



Evidence for a remarkable stasis of chromosome evolution in Malagasy treefrogs (*Boophis*, Mantellidae)

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ABSTRACT

A comparison of chromosome morphology of seventeen species of *Boophis* (Mantellidae, Boophinae) was carried out applying Giemsa, C-, DAPI-, CMA₃- and AgNOR- banding methods. All of the species studied showed a karyotype of $2n = 26$ banded chromosomes. The nucleolar organizer regions (NORs) were interstitially located in a secondary constriction on the long arm of the 6th chromosome pair in all species, except *B. cf. ruficulis* and *B. cf. madagascariensis* in which the NORs were dislocated on the short arms of the 6th pair. Heterochromatin distribution and composition was very variable among the species, displaying C-bands positive or negative both to DAPI and CMA₃, or positive only at one of the two fluorochromes. Taxonomic and phylogenetic implications of these results are discussed.

KEY WORDS: Anura - Mantellidae - *Boophis* - Madagascar - Karyotype - NORs - Heterochromatin.

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INTRODUCTION

Malagasy frogs are included in four families, Hyperoliidae, Microhylidae, Mantellidae, and Ranidae (Vences & Glaw, 2001). According to recent estimates it is likely that the total number of species is at least 250, all of which are endemic, except the introduced *Hoplobatrachus tigerinus* and, possibly, the populations attributed to *Ptychadena mascareniensis* (Glaw & Vences, 1994; Andreone, 1999). However, still the taxonomy and phylogeny of several taxa raise many questions. Most of the species formerly included in the Ranidae (Blommers-Schlösser, 1993), except for the two non-endemic ones, are today considered as a single endemic clade of not fully resolved relationships to African or Asian groups, for which the family name Mantellidae (and three subfamilies: Boophinae, Laliostominae, Mantellinae) is used (Richards & Moore, 1998; Vences *et al.*, 2000; Vences & Glaw, 2001).

Among the most species-rich groups is the genus *Boophis* with currently about 50 nominal species. This genus contains mainly treefrogs which underwent a species-rich adaptive radiation, adapting to forest environments. *Boophis* mainly inhabit the eastern rainforest belt, although a few species of the *B. tephraeomystax* group and *B. occidentalis* also colonized western deciduous forests and open habitats. Despite a certain morphological variety, and a considerable range of body size, *Boophis* constitute a well defined monophyletic unit (Vences *et al.*, 2002). Based on morphological and ecological traits, seven infrageneric phenetic species groups were recognized by Glaw & Vences (1994): the *Boophis albilabris*, *B. luteus*, *B. rappiodes*, *B. difficilis*, *B. goudoti*, *B. microtympanum*, and *B. tephraeomystax* groups. Taxonomic revisions of most of the species groups are badly needed, and few characters have so far been identified that could be used for phylogenetic assessment. Karyology could provide a means for this purpose. In Malagasy frogs, karyological data were not very informative in *Mantella* (Pintak *et al.*, 1998; Odierna *et al.*, 2001), but provided a powerful tool in some groups of *Mantidactylus*: representatives of this genus showed a remarkable variability in presence or absence of acrocentric elements and in the location of NOR, which have been used to assess their relationships (Aprea G. *et al.*, 2002, Abstract in IV Congr. Naz. SHI: 67; Andreone *et al.*, 2003). Some previous studies on the chromosomes of twelve species of the genus *Boophis* were performed using conventional staining (Blommers-Schlösser, 1978), but until now no banding analysis was made for these anurans. To test recent phylogenetic hypotheses in *Boophis*, we conducted a karyological study by means of various banding methods on seventeen species of *Boophis*, thirteen of them analysed for the first time.

MATERIALS AND METHODS

Localities of capture

The specimens for which karyological data are here presented were captured in Madagascar on the occasion of several field-surveys. The localities and references, together with the main researcher(s) and date of capture [between square brackets], are as follows: (1) Ambolokopatrika (Andreone *et al.*, 2000) [Andreone & Aprea, XI-XII.1997]; Besariaka (Andreone *et al.*, 2000) [Andreone, VI.1996]; Masoala (Andreone & Greer, 2002) [Andreone, XI-XII.1998, XI-XII.1999]; Ankaratra (Vences *et al.*, 2002) [M. Vences, III.2001]; Berara (Andreone *et al.*, 2001) [Andreone & Vences, I.2001]; Kirindy (Bloxam *et al.*, 1996) [Andreone, II.1999].

Specimens examined and nomenclature

Vouchers of the analysed specimens are currently deposited in the Museo Regionale di Scienze Naturali (MRSN, Torino, Italy). Their museum number, sex, and locality of capture are reported in Table I. Concerning the taxonomy used, it is worth stressing that this must be considered purely indicative. In fact, it was not possible to attribute reliably most of the collected specimens to any well-known species. This is most evident for some species captured by us in the North of Madagascar, a region that is clearly a source of numerous new, not yet identified species (Andreone, 2004; Vences, 2004). Their specific attribution, as treated in this paper, must be necessarily understood as a 'working taxonomy', and we can anticipate that some of them will be described as new taxa in forthcoming papers.

Preparation techniques

The chromosomes were obtained from intestine, spleen, gonads and lungs using the air drying method as described elsewhere (Odierna *et al.*, 1999). Besides conventional staining (5% Giemsa at pH 7) the following techniques were applied: (1) Ag-NOR banding of the nucleolar organizer regions following Howell & Black (1980); (2) the C+G specific fluorochrome chromomycin A₃ (CMA₃) /methyl green staining according to Sahar & Latt (1980), with a reduced exposure (few seconds) to the non-fluorescent dye, methyl green; (3) C-banding according to Sumner (1972), incubating the slides for 5 min at 45 °C in Ba(OH)₂. Good results were achieved by staining, either separately or sequentially, with CMA₃ and DAPI after hydrolysis in Ba(OH)₂ (Odierna *et al.*, 2001).

The metaphase plates were observed either with a Zeiss PHOM III phase contrast microscope or a Zeiss axioscope epifluorescent microscope. Of each taxon, at least four Giemsa-stained metaphases and two metaphases stained with each of the banding methods used, respectively, were studied.

RESULTS

The studied taxa showed the typical karyotype of ranoid frogs, constituted by thirteen pairs of metacentric or submetacentric chromosomes, with the first five pairs clearly larger than the remaining eight (Fig. 1).

The first and the fifth pairs were metacentric, the second and the third were submetacentric, while the fourth presented a centromeric index close to the submetacentric transition value. The morphology of pairs 6-13, on the other hand, was highly variable. In *B. goudoti*, *B. cf. madagascariensis*, *B. cf. marojezensis*, *B. microtympanum*, *B. occidentalis* and *B. xerophilus* these pairs were all metacentric, while in the remaining studied taxa one of them was submetacentric, occupying a variable position in the karyotypes: in *B. albilabris* and *B. brachybir*, the thirteenth pair was sub-



Fig. 1 - Giemsa stained karyotypes of *B. albilabris* (A), *B. cf. anjanabaribeensis* (B), *B. ankaratra* (C), *B. brachybir* (D), *B. doulioti* (E), *B. goudoti* (F), *B. cf. madagascariensis* (G), *B. cf. mandraka* (H), *B. cf. marojezensis* (I), *B. microtympanum* (J), *B. occidentalis* (K), *B. cf. rappiodes* (L), *B. reticulatus* (M), *B. cf. rufioculis* (N), *B. cf. septentrionalis* (O), *B. xerophilus* (P). Note the 6th NOR bearing pairs reported on the top.

metacentric; in *B. cf. anjanabaribeensis*, *B. ankaratra*, *B. cf. mandraka*, *B. cf. rappiodes*, *B. reticulatus*, *B. cf. rufioculis* and *B. cf. septentrionalis* the seventh pair was submetacentric, in *B. tephraeomystax* the seventh, tenth and thirteenth pairs were submetacentric (Table II).

TABLE I - List of *Boophis* species analysed for karyology, including the species group, museum number for preserved specimens (MRSN = Museo Regionale di Scienze Naturali di Torino, Italy), sex (M = male; F = female), and provenance.

Species	Species-group	no.	Sex	Provenance
<i>B. albilabris</i>	<i>B. albilabris</i>	MRSN A2027	M	Ambolokopatrika
<i>B. albilabris</i>	<i>B. albilabris</i>	Voucher not collected	M	Ambolokopatrika
<i>B. albilabris</i>	<i>B. albilabris</i>	Voucher not collected	M	Ambolokopatrika
<i>B. occidentalis</i>	<i>B. albilabris</i>	MRSN A1998	M	Berara
<i>B. occidentalis</i>	<i>B. albilabris</i>	MRSN A1996	undetermined	Berara
<i>B. cf. anjanabaribeensis</i>	<i>B. luteus</i>	MRSN A4112	M	Ambolokopatrika
<i>B. cf. anjanabaribeensis</i>	<i>B. luteus</i>	MRSN A4111	F	Ambolokopatrika
<i>B. ankaratra</i>	<i>B. luteus</i>	MRSN A4402	M	Ankaratra
<i>B. cf. septentrionalis</i>	<i>B. luteus</i>	MRSN A4113	M	Ambolokopatrika
<i>B. brachybir</i>	<i>B. goudoti</i>	MRSN A4342	M	Ambolokopatrika
<i>B. brachybir</i>	<i>B. goudoti</i>	MRSN A4341	F	Ambolokopatrika
<i>B. goudoti</i>	<i>B. goudoti</i>	MRSN A4109	M	Ankaratra
<i>B. cf. madagascariensis</i>	<i>B. goudoti</i>	MRSN A4292	F	Masoala
<i>B. reticulatus</i>	<i>B. goudoti</i>	MRSN A4186	F	Ambolokopatrika
<i>B. reticulatus</i>	<i>B. goudoti</i>	MRSN A3118	F	Masoala
<i>B. reticulatus</i>	<i>B. goudoti</i>	MRSN A3257	F	Masoala
<i>B. cf. rufioculis</i>	<i>B. goudoti</i>	MRSN A4382	F	Ambolokopatrika
<i>B. cf. marojezensis</i>	<i>B. majori</i>	MRSN A4416	undetermined	Masoala
<i>B. cf. mandraka</i>	<i>B. rappiodes</i>	MRSN A2572	F	Masoala
<i>B. cf. rappiodes</i>	<i>B. rappiodes</i>	MRSN A4286	undetermined	Masoala
<i>B. viridis</i>	<i>B. rappiodes</i>	MRSN A4163	M	Masoala
<i>B. microtypanum</i>	<i>B. microtypanum</i>	Voucher not collected	F	Ankaratra
<i>B. doulioti</i>	<i>B. tephraeomystax</i>	MRSN A4259	M	Kirindy
<i>B. xerophilus</i>	<i>B. tephraeomystax</i>	MRSN A4384	M	Kirindy

The karyological uniformity typical of the genus was also observed in the localization of the nucleolus organizer regions (NORs). In fact, Ag-NOR banding and CMA₃ staining provided evidence for the NORs being located on the long arm, very close to the centromere, of the 6th chromosome pair in almost all of the studied *Boophis*. Only *B. cf. madagascariensis* and *B. cf. rufioculis* were exceptions, displaying NORs on the short arms of the sixth chromosome pair (Fig. 1).

In none of the studied *Boophis* species did we observe chromocentres positive to quinacrine, which would therefore be rich in AT (data not shown in this paper). Furthermore, the C-banding colourations and those sequential in C-banding+CMA₃+ DAPI showed a conspicuous heterogeneity for the heterochromatin, producing a banding pattern which is species-specific (Figs 2-3). These observations are summarized in Table II.

DISCUSSION

Chromosome morphology

It is striking that the adaptive radiation of the ecologically diverse genus *Boophis* occurred without relevant chromosome rearrangements. In fact, all the 13 new species here studied, as well as the other 11 species previously studied by Blommers-Schlösser (1978), showed a karyotype of $2n = 26$ banded elements with the first five

pairs distinctively larger than pairs 6-13. Moreover, the morphology of the pairs 1-5 was highly conserved and rearrangements appeared to have occurred exclusively among the smallest karyotype elements. The greater disposition to rearrangements of the smallest elements had already been evidenced in other taxa (Nanda *et al.*, 2002; Olmo *et al.*, 2002), and is in line with the evidence of the inverse correlation between chromosome length and rate of recombination (Kaback *et al.*, 1992). In *Boophis* pericentromeric inversions very probably are the rearrangements responsible for metacentric/submetacentric transitions that occurred along the chromosome pairs 6-13, and this kind of rearrangement preferentially appears to occur on the chromosomes of the seventh pair and at a lesser rate also on chromosomes of the eleventh and thirteenth pairs (see Table II).

NOR localisation

Loci of NORs also appeared highly conserved along the analysed *Boophis* species. In fact, all of them displayed the nucleolus organizer regions close to centromeric regions of the long arms of the 6th chromosome pair. The shift of NORs, observed in *B. cf. madagascariensis* and *B. cf. rufioculis*, onto the peritelomeric regions of the short arms of the same pairs also could be taken back to a pericentric inversion, thereby providing further evidence that this kind of chromosome

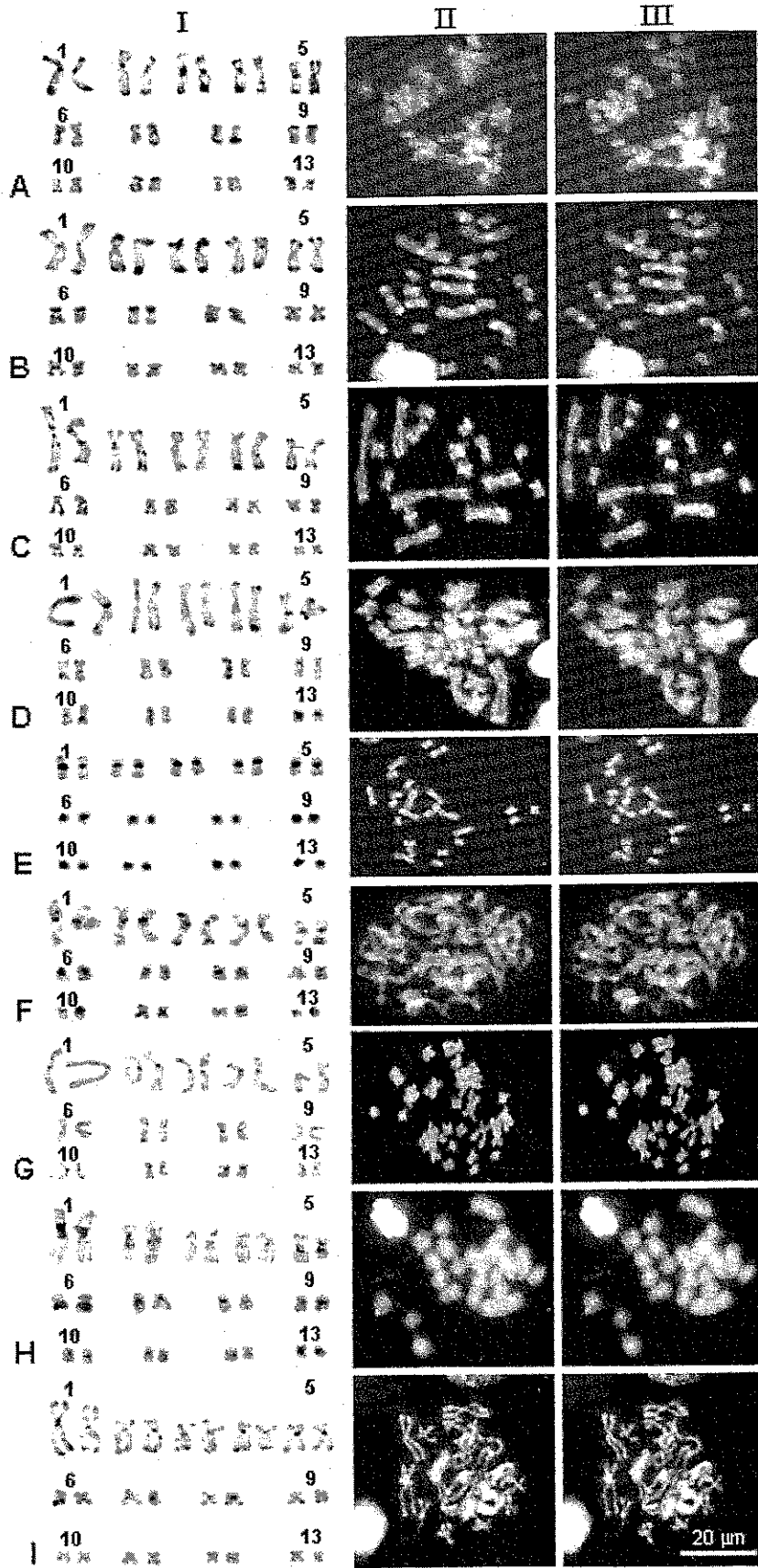


Fig. 2 - C-banded (column I), C-banded+CMA3 (column II)+DAPI (column III) of *B. albilabris* (A), *B. cf. anjanabaribeensis* (B), *B. ankaratra* (C), *B. brachychir* (D), *B. doulioti* (E), *B. goudoti* (F), *B. cf. madagascariensis* (G), *B. cf. mandraka* (H) and *B. cf. marojezensis* (I).

TABLE II - Karyological characteristics of the studied *Boophis* species. Abbreviations: 2n, diploid number; FN, Fundamental Number; Sm, submetacentric; p, short arms; q, long arms; P, present: heavy (h), faint (f); A, absent; +, positive; -, negative.

Species	2n	FN	Sm pairs	NORs 6 th	Heterochromatin				
					Centromeric			Paracentromeric	Telomeric
					P/A	CMA ₃	DAPI		
<i>B. albilabris</i> group									
<i>B. albilabris</i>	26	52	13 th	q	P	+	+	A	P(h)
<i>B. occidentalis</i>	26	52	-	q	P	-	-	A	A
<i>B. luteus</i> group									
<i>B. anjanaharibeensis</i>	26	52	7 th	q	P	+	+	A	P
<i>B. ankaratra</i>	26	52	7 th	q	P(f)	+(2 pairs)	+(2 pairs)	1 st , 2 nd , 5 th	P
<i>B. cf. septentrionalis</i>	26	52	7 th	q	P(f)	-	-	A	P
<i>B. rappiodes</i> group									
<i>B. cf. mandraka</i>	26	52	7 th	q	P	-	-	A	P
<i>B. cf. rappiodes</i>	26	52	7 th	q	P	+	+	1 st , 2 nd , 3 rd	A
<i>B. viridis</i>	26	52	-	q	P(h)		+	+	A-
<i>B. majori</i> group									
<i>B. cf. marojezensis</i>	26	52	-	q	P	-	-	1 st , 2 nd , 5 th	A
<i>B. goudoti</i> group									
<i>B. brachybir</i>	26	52	13 th	q	P(f)	-	-	A	P
<i>B. goudoti</i>	26	52	-	q	P(h)		+	-	AA
<i>B. cf. madagascariensis</i>	26	52	-	p	P(h)	+	+	A	P
<i>B. reticulatus</i>	26	52	7 th	q	P	-	-	A	P
<i>B. cf. rufiocularis</i>	26	52	7 th	p	P	+	+	A	P
<i>B. microtympaanum</i> group									
<i>B. microtympaanum</i>	26	52	-	q	P	-	-	1 st , 2 nd , 3 rd , 5 th	P
<i>B. tepbraeomystax</i> group									
<i>B. doulioti</i>	26	52	7 th th, 11 th , 13 th	q	P	-	-	1 st , 2 nd	P
<i>B. xerophilus</i>	26	52	-	q	P	+	-	A	-

rearrangements in *Boophis* appears exclusively to occur among the smaller pairs of the chromosome complement. Phylogenetically, the deviant state in these two species, both of which are northern representatives of the *B. goudoti* group, can be seen as rather strong argument that they form a monophyletic clade to the exclusion of at least some other members of this species group (see Table I).

Heterochromatin distribution and composition

In contrast to the chromosome morphology and NOR localisation, among the studied species heterochromatin greatly differed both in the distribution and composition. Our results showed the presence of at least three types of heterochromatin: one type endowed with AT and CG rich sequences that was positive to CMA₃ and DAPI; another type endowed with GC rich sequences that was positive only to CMA₃; and a last type endowed with sequences not particularly rich in AT or GC sequences.

However, even if preferentially localised on pericentromeric, paracentromeric and/or telomeric chromosomal regions, the chromosomal distribution of these three classes of heterochromatin greatly differed among the species (see Table II). The observed differences could be the result of selective events of amplification/deletion

of satellite DNA families, of which heterochromatin is notoriously endowed (Redi *et al.*, 2001). However, the addition of paracentromeric heterochromatin occurs preferentially along specific chromosomes pairs, namely 1st, 2nd, 3rd and 5th pairs of the following species: *B. ankaratra*, *B. cf. marojezensis*, *B. microtympaanum*, *B. cf. rappiodes* and *B. doulioti*.

However, the preferential accumulation of specific heterochromatin at certain chromosome sites has been also evidenced in other frogs, such as *Mantella* (Odierna *et al.*, 2001) or Australian myobatrachid species of the genus *Mixophyes* (Schmid *et al.*, 2002).

Taxonomic implications

Our results support the karyological uniformity of *Boophis*, already outlined by Blommers-Schlösser (1978), and extend this uniformity to nucleolus organizer regions, too. The potential of NORs as taxonomic and phylogenetic markers is well known for several anuran taxa (see King 1990), including *Mantella* (Odierna *et al.*, 2001). On the basis of NORs, *Boophis* appears as a monophyletic group, confirming the morphological and molecular data (Glaw & Vences, 1994, 1997; Vences *et al.*, 2002).

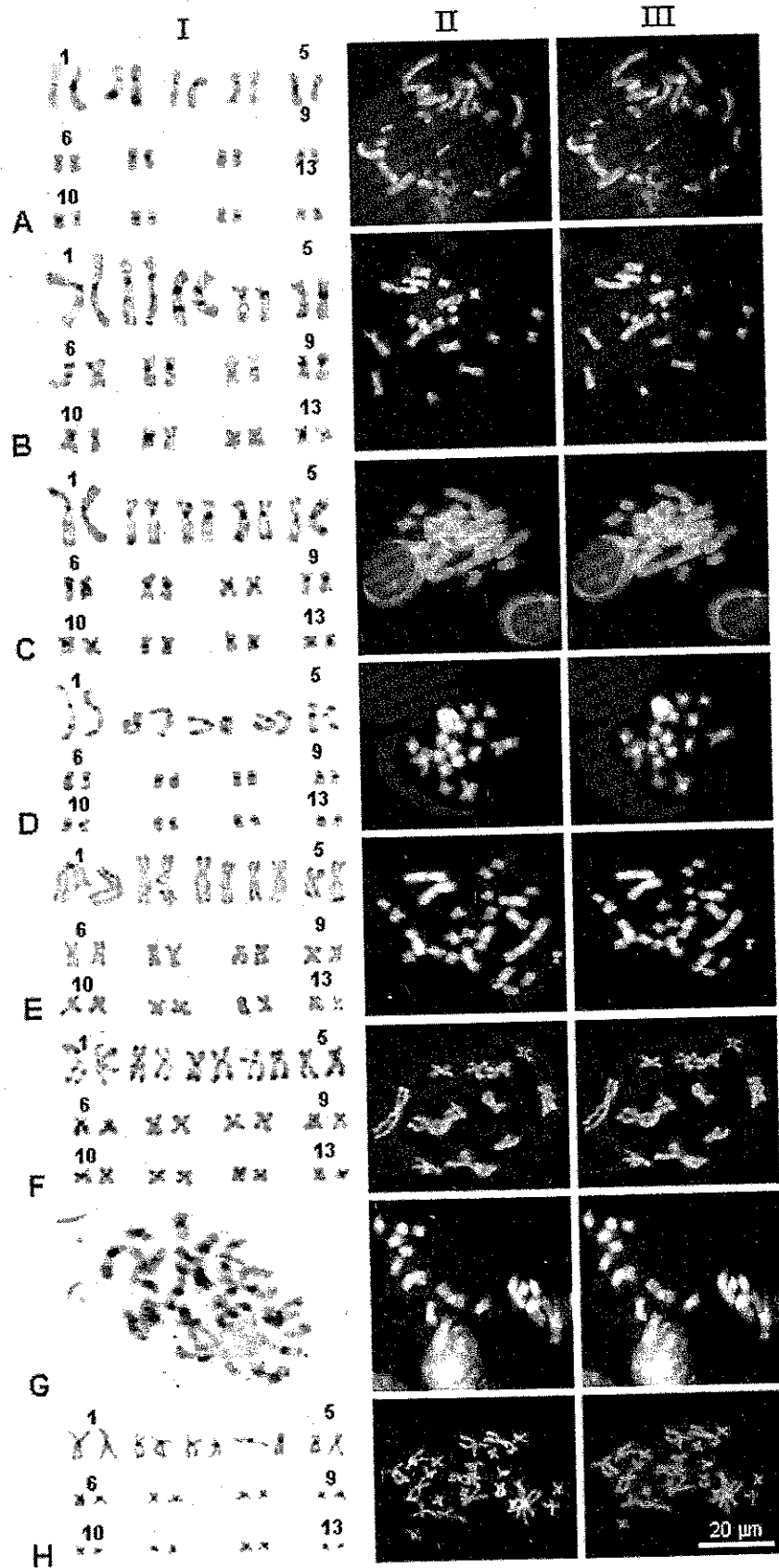


Fig. 3 - C-banded (column I), C-banded+CMA3 (column II)+DAPI (column III) of *B. microtypanum* (A), *B. occidentalis* (B), *B. cf. rappidodes* (C), *B. reticulatus* (D), *B. cf. rufioculcis* (E), *B. cf. septentrionalis* (F), *B. viridis* (G) and *B. xerophilus* (H).

The C-banding produced species specific patterns and appears to be very useful to support the specific separation between taxa which are morphologically very similar. Concerning this, it could be of interest to compare the results for three pairs of sibling species pairs, to test the efficacy of fine karyology and banding in species discrimination: i) *B. albilabris* vs. *B. occidentalis*, ii) *B. doulioti* vs. *B. xerophilus*, iii) *B. cf. anjanaharibeensis* vs. *B. cf. septentrionalis*. For two of these pairs the specific distinction was made possible quite recently, using traditional and molecular data sets: *Boophis albilabris* and *B. occidentalis*, formerly believed to be subspecies of *B. albilabris* (Glaw & Vences, 1994), were recently discriminated by Andreone *et al.* (2002); *B. xerophilus* was recognised as different from the syntopic *B. doulioti* by Glaw & Vences (1997). Karyologically the above three couples of sibling species greatly differ in this distribution and composition of centromeric, paracentromeric and telomeric heterochromatin (see Table II). In detail, *B. albilabris* and *B. cf. anjanaharibeensis* present a CMA₃ and DAPI positive, centromeric heterochromatin, differing from their relative siblings which display a centromeric heterochromatin negative to both the fluorochromes. Furthermore, *B. albilabris* also differs from *B. occidentalis* in showing heavy telomeric C-bands and the 13th chromosome pair shaped as submetacentric. *B. doulioti* differs from *B. xerophilus* by paracentromeric C-bands and has three submetacentric pairs among the smallest elements. Furthermore, centromeric heterochromatin of *B. xerophilus* is CMA₃ positive, while is negative to both the fluorochromes in *B. doulioti*.

Regarding a possible assessment of relationships between species, the heterochromatin data are much more contradictory. The metacentric/submetacentric transition within pairs 6-13 occurred in species belonging both to the same and different species groups, suggesting that this rearrangement may have occurred repeatedly and independently in the different groups. This also applies to heterochromatin, which shows fast diversification also in sibling species. Also the value of the preferential accumulation of heterochromatin in specific paracentromeric regions along the bigger chromosome pairs is questionable, because species belonging to different groups display paracentromeric C-bands. To test the phylogenetic value of these characters and reconstruct their evolution among *Boophis*, more complete analyses of mitochondrial and nuclear DNA sequences will be needed.

CONCLUSIONS

Malagasy treefrogs of the genus *Boophis* show a remarkable karyological uniformity. This refers both to general chromosome morphology and to NOR locations. For instance, no *Boophis* with acrocentric chromosomes has been identified so far, and only two species in our study deviated from the general pattern of NOR loci. In contrast, in *Mantidactylus*, several sub-

genera and species groups regularly display acrocentric chromosomes and a reduction ($2n = 24$) or an increase ($2n = 28 - 30$) of the karyotype (Blommers-Schlösser, 1978; Aprea G. *et al.*, 2002, Abstract in LXIII Congr. Naz. UZI: 121). It is compelling that this variability correlates also with a relevant diversification of NOR positions in some of these groups (Aprea G. *et al.*, 2002, Abstract in LXIII Congr. Naz. UZI: 121; Andreone *et al.*, 2003). It is still uncertain whether this correlation is causal or whether the ecological shifts were somehow related to the origin of chromosomal variability (e.g., Bogart, 1991). It seems clear, however, that the combined study of the phylogeny and karyological variation within the Mantellidae may significantly contribute to understand the factors that lead to karyological stasis in some clades and to high karyological variability in others, and thus to identify the factors that promote karyological rearrangements in nature.

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