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Research Article

Genetic Structure of *Malus sieversii* Population from Xinjiang, China, Revealed by SSR Markers

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Abstract: One hundred and nine *Malus sieversii* accessions from four geographical populations located at Kuerdening in Mohe town, Gongliu County, Jiaowutuohai, in Xinyuan County, Daxigou in Houcheng County of Ily State, and Baerluke Mountain in Yumin County of Tacheng State, Xinjiang Uygur Autonomous Region of China were studied by SSR markers. The purpose of the study was to determine the genetic structure and diversity in these eco-geographical populations with eight pair SSR primers of apple. The results indicated that: an average of 16 bands was detected in the four populations. The percentage of polymorphic bands in Gongliu population (89.06%) was the highest in the four populations. The average Nei's gene diversity index was 0.257 for all the loci. Totally, 128 polymorphic loci were detected and the percentage of polymorphic loci (*P*) was 100%, 88.28%, 84.83%, 87.50%, and 87.12%, respectively, at the species level and Gongliu, Xinyuan, Huocheng, and Yumin population levels. The Nei's gene diversity index (H = 0.2619) and Shannon's information index (I = 0.4082) in the species level were higher than in the population level. The Nei's gene diversity index and Shannon's information index in the four populations were Gongliu ＞ Huocheng ＞ Xinyuan ＞ Yumin. Gongliu population and Xinyuan population were the highest in genetic identity and the closest in genetic distance. Gene flow between the populations was 7.265 based on genetic differentiation coefficient (G_{ST} = 0.064). The UPGMA cluster analysis indicated that the genetic relationships between the Gongliu and Xinyuan population were the closest, and the Yumin population were the farthest with the other three populations. The UPGMA cluster analysis indicated that the four geographical populations located in Gongliu, Xinyuan, Huocheng, and Yumin were relatively independent populations. Concurrently, there was also mild gene exchange between the populations. On the basis of the study of population genetic structure and the highest genetic diversity, Gongliu population should be given a high priority consideration in *Malus sieversii* population's *in situ* germplasm conservation.

Keywords: *Malus sieversii*; SSR marker; population genetic structure

China, one of the oldest breeding centers of *Malus* plants, has extremely abundant germplasm resources. Twenty-one wild *Malus* species originated from China, among which *M. sieversii*, the main progenitor of domesticated apple (*Malus* × *domestica* Borkh.)[1], is mainly distributed in Tianshan Mountains in Central Asia including the Ily Valley of West China, Alma-Ata state, and Taldy-Kurgan state of Kazakhstan, and Issyk state of Kyrgyzstan. The wild fruit forest in Ily, which is located on the slopes of the Ily Valley, one of important sections of Central Asia, is considered to be a peculiar "Ocean climate" broadleaf forest type in Central Asia, with a desert climate[2]. *Malus sieversii*, the dominant tree in the

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wild fruit forest of Ily Valley, is suffering serious destruction [3] and is sharply decreasing in area, from 9,330 hm^2 in 1959 to 1,800 hm^2 in 2005 [4]. It is essential to study population genetic structure and genetic variability, and to evaluate genetic diversity of *M. sieversii* for scientific conservation and effective utilization of *M. sieversii* germplasm. The study on pollen morphology of *Malus sieversii* in Xinyuan, Huocheng, and Central Asia shows that *M. sieversii* from Central Asia is the advanced form, whereas *M. Sieversii* from Xinyuan is the primitive form [5]. Obvious differences have been reported on the isoenzyme diversity among populations of *M. sieversii* of different regions [6]. Feng *et al*. [3] investigated the morphological variations of 132 *M. sieversii* accessions from Daxigou of Houcheng County, Mohe of Gongliu County, and Jiaowutuohai of Xinyuan County, in the Ily State and found that *M. sieversii* were rich in genetic diversity from morphology. Chen *et al*. [7] analyzed the volatile components of 30 *M. sieversii* accessions from Mohe of Gongliu County using GC-MS and concluded that classes and contents of volatile components showed significant differences between seedlings and a wide genetic diversity within the populations.

Genetic diversity is not only one of the most important realms in all countries, but is also an important basis of protection for biology and genetic breeding. In the past two decades, molecular phylogenetics (including population genetics and phylogenetics) from crossing and infiltrating of plant phylogenetics, molecular biology, mathematics, computer science and genetics, and suited date analysis software, for example, AMOVA, offered effective measures for plant classification, phylogeny, molecular evolution, genetic diversity evaluation, and core collection construction, which showed tremendous potential and has made great progress [8–10]. SSR markers are thought to be the perfect markers in studying molecular phylogenetics because of their polymorphic, highly reproducible, and codominant nature [11]. Since 1997, many countries have developed various SSR primers of apple. Three hundred pairs of primers were developed and used in genetic diversity analysis of *Malus* plant, especially apple cultivars [12–16]. Forte *et al*.[17] have analyzed the genetic diversity of 6 *M. sieversii* accessions using the ITS sequence of ribosome and particle matK gene of chloroplasts and testified the genetic diversity of *M. sieversii* in 2002. To date, not much knowledge has been readily available for studying the genetic diversity of *M. sieversii* using molecular markers. In this study, the population genetic structure of 109 *M. sieversii* accessions from different geographical populations were studied by molecular phylogenetics and SSR markers, to evaluate the genetic diversity of *M. sieversii* and to provide molecular evidence for conservation and utilization of *M. sieversii* germplasm.

1 Materials and Methods

1.1 Plant materials

In the present study, 109 accessions were used, which were collected from four populations of *M. sieversii* grown in Kuerdening, Mohe, Gongliu County ($MH_{1\text{-}30}$), Jiaowutuohai, Xinyuan County ($XY_{1\text{-}30}$), and Daxigou, Houcheng County ($HC_{1\text{-}30}$) of Ily State and Baerluke Mountain, Yumin County ($TC_{1\text{-}19}$) of Tacheng State, Xinjiang Uygur Autonomous Region of China (Fig.1). Young leaves collected from these populations were immediately placed in self-ziplocked plastic bags containing silica gel for drying. Each tree sampled was separated by a distance

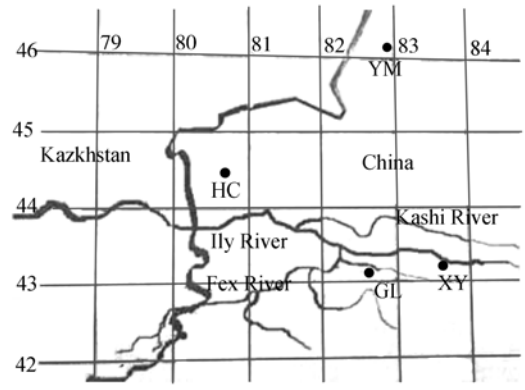

Fig. 1 The four population geographical locations in Xinjiang, China

YM: Yumin county; HC: Huocheng county; GL: Gongliu county; XY: Xinyuan county .

of about 50 m. The experiment was carried out at the Biological Laboratory of Pomology, Shandong Agricultural University from 2005 to 2006.

1.2 Methods

Genomic DNA was extracted as described by Doule and Doyle[18]. SSR-PCR amplifications were performed in a 15 μL volume containing 5 ng of genomic DNA, 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 200 μmol/L each dNTP, 0.2 μmol/L of forward and reverse primers each, and 1 U *Tag* polymerase. SSR primers of apple were developed by Swiss Federal Institute of Technology (ETH) and Horticulture Research International (HRI). DNA amplification was performed in a PTC-100^{TM} thermalcycler (MJ Research, Watertown, Mass., USA) under the following conditions: an initial denaturation at 94℃ for 2 min 30 s followed by four cycles of 94℃ for 30 s, 65℃ for 1 min, and 72℃ for 1 min, and then 30 cycles of 94℃ for 30 s, 60℃ for 1 min, and 72℃ for 1 min. A final cycle of 5 min at 72℃ was included. PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. The gels were silver stained following the protocol of Promega (Promega, Madison, USA).

1.3 Data analysis

The fragment data were inputted in a computer file as a binary matrix, where 0 and 1 coded for absence and presence of a band. NTSYSpc-2.10e (Exeter Software, Setauket, NY) and POPGENE version 1.32 software were used for data analysis [19].

Polymorphism of SSR loci was evaluated by the number of polymorphic bands and the percentage of polymorphic bands. Genetic diversity within the population was estimated by Shannon's Information index (I) [20] and Nei's gene diversity (H) [21]. Gene differentiation between populations was estimated by the coefficient of gene differentiation (G_{ST}) and gene flow (Nm) [22]. The UPGMA (Unweighted pair-group method using arithmetic averages) cluster analyses based on Nei's unbiased genetic distances and simple matching coefficient (SM) were applied between populations [23] and within populations[24], respectively.

2 Results

2.1 Polymorphism of different SSR primers in the *Malus sieversii* population

Eight pairs of primers with high polymorphism and strong-signal bands were selected from 40 pairs of apple SSR primers to amplify genomic DNA of 109 *M. sieversii* accessions. Fig. 2 shows examples of polymorphic bands amplified by prime CH03g12. Table 1 shows the obvious differences in total band-samplified by various primers. The total bands were amplified by eight pairs of primers ranging from 9 for CH05g03 to 21 for CH03h03, with an average of 16 bands per primer pair. Polymorphisms of the same primer pair in different populations were obviously

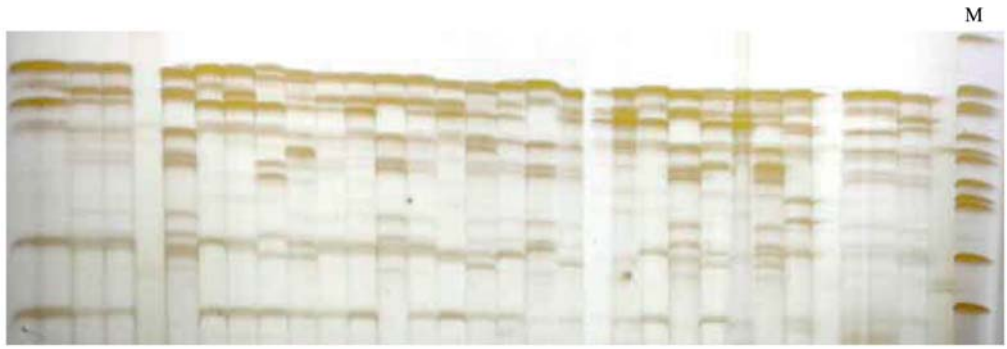

Fig. 2 SSR amplification result of *Malus sieversii* genomic DNA by primer CH03g12

The columns from left to right represent MH_1–MH_{30} and DNA marker (pBR322 DNA / *Msp* Ⅰ).

Table 1 Polymorphism of different SSR primers in the *Malus sieversii* populations and gene differentiation

Primers	Total bands amplified	Gongliu		Xinyuan		Huocheng		Yumin		Nei's gene diversity (*H*)	Coefficient of gene differentiation (G_{ST})
		A	*P* (%)	*A*	*P* (%)	*A*	*P* (%)	*A*	*P* (%)		
CH03g12	15	15	100	14	93.33	15	100	14	93.33	0.394aA	0.062
CH05e03	13	10	76.92	10	76.92	12	92.31	7	53.85	0.128dC	0.037
CH03h03	9	8	88.88	7	77.77	5	55.56	8	88.88	0.208cdBC	0.047
CH05d08	13	11	84.62	7	53.85	10	76.92	6	46.15	0.238bcdBC	0.068
MS06g03	17	16	94.12	17	100	14	82.35	17	100	0.321abAB	0.075
CH04g09	20	15	75	17	85	19	95	18	90	0.281abAB	0.051
CH05g07	21	21	100	19	90.48	17	80.95	16	76.19	0.207bcABC	0.040
CH03d11	20	18	90	19	95	20	95	15	75	0.277abAB	0.066
Average	16	4.25	89.06	13.75	85.93	14	87.5	12.63	78.9	0.257	0.064

A: the number polymorphic bands; *P*: the percentage of polymorphic bands; a, b, c, d indicate the results of Duncan test at 0.05 level; A, B, C indicate the results of Duncan test at 0.01 level.

different. The percentage of polymorphic bands of CH05g03 was the highest in Gongliu population (P = 100%) and the lowest in Yumin population (P = 76.19%). Among the four populations, Gongliu population had the highest average polymorphic bands (14.25) and the percentage of polymorphic bands (P = 89.06%).

Nei's gene diversity (H) in all loci ranged from 0.128 to 0.394 with an average of 0.25. Variance analysis showed that there were significant differences in most loci, highly significant differences in a few loci and no significant differences in a few loci. Gene flow Nm was 7.265 based on G_{ST} at the species level, which indicated that there was a mild gene exchange between populations.

2.2 Genetic diversity of *Malus sieversii* population

The number of polymorphic loci in *M. sieversii* was 128 and the percentage of polymorphic loci was 100% at the species level, which was higher than that at the population level (Table 2). Gongliu population was the highest in the four populations in the number of polymorphic loci, the percentage of polymorphic loci, observed number of alleles, and effective number of alleles. H value of *M. sieversii* at the species level (H = 0.2691), was the highest, followed by Gongliu population (H = 0.25382), Houcheng population (H = 0.2501), Xinyuan population (H = 0.2450), and Yumin population (H = 0.2273). I value of *M. sieversii* at the species level was 0.4082, which was larger than that in the Gongliu population (I = 0.3912), Houcheng population (I = 0.3880), Xinyuan population (I = 0.3770), and Yumin population (I = 0.3482). Variance analysis showed that H (F = 0.335, P > 0.05) and I (F = 0.517, P > 0.05) between populations were not significant. On the basis of the study of all the parameters, the genetic diversity of Gongliu population was the richest among the four populations and an in situ conservation site should be constructed first in this population.

2.3 Genetic identity and genetic distance of *Malus sieversii* between populations

To further elucidate the gene differentiation between populations, Nei's unbiased measure of genetic identity and genetic distance were evaluated (Table 3). I_N ranged from 0.9721 to 0.9854 and D rangedfrom 0.0147 to 0.0283, which indicated that there were high similarity and closer genetic distance between populations. Relationships between populations were further illustrated by a dendrogram, using UPGMA

Table 2 Genetic diversity of *Malus sieversii*

Population	Number of polymorphic loci	Percentage of polymorphic loci (%)	Number of alleles observed (*Na*)	Effective number of alleles (*Ne*)	Nei's gene diversity (*H*)	Shannon's information index (*I*)
Species Level	128	100	2.0000	1.4252	0.2619	0.4082
Gongliu	113	88.28	1.8828	1.4193	0.2538	0.3912
Xinyuan	108	84.38	1.8438	1.4085	0.2450	0.3770
Huocheng	112	87.50	1.8750	1.4035	0.2501	0.3880
Yumin	100	78.12	1.7812	1.3787	0.2273	0.3482

Table 3 Nei's unbiased measures of genetic identity and genetic distance of *Malus sieversii*

Pop. ID	Gongliu	Xinyuan	Huocheng	Yumin
Gongliu	–	0.9854	0.9764	0.9746
Xinyuan	0.0147	–	0.9757	0.9721
Huocheng	0.0239	0.0246	–	0.9750
Yumin	0.0257	0.0283	0.0254	–

Nei's genetic identity (above diagonal) and genetic distance (below diagonal).

algorithm based on Nei's genetic distance (Fig. 3). The dendrogram showed that the Gongliu population and Xinyuan population were closely related to each other in genetic relationship and genetic distance (D = 0.0147). In geographical locations, the Xinyuan population and Gongliu population were located at the southern Ily valley, with closer geographic distance, which further proved that genetic distance was positively correlated with geographical distance.

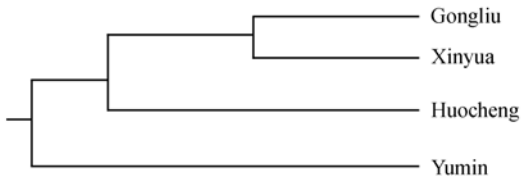

Fig. 3 Dendrogram obtained by UPGMA cluster analysis based on Nei's genetic distances among the four eco-geo graphical groups of *Malus sieversii*

2.4 Clustering analysis of *Malus sieversii* accessions

The dendrogram showed that the 109 accessions of *M. sieversii* were classified into 10 groups at 68.6% similarity coefficient (Fig. 4). Most accessions from the same population were clustered together. The first, the second, and the fifth groups comprised of 24 accessions from the Gongliu population (91%) and Xinyuan population (9%). The third group comprised of four populations, which were divided into three subgroups. The first subgroup comprised of 14 accessions, most accessions from Gongliu population and Xinyuan population and one accession from the Huocheng population. The second subgroup consisted of one accession from the Gongliu population, 20 accessions from the Xinyuan population and 12 accessions from the Huocheng population. The third subgroup comprised of 18 accessions from the Yumin population. The fourth group consisted of one accession from the Gongliu population and three accessions from the Huocheng population. The sixth, the seventh, the eighth, the ninth, and the tenth groups included 16 accessions from the Huocheng population. Clustering analysis showed that the Gongliu population was the nearest to the Xinyuan population in genetic relationship, and the Huocheng population and Yumin population were far from the other three populations. Gongliu population, Xinyuan population, Huocheng population, and Yumin population were relatively independent populations, but there was mild gene exchange between the populations.

3 Discussion

3.1 Population diversity and conservation of *Malus sieversii*

Ily wild fruit forest, which mainly consists of *M.*

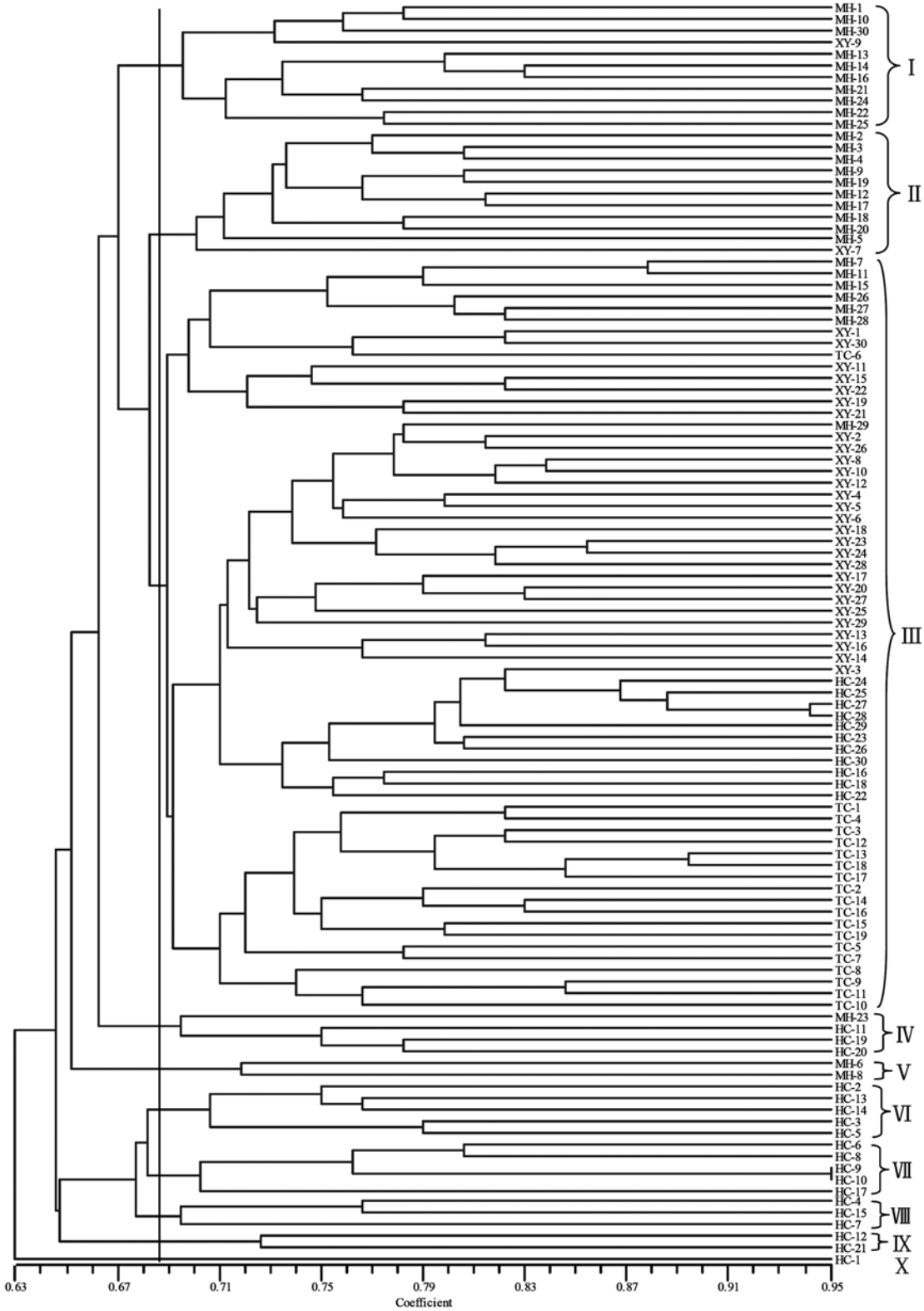

Fig. 4 Dendrogram of 109 *Malus sieversii* based on UPGMA analysis using SM similarity coefficient

sieversii, *Armeniaca vulgaris,* and *Juglans regia*, was considered as a peculiar "Ocean climate" broadleaf forest type in Central Asia[2], of which *M. sieversii* was proposed to be the primary progenitor of the cultivated apple, with a crucial role in domestication of the globally cultivated apple. Therefore, further research on *M. sieversii* is of importance in apple breeding, genetics, evolution, and germplasm conservation.

Liebhard *et al.*[15] detected polymorphism of 140 pairs of primers in eight apple cultivars, of which 6–12 bands were detected using the same eight pairs of primers in this study, with an average of 9.57 bands, but an average of 16 bands were detected in this study. Amplified bands on *M. sieversii* were more than that of cultivated apple, which indicated that *M. sieversii* had more polymorphisms compared to the cultivated apple. Nei's gene diversity (H = 0.2538, 0.2450, and 0.2501) and Shannon's information indexes (I = 0.3912, 0.3770, and 0.3880) of *M. sieversii* were lower than that of wild apricot (H = 0.291, 0.2950, and 0.276; I = 0.443, 0.453, and 0.3880), in the Gongliu population, Xinyuan population, and Huocheng population, respectively. This indicated that *M. sieversii* remained with a relatively high level of genetic diversity, but adaptability and vitality were lower than that of wild apricot[25].

Ily Wild Fruit Forest Resource Research and Development Center and in situ Conservation Site of Agricultural Wild Plants in China were established in Xinyuan County in 2002, where genetic diversity is better protected[26]. In this study, all the parameters including the percentage of polymorphic bands, effective number of alleles, Nei's gene diversity, and Shannon's information index showed that the genetic diversity in the Gongliu population were higher than that of other populations. But the area of *M. sieversii* forest decreased year by year and inherent breeding system and genetic diversity suffered destruction in different degrees in the Gongliu population because of farmland asserting, heavy grazing, and Agrilus mali Matsumura damage[3]. Given that Xinyuan population and Gongliu population had the nearest genetic distance (D = 0.0147), and the higher genetic identity (I_N = 0.9854) lay in the southern Ily valley, therefore, the Gongliu population should be received into the same conservation with the Xinyuan population and be given a high priority for conservation.

3.2 Gene differentiation and gene flow of populations of *Malus sieversii*

Gene differentiation and gene flow are important index to evaluate the population genetic structure. The study of Warren *et al*.[27] shows that *M. sieversii* is an outcrossing species when value of G_{ST} is 0.15, based on the isoenzyme. In the present study, the value of G_{ST} is 0.064 based on the SSR marker for the four populations of *M. sieversii*, indicating that gene differentiation was high within the population and low between the populations (6.4%). Gene flow, the movement of gene within and between populations, is negatively correlated with gene differentiation[28], but is very important for population transfer and plant evolution, and is transferred by pollen and seed between populations, for seed plant[29]. According to the value of *Nm,* if a species over 5.0 belongs to the outcrossing species[30], then *M. sieversii* having the higher gene flow (Nm = 7.265), is likely to be an outcrosing species, but this needs to be proved by the pollination biological experiment. At the same time, the gene flow is mainly transferred by pollen or by seed, and this needs to be the studied further.

3.3 Clustering analysis of *Malus siversii*

The 109 *M. siversii* accessions used in this study were clustered into ten groups. The accessions from the same population were clustered together, which demonstrated that the four populations were relatively independent populations. The third group consisted of four populations, the Gongliu population and Xinyuan population were clustered in the same subgroup, the Xinyuan population and Huocheng population were clustered in the same subgroup, and the third subgroup included only the Yunmin population. The fourth group consisted of the Gongliu population and the Huocheng population. These results showed that the Gongliu population and Xinyuan population were the closest in genetic relationship among the four

populations, followed by the Gongliu population and the Huocheng population. The four populations were clustered using UPGMA, based on Nei's genetic distances. The Gongliu population and Xinyuan population were clustered together, and the Yumin population was far from the other populations. The Yunmin population was the lowest in Nei's identity (I_N = 0.9721–0.9750) and the farthest in genetic distance (D = 0.0254–0.0283), when compared with other populations, which coincided with geographical location.

Baerluke Mountain in Yumin town is a relatively isolated mountain located in the Tancheng State, between the Tianshan Mountain and the Anai Mountain. The other three populations are located in the Ily Valley of Tianshan Mountain, of which the Xinyuan population and the Gongliu population locate at the southern Ily Valley, and Huocheng population locates at the northern Ily Valley. Yunmin population has less chance for gene exchange with other populations because of its geographical location. This further proves that genetic distance is significantly correlated with geographical distance and geographical distance is the most important factor that affects gene exchange. Using the molecular phylogenetics theory, the studied population genetic structure has been widely applied and is of important significance to biological conservation [31]. In this study, the dendrogram constructed using the theory of molecular phylogenetics showed that closer populations in a geographical location can be clustered together, which further proved the reliability of molecular systematics in the genetic structure analysis of *M. sieversii* population .

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中国新疆野苹果[*Malus sieversii* (Lebed.) Roem.]群体遗传结构的 SSR 分析

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摘 要：以中国新疆伊犁地区的巩留县莫合镇库尔德宁、新源县交吾托海、霍城县大西沟和塔城地区的裕民县巴尔鲁克山 4 个种下居群的 109 个新疆野苹果实生株系为材料，利用 8 对苹果SSR引物进行群体遗传结构的研究。结果表明：8 对SSR引物在 4 个居群中可平均扩增出 16 条带，其中巩留县居群多态性带数百分比最高为 89.06%，各位点平均*Nei*基因多样度为 0.257；4 个群体共扩增出 128 个位点，在种级水平及巩留县、新源县、霍城县和裕民县 4 个居群水平多态性位点百分比分别为 100%、88.28%、84.38%、87.50%、78.12%，种级水平*Nei*基因多样度(H = 0.2619) 和香农信息指数(I=0.4082)大于种下居群，4 个种下居群*Nei*基因多样度和香农信息指数比较巩留县＞霍城县＞新源县＞裕民县；巩留县居群和新源县居群遗传一致度最大，遗传距离最近；根据基因分化系数（G_{ST} = 0.064）值，测得的基因流*Nm*为 7.265。UPGMA聚类分析结果表明，巩留县和新源县居群遗传关系最近，霍城县居群次之，裕民县居群远离其他 3 个居群，巩留县、新源县、霍城县和裕民县 4 个居群是相对独立的群体，但同时存在部分基因交流。所有参数分析表明，巩留县遗传多样性最丰富，故在制定原位种质保护计划时应优先考虑巩留县居群。

关键词：新疆野苹果；SSR 标记；群体遗传结构

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