

Chromosome Banding in Amphibia. XXXI. The Neotropical Anuran Families Centrolenidae and Allophrynidae

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Key Words

Allophrynidae · Anura · Centrolenidae · Chromosome evolution · Sex chromosomes

Abstract

The mitotic chromosomes of 11 species from the anuran families Centrolenidae and Allophrynidae were analyzed by means of conventional staining, banding techniques, and in situ hybridization. The amount, location, and fluorochrome affinities of constitutive heterochromatin, the number and positions of nucleolus organizer regions, and the patterns of telomeric DNA sequences were determined for most of the species. The karyotypes were found to be highly conserved with a low diploid chromosome number of $2n = 20$ and morphologically similar chromosomes. The sister group relationship between the Centrolenidae and Allophrynidae (unranked taxon Allocentroleniidae) is clearly corroborated by the cytogenetic data. The existence of heteromorphic $XY\sigma/XX\varphi$ sex chromosomes in an initial stage of morphological differentiation was confirmed in *Vitreorana antisthenesi*. The genome sizes of 4 centrolenid species were determined using flow cytometry. For completeness and for comparative purposes, all previously published cytogenetic data on centrolenids are included.

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During the pre-banding era of cytogenetics, very few studies included tropical glass frogs (family Centrolenidae). These were confined to 4 species (*Cochranella granulosa*, *Espadarana prosoblepon*, *Hyalinobatrachium fleischmanni*, and *Sachatamia albomaculata*) and were carried out using classical squash techniques and uniformly stained chromosomes in the diakinesis stage of male meiosis (table 1). The early studies did demonstrate the existence of a strikingly low chromosome number ($n = 10$, $2n = 20$) in centrolenids. A single further study on somatic tissues of *Vitreorana eurygnatha* revealed that all 10 chromosomes have a biarmed (metacentric or submetacentric) morphology (table 1). No cytogenetic studies have previously been published for the sister taxon of the Centrolenidae, the family Allophrynidae.

Banding analyses were only applied to 2 males and 2 females of the Venezuelan centrolenid species *Vitreorana antisthenesi* which revealed $XY\sigma/XX\varphi$ sex chromosomes in an initial stage of morphological differentiation and unusually large amounts of brightly labeled, AT-rich constitutive heterochromatin in the centromeric and pericentromeric regions of all autosomes and in the X chromosome [Schmid et al., 1989].

A new phylogenetic taxonomy, based on molecular, morphological, anatomical, behavioral, and biogeo-

graphical data, has been proposed for the Centrolenidae [Guayasamin et al., 2008, 2009]. These studies recognized the evolutionary proximity of the families Centrolenidae and Allophrynidae and combined both into the unranked taxon Allocentroleniidae. The family Centrolenidae includes the subfamilies Centroleninae and Hyalinobatrachinae and comprises 11 genera: *Centrolene* (34 species), *Chimerella* (1 species), *Cochranella* (24 species), *Espadarana* (3 species), *Nymphargus* (30 species), *Rulyrana* (8 species), *Sachatamia* (3 species), *Teratohyla* (4 species), *Vitreorana* (8 species), *Celsiella* (2 species), and *Hyalinobatrachium* (27 species). The genus *Ikakogi* (1 species) was described by Guayasamin et al. [2009] and, because it could not be assigned with confidence to either subfamily, was included as incertae sedis in the Centrolenidae. The family Allophrynidae contains the single genus *Allophryne* (3 species). Two of these species were only recently described [Castroviejo-Fisher et al., 2012; Caramaschi et al., 2013].

Glass frogs are distributed throughout the Neotropics, from Mexico to Bolivia, including the Caribbean island of Tobago, with highest species diversity in the northern Andes (Colombia and Ecuador), and with a large hiatus in the lower Amazon basin. An isolated group of species occurs in southeastern Brazil and northeastern Argentina [Duellman, 1977; Cannatella and Duellman, 1982; Guayasamin et al., 2009]. They live mainly in cloud and rain forests and are nocturnal and arboreal. Females deposit egg clutches on the underside of leaves, or on rocks, above running water. After hatching, the tadpoles fall into the water to complete their development. Some species, however, are also known to occasionally breed in ponds. As a further diagnostic character, the skin and flesh of the ventral surfaces are partially or completely transparent owing to the scarcity of pigments. This allows the viscera to be observed through the abdominal wall [Duellman and Burrowes, 1989; Ruiz-Carranza and Lynch, 1991; Duellman and Trueb, 1994; Cisneros-Heredia and McDiarmid, 2007; Kubicki, 2007; Guayasamin et al., 2009]. As pointed out by Guayasamin et al. [2009], glass frogs represent one of the most interesting neotropical anuran groups because of their morphological and ecological characteristics, as well as their phylogenetic and biogeographic complexity.

The 3 described species of the family Allophrynidae occur from the Guiana region of South America (Venezuela, Guyana, Surinam, French Guiana, northern central Brazil) to central Brazil, northeastern Amazonian Peru and the Atlantic rain forest of eastern Brazil [Langone and Se-galla, 1997; Caldwell and Hoogmoed, 1998; Guayasamin

et al., 2009; Castroviejo-Fisher et al., 2012; Caramaschi et al., 2013]. Allophrynids live in forests and flooded forests. They are arboreal or semi-arboreal and nocturnal.

The present study examines the mitotic chromosomes of 10 species in the family Centrolenidae and 1 species in the family Allophrynidae and provides results for several banding techniques and fluorescence in situ hybridization (FISH) experiments that were applied to the chromosomes of 8 species of Centrolenidae. The amount, chromosomal location and composition of the constitutive heterochromatin, the number and location of nucleolus organizer regions (NORs) as well as the patterns of repetitive telomeric DNA sequences were determined. The existence of heteromorphic XY♂/XX♀ sex chromosomes in an initial stage of morphological differentiation was confirmed for *V. antisthenesi*. The genome sizes of 4 centrolenid species were determined using DNA flow cytometry. For completeness and for comparative purposes, all previously published cytogenetic data on centrolenids are included and re-evaluated.

Materials and Methods

Animals

Individuals representing 11 species belonging to the genera *Cochranella* (*C. granulosa*), *Espadarana* (*E. prosoblepon*), *Hyalinobatrachium* (*H. colymbiphyllum*, *H. duranti*, *H. fleischmanni*, *H. fragile*, *H. orientale*, *H. valerioi*), *Vitreorana* (*V. antisthenesi*, *V. eurygnatha*) (family Centrolenidae), and *Allophryne* (*A. ruthveni*) (family Allophrynidae) were collected during various expeditions to Costa Rica, Venezuela, Brazil and the island of Tobago (table 1). The chromosomes of the Venezuelan species were prepared in a temporary cytogenetic laboratory in the field station Estación Biológica de Rancho Grande located in the Henri Pittier National Park, Aragua State. The Costa Rican specimens were prepared in a temporary cytogenetic laboratory in the Departamento de Biología, University of Costa Rica, San José. Tissue obtained for chromosomes was transferred to 1.8-ml plastic tubes (Nunc), stored at 4°C or -20°C and transported to the laboratory in Würzburg (Germany), either packed in dry ice or at room temperature. Frogs collected in Tobago were carried alive to Würzburg where their chromosomes were prepared. Chromosomes of the specimens sampled in Brazil were prepared in the field. All procedures with the living animals strictly conformed to the guidelines established by the Animal Care Committees of the respective countries.

Chromosome Preparations

Mitotic chromosomes were prepared directly from the bone marrow, intestines or the cornea of the eye after in vivo colchicine treatment. Meiotic chromosomes were obtained from testes. Detailed techniques used for squash preparations (cornea) and cell suspensions (bone marrow, intestine, testes), as well as the hypotonic treatment and fixation of the cells have been previously described [Schmid et al., 2010].

Table 1. Species from the families Centrolenidae and Allophrynidae cytogenetically examined in the present study and in previous reports

Species ^a	Country	Specimens ^b	Locality ^c	Geographical coordinates ^d	2n/FN ^e
Family Centrolenidae, subfamily Centroleninae					
<i>Cochranella granulosa</i> *					
Present study	Costa Rica	1♂ 1♂	Carara National Park, Puntarenas Province Río Sucio, Braulio Carrillo National Park, Limón Province	09°44'N/84°37'W 10°09'N/83°58'W	20/40 20/40
Duellman, 1967	Nicaragua	2♂	Finca Tepeyac, 10.5 km N, 9 km E Matagalpa, 960 m, Matagalpa Department	11°58'N/86°05'W	20 [‡] /n.i.
<i>Espadarana prosoblepon</i> *					
Present study	Costa Rica	7♂ 1♀	Alto de la Palma, Limón Province	10°02'N/83°59'W	20/40
León, 1970 & pers. commun.	Costa Rica	n♂	Campus of the University, San José	09°56'N/84°02'W	20 [‡] /n.i.
Duellman, 1967	Panama	1♂	Finca Ojo de Agua, SE slope of Cerro la Pelota, 1440 m, Chiriqui Province	08°54'N/82°42'W	20 [‡] /n.i.
<i>Sachatamia albomaculata</i>					
Duellman, 1967	Panama	1♂	Southern slope of Cerro La Campana, 850 m, Veraguas Province	08°26'N/81°17'W	20 [‡] /n.i.
<i>Vitreorana antisthenesi</i>					
Present study	Venezuela	19♂ 4♀	Henri Pittier National Park, small mountain stream at km 29 on road to Ocumare de la Costa, 650 m, Aragua State	10°24'N/67°45'W	20/40
Schmid et al., 1989	Venezuela	2♂ 2♀	Henri Pittier National Park, small mountain stream at km 29 on road to Ocumare de la Costa, 650 m, Aragua State	10°24'N/67°45'W	20/40 [†]
<i>Vitreorana eurygnatha</i>					
Present study; Bogart, 1973	Brazil	1♂ 1u	Tijuca forest at Bom Retiro, Guanabara, Rio de Janeiro State Teresópolis, Serra dos Órgãos National Park, Rio de Janeiro State	22°53'S/43°13'W 22°22'S/42°45'W	20/40 20/40
Family Centrolenidae, subfamily Hyalinobatrachinae					
<i>Hyalinobatrachium colymbiphylum</i> *					
Present study	Costa Rica	13♂ 3♀ 1♂	Tropical Science Center, Rincón de Osa, 20 m, Puntarenas Province Carara National Park, Puntarenas Province	09°35'N/85°04'W 09°44'N/84°37'W	20/40 20/40
<i>Hyalinobatrachium duranti</i> *					
Present study	Venezuela	2♂	Monte Zerpa, 2200 m, Mérida State	08°37'N/71°10'W	20/40
<i>Hyalinobatrachium fleischmanni</i> *					
Present study	Costa Rica	2♂ 1u	Paraíso, Valle de Orosi, Cartago Province	09°48'N/83°51'W	20/40
León, 1970 & pers. commun.	Costa Rica	n♂	Campus of the University, San José	09°56'N/84°02'W	20 [‡] /n.i.
Duellman and Cole, 1965	Nicaragua	2♂ 1♂	Finca Tepeyac, 10.5 km N, 9 km E Matagalpa, 960 m, Matagalpa Department 16 km S Matagalpa, 660 m, Matagalpa Department	11°58'N/86°05'W 11°55'N/86°05'W	20 [‡] /n.i. 20 [‡] /n.i.
<i>Hyalinobatrachium fragile</i> *					
Present study	Venezuela	1♂ 2u	Henri Pittier National Park, small mountain stream at km 29 on road to Ocumare de la Costa, 650 m, Aragua State	10°24'N/67°45'W	20/40
<i>Hyalinobatrachium orientale</i> *					
Present study	Tobago	3♂ 7u	1.5 km SW Charlotteville	11°19'N/60°32'W	20/40
<i>Hyalinobatrachium valerioi</i> *					
Present study	Costa Rica	1♂	Tropical Science Center, Rincón de Osa, 20 m, Puntarenas Province	09°35'N/85°04'W	20/40
Family Allophrynidae					
<i>Allophryne ruthveni</i> *					
Present study	Brazil	6♂	near A-Ukre on the Rio Xingu, Pará State	07°39'N/51°21'W	20/40

^a Mitotic chromosomes of the species labeled with * are published here for the first time. The identification of *Hyalinobatrachium duranti* is not confirmed. The specimens of *Vitreorana eurygnatha* published by Bogart [1973] are included in the present study.

^b n = Unknown number of male specimens; u = sex unknown.

^c Localities of specimens were obtained from collector's field books, personal communication with the collectors, as well as information from museums where the cytogenetically examined voucher specimens are deposited. m = Meters above sea level.

^d Geographic coordinates were determined either directly in the field using Global Positioning Systems (GPS), or by consulting the 'Directory of Cities and Towns in World' database (<http://www.fallingrain.com/world/>), Google Earth, or are based on extrapolations from geographical maps.

^e n.i. = Not indicated. [‡] Diploid chromosome number inferred from chromosome counts in meiosis. [†] The fundamental number is not explicitly mentioned in the text of the corresponding publication but inferred from the chromosome illustration or text.

Banding Analyses and FISH

Conventional chromosome staining, C-banding, staining with quinacrine mustard, DAPI (4',6-diamidino-2-phenylindole) or Hoechst 33258, fluorescence counterstaining with distamycin A/mithramycin or actinomycin D/DAPI, and labeling of the NORs with AgNO₃ were performed according to the methods in Schmid et al. [2010].

For the detection of the canonical (5'-TTAGGG-3')_n telomeric repeats, a fluorescein-conjugated peptide nucleic acid (PNA) probe (Telomere PNA FISH kit/FITC, K5325; Dako Cytomation, Denmark) was used. Detailed procedures for in situ hybridization and signal detection are provided by the manufacturer.

Microscopic analyses were conducted using Zeiss photomicroscopes III, Zeiss fluorescence microscopes and Zeiss Axiophot microscopes equipped with incident HBO 50W mercury lamp illumination. The various filter combinations necessary for the analyses of metaphases stained with the different fluorochromes or for FISH were described by Schmid et al. [2010].

DNA Flow Cytometry

Blood samples were obtained by cardiac puncture and were immediately fixed in 70% ethanol. The fixed samples were transferred to 1.8-ml plastic (Nunc) tubes and stored at -20°C. Thawed samples were centrifuged (800 rpm, 10 min) in a laboratory centrifuge, and the erythrocyte pellets were resuspended and incubated in 1 ml pepsin solution (0.5% in 0.1 M HCl) at room temperature for 15 min. After the addition of 5 ml staining solution (2 µg/ml DAPI in 0.2 M sodium citrate), the erythrocyte suspensions were kept at room temperature for 3 h [Otto, 1994], and were then mixed with chicken erythrocytes. Flow cytometric analyses were carried out with an epi-illumination flow system (Partec Cell Analyzer CAII) at 365 nm (filter combination KG1/BG38/UG1 for excitation, TK420 as dichromatic mirror, and GG435 as the barrier filter). Several measurements were performed for each sample. The nuclear DNA content of the frogs was calibrated against the known genome size of chicken erythrocytes (2.33 pg DNA/nucleus).

Results

Karyotypes

All 10 centrolenid species analyzed in the present and previous studies, as well as *Allophryne ruthveni* have a conserved diploid chromosome number of 2n = 20 (table 1; figs. 1, 3a). Only male meiotic metaphases, in the stage of diakinesis, were analyzed for *S. albomaculata* [Duellman, 1967], and no more information on this karyotype is available. The other 10 species possess a fundamental number of FN = 40, with 20 bichromosomes (fig. 1). Only a few mitotic metaphases of moderate quality could be obtained for *H. valerioi*, but these were sufficient to evaluate the chromosome morphology (fig. 3). Centrolenid chromosomes have a metacentric or submetacentric morphology (figs. 1, 3a). In most species, chromosome pairs 1–8 decrease slightly in their lengths, with only small size differences between adjacent pairs,

whereas pairs 9 and 10 are distinctly smaller (fig. 1a–h). *H. orientale* is an exception because all 10 chromosome pairs decrease gradually in size (fig. 1i). In the karyotype of *A. ruthveni*, chromosome pairs 1–6 decrease gradually in size and are larger than the pairs 7–9, which share similar lengths. Pair 10 is distinctly smaller (fig. 1j). Distinct nucleolar constrictions are visible in the short arms of chromosomes 2 in *V. antisthenesi* and *H. colymbiphylum*, as well as in chromosomes 1 in *H. durantii* and *A. ruthveni* (fig. 1c, e, f, j). A comparison between the different karyotypes suggests that centrolenid and allophrynid species have maintained a considerable degree of chromosome homeology.

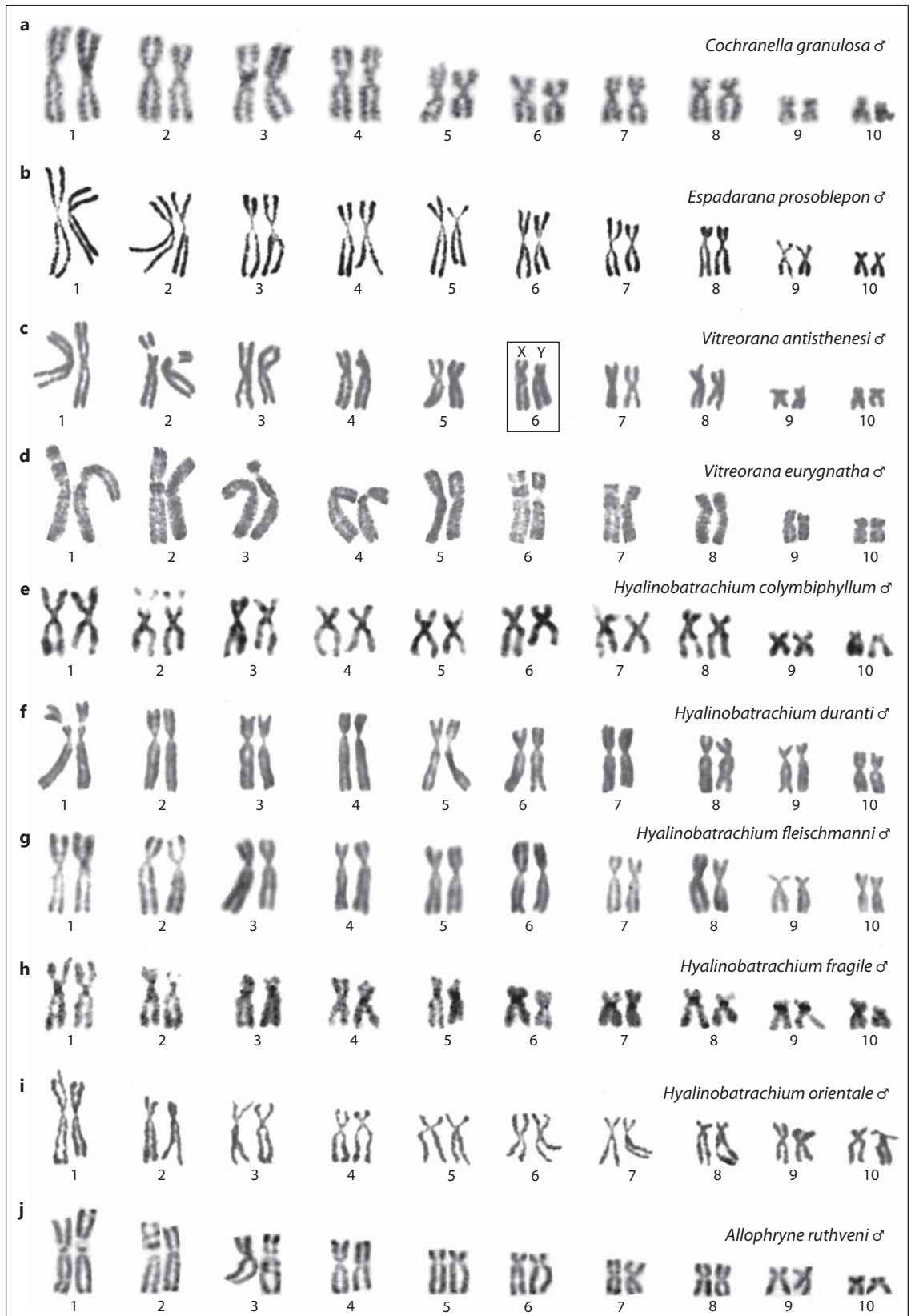
In all 19 male specimens of *V. antisthenesi* examined, chromosome pair 6 was found to be heteromorphic after conventional staining (fig. 1c) and banding analysis (see below). Although both of these chromosomes have about the same length (6.0–6.5 µm), one of them (Y chromosome) is distinctly more submetacentric than the other (X chromosome) in all examined metaphases. The arm ratio (long arm length:short arm length) is approximately 3.5 for the Y chromosome and 2.3 for the X chromosome. In all female individuals, chromosome pair 6 is homomorphic, and both homologs have the same arm ratio of 2.3 as does the X chromosome in the male karyotype. The fact that the heteromorphism of chromosome pair 6 in *V. antisthenesi* is restricted to males demonstrates that the XY♂/XX♀ type of chromosomal sex determination operates in this species. This confirms preliminary results obtained by Schmid et al. [1989] from *V. antisthenesi* that were collected in the same population as the specimens sampled for the present study. There is no evidence for the existence of heteromorphic sex chromosomes in the karyotypes of the other species.

Constitutive Heterochromatin

The patterns of constitutive heterochromatin in centrolenid chromosomes are like those in the karyotypes of most other anurans [Schmid, 1978a, b; Schmid et al., 1987, 2010, 2012]. C-bands are preferentially located in centromeric and pericentromeric regions of the chro-

Fig. 1. Conventionally stained karyotypes of the examined species of Centrolenidae and Allophrynidae. The XY sex chromosomes of *V. antisthenesi* are framed (c). Material was obtained from bone marrow except for *V. eurygnatha* (d) and *A. ruthveni* (j) whose chromosomes were obtained from squashed corneal epithelial cells and photographed unstained using phase-contrast optics.

(For figure see next page.)



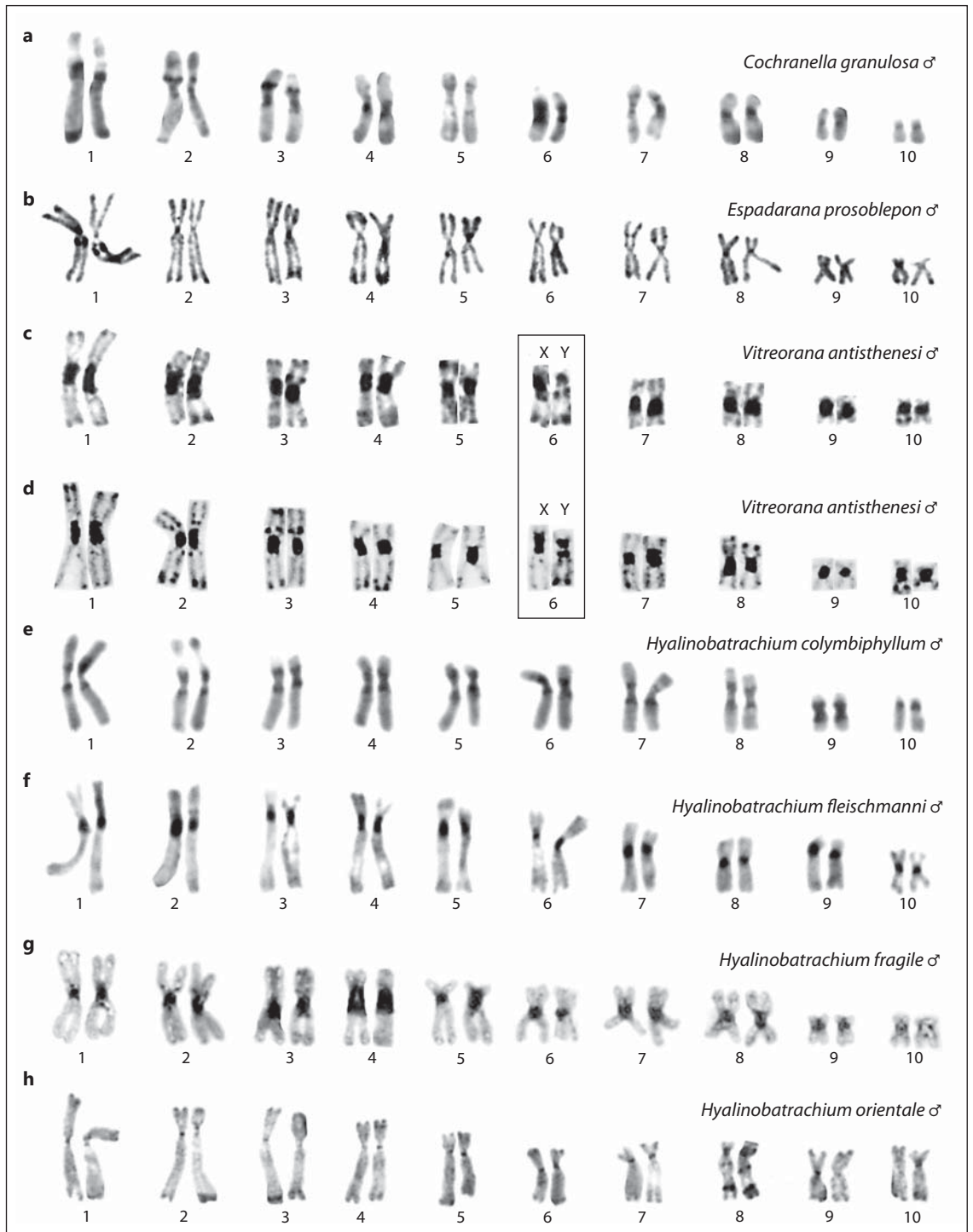


Fig. 2. C-banded karyotypes of the centrolenid species analyzed. Note the large amounts of centromeric and paracentromeric heterochromatin in *V. antisthenesi* (**c, d**), *H. fleischmanni* (**f**) and *H. fragile* (**g**). The heteromorphic XY sex chromosomes of *V. antisthenesi* are framed (**c, d**). Material obtained from bone marrow.

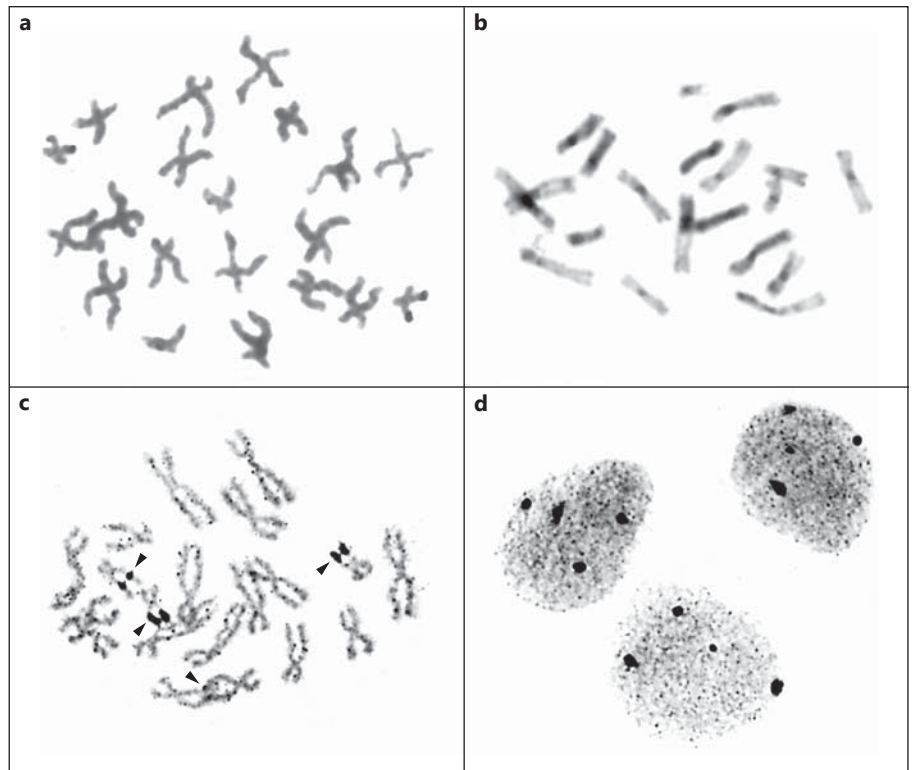


Fig. 3. a–c Metaphase chromosomes of *Hyalinobatrachium valerioi* after conventional staining (a), C-banding (b) and Ag-staining (c). The 4 NORs are marked by arrowheads. d Ag-stained interphase nuclei of *H. valerioi* cells showing the presence of 4 labeled nucleoli. Material obtained from bone marrow.

mosomes (figs. 2, 3b). The small chromosomes usually contain just as much constitutive heterochromatin as do the largest chromosomes. Very large centromeric and pericentromeric C-bands are located in the chromosomes of *V. antisthenesi*, *H. fleischmanni* and *H. fragile* (figs. 2c, d, f, g, 5d). The smallest amounts of centromeric and pericentromeric heterochromatin are found in the karyotypes of *E. prosoblepon* and *H. orientale* (fig. 2b, h). Intercalary C-bands are positioned in the chromosome 1 long arm and chromosome 7 short arm of *E. prosoblepon* (fig. 2b), in the chromosomes 2 and 3 short arms and the chromosomes 1, 2 and 10 long arms of *V. antisthenesi* (fig. 2c, d), and in the chromosome 8 long arm of *H. orientale* (fig. 2h). With the exception of *V. antisthenesi* (fig. 2c, d), the amount of telomeric and NOR-associated heterochromatin in the centrolenid karyotypes is rather small when compared with other anuran species. It should be emphasized that the XY sex chromosomes of *V. antisthenesi* contain the same amounts of centromeric and pericentromeric heterochromatin (figs. 2c, d, 5d). However, in many of the C-banded metaphases, the constitutive heterochromatin in the X appears more darkly stained than in the Y (not shown). Furthermore, the telomeric heterochromatin in

the Y long arm seems to be distinctly larger than in the X long arm (fig. 2d).

As expected from the results obtained in the chromosomes of other anuran families [for reviews, see Schmid et al., 2010, 2012], staining with the fluorochrome quinacrine mustard shows that the large euchromatic segments of centrolenid chromosomes fluoresce with a uniform and moderate intensity (figs. 4, 5a–c). All chromosome regions fluorescing either distinctly brighter or distinctly weaker than the euchromatin consist of constitutive heterochromatin or NORs (as confirmed, respectively, by C-banding and Ag-staining).

The quinacrine-stained karyotype of *V. antisthenesi* is remarkable because of the large amounts of quinacrine-positive (Q^+) heterochromatin in the centromeric and pericentromeric regions of all autosomes and in the X chromosome (figs. 4c, 5a–c). The Y chromosome contains the least amount of Q^+ heterochromatin in the karyotype. Only 2 very small Q^+ bands, one in the centromeric region and the other in the long arm telomeric region can be recognized in the Y. Neither in the X chromosome, nor in the autosomes is Q^+ heterochromatin located in the telomeric regions (figs. 4c, 5a–c). In metaphases prestained with quinacrine and subsequently C-

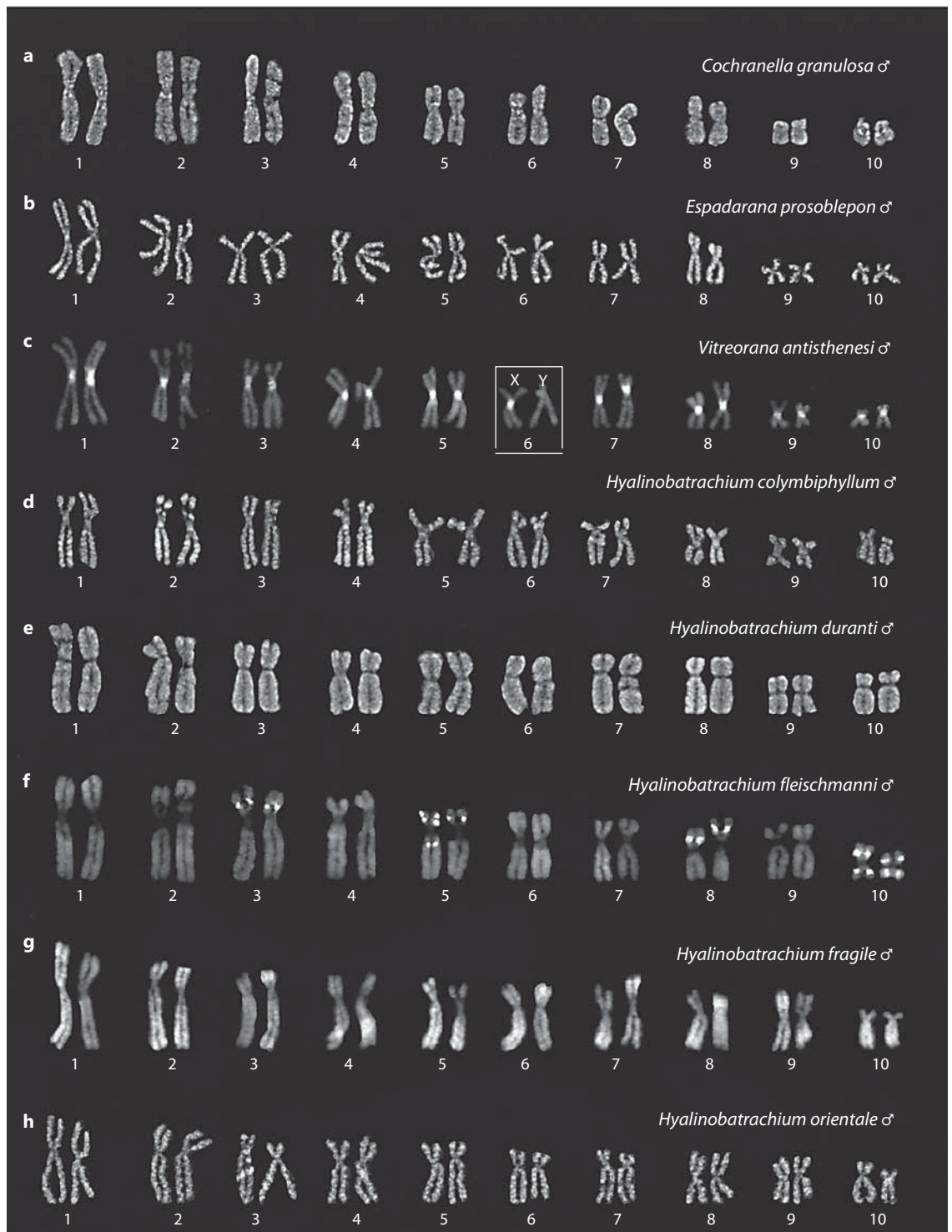


Fig. 4. Karyotypes of the centrolenid species after quinacrine mustard staining. Note the quinacrine-bright centromeric and pericentromeric heterochromatin in all autosomes and in the X chromosome of *V. antisthenesi* (c), and the quinacrine-bright interstitial heterochromatic bands in chromosome pair 10 of *C. granulosa* (a), as well as in the chromosome pairs 3, 5, 8, and 10 of *H. fleischmanni* (f). The heteromorphic XY sex chromosomes of *V. antisthenesi* are framed (c). Material obtained from bone marrow.

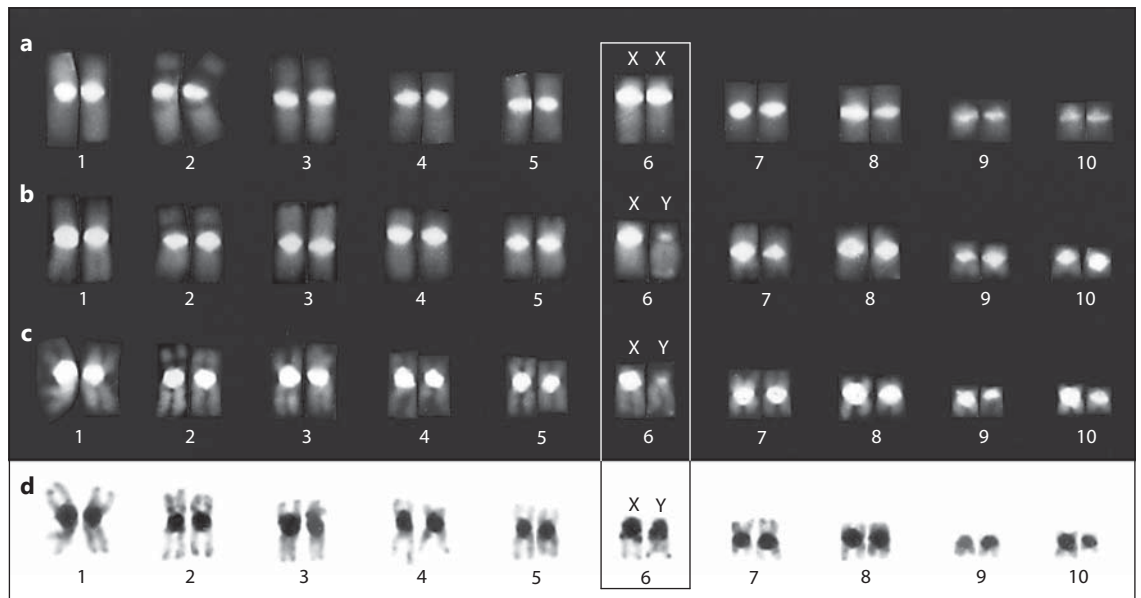


Fig. 5. Karyotypes of female (**a**) and male (**b-d**) *Vitreorana antisthenesi* after quinacrine mustard staining (**a-c**) and C-banding (**d**). The quinacrine-stained chromosomes in **c** were subsequently C-banded in **d**. The sex chromosomes are framed. Note the large amounts of quinacrine-bright centromeric and pericentromeric heterochromatin in all autosomes and in the X chromosome. In

the Y chromosome, all the C-band-positive pericentromeric heterochromatin is quinacrine-negative, whereas the small region of centromeric heterochromatin and the long arm telomeric heterochromatin is quinacrine-positive. Material obtained from bone marrow.

banded, the very bright Q^+ bands in the autosomes and in the X chromosome colocalize with the dark C-bands. In the Y chromosome, however, the centromeric and pericentromeric C-bands are distinctly larger than the Q^+ bands (fig. 5c, d). A close comparison of Q- and C-banded metaphases revealed that only a small band at the Y centromeric region is Q^+ , whereas all of the pericentromeric heterochromatin is not specifically labeled by quinacrine mustard. The comparison of Q-banded Y chromosomes of the 19 males examined showed that there exists a slight size variation of their Q^+ centromeric heterochromatin. Finally, the small telomeric Q^+ band located in the Y long arm (figs. 4c, 5a-c) is labeled by C-bands (figs. 2d, 5d).

In *C. granulosa*, Q^+ heterochromatin is present in both arms of chromosomes 10 (fig. 4a). In the karyotype of *H. fleischmanni*, distinct Q^+ heterochromatin is located in the short arms of chromosomes 3, 5, 8 and 10 as well as in the long arms of chromosomes 5 and 10 (fig. 4f). In one of the males, the Q^+ -band in the chromosome 5 long arm is absent in one of the homologs (fig. 4f), whereas in the other male and an unsexed individual it is present in both homologs (not shown). Other brightly fluorescing Q-

bands were not detected in the karyotypes of the studied centrolenids.

Direct staining of centrolenid chromosomes with the fluorochromes DAPI and Hoechst 33258 or counterstaining with actinomycin D/DAPI yield the same banding patterns as induced by quinacrine. After counterstaining with distamycin A/DAPI, the heterochromatic regions fluoresce slightly more brightly than the euchromatic segments of the chromosomes. AT-rich DNA sequences are known to enhance the intensity of quinacrine, DAPI and Hoechst 33258 fluorescence [Weisblum and de Haseth, 1972; Weisblum, 1973; Comings, 1975; Müller and Gautier, 1975; Lin et al., 1977]. Therefore, heterochromatic regions in amphibian metaphase chromosomes that show bright fluorescence with any of these fluorochromes are believed to be rich in AT base pairs [for reviews, see Schmid et al., 2010, 2012].

As expected from the complementarity of quinacrine and mithramycin staining, the distinct Q^+ heterochromatic regions located in the karyotypes of *V. antisthenesi*, *H. fleischmanni* and *C. granulosa* are always mithramycin-negative. This is especially evident in the chromosomes of *V. antisthenesi* in which the prominent centro-

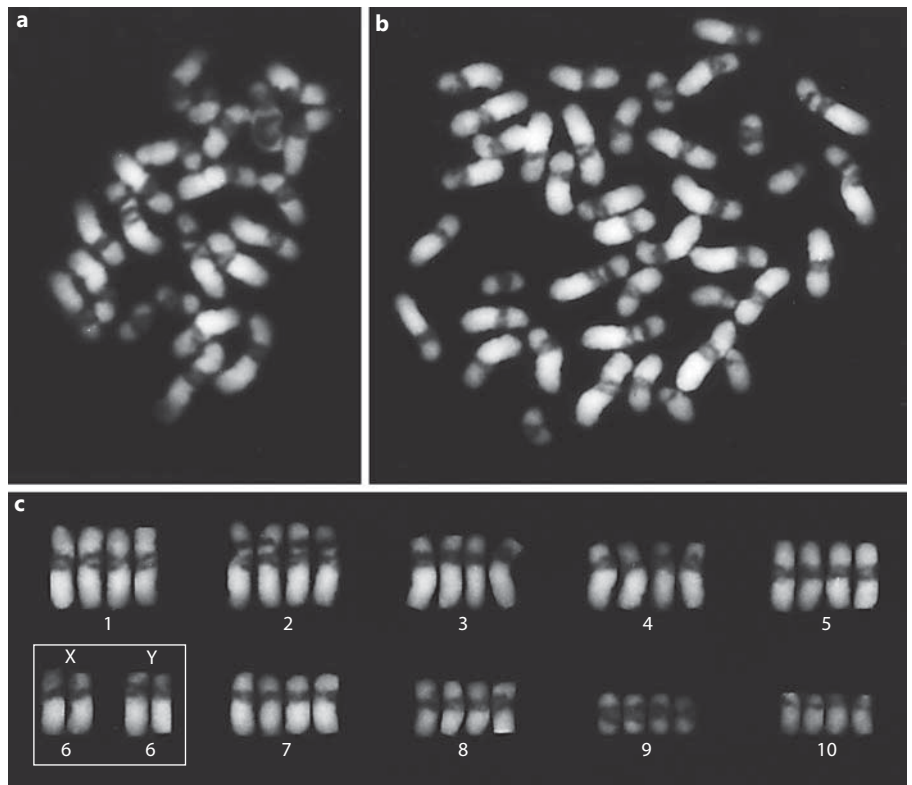


Fig. 6. Diploid (a) and tetraploid (b) spermatogonial metaphases of male *Vitreorana antisthenesi* after distamycin A/mithramycin counterstaining. Chromosomes in b are arranged as a karyotype in c. Note the mithramycin-negative centromeric and pericentromeric heterochromatin in all autosomes and in the sex chromosomes. Material obtained from testes.

meric and pericentromeric heterochromatin in all chromosomes shows a distinctly quenched mithramycin fluorescence (fig. 6).

Nucleolus Organizer Regions

Specific silver (Ag-) staining showed that, with the exception of *H. valerioi*, all analyzed centrolenids have a single pair of NORs in their karyotypes (fig. 7). In most species (*C. granulosa*, *V. antisthenesi*, *H. colymbiphyllum*, *H. duranti*, *H. fleischmanni*, *H. fragile*), the NORs are located in short arm paracentromeric regions of chromosomes 1, 2, 4, and 8. In *E. prosoblepon*, NORs are located in the long arm telomeres of chromosomes 10 (fig. 7b), and in *H. orientale*, they are found in an interstitial position in the long arms of chromosomes 8 (fig. 7h).

In the single specimen of *H. valerioi* that was analyzed, the standard NOR pair is located interstitially in the long arms of chromosomes 9 (fig. 3c). Additionally, ectopic NORs are detected in a telomeric position of 2 other non-homologous chromosomes. Because of the few metaphases available for analysis, the 2 chromosomes with ectopic NORs could not be identified with certainty. As expected, in the bone marrow and intestine cells of this male individual, 4 Ag-stained nucleoli are present (fig. 3d). Accordingly, in the cells of male meiotic pro-

phase, 1 major and 2 smaller nucleoli can be detected (not shown).

Telomeric DNA Sequences

The patterns of telomeric DNA sequences were examined by FISH in 4 species of centrolenids (*C. granulosa*, *V. antisthenesi*, *H. colymbiphyllum*, *H. fleischmanni*). In general, the patterns of chromosomal distribution of the canonical (TTAGGG)_n repeats are consistent with those in the karyotypes of other amphibians and in a variety of other vertebrates [for reviews, see Schmid et al., 2010, 2012]. In all chromosomes, the (TTAGGG)_n repeats are located in the telomeric regions of both chromatids (fig. 8). In the short arms of the smaller chromosomes 9 and 10, the 2 hybridization signals come together very closely and frequently fuse into a single signal (e.g. fig. 8b, left homolog of chromosome pair 10).

In all analyzed specimens of *V. antisthenesi*, distinct heterochromatic intrachromosomal telomeric sequences (het-ITSs) are located in the constitutive heterochromatin adjacent to the NOR in the short arm of chromosome 2, and in the paracentromeric heterochromatin of the long arm in one of the homologs 6 (X chromosome). het-ITSs are not found in the Y chromosome or the other autosomes (fig. 8b).

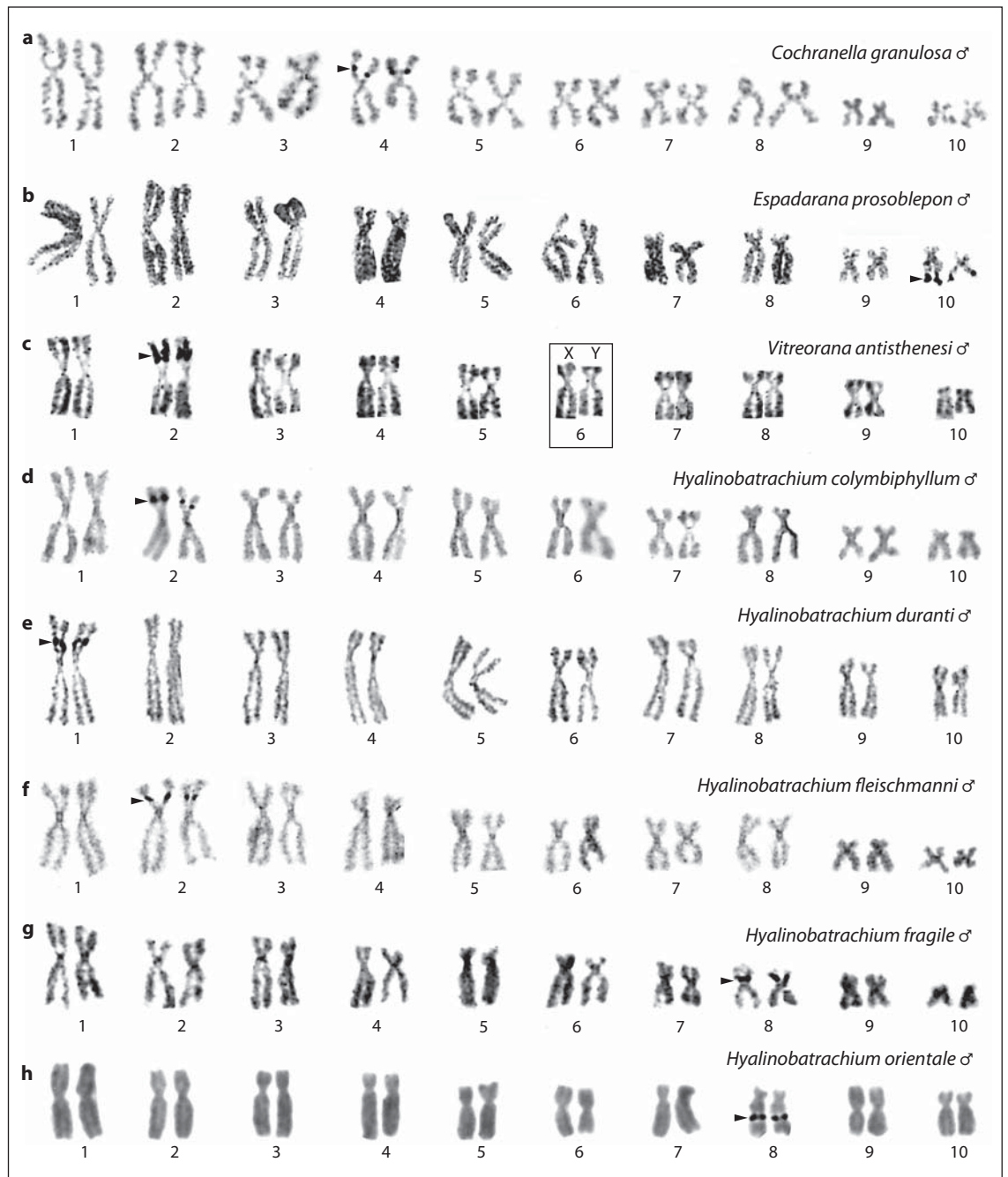


Fig. 7. Ag-stained karyotypes of the centrolenid species examined. The NORs are marked by arrowheads. The heteromorphic XY sex chromosomes of *V. antisthenesi* are framed (c). Material obtained from bone marrow.

Male Meiosis

As in the male meiosis of almost all highly evolved anurans [Morescalchi, 1973; Schmid et al., 2010, 2012], the diakinetid bivalents of centrolenids are strongly contracted, and most of them show a ring-like or dumb-bell-shaped morphology [Duellman and Cole, 1965; Duell-

man, 1967; Schmid et al., 1989]. This results from telomerically located chiasmata. In the pachytene stage of prophase in mammalian male meiosis, a sex vesicle is formed by the XY sex chromosomes. A similar vesicle is not found in amphibians.

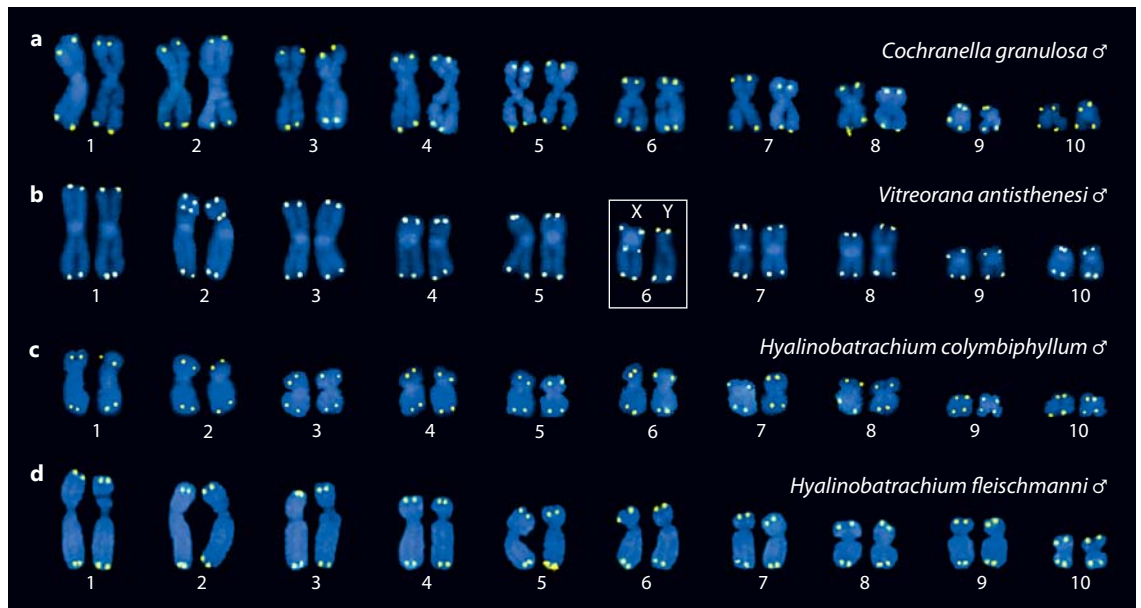


Fig. 8. Karyotypes of 4 centrolenid species showing the FISH patterns of the telomeric (TTAGGG)_n repeats. Note the het-ITs in the short arms of chromosomes 2, and in the long arm pericentromeric heterochromatin of the X chromosome of *V. antisthenesi* (b). The heteromorphic XY sex chromosomes of *V. antisthenesi* are framed. Material obtained from bone marrow.

In *V. antisthenesi*, the diakinetid XY bivalent can easily be identified by the unequally-sized Q⁺ heterochromatin in their centromeric and paracentromeric regions (fig. 9a–e). One half of the bivalent (X) is brightly labeled by quinacrine, and the opposite half (Y) is characterized by a dull fluorescence. In none of the diakineses observed did the XY chromosomes show an end-to-end association like that found in the male meiosis of several other amphibian species possessing heteromorphic XY chromosomes [for reviews, see Schmid et al., 2010, 2012]. Although the morphology and banding patterns of the X and Y chromosomes of *V. antisthenesi* are distinctly heteromorphic, they still show the same pairing configuration as the autosomal bivalents. This could indicate that the euchromatic segments in the XY chromosomes of *V. antisthenesi* are still genetically homologous and that pairing and crossing over occur along them during meiotic prophase. Differentiation among the 9 autosomal bivalents can be achieved by comparing their relative sizes. In the leptotene stage, the Q⁺ heterochromatic chromosome regions can be clearly discerned as individual brightly fluorescing blocks (fig. 9f, g). In contrast to the nuclei of somatic tissues (fig. 10), these heterochromatic regions do not fuse into larger chromocenters. As expected, in the round spermatid nuclei, a maximum of 10

brightly fluorescing heterochromatic regions are present (fig. 9h).

Somatic Interphase Nuclei

In the majority of interphase nuclei from bone marrow and intestine of *V. antisthenesi*, the 20 large centromeric and paracentromeric heterochromatic regions of the chromosomes are fused into a few chromocenters (fig. 10a–d). As was first shown in Hemiptera [Slack, 1938; Schrader, 1941], one of the general features of constitutive heterochromatin is its tendency for different heterochromatic regions to enter into non-specific temporary or permanent associations. In polytene *Drosophila* nuclei, the pericentromeric heterochromatin of all chromosomes becomes permanently fused to form a large chromocenter, and many intercalary heterochromatic bands demonstrate so-called ‘ectopic pairing’ [Slizynski, 1945; Kaufmann and Iddles, 1963]. Ectopic pairing of heterochromatic regions also exists in mammalian interphase nuclei [Hsu et al., 1971; Rae and Francke, 1972], including metaphases of human chromosomes [Schmid et al., 1975, 1981, 1983]. Ectopic pairing occurs most frequently between the heterochromatin of homologous chromosomes but can also occur between non-homologous chromosomes, although the first type is, in statistical

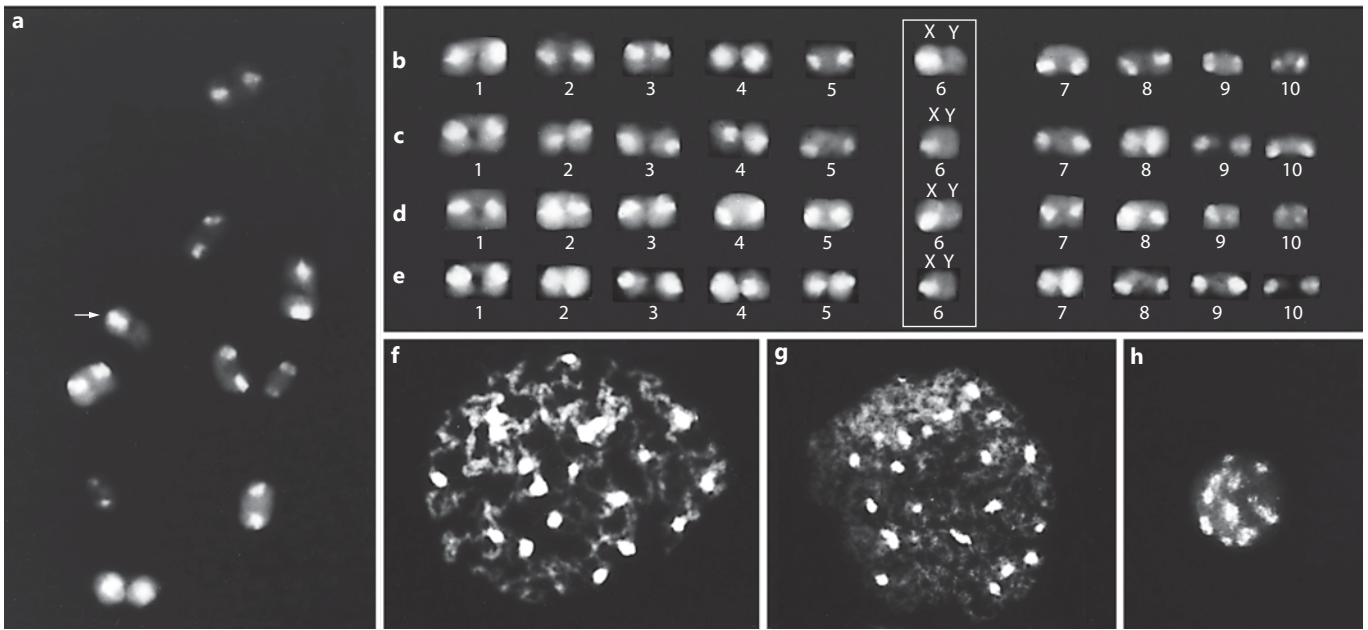


Fig. 9. Diakinesis (**a**) and bivalents (**b–e**) of 4 diakineses of male meiosis in *Vitreorana antisthenesi* after staining with quinacrine mustard. In **a** the XY bivalent is marked by an arrow, in **b–e** the XY bivalents are framed. **f–h** Quinacrine-stained leptotene nuclei (**f, g**) and early spermatid nucleus (**h**) of *V. antisthenesi*. Note the brightly fluorescing heterochromatic chromocenters. Material obtained from testes.

terms, more frequent [Haaf et al., 1986]. The existence of repetitive DNA-containing physical connections between metaphase chromosomes has been demonstrated to occur in mouse and human cell lines by Kuznetsova et al. [2007]. In mitotic metaphase chromosomes of the terraranan frog *Pristimantis fenestratus*, chromatin connections were found between heterochromatic regions in telomere-telomere, centromere-centromere and centromere-telomere configurations [Siqueira et al., 2009]. It seems conceivable, but not yet experimentally proven, that the phenomenon of ectopic pairing is mediated by a complete (or at least partial) homology of the base pairs in the repetitive DNA of heterochromatin.

In some of the bone marrow interphase nuclei of *V. antisthenesi*, the large compact heterochromatic chromocenters have split up into smaller ones with diffuse boundaries (fig. 10e–h). The most plausible explanation is that these nuclei are in the S-phase of the cell cycle in which the compact structure of the constitutive heterochromatin decondenses in order to facilitate replication.

Genome Sizes

The genome sizes of 4 centrolenid species (*V. antisthenesi*, *H. colymbiphyllum*, *H. fleischmanni*, and *H. orientale*) were determined in the present study. All measure-

ments were performed using DNA flow cytometry of DAPI-stained erythrocytes obtained from male specimens. As examples of these measurements, the histograms obtained for *V. antisthenesi* and *H. orientale* are shown in figure 11. *V. antisthenesi* has the lowest genome size (6.20 pg DNA/nucleus), whereas those of *H. colymbiphyllum*, *H. fleischmanni* and *H. orientale* are distinctly higher (10.65, 8.98 and 8.70 pg DNA/nucleus, respectively). The coefficient of variation of the measurements ranged between 2 and 2.7%.

It should be noted that the erythrocytes were stained with the AT base pair-specific DAPI, and not with ethidiumbromide which anneals to double-stranded DNA regardless of its base pair composition. Therefore, erythrocyte nuclei of *V. antisthenesi*, with high amounts of AT-rich repetitive DNA in the Q⁺ constitutive heterochromatin (see above), will be more intensively labeled than those of species depauperate in such sequences or with enriched amounts of GC-repetitive DNA in their heterochromatic chromosome regions. If so, this would lead to a biased, somewhat higher DNA value in *V. antisthenesi*. In spite of this, the results obtained by DNA flow cytometry do allow for general conclusions with respect to genome sizes in the Centrolenidae.

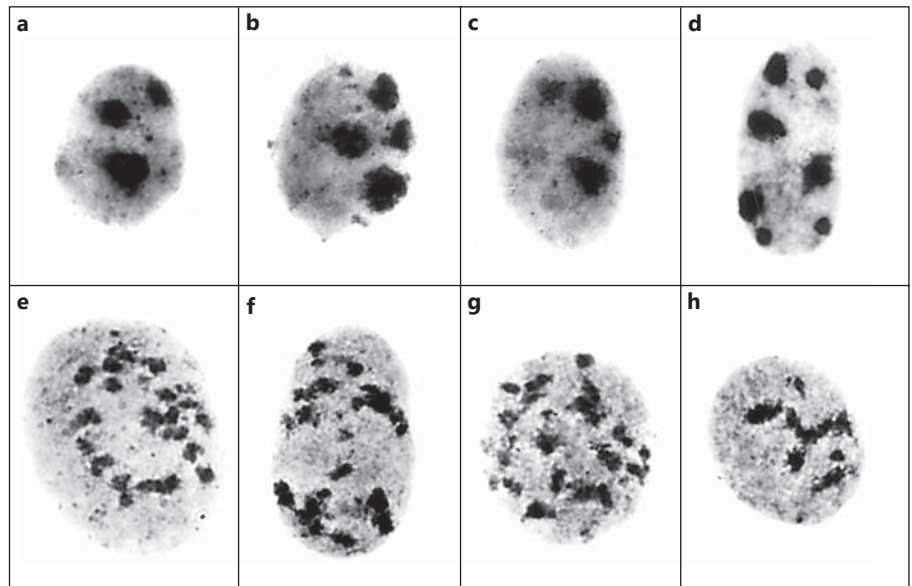


Fig. 10. C-banded interphase nuclei of *Vitreorana antisthenesi*. **a–d** The centromeric and paracentromeric heterochromatic regions of the chromosomes are fused into a few large chromocenters. **e–h** The compact chromocenters have split up and decondensed probably due to replication of the constitutive heterochromatin in the late S-phase. Material obtained from bone marrow.

Discussion

Anuran species with a diploid chromosome number of $2n = 20$ or lower are not very frequent [for reviews, see King, 1990; Green and Sessions, 2007]. The comparative cytogenetic analyses presented here show that the karyotypes of the Centrolenidae and Allophrynidae have been rather conserved during evolution. The minor differences existing between the chromosome lengths in the various species can be easily explained by the occurrence of pericentric inversions, unequal reciprocal translocations, chromosomal shifts, and changes in the amount of constitutive heterochromatin. Such mutations have been successfully fixed in a homozygous condition in the karyotypes since the different species have diverged.

According to Morescalchi [1973], the low chromosome number of $2n = 20$ encountered in the Centrolenidae can be derived from a $2n = 24$ or 22 karyotype found in many South and Central American species of the family Hylidae to which the Centrolenidae seemed to be phylogenetically related [Noble, 1931; Taylor, 1951; Lynch, 1973]. Other studies, however, using molecular techniques [Austin et al., 2002; Biju and Bossuyt, 2003; Darst and Cannatella, 2004; Faivovich et al., 2005; Frost et al., 2006; Grant et al., 2006; Roelants et al., 2007] or phenotypical characters [Ford and Cannatella, 1993; Duellman and Trueb, 1994; Rueda-Almonacid, 1994; Duellman, 2001; Haas, 2003; Burton, 2004; Wiens et al., 2005] could

not resolve with confidence the phylogenetic position of the Centrolenidae within the Neobatrachia.

Since *A. ruthveni* was first described [Gaige, 1926], it has been placed within the families Bufonidae, Centrolenidae, Hylidae, and Leptodactylidae [for a review, see Caldwell and Hoogmoed, 1998] or within its own family Allophrynidae [Savage, 1973, 1986; Goin et al., 1978]. A study using DNA sequence data from 2 mitochondrial genes (16S + 12S ribosomal RNA) suggests that *A. ruthveni* is most closely allied to the family Centrolenidae [Austin et al., 2002]. Further molecular phylogenetic studies analyzing the 16S + 12S rRNA genes and the intervening transfer RNA valine gene [Lourenço et al., 2008], or the mitochondrial 16S + 12S rRNA and *NADH-1* genes, as well as the nuclear genes *c-myc*, *POMC* and *RAG-1* [Guayasamin et al., 2008], have confirmed that *A. ruthveni* is a sister taxon to the family Centrolenidae. This is consistent with Noble [1931] who originally designated *A. ruthveni* as an edentate centrolenid. In conclusion, and as stated by Guayasamin et al. [2009], there are sufficient data to conclude that there is a close evolutionary relationship between the Centrolenidae and Allophrynidae, whereas the relationships of this clade with other anurans are still unclear.

The sister group relationship between the Centrolenidae and Allophrynidae (unranked taxon Allocentrolenidae) is clearly corroborated by the present cytogenetic study which demonstrates a conservation of the diploid chromosome number and a high degree of similarity of

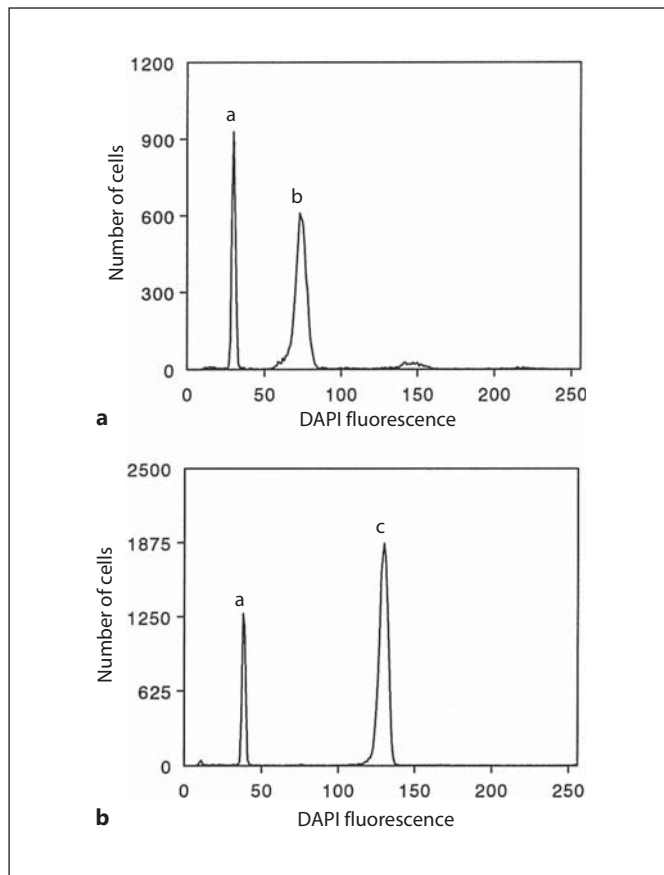


Fig. 11. Histograms obtained by DNA flow cytometry of a mixture of DAPI-stained erythrocytes of chicken and male individuals of *Vitreorana antisthenesi* (a) and *Hyalinobatrachium orientale* (b). The nuclear DNA content is directly proportional to the DAPI fluorescence intensity, expressed as channel numbers on the x-axis. Chicken erythrocytes (peak a) were used as internal standards with a known DNA content of 2.33 pg DNA/nucleus. The nuclear DNA content of the frog erythrocytes is calculated from the ratio of the peak channel number of the frog erythrocytes (peaks b and c) and chicken (peak a) multiplied by the known nuclear DNA amount of the chicken.

chromosome morphology (homeology) in all species examined. The origin of the minor changes existing in the chromosome morphology can be elucidated, at least partially, with the help of modern molecular cytogenetic methods such as cross-species chromosome painting using chromosome-specific DNA probes generated from flow-sorted or microdissected chromosomes that are used as templates for DNA amplification by DOP-PCR. Such experiments have been successfully performed for comparative cytogenetic studies in mammals, birds, reptiles, and fishes [e.g. Reed et al., 1995; Yang et al., 1995,

1999, 2003; Griffin et al., 1999; Campos-Ramos et al., 2001; Phillips et al., 2001; Rens et al., 2001, 2003, 2004, 2006; Harvey et al., 2002; Liu et al., 2002; Guttenbach et al., 2003; Grützner et al., 2004; Nanda et al., 2006, 2007, 2008; Ferguson-Smith and Trifonov, 2007; Henning et al., 2008; Giovanotti et al., 2009; Cioffi et al., 2013].

Previous cytogenetic studies on NORs in Anura clearly demonstrate that the overwhelming majority of species possess only a single 'standard' pair of NORs [Schmid, 1980; King, 1990; Green and Sessions, 2007; Schmid et al., 2010, 2012]. This is also the case in the Centrolenidae with the exception of *H. valerioi*. Intraspecific polymorphisms in the number and location of the NORs have not been detected in the specimens examined. In the karyotypes of the 9 species examined by Ag-staining, the standard NOR pair is located in the chromosomes 1, 2, 4, 8, 9, or 11. Provided that all chromosome pairs of the centrolenids have maintained their evolutionary homeology, the different NOR locations can be best explained by chromosomal shifts which specifically transferred the NORs between the chromosomes without causing gross alterations of their morphologies. The possible molecular mechanism responsible for the NOR transfer has been discussed in detail by Schmid et al. [2010, 2012].

In *V. antisthenesi*, 2 interstitial clusters of the telomeric hexanucleotide (TTAGGG)_n are present in the NOR-associated heterochromatin in the short arm of autosome 2 and in the paracentromeric heterochromatin of the X long arm. Such het-ITSs have also been demonstrated in the karyotypes of a variety of other anuran species. The (TTAGGG)_n repeats in het-ITSs can span up to several hundred kb and are predominantly located in constitutive heterochromatin. It is conceivable that het-ITSs are relics of chromosomal repatterning that occurred during karyotype evolution and shifted telomeric (TTAGGG)_n repeats into internal positions [for reviews, see Schmid et al., 2010, 2012]. Chromosome rearrangements, capable to internalize telomeric (TTAGGG)_n repeats are mainly centric (Robertsonian) fusions, inversions, tandem translocations and insertions. Given the apparent numerical stability of centrolenid karyotypes, centric fusions and tandem translocations seem to be unlikely for the origin of the het-ITSs in *V. antisthenesi*. Thus, either inversions or insertions likely caused these het-ITSs, or alternatively, these (TTAGGG)_n repeats are simply components of the highly repetitive DNA sequences of heterochromatic regions. In this case, a partial or complete sequence identity of the telomeric DNA and the repetitive DNA of the heterochromatic regions is purely coincidental. The (TTAGGG)_n repeat is known to be a component of the

repetitive satellite DNAs of some vertebrates [Southern, 1970; Fry and Salser, 1977; Arnason et al., 1988].

The present data confirm the existence of heteromorphic XY sex chromosomes in *V. antisthenesi*, initially observed by Schmid et al. [1989] in 2 males and females (table 1). Cytogenetically demonstrable sex chromosomes, either of the XY♂/XX♀ or ZW♀/ZZ♂ type, are known to exist but are exceptional in amphibians. According to the Tree of Life database (<http://tolweb.org/Salientia/14938>), there are 172 extant species of Gymnophiona, 557 species of Urodela, and 5,424 extant species of Anura. Although approximately one third of these 6,153 extant amphibians have been karyotyped [listed by Green and Sessions, 2007], only about 5% of those species have demonstrated morphologically distinguishable sex chromosomes. The most recent compilations on anuran sex chromosomes, including those of *V. antisthenesi*, document the existence of only 42 species possessing microscopically detectable sex chromosomes [Schmid et al., 2010, 2012]. The heteromorphic XY sex chromosomes of *V. antisthenesi* are distinguished by several features which, in combination, are not encountered in any of the other anuran sex chromosomes. They can be interpreted as XY chromosomes in a nascent stage of morphological differentiation, still of approximately the same length and only differing slightly in the positions of their centromeres. Chromosome banding analyses, however, reveal a conspicuous heteromorphism. Thus, there is more telomeric heterochromatin located in the Y long arm than in the X long arm. This is in agreement with most cytogenetic and molecular studies on lower vertebrates which demonstrate that the accumulation of repetitive DNA sequences in the Y or W chromosome is the primary step [Singh et al., 1976, 1980, 1981; Jones, 1984; Schmid et al., 2010, 2012]. The centromeric and pericentromeric heterochromatin in the Y chromosome has a higher sensitivity to alkaline Ba(OH)₂ treatment (necessary for the demonstration of C-bands) than the heterochromatin in the X chromosome. Finally, only the centromeric and not the pericentromeric heterochromatin is stained with the AT base pair-specific quinacrine in the Y chromosome, whereas both of these are brightly labeled in the X chromosome and autosomes. The fact that the banding patterns in the X chromosome strongly resemble those in the autosomes of *V. antisthenesi* indicates that it has largely conserved its original organization and that the structurally more complex Y is evolutionarily derived.

According to King [1990], Gregory [2005] and the Animal Genome Size Database (<http://www.genomesize.com>), there are no data on genome sizes for any centro-

lenid or allophrynid species. The values measured in the present study for 4 centrolenids range between 6.20 and 10.65 pg DNA/nucleus. The known genome sizes in the order Anura range from 1.90 to 19.71 pg DNA/nucleus. The frequencies of diploid anuran genome sizes show a clear unimodal distribution, with a value between 8 and 10 pg DNA/nucleus [reviewed in Schmid et al., 2010]. Thus, the genome sizes of the centrolenids seem to fit quite well in this frequency distribution.

The phylogenetic relationships of the Allocentroleniinae within the Neobatrachia has not been resolved. Therefore, it is premature to speculate on the various chromosome mutational events that have led from hypothetical ancestral anuran karyotypes with higher chromosome numbers to the reduced 2n = 20 karyotypes of the Centrolenidae and Allophrynidae. Furthermore, the 11 species analyzed in the present study represent only a very small fraction (7%) of all recognized extant species in the Centrolenidae (145 species) and in the Allophrynidae (3 species). Before general conclusions can be drawn, a representative number of species of all genera must be cytogenetically analyzed.

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