

Original Articles

종 특이 PCR과 PCR-RFLP를 이용한 웅담과 기타 담류의 감별 방법

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Identification of *Fel ursi* and Cattle and Pig Bile Juices by species-specific PCR and PCR-RFLP

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ABSTRACT

Objective : This study developed species-specific PCR and PCR-RFLP to detect the adulteration of *Fel ursi* products with cattle and pig bile juices.

Methods : All the primers for PCR and PCR-RFLP in this study were designed based on nucleotide sequences of cytochrome b genes in the mitochondria.

Results : The species-specific PCR amplified a DNA fragment of 214, 214, 295, and 167 bp from *Fel ursi* product, bear fur, cattle bile juice, and pig bile juice, respectively. The survey using the species-specific PCR indicated that some of commercial *Fel ursi* products were adulterated with cattle and pig bile juices. PCR-RFLP using the restriction endonucleases, *HaeIII* and *HinfI* enabled differentiation among *Fel ursi* product, cattle bile juice, and pig bile juice. Bear furs from two animals showed variations in PCR-RFLP patterns with *HaeIII*.

Discussion : The detection methods of the species-specific PCR and PCR-RFLP could be useful in eliminating adulterated *Fel ursi* products from the market.

Running title : Identification of *Fel ursi* and othe bile juices by PCR.

I. Introduction

Fel ursi, bear bile medicine used for oriental therapy is prepared by concentrating and drying bile juice which is obtained from Asiatic black bear, *Ursus thibetanus* and Brown bear, *Ursus arctos*, which are large mammals of the family Ursidae. The bile juice is often collected from gall bladder by cutting it open or inserting a straw through its canal [1].

The major compositions of *Fel ursi* consist of

alkaline salts of bile acid, cholesterol, and bile pigments. Tauroursodesoxycholic acid is a major component which accounts for 20% of dried bile juice from Asiatic brown bear and can be converted into taurine and ursodesoxycholic acid by hydrolysis. Its other minor components are chenodesoxycholic acid and cholic acid. Ursodesoxycholic acid, a stereoisomer of chenodesoxycholic acid, is a characteristic component of *Fel ursi* and thus its pres-

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ence has been reported to enable differentiation between *Fel ursi* and bile juices of other animals [2].

Many animal and clinical studies showed that *Fel ursi* was effective in preventing and treating bile-related hepatocirrhosis, chronic hepatitis, hepatitis-originated hepatocirrhosis, chronic B-type hepatitis, etc [3-6]. Ursodeoxycholic acid, the major components of *Fel ursi*, was reported to have beneficial effects of liver disease prevention, fever alleviation, detoxification, agitation appeasement, anti-inflammation, antiseptic, blood pressure regulation, anti-oxidation, and anti-stress [7]. *Fel ursi* is not usually used for general therapy, but is one of the essential precious oriental medicines. Especially, the pharmaceutical acupuncture therapy has been introducing *Fel ursi*, ox bezoar, and musk into clinical application [8-9] and their clinical usage frequencies have become significantly higher.

A variety of specific and sensitive polymerase chain reaction (PCR)-based methods have been introduced for species identification [10-12]. Species differentiation is achieved either by the PCR using species-specific primers derived from the gene regions of inter-species sequence diversity [11, 13-15] or by the PCR-restriction fragment length polymorphism (RFLP) in which DNA amplified with consensus PCR is digested with restriction endonucleases [16-18]. The PCR-based methods often targeted cytochrome b gene in mitochondrial DNA [11, 15-18] whose sequencing data is widely available in the genome data bases and which is present in the cell in a much higher copy number than nuclear DNA. The mitochondrial genome sequences of various bear species also have been reported [19, 20].

The international agreement, such as “The Convention on International Trade in Endangered Species of Wild Fauna and Flora” (CITES) are being enforced throughout the world to protect endangered wild animals including bears. Member countries of the agreement are obliged to establish-

ing and enforcing laws to prevent circulation and trade of specimens from the animals. Thus it is extremely difficult to obtain bear bile juice, so adulterated *Fel ursi* products are commercially distributed. However, the regulatory programs to prevent distribution of the adulterated products are not currently established.

This study developed the species-specific PCR and PCR-RFLP in order to detect adulteration of *Fel ursi* products with cattle and pig bile juices.

II. Materials and Methods

1. *Fel ursi* products, bile juices, and bear furs

Fel ursi products and bear bile juices made in China, Mongol, Russia, and Korea were purchased in China and Korea. The cattle and pig bile juices were obtained from a local abattoir in Wonju, Korea. A couple of bear furs were provided by a bear farm in Chungju, Korea.

2. Isolation of genomic DNA

Genomic DNA was isolated using a modified procedure of Wizard Genomic DNA Purification Kit (Promega, USA). Ten mg of the samples were dissolved in 90 μ l of distilled water and 600 μ l of Nucleic Lysis Solution was added and mixed. Three μ l of RNAase Solution was added. The mixture was stirred four to five times during incubation at 37°C for 15 min and then cooled to room temperature for 5 min. 200 μ l of Protein Precipitation Solution was added. The mixture was vortexed for 20 sec, cooled in ice for 5 min, and then centrifuged at 13,000 \times g for 4 min. The supernatant was added into a tube containing 600 μ l of isopropanol. The mixture was vortexed briefly and then centrifuged at 13,000 \times g for 1 min in order to precipitate DNA. The precipitated DNA was washed with 600 μ l of 70% ethanol and then dried. 100 μ l of DNA Rehydrating Solution was added and stored at 4°C for 1 day. After DNA

was dissolved, it was stored at -20°C.

3. Species-specific PCR

The nucleotide sequences of the primer pairs of UT, BT, and SS in Table 1 were determined by using Beacon Designer (Premier Biosoft, USA) for the species-specific PCR based on the nucleotide sequences provided by National Center of Biotechnology Information (NCBI) of cytochrome B genes of *Ursus thibetanus* (AY522429), *Bos taurus* (DQ186273), and *Sus scrofa* (AY534296), respectively. DNA was amplified using Hotstart PCR Premix (Bioneer, Korea) which was added with 0.5 μ M of primer pair and 1 μ l of DNA. The total volume of the reaction mixture was adjusted to 20 μ l by adding distilled water. DNA was amplified in DNA Cyclor (Bio-Rad, USA), which was programmed for initial denaturation at 94°C for 15min, 40 cycles of denaturation at 94°C for 20 sec, annealing at 58°C for 30 sec, and extension at 72°C for 40 sec, and final extension at 72°C for 10 min. The amplified DNA was analyzed on agarose gel electrophoresis.

4. PCR-RFLP analysis

The primer pairs of CYT1 and CYT2 for the PCR-RFLP analysis in Table 1 were designed to have homologous nucleotide sequences of cytochrome B genes for bear products and cattle and pig bile juices, respectively. 0.5 μ M of primer pair and 1 μ l of DNA were added into PCR Premix (Bioneer, Korea) and total volume was made up to 20 μ l by adding distilled water. A DNA Cyclor (Bio-Rad, USA) was programmed for initial denaturation at 94°C for 5 min, 45 cycles of denaturation at 94°C for 20 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min, and final extension at 72°C for 5 min. The amplified DNA was digested with 1 unit of *Hae*III or *Hinf*I at 37°C for 6 hr and subjected to agarose gel electrophoresis.

5. Agarose gel electrophoresis

The DNA samples from species-specific PCR and PCR-RFLP were electrophoresed in 2.5% and 4.0% agarose gel, respectively. The electrophoresis was run in Tris-acetate buffer at 50 V. The gel was stained in 0.5 μ g/ml ethidium bromide and photographed using ultraviolet light illumination. DNA markers were 100bp DNA ladder and 25/50bp DNA ladder (Bioneer, Korea).

III. Results

1. Specificity of the species-specific PCR

The species-specific PCR for detection of adulteration of *Fel ursi* products with cattle and pig bile juices was designed by using the primer pairs of UT, BT, and SS in Table 1 and Hotstart PCR Premix (Bioneer, Korea) described in materials and methods. The nucleotide sequences of the primer pairs derived from the parts of cytochrome B genes heterologous between bear, cattle, and pig, respectively, were determined to differentiate among the three animals and increase sensitivity in detecting DNA.

Fig. 1 showed that the primer pairs, UT, BT, and SS were specific to the *Fel ursi* product, cattle bile juice, and pig bile juice, respectively. DNA fragments of 214, 295, and 167 bp were expected to be amplified from *Fel ursi* product, cattle bile juice, and pig bile juice, respectively. The sizes of the amplified DNA products were similar as those expected from the nucleotide sequences of the cytochrome B genes of each animals (Table 1). Amplification of DNA from the *Fel ursi* product was relatively weaker than those from the cattle and pig bile juices (Fig. 1), which suggested that genomic DNA in the *Fel ursi* product should have been degraded significantly during drying and storage. However, the product was shown to be authentic without adulteration with cattle and pig bile juice.

2. Detection of adulterated *Fel ursi* products by the species-specific PCR

Commercial *Fel ursi* products and bear bile juices made in China, Russia, Mongol, and Korea were examined to determine their adulteration. Some of the *Fel ursi* products and bear bile juices were shown to be adulterated with cattle and pig bile juices in Fig. 2. The bile juice liquid in lane B obtained directly from a bear farm did not show any DNA amplification, which suggested that DNA in the bile juice had been degraded completely during storage. The freeze-dried bear bile juice in lane C and the *Fel ursi* product in lane E showed DNA amplification with both UT and SS, indicating adulteration with pig bile juice. The *Fel ursi* products in lanes G and H showed strong DNA amplification with BT but weak DNA amplification with UT and SS, suggesting that the major component of the products might be cattle bile juices. The *Fel ursi* products in lanes D, F, I, and J showed DNA amplification only with UT, which suggested no adulteration.

3. Application of the species-specific PCR to bear furs

The genomic DNA isolated from bear fur was also used to verify the species-specific PCR and examine the effects of the annealing temperatures in the PCR (Fig. 3). The PCR using the annealing temperature of 58°C amplified the DNA fragment of 214 bp only with UT, but the PCR using the annealing temperature of 54°C amplified nonspecific DNA fragments with both BT and SS as well as the specific DNA fragment with UT. These results suggested that the species-specific PCR using only the primer pair, UT, could detect genomic DNA from bear furs and the annealing temperature was one of the important variables for specific DNA amplification. The annealing temperature of 58°C was used for *Fel ursi* products and bile juices in the species-specific PCR in this study.

4. PCR-RFLP analysis

The PCR-RFLP analysis was also designed to differentiate among *Fel ursi* products, cattle bile juice, and pig bile juices. A DNA fragment of 360 bp was amplified from genomic DNA isolated from all the samples of *Fel ursi* product, bear furs, and cattle and pig bile juices in the PCR step of the PCR-RFLP analysis using the primer pairs of either CYT1 for bear or CYT2 for cattle and pig, as in Fig. 4 and Fig. 5. The size of the DNA expected from the nucleotide sequence of cytochrome b gene was 359 bp (Table 1). However, a nonspecific DNA fragment of 50 bp was also amplified from *Fel ursi* products and bear fur 1 (Fig. 4 lane B, Fig. 5 lane B).

The DNA amplified from *Fel ursi* product and bear furs were digested with *Hae*III and *Hin*FI for the PCR-RFLP analysis. Digestion with *Hae*III of the DNA obtained from *Fel ursi* product and bear furs resulted in two DNA fragments of 285 and 75 bp (Fig. 4 lane E, Fig. 5, lanes F, G). The other smaller DNA fragments seemed to be derived from the nonspecific DNA (Fig. 4 lane E). Digestion with *Hin*FI of the DNA from *Fel ursi* and bear fur 1 and resulted in two DNA fragments of 300 and 60 bp (Fig. 4 lane H, Fig. 5 lane K), but the DNA obtained from bear fur 2 was not cut by *Hin*FI (Fig. 5 lane L).

The DNA amplified from cattle bile juice was cut by *Hae*III and *Hin*FI into a couple of DNA fragments of 285 and 75 bp (Fig. 4 lane F, Fig. 5 lane H) and three DNA fragments of 200, 115, and 45 bp (Fig. 4 lane I, Fig. 5 lane M), respectively, which were similar with the sizes expected from the DNA sequences (Table 2).

The DNA amplified from pig bile juice was digested by *Hae*III into four DNA fragments of 155, 130, 105, and 75 bp (Fig. 4 lane G, Fig. 5 lane I). The cumulative size of the resulted DNA fragments was 565 bp which was longer than the intact DNA size, 359 bp. The DNA fragments of 153, 132, and 75 bp were expected from the nucleotide

sequence of pig cytochrome b gene (Table 2). The amplified DNA was not cut by *HinfI* (Fig. 4 lane J, Fig. 5 lane N), as expected from the nucleotide sequence (Table 2).

IV. Discussion

Trade of fake products of high-priced oriental medicines, such as mountain ginseng, young antler, and *Fel ursi*, has emerged as social and legal problems, for which the oriental medical associations have been asked to set a purgation system. On the other hand, laws for protecting endangered wild animal and prohibiting wild animal farming have been established widely throughout the world. The authentic wild animal products become rare and thus the fake adulterated products are illegally imported and circulated in Korea. So, it is necessary to secure accurate detection methods to evaluate authenticity of *Fel ursi* products and thus to prevent their adulteration with cattle and pig bile juices.

Various molecular techniques have been used for species identification, genetical transformation, hereditary disease diagnosis, disease prediction, etc [21]. The PCR-based analyses enabled breed classification within the same species as well as species identification [10-18]. Thus, species-specific PCR and PCR-RFLP which discern the nucleotide sequences of bear from those of cattle and pig may detect adulteration of *Fel ursi* products with cattle and pig bile juices and prevent distribution of fake adulterated *Fel ursi* products.

The primer pairs, UT, BT, and SS, were designed for the species-specific PCR to specifically detect genomic DNAs of bear, cattle, and pig, respectively, by using the regions of cytochrome b genes the nucleotide sequences of which are diverse among the animals. Hot-start PCR which prevented non-specific annealing of primers to template DNA in the initial stage of the PCR was adopted to increase

specificity of the PCR. The sizes of amplified DNAs were intended to be 100~300 bp to increase sensitivity of the PCR [22].

The amounts of DNA amplified from the *Fel ursi* products were usually less than those from the cattle and pig bile juices in the species-specific PCR in Fig. 1. DNA in the *Fel ursi* products seemed to have been degraded during drying and storage. Since the cattle and pig bile juices used in this study were fresh and frozen-stored, DNA in the bile juices should be intact. However, since PCR was so sensitive as to detect residual amount of DNA, it could detect adulteration of some *Fel ursi* products with cattle and pig bile juices in this study (Fig. 2).

The PCR-RFLP analysis was designed in order to secure DNA markers which enabled detection of adulteration of *Fel ursi* with cattle and pig bile juices following a modified procedure reported by Partis et al. [17]. The nucleotide sequences of the primers, CYT1 and CYT2, were modified to fit closely with the genes of the animals in this study and thus increase amplification efficiency of the PCR step in the PCR-RFLP analysis. The DNA amplified from all the genomic DNA preparations in this study had the size of 360 bp as expected from the nucleotide sequences of cytochrome b gene. However, the PCR of some genomic DNA preparations resulted in smaller DNA fragments which seemed to be amplified nonspecifically.

The DNA fragment sizes in the PCR-RFLP analysis obtained from all the genomic DNA preparations except those from pig bile juices and bear fur 2 in this study were similar as those expected from the nucleotide sequences of cytochrome b genes of the animals. However, the number and cumulative length of DNA fragments obtained by digestion with *HaeIII* of the amplified DNA from pig bile juices in the PCR-RFLP analysis were 4 and 455 bp, respectively which were greater than those expected from the nucleotide sequence (Fig. 4 and Fig. 5, Table 2). The DNA fragments of 155, 130, and 75 bp after digestion with *HaeIII* of the ampli-

fied DNA from pig bile juices in the PCR-RFLP analysis in this study fitted with the fragments of 153, 132, 74 bp expected from the nucleotide sequence. The extra DNA fragment of 105 bp seemed to be originated from other different pig DNA sequences. Partis et al. [17] suggested that the simultaneous amplification of different DNA sequence should result in extra DNA fragments in PCR-RFLP.

The PCR-RFLP pattern of bear fur 2 obtained after digestion with *HinfI* of bear fur 2 was different from those of *Fel ursi* product and bear fur 1 suggested presence of intra-species variation in cytochrome b gene sequences. The intra-species variations of the cytochrome b gene of other animals were reported before [16, 17].

The species-specific PCR and PCR-RFLP analysis in this study enabled specific detection of adulteration of *Fel ursi* products with cattle and pig bile juices. The species-specific PCR was simple straightforward one-step procedure which made advantage of the primers derived from inter-species variable regions of cytochrome b gene and high specificity of hot-start PCR. However, The PCR-RFLP analysis could be used to confirm the results of the species-specific PCR. Even though occurrence of the intra-species variations and anomalous amplification of different sequences in the PCR-RFLP should be investigated further, the distinctive differences in the DNA fragment patterns among bear, cattle, and pig made the PCR-RFLP analysis useful in discriminating among *Fel ursi*, cattle bile juice, and pig bile juice.

V. References

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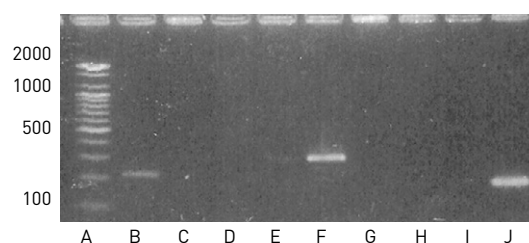


Fig. 1. Species-specific PCR of *Fel ursi* product, cattle bile juice, and pig bile juice. A: 100bp DNA marker, B, C, D; *Fel ursi* product, E, F, G; cattle bile juice, H, I, J; pig bile juice, B, E, H; UT, C, F, I; BT, D, G, J; SS

Table 1. The nucleotide sequences of primer pairs used in this study

Primer pairs	Nucleotide sequences	Amplified DNA (bp)
UT	5'-AGCCACCGCATTT/CATAGGATAT/C-3'	214
	5'-A/GACTGCTGCTAGTGCTAGGATG-3'	
BT	5'-AGGCTCCAACAATCCAACAG-3'	295
	5'-AAGAATTAGGATAGAGAAGGCTAGG-3'	
SS	5'-GAGCTACGGTCATCAAAATCTA-3'	167
	5'-GAGATGTACGGCTGCGAGG-3'	
CYT1	5'-CCA/CTCAAACATT/CTCATCATGATGAAA-3'	359
	5'-GCTCCTCAGAATGATATTTGT/GCCTCA-3'	
CYT2	5'-CCATCAAATATCTCAGCATGATGAAA-3'	359
	5'-GCTCCTCAGAAGGATATTTGGCCTCA-3'	

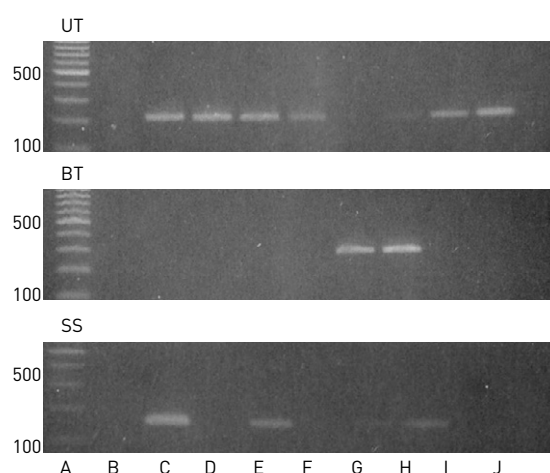


Fig. 2. Species-specific PCR of *Fel ursi* products to detect adulteration with cattle and pig bile juices. A; 100 bp DNA marker, B; bear bile juice. C; freeze-dried bear bile juice, D, E, F, G, H, I, J; *Fel ursi* products

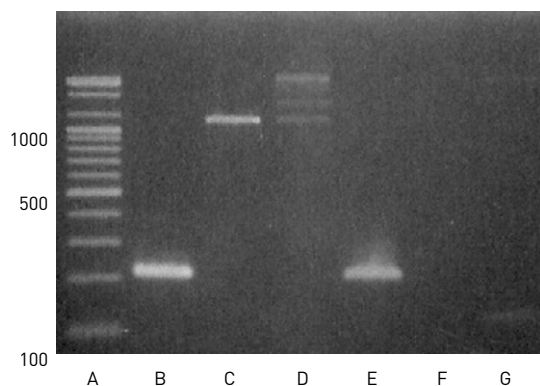


Fig. 3. Species-specific PCR of bear furs using different annealing temperature. A; 100 bp DNA marker, B, E; UT, C, F; BT, D, G; SS, B, C, D; 54°C, E, F, G; 58°C

Table 2. The DNA fragment sizes in base pair inferred from PCR-RFLP in this study and expected from DNA sequences in the data base of NCBI

DNA sources	Hae III		Hinf I	
	PCR-RFLP	DNA sequence	PCR-RFLP	DNA sequence
Bear fur 1	285, 75	285, 74,	295, 65	295, 64
Bear fur 2	285, 75	285, 74	360	295, 64
<i>Fel ursi</i> product	285, 75, 30, 20	285, 74	295, 65	295, 64
Cattle bile juice	285, 75	285, 74	200, 115, 45	196, 117, 46
Pig bile juice	155, 130, 105, 75	153, 132, 74	360	359 (uncut)

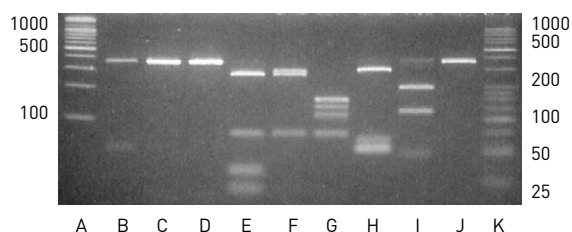


Fig. 4. PCR-RFLP analysis of *Fel ursi* product, cattle bile juice, and pig bile juice. A; 100bp DNA marker, K; 25/50 bp DNA marker, B, C, D; uncut. E, F, G; *Hae* III, H, I, J; *Hinf* I, B, E, H; *Fel ursi* product, C, F, I; cattle bile juice, D, G, J; pig bile juice

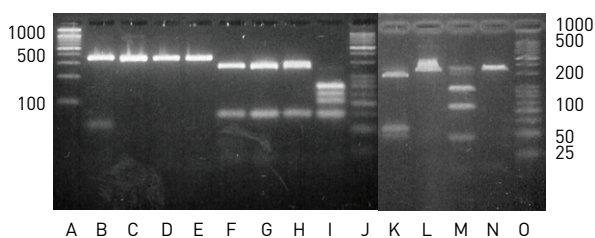


Fig. 5. PCR-RFLP analysis of bear furs, cattle bile juice, and pig bile juice. A; 100bp DNA marker, J, O; 25/50 bp DNA marker, B, C, D, E; uncut. F, G, H, I; *Hae* III. K, L, M, N; *Hinf* I, B, F, K; bear fur 1, C, G, L; bear fur 2, D, H, M; cattle bile juice, E, I, N; pig bile juice