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Cytogenetics of *Gymnogeophagus setequedas* (Cichlidae: Geophaginae), with comments on its geographical distribution

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We provide cytogenetic data for the threatened species *Gymnogeophagus setequedas*, and the first record of that species collected in the Iguaçu River, within the Iguaçu National Park's area of environmental preservation, which is an unexpected occurrence for that species. We verified a diploid number of 2n = 48 chromosomes (4*sm* + 24*st* + 20*a*) and the presence of heterochromatin in centromeric and pericentromeric regions, which are conserved characters in the Geophagini. The multiple nucleolar organizer regions observed in *G. setequedas* are considered to be apomorphic characters in the Geophagini, whereas the simple 5S rDNA cistrons located interstitially on the long arm of subtelocentric chromosomes represent a plesiomorphic character. Because *G. setequedas* is a threatened species that occurs in lotic waters, we recommend the maintenance of undammed environments within its known area of distribution.

Keywords: Chromosomes, Conservation, Iguaçu River, Karyotype, Paraná River.

Fornecemos dados citogenéticos para a espécie ameaçada *Gymnogeophagus setequedas*, e o primeiro registro da espécie coletado no rio Iguaçu, na área de preservação ambiental do Parque Nacional do Iguaçu, a qual é uma área de ocorrência inesperada para esta espécie. Verificamos em *G. setequedas* 2n = 48 cromossomos (4*sm* + 24*st* + 20*a*) e heterocromatina presente nas regiões centroméricas e pericentroméricas, as quais indicam caracteres conservados em Geophagini. Múltiplas regiões organizadoras de nucléolos foram observadas em *G. setequedas* e são consideradas características apomórficas em Geophagini, enquanto cístrons de DNAr 5S simples e localizados intersticialmente no braço longo de cromossomos subtelocêntricos representam uma característica plesiomórfica. Visto que *G. setequedas* é uma espécie ameaçada de extinção que ocorre em águas lóticas, recomendamos a manutenção de ambientes livre de barragens em sua área de distribuição.

Palavras-chave: Cariótipo, Conservação, Cromossomos, Rio Iguaçu, Rio Paraná.

Introduction

Neotropical cichlids are arranged in seven tribes (Sparks, Smith, 2004; Smith *et al*., 2008), including Geophagini. Within that tribe, *Gymnogeophagus* Miranda Ribeiro, 1918 can be diagnosed by two apomorphic traits, the absence of a supraneural bone and the presence of a forward-facing spine of the first dorsal pterygiophore (Reis, Malabarba, 1987). *Gymnogeophagus* currently comprises 17 species, distributed in the Paraná, Paraguay and Uruguay River basins, as well as the coastal basins of southern Brazil and Uruguay (Kullander, 2003; González-Bergonzoni *et al*., 2009; Malabarba *et al*., 2015; Loureiro *et al*., 2016). *Gymnogeophagus setequedas* is the only species of that genus considered threatened in all the Brazilian red lists.

Reis *et al*. (1992) described *Gymnogeophagus setequedas* based on 91 specimens collected in Paraguayan rivers and six from a tributary on the Brazilian margin of the Itaipu Reservoir. Since this description, there have been a number of unsuccessful attempts to collect new specimens from within the known geographical range of the species (Agostinho *et al*., 2004; Pavanelli, Reis, 2008). However, during a recent evaluation of the material deposited in the ichthyological collection of the Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul (MCP) (Porto Alegre, Brazil), eight specimens of *G. setequedas* were identified. These specimens were collected in the Iguaçu River, approximately 200 km from the type locality (Iguazú River at Juan E. O'Leary, Paraguay). More recently, surveys conducted both up and downstream from

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the Iguaçu Falls in the Iguaçu National Park resulted in the collection of 15 specimens of *G. setequedas*.

The fish fauna of the Iguaçu River is highly endemic, due to the presence of the Iguaçu Falls, which form a natural barrier to the dispersal of fish, and includes areas of endemism within the river's lower basin (Vera-Alcaraz *et al.*, 2009b). In this case, the occurrence of *G. setequedas* upstream from the falls was unexpected. For instance, Baumgartner *et al*. (2012), who published a catalog of the fish of the lower Iguaçu River basin, did not register the occurrence of *G. setequedas* in that region.

Abilhoa, Duboc (2004) included *Gymnogeophagus setequedas* among the threatened species of the Brazilian state of Paraná, using its restricted distribution and reduction in its available habitat as justification. Pavanelli, Reis (2008) subsequently included *G. setequedas* in the Brazilian red list of endangered species, and it has been listed as Vulnerable (VU) by the International Union for Conservation of Nature (IUCN). In a subsequent review of the Brazilian list (decree #445 in the Official Federal Gazette of December 17, 2014), the Brazilian Environmental Ministry raised the status of the species to Endangered (EN). Vera-Alcaraz *et al.* (2009a) also listed *G. setequedas* as Endangered (EN) in Paraguay.

The unexpected discovery and collection of new specimens of this species also provided an opportunity to expand cytogenetic knowledge of Geophagini. Previous cytogenetic data are available for species of the genera *Geophagus* Heckel, 1840 (Vicari *et al.*, 2006; Pires *et al.*, 2010; Nakajima *et al.*, 2012; Schneider *et al.*, 2013; Perazzo *et al.*, 2013), *Gymnogeophagus* Miranda Ribeiro, 1918 (Pires *et al.*, 2010), *Apistogramma* Regan, 1913, *Biotodoma* Eigenmann & Kennedy, 1903 and *Satanoperca* Günther, 1862 (Poletto *et al.*, 2010a). In the case of *Gymnogeophagus*, cytogenetic studies have focused on just three species, *G. balzanii* (Feldberg, Bertollo, 1984, 1985a; Roncati *et al*., 2007), *G. gymnogenys* and *G. labiatus* (Pires *et al.*, 2010). These species have a diploid number of 48 chromosomes, a centromeric/pericentromeric distribution of heterochromatin, and simple nucleolar organizer regions (NORs), located predominantly on the first chromosome pair. However, the cytogenetics of *G. setequeda*s has been unknown.

In this study, we present the first basic and molecular cytogenetic data for *G. setequedas*, as well as the 5S and 18S rDNA-FISH for the genus as a whole. We also discuss the recent expansion of the known geographical distribution of *G. setequedas* and the implications of that discovery on its current conservation status.

Material and Methods

Fifteen specimens of *Gymnogeophagus setequedas* were analyzed for cytogenetic characters, comprising six females, six males and three specimens of unidentified sex. The sex determination was made by microscopic analysis of the gonads. Specimens were captured in the Iguaçu River upstream (25°37'13.20"S/54°23'29.20"W: NUP 14913 (6) and NUP 15962 (5)) and downstream from the Iguaçu Falls (25°38'18.72"S/54°28'4.74"W: NUP 14919 (3) and NUP 14933 (1)) (Fig. 1). Two additional lots MCP 22632 (8) and NUP 3122 (6) were verified as *G. setequedas* and plotted on the map to help document the geographic expansion of the species. Institutional abbreviations are: MCP, Museu de Ciências e Tecnologia of the Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, and NUP, Coleção Ictiológica do Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura of the Universidade Estadual de Maringá, Maringá.

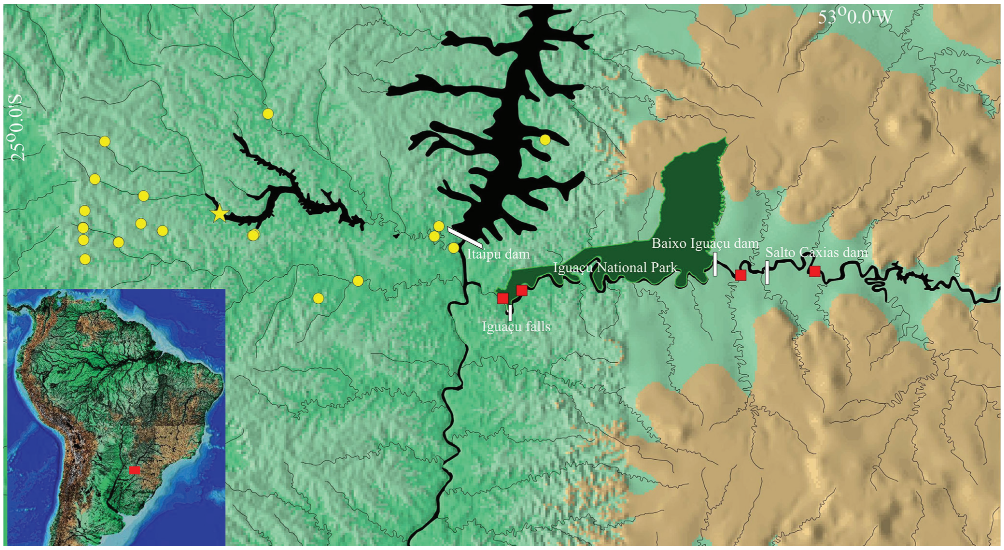

Fig. 1. Partial map of southern South America showing distribution of *Gymnogeophagus setequedas*. Yellow symbols represent lots of specimens from type-material and additional material housed in Swedish Museum of Natural History. The yellow star marks the type-locality (río Iguazú Reservoir, at Juan E. O'Leary, Paraná River basin, Paraguay). Red squares represent the new specimens discovered from the Iguaçu River basin, Brazil. White lines indicate physical barriers to dispersal.

The study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, approved by the Committee on the Ethics of Animal Experiments of the Universidade Estadual do Oeste do Paraná (License Number: Protocol 13/09 - CEEAAP/Unioeste). Individuals were sacrificed with an overdose of clove oil, following Griffiths (2000). Mitotic chromosomes were obtained from kidney cells by direct method of air-drying according to Bertollo *et al*. (1978). Methodologies for detection of NORs by silver impregnation (AgNORs) and heterochromatic regions by technique of barium hydroxide (C-band) followed the protocols of Howell, Black (1980) and Sumner (1972), respectively, with modifications in the step coloration suggested by Lui *et al*. (2012). Physical mapping of 5S rDNA and 18S rDNA sequences was carried out by fluorescence *in situ* hybridization (FISH) in accordance with Pinkel *et al*. (1986) and modifications suggested by Margarido, Moreira-Filho (2008), with probes obtained from *Megaporinus elongatus* (Valenciennes, 1850) (Martins, Galetti, 1999) and *Prochilodus argenteus* Spix & Agassiz, 1829 (Hatanaka, Galetti, 2004), respectively. The 5S rDNA probe was labeled with digoxigenin-11-dUTP (Dig Nick Translation Kit - Roche®, Basel, BS, Switzerland) and the 18S rDNA probe was labeled with biotin-16-dUTP (Biotin Nick Translation Kit - Roche®). Each probe was dissolved at a concentration of 3 ng/µl in a hybridization mixture (50% formamide, 2xSSC, 10% dextran sulfate). Hybridization was carried out in a wet chamber at 37ºC for approximately 16 hours. To detect the signal, the protocol used amplified antidigoxigenina-rhodamine (Roche®) for the 5S rDNA probe and avidin-FITC with anti-avidin biotinylated (Sigma-Aldrich, Buchs, SG, Switzerland) for the 18S rDNA probe. Chromosomes were subsequently counterstained with 4',6-diamidino-2-phenylindol (DAPI) at a concentration of 50 µg/ml. The software DP Controller 3.2.1.276 was used with an Olympus DP 71 digital camera attached to an epifluorescence microscope BX 61 (Olympus America Inc., Center Valley, PA, United States of America) to photograph the slides. To organize the karyotype, we calculated the relation of the arms as proposed by Levan *et al*. (1964).

We also compiled chromosomal data for Cichlidae from this and prior studies, and present those data in a phylogenetic context at the level of genera and in tabular format at the level of species (Tab. 1). Chromosomal data presented for the genera were chosen preferentially for the species included in Smith *et al*. (2008), when chromosomal data for these species were available. Otherwise, we chose a species with the most common chromosomal pattern to represent the genus.

Results

We verified a diploid number of 48 chromosomes (4*sm* + 24*st* + 20*a*) for *Gymnogeophagus setequedas* and observed no differences in karyotype macrostructure either between sexes or between up- and downstream populations (Fig. 2a). The silver impregnation revealed multiple NORs located terminally on the long arm of the subtelocentric chromosome pair 8 and on one of the acrocentric chromosomes of pair 20 (Fig. 2a, in box). Heterochromatin was observed in centromeric/pericentromeric positions in most chromosomes, where it coincided with rDNA sites (Fig. 2b). The 18S rDNA-FISH confirmed the findings of the silver impregnation in both homologous chromosomes of pairs 8 and 20, in addition to highlighting cistrons in the terminal region of the long arm of one of the acrocentric chromosomes of pair 24 (Fig. 2c). The 5S rDNA-FISH revealed cistrons in the interstitial region of the long arm of the subtelocentric chromosome pair 4 (Fig. 2c).

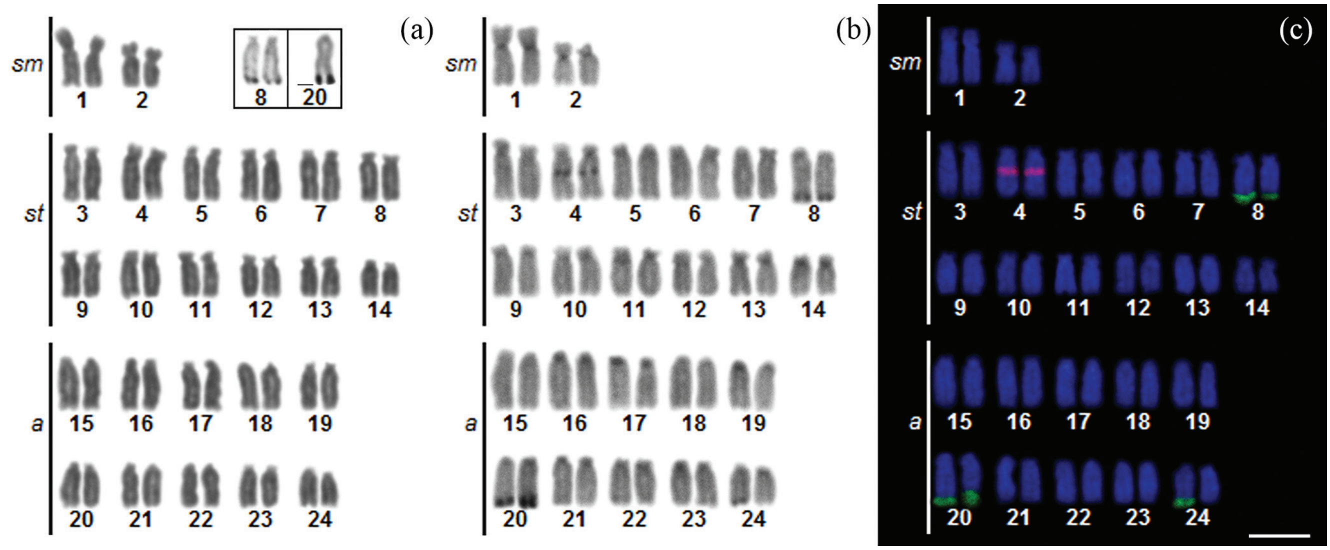

Fig. 2. Karyotypes of *Gymnogeophagus setequedas*: **a.** Giemsa stained; **b.** C-banded; and **c.** after fluorescence *in situ* hybridization with 5S rDNA probe (rhodamine, red) and 18S rDNA probe (FITC, green). NORs bearing chromosomes are boxed in Ag-stained (AgNORs). Scales bar = 5 *µ*m.

Tab. 1. Compilation of cytogenetic data including karyotype formulae, 18S and 5S rDNA for Malagasy, Indian, African and Neotropical cichlid species. 2*n* = diploid number; *m* = metacentric; *sm* = submetacentric; *st* = subtelocentric; *a* = acrocentric; p = short arm; q = long arm; t = terminal position; i = interstitial position; c = centromeric position; chr = chromosomes. References: (1) Nakajima *et al.* (2012); (2) Paiz *et al.* (2014); (3) Perazzo *et al.* (2011); (4) Poletto *et al.* (2010*a*); (5) Poletto *et al.* (2010*b*); (6) Schneider *et al.* (2013); (7) Vicari *et al.* (2006). Classification used follows Smith *et al.* (2008).

Localities **Subfamilies** **Tribes** Species	**2*n***	**Karyotipic formulae**	**18S rDNA**	**5S rDNA**	**Ref.**
Malagasy-Indian species					
Etroplinae					
Etroplus maculatus	46	18*m-sm* + 18*st-a* + 10micro	2chr; t-p; *m-sm*	2chr; i-q; *st-a*	3; 5
African species					
Pseudocrenilabrinae					
Astatotilapia burtoni	40	14*m-sm* + 26*st-a*	2chr; t-p; *st-a*	---	3
Haplochromis obliquidens	44	12*m-sm* + 32*st-a*, 1B or 2B	4chr; t-p; *st-a*	15chr; c; *a*	2; 3
Hemichromis bimaculatus	44	4*m-sm* + 40*st-a*	2chr; t-p; *st-a*	2chr; t-p; *st-a*	3; 5
Oreochromis niloticus	44	2*m-sm* + 42*st-a*	6chr; t-p; *st-a*	6chr; c, i-q; *st-a*	3; 5
Neotropical species					
Cichlinae					
Cichlini					
Cichla monoculus	48	48*st-a*	2chr; t-q; *st-a*	2chr; i-q; *st-a*	6
Retroculini					
Retroculus lapidifer	48	48*st-a*	2chr; t-p; *st-a*	2chr; i-q; *st-a*	3; 5
Astronotini					
Astronotus ocellatus	48	16*m-sm* + 32*st-a*	2chr; i-p; *m-sm*	2chr; i-q; *st-a*	6
Chaetobranchini					
Chaetobranchus flavescens	48	6*m-sm* + 42*st-a*	2chr; i-p; *m-sm*	---	3
Geophagini					
Apistogramma borellii	46	16*m-sm* + 30*st-a*	---	---	3
Biotodoma cupido	48	4*m-sm* + 44*st-a*	2chr; t-p; *m-sm*	---	3
Crenicichla lepidota	48	8*m-sm* + 40*st-a*	2chr; i-p; *m-sm*	4chr; i-q; *st-a*	4
Geophagus brasiliensis	48	6*sm* + 42*st*	2chr; t-p; *st-a*	2chr; i-q; *st-a*	1
Gymnogeophagus setequedas	48	4*sm* + 24*st* + 20*a*	5chr; t-q; *st-a*	2chr; i-q; *st-a*	Present
Satanoperca jurupari	48	4*m-sm* + 44*st-a*	2chr; t-p; *st-a*	2chr; i-q; *st-a*	3; 5
Cichlasomatini					
Acaronia nassa	50	4*m-sm* + 46*st-a*	2chr; i-q; *st-a*	2chr; i-q; *st-a*	6
Aequidens tetramerus	48	12*m-sm* + 36*st-a*	2chr; t-p; *st-a*	4chr; i-q; *st-a*	3; 5
Cichlasoma paranaense	48	6*m-sm* + 42*st-a*	---	---	3
Bujurquina peregrinabunda	50	20*m-sm* + 30*st-a*	2chr; t-p; *m-sm*	2chr; t-q; *st-a*	6
Laetacara dorsigera	44	4*m-sm* + 40*st-a*	2chr; t-p; *st-a*	---	3
Heroini					
Australoheros angiru	48	18*sm* + 30*st-a*	2chr; t-p; *st-a*	2chr; i-q; *st-a*	7
Caquetaia spectabilis	50	12*m-sm* + 38*st-a*	2chr; t-p; *st-a*	4chr; t-p, i-q; *st-a*	6
Heros efasciatus	48	8*m-sm* + 40*st-a*	2chr; t-p; *st-a*	2chr; i-q; *st-a*	3; 5
Hoplarchus psittacus	48	16*m-sm* + 32*st-a*	2chr; t-p; *m-sm*	2chr; t-p; *st-a*	6
Hypselecara coryphaenoides	42	16*m-sm* + 26*st-a*	2chr; i-p; *m-sm*	2chr; i-p; *m-sm*	6
Mesonauta festivus	48	14*m-ms* + 34*st-a*	5chr; t-p; *st-a*	2chr; i-q; *st-a*	3; 5
Pterophyllum scalare	48	16*m-ms* + 32*st-a*	2chr; t-p; *m-sm*	2chr; i-q; *st-a*	6
Symphysodon discus	60	50*m-ms* + 10*st-a*	2chr; t-p; *m-sm*	2chr; t-p; *m-sm*	6
Uaru amphiacanthoides	46	16*m-ms* + 30*st-a*	4chr; t-p; *m-sm*	2chr; i-q; *m-sm*	6

Discussion

Cytogenetic analyses and tendencies of chromosome evolution in the Cichlidae. The diploid number of 48 chromosomes found in *Gymnogeophagus setequedas* is the same as the number observed in congeneric species. However, the karyotype formula for *G. setequedas* (4*sm* + 24*st* + 20*a*) differs from those of its congeners, such as *G. balzanii* (2*m-sm* + 46*st-a*, Roncati *et al.*, 2007), *G. gymnogenys* (4*m* + 44*st-a*; 6*m* + 42*st-a*), and *G. labiatus* (4*m* + 4*sm* + 40*st-a*) (Pires *et al.*, 2010). These differences may reflect structural rearrangements, such as inversions and/or translocations, or may be due to the condensation or reorganization of the chromosomes, as proposed by the respective authors. Similar karyotype formulae have also been found in most other geophagin genera, such as *Geophagus brasiliensis* (6*sm* + 42*st*, Vicari *et al.*, 2006; 4*sm* + 44*st*, Pires *et al*., 2010), *Biotodoma cupido* (4*m-sm* + 44*st-a*) and *Satanoperca jurupari* (4*m-sm* + 44*st-a*) (Poletto *et al.*, 2010a). In *Apistogramma borellii*, however, Poletto *et al.* (2010a) recorded a quite different arrangement (16*m-sm* + 30*st-a*). This arrangement, which includes a large number of meta-submetacentric pairs, is the most differentiated karyotype in the tribe.

Karyotype macrostructure varies considerably in other tribes of the subfamily Cichlinae, such as shown in Tab. 1. Of all the Neotropical cichlids, Smith *et al*. (2008) considered the Heroini as the apical tribe within the Cichlinae (Fig. 3). A diploid set of 48 subtelo-acrocentric chromosomes is considered to be the plesiomorphic condition in the Neotropical group and is observed in *Cichla* Schneider, 1801 (Cichlini) and *Retroculus* Eigenmann & Bray, 1894 (Retroculini) (Poletto *et al.*, 2010a; Valente *et al.*, 2012).

The karyotype macrostructure of *G. setequedas* and its congeners is similar to this ancestral model, but with more subtelo-acrocentric chromosome pairs than meta-submetacentric ones, which appears to be a conserved character in the geophagins.

Silver nitrate impregnation revealed multiple NORs in *G. setequedas*, a pattern also found in one population of *G. gymnogenys* (Pires *et al.*, 2010), but with considerable differences in the NORs distribution. In *G. setequedas*, the NORs are present in a terminal position on the long arm of the subtelo-acrocentric pair 8 and in one acrocentric chromosome of pair 20. In *G. gymnogenys*, by contrast, the NORs are found in an interstitial position on the long arm of the subtelo-acrocentric chromosome pair 3 and a terminal position on the short arm of the subtelo-acrocentric pair 9 (Pires *et al.*, 2010). By contrast, simple NORs were observed in an interstitial position on the short arm of the meta-submetacentric chromosome pair in *G. balzanii* (Roncati *et al.*, 2007), *G. labiatus* (Pires *et al.*, 2010) and *B. cupido* (Valente *et al.*, 2012), and in a terminal position on the short arm of the subtelo-acrocentric chromosome in *Geophagus brasiliensis* (Vicari *et al.*, 2006) and *Satanoperca jurupari* (Poletto *et al.*, 2010a). The presence of simple NORs in the first chromosome pair (meta-submetacentric) is probably a plesiomorphic condition in Neotropical cichlids (Feldberg, Bertollo, 1985b). In this case, multiple NORs in a terminal position of the long arm of the subtelo-acrocentric pair in *G. setequedas* can be considered apomorphic in the Geophagini (Fig. 3), where the location of NORs on different chromosome pairs must be the result of chromosomal rearrangements such as translocations.

The specific implications of the 5S and 18S rDNA-FISH data of *G. setequedas* in comparison with other cichlids. Our FISH data are the first reported for *Gymnogeophagus*, and revealed multiple 18S rDNA cistrons similar to the Neotropical heroins *Mesonauta festivus* and *Uaru amphiacanthoides*, and the African species *Haplochromis obliquidens* and *Oreochromis niloticus* (Pseudocrenilabrinae). However, the number and position of these cistrons vary considerably among species. In *G. setequedas*, they are found in a terminal position on the long arms of the five subtelo-acrocentric chromosomes, whereas in *H. obliquidens*, *M. festivus* and *O. niloticus* these cistrons are located in a terminal position of the short arms of the subtelo-acrocentric chromosomes, in four, five and six pairs, respectively (Poletto *et al.*, 2010a). In the remaining species of the Etroplinae, Pseudocrenilabrinae and Cichlinae, analyses have shown simple 18S rDNA cistrons (Poletto *et al.*, 2010a), which vary in their position (terminal or interstitial) and the chromosome pairs (meta-submetacentric or subtelo- acrocentric) that bear them. *Cichla monoculus* (Cichlini) and *Retroculus lapidifer* (Retroculini) belong to basal cichline genera (Smith *et al.*, 2008), and differ in the location of the 18S rDNA cistrons, which are found in a terminal position on the long arm of the subtelo-acrocentric chromosomes in *C. monoculus* (Schneider *et al.*, 2013) and in a terminal position on the short arm of the subtelo-acrocentric chromosomes in *R. lapidifer* (Poletto *et al.*, 2010a). In *Pseudetroplus maculatus* (Bloch, 1795) (Etroplinae) and *Hemichromis bimaculatus* (Pseudocrenilabrinae), considered here as outgroups, simple 18S rDNA cistrons are located on the short arm of the subtelo-acrocentric chromosomes and represent a plesiomorphic character in the Cichlidae (Fig. 3). In *G. setequedas*, by comparison, multiple cistrons are found in a terminal position on the long arm, indicating that this is an apomorphic character in this species.

The simple 5S rDNA cistrons found in *Gymnogeophagus setequedas* were located in an interstitial position on the long arm of the subtelocentric chromosome pair 4, which is a similar configuration to that found in other geophagin species, such as *Satanoperca jurupari* (Nakajima *et al.*, 2012), *Geophagus proximus* (Schneider *et al.*, 2013) and *G. brasiliensis* (Vicari *et al.*, 2006; Perazzo *et al.*, 2013), and species from closely-related tribes, such as *Cichla monoculus* (Cichlini), *Astronotus ocellatus* (Astronotini) (Schneider *et al.*, 2013), and *Australoheros angiru* (Heroini) (Paiz *et al.*, 2014). However, these cistrons are found in different locations in other species. In *G. brasiliensis* (Perazzo *et al.*, 2013) and *Bujurquina peregrinabunda* (Cichlini) (Schneider *et al.*, 2013), for example, they are found in a terminal position on the long arm of the subtelo-acrocentric chromosomes. In *Hoplarchus psittacus* (Heroini), they are found in a terminal position on the short arm of the subtelo-acrocentric chromosomes, while in *Symphysodon discus* (Heroini), they are in a terminal position on the short arm of the meta-submetacentric chromosomes (Schneider *et al.*, 2013). Multiple 5S rDNA cistrons are uncommon in cichlines, although they have been observed in *Crenicichla lepidota* (Geophagini), on four chromosomes (Perazzo *et al.*, 2011), and in *Aequidens tetramerus* (Cichlasomatini), also on four chromosomes (Nakajima *et al.*, 2012), located in an interstitial position on the long arm of the subtelo-acrocentric chromosomes. In *Caquetaia spectabilis* (Heroini), the cistrons were also found on four chromosomes, located in an interstitial position on the long arm and in a terminal position on the short arm of the subtelo-acrocentric chromosomes (Schneider *et al.*, 2013). Overall, then, simple 5S rDNA cistrons located in interstitial position on the long arm of the subtelo-acrocentric chromosomes, as observed in *G. setequedas* (Fig. 2c), represent a plesiomorphic character (Fig. 3).

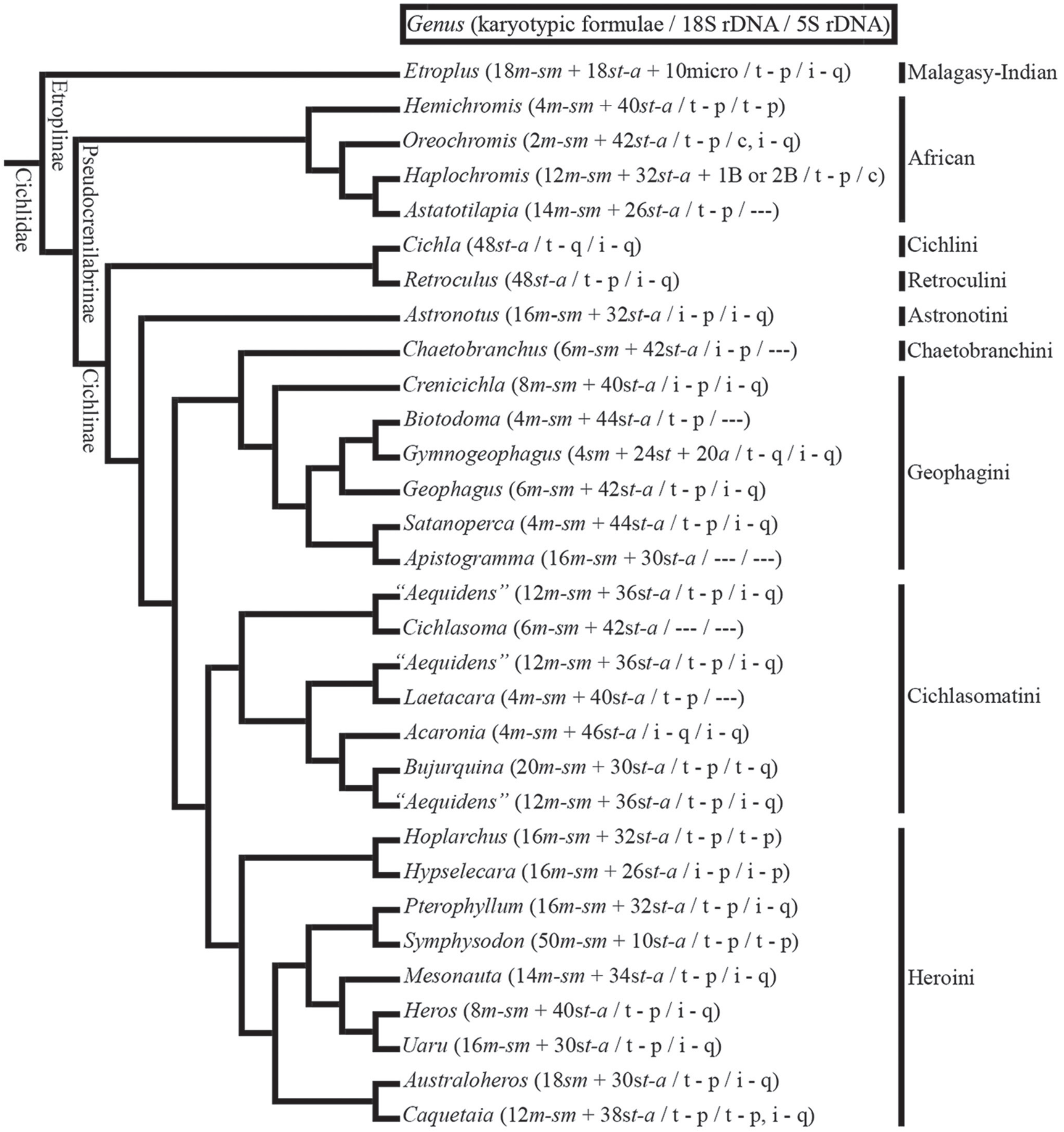

Fig. 3. Phylogeny of Malagasy, Indian, African and Neotropical cichlids showing the karyotypic formulae, 18S and 5S rDNA of a species possessing the most common cytogenetic data for each genus. *m* = metacentric; *sm* = submetacentric; *st* = subtelocentric; *a* = acrocentric; p = short arm; q = long arm; c = centromeric position; t = terminal position; i = interstitial position. The phylogeny is modified from Smith *et al*. (2008).

The presence of heterochromatin in the centromeric and pericentromeric regions of most of the chromosomes of the complement has also been observed in *Gymnogeophagus gymnogenys*, *Geophagus brasiliensis* (Pires *et al.*, 2010), *Biotodoma cupido* and *Geophagus proximus* (Valente *et al.*, 2012) (Geophagini), *Cichla monoculus* (Schneider *et al.*, 2013) (Cichlini), *Cichlasoma facetum* (probably *Australoheros tavaresi* Ottoni, 2012) (Vicari *et al.*, 2006), and the heroin, *A. angiru* (Paiz *et al.*, 2014) (Heroini). This is a common pattern in Neotropical cichlids (Feldberg *et al.*, 2003; Valente *et al.*, 2012). However, while this is a conserved pattern, some variation has been observed, indicating the occurrence of rearrangements during the chromosomal evolution of some groups (Feldberg *et al.*, 2003; Roncati *et al.*, 2007; Perazzo *et al.*, 2013).

Remarks on the conservation status of *G. setequedas*. Since its discovery, the conservation status of *Gymnogeophagus setequedas* has invariably been assigned to a threatened category. The threat has been attributed to lentic waters of the Itaipu Reservoir, which have isolated populations of this rheophilic species, which formerly occurred on tributaries of both banks, in Paraguay and Brazil, and probably in the lower Paraná River proper. After the construction of the reservoir, these populations disappeared. The recent new records of *G. setequedas* in the Iguaçu River extend the geographical distribution of the species from its original description.

Prior to their formal publication herein, these data were also considered in the most recent evaluation of the conservation status of the Brazilian populations, which led to reclassification of *G. setequedas* as endangered in accordance with IUCN (2014) criterion B. Its previously known distribution in the Iguaçu River spanned about 100 km from above the Iguaçu Falls to the middle of the Itaipu Reservoir. That value yields an estimated extent of occurrence (EOO) of just 200 km^2 by multiplying the linear extent of the river where the species occurs by two, according to the IUCN recommendation for the rough calculation of the area of a drainage basin (personal communication with M. F. Tognelli, Program Officer, IUCN | CI Biodiversity Assessment Unit, Global Species Programme, in 2012, when the species was reassessed). The inclusion of the newly discovered lot from Porto Vorá would add a river extension of 225 km^2. Multiplying 225 km^2 by two would result in an addition of 450 km^2 to the extent of occurrence (EOO) of the species, totaling 650 km^2, which is still far below the IUCN threshold of 5,000 km^2 for classifying the species as endangered. The reservoir's severe fragmentation of the population and the continued decline in original extent of occurrence (EOO), area of occupancy (AOO), and area, extent and quality of habitat caused by deforestation, silting and pollution by pesticides, consequences of agriculture and livestock satisfy subcriteria B.1.a(i,ii,iii) and B.1.b(i,ii,iii).

The fact that the known healthy populations of this species occurs only in stretches of the Iguaçu River free of impoundment reinforces the requirement of rapid waters for its survival. Its sporadic capture only twice after building reservoirs, such as Itaipu and Salto Caxias, and its later disappearance detected by monitoring those two large reservoirs confirm its dependence on rapid environments. As the construction of a new hydroelectric power station (Baixo Iguaçu) is already under way between the Caxias Reservoir and the Iguaçu Falls, we strongly recommend keeping the tributaries and the area downstream from the reservoir free of additional dams, to guarantee the long-term survival of the species.

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