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Cytogenetic analysis of four central Amazonian species of *Colostethus* (Anura – Dendrobatidae) with a diploid complement of 22 chromosomes

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Colostethus marchesianus from the type locality and three related species had 2n = 22 chromosomes, which differed from most other *Colostethus* species that have 2n = 24 chromosomes. The species analyzed were morphologically similar and showed a conservative karyotype, although they could be distinguished from each other by their C-banding pattern. Additional NOR sites, heteromorphism in NOR size and heterochromatin, and an additional rDNA site detected by FISH, were observed. These data suggest that chromosomal rearrangements and hetrochromatin-related events may have contributed to the karyotype differentiation of these *Colostethus*.

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According to FROST (2002), the family Dendrobatidae consists of 207 species grouped in nine genera: *Allobates* (1 sp.), *Aromobates* (1 sp.), *Colostethus* (113 spp.), *Cryptophillobates* (1 sp.), *Dendrobates* (37 spp.), *Epipedobates* (28 spp.), *Mannophryne* (12 spp.), *Nephelobates* (9 spp.) and *Phyllobates* (5 spp.).

Colostethus is the largest dendrobatid genus and is considered to be a basal group within the dendrobatids (LYNCH 1982). The species of this genus are widespread in lower Central America, northwestern South America, the Amazon and in some areas of the eastern Andes (DUELLMAN and TRUEB 1986; MYERS et al. 1991). Many *Colostethus* species are very similar in morphology and color pattern and therefore difficult to distinguish from each other.

In a review of the *Colostethus* species from Ecuador, COLOMA (1995) mentioned that populations currently assigned to *C. marchesianus* do not show major morphological differences. However, differences in their announcement calls suggest that more than one species is included in this taxon. In the Brazilian Amazon, the name *C. marchesianus* has also been attributed to some populations of *Colostethus* (A. P. Lima, pers. obs.). Recently, LIMA and CALDWELL (2001) described a species of *Colostethus* with blue digits (*C. caeruleodactylus*) and observed that, in addition to this species, there are other undescribed species morphologically similar to *C. marchesianus* that occur near Manaus in the Amazon. Two of these species are analyzed in the present study and are referred to here as *Colostethus* sp. 1 and *Colostethus* sp. 2, the former frequently being called *C. marchesianus* (HERO 1990; GASCON 1991). Although these species are morphologically very similar, they have distinct calls.

In general, anurans show conserved morphological characteristics, which make the use of such characters difficult in taxonomic and phylogenetic investigations (HILLIS 1991) so that other methods are required. The identification of chromosomal numbers and morphology, and the availability of various staining techniques has provided new data for reassessing anuran systematics (KING 1980; MIURA 1995; LOURENÇO et al. 1999; BUSIN et al. 2001). The cytogenetic information available for the Dendrobatidae is restricted to the number of chromosomes and the morphology of the karayotype. However, chromosome banding studies recently published by AGUIAR JR et al. (2002), KAISER et al. (2003) and VEIGA-MENONCELLO et al. (2003), have helped to clarify some inter- and intrageneric relationships.

In view of the morphological similarity among *Colostethus marchesianus* and the three related species, we have examined the karyotype, NOR localization and C-banding pattern in these frogs in order to assess the usefulness of cytogenetic characteristics in distinguishing these species.

MATERIAL AND METHODS

The material examined consisted of six specimens of *C. marchesianus*, with two males and two females from the type locality at Missão de Taracuá (00°07′56″N, 68°33′03″W) in the state of Amazonas, Brazil, and two males from São Gabriel da Cachoeira, located 150 km east of the type locality, in Amazonas, four specimens of *Colostethus caeruleodactylus* (3 males and 1 female) from the municipality of Careiro, at km 12 on the road to Autazes, state of Amazonas, Brazil (03°,37′,10.4″S, 59°,86′,78.4″W), six specimens of *Colostethus* sp. 1 (5 males and 1 female) from the Reserva Florestal Adolfo Ducke (RFAD), located 25 km from Manaus, Amazonas, (03°,08′S, 60°,04′W) and 10 specimens of *Colostethus* sp. 2 (9 males and 1 female) collected in the same region as the *C. caeruleodactylus* individuals. All specimens were collected and identified by A. P. Lima from February to August 1988 and in February 1999 and 2000 under a permit issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (Proc. No. 02005.001367/99-58-AM). The animals were deposited in the Museu de História Natural "Professor Adão José Cardoso" (ZUEC) at the Universidade Estadual de Campinas or in the herpetological collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus, under the following accession numbers: INPA 10192, 10198, 10201, 10206, 10211, and 10220 (*Colostethus marchesianus*), ZUEC 11633, 11634, 11637 and 11640 (*Colostethus caeruleodactylus*), ZUEC 11806, 11810, 11812, 11814, 11816 and 11818 (*Colostethus* sp. 1), and ZUEC 11707–09, 11711, 11712, 11716, 11719 and INPA 7264–66 (*Colostethus* sp. 2).

Metaphases were prepared from a suspension of intestinal epithelium and testicular cells from animals pre-treated with 2 % colchicine for at least 4 h, as described by KING and ROFE (1976) and SCHMID (1978a). The cells were fixed in methanol/acetic acid fixative (3:1) and the slides were stained with 10 % Giemsa solution for analysis of the chromosome number, or labeled with silver nitrate for nucleolar organizer region (AgNOR) detection, according to HOWELL and BLACK (1980) and for C-banding using the technique of SUMNER (1972), with modifications in the duration of treatment with $Ba(OH)_2$. Fluorescent in situ hybridization (FISH) to identify the ribosomal genes was done according to VIEGAS-PÉQUIGNOT (1992) using a recombinant plasmid (HM 123) containing fragments of *Xenopus laevis* rDNA (MEUNIER-ROTIVAL et al. 1979). This plasmid was biotin-labeled using the nick translation reaction described in the GIBCO protocol. After FISH, the slides were examined with an Olympus BX 60 microscope or a BioRad MRC 1024 UV confocal microscope. The number of metaphases analyzed is shown in Table 1 and the chromosomes were classified according to GREEN and SESSIONS (1991).

Table 1. *Number of silver-stained and C-banded metaphases analyzed from each specimen. ZUEC: Museu de História Natural "Prof. Dr. Adão José Cardoso". INPA: herpetological collections of the Instituto Nacional de Pesquisas da Amazonia.*

Specimen	Metaphases analyzed by		Specimen	Metaphases analyzed by	
	Ag-NOR	C-banding		Ag-NOR	C-banding
C. marchesianus			*Colostethus* sp. 1		
INPA 10192	03	04	ZUEC 11806	04	04
INPA 10198	01	06	ZUEC 11810	06	07
INPA 10201	09	07	ZUEC 11812	26	10
INPA 10206	09	13	ZUEC 11814	06	09
INPA 10211	01	03	ZUEC 11816	—	24
INPA 10220	10	06	ZUEC 11818	09	04
C. caeruleodactylus			*Colostethus* sp. 2		
ZUEC 11633	02	02	ZUEC 11707	10	05
ZUEC 11634	11	02	ZUEC 11708	11	05
ZUEC 11637	05	01	ZUEC 11709	05	06
ZUEC 11640	02	10	ZUEC 11711	02	05
			ZUEC 11712	22	43
			ZUEC 11716	14	06
			ZUEC 11719	03	07
			INPA 7264	01	01
			INPA 7265	01	02
			INPA 7266	04	02

RESULTS

The chromosomal complement in the four species examined was 2n = 22 (Fig. 1), and was confirmed by meiotic chromosome analysis. The karyotypes of *C. marchesianus* and *C. caeruleodactylus* consisted of eight pairs of metacentric chromosomes (1, 2, 5, 6, 8–11), two submetacentrics (3 and 4), and one subtelocentric pair (7). In one specimen of *C. marchesianus* (INPA 10206), a secondary constriction (AgNOR negative) was observed in one of the homologs of pair 7. The karyotypes of *Colostethus* sp. 1 and *Colostethus* sp. 2 were very similar to those of *C. marchesianus* and *C. caeruleodactylus*, differing only in the morphology of pair 3, which was metacentric in these species (Fig. 1 and 5, Table 2). All karyotypes had a bimodal structure (six large and five small pairs).

In *C. marchesianus*, *C. caeruleodactylus* and *Colostethus* sp. 1, the NOR site was located in the interstitial region on the long arm of pair 4. In *Colostethus* sp. 2, the NOR site was detected on the short arm of pair 8; in one specimen (ZUEC 11712), the NOR was heteromorphic between the homologs (Fig. 2). In two specimens of *C. caeruleodactylus*, an

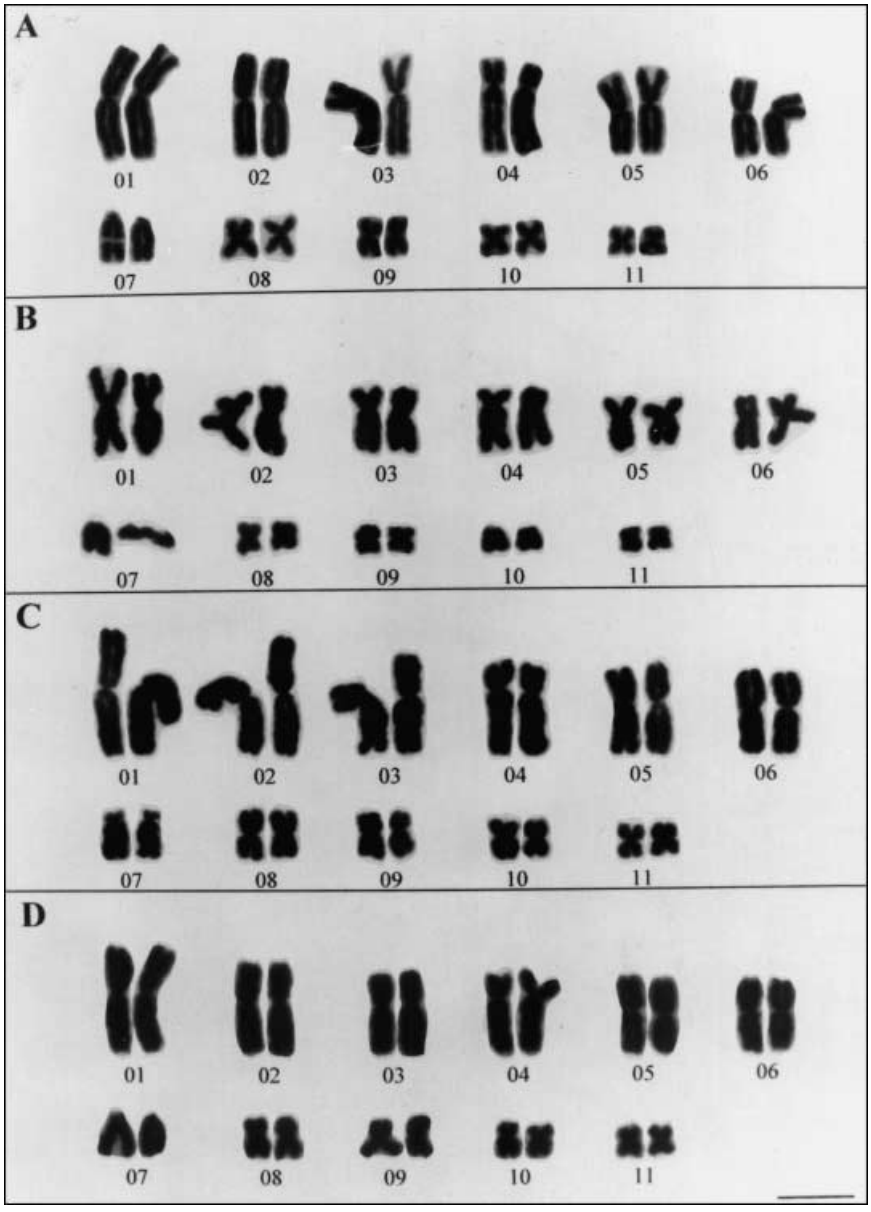

Fig. 1A–D. Giemsa-stained karyotypes of: *C. marchesianus* (**A**), *C. caeruleodactylus* (**B**), *Colostethus* sp. 1 (**C**) and *Colostethus* sp. 2 (**D**). Bar = 10 μm.

Table 2. *Morphometric analysis of the chromosomes of four Colostethus species. Centromeric classification follows that of* Green *and* Sessions *(1991). CH: chromosome, IC: centromeric index, RL: relative length (%), CC: centromeric classification, M: metacentric, SM: submetacentric, ST: subtelocentric. (*): obtained values for one of the homologs of the respective pairs that showed in heteromorphism C-banding size. n: number of measured metaphases.*

Colostethus marchesianus (n = 17)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	16.0	14.2	12.5	12.2	11.1	10.0	6.1	5.5	4.7	4.3	3.3
IC	0.439	0.428	0.374	0.252	0.400	0.426	0.145	0.456	0.447	0.468	0.465
CC	M	M	SM	SM	M	M	ST	M	M	M	M
Colostethus caeruleodactylus (n = 16)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	16.7	14.0	12.5	12.1	10.9	9.6	6.0	5.5	4.8	4.2	3.7
IC	0.414	0.382	0.354	0.260	0.410	0.411	0.132	0.462	0.450	0.460	0.458
CC	M	M	SM	SM	M	M	ST	M	M	M	M
Colostethus sp. 1 (n = 18)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	15.7	13.7	12.5	11.7	11.2	10.4	5.5	5.4	5.1	4.7	4.0
IC	0.488	0.484	0.395	0.314	0.418	0.430	0.175	0.489	0.468	0.427	0.474
CC	M	M	M	SM	M	M	ST	M	M	M	M
Colostethus sp. 2 (n = 39)											
CH	1	2	3	4	5	6	7	8	9	10	11
RL	15.3	13.2	11.7	11.5	10.7	10.1	6.3	6.2	5.7 6.8*	4.9	4.3
IC	0.436	0.429	0.419	0.324	0.435	0.442	0.162	0.461	0.456 0.357*	0.424	0.438
CC	M	M	M	SM	M	M	ST	M	M/SM*	M	M

additional NOR site was detected in the interstitial region on the long arm of pair 1, whereas in one specimen of *Colostethus* sp. 1, an additional NOR site was also detected on the short arm of pair 9. Such extra NOR sites were seen in only one of the homologs. In situ hybridization with an rDNA probe confirmed the location of all NORs detected by silver staining. However, an additional marking on the long arm of pair 5 in one specimen of *Colostethus* sp. 2 (ZUEC 11712), undetected by AgNOR, was also observed (Fig. 3).

C-banding revealed interspecific variations among the karyotypes. C-bands were detected in the centromeric region of all chromosomes in the four species. Pericentromeric, interstitial and terminal bands were also observed, although some stained faintly and were difficult to observe. In the four species, a small block of constitutive heterochromatin was present in the interstitial region on the long arm of pair 7. One specimen of *Colostethus* sp. 2 (ZUEC 11719) showed a heteromorphic C-block in the interstitial region on the long arm of pair 9 (Fig. 4 and 5). This block was detected as a secondary constriction in conventionally and AgNOR stained chromosomes. None of the C-positive blocks was coincident with the NORs in any of the species (Fig. 5).

DISCUSSION

Despite the large number of species in the genus *Colostethus*, only 13 of them have been karyotyped. The studies of Rada de Martínez (1976) and Bogart (1991) were restricted to a description of chromosomal number and morphology using conventional staining, and chromosomal banding data were recently described by Kaiser et al. (2003) and Veiga-Menoncello et al. (2003) for only four *Colostethus* species. Except for *C. chalcopis* (Kaiser et al. 2003) and *C. nidicola* (Veiga-Menoncello et al. 2003), as well as the species studied here all, the other *Colostethus* species have 2n = 24 chromosomes.

The presence of 22 chromosomes indicates that there is karyotypic variability in *Colostethus*. Within the Dendrobatidae, a diploid number of 22 chromosomes has been found only in *Dendrobates opisthomelas* (*Minyobates opisthomelas* – Bogart 1991). Intrageneric variation in chromosome number among dendrobatids has been reported only in *Dendrobates*, with 2n = 18, 20 and 22 chromosomes (León 1970; Rasotto et al. 1987; Bogart 1991). Thus, neither *Dendrobates* nor *Colostethus* appear to be karyotypically conserved. Further analysis with more species should improve our understanding of the relationships between these genera.

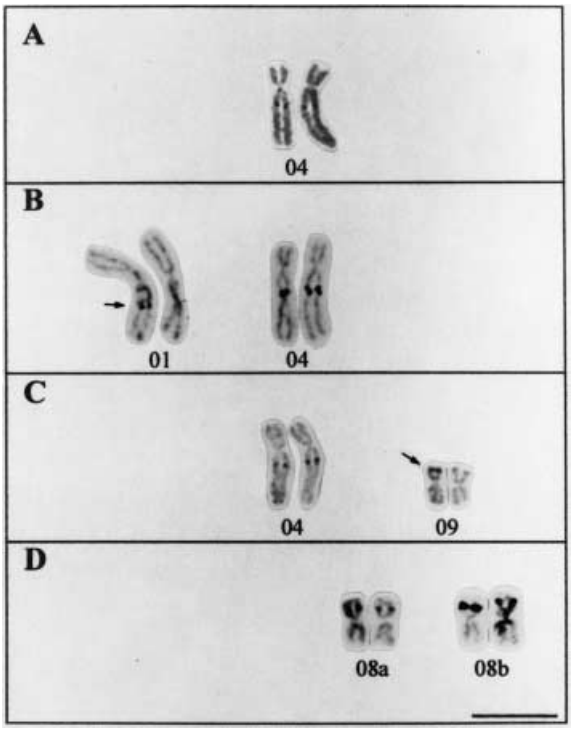

Fig. 2A–D. Silver-stained, NOR-bearing chromosome pairs of: *C. marchesianus* – pair 4 (**A**), *C. caeruleodactylus*- the arrow indicates an additional NOR site on one of the homologs of pair 1, in addition to that of pair 4 (**B**), *Colostethus* sp. 1 – the arrow indicates an additional NOR site on one of the homologs of pair 9, in addition that of pair 4 (**C**). *Colostethus* sp. 2 – pair 8 with homomorphic (a) and heteromorphic (b) NOR sites (**D**). Bar = 10 μm.

Some species belonging to different dendrobatid genera have telocentric chromosomes which suggests centric fusion and fission as possible mechanisms for changes in the chromosomal number in this family, as also found in other anuran groups (BOGART and HEDGES 1995; MIURA et al. 1995; BUSIN et al. 2001). According to BOGART (1991), other chromosomal rearrangements, such as translocations and inversions, are probably involved in the karyotypic evolution of *Colostethus*, since even within the 2n = 24 group some species have no telocentric chromosomes.

The species of *Colostethus* analyzed by BOGART (1991) showed extensive intrageneric variation in their chromosomal morphology, but this was not observed in *C. marchesianus* or in the species related to *C. marchesianus* studied here. The karyotypes of the four species shared some common characteristics with other dendrobatids, including the morphology

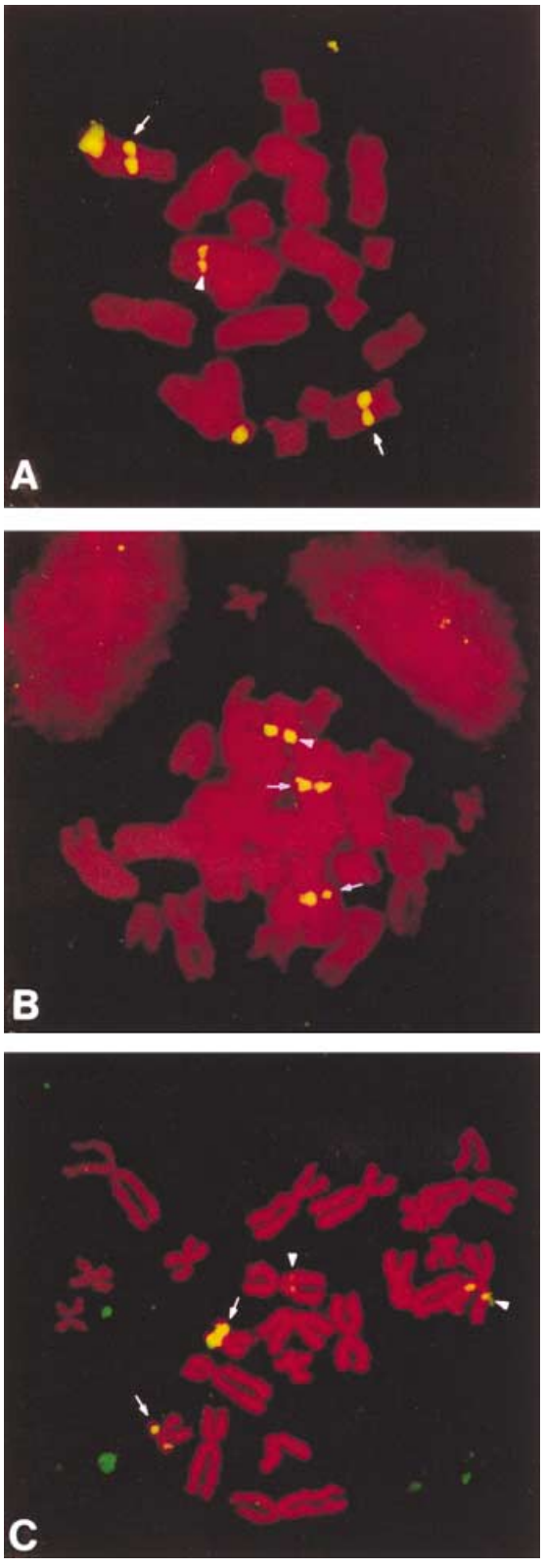

Fig. 3A–C. Mitotic metaphases following FISH with an rDNA probe. *Colostethus caeruleodactylus* – the arrows indicate the NOR sites of pair 4. The arrowheads indicate an additional site on one of the homologs of pair 1 (**A**). *Colostethus* sp.1 – note the NOR on pair 4 (arrows). An additional site can be seen on one of the homologs of pair 9 (arrowhead) (**B**). *Colostethus* sp. 2 – the arrows indicate heteromorphic sites of rDNA on pair 8 and the arrowheads indicate an additional site on pair 5 (**C**).

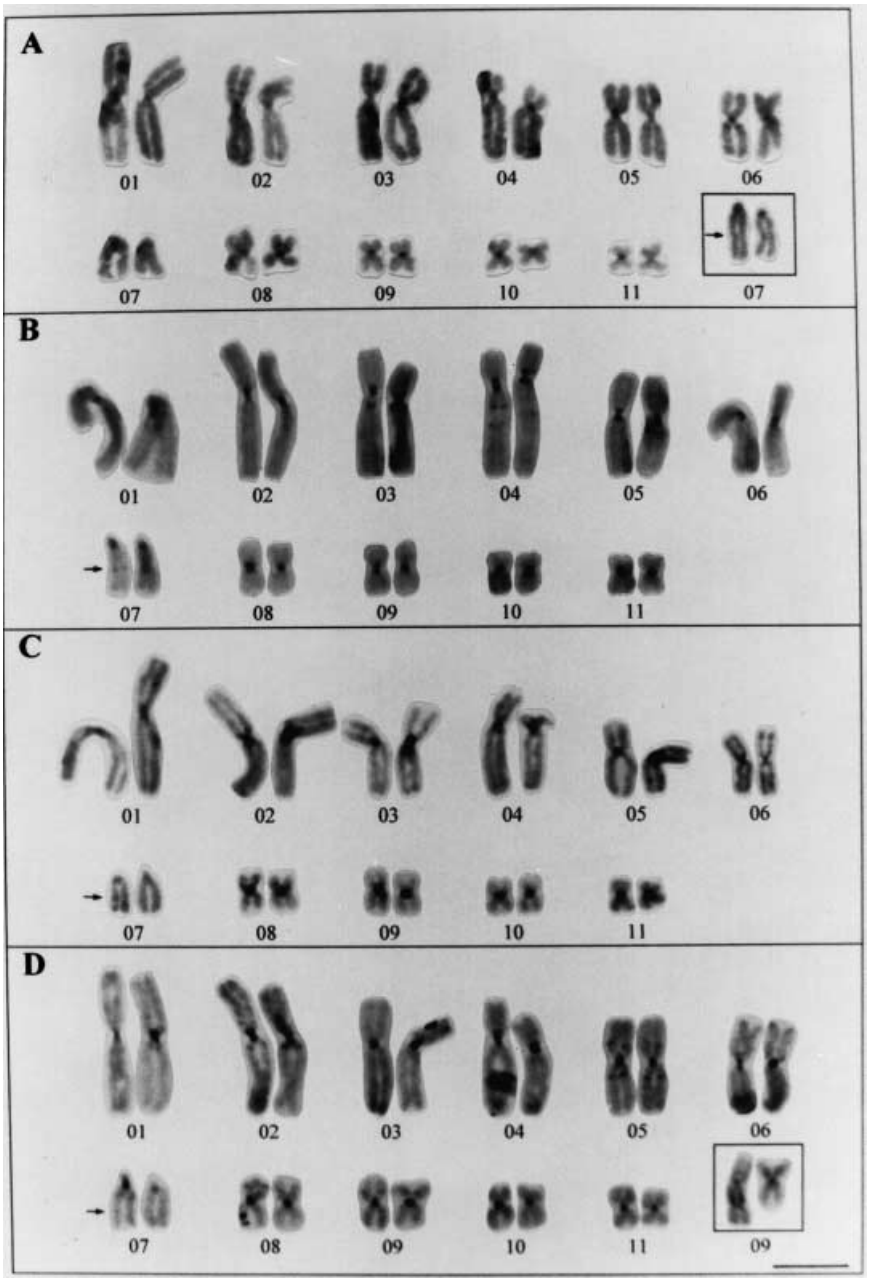

Fig. 4A–D. C-banded karyotypes of: *C. marchesianus* (**A**) – the box shows a faint band on the long arm of pair 7. *C. caeruleodactylus* (**B**), *Colostethus* sp. 1 (**C**) and *Colostethus* sp. 2 (**D**). One of the homologs of pair 9 shows an increase in the amount of heterochromatin (indicated by the square) (**D**). The arrows indicate a faint band on the long arm of pair 7 in the four species. Bar = 10 μm.

of pair 1, which was always metacentric, and a bimodal karyotype.

The karyotypes of *C. marchesianus* and *C. caeruleodactylus* differed from those of *Colostethus* sp. 1 and *Colostethus* sp. 2 only in the morphology of pair 3. Despite differences in the centromeric index, the morphology of this chromosome was similar in the two species. In conventional karyotypical analysis, the difference in the centromeric index was not sufficient to unequivocally distinguish these species.

Despite the great similarity among the four karyotypes, only *Colostethus* sp. 2 could be distinguished from the other species by the NOR location. *Colostethus marchesianus*, *C. caeruleodactylus* and *Colostethus* sp.1 had the NOR site on the long arm of pair 4, whereas *Colostethus* sp. 2 had the NOR on pair 8. According to SCHMID (1982) and SCHMID et al. (1990), variations in NOR location indicate that

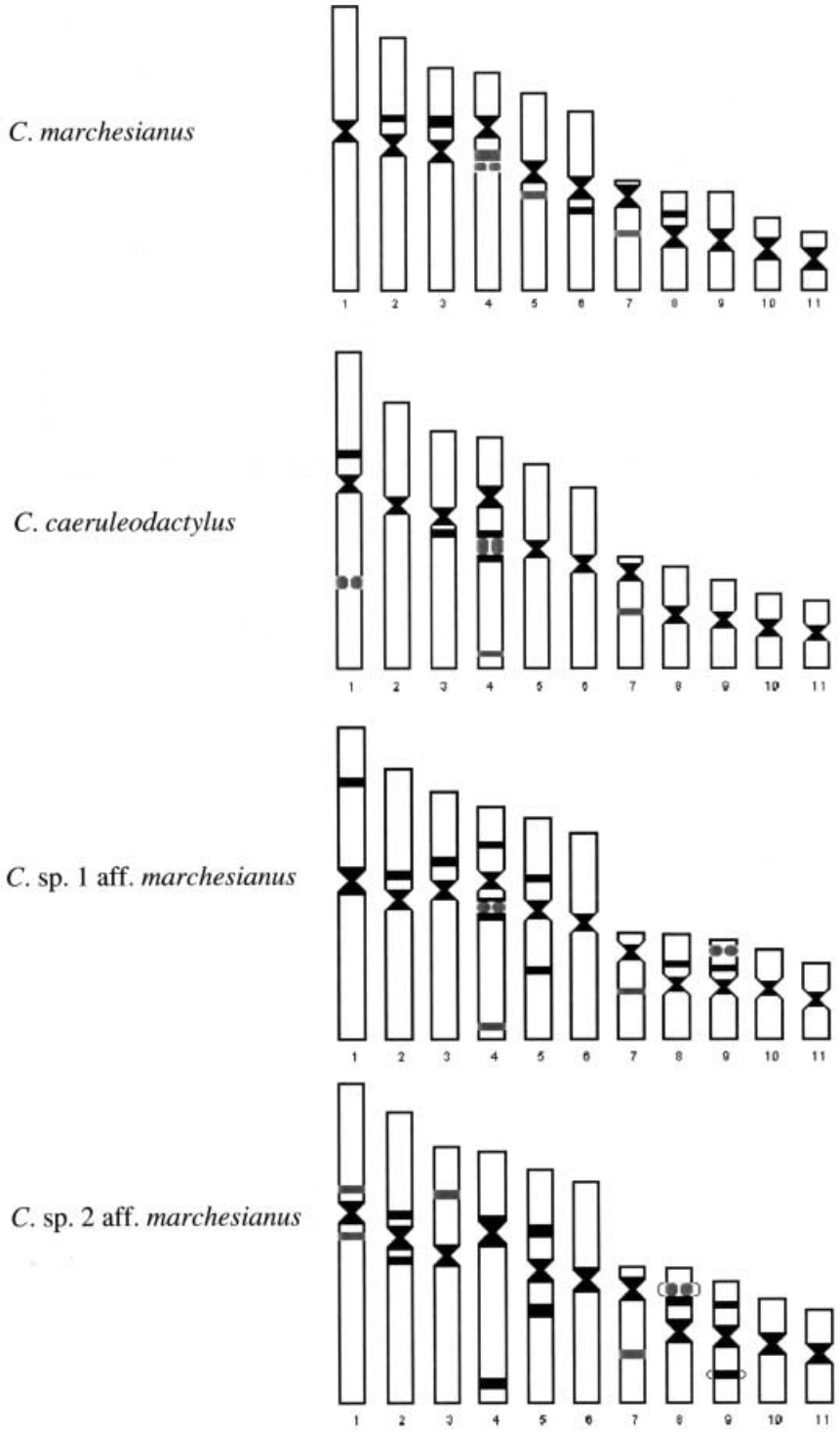

Fig. 5. Representative ideograms of the chromosomal numbers, NOR locations and C-banding patterns of four species of *Colostethus* from the Brazilian Amazon. Solid blocks: dark C-bands. Gray blocks: faint C-bands. Open regions: secondary constrictions. Gray circles: NORs. The parentheses indicate heteromorphic markings.

chromosomal rearrangements occurred during evolution since NOR sites are always located in the same chromosomal region in species of the same or related groups. Our results for the NOR location suggest that the karyotype of *Colostethus* sp. 2 is less conservative than that of other species in this parameter.

Heteromorphism in NOR size is frequent in a large number of anuran species (SCHMID 1982; LOURENÇO et al. 2000; BUSIN et al. 2001). The NOR heteromorphism in *Colostethus* sp. 2 probably resulted from the amplification of some ribosomal sequences in one of the homologs since our results did not indicate a complete duplication similar to that observed in most of the species analyzed by SCHMID (1982). Homomorphism or heteromorphism in Ag-stained NORs was seen in interphase nuclei, as also observed by SCHMID (1980, 1982).

Intraspecific polymorphism in the number and location of NORs has been described in a few anuran species, including *Hyla versicolor*, *Hyla chrysoscelis* (WILEY et al. 1989), *Bufo terrestris* (FOOTE et al. 1991), *Agalychnis callidryas* (SCHMID et al. 1995), *Physalaemus petersi* (LOURENÇO et al. 1998), *Physalaemus cuvieri* (SILVA et al. 1999), *Paratelmatobius poecilogaster* (LOURENÇO et al. 2000), *Pseudis minuta* and *Pseudis* sp. aff. *minuta* (BUSIN et al. 2001). In *C. caeruleodactylus* and *Colostethus* sp. 1, as well as in *A. callidryas* and *B. terrestris*, an additional NOR occurred in one of the homologs. According to SCHMID et al. (1995), these NORs appear to have been excised from or inserted into chromosomes without altering their morphology. FOOTE et al. (1991) suggested some probable mechanisms to explain the origin of this additional NOR site, including NORs functioning as mobile genetic elements, ''orphan'' rDNA copies, and reinsertion errors during ribosomal cistron amplification. However, additional evidence is needed to support such mechanisms.

Other small markers, such as that revealed by in situ hybridization on the long arm of pair 5 in one specimen of *Colostethus* sp. 2, which was undetected by AgNOR, have also been observed in *Hyla versicolor* and *Hyla chrysoscelis* (WILEY et al. 1989). According to SCHMID (1978b), small NORs cannot be detected by AgNOR because of their size. However, SCHMID et al. (1995), KING et al. (1990), FOOTE et al. (1991) and LOURENÇO et al. (1998) reported that in anurans all NORs detected by the AgNOR technique were also detected by in situ hybridization. Hence, a probable hypothesis to explain the additional marker present on pair 5 in specimen ZUEC 11712 of *Colostethus* sp. 2 is the presence of a homologous sequence of some portion of rDNA. In all specimens of *Colostethus* sp. 2, this region also had a C-band, which suggested the transposition of rDNA sequences to the heterochomatic region in this specimen.

Some of the C-band-positive it was useful for distinguishing these species. Thus, *C. marchesianus* had a pericentromeric C-block on the long arm of pair 6, which was not observed in the other species. *C. caeruleodactylus* differed from the other species by the absence of a pericentromeric C-block on pairs 2 and 5, and *Colostethus* sp. 2 had a C-block on the long arm of pair 9 that was characteristic only of this species.

Despite the variability detected in the C-banding pattern, the four species of *Colostethus* examined had a common, faintly staining band on the long arm of pair 7 which could be considered a landmark band for the 22-chromosome *Colostethus* species, as *C. chalcopis* (KAISER et al. 2003) also had this band. Since all species examined here had the same chromosomal number and a similar karyotype, but a different C-banding pattern, it is probable that the transformation of euchromatic segments to heterochromatic ones had a role in the separation of these *Colostethus* species. However, other events related to heterochromatin (and not detectable by the methods used here) may have been involved.

The heteromorphism in C-band size observed on the long arm of one of the homologs of pair 9 in a specimen of *Colostethus* sp. 2 (ZUEC 11719) probably resulted from the amplification of certain repetitive DNA sequences, and may have caused a change in chromosomal morphology. Changes in chromosomal morphology resulting from the addition of heterochromatin have also been reported by KING (1980) for species of *Litoria* (Hylidae).

Despite their similar chromosomal morphology, the species of *Colostethus* examined here were distinguished from each other by their C-banding pattern, and *Colostethus* sp. 2 could also be distinguished from the other species by it NOR location. Moreover, these species could be distinguished from *C. chalcopis* (KAISER et al. 2003) by differences in the position of the centromere of some chromosome pairs, in addition to the NOR location and C-banding pattern.

In conclusion, chromosomal rearrangements and heterochromatin-related events may have been involved in karyotypic differentiation in these species. Further analysis using molecular approaches could be useful for understanding the phylogenetic relationships of the 22-chromosome *Colostethus* species from Central Amazonia and the 22-chromosome *C. chalcopis*.

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