Evaluating the Karyotypic Diversity in Species of *Hyla* (Anura; Hylidae) with 2n = 30 Chromosomes Based on the Analysis of Ten Species

(karyotype / chromosome banding / taxonomy / amphibian)

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Abstract. Ten species of *Hyla* with 2n = 30 from Brazilian fauna were analysed cytogenetically. *Hyla minuta* is the unique presenting all bi-armed metacentric or submetacentric chromosomes in the karyotype, therefore, with the highest FN = 60. The remaining species have a variable number of uni-armed telocentric or subtelocentric chromosomes: *H. cruzi*, *H. elianeae*, and *H. rubicundula* with three pairs (FN = 54), *H. berthalutzae*, *H. elegans*, *H. microps*, and *H. nana* with four pairs (FN = 52), and *H. nahdereri* and *H. sanborni* with five pairs (FN = 50). The uni-armed elements are among pairs 5, 6, 7, 11, 14, and 15, which also appeared with metacentric or submetacentric morphology. The remaining chromosome pairs 1, 2, 3, 4, 8, 9, 10, 12, and 13 were never found to be telocentric or subtelocentric. AgNOR patterns are species-specific, the majority of the species exhibiting a single pair with AgNORs, with the exception of *H. elegans* and *H. nana* with more than one chromosome pair bearing this cytological marker. C banding was obtained in *H. berthalutzae*, *H. cruzi*, *H. elegans*, *H. elianeae*, *H. microps*, *H. minuta*, *H. nahdereri*, and *H. nana*, which showed positively stained centromeric heterochromatin. Our analysis confirms the great karyotypic diversity in the species of *Hyla* with 2n = 30, with no species sharing identical karyotypes.

Among the Anurans, the family Hylidae is one of the most diversified, with about 880 species distributed in four subfamilies (Frost, 2004). In the subfamily Hylinae, the genus *Hyla* alone includes more than 330 representatives, which correspond to about 40% of all known species of hylids. According to the compilation made by King (1990), Kuramoto (1990), and, more recently by Gruber (2002), less than a hundred of species were karyotyped and these studies were, in general, limited to the analyses of mitotic or meiotic chromosomes with standard staining.

The genus *Hyla* has been commonly referred to as conservative, because most of the species share very similar karyotypes, with 2n = 24 and invariable fundamental number of chromosome arms FN = 48. Nevertheless, this is not true for the other group of *Hyla*, which includes species with 2n = 30 chromosomes. In fact, the species analysed thus far (Beçak, 1968; Rabello, 1970; Foresti, 1972; Bogart, 1973; Anderson, 1991; Skuk and Langone, 1992; Kaiser et al., 1996; Medeiros et al., 2003), although conservative in diploid number, have quite variable FN, from 50 to 60, due to a distinct number of bi-armed and uni-armed chromosomes in their karyotypes. Additionally, the idea of variability is highlighted because the species, even those with coincident FN, have uni-armed elements not always corresponding to the same chromosome pairs.

Taking into account the relatively small number of species of *Hyla* with 2n = 30 analysed with banding techniques, the diversity of their karyotype constitution and some unsolved taxonomic and systematic questions, mainly due to the occurrence of cryptic species (Bogart, 1973), we studied cytogenetically a sample of 10 species, some of them for the first time, using standard staining, silver-stained nucleolar organizer region (AgNOR), and C-banding techniques. The obtained data were important to evaluate the extension of the karyotypic variability in the group of *Hyla* with 2n = 30.

Material and Methods

Cytogenetic analysis was performed in *Hyla berthalutzae* Bokermann, *H. cruzi* Pombal and Bastos, *H. elegans* Wied-Neuwied, *H. elianeae* Napoli and Caramaschi, *H. microps* Peters, *H. minuta* Peters, *H. nahdereri* B. Lutz and Bokermann, *H. nana* Boulenger, *H. rubicundula* Reinhardt and Lütken, and *H. sanborni* Schmidt, totalling a sample of 35 specimens, collected in the states of Santa Catarina (SC) and Paraná (PR), both in the Southern region, São Paulo (SP), in the Southeastern, and Goiás (GO), in Central Brazil (Table 1). The

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Abbreviations: AgNOR(s) – silver-stained nucleolar organizer region(s), FN – fundamental number, GO – Goiás, PR – Paraná, SC – Santa Catarina, SP – São Paulo.

Table 1. Species, number and sex, voucher number, and collection locality of the animals

Species	Number and sex	Voucher number	Collection locality
Hyla berthalutzae	1 M	CFBH 7101	Guaraqueçaba, PR
Hyla cruzi	4 M	CFBH 7093-96	Mossâmides, GO
Hyla elegans	3 M	CFBH 7118-20	Ubatuba, SP
Hyla elianeae	5 M	CFBH 7078-79, 7115-17	Rio Claro, SP
Hyla microps	1 M	CFBH 7103	Campos do Jordão, SP
	1 M	CFBH 7104	Ribeirão Branco, SP
	5 M	CFBH 7080-84	São Bento do Sul, SC
Hyla minuta	3 M	CFBH 7097, 7099, 7111	Rio Claro, SP
	1 M	CFBH 7121	Ubatuba, SP
Hyla nahdereri	1 F	CFBH 7107	Rancho Queimado, SC
Hyla nana	2 M	CFBH 7112-13	Rio Claro, SP
Hyla rubicundula	2 M	CFBH 7340, 7397	São João d'Aliança, GO
Hyla sanborni	6 M	CFBH 7085, 7087, 7091-92, 7102, 7122	Rio Claro, SP

M – male, F – female

voucher specimens were deposited in the amphibian collection (CFBH) of the Departamento de Zoologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Rio Claro, SP, Brazil.

Chromosome spreads were prepared directly from bone marrow and liver (Baldissera Jr. et al., 1993) or from intestine (Schmid, 1978). Mitotic chromosomes of *H. nahdereri* were obtained from lymphocytes cultured by the method described in Kasahara et al. (1998). Standard staining was performed with 3% Giemsa solution in phosphate-buffered saline, pH 6.8, and AgNOR and C-banding techniques, according to Howell and Black (1980) and Sumner (1972), respectively. For the majority of the species, the AgNOR technique was performed sequentially to standard staining or after C banding. The bi-armed chromosomes were classified as metacentric or submetacentric, whereas the uni-armed, as subtelocentric or telocentric, by visual inspection. Due to very subtle decreasing in the lengths of the chromosomes in the group of *Hyla* with 2n = 30, with almost no difference in their sizes, the uni-armed chromosomes were arranged in a way to introduce the least amount of variation in the karyotypes of the species.

Results

Description of the karyotypes

The karyograms of the ten species of *Hyla* are presented in Fig. 1, Fig. 2, and Fig. 3. The chromosome morphology in each species and the corresponding FN are indicated in Table 2. With the exception of *H. minuta* with all the chromosomes of metacentric and submetacentric types (FN = 60), the remaining species have telocentric or subtelocentric chromosomes: three pairs in *H. cruzi*, *H. elianeae*, and *H. rubicundula* (FN = 54), four pairs in *H. berthalutzae*, *H. elegans*, *H. microps*, and *H. nana* (FN = 52) or five pairs in *H. nahdereri* and *H. sanborni* (FN = 50).

Specimens of *Hyla microps* from more than one locality were analysed, showing a slight difference in their karyotypes (Fig. 2A and Fig. 2B). The specimen from Campos do Jordão, SP, has four telocentric pairs (5, 6, 7, and 15) instead of three (5, 6, and 15) as found in the animals from São Bento do Sul, SC, and Ribeirão Branco, SP, which on the other hand possess subtelocentric pair 7.

In some species, secondary constriction was observed sporadically in one or in both of the homologues of a unique chromosome pair. Less frequently, it was seen in chromosomes belonging to distinct pairs, like in *H. minuta* from Rio Claro, SP, which exhibited secondary constriction in the two chromosomes 13 and, additionally, in one of the homologues of pairs 3 and 12 (Fig. 2C).

No heteromorphic pair, suggestive of a mechanism of sex-chromosome determination, was noticed in any of the karyotyped specimens.

AgNOR staining

For the majority of the species, there is a single pair of AgNORs (Table 2, Fig. 1, Fig. 2, Fig. 3, and Fig. 4). AgNOR in the pair 1 was observed in *H. nahdereri* (interstitial short arms); in the pair 7 in *H. rubicundula* (interstitial short arms); in the pair 10 in *H. microps* (telomeric short arms); in the pair 11 in *H. elianeae* (telomeric short arms); in the pair 13 in *H. minuta* from Rio Claro, SP (interstitial long arms) and from Ubatuba, SP (telomeric long arms); in the pair 14 in *H. cruzi* (interstitial short arms) and in *H. sanborni* (interstitial long arms); and in the pair 15 in *H. berthalutzae* (telom-

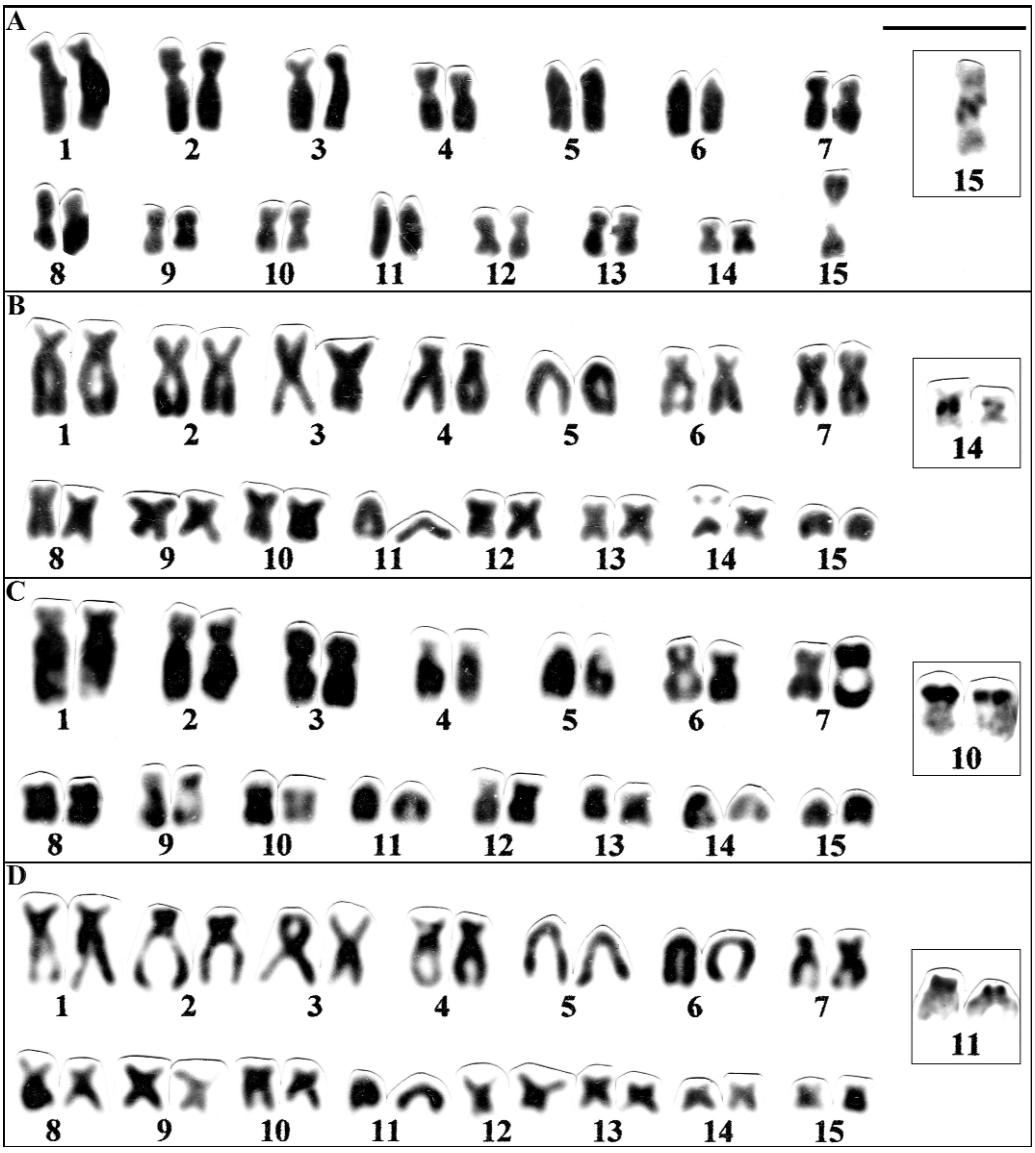

Fig. 1. Standard stained karyotypes. Insets: chromosome pairs from the same metaphases after sequential AgNOR staining. (A) *H. berthalutzae*: AgNOR-bearing chromosomes of pair 15 in association, (B) *H. cruzi*: AgNOR in chromosome pair 14, (C) *H. elegans*: AgNOR in chromosome pair 10, (D) *H. elianeae*: AgNOR in chromosome pair 11. Bar = 10 µm.

eric short arms). The specimens of *H. microps* from Campos do Jordão, SP, and Ribeirão Branco, SP, showed two AgNORs in all metaphases (Fig. 2A), whereas the representatives of this species from São Bento do Sul, SC, exhibited just one stained AgNOR in all metaphases (Fig. 2B).

Hyla elegans (Fig. 1C and Fig. 4B) and *H. nana* (Fig. 3B) exhibited more than one chromosome pair bearing AgNOR. In the former species, AgNORs are found in pairs 10 (telomeric short arms) and 14 (telomeric long arms). In a sample of 35 metaphases from three specimens, the number of AgNORs varied from 1 to 4, according to six distinct patterns: 10 (4 metaphases); 10,10 (9 metaphases); 10,10,14 (16 metaphases); 10,14 (2 metaphases); 10,14,14 (1 metaphase); and 10,10,14,14 (3 metaphases). *H. nana* presents AgNORs in pair 13 (telomeric long arms) and in one of the chromosomes 1 (interstitial short arms). In a sample of 17 metaphases from two specimens, one to three AgNORs per metaphase were noticed, according to four distinct patterns: 13 (6 metaphases); 13,13 (8 metaphases); 1,13,13 (2 metaphases); and 1,13 (1 metaphase).

The majority of the secondary constrictions seen after standard staining corresponded to AgNORs, with the exception of those of the chromosomes 3 and 12 of *H. minuta*.

C banding

C-banding patterns were obtained in *H. berthalutzae*, *H. cruzi*, *H. elegans*, *H. elianeae*, *H. microps* from São

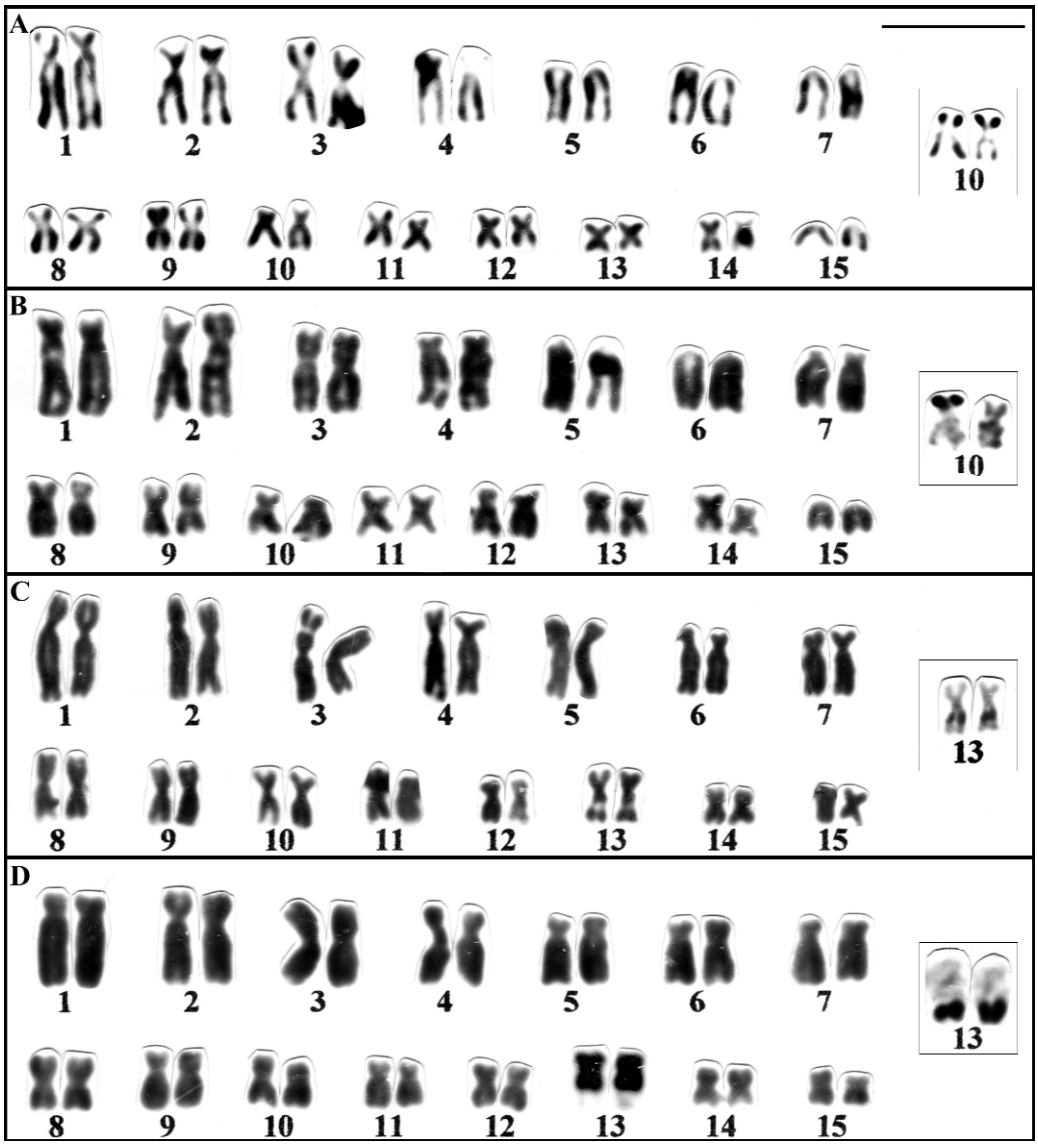

Fig. 2. Standard stained karyotypes. Insets: chromosome pairs from the same metaphases after sequential AgNOR staining (A, C, and D) or from another metaphase with AgNOR (B). (A and B) *H. microps* from Campos do Jordão, SP, and from São Bento do Sul, SC, respectively: AgNOR in both (A) or in one of the homologues (B) of pair 10, (C and D) *H. minuta* from Rio Claro, SP, and Ubatuba, SP, respectively: AgNOR at interstitial (C) and telomeric (D) regions in the long arms of the chromosome pair 13. Bar = 10 μm.

Bento do Sul, SC, *H. minuta* from Rio Claro, SP, and Ubatuba, SP, *H. nahdereri*, and *H. nana*. In all these species, C bands were in the centromeric region of the chromosomes; besides, C-positive staining was occasionally observed in the site of the NOR. Fig. 4 presents the C-banded karyotypes in three of the species.

Discussion

In contrast to what is usually observed in the order Anura, the group of *Hyla* with 2n = 30 is karyologically quite diversified, the majority of the species presenting a variable number of uni-armed chromosomes. Although five is the maximum number of telocentric or subtelocentric pairs ever found in the species, these chromosomes have been described as pairs 3, 5, 6, 7, 8, 10, 11, 12, 13, 14 and 15 in the karyotypes analysed thus far. This fact prompted us to suppose that the karyotypic diversity may be, to a certain extent, due to the difficulties to determine the position of the chromosomes in the karyograms, because they are relatively uniform in size, varying slightly in their lenghts. For this reason, in the construction of the karyograms, we adopted a parsimonious distribution of the uni-armed chromosomes, and our comparative analyses with previous data were performed following the same criterion, in order to ensure a more objective evaluation of the karyotypic variability in the group of *Hyla* with 2n = 30.

According to our analysis, two size groups of uni-armed chromosomes could be recognized. Those of

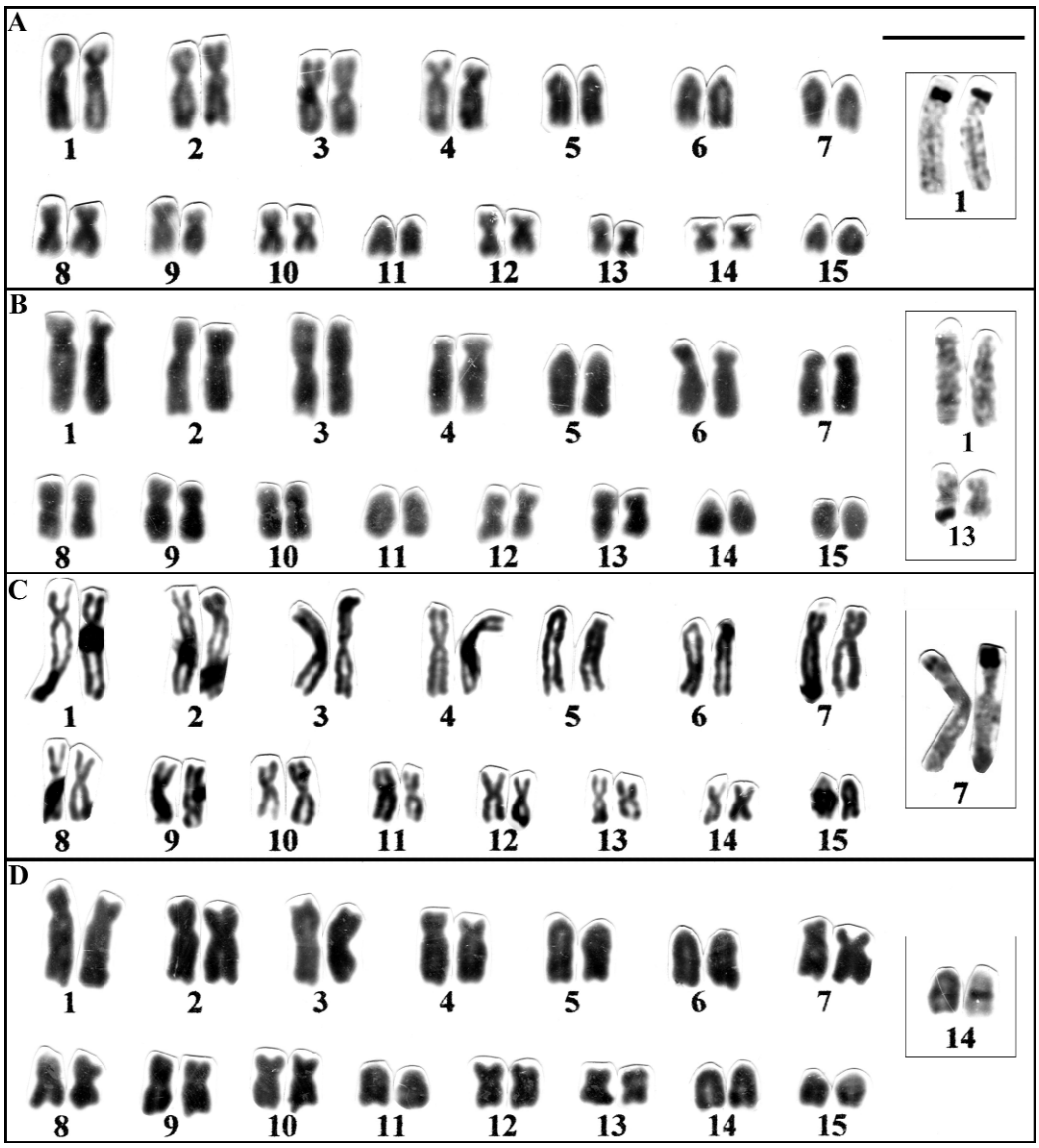

Fig. 3. Standard stained karyotypes. Insets: chromosome pairs from other metaphases with AgNOR (A and C) or from the same metaphases after sequential AgNOR staining (B and D). (A) *H. nahdereri*: AgNOR in chromosome pair 1, (B) *H. nana*: AgNOR in one of the homologues of pair 13, (C) *H. rubicundula*: AgNOR in chromosome pair 7, (D) *H. sanborni*: AgNOR in chromosome pair 14. Bar = 10 μm.

medium size could be arranged as pairs 5, 6 or 7, whereas the small-sized ones, as pairs 11, 14 or 15, such positions corresponding, in general, to the most frequently ascribed to the species karyotyped before. These chromosome pairs also occurred with submetacentric morphology. Several species have coincident FN, and only *H. elegans* and *H. nana* present the same pattern of uni-armed chromosome distribution, but their karyotypes differ by the morphology of pair 12 and by the position of AgNORs. The chromosome pairs 1, 2, 3, 4, 8, 9, 10, 12, and 13 have a relatively uniform morphology of metacentric or submetacentric types, and they were never found to be telocentric or subtelocentric in the karyotypes. Nevertheless, while seven species have each of these chromosomes with a coincident morphology, a slight discrepancy was observed in pairs 3, 10, and 12 of *H. berthalutzae*, *H. microps,* and *H. elegans*, respectively, because they are submetacentric and not metacentric, like in the majority of the species. Therefore, no species of *Hyla* in the sample was observed sharing identical karyotypic constitution.

Hyla cruzi, *H. elegans*, *H. nahdereri*, and *H. elianeae* were karyotyped for the first time. The remaining six species have been analysed before, few of them with banding techniques, and, at first sight, the karyotypes are discrepant in the distinct samples of the same species. Nevertheless, the karyotype of *H. minuta* may be matched with those described by Rabello

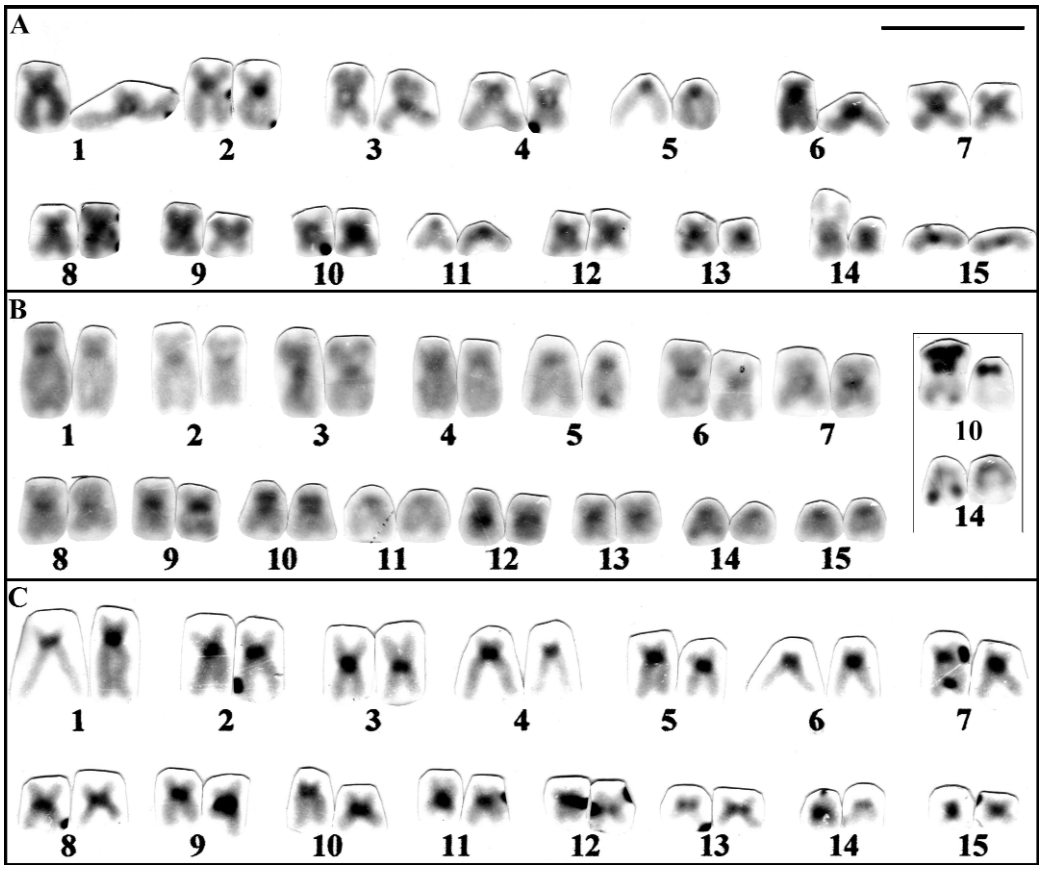

Fig. 4. C-banded karyotypes. (A) *H. cruzi*, (B) *H. elegans*. Inset: chromosome pairs 10 and 14 from the same metaphase after sequential AgNOR staining, with AgNOR in both chromosomes 10 and in one of the homologues of pair 14, (C) *H. minuta* from Rio Claro, SP. Bar = 10 μm.

(1970) and Bogart (1973), for specimens collected in Brazil and also in Peru, in the case of the latter author. The same is true for *H. berthalutzae*, with the same karyotype constitution of the Brazilian specimens from the states of Espírito Santo and Paraná (Foresti, 1972; Skuk and Langone, 1992), despite that the former author had found 2n = 32, due to an extra small pair, probably corresponding to supernumerary chromosomes. All the specimens of *H. microps* from Campos do Jordão, SP (present sample; Beçak, 1968) and from a not mentioned locality of Southeastern Brazil (Bogart, 1973) have similar karyotypes, differing slightly from that of the specimens from São Bento do Sul, SC, and Ribeirão Branco, SP, by a telocentric chromosome pair 7. In these two latter samples, the chromosomes 7 are subtelocentric, exhibiting tiny but characteristically enlarged short arms. Certainly, new populations of *H. microps* should be investigated, to confirm the possibility of geographical karyotypic differences in the species.

Representatives of the sister species *H. nana* and *H. sanborni* from distinct localities have been analysed frequently. The karyotype constitution of *H. nana* may correspond to those described by Rabello (1970) and Medeiros et al. (2003) for specimens from Brazil (locality not mentioned and from the state of São Paulo, respectively), and by Skuk and Langone (1992) for animals from Argentina. The same is true for *H. sanborni,* whose karyotype matches that found previously in another locality of the state of São Paulo (Medeiros et al., 2003). In the past, *H. nana* and *H. sanborni* were confused with each other, but currently they can be separated by their morphology, advertisement calls, and unequivocally by their karyotypes, by possessing four and five pairs of telocentrics, respectively, and also by a quite distinct AgNOR pattern. Medeiros et al. (2003) pointed to an apparently discrepant karyotype found, respectively, by Bogart (1973) for *H. nana* from Argentina, with five telocentric pairs, and by Skuk and Langone (1992) for *H. sanborni* from the state of Paraná, Brazil, with four telocentric pairs, as a consequence of misidentification of the species, although the possibility of interpopulational variation has not been ruled out. We agree with the first presumption, so that the species would correspond, in fact, to *H. sanborni* in the first sample and to *H. nana* in the second, with

Table 2. Chromosome morphology, FN, and AgNOR-bearing chromosomes in Hyla species with 2n = 30 chromosomes

Species	M	S	St	T	FN	AgNOR
H. berthalutzae	8, 9, 10, 12, 13, 14	1, 2, 3, 4, 7		5, 6, 11, 15	52	15
H. cruzi	3, 8, 9, 10, 12, 13, 14	1, 2, 4, 6, 7		5, 11, 15	54	14
H. elegans	3, 8, 9, 10, 13	1, 2, 4, 6, 7, 12		5, 11, 14, 15	52	10, 14
H. elianeae	3, 8, 9, 10, 12, 13, 14, 15	1, 2, 4, 7		5, 6, 11	54	11
H. microps[1]	3, 8, 9, 11, 12, 13, 14	1, 2, 4, 10		5, 6, 7, 15	52	10
H. microps[2]	3, 8, 9, 11, 12, 13, 14	1, 2, 4, 10	7	5, 6, 15	52	10
H. minuta	3, 8, 9, 10, 11, 12, 13, 14, 15	1, 2, 4, 5, 6, 7			60	13
H. nahdereri	3, 8, 9, 10, 12, 13, 14	1, 2, 4		5, 6, 7, 11, 15	50	1
H. nana	3, 8, 9, 10, 12, 13	1, 2, 4, 6, 7		5, 11, 14, 15	52	1, 13
H. rubicundula	3, 8, 9, 10, 11, 12, 13, 14	1, 2, 4, 7		5, 6, 15	54	7
H. sanborni	3, 8, 9, 10, 12, 13	1, 2, 4, 7		5, 6, 11, 14, 15	50	14

M – metacentric, S – submetacentric, St – subtelocentric, T – telocentric
[1]Campos do Jordão, SP
[2]São Bento do Sul, SC, and Ribeirão Branco, SP

apparently no geographical differences in the karyotype constitution of each species, at least in relation to the chromosome data analysed with conventional staining.

Based on the morphological traits and geographical distribution, *H. elianeae* was described as a new species by Napoli and Caramaschi (2000), but it was referred to before as *H. rubicundula*, which on the other hand replaces the erroneous name of *H. elongata* A. Lutz. Our present data on *H. elianeae* and *H. rubicundula* confirm that they can also be identified by their karyotypes because, although exhibiting three pairs of telocentrics, one of them is not coincident, corresponding to the pairs 5, 6, and 11 in the former and to the pairs 5, 6, and 15 in the second species; additionally, the species have AgNORs located in distinct chromosome pairs, 11 and 7, respectively. The karyotype of *H. rubicundula* differs from that presented by Rabello (1970), who described four pairs of uni-armed chromosomes in a male collected in a not specified locality in Brazil. Although karyotypic differences cannot be ruled out, it is important to consider the possibility of misidentification, taking into account that the group of *H. rubicundula* is taxonomically confused, because the species are phenotypically very similar (Napoli and Caramaschi, 1999).

As usual among anurans, the majority of the species of *Hyla* with 2n = 30 have a single pair of AgNORs, generally, located in medium or small-sized chromosomes (Anderson, 1991; Kaiser et al., 1996; Medeiros et al., 2003; present study). It is interesting to remark that few species of our sample have the same chromosomes bearing AgNOR. In the cases in which this cytological marker is in homeologous pairs, AgNORs are not at the same chromosome sites. If coincident, as observed in the chromosomes 1 of *H. nahdereri* and *H. nana*, and in the chromosomes 10 of *H. elegans* and *H. microps*, the species have a distinct number of chromosome pairs bearing AgNORs. This means that each species of our sample is also distinguished unequivocally by the AgNOR patterns, which, therefore, can be considered as species-specific. This finding reinforces that none of the ten species of *Hyla* has identical karyotypes. The distinct sites of AgNORs in the chromosomes 13 of *H. minuta* from two localities might be indicative of geographical differentiation of the karyotypes, but a greater number of specimens should be analysed to confirm or not this possibility. Intraspecific geographical variation is also possible for *H. sanborni,* because in our sample the AgNORs are clearly in the interstitial region of telocentrics, whereas in the specimens from another locality, in the telomeric region (Medeiros et al., 2003).

C-banding patterns, obtained for some species of our sample, confirm previous data of other authors (Anderson, 1991; Kaiser et al., 1996; Medeiros et al., 2003), who also observed heterochromatin predominantly distributed in the centromeric region of the chromosomes. Nevertheless, the presence of additional telomeric or interstitial C bands, as already described in *H. microcephala* and *H. phlebodes* (Kaiser et al., 1996), may be useful to better characterize species or group of species, as well as distinct geographical races of *Hyla* with 2n = 30.

Cytogenetic data of 17 other species of *Hyla* with 2n = 30 are available in the literature (Foresti, 1972; Bogart, 1973; Anderson, 1991; Skuk and Langone, 1992; Kaiser et al., 1996): *H. anceps* A. Lutz, *H. baileyi* Cochran, *H. bipunctata* Spix, *H. branneri* Cochran, *H. decipiens* A. Lutz, *H. ebraccata* Cope, *H. elongata*, *H. labialis* Peters, *H. leali* Bokermann, *H. leucophyllata* (Beireis), *H. marmorata* (Laurenti), *H. meridiana* B. Lutz, *H. microcephala* Cope, *H. oliverai* Bokermann, *H.*

parviceps Boulenger, *H. phlebodes* Stejneger, and *H. rhodopepla* Günther. Four of them (*H. anceps, H. bipunctata*, *H. decipiens*, and *H. microcephala*) have more than one described karyotype, with a distinct number of telocentrics, suggesting geographical karyotypic variation or even misidentification of the species. Only specimens of *H. leali* and *H. microcephala* were described with all bi-armed chromosomes and FN = 60, which is considered the most derived condition among the *Hyla* with 2n = 30 (Bogart, 1973). The remaining species, corresponding to 19 distinct karyotypes, showed FN = 58 (*H. anceps*, *H. bipunctata, H. ebraccata*, *H. elongata*, *H. microcephala*, and *H. rhodopepla*), FN = 56 (*H. baileyi, H. bipunctata, H. decipiens, H. meridiana,* and *H. phlebodes*), FN = 54 (*H. anceps, H. branneri, H. decipiens*, and *H. oliverai*), FN = 52 (*H. leucophyllata* and *H. parviceps*), and FN = 50 (*H. labialis* and *H. marmorata*) due to, respectively, a unique, two, three, four, and five uni-armed pairs. According to a preliminary evaluation, the uni-armed chromosomes in these karyotypes might also be distributed as pairs 5, 6, 7, 11, 14, and 15, if we follow the criterion adopted in the present study, independently to what was established by each author in the karyotype description.

The genus *Hyla*, including species with 2n = 30 and 2n = 24 chromosomes, is an artificial assemblage and, according to Bogart (1973), both groups were probably derived independently from a 2n = 26 chromosome ancestor. In the group with 2n = 30, centric dissociations might be one of the main rearrangements, responsible primarily for the increase in the chromosome number, but subsequent pericentric inversions may have shifted the centromere position, altering the morphology of some chromosome pairs. At present, it is not possible to establish any consistent pattern of chromosome evolution within the group of *Hyla* with 2n = 30, based on the number and position of the uni-armed chromosomes, because further analyses on a greater number of species, using more efficient cytological approaches to confirm the presumptive chromosome homeologies, are still necessary. Certainly, data on the karyotypic diversity may be useful to evaluate the relationships of the species of *Hyla*, along with the molecular phylogenetic analyses, which have been currently performed (Chek et al., 2001; Faivovich et al., 2004).

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