Chromosoma (Berl) (1988) 97:33–42

CHROMOSOMA
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Chromosome banding in Amphibia

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

M. Schmid[1], C. Steinlein[1], W. Feichtinger[1], C.G. de Almeida[1], and W.E. Duellman[2]

[1] Department of Human Genetics, University of Würzburg, Koellikerstrasse 2, D-8700 Würzburg, Federal Republic of Germany
[2] Museum of Natural History and Department of Systematics and Ecology, The University of Kansas, Lawrence KS 66045-2454, USA

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 hylids 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 *Stefania*) (Duellman et al. 1988). These egg-brooding hylid 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 Espiríto 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

Offprint requests to: M. Schmid

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. ovifera* were collected from the branches of bushes. The chromosomes 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-banding 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/FT510/LP520). DAPI and distamycin A/DAPI fluorescence was analyzed under excitation with 360–400 nm UV light (filter combination G365/FT395/LP420). All photographs were taken with Agfaortho 25 films. Several karyotypes for each of the staining techniques 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 chromosomes in the mitotic metaphases of the two specimens examined; these can be arranged in 13 pairs (Fig. 1a, 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 metacentric 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. 1a, d). Chromosomes 2 are distinctly heteromorphic in the karyotype of the male (Fig. 1a). One of these chromosomes (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 homomorphic and have the same length and centromere position 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 constitutive heterochromatin in the pericentromeric regions of all autosomes and the X chromosome (Fig. 1b, e). Smaller heterochromatic bands can also be discerned in some telomeres, 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 careful comparison of 20 karyotypes each of the male and female 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 remarkable because of its extremely low content of constitutive 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 difference between the sex chromosomes is entirely due to the different quantities of heterochromatin in their pericentromeric 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 preparations were in an advanced metaphase stage. As is usual for such metaphases, chromosome condensation was exaggerated 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 chromatids were distinctly separate, whereas the chromatids of most other chromosomes were still adherant at their centromeric regions. This is in agreement with studies on several other animal species that have shown that the centromeres of all chromosomes do not divide simultaneously in colchicine-treated cells, but in an asynchronous, nonrandom 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 chromosomes 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 heterochromatin. Thus, centromeres lacking any detectable heterochromatin, as in the mouse Y chromosome, are always the first to separate, whereas centromeres surrounded by large C-bands, like the Y_2 chromosome of the rat kangaroo (*Potorus tridactylus*) separate late (Vig 1981). The Y chromosome of *G. walkeri* is another example confirming this relationship.

Silver staining of the chromosomes showed the nucleolus organizer 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

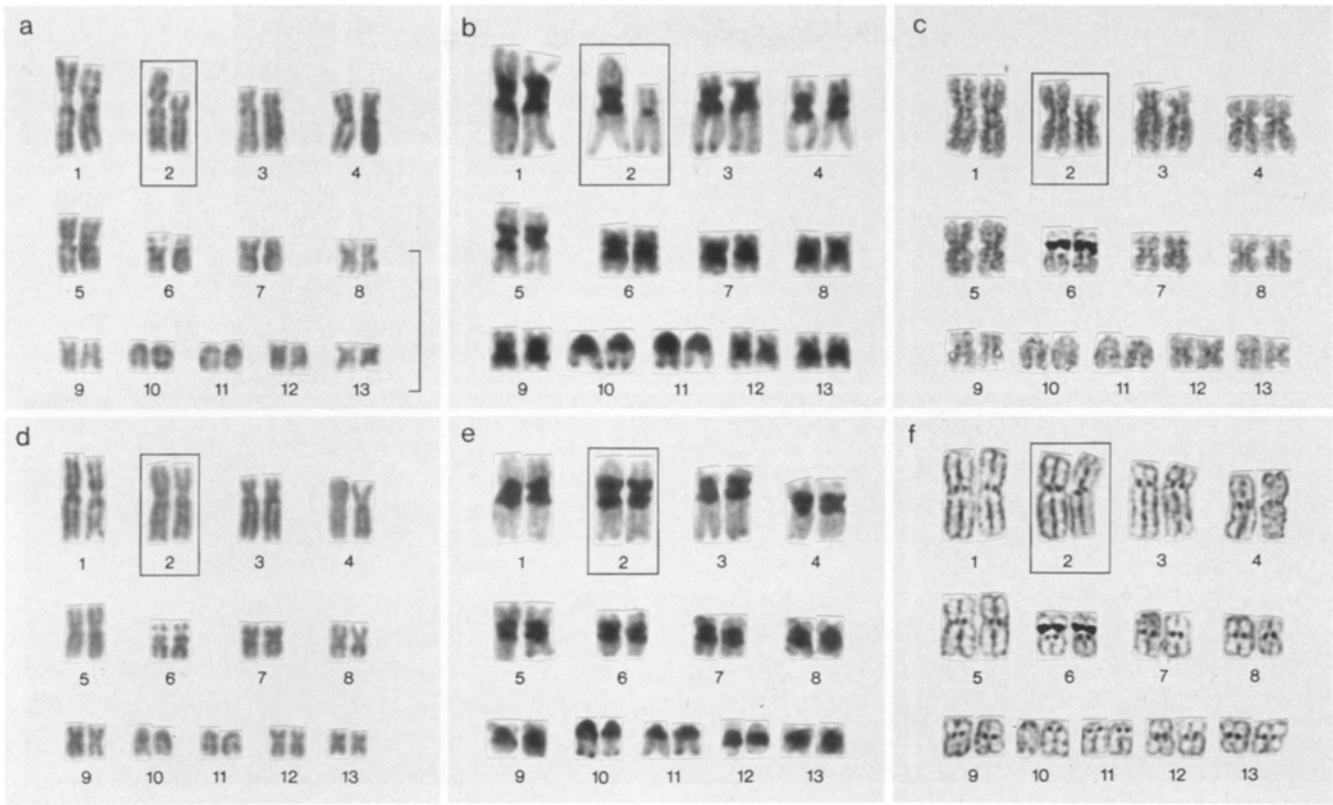

Fig. 1a–f. Karyotypes of male (**a–c**) and female (**d–f**) *Gastrotheca walkeri* after conventional aceto-orcein staining (**a, d**), C-banding (**b, e**) and silver staining (**c, f**). The sex chromosome pairs (no. 2) are *boxed*. In the male (**a–c**) sex chromosomes are heteromorphic (XY), whereas in the female (**d–f**) they are homomorphic (XX). Note in **b** that the Y chromosome has an extremely low content of constitutive heterochromatin. The secondary constrictions located in the short arms of chromosome pairs 6 (**a, d**) are the silver-positive nucleolus organizers (**c, f**). Bar represents 10 μm

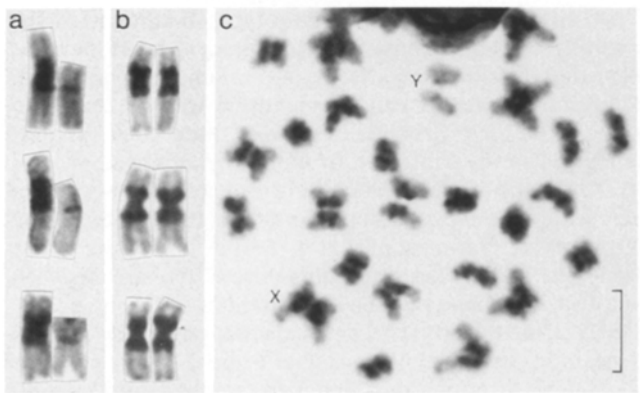

Fig. 2a–c. a, b Selected examples of C-banded sex chromosome (no. 2) pairs of male (**a**) and female (**b**) *Gastrotheca walkeri*. **c** C-banded bone marrow chromosomes of the male in an advanced metaphase stage; the centromeres of the Y are clearly divided, whereas those of most other chromosomes are still unseparated. Bar represents 10 μm

could be noted only in the centromeric heterochromatin of all chromosomes (Fig. 3a, c, e, h). The large bands of pericentromeric heterochromatin in the autosomes and in the X chromosome are not specifically labeled by quinacrine mustard. No quinacrine fluorescence is present in the nucleolus organizer regions in the short arms of chromosome pair 6 (Fig. 3a, c, l). One very small band in the long arm of the X chromosome, immediately at the end of the pericentromeric heterochromatin, also exhibits somewhat weaker quinacrine fluorescence than the remaining chromatin (Fig. 3c, h). Because of its small size, this region cannot be demonstrated in all metaphases. In the mithramycin-stained karyotypes, the entire pericentromeric heterochromatin of the autosomes and the X chromosome is characterized by distinctly reduced fluorescence. However, the centromeric heterochromatin fluoresces with about the same intensity as the euchromatic segments in all chromosomes including the Y (Fig. 3b, d, f, i). As is the rule for Amphibia (Schmid 1980a), the nucleolus organizer regions show the brightest mithramycin labeling in the karyotype (Fig. 3b, d, m). Direct staining of the chromosomes with DAPI or counterstaining the preaparations with distamycin A/DAPI produced a banding pattern intermediate between that obtained after quinacrine and mithramycin staining (compare Fig. 3c, d, o). Whereas the centromeric regions of most chromosomes have brighter fluorescence and the nucleolus organizers weaker fluorescence than the euchromatic segments (quinacrine pattern), the labeling of the pericentromeric heterochromatin of the chromosomes is weaker (mithramycin pattern).

Male meiosis was analyzed in order to obtain data on the pairing arrangement of the XY sex chromosomes. Because the testes were very small and had little meiotic activity, the few diakinetic stages found could be examined only with conventional aceto-orcein staining. The XY pair was differentiated from the autosomal pairs by its relative size.

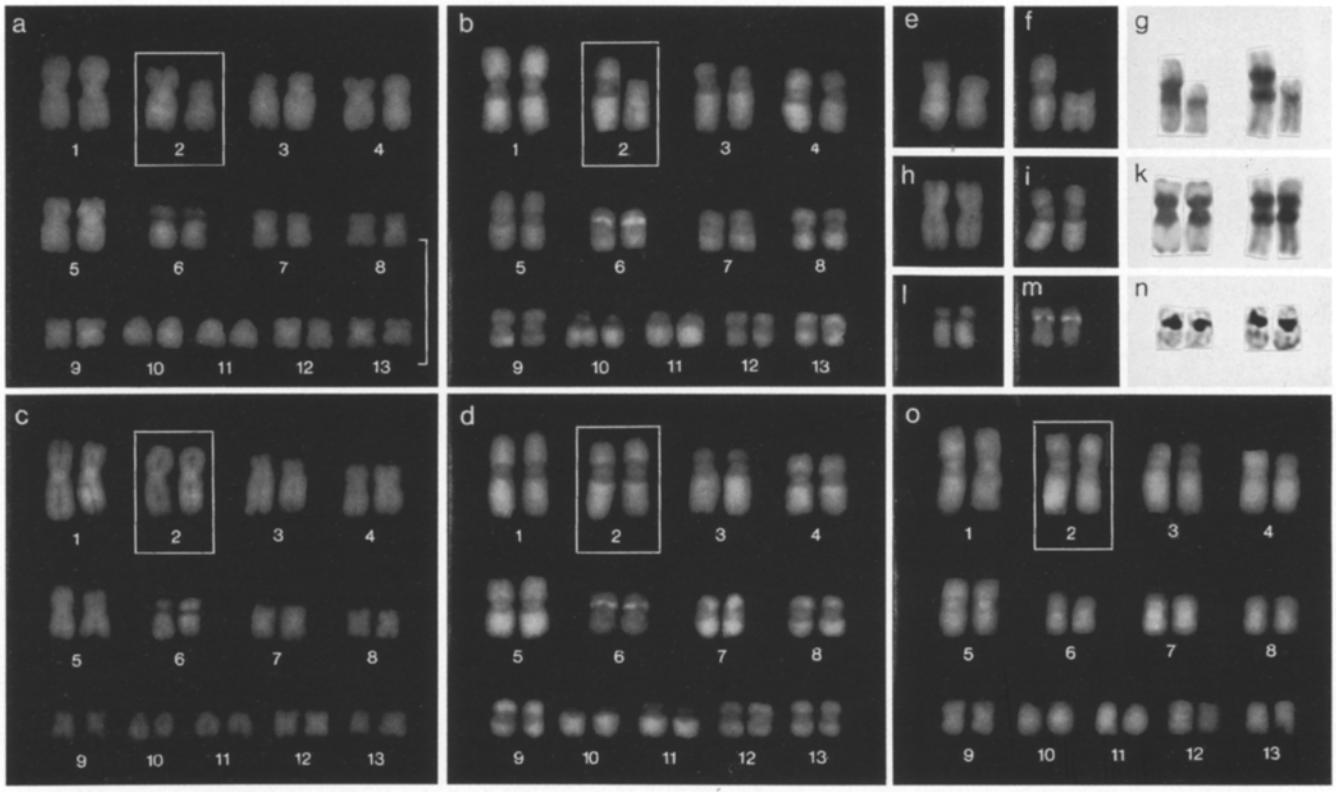

Fig. 3a–o. a–d Karyotypes of male (**a**, **b**) and female (**c**, **d**) *Gastrotheca walkeri* stained with quinacrine mustard (**a**, **c**) and mithramycin (**b**, **d**). The sex chromosome pairs (no. 2) are *boxed*. **e–k** Selected sex chromosome pairs of male (**e–g**) and female (**h–k**) showing quinacrine staining (**e**, **h**), mithramycin staining (**f**, **i**), and C-banding (**g**, **k**). **l–n** Nucleolus organizer carrying chromosome pairs 6 after quinacrine staining (**l**), mithramycin staining (**m**), and silver labeling (**n**). **o** Female karyotype with 4′-6-diamidino-2-phenylindole (DAPI) banding patterns. Bar represents 10 μm

As is characteristic for male meiosis in the highly evolved Anura (Morescalchi 1973), all autosomal bivalents of *G. walkeri* had a ring-like configuration held together by two terminal chiasmata (Fig. 7a–c). Interstitial chiasmata were not determined in any diakinesis. Unexpectedly, the XY sex bivalent no. 2 showed no end-to-end association like the heteromorphic XY sex chromosomes in other vertebrate species, but had the same ring-like form as the autosomal bivalents (see Discussion).

G. ovifera

Only few metaphases of adequate quality were found in the evaluation of the cells of the two small, immature specimens. However, these were sufficient to characterize the karyotype. As in *G. walkeri*, chromosome pairs 1–5 are much longer than pairs 6–13 (Fig. 4a). Pairs 1, 2 and 5 are nearly metacentric, and pairs 3 and 4 are submetacentric. All of the smaller chromosome pairs 6–13 are submetacentric. In contrast to *G. walkeri*, there are no telocentric chromosomes in *G. ovifera*. A secondary (nucleolar) constriction can be recognized in the long arms of the conventionally stained chromosome pair 6 (Fig. 4d). In one of the two juvenile animals examined, the two no. 1 chromosomes are the longest and are definitely homomorphic (Fig. 4a). However, in the other specimen only one chromosome 1 is as long, whereas the other is one-third smaller (Fig. 4h, i). This distinct heteromorphism was evident in all metaphases.

Of all three *Gastrotheca* karyotypes investigated, the constitutive heterochromatin of *G. ovifera* showed the most interesting distribution patterns and staining properties. All 13 chromosome pairs can be identified unequivocally on the basis of their C-bands (Fig. 4a). Interstitial C-bands are located in the short and/or long arms of chromosomes 1–5, 8, 10, and 13 near the pericentromeric C-bands. More interstitial heterochromatin is found in the center of the long arms of chromosomes 3, 4, 6 and 7, the two latter having particularly large amounts. Size differences between homologous C-bands can be recognized in the chromosome pairs 1, 2, 5, 8 and 11. The nucleolus organizer regions in the long arms of chromosomes 6 are C-band negative (Fig. 4a, e). Although the C-banded material from the animal with the heteromorphic chromosome pair 1 was of inferior quality, both chromosomes 1 could be identified in most metaphases. The constitutive heterochromatin in the smaller homolog seems to be restricted to the pericentromeric region (Fig. 4k).

In quinacrine-stained metaphases, all interstitial heterochromatic bands located adjacent to the pericentromeric heterochromatin fluoresce much brighter than the euchromatin (Fig. 4b). These quinacrine-positive regions were detected as very brightly fluorescing and compact chromocenters in the interphase nuclei (Fig. 4c). The quinacrine fluorescence intensity of the other interstitial heterochromatin bands, as well as of the pericentromeric and telomeric heterochromatin, is either the same or weaker than in the euchromatic segments. The nucleolus organizer regions in

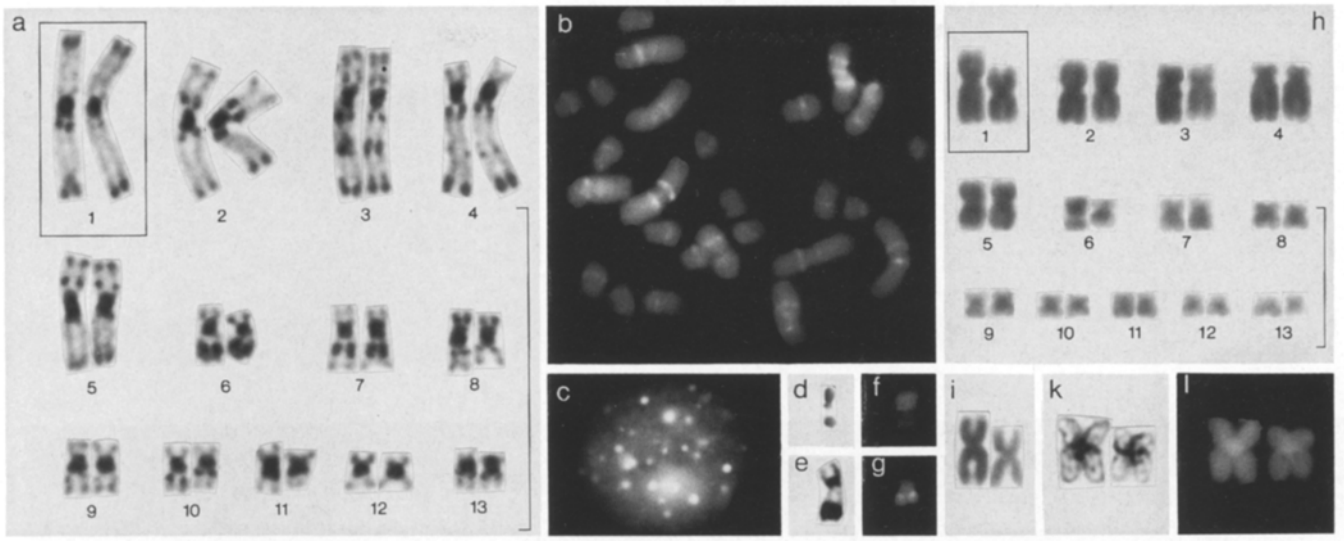

Fig. 4a–l. **a** C-banded karyotype of the juvenile *Gastrotheca ovifera* with a homomorphic chromosome pair 1 (*boxed*). **b** Quinacrine mustard-stained metaphase from the same animal showing brightly fluorescing interstitial heterochromatin. **c** Cell nucleus from the bone marrow; note quinacrine-positive heterochromatic chromocenters. **d–g** Nucleolus organizer carrying chromosomes no. 6 after conventional orcein staining (**d**), C-banding (**e**), quinacrine staining (**f**), and mithramycin staining (**g**). **h** Orcein-stained karyotype of the other juvenile with a heteromorphic chromosome pair 1 (*boxed*). **i–l** Examples of chromosome pairs 1 from the same specimen showing conventional orcein staining (**i**), C-bands (**k**), and mithramycin staining (**l**). Bars represent 10 μm

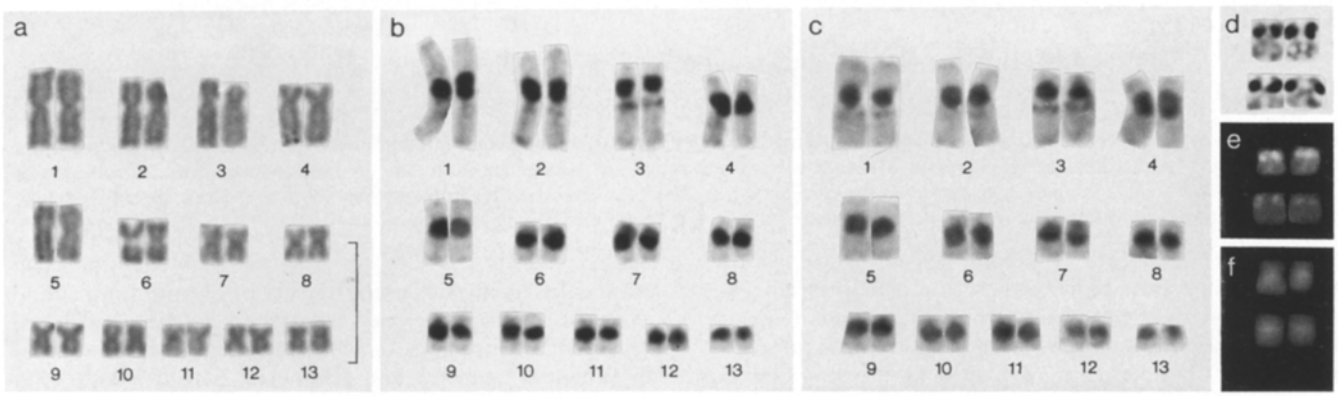

Fig. 5a–f. **a–c** Karyotypes of male (**a**, **b**) and female (**c**) *Gastrotheca fissipes* showing aceto-orcein staining (**a**) and C-bands (**b**, **c**). No heteromorphic sex chromosomes can be recognized. **d–f** Nucleolus organizer carrying chromosome pairs no. 6 after silver staining (**d**), mithramycin staining (**e**), and quinacrine mustard staining (**f**). Bar represents 10 μm

chromosome pair 6 appear as gaps without quinacrine fluorescence (Fig. 4f). After mithramycin staining, the pericentromeric heterochromatin fluoresces slightly brighter than the euchromatin in all chromosomes, whereas the interstitial heterochromatic bands are mithramycin negative. The nucleolus organizer region is distinguished by very bright mithramycin fluorescence with sharp contrast (Fig. 4g). One feature in the metaphases of the animal with the heteromorphic chromosome pair 1 was the localization of a small interstitial, mithramycin-negative heterochromatic region in the long arm of the smaller chromosome 1 adjacent to the mithramycin-positive pericentromeric heterochromatin (Fig. 4l).

G. fissipes

All males and females exhibited $2n=26$ chromosomes. As in *G. walkeri* and *G. ovifera*, the karyotype consists of large chromosome pairs 1–5 and distinctly smaller pairs 6–13 (Fig. 5a). The karyotype of *G. fissipes* shows more similarities with the karyotype of *G. ovifera* than with that of *G. walkeri* (compare Figs. 1d, 4h, 5a). Thus, all chromosomes are either metacentric or submetacentric; telocentric chromosomes were not observed (Fig. 5a). Furthermore, as in *G. ovifera*, chromosome pair 4 is submetacentric (Fig. 4h, 5a), whereas it is nearly metacentric in *G. walkeri* (Fig. 1d). As in *G. walkeri* and *G. ovifera*, the silver-positive nucleolus organizers are located in chromosome pair 6 in the karyotype of *G. fissipes* (Fig. 5d). These demonstrate the characteristic mithramycin-bright fluorescence (Fig. 5e) and negative quinacrine fluorescence (Fig. 5f).

Heteromorphic sex chromosomes could not be identified either by conventionally staining or by C-banding metaphases (Fig. 5a–c). Large pericentromeric C-bands are located in all chromosomes. In addition, small interstitial C-bands are present close to the pericentromeric heterochromatin in the long arms of chromosome pairs 1–3. Distinct telomeric heterochromatin is only found in the short

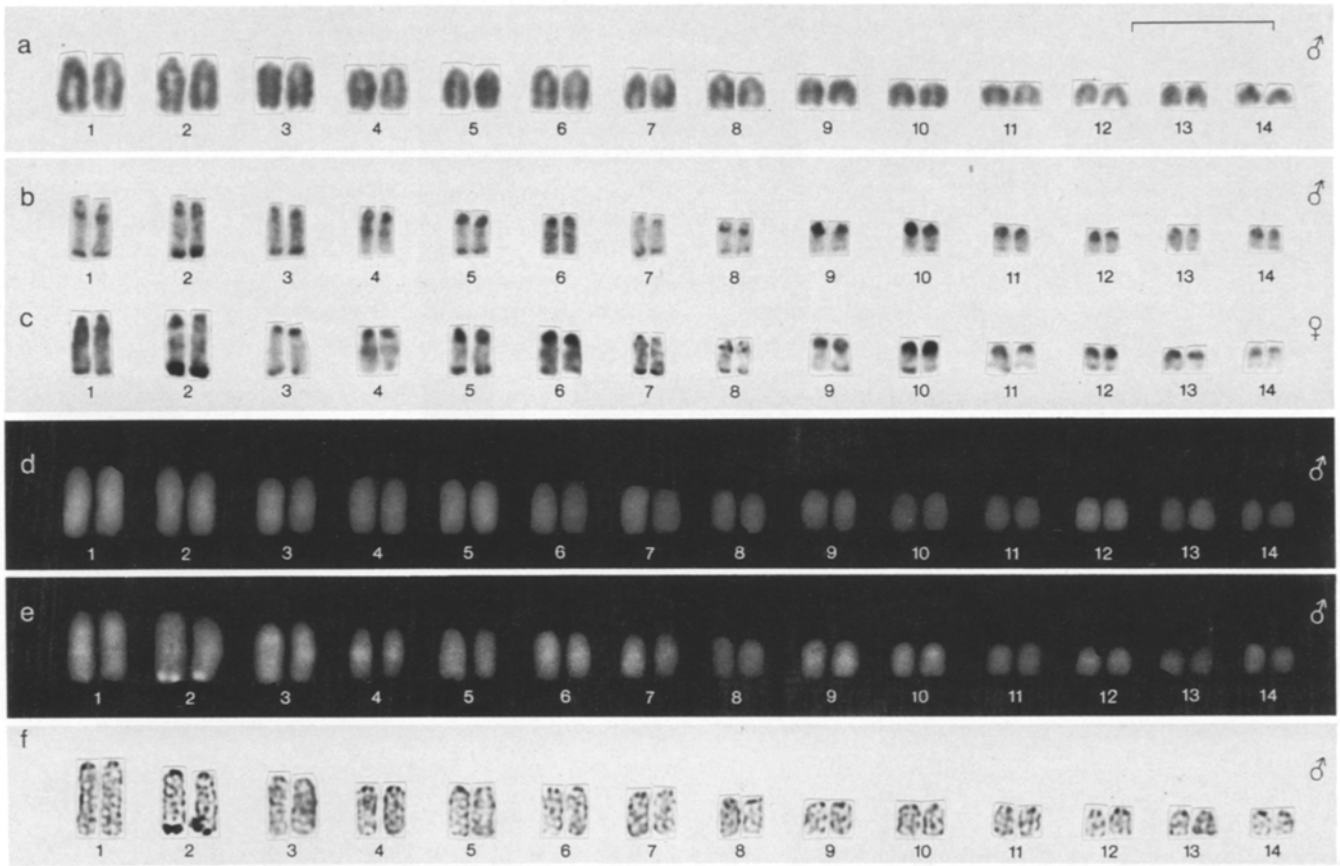

Fig. 6a–f. Karyotypes of *Flectonotus pygmaeus* presenting orcein staining (**a**), C-bands (**b**, **c**), quinacrine mustard staining (**d**), mithramycin staining (**e**), and silver labeling (**f**). Note in **a**–**c** that all chromosomes are telocentric and that no heteromorphic sex chromosomes can be recognized in this species. The nucleolus organizers, which fluoresce brightly after mithramycin staining (**e**) and label specifically with silver (**f**) are located in the telomeres of chromosome pairs 2. Bar represents 10 μm

arm of chromosome pair 7, whereas most other chromosomes exhibit only very faint telomeric C-bands (Fig. 5b, c). There was no conspicuous inter-individual variability in the sizes of the various C-bands. Quinacrine staining yielded uniform fluorescence intensity along the euchromatic arms of all chromosomes and somewhat brighter labeling of the paracentromeric heterochromatin. The interstitial C-bands in the long arms of chromosomes 1–3 fluoresced a little more weakly than the euchromatin. Conversely, after mithramycin staining, all paracentromeric heterochromatin fluoresced somewhat more darkly, and the interstitial heterochromatin in the chromosomes 1–3 a bit more lightly than the euchromatic segments. In the meiotic preparations of the 4 males 13 ring-shaped diakinetic bivalents with terminal chiasmata were observed.

F. pygmaeus

In contrast to the karyotypes of *Gastrotheca*, all the chromosomes of *F. pygmaeus* are very small and telocentric. They can be arranged in 14 pairs of continuously decreasing length (Fig. 6a). All 55 specimens had the same karyotype. Only a few prometaphases from one animal showed a secondary constriction and tiny satellites at the telomeres of the 2nd chromosomes. The C-banded karyotypes of both sexes are presented in Figure 6b, c. The characteristic sizes and positions of the heterochromatic regions permit the identification of all chromosome pairs. Prominent C-bands are located in the telomeric region of chromosome 2 and in the pericentromeric regions of chromosomes 5, 6, 9 and 10; smaller amounts of heterochromatin can be seen at all other centromeres and telomeres. Chromosomes 1, 2, 4, 6, and 8 additionally exhibit an interstitial C-band. This species frequently shows intra- and inter-individual size differences between homologous C-bands, especially those in the telomeric region of chromosome 2.

In quinacrine-stained, as well as in mithramycin-stained preparations, both the euchromatic and the heterochromatic segments fluoresce with uniform intensity (Fig. 6d, e). This is also the case after staining with DAPI or Hoechst 33258 and after counterstaining with distamycin A/DAPI. The only region in the karyotype with bright fluorescence is the mithramycin-positive nucleolus organizer in the telomere of chromosome 2 (Fig. 6e). This location of the nucleolus organizer is confirmed by silver staining (Fig. 6f).

The testes of the mature males contained large numbers of meiotic stages. Particularly interesting is that the bivalents of this highly evolved marsupial frog show all the essential features characteristic for the bivalents of the more primitive Anura. These bivalents are only sligthly spiralized and with a sharp outline in late diplotene. The chiasmata are almost exclusively in an interstitial position (Fig. 7d). As a rule, there is one chiasma per bivalent. Even the diakinetic stage still shows numerous interstitial chiasmata, and their terminalization is never complete (Fig. 7e, f). In comparison, strongly spiralized, ring-like bivalents with termi-

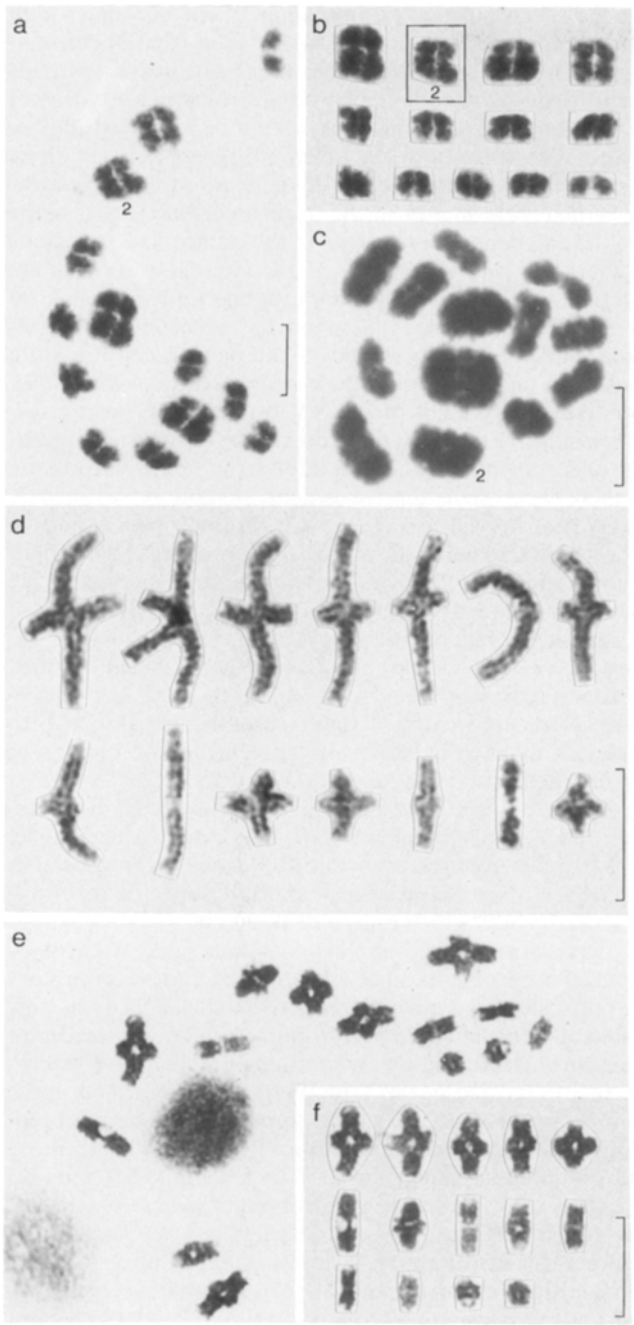

Fig. 7a–f. a–c Conventionally stained bivalents of two diakinetic stages from the male meiosis of *Gastrotheca walkeri*. The bivalents of the cell in **a** were arranged to a karyotype in **b** according to their relative sizes and the positions of their centromeres; the XY bivalent no. 2 is *boxed*. Most bivalents including the XY pair exhibit a ring-like configuration. **d** Bivalents of a late diplotene stage and **e**, **f** diakinetic bivalents from male *Flectonotus pygmaeus* meiosis. The bivalents in **e** were karyotyped in **f** by measuring the areas of the chromosomes. Note in **d** the very slight condensation of the diplotene bivalents and the interstitially located chiasmata, and in **e** the many non-terminalized chiasmata in the diakinetic stage. Bar represents 10 μm

nal chiasmata are always observed in the male meiosis of the other species of the family Hylidae (compare Fig. 7b, f). The high degree of chromosome spiralization reached by the diakinetic bivalents of the advanced anuran families is not achieved in the male meiosis of *F. pygmaeus*. The rod-like appearance of some bivalents in diakinesis is produced by the terminalization of the chiasmata. These bivalents are only loosely connected at their telomeres (Fig. 7e).

Discussion

Genus Gastrotheca

There are currently five studies, including the present report, describing diploid *Gastrotheca* karyotypes. The comparison of the karyotypes of *G. walkeri*, *G. ovifera*, and *G. fissipes* with those of *G. gracilis* (Bogart 1973) and *G. riobambae* (Schmid et al. 1983, 1986) shows a conserved diploid chromosome number of $2n=26$. In all species the karyotypes consist of five large and eight distinctly smaller chromosome pairs. This standard karyotype also exists in *G. pseustes* from Ecuador (M. Schmid et al., in preparation) and in the generalized egg-brooding hylids of the genera *Cryptobatrachus* and *Stefania* (Duellman and Hoogmoed 1984). The homoeology between the various *Gastrotheca* karyotypes is emphasized by the fact that in four of the five species the nucleolus organizer regions are always located in chromosome pair 6, and that there are great similarities in the morphology of the five large chromosome pairs. Only *G. riobambae*, in which the nucleolus organizer was apparently translocated into the short arm of the X chromosome (no. 4), deviates from this pattern (Schmid et al. 1983, 1986). Duellman (1967) reported (as *G. ceratophrys*) an unusual diploid chromosome number of $2n=28$ in *G. cornuta*, which occurs from Costa Rica to Ecuador. However, this result was obtained with the conventional squash technique on testicular tissue. Judging from the photograph of a male diakinesis presented in the study of Duellman (1967), *G. cornuta* could have either 14 bivalents or 12 autosomal bivalents plus 1 XY sex bivalent. The latter situation seems to be more likely and would conform to other *Gastrotheca* karyotypes, but this possible discrepancy can be resolved only when further meiotic and mitotic preparations of *G. cornuta* are analyzed with improved cytogenetic techniques.

Although only one adult pair of *G. walkeri* and two juveniles of *G. ovifera* could be analyzed in the present study, all results indicate the existence of highly differentiated sex chromosomes of the XY♂/XX♀ type in these two species. Both specimens of *G. walkeri* had completely developed gonads with mature oocytes or sperm cells. It is extremely unlikely that the heteromorphism found between the second chromosomes of the male animal represents a simple size variation of the constitutive heterochromatin in an autosomal chromosome pair because (1) none of the remaining chromosome pairs of the male or female showed any size differences between the homologous C-bands; (2) the difference between the second chromosomes is not the result of a continuous variation in the amount of heterochromatin as it can be observed in the C-bands of many vertebrates. This difference is instead based on the complete absence of pericentromeric heterochromatin in the smaller of the two homologs. The differences in size and banding patterns between the no. 1 chromosomes in one of the two specimens of *G. ovifera* also cannot be explained by heterochromatin variability, but by the presence of heteromorphic sex chromosomes. Because neither gonadal nor phenotypical sex identification was possible for these two immature specimens, there is yet no formal proof

of whether the type of sex determination is XY♂/XX♀ or ZW♀/ZZ♂. However, considering that *G. riobambae* (Schmid et al. 1983, 1986), *G. pseustes* (M. Schmid et al., in preparation), and *G. walkeri* (present report) all have the XY♂/XX♀ system, it seems reasonable to infer that the male *G. ovifera* is the heterogametic (XY) and the female the homogametic (XX) sex.

Heteromorphic sex chromosomes are very rare in anurans. Cytologically recognizable sex chromosomes have been demonstrated in only five species, other than those described here. Of these *Pyxicephalus adspersus* and *G. riobambae* possess highly differentiated sex chromosomes (Schmid 1980b; Schmid et al. 1983, 1986). The sex chromosomes of the other three species (*Tomopterna delalandii, Rana esculenta, Eupsophus migueli*) show only minor differences in morphology, amount of heterochromatin, or DNA replication patterns (Schmid 1980b; Schempp and Schmid 1981; Iturra and Veloso 1981). Therefore, the discovery of heteromorphic sex chromosomes in additional species of Anura is important not only to amphibian cytogenetics but for 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 has 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; Ray-Chaudhuri et al. 1971; Singh et al. 1976; Schmid 1983).

Molecular and cytogenetic studies on the ZW♀/ZZ♂ sex chromosomes of snakes have shown emphatically that the W chromosomes are enriched with interspersed repetitive DNA which can be isolated as a satellite (Bkm) fraction in the females (Singh et al. 1976, 1979, 1980). The Bkm DNA is located in high copy number in the W chromosomes of all snake families, including those species in which the ZW chromosomes are still homomorphic (Singh et al. 1980). This suggests strongly that the primary step in the evolution of the W chromosome was the development of specific repetitive DNA sequences (heterochromatinization). Because conserved DNA sequences of the Bkm family are also present in the Y chromosomes of mammals (Jones and Singh 1981), this model can be applied to the evolution of the XY system. Some of the sex chromosomes found so far in Amphibia confirm that heterochromatinization of the Y or W precedes morphological differentiation. In salamanders of the genus *Triturus* (Schmid 1983) and the frog *G. pseustes* (M. Schmid et al., in preparation), sex-specific chromosomes of the XY type have the same length and morphology. However, the long arm telomeres of the Y chromosomes already contain a heterochromatic C-band lacking in the X chromosomes. These small heterochromatic bands reduce the crossover frequencies during male meiosis.

The process of heterochromatinization of the whole Y and W chromosomes by the repeated Bkm sequences is supposed to happen considerably more quickly than the actual morphological divergence of these chromosomes (Jones 1984). As a result, the highly evolved Y and W chromosomes of mammals, birds and many advanced snakes are either completely heterochromatic or contain distinctly more heterochromatin than the X and Z chromosomes. The same is true for the few amphibian species in which highly differentiated Y or W chromosomes have been demonstrated (Schmid 1983). However, *G. walkeri* and *G. ovifera* are the first examples of amphibian Y chromosomes with less accumulated heterochromatin than the X chromosomes. In fact, the Y chromosome of *G. walkeri* contains the least amount of heterochromatin of all chromosomes in the karyotype. Comparison of the banding patterns in X and Y indicates that the heteromorphism between these chromosomes occurred solely by deletion of all pericentromeric heterochromatin in the Y chromosome. Because the lengths and banding properties of the euchromatic segments in the short and long arms of the XY chromosomes are identical, it is assumed that they are genetically homologous as well. This homology between the euchromatic XY regions can also be recognized in the pairing configuration of the sex bivalent during male meiosis of *G. walkeri*. The ring-like arrangement of the XY bivalent and its two terminalized chiasmata found in diakineses indicates that pairing and crossing over occur along the XY euchromatin. If there were no extensive genetic homology between X and Y, end-to-end associated XY chromosomes would be expected in the diakinetic stages. Such rod-like XY configurations, similar to the XY bivalents of male mammals, are formed by the highly heteromorphic XY chromosomes of *G. riobambae* (Schmid et al. 1983). If during meiotic prophase of male *G. walkeri* and *G. ovifera* the X and Y chromosomes pair along their total length, the large pericentromeric heterochromatin of the X chromosome should protrude as an unpaired loop at the centromeric regions of the diplotene and pachytene XY bivalents.

The new category of Y chromosomes detected in *G. walkeri* and *G. ovifera* does not fit the evolutionary model of early heterochromatinization of the Y chromosomes mentioned above. However, possibly sex-specific repetitive DNA sequences which escape demonstration with C-banding have accumulated in these differentiated Y chromosomes. This problem can be addressed by comparative analyses of male and female patterns of satellite DNAs in *Gastrotheca* or by in situ hybridization of Bkm sequences to their chromosomes. Lower amounts of constitutive heterochromatin located in the Y or W chromosomes than in the X or Z chromosomes have been reported in fishes and reptiles. In the lizard *Cnemidophorus tigris*, the X and Y are still homologous along most of their length, but they do not cross over in their pericentromeric regions; C-banding has shown that the Y has considerably less pericentromeric heterochromatin than the X chromosome (Bull 1978). The acrocentric X chromosomes of the turtles *Staurotypus salvini* and *S. triporcatus* have constitutive heterochromatin in the short arms, centromeric regions, and at the secondary constrictions in the long arms; in the smaller Y chromosomes, the heterochromatin is restricted to the centromeric regions (Bull et al. 1974; Sites et al. 1979). The Y chromosomes of the trout *Salmo gairdneri* and *Salvelinus namaycush* are also smaller and contain distinctly less heterochromatin than the X chromosomes; C-banding differences occur in the pericentromeric regions of the XY chromosomes in *S. gairdneri* and in the short arm telomeres of the XY pair in *S. namaycush* (Thorgaard 1977; Lloyd and Thorgaard 1988; Phillips and Ihssen 1985).

All *Gastrotheca* with heteromorphic XY chromosomes (*G. riobambae, G. walkeri, G. ovifera, G. pseustes*) occur in the mountains of northern and western South America. However, *G. fissipes* in which no sex chromosomes were demonstrated, is one of the two species of *Gastrotheca* inhabiting eastern and southeastern Brazil. The Amazon and

Orinoco basins are major geographic barriers between the Andean and Brazilian *Gastrotheca*. The cytogenetic evidence suggests that the evolution of the XY chromosomes occurred in the Andean lineage of *Gastrotheca* after separation from the eastern Brazilian lineage. However, this relationship is not supported by morphological evidence (Duellman 1984). Furthermore, comparative cytogenetic studies on the many Andean species of *Gastrotheca* are required to clarify whether heteromorphic sex chromosomes are indeed restricted to the Andean *Gastrotheca* lineage.

There is an abundance of constitutive heterochromatin in the karyotypes of the three species of *Gastrotheca* examined, especially in the autosomes and X chromosomes of *G. walkeri* and *G. ovifera*. The results of the analyses with fluorochromes permit some conclusions about the base pair composition of the repetitive DNA located in the individual heterochromatic C-bands. AT-rich DNA sequences are known to enhance quinacrine fluorescence intensity (Weisblum 1973), whereas mithramycin preferentially forms complexes with GC-rich DNA (Ward et al. 1965). Comparisons among C-banded, quinacrine- and mithramycin-stained karyotypes of diverse species of anurans have shown that the heterochromatic regions can be classified into two main types: (1) quinacrine-positive C-bands that are always mithramycin negative ($C^+Q^+MM^-$) and (2) conversely, quinacrine-negative C-bands characterized by bright mithramycin fluorescence ($C^+Q^-MM^+$) (Schmid 1980a; Schmid et al. 1987). This relationship between the two categories of constitutive heterochromatin is also seen in the karyotypes of *G. ovifera* and *G. fissipes*. However, the chromosomes of *G. walkeri* show large pericentromeric C-bands in the autosomes and X chromosome which are distinctly MM^- but not Q^+. Furthermore, AT-specific DAPI produces a fluorescence pattern in the karyotype of *G. walkeri* which is similar to that obtained with GC-specific mithramycin. The same has also been observed in *G. riobambae* (Schmid et al. 1983). As yet there is no explanation for these unexpected staining properties. Possibly, not only the AT- or GC-richness of a heterochromatic region is decisive for the preferential binding and differential fluorescence of the fluorochromes, but also the sequence of the AT and GC base pairs within the repetitive DNA.

Genus Flectonotus

The karyotype of *F. pygmaeus* is surprising because it consists of 28 exclusively telocentric chromosomes. Advanced anurans usually have meta- and submetacentric chromosomes, but rarely telocentric ones (Morescalchi 1973). One exception is the species-rich neotropical genus *Eleutherodactylus*, which exhibits karyotypes with low chromosome numbers ($2n=18$, 20, 22, 26) and meta- or submetacentric chromosomes, as well as karyotypes with high chromosome numbers ($2n=30$, 32, 34, 36) and an increasing number of telocentric chromosomes. Because an increase in telocentric chromosomes is always accompanied by a reduction in meta- and submetacentric chromosomes, the development of such karyotypes can be explained by centric fission (Bogart 1973; Morescalchi 1973, 1979). The genus *Flectonotus* contains two species, *F. fitzgeraldi* on the islands of Trinidad and Tobago and *F. pygmaeus* in the mountains of northern Venezuela; both have $2n=28$ chromosomes (Duellman and Gray 1983). There are no recognizable homoeologies between the karyotypes of the genera *Flectonotus* and *Gastrotheca*. These consistent differences imply lengthy independent chromosomal evolution in the two genera. Results of quantitative immunological microcomplement fixation of serum albumins supports the chromosomal data and indicates generic separation of at least 50 million years (Duellman et al. 1988).

In contrast to the karyotypes of *Gastrotheca*, the chromosomes of *F. pygmaeus* do not exhibit the unequivocal alternative of $C^+Q^+MM^-$ or $C^+Q^-MM^+$ heterochromatin. All C-bands present the same fluorescence intensity with quinacrine and mithramycin as the euchromatin. This class of heterochromatin has already been demonstrated in many other anuran genera and has been interpreted as an interspersion of AT- and GC-rich DNA sequences with no preferential binding of any of the fluorochromes (Schmid 1980a).

The male meiosis of *F. pygmaeus* is interesting because of the chiasmata which are located interstitially in the bivalents. Interstitial chiasmata are also exhibited by a few other neotropical hylids (e.g. *Hyla microps*, *H. rubicundula*) which also possess telocentric chromosomes. Thus, as Morescalchi (1973) has outlined, there seems to be a correlation between the presence of interstitial chiasmata in the meiotic bivalents and the occurrence of telocentric chromosomes in the somatic karyotypes.

The marsupial frogs offer many possibilities for studies on the evolution of sex chromosomes, structure of constitutive heterochromatin, and meiotic configurations. The analysis of their genomes should be extended to additional species and should include biochemical and molecular techniques.

Acknowledgements. This study was supported by the Deutsche Forschungsgemeinschaft (Schm 484/2–2 and 484/2–3) and the Universitätsbund Würzburg. The research of W.E. Duellman on marsupial frogs was supported by grants from the National Science Foundation, USA, of which DEB-8219388 is the most recent. We are very grateful to Dr. A. Fernández Badillo (Instituto de Zoología Agrícola, Maracay, Venezuela) for providing facilities at the Estación Biológica de Rancho Grande. C.G. de Almeida is a fellow of the Konrad Adenauer Stiftung (Bonn).

References

Beçak W, Beçak ML, Nazareth HRS, Ohno S (1964) Close karyological kinship between the reptilian suborder Serpentes and the class Aves. Chromosoma 15:606–617

Bogart JP (1973) Evolution of anuran karyotypes. In: Vial JL (ed) Evolutionary biology of the anurans. University of Missouri Press, Columbia, pp 337–349

Bull J (1978) Sex chromosome differentiation: an intermediate stage in a lizard. Can J Genet Cytol 20:205–209

Bull JJ, Moon RG, Legler JM (1974) Male heterogamety in kinosternid turtles (genus Staurotypus). Cytogenet Cell Genet 13:419–425

del Pino EM, Elinson RP (1983) A novel development pattern for frogs: gastrulation produces an embryonic disk. Nature 306:589–591

del Pino EM, Steinbeisser H, Hofmann A, Dreyer C, Campos M, Trendelenburg M (1986) Oogenesis in the egg-brooding frog Gastrotheca riobambae produces large oocytes with fewer nucleoli and low RNA content in comparison to Xenopus laevis. Differentiation 32:24–33

Duellman WE (1967) Additional studies of chromosomes of anuran amphibians. Syst Zool 16:38–43

Duellman WE (1977) Liste der rezenten Amphibien und Reptilien.

Hylidae, Centrolenidae, Pseudidae. In: Mertens R, Hennig W (eds) Das Tierreich 95. de Gruyter, Berlin, pp 1–184
Duellman WE (1984) Taxonomy of Brazilian hylid frogs of the genus Gastrotheca. J Herpetol 18:302–312
Duellman WE, Gray P (1983) Developmental biology and systematics of the egg-brooding hylid frogs, genera Flectonotus and Fritziana. Herpetologica 39:333–359
Duellman WE, Hoogmoed MS (1984) The taxonomy and phylogenetic relationships of the hylid frog genus Stefania. Misc Publ Mus Nat Hist Univ Kansas 75:1–39
Duellman WE, Maness SJ (1980) The reproductive behaviour of some hylid marsupial frogs. J Herpetol 14:213–222
Duellman WE, Maxson LR, Jesiolowski CE (1988) Evolution of marsupial frogs (Hylidae: Hemiphractinae): immunological evidence. Copeia 1988:527–543
Iturra P, Veloso A (1981) Evidence for heteromorphic sex chromosomes in male amphibians (Anura: Leptodactylidae). Cytogenet Cell Genet 31:108–110
Jones KW (1984) The evolution of sex chromosomes and their consequences for the evolutionary process. In: Bennett MD, Gropp A, Wolf U (eds) Chromosomes Today 8:241–255
Jones KW, Singh L (1981) Conserved repeated DNA sequences in vertebrate sex chromosomes. Hum Genet 58:46–53
Lloyd MA, Thorgaard GH (1988) Restriction endonuclease banding of rainbow trout chromosomes. Chromosoma 96:171–177
Macgregor HC, del Pino EM (1982) Ribosomal gene amplification in multinucleate oocytes of the egg brooding hylid frog Flectonotus pygmaeus. Chromosoma 85:475–488
Morescalchi A (1973) Amphibia. In: Chiarelli AB, Capanna E (eds) Cytotaxonomy and vertebrate evolution. Academic Press, London, pp 233–348
Morescalchi A (1979) New developments in vertebrate cytotaxonomy. I. Cytotaxonomy of the amphibians. Genetica 50:179–193
Ohno S (1967) Sex chromosomes and sex-linked genes. Springer, Berlin Heidelberg New York
Phillips RB, Ihssen PE (1985) Identification of sex chromosomes in lake trout (Salvelinus namaycush). Cytogenet Cell Genet 39:14–18
Ray-Chaudhuri SP, Singh L, Sharma T (1971) Evolution of sex chromosomes and formation of W chromatin in snakes. Chromosoma 33:239–251
Schempp W, Schmid M (1981) Chromosome banding in Amphibia. VI. BrdU-replication patterns in Anura and demonstration of XX/XY sex chromosomes in Rana esculenta. Chromosoma 83:697–710
Schmid M (1978) Chromosome banding in Amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in Bufo and Hyla. Chromosoma 66:361–388
Schmid M (1980a) Chromosome banding in Amphibia. IV. Differentiation of GC- and AT-rich chromosome regions in Anura. Chromosoma 77:83–103
Schmid M (1980b) Chromosome banding in Amphibia. V. Highly differentiated ZZ/ZW sex chromosomes and exceptional genome size in Pyxicephalus adspersus (Anura, Ranidae). Chromosoma 80:69–96
Schmid M (1983) Evolution of sex chromosomes and heterogametic systems in Amphibia. Differentiation 23 Suppl:S13–S22
Schmid M, Olert J, Klett C (1979) Chromosome banding in Amphibia. III. Sex chromosomes in Triturus. Chromosoma 71:29–55
Schmid M, Haaf T, Geile B, Sims S (1983) Chromosome banding in Amphibia. VIII. An unusual XY/XX-sex chromosome system in Gastrotheca riobambae (Anura, Hylidae). Chromosoma 88:69–82
Schmid M, Sims SH, Haaf T, Macgregor HC (1986) Chromosome banding in Amphibia. X. 18S and 28S ribosomal RNA genes, nucleolus organizers and nucleoli in Gastrotheca riobambae. Chromosoma 94:139–145
Schmid M, Vitelli L, Batistoni R (1987) Chromosome banding in Amphibia. XI. Constitutive heterochromatin, nucleolus organizers, 18S+28S and 5S ribosomal RNA genes in Ascaphidae, Pipidae, Discoglossidae and Pelobatidae. Chromosoma 95:271–284
Singh L, Purdom IF, Jones KW (1976) Satellite DNA and evolution of sex chromosomes. Chromosoma 59:43–62
Singh L, Purdom IF, Jones KW (1979) Behaviour of sex chromosome-associated satellite DNAs in somatic and germ cells in snakes. Chromosoma 71:167–181
Singh L, Purdom IF, Jones KW (1980) Sex chromosome associated satellite DNA: evolution and conservation. Chromosoma 79:137–157
Sites JW, Bickham JW, Haiduk MW (1979) Derived X chromosomes in the turtle genus Staurotypus. Science 206:1410–1412
Thorgaard GH (1977) Heteromorphic sex chromosomes in male rainbow trout. Science 196:900–902
Vig BK (1981) Sequence of centromere separation: an analysis of mitotic chromosomes from long-term cultures of Potorus cells. Cytogenet Cell Genet 31:129–136
Ward DC, Reich E, Goldberg IH (1965) Base specificity in the interaction of polynucleotides with antibiotic drugs. Science 149:1259–1263
Wassersug RJ, Duellman WE (1984) Oral structures and their development in egg-brooding hylid frog embryos and larvae: evolutionary and ecological implications. J Morphol 182:1–37
Weisblum B (1973) Fluorescent probes of chromosomal DNA structure: three classes of acridines. Cold Spring Harbor Symp Quant Biol 38:441–449

Received April 26, 1988
Accepted by H.C. Macgregor