Chromosome Research **10**: 127–136, 2002.

Ancient tetraploidy and slow molecular evolution in *Scaphiophryne*: ecological correlates of speciation mode in Malagasy relict amphibians

Miguel Vences[1], Gennaro Aprea[2], Teresa Capriglione[2], Franco Andreone[3] & Gaetano Odierna[2]
[1] *Department of Biology (Evolutionary Biology), University of Konstanz, D-78457 Konstanz, Germany. Fax +49 221 9417285, E-mail: m.vences@t-online.de;* [2] *Dipartimento di Biologia Evolutiva e Comparata, Università di Napoli "Federico II", Via Mezzocannone 8, I-80134 Napoli, Italy. Fax +39 081 2535035, E-mail: odierna@dgbm.unina.it;* [3] *Sezione di Zoologia, Museo Regionale di Scienze Naturali, Via G. Giolitti 36, I-10123 Torino, Italy. Fax +39 011 4323331. E-mail: frand@tin.it*

Received 21 September 2001; received in revised form and accepted for publication by Michael schmid 16 November 2001

Key words: allotetraploidy, amphibia, chromosome speciation, mtDNA substitution rates, *Scaphiophryne gottlebei*

Abstract

Karyotypes of three microhylid frog species of the Malagasy relict genus *Scaphiophryne* were studied: *Scaphiophryne gottlebei*, *S. madagascariensis* and *S. spinosa*. The latter two showed a plesiomorphic ranoid karyotype of 2n = 26. In contrast, tetraploidy was demonstrated in *S. gottlebei,* which constitutes an exceptional state among Malagasy amphibians. A combination of different banding techniques and of rDNA-FISH provided evidence for allopolyploidy in the species and for a completed subsequent functional and structural diploidization. Phylogenetic analysis of mitochondrial 16S rDNA sequences revealed a significant deceleration of nucleotide substitution rates in *Scaphiophryne*. The tetraploidy of *S. gottlebei* probably occurred early in their radiation. Ecological and behavioural patterns of *Scaphiophryn*e probably favoured intraspecific gene flow and hybridization events, thereby leading to slow molecular substitution rates and to allopolyploid chromosome speciation in *S. gottlebei.*

Introduction

Explaining patterns of tropical biodiversity has become an important topic in recent works (Moritz *et al.* 2000). Particular attention is paid to the often explosive adaptive radiations which can lead to high numbers of speciation events in short time intervals (e.g. Meyer 1993, Schluter 1996). However, little is known on the evolutionary mechanisms in relict groups which apparently maintain a low species diversity throughout time.

Both in animals and plants, polyploidization is among the most prominent examples of karyological events that can lead to sudden postmeiotic isolation and thereby to the evolution of new species (King 1993). In anuran amphibians, polyploidy is relatively widespread and evolved independently in different lineages. Currently, at least 40 species of polyploid anurans are known, which belong to seven families (Bufonidae, Hylidae, Leptodactylidae, Microhylidae, Myobatrachidae, Pipidae and Ranidae; see King 1990, Kuramoto 1990).

The diversity of the extant Malagasy frog fauna is extraordinary (Glaw & Vences 2000), mainly due to the species richness of a few endemic radiations. However, Madagascar also harbours less-speciose relict groups (Vences *et al.* 2000a). The enigmatic genus *Scaphiophryne* has been considered as a 'living fossil' (Wassersug 1984) and is usually placed into its own subfamily Scaphiophryninae in the cosmopolitan family Microhylidae. Although *Scaphiophryne* are known from most of Madagascar's diverse habitat types, only six nominal species are known so far (Glaw & Vences 1994).

The only karyological information on *Scaphiophryne* has been published by Blommers & Blanc (1972), who reported on the karyotype of a species close to *S. madagascariensis.* In the present paper, we report on tetraploidy in the Malagasy frog *S. gottlebei,* and compare its chromosome characteristics with those of two additional species of the genus, *S. madagascariensis* and *S. spinosa.* Furthermore, we present the results of a molecular phylogenetic analysis of the genus which provides evidence of slow DNA substitution rates in *Scaphiophryne*.

Materials and methods

Taxonomy and voucher specimens

Taxonomy follows Glaw & Vences (1994) with the following changes: usage of *Scaphiophryne madagascariensis* as senior synonym of *S. pustulosa*, of *S. spinosa* as valid species, and recognition of a new species here named *S.* sp. aff. *marmorata*. Vouchers were deposited in the collections of the Museo Regionale di Scienze Naturali, Torino (MRSN); Université d'Antananarivo, Département de Biologie Animale (UADBA); Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn (ZFMK); Zoologische Staatssammlung München (ZSM). Karyological information refers to one female and one juvenile *Scaphiophryne gottlebei* (ZFMK uncatalogued), one female of *S. madagascariensis* (MRSN A2020), and one male and one female of *S. spinosa* (MRSN A2017).

The voucher specimens and EMBL/Genbank accession numbers for the molecular analyses are: (1) Scaphiophryninae: *Scaphiophryne brevis* (Kirindy, AF215384), *Scaphiophryne brevis* (Ifaty, ZSM 577/2000, AJ314808), *Scaphiophryne calcarata* (Ampijoroa, ZSM 715/2001 AJ314811), *Scaphiophryne gottlebei* (no locality, AF215385), *Scaphiophryne* sp. aff. *marmorata* (ZSM 644/2000, AJ314810), *Scaphiophryne madagascariensis* (UADBA-FG/MV 2000.71, AJ314809), *Scaphiophryne spinosa* (no locality, ZSM 1154/2001, AF215383); (2) Dyscophinae: *Dyscophus guineti* (no locality, ZFMK 64133, AF215368); (3) Cophylinae: *Anodonthyla montana* (Andringitra, ZSM 776/2001, AJ314812), *Platypelis grandis* (Nosy Mangabe, ZFMK 66680, AF215381), *Plethodontohyla notosticta* (Nosy Mangabe, ZFMK 66675, AF215380), *Stumpffia grandis* (Nosy Mangabe, ZFMK 66678, AF215386); (4) Microhylinae: *Microhyla ornata* (Vietnam, ZFMK-TZ 52, AF215373), *Microhyla pulchra* (Vietnam, ZFMK-TZ 299, AF215374). *Pelodytes punctatus,* family Pelodytidae (AJ314813), was used as the outgroup.

Karyological methods

Specimens of *S. madagascariensis* and *S. spinosa* were injected in the field with 0.01 ml/g body weight of a 0.1 mg/ml colchicine solution. After two hours, the specimens were sacrificed and intestine and gonads incubated for 30 min in a 0.5% sodium citrate solution, then fixed in a 3:1 ethanol/acetic acid solution; chromosomes were studied using the air drying and scraping method. The specimens of *S. gottlebei* were directly processed in the lab.

In addition to standard methods (5% Giemsa at pH 7), we used the following staining techniques: (1) Ag-NOR banding according to Howell & Black (1980); (2) chromomycin A_3/methylic green staining (CMA_3/MG) according to Sahar & Latt (1980), but with a reduced MG exposure of about 10 s; (3) C-banding according to Sumner (1972); (4) sequential C-banding, CMA_3, and DAPI staining (Odierna *et al.* 2000). Morphometric chromosomal characteristics (relative length, RL; centromeric index, CI) were determined by analysing at least five well-spread metaphase plates of each species.

In-situ hybridization with a digoxigenin-labelled ribosomal unit of *Xenopus laevis* (pXcr7) was performed on the chromosomes of *S. gottlebei*. Hybridization was carried out as described in Capriglione *et al.* 1994. The digoxigenated probe was revealed with fluoresceined antibodies of mouse as suggested by the supplier (Roche).

DNA sequencing and statistics

To sample data on genetic differentiation and phylogenetic relationships among species of *Scaphiophryne*, we sequenced fragments of the mitochondrial 16S rRNA gene of up to 580 nucleotides (nt), using primers and protocols given in Vences *et al.* (2000b). A total of 201 nt, were excluded from the data set as they were either too variable to be reliably aligned or were lacking in one taxon (*Dyscophus*) of which only a sequence of about 400 nt was available. The remaining 379 nt were submitted to analysis using PAUP*, version 4.0b8 (Swofford 2001). MODELTEST 3.04 (Posada & Crandall 1998) was used to estimate the substitution model in the data. Tests of relative molecular substitution rates were conducted using PHYLTEST (Kumar 1996) using different substitution models both based on all substitutions and on transversions only.

Results

Karyotypes of Scaphiophryne spinosa *and* S. madagascariensis

Specimens of these two species had a karyotype of $2n = 26$ biarmed chromosomes (Figure 1A, B). The chromosome pairs 1, 5–7, 9, and 11–12 were metacentric, the remaining pairs were submetacentric (Table 1). The CMA_3/MG staining evidenced the NORs in paracentromeric position on the short arm of the second chromosome pair (Figure 2A, D). Additionally, CMA_3/MG stained (1) the telomeric regions of the long arm of the second chromosome pair in both species, (2) the peritelomeric regions of the long arm of the fourth pair in both species; and (3) the telomeric regions of the seventh pair in *S. spinosa*. C-banding revealed, in both species, a small amount of centromeric heterochromatin, while distinct NOR-associated heterochromatin and telomeric bands on several chromosome pairs were evident. After sequential C + CMA_3 + DAPI banding, a positive staining with both fluorochromes was noted in both species: (1) of the NOR-associated heterochromatin; (2) of telomeric heterochromatin of the second pair; and (3) of the peritelomeric heterochromatin on the long arms of the third pair. Additionally, in *S. spinosa*, both fluorochromes stained the telomeric heterochromatin on the short arm of the seventh pair, while that on the long arm of the same pair was CMA_3-positive only (Figure 2B, C, E, F).

Karyotype of Scaphiophryne gottlebei

Both specimens studied showed metaphase plates with 52 biarmed chromosomes. These were composed of 13 quartets of similar elements (Figure 1C), corresponding in morphology to the 13 pairs of *S. spinosa* and *S. madagascariensis* (Table 1). In two of the four chromosomes of the second quartet, a paracentromeric secondary constriction was usually visible on the short arm; the same region stained positive by AgNOR and CMA_3/MG banding (Figure 2G, H). Additionally, CMA_3/MG-positive bands were also visible: (1) on the paracentromeric region of the short arm of the remaining two chromosomes of the second quartet, although less extended and intensive than on the two AgNOR-positive elements; (2) on the telomeric regions of the long arms of the second quartet; (3) in the peritelomeric regions of the fourth quartet; and (4) in the centromeric region of two chromosomes of the ninth quartet (Figure 2H). C-banding revealed the presence of heterochromatin: (1) in the centromeric regions, distinctly on the quartets 7 and 12 but only very faintly on the remaining quartets; (2) in the telomeric regions of almost all quartets; (3) as a paracentromeric band on the short arm of the second quartet; and (4) as a peritelomeric band on two chromosomes of the first quartet (Figure 2I). *In-situ* hybridization with the rDNA probe resulted in hybridization signals in the paracentromeric regions of the short arms of two elements of the second quartet only (Figure 1J). Sequential C + CMA_3 + DAPI staining resulted in: (1) positive DAPI staining

Figure 1. Giemsa-stained karyotype of *Scaphiophryne madagascariensis* (**A**), *S. spinosa* (**B**) and *S. gottlebei* (**C**).

of the band on two chromosomes of the first quartet; (2) positive CMA_3 staining of the telomeric heterochromatin on the long arm of the seond quartet, the peritelomeric heterochromatin of the fourth quartet, and in the centromeric regions of two chromosomes, respectively, of the seventh and the ninth quartet; and (3) in DAPI- and CMA_3-positive staining of the NOR-associated heterochromatin of the second quartet, the CMA_3 staining being much more expressed in two of the four elements (Figure 2K, L).

Mitochondrial phylogeny and rates of molecular evolution

Maximum likelihood (ML), maximum parsimony (MP) and neighbour-joining (NJ) analyses agreed in placing all *Scaphiophryne* in one monophyletic group (Figure 3) but intrageneric relationships remained unresolved. Analyses including the complete sequences (580 nt) did not result in relevant changes of topology or bootstrap values, neither excluding *Dyscophus* from the analysis nor coding its lacking sequence sections as missing characters. Pairwise sequence divergence among *Scaphiophryne* in the complete 580-nt fragment (mean: 20 substitutions, corresponding to 1.7%) was highest between *S. gottlebei* and *S. brevis* (26–27 substitutions; 4.5–4.7%) and lowest between *S. pustulosa* and *S.* sp. aff. *marmorata* (10 substitutions; 1.7%).

In all analyses, branch lengths of species of *Scaphiophryne* were distinctly shorter compared with those of other taxa, especially cophylines (Figure 3). Independently from the substitution model used, relative rate tests significantly rejected rate constancy ($p < 0.05$) in all comparisons of *Scaphiophryne* with the other microhylids included.

Discussion

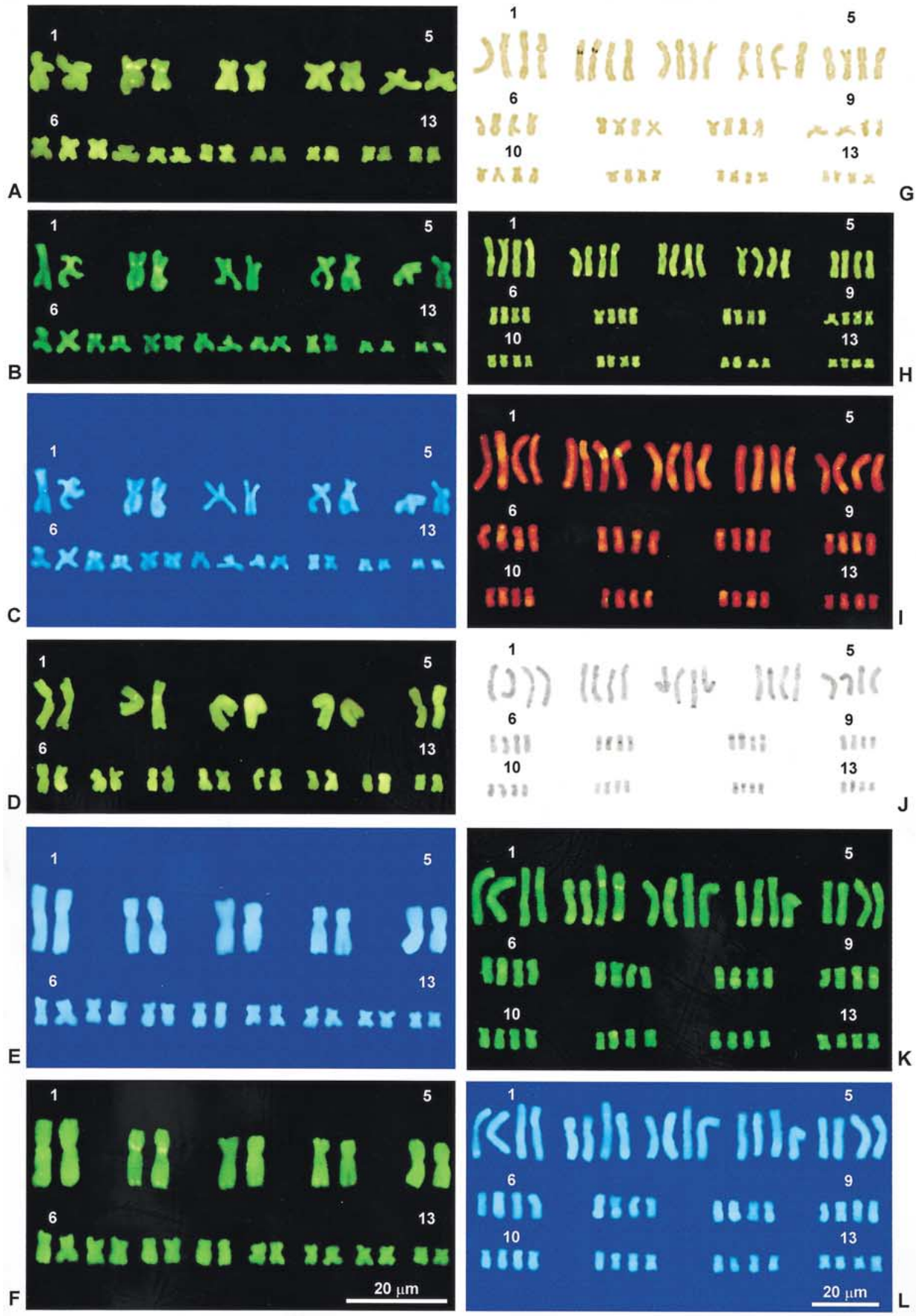
General chromosome morphology and NOR position

Microhylid diploid chromosome numbers range between $2n = 22$ and $2n = 32$. A karyotype of $2n = 26$ biarmed elements, the first five being dis-

Table 1. Relative lengths (RL) and centromeric indices (CI) of the chromosomes of the three species of *Scaphiophryne* studied (mean ± standard deviation).

	S. gottlebei	*S. spinosa*	*S. madagascariensis*
Chromosome 1			
RL	15.5 ± 0.8	14.2 ± 0.7	15.0 ± 0
CI	45.5 ± 3.3	46.8 ± 3.8	42.0 ± 3.7
Chromosome 2			
RL	13.3 ± 1.3	13.0 ± 1.0	12.7 ± 1.1
CI	33.0 ± 4.8	43.5 ± 5.0	43.5 ± 4.6
Chromosome 3			
RL	11.6 ± 0.8	11.7 ± 0.5	12.5 ± 0.7
CI	37.6 ± 3.7	34.4 ± 3.0	33.4 ± 3.3
Chromosome 4			
RL	11.5 ± 0.6	11.1 ± 0.4	12.2 ± 0.3
CI	33.3 ± 2.9	32.6 ± 3.5	29.0 ± 3.8
Chromosome 5			
RL	10.1 ± 0.4	10.0 ± 0.7	10.2 ± 0.5
CI	44.9 ± 2.7	47.0 ± 3.0	43.2 ± 3.3
Chromosome 6			
RL	6.6 ± 1.1	7.0 ± 0.9	6.5 ± 0.8
CI	41.7 ± 3.0	39.1 ± 3.3	42.6 ± 3.2
Chromosome 7			
RL	5.8 ± 0.6	6.9 ± 0.5	5.6 ± 0.8
CI	42.0 ± 2.6	39.0 ± 2.8	45.2 ± 2.0
Chromosome 8			
RL	5.4 ± 0.8	6.2 ± 0.6	5.3 ± 0.5
CI	32.0 ± 3.3	45.0 ± 4.0	33.8 ± 4.2
Chromosome 9			
RL	4.7 ± 0.9	5.3 ± 0.7	4.6 ± 0.6
CI	40.3 ± 3.6	40.6 ± 3.0	36.9 ± 3.5
Chromosome 10			
RL	4.4 ± 1.0	4.7 ± 0.6	4.4 ± 0.6
CI	30.4 ± 3.1	48.3 ± 3.3	34.2 ± 3.3
Chromosome 11			
RL	4.0 ± 0.8	4.4 ± 0.5	4.2 ± 0.6
CI	43.0 ± 3.7	45.6 ± 3.4	41.6 ± 3.2
Chromosome 12			
RL	3.6 ± 0.7	4.1 ± 0.4	3.7 ± 0.6
CI	39.8 ± 3.0	41.8 ± 3.1	35.1 ± 3.0
Chromosome 13			
RL	3.2 ± 0.9	4.0 ± 0.6	3.1 ± 0.5
CI	46.8 ± 3.2	44.4 ± 3.4	40.0 ± 3.3

tinctly larger than the remainder, has been found in 46 of the 65 species studied so far (Kuramoto 1990). This number can be considered as plesiomorphic in ranoid frogs (Morescalchi 1981, Bogart & Tandy 1981, Green 1983), and, in *Scaphiophryne*, is shared by *S. spinosa*, *S. madagascariensis* and *S.* sp. aff. *madagascariensis* (Blommers & Blanc 1972, and this study). Beside general chromosome morphology, conserved patterns among *S. spinosa* and *S. madagascariensis* (and *S. gottlebei*) also include the position of the


A
1
5
6
13
B
1
5
6
13
C
1
5
6
13
D
1
5
6
13
E
1
5
6
13
F
1
5
6
13
20 μm
G
1
5
6
9
10
13
H
1
5
6
9
10
13
I
1
5
6
9
10
13
J
1
5
6
9
10
13
K
1
5
6
9
10
13
L
1
5
6
9
10
13
20 μm

ribosomal cistrons (NOR loci), which are often indicative of relationships at the genus level in frogs (e.g. Odierna *et al.* 2000).

Ancient tetraploidy in Scaphiophryne gottlebei

The data obtained for *Scaphiophryne gottlebei* provide clear evidence for a tetraploid karyotype in this species. The 13 chromosome quartets are homoeologous in dimensions and morphology to the 13 pairs in the other *Scaphiophryne* species studied. In natural bisexual populations of anurans, polyploidy may evolve by two separate mechanisms (Mahony & Robinson 1980, Tymowska 1991): (1) autopolyploidization follows the fusion of diploid gametes caused by the inhibition of meiotic disjunction due to physical agents, such as thermal shocks or hydrostatic pressure, acting simultaneously on both (conspecific) parents (Ferrier & Jailet 1978). (2) In contrast, allopolyploidization occurs by inter-breeding of two species, which induces the somatic duplication of parental genomes in the offspring, and the subsequent production of diploid gametes. Our results strongly suggest that tetraploidy in *S. gottlebei* has been achieved by this latter mechanism. Regarding several characteristics, different states were found in two chromosomes of one quartet, respectively: the DAPI-positive paratelomeric heterochromatin band on two elements of the first quartet and the CMA_3-positive centromeric band on two elements of the seventh and ninth quartets probably reflect the origin of the tetraploid karyotype of *S. gottlebei* from two distinct parental species. These intraquartet differences do not appear to be a byproduct of the 'diploidization' process, since other heterochromatic markers were conserved in all the elements of the concerned quartets: namely the peritelomeric C-band present on the long arms of all the elements of second and fourth quartets and the paracentromeric C-band on the short arms of all the elements of the second quartet.

The process of 'diploidization', which maintains the RNA synthesis in polyploid species at levels similar to diploid species, is usually considered as terminated when the number of NORs is reduced to two (Schmid *et al.* 1985). In *S. gottlebei*, according to our data, the NOR reduction is functional as well as structural. As AgNOR banding provides evidence for functionally active NORs (Schmid 1982), the presence of this stain in two chromosomes of the second quartet indicates a functional diploidization. CMA_3-specifically binds GC-rich DNA sequences, which are common at NOR sites. This method thus stains NORs in ectotherms independently of their functional state (Schmid 1982). The presence of CMA_3 positive regions on the other two elements of the second quartet, and also on two chromosomes of the ninth quartet, therefore indicates the possible presence of further, 'silent' NOR sites. However, the negative result of the above regions to *in-situ* rDNA hybridization allows us to reject this hypothesis, suggesting that also the structural diploidization in *S. gottlebei* has reached an advanced state.

Natural history correlates of speciation in the genus Scaphiophryne

The molecular phylogenetic results did not resolve any distinct clades within *Scaphiophryne*, molecular substitution rates in the genus were decelerated, and pairwise divergences (mean 3.4%) were low. In the 16S rRNA fragment studied, we usually observed pairwise sequence divergences of more than 5% (often 7–9%) among closely related sibling species of other Malagasy anuran groups (personal observations). The data may indicate that the extant *Scaphiophryne* species evolved in a rapid star-like radiation, followed by evolutionary stasis.

Scaphiophryne gottlebei is the only known polyploid amphibian in Madagascar. According to our data, polyploidization in this species is completed, and is therefore not due to a recent speciation event. *Scaphiophryne* are explosive

(*Opposite*) *Figure 2*. Karyotypes of *Scaphiophryne madagascariensis* (A–C), *S. spinosa* (D–F), and *S. gottlebei* (G–L), stained with CMA_3/MG banding (**A**, **D**, **H**), Ag-NOR banding (**G**), rDNA FISH (**I**), C-banding (**J**), C-banding + CMA_3 (**B**, **F**, **K**), C-banding + DAPI (**C**, **E**, **L**). The scale bars in **F** and **L** apply to all figures of the respective column.

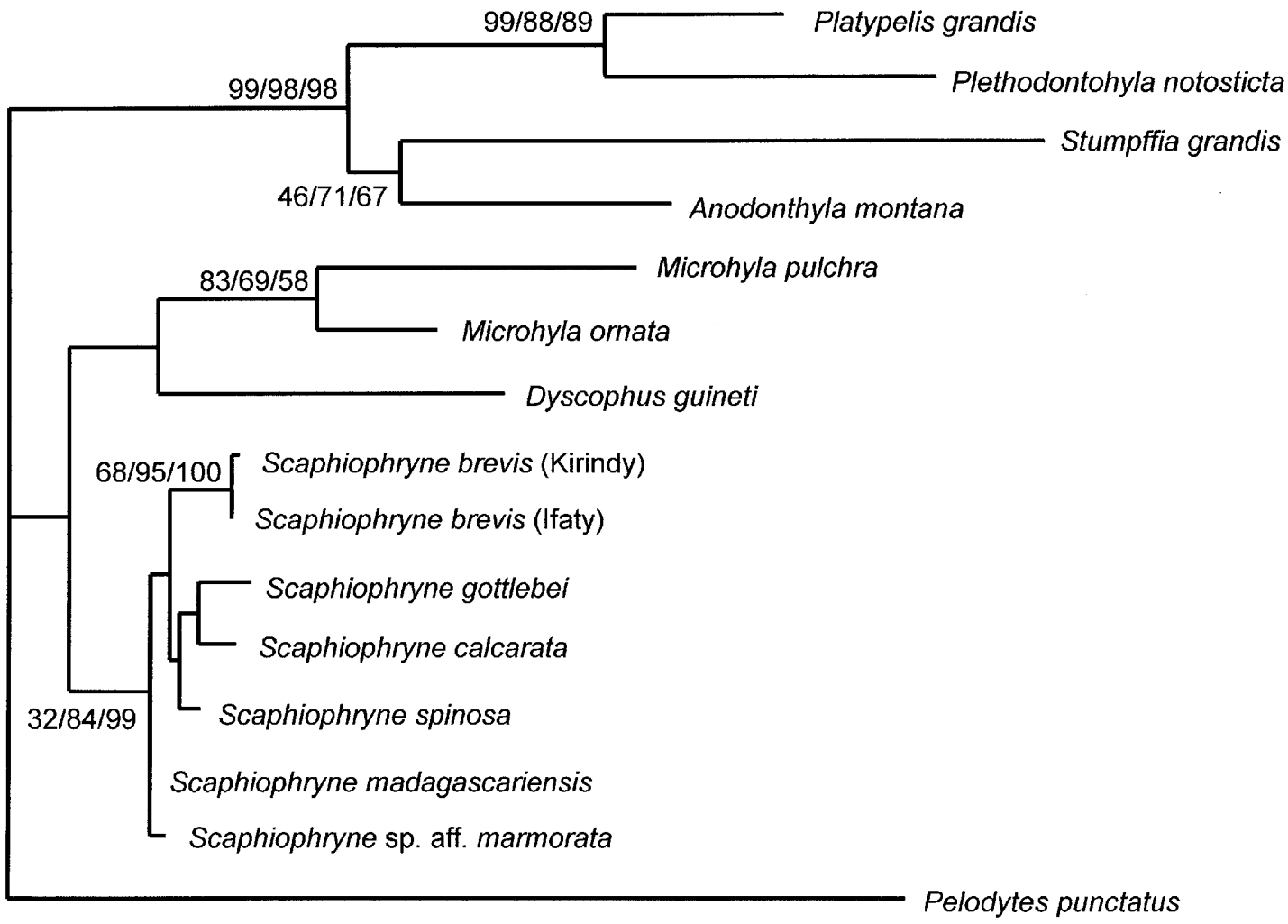


Figure 3. Maximum likelihood phylogram based on partial 16S rDNA sequences, calculated using a general time-reversible substitution model proposed by MODELTEST (gamma shape parameter $\alpha = 0.353$, proportion of invariable sites = 0.299, empirical substitution rates and base frequencies). Numbers are bootstrap values in percentages (150, 2000 and 2000 replicates in ML, MP and NJ analyses) and are shown only for branches which received support >65% in at least two analyses.

breeders which reproduce only during a reduced period each year, usually after the first heavy rainfalls. As far as known, advertisement calls between several species of the genus are similar (at least between *S. madagascariensis, S. spinosa* and *S.* sp. aff. *marmorata*; personal observations). Since they often depend on temporary water bodies, they must also be considered as relatively vagile. These behavioural and ecological predispositions probably contribute to a continuous gene flow between populations, and to a breeding synchronization of all specimens within one population. Such a mechanism would inhibit allopatric as well as sympatric speciation (e.g. Ranker *et al.* 1994). As fixation of mutations is less likely in large gene pools (Avise 2000), a high interpopulational gene flow could possibly explain the deceleration of molecular rates in *Scaphiophryne*. On the other hand, the outlined traits would also favour hybridization when the distribution areas of two species overlap, which may have caused the allotetraploidy of *S. gottlebei*. In this scenario, the peculiar life history traits of *Scaphiophryne* have led to increased interpopulational and interspecific mating, thereby inhibiting mechanisms of allopatric and sympatric speciation but favouring chromosome speciation.

Acknowledgements

We are grateful to G. García (Jersey), F. Glaw (München), S. Lötters (Mainz), V. H. Raherisoa (Antananarivo), J. E. Randrianirina (Antananarivo), A. Rasoamamonjinirina (Antananarivo), K. Schmidt (München), D. Vieites (Vigo) and S. Wanke (Bonn) for their help in obtaining tissue samples and/or specimens for karyological analysis. J. Kosuch (Mainz) contributed some of the DNA sequences. We are grateful to the Malagasy authorities for the permission to collect and export tissue samples and voucher specimens. This work was supported by grants from the Italian Ministero delle Università e Ricerca Scientifica e Tecnologica (PRIN 99), the Deutscher Akademischer Austauschdienst, and the Deutsche Forschungsgemeinschaft (VE 247/1-2). The survey work of F.A. was carried out within a project in collaboration with the Parc Botanique et Zoologique de Tsimbazaza (Antananarivo), supported by the Museo Regionale di Scienze Naturali (Torino).

References

Avise JC (2000) *Phylogeography. The History and Formation of Species.* Cambridge: Harvard University Press.

Blommers R, Blanc CP (1972) Existence d'une variabilité chromosomique intraspécifique chez *Pseudohemisus madagascariensis* Blgr. (Batraciens, Ranidés). *Bull Soc Zool France* **97**: 21–24.

Bogart JP, Tandy M (1981) Chromosome lineages in African ranoid frogs. *Monit Zool Ital* (ns) suppl **15**: 55–91.

Capriglione T, Cardone A, Odierna G, Olmo E (1994) Further data on the occurrence and evolution of satellite families in the lacertid genome. *Chromosome Res* **2**: 327–330.

Ferrier V, Jailet A (1978) Induction of triploidy in the newt *Pleurodeles waltlii* by heat shock or hydrostatic pressure. Interpretation of the different types of ploidy using a chromosomal marker. *Chromosoma* **69**: 47–63.

Glaw F, Vences M (1994) *A Fieldguide to the Amphibians and Reptiles of Madagascar. 2nd Edition, Including Mammals and Freshwater Fish*. Köln: Vences & Glaw Verlag.

Glaw F, Vences M (2000) Current counts of species diversity and endemism of Malagasy amphibians and reptiles. In: Lourenço WR, Goodman SM, eds. *Diversité et Endémisme a Madagascar. Mémoires de la Société de Biogéographie*, Société de Biogéographie, Muséum, Paris, pp 243–248.

Green DM (1983) Evidence of chromosome number reduction and chromosomal homosequentiality in the 24-chromosome Korean frog *Rana dybowskii* and related species. *Chromosoma* **88**: 222–226.

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* **36**: 1014–1015.

King M (1990) Amphibia. In: John B, ed. *Animal Cytogenetics*, Vol 4, *Chordata 2*. Stuttgart: Gebrüder Borntraeger, pp 1–235.

King M (1993) *Species Evolution: the Role of Chromosome Change.* Cambridge: Cambridge University Press.

Kumar S (1996) *PHYLTEST: Phylogeny Hypothesis Testing Software.* Pennsylvania: The Pennsylvania State University.

Kuramoto M (1990) A list of chromosome numbers of anuran amphibians. *Fukuoka Univ Educ Part III* **39**: 83–127.

Mahony MJ, Robinson ES (1980) Polyploidy in the Australian leptodactylid frog genus *Neobatrachus*. *Chromosoma* **81**: 199–212.

Meyer A (1993) Phylogenetic relationships and evolutionary processes in East African cichlids. *Trends Ecol Evol* **8**: 279–284.

Morescalchi A (1981) Karyology of the main groups of African frogs. *Monit Zool Ital* (ns) suppl **15**: 41–53.

Moritz C, Patton JL, Schneider CJ, Smith TB (2000) Diversification of rainforest faunas: an integrated molecular approach. *Ann Rev Ecol Syst* **31**: 533–563.

Odierna G, Andreone F, Aprea G, Arribas O, Capriglione T, Vences M (2000) Cytological and molecular analysis in the rare discoglossid species, *Alytes muletensis* (Sanchiz & Adrover, 1977), and its bearing on archaeobatrachian phylogeny. *Chromosome Res* **8**: 435–442.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics* **9**: 817–818.

Ranker TA, Floyd SK, Trapp PG (1994) Multiple colonizations of *Asplenium adiantum-nigrum* onto the Hawaiian archipelago. *Evolution* **48**: 1364–1370.

Sahar E, Latt SA (1980) Energy transfer and binding competition between dyes used to enhance staining differentation in metaphase chromosomes. *Chromosoma* **79**: 1–28.

Schluter D (1996) Ecological causes of adaptive radiation. *Am Nat* **148** (suppl): S40–S64.

Schmid M (1982) Chromosome banding in Amphibia. VII. Analysis of the structure and variability of NORs in Anura. *Chromosoma* **87**: 327–344.

Schmid M, Haaf T, Schempp W (1985) Chromosome banding in Amphibia. IX. The polyploid karyotypes of *Odontophrynus americanus* and *Ceratophrys ornata* (Anura, Leptodactylidae). *Chromosoma* **91**: 172–184.

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. *Expl Cell Res* **75**: 304–306.

Swofford DL (2001) *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4b8*. Sunderland: Sinauer Associates.

Tymowska J (1991) Polyploidy and cytogenetic variation in frogs of the genus *Xenopus*. In: Green DM, Session SK, eds. *Amphibians Cytogenetics and Evolution*. San Diego: Academic Press, pp 259–297.

Vences M, Glaw F, Kosuch J, Das I, Veith M (2000a) Polyphyly of *Tomopterna* (Amphibia: Ranidae) based on sequences of the mitochondrial 16S and 12S rRNA genes, and ecological biogeography of Malagasy relict amphibian groups. In: Lourenço WR, Goodman SM, eds. *Diversité et Endémisme a Madagascar*. *Mémoires de la Société de Biogéographie*, Société de Biogéographie, Muséum, Paris, pp 229–242.

Vences M, Kosuch J, Lötters S *et al.* (2000b) Phylogeny and classification of poison frogs (Amphibia: Dendrobatidae), based on mitochondrial 16S and 12S ribosomal RNA gene sequences. *Mol Phyl Evol* **14**: 34–40.

Wassersug R (1984) The *Pseudohemisus* tadpole: a morphological link between microhylid (Orton Type 2) and ranoid (Orton type 4) larvae. *Herpetologica* **40**: 138–149.