

Phenotypic and molecular characterization of apple (*Malus × domestica* Borkh) genetic resources in Greece

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ABSTRACT: The phenotypic and genetic analysis of apple (*Malus × domestica* Borkh) genotypes is essential for breeding species. Information on the morphology and genetic structure of apple offers significant help for germplasm maintenance and selection of suitable material to breed superior cultivars. This study shows the results of an investigation on the morphology and the genetic diversity for 19 apple cultivars, which are preserved in an *ex situ* collection in Naoussa, Central Macedonia, Greece. Information was recorded over a 5-year period for 47 traits describing plant morphology and phenotype, as well as leaf and fruit quality. Data were analyzed using the principal component analysis (PCA) method. The Euclidean distance metric and the Ward's agglomeration method were used in an unsupervised hierarchical cluster analysis of all cultivars. The cultivars were grouped into four main clusters, suggesting that the characterized apple collection has a high potential for specific breeding goals. Furthermore, the cultivars were genotyped using seven microsatellite primers. Moderate levels of polymorphism were detected, and 38 distinctive alleles (5.4 alleles per primer pair) were identified. Both multivariate clustering approach (phenotypic data) and the genetic distance clustering approach (genetic data) grouped the apple cultivars according to their type. Hence, these data could be used for protection or patenting processes of existing or new apple cultivars carried out by the EU-Community Plant Variety Office.

Keywords: genetic diversity, genotyping, microsatellite markers, multivariate analysis, phenotyping

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Introduction

Malus × domestica Borkh. (apple) is the crop with the highest economic impact of the Rosaceae family (FAO, 2015) and among the most important fruit crops of temperate zones. Traditionally, genetic diversity in apple species or varieties was assessed with morphological descriptors (Reig et al., 2015), commonly produced by the International Board for Plant Genetic Resources (IBPGR, 1983) and later by the International Union for the Protection of New Varieties of Plants (UPOV, 2005). The multivariate data analysis is an efficient approach to analyze large data of apple qualitative phenotypic traits and is considered the most suitable to identify patterns and relationships among powerful statistical techniques, such as the principal component analysis (PCA) and the cluster analysis (Oliveira et al., 2012; Furones-Pérez and Fernández-López, 2009; Ganopoulos et al., 2015; Mehmood et al., 2014).

However, the use of molecular markers to identify the genetic makeup of a species and a variety has become one of the most important means to characterize and manage germplasm collections of fruit species

(Fernández-Fernández, 2010). Microsatellites, or SSRs, are the DNA markers of choice to uncover the genetic diversity between and within fruit species (Gupta et al., 1996), due to their high polymorphism level, abundance and co-dominant inheritance and reproducibility, as well as relative easiness of analysis (Madesis et al., 2013). Previously, many authors used this technique to determine the genetic diversity in order to identify duplicates and genetic relationships within and between *Malus × domestica* accessions (Garkava-Gustavsson et al., 2013; Gaši et al., 2010; Lassois et al., 2015; Liang et al., 2015; Oraguzie et al., 2005; Patzak et al., 2012; Pina et al., 2014; Urrestarazu et al., 2012). Nevertheless, only a few reports have demonstrated basic and detailed data on adaptability and fruit quality traits of *Malus × domestica* cultivars from different geographical areas, especially from species grown under the Greek climatic conditions.

Hence, this work aimed to: (1) determine the variability of different accessions based on phenotypic and fruit quality traits under the south-eastern Mediterranean basin climatic conditions, (2) identify the most important sources of variability of all accessions analyzed

and the associations between the traits evaluated, (3) fingerprint and characterize the Greek germplasm collection using microsatellite markers of five Greek and 14 international cultivars respectively, (4) investigate the relationships among cultivars based on their genetic and phenotypic distances, and finally (5) provide useful information for breeders to select local Greek varieties of *Malus × domestica* for breeding programs, or for producers aiming to diversify apple production.

Materials and Methods

Plant material

We evaluated 19 cultivars (*Malus × domestica* Borkh) from the apple germplasm collection in Naoussa, Central Macedonia, Greece (40°37'13.8" N 22°06'49.2" E, elevation: 130 m) (Table 1).

Phenotypic analysis

Between 2005 and 2010, 47 qualitative traits were evaluated following the established descriptor lists and scores (IPGRI, 1982; UPOV, 2005): Plant: Vigor: 3: weak 5: medium 7: strong (PV), Plant: Type, 1: columnar 2: ramified (PT), Plant: Habit (columnar types excluded), 1: fastigiated 3: upright 5: spreading 7: drooping 9: weeping (PH), Plant: Type of bearing, 1: on spurs 2: on spurs and long shoots 3: on long shoots (PTB), Dormant one-year-old shoot: Pubescence (on upper half of shoot), 1: absent or very weak 3: weak 5: medium 7: strong 9: very strong (DSP), Dormant one-year-old shoot: Thickness (diameter at centre), 3: thin 5: medium 7: thick (DST), Dormant one-year-old shoot: Length of internode, 1: very short 3: short 5: medium 7: long 9: very long (DSL), Dormant one-year-old shoot: Number of lenticels, 3: few 5: medium 7: many (DSNL), Unopened flower: Colour (balloon

stage), 1: white 2: yellowish and pink 3: light pink 4: dark pink 5: red 6: purple (UFC), Flower: Size (diameter of flower with petals pressed into horizontal position), 3: small 5: medium 7: large (FS), Flower: Arrangement of petals, 1: free 2: touching 3: overlapping (FAP), Leaf: Attitude in relation to shoot, 3: upwards 5: outwards 7: downwards (LAS), Leaf blade: Length, 3: short 5: medium 7: long (LBL), Leaf blade: Width, 3: narrow 5: medium 7: large (LBW), Leaf blade: Ratio length/ width, 3: small 5: medium 7: large (LBW/L), Leaf blade: Shape of incisions of margin, 1: crenate, 2: serrate, Petiole: Length, 3: short 5: medium 7: long (PL), Fruit: Size, 1: very small 2: very small to small 3: small 4: small to medium 5: medium 6: medium to large 7: large 8: large to very large 9: very large (FS), Fruit: Height/ width ratio, 1: very small 3: small 5: medium 7: large 9: very large (FH/W), Fruit: Shape, 1: globose 2: globose to conical 3: broad globose conical 4: flat 5: flat globose 6: conical 7: narrow conical 8: truncate conical 9: ellipsoid 10: ellipsoid conical (ovoid) 11: oblong 12: oblong conical (FSh), Fruit: Ribbing, 1: absent or very weak 3: weak 5: medium 7: strong 9: very strong (FR), Fruit: Crowning at calyx end, 1: absent or very weak 3: weak 5: medium 7: strong 9: very strong (FC), Fruit: Aperture of eye, 1: closed 2: partly open 3: fully open (FAE), Fruit: Size of eye, 3: small 5: medium 7: large (FSE), Fruit: Length of sepal, 3: short 5: medium 7: long (FLS), Fruit: Depth of eye basin, 3: shallow 5: medium 7: deep (FDE), Fruit: Width of eye basin, 3: narrow 5: medium 7: broad (FEW), Fruit: Thickness of stalk, 3: thin 5: medium 7: thick (FTS), Fruit: Length of stalk, 1: very short 3: short 5: medium 7: long 9: very long, (FLS) Fruit: Depth of stalk cavity, 3: shallow 5: medium 7: deep (FDS), Fruit: Width of stalk cavity, 3: narrow 5: medium 7: broad (FWS), Fruit: Bloom of skin, 1: absent or very weak 2:

Table 1 – Apple cultivars and their main fruit traits analyzed in this study.

Cultivar name	Origin	Pedigree	Fruit size	Over color	Intensity of over color	Fruit shape
Delicious	U.S.A.	-	Medium	brown	medium	globose
Achilles	Greece	Natural mutation of 'Firiki'	Medium to large	red	light	oblong conical
Odysseus	Greece	'Mutsu' × 'Firiki', Greece	Medium to large	red	light	oblong conical
Firiki Piliou	Greece	-	Medium to large	red	light	globose to conical
Florina	France	'612-1' × 'Jonathan'	Medium to large	orange	medium	globose to conical
Forlady	Italy	'Forum' × 'Lady Williams'	Medium	red	dark	conical
FuJi Zhen Aztec	New Zealand	'Ralls Janet' × 'Delicious'	Very large	red	dark	ellipsoid
FuJi Kiku 8	Italy	'Ralls Janet' × 'Delicious'	Very large	pink	light	ellipsoid
Gala Buckeye	U.S.A.	Kidd's Orange Red' × 'Golden Delicious'	Medium	orange	light	Flat globose
Gala Brookfield	New Zealand	Kidd's Orange Red' × 'Golden Delicious'	Medium	orange	light	Flat globose
Gold Chief	Italy	'Golden Delicious' × 'Starkcrimson'	Medium to large	orange	light	globose to conical
Golden Reinders	Netherlands	-	Medium	orange	medium	truncate conical
Golden Delicious	U.S.A.	-	Medium	orange	medium	truncate conical
Granny Smith	Australia	-	Medium to large	orange	medium	truncate conical
Mutsu	Japan	'Golden Delicious × Indo'	Large	orange	medium	globose
Mirto	Greece	'Golden Delicious' & 'Granny Smith',	Medium to large	orange	medium	truncate conical
Ozark Gold	U.S.A.	Golden Delicious × (Conrad × Delicious)	Medium	orange	light	conical
Scarlet Spur	U.S.A.	-	Medium to large	red	medium	conical
Super Chief	U.S.A.	-	Medium to large	red	medium	conical

weak 3: strong (FBS), Fruit: Greasiness of skin, 1: absent or very weak 2: weak 3: strong (FGS), Fruit: Ground colour, 1: yellow 2: whitish yellow 3: green yellow 4: whitish green 5: green (FGC), Fruit: Amount of over colour, 1: absent or very low 3: low 5: medium 7: high 9: very high (FAC), Fruit: Over colour, 1: orange 2: pink 3: red 4: purple 5: brown (FOC), Fruit: Intensity of over colour, 3: light 5: medium 7: dark (FIC), Fruit: Pattern of over colour of skin, 1: only solid flush 2: only striped 3: solid flush with indistinct stripes 4: mottled 5: washed out (faded) 6: equally flushed and striped 7: predominantly striped with indistinct flush (FPC), Fruit: Amount of russet around eye basin, 1: absent or very low 3: low 5: medium 7: high 9: very high (FARE), Fruit: Amount of russet on cheeks, 1: absent or very low 3: low 5: medium 7: high 9: very high (FARC), Fruit: Amount of russet around stalk cavity, 1: absent or very low 3: low 5: medium 7: high 9: very high (FARS), Fruit: Size of lenticels, 3: small 5: medium 7: large (FSL), Fruit: Firmness of the flesh (measurement with penetrometer), 1: very soft 3: soft 5: medium 7: firm 9: very firm (FFF), Fruit: Colour of the flesh, 1: white 2: cream 3: yellowish 4: pink 5: greenish (FCF), Fruit in cross-section: Aperture of locules (median through locules), 1: closed 2: partly open 3: fully open (FAL), Time of beginning of flowering (10 % open flowers), 1: very early 3: early 5: medium 7: late 9: very late (TBF), Time of maturity for consumption, 1: very early 3: early 5: medium 7: late 9: very late (TMC), (Table 2). Fruits were handpicked at commercial maturity by a single person to ensure consistency of the maturity grade.

DNA isolation

In order to isolate total genomic DNA, we used dry leaves collected from all localities via the cetyl trimethylammonium bromide (CTAB), as described in Doyle (1987). Both quality and concentration of the extracted DNA were determined by ultraviolet spectrophotometry.

Microsatellite analysis

Seven microsatellite markers (02b1, 28f4, 05g8, CH01E12, CH01H01, CH01F02, CH01H10) were chosen from the marker sets recommended by the ECPGR for apple germplasm and were used in this study (Guilford et al., 1997; Gianfranceschi et al., 1998). The markers used were selected based on their informativeness. We selected the markers with the highest Polymorphic Information Content (PIC) and heterozygosity (He) values and the type of their repeat (di-, tri- or more repeat units). Polymerase Chain Reaction (PCR) amplifications were carried out in a reaction volume of 20 µL containing 20 ng of template DNA, 10x PCR buffer, 200 µM of each dNTP, 10 pmol of each primer (forward primer labelled with FAM, NED, PET and VIC fluorescent dyes) and 1 U of Taq DNA Polymerase. PCR amplifications were performed according to Guilford et al. (1997) and Gianfranceschi et al. (1998). The PCR products obtained

were first run in 2 % agarose gel and then loaded into an ABI PRISM 3730x1 DNA sequencer. The program GENEMAPPER was used to genotype the alleles.

Statistical analysis

The data on 19 apple cultivars, including 47 qualitative traits, were analyzed by the XLSTAT software (version 2014.1). The Principal Components Analysis (PCA) method was used to investigate the simultaneous contribution of all qualitative traits to order the apple cultivars in multivariate space. We used the correlation matrix, as standardization was necessary since the variables were measured in different units (quantitative and qualitative). In addition, the PCA on the correlation matrix separated size from shape more effectively (Somers, 1986). Within the PCA, if factor loadings were above 0.55, they were considered significant, since the number of observations was 47 (see also Mehmood et al., 2014). The three most important principle components were used to construct 2D plots for each case to enable visualization of the results. In the correlation analysis, the Spearman coefficient (non-parametric) was used to assess the correlation between qualitative traits. The dendrogram was constructed using the qualitative trait data. The Euclidean distance was chosen to estimate the genetic dissimilarity component and the Ward's method was used for the agglomerative hierarchical clustering (AHC).

The data on microsatellite markers were used to assess the effective number of alleles (Ne), number of alleles (Na), and expected heterozygosity (He) using POPGEN v.1.32 (Krawczak et al., 2006). PowerMarker 3.25 was used to estimate the Nei's genetic diversity and polymorphism information content (PIC) (Liu and Muse, 2005). The power of discrimination (PD), which estimates the probability that two randomly sampled individuals are discriminated, was evaluated for each locus according to Kloosterman et al. (1993): $PD = 1 - \sum (p_i)^2$ where P_i is the frequency of the i^{th} genotype. The probability of identity ($PI = 1 - \sum p_i^4 + \sum \sum (2p_i p_j)$, where p_i and p_j are the frequency of the i^{th} and j^{th} alleles, respectively, calculates the probability that two randomly drawn diploid genotypes are identical assuming observed allele frequencies and random assortment (Paetkau et al., 1995). The total probability of identity, defined as the likelihood of two varieties sharing the same genetic profile by chance, was also calculated from the individual PI values. PI was calculated by IDENTITY 1.0. The UPGMA algorithm was used for dendrogram construction based on similarities between genotypes that were calculated by the Dice's coefficient (Nei and Li, 1979). GENALEX version 6.501 was used for the Principal Coordinate Analysis (PCoA) (Peakall and Smouse, 2012). The analysis was performed in a multidimensional space with data standardization. The Mantel test was performed using GENALEX version 6.501 to identify possible statistical significant correlations between morphological and genetic distances (Peakall and Smouse, 2012).

Table 2 – List of phenotypic descriptors and their results in 19 apple cultivars analyzed in this study.

Phenotypic traits according to CPVO TP 14/1	Gala Buckeye	Golden Delicious	Granny Smith	Mirto	Fuji Kiku 8	Fuji Zhen Aztec	Mutsu	Ozark Gold	Delicious	Super Chief	Gold Chief	Firiki Piliou	Firiki ax	Firiki od	Florina	Forlady	Gala Brookfield	Golden Reinders	Scarlet spur
PV	5	5	7	3	7	7	7	3	5	5	5	7	7	7	7	5	5	5	5
PT	2	2	2	2	2	2	1	1	2	2	1	1	1	1	2	2	2	2	2
PH	5	3	5	5	5	5	3	3	5	3	3	3	3	3	5	5	5	3	3
PTB	2	2	2	2	2	2	2	2	2	1	2	2	2	2	1	2	2	2	1
DSP	3	1	1	1	5	5	3	5	5	5	3	1	1	1	5	7	3	1	5
DST	5	5	5	5	7	7	7	5	5	5	5	3	3	3	5	7	5	5	5
DSLJ	5	7	5	5	7	7	7	3	3	3	3	5	5	5	7	7	5	7	3
DSNL	3	5	3	3	5	5	3	7	7	5	7	3	5	5	3	5	3	5	5
UFC	3	3	4	4	4	4	5	5	6	4	5	4	5	5	3	3	3	3	4
FS	7	7	7	7	7	7	7	3	5	5	7	5	5	5	5	5	7	7	5
FAP	1	2	2	2	2	2	2	1	2	1	2	1	1	1	2	3	1	2	1
LAS	3	3	7	7	5	5	3	3	3	3	3	3	3	3	3	3	3	3	3
LBL	5	5	7	7	7	7	7	3	5	5	5	5	5	5	5	5	5	5	5
LBW	7	7	7	7	7	7	7	7	5	5	7	3	3	3	5	7	7	7	5
LBW/L	7	5	7	7	5	5	3	7	7	7	5	3	3	3	5	7	7	5	7
LBS	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
PL	7	5	5	5	7	7	3	5	5	5	5	7	7	7	7	7	7	5	5
FS	5	5	6	6	9	9	7	5	6	6	6	5	6	6	6	5	5	5	6
FH/W	3	5	3	3	5	5	5	3	5	3	5	9	9	9	5	5	3	5	3
FSh	5	8	8	8	9	9	1	6	1	6	2	2	12	12	2	6	5	8	6
FR	3	1	1	1	7	1	1	1	3	1	1	3	3	3	1	1	3	1	1
FC	5	3	5	5	3	3	3	5	5	5	3	3	3	3	1	3	5	3	5
FAE	2	1	1	1	3	3	2	1	2	3	2	2	2	2	3	3	2	1	3
FSE	5	3	5	5	7	7	5	5	5	7	5	5	5	5	5	7	5	3	7
FLS	5	3	5	5	5	5	5	5	5	7	3	3	3	3	5	5	5	3	7
FDE	5	5	5	5	5	5	7	5	5	5	5	5	5	5	7	7	5	5	5
FWE	5	3	5	5	5	5	7	5	5	5	3	5	5	5	5	7	5	3	5
FTS	5	5	5	5	7	7	5	7	7	7	5	5	5	5	5	5	5	5	7
FLS	5	5	7	7	5	5	5	1	3	3	5	1	1	1	3	3	5	5	3
FDS	7	3	3	3	5	5	7	5	5	5	3	5	5	5	7	5	7	3	5
FWS	5	5	5	5	5	5	7	5	5	7	5	3	3	3	5	5	5	5	7
FBS	1	1	1	1	2	2	1	1	1	1	1	1	1	1	2	2	1	1	1
FGS	1	1	1	1	2	2	1	2	1	1	1	1	1	1	2	2	1	1	1
FGC	3	2	5	5	4	4	5	3	3	3	2	3	3	3	1	1	3	2	3
FAC	7	3	1	1	3	7	1	3	5	9	3	7	7	7	5	7	7	3	9
FOC	1	1	1	1	2	3	1	1	5	3	1	3	3	3	1	3	1	1	3
FIC	3	5	5	5	3	7	5	3	5	5	3	3	3	3	5	7	3	5	5
FPC	6	1	1	1	2	1	1	6	6	3	1	2	2	2	3	1	6	1	3
FARE	1	3	1	1	3	3	1	3	3	1	3	3	3	3	5	1	1	3	1
FARC	1	3	1	1	1	1	1	3	1	1	3	1	1	1	3	1	1	3	1
FARS	3	3	1	1	3	3	1	3	3	1	3	1	1	1	7	3	3	3	1
FSL	3	5	3	3	5	5	3	5	5	3	5	3	3	3	5	3	3	5	3
FFF	5	7	7	7	9	9	7	5	7	7	5	7	7	7	5	7	5	7	7
FCF	3	3	1	1	2	2	1	3	3	1	3	1	1	1	3	2	3	3	1
FAL	2	2	2	2	2	2	2	2	2	2	2	1	1	1	2	2	2	2	2
TBF	7	3	7	7	5	5	3	3	7	5	3	7	7	7	3	5	7	3	5
TMC	5	5	7	7	9	9	5	5	7	5	5	7	7	7	5	7	5	5	5

Results and Discussion

Phenotypic traits of the cultivars

The most important qualitative traits are shown in Table 1. Morphological diversity was observed based on the qualitative trait shape as defined by UPOV. The fruit shape was predominantly conical ('Forlady', 'Ozark

Gold', 'Scarlet Spur', and 'Super Chief'), although other shapes were observed, such as ellipsoid ('Fuji Zhen Aztec', 'Fuji Kiku 8') and globose ('Delicious' and 'Mutsu'). The main over color observed was orange ('Gala Buckeye', 'Gala', 'Brookfield', 'Gold Chief', 'Golden Reinders', 'Golden Delicious', 'Granny Smith', 'Mutsu', 'Mirto', 'Ozark Gold' and 'Florina'). Two cultivars had

hue of over color: 'Fuji Kiku 8' was pink and 'Delicious' was brown.

Correlation of qualitative traits

All 47 qualitative traits analyzed in this study showed a strong positive linear correlation (data not shown). The most significant positive correlation was between length of stalk and flower size (0.918). In addition, we observed a significant positive correlation between leaf blade length and attitude in relation to shoot (0.805), stalk length and leaf blade width (0.765), eye size and pubescence (0.718), eye aperture and pubescence (0.716) and maturity time for consumption and flesh firmness (0.710). However, significantly high negative correlations between some qualitative traits were observed, including the leaf blade: ratio length/ width and fruit: height/ width ratio (-0.858) as well as fruit ground color and russet amount on cheeks (-0.614). These traits, which showed moderate to high correlations, could be a guide to breeding programs and these germplasm sets could be used to improve certain traits and design efficient breeding programs. Trait correlations are very useful, as breeders could use them to ameliorate simultaneously correlated traits by reducing undesired side effects when the focus is only on one of the correlated traits (Chen and Lübberstedt, 2010).

Multivariate analysis of qualitative variables: PCA and AHC cluster analyses

The most important traits that were included in our developed database were identified by the PCA analysis, which, in this case, allows the multivariate statistical analysis of a large number of variables (Ganopoulos et al., 2015; Ganopoulos et al., 2011). The initial variables were transformed to a decreased number of uncorrelated ones in order to reduce the data, allowing relationships between the individuals and variables to be sufficiently revealed. The distribution of cultivars upon PC-1 and PC-2 reveals the phenotypic variation between the tested cultivars, showing the magnitude of their dispersion along both axes (Figure 1). Using the Kaiser's criterion ("Eigenvalue" > 1) (Kaiser, 1958), seven significant components were obtained, which explained 85.56 % of the total variation (Table 2). Eigenvalues measure the amount of variation in the total sample accounted for by each factor. The first component, which accounted for 19.14 % of the total variation, includes the traits of locules departure, thickness of dormant one-year-old shoot and fruit flesh color. The second component, which explained 16.47 % of the total variation, was defined by the russet amount on cheeks, fruit eye size and width of fruit eye basin. The third component, which explained 15.06 % of the total variation, was mainly linked to traits of pattern of fruit skin over color, leaf blade length and length/width ratio of leaf blade. The fourth component accounted for 13.84 % of the total variation, which, in this case, involved fruit skin bloom, fruit skin greasiness and russet amount around the fruit eye basin. The fifth

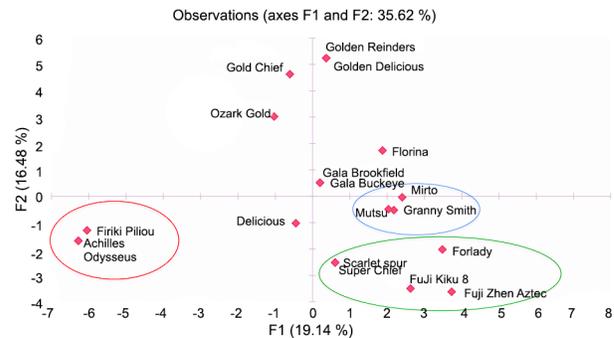


Figure 1 – Two-dimensional PCA plot of the 19 apple cultivars with regard to the first two principal components. Variability explained: F1 (19.14 %) and F2 (16.48 %). The first (F1) and second (F2) PCA axes are based on 47 phenotypic covariates.

component clarified 8.47 % of the total variation regarding fruit stalk thickness and depth of fruit stalk cavity. The sixth component explained 7.58 % of the total variation and included plant habit and depth of fruit eye basin. Finally, the seventh component explained 4.97 % of the total variation and included intensity of over fruit color and plant type (Table 3).

In order to preserve an efficient germplasm inventory, its efficient characterization, both phenotypically and genetically, is a prerequisite. In turn, germplasm characterization facilitates the identification of important phenotypic traits. These traits are commonly linked to genetic data and their use is crucial for breeding programs (Ganopoulos et al., 2015; Ganopoulos et al., 2011). Therefore, the clustering analysis and PCA are key tools to group and evaluate cultivars preserved in GeneBank collections (Iezzoni and Pritts, 1991). The multivariate analysis method has been used to study various germplasm collections of *Prunus* spp. (Ganopoulos et al., 2015; Ganopoulos et al., 2016; Font i Forcada et al., 2014; Khadivi-Khub et al., 2016; Khadivi-Khub et al., 2012; Nikolić et al., 2010; Rakonjac et al., 2010; Ruiz and Egea, 2008) and *Malus × domestica* (Gaši et al., 2011; Pereira-Lorenzo et al., 2003; Reig et al., 2015). Here, the PCA testing revealed that all variables examined showed high scores regarding the first eight principal components. The phenotypic traits that were the main sources of variability in the first principal component are the aperture of locules, thickness of dormant one-year-old shoot and fruit flesh color. These results are in agreement with Pereira-Lorenzo et al. (2003), who studied local apple cultivars in northwestern Spain; Gaši et al. (2011), who studied local and foreign apple cultivars in Bosnia-Herzegovina, and Reig et al. (2015), who studied apple phenotypic diversity in the Germplasm bank of the Ebro Valley area in Spain. Thus, the first seven components explained (85.57 %) of the total variation, which is slightly higher compared with the studies above.

The unsupervised agglomerative hierarchical cluster analysis was used to categorize the data obtained

Table 3 – First seven components from the PCA analysis of 47 qualitative traits in 19 apple cultivars analyzed.

Qualitative trait	F1	F2	F3	F4	F5	F6	F7
Plant: Vigor	0.541	2.565	4.577	1.202	0.401	1.443	0.937
Plant: Type	5.178	0.1	0.295	0.032	1.186	2.795	9.474
Plant: Habit	3.659	1.094	0.114	0.013	5.976	5.489	0.327
Plant: Type of bearing	0.175	0.593	4.423	0.656	0.4	3.535	4.416
Dormant one-year-old shoot: Pubescence	2.803	1.516	3.872	4.437	0.46	0.093	0.93
Dormant one-year-old shoot: Thickness	8.659	0.265	0.147	0.638	0.106	0.146	1.604
Dormant one-year-old shoot: Length of internode	1.426	0.021	6.078	2.154	3.157	0.969	0.724
Dormant one-year-old shoot: Number of lenticels	0.148	0.337	0.617	1.882	13.27	3.255	0.004
Unopened flower: Colour (balloon stage)	1.676	0.672	0.013	0.243	10.089	0.011	12.766
Flower: Size	2.477	0.89	3.779	2.262	1.106	0.239	0.2
Flower: Arrangement of petals	4.781	0.371	3.481	0.877	0.292	0.145	0.126
Leaf: Attitude in relation to shoot	2.52	1.262	3.801	2.027	0.277	3.437	0.003
Leaf blade: Length	2.739	2.08	5.941	1.606	0.01	0.164	0.676
Leaf blade: Width	6.101	2.092	0.41	0.587	0.252	0.615	1.338
Leaf blade: Ratio length/ width	2.843	0.005	5.751	1.697	0.13	3.133	0.971
Leaf blade: Shape of incisions of margin	0	0	0	0	0	0	0
Petiole: Length	0.75	1.847	0.308	3.295	8.773	3.689	0.576
Fruit: Size	0.706	3.755	1.673	0.027	4.643	0.288	4.148
Fruit: Height/ width ratio	3.314	0.06	4.659	4.233	0.13	0.438	0
Fruit: Shape	0.124	0.515	2.973	0.088	0.907	3.588	7.152
Fruit: Ribbing	2.805	1.476	0.091	0.018	3.291	6.23	3.794
Fruit: Crowning at calyx end	0.195	0.178	4.95	7.6	0.055	2.698	0.102
Fruit: Aperture of eye	0.41	5.463	0.714	4.848	0.036	0.417	0.28
Fruit: Size of eye	1.167	8.114	0.826	0.63	0.7	0.043	0.127
Fruit: Length of sepal	3.987	3.104	4.804	0.3	0.029	0.124	0.121
Fruit: Depth of eye basin	1.258	0.062	0.03	3.179	2.066	12.717	0.802
Fruit: Width of eye basin	0.327	6.178	0.156	0.019	1.685	3.703	3.431
Fruit: Thickness of stalk	0.646	2.081	2.434	0.422	8.742	3.567	0.631
Fruit: Length of stalk	5.252	0.705	1.905	3.588	0.472	0.24	0.011
Fruit: Depth of stalk cavity	0.008	1.797	1.99	1.45	7.256	1.437	7.968
Fruit: Width of stalk cavity	5.183	0.012	2.84	0.445	1.656	4.265	0.055
Fruit: Bloom of skin	2.711	1.511	0.873	7.748	0.515	0.398	0.123
Fruit: Greasiness of skin	2.022	0.42	0.022	7.499	0	1.123	0.851
Fruit: Ground color	0.299	3.222	1.092	5.942	0.485	0.112	6.806
Fruit: Amount of over color	1.263	3.017	3.547	1.06	0.571	0.073	7.052
Fruit: Over color	1.211	6.374	0.173	1.086	2.312	0.524	2.539
Fruit: Intensity of over color	4.593	0.27	0.103	0.161	1.021	2.196	8.747
Fruit: Pattern of over color of skin	0.802	0.312	8.178	0.145	1.354	2.622	3.311
Fruit: Amount of russet around eye basin	1.323	1.069	1.193	7.032	1.333	1.823	0.547
Fruit: Amount of russet on cheeks	0.019	9.711	0.02	2.132	0.962	0.028	0.021
Fruit: Amount of russet around stalk cavity	1.697	2.59	0.161	6.216	1.419	4.037	0.621
Fruit: Size of lenticels	0.734	2.993	0.225	4.583	3.71	4.128	1.228
Fruit: Firmness of the flesh	0.245	3.955	4.135	0	4.02	0.21	2.924
Fruit: Color of the flesh	0.597	5.819	0.759	2.468	0.998	4.592	1.153
Fruit in cross-section: Aperture of locules	9.101	0.927	1.144	0.045	0.128	0.086	0.203
Time of beginning of flowering	1.514	3.592	0.017	3.365	3.391	4.08	0.168
Time of maturity for consumption	0.013	5.009	4.705	0.063	0.227	5.052	0.014
Eigenvalue	8.997	7.745	7.081	6.508	3.982	3.56	2.33
Variability (%)	19.14	16.47	15.06	13.84	8.47	7.58	4.97
Cumulative of variability (%)	19.14	35.62	50.68	64.53	73.00	80.59	85.56

in clusters of increasing dissimilarity. The Euclidean distance using the Ward's method for agglomeration was inferred as a metric to estimate the dissimilarity among 19 apple cultivars, based on qualitative data. The relative dendrogram revealed four distinct groups (Figure 2). C1 contained six cultivars ('Gala Buckeye', 'Ozark Gold', 'Delicious', 'Super Chief', 'Gala Brookfield' and 'Scarlet spur'; C2 had six cultivars ('Golden Delicious', 'Mutsu', 'Gold Chief', 'Florina', 'Forlady',

'Golden Reinders'), C3 included four cultivars ('Granny Smith', 'Mirto', 'Fuji Kiku 8' and 'Fuji Zhen Aztec') and C4 contained three cultivars of Firiki group ('Firiki Pilou', 'Achilles' and 'Odysseus'). The maximum distance between the class centroids was observed between C3 and C4 (12.74), followed by C2 and C4 (12.09), C2 and C3 (8.80) and C1 and C2 (8.34). The dendrogram depicted high diversity between apple cultivars, suggesting that an analyzed germplasm collection could be used in

breeding programs as a gene pool carrying important genes for contrasting traits. For example, the cultivars of the Firiki group consisting of 'Firiki Piliou', 'Achilles' and 'Odysseus' were highly diversified from other cultivars. This group is characterized by the following major traits: (1) unopened flower color at the bloom station, (2) leaf blade width, (3) amount of over fruit color, (4) fruit stalk length and (5) thickness of dormant one-year-old shoot. These traits are very important, as they affect both yield and quality of apple fruits and thus are the main traits in breeding programs.

The PCA analysis combined with the unsupervised clustering analysis based on phenotypic and fruit quality traits exposed a wide diversity in apple genetic resources in Greece. In turn, this phenotypic diversity between the apple cultivars is of utmost importance for efficient conservation of traditionally grown plant material.

Molecular characterization using microsatellite markers

The seven microsatellite loci used here amplified 38 alleles (mean 5.42 alleles per locus) showing an average call rate of 99 %. The great level of polymorphism,

which was presented by the seven microsatellites, supported their ability to be used in the diversity analysis. Primer pair ch01f02 showed the highest number of alleles (8 alleles), whereas primer pair 28f4 had the lowest (3 alleles). The number of alleles per locus ranged from 3 to 8, suggesting moderate molecular genetic diversity between the apple cultivars studied (Ferreira et al., 2016).

PIC values ranged from 0.397 for microsatellite locus 28f4 to 0.775 for ch01f02 locus, with an average of 0.644 (Table 4). The highest (0.755) and lowest (0.389) expected heterozygosity (He) values were obtained for ch01f02 and 28f4, respectively, with an average of 0.627. The power of discrimination (PD) showed results from 0.38 (28f4) to 0.86 (ch01f02), with an average of 0.68. Furthermore, the Shannon's information index (I) maximum (1.620) was recorded for ch01f02, while the lowest value (1.299) was recorded for locus 28f4, with an average of 1.335. Our results revealed a decreased number of alleles per locus than those reported previously for apple (Gharghani et al., 2009; Liang et al., 2015; Urrestarazu et al., 2012), which could be attributed to the smaller number of cultivars tested in our study. Moreover, the diversity level of heterozygosity in our collection

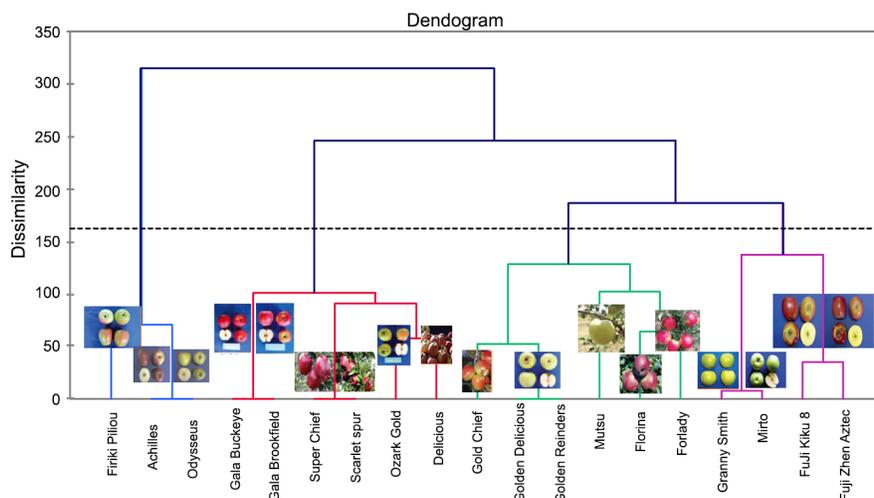


Figure 2 – Dendrogram using agglomerative hierarchical clustering (AHC) for 19 apple cultivars based on 47 qualitative traits.

Table 4 – Genetic diversity parameters for seven polymorphic microsatellites in 19 apple cultivars.

Microsatellite marker	Size range	Species origin	Linkage Group	Na	Ne	I	Ho	He	PIC	PD	F
O2b1	216-238	<i>M. domestica</i>	No position	6	4.011	1.505	0.789	0.751	0.771	0.81	-0.052
ch01h01	111-129	<i>M. domestica</i>	17	6	3.099	1.343	0.684	0.677	0.696	0.76	-0.010
O5g8	111-141	<i>M. domestica</i>	No position	4	1.740	0.813	0.368	0.425	0.437	0.62	0.134
ch01f02	170-208	<i>M. domestica</i>	12	8	4.079	1.620	0.526	0.755	0.775	0.86	0.303
ch01e12	243-283	<i>M. domestica</i>	No position	6	3.374	1.450	0.684	0.704	0.723	0.74	0.028
ch01h10	93-117	<i>M. domestica</i>	8	5	3.282	1.321	0.947	0.695	0.714	0.63	-0.363
28f4	106-120	<i>M. domestica</i>	No position	3	1.900	1.299	0.978	0.389	0.397	0.38	-0.111
Mean	-	-	-	5.42	3.069	1.335	0.711	0.627	0.644	0.68	0.010

Na = Number of alleles; Ne = Effective number of alleles; I = Shannon's information index; Ho = Observed heterozygosity; He = Expected heterozygosity; PIC = Polymorphism information content; PD = Discrimination power; F = Wright's fixation index.

(0.627) is slightly lower compared with heterozygosity (0.73) described for apples from the regions of Asturias, the Basque Country, and Galicia in northern and north-western Spain (Pereira-Lorenzo et al., 2003) and 0.75 in Portuguese apple germplasm collections (Ferreira et al., 2016). In contrast, the diversity observed here was lower than that observed in local cultivars of northeastern Spain (0.82) (Urrestarazu et al., 2012) and 0.80 (Pina et al., 2014), Italy (0.80) (Liang et al., 2015) and Portuguese Azores Islands, (0.81) (Feroni et al., 2012). This result indicates a moderate genetic diversity (Hamrick and Godt, 1996) between the apple germplasm established in a Greek Pomology Institute collection. Possibly, outcrossing and operation of a self-incompatibility system in apple could result in increased levels of heterozygosity and diversity of the cultivars (Mratinić and Akšić, 2012).

The value of the total probability of identity (PI) was low (2×10^{-7}), showing that the seven microsatellites used were powerful enough to discriminate the apple cultivars used. The apple gene pool examined holds significant genetic diversity and demonstrates differentiation between the four genetic clusters identified by the cluster analysis and the PCoA. The results above show that most apple cultivars examined probably have mixed origin and might share common ancestry.

We constructed a dendrogram of genetic similarity based on the SSR profiles by calculating the Dice coefficient in order to confirm the effectiveness of the seven SSRs to distinguish all cultivars. Indeed, Figure 3 shows that two cultivars were not able to be distinguished (cvs. 'Achilles' and 'Odysseus').

The first two coordinates of the PCoA explained 47.59 % of the total variation in a collection of 19 apple cultivars. The first coordinate explains 26.16 % and the second one explains 21.44 % of the total variation, respectively (Figure 4). The results of the UPGMA method were confirmed by the prediction of unique genotypes on two-dimensional multivariate space. Both the unweighted pair-group method with the arithmetic average dendrograms (UPGMA) and the principal coordinate analysis (PCA) plots showed genetic differentiation between the apple cultivars analyzed.

The Mantel test was used to determine the correlation degree of phenotypic and molecular data. The Mantel tests showed no significant correlation between microsatellite markers with the morphology results. Nonetheless, microsatellite markers were not compatible along the clustering analysis with any of the phenotypic traits, possibly because they are not directly associated with the phenotypic traits analyzed (Kalivas et al., 2016). The PCA and the clustering analysis, using various phenotypic data and in combination with molecular data, showed a wide variability in the apple Germplasm Bank from Naoussa, Central Macedonia, Greece. Accurate knowledge of phenotypic and genetic diversity between cultivars is key for the effective use and conservation of traditional material, which faces a high risk of extinction. Moreover, the data could be used

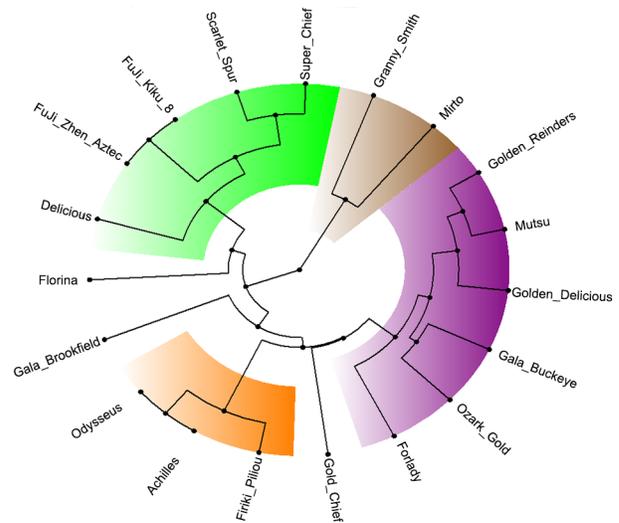


Figure 3 – UPGMA tree showing the genetic relationships between the apple cultivars analyzed in this study.

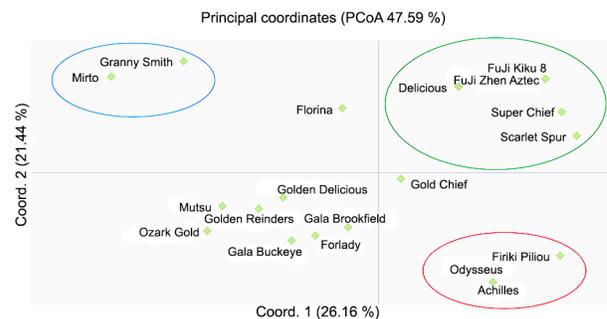


Figure 4 – Principal Coordinate Analysis (PCoA) of the apple cultivars analyzed.

not only for protection, but also in patenting processes of new apple cultivars carried out by the EU-Community Plant Variety Office, such of the PDO cultivar 'Firiki Piliou'. Hence, the cultivars that were comprehensively characterized could be used for gene conservation and for planning an efficient breeding strategy towards favorable apple cultivars with increased adaptation to the Greek climatic conditions and high fruit quality.

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