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Discrimination of *Lonicera japonica* and *Lonicera confusa* using chemical analysis and genetic marker

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ABSTRACT

Objective : *Lonicera japonica* THUNB. a traditional herbal medicine, has been commonly used anti-inflammatory disease. It has been very complicated with respect to its sources on the market. The significant selection of medicine depends on its origin. However, it is difficult to discrimination criteria for confirming *L. japonica* authenticity using the senses. This study was performed to determine the discriminant analysis of *L. japonica and L. confusa.*

Methods : The identification of *L. japonica* and *L. confus*a were performed by the classification and identification committee of the national center for standardization of herbal medicines. And we examined its differences using HPLC and genetic marker analysis.

Results : The analytical pattern of High Performance Liquid Chromatography was determined from the corresponding peak curves ((E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, sweroside). For *L. japonica*, additional unknown peaks were detected at 13.8 min, 20.6 min, and 36.9 min. And, we developed genetic marker using the the tRNA-Leu gene, *trnL-trnF* intergenic spacer and tRNA-Phe region of chloroplast DNA. By the method, 164 bp PCR product amplified from *L. confusa* was distinguished into *L. japonica* and *L. confusa* efficiently.

Conclusion : Base on these results, two techniques provide effective approaches to distinguish *L. japonica* from *L. confusa.*

Key words : *Lonicera japonica*, High Performance Liquid Chromatography, genetic marker, discrimination

Introduction

Lonicera japonica THUNB. (Caprifoliaceae) is commonly used as an anti-inflammatory herbal medicine[1]. It has been very complicated with respect to its sources on the market. Lonicerae Flos is of high medicinal value in traditional Chinese medicine, where it is called jīn yín huā (金銀花). The flowers are double-tongued, opening white and fading to yellow, and sweetly scented. It has antibacterial[2] and anti-inflammatory properties[3,4], and is used to dispel heat and remove toxins, including carbuncles, fevers, influenza and ulcers. Caprifoliaceae Lonicera confusa, shan yín huā (山銀花) in Chinese, is a woody vine, the distribution of the region wide in China's southwestern. The flowers have also been used on the same purpose in China[5]. This plant has a much softer appearance than the more commonly used *L. japonica*.

In Korea Pharmacopeia, Lonicerae Flos is documented as the sources of origin plant[6]. It is called geumeunhwa. So it is an important issue to comprehensively evaluate the different sources of *L. japonica*, so as to ensure the clinical efficacy of these herbal drugs. As the international trade is increased, the import of *L. japonica* from China to Korea has been growing.

Chemical and pharmacological investigations on

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Lonicerae Flos resulted in discovering several kinds of bioactive components, i.e. chlorogenic acid and its analogues[7,8], flavonoids[9], iridoid glucosides[10,11] and triterpenoid saponins[12,13].

Some closely related species of *L. japonica* which have similar morphology but weaker biological activity are also used medicinally. Because there are differences in the amount and distribution of constituents between *L. japonica* and *L. confusa*, the discrimination between the two species essential. The discrimination of *L. japonica* from its adulterants is currently limited to methods of morphology and chemical fingerprinting. So far, many studies have examined the constituents and efficacy of *L. japonica* and *L. confusa*, but molecular genetic reports have been not enough. Besides, with the discrimination based on the gross features such as shape or color reveal the limitation in differentiating between *L. japonica* and *L. confusa*. Noncoding regions of cpDNA are presumably under less functional constraints and evolve more rapidly, so they may provide useful phylogenetic information at lower taxonomical levels and offer molecular marker for species identification[14].

In this study, the chemical analysis of four compounds (Fig. 1)[15], namely: (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, sweroside, were used to qualitatively and quantitatively distinguish different origins of *L. japonica* and *L. confusa*. And, the analytical pattern of HPLC was determined from the corresponding peak curves. In addition, we could obtain a genetic marker between *L. japonica* and *L. confusa* from *trnL-trnF* sequences.

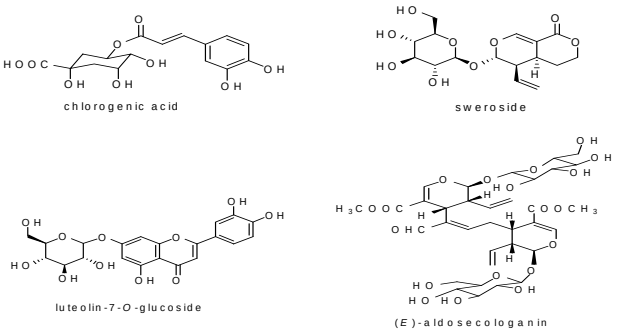

Figure 1. Chemical structures of standards.

Materials and methods

1. Materials

The materials were collected from fresh leaf and purchased from a commercial supplier in Korea and China (Table 1). Fresh leaves were only used as the method of genetic analysis. Samples were deposited in the herbarium of Korea Institute of Oriental Medicine. The identification of species was performed by the classification and identification committee of the national center for standardization of herbal medicines.

Table 1. Plant materials of *L. japonica* and *L. confusa*

No.	Species	Plant tissue	Code No.	Locality	
1		Fresh leaf	89-1-01	Hamyang,	Korea
2		Fresh leaf	89-1-04	Seongnam,	Korea
3		Fresh leaf	89-1-06	Jeju,	Korea
4		Fresh leaf	89-1-12	Jeonju,	Korea
5		Fresh leaf	89-1-13	Jinan,	Korea
6		Fresh leaf	89-1-14	Suwon,	Korea
7		Fresh leaf	89-1-11	Yunnan,	China
8		Fresh leaf	89-1-15	Anguk,	China
9		Dried flower	G-10-14	Andong,	Korea
10		Dried flower	G-10-19	Hongcheon,	Korea
11		Dried flower	G-10-21	Goheung,	Korea
12		Dried flower	G-10-22	Yangyang,	Korea
13		Dried flower	G-10-23	Bonghwa,	Korea
14	*L. japonica*	Dried flower	G-10-24	Gangneung,	Korea
15		Dried flower	G-10-25	Buyeo,	Korea
16		Dried flower	G-10-26	Goheong,	Korea
17		Dried flower	G-10-27	Uiseong,	Korea
18		Dried flower	G-10-28	Seorak,	Korea
19		Dried flower	G-10-29	Cheongsong,	Korea
20		Dried flower	G-10-30	Gunwi,	Korea
21		Dried flower	G-10-31	Uljin,	Korea
22		Dried flower	G-10-02	Henan,	China
23		Dried flower	G-10-16	Shandong,	China
24		Driedflower	G-10-36	Commercial*	Korea
25		Driedflower	R-LJ-2	Commercial*	Korea
26		Driedflower	R-LJ-3	Commercial*	Korea
27		Driedflower	R-LJ-6	Commercial*	Korea
28		Dried flower	S-11-01	Commercial*,	Korea
29	*L. confusa*	Dried flower	S-11-02	Commercial*,	Korea
30		Dried flower	S-11-03	Commercial*,	Korea
31		Dried flower	S-11-04	Commercial*,	Korea
32		Dried flower	S-11-05	Commercial*,	Korea
33		Dried flower	S-11-06	Hebei,	China
34		Dried flower	S-11-07	Hebei,	China
35		Dried flower	S-11-08	Hebei,	China
36		Dried flower	S-11-09	Hebei,	China
37		Dried flower	S-11-10	Hebei,	China

* : Be purchased in the herbs market.

2. Instrument and reagents

Agilent 1100 series HPLC instrument (Agilent Technologies, U.S.A.), equipped with a autosampler, column oven, binary pump, DAD detector and degasser was used. Data was acquired and processed by chemstation software (Agilent Technologies, U.S.A.). All the standards, (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside and sweroside were provided by Prof. S. S. Kang, National Seoul University,

Korea. Their purities were above 97% as determined by HPLC and LC MS/MS analysis.

3. Sample and standard solution preparation for HPLC

Sample preparation was as follows. Approximately 200 mg of the flower buds were pulverized, accurately weighed, and then it was extracted exhaustively with 10 mL of 70% (v/v) ethanol/water in ultrasonication for 2 hr. The residue was re-dissolved in 10 mL of 70% (v/v) ethanol/water for HPLC analysis and then it was filled up to 20 mL with 70% (v/v) ethanol/water. Four identified compounds of (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, and sweroside at the concentration of 2 mg/mL for each were prepared respectively by dissolving in 70% (v/v) ethanol. Each stock solution was diluted with 70% (v/v) ethanol to create seven calibration points (0.5, 5, 10, 25, 50, 100 and 500 ㎍/mL) for the preparation of the calibration curves. All solutions were filtered by a 0.45 ㎛ membrane filter unit prior to HPLC analysis, and were stable at least for 1 week when stored at 4℃.

4. Method validation

The analytical method was validated with respect to the linearity, limit of detection (LOD), limit of quantification (LOQ), and repeatability. The linearity of detection for each analyte was determined using seven different standard solutions (0.5, 5, 10, 25, 50, 100 and 500 ㎍/mL). A calibration was constructed by performing linear regression of peak area vs analyte concentration. The equations and linear ranges are listed in Table 3. The correlation coefficients (r2) of (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, and sweroside ranged 0.9991-0.9999. The limits of detection (LOD) and quantification (LOQ) under the chromatographic conditions were separately determined in six replicate determinations at a signal-to-noise ratio (S/N) of 3. TheLODs (S/N = 3) and LOQs (S/N = 10) obtained for each standard were 0.010-0.075 ㎍/mL and 0.025-0.250 ㎍/mL, respectively. Repeatability was described as the relative standard deviation (RSD) and was evaluated by analysing samples in triplicate. Mean RSDs of each compound for *L. japonica* (No. 9~27 of Table 1) and *L. confusa* (No. 28~37 of Table 1) were calculated (Table 4). The developed analytical method was subsequently applied to the simultaneous determination of the four components in Lonicera spp. extract. The contents of the analytes were determined from the corresponding calibration curves.

5. HPLC condition

The separation was carried out on a YMC-Pack Pro C18 (5 ㎛, 4.6 mm × 150 mm) and for gradient elution, 100% deionized water for A solvent and 100% acetonitrile for B solvent, to which 0.1% formic acid was added respectively at 25℃; the gradient program was used as follows: initial 0-10 min, linear change from A/B (85:15, v/v) to A/B (80:20, v/v); 10-20 min, linear change to A/B (80:20, v/v); 20-22 min, linear change to A/B (75:25, v/v); 22-35 min, linear change to A/B (65:35, v/v); 35-40 min, linear change to A/B (85:15, v/v). The flow rate was set at 0.3 mL/min and the injection volume was 10 ㎕ (Table 2).

Table 2. Solvent gradient condition for HPLC-DAD

Final time (min)	Flow rate (mL/min)	A	B
0	0.3	85	15
10	0.3	80	20
20	0.3	80	20
22	0.3	75	25
35	0.3	65	35
40	0.3	85	15

A : H_2O with 0.1% formic acid,
B : Acetonitrile with 0.1% formic acid.

6. Genomic DNA extraction

The genomic DNA of each sample was extracted according to the manual for the DNeasy® plant Mini kit (QIAGEN, U.S.A.). DNA concentration and purity were determined by spectrophotometry (Nanodrop ND-1000, Nanodrop, U.K.), and electrophoresis in a 1.5% agarose gel with DNA size marker (TaKaRa, Japan). The final concentration of each sample was diluted to approximately 20 ng/㎕ with DEPC (diethyl pyrocarbonate)-DW for PCR amplification.

7. PCR amplification of *trnL-trnF* region, cloning, sequencing

Primers *trnL-trnF* c (5'-CGA AAT CGG TAG ACG CTA CG-3') and *trnF* (5'-ATT TGA ACT GGT GAC ACG AG-3') described by Taberlet *et al.* [16] were used to amplify the *trnL-trnF* region of cpDNA. Total volume of 30 ㎕ reaction mixture contained 10 pmol/㎕ of each primer, 2× premix (Solgent, Korea) and 20 ng template. Reactions were carried out with a PTC-200 (MJ Research, U.S.A.). The temperature cycling paramerters were programmed for on cycle of 5 min. at 95℃, followed by 35 cycles of 30 sec. at 95℃, 30 sec. at 55℃, 1 min. at 72℃, and on cycle of 4 min. at 72℃. PCR products with LoadingSTAR (DyneBio, Korea) were electrophoresed in 1.5% agarose

gel, with a 100 bp DNA size marker (TaKaRa, Japan) in Tris-borate EDTA buffer. The amplified DNA fragments was separated by gel electrophoresis from the agarose gel using the Gel extraction kit (Promega, U.S.A.). These fragments were then subcloned in to the pGEM T-easy vector (Promega, U.S.A.). The nucleotide sequences of the resulting inserted DNA fragments were determined by an automatic DNA sequencer (ABI, 3730 Applied Biosystems, U.S.A.). Sequence alignment analysis was conducted using the DNASTAR® Lasergene® 7.2 software (U.S.A.) and compared with data of NCBI (National Center for Biotechnology Information).

8. Development of genetic markers

We performed sequences alignment analysis of *L. japonica* and *L. confusa* and then designed the LJCF primer (5'-TTC ACG GTC AAT ATC ATT ATT CAT AC-3'), LJCR primer (5'-TTT ACT AGA TAA CTA GGG TCT ATG TC-3'), LCF primer (5'-ATT TCT CAT CCA CCT TAC TTT AC-3') and LCR primer (5'-TGA ATA ATG ATA TTG ACC GTG AAT C-3') on the species-specific site. Total volume of 30 ㎕ reaction mixture contained 5 pmol/㎕ of each primer, 2× premix (Solgent, Korea) and 20 ng template. Reactions were carried out with a PTC-200 (MJ Research, U.S.A.). The temperature cycling paramerters were programmed for on cycle of 5 min. at 95℃, followed by 30 cycles of 20 sec. at 95℃, 30 sec. at 47℃, 60 sec. at 72℃, and on cycle of 5 min. at 72℃. PCR products with LoadingSTAR (DyneBio, Korea) were electrophoresed in 2.0% agarose gel, with a 100 bp DNA size marker (TaKaRa, Japan) in Tris-borate EDTA buffer.

Results

1. Method validation

The analytical method was validated with respect to the linearity, limit of detection (LOD), limit of quantification (LOQ), and repeatability. The linearity of detection for each analyte was determined using seven different standard solutions (0.5, 5, 10, 25, 50, 100 and 500 ㎍/mL). A calibration was constructed by performing linear regression of peak area vs analyte concentration.The equations and linear ranges are listed in Table 3. The correlation coefficients (r2) of (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, and sweroside ranged 0.9991-0.9999. TheLODs (S/N = 3) and LOQs (S/N = 10) obtained for each standard were 0.010-0.075 ㎍/mL and 0.025-0.250 ㎍/mL, respectively. Repeatability was described as the relative standard deviation (RSD) and was evaluated by analysing samples in triplicate (Table 4).

Table 3. Linear range, linear equation, correlation coefficient, limits of detection (LOD), and limits of quantitation (LOQ) for standards

Compounds	Linearrange (㎍/mL)	Linear equation	r2	LOD (㎍/mL)	LOQ (㎍/mL)
(E)-aldosecologanin	0.5-500	y = 51.6795x - 33.61	0.9999	0.010	0.025
Chlorogenic acid	0.5-500	y = 43.5509x - 299.69	0.9991	0.075	0.250
Luteolin 7-O-glucoside	0.5-500	y = 66.626x - 275.65	0.9995	0.050	0.125
Sweroside	0.5-500	y = 52.4410x - 163.89	0.9997	0.025	0.050

Table 4. Mean contents and RSDs of the identified compounds in *L. japonica* and *L. confusa*

Compound	*L. japonica*[a]		*L. confusa*[b]	
	Mean content (mg/g)	Mean RSD (%)	Mean content (mg/g)	Mean RSD (%)
(E)-aldosecologanin	0.37±0.48	3.02±2.40	N.D.[c]	-
Chlorogenic acid	8.09±8.24	5.83±3.86	33.14±6.74	4.50±4.32
Luteolin 7-O-glucoside	0.99±0.30	4.12±3.44	1.00±0.07	4.77±2.91
Sweroside	1.63±2.37	4.80±3.03	N.D.[c]	-

[a] : No. 9~27 of Table 1, [b] : No. 28~37 of Table 1,
[c] : not detected.

2. Quantitative analysis of the identified compounds

A comparative analysis of HPLC-DAD pattern was performed for *L. japonica* and *L. confusa* under the condition where (E)-aldosecologanin, chlorogenic acid, luteolin 7-O-glucoside, and sweroside were used as identified compounds. The result of quantitative analysis is summarized in Table 4. The retention times of each identified compound were as follows; 14.9 min for chlorogenic acid; 17.8 min for sweroside; 31.5 min for luteolin 7-O-glucoside; and 34.7 min for (E)-aldosecologanin. For *L. japonica*, besides the aforementioned retention times, additional unknown peaks were detected at 13.8 min, 20.6 min, and 36.9 min. Upon analysis, two identified compounds and two unknown peaks were detected in both species, in the order of; chlorogenic acid, 14.9 min; unknown peak, 20.6 min; luteolin 7-O-glucoside, 31.5 min; and unknown peak, 36.9 min. A further unknown peak at 13.8 min, sweroside at 18.2 min, and (E)-aldosecologanin at 34.7 min, were only detected in *L. japonica* (Fig. 2).

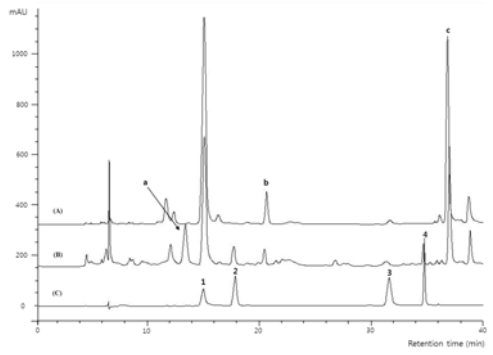

Figure 2. Chromatographies of *L. confusa* (A), *L. japonica*(B) and standard mixture (C). Column: YMC-Pack Pro C18 (5 ㎛, 4.6 mm × 150 mm); temperature of 25℃; DAD detector. Peaks: 1, chlorogenic acid; 2, sweroside; 3, luteolin 7-O-glucoside; 4, (E)-aldosecologanin; a, unknown at 13.8 min; b, unknown at 20.6 min; c, unknown at 36.9 min.

3. Analysis of cpDNA *trnL-trnF* region

As a result of analyzing *trnL-trnF* regions of Lonicerae Flos including whole *trnL* intron of 944-958 bp, 3' exon of *trnL* gene of 44 bp and *trnL-trnF* intergenic spacer of 671-707 bp. *L. japonica* exhibited a length of 947 bp and *L. confusa* exhibited one of 957 bp. Therefore, as a result of analyzing DNA sequences of both *L. confusa* and *L. japonica*, a total of 12 sequence gap (192 bp, 667-671 bp, 736-741 bp) were identified, and were shown to have 98% homology (Fig. 3). Two species were analyzed for their GC content. The result showed that the GC content of *L. japonica* was 35.8%, and the GC content of *L. confusa* was 35.2%.

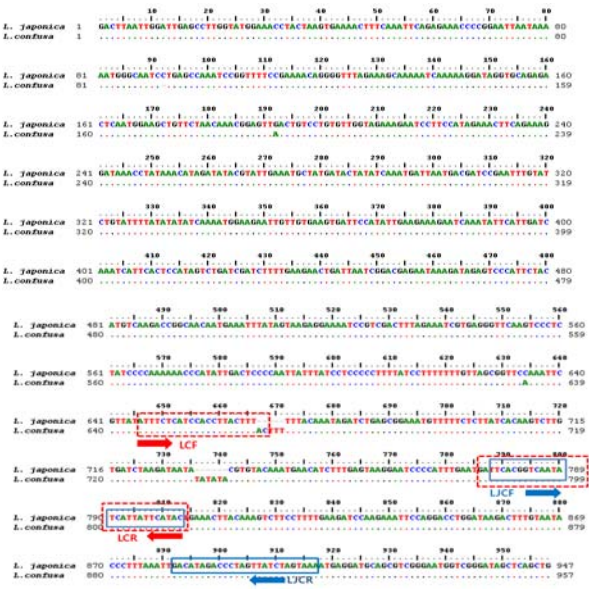

Figure 3. Aligned sequences of the *trnL-trnF* regions in chloroplast DNA. Boxes indicate the position of LJC primers and LJC primers for *L. japonica* and *L. confusa*. Hyphens are gaps required for alignment. Dots in the sequences denote the same nucleotide state as the sequence of *L. japonica*.

4. Detection of LC molecular genetic marker

In order to design markers that distinguish between *L. japonica* and *L. confusa*, an LCF/LCR primer pair that is amplified at 164 bp in *L. confusa*, but not so in *L. japonica*, was identified. The LCF/LCR primer pair was designed to be *L. confusa* specific, based on *trnL-trnF* sequences, and was confirmed to effectively distinguish *L. japonica* from *L. confusa*. At 130 bp, an internal LJCF/LJCR primer pair was amplified to be present in both *L. japonica* and *L. confusa* (Fig. 4). As a results, the listed samples were monitored using the specific band of 164 bp size for its identification (Table 5). Thus, *L. japonica* and *L. confusa* were accurately distinguished using specific primer in *trnL-trnF* region.

Table 5. Identification results of *L. japonica* and *L. confusa* using designed molecular marker

No.	Specific Band (164 bp)	Identification Results	No.	Specific Band (164 bp)	Identification Results
1	-	*L. japonica*	20	-	*L. japonica*
2	-	*L. japonica*	21	-	*L. japonica*
3	-	*L. japonica*	22	-	*L. japonica*
4	-	*L. japonica*	23	-	*L. japonica*
5	-	*L. japonica*	24	-	*L. japonica*
6	-	*L. japonica*	25	-	*L. japonica*
7	-	*L. japonica*	26	-	*L. japonica*
8	-	*L. japonica*	27	-	*L. japonica*
9	-	*L. japonica*	28	+	*L. confusa*
10	-	*L. japonica*	29	+	*L. confusa*
11	-	*L. japonica*	30	+	*L. confusa*
12	-	*L. japonica*	31	+	*L. confusa*
13	-	*L. japonica*	32	+	*L. confusa*
14	-	*L. japonica*	33	+	*L. confusa*
15	-	*L. japonica*	34	+	*L. confusa*
16	-	*L. japonica*	35	+	*L. confusa*
17	-	*L. japonica*	36	+	*L. confusa*
18	-	*L. japonica*	37	+	*L. confusa*
19	-	*L. japonica*			

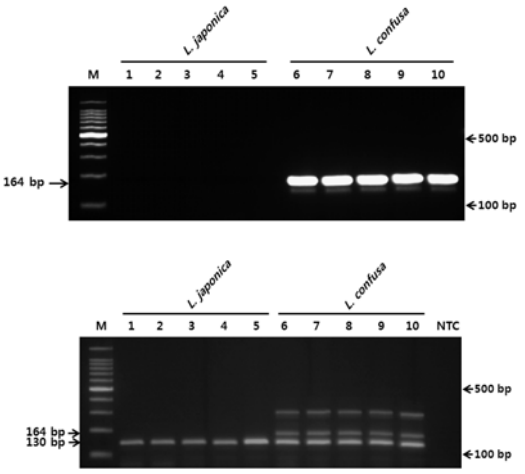

Figure 4. PCR products using species-specific primer designed from *L. japonica* and *L. confusa* (A) and multiplex PCR products using the primer pairs designed from *L. japonica* and *L. confusa* (B) (lane 1-5; number 1~3, 7~8 of Table 1, lane 6-10; number 28~30, 33~34 of Table 1, NTC; no template control, M; 100 bp DNA ladder).

Discussion

In quality assessment of *L. japonica* by HPLC, luteolin 7-O-glucoside was proposed as an index compound[17]. However, luteolin 7-O-glucoside is an inappropriate marker as it is also detected in *L. confusa* and shows a significant deviation among samples. Previously, chlorogenic acid has been used as the chemical marker for the quality evaluation of Lonicerae Flos, owing to its antipyretic and antibiotic property as well as its high content in the herb. However, chlorogenic acid alone could not be responsible for the overall pharmacological activities of Lonicerae Flos. Chlorogenic acid has also been used as the chemical marker for other herbal drugs such as Chrysanthemi Flos[18]. By comparing the overall HPLC profiles of the different samples from Lonicera spp., we found it possible to distinguish clearly the two species in terms of the occurrence and/or relative concentration of iridoid glucosides. *L. japonica* has sweroside as the major iridoid glucosides. A variety of physiologically active compounds (such as luteolin, chlorogenic acid) has been found presented in *L. confusa*. Chlorogenic acid is the main effective constituent *in L. confusa*. Because all four constituents in the crude drugs contains nearby phenolic hydroxyl groups that was employed for their sensitive and selective detection. Therefore, other compounds should also be considered as one of the markers for quality control of Lonicerae Flos. In the comparative analysis between *L. japonica* and *L. confusa* using HPLC-DAD after selection of four index compounds of derived from *L. japonica*, Sweroside and (E)-aldosecologanin were only detected in *L. japonica* and are likely to be used as a discrimination marker to distinguish between *L. japonica* and *L. confusa*. The results of this study were consistent with results of a previous study by Li *et al.*[19] on the development of HPLC methods used to determine the biological origin of Lonicerae Flos using iridoid glucosides such as 7-epi-loganin, sweroside, loganin, 7-epi-vogeloside, and secoxyloganin.

In phylogenetic tree analysis of Lonicerae Flos, the plant was divided into the groups of L. japonica and *L. confusa* (Data not shown). The differentiation between *L. japonica* and *L. confusa* was investigated through the *trnL-trnF* region. When the nucleotide sequences of the *trnL-trnF* region were analyzed in phylogenetic tree, it was divided into two major groups based on genotype classification, though its place-of-origin was not confirmed. The results of homology analysis using the sequences of each sample from *L. japonica* showed more than 98% homology. Thus, this result confirmed that a homology percentage of inter-species nucleotide sequences exceeded 100% for *L. confusa*, the with no sequence variations between individuals identifiable from the samples in this study. However, the variations of base sequences between individuals are likely to be confirmed if more samples, collected from different locations, are analyzed. The analysis of sequence variation in the cpDNA has recently become an effective method of identification of medicinal herbs, and was applied in this case because the discrimination of *L. japonica* from its adulterants is currently limited to methods of morphology and chemical fingerprinting[20]. In addition, when PCR analysis was performed on herbal medicines available in the market, using molecular genetic markers which were designed based on cpDNA, *L. japonica* and *L. confusa* were also distinguished by the amplified 164 bp band. Therefore, *L. japonica* and *L. confusa* were effectively distinguished from one another by using a molecular genetic method.

Conclusion

We attempted to discriminate objectively both *L. japonica* and *L. confusa* using the chemical marker and genetic marker. The results are as follows.

1) This HPLC-DAD method is very suitable for identification between *L. japonica* and *L. confusa* when Sweroside is chosen as chemical marker. Thus, by means of chemical evidence we could rapidly clarify the botanical origin of these crude herbs. Furthermore, it will hopefully be employed to Sweroside within the genus, and within other medicinal plants.
2) The genetic marker obtained by DNA analysis will be useful as an important discrimination standard that can complement current laboratory tests.
3) A combination of the coupled methods would be the ideal in providing confirmative identification and assessment of *L. japonica*.

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