Patterns of ribosomal DNA distribution in hylid frogs from the *Hypsiboas faber* and *H. semilineatus* species groups

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Abstract

We performed a comparative analysis of the nucleolus organizer region (NOR) distribution in the karyotypes of hylid frogs from two different taxonomic groups, *Hypsiboas faber* and *H. semilineatus*. Silver nitrate staining of NORs (Ag-NORs) and fluorescence in situ hybridization (FISH) with a rDNA probe were used to investigate the chromosomal location of rDNA loci in two species. The karyotype of *H. semilineatus* and the Ag-NORs distribution of the four species are presented herein for the first time. After conventional staining, the four species presented very similar karyotypes with 2n = 24, but Ag-NORs analyses revealed species-specific characteristics. *H. albomarginatus*, *H. faber* and *H. semilineatus* had one pair of interstitial Ag-NORs in the short arm of pairs 2, 11, and in the long arm of pair 7, respectively. *H. pardalis* presented telomeric NORs in the long arm of pair 11. Ag-NORs were heteromorphic in three of the species (*H. pardalis*, *H. semilineatus* and *H. albomarginatus*) and FISH confirmed the differential activity of rDNA genes in *H. semilineatus*. In the present study, 2n = 24 karyotypes could be distinguished by their Ag-NORs distribution. Our results further the knowledge about the cytogenetics of hylids from Brazil.

Key words: hylids, *Hypsiboas*, karyotypes, FISH, Ag-NORs, *Hypsiboas semilineatus*, *H. faber*.

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two groups: the *H. semilineatus* group (*H. semilineatus*) and the *H. faber* group (*H. albomarginatus*, *H. faber* and *H. pardalis*). We also intended to verify if the Ag-NORs patterns were consistent with the monophyletic clades identified by molecular and morphological traits. We expected each species to present a distinctive Ag-NORs distribution which would be useful to distinguish individual species or species groups of hylids.

Our sample consisted of 13 specimens of species included in the hylid species groups *H. semilineatus* (*H. semilineatus*) and *H. faber* (*H. albomarginatus*, *H. faber* and *H. pardalis*). The animals were collected in four localities of the state of Espírito Santo, Brazil (Table 1). They were treated with a solution of 0.1% colchicine (1 mL/100 g of body weight) during 4-6 h prior to the sacrifice. Mitotic chromosome preparations were obtained from the intestine by the squash technique described by Bogart (1973a). Briefly, the intestine was immersed for 30 min in distilled water and then fixed in an ethanol/acid acetic solution. The intestine was fragmented with a scalpel onto a glass slide with a few drops of the fixative and then squashed with a glass coverslip. The slide was immersed in liquid nitrogen for about one minute and the coverslip was removed. The slide was then immersed in 90% ethanol for about one minute and air-dried. For conventional staining, the chromosome preparations were hydrolyzed in HCl 1N at 60 °C for 10 min and stained with a 3% Giemsa solution for 10 min. Ag-NORs staining followed Howell and Black (1980). Metaphases of each species were analyzed to determine the diploid number (2n) and the number of chromosome arms (fundamental number, FN). The metaphases were photographed and copies were made in Kodak photographic paper. Ten metaphases per species had their chromosomes measured (in millimeters) to determine the chromosome relative length (RL) and the centromeric ratio (CR), i.e. the proportion between the short and the long arms. We adopted the nomenclature for chromosome morphology proposed by Green and Sessions (1991), which is based on the centromeric ratio. Fluorescence in situ hybridization (FISH) was performed with the biotin-labeled probe HM123, which contains fragments of the 18S and 28S rDNA of *Xenopus leavis* (Meunier-Rotival et al., 1979). *H. semilineatus* and *H. pardalis* metaphases were hybridized with the probe following the protocol described by Viégas-Péquignot (1992).

All specimens exhibited 2n = 24 and FN = 48 (Figure 1a-d), but comparative analyses revealed some differences in the relative length (RL) and chromosome morphologies among the karyotypes of the four species, even though they were all exclusively composed of biarmed chromosomes (Figure 1). No sex chromosomes were observed in any of the karyotypes. The chromosomes varied gradually in size, with pair 1 corresponding to 18-19% and pair 12 corresponding to 2-3% of the genome. Pair 6 of *Hypsiboas faber* was slightly smaller than the same chromosome pair in the other species (Table 2).

The karyotype of *H. semilineatus* (HSE) is described herein for the first time. It is composed of five pairs of metacentric chromosomes (pairs 1, 2, 10, 11 and 12), three pairs of submetacentrics (pairs 4, 5 and 8) and four pairs of subtelocentrics (pairs 3, 6, 7 and 9).

### Table 1 - Analyzed specimens of *Hypsiboas* from the state of Espírito Santo, Brazil.

<table>
<thead>
<tr>
<th>Species group</th>
<th>Species</th>
<th>Animal</th>
<th>Sex¹</th>
<th>Municipality</th>
<th>Number of cells analyzed²</th>
<th>CS</th>
<th>Ag-NOR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>H. faber</em></td>
<td><em>H. albomarginatus</em></td>
<td>LGA 221</td>
<td>M</td>
<td>Anchieta</td>
<td>13</td>
<td>9</td>
<td>22</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>LGA 586</td>
<td>M</td>
<td>Cariacica</td>
<td>10</td>
<td>8</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LGA 587</td>
<td>M</td>
<td>Cariacica</td>
<td>14</td>
<td>6</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td><em>H. faber</em></td>
<td></td>
<td>LGA 202</td>
<td>M</td>
<td>Pedra Azul</td>
<td>14</td>
<td>7</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LGA 1178</td>
<td>M</td>
<td>Pedra Azul</td>
<td>10</td>
<td>4</td>
<td>14</td>
<td></td>
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<td><em>H. pardalis</em></td>
<td></td>
<td>LGA 209</td>
<td>M</td>
<td>Cariacica</td>
<td>22</td>
<td>6</td>
<td>28</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>LGA 208</td>
<td>M</td>
<td>Cariacica</td>
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<td>6</td>
<td>21</td>
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<td></td>
<td></td>
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<td>M</td>
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<td>5</td>
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<tr>
<td></td>
<td></td>
<td>LGA 289</td>
<td>M</td>
<td>Cariacica</td>
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<td>10</td>
<td>26</td>
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<tr>
<td><em>H. semilineatus</em></td>
<td><em>H. semilineatus</em></td>
<td>LGA 132</td>
<td>M</td>
<td>Santa Teresa</td>
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<td>7</td>
<td>22</td>
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<tr>
<td></td>
<td></td>
<td>LGA 133</td>
<td>M</td>
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<td></td>
<td></td>
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<td>ND</td>
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<td></td>
<td></td>
<td>LGA 148</td>
<td>ND</td>
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<td>10</td>
<td>5</td>
<td>15</td>
<td></td>
</tr>
</tbody>
</table>

Total 13 197 94 291

¹M = Male; ND = not determined; ²CS – number of cells analyzed after conventional staining; Ag-NORs – number of cells analyzed after Ag-NOR staining.
pair of subtelocentrics (pair 6). The karyotypes of *H. albomarginatus* from Espírito Santo were similar to those found in specimens from São Paulo identified as *Hyla albomarginata* by Beçak (1968).

*H. pardalis* (HPA) presented five pairs of metacentric chromosomes (pairs 1, 2, 9, 11 and 12), five pairs of submetacentrics (pairs 3, 5, 7, 8 and 10) and two pairs of subtelocentrics (pairs 4 and 6). The karyotype of *Hyla pardalis* from southeastern Brazil previously described by Bogart (1973b) is similar to the one that we observed of *Hypsiboas pardalis* from Espírito Santo.

*H. faber* (HFA) presented four pairs of metacentric chromosomes (pairs 1, 2, 9, and 12), four pairs of submetacentrics (pairs 3, 5, 8 and 10) and four pairs of subtelocentrics (pairs 4, 6, 7 and 11). The karyotype of *Hyla faber*...
from São Paulo described by Beçak (1968) is similar to that described herein for Hypsiboas faber.

A comparative analysis between the four species karyotypes revealed some conserved chromosome pairs (pairs 1, 2, 5, 6, 8, and 12), contrasting with some variable ones (pairs 3, 4, 7, 9, 10 and 11). Some more suggestive differences observed between homologues are more likely due to variations from the squashing technique than to real heteromorphisms. Pair 3 varied from subtelocentric (HSE) to submetacentric (HPA and HFA) and metacentric (HAL). Pair 4 was submetacentric (HSE and HAL) or subtelocentric (HPA and HFA). Pair 7 was submetacentric (HAL and HPA) or subtelocentric (HSE and HFA). Pair 9 was subtelocentric (HSE), submetacentric (HAL) or metacentric (HPA and HFA). Pair 10 was metacentric (HSE and HAL) or submetacentric (HPA and HFA) and pair 11 was metacentric (HSE, HAL and HPA) or subtelocentric (HFA).

Although a single chromosome pair presented an Ag-NOR in all metaphases from all the specimens, it was not the same pair in all species. Pairs 2 and 7 are the NORs-bearing chromosomes in *H. albomarginatus* and *H. semilineatus*, respectively, and present a secondary constriction, not always observed on both homologues (Figure 1e and 1j), which coincided with the location of rDNA gene clusters.

In *H. albomarginatus*, the Ag-NORs were interstitially located at the short arm of pair 2 (Figure 1e-f). In all metaphases both homologues had positive Ag-NORs signals. One specimen (LGA 221) exhibited an additional interstitial Ag-NOR, proximal to the short arm of one homologue of pair 2 (Figure 1g).

*H. pardalis* presented one pair of telomeric Ag-NORs in the short arms of pair 11 (Figure 1h). There was an exact correspondence in the position and size of the NORs after FISH and silver staining (Figure 1i).

All four specimens of *H. semilineatus* presented one interstitial Ag-NOR in the long arm of pair 7 (Figure 1j-k). A conspicuous difference in the size of the Ag-NORs between homologues was observed in all specimens. Despite of the heteromorphism in the Ag-NORs, the size of signals of the rDNA probes observed after FISH were similar on both homologues (Figure 1l).

Both specimens of *H. faber* presented interstitial Ag-NORs in the long arms of pair 11, which showed heteromorphism after silver-nitrate staining (Figure 1m).

Nucleolar organizer regions have been considered important markers for the study of chromosome evolution in amphibians (Lourenço et al., 1998). Although intra-specific variation of NORs location is rare (Schmid, 1978), interindividual heteromorphisms of Ag-NORs size were widely observed in anurans. In an extensive study, Schmid (1982) used silver staining and GC-specific fluorochromes to analyze the NORs of 260 individuals from 23 genera of anurans. In this study, 67% of the animals presented heteromorphic Ag-NORs and in 69% of them the size differences could be attributed to tandem duplications or triplications affecting one of both rDNA clusters.

We observed Ag-NORs heteromorphisms in three of the four species investigated (*H. semilineatus*, *H. albomarginatus* and *H. faber*). The difference in the size of the Ag-NORs by silver staining of *H. semilineatus* was not confirmed by FISH with the rDNA probe, suggesting that the Ag-NORs heteromorphism is caused by differential gene activity (and consequent differential accumulation of ribonucleoproteins). The duplicated Ag-NORs sites in pair 2 of *H. albomarginatus* were also not tested by FISH. We were unable to determine if the Ag-NORs size heteromorphism observed in *H. faber* was due to variation in the number of ribosomal genes or to their differential activity, since FISH results were not obtained.

We observed duplicated Ag-NOR in *H. albomarginatus*. Duplicated Ag-NORs were previously reported by Schmid et al. (1995) in the frog *Agalychnis callidryas*. Possible mechanisms responsible for the dispersion of NOR sites in anuran genomes have been discussed by some authors (Wiley et al., 1989, King 1990, Foote et al., 1991, Schmid et al. 1995, Kaiser et al., 1996). They suggested that NORs transposition could have occurred by the movement of mobile genetic elements closely linked to NOR cistrons, amplifications of “orphan” rDNA cistrons, reinsertion errors during extrachromosomal amplification of ribosomal cistrons, or chromosomal rearrangements such as translocations and inversions involving the segments containing NORs. Oliveira et al. (1996) proposed that pericentric inversions could split NOR cistrons resulting in two Ag-NORs with about half of the regular size.

Considering the similar sizes of each of the duplicate Ag-NORs in *H. albomarginatus*, it is unlikely that pericentric inversions involving the rDNA region occurred in this species. We are thus inclined to attribute the duplication to an intrachromosomal duplication (likely due to mobile elements).

However, it is important to emphasize that the Ag-NOR technique detected heterochromatic regions in some species (Lourenço et al., 1998). Such regions were easily distinguished from the NORs sites by the intensity of silver impregnation, which resulted in black dots in true NORs and in brown sections in heterochromatic regions (Lourenço et al., 1998). We did not observe brownish staining in the extra signal, which allows us to discard that it is a silver-stained heterochromatic region. Moreover, the specimen with the heteromorphic duplicated Ag-NOR was collected in a locality different from that where the animals with non-duplicated Ag-NORs were found. New data on more individuals collected in the same locality would help to verify a possible regional variation.

Although the NORs location may be used to characterize species of amphibians, our analysis revealed that NORs are more useful to discriminate groups of species
rather than species with similar 2n = 24 karyotypes. Anuran species frequently revealed only a single NOR-bearing chromosome pair in diploid karyotypes (Schmid, 1982; Mahony and Robinson, 1986; Anderson, 1991). Schmid (1978; 1982) observed that the NORs usually occur at the same chromosomal location in the karyotypes of the species of the same group or in groups of related species. Exceptions to this rule indicate that rearrangements may have contributed to the species evolution (Schmid, 1978).

We plotted our data on Ag-NORs location on Faivovich et al. (2005) phylogenetic tree and we observed that each monophyletic clade had the ribosomal cistron located in a specific chromosome pair (Figure 2).

**H. pardalis** (present study), **H. faber** (present study) and **H. crepitans** (Gruber et al., 2006), which belong to a monophyletic clade and are included in the **H. faber** group, shared the Ag-NORs at the pair 11. Differences of the relative size of chromosome 11, as specified for **H. pardalis** and **H. faber** in Table 2, are not rare. The variation in the NORs location (terminal in the short arm of a metacentric pair in **H. pardalis**, interstitial in the long arm of a subtelo-centric pair in **H. faber** and interstitial in the long arm of a submetacentric pair in **H. crepitans**) can be explained by a single pericentric inversion in these chromosomes, changing the rDNA cluster from a telomeric to an interstitial position.

**H. albomarginatus** (present paper), also included in **H. faber** group, presented NORs in a different chromosome pair (pair 2). Although we only analyzed conventionally stained chromosomes we do not think that it is possible to misidentify pairs 2 and 11, and we believe that distinct chromosome pairs are NOR-bearing in each of these two clades.

In the **H. pulchellus** group, a sister group of **H. faber**, the species **H. semiguttatus** and **H. joaquini** (Ananias et al., 2004) grouped in a monophyletic clade and shared the presence of a telomeric Ag-NOR in pair 1. **H. prasinus** and **H. pulchellus** (Ananias et al., 2004) form another clade in the **H. pulchellus** group and shared the presence of Ag-NORs in pair 12. **H. guentheri**, **H. bischoffi** (Raber et al., 2004) and **H. marginatus** (Ananias et al., 2004) are arranged in a third monophyletic clade species group and present Ag-NORs in pair 10, at the telomeric position of the long arm of **H. guentheri** and **H. bischoffi**, and at the pericentromeric region in **H. marginatus**. These differences in NORs location can be explained by pericentric inversion.

Schmid (1978, 1982) stated that the NORs were recurrently observed at the same chromosomal location in the karyotypes of species from the same groups and that chromosome translocations could be frequent in the evolution of the group. Our data corroborate this assumption and suggest that: (1) inversions also contributed to chromosome changes and NORs transpositions; (2) Ag-NORs present a pattern in hylid frogs species groups (as observed in the **H. faber** group) or inside clades of species groups (as in the **H. pulchellus** group). These data may be useful in the cytotaxonomic and evolutionary studies of hylids.

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### References


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