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Short Communication

Novel single nucleotide polymorphisms in candidate genes for growth in tilapia (*Oreochromis niloticus*)

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ABSTRACT - The objective of the present work was to identify and validate single nucleotide variations located in candidate genes to growth traits in tilapia (*Oreochromis niloticus*). Two transitions were identified in the promoter region of the growth hormone gene (GH); eight nucleotide changes were identified in introns and promoter region of the IGF-I gene; and a transition (T/C) was identified in the Myogenin gene (MyoG). The highest genotypic frequency (0.8) for GHpA1 and MyoG was found in the GG and TT homozygous individuals, while the highest frequency (0.9) for GHpB1 was observed in the CT heterozygous fish. There was no genotypic frequency in the CC homozygous tilapia for the GHpB1 and MyoG markers. Based on their allelic frequencies, validation as novel single nucleotide polymorphisms (SNP) of those variations located at *O. niloticus* GH and MyoG genes was possible. These new markers will allow their association with growth traits in tilapia to be exploited in order to determine their potential use as assisted-selection markers.

Key Words: assisted selection markers, genotypic frequency, growth hormone gene, myogenin gene

Introduction

Determination of the genetic variability is an essential step for the implementation of genetic improvement programs focused on the selection of faster growing fish with lower feed conversion rates and resistant to diseases (Lupchinski Jr. et al., 2011). Studies of genetic diversity at DNA level represent an expansion field in aquaculture aimed at finding out those DNA variations associated with productive phenotypes, in order to use them as tools for assisting the offspring selection at any early stage and predict their productive performance (Na-Nakorn and Moeikum, 2009). This strategy is known as gene-assisted selection (GAS) (De-Santis and Jerry, 2007). Application of GAS requires molecular markers. Currently, many aquatic species are improving their productive traits SNP as molecular markers from GAS (De-Santis and Jerry, 2007). Fish growth is an important productive trait and identification of SNP for growth performance located in the somatotropic axis genes has been achieved for different species (De-Santis and Jerry, 2007). There are some candidate genes associated with growth in different species of fish such as the growth hormone (GH) (Blanck et al., 2009; De-Santis and Jerry, 2007), insulin-growth factor I (IGF-1) (De-Santis and Jerry, 2007; Thompson et al., 2010), and myogenin (MyoG) (Ulloa et al., 2013; Zou and Liu, 2015) genes, which were selected for this work. The growth hormone plays an important role in the stimulation of somatic growth in teleost (Tao and Boulding, 2003), while IGF-I is a mediator of the endocrine action of hormonal growth and is able to stimulate the synthesis of DNA and subsequent cell division in many cell types (Wood et al., 2005). Myogenin belongs to the family of myogenic regulatory factors: MRF (MyoD, Myf-5, Myogenin, and MRF- 4); this gene is a specific transcription factor in muscle basic-helix-loop-helix involved in coordination of skeletal muscle development and also participates in the myogenesis and its repair (Wu et al., 2012).

Among the most exploited fish species, tilapia is considered the one with best conditions for freshwater aquaculture (Lupchinski Jr. et al., 2011). However, tilapia production depends on the improvements in the farming systems that allow the reduction of production costs increasing productivity. To date, there are no reports on molecular markers allowing the GAS application for

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growth traits in tilapia. Therefore, the aim of the present work was to search genetic variation and single nucleotide polymorphisms (SNP) in three of the main genes related to growth in tilapia (*O. niloticus*), and their validation.

Material and Methods

Fifteen females from three commercial hatcheries in Mexico (H1 = 5, H2 = 5, and H3 = 5) were used to evaluate the experimental discovery population to identify genetic variability in *O. niloticus* (H1: *O. niloticus*, Stirling; H2: *O. niloticus,* Chitralada, and H3: *O. niloticus*, Egypt). Blood samples were extracted from the caudal fin, and DNA was isolated using the Genelute Mammalia Genomic DNA commercial kit (Cat.G1N350, Sigma - Aldrich). The specific primers were designed to amplify coded regions of three genes and promoter regions for GH and IGF-I genes using the Primer Select V7.0.0 program from Lasergene Suite, DNASTAR. We used sequences available from public database to develop tilapia-specific primer (GH, GenBank accession M84774.1; IGF-I, GenBank accession AF033797; and MyoG, GenBank accession GU246717) which was re-sequenced. DNA amplification was carried out by polymerase chain reaction (PCR), using 50 ng DNA, 1.25U Taq DNA polymerase (Promega Com, Madison, WI, USA), and 0.4 mM dNTPs. Different concetrations of $MgCl_2$ and alignment temperatures were used for each primer-pair (Table 1). Purified PCR products were bidirectional-sequenced in an automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems®). Once nucleotide sequences were obtained, sequence alignments and SNP identification were achieved using PolyPhred software (Nickerson et al., 1997).

The progeny from the experimental discovery population was used for validation of the observed genetic variations in the GH and MyoG genes. Eighty-six juveniles of tilapia from the discovery population were used for gene validation. The population was genotyped using allelic discrimination assays designed and synthesized in Applied Biosystems® (Table 2). Because of the location of the variations found in the promoter region of the IGF-I gene, it was not possible to design assays of allelic discrimination that would allow their validation. All samples were standardized using 0.25 µL DNA (25 ng), 6.25 µL Taqman PCR master mix (Applied Biosystems), and 0.3125 µL mixture of probes. Each assay was conducted on an individual basis in optical 96 well-plates in ABI PRISM® 7000 equipment (Sequence Detection System), under the following conditions: one cycle at 50 °C/2 min and 95 °C/10 min, and 40 cycles of two steps at 92 °C/15 s and 60 °C/1 min.

Table 1 - Primers used for resequencing of GH, IGF-I, and MyoG genes in tilapia

GEN	Primers (sense/antisense) → 5'3'	Size of PCR product	PCR condition		
			T_m ºC[1]	$MgCl_2$[2]	Region
GH_1	A:ATGTTGGATGTCAGAGCACTTT B:TGAAAAAGACCAAATGTTACC	545pb	60	1.5	Exon 1, 2, Intron 1, 2
GH_3	A:ACACAGGACAGAAGAGCGCATACTGAAGACTA B:CTGCTGGTTGAGGGAGGAC	552pb	60	1.5	Exon 5, 6, Intron 4
GHp	A:GTCGACCTTTATTTCAGA B:GCTCAGAGTTTTTGCTTTTA	466pb	55	1.5	Promoter
IGF-I_1	A:CTTGGACGAGTAGGAGGCAAATG B:GAAATACAAGCAAGCGATAAGAA	447pb	65	1.5	Exon 1, Intron 2
IGF-I_2	A:ACTCTTTTTCCGATGATGCTGA B:GAAATAAAAGCCTCGCTCTCCAC	233pb	65	1.5	Exon 2
IGF-I_3	A:AATAAACCAA CAGGCTATGGC B:TCGCCTGCTTTGAAACTCTTATG	568pb	65	1.5	Exon 3, 4, Intron 3
IGF-I_4	A:AAATGT GTTTTATTTTGTTTGC B:TTGTTTTACAGTGAACCATTC	221pb	55	1.5	Exon 5
$IGFp_1$	A:ATGATGATCGCTTTTGACC B:GGCACCGTGTATCTGACCA	789pb	55	1.5	Promoter
$IGFp_2$	A:GCCAAATTACGCACAACAG B:AATACAAGCAAGCGATAAGAAT	696pb	55	1.5	Promoter
$MyoG_1$	A:ACACAGGACA GAAGAGCGCA TACTGAAGACTA B:CTGCTGGTTGAGGGAGGAC	556pb	55	1.5	Exon 1, Intron 1
$MyoG_3$	A:AATAAACCAA CAGGCTATGGC B:TCGCCTGCTTTGAAACTCTTATG	590pb	60	3.0	Exon 3, Intron 4

[1] Annealing temperature during PCR.
[2] $MgCl_2$ concentration (mM).

ABI PRISM 7000 program (Real-Time Sequence Detection Software) was used for the analysis of each genotype. Results were analyzed with ABI PRISM® 7000 (Real-Time Sequence Detection Software). The genotype of each sample was confirmed by visual inspection. Allelic and genotypic frequencies for three markers (GHpA1 A/G, GHpB1 C/T, and MyoG C/T) were generated according to Falconer and Mackay (2001; Figure 1).

Results and Discussion

A candidate genes approach was used to search and validate genetic variation with potential to be associated with growth traits in tilapia. Some studies support this approach as an effective tool for the discovery of SNP in genes that regulate specific traits (Primmer et al., 2002; De-Santis and Jerry, 2007; Hemmer-Hansen et al., 2011).

In GH gene, a 1.563 pb region was re-sequenced (100% promoter region, 66% exons, and 42% introns), and two transitions in the promoter region were found: one located at −284 (A/G), and other at −271 (C/T). Also, the 2,944 pb of the complete IGF-I gene was re-sequenced. A total of eight nucleotide changes were identified in discovery population (six transversions and two transitions), four located in the introns (T/A, G/A, C/G, and T/G), and four in the promoter region (G/C, T/A, C/G, and C/T). The average number of polymorphisms present in introns was higher compared with exons and promoter regions. A 1,146 bp region from MyoG gene was re-sequenced and included 66% of the gene coded region. This was achieved in six individuals where a transition in the non-coding region (C/T) was identified. A total of 11 nucleotide variations were identified in the discovery population. Insulin growth factor I gene showed the highest genetic variability, followed by GH and MyoG genes. For GH and MyoG genes, observed variations were validated with minor allelic frequencies greater than 1% (Table 3).

Several studies in fish species have supported the identification, validation, and association of SNP located in candidate genes for growth, maturation, and reproduction traits. The validated markers found in this study for *O. niloticus* will allow a better understanding of heritability and molecular basis of this complex trait (Diopere et al., 2013).

Table 2 - Primers and probes used for validation of markers GHpA1 A/G, GHpB1 C/T, and MyoG C/T

SNP	Primer forward	Primer reverse	Probe
GHpA1	GGAGTTTTTGAAAACTTTACATTAGATCTCCTTT	CACACCGCTGGTGACTAAAGT	VIC:TCTGACATCCAGCATGTT FAM:CTGACATCCAACATGTT
GHpB1	GGAGTTTTTGAAAACTTTACATTAGATCTCCTTT	ACATTAACACCTCAATCATCATATTGAT GCT	VIC:TGGTGACTAAAGTTTCTGAC FAM:TGGTGACTAAAGTCTCTGAC
MyoG	AGGGAAAGCATTTGAAGAGGAAATCTTAA	CTGTTAGGCTTGCCTCCAGATTTAT	VIC:AGGAATGACAAATCCAG FAM:AGGAATGACAGATCCAG

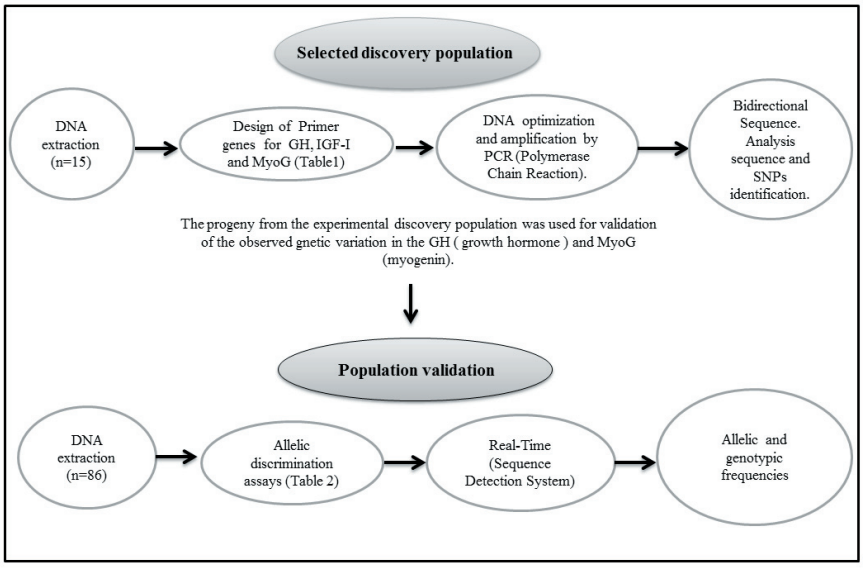

Figure 1 - Procedures for selected discovery population and validation for candidate growth genes in *O. niloticus*.

Table 3 - Genotypic and allelic frequencies for markers GHpA1 A/G, GHpB1 C/T, and MyoG C/T in tilapia

SNP	Genotype/allele	Frequency	H1	H2	H3
GHpA1 A/G	AA	0.1	0.08	0.1	0.03
	GA	0.1	0.12	0.2	0.17
	GG	0.8	0.8	0.7	0.8
	A	0.2	0.1	0.2	0.2
	G	0.8	0.9	0.8	0.8
GHpB1 C/T	CC	-	-	-	-
	CT	0.9	0.9	0.8	0.9
	TT	0.1	0.1	0.2	0.1
	C	0.4	0.6	0.4	0.4
	T	0.6	0.4	0.6	0.6
MyoG C/T	CC	-	-	-	-
	CT	0.2	0.2	-	0.4
	TT	0.8	0.8	-	0.6
	T	0.9	0.9	1	0.8
	C	0.1	0.1	-	0.2

Conclusions

Novel and validated SNP obtained in this study will allow the evaluation of their association in tilapia growth traits in order to determine their potential use as assisted-selection markers.

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