Biological and Applied Sciences

Vol.59: e16160102, January-December 2016
http://dx.doi.org/10.1590/1678-4324-2016160102
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Genetic Variability of *Brycon hilarii* in a Repopulation Program

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ABSTRACT

Brycon hilarii, *popularly called piraputanga in Brazil, is a species distributed throughout the whole basin of the river Paraguay. In recent years, the species has been on a repopulation program due to its remarkable decline as a wild species in the region. Assessment of the genetic diversity of broodstock and fingerling stocks in repopulation programs is basic to avoid genetic impacts on wild populations. The genetic variability of the wild population and of the broodstock and fingerling stocks of* B. hilarii *in a repopulation program in the river Itiquira MT Brazil will be determined. Seven microsatellite loci produced 52 polymorphic alleles and heterozygosity revealed rates between 0.5794 and 0.7204.* F_{IS} *did not register any endogamy in the broodstock but it was present in fingerlings and wild populations. Intra- and inter-specific genetic variability rates were higher within each combination but not between groups. Grouping in fingerling groups had a lower density when compared to the others. There is a higher genetic proximity between the natural population and broodstock (0.0237) when the distance between populations was analyzed, even though the two were greatly distant from the fingerling group (0.2622 – 0.2617). Results show that the wild population and the broodstock had high genetic variability and low genetic divergence; contrastingly, fingerlings showed mild genetic variability and great divergence when compared to other groups, indicating that they were not adequately constituted.*

Key words: conservation genetics, microsatellites, piraputanga

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INTRODUCTION

Evolutionary characteristics and the integration of anthropic factors resulted in the homogenization of genetic composition and stock reduction of the wild *Brycon hilarii*. Popularly known as *piraputanga* in the Brazil, the rheophile fish species is distributed throughout the basin of the river Paraguay and highly appreciated for commercial and sports fishing (Reys et al. 2009). However, populations of *B. hilarii* have decreased throughout the basin (Catella et al. 2014), probably due to excessive fishing and anthropic activities, such as the construction of hydroelectric dams, transposition of the riverbed and pollution.

Repopulation programs may not only help in the reconstruction of populations but even minimize impacts on the abundance of several fish species caused by excessive fishing and the destruction of their natural habitat. However, genetic repercussions caused by the introduction of other specimens over the wild population structure should be taken into account (Laikre et al. 2010; González-Wangüemert et al. 2012). The genetic characteristics of fish groups released in certain environments through repopulation program are highly relevant due to the genetic impact on wild populations. Genetic interaction among the fish released and wild populations may cause loss of alleles, especially those related to adaptation (Povh et al. 2008).

Molecular markers, especially microsatellites, have been widely used for information on the genetic variability of broodstocks and wild populations (Povh et al. 2011; Almeida et al. 2013; Lopera-Barrero et al. 2015). Microsatellites warrant a more specific analysis in population structures since they have high genetic mutation rates when compared to other DNA parts. They are actually the best for studies on mating and genic flow (Yue and Orban 2002).

Current assay determines the genetic variability of a wild population, broodstock and fingerlings of *Brycon hilarii* used in a repopulation program in the river Itiquira MT Brazil, by microsatellite markers.

MATERIALS AND METHODS

Samples were retrieved from the caudal fins of 30 *Brycon hilarii*, a broodstock used in repopulation reproductions, 96 fingerlings for repopulation and 30 specimens caught from the mid-river Itiquira MT Brazil, where repopulations programs were undertaken. The 30 broodstocks came from a fish-breeding stock in the municipality of Santo Antônio do Leverger MT Brazil (15° 51'S; 56º 04' W).

DNA extraction of samples from fragments of the caudal fin (0.5 cm^2) followed protocol by Lopera-Barrero et al. (2008). Samples were collected in 1.5 L microtube, to which were added 550 μL of a lysis buffer (50 mM Tris-HCl; 50 mM EDTA; 100 mM NaCl and 1% SDS) and 7 μL of proteinase K (200 μg/mL). Samples were kept in a warm bath overnight at 50ºC. DNA was then purified with 400 μL sodium chlorate (5 M) and centrifuged for 10 min at 14000 rpm; it was later precipitated with 700 μL iced absolute ethanol. Precipitate DNA was washed in ethanol 70%, re-suspended in TE (10 mM Tris and 1 mM EDTA), treated with 6 μL RNAse (30 μg/mL), and kept in a warm bath at 37ºC for 40 min.

DNA was counted in a Shimadzu spectrophotometer (Shimadzu Corporation, Kyoto, Japan), absorbance 260 nm, while samples were standardized for a concentration of 10 ng/μL. DNA quality was checked by agar gel electrophoresis gel 1%, in buffer TBE 1X (500 mM Tris-HC1, 60 mM boric acid and 83 mM EDTA) for 1 h at 70 volts. The gel was monitored under ultraviolet radiation after exposure to ethidium bromide (0.5 μg/ml) for one hour.

Seven microsatellite loci (Bh5, Bh6, Bh8, Bh13, Bh15, Bh16, Bh17), described by Sanches and Galetti Jr (2006), were amplified (Table 1). DNA was amplified in a 20 μL reaction volume with buffer 1X Tris-KCl, 2.0 mM $MgCl_2$, 0.8 μM of each primer (forward and reverse), 0.2 mM of each dNTP, one unit of Platinum Taq DNA Polymerase and 20 ng DNA. DNA was first de-natured at 94ºC for four minutes and then 30 consecutive circles were performed, made up of 30 initial seconds at 94ºC, one minute annealing (Bh5: 52ºC; Bh6, Bh8, Bh13, Bh15 and Bh17: 56ºC; and Bh16: 58ºC) and one extension minute at 72ºC. There was a final

extension at 72ºC for 10 minutes. The reactions were amplified in a Bio-Rad thermocycler (BIO-RAD, Hercules CA USA) and amplified samples underwent polyacrylamide gel electrophoresis 10% (acrylamide:bisacrylamide – 29:1) de-naturing (6 M urea) for 12 hours at 15 mA. Staining by silver nitrate visualized the alleles (Bassam et al. 1991). Gel underwent three types of solution: the first was constituted by a fixation solution (10% ethanol and 0.5% acetic acid) for 20 minutes; the second was an impregnation solution (6 mM silver nitrate) for 10 minutes; the third was a revelation solution (0.75 M NaOH and 0.22% formaldehyde 40%), photographed by Canon CoolPix 5200. Size of fragments was estimated by comparing with standard 10, 50 and 100 bp ladder (Invitrogen, USA).

Observed (Ho) and expected (He) heterozygosity, number of migrants (Nm), fixation index (F_{IS}) and Hardy-Weinberg equilibrium (Wright 1978) were calculated by GENEPOP 1.2 (Raymond and Rousset 1995), while PopGene 1.31 calculated allele frequency, distance and genetic identity and true number of alleles (Âe) (Yeh et al. 1999). MEGA 5.2.2 (Tamura et al. 2011) prepared the dendrogram, while Weir and Cockerham´s method (1984) with FSTAT 2.9.3 (Goudet 2001) estimated rates of genetic differentiation (F_{ST}). Arlequin 3.0 (Excoffier et al. 2005) determined genetic differentiation by F_{ST} estimates (Weir and Cockerham 1984) and for Analysis of Molecular Variance (AMOVA) (Excoffier et al. 1992).

RESULTS AND DISCUSSION

The seven microsatellite loci provided 52 polymorphic alleles. Allele size ranged between 125 bp (Bh13 and Bh15) and 233 bp (Bh8). At the most, twelve alleles were observed for locus Bh8 and a minimum of three alleles for Bh15. There was a minimum of one allele (Bh15) and a maximum of seven alleles (Bh17 and Bh8) within each group of wild population and fingerlings. The former had the greatest number of alleles (4.3), followed by those from the broodstock (4.2) and fingerling stocks (4.1) (Table 1).

Table 1 - Characterization of microsatellite loci, sequence of loci, size and number of alleles (bp) and number of alleles per locus of wild population (WP), broodstock (BS) and fingerlings (FG) of *Brycon hilarii.*

Locus	Sequence	Size of alleles (bp)	N. of alleles	WP	BS	FG
Bh5	F:CTTCCACTCATACCGGCACT R:ACATCTGGCATTAGGCATAG	202-212	3	-	-	3
Bh6	F: GCGTTGCGTGTGTATGTTAA R:AGAGGTGTCCACAAAGTTTT	150-188	9	4	4	4
Bh8	F:CCATGGCTCAACACAGATA R:TGTACGAATCCTGAAATGCT	142-233	12	7	6	5
Bh13	F:AGCAATTTAAGCAAGTGAAG R:GCGTCGGAGCAGTAGTTATA	125-185	8	4	4	5
Bh15	F:GAGAGCATTGTCAGGATTTA R:ACTAATGACTGCTACTGCGG	125-142	4	1	3	2
Bh16	F:CCTCCAATGAAAACAGTGCG R:ACGACTTAGCCACCCACCCT	141-163	6	4	4	3
Bh17	F:GTCAGCACTCAGCACATAGC R:AGAGAGCCTGAAAGTGAGTC	152-222	10	6	4	7
TOTAL		125-233	52	4.3	4.2	4.1

Allele size and number per locus in current assay were similar to size and number for *B. hilarii* reported by Sanches and Galetti Jr (2006). The number of alleles for the three genetic groups under analysis was higher than that registered by Lopera-Barrero et al. (2014) for broodstock and wild population of *B. hilarii*, by Lopera-Barrero et al. (2015) for broodstock and wild population of *Prochilodus lineatus*, and by Povh et al. (2011) for broodstock and fingerling stocks of *Piaractus mesopotamicus*, respectively with 11, 44 and 31 alleles. Therefore, the number of alleles in current assay is adequate when compared to that in similar experiments.

The three exclusive alleles in locus Bh5 for fingerling stocks demonstrate that the group´s ancestry manifests genetic difference when contrasted to broodstock and wild population analyzed. Most probably the fingerling stock group had been formed by an ancestry that was not highly representative of the broodstocks and that adopted reproduction management should have benefitted the ancestry that failed to be represented in the broodstock sampling.

Low frequency alleles (< 0.1000) were reported in the three groups, with a higher percentage in the wild population (9 alleles) when compared to the stock of broodstocks (7 alleles) and fingerlings (6 alleles); the lowest allele frequency (0.0053) was registered in locus Bh8 (145 bp) and the highest (0.7384) in locus Bh16 (145 bp), in the fingerling stock. Twenty-one exclusive alleles (alleles of locus Bh5) were reported in the fingerling stock, five in the wild population and four in the broodstock (Table 2). Since no alleles were reported in loci Bh6 and Bh17, a lack of equilibrium was detected in the link between the alleles of the three groups.

Table 2 - Characterization of allele frequency of wild population (WP), broodstock (BS) and fingerlings (FG) of *Brycon hilarii.*

Locus	Size (bp)	WP	BS	FG
BH5	212	0.0000	0.0000	0.3882
	206	0.0000	0.0000	0.1842
	202	0.0000	0.0000	0.4276
BH6	150	0.0000	0.0000	0.1022
	158	0.0000	0.0741↓	0.0000
	160	0.0000	0.0000	0.4946
	162	0.0862↓	0.0000	0.0000
	164	0.0000	0.0000	0.1667
	166	0.0000	0.0000	0.2366
	173	0.5862	0.1296	0.0000
	185	0.0517↓	0.2963	0.0000
	188	0.2759	0.5000	0.0000
BH8	142	0.0000	0.1207	0.0000
	145	0.0000	0.0000	0.0053↓
	150	0.3500	0.1207	0.0947 ↓
	157	0.0333 ↓	0.0000	0.0000
	190	0.0000	0.0690↓	0.0000
	195	0.1167	0.1379	0.0000
	198	0.1000	0.0690↓	0.0000
	200	0.0000	0.0000	0.1316
	203	0.3333	0.4828	0.0000
	205	0.0000	0.0000	0.6053
	210	0.0167↓	0.0000	0.1632
	233	0.0500↓	0.0000	0.0000
BH13	125	0.1071	0.2778	0.0000
	130	0.0000	0.0000	0.1702
	150	0.2143	0.2778	0.3032
	155	0.2857	0.2037	0.0000
	158	0.3929	0.2407	0.0000
	165	0.0000	0.0000	0.3298
	175	0.0000	0.0000	0.1383
	185	0.0000	0.0000	0.0585↓
BH15	125	0.0000	0.0172↓	0.0000
	130	0.0000	0.0000	0.5798
	136	0.3621	0.3621	0.0000
	138	0.0000	0.0172↓	0.4202
BH16	141	0.0000	0.0000	0.1512
	145	0.0000	0.0000	0.7384
	147	0.0227↓	0.1818	0.1105
	150	0.4545	0.4773	0.0000

	155	0.4773	0.3182	0.0000
	163	0.0455↓	0.0227↓	0.0000
BH17	152	0.0000	0.0000	0.1223
	160	0.2143	0.1429	0.0957↓
	165	0.0714↓	0.0000	0.0000
	170	0.1964	0.0536↓	0.1596
	178	0.0000	0.0000	0.2181
	180	0.3036	0.3929	0.2979
	193	0.0179↓	0.0000	0.0000
	200	0.0000	0.0000	0.0266↓
	212	0.0000	0.0000	0.0798↓
	215	0.1964	0.4107	0.0000

↓Low frequency alleles.

Alleles´ frequency size between the broodstocks and the wild population remained stable. Although locus Bh6 has the most discrepant allele frequencies among broodstock and wild population, most alleles of the other loci had similar frequencies among these groups. Three out of the four alleles were similar in locus Bh16 for broodstock and wild population, corroborating the genetic similarity between the two groups. Locus Bh13 had a similar situation, or rather, all the alleles in the broodstocks and wild population were similar, with similarity in only one allele in fingerlings (150 bp – 0.3032). A greater number of exclusive alleles in fingerlings reveals genetic difference of the group when compared to broodstocks and wild population.

Data of mean observed heterozygosity (Ho) indicate a greater genetic variability in the wild population and broodstock (0.6290; 0.7204) when compared to the fingerling stock (0.5794). The low genetic variability of the wild population when compared to the broodstock may be due to the appearance of nil alleles in two loci (Bh6 and Bh17), revealing a lower occurrence of heterozygosity which, albeit with small intensity, may provide a distancing from the Hardy-Weinberg´s equilibrium percentage which is not expected in wild populations. Only Bh8 among the loci studies complied with the expected Hardy-Weinberg´s equilibrium in the three populations, forming a higher percentage of heterozygotes (Table 3).

Table 3 - Observed heterozygosity (Ho), expected heterozygosity (He), fixation index (F_{IS}), Hardy-Weinberg equilibrium test (HWE), number of alleles per locus (NA) and allele richness ($\mathbf{A_R}$) of wild population (WP), broodstock (BS) and fingerlings (FG) of *Brycon hilarii*.

Group	Locus	Ho	He	F_{IS}	HWE	NA	A_R
	Bh5	--	--	--	--	--	--
	Bh6	0.3448	0.5802	0.4099	**	4.0	3.9
	Bh8	0.7667	0.7514	-0.0207	NS	7.0	6.6
WP	Bh13	0,6071	0.7195	0.1586	**	4.0	4.0
	Bh15	0.4483	0.4701	0.0471	**	1.0	2.0
	Bh16	1.0000	0.5761	-0.7667	NS	4.0	4.0
	Bh17	0.6071	0.7935	0.2382	**	6.0	5.7
	Mean	0.6290	0.6485	0.0110	**	Σ=26.0	4.33-4.36
	Bh5	--	--	--	--	--	--
	Bh6	0.5185	0.6520	0.2078	**	4.0	3.9
	Bh8	0.7931	0.7217	-0.1009	NS	6.0	5.9
BS	Bh13	0.8519	0.7603	-0.1230	NS	4.0	4.0
	Bh15	0.5517	0.5130	-0.0769	NS	3.0	3.9
	Bh16	1.0000	0.6522	-0.5529	NS	4.0	4.0
	Bh17	0.6071	0.6656	0.0893	**	4.0	3.9
	Mean	0.7204	0.6608	-0.0927	NS	Σ=25.0	4.17-4.26
	Bh5	0.3026	0.6367	0.5264	**	3.0	3.0
	Bh6	0.7849	0.6647	-0.1820	NS	4.0	4.0
	Bh8	0.7263	0.5838	-0.2458	NS	5.0	4.8
FG	Bh13	0.7447	0.7518	0.0095	**	5.0	5.0
	Bh15	0.4787	0.4899	0.0229	**	2.0	2.0
	Bh16	0.0930	0.4222	0.7807	**	3.0	3.0

Bh17	0.9255	0.8114	-0.1416	NS	7.0	7.0
Mean	0.5794	0.6229	0.1100	**	Σ=29.0	4.14-4.11

**=significant deviation; NS= non-significant deviation

Sanches and Galetti Jr (2012) analyzed populations of *B. hilarii* collected in several different places of the sub-basin of the river Miranda (river Paraguay basin) by seven microsatellite loci and reported two genetic populations. However, loci Bh6 and Bh13 failed to comply with the expected Hardy-Weinberg equilibrium. In fact, there was a deficit in heterozygosity, corroborating results in the wild populations reported in current analysis (Table 3). On the other hand, the inability of separating near-sized alleles, made difficult by stutter bands in the di-nucleotide microsatellite electrophorese, may have also decreased heterozygosity rates (Hassanien and Gilbey 2005; Shyamala et al. 2014). In the case of the fingerling group, mean rates for Ho reached 0.5794 and deviants in the Hardy-Weinberg equilibrium were reported in loci Bh5, Bh13, Bh15 and Bh16 with heterozygous deficits, corroborated by the mean rate of the fixation index (F_{IS}= 0.1100). The above results revealed a close genetic factor among these specimens, perhaps due to reproduction management, already indicated by Lopera-Barrero et al. (2014), or to the control of cannibalism, as underscored by Borba et al. (2006). The two factors may have reduced the effective number of broodstocks and genetic variability.

The use of small numbers of broodstocks (with specimens in total spawning) and non-intentional selection (selection of broodstocks with certain specific characteristics) are usually the main factors that decrease genetic variability and increase endogamy in fingerlings. Further, in the case of inadequate management, possible cannibalism by post-larvae *B. hilarii* during the early phase (usually 36 – 96 hours old) may have decreased the effective number of broodstocks and, consequently, genetic variability.

A selection of beneficent characteristics for breeding, such as fish selection merely based on external characteristics (male: semen release after encephalic-caudal pressure; female: swelled belly and a reddened urogenital orifice), maturation stage of oocytes (migrated nucleus), regardless of parenthood, variability and genetic divergence, and the effective number of broodstocks (Povh et al. 2011) may greatly reduce genetic variability within a single generation and decrease fingerlings´ viability within the natural environment (Christie et al. 2012). Povh et al. (2008) studied populations of pacu (*P. mesopotamicus*) for genetic monitoring and registered high rates of genetic variability in fingerlings when compared to broodstocks on the same site. This fact demonstrates that management in fish breeding maintained the ancestors´ genetic base. Since the above has not been observed in current assay, the fingerling sampling evaluated may reveal that repopulation may be including gene pools different from those from natural populations, or the absence of important genes for the feasibility of fish in the environment.

Fixation Index (F_{IS}) provided negative rates (excess of heterozygotes) in six loci of broodstock population, averaging -0.0927, albeit with a non-significant Hardy-Weinberg equilibrium, which indicated absence of endogamy (Table 3). In the wild population, average F_{IS} was positive (0.0110), with significant Hardy-Weinberg equilibrium, which indicated mild endogamy, perhaps related to the decrease in size of *B. hilarii* population. In fact, the capture of the species decreased in the last few years (Catella et al. 2014).

With regard to allele richness (A_R) (Table 3), there was a slight variation in the number of alleles in all groups studied (wild population A_R =4.33 – 4.36), (broodstocks A_R = 4.17 – 4.26), (fingerlings A_R = 4.14 – 4.11). Purcell et al. (2012) also reported higher allele richness in the wild population of *Gambusia affinis* (A_R = 11.4–12.3) when compared to that of introduced populations (A_R = 4.5–7.7). Decrease in allele richness in broodstocks may cause the animals to lose or limit their capacity to respond to changes in the natural environment (Christie et al. 2012).

AMOVA results show that most variability lies within each population (97.62; 73.77; 73.83), in the three groupings (wild population x broodstocks; wild population x fingerlings; broodstocks x fingerlings), respectively (Table 4). Rates are more representatives in the grouping wild population x broodstocks, and indicate that the two form a single genetically structured population. Comparison data between wild population and broodstocks with fingerlings reveal that fingerlings have a basic genetic structure that is different from natural populations and from broodstocks. There may have been a decrease in the true number of broodstocks (N_e) due to reproduction management or to cannibalism-control management, or even to the

fact that the sample of the broodstocks was not representative.

Table 4 - Analysis of molecular variance (AMOVA) of different groupings with wild population, broodstock and fingerlings of *Brycon hilarii*.

Grouping	FV	SMQ	CV	%V
Wild population	EG	1.442	0.01426	2.38
x	DG	69.133	0.58588	97.62
Broodstock	Total	70.575	0.60014	100.00
Wild population	EG	52.538	0.55748	26.23
x	DG	392.053	1.56821	73.77
Fingerlings	Total	444.591	1.62149	100.00
Broodstock	EG	51.708	0.54863	26.17
x	DG	386.903	1.54761	73.83
Fingerlings	Total	438.611	2.09624	100.00

EG: between groups; DG: within groups. *P>0.05.

AMOVA data, confirmed by Wright´s genetic differentiation rates (1978) and the coefficient of ancestrality (F_{ST}) (Table 5), revealed that fingerlings demonstrated high genetic differentiation from the other groups. They may be a single population unit, since the group had not been constituted with enough genetic variability. The genetic identity in the groupings wild population x fingerlings (0.2154) and broodstocks x fingerlings (0.2169) and their identities (1.535 and 1.5282 respectively) reinforce the slight genetic closeness of natural populations and broodstocks with the fingerlings (Table 5).

Table 5 - Coefficient of ancestrality (F_{ST}), Wright´s genetic differentiation (1978) (Wright), genetic identity (GI), genetic distance (GD) and number of migrants (Nm) of different groupings of wild population, broodstock and fingerlings of *Brycon hilarii*.

Group	Wild population x Broodstock	Wild population x Fingerlings	Broodstock x Fingerlings
F_{ST}	0.02377	0.26226	0.2617
Wright	Small differentiation	Big differentiation	Big differentiation
GI	0.8662	0.2154	0.2169
GD	0.1424	1.5353	1.5282
Nm	6.1021	0.7851	0.800

Since lowest F_{ST} rate occurred between the wild population and the brookstocks (0.02377), only a small genetic differentiation was extant (Wright 1978). The rate of genetic identity (0.8662) and genetic distance (0.1424) for the same group revealed genetic relationship. A higher rate for the number of migrants (6.1021) has been reported in the grouping (wild population x broodstocks), probably caused by the evidence of genic flux of these populations (Table 5). Dendrogram (Fig. 1) shows the genetic relationship between the three groups (wild population, broodstocks and fingerlings) and the shortest distance between the wild population and the broodstocks.

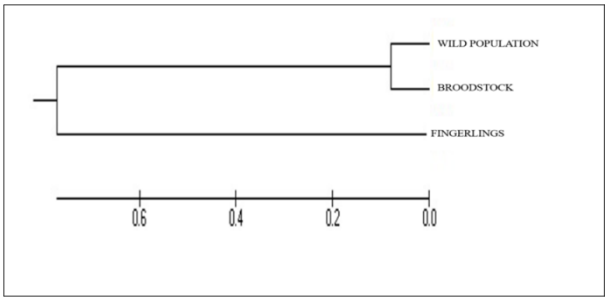

Figure 1 - Dendrogram of groups wild population (WP), broodstock (BS) and fingerlings (FG) of Brycon hilarii, based on Nei´s genetic distance (Nei, 1972).

Results show that the wild population of *B. hilarii* should be further analyzed, since a decrease in heterozygosity may occur even with the best

variability levels, due to the introduction of animals with low genetic diversity coupled to reduction in population levels. Lower variability will produce not only several changes in allele frequencies, with a subsequent Hardy-Weinberg imbalance, but also disorders in the populations´ genic links.

Analyses of fish broodstocks revealed that the animals kept intact their genetic base. It is highly recommended that, due to the fingerlings´ genetic variability, further analyses should be performed with a larger sample of *B. hilarii*. Further, the species´s reproduction management should also be investigated by assessing the number of broodstocks, type of reproduction system (extrusion or semi-natural) since reproduction management and control of cannibalism may directly affect the offspring´s genetic variability.

Results in current study revealed the profile of fingerling stocks used in the repopulation of *B. hilarii* in the river Itiquira MT Brazil and recommended that new reproduction management and control of cannibalism should be taken for the preservation of the species, coupled to the maintenance of the genetic integrity of the population in its natural environment.

CONCLUSIONS

Genetic variability in the natural population and in broodstocks was high, albeit moderate in the fingerling stock. Genetic distancing was small between the wild population and the broodstocks, both of which had great genetic differentiation with the fingerling group.

ACKNOWLEDGMENTS

The authors would like to thank Group Bom Futuro and Nortox for their financial support.

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Received: May 07, 2016;
Accepted: June 01, 2016