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SSR diversity and hybridization of wild apples (*Malus* spp.) growing in the Guadarrama and Ayllón mountain ranges (Central Spain)

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Abstract

Aim of study: The crab apple tree (*Malus sylvestris* (L.) Mill.) is a wild crop relative of the apple tree (*M. domestica* Borkh.). Hybridization and genetic exchange between these species has been studied in some European regions, but there is no record in Spain. This work aimed to characterise a set of 330 feral and crab apples.

Area of study: Guadarrama and Ayllón mountain ranges (Central Spain).

Material and methods: We used 12 microsatellites to detect intermediate profiles. To do so, diversity, multivariate and population structure Bayesian analyses were performed on the sample, adding a total of 28 crab apples, feral and widespread apples varieties as references.

Main results: We found a large molecular diversity in this *Malus* germplasm, scoring a mean of 28.58 alleles per locus (A); an observed heterozygosity (H_o) of 0.80 and a very low value of inbreeding coefficient (F_{is} =0.06). On the other hand, we found from our Bayesian population analysis three populations (one per species and a third one very admixed) apparently not spatially correlated and a substantial level of intermediate genetic profiles, as around 47% of the feral trees and 35% of crab apples may be hybrids.

Research highlights: Connectivity in the crab apple genetic pool is still functional and interspecific gene flow may be relevant. Nevertheless, further conservation measures and research must be carried to understand the population dynamics between both species.

Additional key words: crab apple; feral apple; hybridization; simple repeat sequences; wild crop relatives

Abbreviations used: A (number of alleles per locus); A_e (effective alleles); B (rare alleles); F_{is} (inbreeding coefficient); G_o (observed genotypes); G_p (possible genotypes); H_e (expected heterozygosity); H_o (observed heterozygosity); PCoA (principal coordinates analysis); P_{ID} (probability of identity); R (representativeness); SSR (Simple Sequence Repeats); UPGMA (unweighted pair group method with arithmetic mean); WCR (Wild Crop Relatives)

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Introduction

The apple tree, *Malus domestica* Borkh., and the European crab apple, *M. sylvestris* (L.) Mill. (Fig. 1) are two deciduous trees present in Europe (Terpó, 1981; Aedo et al., 1998; GBIF, 2023). *M. sylvestris* occupy many habitats in the temperate areas of Europe, but in Spain, it is nearly restrained to mountainous localities, such as the Central System (Aedo et al., 1998; Schnitzler et al., 2014).

As a domesticated species, *M. domestica* exists thanks to the combination of four *Malus* species. The Sievers apple, *M. sieversii* (Ldb.) M. Roem, is alleged to be the main parental species, whereas the European crab apple, the oriental crab apple (*Malus orientalis* Uglitzk) and the Siberian crab apple (*Malus baccata* (L.) Borkh) are considered secondary contributors (Cornille et al., 2012; Schnitzler et al., 2014; Spengler, 2019).

Although the crab apple is well known by inhabitants of rural communities (Kalle & Sõukand, 2012; Cornara et al., 2014; Tardío et al., 2021), nowadays it can be considered a threatened plant in some regions, due to climate change, habitat fragmentation and introgression (Ellstrand et al., 1999; Bleeker et al., 2007; Cornille et al., 2019). In fact, it has been reported for the crab apple and the Siever's apple that intense agricultural practices in foothills and other natural lands are hazardous for wild crop relatives (WCR) conservation (Jacques et al., 2009; Schnitzler et al., 2014; Omasheva et al., 2017). This scenario led some authors to assume that the number of "authentic" crab apples may be reduced in Europe (Ruhsam et al., 2019).

Several *Malus* species are considered endangered (UICN, 2021). In the case of *M. sylvestris*, it is listed in some European areas (Reim et al., 2013b), including the region of Madrid (Comunidad de Madrid, 2012) and other adjacent territories of Central Spain. Cattle management is important for seedling germination and survival of crab apples (Buttenschøn & Buttenschøn, 1999), so human intervention seems to be necessary for the preservation of this wild species in those cattle landscapes (Spengler, 2019).

The Iberian Peninsula has been considered a refuge of the last ice age for many European species, included *M. sylvestris*. However, as far as we know there are no local or global studies about this species diversity and its hybridization with the cultivated apple in the region. In general, the *Malus* germplasm diversity is very large, at least in the species close to apple domestication, as the oriental apple (Volk et al., 2009) and the Sievers' apple (Richards et al., 2009) and in other not proved to be related, as *M. fusca* (Raf.) C.K. Schneid. (Routson et al., 2019), *M. hupehensis* (Zhang et al., 2009), *M. kirgishorum* Al.Fed. & Fed., *M. niedzwetzkyana* Dieck ex Koehne (Uzun et al., 2019) and *M. baccatta* (L.) Borkh. (Raja et al., 2022).

Regarding crab apple diversity, in Europe there may exist three to five low differentiated populations, according to Cornille et al. (2013a, 2015). Local european studies are mainly from Central Europe, and show a very variable proportion of hybrids with the apple tree: 5% in east France (Schnitzler et al., 2014); 11% in Central Europe (Coart et al., 2006); 13%, 37% and 40% in Saxony, Germany (Reim et al., 2013b, 2020); 14% in Germany and Luxembourg (Wagner et al., 2014); between 10 and 20% in Denmark (Larsen et al., 2006) and 26% in northern Britain (Ruhsam et al., 2019). Such differences may be due also to sample size and the molecular characterization employed method. As a result, it is highly necessary to quantify such diversity in Spain before any conservation measure were to be

Figure 1. Feral *Malus domestica* (larger leaves, left) and *Malus sylvestris* (smaller leaves, right), growing wild in Central Spain.

Figure 2. Location of Guadarrama (ellipse) and Ayllón (circle) mountain ranges; Alcaraz mountain range (southern square); Huesca Pre-Pyrenees (northern square) and the IMIDRA apple collection (yellow star).

implemented (Reim et al., 2013b; Ruhsam et al., 2019; Buiteveld et al., 2021).

The objective of our molecular analysis is to screen the genetic diversity of the crab apple and the feral apple from the Guadarrama and Ayllón mountain ranges (Central Spain) and to detect the possible hybrids between both species for helping in the management of *M. sylvestris*.

Material and methods

Plant material

A total of 330 feral apples (*M. domestica*) and crab apples (*M. sylvestris*) were sampled during field prospections at the Guadarrama and Ayllón mountain ranges from July 2016 to May 2019 (Fig. 2; Central system, Spain). Identification of wild individuals consisted mainly in an *in situ* evaluation of organ size and pubescence (Terpó, 1981; Aedo et al., 1998; Reim et al., 2012). In addition, we included 28 individuals as references to anchor subsequent analysis. Those references were thirteen crab apples and three feral apples sampled in two different Spanish locations (Sierra of Alcaraz and the Pre-Pyrenees of Huesca) and 12 apple cultivars maintained at 'La Isla' Experimental Station (Arganda del Rey, Madrid). These apple cultivars consisted of seven traditional apple landraces from rural regions of Madrid named 'Camuesa' (CABU1), 'del Ortel' (ORMO3), 'Hojancas' (MHPR2), 'Pero Pardo' (PPPU1, PPPU2), 'de Rosa' (RORA1; RORA2); and five widespread apple varieties ('Fuji Aztec', 'Gala Buckeye', 'Golden Delicious', 'Granny Smith' and 'Reineta Blanca'), many of them tested in previous research (Arnal et al., 2020).

DNA extraction, amplification and sequencing

Molecular genotyping was performed using 12 SSR loci (or microsatellites) already contrasted in the apple tree (HiDRAS, 2020): CH01f02, CH01h01, CH01h10, Ch02b10, CH02c09, CH02c11, CH02d08, CH03d12, CH04c07, CH04e05, CH05c06 and CH05f06. The samples of DNA were obtained from young leaves through an adapted protocol of DNeasy 96 Plant Kit® (Qiagen, Hilden, Germany) and dilution of purified DNA to 5 µg/mL in ultrapure water. DNA amplification was performed using the multiplexes mixtures A and B designed by Urrestarazu et al. (2012). CH03d12, Ch02b10 and CH05c06 loci were individually amplified within the same conditions of multiplex A having the forward primer labelled with a 5´WELLRED fluorescent dye. All PCR amplification was performed in a 'BIO-RAD T100TM' Thermal Cycler (BIO-RAD Laboratories). Those young leaves were collected from March to May 2019 and conserved frozen until DNA extraction.

PCR amplicons analysis was performed using capillary electrophoresis in an ABI Prism 3500 for multiplexes A and B. A CEQ 8000 Genetic Analysis System (Beckman Coulter, 2004) was used for the three SSR amplified individually. Allele molecular weights were automatically assigned using 'GeneMapper 5' (Thermo Fischer Scientific) and the CEQ 8000 Fragment Analysis software, version

9.0, according to the recommendations of the manufacturer (Beckman Coulter Inc., Brea, CA, USA).

At this stage, samples considered triploid trees were removed according to the criterion of Urrestarazu et al. (2012), as it was not possible to know their true genotype in the product amplification (a heterozygous individual with two peaks can be AAB or ABB).

Molecular diversity analysis

Diversity parameters were all calculated manually in a spreadsheet following the formulas and procedures from several autors (Nei, 1978; Tessier et al., 1999; Waits et al., 2001; Arnal et al., 2020) and included: the number of alleles per locus (A), rare alleles (B), effective alleles (A_e), observed heterozygosity (H_o), expected heterozygosity (H_e), inbreeding coefficient (F_{is}) probability of identity (P_{ID}), observed genotypes (G_o), possible genotypes (G_p) and representativeness (R).

Multivariate analysis: principal coordinate analysis and UPGMA tree on genetic distances

A principal coordinates analysis (PCoA) and a tree on genetic distances (or cluster) analysis were calculated in 'R Studio' 1.0.143. After preparing the raw data table containing sample alleles, the *loci2genind* function from the R package 'pegas' (Paradis, 2010) was used to adapt data to further analyses. For PCoA we used the R package 'adegenet' (Jombart & Ahmed, 2008). For cluster analysis, genotypes were classified with the Nei's distance (Nei, 1973) through the unweighted pair group method with arithmetic mean (UPGMA) (Sokal & Michener, 1958) using the function *aboot* from the package 'poppr' considering 10,000 replications (Kamvar et al., 2014). The graphical output from PCoA and cluster analysis was exported in svg and improved in Inkscape, an open-source vector graphics editor.

Bayesian analysis of populations

To detect population structure, a systematic Bayesian clustering approach was applied using STRUCTURE 2.3.4 (Pritchard et al., 2000). Two to 10 populations (*k*) were tested. The programme assigned genotypes to the group where the highest membership was obtained, considering such affinity with the probability of membership (*qI*) above or equal to 0.8. For testing, twenty runs per *k* were evaluated, consisting of a burning period length of 30,000 steps followed by 1,000,000 Monte Carlo Markov Chain (MCMC) replicates (Porras-Hurtado et al., 2013). The Evanno's ΔK statistic from Structure Harvester (Earl & Von Holdt, 2012) computed the optimum value for *k* populations. Finally, we plotted the results from the cluster and Bayesian analysis in ArcMAP 10.2 to detect visually any possible spatial structure between species and individuals.

Results

Diversity analysis

A total of 330 wild *Malus* individuals (117 from feral *M. domestica* and 213 from *M. sylvestris*) from Central Spain were successfully characterised. For both species more than one allele in each microsatellite loci was amplified and similar ranges for allele size were shared. Only 328 individuals were considered in the diversity analysis, as two feral apples (from two distant locations) were triploid. Mean values and ranges of A, B, A_e, H_o, H_e, F_{is}, G_o, G_p, R and P_{ID} for feral apples, crab apples and for the whole study are shown in Table 1.

In general, the allele diversity analysis showed that feral apple diversity was slightly lower than that shown by the crab apple. For example, the A value of the 328 wild individuals was a 25% higher considering the feral apples but only an 8% higher regarding the crab apple; and mean G_o value was 46.08 genotypes per allele in feral apples against the 66.58 genotypes per allele found in the crab apple (Fig. 3). In addition, Fis was very lower in crab apples, leading to consider very low inbreeding events. Nevertheless, values of B, H_o, H_e and P_{ID} were similar between species or even slightly higher in feral apples.

PCoA and UPGMA on genetic distances

A PCoA was performed with 355 individuals (wild *Malus* profiles plus the references), obtaining three differenced groups. The two first principal coordinates (PCo 1 and PCo 2) gathered the 48.17% and 16.81%, respectively (Fig. 4), not existing a clear pattern neither in the distribution of references nor in the geographical relationship of wild individuals. Although PCo 1 accumulated almost 50% of the variance, PCo 2 was that best separated the two species. As a result, according to PCo 2, 69 individuals shown an intermediate molecular profile; being 50 of them (22%) feral apples mixed with crab apples and the last 19 (16%), crab apples mixed with feral apples.

As shown in Fig. 5, cluster analysis results were quite similar. Feral and crab apples references were classified with feral apples and crab apples from the study area, respectively. Furthermore, no clear spatial structure were found, except for 2 to 5 individuals of crab apple from five small areas (at a municipality scale) that were distributed along the study area. Nevertheless, the dendrogram computed from Nei's matrix revealed that crab apples were nested below the feral apple and many crab

Table 1. Summary of the genetic diversity of the 328 diploid specimens studied. A, number of alleles per locus; A_e, number of effective alleles; B, number of rare alleles per locus; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{is}, inbreeding coefficient; G_o, observed genotypes; R, representativeness; P_{ID}, probability of identity. In parantheses, the microsatellite referred to the number beside

		Feral apples (*M. domestica*) N=115	**Crab apples (*M. sylvestris*) N=213**	**Total N=328**
A	Mean	21.33	26.08	28.58
	Range	12 (CH01h10) - 31 (CH02b10)	11 (CH01h10) - 43 (CH02b10)	13 (CH01h10) - 46 (CH02b10)
A_e	Mean	7.81	6.81	7.74
	Range	12.69 (CH02b10) - 4.18 (CH04e05)	13.43 (CH02b10) - 2.36 (CH04e05)	2.90 (CH04e05) - 14.74 (CH02b10)
B	Mean	15.33 (72% A)	20.83 (80% A)	22.25 (78% A)
	Range	6 (CH01h10) - 26 (CH05c06)	4 (CH01h10) - 36 (CH02b10)	6 (CH01h10) - 38 (CH02b10)
H_o	Mean	0.78	0.78	0.80
	Range	0.58 (CH04c07, CH04e05) - 0.98 CH02c11	0.62 (CH05c06) - 0.95 (CH02c11)	0.64 (CH05c06) - 0.96 (CH02c11)
H_e	Mean	0.86	0.83	0.85
	Range	0.93 (CH02b10) - 0.76 (CH04e05)	0.93 (CH02b10) - 0.58 (CH04e05)	0.96 (CH02b10) - 0.66 (CH04e05)
F_{is}	Mean	0.09	0.04	0.06
	Range	-0.07 (CH02c11) - 0.11 (CH04c07)	-0.32 (CH04e05) - 0.27 (CH05c06)	-0.15 (CH01h10) - 0.26 (CH05c06)
G_o	Mean	46.08	66.58	86.25
	Range	25 (CH04e05) - 63 (CH02b10)	32 (CH01h10) - 112 (CH01f02)	37 (CH01h10) - 136 (CH01f02, CH02b10)
R (%)	Mean	21.52	24.41	24.23
	Range	10.75 (CH05c06) - 34.62 (CH01h10)	9.79 (CH04e05) - 48.48 (CH01h10)	9.88 (CH04e05) - 46.67 (CH05f06)
P_{ID}	Mean	0.059	0.080	0.063
	Overall	1.01×10^{-15}	1.32×10^{-14}	8.85×10^{-16}

apple clusters showed a relatively larger Nei's distance compared to feral apples, so molecular diversity may be similar between the two species. Regarding to hybrids, as clusters contained individuals from both species at any scale and *M. domestica* individuals nested almost all *M. sylvestris* individuals, it was not possible to determinate even an approximate value of possible intermediate genetic profiles.

Bayesian population analysis

The Bayesian population analysis for 128 feral apples and 225 crab apples performed by STRUCTURE showed that the optimal *k* value was *k* = 3, with an Evanno ΔK of 34.53 (Fig. 6a). Although no spatial structure was observed between species (Fig. 6b), individuals were separated satisfactorily. The first and third population included respectively apple trees (96%) and crab apples (94%) whereas the second population were formed by 44% of apple trees and 56% of crab apples. Regarding to references, apple international varieties were included in the first population; the traditional cultivars and feral apples fell in the second population and crab apples were distributed mainly in the third population, with some individuals in the second one. A total of 12 individuals (4 feral apples and 8 crab apples) could not assigned to any population. In this way, Bayesian analysis considered 148 individuals with intermediate profile or hybrids, of which 60 were apple trees (46.88%) and 78 crab apples (34.66%).

Discussion

Authors as Hokanson et al. (1998) and Liebhard et al. (2002) used microsatellites for fingerprinting apple cultivars. Others have found that microsatellites were effective to approach *M. sylvestris* diversity (Reim et al., 2013a; Ruhsam et al., 2019) and other worldwide wild crab apples (Omasheva et al., 2017; Routson et al., 2019; Raja et al., 2022). That effectiveness helps in *Malus* germplasm conservation, as microsatellites can overcome the difficulties of phenotyping, as feral *M. domestica* may show similar traits to the crab apple (Kišek et al., 2021), although the study of fragment lengths may lead to a underestimate the genetic variability due to homoplasy, defined here as the converge of the same fragment length from different lineages (Šarhanová et al., 2018).

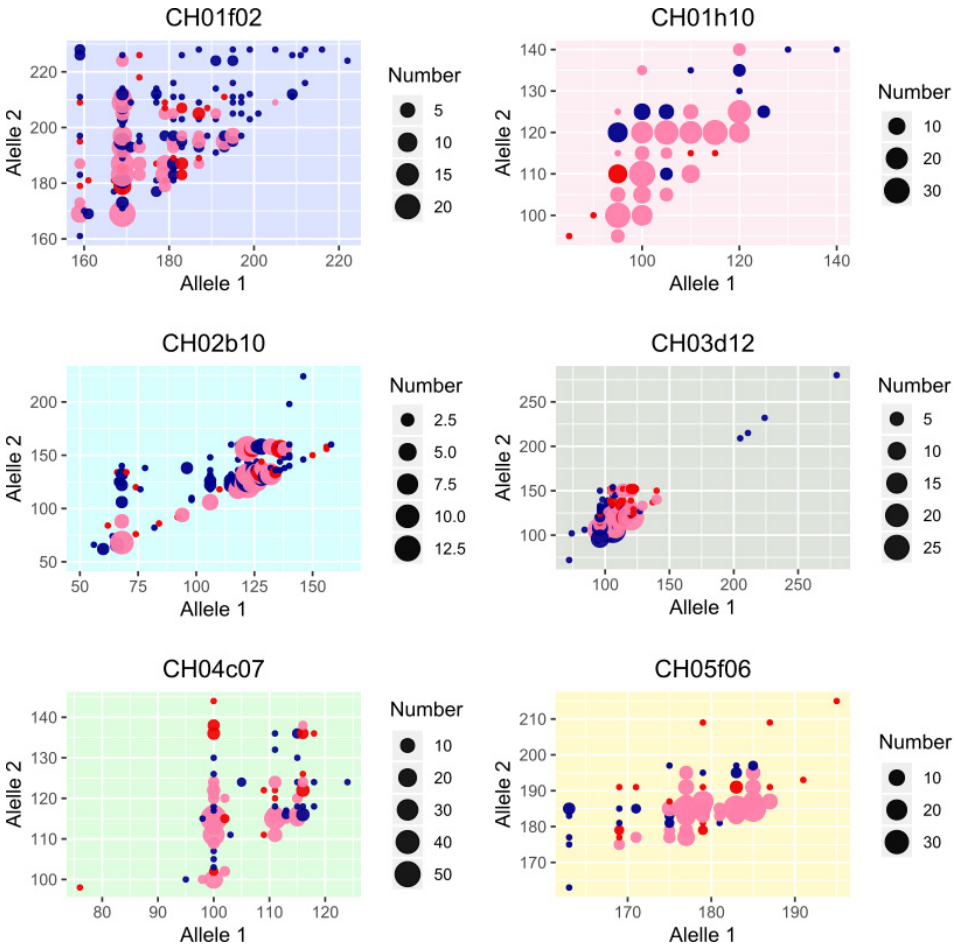

Figure 3. Distribution and frequency of the observed genotypes (G_o) in CH01f02, CH01h10, CH02b10, CH03d12, CH04c07 and CH05f06 for the wild *Malus* individuals (N=328) plotted in R studio with the package 'ggplot2' in R Studio. Red, feral apples; blue, crab apple; pink, both species. We assumed homozygosis in one-peak outputs from the sequencer.

Genetic diversity

According to our results, a great diversity was found with the 12 nuclear SSR used, both in the whole study and in each species separately. The number of alleles per SSR was high, but it was not possible to assign different allelic ranges for each SSR and species. This circumstance is in accordance with our PCoA, as it was PCo 2 who included the factor species. As a result, it seems unaffordable to unravel with our microsatellites the contribution of each species in hybrid offsprings from *M. domestica* × *M. sylvestris* (Estoup et al., 1999; Larsen et al., 2006).

We expected that molecular diversity of feral apples would have been higher than the one of *M. sylvestris* for three reasons. First, for the essayed and demonstrated informative power of the applied SSR in cultivated apples. Second, since *M. domestica* genome is inherited from four different *Malus* species from Eurasia (Cornille et al., 2014, 2019; Harris et al., 2002), its genetic diversity may be presumed to be higher. Third, our previous morphological work in this study area (Arnal et al., 2023) pointed out to a greater diversity in feral apples. Nevertheless, the present results have shown that *M. sylvestris* molecular diversity was slightly higher than those of feral apples, similarly to Reim et al. (2013a), who found more alleles in the 'true' crab apple set. The presence of more alleles in our WCR than in the feral apple may be due to restrictions in feral germplasm. For example, there may be non-viable alleles or genotype combinations, as the lowest values of G_o and R were obtained in the feral *M. domestica* pool.

The mean A value in the crab apple per loci was 26.08, ranging from 11 (CH01h10) to 43 alleles (CH02b10). Considering wild *Malus* species, our results are higher to those obtained in Larsen et al. (2006), Reim et al. (2013a), Omasheva et al. (2017), Bitz et al. (2019), Uzun et al. (2019) and Yu et al. (2019). In addition, the A value varied between microsatellites, but diversity of some SSR was similar between studies and species. Considering only the crab apple studies, herein we found hat CH01f02 and CH01h01 were among the most diverse, as in other studies (Reim et al., 2013a; Heinonen & Bitz, 2019). Finally, we found low diversity in CH04e05, which is in agreement with several

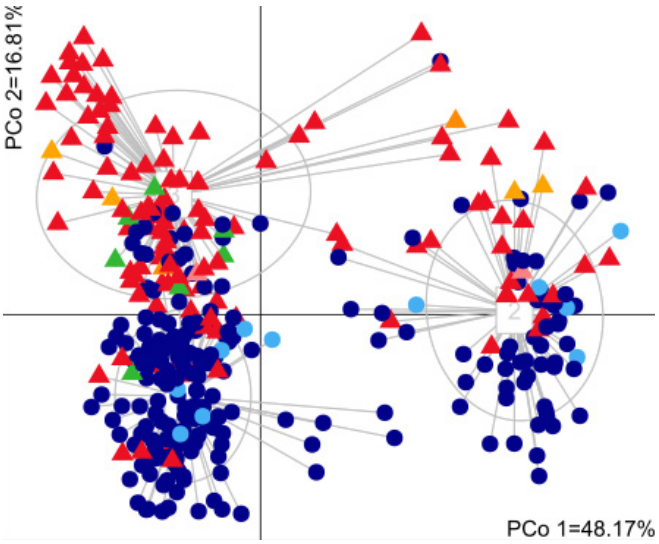

Figure 4. Principal Coordinates Analysis (PCoA) based on 12 SSR loci from 355 Malus diploid genotypes. Dark blue and red, crab apples and feral apples from the study area, respectively; light blue and pink, crab apples and feral apples from Alcaraz mountains and Huesca Pre-Pyrenees respectively; orange, reference apple varieties; green, apple landraces.

studies (Larsen et al., 2006; Reim et al., 2013a; Bitz et al., 2019). Reduced diversity on CH04e05 may be due to amplification problems or the presence of null alleles, so it may not be sufficiently informative for crab apples (Reim et al., 2013a; Ruhsam et al., 2019).

Other diversity parameters, as A_e, showed a similar behaviour that one found in other crab apple studies (Reim et al., 2013a; Omasheva et al., 2017; Uzun et al., 2019; Yu et al., 2019), as our A_e value was very low in comparison to A (~ 26%), with a notable range between microsatellites. In the case of heterozygosis, the mean observed heterozygosity ($\overline{H}_o$) and mean expected heterozygosity ($\overline{H}_e$) in our study were 0.80 and 0.85, respectively, with a high variation among SSR. Our values were similar or slightly higher than the other studies (Larsen et al., 2006; Reim et al., 2013a; Schnitzler et al., 2014; Cornille et al., 2015; Bitz et al., 2019), even only concerning the individuals sampled in the southern Iberian Peninsula in the study of Cornille et al. (2015). Such values of heterozygosity in central Spain may agree with previous research (Cornille et al., 2013a, 2015), who explained that recolonization started from southwest Europe (southern France and Italy) and the southeast (the Balkans and the Carpathian mountains) to Scandinavia. In consequence, the crab apple recolonization after the last glaciation was similar to other iconic European species, such as *Fagus sylvatica* L. (Carrión, 2015), *Fraxinus excelsior* L. (Heuertz et al., 2004) and several oak species (Petit et al., 2002).

In multivariate analysis, PCoA and cluster analysis pointed out a high diversity in the studied crab apple germplasm, although it was no possible to find a clear differentiation between the two species, especially in cluster analysis. Regarding strictly to our dendrogram, *M. domestica* tended to show larger Nei distances than the crab apple, being the second species nested inside *M. domes-*

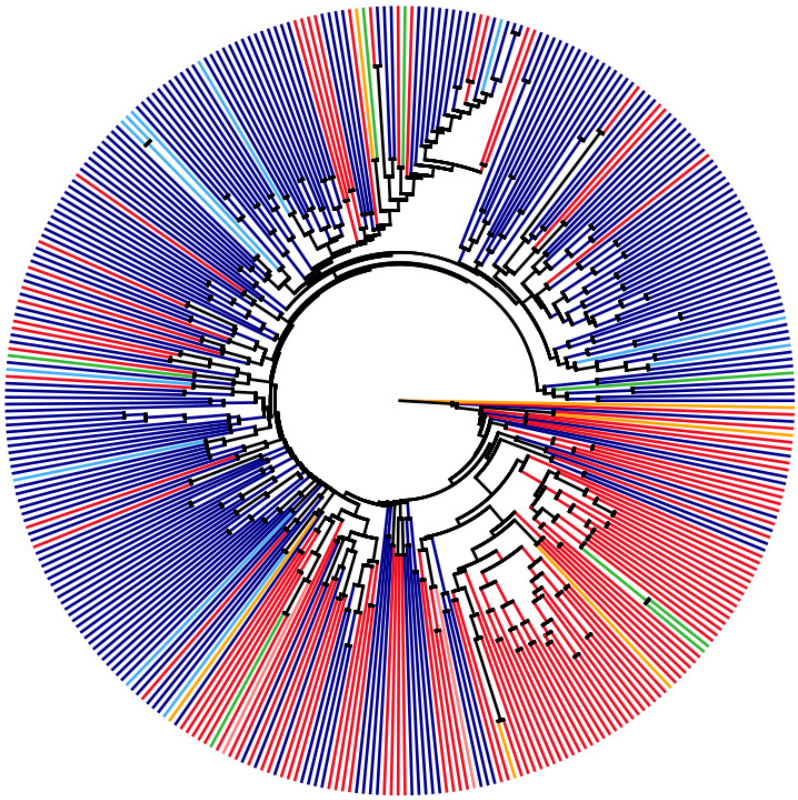

Figure 5. Dendrogram based on a unweighted pair group method with arithmetic mean (UPGMA) cluster analysis with Nei's distance for 358 *Malus* individuals through 12 SSR loci. Dark blue and red, crab apples and feral apples from the study area, respectively; light blue and pink, crab apples and feral apples from Alcaraz mountains and Huesca Pre-Pyrenees respectively; orange, reference apple varieties; green, apple landraces.

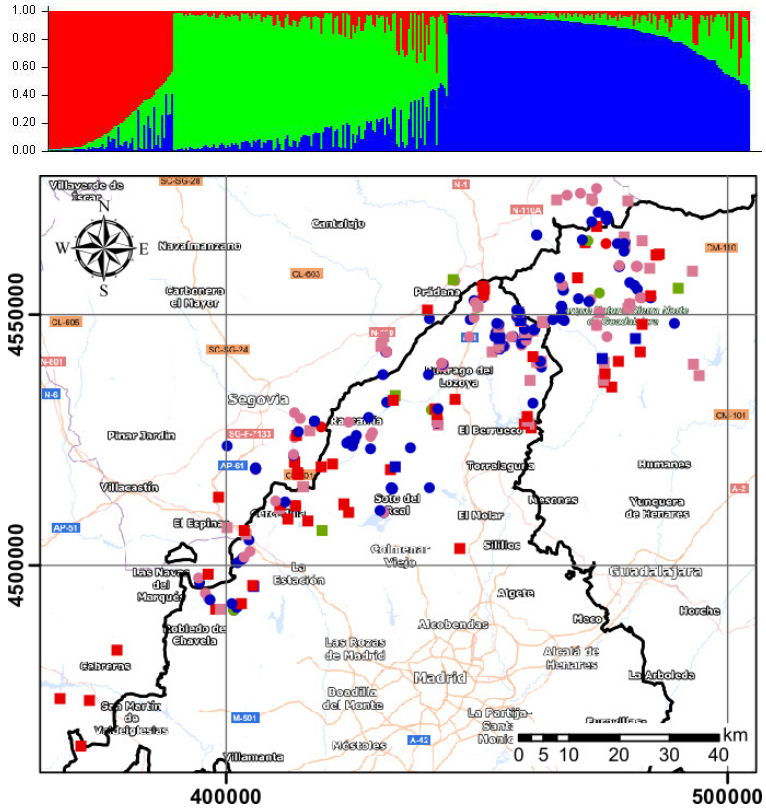

Figure 6. Bayesian population structure (k =3) of 355 *Malus* diploid individuals through 12 SSR (a). Spatial distribution of the individuals in the study area, Central Spain (b): squares, feral apples; circles, crab apples; blue, structure population 1; pink, population 2; red, population 3; green, individuals that could not be assigned to any population.

tica clusters (Wagner et al., 2014). This dominance of *M. domestica* over *M. sylvestris* is similar to other wild crab apples, including the Siever's apple (Reim et al., 2013a; Omasheva et al., 2017; Bitz et al., 2019).

Population structure

The Bayesian analysis of populations, using the ΔK, obtained three populations, showing that *M. domestica* largely predominated in population 1 and *M. sylvestris* in population 3, being both *M. domestica* and *M. sylvestris* references classified in populations 1 and 3, respectively. Such results suggests a clear separation between species, as obtained in other research with SSR markers (Reim et al., 2013a; Omasheva et al., 2017; Bitz et al., 2019), so it is important to study different statistical approaches to evaluate population structure.

Studied *Malus* germplasm had no clear spatial structure in the study area. This result supports other local or regional molecular studies of *M. sylvestris* (Cornille et al., 2013a; Schnitzler et al., 2014; Wagner et al., 2014; Ruhsam et al., 2019; Reim et al., 2020). Nevertheless, other research works in several *Malus* species have also found different degrees of population structure. In the paneuropean study of crab apples performed by Cornille et al. (2015), a mild differentiation in five populations was found in Europe. In the Bayesian analysis of populations performed by Bitz et al. (2019) a certain structure was also found. Furthermore, Omasheva et al. (2017), did report differentiated Siever apple populations; and Routson et al. (2019) also discussed certain level of population differentiation in *M. fusca*. However, although all those works used SSR, they have different geographic scales.

The absence or low population structure in our crab apple may be explained by several factors. First, because *Malus* is self-incompatible, so individuals are forced to cross with others. Second, pollination is entomophile, so crab apples take benefit both from the prodigious number of flowers that bees can visit and the distances assumed by their pollinators, which varies in a range from 300 m to 10 km, according to Beekman & Ratnieks (2000), Larsen & Kjær (2009) and Reim et al. (2017). Third, because seed dispersal is zoochorous, mainly through cattle and other large livestock (Buttenschøn & Buttenschøn, 1999; Spengler, 2019). Fourth, because there existed an intense transhumance and traditional cattle movement along the *old livestock trails* (Millán González, 2011), which even attenuated the altitude effect, found as a controversial factor (Omasheva et al., 2017; Yousefzadeh et al., 2020).

As a result, and considering also our very low F_{is} value, although low species density and habitat fragmentation may be threatening our natural landscapes, links between subpopulations are still maintained (Reim et al., 2020). Nevertheless, conservation planning may be still necessary via *ex situ*, reintroduction (Ellstrand et al., 2013; Cornille et al., 2015; Reim et al., 2020) and especially the sustainable management of cattle and livestock (Ruhsam et al., 2019; Spengler, 2019).

Hybridisation

Of the subset of individuals fully characterised, a 46% of feral apples and a 35% of crab apples showed intermediate genotype profiling with *M. sylvestris* and *M. domestica*, respectively, so they are probably hybrids. For *M. sylvestris*, our result is included in the range of values found in other molecular studies with this species, between the minimum of 5% (Schnitzler et al., 2014), and the maximum, a 40% (Reim et al., 2013a). The closeness of our result to the upper limit may be explained by the land use in the sampled territory, which is dominated by forest edges, meadows and other abandoned agricultural areas. In fact, Jacques et al. (2009) recognised that hybrids were more common close to farmlands, whereas only a 20% of crab apples from forests were hybrids.

The relative large diversity herein described in feral apples, with several levels of admixture, and their low population structure can be explained with two reasons. First, due to the high pressure of cattle transit for centuries via the old *livestock trails* mentioned above, which moves genotypes from cultivated areas to other open semi-natural landscapes as meadows, pastures, drinking troughs, resting places, sheepfolds and other localities; bringing apple seeds to perfect conditions to germinate, especially in direct sunlight areas (Buttenschøn & Buttenschøn, 1999). The second reason is anthropic, because humans dispose of and throw away eaten apples along roads edges and paths, according to van Slageren (1994) and Ruhsam et al. (2019). Therefore, Ruhsam et al. (2019) suggest that in natural areas close to places with a minimal anthropic pressure, presumed hybrid apple trees are actually apple trees descended from apple trees feralled from seeds. Such discussion was exposed previously by Schnitzler et al. (2014), who reported that most hybrids were found near forest roads. We agree with this hypothesis, as we observed that many feral apples appeared in environments linked to agroforestry, both abandoned and in use.

Nevertheless, we do not know the actual effect of those feral apples on the population dynamics of the crab apple, as their interaction is unknown. In fact, the reproductive barriers between species have not been deeply evaluated. For example, the crab apple may have a preference for pollen of its own species, as occurs in other species. For instance, females of *Populus nigra* have more affinity to pollen from the same species, even in the presence of pollen from other species that can form viable hybrids (Vanden Broeck et al., 2005). In addition, Larsen et al. (2006) reported that hybridization in their area is not so frequent as crab apple blooms earlier (among other reasons). Blooming differences are also cited in Reim et al. (2013a), although there exist some overlap, depending on the year. Furthermore, it is important to elucidate at which extent hybrids are capable to cross with themselves or with the parental populations, as hybrids are expected to have reduced fertility and consequently they may not participate in genetic dynamics. For example, the first generation of the hybrids between *Populus* sp. and a *P. nigra* female originates populations that do not re-hybridize with their parents (Vanden Broeck et al., 2005).

Despite the reported negative effects of cultivated apple introgression on crab apples, hybridization is an important component in evolution and speciation (Ellstrand et al., 1999). In the context of the Hardy-Weinberg law (Hardy, 1908; Weinberg, 1908), a reduced gene flow can be a powerful force, interacting with mutation, drift and selection (Ellstrand et al., 1999), assisting the crab apple in the face of environmental conditions derived from climate change (Omasheva et al., 2017). In addition, we also propose that the presence of feral apples may help to increase the biodiversity and quality of many habitats at least due to the palatability of their fruits for large mammals.

Nevertheless, we are also in agreement with Todesco et al. (2016), who discuss that more research should be performed, as an excess of introgression may whither the integrity of the species and its adaptive ability (Bleeker et al., 2007; Bitz et al., 2019), reducing their interest as a genetic resource for food and agriculture. In addition, due to the possible presence of homoplasy, maybe a new study using SNPs (Single Nucleotide Polimorphisms) could clarify such genetic relationships.

Conclusions

The *Malus* gene pool found in central Spain is very diverse, although described variability is larger in the crab apple. Due to a lack of spatial structure, *Malus* diversity is contained in two populations, one for each species. In addition, there exist a great gene flow between species. Considering the Bayesian analysis, around the 47% of feral apples and the 35% of crab apples have shown intermediate genetic profiles. Finally, although habitat fragmentation may not be affecting severely the crab apple diversity in the study area yet, it is highly recommendable to determinate the long term effect of feral *M. domestica* on population dynamics and to consider the crab apple in the management of natural areas in the Central System. Such protection may be beneficial in the areas already designed by the law regulation or even in new localities.

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