

Notas Científicas

Optimization of heterologous microsatellites in piracanjuba

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Abstract – The objective of this work was to evaluate 41 microsatellite markers for heterologous amplifications in piracanjuba (*Brycon orbignyanus*). Some markers were tested for the first time. Loci were optimized for PCR conditions and applied to a sample of 49 individuals. Thirty-one loci resulted in PCR product formation, whereas ten loci yielded intelligible polymorphic patterns in the evaluated sample and can be used for amplifications in this species. From the evaluated markers, four loci (BoM1, BoM13, Bh6, and Bh16) are valid to be applied in the study of piracanjuba.

Index terms: *Brycon orbignyanus*, aquaculture, hatchery, molecular markers.

Otimização de microssatélites heterólogos em piracanjuba

Resumo – O objetivo deste trabalho foi avaliar 41 marcadores microssatélites para amplificação heteróloga em piracanjuba (*Brycon orbignyanus*). Alguns marcadores foram testados pela primeira vez. Os loci foram otimizados para condições de PCR e aplicados a uma amostra de 49 indivíduos. Trinta e um loci resultaram em formação de produtos de PCR, enquanto dez loci resultaram em padrões polimórficos inteligíveis na amostra avaliada e podem ser usados para amplificações nessa espécie. Dos marcadores avaliados, quatro loci (BoM1, BoM13, Bh6 e Bh16) são válidos para aplicação em estudos em piracanjuba.

Termos para indexação: *Brycon orbignyanus*, aquicultura, procriação, marcadores moleculares.

Piracanjuba is a common name for *Brycon orbignyanus* (Valenciennes, 1849), a Neotropical freshwater species from the Prata river basin, in southeastern South America. It is a highly valued omnivorous species, with reasonable acceptability for hatchery conditions (Nogueira et al., 2014), and is listed as threatened by Rosa & Lima (2008). So far, the only microsatellite markers available for the study of this species have been heterologous markers. Originally, seven microsatellite loci isolated from *B. opalinus* (Barroso et al., 2003) were successfully reported in cross-amplifications with *B. orbignyanus*. Sanches & Galetti Jr (2006) described cross-amplifications in *B. orbignyanus* with seven markers isolated from *B. hilarii*. Later, four loci from *B. opalinus* (BoM1, BoM2, BoM7, and BoM13) were used in *B. orbignyanus* by Rodriguez-Rodriguez et al. (2010) and five (BoM1, BoM2, BoM5, BoM7, and BoM13) by Lopera-Barrero et al. (2010) in breeding stocks for population enhancement programs. More recently,

Ashikaga et al. (2015) evaluated the genetic structure of free-living populations of *B. orbignyanus* using four heterologous loci (BoM6 and BoM13, along with Bh5 and Bh13).

The objective of this work was to evaluate 41 microsatellite markers for heterologous amplifications in piracanjuba.

Heterologous microsatellite loci described for five other characid species were evaluated: eight from *Salminus brasiliensis* (Rueda et al., 2011); nine from *S. hilarii* (Silva & Hilsdorf, 2011); ten from *S. franciscanus* (Agata et al., 2011); seven from *B. opalinus* (Barroso et al., 2003); and seven from *B. hilarii* (Sanches & Galetti Jr, 2006). A sample of 49 different free-living *B. orbignyanus* individuals, captured for broodstock formation in the Itutinga hydroelectric power plant, in the state of Minas Gerais, Brazil, were analyzed. DNA was extracted from ethanol-fixed caudal fin with 5% chelex 100, 0.1% SDS, and 2.0 µL (10 mg mL⁻¹) proteinase K.

Polymorphic loci were optimized for PCR conditions, mainly testing different Mg^{++} and *Taq* DNA polymerase concentrations (0.25–1.0 U), followed by primer annealing temperature gradient (8–12°C) PCR assays. Fixed variables, per reaction, included buffer (50 mmol L⁻¹ KCl; 10 mmol L⁻¹ Tris-HCl, pH 8.0; 1% Triton X-100), 5.0 pmol of each primer, and dNTP (100 µmol L⁻¹ of each kind). Products were scored through 12% polyacrylamide gel electrophoresis – 4.5 V/cm, for 15 hours, using 25 bp DNA ladders. Results were analyzed using the Genepop software (Laboratoire de Genetique et Environnement, Montpellier, France) for Hardy-Weinberg equilibrium (HWE) expectations and for linkage disequilibrium. Further analyses were conducted with the Micro-Checker software (Norwich Research Park, Norfolk, United Kingdom).

PCR optimization of 41 potential heterologous microsatellite loci revealed 10 polymorphic markers, 9 monomorphic, and 22 with unsatisfactory results or showing no amplification at all. These polymorphic loci amounted to 43 different alleles, varying from two to seven alleles per locus (average of 4.3 ± 1.07). All seven tested loci from *B. opalinus* and from *B. hilarii* showed positive amplifications, although only four from each species were consistent and polymorphic. The BoM1, BoM13, Bh6, and Bh16 loci were shown to be in HWE (Table 1). The BoM2 and BoM6 loci departed from HWE and actually showed heterozygote excess. Possible causes for heterozygote excess are the artifactual scoring of nonspecific and stuttering bands (Oosterhout et al., 2004), as well as the small effective population size (Waples, 2015). The results from the null allele detection analysis ruled out null alleles at the BoM1, BoM2, BoM6, BoM13, Bh6, and Bh16 markers. This result shows the presence of null alleles in appreciable estimated frequencies (>0.17) in the remaining polymorphic loci; however, these estimates should be taken with skepticism, since the Micro-Checker (Norwich Research Park, Norfolk, United Kingdom) implementation only considers the concurrent hypothesis of Wahlund effect if heterozygote deficiency is found over all loci, which is arguably flawed (Waples, 2015).

An important challenge for population geneticists is to distinguish among the various factors that can cause HWE departure (Waples, 2015). In this regard, many genetic and non-genetic (e.g., artifactual PCR

amplifications and sampling design) variables can act alone or in various combinations. However, this species' history of sharp population decline (Rosa & Lima, 2008), intensive hatchery and broodstocking favors a scenario of multiple violations of HWE assumptions, such as nonrandom mating, mixed stocks, and severe random genetic drift. Therefore, these results should be seen as reflecting the nature of the evaluated sample alone, in which the following heterologous markers were found to be useful in monitoring *B. orbignyanus*: BoM1, BoM13, Bh6, and Bh16. It is important to note that if a Bonferroni correction was applied, given the number of multiple tests performed, i.e., 75, even under the new significance value of ~ 0.0006 , only BoM6 would pass the criteria for HWE. Lopera-Barrero et al. (2010) using the BoM1, BoM2, BoM7, and BoM13 loci, and Rodriguez-Rodriguez et al. (2010) using all of these and the BoM5 locus observed polymorphism for BoM7 but did not use the BoM6 locus.

Recently, Ashikaga et al. (2015) reported BoM6 polymorphic amplifications in different stocks from the Alto Paraná river basin, detecting null alleles at the BoM6 and BoM13 loci. According to Lopera-Barrero (2010), BoM5 is monomorphic, although Rodriguez-Rodriguez et al. (2010) observed otherwise. These varying results among different stocks seem to show that many of these departures from HWE are possibly caused by spawning practices, limited number of matrices, and subsequent population bottlenecks that lead to allele fixation in some stocks (Povh et al., 2011).

The Sfra04, Sm25, Bh5, and Bh13 loci showed departure from HWE in the direction of lack of heterozygotes in the assessed sample. This could be caused by: the segregation of null alleles, arguably more likely to occur in more distantly-related species; increased endogamy in hatchery stocks; or due to the presence of genetic population substructuring (Waples, 2015), as would be expected in a mixed-stock scenario, in which distinct migratory runs are sampled to form broodstocks in the hatchery operation. Since other markers were found to be in HWE, the null allele hypothesis cannot be ruled out as a possible explanation, even though there would be no Wahlund effect for loci with the same allelic frequencies among different stocks. Therefore, these loci should be carefully analyzed before downstream use in genetic monitoring of *B. orbignyanus*, in a case-by-case basis,

Table 1. Cross-species microsatellite amplifications in a *Brycon orbignyanus* stock⁽¹⁾.

Locus ⁽²⁾	Motif	Annealing temperature (°C)	Observed number of alleles	Fragment size range (bp)	H _o /H _e	F _{is}	Hardy-Weinberg equilibrium (p values)		
							Exact test	Heterozygote deficit	Heterozygote excess
Sfra04	Mixed	54	3	153–163	0.22/0.60	0.64	0.0000*	0.0000*	-
Sm25	Di-	52	2	200–220	0.18/0.40	0.54	0.0003*	0.0003*	-
BoM1	Di-	59	4	146–198	0.63/0.69	0.08	0.3622	-	-
BoM2	Di-	58	2	131–149	0.50/0.37	0.33	0.0233	-	0.0198*
BoM6	Mixed	63	6	134–172	0.85/0.78	0.09	0.0034*	-	0.0087*
BoM13	Di-	55	4	160–186	0.59/0.70	0.16	0.4897	-	-
Bh5	Di-	58	5	199–225	0.45/0.72	0.36	0.0001*	0.0000*	-
Bh6	Di-	59	7	162–218	0.65/0.66	0.01	0.1685	-	-
Bh13	Di-	55	6	142–165	0.36/0.80	0.54	0.0001*	0.0000*	-
Bh16	Tri-	63	4	140–166	0.48/0.40	0.20	0.4354	-	-

⁽¹⁾Di-, dinucleotide; Tri-, trinucleotide; H_o, observed heterozygosity; H_e, heterozygosity; and F_{is}, inbreeding coefficient. ⁽²⁾Sfra, *Salminus franciscanus* loci (Agata et al., 2011); Sm, *Salminus brasiliensis* loci (Rueda et al., 2011); BoM, *Brycon opalinus* loci (Barroso et al., 2003); and Bh, *Brycon hilarii* loci (Sanches & Galetti Jr, 2006).

if homozygote excess is found. The Bh08 and Bh15 loci, together with four markers from *S. franciscanus* (Sfra03, Sfra13, Sfra15, and Sfra18) and three from *S. brasiliensis* (Sm10, Sm33, and Sm41), exhibited monomorphic amplifications in *B. orbignyanus* in the evaluated sample, although it is possible that researchers could find alternative results over other studied stocks.

Out of 45 possible pair-wise polymorphic loci combinations, five loci pairs showed linkage disequilibrium: BoM1 and BoM13 ($p=0.0006$); BoM1 and Bh13 ($p=0.016$); BoM2 and Bh5 ($p=0.001$); BoM2 and Bh6 ($p=0.016$), and Bh5 and Bh6 ($p=0.002$). Among several possible explanations for the nonrandom association of alleles from different loci are the presence of sample substructure, random genetic drift, and selection and physical linkage between genes.

The rate of success for cross-amplifications is directly related to the phylogenetic proximity between involved species. This seems to be apparent in the obtained results, considering the positive response and validation between cross-genus amplification from *Salminus* in *Brycon* species. Combined with the current literature, these results will assist the informative use of heterologous markers for the management of the genetic diversity in this threatened migratory species, with great potential value for aquaculture.

Cross-species microsatellite amplifications were optimized, and a set of four heterologous markers for *B. orbignyanus* are validated in the assessed sample and, therefore, are considered informative.

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