

Genetic Diversity and Conservation Challenges of The European Crab Apple (*Malus sylvestris* (L.) Mill.) in the Northwestern Balkans

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ABSTRACT

This study investigates the genetic diversity and introgression dynamics of European crab apple (*Malus sylvestris* (L.) Mill., Rosaceae) populations in the northwestern Balkans. A total of 175 trees were analysed, including 157 putative wild individuals from 21 natural populations and 18 domesticated apples (*M. ×domestica* (Suckow) Borkh.) as out-groups. Genetic variation was assessed using ten nuclear microsatellite loci and Bayesian cluster analysis. The results revealed high allelic richness (average $A = 25.2$) and high genetic diversity ($H_T = 0.76$) with moderate population differentiation ($F_{ST} = 0.14$). Observed heterozygosity was generally lower than expected, suggesting potential effects of introgression and genetic drift. Bayesian assignment classified 60.58% of all sampled individuals as pure crab apples, 21.71% as hybrids, and 17.71% as domesticated apples. However, when excluding domesticated individuals, the proportion of pure crab apples within natural populations increased to 67.5%. Populations located in densely forested and sparsely populated regions exhibited higher genetic integrity, whereas those in fragmented and human-influenced landscapes showed increased hybridization. These findings highlight the role of human-mediated factors, such as supplemental feeding of wildlife, discarded apples, and the use of non-verified forest reproductive material, in facilitating gene flow from domesticated to wild apples. Conservation strategies should therefore favour genetically verified propagating material, protect populations with high genetic integrity, and mitigate human-induced pressures that promote hybridization between wild and domesticated apples.

Keywords:

conservation of genetic resources, genetic admixture, hybridization, microsatellite (SSR)

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INTRODUCTION

The European crab apple (*Malus sylvestris* (L.) Mill.) is the only native representative of the genus *Malus* Mill. in Europe. As a member of the Rosaceae family, it shares ecological and biological traits with other fruit-bearing species, yet stands out due to its unique evolutionary history and limited distribution. Genetic studies across the continent reveal a pronounced population structure shaped by post-glacial dynamics, with western populations forming a distinct cluster from those in Eastern Europe. Within Eastern Europe, two major genetic groups have been identified – one in the Carpathians and another in the Balkans (Cornille et al. 2013). This pattern suggests that Balkan populations, including those in the northwestern Balkans, originate from a unique glacial refugium, underscoring their significance as a distinct genetic lineage. Despite its ecological and evolutionary importance, *Malus sylvestris* remains rare and scattered across natural habitats. Nevertheless, it plays a vital role in forest biodiversity, contributing to ecosystem functioning through insect-mediated pollination and seed dispersal by mammals, birds, watercourses, and human activities (Wagner et al. 2014).

The species exhibits high heterozygosity and allelic richness (e.g. Larsen et al. 2006, Reim et al. 2013, Schnitzler et al. 2014), indicating a strong evolutionary potential and considerable genetic resources, despite its fragmented distribution. This genetic diversity is essential not only for the long-term adaptability of the species, but also for providing valuable traits, such as disease resistance, that can improve the breeding of domestic apples (Kišek et al. 2021). The conservation of this diversity is particularly important in the Balkans, where historically isolated and locally adapted populations may harbour unique alleles of high conservation and breeding value.

One of the most pressing conservation concerns for *Malus sylvestris* is its ongoing hybridization with the domestic apple (*M. × domestica* (Suckow) Borkh.) (Kleinschmit et al. 2012, Ruhsam et al. 2019, Kišek et al. 2021). Domesticated apples originated from their wild ancestors in Central Asia, particularly *Malus sieversii* (Ledeb.) M. Roem., and spread westward along the Silk Road, where they interbred with native *Malus sylvestris* populations upon reaching Europe. This early gene flow introduced adaptive traits into cultivated apples and significantly shaped the genetic makeup of modern cultivars, a legacy still evident today (Forte et al. 2002, Ignatov and Bodishevskaya 2011, Cornille et al. 2012, 2014, Duan et al. 2017). Currently, the gene flow is largely reversed: alleles from domesticated apples are infiltrating wild populations, blurring species boundaries and threatening the genetic integrity of *Malus sylvestris* (Wagner et al. 2014, Ruhsam et al. 2019). Hybridization is facilitated by the absence of strong reproductive barriers, as both species are genetically compatible and share a self-incompatible mating system that promotes cross-pollination (Euforgen 2024). From an evolutionary standpoint, *Malus sylvestris* is therefore involved in a long-term bidirectional gene flow with cultivated apples, making it one of several wild fruit tree species historically and currently involved in interspecific hybridization, along with pear and cherry (Ganopoulos 2013, Vidaković et al. 2024). Importantly, introgression does not necessarily imply ecological degradation; in many wild populations,

individuals remain predominantly descended from *Malus sylvestris*, suggesting that domesticated alleles are being gradually integrated into wild gene pools.

Nevertheless, ongoing introgression poses significant challenges to species conservation. It diminishes the evolutionary potential of wild populations and complicates the identification of genetically pure individuals. Morphological traits alone are insufficient to distinguish pure wild apples from hybrids (Kišek et al. 2021), making molecular tools essential for accurate identification and monitoring. In addition to genetic “flooding”, habitat loss and fragmentation further endanger the species by creating small, isolated populations that are more vulnerable to inbreeding, genetic drift, and environmental change (Larsen et al. 2006, Jacques et al. 2009, Reim et al. 2012). These threats are intensified by poor natural regeneration, a narrow genetic base, and high susceptibility to grazing (Stephan et al. 2003, Reim et al. 2013, Wagner et al. 2014).

Human activities contribute to both increased hybridization and the wider dispersal of cultivated apple genes. Seeds from cultivated apples are frequently introduced into natural ecosystems via discarded fruit, artificial feeding of wildlife, or zoochory (Fležar et al. 2019, Pereira et al. 2021), thereby enhancing opportunities for gene flow between domesticated and wild populations. Similar patterns of genetic introgression have been documented in other wild fruit species, such as *Pyrus pyrausta* (L.) Burgsd. (Vidaković et al. 2024) and *Prunus avium* (L.) L. (Ganopoulos 2013).

In this study, we examine the genetic diversity of *Malus sylvestris* populations in the northwestern Balkans, with a particular focus on the extent of introgression of *Malus × domestica*. Our results provide new insights into the genetic structure of wild apple populations and contribute to the development of effective conservation strategies aimed at preserving this ecologically important and increasingly threatened species.

MATERIALS AND METHODS

Study Area and Sampling

To evaluate the status of the European crab apple in a wider geographical area, we included populations from the northwestern Balkans in our analysis. All trees sampled in natural habitats were classified as putative European crab apples based on morphological criteria. Particular attention during selection and sampling was given to leaf shape, leaf size, and the presence of pubescence on the underside of the leaves (Kišek et al. 2021). We analysed 157 putative European crab apple trees from 21 natural populations (Table 1). In addition, 18 domesticated apple trees (*M. × domestica*) were included as out-group genotypes (Kišek et al. 2021).^{†1}

Genetic Analysis

A total of 175 trees were included in the genetic analysis. Young leaves were collected from each tree and immediately preserved in silica gel for further processing. Genomic DNA was extracted from approximately 20 mg of dried leaf tissue using the DNeasy® Plant Mini Kit (Qiagen) according to the manufacturer’s protocol. All individuals were genotyped using two multiplex polymerase chain reactions (PCR) at ten nuclear microsatellite loci as described by Kišek et al. (2021).

Table 1 Main characteristics of the populations.

Pop. No.	Country ID	Population name	No. of trees
1	SI	Ajdovščina	4
2	SI	Boč	9
3	SI	Dole	14
4	SI	Gabrovka	7
5	SI	Kočevje	22
6	SI	Koroška	8
7	SI	Kozjansko	4
8	SI	Logatec	12
9	SI	Postojna	11
10	BIH	Kozara	6
11	HR	Ogulin	2
12	HR	Zalesina	5
13	HR	Dinara	7
14	HR	Strahinjčica	12
15	HR	Psunj	5
16	HR	Brinje	5
17	HR	Bilogora	2
18	HR	Pazin	5
19	HR	Vinkovci	5
20	HR	Moslavačka gora	10
21	HR	Perušić	2
22	SI	<i>Malus × domestica</i>	18
Total			175

Multiplex PCRs were performed according to the protocol of Patocchi et al. (2008). PCR products were genotyped using an ABI3130XL automated sequencer (Applied Biosystems).

Genetic variability indicators were calculated in GenAlEx 6.5 (Peakall and Smouse 2012). For each microsatellite locus, the total number of alleles, the range of allele sizes, and gene diversity (H_T) were determined to evaluate the

overall polymorphism. Allelic richness (A_R) was calculated using a rarefaction approach to account for differences in sample size among populations, standardizing to the smallest sample size ($N = 5$ individuals), following the method implemented in SPAGeDi (Hardy and Vekemans 2002). We also calculated the average number of observed alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E) and Wright's inbreeding coefficient (F_{IS}) for all loci.

We used the Bayesian clustering method (also known as the Bayesian analysis of population structure) in the programme STRUCTURE 2.3.4 to genetically identify the specimens and assign individual trees to groups (Pritchard et al. 2000). The Bayesian sorting parameters in STRUCTURE 2.3.4 were 20,000 burn-in periods and 10,000 Markov chain Monte Carlo replicates, under the admixture model with correlated allele frequencies. Analyses were run for population structure models, assuming $K = 1$ to $K = 10$ distinct clusters. Evanno's delta K (ΔK) value (Evanno et al. 2005, Li and Liu 2018), which identifies the most relevant number of clusters by determining the point beyond which there is no further increase in likelihood, was greatest for $K = 2$ ($\Delta K = 38,843$) (Supplementary material Figure S1, Table S1). To distinguish between tree groups, we defined a hybrid coefficient Q_E as the individual membership coefficient (Q -value) obtained from STRUCTURE for $K = 2$, reflecting the proportional assignment of each individual to the inferred genetic clusters (Reim et al. 2013, Wagner et al. 2014, Coart et al. 2003). Individuals were classified using commonly applied threshold values: $Q_E < 0.2$ was assigned to European crab apples, $Q_E > 0.8$ to domesticated apples, and intermediate values ($0.2 < Q_E < 0.8$) were considered admixed (hybrid) individuals.

RESULTS

Allelic Diversity of Microsatellite Loci

High frequencies of null alleles (> 0.5) can significantly affect the results of genetic analyses (Huang et al. 2016). In our study, where their presence was predicted using Cervus 3.0.7 software (Kalinowski et al. 2007), the observed frequencies were low (data not shown), and all loci showed high polymorphism. Therefore, we concluded that their

Table 2 Indicators of genetic variability for all 157 European crab apple trees sampled in nature.

Locus	N	RA	A	A_E	H_T	F_{IS}	F_{ST}
CH01h10	151	88–166	24	5.39	0.78	0.06	0.14
CH04c07	154	88–190	21	4.63	0.75	0.12	0.13
CH01h01	150	73–135	22	4.50	0.75	0.35	0.14
CH02b03b	157	69–110	23	5.16	0.77	0.14	0.16
CH02b12	153	110–174	33	5.79	0.75	0.17	0.16
MS06g03	156	148–198	26	5.48	0.75	0.27	0.20
CH02b07	157	92–127	16	5.09	0.77	-0.10	0.13
CH02c11	157	195–246	28	3.07	0.66	-0.42	0.08
CH03d11	157	92–179	30	6.52	0.83	-0.01	0.12
CH02a10	156	132–176	29	6.68	0.84	0.14	0.11
average			25.2	5.23	0.76	0.07	0.14

N = number of trees; RA = range of allele lengths (bp); A = total number of alleles; A_E = effective number of alleles; H_T = total genetic diversity; F_{IS} = Wright's inbreeding coefficient; F_{ST} = fixation index

presence did not significantly affect the results, and all loci were included in further analyses.

Among the 157 trees analysed, we identified 252 different alleles at ten microsatellite loci (Table 2). The highest number of alleles was found at locus CH02b12 (33), and the lowest at locus H02b07 (16). The average number of alleles per locus was $A = 25.2$, while the mean number of effective alleles (A_e) was 5.23. The total genetic diversity (H_T) was 0.76, with the highest value at locus CH02a10 ($H_T = 0.84$) and the lowest at locus CH02c11 ($H_T = 0.66$). The fixation index (F_{ST}) was 0.14, and the inbreeding coefficient (F_{IS}) was 0.07.

Genetic Diversity Within Populations

The observed heterozygosity (H_O) in the populations is high, averaging 0.71 (Table 3). The expected heterozygosity at the

loci (H_E) ranges from 0.34 (CH02c11, Psunj) to 0.92 (MS06g03, Kočevje). A comparison between the observed (H_O) and expected (H_E) heterozygosity in the populations shows a deficit of heterozygotes in most populations ($F_{IS} > 0$), except in the populations of Ajdovščina, Kozjansko, Ogulin, Brinje, Bilogora, and Perušić.

Private alleles (A_{PRIV}) are present in all populations except in the Bilogora and Perušić populations. This is most likely due to the small number of trees analysed in these populations (only two). The probability of detecting private alleles in such small samples is lower than in populations with a larger number of analysed trees. The number of private alleles per locus population is between 0 and 8, with allele frequencies varying between 0.02 and 0.25.

Table 3 Statistics of genetic variation within populations at ten microsatellite loci.

Population	N	A	AR	APRIV	HO	HE	FIS
Ajdovščina*	4	5.4	/	7	0.80	0.76	-0.05 ns
Boč	9	9.2	6.57	3	0.78	0.83	0.06 ns
Dole	14	10.5	6.37	3	0.74	0.84	0.12 ns
Gabrovka	7	7.2	6.17	4	0.66	0.81	0.18 ns
Kočevje	22	13.7	6.68	8	0.72	0.86	0.16 ns
Koroška	8	8.4	6.52	4	0.65	0.82	0.21 ns
Kozjansko*	4	4.7	/	2	0.76	0.71	-0.09 ns
Logatec	12	9.5	6.14	2	0.63	0.82	0.23 ns
Postojna	11	9.8	6.51	5	0.71	0.84	0.14 ns
Kozara	6	6.4	5.90	1	0.65	0.79	0.17 ns
Ogulin*	2	3.1	/	1	0.75	0.63	-0.19 ns
Zalesina	5	5.5	5.50	1	0.72	0.75	0.05 ns
Dinara	7	6.9	5.80	3	0.67	0.79	0.14 ns
Strahinjčica	12	9.9	6.31	5	0.63	0.82	0.22 ns
Psunj	5	5.8	4.50	3	0.72	0.74	0.04 ns
Brinje	5	4.7	4.40	2	0.79	0.69	-0.12 ns
Bilogora*	2	2.5	/	0	0.70	0.49	-0.42 ns
Pazin	5	5.9	5.90	2	0.70	0.79	0.11 ns
Vinkovci	5	5.8	5.80	4	0.62	0.75	0.19 ns
Moslavačka gora	10	9.0	6.41	5	0.62	0.84	0.24 ns
Perušić*	2	3.3	/	0	0.80	0.66	-0.17 ns
average	Σ 157	7.01	5.97	3.01	0.71	0.76	0.06

N = number of trees; A = total number of alleles; AR = allelic richness; APRIV = number of private alleles; HO = observed heterozygosity; HE = average expected heterozygosity; FIS = the deficiency or excess of average heterozygosity. Significant levels are adjusted using Bonferroni according to Rice (1989) (ns $P > 0.05$;

* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$; *** $P < 0.001$).

Statistics for populations marked with an asterisk (*) ($N < 5$) should be interpreted with caution, as the small sample size may limit the reliability of the estimates.

Genetic Structure of Trees

In order to assess the extent of genetic variability in natural populations of the European crab apple, domesticated apple trees were included in the analysis as a reference for comparison and calibration. This group originated from a

previous study (Kišek et al. 2021). Genetic clustering was performed using the Bayesian approach implemented in STRUCTURE 2.3.4 (Pritchard et al. 2000). A total of 175 trees were analyzed (Table 1). Based on the Q coefficient values, the trees were categorized into three groups: European crab apples, hybrids, and domesticated apples (Figure 1).

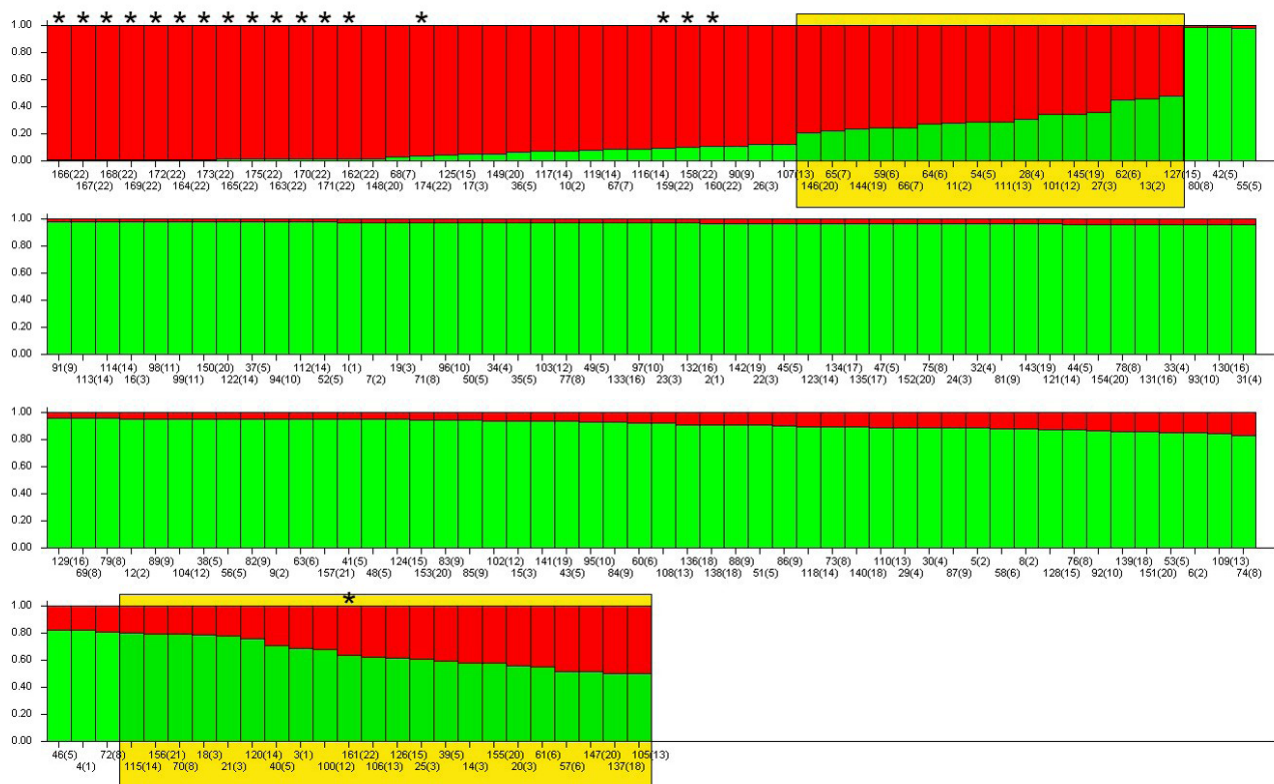


Figure 1 The genetic mixture of the 175 analyzed specimens of the European crab apple and the domesticated apple. The diagram was created with the STRUC-TURE program based on the analysis of 10 microsatellite loci; the number of classes (K) = 2; the Y-axis contains the admixture coefficient; the green part of the column represents the probability of each individual being assigned to the European crab apple genetic group; and the red part of the column represents the probability of each individual being assigned to the domesticated apple genetic group. The yellow frame highlights individuals classified as hybrids, based on their intermediate admixture coefficient, which indicates mixed genetic ancestry between the two clusters. Asterisk (*) above the bars denote individuals identified as *Malus x domestica*.

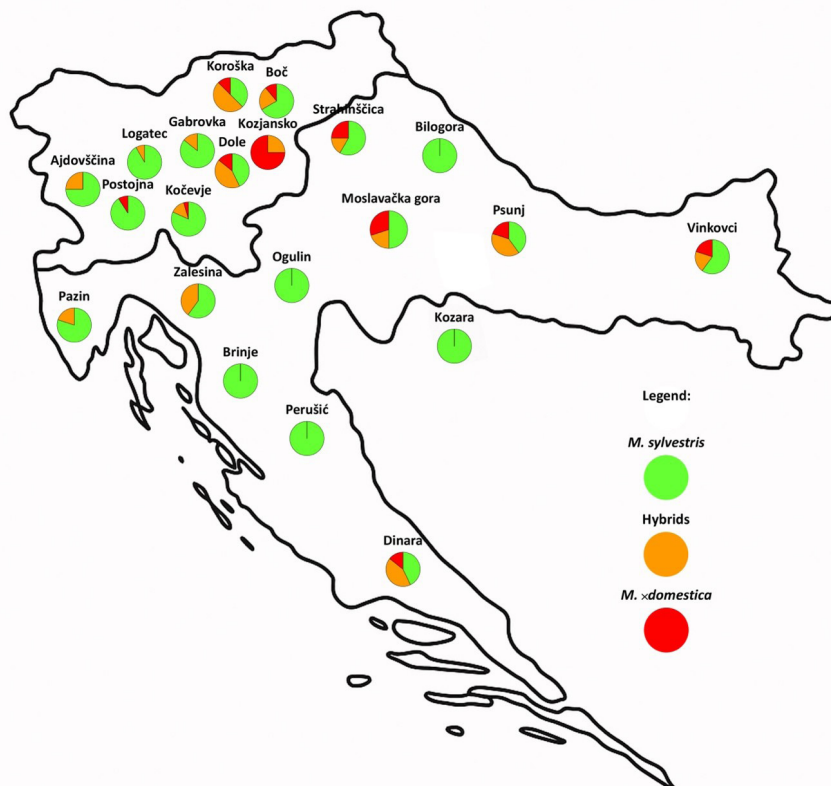


Figure 2 Proportions of genetically identified European crab apples, hybrids and domesticated apples for 157 trees, sampled in NW Balkans.

Of the 175 trees examined, 106 (60.6%) were classified as European crab apples, 38 (21.7%) as hybrids, and 31 (17.7%) as domesticated apples using the Bayesian clustering method. However, when considering only individuals sampled from natural populations (i.e. excluding domesticated apples), the proportion of pure crab apples increased to 67.5%. Figure 2 shows the proportions of crab apples, hybrids and domesticated apples in the studied populations. In five populations (Kozara, Ogulin, Brinje, Bilogora, and Perušić), all trees were identified as crab apples. Of particular note are three large populations – Kočevje, Logatec, and Postojna – in which the proportion of true crab apples is over 80% or even 90%. Particularly noteworthy is the Kočevje population, in which pronounced morphological variability was observed during sampling. This observation initially suggested a higher proportion of hybrids or feral domesticated apples in the sample.

DISCUSSION

Our genetic analysis of *Malus sylvestris* populations in the northwestern Balkans provides new insights on their conservation status, emphasizing the delicate balance between preserving genetic integrity and acknowledging the evolutionary significance of introgression. The results revealed high genetic diversity and moderate population differentiation, consistent with the species' broad distribution, outcrossing reproductive system, and animal-mediated seed dispersal (Euforgen 2024). A general deficit in heterozygosity suggests possible effects of genetic drift, inbreeding, or introgression from cultivated apples. These patterns are consistent with previous findings (Kišek et al. 2021) and indicate that the species maintains substantial genetic variability despite localized pressures.

A clear geographical pattern emerged, showing that the conservation status of European crab apple populations is closely linked to human population density and forest cover. Populations in sparsely populated, densely forested regions such as Kočevje and other parts of the Dinaric Alps exhibited higher genetic integrity, with a greater proportion of individuals classified as genetically pure European crab apples. An exception to this trend is the Dinara population, which, despite being located in a relatively intact forest landscape and distance from cultivated orchards, showed clear signs of genetic admixture. This unexpected result may be explained by historical land use practices in the area, including extensive livestock grazing and seasonal human activity. It is possible that domesticated apple fruits were introduced into wild either through feeding of domesticated animals or by shepherds and workers who consumed cultivated apples and inadvertently dispersed their seeds into the surrounding habitat.

In contrast, populations from the northeastern part of the study area, which is characterised by greater forest fragmentation and increased anthropogenic pressure from agricultural land use, urban expansion, and a higher proportion of managed landscapes, showed a higher degree of admixture with domesticated apples and hybrids. Similar patterns have been reported in northern England, where hybrid individuals were more common in areas of intensive land use (Ruhsam et al. 2019). This is consistent with expectations, as there are no strong biological barriers preventing

cross-pollination between wild populations and cultivated apple varieties.

Multiple anthropogenic factors contribute to this genetic mixing, many of which are often overlooked in forest management and conservation planning. Beyond habitat loss and fragmentation, subtle human activities directly affect wild populations, particularly fruit tree species. One such influence is the widespread practice of feeding wild animals with cultivated fruits, especially during winter months in Slovenia and Croatia (Fležar et al. 2019, Pereira et al. 2021). These fruits often contain seeds that, once discarded by animals, can germinate in natural habitats and initiate hybridization. A similar dynamic occurs during summer and autumn, when wild animals forage in orchards for fallen fruit, increasing contact between wild and cultivated genotypes. In addition, apple cores discarded by humans in natural areas contribute to the gene flow. Such seemingly trivial actions can have disproportionate effects, especially in regions with high visitor numbers.

The constant presence of cultivated or feral apple trees in the agricultural landscape further exacerbates the problem. These trees act as persistent sources of pollen and seeds. Pollinators and the wind can transport pollen from cultivated trees to wild populations, while frugivorous animals disperse seeds across the landscape, increasing the likelihood that hybrid progeny will establish near or within natural populations. This type of gene flow is especially concerning because it is subtle and long-term, operating across generations and gradually altering genetic composition of wild apple populations.

Another critical factor is the use of inappropriate forest reproductive material. In the past, seedlings of unclear or hybrid origin were often used for afforestation and reforestation, introducing foreign genes into wild populations (Kišek et al. 2021). These inadequately regulated practices pose a significant threat to the genetic purity of *Malus sylvestris* and may lead to long-term shifts in the species' genetic identity.

While these anthropogenic influences may appear marginal at first sight, their cumulative and long-term effects on the genetic integrity and diversity of wild apple populations are substantial. Recognizing and addressing these influences is essential for the effective conservation and sustainable management of European wild apple genetic resources. Integrating genetic monitoring with habitat protection and public awareness will be essential to preserve this ecologically valuable and increasingly vulnerable species.

CONCLUSIONS

This study highlights the complex interplay between natural landscape features and human activities in shaping the genetic structure of European crab apple populations. Forested and less populated areas serve as refugia for genetically intact lineages, while regions under stronger anthropogenic influence, such as Eastern Slovenia, exhibit increased hybridization. These results underline the importance of protecting genetically pure populations, minimizing human-induced gene flow, and ensuring the use of genetically appropriate reproductive material in forest management.

At the same time, the ecological consequences of hybridization between cultivated and wild forms remain poorly

understood. Introgression may enhance ecological plasticity and introduce adaptive traits, but it can also disrupt locally adapted interactions and compromise ecosystem stability. Selective breeding of resilient cultivars offers potential to support the adaptive capacity of natural populations, particularly in ecosystems facing environmental stresses. However, the long-term effects of such genetic exchange on the persistence and integrity of wild *Malus sylvestris* remain uncertain.

Ultimately, tree populations shaped by centuries of human interaction are an integral part of both natural and cultural landscapes. Recognizing their dual role – as reservoirs of genetic diversity and living witnesses to millennia of coexistence – is essential for developing conservation strategies that protect *Malus sylvestris* while sustaining the ecological processes that support forest resilience.

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SUPPLEMENTARY MATERIAL

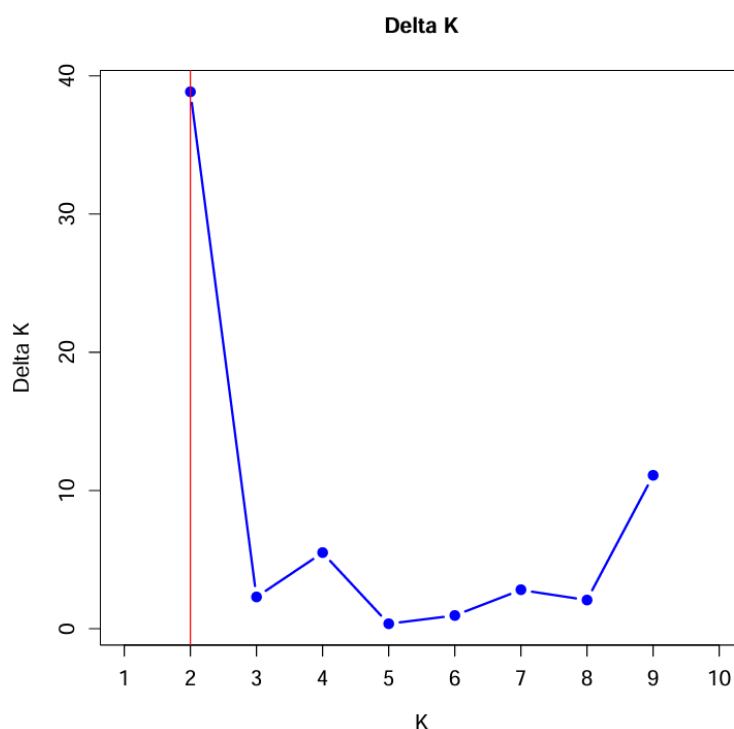


Figure S1 Delta K values calculated by Evanno's method detecting the most relevant number of K = 2 clusters.

Table S1 Results of Evanno's method for determining the optimal number of clusters (K).

K	Reps	Mean LnP(K)	Stdev LnP(K)	Ln' (K)	Ln' (K)	Delta K
1	5	-9275.32000	2.28626	NA	NA	NA
2	5	-8942.72000	6.16579	332.60000	239.50000	38.84334
3	5	-8849.62000	23.38604	93.10000	53.62000	2.29282
4	5	-8810.14000	15.19023	39.48000	83.48000	5.49564
5	5	-8854.14000	152.07389	-44.00000	54.86000	0.36075
6	5	-8843.28000	139.12296	10.86000	133.20000	0.95743
7	5	-8699.22000	115.38495	144.06000	323.38000	2.80262
8	5	-8878.54000	250.42373	-179.32000	519.80000	2.07568
9	5	-8538.06000	46.11039	340.48000	510.88000	11.07950
10	5	-8708.46000	220.14077	-170.40000	NA	NA