Journal of Medicinal Plants Research Vol. 6(19), pp. 3596-3602, 23 May, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR12.371
ISSN 1996-0875 ©2012 Academic Journals

Full Length Research Paper

ISSR and SRAP markers in the genetic relationship analysis among *Pinellia* in China

Lingling Liu, Zaijun Yang, Shuhong Wei, Zhongming Ouyang, Kai Wu and Zhengsong Peng*

Key Laboratory of Southwest China Wildlife Resources Conservation, College of Life Science, China West Normal University, Nanchong, Sichuan 637009, China.

Accepted 22 March, 2012

The genetic relationship among five species of *Pinellia* endemic in China was examined by inter-simple sequence repeat (ISSR) and sequence-related amplified polymorphism (SRAP) markers. Twelve ISSR primers amplified a total of 569 DNA fragments, of which 504 (88.58%) were polymorphic, whereas 38 SRAP primer combinations amplified 1826 fragments, of which 1431 (78.37%) were polymorphic. Cluster analysis showed that ISSR, SRAP and ISSR + SRAP dendrograms of the five species of *Pinellia* generally exhibited similar clustering patterns. *Pinellia ternata*, *Pinellia cordata*, *Pinellia integrifolia* and *Pinellia peltata* were clustered into group I, while *Pinellia pedatisecta* was in group II. Group I comprises two subgroups, that is, subgroups A and B. *P. ternata* and *P. integrifolia* were clustered into subgroup A, while *P. cordata* and *P. peltata* were clustered into subgroup B. The results suggested that *P. cordata* has the closest genetic relationship with *P. peltata*, and *P. ternata* is closely related to *P. integrifolia*. *P. pedatisecta* is a sister group to the other species. The results of this study might be useful for guiding the exploitation and conservation of *Pinellia* in China.

Key words: *Pinellia*, genetic relationship, inter-simple sequence repeat (ISSR), sequence-related amplified polymorphism (SRAP).

INTRODUCTION

Pinellia Tenore is a small Eastern Asian genus of the Araceae, with only 7 perennial herbaceous species (Mayo et al., 1997), and mainly distributed in China, Korea and Japan. Among the 5 species of *Pinellia*, only *Pinellia ternata* (Thunb.) Breit. was not endemic in China. The center of distribution was located in Central and Eastern China (Yi, 2002). All *Pinellia* species were seasonally dormant herbs growing in woodlands and forests, on rocks with dripping water, or among crops as weeds (e.g., *P. ternata*). *P. ternata* are important traditional Chinese medicinal plants recorded in Chinese herb classics more than 2000 years ago (Li, 1976) and used for the treatment of viper bites, lumbago, allergic reaction and externally to treat traumatic injury, abscesses, neck sarcoma, breast mastitis and uterine cancer (Li, 1976; Luo, 1979). *P. ternata* has been embodied by the Pharmacopoeia of the People's Republic of China (2005), and the Hubei, Jiangsu, Henan, Shanxi, Shandong and Sichuan provinces are the geo-authentic producing areas.

Pinellia genus is one of the evolutionary taxa in Araceae. Some studies of the genetic relationship in *Pinellia* had been carried out (Cheng et al., 2006; Li et al., 2000, 1999, 2008). However, most studies are limited to a comparison between two or three species, and most of them are based on morphological analysis. Therefore, there has been a lack of systematic study on the genetic relationship among the 5 species of *Pinellia* in China.The inter-simple sequence repeat (ISSR) marker involves polymerase chain reaction (PCR) amplifications of DNA using a primer composed of a microsatellite sequence anchored at 3' or 5' end by 2 to 4 arbitrary nucleotides (Zietkiewicz et al., 1994). The PCR amplification of the DNA has proven to be a rapid, simple and inexpensive way to assess the structure and genetic diversity (Culley et al., 2007; Gonzàlez A et al., 2005; Nan et al., 2003; Jian et al., 2002) to analyze genetic relationships among cultivars (Martins et al., 2003) and to study the evolutionary processes (Sun et al., 2005). Sequence-related amplified polymorphism (SRAP) is a newer molecular

*Corresponding author. E-mail: pzs8833@163.com.

Table 1. Location of five species of *Pinellia* (family Araceae).

S/N	species	Origin	Voucher
1	*P. ternata*	Nanchong, Sichuan province	Zhengsong Peng
2	*P. peltata*	Wenzhou, Zhejiang province	Zaijun Yang
3	*P. cordata*	Lushan, Jiangxi province	Zaijun Yang
4	*P. inlegrifolia*	Xuyong, Sichuan province	Zaijun Yang
5	*P. Pedatisecta*	nanchuan, chongqing	Zhengsong Peng

Table 2. Primer sequence used for ISSR and SRAP analysis in this study.

ISSR primer(5' →3')		SRAP primer			
		Forward primer(5' →3')		Reverse primer (5' →3')	
807	$(AG)_8T$	me1	5′TGAGTCCAAACCGGATA 3′	em1	5′GACTGCGTACGAATTAAT 3′
824	$(TC)_8G$	me2	5′TGAGTCCAAACCGGAGC 3′	em2	5′GACTGC·GTACGAATTTGC 3′
825	$(AC)_8T$	me3	5′TGAGTCCAAACCGGAAT 3′	em3	5′GACTGCGTACGAATTGAC 3′
826	$(AC)_8C$	me4	5′TGAGTCCAAACCGGACC 3′	em4	5′GACTGCGTACGAATTTGA 3′
834	$(AG)_8YT$	-	-	em5	5′GACTGCGTACGAATTAAC 3′
836	$(AG)_8YA$	-	-	em6	5′GACTGCGTACGAATTGCA 3′
841	$(GA)_8YC$	-	-	em7	5′GACTGCGTACGAATTCAA 3′
845	$(CT)_8RG$	-	-	em8	5′GACTGCGTACGAATTCTG 3′
855	$(AC)_8YT$	-	-	em9	5′GACTGCGTACGAATTCGA 3′
856	$(AC)_8YA$	-	-	em10	5′GACTGCGTACGAATTCAG 3′
857	$(AC)_8YG$	-	-	em11	5′GACTGCGTACGAATTCCA 3′
873	$(GAC A)_4$	-	-	em12	5′GACTGCGTACGAATTATT 3′

marker first introduced by Li and Quiros (2001). The SRAP technique consists of preferential amplification of open reading frames (ORFs) using PCR. SRAP had been applied extensively in genetic linkage map construction, genetic diversity analysis and comparative genetics of different species and other fields (Li and Quiros, 2001; Ferriol et al., 2003; Guo and Luo, 2006; Ding et al., 2008).

In the present study, we systematically investigate the genetic relationship among the five species of *Pinellia* in China using both ISSR and SRAP markers, and estimate the genetic base of these germplasm to be used in future conservation and breeding programs.

MATERIALS AND METHODS

Plant

A total of five *Pinellia* spp. were collected from their main natural growing regions in different provinces of China and were then planted in the botanical garden of China West Normal University, Nanchong, China. The details on sample collection are given in Table 1. Young leaves were collected from the field-grown plants and stored at -80°C prior to DNA extraction.

DNA extraction

Total genomic DNA of fresh young leaves were extracted using the cetyltrimethylammonium bromide method described by Sambrook et al. (2001), and the quality confirmed by 1.0% (w/v) agarose gel electrophoresis. DNA concentrations were determined with a Photometer Nanodrop-2000C (Thermo, America), and samples were diluted to 50 ng/µl for PCR amplification.

ISSR-PCR amplification

One hundred ISSR primers biosynthesized from the Shanghai Sangon Biological Engineering Technology and Service Company were screened initially. Twelve primers with good and clear amplified bands were selected for genetic diversity analysis (Table 2). ISSR amplification was carried out using PCR instrument MyCycler (Bio-Rad, America) in 10 µl reaction volumes containing 5 µl of 2×Taq PCR MasterMix (Tiangen Biotech, China), 3.7 µl of ddH_2O, 1 µl of DNA (50 ng/µl) and 0.8 µl of primer (10 ng/µl). PCR amplification was performed under the following conditions: initial 5 min at 94°C, followed by 38 cycles of 30 s at 94°C, 30 s annealing at 55°C or 52°C and 90 s extension at 72°C and a final 8 min extension at 72°C. The PCR products were separated on 8% denatured polyacrylamide gels. The gels were run in 1 × (tris-borate-ethylenediaminetetraacetic acid (EDTA) (TBE)) buffer (0.09 M Tris-borate and 0.002 M EDTA) at 300 V for 4 h. The products were visualized by silver staining using the method

Table 3. Genetic diversity of five species of *Pinellia* revealed by ISSR, SRAP and combined ISSR and SRAP.

Species	Total bands	Polymorphic bands	Percentage of polymorphic bands (PPB)	Nei's gene diversity (H)	Shannon's information index (I)
ISSR					
P. ternata	108	95	87.96	0.2229	0.3768
P. peltata	103	90	87.38	0.2203	0.3751
P. cordata	115	102	88.70	0.2391	0.3956
P. inlegrifolia	111	98	88.29	0.2306	0.3854
P. Pedatisecta	132	119	90.15	0.2687	0.4364
Average	113.8	100.8	88.58	0.2363	0.3939
SRAP					
P. ternata	388	309	79.63	0.3196	0.4975
P. peltata	382	303	79.31	0.3001	0.4741
P. cordata	371	292	78.70	0.3015	0.4715
P. inlegrifolia	324	245	75.61	0.2704	0.4357
P. Pedatisecta	361	282	78.11	0.3091	0.4781
Average	365.2	286.2	78.37	0.3001	0.4714
Combined ISSR and SRAP					
P. ternata	496	404	81.45	0.2436	0.4033
P. peltata	485	393	81.03	0.2449	0.4024
P. cordata	486	394	81.06	0.2435	0.4014
P. inlegrifolia	435	343	78.85	0.2210	0.3739
P. Pedatisecta	493	401	81.34	0.2477	0.4106
Average	479	387	80.79	0.2401	0.3983

described by Bassam et al. (1991). The ISSR-PCR was repeated three times.

SRAP analysis

Forty-eight random SRAP primer combinations (Sangon Biotech, Shanghai) (Li, 2001) were selected for SRAP analysis (Table 2). SRAP amplifications were performed in 20 µl reaction volumes containing 10 µl 2×Taq PCR MasterMix (Tiangen Biotech, China), 50 ng genomic DNA and 10 ng of each primer. Amplification conditions were as follows: initial 5 min at 94°C, 5 cycles of 94°C for 1 min, 35°C for 1 min and 72°C for 1 min; 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min; followed by a final 10 min extension at 72°C. Amplification products were fractionated by electrophoresis in 8% (w/v) denaturing polyacrylamide gel at 300 W for 4 h and were visualized by silver staining. The SRAP analysis was repeated three times.

Data analyses

The amplified DNA fragments were recorded as either 1 or 0, representing the presence or absence of the band, respectively. Data analyses were performed using the numerical taxonomy multivariate analysis system (NTSYS-pc), version 2.10 software package (Exeter Software, Setauket, New York). The genetic similarity coefficient was calculated using Jaccard's coefficient by the SIMQUAL program (Rohlf FJ, 2000). The genetic diversity parameters, including the percentage of polymorphic bands (PPB), Shannon's information index (I) (Shannon CE, Weaver W, 1949) and Nei's gene diversity (H) (Nei, 1973) were obtained at species level. A dendrogram (Sokal and Michener, 1958) was constructed later based on the genetic similarity matrix by the unweighted pair group method with arithmetic average (UPGMA) (Sneath and Sokal, 1973).

RESULTS

ISSR analysis

Twelve ISSR primers amplified a total of 569 scorable bands (Table 3), of which 504 were polymorphic and accounted for 88.58%. The number of bands varied from 3 (UBC825) to 13 (UBC841), with an average of 8 polymorphic fragments per primer. Figure 1 represents the extent of polymorphism observed among the five species as revealed by UBC841. Genetic similarities among the 5 species ranged from 0.5949 to 0.7569 (Table 4). Figure 3a shows the dendrogram based on the ISSR data. UPGMA grouped the 5 species into two main clusters at a similarity index value of 0.6482. Cluster I comprised 4 species that were delineated into two sub-clusters. Sub-cluster I comprised 2 genotypes, with species *P. ternata* and *Pinellia integrifolia* (with a 0.7569 similarity coefficient) appearing to be closely related

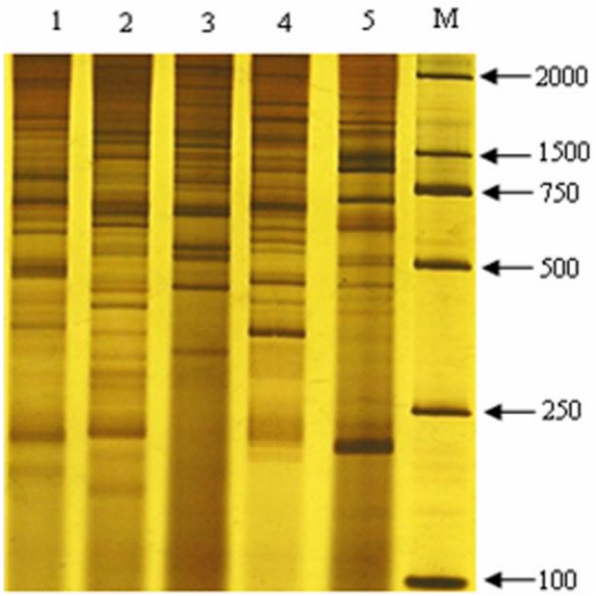

Figure 1. ISSR amplified result of *Pinellia* by primer UBC841. The line from left to right were 1, *P. ternate*; 2, *P. peltata*; 3, *P. cordata*; 4, *P. inlegrifolia;* 5*, P. pedatisecta;* M is DL2000 DNA marker.

Table 4. Genetic distance and similarity revealed by ISSR, SRAP and combined ISSR and SRAP.

Species	*P. ternata*	*P. peltata*	*P. cordata*	*P. inlegrifolia*	*P. pedatisecta*
ISSR					
P. ternata	****	1.2572	1.2214	0.6528	0.9983
P. peltata	0.6505	****	0.7623	1.0886	1.3576
P. cordata	0.6343	0.7292	****	1.1480	1.1293
P. inlegrifolia	0.7569	0.6713	0.6412	****	1.0826
P. Pedatisecta	0.6481	0.5949	0.6111	0.6111	****
SRAP					
P. ternata	*****	0.5700	0.5633	0.5479	0.6468
P. peltata	0.6852	*****	0.4768	0.5761	0.5954
P. cordata	0.6927	0.7312	*****	0.5806	0.6450
P. inlegrifolia	0.7162	0.7077	0.7115	*****	0.7786
P. Pedatisecta	0.6645	0.6861	0.6729	0.6513	*****
Combined ISSR and SRAP					
P. ternata	*****	0.6778	0.6728	0.5857	0.7180
P. peltata	0.6798	*****	0.5368	0.6744	0.7338
P. cordata	0.6818	0.7293	*****	0.6992	0.7456
P. inlegrifolia	0.7266	0.6965	0.6892	*****	0.8387
P. Pedatisecta	0.6658	0.6598	0.6564	0.6477	*****

Genetic distance (above diagonal) and genetic similarity (below diagonal).

genetically, while sub-cluster II comprised 2 genotypes, with species *Pinellia peltata* and *Pinellia cordata* (with a 0.7292 similarity coefficient) appearing to be closely related genetically. Cluster II consisted of one species

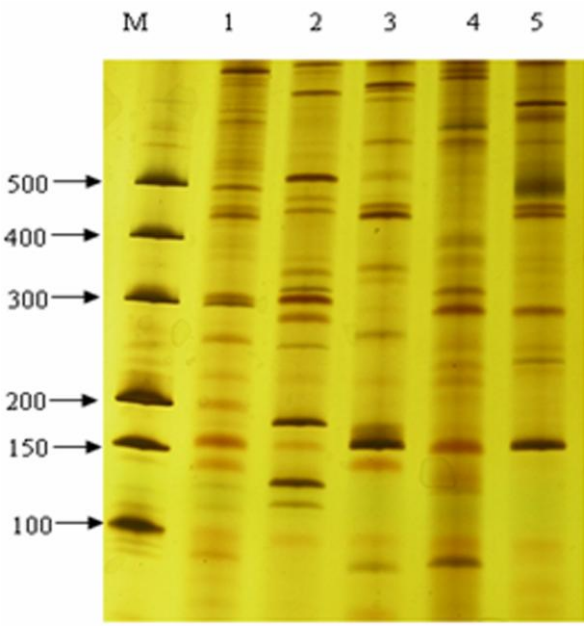

Figure 2. SRAP amplified result of *Pinellia* by primer ME1-EM9. The line from left to right were 1, *P. ternate*; 2, *P. pedatisecta*; 3, *P. cordata*; 4, *P. inlegrifolia;* 5*, P. peltata;* M is DL500 DNA marker.

(*Pinellia pedatisecta*) that appeared to be distinct from all the other species. The lane numbers correspond to the species listed in Table 3. In the case of ISSR analysis, the PPB varied from 87.38 (*P. peltata*) to 90.15% (*P. pedatisecta*) within each species, and the mean value of H was 0.2363, ranging from 0.2203 to 0.2687. The value of I showed similar trends, ranging from 0.3751 to 0.4364.

SRAP analysis

A total of 1826 bands, of which 1431 (78.37%) were polymorphic, were scored from 5 species using thirty eight SRAP primers. The extent of polymorphism revealed by the ME1-EM9 primer is as shown in Figure 2. Percentage polymorphism ranged from 53.85% (ME1-EM7) to a maximum of 95.83% (ME2-EM12), with an average of 82.74%. The number of bands varied from 13 (ME1-EM7) to 28 (ME2-EM11), with an average of 21.70 polymorphic fragments per primer. Genetic similarities among the 5 species ranged from 0.6513 to 0.7312 (Table 4). Figure 3b shows the dendrogram based on the SRAP data. UPGMA grouped the 5 genotypes into two main clusters at a similarity index value of 0.5750. Cluster I comprised 4 species that were delineated into two sub-clusters. Sub-cluster I comprised 2 species, *P. ternata* and *P. integrifolia* (with a 0.7162 similarity coefficient), which appeared to be closely related genetically, while sub-cluster II comprised 2 species, *P. peltata* and *P. cordata* (with a 0.7312 similarity coefficient), which appeared to be closely related genetically. Cluster II consisted of one species (*P. pedatisecta)* that appeared to be distinct from all the other genotypes. The lane numbers correspond to the genotypes listed in Table 3. In the case of SRAP analysis, the PPB varied from 85.61 to 79.63% within each population, and the mean value of H was 0.3001, ranging from 0.2704 to 0.3196. The value of I showed similar trends, ranging from 0.4357 to 0.4975.

Combined ISSR and SRAP analysis

Analysis of combined ISSR and SRAP data shows genetic similarities among all the 5 genotypes ranging from 0.6477 to 0.7293 (Table 4). The structure of the tree is very similar to that from SRAP analysis. UPGMA grouped the 5 genotypes into two main clusters at a similarity index value of 0.6864. Cluster I comprised 4 species that were delineated into two sub-clusters. Sub-cluster I comprised 2 genotypes, species *P. ternata* and *P. integrifolia* (with a 0.7266 similarity coefficient), which appeared to be closely related genetically, while sub-cluster II comprised 2 genotypes, species *P. peltata* and *P. cordata* (with a 0.7293 similarity coefficient), which appeared to be closely related genetically. However, cluster II consisted of only one species (*P. pedatisecta)* that appeared to be distinct from all the other species. In the case of combined ISSR and SRAP analysis (Figure 3c), PPB varied from 78.85 (*P. integrifolia*) to 81.45% (*P. ternata*) within each species, and the mean value of H was 0.2401, ranging from 0.2210 to 0.2477. The value of I ranged from 0.3739 to 0.4106.

DISCUSSION

In this study, ISSR and SRAP markers were applied to assess the level and pattern of genetic relationship among the five species of *Pinellia*. The results revealed that ISSR and SRAP methodologies were highly effective in demonstrating the genetic diversity among the five species of *Pinellia*. The percentage of polymorphic band (PPBs = 78.37%) generated by SRAP primer were lower than ISSR (PPBs = 88.58%). However, the average Nei gene diversity (Hs = 0.3001) and Shannon's information index (Is = 0.4714) at species level generated by SRAP makers were higher than that of ISSR analysis (Hs = 0.2363, Is = 0.3939). The possible reason was that the ISSR and SRAP techniques target different parts of the genome. ISSR marker amplification targets are located in the region between simple sequence repeats (SSRs), whereas microsatellite DNA is not a general transcription region. In the case of SRAP, ORF, including the intron(s) and promoter region, is the amplification target. Therefore, combining ISSR and SRAP analysis were effective and reliable for accurately assessing the genetic relationship of *Pinellia*.

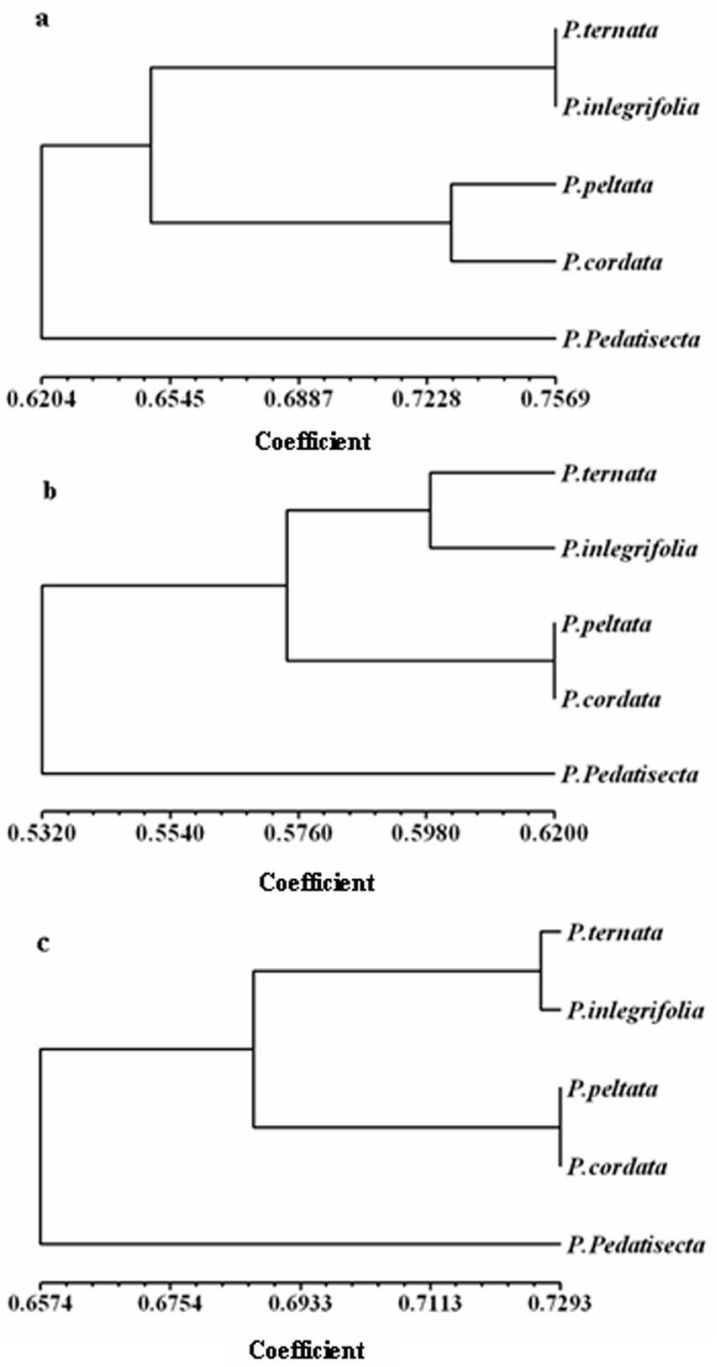

Figure 3. Dendrogram obtained by UPGMA cluster based on data from ISSR and SRAP analysis. (a) ISSR analysis; (b) SRAP analysis; (c) combined ISSR and SRAP analysis.

ISSR, SRAP and ISSR + SRAP dendrograms of the five species in *Pinellia* generally exhibited similar clustering patterns. For example, species *P. ternata* and *P. integrifolia* clustered together in each case, the same with species *P. peltata* and *P. cordata*. However, small differences were observed. For example, in the dendrogram obtained by ISSR, subgroup I (species *P. ternata* and *P. integrifolia*) appeared to be more closely related genetically. However, in the dendrogram obtained by SRAP, subgroup II (species *P. peltata* and *P. cordata*) had close relationship. The dendrogram obtained by the combined ISSR + SRAP had a similar cluster pattern with those generated by SRAP markers.The cluster results based on ISSR, SRAP and combined ISSR + SRAP

analyses showed that the species obviously clustered into two major groups. *P. ternata*, *P. cordata*, *P. integrifolia* and *P. peltata* were clustered into group I, while *P. pedatisecta* was in group II. Group I comprises of two subgroups, namely, subgroups A and B. *P. ternata* and *P. integrifolia* were clustered in subgroup A, while *P. cordata* and *P. peltata* were clustered in subgroup B. The results suggested that *P. pedatisecta* is a sister group to the other species, and supported the results of Chen et al. (2006) and Yi et al. (2002). *P. pedatisecta* and the other species of *Pinellia* have significant differences in plant morphological and pollen grain characteristics. For example, in plant morphology, *P. pedatisecta* is the only species of *Pinellia* that always have pedate leaf blades and lack the transverse septum inside its spathe. In pollen grains, *P. pedatisecta* has small pollen and scarce long spines on exine (Li, 1999). *P. cordata* and *P. peltata* had close relationships, which was consistent with the results of Chen et al. (2006). Palynological evidence indicated that *P. cordata* and *P. peltata* had similar pollen grain morphologies, size and exine structure (Li et al., 2000). In the present study, *P. ternata* and *P. integrifolia* had a close relationship. Morphologically, *P. ternata* differed from *P. integrifolia*; especially since *P. ternata* had 3-foliolate leaves, while *P. integrifolia* leaf blade was ovate or oblong. However, *P. ternata* and *P. integrifolia* had similar leaf shape, namely, ovate or oblong with entire margin in the seedling stage. To conclude, the results of the present study show that the SRAP and ISSR technique is an effective and reliable additional method for analyzing the genetic relationship and similarities among *Pinellia* species.

ACKNOWLEDGEMENTS

This study was supported by the Foundation of Key Laboratory of Southwest China Wildlife Resources Conservation (Ministry of Education), China West Normal University, P. R. China (XNYb01-4) and Scientific Research Fund of SiChuan Provincial Education Department (2006ZD025, 08ZA124).

REFERENCES

Bassam BJ, Caetano-Anolles G, Gresshoff PM (1991). Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem., 196: 80-83.

Cheng YC, cheng B, xue M, chen L (2006). Relationships of Five Pinellia Species (Araceae) Inferred from AFLP Data. Acta Bot. Yunnanica, 28(6): 559.

Culley TM, Sbita SJ, Wick A (2007). Population genetic effects of urban habitat fragmentation in the perennial herb Viola pubescens (Violaceae) using ISSR markers. Ann. Bot., 100: 91–100.

Ding G, Zhang DZ, Ding XY, Zhou Q, Zhang WC, Li XX (2008). Genetic variation and conservation of the endangered Chinese endemic herb Dendrobium officinale based on SRAP analysis. Plant Syst. Evol., 276:149–156.

Ferriol M, Pico B, Nuez F (2003). Genetic diversity of a germplasm collection of Cucurbita pepo using SRAP and AFLP markers. Theor. Appl. Genet., 107:271–282.

Gonzàlez A, Wong A, Delgado-Salinas A, Papa R, Gepts P (2005). Assessment of inter simple sequence repeat markers to differentiate sympatric wild and domesticated populations of common bean. Crop Sci., 45:606–615.

Guo DL, Luo ZR (2006). Genetic relationships of some PCNA persimmons (Diospyros kaki Thunb.) from China and Japan revealed by SRAP analysis. Genet. Resour. Crop Evol., 53:1597–1603.

Jian SG, Shi SH, Zhong Y (2002). Genetic diversify among south China Heritiera littoralis detected by inter-simple sequence repeats (ISSR) analysis. J. Genet. Mol. Biol., 13(4):272–276.

Li G, Quiros CF (2001). Sequence-related amplified polymorphism (SRAP), a new marker system based on a simple PCR reaction: its application to mapping and gene tagging in Brassica. Theor. Appl. Genet., 103:455–461.

Li H (1976). The 'tian-nan-xing', 'hu-zhang' and 'ban-xia' in Chinese herbalogies. Acta Bot. Yunnanica, 2:13–26.

. Ll L (1999). Micromorphological Characteristics and Enzyme Analysis of Some Chinese Pinellias and Their Taxonomic Significance. Acta Bot. yunnanica, 21(4):442-448.

Li L, chen M, zhang M, sun M, liao ZH (2008). The Analysis of Germplasms Evaluation of Four Different Leaf Types and Characters of Pinellia ternata and Pinellia pedatisecta by ISSR. J. Southwest China Normal Univ. (Natural Science Edition), 33(11):86-89.

Li MW, Yu BJ, Gu DX, Liu YL (2000). Comparison of pollen grains of pinellia and their tuber protein in alcohol. J. Wuhan Bot. Res., 18(3):250.

Luo HS, Zhou DH (1979). Brief introduction of Chinese medicine commonly used as anti-tumor. J. New. Chin. Med., 4: 53–54.

Martins M, Tenreiro R, Oliveira MM (2003). Genetic relatedness of Portuguese almond cultivars assessed by RAPD and ISSR markers. Plant Cell Rep. 22: 71–78.

Mayo SJ, Bogner J, Boyce PC (1997). The genera of Araceae. Royal Botanic Gardens, Kew. Pp. 91-288.

Nan P, Shi SH, Peng SL (2003). Genetic diversity in Primula obconica (Primulaceae) from central and south-west China as revealed by ISSR markers. Annal. Bot., 91(3): 329–333.

Nei M (1973). Analysis of gene diversity in subdivided populations. Proc. Nat.l Acad. Sci., USA 70:3321–3323.

Rohlf FJ (2000). NTSYS-PC: numerical taxonomy and multivariate analysis system, version 2.1. , New York, USA.

Sambrook J, Russell DW (2001). Molecular cloning:A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Shannon CE, Weaver W (1949). The mathematical theory of communication. University of Illinois Press, Urbana.

Sneath PH, Sokal RR (1973). Numerical Taxonomy: The Principal and Practice of Numerical Classification. W. H. Freeman and Company, San Francisco.

Sokal RR, Michener CD (1958). A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull., 28:1409–1438.

Sun JH, Li ZC, Jewett DK (2005). Genetic diversity of Pueraria lobata (Kudzu) and closely related taxa as revealed by intersimple sequence repeat analysis. Weed Res., 45(4):255–260.

The State Pharmacopoeia Commission of P.R. China (2005). Pharmacopoeia of the People's Republic of China. China Medical Science and Technology Press, beijing. pp. 78.

Yi TS (2002). Phylogeny and biogeography of Pinellia (Araceae) with special reference to phylogenetic problems of Remusatia. PH.D. Tthises, Kunming Institute of Botany, kunming, China.

Zietkiewicz E, Rafalski A, Labuda D (1994). Genome fingerprinting by simple sequence repeats (SSR)-anchored PCR amplification. Genomics, 20: 176–183.