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Cross-amplification of heterologous microsatellite markers in *Rhamdia quelen* and *Leporinus elongatus*

Amplificação cruzada de marcadores microssatélites heterólogos em *Rhamdia quelen* e *Leporinus elongatus*

Nelson Mauricio Lopera-Barrero[1*]; Fernanda Tanamati[2]; Maria del Pilar Rodriguez-Rodriguez[3]; Jayme Aparecido Povh[4]; Angela Rocio Poveda-Parra[5]; Rodrigo Alejandro Arellano Otonel[6]; Pedro Luiz de Castro[7]; Elenice Souza dos Reis Goes[7]; Pâmela Juliana Furlan[8]; Ricardo Pereira Ribeiro[9]

Abstract

Native fish species in Brazil are an asset in fish farming, but their natural stocks have been significantly reduced in recent years. To mitigate this negative impact, studies on fish conservation are being conducted and genetic tools for the discrimination of population parameters are increasingly achieving great importance. Current analysis evaluates a set of microsatellite heterologous primers in the jundiá (*Rhamdia quelen*) and in the piapara (*Leporinus elongatus*). Samples from the caudal fin of 15 broodstock from each species were analyzed. DNA extraction was performed with NaCl protocol and the integrity of the extracted DNA was checked with agarose gel 1%. Twenty primers developed for *Piaractus mesopotamicus*, *Colossoma macropomum*, *Prochilodus lineatus*, *Brycon opalinus* and *Oreochromis niloticus* were evaluated. Cross amplification of four primers of the *B. opalinus* and *P. lineatus* species (BoM12, Pli43 and Pli60 in *R. quelen* and BoM2, Pli43 and Pli60 in *L. elongatus*) was assessed. Primers of *P. mesopotamicus*, *C. macropomum* and *O. niloticus* showed no cross amplification in the two species analyzed. Results revealed the possibility of using the four amplified heterologous primers in genetic studies for *R. quelen* and *L. elongatus*.

Key words: Jundiá, piapara, heterologous *primers*

[1] Prof. Dr., Deptº de Zootecnia, Programa de Pós-Graduação em Ciência Animal, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: nmlopera@uel.br
[2] Discente do Curso de Doutorado, Programa de Pós-Graduação em Zootecnia, Universidade Estadual Paulista, UNESP, Jaboticabal, SP, Brasil. E-mail: ftanamati@hotmail.com
[3] Pesquisadora, Universidade Federal dos Vales de Jequitinhonha e Mucuri, UFVJM, Diamantina, MG, Brasil. E-mail: rodrigpilar@gmail.com
[4] Prof. Dr., Universidade Federal de Mato Grosso do Sul, UFMS, Cidade Universitária, Campo Grande, MS, Brasil. E-mail: jayme.peixegen@gmail.com
[5] Pós-Doutoranda, Deptº de Zootecnia, UEL, Londrina, PR, Brasil. E-mail: angelapovedaparra@hotmail.com
[6] Prof. Dr., Centro Universitário Filadelfia, UNIFIL, Londrina, PR, Brasil. E-mail: otonel.rodrigo@gmail.com
[7] Discentes do Curso de Pós-Doutorado, Programa de Pós-Graduação em Zootecnia, Universidade Estadual de Maringá, UEM, Maringá, PR, Brasil. E-mail: pedrocastro.zoo@hotmail.com; elenicesreis@yahoo.com.br
[8] Discente do Curso de Mestrado, Programa de Pós-Graduação em Ciência Animal, UEL, Londrina, PR, Brasil. E-mail: pamela.furlan@outlook.com
[9] Prof. Dr., Deptº de Zootecnia, Programa de Pós-Graduação em Zootecnia, UEM, Maringá, PR, Brasil. E-mail: rpribeiro@uem.br
[*] Author for correspondence

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Resumo

As espécies nativas de peixes no Brasil tem alto potencial para utilização dentro da piscicultura, porém, seus estoques naturais vêm sofrendo redução nos últimos anos. Para diminuir esse impacto negativo, estudos voltados à conservação estão sendo realizados e cada vez mais, a obtenção de ferramentas genéticas que permitam a discriminação de parâmetros populacionais toma maior importância. Objetivou-se através do presente estudo avaliar um conjunto de *primers* microssatélites heterólogos em jundiá (*Rhamdia quelen*) e piapara (*Leporinus elongatus*). Foram analisadas amostras de nadadeira caudal de 15 reprodutores de cada espécie. A extração de DNA foi realizada utilizando o protocolo com NaCl. A integridade do DNA extraído foi checada em gel de agarose 1%. Foram avaliados 20 *primers* desenvolvidos para as espécies: *Piaractus mesopotamicus*, *Colossoma macropomum*, *Prochilodus lineatus*, *Brycon opalinus* e *Oreochromis niloticus*. Foi observada amplificação cruzada de quatro *primers* das espécies *B. opalinus* e *P. lineatus* (BoM12, Pli43 e Pli60 em *R. quelen* e BoM2, Pli43 e Pli60 em *L. elongatus*). Os *primers* de *P. mesopotamicus*, C. *macropomum* e *O. niloticus* não mostraram amplificação cruzada nas duas espécies analisadas. Os resultados indicaram a possibilidade de uso dos três *primers* heterólogos amplificados em estudos genéticos em *R. quelen* e *L. elongatus*.

Palavras-chave: Jundiá, piapara, *primers* heterólogos

During the last fifty years, fish production has been constantly on the increase worldwide at a mean yearly rate of 3.2%, exceeding world population rate by 1.6% (FAO, 2014). According to data retrieved from the Ministério da Pesca e Aquicultura (2013), fish culture increased from 479,398 tons in 2010 to 628,704 tons in 2011 (31.1%). *Rhamdia quelen* (jundiá) and *Leporinus elongatus* (piapara) are the two main species of great importance produced in Brazil (IBGE, 2013). *R. quelen* and *L. elongatus* are economically important species, with great acceptance on the market (SIGNOR et al., 2013). *R. quelen* is a fast-growing fish species, featuring easy reproduction and savory meat. It lacks intramuscular spines and adapts itself to different breeding systems with great commercial assets (MONTANHA et al., 2011). Similarly, *L. elongatus* has the best physiological traits for fish culture, including an increase of 1 kg in 12 months in fishponds, which make it an excellent fish species for production (MARTINS et al., 2003).

However, eutrophication, pollution, the building of hydroelectric plants and the introduction of exotic fish species have reduced biodiversity in Brazilian water ecosystems and in several countries (AGOSTINHO et al., 2005). Since economically and biologically important species have had their natural stocks decreased, genetic studies for the conservation of economically relevant populations or species provide data that foreground adequate management and conservation in fish populations (GOMES et al., 2013).

Simple Sequence Repeats (SSR) or microsatellites are small DNA sequences, with one to eight nucleotides in length, repeated in tandem (ALAM; ISLAM, 2005). Markers are employed to study genetic variability and answer such issues as population´s real size, migration rate, mating systems and the conservation of genetic resources (BRONDANI et al., 2003; ABDUL MUNEER, 2014). The microsatellites main advantages are their co-dominant transmission (heterozygotes may be differentiated from homozygotes), high polymorphism, hypervariability, specificity for a given place and use in several animal species (ABDUL MUNEER et al., 2009).

Although microsatellite markers are very handy in genetic analysis, no studies on *R. quelen* and *L. elongatus* exist foregrounded on these markers. This is mainly due to the lack of specific primers. Consequently, several researchers used sequenced primers of other species, or rather heterologous

primers (OLIVEIRA et al., 2006; RAMOS et al., 2012) or other molecular markers (GOMES et al., 2008; LOPERA-BARRERO et al., 2008a, 2015; RAMOS et al., 2012). Studies that identify and characterize more primers for the two species are required. Current analysis verifies the cross-amplification of heterologous microsatellite primers in *R. quelen* and *L. elongatus*.

Samples of caudal fins from 30 broodstocks of *R. quelen* and *L. elongatus* (15 of each) were retrieved from two fish farms in the western region of the Paraná State, Brazil. DNA was extracted following method by Lopera-Barrero et al. (2008b) and quantified in a Shimadzu spectrophotometer (Shimadzu Corporation, Kyoto, Japão), wavelength 260 nm, and diluted in TE for a concentration of 20 ng/μL. DNA integrity was checked in agarose gel 1%, revealed with 0.5 μg/mL ethidium bromate and image caught by L-pix system (Loccus Biotecnology, USA).

DNA was amplified in a 16 μL final reaction volume employing 1X buffer Tris-KCl, 2.5 mM of $MgCl_2$, 0.8 μM of each primer (forward and reverse), 0.2 mM of each dNTP, a unit of Platinun *Taq* DNA Polymerase and 30 ng DNA. Twenty primers described for the species *Prochilodus lineatus*, *Brycon opalinus*, *Piaractus mesopotamicus*, *Colossoma macropomum* and *Oreochromis niloticus* were amplified for *R. quelen* and *L. elongatus* (Table 1).

Reactions were performed in Eppendorf Mastercycler Gradient thermocycler with two different cycles to identify the most adequate amplification temperature for each species. The amplification of primers described for *P. mesopotamicus, P. lineatus* and *O. niloticus* was performed according to the following conditions: initial denaturation at 94ºC for 4 minutes; followed by 30 cycles (30 seconds denaturation at 94ºC; 30 seconds annealing and one minute extension at 72ºC) and a final extension at 72ºC for 10 minutes. The conditions for primers of *C. macropomum* and *B. opalinus* were: 94ºC for four minutes; 30 cycles (one minute denaturation at 94ºC; one minute annealing and one minute extension at 72ºC) with a final extension at 72ºC for 10 minutes.

Amplified samples underwent polyacrylamide gel electrophoresis 10%, denaturation (6 M urea) and buffering in TBE 1X (90 mM Tris-Borate and 2 mM EDTA) at 180 volts for eight hours. Silver nitrate was used to visualize microsatellite alleles, following Bassam et al. (1991). After the visualization of the bands, the gels were photographed with a digital camera Nikon Coolpix (S520) and the size of the alleles was calculated by a 100-bp DNA ladder (Invitrogen).

Only three heterologous primers analyzed in *R. quelen* (BoM12, Pli43 and Pli60) showed amplification standards. Annealing temperatures ranged between 45ºC and 69ºC, whilst molecular weight of the alleles varied between 170 pb and 490 pb. Primer BoM12 had three polymorphic alleles (450 pb, 480 pb and 490 pb). Amplification quality was best at 47ºC, 48ºC and 49ºC. The amplification of two monomorphic alleles (180 bp - 190 bp and 170 bp - 180 bp, respectively) was reported for primers Pli43 and Pli60. Best definition in Pli43 was shown at 69ºC, whereas there were no significant differences for Pli60 at 65ºC, 66ºC, 67ºC, 68ºC and 69ºC. Primers of *P. mesopotamicus*, C. *macropomum* and *O. niloticus* did not show any cross-amplification in *R. quelen*, with only a non-specific and difficult-to-read amplification of gels.

Table 1. Primers, Forward and Reverse sequence, expected and reported sizes of alleles and best amplification temperature for heterologous primers in *R. quelen* and *L. elongatus*.

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Primer	Sequence	Expected size	Reported size (pb) *R. quelen*	Best temperature (ºC) *R. quelen*	Reported size (pb) *L. elongatus*	Best temperature (ºC) *L. elongatus*	References
Pli1	F: TGACTGTGAACACGGTCACGC R: ACACAAGTAGAACATACCTCTG	140-295	Without amplification	-	-	-	HATANAKA et al., 2002
Pli30	F: GATGTCGGTTCTTGTACAGTGGTG R: AGCTGCTGAGGATTCTGGGTCAC	162-360	Without amplification	-	-	-	YAZBECK; KALAPOTHAKIS, 2007
Pli43	F: AGTCCACTCCTTAGGCGAGTGAG R: ATAGACGGGCATGTGTCACAGCT	186-240	182-195	69	190-200-215-228	65 e 66	
Pli60	F: GCTAGGACGGTTAGCGTCCACTG R: CGACACGTACATCATTACCTCGG	130-228	170-187	65, 66, 67, 68 e 69	190-202	67-68	
BoM2	F: CTGGGCAGCGGAAGAG R: CCCACATCTCTCCCCTTCG	162-242	-	-	330-372	55 e 56	BARROSO et al., 2003
BoM5	F: CGACCACAATAGGATTAGGG R: CTGGAGTTTGTGTGTGGA	117-151	Without amplification	-	-	-	
BoM6	F: GGAGTTTGTGTGTGGAGACCGAG R: GCACGCAGACACCAGA	142-178	Without amplification	-	-	-	
BoM12	F: GCAGCAGAAAGAAACAG R: CGGGGAGATTTCAACCT	78-118	452-493	47, 48 e 49	-	-	
Pme5	F: CAGAGCATCTGGAGGGACAT R: TCTGAGACACTGATATCTAAACACACA	182-200	Without amplification	-	-	-	CALCAGNOTTO et al., 2001
Pme14	F: ACCGTTATGCCCTACCCTTC R: GCGTTCTAGACAGAACTCATGC	195-208	Without amplification	-	-	-	
Pme28	F: CCCAGAAGAGTGGAAGCTGT R: TGGTGGGAATTGACAAGAAA	209-227	Without amplification	-	-	-	
Pme32	F: GCGAGAAATCTGCCTGTGAC R: AGGAGGGCATCATGGAGAA	242-247	Without amplification	-	-	-	

... Continuation

Cm1C8	F: AGCATGTGTGGAACGTAGGG R: CTGCCGATCACAGCACTAGA	239-273	Without amplification	-	-	-	SANTOS et al., 2009
Cm1D1	F: GCAAATGTGCACACCAATAG R: GCAGAAGGTGAAGAGTCTTGTG	179-209	Without amplification	-	-	-	
Cm1A11	F: CCAGCGGGTTAAAGCCTAC R: GCAGCCTCACTGATACGTTG	230-276	Without amplification	-	-	-	
Cm1B8	F: CACAACCCACCTGTTTGATT R: CTAATAACAAACCTACTTCCACTTCTC	141-169	Without amplification	-	-	-	
UNH108	F: GGGATCAGCTGTTAAGTTT R: TGAGTTGATTATTAATTTCTGA	130-148	Without amplification	-	-	-	KOCHER et al., 1998
UNH154	F: ACGGAAACAGAAGTTACTT R: TTCCTACTTGTCCACCT	184-290	Without amplification	-	-	-	
UNH162	F: CAGACACAGCAGAGGAT R: TGATAAGTAATTCATCTGTTTG	140-170	Without amplification	-	-	-	
UNH163	F: AGCAATCAGCTGTCATC R: CTTTCCTTTTTTAACGTAATTTAAT	120-160	Without amplification	-	-	-	

Further, *L. elongatus* had only three heterologous primers (BoM2, Pli43 and Pli60) with amplification standards. Annealing temperatures varied between 52ºC and 69ºC and variation in molecular weight of the alleles ranged between 190 bp and 370 bp. Primer BoM2 revealed two polymorphic alleles (330 bp and 370 bp). Due to amplification quality, some samples failed to amplify and repetitions occurred for 52ºC (two) and 53ºC (one). Low-definition repetitions revealed amplification standards, although the best definition in this primer occurred at 55ºC and 56ºC. Primer Pli43 showed four polymorphic alleles (190 bp, 200 bp, 210 bp and 220 bp) and the best definition of alleles occurred at 65ºC and 66ºC. Moreover, two monomorphic alleles (190 pb and 200 pb) occurred in primer Pli60. Temperatures failed to have any significant difference, although the best definition occurred at 67ºC and 68ºC. Similarly, as reported for *R. quelen*, the primers of *P. mesopotamicus*, *C. macropomum* and *O. niloticus* failed to show any cross- amplification in *L. elongatus*.

It should be emphasized that allele size in the primers BoM2 and BoM12 exceeded the expected size of the allele. Transference success of primers between the species depends on the degree of genetic conservation of the sites that flank the microsatellite and its evolution stability (ABDUL MUNEER, 2014). Lack of conservation or the occurrence of null alleles may explain the difference in BoM2 and BoM12 alleles. Further research must be undertaken to elucidate the genetic behavior of primers in the studied species.

The use of microsatellite primers in cross amplification is related to the phylogenetic distance among specimens. The technique is efficient in species of the same genus (SUN et al., 2012) but less efficient among families of the same order or between different genera (PENTEADO et al., 2011). However, tests may be applied between specimens of different taxonomies and reported acceptable results, demonstrating the transference of primers among fish species. For instance, Formiga et al. (2010) cross-amplified 36 microsatellite primers developed for *Brachyplatystoma rousseauxii* (dourada) in six species of the same genus (*B. vaillantii, B. filamentosum, B. capapretum, B. tigrinum, B. platynemum, B. juruense*) and obtained 17 amplified primers in all the species. Similarly, Olivatti et al. (2011) tested 46 primers developed for species of the neotropical ichthyofauna species (*Prochilodus argenteus, P. costatus, Leporinus macrocephalus, Astyanax fasciatus* and *Poecilia reticulata*) and obtained eight amplified primers for *L. friderici*. Lopera-Barrero et al. (2014) used the heterologous primers of *Brycon opalinus* to evaluate the genetic variability of *Brycon orbignyanus* matings and reported cross-amplification of the four primers with 11 alleles.

Cross-amplification of primers BoM12, Pli43 and Pli60 was reported in *R. quelen* and of primers BoM2, Pli43 and Pli60 in *L. elongatus*. Results demonstrate their use in genetic studies of the species until species-specific primers are obtained.

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