RESEARCH ARTICLE

Chromosome analysis of five Brazilian species of poison frogs (Anura: Dendrobatidae)

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Abstract

Dendrobatid frogs have undergone an extensive systematic reorganization based on recent molecular findings. The present work describes karyotypes of the Brazilian species *Adelphobates castaneoticus*, *A. quinquevittatus*, *Ameerega picta*, *A. galactonotus* and *Dendrobates tinctorius* which were compared to each other and with previously described related species. All karyotypes consisted of $2n = 18$ chromosomes, except for *A. picta* which had $2n = 24$. The karyotypes of the *Adelphobates* and *D. tinctorius* species were highly similar to each other and to the other $2n = 18$ previously studied species, revealing conserved karyotypic characteristics in both genera. In recent phylogenetic studies, all *Adelphobates* species were grouped in a clade separated from the *Dendrobates* species. Thus, we hypothesized that their common karyotypic traits may have a distinct origin by chromosome rearrangements and mutations. In *A. picta*, with $2n = 24$, chromosome features of pairs from 1 to 8 are shared with other previously karyotyped species within this genus. Hence, the *A. picta* data reinforced that the C-banding pattern and the NOR location are species-specific traits in the genus *Ameerega*. Moreover, the *Ameerega* monophyletism proposed by previous phylogenetic studies indicates that the karyotypic differences among species in this genus result from a long divergence time.

[Rodrigues P. C., Aguiar O., Serpieri F., Lima A. P., Uetanebaro M. and Recco-Pimentel S. M. 2011 Chromosome analysis of five Brazilian species of poison frogs (Anura: Dendrobatidae). *J. Genet.* **90**, 31–37]

Introduction

Recent molecular studies have contributed to a revision in the family Dendrobatidae and resulted in extensive taxonomic change (Grant *et al.* 2006). Currently, the family Dendrobatidae consists of 165 species grouped in three subfamilies: Colostethinae, Dendrobatinae and Hiloxalinae. The subfamily Colostethinae comprises of four genera: *Ameerega* (27 spp.), *Colostethus* (19 spp.), *Epipedobates* (6 spp.) and *Silvertoneia* (3 spp.). The subfamily Dendrobatinae contains six genera: *Adelphobates* (3 spp.), *Dendrobates* (5 spp.), *Excitobates* (2 spp.), *Minyobates* (1 spp.), *Oophaga* (9 spp.), *Phyllobates* (5 spp.) and *Ranitomeya* (27 spp.). The subfamily Hyloxalinae consists of the genus *Hyloxalus* (57 spp.) (Frost 2008). The Dendrobatidae species have diurnal activity and a typical reproductive behaviour that includes biparental care. Some of the species show aposematism (Grant *et al.* 2006), and mimetism is also observed (Myers *et al.* 1991; Zimmermann and Zimmermann 1988; Santos *et al.* 2003).

Species of the genus *Adelphobates* are found in central and lower Amazon drainage basin of Peru and Brazil. The *Ameerega* genus is distributed in the northern and northwestern regions of Amazon, in the states of Mato Grosso do Sul and Goiás, Brazil, extending west towards the foothills of the Andes from Bolivia to Venezuela, and in Panama (Frost 2008). The genus *Dendrobates* is found from South

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Keywords. C-banding; chromosomes; cytogenetics; dendrobatids; karyotype; NOR.

Nicaragua to Costa Rica, Panamá, Colombia, Guyanas and adjacent Brazil.

Studies on morphology, behaviour, sperm ultrastructure, cytogenetics and molecular analyses have been used to study dendrobatids in an attempt to elucidate their intra-family and inter-family relationships (Toft 1995; Zimmermann and Zimmermann 1988; Aguiar *et al.* 2002, 2003, 2004a,b; Garda *et al.* 2002; Veiga-Menoncello *et al.* 2003a,b, 2006a,b, 2007).

Cytogenetic data have proven to be valuable tools in systematic studies of anuran groups (Bogart 1991; Lourenço *et al.* 1999, 2000, 2008; Busin *et al.* 2001; Aguiar *et al.* 2002; Medeiros *et al.* 2003; Rosa *et al.* 2003; Veiga-Menoncello *et al.* 2003a, b, 2006a; Siqueira *et al.* 2008). So far, a large number of dendrobatid species have been described, but numerous questions about their chromosome characteristics remain unclear. Further, cytogenetic studies are needed to contribute to addressing taxonomic and phylogenetic questions in this family.

In the present work, the karyotypes, nucleolar organizing region (NOR) sites and heterochromatin distribution were analysed in the Brazilian species *A. castaneoticus*, *A. quinquevittatus*, *A. galactonotus*, *Ameerega picta* and *Dendrobates tinctorius* which were compared to each other and with those previously analysed, aiming to improve knowledge on the systematics and relatedness of these species.

Materials and methods

The Brazilian poison frog specimens examined in this work consisted of 10 specimens of *A. quinquevittatus* from Abunã, RO (03°24′S; 61°29′W), 20 specimens of *A. castaneoticus* from Santarém, PA (03°08′S; 54°50′W) and 18 specimens of *Ameerega picta* from Serra da Bodoquena, MS (20°41′S; 56°44′W), Mato Grosso do Sul. The specimens were collected and identified by A. P. Lima and M. Uetenabaro under the permit issued by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, License 178/05 – RAN/IBAMA, 02001.004580/02-08 and 13818-1). The exact origin of the six specimens of *A. galactonotus* and three of *Dendrobates tinctorius* is not known, as they were donated by IBAMA from an illegal capture. The voucher specimens were deposited in the Zoology Museum, Prof. Adão José Cardoso (ZUEC) in the State University of Campinas, Brazil, under the accession numbers: 12473–12478 (*A. galactonotus*), 13542–13551 (*A. quinquevittatus*), 13521–13523 (*A. castaneoticus*), 13512–13514 (*D. tinctorius*) and 12481–12498 (*A. picta*).

Metaphases were obtained from intestinal epithelium cell suspensions of animals pretreated with 2% colchicine for at least 4 h, according to King and Rofe (1976) and Schmid (1978). Cells were fixed in methanol : acetic-acid (3:1) and the slides were stained with 10% Giemsa solution for analysis of chromosome number and morphology, or stained

Table 1. Morphometric parameters of the dendrobatid karyotypes are described here. Classification according to Green and Session (1991).

N°	1	2	3*	4	5	6	7	8	9	10	11	12
Adelphobates castaneoticus												
RL%	20.58	19.44	13.6/ 13.97	13.34	11.5	9.66	4.64	3.99	3.21			
AR	1.32	1.17	2.56/ 2.31	1.56	2.3	1.51	1.13	1.22	1.16			
CP	M	M	SM	M	SM	M	M	M	M			
Adelphobates galactonotus												
RL%	20.23	19	13.21	13.03	11.25	9.41	5.77	4.43	3.67			
AR	1.45	1.16	2.16	1.44	2.48	1.60	1.34	1.02	1.03			
CP	M	M	SM	M	SM	M	M	M	M			
Adelphobates quinquevittatus												
RL%	20.69	19	13.42	12.84	11.6	9.15	5.1	4.39	3.77			
AR	1.3	1.37	2.34	1.57	2.1	1.4	1.1	1.08	1.06			
CP	M	M	SM	M	SM	M	M	M	M			
Dendrobates tinctorius												
RL%	20.87	18.81	12.79	12.98	10.71	9.96	5.73	4.78	3.35			
AR	1.36	1.24	2.67	1.49	1.92	1.46	1.26	1.19	1.08			
CP	M	M	SM	M	SM	M	M	M	M			
Ameerega picta												
RL%	17.01	13.29	12.28	11.35	10.81	10.18	5.50	4.51	4.28	3.99	3.57	3.40
AR	0.46	0.40	0.28	0.19	0.37	0.40	0.45	0.45	0.28	–	–	0.24
CP	M	M	SM	ST	M	M	M	M	SM	T	T	ST

RL, relative length; AR, arm ratio; CP, centromere positiom; M, metacentric; SM, submetacentric; ST, subtelocentric; T, telocentric; *heteromorphic pair 3.

with silver nitrate for detection of nucleolar organizer region (AgNOR) according to Howell and Black (1980). C-bands were detected using the technique described by Sumner (1972) with modifications in the alkaline treatment regarding incubation time and barium hydroxide temperature. Slides were examined under an Olympus BX60 (Tokyo, Japan) optical microscope. Chromosomes were classified according to the nomenclature of Green and Session (1991).

Results

A diploid number, $2n = 18$ chromosomes, was observed in *A. castaneoticus, A. galactonotus*, *A. quinquevittatus* and *Dendrobates tinctorius*. Karyotypes are highly similar among these species, consisting of two large, four medium-size and three small pairs of chromosomes. The chromosomes were metacentric except for pairs 3 and 5, which were

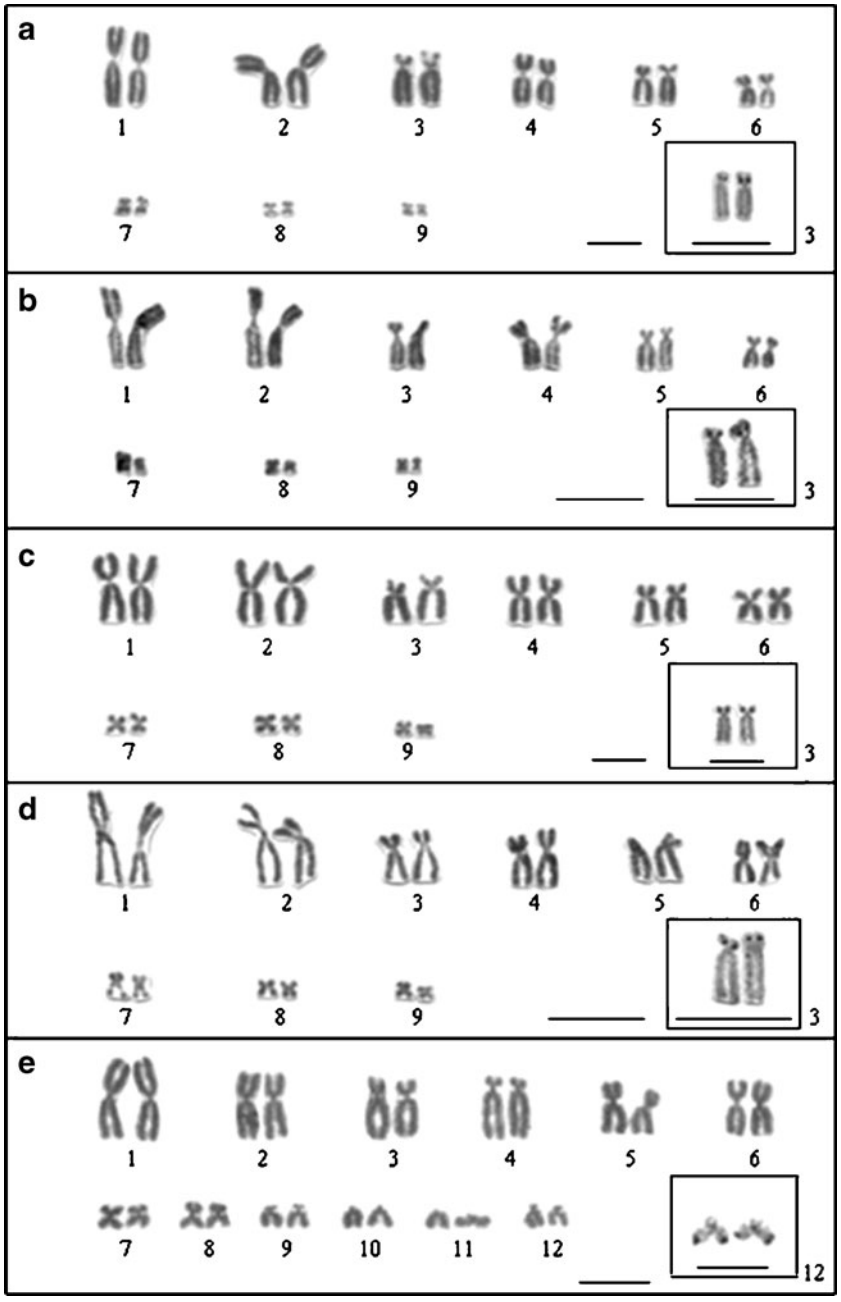

Figure 1. Giemsa-stained karyotypes of Dendrobatidae species: (a) *Adelphobates castaneoticus*; (b) *A. quinquevittatus*; (c) *A. galactonotus*; (d) *Dendrobates tinctorius*; (e) *Ameerega picta*. Bars: A = 4.5 μm; B and D = 2 μm; C = 3.4 μm; E = 4 μm. Insets: NOR-bearing chromosomes submitted to the AgNOR technique. Bars: A and D = 2.5 μm; B = 2.2 μm; C and E = 4 μm.

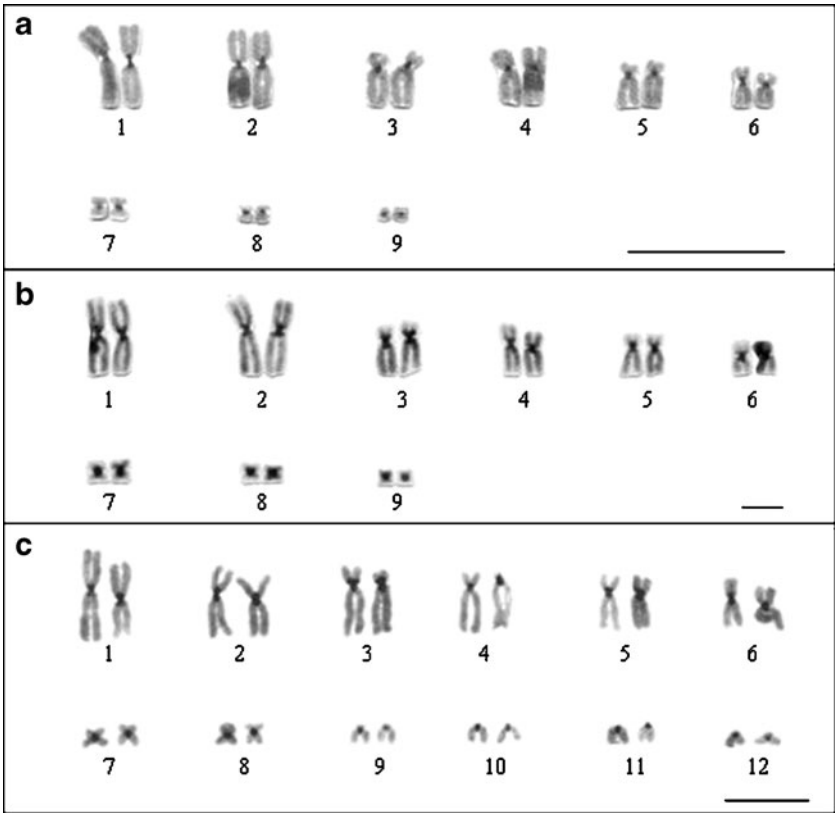

Figure 2. C-banded karyotypes of (a) *Adelphobates castaneoticus*, (b) *A. quinquevittatus* and (c) *Ameerega picta*. Bars: A and C = 3.5 μm; B = 3 μm.

submetacentric (table 1). In all species, NORs were located in the interstitial region of the short arm of chromosome 3 (figure 1, A–D). Heteromorphic NORs were detected in several individuals of *A. castaneoticus* (figure 1B). In a few *D. tinctorius* metaphases, there was a secondary constriction on the long arm of pair 7, which sometimes changed the chromosome morphology (figure 1D).

The karyotype of *Ameerega picta* consisted of $2n = 24$ chromosomes, with six large pairs and six small pairs. Pairs 1, 2, 5, 6, 7 and 8 were metacentric, 3 and 9 submetacentric, 4 and 12 subtelocentric and pairs 10 and 11 were telocentric (table 1). The NOR was located on the telomeric region of the long arm of pair 12 (figure 1E).

C-bands were detected only in *A. quinquevittatus*, *A. castaneoticus* and *Ameerega picta*. Centromeric heterochromatin was present in all chromosomes but in *A. quinquevittatus* and *A. picta*, the centromeric bands were larger than in *A. castaneoticus* (figures 2 and 3, A, B & E). Additionally, an interstitial faint band on the long arm of pair 10 was observed in *A. picta* (figures 2 and 3E).

Discussion

Karyotypes of *A. castaneoticus*, *A. galactonotus*, *A. quinquevittatus* and *D. tinctorius* are very similar to those of *D. auratus* (Rasotto *et al.* 1987) and *D. truncatus* (Bogart 1991). All of them have the same diploid number and similar chromosome morphology, suggesting karyotypic conservation among these $2n = 18$ dendrobatid genera. The only difference detected between the *Adelphobates* karyotypes and those of *D. auratus* and *D. truncatus* was an inversion in the position of pairs 3/4 and 5/6 in the karyogram. This difference is most likely due to an experimental error in chromosome measurements, since they are highly similar in size.

Bogart (1991) reported $2n = 20$ chromosomes in the karyotype of specimens identified as *D. quinquevittatus* (currently *A. quinquevittatus*). Therefore, those specimens are karyotypically distinct from the $2n = 18$ species analysed in the present work. The specimens analysed by Bogart, which were from Peru, were not actually *D. quinquevittatus* since this species is restricted to the state of Rondônia (Bacia do Rio Madeira) and subjacent areas in the state of Amazonas, Brazil (Martins and Haddad 1990). Those specimens (Bogart 1991) were indeed *Ranitomeya vanzolinii* as afterwards referred by Grant *et al.* (2006).

Karyotypes of species within the previous *Dendrobates* genus had been described as $2n = 18$ and $2n = 20$. After the taxonomic revision by Grant *et al.* (2006), these diploid numbers are currently found in the genera *Dendrobates* and *Adelpohobates* ($2n = 18$), as well as *Oophaga* and *Ranitomeya* ($2n = 20$). According to Rasotto *et al.* (1987) and

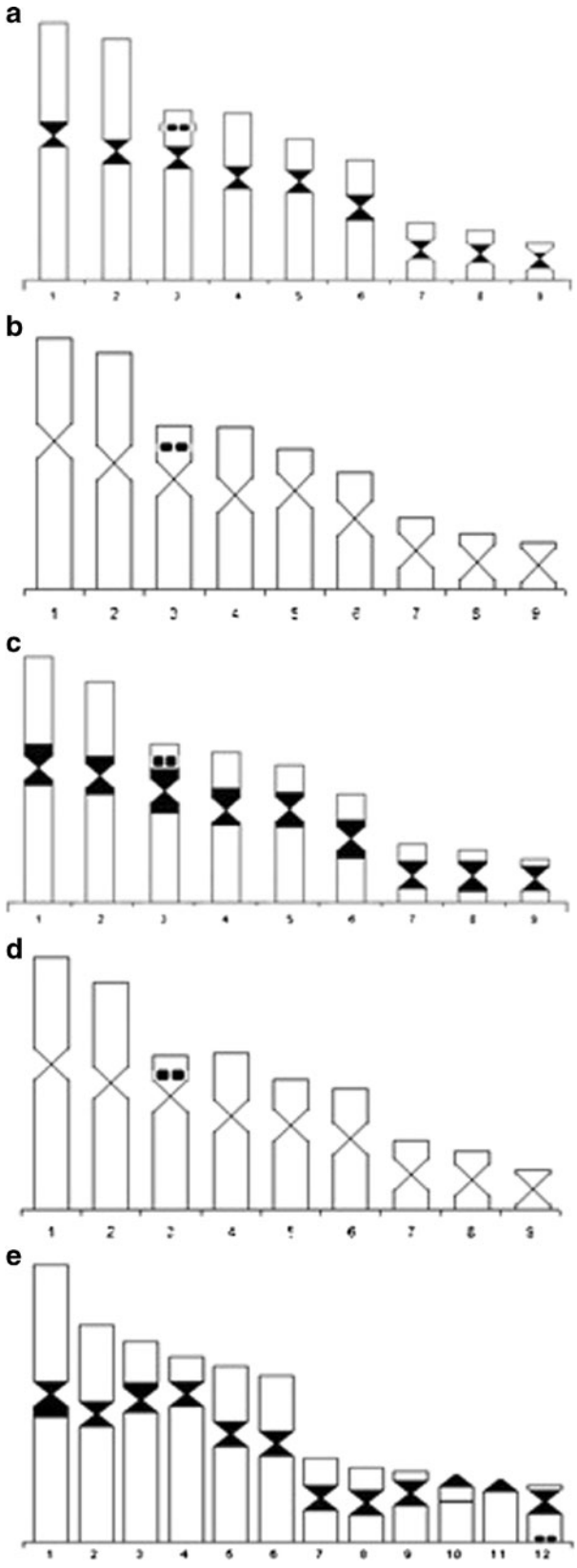

Figure 3. Ideograms of (a) *Adelphobates castaneoticus*, (b) *A. quinquevittatus*, (c) *A. galactonotus*, (d) *Dendrobates tinctorius* and (e) *Ameerega picta* karyotypes. Dark areas, C bands; dark circles, NORs; dotted areas, secondary constriction; parenthesis indicates NOR heteromorphism.

Bogart (1991), the following species have $2n = 20$ and a bimodal karyotype with six large pairs (1 to 6) and four small pairs (7 to 10) of chromosomes: *Ranitomeya vanzolinii* (previously *D. vanzolinii*) from Peru; *Oophaga pumilio* (previously *D. pumilio*); *O. histrionica* (previously *D. histrionicus*); and *O. granulifera* (previously *D. granulifer*). Comparing the karyotypes of the species with $2n = 20$ with those species showing $2n = 18$ (currently *Dendrobates* and *Adelphobates*), it is evident that $2n = 18$ karyotypes show no bimodal character and subtelocentric or telocentric chromosomes.

Surprisingly, the chromosome data applied to an adapted cladogram (figure 4) from the phylogenetic tree reported by Grant *et al.* (2006) showed the genera *Dendrobates* ($2n = 18$) and *Oophaga* ($2n = 20$) as more closely related to each other than the *Dendrobates* to *Adelphobates* ($2n = 18$). The two latter have the same number and morphology of chromosomes, as well as equal NOR location. In the same manner, *Oophaga* ($2n = 20$) and *Ranitomeya* ($2n = 20$ and 22) have similar chromosome morphology and were more distantly related to each other than are *Oophaga* to *Dendrobates*. However, since $2n = 24$ is found in the ancestral species *Phyllobates lugubris*, as indicated in the cladogram by Grant *et al.* (2006), it appears that the chromosome evolution in this clade occurred through reduction in chromosome number, leading to karyotypes with 22, 20 and 18 chromosomes in such related species. Therefore, mapping chromosome number in the recent phylogeny, assumed here as the better hypothesis, may result in a confounding factor about their relationships, since the karyotypes $2n = 18$ (*Adelphobates* and *Dendrobates*), $2n = 20$ (*Oophaga*) and $2n = 20$ and 22 (*Ranitomeya*) most likely might have resulted from diverse chromosome rearrangements and mutations that were not morphologically detectable in with the so far applied techniques.

The diploid chromosome number of *A. picta* ($2n = 24$) was previously observed in other *Ameerega* species by Bogart (1991) and Aguiar *et al.* (2002). In addition, the *A. picta* chromosome morphology of the specimens analysed in the present work was very similar to another population that was described by Bogart (1991). Detectable differences were limited to a few chromosomes with inverted position in the karyogram. Although they have the same diploid number,

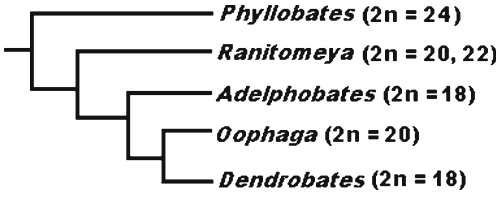

Figure 4. Simplified cladogram adapted from the phylogenetic tree seen in figure 78 of the Dendrobatoidea taxonomy proposed by Grant *et al.* (2006) with diploid numbers mapped on the Dendrobatidae clade.

A. picta differs from the other *Ameerega* species in the morphology of pairs from 9 to 12. The morphology of chromosomes from 1 to 8 is quite conserved in all species of this genus. The *A. picta* C-band pattern was highly similar to *A. flavopicta* (Aguiar *et al.* 2002), except for the interstitial band in the long arm of pair 10 that was observed only in *A. picta*.

The *Ameerega* species have the same diploid number ($2n = 24$), similar chromosome morphology and C-bands. Even though, their karyotypes can be distinguished by the morphology of pairs 9 to 12 and by the NOR location. In *A. picta* and *A. flavopicta* species, the NOR is located on the telomeric region of pair 12 (Aguiar *et al.* 2002). However, the morphology of this pair of chromosomes is slightly distinct, being telocentric in *A. flavopicta* and subtelocentric in *A. picta*. The NOR location has been reported as a species-specific characteristic in *Ameerega* (Aguiar *et al.* 2002).

The heterochromatin distribution has also been shown to be a species-specific trait in *Ameerega* (Aguiar *et al.* 2002), preventing the recognition of the homologies which could indicate relationships among the species already studied. Also, recent findings by Grant *et al.* (2006) indicated monophyletism in *Ameerega*. Hence, the karyotypic variability suggests a considerable time of divergence leading to the current karyotypic distinction among *Ameerega* species. The variation in NOR location reinforces this hypothesis. The cytogenetic characteristics of *A. picta* easily distinguished it from *A. hahneli* and *A. flavopictus*, which were considered in the past as synonyms of *A. picta*.

The cytogenetic data described in this work contribute to further understand the chromosome evolution in Dendrobatidae clade and are potentially useful as taxonomic traits for additional studies in this family.

Acknowledgements

Authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo, Brazil, for financial support (FAPESP, grant 02/12139-9 to SMRP and a scholarship 05/05132-6 granted to PCR), and to Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil (CNPq, grant 300660/2005-7 to APL).

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Received 15 February 2010, in received form 5 July 2010; accepted 16 July 2010
Published on the Web: 19 May 2011