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A comparative study of *Lonicera japonica* with related species: Morphological characteristics, ITS sequences and active compounds

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ABSTRACT

Morphological characteristics, ITS sequences, and active compounds have been used to differentiate between species of *Lonicera* used in the traditional Chinese medicines Flos Lonicerae Japonicae (FLJ) and Flos Lonicerae (FL). FLJ includes *L. japonica* whereas FJ includes *Lonicera macranthoides*, *Lonicera hypoglauca*, *Lonicera confusa* and *Lonicera fulvotomentosa*. FLJ could be distinguished from FL using four quantitative and 10 qualitative characters, ITS sequences, chlorogenic acid and luteoloside contents. Analyses revealed that *L. japonica* was very different from the other species. The results have implications for the identification and quality control of species of *Lonicera* used medicinally, suggesting that species should not be interchanged in medicinal preparations.

1. Introduction

Flos Lonicerae Japonicae (FLJ), the flower buds of *Lonicera japonica* Thunb. (Caprifoliaceae), have been used as a common traditional Chinese medicine for the treatment of exopathogenic wind-heat, epidemic febrile diseases, sores, carbuncles, furuncles, and some infectious diseases (Committee for the Pharmacopoeia of PR China, 2010; Shang et al., 2011). Recently, FLJ has been used extensively to prevent and treat some serious human and veterinary viral diseases including SARS coronavirus and H1N1 (Swine) flu virus. Therefore, FLJ has been called the 'Plant antibiotic' (Jiao, 2009). It has also been used as a food and as a healthy beverage, e.g. the famous Chinese herbal tea of 'Wang Lao Ji', all over the world (Wang, 2010). The flower buds from several species of *Lonicera*, such as *L. macranthoides* Hand. Mazz., *L. fulvotomentosa* Hsu., *L. hypoglauca* Miq., *L. confusa* DC., and *L. similis* Hemsl., are also considered as alternatives to FLJ in folk medicines and are described as 'Jin Yin Hua' (alternative name of FLJ) in the Chinese Pharmacopoeia (Xu et al., 1988; Committee for the Pharmacopoeia of PR China, 2000). Before 2005, *L. japonica* and some related species were described as the original plants of FLJ, and they were not distinguished in practical applications. Since 2005, Flos Lonicerae (FL), the flower buds of *L. macranthoides* Hand. Mazz., *L. hypoglauca* Miq., and *L. confusa* DC. have been listed in the Pharmacopoeia of the People's Republic of China (PPRC) (Committee for the

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Table 1
Species, code, locality, latitude and longitude of 30 *Lonicera* germplasm samples used in this study.

Species	Germplasm	Code	Locality (county/city, province)	Latitude/longitude	GenBank accession number
Lonicera japonica Thunb.	Cultivar with yellow flowers	Lj_JZ	Jingning, Zhejiang	N27°58′/E119°37′	KF160894
		Lj_PS1	Pingyi, Shandong	N35°30′/E117°38′	KF160897
		Lj_BA	Bozhou, Anhui	N33°50′/E115°46′	KF160899
		Lj_SJ	Shuyang, Jiangsu	N34°06′/E118°45′	KF160901
		Lj_JJ	Jiujiang, Jiangxi	N29°42′/E115°59′	KF160903
		Lj_XXH	Xinxiang, Henan	N35°18′/E113°55′	KF160905
		Lj_QG	Qingyuan, Guangdong	N23°41′/E113°03′	KF160907
		Lj_TH	Tianmen, Hubei	N30°39′/E113°09′	KF160908
		Lj_XTH	Xingtai, Hebei	N37°04′/E114°29′	KF160909
		Lj_PS2	Pingyi, Shandong	N35°30′/E117°38′	KF160895
	Wild	Ljw_PZ	Panan, Zhejiang	N29°03′/E120°26′	KF160912
		Ljw_QZ	Qingyuan, Zhejiang	N27°37′/E119°03′	KF160890
		Ljw_SF	Shouning, Fujiang	N27°27′/E119°30′	KF160892
		Ljw_LZ	Liandu, Zhejiang	N28°26′/E119°54′	KF160893
		Ljw_JZ1	Jingning, Zhejiang	N27°58′/E119°37′	KF160896
		Ljw_JZ2	Jingning, Zhejiang	N27°58′/E119°37′	KF160898
		Ljw_QZ	Qingtian, Zhejiang	N28°08′/E120°17′	KF160904
Lonicera japonica Thunb. var. chinensis	Cultivars with red flowers	Ljv_PS1	Pingyi, Shandong	N35°30′/E117°38′	KF160886
		Ljv_PS2	Pingyi, Shandong	N35°30′/E117°38′	KF160891
		Ljv_BA	Bozhou, Anhui	N33°50′/E115°46′	KF160900
		Ljv_JJ	Jiujiang, Jiangxi	N29°42′/E115°59′	KF160902
		Ljv_XXH	Xinxiang, Henan	N35°18′/E113°55′	KF160906
Lonicera macranthoides Hand. Mazz.	Cultivars	Lm_LH1	Longhui, Hunan	N27°07′/E111°01′	KF160887
		Lm_LH2	Longhui, Hunan	N27°07′/E111°01′	KF160888
		Lm_LH3	Longhui, Hunan	N27°07′/E111°01′	KF160889
	Wild	Lmw_LH	Longhui, Hunan	N27°07′/E111°01′	KF160915
Lonicera hypoglauca Miq.	Wild	Lhw_LZ1	Liandu, Zhejiang	N28°26′/E119°54′	KF160910
		Lhw_YZ	Yongjia, Zhejiang	N28°09′/E120°41′	KF160911
		Lhw_LZ2	Liandu, Zhejiang	N28°26′/E119°54′	KF160914
Lonicera fulvotomentosa Hsu.	Wild	Lfw_QG	Qianxinan, Guizhou	N25°05′/E104°54′	KF160913

Pharmacopoeia of PR China, 2005). In the 2010 PPRC, *L. fulvotomentosa* was also recorded as one of the original plants of FL. However, to date the original plants of FL and other *Lonicera* species have continued to be misused as FLJ in medicines, foods, and healthcare products in China.

The commercial value of FLJ in herbal medicine trading markets has increased over 400% in recent years, and over 30% of current traditional Chinese medicine prescriptions contain FLJ (Yuan et al., 2012). This increasing demand has resulted in large areas of *L. japonica* and related *Lonicera* species being cultivated with various cultivars and landraces (Hu et al., 2011), and many wild *Lonicera* species are also harvested as medicines or foods. However, FLJ and FL from different species, cultivars, landraces, and wild germplasm have entered the market, causing a confusing array of medicinal materials with very different qualities. This is detrimental to the development of the *Lonicera*-related industry. Therefore, there is an urgent need to establish a simple and effective approach to differentiating the complex original plants of FLJ and FJ.

Previous studies on the identification of *L. japonica* and related species have mainly focused on their morphology and histology (Xu, 1979; Xu et al., 1979; Pu et al., 2002), chemical components (Hu et al., 2011; Shang et al., 2011; Yuan et al., 2012), and various genetic molecular markers, such as RFLP (Wang et al., 2007), diagnostic PCR (Peng et al., 2009), and the psbA-trnH intergenic spacer (Sun et al., 2011). Xu (1979) described (with 15 illustrations) the botanical origin of 14 species and one subspecies of *Lonicera*. Glandular and non-glandular hairs were discovered as the distinguishing pharmacognostic characteristics of 20 *Lonicera* species (Xu et al., 1979, 1981). Floral morphology of *L. japonica* varied from that of *L. hypoglauca*, but not within *L. japonica* (Pu et al., 2002). HPLC analysis showed that 'Nanjiang', a variation of *L. similis*, had the highest content of chlorogenic acid of six different *Lonicera* species (Hu et al., 2011). However, the chemical components and contents of FLJ are often influenced by different habitat, harvesting time, medicinal parts, extraction methods, and whether the flowers are fresh or dry (Shang et al., 2011). Molecular markers mainly including intergenic spacers of ITS and psbA-trnH were found to identify *L. japonica* and related species (Wang et al., 2007; Peng et al., 2009; Chen et al., 2010; Sun et al., 2011).

The genetic relationships among *L. japonica* and related species are unclear when using individual morphological, chemical and molecular markers, especially for cultivars, landraces and wild germplasm within species. The effective identification and quality control of *L. japonica* and its related species or various germplasm within *L. japonica* still remain to be explored.

The objectives of this study were to (i) reveal the genetic relationships among 30 *Lonicera* germplasm samples through morphological characteristics, ITS sequences and active compounds; (ii) analyze the correlations among the morphological characteristics, ITS sequences and active compounds of these *Lonicera* germplasm samples; (iii) illustrate the differences between medicinal species of *Lonicera*, and confirm an effective and exercisable protocol to identify FLJ and FJ. Understanding

the systematic differences between *L. japonica* and related species could provide valuable information to ensure their quality and therapeutic effects.

2. Materials and methods

2.1. Plant materials

Thirty *Lonicera* germplasm samples including 22 *L. japonica*, four *L. macranthoides*, three *L. hypoglauca* and one *L. fulvotomentosa* collected from 11 provinces of China were investigated (Table 1; Fig. 1). Among *L. japonica* germplasm, 10 cultivars with yellow flowers (Lj_JZ–Lj_PS2), five cultivars with red flowers (Ljv_PS1–Ljv_XXH), and seven wild germplasm (Ljw_PZ–Ljw_QZ) were included. All *Lonicera* germplasm are planted and stored in the Nursery of Chinese Herbal Medicine in the Lishui Institute of Forestal Sciences, Zhejiang Province.

2.2. Determination of morphological characteristics

Fourteen morphological characteristics including four quantitative characters (leaf length, leaf width, leaf length/width ratio, petiole length), and 10 qualitative characters (leaf type, leaf surface coat, abaxial leaf surface coat, leaf margin coat, leaf vein coat, leaf glandular spots, branch type, branch color, branch coat, branch glandular spots) were determined for all selected *Lonicera* germplasm samples (Table 2). Five to seven plants were selected from each germplasm sample and the quantitative characters of 10 fully developed leaves were measured for each plant. Qualitative characters were observed and described as numerical values from 0–4 (Table 2).

2.3. PCR amplification and ITS sequence analysis

The total genomic DNA was extracted from gel-dried leaves using a modified CTAB method (Wang et al., 2008). The entire ITS sequences, including ITS1, 5.8SrDNA gene and ITS2, were amplified using universal primers ITS1 'AGAAGTCGTAACAAGGTTTCCGTAGG' and ITS4 'TCCTCCGCTTATTGATATGC' for all *Lonicera* germplasm samples (Zhu et al., 2010). The reaction mixture (50 μL) for PCR consisted of 25 ng total genomic DNA, 0.25 mM each of dNTP, 0.2 mM $MgCl_2$, 1 μM of each forward and reverse primer, 1× buffer, 10% (v/v) DMSO, and 0.03 U Ex *Taq* polymerase (TaKaRa). The ITS sequence was amplified at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 50 °C for 2 min, 72 °C for 1 min, and a final extension at 72 °C for 10 min in a Mastercycler Gradient PCR (Eppendorf, Germany). All amplified products were detected by electrophoresis on 1.5% agarose gels in 1× TAE buffer (100 V for 35 min), and purified and sequenced by the Shanghai Invitrogen Company. After manual

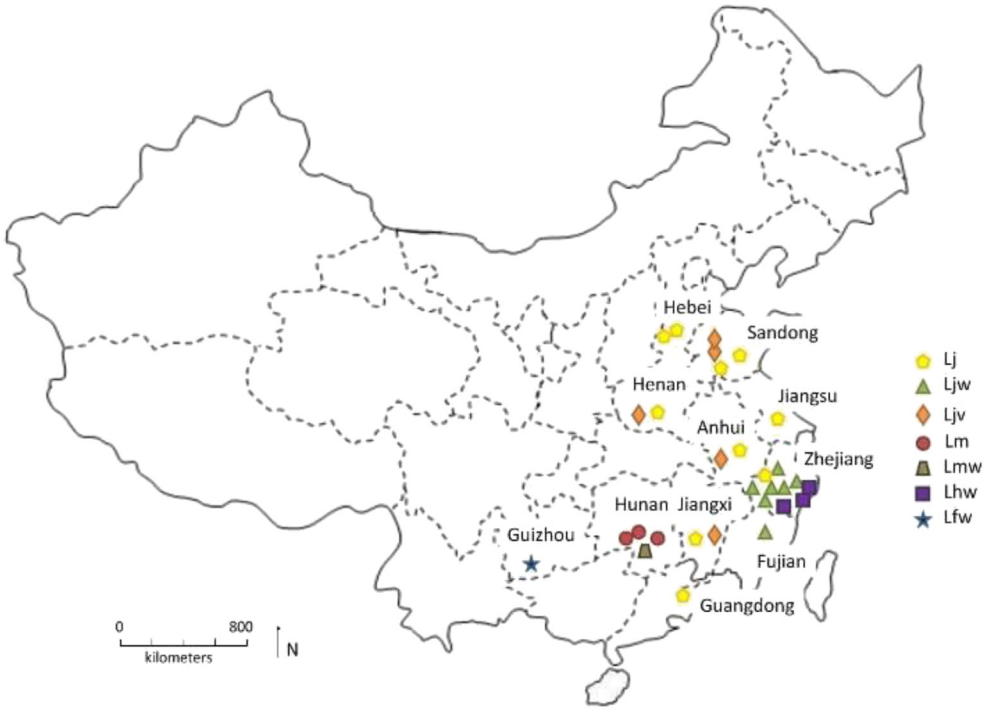

Fig. 1. Geographic distribution of the 30 *Lonicera* germplasm samples. Germplasm codes refer to Table 1.

Table 2
Fourteen morphological characteristics of 30 *Lonicera* germplasm samples.

Code	Leaf traits										Branch traits			
	Leaf length (cm)	Leaf width (cm)	Leaf length/width ratio	Petiole length (cm)	Leaf type[a]	Leaf surface coat[b]	Abaxial leaf surface coat[c]	Leaf margin coat[d]	Leaf vein coat[e]	Leaf gland spots[f]	Branch type[g]	Branch colour[h]	Branch coat[i]	Branch glands[j]
Lj_JZ	5.1 ± 0.55 k–n*	3.0 ± 0.20 g–i	1.7 ± 0.22 j–o	0.6 ± 0.11 e–k	0	0	1	1	1	0	0	2	1	0
Lj_PS1	5.0 ± 0.47 l–n	3.5 ± 0.36 e–g	1.4 ± 0.15 o	0.5 ± 0.05 g–l	0	0	1	1	1	0	0	2	1	0
Lj_BA	6.0 ± 0.60 h–k	3.6 ± 0.22 d–f	1.7 ± 0.17 k–o	0.6 ± 0.09 g–l	0	0	1	1	1	0	0	2	1	0
Lj_SJ	7.3 ± 0.55 fg	4.5 ± 0.37 ab	1.6 ± 0.09 l–o	0.7 ± 0.06 d–j	0	0	1	1	1	0	0	2	1	0
Lj_JJ	5.8 ± 0.51 h–l	3.7 ± 0.64 de	1.6 ± 0.23 m–o	0.6 ± 0.19 f–k	0	0	1	1	1	0	0	2	1	0
Lj_XXH	5.3 ± 0.66 i–n	3.6 ± 0.32 d–f	1.5 ± 0.20 o	0.5 ± 0.03 i–l	0	0	1	1	1	0	0	2	2	0
Lj_QG	4.6 ± 0.81 n	3.0 ± 0.41 g–i	1.5 ± 0.12 no	0.5 ± 0.06 i–l	0	0	1	1	1	0	0	2	1	0
Lj_TH	6.3 ± 0.55 g–i	3.9 ± 0.44 c–e	1.6 ± 0.05 l–o	0.5 ± 0.10 j–l	0	0	1	1	1	0	0	2	1	0
Lj_XTH	5.4 ± 0.35 i–n	3.5 ± 0.39 e–g	1.6 ± 0.16 m–o	0.5 ± 0.11 g–l	0	0	1	1	1	0	0	2	1	0
Lj_PS2	4.7 ± 1.18 mn	3.1 ± 0.70 f–h	1.5 ± 0.16 no	0.6 ± 0.11 g–l	0	0	1	1	1	0	0	2	1	0
Ljv_PS1	5.0 ± 0.22 k–n	2.8 ± 0.20 hi	1.8 ± 0.09 i–m	0.5 ± 0.04 kl	1	0	0	1	1	0	1	3	2	0
Ljv_PS2	5.3 ± 0.41 j–n	2.8 ± 0.21 hi	1.8 ± 0.03 h–l	0.6 ± 0.08 g–l	1	0	0	1	1	0	1	3	2	0
Ljv_BA	5.4 ± 0.47 i–n	2.8 ± 0.31 hi	1.9 ± 0.07 g–j	0.4 ± 0.03 l	1	0	0	1	1	0	1	3	2	0
Ljv_JJ	5.4 ± 0.32 i–n	2.8 ± 0.10 hi	1.9 ± 0.12 g–k	0.5 ± 0.12 kl	1	0	0	1	1	0	1	3	2	0
Ljv_XXH	5.5 ± 1.35 i–n	2.8 ± 0.28 hi	2.0 ± 0.52 e–i	0.6 ± 0.13 g–l	1	0	0	1	1	0	1	3	1	0
Ljw_PZ	5.5 ± 1.10 i–n	3.1 ± 0.83 f–i	1.9 ± 0.21 g–l	0.7 ± 0.34 c–g	0	0	0	1	1	0	0	0	2	0
Ljw_QZ	5.4 ± 0.37 i–n	2.8 ± 0.24 hi	2.0 ± 0.08 f–i	0.5 ± 0.05 kl	1	0	0	1	1	0	0	0	3	0
Ljw_SF	5.7 ± 0.47 i–m	2.9 ± 0.13 hi	1.9 ± 0.12 g–j	0.8 ± 0.16 c–f	1	0	1	1	1	0	0	0	3	0
Ljw_LZ	5.6 ± 0.19 i–n	2.7 ± 0.15 hi	2.1 ± 0.09 e–h	0.7 ± 0.11 c–h	1	0	1	1	1	0	0	0	3	0
Ljw_JZ1	6.2 ± 0.68 h–j	3.5 ± 0.57 e–g	1.8 ± 0.11 i–n	0.7 ± 0.28 d–i	1	0	0	1	1	0	0	1	3	0
Ljw_JZ2	6.6 ± 0.54 f–h	2.8 ± 0.24 hi	2.4 ± 0.21 b–d	0.7 ± 0.32 c–g	1	0	1	1	1	0	0	1	3	0
Ljw_QZ	5.6 ± 1.13 i–m	2.6 ± 0.48 i	2.2 ± 0.41 de	0.8 ± 0.15 b–d	1	0	1	1	1	0	0	0	1	0
Lmw_LH	7.4 ± 0.44 ef	2.9 ± 0.38 hi	2.6 ± 0.24 ab	0.6 ± 0.05 g–l	0	1	1	1	1	0	0	1	3	0
Lfw_QG	9.7 ± 1.13 bc	3.5 ± 0.63 e–g	2.8 ± 0.27 a	0.8 ± 0.11 c–e	1	1	1	1	1	0	1	0	4	0
Lhw_YZ	8.4 ± 0.76 de	3.8 ± 0.52 c–e	2.2 ± 0.19 d–f	0.9 ± 0.11 a–d	1	0	0	1	1	2	0	1	3	0
Lhw_LZ1	8.6 ± 1.01 d	4.0 ± 0.61 b–e	2.2 ± 0.17 d–f	0.7 ± 0.15 c–g	1	0	2	0	2	1	0	0	1	1
Lhw_LZ2	9.6 ± 1.26 c	4.3 ± 0.40 bc	2.3 ± 0.20 c–e	0.8 ± 0.14 c–e	1	0	2	1	2	1	0	1	1	0
Lm_LH1	11.2 ± 1.16 a	4.5 ± 0.50 ab	2.5 ± 0.20 a–c	1.0 ± 0.29 a	1	0	2	0	2	1	0	2	0	0
Lm_LH2	10.0 ± 0.35 bc	4.8 ± 0.36 a	2.1 ± 0.09 e–g	0.9 ± 0.08 a–c	1	0	2	0	2	0	0	2	0	0
Lm_LH3	10.5 ± 0.89 ab	4.1 ± 0.23 b–d	2.6 ± 0.14 ab	1.0 ± 0.03 ab	1	0	2	0	2	0	0	2	0	0

[a]: 0 papery, 1 stiffly papery. [b], [c], [d], [e]: 0 glabrous, 1 strigose, 2 tomentose. [f]: 0 no gland spots, 1 white gland spots, 2 orange glandular spots. [g]: 0 hollow, 1 solid. [h]: 0 green turn to reddish brown, 1 reddish brown, 2 green, 3 purplish red. [i]: 0 glabrous, 1 strigose, 2 densely hispid, 3 down to glabrous, 4 coated with fulvous hair. [j]: 0 no glandular spots, 1 with glandular spots. Germplasm codes refer to Table 1 k–n*: klmn.

correction, the nucleotide sequences were aligned using MEGA 5.0 software (Koichiro et al., 2011). Genetic distances among the 30 germplasm samples were calculated based on Kimura's two-parameter model using MEGA 5.0. Gaps were treated as missing data. Phylogenetic trees were constructed using the UPGMA method, and the bootstrap values for the interior nodes were performed with 1000 replicates. The ITS sequence of *L. japonica*, *L. macranthoides* (JQ731716), *L. confusa* (FJ774986), *L. fulvotomentosa* (FJ774988), *L. hypoglauca* (FJ372916) and *L. similis* (FJ774987) from GenBank were also analyzed for constructing the phylogenetic trees.

2.4. Restriction endonuclease digestion reaction

Restriction endonuclease analysis was performed by DNAman to find appropriate endonucleases for the digestion reaction. The selected restriction endonuclease *Cfr* I was used to digest the amplified ITS sequence of FLJ and FL. The digestion reaction was performed in a 20 μL reaction mixture containing 2 μL 10× buffer, 10 μL PCR products, 5 U restriction endonuclease *Cfr* I, and ddH_2O. Then the mixture was placed in a 37 °C water bath overnight. The enzyme-digested product was detected by 2% agarose gel electrophoresis. The electrophoretogram was used to identify FLJ and FL.

2.5. Determination of active components

The chlorogenic acid and luteoloside contents were determined by the high-performance liquid chromatography (HPLC) method according to the current PPRC (Committee for the Pharmacopoeia of PR China, 2010). HPLC was conducted by an Agilent 1100 system with an Elit C18 chromatographic column (250 mm × 4.6 mm, 5 μm). To determine the chlorogenic acid content, 0.5 g of each sample powder was accurately weighed and extracted with 50 mL of 50% methanol by ultrasonication for 30 min (250 W, 35 kHz). The extract was filtered, and 5 mL of filtrate was supplemented with 50% methanol to 25 mL. Ten μL of the filtrate was injected into the HPLC system for chlorogenic acid analysis at 327 nm with a flow rate of 1.0 mL/min. The mobile phase contained acetonitrile/0.4% phosphate (13:87, V/V). To determine the luteoloside content, 2 g of each sample powder was accurately weighed and extracted with 50 mL of 70% alcohol by ultrasonication for 1 h (250 W, 35 kHz). Ten μL of the filtrate was injected into the HPLC system for luteoloside analysis at 350 nm with a flow rate of 1.0 mL/min. The mobile phase contained acetonitrile (A) and 0.5% glacial acetic acid (B).

2.6. Statistical analysis

The mean values, standard deviations of morphological characteristics, chlorogenic acid and luteoloside contents were calculated using the statistical package SPSS v17.0 (SPSS Inc., USA). The differences among the germplasm samples were compared by the Minimum Significant Difference method (LSD). Cluster analysis of morphological characteristics and active components were conducted using the average linkage method in SAS 9.1 and SPSS v17.0 software, respectively. Principal component analysis (PCA) was performed using SPSS v17.0.

Distance matrixes of morphological and componential data were carried out by SPSS v17.0. The correlation analysis among the morphological characteristics, ITS sequences and active components of *Lonicera* germplasm samples were performed by a Mantel test using GenAlEx 6.5 software (Peaktall and Smouse, 2012).

3. Results

3.1. Morphological analysis

The morphological characteristics were significantly different among the 30 *Lonicera* germplasm samples (Table 2). Cluster analysis showed that they were divided into five clusters: I) 22 *L. japonica*, II) a wild *L. hypoglauca* of Lhw_YZ, III) a wild *L. fulvotomentosa* of Lfw_QG and a wild *L. macranthoides* of Lmw_LH, IV) another wild *L. hypoglauca* of Lhw_LZ1, V) three *Lonicera macranthoides* cultivars of Lm_LH1-3 and the other wild *L. hypoglauca* of Lhw_LZ2 (Fig. 2).

All the 22 *L. japonica* germplasm samples formed cluster I with three subgroups: a) yellow flower cultivars, b) wild germplasm, c) red flower cultivars. Germplasm in subgroup a were characterized by papery and oval leaves with a length/width ratio of about 1.5, and green hollow branches. Germplasm in subgroup b were characterized by stiffly papery, glabrous and strigose leaves, and green and reddish-brown hollow branches. Germplasm in subgroup c were characterized by stiffly papery and glabrous leaves, and purplish red and solid branches. The inter-germplasm differences of wild *L. japonica* were higher than those of cultivars with red flowers. Details are described in Table 2. The PCA analysis of morphological characteristics also showed the same pattern: the 22 *L. japonica* germplasm samples were separated into three groups of A, B and C (Fig. 3L), corresponding to the subgroups of a, b and c in Fig. 2.

Apart from the 22 *L. japonica* germplasm samples, another eight *Lonicera* germplasm samples formed four distantly related clusters in the dendrogram based on morphological characteristics (Fig. 2). PCA analysis demonstrated that the eight *Lonicera* germplasm samples were separated into two groups (Fig. 3R), corresponding to cluster II, III, and cluster V, IV in Fig. 2. The germplasm in cluster II and III showed similar morphological characteristics such as large, oblong leaves, strigose leaf margins and veins, and specificities such as the coating of fulvous hair of Lfw_QG, papery and strigose leaves of Lmw_LH, and orange glandular spots of the abaxial leaf surface of Lhw_YZ. The germplasm in clusters IV and V were specialized with a dense

tomentose leaf with a length of 9–12 cm. However, Lm_LH1–Lm_LH3 with green and glabrous branches were distinguished from Lhw_LZ1 and Lhw_LZ2.

3.2. ITS sequence analysis

The total length of ITS sequence of the 30 *Lonicera* germplasm samples was 722–723 bp, with one insertion or deletion of base pairs, of which 16 nucleotide sites were variable sites, i.e. the average divergence rate of the ITS sequence was 2.2%. The phylogenetic tree constructed based on ITS sequences of the 30 *Lonicera* germplasm samples showed that two large, distinct clusters matched with the FLJ and FL, respectively (Fig. 4). In the FLJ cluster, five *L. japonica* cultivars with red flowers (Ljv) were closer to the wild *L. japonica* germplasm (Ljw) than cultivars with yellow flowers (Lj), although the genetic relationships among all 22 *L. japonica* germplasm samples were very close. The FJ cluster was composed of the eight germplasm of FL and five *Lonicera* species (*L. macranthoides*, *L. confusa*, *L. fulvotomentosa*, *L. hypoglauca* and *L. similis*). Three *L. macranthoides* cultivars (Lm_LH1–Lm_LH3) were very different from their wild germplasm (Lmw_LH). However, they had a close genetic relationship with the wild germplasm of *L. hypoglauca* (Lhw_YZ, Lhw_LZ1, Lhw_LZ2), which was similar to that according to the morphological characteristics. The correlation analysis also showed a significant correlation ($r = 0.754$, $P < 0.01$) between ITS sequences and morphological characteristics of all the 30 *Lonicera* germplasm samples, suggesting consistency of morphological characteristics and the ITS sequences.

3.3. Active components analysis

Chlorogenic acid contents were significantly different between FLJ and FL (Table 3). The chlorogenic acid content of the *L. macranthoides* cultivar was significantly higher than that of *L. japonica*. The highest chlorogenic acid contents were detected in *L. macranthoides* cultivars of Lm_LH2 (5.55%) and Lm_LH1 (4.36%), while *L. japonica* cultivars with red flowers of Ljv_PS2 (0.95%) and Ljv_PS1 (0.99%) had the lowest content. The luteoloside contents were also significantly different between FLJ and FL, ranging from 0.039% (Ljw_LZ) to 0.187% (Lj_SJ). The order in luteoloside content was *L. japonica* cultivars with yellow flowers (e.g., Lj_SJ) > *L. japonica* cultivars with red flowers (e.g., Ljv_PS1) > *L. macranthoides* cultivars (e.g., Lm_LH1) > *L. japonica* wild germplasm (e.g., Ljw_LZ).

FL and FLJ could be divided into two clusters based on the contents of chlorogenic acid and luteoloside. Two *L. macranthoides* cultivars, Lm_LH1 and Lm_LH2, formed FL cluster II and were obviously distant from the FLJ cluster that contained *L. japonica* germplasm. The distinction in contents of active components between FLJ and FL was similar to those in the morphological characteristics and ITS sequence. Correlation analysis demonstrated that chlorogenic acid and luteoloside contents among different *Lonicera* germplasm samples was significantly correlated with morphological characteristics ($r = 0.619$, $P < 0.01$) and the ITS sequence ($r = 0.727$, $P < 0.01$).

3.4. Molecular identification of FLJ and FL

A mutation that appeared around site 541 bp of the ITS sequence between FLJ and FL was used for the digestion reaction. Restriction endonuclease analysis revealed that endonuclease *Cfr* I could be used for discrimination of FLJ and FL. The endonuclease recognized site C^GGCCG existed in FLJ and FL owned CGGCTG in homologous sites. The digested reaction

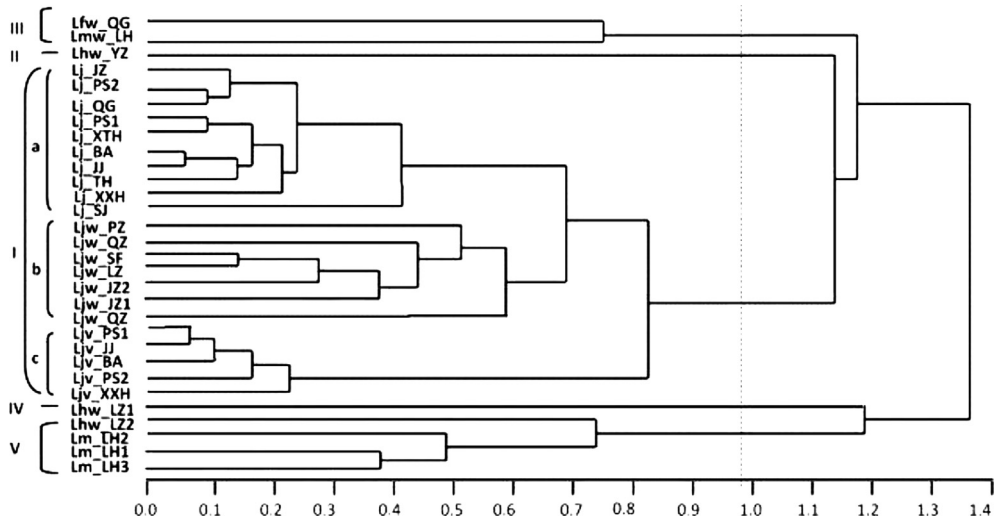

Fig. 2. Dendrogram of 30 *Lonicera* germplasm samples based on morphological characteristics. Germplasm codes refer to Table 1.

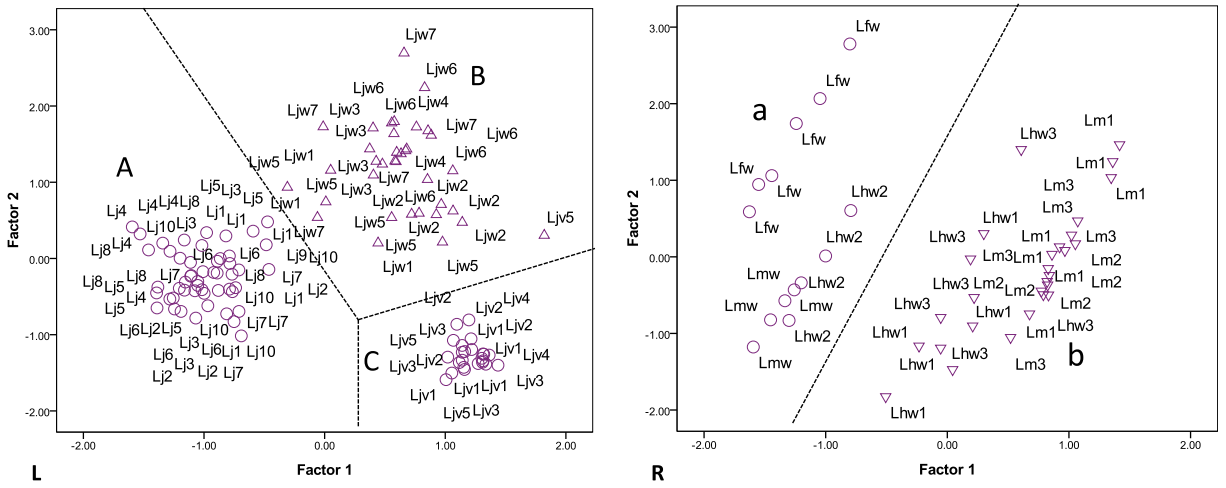

Fig. 3. Principal component analysis of morphological characteristics variation of 22 *L. japonica* germplasm (L), and eight *Lonicera* germplasm samples except for *L. japonica* (R). Germplasm codes refer to Table 1. Lj1–Lj10 represent Lj_JZ–Lj_PS2, Ljw1–Ljw7 represent Ljw_PZ–Ljw_QZ, Ljv1–Ljv5 represent Ljv_PS1–Ljv_XXH, Lm1–Lm3 represent Lm_LH1–Lm_LH3, Lmw represent Lmw_LH, Lhw1–Lhw3 represent Lhw_LZ1–Lhw_LZ2, Lfw represent Lfw_QG. Each triangle or cycle represents an individual.

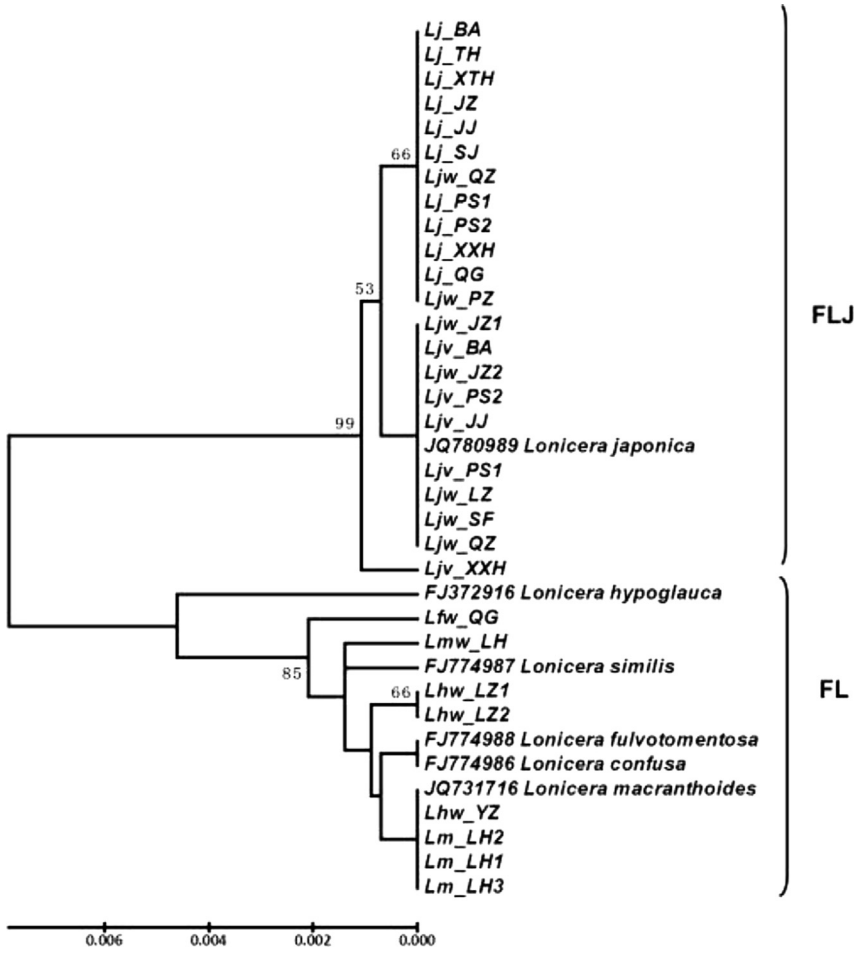

Fig. 4. UPGMA dendrogram showing the genetic relationships of *Lonicera japonica* and related species based on ITS sequences. The numbers between nodes indicate percentages of bootstrap support. Germplasm codes refer to Table 1.

resulted in the 722–723 bp ITS PCR products of FLJ being digested into two fragments around 500 bp and 200 bp using endonuclease *Cfr* I; meanwhile, the FL remained unchanged. Consequently, the FLJ can be identified from FL.

4. Discussion

In 1963 the PPRC recorded that the origin plant of FLJ was *L. japonica* Thunb. (Wang et al., 2009). Three *Lonicera* species, *L. hypoglauca* Miq., *L. confusa* DC. and *L. dasystyla* Rhed., were also included in the PPRC with *L. japonica* as the origin plant of FLJ since 1977. In 2005, the PPRC changed the rule once more, and although *L. japonica* was retained as the origin plant of FLJ, new medicinal material of FL from the flower buds of *L. macranthoides*, *L. hypoglauca* and *L. confusa* were recorded. *Lonicera fulvotomentosa* was included as one of the origin plants of FL in the latest PPRC (Committee for the Pharmacopoeia of PR China, 2010). The repeated changes in the rules of FLJ caused confusion about the origin plants of FLJ and FJ. The morphological characteristics of several *Lonicera* species were described in the Flora of China (Xu et al., 1988). However, it is usually difficult to identify closely related species of *Lonicera* based on traditional classification owing to their similar morphological characteristics (Sun et al., 2011). Generally, the morphological characteristics were only qualitatively described with illustrations (Xu, 1979; Xu et al., 1979). In this study, multi-character joint analysis showed that *L. japonica* could be distinguished from *L. macranthoides*, *L. hypoglauca* and *L. fulvotomentosa* based on four quantitative and 10 qualitative characters. *L. japonica* has been domesticated from wild species and cultivated for hundreds of years mainly in the Shandong and Henan provinces of China. Many *L. japonica* germplasm with obvious different morphological characteristics, yield and quality were formed after long-term natural and artificial selection (Zhang, 2005). In this study, three kinds of *L. japonica* germplasm, yellow flowers cultivars, wild germplasm, and red flowers cultivars were also identified with morphological characteristics (see Table 2).

Previous research found a suitable DNA marker (psbA-trnH intergenic spacer) for the identification of the botanical origins of FLJ and FL by testing the genetic differences of *L. japonica* and its closely related species (Sun et al., 2011). Chen et al. (2010) proposed that the ITS2 region was the most promising universal DNA barcode for authenticating medicinal plants. A cleavage rate of ITS PCR products by *Eco*N I can be used to classify geo-authentic *L. japonica* from different geographical origins (Wang et al., 2007). However, the discrimination power of these sequences was not enough if different cultivars and wild germplasm of *L. Japonica* were included. We clarified the genetic relationships among FLJs (from different *L. japonica* germplasm samples) and FL (*L. macranthoides*, *L. hypoglauca*, *L. fulvotomentosa* and *L. confusa*), and distinguished the origins of FLJ and FL based on an ITS sequence. Furthermore, FLJs can be identified from FL by *Cfr* I digested reaction of the ITS PCR products.

Phytochemical and pharmacological research showed that more than 140 chemical compounds have been isolated from *L. japonica* (Shang et al., 2011). Among these compounds, chlorogenic acid and luteoloside have been chosen as biomarkers to characterize the quality of FLJ by the PPRC (Chinese Pharmacopoeia Commission, 2010). However, the active components of FLJ varied in contents along with different *L. japonica* germplasm. Chlorogenic acid and luteoloside contents were very different among the *L. japonica* germplasm samples of the current study, and only 10 were up to a grade of $\geq$1.5% chlorogenic acid and $\geq$0.05% luteoloside as decided by the 2010 PPRC. The FLJ from wild *L. japonica* germplasm had a high chlorogenic acid content and relatively low luteoloside content. Yuan et al. (2012) reported that the chlorogenic acid, luteoloside, quercitin, and isopropyl laurate contents are higher overall in red FLJ flower buds compared with those of FLJ. We detected that the chlorogenic acid and luteoloside contents were different in red FLJ from the four cultivars with red flowers, and two red flower

Table 3
Chlorogenic acid and luteoloside contents of FLJ (*L. japonica*) and FL (*L. macranthoides*).

Medicinal name	Code	Chlorogenic acid (%)	Galuteolin (%)
Flos Lonicerae Japonicae	Lj_JZ	1.04 ± 0.075 h	0.075 ± 0.0077 ef
(FLJ)	Lj_PS1	1.93 ± 0.017 fg	0.074 ± 0.0013 ef
	Lj_BA	2.07 ± 0.009 fg	0.176 ± 0.0127 ab
	Lj_SJ	2.60 ± 0.035 e	0.187 ± 0.0063 a
	Lj_JJ	2.73 ± 0.086 e	0.167 ± 0.0027 abc
	Lj_XXH	2.25 ± 0.011 f	0.063 ± 0.0023 fg
	Lj_TH	2.09 ± 0.025 fg	0.071 ± 0.0032 ef
	Lj_PS2	3.18 ± 0.02 cd	0.165 ± 0.0666 abc
	Ljv_PS1	0.99 ± 0.008 h	0.137 ± 0.0063 d
	Ljv_PS2	0.95 ± 0.012 h	0.145 ± 0.0027 cd
	Ljv_BA	1.23 ± 0.035 h	0.181 ± 0.0061 ab
	Ljv_JJ	2.23 ± 0.051 f	0.156 ± 0.0083 bcd
	Ljw_QZ	1.26 ± 0.011 h	0.088 ± 0.0013 ef
	Ljw_SF	3.26 ± 0.02 c	0.041 ± 0.0007 g
	Ljw_LZ	2.89 ± 0.012 de	0.039 ± 0.0010 g
	Ljw_JZ1	3.27 ± 0.011 c	0.042 ± 0.0020 g
	Ljw_JZ2	1.87 ± 0.115 g	0.088 ± 0.0202 ef
	Ljw_QZ	2.16 ± 0.005 fg	0.095 ± 0.0103 e
Flos Lonicerae (FL)	Lm_LH1	4.36 ± 0.292 b	0.072 ± 0.0117 ef
	Lm_LH2	5.55 ± 0.802 a	0.098 ± 0.0086 e

Mean values with alphabetical suffices within the same column are statistically different at the significance level of 0.05 based on the protected least significant difference test. Germplasm codes refer to Table 1.

cultivars had the lowest chlorogenic acid contents. The active components of FLJ have been shown to vary with habitat (Xing et al., 2003), harvesting time (Xu et al., 2010), processing (Huang et al., 2009), and so on. Whether the labile and inconsistent chemical components are appropriate biomarkers for identifying and quality control of FLJ should be studied further.

The active components, which are secondary metabolites produced in the secondary metabolism process, differ between species of *Lonicera* (Xu et al., 1988; Hu et al., 2011). The chlorogenic acid contents of *L. japonica, L. confusa*, *L. hypoglauca*, *L. macranthoides*, *L. acuminate* and *L. similis* were 5.06%, 3.25%, 2.11%, 5.72%, 3.06% and 7.66%, respectively (Hu et al., 2011). Chlorogenic acid content (2.80%–5.08%) of *L. macranthoides* is higher than that of FLJ, and luteoloside is relatively low (0.04%–0.06%) in *L. macranthoides* (Li et al., 2007). Recently, chlorogenic acid and luteolin were found in other medicinal plants, where the contents were higher than *L. japonica* (Committee for the Pharmacopoeia of PR China, 2010; Shang et al., 2011). In this study, two cultivars of *L. macranthoides* are up to the grade of chlorogenic acid and luteoloside contents for FLJ as decided by the PPRC. The results also confirm the conclusion that employing chlorogenic acid and luteolin to control the quality of FLJ lacks specificity (Shang et al., 2011). The specific effective component Macranthoidin B of FJ may be more suitable for identifying and evaluating the FLJ and related preparations blended with FJ (Lin et al., 2013).

Pharmacological studies have demonstrated that both FLJ and FL possess wide efficacies as anti-inflammatory (Cui et al., 2007; Yang et al., 2009), anti-viral (Wang et al., 2011), anti-bacterial (Li and Zhao, 2010; Liu and Li, 2012), anti-oxidant (Wang et al., 2010), hepatoprotective (Hu et al., 2008) and anti-tumor (Zhang et al., 2007) medicines. The similar pharmacological actions of FLJ and FL might be due to their similar chemical components, such as phenolic acids (e.g. chlorogenic acid), flavonoids (e.g. luteoloside), saponins and iridoids (Shang et al., 2011; Liu et al., 2013). However, a couple of chemical components differ between FLJ and FL, for example macranthoidin B is present in *L. macranthoides*, *L. hypoglauca*, *L. fulvotomentosa* and *L. confusa*, but not in *L. japonica* (Lin et al., 2013). The distinct components and contents of FLJ and FL might affect their efficacies by the interactions of chemical components.

In conclusion, the morphological characteristics, ITS sequences and active components have advantages in clarifying the genetic relationships among *L. japonica* and closely related species of *L. macranthoides*, *L. hypoglauca*, *L. fulvotomentosa* and *L. confusa*, and in the identification of FLJ (*L. japonica* germplasm) from FL (*L. macranthoides*, *L. hypoglauca*, *L. fulvotomentosa* and *L. confusa*). FLJs are easy to identify from FL by the *Cfr* I digested reaction of the ITS PCR products. All of the analyses revealed that *L. japonica* differed from its related species, implying caution in replacing or blending the traditional Chinese medicines of FLJ and FL.

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