Karyotype diversity between species of *Crenicichla* (Perciformes, Cichlidae) from different Brazilian hydrographic basins

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Abstract

*Crenicichla* is the largest genus in the Cichlidae family in South America. The genus includes 100 valid species that are popularly known in Brazil as *jacundás* or *joaninhas* and are widely distributed in rivers east of the Andes. Cytogenetic analyses were carried out on seven species in this genus. All species showed a diploid number of 48 with interspecific differences in karyotype formulas and AgNORs located in interstitial position on the short arm of the largest metacentric pair, except for the two populations from *C. britskii*. Population A showed terminal markings on the long arm of the fifth pair of the complement, and population B showed up to two marked chromosome pairs. FISH with an 18S rDNA probe was coincident with AgNORs and CMA, except for pair 6 from population B of *C. britskii* that did not presented positive CMA sites. This work presents first cytogenetic data for *C. haroldoi*, *C. maculata*, and *C. punctata*, and the results show karyotypic patterns similar to those in the literature. However, the diversity found in populations of *C. britskii* represents new information about the evolution of the karyotype of the Cichlidae family, which has been conservative. Furthermore, the data could assist in phylogenetic studies of *Crenicichla*.

Keywords: Chromosome banding, fish cytogenetics, Geophaginae, ribosomal DNA.

Received: February 20, 2018; Accepted: July 17, 2018.

Introduction

The Cichlidae family includes a wide variety of fish species and is one of the largest in Perciformes. There are approximately 1706 valid species (Eschmeyer and Fong, 2018), and the group is considered highly specialized (Kullander, 1998). Through cladistics morphological analyses, Kullander (1998) verified that this family is a monophyletic group and showed a dichotomy between “Old World” and “New World” cichlids.

Stiassny (1991) first recognized the monophyletism of Neotropical cichlids, which include more than 406 valid species (Kullander, 2003). This was later confirmed by phylogenetic relationships based on molecular data (Farias et al., 1999; López-Fernández et al., 2010) and combinations of morphological and molecular data (Farias et al., 2000; López-Fernández et al., 2005; Smith et al., 2008). Among Neotropical cichlids, the genus *Crenicichla* is one of the most numerous, with 100 valid species described (Froese and Pauly, 2018). The pike cichlids are easily recognized by their elongated body, large mouth, and prognata. These cichlids mostly occur in tropical and subtropical regions of South America, from the coastal drainages of Venezuela and Guiana to the Plata River in Argentina (Kullander and Lucena, 2006).

This genus has been studied extensively from a cytogenetic point of view, with the first work conducted by Oyhenart-Perera et al. (1975) on *Crenicichla sexatilis*. Since then, several studies have been carried out, and the majority identify only the diploid number (2n), with a total of 19 species analyzed to date presenting a conserved 2n equal to 48, according to cytogenetic surveys performed by Feldberg et al. (2003) and Benzaquem et al. (2008). Only *Crenicichla* sp. does not present 48 chromosomes, showing 2n=46 (Rezende et al., 1996). The phylogenetic position of *Crenicichla* within the family is quite controversial, sometimes being assigned to the clade Cichlinae (Stiassny, 1991; Kullander, 1998) and sometimes to the clade Geophaginae (Farias et al., 2000; López-Fernández et al., 2005; Landim, 2006; Smith et al., 2008).

Thus, the aim of this work was to perform conventional and molecular cytogenetic analyses of seven pike cichlids species: *Crenicichla britskii*, *C. lepidota*, *C. niederleinii*, *C. semifasciata*, *C. punctata*, *C. haroldoi*, and *C. maculata*. The results provide the first karyotypic information for the last three species. The data presented could be used as an additional tool for phylogenetic studies and help to better define relations within the genus, as well as improve the understanding of the karyotype evolution of the group.
Materials and Methods

The seven species studied were collected from four Brazilian hydrographic basins (Table 1). The specimens were deposited in the Museum of Zoology at the State University of Londrina, Parana, Brazil. For convenience, different populations of C. britskii were called population A (Taquari) and population B (Paranapanema), as shown in Table 1.

Mitosis was stimulated by the injection of yeast suspension in animals, as described by Lee and Elder (1980). Mitotic chromosomes were obtained by direct preparation by removing the anterior kidney according to the methodology proposed by Bertollo et al. (1978), and slides for conventional analysis were stained with 5% Giemsa stain in phosphate buffer at pH 6.8. The morphology of the chromosomes was determined based on the ratio of arms, as proposed by Levan et al. (1964). For determination of the fundamental number (FN), the metacentric (m) and sub-metacentric (sm) chromosomes were considered biarmed and the sub-telo-acrocentric (st-a) uniarmed.

Nucleolar organizer regions (NORs) were detected by impregnation with silver nitrate according to the technique described by Howell and Black (1980). GC- and AT-rich sites were detected with chromomycin A3 (CMA3) and 4', 6-diamino-2-phenylindole (DAPI) according to Schweizer (1980). Fluorescence in situ hybridization (FISH) was performed according to the protocol from Pinek et al. (1986) with modifications according Gouveia et al. (2013) using a 18S rDNA probe (Hatanaka and Galetti Jr, 2004). Finally, the slides were analyzed on an epifluorescence microscope (Leica DM2000), which was attached to a digital camera. Metaphase images were captured using Leica Application Suite version 3.1.0. (Leica Microsystems).

Results

All species analyzed showed a diploid number (2n) of 48 chromosomes, but four different karyotype formulas among species were observed: 6m+4sm+38st-a and FN=58 for C. haroldoi (Figure 1a), 4m+6sm+38st-a and FN=58 for C. britskii, C. niederleinii, and C. punctata (Figure 1b-d and Figure 2c), 6m+42st-a and FN=54 for C. maculata and C. lepidota (Figure 2a,b), and 4m+44st-a and FN=52 for C. semifasciata (Figure 2d).

AgNORs were located on a pair of chromosomes for all species (Figure 1a,b,d and Figure 2a-d), except for population B from C. britskii, which showed up to two marked chromosome pairs (Figure 1c). Population A of C. britskii showed terminal markings on the long arm of the fifth pair of the complement (sm) (Figure 1b). All other species showed NORs in an interstitial location on the short arm of the largest metacentric pair (boxes in Figure 1a,d and Figure 2a-d).

The AgNORs were coincident with the secondary constrictions observed by Giemsa staining. Exceptions were observed in C. britskii. In population A, the secondary constriction observed in pair 20 was not a positive AgNOR, only the constriction of pair 5 (Figure 1b, box). In population B, pair 5 showed a heteromorphism of NORs in the long arm coincident with the secondary constriction, and pair 6 showed a heteromorphism of NORs in the short arm that was not coincident with secondary constriction (Figure 1c, box).

For all species of Crenicichla the FISH analysis with the 18S rDNA probe was coincident with AgNORs (Figures 1 and 2).

Staining with CMA3 showed fluorescent markings coinciding with the NORs in all species analyzed (Figures 1 and 2), except pair 6 from population B of C. britskii. In this population, there was an additional positive CMA3 pair (pair 1), as shown in Figure 1c. Size heteromorphism with CMA3 occurred in pair 5 of C. britskii from population B and in pair 1 of C. niederleinii and C. maculata, as evidenced by Giemsa staining and with the 18S rDNA probe (Figure 1c,d, Figure 2a, Table 2). In DAPI staining, the NORs did not show fluorescent signals, appearing only as a negative band (Figures 1 and 2).

Table 1 - Collection sites and hydrographic basins of Crenicichla specimens analyzed. MS = Mato Grosso do Sul; PR = Paraná; RS = Rio Grande do Sul

<table>
<thead>
<tr>
<th>Species</th>
<th>Collection sites</th>
<th>Hydrographic basins</th>
<th>Number of individuals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crenicichla britskii</td>
<td>Taquari stream-PR (A) 23º10'45.2''S 50º56'30.9''W</td>
<td>Paranapanema river</td>
<td>7M,6F</td>
</tr>
<tr>
<td></td>
<td>Paranapanema-SP (B) 22º42'30.3''S 51º04'08.4''W</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. haroldoi</td>
<td>Pavão stream / PR</td>
<td>Paranapanema river</td>
<td>2M,2F</td>
</tr>
<tr>
<td>C. niederleinii</td>
<td>Três Bocas stream-PR 23º23'06.6'S 51º04'35.8''W</td>
<td>Paranapanema river</td>
<td>2M,5F</td>
</tr>
<tr>
<td>C. lepidota</td>
<td>Miranda river-MS 19º34'38.01'S 57º01'06.63'W</td>
<td>Paraguai river</td>
<td>1M,2F</td>
</tr>
<tr>
<td>C. semifasciata</td>
<td>Miranda river-MS 19º34'38.01'S 57º01'06.63'W</td>
<td>Paraguai river</td>
<td>1F</td>
</tr>
<tr>
<td>C. maculata</td>
<td>Maquiné river-RS 29º30'10.4'S 50º12'31.8''W</td>
<td>Tramandai river</td>
<td>2M,4F</td>
</tr>
<tr>
<td>C. lepidota</td>
<td>Barra do João Pedro-RS 29º46'21.2'S 50º05'08.0''W</td>
<td>Tramandai river</td>
<td>3M,3F,3?</td>
</tr>
<tr>
<td>C. punctata</td>
<td>Saco da Alemoa and river Forqueta-RS 29º22'08.0'S 52º03'30.0''W</td>
<td>Laguna dos Patos System</td>
<td>2M,5F</td>
</tr>
</tbody>
</table>

Total of individuals: 50

Karyotype diversity in *Crenicichla*

**Figure 1** - Karyotype and chromosome pairs with silver nitrate staining, FISH with 18S rDNA probe and CMA3/DAPI in: *Crenicichla haroldoi* (a), *C. britskii*, populations A (b) and B (c), and *C. niederleinii* (d), respectively. In the boxes are secondary interstitial constrictions in the short arm of the first metacentric pair (a, d) and in the long arm of the fifth pair (b, c).
Figure 2 - Karyotype and chromosome pairs with silver nitrate staining, FISH with 18S rDNA probe and CMA3/DAPI in: Crenicichla maculata (a), C. lepidota (b), C. punctata (c) and C. semifasciata (d), respectively. In the boxes are secondary interstitial constrictions in the short arm of the first metacentric pair.
Discussion

These are the first cytogenetic data for *C. haroldoi*, *C. maculata* and *C. punctata*. Along with data for *C. lepidota*, *C. niederleinii*, *C. semifasciata*, and *C. britskii*, all results presented a conserved diploid number (2n=48), corroborating data from the literature (Feldberg et al., 2003; Benzaquem et al., 2008). Thus far, all species of *Crenicichla* have shown this pattern, except *Crenicichla* sp. studied by Rezende et al. (1996), which presented 2n=46. The FN is also consistent with the variations of 52 to 64 found in the literature (Pires, 2013). Despite the conservation of the diploid number, variations in the karyotype formulae were found in *C. semifasciata*, *C. niederleinii* and *C. britskii* in relation to other populations of these species (Feldberg and Bertollo, 1985a,b; Martins et al., 1995; Benzaquem et al., 2008; Poletto et al., 2010). Such differences can be attributed to pericentric inversion events, which play an important role in the karyotype diversity of these species, as suggested by Feldberg and Bertollo (1985a).

According to Thompson (1979), the cichlids have 48 chromosomes of the subtelo-acrocentric type in basal species, where the presence of meta-submetacentric chromosomes would mean a derived karyotype. Furthermore, a greater presence of acrocentric chromosomes indicates a more ancestral karyotype. This hypothesis is shared by Feldberg et al. (2003), who consider the genus *Crenicichla* to be more derived because of the presence of meta- and submetacentric chromosomes. Considering this information, the genus *Crenicichla* is closer to Geophaginae, since the clade Cichlinae would be more ancestral because it presents mainly species with only subtelo-acrocentric chromosomes, as in the genus *Cichla* (Poletto et al., 2010).

Another characteristic shared between the species analyzed, except for population A of *C. britskii*, was the presence of a secondary interstitial constriction on the first chromosome pair. This seems to be a chromosome characteristic of this genus and perhaps a cytotaxonomic marker, because it is also observed in *C. lacustris*, *C. semifasciata*, and *C. vittata* (Feldberg and Bertollo, 1985a,b), *C. lepidota* (Martins et al., 1995; Perazzo et al., 2011; Poletto et al., 2010), *Crenicichla* sp., *C. niederleinii* (Loureiro et al., 2000), *C. iguassuensis* (Mizoguchi et al., 2007), and *C. reticulata* (Benzaquem et al., 2008). This particular chromosome of the genus is another characteristic and makes this group similar to the clade Geohaginaceae, since other genera of this clade also present this type of chromosome, such as *Gymnogeophagus balzani* (Feldberg and Bertollo, 1984; Roncati et al., 2007), *Gymnogeophagus labiatus* (Pires et al., 2010); *Geophagus surinamensis* (Feldberg and Bertollo, 1985a), and *Geophagus proximus* (Valente et al., 2012).

Interestingly, population A of *C. britskii* did not show this constriction in the interstitial region but in the terminal region of the long arm of a submetacentric chromosome pair. Another interesting fact is that both populations of *C.
Crenicichla britskii presented a secondary constriction in the long arm in pair 20 (population A) and pair 5 (population B). The occurrence of these additional secondary constrictions has never been reported and may indicate a differential characteristic for this species.

The presence of a simple interstitial NOR in the first chromosome pair in all species, except Crenicichla britskii, and coincident with the secondary constriction, is well conserved in this genus, as reported by Loureiro et al. (2000), Roncati et al. (2007), Benzaquem et al. (2008) and Valente et al. (2012), among others. This trait varies only in the type of chromosomes, which may be metacentric (Martins et al., 1995; Loureiro et al., 2000; Mizoguchi et al., 2007), or submetacentric (Martins et al., 1995).

Occurrence of multiple NORs in population B of C. britskii may indicate that this population presents characteristics that are more derived in relation to the same species studied by Benzaquem et al. (2008) from another locality, which showed only a pair of NOR. This multiple pattern was previously reported in the genus, but only in C. lepidota from the region of Puerto Rico in the Paraná River basin (Martins et al., 1995), which is a different situation from that found in C. lepidota in the present study.

All analyzed species of Crenicichla, except population B of C. britskii, showed only a pair of chromosomes with ribosomal cistron 18S, thus corroborating the data obtained by the impregnation of silver nitrate and the ancestral condition proposed by Feldberg et al. (2003). The hybridization signals were located interstitially on the short arm of the largest chromosome pair of the complement, similar to previously reported for C. lepidota (Perazzo et al., 2010; Poletto et al., 2010), the only species of the genus to date with results of in situ hybridization.

Size heteromorphism in the NORs, as found in pair 5 in C. britskii (population B), C. niederleinii and C. maculata, may be the result of irregular crossover or differential amplification of this region among the homologous chromosomes. This has previously been proposed for other fishes, including Cichlidae (Pires et al., 2008; Gross et al., 2010; Poletto et al., 2010). The staining with CMA3 fluorochrome evidenced fluorescent signals coincident with the NORs for the seven species, indicating the predominance of GC bases. However, population B of C. britskii again presented a distinct pattern with only one of the nucleolar pairs (pair 5) as CMA3 positive. NORs were negative for DAPI, thus revealing a scarcity of AT bases. The data with fluorochromes coincide with those reported for the genus by Loureiro et al. (2000), Perazzo et al. (2011), Mizoguchi et al. (2007), and Valente et al. (2012).

Among the species analyzed, C. britskii presented unique characteristics, despite having the same diploid number as the others members of the genus. The cytogenetic differences observed among the two populations of C. britskii may have resulted from geographic isolation between them. Ploeg (1991) also studied this species and found that it was endemic to the basin of Alto Paraná. This endemism resulted from the small displacement capacity of these fish: because they are highly territorial, they generally do not perform extensive migration throughout their life cycle and remain isolated (Castro, 1999).

According to Oliveira et al. (1988), populations that have less mobility and fewer individuals are more unstable in relation to their karyotype macrostructure. Gene flow is smaller, thus providing a higher rate of fixation of some chromosomal abnormality. This may be happening with the two populations of C. britskii, where geographic isolation would facilitate the establishment of chromosomal rearrangements and lead to a process of speciation. The population of C. britskii from the Paranapanema River has characteristics that are more derived when compared with the population from the Taquari Stream.

The results for the other species of Crenicichla show that karyotype patterns were similar to those found in the literature (Benzaquem et al., 2008), indicating a conservative trend in chromosome evolution in this group of fish. However, the karyotype diversity found in populations of C. britskii provides new information about the karyotype evolution of the Cichlidae family. The cytogenetic characteristics that are particular to Crenicichla can be an important tool for phylogenetic studies in this group of fish, such as the largest pair of complement with secondary interstitial constriction and the presence of meta/sub metacentric chromosomes in the karyotype. This places the genus Crenicichla in the clade Geophaginae, which corroborates the phylogeny proposed by López-Fernández et al. (2005) and Smith et al. (2008).

Acknowledgments

The authors thank Prof. Dr. Luiz Roberto Malabarba of the Zoology Laboratory at the Universidade Federal do Rio Grande do Sul (UFRGS), for the identification of specimens. This research was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The research received permission from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) to collect fish specimens.

Conflicts of interest

The authors have no conflicts of interest to declare.

Author contributions

ALD, LBP conceived and designed the study; LGC, LBP collected the samples; LBP, performed the cytogenetic analysis; LBP, MCU, wrote the manuscript and designed the figures, all authors read and approved the final version.
References


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Associate Editor: Catarina S. Takahashi

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