Genetic diversity analysis of different populations of Epimedium koreanum Nakai (Berberidaceae) based on inter simple sequence repeat molecular markers

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Research Article

Keywords: Epimedium koreanum, inter simple sequence repeat markers, genetic diversity

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1235795/v1

Abstract

As one of the medicinal herbs recorded in the Chinese Pharmacopoeia, *Epimedium koreanum* is a traditional and edible herbal plant with important medicinal and economic values in China. Currently, the plant is harvested in the wild to meet the market demand because of the poor cultivation strategies of *E. koreanum*, and it has been collected without restriction for a long time, leading to a large reduction of wild resources. The study of genetic diversity of wild populations of *E. koreanum* would be beneficial for the conservation and development of this species. This study was aimed at analyzing the genetic diversity of 134 plant samples from 14 wild populations in the Jilin and Liaoning Provinces using seven selected primers from inter simple sequence repeat (ISSR) markers. The results showed that *E. koreanum* had a high level of genetic diversity and genetic variation intra population rather than inter population, with low gene flow between populations (Ht=0.3034, Hs=0.2189, Gst=0.2784, Nm=1.2960). The above results obtained from the POPGENE software analysis were in general agreement with the AMOVA analysis. A total of 14 *E. koreanum* populations were classified into two major taxa and five subclassifications. The Mantel test showed a low correlation between genetic and geographic distances. Based on these genetic results, conservation strategies considering the genetic diversity of *E. koreanum* are needed for areas with low genetic diversity, such as the establishment of nature reserves and appropriate artificial cultivation to maintain the population size.

1 Introduction

Epimedium koreanum is a perennial herb of the genus *Epimedium* in the family Berberaceae (Ma et al. 2011) and is found in China, Japan, Korea, and Russia (Ying et al. 2000;Stearn et al. 2002). The aboveground parts of the plant are very distinctive, with only three stems and three leaves on each stem (the whole plant has nine leaves), which is referred to as "San Zhi Jiu Ye Cao" in China (Administration Bureau in Traditional Chinese Medicines (China). 1998). The *Epimedium* genus is characterized by an evolutionarily conserved genome and high interspecific genetic similarity (Takahashi et al. 1989;Yan et al. 2016; Zhang et al. 2018), and plants of this genus are diploid (2n=2x=12), with karyotype characteristics according to Stebbins' classification—all chromosomes having a pair of satellites. Because the seed cultivation technology is not developed, the propagation of *E. koreanum* relies mainly on rhizome division (Mihaljević et al. 2009), which has led to it being a vulnerable species.

Notably, *E. koreanum* is one of the herbal medicinal plants recorded in the Chinese Pharmacopoeia as *Epimedium* (National Commission of Chinese Pharmacopoeia. 2020). It grows mainly in the northeastern provinces of China. *Epimedium* is a commonly used Chinese herb that has been known for 2,000 years for its effectiveness in tonifying kidney yang, strengthening muscles and bones, and combating rheumatism (Li et al.1991). Previous experiments have proven that *E. koreanum* extract has a variety of biological activities, such as antibacterial, antioxidative, and anti-osteoporosis effects, among others (Meng et al. 2005). Even in modern Chinese medicine, it is utilized into tablets, capsules, and other drugs (Oh et al. 2015), such as Xianling Bone Capsules, which has been approved by the State Food and Drug Administration of China since 2002 and consists of six herbs, of which *Epimedium* accounts for 70% by weight (Cheng et al. 2013). Due to its medicinal properties, *Epimedium* is very popular in China and the herb is in high demand. Like most traditional Chinese herbs, medicinal plants of the genus *Epimedium* are not cultivated but collected from the wild (Guo et al. 2003), thus leading to a shortage in the number of wild *E. koreanum* plants in northeast China annually. Only a few conservation studies have been performed on this plant.

Many molecular marker methods are present for plant genetic diversity research, such as randomly amplified polymorphic DNA, simple sequence repeat, amplified fragment length polymorphism, and next-generation sequencing (Mondini et al. 2009). Inter simple sequence repeat markers (ISSR) combine the advantages of the aforementioned markers (Henareh et al. 2016), such as good stability, high reproducibility, rich polymorphism, simple operation, low cost, and good safety measures. They can also be used for organisms for which the genetic information available is insufficient to conduct a study (Mohammadi et al. 2020). ISSR is a PCR-based dominant marker, generally using primers of 16 to 25 bp in length, which can be dinucleotide, trinucleotide, tetranucleotide, or pentanucleotide (Zietkiewicz et al. 1994), and its usefulness as a molecular marker in plant genetic studies has been well established (Nidhal et al. 2016; Tiwari et al. 2017;Azizi et al. 2018).

Successful application of ISSR techniques has been reported for *Epimedium brevicornum* (Duan et al. 2021), and *Epimedium elatum* Morr & Decne (Lone et al. 2017), and this paper is the first report on *E. koreanum*. We selected ISSR markers to analyze the genetic diversity and genetic structure of 134 samples collected from 14 wild populations, and we believe that the results of our study provide valuable information for conservation strategies of *E. koreanum*.

2 Materials And Methods

2.1 Epimedium koreanum Populations

First, a comprehensive literature review was performed on *E. koreanum*. We have compiled information on the distribution of *E. koreanum* in the field after field surveys and interviews with local farmers. The geographic distribution and location information of *E. koreanum* was subsequently assembled. Through extensive investigation in the south of the Jilin and Liaoning provinces, we selected 14 natural populations. Young leaf tissue was collected from 134 individuals from 14 natural populations. The number of samples per population ranged from seven to ten individuals (mean=9.57). Details, such as population name, longitude, latitude, altitude, and sample size are provided in Table 1.

Table 1
Details of the natural populations used in this study

Population	Location	Longitude°	Latitude°	Elevation/m	Sample size
YL	Yaoling village, Jilin	125°34'4.800"E	41°33'21.600"N	483.28	10
QHZ	Qinghe town, Jilin	125°55'48.000"E	41°25'48.000"N	464.91	10
XJZ	Xijaing town, Jilin	125°44'9.600"E	41°27'7.200"N	375.99	10
HML	Humaling village, Jilin	125°41'6.000"E	41°35'34.800"N	471.03	10
DLZ	Dalu town, Jilin	125°45'36.000"E	40°59'42.000"N	376.00	10
DLZC	Dalizi village, Jilin	126°49'8.400"E	41°44'56.400"N	376.37	7
XQGM	Xiaoqinggoumen village, Liaoning	126°12'32.400"E	41°11'20.400"N	290.10	10
HKED	Hekouerdui village, Liaoning	125°50'27.600"E	41°39'32.400"N	377.61	10
CYZ	Caiyuan town, Jilin	125°44'52.800"E	41°19'4.800"N	369.20	10
WNS	Wunvshan village, Liaoning	125°24'14.400"E	41°18'54.000"N	489.67	10
DLX	Donglai town, Jilin	126°9'46.800"E	41°37'4.800"N	563.80	10
MYH	Mayihe town, Jilin	127°6'46.800"E	41°49'13.080"N	561.78	7
DYH	Dayahe village, Liaoning	125°6'50.400"E	41°9'46.800"N	406.58	10
EPDZ	Erpengdianzi village, Liaoning	125°30'0.000"E	41°10'40.800"N	337.20	10

2.2 DNA Extraction and Detection

Total genomic DNA was extracted from dry leaf tissues of 134 plants using the plant genomic DNA extraction kit (Tiangen, China) following the manufacturer's instructions, with a few changes in the leaf grinding process. An appropriate amount of PVP-40 was added while grinding leaves with liquid nitrogen. The DNA quality was checked using 1% agarose gel electrophoresis that was performed at 110 V for 40 min. The gel was visualized using the GelRed staining under UV light. DNA concentration and purity were analyzed using the BioSpec-nano spectrophotometer (Shimadzu Corporation, Kyoto, Japan). After detection, the DNA was stored at -20℃ until use.

2.3 ISSR Primer Screening and Polymerase Chain Reaction (PCR) Amplification

Seven ISSR primers (Table 2), previously screened from 100 primers published by the University of British Columbia (Canada), were synthesized by Shanghai Bioengineering Co., Ltd. (Shanghai, China) with consistent amplification, good polymorphism, and well-defined DNA segments. PCR amplification was performed using the final volume of 20 μL containing 27 ng of template DNA, 3.5 μL of 10 $\mu mol \cdot L^{-1}$ primers, 12 μL of 2×Taq Master mix (Tiagen, China), and 1.5 μL of double-distilled H_2O (ddH_2O). PCR was performed in a Biometra cycler (Germany) using the following settings: initial denaturation at 94°C for 4 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 42.6–55.7°C for 40 s, and extension at 72°C for 40 s. Afterwards, a final extension was performed at 72°C for 5 min and stored at 4°C. The PCR products were analyzed using 1% agarose gel electrophoresis.

Table 2
ISSR primers and amplification results

Primers	Sequence (5′-3′)	Annealing temperature (°C)	Total number of bands	Number of polymorphic bands	Polymorphism (%)
UBC 822	TCTCTCTCTCTCTCTCA	47.3	11	11	100
UBC 844	CTCTCTCTCTCTCTCTRC	45.6	11	11	100
UBC 815	CTCTCTCTCTCTCTCTG	42.6	9	8	88.89
UBC 813	CTCTCTCTCTCTCTCTT	49.4	11	10	90.91
UBC 854	TCTCTCTCTCTCTCTCRG	50.3	14	13	92.86
UBC 824	TCTCTCTCTCTCTCTCG	55.7	11	10	90.91
UBC 853	TCTCTCTCTCTCTCTCRT	53.9	13	11	84.61

2.4 Optimization of Annealing Temperature

The Tm value of primers was close to the annealing temperature, however was not the optimal annealing temperature. Selecting a high annealing temperature improves the specific binding between primers and the DNA template, as the PCR products may be reduced at low annealing temperatures. The optimal annealing temperature is critical in obtaining the best amplification effects. In the current study, we set eight temperature gradients based on the primer Tm value using a gradient PCR instrument (analytik jena, Germany). Moreover, gradient temperature PCR amplification experiments were performed to determine the optimal annealing temperature.

2.5 Data Analysis

The data generated via the seven ISSR primers on 134 *E. koreanum* samples were either; 1 (presence) and 0 (absence) of amplified DNA segments, and the "0, 1" binary matrix was established and entered into Excel software. Genetic parameters containing the polymorphic loci ratio, observed number of alleles (Na), effective number of alleles (Ne), Nei's gene diversity index (H), and Shannon information index (I) were calculated using POPGENE32 v.1.32 (Shi et al. 2020) software. The total genetic diversity (Ht), mean within-population genetic diversity (Hs), genetic differentiation coefficients among different populations (Gst), and gene flow number (Nm, Nm = 0.5 (1 - Gst)/Gst) were also analyzed using POPGENE32. NTSYSpc2.10e was used to examine the genetic similarity coefficients among the 14 populations (Rohlf. 2000). The clustering map was established using MEGA5.0 (Xu et al. 2018) based on the unweighted pair group method with arithmetic mean according to the genetic similarity coefficient. The correlation between populations of genetic distances and geographical distances was analyzed with the Mantel test using TFPGA1.3 (tools for population genetic analyses) (Miller. 1997). Geographical distance was calculated using the longitude and latitude coordinates between the 14 populations. The Arlequin 3.1.1 software was used to analyze the analysis of molecular variance (AMOVA) to assess the variation between and within the populations (Zhou et al. 2020).

3 Results

3.1 DNA Profiling Analysis

After detecting the DNA template using 1% agarose gel electrophoresis and spectrophotometry, bands showed no obvious tailing, and the OD_{260}/OD_{280} value was between 1.7–1.9. These results indicated that the quality of the DNA template was satisfactory for PCR amplification. In the current study, the seven ISSR primers produced 80 bands; of which, 74 bands were polymorphic and six were monomorphic (92.5% polymorphism). The number of amplification sites per primer ranged from 9–14, and the ratio of polymorphic sites ranged from 84.61–100%. Primer UBC853 generated the lowest ratio of polymorphic sites, and primers UBC844 and UBC822 yielded the highest ratio of polymorphic sites. A representative amplification result using the primer UBC853 was established (Fig. 1). The amplification results revealed high levels of polymorphism and abundant genetic diversity among the populations (Table 2).

3.2 Genetic Diversity of Epimedium koreanum

The genetic diversity parameters of 14 populations were calculated using POPGENE32 according to the "0, 1" matrix (Table 3). The observed number of alleles (Na) ranged from 1.5125 to 1.8000, with an average of 1.6438, and the effective number of alleles (Ne) ranged from 1.2685 to 1.4940, with an average of 1.3747; Nei's gene diversity index (H) was between 0.1635–0.2825, and the Shannon information index (I) ranged from 0.2506 to 0.4248. According to genetic diversity parameters, the highest and lowest genetic diversity was found in the WNS population (H=0.2852, I=0.4248, PPB=80%) and the DLZC population (H=0.1635, I=0.2506, PPB=51.25%), respectively. The results demonstrated abundant genetic diversity among the *E. koreanum* populations. According to the ISSR data, the following parameters were: Ht=0.3034, Hs=0.2189, Gst=0.2784, Nm=1.2960. Almost 72.16% of the total genetic variation was produced within populations, rather than among populations. The Nm among the populations of *E. koreanum* was 1.2960, indicating a low degree of gene flow among the 14 populations of *E. koreanum*.

Table 3
Genetic diversity of *Epimedium koreanum* populations

Population	Na	Ne	H	I	PPB (%)
YL	1.6375	1.3693	0.2155	0.3243	63.75
QHZ	1.7250	1.4394	0.2535	0.3782	72.50
XJZ	1.6250	1.3762	0.2182	0.3258	62.50
HML	1.6500	1.3731	0.2231	0.3358	65.00
DLZ	1.5875	1.3060	0.1819	0.2779	58.75
DLZC	1.5125	1.2685	0.1635	0.2506	51.25
XQGM	1.6625	1.3872	0.2245	0.3371	66.25
HKED	1.6375	1.3710	0.2178	0.3275	63.75
CYZ	1.6875	1.4008	0.2341	0.3522	68.75
WNS	1.8000	1.4940	0.2852	0.4248	80.00
DLX	1.6500	1.3713	0.2176	0.3283	65.00
MYH	1.5625	1.2934	0.1794	0.2751	56.25
DYH	1.6250	1.4167	0.2346	0.3450	62.50
EPDZ	1.6500	1.3789	0.2159	0.3237	65.00
Mean	1.6438	1.3747	0.2189	0.3290	64.37

3.3 Genetic relationship among populations of Epimedium koreanum

The genetic similarity coefficient and genetic distance were generated by NTSYSpc2.10e with the “0, 1” matrix (Table 4). The larger the genetic similarity coefficient, the closer the genetic relationship. Consequently, a small genetic similarity coefficient indicates a greater kinship. In the current study, the genetic similarity coefficient ranged from 0.7441 to 0.9700, and the genetic distance ranged from 0.0304 to 0.2955. The highest genetic similarity coefficient was produced between the DLZC and XQGM populations; accordingly, the genetic distance was the lowest. The lowest genetic similarity coefficient was generated between the XJZ and DLX populations; therefore, the genetic distance was the highest. A Mantel test was performed to assess the correlation between genetic distance and geographical distance. Fig. 2 displays a low correlation between the genetic distance and geographical distance.

A dendrogram cluster based on unweighted pair group method with arithmetic mean was established using the genetic similarity coefficient (Fig. 3). According to the map, the 14 populations of *E. koreanum* were divided into two major groups at the coefficient of around 0.87 (Group I and Ⅱ). Group I contained populations of WNS, DLX, and MYH. Group II could be further separated into two groups, called Ⅱa and Ⅱb based on the results of software analysis. Group IIb contained a single sample (XJZ). Group Ⅱa can also be divided into three subgroups, namely Ⅱa1, Ⅱa2, and Ⅱa3.

Table 4
Genetic similarity coefficient (above) and genetic distance (below) matrix for *Epimedium koreanum* by inter simple sequence repeat markers

	YL	QHZ	XJZ	HML	DLZ	DLZC	XQGM	HKED	CYZ	WNS	DLX	MYH	DYH	EPDZ
YL	****	0.9402	0.8014	0.9055	0.864	0.8908	0.8966	0.8672	0.882	0.8785	0.8224	0.8638	0.8567	0.8959
QHZ	0.0617	****	0.8674	0.9321	0.9128	0.926	0.9291	0.9066	0.9177	0.8951	0.8555	0.8846	0.8869	0.9156
XJZ	0.2214	0.1423	****	0.9266	0.919	0.865	0.8905	0.8708	0.8794	0.8123	0.7441	0.7613	0.8465	0.8407
HML	0.0993	0.0703	0.0763	****	0.9588	0.9497	0.9582	0.9221	0.9345	0.8802	0.8334	0.8758	0.9058	0.927
DLZ	0.1461	0.0912	0.0844	0.0421	****	0.9357	0.9499	0.9178	0.9064	0.8499	0.8085	0.8266	0.8851	0.9044
DLZC	0.1156	0.0769	0.145	0.0516	0.0664	****	0.97	0.9012	0.9078	0.8646	0.8157	0.8536	0.8786	0.9018
XQGM	0.1092	0.0735	0.116	0.0427	0.0514	0.0304	****	0.9256	0.9135	0.8799	0.8244	0.8522	0.914	0.9233
HKED	0.1425	0.098	0.1383	0.0811	0.0858	0.1041	0.0773	****	0.8964	0.8797	0.8291	0.8324	0.9166	0.9069
CYZ	0.1256	0.0859	0.1286	0.0677	0.0982	0.0967	0.0904	0.1093	****	0.8737	0.8329	0.8417	0.8619	0.8818
WNS	0.1296	0.1108	0.2079	0.1276	0.1626	0.1454	0.128	0.1281	0.135	****	0.9236	0.9145	0.8527	0.8735
DLX	0.1955	0.1561	0.2955	0.1822	0.2126	0.2037	0.193	0.1875	0.1828	0.0795	****	0.9358	0.8518	0.8482
MYH	0.1464	0.1226	0.2728	0.1326	0.1905	0.1582	0.16	0.1835	0.1724	0.0894	0.0663	****	0.8441	0.8806
DYH	0.1547	0.12	0.1666	0.0989	0.122	0.1294	0.09	0.0871	0.1486	0.1594	0.1605	0.1694	****	0.952
EPDZ	0.11	0.0882	0.1735	0.0758	0.1005	0.1033	0.0798	0.0977	0.1257	0.1352	0.1646	0.1272	0.0491	****

3.4 AMOVA Analysis of Populations of Epimedium koreanum

AMOVA was used to analyze the molecular differences. It can discriminate between various genetic structures and conduct statistical tests by classifying and dividing populations at different levels. Accordingly, the proportion of variation in the total variation can be calculated among and within populations or among individuals. In the current study, the AMOVA analysis of populations of *E. koreanum* was performed using Arlequin 3.1.1. As shown in Table 5, 73.84% genetic variation was observed within the population, and 26.16% was among the population. This result is consistent with the results of POPGENE32. The variation in genetic structure within populations of *E. koreanum* was significantly higher than that among populations.

Table 5
Analysis of molecular variance for 134 individuals from 14 populations

Source	Degree of freedom	Sum of squares	Variance	Variance%	Significance
Among populations	13	602.483	3.74196	26.16	P<0.01
Within populations	120	1267.443	10.56202	73.84	P<0.01
Total	133	1869.925	14.30399	100	

4 Discussion

Genetic variation and evaluation of the genetic relationships of medicinal plants are major components in the investigation of medicinal plant resources (Xiao et al.2020). According to modern genetics, the higher the genetic diversity of organisms, the wider the population distribution and environmental adaptability (Yan et al. 2019). The study of genetic diversity on the adaptation of organisms to an ever-changing environment, particularly that of anthropogenic habitat destruction, is very important (Hsu et al. 2015). ISSR molecular markers are widely used to assess genetic diversity because of their easy application and interpretation of the results. In this study, ISSR molecular markers were used to investigate the genetic diversity of 134 individuals from 14 populations of *E. koreanum*. The amplification of seven primers showing rich polymorphisms demonstrated that this marker was effective. Compared with the other four species of *Epimedium*, for instance, *Epimedium pubescens* (H=0.1298, I=0.1935 for ISSR), *Epimedium stellulatum* (H=0.088,

I=0.126 for ISSR), *Epimedium acuminatum* (H=0.2075, I=0.3135 for ISSR), *Epimedium brevicornu* (H=0.2494, I=0.3881 for ISSR), our results were relatively high. The genetic diversity of *E. koreanum* was slightly higher than that of *Epimedium acuminatum*, slightly lower than that of *Epimedium brevicornu*, and significantly higher than that of *Epimedium pubescens* and *Epimedium stellulatum* (Liu et al. 2017; Guan et al. 2018).

Gene flow refers to the movement of hereditary substances among populations through migration, seeds, and pollen transmission. From the perspective of Wright et al. (1935): when Nm <1, the population is severely fragmented; when 1<Nm<4, gene exchange can be performed normally; when Nm>4, gene exchange can occur frequently. The analysis results among 14 *E. koreanum* populations demonstrated a high level of gene flow. If genetic differences in populations are mainly the result of mating systems and seed dispersal, the genetic distance between populations should be positively correlated with geographical distance. Nevertheless, the Mantel test indicated that the distribution of genetic diversity of populations was not completely determined by geographical distance (Jiang et al. 2018). The phylogenetic tree showed the genetic relationships between populations. In general, populations from closer locations tended to cluster together. However, this was not always the case. In this study, populations with closer geographical distances also had a high genetic diversity.

- *E. koreanum* is an endemic medicinal resource that grows only in the northeast areas of China. During our field investigation, we found that the population size gradually reduced owing to excessive exploitation (Wang et al. 2019). Thus, it became necessary to formulate a protective strategy based on the results of the current research. First, excessive collection and anthropogenic destruction must be prohibited (Bruni et al. 2013). Secondly, nature reserves should be established and appropriate artificial cultivation techniques should be performed in areas with low *E. koreanum* genetic diversity, such as MYH and DLZC populations, to maintain a sufficient population.

Currently, there are few studies on genetic diversity of *E. koreanum*, and our study is only a beginning. The molecular marker techniques for research and analysis of this species can be continuously improved. For example, the results of this study could be made more representative by expanding the distribution of the number of test samples, and the error of the results can be reduced by improving the resolution of agarose gel electrophoresis. Moreover, it is possible to extend the depth and breadth of research on the use of ISSR markers in *E. koreanum* regarding the following aspects—excellent germplasm selection and breeding, kinship delineation, plant diversity conservation, and sustainable utilization research.

Authorship: The authors confirm contribution to the paper as follows: study conception and design: DaCheng Jiang, JingLei Xiao; data collection: LuSheng Rong, FangFang Meng; analysis and interpretation of results: Kun Guo, YingZhe Wang; draft manuscript preparation: Kun Guo, YingZhe Wang. All authors reviewed the results and approved the final version of the manuscript.

Abbreviations

RAPD Randomly amplified polymorphic DNA

SSR Simple sequence repeat

AFLP Amplified Fragment Length Polymorphism

Tm value Primer melting temperature

Declarations

Authorship: The authors confirm contribution to the paper as follows: study conception and design: DaCheng Jiang, JingLei Xiao; data collection: LuSheng Rong, FangFang Meng; analysis and interpretation of results: Kun Guo, YingZhe Wang; draft manuscript preparation: Kun Guo, YingZhe Wang. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgement:

Funding Statement: This work was supported by Jilin Province Science and Technology Development Plan (Grant No.20200404001YY).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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Figures

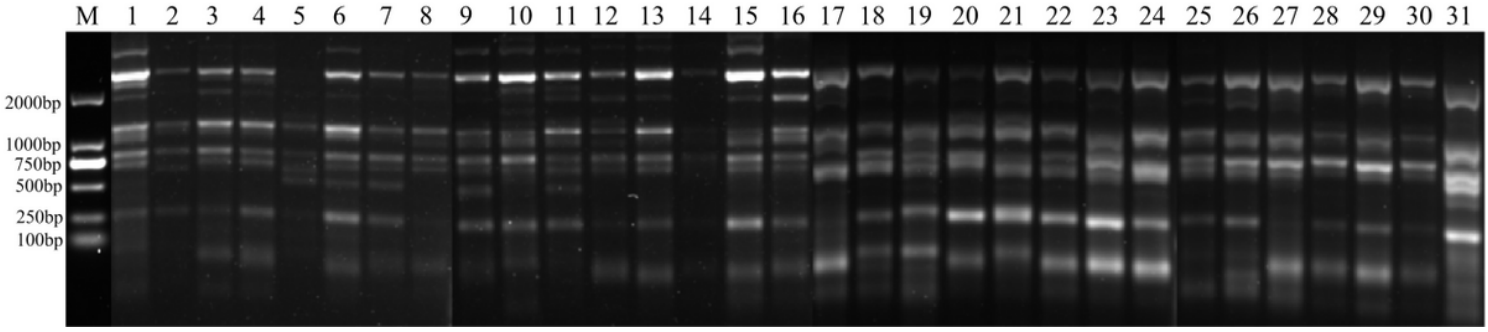

Figure 1

Representative agarose gel electrophoresis image with the inter simple sequence repeat primer UBC853. “M” is the DNA ladder 2000 bp molecular weight marker. (samples 1–8 were from the YL population; samples 9–16 were from the QHZ population; samples 17–24 were from the XJZ population; samples 25–31 were from the HML population)

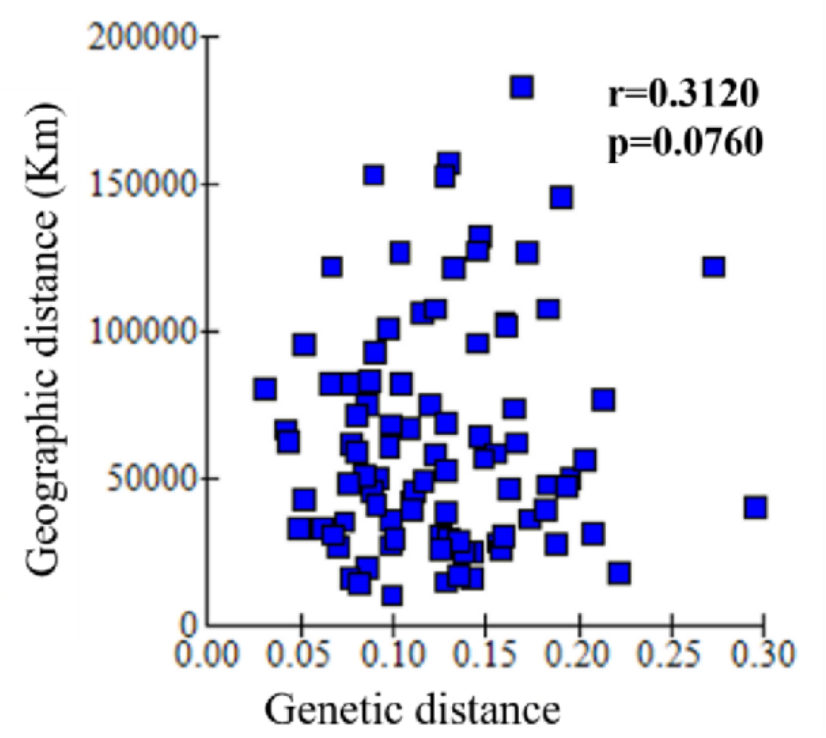

Figure 2

Correlation analysis between geographical and genetic distances

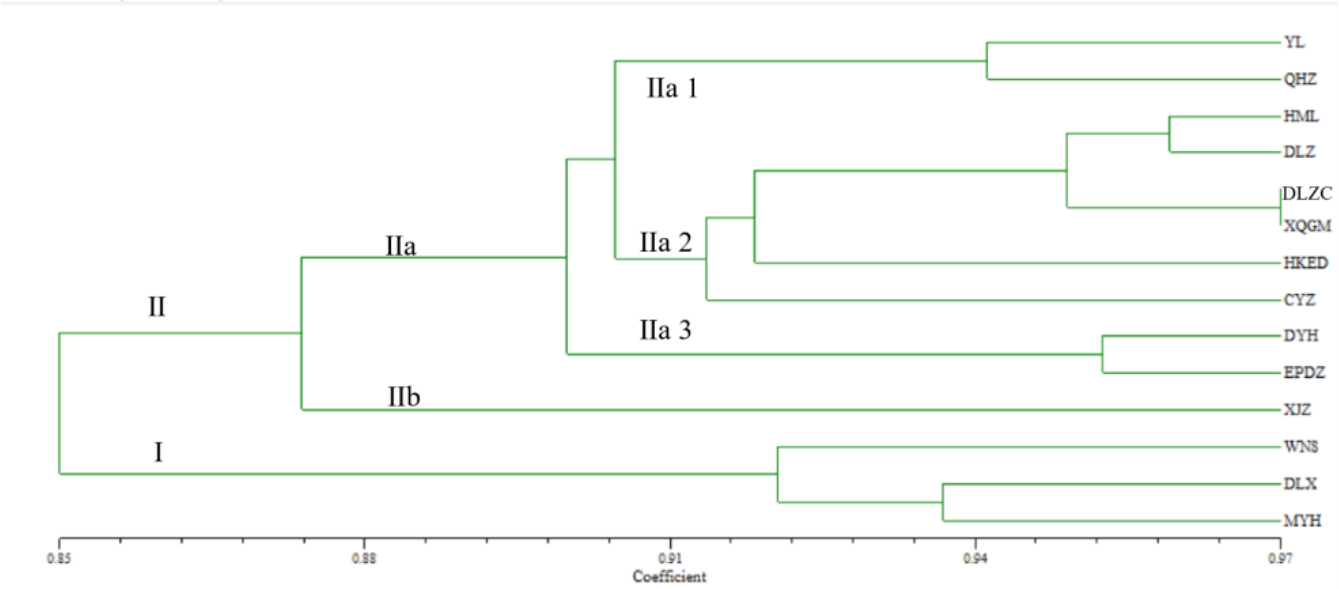

Figure 3

Cluster diagram of *Epimedium koreanum* populations from inter simple sequence repeat marker