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Characterization and Tissues Distribution of Vinculin, Agouti-relating Protein and Melanocortin 4 Receptor Genes in Rainbow Trout, *Oncorhynchus mykiss*

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ABSTRACT : As in the *O. mykiss* electrophoretic profiles of RNA, the signals of each RNA sample from 9 individual tissues such as liver, muscle, brain, heart, pituitary gland, kidney, intestine, spleen and gill similar to positive control were obtained. The tissue distributions of the complimentary DNA (cDNA) of *O. mykiss* four genes were analyzed using quantitative real-time PCR with primer sets for tissue expression analysis. In this rainbow trout species, author obtained bands of various sizes, ranged from 700 bp to 1,400 bp. A dissociation curve was made at the end of each run to make sure that there was no non-specific amplification. Supplementarily , the Ct of each DNA was compared. The Ct values of vinculin with rainbow trout tissues were determined in a manner similar to those for agouti-related protein (AgRP) and melanocortin receptors (MC4R I and MC4R II). Further, obtained Cts for standard curve of each DNA were affected by specific product (vinculin, AgRP and MC4R II genes). After several experiments with four individual genes of rainbow trout, author estimated a variation ratio of the mean Ct value of the DNA extracted using the comparative CTt method was 37.27, and the standard deviation was 5.33. The correlation coefficient between the Ct values and the concentration of cDNA was −0.98, −0.99, −0.91 and −0.86, respectively (vinculin, AgRP, MC4R I and MC4R II genes). Since this correlation showed high linearity, the straight line obtained was used as a standard for the *O. mykiss* tissues reared in aquarium. A PCR efficiency of 100% is ideally achieved when the slopes are close to the theoretical value of −3.31. According to quantification method, the results of quantification are strongly affected by the DNA fragmentation. The size of most DNA fragments obtained from various tissues of rainbow trout used in the experiment was approximately 100 bp. According to the four slopes, an efficiency of nearly 100% was estimated for four genes detection methods. Additionally, further analysis with more individuals and primers will be required to fully establish optimization in rainbow trout.

Key words : Agouti-relating protein, Melanocortin 4 receptor, mRNA, Tissue distribution, Vinculin.

INTRODUCTION

The melanocortin system includes both agonist (α-melanocyte stimulating hormone, α-MSH) and antagonist peptides (agouti-related peptide, AGRP) (Della-Fera & Baile, 2005). Increased melanocortin receptor stimulation following leptin administration plays an important role in leptin-induced hypophagia and increased sympathetic nervous system activity and is partly responsible for leptin-induced weight loss.

The specific melanocortin receptors, MC3R and MC4R, are directly linked to metabolism and body weight controls (Jackson et al., 2005), have a role in regulating energy homeostasis and obesity in human (Xiang et al., 2006). These receptors are activated by the peptide hormone α-MSH and antagonized by the agouti-related protein (AGRP). The hypothalamic melanocortin receptors, MC3R and MC4R, play a central role in the regulation of food intake and metabolism (Vaisse et al., 1998; Wilson et al., 1999). Deletion of the MC4R gene in mice results in hyperphagia, obesity and symptoms equivalent to that of adult onset diabetes (Huszar et al., 1997). Diminished hypothalamic melanocortin receptors, increased Agrp expression, and potential rewiring of brain circuits may underlie the exacerbated obesity (Li et al., 2007).

AGRP exhibits agonistic properties on the endocytosis

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pathway of melanocortin receptors in human (Breit et al, 2006). Vinculin, a highly conserved 117-kDa monomeric intracellular protein (1,066 residues), plays crucial roles in the maintenance and regulation of cell shape, adhesion, and migration (Geiger, 1979; Jockusch et al., 1995) and is essential to any physiological, genetic and pathological processes such as wound healing (Kris & DeMali, 2004), muscle development (Barstead & Waterston, 1991; Bass et al., 1999), and embryonal carcinoma (Samuels et al., 1993).

Fish agouti-family genes have also been identified in some fish such as goldfish, zebra fish and puffer (Cerdà-Reverter & Peter, 2003; Song et al, 2003; Kurokawa et al., 2006). The goldfish, *Carassius auratus*, agouti-signaling protein (ASIP) had an antagonistic function to a-MSH in skin (Cerdá-Reverter et al., 2005) and goldfish and zebrafish, *Danio rerio*, agouti-relating protein (AGRP) are related to metabolic state in the brain (Cerdá-Reverter & Peter, 2003; Song et al, 2003), as is mammalian AGRP (Volkoff et al., 2005).

Despite the importance of these systems in various organisms' health, there are as yet no basic informations reported for any of the members of agouti-relating protein, melanocortin 4 receptor and vinculin genes in rainbow trout, *Oncorhynchus mykiss* or coldwater fishes. Quantitative real-time polymerase chain reaction (qRT-PCR) technology is based on the detection and quantification by a high-quality optical detection instrument of a fluorescence reporter (Lopez & Pardo, 2005; Koskenniemi et al., 2007; Solstad et al., 2007). In the present study, author has used quantitative real-time PCR to study the characterization and tissues distribution of agouti-relating protein, melanocortin 4 receptor and vinculin genes in rainbow trout, *Oncorhynchus mykiss*.

MATERIALS AND METHODS

1. Preparation of Samples

Tissue samples were collected from the aquarium of the National Center for Cool and Cold Water Aquaculture, USDA. Rainbow trout (20 ㎝ long, 1-year-old fingerlings) were dissected under 100 ㎎/㎏ tricaine methane sulfonate anaesthesia. Liver, muscle, brain, heart, pituitary, kidney, intestine, spleen and gill tissues, respectively, were excised as previously described (Kurokawa et al, 2006). The fish samples (1～3 ㎝) were washed twice in TE buffer, and stored in the freezer at －80℃ until the experiment was performed.

2. RNA Isolation

In this study, the following methods for RNA extraction were used: add 50 to 100 ㎎ of fresh or flash frozen tissues (for muscle or other tough tissues, chop to facilitate homogenization) to 1 ㎖ of TRI REAGENT™ (Trizol, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Use Mix-a-Mill (Ekurt Retsch GmbH, Haan, Germany) to homogenize tissues in TRI REAGENT with one steel bead at 20 rpm and centrifuge at 12,000×g for 10 min at 4℃ to remove insoluble material (Beckman Coulter Inc., Fullerton, CA, USA). Transfer supernatant to a clean 1.5 ㎖ microfuge tube, incubate for ～5 min at room temperature and 200 ㎕ of chloroform per ㎖ of TRI REAGENT to each tube, and then centrifuge at 12,000×g for 15min at 4℃ to obtain clear upper aqueous phase (RNA). Add 500 ㎕ of isopropanol per ㎖ of TRI REAGENT and centrifuge at 12,000×g for 10 min at 4℃ to obtain the white tear shaped pellet. Remove the supernatant and wash pellet with 1 ㎖ of 75 % ethanol per ㎖ of TRI REAGENT. If the RNA pellets float, perform the wash in 75 % ethanol at 12,000×g at 4℃ and RNA can be stored at －20℃. Perform DNase Treatment (Promega RQ1 RNase-free DNase). Re-extraction was performed twice to remove residual DNase using 500 ㎕ of Tri-Reagent. The concentration and purity was measured by NanoDrop (ND-1000 spectrophotometer, USA) in duplicate.

3. RNA Dilution and Gel Electrophoresis

The volume of RNA at 2 ㎍/㎕ was calculated and stored diluted RNA samples at －80℃ until ready to use. The quality of the RNA was further investigated by agarose gel electrophoresis. The diluted RNA products were generated on 1.5% agarose (IBI Shelton Scientific INC, Shelton, CT, USA) gel containing 2% ethidium bromide, using a running buffer of 1X TAE (20 mM Tris; 9 mM acetic acid; 0.4 mM EDTA). The 100 bp step Ladder DNA markers (Promega, Madison,

WI, USA) were used as a DNA molecular weight marker. The gels were photographed over ultraviolet light using a Alpha Imager (Alpha Innotech Fluorchem Camera, USA).

4. Reverse Transcription Reaction

12 ㎕ of NF-water (Invitrogen Corp., Grand Island, NY, USA) was added to PCR tubes containing 2 ㎕ (1 ㎍) of random primers (Promega, Madison, WI, USA) and 2 ㎍ (1 ㎕) of diluted RNA and heated at 70℃ for 5 min and cool samples to 4℃ in thermocycler (MJ Research Inc, Waltham, MA, USA). Samples were Incubated in thermocycler with the following program (37-1 hr, check program): at 37℃ for 1 hr; 95℃ for 5 min; 4℃ for ever.

Reverse transcription reaction were carried out using 16 ㎕ of master mix volumes that consisted of 1 ㎕ of template DNA, 2 ㎕ of each primer pair, 13 ㎕ of master mix (containing MMLV PCR buffer, dNTP, rRNasin, NF-Water, and MMLV-RT) (Promega, Madison, WI, USA) according to the manufacturer's instructions. Samples cDNA were stored at −20℃ until ready for real-time PCR.

5. Quantitative Real-Time PCR

Real-time reactions were carried out with PCR master mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The tissue distributions of the cDNA of *O. mykiss* three genes were analyzed using quantitative real-time PCR with primer sets for tissue expression analysis as described in Table 1. Samples were run in duplicates and elongation factor 1 alpha (EF1alpha) was used as endogenous control and this was found to be suitable as endogenous control, although there were some differences in the degree of expression. Each 15 ㎕ PCR reaction contain 2 ㎕ cDNA, 0.5 ㎕ primer mix (25 ㎛ forward+25 ㎛ reverse), 7.5 ㎕ SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA) and 5 ㎕ NF-water. 2 ㎕ cDNA samples were loaded into a used PCR plate in an organized, logical arrangement. Provided cDNA sample was used as a positive control and load 2 ㎕ NF-water as a negative control. It was careful not to cause air bubbles above the surface of the plate. Fifteen microliters of a reaction mixture was added into a plate and centrifuged at 100 rpm for 1 min at 4℃. The plates were loaded into the ABI Prism 7900 HT sequence detection systems (Applied Biosystems, Foster City, CA, USA). Amplification was performed using a total of reaction volume of 15 ㎕ in MicroAmp Optical 96-well reaction plate (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: one cycle at 50℃ for 2 min, at 95℃ for 10 min; 40 cycles of denaturation at 95℃ for 15 s and annealing/extension at 60℃ for 1 min, at 95℃ for 15 s, and 60℃ for 15 s. PCR efficiency was calculated from each linear regression of standard curves.

RESULTS AND DISCUSSION

1. RNA Extraction and cDNA Reverse Transcription Reaction

In order for the efficient RNA extraction, the RNA extraction method was carried out using three rainbow trout reared

Table 1. Primer sequences used for RT-PCR amplification of vinculin, agouti-relating protein (AgRP), melanocortin 4 receptor I (MC4R I) and melanocortin 4 receptor II (MC4R II) genes in rainbow trout, *Oncorhynchus mykiss*

	Forward	Reverse
Vinculin	CAGCCCCAGATGTTAGTGGT	AGAGTTCTCCACCTCCCGTT
AgRP	AGGGAAACCCGGAAGACTGTT	GCTACAGGCTCGTGTTTCTGAAA
MC4R I	TGGGAACCCTCAACAAAGAC	TCCCCAGTGTGAGAAAGACC
MC4R II	TGAATTCCACAGACCACCAA	TTTGTTGAGGGTTCCCAGAG

AgRP: agouti-relating protein.
MC4R I: melanocortin 4 receptor I.
MC4R II: melanocortin 4 receptor II.

in aquarium, which was collected from NCCCWA in USA. Ribosomal RNA genes are widely used for phylogenetic analysis in fish and/or shellfish. In the present study, electrophoretic profiles RNA isolated from individual American rainbow trout (*O. mykiss*) were shown in Fig. 1. Total RNA was extracted from various tissues and reverse transcribed. As in the *O. mykiss* electrophoretic profiles of RNA, the signals of each RNA sample from 9 individual tissues such as liver, muscle, brain, heart, pituitary gland, kidney, intestine, spleen and gill similar to positive control were obtained. In this rainbow trout species, author obtained bands of various sizes, ranged from 700 bp to 1,400 bp.

Complimentary DNA (cDNA) solutions were used as the template for quantitative real-time PCR assay along with vinculin, agouti-relating protein (AgRP) and melanocortin 4 receptor II (MC4R II) genes (Fig. 2, 3 and 4). DNA was extracted from the serial dilutions of *O. mykiss* tissues (250, 500, 1,000, and 2,000). The Ct values of vinculin with rainbow trout tissues were determined in a manner similar to those for AGRP, MC4R I and MC4R II (Fig. 5). Further, obtained Cts for standard curve of each DNA were affected by specific product (vinculin, AgRP and MC4R II genes). After several experiments with four individual genes of rainbow trout,

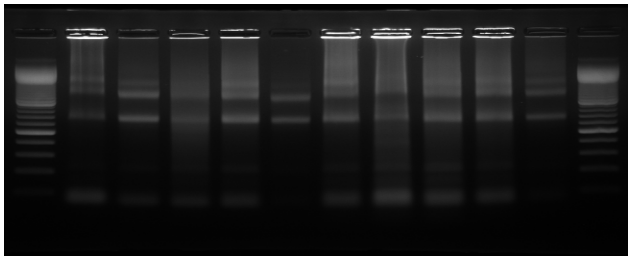

Fig. 1. Electrophoretic profiles of RNA isolated from individual American rainbow trout (*O. mykiss*) (lanes 2～10) and positive (lane 11). Each lane shows RNA samples from 9 individuals. Lane 01: molecular marker, lane 02: liver, lane 03: muscle, lane 04: brain, lane 05: heart, lane 06: pituitary gland, lane 07: kidney, lane 08: intestine, lane 09: spleen, lane 10: gill, lane 11: positive sample, lane 12: 100 bp step DNA ladder molecular marker. 28S rRNA, 18S rRNA, 5S rRNA and degraded RNA.

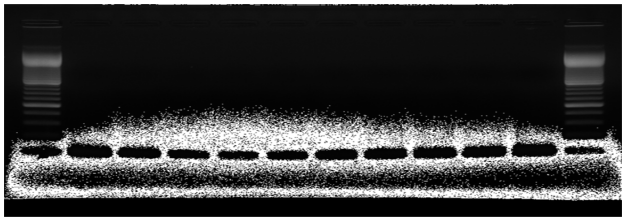

Fig. 2. RT-PCR analysis of mRNA isolated from American rainbow trout (*O. mykiss*) liver (lanes 02～06) and muscle (lanes 07～11) was amplified by vinculin primer. PCR was carried out for 40 cycles using primers specific for agouti-relating peptides. Amplification products were generated via electrophoresis on 1.75% agarose gel containing ethidium bromide. The 100 bp DNA Ladder (lanes 01 and 12) was used as a DNA molecular weight marker. Lane 01: molecular marker, lane 02 (dilution 2,000), lane 03 (dilution 1,000), lane 04 (dilution 500), lane 05 (dilution 250), lane 06: positive cDNA, lane 07: (dilution 2,000), lane 08 (dilution 1,000), lane 09 (dilution 500), lane 10 (dilution 250), lane 11: positive cDNA, lane 12: molecular marker (100 bp step ladder).

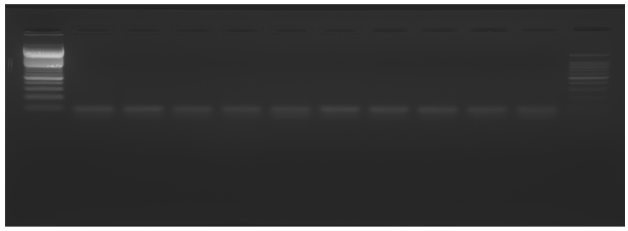

Fig. 3. RT-PCR analysis of mRNA isolated from American rainbow trout (*O. mykiss*) liver (lanes 02～06) and muscle (lanes 07～11) was amplified by agouti-relating peptides primer. PCR was carried out for 40 cycles using primers specific for agouti-relating peptides. Amplification products were generated via electrophoresis on 1.75% agarose gel containing ethidium bromide. Lanes 01 and 12 are 100 bp step Ladder molecular markers. Lanes 01 and 12 are 100 bp step Ladder molecular markers. Lane 02: liver (× 2,000), lane 03: liver (× 1,000), lane 04: liver (× 500), lane 05: liver (× 250), lane 06: positive cDNA, lane 07: muscle (× 2,000), lane 08: muscle (× 1,000), lane 09: muscle (× 500), lane 10: muscle (× 250), lane 11: positive cDNA.

Fig. 4. RNA isolated from American rainbow trout (*O. mykiss*) liver (lanes 02～06) and muscle (lanes 07～11) was amplified by melanocortin receptor MC4RII primer. Amplification products were generated via electrophoresis on 1.75% agarose gel containing ethidium bromide. Lanes1 and 12 are 100 bp step Ladder molecular markers. Lane 02: liver (× 2,000), lane 03: liver (× 1,000), lane 04: liver (× 500), lane 05: liver (× 250), lane 06: positive cDNA, lane 07: muscle (× 2,000), lane 08: muscle (× 1,000), lane 09: muscle (× 500), lane 10: muscle (× 250), lane 11: positive cDNA.

author estimated a variation ratio of the mean Ct value of the DNA extracted using the comparative CT method was 37.27, and the standard deviation was 5.33 (Fig. 5). The correlation coefficient between the Ct values and the concentration of cDNA was −0.98, −0.99, −0.91 and −0.86, respectively (vinculin, AgRP, MC4R I and MC4R II genes) (Fig. 3). Since this correlation showed high linearity, the straight line obtained was used as a standard for the *O. mykiss* tissues reared in aquarium.

PCR efficiency was calculated from each linear regression of standard curves. A PCR efficiency of 100% is ideally achieved when the slopes are close to the theoretical value of −3.31. According to the slopes shown in Fig. 5, an efficiency of nearly 100% was estimated for four genes detection methods. The slopes from the target and endogenous genes of the detection method should be identical to those of the other authors (Lopez and Pardo et al., 2005). They insisted that if necessary, normalization factors should be added to the equations to work in reliable conditions. The standard curves with agouti-relating protein (AGRP) and vinculin genes showed the same behavior. In addition, the behavior of melanocortin 4 receptor I (MC4R I) and melanocortin 4

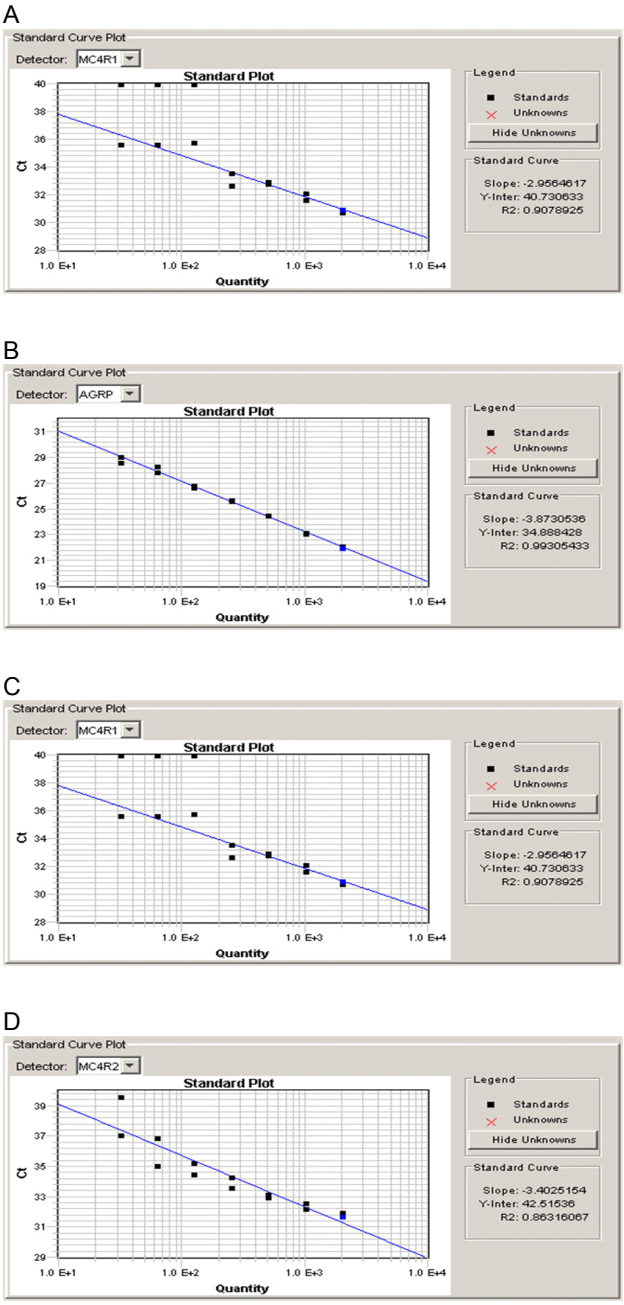

Fig. 5. Standard curve plots of cDNA amplified by real-time PCR machine (7900 HT sequence detection systems) using vinculin (A), agouti-relating peptides (B), melanocortin receptor MC4R1 (C) and melanocortin receptor MC4R2 (D) primers, respectively. Ct values of cDNA extracted with the nine tissues from rainbow trout reared in aquarium, which was collected from NCCCWA in USA. Assays performed in duplicate.

receptor II (MC4R II) was identical. The regression lines cross the y-axis at different Ct values, indicating different behaviors of a group such as AGRP and vinculin, the other group such as MC4R I and II at the same template concentration. A dissociation curve was made at the end of each run to make sure that there was no non-specific amplification (Fig. 6). According to quantification method, the results of quantification are strongly affected by the DNA fragmentation. The size of most DNA fragments obtained from various tissues of rainbow trout used in the experiment was

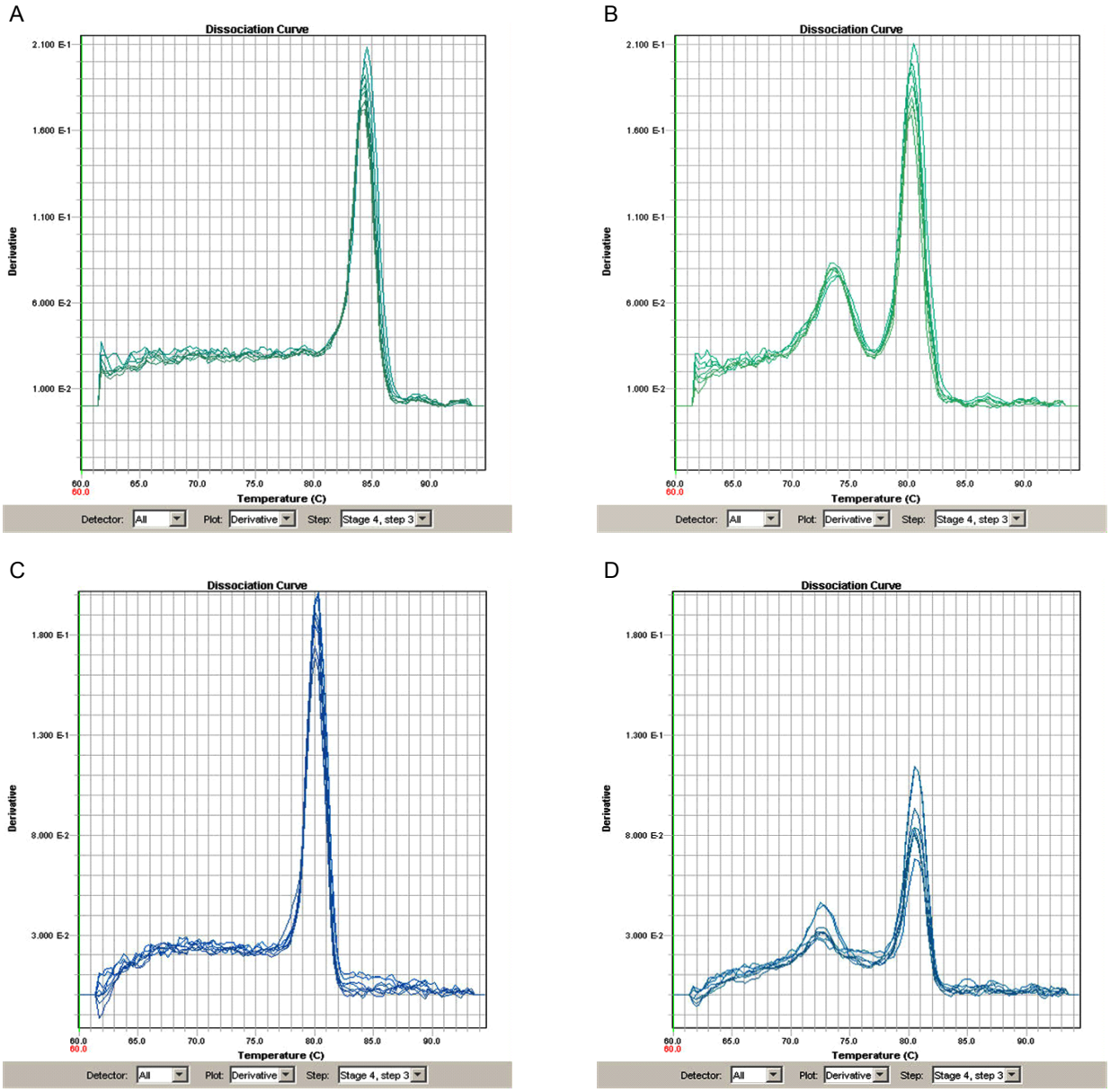

Fig. 6. Dissociation curves of nine tissues of rainbow trout amplified by real-time PCR machine (7900 HT sequence detection systems) using vinculin (A), agouti-relating peptides (B), melanocortin receptor MC4R1 (C) and melanocortin receptor MC4R2 (D) primers, respectively. Nine tissues are liver, muscle, brain, heart, pituitary gland, kidney, intestine, spleen, and gill of rainbow trout reared in aquarium, which was collected from NCCCWA in USA.

approximately 100 bp. In case of canned tuna, the size of the fragment was smaller than 200 bp (Quinteiro et al., 1998; Lopez & Pardo, 2005). In addition, the real-time PCR method will be validated using 20 commercial samples with optimal results in all cases (Lopez & Pardo, 2005). Accordingly, further analysis with more individuals and primers will be required to fully establish optimization in rainbow trout.

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