

Unusual primitive heteromorphic ZZ/ZW sex chromosomes in *Proceratophrys boiei* (Anura, Cycloramphidae, Alsodinae), with description of C-Band interpopulational polymorphism

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We performed cytogenetic analyses on specimens from three population samples of *Proceratophrys boiei* from southeastern and northeastern Brazil. We stained chromosomes of mitotic and meiotic cells with Giemsa, C-banding and Ag-NOR methods. All specimens of *P. boiei* presented a karyotype with a full chromosome complement of $2n=22$, metacentric and submetacentric. We observed the secondary constriction within the short arm of pair 8, which was in the same position of the nucleolus organizer region (NOR). NOR heteromorphism was observed within two specimens from the municipality of Mata de São João (northeastern Bahia State). The C-banding evidenced an unusual heterochromatic pattern in the genome of *P. boiei*. In the southernmost population samples (São Paulo State), we observed large blocks of heterochromatin in the centromeric regions of all chromosomes, whereas the northernmost samples (Bahia State) presented a small amount of constitutive heterochromatin. We suppose that this geographic variation in heterochromatin quantities could be due to heterochromatinization of some chromosome regions in the genome of the São Paulo samples. Furthermore, females from São Paulo presented, within chromosome pair 1 from C-banded karyotypes, one homologous chromosome almost heterochromatic, whereas males had heterochromatin restricted to the centromeric region. This unusual heterochromatic arrangement led us to assume that *P. boiei* owns a ZZ/ZW type of sexual determination system. This finding is very important, as this is the first record of ZZ/ZW sex chromosomes within Cycloramphidae. We believe that the cytogenetic differences found between southeastern and northeastern Brazilian population samples of *P. boiei* strongly supports the existence of a species complex under the name *P. boiei*, and the requirement of taxonomic and systematic reviews by morphological, bioacoustical, molecular, and cytogenetic data could define this taxonomic issue in the future.

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The Neotropical frog genus *Proceratophrys* Miranda-Ribeiro, 1920 currently comprises 17 species occurring in Brazil, Argentina and Paraguay (CRUZ et al. 2005; FROST 2007). *Proceratophrys boiei* is the most widespread Brazilian species of the genus, inhabiting the Atlantic Forest Biome from southern to northeastern Brazil (see MORELLATO and HADDAD 2000 for the range extension of the Brazilian Atlantic Forest). Our analyses on the external morphology of 20 specimens of *P. boiei* from the municipality of Mata de São João, northern coastline of the State of Bahia, northeastern Brazil, revealed differences in head proportions between these specimens and those measured by IZECKSOHN and PEIXOTO (1981) from southeastern Brazil (head length/snout-vent length: 0.435–0.559 and 0.333–0.404, respectively; head length/head width: 0.843–0.971 and 0.714–0.778, respectively). CARNAVAL (2002), conducting a study of phylogeography on four

anuran species in the Atlantic Forest fragments of northeastern Brazil, which included *P. boiei*, found an intraspecific genetic variation in haplotypes from some geographic population samples. These morphological and genetic variations among geographic samples of *P. boiei* point to a possible species complex under this specific name.

We considered that more comprehensive data on the cytogenetics of *P. boiei* could contribute to the understanding of the geographic variation of this taxon, as cytogenetics of the frog genus *Proceratophrys* is poorly known and restricted to the description of a $2n=22$ karyotype for *P. boiei* and *P. appendiculata* (BEÇAK et al. 1970; SILVA et al. 2003), and to the localization of the nucleolar organizer region in *P. boiei* (SILVA et al. 2003). This issue led us to compare karyotypes of specimens of *P. boiei* from population samples of the State of São Paulo, southeastern Brazil, with that from

the municipality of Mata de São João, State of Bahia, northeastern Brazil (respectively, ca 270 and 1210 km distance from the municipality of Rio de Janeiro, type locality of *P. boiei*).

The lack of cytogenetic information on the frog genus *Proceratophrys* is not a particular issue and could be acknowledged to anurans in general. KING (1990) stated that less than 25% of frog species were chromosomally examined, and only 17 species had their sex chromosomes microscopically recognized. Within these 17 species, 10 presented XX/XY sex chromosomes, 6 had the mechanisms of the ZZ/ZW chromosome type described, and only one species owned a very exceptional sex determination system, possibly associated to a supernumerary univalent W chromosome, leading to the rare WO/OO mechanism (reviewed by SCHMID and STEINLEIN 2001). Moreover, heteromorphic sex chromosomes of the ZZ/ZW type were mainly characterized for non Neotropical anurans, as for *Pyxicephalus adspersus* (SCHMID 1980b), *Buergeria buergeri* (OHTA 1986; SCHMID et al. 1993), *Leiopelma hamiltoni* (GREEN 1988), and *Crinia bilingua* (MAHONY 1991), and only for two Neotropical frogs of the genus *Eleutherodactylus* (SCHMID et al. 2002). Therefore, the finding and description of an additional Neotropical species owning heteromorphic ZZ/ZW sex chromosomes, *P. boiei*, is very important.

The purposes of the present paper are to describe (1) an unusual primitive heteromorphic type of ZZ/ZW sex chromosome in *Proceratophrys boiei*, and (2) a C-band interpopulation polymorphism between two distinct geographic samples of *P. boiei*.

MATERIAL AND METHODS

Specimens used in the account are housed in the Coleção Célio Haddad (CFBH), deposited in the Depto de Zoologia, Instituto de Biociências, Univ. Estadual Paulista, Rio Claro, São Paulo, Brazil (CFBH), and Museu de Zoologia (UFBA), Depto de Zoologia, Instituto de Biologia, Univ. Federal da Bahia, Salvador, Bahia, Brazil.

We analyzed 17 specimens of *Proceratophrys boiei* (13 females, 1 male, 3 juveniles) from three Brazilian localities: 7 females from the Municipality of Santa Branca, State of São Paulo (23°23'48"S; 45°53'02"W); 1 male and 1 female from Parque Natural Municipal da Serra do Itapety, Municipality of Mogi das Cruzes, State of São Paulo (23°31'22"S; 46°11'18"W); and 5 females and 3 juveniles from the Municipality of Mata de São João, State of Bahia (12°33'59"S; 38°02'18"W). We transported these specimens alive to the laboratory, where they were processed. We obtained the

chromosome preparations by three different procedures: (1) intestinal epithelium and testicular cells – animals pretreated with 1% colchicine solution for at least 4 h (SCHMID 1978a); (2) cytological preparations of bone marrow, liver, spleen and testis – animals previously submitted to Phytohemagglutinin-P (Cultilab) and 0.01% colchicine treatments, in the proportion of 0.1 ml 10 g⁻¹ animal weight, during 48 h and 6 h, respectively (BAKER et al. 1971; WILEY 1982; BALDISSERA JR. et al. 1993); and (3) lymphocyte cultures (KASAHARA et al. 1998). We stained the slides with 10% Giemsa solution for chromosome morphological studies, detected the constitutive heterochromatin (C-banding) following SUMNER (1972), and performed the nucleolus organizer regions (NOR) by silver nitrate staining (Ag-NORs) (HOWELL and BLACK 1980). We identified the chromosomes as metacentric, submetacentric, and subtelocentric by visual inspection.

RESULTS

We encountered a diploid number of 22 chromosomes in mitotic cells in both sex, and 11 bivalents in meiotic cells in the male of *P. boiei*. Chromosome pairs 1, 2, 4, 5, 6, 7, 8, 9, 10, and 11 metacentric; pair 3, submetacentric. Pairs 1–5 distinctly larger than pairs 6–11; slight size decrease in chromosome length from pairs 1–5 and 6–11; pair 8 with a secondary constriction in the interstitial position of the short arm (Fig. 1a, 1c and 1e), in which is located the NOR (Fig. 1b, 1d and 1f); heteromorphic NORs in two specimens from Mata de São João (Fig. 1f). All population samples had an unusual constitutive heterochromatin pattern after C-banding treatment. Females from Mogi das Cruzes and Santa Branca (both in São Paulo State, southeastern Brazil) had conspicuous autosomes, characterized by large quantities of constitutive heterochromatin in centromeric regions after C-banding treatment (Fig. 2a–b), and often with a strong C-band in one of the chromosomes of pair 1 within metaphasic cells, except by the euchromatic terminal region (Fig. 2a–b, 2d–e, Table 1). The only adult male analyzed from southeastern Brazil (Mogi das Cruzes, São Paulo State) lacked heterochromatic C-bands in the chromosome pair 1 (Fig. 2a, 2d), denoting that this chromosome pair must represent the ZZ/ZW sex chromosomes, and thus a sex determining system for *P. boiei*. On the other hand, specimens from northeastern Brazil (Mata de São João, Bahia State) presented an unusual C-band pattern in all chromosomes, characterized by small amount of constitutive heterochromatin and heterochromatic bands in centromeric positions (Fig. 2c).

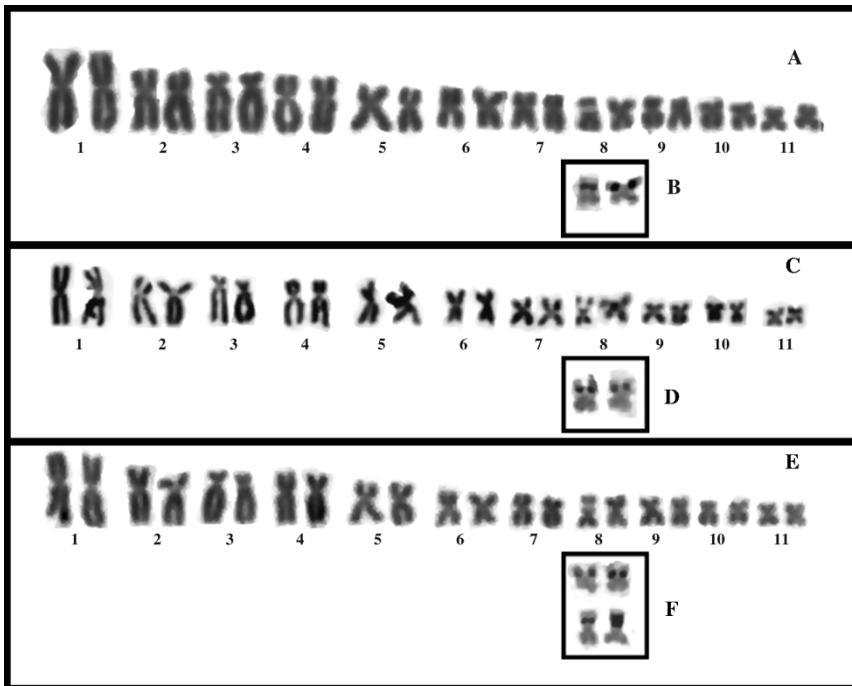


Fig. 1A–F. Comparisons of giemsa stained karyotypes (A, C and E) and NOR bearing chromosomes (B, D and F) of *Proceratophrys boiei*. A, male from Mogi das Cruzes population sample; C, female from São Paulo population samples; E, female from Bahia population sample. Note the secondary constriction of the pair 8 chromosomes, which coincides with the Ag-NOR site in homomorphic (B and D) and homomorphic/heteromorphic state (F).

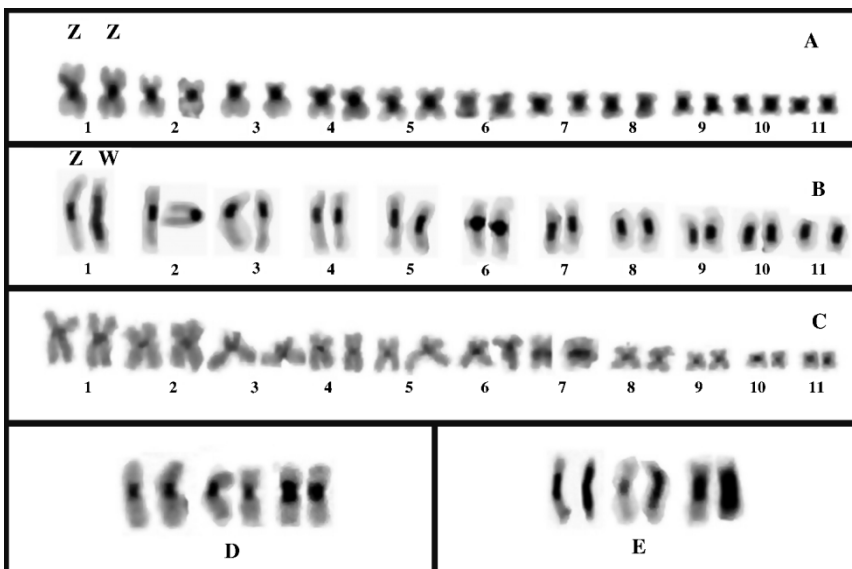


Fig. 2. C banded karyotypes of *Proceratophrys boiei*. A, male from Mogi das Cruzes population sample; B, female from São Paulo population sample; C, female from Bahia population sample. Chromosome pair 1 from females of São Paulo samples comprises sex chromosomes. Note the large amounts of heterochromatin in the karyotypes of the population samples from southeastern Brazil (Mogi das Cruzes and Santa Branca, São Paulo State) and an unusual C-band pattern in the karyotype of the population sample from northeastern Brazil (Mata de São João, Bahia State). D, ZZ sex chromosomes of male. E, ZW sex chromosomes of female.

Table 1. Number of metaphasic cells with W chromosomes in the three population samples analyzed. SE, southeastern Brazil; NE, northeastern Brazil; SP, São Paulo State; BA, Bahia State.

Population samples	Geographic distribution	Number of females	Number of metaphasic cells with W chromosomes
Santa Branca, SP	SE	07	154
Mogi das Cruzes, SP	SE	01	38
Mata de São João, BA	NE	05	0

DISCUSSION

Heteromorphic sex chromosomes

Sex chromosomes in amphibians are rare, but when present, the conventionally stained karyotypes often show heteromorphic discernable XY/XX or ZZ/ZW chromosomes (SCHMID et al. 1991; SCHMID and STEILEIN 2001). Nevertheless, we only identified the sex chromosomes ZZ/ZW in *P. boiei* after the C-banding treatment. This is an unexpected result, and only reported for *Leiopelma hamiltoni* and *Rana tigrina*, in which the centromeric region of one of the homologous chromosomes was enlarged in females (CHAKRABARTI et al. 1983; GREEN 1988). Nevertheless, this result is not unique for the ZZ/ZW type of sex determination. In some species with the XY sex determination system the observation of sex chromosomes only could be seen after applying the technique of C-band, as registered for salamanders of the genus *Triturus* (SCHMID et al. 1979) and for the treefrog *Gastrotheca pseustes* (SCHMID et al. 1990).

We only observed ZZ/ZW sex chromosomes within the São Paulo population samples of *P. boiei* (absent in the Bahia sample). The relative lengths of W and Z chromosomes are similar, but the W chromosome is completely heterochromatic in São Paulo samples. The same chromosome pair 1 in Bahia sample may remain in an initial stage of morphological differentiation, not identified by the cytogenetic methods used herein. Similar results was observed in *Pyxicephalus adspersus*, in the closely related *Tomopterna delalandii* (SCHMID 1980b), and in *Rana tagoi* (RYUZAKI et al. 2006). The presence of distinct ZZ/ZW sex chromosomes in *P. boiei*, with chromosome W almost totally heterochromatic, suggests that heterochromatinization is a process that precedes the W and Y morphological differentiation. Therefore, the discovery of several stages of morphological differentiation of sex chromosomes in anuran species is important not only to amphibian cytogenetics, but also to the understanding of evolution of sex chromosomes in vertebrates. The discovery of several stages of morphological differentiation of sex chromosomes in fishes, amphibians, and reptiles permitted the determination of evolutionary series of karyotypes, in which the increasing

structural complexity of the sex chromosomes can be reconstructed (BEÇAK et al. 1964; OHNO 1967; RAYCHAUDHURI et al. 1971; SINGH et al. 1976, 1980; SCHMID and STEINLEIN 2001; ODIERNA et al. 2007).

Systematic considerations

The diploid number of 22 chromosomes and the chromosome morphology (metacentric and submetacentric chromosomes) found for *Proceratophrys boiei* in the present study are common within Cycloramphidae. Our results are, in part, similar to that found by SILVA et al. (2003) for *P. boiei* from the Municipality of Ribeirão Branco, São Paulo State, and by BEÇAK et al. (1970) for *P. appendiculata* ($2n=22$; chromosome morphology similar to *P. boiei*). According to KING (1990), closely related species have more similar karyotypes than distinctly related species, which led other researches to use the number of chromosomes as a primary difference between taxa in anuran systematic studies (LYNCH 1971; HEYER 1975). Following this premise, the similar karyotypes observed for *P. boiei* and *P. appendiculata* indicates a close relationship between these two species, which accords with IZECKSOHN et al. (2005) in joining them into a natural species group.

Another important feature for studies of phylogenetic relationships and chromosome evolution is the structure, number, and position of the nucleolus organizer region (NOR). We observed heteromorphic NORs only two specimens from Mata de São João (northernmost distribution range). Heteromorphic NORs are common within anurans (SCHMID 1978a, 1978b, 1980a, 1980b, 1982; LOURENÇO et al. 2000; BUSIN et al. 2006), and in most cases attributed to NOR amplification. Nevertheless, deletion events may be the sources of NOR heteromorphism (SCHMID 1982).

Concerning the number and position of NORs, they are usually conservative for species or populations, although intraspecific variability has been documented (RUIZ et al. 1981; WILEY et al. 1989; FOOTE et al. 1991; SCHMID et al. 1995; LOURENÇO et al. 2000; SILVA et al. 1999; MEDEIROS et al. 2003; CAMPOS et al. 2006). The presence of a specific chromosome pair bearing the NOR in diploid

karyotypes, as herein observed for *P. boiei*, is common for anuran families (SCHMID 1982; MAHONY and ROBINSON 1986), which led KING et al. (1990) to suggest the presence of a single pair of NORs in diploid karyotypes as the ancestral condition in Anura, a hypothesis previously proposed by SCHMID (1978a) for bufonids and hylids. In our research, the NOR always was positioned in chromosome pair 8, but SILVA et al. (2003), studying specimens of *P. boiei* from Ribeirão Branco (São Paulo State), observed the NOR only in pair 4, which implies in distinct NOR sites among local populations recognized as *P. boiei*. However, fixed NOR sites for different populations of the same species are rare, and was only reported for *Dendropsophus ebraccatus* (KAISER et al. 1996). Two explanations could be assumed for the NOR site variation among population samples recognized as *P. boiei* in the State of São Paulo: the distinct chromosome bearing the NOR is (1) merely a function of the NOR activity (MACGREGOR and SHERWOOD 1979), and (2) a result of translocations or insertions (WILEY et al. 1989; FOOTE et al. 1991; SCHMID et al. 1995; KAISER et al. 1996). If we assume that the second explanation is the source of variation for the geographic samples recognized as *P. boiei* within the State of São Paulo, our results could be considered, at least, as an indicative of an incipient speciation. Additional data at the molecular level will be useful to make a more detailed assessment.

Interspecies comparisons of C-band pattern are of basic importance for citotaxonomic studies. Regarding our samples of *P. boiei*, the C-banding treatment revealed larger quantity of heterochromatin within the southernmost population samples (São Paulo State). This geographic variation in heterochromatin quantities could be explained by the heterochromatinization of some chromosome regions in the genome of the São Paulo samples. However, KING (1990) emphasized that is uncommon to find two related species with similar heterochromatin distributions, and also for interpopulation variations, where the most common observed situation is the same C-band pattern within populations, like that reported for *Hypsiboas semiguttatus* (ANANIAS et al. 2004). Thus, the C-band interpopulation variation observed for *P. boiei* is unexpected and point out to the existence of two distinct species, until now recognized as *P. boiei*, one from southeastern Brazil and another from northeastern Brazil. In spite of this, the C-banding pattern was recognized in literature as identical for *Chaunus schneideri* (as *Bufo paracnemis* in KASAHARA et al. 1996), *Crossodactylus* (AGUIAR JR et al. 2003), and species of the *Physalaemus beligonigerus* group (AMARAL et al. 2000). Moreover, SCHMID et al.

(1987) recognized differences in the amount and position of a constitutive heterochromatin within two distinct populations of *Alytes obstetricans*; MIURA (1995) found two distinct karyological groups within 11 population samples of *Bufo japonicus*, based on their C-banding pattern; SILVA et al. (2000) found similar results in population samples of *Leptodactylus fuscus* and *L. acellatus*; CAMPOS et al. (2006) found distinct C-banding patterns in *Eleutherodactylus binotatus* from three localities; and ANANIAS et al. (2007) recognized differences in the amount of heterochromatin in *Eupemphix nattereri*. It is important to note that an anuran species with widespread range distribution, as *C. schneideri*, *L. ocellatus*, and *L. fuscus*, has in general a wide variety of morphologic, bioacoustic, and genetic geographic variation among its local populations, which may represent, in fact, more than one taxonomic entity (see HEYER 1978, 1984 for examples of anuran taxonomic revisions that dealt with cases of species complexes). Therefore, the cytogenetic variation of widespread species previously assumed to be intraspecific could be interespecific.

MERCADAL DE BARRIO and BARRIO (1993) compared the species of *Proceratophrys* and argued that the genus needs a taxonomic revision. We agree with that authors and believe that the cytogenetic differences found between southeastern and northeastern Brazilian population samples of *P. boiei* strongly supports the existence of a species complex under the name *P. boiei*, and specifically, a distinct taxonomic entity in northeastern Brazil.

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