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Full Length Research Paper

Internal transcribed spacer-based identification of *Bupleurum* species used as sources of medicinal herbs

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Bupleuri Radix is a medicinal herb that is widely used in Asia, and several *Bupleurum* species are used as a source of active ingredients for traditional Chinese medicines. Among these species, *Bupleurum longiradiatum* Turcz., is toxic and is not used as a medicinal herb. Because *B. longiradiatum* is morphologically similar to other *Bupleurum* species, it is important to distinguish between them. In other to address this problem, we developed a polymerase chain reaction (PCR) method based on internal transcribed spacer (ITS) nucleotide sequences to discriminate *B. longiradiatum* from *Bupleurum falcatum* L. and *Bupleurum chinense* DC., which are the most frequently used medicinal *Bupleurum* species. The discriminatory power of the method was verified by gel and capillary electrophoresis. Three PCR primers specific to *B. longiradiatum* were developed for use in this assay to enable us reveal the four *B. chinense* haplotypes.

Key words: *Bupleurum longiradiatum*, medicinal herb, molecular identification, internal transcribed spacer.

INTRODUCTION

Bupleuri Radix is a medicinal herb that is used extensively in Korea, China and Japan. It possesses numerous pharmacological activities, including antipyretic, analgesic, immuno-modulatory, anti-inflammatory, antiallergic, anticlotting, antiatherosclerotic, and antitussive effects (Park et al., 2000; Kim and Park, 2001). Its major constituents include triterpenoid glycosides (saikosaponins), essential oils and polysaccharides. The saikosaponins are the principal bioactive components (Hsu et al., 2000; Chiang et al., 2003) and saikosaponin-a and saikosaponin-d are the dominant derivatives (Tian et al., 2009). Bupleuri Radix is known differently in Korea, China and Japan. The Japanese pharmacopoeia states that Bupleuri Radix is derived from *Bupleurum falcatum* L. (Apiaceae) (Ministry of Health, Labour and Welfare, 2006). The Korean Herbal Pharmacopoeia recognizes *B. falcatum* and *B. chinense* DC as Bupleuri Radix (Korea Food and Drug Administration, 2007). The Chinese Pharmacopoeia Commission confirms *B. chinense* and *B. scorzonerifolium* Willd. as Bupleuri Radix (Chinese Pharmacopoeia Commission, 2010).Thirty-six *Bupleurum* species and varieties are commonly used as Bupleuri Radix in various areas (Pan, 1996; Yang et al., 2007). Bupleurotoxin and acetylbupleurotoxin bestow toxic properties upon *B. longiradiatum* Turcz (You et al., 2002). Therefore, China prohibits the use of *B. longiradiatum* as a herbal drug source. *B. longiradiatum* Turcz. can be confused with more than 20 other *Bupleurum* species

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Table 1. *Bupleurum* plant materials.

Scientific name	Collection location	Collection date	Voucher no	Figure 2 Lane#
	Jinan and Korea	2002.5	G039005	1
	Sancheong and Korea	2006.9	G039022	2
B. falcatum L.	Pohang and Korea	2006.10	G039023	3
	Suwon and Korea	2007.7	G039029	4
	Toyama and Japan	2003.12	G039062	5
	Inner Mongolia and China	2004.8	G039012	6
	Jeonju and Korea	2006.6	G039019	7
B. chinense DC.	Sichuan and China	2007.6	G039026	8
	Qinghai and China	2007.7	G039027	9
	Gansu and China	2004.4	G039063	10
	Jeonju and Korea	2002.5	G039038	11
	Jeonju and Korea	2004.6	G039039	12
B. longiradiatum Turcz.	Pyeongchang and Korea	2006.8	G039047	13
	Seongnam and Korea	2007.6	G039048	14
	Seongnam and Korea	2007.6	G039053	15

used as Bupleuri Radix because of similarities in root shapes (Yang et al., 2007; Huang et al., 2009). Processing of medicinal plants leads to changes in their morphology and chemical constituents (Hon et al., 2003; Yang et al., 2007).

Various methods of discriminating medicinal herbs by using genetic tools facilitate accurate identification, despite such changes. For example, the nucleotide sequence of the ITS has been used successfully in studies aimed to define phylogenetic relationships among species and related genera (Wang et al., 2007; Lee et al., 2008; Lin et al., 2008; Ryuk et al., 2010; Sun et al., 2010). Up to date, more publications have revealed that ITS2 can serve as a novel universal barcode for the identification of a broader range of medicinal plant species and genera (Chen et al., 2010; Yao et al., 2010), but no method was reported to accurately identify medicinal plant of a species. ITS sequence analysis has also been used to identify *Bupleurum* species in China, (Yang et al., 2007; Xie et al., 2009), in a Korean study of *Bupleurum* species (including a native species) phylogeny (Moon et al., 2009), and for rapid discrimination of 3 *Bupleurum* species with a sequence-specific oligonucleotide probe (SSOP) array (Lin et al., 2008). These studies identified species by simply comparing their ITS nucleotide sequences, but more simpler and effective methods for discrimination of *Bupleurum* species are necessary. Diagnostic analysis of PCR amplification products is typically achieved with gel electrophoresis (GE). Because capillary electrophoresis (CE) can improve resolution compared to GE, this technique more accurately discriminates PCR products. CE has been proven to be very useful for identification of soil bacteria (King et al., 2005), detection of genetically modified organisms during food production (Kim et al., 2005), and plant nitrogen-glycan estimation using high-throughput quantitative techniques (Lee et al., 2009). In this study, we developed a reliable deoxyribonucleic acid (DNA) markers based on the ITS sequence to discriminate *B. longiradiatum* from *B. falcatum* and *B. chinense* and consummated the phylogenetic analysis of *Bupleurum* genus. The use of specific primers allowed us to unambiguously differentiate the PCR products of *B. longiradiatum* from those of other *Bupleurum* species by GE and CE, we first identify the most frequently used medicinal *Bupleurum* species by ITS sequences and pave way for further phylogenetic and/or evolutionary studies on *B. longiradiatum* and other *Bupleurum* species.

MATERIALS AND METHODS

Plant materials

The plant materials were collected from various locations in Korea, China and Japan. Prof. Je Hyun Lee of the Dongguk University identified the samples on the basis of their morphologies. The voucher samples were deposited with the Korea Institute of Oriental Medicine (Table 1). Photographs of representative dried samples are shown in Figure 1.

DNA extraction

Plant materials were frozen with liquid nitrogen and milled into a fine powder. Total cellular DNA was extracted from the powder using a Nucleospin® PlantII kit (MACHEREY-NAGEL GmbH). Genomic DNA concentrations were measured using a model

Figure 1. Photographs of representative dried samples of three *Bupleurum* species.

ND-1000 spectrophotometer (NanoDrop).

PCR analysis

PCR reactions were performed in 25 µl volumes containing 20 ng genomic DNA, 2× PCR Pre-Mix (Solgent), 10 pmol primers ITS1 and ITS4 (White et al., 1990), and distilled deionized water. Reaction conditions were 95°C for 4 min; 35 cycles at 95°C for 30 s, 55°C for 40 s, 72°C for 1 min and 72°C for 5 min, using a DNA Engine® apparatus (Bio-Rad). The desired product migrated as an approximately 700- bp band through a 1.5% (w/v) agarose gel, which was visualized by LoadingSTAR (Dynebio) staining under ultraviolet illumination. PCR products were subsequently purified using a NucleoSpin® Extract II Kit (MACHEREY-NAGEL GmbH).

Nucleotide sequencing and design of specific primers

PCR products were subcloned using a pGEM-T Easy Vector System (Promega), was used to transform *Escherichia coli* HIT™-DH5α and red blood cells (RBC). Plasmid DNAs were isolated using a Plasmid Mini-prep Kit (Solgent). The nucleotide sequences of the cloned PCR product were determined using 5 samples isolate from different colonies by Solgent Company Limited. The forward and reverse strands of all samples were sequenced. BioEdit version 7.0.5 (Hall, 1999) was used for multiple sequence alignments. Based on *Bupleurum* species ITS nucleotide sequences, a 20-mer BL-F primer (forward primer), 34-mer BL-R primer (reverse primer), and a 20-mer B-I-F primer (forward primer) were designed to discriminate *B. longiradiatum,* from *B. falcatum*, and *B. chinense*. The ITS4 primer was also used as a reverse primer. Primer locations (boxed) and their sequences are presented in Figure 2 and Table 2, respectively.

Specific PCR and capillary electrophoresis

PCR amplifications to be analyzed by GE were performed in reactions using 20 µl volume containing 10× buffer (750 mM Tris-HCl pH 8.8, 200 mM $(NH_4)_2SO_4$, 0.1% (v/v) Tween®20), 200 µM deoxynucleotide triphosphates (dNTPs), 2 mM $MgCl_2$, 5 ng genomic DNA, 10 pmol each primer (Table 2), and 1 U Thermoprime *Taq* DNA polymerase (Thermo Fisher Scientific Inc.). PCR conditions were 94°C for 7 min; 35 cycles at 94°C for 30 s, 52 for 30 s and 72°C for 1 min and 72°C for 7 min. PCR was conducted using a DNA Engine® apparatus (Bio-Rad). Amplified products separated in a 2% (w/v) agarose gel, were stained with LoadingSTAR (Dynebio), and were analyzed using a U: Genius (Syngene). PCR amplifications analyzed by CE were performed in 25 µl volumes containing 1 ng genomic DNA, 2.5 µl 10× reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% (w/v) gelatin) (Applied Biosystems), 0.2 mM dNTP mixture, 10 pmol each primer, and 1.25 U AmpliTaq Gold® Polymerase (Applied Biosystems). The 5′-end of each reverse primer was fluorescently labeled with JOE (6-carboxy-4′, 5′-dichloro-2′, 7′-dimethoxy-fluorescein) or FAM (9-carboxy fluorescein) (Table 2). PCR conditions were 95°C for 12 min; 30 cycles at 94°C for 30 s, 51°C for 30 s, and 72°C for 30 s; and 72°C for 7 min using a DNA Engine® (Bio-Rad). The fluorescent PCR products were mixed with 14.7 µl Hi-Di™ Formamide (Applied Biosystems) and 0.3 µl Genescan-500 ROX™ size standards (Applied Biosystems), processed by CE using an ABI PRISM® 310 genetic analyzer (Applied Biosystems), were viewed using GeneScan 3.1 software (Applied Biosystems).

RESULTS AND DISCUSSION

The sequences (655 bp) of all *B. falcatum* samples were identical and were represented as ITS1 exon (225 bp), 5.8S exon (163 bp), and ITS2 exon (267 bp) (Figure 2). The sequence was identical to that of *B. falcatum* (Gen Bank accession number, AJ131344). Nucleotide sequencing of *B. chinense* revealed 4 haplotypes designated hap1, 2, 3, and 4, based on the differences at 8 positions (Table 3). The hap4 nucleotide sequence is 657 bp length because of the insertion of G at positions 206 and 207 bp, which is absent in other haplotypes (hap1, 2, and 3). The *B. chinense* ITS1 hap1, 2, and 3 exons each are 225 bp length, and that of hap4, 227 bp length. The 5.8S and ITS2 exons are 163 and 267 bp length, respectively (Figure 2). Sequences of the 4 haplotypes have been deposited in GenBank as

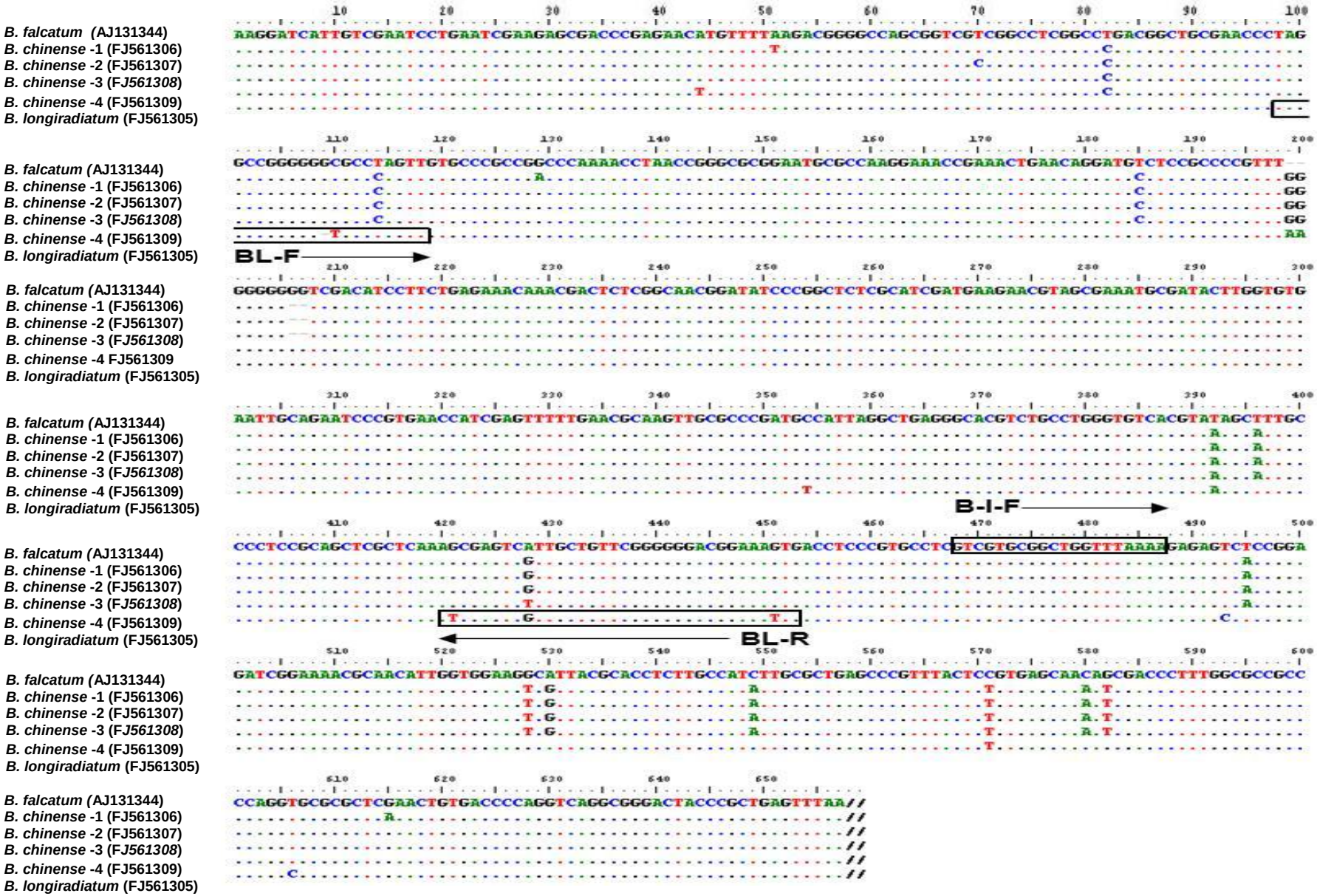

Figure 2. Nucleotide sequence comparisons of *Bupleurum* species' DNA amplified using ITS1/ITS4 primers. Bold boxes indicate sequences used to design specific primers for *B. longiradiatum* Turcz.

Table 2. PCR primers for ITS amplification and identification of *B. longiradiatum.*

Primer sequence (5′→3′)		Fluorophore	Reference
	Amplification of ITS region		
ITS1	TCC GTA GGT GAA CCT GCG G		White et al. (1990)
ITS4	TCC TCC GCT TAT TGA TAT GC		White et al. (1990)
	***B. longiradiatum*-specific primers**		
BL-F	TAG GCC GGG GGT GCC TAG TT		
BL-R	CAA TTT CCG TCC CCC CGA ACA GCA ACG ACT CGA T	JOE (green)	
B-I-F	GTC GTG CGG CTG GTT TAA AA		
ITS4	TCC TCC GCT TAT TGA TAT GC	FAM (blue)	White et al. (1990)

Table 3. *B. chinense* sequence haplotypes.

Haplotype	Position (bp)								
	44	**51**	**70**	**119**	**206**	**207**	**428**	**615**	**Accession no**
B. chinense hap1	A	T	T	A	-	-	G	A	FJ561306
B. chinense hap2	A	A	**C**	G	-	-	G	G	FJ561307
B. chinense hap3	A	A	T	G	-	-	G	G	FJ561308
B. chinense hap4	T	A	T	G	G	G	T	G	FJ561309

Table 4. *Bupleurum* species sequence characterization.

Species	Accession no	Code	Sequence identity (%)						% GC
			BF	**BC-1**	**BC-2**	**BC-3**	**BC-4**	**BL**	
B. falcatum	AJ131344	BF	-						58.63
B. chinense hap1	FJ561306	BC-1	98	-					58.32
B. chinense hap2	FJ561307	BC-2	98	99	-				58.78
B. chinense hap3	FJ561308	BC-3	99	99	99	-			58.63
B. chinense hap4	FJ561309	BC-4	98	99	99	99	-		58.60
B. longiradiatum	FJ561305	BL	99	97	97	98	97	-	58.08

B. chinense hap1 (FJ561306), *B. chinense* hap2 (FJ561307), *B. chinense* hap3 (FJ561308), and *B. chinense* hap4 (FJ561309). All *B. longiradiatum* ITS nucleotide sequences were identical. The *B. longiradiatum* sequence differed at 5 positions when compared with that of *B. longiradiatum* (accession number, AY551291), and was, therefore, deposited in GenBank as a new *B. longiradiatum* nucleotide sequence (accession number, FJ561305). The hap1, 2, 3, and 4 sequences were found to be 98 to 99% and 99% identical, for *B. falcatum* versus *B. chinense* and for *B. falcatum* versus *B. longiradiatum*, respectively. Thus, *B. falcatum*'s sequence was more closely related to that of *B. longiradiatum* than *B. chinense*. The lowest sequence identity (97%) determined was between *B. chinense* hap1 and *B. longiradiatum. B. longiradiatum*'s (58.08%) and *B. chinense*'s (58.78%) hap2 GC contents were the lowest and the highest, respectively (Table 4).

Comparison of ITS nucleotide sequences revealed 2 positions (199 and 200 bp) at which all 3 species differed were *B. falcatum*, single base deletion; *B. chinense*, 2 G's, and *B. longiradiatum*, 2 A's. Within these sites, 9 *B. longiradiatum* nucleotides differed from those of *B. falcatum* and *B. chinense* sequences (Table 5). The nucleotide sequences within the chloroplast *rbc*L region were also confirmed and displayed less variability than those of the ITS. Therefore, they were deemed inappropriate for identifying *Bupleurum* species. Furthermore, the highly variable ITS region is more effective for distinguishing *Bupleurum* species. We then designed BL-F, BL-R, and B-I-F primers based on the 9 *B. longiradiatum* specific stretches to discriminate *B. longiradiatum* from *B. falcatum* and *B. chinense* (Table 2). PCR reactions employing 15 samples were listed in Table 1, in combination with these primers, generated *B. longiradiatum*-specific bands, 204 and 351 bp length (Figure 3, Lanes 11 to 15) and unique *B. falcatum* (206 bp, Figure 3, Lanes 1 to 5) and *B. chinense* (Figure 3,

Table 5. Comparison of *Bupleurum* sequences.

Species	Position (bp)										
	82	**109**	**110**	**114**	**185**	**199**	**200**	**354**	**392**	**396**	**421**
B. falcatum	T	G	C	T	T	-	-	C	T	T	G
B. chinense	C	G	C	C	C	G	G	C	A	A	G
B. longiradiatum	T	-	T	T	T	A	A	T	A	T	T
Species	451	493	495	528	530	549	571	580	582	606	
B. falcatum	G	T	T	G	A	C	C	C	G	T	
B. chinense	G	T	A	T	G	A	T	A	T	T	
B. longiradiatum	T	C	T	G	A	C	T	C	G	C	

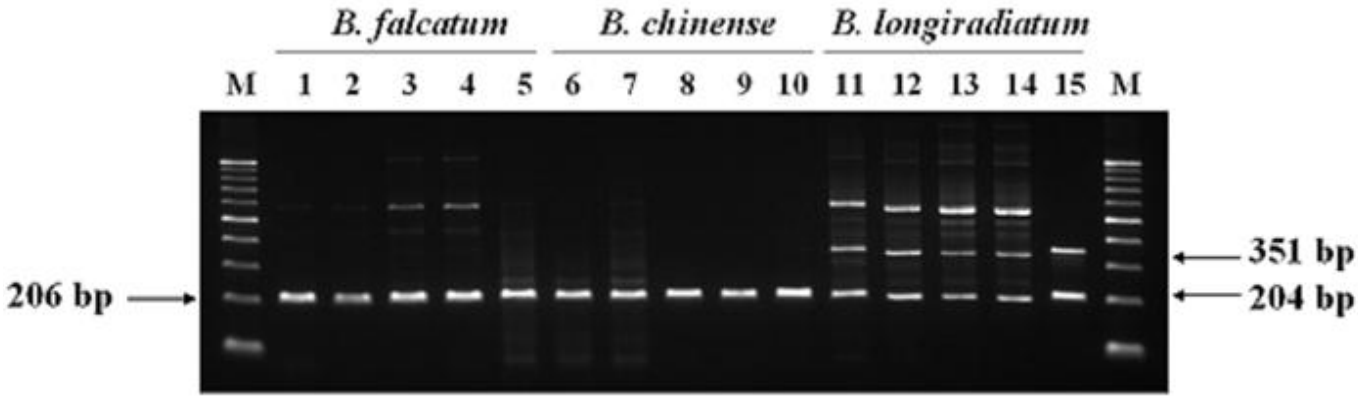

Figure 3. PCR Products Generated with *B. longiradiatum*-specific Primers. Lanes 1 to 15, *Bupleurum* species listed in Table 1. M = 100 bp DNA size standard ladder.

Lanes 6 to 10) bands. By using fluorescently labeled *B. longiradiatum*-specific primers (Table 2), we compared CE and GE. Reactions using templates of the DNA of *B. falcatum* and *B. chinense* DNAs as listed in Table 1 yielded specific 206 bp products, in contrast to the 2 specific *B. longiradiatum* 204 and 351 bp peaks (Figure 4).

The amplified products were similar in size as determined by both CE and GE analyses, confirming the suitability of the *B. longiradiatum*-specific primers for differentiating *Bupleurum* species. Five *Bupleurum* species growing naturally in Korea were classified into 3 groups based on their external morphologies and number of somatic chromosomes (Ahn et al., 2008). *B. falcatum* and *B. longiradiatum* could further be discriminated from each other by microscopic analysis of their external root morphologies (Yang et al., 2004). However, because the external appearance or constituents of medicinal plants are influenced by various factors such as growth environment, harvesting time, and process flow (Yang et al., 2007; Hon et al., 2003; Tristezza et al., 2009), it is necessary to objectively and unambiguously differentiate *Bupleurum* species based on the molecular genetic information. Accordingly, to determine whether *B. longiradiatum* could be molecularly differentiated from *B. falcatum* and *B. chinense*, primers were developed for routine GE. The results demonstrated that an ITS-PCR method using these primers is useful for rapid and efficient discrimination of *Bupleurum* species. Although GE is relatively simple to perform, the technique is not easy to determine the exact size of PCR products or resolve similar sized bands. However, incorporating an internal size marker in CE can virtually eliminate the influence of external parameters, thereby permitting highly reproducible and accurate size determination of PCR products (Tristezza et al., 2009).

Even a single-base pair difference can be detected by CE-based genotyping of *Plasmodium falciparum* (Liljander et al., 2009). Recently He et al. (2011) reported that *Angelica anomala* and *Angelica dahurica* are closely related and nested in this phylogeny tree. *A. dahurica* shown the high similarity with *A. anomala* than any other species. Therefore, we sought to develop a GE-based method capable of identifying *B. longiradiatum* swiftly, accurately, and economically and to enhance PCR accuracy based on CE using fluorescently labeled compounds. Further studies should aim to simultaneously identify multiple herbal plants by using CE and primers labeled with dyes that fluoresce at different wavelengths.

In conclusion, ITS nucleotide sequence variation can be used to distinguish between *B. falcatum, B. chinense*, and *B. longiradiatum.* Furthermore, *B. longiradiatum* can

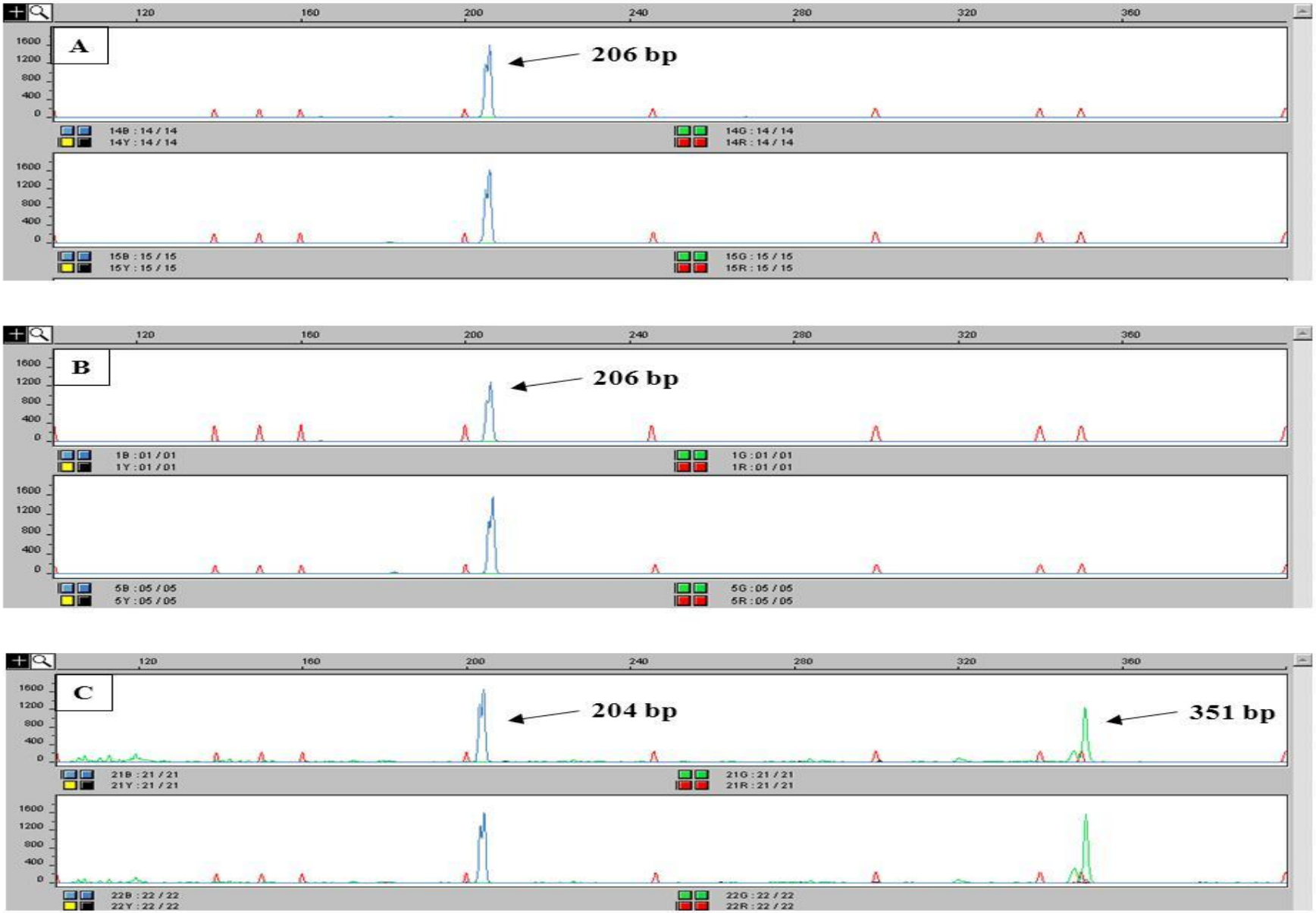

Figure 4. Capillary Electrophoresis Analysis of *B. longiradiatum*-specific PCR products; A *B. falcatum*; B *B. chinense*, and C *B. longiradiatum.*

be unambiguously identified using specific primers. GE and CE verified the specificity of this method, which may also prove to be useful in identifying other medicinal herbs that will not be easy to distinguish on the basis of their external appearance.

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