Organization and Distribution of Repetitive DNA Classes in the *Cichla kelberi* and *Cichla piquiti* Genome

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Summary The family Cichlidae is considered a non-Ostariophysi freshwater fish family with diverse and geographical distribution wide in the world; nevertheless there is scarce cytogenetic information for the group. Accordingly, we aim to characterize *Cichla kelberi* and *Cichla piquiti* specimens by conventional cytogenetic (Giemsa, Ag-NOR, Banding C) and cyto-molecular (FISH with 5S, 18S, Rag1, Rex3, Rex6 and telomeric probes) markers. In both species, cytogenetic analyses showed $2n=48$ chromosomes (all of the subtelocentric/acrocentric type), NORs located terminally on the second chromosome pair and heterochromatic blocks in the centromeres of some chromosomes. The location of the 18S ribosomal gene confirms what was previously observed with impregnation with silver nitrate, while the 5S rDNA is in interstitial position on the third chromosome pair. The telomeric probe, as expected, was present only in telomeric regions of all chromosomes. The Rag1, transposable elements, Rex3 and Rex6 present dispersed sites throughout most of the chromosomes, not characterizing a definite pattern of distribution. Thus, the present study adds relevant information about the cytogenetics and behavior of different repetitive DNA families in *C. kelberi* and *C. piquiti*, which require future studies to understand the gene behavior and evolution in Neotropical fish, especially in the genus *Cichla*.

Key words Ribosomal gene, Neotropical fish, Transposable element, *Cichla kelberi*, *Cichla piquiti*.

Eukaryotic organisms present in their chromosomes large fraction of repetitive DNA segments, which apparently are not found in prokaryotes individuals, and the use of these sequences as chromosomal markers have revealed significant information on the characterization of biodiversity and evolution of ichthyofauna (Vicari *et al.* 2010) and a greater knowledge of the organization, diversification, evolution and function of repetitive sequences in the genomes of different vertebrate groups (Chalopin *et al.* 2015, Gursel *et al.* 2003). Furthermore, different studies have shown that repetitive sequences are not distributed at random in the genome of many organisms (Bueno *et al.* 2013), but are connected to intergenic regions, heterochromatic, and in some cases involve transposable elements (Martins *et al.* 2013).

Among the species of cichlids, many cytogenetic studies indicate that the majority presence of 48 chromosomes of the subtelocentric/acrocentric type is a conserved characteristic among Perciformes (Accioly *et al.* 2012, Accioly and Molina 2008, Kushwaha *et al.* 2011, Merlo *et al.* 2012, Motta Neto *et al.* 2012, Nirchio *et al.*

2007), including the species of the genus *Cichla*, which is composed of 15 species and is widely distributed in neotropical regions (Kullander and Ferreira 2006). However, cytogenetic and cyto-molecular studies of the Brazilian species *Cichla kelberi* and *Cichla piquiti* are scarce.

Despite cichlids apparently constituting a well-established phylogenetic unit, they still have presented a number of differences between their clades, indicating a number of query about the chromosomal mechanisms involved in the karyotype diversification of this group (Farias *et al.* 2001, Stiassny 1987). With the significant progress of research based on chromosomal characters, we expect a greater phylogenetic knowledge. Therefore, to achieve these expectations, several populations of different species of cichlids, such as populations of *Cichla kelberi* and *Cichla piquiti*, still require basic cytogenetic and cyto-molecular information, since this prior knowledge helps to understand unfilled gaps for phylogenetic and evolutionary inferences (Oliveira *et al.* 2009, Stiassny 1991). In this sense, the objective of the current study was to characterize cytogenetically the species *Cichla kelberi* and *Cichla piquiti* and repetitive DNAs probes, seeking a better understanding of different fami-

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lies of repetitive DNA behavior and the distribution of species that compose this important group of fishes.

Materials and methods

Sampling and mitotic chromosomes

Fifteen individuals of the species *Cichla kelberi* and *Cichla piquiti* were sampled from natural populations of the Tietê River basin of São Paulo State, Brazil. Following morphological identification, the specimens were subjected to stimulation by mitosis according to the protocol proposed by Oliveira *et al.* (1988). The chromosome preparations and cytogenetic analysis were performed according to Foresti *et al.* (1993) based on direct preparations of kidney cell suspensions. The C-banding technique was applied according to Sumner (1972) with some adaptations. The chromosome morphology was determined on the basis of the arm ratio according to Levan *et al.* (1964), and chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) and arranged in descending order of size for the karyotype organization. After cytogenetic analysis of the specimens, the top 10 preparations of each sampled site were used for the application of FISH.

Repetitive probes and FISH experiments

For labeling of the probes and amplification, we used a primer (Table 1). PCR reactions were performed in a total volume of 25 μ L containing 150 μ M of each dNTP (dATP, dTTP, dGTP and dCTP), 1.5 mM MgCl₂, 1 $\times$ Taq DNA buffer (20 mM Tris-HCl, pH 8.4 and 50 mM KCl), 0.5 unit (U) of Taq Polymerase (Invitrogen), 0.2 μ M of each primer, 0.1 nmol μ L⁻¹ fluorescer and 10–50 ng of genomic DNA. Products were performed by electropho-

resis on agarose gel stained with SYBR[®] Safe DNA gel stain, visualized with UV illumination and images were captured by a digital camera (OLYMPUS, CAMEDIA, C-5060 5.1 Megapixel). The hybridization conditions were the same for every specimen analyzed, and high stringency conditions were applied following the procedures described in Pinkel *et al.* (1986). The probes were labeled with biotin-16-dUTP and/or digoxigenin-11-dUTP, and hybridization signals were developed using avidin-FITC and/or anti-digoxigenin-rhodamine. The images were captured by a digital camera attached to a fluorescence microscope (Olympus BX50). Approximately 20 metaphase spreads of each individual were analyzed to confirm the signals obtained. The assembly of karyotypes, maintenance, and the brightness and contrast of the images were characterized using the Adobe Photoshop CS5 software.

Results and discussion

In general terms, cichlid representatives usually present 24 chromosome pairs, a pattern considered conserved for the group (Feldberg *et al.* 2003). However, some African representatives have $2n=44$ chromosomes, and fundamental numbers (NF) ranging from 44 to 88. American representatives have presented a modal diploid number of 48 chromosomes, with NF from 44 to 118. In our results, we observed $2n=48$ chromosomes of subtelocentric/acrocentric type in all specimens analyzed of *C. kelberi* and *C. piquiti* (Fig. 1), corroborating previous analyses reported for both species as the modal and fundamental number (Poletto *et al.* 2010, Teixeira *et al.* 2009, Valente *et al.* 2012).

The presence of 48 subtelocentric/acrocentric chro-

Table 1. List of primers used for amplification and labeling of the probes in this manuscript.

Primers	Sequence	References
18S F	5'-TACGCCGATCTCGTCCGATC-3'	Hatanaka and Galetti Jr. 2004
18S R	5'-CAGGCTGGTATGGCCGTAAGC-3'	Hatanaka and Galetti Jr. 2004
5S F	5'-TCAACCAACACAAAACATTGGCAC-3'	Pendás <i>et al.</i> 1994
5S R	5'-TAGACTTCTGGGTGGCCAAGGAATCA-3'	Pendás <i>et al.</i> 1994
Rag1 F	5'-GGCTCTCTGGATGGTCTTCCT-3'	Present study
Rag1 R	5'-ACACTTCYCCAATYTCATCCTGGA-3'	Present study
Rex3 F	5'-TACGGA GAAACCCA TTT CG-3'	Volff <i>et al.</i> 2000
Rex3 R	5'-AAA GTT CCT CGG TGG CAAGG-3'	Volff <i>et al.</i> 2000
Rex6 F	5'-TAA AGC ATACATGGAGCGCCAC-3'	Volff <i>et al.</i> 2001
Rex6 R	5'-GGT CCTCTACCA GAGGCC TGGG-3'	Volff <i>et al.</i> 2001
Telomeric F	(TTA GGG) _n	Ijdo <i>et al.</i> 1991
Telomeric R	(CCC TAA) _n	Ijdo <i>et al.</i> 1991

Fig. 1. Ideogram of *Cichla kelberi* and *Cichla piquiti* showing the main cytogenetic markers, and localization of telomeric and ribosomal probes used in this paper. Bar=10 μ m.

mosomes in most Perciformes and also in *Cichla* suggests that this genus maintains basal characteristics that originated from the family Cichlidae. Accordingly, the ancestral karyotype of cichlids would be represented by $2n=48$ acrocentric chromosomes, ergo, any difference in this condition would represent an evolutionary derivation of this characteristic (Feldberg and Bertollo 1985), which can be attributed to structural rearrangements mechanisms, commonly connected to pericentric inversions and fusions and fissions centric (Feldberg *et al.* 2003).

The C-banding revealed the presence of heterochromatic blocks located in the centromeric portion of all chromosomes of *C. kelberi* e *C. piquiti* (Fig. 1), corroborating several cichlid studies, e.g., *Cichlasoma facetum* (Vicari *et al.* 2006), *Astronotus ocellatus* (Mazzuchelli and Martins 2009), *Geophagus brasiliensis* (Pires *et al.* 2008), *Laetacara prope dorsigera* (Martins-Santos *et al.* 2005), *Geophagus* cf. *proximus* (Rocha *et al.* 2013), among other species (Poletto *et al.* 2010), supporting the idea that constitutive heterochromatin in centromere is distributed preferentially in the majority species. Moreover, it suggests that the presence of centromeric heterochromatin blocks is a widely distributed and plesiomorphic condition in cichlids.

The Ag-NOR technique revealed the presence of single nucleolar sites in both species reported, located in the terminal region of the second chromosome pair (Fig. 1), a pattern previously observed by Teixeira *et al.* (2009) and Valente *et al.* (2012), and later confirmed by FISH with the 18S ribosomal gene in both species (Fig. 1). Despite this, there are heteromorphic cases relative to NOR site size and position involving both homologous chromosomes, indicating a difference in the quantity of rDNA between these two chromosomes bearing NOR and a difference in the activity of these genes in the same chromosomes (Feldberg and Bertollo 1985).

Similarly, the 5S rDNA gene is present only in the third chromosome pair, however, in position pericen-

tromeric (Fig. 1). This location appears to offer some organization advantage of this gene in the genome, since many fish species belonging to distinct groups reported the same chromosomal position of 5S rDNA gene (Martins and Galetti Jr. 2001). In addition, our results support the analysis of other species of this group (Poletto *et al.* 2010, Teixeira *et al.* 2009) and indicate that during the evolutionary history of this genus, this gene family remained apparently unchanged relative to their chromosomal position, present in a single pair of acrocentric chromosomes. Thus, as new species and populations are analyzed, examples that contradict this proposal can be found. A FISH with telomeric probes indicated just terminal sites in the *C. kelberi* and *C. piquiti* chromosomes (Figs. 1 and 2), probably indicating that evolutionary processes involving these sequences did not occur in the karyotype evolution of these species.

Another interesting class of repetitive DNA is the transposable elements (TEs). The TEs with better known distribution are the non-LTR retrotransposons, which include the Rex family (Ferreira *et al.* 2011a, 2011b, Gross *et al.* 2009, Valente *et al.* 2011). In fish, these elements can be distinctly organized and may be compartmentalized in euchromatic/heterochromatic regions, or scattered over the genome. In *C. kelberi* and *C. piquiti*, for example, we noted that the Rex3 and Rex6 elements are scattered on the majority of chromosomes of both species (Fig. 1), not characterizing a definite pattern of distribution.

In review, Ferreira *et al.* (2011a) highlight these characteristics in 32 fish species, among them, *Hypostomatinae*, *Erythrinus*, *Astyanax*, *Hypostomus*, cichlids and Antarctic fishes. Such a similarity, widely observed in fish, could suggest a joint evolution or even similar evolutionary mechanisms of these TEs, as suggested by Ferreira *et al.* (2011b) for Loricariidae. Thus, an extensive form of these elements are incorporated in the genomes, which could facilitate the origin of structural alterations (Ozouf-Costaz *et al.* 2004), resulting in

Fig. 2. Mapping of the telomeric sequences, Rag1, transposable element Rex3 and Rex6, and rDNA 5S (red) and 18S (green) in the somatic metaphases from individuals of *Cichla kelberi* and *Cichla piquiti* after *in situ* hybridization. Bar=10 μ m.

intra- and interspecific polymorphisms and numerical variation of copies, subsequently causing gene expression alteration (Capy 1998, Ferreira *et al.* 2011b).

The recombination of the V, D, and J gene segments, which form the genes encoding the V regions of Igs and T-cell receptors, is promoted by two closely-linked genes, Rag 1 and Rag 2, which also can cause expression alterations. According to Schatz *et al.* (1992) and Oettinger (1992), the coding region of Rag1 is found within an exon, and therefore it would be an indicator of the presence of rearrangements of the genes related to the immune system in cyclostomes, primitive vertebrates (Greenhalgh and Steiner 1995). This information can be critical to answer questions directly related to the immune response, since a huge number of antigens exist and each lymphocytes expresses a structurally different antigen receptor to combat them (Nishana and Raghavan 2012). It was noted in *C. kelberi* and *C. piquiti* that the Rag1 are scattered over the majority of chromosomes (Fig. 2), not characterizing a definite pattern of distribution. A possible hypothesis is that an evolutionary mechanism is occurring related to such genes in both species, probably due to the constant contact of antigens with species of fish in general, mainly cichlids.

Since previous studies were performed for the genus *Cichla* by Poletto *et al.* (2010), Teixeira *et al.* (2009) and Valente *et al.* (2012), these results contribute to the understanding of multigenic family evolution and reinforce classical cytogenetic data previously reported. This increases the knowledge of the chromosomes of this family, which has a wide distribution in Brazilian river basins, and for presenting recent description, work is needed to supplement the various areas of study.

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