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Obtaining microsatellite markers for Pseudoplatystoma reticulatum using heterologous primers

Obtenção de marcadores microssatélites para Pseudoplatystoma reticulatum a partir de primers heterólogos

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Highlights

Heterologous primers were successfully transferred to Pseudoplatystoma reticulatum.
Three highly-informative heterologous primers were obtained.
Heterologous primers will contribute to genetic studies of the species.

Abstract

Studies on genetic composition in fish populations contribute to conservationist practices and inbreeding control in fish stocks. To this end, molecular tools such as microsatellite markers (SSRs) are often used, but they are expensive and time-consuming to develop. A species-specific heterologous marker emerges as an alternative, which can be used in taxonomically related species in a fast way. Our goal was to test SSRs markers of *Brachyplatystoma rousseauxi* and *Pseudoplatystoma punctifer* in *P. reticulatum* in an unprecedented way. For this purpose, DNA was extracted from fragments of the caudal fin of 222 *P. reticulatum* adults, using a NaCl-based method. Then, DNA samples were amplified by Polymerase Chain Reaction (PCR) using six markers, four from B. rousseauxi (BR38, 47, 51, and 61) and two from *P. punctifer* (PPU13 and PPU15). Two primers showed non-specific amplification and were disregarded (BR38 and PPU13). In the remaining four

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primers, the number of alleles per locus varied between two (BR47) to sixteen (BR51), and the average size of alleles was between 142 and 400 bp. Mean effective number of alleles per locus ranged from 10,650 (BR51) to 1,784 (BR47), with null or low-frequency alleles in all studied loci. Observed heterozygosity ranged from 0.299 (BR47) to 0.640 (BR51) and was always lower than the expected heterozygosity. Hardy-Weinberg balance was significant ($p < 0.05$) in all loci, and inbreeding coefficient (FIS) was always positive. Polymorphic Information Content (PIC) confirmed the efficiency of the markers since they had moderate (BR47) to high levels of information (BR51, BR61, and PPU15). Transferability test showed that the heterologous microsatellite molecular markers, originally for *B. rousseauxi* and *P. punctifer,* were efficient in *P. reticulatum,* producing three primers with high information content. Therefore, these markers can be safely used in future population studies of this species.
Key words: *Brachyplatystoma rousseauxi.* Cachara. Genetic variability. Heterologous markers. Population genetics. *Pseudoplatystoma punctifer.*

Resumo

Estudos acerca da composição genética de populações de peixes contribuem para a elaboração de práticas conservacionista, bem como para controle da consanguinidade em estoques de piscicultura. Neste sentido, ferramentas moleculares como os marcadores microssatélites (SSR's) são comumente utilizados, contudo, seu desenvolvimento corresponde a um processo caro e demorado. Surge como alternativa a utilização dos marcadores heterólogos, originalmente desenvolvidos para uma espécie, mas que podem ser utilizados de maneira rápida em outras taxonomicamente relacionadas. O objetivo desta pesquisa foi testar de maneira inédita a utilização de marcadores SSR's de *Brachyplatystoma rousseauxi* e *Pseudoplatystoma punctifer* em *P. reticulatum.* Para tanto, foram extraídas amostras de DNA a partir de fragmentos de nadadeira caudal de 222 indivíduos adultos de *P. reticulatum* por metodologia a base de NaCl. As amostras foram então amplificadas por Reação em Cadeia da Polimerase (PCR) utilizando-se seis marcadores, quatro de *B. rousseauxi* (BR38, 47, 51 e 61) e dois de *P. punctifer* (PPU13 e PPU15). Dois primers apresentaram amplificação inespecífica e foram desconsiderados (BR38 e PPU13). Entre os quatro restantes, o número de alelos por locus variou entre dois (BR47) a dezesseis (BR51), com tamanho médio de alelos entre 142 e 400 pb. O número efetivo médio de alelos por locus variou de 10,650 (BR51) à 1,784 (BR47), com alelos nulos ou de baixa frequência presentes em todos os loci estudados. Os valores de heterozigosidade observada variaram de 0,299 (BR47) à 0,640 (BR51), e foram sempre menores que os de heterozigosidade esperada. O Equilíbrio de Hardy-Weinberg foi significativo ($P < 0,05$) em todos os locus e o coeficiente de endogamia Fis sempre apresentou valores positivos. Os valores de Conteúdo de Informação Polimórfica (PIC) confirmaram a eficiência dos marcadores utilizados, já que possuíram de moderado (BR47) a alto nível de informação (BR51, BR61 e PPU15). O teste de transferibilidade de marcadores moleculares microssatélites heterólogos, originalmente desenvolvidos para *B. rousseauxi* e *P. punctifer* em *P. reticulatum* se mostrou eficiente, produzindo três primers com alto nível de informação, fato que garante sua utilização segura em estudos populacionais futuros voltados à esta espécie.
Palavras-chave: *B. rousseauxi.* Cachara. Variabilidade genética. Genética populacional. Primers Heterólogos. *P. punctifer.*

Introduction

In recent years, Brazil has gained prominence due to its considerable growth in fish production. According to the 2020 yearbook of the Brazilian Association of Fish Farming [Peixebr] (2020), the country went from 578,000 tons in 2014 to 758,006 tons in 2019 (i.e., a rise of 31%). Among the species produced, *Pseudoplatystoma reticulatum,* popularly known as *'cachara,'* is particularly important for its noble meat, high-quality fillet, and few intramuscular bones (Campos, 2010).

Despite its high demand, reproductive management of this species still needs to be improved in fish farms. For instance, it is difficult to maintain a considerable number of breeders in fish ponds, as well as high prolificacy. Often, many fingerlings are obtained from a few couples, with difficult individual control of pedigree. This directly interferes with the effective number of animals mated, increasing within-shoal inbreeding (Povh et al., 2010; Lopera-Barrero et al., 2016). High inbreeding rates can lead to inbreeding depression, which is characterized by reduced growth and reproductive capacity, as well as decreased offspring survival due to loss of adaptive gene pools (Ribeiro et al., 2016).

Molecular tools, such as microsatellite markers or Simple Sequence Repeats (SSRs), have been used today to study genetic variability in fish populations (Lopera-Barrero et al., 2016). According to Prado et al. (2014), in all, 16 specific SSR markers have already been developed for *Pseudoplatystoma reticulatum.* However, the same authors report that population studies require the collection of as much information as possible, thus increasing the number of analyzed loci.

Developing new SSR markers is a costly and time-consuming process (Abdul-Muneer, 2014). In this sense, another alternative would be cross-taxon conservation and transferability of genic SSR markers (Abdul-Muneer, Gopalakrishnan, Shivanandan, Basheer, & Ponniah, 2011). In this way, markers developed for one species can be used in another by amplification of homologous loci (Sudheer et al., 2011). Thus, SSR marker transferability is an effective approach to save time and money, especially in species not yet sequenced as a large part of the Siluriformes (Maduna, Rossouw, Roodt-Wilding, & Merwe, 2014). SSR marker transferability studies among species of the genus *Pseudoplatystoma* have already been carried out. For example, Carvalho, Seerig, Brasil, Crepaldi and Oliveira (2013) used this technique to identify hybrids of *P. corruscans* and *P. reticulatum.* Latterly, Albuquerque, Oliveira, Rodriguez-Rodriguez and Ribeiro (2020) successfully tested the transferability of eight *P. corruscans* and *P. punctifer* markers in *P. reticulatum.* Our research aimed to unprecedentedly evaluate the transferability of specific SSR markers of *Brachyplatystoma rousseauxi* and *P. punctifer* in *P. reticulatum.*

Materials and Methods

The procedures used in this study were approved by the Ethics Committee on Animal Use (CEUA) of the State University of Maringá, under number 9710100918.

Sampling

Caudal fin fragments of 222 *Pseudoplatystoma reticulatum* adults were

obtained from a commercial breeding stock in the city of Cuiabá, Mato Grosso State, Brazil (Latitude: 15° 35′ 56″ South, Longitude: 56° 5′ 42″ West). Samples were placed in 1.5-mL microtubes containing 70% ethyl alcohol. Then, they were sent to the Laboratory of Molecular Biology of the PeixeGen research group, at the State University of Maringá, Paraná, Brazil, where they were kept in a freezer (-20 °C) until processing.

DNA extraction

Total DNA was extracted according to a NaCl-based method (Lopera-Barrero et al., 2008). Accordingly, caudal fin fragments (0.5 cm²) were individually placed in 1.5-mL microtubes. Then, a digestion solution was added, which was composed of 550-µL lysis buffer (50 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl), 160-µL SDS (1%), and 10-µL proteinase K (200 µg mL^{-1}). Then, the samples were incubated in a water bath at 50 °C for 12 h. After incubation, digestion products were removed by adding 600-µL NaCl solution (5M) and centrifuging (for 10 min at 12,000 rpm), then the supernatant was collected. Afterwards, DNA was precipitated by adding 700-µL absolute ethyl alcohol, previously chilled and incubated for 1 h at -20 °C. DNA pellets were obtained after another centrifugation (10 min at 12,000 rpm), discarding the supernatant. DNA was then diluted in 80-µL TE buffer (10 mM Tris pH 8.0 and 1 mM EDTA) and treated for RNA removal by treatment with 7-µL RNAse (30 µg mL^{-1}) in a 37 °C water bath for 1 h, and then stored in a freezer (-20 °C).

Total DNA integrity and standardization

Total DNA was quantified on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 260 nm, and samples were diluted to 10 ng/µL final concentration. DNA integrity was verified by horizontal electrophoresis on a 1% agarose gel at 70 V for 120 min. DNA fragments were observed under UV using the L-PIX system (LOCCUS biotechnology), with a standard of 100-bp known bases (Invitrogen, Carlsbad, California, EUA).

Amplification of microsatellite primers

A total of six SSR markers were evaluated, four originally developed for *Brachyplatystoma rousseauxi* (Batista, Farias, Formiga-Aquino, Sousa, & Alves-Gomes, 2010) and two for *Pseudoplatystoma punctifer* (Saulo-Machado et al., 2011). Table 1 shows sequence information, annealing temperature, and GenBank accession number.

Table 1
Characteristics of the microsatellite molecular markers used

Marker	Sequence 5′ - 3′	AT (°C)	GenBank	Species	Refence
BR38	F - AGTTCCTTCTCGTTCCCCTTC R - ATCTCCCACTCTCTCTGGCTC	62	GQ903737	*B. rousseauxii*	Batista et al., 2009
BR47	F - TCAGTGTGTGTGTGACTGTTG R - GCTCCTCTTGTTTCACTTTC	59	GQ903739		
BR51	F - GTTACACATGGTCGCTGGTG R - GTTCATTCTCTTCGGCTTCG	60	GQ903743		
BR61	F - CTGTCGAAAACATGAGGCAG R - GACATCAGAGCGAAGCACAC	65	GQ903749		
PPU13	F - TCTCAGGGGCCATTCTCA R - ATCAATTCCCAGCCGGAG	60	HQ317856	*P. punctifer*	Saulo-Machado et al., 2011
PPU15	F - GAGCGCCCAAGGTTCAC R - GGCCAAAGTAACAGGCCA	60	HQ317858		

AT, Annealing temperature.

Amplification reactions were carried out to a 16-µL final volume, using 1X Tris-KCl buffer, 2.5 mM $MgCl_2$, 0.8 µM each primer, 0.4 mM dNTP, one Platinum Taq DNA Polymerase, and 30-ng target DNA. The reactions were performed in a Veriti® thermal cycler (Applied Biosystems, Foster City, California, USA). First, DNA was denatured at 94ºC for four minutes, followed by 35 30-second denaturation cycles at 94 ºC, 30-second annealing temperature (specific for each primer), 1-minute extension at 72 ºC, and a final extension at 72ºC for 10 minutes.

The set containing 8-µL amplified products and 4-µL running buffer (40% sucrose and 0.25% bromophenol blue) was subjected to vertical electrophoresis on 10% polyacrylamide gel (acrylamide: bisacrylamide - 29: 1) with denaturant (6 M urea) in a 0.5X TBE buffer solution (90 mM Tris-Borate, 2 mM EDTA) at 180 V (250 mA) for 420 minutes (seven hours). Alleles were visualized by staining with silver nitrate, following a method adapted from Bassam, Caetano-Anollés and Gresshoff (1991). The gel was fixed in a solution with 10% ethanol and 0.5% acetic acid for 20 min, then in 6 mM silver nitrate for 30 min, and finally immersed in a developing solution (0.22% 0.75-M NaOH and 1-mL 40% formaldehyde). After development, the gels were photographed and submitted to Adobe Photoshop CC software (64-bit version) for alignment. Allele size was determined using known bases of 100 bp and 50 bp (Invitrogen, Carlsbad, California, USA). The alleles obtained were arranged in data matrices, which were subjected to population statistics.

Statistical analyses

Allele frequency (Fa), size (Sa), number (Na), and effective number (Ne), as well as observed heterozygosity (Ho), expected heterozygosity (He), inbreeding coefficient (FIS), and Hardy-Weinberg balance test (HW) were estimated for each locus by GenAlex computer software version 6.5 (Peakall & Smouse, 2012).

Polymorphic information content (PIC) was calculated by software Cervus 3.0.7 (Kalinowski, Taper, & Marshall, 2007), following a scale proposed by Botstein, White, Skolnick and Davis (1980), wherein *loci* can be highly (PIC > 0.500), mildly (0.250 < PIC < 0.500), and poorly (PIC < 0.250) informative. Additionally, the presence of null alleles (p <0.05) was tested using the Micro-Checker software (Oosterhout, Hutchinson, Wills, & Peter, 2004).

Results and Discussion

As microsatellite locus markers are time-consuming and expensive to develop, mainly in species not fully sequenced (e.g., *P. reticulatum*), heterologous markers originally developed for taxon-related species are an excellent alternative for genetic variability studies. In our study, we successfully amplified four out of the six tested primers, three of which were highly informative. These can, therefore, be used safely in future genetic studies for *P. reticulatum.*

Good SSR marker transferability results have already been reported for fish species, but their amplification quality depends on the degree of genetic conservation of DNA binding sites. In other words, it has a direct relationship with the phylogenetic distance between species, thus being less efficient between distant families or genera (Sun, Li, Xu, & Wang, 2012; Abdul-muneer, 2014). Mantovani (2018) demonstrated that primers originally developed for *Brachyplatystoma rousseauxi, Brycon hilarii,* and *P. punctifer* can be efficiently used in commercial populations of Leiarius marmoratus. Carvalho et al. (2013) used the same technique to identify hybrids of *P. corruscans* and *P. reticulatum.* Moreover, Albuquerque et al. (2020) have recently had success by testing transferability of eight primers of *P. corruscans* and *P. punctifer* in *P. reticulatum.*

In our study two out of the six tested markers showed poor specific amplification. Therefore, they were disregarded in statistical analysis. PPU13 showed unclear amplification, while BR38 revealed a monomorphic band pattern. Conversely, four markers were successfully amplified and showed transferability to *Pseudoplatystoma reticulatum* population, with band polymorphism and clarity. Three of these were originally developed for *Brachyplatystoma rousseauxi* (BR47, BR51, and BR61) and one for *P. punctifer* (PPU15) (Table 2).

Allele number per locus ranged from two (BR47) to sixteen (BR51), averaging 10.5. Yet allele size varied between 142 bp (BR47) and 400 bp (PPU15) (Table 2). Average effective allele number per locus was 6,759, ranging from 10,650 (BR51) to 1,784 (BR47). Such a wide difference between allele number and effective number revealed the presence of null or low-frequency alleles in all studied loci (Table 2).

Observed heterozygosity ranged from 0.299 in BR47 to 0.640 in BR51, always lower than expected heterozygosity (0.766 on average: from 0.439 in BR47 to 0.906 in BR51) (Table 2). Hardy-Weinberg balance was significant (p <0.05) in all loci. Inbreeding coefficient (FIS) was always positive and ranged from 0.294 in BR51 to 0.471 in PPU15. Lastly, based on PIC, we could notice that BR47 (0.343) was mildly informative, while BR51 (0.898), BR61 (0.814), and PPU15 (0.872) were highly informative (Table 2).

Table 2
Parameters of genetic variability obtained from heterologous amplification in samples of *P. reticulatum*

Marker	Na	Size (pb)	Ne	Ho	He	EH-W	FIS	PIC
BR38	MA	-	-	-	-	-	-	-
BR47	2	142 - 162	1,784	0,299	0,439	0,000*	0,320	0,343
BR51	16	221 - 383	10,650	0,640	0,906	0,000*	0,294	0,898
BR61	11	224 - 280	6,053	0,524	0,835	0,000*	0,372	0,814
PPU13	NA	-	-	-	-	-	-	-
PPU15	13	300 - 400	8,549	0,468	0,883	0,000*	0,471	0,872
Mean	10,5	-	6,759	0,483	0,766	0,000*	0,364	0,732

Na, number of alleles; MA, monomorphic amplification; NA, Non-specific amplification; bp, fragment size in base pairs; Ne, effective number of alleles; Ho, observed heterozygosity; He, expected heterozygosity; EH-W, Hardy Weinberg Equilibrium (p values); Fis, inbreeding coefficient; PIC, polymorphic information content. * Significant at the 1% level ($p < 0.01$).

Allele number and size in our study were different from those observed in other Pimelodidae species for the same locus. We obtained two alleles (142 bp and 162 bp) for the BR47 locus, while Mantovani (2018) reported three (136 bp to 150 bp) in *L. marmoratus.* In our study, BR51 produced 16 alleles (221 bp to 383 bp). This is twice the finding of Batista et al. (2009) in *B. rousseauxi,* who found eight alleles (263 bp to 285 bp). For BR61, we found 11 alleles (224 bp to 280 bp), whereas Ochoa et al. (2015) found an average of 16 alleles for six *Brachyplatystoma platynemum* populations, with sizes between 261 bp and 271 bp. Lastly, PPU15 produced 13 alleles (300 bp to 400 bp), just as the findings by Saulo-Machado et al. (2011) in *P. punctifer.* According to Prado et al. (2014), differences in mean allele number and their respective sizes can be attributed to factors such as intrinsic genetic traits of each species, population structure or selective pressures (mutations, gene flow, among others) in the evaluated regions. Thus, despite the differences with other species of the same family, our dataset highlighted that amplification was correct and informative.

Although our focus was on validating the transferability of heterologous primers, some measures of intra-population genetic variability such as heterozygosity indices, inbreeding coefficient, and Hardy-Weinberg balance deviation can reveal heterozygote deficit caused by several factors such as Wahlund (proportion of heterozygotes lower than the Hardy-Weinberg expectation), presence of null or low-frequency alleles, inbreeding, genotyping or sampling errors, or a combination of these factors (Calcagnotto & DeSalle, 2009). Expected heterozygosity means were higher than the observed in all loci, which evidenced a heterozygous deficit, as demonstrated by positive inbreeding coefficients (FIS). Recent studies using natural populations of *P. reticulatum* (Prado et al., 2014) and *R. quelen* have indicated similar results (Virmond, Conceição, Amaral, & Virmond, 2017). Hardy-Weinberg balance deviations were significant for all the studied

loci, which might have been influenced by the heterozygous deficit.

In genetic studies, the PIC described by Botstein et al. (1980) is a marker quality indicator (segregation, population identification, and paternity control). Accordingly, markers with PIC values above 0.5 are deemed highly informative, between 0.25 and 0.50 mildly, and below 0.25 poorly informative. In our study, the PIC values revealed that the studied markers had informative content from moderate to high and are therefore a great tool for population genetic studies of *P. reticulatum* (Castro et al., 2017; Souza et al., 2018).

Conclusion

The results for transferability test showed that heterologous microsatellite molecular markers originally developed for *Brachyplatystoma rousseauxi* and *Pseudoplatystoma punctifer* were efficient in *Pseudoplatystoma reticulatum,* producing three primers with a high level of information. Therefore, they can be safely used in future population studies of this species.

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