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RESEARCH ARTICLE

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S-allele frequency and genetic diversity of *Malus orientalis* Uglitzk along an altitudinal gradient in the Hyrcanian forest

Hamed Yousefzadeh[1,*], Rasta Rajaei[2], Bjarne Larsen[3], Hamid Bina[2] and Gregor Kozlowski[4, 5]

[1]Tarbiat Modares University, Faculty of Natural sciences, Department of Environment Science, Tehran, Iran. [2]Tarbiat Modares University, Faculty of Natural sciences, Department of Forestry, Tehran, Iran [3]University of Copenhagen, Department of Plant and Environmental Sciences, Thorvaldsensvej 40, [3]rd Floor, 1871, Frederiksberg C, Denmark. [4]University of Fribourg, Department of Biology and Botanic Garden, Chemin du Musée 10, CH-1700 Fribourg, Switzerland. [5]Natural History Museum Fribourg (NHMF), Chemin du Musée 6, CH-1700 Fribourg, Switzerland

Abstract

Aim of study: The Caucasian apple (*Malus orientalis* Uglitzk.) is distributed throughout the Hyrcanian forest. Self-incompatibility (SI) is one of the most important plant strategies to prevent self-fertilization, but the genetic basis of this system has never been studied in Caucasian apple. Investigating the genetic diversity of Caucasian apple along an elevation gradient is the second aim of this study.

Area of study: Three populations of Caucasian apple along an altitudinal gradient in northern Iran were studied.

Material and methods: Here, we evaluated the S-allele frequency and genetic diversity of three populations of *Malus orientalis* using SSR markers.

Main results: In total, 18 S-alleles were identified in three populations, and a positive trend was detected between S-allele frequency and altitude, which is consistent with the positive correlation with genetic diversity. Overall, the genetic differentiation among populations was high, and four distinct groups were determined among three altitudinal populations.

Research highlights: Despite the small number of individuals and low genetic diversity of the populations, the S-allele frequency of Caucasian apple in Hyrcanian forests is high, and these resources have potential use in apple breeding programs.

Keywords: *Malus orientalis*; S-RNase alleles; Genetic diversity; Mate availability; Conservation strategy; Hyrcanian forest.

Authors' contributions: Conceived, designed and performed the experiments: HY and RR. Analyzed the data: HY, RR and HB. Contributed reagents/materials/analysis tools: HY, BL and GK. Wrote the paper: HY, RR and BL. All authors read and approved the final manuscript.

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Correspondence should be addressed to Hamed Yousefzadeh: h.yousefzadeh@modares.ac.ir; hamed.yousefzadehseraj@gmail.com

Introduction

Apple maintains a gametophytic self-incompatibility (GSI) system that is controlled by a single multi allelic locus (S-locus) (Kobel, 1939; De Nettancourt, 2013). Here, if the pistil is pollinated with pollen with a matching S-allele, pollen tube growth into the style is blocked, preventing fertilization from occurring (Hoebee *et al.*, 2011; Matsumoto, 2014). On the other hand, in cases where cross-pollination involves a pistil and pollen with different S-alleles, pollen tube growth is not blocked by the GSI system, thus allowing pollen tube growth. Hence, to ensure the long-term survival of apple populations, a high diversity of S-alleles is needed to obtain a sufficient population size for mate availability and production of viable seeds.

The Caucasian apple (*Malus orientalis* Uglitzk.) (Komarov, 1939) occurs in the Transcaucasian region (Tur-

key, Armenia, Georgia, and southwestern Russia) but little is known about its ecology (Cornille *et al.*, 2013). *M. orientalis* is one the fourth crabapple species which are considered to be the closest relatives of *Malus domestica* L., with which they are fully interfertile (Zohary & Hopf, 2000). This tree is distributed throughout the Hyrcanian forest, from the Arasbaran (northwest) and Zagroasian forest (west of Iran). Caucasian apple is usually dispersed as single trees or populations in its natural habitat, where it can reach approximately 10 meters in height and a maximum age of 80-90 years. Caucasian apple is found at most altitudes up to 2400 meters above sea level in the Hyrcanian forest, though it is rarely observed at sea level (Amirchakhmaghi, 2018). The Hyrcanian forest maintains significant biodiversity, which is currently in decline (Colagar *et al.*, 2016; Zarandian *et al.*, 2016), as approximately 60% of the area is managed for timber production and the remaining area is partly deforested (Salehi *et al.*, 2007). Due to continuous deforestation caused by human population growth (urbanism) and industrialization, there is an urgent need to evaluate the level of diversity and thereby assess the vulnerability of species in this ecosystem. This may facilitate future conservation strategies for sustainable forest management (Fussi *et al.*, 2016).

The S-allele frequency and diversity of Caucasian apples in Hyrcanian forests have not been reported thus far. Moreover, genetic diversity studies relating to horticultural and wild crop trees along altitudinal gradients are rare, particularly those pertaining to the effects of climatic variables on the genetic variation and structure of horticultural trees in forests. Additionally, diversity in plant genetic resources (PGR) provides an opportunity for plant breeders to develop new and improved cultivars with desirable characteristics.

Most studies to date have focused on describing the S-alleles in *M. domestica* cultivars (*e.g.*, Halász *et al.*, 2011; Kim *et al.*, 2007; Larsen *et al.*, 2016; Long *et al.*, 2010; Matsumoto, 2014). Many studies have focused on national collections, such as that by Nybom *et al.,* (2008), who considered S-allele frequency in 104 apple cultivars grown mainly in Northern Europe and revealed that the most common S-allele was S7 (18%), followed by S3 (17%), S5 (14%), and S1 and S2 (both at 11%). Heo *et al.,* (2012) detected nine different S-alleles in Korean apple cultivars and found that the most common S-allele was S3, followed by S9 and S1.

The aim of this study was to evaluate the genetic diversity of Caucasian apple in Hyrcanian forests growing on an elevation gradient. Furthermore, we aimed to unravel the S-allele frequency and diversity at each altitude to evaluate the eventual vulnerability of Caucasian apple populations.

Materials and Methods

Leaf sampling and DNA extraction

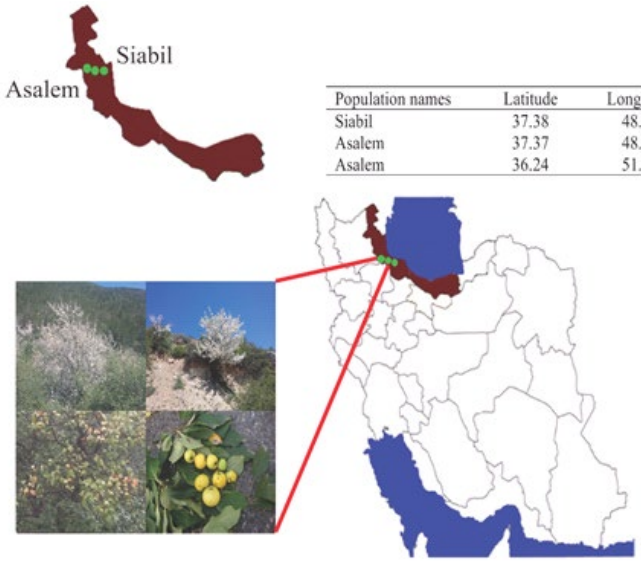
Three wild populations of Caucasian apple were selected for sampling across an elevation gradient in the western part of the Hyrcanian Forest (Fig. 1). Leaf samples were collected from 26 trees from three locations with at least 50 m between each sampled tree. The leaves were stored in containers on dry ice until further analysis. DNA extraction was performed as described by Murray & Thompson (1980) with significant modifications (Janfaza *et al.*, 2017).

Identification of S-alleles

To amplify the S-alleles, two methods were used: 1) the specific primers described by Broothaerts (2003) and Long *et al.,* (2010); and 2) general primers described by Larsen *et al.,* (2016). PCR products were sequenced using the Sanger method (Bioneer Company). The Shannon index was calculated for each population by PAST software (Hammer *et al.*, 2001). The proportion of S-allele i relative to the total number of S-alleles (pi) was calculated, and then multiplied by the natural logarithm of this proportion (lnpi). The resulting product is summed across S-alleles, and multiplied by -1. To calculate the S-allele ratio per population we divided the number of variety of alleles in each population by the number of its individuals.

SSR amplification, genetic diversity and structure analysis

SSR amplification was performed using six primers described by Hokanson *et al.,* (2001) (Table 1). PCR products were loaded on 8% polyacrylamide gels to visualize the amplified bands and enable scoring (Colagar *et al.*, 2016). The software GenAlEx 6.501 was used to perform AMOVA using 999 randomizations, as well as to calculate the fixation index (Fst), the amount of genetic flow (Nm) and other parameters, such as the percentage of polymorphic loci and analysis of molecular variance. Allelic richness (AR) and private allele richness (PAR) were calculated using hp-rare 1.0 computer program (Kalinowski, 2005). The population structure was analyzed using the software STRUCTURE 2.3.4 (Pritchard *et al.*, 2000). The optimal number K, the number of subgroups, was calculated empirically. The simulation was conducted with a duration of 100,000 burn-in and 500,000 Markov Chain Monte Carlo (MCMC) repeats. A number, K, from 2 to 6 was considered for each subgroup to increase accuracy. Structure Harvester v. 0.6.94 was used to calcula-



Population names	Latitude	Longitude	Altitude
Siabil	37.38	48.58	50
Asalem	37.37	48.46	1020
Asalem	36.24	51.49	1980

Figure 1. Populations of *Malus orientalis* studied along an altitudinal gradient in the western part of the Hyrcanian forest.

te the number of subgroups (K) and optimal K-number. Furthermore, genetic relationships among detected populations were considered by principal coordinate analysis (PCoA) in GenAlEx 6.501. DAPC (Discriminant Analysis of Principal Components) from the *adegenet* package in R (Jombart 2008; Jombart & Ahmed 2011) was used, complemented, with Bayesian clustering, methods in STRUCTURE.

Results

S-allele identification and diversity

In total, 18 S-alleles were identified in three populations (Table 1). Five (19.23%) of the 26 individuals had three S-alleles, including one individual from the lower population, three from the middle population and one from the upper population. The S1 allele, with 24% frequency, was the most common allele, followed by S6 and S19, which were present in all populations. We identified S23 in four trees of middle elevation, while S23 was not observed in the upper population and was only recognized in one tree of the lower population (Fig. 2). In the lower, middle and upper populations, 10, 12 and 10 different S-alleles were identified, respectively. The diversity of S-alleles showed a positive correlation with altitude, which is consistent with the positive correlation between altitude and observed heterozygosity (Fig. 3a).

Genetic diversity using SSR markers

The highest and lowest mean number of alleles, effective alleles and expected heterozygosity rates were observed in the middle and lowest populations, respectively

Table 1. Sequences of SSR primers used in this study

Annealing time	Primer sequence	Size	Primer name
65	F: GAGGCAAGTGACAAAGAAAGATG R:AAAATGTAACAACCCGTCCAAGTG	182-252	**P3-GD162**
55	F:ACAGCAAGGTGTTGGGTAAGAAGGT R:TGCGGACAAAGGAAAAAAAAAAGTG	213-268	**P4-GD100**
55	F:CGGCGGAAAGCAATCACCT R:GCCAGCCCTCTATGGTTCCAGA	131-203	**P5-GD96**
65	F:TCCCGCCATTTCTCTGC R:AAACCGCTGCTGCTGAAC	114-170	**P6-GD147**
55	F:TTGAGGTGTTTCTCCCATTGGA R:CTAACGAAGCCGCCATTTCTTT	135-200	**P7-GD12**
65	F:GGCACCCAAGCCCCTAA R:GGAACCTACGACAGCAAAGTTACA	123-189	**P8-GD142**

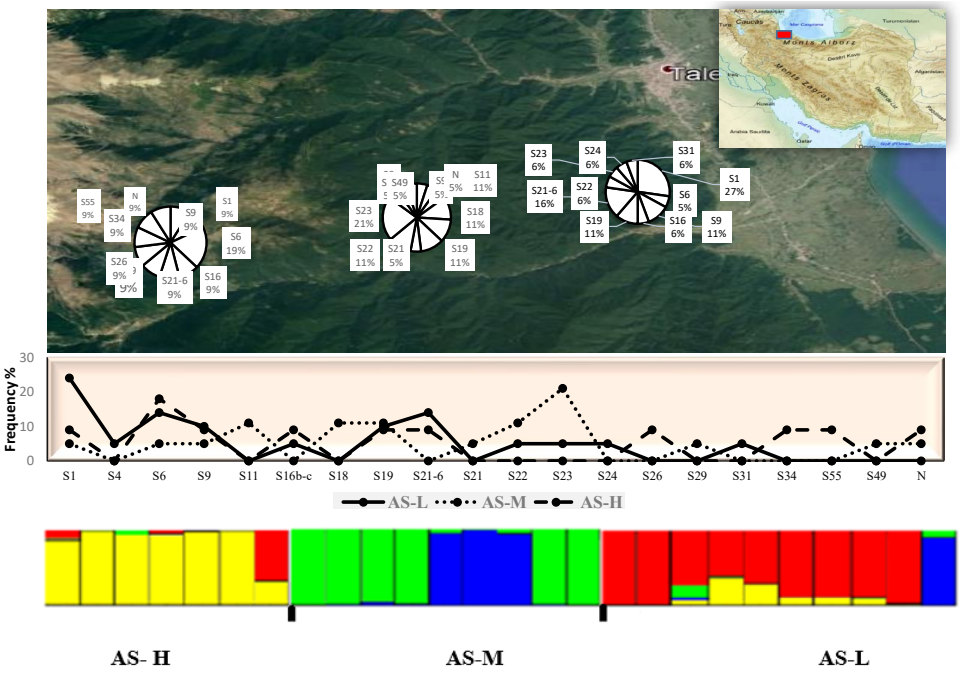


Figure 2. (a) S-allele frequency in three studied populations; (b) comparison of each altitude in terms of each S allele type; (c) structural analysis results (Q plot) for each population. The most likely number of genetic groups in K=4. The bar represents the proportion of each individual assigned to each of the inferred K values (A).

(Table 2). Maximum allelic richness and private allelic richness of 8.28 and 7.66, respectively, were observed at middle altitude. The highest mean heterozygosity (0.524) was observed in the upper population. Among the 18 tests (six positions for each population), eight tests showed a significant ($P < 0.05$) deviation from Hardy-Weinberg equilibrium. There was a positive trends between the S-allele frequency and observed heterozygosity with the elevation of populations (Fig. 3a), with increasing elevation, both of them increased. Additionally, a large gap between the observed and expected heterozygosity was detected for the middle population (Fig. 3b). Shannon index was not different among all populations based on S-allele frequency, while this value for SSR markers was bigger than in the middle elevation (Fig. 3c).

Genetic differentiation and structure

Overall, the genetic differentiation (Fst) among populations was 0.23, while the average genetic flow (number of migrants; Nm) was 0.8. AMOVA revealed that 24% of the total variation was found among populations, while the rest (76%) was within populations (Table 3). The middle elevation Asalem population was genetically distant from the lower and upper populations. In fact, based on PCoA and DAPC, three different groups were identified, but the lower and upper populations were closer in comparison to the middle elevation population (Fig. 4). Only one tree from the middle elevations was grouped with trees from the low elevation. Structural analysis (K optimum= 4) revealed that the studied populations were divided into four genetically distinct groups, and in the middle population, two of the subgroups were clearly observed (Fig. 2c).

Discussion

Diversity, frequency and distribution of S-alleles

In the present study, 18 different S-alleles were identified in three apple populations.

Among the identified S-alleles in this study, S1 and S6 had the highest frequency at 12.3% and 10.5%, respectively. S19 and S23 were the second most abundant S-alleles, with 8.7% of the studied samples. Rahemi *et al.* (2010) suggested that geographical positions and altitudinal differences of wild almond and other *Prunus* populations influence the diversity and differentiation at these loci. The S-alleles identified in *M. orientalis* are consistent with the S-alleles in cultivated *M. domestica* cultivars, which suggests cross-compatibility between the two species. The diversity maintained by wild populations of M. orientalis might therefore be relevant in future breeding programs to increase the genetic diversity in *M. domestica*.

Based on Vekemans *et al.,* (1998), approximately 8-10 different S-alleles in each population are sufficient for

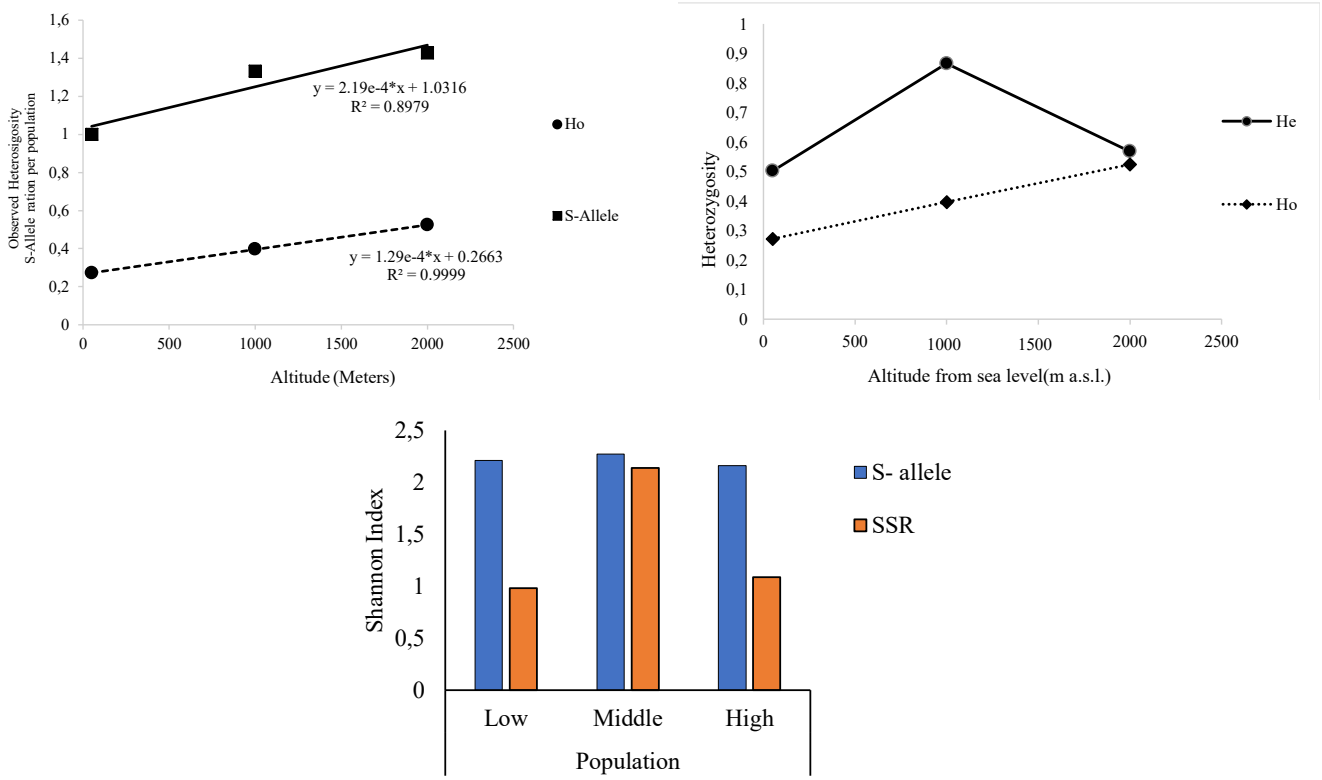


Figure 3. (a) Correlation between observed heterozygosity and S-allele frequency with the altitude of the population; (b) He and Ho trends along altitudinal gradients of studied populations; (c) comparison of Shannon index values with SSR marker and S-allele frequency results.

successful pollination (mate availability index= 1) and production of viable seeds. Accordingly, populations with less than five different S-alleles exhibit successful mating rates lower than 90% (Vekemans *et al.*, 1998).

Loss of genetic diversity and consequent loss of the S-allele in populations, especially in species with small size and patchy distribution, such as wild *M. orientalis*, can significantly increase the risk of those populations becoming endangered in a short time (Frankham, 2005). In our study, although the number of trees per population was less than 10, at least 8 S-allele types per population were detected. According to the predictions of Gutfitti balanced models (Vekemans *et al.*, 1998; Wright, 1939) and what has been reported about wild populations of wildflowers with the Gutfitti self-incompatibility system in previous studies (*e.g.*, Hoebee *et al.*, 2011; Holderegger *et al.*, 2008), mates were available for more than 90% for the studied populations. Despite the results of other studies that found reduced fertilization success in small populations of SI species (*e.g.*, Fischer *et al.,* 2003; Willi *et al.*, 2005), in line with Vekemans *et al.,* (1998), for the populations under study, more than 80% successful seed germination has been reported (Karimi, 2015).

In fact, the high frequency and diversity of the S-allele, even in small populations, can increase the chance of successful pollination and reduce concern about mate availability (Holderegger *et al.,* 2008; Hoebee *et al.,* 2011). Hence, based on this research, there is no concern about

Table 2. Genetic diversity in three *Malus orientalis* populations based on SSR markers

PAR	AR	PPL	F	He	Ho	I		Population
1.27	3.51	83.33	0.432	0.502	0.272	0.981	Mean	AS-L
			0.140	0.118	0.079	0.251	SE	
7.66	8.28	100.00	0.545	0.866	0.397	2.146	Mean	AS-M
			0.121	0.012	0.110	0.085	SE	
1.46	3.85	100.00	0.130	0.568	0.524	1.090	Mean	AS-H
			0.194	0.096	0.115	0.228	SE	

Ho = observed heterozygosity; He = expected heterozygosity; F = F-values; PPL =Percentage of Polymorphism; AR = Allelic Richness; PAR = Private Allelic Richness

Table 3. Analyses of molecular variance (AMOVA) for the studied populations

Source	df	SS	MS	Est. Var.	%	PhiPT	Nm
Among Pops	2	50.207	25.104	2.127	24%		
Within Pops	23	157.870	6.864	6.864	76%	0.237**	0.8
Total	25	208.077		8.991	100%		

**- Significant at the 0.01 level. Squared deviations (SS); mean squared deviations (MS), variance component estimates (Est. Var.), the percentage of the total variance contributed by each component and gene flow (Nm).

mate availability in *Malus orientalis* populations and, consequently, in production of viable seeds in Hyrcanian forests. Furthermore, seed germination of these same populations was previously studied by Karimi (2015), who indicated that seed germination was over 80%.

Genetic diversity and structure

Here, we studied the S-alleles and genetic diversity in three populations of *M. orientalis* in the Hyrcanian forest of Iran. The number of trees included here is too low to give a true description of the actual diversity in a large and diverse forested area. However, it may contribute to an understanding of the diversity in *M. orientalis* populations of the area, and the S-allele diversity is especially important for the successful reproduction of this species in its natural populations. In this study, allelic richness (AR) showed a slight difference between the lower and upper Asalem populations, which showed AR values of 3.51 and 3.85, respectively, but in the middle Asalem population, the AR (8.28) was much higher. The high density of summer villages and the transfer and cultivation of domestic and improved cultivars for domestic use can be one of the reasons for the high allelic richness of Caucasian apples in the western parts of the Hyrcanian forest.

The observed heterozygosity for populations under study, west of Hyrcanian forest, varied from low (0.22 and 0.39 in lower and middle populations) to medium (0.52 in upper population). The observed heterozygosity showed an increasing trend with altitude (0.22, 0.39, and 0.52), in agreement with results from Goto et al. (2009), which revealed that there is a positive correlation between altitude and genetic diversity parameters, and as altitude increases, the observed heterozygosity increases.

High concentration of human populations, changes in land use and grazing in the low and middle elevations of Hyrcanian forests (Saeei, 1950; Tohidifar et al., 2016) are the main reasons for the sharp decrease in the genetic diversity of most plant species. Additionally, the loss of genetic diversity (gap between Ho and He) can be a consequence of repeated artificial selection of apple trees by indigenous people.

The habitats of the studied populations are under strong pressure from habitat degradation due to human activities (agriculture and urbanization). Land use change is much higher at low elevations than at middle elevations, although the main habitat of *M. orientalis* is above the timber line (>1800 m a.s.l) of Hyrcanian forest. The relatively low gene flow (Nm = 0.8) and consequently relatively high Fst (0.23) among the studied populations can be attributed to various reasons. A clearly distinct genetic structure was observed among populations under study, especially between high and low populations. An important factor in these findings is no doubt the phenological separation. There is approximately one-month interval between the flowering time of *M. orientalis* in highland

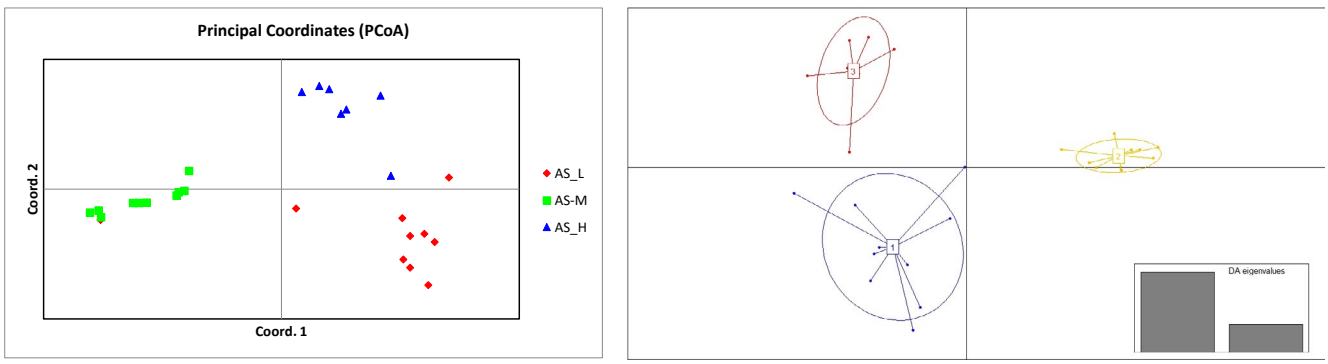


Figure 4. Principal coordinate analysis of three populations of Caucasian apple along an altitudinal gradient; (b) Discriminant analysis of principal components (DAPC) with populations used as clusters: 1: AS _L; 2: AS_M; 3: AS_H populations.

and lowland populations in the Hyrcanian forest (Amirchakhmaghi *et al*., 2018). The studied populations are separated from each other by approximately 1000 meters in altitude, and their flowering times do not overlap. Thus, as noted by Alberto *et al.*, (2018), the different flowering times of populations can increase their genetic differentiation. The most important bee for pollination of apple is *Apis mellifera* (Visscher & Seeley, 1982). The effective pollination distance of this bee is a maximum of 6 km (Visscher & Seeley, 1982). Since our study locations were separated horizontally by at least 20 km, no major gene flow among populations was expected.

Additionally, a positive trend was observed between genetic diversity (Ho and AR) and the altitude of the habitat. Similarly, a strong association between altitude and genetic differentiation (Yousefzadeh *et al.*, 2018; Nazarzadeh *et al.*, 2020) or genetic diversity (Mathiasen & Premoli 2013; Shirmohammadli et al., 2018; Meng *et al.*, 2019) has been reported in many published works. Ohsawa & Ide (2008) expressed that altitudinal climatic gradients can influence the distribution of genetic variation within and among plant populations. In fact, knowing the genetic diversity of a plant species across elevation gradients is important to obtain a suitable strategy for conservation (Hahn *et al*., 2012)

Conclusion

The study demonstrated a high diversity of S-alleles in *M. orientalis* from three different study sites in the Hyrcanian forest. Despite the small number of individuals studied here, the findings suggest high S-allele diversity. Low genetic diversity and the presence of a gap between He and Ho in the populations under study can definitely affect the species' ability to adapt to changing environmental conditions and thus may increase the risk of genetic erosion of *M. orientalis* in Hyrcanian forests. This research is a preliminary study, and it is recommended that stronger conclusions be drawn in the future by using more SSR primers and more accurate coding methods, such as capillary sequencing methods, as well as adding several additional gradients to the study.

The current S-allele study might also be of interest for future breeding programs aimed at utilizing the diversity of natural populations to increase the diversity of ornamental or edible cultivated apple breeding materials.

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