Malus sieversii: A Diverse Central Asian Apple Species in the USDA-ARS National Plant Germplasm System

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Abstract. There are several Central Asian *Malus* species and varieties in the USDA-ARS National Plant Germplasm System (NPGS) apple collection. *Malus sieversii* is the most comprehensively collected species native to Central Asia. Other taxa such as *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, *M. pumila*, and *M. pumila* var. *niedzwetzkyana* have primarily been donated to the collection by other institutions and arboreta. We sought to determine if genetic and/or phenotypic differences among the individuals that make up the gene pools of these taxa in the NPGS exhibit unique characteristics. Genetic data, based on microsatellite analyses, suggested that the diversity within each taxa is significantly greater than that among taxa. Trait data also revealed very few differences among taxa, the primary characteristic being the dark red fruit coloration and tinted flesh color of the accessions assigned to *M. pumila* var. *niedzwetzkyana* resulting from a known single-gene mutation in anthocyanin production. We found that *M. sieversii* is a highly diverse species with a range in genetic and phenotypic trait variation that includes the characteristics of the other Central Asian taxa of interest. We conclude that the gene pools that comprise the accessions within the NPGS Central Asian *Malus* collection are highly overlapping with respect to both phenotypic traits and genotypic characters.

The mountains of Central Asia have forest species that include *Malus sieversii* (Ledeb.) M. Roem., a highly diverse apple crop wild relative. The Russian scientist Vavilov explored the forests of Central Asia in the 1920s and made note of the wide range of *M. sieversii* phenotypes, suggesting that this region, including Kazakhstan, Kyrgyzstan, Tajikistan, and western China, is a center of origin for the domesticated apple (Luby et al., 2001; Wan et al., 2011; Yan et al., 2008). *Malus sieversii* grows in a wide range of habitats, including areas with hot summers and short winters as well as those with long, severe winters in the Tien Shan Mountains (Dzhangaliev, 2003). Wild populations are found in montane, scrub, humid as well as dry continental forests and in diverse stream habitats in xeric areas (Forsline et al., 2003). *Malus sieversii* trees are phenotypically diverse, offering variation in the time of flowering, quantity and quality of fruit, ripening period, fruit biochemical composition, tree architecture, winter and

drought tolerance, and disease resistance (Dzhangaliev, 2003; Yan et al., 2008; Zhou, 1999).

The U.S. Department of Agriculture (USDA) sponsored four plant exploration trips in Central Asia to collect wild apples between 1989 and 1996. Participants on those trips sought clones with unusual phenotypes and collected seeds from trees growing in diverse habitats, primarily in Kazakhstan. Over 1000 seedlings from the wild-collected seeds have been grown in Geneva, NY, in the USDA-ARS NPGS apple collection. Most of the seedlots were assigned to the *M. sieversii* species, although some have been labeled as *Malus sieversii* var. *kirghisorum* (Al. Fed. & Fed.) Ponomar. The repository also has a number of accessions that have been classified as Central Asian taxa, namely *Malus sieversii* var. *turkmenorum* (Juz. & Popov) Ponomar. and *Malus pumila* Mill. *Malus pumila* var. *niedzwetzkyana* Dieck. was formerly listed as a variety but in 2010 was reclassified as *M. pumila*. Many of these accessions were donated to the repository from other gene banks or arboreta. In addition to these species/varieties, the literature describes a number of variations in nomenclature for Central Asian *Malus* species, subspecies, and varieties including: *M. niedzwetzkyana*,

M. dasyphylla, and *M. kirghisorum*. In the Genetic Resources Information Network (GRIN; USDA, ARS, National Genetic Resources Program, 2013), these are synonymous with *M. pumila* var. *niedzwetzkyana*, *M. pumila*, and *M. sieversii* var. *kirghisorum*, respectively. Dzhangaliev (2003) summarized the *Malus* species native to Kazakhstan. *M. sieversii* and *M. niedzwetzkyana* are most similar to cultivated apple with *M. niedzwetzkyana* as a botanical variety of *M. dasyphylla*. Furthermore, *M. kirghisorum* is described as distinct from *M. sieversii* because it grows adjacent to relic *Juglans regia* forests as part of a mesophilous Turgai forest flora with a limited range in Dzhangaliev and Zailiyskei Alatau (Dzhangaliev, 2003).

Based on molecular and phenotypic evidence, *M. sieversii* is likely a progenitor species of the domesticated apple, *Malus ×domestica* (Harris et al., 2002; Velasco et al., 2010; Zhou and Li, 2000). In fact, *M. sieversii* and *M. ×domestica* have sometimes been coclassified as *M. pumila* (Harris et al., 2002; Juniper and Mabblerley, 2006; Phipps et al., 1990, 1991). Although there are data suggesting similarities between these species, genetic evidence suggests that they can be genetically distinguished based on microsatellite markers (Gross et al., 2012). In fact, putative hybrids between *M. sieversii* and *M. ×domestica* can also be identified, thus further supporting the differentiation of these species (Gross et al., 2012). Other species such as *M. orientalis*, *M. sylvestris*, and *M. prunifolia* likely played a role in *Malus* domestication (Coart et al., 2006; Cornille et al., 2012; Forte et al., 2002; Gross et al., 2012; Luby, 2003; Robinson et al., 2001; Velasco et al., 2010). Single nucleotide polymorphism transferability was ≈26% from *M. ×domestica* to either *M. sieversii* or *M. sylvestris*, supporting the evidence that there are significant genomic differences among *M. ×domestica* and its likely progenitor species (Micheletti et al., 2011).

Previously, we assessed the diversity of the *M. sieversii* collection in the USDA-ARS NPGS using microsatellite markers. Collection sites were significantly differentiated and had unique alleles; however, differentiation within individual families was more than three times the level of that among sites. Differentiation was congruent with geographical location with southwestern collection sites being more admixed and more diverse than the northern sites (Richards et al., 2009b). These data supported results published previously that used isozymes to determine within- and among-population differences in *M. sieversii* collections (Lamboy et al., 1996).

Core collections were also proposed using available microsatellite and quantitative trait data for the *M. sieversii* trees from Kazakhstan in the NPGS. Three sets of 35 trees, representative of Site 6, Site 9, and “other” collection sites, were identified as core collections that capture greater than 95% of the measured allelic and phenotypic diversity

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(Forsline et al., 2003; Richards et al., 2009a; Volk et al., 2005).

Malus sieversii accessions in the NPGS have been evaluated for a number of fruit, biotic, and abiotic stress resistance traits both on-site in Geneva, NY, and by collaborators. Physiological and phenological traits such as high chilling requirement, early fruit maturity, short juvenility, dwarf growth habit, late flowering, stooling ability, and vigor were of particular interest to breeding programs (Bassett et al., 2011; Fazio et al., 2009; Forsline and Aldwinckle, 2004; Forsline et al., 2003; Luby et al., 2001, 2002). In addition, sources of disease and insect resistance have been identified for apple scab (*Venturia inaequalis*), fireblight (*Erwinia amylovora*), cedar apple rust (*Gymnosporangium juniper-virginianae*), *Rosellinia necatrix*, *Helicobasidium mompa*, woolly apple aphid (*Eriosoma lanigerum*), apple leaf curling midge (*Dasineura mali*), soilborne apple replant pathogens, and apple maggot (*Rhagoletis pomonella*) (Fazio et al., 2009; Forsline and Aldwinckle, 2004; Forsline et al., 2003; Luby et al., 2001, 2002). Characterized fruit traits include aroma, flavor, size, color, weight, texture, soluble solids, pH, and

volatile components (Bai et al., 2012; Fazio et al., 2009; Forsline and Aldwinckle, 2004; Forsline et al., 2003; Luby et al., 2001, 2002). Some traits even differed by collection site such as the later harvest time and an increased level of fireblight resistance in Site 6 and larger, red fruit more common in Site 9 (Forsline and Aldwinckle, 2004).

The large *M. sieversii* collection in the NPGS provided us with an opportunity to assess the relationship among accessions collected and labeled as *M. sieversii* to those that have been classified as related species/varieties: *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, *M. pumila*, and *M. pumila* var. *niedzwetzkyana*. We use genotypic and phenotypic data to determine if the gene pools in the NPGS that comprise these taxa can be distinguished from one another.

Materials and Methods

Malus sieversii and several accessions of *M. sieversii* var. *kirghisorum* seeds were collected from forests in the Tien Shan Mountains in Kazakhstan and Kyrgyzstan in the

1990s (Forsline et al., 2003; Luby et al., 2001). Seeds were germinated and planted as own-root seedlings within an orchard setting. Clones representing individuals of the other Central Asian taxa were provided to the NPGS by donors and are maintained as grafted trees within the collection (Table 1). A total of 99 *M. sieversii*, nine *M. sieversii* var. *kirghisorum*, three *M. sieversii* var. *turkmenorum*, eight *M. pumila*, and three *M. pumila* var. *niedzwetzkyana* trees were available for inclusion in this research.

Genetic comparisons among accessions assigned to the five *Malus* species/varieties were performed using microsatellite markers. DNA extraction, polymerase chain reaction amplifications, and allele identifications have been described previously (Volk et al., 2005). Two sets of data were available. A total of 18 simple sequence repeat (SSR) loci (GD12, GD15, GD96, GD142, GD147, GD162, CH01h01, CH01f02, CH01d08, CH01f07a, CH05e03, CH02d12, CH02b10, COL, NH009b, CH01d09, NZ28f4, NH015a; Guilford et al., 1997; Hokanson et al., 1998; Liebhard et al., 2002; Yamamoto et al., 2002) were available to compare 111 accessions

Table 1. National Plant Germplasm System accessions of *Malus pumila*, *M. pumila* var. *niedzwetzkyana*, *M. sieversii* var. *kirghisorum*, and *M. sieversii* var. *turkmenorum* for which phenotypic and/or genotypic data were available.

Taxon	Accession	Local number	SSR loci available (no.)	Admixture (Gross et al., 2012)	Original collection country	Donor
<i>Malus pumila</i>	PI 323617	GMAL 3163	5		Afghanistan	Arnold Arboretum
<i>Malus pumila</i>	PI 588827	GMAL 140	5			Finland (through Agriculture Canada)
<i>Malus pumila</i>	PI 588916	GMAL 244	5			Morton Arboretum
<i>Malus pumila</i>	PI 589166	GMAL 1016	5			Cornell University
<i>Malus pumila</i>	PI 590096	GMAL 3370	5			Arnold Arboretum
<i>Malus pumila</i>	PI 590097	GMAL 3378	5			Arnold Arboretum
<i>Malus pumila</i>	PI 590098	GMAL 3379	5			Arnold Arboretum
<i>Malus pumila</i>	PI 594106	GMAL 3223	5			Beijing Botanical Garden
<i>Malus pumila</i>	PI 589225	GMAL 1223	5		Turkestan/southwest Siberia	Arnold Arboretum (through Cornell University)
<i>Malus pumila</i> var. <i>niedzwetzkyana</i>						District of Columbia
<i>Malus pumila</i> var. <i>niedzwetzkyana</i>	PI 589287	GMAL 1389	5			
<i>Malus pumila</i> var. <i>niedzwetzkyana</i>	PI 589857	GMAL 2613	5			University of Maine
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 589373	GMAL 1740	18			Morton Arboretum
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 589380	GMAL 1750	18			Morton Arboretum
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 590043	GMAL 3158	18			Arnold Arboretum
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 590094	GMAL 3367	18	x		Arnold Arboretum
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 613854	GMAL 2288.a1	18	x		Vavilov Research Institute of Plant Industry
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 613855	GMAL 2288.b1		x		Vavilov Research Institute of Plant Industry
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 613968	GMAL 4445	18	x	Kazakhstan	USDA collection trip
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 633798	GMAL 3574	18		Kyrgyzstan	USDA collection trip
<i>Malus sieversii</i> var. <i>kirghisorum</i>	PI 657001	GMAL 3583	18		Kyrgyzstan	USDA collection trip
<i>Malus sieversii</i> var. <i>turkmenorum</i>	PI 594104	GMAL 2251	5			Vavilov Research Institute of Plant Industry
<i>Malus sieversii</i> var. <i>turkmenorum</i>	PI 613851	GMAL 2251	5			Vavilov Research Institute of Plant Industry
<i>Malus sieversii</i> var. <i>turkmenorum</i>	PI 613852	GMAL 2251	5			Vavilov Research Institute of Plant Industry

SSR = simple sequence repeat; USDA = U.S. Department of Agriculture.

assigned to *M. sieversii* with nine accessions assigned to *M. sieversii* var. *kirghisorum*. Another data set with five SSR loci (GS12, GD15, GD142, GD147, GD162) was used to compare 61 *M. sieversii* accessions with three *M. sieversii* var. *turkmenorum*, eight *M. pumila*, and three *M. pumila* var. *niedzwetzkyana* accessions. Analyses of multivariate (AMOVA) determined the among- and within-population variation for each data set. DARwin software was used to graphically display the interindividual differences among accessions within each data set. Genetic distances were calculated using a simple matching coefficient.

Bayesian structure analyses were performed to determine if individuals could be assigned to groups that corresponded to species, subspecies, or varieties without a priori taxa definitions (Pritchard et al., 2000). Individuals in the five SSR and 18 SSR data sets were analyzed independently.

Phenotypic data were primarily collected for a single season for each accession between 1996 and 2003, when the trees were mature. Phenotypic data were collected according to descriptors that are described in the GRIN database (USDA, ARS, National Genetic Resources Program, 2013). Trait data for each descriptor were compared across the five *Malus* species/varieties. Categorical data were analyzed using χ^2 analyses and likelihood ratio tests to determine significance. Quantitative data were compared using analyses of variance and Tukey-Kramer honestly significant difference means comparisons.

Results

Malus sieversii is a broadly defined species native to Kazakhstan, Kyrgyzstan, Tajikistan, Uzbekistan, and China. Many accessions of this species are accessible through the NPGS as a result of collection trips to Central Asia in the 1990s. In contrast, fewer individuals represent *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, *M. pumila*, and *M. pumila* var. *niedzwetzkyana* in the NPGS (Table 1). Interindividual distances, genetic assignments, and genetic differentiation analyses were used to identify differences among the designated gene pools.

All three of the *M. pumila* var. *niedzwetzkyana* accessions in the data set had identical scores for the five SSRs tested. It is likely that these are clones of the same individual. AMOVA results for the 18 SSR data sets revealed no statistical differences between the microsatellite scores for *M. sieversii* and *M. sieversii* var. *kirghisorum*. Using AMOVA, we found 2% of the variation explained differences among varieties (Table 2). There were also no differences among *M. sieversii* accessions and *M. sieversii* var. *turkmenorum*, *M. pumila*, and *M. pumila* var. *niedzwetzkyana* accessions in the NPGS. Like in the 18 SSR data sets, most of the variation (91%) is attributed to within-taxa and a small portion (9%) is among the taxa (Table 3).

A dissimilarity index as determined by using DARwin software was used to construct an unrooted neighbor joining tree with

Table 2. Comparison of *Malus sieversii* and *Malus sieversii* var. *kirghisorum* apple accessions in the National Plant Germplasm System using data for 18 microsatellites.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among species/varieties	1	11.3	0.14	1.91
Within species/varieties	232	1662	7.16	98.09

Table 3. Comparison of *Malus sieversii*, *M. sieversii* var. *turkmenorum*, *Malus pumila*, and *M. pumila* var. *niedzwetzkyana* accessions within the National Plant Germplasm System using data for five microsatellites.

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among species/varieties	3	14	0.18	8.91
Within species/varieties	140	254.7	1.82	91.09

Fig. 1. Graphical representation from DARwin analyses. Each sample is represented as a terminal node in the neighbor-joining tree for (A) *Malus sieversii* (unlabeled) and *M. sieversii* var. *kirghisorum* (K) in the 18 simple sequence repeat (SSR) data set and (B) *M. sieversii* (unlabeled) and *M. pumila* (P), *M. pumila* var. *niedzwetzkyana* (N), and *M. sieversii* var. *turkmenorum* (T) for the five SSR data sets.

the 18 SSR and five SSR sets of data (Fig. 1). Individuals from each species/variety were identified. For both data sets, the non-*M. sieversii* individuals were dispersed throughout the trees rather than occurring together. This suggests that the genetic diversity encompassed by *M. sieversii* is broader than that of the other species and varieties.

Results from structure analysis showed no significant discontinuity. Using either the 18 locus data set to separate *M. sieversii* var. *kirghisorum* variety for *M. sieversii* or using the five locus data to partition the *M. pumila* accessions, results indicated no supportable partition among the named taxa. Admixture models with or without taxon identity as priors resulted in the same lack of separation. Admixture was evenly distributed among each of the named taxa indicating that linkage disequilibrium has not built up among these accessions (data not shown).

Image data are available for many of the accessions included in these analyses (Supplementary Fig. 1). Overall, the images of *M. sieversii*, *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, and *M. pumila* are similar and overlapping in the range of variation observed for each taxon. In contrast, *M. pumila* var. *niedzwetzkyana* is distinct. These apples are dark red to purple and

exhibit some degree of red coloration in the flesh.

Many of the phenotypic data for the NPGS apple collection are categorical; however, data for fruit length, fruit width, overcolor, and soluble solids were collected quantitatively. *Malus sieversii* had the smallest average fruit length (39.7 ± 0.6 mm) and width (44.2 ± 0.7 mm) among the species and varieties compared (Table 4). Statistically, the fruit length was the same for all the species/varieties except *M. pumila* var. *niedzwetzkyana*, which had larger length and width. In addition, the *M. sieversii* var. *kirghisorum* fruit were wider than the *M. sieversii* fruit. The overcolor percentage of the *M. sieversii* fruit was lower than that of the *M. pumila* and the solid-colored *M. pumila* var. *niedzwetzkyana*. *M. sieversii* had the same soluble solids as the accessions representing other species/varieties (Table 4).

Chi square analyses among NPGS accessions assigned to *M. sieversii*, *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, *M. pumila*, and *M. pumila* var. *niedzwetzkyana* revealed no significant differences for the following traits: calyx persistence, calyx basin, fruit flesh flavor, fruit flesh firmness, fruit juiciness, fruit firmness, fruit flesh oxidation, fruit weight, fruit ground

Table 4. Quantitative phenotypic trait values among Central Asian *Malus* species/varieties.

	Fruit length (mm)	Fruit width (mm)	Overcolor (%)	Soluble solids (%)
<i>Malus sieversii</i>	39.7 ± 0.6 b	44.2 ± 0.7 b	28 ± 3 c	11.9 ± 0.1 ab
<i>Malus sieversii</i> var. <i>kirghisorum</i>	46.0 ± 5.3 b	53.3 ± 4.9 a	51 ± 10 bc	13.1 ± 0.5 a
<i>Malus sieversii</i> var. <i>turkmenorum</i>	45.8 ± 11.8 ab	54.0 ± 12.0 ab	70 ± 5 abc	11.9 ± 1.2 ab
<i>Malus pumila</i>	44.3 ± 2.8 b	51.6 ± 3.6 ab	56 ± 8 ab	12.4 ± 0.8 ab
<i>Malus pumila</i> var. <i>niedzwetzkyana</i>	59.0 ± 1.3 a	62.5 ± 2.4 a	94 ± 1 a	10.2 ± 0.4 b

Means, standard errors, and significant differences between *Malus* species/varieties were determined using Tukey means separation tests ($P < 0.05$).

color, harvest season, overcolor pattern, top fruit shape, stem cavity, fruit russet location, and fruit bloom.

Some traits differed among the Central Asian species/varieties. Several fruit color traits, including fruit flesh color and fruit overcolor, were significantly different for *M. pumila* var. *niedzwetzkyana* than the four other species/varieties. The fruit flesh color of *M. pumila* var. *niedzwetzkyana* was cream and red, red, or rose, whereas most of the other fruit flesh colors were shades of white and cream. Likewise, the fruit overcolor of *M. pumila* var. *niedzwetzkyana* accessions was dark red or purple-red and the other fruits were mostly green, yellow, orange, brown, or red. In addition, the fruit of *M. pumila* var. *niedzwetzkyana* were classified as conical, whereas those of the other species/varieties were mostly globose or flat.

With respect to fruit weight, a higher proportion of *M. sieversii* had small fruit (less than 50 g) than the accessions of the other four taxa. *M. sieversii* also had more variable fruit shape and size uniformity than the other types. This might be indicative of the large sample size available for *M. sieversii* phenotypic measurements and that this species is generally diverse for many traits.

Discussion

The NPGS collection of *M. sieversii* is a valuable resource for research and breeding programs. The accessions within this collection display wide range of phenotypic and genetic diversity and have the potential to provide novel alleles for key breeding traits. The collection has relatively few accessions that represent other related taxa, including *M. pumila*, *M. pumila* var. *niedzwetzkyana*, *M. sieversii* var. *kirghisorum*, and *M. sieversii* var. *turkmenorum*. The purpose of this research was to determine if the gene pools that comprised these other taxa overlapped significantly with those designated as *M. sieversii*.

Phenotypic data exhibit a high level of similarity among Central Asian *Malus* taxa in the NPGS. The one exception is the three identical clones that were formerly classified as *M. pumila* var. *niedzwetzkyana*. These are unique with dark red skin and tinted flesh. *M. pumila* var. *niedzwetzkyana* has been examined previously (Chagné et al., 2013; Van Nocker et al., 2012). Nearly all of the red-fleshed apple cultivars in collections worldwide all could all be traced back to

a mutation in a transcriptional regulator of anthocyanin biosynthesis. This specific mutation confers the red-fleshed trait and the presence alone of this allele.

Malus pumila is a taxon that includes *M. ×domestica* and *M. sieversii* (Harris et al., 2002; Juniper and Mabberley, 2006). Although these species do exhibit similarities, genetic data suggest that they are distinct (Gross et al., 2012). Our analyses do not differentiate the accessions of *M. pumila* in the NPGS from those of *M. sieversii*.

The taxonomy of the Central Asian wild apple species is complex. Many species have been identified in the literature and then subsequently renamed (Korban and Skirvin, 1984). In some cases, accessions that are donated to the NPGS from other institutions have retained their original species classifications. As a result, the few numbers of accessions of these taxa can lead curators to believe that they have some species that may be underrepresented in their collections. Careful assessments of the validity of the assignments to taxa may clear up these misconceptions. Accessions that may be viewed as particularly novel based on their taxonomic identities may in fact be quite similar to those already represented in the collection.

The results from this research assert that the *M. sieversii* collection in the NPGS displays a range of genotypic and phenotypic diversity that encompasses that of related taxa *M. pumila*, *M. pumila* var. *niedzwetzkyana*, *M. sieversii* var. *kirghisorum*, and *M. sieversii* var. *turkmenorum*. The gene pools represented by the individuals within these taxa are highly overlapping.

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Supplementary Fig. 1. Fruit images from Genetic Resources Information Network for *Malus pumila*, *M. pumila* var. *niedzwetzkyana*, *M. sieversii* var. *kirghisorum*, *M. sieversii* var. *turkmenorum*, and *M. sieversii*.

Supplementary Fig. 1. Continued.

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Malus pumila var. *niedzwetzkyana*

Malus sieversii var. *kirghisorum*

Malus sieversii var. *turkmenorum*

Malus pumila

Supplementary Fig. 1. Continued.