Genetic Diversity and Population Structure of Sardinian Myrtle (*Myrtus communis* L.) Selections as Obtained by AFLP Markers

Sara Melito

Department of Agriculture, University of Sassari, 07100 Sassari, Italy

Angela Fadda¹

Institute of Science of Food Production, National Research Council, 07100 Sassari, Italy

Emma Rapposelli

Department for Fruit and Forestry Research of AGRIS–Sardinia, Cagliari, Italy

Maurizio Mulas

Department of Nature and Land Sciences, Via De Nicola 9, University of Sassari, Italy

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Abstract. Myrtle is an aromatic plant typical of the Mediterranean maquis. It is widely exploited in pharmaceutical, cosmetic, and food industries, whereas in Italy, it is mainly used for the production of the typical liqueur. Amplified fragment length polymorphism (AFLP) markers were used to evaluate the genetic variability of some Sardinian *Myrtus communis* L. candidate cultivars. The AFLP selective amplification produced 138 reproducible AFLP fragments, 96% of which were polymorphic. STRUCTURE analysis divided the myrtle accessions into two main genetic groups ($K = 2$). The two clusters showed different numbers of individuals. Most of the individuals belonged to Cluster B, whereas only eight genotypes were attributed to Cluster A. Unweighted pair group method with Arithmetic Mean (UPGMA) dendrogram segregated all the myrtle cultivars into five main groups, displaying a partial congruence with the division observed by STRUCTURE analysis. The analysis of the genetic diversity distribution in a candidate cultivar selection displayed a geographical gradient of myrtle from north to south, which reflects the Sardinian shape, and from west to east, which reflects the Sardinian mountain distribution.

Myrtle (*Myrtus communis* L.) is an ever-green shrub typical of the Mediterranean maquis. Myrtle is an aromatic plant rich in essential oils used in pharmaceutical, cosmetic, and food industries. Its characteristics have long been known; in the past, myrtle was used as flavoring agent and in folk medicine for its anti-inflammatory and anti-septic properties. The medical literature have confirmed the antimicrobial properties (Deriu et al., 2007; Gündüz et al., 2009) and assessed the apoptotic activity against cancer cells of myrtucommulone, a nonprenylated acylphloroglucinol present in myrtle leaves (Tretiakova

et al., 2008). Furthermore, recent data highlighted the good antioxidant properties of myrtle berry extracts and their use as food preservatives was also suggested (Tuberoso et al., 2010). In the last years, myrtle has been appreciated as an ornamental plant as a result of the new interest of consumers in Mediterranean flora. Despite its manifold uses, in Sardinia, myrtle plant is mainly associated with the typical liqueur that is produced through maceration of its berries and leaves. At present, the production of the myrtle liqueur accounts for ≈ 4 million liters per year (homemade and industry production) but it is steadily increasing because of its success at the national and international levels (Mulas, 2012). The enhanced interest on myrtle liqueur as well as the restriction for certified producers to use only Sardinian myrtle has given rise to an increase in the demand for Sardinian raw material. At present, the bulk of the raw material comes from natural populations, which are therefore increasingly subjected to human pressure and genetic erosion (Mulas et al., 1999; Mulas and Deidda, 1998). In the last 10 years a domestication program has been carried out with the aim to supply to

both the liqueur and pharmaceutical industries raw material with known characteristics and to preserve the natural populations (Mulas and Deidda, 1998; Mulas and Cani, 1999). With these purposes, some ecotypes, interesting for biomass and fruit production, were collected from Sardinian natural populations and planted in a repository at the “Antonio Milella” experimental station of the University of Sassari, in central–western Sardinia (Mulas, 2012; Mulas et al., 2002).

Germplasm genetic identification and characterization is an important step for the conservation and the use of plant genetic resources. DNA-based markers have been widely used to assess the genetic diversity of plant cultivars (Amar et al., 2011; Kalia et al., 2011; Osman et al., 2012) and to study the relationships among the genetic, morphological, chemical, and ecological variables (Melito et al., 2013a). Previous studies on Mediterranean myrtle populations demonstrated that intersimple sequence repeat and AFLP could discriminate the genotypes according to their geographic origin (Agrimonti et al., 2007; Bruna et al., 2007; Melito et al., 2013b). Among the genetic markers, the AFLPs have some advantages: a high level of reproducibility and the possibility to screen a large number of loci simultaneously without any sequence information. Concerning myrtle, much efforts have been dedicated to the characterization of its secondary metabolites (essential oils and polyphenols) (Mulas and Melis, 2011; Tuberoso et al., 2010), whereas little information is available on the environmental factors influencing the myrtle genetic diversity in Sardinia. For a new crop such as myrtle, the knowledge of the relationships between genetic resources and factors affecting the adaptability of the plant to different environmental conditions is necessary for the optimization of its management. In the present work, AFLP markers were applied with the aim to characterize from a genetic point of view some myrtle accessions previously selected for their fruit and biomass production properties. The genetic traits and the population genetic structure analysis were used to investigate the interrelationships between the genetic variation and the environmental factors with the aim to provide information about genetic diversity and structure distribution in different environmental conditions.

Material and Methods

Plant material and sampling sites. In this study, we analyzed a candidate cultivar collection of 33 *Myrtus communis* L. subsp. *communis* from Sardinia and one *Myrtus communis* subsp. *tarentina* (L.) Arcangelii (SAS1). The Sardinian genotypes studied were selected from the collection of the Department of Nature and Land Sciences of the University of Sassari, located at the “Antonio Milella” experimental station in Oristano in the midwest coast of Sardinia, Italy (lat. 39°53' N, long. 8°37' E, 11 m above sea level, 10 km from the sea). Such a collection is the result of a selection program, based on few morphological traits (see subsequently),

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¹To whom reprint requests should be addressed; e-mail angela.fadda@ispa.cnr.it.

aimed at identifying, throughout Sardinia, genotypes with good biomass and fruit production (Mulas et al., 2002). The origin of each accession, the accession code, the longitude, the latitude, the altitude, and the soil type of each collection area are reported in Table 1.

From each individual, young branches roughly 10 cm in length were collected, placed on ice in plastic bags, and taken to the laboratory. Samples were stored at -20 °C until the DNA extraction was performed.

Morphological analysis. Morphological and biometrical data were recorded for each

accession. Thirty offshoots for each ecotype were selected and the following traits were measured: shoot length (cm), internode number per shoot (n), internode length (cm), leaf length, and width (cm). Pairwise correlation (from a multivariate platform) was performed among the morphological and biometrical data using JMP 7 software (SAS Institute, Cary, NC).

DNA extraction and AFLP procedure. Total genomic DNA was extracted from 100 mg of young leaves using the DNeasy® Plant mini kit (QIAGEN, Hilden, Germany) following the supplier's instructions. DNA

quality and concentration were estimated by spectrophotometric analysis and by comparison, on a 0.8% (w/v) agarose gel, with a known amount of λ DNA.

AFLP analysis was carried out according to Vos et al. (1995) with few minor modifications. Briefly, genomic DNA (250 ng) was double-digested for 1 h at 37 °C in a final volume of 40 μ L with *EcoRI* (5U) and *MseI* (5U) in 10 $\times$ Restriction/Ligation (R/L) buffer (100 mM Tris base, 100 mM MgAc, 500 mM KAc, 50 mM DTT, 100 ng/ μ L bovine serum albumin). Ten microliters of ligation mix (5 μ M *EcoRI* adapters + 1A, 50 μ M *MseI* adapters + 1C, 10 mM adenosine-5'-triphosphate, 1U *T4* ligase) were added to the restriction mix and incubated for 3 h at 37 °C. Restriction enzymes and *T4* ligase were purchased from New England Biolabs (Ipswich, MA). Five microliters of R/L mixture (diluted 1:9) were used in 20 μ L of the pre-amplification mix. The pre-amplification was conducted by adding 1.5 mM MgCl₂, 10 $\times$ Buffer, 10 mM dNTPs, 2.75 μ M *EcoRI* Primer, 2.75 μ M *MseI* Primer, and 1 U *Taq* polymerase.

The pre-amplification polymerase chain reaction (PCR) product was diluted 1:4 with sterile water and 5 μ L were used as a template for selective PCR by adding 10 $\times$ PCR Buffer, 1.5 mM MgCl₂, 10 mM dNTPs, 0.46 μ M *EcoRI* + 3N primer, 2.75 μ M *MseI* + 3N primer, and 1 U *Taq* polymerase in 20- μ L final volume. Four *EcoRI/MseI* primer combinations with three selective nucleotides were used (Table 2).

All PCRs were performed using Platinum® *Taq* DNA Polymerase High Fidelity. Pre-amplification and selective amplification cycles were carried out according to Vos et al. (1995).

AFLP-PCR products were separated by electrophoresis on 6% denaturing polyacrylamide gels along with the 100-bp DNA Ladder 100 (Invitrogen Life Technologies) to determine the sample band sizes. Gels were silver-stained according to Bassam et al. (1991) with the following modifications: 20 min fixer solution (10% glacial acetic acid, two 5-min washes in distilled water), 30 min staining in 0.2% silver nitrate, 3 mL·L⁻¹ formaldehyde, 10-s wash in distilled water, and revelation in a 4 °C pre-cooled solution containing sodium carbonate (30 g·L⁻¹), formaldehyde (0.35 mL·L⁻¹), and sodium thiosulfate (0.05 g·L⁻¹). The gel development

Table 1. Characteristics of myrtle sampling localities.^z

Sampling localities	Code	Coordinates		Altitude (m)	Soil type
		Latitude	Longitude		
Siniscola	Sin2	40°34'39" N	9°41'23" E	20	Schist
Telti	Tel2	40°52'47" N	9°21'20" E	332	Granite
Olia Speciosa	Sbd1	39°16'38" N	9°31'36" E	239	Granite
	6_2	39°16'38" N	9°31'36" E	239	Granite
Bosa	Bos1	40°20'06" N	8°22'51" E	13	Sandstone
	Bos2				
Budoni	Bud1	40°42'11" N	9°42'35" E	24	Granite
Uta-Monte Arcosu	Cpt3	39°11'20" N	8°56'08" E	54	Granite/schist
	Cpt4				
	Cpt5				
	Cpt6				
Cuglieri	Cug11	40°11'18" N	8°33'57" E	284	Basalt
Isili	Isl1	39°44'23" N	9°06'61" E	523	Basalt
Laconi	Lac1	39°51'07" N	9°03'09" E	637	Schist
	Lac10				
	Lac11				
	Lac3				
Monti	Lac31				
	Mon2	40°48'04" N	9°19'29" E	296	Granite
	Mon4				
Orosei	Oro2	40°22'21" N	9°41'50" E	19	Granite
	Ors2				
	Ors3				
Parco dei sette fratelli	Psfl	39°20'29" N	9°13'04" E	423	Granite
	Psf4				
Rumanedda	Rub	40°40'56" N	8°21'38" E	100	Limestone
	Rum15				
	Rum20				
	Rum12				
	Rum13				
	Rum14				
	Rum3				
	Rum4				
	Rum6				

^zFor each accession the sampling location with geographical coordinates, the identity code, the altitude, and the soil type are reported.

Table 2. Primer sets used for pre-selective and selective amplification for amplified fragment length polymorphism fingerprinting.^z

Primer sequence for pre-selective amplification	Primer sequence for selective amplification	Monomorphic alleles (%)	Unique alleles (%)
<i>EcoRI</i> + 1A 5'-GACTGCGTACCAATTCA-3'	<i>EcoRI</i> 01 GACTGCGTACCAATTCAAC	14	20
<i>MseI</i> + 1C 5'-GATGAGTCCTGAGTAAC-3'	<i>MseI</i> 01 GATGAGTCCTGAGTAACAT		
	<i>EcoRI</i> 01 GACTGCGTACCAATTCAAC		
	<i>MseI</i> 02 GATGAGTCCTGAGTAACAT		
	<i>EcoRI</i> 01 GACTGCGTACCAATTCAAC		
	<i>MseI</i> 03 GATGAGTCCTGAGTAACAT		
	<i>EcoRI</i> 03 GACTGCGTACCAATTCAAC		
	<i>MseI</i> 03 GATGAGTCCTGAGTAACAT		
	<i>EcoRI</i> 02 GACTGCGTACCAATTCAAC	ND	ND
	<i>MseI</i> 02 GATGAGTCCTGAGTAACAT		

^zNumber of fragment, percentage of monomorphic, and unique alleles are also reported.

ND = no data.

was stopped by incubating in 10% acetic acid for 1 min. Gels were rinsed for 5 min and dried overnight at room temperature. PCR amplification was repeated twice to ensure the consistency of the AFLP profile.

Dried polyacrylamide gels were manually analyzed for presence (1) or absence (0) of bands. Only clearly amplified fragments were scored; bands with a weak signal or blurred appearance were not considered.

Data analysis. Genetic population structure was investigated using the Bayesian clustering model implemented in STRUCTURE 2.3.3 (Pritchard et al., 2000). The software was run without a priori information on population membership, assuming admixture and correlated allele frequencies and a recessive genotype mode. Cluster numbers (K) ranged from one to 10, and for each K , 20 replicate chains of 200,000 Markov Chain Monte Carlo interactions with a length of burn-in period of 100,000 were run. The attribution of each sample to a specific cluster was based on a coefficient of membership (Q). The optimal K was calculated according to Evanno et al. (2005).

Estimation of polymorphic alleles number, genetic diversity value (H_e), and fixation index (F_{ST}) for each population considered were calculated by Arlequin Version 3.5.1.2 (Excoffier et al., 2005).

The analysis of population divergence was carried out by analysis of molecular variance (AMOVA) as implemented in Arlequin Version 3.5.1.2 (Excoffier et al., 2005).

Population genetic distance matrix (Nei, 1973) was performed with Arlequin to explore the pairwise relationships among the *M. communis* accessions analyzed. A further analysis based on distance matrix among candidate cultivars was performed to discriminate plants according to their sampling site altitude.

A phylogenetic tree was designed based on UPGMA clustering (Nei, 1978) using TFPGA Version 1.3 software. Spearman correlation analysis was performed by JMP 7 software (SAS Institute, Cary, NC) to assess the relationships among environmental variables (altitude and soil), morphological traits, and population genetic cluster assignment coefficient of membership (Q).

Results and Discussion

Morphological traits. Previous works (Mulas et al., 2002; Mulas and Cani, 1999) highlighted the high variability of Sardinian myrtle accessions based on morphological parameters. The pool of candidate cultivars studied in this article is the result of a selection work started with the mass selection from the wild populations of 130 accessions. All accessions were characterized by high spring shoot length, leaf size, internode number, and length. Those characters were related to fruit and biomass production. In fact, spring shoot vigor and length are linked to a strong possibility to observe abundant flower bud differentiation in the basal

internodes. Moreover, a well-defined spring blossom flux avoids multiple plant flowering fluxes with negative effects on final yield (Mulas and Cani, 1999; Mulas and Fadda, 2004). In this article, we studied the correlations among such morphological traits as parameters associated with fruit or biomass production.

The candidate cultivar selections analyzed showed high positive correlations (Pearson; $P < 0.0001$) among leaf (length and width) and internodes (length and number) (Table 3). Those findings were useful to confirm the effectiveness of the phenotypic selection process and to have a first basis to evaluate the effects in term of possible loss of genetic variability.

AFLP patterns. The AFLP profiling has been used to discriminate between closely related genotypes belonging to the same species and to evaluate the genetic diversity in wild species (Baldoni et al., 2006; Christensen et al., 2011; Erre et al., 2010; Turpeinen et al., 2001). The assessment of genetic diversity could be considered a useful instrument to identify selected genotypes for fruit or biomass yield in a breeding program.

The AFLP analysis of the myrtle candidate cultivars produced more than 100 fragments for the primer set, ranging from 40 to 700 bp. Fragments from 50 to 500 bp were manually scored for 138 reproducible amplicons. Each primer generated a specific number of polymorphic bands. Primers set *EcoRI* 1/*MseI* 01 and *EcoRI* 1/*MseI* 03 generated the highest number of fragments (59 for each primer combination); the other two primer combinations, *EcoRI* 01/*MseI* 02 and *EcoRI* 03/*MseI* 03, produced, respectively, 15 and five fragments. Overall, we identified 14% of monomorphic markers and 20% of unique AFLP fragments (Table 2). All the unique amplicons were found in *EcoRI* 1/*MseI* 01 and *EcoRI* 1/*MseI* 03 primer combinations. To minimize the AFLP scoring errors, faint polymorphic bands were excluded. Control genotypes were used to align different run gels in the same primer combination and finally 10% of the samples were twice scored to assess the reproducibility of the scoring.

The analyzed myrtle candidate cultivars showed a high level of polymorphic markers (96%). Similarly, in Tunisian myrtle populations, a high percentage of polymorphic loci was identified with random amplified polymorphic DNA and isozymes (Messaoud et al., 2006, 2007).

Population genetic structure. STRUCTURE analysis identified two main genetic groups ($K = 2$) referred as Cluster A (CA) and Cluster B (CB) (Fig. 1). The maximum likelihood value was observed at $K = 2$ with a $\Delta K = 35.48$ (Fig. 1A). The coefficient of membership (Q) of each genotype is shown in Figure 1B. The two clusters showed different numbers of individuals. Most of the individuals belonged to Cluster B, whereas only eight genotypes were attributed to Cluster A. In addition, a group of ecotypes (LAC11, SIN2, RUM12, RUM15, and ORS2) showed a coefficient of membership lower than 0.60. These myrtle accessions were not assigned to any specific genetic cluster and were defined as an “admixture.” These admixed individuals may be the result of the gene flow between the two genetic clusters identified. The genotype SAS1, which is the only genotype of the subspecies *tarentina*, belonged to the smaller group, and it did not cluster separately as it is expected. Furthermore, a deeper analysis of the ΔK trend revealed a second pick at $K = 3$ (Fig. 1A). This further division was explored for its high informative value. At $K = 3$, the composition of Clusters A and B changed. Cluster A composition was quite respected except for two accessions: PSF4 (that became an admixed individual) and BUD1 (assigned to the new Cluster III). Cluster B underwent the deepest variation in genetic cluster assignment: more than 50% of the individuals were attributed to the “admixed group,” whereas two of the admixed individuals (ORS2 and RUM15) were grouped into the new genetic cluster (III) identified. LAC11, SIN2, and RUM12 maintained an admixed genetic composition because of their low coefficient of membership ($Q < 0.6$) (Fig. 1B).

A detailed picture of myrtle accessions relationships was also studied by UPGMA clustering using Nei's (1978) index as distance matrices. The UPGMA dendrogram showed a partial congruence with the division observed with STRUCTURE analysis (Fig. 2). Five main groups can be identified in Figure 2. Branch I grouped all myrtle accessions belonging to Cluster B and it has clearly separated from the other myrtle accessions. Branch II and III included most of the Cluster A members. The accessions PSF4 and BUD1, assigned to Clade II, were the only exception to the cluster assignments obtained at $K = 2$. In addition, two genotypes, CPT6 and SAS1,

Table 3. Pairwise correlations coefficients among morphological traits of myrtle plants.^z

Variable	By variable	Correlation coefficient	Significance (P)
Internode L	Internode N	-0.5032	0.0024
Leaf L/W	Leaf W	-0.5315	0.0012
Leaf L	Internode N	-0.4174	0.0141
Leaf L	Internode L	0.7821	<0.0001
Leaf W	Internode L	0.6807	<0.0001
Leaf W	Leaf L	0.8022	<0.0001
Internode N	Shot L	0.7874	<0.0001

^zShoot length, internode length and number, leaf length and width, and their ratio were analyzed. L = length; W = width; N = number.

were grouped in two separated and independent clusters.

The genotype SAS1, classified as *M. communis* subsp. *tarentina*, clustered separately into one independent branch. These data clearly showed the *M. communis* subsp. *communis* and the *Myrtus communis* subsp.

tarentina division, thus confirming the reliability of the AFLP genotyping.

Genetic diversity. The genetic diversity (H_e) of the myrtle core collection studied ranged from 0.239 to 0.420. The observed average heterozygosity ($H_e = 0.314$), along with the high number of polymorphic loci

(96%), suggests a high genotypic richness of the original collected selections. Monti selection exhibited the lowest H_e , whereas the highest value was assigned to Bosa myrtle selections. The levels of H_e reported in this study were higher than those observed by Agrimonti et al. (2007) in other Sardinian myrtle populations and by Messaoud et al. (2006) in Tunisian myrtle populations. Such low levels of genetic diversity reported in these works could be the result of a greater sample size and of a different collection strategy of the analyzed populations. Indeed, it should be emphasized that in our work, plants were not randomly selected but the selection was performed according to plant fruit and biomass productivity.

The levels of genetic diversity were confirmed by the F_{ST} values. These were reported as pairwise distance matrix and displayed in Table 4. Mean F_{ST} value, calculated among all myrtle candidate cultivars, was quite low (0.065), showing a small genetic distance among populations collected in the original sampling localities. The highest genetic distance was found between myrtle plants collected in Monti (north Sardinia) and Capoterra (south Sardinia) and between plants collected in Monti and Laconi (central Sardinia) (F_{ST} value of 0.146 and 0.181, respectively). By contrast, the lowest genetic distance was recorded between Bosa and Olia Speciosa populations ($F_{ST} = -0.016$). The F_{ST} values found in sampling sites with the highest genetic distance demonstrated that populations were genetically different but not geographically isolated, thus suggesting a possible gene flow among these populations. These results pointed out that a geographical trend in the genetic differentiation among Sardinian myrtle populations may be present. Physical barriers such as mountains might influence the pollen dispersion, modifying the genetic exchanges between different sampling sites. The AMOVA was used to analyze the sources of genetic variance among or intra populations level (Table 5). Our AFLP-based AMOVA studies show that most of the genetic variation in myrtle is distributed within populations rather than among them, indicating a relatively restricted population differentiation as expected in mixed mating species. As shown in Table 5, 93.54% of the variability was within the population, whereas only 6.46% of the AFLP diversity was distributed among populations. According to Migliore et al. (2012), the homogeneity among Sardinian myrtle populations is the result of very recent expansion of this plant in Sardinia.

The AMOVA analysis was also used to explore the partition of the molecular variance at the cluster divisions identified by structure ($K = 2$; $K = 3$). As expected, at $K = 2$, the AMOVA revealed an increase of the percentage of genetic variation calculated within populations (93.91%) This trend is also confirmed by the F_{ST} value, which indicated the strongest genetic differentiation at $K = 2$ ($F_{ST} = 0.0538$) (Table 5). Taken together, these results suggest a low genetic

Fig. 1. Population structure of 33 *Myrtus communis* L. candidate cultivars. (A) Estimation of ΔK value obtained with STRUCTURE analysis; (B) myrtle accessions genetic group assignment based on Bayesian clustering model analysis. The average coefficient of membership (Q) was calculated considering the partition of myrtle accessions into 2 ($K = 2$) and 3 ($K = 3$) clusters.

Fig. 2. Unweighted pair group method with averages cluster dendrogram, based on Nei's genetic distance, showing the genetic relationships among the 33 *M. communis* L. candidate cultivar selections.

distance among populations. These outcomes are compatible with the proportion of out-crossing of myrtle plants. Indeed, according to Gonzles Varo et al. (2009), the differentiation among plant populations might be influenced by the plant mating pattern. As already reported, myrtle is mainly an out-crossing species with a proportion of self-pollination (Mulas and Fadda, 2004). A large variation of mating patterns has been observed among populations (Gonzles Varo et al., 2009, 2010).

Genetic diversity and environmental parameters. The understanding of the relationships between the genetic diversity distribution and the ecological variables has a major role in biodiversity conservation strategies. In the case of myrtle, evidence on the impact of ecological parameters on the genetic variability may provide useful information about the adaptability of the selected accessions to different environmental and agronomical conditions. Altitude produces ecological barriers against the gene flow acting on the genetic selection and differentiation.

Local genetic differentiation, as a result of the altitude gradient, has been reported in different wild herbaceous species such as *Helichrysum italicum* (Melito et al., 2013a) and *Elymus nutans* (Yan et al., 2009), whereas little information is available about shrub and tree species. To the extent of our knowledge, little information is available on ecological factors affecting population genetic structure and distribution in *Mirtaceae* family, and almost no studies have been performed on *M. communis*.

In Sardinia, myrtle grows up to 800 m above the sea level (Mulas et al., 2002); however, the plants under study were collected up to a maximum altitude of 637 m. For this reason, we decided to study the relationships between the model-based clusters and the genetic distribution in three different altitude levels (less than 100 m, 100 to 200 m, greater than 200 m a.s.l.).

Plants belonging to Cluster A showed a greater distribution at high altitudes (Fig. 3B). Conversely, Cluster B members showed an opposite trend: individuals belonging to this group decreased their presence progressively with altitude (Fig. 3B). When evaluated with a partition of the plants into three genetic clusters ($K = 3$), it was observed that plants of the first (I) group were most common at altitudes above 200 m, whereas plants of the second group (II) showed their greatest distribution at altitudes lower than 100 m. The third genetic group, mainly identified as a partition of CB (Fig. 3B), revealed its maximum presence at altitudes ranging from 101 m to 200 m a.s.l., whereas it was less represented at altitudes from less than 100 m and greater than 200 m a.s.l (Fig. 3B). The correlations between altitude and genetic cluster assignment (Q) were statistically significant at $K = 2$ (Pearson, $P = 0.0285$), whereas at $K = 3$, only Cluster I showed a significant correlation with altitude ($P = 0.0117$). This outcome was also confirmed by

Table 4. Pairwise genetic distance matrix (F_{ST}) among different localities with more the one myrtle accessions.

	Olia Speciosa	Bosa	Capoterra	Laconi	Monti	Orosei	Sette fratelli	Rumanedda
Olia Speciosa	0							
Bosa	-0.0162	0						
Capoterra	-0.0379	0.0344	0					
Laconi	0.0915	0.1427	0.1147	0				
Monti	0.0993	0.0444	0.1459^a	0.1813	0			
Orosei	-0.0731	0.0597	0.0301	0.0866	0.0596	0		
Sette fratelli	-0.0541	0.0588	0.0489	0.0197	0.0541	-0.0152	0	
Rumanedda	-0.0576	0.0973	0.1005	0.0479	0.1215	0.0209	0.0497	0

^aIn bold are indicated the two highest genetic distances.

Table 5. Partition of genetic variation determined by AMOVA analysis.^z

	Sources of variation	df	Sum of squares	Variance components	Percentage of variation	F_{ST}
Overall	Among populations	7	158.982	1.26941	6.46	0.065
	Within populations	21	385.811	18.37196	93.54	
	Total	28	544.793	19.64137		
Altitude	Among populations	2	40.348	0.33019	1.96	0.0196
	Within populations	31	511.299	16.49351	98.04	
	Total	33	551.647	16.82370		
$K = 2$	Among populations	1	28.731	0.93910	5.38	0.0538
	Within population	24	396.538	16.52244	94.62	
	Total	25	425.269	17.46154		
$K = 3$	Among populations	2	49.597	0.795	4.68	0.0468
	Within population	31	502.050	16.195	95.32	
	Total	33	551.647	16.990		

^zAltitude and population genetic structure at $K = 2$ and $K = 3$ were considered as sources of molecular variance. The overall AMOVA was also evaluated.

AMOVA = analysis of molecular variance.

Fig. 3. Distribution of myrtle population clusters, as identified by STRUCTURE, according to geographical origin (A), and to altitude classes (B). Cluster membership was based on average coefficient of membership (Q) calculated considering the population genetic structure at $K = 2$ and at $K = 3$. Colors refer to clusters: at $K = 2$ deep gray = Cluster A, pale gray = Cluster B; at $K = 3$ deep gray = Cluster I, pale gray = Cluster II, white = Cluster III.

the AMOVA analysis (Table 5), which showed that, when the molecular variance was calculated according to the altitude classes, the greatest part of the genetic variance was assigned “within populations” rather than among them (98.04%). These results suggested a high adaptability of the plants under study to the different environmental conditions found at the altitude classes considered.

Our results confirm that environmental factors may act in the selection processes, suggesting that selection for adaptation at different altitudes levels may occur.

By contrast, when calculated independently from the cluster partition, pairwise F_{ST} distance matrices showed a high level of genetic distance among the AFLP profile of individuals growing at the lowest and highest altitudes (data not shown).

The geographic distribution of hills and mountains in Sardinia prompted interesting questions on the relationships between genetic diversity distribution and the habitat contests. In Figure 3 is reported the distribution of the genetic variance considering the two cluster partition identified ($K = 2$ and $K = 3$), expressed as a coefficient of membership Q for each myrtle population.

At $K = 2$, the distribution of the genetic clusters did not show any geographical trend. Indeed, the geographic distribution of the genetic clusters identified at $K = 3$ showed that the genetic group III increased its representativeness from west to east (Fig. 3A). This result was confirmed by the two $\times$ two contingency test performed between genetic clusters assignment and geographic origin (data not shown) (χ^2 , Pearson 0.05, $P = 0.0182$). Similarly, the genetic clusters displayed a different distribution from north to south (χ^2 , Pearson 0.05, $P = 0.0032$). The geographical trend observed in the distribution of the three clusters may be partially influenced by the position of the Sardinian mountains (distribution west–east) and by the long distance from north to south, which may have limited the gene flow. These data are compatible with the hypothesis of isolation by distance. These results are different from those obtained by Agrimonti et al. (2007), who found no significant correlations between the genetic variability and the geographical distance among myrtle populations sampled in Sardinia. In our opinion, such differences could lie on a lower size of our sample and difference in sampling strategy applied by these authors.

Conclusions

In Sardinia, myrtle populations are often subjected to overexploitation resulting from the intensive harvest of fruits and biomass for liqueur production (Mulas et al., 1999). Our results revealed a low genetic differentiation among populations, whereas a high genetic variability was observed within populations. These outcomes provide evidence of a certain resistance of the populations studied to genetic erosion as a result of excessive human pressure. Nevertheless, myrtle cultivation has

been highly recommended to preserve natural populations (Mulas, 2012; Melito et al., 2013b). The measurement of the genetic diversity and the correlations between genetic variability and environmental factors may have important implications for the cultivation of the species. The genetic diversity and the structure of myrtle populations reported in this work demonstrated a different adaptability of the accession studied to environmental factors. The genetic clusters identified at two different levels of partition ($K = 2$ and $K = 3$) showed that altitude represents an important factor acting on the myrtle population genetic diversity. Furthermore, a geographical trend was observed in the differentiation among Sardinian myrtle populations.

This work represents the first attempt to characterize, from a genetic point of view, some candidate cultivars selected, within the Sardinian germplasm, for fruit and biomass properties and production. Further studies will be necessary to build association maps of genetic, chemical, and agronomical traits.

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