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Hybridisation of *Malus sylvestris* (L.) Mill. with *Malus* × *domestica* Borkh. and Implications for the Production of Forest Reproductive Material

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Abstract: This study focuses on the morphological and genetic characteristics of European crab apple (*Malus sylvestris* (L.) Mill.) and the occurrence of hybrids in its populations. We analyzed a total of 107 putative European crab apple trees in Slovenia: 92 from nine natural populations, five from a seed stand and 10 from a stand of unnatural origin. We also included 18 domesticated apple trees (*Malus* × *domestica* Borkh.) and two Japanese flowering crab apple trees (*Malus floribunda* van Houtte) as outliers. The trees were classified into groups of European crab apples, hybrids and domesticated apples according to their morphological and genetic characteristics. Classification based on morphological traits produced different results (58.75% European crab apple, 37.11% hybrids and 4.14% domesticated apple) compared to those based on genetic analysis (70.10% European crab apple, 21.64% hybrids and 8.26% domesticated apple). When genetic and morphological characteristics were combined, only 40.20% of the trees were classified as European crab apple, and an additional group of feral cultivars of domesticated apples (6.18%) was identified. The analysis revealed that hybridization with domesticated apple is taking place in all studied natural European crab apple populations; however, hybrids and feral cultivars only occur to a limited extent. When introducing European crab apple into forests in the future, only genetically verified forest reproductive material obtained exclusively from suitable seed stands should be used.

Keywords: hybridization; morphology; microsatellite (SSR); genetic admixture; seed object

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1. Introduction

European crab apple (*Malus sylvestris* (L.) Mill.) is a tree species that occurs individually in forests and is the only indigenous species of the genus *Malus* Mill. in Europe. It is a light-demanding species and prefers to grow as an individual specimen, mostly on forest edges [1]. Its fruit is an important source of food for a number of forest animals. Because of its contribution to the rapid regeneration of forests, European crab apple has a large impact on the stability of the forest ecosystem in the event of a major disruption [2]. Global environmental changes will affect tree species in a variety of ways [3,4], and tree species with limited ranges and low genetic variation are expected to be more sensitive to these changes [5]. Natural disturbances in forests (wind and snow storms, sleet, pests) are expected to increase in frequency and intensity due to climate change. The proportion of damaged forest areas will likely increase. The resulting increase in forest clearings and forest edges will create good ecological conditions for the growth of European crab apple. European crab apple enhances the biodiversity and adaptive potential of forests [2]. In addition, some characteristics which are important for modern apple production are only present in feral cultivars of the genus *Malus* [6], and the preservation of natural populations of European crab apple will be crucial in different maintenance breeding programs of domesticated apple (*Malus* × *domestica* Borkh.) in the future [7,8]. European crab apple

has many genes that reduce the susceptibility of the domesticated apple to many types of diseases and pests, i.e., resistance to the fungi *Penicillium expansum* Link. (blue mold) and *Colletotrichum acutatum* J. H. Simmonds (bitter rot) [8], which cause a number of problems in modern apple production.

M. sylvestris is defined in the IUCN Red List of threatened species as a data-deficient species (abbr. DD) [9]. It became a rare and endangered species mainly due to shrinking habitat, fragmentation of populations [10–12] and possibly hybridization with cultivated apple (*M.* × *domestica*). European crab apple has had a significant impact on the 'evolution' of the domesticated apple [13–15]. The domesticated apple was primarily domesticated in the mountains of Central Asia (Tian Shan), with the species *Malus sieversii* (Ledeb.) M. Roem. playing an important role in its creation. It spread to Europe across the Silk Road, where it encountered European crab apple [15]. Today in Europe, the domesticated apple is genetically more similar to European crab apple than the species *M. sieversii* [15,16]. Therefore, European crab apple and the domesticated apple are closely related tree species given the ongoing occurrence of hybridization and introgression.

Hybridization between wild species and their cultivated relatives is likely to reduce the fitness of wild populations and can lead to gene swamping [17] and even to extinction [18,19]. It can seriously threaten the persistence of wild taxa [20]. Crop-to-wild gene flow in apples has been shown to be responsible for a decrease in the fitness of populations of European wild apple in Germany [21]. According to some authors [22–27], hybridization between *M. sylvestris* and *M.* × *domestica* is very common, and there are no genetic barriers between *M. sylvestris* and *M.* × *domestica* [10,11,28]. On the other hand, some research performed at a local geographic scale and based on genetic analysis indicates that gene flow between *M. sylvestris* and *M.* × *domestica* has been rare [7,10,29,30]. Similarly, a significant number of hybrids between *M. sieversii* and cultivated apples have been found, suggesting frequent crop-to-wild gene flow for this species [31].

Crops and their wild relatives very often remain interfertile, which can lead to introgression if the two taxa remain geographically close or come into secondary contact [32]. Among the most direct negative consequences of crop-to-wild gene flow are the loss of wild population integrity [33] and the reduction of the fitness of wild species [20]. Gene flow is highly idiosyncratic and it would be very difficult to completely prevent the spread of crop genes [34]. Local human food production can directly influence gene flow from crops to wild plants [35] and therefore this phenomenon has been found in many edible tree species, e.g., between domesticated almond (*Prunus dulcis* (Mill.) D. A. Webb) and wild almond (*Prunus orientalis* (Mill.)) [36], between domesticated pear (*Pyrus communis* L.) and wild pear (*Pyrus pyraster* L. Burgsd.) [37], between different cherry species (*Prunus avium* L. and *Prunus fruticosa* Pall.) [38], between cultivated and wild apricots (*Prunus armeniaca* L.) [39], between cultivated and wild common walnut (*Juglans regia* L.) [40] and many other species. Hybridization has also been demonstrated in some typical forest tree species, e.g., between field elm (*Ulmus minor* Mill.) and Siberian elm (*Ulmus pumila* L.) [41] and between Canadian poplar (*Populus* × *canadensis* Moench.) and black poplar (*Populus nigra* L.) [42]. Accurate knowledge of the hybridization process is essential for planning conservation measures.

In order to ensure appropriate silvicultural treatment, it is essential to determine which morphological traits enable accurate identification of individual specimens. In nature, hybrids of European crab apple and the domesticated apple occur, as do feral cultivars which are morphologically very similar to hybrids. In this study, trees growing from the seed of the domesticated apple were considered feral cultivars. Feral cultivars show morphological characteristics of European crab apples, but according to their genetic structure, they are instead classified as domesticated apples. Viršček Marn and Stopar [6] found that 90% of adult trees had the morphological characteristics of their ancestors, i.e., the species from which the domesticated apple descended.

In morphological descriptions, European crab apple leaves are typically described as 4–10 cm long and 4–5 cm wide, with a non-pubescent underside [43]. The leaves of the

domesticated apple are typically described as longer than 11 cm and wider than 5.5 cm, with a pubescent underside [15]. The transition in morphological characteristics between European crab apple and the domesticated apple is ongoing, and so far, there are no clear morphological differences that would enable the reliable identification of European crab apple, hybrids or feral cultivars of the domesticated apple in the field. Many studies on the morphological characteristics of European crab apple place particular emphasis on the pubescence of the underside of the leaves [11,12]. As reported, pubescence alone does not appear to be a very effective means of distinguishing between crab apple trees and hybrids [11,12,44]. In order to facilitate the identification of European crab apple in nature, it is important to identify its typical distinctive morphological characteristics. Many individual trees identified as *M. sylvestris* may in fact represent various stages of hybridization between *M. sylvestris* and *M. domestica*. Precise identification requires very careful examination to determine whether a particular tree is pure *M. sylvestris*, a feral cultivar of *M.* × *domestica* or a hybrid between these two species. Comparison between morphological and genetic analyses of European crab apple and domesticated apple could provide a better understanding of the hybridization process and lead to new silvicultural measures which could improve the status of European crab apple in the future.

The research objectives were: (a) to determine the proportion of hybrids and feral cultivars of the domesticated apple in natural populations of European crab apple in Slovenia by means of genetic analysis and to determine the extent of ongoing hybridization between the two species; (b) to compare the results of morphological and genetic analyses of European crab apple and to determine the key morphological characteristics that could reliably differentiate between hybrids, European crab apple and feral cultivars of the domesticated apple; and (c) to examine the quality of the forest reproductive material (FRM) of European crab apple in a sample seed stand and, if necessary, to determine measures for improving it.

2. Materials and Methods

2.1. Area of Research and Sampling

We analyzed 107 putative European crab apple trees in Slovenia: 92 individuals from nine natural populations, five from a seed stand (representing the entire seed object) and 10 from a stand of unnatural origin. Additionally, 18 domesticated apples (*M.* × *domestica*) and two Japanese flowering crab apples (*Malus floribunda* van Houtte) were analyzed as out-group genotypes. A total of 127 trees were analyzed (Figure 1). In selecting and sampling putative European crab apple, we paid particular attention to the shape and size of the leaves and hairiness of the undersides of the leaves.

The domesticated apple includes trees that belong to a group of older cultivars (local names: 'Pisani Kardinal', 'Dolenjska Voščenka', 'Boskopski Kosmač', 'Štajerski Pogačar', 'Sladka Jabka', 'Kanadka', 'Carjevič') as well as newer varieties (local names: 'Fuji', 'Gala', 'Elstar', 'Majda', 'Lonjon', 'Topaz') [45]. Trees from the seed stand were selected as samples to examine the quality of European crab apple FRM. The seed stand is a certified European crab apple [46] seed stand at Tolsti vrh in Slovenske Konjice (46.18 N, 15.26 E), identification number 213. This is the only registered seed stand of European crab apple in Slovenia.

2.2. Morphological Analysis

Samples from all 127 trees were included in the morphological analysis. From the short shoots, we collected 50 undamaged and fully developed leaves per tree. Tree sampling took place in the summer of 2016. All leaves were dried and herbarized. After herbarization, they were scanned with a Microtek ScanMaker 9800XL Plus scanner (Microtek International, Hsinchu, Taiwan) and Microtek ScanWizard Pro (version 5.20, Hsinchu, Taiwan) scanning software. The measured morphological characteristics of the leaves were leaf area (A), leaf circumference (LC), W/LL ratio (W/LL), shape coefficient (SC), leaf length (LL), leaf width (W), position of maximum leaf width (PW%), width at 70% and 80% of leaf length (W70%, W80%), angle at 5% and 15% of leaf length (A5%, A15%) and petiole length (PL). The

shape coefficient is a numerical value that ranks the leaf shape between circular (shortest circumference for a given area) and filiform (longest circumference for a given area). The measured morphological characteristics are shown in Table 1. The morphometric analysis was performed with Winfolia software Pro 2014 from Regent Instruments (version 2014, Quebec, QC, Canada).

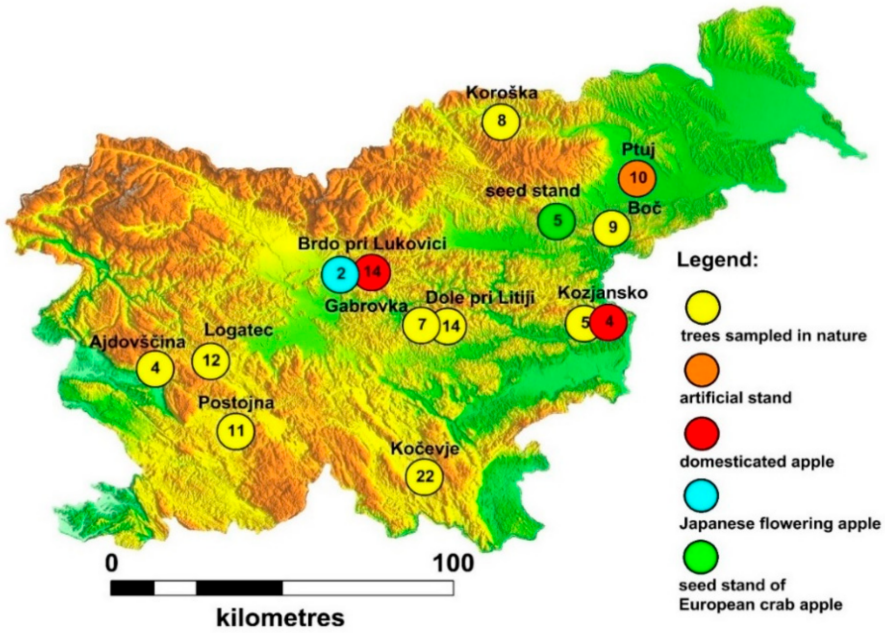

Figure 1. Locations of the analyzed populations of European crab apple in Slovenia with the number of analyzed trees shown for each population. The yellow circles indicate natural populations, the orange circle indicates a stand of unnatural origin (Ptuj population), the red circles indicate sampling locations of domesticated apple, the blue circle indicates the sampled Japanese flowering crab apple trees and the green circle indicates the European crab apple seed stand.

Table 1. Measured morphological traits of leaves.

Morphological Trait	Abbreviation
Leaf area	A (cm^2)
Leaf circumference [1]	LC (cm)
Leaf width/Leaf length ratio [1]	W/LL
Shape coefficient [1]	SC
Leaf length	LL (cm)
Leaf width	W (cm)
Position of max. leaf width (%)	PW%
Width at 70%	W70% (cm)
Width at 80%	W80% (cm)
Angle at 5% length	A5% (°)
Angle at 15% length	A15% (°)
Petiole length	PL (cm)

[1] morphological traits excluded from principal component analysis (PCA) analysis, as these are synthesis variables.

Principal component analysis (PCA) based on the morphological traits of the leaves was performed. The aim of this analysis was to combine variables (morphological traits) into independent synthetic variables that explained most of the variation. Varimax rotation was used in the method. PCA analysis was performed using SPSS (version 21.0, Armonk, NY, USA) [47]. Nine leaf morphological traits were included in the analysis. Due to multicollinearity, some traits were excluded from the analysis (highly correlated variables) (Table 1).

Cluster analysis was performed for morphological identification of the analyzed specimens. First, a dissimilarity distance matrix was created using DARwin software (version 6.0, Montpellier, France) [48], and then a dendrogram was constructed based on this matrix. We used Ward's clustering method of hierarchical grouping. We performed a nonparametric Kruskal–Wallis *H* test in SPSS (version 21.0, Armonk, NY, USA) [47] to determine if there were statistically significant differences between groups.

2.3. Genetic Analysis

All 127 trees were also included in the genetic analysis. For the purpose of the genetic analysis, the leaves were immediately stored on silica gel. A Qiagen set (DNeasy® Plant Mini Kit) was used to isolate DNA from the leaves [49]. DNA concentration was measured with a NanoVue spectrophotometer GE Healthcare Life Sciences (Cytiva, Cardiff, UK). DNA was stored at −20 °C. We analyzed 10 microsatellites, which were combined into three multiplex PCR reactions (Table 2). All microsatellites had already been used on European crab apple in previous research [15–20]; see Table 2. The PCR profiles (temperature, duration and number of cycles) for individual multiplex reactions were summarized according to Patocchi et al. [50]. PCR reactions were performed in an Mastercycler nexus thermal cycler (Eppendorf, Hamburg, Germany) whereas PCR products readings were done with an ABI3130XL automated sequencer (Applied Biosystems).

We used the Bayesian clustering method (also called Bayesian analysis of population structure) in the STRUCTURE 2.3.4 program. We genetically identified specimens and assigned individual trees to groups [51]. The Bayesian sorting parameters in STRUCTURE 2.3.4. were as follows: 20,000 burn-in periods and 10,000 Markov chain Monte Carlo replicates. We used the admixture model with correlated alleles. Analyses were run for population structure models, assuming K = 1 to K = 5 distinct clusters. Evanno's delta K (ΔK) statistic, which is designed to identify the most relevant number of clusters by determining the number of clusters beyond which there is no further increase in likelihood [52], was greatest for K = 2 (ΔK = 28,955) (see at Figure S1, Table S1). To distinguish between groups of trees, we determined a coefficient Q_E, which expresses the proportional assignment of an individual to each cluster [28,30,44]. Based on the comparison of the morphological and genetic data set within the entire collection of trees, a coefficient of admixture was chosen. The correlation was highest when a threshold $Q_E > 0.8$ was used. The hybrid coefficient $Q_E > 0.8$ represents the group of European crab apple, $0.2 < Q_E < 0.8$ the group of hybrids and $Q_E < 0.2$ the group of trees defined as domesticated apples.

Table 2. Loci studied, allele size range, nucleotide sequence, repeated motif, number of alleles per locus, PIC values (polymorphic information content) and studies in which a particular locus was previously studied. (* PCR multipleks 1, ** PCR multipleks 2, *** PCR multipleks 3).

Locus Name	Allele Size Range	Nucleotide Sequence of the forward and Reverse Primer (5′→3′)	Repeated Motif	Number of Alleles per Locus	PIC Value	Studies in which the Locus Was Previously Studied
CH01h10 *	94–114	tgc aaa gat agg tag ata tat gcc a agg agg gat tgt ttg tgc ac	(ag)21	5	0.91	[10,28,44,53,54]
CH04c07 *	98–135	ggc ctt cca tgt ctc aga ag cct cat gcc ctc cac taa ca	(ga)	8	0.87	[10,28,53]
CH01h01 *	114–134	gaa aga ctt gca gtg gga gc gga gtg ggt ttg aga agg tt	(ag)25.5	6	0.85	[10,28,44,53]
CH02b03b **	77–109	ata agg ata caa aaa ccc tac aca g gac atg ttt ggt tga aaa ctt g	(ga)22	8	0.90	[23,54]
CH02b12 **	101–143	ggc agg ctt tac gat tat gc ccc act aaa agt tca cag gc	(ga)26	13	0.92	[44,53]

Table 2. *Cont.*

Locus Name	Allele Size Range	Nucleotide Sequence of the forward and Reverse Primer (5′→3′)	Repeated Motif	Number of Alleles per Locus	PIC Value	Studies in which the Locus Was Previously Studied
MS06g03 **	154–190	cgg agg gtg tgc tgc cga ag gcc cag ccc ata tct gct	(ga)	9	0.93	[23,53]
CH02b07 ***	180–202	cca gac aag tca tca caa cac tc atg tcg atg tcg ctc tgt tg	(ga)	7	0.90	[10,54]
CH02c11 ***	219–239	tga agg caa tca ctc tgt gc ttc cga gaa tcc tct tcg ac	(ga)	7	0.70	[10,28,54]
CH03d11 ***	115–181	acc cca cag aaa cct tct cc caa ctg caa gaa tcg cag ag	(ga)	6	0.91	[54]
CH02a10 ***	143–177	atg cca atg cat gag aca aa aca cgc agc tga aac act tg	(ga)	6	0.94	[54]

Genetic variability indicators were calculated in GenAlex 6.0 [55] for all groups of trees. We calculated the range of allele lengths (RA), the number of different alleles by loci (A), the effective number of alleles (A_E), total genetic diversity (H_T), inbreeding coefficient (F_{IS}), fixation index (F_{ST}), and observed (H_O) and expected (H_E) heterozygosity.

We were also interested in how morphological and genetic classifications matched with respect to trees that are commonly considered European crab apples based on field identification using the most commonly used identification traits. For this purpose, classification based on morphological data was compared with classification based on genetic data for the 92 trees sampled in the field and five trees sampled in the seed stand. We also calculated the Spearman correlation coefficient between genetic and morphological classification in order to determine the extent to which these two classifications correlated.

3. Results

3.1. Morphological Identification of Trees

Using principal component analysis (PCA), we gained insight into the basic patterns of distribution and grouping of individual trees. The first three components explain 88.10% of the total variance. The first component explains 43.65% and the second 25.96% of the total variance (Figure 2). The loading plots for each morphological character are available in Figure S2. The first component is most influenced by the leaf width at 70% and 80% of the leaf surface length (W70% and W80%) and the second at 5% and 15% of the leaf surface length (A5% and A15%) (Table 3). Figure 2 shows domesticated apple trees (red dots), which are characterized by larger and more elongated leaves of an elliptical shape and a more rounded leaf base. In the opposite direction (yellow dots) there are trees from natural populations. These trees have smaller leaves; the shape of the leaves is rounder with a wedge-shaped bottom of the leaf surface. The transition in morphological characteristics is ongoing between the two species.

The 127 analyzed trees were grouped using Ward's clustering method of hierarchical grouping in DARwin software (version 6.0, Montpellier, France) 6.0 software according to the morphological similarities in the dendrograms, as shown in Figure 3. Group 1 (yellow) included a group of 61 trees (48.03%), consisting mainly of trees from natural populations. Group 2 (orange) consisted of 36 trees (38.34%), which were morphologically between wild and domesticated apples and could be treated as a hybrids and domesticated apples. The most mixed was Group 3 (red), consisting of 30 trees (23.62%), of which 18 were domesticated apple, two were Japanese flowering crab apple and 10 were European crab apple trees collected in the natural environment.

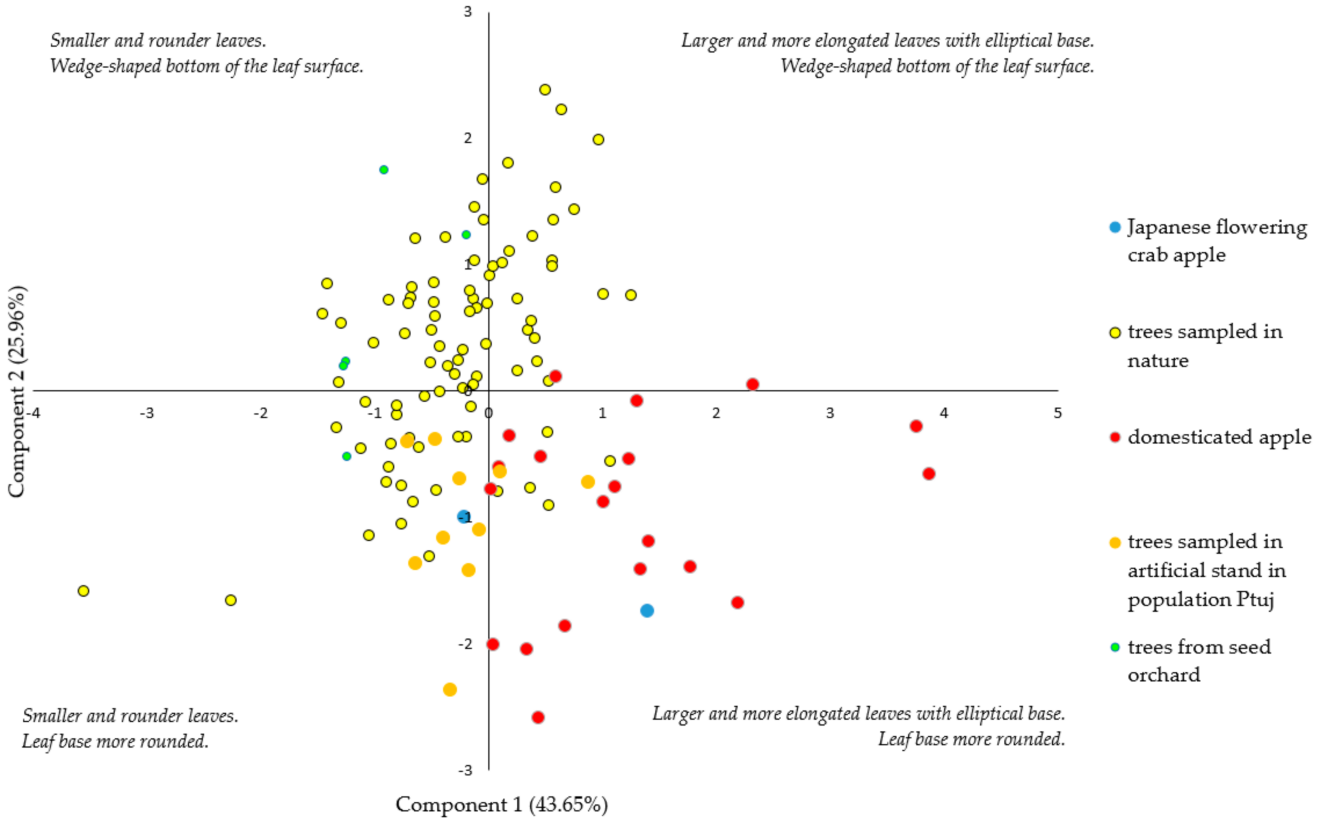

Figure 2. PCA analysis based on morphological traits of leaves: dispersion of individual trees along the first and second components. The yellow dots represent trees sampled in the natural environment, the orange dots represent trees from the artificial stand from the Ptuj population, the red dots represent domesticated apple trees, the green dots represent trees from the seed stand and the blue dots represent Japanese flowering crab apple trees.

Table 3. Correlations between morphological characteristics and the first three synthetic components for trees.

Morphological Characteristics	Component 1	Component 2	Component 3
Leaf area (A)	0.944	−0.189	0.093
Leaf length (LL)	0.739	−0.597	0.001
Leaf width (W)	0.305	0.017	0.916
Max. leaf width (PW%)	−0.101	−0.025	0.928
Width at 70% (W70%)	0.933	0.183	0.221
Width at 80% (W80%)	0.900	0.189	0.204
Angle at 5% length (A5%)	−0.028	0.949	−0.048
Angle at 15% length (A15%)	0.008	0.986	0.050
Petiole length (PL)	0.696	−0.184	−0.228
Eigenvalues	9.92	2.33	1.66
% of variability (%)	43.65	25.96	18.48
Cumulative variability (%)	43.65	69.62	88.10

Table 4 presents the average values and variation coefficients based on individual morphological characteristics for certain groups of sample trees, which were determined using DARwin software (version 6.0, Montpellier, France). Trees classified as Group 2 had the roundest shaped leaves with a wedge-shaped leaf base (large angle at 5% and 15% of leaf length) (A5% and A15%), in contrast to Group 3, with the most rounded leaf base and a more elongated leaf shape. Group 1 trees had slightly more elongated leaves than the putative hybrids from Group 2. Trees in Group 2 had the shortest leaves (LL = 5.66 cm), followed by Group 1 (LL = 6.07 cm) and Group 3 (LL = 7.62 cm). The length of the petiole

(PL) was longest in Group 3 (SL = 3.82 cm), followed by Group 2 (SL = 3.20 cm) and Group 1 (SL = 2.95 cm).

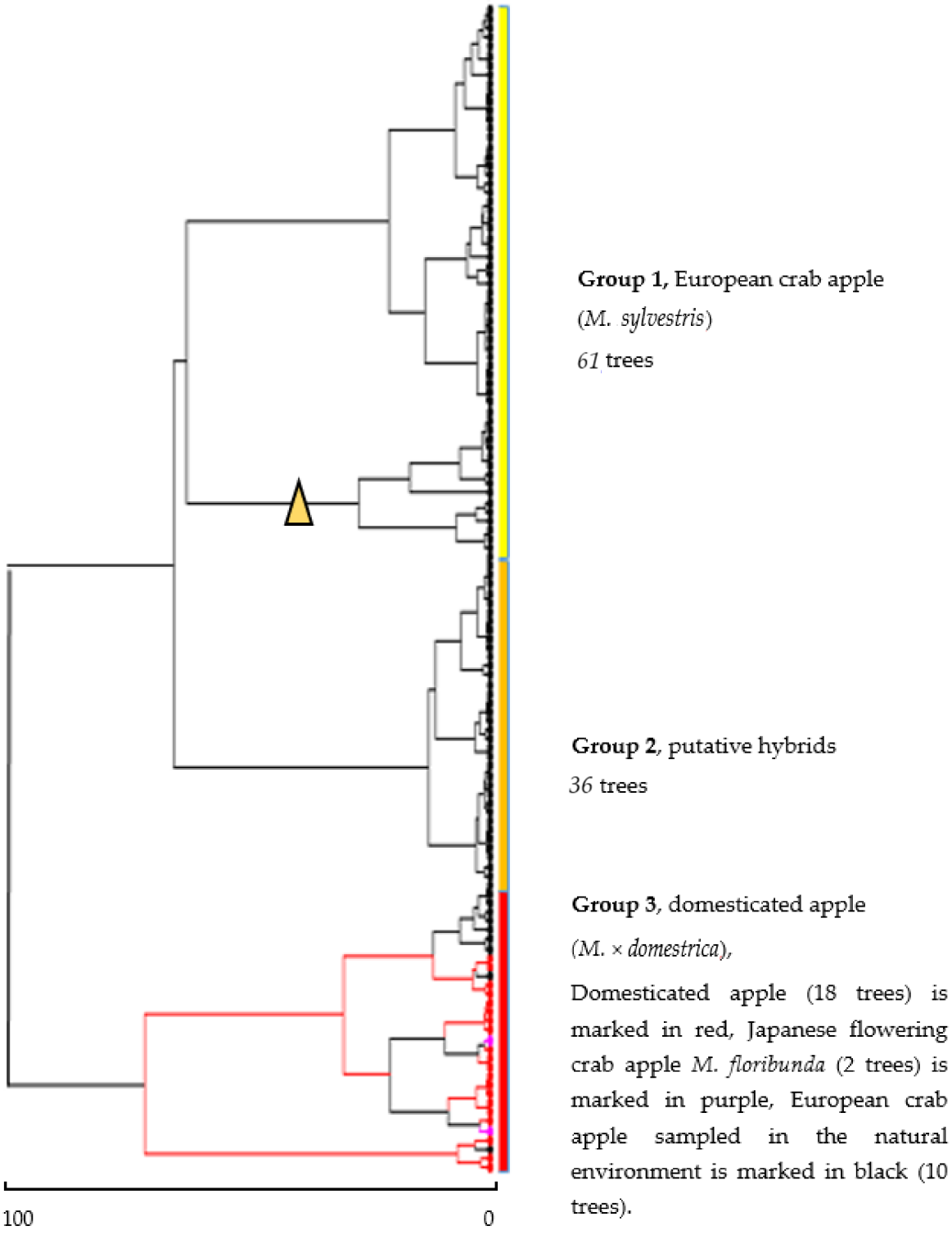

Figure 3. Ward's clustering dendrogram of the 127 analyzed trees.

Table 4. The average values, coefficients of variation (CV (%)) and mean squared error by individual morphological characteristics for groups of sample trees determined using the DARwin software (version 6.0, Montpellier, France) using Ward's clustering method of hierarchical grouping. The result of the H-test (Kruskal–Wallis test) for individual morphological traits (* $0.01 < p < 0.05$; *** $p < 0.001$) is presented.

	Group 1			Group 2			Group 3			*p*
	Average	CV (%)	MSE	Average	CV (%)	MSE	Average	CV (%)	MSE	
A (cm^2)	16.04	20.03	3.21	17.01	13.42	2.28	23.51	26.53	6.24	<0.001 ***
LC (cm^2)	17.25	10.45	1.80	16.99	6.33	1.08	25.24	18.77	4.74	<0.001 ***
W/LL	0.60	10.37	0.60	0.74	7.76	0.60	0.44	13.53	0.6	<0.001 ***
FC	0.67	8.46	0.06	0.74	5.47	0.04	0.50	22.24	0.11	<0.001 ***
LL (cm)	6.08	10.98	0.67	5.66	7.48	0.42	7.62	11.95	0.91	<0.001 ***
W (cm)	4.57	9.74	0.45	4.54	5.15	0.23	4.41	14.17	0.62	<0.001 ***
PW%	44.67	15.95	7.13	45.41	5.15	2.34	38.17	15.71	5.99	0.005 *
W70% (cm)	3.10	12.67	0.39	3.45	8.55	0.30	3.61	16.97	0.61	<0.001 ***
W80% (cm)	2.37	15.67	0.37	2.72	11.33	0.31	2.86	18.47	0.53	<0.001 ***
A5% (°)	57.91	12.06	6.99	67.95	6.28	4.27	53.55	13.50	7.23	<0.001 ***
A15% (°)	52.22	7.00	3.65	59.09	4.35	2.57	48.72	9.54	4.65	<0.001 ***
SL (cm)	2.95	19.88	0.59	3.20	10.13	0.32	3.82	16.51	0.63	<0.001 ***

3.2. Genetic Identification of Trees

The 127 sampled trees were analyzed using the Bayesian method in the STRUCTURE) program (version 2.3.4., Oxford, MS, USA) [51] based on 10 microsatellites. Figure 4 displays the genetic admixture of individual analyzed trees. Based on the admixture coefficient $Q_E > 0.8$, 71 trees (55.90%) were classified as Group 1 (European crab apple), 25 trees (19.68%) as Group 2 (hybrids) and 31 trees (24.40%) as Group 3 (domesticated apples).

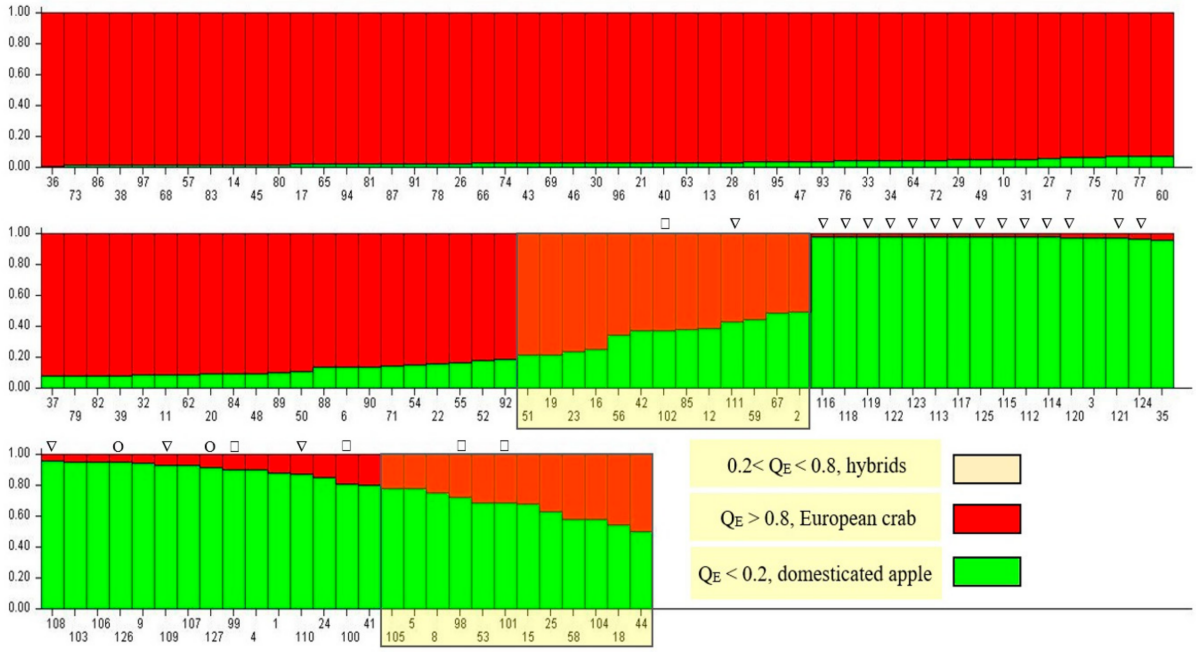

Figure 4. The genetic admixture of the 127 analyzed specimens of European crab apple, domesticated apple and Japanese flowering crab apple. The graph was created in the STRUCTURE 2.3.4 program based on the analysis of 10 microsatellite loci; the number of classes (K) = 2; the Y-axis contains the admixture coefficient; the red part of the column represents the probability of each individual being assigned to the genetic group of European crab apple; and the green part of the column represents the probability of each individual being assigned to the genetic group of the domesticated apple. Markings at the top of the bar are as follows: □ trees from seed orchard, ∇ domesticated apple, O Japanese flowering crab apple.

Genetic variability indicators were calculated for the entire group of trees sampled in nature and for the genetically determined Group 1 (the group of European crab apples),

Group 2 (group of hybrids) and Group 3 (domesticated apples) (Table 5). The average number of alleles (A) within all trees sampled in nature was A = 22.10. In Group 1, A = 19.70; in Group 2, A = 14.00; and in Group 3, A = 11.90. The average number of effective alleles (A_E) was highest in Group 1 (A_E = 6.77), followed by Group 2 (A_E = 3.39) and Group 3 (A_E = 2.67). As expected, total genetic diversity (H_T) was highest in Group 1 (H_T = 0.87), followed by Group 2 (H_T = 0.86) and Group 3 (H_T = 0.81). The largest total genetic diversity in the group of trees sampled in nature had a locus of CH02a10 (H_T = 0.93) and the lowest had a locus of CH02c11 (H_T = 0.71). The fixation index value (F_{ST}) for trees sampled in nature amounted to F_{ST} = 0.08. If the group is divided into Groups 1, 2 and 3, the F_{ST} index increases to F_{ST} = 0.16 for Group 1 (European crab apple), F_{ST} = 0.26 for Group 2 (hybrids) and F_{ST} = 0.38 for Group 3 (domesticated apple trees). These high values imply prominent genetic differentiation between classes, taking into account the fixation of different alleles.

Table 5. Indicators of genetic variability for the whole group of European crab apple trees sampled in nature and for the groups of European crab apple, hybrids and domesticated apple trees after determining the identity of the trees with the STRUCTURE 2.3.4 program *.

Trees Sampled in Nature									
	N	***SR***	***A***	A_E	H_T	F_{IS}	F_{ST}	H_O	H_E
CH01h10	95	88–166	23	6.63	0.92	0.13	0.09	0.72	0.83
CH04c07	96	88–190	21	5.79	0.89	0.16	0.10	0.68	0.81
CH01h01	92	100–135	15	4.93	0.85	0.34	0.07	0.53	0.79
CH02b03b	97	71–110	21	6.59	0.91	0.18	0.08	0.68	0.83
CH02b12	95	110–174	29	6.84	0.93	0.35	0.10	0.54	0.84
MS06g03	96	148–198	25	6.74	0.93	0.34	0.11	0.55	0.83
CH02b07	97	92–127	15	6.33	0.90	−0.03	0.07	0.86	0.83
CH02c11	97	195–246	22	3.08	0.70	−0.39	0.05	0.93	0.67
CH03d11	97	95–179	25	7.70	0.92	0.02	0.07	0.84	0.86
CH02a10	96	133–176	25	7.27	0.93	0.26	0.09	0.63	0.85
average			22.1	6.19	0.89	0.14	0.08	0.70	0.81
Group 1 (European Crab Apple *)									
CH01h10	70	88–166	21	7.20	0.89	0.00	0.19	0.72	0.72
CH04c07	71	94–190	17	6.40	0.86	−0.02	0.19	0.71	0.70
CH01h01	70	100–135	15	5.70	0.83	0.11	0.17	0.62	0.69
CH02b03b	71	73–109	19	6.90	0.89	0.01	0.20	0.70	0.71
CH02b12	69	110–174	24	7.00	0.91	0.18	0.15	0.63	0.77
MS06g03	70	150–198	22	7.10	0.92	0.21	0.20	0.59	0.74
CH02b07	71	92–127	14	6.50	0.86	−0.05	0.13	0.79	0.76
CH02c11	71	195–246	17	5.80	0.71	−0.50	0.08	0.98	0.66
CH03d11	71	95–179	25	7.50	0.92	−0.03	0.14	0.82	0.79
CH02a10	71	133–176	23	7.60	0.93	0.28	0.14	0.57	0.80
average			19.70	6.77	0.87	0.02	0.16	0.71	0.73
Group 2 (Hybrids *)									
CH01h10	23	88–117	15	3.64	0.91	−0.12	0.23	0.78	0.69
CH04c07	23	88–125	14	3.28	0.86	−0.25	0.24	0.83	0.66
CH01h01	22	104–133	9	2.58	0.80	0.35	0.45	0.29	0.44
CH02b03b	24	71–110	15	3.79	0.90	0.06	0.34	0.56	0.59
CH02b12	24	110–151	19	4.20	0.92	0.08	0.22	0.65	0.71
MS06g03	24	148–189	12	3.09	0.89	0.01	0.28	0.64	0.65
CH02b07	24	98–127	12	3.88	0.87	−0.22	0.23	0.82	0.67
CH02c11	24	195–236	12	2.41	0.64	−0.52	0.14	0.84	0.55
CH03d11	24	95–129	16	3.58	0.84	−0.26	0.20	0.85	0.68
CH02a10	24	139–169	16	3.46	0.92	−0.04	0.26	0.71	0.68
average			14.00	3.39	0.86	−0.09	0.26	0.70	0.63
Group 3 (Domesticated Apple *)									
CH01h10	30	88–111	10	2.46	0.83	−0.38	0.49	0.59	0.42
CH04c07	30	92–121	12	2.57	0.81	−0.03	0.55	0.38	0.36
CH01h01	28	104–131	8	2.17	0.83	−0.07	0.48	0.46	0.43
CH02b03b	30	71–103	9	2.54	0.84	−0.22	0.42	0.59	0.49
CH02b12	29	110–155	17	3.17	0.87	−0.02	0.45	0.49	0.48
MS06g03	30	153–189	12	2.73	0.82	−0.25	0.37	0.65	0.52
CH02b07	30	92–127	10	2.73	0.78	−0.39	0.32	0.74	0.53
CH02c11	30	195–238	13	2.36	0.68	−0.68	0.18	0.94	0.56
CH03d11	30	108–179	14	2.93	0.84	−0.38	0.26	0.85	0.62
CH02a10	29	139–176	14	2.99	0.85	−0.38	0.27	0.86	0.62
average			11.90	2.67	0.81	−0.28	0.38	0.65	0.50

N, number of trees; SR, size range; A, number of different alleles; A_E, effective number of alleles; H_T, total genetic diversity; F_{IS}, inbreeding coefficient; F_{ST}, fixation index; H_O, observed heterozygosity; H_E, expected heterozygosity.

The inbreeding coefficient (F_{IS}) was F_{IS} = 0.02 in Group 1, F_{IS} = −0.09 in Group 2 and F_{IS} = −0.28 in Group 3. By analyzing the trees, we found that there were two clearly separated gene pools (Group 1 and Group 3) representing European crab apple and domesticated apple trees, respectively, with many trees having a mixture of genes from both species. We also found high values of genetic distance between European crab apple and domesticated apple (D = 0.489), which together with the fixation index value (F_{ST} = 0.40) suggest significant genetic differentiation between the two species.

The results of the genetic admixture analysis of 18 domesticated apple trees are as follows: older cultivars (local names: 'Boskopski Kosmač', 'Sladka Jabka', 'Kanadka') demonstrated a slightly higher share of gene admixture with European crab apple (Q_E = 0.127; Q_E = 0.068; Q_E = 0.042) than younger cultivars (local names: 'Elstar', 'Lonjon', 'Gala') (Q_E = 0.016; Q_E = 0.016; Q_E = 0.015).

3.3. Comparison of Morphological and Genetic Identification of Trees

We compared the classifications of 92 trees sampled in natural populations from the natural environment and five trees from the seed stand based on morphological (Ward's clustering method of hierarchical grouping in the DARwin software (version 6.0, Montpellier, France)) and genetic data (Bayesian method in STRUCTURE 2.3.4) (Table 6). The Spearman correlation coefficient between genetic and morphological data was ρ = 0.502. We found that 40.20% of trees were identified as European crab apple based on both genetic and morphological classification. In total, 70.10% of trees were classified into Group 1 based on genetic traits (defined as the European crab apple) (of which, based on morphological analysis, 40.20% of trees fell into Group 1 and 29.90% into Group 2). Twenty-one specimens (21.64%) were genetically classified into Group 2 (hybrids), of which, based on morphological analysis, 12.37% were classified as Group 1 (European crab apple), 7.21% as Group 2 (hybrids) and 2.06% as Group 3 (domesticated apple) trees. Among the analyzed trees, there were also six (6.18%) trees sampled in the natural environment which genetically belonged to the group of domesticated apples but, based on morphological characteristics, fell into the European crab apple classification. We identified these trees as possible feral cultivars.

Table 6. Comparison between tree classifications in three identification groups based on genetic data with identification based on morphological data. The analysis included 97 trees, which were sampled in nature and the seed stand.

	Tree Identification Based on Genetic Data (Bayesian Method)		**Tree Identification Based on Morphological Data (Ward Method)**			
	Trees	**(%)**	**Group 1 (European Crab Apple Trees) (%)**	**Group 2 (Hybrids) (%)**	**Group 3 (Domesticated Apple Trees) (%)**	**Total (%)**
Group 1 (European crab apple)	68	70.10%	39 (40.20%)	29 (29.90%)	0 (0.00%)	70.10%
Group 2 (hybrids)	21	21.64%	12 (12.37%)	7 (7.21%)	2 (2.06%)	21.64%
Group 3 (domesticated apple)	8	8.26%	6 (6.18%)	0 (0.00%)	2 (2.06%)	8.26%
Total	97	100				

Genetic analysis of 10 trees from the stand of unnatural origin (Ptuj population) showed that four trees were classified into Group 3, three trees into Group 2 and three trees into Group 1. Based on morphometric analysis, six trees were classified into Group 3, of which three trees were also genetically classified into Group 3, two trees into Group 1 and

one tree into Group 2. Of the four trees morphologically classified into Group 1, one tree was classified into Group 1, two trees into Group 2 and one tree into Group 3.

All five trees from the seed stand were classified into Group 1 according to their morphological characteristics. Genetic analysis classified two trees into Group 3 and three trees into Group 2. The two trees are likely feral cultivars of domesticated apple—according to genetic classification, they belong to the group of domesticated apple, whereas morphologically the two trees are classified into Group 1 (European crab apple).

4. Discussion

4.1. Admixture Analysis and Genetic Identification of Trees

When comparing classifications based on genetic and morphological data, we included only European crab apples from natural populations (92 trees from natural populations and five trees from a seed stand). Of the trees from natural populations, 70.10% formed a homogeneous group, which with the help and support of morphometric analysis could be called the pure crab apple group. This share is comparable to research from Scotland (70.00% European crab apple) [56], Germany and Luxembourg (82.80% European crab apple) [30] and the general European area (72.90% European crab apple) [35]. The share of hybrids (Group 2) in populations in Slovenia was 21.64%, which is also comparable to recent research from Scotland (26.00% hybrids) [56] and Germany and Luxembourg (13.90% hybrids) [30] and correlates with European research reporting 23.10% hybrids among analyzed trees [35]. All of the above-mentioned studies also report a smaller proportion of trees exhibiting characteristics of European crab apple according to morphological characteristics but which were genetically classified as domesticated apple (feral cultivars). The proportion of feral cultivars in other studies ranged from 3.00% to 6.30% [28,30,35,56], whereas in our study this proportion was 6.18%. Direct comparison of research results should be done with caution, as the number of trees analyzed and sampling methods vary slightly. However, the similarities between the five studies are most likely not a coincidence. Thus, it is evident that gene flow and a hybridization process between European crab apple and domesticated apple is occurring in all the populations we have studied. Since there are mechanisms that prevent hybridization between the two species to some extent, hybridization is relatively limited (the percentage of hybrids ranges from 13.90% to 26.00%), but it is still effective in the long term and should not be overlooked. As crop-to-wild hybridization and introgression are known to be major threats to the integrity of endangered wild populations of European crab apple, all measures should be taken to prevent them in the future if the conservation of a species' gene pool is our main goal. On the other hand, from a species conservation point of view, it would also be interesting to know whether hybrids have lower or higher fitness than European crab apple trees. This was not the focus of our study, but other studies have suggested that hybrids in a natural environment are unlikely to show reduced fitness in the early growth stages [20,57]. Certain unexplained flaws of genetic inheritance are also indicated by the very similar proportion of feral cultivars in different studies (ranging from 3.00% to 6.50%), which would be interesting to investigate in more detail in the future.

Genetic barriers to hybridization between wild and cultivated apples have not been found [26]. Some authors report that a significant disjunction in flowering time observed in the field and the geographical distance between wild and cultivated trees are the most important factors in the absence of hybridization [10,57]. In the case of European crab apple, this is not obvious, as the trees tend to grow singly, usually on forest edges and clearings adjacent to an agricultural area, in close proximity to widespread cultivated crops of domesticated apple. In a very geographically varied landscape with rapid changes in elevation and relief, the ecological conditions are very diverse and therefore the differences in flowering between European crab apple and domesticated apple are less pronounced and may even overlap. Important pollinators of apples are also able to travel several kilometers to pollinate flowers [58] and therefore high pollen dispersal distances have negative effects on hybridization frequency [58].

The results of the analysis of genetic admixture in domesticated apple species are consistent with the results of research from Germany and Luxembourg [30]. Namely, older cultivars showed a higher share of gene mixing with European crab apple (Q_E = 0.09) than newer ones (Q_E = 0.06). The differences between older and newer cultivars were even greater in our study. This finding seems logical and is in line with expectations. Indeed, a longer coexistence period necessarily leads to greater interaction between species. When barriers to sexual reproduction are not 100% tight, as in our case, interspecific interbreeding and mixing of genes occurs.

4.2. Comparison of Morphological and Genetic Traits

Morphological analysis (PCA) revealed significant differences in the shape of the leaves between European crab apple and domesticated apple. Domesticated apple typically had an elongated and elliptical leaf shape with larger leaves (an average of 7.63 cm long), and the bottom of the leaf surface was rounded. The leaves were usually hairy on the underside. European crab apple typically had ovate leaves with a wedge-shaped leaf base and smaller leaves (an average of 6.08 cm long). Hybrids had rounded leaves (an average of 5.66 cm long) with a wedge-shaped bottom of the leaf surface. These differences have not been addressed or discussed in previous research.

We compared classifications based on morphological and genetic data for 92 trees from natural populations and five trees from a seed stand. The trees identified as European crab apple on the basis of morphological and genetic data were scattered throughout the populations. In both morphological and genetic classifications, Group 1 represented European crab apple, Group 2 represented hybrids and Group 3 represented domesticated apple. Genetic analysis identified 70.10% of trees as European crab apple. This proportion is comparable to two studies of European crab apple from Scotland, in which 68.00% [56] and 70.00% of trees [59] were correctly identified. Classification based on genetic data identified more trees belonging to the European crab apple group (70.10%) than that based on morphological data (58.75%). Larsen et al. [10] and Coart et al. [44] reported similar findings in studies in Denmark and Belgium. However, Gross et al. [25] reported 20.00% incorrectly classified European crab apple trees, which were either hybrids or feral cultivars of domesticated apple. In contrast, Reim et al. [28] found that based on morphological data, a larger proportion of trees was classified into the European crab apple group than on the basis of genetic analysis. The high variability of morphological traits is a familiar phenomenon, and inconsistency with genetic data has been described several times [60,61]. Spontaneous hybridization between Rosaceae fruit crops and their wild relatives has led to intermediate phenotypes that are known to occur throughout Europe, where wild populations have been in contact with cultivated genotypes for centuries [44]. Reverse hybridization of first-generation specimens with parent plants results in offspring that are far more similar to one of the parent species. When hybrids are crossed again with the parent population of European crab apple, many morphological traits are no longer associated with the parent population but are the result of simple genetic inheritance [10].

In our research, a portion of the morphologically classified European crab apples were genetically classified into the group of domesticated apple trees (6.18%). These were in fact feral cultivars of domesticated apple, in which the typical characteristics of the species from which the domesticated apple developed were conspicuous. Domesticated apple is a hybrid between different species of the genus *Malus* [13–16] and in some cases the genes of feral cultivars are so strong that the specimen cannot be identified as domesticated apple on the basis of morphological traits only. Such trees cannot be identified in nature and are the most problematic specimens in preserving the European crab apple gene pool.

The results of the analysis of trees from the stand of unnatural origin in the Ptuj population confirmed the previous assumptions [62] that the sampled stand from Ptuj does not consist of genetically pure European crab apple trees. The genetic structure of the Ptuj population was heterogeneous, and the composition of this population was extremely diverse from a genetic point of view. The stand is of artificial origin, and it is

clear that seedlings which have been introduced into the forest area are not genetically suitable and are most likely domesticated apple seedlings. The most commonly used seed for apple seedlings is from the fruits of trees recognized by nurseries as European crab apple. European crab apple seeds have better germination than the seeds of domesticated apple [45], and rootstocks in nurseries are thus obtained from the seeds of trees that are identified as European crab apple. If seeds were collected from several different trees, it is of course possible that some of the trees would be feral cultivars or hybrids. In order to avoid such situations in the future, it is necessary to follow recommendations for obtaining appropriate FRM. The high potential for crop-to-wild gene flow in apples needs to be considered in the implementation of in situ and ex situ actions for the conservation of the genetic resources of wild apple [23].

4.3. Implications for the Production of Forest Reproductive Material

We found that the analyzed trees from the seed stand were not genetically suitable, as they contained a large proportion of the domesticated apple genome. In addition, the number of seed trees alone was too small to provide sufficient genetic diversity and adequate adaptive potential for the forest reproductive material. The recommended number of trees for maintaining the adaptive potential of the species and sufficient variability of populations is 30 to 50 in most tree species [63]. In the future it will be important to consider increasing the number of trees and seed stands of European crab apple in Slovenia.

In addition, adult trees in the European crab apple seed stand grow in the immediate vicinity of domesticated apple trees, which are very likely also pollinators of European crab apple. Based on their research results, Graudal et al. [64] stated that at least a 500-m-wide strip with no other trees that could be a source of pollen for European crab apple pollination is required around European crab apple seed trees. This is not assured in the case of the analyzed seed stand. The production of European crab apple seedlings from uncontrolled and unsupervised trees is not recommended [54,58,65]. Reim et al. [58] even suggest that European crab apple seedlings should be created only by controlled crossing to ensure genetically pure European crab apple, but such a measure is costly.

For the long-term preservation of European crab apple, it is necessary to introduce genetically pure European crab apple trees into the forest area. Populations need to be protected by reducing interspecific gene flow and replanting genotypes free from introgression [20]. Only the use of genetically diverse and pure seed will enable sufficient protection of the species' gene pool. The most appropriate way to obtain suitable European crab apple FRM would be to establish a suitable European crab apple seed stand, or several stands, which fully meet the abovementioned conditions. Another less convenient solution would be to identify suitable (and genetically verified) groups of trees that could be used in the event that seed stands of European crab apple do not bear fruit. As a last resort, a network of plus trees could be established, taking into account the specific characteristics of European crab apple discussed in this study. The most important step in establishing groups of trees or a network of European crab apple plus trees is the selection of high-quality trees. All trees determined as seed trees or plus trees should be genetically analyzed and certified as European crab apple trees.

The most suitable areas for the establishment of a European crab apple seed stand are those with a high degree of forest cover and a low human population, where the probability of the presence of domesticated apple trees is low. In our research, we highlight the area of Kočevje as the most suitable area for the establishment of a new European crab apple seed stand, as it is one of the largest preserved forest landscapes in Central Europe.

5. Conclusions

We can conclude that European crab apple is a tree species with extremely complex morphological characteristics and genetic structure. Among the trees identified in nature as putative European crab apple, only 70.10% were also confirmed to be European crab apple trees through genetic analysis. When comparing identification based on genetic

and morphological data, only 40.20% of trees were classified as European crab apple by both methods. The results indicate the difficulty and unreliability of the identification of European crab apple trees in the field, which makes it difficult to implement the silvicultural measures necessary to preserve the species. We found hybrids and feral cultivars of domesticated apple in all European crab apple populations, which means that even greater importance should be placed on maintaining pure European crab apple in its natural environment. The origin of forest reproductive material is extremely important when introducing it into the forest area. It should be genetically verified and obtained exclusively from suitable and verified seed stands.

Supplementary Materials: The following are available online at https://www.mdpi.com/1999-4907/12/3/367/s1, Figure S1: Delta K values calculated by Evanno's method detecting K = 2, Table S1: The Evanno table output, Figure S2: A loading plot for each morphological characteristics.

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