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Validation of SSR-tetranucleotide multiplex panel for kinship evaluation in tilapia breeding programs[1]

Validação de painel multiplex SSR-tetranucleotídeo para avaliação de parentesco em programas de seleção de tilápias

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ABSTRACT - This study aimed to develop PCR assays, reaction combinations, and validation of tetranucleotide SSR *loci* for tilapia, to minimize the impact of erroneous allele inferences on genotype determination of this marker. Microsatellites containing tetranucleotide repeats were obtained from tilapia genome, version 2.1, avoiding *loci* in the same linking group. Primers were designed for different fragment sizes, and fluorescence added to each *locus*. A total of 10 *loci* were amplified, separately and in combination, and loaded into a single capillary sequencer panel. Alleles were amplified without stutters and easily interpreted. PCR amplifications of DNA repeatedly extracted from samples, and genotyping at different PCR rounds were performed to infer allele signaling errors. The panel obtained in this study is currently used in kinship analyses and pedigree corrections in a tilapia breeding program.

Key words: Genetic variability. Genotyping. Microsatellite markers.

RESUMO - O objetivo deste trabalho foi desenvolver ensaios de PCR e combinação de reações e validação de *loci* SSR de tetranucleotídeos para tilápias, visando minimizar o impacto de inferências errôneas de alelos na determinação dos genótipos deste tipo de marcador. Os microssatélites contendo repetições de tetranucleotídeos foram obtidos da versão 2.1 do genoma de tilápia, evitando a escolha de *locus* no mesmo grupo de ligação. *Primers* foram desenhados para diferentes tamanhos de fragmentos e fluorescências adicionadas a cada *locus*. Um total de 10 *loci* foram amplificados em separado e em combinações e carregados conjuntamente em um único painel em sequenciador capilar. Os alelos foram amplificados sem *stutter* e facilmente interpretados. Amplificações de PCR de DNA extraído de amostras repetidas e genotipagem em diferentes rodadas de PCR foram utilizadas para inferência de erros de assinalamento de alelos. O painel obtido neste estudo está sendo empregado na análise de parentesco e correção de pedigree em um programa de melhoramento genético desta espécie.

Palavras-chave: Genotipagem. Marcadores microssatélites. Variabilidade genética.

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INTRODUCTION

Kinship analysis is influenced by errors in pedigree determination and genotyping. Uncertain estimates of true kinship relationships caused by these errors may contribute to incorrect management decisions in genetic selection programs (POMPANON *et al*., 2005; PUTNAN; IVY, 2014). In aquaculture, especially in tilapia culture, information regarding pedigree, and the evaluation of generational genetic diversity is often difficult, given the large number of offspring per spawning, resulting in many animals per family in selection programs (TIBIHIKA *et al*., 2019; YANG *et al*., 2014).

Therefore, molecular markers, like SSR (Simple Sequence Repeat) or microsatellites and SNP (Single Nucleotide Polymorphism), have been widely used to correct possible genealogical registration errors, supporting previous studies based on phenotype alone, as well as reducing endogamic effects and loss of variability of genetically improved strains (PUTNAM; IVY, 2014; TURCHETTO-ZOLAT *et al*., 2017). Some advantages of SNPs over SSRs have made them more popular over recent years (PUTMAN; CARBONE, 2014). Some of the advantages cited include the greater abundance in genomes, higher number of data obtained, lower rate of genotyping errors, and lower costs per simple genotype (ZHAN *et al*., 2017). Conversely, SSRs markers are more powerful than SNPs in detecting mixtures, with improved accuracy determination as a higher proportion of errors can be detected in pedigree analysis with many alleles per *locus* (GUICHOUX *et al*., 2011). However, when considering cost, improved discrimination power, and the genotyping of microsatellites through next generation sequencing (NGS), this type of marker out-performs SNPs (JOSHI; RAM; LOHANI, 2017; PUCKETT, 2017).

However, for SSRs markers to be effective, a need for dinucleotide substitution with tetranucleotides exists, given the advantage of tetranucleotides in reducing genotyping errors and greater accuracy in genotype inference (JONES *et al*., 2010).

Based on this, we aimed to develop and validate a multiplex SSR-tetranucleotide panel for tilapia, aimed at reducing genotyping errors and improving the estimation of breeding relationships in tilapia selection programs.

MATERIAL AND METHODS

Sample Collection and DNA Extraction

A total of 70 caudal fin samples from the first and second generation of the genetic bank of Copacol Cooperative and the Rei da Tilapia Company were used for analyses. Extraction of genomic DNA was performed through organic precipitation by NaCl and TNE1 buffers (5 mL Tris HCl pH 8.0, 10 mL EDTA, 1 mL NaCl, 84 mL ultrapure water), TNE2 (5 mL Tris HCl pH 8.0, 10 mL EDTA, 1 mL NaCl, 10 mL SDS 20%, 74 mL ultrapure water), and Proteinase K (20 mg/mL) for cell- and protein lysis, respectively. DNA concentrations and quality were estimated with 1% agarose gel electrophoresis, in which intact bands absent of any signs of degradation and/or contamination were used (HUYNH *et al*., 2017) and stained with GelRed (Biotium, USA) for visualization in a UV light transilluminator.

Primer design

Using the *Oreochromis niloticus* genome from the GenBank database of the National Center for Biotechnology Information - NCBI (assembly accession GCA_001858045.3, ncbi.nlm.nih.gov), matching microsatellite sequences were searched.

After screening the genome for primer designs, the following parameters were established using the Primer3 software: melting temperature (Tm) between the forward and reverse primers of less than 3 °C, GC content lower than 60%, primer size preferably 18 to 24 bases, repetition of the motif sequence at 8 to 12 times, choice of *loci* microsatellites containing only tetranucleotides, and amplicon size of less than 450 bp. Additionally, for multiplex viability, *loci* of different molecular sizes were recommended (KORESSAAR; REMM, 2007; UNTERGASSER *et al*., 2012).

Of the potentially amplifiable *loci* identified by the software, microsatellites were elected in linkage groups 01, 02, 05, 06, 07, 08, and 10. BLASTn (Basic Local Alignment Search Tool for nucleotide) was used to determine the alignment matching in pairs, in which E values equal to 0 were identified. This way only primers originating from unique sequences of matches in the genome were selected to establish primer specificity. Finally, 10 *loc*i that best fit the established criteria were synthesized for multiplex construction.

Amplification of SSR and validation

PCR were performed on all animals, at a 25 µL volume, with 10-50 ng DNA template, 1 U Red Jumpstart Taq polymerase (Sigma-Aldrich, USA), 200 µM dNTPs (Sigma-Aldrich, USA), and forward and reverse primers at 5 pmoles/µL (IDT DNA, USA). Amplification was performed on a GeneTouch thermal cycler (Bioer, China) with the following conditions: denaturation at 94 °C for 5 min, followed by 30–35 cycles of 94 °C for 30 s, annealing according to Table 1 for 30 s, elongation at 72 °C for 40 s, and final extension at 72 °C for 10 min.

After confirming the amplification by electrophoresis in 2.5% agarose-gel, validation of the SSR sequence was performed by sequencing. Reverse primers of the OniUFPel01, 02, 03 and 06 *loci* were marked with 6-FAM fluorescence, OniUFPel04, 05, and 07 with HEX fluorescence, and OniUFPel08, 09, and 10 with NED fluorescence.

To establish the validation of the proposed multiplex panel, UNH104, UNH148, UNH160, UNH178, UNH208, UNH222, UNH934, ISP, PRL1, and PRL2 *loci* SSR dinucleotides were amplified for comparison (DIAS *et al*., 2016; RUTTEN *et al*., 2004). For both di- and tetranucleotides, the allele sizes were estimated in capillary electrophoresis in an ABI3730xl System sequencer and compared to the pattern size 400HD (Macrogen, Korea). Allele sizing was performed with the Peakscanner program (Fisher Biotech, USA), publicly available from the internet. The evaluation of polymorphic information content (PIC) per *locus* for the tetra panel was calculated using the software Cervus 3.0.

RESULT AND DISCUSSION

The SSR-tetranucleotide primers designed with Primer3 (Table 1) were effective and produced clear bands with expected *in silico* analysis size. After confirmation of the amplified sequences (Figure 1), the analysis allowed the establishment of multiplex PCR according to allele size and combining FAM, HEX, and NED fluorescence.

Polymorphic information content (PIC) refers to the power of the molecular marker in determining existing polymorphisms in the study population. The index ranges from 0 to 1, where PIC > 0.5 are considered highly informative, 0.5 to 0.25 as reasonably informative, and PIC < 0.25 as less informative (BOTSTEIN *et al*., 1980).

As shown by the PIC listed in Table 1, all markers selected for the composition of the multiplex were highly polymorphic, as shown in Figure 2, in which it exemplifies different genotypes observed for a portion of analyzed samples of the OniUFPel09 *locus*, with similar efficacy as a tool for differentiating between individuals.

Table 1 - Loci characteristics, including name, primer sequences, annealing temperature (TA), repeat motif, expected size, linkage group from the design with Primer3, and polymorphic information content (PIC)

Locus	Primer sequence (5´ →3´)	TA (°C)	Repeat motif	Expected size (bp)	LG	PIC
OniUFPel01	F:TGAAGCTACAACTTTGAAAACCA R: TGTGTTAGGGTTTCACTCCCA	54.0	$(AATG)_8$	90-100	1	0.81
OniUFPel02	F:TTGCTAACGTATGTGTTTTAAAGT R:GCGTTAACTGATCACCACACT	52.6	$(CTTT)_1$1	154	2	0.89
OniUFPel03	F:TATGGTCGGAAGGGTAAGACG R:CTCTTGGAAGAGTCTCTGTGGT	53.5	$(AATC)_9$	220	6	0.67
OniUFPel04	F: GGGGCACTCTAGCACAATGA R: CTACCAAGTGAGCATGAAATGT	59.8	$(ATGG)_{12}$	290	7	0.86
OniUFPel05	F: TGGTGGGGTTTTTGAAGGCT R: GGAGCATTACCGCCTCCTAC	57.4	$(AAAC)_{12}$	172	5	0.90
OniUFPel06	F: AGAACAACACATGTCGGGGA R: ACTGGTGTGCAGAGTACCAC	57.4	$(AAAT)_8$	330	6	0.55
OniUFPel07	F: TCCTTACCATACCTTTGTGTGC R: TCCTGCACTGTTCCTAGTGGTT	58.3	$(AATG)_8$	122	7	0.94
OniUFPel08	F: ACTGGCCTGAAAGTGAGTGA R: CGGCGGTACATGTATTCCGT	57.4	$(GAAA)_{12}$	227	8	0.94
OniUFPel09	F: GCTGGCAGCCTTAACCCAA R: TGTTCCTTGGACTTTCGCACT	54.4	$(AATC)_{12}$	126	1	0.83
OniUFPel10	F: GATTCGGTATCGCTGGGAACT R: ACTCTCGATTGTGCTCCTGA	58.9	$(AATC)_{12}$	334	10	0.92

Figure 1 - Electropherogram showing confirmation of SSR sequences of OniUFPel01(A), OniUFPel02(B), and OniUFPel06(C), with the AATG, CTTT, and AATC repeat motifs, respectively

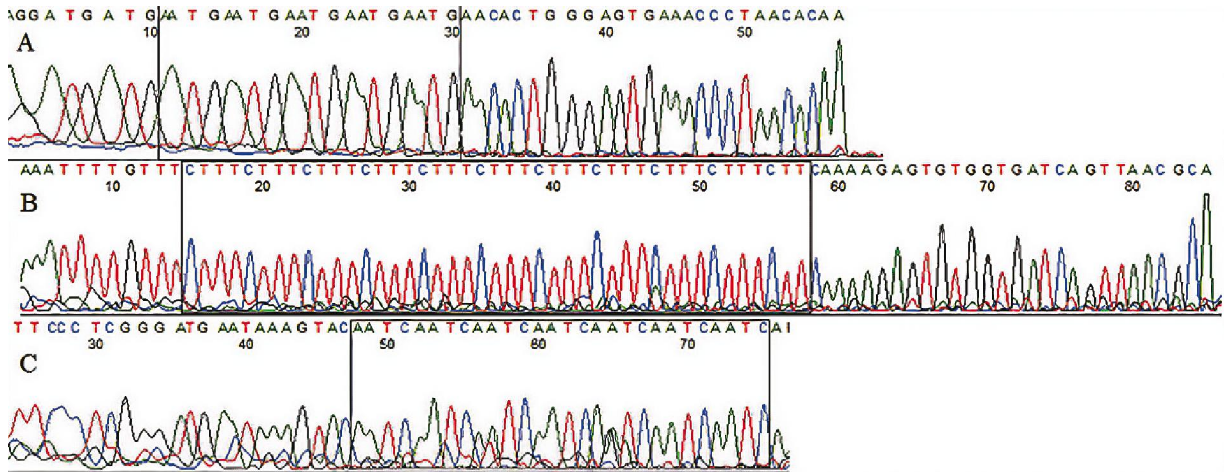
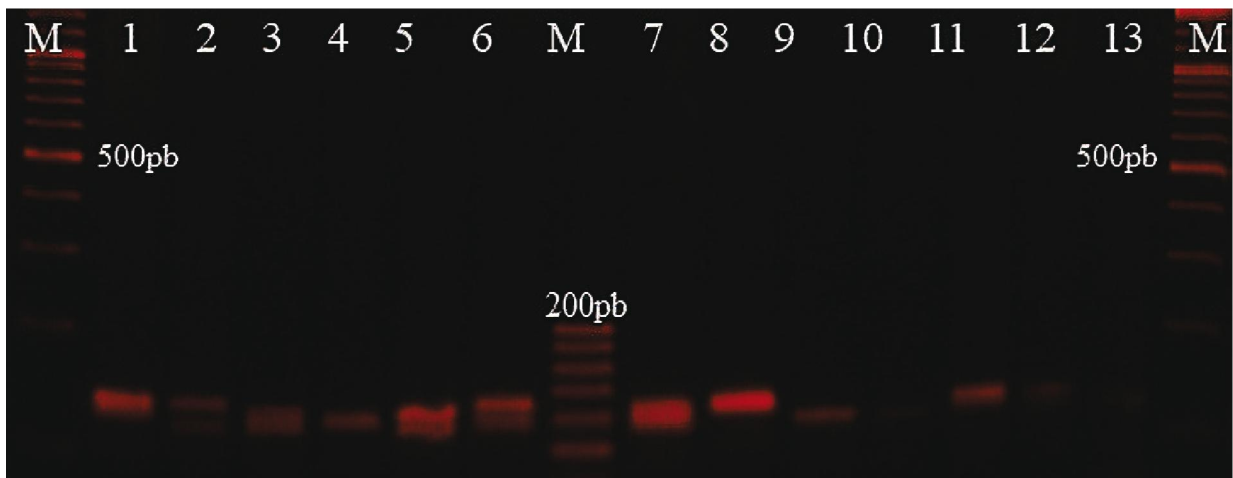


Figure 2 - Electrophoretic profile in 2.5% agarose gel, illustrating the polymorphism observed in the tetranucleotide OniUFPel09 (expected size of 126 base pairs). M = molecular weight marker, 1 to 13 = amplified samples. Samples: 2, 5, 6, 12 were heterozygotes; samples: 4, 8, 9, 10, 11, 13 were homozygotes, and samples: 1, 3, 7 were not susceptible to agarose gel determination (the mentioned electrophoresis contains a sample of the analyzed data for exemplification)

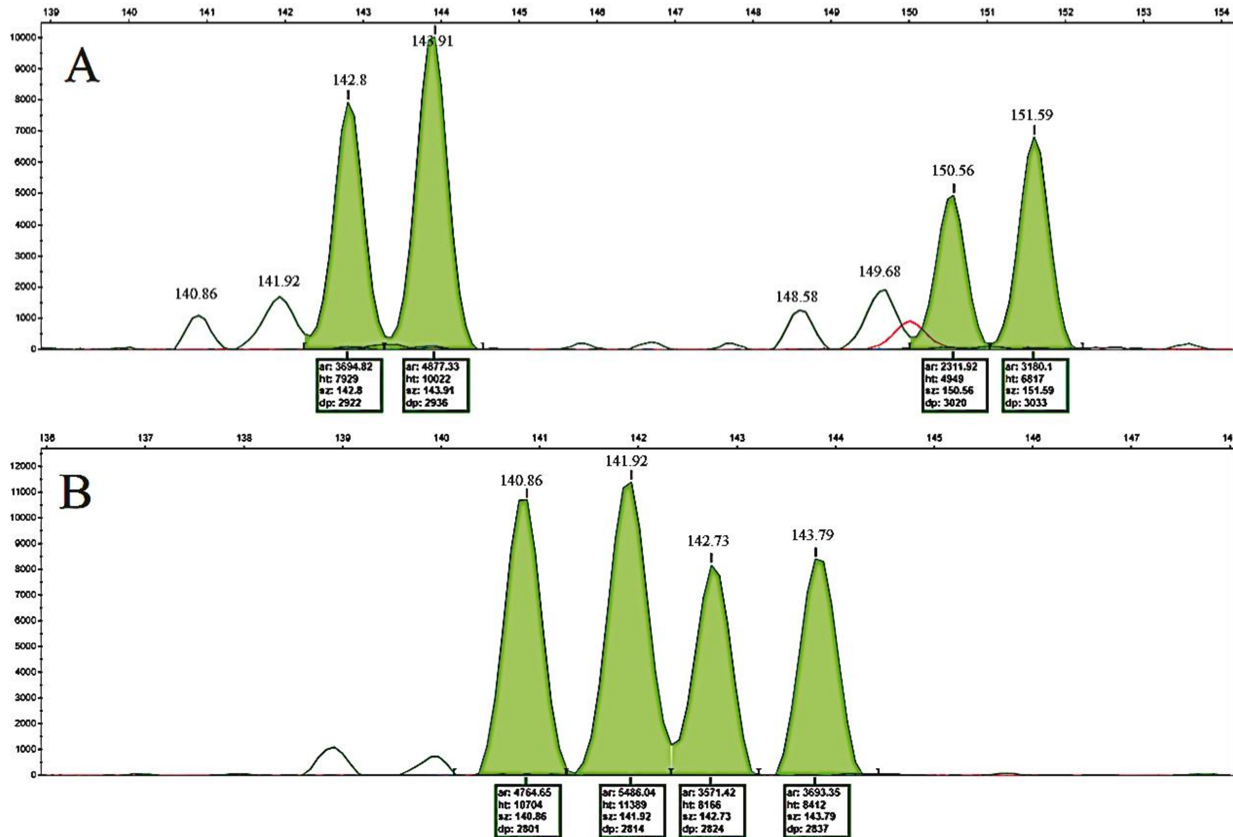


SSR markers are characterized by high mutation rates ranging from 10^{-4} to 10^{-3} per generation, leading to the codominant and highly discriminating characteristics (AMOUSSOU *et al*., 2019; BAGSHAW, 2017).

According to Lee-Montero *et al*. (2013), marker validation is achieved by evaluating the polymorphism and possible genotyping errors through comparison tests, thus establishing nomenclature patterns and procedures for use.

Stutter band patterns for dinucleotides can be observed by capillary electrophoresis in Figure 3A and 3B. From the RFUs (Relative Fluorescence Units), the size peaks of 140.86 and 141.92 indicates minor alleles, while 148.58 and 149.68 peaks can be discarded as alleles, since their values are lower than 15% of that of major peaks (SULLIVAN *et al*., 1992). Considering this, two peaks with 142.8 and 150.56 on each allele can still be seen. However, by comparing RFUs it can be inferred that the genotype of the reproducer is 144–152 (Figure-3A).

Figure 3 - Electropherogram of the dinucleotide UNH178 marked with HEX fluorescence. The column on the left represents the RFU. The upper line indicates the size in base pairs. A = Heterozygous reproducer, B = Stutter band reproducer with allele confounding



Conversely, the pattern observed in Figure 3B could not be precisely defined, since 4 peaks with values of 140.86, 141.92, 142.73, and 143.79 were observed. Therefore, this reproducer could be classified as both containing the genotypes 140–142, and 142-144, demonstrating the potential confusion in the interpretation of this information.

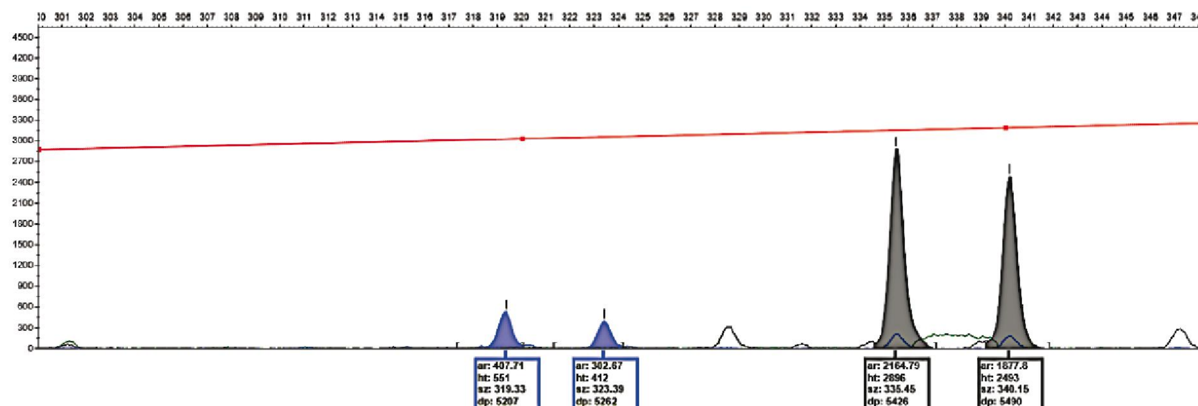
Recent reports (HAMOY *et al*., 2011; JONES *et al*., 2010; OLAFSSON *et al*., 2010) highlighted the complexity of analyzing dinucleotide patterns in the laboratory, given the instability in PCR amplification which commonly promotes the compromise of genotyping results.

Figure 4 illustrates the electrophoretic patterns of two *loci* contained in the proposed multiplex, in which it is possible to observe the absence of a stutter pattern for SSR-tetranucleotides. Alleles 319 and 323 are clearly identified at OniUFPel06, and alleles 336 and 340 at OniUFPel10.

Traditionally, microsatellite studies for population analyses include di-, tri-, or tetra-nucleotides repeats, i.e. two, three, or four base pair repeats. However, the evaluation of tetranucleotide microsatellites in particular has been pursued, since the minimum expected allele differentiation is four base pairs, which favors the visualization and interpretation of these polymorphisms in addition to the lower prevalence of stutter bands compared with dinucleotides (JONES *et al*., 2010; PIMENTEL *et al*., 2018).

Zhan *et al*. (2017) recently developed the MEGASAT software for microsatellite genotyping using NGS. According to the authors, the genotyping of appropriate sets of microsatellite *loci* results in high quality data generation with reduced genotyping errors and extremely low cost. Therefore, the appropriate marker choice is imperative to obtain accurate and reproducible estimates of population structure, genetic diversity, or individual markers (MILLER *et al*., 2019).

Figure 4 - Electropherogram of the tetranucleotide OniUFPel06 marked with FAM fluorescence (blue) and OniUFPel10 marked with NED fluorescence (black). The column on the left represents the RFU. The upper line indicates the size in base pairs.



CONCLUSIONS

1. The 10 microsatellite tetranucleotide *loci* evaluated proved to be effective for tilapia genotyping, expressed by specific amplification of alleles containing a high rate of polymorphic information, as well as the ability to accurately detect genotypes;
2. The microsatellite panel proposed in this study can be used to obtain kinship estimates and to monitor inbreeding. Therefore, presenting a useful tool in guiding mating in tilapia breeding programs.

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