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A Phylogenetic Analysis of Cordyliformes (Reptilia: Squamata): Comparison of Molecular and Karyological Data

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A karyological study and the analysis of two mitochondrial genes were conducted in several species of cordyliformes from Madagascar. Both studies confirmed the monophyly of cordyliformes. The karyological conservatism and small genetic distance among mitochondrial genes observed in these species are in line with the hypothesis that all cordyliformes should be included in a single family. The two studies yielded contrasting results with regard to the relationships between the Malagasy species and the Cordylidae and Gerrhosauridae. Nonetheless, the consistency between the data from mitochondrial gene analysis and those of taxonomic studies based on morphological characters is more in favor of an affinity between Malagasy Zonosaurinae and Gerrhosauridae and suggests that the karyological similarities between the former and the Cordylidae may be due to plesiomorphy. Interesting, though not conclusive, data were also obtained on interspecific relationships among Zonosaurinae. © 2002 Elsevier Science (USA)

Key Words: **cordyliformes; molecular phylogeny; 12S rDNA; 16S rDNA; systematics; chromosomes.**

INTRODUCTION

Cordyliformes are a clade of scincomorph lizards that includes two families: Cordylidae, restricted to sub-Saharan Africa, and Gerrhosauridae, also found in Madagascar.

Whereas several researchers agree on the monophyly of cordyliformes and their relationships with Scincidae (Rieppel, 1980; Estes *et al.*, 1988; Lang, 1991; Miehle and Bauer, 1993; Mouton and van Wyik, 1997), there is some controversy on whether Cordylidae and Gerrhosauridae should be placed in a single (McDowell and Bogert, 1954; Romer, 1956; Underwood, 1957; Wermuth, 1968) or in separate (Hoffstetter, 1962; Lang, 1991; Mouton and van Wyik, 1997) families.

Poorly studied cordyliformes include the Zonosaurinae of Madagascar, which are generally placed in the Gerrhosauridae. They consist of two genera: *Tracheloptychus*, made up of two species found only in the dry southern regions of Madagascar, and *Zonosaurus*, whose species inhabit several biota of Madagascar (Glaw and Vences, 1994).

There are few cytogenetic and molecular studies on cordyliformes (Olmo *et al.*, 1979, 1995; Olmo and Odierna, 1980; De Smet, 1981; Aprea *et al.*, 1995). The chromosome number has been established in 11 species of Cordylidae and 4 of Gerrhosauridae from continental Africa (Olmo *et al.*, 1979; Olmo and Odierna, 1980; De Smet, 1981); all but 2 of these species share a similar karyotype with 12 biarmed macrochromosomes and 22 microchromosomes (Table 1). A single molecular study on the presence and characteristics of some highly repetitive DNA in 5 species of African cordyliformes (Olmo *et al.*, 1986) is available.

We report the results of a karyological study conducted with traditional staining and banding methods and of a comparative analysis of the mitochondrial genes for 12S and 16S ribosomal RNA (rDNA) performed in various Zonosaurinae species, two species of *Gerrhosaurus*, and one of *Cordylus*. These mitochondrial genes have been a powerful tool in phylogenetic studies and have provided information on the systematics of several scincomorph species (Gonzalez *et al.*, 1996; Fu, 1998; Harris *et al.*, 1998a,b; Kizirian and Cole, 1999; Honda *et al.*, 1999, 2000).

MATERIAL AND METHODS

Twenty-one specimens of 11 species of cordyliformes were included in the study. Specimens of the following species were collected in Madagascar during a field survey (by F. Andreone and G. Aprea): *Tracheloptychus madagascariensis*, *Zonosaurus trilineatus*, *Z. ornatus*, *Z. madagascariensis*, *Z. brygooi*, and *Z. rufipes* (voucher specimens were deposited at the Museo Regionale di Scienze Naturali, Sezione di Zoologia,

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TABLE 1

Karyological Features of the Cordyliformes Studied to Date

Species	Karyotype	NOR (s.c.)	Ag-NOR	Ref.
Cordylidae				
Cordylus cataphractus	0, 24, 22			1
	2, 20, 22	parac. 2q		2
	1, 22, 22	parac. 2q		2
Cordylus giganteus	2, 20, 22	parac. 2q		2
	2, 16, 24	subtelo 1q		3
Cordylus rhodesianus	12, 0, 22 2nd sm			2
Cordylus polyzonus	12, 0, 22 2nd sm			2
Cordylus tropidosternum	12, 0, 22 2nd sm			2
	12, 0, 22 2nd sm		micro	4
	12, 0, 22 2nd sm			3
Cordylus vittifer	12, 0, 22 2nd sm			2
	12, 0, 22 2nd sm			3
Cordylus warreni	12, 0, 22 2nd sm			2
Platysaurus guttatus	12, 0, 22 2nd m			2
	12, 0, 22 2nd m	subtelo 2p		3
Platysaurus intermedius	12, 0, 22 2nd m			2
Platysaurus minor	12, 0, 22 2nd m	subtelo 2p		3
Pseudocordylus melanotus	12, 0, 22 2nd sm			2
	12, 0, 22 2nd sm			3
Gerrhosauridae				
Gerrhosaurinae				
Gerrhosaurus flavigularis	12, 0, 24 2nd m			1
	12, 0, 22 2nd m	subtelo1, 2, 3q		3
	12, 0, 22 2nd m		subtelo 2q	4
Gerrhosaurus major	12, 0, 22 2nd m			2
	12, 0, 22 2nd m	subtelo1, 2q		3
	12, 0, 22 2nd m		subtelo 2q	4
Gerrhosaurus validus	12, 0, 22 2nd m			2
Tetradactylus seps	12, 0, 22 2nd m			2
Zonosaurinae				
Tracheloptychus madagascariensis	12, 0, 22 2nd sm		micro	4
Zonosaurus brygooi	12, 0, 22 2nd sm		micro	4
Zonosaurus karsteni	12, 0, 22 2nd sm		micro	4
Zonosaurus laticaudatus	12, 0, 22 2nd sm		micro	4
Zonosaurus madagascariensis	12, 0, 22 2nd sm		micro	4
Zonosaurus ornatus	12, 0, 22 2nd sm		micro	4
Zonosaurus rufipes	12, 0, 22 2nd sm		micro	4
Zonosaurus trilineatus	12, 0, 22 2nd sm		micro	4

Note. Karyotype: karyotypical formula, the first digit represents the number of biarmed macrochromosomes, the second digit the number of uniarmed macrochromosomes, and the third digit the number of microchromosomes. The morphology of 2nd pair macrochromosomes is reported in the same column: m, metacentric; sm, submetacentric. NOR (s.c.), position of secondary constrictions; p, short arm; q, long arm. Ag-NOR, position of nucleolus organizer region evidenced by the Ag-NOR technique. Ref, references, 1, Matthey, 1931; 2, Olmo and Odierna, 1980; 3, De Smet, 1981; 4, present paper.

Torino, Italy). Individuals of 2 additional Malagasy species, *Z. karsteni* and *Z. laticaudatus* (voucher specimens deposited at the Museo Zoologico "La Specola" Università di Firenze, Italy), were bought from a specialized dealer together with specimens of *Cordylus tropidosternum*, *Gerrhosaurus major*, and *G. flavigularis* (available at the Dipartimento di Biologia Evolutiva e Comparata, Università di Napoli, Italy).

Chromosome Analysis

After injecting colchicine (0.05 mg/ml; 0.1 ml/10 g body weight) 1 h before sacrifice, the chromosomes were obtained by scraping and air-drying the intestine, gonads, spleen, and lungs of each specimen. They were stained with 5% Giemsa for morphological analysis. Ag-NOR banding was performed according to Howell and Black (1980).

Analysis of 16S and 12S Sequences

Total DNA was extracted according to Jeffreys and Flavell (1977) from *Z. karsteni*, *Z. ornatus*, *Z. trilineatus*, *Z. rufipes*, *Z. madagascariensis*, *T. madagascariensis*, *G. major*, *G. flavigularis*, *C. tropidosternum*, and *Chalcides chalcides* of the family Scincidae, which was

used as the outgroup. For PCR amplification of 16S rDNA, we used the oligonucleotide primers designed by Palumbi *et al.* (1991), except in two cases (*G. flavigularis* and *Z. rufipes*), where we had to devise a new forward primer: 5′-CTGACCGTGCAAAGGTAGCGTA-ATCACT-3′. For 12S rDNA we used the primers designed by Kocher *et al.* (1989). Amplification conditions were as follows (30 cycles): 94°C, 1 min (denaturation); 55°C, 1 min (annealing); 72°C, 2 min (elongation). The amplified DNA was directly sequenced on an automated DNA sequencer (ABI PRISM 310, from PE Biosystems). Alignments were performed with the CLUSTAL W program (Thompson *et al.*, 1994) set at default parameters. To improve data reliability, we took into account secondary structural information where possible (Gutell *et al.*, 1993; Hickson *et al.*, 1996), and we used different alignments obtained with different gap open penalty and gap extension parameters, which were changed from 5 to 25 and from 0.05 to 7.5, respectively. However, since their results were virtually identical, the phylogenetic trees derived from these further two studies are not shown. CLUSTAL W is available at site ftp.ebi.ac.uk.

The trees were produced with maximum-parsimony (MP) and maximum-likelihood (ML) with PAUP 4.0 beta version (Swofford, 1998). The MP tree was produced following heuristic search by attributing equal weight to transitions and transversions and by transversion parsimony. The search was carried out with equal character weighting and random stepwise addition with 10 replications, and only minimal trees were retained; bootstrap values, indicating robustness of nodes, refer to 1000 replications. The ML trees were obtained with the quartet puzzling algorithm with exact parameter estimates and with the number of puzzling steps set at 1000. Gaps were not taken into account in these studies.

The alignment with 9 sequences of cordyliformes based on 16S and 12S rRNA was 950 nucleotides long and contained 188 parsimony-informative sites, whereas the alignment with 24 sequences (of which 19 came from GenBank) based on 16S rRNA, was 599 nucleotides long and contained 224 parsimony-informative sites. The alignments can be obtained from the authors. The nucleotide sequences have been deposited in GenBank (Accession Nos. AS416917 to AS416936).

RESULTS

Chromosomes

All species have 12 biarmed macrochromosomes and 22 microchromosomes (Fig. 1). All the macrochromosomes of the two *Gerrhosaurus* are metacentric, whereas in the Malagasy Zonosaurinae species and *C. tropidosternum* the 2nd pair chromosomes are submetacentric. Microchromosome morphology was not always easily identifiable; only in the more decondensed chromosomes could biarmed and uniarmed microchromosomes be distinguished. A secondary constriction was noted in the two *Gerrhosaurus* at subtelomeric level on the long arm of the 2nd pair macrochromosomes, whereas in *C. tropidosternum*, *T. madagascariensis*, and all *Zonosaurus* species a secondary constriction was observed in the larger microchromosomes. Ag-NOR staining evidenced that the nucleolus organizer region (NOR) is located at the level of these secondary constrictions (Fig. 1).

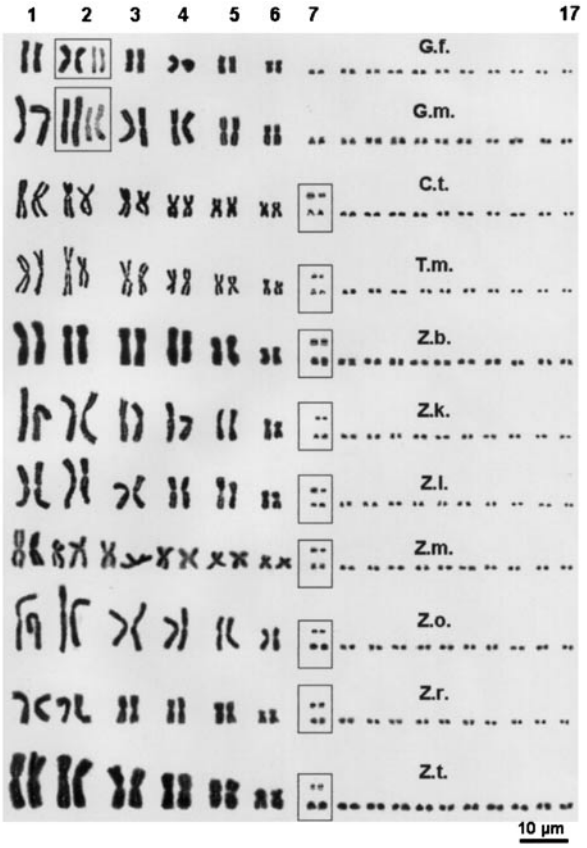

FIG. 1. Karyotypes of various species of cordyliformes from Madagascar and continental Africa. G.f., *Gerrhosaurus flavigularis;* G.m., *G. major;* C.t., *Cordylus tropidosternum;* T.m., *Trachelopty-chus madagascariensis;* Z.b., *Zonosaurus brygooi;* Z.k., *Z. karsteni;* Z.l., *Z. laticaudatus;* Z.m., *Z. madagascariensis;* Z.o., *Z. ornatus;* Z.r., *Z. rufipes;* Z.t., *Z. trilineatus.* NOR-banding chromosomes stained by conventional methods and Ag-NOR methods are framed.

Mitochondrial DNA

The lengths of the 12S partial sequences range from 383 to 391 nucleotides, whereas the 16S partial sequences range from 512 to 525 nucleotides except for *Z. rufipes* and *G. flavigularis* (426 and 428 nucleotides, respectively) due to the utilization of a more internal primer.

Analysis of the mitochondrial genes for 12S and 16S rRNA (Fig. 2) clearly indicates that the Zonosaurinae

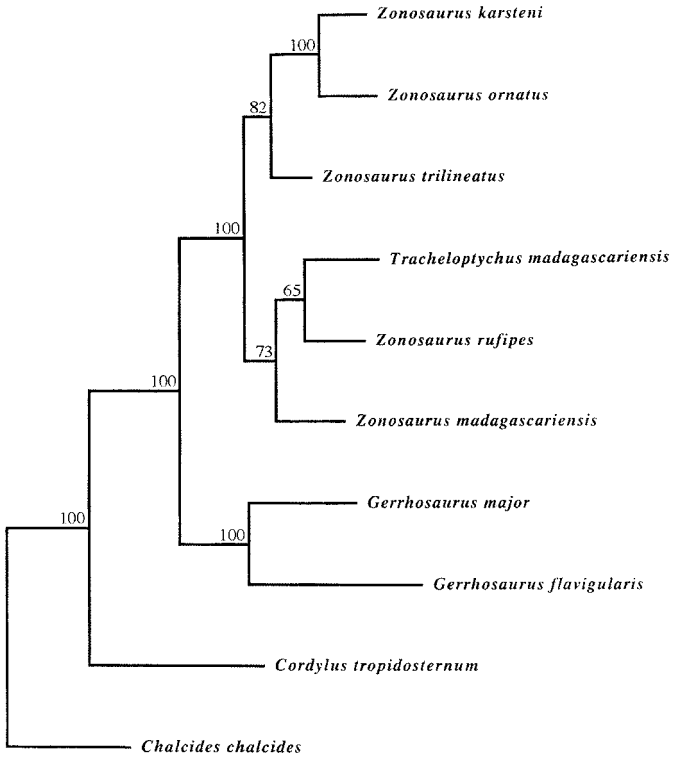

FIG. 2. Maximum-likelihood tree calculated with the quartet puzzling algorithm based on partial sequences of mitochondrial 12S and 16S rRNA genes. The numbers represent the percentage of quartet puzzling support values.

included in this study constitute a homogeneous lineage sustained by a high quartet puzzling support value and that they are more similar to the two *Gerrhosaurus* than to *C. tropidosternum*. This affinity is supported by a high support value.

Within Zonosaurinae, ML analysis evidenced two clusters sustained by a high support value: one includes *Z. karsteni, Z. ornatus,* and, in a more external position, *Z. trilineatus;* the other includes *T. madagascariensis, Z. rufipes,* and *Z. madagascariensis.* Similar results were obtained with MP and analysis of the sole transversions (data not shown).

The comparison with species belonging to other scincomorph families confirmed the monophyly of cordyliformes, since the affinity among *Zonosaurus, Gerrhosaurus,* and *Cordylus* is sustained by a high bootstrap value (Fig. 3). In addition, the genetic distance among cordyliformes is always smaller than the distance between these and other scincomorph families (Table 2).

DISCUSSION

Both the cytogenetic study and the analysis of mitochondrial DNA confirmed the monophyly of cordyliformes already suggested by Lang (1991) based on morphological data. In addition, the significant karyological conservatism and the small genetic distance among mitochondrial sequences are more consistent with the hypothesis that Cordylidae and Gerrhosauridae belong to a single family (McDowell and Bogert, 1954; Romer, 1956; Underwood, 1957; Wermuth, 1968).

By contrast, the two studies seem to disagree on the relationships between Zonosaurinae and the continental African species. Like the morphological data, mitochondrial sequences showed greater affinity between the Malagasy species and the two *Gerrhosaurus,* whereas the karyological data would appear to indicate that the former are closer to Cordylidae. In Gerrhosaurinae the 2nd pair macrochromosomes are always metacentric and in *G. flavigularis* and *G. major* the NOR is subtelomeric. By contrast, in Zonosaurinae

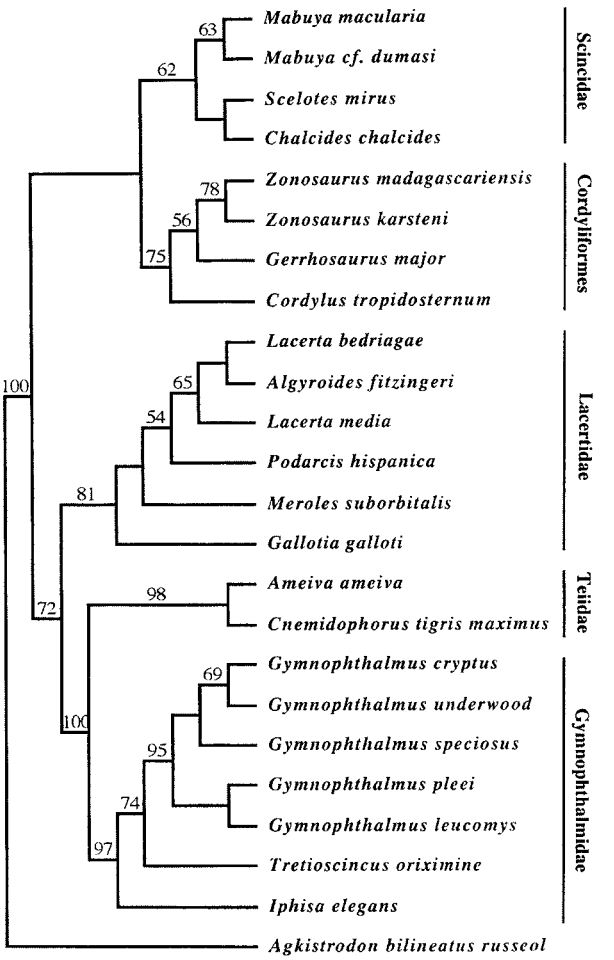

FIG. 3. Phylogenetic relationships among 23 Scincomorpha based on partial sequences of mitochondrial 16S rRNA gene. Maximum-parsimony tree resulting from a heuristic search (length = 916; consistency index = 0.511; retention index = 0.612; rescaled consistency index = 0.313). Numbers represent the percentage of 1000 bootstrap replications where a given node appeared. Only bootstrap values >50% are shown. The tree was rooted with a user-specified ougroup (*Agkistrodon bilineatus russeol*).

TABLE 2

Minimum and Maximum Genetic Distance within and between the Main Scincomorph Taxa

	ID	1	2	3	4	5
1. Cordyliformes	8.0–13.8	—	20.2–25.8	16.5–22.3	14.0–16.4	21.1–23.5
2. Gymnophthalmidae	2.6–12.2		—	17.4–22.2	20.5–25.6	14.5–17.1
3. Lacertidae	8.9–17.5			—	15.1–21.7	20.1–22.9
4. Scincidae	8.1–10.5				—	19.6–24.4
5. Teiidae						—
	9.1					

Note. ID, internal distance.

they are submetacentric, as in *C. tropidosternum* and in the majority of Cordylidae, and NOR is located on a pair of microchromosomes as in *C. tropidosternum*. However, in some cordylid species of the genus *Platysaurus* the 2nd pair macrochromosomes are also metacentric, and the position of secondary constrictions is not identical in the various cordylid species studied to date (Table 1), even though the presence of secondary constrictions does not always entail the presence of NOR (King, 1990). Moreover, the location of NOR on microchromosomes is quite common in reptiles, and in several families of squamates it is observed in species considered primitive (Olmo *et al.*, 1991; Porter *et al.*, 1991). The karyological similarities between Zonosaurinae and some Cordylidae could thus be plesiomorphic features and the karyotype of these cordyliformes might be the closest to the ancestral karyotype in the whole lineage. Interestingly, its gross morphology is identical to that of *Crocodilurus lacertinus*, one of the species considered karyologically more primitive in another scincomorph family, Teiidae (Gorman, 1970).

The considerable conservatism of the cordylid karyotype suggests that the various steps of the evolution of this taxon, such as the divergence between Cordylidae and Gerrhosauridae and the subsequent separation of the Malagasy forms, occurred without karyotype modifications and that the few chromosome changes observed so far arose separately in the different families and subfamilies.

The comparative analysis of mitochondrial genes also provided interesting data on interspecific relationships among Zonosaurinae by identifying a group including *Z. karsteni*, *Z. ornatus*, and *Z. trilineatus* and another including the other two species of *Zonosaurus* and *T. madagascariensis*. These relationships agree only partially with those suggested by Lang (1990) based on morphological and biogeographic studies. The position of *T. madagascariensis*, which morphologically should be attributed to a distinct genus but is instead placed among *Zonosaurus* by mitochondrial DNA analysis, is especially intriguing. It is impossible to draw definite conclusions solely on the basis of these data. Indeed, given the restricted data set used, Lang himself (1990) considered his own phylogenetic hypothesis preliminary. Results indicate that the systematic relationships among the various Zonosaurinae species are far from being clear and that even the distinction of Malagasy species into two genera may be unwarranted.

In conclusion, karyological and molecular studies seem to be able to provide valuable data on the systematics of cordyliformes and should be extended by increasing the number of species studied, by using the more informative cytological techniques, such as banding methods, and by analyzing other mitochondrial and nuclear genes.

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