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Detection of Genetically Modified Coho Salmon Using Polymerase Chain Reaction (PCR) Amplification

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A PCR-based protocol for the identification of genetically modified salmon carrying a growth hormone transgene was developed. Several primer pairs were examined, and the primers that gave consistent results were selected to conduct routine testing. Comparison among several DNA extraction procedures, as well as different buffer compositions, led to the adoption of TriZol as the method of choice. Low potassium and high magnesium chloride concentrations were very important in the overall success of the PCR reaction, whereas buffer pH, ranging from 8.3 to 9.2, had little impact on the amplification reaction. The optimal primer annealing temperature was 52 °C. Although fish muscle tissues were the primary source for DNA samples, detection of the transgene was also possible in bones, skin, fins, and other organs. No benefits were achieved by the addition of additives such as dimethyl sulfoxide and betaine to the PCR reaction. This optimized PCR method was used to identify all samples tested (61 samples and 17 controls) with 100% accuracy.

KEYWORDS: Genetically modified fish; genetically modified food; GMO; Polymerase Chain Reaction; PCR

INTRODUCTION

To increase the efficiency of production of aquaculture, research efforts are underway to enhance the growth and feed conversion of several fish species by producing transgenic strains containing modified growth hormone gene constructs. Indeed, dramatic growth enhancement has been achieved in a number of salmonids, including Atlantic salmon (*1*, *2*), coho and other Pacific salmonids (*3*, *4*), rainbow trout (*5*), and char (*6*). The potential use of transgenic fish has prompted considerable debate and research on issues associated with both food safety and ecological impacts. This technology has also raised consumer concerns regarding genetically modified organisms (GMOs), resulting in the need to distinguish between GMO- and non-GMO-containing food. In this model study, we used a transgenic Pacific coho salmon (*Oncorhynchus kisutch*) produced by introducing a DNA construct containing the sockeye salmon type I growth hormone (GH) gene (*3*, *4*). The growth hormone coding regions in these transgenes have been fused to the salmon metallothionein-B gene promoter and are designed to (1) allow expression of GH from all tissues in the salmon (rather than just the pituitary gland as occurs in normal fish) and (2) elevate circulating levels of the growth hormone in the blood of the GM fish. Dramatic growth enhancement has been achieved, with the average transgenic salmon being 10 times larger than the nontransgenic controls and a maturation time half that of normal fish. Fish species other than salmonids have also been genetically modified, including goldfish (*7*), tilapia (*8*), catfish (*9*, *10*), and carp (*11*). In this study we concentrated on the detection of GM coho salmon as a model system.

MATERIALS AND METHODS

Chemicals and Enzymes. All chemicals were of molecular biology grade. *Taq* DNA polymerase, DNAzol, and TriZol were purchased from Invitrogen, and titanium *Taq* DNA polymerase was purchased from Clontech. To optimize the amplification reaction, 12 different buffer combinations were evaluated. All buffers contained 12 mM Tris-HCl (pH 8.3−9.2) and various concentrations of $MgCl_2$ and KCl (**Table 1**).

Transgenic fish, as well as wild-type controls, were obtained from a contained transgenic fish facility at the Department of Fisheries and Oceans, West Vancouver Laboratory. Two-year-old transgenic salmon were used. Both 2- and 5-year-old wild-type salmon were used as age and weight controls, respectively.

DNA Primers. A pair of primers for the detection of the transgenic fish (MT1 and GH19) were designed to span the junction between the metallothionein promoter and the growth hormone gene, respectively (*12*, *13*): GH19, 5′ GTT AAA TTG TAT TAA ATG GT 3′; and MT1, 3′ CTG ATT AAG TTT TGT ATA GT 5′. These primers amplify a product of 427 base pairs (bp). Primers (GH5 and GH7) spanning 158 bases, and located within exon 5 of the salmon growth hormone gene,

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Table 1. Different Buffer Formulations Used for the Amplification of Transgenic Salmon[a]

buffer	[Tris-HCl] pH 8.3	[Tris-HCl] pH 8.8	[Tris-HCl] pH 9.2	[KCl]	[EDTA]	[$MgCl_2$]
1	12			25	0.25	1.5
2	12			75	0.25	1.5
3	12			25	0.25	3.5
4	12			75	0.25	3.5
5		12		25	0.25	1.5
6		12		75	0.25	1.5
7		12		25	0.25	3.5
8		12		75	0.25	3.5
9			12	25	0.25	1.5
10			12	75	0.25	1.5
11			12	25	0.25	3.5
12			12	75	0.25	3.5

[a] All concentrations are given in millimolar.

were synthesized (*13*) and used as positive amplification controls in all PCR experiments. The primer sequences used were as follows: GH5, 5′ AGC CTG GAT GAC AAT GAC TCT C 3′; and GH7, 5′ CTT ATG CAT GTC CTT CTT G 3′.

PCR Reaction. All initial Polymerase Chain Reactions (PCR) on muscle tissue were performed in a volume of 50 μL of PCR buffer supplied by the manufacturers, containing 1× cresol/sucrose solution (10× stock: 1 mM cresol red, 0.2 g/mL sucrose), 5 μL of DNA (1−2 ng), 1 μM primer mix, 1.8 mM $MgCl_2$, 0.2 mM dNTPs, and 2.5 units of *Taq* polymerase in a 96 well gradient Robocycler (Stratagene). The DNA was amplified for 35 cycles as follows: 94 °C for 50 s, 54 °C for 50 s, and 72 °C for 70 s (*14*). In subsequent experiments the concentrations of $MgCl_2$ and KCl and the pH were changed to 3.5 mM $MgCl_2$, 75 mM KCl, and pH 8.3.

Agarose Gel Electrophoresis. Gel electrophoresis was carried out in 2% agarose (SeaKem, LE, BMA, Rockland, ME) containing 0.1 μg/mL ethidium bromide using TBE buffer. A 100 bp DNA ladder was used as a molecular weight marker (AmpliSize, Bio-Rad, Mississauga, ON, Canada).

DNA Extraction. The effects of different DNA extraction procedures on the amplification experiments were compared. For this study, four methods of DNA purification were assessed and compared to two commercially available DNA extraction kits.

Cetyltrimethylammonium Bromide (CTAB) Method. Muscle tissues were homogenized and the DNA was extracted according to previously published procedures (*15*, *16*). One hundred milligrams of fish muscle tissue was homogenized in 500 μL of CTAB buffer (2% CTAB, 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl), incubated at 65 °C for 30 min, and then centrifuged at a maximum speed of 15000*g* for 10 min. Two hundred microliters of chloroform was added to the supernatant, and the mixture was vortexed and spun at the same speed as before. The supernatant was precipitated with 2 volumes of 0.5% CTAB in 40 mM NaCl, pH 8.0. The DNA was incubated at room temperature for 1 h, pelleted by centrifuging for 5 min, and then resuspended in 350 μL of 1.2 M NaCl in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA). An equal volume of chloroform was added. The samples were vortexed and centrifuged as before. The DNA was then precipitated with 0.7 volume of 2-propanol. The DNA was pelleted, washed, dried, and suspended in TE buffer at a concentration of 100−200 ng/mL. Five microliters of the DNA suspension was used in the PCR.

Proteinase K Extraction Method. DNA was extracted from muscle tissue according to a modification of a previously published method (*17*). One hundred milligrams of salmon muscle tissue was homogenized in 500 μL of TE buffer containing 500 μg/mL proteinase K. The homogenate was incubated at 37 °C for 1 h. An equal volume of phenol−chloroform solution (50% phenol, 49% chloroform, and 1% isoamyl alcohol) was added to the homogenate, and the mixture was vortexed for a few seconds and centrifuged at maximum speed for 3 min. An equal volume of 2-propanol was added to the aqueous phase, and the DNA was precipitated by centrifugation at maximum speed for 10 min. The DNA was suspended in 500 μL of TE buffer and reprecipitated by the addition of 125 μL of 5% CTAB and 0.5 M NaCl in 20 mM Tris-HCl, pH 8.5. The DNA was incubated at 65 °C for 5 min followed by centrifugation for 10 min at maximum speed. The DNA pellet was suspended in 500 μL of 1.2 M NaCl in TE buffer and precipitated by addition of an equal volume of 2-propanol. The purified DNA was suspended in 100 μL of TE buffer. The DNA was adjusted to the same concentration as above.

DNAzol Method. One hundred milligrams of salmon muscle tissue was homogenized in 500 μL of DNAzol (Invitrogen). Another 500 μL of DNAzol was added and vortexed. The homogenate was centrifuged at 10000*g* for 10 min. An equal volume of 100% ethanol was added, and the DNA was precipitated by incubation at room temperature for 3 min followed by centrifugation for 2 min at 4000*g*. The DNA pellet was washed twice with 75% ethanol, air-dried, and suspended in 100 μL of 8 mM NaOH according to the manufacturers' recommendation.

TriZol Method. One hundred milligrams of salmon muscle tissue was homogenized in 1.0 mL of TriZol and incubated at 65 °C for 10 min. One hundred microliters of chloroform was added to the homogenate, and the mixture was vortexed and centrifuged at maximum speed for 10 min. The interphase was carefully removed and resuspended in 200 μL of TE buffer. An equal volume of phenol−chloroform was added, vortexed, and centrifuged as above for 3 min. An equal volume of 2-propanol was added to the aqueous phase. The DNA was precipitated, dried, and then suspended in 100 μL of TE buffer and adjusted to the same concentration as above.

Commercial Kits for DNA Extraction. Two commercially available kits, Wizard (Promega) and NucleoSpin (Clontech), were used according to the manufacturers' recommendations. The final DNA was eluted in 100 μL of TE buffer. The concentration of DNA obtained from all extraction methods was adjusted to 100−200 ng/ mL.

RESULTS AND DISCUSSION

DNA Extraction. DNA of high quality is essential for the success of the PCR procedure. The quantity of DNA is also important especially for the detection of low-copy genes or transformed products in food mixed with natural food. All of the extraction procedures used in this study produced a relatively large amount of DNA. The average amount of DNA extracted from 100 mg of muscle tissue varied depending on the extraction procedure. The average amount of DNA was as high as 60 μg/100 mg of muscle tissue using the CTAB method and as low as 15 μg/100 mg of tissue when the TriZol method was used. The DNAzol method produced an average of 20 μg of DNA/100 mg of muscle tissue. The amount of DNA obtained using the commercially available kits was also comparable to that obtained with the other methods used, ranging from 20 μg/100 mg using the Wizard kit to 50 μg/100 mg of muscle tissue with the NucleoSpin kit. DNA extracted from muscle tissue using different procedures gave comparable results after PCR amplification using the primers MT1 and GH19 as assessed by agarose gel electrophoresis of the amplified products (**Figure 1**). When the DNA was extracted from other tissues, such as fins, bones, scales, eggs, slime, skin, and blood, only the TriZol method produced amplification products from all of the tissues tested (**Figure 2**). All other procedures failed to produce amplification products from blood, fins, and/or scales (data not shown). As a result of these experiments, the TriZol method was adopted as the method of choice for all subsequent experiments due to the low price, simplicity, and speed associated with it.

PCR Amplification. Amplifying the salmon growth hormone gene using GH5 and GH7 primers was easily achieved using *Taq* polymerase. The amplification of the transgene was more challenging and led us to try different *Taq* polymerase enzymes. The titanium *Taq* was tried and found to give the most consistent amplification results. The buffer conditions for amplification of the transgene using primers GH19 and MT1 were critical

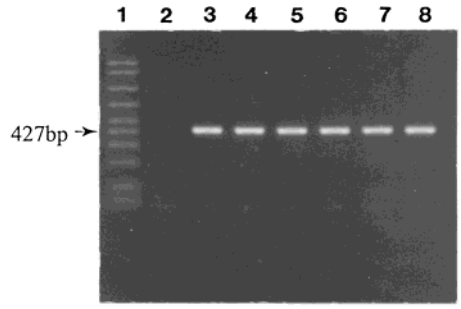

Figure 1. PCR amplification product, using primers GH19 and MT1, from transgenic salmon using DNA extracted by different extraction procedures: (lane 1) DNA marker (100 bp ladder, AmpliSize, Bio-Rad); (lane 2) PCR products from DNA extracted from 2-year-old wild-type salmon muscle tissue using the Trizol method; (lanes 3–8) PCR products obtained from DNA extracted from transgenic fish muscles using PK, CTAB, DNAzol, TriZol, Wizard kit, and Nucleospin methods, respectively.

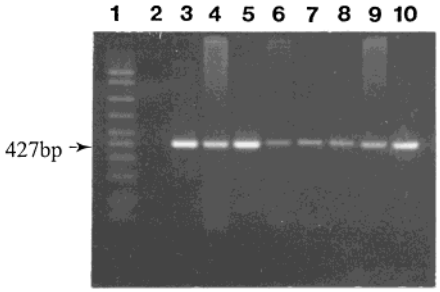

Figure 2. PCR results obtained from amplification of DNA extracted from different parts of the transgenic fish organs using the Trizol method for the purification of DNA: (lane 1) DNA marker (100 bp ladder, AmpliSize, Bio-Rad); (lanes 2–10) amplification results from wild coho negative control, tissues from genetically modified coho muscle, fins, bones, scales, eggs, slime, skin, and blood, respectively.

for obtaining consistent PCR amplification. *Taq* DNA polymerase (Invitogen) and titanium *Taq* (Clontech) gave excellent results when they were used together with the buffer supplied with the titanium *Taq*. Neither polymerase produced any PCR products when used with buffer supplied with the *Taq* polymerase (data not shown). To further study this observation, a series of buffers were assessed for their effect on DNA amplification. Optimum amplification results were achieved when the pH buffer was adjusted to 8.3. It was also evident that a combination of a high concentration of potassium (75 mM) and a low concentration of $MgCl_2$ (1.5 mM) inhibited the PCR when buffers of high pH were used. On the other hand, a potassium concentration of 25 mM and a high magnesium chloride concentration (3.5 mM) gave the best amplification products as assessed by agarose gel electrophoresis (**Figure 3**) and was the only combination that gave a positive product when high pH buffers were used.

The effect of annealing temperature on the overall PCR results was evaluated to optimize the PCR conditions. When the transgene primers were used, annealing temperatures ranging between 51 and 55 °C gave comparable amplification products. On the basis of the concentration of the amplification products, the best annealing temperature was found to be 52 °C (**Figure 4A**). In the case of the amplification of the growth hormone positive control, the annealing temperature range was much broader, ranging from 51 to 61 °C (**Figure 4B**) but with optimum results obtained between 51 and 55 °C.

Using the optimized protocol, a double-blind test was performed on 78 samples containing different age and size

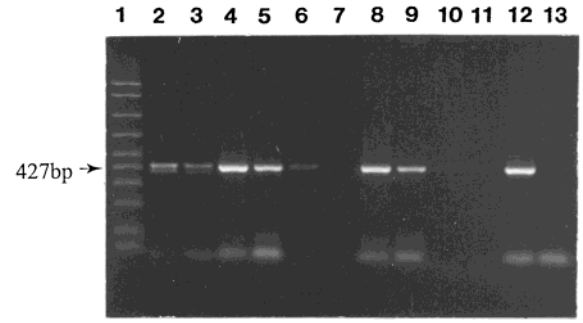

Figure 3. PCR amplification results using different buffers: (lane 1) DNA marker (100 bp ladder, AmpliSize, Bio-Rad); (lanes 2–13) PCR products obtained by using buffers 1–12 (see **Table 1** for buffer composition).

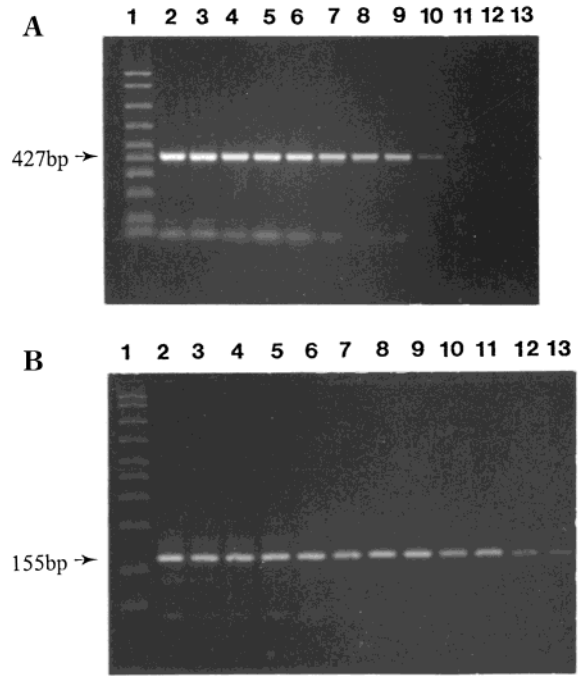

Figure 4. Effect of annealing temperature on amplification results using the targeted transgenic gene: (A) PCR products obtained from DNA purified from transgenic fish tissue using the specific target primers (GH19 and MT1) [lane 1, DNA marker (100 bp ladder, AmpliSize, Bio-Rad); lanes 2–13, different annealing temperatures ranging from 51 to 62 °C, respectively]; (B) PCR products obtained from DNA purified from transgenic fish tissue using the growth hormone positive control primers (GH5 and GH7) [lane 1, DNA marker (100 bp ladder, AmpliSize, Bio-Rad); lanes 2–13, different annealing temperatures ranging from 51 to 62 °C, respectively].

controls (17 wild-type negative controls and 61 GM positive samples). Some of the test results are shown in **Figure 5**. To improve primer fidelity, some additives such as dimethyl sulfoxide, formamide, and betaine were mixed with the amplification buffers. These chemicals had no positive effect on the PCR reaction and, in some cases, had inhibitory effects (data not shown).

Conclusion. Consumer concerns regarding GMOs have created a demand for labeling foods derived from transgenic products. In anticipation of voluntary or mandatory labeling, tests are being developed to identify GM food. Protein-based tests such as ELISA can directly detect expressed proteins. However, there are problems associated with this method, such as cross-reactivity, speed, sensitivity, and false-positive results. When homologous proteins are being detected, quantitative

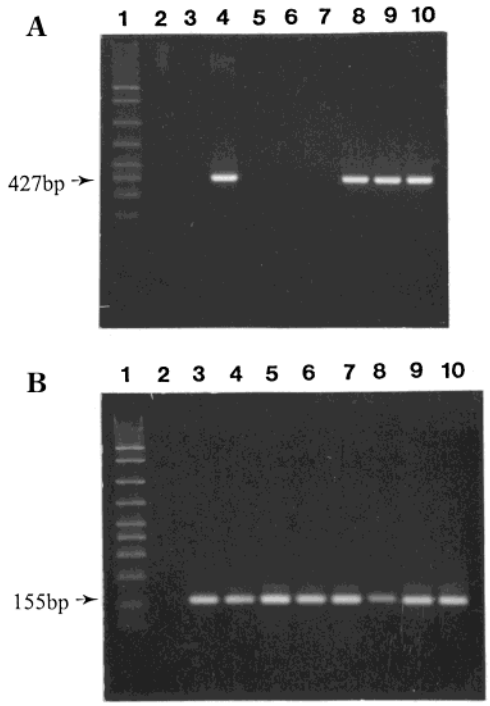

Figure 5. Amplification results from DNA extracted from different fish samples using the transgenic primers GH19 and MT1 (A) and the growth hormone primers GH5 and GH7 (B): (A) (lane 1) DNA marker (100 bp ladder, AmpliSize, Bio-Rad); (lane 2) sample with no template; (lanes 4 and 8–10) DNA extracted from transgenic fish; (lanes 3 and 5–7) amplification products from DNA obtained from wild-type salmon using primers GH19 and MT1; (B) corresponding results from the same DNA samples using growth hormone gene and primers GH5 and GH7 as positive control for the PCR reaction.

rather than qualitative criteria must be used. As an alternative, the PCR assay described herein can be both sensitive and specific. The assay described was specific for the amplification of a gene construct present in transgenic coho salmon and was specific by the absence of the PCR product from the wild-type fish. Further potential advantages of the PCR test are its speed and sensitivity. The assay was sensitive enough to detect the transgene in as little as 1 ng of total DNA. The PCR test can be performed in a few hours, using tissues from all parts of the fish including blood, bones, scales, slime, and other internal organs; thus, it is possible to perform the detection test without damaging or reducing the market value of the target fish. The transgene was detected in all controls including same age and same size controls, which were part of the double-blind test. Even though commercially purchased kits gave good results, and were relatively easy to use, they are expensive and the results obtained using such kits were not better than those obtained with the methods described in this study. The method for DNA extraction chosen in this study can overcome some of the other drawbacks associated with commercially available kits such as the high cost and the scaling up of purification procedures, which is almost impossible to perform using commercially available kits, and this could be a problem when the concentration of the target DNA is very small and larger amounts of tissues are needed to obtain adequate amounts of DNA.

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