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Notes and Comments

Microsatellite multiplex systems for *Brycon orbignyanus* (Characiformes: Bryconidae)

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The Neotropical migratory (*piracema*) fish species *Brycon orbignyanus* (Valenciennes, 1850), commonly known as piracanjuba, was once considered one of the most conspicuous resources for fisheries in the La Plata River Basin, the second largest in South America, but today is considered a threatened species, almost extinct in the wild (Machado et al., 2008; Oliveira et al., 2017). It suffers from several pressures such as intense hydroelectric exploitation in the basin, pollution, deforestation of riverine vegetation and introduction of species (Oliveira et al., 2017). Its omnivorous habits and fast growth curve under domestication (Sipaúba-Tavares et al., 2008; Nogueira et al., 2014) makes this fish a good candidate for the development of aquaculture programs, which could guarantee its *ex situ* cultivation and help *in situ* conservation initiatives. Hatchery production of this species, targeting stocking efforts in areas affected by dam introduction, is presumed to be important in helping maintaining small population remnants in some isolated points in the basin, but this is still unaddressed in rigorous scientific studies. Such scenario brings about the importance of the development of tools for the rapid assessment of genetic diversity and structure, in order to identify the impacts over dwindling stocks and to evaluate the efficiency of fish stocking activities, in this important species.

Microsatellite markers are considered useful and effective tools for these purposes and have been demonstrated to be efficient in stock selection and reintroduction programs (*e.g.* Attard et al., 2016). *B. orbignyanus* could benefit from the development of advanced breeding and rearing technologies, in which microsatellite diversity assessment systems could be valuable resources (Lopera-Barrero, 2009). Despite its status, species-specific microsatellite markers were lacking in *B. orbignyanus* until recent years, which led to the use of heterologous loci for breeding, genetic diversity and conservation studies in this species (*e.g.* Rodriguez-Rodriguez et al., 2010; Lopera-Barrero et al., 2010, 2014; Ashikaga et al., 2015; Carmo et al., 2015; Castro et al., 2017). This yields a restricted number of usable loci and can lead to increasing artefactual noise in the analyses (Carmo et al., 2015).With the unveiling of the first 29 microsatellite loci from *B. orbignyanus* (Arias et al., 2016), by the application of next-generation sequencing (NGS) pipelines (Yazbeck et al., 2018), more reliable genetic analysis systems for this species can now be developed, in order to accelerate the profiling of extant and cultivated stocks in this threatened species. Recently, seven polymorphic microsatellite loci were also developed by means of traditional cloning methods, involving a microsatellite-enriched library prepared with hybridization capture process, and their transferability assayed, along five other new markers, in *Brycon gouldingi* and *B. falcatus* (Souza et al., 2018).

Combining (*i.e.* multiplexing) polymerase chain reactions (PCR) of different loci, simultaneously into a single assay, provides the advantage of making the process more economically efficient. Multiplex systems of microsatellite loci are particularly useful for making it practical to perform parentage analysis in fish, an important step in the evaluation of the efficiency of stock supplementation, selective breeding programs and hatchery management (Abdul-Muneer, 2014; Yue and Xia, 2014). Here we present the design, development and validation of microsatellite multiplexes for *B. orbignyanus*.

Candidate multiplex systems were evaluated from the group of microsatellite markers described in Arias et al. (2016). For combined primers selection we developed an in-house program, written in Python, based on graph theory, with the aid of the igraph library to automatically sort markers based on its reported range of fragment amplification length: each microsatellite marker is represented as a vertex in the graph and pairs of markers bearing mutually exclusive observed amplification ranges being connected by edges. This permitted us to create lists of possible compatible sets of primers. Further, this compatibility was verified with Primer3 (Untergasser et al., 2012) to avoid heterodimers and used as a further criterion to select putative marker groups for empirical testing. We newly analyzed the extracted DNA from a subsample of 36 individuals, out of the 49 present in Arias et al. (2016). We used the initial PCR conditions for each individual DNA marker, described in the supplemental information for *B. orbignyanus*– Electronic Supplementary Material 16 – *in* Arias et al. (2016), and tested the multiplex systems by doubling buffer concentration, adding TBT (Trealose-BSA-Tween 20 – Samarakoon et al., 2013), assaying varying primers and $MgCl_2$ concentrations, following the guidelines presented in Green and Sambrook (2012).

The empirical test for each proposed multiplex system included the elimination of loci which, despite being theoretically compatible in range of amplified fragment

size, resulted in unspecific amplification of stutter or ghost bands, confusing straight genotyping. Each reaction was standardized with an annealing temperature of 50ºC, 1 U of *Taq* DNA polymerase and a final concentration of 0.1 mM of dNTPs; 100 mM KCl; 25 mM Tris-HCl pH 8.4; 0.2% Triton X-100; 300 mM trehalose; 400 µg/ml nonacetylated BSA and 0.4% Tween-20 for a multiplex volume of 10 µl. This was followed by adjustments in primers and enzymatic co-factor (Mg^{++}) concentrations. Amplification results were resolved using polyacrylamide gel electrophoresis (PAGE), using 10% gels and applying 4.5 $V \cdot cm^{-1}$, for 15 h. Conformity with Hardy-Weinberg Expectations (HWE) was verified with the exact test implemented on Genepop, according to a Holm-Bonferroni significance correction and other general variability descriptors (number of alleles, observed and expected heterozygosity, locus inbreeding coefficient, Shannon's information index) were assessed with GenAlex.

A set of six individual PCR assays, biplexes and triplexes, including a total of 16 different *B. orbignyanus* microsatellite loci (multiplexes 1 through 6) were developed and tested (as shown in Table 1). Genetic diversity results were obtained through its application in a broodstock sample (as shown in Table 2). The observed number of alleles ranged from 2 to 9, the expected heterozygosity varied from 0.24 to 0.86, and Shannon information's index ranged from 0.41 to

Table 1. Microsatellite multiplex systems for *B. orbignyanus*. Table shows the concentrations of variable components for PCR ($MgCl_2$ and each primer), Na=Number of alleles.

System	Locus	GenBank accession	Repeat sequence	$MgCl_2$ (mM)	Primer (mM)	Expected/observed range of product length (bp)	Na
Multiplex 1	Borb01	KT827795	ATCT	2.5	0.25	100-135	8
	Borb25	KT827811	AC		0.25	262-318	9
	Borb33	KT827815	AC		0.15	68-83	3
Multiplex 2	Borb04	KT827796	ATAG	3	0.1	118-123	2
	Borb11	KT827801	ATT		0.25	147-178	8
	Borb39	KT827820	AC		0.25	94-101	2
Multiplex 3	Borb07	KT827798	ATA	2.5	0.25	142-152	4
	Borb30	KT827814	CA		0.15	79-89	4
	Borb46	KT827823	CA		0.25	121-131	3
Multiplex 4	Borb08	KT827799	ATT	2.5	0.25	114-140	8
	Borb15	KT827805	ATT		0.25	147-179	9
	Borb24	KT827810	AC		0.15	96-104	4
Multiplex 5	Borb09	KT827800	AAT	3.5	0.35	126-144	5
	Borb36	KT827818	AG		0.35	89-109	6
Multiplex 6	Borb17	KT827807	ATA	3.5	0.35	131-151	6
	Borb44	KT827822	AG		0.35	88-110	5

Table 2. Diversity results from the new *B. orbignyanus* microsatellite multiplexes.

System	Locus	I	Ho	He	F	P-value	HWE
Multiplex 1	Borb01	1.81	0.47	0.81	0.42	0.0000	No
	Borb25	1.95	0.97	0.81	-0.19	0.0329	Yes
	Borb33	0.77	0.47	0.44	-0.08	0.2916	Yes
Multiplex 2	Borb04	0.41	0.23	0.24	0.07	0.5261	Yes
	Borb11	1.90	0.66	0.83	0.21	0.0006	No
	Borb39	0.49	0.33	0.31	-0.06	1	Yes
Multiplex 3	Borb07	1.11	0.19	0.63	0.69	0.0000	No
	Borb30	1.33	0.28	0.72	0.61	0.0000	No
	Borb46	0.99	0.19	0.59	0.67	0.0000	No
Multiplex 4	Borb08	1.77	0.58	0.79	0.26	0.0008	No
	Borb15	2.07	0.41	0.86	0.53	0.0000	No
	Borb24	0.99	0.44	0.52	0.14	0.0650	Yes
Multiplex 5	Borb09	1.28	0.64	0.65	0.02	0.0588	Yes
	Borb36	1.62	0.53	0.78	0.32	0.0000	No
Multiplex 6	Borb17	1.66	0.61	0.79	0.22	0.0108	Yes
	Borb44	1.30	0.58	0.69	0.15	0.0192	Yes

I= Shannon's Information Index; Ho=Observed heterozygosity; He=Expected heterozygosity; F=Fixation index; P-value=exact probability value; HWE=Hardy-Weinberg Expectations.

2.07, respectively for marker Borb04 present in Multiplex 2 and marker Borb15, from Multiplex 4. Most pronounced value of F (Sewall Wright's inbreeding coefficient) was found at locus Borb07, followed by Borb46, Borb30, Borb15 and Borb01. Half the multiplexed loci conformed to the Hardy-Weinberg expectations in the sample, after a Holm-Bonferroni significance correction (α=0.0008).

The six combinations of molecular DNA markers for performing multiplex microsatellite PCR in *B.* orbignyanus validated here encompass around 44% of all specific polymorphic microsatellites so far validated for this species (Arias et al., 2016; Souza et al., 2018). These multiplex systems potentially increase the rate the genetic analysis of these 16 loci in approximately 2.5 times (*i.e.* 16 markers in six reactions) and provide the first multiplex class of specific DNA markers for *B. orbignyanus*. The results found on the diversity analysis of the multiplex sets reveal a similar pattern as observed in Arias et al. (2016), which analysed more fish (N=49) and more markers (N=29). Only a single allele (#03) was not detected at one locus (Borb07), due to the smaller sample analyzed here (N=36 fish). Lack of conformation with HWE is probably due to stock genetic substructuring, because of broodstock formation, which typically draws from alternative population sources, leading to a pronounced Wahlund effect (all deviations are due to excess of homozygous genotypes). Besides, it has been argued that purging data for the lack of HWE conformation (when the cause is not a PCR artifact) can do more harm than good (*e.g.* Allendorf et al., 2013). Future full families tests can screen out fortuitous null alleles. Our systems balance both class of loci and presents four times more markers than in a conservation genetics analysis recently carried out in this fish (Ashikaga et al., 2015). Taken together, these results attest to the systems' usefulness in genetic diversity studies and applications.

The development of multiplex systems in PAGE, rather than capillary electrophoresis (CE), severely hampers the assembling of assays accumulating more loci in a single reaction (*e.g.* Wang et al., 2016). Nevertheless, it favors the use of loci with amplified fragment profile promptly detectable with the use of simple electrophoresis rigs, generally available to small laboratories and easily adaptable to hatchery operations.

Although radically new alternative approaches have been recently revealed for the massively parallel screening of microsatellite loci, conjugating CRISPR-Cas 9 and NGS technologies (Shin et al., 2017) – along with its promised dramatic drop in the cost of molecular variability analysis in non-model species – PCR based marker screening will still provide a way for rapid access of genetic information in simple laboratories, hatcheries and conservation initiatives focusing on piracanjuba.

The multiplex systems presented here constitute the first combined specific PCR markers for this important Neotropical fish. It will assist in alleviating labor and reducing material costs of routine DNA analysis in *B. orbignyanus*.

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