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Chromosome banding in Amphibia

XXI. Inversion polymorphism and multiple nucleolus organizer regions in *Agalychnis callidryas* (Anura, Hylidae)

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Abstract. Cytogenetic analyses were performed on several populations of the Central American tree frog *Agalychnis callidryas*, using conventional methods and banding techniques. The karyotype of this species is distinguished by an inversion polymorphism in chromosome 9, which is either submetacentric or telocentric. The populations examined are in Hardy-Weinberg equilibrium with respect to the two alternative morphs of chromosome 9. This is the first report of the occurrence of an intrapopulational chromosomal inversion polymorphism in the order Anura. In male meiosis, the two chromosomes 9 form a bivalent exhibiting a ring-like pairing configuration with terminal chiasmata in both arms, regardless of whether the paired homologs are heteromorphic or homomorphic. Furthermore, individual specimens of *A. callidryas* exhibit one or two unexpected 18S + 28S ribosomal RNA gene clusters, in addition to the standard nucleolus organizers. The chromosomal localization of these extra nucleolus organizers is identical in all metaphases from the same specimen and shows a specific intraindividual pattern. The karyotype evolution in the phyllomedusine hylids, the structure of the various classes of heterochromatin, and the occurrence and possible origin of the rare inversion polymorphisms and multiple nucleolus organizers in *A. callidryas* and a few other amphibian species are discussed.

The tree frogs of the genera *Agalychnis* (8 species), *Pachymedusa* (1 species), and *Phyllomedusa* (33 species) form the subfamily Phyllomedusinae of the highly evolved anuran family Hylidae. These genera have a geographic range extending from Mexico throughout Central America to tropical South America. Details on the biology of these interesting Amphibia have been compiled by Duellman and Trueb (1986). In contrast to numerous cytogenetic studies on other Hylidae (for review, see King, 1990), only limited and preliminary data about the chromosomes of Phyllomedusinae are available to date. Thus, in two species of *Agalychnis* and in three species of *Phyllomedusa* merely the chromosome number of 2n = 26 is known from classical squash preparations of testicular tissue (Duellman and Cole, 1965; León, 1970). In only three further *Phyllomedusa* species have more precise karyotype analyses been performed using improved cytogenetic techniques (Beçak et al., 1970; Bogart, 1973; Batistic et al., 1975), and only the chromosomes of the single extant *Pachymedusa* species have been examined with banding methods (Schmid, 1980a, b).

The work presented here describes the results of conventional staining, differential banding, and in situ hybridization with ribosomal DNA applied to mitotic and meiotic chromosomes of *Agalychnis callidryas*. This species exhibits an unusual chromosomal polymorphism due to a pericentric inversion in chromosome 9, an unexpected pairing configuration of bivalent 9 in the meiosis of males heterozygous for this inversion, and a rare intraspecific variability in the number and location of the 18S + 28S ribosomal RNA genes.

Agalychnis callidryas (Fig. 1) is one of the most prevalent and widely distributed species of the genus. It has a continuous distribution from the Atlantic lowlands of Veracruz and Oaxaca in Mexico, southeastward along the Caribbean lowlands to central Panama, and in the Pacific lowlands of southern Costa Rica and eastern Panama (Duellman, 1977). The animals are found close to slowly flowing brooks or ponds and on the branches of bushes and trees up to 5 m above the ground. As is characteristic of all Phyllomedusinae, the females deposit their egg clutches on the vegetation over the water, into which the hatching tadpoles drop (Duellman, 1970). *Agalychnis calli-*

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dryas is distinguished by a striking phenotype (Fig. 1): yellow-green to dark-green dorsal skin, conspicuous blue and/or yellow bars on the flanks, very often orange feet, and a bright, uniform red iris with a vertically elliptical pupil. The adult animals grow to a snout-vent length of 76 mm (Duellman, 1970; Weimer et al., 1993).

Materials and methods

Animals

Twenty-seven specimens of *A. callidryas* (22 males and 5 females) were collected from September to November 1991 in Costa Rica. Nineteen of these animals (15 males and 4 females) were found in the Atlantic (Caribbean) lowlands, and the remaining eight individuals (7 males and 1 female) in the Pacific lowlands. The collection localities are listed in Table I. All animals were caught at night either from the branches of bushes and trees in the close vicinity of ponds or crossing roads and paths during rainfalls. The chromosomes were prepared in a laboratory of the Centro de Investigación en Biología Celular y Molecular, University of Costa Rica, San José. The material obtained was transferred to plastic tubes (Nunc) immediately after fixation, stored at 4 °C, and transported to Würzburg packed in dry ice.

Chromosome preparation and banding analyses

Mitotic chromosomes were prepared directly from the bone marrow and intestines of all specimens after in vivo colchicine treatment. Meiotic chromosomes were obtained from the testes of 14 males. The techniques used for the preparation of cell suspensions, hypotonic treatment, and fixation of the cells have been described previously (Schmid, 1978; Schmid et al., 1979). Conventional chromosome staining, C-banding, staining with quinacrine mustard, fluorescence counterstaining with distamycin A/DAPI (4′,6-diamidino-2-phenylindole), distamycin A/mithramycin, and labeling with $AgNO_3$ were performed according to Schmid et al. (1983) and Schweizer (1976). At least five karyotypes for each of the differential staining techniques used, and 20 $AgNO_3$-stained karyotypes were analyzed from each of the 27 animals examined. Determination of the conformity in their banding patterns and interindividual differences of certain chromosomes and/or bands was facilitated by arranging the karyotypes in parallel rows.

Localization of 18S + 28S ribosomal DNA

The hybrid plasmid pXlr 101A, containing inserted ribosomal RNA gene sequences from *Xenopus laevis* (Morgan et al., 1980), was used as a source of ribosomal DNA. Nonfluorescent in situ hybridization with biotinylated pXlr 101A and detection of the hybridized probe with streptavidin-horseradish peroxidase and DAB (3,3′-diamidinobenzidine tetrahydrochloride) were carried out as described previously (Schmid et al., 1993).

Gonadal sex identification

The cytogenetic analyses were preceded by microscopical identification of the sex of all animals. The gonads were dissected, hypotonically treated in distilled water for 35 min, and fixed in 1:3 acetic acid:methanol. The fixed material was cut into small pieces, transferred onto prewarmed (40 °C) slides lying on a hot plate, and overlaid immediately with a drop of 50% acetic acid. The fragments were siphoned up and down until the acetic acid had completely evaporated and the tissue fragments had dissociated to form a homogeneous cell suspension. Finally, the preparations were stained in Giemsa solution and inspected for the presence of male or female germ cells.

Results

Conventional chromosome staining

The diploid chromosome number of *A. callidryas* is 2n = 26 (Fig. 2a, c, e). All individuals have the following chromosome pairs in common: seven large submetacentric pairs (1–7), three medium-sized submetacentric pairs (8, 10, and 11), a small metacentric pair (12), and a submetacentric pair (13). In all animals examined, a distinct secondary constriction was located in the short arm of both homologs of chromosome 5. The population of *A. callidryas* occurring in the Caribbean lowlands of southeastern Costa Rica is polymorphic for an apparent pericentric inversion in chromosome 9, which can have either a submetacentric or telocentric morphology. In Fig. 2 and Table I these chromosomes are designated as 9a (submetacentric type) and 9b (telocentric type). As expected from inversions, chromosomes 9a and 9b are equal in length (Fig. 2c). Both males and females may be either homozygous for the submetacentric

Fig. 1. Adult male of *Agalychnis callidryas* from Cahuita in the Caribbean lowlands of Costa Rica.

Table I. Collection localities of *Agalychnis callidryas* in Costa Rica, number of animals analyzed, and morphology of their chromosome pairs 9

Collection locality	Number and sex of animals	Animals displaying the different chromosome pairs 9[a]		
		aa	ab	bb
Atlantic lowlands				
Cahuita	12 ♂	8	3	1
(9°47′N, 82°53′W)	4 ♀	3	1	
Valle de las Rosas	2 ♂		2	
(9°40′N, 82°58′W)				
Valle de Talamanca	1 ♂		1	
(9°35′N, 82°56′W)				
Pacific lowlands				
Quepos	2 ♂	1	1	
(9°25′N, 84°10′W)				
Rincón	1 ♂	5		
(8°42′N, 83°30′W)	1 ♀	1		

[a] a = Submetacentric morph of chromosome 9; b = telocentric morph (see Fig. 2).

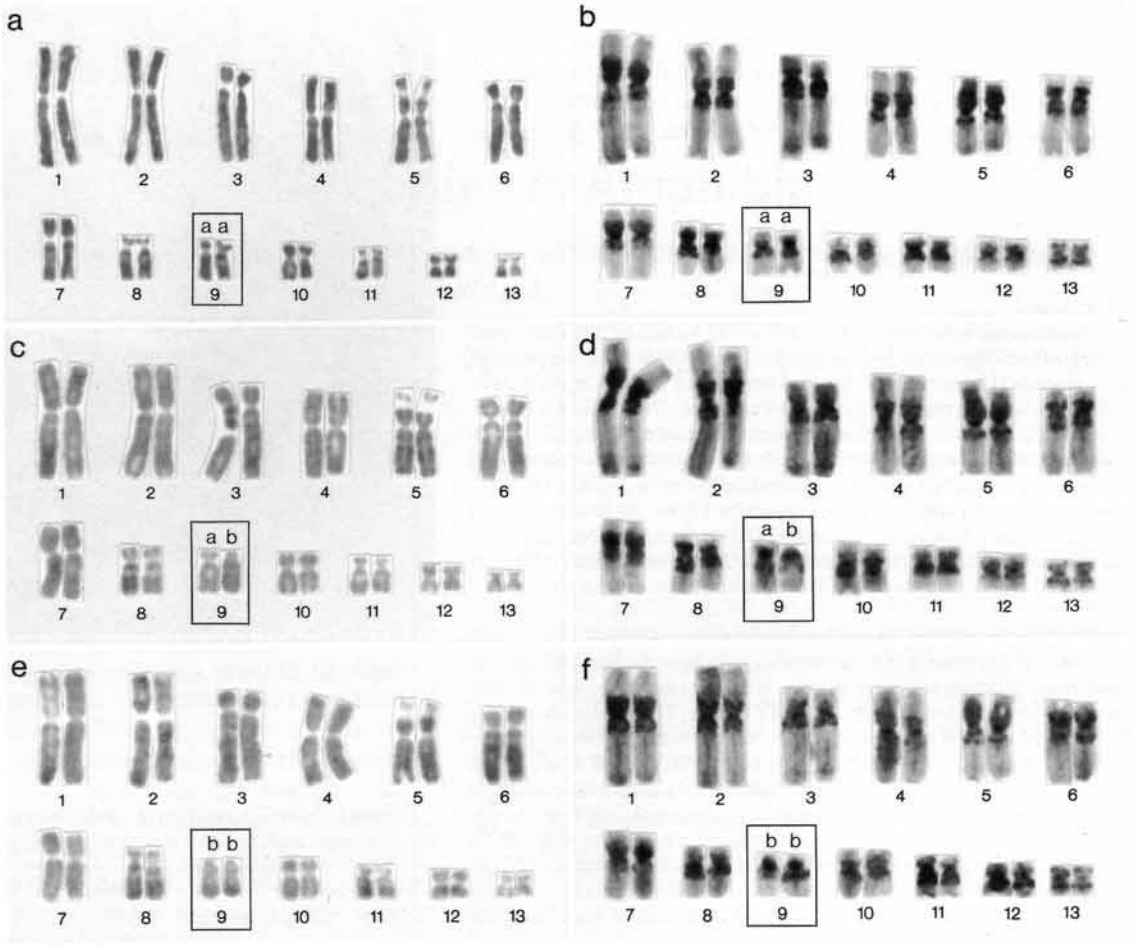

Fig. 2. Karyotypes of *Agalychnis callidryas* homozygous for the submetacentric 9a chromosome (**a**, **b**), heterozygous for the submetacentric 9a and telocentric 9b chromosomes (**c**, **d**), and homozygous for the telocentric 9b chromosome (**e**, **f**) after conventional Giemsa staining (**a**, **c**, **e**) and C-banding (**b**, **d**, **f**). The chromosome 9 pairs are framed. Note also the secondary constriction in the short arms of chromosome pair 5 (**a**, **c**, **e**) and the large amounts of centromeric and pericentromeric heterochromatin in all of the chromosomes (**b**, **d**, **f**).

chromosome 9a (Fig. 2a) or heterozygous for the submetacentric 9a and telocentric 9b chromosome (Fig. 2c). A single (male) individual homozygous for the telocentric 9b chromosome is present in the material collected (Fig. 2e, Table I). In this population, the submetacentric 9a chromosome has a significantly higher frequency (76%) than the telocentric 9b variant (24%). Although the number of animals examined so far is low, the cytogenetic data available (Table I) can be used to show that this population of *A. callidryas* is in Hardy-Weinberg equilibrium with respect to the three possible karyomorphs (9a9a, 9a9b, and 9b9b).

Among the eight *A. callidryas* specimens collected in the Pacific lowlands of Costa Rica, only one male animal had the heteromorphic 9a9b karyomorph; all others exhibited the homomorphic 9a9a constitution (Table I). The frequency of occurrence of the chromosomal inversion in the Pacific lowlands can only be determined after more specimens from this population have been examined.

Banding analyses of chromosomes

In the karyotype of *A. callidryas* large amounts of constitutive heterochromatin are located in the centromeric and pericentromeric regions of all chromosomes (Fig. 2b, d, f). A more detailed examination shows that these darkly stained blocks are composed of subsets of individual C-bands. The short arms of chromosome pair 5 are apparently completely heterochromatic, with a more prominent C-band closely adjacent to or within the secondary constriction. Furthermore, an interstitial C-band is located in the long arms of chromosomes 13. Faintly stainable heterochromatin can be recognized in the telomeric regions of the long arms of chromosomes 1–3, 5, and 6, as well as in the short arms of chromosomes 13. Size variants between homologous C-bands are common in this amphibian species (Fig. 2b, d, f).

The submetacentric 9a chromosome and its telocentric 9b variant show a centromeric and long-arm pericentromeric C-band (Fig. 2b, d, f). In both chromosomes the euchromatic region between these two heterochromatic bands has the same

size (compare the banding patterns between chromosomes 9a and 9b in Fig. 2d). It can therefore be concluded that the two breaks preceding the inversion must have been situated proximal to the long-arm pericentromeric C-band and close to the short-arm telomere in chromosome 9a.

In quinacrine-stained metaphases, the fluorescent intensity of all the centromeric heterochromatin is distinctly weaker than that of the euchromatic chromosome segments, whereas the pericentromeric C-bands have either the same or a slightly brighter fluorescent intensity than the euchromatic segments (Fig. 3a). The long-arm interstitial C-band in chromosome 13 is brightly fluorescent. The short-arm secondary constriction in chromosome 5 exhibits no quinacrine fluorescence at all (Fig. 3a). Following distamycin A/DAPI counterstaining, all the pericentromeric C-bands are characterized by extremely bright fluorescence (Fig. 3b). Counterstaining with distamycin A/mithramycin produces brighter fluorescence of the centromeric heterochromatin; the pericentromeric C-bands, however, exhibit weaker fluorescence than the euchromatic regions (Fig. 3c). The short-arm secondary constriction in chromosome 5 is distinguished by the brightest mithramycin fluorescence in the karyotype, which identifies it as the nucleolus organizer region (NOR) (Schmid, 1980b, 1982). The results obtained by the various fluorochrome stainings on the mitotic chromosomes of *A. callidryas* follow the general rule of complementarity of the AT and GC base pair-specific fluorochromes used (Schweizer, 1976; Schmid 1980a; Schmid and Guttenbach, 1988).

Silver labeling shows that in all *A. callidryas* specimens examined, an NOR is located in the short-arm secondary constriction of chromosome pair 5 (Fig. 4a). Five specimens, collected at three different sites in the Atlantic lowlands (Table I), exhibit one or two unexpected additional NORs in their karyotypes (Fig. 4b–f). The number and chromosomal position of the additional NORs are characteristic for each of these individuals and consistent in all metaphases studied. The chromosomes carrying those NORs are 2, 3, 5, and 6 (Fig. 4). The extra NORs occur in only one of the homologs each, in an interstitial position (Fig. 4b, e, f), at the telomeric region (Fig. 4c, d), or in a paracentromeric location (Fig. 4f). They appear to have been inserted into the chromosomes without changing their arm ratio considerably. In one of the animals, two additional NORs are both located in the same homolog of chromosome 2 (Fig. 4e). The *X. laevis*-derived 18S + 28S ribosomal DNA probe was hybridized in situ to metaphases of these five *A. callidryas*. In each case, an exact correspondence between the hybridization pattern and the chromosomal position of the silver-stained, transcriptionally active NORs is found. The silver-positive NORs always display hybridization with the rDNA probe, and there is no hybridization signal at a locus that does not label with silver. Some examples of NOR-carrying chromosomes hybridized in situ with the biotinylated pXlr 101A probe are depicted in Fig. 5.

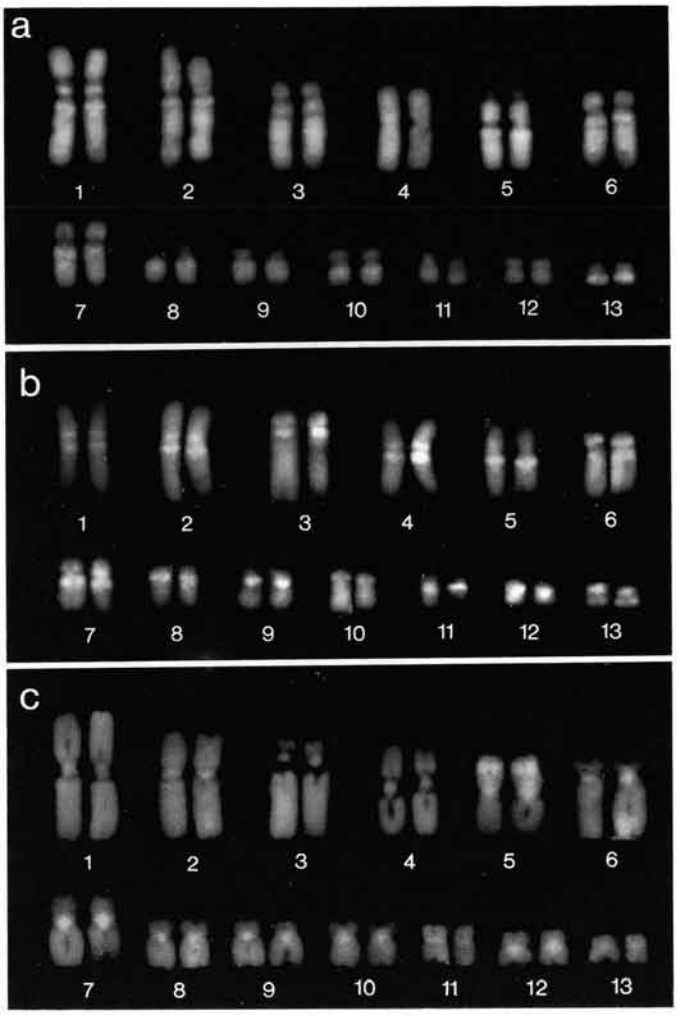

Fig. 3. Karyotypes of *Agalychnis callidryas* stained with quinacrine (**a**), distamycin A/DAPI (**b**), and distamycin A/mithramycin (**c**). The centromeric heterochromatin (compare Fig. 2b, d, f) is quinacrine- and DAPI-negative (**a**, **b**), but mithramycin-positive (**c**). The pericentromeric heterochromatin shows moderate to bright quinacrine labeling (**a**), very bright DAPI fluorescence (**b**), but no mithramycin binding (**c**). Note also the enhanced mithramycin fluorescence of the nucleolus organizer region in the short arms of chromosome pair 5 (**c**).

Male meiosis

The diplotene and diakinesis stages of the first meiotic division were examined in nine male *A. callidryas* specimens after conventional Giemsa staining. The sample includes four specimens homozygous for the submetacentric chromosome 9a (Fig. 2a), four individuals heterozygous for the submetacentric 9a and telocentric 9b chromosomes (Fig. 2c), and the single male homozygous for the telocentric 9b chromosome (Fig. 2e). As in most other species of highly evolved Anura (Morescalchi, 1973; Schmid et al., 1988), the diakinetic bivalents of *A. callidryas* are strongly condensed with terminal chiasmata in both arms, producing a ring-like morphology (Fig. 6a–c). In most of the diakineses analyzed, both the males with the homomorphic 9a9a and 9b9b karyomorphs, as well as the males with the heteromorphic 9a9b constitution, exclusively possess bivalents with this ring-like pairing configuration (Fig. 6a–c).

Interstitially or pericentromerically located chiasmata are not present, either in the larger or smaller bivalents. Although the bivalents of the larger chromosome pairs (1–7) can be very well differentiated from those of the medium-sized and smaller

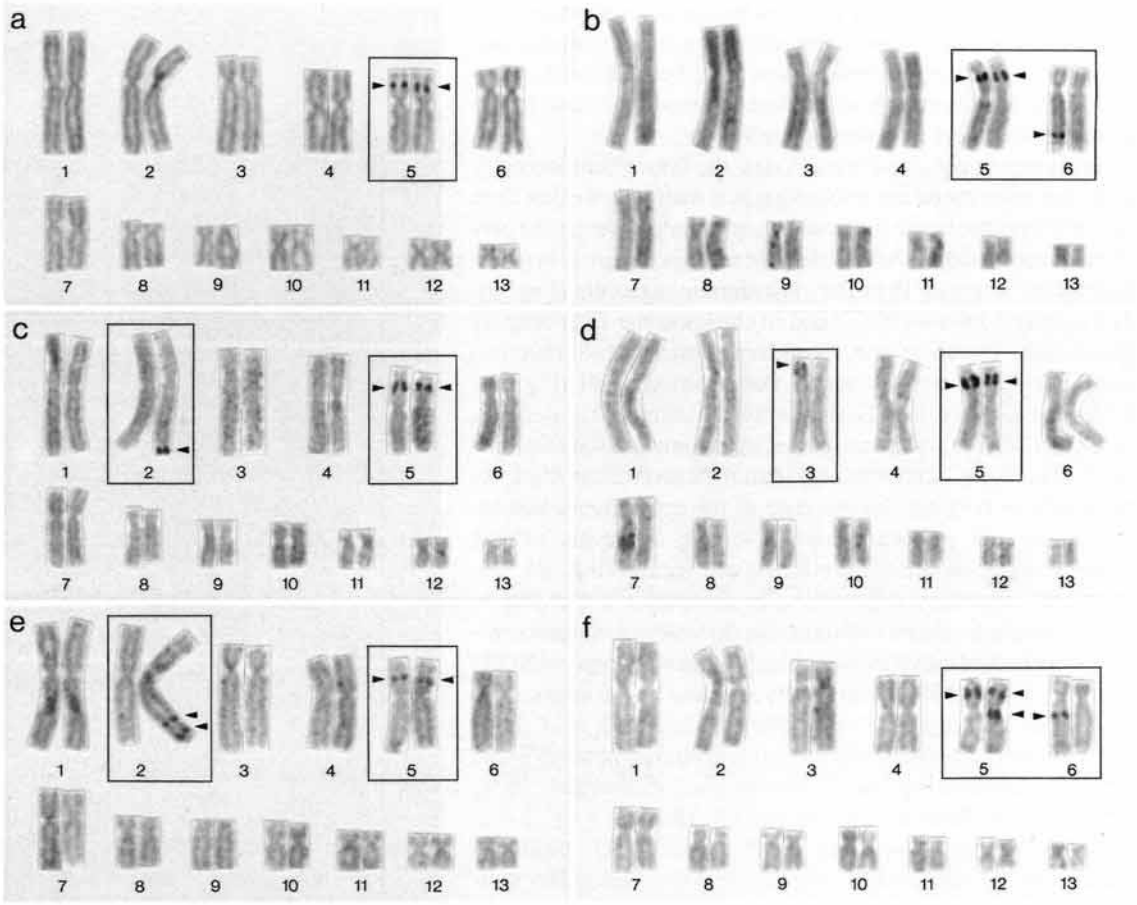

Fig. 4. Silver-stained karyotypes of seven individuals of *Agalychnis callidryas* collected in the Caribbean lowlands of Costa Rica. The nucleolus organizer region (NOR)-carrying chromosome pairs are framed, and the NORs are marked by arrowheads. (**a**) Standard karyotype with one pair of NORs in the secondary constriction of the short arms of chromosome pair 5; these NORs are present in all specimens. (**b–f**) Karyotypes with either one (**b**, **c**, **d**) or two (**e**, **f**) additional NORs. Note that the extra NORs are located in only one of the homologs 2, 3, 5, or 6, each. The individuals in **b**, **c**, **d**, and **f** are homozygous for the submetacentric 9a chromosome, the specimen in **a** is heterozygous for the submetacentric 9a and telocentric 9b chromosomes, whereas the animal in **e** is homozygous for the telocentric 9b chromosome.

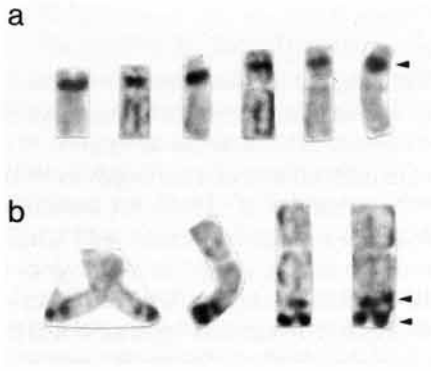

Fig. 5. Selected examples of chromosomes 5 (**a**) and 2 (**b**) from mitotic metaphases of *Agalychnis callidryas* after in situ hybridization with the biotinylated probe pXlr 101A (18S + 28S rDNA). The arrowheads indicate the sites of rDNA hybridization. The rDNA cluster in the short arms of chromosomes 5 (**a**) is the usual nucleolus organizer region (NOR) present in all specimens (compare with Fig. 4a–f), whereas the duplicated hybridization signal in the distal long arms of chromosome pair 2 (**b**) corresponds to the two additional NORs found in a single animal (see Fig. 4e).

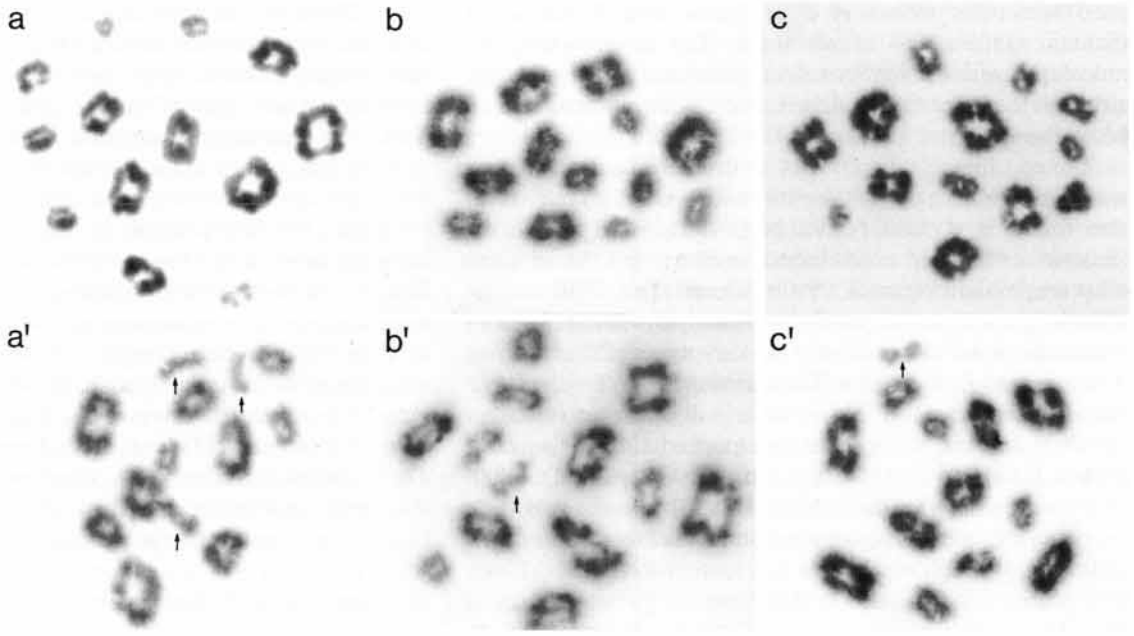

Fig. 6. Giemsa-stained diakineses from the meiosis of *Agalychnis callidryas* males homozygous for the submetacentric 9a chromosome (**a**, **a'**), heterozygous for the submetacentric 9a and telocentric 9b chromosomes (**b, b'**), and homozygous for the telocentric 9b chromosome (**c**, **c'**). In **a–c** all 13 bivalents exhibit a ring-shaped pairing configuration with telomeric chiasmata in both of their arms. In **a'–c'** several of the medium-sized and/or small bivalents (arrows) have attained a rod-like pairing arrangement due to terminalization of the chiasma in one of their arms.

pairs (8–13), the individual bivalents cannot be identified with certainty. After terminalization of the chiasmata, the middle-sized and small bivalents (8–13) preferentially open at one of their ends and assume a rod-like configuration (Fig. 6a'-c', Table II). The linear, end-to-end association of these bivalents persists until anaphase.

In the diakineses of the males with the heteromorphic 9a9b karyomorph, the number of such end-to-end-associated mid-sized bivalents is no larger than that found in the diakineses of males with the homozygous 9a9a or 9b9b constitution (Table II). Neither are bivalents with inversion loops observed in the diplotene and diakinesis stages of these males, nor in the anaphase acentric fragments or anaphase bridges. This leaves the conclusion that meiotic pairing and recombination between the heteromorphic submetacentric 9a and telocentric 9b chromosomes proceed just as smoothly as that between the two homomorphic submetacentric 9a chromosomes and between the two homomorphic telocentric 9b chromosomes.

Table II. Frequencies of middle-sized and small bivalents (chromosome pairs 8–13) with a ring-like or rod-like pairing configuration during the diakinesis stage in male meiosis of *Agalychnis callidryas* (see Fig. 6)

Specimen	Morphology of chromosome pair 9[a]	Number of diakineses analyzed	Number of bivalents showing	
			ring-like shape	rod-like shape
♂1	aa	28	164	4
♂2	aa	26	152	4
♂3	aa	28	160	8
♂4	aa	27	156	6
♂5	ab	15	88	2
♂6	ab	20	114	6
♂7	ab	30	173	7
♂8	ab	17	98	4
♂9	bb	25	147	3

[a] a = Submetacentric morph of chromosome 9; b = telocentric morph (see Fig. 2).

Discussion

The present study on *A. callidryas* is the first comprehensive chromosome banding analysis on one of the 42 species of the neotropical Phyllomedusinae. Some few data on banded karyotypes have been obtained only from two further species from this subfamily (*Agalychnis annae* and *Pachymedusa dacnicolor*) (Schmid, 1980a, b, and unpublished observations). All the remaining cytogenetic examinations on six *Phyllomedusa* species were performed exclusively on conventionally stained chromosomes (Duellman and Cole, 1965; Beçak et al., 1970; León, 1970; Bogart, 1973; Batistic et al., 1975). The Phyllomedusinae are distinguished by a largely conservative karyotype. With the exception of several tetraploid populations of *Phyllomedusa burmeisteri* in Brazil with 4n = 52 (Beçak et al., 1970; Batistic et al., 1975), all species examined have a diploid chromosome number of 2n = 26 and similar chromosome morphologies. The karyotype of *A. callidryas,* with its seven large and six small submetacentric to metacentric chromosome pairs and a distinct demarcation in size between pairs 7 and 8, is in very

good agreement with that of *A. annae* and *P. dacnicolor* (Schmid, 1980b, and unpublished). The chromosomal homoeologies with the *Phyllomedusa* species are just as apparent, although the latter show a demarcation in chromosome length between pairs 6 and 7 (Bogart, 1973).

The considerable amounts of centromeric and pericentromeric heterochromatin in the chromosomes of *A. callidryas* are also found in *A. annae*, and particularly in *P. dacnicolor* (Schmid, 1980b and unpublished observations). As in many other amphibians (Schmid, 1980b; Schmid et al., 1990), quinacrine-bright and, consequently, AT-base pair-rich heterochromatic regions are also present in the karyotypes of *A. callidryas, A. annae,* and *P. dacnicolor.* The abundancy of AT-rich constitutive heterochromatin in Amphibia is in contrast to all other vertebrate classes, where quinacrine-positive C-bands are rather rare (Jalal et al., 1974; Schmid and Guttenbach, 1988). Counterstaining with distamycin A/DAPI selectively reveals the pericentromeric heterochromatin in all chromosomes of *A. callidryas.* It seems reasonable that a special class of AT-rich heterochromatin is located in these brightly fluorescing bands, since the most likely event in the sequential distamycin A/DAPI staining appears to be a competitive interaction between distamycin A and DAPI for specific subsets of AT-rich DNA sequences (Schweizer, 1981).

The demonstration of the inversion polymorphism in chromosome 9 of *A. callidryas* is an important karyological finding. It is generally acknowledged that inversions have been of major importance during the transspecific evolution of amphibian genomes (for review, see King, 1990). However, not a single previous report has been issued to date on the occurrence of structurally variable chromosomes involved in the inversion polymorphisms on the level of whole populations in the order Anura (frogs and toads). However, such intra- and interpopulational inversion polymorphisms are well known from several species of the order Urodela (salamanders and newts). Thus, in a wild population of the American newt, *Notophthalmus viridescens,* 15 females out of a total of 94 were found to be heterozygous for a paracentric inversion comprising almost the complete long arm of chromosome 11 (Hartley and Callan, 1977). The inversion was recognized in lampbrush chromosomes from oocytes because it transferred the sequential landmark loops, normally located close to the telomere, to a position neighboring the centromere. In bivalents 11 from females heterozygous for the inversion no chiasmata were demonstrated between the mutually inverted long-arm pairs, presumably because they failed to synapse. A more complex polymorphism was detected by Bucci-Innocenti et al. (1983) in chromosome 12 of the Italian newt, *Triturus italicus,* in a thorough study on 1,059 individuals (417 adults and 642 larvae). This polymorphism consisted of a size variant of an interstitial C-band and two different independent pericentric inversions, both including the centromere and the same interstitial C-band. Heterozygous animals were obtained in 15 of the 21 populations examined, the latter covering nearly the entire geographic range of the species. With regard to the double-inversion polymorphism, a statistical analysis showed that in nine of these populations from which enough specimens could be collected, the three alternative morphs of chromosome 12 were in Hardy-Weinberg equilibrium. In male meiosis, pairing and chiasma formation were not affected when both homologs 12 were largely homomorphic, but in heterozygous males the bivalent 12 was always rod-like and unichiasmatic. Analogously, in heterozygous females the lampbrush bivalent 12 showed only a single chiasma between the paired heteromorphic homologs, as expected from the extent of the pericentric inversion. The final example for the unequivocal existence of intrapopulational inversion polymorphisms in Urodela is offered by the North American salamander *Aneides ferreus* (Kezer and Sessions, 1979; Sessions and Kezer, 1987). Populations of *A. ferreus* in western Oregon and extreme northern California (defined as the *ferreus* I group) are chromosomally polymorphic for a pericentric inversion in chromosome 13 which has either a telocentric (T) or a subtelocentric (ST) morphology. These alternative morphs of chromosome 13 are in Hardy-Weinberg equilibrium over the major part of the geographic range, where both males and females may be either homozygous (T/T or ST/ST) or heterozygous (T/ST). In male meiosis the chromosomes 13 form a bivalent that shows a conspicuous absence of chiasmata from at least half of the bivalent length, regardless of whether the paired homologs are heteromorphic (T/ST) or homomorphic (T/T or ST/ST). However, occasional instances of anaphase bridges have been observed, suggesting the existence of recombination events between the mutually inverted chromosome segments that can lead to aberrant dicentric chromosomes (Sessions and Kezer, 1987).

The cases of inversion polymorphisms in urodelan species listed above all have in common that the structurally variable chromosomes apparently form normal bivalents in which chiasmata are restricted to regions outside the rearranged chromosome segments. Therefore, these inversions do not result in meiotic disturbances in the heterozygous carriers and so cannot act as reproductive barriers (producing, e.g., reduced fertility and/or chromosomally unbalanced, lethal offspring). The lack of reproductive barriers due to chromosomal rearrangements is a prerequisite for the spreading of a new chromosome type and for the formation of new populations that are not separated reproductively from the ancestral population (Hedrick, 1981). The pericentric inversion in chromosome 9 of *A. callidryas* described in this study also does not cause a selective disadvantage in the meiosis of heterozygous animals with the 9a9b karyomorph. This is completely understandable, because in the meiosis of *A. callidryas,* as in other highly evolved Anura, the chiasmata are restricted to the telomeric regions of the bivalents. This means that the likelihood for a recombination between the mutually inverted segments in the 9a and 9b homologs and of any aberrant meiotic products resulting from this is very small. The absence of crossing-over between the inverted chromosome segments is a promoting factor for the maintenance of a normal degree of fertility in heterozygous *A. callidryas* animals and the successful spreading of the inverted chromosome 9b. Although only a single specimen with the heteromorphic 9a9b karyotype was demonstrated in the Pacific lowland population of Costa Rica in the present study, this can be related to the relatively small number of animals collected in this region (Table I).

The ancestral stock of the genus *Agalychnis* dispersed northward from South America through the Central American corridor during the Paleocene (Savage, 1982; Duellman and Trueb, 1986). The geographic range of *A. callidryas* is continuous from eastern and central Panama along the Atlantic and Pacific lowlands of Costa Rica, Nicaragua, Honduras, and Guatemala up to southern Mexico (Duellman, 1977). In Costa Rica, the Atlantic and Pacific populations of the species are geographically separated from each other by the Central American Cordilleras. Nevertheless, the inverted 9b chromosome occurs in both populations. It could be concluded from this that the inversion already existed in the ancestors of *A. callidryas* prior to the geographic separation of the various populations or else the inversion first occurred in one of the northern lowland populations and then spread southward along the Atlantic and Pacific slopes of the Cordilleras. An extensive cytogenetic analysis of *A. callidryas* and its closely related species throughout their entire area of distribution in Central America and Mexico could solve this question.

The other exceptional finding in *A. callidryas* is the variability in the number and locations of the 18S + 28S rRNA genes. The results of in situ hybridization experiments and silver-staining studies on numerous amphibians have supported the supposition that the ribosomal RNA genes are conservatively confined to a few chromosomal NORs, this being a constant karyological feature within a species. Most primitive and highly evolved Anura present only one pair of NORs in their karyotypes, and in closely related species the NORs are almost always located in homoeologous chromosomes (for reviews, see Schmid 1980b, 1982; King, 1990; Schmid et al., 1990). It is therefore remarkable that five (approximately 25%) of the *A. callidryas* specimens from the Atlantic lowland populations possess one or two NORs in addition to the standard NORs normally present in chromosome pair 5. In spite of the variation in number and position of NORs between these five specimens, the pattern is constant for each animal. All of the metaphases of any of these five *A. callidryas* specimens show a characteristic intraindividual NOR distribution.

Intraspecific polymorphisms in the number and locations of NORs have been discovered in five further amphibian species to date. In 11 of 55 specimens of the North American toad *Bufo terrestris* from various populations in eastern North Carolina, Foote et al. (1991) found one to three NORs in addition to the standard NOR in chromosome 1. As in *A. callidryas,* the extra NORs occurred in only one of the homologs in all of these *B. terrestris* specimens and had a paracentromeric, an interstitial, or a telomeric location. In the North American tree frog *Hyla chrysoscelis* and its tetraploid sibling species, *H. versicolor,* the most common NOR is located in chromosome 6. After examination of a total of 236 *H. chrysoscelis* specimens from 34 populations in 15 U.S. states and 100 *H. versicolor* specimens from 15 populations in 12 U.S. states, Wiley et al. (1989) discovered the existence of 13 additional NORs in *H. chrysoscelis* and 4 extra NORs in *H. versicolor.* As in *A. callidryas,* in most of these *Hyla* karyomorphs the NORs appeared to have been precisely excised from or inserted into the chromosomes without altering their morphology. Only in two of the *H. chrysoscelis* karyomorphs did it appear that a paracentric or pericentric inversion relocated the NOR within the same chromosome. Intraspecific variability of the number and location of NORs has also been reported for the North American newt *N. viridescens* (Hutchison and Pardue, 1975) and for the European newt *Triturus vulgaris meridionalis* (Nardi et al., 1977). In the last species it was shown that the additional NORs are inherited in simple Mendelian fashion (Batistoni et al., 1978).

Reciprocal translocations and/or inversions could hardly have been responsible for the multiple NORs in *A. callidryas,* since they would have altered the karyotypes in very different ways. As discussed by Wiley et al. (1989) and Foote et al. (1991) for *H. chrysoscelis, H. versicolor,* and *B. terrestris,* possible mechanisms involved in the origin of such NOR polymorphisms as detected in *A. callidryas* include: (1) mobile genetic elements closely linked to NORs and carrying them when they transpose (Syvanen, 1984), (2) amplification of "orphon"-like 18S + 28S rDNA cistrons (Childs et al., 1981), and (3) reinsertion errors during the extrachromosomal amplification of 18S + 28S rDNA during amphibian oogenesis (Buongiorno-Nardelli et al., 1972; Hourcade et al., 1974). Whether the NOR variability is in fact produced by one of these phenomena or whether it is due to a hitherto unknown genetic process can only be decided by the application of molecular techniques.

In the *A. callidryas* populations available for the present study, the multiple additional NORs were demonstrated exclusively in specimens from the Atlantic lowland populations of Costa Rica, but they seem to be absent in the animals collected in the Pacific lowlands, which exhibited only the standard NORs in chromosome pair 5. Analysis of further populations of *A. callidryas* will reveal whether the NOR polymorphisms are restricted to populations living to the north of the Central American Cordilleras.

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