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Original Article

Morphometric and Molecular Analysis of the Three *Arbutus* Species of Greece

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Abstract

Arbutus andrachne, *A. unedo* and *A.* × *andrachnoides* found in the Greek macchia are promising species for reforestations, ornamental use, as well as for medicinal use and the food industry. Morphological traits and molecular markers (RAPD) were used to identify and distinguish these *Arbutus* species to facilitate their exploitation. Since there are no descriptors established for *Arbutus* spp., 23 qualitative morphological characteristics of crown, foliage, bark, flowering, fruiting, and four quantitative morphological characteristics of leaf and fruit were selected and used to define differences and similarities between sampled individuals of *A. andrachne, A. unedo* and individuals with intermediate characteristics sampled as *A.* × *andrachnoides*. Twenty eight individuals representative of three *Arbutus* taxa were sampled in two typical macchia forest areas of the prefecture of Attica, Greece. Cluster analysis based on morphological characteristics separated the individuals in three distinct groups, and this was confirmed by molecular analysis. The intermediate form was indicated as *A.* × *andrachnoides*, a natural hybrid of *A. andrachne* and *A. unedo.* Fifteen 10-mer oligonucleotide arbitrary primers used to amplify genomic DNA generated 166 reproducible polymorphic fragments, which revealed that *A.* × *andrachnoides* has higher degree of genetic similarity with *A. andrachne* than *A. unedo*. The applied morphometric characteristics are suggested as a basis to develop a complete list of discriminating descriptors for *Arbutus* genus.

Keywords: *Arbutus* spp**.**, molecular markers, morphological traits, PCR, RAPD, strawberry tree, UPGMA

Introduction

Three *Arbutus* species (*Arbutus andrachne* L., *Arbutus unedo* L. and *Arbutus* × *andrachnoides* Link, f. Ericaceae), typical members of the Greek macchia, are promising for exploitation in forestry, landscape, horticulture and pharmaceutical industry. *A. unedo* (strawberry tree) and *A. andrachne* (Eastern or Greek strawberry tree) may be a vicariant pair, as it is isolated geographically from other *Arbutus* species (Torres *et al*., 2002; Blondel *et al*., 2010). *A. unedo* is used and *A. andrachne* has the potential for use as an ornamental shrub in the urban and suburban landscape (Bertsouklis and Papafotiou, 2013), while both are recommended species for reforestations in Mediterranean region, where fires are common, as they are characterized by vigorous re-sprouting after fire and overgrazing (Konstantinidis *et al*., 2006; Moreno-Jiménez, 2008; Kazanis *et al*., 2012). They both have high medicinal value (Diba *et al*., 2010; González *et al*., 2010; Afifi-Yazar *et al*., 2011; Mendes *et al*., 2011; Etxeberria *et al*., 2012; Abidi *et al*., 2015; Güler *et al*., 2015), while strawberry tree fruits are an excellent source of vitamin C, dietary fiber and total available carbohydrates, sugars, potassium and secondary metabolites, such as phenolic compounds, being poor in lipids and Na, with ascorbic acid and fat-soluble antioxidants (Ruiz-Rodríguez, 2011; Mosele *et al*., 2016) of higher values than other red small fruits (Guerreiro *et al*., 2013). They are also used for preparing liqueurs and aromatic traditional distillate (Soufleros *et al*., 2005; González *et al*., 2011; Botelho *et al*., 2015). Strawberry tree fruits can be stored at low temperature preserving their quality (Guerreiro *et al*., 2013; Fadda *et al*., 2015) and they could be introduced in the market as fruits and/ or as a source of bioactive compounds for dietary supplements or functional foods used as components in yoghurts, in cereal bars, in pastry making and confectionery, as flavors or in pieces, like other berries (Ayaz *et al*., 2000; Alarcão-e-Silva *et al*., 2001; Miguel *et al*., 2014).

A. × *andrachnoides* Link is also native of the eastern Mediterranean region including Greece (Arabatzis, 2001; Torres *et al*., 2002), it is considered a natural hybrid form between *A. unedo* and *A. andrachne* and it is very difficult to distinguish from *A. andrachne* (Bačić *et al*., 1992; The European Garden Flora, 1997). Apart from forestry and

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landscape use it is recommended particularly for cut foliage production because its growth speed is higher than the other two species, has lower production of flowers and fruits and prolonged (90-d) vase life (Cervelli *et al*., 2012).

Individuals with intermediate morphological characteristics are not necessarily hybrids. Morphological traits in combination with molecular markers (RAPDs) are used to assess genetic variability in plant species or to identify hybrids (Yüzbaşıoğlu *et al*., 2008; Hršak *et al*., 2011; Ferreyra *et al*., 2013; Song *et al*., 2015). In recent years RAPD have been used for studies between different *A. unedo* genotypes (Takhrouni and Boussaid, 2010; Lopes *et al*., 2012; Gomes *et al*., 2013). There are also a few references on morphological traits used for diversity studies in *A. unedo* in Italy (Mulas *et al*., 1998), Turkey (Celikel *et al*., 2008) and Portugal (Lopes *et al*., 2012). Koukos *et al*. (2015) studied the surface microscalpture and waxes of Greek *A. unedo* and *A. andrachne,* this being the only information on genetic diversity of Greek *Arbutus* spp.

The objective of the present study was to test both morphological traits (quantitative and qualitative) and molecular markers in order to develop a set of proper descriptors to identify and distinguish *Arbutus* species found in Greece. The results could be used both for conservation strategies and cultivation of specific genotypes with particular characteristics.

Materials and Methods

Sampling areas and protocol for sample plants selection

Individuals, representatives of the two *Arbutus* species, *A. unedo* and *A. andrachne*, as well as individuals with intermediate characteristics, were sampled for identification in the years 2011-2013 (monthly recordings from October to May), at two typical macchia forests of the prefecture of Attica, Greece, one in Kalamos and another in Varympompi, at 450 and 400 m altitude, respectively. In macchia forests of Greece *A. unedo* and *A. andrachne* are found mixed, in large populations, while individuals with intermediate characteristics believed to be their natural hybrid *A.* × *andrachnoides* are randomly occurring between these parental species. In each forest a sampling area of about 2 km^2 was selected. The geographic coordinates (vertices of a quadrangle) of the Kalamos sampling area were: 38°16'04.2"N - 23°51'46.9"E, 38°15'50.8"N - 23°51'43.1"E, 38°15'52.7"N, 23°52'07.1"E, 38°16'09.6"N - 23°52'14.4"E and those of the Varympompi were: 38°08'02.5"N - 23°47'48.9"E, 38°08'13.9"N - 23°47'58.3"E, 38°08'30.5"N - 23°47'43.0"E, 38°08'07.4"N - 23°47'36.5"E.

In each area five *A. unedo* and five *A. andrachne* shrubs, as well as four shrubs with intermediate characteristics were selected being 2-3 m in height and 2-3 m in diameter (at 150 cm from the ground). The shrubs were re-sprouted after fires that occurred in 1990 at Kalamos and 1986 in Varympompi. The selected shrubs were at least 100 m apart from each other and were found along the existing paths (five - six paths) of each sampling area at 0-50 m distance left and right of the path.

Phenotypic classification to three groups

The identification of the sampled individuals was followed by the classification into three groups including individuals with typical morphological characteristics of *A. andrachne* (Kalamos: no 1-5, code $A_{1\text{-}5}$ K, Varympompi: no 6-10, code $A_{1\text{-}5}$ V), individuals with typical morphological characteristics of *A. unedo* (Kalamos: no 11-15, code $U_{1\text{-}5}$ K, Varympompi: no 16-20, code $U_{1\text{-}5}$ V), and individuals with intermediate phenotype (Kalamos: no 21-24, code $H_{1\text{-}4}$ K, Varympompi: no 25-28, code $H_{1\text{-}4}$ V), which were assigned to the hybrid group (H).

Morphological traits

A total of 27 morphological traits of crown, leaf, flower and fruit were recorded for each individual; 23 qualitative descriptors (1-23) and five quantitative (24-27) were used (Table I) based on descriptors used for other woody plant species including those used by Lopes *et al*. (2012) for *A. unedo*. Hundred leaves from each individual (10 replications of 10 leaves each) were used to estimate length/width and leaf area (LI-3100 scanner, LI-COR Biosciences, USA). Mature, fully expanded leaves derived from nodes six to nine, of one year old shoots (15 cm length) were selected in early November from the SE-side of the shrubs. Hundred mature fruits derived from each individual were used to estimate fruit diameter. For 24 to 27 characteristics took place customization of characteristics (values 1, 2, 3).

DNA extraction

Young leaves were collected in early May from each individual, transported in dry ice and stored at -80 °C. Genomic DNA was extracted from 0.1 g of leaves using the Dneasy Plant Mini Kit (Qiagen). Amplification reactions were performed according to Stavrakakis and Biniari (1998), using the same PCR apparatus. Fifteen 10-mer oligonucleotide arbitrary primers were used (Table 2) for the amplification of RAPD sequences out of which 12 primers that could generate discernible and reproducible bands was selected for formal amplification. All reactions were repeated at least twice with independently isolated genomic DNA as templates.

Gel electrophoresis

Aliquots (20 μl) of the RAPD products were analyzed in 2% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8). After staining in ethidium bromide (1 μg ml^{-1}) the gels were photographed on a Gel Doc 1000 (Biorad). A mix of λ DNA Hind III × *Hae* III was used as size marker. All of the reactions were repeated at least twice with independently isolated genomic DNA as templates.

Data analysis

The matrix of average taxonomic distance for individuals was computed using the Euclidean distance coefficient. Cluster analysis was conducted on the taxonomic distance matrix with the Unweighted Pair Group Method based on Arithmetic Average (UPGMA) and the dendrograms were generated based on the genetic distance matrix. One-way ANOVA was used for determination of the differences between the mean values of the leaf traits. Genetic similarities were calculated using the Simple Match (SM) and Jaccard (JAC) coefficients described by Sokal and Sneath (1963). The RAPD data were used to generate two genetic distance matrices and dendrograms were generated using the same methods used with the morphologic data.

Principal coordinate analysis (PCA) was used in order to verify cluster analysis and to assist in visualizing the data. Mantel test (Mantel, 1967) was used to estimate the correlation between the distance matrices resulting from morphological, RAPD and combined analyses. Statistical analysis of morphological and RAPD markers was conducted by the software NTSYS-pc version 2.11f (Rolf, 1992).

Results and Discussion

Morphological characteristics

In the two study areas a few individuals with a mixture of intermediate morphological characteristics between *A. unedo* and *A. andrachne* were found, resembling more to *A. andrachne*. The distinction was mainly based on the morphology of the bark that was smooth and cinnamon-red in color, peeling in small strips, every year, with high rate, revealing a grey-green internal like the bark of *A. andrachne,* with the difference that *A. andrachne* bark was peeling in long strips (Table I). The bark of *A. unedo* was totally different being rough, ash-grey and occasionally peeling in small flakes revealing a chestnut-colored internal; *A. unedo* individuals were strong branching from base in contrary to other individuals.

The leaves of plants with intermediate morphological characteristics were leathery and elastic, shiny, with dark green colour on the upper side, like *A. andrachne* leaves; *A. unedo* leaves were membranaceous-rough, not shiny with green colour of upper side. Intermediate individuals had serrate leaves similar to *A. unedo* and their shape was either like *A. unedo* (elliptical to obovate) or *A. andrachne* (elliptical to oblong lanceolate). Petiole length and fruit diameter of intermediate individuals were also counted between *A. andrachne* and *A. unedo*. These characteristics of plants with intermediate characteristics match with descriptions of *A.* × *andrachnoides* the natural hybrid between *A. andrachne* and *A. unedo* (Arabatzis, 2001).

It was also observed that the flowering period of intermediate individuals was either like *A. andrachne* (January-April) or like *A. unedo* (October-January). Fruiting time was the same (October-January) in all species. Fruits of intermediate individuals were spherical, red-orange, with slightly granular surface like *A. andrachne* in contrast to granular surface of *A. unedo*. Fruit taste of intermediate individuals was sweet, like *A. unedo,* while *A. andrachne* was astringent.

Leaves of *A. andrachne* were larger than those of individuals with intermediate characteristics and *A. unedo* leaves were the smallest of all. Locality did not affect leaf length and leaf area (Table 3). Principal coordinate analysis confirmed the results and four components have been arranged in a decline series of their importance, explaining 98.92% of the total variability among the different individuals. All suggested descriptors (Table 1) that are involved in the same principal component are strong correlated. Descriptors 2 (vegetation density), 4 (branching), 5 (bark surface), 6 (external bark colour), 7 (bark peeling), 8 (peeling time), 9 (peeling rate), 10 (internal bark colour), 12 (leaf texture), 13 (boat-shaped leave), 16 (leaf colour of upper side), 21 (fruit surface) and 27 (fruit diameter) contributed better to variability compared to descriptors 17 (flowering) or 26 (fruit diameter); qualitative characteristics were strong correlated while quantitative characteristics were found to be more variable (Table 4, Fig. 1).

Cluster analysis separated the individuals according to their morphological characteristics in three branches; individuals H were found to be distinct from A or U but closer to A (Fig. 2) and the Mantel test comparing Euclidean values matrix and cophenetic matrix gave very high value (r=0.965). The analysis of the above data indicated that most probably the individuals with intermediate characteristics are the hybrid form *A.* × *andrachnoides*.

Table 1. Morphological traits of *Arbutus* individuals

Code	Morphological Trait (descriptor)	Score code - descriptor state		
1	Growth Habit	1. Shrub	2: Tree	
2	Vegetation Density	1: Medium	2: Strong	
3	Regeneration after pruning	1: High	2: Little	
4	Branching from base	1: Medium	2: Strong	3: Very strong
5	Bark surface	1: Smooth	2: Rough	
6	Bark colour (external)	1: Cinnamon-red	2: Ash-grey	
7	Bark peeling in	1: Long strips	2: Short strips	3: Small flakes
8	Peeling every year	1: Yes	2: No	
9	Peeling rate	1: High	2: Low	
10	Bark colour (internal)	1: Grey-green	2: Chestnut	
11	Leaf persistency	1: Evergreen	2: Deciduous	
12	Leaf texture	1: Leathery-elastic	2: Membranaceous-tough	
13	Boat-shaped leave	1: Yes	2: No	
14	Leaf shape	1: Elliptical to oblong lanceolate	2: Elliptical to obovate	3: Intermediate
15	Leaf margin	1: Entire	2: Serrate	3: Lightly serrate
16	Leaf colour of upper side	1: Green	2: Light green	
17	Flowering	1: October-January	2: January-April	
18	Flower colour	1: White-greenish	2: Other	
19	Fruiting	1: October-January	2: Other	
20	Fruit shape	1: Spherical	2: Elliptical	
21	Fruit surface	1: Granular	2: Lightly granular	
22	Fruit colour	1: Orange - red	2: Other	
23	Fruit taste	1: Sweet	2: Astringent	
24	Leaf ratio (x/y) (cm)	1: 1.32-1.78	2: 1.78-2.24	3: 2.24-2.70
25	Leaf surface (cm^2)	1: 10.30-15.00	2: 15.10-19.80	3: 19.90-24.60
26	Petiole length (cm)	1: 0-1	2: 1-2	3: 2-3
27	Fruit diameter (cm)	1: 1.10-1.40	2: 1.40-1.90	3: 1.90-2.90

Table 2. Primer sequences, number of bands per primer, number of polymorphic bands per primer and the approximate band size range

Primer	Nucleotide Sequence (5′ to 3′)	No. of bands	No. of polymorphic bands	Percentage of polymorphic bands (%)
1224*	CAGGCCCTTC	15	10	66.67
1225*	AGGTGACCGT	9	6	66.67
1226*	CGCAGGATGG	8	4	50.00
1227*	GTGTGCCCCA	11	4	36.36
OPF-01	ACGGATCCTG	21	12	57.14
OPF-02	GAGGATCCCT	17	12	70.58
OPF-03	CCTGATCACC	16	10	62.50
OPF-04	GGTGATCAGG	16	11	68.75
OPF-06	GGGAATTCGG	13	9	69.23
OPM-11	GTCCACTGTG	12	6	50.00
OPM-14	AGGGTCGTTC	7	5	71.43
OPM-18	CACCATCCGT	21	16	76.19
	Total:	166	105	63.25
	Mean:	13.83	8.75	

* Primers were obtained from IBBM (University of Crete, Greece)

Table 3. Leaf traits of 28 individuals selected from two different areas (mean values)

Trait (Leaf)	Source	F (p = 0.05)	Species (S)			Locality (L)	
			A	H	U	K	V
Length (cm)	L S L x S	NS * NS	6.4 b	6.7 a	6.3 b	6.5 a	6.4 a
Width (cm)	L S L x S	NS * *	4.2 a	3.2 b	2.8 c	3.4 a	3.4 a
Length/Width	L S L x S	NS * *	1.5 c	2.1 b	2.3 a	2.0 a	2.0 a
Area (cm^2)	L S L x S	NS * NS	215.5 a	159.4 b	125.7 c	163.2 a	170.6 a

A: *A. andrachne*; H: Intermediate individuals; U: *A. unedo*; K: Kalamos, V: Varympompi
*p < 0.05, NS: p > 0.05. Values followed by different lowercase letter within each trait are significantly different at the 5% level, determined by the one-way Anova

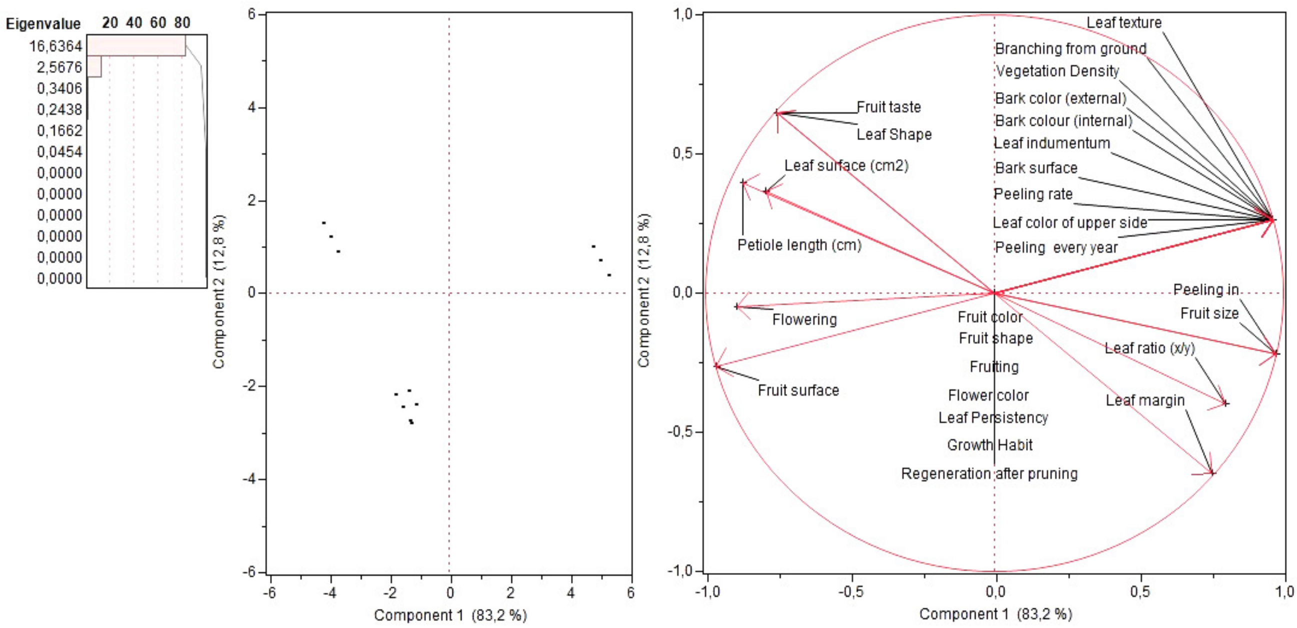

Fig. 1. Evaluation of the descriptors and their contribution to the variability of the individuals studied

Molecular analysis

For the molecular analysis and the identification of the individuals studied, 12 primers were used which proved to be highly polymorphic (63.5% polymorphism) producing a total of 166 amplified markers. Higher percentage of polymorphism was presented by primers OPM-18 and OPM-14 (76.19% and 71.43% respectively, Table 2). Primers could classified in three groups (Table 2) with:

Table 4. Evaluation of the descriptors and their contribution to the variability of the individuals studied

Principal Components			
1	2	3	4
% Contribution to variability			
83.18	12.83	1.7	1.21
Eigenvalue			
16.63	2.57	0.34	0.24
Related Descriptors			
2	14	24	17
4	15	25	26
5	23		
6			
7			
8			
9			
10			
12			
13			
16			
21			
27			

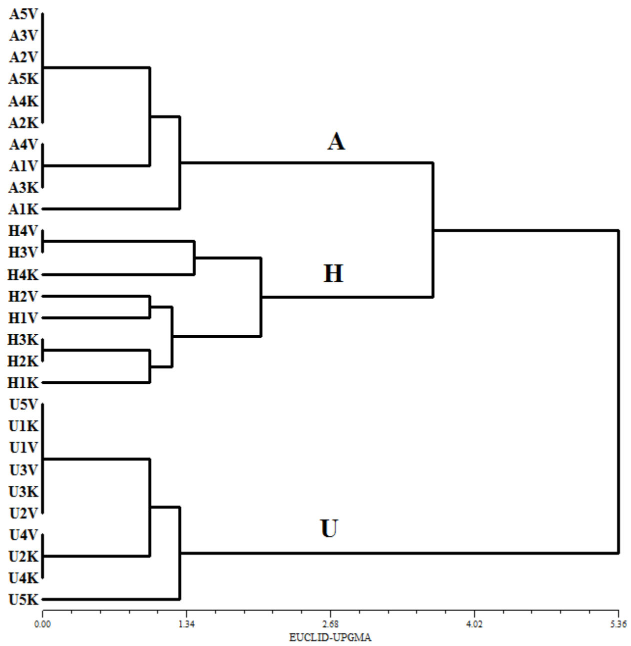

Fig. 2. UPGMA dendrogram of 28 *Arbutus* individuals based on Euclidean distance of morphological character analysis. Individual names: A:*A. andrachne*, U:*A. unedo,* H:*Arbutus* spp. with intermediate morphological characteristics, K:Kalamos, V:Varympompi, 1-5:number of individual

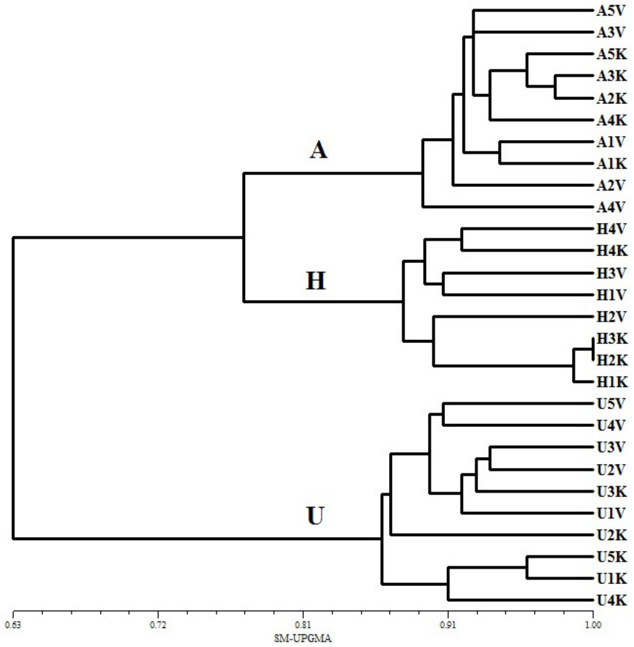

Fig. 3. UPGMA dendrogram of 28 *Arbutus* individuals based on RAPD molecular analysis for SM, showing genetic similarity between individuals studied. Individual names: A:*A. andrachne*, U:*A. unedo,* H:*Arbutus* spp. with intermediate morphological characteristics, K:Kalamos, V:Varympompi, 1-5: number of individual

a) high polymorphic ability (more than 15 bands: 1224, OPF-01, OPF-02, OPF-03, OPF-04, OPM-18). Primers OPF-01, OPF-02 and OPM-18 were suitable to identify individuals of *A. unedo;*

b) medium polymorphic ability (10-15 bands: primers 1227, OPF-06, OPM-11). Primer OPF-06 was suitable to identify *A. andrachne* individuals;

c) *low polymorphic ability* (7-10 bands, primers 1225, 1226, OPM-14). Primers OPM-14 and 1225 were suitable to identify *A. unedo* individuals.

The primers 1225 and 1227 proved much more useful in differentiating individuals as they generated more polymorphic DNA fragments. As expected there was genetic variation among the individuals studied and they were grouped in three different branches (Fig. 3, 4) consistently with our observations based on morphological characteristics.

Individuals with intermediate morphological characteristics from Kalamos (I=0.98-1.00) grouped in one branch, something that allows the hypothesis that belong to another species, at least for the primers that were used, possibly *A.* × *andrachnoides* (Fig 1). The genetic similarity between *A. andrachne* individuals from two different sampling areas was high (I=0.94). The higher genetic similarity found between individuals with intermediate characteristics and *A. andrachne* individuals (I=0.69-0.73) was consistent with the bigger

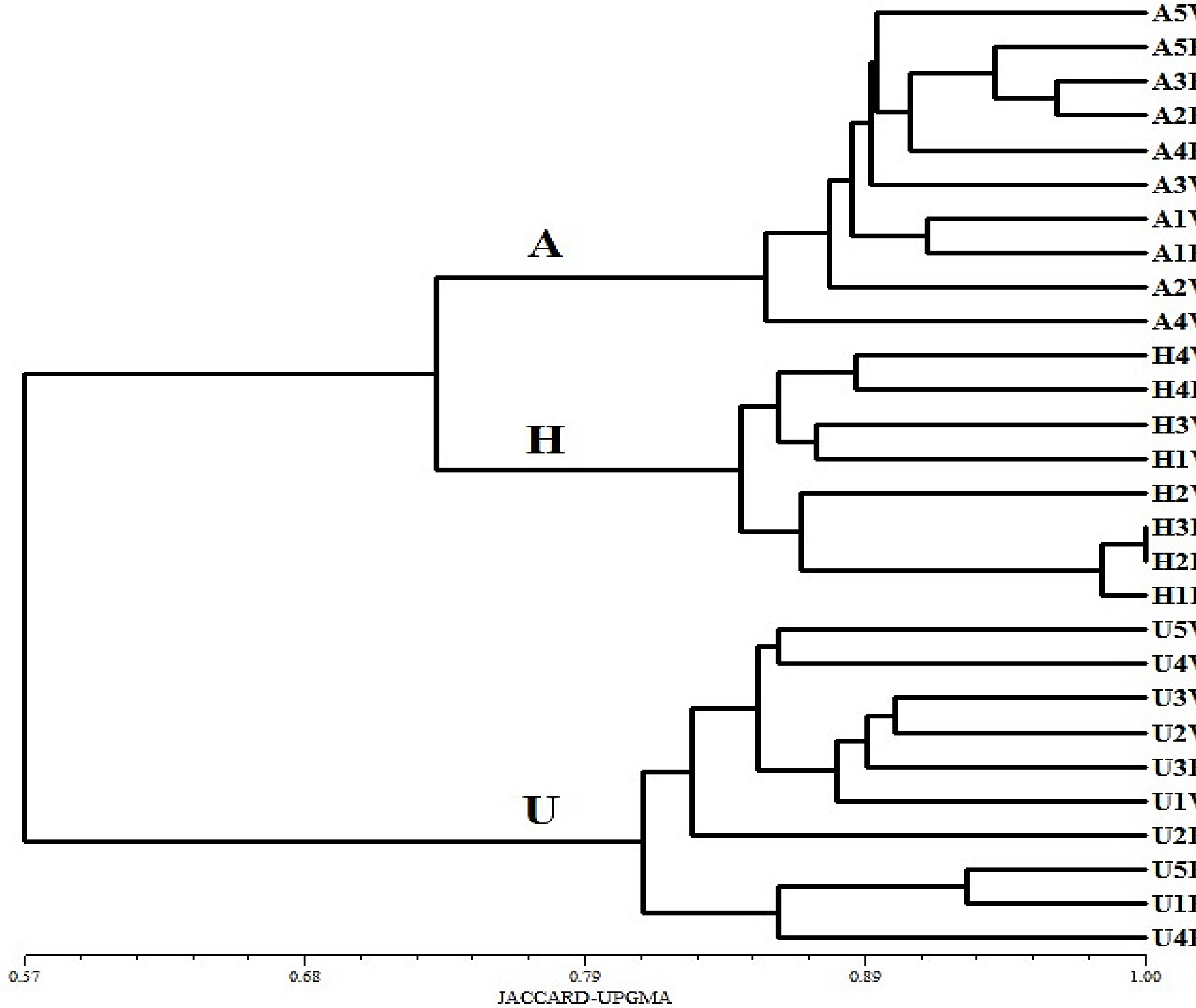

Fig. 4. UPGMA dendrogram of 28 *Arbutus* individuals based on RAPD molecular analysis Jaccard, showing genetic similarity between individuals studied. Individual names: A:*A. andrachne*, U:*A. unedo*, H:*Arbutus* spp. with intermediate morphological characteristics, K:Kalamos, V:Varympompi, 1-5:number of individual

phenotypic resemblance between these two species. On the contrary genetic similarity between *A. unedo* individuals and individuals with intermediate characteristics was lower (I=0.52-0.60), consistently with their lower phenotypic resemblance. Low degree of polymorphism was observed in each species population and differentiation among populations was not related to geographical distance similar to Takrouni and Boussaid, (2010) and Gomes *et al.* (2013), while moderate differentiation was recorded between or within *A. unedo* populations in Portugal Lopes *et al.* (2012).

Association between matrices of morphological and genetic distances were investigated (Mantel test) and the results of the tests showed significant correlation (r=0.958 and r=0.951 for SM and JAC, respectively).

Thus, individuals sampled as *A.* × *andrachnoides* showed both a mixture of *A. andrachne* and *A. unedo* morphological characteristics and intermediate characteristics that complicated their identification as hybrids. However the suggested descriptors in combination with molecular markers proved a useful tool to assess the hybrid identity of these individuals. Combination of morphological traits with RAPD has been successfully used for differentiation of other natural hybrids and clones, too (Yüzbaşıoğlu *et al.*, 2008; Stavrakaki and Biniari, 2016). The results of this research are supported by the fact that plants derived by micropropagation of individuals with intermediate characteristics defined as *A.* × *andrachnoides* (Bertsouklis and Papafotiou, 2011) were morphologically identical after three years ex vitro growth to the mother plant found in Kalamos (unpublished data) revealing that the hybrid was defined correctly.

A total of morphological characteristics that proved for first time suitable for identification of the hybrid form of the two Greek *Arbutus* species, RAPD profiles, the resulting similarity and the dendrogram, led to the conclusion that most probably individuals with intermediate morphological characteristics from Kalamos is another species, possibly the one reported in the bibliography as a natural hybrid between *A. unedo* and *A. andrachne*, named *A.* × *andrachnoides*. All three *Arbutus* species found in Greece have or could be introduced for various uses in forestry, landscape, medicinal and horticultural industry, as mentioned in the introduction section. Furthermore the *in vitro* propagation of these species has been successfully achieved both by axillary bud proliferation of adult plant (Mereti *et al*., 2002; Bertsouklis and Papafotiou, 2009; Gomes *et al*., 2010; Mohamed El-Sayed *et al*., 2010; Bertsouklis and Papafotiou, 2011) and from seedlings (Papafotiou *et al*., 2013), so the most suitable clones could be propagated. All these techniques would be very useful tools for breeding programs involving *Arbutus* species or for developing effective conservation strategies. Santiso *et al*. (2015) reported that *A. unedo* maintains the ability to evolve despite low genetic differentiation and stabilizing selection. The present study, proposing appropriate morphological characteristics in combination with selected molecular markers for distinguish individuals of three *Arbutus* species, is a step in achieving these goals for *Arbutus* spp. in Greece and other counties, particularly those in which *Arbutus* species are endangered or threatened, as *A. andrachne* in Ukraine, Albania, Jordan and Georgian SSR (Al-Tellawi, 1989; Garzuglia, 2006; Melia *et al*., 2012), *A. unedo* in Slovenia (Garzuglia, 2006) and *A.* × *andrachnoides* in Croatia (Šatović, 2004).

Conclusions

The present work provides both morphological traits and molecular markers (RAPD) to differentiate the three *Arbutus* species found in Greece, particularly useful for identification of the hybrid *A.* × *andrachnoides* from the parental species *A. andrachne* and *A. unedo,* and in this way to facilitate their exploitation in horticultural and pharmaceutical industry. The applied morphometric characteristics are suggested as a basis to develop a complete list of discriminating descriptors for *Arbutus* genus.

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