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Utilization of Real-Time PCR to Detect Rangifer Cornu Contamination in Cervi Parvum Cornu

Young Hun Shim[1], Rack Seon Seong[1], Dong Sup Kim[1], Shin Jung Kang[2], Seung Yeup Chang[1], and Hyoung Jin Kim[3]

[1]National Institute of Food and Drug Safety Evaluation, Cheonwon 363-951, Korea, [2]Korea Food & Drug Administration, Cheonwon 363-951, Korea, and [3]College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea

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Cervi Parvum Cornu (CPC) is a well-known ethnopharmacological source, whereas Rangifer Cornu (RC) is not considered to be a major source. CPC is distributed in sliced form. Addition of RC to CPC has become an issue in CPC distribution because the appearance of sliced RC is not different from sliced CPC. Therefore, a real-time polymerase chain reaction (PCR) method was developed in this study to detect contaminating RC in CPC. The C-VIC and R-FAM primer/probe sets were designed to specifically amplify CPC and RC DNA, respectively. The specificities and sensitivities of real-time PCR using two primer/probe sets and the applicability of the real-time PCR to powder mixtures, which involved mixtures of powdered CPC and powdered RC in diverse ratios, were evaluated. Real-time PCR using C-VIC and R-FAM primer/probe sets specifically and sensitively amplified both CPC and RC DNA. Furthermore, real-time RCR sensitively detected RC DNA in the powder mixtures of CPC and RC. These results indicate that this real-time PCR method using two primer/probe sets is sufficiently applicable for the detection of contaminant RC in CPC.

Key words: Cervi parvum cornu, Rangifer cornu, Real-time PCR

INTRODUCTION

Cervi Parvum Cornu (CPC) is a famous Korean traditional medicine derived from the young antlers of male deer (Kim et al., 2005). CPC, which is velvet antler or Nokyoung, has been used for neurosis, enriching vital energy, strengthening the kidney, and prolonging life to treat thousands of people every year (Kim and Lim, 1999). Only the young antlers derived from the *Cervus* genus of the *Cervinae* subfamily of the *Cervidae* family are defined as CPC in Korean Herbal Pharmacopoeia (KFDA, 2007). The ash content of CPC is the most important parameter that determines CPC quality (Cho et al., 2001). Meanwhile, Rangifer Cornu (RC) is the antler derived from the *Rangifer* genus of the *Odocoileinae* sub-family of the *Cervidae* family (Schaefer and Mahoney, 2001). The *Rangifer tarandus* is the only species of the *Rangifer* genus (Shah et al., 2008). Both males and females *Rangifer* genus have antlers, while only males of the *Cervus* genus have antlers (Shah et al., 2008). The RC has not been considered as drug material because the associated ash content does not meet the specified minimum.

There are apparent distinctions in appearance between the *Cervus* and *Rangifer* genus. However, the slice forms of these antlers exhibit no discernible difference (Fig. 1A). Therefore, the appearance of slice RC demonstrates characteristics of being a CPC replica. Furthermore, detection of any contaminant RC in CPC is particularly difficult because CPC is distributed in slice form. Accordingly, the potential confusion between CPC and RC has been a significant issue in CPC distribution. Recently, the diverse effects of CPC were scientifically proven, which include anti-inflammatory, anti-whiplash, anti-stress, anti-aging, and anti-bone resorption activity (Kim and Lim, 1999; Kim et al., 1999, 2005, 2008). Therefore, CPC is assumed to have novel factors that enhance biological performance. However, there is no method to detect contaminating

Correspondence to: Young Hun Shim, National Institute of Food and Drug Safety Evaluation, Cheonwon 363-951, Korea
Tel: 82-43-719-4162, Fax: 82-43-719-4150
E-mail: justman@korea.kr

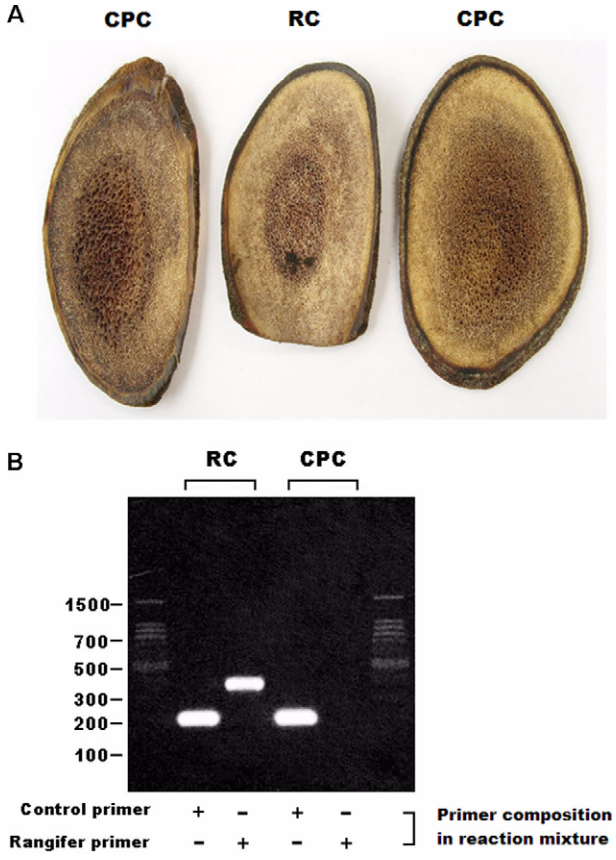

Fig. 1. Identification of CPC and RC. (**A**) The appearance of sliced CPC and sliced RC and (**B**) the identification by traditional PCR. Traditional PCR was performed as described in the Materials and Methods section. (**B**) is representative of identification with traditional PCR, which tested 50 CPC samples and 6 RC samples (Table I).

RC in CPC or to definitely distinguish CPC and RC, despite the increasing focus on the study of CPC.

Real-time polymerase chain reaction (PCR) offers rapid and quantitative analysis for detection of target material (He et al., 2007). There are two kinds of real-time PCR methods: the Taqman probe system and the SYBR Green dye system. The SYBR Green dye system has advantages, such as the fact that a specific probe is not required, and therefore, reduction of the cost of analysis is possible (Maeda et al., 2003). However, multiplex PCR is impossible because amplification is not based on a particular sequence of target gene. The Taqman probe system is prepared on the basis of a specific sequence of target gene. Therefore, only particular amplification products can be detected in the course of PCR analysis, which results in highly effective data collection (Cao et al., 2007; Chapela et al., 2010). In addition, individual probes with different fluorescent colors can be constructed, which enables multiplex PCR analysis with the use of several probes simultaneously (Wittwer et al., 2001; Hohne et al., 2002; Cao et al., 2007; Chapela et al., 2010; Herrero et al., 2010).

In this study, we utilized multiplex real-time PCR based on the Taqman probe system and designed C-VIC and R-FAM primer/probe sets to distinctly amplify CPC and RC DNA to develop a sensitive method to detect contaminating RC in CPC. The specificity, sensitivity, and application of this real-time PCR method to the CPC and RC powder mixture were evaluated.

MATERIALS AND METHODS

Materials

In this study, CPC samples from New Zealand, China, and Russia and RC samples from Canada were used (Table I). To confirm the origin of these CPC and RC samples, analysis certificates for samples issued by the regulatory authority of the exporting country were carefully examined. The CPCs were confirmed as *Cervus elaphus, Cervus nippon* and *Cervus* sp., and RCs were derived from *Rangifer tarandus*. *Cervus* sp. refers to the *Cervus* species that were not described in detail on the certificates. In this study, 50 CPC and

Table I. Identification of RC and CPC by traditional PCR and real-time PCR. A total of 50 samples of CPC and six samples of RC were analyzed with each method.

Samples	Species	n[a]	Country	PCR[b]		Real-time PCR[c]	
				Control primer	Rangifer primer	C-VIC	R-FAM
RC	Rangifer tarandus	6	Canada	positive	positive	negative	positive
CPC	*Cervus elaphus*	31	New Zealand	positive	negative	positive	negative
	Cervus nippon	9	China	positive	negative	positive	negative
	Cervus sp.[d]	10	Russia / China	positive	negative	positive	negative

[a]Sample number
[b]Traditional PCR was performed to identify the origin prior to real-time PCR, and the representative results are presented in Fig. 1B.
[c]Representative results are presented in Fig. 3.
[d]The detailed species was not described in the certificate.

six RC samples were tested for the evaluation of real-time PCR specificity. The origin of CPC and RC samples were primarily confirmed by the expert and assessed again by traditional PCR analysis, as described below (Fig. 1B and Table I).

Extraction of DNA

CPC and RC samples were reduced to powder using a sterilized mortar and pestle for efficient DNA isolation. For extraction of total DNA, 100 mg of the powder was used. The reaction was performed at 60°C for 1 h after addition of 700 μL of CTAB solution (50 mM Tris·HCl, 0.7 M NaCl, 50 mM EDTA, and 140 mM β-mercaptoethanol) and 20 μL of proteinase K solution (not less than 600 mAU/mL; Sigma Co.) to the powder. Total DNA from each sample was extracted using the previously described phenol/chloroform/isoamyl alcohol (25:24:1) method with an ethanol wash (Kang and Yang, 2004; Chakraborty et al., 2006).

Identification of CPC and RC by traditional PCR

Traditional PCR was performed to reconfirm the origins of CPC and RC samples. The control primer amplifies a common DNA sequence in mammals, and the *Rangifer* primer amplifies a RC-specific DNA sequence, which is residues 346 - 731 of the *Rangifer tarandus* mitochondrial D-loop (Fig. 2B). In this study, two kinds of control primers were used for the traditional PCR. The control primer-1 and 2 were used in Fig. 1B and Fig. 5A, respectively. The detailed sequence of the control primers-1 is as follows: sense primer, 5'-CAG CCA CCG CGG TCA TAC-3'; antisense primer, 5'-GCA TAG TGG GGT ATC TAA TCC CA-3'. The detailed sequence of the control primer-2 is as follow: sense primer, 5'-TTT CAT GTT TCC TTG CGG TAC-3'; antisense primer, 5'-AAA GCA CGG CAC TGA AGA TGC-3'. The detailed sequence of the Rangifer primers is as follows: sense primer, 5'-CCC CAT GCT TAT AAG CAA GTA CTT GA-3'; antisense primer, 5'-AGC ATC CCC CCA AAA ATT AAA AA-3'.

Design of the primer/probe set for real-time PCR

Mitochondrial D-loop DNA sequences of the *Cervus* genus (*Cervus elaphus, Cervus nippon and Cervus canadensis*) and *Rangifer* genus (*Rangifer tarandus*) were analyzed to design primer/probe sets that specifically react with CPC and RC DNA. The Cervus genusspecific nucleotide sequence was analyzed with

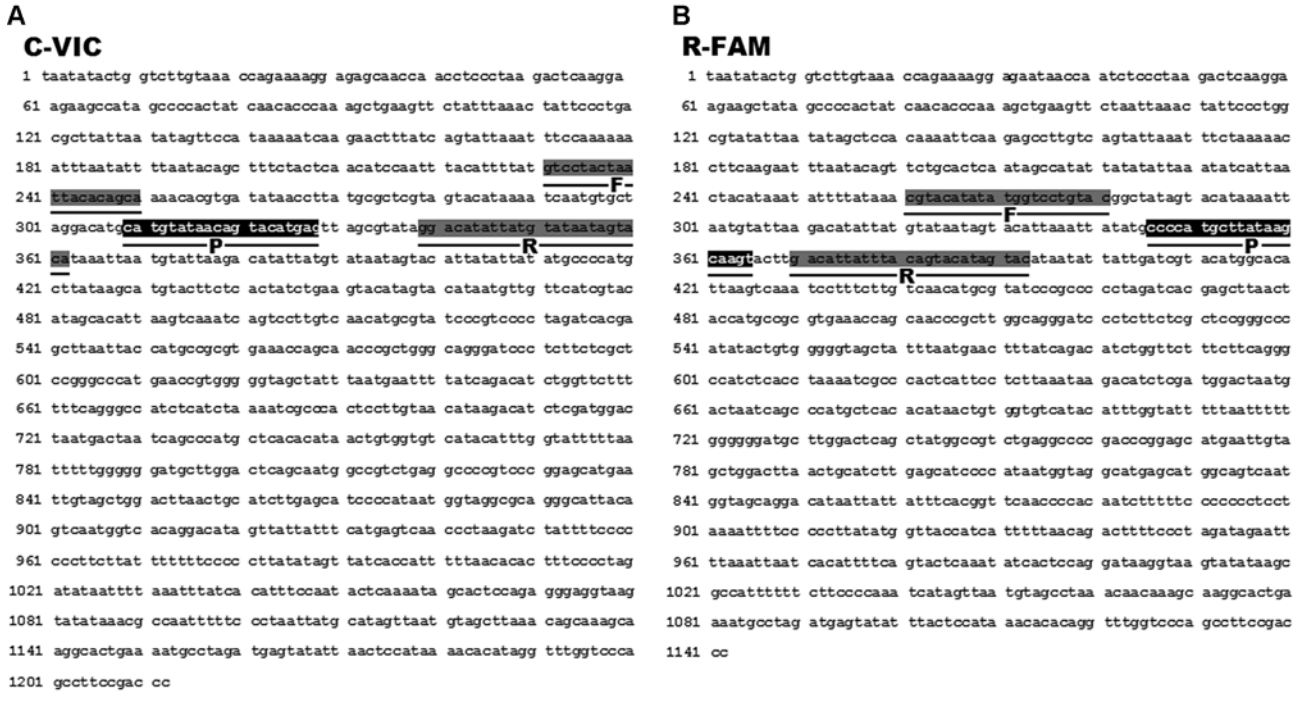

Fig. 2. The binding residues of C-VIC (**A**) and R-FAM (**B**) to mitochondrial D-loops of *Cervus* and *Rangifer* genus. Gene accession numbers for (**A**) and (**B**) are AY970667 (*Cervus canadensis*) and AY970667 (*Rangifer tarandus*), respectively. The gray boxes indicate primer-binding residues, and black boxes indicate probe-binding residue. The letters F, P, and R indicate residues of the forward primer, probe, and reverse primer, respectively. The binding residues of (**A**) are common binding residues found in *Cervus* sp., including *Cervus elaphus*, *Cervus nippon*, and *Cervus canadensis*.

gene accession numbers AF016979 (*Cervus elaphus*), AF016974 (*Cervus nippon*), and AY970666 (*Cervus canadensis*). *Rangifer* genus-specific nucleotide sequence was analyzed with gene accession number AY970667 (*Rangifer tarandus*). The detailed sequence of the primer/probe set for amplification of CPC DNA (C-VIC) is as follows: sense primer, 5'-GTC CTA CTA ATT ACA CAG CA-3'; antisense primer, 5'-TGT ACT ATT ATA CAT AAT ATG TCC-3'; Taqman probe, 5'-CAT GTA TAA CAG TAC ATG AG-3' (Fig. 2A). The detailed sequence of the primer/probe set for amplification of RC DNA (R-FAM) is as follows: sense primer, 5'-CGT ACA TAT ATG GTC CTG TAC-3'; antisense primer, 5'-GTA CTA TGT ACT GTA AAT AAT GTC-3'; Taqman probe, 5'-CCC CAT GCT TAT AAG CAA GT-3' (Fig. 2B). The 5 ends of the probes of C-VIC and R-FAM were labeled with VIC and FAM as the reporter fluorescent dye, respectively, to distinguish CPC and RC fluorescent signals. The 3 ends of the probes were labeled with the fluorescent dye 6-carboxy-tetramethyl-rhodamine (TAMRA) as the quencher. These primer/probe sets were synthesized by Applied Biosystems (Applied Biosystems).

Real-time PCR reaction

For each real-time PCR reaction, 4 μL of primer/probe set (PowerAmp™ Real-time PCR MasterMix M0103, Kogenebiotech Co.), 2 μL of DNA extracted from CPC or RC (detailed concentrations of DNAs were described below), 10 μL of master mix (PowerAmp™ Norkyong ID RN100, Kogenebiotech Co.), and 4 μL of distilled water were added to 0.5 mL of real-time PCR tube. The total master mix volume was 20 μL. The PCR protocol used was the following: initial denaturation, 50°C for 2 min; activation, 95°C for 10 min; denaturation, 95°C for 15 sec; annealing/elongation, 60°C for 1 min. To amplify the target DNA, 40 cycles of PCR (from denaturation to annealing/elongation) were performed. The real-time PCR was performed on the Chromo 4 real-time PCR machine (Bio-Rad). Fluorescent signals of the two probes were monitored throughout the entire amplification using Opticon monitor version 3 software (Bio-Rad).

Evaluation of real-time PCR specificity and sensitivity

For evaluation of the real-time PCR specificity, 50 samples of CPC and six samples of RC were tested (Table I). The final DNA concentration of each sample was adjusted to 2 ng/μL, and CPC and RC DNA were simultaneously reacted with C-VIC or R-FAM. For the evaluation of sensitivity, final DNA concentrations of CPC and RC were 20, 2, 1, 0.5, 0.2, 0.1 and 0.02 ng/μL, respectively. The CPC and RC preparations were simultaneously amplified by the real-time PCR using C-VIC or R-FAM.

Evaluation of real-time PCR applicability

A total of 30 mg of powder was used to prepare the RC and CPC powder mixture. The mixture ratios (RC : CPC) were 50 : 50, 40 : 60, 30 : 70, 23 : 77, 17 : 83, 10 : 90, and 3 : 97. For example, 0.9 g of powdered RC and 29.1 g of powdered CPC were mixed to prepare the 3 : 97 ratio. The prepared mixtures of DNA were extracted as described above, and final DNA concentrations were adjusted to 5 ng/μL. C-VIC and R-FAM were simultaneously reacted with the RC and CPC DNA mixtures for the real-time PCR.

RESULTS

Identification of CPC and RC origins

The origin of CPC and RC samples were confirmed by an expert and corroborated using traditional PCR (Table 1). Fig. 1B is representative of the confirmation using traditional PCR. The *Rangifer* primer reacted specifically with RC, while the control primer reacted with both RC and CPC (Fig. 1B). This result indicates that the origin of RC is apparently different from CPC. Meanwhile, amplification by the control primer indicates that the extraction conditions for CPC and RC produced quality DNA for real-time PCR. We selected the qualified CPC and RC (50 samples of CPC and 6 samples of RC), and then these samples were used to evaluate real-time PCR specificity.

Specificity and sensitivity of real-time PCR

A total of 50 CPC and six RC samples were used in the real-time PCR specificity test (Table I). Fig. 3 is representative of the specificity test. As shown in Fig. 3A and B, CPC DNAs were specifically amplified by real-time PCR using C-VIC, and RC DNA was specifically amplified using R-FAM. In addition, non-specific amplified signals were not observed with real-time PCR. In the sensitivity analysis, CPC DNA was distinctly amplified using C-VIC when the final concentration of CPC DNA was greater than 0.2 ng/μL (Fig. 4A). RC DNA was distinctly amplified by the reaction using R-FAM when the final concentration was greater than 0.02 ng/μL (Fig. 4B). Approximately 30 ng of DNA could be obtained when 30 mg powdered CPC or RC was used for DNA isolation. Therefore, the sensitivity of the real-time PCR was sufficient. Consequently, real-time PCR using C-VIC and R-FAM exhibited superior specificity and sensitivity for CPC and RC, respectively.

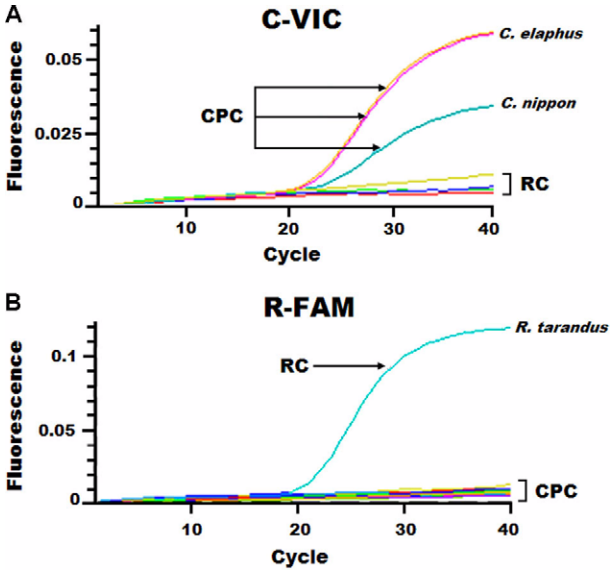

Fig. 3. Evaluation of real-time PCR specificity using C-VIC and R-FAM primer/probe sets for CPC and RC. These analyses included two samples of *Cervus elaphus*, one sample of *Cervus nippon*, and six samples of RC to evaluate the C-VIC specificity for CPC (**A**), and the six samples of CPC included three samples of *Cervus elaphus*, two samples of *Cervus nippon*, and one sample of *Cervus* sp. with one sample of RC to investigate R-FAM specificity for RC (**B**). Final DNA concentrations from CPC and RC were adjusted to 2 ng/μL. These results are representatives of the specificity analysis that tested 50 CPC samples and 6 RC samples (Table I).

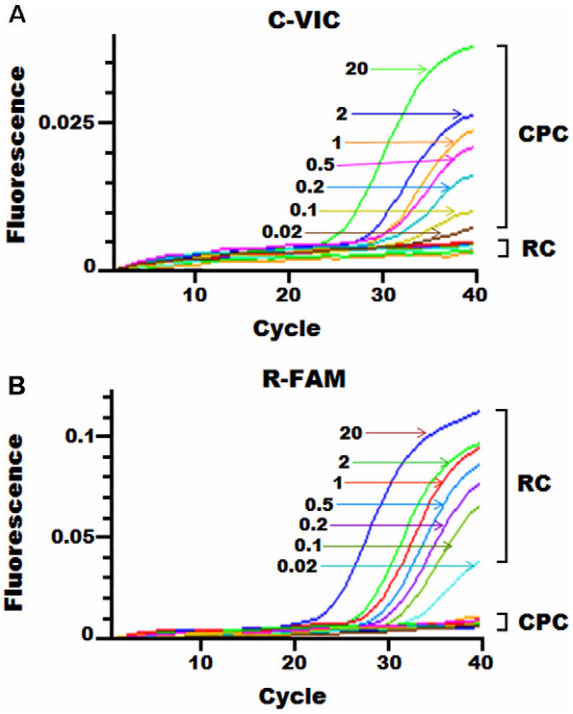

Fig. 4. Evaluation of real-time PCR sensitivity using C-VIC and R-FAM primer/probe sets for CPC and RC. Final DNA concentrations isolated from CPC and RC were adjusted to 20, 2, 1, 0.5, 0.2, 0.1, and 0.02 ng/μL, respectively. Real-time PCR using C-VIC (**A**) and R-FAM (**B**) were performed with DNA preparations of CPC and RC, respectively.

Specificity of real-time PCR for wild market CPC

Wild market CPCs were purchased for this study. RCs originated in Canada were used as a control. Prior to real-time PCR, CPC and RC origins were identified by experts and then qualified by traditional PCR (Fig. 5A). As shown in Fig. 5A, The *Rangifer* primer reacted specifically with RC DNAs (S2 and S6), while the control primer reacted with both RC (S2 and S6) and CPC DNAs (S1, S3, S4 and S5). The wild market CPC and RC were specifically amplified by real-time PCR using C-VIC and R-FAM primer/probe sets, respectively (Fig. 5B and C). These results indicate that real-time PCR is applicable in the identification of wild market samples.

The applicability of real-time PCR

Powdered CPC and RC were mixed in diverse ratios to evaluate the applicability of real-time PCR. As shown in Fig. 6, CPC and RC DNA in all prepared mixtures were successfully amplified. The fluorescence values for amplified RC DNA were apparently higher than those of CPC DNA, such that the amplification signals for CPC and RC did not overlap. Furthermore, real-time PCR successfully detected various ratios of RC in CPC. In particular, the reduced RC signal was not observed even though the contamination ratio of RC was 3% (see blank box). Therefore, real-time PCR exhibited superior sensitivity in amplification of RC DNA in the powder mixture. These results indicate that multiplex real-time PCR using C-VIC and R-FAM has sufficient applicability for detection of contaminant RC in CPC.

DISCUSSION

In the study of ethnopharmacological sources, contaminants with similar appearance to the original source hinder the accurate evaluation of the original sample. However, there has been little research on the detection of potential contaminants. In this study, we developed a sensitive real-time PCR method to detect contaminating RC in CPC and confirmed the applicability of this method. Unlike traditional PCR methods, real-time PCR allows for detection of DNA amplification during the reaction (Cao et al., 2007). Furthermore, real-time PCR did not require post-PCR procedures, such as agarose gel electrophoresis (Jebbink et

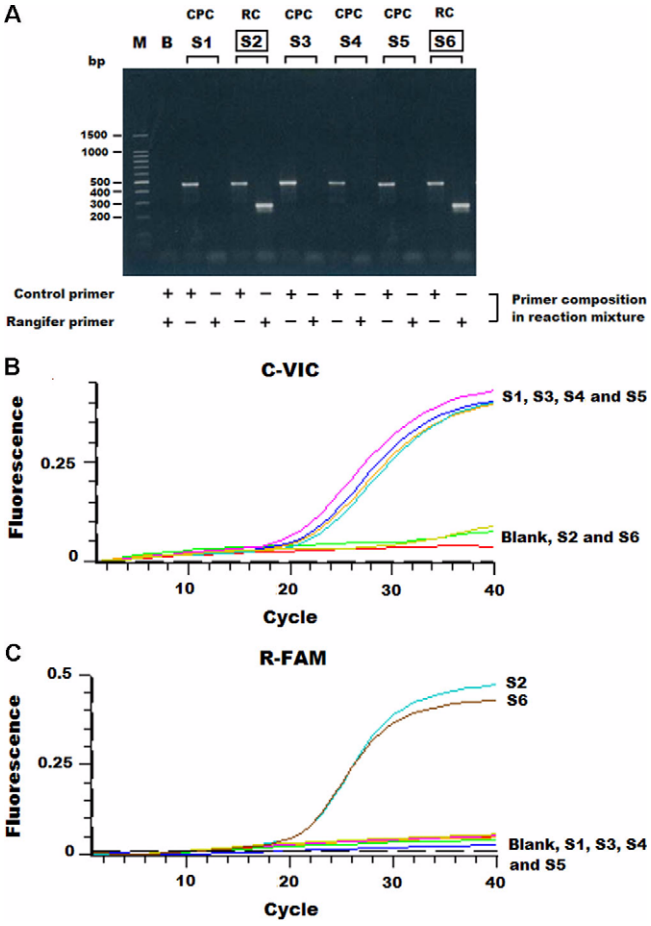

Fig. 5. Evaluation of real-time PCR specificity for CPC obtained from wild market. The results of traditional PCR using control and rangifer primers are presented in (**A**). M and B indicate the 100 bp ladder and blank, which contained a reaction mixture with primers. S1, S3, S4 and S5 are CPC samples obtained from wild market. S2 and S6 (blank boxes) are RC samples originated in Canada. The results of real-time PCR using C-VIC and R-FAM are presented in (**B**) and (**C**), respectively. Final DNA concentrations from CPC and RC were adjusted to 2 ng/μL.

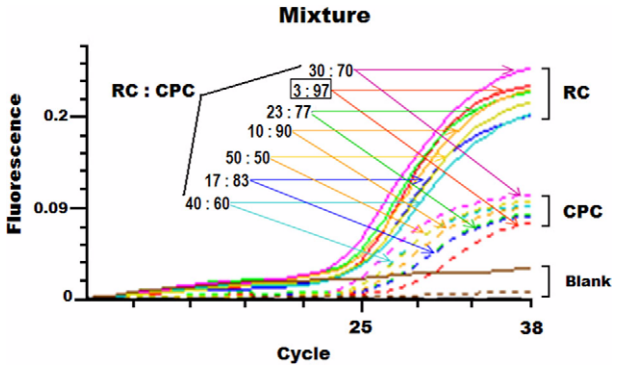

Fig. 6. Evaluation of the applicability of this real-time PCR method. CPC and RC were powdered to prepare the mixture. The ratio of RC and CPC at 50: 50, 40: 60, 30: 70, 23: 77, 17: 83, 10: 90, and 3: 97, and the total amount of powdered mixture was adjusted to 30 mg. DNA was isolated from these preparations. For real-time PCR, C-VIC and R-FAM reacted simultaneously with each DNA preparation, and the final DNA concentrations were adjusted to 5 ng/μL.

al., 2003; Valasek and Repa, 2005). Therefore, rapid and simple detection of the target gene is possible. Multiplex real-time PCR, which was introduced in this study, has the advantages of minimizing time and cost because the simultaneous amplification of more than one target sequence is performed in a single reaction (Wittwer et al., 2001; Valasek and Repa, 2005). Our real-time PCR method demonstrated superior specificity and sensitivity for both CPC and RC, and the method also exhibited sensitive signals in amplification of contaminating RC DNA in the powdered mixture of CPC and RC. However, the purity and concentration of DNA isolated from CPC and RC is of great consideration for the successful detection of contaminating RC DNA. We confirmed that quality results could be obtained when the highly purified DNA exhibited values of 1.7 - 1.8 for the A260/A280 ratio and the final DNA concentration in the reaction mixture was 2 - 7 ng/μL. In addition, long-term storage of CPC and RC in a deep-freezer resulted in a drastic decrease in amplification signals of the target DNA sequences.

The mitochondrial DNA sequence has been used to investigate the phylogeny of many species (Xie et al., 2006). The mitochondrial D-loop region is particularly useful for the investigation of intraspecific genetic differentiation (Sbisa et al., 1997; Sakai et al., 2003; Xie et al., 2006). The mitochondrial D-loop region was selected to design primer/probe sets that would specifically react with CPC and RC DNA because both the *Cervus* and *Rangifer* genus are from the *Cervidae* family. Our results obtained using real-time PCR demonstrated that the amplification of *Cervus*- and *Rangifer*-specific D-loop residues are useful to distinguish CPC and RC, which are separated by intraspecific genetic differentiation.

The identification of sources used in traditional medicine has largely depended on visual evaluation. Such evaluation is based on comparing visual parameters and microscopic properties with standard reference material (Shim et al., 2005). However, these criteria are subjective, and substitutes or adulterants may closely imitate the genuine material (Shim et al., 2005). Contaminations by substitutes and adulterants should be sensitively examined to accurately identify genuine material. Recently, Shin et al. demonstrated that the mitochondrial D-loop region is effective for discrimination of CPC and RC using the restriction fragment length polymorphism (RFLP) (Shin et al.,

2008), which indicates that the mitochondrial D-loop region of the deer antler has a genus-specific DNA sequence. The real-time PCR method employed herein has several advantages, such as being a high-throughput and simple procedure, while the RFLP method is tedious, laborious, and requires optimization of reaction conditions (Wilson et al., 2005). Meanwhile, real-time PCR has been used primarily for the detection of pathogens in food, analysis of gene expression, and diagnosis by detection of pathogen-specific gene (Malorny et al., 2004; Nitsche et al., 2004; Ahmed et al., 2008). However, there has been no previous attempt to utilize real-time PCR to detect contaminants in traditional medicine.

In this study, we demonstrated that a real-time PCR method could be utilized to detect contaminants in traditional medicine sources. Furthermore, our real-time PCR method for detection of contaminating RC in CPC should facilitate the further study of CPC.

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