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An ISSR-based genetic diversity analysis of *Malus sieversii* in Tienshan Mountains in Xinjiang, China and Kyrgyzstan

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Abstract: *Malus sieversii* constitute a valuable genetic resource in wild apple ecosystems. The aim of this study was to use inter-simple sequence repeat (ISSR) primers as an accessible tool to investigate the genetic diversity in *Malus sieversii* species. The experimental materials include 34 samples from Kyrgyzstan and Xinjiang of China. A total of 125 bands and 98 polymorphic bands were amplified using 47 ISSR primers. The polymorphism rate was 78.4%. The genetic similarity coefficient of Kyrgyzstan and Xinjiang of China population was 0.68; the genetic similarity coefficient of various populations in Xinjiang was 0.72~0.94. The samples in same population got into a category, but some samples in faraway geographic locations have cross clustering. Geographical isolation hindered the gene exchange of *Malus sieversii* in different populations for a long time, and *Malus sieversii* developed along the natural selection environment direction and generate genetic differentiation after that.

Keywords: *Malus sieversii*; ISSR; Genetic diversity

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Introduction

Malus sieversii (Ledeb) Roem. is also known as Tienshan or Xinjiang wide apple, which is not only precious tertiary relict species [1], but also an important part of the world apple gene pool. *Malus sieversii* mainly distributed in Yili and TaCheng of XinJiang in China. In addition, kazakhatan, kyrgyzatan, tajikistan and other countries of central Asia has distribution of *Malus sieversii*. Early studies of *Malus sieversii* mainly focused on the origin and evolution [2], geographical distribution, systematic

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classification and so on. Among genetic diversity research, polyphenols [3,4] and fruit volatile compounds [5,6] has been studied. However, information regarding their genetic diversity remains poor.

The development of molecular markers provide a new method for screening and identification of germplasm resources. So far, SSR (Simple Sequence Repeats) [7] and RAPD (Random Amplified Polymorphic DNA) [8] technology has been applied to the genetic structure of *Malus sieversii*. ISSR (inter-simple sequence repeat) [9-26] is a kind of molecular marker technology with more stability and repeatability. So, ISSR was used to study the genetic diversity of *Malus sieversii*, which could provide theoretical basis for germplasm conservation and utilization.

Materials and Methods

The experimental materials included 34 samples representing 6 populations that distributed in Kyrgyzstan and China, which were collected from May 2012 to July 2013, including 6 materials from Balykchy and Karakol of Kyrgyzstan and 28 materials from Xinjiang of China（the geographical location of the samples sites is shown in Figure 1 and Table 1）. All populations are wild ancient wild *M. sieversii* distribution areas, and there is no distribution of cultivated species. All samples were taken from selected trees, which we survey and make a record every year. The distance of two populations was more than 60 kilometers. Choose appropriate amount of leaves without plant diseases and insect pests and make a record of the sampled tree with GPS system. The leaves were kept in Hermetic bag with silica gel, which is replaced every two days until completely dry.

Figure 1: Distribution of *M. sieversii* in kyrgyzstan and China. Yili valley occupied a triangle region that distributed with *M. sieversii* in China, which distributed Nalati, Xinyuan, Gongliu, Daxigou populations from east to west.

The samples were ground with PVP (Polyvinyl Pyrrolidone) and dissociated with 10%CTAB (Cetyltrimethyl Ammonium Bromide) and 0.7M NaCl in the supernatant, deposited with $C_2H_3NaO_2$ (Sodium Acetate) and precooling C_3H_8O (Isopropyl Alcohol). The concentration and purity of DNA were assessed with Nanodrop 2.1 and 0.8% agarose gel electrophoresis. A total of 47 ISSR primers sequences were selected on the basis of University of British Columbia Biotechnology and synthesized by BGI (Supplementary Table 1). The ISSR-PCR reaction system used in the present study followed the technique described by Weisheng[27]: Each 20μl reaction solution included 14.4μl double distilled water, 2.0μl 10×buffer (including Mg^{2+}), 1μl primer (10nM), 1μl DNA template (200ng/μl), 0.5μl dNTPs (2.5nM), 0.1μl TaqDNA polymerase (5 units/μl). Amplification procedure were as follows: Initial denaturation at 94℃ for 5min; followed by denaturation at 94℃ for 30s, annealing at 50℃-60℃ (depending on the primer) for 1 min, extension at 72℃ for 8 min, 35cycles; 72℃ extended 7 min; Save 4℃.

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Table1: The main distribution of the 34 samples in kyrgyzstan and China.

Number	Materials	Location	Time	Elevation （m）
1	JY1	Balykchy and Karakol of kyrgyzstan	2012.7.20	1645.0
2	JY2	Balykchy and Karakol of kyrgyzstan	2012.7.20	1650.0
3	JY3	Balykchy and Karakol of kyrgyzstan	2012.7.20	1659.0
4	JY4	Balykchy and Karakol of kyrgyzstan	2012.7.20	1666.0
5	JY5	Balykchy and Karakol of kyrgyzstan	2012.7.22	1850.0
6	JY6	Balykchy and Karakol of kyrgyzstan	2012.7.22	1900.0
7	EY1	Emin in China	2012.5.7	1220.1
8	EY2	Emin in China	2012.5.7	1357.2
9	EY3	Emin in China	2012.5.7	1388.1
10	TY1	TuoLi in China	2012.5.7	888.3
11	TY2	TuoLi in China	2012.5.7	900.1
12	TY3	TuoLi in China	2012.5.7	821.6
13	TY4	TuoLi in China	2012.5.7	831.7
14	TY5	TuoLi in China	2012.5.7	841.5
15	NY1	NaLaTi in China	2012.5.4	1489.8
16	NY2	NaLaTi in China	2012.5.4	1489.5
17	NY3	NaLaTi in China	2013.4.23	1489.9
18	GY1	GongLiu in China	2012.5.4	1386.6
19	GY2	GongLiu in China	2012.5.4	1423.3
20	GY3	GongLiu in China	2012.5.4	1424.4
21	GY4	GongLiu in China	2012.5.4	1437.6
22	GY5	GongLiu in China	2012.5.4	1442.6
23	XY1	XinYuan Germplasm Nursery in China	2013.5.3	1254.5
24	XY2	XinYuan Germplasm Nursery in China	2012.5.3	1257.9
25	XY3	XinYuan Germplasm Nursery in China	2012.5.3	1338.4
26	XY4	XinYuan Germplasm Nursery in China	2012.5.3	1432.6
27	XY5	XinYuan Germplasm Nursery in China	2012.5.3	1411.2
28	XY6	XinYuan Germplasm Nursery in China	2012.5.5	1390.0
29	XY7	XinYuan Germplasm Nursery in China	2012.5.5	1456.3
30	XY8	XinYuan Germplasm Nursery in China	2012.5.5	1465.5
31	XY9	XinYuan Germplasm Nursery in China	2012.5.5	1424.4
32	W1	XinYuan Germplasm Nursery in China	2013.7.29	1932.0
33	W2	XinYuan Germplasm Nursery in China	2013.7.29	1932.0
33	HR	XinYuan Germplasm Nursery in China	2013.7.29	1250.5

ISSR - PCR products were test by 4% polyacrylamide gel electrophoresis with 90V voltage in 2xTBE buffer. The bands were stained with silver nitrate, and Bio-Rad Gel Imaging took photos (BIO-RAD,

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America). All of the above repeated three times. Clear bands were marked with "1", and others marked with "0". The data construct binary data matrix in Microsoft Excel, and did clustering analysis with UPGMA method of NTsys2.1 software. Genetic similarity coefficient (GS) were calculated according to the formula N_{XY}/GS=2 (NX + NY), where N_X and N_Y was amplified fragments number of strains X and Y,N_{XY} is the common bands of two strains.

Results

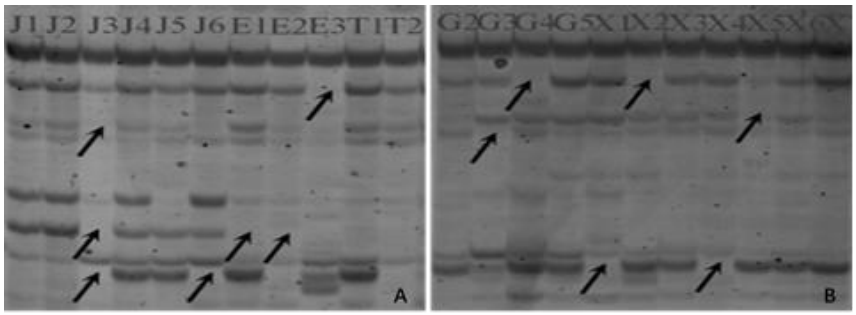
ISSR polymorphism analysis

We identified 7 efficient ISSR primers with polymorphic from 47 ISSR primers. The criteria for selection of markers as follows: clear bands were marked with "1", and others marked with "0". By statistics, every primer could amplificated 9-26 bands and the average number of bands is 14. Among them, the primer (TG)8AC26 amplificated the most loci for 26. The primer (AT)8TC amplificated the least loci for 9. All amplified fragment sizes were between 200 and 1500bp. The highest percentage of polymorphic is primer (CA)8AT and (CA)8GT followed 100%. The primer CATGGTGTTGGTCATTGTTCCA polymorphic percentage is 82.3%, and a minimum of polymorphic percentage is 44.4% of (AT)8TC (Table 2). 7 primers amplificated 125 loci in total. The polymorphism loci was 98, and polymorphism percentage is 78.4%. Figure 1 showed the ISSR-PCR result of 11 *Malus sieversii* electrophoresis with (CA)8GT and CATGGTGTTGGTCATTGTTCCA.

Table 2: The sequences of primers and their polymorphism.

Primer sequence	The number of bands	Polymorphic bands	The percentage of polymorphic bands
(CA)8AT	19	19	100%
(CA)8GT	17	17	100%
(AG)8T	22	12	54.5%
(AT)8TC	9	4	44.4%
(TG)8AC	26	17	65.3%
(GATA)4	15	12	80.0%
CATGGTGTTGGTCATTGTTCCA	17	14	82.3%

Figure 1: ISSR-PCR results of 11 *Malus sieversii*. (A) ISSR-PCR amplification results of (CA)8GT. (B) ISSR-PCR amplification results of CATGGTGTTGGTCATTGTTCCA.

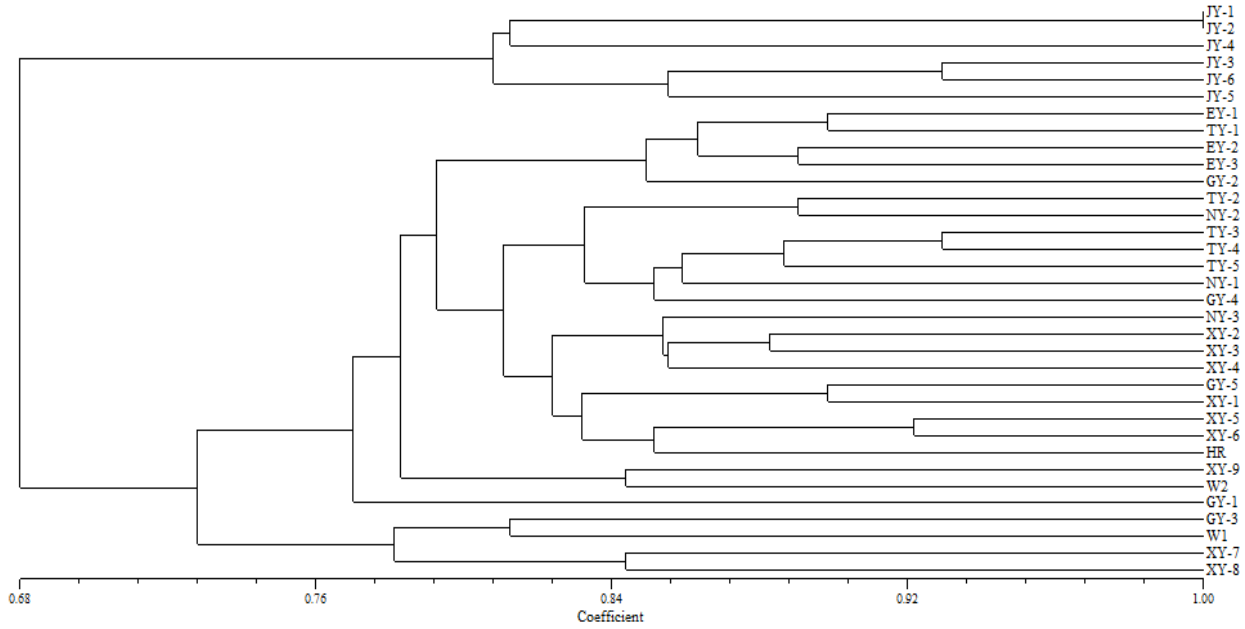


DOI: https://doi.org/10.36811/ijpsh.2019.110004 **IJPSH: January-2019: Page No: 40-48**

Cluster analysis results

Based on ISSR-PCR statistics, clustering analysis diagram built using NTsys2.1 software system (Figure 2). GS（Genetic similarity coefficient）of *Malus sieversii* is 0.68 to 1.00. Among of them, GS of JY-1 and JY-2, JY-3 and JY-6, TY-3 and TY-4, XY-5 and XY-6 is above 0.92, which revealed higher sequence homology and more close genetic relationship. Did the first hierarchy in the GS=0.71, 34 *Malus sieversii* germplasms were divided into two categories, the first is *Malus sieversii* of kyrgyzstan and the second is *Malus sieversii* in XinJiang of China. Did the seconed hierarchy in the GS = 0.76, different of germplasms divided into three categories. *Malus sieversii* of Kyrgyzstan is a separately category, and a small category included XY-7 and XY-8 in XinYuan and "The king of *Malus sieversii*" and GY-3 in Gong Liu. The last category is *Malus sieversii* of other regions. The result showed that *Malus sieversii* has abundant genetic variation with a variety of complex natural environment influence, especially long-term geographical isolation.

Figure 2: The dendrogram of *Malus sieversii* based on ISSR cluster analysis. The value on the coordinate axis is the genetic similarity coefficient, 1.00, 0.92, 0.84, 0.76 and 0.68 means 100%, 92%, 84%, 76%, 68% genetic similarity coefficient.



Discussion

Kyrgyzstan is located in the northeast of central Asia, which east and southeast is China. *Malus sieversii* distributed in XinJiang of China along the Tienshan and Kyrgyzstan and Kazakhstan and so on. In this study, the results show that *Malus sieversii* of Kyrgyzstan and XinJiang together into their respective category, and both genetic similarity coefficient was 0.72. Genetic similarity coefficient of above is lower than that inside of population, which indicated *Malus sieversii* of Kyrgyzstan and XinJiang have relative relationships. These populations may be existed in a similar form before, but they produced geographical isolation after the change of geographical environment, such as the block of Tienshan. Durable geographical isolation cut off genetic exchange, which made the populations use different resources to grow and reproduce in their respective geographic areas, and occurred genetic differentiation. Therefore, to some extent, Tienshan plays a key

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role in enriching the genetic diversity of *Malus sieversii.*

Xinjiang is broad, and the distance between YiLi and TaCheng is more than 700 kilometers. In this study, the majority of samples within a region belong to a class, such as EY-2 and EY-3, TY-3 and TY-4, XY-5 and XY-6. HR from XinYuan with XY-5, XY-6 together into a category; W-1 (The oldest *Malus sieversii*) and W-2 (The second oldest *Malus sieversii*) from XinYuan respectively with GY-3 and XY-9 together into a category. Both of XinYuan and GongLiu belong to Yili region, which geographic distance is closer than that between TaCheng and Yili. So, they have more opportunities to exchange genes, and facilitated higher genetic similarity coefficient and closer relatives. TuoLi and NaLaTi respectively belong to TaCheng and YiLi prefecture and geographic distance is far, but TY-2 and NY-2 together into a category and the genetic similarity coefficient is 0.88. This result may be connected with complex environment conditions such as the spread of insects, the influence of climate and human factors. Genetic diversity reflected the difference of genetic basis and genetic improvement. High genetic diversity is the basis of maintaining the long-term survival of species [28]. The genetic similarity coefficient of all *Malus sieversii*

resources in the study is 0.68 to 1.00, which provides a certain theoretical basis for the development and utilization of new varieties, and lay the foundation for the germplasm resources protection or utilization.

Conclusion

ISSR molecular marker showed a higher level of polymorphism genotype differences, which was suitable for genetic diversity analysis and genetic relationship of *Malus sieversii*. ISSR-PCR has cleared about genetic similarity coefficient of *Malus sieversii* in different geographical area, which was advantageous to *Malus sieversii* germplasm resources utilization and protection, genetic research and breeding. The clustering analysis of *Malus sieversii* showed that the geographical isolation block gene exchange, and made each isolated population have firmly genetic stability. So, they developed along the appropriate direction in the natural selection of environment and rich genetic diversity all the time (Supplementary Table 1).

Supplementary Table 1 ISSR Primer Sequences

No.	Number	Primer Sequences	Tm
ISSR1	UBC 846	CA CA CA CA CA CA CA CAAT	54℃
ISSR2	UBC 846	CA CA CA CA CA CA CA CAGT	56℃
ISSR3	UBC 847	AGAGTTGGTAGCTCTTGATC	53℃
ISSR4	UBC 834	AG AG AG AG AG AG AG AGGT	54℃
ISSR5	UBC 801	AT AT AT AT AT AT AT ATT	36℃
ISSR6	UBC 802	AT AT AT AT AT AT AT ATG	38℃
ISSR7	UBC 803	AT AT AT AT AT AT AT ATC	38℃
ISSR8	UBC 804	TA TA TA TA TA TA TA TAA	36℃
ISSR9	UBC 805	TA TA TA TA TA TA TA TAC	36℃
ISSR10	UBC 806	TA TA TA TA TA TA TA TAG	36℃

DOI: https://doi.org/10.36811/ijpsh.2019.110004 **IJPSH: January-2019: Page No: 40-48**

ISSR11	UBC 807	AG AG AG AG AG AG AG AGT	50℃
ISSR12	UBC 808	AG AG AG AG AG AG AG AGC	52℃
ISSR13	UBC 809	AG AG AG AG AG AG AG AGG	52℃
ISSR14	UBC 810	GA GA GA GA GA GA GA GAT	50℃
ISSR15	UBC 811	GA GA GA GA GA GA GA GA C	52℃
ISSR16	UBC 812	GA GA GA GA GA GA GA GAA	50℃
ISSR17	UBC 813	CT CT CT CT CT CT CT CTT	50℃
ISSR18	UBC 815	CT CT CT CT CT CT CT CTG	52℃
ISSR19	UBC 821	CA CA CA CA CA CA CA CAT	50℃
ISSR20	UBC 828	TG TG TG TG TG TG TG TGA	50℃
ISSR21	UBC 829	TG TG TG TG TG TG TG TGC	52℃
ISSR22	UBC 830	TG TG TG TG TG TG TG TGG	52℃
ISSR23	UBC 832	AT AT AT AT AT AT AT ATTC	38℃
ISSR24	UBC 833	AT AT AT AT AT AT AT ATTG	38℃
ISSR25	UBC 840	GA GA GA GA GA GA GA GATT	52℃
ISSR26	UBC 847	CA CA CA CA CA CA CA CAGC	56℃
ISSR27	UBC 851	GT GT GT GT GT GT GT GTCG	56℃
ISSR28	UBC 854	TC TC TC TC TC TC TC TCAG	54℃
ISSR29	UBC 855	AC AC AC AC AC AC AC ACCT	52℃
ISSR30	UBC 856	AC AC AC AC AC AC AC ACTA	52℃
ISSR31	UBC 857	AC AC AC AC AC AC AC ACCG	56℃
ISSR32	UBC 858	TG TG TG TG TG TG TG TGAG	54℃
ISSR33	UBC 859	TG TG TG TG TG TG TG TGAC	54℃
ISSR34	UBC 855	TG TG TG TG TG TG TG TGAA	52℃
ISSR35	UBC 861	ACC ACC ACC ACC ACC	50℃
ISSR36	UBC 862	AGC AGC AGC AGC AGC	50℃
ISSR37	UBC 863	AGT AGT AGT AGT AGT	40℃
ISSR38	UBC 864	ATG ATG ATG ATG ATG	40℃
ISSR39	UBC 865	CCG CCG CCG CCG CCG	60℃
ISSR40	UBC 866	CTC CTC CTC CTC CTC	50℃
ISSR41	UBC 872	GATA GATA GATA GATA	40℃
ISSR42	UBC 874	CCCT CCCT CCCT CCCT	56℃
ISSR43	UBC 875	CTAG CTAG CTAG CTAG	48℃
ISSR44	UBC 876	GATA GATA GATA GATA	40℃
ISSR45	UBC 892	TAGATCTGATATCTGAATTCC	50℃
ISSR46	UBC 899	CATGGTGTTGGTCATTGTTCCA	56℃
ISSR47	UBC900	ACTTCCACAGGTTAACACA	47℃

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