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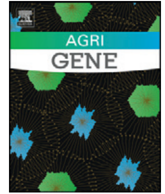

Association between myostatin gene (*MSTN-1*) polymorphism and growth traits in domesticated rainbow trout (*Oncorhynchus mykiss*)

Sajad Nazari [a,*], Valiolah Jafari [a], Mohammad Pourkazemi [b], Hamed Kolangi Miandare [a], Hossein Ali Abdolhay [b]

[a] Gorgan University of Agriculture Sciences and Natural Resources, Faculty of Fisheries, Golestan, Iran
[b] Iranian Fisheries Sciences Research Institute (IFSRI), Agricultural Research, Education and Extension Organization (AREEO), Tehran, Iran

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ABSTRACT

In this study, the polymorphism of myostatin gene (*MSTN-1*) as a candidate gene for quantitative traits was analyzed in the rainbow trout (*Oncorhynchus mykiss*). The association between the *MSTN-1* polymorphisms and growth traits were also studied. Forty broodstock parents of rainbow trout from two farms including Yasuj and Shahrkord were selected and tagged. These spawning were mated and then juveniles were raised at the ponds until 5 months post-hatching. Two single nucleotide polymorphisms (SNPs: *g.660T > C* and *g.1904C > A*) in intron 1 and 2 regions of the *MSTN-1* gene in rainbow trout were determined by DNA sequencing and PCR-single stranded conformation polymorphism (PCR-SSCP) methods. The assessment results demonstrated that these two site mutations are synonymous mutations and therefore 6 haplotypes were revealed in these samples ($n = 120$). Moreover, One way ANOVA of the SNPs and growth traits revealed the genotypes of SNP *g.1904C > A* are associated with the length and body weight at 90 and 150 days of age ($P < 0.05$), whereas no significant association was found between the SNP *g.660T > C* and growth trait parameters. These results provide evidence that SNPs in *MSTN-1* gene may be associated with length and body weight during the early stages of life (from hatching to 5 months of age), therefore *MSTN-1* gene may be used for rainbow trout breeding program.

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1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) aquaculture is becoming increasingly popular in Iran. It has rapidly become extensively beneficial for the Iranian farmers economy. As production increases, more efficient procedures for breeding practices are required. The practice of marker-assisted selection (MAS) needs sets of genetic markers linked to desirable traits to manage breeding decisions for producing of aquaculture species (Sonesson, 2007). Indeed, MAS has proven to be an effective program for the development of aquaculture and animal species with dramatic improvements in the traits (Thomas et al., 2002; Gjedrem, 2012; Vandeputte and Haffray, 2014). The rainbow trout (*Oncorhynchus mykiss*) aquaculture is extensive for economic, and harvest plans, but the application of molecular genetic markers to promote quantitative traits are currently inadequate.

Single nucleotide polymorphisms (SNPs) have become progressively established in the field of molecular ecology (Campbell et al., 2009; Ogden et al., 2013), fish genetic studies (Hemmer-Hansen et al., 2011; Palti et al., 2011; Yang et al., 2016) and aquaculture improvement (Palti et al., 2011; Houston et al., 2012; Poćwierz-Kotus et al., 2014). Compared to the commonly used single sequence repeats (SSRs), SNPs acquire many characteristics that make them interesting for genetic association studies (Vera et al., 2013; Zhang et al., 2015; Liu et al., 2016). SNPs are abundant, widely distributed throughout the animal genome, more often found than SSRs in coding regions, a critical cause of variation in genes, promoter and regulatory regions (Vong et al., 2003; Tian et al., 2014). Within the coding regions, a SNP is either non-synonymous and results in an amino acid sequence change, or it is synonymous and does not alter the amino acid sequence (Liu and Cordes, 2004; Komar, 2007). Correlation analysis between SNP markers and traits reaching significant level may imply the existence of relation between the markers and certain traits (Lynch and Walsh, 1997; Doerge, 2002). Hence, the selective breeding based on phenotype can progress to genotype-assisted selection (Sonesson, 2007). For MAS to become useful for rainbow trout trait selection, closely associated genetic markers must first be identified from a dense panel of markers across the genome.

Myostatin (*MSTN*), earlier named as growth differentiation factor (GDF-8), is a member of the transforming growth factor-b (TGF-b) superfamily which includes a number of factors that mediate basic actions in cell growth and development through signal transduction (McPherron et al., 1997; Langley et al., 2002; Lee, 2004). In comparison to mammalian, the myostatin expression in fish occurs in different types of tissues, including skeletal muscle, gills, skin, brain and ovary (Ostbye et al., 2001; Roberts and Goetz, 2001; Rodgers et al., 2001; Ko et al.,

* Corresponding author.
E-mail address: nazarisajad92@gmail.com (S. Nazari).

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2007; Tang et al., 2010), offering that the functional act of fish myostatin may not be limited to muscle growth process, but may have supplementary functions. Moreover, two myostatin genes have been observed in rainbow trout resulting from salmonid genome tetraploidisation (Rescan et al., 2001). Salmonid myostatin involves three exons and two introns (Roberts and Goetz, 2001; Joulia et al., 2003). Several *MSTN* gene variants have been also addressed in fishes (Maccatrozzo et al., 2001; Roberts and Goetz, 2003; Wang et al., 2011; Peñaloza et al., 2013).

MSTN gene has also been detected in other aquatic species, such as Sea urchin (Lapraz et al., 2006), shrimp (Qian et al., 2013) and scallop (Kim et al., 2004; Meng et al., 2015), and its functions in muscle growth regulation were increasingly examined. Various polymorphisms have been determined in the *MSTN* gene (Rescan et al., 2001; Kim et al., 2004; Rodgers and Garikipati, 2008; Stinckens et al., 2010) implying that the *MSTN* gene is highly variable. Clearly, increasing the number of nucleotide variations, which are significantly associated with growth traits, have been described in *MSTN* in many commercial species, providing more functional genetic markers for the genetic improvement of aquatic species (Yu et al., 2010; Liu et al., 2012; Sun et al., 2012; Peñaloza et al., 2013; Meng et al., 2015).

In Iranian rainbow trout breeds, sequence variants of *MSTN* gene have been poorly described. The significance of *MSTN* SNPs on rainbow trout growth performance urges the demand for a thorough characterization of *MSTN* polymorphisms among farm populations. The aim of this study is to investigate the association between polymorphisms within *MSTN-1* gene and quantitative traits to examine the potential for marker assisted selection.

2. Materials and methods

2.1. Broodstock selection and spawning

Forty healthy brood rainbow trout from two farms (Yasuj and Shahrkord) were used in this experiment. The stripping process has been characterized by Stickney (1991). The spawning were mated in a partial factorial design (each of a group of males mated to each of a group of females) (Martinez et al., 1999; Kause et al., 2005) and a total of 22 families were created. These families originate from 20 male and 20 female broodstock following partial factorial mating design. The mating ratio was 1:2–3, with one male used to fertilize eggs from two or three females (Martinez et al., 1999). Some fish were found to be still sexually immature, particularly females, thus limiting the planned sample size. All broodstock were biopsied to enable them to be genotyped for further analysis. The families were reared independently up to the eyed stage at which point the eggs from each family were divided into three to generate three communal replication groups.

The ripen brood fish were spawned after anesthetizing them with clove oil essence at 200 mg L^{-1}. The fertilization of eggs was carried out according to the conventional produced approach explained for trout eggs (Estay et al., 1994). The embryonic development, ova handling and fry rearing have been described by Estay et al. (1994) and Stickney (1991). The dead eggs were eliminated cautiously 24 h post-incubation and the remaining eggs were left untouched until the eyed-eggs phase. Water quality parameters including of dissolved oxygen, temperature, pH, hardness were 9 ± 0.5 mgL^{-1}, 10.5 ± 0.5 °C, 7.8 ± 0.3, and 165 ± 2.6 mgL^{-1}, respectively. Other hatchability parameters consisting inlet water flow (8.8–9.5 $L\cdot min^{-1}$ for the eggs and 52–55 $L\cdot min^{-1}$ for the larvae), protection of light during hatching period and no manipulation of eggs were accomplished during the study. The fish were hand-fed eight times daily with commercial pellets (Kimiagaran Company) at the beginning of the research and reached four times daily during continuous growth.

All experimental fish were maintained under constant environmental conditions and were fed according to the management practices of the fishery farm. Rearing conditions such as numbers per tank, densities, flow rates, type of feed, feeding levels and methods were standardized over all families, according to commercial standards. A hand-feeding method was used with feeding levels being standardized according to a feed table on the basis of water temperature and size of fish. Groups were kept separately in randomly allocated tanks through the experiment. Water temperature (near constant temperature 11 ± 0.5 °C), dissolved oxygen levels (9 ± 0.5 mgL^{-1}) and mortality were documented on a daily basis during the study. Four growth traits (body weight, total length, body length and body height) were recorded for statistical analysis. The standardized application of commercial production conditions was regarded as very important during assessment in order to minimize environmental variation and possible genotype-environment interactions (Gjerde et al., 1996; Kause et al., 2005). The 120 individuals were taken from the families in the farms based on growth performance. Individuals were then genotyped as growth-rate extreme classes (top and bottom families within the farms) for the SNP trait association study. Each family contributed 8 to 10 individuals to the 120 individuals genotyped. The sampling procedure used was first to anaesthetize the fingerlings, then a clean scalpel blade was used to cut a 4 mm of the caudal fin. Then the finclip samples were placed in a previously labelled 1.5 mL microtube and stored in 98% ethanol. All the samples were transported to the genetic laboratory for molecular analysis at Coldwater Fishes Genetic and Breeding Research Center (CFGB) in Yasuj.

2.2. Primer design

To elucidate single nucleotide polymorphisms (SNPs) from the part of myostatin gene, we designed four PCR primer pairs. PCR-SSCP analysis was employed based on the published myostatin gene sequence (GenBank accession No: NM-1124282). Rescan et al. (2001) reported that two distinct myostatin genes (*MSTN-1* and *MSTN-2*) are presented in the rainbow trout genome. In our study, specific primers were designed to recognize target regions within the rainbow trout myostatin 1 gene (Fig. 1). The sequences of all primer pairs used are listed in Table 1.

2.3. DNA extraction and sequencing of myostatin gene

The total genomic DNA was extracted using the protocol from Pourkazemi et al. (2012). Between three and six individuals from each populations or strain were first analyzed by direct sequencing. PCR amplifications were performed out in 20 µl reactions volumes (1× reaction buffer, 1 mM dNTP, 1.2 mM MgCl2, 0.8 µM of each primer, 0.3 U Taq DNA polymerase, and nearly 100 ng DNA template). Thermal cycling included 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, annealing at 55–61 °C for 30 s, and an extension temperature of 72 °C for 1 min. This was then followed by a final extension of 72 °C for 5 min. For each individual, sequencing reactions were performed using both forward and reverse primers, resulting in different fragments (286, 227, 384 and 265 bp in length). Amplification was carried out in Bio-Rad S1000 thermal cycler (Bio-Rad, USA). PCR fragments of the predicted size were cut and purified from gels with an agarose

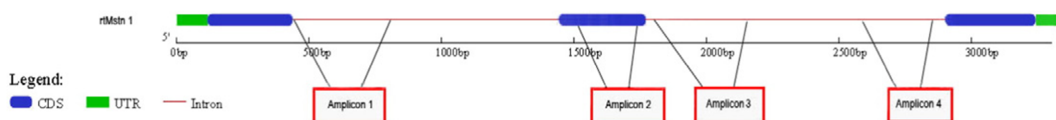

Fig. 1. The amplicons and genomic structure of the rainbow trout *MSTN-1* gene.

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