Novel microsatellite markers for the endangered neotropical fish *Brycon orbignyanus* and cross-amplification in related species

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**ABSTRACT**

*Brycon orbignyanus* is a neotropical fish found in several South American countries. This species has high ecological importance and is included in the list of fish threatened with extinction. In this study, we report the development and characterisation of 12 microsatellite primers of *B. orbignyanus* for future genetic studies. Screening of the 12 markers in a sample of 35 individuals identified seven polymorphic loci and 22 alleles, with one to three alleles per locus. The polymorphic information content ranged from 0.054 to 0.542, with four loci showing significant deviation from Hardy-Weinberg equilibrium. Two loci possessed null alleles. Cross-species amplification tests in two closely related species showed that 11 loci are transferrable to *Brycon gouldingi* (*B. gouldingi*) and nine are transferrable to *B. falcatus*. The microsatellite primers designed may be used for genetic variability and population structure studies in wild populations, fish farms and restocking programmes of *B. orbignyanus* and the two related species.

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**Introduction**

*Brycon orbignyanus* (Characiformes: Characidae) (Valenciennes, 1850) or Piracanjuba, is a neotropical migrant fish distributed along the La Plata River basin, covering several South American countries (Reis et al. 2003; Antunes et al. 2010). This species has an excellent growth rate and is highly valued by consumers. Therefore, it is suitable for captive breeding (Borba et al. 2006) and sport fishing from fish farms. However, anthropic factors, including overfishing, pollution and habitat fragmentation for hydroelectricity generation have caused a significant decrease in the natural populations of this species (Ashikaga et al. 2015; Lima 2017), making it critically endangered in specific locations (Lima 2017).

The occurrence of these factors underlies the need for genetic monitoring of natural stocks, thereby enabling biodiversity and ecosystem conservation (Van Herwerden et al. 2006), and guiding management activities towards captive breeding and conservation management programmes (Ribeiro et al. 2016). Despite the economic and ecological importance of this species, few studies have been conducted to estimate the genetic variability in natural populations or restocking programmes. One reason is the difficulty of using molecular markers in *B. orbignyanus*, owing to the lack of data on the standardisation of species-specific markers, which limits studies on genetic stocks.

Microsatellite markers are widely used for genetic studies in fish, mainly due to their ease of use and high resolving power, which makes them valuable for analyses of population structure (Abdul-Muneer 2014). However, previous data on the genome of the species being studied are necessary, and the use of microsatellites is restricted when species-specific primers are not available for genetic studies. Recently, the effort to use heterologous loci in *B. orbignyanus* has hampered the work of researchers, limiting the number of loci used (Lopera-Barrero et al. 2014; Ashikaga et al. 2015), due to the occurrence of null alleles (Henriques et al. 2017). Thus, efforts that enable the design and use of molecular markers to evaluate genetic variability in populations and guide conservation programmes are valuable.
Considering the difficulty of using heterologous primers, and the economic and ecological importance of *B. orbignyanus*, the objective of this study was to develop and characterise microsatellite primers specific for *B. orbignyanus*, and to assess cross-species amplification in two-related species (*Brycon Gouldingi* and *Brycon falcatus*).

**Materials and methods**

The methodologies were approved by the Ethics Committee on the use of Animals of the State University of Londrina (CEUA_UEL n. 11679.2017.46). The genomic DNA of *B. orbignyanus* was extracted from the caudal fin according to the protocol described by Lopera-Barrero et al. (2008). DNA quality was assessed by agarose gel (1%) electrophoresis in TBE 1X buffer, run for 2h at 80 V, and subsequent staining with SYBR Safe™ DNA Gel Stain (Invitrogen, Carlsbad, CA).

A microsatellite-enriched library was prepared using the hybridisation capture method, according to the protocol described by Billotte et al. (1999), with the probes (AGA)$_5$, (CT)$_8$ and (GT)$_8$ in the enrichment step. Briefly, genomic DNA (5 μg) from *B. orbignyanus* was digested with 50 U of the type II restriction enzyme *Rsa*1 and the resulting fragments were ligated to the single-strand adaptors (10 μM each) *Rsa*21 (5’-CTCTT GCTTACGCGTGGACTA-3’) and *Rsa*25 (5’- TAGTCCACG CGTAAGCAAGGCACA-3’) with T4 DNA ligase (Promega, Madison, WI). Ligation was performed by incubation at 20 °C for 2h. Fragments with AGA, CT and GT repeats were selected by hybridisation with biotinylated oligonucleotides, complementary to the microsatellite sequences, and recovered using streptavidin-coated magnetic beads (Thermo Fisher Scientific, Waltham, MA). The microsatellite-enriched fragments were amplified by PCR with the primers *Rsa*21, cloned into the pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent *Escherichia coli* JM109 cells (Promega). Plasmids from single colonies were isolated and the inserts were sequenced using the sequencing kit Big Dye Terminator version 3.1 (Applied Biosystems, Foster City, CA) and an automated sequencer (ABI 3500XL, Applied Biosystems).

The software MEGA version 6.0 (Tamura et al. 2013) was used to read the sequences. The primers were designed using the software Primer3 version 0.4.0 (Rozen and Skaltsky 2000). The designed primers were tested in three *B. orbignyanus* specimens for PCR standardisation. An annealing temperature of 55 °C resulted in the best visualisation resolution on polycrylamide gel (stained with silver nitrate) for all primers tested.

The microsatellite primers designed were amplified in 10 μL PCR reactions containing 4.5 μL 0.9 × GoTaq Green Master (Promega), 0.08 μL 5 pmol forward primer and 0.32 μL 5 pmol reverse primer, 0.32 μL 0.5 μM M13 primer labelled with FAM, HEX, NED or PET probes (Applied Biosystems), 30 ng genomic DNA and 2.78 μL ultrapure water (Schuelke 2000). The following PCR conditions were used: 94 °C for 4 min for initial denaturation, followed by 35 cycles at 94 °C for 1 min, 55 °C for 45 s, 72 °C for 1 min and lastly, a final extension at 72 °C for 10 min. The PCR products were subjected to electrophoresis in an ABI 3500xL automated sequencer (Applied Biosystems) using GeneScan 600 Liz dye (Thermo Fisher Scientific) as a size standard.

A population sample with 35 *B. orbignyanus* specimens from the Tietê River was used to validate the microsatellite primers. The specimens were collected from stock at the hydrobiology station (21°19’01.2”S and 49°47’22.8”W) located in the city of Promissão, in São Paulo, Brazil. Cross-species amplification was tested in eight individuals of each of the two *Brycon* species tested: *B. gouldingi* and *B. falcatus*, from the Araguaia river basin (15°33’21.52”S and 52°14’47.79”W), in Mato Grosso, Brazil. DNA extraction and PCR were performed according to the conditions mentioned above.

Observed (*H*$_o$) and expected (*H*$_e$) heterozygosity, and deviations from the Hardy–Weinberg equilibrium were calculated for each locus using the software GenAlex version 6.5 (Peakall and Smouse 2012). The polymorphic information content (PIC) was calculated using the software Cervus version 3.0.7 (Kalinowski et al. 2007). The inbreeding coefficient ($F$$_I$) was calculated using the software FSTAT version 2.9.3 (University of Lausanne, Lausanne, Switzerland) (Goudet 2005), and the linkage disequilibrium ($p < .05$) was assessed using the software Arlequim version 3.5 (Excoffier and Lischer 2010). The presence of null alleles was tested using the software Micro-Checker (Van Oosterhout et al. 2004).

**Results**

Ninety-six clones were sequenced, of which 18 had microsatellite regions and were selected for primer design. Primer screening identified 12 loci with potential for cross-amplification; these were selected for characterisation and registered in GenBank (accession numbers MFS10255–MFS10266). A total of 22 alleles were obtained from the 12 loci amplified among the 35 *B. orbignyanus* individuals analysed, of which seven
loci were polymorphic (Borg9, Borg10, Borg25, Borg54, Borg56, Borg59 and Borg65) (Table 1). Allele size ranged from 139 (Borg59) to 413 (Borg9) and the number of alleles per locus ranged from one (Borg4, Borg12, Borg13, Borg17 and Borg55) to three (Borg9, Borg59 and Borg65). The Borg13 locus, although monomorphic for B. orbignyanus samples, was polymorphic in the two species tested for cross-species amplification, with two alleles in B. falcatus and three (Borg9, Borg12 and Borg65) to three (Borg9, Borg54 and Borg65). The Borg13 locus, although monomorphic for B. orbignyanus samples, was polymorphic in the two species tested for cross-species amplification, with two alleles in B. gouldingi and three in B. falcatus. All 12 loci showed transferability to B. gouldingi or B. falcatus. However, one (Borg55) and three (Borg10, Borg12 and Borg65) loci failed to amplify in B. gouldingi and B. falcatus, respectively. Six loci were polymorphic to B. gouldingi (Borg9, Borg 13, Borg25, Borg56, Borg59 and Borg65) and two were polymorphic to B. falcatus (Borg13 and Borg59) (Table 2).

The mean values of observed (H_o) and expected heterozygosity ranged from 0.000 (Borg10 and Borg54) to 0.958 (Borg65), and from 0.056 (Borg54) to 0.612 (Borg59), respectively. The inbreeding coefficient (FIS) was positive and significant (p < .05) in three loci (Borg9, Borg10 and Borg54) and negative and significant (p < .05) in four loci (Borg25, Borg56, Borg59 and Borg65). The PIC ranged from 0.054 (Borg54) to 0.542 (Borg65). Deviation from Hardy–Weinberg equilibrium (p < .05) occurred at the Borg10, Borg54, Borg59 and Borg65 loci (Table 1). Null alleles (p < .05) were observed in the Borg10 and Borg54 loci. Linkage disequilibrium of Borg9 with Borg10, Borg56 and Borg59; Borg10 with Borg54, Borg56, Borg59 and Borg65; Borg25 with Borg59; and Borg59 with Borg59 was detected (p < .05).

### Discussion

The deviation from Hardy–Weinberg equilibrium observed for Borg10 and Borg54 can be attributed to the presence of null alleles at these loci, which might have led to the erroneous detection of homozygous genotypes and thus the underestimation of heterozygotes (Aung et al. 2010). In addition, linkage disequilibrium showed genetic drift and suggested deviation from the Hardy–Weinberg equilibrium, particularly when considering small populations, where the effect of drift accentuates random changes in allele frequencies over time (Frankham et al. 2014). In addition, the positive and significant FIS in three loci (heterozygote deficit) can suggest inbreeding; however, the four negative values suggest that this did not occur (Okazaki et al. 2017).

The genus *Brycon* includes more than 40 species, which are widespread in the South and Central American basins, including the Amazonas, Paraná, Paraguay and Araguaia basins (Reis et al. 2003; Antunes et al. 2010), which are key components of the continental ichthyofauna. However, to date, genetic studies of species from the Araguaia basin, including *B. gouldingi* and *B. falcatus*, are scarce. This study designed 12 markers with potential for use in three *Brycon* species, including one with high economic and ecological importance (*B. orbignyanus*). Although five loci were monomorphic, it is presumed that evaluation in populations from different basins will reveal additional polymorphic loci.

### Table 1. Characterisation of seven polymorphic microsatellite loci genotyped in *Brycon orbignyanus*.

| Locus/ GeneBank | Primer sequences (5’–3’) | Allele size, bp | Repeat Motif | Na | H_o | H_e | FIS | H_e (p) | PIC |
|----------------|--------------------------|-----------------|--------------|----|-----|-----|-----|--------|-----|
| Borg9/          | F: CAGTCTGGCACTAATCCTC  | 395–413         | (TG)_4(TA)_4(CGT)_2  | 3  | 0.147 | 0.189 | 0.236* | 0.228 | 0.179 |
| MF510256        | R: AAGGTGCTTTGAGTGATGCC  | 198–248         | (TA)_4(CGT)_2(CA)_2 | 2  | 0.000 | 0.057 | 1.000* | 0.000* | 0.055 |
| Borg10/         | F: GAGTACACATGACATGCTC  | 286–288         | (GA)_4          | 2  | 0.348 | 0.287 | −0.189* | 0.313 | 0.246 |
| MF510257        | R: TTCGATGGCCTTTGAAGGG  | 139–188         | (TTTA)_3(TTCCCT) | 3  | 0.567 | 0.512 | −0.091* | 0.000* | 0.411 |
| Borg54/         | F: ACTCACACTCTGCTTGCTT  | 332–338         | (CT)_4(CTT)_4    | 2  | 0.471 | 0.360 | −0.294* | 0.073 | 0.295 |
| MF510262        | R: AACGACCCCTAATCCTAATCCC | 166–172      | (CTT)_2(CTT)_2   | 3  | 0.567 | 0.512 | −0.091* | 0.000* | 0.411 |
| Borg56/         | F: ATCTGCAACAGGGGAACTA  | 281             | (TTAA)_3(TTAA)_3 | 3  | 0.598 | 0.612 | −0.551* | 0.000* | 0.542 |
| MF510265        | R: AAAGCACCTAATCCTAATCCC | 286–293       | (CTT)_2(CTT)_2   | 3  | 0.598 | 0.612 | −0.551* | 0.000* | 0.542 |

Bp: base pairs; Na: number of alleles; H_o: observed heterozygosity; H_e: expected heterozygosity; Hardy–Weinberg equilibrium; FIS: inbreeding coefficient; PIC: polymorphic information content

*Significant at p < .05.

### Table 2. Cross-species amplification of microsatellite loci from *Brycon orbignyanus*.

| Species     | Locus | Na | Allele size, bp | bp |
|-------------|-------|----|----------------|----|
| Brycon gouldingi | Borg9 | 2  | 401–413     |    |
|            | Borg13| 2  | 349–401     |    |
|            | Borg25| 2  | 286–288     |    |
|            | Borg56| 3  | 328–338     |    |
|            | Borg59| 2  | 166–169     |    |
|            | Borg65| 3  | 227–281     |    |

| Species     | Locus | Na | Allele size, bp | bp |
|-------------|-------|----|----------------|----|
| Brycon falcatus | Borg9 | 2  | 401–413     |    |
|             | Borg13| 2  | 349–401     |    |
|             | Borg25| 2  | 286–288     |    |
|             | Borg56| 3  | 328–338     |    |
|             | Borg59| 2  | 166–169     |    |
|             | Borg65| 3  | 227–281     |    |

Na: number of alleles; bp: base pairs
River pollution, deforestation and particularly the construction of dams to generate hydroelectricity, contribute to the decline in natural populations of *B. orbignyanus*, making the species threatened in key Brazilian and Argentinian river basins (Lima 2017). Therefore, this species is of great interest for conservation programmes on the South American continent. The microsatellite markers developed and validated in this study will facilitate genetic diversity and population structure studies, and contribute to the production of valuable data for *B. orbignyanus* conservation and management programmes. The validation of those markers in the two related species (*B. gouldingi* and *B. falcatus*) will enable further genetic studies to be carried out on those species.

**Conclusions**

These new microsatellite primers can be used to analyze genetic diversity and structure in restocking programmes, natural stocks and fish farms of *B. orbignyanus*. Cross-amplification of these primers was validated for two species of Brycon (*B. gouldingi* and *B. falcatus*).

**Ethical Approval**

The methodologies used in this study complied with the ethical principles postulated by the National Council for the Control of Animal Experimentation, and were approved by the Ethics Committee on the use of animals of the State University of Londrina (CEUA_UEL n. 11679.2017.46).

**Geolocation information**

The hydrobiology station (21°19’01.2”S and 49°47’22.8”W) is located in the city of Promissão, state of São Paulo, Brazil. Araguaia River (15°53’21.52”S and 52°14’47.79”W) is located in the state of Mato Grosso, Brazil.

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**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**

Abdul-Muneer PM. 2014. Application of microsatellite markers in conservation genetics and fisheries management: recent advances in population structure analysis and conservation strategies. Genet Res Int. 2014:1–11.

Antunes RSP, Gomes VN, Prioli SMAP, Prioli RA, Júlio HF, Prioli LM, Agostinho CS, Prioli AJ. 2010. Molecular characterization and phylogenetic relationships among species of the genus Brycon (characiformes: characidae) from four hydrographic basins in Brazil. Genet Mol Res. 9:674–684.

Ashikaga FY, Orsi ML, Oliveira C, Senhorini JA, Foresti F. 2015. The endangered species Brycon orbignyanus: genetic analysis and definition of priority areas for conservation. Environ Biol Fishes. 98:1845–1855.

Aung O, Nguyen TTT, Poompuang S, Kamonrat W. 2010. Microsatellite DNA markers revealed genetic population structure among captive stocks and wild populations of mrigal, Cirrhinus cirrhosus in Myanmar. Aquaculture. 299:37–43.

Billotte N, Lagoda PJR, Risterucci AM, Baurens FC. 1999. Microsatellite-enriched libraries: applied methodology for the development of SSR markers in tropical crops. Fruits. 54:277–288.

Borba MR, Fracalossi DM, Pezzato LE. 2006. Dietary energy requirement of piranajuba fingerlings, Brycon orbignyanus, and relative utilization of dietary carbohydrate and lipid. Aquac Nutr. 12:183–191.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour. 10: 564–567.

Frankham R, Bradshaw CJA, Brook BW. 2014. Genetics in conservation management: revised recommendations for the 50/500 rules, red list criteria and population viability analyses. Biol Conserv. 170:56–63.

Goudet J. 2005. FSTAT: a program to estimate and test gene diversities and fixation indices (version 2.9.3.2). Lausanne: UNIL – Department of Ecology and Evolution. Available from: https://www2.unil.ch/popgen/softwares/fstat.htm.

Henriques R, Nielsen ES, Durholtz D, Japp D, Heyden SVD. 2017. Genetic population sub-structuring of kingklip (genypterus capensis – ophidiidae), a commercially exploited demersal fish off South Africa. Fish Res. 187:86–95.

Kalinowski ST, Taper ML, Durholtz D, Japp D, Heyden SVD. 2015. The endangered species Brycon orbignyanus: genetic characterization and conservation strategies. Genet Res Int. 2014:1–11.

Lima FC. 2017. A revision of the cis-andean species of the genus Brycon Müller & Troschel (characiformes: characidae). Zootaxa. 4222:1–198.

Lopera-Barrero NM, Povh JA, Ribeiro RP, Gomes PC, Jacometo CB, Silva TL. 2008. Comparison of DNA extraction protocols of fish fin and larvae samples: modified salt (NaCl) extraction. Cienc Invest Agrar. 35:65–74.

Lopera-Barrero NM, Alvarez CAR, Rodriguez-Rodriguez MDP, Povh JA, Vargas L, Streit Junior DP, Sirol RN, Ribeiro RP. 2014. Genetic diversity and paternity of Brycon orbignyanus offspring obtained for different reproductive systems. Semin Cienc Agrar. 35:541–554.

Okazaki TI, Halleran EM, ResendeEK, Hilsdorf AWS. 2017. Genetic characterization of Brycon hilarii (characiformes)
populations within the pantanal: aspects of their conservation within a globally important neotropical wetland. J Ichthyol. 57:434–444.

Peakall R, Smouse PE. 2012. GenALEX 6.5: genetic analysis in excel. Population genetic software for teaching and research—an update. Bioinformatics. 28:2537–2539.

Reis RE, Kullander SO, Ferraris CJ. 2003. Checklist of the freshwater fishes of South and Central America. Porto Alegre: Edipucrs.

Ribeiro RP, Rodriguez-Rodriguez MP, Resende EK, Souza FP, Povh JA, Poveda-Parra AR, Goes ESR, Galo JM, Bernardo M, Lopera-Barrero NM. 2016. Genetic characteristics of Tambaqui broodstocks in the state of Rondônia, Brazil: implications on production and conservation. Semin-Cienc Agrar. 37:2375–2385.

Rozen S, Skaletsky HJ. 2000. PRIMER 3 on the WWW for general users and for biologist programmers. In: Bioinformatics methods and protocols: methods in molecular biology. Berlin, Germany: Springer.

Schuelke M. 2000. Na economic method for the fluorescent labeling of PCR fragments. Nature Biotech. 18:233–234.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 30:2725–2729.

Van Herwerden L, McIlwain J, Al-Oufi H, Al-Amry W, Reyes A. 2006. Development and application of microsatellite markers for scomberomorus commerson (perciformes; teleostei) to a population genetic study of Arabian Peninsula stocks. Fish Res. 79:258–266.

Van Oosterhout C, Hutchinson WF, Wills DPM, Shipley P. 2004. MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes. 4:535–538.