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The Application of DNA Chip Technology to Identify Herbal Medicines: an Example from the Family Umbelliferae

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Abstract – Comparative molecular analysis has been frequently adopted for the authentication of herbal medicines as well as the identification of botanical origins. Roots and rhizomes of the family Umbelliferae have been used as traditional herbal medicines to relieve various symptoms such as inflammation, neuralgia and paralysis in countries of East Asia. Since most herbal medicines of the Umbelliferae roots or rhizomes are generally supplied in the form of dried slices, morphological examination does not often provide sufficient evidence to identify the botanical origin. Using species-specific probes developed by the comparative analysis of nrDNA ITS sequences, a DNA chip was developed to identify herbal medicines for 13 species in the Umbelliferae. The developed DNA Chip proves its potential as a rapid, sensitive and effective tool for authenticating herbal medicines in future.
Keywords – Authentication, DNA chip, Herbal medicines, ITS, Umbelliferae

Introduction

Many species in the family Umbelliferae are used in the wide range of purposes from food and fodder to spices, medicines, and even perfumery.[1] Especially, in countries of East Asia, roots and rhizomes of many umbellifers have been often used as herbal medicines to deal with various symptoms such as gastrointestinal complaints, inflammation, neuralgia, and paralysis.[2-6]

The essential prerequisite to ensure the quality of herbal medicines is the precise authentication of herbal materials through the correct identification of botanical origin. In general, the identification of medicinal plants requires the morphological examination for both macroscopic and microscopic features. Chemical profiles are also referred to as practical criterion for the quality control and standardization of herbal medicines in pharmacopoeias.[3-7]

However, the morphological examination does not always provide sufficient evidence for discriminating herbal medicines of umbellifers because they are provided commonly in dried slices of roots or rhizomes, and many closely related species are often very similar in morphology for both external and internal structure. Likewise, the chemistry does not often work for confirming botanical identity because the chemical composition is also affected by the growing condition of plants, and the process and storage of plant materials. Under some circumstances in the family Umbelliferae, the botanical sources are different for the herbal medicines with same name among Korea, Japan and China, which causes confusions in the distribution process at Korean markets.

Remarkable advances in molecular biological techniques have made comparative analysis of DNA molecules applied in various scientific fields. Especially, with the introduction of PCR technology, the DNA analysis has been proved as a powerful tool to identify species and to elucidate phylogenetic relationships in plant taxonomy. As the accumulation of DNA sequence data have been accelerated in global scale, DNA-barcoding system was proposed to facilitate the identification of species.[8-11] Even if various DNA regions (e.g., *matK*, *rbcL*, *psbA-trnH*, and nrDNA ITS) have been suggested for the DNA barcode system of plants, previous studies on the family Umbelliferae have clearly demonstrated that nrDNA ITS should serve well as a molecular marker to distinguish species in the family.[12-15]

DNA chip technology is an emerging innovation for the massive screening of variations in DNA sequences. In the application for authenticating herbal medicines, DNA chip is designed by immobilizing species-specific probes on solid plate (e.g., microscope glass slides) in microarray. Target DNAs from the sample to be examined are

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Table 1. Plant materials used in the study. Voucher specimens are kept in the herbarium of Natural Products Research Institute of Seoul National University (NPRI)

Species	Herbal Medicine	Ref.[a]	Collection and voucher	Abbr.
Angelica acutiloba (Sieb. et Zucc.) Kitag.	Angelicae Radix	JP	Korea: Kangwon Prov.: Pyongchang, 23 Jul 2008, Suh & Choi DC03	AA
A. dahurica Bentham et Hooker f. var. *dahurica*	Angelicae Dahuricae Radix	CP, JP, KP	Korea: Seoul: Kyungdong Herbal Market, 13 Mar 2009, Choi DC11	ADD
A. dahurica Bentham et Hooker f. var. *formosana* Shan et Yuan	Angelicae Dahuricae Radix	CP, KP	China: Anguo City: Chinese Traditional Medicine Market, 8 Nov 2008, Choi DC08	ADF
A. gigas Nakai	Angelica Gigantis Radix	KP	Korea: Seoul: Kyungdong Herbal Market, 13 Mar 2009, Choi DC12	AG
A. sinensis (Oliv.) Diels	Angelicae Sinensis Radix	CP	China: Anguo City: Chinese Traditional Medicine Market, 8 Nov 2008, Choi DC09	AS
Bupleurum falcatum L.	Bupleuri Radix	CP, JP, KP	Korea: Jeonnam Prov.: Gurae, 15 Jul 2008, Suh & Choi DC01	BF
Cnidium officinale Makino	Cnidii Rhizoma	JP, KP	Korea: Gyunggi Prov.: Soowon, 8 Sep 2008, Suh & Choi DC07	CO
Glehnia littoralis Fr. Schmidt ex Miguel	Glehniae Radix et Rhizoma	CP, JP, KP	Korea: Jeonnam Prov.: Yeosoo, 15 Jul 2008, Suh & Choi DC02	GL
N. incisum Ting	Notopterygii Radix et Rhizoma	CP, JP, KP	China: Anguo City: Chinese Traditional Medicine Market, 8 Nov 2008, Choi DC10	NI
Ostericum koreanum Maxim. cv. 'North'[b]	Osterici Radix et Rhizoma	KP	Korea: Gyunggi Prov.: National Arboretum, 25 Aug 2008, Suh & Choi DC06	OKN
O. koreanum Maxim. cv. 'South' [b]	Osterici Radix et Rhizoma	KP	Korea: Gyungbuk Prov.: Youngcheon, 7 Aug 2008, Suh & Choi DC04	OKS
Peucedanum japonicum Thunb.	Peucedani Japonici Radix	KHP	Korea: Gyungbuk Prov.: Youngjoo, 7 Aug 2008, Suh & Choi DC05	PJ
Saposhnikovia divaricata (Turcz.) Schischk.	Saposhnikoviae Radix	CP, JP, KP	Korea: Seoul: Kyungdong Herbal Market, 13 Mar 2009, Choi DC13	SD

[a]CP, JP and KP indicate the Pharmacopeia of China, Japan, and Korea, respectively, and KHP stands for the Korean Herbal Pharmacopoeia. [b]The botanical origin of Osterici Radix et Rhizoma is described to be *Ostericum koreanum* Maxim. in the Pharmacopeia of Korea, but two cultivars, cv. 'North' and cv. 'South', are recognized in cultivation.

amplified with PCR primers that fluorescent dyes are incorporated. A positive hybridization with complementary probe will be visualized by the detection of the fluorescence signal using the microscopic scanner.[7,16]

In this study, by using species-specific probes developed on the basis of nrDNA ITS sequences, a DNA chip was developed to identify the botanical origin of 13 herbal medicines in the Umbelliferae, which are commonly used in Korea, Japan and China.[3-6] The developed DNA Chip proved to be a rapid, sensitive and effective method for the identification of herbal drugs tested from the family Umbelliferae. DNA chip technology certainly has the potential as the future choice for the large-scale identification of herbal medicines.

Experimental

Plant materials – The sequences of nrDNA ITS were determined for 13 herbal medicines of the family Umbelliferae listed in Pharmacopoeias of Korean, Japan and China (Table 1).[3-6] Voucher specimens were kept in the herbarium of Natural Products Research Institute of Seoul National University (NPRI).

DNA extraction – Genomic DNA was extracted from dried herbal medicines using the DNeasy Plant Mini Kit (QIAGEN, Germany) according to the vendor's protocol with minor modification. The initial incubation at 60 ºC was extended to 2 hrs. The final DNA extracts were confirmed on 0.7% agarose gel containing 0.5 mg/mL ethidium bromide under UV illumination by the comparison

with the standard marker of λ DNA digested with *Hin*d III (Takara Bio Inc., Japan).

PCR amplification – The region of nrDNA ITS including complete ITS 1, ITS 2 and 5.8S gene was amplified by conventional PCR using primers ITS4 and ITS5.[17] PCR was carried out with TaKaRa Ex Taq (Takara Bio Inc.) according to vendor's recommendation with slight modification. The reaction mixture contained 0.2 Unit of Ex Taq polymerase (Takara Bio Inc.), 1 × Ex Taq Buffer (Takara Bio Inc.), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.2 μM each primer and 20 - 100 ng template DNA in the final volume of 50 μL. The thermal profile for the reaction was composed with the initial denaturation for 5 min at 95 ºC, 40 cycles consisted of denaturation for 30 sec at 94 ºC, annealing for 30 sec at 53 ºC, and extension for 1 min at 72 ºC, and the final extension for 7 min at 72 ºC. The amplified products were examined on 1% agarose gel containing 0.5 μg/mL ethidium bromide under UV illumination by the comparison with Gene Ruler 100 bp ladder marker (Fermantas Co., Germany). The PCR products were further purified with High Pure PCR Product Purification Kit (Roche, Germany) before sequencing.

DNA sequencing and sequence analyses – The sequences of nrDNA ITS were determined by the dideoxy chain termination method[18] using an ABI PRISM 3730XL automated sequencer with the BigDye Terminator Cyclic Sequencing Ready Reaction Kit (Applied Biosystems, CA). Primers ITS4 and ITS5 were also used as sequencing primers and sequences were confirmed on both DNA strands. Proofreading and editing of sequences were performed using Sequencher 4.5 (Gene Codes Corp., MI). Sequences were aligned initially with CLUSTRAL X 1.64b[19] with default setting, and then finally adjusted with the naked eye. Boundaries of the coding (i.e., 18S, 5.8S, and 26S rDNA) and spacer regions were determined in comparison to previously reported ITS sequences in GenBank nucleic acid sequences database. Sequence divergences were calculated by pairwise comparison using Kimura's two parameter method.[20]

Preparation of DNA chip – From the aligned sequence matrix, distinct DNA region in size of 20 - 25 bp, of which sequence was unique to each species, was identified for designing sequence-specific oligonucleotide probe. The 5' end of each probe was tailed with 15 poly-T to facilitate the accessibility of spotted probes to target DNA. The synthesized probes were diluted in the concentration of 100 pmol/μL and then mixed with the equivalent amount of GenoCheck Platinum Spotting Solution (GenoCheck, Korea) for printing. The prepared probes were printed on 25 × 75 mm silylated microscope glass slide with Prosys GANTRY SYSTEM (Cartesian Technologies, Inc., CA) by 300 μm intervals. Each probe was spotted in duplicates on the microscope slide. After being dried for 12 hrs at room temperature, the printed slides were washed once with 0.1% SDS solution for 1 min, and then rinsed two times with distilled water for 1 min each to remove unbounded oligonucleotide probes. The washed slides were treated with sodium borohydride solution ($NaBH_4$ 1 g: phosphate buffer saline 300 ml: ethanol 100 ml) for 5 min, rinsed two times with distilled water for 5 min, dried by centrifugation at 800 rpm for 3 min, and stored at room temperature until use.

Labelling PCR products and hybridization – The nrDNA ITS regions were amplified in the same conditions of previous PCR except for using the primers ITS4 and ITS5,[17] which were chemically labelled with Cyanine 3. After the denaturation by heating at 99°C for 3 min and then immediate chilling on ice, 10 μL of the fluorescent labelled PCR product was diluted with the addition of 90 μL GenoCheck Platinum Hybridization Solution (GenoCheck, Korea). The hybridization mixture was applied directly onto slides and covered with a coverslip (Sigma Aldrich Co., Germany). After the hybridization at 60 °C for 1 hr, the slides were washed consecutively in the solutions of 1 × SSC with 0.1% sarcosyl, 1 × SSC, and then 0.1 × SSC for 5 min each. The coverslip was removed in the first washing step. Finally, the slides were dried by the centrifugation at 800 rpm for 3 min. The hybridized slides were scanned with Axon Chip Scanner and each spot was quantified using GenePix Pro version software (Axon Instruments, CA).

Results and Discussion

Analyses of ITS sequences – The sequences of ITS 1 and ITS 2 for 13 plants in the family Umbelliferae ranged from 215 bp to 217 bp and from 217 bp to 223 bp in size, respectively. The size of 5.8S coding region was 162 bp. The size of ITS 1, ITS 2 and 5.8S coding region for the umbellifers examined in this study lies within the range of those reported previously in the family.[12-14,21] The G+C contents was 53.7-62.3% for ITS 1, and 54.3-59.5% for ITS 2.

Sequence alignment required 7 independent indels for ITS 1, and 11 independent indels for ITS 2. The length of indels was 1 bp for 16 instances, and 2 bp for 2 instances throughout ITS 1 and ITS 2 (Fig. 1). The range of sequence divergence (Kimura's $K \times 100$) of ITS 1 was from 0.5% to 49.7%, with the highest values between BF and GL,

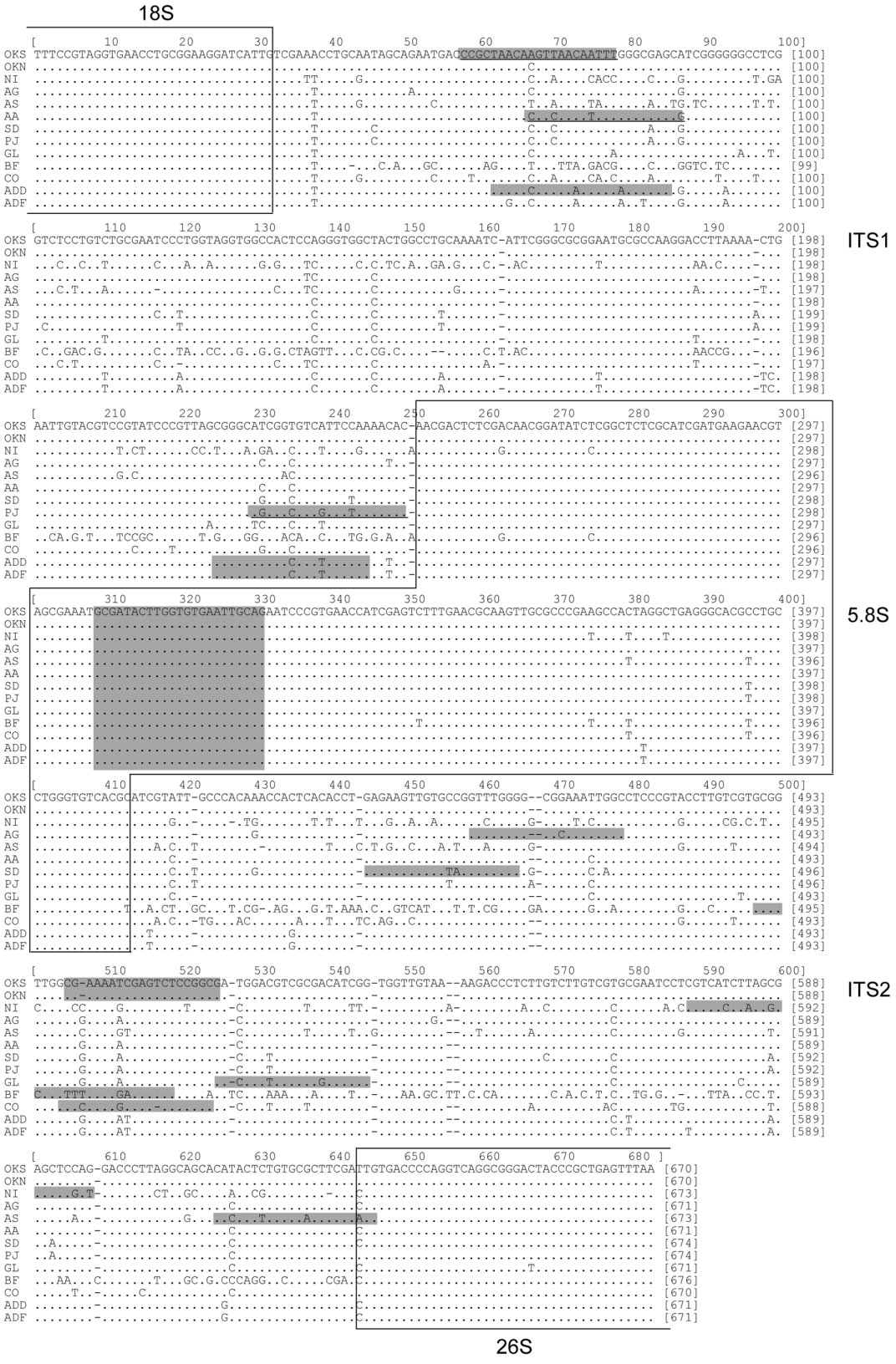

Fig. 1. Aligned sequences of nrITS region from 13 herbal medicines. The locations of probes are shaded. Sequences are underlined in cases the complementary oligonucleotides were adopted for probes. The probe nested in the 5.8S coding region was used as positive control. See Table 1 for the abbreviations of species names.

Table 2. Numbers of substitutions per 100 sites (%; Kimura's $K \times 100$) of ITS sequences in the family Umbelliferae. Values for ITS 1 are shown below diagonal, and ITS 2 above diagonal. See Table 1 for the abbreviations of species names

	OKS	OKN	NI	AG	AS	AA	SD	PJ	GL	BF	CO	ADD	ADF
OKS	–	0.0	21.8	3.3	13.0	2.3	6.6	4.7	4.7	47.4	13.6	3.7	4.2
OKN	0.5	–	21.8	3.3	13.0	2.3	6.6	4.7	4.7	47.4	13.6	3.7	4.2
NI	28.7	28.0	–	21.6	21.5	21.0	22.2	22.8	22.9	50.8	24.7	24.9	25.6
AG	4.8	4.3	25.3	–	13.7	2.8	5.2	4.2	4.3	45.2	14.2	5.2	5.7
AS	14.5	14.5	23.5	12.8	–	12.5	15.0	14.1	14.1	46.4	11.4	14.6	15.2
AA	4.3	3.8	24.7	2.4	11.7	–	4.2	2.3	2.3	46.2	13.0	5.2	5.7
SD	6.8	6.3	25.4	5.3	13.9	3.8	–	2.3	4.7	48.2	15.1	7.6	8.1
PJ	7.3	6.8	26.7	5.8	15.1	4.3	1.4	–	2.8	48.3	13.1	5.7	6.2
GL	6.8	6.3	26.0	5.8	15.1	5.3	8.4	8.4	–	48.0	13.5	6.7	7.2
BF	47.4	47.6	40.1	45.7	43.9	45.6	44.1	44.1	49.7	–	53.3	48.8	49.6
CO	12.7	12.2	21.5	11.1	9.5	10.6	10.6	12.3	12.7	45.8	–	16.4	17.0
ADD	8.4	7.8	26.7	6.3	16.2	6.8	8.4	8.4	8.4	47.7	16.3	–	0.5
ADF	9.4	8.9	28.2	7.4	17.4	7.9	9.4	9.4	9.5	49.7	17.5	0.9	–

Table 3. Oligonucleotide probes for DNA chip to identify herbal medicines of the family Umbelliferae

Probe	Probe sequence[a]	Target species[b]	Location[c]
OKS-3-A	T(15)- AAATTGTTAACTTGTTAGCGG[d]	OKS	58 - 78
OKN/S-4	T(15)- CGAAAATCGAGTCTCCGGCG	OKN or OKS	505 - 525
NI-2	T(15)- CGTCACCTAAGGGAGCTCGAT	NI	588 - 608
AG-3	T(15)- GTTTGGGGCGCAAATTGGC	AG	459 - 479
AS-4	T(15)- CACACTTTGTGCACTTCGAATG	AS	625 - 646
AA-2-A	T(15)- CGCTCGCCCAAAATGTTGACG[d]	AA	67 - 87
SD-3	T(15)- GAGAAGTTGTGTAGGTTTGGG	SD	445 - 465
PJ-2-A	T(15)- GTGTTTTAGAACGACGCCGCT[d]	PJ	230 - 250
GL-5	T(15)- GACGGATGTCGCGGCATCGG	GL	525 - 545
BFK-2	T(15)- GCGGCTGGTTTAAAAGAGAGT	BF	497 - 519
CO-3	T(15)- GCGCAAAAGCGAGCTCCGGC	CO	504 - 524
ADD-4	T(15)- TAACACGTTAAAAATTTAGGCGAG	ADD	62 - 85
ADD/F	T(15)- CGGGCATCGGCGTCTTTCCAA	ADD or ADF	225 - 245
P.C-2	T(15)- GCGATACTTGGTGTGAATTGCAG	Positive Control	309 - 331

[a]The 5' end of probes was tailed with 15 poly-T for facilitating target DNA to access spotted probes. [b]See Table 1 for the abbreviations of species names. [c]Numbers indicate the location of probes in the aligned sequence data matrix shown in Fig. 1. [d]Complementary oligonucleotides were adopted as probes.

and between BF and ADF (Table 2). The lowest divergence was 0.5% between two cultivars of OK, which resulted from the difference of 1 bp, and the divergence between two subspecies of AD was 0.9%. Among 4 species of the genus *Angelica*, the interspecific variation of ITS 1 sequences ranged from 2.4% to 17.4%. The sequence divergence of ITS 2 ranged from 0.0% to 50.8%, with the highest value between BF and NI. The sequences of ITS 2 were identical for two cultivars of OK, and the divergence of ITS 2 between two subspecies of AD was 0.5%. The interspecific variation of ITS 2 sequences in *Angelica* ranged from 2.8% between AA and AG to 15.2% between AS and ADF. Overall for both ITS 1 and ITS 2 sequences, BF showed the highest divergence in comparison to other species, which suggested that BF should be the most distantly related to other umbellifers analyzed in this study.

Probe design – Based on the aligned sequences of nrITS region (Fig. 1) and previously reported database of GenBank of NCBI, unique sequences of about 20-mer in size were searched for each species to design species-specific probes. Initial analysis produced 78 possible

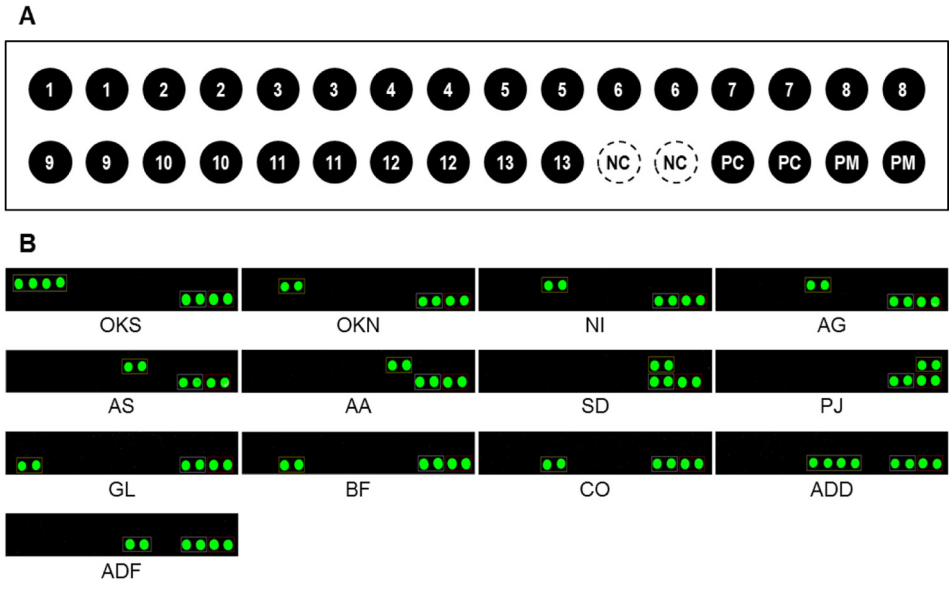

Fig. 2. A. Probe arrangement on microscope slide. 1. OKS-3-A; 2. OKS/N-4; 3. NI-2; 4. AG-3; 5. AS-4; 6. AA-2-A; 7. SD-3; 8. PJ-2A; 9. GL-5; 10. BF-2; 11. CO-3; 12. ADD-4; 13. ADD/F; NC. Negative control; PC. Positive control; PM. Position marker. See Table 3 for the abbreviations of probes. **B.** Fluorescence emission pattern detected by scanner after the hybridization with PCR products using fluorescence-labelled primers. See Table 1 for the abbreviations of species names.

species-specific proves, and 13 out of them, of which melting temperature was in the range of 48.5 ºC - 60 ºC, were finally chosen to manufacture DNA chip for authenticating herbal medicines. In addition, the region with identical sequences for all species was selected for positive control, which was nested in the 5.8S coding region (Fig. 1, Table 3).

The sequence variation in ITS 1 and 2 between two cultivars of *Ostericum koreanum* (OKN and OKS) and between two subspecies of *Angelica dahurica* (ADD and ADK) was very low, 0.3% and 0.7%, respectively. The sequences of OKN and OKS were different by single nucleotide, and the sequences of ADD and ADF were different by three nucleotides (Fig. 1, Table 2). For those showing very little sequence divergence such as two cultivars of *O. koreanum*, OKN and OKS, of which sequences were different from each other by single nucleotide, a set of two probes were designed to discriminate target samples at two different taxonomic levels. The probes OKN/S-4 and OKS-3-A were designed to discriminate target samples at species and cultivar level, respectively. The probe OKN/S-4 was located at 505 - 525 in aligned sequences, where both cultivars OKN and OKS shared identical sequences. The probe OKN/S-4 discriminated both OKN and OKS from the rest. The probe OKS-3-A was located at 58-78, where the sequences of OKN and OKS were different by single nucleotide. The probe OKS-3-A could distinguish OKS from OKN (Fig. 1, Table 3)

The positive hybridization with the probe OKN/S-4 would suggest that the target sample should be either OKN or OKS. On the other hand, the probe OKS-3-A would yield the positive hybridization only with OKS. Therefore, if the target sample hybridized with both probes OKN/S-4 and OKS-3-A, it should be OKS. However, OKN should show positive hybridization only with the probe OKN/S-4. By the same token, the probe ADD/F was designed to work for both subspecies of *Angelica dahurica* (ADD and ADF), but the probe ADD-4 was designed exclusively for ADD (Fig. 1, Table 3).

Identifying herbal medicines with DNA chip – DNA chip was manufactured by immobilizing the synthesized probes on microscope glass slide by 300 nm intervals in duplicates (Fig. 2A, Table 3). Positive hybridization reactions were detected by fluorescence emission when PCR products from target samples using Cyanine 3-labelled primers ITS4 and ITS5 were applied to the DNA chip (Fig. 2B). Emission patterns of fluorescence visualized by scanner clearly identified the target samples in comparison with the arrangement of probes on DNA chip (Fig. 2). Especially, single nucleotide polymorphism shown in two cultivars of *O. koreanum*, OKN and OKS, was successfully detected by DNA chip technology. When the target sample reacted with both probes, OKS-3-

A and OKN/S-4, it should be OKS because OKS-3-A was designed to work exclusively for OKS. On the other hand, when the target sample did not react with OKS-3-A but with OKN/S-4, it should be OKN. The same principle was applied to discriminate two subspecies of *A. dahurica*, ADD and ADF (Fig. 2, Table 3).

A recent study claimed that the plant known as *O. koreamum* should be treated as a new species, *Angelica reflexa* B.Y.Lee.[22] The name of *O. koreamum* should be rejected because it is apparent that *O. koreamum* is the synonym of *O. grosseserratum*.[22-25] Even though the clarification of taxonomic status of *O. koreanum* is out of the scope in this study, the citation of *O. koreanum* in Korean Pharmacopoeia must be reconsidered.

The developed DNA chip in this study proved to be a rapid, sensitive and effective method for identifying herbal materials of the family Umbelliferae. This study has demonstrated that DNA chip technology has much potential as an effective tool for the large-scale authentication of herbal medicines.

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