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Full Length Research Paper

Genetic diversity of *Ligusticum chuanxiong* Hort. based on inter simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) analyses

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***Ligusticum chuanxiong* Hort. (Apiaceae) is a well-known traditional Chinese medicinal herb with hemodynamic and analgesic effects. The species is known to undergo strict asexual reproduction via clonal propagation, and all known occurrences of the species are under cultivation practices. In this study, genetic diversity of *L. chuanxiong* throughout most of its cultivated range in China was assessed. Two hundred and sixty-six individuals representing 14 populations of *L. chuanxiong* were screened for ISSR and AFLP markers. ISSR analysis detected polymorphisms in 35% of the observed bands generated from 10 primer pairs. Two hundred and fifty-six AFLP fragments were obtained with six primer combinations, with a 29.3% level of polymorphism. These results indicated low levels of genetic variation in cultivated populations. Independent UPGMA analyses were performed for ISSR and AFLP data. The dendrograms generated from both data sets showed a close alliance among Sichuan Province populations, and Mantel tests revealed the presence of a moderate spatial geographic gradient among all populations representing five Provinces. Overall, our results suggested that mating system and anthropogenic effects play an important role in shaping the genetic diversity and structure of *L. chuanxiong*.**

Key words: *Ligusticum chuanxiong* Hort., Genetic diversity, inter simple sequence repeat, amplified fragment length polymorphism.

INTRODUCTION

The dried rhizome of *Ligusticum chuanxiong* has been used for the clinical treatment of headache, rheumatic arthralgia and coronary heart diseases in China over hundreds of years (Pharmacopoeia of China, 2005). The species is propagated by clonal vegetative growth, which comprised of the following three steps: 1) The immature rhizomes are separated and removed from the soil in January; 2) the rhizomes are then transplanted to an altitude of 900 - 1300 m; and 3) following four months of growth, the rhizomes are separated and removed from the soil and sun dried. To ensure continued propagation success, stipes are removed, wrapped and transplanted back to the soil between July and August. This is the traditional propagation method and is still practiced today.

So, the species undergo strict asexual reproduction via clonal propagation, and all known occurrences of the species are under cultivation practices. *L. chuanxiong* is primarily in Sichuan, Gansu, Jiangxi, Jilin and Jiangsu

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Abbreviations: AFLP, Amplified fragment length polymorphisms; **ISSR,** inter simple sequence repeats; **CTAB,** cetyltrimethyl ammonium bromide; **PPB,** percentage of polymorphic bands; **UPGMA,** unweighted pair group method with arithmetic mean; **AMOVA,** analyses of molecular variance; **RAPD,** random amplified polymorphic DNA; **PCR,** polymerase chain reaction; **Ht,** total genetic diversity; **Hs,** genetic diversity within populations; **Gst,** genetic differentiation among populations.

Table 1. Population acronyms, geographic locations and sample size for *L. chuanxiong* populations.

Population	Sample site	Longitude(E)/ latitude(N)	Altitude (m)	Sample size
PX	Pixian, Sichuan Province, China	103°45′/ 30°51′	550	20
ZT	Zitongcun, Chongzhou, Sichuan Province, China	103°42′/ 30°45′	548	20
LJ	Liaojiazhen, Chongzhou, Sichuan Province, China	103°42′/ 30°43′	548	20
SY	Shiyang, Dujiangyan, Sichuan Province, China	103°37′/ 30°50′	600	20
DJ	Xudu, Dujiangyan, Sichuan Province, China	103°39′/ 30°49′	610	20
CY	Cuiyuehu, Dujiangyan, Sichuan Province, China	103°37′/ 30°51′	610	20
XD	Xindu, Sichuan Province	104°00′/ 30°52′	539	20
GX	Gexianshan, Pengzhou, Sichuan Province, China	103°59′/ 30°09′	610	20
PZ	Aoping, Pengzhou, Sichuan Province, China	104°01′/ 30°06′	595	20
SF	Shifang, Sichuan Province, China	104°11′/ 31°06′	510	20
NT	Nantong, Jiangsu Province, China	120°50′/ 32°01′	5	20
HT	Huating, Gansu Province, China	106°44′/35°07′	1800	15
RC	Ruichang, Jiangxi Province, China	115°37′/ 2 9°43′	513	15
LJ	Longjing, Jilin Province, China	129°25′/ 42°.98′	110	16

provinces. Historical records indicate that *L. chuanxiong* was originally planted in Sichuan Province. It is widely accepted that population genetic variation is influenced by some factors, such as historical events, breeding system, genetic drift and natural selection (Barrett et al., 1992). The artificial cultivation could affect genetic variations between and within artificial populations by breeding system and human behaviors. Despite hundreds of years of clonal propagation of *L. chuanxiong* in China, there is little information available for genetic variation within and among artificial populations. So, large-scale data on the genetic diversity and population structure of *L. chuanxiong* are needed for setting up suitable guidelines for future domestication programs aimed at preserving diversity and inbreeding.

Studies of genetic diversity within and among plant populations have applied a variety of molecular markers, including random amplified polymorphic DNAs (RAPDs), inter-simple sequence repeats (ISSRs) and amplified fragment length polymorphisms (AFLPs) (Bouza et al., 2002; Behera et al., 2008; Li et al., 2005). ISSR is a marker of choice in population genetic studies, especially in detecting clonal diversity and can be used to fingerprint closely related individuals (Zietkiewicz et al., 1994; Wolfe et al., 1998; Esselman et al., 1999). AFLP analysis generates DNA fingerprints with a large number of genetic markers.

These data can be used as a viable alternative to analyze morphological and biochemical trait. Furthermore, previous studies have indicated that AFLPs provided better resolution in discerning phylogenetic relationships relative to isozymes, nuclear RFLPs and chloroplast DNAs (Sharma et al., 1996). In this study, we employed ISSR and AFLP makers to investigate the levels of genetic diversity in *L. chuanxiong* throughout most of its major cultivated range in China.

MATERIALS AND METHODS

Study populations and sampling

The locations of 14 populations of *L. chuanxiong* representing the range of cultivation in China are shown in Table 1. Young leaves were sampled from 266 individuals from 14 populations. Individual samples were separated by a distance of more than 5 m to increase the likelihood of sampling inter-individual variation and decrease the likelihood of sampling clones of the same individual plant within populations. The voucher specimens of these samples were deposited at the Department of Life Sciences, Sichuan University and their identities were authenticated according to the pharmacognostic standard documented in China Pharmacopoeia (2005).

DNA extraction

Leaf tissues from each individual were placed on silica gel in separate zip-lock plastic bags until DNA extraction. Genomic DNA was extracted from approximately 0.1 g of leaf tissue using a modified cetyl trimethylammonium bromide (CTAB) method (Doyle, 1991) with minor modifications. Procedures were performed in 1.5 mL eppendorf tubes. Briefly, about 0.1 g of leaf tissue was grounded in liquid nitrogen and suspended in 500 µL of 2% CTAB buffer containing 0.5% β-mercaptoethanol, followed by incubating at 65°C for 60 min and centrifuging at 10 000 × *g* for 10 min at 4°C. The supernatant was added to 500 µL of chloroform: isoamylalcohol (24:1, v/v), and the sample was shaken gently for 1 min and centrifuged at 7000 × *g* for 10 min at 4°C. The supernatant was extracted once more and then mixed with 2/3 volume ice-cold isopropanol. DNA was recovered as a pellet by centrifugation at 10,000 × *g* for 5 min at 4°C, washed with 300 µL of ice-cold ethanol twice, dried in the air, and then dissolved in 200 µL of 1× TE buffer. DNA quality and quantity were checked in 0.8% agarose gels.

ISSR analysis

ISSR primers produced by the Biotechnology Laboratory, University

Table 2. Primer sequences and the number of bands generated from ISSR analysis.

Primer	Sequence of primer (5'–3')	Total number of band	Number of polymorphic band
UBC825	$(AC)_8T$	13	6
UBC835	$(AG)_8YC$	9	4
UBC836	$(AG)_8YA$	14	7
UBC842	$(GA)_8YG$	12	5
UBC865	$(CCG)_6$	9	5
UBC866	$(CTC)_6$	10	0
UBC867	$(GGC)_6$	7	1
UBC876	$(GAT A)_2(G ACA)_2$	12	4
UBC879	$(CTT CA)_3$	11	2
UBC880	(GGA GA)3	8	3
Total		106	37
Average		10.6	3.7
% Polymorphism	35%		

Y= C,T.

Table 3. AFLP bands generated by selective amplification based on six primer pairs.

Primer code	Primer pairs	No. of bands scored	No. of polymorphic bands
1	E-AAG/M-CAG	38	14
2	E-ACA/M-CAA	48	11
3	E-ACA/M-CAG	50	12
4	E-ACT/M-CTG	40	10
5	E-ACG/M-CTA	38	13
6	E-AGG/M-CAA	42	15
Average		44	12.5
Species		256	75
% Polymorphisms	29.3%		

of British Columbia were tested using polymerase chain reaction (PCR). One-hundred ISSR amplifications were performed in a Biometra® Tpersonal PCR system using the following parameters: initial step of 4 min at 94°C; followed by 40 cycles at 94°C for 30 s, 46°C for 45 s, and 72°C for 2 min; and a final 7 min extension step at 72°C.

Reactions were performed in a 20 µL volume, using 40 ng of template DNA, 1 mM of primer, 1 U of Taq DNA polymerase (Pharmacia, Biotech), and 0.2 mM of each dNTP (100 mM dNTP Set, Life Technologies) in a reaction buffer containing 10 mM Tris–HCl pH 9.0, 50 mM KCl and 1.5 mM $MgCl_2$. The amplification products were separated by electrophoresis against a 100 bp molecular marker in 2% agarose gels and visualized by ethidium bromide staining. Photodocumentation was obtained for each gel. Ten ISSR primers (Table 2) revealed clear and reproducible banding patterns and were subsequently chosen for amplifying all DNA samples after amplification conditions were optimized.

AFLP analysis

The AFLP analysis was performed using six primer combinations (Table 3), using the GibcoBRL AFLP Analysis System (Gaithersburg, Md.) with minor modifications. Genomic DNA (400 ng) was digested with *Eco*RI and *Mse*I at 37°C for 4 h. Following heat inactivation of the restriction endonucleases, the genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters overnight at 16°C to generate template DNA for amplification. PCR was performed in two conescutive reactions. Template DNA was first preamplified using AFLP primers, none of which contained selective nucleotides. The preamplification reaction products were then used as templates at a 1:10 dilution for selective amplification using two AFLP primers, containing three selective nucleotides. *Eco*RI primers were labeled using a modified silver staining protocol. The final PCR products were separated on a 6% denaturing poly acrylamide gel in 1×TBE buffer, followed by silver staining and photodocumentation (Li et al., 2005).

Data analysis

ISSR and AFLP amplification fragments were scored as presence (1) or absence (0) data, and coded into a binary data matrix. Only unambiguous distinct and reproducible fragments were coded for both marker types. The binary data matrix of AFLP and ISSR genotypes was analyzed in POPGENE version 1.32 (Yeh et al., 1997) to calculate the percentage of polymorphic bands (PPB). The genetic structure was investigated using Nei's gene diversity statistics (Nei et al., 1972), including total genetic diversity (Ht), genetic diversity within populations (Hs), and the relative magnitude of genetic differentiation among populations (Gst = (Ht-Hs)/Ht). A nonparametric analysis of molecular variance (AMOVA) was also

Table 4. Analysis of molecular variance (AMOVA) within/among populations from 106 ISSR bands.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P* - value
Among populations	13	3509.769	14.50318 Va	53.58	P < 0.0001
Within populations	252	3197.849	12.56514 Vb	46.42	P < 0.0001

P - value after 5000 permutations. d.f., degree of freedom.

applied to calculate genetic structure and variability among populations, and 5000 permutations were set for significance testing (Excoffier et al., 1992). In order to test for a correlation between genetic and geographic distances, a Mantel test was performed with 5000 random permutations (Miller et al., 1997).

The genetic relationships among populations were examined as follows: an unweighted paired group method of cluster analysis with arithmetic averages (UPGMA) based on Nei's genetic distance (D) for each method (ISSR and AFLP) was used to generate two dendrograms (Nei et al., 1972); branch support for all analyses was determined using bootstrap analysis (10 000 replicates) (Hillis et al., 1993).

RESULTS

ISSR analysis

A total of 106 markers were analyzed (Table 2). The size of amplified DNA fragments ranged from 200 - 1900 bp. The number of bands scored for each primer varied from 7 [$(GGC)_6$] to 14 [$(AG)_8$YA], with a mean number of 10.6 markers per primer. Thirty-nine polymorphic bands were observed, and ranged from 0 [$(CTC)_6$] to 7 [$(AG)_8$YA] per primer. Each primer generated an average of 3.7 polymorphisms. Computation of genetic diversity resulted in an average of 35% polymorphic bands (PPB) over all *L. chuanxiong* populations.

Ht was 0.1056 and Hs was 0.0458. The coefficient of Gst was 0.57, that is 57% of the genetic variation was among populations. AMOVA analysis further revealed significant ($p < 0.001$) genetic differences among the 14 populations. Of the total genetic diversity, 53.58% was attributable to diversity among populations and 46.42% within populations (Table 4).

UPGMA cluster analysis showed geographic structuring of some populations. PZ and GX were clustered with 83% bootstrap support; and SY, DJ and CY were clustered with a 81% bootstrap value. Moreover, the ten populations from Sichuan Province were clustered with a 56% bootstrap value (Figure 1A). However NT population from Jiangsu Province and the ten populations from Sichuan Province were clustered.

AFLP analysis

Scored fragments ranged from 100 - 1000 bp and the number of markers per primer combination ranged from 38-50. AFLP data detected a 29.3% level of polymorphism, with an average of 12.5 polymorphic bands per primer combination.

The Ht was 0.0994 and Hs was 0.0351 while Gst was 0.6470. These results indicated a nearly 65% level of genetic variation among *L. chuanxiong* populations. Significant ($p < 0.0001$) genetic differences were revealed among all 14 populations based on AMOVA. Of the total genetic diversity, 61.61% was attributable to among population differences and 38.39% within populations (Table 5).

UPGMA cluster analysis based on AFLPs showed a higher level of geographic structuring (Figure 1B) compared with ISSR data. The 14 populations were separated into two main groups (I and II); Group I comprised of the Sichuan Province populations (64% bootstrap value), and Group II of those populations occupying other geographic regions (74% bootstrap support).

The correlation coefficient (r) between genetic and geographical distance (not shown) using Mantel's test for all populations was 0.5314 ($p = 0.0160$). This result suggested a moderate spatial correlation between genetic distance and geographic distribution.

DISCUSSION

Genetic variation patterns

The results of this study detected similar levels of polymorphism in ISSR and AFLP data (35 and 29.3%, respectively), demonstrating low levels of genetic variation within and among populations (Li et al., 2004). Furthermore, this study demonstrated the reliability of ISSR and AFLP approaches for assessing the genetic diversity in *L. chuanxiong.* At the species level, PPB was lower than that reported in other clonal species (e.g. *Potamogeton maackianus*; PPB = 52.7%) (Li et al., 2004). However, other clonal species have been shown to exhibit extremely low or no genetic variation (e.g. *Eichhornia crassipes*) due to a founder effect following colonization of a new region (Li et al., 2006). Compared with other clonal reproducing species, *L. chuanxiong* maintains moderate levels of genetic diversity.

Two explanations are proposed to explain these results. First, the genetic diversity of the species may be very low to begin with and clonal propagation exacerbated genetic uniformity among and within the introduced populations. *L. chuanxiong* is widely planted in China, and Sichuan Province is considered its point of origin based on thousands of years of historical records of its use and

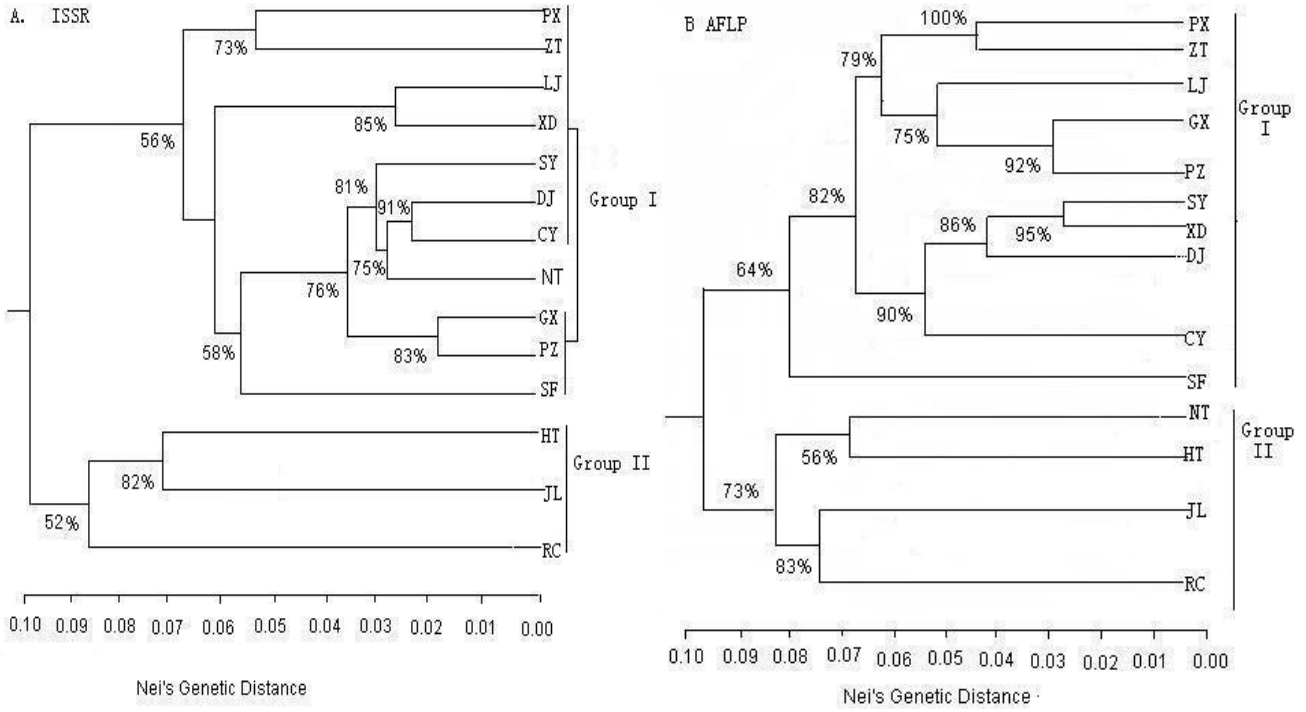

Figure 1. UPGMA dendrograms for populations of *L. chuanxiong* based on Nei's genetic distance using ISSRs (A) and AFLPs (B). Bootstrap support is indicated at each node.

Table 5. Analyses of molecular variance (AMOVA) with Euclidean distance matrix from 266 individuals using 256 AFLP bands.

Source of variation	d.f.	Sum of squares	Variance component	Percentage of variation	*P* - value
Among population	13	838.445	3.86380	61.61	P < 0.0001
Within population	252	547.643	2.40764	38.39	P < 0.0001

P - value after 5000 permutations. d.f., degree of freedom.

cultivation. The species may have always possessed lower levels of genetic variability compared with congeners or other clonal species. Species propagation is purely by clonal reproduction, and seed production has never been observed. Furthermore, flowering or normal flower development is rarely seen, suggesting sexual reproduction does not play a role in the life history of *L. chuanxiong*. Strict asexual reproduction results in a loss of genetic variability, and likely contributes to the genetic uniformity in the species. Second, in the recent past, the price of medicinal materials of *L. chuanxiong* fell, and habitats were destroyed due to human disturbance. Habitat loss resulted in a dramatic decline in the number of extant populations. This loss of populations could have led to a decrease in genetic variability even in the absence of sexual reproduction. Therefore, the low levels of genetic variability in *L. chuanxiong* may be attributed to both the breeding system and anthropogenic effects.

Gst and AMOVA results indicated that within-population variability was lower than between-population variability (Gst = 0.65 for AFLPs and 0.57 for ISSRs). Due to the presumed strict asexual reproduction, these results were expected. In general, Gst ranges from 0.5 - 0.59 for inbreeding species (Hamrick and Godt, 1989). These results indicated a higher level of genetic variation among *L. chuanxiong* populations.

UPGMA cluster analysis generated different graphic representations of *L. chuanxiong* population genetic structure based on ISSR and AFLP data (Figure 1A and B). Nevertheless, in spite of the incongruent results, certain trends were evident. Thirteen of the 14 populations clustered into two main groups (NT the exception). Group I comprised of the 10 Sichuan province populations; and Group II, three populations from Gansu, Jilin, Jiangxi provinces. The Mantel's test suggested a spatial correlation between genetic distance and geographic location, although this result was only moderately supported. The NT population was introduced from Sichuan Province four decades ago. In its new environment, *L. chuanxiong* may have accumulated somatic mutations through clonal growth, placing it in an equivocal position on the dendrogram. Additional in-

consistencies between ISSR and AFLP results for the 10 Sichuan province populations may be explained by passed introductions to the region, perhaps 100s of years ago. Unfortunately, we can only infer population history and are left with unresolved relationships between genetic structure and geographic distance. Since the species has been cultivated for thousands of years, geographic distance may not be the only or most important factor affecting the genetic structure of a population, particularly as human activity becomes more frequent and disruptive (Li et al., 2004). Anthropogenic change, a long history of domestication, asexual reproduction and a clonal growth habit may all have influenced the genetic structure of *L. chuanxiong*.

Artificial cultivations

Given *L. chuanxiong* is currently experiencing habitat loss due to human activity, we must propose an appropriate strategy now for conserving the genetic resources in this valuable medicinal plant. The first would consist of a conservation plan that defines areas free from signficant disruption. A stable environment suitable for population growth and breeding is of great importance. The second management strategy would be a program of samples intensively from within all populations to fulfill the objective of capturing most of the detected genetic variability. The knowledge of genetic diversity may redound to the domestication program for genetic improvement such as increase the amount of some certain medical component, enhance the adaptability of some populations in order to accelerate its worldwide usage. Thirdly, it is necessary to design an availability strategy for reducing the impact of medical market, especially while the price of medicinal materials of *L. chuanxiong* falls.

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