Chromosome banding in Amphibia

XIII. Sex chromosomes, heterochromatin and meiosis in marsupial frogs (Anura, Hylidae)

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Abstract. The chromosomes of the South American marsupial frogs *Gastrotheca fissipes*, *G. ovifera*, *G. walkeri* and *Flectonotus pygmaeus* were analyzed by means of conventional and various banding techniques. The karyotypes of *G. ovifera* and *G. walkeri* are characterized by highly differentiated XY/XX sex chromosomes. Whereas the X chromosomes and autosomes contain large amounts of constitutive heterochromatin, extremely little heterochromatin is located in the Y chromosomes. This is in contrast to all previously known amphibian Y chromosomes and the Y chromosomes of most other vertebrates. In the male meiosis of *G. walkeri*, the euchromatic segments of the heteromorphic XY chromosomes show the same pairing configuration as the autosomal bivalents. The karyotype of *F. pygmaeus* is remarkable for the unique presence of telocentric chromosomes and the high frequency of interstitially located chiasmata in the meiotic bivalents. The evolution of the karyotypes and sex chromosomes, the structure of the various classes of heterochromatin and the data obtained from meiotic analyses of the marsupial hyliids are discussed.

Introduction

The egg-brooding marsupial frogs are especially significant biologically because of their unique mode of reproduction. After external fertilization, the eggs develop in a dorsal pouch on the females. In some species, the embryos complete their development within the maternal brood pouch and hatch as froglets, whereas in others the eggs hatch as tadpoles and complete their development in standing water (Duellman and Maness 1980). Further properties unique to the marsupial frogs are: (1) they produce the largest eggs in Anura, up to 10 mm in diameter (del Pino et al. 1986); (2) the oocytes of some species contain many nuclei during early oogenesis (Macgregor and del Pino 1982); (3) an embryonic disk from which the body of the embryo originates is formed during gastrulation (del Pino and Elinson 1983); and (4) the developing embryos are enclosed in enormous bell-shaped gills which function as vascular wrappings (Duellman and Maness 1980; Wassersug and Duellman 1984). Marsupial frogs belong to the family Hylidae and constitute the subfamily Hemiphractinae (genera *Cryptobatrachus*, *Flectonotus*, *Fritziana*, *Gastrotheca*, *Hemiphractus*, and *Stieflania*) (Duellman et al. 1988). These egg-brooding hyliid frogs are restricted to Central and South America (Duellman 1977).

There have been few cytogenetic studies on marsupial frogs. Previously performed analyses on the karyotype of *Gastrotheca riobambae* from Ecuador demonstrated highly heteromorphic sex chromosomes of the XX/XY type (Schmid et al. 1983, 1986). The X chromosome is distinctly smaller than the Y chromosome and contains the unique nucleolus organizer region of the karyotype. This results in a rare sex-specific difference in the number of 18S and 28S ribosomal RNA genes. Female (XX) animals have twice the number of ribosomal RNA genes than do male (XY) animals. The fact that the X is smaller than the Y is also an exceptional situation among vertebrates. *G. riobambae* was furthermore the first anuran species in which the occurrence of a typical sex bivalent with end-to-end paired XY chromosomes was demonstrated in male meiosis (Schmid et al. 1983).

These results indicate the necessity for further cytogenetic examination of marsupial frogs. One particular aspect which needs clarification is whether heteromorphic sex chromosomes, which are known to be an exception in Amphibia (for review see Schmid 1983), also exist in other species of marsupial frogs. With the help of colleagues in Venezuela and Brazil, specimens of *G. fissipes*, *G. ovifera*, *G. walkeri*, and *Flectonotus pygmaeus* were collected for the present study. Determination of karyotypes by all available banding techniques resulted in distinguishing highly differentiated XY sex chromosomes, an unusual structure of the Y chromosome, and an unexpected pairing configuration between X and Y in male meiosis in two species.

Material and methods

*Animals.* Eight mature specimens of *G. fissipes* (four males, four females) were collected 100 km north of Vitoria (Estado Espírito Santo, Brazil) in March 1986. The animals were found in large terrestrial bromeliads in the xerophytic shore vegetation, only 200 m from the Atlantic coast. The frogs were brought to the Department of Genetics, University of Campinas (São Paulo) and immediately used for chromosome preparations. One adult male and 1 female each of *G. walkeri*, 2 juveniles of *G. ovifera*, and 55 mature *F. pygmaeus* (29 males, 26 females) were caught from May to July 1987 in the vicinity of the Estación Biológica de Rancho Grande (Estado Aragua, Venezuela). This station is at an elevation of 1100 m in the cloud forest of the Henri
Pittier National Park in the Cordillera de la Costa. Most of the *F. pygmaeus* were found in arboreal bromeliads up to 10 m above the ground, whereas *G. walkeri* and *G. ovi-
fera* were collected from the branches of bushes. The chromo-
somes were prepared in a temporary laboratory in the field station. The chromosome material obtained in both Brazil and Venezuela was transferred into tightly closed plastic tubes immediately after fixation, stored at 4°C and transported to Würzburg on dry ice. Although the time interval between chromosome fixation in South America and banding analysis in Würzburg was as long as 25 weeks for some samples, the quality of the preparations was not reduced.

**Chromosome preparations.** Mitotic chromosomes were obtained from the bone marrow and intestine of all animals after in vivo colchicine treatment. Meiotic chromosomes were prepared from the testes of adult males. The techniques used for the preparation of cell suspensions, hypotonic treatment and fixation of the cells, and for splashing the material onto slides have been described previously (Schmid 1978; Schmid et al. 1979).

**Chromosome banding.** Conventional chromosome staining. C-bandning and quinacrine, mithramycin 4'-6-diamidino-2-phenylindole (DAPI), distamycin A/DAPI, Hoechst 33258, and silver staining were performed on the metaphases of all the specimens according to Schmid et al. (1983).

**Photography and analysis of banding patterns.** All metaphases of satisfactory quality were photographed. Zeiss III photomicroscopes were used for bright field microscopy. Fluorescence microscopy was performed with Zeiss fluorescence microscopes equipped with incident HBO 50 W mercury lamp illumination. Specific quinacrine and mithramycin fluorescence was selectively obtained by exciting with UV light in the 450–490 nm range (filter combination BP450–490/FTS10/LP520). DAPI and distamycin A/DAPI fluorescence was analyzed under excitation with 360–400 nm UV light (filter combination G365/FT395/ LP520). All photographs were taken with Agfaortho 25 films. Several karyotypes for each of the staining tech-
niques used were prepared from each of the animals. All karyotypes were laid out in parallel rows. This permitted the determination of conformity in the banding patterns, inter-individual variation of specific bands, and the occurrence of heteromorphic sex chromosomes.

**Results**

**G. walkeri**

Conventional aceto-orcein staining revealed 26 chromo-
somes in the mitotic metaphases of the two specimens exam-
ined; these can be arranged in 13 pairs (Fig. 1 a, d). Pairs 1–5 are distinctly longer than pairs 6–13. Pairs 1, 4 and 5 are nearly metacentric, and pair 3 is submetacentric. Of the smaller chromosomes, pairs 6–9, 12 and 13 are meta-
centric or slightly submetacentric, and pairs 10 and 11 are telocentric. A secondary constriction is present in the middle of the short arms of chromosome pair 6 in both sexes (Fig. 1 a, d). Chromosomes 2 are distinctly heteromorphic in the karyotype of the male (Fig. 1a). One of these chromo-
somes (X) has the same size as chromosome 1 and is almost metacentric. The other chromosome (Y) is one-third smaller and submetacentric. In the female, chromosomes 2 are ho-
omorphic and have the same length and centromere posi-
tion as the X chromosome of the male animal (Fig. 1d). In all metaphases analyzed, the X and Y chromosomes showed the same degree of condensation as the autosomes.

The C-banded karyotype exhibits large amounts of con-
stitutive heterochromatin in the pericentromeric regions of all autosomes and the X chromosome (Fig. 1b, e). Smaller heterochromatic bands can also be discerned in some telo-
meres, especially in those of pairs 10 and 11. Constitutive heterochromatin located in interstitial positions apparently does not occur in the chromosomes of this anuran. A care-
ful comparison of 20 karyotypes each of the male and fe-
male revealed no conspicuous inter-individual differences in the sizes or positions of the C-bands. Furthermore, no heteromorphism of the pericentromeric heterochromatin could be distinguished in the homologous X chromosomes of the female (Figs. 1e, 2b, 3k). The Y chromosome is re-
markable because of its extremely low content of constitut-
ive heterochromatin (Figs. 1b, 2a, 3g). This permitted its unequivocal differentiation from all other chromosomes in every metaphase analyzed. Besides a small C-band exactly in the centromeric region and very faint C-bands in both telomeres, the Y is completely euchromatic. The size differ-
ence between the sex chromosomes is entirely due to the different quantities of heterochromatin in their pericentro-
meric regions. The euchromatic segments in both the short and the long arms of the X and Y chromosomes are of the same lengths (Figs. 1b, 2a, 3g).

Some of the C-banded cells found in bone marrow prepara-
tions were in an advanced metaphase stage. As is usual for such metaphases, chromosome condensation was exagger-
tated by the effect of colchicine. It was noticed in all of these cells that the centromere of the Y chromosome was among the first to divide (Fig. 2c). Therefore, the two Y chromo-
tids were distinctly separate, whereas the chroma-
tids of most other chromosomes were still adherent at their centromeric regions. This is in agreement with studies on several other animal species that have shown that the cen-
troneres of all chromosomes do not divide simultaneously in colchicine-treated cells, but in an asynchronous, non-
random and rather specific sequence (for references see Vig 1981). Such sequences of centromere division do not appear to be artifacts of colchicine or hypotonic treatment, and are believed to reflect the ordered separation of chromo-
somes at the metaphase-anaphase transition. In the species previously studied, centromere separation is not influenced by chromosome length or centromere position, but seems to be controlled by the amount of centromeric heterochro-
matin. Thus, centromeres lacking any detectable hetero-
chromatin, as in the mouse Y chromosome, are always the first to separate, whereas centromeres surrounded by large C-bands, like the Y2 chromosome of the rat kangaroo (*Potoros triactylythus*) separate late (Vig 1981). The Y chromo-
some of *G. walkeri* is another example confirming this rela-
tionship.

Silver staining of the chromosomes showed the nucleolus organ region to be located within the secondary (nucleolar) constriction in the short arms of chromosome pair 6 (Figs. 1c, f, 3n). Both animals had about equally large silver blocks in the homologous nucleolus organizers. The chromosomes fluoresce with uniform intensity after quinacrine mustard staining. Slightly brighter fluorescence