Conserved karyotypes in the *Hyla pulchella* species group (Anura, Hylidae)

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Ananias, F., Garcia, P. C. A. and Recco-Pimentel, S. 2004. Conserved karyotypes in the *Hyla pulchella* species group (Anura, Hylidae). — *Hereditas 140*: 42–48. Lund, Sweden. ISSN 0018-0661. Received August 19, 2003. Accepted November 18, 2003

Cytogenetic analyses were done on specimens of *Hyla marginata* and on three populations of *H. semiguttata* differing in morphology and in the physical parameters of their advertisement call, as well as in individuals of *Hyla* sp. (aff. *semiguttata*). All specimens had 2n = 24 chromosomes with a morphology very similar to that of other 24-chromosome *Hyla* species. *Hyla semiguttata* and *H. marginata* showed the same C-banding pattern but were distinguished by the location of the NOR on pair 1 in *H. semiguttata* (in the three populations) and *Hyla* sp. (aff. *semiguttata*), and on pair 10 in *H. marginata.* The *H. semiguttata* populations did not differ cytogenetically, despite variations in their morphology and advertisement calls. Similarly, *H. semiguttata* and *H. p. joaquini* studied previously had identical C-banding patterns and NOR locations, suggesting that they are very closely related.

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According to Lutz (1973), the *Hyla pulchella* group, previously known as *Hyla raddiana*, occurs in Brazil, Uruguay and Argentina. The group was defined by Duellman et al. (1997) as consisting of the following species, excluding the related species of the *Hyla circumdata* group: *Hyla pulchella pulchella* Duméril and Bibron, 1841; *H. pulchella joaquini* Lutz, 1968; *H. pulchella cordobae* Barrio, 1965; *H. pulchella riojana* Koslowski, 1895; *H. andina* Müller, 1924; *H. semiguttata* Lutz, 1925; *H. marginata* Boulenger, 1887; *H. prasina* Burmeister, 1856; *H. cymbalum* Bokermann, 1963; *H. albonigra* Nieden, 1923; *H. balzani* Boulenger, 1898; *H. marianitae* Carrizo, 1992; *H. melanopleura* Boulenger, 1912 and *H. palaestes* Duellman, De La Riva and Wild, 1997. Faivovich (1996) and Caramaschi and Cruz (2000) included *H. caingua* Carrizo, 1990 and *H. ericae* Caramaschi and Cruz, 2000 in the *pulchella* group. In addition, Garcia et al. (unpubl.) raised the subspecies *H. p. joaquini* to the full species category because of its larger size, robust arms and distinct acoustic parameters compared to *Hyla p. pulchella*.

The characterization of the *pulchella* group is difficult. The species considered to be part of this group have the following characters: (1) a moderately robust body and a proportionally long, wide head, (2) flanks and inner thigh areas with a pale coloration and black bars or reticulations, or dark thighs and flanks with pale spots, (3) males with hypertrophied forearms but a well developed prepolex terminating in a spine, (4) an advertising call consisting of a series of "bell type" notes, (5) reproduction in flowing water, and (6) a brown, green or gray dorsal color, generally with dark spots, reticulations or transversal bars (Duellman et al. 1997).

In an attempt to clarify the relationships within the large *pulchella* group, Garcia et al. (2001) suggested that the species *H. marginata*, *H. semiguttata*, *H. p. joaquini* and *H. ericae* form a subgroup within the *Hyla pulchella* group based on certain common characteristics, including the absence of stains or dark bars on the flanks and on the inner surface of the thighs, long, multi-pulsed acoustic notes, and reproduction in creeks.

Hyla marginata is found in the southern Brazilian states of Rio Grande do Sul and Santa Catarina (Garcia et al. 2001) and *H. semiguttata* Lutz in the states of Rio Grande do Sul, Santa Catarina and Paraná in Brazil, as well as in northwestern Argentina (Lutz 1925; Cei and Roig 1961; Lutz 1973; Braun and Braun 1980). The relationship between *H. marginata* Boulenger and *H. semiguttata* Lutz within the *pulchella* group is unclear. Lutz (1973) suggested that *H. marginata* was similar to *H. p. joaquini* in some characters. Langone (1993) considered *H. semiguttata* and *H. p. joaquini* synonymous with *H. margin-*

ata. Morphological differences have been observed in specimens of *H. semiguttata* from southern of Brazil and northwestern Argentina (P. C. A. Garcia, pers. obs.).

Garcia and Haddad (1999) reported the existence of different populations which they referred to as belonging to the *marginata/semiguttata* complex. Analysis of the advertising calls of populations of *H. semiguttata* and *H. marginata* showed that *H. marginata* had call parameters that differed from those of *H. semiguttata.* All populations of *H. semiguttata* studied so far (Misiones, Argentina; Cambará do Sul and São Francisco de Paula, RS, and Piraquara, PR, Brazil) show significant differences in their call patterns, which suggests the existence of more than one species under the same name.

Considering the difficulty in defining *H. marginata* and *H. semiguttata*, as well as the uncertain relationships among species of the *pulchella* group and between this and other groups (*polytaenia* and *circumdata*), the aim of this study was to compare cytogenetically three populations of *H. semiguttata* and one population of *H. marginata* in order to clarify some of these issues.

MATERIAL AND METHODS

Specimens of *H. marginata*, *H. semiguttata* (populations of Cambará do Sul, São Francisco de Paula and Piraquara, Brazil) and *Hyla* sp. (aff. *semiguttata*) from Argentina were collected and deposited in the collection of the Dept of Zoology of the State University of São Paulo (UNESP), Rio Claro, SP, Brazil (Table 1).

Chromosomal preparations were obtained after intraperitonial injection of aqueous solution of 2% colchicine (0.02 ml g^{-1} of body weight). After at least 4 h the intestines and testes were removed to prepare the cell suspensions (Schmid 1978; Schmid et al. 1979). Metaphase preparations stained with 10% Giemsa solution were photographed with an Olympus BX60 microscope. The chromosomes were classified according to their centromere position based on the nomenclature and centromeric index proposed by Green and Session (1991).

The constitutive heterochromatin pattern and NOR localization were assessed using the C-banding (Sumner 1972) and Ag-NOR (Howell and Black 1980) techniques, respectively.

RESULTS

Karyotypes

All populations of *H. marginata*, *H. semiguttata*, and *Hyla* sp (aff. *semiguttata*) had 2n = 24 chromosomes. Pairs 1, 2, 8, 11 and 12 were metacentric, pairs 3, 5, 7, 9 and 10 were sub-metacentric, and pairs 4 and 6 were sub-telocentric (Fig. 1 and 4; Table 2). *H. marginata* had secondary constrictions in the centromeric region of the long arms of pair 10. In *H. semiguttata* (three populations) and *Hyla* sp. (aff. *semiguttata*), such constrictions occurred in the telomeric region of the short arm of pair 1 in some metaphases (Fig. 1 and 4).

C-banding pattern

The same heterochromatin pattern was observed in *H. marginata*, *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*) (Fig. 2 and 4) using the C-banding method. The centromeric regions of all chromosomes were labeled. A strong heterochromatic band was observed on the long arm of pair 10, as well as in the telomeric region of the long arm in pair 1.

Nucleolar oganizing region – (NOR)

The NOR in *H. marginata* was located on the long arm of pair 10, coincident with the secondary constriction and the heterochromatin block. In *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*), the NOR occurred in the telomeric region on the short arm in pair 1 (Fig. 3 and 4).

DISCUSSION

The diploid chromosome number of 2n = 24 is common in the order Anura and occurs in species of

Table 1. *Number of specimens, collection site in Brazil and Argentina and Museum catalogue number of the examined specimens. RS = Rio Grande do Sul State; SC = Santa Catarina State; PR = Paraná State; BR = Brazil.*

Species	Number of specimens	Collection site	Museum acession numbers
H. semiguttata	12 males	Cambará do Sul, SC; BR	3114–3122 and 3126–3128
	7 males	São Francisco de Paula, RS, BR	3139–3145
	4 males	Piraquara, PR; BR	3704–3707
Hyla sp. (aff. *semiguttata*)	4 males	Misiones, Argentina	4908, 3446, 4909 and 4910
H. marginata	8 males	São Francisco de Paula, RS, BR	3090–3094 and 3819–3821

Fig. 1a–e. Karyotypes of *H. marginata* (**a**), *H. semiguttata* from São Francisco de Paula, RS. (**b**), Cambará do Sul, RS. (**c**) and Piracuara, PR. (**d**) and *Hyla* sp. (aff. *semiguttata*) (**e**) after Giemsa staining. Bar = 5 μm.

several families (Kuramoto 1990). Most species belonging to the genus *Hyla* show 2n = 24 or 2n = 30 chromosomes (Beçak 1968; Rabello 1970; Bogart 1973; Kuramoto 1990; Anderson 1991; Skuk and Langone 1992; Baldissera et al. 1993; Kaiser et al. 1996), which suggests a dichotomy within the genus *Hyla,* despite the fact that there are also other diploid numbers such as 2n = 18, 20, 26, 30, 32 and 34 (Kuramoto 1990; Baldissera et al. 1993). According to Miura (1995), the appearance of *Hyla* species with 2n = 24 chromosomes can be related to a common ancestor with 2n = 26, and Bogart (1973) suggested that species with morphologically similar karyotypes can be considered to share a common ancestor. One of the possible mechanism to explain the change from 2n = 26 to 2n = 24 chromosomes may be related to centric fusion (Morescalchi 1990). The *Hyla* species with 2n = 18, 20 and 22 chromosomes probably had their origin in the karyotype with 2n = 24 chromosomes (Bogart 1973). For the 30-chromosomes *Hyla*, centric dissociation probable is responsible for the increase in number and pericentric inversion have shifted the position of the centromeres in many cases (Bogart 1973; King 1990). As stated by Bogart (1973) the 30-chromosomes *Hyla* and the 24-chromosome *Hyla* were probably independently derived from a 26-chromosome ancestor.

Despite the differences in external morphology among *H. semiguttata* populations, *H. marginata* and *Hyla* sp. (aff. *semiguttata*), these species show a very conserved chromosomal morphology. The karyotypes of *H. marginata*, *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*) were very similar to other species of the *pulchella* group (*H. p. pulchella, H. caingua, H. prasina, H. p. joaquini*) (Baldissera et al. 1993; Ananias 1996) and to *H. guentheri* and *H. bischoffi* (Raber 2000), as well as to some neotropical and holoartic *Hyla* species (Bogart 1973; Anderson 1991).

Comparison of the constitutive heterochromatin pattern of the three species studied with those previously described for the *pulchella* group and related species revealed that there were some common C-bands in most of them. *H. prasina* (Baldissera et al. 1993; Ananias 1996), *H. p. joaquini* (Ananias 1996), *H. guentheri* and *H. bischoffi* (Raber 2000) had the same telomeric band in pair 1 also found in

Table 2. *Morphometric data of mitotic chromosomes of Hyla semiguttata, Hyla sp. (aff. semiguttata) and H. marginata.*

Chromosome number												
	1	2	3	4	5	6	7	8	9	10	11	12
H. semiguttata (São Francisco de Paula, Brasil)												
AR ± SD	1.15 ± 0.01	1.58 ± 0.15	2.70 ± 0.03	3.26 ± 0.06	2.50 ± 0.23	4.16 ± 0.20	1.85 ± 0.12	1.10 ± 0.10	1.86 ± 0.03	1.73 ± 0.14	1.10 ± 0.02	1.05 ± 0.02
CI ± SD	0.48 ± 0.01	0.39 ± 0.02	0.30 ± 0.01	0.23 ± 0.03	0.31 ± 0.02	0.19 ± 0.01	0.34 ± 0.01	0.49 ± 0.02	0.35 ± 0.03	0.36 ± 0.01	0.49 ± 0.02	0.49 ± 0.05
H. semiguttata (Cambará do Sul, Brasil)												
AR ± SD	1.10 ± 0.09	1.60 ± 0.05	3.00 ± 0.10	3.35 ± 0.02	2.70 ± 0.05	4.18 ± 0.03	1.87 ± 0.05	1.10 ± 0.02	1.83 ± 0.01	1.74 ± 0.04	1.07 ± 0.03	1.03 ± 0.03
CI ± SD	0.48 ± 0.05	0.39 ± 0.01	0.31 ± 0.01	0.23 ± 0.05	0.30 ± 0.05	0.19 ± 0.01	0.34 ± 0.02	0.49 ± 0.01	0.35 ± 0.05	0.37 ± 0.02	0.48 ± 0.01	0.49 ± 0.05
H. semiguttata (Piracuara, Brasil)												
AR ± SD	1.11 ± 0.04	1.61 ± 0.02	2.90 ± 0.05	3.30 ± 0.04	2.65 ± 0.10	4.15 ± 0.07	1.86 ± 0.08	1.10 ± 0.07	1.87 ± 0.01	1.73 ± 0.04	1.08 ± 0.05	1.04 ± 0.03
CI ± SD	0.49 ± 0.02	0.39 ± 0.01	0.31 ± 0.03	0.23 ± 0.02	0.29 ± 0.06	0.19 ± 0.02	0.36 ± 0.03	0.49 ± 0.03	0.36 ± 0.02	0.36 ± 0.05	0.50 ± 0.01	0.49 ± 0.03
Hyla sp. (aff. *semiguttata*) (Misiones, Argentina)												
AR ± SD	1.12 ± 0.03	1.63 ± 0.02	3.00 ± 0.03	3.40 ± 0.05	2.85 ± 0.20	4.16 ± 0.10	1.90 ± 0.01	1.10 ± 0.05	1.92 ± 0.10	1.72 ± 0.05	1.06 ± 0.02	1.03 ± 0.02
CI ± SD	0.50 ± 0.09	0.40 ± 0.02	0.32 ± 0.05	0.26 ± 0.01	0.30 ± 0.05	0.19 ± 0.03	0.37 ± 0.05	0.49 ± 0.01	0.36 ± 0.01	0.37 ± 0.10	0.48 ± 0.01	0.49 ± 0.05
H. marginata (São Francisco de Paula, Brasil)												
AR ± SD	1.14 ± 0.04	1.65 ± 0.05	3.00 ± 0.15	3.40 ± 0.06	2.85 ± 0.16	4.17 ± 0.10	1.86 ± 0.12	1.10 ± 0.10	1.90 ± 0.19	1.72 ± 0.05	1.11 ± 0.03	1.05 ± 0.02
CI ± SD	0.49 ± 0.01	0.38 ± 0.02	0.31 ± 0.03	0.25 ± 0.01	0.30 ± 0.02	0.19 ± 0.01	0.34 ± 0.05	0.48 ± 0.02	0.35 ± 0.02	0.37 ± 0.03	0.49 ± 0.02	0.48 ± 0.01
Type	m	m	sm	st	sm	st	sm	m	sm	sm	m	m

AR = arm ratio; CI = centromeric index; SD = standard deviation; m = metacentric, sm = submetacentric and st = subtelocentric.

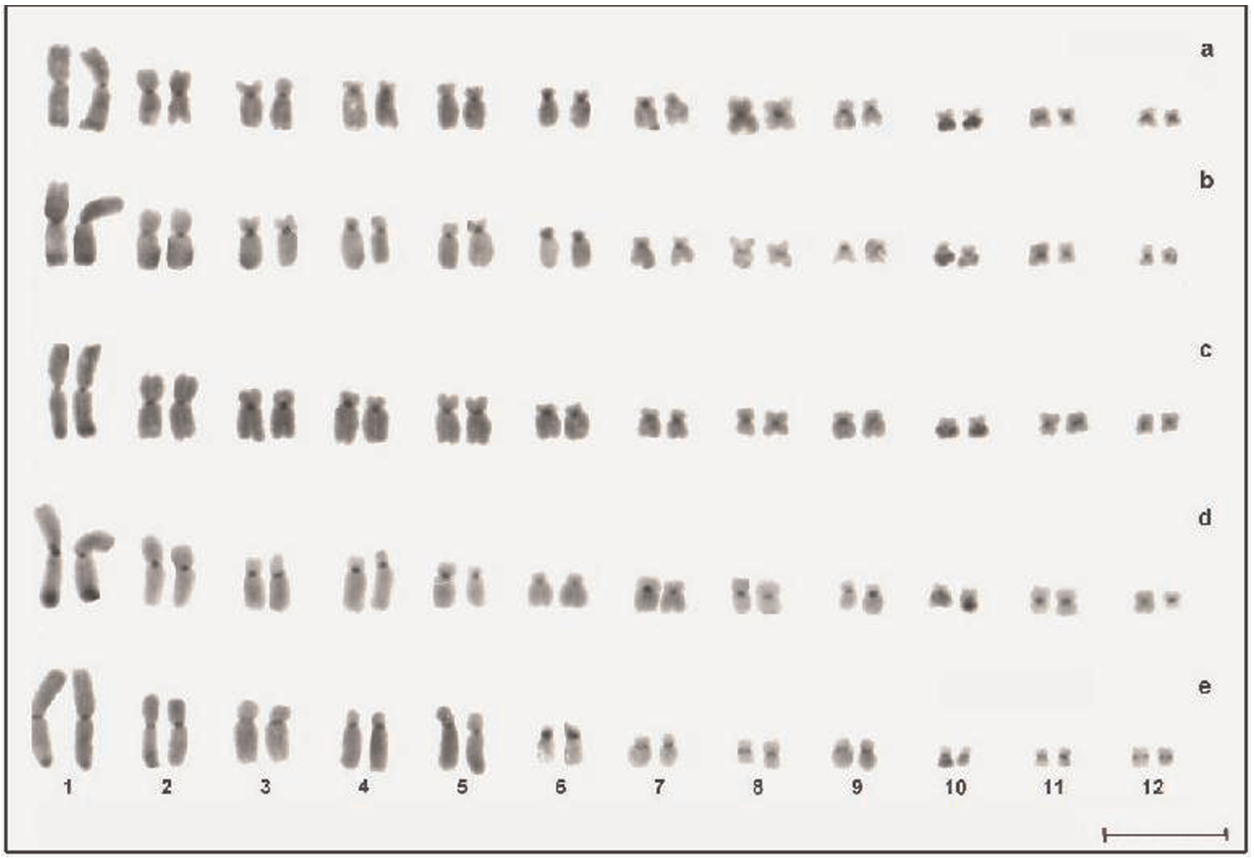

Fig. 2a–e. C-banded karyotypes of *H. marginata* (**a**), *H. semiguttata* from São Francisco de Paula, RS. (**b**), Cambará do Sul, RS. (**c**) and Piracuara, PR. (**d**) and *Hyla* sp. (aff. *semiguttata*) (**e**). Bar = 5 μm.

H. marginata, *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*). However, this band is absent in *H. caingua* and *H. p. pulchella* (Ananias 1996).

Although *H. marginata*, *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*) are morphologically different, their constitutive heterochromatin patterns were the same, confirming that they are very closely related. In addition, *H. p. joaquini* (São Joaquim – type location) (Ananias 1996) had the same heterochromatin pattern observed in *H. marginata*, *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*), suggesting that they are more closely related to each other than to other species of the group. Kasahara et al. (1996) reported similar results in three species of *Bufo* which had striking morphological differences but indistinguishable C-band pattern, typical of species of the *marinus* group, but different from species in other groups (Schmid 1978, 1980, 1982; Matsui et al. 1985; Schmid and Almeida 1988; Schmid and Guttenbach 1988; Herrero et al. 1993). *H. marginata* differed from *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*) in the localization of the NOR. The

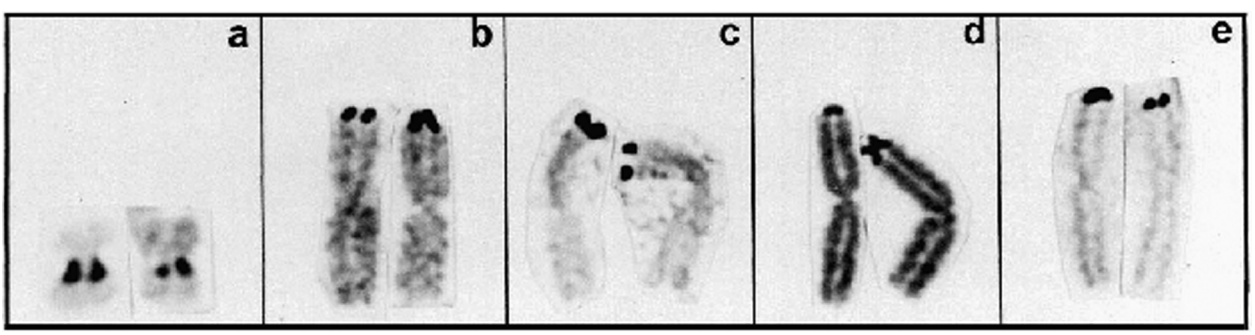

Fig. 3a–e. Silver-stained NOR of *H. marginata* (**a**), *H. semiguttata* from São Francisco de Paula, RS. (**b**), Cambará do Sul, RS. (**c**) and Piracuara, PR. (**d**) and *Hyla* sp. (aff. *semiguttata*) (**e**).

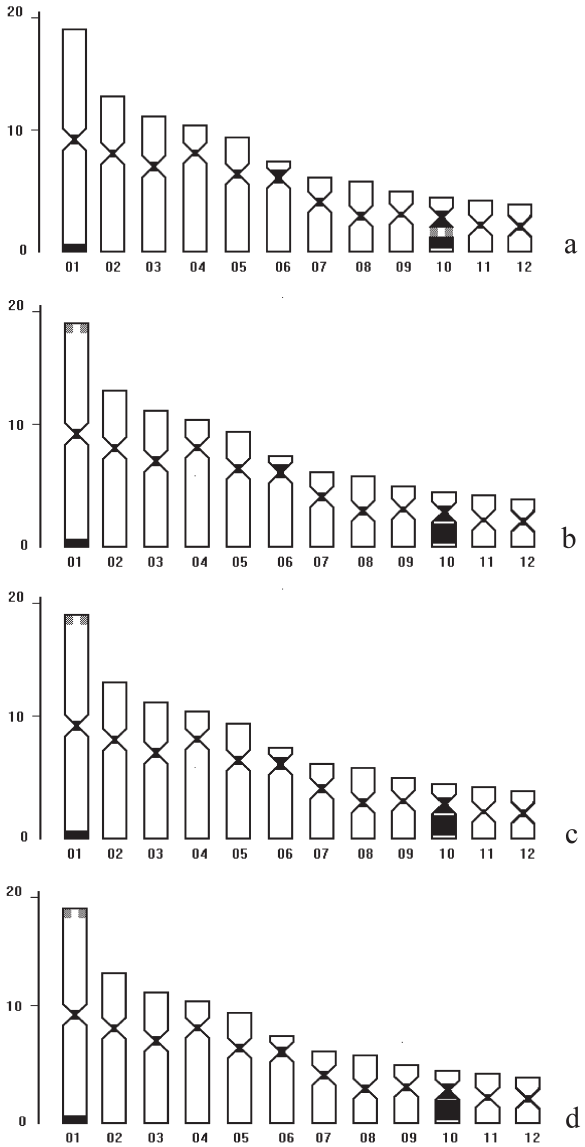

Fig. 4a–d. Idiograms of *H. marginata* (**a**), *H. semiguttata* (**b**) and *Hyla* sp. (aff. *semiguttata*) (**c**) (analyzed in the present work) and *H. p. joaquini* (**d**) (Ananias et al., unpubl.). Solid blocks denote heterochromatin, opened areas represent the secondary constriction and shaded circles denote NORs.

Ag-NOR staining in the telomere of pair 1 in *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*) and in the near of pericentromeric area of pair 10 in *H. marginata* may have arisen through translocations. *H. p. joaquini* also had an NOR in the telomeric region of pair 1, suggesting a similarity to *H. semiguttata* and *Hyla* sp. (aff. *semiguttata*).

According to Garcia and Haddad (1999), there are differences in the advertisement call among the three populations of *H. semiguttata.* However, cytogenetic analysis of these populations provides no evidence to support the hypothesis of their belonging to different *taxa*. On the other hand, the synonymization of *H. semiguttata* and *H. p. joaquini* to *H. marginata*, as suggested by Langone (1997), was not confirmed since only *H. semiguttata* and *H. p. joaquini* can be mistaken cytogenetically.

In conclusion, the biological differences within the *Hyla* species and populations of *H. semiguttata* studied here were not reflected in the chromosomal structure of these species, even though Goldman and Barton (1992) suggested that genetic changes should be seen in the populational structure and should influence speciation and diversification. However, this lack of chromosomal variation does not mean that there is currently no speciation in progress. Our results also show that the relationship among *H. marginata*, *H. p. joaquini* and *H. semiguttata* and their populations may be better understood through molecular DNA analysis.

Acknowledgements – The authors thank Dr. Célio F. B. Haddad for his help in collecting some specimens and Klélia Aparecida de Carvalho for her technical expertise. P.C.A.G. was supported by a scholarship from FAPESP (process No. 98/06087-9).

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