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Morphological characterization of 23 *Malus domestica* Borkh cultivars from central Spain

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Abstract: The purpose of this work was to morphologically characterize an apple tree collection composed of 67 individuals from 41 accessions belonging to 23 old Spanish apple cultivars (*Malus domestica* Borkh) alongside 9 reference cultivars. The studied germplasm was collected previously in rural areas of central Spain (Sierra Norte de Madrid and Tagus river basin) and it was analyzed through 67 descriptors mainly from IBPGR and UPOV. We found a very high morphological diversity in the studied old apple cultivars, as 48% of the descriptors (most of them devoted to fruit traits) were significantly different between types of cultivars. In addition, the sample cultivars resulted clearly distinct from reference cultivars in multivariate analysis. In general, no particular structure was found in old cultivars, but a strong differentiation of 'Agridulce' and 'Hojancas' is reported due to their bigger fruits. Our results support the molecular analysis and call for further analysis of the local apple germplasm and long-term conservation actions.

Keywords: Apple genetic resources, old apple cultivars, phenotype, multivariate analysis

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Introduction

The apple tree (*Malus domestica* Borkh.) is the most important temperate fruit tree crop, with more than 126 million tonnes harvested worldwide in 2020 (FAO, 2022). Such production is in line with the Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture (FAO, 2010), which reported *Malus* L. genetic resources to be among the largest *ex situ* collections. Morphological characterization of *Malus* has been essential for an adequate description of germplasm collections, for breeding programmes (Božović *et al*, 2015) and taxonomic studies (Höfer *et al*, 2014; Wagner *et al*, 2014). Currently, although the information provided by genetic markers (such as microsatellites) is preferred against phenotyping due to their stability and economy (Reddy *et al*, 2002; Ban *et al*, 2014), the study of agricultural germplasm by morphological traits is still relevant and useful in diversity analysis (Božović *et al*, 2015; Király *et al*, 2015; Kumar *et al*, 2018).

As a result, apple morphological descriptions were conducted in Bosnia and Herzegovina (Gaši *et al*, 2011), Canada (Watts *et al*, 2021), Hungary (Király *et al*, 2015), India (Dolker *et al*, 2021), Iran (Farrokhi *et al*, 2013), Italy (Martinelli *et al*, 2008), the Kashmir Valley (Dar *et al*, 2015), Macedonia (Kiprijanovski *et al*, 2020), Montenegro (Božović *et al*, 2015), Serbia (Mratinić *et al*, 2012) and Turkey (Karatas, 2022), reporting high morphological diversity. The most common morphological descriptors used in those works belong to international guidelines such as IBPGR (1982) and UPOV (2005) and they focus on fruit characteristics because sensorial characteristics and consumer demand focus on fruits (Pereira-Lorenzo *et al*, 2018).

In Spain, several studies also reported great phenotypic apple diversity (Royo and Itoiz, 2004; Ramos-Cabrer *et al*, 2007; Santesteban *et al*, 2009; Pérez-Romero *et al*, 2015), but they did not include old cultivars from some central regions. This lack of informa-

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tion should be filled, as some of these undescribed old apple cultivars are valued for their sensorial qualities, others are suspected to be exclusive to central Spain, and the abandonment of rural landscapes threatens their survival (Aceituno-Mata, 2010; Comunidad de Madrid, 2018; Arnal *et al*, 2020).

This study aims to quantify the morphological diversity of old apple cultivars growing in rural areas of central Spain, compare it with a previous genetic study with simple sequence repeats (Arnal *et al*, 2020) and see whether the old traditional cultivars were distinguishable by morphological descriptors. This work also provides the foundations for further agronomic and sensorial studies to complete the Spanish apple morphological description and provide valuable information that will increase the knowledge of apple genetic resources.

Material and Methods

Plant material

A collection of 67 individuals from 41 accessions belonging to 23 old Spanish apple cultivars was evaluated to assess their morphological diversity (Table 1). The collection is located in Arganda del Rey (Madrid) and belongs to the Instituto Madrileño de Investigación y Desarrollo Rural, Agrario y Alimentario IMIDRA (Figure 1). For each accession, in 2009 two scions were grafted onto seedlings of *M. domestica* in a frame of 5m, being the aisles oriented in the SW-NE direction. Two individuals of nine main reference varieties and sports (grafted in rootstocks from the same nursery) curated in the same orchard and environmental conditions were included as controls: 'Fuji Aztec', 'Fuji Kiku 8', 'Gala Buckeye', 'Gala Schniga', 'Golden Delicious', 'Golden Reinders', 'Granny Smith', 'Reineta Blanca' and 'Verde Doncella' (Table 2). Those references were selected because they are widespread in Spain (Iglesias *et al*, 2009).

The annual maintenance of the collection was conducted as follows: goblet pruning at the end of autumn, soil amendment at the beginning of winter and a preventive application of pesticides against aphids at the end of spring. Trees were irrigated every two weeks from May to September to reduce water stress during summer.

Morphological descriptors

A set of 67 morphological descriptors (25 quantitative and 42 qualitative, of which 3 were discrete, 23 nominal and 16 ordinal) were assessed on for 4 organs: 8 descriptors on winter 1-year-old wooden branches (or shoots), 15 on leaves, 16 on flowers and 28 on fruits (Table 3). The descriptors were obtained from IBPGR (1982), UPOV (2005) and Urbina and Dalmases (2014) and new descriptors and further categories in some qualitative traits were also considered, such as watercore (Arnal, 2021). Ten (10) to 20 fruits, 20 leaves, 10 flowers and 20 shoots were collected from different orientations of the tree crown in two years (except flowers) from summer 2016 to autumn 2019 and stored at 4–7°C until processing. In particular, leaves and shoots were collected in 2016 and 2017, and flowers and fruits through the four years of the study.

Continuous descriptors of shoots, flowers and fruits were measured manually using a JP Selecta model 5900601 digital caliper with a precision of 0.01mm. Leaf quantitative descriptors were captured with ImageJ (Schneider *et al*, 2012), so leaves were previously scanned attached to a 2D-scale. Finally, apple fruit weight (ten per individual) was registered with a Sartorius CP 2202 S digital scale with a precision of 0.01g.

Data analysis

Phenotypic diversity. Arithmetic means for 25 quantitative, medians for 16 discrete and ordinal, and modes for 26 nominal descriptors were calculated to obtain the central values by accession and cultivar. All qualitative descriptors were translated into a numerical value to meet computing requirements. Student's t- and Cohen's d-tests were conducted to identify ifferences between types of cultivar descriptors (references and old apple cultivars). Lastly, Tukey's HSD test was performed to detect different groups among old apple cultivars. The significance level (α) was set at 0.05.

Correlations. A correlation matrix was calculated to explore significant correlations between descriptors in old traditional cultivars. Correlations between continuous descriptors were done with Pearson, whereas the rest were computed with Spearman. No correlations between nominal descriptors were performed. Descriptors with no variance were removed at this stage.

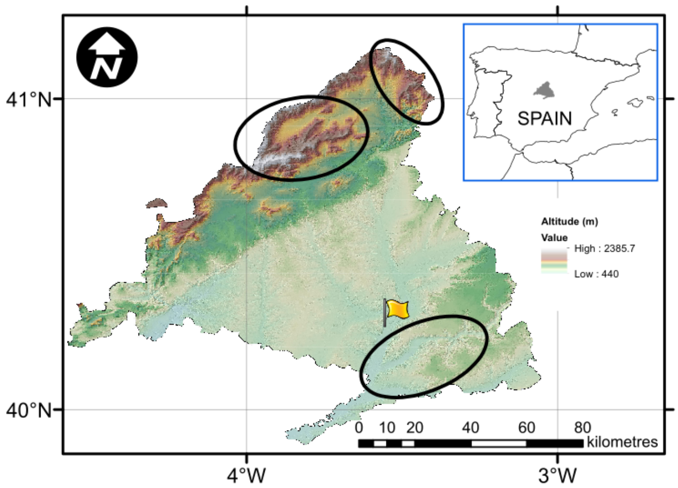

Figure 1. Collection sites of the old apple cultivars from central Spain. The two upper ellipses indicate accessions from Sierra Norte de Madrid and the lower one, from the Tagus River basin. The yellow flag indicates the location of the IMIDRA collection. MDT25 2015 and BDLJE 2018, CC-BY 4.0 ign.es.

Table 1. Cultivar name, individual, accession andcollection site of the 23 old apple cultivars sampled in rural areas of central Spain.

Old cultivar name	Individual/s	Accession	Collection site
Amarillo de El Paular	APRA1/2	APRA	Rascafría
Camuesa	CABU1/2	CABU	Bustarviejo
	CAPR1/2	CAPR	Prádena del Rincón
Camueso tardío	CTAP1/2	CTAP	Puebla de la Sierra
Camueso temprano	CTEP1	CTEP	Puebla de la Sierra
de Chapa	CHCA1/2	CHCA	Canencia
	CHVA1	CHVA	Valdemanco
Esperiega	ESPU1/2	ESPU	Puebla de la Sierra
Agridulce	MAMO1/2	MAMO	Montejo de la Sierra
Hojancas	MHHO1/2	MHHO	Horcajuelo de la Sierra
	MHPR1/2	MHPR	Prádena del Rincón
del Ortel	ORCA1/2	ORCA	Canencia
	ORMO3	ORMO	Morata de Tajuña
Pero de Aragón	PAHO1/2	PAHO	Horcajuelo de la Sierra
	PAPR1/2	PAPR	Prádena del Rincón
	PAPU1/2	PAPU	Puebla de la Sierra
Pepita de melón	PECA1/2	PECA	Canencia
	PEHI1/2	PEHI	La Hiruela
	PEVA2	PEVA	Valdemanco
Pero gordo	PGHI2	PGHI	La Hiruela
Pero pardo	PPHI1	PPHI	La Hiruela
	PPMO1/2	PPMO	Montejo de la Sierra
	PPPU1/2	PPPU	Puebla de la Sierra
Pero real	PRBU1/2	PRBU	Bustarviejo
	PRHI1/2	PRHI	La Hiruela
Rabudas	RAHI1/2	RAHI	La Hiruela
Reineta	REHO11	REHO1	Horcajuelo de la Sierra
	REHO21	REHO2	
	REMO1	REMO	Montejo de la Sierra
	REPR1/2	REPR	Prádena del Rincón
Rojillo	RJHO1/2	RJHO	Horcajuelo de la Sierra
Rojo	RJPR1/2	RJPR	Prádena del Rincón
	RJPU1/2	RJPU	Puebla de la Sierra
de Rosa	RORA1/2	RORA	Rascafría
Rojillo temprano	RTEV1	RTEV	Valdemanco
San Felipe	SFCA1	SFCA	Carabaña
Temprano	TEPI1	TEPI	Pinilla del Valle
Verde Doncella	VDCA1/2	VDCA	Canencia
	VDHO1/2	VDHO	Horcajuelo de la Sierra
	VDTI1	VDTI	Tielmes

Table 2. Reference cultivars curated in the IMIDRA collection.

Reference cultivar name	Accession number	Origin
Fuji Aztec	4	Worldwide cultivar
	7	
Fuji Kiku 8	11	Worldwide cultivar
	14	
Gala Buckeye	115	Worldwide cultivar
	117	
Gala Schniga	109	Worldwide cultivar
	113	
Golden Delicious	104	Worldwide cultivar
	108	
Golden Reinders	101	Worldwide cultivar
	203	
Granny Smith	206	Worldwide cultivar
	210	
Reineta Blanca	211	Worldwide cultivar
	215	
Verde Doncella	307	National cultivar
	311	

Multivariate analysis. A principal coordinate analysis (PCoA) was performed to visualize the possible groups of cultivars and detect the descriptors that better describe the differences among individuals. In the cluster analysis, a distance matrix between accessions was calculated with Nei's distance (Nei, 1973) and the dendrogram was plotted using the unweighted pair group method with arithmetic mean (UPGMA) hierarchical agglomerative method (Sokal and Michener, 1958).

Analysis computing. Statistics were performed in R Studio v.3.4.1 (R Core Team, 2017) and a set of packages: 'effsize' for Cohen's d-test Torchiano (2018), 'corrplot' (Wei and Simko, 2017) for the correlation matrix and 'agricolae' (De Mendiburu, 2019) for Tukey HSD. Multivariate analysis was computed with an adapted version of the 'MorphoTools' script (Koutecký, 2015).

Results

Phenotypic diversity

Thirty-two morphological descriptors (48% of the total; 19 continuous and 13 non-continuous), showed significant differences between references and old apple cultivars (Table 4). In general, old apple cultivars registered lower quantitative values than references, but level frequencies in many qualitative descriptors were less skewed.

Shoots, leaves and flowers from both types of cultivars were similar, as there were only 14 significant descriptors out of 39 (36%). Some significantly different descriptors found in those organs were the width of the apical bud (ShW; $P = 6.63 \times 10^{-14}$), the petal length (FlPetL; $P = 0.0012$) and the petal width (FlPetW; $P = 0.023$).

In fruit, 18 out of 28 descriptors were significantly different (64%). The calyx opening diameter (FrCCD; $P = 4.26 \times 10^{-24}$), fruit peduncle length (FrPedL; $P = 5.57 \times 10^{-37}$), and peduncle width (FrWP; $P = 7.37 \times 10^{-18}$) stood out by their significance and effect size (Cohen's d). In fact, it was observed that the peduncles from old apple cultivars were around 1cm shorter than those from references (Figure 2a). Other important quantitative descriptors such as the peduncular cavity width (FrSCW; $P = 0.0163$), the calyx cavity width (FrCCW; $P = 0.0163$), fruit length (FrL; $P = 0.0026$) and fruit weight (FrM; $P = 0.0275$) were significant, but the effect size (Cohen's d) was not large. Alternatively, no significant differences existed in fruit width (FrW; $P = 0.2081$). In the fruit qualitative descriptors, the depth of the calyx cavity (FrCCDep) was 'intermediate' in old apple cultivars and 'strong' in references, with significant differences ($P = 0.006$), describing more diversity in old apple cultivars, as it was relatively easy to find apples with a 'weak', 'intermediate', 'strong', or 'very strong' calyx. Regarding over colour (FrUpCol), it was found that old apple cultivars had significantly less over colour than references ($P = 2.69 \times 10^{-06}$). Nevertheless, among apples with cheeks, 'red' and 'yellow' were the most abundant colours.

Means, medians and modes were also computed by cultivar. As a result, means of quantitative descriptors from references were contained in the Tukey's HSD groups of old apple cultivars. The average fruit length (FrL) of the apples was in the 43–63mm range and the fruit width (FrW) was between 50 and 81.5mm. Regarding fruit weight (FrM), apples weighed 125g on average. Their shape (FrShp) was mostly conical (sum of 'conical globose', 'conical oblong' and 'conical truncated'), with a minority of ellipsoidal and flat globose shapes.

The cultivar 'Agridulce' showed significant larger sizes than the other old apple cultivars and even references, as its measurements belonged to the 'a' group of Tukey's HSD in 17 out of the 25 quantitative descriptors (almost 50%), such as fruit length (FrL), fruit weight (FrM) and fruit width (FrW). 'Hojancas' and 'Pero gordo' also tended to have larger organs. The rest of the cultivars presented intermediate size organs, except 'Esperiega', and 'San Felipe', which showed small organs.

Correlations

There was significant correlation in 44 out of 67 descriptors studied, as the total average significant correlation was 0.43 (Figure 3). Shoot colour (ShCol) was removed as no variance was detected. By organ, the means of correlation was 0.37 in the shoot, 0.54 in the leaf, 0.42 in the flower, and 0.44 in the fruit. Correlations within each organ were mainly positive, such as leaf area

Table 3. Morphological descriptors evaluated in 23 old apple cultivars curated at the IMIDRA collection. Type of descriptor (C, Continuous;D, Discrete; N, Nominal; O, Ordinal). The hash (#) indicates that the descriptor has been altered (by adding or removing some levels of the published descriptors). Sources:1, IBPGR (1982); 2, UPOV (2005); 3, Urbina and Dalmases (2014); 4, this paper.

Organ	Descriptor name	Code	Type	Levels	Source
Shoot	Pubescence on the apical bud	ShBPub	O	0, glabrous; 1, intermediate; 2, tomentose	3
	Shoot colour	ShCol		1, brown; 2, reddish brown, 3; green; 4, grey; 5, purple; 6, red; 7, brown reddish; 8, light brown	2
	Shoot diameter (mm)	ShDia	C	–	2
	Length of the apical bud (mm)	ShL	C	–	3
	Lenticels#	ShLent	O	1, very few; 3, few; 5 frequent; 7, densely populated	2, 3
	Apical shoot shape#	ShShp		1, semispherical; 2, ovoid; 3 intermediate; 4, conical	3
	Pubescence on shoot	ShSPub	O	0, glabrous; right, 9, tomentose	2
	Width of the apical bud (mm)	ShW	C	–	4
Leaf	Leaf area (cm2)	LeArea	C	–	4
	Asymmetry of the leaf blade	LeAsim		0, symmetric; 1, asymmetric	4
	Shape of the base of the leaf blade	LeBas		1, cuneate; 2, rounded cuneate; 3, rounded; 4, asymmetric; 5, cordate; 7, truncated	3
	Petiole colour	LeCol		1, purple; 2, green and purple; 3, green	2
	Leaf edge shape	LeEdg		1, crenate; 2, bicrenate; 3, serrate-1; 4, serrate-2; 5, biserrate-2; 6, biserrate-1; 7, triserrate	2, 3
	Foliar blade folding	LeFold		1, folded; 2, turned; 3, convex; 4, ondulate; 5, flat	3
	Leaf blade length (cm)	LeL	C	–	2, 3
	Maximum width of the leaf blade (cm)	LeMWL	C	–	4
	Petiole length (cm)	LePetL	C	–	3
	Pubescence on the reverse	LePub	O	0, not pubescent; 1, pubescent at the base of the midrib; 3, little pubescent; 5, pubescent; 7, very pubescent; 9, tomentose	2, 3
	Leaf blade shape	LeShp	O	1, ovate; 2, elliptical; 3, obovate	4
	Leaf petiole stipules	LeSti		1, rudimentary; 3, short filiform; 5, long filiform; 7, narrow foliar; 9, wide foliar	3
	Leaf apex length (mm)	LeTip	C	–	3
	Shape of the leaf apex	LeTipShp		2, rounded; 3, acute; 5, mucronate; 7, acuminated; 9, cuspidate	4
	Leaf blade width (cm)	LeW	C	–	2, 3

Continued on next page

Table 3 continued

Flower	Androecium length (mm)	FlAnd	C	–	4
	Dominance of the gynoecium over androecium	FlDom	O	1, dominated; 2, balanced; 3, dominant	2
	Gynoecium length (mm)	FlGin	C	–	3
	Pedicel colour	FlPedCol	O	1; purple; 2, purple and green; 3, green	4
	Flower pedicel length (mm)	FlPedL	C	–	4
	Petal colour	FlPetCol		0, white; 1, pink white; 2, purple white; 3, purple	3
	Petal length (mm)	FlPetL	C	–	2, 3
	Number of petals	FlPetN	D	Integer counting	3
	Petal width (mm)	FlPetW	C	–	2, 3
	Pubescence on pedicel	FlPub	O	1, glabrous; 2, slightly pubescent; 3, tomentose	4
	Relative position of the petals	FlRPP	O	0, free; 1, tangent; 2, overlapped	2, 3
	Sepal length (mm)	FlSepL	C	–	4
	Flower shape	FlShp		1, flat turned; 2, turned cupuliform; 3, cupuliform; 4, slightly cupuliform; 5, flat	3
	Flower pedicel stipules	FlSti		0, no present; 1, present	4
	Type of petal	FlTyp		0, flat; 1, wavy; 2, concave; 3, convex	4
	Welding point of the stamens	FlWeld	O	1, welded at the base; 2 welded in pairs up to a certain height; 3 completely welded	3
Fruit	Calyx opening diameter (mm)	FrCCD	C	–	2, 3
	Depth of the calyx cavity#	FrCCDep	O	0, external; 1, very weak; 2, weak 3, intermediate; 4, strong; 5, very strong	2, 3
	Length of the calyx cavity (mm)	FrCCL	C	–	2, 3
	Shape of the opening of the calyx cavity	FrCCShp		0, without sepals; 1, convergent; 2, partially extended or extended; 3, erect	2, 3
	Calyx cavity width (mm)	FrCCW	C	–	2, 3
	Over colour distribution#	FrDisCol	O	0, uniform (no cheeks); 1, blurred; 2, blurred and stripped; 3, stripped	1, 3
	Opening of the calyx cavity#	FrEye		0, closed; 1 open	3
	Flattening#	FrFlat	O	1, dominated; 2, balanced; 3 dominant	2, 3
	Surface colour	FrGroCol		1, green; 2, light green; 3, yellowish green; 4, light yellow and 5, yellow	1, 2, 3
	Opening of the locules	FrHea	O	0, closed; 1, semi-open; 2 open	2, 3
	Fruit length (mm)	FrL	C	–	2
	Number of loculi	FrLoc	D	Integer counting	1

Continued on next page

Table 3 continued

	Fruit weight (g)	FrM	C	–	1, 3
	Pulp colour	FrMCol		0, white; 1, greenish white; 2, green; 3, yellowish green; 4, white-yellowish; 5, Greenish yellow; 6, yellow	2, 3
	Fruit peduncle length (mm)	FrPedL	C	–	2, 3
	Russeting in the calyx cavity#	FrRCC		0, no russeting; 1, russeting	2, 3
	Russeting on fruit faces#	FrRF		0, no russeting; 1, russeting	2, 3
	Ribs	FrRib	O	from 1, absent; to 5, very prominent	2, 3
	Russeting in the peduncular cavity#	FrRS		0, no russeting; 1, russeting	2, 3
	Peduncular cavity length (mm)	FrSCL	C	–	2, 3
	Peduncular cavity width (mm)	FrSCW	C	–	2, 3
	Number of seeds	FrSeed	D	Integer counting	4
	Fruit shape	FrShp		1, Globose 2, conical globose; 3, wide conical globose; 4, flat; 5, flat globose; 6, conical; 7, narrow conical; 8, conical truncate; 9 ellipsoidal; 10, conical ellipsoidal; 11, oblong; 12, conical oblong; 13, asymmetric	1, 2, 3
	Over colour	FrUpCol		0, without over colour (no cheeks); 1, white; 2, yellowish; 3, yellow; 4, orange; 5, reddish-pink; 6, red; 7, purple; 9, brown	1, 2, 3
	Vitrification (or watercore)	FrVitr		0, absent; 1, present	4
	Fruit width (mm)	FrW	C	–	2
	Bloom of skin	FrWax	O	1; weak; 2, moderate; 3, intense	2, 3
	Peduncle width (mm)	FrWP	C	–	2, 3

(LeArea) with leaf width (LeW; 0.91), petal length (FlPetL) with petal width (FlPetW; 0.81) and fruit weight (FrM) with fruit width (FrW; 0.92) and fruit length (FrL; 0.71).

Some significant correlations were negative, such as the one found between the fruit peduncle length (FrPedL) and peduncle width (FrWP; -0.68, Figure 2b), and the length of the apical bud (ShL) with peduncle width (FrWP; -0.63). Correlations between two different organs were also positive, for example between the fruit peduncle length (FrPedL) and flower pedicel length (FlPedL; 0.86) and with length of the apical bud (ShL; 0.72). We found some negative significant correlations, such as the ones that involved the flower pedicel length (FlPedL) with peduncle width (FrWP; -0.63) and with the length of the apical bud (ShL; -0.55).

Multivariate analysis

The two multivariate analyses showed that the morphological diversity of apple references was lower than the one from old cultivars. Results from PCoA (Table 5) showed that the first three PCos explained 31% of the variability assessed, with seven coordinates necessary to reach 50% variability. By coordinates, PCo 1 represented 14% of the variance, PCo 2 10%, and PCo 3 7%. The most important correlations in PCo 1 were negative: outstanding fruit weight (FrM; -0.76), fruit length (FrL; -0.75), leaf area (LeArea; -0.75), peduncular cavity width (FrSCW;-0.74) and leaf width (LeW; -0.71). In PCo 2, the balance between negative and positive correlations was similar, with important correlations for peduncle width (FrWP; -0.77), peduncular cavity width (FrSCW; -0.740), leaf width (LeW; -0.71), length of the apical bud (ShL; 0.70), flower pedicel length (FlPedL; 0.67) and lenticels (ShLent; 0.62). In PCo 3, the strongest positive correlations involved the maximum width of the leaf blade (LeMWL; 0.65), leaf length (LeL; 0.54), and apical shoot shape (ShShp; 0.49). Among the negative correlations in PCo 3, most noticeable were the width of the apical bud (ShW; -0.51) and over colour (FrUpCol; -0.48). In the plot that represents apple individuals in PCo 1 and PCo 2 (24% of variance), we detected that clonal replicates of references were closely grouped while old apple cultivars appeared separated from apple references, but no further structure was detected in the traditional pool (Figure 4). Despite their low structure in the plot, 'Agridulce' (MAMO1 and MAMO2) and 'Hojancas' (MHHO1) were clearly distinct from the rest of the cultivars thanks to quantitative descriptors such as leaf area (LeArea), fruit weight (FrM) and fruit width (FrW). Finally, 'Verde Doncella' with VDCA, VDHO and VDTI clustered together.

In the cluster analysis, the two 'Agridulce' individuals (MAMO1 and MAMO2) split off very early from the rest of the individuals (Figure 5). They were followed by a 'Rojillo temprano' (RTEV1), two 'Pero de Aragón' (PAPR2 and PAPU1), a 'Camueso tardío' (CTEP1), a 'Pero gordo' (PGHI2), and a 'Temprano' (TEPI1). The rest of the dendrogram was structured in three clusters. The first cluster was composed of two 'peros' (PPPU2 and PRBU2), two 'reineta' ('Reineta Blanca' and REPR2), 'Hojancas' (MHHO1), and one 'Camueso' (CAPR1). The second cluster contained 28 individuals, including the two reference 'Verde Doncella' (VDHO2). Here also appeared a pool consisting of the cultivar 'Esperiega' (ESPU1, ESPU2), 'de Chapa' (CHVA1, CHCA1, and CHCA2) and 'Pepita de melón' (PECA2, PEVA2), as well as a group that nested the cultivars 'Rojo' and 'Rojillo' (RJHO1, RJPU1, RJPU2, and RJPR2). This second cluster also included a 'Camuesa' (CABU2) and the three remaining individuals of 'Pepita de melón' (PEHI1, PEHI2, and PECA1). The third cluster was composed by references ('Fuji', 'Gala', 'Golden', and 'Granny Smith'), as well as one 'Reineta' (REPR1) and two 'Pero de Aragón' (PAPR1 and PAPU2).

Discussion

Phenotypic diversity

The results of the present morphological analysis of shoots, leaves and flowers were similar to other morphological studies (Božović *et al*, 2015; Hassan *et al*, 2017). Fruit size measures were highly variable, especially fruit length (FrL), fruit weight (FrM) and fruit width (FrW). Although averages of these three descriptors were similar to results reported by other works, the registered range was larger than those described in studies by Mratinić *et al* (2011), Özrenk *et al* (2011), Király *et al* (2012), Božović *et al* (2015), Pérez-Romero *et al* (2015) and Posadas-Herrera *et al* (2018).

According to Pereira-Lorenzo *et al* (2003), Gaši *et al* (2011), Božović *et al* (2015) and Pérez-Romero *et al* (2015), quantitative descriptors related to apple cavities (FrSCL, FrSCW, FrCCL, FrCCW) are informative because they are genetically controlled. Those four descriptors detected statistically significant differences among studied cultivars, but such significance may be due to their correlation to fruit length (FrL) and fruit width (FrW). In fact, the depth of the calyx cavity (FrCCDep), a qualitative descriptor that relativizes the calyx cavity width and the calyx cavity length with the global size of the fruit, showed an intermediate diversity, since the depth of this cavity was found to be 'intermediate' in many of our studied old apple cultivars, similarly to Božović *et al* (2015). Furthermore, no 'external' cavity was found neither in our collections nor in Božović *et al* (2015), being 'Sisa' the unique old apple cultivar reported with such characteristic (Zovko *et al*, 2010).

Related to fruit shape, our results agree with Božović *et al* (2015), since their predominant shapes were 'conical' and 'obloid', with some presence of 'ellipsoidal' and 'globose'. Nevertheless, not all studies reported conical shapes as dominant, as Pırlak *et al* (2003) found that the 'flat', 'conical' and 'spherical' shapes were all abundant. Similarly, Hassan *et al* (2017) reported predominantly the shapes 'globose', 'obloid'

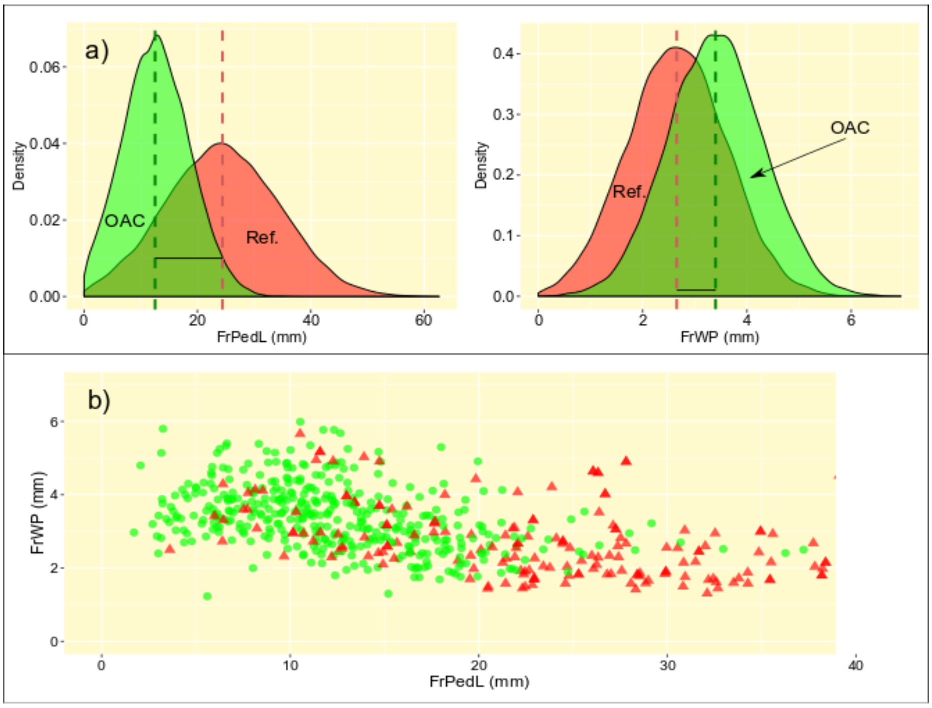

Figure 2. Diversity in the morphological descriptors of fruit peduncle length (FrPedL) and peduncle width (FrWP): a) density model of fruit peduncle length (FrPedL; left) and peduncle width (FrWP; right) from each mean and variance. Curves were computed with the rnorm(20000, $\bar{x}$, s^2) code from R. Red, reference cultivars (Ref.); Green, old apple cultivars (OAC); b) significant negative correlation between both continuous descriptors. Red triangles, reference cultivars; green circles, old apple cultivars.

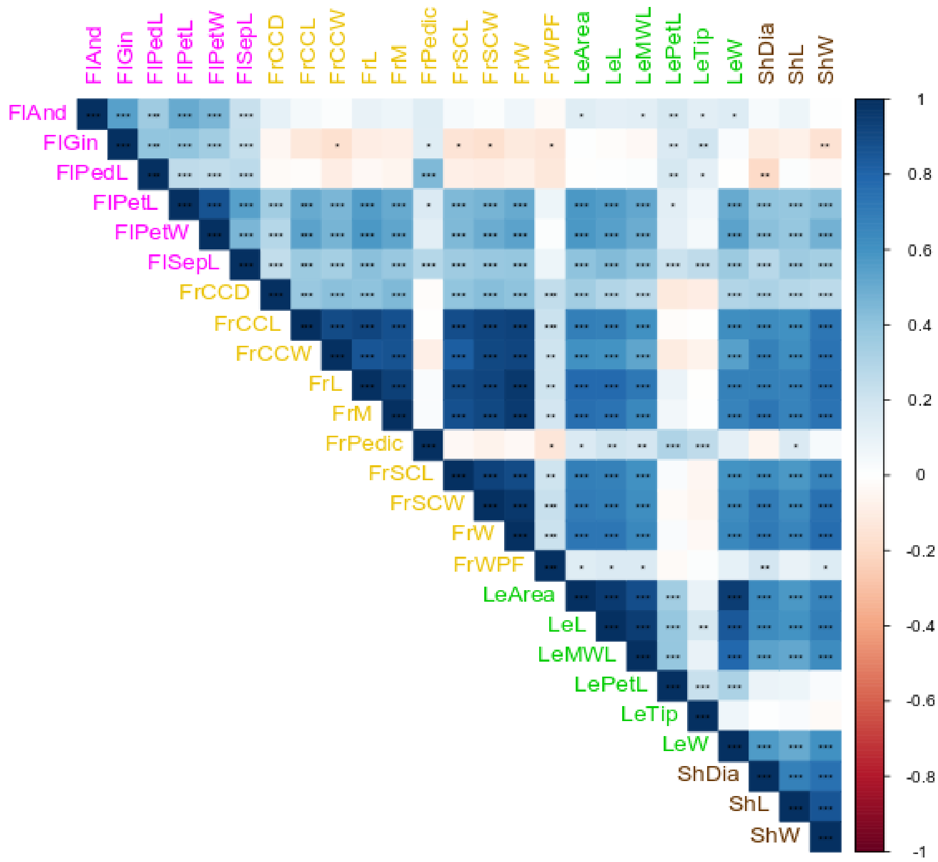

Figure 3. Correlation matrix plot for continuous and ordinal apple descriptors assessed on old traditional apple cultivars. Shoot colour (ShCol) could not be computed due to a lack of variation at a tree level. Descriptors are coded as in Table 3. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$; no asterisk, non-significant correlation.

Table 4. Central statistics of the 32 significant descriptors analyzed by type of cultivar (references and old apple cultivars). Descriptor codes are as used in Table 3. C, continuous; D, discrete; N, nominal; O, ordinal; Ref., reference cultivar; OAC, old apple cultivar; Sig., significance; ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05.

Organ	Descriptor	Type	Mean		Median		Mode		P-value	Sig.	Cohen's d
			Ref.	OAC	Ref.	OAC	Ref.	OAC			
Shoot	ShCol	N					Brown		2.69×10^{-5}	***	-
	ShDia	C	4.95±0.06	6.81±0.07					8.74×10^{-94}	***	Large
	ShL	C	6.75±0.08	5.41±0.05					1.74×10^{-41}	***	Large
	ShW	C	4.54±0.05	4.13±0.04					6.63×10^{-14}	***	Medium
Leaf	LeFold	N					Folded		0.003	**	-
	LeMWL	C	4.48±0.17	4.72±0.07					0.013	*	Small
	LeTip	C	0.62±0.03	0.68±0.01					0.006	**	Small
Flower	FlAnd	C	10.46±0.53	9.96±0.24					0.001	**	Small
	FlPedL	C	17.43±0.93	8.66±0.25					2.20×10^{-16}	***	Large
	FlPetL	C	23.28±1.18	21.85±0.52					0.001	**	Small
	FlPetN	D			Five				0	***	-
	FlPetW	C	16.27±0.82	15.58±0.38					0.023	*	Small
	FlSepL	C	7.54±0.39	7.01±0.17					0.014	*	Small
	FlShp	N					Flat		0.034	*	-
Fruit	FrCCD	C	5.88±0.22	3.81±0.13					4.26×10^{-24}	***	Large
	FrCCDep	O			Strong	Intermediate			0.006	**	-
	FrCCL	C	11.93±0.38	10.44±0.26					3.47×10^{-10}	***	Medium
	FrCCW	C	24.82±0.76	24±0.57					0.016	*	Negligible
	FrDisCol	O			Blurred	Uniform			0.012	*	-
	FrGroCol	N					Yellow		2.46×10^{-10}	***	-
	FrL	C	57.35±1.71	55.57±1.29					0.003	**	Small
	FrLoc	D			Five				2.20×10^{-16}	***	-
	FrM	C	131.87±4.47	124.37±3.27					0.028	*	Negligible
	FrMCol	N					Greenish-yellow		6.12×10^{-05}	***	-
	FrPedL	C	24.36±1.01	12.54±0.38					5.57×10^{-37}	***	Large
	FrRS	N					Presence		0.017	*	-
	FrSCL	C	2.42±0.47	3.64±0.29					7.67×10^{-20}	***	Medium
	FrSCW	C	29.71±0.91	27.94±0.67					2.64×10^{-05}	***	Small
	FrUpCol	N					Absent		2.69×10^{-06}	***	-
	FrVitr	N					Absent		-	***	-
	FrWax	O			Very intense				0.011	*	-
	FrWP	C	2.65±0.10	3.42±0.09					7.37×10^{-18}	***	Large

and 'ellipsoid', finding only one conical old apple cultivar. Although a great morphological diversity in apple shapes is generally reported, probably some of these differences could be attributed to the high subjectivity of this descriptor (Currie *et al*, 2000).

Discrepancies in shape should have affected other descriptors such as flattening (FrFlat), but our results were similar to those reported in other collections (Božović *et al*, 2015; Salkić *et al*, 2017), leading us to consider that old apple cultivars are, in general, wider than longer. We also support this conclusion, as although fruit length (FrL) was larger in references, no significant differences between types of cultivars were found in fruit width (FrW).

Apple skin colour is supposed to be a distinctive trait in apple cultivars. The fruit ground colour (FrGroCol) ranged in our collection from 'greenish white', 'green', 'greenish yellow' to 'yellow', similarly to Božović *et al* (2015), Mišić (2002) and Zovko *et al* (2010). Regarding over colour (FrUpCol), apples herein described were mainly cheekless, as the most common level for this descriptor was 'absent'. Therefore, the studied old apple cultivars have a more uniform colour than those reported in the collection of Božović *et al* (2015) and Šebek (2013). Concerning our cheeked apples, the most common colour was 'red', as in Mratinić *et al* (2012) and Božović *et al* (2015).

Correlations

Many of the significant correlations computed were logical, supporting the botanical description of the apple tree (Terpó, 1981; Aedo *et al*, 1998) and agree

Table 5. Correlation coefficients between the first three principal coordinates (PCo) and the morphological descriptors, abbreviated as in Table 3. Eigenvalues below -0.5 and above 0.5 are highlighted in bold.

Descriptor	PCo 1	PCo 2	PCo 3	Descriptor	PCo 1	PCo 2	PCo 3
FlAnd	**-0.59**	0.08	-0.04	FrRib	0.12	-0.09	-0.15
FlDom	-0.26	-0.45	-0.06	FrRS	0.03	-0.21	-0.21
FlGin	**-0.66**	-0.34	-0.03	FrSCL	**-0.68**	0.45	0.07
FlPedCol	-0.13	0.39	-0.22	FrSCW	**-0.74**	-0.26	-0.08
FlPedL	**-0.61**	**0.51**	-0.30	FrSeed	0.10	**0.55**	-0.14
FlPetCol	-0.13	0.23	0.14	FrShp	-0.26	**0.53**	0.25
FlPetL	**-0.60**	-0.01	0.06	FrUpCol	0.08	0.43	-0.48
FlPetN	0.24	-0.15	0.12	FrVitr	0.22	-0.12	0.19
FlPetW	**-0.69**	-0.22	-0.05	FrW	**-0.70**	-0.41	-0.10
FlPub	-0.01	0.07	0.21	FrWax	-0.14	-0.12	0.22
FlRPP	-0.12	-0.34	-0.07	FrWP	0.11	**-0.77**	-0.03
FlSepL	**-0.68**	0.04	0.14	LeArea	**-0.75**	-0.19	0.29
FlShp	-0.13	-0.05	0.38	LeAsim	-0.03	0.10	0.24
FlSti	-0.30	-0.03	-0.30	LeBas	-0.18	-0.44	-0.29
FlTyp	-0.16	-0.14	-0.23	LeCol	-0.22	-0.26	0.15
FlWeld	0.04	0.07	0.29	LeEdg	-0.40	-0.09	0.24
FrCCD	**-0.55**	0.07	-0.44	LeFold	-0.09	-0.06	-0.004
FrCCDep	-0.15	0.18	0.20	LeL	**-0.54**	0.09	**0.54**
FrCCL	**-0.63**	0.12	-0.05	LeMWL	-0.43	0.15	**0.65**
FrCCShp	-0.18	-0.02	0.10	LePetL	-0.04	0.24	0.38
FrCCW	**-0.52**	-0.31	-0.15	LePub	0.19	-0.39	-0.16
FrDisCol	0.10	0.37	-0.37	LeShp	-0.03	0.21	0.44
FrEye	0.08	-0.04	-0.29	LeSti	-0.49	-0.08	-0.47
FrFlat	0.12	**-0.64**	-0.36	LeTip	-0.13	-0.22	0.17
FrGroCol	0.02	0.18	0.40	LeTipShp	-0.17	-0.21	-0.20
FrHea	-0.26	-0.25	-0.13	LeW	**-0.71**	-0.31	0.09
FrL	**-0.75**	0.06	0.17	ShCol	0.29	-0.05	0.18
FrLoc	-0.07	-0.07	0.16	ShDia	0.10	**-0.59**	0.36
FrM	**-0.76**	-0.33	-0.03	ShL	-0.29	**0.70**	-0.30
FrMCol	-0.20	0.46	0.09	ShLent	-0.23	**0.62**	-0.19
FrPedL	**-0.57**	**0.67**	-0.14	ShShp	0.03	-0.13	0.49
FrRCC	-0.21	-0.35	-0.24	ShSPub	0.16	0.19	0.01
FrRF	0.03	0.07	-0.14	ShW	-0.12	-0.002	**-0.51**

with Ganopoulos *et al* (2018) and Farrokhi *et al* (2013), as the strongest correlations occurred among quantitative descriptors and in the same organ. Also, we obtained a strong correlation between leaf area and descriptors related to fruit size, as mentioned by Migicovsky *et al* (2018).

A significant correlation, important in breeding, was detected between the fruit peduncle length (FrPedL) and peduncle width (FrWP). Salkić *et al* (2017) consider that short peduncles are not desirables. We agree with these authors, as probably short and wide peduncles may suffer from lack of growth space, causing some injuries to the fruits and decreasing their commercial quality (Figure 6). Although this behaviour was not deeply studied in this morphological characterization, our observations suggest that correlation analyses help breeders select descriptors that have a lever effect on genetic improvement (Chen and Lübberstedt, 2010; Ganopoulos *et al*, 2018).

Multivariate analysis

The PCoA decomposed the variance of the morphological descriptors analyzed. The sedimentation rate along the PCo is almost identical to the Spanish study of Pereira-Lorenzo *et al* (2003), but it was slower compared to other collections (Gaši *et al*, 2011; Božović *et al*, 2015; Ganopoulos *et al*, 2018). For example, our PCo 1 only gathers 14% of the variance, whereas PCo 1 from Gaši *et al* (2011) gathered almost 30% with 18 descriptors. A slow sedimentation rate does not necessarily indicate that our collection is more diverse than others. Probably, the greater the number of descriptors and accessions analyzed, the slower the sedimentation process tends to be. In fact, our study analyzed the largest number of descriptors, followed by Pereira-

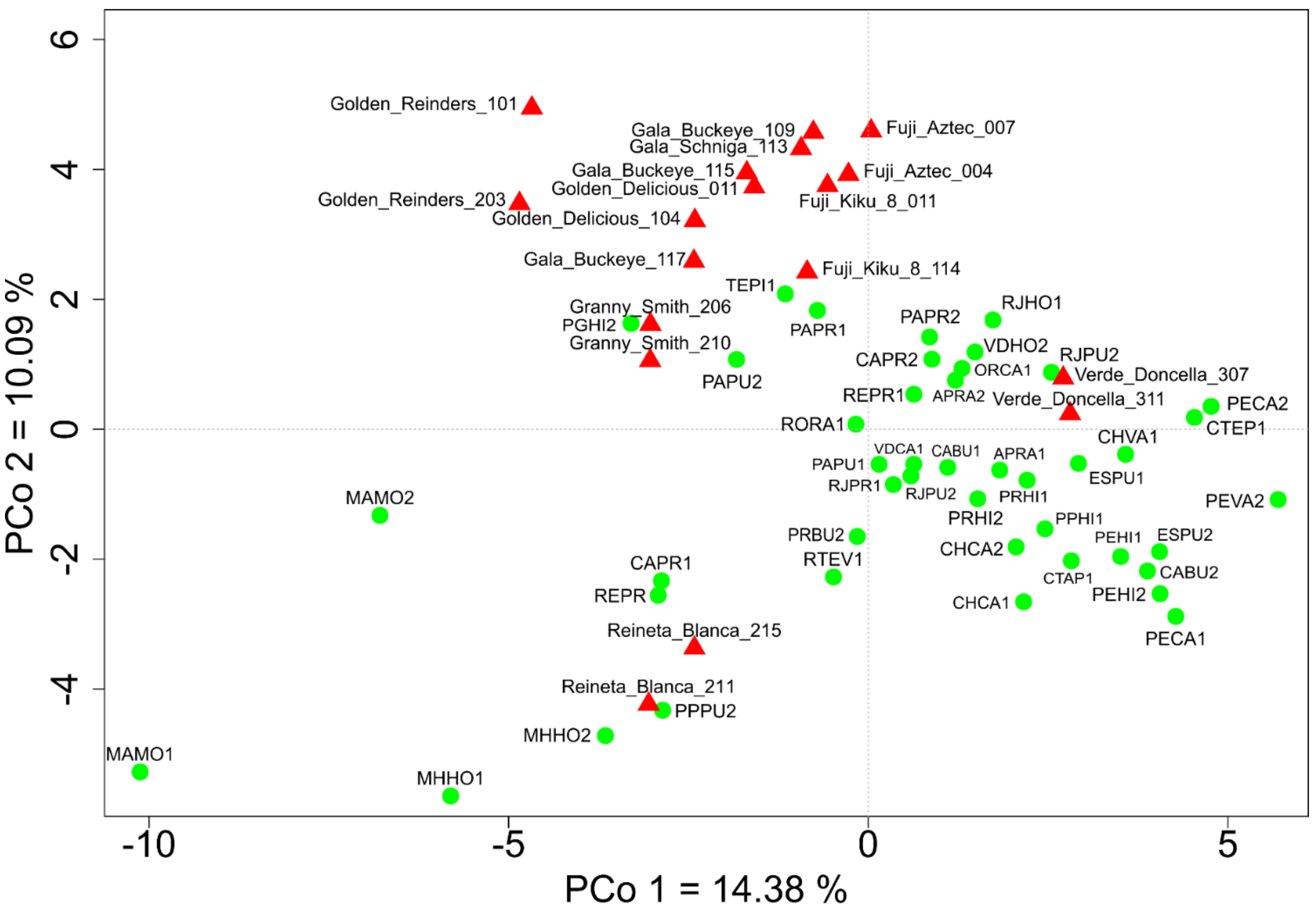

Figure 4. Plot between PCo 1 and PCo 2 for all 67 old apple cultivar individuals and 18 reference individuals from 'Fuji', 'Gala', 'Golden', Granny Smith', 'Reineta Blanca' and 'Verde Doncella' based on morphological descriptors. Red triangles, reference cultivars; green circles, old apple cultivars.

Lorenzo *et al* (2003), who used 49 descriptors in 350 trees.

Regarding PCo eigenvalues, fruit descriptors were usually predominant, especially fruit weight and size. Our results were very similar to other studies, such as Božović *et al* (2015), Gaši *et al* (2011), Farrokhi *et al* (2013) and Pereira-Lorenzo *et al* (2003). Some results reported from other collections do not totally agree with ours. This is the case of Ganopoulos *et al* (2018) where they highlighted other types of fruit characteristics, such as the number of loculi (FrLoc), pulp colour (FrMCol), russeting on fruit faces (FrRF) and calyx opening diameter (FrCCD). The importance of fruit descriptors in the total variance can be explained because it is the organ where selection is performed (Šebek, 2013; Božović *et al*, 2015; Dar *et al*, 2015; Pérez-Romero *et al*, 2015; Salkić *et al*, 2017; Posadas-Herrera *et al*, 2018).

Results from PCoA are consistent with the cluster analysis, as both detect a high morphological diversity in the old apple cultivars. Differences may be due to the lower number of references analyzed, as 'Gala', 'Golden', 'Fuji', and 'Granny Smith' are few, but they are the most widespread varieties in Spain and represent almost the whole apple production (Iglesias *et al*, 2009). In addition, we could discriminate references from old apple cultivars and we found an early separation of 'Agridulce', the presence of two clusters composed of 'de Chapa', 'Esperiega', 'Pepita', and 'Camuesa' and 'Pepita', respectively, and the inclusion of 'Verde Doncella' within old apple cultivars. The closeness of 'Verde Doncella' to other traditional apples is congruent with its breeding history, as this cultivar is autochthonous to Spain (Iglesias *et al*, 2009; Urrestarazu *et al*, 2012; Pina *et al*, 2014).

We did not find further classification in old apple cultivars, as for instance we could not separate 'camuesas' from 'peros'. A clear separation of references from old traditional cultivars without a strong structure has been reported before (Božović *et al*, 2015; Ganopoulos *et al*, 2018), indicating that old apple germplasm is different from references, in contrast with Posadas-Herrera *et al* (2018) and Király *et al* (2015), who could not differentiate between both type of cultivars. Regarding descriptors, no single descriptor can distinguish among cultivars, but some of them may be informative and should be considered in cultivar classification, such as the flower pedicel length (FlPedL), depth of the calyx cavity (FrCCDep), fruit peduncle length (FrPedL) and peduncle width (FrWP).

Difficulties in old apple cultivar classification are probably due to boundaries among old apple cultivars being more diffuse than in modern cultivars, whose

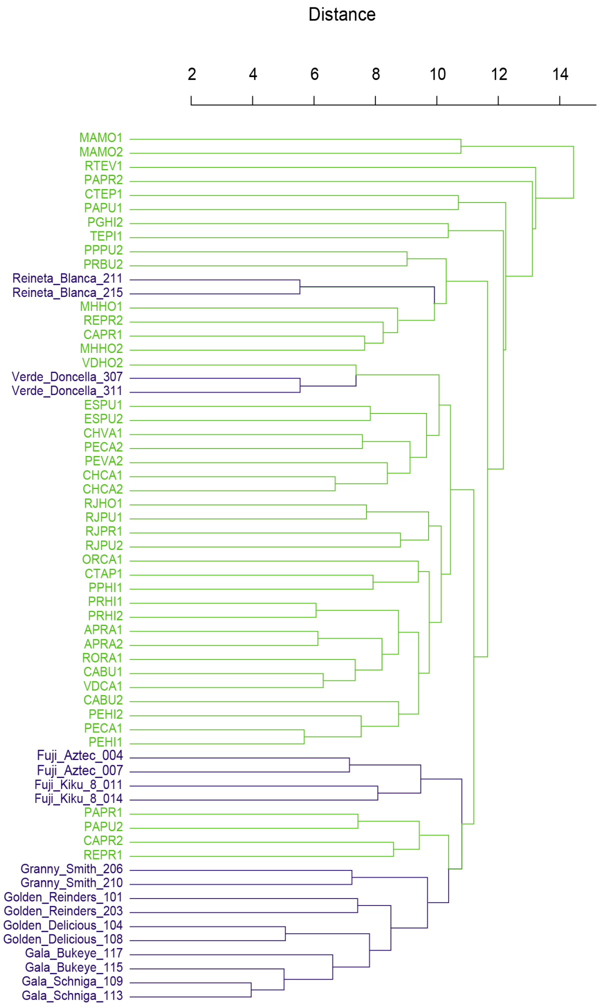

Figure 5. Dendrogram constructed based on unweighted pair group method with arithmetic mean (UPGMA) cluster analysis calculated with the Euclidean distance for all 67 old apple individuals and 18 reference individuals from 'Fuji', 'Gala', 'Golden', Granny Smith', 'Reineta Blanca' and 'Verde Doncella' based on 67 morphological descriptors. Purple, references; green, old apple cultivars. Accession and individuals are numbered as in Table 1 and Table 2.

genealogy is always known (Noiton and Alspach, 1996; Laurens, 1999). For example, 'Crisp Pink' (Pink Lady) derives genetically from 'Golden' and 'Lady Williams' (Iglesias *et al*, 2009). The lack of information on the origin of old apple cultivars is also accompanied by homonymies. Probably, any morphological or sensory trait may be enough to link two cultivars not necessarily parented. For example, flattened apples may evoke a 'reineta', as Martinelli *et al* (2008) found that 'Reineta grigia' was not really a 'reineta'. Something similar was reported by Mratinić and Fotirić (2012), who informed that some accessions named 'Šerbetka' (which means 'too sweet') were later clustered separately.

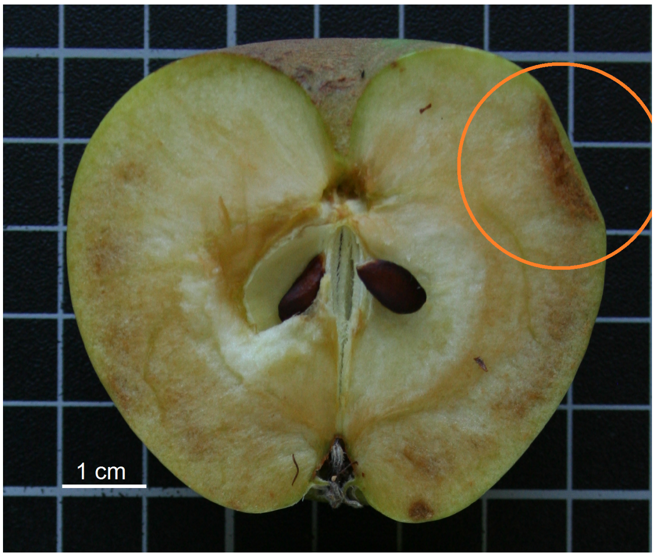

Figure 6. Apple damaged due to lack of growth space, possibly triggered by the presence of a short or wide peduncle.

Support of SSR molecular data in old apple cultivars identities

Morphological and DNA characterization are two complementary techniques, although conclusions about diversity and parental analysis are more robust with molecular analysis (Király *et al*, 2012). Therefore, we recently published a molecular analysis of the same accessions in this study based on 13 microsatellites (Arnal *et al*, 2020) in which we reported germplasm with breeding potential that should be further considered. Interestingly, PCoA and clustering analysis between the two studies are very comparable, as both clearly differentiate references from old apple cultivars and in general no further groups could be defined.

Our two studies pointed out two singular old apple cultivars ('Agridulce' and 'Hojancas'), which may derive from 'reineta'. In the morphological study, these two old cultivars (especially 'Agridulce') showed traits that differentiated them better than microsatellites, as their differential morphology allowed us to segregate them even earlier than in the molecular study. In consequence, they could be considered for *ex situ* conservation and further studies. Moreover, both methodologies closely related 'Camuesa', 'de Chapa' and 'Pepita'. Also, 'Rojillo' and 'Rojo', which seemed synonyms in our molecular analysis, showed similar morphological profiles, as they appeared together in the multivariate analysis. In contrast, the present morphological study does not gather all triploids in a cluster, nor detect the two groups of 'peros' found with microsatellites, as PGHI2, PAPU1, and PAPU2 fell each one in different clusters and one of them (PAPU2) was closely related to PAPR1.

In conclusion: 1) a great morphological diversity of old apple cultivars was detected in rural areas of central Spain; 2) the presented results confirm our previous analysis with microsatellites; 3) both approaches will help to better understand Spanish and global apple genetic resources; 4) the described collection contains two old apple cultivars ('Agridulce' and 'Hojancas') with a very distinct morphology, which may deserve further studies (such as flowering and ripening times, productivity, resistance to pests, etc.); 5) two old apple cultivars ('de Chapa' and 'Pepita') may be a variation of 'Camuesa', and 6) the cultivars 'Rojillo' and 'Rojo' are likely synonyms.

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Author contributions

A. Arnal, J. Tardío and A. Lázaro designed the research. A. Arnal sampled the individuals with the help of J. Tardío and A. Lázaro. A. Arnal adapted the R Code and analyzed the data. A. Arnal, J. Tardío and A. Lázaro wrote the manuscript. A. Arnal, J. Tardío and A. Lázaro revised the manuscript.

Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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