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Ecological Distribution, Reproductive Characteristics, and In Situ Conservation of *Malus sieversii* in Xinjiang, China

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Abstract. **As a wild apple species native to central Asia, *Malus sieversii* (Ledeb.) Roem. is distributed in a wide region covering most of the Tienshan Mountains. *Malus sieversii* is a useful genetic pool for apple breeding since rich with diversity. In this paper, we first describe the species range of this endangered species. We then describe an in situ reserve that has been established. We also investigated some reproductive characteristics of *M. sieversii* including pollen germination, seed dormancy, and seed viability. Both stratification and seedcoat removal efficiently released seed dormancy and accelerated seed germination. Pollen germination rate is around 60%. Our data suggest that injurious insects and human activities, rather than reproductive characters, limit the renewal of *M. sieversii.***

Malus sieversii (Ledeb.) Roem. is a wild apple species native to Central Asia. Its provenance includes the region of the Tienshan Mountains, extending from China to Kyrgyzstan and Kazakhstan. It has been recognized as one of the major progenitors of *Malus ×domestica*, the domesticated apple (Forsline and Aldwinckle, 2004). *Malus sieversii* is a very diverse species, exhibiting many of the qualities of *M. ×domestica* (Geibel et al., 2000; Volk et al., 2009). As a diploid primary crop wild relative with desirable traits as a scion and as a rootstock, *M. sieversii* is a valuable resource for plant breeders (Yan et al., 2008). In China, *M. sieversii* is distributed mainly in Xinjiang Uygur Autonomous Region in the western Tienshan Mountains.

Wild populations produce many fruits as a result of insect pollination. Preliminary studies revealed that pollen vitality is high and the average pollen vigor is as high as 64%. However, the self-pollination levels are low (Liu et al., 2008). The exact pollen germination percentage in different populations of *M. sieversii* remains unclear. Seed germination is another major aspect for plant reproduction. Relieving seed dormancy successfully is critical for fruit breeding. Evidence showed that seeds of apple trees do not germinate without stratification (Sińska, 1989). Naked stratification and sand stratification were used to remove seed dormancy and to accelerate germination (Sińska, 1989). This stratification method takes a long time, usually 2–3 months, to relieve seed dormancy. We sought to shorten the time for seed germination.

Due to interference of human activities and natural disasters, the natural population and distribution area of *M. sieversii* is reduced, and it is critical to protect this precious genetic resource (Volk et al., 2005). Conservation of plant genetic resources is achieved by protection of populations in nature (in situ) or by preservation of samples in gene banks (ex situ) (Cohen et al., 1991). The latter are essential for users of germplasm who need ready access, although it costs more money and time. Ex situ conservation of *M. sieversii* has been established in the United States using *M. sieversii* seeds collected from the wild in Kazakhstan and the Kyrgyz Republic (Volk et al., 2005). In situ conservation, which can be more economical, should also be considered as a conservation strategy.

To determine the distribution of geographical populations of *M. sieversii* and its growth status in Xinjiang, we made field practice for six times. After recording and analyzing the data, we drew a map for distribution of *M. sieversii* in Xinjiang. This map is the firsthand data for its detailed distribution, which is important for geographic research of the wild apple trees. We also set a garden for in situ conservation and renewal of *M. sieversii*. This is one of the few gardens for wild apple tree protection and utilization in the world. Moreover, we investigated reproductive characters of *M. sieversii*, including vigor of pollen and seed. These experimental data and observation of growth status in *M. sieversii* suggest that injurious insects and human activities, rather than reproductive characters, limit the renewal of *M. sieversii*.

Materials and Methods

Field investigation. Field investigations were performed twice a year in 2012, 2013, and 2014. Flower buds were collected between late April and early May and seeds were collected between late July and early August from collection sites in the following Xinjiang counties: Huocheng (Daxigou), Gongliu, Xinyuan (preserved base), and Nalati (in Xinyuan). In Emin and Tuoli counties, we investigated the distribution of *M. sieversii*.

In situ conservation. To protect *M. sieversii* in situ, a barbed wire fence was placed around an area of 700 hm^2 in Xinyuan, at an elevation of around 1400 m, N:43°37′90′′, E:83°58′01′′, altitude 1291.30 m. This site includes seven valleys, and all these valleys were distributed with *M. sieversii*.

Pollen and seed germination. In April of 2012, 2013, and 2014, we collected floral buds of *M. sieversii* at the sample sites. Flower buds were transported to Tianjin, China, and pollen were collected when the flowers opened.

Culture medium for pollen germination was 10% sucrose, 0.01% boric acid, and 0.01% agar. Pollen were scattered evenly on sterilized culture medium on a clean slide and germinated at 25 °C. The shape and germination of pollen was observed using an Olympus BX53 microscope (Olympus, Japan, Tokyo). The pollen germination rate was calculated by randomly selecting no fewer

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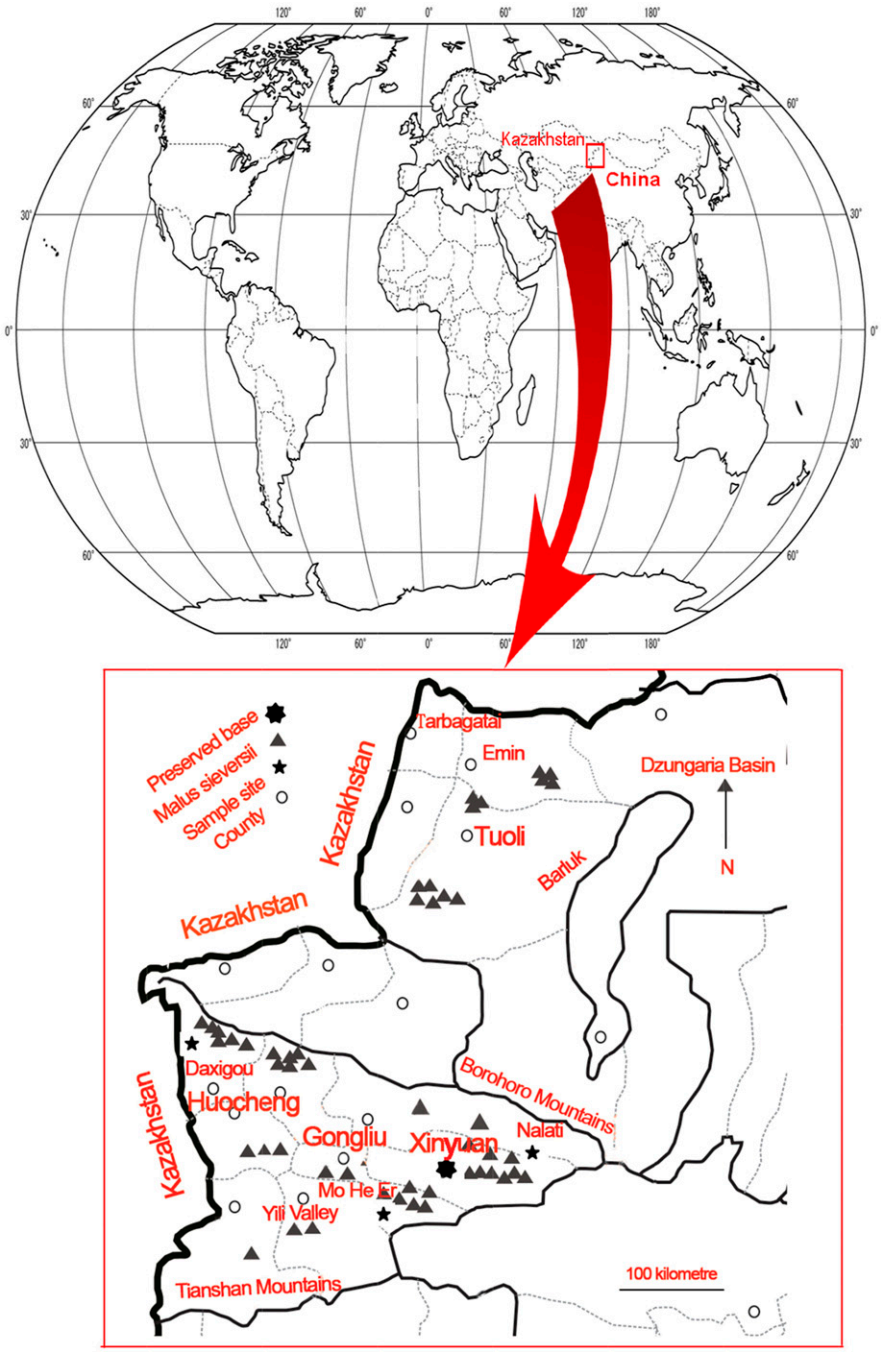

Fig. 1. Distribution of *Malus sieversii* in Xinjiang. Yili valley occupied a triangle region that distributed with *M. sieversii*.

than 600 pollen grains. Pollen germination rate (%) = pollen germination number of each field vision/total pollen number of each field × 100%.

Images were taken using a SPOT-idea camera (Sterling Heights, MI), and analyzed using Image Pro Plus software (Media Cybernetics, Inc., Rockville, MD).

Seed viability. Nearly 5000 seeds from 50 wild *M. sieversii* trees were collected from different sites over six collection trips (2012–14). Seeds were stored at –20 °C after collection until use. To break seed dormancy, we put seeds into plastic bags full of wet sand (sand moisture content 14.26%) and stored samples either at 4 °C or buried under the ground at a depth of 40–50 cm outdoors in winter from December to February in the yard in Tianjin Agricultural University (N:39°89′13″, E:117°09′32″, elevation: 12 m, average temperature is 2.2 °C in December, –0.85 °C in January, 1.4 °C in February). After the treatments, the seeds began to germinate and were moved to a 25 °C incubator without light for the germination assay after surface sterilized with 70% alcohol. The seed germination process was completed using wet filter paper in petri dishes without illumination and the germination rate was calculated. Duration time (from the beginning of the dormancy-breaking treatment to the time in which the last seed germinated), germination vigor (peak ratio of maximum seeds germinated and the total seed number during germination), and germination rate (the ratio of all seeds germinated and the total seed number in the end of germination, lasting till duration time) were recorded.

Results

Malus sieversii is discontinuously distributed in the Tienshan Mountains. Malus sieversii is a deciduous broad-leaf tree that grows in the temperate zone with a rainy growing season and winters that are not severely cold. In Xinjiang, *M. sieversii* is distributed mainly in the Yili valley, but also in the southern region of the Tarbagatai Mountains and in the west of the Barluk Mountains. Six collection trips were performed, targeting seven distinct areas of Xinjiang. The observed native populations of *M. sieversii* are discontinuously distributed (Figs. 1 and 2).

This distribution suggests that the species range is affected by local climate conditions. Wild *M. sieversii* populations were primarily localized to west-deflected warm, moist air flows specific to the centrally region of the Tienshan Mountains and western Dzungaria Basin. The damp climate in Yili valley is suitable for the growth of *M. sieversii*. The average annual precipitation is over 500 mm and the lowest temperature is –10 °C. The wild apple tree formed pure stands, mixed with *Prunus armeniaca* Lam. and *Picea schrenkiana* Fisch et Mey. var. *tianschanica (Rapi)* Cheng et Fu.

The Nalati Grassland is located to the east of the town of Nalati in Xinyuan County, at an elevation of 1487 m. The surrounding Tienshan Mountains extend in "V" shape to the west, with fragmentary distribution of *M. sieversii* on the sunny southern slopes. Associated plants include *P. armeniaca* Lam., *Caragana fruten* (L) koch, and *Populus euphratica* Oliv. Few plants were grown on shady slope. Due to the heavy grazing in this area, cover plants were less than usual (Fig. 2A).

The *M. sieversii* population in Mo He Er, Gongliu occupied 2100 hm^2, altitude 1100–1630 m. This population is located at the foot of the Tienshan Mountains, south of Yili River, with inclined topography from southeast to northwest. The trees are scatted and distributed in a narrow and long belt along the slope (Fig. 2B).

Malus sieversii occupies an area in Emin of 280 hm^2, elevation 1040–1450 m. The temperature in this region is lower than that in others, and the climate is drier. The apple trees are distributed in a scattered pattern and are shorter (Fig. 2C).

The natural reserve in Tuoli protects the wild fruit trees in this region. This region covered 650 hm^2, elevation 700–1500 m. *Prunus amygdalus* Batsch is the major accompanying plant of *M. sieversii*, growing together in forests. In the low elevation

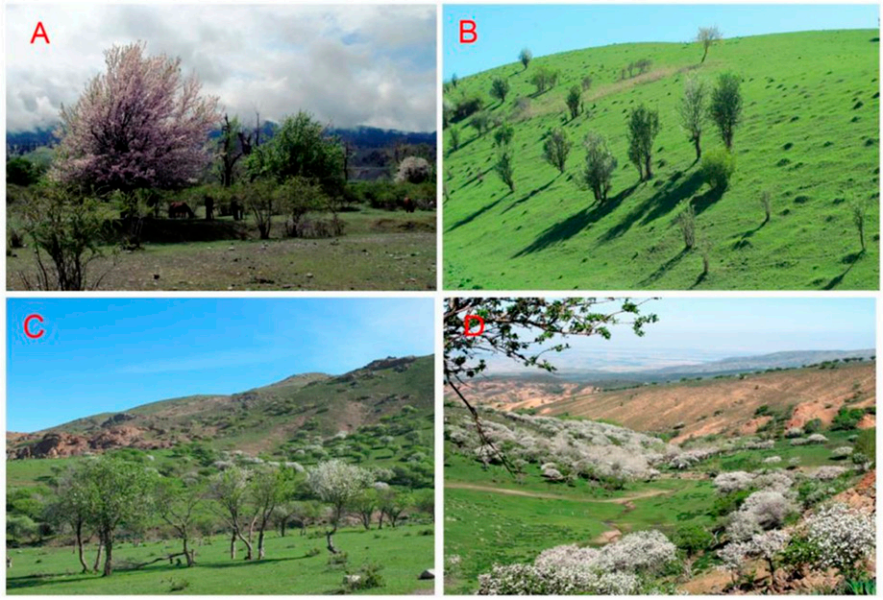

Fig. 2. Different populations of *Malus sieversii* in Xinjiang. (**A**) Nalati, Xinyuan, (**B**) Mo He Er, Gongliu, (**C**) Emin, and (**D**) Tuoli.

Table 1. Geographic Coordinates of each *Malus sieversii* collection site. Geographic coordinates were recorded according to field investigation and climatological data.

Distribution site	Position			Precipitation (mm)	Annual temperature		
	Latitude	Longitude	Elevation (m)		Lowest (°C)	Avg (°C)	Highest (°C)
Nalati	N:43°30′83″	E:84°10′94″	H:1,487	271	24.50	9.81	–2.92
Mo He Er	N:43°15′22″	E:82°52′58″	H:1,530	253	24.17	9.16	–5.17
Emin	N:46°21′04″	E:83°58′37″	H:1,040	220	22.58	7.66	–7.92
Tuoli	N:46°08′31″	E:83°32′40″	H:960	390	20.75	6.54	–7.58
Daxigou	N:44°42′72″	E:80°78′81″	H:1,181	220	18.50	10.89	–3.75

Fig. 3. Preserved base of *Malus sieversii* in Xinyuan County. (**A**) View of the base. (**B**) The gate and fence of the garden. (**C**) Biannual/triennial seedlings transplanted in the garden.

Table 2. Survival rate of seedlings and transplanting of *M. sieversii* in different years. Survival rate of 5-year-old plant in 2012 reached to 88.50%.

Yr	Seedling	2-yr-old plant	5-yr-old plant
2011	19.00%	70%	N/A
2012	N/A	45.90%	88.50%
2013	N/A	N/A	50.00%

N/A = not applicable.

region, the ground was sparsely covered with grass (Fig. 2D). Geographic coordinates of each region for collection of *M. sieversii* are listed in Table 1.

In situ conservation of Malus sieversii promotes its growth and renewal of its population. Our in situ conservation of *M. sieversii* focused on establishing and utilizating a preserved base in Xinyuan County of XinJiang YiLi Kazak Autonomous Prefecture (Fig. 3A). This base covered an area of 700 hm^2, with an elevation of 1240–1650 m. In the base, we set aside a garden to regenerate *M. sieversii* (Fig. 3B and C).

Artificial regeneration of the wild *M. sieversii* tree was performed mainly through seedling propagation and by transplanting. Threats in the external environment, such as cattle consumption or human trampling, could reduce its ability of natural *M. sieversii* regeneration. We set aside a garden in the preserved base with an area of 5 hm^2, 1476 m, N:43°37′53″, E:83°53′50″ in May 2011. At this time, 20 seedlings were transplanted into the garden, and only four trees survived. In Oct. 2011, 200 seedlings were transplanted, and the survival rate was 70%. Moreover, we sowed 3000 seeds in 300 holes, 10 seeds/hole (survival rate is 20%), as a supplement. Transplanting in autumn had a higher survival rate than that of in spring, perhaps due to the higher rainfall levels. In May 2012, 249 two- or three-year-old seedlings were transplanted, of which 135 plants survived. In May 2013, 500 two-year-old seedlings were transplanted into the in situ garden and 135 trees survived (survival rate was 46%). One hundred three-year-old plants and 260 five-year-old plants were transplanted in Oct. 2013 (survival rate was 50% and 71%, respectively) (Table 2).

Despite our efforts to select a site that minimal threats, the trees in the in situ reserve suffered from both diseases and pests. *Agrilus mali* Mats., jewel beetle from *Buprestidae*, caused great damage to *M. sieversii* (Fig. 4A), and resulted in a 30% reduction of the total planting in the garden. The spatial distribution pattern of the larvae of *A. mali* was aggregate (Liu et al., 2007). Larva of *A. mali* consumed phloem of the wild apple tree, causing the injured branch to undergo necrosis (Fig. 4B and C). With adult emergence, the branch desiccated. The pest diffused seriously, especially in lower elevation regions of the in situ site. *Agrilus mali* is extremely injurious to the wild apple trees and tends to destroy them. In 2005, we found that *A. mali* started to damage individual cultivated apple trees in a farmer's orchard. In 2006, it was diffused among wild apple trees. In 2007, parts of the branches were dried and withered. By cutting and burning the dried branches, the trees grew better with fewer diseased plants in 2008. In Spring 2009, we performed pesticide control on large amount of branches *M. sieversii*, injecting 40% Omethoate 10 × solutions via drilling-holes on xylems of stems to kill larva. Moreover, pesticide was used to control adult stages of the insect as well. Biological control (parasitic wasps) were been used during 2011–12 to successfully control *A. mali* in the garden. Wild populations seriously suffered from the insect.

Pollen germination revealed high vigor of male reproduction. Pollen grains of *M. sieversii* vary in shape and size. Dry pollen are oblong oval (Fig. 5A), and the pollen grains become spherical after absorbing water in the culture medium (Fig. 5B). The length of the polar axis is longer than the equatorial axis. The length of polar axis is between 31 and 45 µm, while the equatorial axis is between 28 and 41 µm. The ratio of polar axis and equator is between 1.0 and 1.3 (Table 3). According to the length of polar axis, equatorial axis and the ratio, the pollens can be divided into oval or bean shaped.

We used in vitro germination assays to assess pollen viability. After germinating 2, 3, and 4 h, pollen germination rate and length of pollen tube were observed and calculated (Fig. 5C and D; Table 4).

The pollen germination level was as high as 59%, and the length of pollen tube was 250 µm in 2 h. At 3 h, the germination level was similar to that of 2 h (59%), and the pollen tube length was 277 µm. After 4 h, the pollen germination level was 69%, and the pollen tube length was 566 µm, significantly higher than germination levels and pollen tube lengths than those observed at 2 and 3 h.

Fig. 4. Wild apple trees suffered by *Agrilus mali*. (**A**) The forest. (**B** and **C**) Injured branches.

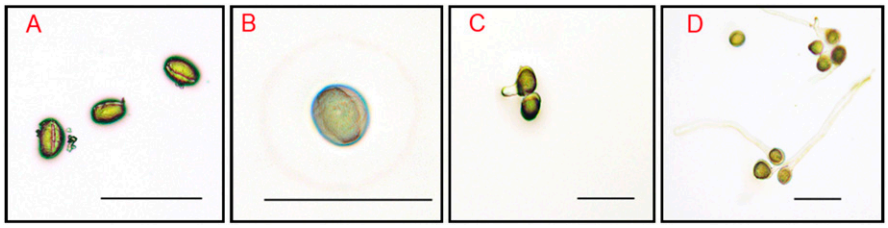

Fig. 5. Process of pollen germination. (**A**) Dried pollen. (**B**) Swelled pollen. (**C**) Pollens start to germinate. (**D**) Elongation of pollen tubes. Bars = 50 µm.

Table 3. *Malus sieversii* pollen length of polar axis and equator axis. Samples from different populations were used in the measurement of pollens.

Sample site	Polar axis (µm)	Equator axis (µm)	Ratio (%)
Na La Ti	38.186	35.293	1.105
Mo He Er	35.972	30.769	1.172
Emin	38.148	35.020	1.092
Tuoli	43.360	38.074	1.140

Table 4. Pollen germination rate and tube length.

Germination time (h)	Pollen germination level (%)	Pollen tube length (µm)
2	58.9	250.0
3	59.1	276.9
4	69.0**	566.3**

t test was used for statistical analysis, **significantly different from 2/3 ($P < 0.01$, n = 20).

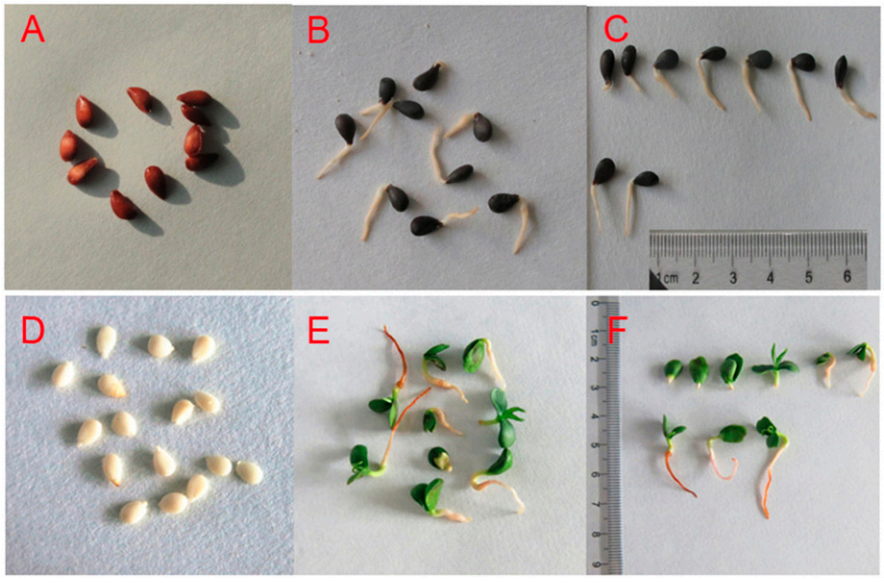

Fig. 6. *Malus sieversii* seeds (2 years old) with seedcoats (**A**) and with seedcoats removed (**D**). Seeds were stratified at 4 °C for 90 d before germination (**B** and **C**) or germinated immediately following seedcoat removal (**E** and **F**). Images were taken 5 d after germination (**B**–**C** and **E**–**F**).

Both stratification and seedcoat removal break seed dormancy and accelerate seed germination. Seeds stored at 4 °C in wet sand and those buried outside in the winter successfully germinated (Fig. 6A–C). When stored at 4 °C, germination level of 2-year-stored seeds and 3-year-stored seeds was 95% and 96%, respectively. The seeds started to germinate after a 60 d the stratification treatment. Total seed germination duration was 69 and 70 d, the vigor of germination were 46% and 45% in seeds that were stored 2 and 3 years, respectively (Table 5). The germination status for seeds buried under earth was similar to that above, except it took longer to break the dormancy (85 and 87 d, Table 5).

We also tested the germination status for the seeds harvested in the current year (without stratification treatments) and did not observe any germination. To accelerate seed germination, we removed the seedcoat from sets of seeds (Fig. 6E and F). The germination percentage is 96%, similar to that of stratified seeds.

Discussion

Plant geographic distribution varies with climate (Kelly and Goulden, 2008). In ancient times, *M. sieversii* was grown and flourished in the Yili valley and in Kyrgyzstan and Kazakhstan with the protection of Tienshan Mountains. Currently, climate is a major factor influencing the natural distributions of wild apple trees. In China, *M. sieversii* does not thrive in cold regions, which limit its distribution. According to our field investigation, the regions suitable for the growth of *M. sieversii* are low elevation regions that are humid and warm.

Seed germination is very important for plant reproduction and prerequisite for in situ conservation. We compared three different methods of seed germination. Stratification (4 °C storage) resulted in the highest levels of seed germination. Primary seed dormancy is initiated during development. Developing seeds rarely germinate, and when precocious germination does occur, it is frequently associated with deficiencies in ABA synthesis or sensitivity (Bewleyl, 1997; Finch-Savage and Leubner-Metzger, 2006). With cold treatment, gibberellins release (coat) dormancy and promote germination (Kucera et al., 2005; Leubner-Metzger, 2001). Seedcoat removal is also a good method quickly germinating some types of seeds, although the germination levels may be decreased. The rapid germination may be related to the removal of hormones that accumulate in the seedcoat.

Different populations of *M. sieversii* in Xinjiang showed nonhomogeneous distribution. Natural renewal of seedlings and suckering plants was not adequate to recover the populations. In recent years, vegetation destruction caused by farming-related activities has resulted in a decline in the vigor of wild apple trees. Large amount of fruits were collected every autumn for the use of fruit

Table 5. Germination of 2 and 3 year's seed of *Malus Sieversii*. 4 °C treatment was performed in refrigerator with seeds in wet sand. The stratification treatment was performed by burying seeds outdoors between December and February Duration refers to the time from the beginning of the dormancy-breaking treatment to the time in which the last seed germinated. Germination vigor refers to the peak ratio of maximum seeds germinated and the total seed number during germination. Germination rate refers to the ratio of all seeds germinated and the total seed number in the end of germination, lasting till duration time.

Treatment	Storage time (y)	Days to germinate (d)	Duration (d)	Germination vigor (%)	Germination rate (%)
4 °C	2	61	69	46	95
4 °C	3	63	70	45	94
Stratification	2	70	85	51	96
Stratification	3	70	87	47	94

processing enterprises, which may have affected the growth and renewal of *M. sieversii* populations. Moreover, the excessive human activities such as land reclamation, overgrazing, and over-cutting damaged both mature and young plants, and affected its natural propagation. Biological factors (mainly insects) may also aggravate natural changes in the growth environment of *M. sieversii* (Yan et al., 2008).

Effective methods for protection of *M. sieversii* are necessary to improve growth environment and maintain the balance of population structure and function. Enclosure is a suitable in situ protective measure. According to our experience, the damage caused by *A. mali* was more serious in low elevation area than that in high elevation area. Therefore, we set up an enclosure in a high elevation area to reduce the possible pernicious influence by injurious insects and human activities.

In summary, our results showed that there were not significant differences among the seed and pollen germination levels from different populations of *M. sieversii*. Both stratification and seedcoat removal efficiently released seed dormancy successfully accelerated seed germination. We could protect endangered *M. sieversii* populations resource by enclosure in situ. Our data suggest that injurious insects and human activities, rather than reproductive characters, limit the renewal of *M. sieversii*.

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