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Cytosystematics of hyperoliid frogs: Phylogeny of *Heterixalus*, low karyotypic variability in hyperoliines and separate phylogenetic position of *Leptopelis*

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Abstract
Karyotypes of 13 species in the anuran family Hyperoliidae are described based on conventional staining, C-banding, Ag-NOR-banding and staining with fluorochromes (CMA_3 and DAPI). The nine studied species of the Malagasy genus *Heterixalus*, as well as African species of *Acanthixalus*, *Hyperolius* and *Kassina*, had a karyotype of $2n=24$ biarmed chromosomes with NORs on the ninth chromosome pair, whereas the sole species of *Leptopelis* studied had $2n=24$ with one telocentric pair and NORs on the fifth pair. These data confirm the isolated position of *Leptopelis*, which according to molecular data does not form a clade with other hyperoliids. Details of NOR location, relative chromosome size and heterochromatin distribution suggest a phylogenetic hypothesis, within *Heterixalus*, that is largely though not completely in agreement with bioacoustic and molecular data sets: ((*betsileo*, *tricolor*, *variabilis*, (*andrakata*, (*alboguttatus*, *boettgeri*))), (*rutenbergi*, (*luteostriatus*, *punctatus*))). In general, hyperoliines seem to be characterized by evolutionary stability in chromosome number and NOR-bearing chromosomes, although some rearrangements such as inversions and translocations occurred in their evolution.

Keywords: *Amphibia, Hyperoliidae, chromosomes, NOR,* Heterixalus, Leptopelis, *phylogeny*

Introduction

Hyperoliid frogs are endemic to Africa, Madagascar, and the Seychelles. Their phylogeny and systematics have been quite intensively studied. Besides the most influential work of Drewes (1984), recent contributions were published by Channing (1989), Richards & Moore (1998), Schiøtz (1999), Vences et al. (2003a), and Drewes & Wilkinson (2004). Mitochondrial and nuclear DNA phylogenies have indicated that *Leptopelis* is the most divergent genus and does not even form a monophyletic group with the other hyperoliids (Emerson et al. 2000; Vences et al. 2003a; Van der Meijden et al. 2004). Consequently, this genus has recently been proposed to belong to a redefined family Arthroleptidae (Frost et al. 2006). Also, there is strong evidence that the Seychellean *Tachycnemis* is the sister group of the Madagascan genus *Heterixalus* (Richards & Moore 1998; Vences et al. 2003a), and the São Tomé and Principe endemic *Nesionixalus* is nested within the large genus *Hyperolius* and thus a junior synonym (Drewes & Wilkinson 2004). However, the limited molecular data available so far (Vences et al. 2003a; Drewes & Wilkinson 2004) were unable to clarify the phylogenetic affinities of a number of species-poor enigmatic hyperoliid genera from tropical Africa, such as for example the Wax frogs, *Cryptothylax*, or the tree-hole breeding African wart frogs, *Acanthixalus*.

Karyological data are known to bear potential for systematic studies in amphibians (King 1990), and they may also be relevant to understand mechanisms and rates of speciation in these animals (e.g. Bogart & Hedges 1995; Vences et al. 2002). Among the about 250 species of hyperoliids currently, chromosome information is only available for 42 species (summarized in Table I). All these karyotypes were described based on conventional staining, except for

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Table I. Summary of previous knowledge on chromosome numbers of hyperoliid frogs. For detailed references, see King (1990). *Semnodactylus weali* was originally considered to belong to the genus *Kassina* and its karyotype described as *Kassina weali.*

	No. species studied	No. chromosomes ($2n$)	No. sp. applied banding
Hyperolius	16	24	–
Heterixalus	2	24	–
Opisthothylax	1	24	–
Tachynemis	1	24	–
Afrixalus	6	24	–
Kassina	2	24	–
Semnodactylus	1	24	1 (Ag-NOR, CMA_3)
Phlyctimantis	1	24	–
Leptopelis	11	22–24–30	1 (Ag-NOR, CMA_3)

two species (one *Semnodactylus*, one *Leptopelis*) in which banding techniques to identify the nucleolus organizer regions (NOR) were also performed (reviewed in King 1990).

Blommers-Schlösser (1978) described the karyotype of the endemic Malagasy hyperoliids *Heterixalus betsileo* and *H. madagascariensis* (as *H. tricolor*). These had $2n=24$ biarmed chromosomes, the first five pairs being distinctly larger than the remaining seven pairs. Pairs 2, 3 and 4 were submetacentric, the latter close to subtelocentric state, and the remaining pairs were all metacentric. These chromosomal characteristics were considered to be the basal state in hyperoliids (Bogart & Tandy 1981) and were observed in all genera and species examined to date, with the exception of *Leptopelis*. In this genus, the chromosome number is variable, with species having a larger ($2n=30$) or smaller ($2n=22$) number of chromosomes, and a variable number of telocentric elements. In *Leptopelis bocagei*, the position of the NORs was peritelomeric on the long arm of the fifth chromosome pair (Schmid 1980), whereas in *Semnodactylus wealii* they were telomeric on the long arm of the ninth pair (Schmid 1978).

The present paper aims at contributing to the knowledge of hyperoliid and leptopelid karyology, focusing mainly on the Malagasy genus *Heterixalus* for which we studied 9 out of 12 known species, as well as on 4 African hyperoliids: *Acanthixalus spinosus*, *Hyperolius* cf. *viridiflavus*, *Kassina maculata* and *Leptopelis calcaratus*. Hence, we provide previously undescribed karyotypes for seven species of *Heterixalus*, for *Acanthixalus*, and for one species of *Leptopelis*. We extend previous studies to include also banding analyses (Ag-NOR, C-banding, C- and Alu I banding, $+CMA_3$+DAPI), and provide the first such data for the genera *Acanthixalus*, *Heterixalus*, *Hyperolius* and *Kassina*. We use the novel data to test the hypothesis of conserved karyotypic structure in hyperoliines vs. *Leptopelis*, and explore the utility of chromosome data to asses the intrageneric phylogeny of *Heterixalus*.

Materials and methods

We examined chromosome preparations of the following specimens: *Heterixalus alboguttatus*, one specimen from Ranomafana, Madagascar; *H. andrakata*, one male from Sambava, Madagascar; *H. betsileo*, one male from Manjakatompo, Madagascar (MRSN A4561); *H. boettgeri*, one specimen from near Tolagnaro, Madagascar; *H. luteostriatus*, two males from Betsimipoaka, Sahamalaza peninsula, Madagascar (MRSN A4552, A4550); *H. punctatus*, one male from Sambava, Madagascar; *H. rutenbergi*, four males and one female, from Madagascar (no precise locality; GA 01–05); *H. tricolor*, two males from the type locality Nosy Be, Madagascar; *H. variabilis*, one male and one female from Ambanja, Madagascar (MRSN A4556, A4555); *Acanthixalus spinosus*, one male from Cameroon; *Hyperolius* cf. *viridiflavus*, one female (from Kafue River, Zambia), *Kassina maculata*, one female from Natal, South Africa; *Leptopelis calcaratus*, two males from Cameroon (ZFMK uncatalogued) (collection acronyms are GA, Gennaro Aprea fieldnumbers, specimens to be deposited in the Museo Regionale di Scienze Naturali, Torino, Italy, MRSN, ZFMK, Zoologisches Forschungsmuseum A. Koenig, Bonn, Germany).

Heterixalus specimens were processed during fieldwork in Madagascar in 2000–2003. Specimens were injected with 0.1 ml/10 g body weight of a 0.5 mg/ml colchicine solution. One hour later specimens were euthanized by immersion in a MS 222 solution, and intestine, lungs, spleen and gonads removed. These organs were incubated for 30 min in a solution of sodium citrate (0.5%) and fixed in 3:1 methanol and acetic acid. The fixed material was preserved at 4°C and transferred to the laboratory in Naples. African hyperoliids were transferred alive to Naples and processed there.

Chromosomes were revealed using the air-drying method. They were studied with conventional methods (5% Giemsa at pH 7) and subsequently with banding techniques: Ag-NOR following Howell & Black (1980); chromomycin A_3 (CMA_3)/methyl green according to Sahar & Latt (1980) but reducing the time of exposure to the non-fluorescent methyl green to a few seconds only; C-banding according to Sumner (1972) but incubating the preparations

in barium hydroxide at 45°C for 5 min; in-situ digestion with the *Alu* I endonuclease following Mezzanotte et al. (1983). In addition we performed a sequential treatment of the preparations: after hydrolysis with $Ba(OH)_2$ or *Alu* I digestion, chromosomes were stained with CMA_3 and DAPI (Odierna et al. 1999).

Results

All *Heterixalus* species studied had a similar general chromosome morphology: $2n=24$ biarmed elements of decreasing size. Relative chromosome lengths and centromeric indices for all species studied are summarized in Table II. In most species, the first chromosome pair was distinctly larger than pairs 2–5, and the second, third and fourth pairs were submetacentric (Figure 1; Table II). In some species, also a variable number of the elements 7–12 was submetacentric (Table I). *Heterixalus luteostriatus* and *H. punctatus* differed in that their second pair was metacentric rather than submetacentric, and of similar length as the first pair (Figure 1). *Acanthixalus spinosus, Hyperolius* cf. *viridiflavus* and *Kassina maculata* had karyotypes similar to those found in *Heterixalus* (Figure 1). *Leptopelis calcaratus* had $2n=24$ chromosomes, all biarmed except the 12th pair that was telocentric (Figure 1). In *Kassina*, the 12th chromsome pair was strongly submetacentric (centromeric index 24.2 ± 2.8; Table II), but clearly biarmed in contrast to *Leptopelis* where it was fully telocentric.

Loci of NORs, as individuated from Ag-NOR- and CMA_3-staining, were located in pericentromeric position on the long arm of the fifth chromosome pair in *Leptopelis calcaratus*, whereas they were on the ninth chromosome pair in all other hyperoliids studied. However, the exact NOR position was variable: in telomeric position on the short arm in *Acanthixalus*; telomeric on the long arm in *Kassina* and *Hyperolius*; peritelomeric on the long arm in most *Heterixalus*, and interstitially on the short arm in *Heterixalus luteostriatus*, *H. rutenbergi* and *H. punctatus*.

The various banding techniques revealed further differences, also among those karyotypes of similar general morphology (Figures 2–4). *Heterixalus luteostriatus* had mostly telomeric C-bands, *H. alboguttatus* and *H. boettgeri* had paracentromeric bands on almost all chromosomes, *H. andrakata* had paracentromeric bands on only some chromosomes, and the other *Heterixalus* species had centromeric bands on all chromosomes. Further differences were observed after the various fluorochrome staining methods and are summarized in Table III. *Acanthixalus* had solid centromeric and telomeric C-bands on all chromosomes, and a strong band that made up most of the distal part of the long arm of the sixth chromosome pair. These bands were negative to both fluorochromes (CMA_3 and DAPI). *Hyperolius* had largely telomeric heterochromatin that was CMA_3- and DAPI-negative as well. *Kassina maculata* had centromeric C-bands on all chromosomes which were CMA_3-positive subsequent to C-banding but negative to both fluorochromes after *Alu* I digestion. *Leptopelis* had centromeric and telomeric C-bands on all chromosomes, the centromeric bands being negative to CMA_3 and DAPI subsequent to C-banding but positive to both stains after *Alu* I digestion.

Discussion

Tempo and pattern of chromosome evolution

Our study provides evidence that in hyperoliid frogs (excluding *Leptopelis* which we consider as not belonging to the hyperoliidae; Frost et al. 2006) some chromosomal rearrangements have taken place, although these did not result in changes in chromosome number or in NOR-bearing chromosomes. Inversions are most likely responsible for the relocation of the NORs from being terminal on the long arm (*Hyperolius*, *Kassina*) to terminal on the short arm (*Acanthixalus*), or from peritelomeric to interstitial on the long arm, within *Heterixalus*. The larger size of the second chromosome pair in *Heterixalus luteostriatus* and *H. punctatus* is probably a result of a translocation of genomic material from the first to the second chromosome pair. Numerous events of deletion/amplification, minute insertions and amplification of specific families of satellite DNA may explain the large variability in the distribution of heterochromatin among the species; this genomic material, largely consisting of repetitive DNA and mainly localized in the centromeric or telomeric regions, is not subjected to meiotic constraints and therefore variable even within groups of closely related species (John 1988; King 1990).

Among anurans, several lineages are known to be characterized by a remarkable evolutionary stability of their karyotype (e.g. *Boophis* and *Mantella*, Blommers-Schlösser 1978; Pintak et al. 1998; Odierna et al. 2001; Aprea et al. 2004; or *Bufo*, e.g. Baldissera et al. 1999) while others have a very high rate of chromosomal change (e.g. *Eleutherodactylus* and some lineages of *Mantidactylus*, Bogart & Hedges 1995; Andreone et al. 2003). Apparently,

Table II. Relative lengths (±SD; upper value) and centromerix indices (±SD; lower value) of chromosomes 1–12 in hyperoliid karyotypes studied.

Chromosome	1	2	3	4	5	6	7	8	9	10	11	12
H. alboguttatus	14.7±0.5	11.8±0.7	11.4±0.8	10.5±0.4	10.5±0.6	9.1±0.7	6.8±0.5	6.3±0.6	5.3±0.5	4.9±0.7	4.7±0.7	4.6±0.8
	48.8±4.7	34.2±3.2	32.4±3.8	32.5±3.0	48.7±2.9	47.5±3.0	48.0±3.7	45.4±3.3	42.9±4.5	41.7±5.0	41.6±3.6	44.6±2.8
H. andrakata	16.5±0.6	10.6±0.8	10.6±0.4	10.3±0.7	10.3±0.5	7.0±0.6	6.5±0.6	6.5±0.5	6.4±0.7	5.7±0.8	5.2±0.6	4.4±0.6
	47.9±4.1	30.8±3.9	35.6±4.0	33.8±4.6	40.1±3.5	44.8±4.0	45.9±3.9	47.5±4.3	48.8±4.9	45.6±4.4	43.8±4.1	45.6±3.9
H. betsileo	14.3±0.5	11.5±0.6	11.4±0.5	9.8±0.6	9.8±0.7	7.4±0.9	6.9±0.5	6.6±0.7	6.1±0.9	6.0±0.6	5.2±0.7	5.0±0.6
	46.8±3.9	31.8±3.7	34.9±2.7	30.9±4.0	41.4±3.5	43.8±3.5	43.9±3.8	45.8±3.0	42.9±4.3	46.8±2.8	43.8±3.0	47.2±3.7
H. boettgeri	15.0±0.5	12.2±0.7	11.0±0.6	10.2±0.8	10.2±0.5	8.4±0.6	6.3±0.8	6.1±0.4	5.5±0.8	5.5±0.7	5.3±0.5	4.3±0.4
	43.2±4.6	30.8±3.1	34.9±3.6	32.7±3.2	45.6±4.3	44.7±3.6	44.0±3.9	43.8±4.3	46.9±4.4	47.3±3.7	38.8±4.0	45.3±3.3
H. luteostriatus	13.7±0.6	11.0±0.5	10.7±0.8	10.3±0.5	10.3±0.7	8.3±0.5	6.5±0.6	6.4±0.8	6.0±0.9	5.9±0.8	5.8±0.6	5.1±0.6
	41.9±3.9	39.9±4.0	31.8±3.7	32.9±3.5	44.2±2.0	43.8±2.9	46.5±3.2	39.9±3.1	47.2±3.9	44.2±3.5	40.1±2.9	46.3±3.5
H. punctatus	13.7±0.6	11.3±0.7	10.9±0.6	10.1±0.5	9.9±0.6	8.3±0.8	7.9±0.6	6.4±0.6	6.1±0.7	5.9±0.5	4.8±0.6	4.7±0.8
	44.0±3.0	40.4±4.2	30.0±3.5	34.2±2.9	40.3±3.2	44.8±3.0	45.3±2.6	43.9±3.3	45.0±3.0	47.5±2.9	39.7±2.7	46.8±3.1
H rutenbergi	14.4±0.7	12.0±0.8	11.4±0.6	9.5±0.7	9.3±0.8	6.9±0.6	6.8±0.7	6.3±0.5	6.2±0.4	5.9±0.6	5.7±0.5	5.6±0.4
	43.2±3.2	33.8±3.0	32.7±2.8	31.9±3.0	42.5±3.4	43.2±2.8	44.8±3.8	46.7±3.6	45.3±4.0	42.8±2.9	46.0±3.2	44.8±3.5
H. tricolor	13.6±0.5	12.7±0.6	12.1±0.7	9.7±0.6	9.1±0.5	8.4±0.8	7.6±0.6	6.5±0.5	5.2±0.7	5.8±0.9	5.0±0.6	4.3±0.5
	41.9±3.0	32.8±2.9	34.8±3.8	32.0±3.5	42.8±3.1	44.5±2.9	45.7±2.2	46.3±3.8	43.8±4.0	48.4±2.9	43.2±3.0	44.8±2.9
H. variabilis	13.1±0.4	10.4±0.4	10.1±0.7	9.6±0.6	9.4±0.7	9.1±0.5	7.8±0.7	7.2±0.8	6.5±0.6	6.2±0.7	5.8±0.6	5.0±0.6
	43.8±3.8	32.5±3.0	32.0±2.7	31.5±3.0	44.3±2.7	45.8±3.3	46.9±3.2	47.6±2.9	43.8±4.1	44.9±2.9	45.0±3.5	46.8±2.5
A. spinosus	15.3±0.6	11.5±0.6	10.4±0.5	10.0±0.6	9.3±0.5	8.6±0.7	8.0±0.6	6.1±0.6	5.9±0.8	5.8±0.4	5.1±0.6	4.0±0.5
	40.0±3.2	35.8±3.0	33.8±2.9	34.9±2.7	45.3±3.3	45.2±2.8	42.8±3.2	44.7±3.8	45.3±2.7	40.6±3.0	43.5±4.1	38.9±3.5
Hy. cf. *viridiflavus*	15.0±0.5	10.9±0.8	10.8±0.7	9.7±0.6	9.5±0.6	8.0±0.7	6.9±0.5	6.7±0.8	6.3±0.7	5.8±0.6	5.4±0.7	5.0±0.5
	43.4±3.3	32.0±3.2	29.4±2.8	31.2±3.3	41.1±2.8	42.6±3.0	42.2±2.0	44.4±3.5	46.8±3.4	41.9±3.7	43.3±2.9	45.0±3.0
K. maculata	13.0±0.6	11.5±0.7	10.4±0.6	9.9±0.6	9.9±0.5	7.9±0.4	7.8±0.6	6.3±0.7	6.3±0.6	6.2±0.5	6.1±0.4	4.7±0.8
	45.2±2.8	30.2±2.8	30.9±3.0	32.1±3.1	43.6±2.9	43.4±2.9	46.8±2.7	48.6±3.9	41.9±3.6	38.0±3.2	46.3±3.	24.2±2.8
L. calcaratus	15.5±0.7	11.5±0.5	11.0±0.6	10.4±0.5	10.3±0.7	7.7±0.6	6.4 ±0.8	6.0±0.5	5.7±0.6	6.1±0.4	6.0±0.8	3.4±0.7
	45.3±2.7	26.9±3.8	29.0±3.4	36.0±2.8	43.2±3.8	44.9±2.	43.3±2.0	43.5±3.8	42.9±3.9	36.8±2.5	46.3±3.6	0.0

Figure 1. Giemsa stained karyotypes of the 13 studied hyperoliids. **A**, *Heterixalus alboguttatus*; **B**, *H. andrakata*; **C**, *H. betsileo*; **D**, *H. boettgeri*; **E**, *H. luteostriatus*; **F**, *H. punctatus*; **G**, *H. rutenbergi*; **H**, *H. tricolor*; **I**, *H. variabilis*; **J**, *Acanthixalus spinosus*; **K**, *Hyperolius* cf. *viridiflavus*; **L**, *Kassina maculata*; **M**, *Leptopelis calcaratus*. The Ag-NOR banded pairs are reported above the corresponding Giemsa stained pairs.

Figure 2. C-banded karyotypes of the 13 studied studied hyperoliids. **A**, *Heterixalus alboguttatus*; **B**, *H. andrakata*; **C**, *H. betsileo* **D**, *H. boettgeri*; **E**, *H. luteostriatus*; **F**, *H. punctatus*; **G**, *H. rutenbergi*; **H**, *H. tricolor*; **I**, *H. variabilis*; **J**, *Acanthixalus spinosus*; **K**, *Hyperolius* cf. *viridiflavus*; **L**, *Kassina maculata*; **M**, *Leptopelis calcaratus*.

a low rate of chromosomal evolution also characterizes the Hyperoliidae. All species studied to date have a karyotype of $2n=24$ biarmed chromosomes (Morescalchi et al. 1970; Blommers-Schlösser 1978; Schmid 1978, 1980; Bogart & Tandy 1981; this study), and those studied with Ag-NOR banding techniques, belonging to the genera *Acanthixalus*, *Heterixalus*, *Hyperolius*, *Kassina* and *Semnodactylus*, have NORs located on the ninth chromosome pair (Schmid 1978, 1980; this study). Our study provides evidence that, nevertheless, events such as inversions and translocations do occur in hyperoliines, but apparently did not or rarely affect the stability of chromosome number and NOR location.

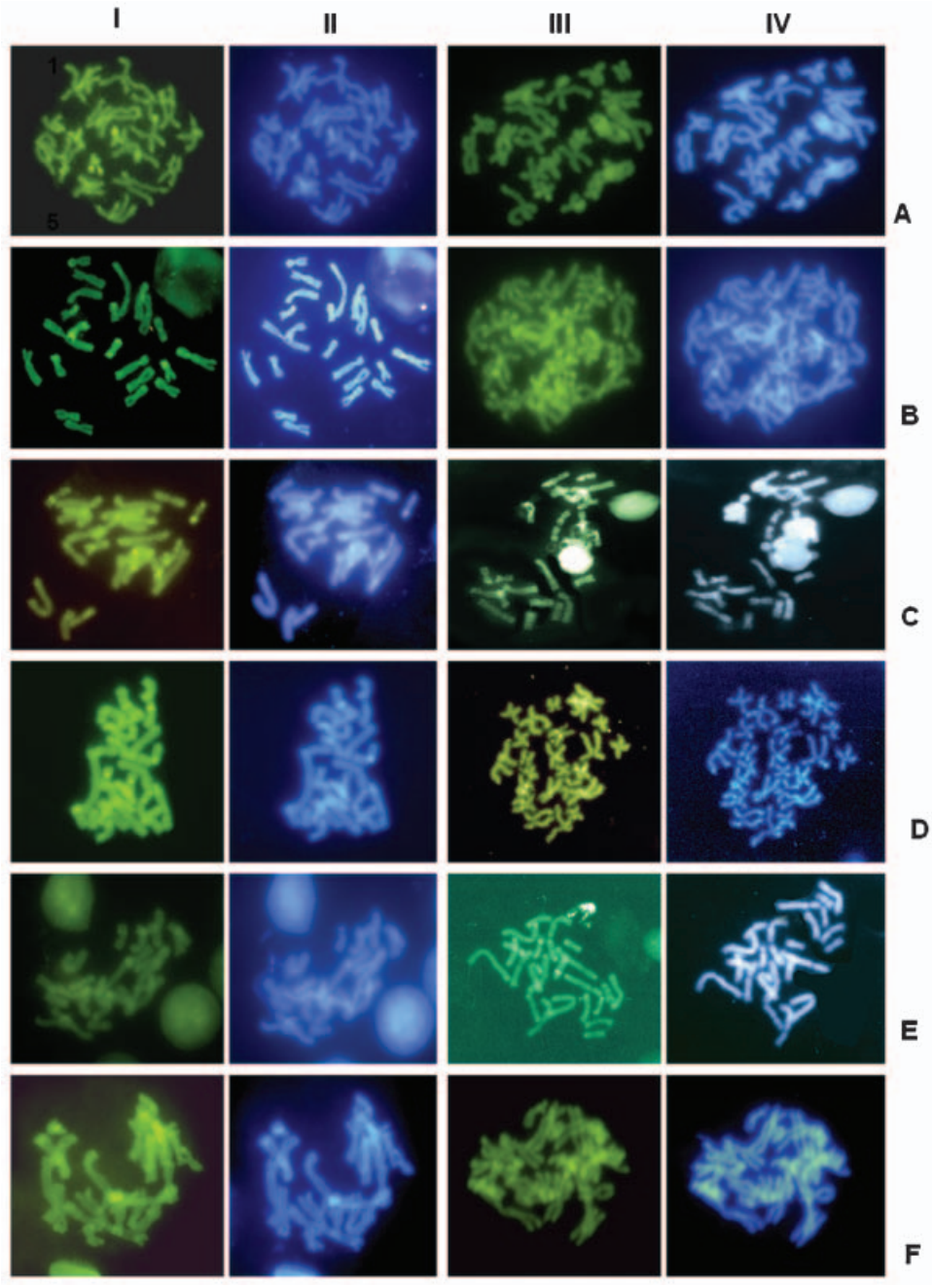

Figure 3. Sequential C-banding (rows I and II) or Alu I (rows III and IV)+CMA_3 (rows I and III)+DAPI (rows II and IV) of: **A**, *H. alboguttatus*; **B**, *H. andrakata*; **C**, *H. betsileo*; **D**, *H. boettgeri*; **E**, *H. luteostriatus*; and **F**, *H. punctatus*. The colour version of this figure is available online.

Deciphering the factors that influence the frequency of chromosomal rearrangements, and their influences on the karyotype, in different lineages of frogs appears to be a fruitful field of study.

Cytosystematics

The low variability in general chromosome number and NOR location encountered among congeneric taxa of hyperoliids, and the absence of individual differences in the species of *Heterixalus* where more than one individual was studied (*Heterixalus luteostriatus*, *H. rutenbergi*, *H. tricolor*, *H. variabilis*), indicates that the obtained karyotypes are likely to represent the typical pattern for each species, despite the low number of individuals available to us for several species (in several cases a single specimen only). Since no sex chromosomes were

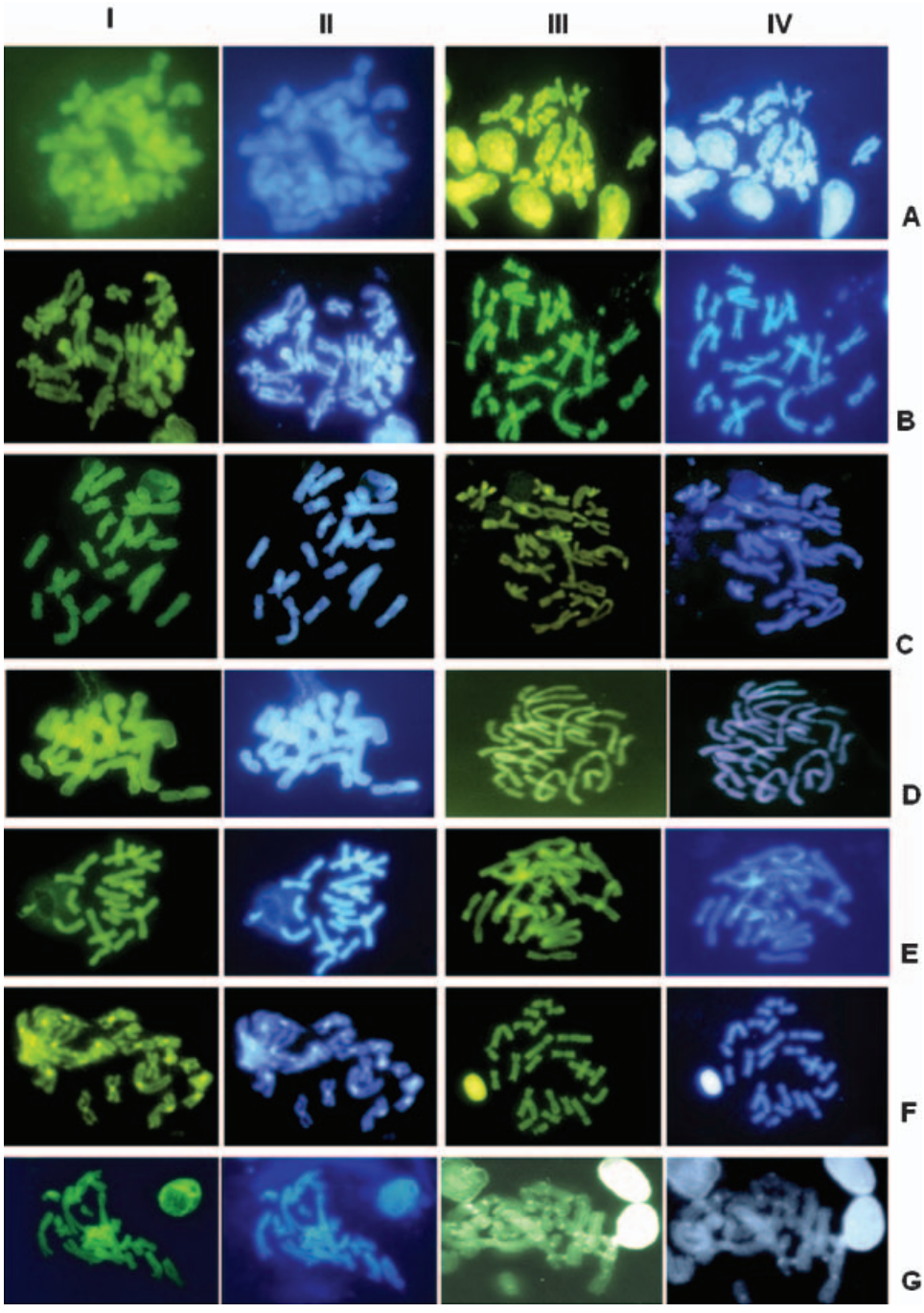

Figure 4. Sequential C-banding (rows I and II) or Alu I (rows III and IV)+CMA_3 (rows I and III)+DAPI (rows II and IV) of **A**, *H. rutenbergi*; **B**, *H. tricolor*; **C**, *H. variabilis*; **D**, *Acanthixalus spinosus*; **E**, *Hyperolius* cf. *viridiflavus*; **F**, *Kassina maculata*; and **G**, *Leptopelis calcaratu*s. The colour version of this figure is available online.

detected in the species where males and females were observed (*Heterixalus rutenbergi*, *H. variabilis*), and sex chromosomes are in general rare in amphibians (King 1990), we are confident that no bias due to sexual dimorphism in karyotype is present in our data set, allowing for some systematic considerations.

The general karyotypes found in the hyperoliid species studied herein agree with those described for 16 species of *Hyperolius* (Bogart & Tandy 1981), and for one *Semnodactylus* and two *Kassina* (Morescalchi et al. 1970; Schmid 1978, 1980; Bogart & Tandy 1981). Also the karyotype of *Leptopelis calcaratus* agrees with the data of Bogart & Tandy (1981) for

Table III. Summary of karyotype data resulting from the present study. Abbreviations used: Cen=centromeric; Tel=Telomeric; Pcen=Paracentromeric; m=metacentric; sm=submetacentric; p→int=NORs interstitial on short arm; q→stel=NORs peritelomeric on long arm; p→tel=NORs telomeric on short arm; q→tel=NORs telomeric on long arm; p→cen=pericentromeric on long arm; Neg=negative; pr/prs=pair(s).

Species	2*n*	2nd pair	NORs	C-banding+Giemsa	Paracentromeric C-bands	C-banding+CMA_3+DAPI	Alu I-banding+CMA_3+DAPI	NOR associated heterocromatin
Heterixalus								
H. alboguttatus	24	sm	9th; q→stel	Cen (+5 prs), Tel (+4 prs)	Pairs 2,3,5,6,7,8	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
H. andrakata	24	sm	9th; q→stel	Cen (+all prs), Tel±	Pairs 5 and 8	Neg +(3 prs)	Cen−, T+Neg	C±, CMA_3+, Alu I+
H. betsileo	24	sm	9th; q→stel	Cen (+8 prs), Tel (+2 prs)	None	Cen (+2 prs) Neg	Neg Neg	C±, CMA_3+, Alu I+
H. boettgeri	24	sm	9th; q→stel	Cen (+1 pr, Tel (+10 prs)	Pairs 3,5,6, 8	Pcen+Pcen+	Pcen+Pcen+	C+, CMA_3+, Alu I+
H. luteostriatus	24	m	9th; p→int	Cen (+2 prs), Tel (+all prs)	None	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
H. punctatus	24	m	9th; p→int	Cen (+9 prs), Tel−	None	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
H. rutenbergi	24	sm	9th; p→int	Cen (+all pairs), Tel−	None	Neg Neg	Neg Neg	C±, CMA_3+, Alu I+
H. tricolor	24	sm	9th; q→stel	Cen (+all pairs), Tel (+2 prs)	None	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
H. variabilis	24	sm	9th; q→stel	Cen (+4 prs), Tel (+10 prs)	None	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
Acanthixalus								
A. spinosus	24	sm	9th; p→tel	Cen (+all prs), Tel (++5th pr)	None	Neg Neg	Neg Neg	C−, CMA_3−, Alu I−
Hyperolius								
H. cf. *viridiflavus*	24	sm	9th; q→tel	Cen (+2prs), Tel (+7 prs)	None	Neg Neg	Neg Neg	C+, CMA_3+, Alu I+
Kassina								
K. maculata	24	sm	9th; q→tel	Cen (+all prs), Tel (+3 prs)	None	Cen+Cen+	Neg Neg	C−, CMA_3−, Alu I−
Leptopelis								
L. calcaratus	24	sm	5th; p→cen	Cen (+all prs),Tel (+all prs)	Pair 1	Neg Neg	Cen+ Cen+	C−, CMA_3−, Alu I−

this species. In conjunction with the published data, our results from general chromosome morphology, NOR location, and banding are informative to assess the systematics of hyperoliid frogs in various respects.

Available molecular phylogenies for the genus *Heterixalus* are inconclusive (Vences et al. 2003a). At present, we are in the process of analysing larger and more comprehensive data sets (ca. 2000 bp data set of two nuclear and three mitochondrial genes; Wollenberg, Glaw, Meyer and Vences, in preparation). However, also this data set does not resolve satisfyingly several basal relationships within the genus. It therefore seems reasonable to explore whether the karyological data may be informative to help reconstructing intrageneric *Heterixalus* phylogeny. Of the other hyperoliid genera studied here, *Hyperolius* is the one most closely related to the Malagasy *Heterixalus* (Frost et al. 2006), which are most probably a monophyletic group (Vences et al. 2003a). The fact that the NORs are in terminal position in *Hyperolius* as well as in the more distantly related *Kassina* lead us to consider this position as plesiomorphic in hyperoliines. A similar (subterminal) position is observed in *Heterixalus alboguttatus*, *H. andrakata*, *H. betsileo*, *H. boettgeri*, *H. tricolor* and *H. variabilis*, and we hypothesize that it represents the ancestral state for *Heterixalus*. A modification occurred by a pericentromeric inversion to the interstitial position on the short arm in *H. luteostriatus*, *H. punctatus* and *H. rutenbergi*, which would characterize these three species as monophyletic group. Of these species, *H. luteostriatus* and *H. punctatus* further share the metacentric state and larger size of the second chromosome pair.

The remaining *Heterixalus* can be divided in two subgroups in which the heterochromatin is distributed in (1) mainly pericentromeric bands (*H. alboguttatus*, *H. andrakata*, *H. boettgeri*) or (2) mainly centromeric bands (*H. betsileo*, *H. tricolor*, *H. variabilis*). In the first subgroup, *H. alboguttatus* and *H. boettgeri* further share more homologous chromosome pairs with paracentromeric bands than each of them with *H. andrakata*.

Summarizing, the phylogenetic relationships among *Heterixalus* suggested by our interpretation of the chromosomal data are ((*betsileo*, *tricolor*, *variabilis*, (*andrakata*, (*alboguttatus*, *boettgeri*))), (*rutenbergi*, (*luteostriatus*, *punctatus*))). This hypothesis is in relatively good agreement with bioacoustic data as summarized by Glaw & Vences (1993, 1994), and molecular data (Vences et al. 2003a, and unpublished data). The close relationships of *H. alboguttatus* and *H. boettgeri* are obvious from all available data sets, and also supported by the karyological characters. The close relationships of *H. tricolor* and *H. variabilis* as suggested by the karyological characters are also well assessed by molecular and bioacoustic means, and indeed *H. variabilis* may be a junior synonym of *H. tricolor* (Glaw & Vences 1993). The similarity of advertisement call structure would predict a closer relationship of *H. luteostriatus* with *H. boettgeri* and *H. alboguttatus*, but molecular data so far failed to confirm this, in agreement with the distinctly different karyotypes of these species. Interestingly, the possibility of relationships between *H. punctatus* and *H. rutenbergi*, as inferred from the chromosomal characters, is also indicated by unpublished comprehensive molecular data. The divergent karyotype of *H. andrakata* appears to confirm its status as distinct taxon, despite its very obvious and close relationships (by bioacoustic and genetic data) to *H. tricolor* and *H. variabilis*.

As a second cytosystematic aspect, the isolated position of *Leptopelis* in a position unrelated to hyperoliids is supported by several lines of evidence. First, representatives of this genus have a variable chromosome number, although the species studied here, *L. calcaratus*, agrees with the other hyperoliids in its diploid complement of $2n=24$. Second, all *Leptopelis* studied so far had at least one telocentric chromosome pair, a state that so far is unknown in other hyperoliids (*Kassina* having a strongly subtelocentric but not telocentric twelfth pair). Third, the NOR position on the fifth chromosome pair differs from all other hyperoliids. These data confirm that *Leptopelis* is the most deviant of the genera studied here, and are in agreement with the hypothesis that this genus is more closely related to astylosternid and possibly arthroleptid frogs (Emerson et al. 2000; Vences et al. 2003a, 2003b; Frost et al. 2006). However, karyology seems to provide little information in this respect. Arthroleptids are characterized by a strong chromosome reduction ($2n=18$, 16 and 14), whereas the two astylosternids studied have higher numbers ($2n=28$ in *Nyctibates*, and $4\times=54$ in *Astylosternus diadematus*) (references in King 1990), of which the presumably tetraploid numbers in *Astylosternus* are in need of confirmation. The location of NORs has proven to be of high significance for the assessment of phylogenetic relationships and systematics of amphibians (King 1990), including Malagasy frogs (Andreone et al. 2003; Aprea et al. 2004), but no banding has been performed so far to localize the NORs in representatives of the Astylosternidae or Arthroleptidae.

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