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Original article

Development and characterization of fifteen polymorphic microsatellite loci in *Bryconamericus* aff. *iheringii* (Teleostei: Characidae) and cross-amplification in related Characidae species

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Data on 15 novel microsatellite loci from the Neotropical fish *Bryconamericus* aff. *iheringii* are presented here. Analyses of 32 individuals from four different streams revealed 192 different alleles, ranging from four to 32 alleles per locus (mean of 12.8 per locus). Observed and expected heterozygosities ranged from 0.094 to 0.813 and 0.205 to 0.952, respectively. These loci showed high polymorphic information content and will be a resource for genetic studies of *B.* aff. *iheringii*. Furthermore, several loci also amplified other small Neotropical Characidae (*Piabarchus stramineus* and *Piabina argentea*) and should be useful for these species.

Keywords: Genetic diversity, Lambarizinho, Molecular markers, Neotropical fish, SSR.

Um total de quinze novos locos microssatélites é aqui apresentado para o pequeno peixe Neotropical *Bryconamericus* aff. *iheringii*. A análise de 32 indivíduos provenientes de quatro ribeirões diferentes revelou 192 alelos diferentes, variando de quatro a 32 alelos por loco (média de 12,8 por loco), e heterozigozidades observada e esperada variando de 0,094 a 0,813 e 0,205 a 0,952, respectivamente. O conjunto de locos obtido mostrou alto conteúdo de informação polimórfica e bom potencial para estudos genéticos de *B.* aff. *iheringii*, além disso vários locos amplificaram para outras espécies de pequenos Characidae neotropicais (*Piabarchus stramineus* and *Piabina argentea*).

Palavras-chave: Diversidade genética, Lambarizinho, Marcadores moleculares, Peixe Neotropical, *SSR*.

Introduction

Bryconamericus Eigenmann, 1907 is one of the most diverse genera of the Characidae family, occurring in many river systems in of the Neotropical region, from Costa Rica to Argentina (Vari, Siebert, 1990; Lima *et al*., 2003). They are small omnivorous fish (not exceeding 10 cm in length) and inhabit a range of environments, including streams, rivers and lakes (Britski *et al.*, 1988). Species of this genus are important food sources for piscivorous fish (Britski *et al.*, 1988) throughout the range of environments, and in the Paraná River basin (one of the main drainage systems in South America) the genus is a prey item of several piscivorous fish (Hahn *et al.*, 1997; Lowe-McConnell, 1999).

Bryconamericus aff. *iheringii* (Boulenger, 1887), regionally known as pequira or lambarizinho, is a member of this genus distributed in the upper reaches of the Paraná River basin (Graça, Pavanelli, 2007). Studies have suggested that *B.* aff. *iheringii* from the Paraná basin may constitute a distinct species from the *B. iheringii* found in the Laguna dos Patos system, Southern Brazil (Langeani *et al.*, 2005, 2007). However, *B.* aff. *iheringii* populations are commonly found throughout the upper Paraná sub-basins, including rivers and small streams (Langeani *et al*., 2007; Costa *et al*., 2013; Cetra *et al.*, 2016).

Fish less than 150 mm, such as *B.* aff. *iheringii* (maximum total length of 60 mm, Graça, Pavanelli, 2007), often dominate the ichthyofauna of Neotropical streams (Castro,

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1999; Winemiller *et al.*, 2008). The high prevalence of small fish dominating the ichthyofauna raises questions about the evolutionary and population aspects of these species. According to Castro (1999), the small size of these species usually contributes to a low movement capacity along the drainages. This reduced movement capacity could be one of the underlying explanations for the many endemic species and small-scale population structure found in Neotropical areas (Castro *et al.*, 2003; Ferreira *et al.*, 2016). However, genetic data for these species are largely lacking (Sofia *et al.*, 2006, 2008; Ferreira *et al.*, 2016). Biological data including genetic analyses of small fish, such as *Bryconamericus* spp., are essential for understanding biological and evolutionary properties, as well as for the management and conservation of the ichthyofauna of Neotropical streams (Agostinho *et al.*, 2005; Dudgeon *et al.*, 2006). Microsatellites are highly variable genetic markers suitable for cost-effective population genetic studies. However, particularly in the case of Neotropical fish species, the lack of suitable genetic markers has been one of the main obstacles to the development of more complete genetic studies involving this diverse group of fish. Thus, the present study aimed to identify and develop polymorphic microsatellite loci for *Bryconamericus* aff. *iheringii* and assess cross-species transferability to four other small Characidae from the Neotropical region.

Material and Methods

Microsatellite loci were obtained from an enriched genomic library using the methods described by Billotte *et al.* (1999) with some minor modifications (*cf.* Ferreira *et al.*, 2013). The required genomic DNA was extracted from a sample of *Bryconamericus* aff. *iheringii* using the method of Almeida *et al.* (2001). Briefly, 5 µg was digested using 50 U of RsaI endonuclease and specific adapters *Rsa*I-21 (5′CTCTTGCTTACGCGTGGACTA3′) and *Rsa*I-25 (5′TAGTCCACGCGTAAGCAAGAGCACA3′) were linked to digested DNA using 5U of T4 DNA ligase (Invitrogen; www.invitrogen.com) in reaction buffer, including 10µM of each adapter in a final volume of 200µl and incubated at 20°C for 2 h. Fragments with putative microsatellite sequences were obtained using three probes bound to biotin: $(AGA)_5$, $(CT)_8$ and $(GT)_8$. These fragments were amplified by Polymerase Chain Reaction and cloned into pGem-T Easy (Promega; www.promega.com) vectors using 5µl of amplification product, 50 ng of vector and 1 U of T4 DNA ligase in reaction buffer at 4°C (overnight). Cloning products were used to transform *Escherichia coli* (DH5- α lineage) cells.

A total of 96 positive clones were amplified using M13 primers (F and R) and subsequently sequenced using a Big Dye Terminator 3.1 sequencing kit (Applied Biosystems; www.appliedbiosystems.com) on an automated sequencer (ABI3500 xL, Applied Biosystems). BioEdit v.7.0 software (Hall, 1999) was used to visualize sequences and the Primer3 program (Rozen, Skaletsky, 2000) to generate microsatellite locus specific primers. Tests for potential hairpin structures and primer-dimers were conducted in AutoDimer software (Vallone, Butler, 2004).

Microsatellite validation was performed on a panel consisting of 32 individuals of *B.* aff. *iheringii* sampled from four streams (eight individuals per stream) in the Cinzas River basin (Grande - 23°16’00.70”S 50°27’58.06”W, Antas - 23°04’10.01”S 50°23’48.32”W, Barreiro- 23°38’44.97”S 50°23’32.32”W, and Bonito - 23°23’42.90”S 50°10’8.61”W streams), Southern Brazil. Cross-species amplification was trailed on extracted DNA from four other small Characidae species (five individuals per species), *Piabarchus stramineus* (Eigenmann, 1908), *Piabina argentea* Reinhardt, 1867, *Serrapinnus notomelas* (Eigenmann, 1915), and *Hyphessobrycon eques* (Steindachner, 1882), captured in the same drainages. Voucher specimens were deposited in the Museu de Zoologia da Universidade Estadual de Londrina (MZUEL) fish collection under catalog numbers, as follows: MZUEL 6457 (*Bryconamericus* aff. *iheringii*), MZUEL 6460 (*Piabarchus stramineus*), MZUEL 6466 (*Piabina argentea*), MZUEL 6467 (*Serrapinnus notomelas*) and MZUEL 6468 (*Hyphessobrycon eques*).

Microsatellite fragment length polymorphisms were analyzed on an automatic sequencer ABI PRISM 3500 xL, using GeneScan 600 Liz (Applied Biosystems) as standard molecular weight. Fluorescent labeling of fragments followed the method of Schuelke (2000), which includes an additional M13 sequence (5′- TGTAAAACGACGGCCAGT-3′) on the 5′ end of the forward primer.

PCR reactions were conducted in a total volume of 5µl, including 1 X GoTaq Master Mix (Promega), 10 ng of template DNA, 1% glycerol, 0.125 µM labeled M13 primer (either FAM, HEX or NED, Applied Biosystems, CA), 0.125 µM reverse primer, and 0.0125 µM forward primer. Amplifications were performed with an initial denaturation step at 94 °C for 4 min, followed by 35 cycles at 94°C for 40s, 48°C, 52°C, 56°C, or 60°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 30 min.

Genotyping was performed using the GeneMarkerv.1.85 program (SoftGenetics; www.softgenetics.com). Cervus v.3.0 (Marshall *et al.*, 1998) was used to calculate the number of alleles (k), observed (H_o) and expected (H_e) heterozygosities, and polymorphic information content (PIC). The software package GENEPOP 4.0.10 (Raymond, Rousset, 1995; Rousset, 2008, using default settings) was used to test for conformance to the Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD) and resulting probability values were corrected for multiple tests using the sequential Bonferroni correction (Rice, 1989). The genetic identity (I) and paternity exclusion (Q) probabilities (Paetkau *et al.*, 1995; Weir, 1996) were estimated using Identity 1.0 (Wagner, Sefc, 1999, default settings). Endogamy coefficient (F_{IS}) was estimated using the program Arlequin 3.5 (Excoffier, Lischer, 2010). Tests (default settings) for the presence of null alleles and genotyping errors caused by large allele dropout and stutter were assessed using the programme MicroChecker v.2.2.3 (Van Oosterhout *et al.*, 2004).

Results

Analyses of chromatograms for the 96 clones confirmed the presence of microsatellite repeats in a total of 46 sequenced clones. Based on sequence quality, microsatellite repeat structure, and primer suitability 15 loci (almost all dinucleotide repeats) were selected for further testing. These 15 loci all amplified successfully, showing polymorphisms that could be genotyped unambiguously. Information for these loci was submitted to the GenBank (accession numbers: MF693815-MF693829, Tab. 1).

A total of 192 alleles were detected across the 15 microsatellite loci (mean of 12.8 per locus), ranging from four (*Bih26*) to 32 (*Bih16*) alleles per locus (Tab. 1).

The *Bih42* locus showed the lowest observed (H_o = 0.094) and expected (H_e = 0.205) heterozygosities while the *Bih16* showed the highest observed and expected heterozygosity (H_o = 0.813 and H_e = 0.952, respectively). The mean Polymorphic Information Content (PIC) for the 15 loci was 0.686. Following a scale proposed by Botstein *et al.* (1980), 12 loci (*Bih13*, *Bih16*, *Bih19*, *Bih22*, *Bih30*, *Bih31*, *Bih40*, *Bih44*, *Bih45*, *Bih47*, *Bih76,* and *Bih77*) were highly informative, with PIC values > 0.5, two loci (*Bih26* and *Bih73*) were moderately informative (PIC > 0.25 and < 0.5), and only *Bih42* showed low informative potential, PIC < 0.25 (Tab. 1). This set of loci demonstrated a very low value of genetic identity combined probability ($4.7x10^{-6}$) and high combined probabilities of paternity exclusion (0.999), indicating high discriminatory power for population genetic studies.

Tab. 1. Description of 15 polymorphic microsatellite loci isolated from Neotropical fish *Bryconamericus* aff. *iheringii*. *T*a optimal annealing temperatures; *k,* number of alleles; allele size range (bp); H_o, observed heterozygosity; H_e, expected heterozygosity estimated from 32 individuals; PIC, polymorphic information content; *Q*, paternity exclusion probability; *I*, probability of genetic identity; F_{IS}, endogamy coefficient; GenBank accession numbers. *Significant value of endogamy coefficient (F_{IS}).

Locus name	Sequence repeat	Primer sequences (5' - 3')	*T*a (°C)	*k*	Allele size range (bp)	H_o	H_e	PIC	(*Q*)	(*I*)	F_{IS}	GenBank accession
Bih13	$(CA)_{14}$	F: CGCTCATTCAAGGTGAGAGTC R: CTACGCCTTCCTACGCTGAG	56	22	184 - 258	0.719	0.933	0.929	0.864	0.285	0.244*	MF693815
Bih16	$(GT)_{17}$	F: TAAGGAGAGCGTCAGAATCC R: CAGCAGCTCAGCTCTTCATC	56	32	201 - 351	0.813	0.952	0.950	0.903	0.275	0.162*	MF693816
Bih19	$(AC)_{14}$	F: GGCATAAGGAATCCAGCATATC R: GTAACCGCTGATCACTAATAAGG	56	24	169 - 243	0.781	0.930	0.926	0.858	0.287	0.175*	MF693817
Bih22	$(AG)_{13}$ GG $(AG)_3$	F: GGTCCTGCACTCGCTGTTAG R: GCCAGCCAACGGTTAGAG	52	19	211 - 281	0.375	0.853	0.840	0.852	0.336	0.570*	MF693818
Bih26	$(GT)_4$A $(AG)_5$	F: GAACTGCACGTTACATTCAGGA R: CTCAGCACTTCTGCCTGAC	52	4	175 - 197	0.250	0.514	0.465	0.285	0.658	0.524*	MF693819
Bih30	$(TG)_{14}$	F: CTCCACGGCTGAAACAATG R: CCAGCGAATAATCAGTCTTCC	48	7	159 - 187	0.414	0.601	0.551	0.440	0.493	0.327*	MF693820
Bih31	$(GT)_{14}$	F: GCATTGATATCCTGCCATCC R: TCTACCTGTCCATCGTGTCC	52	19	182 - 260	0.310	0.893	0.885	0.903	0.303	0.662*	MF693821
Bih40	$(TG)_2$C $(TG)_{12}$	F: ACAGCGCATTCCTCCATTC R: AATCGACACGCTGCCAAG	48	12	201 - 235	0.500	0.712	0.686	0.516	0.449	0.312*	MF693822
Bih42	$(AC)_{14}$AA $(AC)_3$	F: GAGCCGAACGCTGATGAC R: CCTCCAGACTTGACTACACCAC	52	7	108 - 174	0.094	0.205	0.201	0.113	1.121	0.552*	MF693823
Bih44	$(GT)_5$ TAACACAG $(GT)_8$	F: TCCTCTCATAGCGCTCTCTTC R: GCTTCACGTTACACCTGGAC	48	6	138 - 166	0.500	0.730	0.693	0.510	0.429	0.329*	MF693824
Bih45	$(GA)_6(GT)_{14}$	F: CCGGATCAGCAGAACACAG R: CAGGTCAACTACAACGACACC	60	8	207 - 235	0.438	0.750	0.721	0.551	0.414	0.429*	MF693825
Bih47	$(TTAT)_5(CT)_9$	F: CTCCTCCGATACCTGTGAACTC R: CCAGCAGATACACAACACAGC	52	7	126 -160	0.281	0.571	0.540	0.363	0.595	0.518*	MF693826
Bih73	$(GT)_{17}$	F: CGCAAGCTTATGGTGAATCAG R: CAATGCACGTCATTCTGC	48	9	220 - 268	0.188	0.427	0.416	0.267	0.779	0.571*	MF693827
Bih76	$(AC)_{19}$	F: CTGCATTACTCGGTAGGTTATATG R: CCTGTGCAATTAAGGAACTGTG	52	10	164 - 190	0.500	0.806	0.779	0.619	0.368	0.393*	MF693828
Bih77	$(AC)_8(TG)_2$ TTA$(AC)_5$	F: TGCCAACTTCATTAGAGCTTACAG R: CAGTTATATTCCTCACCTGATAGGAC	52	6	182 - 192	0.469	0.755	0.721	0.543	0.409	0.392*	MF693829
All loci				12.8		0.442	0.709	0.686	0.999	$4.7x10^{-6}$	0.367*	

Tab. 2. Cross-amplification of the 15 polymorphic loci in four small Characidae species. *N,* sample size. The number in brackets represents the number of alleles. The superscript numbers are the number of individuals that successfully amplified per locus. - represents no amplification product.

Primers Species	*N*	Microsatellite loci														
		Bih13	Bih16	Bih19	Bih22	Bih26	Bih30	Bih31	Bih40	Bih42	Bih44	Bih45	Bih47	Bih73	Bih76	Bih77
		Allele size range (bp)														
Piabarchus stramineus	5	162-172	202-216	156-168	208-220	-	179-187	-	210-238	-	163	225-229	132-164	219-225	-	190
		(3)[4]	(5)[5]	(4)[5]	(3)[4]	-	(2)[5]	-	(4)[5]	-	(1)[5]	(3)[5]	(6)[5]	(3)[5]	-	(1)[5]
Piabina argentea	5	190-198	190-228	153-175	231-241	-	169-221	-	206-230	135-159	157-175	197-251	134-1508	212-244	172	185-199
		(4)[5]	(5)[5]	(5)[5]	(3)[5]	-	(6)[5]	-	(5)[5]	(4)[5]	(7)[5]	(4)[5]	(4)[5]	(3)[5]	(1)[5]	(5)[5]
Serrapinnus notomelas	5	-	205-229	-	212	-	220-240	-	166-184	-	-	195-223	-	262	-	-
		-	(3)[3]	-	(1)[5]	-	(6)[5]	-	(4)[3]	-	-	(2)[5]	-	(1)[5]	-	-
Hyphessobrycon eques	5	-	-	-		-	203-237	-	220	131-133	-	-	-	-	-	-
		-	-	-		-	(8)[5]	-	(1)[5]	(2)[5]	-	-	-	-	-	-

Discussion

As expected, the mixture of individuals from different streams (possibly composing different populations) resulted in deviations from the Hardy-Weinberg equilibrium (HWE) and significant F_{IS} values for all loci. This is likely an indication of a Wahlund effect caused by including elements of multiple genetic units in a single panel causing excess homozygosity and significant F_{IS} estimates (Freeland, 2005). From the inflated homozygosity estimates, MicroChecker suggested null alleles were present at all loci. However, it is unlikely that the excess of homozygosity was caused by null alleles as it would require all analysed loci to be affected by null alleles. Instead we favour the hypothesis that the samples were derived from genetically distinct units and the pooling of these units caused a Wahlund effect. After correction for multiple tests (sequential Bonferroni correction, k= 105), thirteen loci combinations showed linkage disequilibrium, the locus *Bih73* was linked to loci *Bih30*, *Bih44*, *Bih45*, *Bih47*and *Bih77*, the locus *Bih45* linked to loci *Bih31*, *Bih40* and *Bih42*, the locus *Bih76* linked to loci *Bih16* and *Bih22*, the locus *Bih77* showed linkage with loci *Bih30* and *Bih44*, and the locus *Bih44* with *Bih30.*

The novel microsatellite loci developed in the present study constitute a highly variable marker set suitable for genetic studies of *Bryconamericus* aff. *iheringii*. Moreover, successful cross-species amplifications of some loci indicate that these microsatellites can be used for genetic studies of other small Characidae; *Piabina argentea* (12 polymorphic loci) and *Piabarchus stramineus* (nine polymorphic loci) (Tab. 2).

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