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, kazakhstan, kyrgyzzatan, 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
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.

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 C6H5NaO2 (Sodium Acetate) and precooled C3H8O (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 Mg2+), 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°C for 5min; followed by denaturation at 94°C for 30s, annealing at 50°C-60°C (depending on the primer) for 1 min, extension at 72°C for 8 min, 35cycles; 72°C extended 7 min; Save 4°C.

**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.
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**Table 1:** 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 | 1342.6        |
| 27     | XY5       | XinYuan Germplasm Nursery in China | 2012.5.3 | 1411.2        |
| 28     | XY6       | XinYuan Germplasm Nursery in China | 2012.5.3 | 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        |
| 34     | HR        | XinYuan Germplasm Nursery in China | 2013.7.29 | 1250.5        |

ISSR - PCR products were tested 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,
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 CATGGTGTTGGTCAATTGTTCCA 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 CATGGTGTTGGTCAATTGTTCCA.

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%                               |
| CATGGTGTTGGTCAATTGTTCCA | 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 CATGGTGTTGGTCAATTGTTCCA.
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 second 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.](image)

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 role in the geographical isolation of *Malus sieversii*.
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 CA AT | 54°C |
| ISSR2 | UBC 846  | CA CA CA CA CA CA CAGT   | 56°C |
| ISSR3 | UBC 847  | AGAGTTGGTAGCTTGTGATC      | 53°C |
| ISSR4 | UBC 834  | AG AG AG AG AG AG AG AG GT | 54°C |
| ISSR5 | UBC 801  | AT AT AT AT AT AT AT AT T | 36°C |
| ISSR6 | UBC 802  | AT AT AT AT AT AT AT AT G | 38°C |
| ISSR7 | UBC 803  | AT AT AT AT AT AT AT TC   | 38°C |
| ISSR8 | UBC 804  | TA TA TA TA TA TA TAA    | 36°C |
| ISSR9 | UBC 805  | TA TA TA TA TA TA TAC    | 36°C |
| ISSR10| UBC 806  | TA TA TA TA TA TA TAG    | 36°C |
| ISSR   | UBC  | Primer Sequence       | Temperature |
|--------|------|-----------------------|-------------|
| ISSR11 | UBC 807 | AG AG AG AG AG AG AG AGT | 50°C        |
| ISSR12 | UBC 808 | AG AG AG AG AG AG AG AGC | 52°C        |
| ISSR13 | UBC 809 | AG AG AG AG AG AG AG AGG | 52°C        |
| ISSR14 | UBC 810 | GA GA GA GA GA GA GA GAT | 50°C        |
| ISSR15 | UBC 811 | GA GA GA GA GA GA GA GA C | 52°C        |
| ISSR16 | UBC 812 | GA GA GA GA GA GA GA GAA | 50°C        |
| ISSR17 | UBC 813 | CT CT CT CT CT CT CT CTT | 50°C        |
| ISSR18 | UBC 815 | CT CT CT CT CT CT CT CTG | 52°C        |
| ISSR19 | UBC 821 | CA CA CA CA CA CA CA CA CAT | 50°C        |
| ISSR20 | UBC 828 | TG TG TG TG TG TG TG TGA | 50°C        |
| ISSR21 | UBC 829 | TG TG TG TG TG TG TG TGC | 52°C        |
| ISSR22 | UBC 830 | TG TG TG TG TG TG TG TGG | 52°C        |
| ISSR23 | UBC 832 | AT AT AT AT AT AT AT ATTC | 38°C        |
| ISSR24 | UBC 833 | AT AT AT AT AT AT AT ATTG | 38°C        |
| ISSR25 | UBC 840 | GA GA GA GA GA GA GA GATT | 52°C        |
| ISSR26 | UBC 847 | CA CA CA CA CA CA CA CA CAGC | 56°C        |
| ISSR27 | UBC 851 | GT GT GT GT GT GT GT GTGCG | 56°C        |
| ISSR28 | UBC 854 | TC TC TC TC TC TC TC TC TC CAG | 54°C        |
| ISSR29 | UBC 855 | AC AC AC AC AC AC AC ACCT | 52°C        |
| ISSR30 | UBC 856 | AC AC AC AC AC AC AC ACTA | 52°C        |
| ISSR31 | UBC 857 | AC AC AC AC AC AC AC ACCG | 56°C        |
| ISSR32 | UBC 858 | TG TG TG TG TG TG TG TGAG | 54°C        |
| ISSR33 | UBC 859 | TG TG TG TG TG TG TG TGAC | 54°C        |
| ISSR34 | UBC 855 | TG TG TG TG TG TG TG TGAA | 52°C        |
| ISSR35 | UBC 861 | ACC ACC ACC ACC ACC | 50°C        |
| ISSR36 | UBC 862 | AGC AGC AGC AGC AGC | 50°C        |
| ISSR37 | UBC 863 | AGT AGT AGT AGT AGT | 40°C        |
| ISSR38 | UBC 864 | ATG ATG ATG ATG ATG | 40°C        |
| ISSR39 | UBC 865 | CCG CCG CCG CCG CCG | 60°C        |
| ISSR40 | UBC 866 | CTC CTC CTC CTC CTC CTC | 50°C        |
| ISSR41 | UBC 872 | GATA GATA GATA GATA GATA | 40°C        |
| ISSR42 | UBC 874 | CCCT CCCT CCCT CCCT | 56°C        |
| ISSR43 | UBC 875 | CTAG CTAG CTAG CTAG CTAG | 48°C        |
| ISSR44 | UBC 876 | GATA GATA GATA GATA | 40°C        |
| ISSR45 | UBC 892 | TAGATCTGATATCTGAAATTCC | 50°C        |
| ISSR46 | UBC 899 | CATGGGTGTGTCATTGTTCAGCA | 56°C        |
| ISSR47 | UBC 900 | ACTTCCACAGGTTACACA | 47°C        |
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