

## MOLECULAR AND KARYOLOGICAL DATA ON THE SOUTH ASIAN RANID GENERA *INDIRANA*, *NYCTIBATRACHUS* AND *NANNOPHRYS* (ANURA: RANIDAE)

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(with four text-figures)

**ABSTRACT.**— Phylogenetic relationships of the endemic south Asian frogs of the genera *Indirana*, *Nyctibatrachus* and *Nannophrys* were studied using DNA sequences (a total of 880 bp) of the mitochondrial 16S and 12S rRNA genes. The topology of the obtained cladograms was largely unresolved, indicating a star-like radiation of the main ranid lineages. No molecular affinities were found between the south Asian taxa and Malagasy ranids. *Nannophrys* was positioned as sister group of *Euphlyctis* in all analyses. This grouping, which was supported by moderate to high bootstrap values, indicates that *Nannophrys* is an offshoot of Asian ranids, and not related to the South African cacosternines. Karyotypes were obtained for *Nannophrys ceylonensis* ( $2n = 26$ ), *N. marmorata* ( $2n = 26$ ), *Indirana* sp. ( $2n = 30$ ) and *I. cf. leptodactyla* ( $2n = 24$ ). The  $2n = 30$  karyotype of *Indirana* sp. was so far unknown in ranids; it may represent a transitory stage in a process of karyotype reduction by means of centric fissions which produce telocentric chromosomes, and their subsequent fusion.

**KEY WORDS.**— Amphibia: Ranidae: *Indirana*, *Nannophrys*, *Nyctibatrachus*; mitochondrial DNA; karyotypes; phylogeny; chromosomal evolution.

### INTRODUCTION

The anuran family Ranidae is a speciose group (750 nominal species) of largely unsolved phylogenetic relationships. There is little agreement in the subfamilial partition of ranids, and different schemes have been proposed (e.g., Duellman and Trueb, 1986; Laurent, 1986; Dubois, 1992; Blommers-Schlösser, 1993). Relationships of three genera of ranids endemic to south Asia (India and Sri Lanka), *Indirana*, *Nyctibatrachus* and *Nannophrys*, are especially enigmatic (Blommers-Schlösser, 1993).

While *Nannophrys* has been revised by Clarke (1983) and Dutta and Manamendra-Arachchi (1996), no recent comprehensive

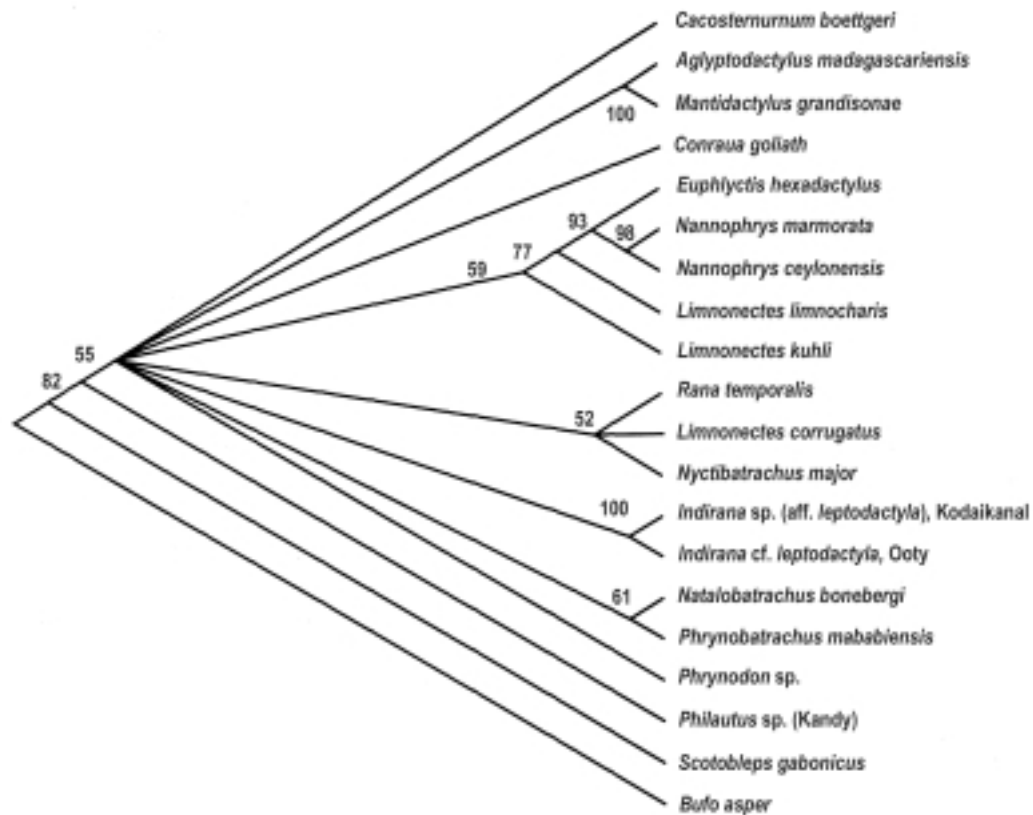
works have focused on *Indirana* and *Nyctibatrachus*. Their phylogeny has so far only been addressed by Blommers-Schlösser (1993), and no karyological data are available on any of the three genera (King, 1990; Prakash, 1998). In the present paper, we present preliminary molecular data on their relationships, and describe the karyotypes of two species of *Indirana* and two species of *Nannophrys*.

### MATERIALS AND METHODS

We sequenced fragments of the mitochondrial 16S and 12S rRNA genes homologous to bp 4012-4561 and bp 2544-2909 of the *Xenopus laevis* mt genome (Roe et al. 1985). Taxa studied

**TABLE 1:** List of specimens used for analysis, and of GenBank accession numbers of the 16S and 12S rRNA genes. The 12S sequence of *Bufo asper* (marked with an asterisk) was obtained from GenBank. Museum acronyms used are MNHN (Muséum National d'Histoire Naturelle, Paris) and ZFMK (Zoologisches Forschungsinstitut und Museum A. Koenig, Bonn). Subfamilial attribution of genera follows a modified consensus scheme of Dubois (1992) and Blommers-Schlösser (1993).

Family	Species	Origin	Collection number	Genbank 16S	Genbank 12S
Bufo	<i>Bufo asper</i>	Tanak Masa island, West Sumatra (16S)	-	AF124109	U52733*
Astyrodermatidae	<i>Scotobleps gabonicus</i>	Nlonako, Cameroon	ZFMK 69155	AF215341	AF215141
Ranidae	<i>Aglyptodactylus madagascariensis</i>	Andasibe, Madagascar	ZFMK 64137	AF215330	AF215179
Ranidae (Mantellinae)	<i>Manitidactylus grandisonae</i>	Ambato, Madagascar	ZFMK 66669	AF215315	AF215149
Ranidae (Cacosterninae)	<i>Cacosternum boettgeri</i>	Bredell, South Africa (12S); Hardap, Namibia (16S)	ZFMK 66727	AF215414	AF124096
Ranidae (Raninae)	<i>Conraua goliath</i>	Cameroon	ZFMK 64829	AF215388	AF215190
Ranidae (Raninae)	<i>Euphylyctis hexadactylus</i>	Unawatuna, Sri Lanka	-	AF215389	AF215191
Ranidae (Raninae)	<i>Limnonectes corrugatus</i>	Kandy, Sri Lanka	MNHN 2000.616	AF215393	AF215195
Ranidae (Raninae)	<i>Limnonectes limnocharis</i>	Laos	MNHN 1997.3932	AF215416	AF215210
Ranidae (Raninae)	<i>Limnonectes kuhli</i>	Laos	MNHN 1997.3904	AF215415	AF215209
Ranidae (Raninae)	<i>Nannophrys ceylonensis</i>	Kitulgala, Sri Lanka	MNHN 2000.627	AF215394	AF215197
Ranidae (Raninae)	<i>Nannophrys marmorata</i>	Laggalla, Sri Lanka	MNHN 2000.621	AF215395	AF215196
Ranidae (Raninae)	<i>Rana (Sylvirana) temporalis</i>	near Kodaikanal, India	ZFMK uncatalogued	AF215390	AF215192
Ranidae (Petropedetinae)	<i>Natalobatrachus bonebergi</i>	The Haven, South Africa	ZFMK 66443	AF215396	AF215198
Ranidae (Petropedetinae)	<i>Phrynobatrachus mababiensis</i>	Rundu, Namibia	ZFMK 68821	AF215399	AF215200
Ranidae (Petropedetinae)	<i>Phrynodon</i> sp.	Kodmin, Cameroon	ZFMK 67324	AF215400	AF215201
Ranidae (Ranixalinae)	<i>Indirana cf. leptodactyla</i>	near Ooty, India	ZFMK uncatalogued	AF215392	AF215194
Ranidae (Ranixalinae)	<i>Indirana</i> sp. (aff. <i>leptodactyla</i> )	Kodaikanal, India	ZFMK uncatalogued	AF215391	AF215193
Ranidae (Nyctibatrachinae)	<i>Nyctibatrachus major</i>	near Ooty, India	ZFMK uncatalogued	AF215397	AF215199
Ranidae (Rhacophorinae)	<i>Phyllanthus</i> sp.	Kandy, Sri Lanka	MNHN 2000.623	AF215350	AF215182



**FIGURE 1:** Neighbor-joining bootstrap consensus tree (HKY85-distances; shape factor 0.5) of south Asian taxa in comparison to African, Malagasy and Asian ranids, based on 509 bp of 16S rRNA and 371 bp of 12S rRNA gene sequences. Numbers are bootstrap values in percent (2000 replicates). *Bufo asper* was used as an outgroup.

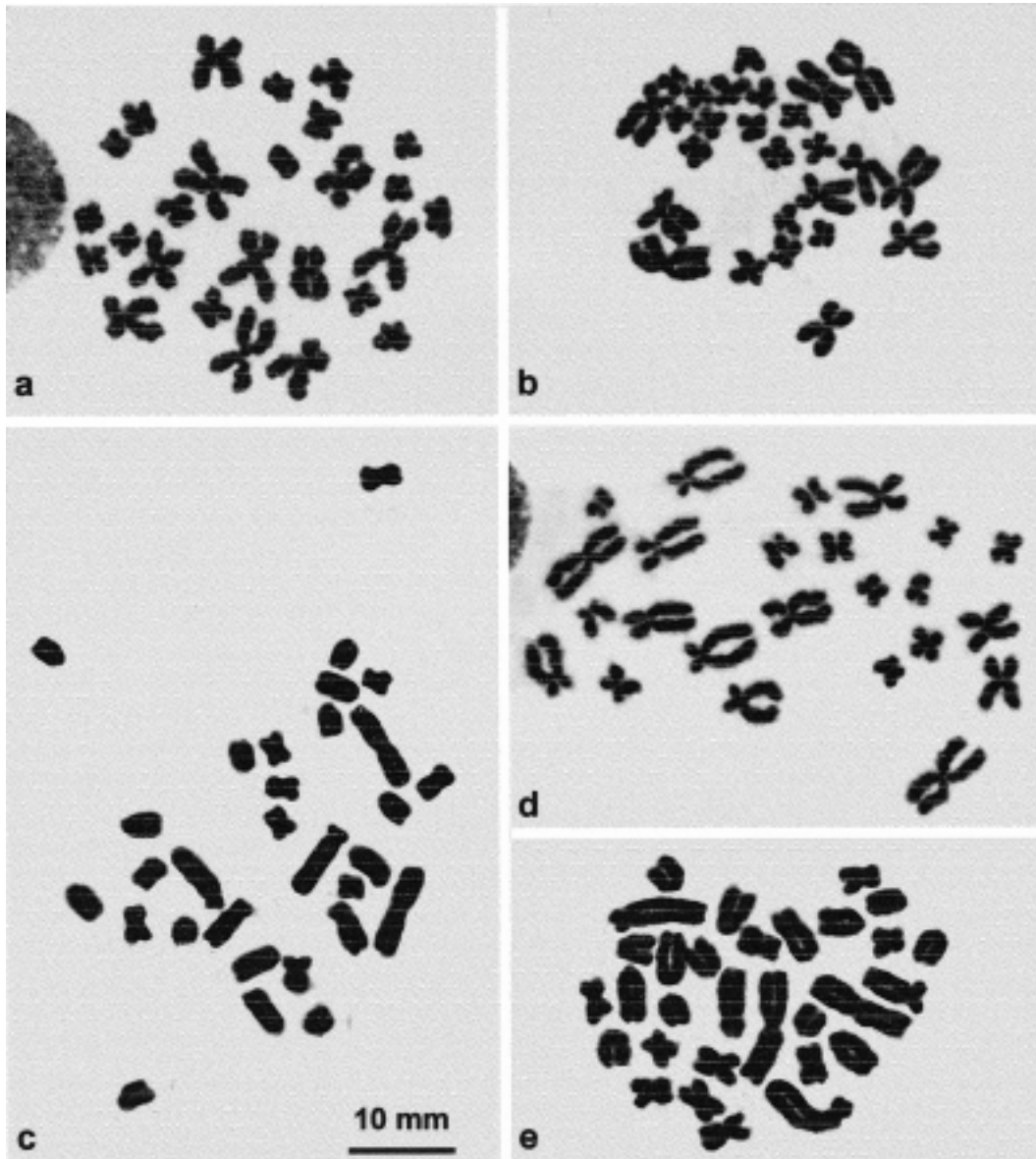
are listed in Table 1. Details of primers, cycling protocols and methodology of analyses are given in Vences et al. (2000).

A female of each *Nannophrys ceylonensis* and *N. marmorata*, a female of *Indirana* cf. *leptodactyla*, and a male and a female of *Indirana* sp. (aff. *leptodactyla*, from Kodaikanal) were available for karyological analysis. Each specimen was injected with a dose (0.01 ml/g of body weight) of a 0.5 mg/ml colchicine solution and sacrificed two hours later, after anaesthesia with tricaine metasulfonate. Chromosomes were taken from intestine, spleen, lungs and (in males) testes, employing the air drying and scraping method described by Odierna et al. (1999). Standard chromosome staining was conducted by means of a solution of Giemsa 5% at pH 7. Of each specimen at least 25 metaphase plates were counted

and 6 of them were karyotyped. Relative length (R.L.; percentage ratio between the length of each chromosome and the total length of all the chromosomes) and centromeric index (C.I.; ratio between the short arm and total length of a chromosome) were determined in all specimens. Chromosome nomenclature follows the specifications of Green and Session (1991).

## RESULTS

*Phylogenetic analysis of DNA sequences.* - After exclusion of 54 bp of one hypervariable region of the 16S rRNA gene fragment, 509 bp of this fragment and 371 bp of the 12S fragment were available for analysis. The maximum parsimony (MP; not shown) and neighbor-joining (NJ) trees obtained show inconsistent topologies, and few lineages are corroborated by bootstrap values > 50% (Fig. 1). Position of some taxa appears to be



**FIGURE 2:** Giemsa stained metaphase plates of *Nannophrys ceylonensis* (a), *N. marmorata* (b), *Indirana* sp. (female) (c), *I. cf. leptodactyla* (d) and *I. sp.* (male) (e). The scale bar in c refers to all the images.

almost random in a comparison of the results based on different algorithms and different gene fragments. The three endemic south Asian genera are not arranged as monophylum in any cladogram. All trees agree in placing the two Malagasy ranids as monophyletic. The two species of *Nannophrys* are solidly positioned as monophylum in all analyses, as are the two species of *Indirana*. The single aspect of the topol-

ogy informative regarding the position of the south Asian genera is the consistent clustering of *Nannophrys* with *Euphlyctis hexadactylus*. This monophyletic group is supported by the 16S data (bootstrap support: NJ 84%, MP 72%), the 12S data (NJ 58%, MP 50%), and the combined analysis (NJ 91%, MP 63%). No affinities between *Nannophrys* and the African *Cacosternum* were detected.

TABLE 2: Relative chromosome lengths (R.L.) and centromeric indices (C. I.) of specimens studied karyologically.

Chrom.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
<i>Iridirana</i> sp., male															
<b>R.L.</b>	14.2 ±0.8	12.6 ±0.6	9.5 ±0.6	7.9 ±0.7	7.0 ±0.4	6.0 ±0.8	5.6 ±0.4	5.6 ±0.9	5.1 ±0.7	5.1 ±0.6	5.0 ±0.6	4.9 ±0.7	4.7 ±0.7	4.0 ±0.6	3.8 ±0.8
<b>C.I.</b>	48.5 ±3.3	14.0 ±4.4	23.2 ±3.0	0	0	0	41.7 ±4.4	46.5 ±3.1	0	47.6 ±3.6	0	41.9 ±3.7	0	0	40.0 ±4.4
<i>Iridirana</i> sp., female															
<b>R.L.</b>	14.6 ±0.5	12.9 ±0.7	8.5 ±0.8	8.3 ±0.5	6.9 ±0.4	6.4 ±0.6	6.4 ±0.8	6.3 ±0.6	6.1 ±0.4	5.4 ±0.5	4.8 ±0.4	4.8 ±0.4	4.8 ±0.5	4.8 ±0.4	4.0 ±0.4
<b>C.I.</b>	47.3 ±3.5	13.0 ±4.1	24.2 ±3.2	0	0	0	45.0 ±4.2	42.3 ±3.5	0	47.4 ±3.3	0	44.3 ±3.2	0	0	38.5 ±4.9
<i>Iridirana</i> cf. <i>leptodactyla</i> , female															
<b>R.L.</b>	15.1 ±0.8	12.9 ±0.6	11.9 ±0.6	12.2 ±0.7	10.6 ±0.4	9.4 ±0.8	5.8 ±0.4	5.0 ±0.9	4.8 ±0.7	4.8 ±0.6	4.6 ±0.6	3.9 ±0.7	-	-	-
<b>C.I.</b>	43.2 ±4.3	19.6 ±4.0	36.6 ±3.6	28.7 ±3.5	20.2 ±3.8	41.2 ±4.6	39.8 ±4.3	46.3 ±3.7	31.8 ±3.0	45.2 ±3.2	49.1 ±3.9	36.5 ±4.0	-	-	-
<i>Nannophrys</i> <i>ceylonensis</i> , female															
<b>R.L.</b>	14.1 ±0.7	13.9 ±0.8	11.5 ±0.5	11.2 ±0.7	10.1 ±0.9	6.2 ±0.7	5.9 ±0.6	5.2 ±0.8	5.2 ±0.5	4.4 ±0.5	4.4 ±0.8	4.1 ±0.6	3.8 ±0.8	-	-
<b>C.I.</b>	43.9 ±3.8	39.5 ±4.6	30.8 ±4.0	34.9 ±3.5	40.4 ±4.0	33.2 ±4.2	43.7 ±3.3	47.9 ±4.7	48.2 ±4.0	35.5 ±3.6	37.0 ±3.8	40.5 ±4.1	32.0 ±3.7	-	-
<i>Nannophrys</i> <i>marmorata</i> , female															
<b>R.L.</b>	15.7 ±0.5	13.4 ±0.9	11.7 ±0.7	11.0 ±0.7	10.5 ±0.6	5.5 ±0.8	5.5 ±0.5	4.9 ±0.9	4.8 ±0.6	4.4 ±0.5	4.3 ±0.7	4.2 ±0.6	4.1 ±0.6	-	-
<b>C.I.</b>	42.3 ±3.1	39.9 ±4.0	35.3 ±3.6	40.3 ±3.9	42.0 ±3.8	36.5 ±4.0	41.0 ±3.8	42.0 ±4.0	49.0 ±4.4	37.0 ±3.8	45.9 ±4.1	42.2 ±3.7	36.5 ±3.9	-	-

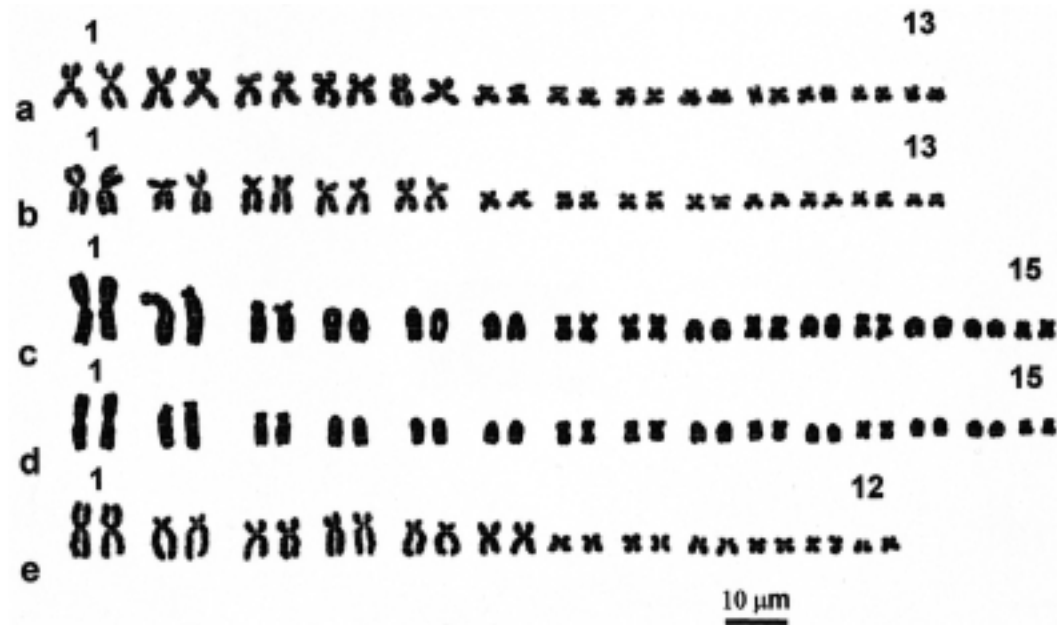


FIGURE 3: Karyotypes of *Nannophrys ceylonensis* (a), *N. marmorata* (b), *Indirana* sp. (male) (c), *I.* sp. (female) (d), and *I.* cf. *leptodactyla* (e).

*Karyotypes.*- Metaphase plates and karyotypes of the studied species are shown in Figs. 2-3. Relative chromosome lengths and



FIGURE 4: Chromosomes of *Indirana* sp. (b) arranged to show how three centric fusion events could give place to a karyotype of  $2n = 24$  chromosomes like that of *Indirana* cf. *leptodactyla* (a); in this case by forming the "new" chromosomes 3, 4, and 6.

centromeric indices are given in Table 2. *Nannophrys ceylonensis* and *N. marmorata* possess  $2n = 26$  biarmed chromosomes, with the first five pairs distinctively larger than the other eight pairs. Chromosome pairs 1, 2, 5, 7, 8, 9 and 12 of *N. ceylonensis* are metacentric, while the other six pairs are submetacentric. In *N. marmorata*, chromosome pairs 1, 2, 4, 5, 7, 8, 9, 11 and 12 are metacentric, while the other four pairs are submetacentric. *Indirana* sp. has  $2n = 30$  chromosomes, 16 are biarmed and 14 uniarmed. Chromosome pairs 1, 7, 8, 10, 12 and 15 are metacentric, pairs 2 and 3 are subtelocentric and pairs 4, 5, 6, 9, 11, 13 and 14 are telocentric. *Indirana* cf. *leptodactyla* has  $2n = 24$  biarmed chromosomes. The pairs 1-6 are distinctly larger than the pairs 7-12. Chromosome pairs 1, 7, 8, 10 and 11 are metacentric, pairs 3, 4 and 12 are submetacentric, pairs 2 and 5 are subtelocentric.

#### DISCUSSION

*Phylogenetic relationships.*- The studied gene fragments do not adequately resolve the relationships between the taxa studied. The respective lineages may have similar ages, and possibly radiated in a relatively short period, rendering the

phylogenetic signal in the analysed sequences weak. The lack of conspicuous molecular relationships between south Asian and Malagasy taxa indicates that it is not probable that both groups have a common ancestor which lived in isolation for at least 30 my on the Cretaceous Madagascar-India continent (see Barron et al., 1981; Duellman and Trueb, 1986; Blommers-Schlösser, 1993).

All MP and NJ analyses of the 16S, 12S, and combined data sets agreed in placing *Euphlyctis* as sister group of *Nannophrys*, in agreement with the generalized karyotype of both ( $2n = 26$  according to King 1990; Prakash, 1998; and this study). Although the two genera may not be direct sister groups, they almost certainly are closely related to each other. This is surprising considering their morphological distinctiveness. However, most of the characters which make up the conspicuous general appearance of *Nannophrys* and of *Euphlyctis* are clearly related to their habits. *Euphlyctis* are largely aquatic species, mainly inhabiting lotic water bodies. In contrast, *Nannophrys* are highly derived (see Clarke, 1983), dorso-ventrally compressed anurans specialized for a life in humid crevices along brooks and in waterfalls. The molecular data suggest rejection of Blommers-Schlösser's (1993) inclusion of *Nannophrys* in her otherwise strictly African subfamily Cacosterninae, as well as of Dubois' (1992, 1999) proposal of inclusion of *Nannophrys*, *Nyctibatrachus* and *Indirana* in an endemic south Asian subfamily Ranixalinae.

*Chromosomal evolution.*- According to King (1990), the vast majority of ranid frogs have a karyotype of  $2n = 26$ , with all chromosomes biarmed and the first five pairs distinctly larger than the remaining eight pairs. The two studied *Nannophrys* show this typical ranid karyotype. Differences between both species are found in the 4<sup>th</sup> and 11<sup>th</sup> chromosome pairs which are metacentric in *N. marmorata* and submetacentric in *N. ceylonensis*. Two pericentric inversions may account for these difference.

In contrast, the karyotype of the two studied *Indirana* species, displaying 24 or 30 chromo-

somes, deviates from the standard ranid karyological formula. A more reduced diploid number of  $2n = 24$  chromosomes as observed in *Indirana* cf. *leptodactyla* is known from a number of ranid groups, including petropedetines and some mantellines (King, 1990). Among the Raninae, it is found in all *Ptychadena* karyotyped so far, and in a few *Rana* and *Limnonectes*. On the other hand, the diploid number of  $2n = 30$ , with unarmed and biarmed chromosomes displayed by *Indirana* sp. is a novelty among the Raninae, and in the Ranidae this diploid number has so far been only found in a Malagasy *Mantidactylus* species, *M. cf. femoralis* (Apra et al., 1998).

The  $2n = 24$  (or 22 or 20) biarmed chromosome karyotype is considered a derived condition from the  $2n = 26$  biarmed chromosome state (Morescalchi, 1981; Bogart and Tandy, 1981; Green, 1983). According to these authors this reduction could have occurred by means of a process involving (a) inversions of biarmed chromosomes to form telocentric chromosomes and (b) their subsequent fusion. This evolutionary model has been preferred over an alternative way to reach the reduction to  $2n = 24$  chromosomes, namely by (a) production of telocentric elements by means of fissions of metacentric elements, and (b) subsequent centric fusions of these new telocentric chromosomes. So far this alternative model suffered from the lack of Raninae species possessing transitional karyotypes with a diploid number higher than  $2n = 26$  chromosomes and telocentric elements. The karyotype of *Indirana* sp., with  $2n = 30$  chromosomes and 7 telocentric pairs, could represent a transitional state in the process of chromosome reduction. Three fusions involving six telocentric pairs could lead to a karyotype of  $2n = 24$  chromosomes as found in *I. cf. leptodactyla* (Fig. 4). Additionally, a pericentric inversion of one telocentric pair (tentatively the 9<sup>th</sup> pair, see also Fig. 4) not involved in centric fusions must also be assumed in this scenario. If it is true that *Indirana* species possess evolving karyotypes by means of centric fissions which produce telocentric chromosomes, and their subsequent fusions, such

processes may also favour speciation events within the genus.

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