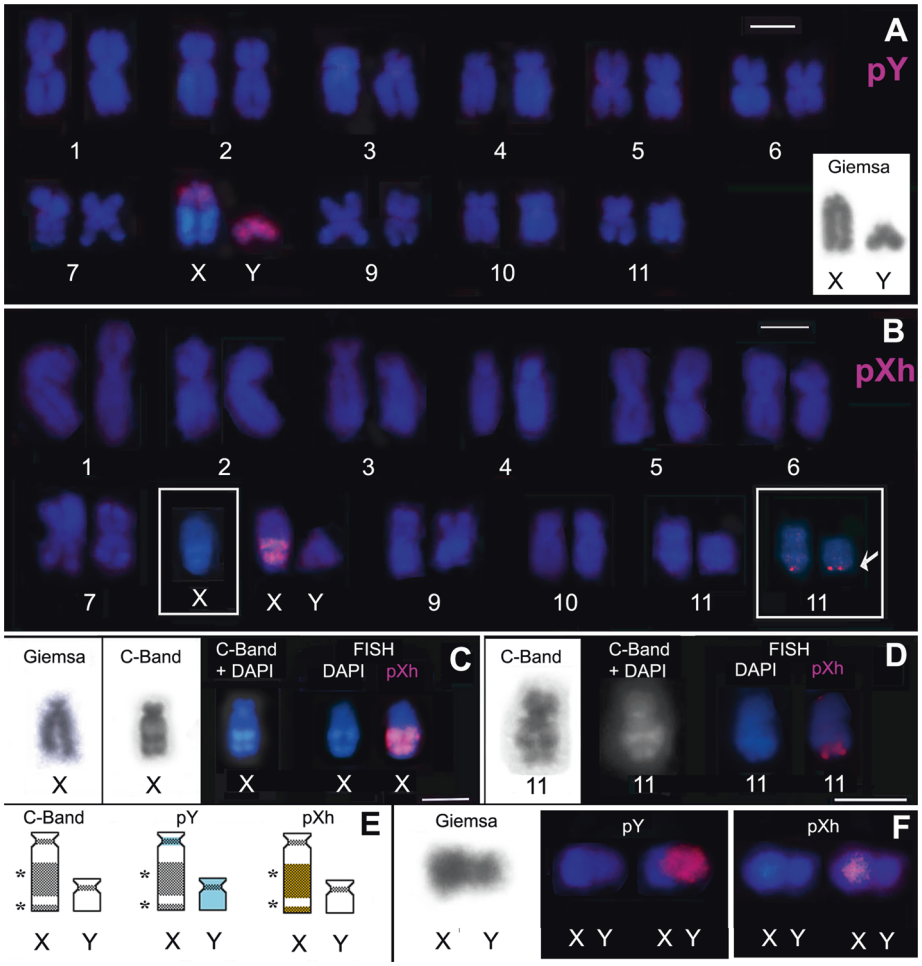

Fig. 3 In situ hybridization of the pY and pXh probes. *Engystomops freibergi* karyotypes hybridized with the probes pY (**A**) and pXh (**B**). In the insets in **B**, the X chromosome is shown stained with DAPI, and chromosome pair 11 is shown with a darker DAPI image and a brighter probe image. Note that the regions detected with the pXh probe coincide with the DAPI heterochromatic bands of the long arm of the X chromosome and with the terminal region of chromosome 11 (arrow). **C** X chromosome of *Engystomops freibergi* stained with Giemsa, C-banded, subjected to DAPI staining after C-banding, and hybridized with the pXh probe. In comparing **B** and **C**, note the intra-populational variation observed with respect to the size of the Xq heterochromatic bands. **D** Chromosome 11 subjected to C-banding, DAPI staining after C-banding, and hybridization with the pXh probe. Note that the probe signal is terminally located in the long arm and not coincident with the interstitial heterochromatic band. Bars = 4 µm. **E** Scheme depicting the regions detected by C-banding (black) and by the pY (blue) and pXh (yellow) probes. *Intra-populational variation is observed with respect to the size of these non-centromeric heterochromatic bands. **F** Meiotic XY bivalents stained with Giemsa and hybridized with the pY and pXh probes.

found between the French Guiana + Amapá clade and the remaining seven major clades of the Amazonian *Engystomops* (SM4).

The French Guiana + Amapá clade (Clade F in SM3) was recovered as a sister of all the other lineages/species of the Amazonian *Engystomops* species complex (SM3). However, the support of the clade with all the Amazonian *Engystomops* except the French Guiana and Amapá exemplars was not high (posterior probability of 0.8; SM3).

The lineage of the specimens from Pará was recovered as the sister group of *E. freibergi* but with low statistical support (posterior probability of 0.51; SM3). Regarding the *E. petersi* species complex, we recovered Clades A–D previously recognized by Funk et al. (2012). A highly supported clade composed of *E. petersi* (Clade C), *E.* "*magnus*" (Clade A), and Clade B was recovered as a sister group of *E. "selva"* (Clade D) (SM3).

Based on this phylogenetic hypothesis, the ancestral state reconstruction of sex chromosomes favors the presence of heteromorphic X and Y chromosomes in the common ancestor of all the Amazonian *Engystomops* (53% posterior probability, arrow in Fig. 5) in contrast to homomorphic state and heteromorphic ZW chromosomes (24% and 23% posterior probability, respectively). Therefore, the evolutionary scenario with the highest posterior probability posits that the XY heteromorphic condition was secondarily lost, as it is not found in *E.* "*selva*" and *E.* "*magnus*" (Fig. 5).

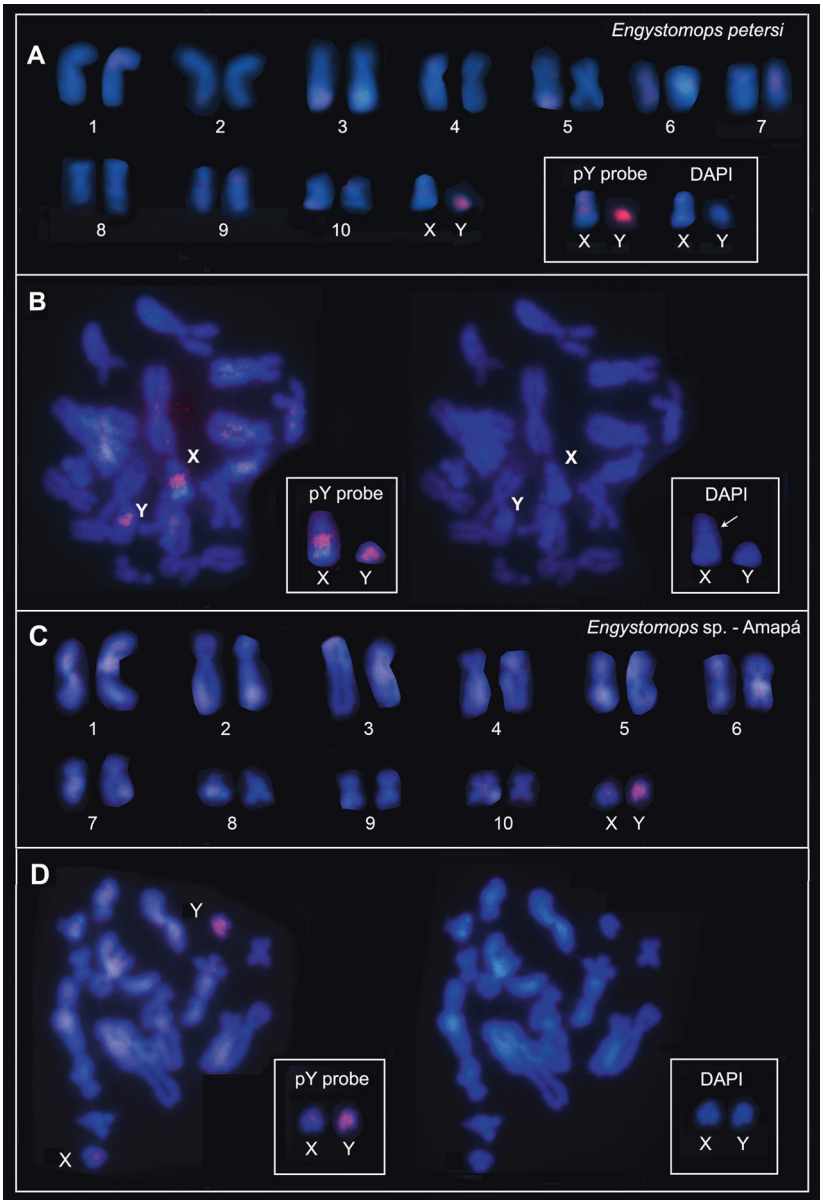

Fig. 4 Cross species in situ hybridization of the pY probe. Metaphases of *Engystomops petersi* from Puyo (**A, B**) and *Engystomops* sp. from Amapá (**C, D**) hybridized with the pY probe. Note that Y chromosome of *E. petersi* is wholly painted by this probe in **A, B**. In inset in **A**, the sex chromosome pair of *E. petersi* is shown under a higher level of brightness to better visualize the probe signal in the X chromosome. In **B**, a metaphase of *E. petersi* with an evident probe signal between the NOR constriction (DAPI-negative segment indicated by an arrow) and the distal DAPI-positive heterochromatic band of the X chromosome. In the metaphase of *Engystomops* sp. from Amapá (**C, D**), only one of the 11th-pair homologs is entirely painted by the Y probe, which supports its identification as a Y chromosome. Note that only a small signal of the Y probe is present in the homolog of pair 11 that bears two notorious non-centromeric DAPI-positive bands (chromosome tentatively identified as the X chromosome —see text for details).

Discussion

Engystomops sp. from Amapá, a representative of the Amazonian *Engystomops* Clade F, fills a gap in cytogenetic data

Previous studies recognized that specimens from French Guiana composed a well-supported clade of the Amazonian *Engystomops* named Clade F, which was genetically highly divergent from all the remaining clades inferred for this genus (Funk et al. 2012). However, morphological and acoustic data remained unavailable for these specimens, and Clade F was considered an unconfirmed candidate species (Funk et al. 2012). Here, we found that Clade F is not restricted to French Guiana, since specimens from the Brazilian municipality of Lourenço (state of Amapá, Brazil) clustered in the same clade as specimens from French Guiana, with low genetic divergence between the samples from both areas. Thereby, the geographical distribution of Clade F is larger than previously suspected, and by describing the karyotype of specimens from Amapá, cytogenetic data were made available for an unnamed species of the Amazonian *Engystomops* that is of fundamental importance for evolutionary studies in this group.

The karyotype of *Engystomops* sp. from Amapá is composed of 22 chromosomes, which is the same diploid number reported for all the remaining species of the Edentulus Clade (Targueta et al. 2010; Morescalchi and Gargiulo 1968). Moreover, it is similar to the *E. freibergi* and *E. petersi* karyotypes (Targueta et al. 2010), especially

from chromosome pairs 1–6. In *Engystomops* sp., the subtelocentric chromosome pair 8 bears an NOR on its short arm and has a non-centromeric C-band on the long arm, which makes it similar to one of the morphs of X chromosome found in *E. petersi* (Targueta et al. 2010). However, pair 8 of *Engystomops* sp. did not have any chromosomes detected by the *E. freibergi* Y probe. Instead, this Y probe strongly painted one of the chromosomes of pair 11 of *Engystomops* sp., which was a heteromorphic pair with respect to the C-banding pattern in all the males we analyzed. Chromosome 11 that was painted by the pY probe bears a faint terminal C-band in the long arm. In contrast, chromosome 11 that showed only a small pericentromeric pY probe signal bears a terminal C-band on its long arm, as do the X chromosomes of *E. freibergi* and *E. petersi*, and an interstitial C-band, similar to the X chromosome of *E. freibergi*.

Although no females of *Engystomops* sp. were analyzed, the exclusive complete painting of one chromosome 11 and the C-banding heteromorphism of chromosome pair 11 of all males support the hypothesis that pair 11 is the sex chromosome pair of *Engystomops* sp. from Amapá. Further analyses of female specimens, however, are still necessary to corroborate this hypothesis.

Another relevant finding regarding the cytogenetics of *Engystomops* sp. from Amapá refers to the polymorphisms of chromosome 8. Even though only few males were analyzed, three distinct morphs of chromosome 8 were detected, which differed with respect to the NOR size or C-banding pattern. The two morphs of chromosome 8 that differed by the location of a non-centromeric C-band on the long arm (interstitial or terminal) may be the result of a chromosome paracentric inversion involving this heterochromatic region. This finding denotes a high level of chromosome variation in this population, for which the causes should be explored in further studies.

In our phylogenetic analysis, Clade F including Amapá specimens was inferred as the sister group of a clade composed of all the remaining Amazonian *Engystomops*. Such inference agrees with the results obtained by Funk et al. (2012) based on nuclear genes but not with the inference from mitochondrial sequences by the same authors, in which Clade F (French Guiana Lineage) was recovered as the sister group of *E. freibergi*, although with no statistical support. Considering that in the latter case, Funk and colleagues used the mitochondrial H1 fragment and Bayesian approach (in addition to maximum-likelihood analysis), as we did in our analysis, we may conclude that the divergence most likely relies on the inclusion of the samples from Amapá. Although further studies with expanded specimen and gene samplings are still necessary for a better evaluation of the phylogenetic relationships in *Engystomops*, the available data enabled some interesting inferences about sex chromosome evolution in this genus, as we discuss below.

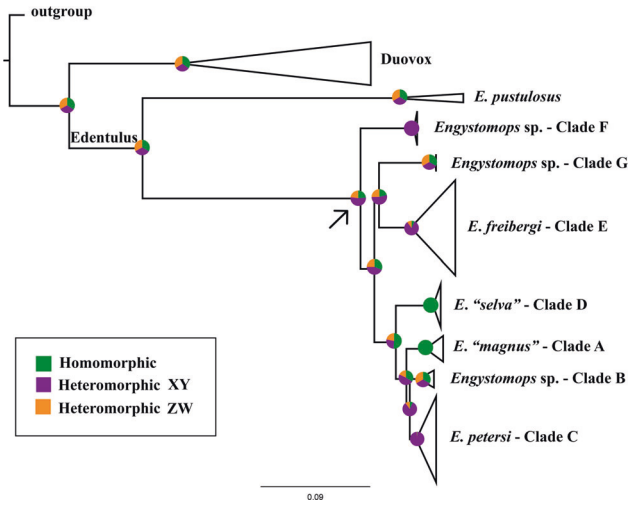

Fig. 5 Ancestral reconstruction of sex chromosome conditions in the genus *Engystomops*. Three states were considered for this analysis: homomorphic chromosomes (green), heteromorphic X and Y chromosomes (purple), and heteromorphic Z and W chromosomes (orange). The arrow indicates the most recent common ancestor of all the Amazonian *Engystomops*.

Sex chromosome evolution in the Amazonian *Engystomops*

Though chromosome painting has been used in studies of the sex chromosomes of several organisms (e.g., Hassanane et al. 1998; Shibata et al. 1999; Marchal et al. 2004; Diniz et al. 2008; Wang et al. 2009; Cioffi et al. 2011; Henning et al. 2011; Kawagoshi et al. 2012), it has only been modestly employed to study anuran chromosome evolution. To date, only five papers have reported satisfactory data from chromosome painting (Krylov et al. 2010; Gruber et al. 2014; Uno et al. 2015; Knytl et al. 2017; Targueta et al. 2018), and none focused on sex chromosomes. Here, we obtained a high-quality chromosome probe from the Y chromosome of *Engystomops freibergi*, which enabled valuable comparisons. This probe painted the Y chromosome of *E. freibergi* (Acre) and detected the entire Y chromosome of *E. petersi* and one entire chromosome 11 of male *Engystomops* sp. from Amapá (inferred to be its Y chromosome). Therefore, we could hypothesize that the Y chromosomes of *Engystomops freibergi*, *E. petersi*, and *Engystomops* sp. from Amapá share the same evolutionary origin. Based on the phylogenetic inferences, in which Clade F (*Engystomops* sp. from Amapá included) is the sister group of a clade that includes *E. freibergi* and *E. petersi*, and on the ancestral reconstruction of sex chromosome states, we may infer that sex chromosome differentiation occurred before the

split between these two major clades, in the common ancestor of the Amazonian *Engystomops*.

The supposed sex chromosome pair of *Engystomops* sp. from Amapá shows the lowest level of heteromorphism when compared to the sex chromosomes of *E. freibergi* and *E. petersi* (Targueta et al. 2010). Considering that *E. petersi* (Clade C in Fig. 5) is more closely related to *E. freibergi* (Clade E in Fig. 5) than *Engystomops* sp. from Amapá (included in Clade F in Fig. 5), one may hypothesize that pair 11 of *Engystomops* sp. represents an early stage of sex chromosome differentiation when compared to the sex chromosomes of *E. freibergi* and *E. petersi*.

The first evolutionary steps leading to heteromorphic sex chromosomes involve the acquisition of a sex-determining gene in one homolog, followed by the suppression of chromosomal recombination (Ohno 1967; Charlesworth 1991; Charlesworth et al. 2005). Heterochromatin accumulation is not only a cause but also a consequence of the suppression of crossing over during sex chromosome differentiation (Singh et al. 1976; Bull 1983; Charlesworth 1991; Kejnovsky et al. 2009). In most cases, heterochromatin accumulation occurs in the chromosome exclusive to the heterogametic sex (W or Y) (examples in Ray-Chaudhuri et al. 1971; Singh et al. 1980; Chakrabarti et al. 1983; Schmid et al. 1983; 2002; Green 1988; Mahony 1991; Ananias et al. 2004; Busin et al. 2008; Vicari et al. 2008; Nascimento et al. 2010), and heterochromatin accumulation is associated with the degeneration/differentiation of these chromosomes (Schmid 1980; Schemberger et al. 2011). However, this is not the case in the species complex *Engystomops freibergi-Engystomops petersi*, in which heterochromatin is accumulated not in the chromosome exclusive to the heterogametic sex (Y chromosome) but in its partner (X chromosome) [Targueta et al. (2010) and present data]. Although this is a less frequent condition, it has also been observed in other anuran species (Schmid et al. 1993; Miura 1994; Cuevas and Formas 1996; Sumida 1997).

The pXh probe produced in this study detected the interstitial and terminal heterochromatic regions of the long arm of the *Engystomops freibergi* X chromosome but showed no hybridization signal in the Y counterpart. The detection of X-specific heterochromatin indicates differences in the repetitive DNA content between the X and Y chromosomes, which may have resulted in and/or reinforced the lack of recombination between ancestral sex chromosomes, as discussed above. Therefore, it is likely that these heterochromatic regions played an important role throughout sex chromosome differentiation in *E. freibergi*.

The additional detection of a small terminal heterochromatic region on the long arm of *Engystomops freibergi* autosomal pair 11 by the pXh probe suggests some level of nucleotide similarity between the sequences that compose this autosomal heterochromatin and those found in the large non-centromeric heterochromatic Xq bands. Considering that (i) a telomeric probe did not detect this terminal region of 11q more brightly than the other telomeres and (ii) no other terminal region, apart from those on the long arm of Xq and 11q, was detected by the pXh probe (which also detected the interstitial band in Xq), we may exclude the hypothesis that the similarity between the terminal bands of 11q and Xq is restricted to telomeric sequences. The presence of repetitive sequences found in one of the sex chromosomes in autosomes has been previously reported for some organisms. In the medaka fish *Oryzias hubbsi*, for example, the repetitive element *Bst*NI, which mapped to the W chromosome, is also present in two autosomal pairs but absent on the Z chromosome (Takehana et al. 2012). The same repetitive DNA sequence was also found in a closely related species, *Oryzias javanicus*, but this element is restricted only to an autosomal pair. Based on these findings, the authors proposed that this repetitive element originated in the common ancestor of these two species and was highly amplified in *O. hubbsi*, particularly on the W chromosome (Takehana et al. 2012).

In contrast to the pXh probe, which hybridized exclusively with one of the sex chromosomes, the pY probe detected the centromere/pericentromere regions of the subtelocentric X chromosome in addition to the entire Y chromosome of *Engystomops freibergi*, suggesting that the areas near the centromere of the X chromosome share similar DNA sequences with the Y counterpart. The analysis of meiotic chromosomes revealed the presence of chiasma between the short arms of the sex chromosomes, indicating that these chromosome arms might include or represent pseudo-autosomal regions as defined by Burgoyne (1982). Therefore, we may hypothesize that the regions detected by the pY probe in the X chromosome have counterparts in the Y chromosome and may correspond to segments derived from an ancient autosomal pair.

Using cytogenetic techniques, Targueta et al. (2010) observed that the X chromosome of *Engystomops petersi* from Puyo and pair 11 of *E. "magnus"* from Yasuní did not exhibit any interstitial heterochromatic band in the long arm, differing from the X chromosome of *E. freibergi*, even though all of these chromosomes have a common terminal heterochromatic band in the long arm. In *Engystomops* sp. from Amapá, the homolog of pair 11 inferred to be the X chromosome (see discussion above) shows a conspicuous interstitial heterochromatic region in addition to a terminal region, similar to the X chromosome of *E. freibergi*, although these chromosomes deeply differ in chromosomal length and interstitial C-band size. The pXh probe constructed from the heterochromatic blocks of the *E. freibergi* X chromosome did not detect neither the heterochromatic bands in the X chromosome of *E. petersi*

nor the heterochromatic bands in pair 11 of *E. "magnus"* from Yasuní and *Engystomops* sp. from Amapá. These results suggest that the X chromosomes of *E. freibergi* and *E. petersi*, the supposed X chromosome of *Engystomops* sp. from Amapá and chromosome 11 of *E. "magnus"* differ not only in the position and size of non-centromeric heterochromatic bands but also in their molecular compositions.

Despite this finding, it is still not possible to determine whether the heterochromatic non-centromeric regions of the similar X chromosomes of *Engystomops freibergi* and *E. petersi* shared a common origin or emerged independently after the evolutionary divergence of these two species. The evolution of repetitive DNA sequences as transposable elements and satellite DNA, which are the major components of heterochromatic regions, is very complex and might occur at a very rapid rate. Different satellite DNA families might exhibit distinct levels of variability depending on the ratio between mutation and homogenization/fixation rates, and divergence between satellite DNA sequences can be detected at different taxonomic levels, including the species level (e.g., Subirana et al. 2015; Kirov et al. 2017; Souza et al. 2017; for a review, see Ugarkovic and Plohl 2002). Therefore, even in the scenario in which the heterochromatin regions of the *Engystomops freibergi* and *E. petersi* X chromosomes share a common origin, these blocks could rapidly lose their sequence conservation after the evolutionary split of both species.

Finally, the evolutionary scenario that has the highest posterior probability on the ancestral reconstruction of the sex chromosomes evolution combined with the chromosome painting results showed that a secondary loss of sex chromosome heteromorphism also took place in the Amazonian *Engystomops*. Considering that (i) the heteromorphic sex chromosomes of *Engystomops freibergi*, *E. petersi*, and *Engystomops* sp. from Amapá share a common evolutionary origin, as discussed above; (ii) *E. "magnus"* and *E. "selva"* are not sister species, and with *E. petersi*, they compose the sister group of a clade that includes *E. freibergi*; and (iii) *Engystomops* sp. from Amapá is the sister taxon of all the remaining Amazonian *Engystomops* (Fig. 5), the absence of heteromorphic X and Y chromosomes in *E. "magnus"* and *E. "selva"* (Targueta et al. 2010) is likely explained by loss of sex chromosome heteromorphism. The ancestral state reconstruction analysis assigned to the most recent common ancestor of *E. "selva"*, *E. "magnus"*, *Engystomops* sp. - Clade B, and *E. petersi* had a higher posterior probability (51%; Fig. 5) of having homomorphic sex chromosomes than heteromorphic X and Y (27%; Fig. 5). In this scenario, the heteromorphic sex chromosomes of *E. petersi* and *E. freibergi* would have arisen independently from those of *Engystomops* sp., and *E. "magnus"* and *E. "selva"* would share an ancestral state of no sex chromosome heteromorphism. The chromosome painting of the Y chromosome of *E. petersi*, *E. freibergi*, and *Engystomops* sp. with the same probe, however, does not corroborate such a hypothesis because it demonstrated that the Y chromosomes of these species share some similarities, which would be resulted from a common ancestral Y chromosome. In addition, the noticeable differences between the karyotypes of *E. "magnus"* and *E. "selva"* (Targueta et al. 2010) also suggest an alternative scenario, in which sex chromosome heteromorphism was lost independently in the lineages of *E. "magnus"* and *E. "selva"*.

It is worth noting that *Engystomops coloradorum*, as a species of the Duovox Clade, probably exhibits a ZZ/ZW sex system, in which the W chromosome could be differentiated from the Z chromosome by the presence of NOR (Targueta et al. 2012). If this hypothesis is confirmed after a larger specimen sample is analyzed, then *Engystomops* will present two different sexual systems, which would be indicative of sex chromosome turnover involving the assignment of a new sex-determining gene.

General remarks

In Anura, homomorphic sex chromosomes are more common than heteromorphic sex chromosomes, but evolutionary reversions from heteromorphic to homomorphic conditions have been little documented and restricted to few taxonomic groups. One exhaustive study refers to the ranid frog *Glandirana rugosa*, for which chromosomal rearrangements involved in several sex chromosome transformations and transitions between homomorphic and heteromorphic conditions were recognized and are summarized in Miura and Ogata (2013). More recently, using a genomic approach to infer homology between sex-linked RAD tags and *Xenopus tropicalis* chromosomes, Jeffries et al. (2018) suggested the occurrence of at least 13 events of chromosome turnovers in Ranidae within a period of time of 55 million years. For the hylid genus *Pseudis*, Gatto et al. (2016, 2018) suggested some evidence of the loss of an incipient heteromorphic condition based on the analysis of satellite DNA (PcP190 satellite DNA).

Here, we infer that two reversions from heteromorphic to homomorphic conditions and one transition between XX/XY and ZZ/ZW systems have occurred since the diversification of the two clades of the leptodactylid genus *Engystomops* dated to 16 million years ago (Weigt et al. 2005). It is worth mentioning that previous population studies have evidenced the occurrence of several introgression events between species/clades of this genus (Funk et al. 2012), and we can suppose that such a phenomenon may have had important role in the rapid rate of sex chromosome evolution observed in this genus.

Engystomops frogs, therefore, are excellent candidates for further studies about sex-linked markers and evolutionary processes involved in sex chromosome modifications, which remain poorly understood in lower vertebrates.

Data archiving

Data produced in this research were deposited in Genbank under the accession numbers: MK347220; MK347221; MK347222 (SM1).

Acknowledgements The authors are thankful for the access to equipment and assistance provided by the National Institute of Science and Technology on Photonics Applied to Cell Biology (INFABIC) at the State University of Campinas, the National Institute of Science and Technology on Ecology, Evolution and Biodiversity Conservation (INCT EECBio), and the Laboratory of Genetics and Biodiversity, both at the Federal University of Goiás. The authors thank Janaína Reis Ferreira Lima for her help with field collection. LBL thanks Dr Antonio Baca Sánchez for his important help in the initial assays of chromosome microdissection. This study had the financial support of Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP #2009/02405-2; #2008/11326-6) and Fundo de apoio ao ensino, pesquisa e extensão da Unicamp (FAEPEX #519292/435/11).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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