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Molecular authentication of multi-species honeysuckle tablets

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ABSTRACT. Authenticating multi-species original raw materials in commercial formulations is difficult. Jin Yin Hua and Shan Yin Hua, both classified as raw honeysuckle materials in the Chinese Pharmacopoeia, are used in various medicines. Differentiating one variety from another is difficult based on chemical analysis. We developed molecular authentication of multi-species original honeysuckle in 3 brands of commercial tablets using allele-specific PCR. All 3 tablets contained both Jin Yin Hua and Shan Yin Hua. We also built a PCR-enzyme digestion method and enzymatic mutation detection in the PCR fragments of *psbA-trnH* and *trnL-trnF*, and the restriction endonucleases *Hin*fI and *Nla*IV, respectively. The PCR-enzyme digestion method produced the same result as the allele-specific PCR. Sequence and phylogenetic analyses show that the tablets YXC and YQJ contained *Lonicera japonica* and *L. macranthoides* as original raw materials, and LYG contained *L. japonica*, *L. hypoglauca*, and *L. macranthoides*.

Key words: SNP genotyping; Restriction endonuclease; Honeysuckle; Chloroplast sequences; Commercial tablets

INTRODUCTION

Raw materials are primarily used in producing commercial tablets in Chinese material medicine. The compounds contained in original raw materials consisting of multiple species are different from those in materials composed of a single species. Differences in contents of tablets can be due to the use of different species and can result in different efficacy.

Honeysuckles are used in herbal medicines, and they possess biological and pharmaceutical properties such as anti-bacterial, anti-inflammatory, anti-viral, liver protectant, anti-angiogenic, and anti-nociceptive activities (Ku et al., 2009; Rahman and Kang, 2009; Ryu et al., 2010; Kang et al., 2010). Five species of honeysuckle, namely, *Lonicera* (L.) *japonica*, *L. hypoglauca*, *L. macranthoides*, *L. confuse*, and *L. fulvotomentosa*, are recorded in the Chinese Pharmacopoeia (Commission, 2010). Only *L. japonica* was indicated as Jin Yin Hua, and 4 other species represented Shan Yin Hua (Commission, 2010). Chlorogenic acid is used in evaluating the chemical quality of these 5 species, and both dipsacoside B and macranthoidin B are used only to evaluate *L. hypoglauca*, *L. macranthoides*, *L. confuse*, and *L. fulvotomentosa* (Chai et al., 2005; Shang et al., 2011). However, the similarities in morphological and biochemical characteristics prevent the application of chemical analysis in determining which species are used in producing a commercial tablet (Pu et al., 2002).

Chloroplast sequences are candidates for species recognition (Kress et al., 2005). Sun et al. (2011) published the sequences of *rbcL*, *matK*, *psbA-trnH*, and *trnL-trnF* of *L. japonica* and its closely related species. We collected and compared 1002 chloroplast sequences of *Lonicera* Linn. from the GenBank database and obtained 1 SNP (C/A) in trnL-trnF and 2 SNP (G/A, A/T) in psbA-trnH among *L. japonica* and its 18 closely related species, which include *L. hypoglauca*, *L. macranthoides*, *L. confuse*, and *L. fulvotomentosa*. Based on the SNP in *trnL-trnF*, we designed an allele-specific polymerase chain reaction (PCR) method for the molecular authentication of *L. japonica* medicinal materials (Jiang et al., 2012).

In this study, we also developed a PCR-enzyme digestion method for the molecular authentication of *L. japonica* medicinal materials based on the SNP in *psbA-trnH*. Three types of commercial tablets in Chinese material medicine that contain honeysuckle were initially submitted to molecular authentication using allele-specific PCR and a PCR-RFLP method. *trnL-trnF* sequences from the DNA of the commercial tablets were analyzed to determine which honeysuckle species were used in producing the tablets.

MATERIAL AND METHODS

Sample collection

Samples of honeysuckle including *L. japonica*, *L. hypoglauca*, *L. macranthoides*, *L. confuse*, and *L. fulvotomentosa* were collected from Guangxi, Henan, Shandong, Jiangsu, Beijing, and Anhui Provinces in China. All samples collected were identified by a taxonomist (Table 1). Three types of tablet samples, namely, Fu Fang Yu Xing Cao Pian, Lin Yang Gan Mao Pian, and Yin Qiao Jie Du Pian, were purchased from different pharmacies (Table 2).

Table 1. Plant samples used in the study.

No.	Species	Source	Numbers	Collection date
1	*Lonicera japonica*	Linyi, Shandong	12	2011.7.6
2	*L. japonica*	Lianyungang, Jiangsu	12	2011.7.4
3	*L. japonica*	Fengqiu, Henan	12	2011.7.1
4	*L. japonica*	Xinmi, Henan	6	2011.7.2
5	*L. japonica*	Jinan, Shangdong	5	2012.6.1
6	*L. japonica*	Hefei, Anhui	1	2011.9.7
7	*L. japonica*	Nanning, Guangxi	4	2011.4.21
8	*L. japonica*	Guiling, Guangxi	2	2011.4.22
9	*L. japonica*	Beijing	7	2012.5.25
10	*L. japonica*	Nanjing, Jiangsu (processing)	10	2011.11.12
11	*L. japonica*	Beijing (processing)	25	2012.3.7
12	*L. hypoglauca*	Nanning, Guangxi	6	2011.4.21
13	*L. hypoglauca*	Chongzuo, Guangxi	6	2011.4.24
14	*L. hypoglauca*	Guilin, Guangxi	12	2011.4.22
15	*L. confusa*	Guilin, Guangxi	3	2011.4.23
16	*L. macranthoides*	Nanning, Guangxi	6	2011.4.21
17	*L. macranthoides*	Guilin, Guangxi	5	2011.4.22
18	*L. macranthoides*	Linyi, Shandong	6	2012.5.20
19	*L. fulvotomentosa*	Nanning, Guangxi	6	2011.4.21
20	*L. maackii*	Beijing	5	2012.3.9
21	*L. maackii*	Hefei, Anhui	1	2011.9.7
22	*L. kawakamii*	Beijing	1	2012.6.22
23	*L. tatarica* var. *tatarica*	Beijing	4	2012.3.9
24	*L. tatarica* cv. '*Fanguo*'	Beijing	4	2012.3.9
25	*L. microphylla*	Beijing	1	2012.6.22
26	*L. ferdinandii*	Beijing	1	2012.6.22
27	*L. similis*	Linyi, Shandong	1	2012.5.25
28	*L. tragophylla*	Linyi, Shandong	1	2012.5.25

Table 2. Tablet samples used in the study.

No.	Tablet*	Raw materials
YXC	Fu Fang Yu Xing Cao Pian	Honeysuckle, chameleon, weeping forsythia
LYG	Lin Yang Gan Mao Pian	Honeysuckle, platycodon root
YQJ	Yin Qiao Jie Du Pian	Honeysuckle, platycodon root

*Sequences in Supplementary material.

Genomic DNA extraction and PCR amplification

The genomic DNA was isolated from the sample (25 mg each) using the modified CTAB method (Li et al., 2007). Total genomic DNA was used in the allele-specific PCR according to the protocol established by Jiang et al. (2012). PCR was performed by adding 2.5 µL 10X Ex Taq buffer, 2 µL 10 mM dNTPs, 0.5 U Ex Taq (Takara, China), and 10 ng DNA per reaction volume as well as 0.2 pmol of each forward and reverse primer. Total genomic DNA was also used in PCR to amplify trnL-trnF and psbA-trnH from *L. japonica* and its closely related species, respectively. The universal primers, sequences, and PCR conditions used in the amplification are presented in Table 3.

Development of the PCR-enzyme digestion method

Sequence assembly and consensus sequence generation were performed using BioEdit v. 7.1.3. The analysis of restriction endonuclease sites was performed using Primer Premier

5.0 (Primer, Canada). The amplified PCR products of *trnL-trnF* and *psbA-trnH* were digested with *Nla*IV (recognition: GGNN^CC) and *Hin*fI (recognition: G^ANTC), respectively.

Table 3. Universal gene specific primers, sequences and their reaction conditions.

Primer	Name	Sequence 5'→3'	Reaction conditions
Lonicera japonica primer	Lj-1F	GTTGACTGTCCTGTGTTGGT	94°C 5 min,
	Lj-1R	TGAGAAATATAACGAATTTAG	94°C 30 s, 54°C 30 s, 72°C 45 s, 35 cycles, 72°C 7 min
Non-*Lonicera japonica* primer	Lj-2F	TATCCTTTTTTTGTTAGCGGTTGA	94°C 5 min,
	Lj-2R	CTATCCCGACCATTCCC	94°C 30 s, 54°C 30 s, 72°C 45 s, 35 cycles, 72°C 7 min
Universal primers 1	trnL	CGAAATCGGTAGACGCTACG	94°C 5 min,
	trnF	ATTTGAACTGGTGACACGAG	94°C 30 s, 54°C 30 s, 72°C 45 s, 30 cycles*, 72°C 7 min
Universal primers 2	psbA	GTTATGCATGAACGTAATGCTC	94°C 5 min,
	trnH	CGCGCATGGTGGATTCACAATCC	94°C 30 s, 56°C 30 s, 72°C 30 s, 30 cycles*, 72°C 7 min

*36 cycles for tablet samples.

Cloning, sequencing, and sequence analysis

The amplified PCR products of psbA-trnH were cloned into pMD19-T vector (Takara, Japan). Ten clones were randomly selected from each sample and directly sequenced at Beijing Genomics Institute, China, using a 3730XL sequencer (Applied Biosystems, USA). Sequence assembly and consensus sequence generation were performed using DNA STAR (Madison, WI, USA). The boundary of the psbA-trnH clones was determined according to the annotations of similar sequences in GenBank. To perform a phylogenetic study, the sequences of the DNA regions were aligned using ClustalW (Thompson et al., 1994), and the genetic distances were computed using MEGA 5.0 (Tamura et al., 2011) in accordance with the Kimura 2-parameter model.

RESULTS AND DISCUSSION

In recent years, several technologies such as morphology, chemical analysis (Xie et al., 2006), biological activity assay (Bai et al., 1997; Liu et al., 2006), and DNA molecular markers (Yip et al., 2007; Al-Qurainy et al., 2011; Guo et al., 2011) have been developed for authenticating raw herbal materials. With their characteristics of non-reliance on morphology, reproducibility, extreme reliability, and easy manipulation, DNA-based molecular tools are urgently needed in authenticating raw herbal materials. Authentication is essential for the standardization of Chinese medicines (Zhao et al., 2006). Authentication of raw materials is also required for commercial tablets in Chinese material medicine to maintain the quality of these tablets. However, few studies have focused on such molecular authentication. Although many studies have explored the identification of raw honeysuckle using DNA-based molecular tools, such as DNA barcoding (Sun et al., 2011), PCR-RFLP (Peng et al., 2010) and ITS sequencing (Hu et al., 2012), they have not focused on how to authenticate honeysuckle in tablets.

Coghlan et al. (2012) developed a high-throughput sequencing approach to authenticate the animal and plant composition of 15 traditional commercial Chinese medicine preparations, but this approach was too expensive and time-consuming. In this study, 3 types of commercial tablets containing honeysuckle were purchased from pharmacies and authenticated through the allele-specific PCR method. The results showed that all 3 types of commercial tablet contained *L. japonica* and its closely related species (Figure 1).

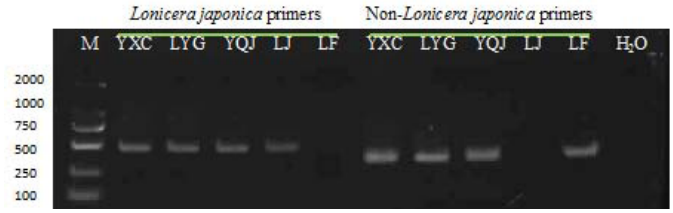

Figure 1. Allele-specific PCR using *Lonicera japonica* primers and non-*Lonicera japonica* primers. YXC, LYG, and YQJ = 3 kinds of tablet samples, FFYXCP, LYGMP, and YQJDP. LJ = *Lonicera japonica* medical material; LF = *L. fulvotomentosa* medical material. *Lane M* = DL2000 marker.

To verify the results, we developed an authentication method based on PCR-enzyme digestion. Based on the SNP (C/A) in *trnL-trnF* and SNP (G/A) in *psbA-trnH* between *L. japonica* and its closely related species, special restriction endonuclease sites, *Nla*IV and *Hin*fI, were detected in chloroplast sequences of *L. japonica*. *trnL-trnF* and *psbA-trnH* fragments of 165 honeysuckle samples were amplified, and their PCR products were digested by *Nla*IV and *Hin*fI. The results showed that PCR-*Nla*IV and *Hin*fI digestion could be used in authenticating *L. japonica* and its closely related species (Figure 2). The tablets' PCR fragments amplified by *trnL-trnF* and *psbA-trnH* were digested by *Nla*IV and *Hin*fI, respectively (Figure 3). The results were similar to those of the allele-specific PCR method.

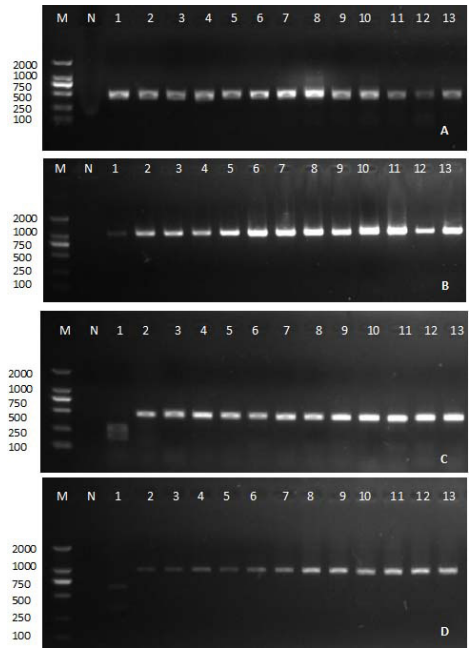

Figure 2. Authenticating honeysuckle samples using PCR-enzyme digestion. **A.** *psbA-trnH* fragment; **B.** *trnL-trnF* fragment; **C.** *psbA-trnH* fragments digested by *Hin*fI; **D.** *trnL-trnF* fragments digested by *Nla*IV. *Lane M* = DL 2000 marker; *lane N* = ddH_2O as negative control; *lane 1 = Lonicera japonica*; *lane 2 = L. hypoglauca*; *lane 3 = L. confusa*; *lane 4 = L. macranthoides*; *lane 5 = L. maackii*; *lane 6 = L. fulvotomentosa*; *lane 7 = L. kawakamii*; *lane 8 = L. tatarica* var. *tatarica*; *lane 9 = L. tatarica* cv. '*Fanguo*'; *lane 10 = L. microphylla*; *lane 11 = L. ferdinandii*; *lane 12 = L. similis*; *lane 13 = L. tragophylla*.

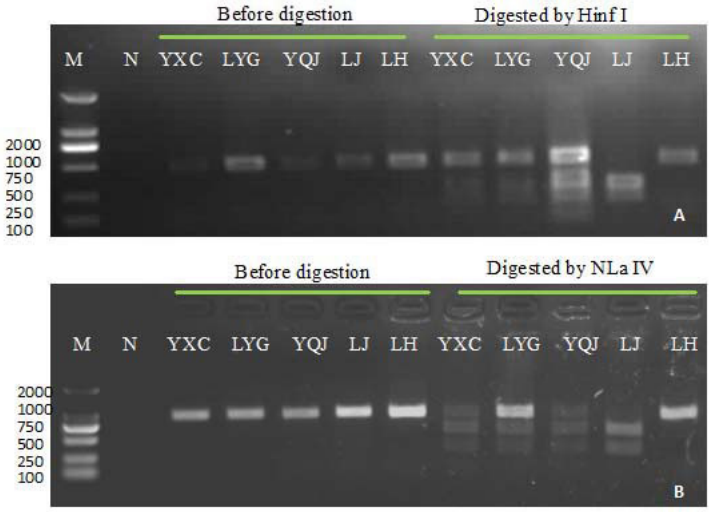

Figure 3. Endonuclease restriction analysis of 3 tablet samples. **A.** *psbA-trnH* PCR productions digested by *Hin*fI; **B.** *trnL-trnF* PCR productions digested by *Nla*IV. *Lane M* = DL 2000 marker; *lane N* = ddH_2O as negative control; YXC, LYG, and YQJ = 3 kinds of tablet samples, FFYXCP, LYGMP, and YQJDP; LJ = *Lonicera japonica*; LH = *L. hypoglauca*.

However, aside from *L. japonica*, other species used in producing commercial tablets could still not be distinguished. Thus, we selected an accepted chloroplast sequence psbA-trnH and obtained a mixed PCR production from the DNA of the tablet. The mixed PCR production was cloned into PMD19-T vector and 10 single PCR clones were randomly selected and sequenced. Honeysuckle species in the tablets were distinguished using sequence and phylogenetic analyses.

Phylogenetic analysis showed that 2 clones in YXC, 4 clones in LYG, and 3 clones in YQJ were clustered with *L. japonica*, 8 clones in YXC with *L. confuse*, 4 clones in LYG with *L. hypoglauca* and *L. macranthoides*, and 7 clones in YQJ with *L. macranthoides* and *L. confuse* (Figure 4).

BLAST results also revealed 10 clones in YXC, 8 clones in LYG, and 10 clones in YQJ annotated with honeysuckle species. Further analysis showed that 2 clones in YXC and 3 clones in YQJ were annotated with *L. japonica* and the rest of the clones with *L. macranthoides*. Five clones in LYG were annotated with *L. japonica*, 1 clone with *L. hypoglauca*, and 2 clones with *L. macranthoides* (Table 4).

In conclusion, we suggest that the honeysuckle species *L. japonica* and *L. macranthoides* can be used in YXC and YQJ, and *L. japonica*, *L. hypoglauca*, and *L. macranthoides* can be used in LYG. Although these species have similar compositions, they differ slightly in terms of the content of active compounds (Pu et al., 2002; Ren et al., 2008). The content of chlorogenic acid is lower in *L. japonica* than in *L. macranthoides* and *L. hypoglauca* (Zhou and Tong, 2003; Chen et al., 2005). However, chlorogenic acid was the only compound used in evaluating the quality of the tablets, and the price of *L. japonica* was higher than that of other species. Thus, considering the suitable chlorogenic acid content and the production cost, mixed species of original honeysuckle were used in producing the tablets, thus making it difficult to distinguish the species used in these tablets by chemical analysis.

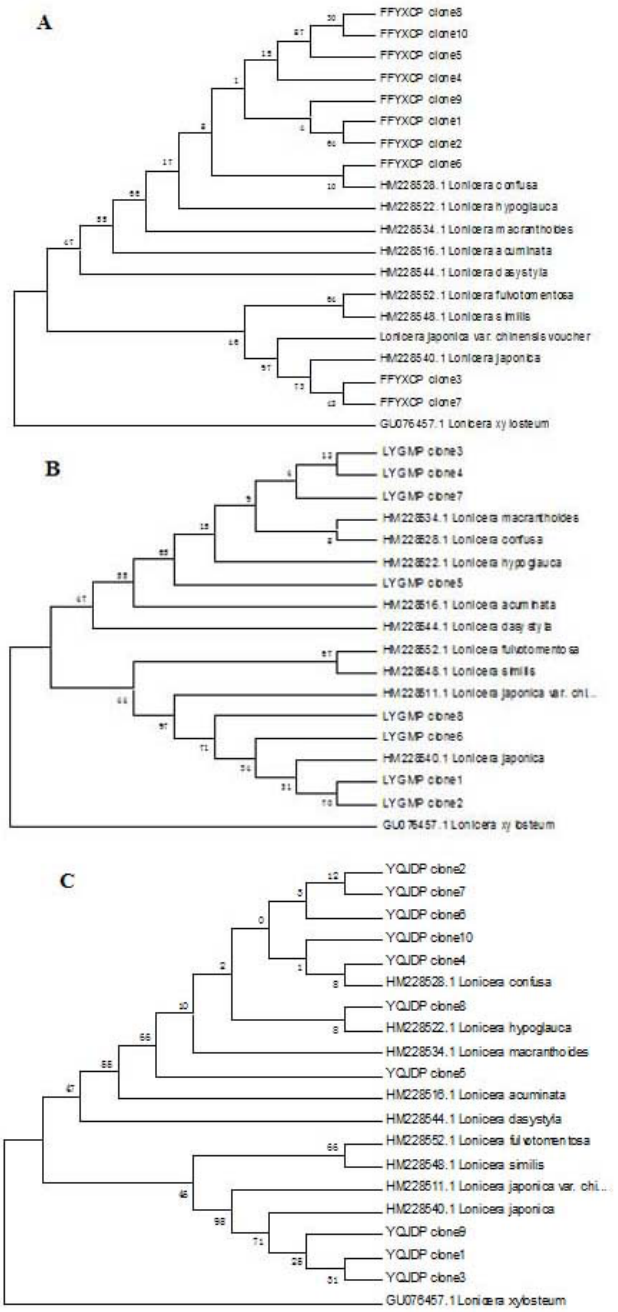

Figure 4. Phylogenetic analysis of psbA-trnH clones from 3 kinds of tablet samples and psbA-trnH sequences of *Lonicera japonica*, *L. confuse*, *L. hypoglauca*, *L. macranthoides*, *L. acuminata*, *L. dasystyla*, *L. fulvotomentosa*, *L. similis*, *L. xylosteum* from GenBank. **A.** Tablet Fu Fang Yu Xing Cao Pian (FFYXCP); **B.** tablet Ling Yang Gan Mao Pian (LYGMP); **C.** tablet Yin Qiao Jie Du Pian (YQJDP).

Table 4. BLAST results of honeysuckle in three tablets.

No.	Tablet	Total clones			Honeysuckle clones		
		Total	Honeysuckle	Others	Total	*L. japonica*	Others
YXC	Fu Fang Yu Xing Cao Pian	10	10	0	10	2	8
LYG	Lin Yang Gan Mao Pian	10	8	2	8	5	3
YQJ	Yin Qiao Jie Du Pian	10	10	0	10	3	7

We provide here a sequencing method to detect the species in complex prescriptions and to evaluate the quality of commercial tablets.

Supplementary material

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