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Comparative cytogenetics of cichlid fishes through genomic in-situ hybridization (GISH) with emphasis on *Oreochromis niloticus*

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Abstract Cichlidae is the most species-rich freshwater family of Perciformes and has attracted the attention of aquarium hobbyists, aquaculturists, and sport fisherman. *Oreochromis niloticus* is very important in aquaculture today and is currently used in varied areas of study as an 'experimental model'. *Oreochromis niloticus* has been characterized using classical and molecular cytogenetic techniques, with special attention paid to heterochromatin structure and the identification of sex chromosomes. In this study, we compare the genome of *O. niloticus* with that of other cichlids from Africa and South America using genomic in-situ hybridization (GISH). Our results show that at least some elements comprising the pericentromeric heterochromatin of Nile tilapia are species-specific and that the sequence of the majority of the long arm of the largest chromosome pair is conserved within the tilapiine group, which is composed of the genera *Tilapia*, *Oreochromis*, and *Sarotherodon*. It is suggested that the extensive regions of repeated DNA in the largest chromosome pair of *O. niloticus* resulted from chromosome rearrangement or accumulation caused by recombination suppression during the evolutionary history of the tilapiines.

Keywords Cichlidae · chromosome · heterochromatin · evolution · repeated DNAs · genome

Abbreviations

2n	diploid number
BAC	bacterial artificial chromosome
C buffer	coupling buffer
Cot-1 DNA	DNA enriched for highly and moderately repetitive DNA sequences
FISH	fluorescence in-situ hybridization
FITC	fluorescein isothiocyanate
GISH	genomic in-situ hybridization
LINE	long interspersed nuclear elements
m/sm	meta-submetacentric chromosome

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NCBI	National Center for Biotechnology Information
SINE	short interspersed nuclear elements
SSC	standard saline citrate
st/a	subtelo-acrocentric chromosomes
st	subtelocentric chromosomes
Tc1	transposon of *Caenorhabditis elegans*

Introduction

Cichlids represent the most species-rich group of Perciformes, and indeed all vertebrates, and comprise more than 3000 living species (Nelson 2006). The African continent has the greatest number of species (more than 1500) and the highest diversity in this family is found in the Great Lakes of East Africa (Victoria, Tanganyika, and Malawi) (Trewavas 1983; Genner and Turner 2005). For instance, approximately 500 species of cichlids that colonized Lake Victoria have evolved from only a few ancestral species in the last 100 000 years (Verheyen et al. 2003). Thus, cichlid fishes have attracted increasing scientific interest because of their rapid adaptive radiation, which has lead to their extensive ecological diversity and enormous importance to tropical and subtropical aquaculture (Kocher 2004).

The family Cichlidae represents a monophyletic group and the limits and interrelationships of all four subfamilies (Etroplinae (Indian), Ptychochrominae (Malagasy), Cichlinae (Neotropicals), and Pseudocrenilabrinae (African)) are well supported by molecular and morphological data (Smith et al. 2008). The African (Pseudocrenilabrinae) and Neotropical (Cichlinae) cichlids are both monophyletic and represent sister groups (Smith et al. 2008). The African cichlids are often distributed into pelmatochromine, haplochromine, and tilapiine groups (Lowe-McConnell 1999), but these groups are not recognized as valid taxonomic units. The Neotropical cichlids (Cichlinae) are monophyletic and are composed of 51 genera and 406 described species (Kullander 1998, 2003). The most recently proposed phylogeny of the group denotes the tribes Cichlini, Retroculini, Astronotini, Chaetobranchini, Geophagini, Cichlasomatini, and Heroini as part of the Cichlinae clade (Smith et al. 2008).

Although approximately 70 species of cichlids are referred to as 'tilapia', only *Oreochromis niloticus*, *Oreochromis mossambicus*, and *Oreochromis aureus*, and their hybrids, have great importance in world fisheries. Nowadays, the Nile tilapia, *O. niloticus*, represents one of the most widely farmed freshwater fish in the world (FAO 2006). Although the *O. niloticus* genome is being completely sequenced (The International Cichlid Genome Consortium 2006), the knowledge of the Nile tilapia genome is rather preliminary, and far behind that of pufferfish (*Tetraodon nigroviridis*) (Jaillon et al. 2004), zebrafish (*Danio rerio*) (Meli et al. 2008), and medaka (*Oryzias latipes*) (Kasahara et al. 2007). In this way, molecular cytogenetic data of *O. niloticus* can be integrated to DNA sequences providing a better landscape of the genome, not yet clearly defined even in the completely sequenced genomes.

Despite the morphological and ecological diversity of cichlid fishes, chromosome information is known for 135 species, but most cytogenetic data are only related to the determination of haploid/diploid chromosome number. The African cichlids have a modal diploid chromosome number of 44, whereas the Neotropical cichlids have a modal diploid number of 48 (Feldberg et al. 2003). The mechanisms of chromosome evolution active during the karyotype diversification of cichlids are still uncertain. However, the application of molecular cytogenetic techniques shows promise for the clarification of chromosome evolution in this group.

Since the first publication of in-situ hybridization of DNA sequences (Pardue and Gall 1969), many variations of this technique have been described. Genomic in-situ hybridization (GISH) represents one of these variations and utilizes the genomic DNA of one organism as a probe to target DNA of another organism. GISH technology has been applied to several areas of investigation in cytogenetics including chromosome disposition, genomic identification, recognition of parts of genomes, B chromosomes, and comparative cytogenetics and genomics (revised by Stace and Bailey 1999; Svartman and Vianna-Morgante 1999). In order to investigate the organization of cichlid genomes, we utilized GISH for comparative cytogenetic analysis with emphasis on *O. niloticus*. Considering that the genome of *O. niloticus* is being completely sequenced (The International Cichlid Genome Consortium 2006), molecular cytogenetic data are certainly welcome as a contribution to the understanding the Nile tilapia genome and will be important for the further expansion of knowledge on cichlid biology.

Materials and methods

Biological samples, DNA extraction, and chromosome preparation

The species used in this study and their geographic origin are described in Table 1. *Oreochromis niloticus* was the reference species and its DNA was used as probes against its own chromosomes. The DNA of the other cichlids was used as a block to discourage the hybridization of nucleotide sequences common to *O. niloticus* and the other cichlid genomes.

Genomic DNA was extracted from species listed in Table 1 according to standard phenol–chloroform procedures (Sambrook and Russel 2001). Mitotic chromosomes of *O. niloticus* were prepared from anterior kidney cells with *in vivo* colchicine treatment (Bertollo et al. 1978) and were subjected to GISH according to the protocol described bellow.

Blocking DNAs and probes

The amount of blocking DNA used for GISH varies from 1 to 100 times the amount of probe DNA (revised by Stace and Bailey 1999). In the present study, the quantity of blocking DNA was optimized using DNA from *Geophagus brasiliensis*. To establish the ideal probe/blocking DNA ratio in the GISH experiments, the amount of blocking DNA was tested from 10 μg to 50 μg (10 to 50 times the amount of probe DNA). In these experiments the amount of probe was maintained constant (1 μg). In the end, 45 μg of blocking DNA proved optimal for generating species-specific signals in the genome of *O. niloticus* and was used in all experiments. Experiments utilizing blocking DNA from the *O. niloticus* genome served as positive controls for the blocking DNA. Experiments involving hybridization without blocking DNA served as positive controls for DNA probes.

DNA samples used for blocking were fragmented to 200–500 bp segments by autoclaving at 98 kPa (1 kgf/cm^2) for 12 min. The DNAs used as probes and for blocking were quantified by spectrophotometry using the Thermo Scientific (Waltham, MA, USA) NanoDropTM 1000.

The bacterial artificial chromosome (BAC) clone C4E09, which contains repeated DNAs of the *O. niloticus* genome (Ferreira and Martins 2008), was used as a probe to establish a correlation between the distribution of repeated DNAs and the GISH results. This BAC was identified among thousands of clones of a genomic library of *O. niloticus*. Shotgun sequencing of BAC C4E09 identified several repeated sequences, including LINE retrotransposons and satellite DNAs conserved in other cichlids and dispersed sequences conserved in other fish species (Ferreira and Martins 2008).

Genomic in-situ hybridization (GISH)

Oreochromis niloticus DNA was used as a probe in all experiments after nick translation labeling with biotin-

Table 1 Species analyzed and their origin

Species	Application	Origin of specimens
African cichlids		
Oreochromis niloticus	Probe, chromosome preparation and blocking DNA	Tietê River, Botucatu, SP, Brazil
Oreochromis karongae	Blocking DNA	Institute of Aquaculture, University of Stirling, Stirling, Scotland
Oreochromis aureus	Blocking DNA	Institute of Aquaculture, University of Stirling, Stirling, Scotland
Sarotherodon galilaeus	Blocking DNA	Institute of Aquaculture, University of Stirling, Stirling, Scotland
Haplochromis obliquidens	Blocking DNA	Aquarium Shop, Botucatu, SP, Brazil
Hemichromis bimaculatus	Blocking DNA	Aquarium Shop, Botucatu, SP, Brazil
South American cichlids		
Astronotus ocellatus	Blocking DNA	Tietê River, Botucatu, SP, Brazil
Geophagus brasiliensis	Blocking DNA	Tietê River, Botucatu, SP, Brazil
Crenicichla sp.	Blocking DNA	Araguaia River, São Félix do Araguaia, MT, Brazil
Aequidens tetramerus	Blocking DNA	Araguaia River, São Félix do Araguaia, MT, Brazil

14-dATP (BioNick™ Labeling System; Invitrogen, San Diego, CA, USA) according to the specifications of the manufacturer. Biotin-14-dATP was also applied in the labeling of BAC C4E09. The chromosomal DNA was denatured in 70% formamide / 2× SSC (pH 7.0) for 42 s at 67°C. Hybridization mixtures containing 1 μg of biotin-labeled DNA probe, 45 μg of blocking DNA, 10 mg/ml dextran sulfate, 2× SSC, and 50% formamide in a total volume of 30 μl were denatured for 5 min at 95°C and dropped onto the slides. The hybridization was performed overnight at 37°C in a 2× SSC moist chamber. Detection of hybridized probes was carried out with 0.07% avidin-FITC (fluorescein isothiocyanate) conjugate (Sigma, St. Louis, MO, USA) in C buffer (0.1 M $NaHCO_3$ pH 8.5, 0.15 M NaCl) for 30 min. The signal was amplified by incubating samples with 5% anti-avidin biotin conjugate (Sigma) in blocking buffer (4× SSC, 0.5% Triton, and 1% nonfat dried milk) for 10 min by another application with avidin-FITC. Both incubations were conducted in a 2× SSC moist chamber at 37°C. After each amplification step, the slides were washed three times for 2 min each in blocking buffer at 45°C. Chromosomes were counterstained with propidium iodide 0.2% diluted in antifade (Vector, Burlingame, CA, USA).

GISH signal and statistical analysis

Hybridized chromosomes were analyzed under an Olympus BX 61 microscope and the images were captured with a digital camera (Olympus DP71) and Image-Pro MC 6.0 software. Chromosomes were arranged by decreasing size according to Ferreira and Martins (2008) and analyzed with Adobe Photoshop CS2. The chromosome arms were measured twice with the software's Image Tool. Three patterns of signals were considered based on signal intensity: strong, medium, and low. The signal intensity (including the three patterns) was estimated in relation to whole chromosomes with the software Image Tool. The whole chromosome extension of *O. niloticus* was defined as 100% and the intensities of signals in hybridized segments were presented as a percentage of the *O. niloticus* genome hybridized after blocking. The evolutionary divergence between *O. niloticus* and each species whose DNA was used for blocking was determined by measuring the horizontal branches of the phylogeny with a pachymeter as proposed by Smith et al. (2008). Branch lengths were converted to values representing the number of transformations (10 transformations = 3.49 mm in the bar of calibration as stated by Smith et al. (2008)). These transformations were related to genetic and morphological data (Smith et al. 2008). Furthermore, the genetic distance between cichlid species was determined based in the *p* distance analysis from mitochondrial and nuclear genes obtained at National Center for Biotechnology Information (NCBI) (see Supplementary Material S1), using the PAUP 4.0b program (Swofford 1998). The number of transformations and the genetic distance data were plotted separately against the percentage of GISH hybridization; the coefficient of correlation of Pearson (r), the coefficient of determination (R^2), and the relationship between the two variables were determined through regression analysis ($y = a + bx$).

Results and discussion

In the present study, chromosomal DNA from *O. niloticus* was used as a reference for genomic in-situ hybridization. For blocking, we utilized DNA from the African cichlids *O. niloticus* (positive control), *O. aureus*, *O. karongae*, *Sarotherodon galilaeus*, *Hemichromis bimaculatus*, and *Haplochromis obliquidens*; and from the South American cichlids *Astronotus ocellatus*, *Crenicichla* sp., *Geophagus brasiliensis*, and *Aequidens tetramerus*. In general, GISH patterns were similar to results obtained for the distribution of repeated DNAs and heterochromatin in the genome of *O. niloticus*, evidenced by hybridization of the BAC-probe clone C4E09 which is enriched for repeated sequences (Fig. 1a). The distribution of repeated elements was previously described for *O. niloticus* and showed a preferential localization in the pericentromeric region, in the distal part of the long arm of the largest chromosome pair, and, less intensely, in the subtelomeric regions (Ferreira and Martins 2008). These results suggest genomic conservation of a majority of the euchromatic regions in all Cichlidae species studied. This correlation among GISH results and the distribution of repeated elements was also observed for other organisms (revised by Stace and Bailey 1999).

The most distinct regions of the *O. niloticus* genome are found in the pericentromeric regions of

most chromosomes and in the long arm of the largest chromosome pair. However, there are small differences in the GISH signals generated by the different species-specific blocking DNAs (Figs. 1 and 2).

Pericentromeric DNA contains the most rapidly evolving DNA sequences in eukaryotic genomes—differing even between closely related species (Haaf and Willard 1997; Csink and Henikoff 1998; Murphy and Karpen 1998). The pericentromeric regions are associated with a rapid evolutionary rate due to localized recombination suppression that leads to the accumulation of repeated sequences (Charlesworth et al. 1994; Topp and Dawe 2006). The formation of heterochromatin in association with repetitive sequences is crucial for the functional organization of chromosomal structures such as centromeres. Our results indicate that the pericentromeric heterochromatin of *O. niloticus* is species-specific. The heterochromatin of centromeric regions is composed of repetitive DNA elements (Grewal and Jia 2007). Furthermore, although GISH does not allow the determination of specific sequences, it seems clear that the differential hybridization pattern we obtained represents the presence of repeated DNA sequences such as satellite DNA and transposable elements.

The largest chromosome pair seems to be conserved among the tilapiines *O. niloticus*, *O. karongae*, *O. aureus*, and *S. galillaeus* (Figs. 1 and 2). On the other hand, remarkable differences in the largest chromosome of *O. niloticus* were observed, evidenced by the hybridization pattern observed with blocking DNAs of non-tilapiines and South American cichlids. These data indicate that the genomes of the three tilapiines (*O. karongae*, *O. aureus*, and *S. galillaeus*) used for blocking are more similar to *O. niloticus* than the others cichlids. This result is in

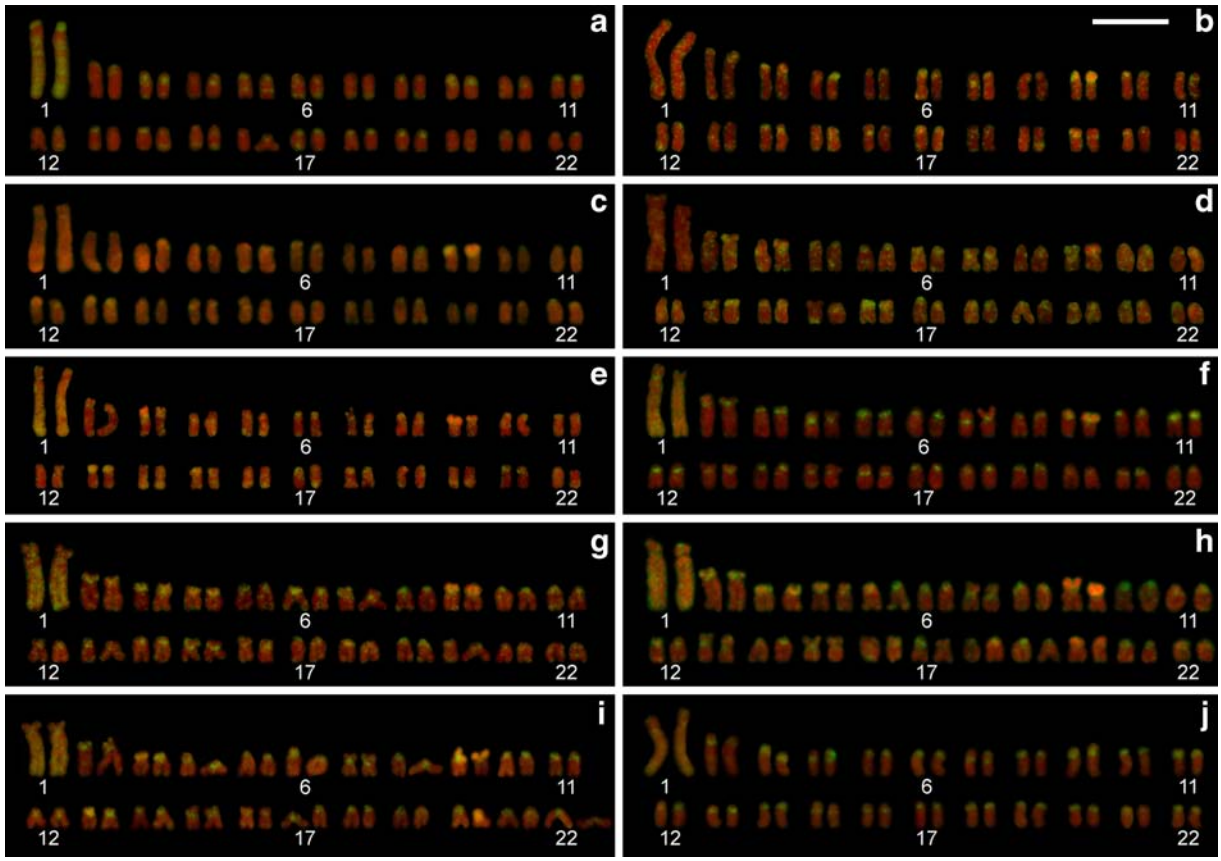

Fig. 1 In-situ hybridization of *Oreochromis niloticus* chromosomes using the BAC clone C4E09 as probe **(a)**, and blocking DNAs from *O. aureus* **(b)**, *O. karongae* **(c)**, *Sarotherodon galilaeus* **(d)**, *Hemichromis bimaculatus* **(e)**, *Haplochromis obliquidens* **(f)**, *Astronotus ocellatus* **(g)**, *Crenicichla* sp. **(h)**, *Geophagus brasiliensis* **(i)** and *Aequidens tetramerus* **(j)**. Scale bar represents 5 μm

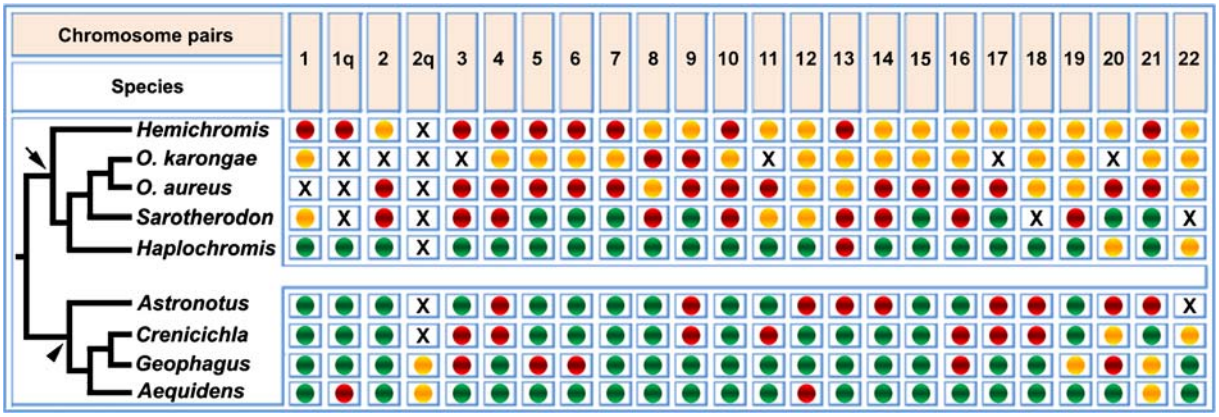

Fig. 2 Relationship between the chromosome signals generated by GISH experiments. The genomes of the species in the cladogram (Smith et al. 2008) were used as blocking DNA. The number at the top refers to each chromosome pair of *Oreochromis niloticus*. The long-arm (q) information for chromosomes 1 and 2 is also indicated. Green, strong signal evidenced; red, signal of medium intensity; yellow, weak signal evidenced; X, no signal. The African and South American cichlid clades are indicated by an arrow and arrowhead, respectively

agreement with the proposed phylogeny for this family (Smith et al. 2008). Although *O. karongae* has a karyotype composed of 2n=38 chromosomes (Harvey et al. 2002) and the other tilapiines analyzed herein posses 2n=44 (Majumdar and McAndrew 1986), the karyotype differences do not seem to reflect remarkable genomic differences among these species, at least in relation to the repeated genomic segments. In fact, the existence of hybrids of *O. niloticus* and *O. karongae* (Harvey et al. 2002) indicates a close genetic relationship among these species. Viable interspecific hybrids between *O. niloticus* and *O. aureus* have also been reported (Pruginin et al. 1975; Hulata et al. 1983, 1993). In the same way, hybrids among *Sarotherodon*, *Tilapia*, and *Oreochromis* have also been obtained (Heinrich 1967; Fishelson 1988; Rana et al. 1996).

Genomic DNA of the African cichlids *Hemichromis bimaculatus* and *Haplochromis obliquidens* did not block the DNA of the largest pair of *O. niloticus* (Figs. 1 and 2) even though these species are somewhat related to the tilapiines (Smith et al. 2008). The tilapiines have a typical karyotype pattern (represented here by *O. niloticus*) composed of one pair (the largest) of subtelocentric (st), a few meta-submetacentric chromosomes (m/sm), and several subtelo-acrocentric (st/a) chromosomes (Majumdar and McAndrew 1986). On the other hand, the karyotypes of non-tilapiine African cichlids are composed of a typical large m/sm, a large st/a, several small m/sm, and several small st/a (Poletto, AB et al. in preparation). Our results indicate that the largest chromosome pair of tilapiines has exclusive genomic sequences not evidenced in the genome of other cichlids. In addition, although the tilapiines posses different sex chromosome systems (*O. niloticus*, XY; *O. aureus* and *O. karongae,* ZW) (Cnaani et al. 2008), such variation does not mean major differences in the distribution of repeated genomic DNAs as here visualized by GISH.

The largest chromosome pair of *O. niloticus* is enriched in repeated DNA sequences (Harvey et al. 2003; Ferreira and Martins 2008). It is also believed to be the sex chromosome of this species, evidenced by the unpaired segments of DNA in the terminal region of the long arm of male heterogametic meiotic cells (Carrasco et al. 1999; Griffin et al. 2002). The restriction of recombination in the heterogametic genotype between regions containing the sex-determining genes is a general characteristic of sex chromosome differentiation (Solari 1994). When recombination is limited in this way, an accumulation of repeated DNA sequences is expected (Topp and Dawe 2006). Several classes of repeated DNAs were identified in the largest chromosome of *O. niloticus* including LINES (Oliveira et al. 1999), SINES (Oliveira et al. 2003), telomeric sequences (Chew et al. 2002), Tc1-like transposons (Harvey et al. 2003), and several non-classified transposon elements (Ferreira and Martins 2008). FISH analysis using Cot-1 DNA (DNA enriched for highly and moderately repetitive DNA sequences) confirms that this chromo-

Table 2 Number of transformations according to Smith et al. 2008 and percentage extent of GISH-hybridization between different cichlid species and *O. niloticus*

Blocking species	Number of transformations	Percentage of hybridization
Sarotherodon	41.604	15.14
Hemichromis	80.946	21.19
Haplochromis	85.301	26.79
Astronotus	204.112	24.36
Crenicichla	261.304	26.63
Aequidens	251.361	28.36
G. brasiliensis	266.576	32.34

some is rich in repeated elements (Ferreira and Martins 2008). Moreover, C-banding techniques confirmed the presence of heterochromatin along the entirety of the long arm of chromosome 1 (Majumdar and McAndrew 1986), which is supposed to contain repeated DNAs. The accumulation of repeated DNAs in the largest chromosome of *O. niloticus* could also be a result of chromosome fusions that appear to have been involved in the origin of this chromosome pair (Oliveira et al. 1999). On the other hand, linkage mapping analyses have detected that the locus of sex determination in *O. niloticus* is located in a small chromosome pair (linkage group 1) and not in the largest chromosome (Lee et al. 2003; Lee and Kocher 2007; Cnaani et al. 2008). These data represent a paradox with no clear resolution yet.

Besides major differences related to the first chromosome pair and centromeric region, minor variations were observed with blocking DNAs of different cichlid species. The use of blocking DNA of *Haplochromis obliquidens* evidenced stronger signals compared to the other African cichlids. It is interesting because *Oreochromis* is more related to *Haplochromis* than to *Hemichromis*. Although *Haplochromis* is considered to be closely related to tilapiines (Smith et al. 2008), our results suggest that the genome of *O. niloticus* is more similar to that of *Hemichromis* than to that of *Haplochromis*. The comparative analyses of GISH-signals blocked with DNA from South American cichlids reveal a similar pattern to the blocking with *Haplochromis* genomic DNA (Figs. 1 and 2). *Haplochromis* is characterized as a highly diverse group compared with other African cichlids (Kornfield et al. 1979; Liem 1991). We speculate that the differential pattern of hybridization observed for *Haplochromis* could be related to the high genetic diversity observed in this genus (Turner 2007).

GISH blocked with DNA from different South American cichlid species revealed a similar hybridization pattern for all of them (Figs. 1 and 2). With the exception of the pericentromeric heterochromatin and

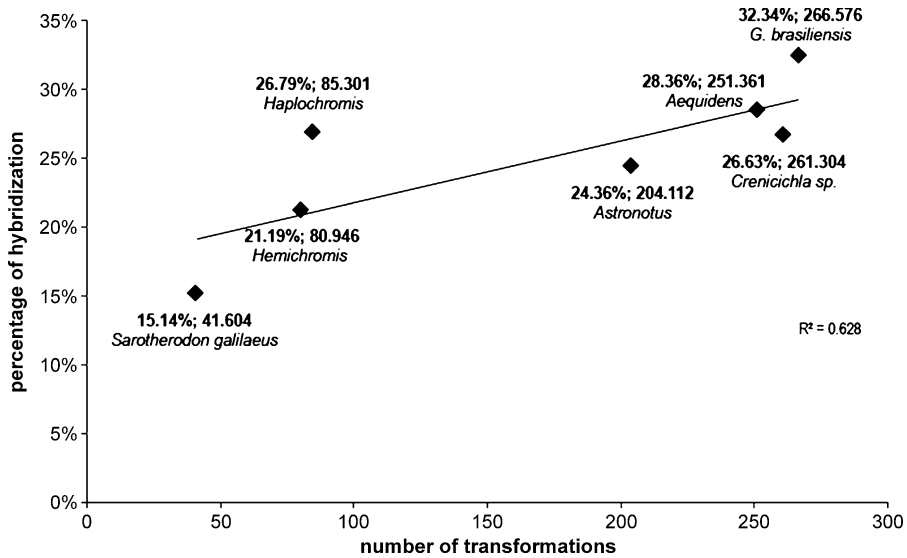

Fig. 3 Correlation between the number of transformations (Smith et al. 2008) among cichlid species and the percentage of hybridization in the chromosomes of *Oreochromis niloticus*. R^2 indicates the coefficient of determination

the largest chromosome pair, the remaining chromosome extensions of *O. niloticus* were blocked and thus no signals were observed. This hybridization pattern is similar to the results obtained when *H. obliquidens* DNA was used for blocking.

Comparative analysis of transformations based on the phylogeny of Smith et al. (2008) and the extent of hybridization shows some correlation between the divergence of Cichlidae groups and the level of GISH hybridization (Table 2, Fig. 3). Similar results were also obtained after the correlation of GISH and genetic distance analysis (see Supplementary Material S2). Furthermore, among the South American cichlids that were analyzed; *Geophagus brasiliensis, Crenicichla* sp. and *Aequidens tetramerus* exhibited less effective blocking and generated higher hybridization values (Table 2, Figs. 1, 2, and 3). These data could be explained by the fact that Geophagini (which includes *Geophagus brasiliensis* and *Crenicichla* sp.) presents a high evolutionary rate (Farias et al. 1999, 2000, 2001; López-Fernández et al. 2005; Smith et al. 2008). Overall, these data support the use of GISH for comparative genomics.

Although cytogenetics has been neglected as a tool for understanding genome structure and evolution, our data support the use of GISH for comparative genomics. GISH is an inexpensive technique that does not require DNA cloning and sequencing and can be helpful to investigate several genomic aspects including sex chromosomes, B chromosomes, chromosome rearrangements, and genomic evolution. In this way, cytogenetic data can be integrated with nucleotide sequences and other genetic/genomic data to determine the most parsimonious scenario for the complex evolutionary history of fishes.

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