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ScienceDirect
Zoologischer Anzeiger 246 (2007) 23–41
ZOOLOGISCHER ANZEIGER
www.elsevier.de/jcz

Karyological evolution and systematics of Malagasy microhylid frogs

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Received 8 September 2005; received in revised form 13 October 2006; accepted 24 October 2006
Corresponding Editor: D.G. Homberger

Abstract

Microhylid frogs are a group of largely unresolved phylogeny, and diverse data sets are needed to improve the evolutionary understanding of these amphibians. We here report karyological data for 22 species of this family, belonging to the Malagasy genera *Anodonthyla*, *Cophyla*, *Platypelis*, *Plethodontohyla*, *Rhombophryne*, and *Stumpffia* (Cophylinae); *Scaphiophryne* and *Paradoxophyla* (Scaphiophryninae); and *Dyscophus* (Dyscophinae); and the Asian genera *Calluella* and *Ramanella* (Microhylinae). All species studied have $2n = 26$ chromosomes, most of which are metacentric or submetacentric. Chromosome morphology, banding pattern, and position of the nucleolar organizer regions (NORs) provide relevant characters for the understanding of the phylogeny and systematics of these frogs. The species of the Cophylinae are characterized by a subtelocentric or telocentric fourth chromosome pair (submetacentric only in *Anodonthyla*), which can be seen as a synapomorphy for this subfamily. Shifts in NOR position within the Cophylinae are frequent and agree with recent mitochondrial DNA data, corroborating the non-monophyly of the genus *Plethodontohyla.* Changes of NOR position and chromosome morphology (i.e., occurrence of subtelocentric and telocentric elements) were also common in this subfamily, possibly being related to their faster mitochondrial substitution rate and high species diversity. The ninth chromosome pair of the examined specimens of *Dyscophus guineti*, all juveniles, is heteromorphic. In this pair, one of the two chromosomes is longer due to the addition of two heterochromatic segments, raising the possibility that one chromosome of this pair may be a sex chromosome.

Keywords: Scaphiophryninae; Cophylinae; Dyscophinae; Chromosome evolution; NORs; Heterochromatin; Madagascar

1. Introduction

Despite a recent rush of studies of the Malagasy amphibian fauna, some of the major subgroups are still incompletely known. This applies also to the family Microhylidae, which is represented in Madagascar by three subfamilies: The Dyscophinae, containing the Malagasy endemic genus *Dyscophus* and previously the Asian *Calluella* (which has recently been included in the Microhylinae); the endemic Scaphiophryninae, with the genera *Paradoxophyla* and *Scaphiophryne*; and the endemic and diverse Cophylinae with the genera *Anodonthyla*, *Cophyla*, *Madecassophryne*, *Platypelis*,

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doi:10.1016/j.jcz.2006.10.001

Plethodontohyla, *Rhombophryne*, and *Stumpffia* (Blommers-Schlösser and Blanc 1991; Glaw and Vences 1994; Andreone et al. 2005).

The family Microhylidae is a widely distributed group occurring in North and South America, Africa and Madagascar, and Asia to North Australia. The phylogenetic relationships of major microhylid subclades are still largely unknown (Van der Meijden et al. 2004). Many microhylids are characterized by a specialized filter-feeding tadpole morphology, although some have special derived reproductive modes, such as direct development in Papuan species and non-feeding tadpoles in the African *Hoplophryne* and the Malagasy cophylines (Duellman and Trueb 1986). The Malagasy *Scaphiophryne* species have a tadpole morphology that was interpreted as intermediate between the ranoid and the microhylid type (Wassersug 1984), which may be indicative of a phylogenetically basal position or of a morphological character state reversal.

To improve the understanding of microhylid evolution, new sets of independent characters are needed, which can complement the currently available morphological characters and molecular data (Van der Meijden et al. 2004; Andreone et al. 2005). As an example, the combined analysis of the mitochondrial substitution rates in *Scaphiophryne* with the tetraploid karyotype of *Scaphiophryne gottlebei* by Vences et al. (2002) lead to the hypothesis of increased incidence of allotetraploid species formation and decreased rate of molecular evolution in these explosively pond-breeding frogs, in which hybridization and large population sizes may not be unusual. Karyological studies on the microhylids of Madagascar are currently limited to five publications (Blommers 1971; Blommers and Blanc 1972; Blommers-Schlösser 1976; Vences et al. 2002; Andreone et al. 2006), in which the basic karyotype morphology of 16 species were described (Table 1). Our study contributes to the understanding of chromosomal evolution of Malagasy microhylid frogs by describing the karyotypes of 20 species of all genera occurring in Madagascar, except for *Madecassophryne*, but including the genera *Cophyla*, *Rhombophryne*, and *Stumpffia*, for which chromosomal data are not available so far. Because chromosome banding data of mainland African and Asian microhylids are available only for *Kaloula pulchra* (Schmid 1978) and *Phrynomantis bifasciatus* (Schmid

Table 1. Voucher specimen numbers and locality data

Species	Subfamily	Specimens examined	Locality	Voucher numbers
Anodonthyla montana	Cophylinae	1 female	Andringitra	ZMA 19516 or 19517
Anodonthyla moramora	Cophylinae	1 specimen	Vohiparara	uncatalogued (GA 427)
Cophyla phyllodactyla	Cophylinae	1 male	Nosy Be	Not preserved
Paradoxophyla palmata	Scaphiophryninae	1 male	Fierenana	MRSN A2518
Paradoxophyla tiarano	Scaphiophryninae	1 female	Masoala	MRSN A2525
Platypelis grandis	Cophylinae	2 females	Masoala	MRSN A2630
Platypelis grandis	Cophylinae	1 male	Vohidrazana	ZMA 19652
Platypelis grandis	Cophylinae	1 female	Ranomafana	ZMA 19422
Platypelis sp. aff. *mavomavo*	Cophylinae	2 males	Tsaratanana	MRSN A2630
Platypelis tuberifera	Cophylinae	2 males, 1 juvenile	Ambolokopatrika	Not preserved
Plethodontohyla alluaudi	Cophylinae	1 male	Andasibe	ZSM 3/2002
Plethodontohyla laevipes	Cophylinae	1 female	Tsaratanana	FAZC 11063
Plethodontohyla mihanika	Cophylinae	1 male	Masoala	MRSN A4594
Plethodontohyla sp. aff. *minuta*	Cophylinae	1 female	Masoala	MRSN A3656
Plethodontohyla tuberata	Cophylinae	1 male	Ankaratra	MRSN A4623
Rhombophryne testudo	Cophylinae	2 males	Nosy Be	Not preserved
Scaphiophryne boribory	Scaphiophryninae	3 males, 2 females	Fierenana	ZSM 153/2002 and unpreserved vouchers
Scaphiophryne calcarata	Scaphiophryninae	2 males	Tolagnaro	ZSM 115-116/2002
Scaphiophryne calcarata	Scaphiophryninae	1 juvenile	Isalo	ZSM 118/2002
Stumpffia gimmeli	Cophylinae	1 specimen	Manongarivo	ZMA 19572
Stumpffia cf. *grandis*	Cophylinae	1 specimen	probably Fierenana	Not preserved
Stumpffia sp.	Cophylinae	1 specimen	Ranomafana	ZMA 19420
Dyscophus guineti	Dyscophinae	3 juveniles	Captive bred	Not preserved
Calluella guttulata	Microhylinae	1 male	Thailand	ZSM 434/2002d
Ramanella cf. *obscura*	Microhylinae	1 male	Sri Lanka	MNHN 2000.628

All localities are in Madagascar (see Fig. 1), except when stated otherwise. Collection acronyms are as follows: MNHN, Museum National d'Histoire Naturelle, Paris, France; MRSN, Museo Regionale di Scienze Naturali, Torino, Italy; ZMA, Zoological Museum Amsterdam, Netherlands; ZSM, Zoologische Staatssammlung München, Germany. FAZC refers to field numbers of F. Andreone (specimens to be catalogued in MRSN), GA to field numbers of G. Aprea.

1980), we extended our analysis to two Asian species, the Sri Lankan endemic *Ramanella* cf. *obscura* and the south-east Asian *Calluella guttulata* which has been hypothesized to be related to the Malagasy *Dyscophus*.

2. Materials and methods

Individuals of a few taxa (*Dyscophus guineti*, *Calluella guttulata*, *Ramanella* cf. *obscura*) were available alive in the laboratory of the Università di Napoli Federico II, Dipartimento di Biologia Strutturale e Funzionale. They were injected with 0.1 ml per 10 g of body weight of colchicine solution (0.5 mg/ml) and euthanized in a 1% MS 222 solution 2 h later. The intestine, spleen, kidneys, lungs, and gonads were extracted from the specimens and incubated for 30 min in a solution of one part 0.5% sodium citrate and one part 0.56% potassium chloride. The tissues were subsequently fixed for 30 min in methanol + acetic acid, 3:1. Chromosomes were obtained using the air drying and scraping method. In brief, the organs were scraped on a sieve; cells collected with freshly prepared fixative (methanol and acetic acid, 3:1), and a drop of the obtained cell suspension was put on a cleaned slide. All other specimens were processed in the field upon capture, using the same methods, but transferring the specimens while in the fixative to the lab, where their processing was completed.

The chromosomes were stained both with the standard method (5% Giemsa at pH 7) and using the following banding techniques: (1) Ag-NOR banding (Howell and Black 1980); (2) chromomycin A3/methyl green staining (CMA_3) according to Sahar and Latt (1980); (3) Q-banding and DA/DAPI as suggested by Schmid (1978); (4) C-banding according to Sumner (1972); and sequential C- or AluI-banding + CMA_3 + DAPI as reported by Odierna et al. (1999).

The karyotype of *Paradoxophyla palmata* has already been published by Blommers-Schlösser (1976) under the name "*Dyscophus* sp.". Andreone et al. (2006) provided more detailed chromosome data for *P. palmata* and *P. tiarano* to diagnose *P. tiarano*, which is described in that paper as a new species. We here redescribe the chromosome morphology of these two species to allow for a better comparison with the species of *Scaphiophryne*.

Voucher specimens used for the present study are listed in Table 1, their collecting localities are listed in Table 1 and shown in Fig. 1.

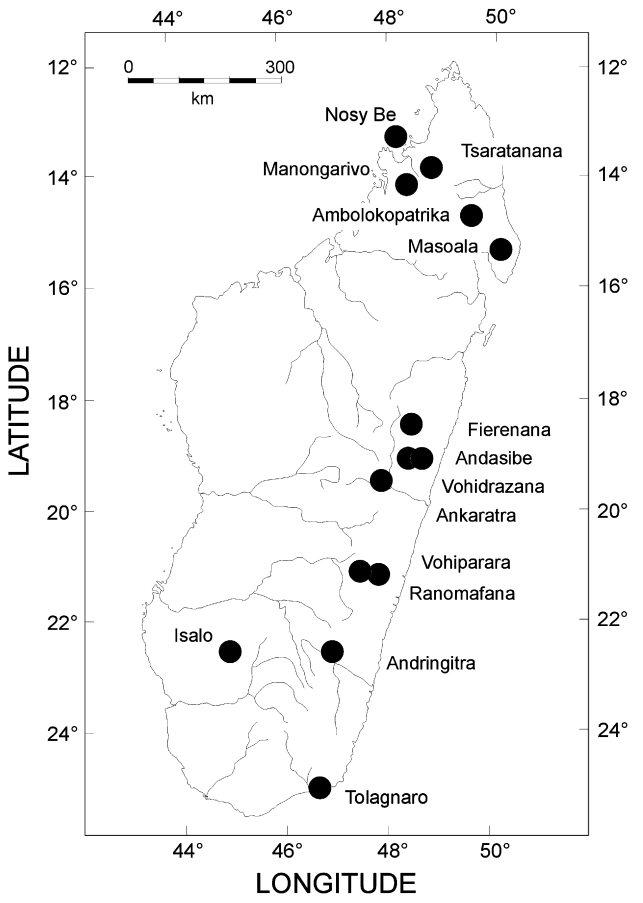

Fig. 1. Map of Madagascar showing the collecting localities of specimens examined in the present study as listed in Table 1.

3. Results

3.1. Genera *Scaphiophryne* and *Paradoxophyla* (subfamily Scaphiophryninae)

Two species of *Scaphiophryne* (*S. boribory* and *S. calcarata*) and two species of *Paradoxophyla* (*P. palmata* and *P. tiarano*) had a karyotype of $2n = 26$ with two arms on all chromosomes ($Fn = 52$) (Fig. 2). The NORs in all species were located in a paracentromeric position on the short arm of the second chromosome pair (Fig. 2), which was also confirmed by CMA_3 staining (not shown). By C-banding and sequential C-banding + CMA_3 + DAPI staining nucleolus-associated heterochromatin was visible in both *Scaphiophryne*, which stained also positive with CMA_3 in both species, and with DAPI in *S. calcarata*. In *S. calcarata* no other heterochromatin was observed, whereas *S. boribory* had CMA_3-positive heterochromatin in the telomeric regions of almost all chromosomes. In *Paradoxophyla palmata* and *P. tiarano*, the nucleolar heterochromatin was evident as well. *P. palmata* had heterochromatin distributed along the telomeric regions, whereas *P. tiarano* had it distributed mainly in the centromeric regions of chromosome pairs 6–13 (Fig. 2).

3.2. Genus *Anodonthyla* (subfamily Cophylinae)

Two species of this genus, *Anodonthyla montana* and *A. moramora*, have a karyotype of $2n = 26$ biarmed chromosomes, with chromosome pairs 2–4 being submetacentric and the others metacentric (Fig. 3), similar

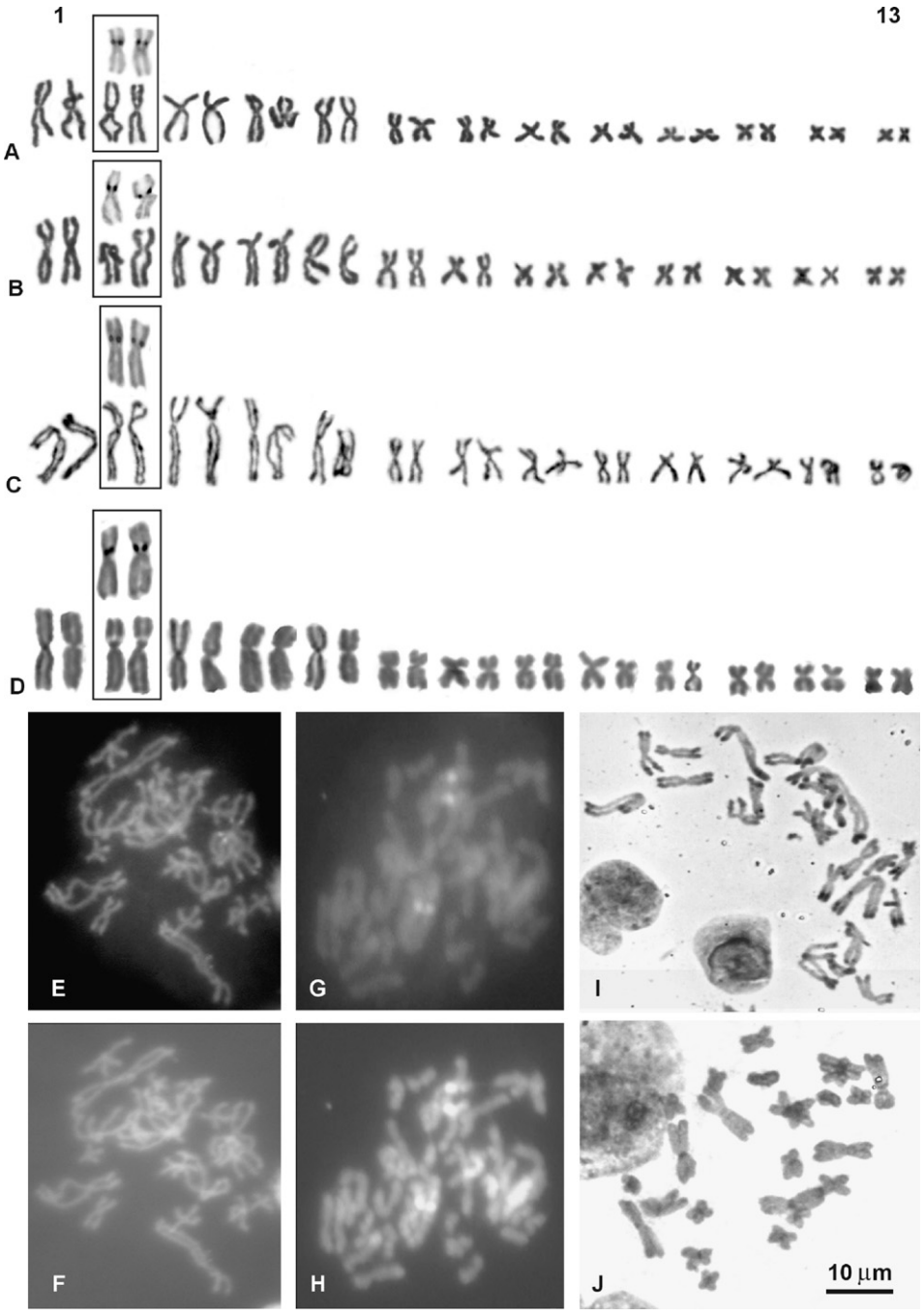

Fig. 2. Giemsa-stained karyotypes (A–D) and metaphase plates (E–J), stained with C-banding and Giemsa (I, J); C-banding, Giemsa, and CMA_3 (E, G); C-banding, Giemsa, CMA_3, and DAPI (F, H) of scaphiophrynine microhylids. *Scaphiophryne boribory* (A, E, F); *S. calcarata* (B, G, H); *Paradoxophyla palmata* (C, I); and *P. tiarano* (D, J). The scale bar in (J) refers to all images. NOR-bearing pairs are framed, with the Ag-NOR-banded picture of each pair above the Giemsa-stained picture.

to the condition reported for *A. boulengeri* by Blommers-Schlösser (1976) (see Table 2). The NORs were localized in both species on the sixth chromosome pair, but in *A. montana* on the short arms and in *A. moramora* on the long arms (Fig. 3). C-banding revealed heterochromatin only in the centromeric position in *A. montana*, which resulted to be CMA_3 positive. In *A. moramora*, C-banding revealed NOR-associated heterochromatin and bands in the centromeric regions of all chromosomes that turned out to be DAPI positive (Fig. 3).

3.3. Genus *Cophyla* (subfamily Cophylinae)

The studied specimen of *Cophyla phyllodactyla* from the type locality Nosy Be had $2n = 26$ chromosomes, all

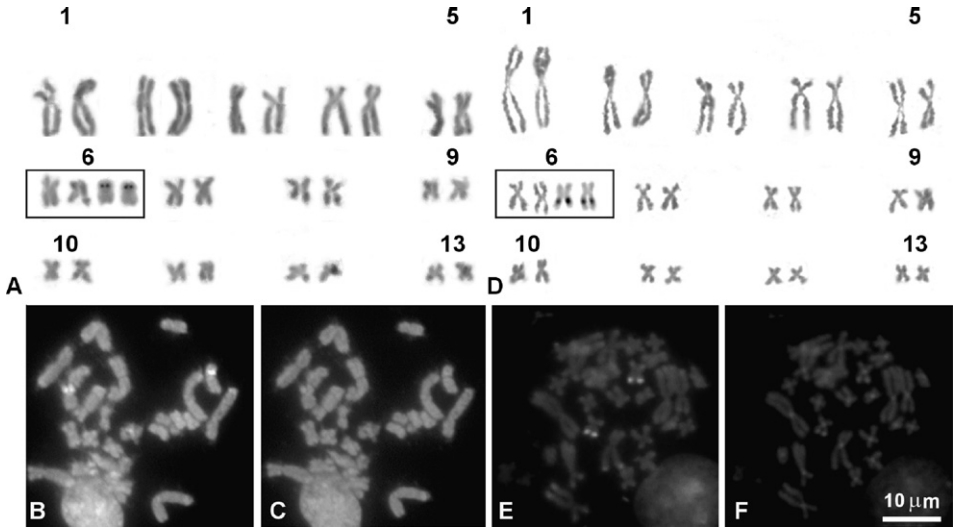

Fig. 3. Karyotypes (A, D) and metaphase plates (B–C, E–F) of *Anodonthyla montana* (A–C) and *A. moramora* (D–F). Metaphases are shown after C-banding and CMA_3 -staining (B, E) and after C-banding, CMA_3-staining, and DAPI-staining (C, F). The NOR-bearing pairs in (A) and (D) are framed, with the Ag-NOR-banded picture of each pair to the right of the Giemsa-stained picture. The scale bar in (F) refers to all images.

biarmed, except for the telocentric fourth chromosome pair ($Fn = 50$). The chromosome pairs 2, 3, 7, and 11 were submetacentric, the other biarmed pairs were metacentric. The NORs were on the long arm of the chromosomes of the fifth pair. C-banding identified only the NOR-associated heterochromatin, which turned out to be CMA_3 positive (Fig. 4).

3.4. Genus *Platypelis* (subfamily Cophylinae)

The three studied species of this arboreal cophyline genus, *Platypelis grandis*, *P.* sp. aff. *mavomavo*, and *P. tuberifera*, had a karyotype of $2n = 26$. The fourth chromosome pair was telocentric in all three species, and the 12th pair was telocentric in *P. grandis* (Fig. 5). The other chromosome pairs were metacentric, except for pairs 2 and 3 in *P. grandis* and 3, 12, and 13 in *P. tuberifera*, which were submetacentric. The NORs were located on the fourth chromosome pair, constituting the very short arms of this telocentric element. In the three species, we identified distinct NOR-associated heterochromatin, which was CMA_3 positive. Additional C-bands were as follows: None in *P.* sp. aff. *mavomavo*; present in the centromeric regions of almost all DAPI-positive chromosomes in *P. tuberifera*; present in a telomeric position on all or most pairs 1–5, and a centromeric band on the second pair in *P. grandis* (Fig. 5).

3.5. Genus *Plethodontohyla* (subfamily Cophylinae)

We examined five species of this terrestrial–fossorial cophyline genus: *Plethodontohyla alluaudi*, *P. laevipes*, *P. mihanika*, *P. tuberata*, and *P.* sp. aff. *minuta.* In agreement with previous data (Blommers-Schlösser 1976), the species examined had a karyotype of $2n = 26$ with a subtelocentric fourth chromosome pair. In *P. alluaudi*, the chromosomes of the eleventh pair were subtelocentric, too. *P. tuberata* and *P.* sp. aff. *minuta* had telocentric chromosomes of the 10th and eighth pair, respectively. The other chromosomes were all metacentric or submetacentric (Fig. 6; see Table 2 for details). The NORs were located on the long arms of the sixth chromosome pair in *P. mihanika*, *P.* sp. aff. *minuta*, and *P. tuberata*, whereas in *P. alluaudi* and *P. laevipes,* they were in a peritelomeric position on the long arm of the second chromosome pair (Fig. 6). After C-banding, the NOR-associated heterochromatin and centromeric regions of almost all chromosomes presented C-positive bands in all species studied. These centromeric bands were DAPI positive in *P. alluaudi* and *P. tuberata*, and CMA_3 and DAPI negative in *P. laevipes* and *P.* sp. aff. *minuta.* In *P. mihanika,* only two of the centromeric bands were DAPI positive. In these species, also the peritelomeric regions of one of the larger chromosome pairs (probably the fifth pair) was DAPI positive (Fig. 7).

3.6. Genus *Rhombophryne* (subfamily Cophylinae)

The two studied specimens of this fossorial cophyline from its type locality Nosy Be had a karyotype of $2n = 26$ chromosomes, of which all were metacentric, except those of the fourth chromosome pairs, which were at the borderline between subtelocentric and telocentric, and those of the second and

Table 2. Available chromosomal data on Malagasy microhylids, from the works of (a) Blommers (1971); (b) Blommers and Blanc (1972); (c) Blommers-Schlösser (1976); (d) Vences et al. (2002); (e) Andreone et al. (2006); and (f) the data obtained in this study

Species	Subfamily	2*n*	NOR	sm	st	t	Reference
Paradoxophyla palmata	Scaphiophryninae	26	2 (short)	2; 3; 4; 8; 10; 13	—	—	e
Paradoxophyla palmata	Scaphiophryninae	26	—	4; 8; 9	—	—	c
Paradoxophyla tiarano	Scaphiophryninae	26	2 (short)	2; 3; 4; 8; 10; 13	—	—	f
Scaphiophryne boribory	Scaphiophryninae	26	2 (short)	2; 3; 4; 8; 10; 13	—	—	f
Scaphiophryne calcarata	Scaphiophryninae	26	2 (short)	2; 3; 4; 8; 10; 13	—	—	f
Scaphiophryne gottlebei	Scaphiophryninae	52 (4*n*)	2 (short)	2, 3, 4, 8, 10, 13	—	—	d
Scaphiophryne madagascariensis	Scaphiophryninae	26	—	—	—	—	b
Scaphiophryne madagascariensis	Scaphiophryninae	26	2 (short)	2, 3, 4, 8, 10, 13	—	—	d
Scaphiophryne spinosa	Scaphiophryninae	26	2 (short)	2, 3, 4, 8, 10, 13	—	—	d
Anodonthyla boulengeri	Cophylinae	26	—	2; 3; 4	—	—	c
Anodonthyla montana	Cophylinae	26	—	2; 3; 4	—	—	a
Anodonthyla montana	Cophylinae	26	6 (short)	2; 3; 4	—	—	f
Anodonthyla moramora	Cophylinae	26	6 (long)	2; 3; 4	—	—	f
Cophyla phyllodactyla	Cophylinae	26	5 (long)	2; 3; 7; 11	—	4	f
Platypelis barbouri	Cophylinae	26	—	2; 3	4; 11	—	c
Platypelis grandis	Cophylinae	26	—	2; 3; 13	4	—	c
Platypelis grandis	Cophylinae	26	4 (short)	2; 3	—	4; 12	f
Platypelis sp. aff. *mavomavo*	Cophylinae	26	4 (short)	3; 12; 13	—	4	f
Platypelis pollicaris	Cophylinae	26	—	2; 3; 8; 13	4	—	c
Platypelis tuberifera	Cophylinae	26	—	2; 3; 8; 13	4	—	c
Platypelis tuberifera	Cophylinae	26	4 (short)	2; 3	—	4	f
Plethodontohyla alluaudi	Cophylinae	26	—	2; 3; 7; 10; 13	4	—	c
Plethodontohyla alluaudi	Cophylinae	26	2 (long)	2; 4	4; 11	—	f
Plethodontohyla laevipes	Cophylinae	26	2 (long)	2; 4;10; 11	4	—	f
Plethodontohyla notosticta	Cophylinae	26	—	2; 3; 7; 8; 10	4; 12; 13	—	c
Plethodontohyla mihanika	Cophylinae	26	6 (long)	2; 3	4	—	f
Plethodontohyla sp. aff. *minuta*	Cophylinae	26	6 (long)	2; 3	4	8	f
Plethodontohyla tuberata	Cophylinae	26	—	2; 3; 6; 9; 10; 11	4; 13	—	c
Plethodontohyla tuberata	Cophylinae	26	6 (long)	2; 3	4	10	f
Rhombophryne testudo	Cophylinae	26	2 (short)	2; 3	4	—	f
Stumpffia gimmeli	Cophylinae	26	8 (short)	2; 3; 4	—		f
Stumpffia cf. *grandis*	Cophylinae	26	6 (short)	2; 3; 4; 8	—		f
Stumpffia sp.	Cophylinae	26	1 (short)	2; 3; 4	—	8	f
Dyscophus antongili	Dyscophinae	26	—	2; 7	—	—	c
Dyscophus guineti	Dyscophinae	26	—	2; 4; 11	—	—	c
Dyscophus guineti	Dyscophinae	26	6 (long)	2 ;3; 4;10	—	—	f
Dyscophus insularis	Dyscophinae	26	—	2; 3; 12	—	—	c
Ramanella cf. *obscura*	Microhylinae	26	6 (long)	3; 4	6	—	f
Calluella guttulata	Microhylinae	26	6 (long)	2; 3; 4; 6	—	—	f

Species names were partly adjusted to current taxonomy (Blommers-Schlösser and Blanc, 1991; Frost et al., 2006). The three columns indicate which chromosome pairs were identified as submetacentric (sm), subtelocentric (st), or telocentric (t). All other chromosome pairs were metacentric. The chromosomes of *Paradoxophyla palmata* in Blommers-Schlösser (1976) was described here as belonging to ''*Dyscophus* sp.''. The data of Blommers and Blanc (1972) who recorded an all-metacentric karyotype with intraspecific variation in Scaphiophryne madagascariensis (an extra chromosome pair and two acrocentric pairs in a single specimen) are probably not directly comparable with our assessment of metacentric vs. submetacentric states.

third chromosome pairs, which were submetacentric (Fig. 8). The NORs were in a pericentromeric position on the short arm of the second chromosome pair. The heterochromatin was indistinct and could be identified only on some chromosome pairs in a centromeric position, CMA_3 and DAPI negative (Fig. 8).

3.7. Genus *Stumpffia* (subfamily Cophylinae)

The three studied species of this terrestrial cophyline genus, *Stumpffia* cf. *grandis*, *S. gimmeli*, and *S.* sp., had a karyotype of $2n = 26$ chromosomes, the first five chromosome pairs being distinctly larger than the chromosome pairs 6–13. Furthermore, the first pair

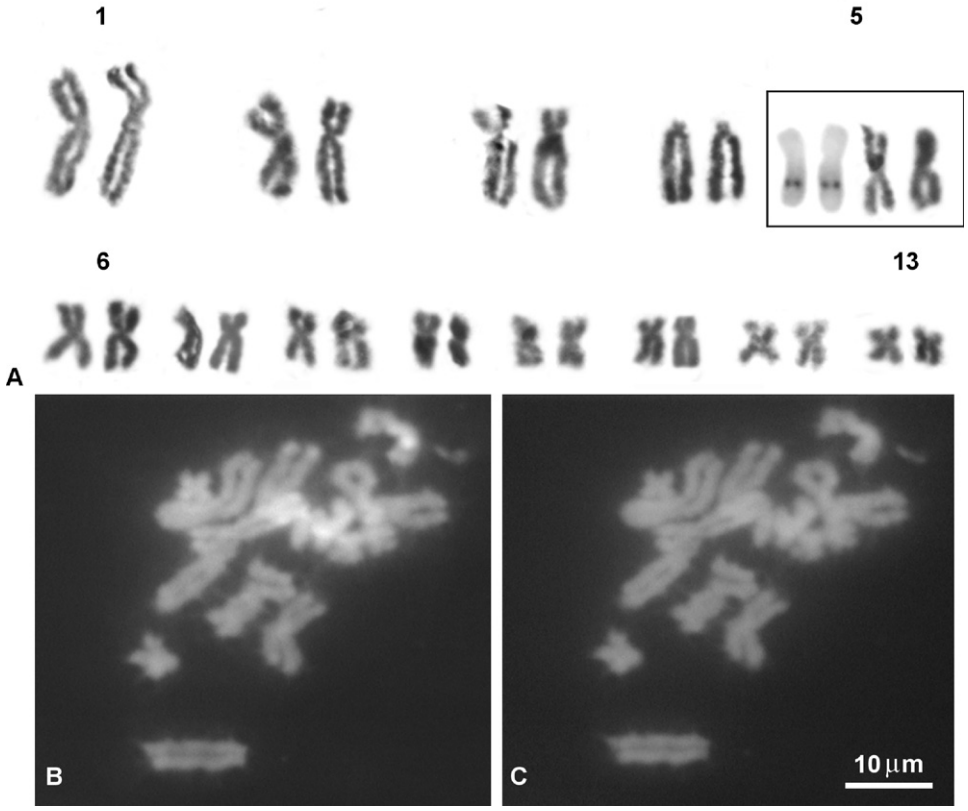

Fig. 4. Karyotype (A) and metaphase plates (B, C) of *Cophyla phyllodactyla* after C-banding and CMA_3-staining (B) and after C-banding, CMA_3-staining, and DAPI-staining (C). The NOR-bearing pair in (A) is framed, with the Ag-NOR-banded picture of the pair to the left of the Giemsa-stained picture. The scale bar in (C) refers to all images.

was distinctively bigger than the second pair. The three species had a subtelocentric fourth chromosome pair, and the chromosome pairs 2 and 3 were submetacentric. *Stumpffia* cf. *grandis* also had submetacentric chromosomes of pair 8. *Stumpffia* sp., in addition, had a submetacentric chromosome pair 12. In *S. gimmeli*, the chromosomes of the eighth pair were telocentric. The NORs in *S.* cf. *grandis* were on the short arm of the sixth chromosome pair, in *S. gimmeli* the NORs were in a subterminal position on the telocentric eighth chromosome pair, and in *S.* sp. the NORs were close to the centromeric region on the short arm of the first chromosome pair. The various banding techniques revealed only the NOR-associated heterochromatin (Fig. 9).

3.8. Genus *Dyscophus* (subfamily Dyscophinae)

Three juvenile specimens of *Dyscophus guineti* were available for examination. Unfortunately, their sex could not be reliably determined, despite thorough examination of the gonads, and we obtained chromosomes suitable for the present study from only two specimens. In agreement with the data presented by Blommers-Schlösser (1976), this species had $2n = 26$ chromosomes, of which the chromosome pairs 1, 5–9, and 11–13 were metacentric and the chromosome pairs 2, 3, 4, and 10 were submetacentric. The size transition from the fifth to the sixth chromosome pair was much less dramatic than in the other cophyline, microhyline, and scaphiophrynine genera considered in our study, and the chromosome pairs 6–9 were all of similar length. The 12^{th} chromosome pair turned out to be heteromorphic in two specimens, with one chromosome larger than the other (Fig. 10). This heteromorphic condition was not immediately obvious through conventional staining, which may explain why it has not been recognized by Blommers-Schlösser (1976).

The NORs were revealed in subterminal position of one of the chromosome pairs 6–9, which we tentatively report as the sixth (Fig. 10). Q-banding DA/DAPI treatment resulted in uniform staining of all chromosomes, indicating the absence of AT-rich regions. CMA_3 staining confirmed the NOR location and revealed CMA_3-positive regions in a centromeric position on all chromosomes, except for the heteromorphic chromosome pair 12, which had an intense telomeric band on both chromosomes, and the longer chromosome had a double band with an intercalated CMA_3-negative region on the telomeres of the short arm. C-banding revealed distinct bands in a centromeric position of all

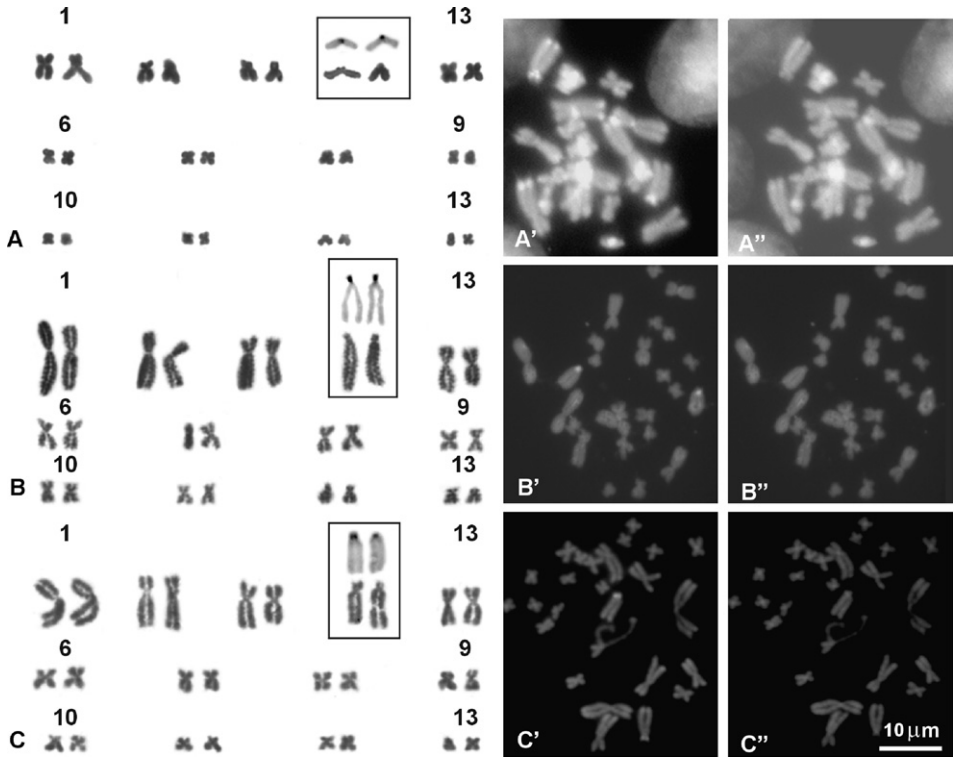

Fig. 5. Karyotypes and metaphase plates of *Platypelis grandis* (A); *P.* sp. aff. *mavomavo* (B); and *P. mihanika* (C). Metaphases are shown after C-banding and CMA_3-staining (A′, B′, and C′) and after C-banding, CMA_3-staining, and DAPI-staining (A″, B″, and C″). The NOR-bearing pairs in (A), (B), and (C) are framed, with the Ag-NOR-banded picture of each pair above the Giemsa-stained picture. The scale bar in (E) refers to all images.

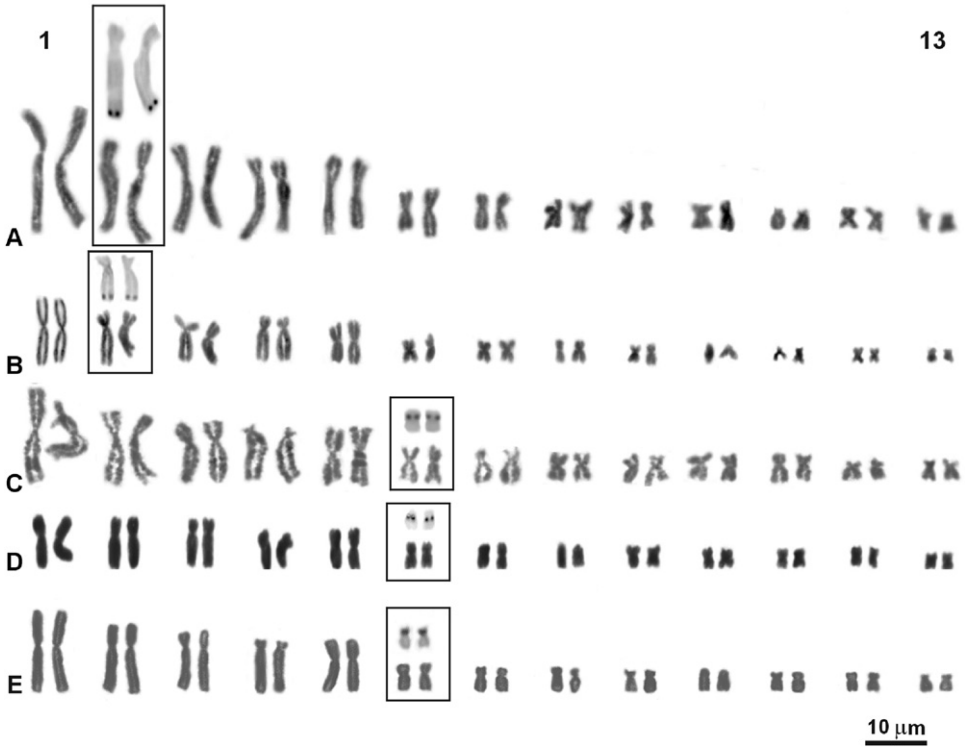

Fig. 6. Karyotype of *Plethodontohyla alluaudi* (A); *P. laevipes* (B); *P. mihanika* (C); *P.* sp. aff. *minuta* (D); and *P. tuberata* (E). The NOR-bearing pairs are framed, with the Ag-NOR- banded picture of each pair above the Giemsa-stained picture. The scale bar in (E) refers to all images.

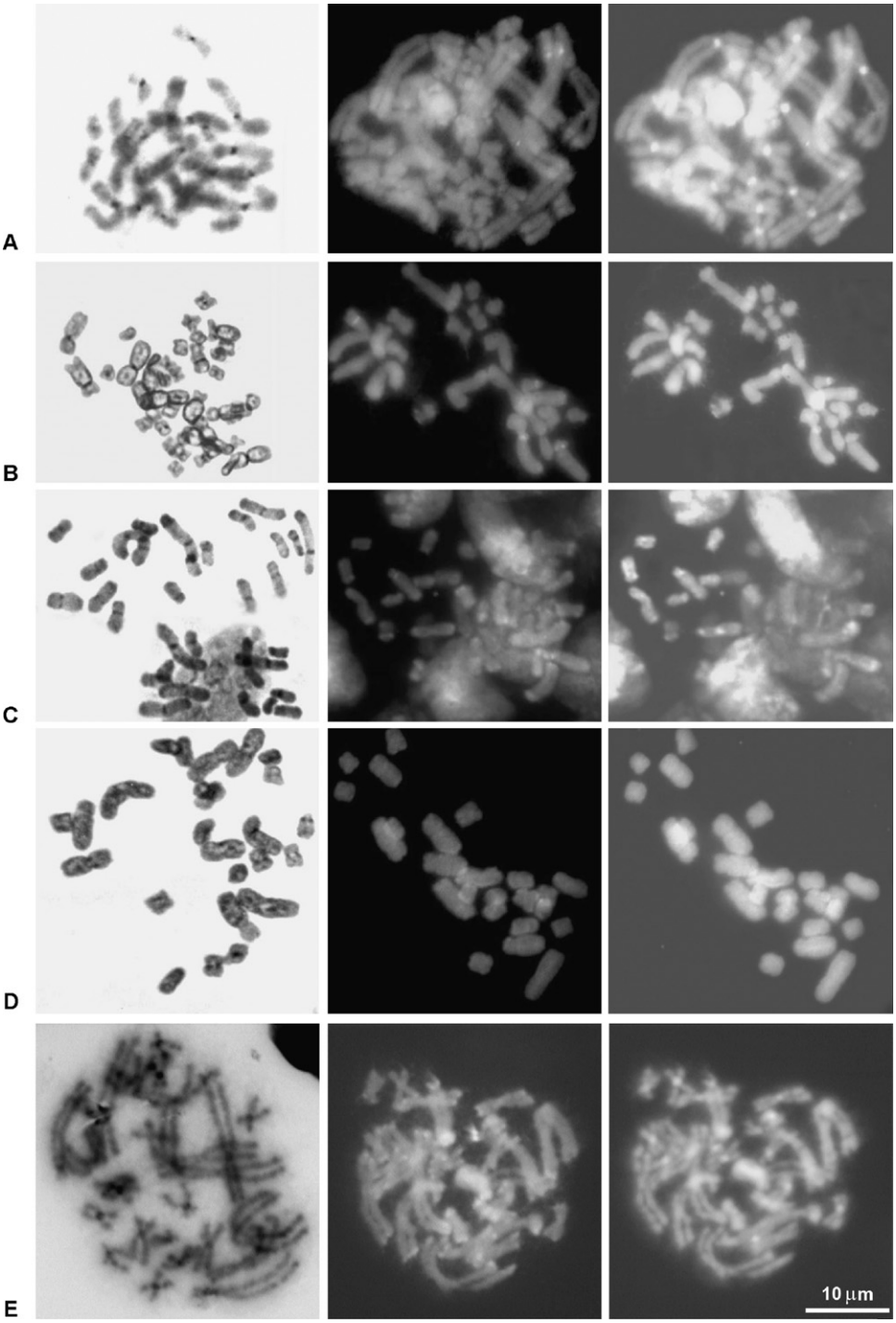

Fig. 7. Metaphase plates of *Plethodontohyla alluaudi* (A); *P. laevipes* (B); *P. mihanika* (C); *P.* sp. aff. *minuta* (D); and *P. tuberata* (E). Metaphases are shown after C-banding and Giemsa-staining (left); after C-banding and CMA_3-staining (center); and after C-banding, CMA_3-staining, and DAPI-staining (right). The scale bar in (E) refers to all images.

chromosome pairs and in a telomeric position on at least seven pairs (Fig. 10). *Alu* I treatment digested the telomeric bands on all but one chromosome pair, whereas the centromeric bands were still visible after digestion. The *Alu* I-resistant telomeric band probably corresponds to the NOR-associated heterochromatin (Fig. 10). After sequential C-banding + CMA_3 + DAPI staining, both the centromeric and the telomeric bands were CMA_3 positive as well as DAPI positive, with the centromeric bands staining more intensely with CMA_3 and the telomeric bands more intensely with DAPI. Also on the heteromorphic chromosome pair, the heterochromatin was stained positively with the two fluorochromes. CMA_3 and DAPI staining after *Alu* I

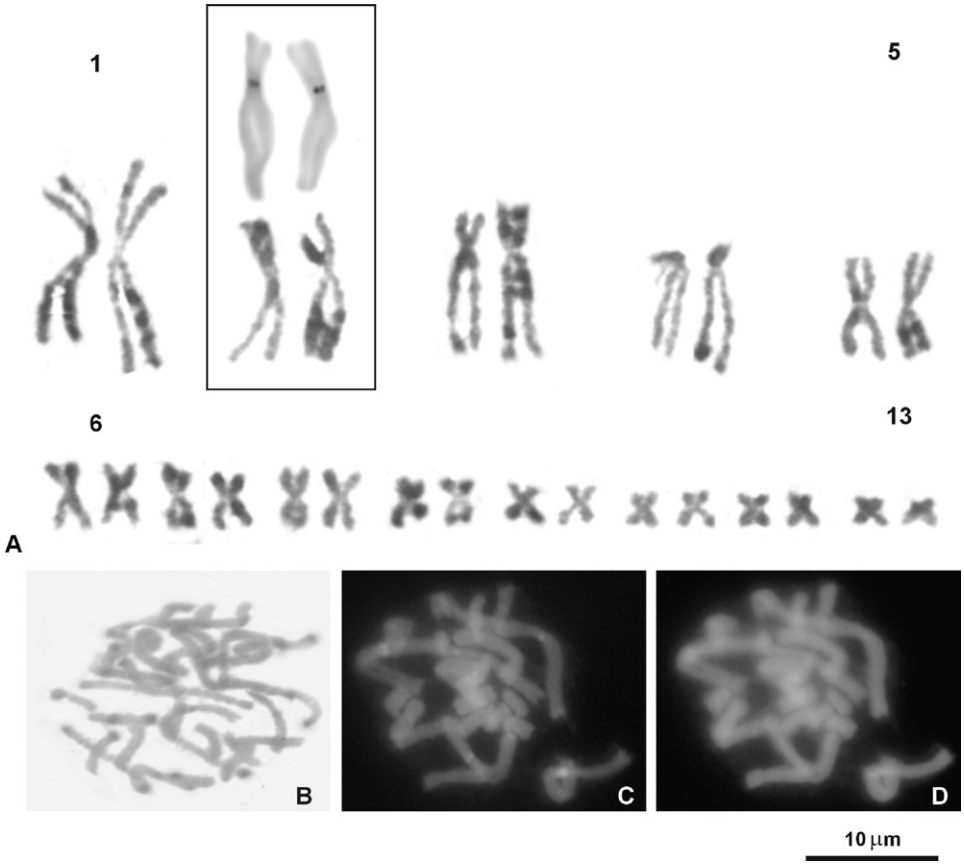

Fig. 8. Karyotype (A) and metaphase plates after C-banding and Giemsa-staining (B), after C-banding and CMA_3-staining (C), and after C-banding, CMA_3-staining, and DAPI-staining (D) of *Rhombophryne testudo*. The NOR-bearing pair in (A) is framed, with the Ag-NOR-banded picture of the pair above the Giemsa-stained picture. The scale bar refers to all images.

digestion obtained comparable results, except for the telomeric regions that were uniformly stained (Fig. 10). The CMA_3-banded karyotype and the various staining characteristics of the heteromorphic pair are shown in Fig. 11.

3.9. Genus *Calluella* (subfamily Microhylinae)

The studied male specimen of this Asian species, *Calluella guttulata*, had a karyotype of $2n = 26$ chromosomes, of which chromosome pairs 1, 5, and 7–13 are metacentric and chromosome pairs 2–4 and 6 were submetacentric (Fig. 12). As in *Dyscophus guineti*, the size transition between the fifth and sixth chromosome pairs was not distinct, but unlike in *Dyscophus*, the chromosome pairs 7–9 were distinctly smaller. Furthermore, the apparent increased length of the sixth chromosome pair could be a consequence of the presence of a secondary constriction in the pericentromeric position on the long arm, corresponding to the position of the NORs. Q-banding was uniform on all chromosomes. C-banding and *Alu* I digestion revealed faint centromeric bands on almost all chromosomes and distinct C-bands in the paracentromeric position on the short arm of the second chromosome pair. In addition, a distinct NOR-associated C-band was found in a pericentromeric position on the sixth chromosome pair. Both of these paracentromeric bands were *Alu* I resistant, as was one of the centromeric bands, probably on the eighth chromosome pair. The centromeric bands were CMA_3 and DAPI negative, whereas the NOR-associated heterochromatin consisted of an upper CMA_3-positive band and a lower DAPI-positive band. The paracentromeric band on the second pair was DAPI positive. After *Alu* I digestion the NOR-associated heterochromatin (DAPI positive after C-banding) was DAPI negative. Furthermore, the centromeric band on the eighth chromosome pair was DAPI positive and CMA_3 negative after *Alu* I digestion (Fig. 12).

3.10. Genus *Ramanella* (subfamily Microhylinae)

The male specimen studied from this representative of the Microhylinae had a karyotype of $2n = 26$ chromosomes, which were metacentric (pairs 1, 5, 7–13), submetacentric (pairs 2–4, 6), or subtelocentric (pair 6). The NORs were

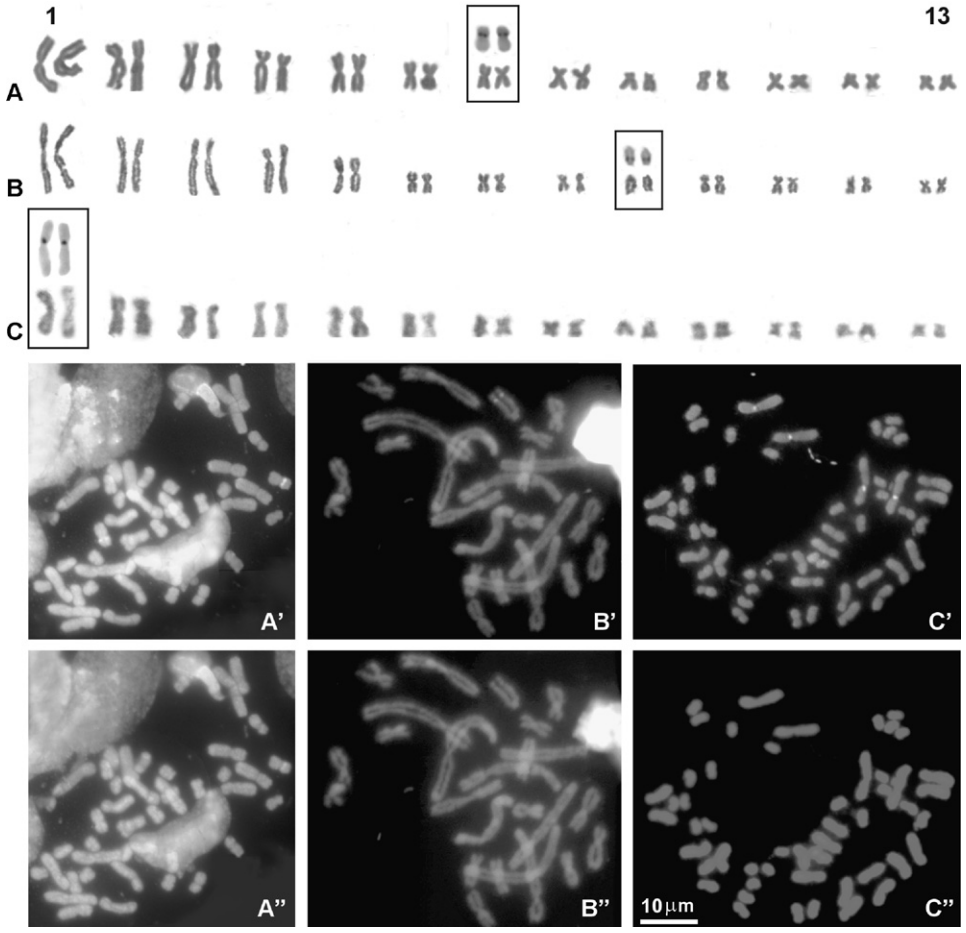

Fig. 9. Karyotypes and metaphase plates of *Stumpffia* cf. *grandis* (A), *S. gimmeli* (B), and *S.* sp. (C) after C-banding and CMA_3-staining (A′, B′, and C′), and after C-banding, CMA_3-staining, and DAPI-staining (A′, B′, and C′). The NOR-bearing pairs in (A), (B), and (C) are framed, with the Ag-NOR-banded picture of each pair above the Giemsa-stained picture. The scale bar in (C″) refers to all images.

located very close to the centromeres on the long arm of the sixth pair. CMA_3 staining confirmed this position of the NORs as obtained by Ag-NOR staining and, in addition, revealed CMA_3-positive regions paracentromerically on the long arm of the second chromosome pair and on at least two of the chromosome pairs 6–13 (Fig. 13). C-banding and sequential C-banding + CMA_3 + DAPI staining revealed strong centromeric C-bands on all chromosomes, which turned out to be DAPI positive, and distinct NOR-associated heterochromatin that was CMA_3 positive. The paracentromeric regions that stained positive with CMA_3 were also slightly CMA_3 positive using the sequential staining method (Fig. 13).

4. Discussion

4.1. Karyological characters and the systematics of Malagasy microhylids

The Malagasy microhylids studied to date are characterized without exception by a karyotype of $2n = 26$ chromosomes (or $4n = 52$ in *Scaphiophryne gottlebei*), all or a great majority of which are meta- or submetacentric, and the first five chromosome pairs are almost always distinctly larger than the remaining pairs. Based on its universal occurrence in many groups of ranoid frogs, such a karyotype has been considered to be ancestral in this lineage (King 1990), to which also the microhylids belong (Van der Meijden et al. 2004). The definition of karyological character states in amphibians as plesiomorphic or apomorphic is, in general, problematic, since no comprehensive and formal attempts have so far been undertaken either to reconstruct anuran phylogeny with chromosome characters in a cladistic approach, or to trace chromosomal character state transformations along a well-resolved tree based on other characters. Based on the current prevalent opinion on this subject, we here state preliminary hypotheses which should be tested cladistically once more complete data sets of chromosome characters become available.

As will be discussed below, the location of NORs appears to be a potentially useful phylogenetic marker

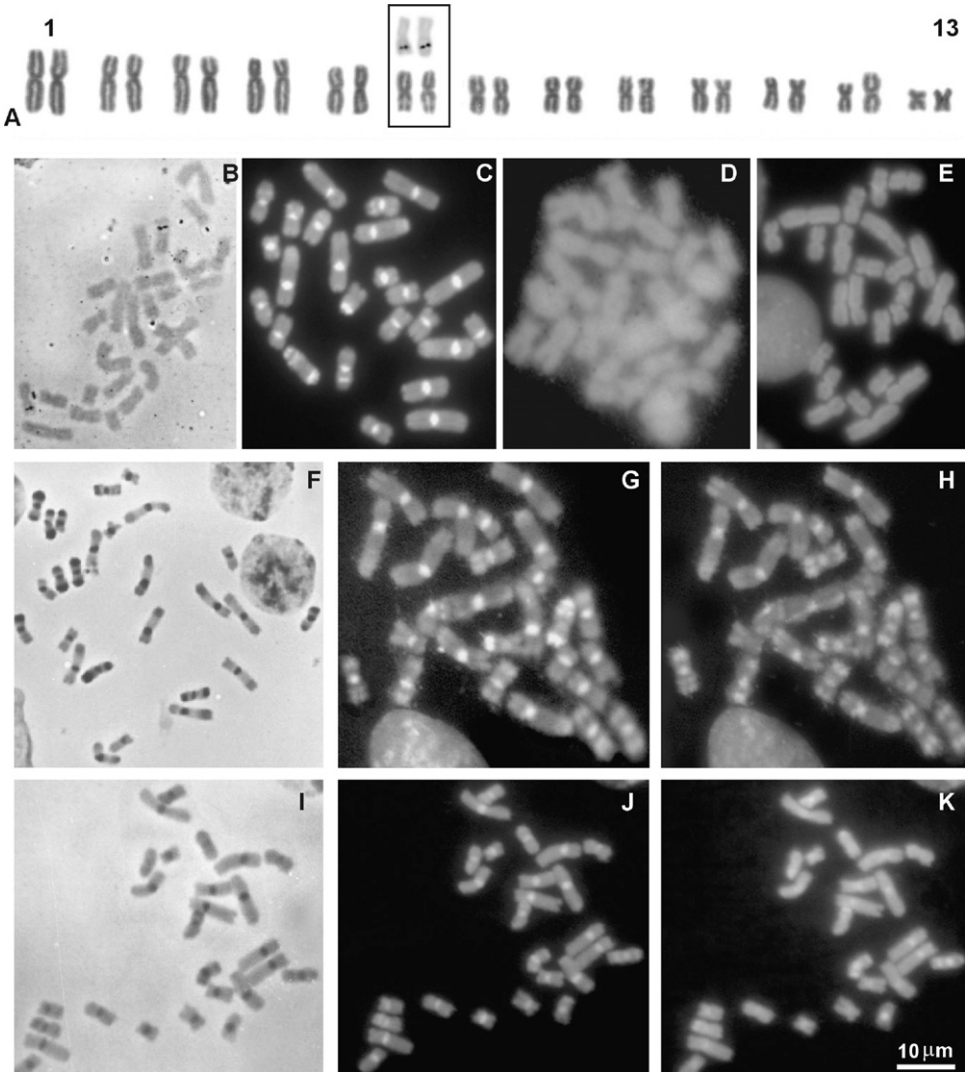

Fig. 10. Karyotype (A) and metaphase plates of *Dyscophus guineti,* stained with Ag-NOR (B); CMA_3/MG (C); Q-banding (D); DA/DAPI (E); C-bandig and Giemsa (F) C-banding and CMA_3 (G); C-banding, CMA_3, and DAPI (H); Alu I and Giemsa (I); Alu I and CMA_3 (J); Alu I, CMA_3, and DAPI (K). The NOR-bearing pair in (A) is framed, with the Ag-NOR-banded picture of the pair above the Giemsa-stained picture. The scale bar refers to all images.

to define genus-level clades of microhylids. However, comparative data on this character from non-Malagasy microhylids are very scanty and, so far, available only for *Kaloula pulchra* with NORs on the fifth pair (Schmid 1978), for the African *Phrynomantis bifasciatus* with NORs on the sixth pair (Schmid 1980), and for *Ramanella* cf. *obscura* with NORs on the sixth pair (this paper). An unambiguous identification of synapomorphies in NOR position among microhylids is, therefore, not yet possible.

All representatives of the Scaphiophryninae studied (*Scaphiophryne* and *Paradoxophyla)* are characterized by a very uniform karyotype. Notable is also that the NOR position on the short arms of the second chromosome pair is conserved in all species of both genera studied so far (see also Vences et al. 2002). *Paradoxophyla palmata* was initially described as belonging to the genus *Microhyla* in the subfamily Microhylinae (Guibé 1974), but was later transferred to the Scaphiophryninae based on the presence of a vomer and sphenethmoid that are undivided (versus divided in *Microhyla*), and without a detailed phylogenetic analysis of these osteological characters (Blommers-Schlösser and Blanc 1991). While *Scaphiophryne* have unique tadpoles that are intermediate between the microhylid and the ranid type, with a horny beak-like ranids, but without labial teeth-like microhylids (Wassersug 1984), the tadpoles of *Paradoxophyla* are of the typical specialized filter-feeding microhylid type (Blommers-Schlösser and Blanc 1991; Glaw and Vences 1994; Mercurio and Andreone 2006). The morphological analysis of Haas (2003) failed to place *Scaphiophryne*

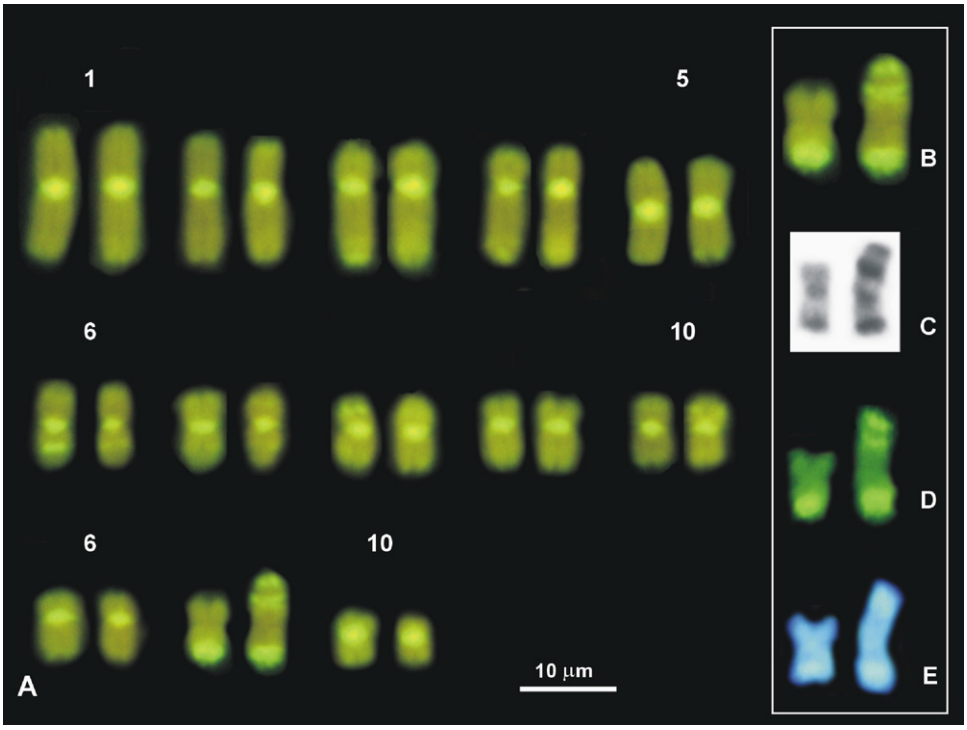

Fig. 11. Karyotype of *Dyscophus guineti* after CMA_3/MG-staining. The inserted picture shows enlarged views of the heteromorphic chromosome pair after CMA_3/MG-staining (A); C-banding (B); C-banding and CMA_3-staining (C); and C-banding, CMA_3-staining, and DAPI-staining (D). The scale bar refers to all images.

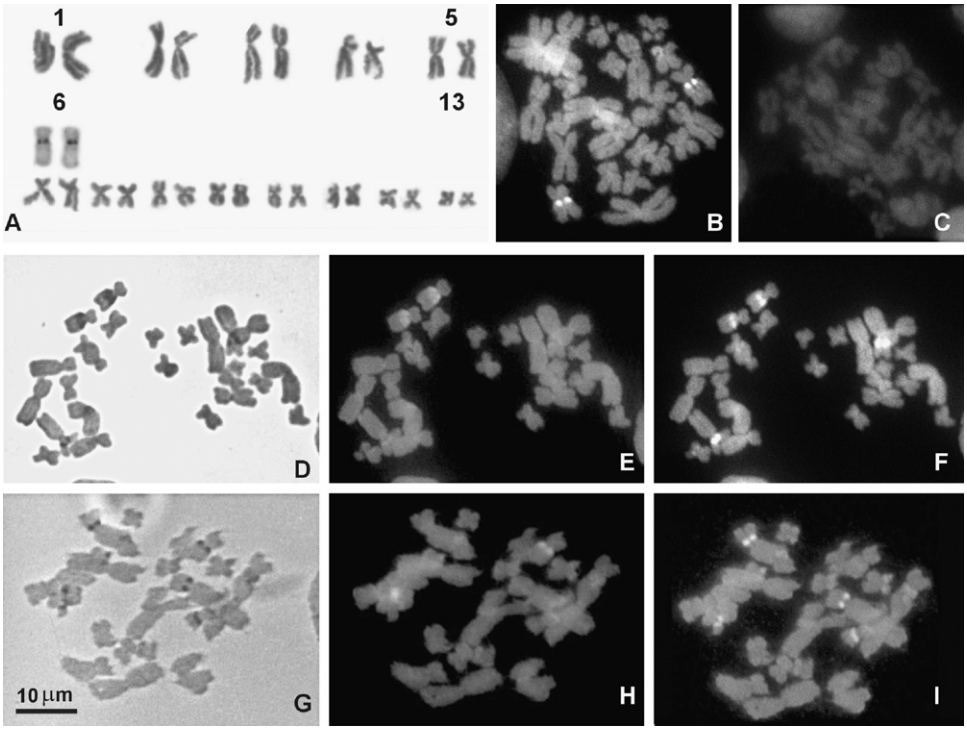

Fig. 12. Karyotype of *Calluella guttulata* (A) and metaphase plates after staining with CMA_3/MG (B); Q-banding (C); C-banding and Giemsa (D); C-banding and CMA_3 (E); C-banding, CMA_3, and DAPI (F); Alu I and Giemsa (G); Alu I and CMA_3 (H); and Alu I, CMA_3, and DAPI (I). The NOR-bearing pair in (A) is shown with the Ag-NOR-banded picture above the Giemsa-stained picture. The scale bar refers to all images.

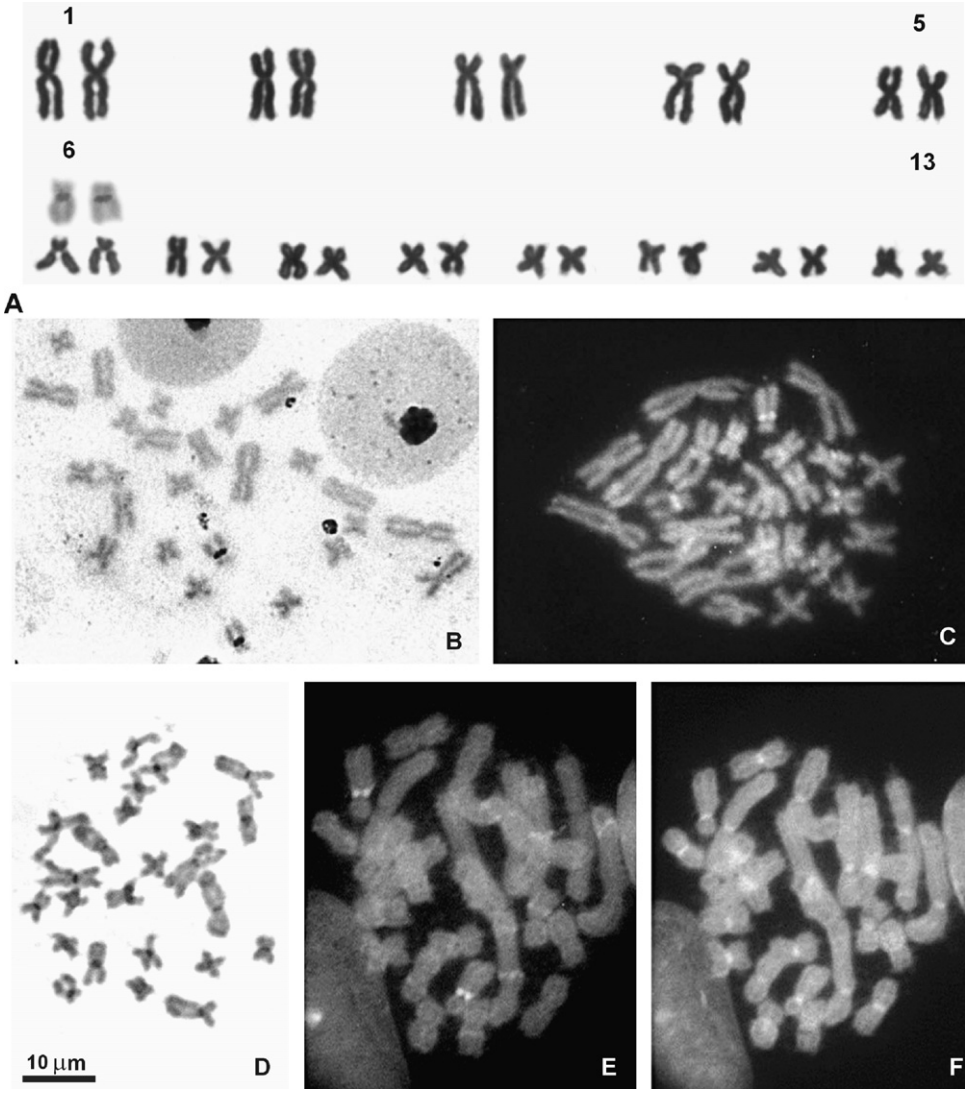

Fig. 13. Karyotype of *Ramanella* cf. *obscura* (A) and metaphase plates stained with Ag-NOR (B); CMA_3/MG (C); C-banding and Giemsa (D); C-banding and CMA_3 (E); C-banding, CMA_3, and DAPI (F). The NOR-bearing pair in (A) is shown with the Ag-NOR-banded picture above the Giemsa-stained picture. The scale bar refers to all images.

and *Paradoxophyla* in a monophyletic group, and Frost et al. (2006) transferred the genus into ''Microhylidae *incertae sedis*''. Unpublished molecular data of A. van der Meijden and M. Vences instead corroborate scaphiophrynine monophyly. The chromosomal similarities between these two genera could be an important argument supporting their phylogenetic relatedness, although for the time being it is not possible to polarize reliably the chromosomal character states and define clear karyological synapomorphies for the Scaphiophryninae.

The Cophylinae are characterized by a fourth chromosome pair that is usually subtelocentric or in some cases even telocentric, such as in *Cophyla phyllodactyla* and in some species of *Platypelis*, and *Plethodontohyla.* Only in *Anodonthyla* are chromosomes of the fourth pair submetacentric, though close to being subtelocentric. If the typical ranoid karyotype, as described above, were indeed ancestral in the lineage leading to cophylines, then the origin of subtelocentric elements in this group could be seen in pericentric inversions of the metacentric elements of a putative cophyline ancestor, and the state seen as synapomorphy of the group.

The location of NORs is likely to bear information for cophyline systematics, although a thorough polarization of character states is not possible at present. First, general NOR placement is uniform in the included species of *Anodonthyla* (NORs on the sixth chromosome pair) and *Platypelis* (on the fourth pair), respectively. NOR position in *Cophyla* (on the fifth chromosome pair) differs from that in *Platypelis*, which supports the recognition of *Cophyla* as a separate genus despite a great similarity to *Platypelis* in external morphology.

The two distinctly different NOR positions in *Plethodontohyla* (on the second pair Versus the sixth pair) agrees with molecular results (Andreone et al. 2005) that placed the corresponding species into two different lineages. The similar NOR position on the second pair in *Rhombophryne testudo* and *Plethodontohyla* group 2 (in our study represented by *P. alluaudi* and *P. laevipes*) agrees with the phylogenetic position of *R. testudo* nested within *Plethodontohyla* group 2 based on molecular data by Andreone et al. (2005). In all three species of *Stumpffia* examined here, the NORs were found at different positions, but it might be a shared characteristic of *Stumpffia* karyotypes that the chromosomes of the first pair was distinctly larger than those of the second pair.

It is possible to develop a hypothesis of chromosomal evolution in cophylines, but this requires several assumptions. First, we will base our conclusion on the maximum likelihood tree of Andreone et al. (2005), but it needs to be kept in mind that several basal nodes in that tree were poorly resolved. Second, we will assume that a position of NORs on the sixth pair is the ancestral state for cophylines, based on the fact that this character is found in most outgroups (i.e., *Phrynomantis*, *Caluella*, *Ramanella*, and *Dyscophus*). Third, we will assume that the ancestral state in the lineage leading to cophylines was a meta- or submetacentric fourth chromosome pair as found in other microhylids. Under these assumptions, the telocentric or subtelocentric fourth chromosome pair should be seen as a synapomorphy of the Cophylinae, the NOR location on the second chromosome as a synapomorphy of *Plethodontohyla* group 2 and *Rhombophryne*, the NOR location on the fourth pair as derived for *Platypelis*, the NOR location on the fifth pair as derived for *Cophyla*, and the NOR location on the first and eighth pairs as autapomorphies for two species of *Stumpffia*, respectively (Fig. 14).

Irrespective of whether or not this evolutionary scenario explains the origin for the NOR positions encountered in the Cophylinae, the high incidence of change in this character is a striking difference with respect to other frog lineages, such as the scaphiophrynines (see above) or the mantellid genus *Boophis* from Madagascar, in which changes in the NOR position are very rare (Aprea et al. 2004). Various mechanisms may be responsible for these NOR translocations, such as cryptic structural rearrangements, minute insertions, reintegration of rDNA genes amplified during ovogonial auxocytosis, or the activation of silent sites (Nardi et al. 1977; Schmid 1978; King 1980; Mahony and Robinson 1986; Schmid and Guttenbach 1988). However, the fact that also other modifications of chromosomal morphology seem to be frequent in cophylines (as evidenced from the numerous occurrences of subtelocentric or telocentric chromosome pairs in addition to those on the fourth one; Table 2) may indicate that, in general terms, chromosome restructuring in this lineage is particularly intense, which may relate to their relatively fast mitochondrial evolution (Vences et al. 2002) and high cryptic species diversity (Andreone et al. 2005). Indeed, the genus *Eleutherodactylus*, another anuran lineage of high species diversity, is also characterized by a high rate of chromosomal change (Bogart and Hedges 1995).

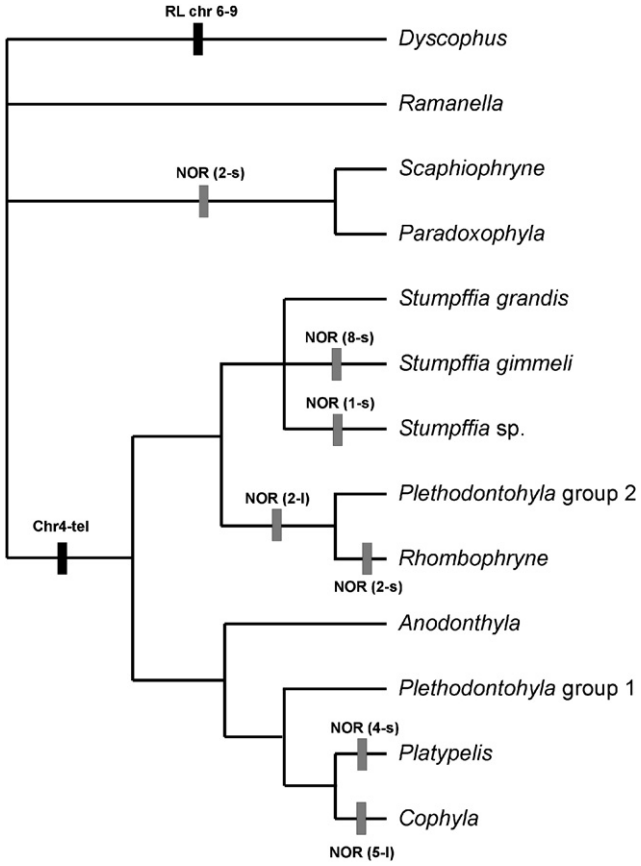

Fig. 14. Phylogenetic tree of the Malagasy microhylids, based on mitochondrial data of Andreone et al. (2005) and Van der Meijden et al. (2004). The changes in NOR position and major chromosome morphology are plotted on the tree, assuming a parsimonious hypothesis of minimal number of changes. The hypothesized changes in chromosome morphology rely on the assumption that a karyotype of $2n = 26$ with all metacentric or submetacentric chromosomes, and the chromosome pairs 1–5 clearly larger than the chromosome pairs 6–9, is the ancestral state. Based on the fact that the microhylines *Ramanella* and *Caluella*, the phrynomerine *Phrynomantis bifasciatus,* the dyscophine *Dyscophus*, and many cophylines have the NORs on the 6th chromosome pair (Schmid, 1980; this paper), we consider this condition as the ancestral condition as well. Characters are coded as follows: RL chr 6–9, shift in the relative length of chromosome pairs 6–9 without distinct size transition between pairs 5 and 6; chr 4-tel, subtelocentric or telocentric 4th chromosome pair (submetacentric close to subtelocentric in *Anodonthyla*). NOR position is given as chromosome pair number and short (s) or long (l) branch.

The studied species originally included in the subfamily Dyscophinae (i.e., Caluella and Dyscophus) are characterized by a position of the NORs on the sixth chromosome pair. However, because this location is also observed in many cophylines, in which it might be the ancestral state (Fig. 14), in the phrynomerine *Phrynomantis*, and in the microhyline *Ramanella*, it cannot be seen as an unequivocal synapomorphy uniting the Malagasy genus *Dyscophus* and the Asian genus *Calluella*. One of the characteristics of the *Dyscophus* karyotype is the lack of a distinct size gap between the first five chromosome pairs and the remaining eight pairs. Our results indicate that this may be caused by phenomena of amplification of satellite DNA, because the various banding techniques provided evidence for large blocks of heterochromatin, known to be made up largely of satellite DNA, in telomeric position on at least four of the chromosome pairs 6–13. Although some size particularities were also found in the karyotype of *Calluella*, it is uncertain whether these can be seen as synapomorphy of the two genera.

4.2. Genus-level classification of cophylines

Andreone et al. (2005), based on their mitochondrial phylogeny, found evidence for non-monophyly of the genus *Plethodontohyla* but refrained from taxonomic conclusions because only a small portion of the species of *Plethodontohyla* had been studied and could be assigned to either clade. They distinguished two groups within *Plethodontohyla*: a first clade, *Plethodontohyla* group 1, that possibly is related to *Platypelis* and *Cophyla* and contains the type species of the genus, *Plethodontohyla notosticta*; and a second clade, sister to *Stumpffia* and named *Plethodontohyla* group 2, that also includes *Rhombophryne testudo*. Frost et al. (2006), based on the published data of Andreone et al. (2005), transferred the three species known at the time to belong to *Plethodontohyla* group 2 (*P. alluaudi*, *P. coudreaui*, and *P. laevipes*) to the genus *Rhombophryne*. We discovered the partition of *Plethodontohyla sensu lato*, which was proposed by Frost et al. (2006) while the present paper was in review, but we decided not to adopt the taxonomic changes here. The chromosomal data presented herein reinforce the polyphyly of the genus *Plethodontohyla sensu lato* and fully agree with the molecular data of Andreone et al. (2005). Of the species studied herein, *Plethodontohyla mihanika*, *P.* sp. aff. *minuta*, and *P. tuberata*, with NORs on the sixth chromosome pair, belong to *Plethodontohyla* group 1, whereas *P. alluaudi* and *P. laevipes* belong to *Plethodontohyla* group 2 and have NORs on the second chromosome pair, similar to *Rhombophryne testudo*.

Our own novel, but yet to be published molecular data of a near-complete taxon sampling will provide the basis for a more stable classification of cophylines and will assign all species of *Plethodontohyla sensu lato* either to the genus *Plethodontohyla* or to the genus *Rhombophryne*, which will be expanded to include the species of *Plethodontohyla* group 2. Furthermore, during inventory work in progress, we have identified several undescribed taxa (not included here) that may also render the genus *Stumpffia* non-monophyletic, further supporting the need for a comprehensive reassessment of cophyline classification, instead of partial and incomplete generic reassignments as data on a few taxa at a time are generated.

4.3. Heterochromatin in Malagasy microhylids

Analyses of C-banded karyotypes carried out on a range of amphibian species, including some that are congeneric and others that have near-identical karyotypes with respect to chromosome number, shape, and size, have shown differences in the distribution and size of C-bands. Until now, it has been impossible to find any two amphibian species, even if very closely related, that had identical C-band patterns (e.g., Morescalchi 1983). This situation also applies to the microhylid species investigated here, each of which displaying a unique C-banding pattern. Within Malagasy microhylids, heterochromatin often is abundant on telomeric regions, while centromeric heterochromatin generally is present as faint C-bands and only on a few chromosomes. Only *Dyscophus* deviates from this pattern, containing heterochromatin in relevant quantities in centromeric and telomeric positions. This heterochromatin is known to be composed of different families of highly repetitive DNA (John 1988) and, in *Dyscophus*, has turned out to be highly heterogeneous. It contains at least six different heterochromatin types, namely two types of centromeric heterochromatin, three types of telomeric heterochromatin, and the NOR-associated heterochromatin: (1) centromeric *Alu* I resistant and intensely CMA_3- and DAPI-positive heterochromatin present on all but the heteromorphic chromosome pair; (2) centromeric *Alu* I sensible and CMA_3- and DAPI-negative heterochromatin present only on the heteromorphic chromosome pair; (3) telomeric heterochromatin on the five largest chromosome pairs, appearing as a grey C-band and as *Alu* I sensible and slightly CMA_3-positive heterochromatin; (4) telomeric heterochromatin on chromosome pairs 6–13, except for the heteromorphic pair, appearing as an intense C-band and as *Alu* I sensible, CMA_3- and DAPI-positive heterochromatin; (5) telomeric heterochromatin on the heteromorphic chromosome pair, appearing as *Alu* I sensible and as intensely CMA_3- and DAPI-positive hetrochromatin; and (6) NOR-associated heterochromatin, appearing as *Alu* I resistant and CMA_3-positive heterochromatin.

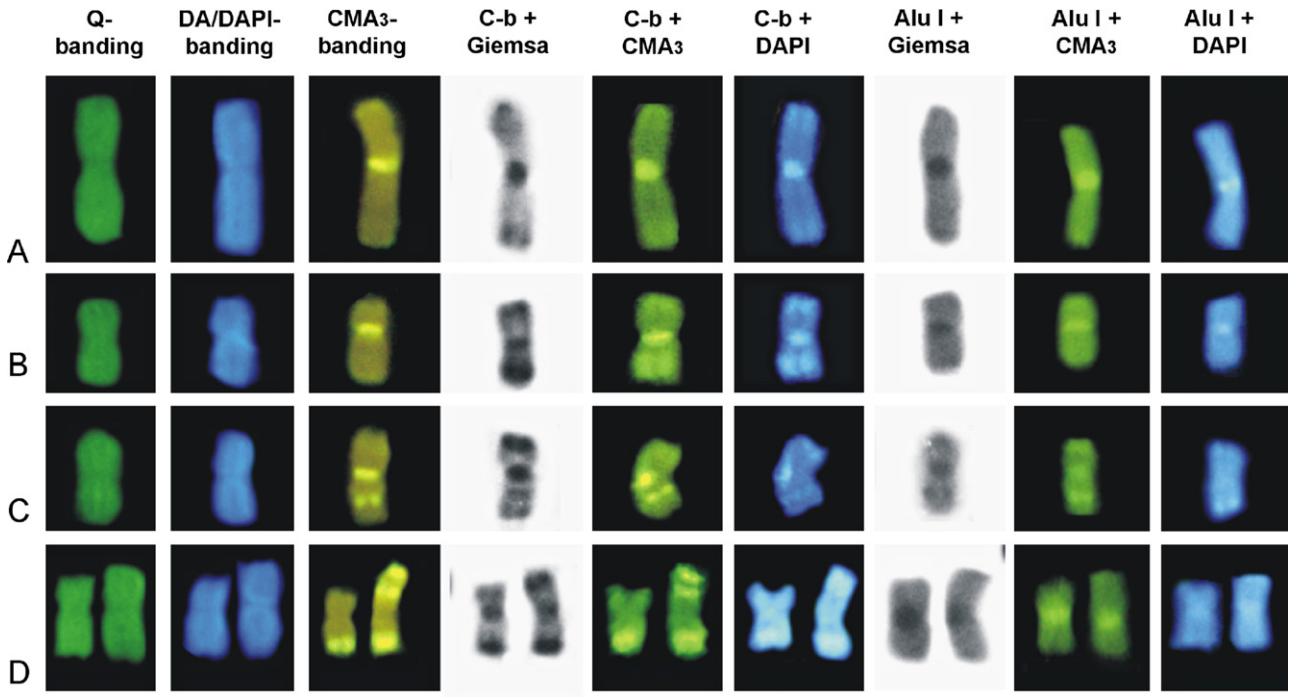

Fig. 15. Different types of heterochromatin in *Dyscophus guineti,* as observed on chromosome pairs 1–5 (A; represented by chromosome 1); on chromosome pairs 7–11 and 13 (B; represented by chromosome 8); on the NOR-bearing sixth chromosome pair (C); and on the heteromorphic 12th chromosome pair (D) after the various staining techniques as given on top of each column.

Two aspects should be stressed in *Dyscophus*. One concerns the fact that DA/DAPI and Q-banding, both of which are AT-specific fluorochromes, resulted in a uniform stain of all chromosomes (Fig. 15). A similar result was obtained by Schmid et al. (2003) by Q-banding in the Australian frog genus *Mixophyes*, indicating that the fluorescence of chromosomes after Q-banding does not only depend on the presence of AT-rich regions, but also on the incidence of interspersed GC-base pairs, on protein effects, and on the degree of condensation of the chromatin. Our results allow the extension of these conclusions to the CMA_3 and DAPI staining and indicate that the heterochromatin of *Dyscophus guineti* contains at least two families of satellite DNA, namely one that is GC rich and is accessible to CMA_3 stain independent from hydrolysis because it is not strongly spiralized, and another one that is AT-rich and and is accessible to DAPI staining only after hydrolysis because it is strongly spiralized possibly because AT-rich regions result in a stronger curvature of the DNA strand (Radic et al. 1987).

The second aspect that should be stressed in *Dyscophus* concerns the heteromorphic pair, whose chromosomes are endowed with telomeric and centromeric heterochromatin that differs from that displayed on the other chromosome pairs. These heterochromatin peculiarities, in addition to the encountered chromosome heteromorphism in the specimens examined, suggest that the heteromorphic pair indeed is a pair of sex chromosomes. However, considering the currently limited number of analyzed specimens and the impossibility to identify their sex, more data from mature specimens of various populations and co-generic species are required to draw definitive conclusions on the presence of sex chromosomes in *Dyscophus guineti* and possibly in other species of this genus, and on the identity of one of the heteromophic chromosomes as possible sex chromosome.

Acknowledgements

This study would not have been possible without the assistance and patience of many friends, colleagues, and students in the field. We are particularly indebted to Kathrin Glaw, Alexander Kupfer, Marta Puente, Liliane Raharivololoniaina, Jasmin Randrianirina, Meike Thomas, and David R. Vieites. This study was carried out in the framework of cooperation accords between the Parc Botanique et Zoologique de Tsimbazaza and the Museo Regionale di Scienze Naturali, Torino, and between the Institute for Biodiversity and Ecosystem Dynamics at the University of Amsterdam, the Zoologische Staatssammlung München, and the Département de Biologie Animale de l'Université d'Antananananarivo. We are grateful to the Malagasy authorities for research and export permits, and to the Association Nationale de Gestion des Aires Protegées

ANGAP for the permit of working in nature reserves. This work was supported by grants of the Deutsche Forschungsgemeinschaft and the Volkswagen Foundation to M.V. and F.G., and of the Università degli Studi di Napoli Federico II to G.A and G.O.

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