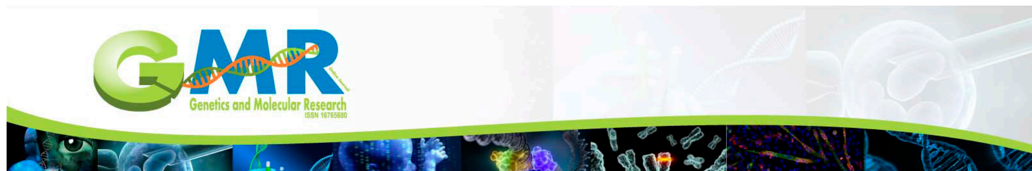

Analysis of the genetic diversity of *Lonicera japonica* Thumb. using inter-simple sequence repeat markers

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ABSTRACT. Inter-simple sequence repeats (ISSRs) were used to analyze the genetic diversity of 21 accessions obtained from four provinces in China, Shandong, Henan, Hebei, and Sichuan. A total of 272 scored bands were generated using the eight primers previously screened across 21 accessions, of which 267 were polymorphic (98.16%). Genetic similarity coefficients varied from 0.4816 to 0.9118, with an average of 0.6337. The UPGMA dendrogram grouped 21 accessions into two main clusters. Cluster A comprised four *Lonicera macranthoides* Hand. Mazz. accessions, of which J10 was found to be from Sichuan, and J17, J18, and J19 were found to be from Shandong. Cluster B comprised 17 *Lonicera japonica* Thumb. accessions, divided into the wild accession J16 and the other 16 cultivars. The results of the

principal component analysis were comparable to the cluster analysis. Therefore, the ISSR markers could be effectively used to distinguish interspecific and intraspecific variations, which may facilitate identification of *Lonicera japonica* cultivars for planting, medicinal use, and germplasm conservation.

Key words: Genetic diversity; *Lonicera japonica* Thumb.; Inter-simple sequence repeat

INTRODUCTION

Lonicera japonica Thumb. has been used for thousands of years as traditional Chinese medicine owing to its bacteriostasis (Tang et al., 2008), antitumor (Liu et al., 2012), antioxidant (Gong et al., 2006), anti-endotoxin (Yang et al., 2012), anti-inflammatory (Kang et al., 2004; Chen et al., 2012), antiviral (Yan et al., 1998), pro-immune (Cui, 2011), and blood sugar-lowering effects (Liang et al., 2011). In commercial production, many species from the family Caprifoliaceae, including *Lonicera similis* Hemsl., *Lonicera macranthoides* Hand.-Mazz., *Lonicera acuminata* Wall., and *Lonicera hypoglauca* Miq., have been applied as *Lonicera japonica*. The Chinese pharmacopoeia stipulates that *Lonicera japonica* Thumb. must be the only source of this plant, whereas different cultivars are the main source of commercial herbs of *Lonicera japonica*.

In the past four decades, researchers have developed several molecular marker techniques, including inter-simple sequence repeat (ISSR) (Zietkiewicz et al., 1994). ISSR is rapid, inexpensive, simple, and efficient DNA marker method that does not require prior knowledge of the DNA sequence or large amounts of starting DNA template. ISSR is widely used for germplasm identification, fingerprint construction, and genetic diversity and relationship analyses (Charters et al., 1996; Blair et al., 1999; Chen et al., 2008; Smolik et al., 2010; Wang et al., 2012; Wang et al., 2013; Li et al., 2013; Sunar et al., 2016). In this study, we aimed to establish a method for identification of real *Lonicera japonica* for planting, medicinal use, and germplasm conservation by analyzing the genetic diversity of 17 *Lonicera japonica* accessions (including 16 cultivar accessions and one wild accession) and four *Lonicera macranthoides* accessions.

MATERIAL AND METHODS

Plant sample collection and extraction of DNA

In this study, seedlings were collected from different provinces in China, Shandong, Henan, Hebei, and Sichuan (as listed in Table 1) and planted in experimental plots in the Institute of Mountain Hazards and Environment, Chinese Academy of Sciences. Total genomic DNA was extracted from young leaves using the CTAB (hexadecyltrimethylammonium bromide) method, with slight modifications. DNA quality and quantity were evaluated on 2% agarose gels and a Nanodrop 2000 ultramicro-spectrophotometer. The final concentration of all DNA samples was adjusted to 20 ng/μL, and samples were stored at -20°C for further analysis.

Table 1. Sources of ISSR samples.

No.	Sources	Species	No.	Sources	Species
J1	Shangdong	*L. japonica*	J12	Shangdong	*L. japonica*
J2	Shangdong	*L. japonica*	J13	Shangdong	*L. japonica*
J3	Shangdong	*L. japonica*	J14	Shangdong	*L. japonica*
J4	Shangdong	*L. japonica*	J15	Henan	*L. japonica*
J5	Shangdong	*L. japonica*	J16	Sichuan	*L. japonica*
J6	Shangdong	*L. japonica*	J17	Sichuan	*L. macranthoides*
J7	Shangdong	*L. japonica*	J18	Sichuan	*L. macranthoides*
J8	Shangdong	*L. japonica*	J19	Sichuan	*L. macranthoides*
J9	Shangdong	*L. japonica*	J20	Sichuan	*L. japonica*
J10	Shangdong	*L. macranthoides*	J21	Hebei	*L. japonica*
J11	Shangdong	*L. japonica*			

Amplification of DNA by ISSR-polymerase chain reaction (PCR)

ISSR amplification was performed in a 20-μL volume containing 10 μL 2X PCR Mix, 3 μL genomic DNA, 0.8 μL 10 μM primers, and 6.2 μL sterile water. The amplification reactions were carried out under the following conditions: 5 min at 94°C, followed by 30 s at 94°C, 30 s at the annealing temperature (specific to each primer), 90 s at 72°C for 36 cycles, and 7 min at 72°C. PCR products were separated by electrophoresis using 6% polyacrylamide gels, stained with silver nitrate, and imaged using a digital camera. Eight primers (Table 2) from a total of 27 ISSR primers tested (published by Columbia University) were selected, and their respective annealing temperatures were optimized for ISSR amplification. PCR amplification of all selected primers was repeated in triplicate.

Statistical analysis

ISSR-amplified fragments with the same mobility according to molecular weight (bp) were scored manually for band presence (marked as 1) or absence (marked as 0). The percentage of polymorphic bands was measured using the POPGENE 32 program for genetic diversity analysis. A dendrogram was constructed based on SM similarity coefficients using the unweighted pair group method with arithmetic mean (UPGMA) with the SAHN module of NTSYS-pc 2.10. A principal coordinate analysis (PCA) was used to construct a two-dimensional array of eigenvectors in the DCENTER module of NTSYS-pc 2.10 (Rohlf, 2000).

RESULTS

Genetic diversity

A total of 272 scored bands were generated using eight primers previously screened across the 21 accessions. The number of amplified bands per primer ranged from 24 (UBC815) to 41 (UBC840; Table 2). Of the 272 amplified bands, 267 were polymorphic (98.16%). The percentage of polymorphic bands across the primers ranged from 95.83%

(UBC815) to 100% (UBC808, UBC840, and UBC841; Table 2). The band sizes ranged from approximately 200 to 2500 bp.

Table 2. ISSR primers used in this study and some summary results.

Primer	Sequence (5'-3')	T (°C)	No. of bands scored	No. of polymorphic bands	Percentage of polymorphic bands (PPB)
UBC 808	$(AG)_8C$	52.2	26	26	100.00
UBC 811	$(GA)_8C$	47.2	39	38	97.44
UBC 815	$(CT)_8G$	49.2	24	23	95.83
UBC 827	$(AC)_8G$	53.4	36	35	97.22
UBC 834	$(AG)_8YT$	48	31	30	96.77
UBC 835	$(AG)_8YC$	49.2	38	37	97.37
UBC 840	$(GA)_8YT$	47.2	41	41	100.00
UBC 841	$(GA)_8YC$	48	37	37	100.00
In total			272	267	98.16

Genetic similarity coefficients

The SM similarity coefficients across 21 accessions ranged from 0.4816 to 0.9118 (as listed in Table 3), with an average of 0.6337. The maximum similarity coefficient was observed between J17 and J18 (0.9118), whereas the minimum similarity coefficient was observed between J5 and J17 (0.4816).

Cluster analysis

Figure 1 shows the UPGMA tree constructed from 21 accessions. When the similarity coefficient was 0.54, the UPGMA dendrogram grouped the 21 accessions into two main clusters. Cluster A comprised four *Lonicera macranthoides* accessions, and cluster B comprised 17 *Lonicera japonica* accessions. When the similarity coefficient was 0.58, cluster A could be divided into two subclusters: the A1 subcluster, including accessions J17, J18, and J19; and the A2 subcluster, including accession J10. Accession J10 from Sichuan was distinguished from accessions J17, J18, and J19 from Shandong. When the similarity coefficient was 0.61, 17 accessions in cluster B could be divided into two subclusters: the B1 subcluster, including accessions J2, J4, and J11; and the B2 subcluster, including the other 14 accessions. When the similarity coefficient was 0.68, subcluster B2 could be subdivided into two groups: group B21, including the wild accession J16; and group B22, including the other 13 accessions.

PCA

The SM genetic similarity coefficients of 21 accessions were used to carry out PCA of a two-dimensional array of eigenvectors (Figure 2). The results of the PCA were comparable to the cluster analysis. The first three components explained 43.05% of the total variation.

Table 3. Genetic similarity coefficients using SM similarity coefficients based on ISSR data.

	J1	J2	J3	J4	J5	J6	J7	J8	J9	J10	J11	J12	J13	J14	J15	J16	J17	J18	J19	J20	J21
J1	1.0000																				
J2	0.6250	1.0000																			
J3	0.7132	0.6471	1.0000																		
J4	0.5882	0.8309	0.6544	1.0000																	
J5	0.6875	0.6360	0.7169	0.6507	1.0000																
J6	0.6471	0.6029	0.8088	0.6324	0.7169	1.0000															
J7	0.6176	0.6176	0.6176	0.5588	0.6213	0.6354	1.0000														
J8	0.6544	0.6103	0.7794	0.6176	0.6801	0.8676	0.5882	1.0000													
J9	0.6618	0.6029	0.7500	0.5882	0.7096	0.7794	0.5956	0.8015	1.0000												
J10	0.5257	0.5478	0.5257	0.5110	0.5588	0.5404	0.5846	0.5036	0.5846	1.0000											
J11	0.5809	0.7794	0.6250	0.7941	0.6287	0.6250	0.6176	0.6324	0.5956	0.5037	1.0000										
J12	0.6544	0.6471	0.6691	0.6029	0.6801	0.6691	0.6354	0.6397	0.6765	0.5625	0.6691	1.0000									
J13	0.6434	0.6360	0.7463	0.6287	0.6544	0.8051	0.6581	0.7423	0.8022	0.5735	0.6434	0.7096	1.0000								
J14	0.6250	0.6103	0.6985	0.6029	0.6287	0.7868	0.6250	0.7279	0.6912	0.5331	0.6176	0.6544	0.8566	1.0000							
J15	0.5441	0.5551	0.5184	0.5404	0.4926	0.5184	0.7353	0.5110	0.5478	0.5882	0.5037	0.5331	0.7610	0.6945	1.0000						
J16	0.5368	0.5625	0.5184	0.5772	0.5000	0.5404	0.6103	0.5404	0.5478	0.5882	0.5257	0.5478	0.7022	0.7132	0.5809	1.0000					
J17	0.5331	0.5368	0.5221	0.5380	0.4816	0.5294	0.5184	0.5147	0.5147	0.5515	0.4926	0.5074	0.5441	0.5404	0.5110	0.6213	1.0000				
J18	0.5882	0.6029	0.7279	0.5735	0.6287	0.7647	0.5699	0.8088	0.7500	0.5037	0.6029	0.6691	0.5515	0.5478	0.5551	0.6066	0.9118	1.0000			
J19	0.6544	0.5956	0.8059	0.5956	0.6949	0.6691	0.5221	0.6691	0.6765	0.5331	0.6176	0.8462	0.5625	0.5441	0.5294	0.6103	0.8640	0.8493	1.0000		
J20	0.6581	0.5846	0.7684	0.5846	0.6838	0.7390	0.7610	0.7684	0.7390	0.5394	0.5993	0.6581	0.6985	0.6581	0.8417	0.5699	0.5441	0.5735	0.5404	1.0000	
J21	0.6176	0.5956	0.7574	0.5956	0.6801	0.7279	0.7279	0.7500	0.7794	0.5541	0.5882	0.6544	0.6801	0.6471	0.8162	0.5735	0.5404	0.5625	0.5294	0.9081	1.0000

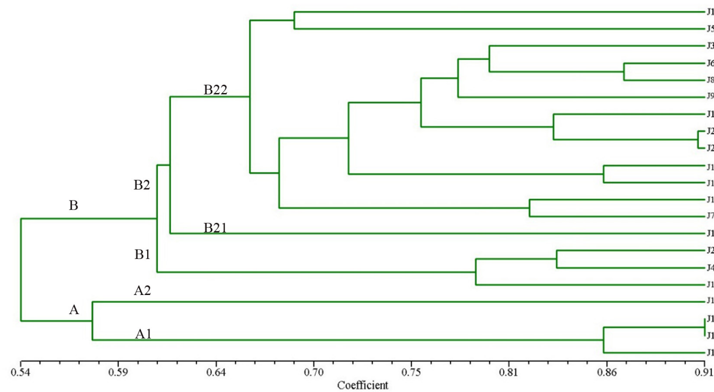

Figure 1. Dendrogram relationship of 21 accessions.

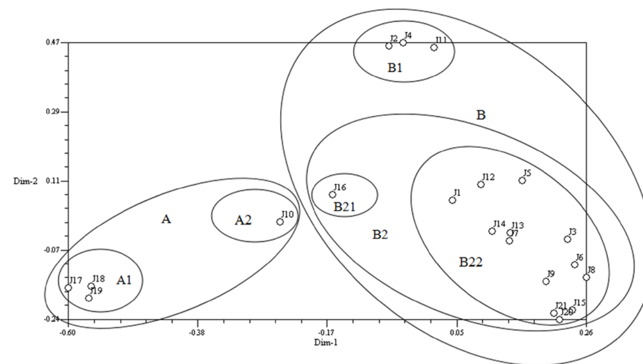

Figure 2. Two-dimensional plot (with vectors) of PCA of 21 accessions using ISSR.

DISCUSSION AND CONCLUSION

Molecular techniques have become a major tool for systems biologists, and they are useful for solving taxonomic problems at species and population levels. ISSR is widely used for germplasm identification and genetic diversity, genetic variation, and genetic relationship analyses.

When the genetic similarity coefficient is small, the genetic relationship is more distant. The similarity coefficient among accessions J17, J18, and J19 was very high, suggesting that these accessions belonged to the same species. However, for commercial applications, this variety was divide into three groups. Analysis of the UPGMA dendrogram clearly distinguished *Lonicera japonica* and *Lonicera macranthoides*, demonstrating that the ISSR markers could be effectively used to distinguish interspecific variations. In the *Lonicera japonica* cluster, the wild accession (J16) was distinguished from the other cultivars, demonstrating that the ISSR markers could also be effectively used to distinguish intraspecific variation. Therefore, these results provided insights into approaches for the selection of real *Lonicera japonica* germplasms for planting and established the wild germplasm is a new source for breeding.

In cluster A, accession J10 from Sichuan was distinguished from accessions J17, J18, and J19 from Shandong. The differences between the two subclusters may be explained by

variations in soil and climatic conditions among regions. This is consistent with the results reported by Sun et al. (2013) and Han et al. (2013). In cluster B, subcluster B1 (including accessions J2, J4, and J11) shared common phenotypic features with young branches of *Euonymus atropurpureus* and young leaves of eggplant and exhibited red corolla. The other 14 accessions in B2 had white corolla. These results showed that the appearance of *Lonicera japonica* could be used as morphological classification features and that the ISSR results supported the morphological classification.

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