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Association of *MyoD1a* and *MyoD1b* gene polymorphisms and meat quality traits in rainbow trout

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ABSTRACT. In this study, we identified myogenic regulatory factors (*MRFs*) and analyzed the correlation between *MRFs* and meat quality in rainbow trout. The *MyoD1a* and *MyoD1b* genes were cloned from rainbow trout using a homology cloning method. Introns 1 and 2 in the *MyoD1a* and *MyoD1b* genes were cloned and submitted to GenBank (accession Nos. FJ623462 and FJ793566). Polymorphisms of *MyoD1a* and *MyoD1b* genes were analyzed using single-strand conformation polymorphism and sequencing, respectively. Two single nucleotide polymorphisms were detected in the *MyoD1* gene, located at 129G→A in exon 1 and 37 G→A in exon 2. The 37 G→A mutation in exon 2 induced the R185K amino acid change in the polypeptide chain. Seven single nucleotide polymorphisms in the *MyoD2* gene were detected, including 218T→C, 224T→C, 242A→C, 246T→A, 248T→C, 305T→C, and 329C→T. The 246T→A mutation in exon 1 induced the R83K change in the polypeptide chain. In the S3 fragment, meat quality traits of genotypes AA and AB significantly differed from those of genotype BB ($P < 0.05$). In the S5 fragment, meat

quality traits of the genotypes AA and AC were significantly different from the genotypes BB and BC ($P < 0.05$). These results indicate that the *MyoD1a* and *MyoD1b* genes have an important influence on meat quality or were linked to the major genes in these strains. These genes can be used to control muscle fiber traits in rainbow trout, and the mutations in the S3 and S5 fragments can be used as molecular markers for selecting rainbow trout with better meat quality traits.

Key words: Meat quality; Myogenic regulatory factor; Rainbow trout; Myogenic determining factor; Polymorphism

INTRODUCTION

The myogenic regulatory factors (*MRFs*) are a family of vertebrate proteins that are potent transcription factors for muscle genes (Pownall et al., 2002). The myogenic determining factor (*MyoD*) gene belongs to the *MRF* gene family, which initiates and maintains the differentiation and development of skeletal muscle during myogenesis (Weintraub et al., 1993). In higher vertebrates, the *MRF* gene family includes 4 members, *MyoD*, *Myf5*, myogenin, and MRF4, which together induce the differentiation and development of pre-myoblasts to form muscle fiber (Olson et al, 1991; Buckingham, 1992). *Myf5*, in combination with *MyoD1*, determine the muscular lineage and is the first factor in this family to be expressed in the embryo (Braun et al., 1989, 1990). The presence of a very mild muscle phenotype in mice containing null mutations in *MyoD1* likely occurred because of a compensatory effect of *Myf5* (Rudnicki et al., 1992). The double-mutant mice died soon after birth because of a lack of muscle structure, highlighting the importance of these 2 proteins in establishing the myogenic lineage (Rudnicki et al., 1993). Over-expression of the *MyoD1* gene inhibits the proliferation process of myoblasts and promotes the differentiation of myoblasts to form mature muscle fiber cells (Kablar et al., 1997). Myogenin gene activity was affected by the *MyoD1* gene, and indirectly affected the terminal differentiation process of muscle cell (Weintraub, 1993). Previous studies suggested that myogenin and *MyoD1* are also involved in establishing and maintaining the slow-twitch and fast-twitch mature muscle fiber phenotype; myogenin was expressed at higher levels compared with *MyoD1* in slow-twitch muscles, whereas the opposite was true for fast-twitch muscles (Hughes et al., 1993; Voytik et al., 1993). Muscle fiber characteristics play a key role in meat quality (Picard et al., 2002). All *MyoD* in rainbow trout and Atlantic salmon are thought to be *MyoD1* (a, b, c), whereas *MyoD2* exists only in non-salmonid fish species (Macqueen and Johnston, 2008). The cDNA sequences of *MyoD1a* and *MyoD1b* were not products of true alleles, but originate from 2 loci that were likely duplicated during the tetraploidization of the whole salmonid genome. The *MyoD1b* gene shows high conservation with the *MyoD1a* gene in the basic helix-loop-helix domain, and their respective coding regions are located at the 3'- and 5'-ends of genes on the nucleotide level. The *MyoD1a* and *MyoD1b* genes in rainbow trout were found to be co-expressed, but the large differences in the coding region and non-coding region indicated that they come from different loci (Rescan and Gauvry, 1996).

New types of molecular markers are needed for the selection of meat production ability and meat quality in live animals. A recent correlation study on *MyoD1* gene and meat quality traits was mainly concentrated in pig (Knoll et al., 1997). Intron 1 of the *MyoD1* gene contains a *Dde*I polymorphic locus in the pig, which plays a role in increasing muscle fiber

area, lean meat percentage, carcass length, loin eye area, and leg proportion (Knoll et al., 1997). However, this mutant can also lead to meat quality deterioration. Kim et al. (2009) identified a new single nucleotide polymorphism (SNP) site in the 5'-upstream region of the myogenin gene (nucleotides C and T). A total of 252 pigs of 3 breeds were genotyped by polymerase chain reaction (PCR)-restriction fragment length polymorphism using *Bsp*CNI (restriction enzymes). Additionally, the pigs were genotyped for the previously detected *Msp*I site in the 3'-flanking region (alleles A and B). The CCBB diplotype showed the highest frequency among the breeds, followed by TCBB and CCAB. Association analysis performed for the markers showed that the TCBB diplotype had desirable effects on the total number of fibers ($P < 0.002$), fiber cross-sectional area ($P < 0.06$), and loin eye area ($P < 0.001$) compared to the other diplotypes. Correlation analysis of the *MyoD1a* and *MyoD1b* genes with fish meat quality has not been previously reported. In this study, the *MyoD1a* and *MyoD1b* gene sequences of rainbow trout and Atlantic salmon (Rescan and Gauvry, 1996) were used to analyze polymorphisms of genes, explore the distribution of different genotypes, and further reveal the functions of genes. The relationship between the *MyoD1a* and *MyoD1b* genes in rainbow trout and meat quality traits will be examined in further studies, and molecular genetic markers will be identified, thus providing a basis for molecular marker-assisted selection.

MATERIAL AND METHODS

Samples

One hundred healthy rainbow trout, with body weights of approximately 130-150 g, were provided by the Bohai Experiment Station of Cold-Water Fish, Heilongjiang River Fishery Research Institute of Chinese Academy of Fishery Sciences. The muscle from the dorsal fin base was selected for further analysis of meat quality.

DNA isolation

Genomic DNA was extracted from fin samples of rainbow trout using the Qiagen DNAeasy kit (Hilden, Germany). The quality and concentration of DNA were assessed by agarose gel electrophoresis and measured using an ultraviolet RNA/DNA spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China). Finally, DNA was adjusted to 50 ng/μL and was stored at 4°C until future use.

Primer design, PCR amplification, and sequencing

According to reported DNA sequences of rainbow trout (GenBank accession Nos. X75798, Z46924) and Atlantic salmon (GenBank accession Nos. AJ557148, AJ557149), the primers M1-M5 were designed to amplify the DNA sequences of the *MyoD1a* and *MyoD1b* genes. According to DNA sequences obtained, the 11 pairs of primers (S1-S11) were designed for PCR-single-strand conformation polymorphism (SSCP) analysis. The primer names, primer sequences, and length of products amplified are shown in Table 1.

Table 1. Primers of *MyoD1a* and *MyoD1b* used for rainbow trout.

Gene	Primer name	Primer sequence (5'-3')	Location	Product length	Annealing temperature
MyoD1a	M1	F-GAAGGCGACTGAGCAAGGTG R-GGGACAGGCAGAGGTAT	Exon 1	393	47°C
	M2	F-ACGGAATGGTGAGAAACT R-GGGACAGGCAGAGGTAT	Intron 1 Exon 2	621	60°C
	S1	F-CGTCTACTAACCCAAACC R-ACCATTCCGTCTGAGC	Exon 1	187	57°C
	S2	F-AACTGCTCAGACGGAA R-TTGGTGGACAAGACTGA	Intron 1	178	55°C
	S3	F-GACGGAGAAACAAGTAT R-CCACATCATAGCAAAAC	Exon 2 Intron 2	241	57°C
	S4	F-GAGTATATTTGACCCAG R-CAGAGTTCTTCTTGTG	Intron 2	235	50°C
MyoD1b	M3	F-TGTGACAAATACAGAGCC R-TATCCGATTGGTAGTTCC	Exon 1	721	47°C
	M4	F-AACTGCTCAGACGGAA R-TCGTTGAAGTAGGTGC	Exon 2	568	47°C
	M5	F-TCGCCGAAACTCCAAAT R-GCCATCCTCTTCTTCTACT	Intron 2 Exon 3	569	46°C
	S5	F-GTGAGATGGAGTTGTCG R-TCCCGCATAGTAGCAG	Exon 1	283	50°C
	S6	F-CGAAGACGAGCACATC R-TCTCCACCTTGGGAAG	Exon 1	218	50°C
	S7	F-CGAGAACCTGAAGAGA R-AGAGCAGTTGGACTGT	Exon 1	193	50°C
	S8	F-CATCCAGTCCACAGTC R-TATCCGATTGGTAGTTCC	Intron 1	192	53°C
	S9	F-GGAACTACCAATCGGA R-GCCTAACAAGTCACAAT	Exon 2 Intron 1, 2	256	56°C
	S10	F-AAGACCCTTGGCAGACAT R-CCGTTTCGCTCAGGATA	Intron 2	251	53°C
	S11	F-ATCTATCCTGAGCGAA R-GCCATCCTCTTCTTCTA	Exon 3	200	49°C

F = forward primer; R = reverse primer.

DNA sequences of the *MyoD1a* and *MyoD1b* genes were amplified using the M1-M5 primers. The 25-μL PCR mixture contained 0.1 μL of each primer, 2.5 μL 10X PCR buffer, 2.0 μL dNTP, 0.2 μL Taq polymerase (Takara, Shiga, Japan), and 1 μL DNA template. PCRs were performed on PCR machine (PTC-200; MJ Research, Waltham, MA, USA). The PCR conditions were as follows: pre-denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 54.8°-60.1°C (Table 1) for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The products were cloned into the pUCm-T cloning vector and sequenced by a commercial sequencing service.

PCR-SSCP analysis

Polymorphisms in the *MyoD1a* and *MyoD1b* genes were identified using the SSCP method. First, 2 μL PCR product was mixed with 6 μL sample buffer (9.8 mL formamide, 0.2 mL 0.5 M EDTA, pH 8.0, 0.2 mL glycerol, 0.025% bromophenol blue, 0.025% xylene blue), and then the gel was quickly placed in ice and cooled for 10 min after denaturation for 10 min at 98°C. Fragments were subjected to electrophoresis for 12 h at a constant voltage of 120 V at room temperature and then silver-stained.

Analysis of meat quality traits

Samples were paraffin-sectioned for analysis of muscle fiber density and muscle fiber diameter. The fresh muscle in the dorsal fin base of rainbow trout was fixed in Bouin's fixative for 24 h. After dehydration in a series of increasing concentrations of ethanol, specimens were embedded in paraffin, cut into 6-μm thick transverse sections, and stained with hematoxylin-eosin. The sections were examined under a Moctic DM-BA300-B microscope (Hong Kong, China) and analyzed using Motic Images Advanced 3.2. The cross-sectional areas (Si) of muscle fibers were measured at random and the single diameter (Ds) and average diameter (Da) of muscle fibers were calculated using the following equation:

$$Ds = 2(Si/\pi)^{0.5} \qquad Da = \sum Di/50 \qquad \text{(Equation 1)}$$

Dry matter content of fresh muscle was measured using the drying temperature dehydration method. The protein content of fresh muscle was measured using the Micro-Kjeldahl method. Intramuscular fat content of fresh muscle was measured by Soxhlet extraction. Crude ash content of fresh muscle was measured using the conventional muffle furnace method (550°C) (Liang et al., 2009).

Statistical analysis

The distribution of the genotypes was tested for Hardy-Weinberg equilibrium as described by Falconer and Mackay (1996). Associations between genotypes and meat quality traits were evaluated using SPSS13.0 (SPSS, Inc., Chicago, IL, USA).

RESULTS

PCR amplification of *MyoD1a* and *MyoD1b* genes

The primers M1 and M2 amplified approximately 400-bp (Figure 1a) and 600-bp (Figure 1b) fragments. The 751-bp *MyoD1a* gene sequence was obtained by sequencing, comparison, and splicing, and included 129 bp of intron 1 and 296 bp of intron 2. The primers M3, M4, and M5 amplified approximately 750-bp (Figure 1c) and 600-bp fragments (Figure 1d and 1e). The length of the 1471-bp *MyoD1b* gene sequences was obtained through sequencing, comparison, and splicing, and included 271 bp of intron 1 and 197 bp of intron 2. Introns 1 and 2 of the *MyoD1a* and *MyoD1b* genes were cloned and submitted to GenBank with accession Nos. FJ623462 for *MyoD1a* and FJ793566 for *MyoD1b*. The 11 pairs of SSCP primers used for SSCP analysis showed good amplification. The figures of agarose detection were omitted.

Polymorphism analysis of *MyoD1a* and *MyoD1b* genes

The PCR-amplified fragment of primer S2 showed 3 genotypes (AA, BB, and AB) (Figure 2), and the homozygous individuals of the 2 genotypes were sequenced and showed an A/G mutation at position 129 bp of the genotype AA sequence in intron 1 of the *MyoD1a* gene. The genotype BB sequence showed consensus with the sequence of the *MyoD1a* gene.

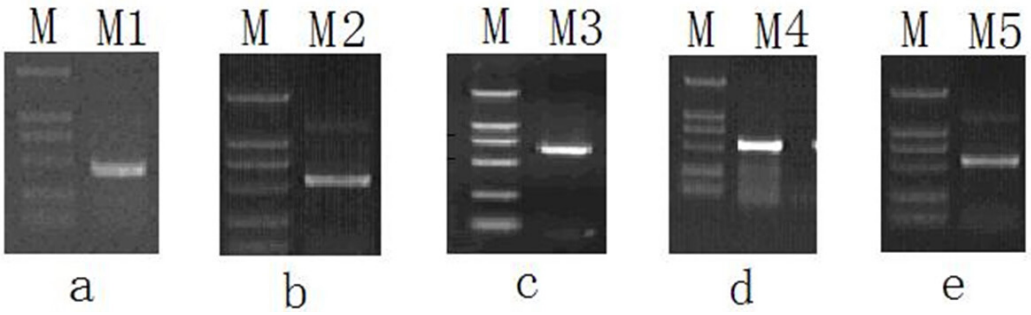

Figure 1. PCR amplification using primers M1, M2, M3, M4, and M5. **a.** 400 bp; **b.** 600 bp; **c.** 750 bp; **d.** 600 bp; **e.** 600 bp.

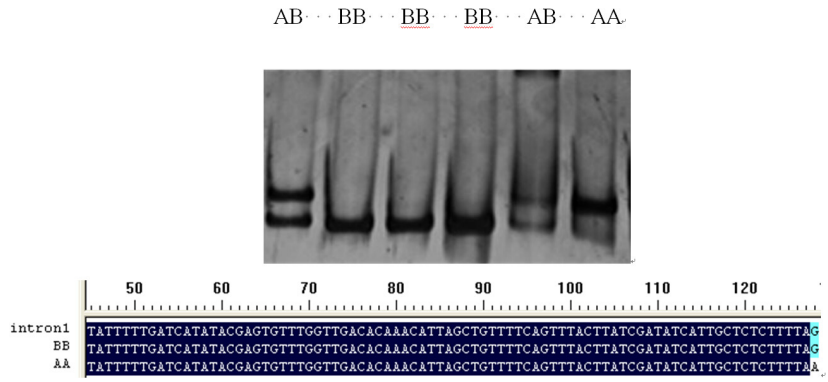

Figure 2. PCR-SSCP pattern and sequence comparison of different genotypes using the S2 primer.

The PCR-amplified fragment of primer S3 showed 3 genotypes (AA, BB, and AB) (Figure 3), and the homozygous individuals of the 2 genotypes were sequenced and showed an A/G mutation at position 37 bp of the genotype AA sequence in exon 1 of the *MyoD1a* gene (Figure 3). This mutation induced an R185K amino acid change in the protein polypeptide chain (Figure 3).

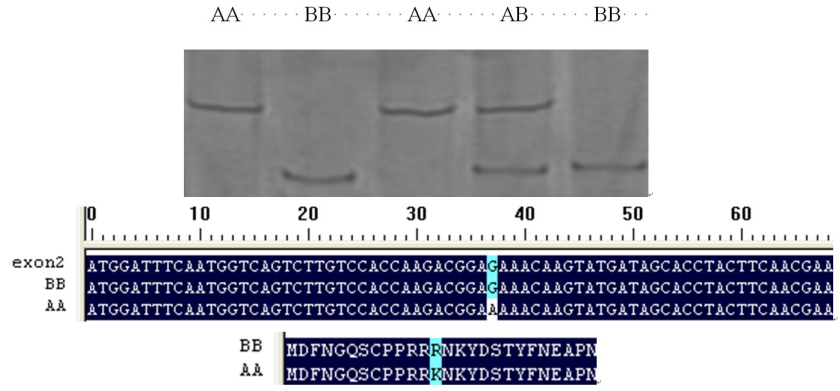

Figure 3. PCR-SSCP pattern and sequence comparison of different genotypes using the S3 primer.

The PCR-amplified fragment of primer S5 showed 4 genotypes (AA, BB, AC, and CC) (Figure 4), and the homozygous individuals of the 3 genotypes were sequenced, revealing 5 mutations, including 3 T/C mutations at positions 218, 224, and 248 bp, an A/C mutation at position 224, and a T/A mutation at position 246 bp in exon 1 of the *MyoD1b* gene (Figure 4). The T247A mutation induced an R83K amino acid change in the protein polypeptide chain (Figure 4).

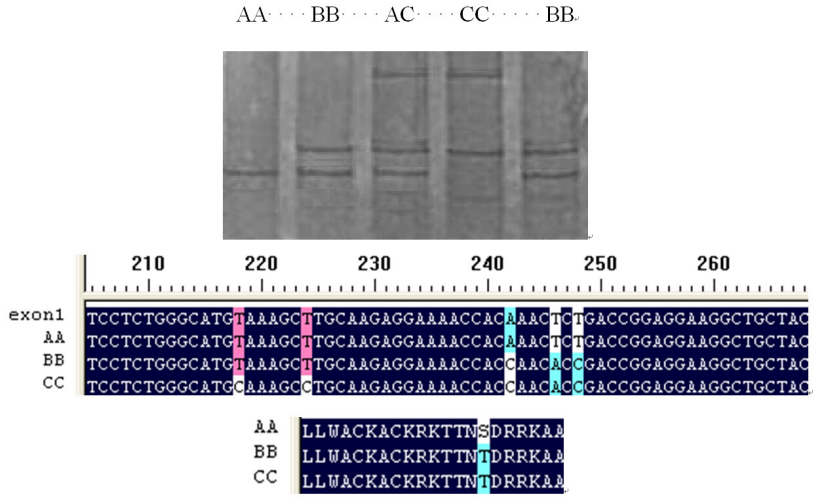

Figure 4. PCR-SSCP pattern and sequence comparison of different genotypes using the rainbow trout S5 primer.

The PCR amplified fragment of primer S6 showed 6 genotypes (AA, BB, CC, AB, AC, and BC) (Figure 5). The homozygous individuals of the 3 genotypes were sequenced, revealing 2 mutations, including a T/C mutation at position 305 bp and a C/T mutation at position 329 bp in exon 1 of the *MyoD1b* gene (Figure 5).

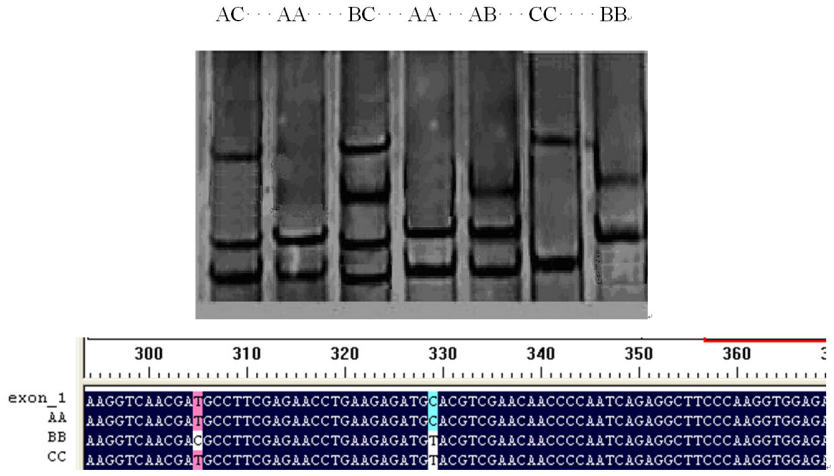

Figure 5. PCR-SSCP pattern and sequence comparison of different genotypes using the S6 primer.

Gene and genotype frequency

The gene and genotype frequencies of the S2, S3, S5, and S6 polymorphic loci were analyzed (Table 2). In the S2 and S5 loci, allele A was the dominant allele. In S3 and S6, the genotypes were evenly distributed.

Table 2. Allele, genotype frequencies, and statistical values at loci of S2, S3, S5, and S6 in *MyoD1a* and *MyoD1b* genes.

Gene	Primers	Genotype frequency						Allele frequency			H_O	H_E	N_E	PIC
		AA	AB	BB	AC	BC	CC	A	B	C				
MyoD1a	S2	0.620 (62)	0.230 (23)	0.150 (15)				0.735	0.265		0.610	0.390	1.64	0.314
MyoD1a	S3	0.340 (34)	0.250 (25)	0.410 (41)				0.465	0.535		0.502	0.498	1.99	0.374
MyoD1b	S5	0.490 (49)	0.000	0.090 (9)	0.270 (27)		0.150 (15)	0.625	0.09	0.285	0.480	0.520	2.08	0.449
MyoD1b	S6	0.130 (13)	0.330 (33)	0.140 (14)	0.100 (20)	0.090 (9)	0.110 (11)	0.395	0.350	0.255	0.329	0.671	3.03	0.582

*Number of individuals are shown in parentheses.

Genetic polymorphism analysis in different loci of *MyoD1a* and *MyoD1b* genes

Polymorphic information content (PIC) of S2, S3, and S5 showed moderate polymorphism, $0.25 < PIC < 0.5$. The S6 locus of the *MyoD1b* gene showed a high level of polymorphism (PIC = 0.582). This result indicates that this locus has a higher degree of genetic variability and larger selective potential than other loci.

Association between SNPs and meat quality traits of rainbow trout

One hundred healthy rainbow trout were screened using PCR-SSCP. The different genotypes produced by the S2 and S6 loci were not significantly associated with meat quality traits ($P > 0.05$). Table 3 shows that the crude protein at the S3 locus was not significantly different ($P > 0.05$). However, the muscle fiber density and dry matter content of genotypes AA and AB were significantly higher than of genotype BB ($P < 0.05$), and the muscle fiber diameter was significantly lower than in genotype BB ($P < 0.05$).

Table 3. Effect of different genotypes in S3 and S5 on meat quality traits in rainbow trout.

Traits	Genotype of S3			Genotype of S5			
	AA	AB	BB	AA	AC	BB	CC
Dry matter (%)	31.21 ± 1.52^a	30.82 ± 2.20^a	28.17 ± 1.89^b	31.56 ± 1.32^a	30.45 ± 1.78^a	28.97 ± 1.90^b	29.53 ± 1.11^b
Protein (%)	17.29 ± 0.62	17.63 ± 0.55	16.58 ± 0.31	17.33 ± 0.21	17.74 ± 0.11	17.01 ± 0.87	17.56 ± 0.44
Intramuscular fat content (%)	11.24 ± 0.50	10.67 ± 0.41	12.11 ± 0.67	11.67 ± 0.39	10.99 ± 0.36	11.32 ± 0.41	11.01 ± 0.25
Ash (%)	2.49 ± 0.04	2.45 ± 0.03	2.46 ± 0.04	2.52 ± 0.03	2.56 ± 0.04	2.54 ± 0.03	2.51 ± 0.03
Muscle fiber density (N/mm^2)	293.63 ± 46.33^a	268.97 ± 79.14^a	241.32 ± 56.25^b	269.22 ± 56.12	271.02 ± 63.83^a	257.73 ± 42.78^b	256.54 ± 50.88^b
Muscle fiber diameter (μm)	29.67 ± 2.63^a	30.12 ± 1.98^a	32.53 ± 1.14^b	28.14 ± 2.35^a	27.75 ± 1.65^a	30.87 ± 1.05^b	31.17 ± 1.83^b

Different letters indicate significant difference at 0.05 levels.

In S5 locus, the crude protein, intramuscular fat, and ash among different genotypes were not significantly different ($P > 0.05$; Table 3). However, dry matter content and muscle fiber density of genotypes AA and AC were significantly higher than in genotypes BB and CC ($P < 0.05$). Muscle fiber diameters of genotypes BB and CC were significantly greater than in genotypes AA and AC ($P < 0.05$; Table 3).

DISCUSSION

The aim of breeding programs has changed from increasing yield to improving meat quality-related traits (van Wijk et al., 2005). A study examining the association of these traits and candidate genes increased the understanding of the genetic basis of productive traits (Óvilo et al., 2006). The development process of muscle is controlled by the *MyoD* gene family (Massari and Murre, 2000). *MRFs* play important roles in muscle precursor cell shape, as well as muscle fiber formation, until individual functions mature after birth (te Pas and Visscher, 1994). The *MyoD1* gene was first identified in the human genome (Davis et al., 1987). Two distinct *MRFs* with homology to *MyoD* (*MyoD1a* and *MyoD1b* genes) in rainbow trout were isolated by Rescan in 1996. In this study, the intron 1 and 2 sequences of the *MyoD1a* and *MyoD1b* genes were cloned and submitted to GenBank (accession Nos. FJ623462 and FJ793566**)**.

Numerous studies have examined *MyoD1* gene polymorphisms, mainly focusing on pigs (Knoll et al., 1997; Urbański and Kuryl, 2004; Wyszyńska-Koko et al., 2006; Kim et al., 2009), cattle (Tian et al., 2007), and other animals (Zhang et al., 2007); however, there have been no reports in fish. In the pig, 2 polymorphic loci (C489T and G566C) were detected at 333 and 62 bp of exon 1, and the G566C mutation caused an exchange of arginine to proline (Urbański and Kuryl, 2004). In cattle, intron 2 of the *MyoD1* gene was found to contain 2 polymorphic loci, C39T and C112G (Tian et al., 2007). Seven SNP loci in introns were identified in the *MyoD* gene using the PCR-SSCP technique and sequencing in *Micropterus salmoides* (Yu et al., 2009). In this study, 2 SNP loci of the *MyoD1a* gene were detected, located at 129G→A in exon 1 and 37G→A in exon 2. The 37G→A mutation in exon 2 induced an R185K amino acid change in the protein polypeptide chain. Seven SNPs in the *MyoD1b* gene were detected and located at 218T→C, 224T→C, 242A→C, 246T→A, 248T→C, 305T→C, and 329C→T. A 246T→A mutation in exon 1 induced an R/K change in 83 amino acids of the protein polypeptide chain. Most SNPs in the genome were located in intron regions because exons are relatively conserved. In this study, some mutations were identified in exons of the *MyoD1b* gene. The phenomenon indicates that the *MyoD1b* gene disappeared during evolution through non-exon conservation; the *MyoD* gene in rainbow trout is rich in polymorphisms and the genetic variability is high. Furthermore, the S6 locus showed high PIC (0.582), indicating that the locus had a higher degree of genetic variability and a larger selection potential.

In pig, the TCBB diplotype of the myogenin gene had desirable effects on the total number of fibers, fiber cross-sectional area, and loin eye area compared to the other diplotypes. Moreover, the diplotype had the highest pH value in muscle and all meat quality traits were near the upper limit of the normal range, including the traits reddish pink, firm, and non-exudative pork (Kim et al., 2009). Intron 2 of the *MyoD* gene was associated with carcass traits in cattle (Tian et al., 2007). However, in goat, the *MyoD* gene was found to be correlated with body size traits (Zhang et al., 2007). The association between the *MyoD* gene and meat quality traits has not been reported previously in fish. In this study, the mutated polymorphisms were examined using correlation analysis to determine their influence on meat quality traits in rain-

bow trout. The results indicated that different genotypes in the S3 and S5 loci had significant impacts on muscle fiber density, muscle fiber diameter, and dry matter indicators ($P < 0.05$). The SNPs in the *MyoD1a* and *MyoD1b* genes in rainbow trout were associated with muscle fiber traits and may play important roles in the meat quality traits of rainbow trout. Thus, these genes can be used as candidate genes to affect the muscle fiber traits of rainbow trout.

Molecular marker-assisted selection is used widely in upland cotton (Guo et al., 2005), rice (Sun et al., 2006), wheat (Anderson, 2007), sorghum (Knoll and Ejeta, 2008), and roses (Biber et al., 2009). Particularly, in upland cotton, the pyramiding of 2 quantitative trait loci controlling high fiber strength identified through marker-assisted selection greatly improved the selection efficiency of cotton fiber strength (Guo et al., 2005). In dairy cattle, breeding across-family marker-assisted selection using selective genotyping strategies has been reported previously (Ansari-Mahyari et al., 2008). However, in fish, this method is rarely used. Only in Chinese shrimp, microsatellite markers, including simple sequence repeats were examined for their usefulness in breeding (Dong et al., 2006). Thus, SNPs in the S3 and S5 loci should be examined in larger populations and used for molecular marker-assisted selection for meat quality traits in rainbow trout.

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