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Chromosome banding in Amphibia. XV. Two types of Y chromosomes and heterochromatin hypervariabilty in *Gastrotheca pseustes* (Anura, Hylidae)

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Abstract. The chromosomes of the newly discovered South American marsupial frog *Gastrotheca pseustes* were analyzed by conventional methods and by various banding techniques. This species is characterized by XY♂/XX♀ sex chromosomes and the existence of two different morphs of Y chromosomes. Whereas in type A males the XY^A chromosomes are still homomorphic, in type B males the Y^B chromosome displays a large heterochromatic region at the long arm telomere which is absent in the X. In male meiosis, the homomorphic XY^A chromosomes exhibit the same pairing configuration as the autosomal bivalents. On the other hand, the heteromorphic XY^B chromosomes form a sex bivalent by pairing their short arm telomeres in a characteristic end-to-end arrangement. Analysis of the karyotypes by C-banding and DNA base pair-specific fluorochromes reveals enormous interindividual size variability of the autosomal heterochromatin.

Introduction

The marsupial frogs of the genus *Gastrotheca* belong to the diverse family Hylidae and are confined to Central and South America. To date, 42 species have been identified, most of them living in the South American Andes and associated Cordilleras (Duellman 1977; Duellman et al. 1988). Because of the limited availability of marsupial frogs, only three previous cytogenetic studies on *Gastrotheca* chromosomes have been performed (Schmid et al. 1983, 1986, 1988). The results obtained in these analyses demonstrated that exceptional XY chromosomes have evolved in these Anura. Thus, the karyotype of *G. riobambae* from Ecuador is distinguished by highly heteromorphic XY chromosomes. The X is considerably smaller than the Y and carries the sole nucleolus organizer (NOR) of the karyotype. This leads to a rare sex-specific difference in the number of 18S + 28S ribosomal RNA genes: female (XX) animals possess twice as many ribosomal RNA genes as male (XY) animals. The fact that the X is smaller than the almost completely heterochromatic Y is also a rare situation in vertebrates. Furthermore, *G. riobambae* was the first species of Anura in which end-to-end associated XY chromosomes were found in male meiosis (Schmid et al. 1983, 1986).

Two other species, *G. walkeri* and *G. ovifera* from Venezuela, are also characterized by highly differentiated XY chromosomes. However, in both species the X chromosomes are larger than the Y chromosomes. Whereas the X chromosomes and the autosomes contain large amounts of constitutive heterochromatin, extremely little heterochromatin is located in the Y chromosomes. This is in contrast to all other known amphibian Y chromosomes and the Y chromosomes of most vertebrates (Schmid et al. 1988).

These data indicated the necessity to continue cytogenetic investigations on more of these interesting marsupial frogs. Because the presence of heteromorphic sex chromosomes is exceptional in other Amphibia (for review see Hillis and Green 1990; Schmid et al. 1990), it is necessary to determine if the existence of differentiated sex chromosomes is the rule in *Gastrotheca*. The present work describes the results on the newly discovered species *G. pseustes* (Duellman and Hillis 1987) from the Andean Cordilleras in Ecuador. This species is unique in having two different coexisting types of Y chromosomes and hypervariability of the constitutive heterochromatin.

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Materials and methods

Animals and taxonomy. Ninety-eight mature animals, all classified as *G. riobambae*, were obtained from a herpetological animal dealer. According to the information from the collectors in South America, all specimens were caught in geographically unspecified localities of the province of Loja, in southern Ecuador. Cytogenetic analyses showed that only 38 of these marsupial frogs actually had the characteristic *G. riobambae* karyotype described in detail previously (Schmid et al. 1983, 1986; Schmid and de Almeida 1988). The other 60 animals, although phenotypically indistinguishable from the former by nontaxonomists, had a completely different karyotype with regard both to chromosome morphology and banding patterns. Because both species were so alike, it was necessary to perform an electrophoretic analysis of those allozymes which were diagnostic for several of the Ecuadorian *Gastrotheca* (Duellman and Hillis 1987). Further differences between the two species were defined by flow cytometric determination of their nuclear DNA contents and AT or GC base pair contents.

Allozyme electrophoresis. Muscle samples from the frozen specimens previously used for cytogenetic analysis were homogenized for allozymic analysis. Although the limited samples of available tissue did not permit an examination of all 25 loci examined by Duellman and Hillis (1987), 5 loci (which together are diagnostic for the five species previously confused with *G. riobambae* in southern Ecuador) were examined in each specimen. Of these loci, Duellman and Hillis (1987) found that glucose-6-phosphate dehydrogenase (G-6-pdh) was diagnostic for *G. riobambae*, lactate dehydrogenase-1 (Ldh-1) was diagnostic for *G. pseustes* and *G. psychrophila*, Ldh-2 could be used to distinguish *G. litonedis* and *G. monticola* from *G. riobambae*, *G. pseustes*, and *G. psychrophila*, superoxide dismutase-1 (Sod-1) was diagnostic for *G. litonedis*, and Sod-2 could be used to distinguish *G. litonedis* and *G. monticola* from *G. pseustes* and *G. riobambae* (see Table 1). Gels were made from 12% starch (Electrostarch), and the techniques for starch gel electrophoresis and histochemical staining were as described by Duellman and Hillis (1987). Samples of known identity (of each species) from the study of Duellman and Hillis (1987) were run as standards on the gels.

Flow cytometry. From one male each of both *Gastrotheca* species, blood samples were obtained by cardiac puncture using a heparinized syringe. The erythrocytes were washed twice in PBS and stained in ethidium bromide, Hoechst 33258, or chromomycin A_3 buffers according to Kubbies and Friedl (1985). To the ethidium bromide buffer was added 50 IU/ml RNase A. Aliquots of 4×10^5 cells were stained for at least 30 min at 4° C in the respective staining buffers. Chicken erythrocytes were added as an internal standard. Flow cytometry was performed with an ICP 22 (Ortho). Histograms were recorded with a type 2103 pulseheight analyzer (Ortho) interfaced to a PDP 11/23 microcomputer (Digital). Fluorescence means, coefficients of variation, and standard fluorescence ratios were determined by automated curve fitting (Kubbies and Rabinovitch 1983). The calculation of the DNA contents and the AT or GC base pair contents from the flow cytometry histograms was done according to Kubbies and Friedl (1985) using the known values for chicken erythrocytes (DNA content, 2.4 pg; AT content, 57% Shapiro 1976a, b) as a base.

Chromosome preparation and banding. Mitotic chromosomes were prepared directly from bone marrow and meiotic chromosomes were obtained from the testes after in vivo colchicine treatment. The techniques used for the preparation of the tissues, hypotonic treatment, and fixation of the cells have been described elsewhere (Schmid 1978). Conventional staining of the chromosomes, C-banding, staining with quinacrine, 4'-6-diamidino-2-phenylindole (DAPI), and counterstaining with distamycin A/mithramycin were done on the mitotic metaphases of all 60 animals according to Schmid et al. (1983) and Schweizer (1976). The meiotic preparations were conventionally stained or C-banded. For all of the animals, at least ten mitotic karyotypes were prepared for each of the staining techniques employed. All karyotypes were laid out in parallel rows: this system permits proof of the conformity in banding patterns, demonstration of heteromorphic sex chromosomes, and facilitates the recognition of interindividual variation of specific bands.

Table 1. Enzyme loci examined in the present study and by Duellman and Hillis (1987) to distinguish among Ecuadorian species of *Gastrotheca* that have been confused with *G. riobambae*[a]

Locus[b]	Species				
	litonedis	*monticola*	*pseustes*	*psychrophila*	*riobambae*
G-6-phd	*c*	*c*	*c*	*c*	*b*
Ldh-1	*g*	*g*	*c*	*a*	*d, g*
Ldh-2	*g*	*g*	*f*	*f*	*f*
Sod-1	*c*	*a*	*a*	*a*	*a*
Sod-2	*g*	*d*	*c, i*	*d, i*	*i*

[a] Allelic designations follow Duellman and Hillis (1987)
[b] G-6-pdh, glucose-6-phosphate dehydrogenase; Ldh, lactate dehydrogenase; Sod, superoxide dismutase

Results

Allozyme electrophoresis

The allozymic analysis demonstrated that two species were present in the sample of *Gastrotheca*: *G. riobambae* and *G. pseustes*. Specimens of *G. riobambae* were distinguishable on the basis of the presence of the *b* allele at G-6-pdh and the *g* allele at Ldh-1. Specimens of *G. pseustes* were distinguishable on the basis of the *c* allele at G-6-pdh and the *c* allele at Ldh-1. All specimens had the *f* allele at Ldh-2, the *a* allele at Sod-1, and the *i* allele at Sod-2. These combinations of characters were sufficient to determine that *G. pseustes* and *G. riobambae* were the only species present in the sample, and to discriminate between the two species.

Nuclear DNA contents, chromosome lengths, and AT/GC base pair contents

The total nuclear DNA content in *G. pseustes* erythrocytes as determined by flow cytometry is 7.07 pg, whereas that in *G. riobambae* erythrocytes is 8.47 pg. Measurements of chromosome lengths also show that *G. pseustes* has the smaller genome. These measurements were performed on five high-quality C-banded karyotypes each from the mid-metaphase stage of the two species. The total chromosome lengths in the haploid male karyotypes (12 autosomes + XY sex chromosomes) amount to 55.8 μm in *G. pseustes* and to 76.0 μm in *G. riobambae*. The nuclear DNA values of *G. pseustes* and *G. riobambae* lie within the general unimodal distribution of the genome sizes of diploid hylid species, which ranges from a minimum of 3 pg to a maximum of 10 pg with a modal value between 4 pg and 8 pg (for review see Olmo

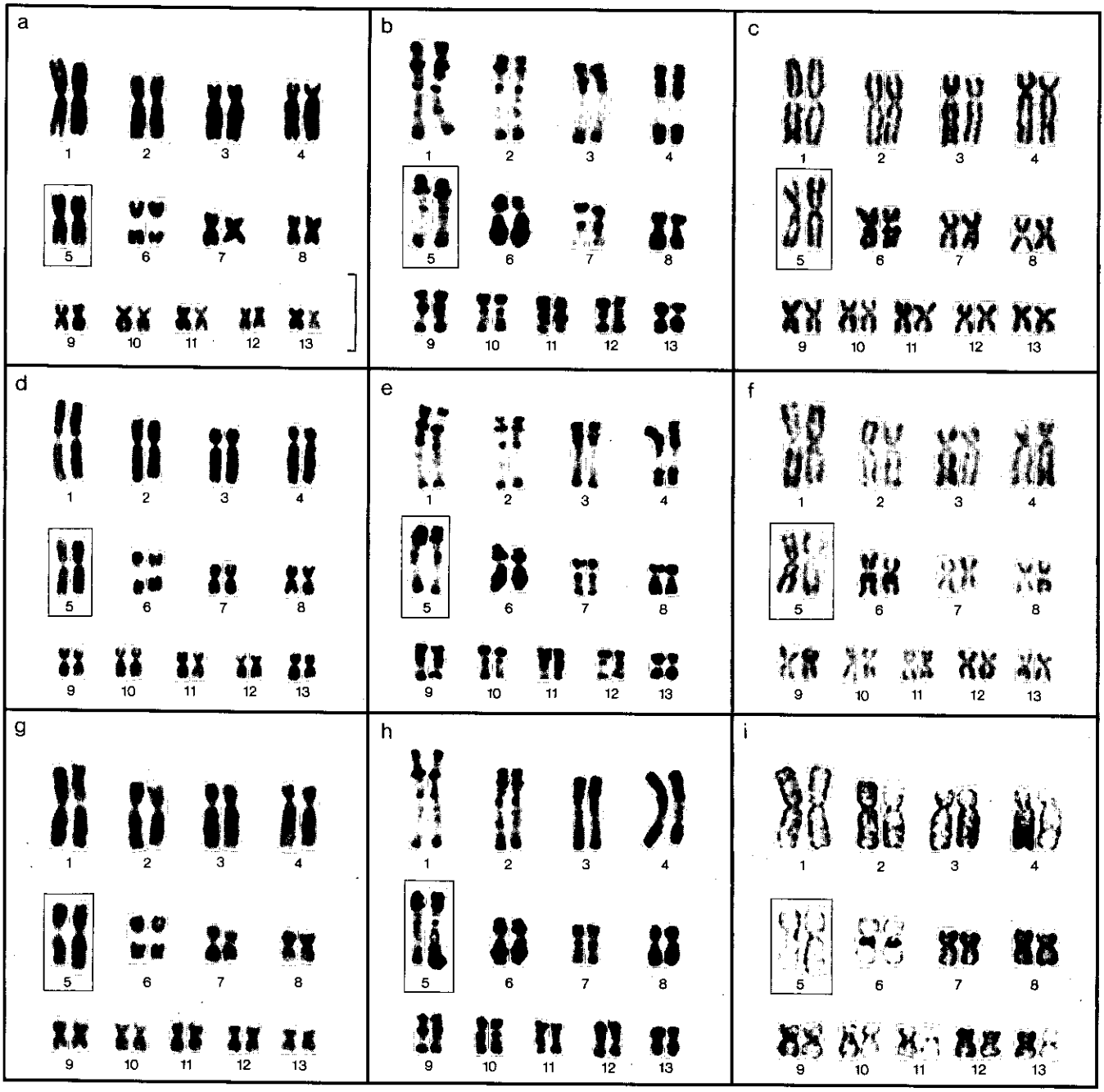

Fig. 1a–i. Karyotypes of female (**a–c**), type A male (**d–f**), and type B male (**g–i**) *Gastrotheca pseustes* after conventional Giemsa staining (**a, d, g**), C-banding (**b, e, h**), and silver staining (**c, f, i**). The sex chromosme pairs 5 are *boxed*. In the female (**a–c**) and the type A male (**d–f**), the sex chromosomes are homomorphic (XX and XY^A, respectively), whereas in the type B male (**g, h, i**) they are heteromorphic (XY^B). The Y^B long arm is distinctly larger than the X long arm and carries a prominent telomeric C-band which is absent in the X. Bar in **a** represents 10 μm

1973). There is only one other report in the literature concerning the genome sizes in the genus *Gastrotheca*. Using Feulgen cytophotometry, Goin et al. (1968) found that the DNA content in hepatocytes of an unidentified *Gastrotheca* female was 7.3 pg, and this value corresponds fairly well with those obtained in the present study.

The AT(GC) base pair contents in the DNAs of *G. pseustes* and *G. riobambae* are almost identical. The ratios of fluorescence intensities of the marsupial frog erythrocytes relative to the internal standard (chicken erythrocytes) derived from the flow cytometry histograms yield AT(GC) contents of 54.87% (45.13%) for *G. pseustes* and 54.51% (45.49%) for *G. riobambae*.

Conventional staining of chromosomes

This and the following sections describe exclusively the cytogenetic data of *G. pseustes*; all features of the *G.*

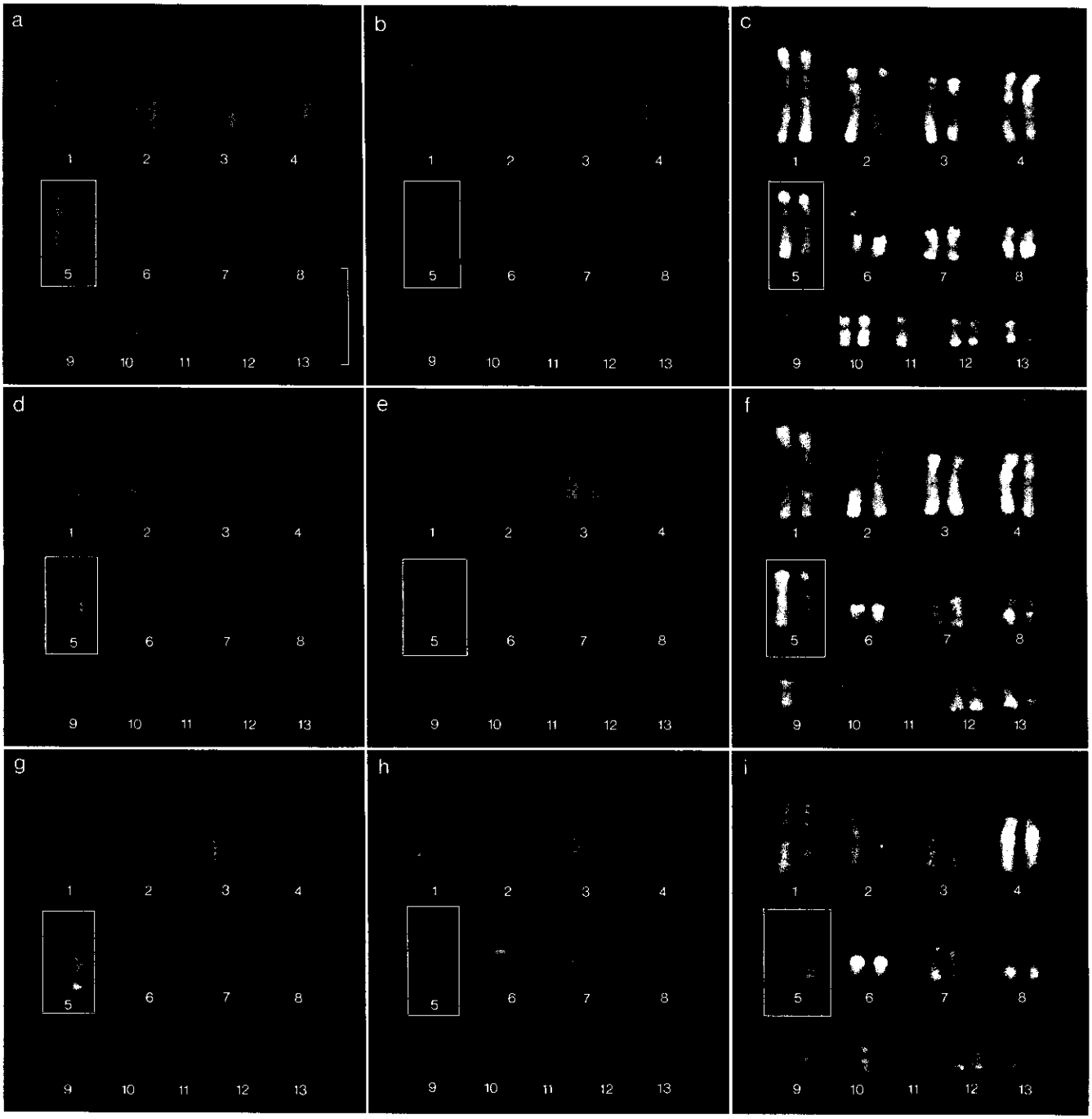

Fig. 2a–i. Karyotypes of female (**a–c**), type A male (**d–f**), and type B male (**g–i**) *Gastrotheca pseustes* stained with quinacrine mustard (**a, d, g**), 4′-6-diamidino-2-phenylindole (DAPI) (**b, e, h**), and distamycin A/mithramycin (**c, f, i**). The sex chromosome pairs 5 are *boxed*. Note the heteromorphic XY^B chromosome pairs in (**g–i**). The telomeric heterochromatin in the Y^B long arm is quinacrine and DAPI positive, but mithramycin negative. Bar in **a** represents 10 μm

riobambae karyotype have been described previously (Schmid et al. 1983, 1986; Schmid and de Almeida 1988). All 34 females and 26 males of *G. pseustes* exhibited 26 biarmed chromosomes which can be arranged in 13 pairs (Fig. 1a, d, g). Pairs 1–5 are distinctly longer than pairs 6–13. Pairs 1 and 5 are nearly metacentric; pairs 2–4 have a submetacentric morphology. The smaller pairs 6–13 are metacentric to submetacentric. A distinct secondary constriction is located in the pericentromeric region of the long arms of chromosome pair 6 in both sexes (Fig. 1a, d, g).

In all 34 females and in 11 of the 26 males (type A males) chromosomes 5 have a homomorphic appearance (Fig. 1a, d). However, in the remaining 15 male specimens (type B males), chromosomes 5 are heteromorphic (Fig. 1g). One of these chromosomes has the same size and nearly metacentric morphology as the chromosomes 5 of the females and the type A males

(compare left homolog 5 in Fig. 1g with chromosomes 5 in Fig. 1a, d). The other chromosome 5 shows a distinctly larger long arm and is therefore submetacentric (right homolog in Fig. 1g). The fact that this heteromorphism of pair 5 is found in about 57% of the males and in none of the females indicates that (1) the XY♂/XX♀ sex determining mechanism operates in *G. pseustes* and (2) apparently two morphs of Y chromosomes (referred to as Y^A and Y^B) coexist in this species.

Banding analyses

The C-banded karyotype of *G. pseustes* exhibits a large number of constitutively heterochromatic regions (Fig. 1b, e, h). All 13 chromosome pairs can be identified unequivocally by their characteristic C-band patterns. Constitutive heterochromatin is present in all centromeric and telomeric segments. Furthermore, interstitial and/or pericentromeric C-bands can be recognized in most chromosomes. A diagrammatic representation of the maximum number of heterochromatic regions determined in mid-metaphase *G. pseustes* chromosomes and the C-band designations is shown in Fig. 4.

With regard to the sex chromosome pair 5, the homomorphic XX pairs of the females (Fig. 1b) display the same C-band patterns as the homomorphic XY^A pairs of the type A males (Fig. 1e). The heteromorphism in the XY^B pairs of the type B males can be traced to a prominent telomeric C-band in the Y^B long arm (Fig. 1h). In Fig. 3a, c, e the C-banded XX, XY^A and XY^B chromosome pairs 5 of ten different females, type A males, and type B males are arranged. It is apparent that no consistent structural differences between the X and Y^A chromosomes (Fig. 3c) can be demonstrated with this banding technique. Although minor size differences between the homologous C-bands located interstitially in the middle of the short arms of a few XY^A pairs can be recognized, these heterochromatin variants also exist in chromosome pairs 5 of females and type B males (see below). The telomeric C-bands in the Y^B long arms appear to be the same size in all type B males (Fig. 3e). They have a homogeneous structure and are terminally added to the long arm, which explains the unequal lengths of X and Y^B.

Silver staining of the *G. pseustes* chromosomes showed that the NOR is located within the secondary (nucleolar) constriction in the long arms of chromosome pairs 6 (Fig. 1c, f, i). Following quinacrine staining, the NOR and most of the heterochromatic bands fluoresce distinctly weaker than the euchromatic chromosome segments (Figs. 2a, d, 3b, d). Only the telomeric heterochromatin the Y^B long arm of type B males is distinguished by brighter fluorescence that the remaining chromatin (Figs. 2g, 3f). Nearly identical banding patterns are induced by staining the metaphases with DAPI (Fig. 2b, e, h). However, in contrast to quinacrine fluorescence, which labels the long arms of the X, Y^A, and Y^B chromosomes uniformly (Figs. 2a, d, g, 3b, d, f), a heterochromatic band with clearly reduced fluorescence intensity appears in the proximal third of these chromosome arms after DAPI staining (Fig. 2b, e, h). This category of DAPI-negative heterochromatin is very special, because there is only a hint of it in a few metaphases after C-banding (Figs. 1b, e, h, 3a, c, e). As with quinacrine banding, the telomeric heterochromatin in the Y^B long arm displays enhanced fluorescence after DAPI staining (Fig. 2h).

After distamycin A/mithramycin counterstaining, the NORs in the long arms of chromosomes 6 display the brightest labeling (Fig. 2c, f, i). Positive mithramycin staining is also present in the NOR-associated heterochromatin, in the prominent C-bands of chromosomes 8 and 13, and in the telomeric C-bands of most chromosomes (compare Fig. 1b, e, h with Fig. 2c, f, i).

As expected from the complementarity of quinacrine and mithramycin banding (Schweizer 1976; Schmid

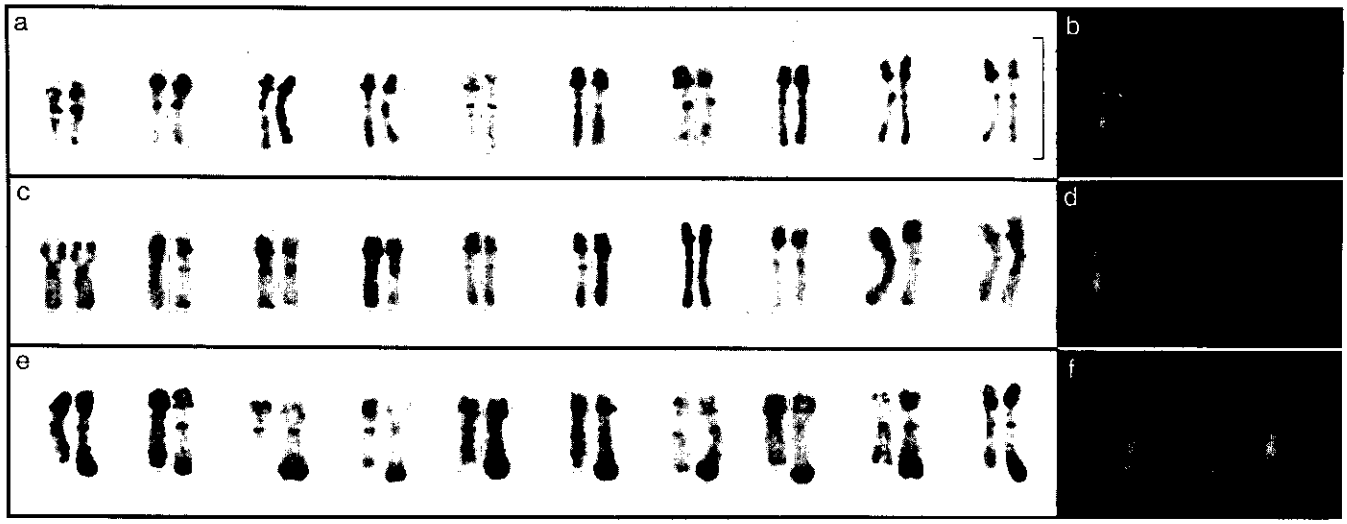

Fig. 3a–f. Selected sex chromosome pairs 5 of 13 females (**a, b**), type A males (**c, d**), and type B males (**e, f**) of *Gastrotheca pseustes* after C-banding (**a, c, e**) and quinacrine mustard staining (**b, d, f**). All the XX pairs of the females and the XY^A pairs of the type A males are homomorphic (**a–d**), whereas the XY^B pairs of all type B males are distinctly heteromorphic (**e, f**). Bar in **a** represents 10 μm

1980), the quinacrine-positive telomeric heterochromatin in the Y^B long arm is mithramycin negative (Fig. 2i). However, unexpectedly the conspicuous quinacrine-negative heterochromatin located interstitially in the middle of the short arms of chromosomes 1, 2, 5, and 6 (Fig. 2a, d, g) also does not react with mithramycin (Fig. 2c, f, i). As yet there is not satisfactory explanation for this peculiar staining property. It is feasible that in this type of heterochromatin both quinacrine binding AT-rich DNA sequences and mithramycin binding GC-rich DNA sequences are interspersed with each other in such an order that none of the base-pair specific fluorochromes reveals differential banding (Schmid 1980).

Lengths of sex chromosomes in mitotic metaphases

The length measurements confirm that the Y^B chromosome is distinctly larger than the X chromosome (Table 2). With a length of 5.97 μm, the Y^B is in fact the second largest chromosome in *G. pseustes*. In contrast, the X chromosome is only 5.04 μm. The relative lengths of the sex chromosomes of *G. pseustes* and *G. riobambae* are almost identical. Thus, the Y^B chromosome of *G. pseustes* constitutes 10.69% of the haploid male karyotype (Table 2) and that of *G. riobambae* 11.5% (Schmid et al. 1983). The relative lengths of the X chromosomes of *G. pseustes* and *G. riobambae* are 9.03% and 9.13%, respectively. It is conceivable that these similar values reflect an evolutionarily conserved, ancestral homoeology of the XY chromosomes in recent *Gastrotheca*.

Male meiotic chromosomes

As is characteristic for male meiosis of almost all species belonging to the more highly evolved anuran families (Morescalchi 1973), the diakinetic bivalents of *G. pseustes* are also strongly contracted. Their ring-like configuration is caused by the telomerically located chiasmata (Fig. 5).

In type A males, the homomorphic XY^A chromosomes exhibit the same typical ring-like pairing configuration as the autosomal bivalents (Fig. 5a, b). However, in all type B males the XY^B chromosomes are distinguished by an end-to-end association (Fig. 5c–g) like that occurring in the male meiosis of other amphibian species with heteromorphic XY chromosomes (for review see Schmid et al. 1990). C-banding reveals that the homomorphic short arms of the X and Y^B, identified by their dark telomeric heterochromatin, achieve this end-to-end association (Fig. 5c–g). The heteromorphic XY^B long arms always remain separated. In 1 of the 200 diakineses examined, even end-to-end association between the XY^B chromosomes was disrupted (Fig. 5h).

Variability of constitutive heterochromatin and nucleolus organizers

Of all species previously covered in this series on chromosome banding in Amphibia, *G. pseustes* shows the highest incidence of polymorphic heterochromatic regions. To simplify the description of these variants, the C-band nomenclature shown in Fig. 4 is used. Extreme variability occurs in C-bands 4p2 (Fig. 6f, h), 6p1 (Fig. 6k), 7p1 (Fig. 6b, h, m), 8q1 (Fig. 6d, f), 10p1 (Fig. 6i) and 13p1 (Fig. 6m). The size increases of C-bands 4p2, 7p1, and 8q1 can be so great that heteromorphism between the homologous chromosomes is visible even after conventional staining (Fig. 6a, c, e, g).

The polymorphic region 8q1 is particularly remarkable. In many females, type A males and type B males, this heterochromatic region is twice as large in one of the no. 8 homologs than in the other (Fig. 7a, b, e–g). After C-banding, the amplified heterochromatin appears uniformly darkly stained (Fig. 7b, f, g). This GC-rich heterochromatin displays quinacrine-negative fluores-

Table 2. Absolute lengths, relative lengths, and arm ratios of *Gastrotheca pseustes* chromosomes[a]

Chromosome	Length (μm)			Percentage of haploid karyotype	Arm ratio[b]
	Short arm	Long arm	Total		
1	3.33±0.21	3.72±0.28	7.05±0.51	12.63	1.12
2	2.27±0.17	3.55±0.25	5.81±0.42	10.41	1.56
3	1.84±0.27	3.69±0.26	5.52±0.53	9.89	2.00
4	1.48±0.15	4.11±0.18	5.59±0.33	10.00	2.78
5(X)	2.21±0.24	2.83±0.19	5.04±0.43	9.03	1.28
5(Y^B)	2.47±0.18	3.50±0.11	5.97±0.32	10.69	1.42
6	1.26±0.20	2.30±0.31	3.56±0.51	6.38	1.83
7	1.52±0.20	1.56±0.24	3.08±0.44	5.52	1.03
8	1.30±0.26	1.48±0.25	2.78±0.51	4.98	1.14
9	1.14±0.14	1.52±0.28	2.66±0.42	4.77	1.33
10	1.08±0.20	1.48±0.13	2.56±0.33	4.59	1.37
11	1.17±0.14	1.12±0.13	2.29±0.27	4.10	0.96
12	0.87±0.15	1.17±0.14	2.04±0.29	3.65	1.35
13	0.89±0.16	1.09±0.17	1.88±0.33	3.37	1.23

[a] The chromosomes of five high-quality C-banded metaphases from the bone marrow of one type B male were used for the measurements
[b] Arm ratio = length of long arm: length of short arm

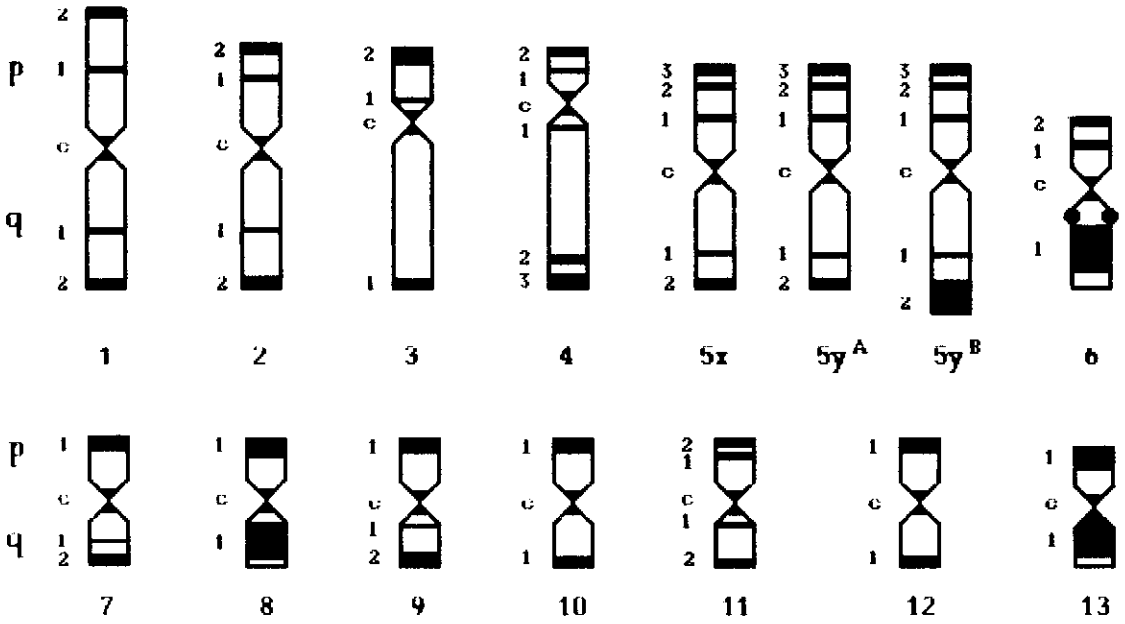

Fig. 4. Diagrammatic representation of the maximum number of C-bands determined in the metaphase chromosomes of *Gastrotheca pseustes*. *White sectors* euchromatin; *black sectors* constitutive heterochromatin; *black circles* nucleolus organizer region. The numbers to the left of the chromosomes refer to the nomenclature for the C-bands used in the text. Further abbreviations are: *c* centromere; *p* short arm; *q* long arm

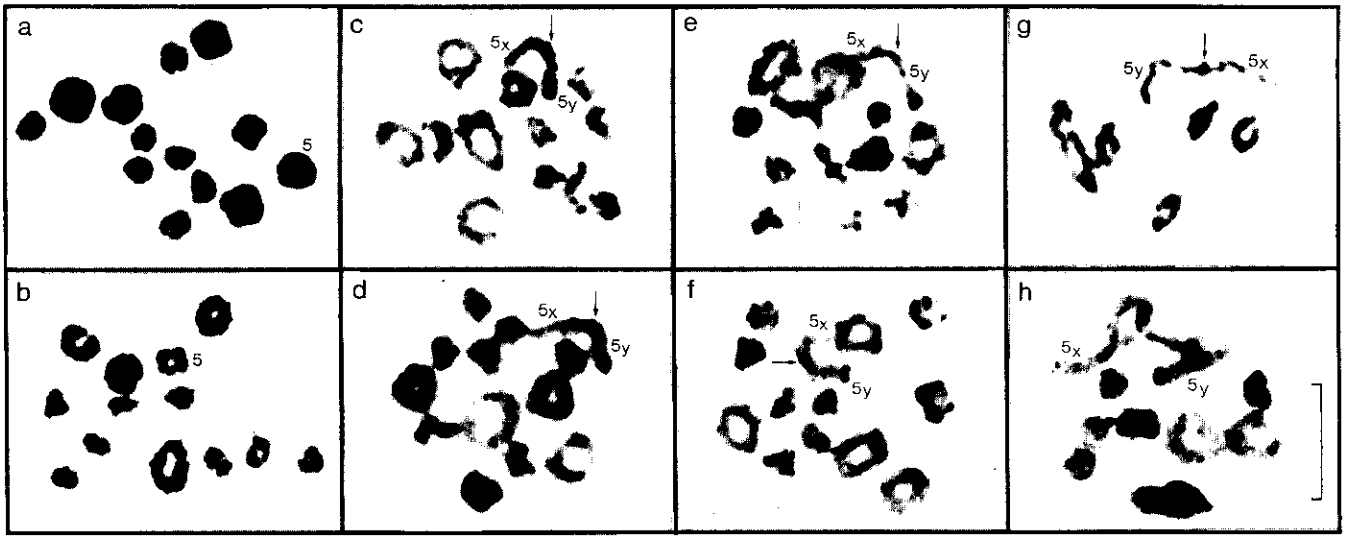

Fig. 5a–h. Diakinetic stages from the meiosis of type A males (**a, b**) and type B males (**c–h**) of *Gastrotheca pseustes*. **a, b** Conventional Giemsa staining; all bivalents of the type A males exhibit a ring-like configuration. The XY^A bivalent 5 was differentiated from the autosomal pairs by its relative size. **c–h** C-banding; the autosomal bivalents of the type B males show the characteristic ring-like arrangement whereas the heteromorphic XY^B chromosomes have a conspicuous end-to-end association, their heterormorphic long arms frequently pointing in opposite directions. The *arrows* indicate the end-to-end associated XY^B short arms. In **h** the unique diakinesis found with completely separated X and Y^B chromosomes is depicted. Bar in **h** represents 10 μm

cence (Fig. 7c) and labels brightly with mithramycin (Fig.7d). Furthermore, quinacrine banding reveals an alternating sequence of two large dark and two smaller, bright bands (Fig. 7h). Correspondingly, in mithramycin-stained preparations, two large bright bands alternate with two small dark bands (Fig.7i). This arrangement can best be explained by assuming a duplication of the complete long arm of chromosome no. 8.

It should be pointed out that all of the heterochromatin variants described above occur in male as well as in female karyotypes. This is in clear contrast to the large telomeric C-band in the Y^B long arm (band 5q2), which is restricted to type B males. A further difference is that the heteromorphic XY^B arm never participate in meiotic pairing, whereas heteromorphisms between homologous autosomes caused by heterochromatin vari-

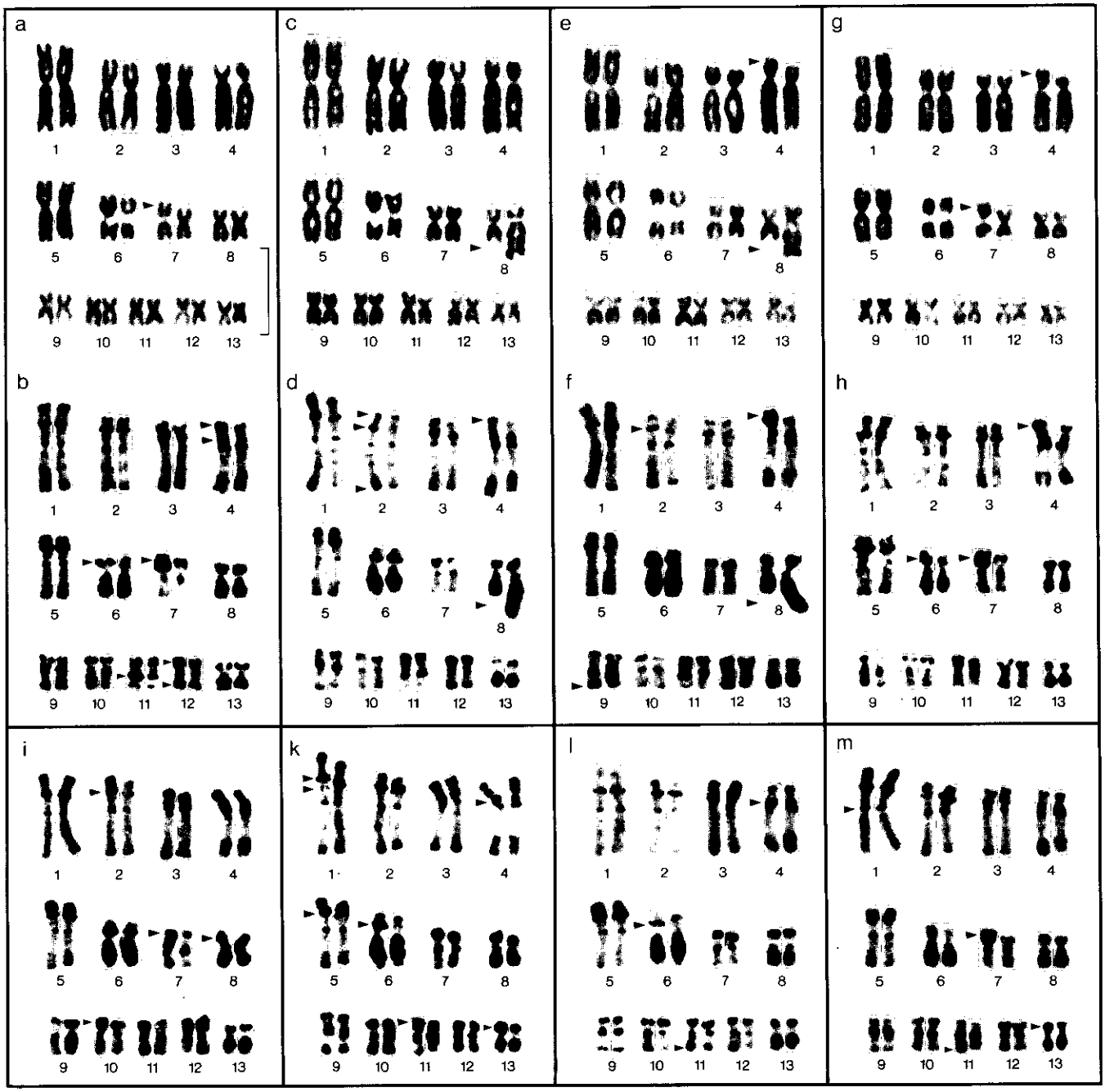

Fig. 6a–m. Karyotypes of seven female individuals of *Gastrotheca pseustes* after conventional Giemsa staining (**a, c, e, g**) and C-banding (**b, d, f, h, i–m**) illustrating the high variability of heterochromatic regions (*arrowheads*). One Giemsa-stainind and one C-banded karyotype is shown for each of the first four females (**a–h**). Note that several of the C-band heteromorphism between the homologous chromosomes can also be recognized as considerable size differences between the conventionally stained homologs (*arrowheads*). Bar in **a** represents 10 μm

ation apparently do not interfere with pairing and terminal chiasmata formation (Fig.5c–h).

In 40 *G. pseustes*, the NORs in chromosome pair 6 are highly heteromorphic. In 25 of the 40 animals with unequal-sized NORs, the heteromorphism is due to a tandem duplication in one of the two NORs (Fig. 7k–p). In such NORs two silver blocks, which often fuse with each other, are located in both chromatids within the nucleolar constriction (Fig. 7l, o). After distamycin A/mithramycin banding, the duplicated NORs can be identified by their double fluorescing signals, whereas quinacrine staining results in strongly reduced fluorescence (Fig. 7m). In conventionally stained preparations, it is possible to demonstrate a small, dense chromatin region within the nucleolar constriction dividing the latter into two halves (Fig.7k). This becomes more evident when the photographs are overexposed (Fig. 7n).

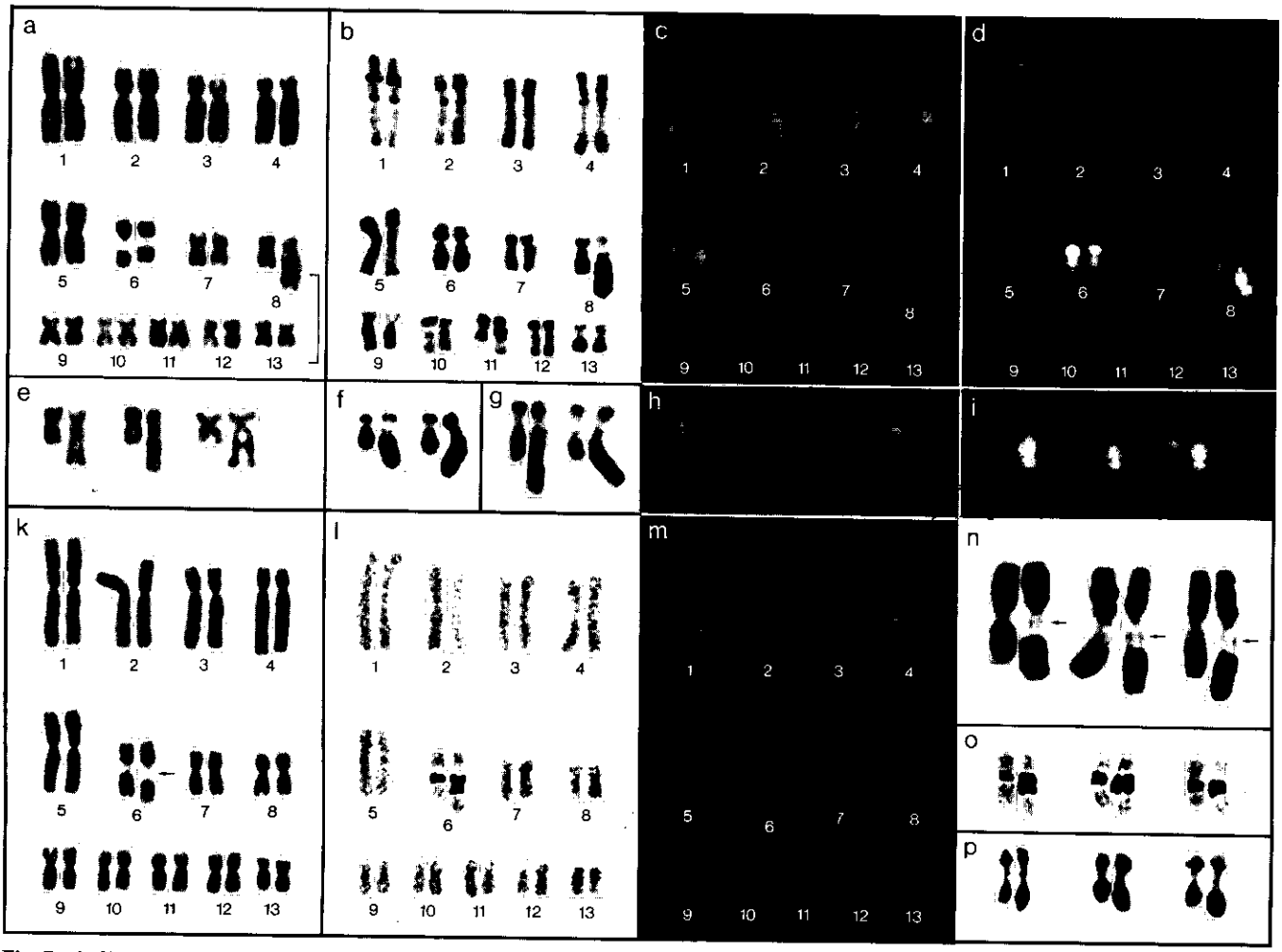

Fig. 7a–i. Karyotypes and selected chromosome 8 pairs from a female of *Gastrotheca pseustes* with a tandem duplication of the long arm in one the no. 8 homologs. **a, e** Giemsa staining; **b, f, g** C-bands; **c, h** quinacrine staining; **d, i** distamycin A/mithramycin. **k–p** Karyotypes and selected chromosome 6 pairs of another female individual of *G. pseustes* showing a tandem duplication of the nucleolus organizer region in one of the no. 6 homologs. **k, n** Giemsa staining; **l, o** silver staining; **m** quinacrine staining; **p** C-banding. The chromosome pairs in **n** are from photographic overexposures in order to demonstrate the small, dense chromatin region within the achromatic nucleolar constriction which divides the nucleolus organizer into two equal halves (*arrows*); this dense chromatin region can hardly be distinguished in karyotypes obtained from prints with automatic exposure **k**. Bar in **a** represents 10 μm

Discussion

Phylogenetic relationships

It is known that superficially similar or indistinguishable populations of certain anuran species actually represent more than one taxon. Cryptic species have been discriminated on the basis of advertisement calls (e.g. *Hyla chrysoscelis* and *H. versicolor*; Johnson 1959), chromosomes (e.g. *Odontophrynus, Xenopus*; Beçak et al. 1970; Tymowska and Fischberg 1973), allozymes, albumin evolution and osteological features (e.g. *Gastrotheca*; Duellman and Hillis 1987; Duellman et al. 1988; Duellman and Trueb 1988). Scanlan et al. (1980) first reported what appeared to be a cryptic species within individuals identified as *G. riobambae*. Subsequent morphological comparisons, allozymic analysis, and immunological studies on albumin evolution convincingly demonstrated that *G. "riobambae"* was a composite of several species. These included the true *G. riobambae*, as well as *G. espeletia, G. litonedis, G. ruizi*, and *G. pseustes* (Duellman and Burrowes 1986; Duellman and Hillis 1987; Duellman et al. 1988). With the exception of *G. pseustes*, all of these species belong to the *G. plumbea* group. However, *G. pseustes*, which has been universally confused with *G. riobambae* because of their extreme phenotypic similarities, is clearly a member of the *G. marsupiata* group. In the Andes of Ecuador, *G. pseustes* is the northernmost species of the *G. marsupiata* group, and the only member of this species group living to the north of the Huancabamba Depression, which is the southern boundary of the *G. plumbea* group. *G. pseustes* is broadly parapatric with *G. riobambae* of the *G. plumbea* group. In the northern part of its range, *G. pseustes* occurs at elevations in excess of 3,000 m, whereas *G. riobambae* in confined to intermontane basins and the Andean

slopes, usually at elevations less then 3,000 m (Duellman and Hillis 1987). The results obtained by the present approach confirm that *G. pseustes* is a separate species and again demonstrate the value of cytogenetics in amphibian systematics.

Sex chromosomes

The fact that the conspicuous Y^B chromosome of *G. pseustes* is restricted to about half of the male individuals (type B males), whereas chromosome pair no. 5 in the females is homomorphic proves that these are sex chromosomes of the XY♂/XX♀ type. Although *G. pseustes* shares the XY♂/XX♀ system of sex determination with the other *Gastrotheca* species, it is the first amphibian species in which two morphs of Y chromosomes have been demonstrated (for review see Schmid et al. 1990). A comparable case is known only for the ZW♀/ZZ♂ type of the North American plethodontid salamander *Aneides ferreus*. Extensive cytogenetic studies have revealed that in the populations of northern California two different morphs of the W chromosome, telocentric or metacentric, coexist (Sessions and Kezer 1987).

Both the Y^A and Y^B chromosomes of *G. pseustes* are in a primitive stage of morphological differentiation. The Y^A is even indistinguishable from the X regardless of the banding techniques used. The only difference recognized between Y^A and Y^B is the amplified telomeric heterochromatin in the Y^B long arm. It is extremely unlikely that this prominent C-band in the Y^B is merely a size variant of autosomal heterochromatin like those found in most of the other C-bands in the *G. pseustes* karyotype, instead of genuine sex-specific heterochromatin. The reasons are that: (1) none of the 34 females examined shows any size differences between the homologous C-bands in the telomeric regions of chromosome pair no. 5; (2) in contrast to most of the other polymorphic heterochromatic regions, the telomeric C-band in the Y^B long arm is the same size in all type B males; (3) the Y^B-specific heterochromatin differs from all other heterochromatic sites by its quinacrine-bright fluorescence.

It might be argued that the type A males of *G. pseustes* are really not genetic males with homomorphic XY^A chromosomes at all, but rather sex-reversed females with conventional XX chromosomes. This argument is contradicted by several observations. All 11 type A males had perfectly developed testes with normal meiotic stages and mature sperm cells, but without remnants of ovarian cortices. All animals were collected as adults in the field; such a high rate of spontaneous sex-reversals under natural conditions in 11 of 45 females (24%) has never been reported for Amphibia. Furthermore, experimental sex-reversal in Hylidae achieved by administration of steroid hormones has shown that reversal of genetic males into females using estradiol occurs easily; however, sex-reversal of genetic females into males with testosterone meets resistance (Kawamura and Nishioka 1977). Finally, the result of mating these potential sex-reversed (XX) females of *G. pseustes* with the normal (XX) females should produce female offspring exclusively; such all-female progenies have never been observed in this marsupial frog (unpublished data).

Comparative molecular and cytogenetic studies on the ZW♀/ZZ♂ sex chromosomes of reptiles have shown that the primary step in the evolution of heteromorphic sex chromosomes was the accumulation of specific repetitive DNA sequences (heterochromatinization) in the W chromosome (Singh et al. 1976, 1979, 1980). This means that molecular changes in the DNA sequences of the ancestral, primitive W chromosomes preceded their actual morphological differentiation, such as inversions, deletions or duplications. Observations on some of the sex chromosomes found so far in Amphibia also suggest that progressive heterochromatinization of the W and Y was at least one of the evolutionary channels by which the differentiation of heteromorphic sex chromosomes was initiated. For example, the XY chromosomes in the males of several salamander species of the genus *Triturus* still have the same lengths and centromere positions. However, the long arm telomeres of the Y chromosomes already contain a heterochromatic region lacking in the X chromosomes (Schmid et al. 1979). The Y^A and Y^B chromosomes detected in *G. pseustes* impressively illustrate two successive stages of Y chromosome differentiation caused by accumulation of heterochromatin. No similar cases have been found among other Amphibia, or among any other lower vertebrates. No statement can be made on whether these two morphs of Y chromosomes coexist within the same population of *G. pseustes*, or whether they only occur in geographically separate populations. This would require exact data on the geographic sites in which the individual animals were obtained, and these were unfortunately not available for the specimens investigated. In any case, *G. pseustes* confirms the conclusion previously reached that marsupial frogs are especially suitable for the study of evolutionary series in which the increasing structural complexity of the sex chromosomes can be reconstructed.

Male meiosis and sex bivalents

Comparative studies have shown that in most of the more highly evolved anuran families, including the Hylidae, the diakinetic bivalents of male meiosis exhibit a strongly spiralized, ring-like appearance (Morescalchi 1971, 1973). The characteristic ring-shaped configuration of the autosomal bivalents is produced by the presence of one chiasma at each end of the bivalents and by the lack of interstitial chiasmata. This also holds true for the male meiosis of *G. pseustes* and of the other species of *Gastrotheca* previously investigated (Schmid et al. 1983, 1988).

The genetic homology existing between a pair of sex chromosomes is also expressed by the extent of synapsis in the sex bivalents. The more structural differences there are between the sex chromosomes, the less is their contact during meiosis. In *G. riobambae*, homology between the highly heteromorphic XY chromosomes is almost completely lost. In diakinesis, long, stretched sex bivalents are found, like those occurring in the male meiosis of mammals. The sex bivalent consists of end-to-end paired XY chromosomes (Schmid et al. 1983). The present study has shown that, as expected, the homomorphic XY^A chromosomes pair like the autosomes in the type

A males of *G. pseustes*. In contrast, in the type B males the heterochromatic band at the long arm telomere of the Y^B chromosomes completely suppresses crossing over between the long arms of the X and Y. This leads to the assumption that the telomeric Y^B-specific heterochromatin has an inhibitory effect on synapsis and/or crossing over in the XY^B long arms. However, it cannot be excluded that there are further structural differences in the XY^B long arms which are not detectable cytologically. In either case, the pairing failure betwen the long arms of the XY^B chromosomes is a promoting factor for further differentiation toward highly heteromorphic sex chromosomes. The XY^B short arms still pair in the same way as the autosomes. It may be concluded from this that the Y^B-specific heterochromatin does not influence synapsis and crossing over beyond the centromeres. An identical situation has been found in the male meiosis of *Triturus* species that have a small heterochromatic region at the telomere of the Y long arm (Schmid et al. 1979).

In contrast to the XY^B pair, the very frequent heteromorphisms between homologous autosomal C-bands of *G. pseustes* do not produce achiasmatic bivalents in the male meiosis. Neither the considerable size diifferences of interstitial nor of terminal C-bands seem to interfere with the formation of the typical telomeric chiasmata in the ring-shaped autosomal bivalents. This is a further indication that the Y^B heterochromatin does not constitute a simple amplification of heterochromatin, but that it consists of novel DNA sequences not present in the X chromosome. Molecular techniques will need to be used in order to obtain exact information on the structure of the interesting sex chromosomes of these marsupial frogs.

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CHROMOSOMA

Evolutionary changes in the organization of the major LCP gene cluster during sex chromosomal differentiation in the sibling species *Drosophila persimilis, D. pseudoobscura* and *D. miranda*

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Abstract. The major larval cuticle protein (LCP) genes I–IV of *Drosophila melanogaster* are clustered on the right arm of the second chromosome. By cross-hybridization we cloned the corresponding genes from three different members of the *obscura* group: *D. persimilis*, *D. pseudoobscura* and *D. miranda*. In *D. pseudoobscura* and *D. persimilis* the gene cluster maps to autosome *3*. In contrast, in *D. miranda* it was found on the *X2* and *Y* sex chromosome. Hence, this exceptional karyotypic situation offers a unique opportunity to analyse the molecular processes underlying the phenomenon of chromosome degeneration. Comparison of LCP genes I–IV in the *X2* and *Y* chromosomal region in *D. miranda* revealed extensive DNA rearrangements at the latter. The *Y* chromosomal LCP cluster is characterized by DNA insertions which are absent in the corresponding *X2* chromosomal DNA, suggesting that these DNA sequences must have invaded this area. In addition, part of the analysed *Y* chromosomal region is duplicated.

Introduction

The major larval cuticle protein (LCP) genes I–IV of *Drosophila melanogaster* are clustered in a segment of about 8 kb at 44D on the right arm of chromosome *2* (Snyder et al. 1981). They represent a set of coordinately expressed genes which are synthesized and secreted by the epidermal cells of late third instar larvae (Fristrom et al. 1978). Based on chromosomal homologies (Patterson and Stone 1952; Steinemann 1982b; Steinemann et al. 1984) the right arm of chromosome *2* of *D. melanogaster* is homologous to chromosome *3* in *D. pseudoobscura* and *D. persimilis*, two sibling species from the *obscura* group. According to the terminology of chromosome elements (Muller 1940; Sturtevant and Novitsky 1941) these chromosomal arms correspond to chromosome element *C* (see Fig. 1).

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As a result of chromosomal rearrangements in the third member of this sibling species group, *D. miranda*, the males of this species show only nine chromosomes while females have ten chromosomes per diploid chromosomal set. This intriguing chromosomal constitution in *D. miranda* males results from the translocation of chromosome element *C* to the *Y* chromosome (MacKnight 1939; Steinemann 1982a, 1984). As a consequence of this chromosomal rearrangement a metacentric neo-*Y* chromosome and a monosome, designated the *X2* chromosome, characterize the *D. miranda* karyotype. This sex chromosome pair in *D. miranda* is homologous to the *2R* chromosome of *D. melanogaster* (Fig. 1). Hence, LCP genes I–IV should be located on the X2 and Y chromosome.

Both the *X2* and *Y* chromosome reveal the characteristic cytological features of sex chromosomes. In the polytenized male karyotype the *X2* chromosome shows the characteristic puffy appearance and pale staining behaviour of *X* chromosomes. In parallel the polytenized *Y* chromosome has adapted to a more heteropycnotic morphology, indicating that it is in the process of degeneration (Muller 1918, 1932). From uridine incorporation studies it has been concluded that the *X2* chromosome is partially dosage compensated (Strobel et al. 1978). This suggests that at least some of the genes on the *D. miranda Y* chromosome are inactive.

According to the hypothesis of Muller (1928, 1932) the differentiation of the sex chromosomes is due to the successive degeneration of the *Y* chromosome as a consequence of its permanent heterozygosity. Thus, degeneration is thought to be due to mutations of genes to recessive or completely inactive alleles. Such mutations on the *Y* are effectively neutral because they can never become homozygous. Alternative genetic models for the evolution of reduced genetic activity along the *Y* chromosome are based on the principle of Muller's ratchet (Charlesworth 1978) and on the process of genetic hitchhiking (Rice 1987). The accumulation of nonfunctional genes on the *Y* chromosome, leading eventually to a genetically inert and structurally heteropycnotic