Two karyotypes and heteromorphic sex chromosomes in *Physalaemus petersi* (Anura, Leptodactylidae)

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Abstract: Cytogenetic analyses were performed on specimens from two populations of *Physalaemus petersi* from three locations in Brazilian West Amazon. Chromosomes from the testis and intestinal epithelium were stained conventionally with Giemsa or C-banded. All animals studied showed a full chromosome complement of $2n = 22$, but two distinct karyotypes (I and II) were detected among specimens from one of the populations. Karyotype I specimens showed a XX/XY sex chromosome system and C-band polymorphism. Bivalent chromosomes with heterozygous C-banding frequently lacked chiasmata in the region of this heterochromatin during the first meiotic division. The less common karyotype (II) had a heteromorphic pair of chromosomes, but the relationship of this pair to sex determination could not be elucidated because of the absence of female specimens. Karyotype II was observed in males whose call differed from those of other males in the same population, suggesting that a reevaluation of the taxon *P. petersi* may be necessary. These results suggest that, in these populations, karyological evolution occurs faster than anatomical evolution.

Résumé : Nous avons procédé à des analyses cytogénétiques sur des individus de deux populations de *Physalaemus petersi* provenant de trois localités dans l'ouest de l'Amazonie brésilienne. La coloration conventionnelle au Giemsa ou la technique de coloration des bandes C ont permis d'établir le complément chromosomique des chromosomes testiculaires et intestinaux. Tous les animaux étudiés avaient un complément chromosomique de $2n = 22$, mais deux caryotypes distincts (I et II) ont été observés au sein de l'une des populations. Chez les spécimens à caryotype I, le système de chromosomes sexuels était de type XX/XY et il y avait polymorphisme des bandes C. Dans les chromosomes bivalents avec système des bandes C hétérozygote, les chiasmas étaient souvent absents dans la région de l'hétérochromatine au cours de la première division méiotique. Dans le caryotype II, moins fréquent, nous avons constaté la présence d'une paire de chromosomes hétéromorphes, mais la relation entre cette paire de chromosomes et la détermination du sexe n'a pu être élucidée à cause de l'absence de femelles. Le caryotype II a été observé chez des mâles dont le cri différait de celui des autres mâles de la même population, ce qui semble démontrer la nécessité de réévaluer le taxon *P. petersi*. Ces résultats indiquent que, chez ces populations, l'évolution caryologique semble plus rapide que l'évolution anatomique.

[Traduit par la Rédaction]

Introduction

Cytogenetic data constitute important information for phylogenetic analyses, especially of members of the Anura, which contains several species that show poor morphological differentiation (Bogart and Wasserman 1972; Batistic et al. 1975; Hillis 1991). Heteromorphic sex chromosomes are rare in Anura and apparently represent a derived state. Among the anuran species whose chromosomes have been studied, only 21 cases of heteromorphic sex chromosomes have been reported (see reviews by Schmid et al. 1991, 1992; Nishioka et al. 1993; Miura 1994*a*, 1994*b*; Cuevas and Formas 1996). Fourteen of these are XX/XY sex chromosome systems and 5 are ZZ/ZW. *Leiopelma hochstetteri* has a OO/WO system (Green 1988) and *Eleutherodactylus maussi* has the $XXAA/XXA^y$ multiple system (Schmid et al. 1992). Different stages of differentiation occur among these anurans, independently of the taxonomic status of the species (Schmid et al. 1992). Schmid et al. (1991) analyzed the available cytogenetic data and suggested that the sex chromosomes of amphibians are in a primitive state of morphological differentiation. These characteristics make anuran sex chromosomes very interesting material for phylogenetic analyses and studies of karyotype evolution.

The genus *Physalaemus* Fitzinger encompasses about 33 species, the inter- and intra-generic relationships of which are still unclear, as is the monophyly of the genus (Cannatella and Duellman 1984). However, Cannatella and Duellman (1984) recognized four species, *Physalaemus petersi* (*Physalaemus freibergi* designated as a junior synonym), *Physalaemus pustulosus*, *Physalaemus pustulatus*, and *Physalaemus coloradorum*, as a monophyletic group. The monophyly of the P. pustulosus species-group was supported by Cannatella et al. (1998), although they recognized *P. freibergi* and three

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Table 1. Morphometric analysis of karyotype I in *Physalaemus petersi*.

Chromosome No.	RL ± SD	AR ± SD	CI ± SD	Classification
1	0.14±0.01	1.23±0.10	0.45±0.02	m
2	0.13±0.01	1.76±0.24	0.36±0.03	sm
3	0.10±0.01	3.72±0.38	0.22±0.02	st
4	0.10±0.00	2.65±0.26	0.27±0.02	sm
5	0.10±0.01	1.17±0.12	0.46±0.04	m
6	0.08±0.01	1.28±0.13	0.44±0.03	m
7	0.08±0.01	1.86±0.28	0.35±0.04	sm
8	0.08±0.01	4.29±0.67	0.19±0.03	st
9	0.07±0.01	1.87±0.29	0.35±0.04	sm
10	0.06±0.01	1.30±0.15	0.44±0.02	m
X*	0.05±0.01	1.24±0.13	0.45±0.03	m
X′	0.06±0.00	1.36±0.15	0.41±0.02	m
X″	0.04±0.00	1.04±0.06	0.49±0.01	m
Y	0.04±0.00	4.81±0.81	0.17±0.05	st

Note: Relative length (RL), arm ratio (AR), centromeric index (CI), and classification of the chromosomes of karyotype I are shown. These data were obtained from 26 metaphases from 10 specimens (9 males and the female ZUEC 9610). The X chromosome data for males are indicated by X* and those of the different X chromosome morphs of the female ZUEC 9610 by X′ and X″. The relative lengths of the autosomes and X* and X′ are relative to the haploid genome length, which includes the male and female sex chromosomes mentioned above, respectively. The relative lengths of X″ and Y are based on the homologous X chromosome. SD, standard deviation; m, metacentric; sm, submetacentric; st, subtelocentric.

other species (*Physalaemus* sp. A, *Physalaemus* sp. B, and *Physalaemus* sp. C) as additional species in this group. The only available cytogenetic information for this group is the chromosome analysis of *P. pustulosus* (León 1970). To provide additional information for the phylogenetic revision of this genus, which is being done by others, we performed a cytogenetic analysis of *P. petersi* (Jiménez de la Espada), and we describe some chromosomal heteromorphisms. This species is widely distributed in the upper Amazon Basin, but also occurs in the lower Amazon Basin and in the eastern Guianan region (Cannatella and Duellman 1984; Frost 1985). Based on the results obtained, we propose a reevaluation of the taxon *Physalaemus petersi*.

Materials and methods

Animals

Specimens of *P. petersi* were obtained from two Amazonian ecological reserves in Acre State, Brazil. Ten males were collected from the municipality of Restauração (Marechal Thaumaturgo) in the Alto Juruá Reserve in February 1994 and January 1996. Two females and 9 males were obtained from the Tejo River estuary in the same reserve, and 15 specimens (12 males and 3 females) were obtained from the Forest Reserve of Humaitá in December 1994 and April 1995. All animals were deposited in the Zoology Museum at the State University of Campinas (ZUEC).

Chromosome preparations and techniques

Chromosome preparations were obtained from a suspension of intestinal and testis cells from animals treated with colchicine for at least 4 h, as described by Schmid (1978*a*) and Schmid et al. (1979), and stained conventionally with Giemsa solution or C-banded (King 1980). Chromosomes were classified according to Green and Sessions (1991).

Results

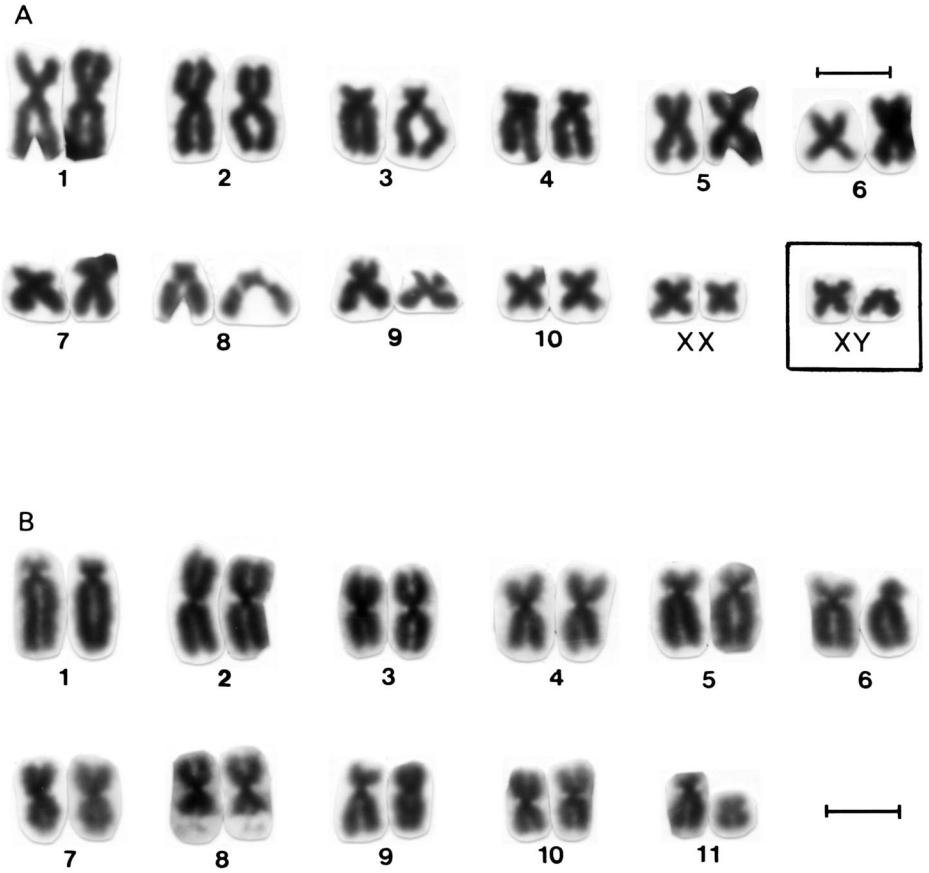
Two very different karyotypes were found among the *P. petersi* specimens. To facilitate their description, the most frequent karyotype in the sample analyzed is referred to as karyotype I and the other as karyotype II.

Karyotype I

The females and all males except three from the Alto Juruá Reserve (ZUEC 9586–9589, 9620, 9624, 9625, 9639, 9641–9645, 9647, 9649, 9650, 9652, and 9653), as well as all specimens from the Humaitá Reserve (ZUEC 9593, 9596–9598, 9602, 9603, 9610, 9613, and 9628–9634), had the same karyotype: chromosome pairs 1, 5, 6, and 10 of this karyotype were metacentric, pairs 2, 4, 7, and 9 were submetacentric, and pairs 3 and 8 were subtelocentric in both sexes (Figs. 1A, 2, 3, and 5, Table 1). The 11th chromosome pair was homomorphic and metacentric in females, while it was heteromorphic and consisted of a metacentric and a subtelocentric chromosome in males.

The C-banding pattern of six individuals, five from the Humaitá Reserve (Figs. 2A–2C, 2E, and 2F) and one from the Alto Juruá Reserve (Fig. 2D), was determined unequivocally. The centromeric regions of all the chromosomes contained C-banded heterochromatin. Telomeric and interstitial bands were also found in this karyotype, but their occurrence was variable. Five C-banding patterns were observed in the specimens examined. Although good C-banding results were obtained for only one specimen from the Alto Juruá Reserve, it was still possible to observe interindividual variability in the banding pattern among the specimens from this reserve. C-banding also revealed an interesting interstitial region of a chromosome 8 of specimens ZUEC 9625 and ZUEC 9643. In some C-banded metaphases, this region appeared as a large constriction, whereas in others it appeared

Fig. 1. Karyotypes I (A) and II (B) of *Physalaemus petersi*, stained with Giemsa. (A) The female karyotype, obtained from specimen ZUEC 9620. The inset shows the sex chromosomes of male ZUEC 9641. Scale bar = 5 μm. (B) Metaphase chromosomes of group II specimen ZUEC 9585. Scale bar = 5 μm.



as a C-band that stained blue (Fig. 2D). Chromosomes X and Y had no noncentromeric C-banded heterochromatin.

Comparison of the homologous X chromosomes of 24 metaphases from female ZUEC 9610 showed morphological differences in all metaphases analyzed (Fig. 2A). However, no difference between these two morphs of chromosome X, termed X′ and X′′, could be detected by C-banding.

The analysis of C-banded meiotic chromosomes from most individuals with a C-banding pattern that was recognized in mitotic metaphases showed that there were no chiasmata between the arms of chromosome pairs 8, 9, and 10, which were heterozygous for noncentromeric C-bands (Figs. 3A–3E, 3B–3E, and 3E, respectively). There were also no chiasmata between the long arms of chromosome 10, which were homozygous for a telomeric C-band in diakinetic cells of one individual (Fig. 3A). However, such meiotic pairing of this chromosome was not seen in two other specimens that were also homozygous for this C-band (Figs. 3B and 3D). Chromosomes X and Y showed an abnormal association in all diakinetic cells examined (Fig. 3).

Karyotype II

Three males from the municipality of Restauração in the Alto Juruá Reserve (ZUEC 9584, 9585, and 9654), two collected in March 1994 and the other in January 1996, had an unusual karyotype. It consisted of six metacentric pairs of chromosomes (2, 3, 4, 7, 8, and 10), two submetacentric pairs (5 and 9), one subtelocentric pair (1), and one heteromorphic pair (11) with one metacentric and one submetacentric chromosome (Figs. 1, 4, and 5, Table 2). No female of this karyotypic group was found.

For only one of these three males could the C-banding pattern be determined. C-banded heterochromatin was detected in all of the centromeric regions of this karyotype, as well as in the telomeric region of chromosome pairs 4 and 8 and in one chromosome 9 (Fig. 4).

Discussion

Systematic considerations

The chromosome number observed in both karyotypes of *P. petersi*, 22, agrees with that of other *Physalaemus* species (see references in Kuramoto 1990). Both karyotypes also had a large amount of C-banded heterochromatin, which agrees with the data for C-banding in other leptodactylids (King 1991). Although relatively few leptodactylid species have been C-banded, the large heterochromatic portion of the karyotype of these species has been clearly documented.

Karyotypes I and II of *P. petersi* clearly differed from each other in the morphology of the chromosomes and their C-banding patterns. The polymorphic nature of the heterochromatin of these karyotypes impaired the interpretation of C-bands as species-specific chromosome markers. As a result,

Fig. 2. C-banding patterns of metaphase (A–D) and prometaphase (E and F) chromosomes of six specimens from group I. (A) Specimen ZUEC 9602. The inset shows the sex chromosomes of female ZUEC 9610. (B) Specimen ZUEC 9597. The arrow indicates a telomeric C-band in the short arm of chromosome 10 that was seen only in this individual. (C) Specimen ZUEC 9610. (D) Specimen ZUEC 9625. The inset presents a more decondensed chromosome pair 8 of the same specimen and shows unusual behavior of the interstitial C-band in one of these chromosomes (arrow). (E) Specimen ZUEC 9632. (F) Specimen ZUEC 9633. Scale bar = 5 μm.

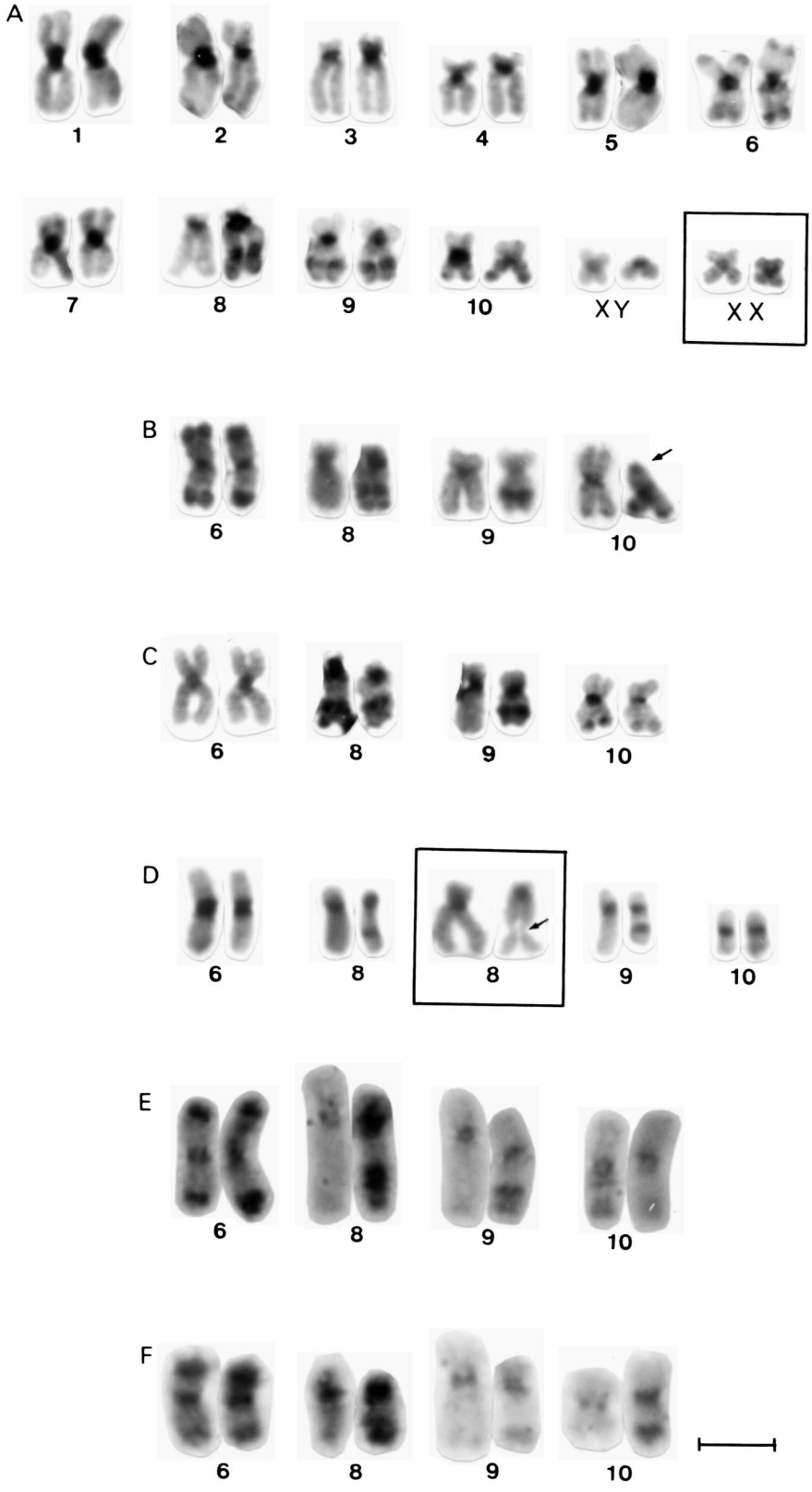
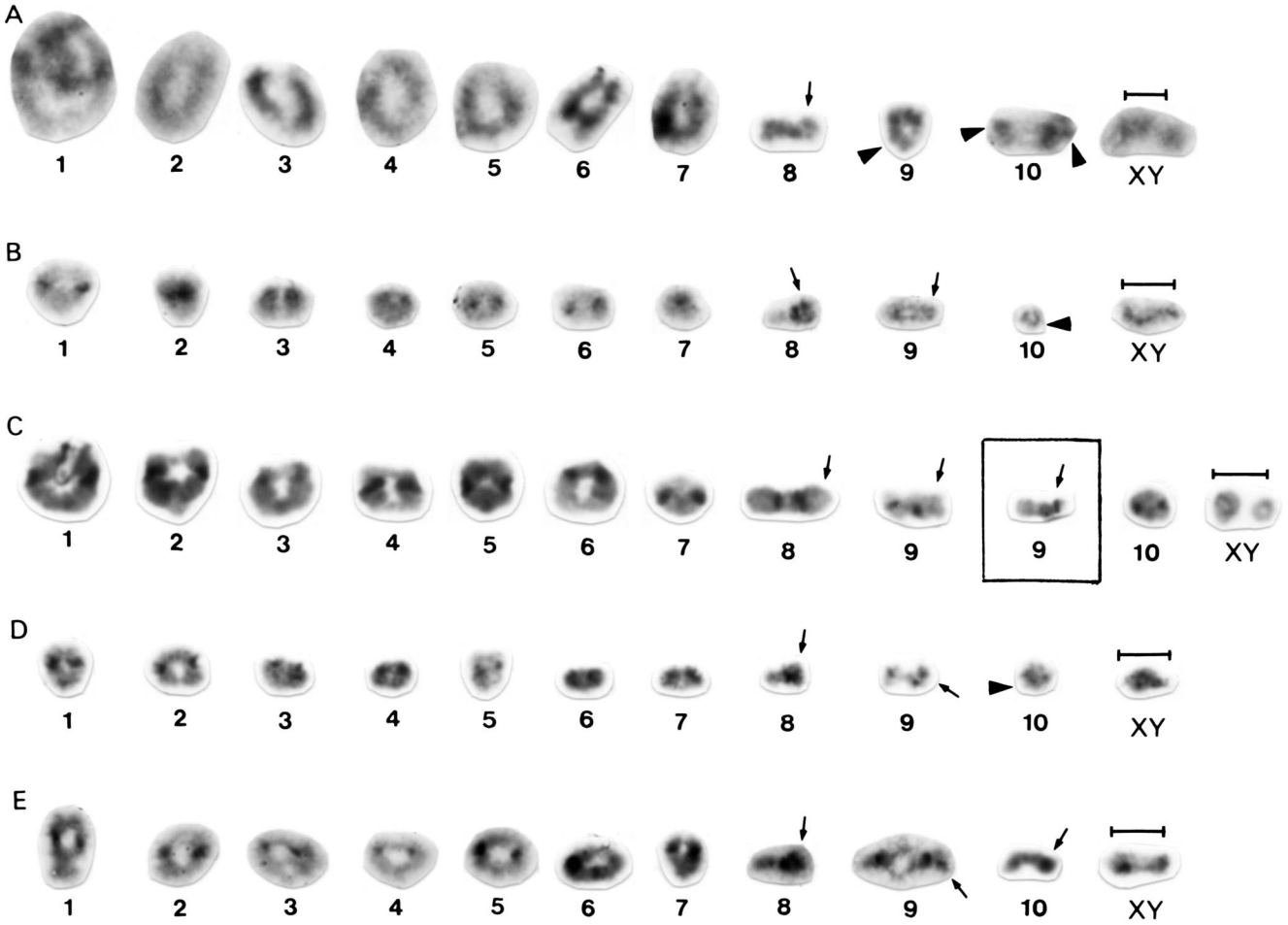


Fig. 3. C-banded diakinetic chromosomes of spermatocytes from group I specimens. (A) Specimen ZUEC 9602. (B) Specimen ZUEC 9597. (C) Specimen ZUEC 9625. (D) Specimen ZUEC 9632. (E) Specimen ZUEC 9633. Another chromosome pair 9 that clearly shows the interstitial C-band is outlined in C. The arrows indicate heterozygous noncentromeric C-bands and the arrowheads indicate homozygous noncentromeric C-bands of chromosome pairs 8, 9, and 10. The interstitial heterochromatin of chromosome 8 appears as a constriction in C. Scale bars = 5 μm.



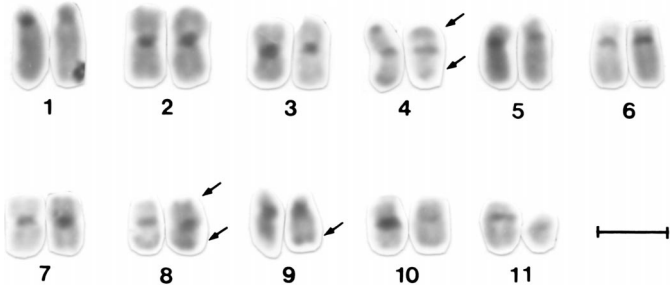
it was not possible to identify homologies between karyotypes I and II with the cytogenetic techniques used here.

The occurrence of these easily distinguished karyotype groups among animals identified as *P. petersi* suggests the presence of cryptic species and agrees with the observation of different calls between these groups (A.J. Cardoso, personal observation). These findings also indicate that in these anurans, the rate of evolution of chromosomes is higher than that of some morphological characters, which shows the relevance of cytogenetic studies of this group.

The chromosomal morphology of *P. petersi* karyotype I resembles that of other karyotypes of *Physalaemus* spp. (Beçak et al. 1970; Denaro 1972; De Lucca et al. 1974), although no heteromorphic sex chromosomes have been observed in the latter. Another clear difference in the karyotype I of *P. petersi* is seen in chromosome pair 8. *Physalaemus petersi* showed considerable differences in karyotype II from other *Physalaemus* spp.

Morphological evaluation has suggested proximity between the genera *Physalaemus* and *Pleurodema* (Lynch 1971). Indeed, karyotype I of *P. petersi* exhibits some similarities with the karyotypes of *Pleurodema thaul* and

Fig. 4. C-banded metaphase chromosomes of group II specimen ZUEC 9654. The arrows indicate noncentromeric C-bands. Scale bar = 5 μm.



Pleurodema brachyops, which have also been studied by C-banding (Schmid et al. 1993). These similarities include the same chromosome number ($2n$ = 22) and similar relative lengths and morphologies of the chromosomes, as well as large amounts of heterochromatin that differ considerably in C-band localization. Heteromorphic sex chromosomes have not been detected in either *P. thaul* or *P. brachyops*.

Fig. 5. Idiograms of karyotypes I (A) and II (B) of *Physalaemus petersi*. Black areas denote dark C-bands, shaded areas denote C-bands showing unusual behavior, white areas denote secondary constrictions, and brackets denote highly polymorphic regions.

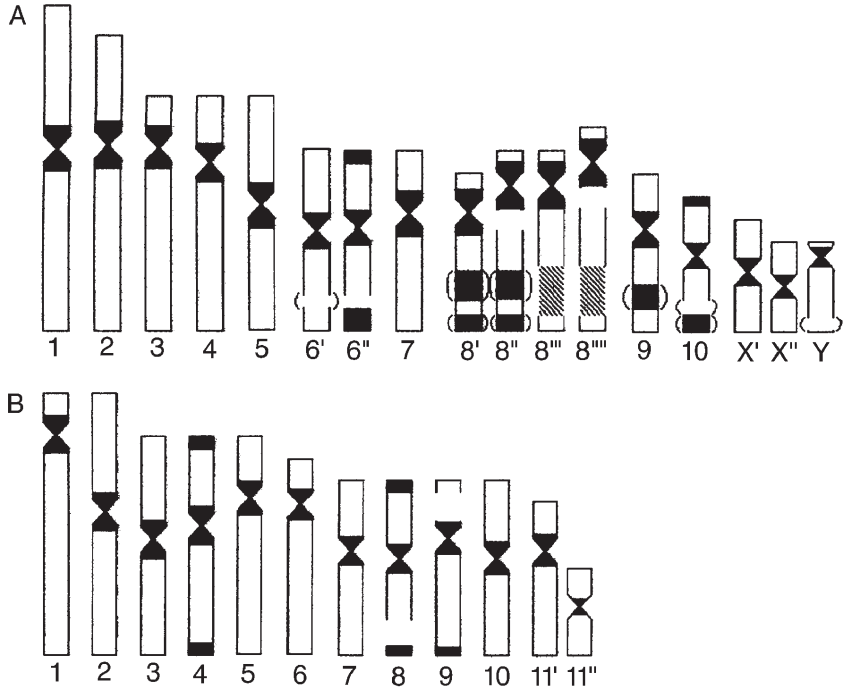


Table 2. Morphometric analysis of karyotype II in *P. petersi*.

Chromosome No.	RL ± SD	AR ± SD	CI ± SD	Classification
1	0.12±0.01	5.54±0.50	0.15±0.01	st
2	0.12±0.01	1.23±0.06	0.45±0.01	m
3	0.10±0.01	1.13±0.12	0.47±0.03	m
4	0.10±0.00	1.48±0.40	0.41±0.06	m
5	0.10±0.00	2.57±0.40	0.29±0.02	sm
6	0.09±0.01	3.42±0.81	0.24±0.03	st
7	0.08±0.01	1.53±0.33	0.40±0.04	m
8	0.08±0.00	1.22±0.17	0.44±0.03	m
9	0.08±0.01	2.12±0.22	0.33±0.02	sm
10	0.08±0.00	1.23±0.19	0.45±0.04	m
11′	0.07±0.01	2.06±0.40	0.32±0.05	sm
11″	0.04±0.01	1.31±0.16	0.43±0.03	m

Note: Relative length (RL), arm ratio (AR), centromeric index (CI), and classification of the chromosomes of karyotype II are shown. These data were obtained from 12 metaphases from three males. The haploid genome length is based on chromosome 11′. The length of chromosome 11″ was estimated from chromosome 11′. For an explanation of abbreviations see Table 1.

Heteromorphic sex chromosomes

An XX/XY sex-determination system was observed in karyotype I and represents another rare case of heteromorphic sex chromosomes in Anura. Among the Leptodactylidae, only four cases in the subfamily Telmatobiinae have been described. Three of them, *Eupsophus roseus*, *Eupsophus migueli* (Iturra and Veloso 1989), and *Eupsophus insularis* (Cuevas and Formas 1996), have an XX/XY system, whereas *Eleutherodactylus maussi* has a multiple sex chromosome system ($XXAA/XXA^y$) (Schmid et al. 1992). Cuevas and Formas (1996) suggested that the XX/XY sex-determination system has evolved at least twice within the Telmatobiinae.

Since this is the first report of heteromorphic sex chromosomes in the genus *Physalaemus* (see references in Kuramoto 1990), and considering the derived state conferred on heteromorphic sex chromosomes (John 1988), we conclude that sex-chromosome differentiation occurs at the species level in this group. Other studies, such as that by Mahony (1991) on *Crinia bilingua* (Myobatrachidae), have also indicated recent sex-chromosome differentiation in Anura.

Chromosomes X and Y of karyotype I were highly differentiated, with different arm ratios and abnormal association in diakinesis. A pericentric inversion may be involved in the differentiation of these chromosomes. However, the tech-

niques used here do not rule out other processes that do not involve this kind of inversion. Further characterization of the chromosomes and analysis of the synaptic complex could answer this question.

It has been proposed that pericentric inversions are responsible for initiating sex-chromosome differentiation in different groups (see review by John 1988), but they are not considered to have been involved in the initial stages of sex-chromosome differentiation in anurans.

Although the involvement of constitutive heterochromatin in sex-chromosome differentiation is widely accepted, and heterochromatin heteromorphism is considered by several authors to be responsible for initiating such processes, no C-band heteromorphism was detected between the sex chromosomes of *P. petersi*. However, segments of repetitive DNA not detectable by C-banding techniques can occur in these chromosomes.

The dimorphism in relative length and centromeric index between the X chromosomes of the female ZUEC 9610 does not reflect differential chromosomal condensation, since such behavior was not detected in the other three females. Alternatively, chromosomes X′ and X′′ probably represent two morphs of X chromosomes. This hypothesis is strengthened by the observation that the average chromosome X arm ratio in males was intermediate between the averages for X′ and X′′ (Table 1), since there could be males with either X′ or X′′. There was no chromosomal evidence that dosage compensation occurred in females of these *P. petersi* groups, which agrees with data obtained for other amphibians (Schmid et al. 1991).

The relationship of the heteromorphic chromosome pair in karyotype II to sex determination could not be elucidated because no female of this karyological group was analyzed.

Heterochromatin heteromorphisms

High variability in C-band number and location was observed in karyotype I of *P. petersi*. Also, C-banding revealed the interesting behavior of a band located interstitially on the long arm of one chromosome 8 in specimens ZUEC 9625 and ZUEC 9643 (Fig. 2D). The use of fluorochromes such as mithramycin and DAPI (4′,6-diamidino-2-phenylindole) could provide additional information on the mechanism responsible for this phenomenon.

When karyotype II considered, C-banding showed an interesting hetermorphism in chromosome pair 9. The existence of interindividual variability could not be determined because only one specimen of this group was C-banded.

The high intraspecific variability detected in *P. petersi* represents an unusual finding in Anura. Although heteromorphism in C-band size has been reported for several anuran species (Schmid 1978*a*, 1978*b*, 1980*a*, 1980*b*; King 1980; Schmid et al. 1993), variations in C-band number and location have been detected in relatively few species, among which are *Rana ridibunda* (Schmid 1978*b*) and *Pyxicephalus adspersus* (Schmid 1980*b*). In these species, paracentric inversions appear to explain the occurrence of heteromorphic chromosomes. Schmid et al. (1995) described a pericentric inversion polymorphism in *Agalychnis callidryas* that resulted in two morphs with distinct morphologies, although the number and size of their C-bands did not differ. The mechanisms responsible for the appearance of C-band heteromorphisms in karyotype I of *P. petersi* were not identified.

The analysis of C-banded diakinetic chromosomes showed the influence of heterozygous C-bands on the distribution of chiasmata in the karyotype I group. The heterozygosity of the noncentromeric C-bands of chromosomes 8, 9, and 10 prevented pairing and (or) crossing over between these C-band regions in all the diakinetic cells examined. The homozygosity of the telomeric C-band of the long arm of chromosome 10 also prevented the pairing of homologous long arms, although such an influence was not always observed. These results corroborate the finding that homozygous C-bands have less influence on meiotic pairing than the heterozygous C-bands do (see the review by John 1988). Thus, analysis of a larger number of diakinetic chromosomes could reveal meiotic association not yet observed in pairs 8 and 9 and also in pair 6, which are homozygous for noncentromeric C-bands.

Such meiotic behavior probably minimizes the homogenization between homologous chromosomes and contributes to the maintenance of C-band polymorphism in this group. Alternatively, some ecological characteristics may also be involved in this elevated polymorphism in *P. petersi*.

The polymorphic nature of the C-bands limits their usefulness for phylogenetic analysis because of the difficulties in recognizing homologous characters unequivocally. The use of fluorochromes and other molecular techniques may facilitate the study of homologous C-bands. According to Amorim (1994), the concept of synapomorphy does not apply to characters that show polymorphism, which suggests that difficulties may be encountered in the use of polymorphic characters in phylogenetic studies. Rocha and El-Hani (1996) summarized the possible evolutionary history of polymorphic characters and concluded that these problems are more marked in studies dealing with terminal taxa, whose character states represent part of the polymorphism of the shared ancestral population.

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