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BIOLOGY

Cross-amplification of heterologous microsatellite markers in Piracanjuba

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ABSTRACT: *Brycon orbignyanus, popularly known in Brazil as piracanjuba, is a fish with great economic value but whose natural population drastically decreased in number during the last years. In this context, genetic variability studies of natural stocks and in restocking programs are fundamental for the adoption of conservation measures. Current analysis verifies the cross-amplification of heterologous primers in B. orbignyanus. Fifty-two primers of the species Brycon opalinus, Brycon hilarii, Brycon insignis, Prochilodus sp., Piaractus mesopotamicus, Colossoma macropomum and Oreochromis niloticus were tested. Primers with the best reproducibility were applied to a sample of 20 individuals and the genetic parameters were calculated. Nine primers provided good results for cross-amplification with B. orbignyanus, involving (BoM5 and BoM13) of Brycon opalinus, (Bh5, Bh6, Bh8, Bh13 and Bh16) of Brycon hilarii, (Bc48-10) of Brycon insignis and (Par80) of Prochilodus argenteus. Primers of Piaractus mesopotamicus, Colossoma macropomum and Oreochromis niloticus failed to provide amplification or provided non-specificity. Results demonstrated the possibility of using primers of different species and genera of B. orbignyanus, facilitating genetic studies on the species.*
Key words: *Brycon orbignyanus, conservation, molecular markers, heterologous primers.*

Amplificação cruzada de marcadores microssatélites heterólogos em piracanjuba

RESUMO: *A piracanjuba (Brycon orbignyanus) é um peixe de grande valor econômico que nos últimos anos tem apresentado uma redução drástica em suas populações naturais. Nesse contexto, estudos de variabilidade genética dos estoques naturais e nos programas de repovoamento são fundamentais para adoção de medidas conservacionistas. O objetivo do presente trabalho foi verificar a amplificação cruzada de primers heterologos em B. orbignyanus. Foram avaliados um total de 52 primers das espécies Brycon opalinus, Brycon hilarii, Brycon insignis, Prochilodus sp., Piaractus mesopotamicus, Colossoma macropomum e Oreochromis niloticus. Os primers com melhor reprodutibilidade foram aplicados a uma amostra de 20 indivíduos e os parâmetros genéticos foram calculados. Nove primers apresentaram resultados satisfatórios de amplificação cruzada com B. orbignyanus, sendo das espécies Brycon opalinus (BoM5 e BoM13), Brycon hilarii (Bh5, Bh6, Bh8, Bh13 e Bh16), Brycon insignis (Bc48-10) e Prochilodus argenteus (Par80). Os primers de Piaractus mesopotamicus, Colossoma macropomum e Oreochromis niloticus não apresentaram amplificação ou apresentaram inespecificidade. Os resultados revelaram a possibilidade da utilização de primers de diferentes espécies e gênero em B. orbignyanus, o que facilita a realização de estudos genéticos nessa espécie.*
Palavras-chave: *Brycon orbignyanus, conservação, marcadores moleculares, primers heterólogos.*

INTRODUCTION

Brycon orbignyanus (Valenciennes, 1849), or piracanjuba, is a fish originally native to the Uruguay and Paraná river basins. It is omnivorous but easily feeds on an artificial diet when bred in fish ponds. Its meat is greatly appreciated. However, the natural stocks of the species had decreased drastically due to human activities such as dam construction, overfishing and deposition of contaminated wastes in Brazilian rivers. In fact, it is on the list of endangered fish (ROSA & LIMA, 2008).

Consequently, genetic studies are greatly relevant to monitor the species since they may be the basis of conservation and production programs (RIBEIRO et al., 2016). Microsatellite markers are currently widely used for assessment since they provide a great amount of information due to high polymorphism and their co-dominant characteristics (ABDUL-MUNEER, et al., 2014). However, the

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marker requires previous information on the genome (ABDUL-MUNEER, et al., 2014), limiting the use of the technique for specimens with no species-specific primers, as is the case for *B. orbignyanus*.

There are two solutions for the above issue: the construction of species-specific microsatellite primers or the transferability of primers between the species (heterologous primers). The first solution is time-consuming and costly, limiting the construction of the markers (PENTEADO et al., 2011). Alternatively, primers of related species or phylogenetically close species may be transferred through the annealing of microsatellite sequences (MIA, 2005).

In the case of the genus *Brycon* sp., several studies were successful for primer transferability between species. SANCHES & GALETTI (2006) identified cross-amplified primers of *B. hilarii* for the five species of *Brycon* sp., including *B. orbignyanus*. LOPERA-BARRERO et al. (2014) detected transferability of *B. opalinus* for broodstocks and fries of *B. orbignyanus* bred in fish ponds, similar to ASHIKAGA et al. (2015) for natural populations. However, due to the restricted number of research on the species, scanty information exists on the use of heterologous primers among *Brycon* species and much less among different genera.

Current analysis verifies the cross-amplification of microsatellite markers of eight fish species (*Brycon opalinus, Brycon hilarii, Brycon insignis, Prochilodus argenteus, Prochilodus lineatus, Piaractus mesopotamicus, Colossoma macropomum* and *Oreochromis niloticus)* in *B. orbignyanus*.

MATERIALS AND METHODS

Samples of the caudal fin (approximately 0.5cm²) of five *B. orbignyanus* specimens from the restocking center of the AES-Tietê were collected. DNA was extracted following methodology by LOPERA-BARRERO et al. (2008). Total DNA concentration was assessed by measuring samples by spectrophotometry PICODROP® (Picodrop Limited, Hinxton, UK). Samples were diluted in a concentration 20ng μL^{-1}. DNA integrity was evaluated in agar gel 1%, stained with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad CA, USA). Electrophoresis was performed in buffer TBE 0.5 X (250mM Tris-HC1, 30mM boric acid and 41.5mM EDTA) for one hour, at 70V. Gel was observed in an UV trans-illuminator and image was photographed with Kodak EDAS (Kodak 1D Image Analysis 3.5).

Fourteen loci of microsatellite regions developed for the genus *Brycon* were tested for the cross-amplification of the primers: six were described by BARROSO et al. (2003) for *B. opalinus* (BoM1, BoM2, BoM5, BoM6, BoM7 and BoM13), seven by SANCHES & GALETTI (2006) for *B. hilarii* (Bh5, Bh6, Bh8, Bh13, Bh15, Bh16 and Bh17) and one by MATSUMOTO & HILSDORF (2009) for *B. insignis* (Bc48-10). The following 38 primers were also tested: eleven primers of curimba (*Prochilodus sp*): Par12, Par14, Par15, Par21, Par43, Par80, Par82 (*Prochilodus argenteus*), Pli01, Pli30, Pli60 and Pli43 (*Prochilodus lineatus*) (YAZBECK & KALAPOTHAKIS, 2007; BARBOSA et al., 2008); eight primers of the pacu (*Piaractus mesopotamicus*): Pme2, Pme4, Pme5, Pme14, Pme20, Pme21, Pme28 and Pme32 (CALCAGNOTTO et al. 2001); 10 primers of the tambaqui (*Colossoma macropomum*): CmA8, CmA11, CmB8, CmC8, CmD1, CmE3, CmF4, CmF5, CmF7 and CmH8 (SANTOS et al., 2009); nine primers of the tilapia (*Oreochromis niloticus*) UNH 104, UNH 108, UNH 136, UNH 140, UNH 159, UNH 160, UNH 162, UNH 163 and UNH 169 (LEE & KOCHER, 1996).

Amplification was performed for a final 15µL reaction volume with 1X of buffer Tris-KCl, 2.0mM of $MgCl_2$, 0.8µM of each primer (forward and reverse), 0.4mM of each dNTP, one unit of Platinum Taq DNA Polymerase and 20ng of DNA. Primers described for *P. mesopotamicus, P. lineatus* and *O. niloticus* were amplified as follows: DNA was denatured at 94ºC for four minutes, followed by 30 cycles of 30 seconds for the initial denaturation at 94ºC; 30 seconds of annealing (variable temperature for each primer) and a 60-second extension at 72ºC; a final extension at 72ºC for 10 minutes was done. In the case of primers for *C. macropomum, B. opalinus, B. hilarii* and *B. insignis*, amplification conditions were: initial denaturation at 94ºC for four minutes; thirty cycles of denaturation at 94°C for 60 seconds; 60 seconds for annealing (variable temperature for each primer) and 60 seconds extension at 72ºC; final extension at 72ºC for 10 minutes.

Amplified samples underwent polyacrylamide gel electrophoresis 10% (acrylamide: bisacrylamide - 29:1) denaturant (6M urea) and placed in a buffer TBE 0.5X with 180V and 250mA for eight hours. Staining by silver nitrate was used to visualize microsatellite alleles. Consequently, gel underwent fixation solution (10% ethanol and 0.5% acetic acid) for 20 minutes, followed by a solution of 6mM of silver nitrate for 30 minutes and revealed in a solution with 0.75M NaOH and 0.22% formaldehyde

40%, and photographed by Nikon CoolPix 5200 for later analyses. Allele size was calculated by Kodak EDAS-290 with 50 and 100bp DNA ladder. Primers with good cross-amplification results were amplified for 20 specimens of *B. orbignyanus* for the calculation for genetics parameters with the same methodology described previously.

The allele frequency and fixation index (Fis) were calculated using FSTAT 2.9.3 software (GOUDET, 2005). The presence of null alleles was tested by the Micro-Checker software (VAN OOSTERHOUT et al., 2004). Number of Alleles (Na), number of Effective Alleles (Ne), Observed Heterozygosity (Ho), Expected Heterozygosity (He), Inbreeding coefficient (Fis) and HardyWeinberg equilibrium (P>0,05) was calculated by GenAlex 6.5 (PEAKALL & SMOUSE, 2012). Polymorphic information content (PIC) was calculated by Cervus 3.0.7 (KALINOWSKI et al., 2007).

RESULTS

Nine out of the 52 heterologous primers had good cross-amplification results for *B. orbignyanus*, or rather, eight derived from fish of the genus *Brycon* (*B. opalinus*: BoM5 and BoM13; *B. hilarii*: Bh5, Bh6, Bh8, Bh13 and Bh16, and *B. insignis*: Bc48-10) and one derived from *Prochilodus argenteus* (Par80). Allele size ranged between 76bp (Bc48-10) and 225 bp (Bh5) (Table 1). Excepting Par80, all primers of *P. argenteus*, *P.lineatus*, *P. mesopotamicus*, *C. macropomum* and *O. niloticus* either lacked amplification or did not show any specificity. The presence of null alleles was verified at Bh8 loci.

Number of alleles per locus ranged from two (Bh6, BoM5 and Par80) to four (Bc48-10). The mean value for the expected heterozygosity (He) was higher than observed heterozygosity (Ho). The coefficient inbreeding was positive and significative ($P<0.05$) in five loci (Bh5, Bh8, Bh13, Bh16 and Bc48-10), and negative and significative in four (Bh6, BoM5, BoM13 and Par80). A deviation from Hardy-Weinberg equilibrium ($P<0.05$) was observed in tree four (Bh8, Bh13, Par80 and Bc48-10). Polymorphic information content (PIC) varied from 0,215 (Bh5) to 0.609 (Bc48-10) (Table 2).

DISCUSSION

The size of alleles produced by primers derived from the genus *Brycon* was similar to that in previous research , as *B. hilarii* (SANCHES & GALETTI, 2006; BIGNARDI et al., 2016), *B. insignis* (MATSUMOTO & HILSDORF, 2009) and *B. orbignyanus* (LOPERA-BARRERO et al., 2014); however, the number of alleles was lower than reported by these studies. The primer Par80 (*P. argenteus*) had the same result, albeit, a different genus (BARBOSA et al., 2008; LOPERA-BARRERO et al., 2016a). Low number of alleles is related to transferability between these species. The genetics indices (Ho, He, Fis and Hw equilibrium) are very variable in the literature. In wild population, broodstock and fingerlings of *B. hilarii*, BIGNARDI et al. (2016) observed great variation of these parameters by locus. Similar was reported by Lopera-Barrero et al. (2016a) in wild populations of *P. lineatus* through the Par80. Our

Table 1 - Caracterization of Locus, Motif, Repetition, Species, Annealing temperature (TAºC), Fragment size – bp (Frequency) and Polymorphic information content (PIC) of microsatellite primers used.

Locus	Motif	Repetition	Species	TA ºC	Fragment size - bp (Frequency)	PIC
Bh5	Di-	$(CA)_{13}$	*B. hilarii*	56	195 (0.063); 202 (0.875); 225 (0.063)	0.215
Bh6	Di-	$(CA)_{14}$	*B. hilarii*	56	178 (0.853); 190 (0.147)	0.219
Bh8	Tri-	$(GAT)_5$	*B. hilarii*	56	182 (0.325); 190 (0.575); 200 (0.1)	0.475
Bh13	Di-	$(AT)_7$	*B. hilarii*	56	160 (0.472); 165 (0.222); 170 (0.306)	0.561
Bh16	Tri-	$(TAA)_8$	*B. hilarii*	56	160 (0.821); 165 (0.107); 170 (0.071)	0.286
BoM5	Complex	$(AC)_4T(AC)_{10}AT(AC)_5$	*B. opalinus*	53	105 (0.816); 120 (0.184)	0.255
BoM13	Di-	$(CT)_{11}$	*B. hilarii*	47	160 (0.031); 165 (0.219); 170 (0.750)	0.334
Par80	Di-	$(CT)_{37}$	*P. argenteus*	52	175 (0.605); 182 (0.395)	0.364
Bc48 -10	Di-	(CA)	*B. insignis*	65	76 (0.115); 80 (0.462); 85 (0.115); 90 (0.308)	0.609

Di-: Dinucleotide; Tri-:Trinucleotide; bp: base pairs.

Table 2 - No. Alleles (Na), No. Effective Alleles (Ne), Allelic richness (Ar), Shannon Index (I), Observed Heterozygosity (Ho), Expected Heterozygosity (He), Inbreeding *coefficient* (Fis) and HardyWeinberg equilibrium (p values) per locus.

Locus	Na	Ne	Ho	He	Fis	Hw
Bh5	3.000	1.293	0.125	0.227	0.474*	0.065
Bh6	2.000	1.335	0.294	0.251	-0.143*	1.000
Bh8	3.000	2.241	0.250	0.554	0.566*	*0.000
Bh13	3.000	2.734	0.500	0.634	0.239*	*0.002
Bh16	3.000	1.446	0.214	0.309	0.339*	0.153
BoM5	2.000	1.430	0.368	0.301	-0.200*	1.000
BoM13	3.000	1.636	0.438	0.389	-0.094*	0.320
Par80	2.000	1.915	0.789	0.478	-0.636*	*0.010
Bc48-10	4.000	2.991	0.538	0.666	0.229*	*0.002
Mean	2.778	1.891	0.391	0.423	0.086*	0.284

*significant deviation (P<0.05).

results are close to those observed by these authors and showed moderate genetic variability. The mean value of He was higher than the value of Ho, which likely inferred the significant deviation in the Hw, indicating a heterozygous deficit through the Fis coefficient in most of loci.

The PIC is an important parameter in primer evaluation. According scale proposed by BOTSTEIN et al. (1980), the loci can be highly (PIC>0.500), moderate (0.250-0.500) or low informative (<0.250). In current study, two loci were highly informative (Bh13 and Bc48-10), five were moderate informative (Bh8, Bh16, BoM5, BoM13 and Par80) and only two were low informative (Bh5 and Bh6). These results are important to select the more informative loci for population analyses.

According to ABDUL MUNEER (2014), heterologous primers may be successfully used in several fish species and the amplification quality depends on the degree of genetic conservation of the sites that border on the microsatellite regions. Conversely, these primers increase error chances during annealing of sequences (resulting in null alleles) (CHAPUIS & ESTOUP, 2007) and make difficult the application to phylogenetically distant species. However, the presence of null alleles was observed only in Bh8, and probably should not have affected genetic variability in this case. The absence of conservation of microsatellite sites may probably explain the lack of amplification for the primers of the species *P. lineatus, P. mesopotamicus*, *C. macropomum* and *O. niloticus*. Similarly, greater closeness between specimens of the genus *Brycon* provided satisfactory amplification standards.

Recent studies have shown that the transferability of microsatellite primers is not limited to species level and may occur between different genera. LOPERA-BARRERO et al. (2016b) detected amplification in *Leporinus elongatus* with primers of *B. opalinus* (BoM5) and *P.lineatus* (Pli43 and Pli60). CARMO et al. (2015) reported positive results for *B. orbignyanus* by primers of *Salminus brasiliensis* and *S. franciscanus*. However, transferability between genera or families is more difficult due to the genetic distance between specimens (PENTEADO et al., 2011; LOPERA-BARRERO et al., 2016b).

Similar to genus *Brycon*, it has been shown that different species of *Prochilodus* share transferability of microsatellite primers (BARBOSA et al., 2008). However, current analysis has shown for the first time that cross-amplification was possible between the genera through Par80 (*P. argenteus*). The above results are due to a greater genetic proximity between these fish and guarantee the success of cross-amplification. Further, since allele size is similar to that in fish of the genus *Prochilodus*, the idea is underscored for the conservation of microsatellite sites throughout the evolution process which caused the correct pairing of nitrogen bases providing adequate amplification pattern.

In the case of restocking programs, the validation of methodologies that contribute towards studies on wild populations or on broodstocks is highly relevant for the implantation and improvement of conservational measures. Employment of heterologous primers within this context is an opportunity for the study of species with no specific primers. The latter´s development is time-consuming

and costly. Current study demonstrated that the use of heterologous primers of the different species and genera in *B. orbignyanus* is possible. Further studies are required to prove the viability of these markers especially with regard to the possibility of inter-species cross-amplification or till the development of species-specific primers.

CONCLUSION

Cross-amplification of nine primers derived from *Brycon opalinus* (BoM5 and BoM13), *Brycon hilarii* (Bh5, Bh6, Bh8, Bh13 and Bh16), *Brycon insignis* (Bc48-10) and *Prochilodus argenteus* (Par80) were validated for *B. orbignyanus*. These results will aid the analyses of genetic diversity and structure population for *B. orbignyanus.*

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

Methodologies employed were approved by the Committee for Ethics in the use of animals of the Universidade Estadual de Londrina (CEUA_UEL nº17156.2012.50).

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