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Morphological, chemical, and genetic diversity of wild myrtle (*Myrtus communis* L.) populations in Sicily

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Abstract: *Myrtus communis* L. is a shrub widespread in the Mediterranean area. The interest in this species is growing, mainly due to its pharmacological and aromatic properties. The overexploitation of wild populations induced increasing degradation of plant cover with serious risk of loss of genetic diversity. This research explored the morphological, chemical, and genetic diversity of wild myrtle populations in Sicily, with the aim to provide a first characterization of a core collection of 36 accessions from 7 localities for future domestication programs. Amplified fragment length polymorphism fingerprinting generated 152 polymorphic fragments. STRUCTURE analysis identified three genetic clusters (A, B, and C) corresponding to specific geographical origin. Analysis of molecular variance estimated a quite high overall fixation index (F_{ST} = 0.332). Misilmeri and Ispica were the more divergent populations (F_{ST} = 0.502), while M. Pellegrino and Scopello revealed the lowest F_{ST} (0.153). The relationships between genetic, morphological, and biometric data were investigated. Significant correlation between genetic clusters and bush shape/plant growth behavior was found (P < 0.005). Moreover, morphological traits such as leaf, fruit, and seed size were significantly correlated to Clusters B and C. Leaves' secondary metabolite profiles were evaluated based on antioxidant activity and total tannin and phenol concentrations. High antioxidant activity differences were recorded using DPPH (21.4–35.5 mmol Trolox/100 g DW) and ABTS (24.2–39.5 mmol Trolox/100 g DW) methods. A low variability was observed among populations regarding phenol (2466–3800 mg catechin equivalents/100 g DW) and total tannin contents (93.9–262.3 mg catechin equivalents/100 g DW). Results indicated that multiple approaches based on genetic, morphological, and chemical traits might allow the characterization of natural myrtle diversity.

Key words: Amplified fragment length polymorphism, genetic resources, leaf antioxidant properties, myrtle, molecular markers

1. Introduction

Myrtus communis L. is a key shrub of the Mediterranean maquis, widely spread in the Mediterranean area (Mendes et al., 2001) and in the Middle East (Zilkah and Goldschdmidt, 2014). Ancient Mediterranean populations largely used myrtle for its ornamental and aromatic value (Agrimonti et al., 2007). Today the species is probably better known for its medicinal proprieties and uses in food industries (Gastaldo, 1987; Flamini et al., 2004; Barboni et al., 2010). More recently the pharmacological proprieties of its essential oil have been deeply explored. Antimicrobial properties (Deriu et al., 2007; Gündüz et al., 2009; Cannas et al., 2013), antihyperglycemic activities (Sepici et al., 2004; Onal et al., 2005), and antioxidant activity and fatty acid composition (Serce et al., 2010) have been also reported. A recent ethnobotanical study conducted by Leto et al. (2013) showed effective medicinal use in both Italy (Sicily, Tuscany, and Sardinia) and Tunisia. In addition, the essential oils extracted from leaves are used in the perfume and food industries (Mulas et al., 1998), while leaves and berries are mainly used as sources of antioxidants (Tuberoso et al., 2007) and for liqueur production (Mulas and Cani, 1999). Considering the high commercial value of this species, and the success of liqueur production, the demand for raw material in processing industries is increasing. Most of the myrtle biomass (leaves and berries) is harvested from wild plants without consideration of the reduction of natural biodiversity. Consequently, the natural populations are progressively decreasing in number and size (Messaoud

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et al., 2006). Furthermore, berry production of natural populations is highly affected by meteorological conditions; consequently, fruit quality and quantity are often insufficient to meet the amount and the qualitative standard required by industries (Mulas and Fadda, 2004). Conservation programs appear indispensable to plan an efficient exploitation of the species that follows the growing request for myrtle biomass. Germplasm characterization represents a crucial step for conservation strategies and plant genetic resource use. Molecular markers have been largely used to assess the genetic diversity of wild species (Martinelli et al., 2008; Minnocci et al., 2010; Messaoud et al., 2011; Melito et al., 2013b; Dettori et al., 2014), and to explore the relationships among genetic, morphological, and ecological factors (Melito et al., 2013b, 2014). The exploration of myrtle genetic diversity of the Mediterranean Basin and the Middle East has been mainly carried out using random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and amplified fragment length polymorphism (AFLP) molecular markers (Messaoud et al., 2006; Agrimonti et al., 2007; Serçe et al. 2008; Melito et al., 2013a, 2014). Previous investigations of Sardinian myrtle wild populations and candidate cultivar collections (Melito et al., 2013a, 2014) showed that dominant molecular markers such as ISSR and AFLP can discriminate genotypes based on their geographical origin and ecological distribution. In addition, AFLP genotyping has been successfully used to study the genetic diversity among ecotypes from the Mediterranean area (Bruna et al., 2007). In this area, *M. communis* from Sardinia and Calabria presents a great level of biodiversity in morphological and genetic traits (Agrimonti et al., 2007), while fragmented information is still available for Sicilian myrtle population.

AFLP fingerprinting has been largely used to explore the genetic diversity and population structure of natural species under potential risk of genetic erosion (Schmidt and Jensen, 2000; Juan et al., 2004). Moreover, this marker system represents a useful technique to screen a large number of loci in species with reduced genetic information such as *M. communis*. The exploration of the genetic, chemical, and morphological diversity represents a fundamental step to study myrtle fitness, to improve the biomass production, and to plan future conservation strategies, in order to preserve the Mediterranean maquis ecosystem. In addition, the evaluation of plant diversity constitutes an important resource for agroindustrial purposes. To develop a core collection of local selected myrtle accessions from Sicily, a preliminary investigation of the natural germplasm was developed. In the present study, we report the population genetic diversity and structure of seven myrtle populations, as well as the correlation among morphological (biometric), chemical, ecological, and genetic characters.

2. Materials and methods

2.1. Plant material and sampling sites

Morphological, chemical, and genetic diversity was assessed in 36 *Myrtus communis* L. leaf and fruit samples belonging to 7 populations (Table S1). The Sicilian genotypes studied were collected from 7 localities and stored in a collection orchard located at the experimental station "Orleans" of the Department of Agricultural and Forest Sciences of the University of Palermo (Italy), located at Palermo (38°06′26.20″N, 13°20′56.00″E; 31 m a.s.l.). The genotypes' studied characteristics are reported in Table S1. The collection is the result of a germplasm study, based on the exploration of different localities, aimed at identifying the natural variability of the wild myrtle populations of Sicily. Meteorological data relevant for each site were provided by the Sicilian Agrometeorological Information Service (Italian acronym: SIAS) and derived from facilities located close to each site (Table S2). Monthly precipitation and temperature (average, maximum, and minimum) of historical series (2003–2013) were considered.

2.2. AFLP analysis

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. AFLP analysis was carried out according to Vos et al. (1995) using 250 ng of genomic DNA. Three Eco*R*I/Mse*I* primer combinations (E-AAC/M-CAT; E-AAC/M-CTG; E-AAC/M-CTA) with three selective nucleotides were used in this study. All polymerase chain reactions (PCRs) were performed using Platinum *Taq* DNA Polymerase High Fidelity. Preamplification 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, USA) for sample band size determinations. Gels were silver-stained according to Bassam et al. (1991). Polyacrylamide gels were manually analyzed and presence (1) or absence (0) was recorded for each band scored. Bands with a weak signal or blurred appearance were excluded. Samples for each primer pair were run on the same gel, allowing for fast and accurate manual scoring.

2.3. Genetic data analysis

Population structure was investigated using STRUCTURE 2.3.3 software (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 1 to 10 and were explored for each K. Twenty replicate chains of 200,000 Markov chain Monte Carlo interactions were run. A burn-in period of 100,000 interactions followed by an additional 500,000 interactions was run. The attribution of each sample to a specific cluster

was based on a coefficient of membership ($Q > 0.7$). The optimal K was calculated according to Evanno et al. (2005). Estimation of genetic diversity value (*He*), fixation index (F_{ST}), and analysis of molecular variance (AMOVA) were calculated by Arlequin version 3.5.1.2 (Excoffier et al., 2005). A phylogenetic analysis, based on UPGMA clustering (Nei, 1973; Nei and Li, 1979), was performed using TREECON software (Van de Peer and de Wachter, 1994).

2.4. Morphological analysis

During the 2011–2013 seasons, the following characteristics were measured individually in each accession collected in the experimental orchard: fruit length and width, fresh and dry weight, number of seeds per fruit, fruit and seed weight, pulp/seed ratio, and leaf length and width. The descriptor list proposed by Mulas and Cani (1999) was used as a reference. A hierarchical cluster analysis based on Ward's method was run using XLSTAT 2007 software (Addinsoft, France).

2.5. Chemical composition and antioxidant activity analysis

Leaves from each genotype were freeze-dried and ground. One gram of the lyophilized sample was extracted with 25 mL of methanol using a homogenizer (Ultra-Turrax, T25 Basic IKA, Germany) at 13,000 rpm for 2 min. Homogenates were centrifuged (10 min at 6000 × *g*) and the organic extracts were filtered with Whatman no. 4 filter paper. Three separate extractions were carried out for each genotype. Methanolic extracts were used for assessment of antioxidant activity, total phenols, and total tannins. Antioxidant activity was evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) methods according to Surveswaran et al. (2007). Briefly, for DPPH assay, 0.1 mL of diluted methanolic extract (1:10 in water) was mixed with 3.9 mL of 60 μM DPPH and stored in the dark for 120 min. DPPH absorbance was recorded at 515 nm. For ABTS assay, 3.9 mL of the ABTS radical solution was mixed with 100 μL of methanolic extracts appropriately diluted. The spectrophotometric readings at 734 nm were carried out after 120 min. For both assays, absorbance was recorded with an Agilent spectrophotometer (8453 UV-Visible Spectrophotometer, Agilent Technologies, USA), and results were expressed as TEAC units (mmol Trolox equivalents/100 g dry sample) using a Trolox calibration curve (3–15 μM; DPPH: $R^2 = 0.992$; ABTS: $R^2 = 0.998$). The total phenolic content was assayed using the Folin–Ciocalteu assay according to Singleton and Rossi (1965), with some modifications. The diluted extracts (0.1 mL) were added to 15 mL of deionized water and 1.25 mL of Folin–Ciocalteu reagent. Before adding 2.5 mL of 20% sodium carbonate (Na_2CO_3) solution, the mixture was shaken and allowed to stand for 6 min, and then it was adjusted with water to a final volume of 25 mL. After incubation for 120 min at room temperature, the absorbance was read at 750 nm. Results were expressed as catechin equivalents (CE) (mg/100 g dry sample) using catechin as an external standard (0.001–0.01 mg/mL, $R^2 = 0.992$). Tannins were measured by vanillin assay as reported by Fadda and Mulas (2010). Sample absorbance was detected at 500 nm and tannin concentration was calculated by means of a calibration curve with pure catechin (1–6 μg/mL, $R^2 = 0.998$). Results were expressed as milligrams of catechin per 100 grams of dry sample.

2.6. Statistical analysis

Correlations between experimental findings (antioxidant activity, total phenolics and tannins, altitude of growing localities, meteorological information, and plant biometric data) and the genetic coefficient of membership (Q) were calculated. Pearson's chi-square test for 2 × 2 contingency tables was performed for the categorical variables. All variables were standardized for the analysis, and the studies were carried out using JMP 7 software (SAS Institute, USA). Correlations between biometric and genetic distance matrices were explored by Mantel test using XLSTAT 2007 software. For total phenols, tannins, and antioxidant activity analysis of variance (one-way ANOVA) was performed using StatGraphics software (version XV, Manugistics, USA). Comparisons of means were carried out according to Duncan's multiple range test at $P \leq 0.05$.

3. Results and discussion

3.1. Population genetic structure

To protect the natural myrtle populations, the assessment of genetic diversity is a necessary step to prevent genetic erosion events. In Sardinia and in Calabria, for instance, molecular markers have been used to evaluate the genetic variability among and within natural myrtle populations (Agrimonti et al., 2007; Melito et al., 2013a). Molecular markers, such as ISSR and AFLP, have been used to investigate the shape of genetic diversity and the gene flow of several plant species (Barcaccia et al., 1999; Tomkins et al., 2001; Portis et al., 2005). In the same way, wild myrtle populations and candidate cultivar selections from the Mediterranean Basin have been studied by means of AFLP markers (Bruna et al., 2007; Albaladejo et al., 2009; Melito et al., 2014; Nora et al., 2015). The estimation of the genetic diversity represents a preliminary step to plan future breeding programs to increase fruit and biomass yield and to individuate markers associated with important agronomical traits.

The AFLP analysis of myrtle accessions produced overall 152 reproducible fragments ranging from 50 to 500 bp. STRUCTURE analysis and the ΔK method (Evanno et al., 2005) (Figure S1) revealed three main genetic groups:

Cluster A (most of the Misilmeri samples), Cluster B (Scopello, Ribera, Sciacca, and M. Pellegrino), and Cluster C (Ispica and Ribera) (Figures 1a and 1b). More than 88% of the genotypes had $Q > 0.7$, and only 4 samples (MRT7 M. Pellegrino, MRT2 Sciacca, MRT5 Misilmeri, MRT5 Scopello) displayed a lower Q. These genotypes, defined as "admixed", were not assigned to any specific genetic group and were excluded from the successive investigations (Figure 1a). These plants are probably the result of gene flow, generated by crossing events, among the three

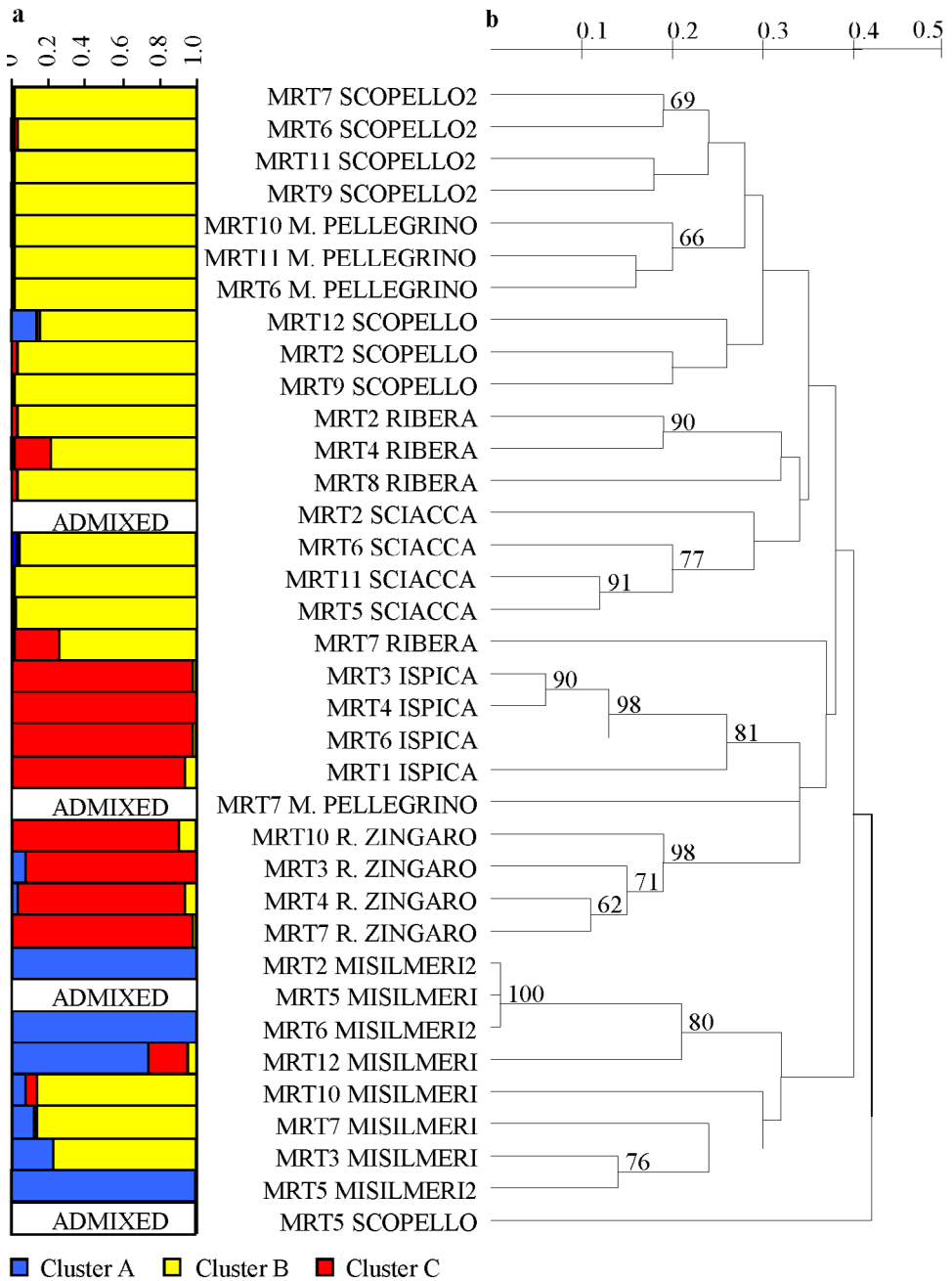

Figure 1. a) Population genetic structure of 36 wild *Myrtus communis* plants from 7 different localities in Sicily. Results based on $K = 3$ partition using the Bayesian clustering model analysis implemented in the STRUCTURE program (Pritchard et al., 2000). Each individual was assigned to one of the three genetic clusters (A, B, C) based on the coefficient of membership ($Q > 0.7$). Four myrtle plants defined as admixed were not assigned to any specific genetic group because of their low coefficient of membership. b) A UPGMA-based dendrogram showing the genetic relationship among myrtle samples was also run. The 1000 replicate bootstrap support fractions are indicated for the higher nodes.

genetic clusters identified. A similar feature was previously shown in Sardinian wild populations and candidate clone selections (Melito et al., 2013a, 2014), where few admixed individuals were identified by Bayesian clustering model analysis. Comparable results were obtained by exploring the myrtle accession relationship with the UPGMA clustering method (Nei and Li, 1979) (Figure 1b). The dendrogram showed a general congruence with the Bayesian clustering model, as inferred by STRUCTURE. In order to explore the geographical distribution of the three genetic clusters, the average *Q* was evaluated for each population. The distribution of the three genetic clusters (A, B, and C) presents a specific local assignment as shown in Figure 2. In Cluster A only the Misilmeri population was predominant, while Cluster B included Scopello, M. Pellegrino, Sciacca, and Ribera sites and C included myrtle plants from Ispica and R. Zingaro. Besides the geographical localization, other environmental factors might influence the genetic group distribution in Sicily, such as the altitude. Relationships between altitude level and genetic differentiation have been only partially explored in the family Myrtaceae. In *Metrosideros polymorpha*, for instance, only limited differentiation was observed along altitudinal gradients (Aradhya et al., 1993), while in a Sardinian myrtle collection from different environmental conditions, a significant correlation between genetic clusters and the altitude levels of each sampling site was shown (Melito et al., 2014). To explore whether the genetic diversity distribution of the wild Sicilian myrtle population was influenced by the altitude gradient, the correlation between *Q* and altitude level was analyzed. A significant correlation was found with Cluster C (Pearson, $P = 0.0163$). Considering that this research represents a preliminary exploration of the myrtle genetic diversity distribution in Sicily, we are aware that the sample sites as well as the altitude levels explored are limited. Further investigation will be conducted in order to deeply explore this trend across more divergent sampling sites.

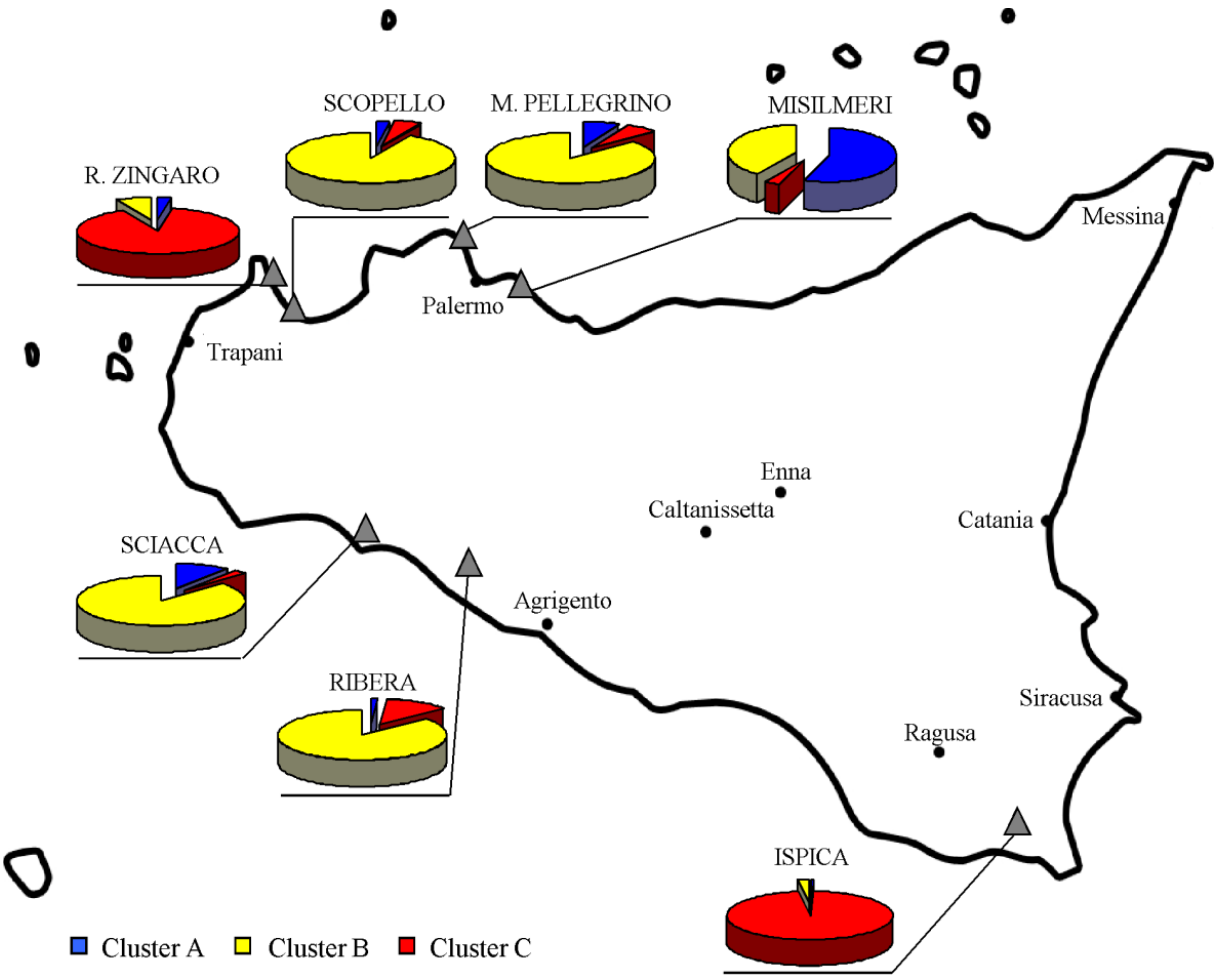

Figure 2. Distribution of Sicilian myrtle population's genetic clusters, as evaluated by STRUCTURE, based on the geographical origin. Each individual pie chart shows the average coefficient of membership for each population at $K = 3$.

Ecological variables could influence the genetic diversity distribution in myrtle. Indeed, the exploration of the environmental factors correlated to the genetic cluster is a fundamental step to identify candidate accession with interesting agronomical traits for future breeding programs. Climatic conditions of specific sampling sites are considered relevant parameters for fruit quality and plant biomass production. Relevant meteorological data for the collection sites are given in Table S3. Pairwise correlation between climatic parameters and genetic clusters identified a few important factors that influence the distribution of the genetic diversity (Table S4). The average rainfalls (mm) of the winter months of January and March are positively correlated to the *Q* of Cluster A (Spearman, P = 0.01), while the average maximum temperatures of December and February are negatively correlated. The average temperature of the period between May and October (with the exception of September) positively correlated with Cluster B; summer average temperature (May to August), average minimum temperature of July, and average rain precipitation of October all negatively correlated with Cluster C (Table S4).

3.2. Genetic diversity

The observed *He* values ranged from 0.148 to 0.251, with an average *He* of 0.210. R. Zingaro showed the lowest *He* value, while Scopello had the highest score. The genetic diversity expressed as *He* reported in this research is comparable to the *He* reported by Messaoud et al. (2006) in Tunisian myrtle populations. However, our results were lower than in a previous work conducted in Sardinia by Melito et al. (2014). The apparent discrepancy between the *He* values of these two Italian islands could be the consequence of different sampling strategies. In Sardinia, the genetic diversity was recorded in a candidate clone selection from all over Sardinia, while in our case we are considering the population genetic diversity in a natural myrtle population without any a priori collection strategy. ANOVA and Wilcoxon tests revealed a significant *He* difference among the Sicilian populations (P = 0.00008; P < 0.0001). A similar result was found considering the genetic cluster assignment (P > 0.0001), while no correlation was found between *He* and altitude level. The absence of correlation between *He* and altitude level could be the result of the low variability of environmental conditions: the altitude gradient in fact ranged from 44 to 236 m a.s.l. The genetic diversity was also explored by F_{ST} value. An F_{ST} pairwise distance matrix was calculated with Arlequin and the results are shown in Table 1. The overall average F_{ST} indicated a quite high level of genetic divergence among the seven myrtle populations studied (F_{ST} = 0.332). These data were quite comparable to the F_{ST} values recorded in *M. communis* populations from upper semiarid and subhumid bioclimatic regions in Tunisia (Messaoud et al., 2006). Misilmeri and Ispica plants present the highest genetic distance (F_{ST} = 0.502), while M. Pellegrino and Scopello had the lowest genetic distance with an F_{ST} value of 0.153. These populations showed different genetic cluster assignments (Figure 2). These differences are the results of a differential gene flow among the populations. Geographical distance and physical barriers could have negatively influenced the pollen dispersion, causing limitation of genetic exchange between sampling sites.

Table 1. Pairwise F_{ST} matrix among the seven Sicilian myrtle populations. In bold are indicated the highest and lowest F_{ST} values. All comparisons were significant after 1000 random permutation tests (P < 0.05).

	Ispica	M. Pellegrino	Misilmeri	R. Zingaro	Scopello	Ribera	Sciacca
Ispica	0.000						
M. Pellegrino	0.269	0.000					
Misilmeri	**0.502**	0.221	0.000				
R. Zingaro	0.406	0.360	0.375	0.000			
Scopello	0.398	**0.153**	0.331	0.460	0.000		
Ribera	0.329	0.190	0.342	0.429	0.240	0.000	
Sciacca	0.348	0.201	0.303	0.489	0.264	0.240	0.000

The Misilmeri and Ispica populations, which showed the highest F_{ST} value, are localized in two opposite sites of the island (Figure 2) and are probably subjected to isolation by distance. In contrast, the low F_{ST} found between M. Pellegrino and Scopello indicated high gene flow among individuals belonging to the two nearby sampling sites (Figure 1). Based on this finding, the F_{ST} pairwise distance matrix was also estimated among the three genetic clusters identified (data not shown). Cluster A, mainly consisting of the Misilmeri site, was highly differentiated in comparison to Clusters B and C (F_{ST} values respectively of 0.477 and 0.463); in contrast, a much lower differentiation was found between Clusters B and C (F_{ST} = 0.217). The F_{ST} pairwise distances among populations assigned to Clusters A, B, and C were smaller than those found among the 7 myrtle populations. AMOVA was run to explore the genetic variance distribution among and between myrtle populations. Most of the genetic variation was found within populations (66.84%), while a lower value was detected among the 7 myrtle populations (33.16%) (Table 2). In addition, to evaluate the population genetic structure's contribution to the genetic variance, AMOVA was also performed at K = 3 genetic partition. Again in this case, a similar distribution of genetic variance was found within (65.95%) and among (34.05%) the genetic clusters. In addition, at K = 3, AMOVA revealed a total F_{ST} value almost equal to that of the overall population (F_{ST} = 0.340 and F_{ST} = 0.332, respectively) (Table 2). These results showed that, despite a significant part of variation being attributed to the difference among populations and among the three genetic clusters identified (33.05 and 34.05), the main source of variance is at the intrapopulation level. These findings suggest that, as with other Myrtaceae species, myrtle is an outcrossing species with a proportion of self-pollination (Lughadha and Proenca, 1996; Mulas and Fadda, 2004). AMOVA and the F_{ST} data are compatible with prevalent pollination by pollinators such as coleopterans. In this case, the limited mobility of the insects induced more genetic exchange among individuals of the same populations and geographically neighboring populations (Agrimonti et al., 2007).

3.3. Morphological data

Results of biometric characters of fruits and leaves are reported in Tables 3a and 3b. Fruit length ranged from 7.18 to 9.03 mm and fruit width between 5.74 and 8.22 mm. Length/width ratio was between 1.10 and 1.39. The smallest fruits were observed in the Ribera and M. Pellegrino accessions with 0.25 g of fresh weight and 0.09 g of dry weight, while the largest fruits were from R. Zingaro with 0.34 g and 0.13 g respectively of fresh and dry fruit weight (Table 3a). The largest fruits also had the highest number of seeds (18.43) and showed the highest pulp/seed ratio (5.88). The smallest fruits had the lowest values of 10.34 and 11.87 seeds per fruit and low pulp/seed ratios (3.81 and 3.71). The seed weight per fruit was quite constant, ranging between 0.04 and 0.05 g. The largest leaves were observed in the Sciacca accessions with 34.50 mm of length and 14.08 mm of width, while the smallest were from R. Zingaro with 26.42 mm of length and 9.88 mm of width (Table 3b).

A more specific analysis was performed to cluster the 36 *M. communis* accessions based on the biometric data shown in Tables 3a and 3b (Figure 3). Overall, a high level of similarity among the plants was observed (coefficient of similarity: >0.92). Results revealed two principal groups that diverge at a similarity of 0.92. The smallest group (I) contains 6 plants that further clustered in two subgroups (A, B). The first contains myrtle accessions mainly from Scopello, and the second subcluster included two accessions from M. Pellegrino and Ispica. Most of the plant samples were instead clustered in group II, where two principal subgroups can be identified (C, D), which collected individuals from all the explored localities. The dendrogram did not show any correspondence between plant and geographical origin of the myrtle accessions. This result could be in part explained considering the high

Table 2. Partition of genetic diversity determined by AMOVA analysis. The overall AMOVA and the population genetic structure at K = 3 were considered as sources of molecular variance.

Partition	Sources of variation	d.f.	Sum of squares	% of variation	F_{ST}
Overall	Among populations	6	254.71	33.16	0.332
	Within populations	29	351.88	66.84	
	Total	35	606.58		
K = 3	Among clusters	2	143.35	34.05	0.340
	Within clusters	29	385.78	65.95	
	Total	31	529.13		

Table 3. Biometric characters of myrtle fruits (a) and leaves (b) as observed in Sicilian myrtle populations.

a.

Population	Fruit length (mm)	Fruit width (mm)	Fruit length/ width ratio	Fruit fresh weight (g)	Fruit dry fruit weight (g)
Ribera	7.22 ± 0.26	5.76 ± 0.43	1.26 ± 0.08	0.25 ± 0.03	0.09 ± 0.01
Ispica	8.15 ± 0.51	5.85 ± 0.27	1.39 ± 0.03	0.31 ± 0.05	0.12 ± 0.02
Misilmeri	7.44 ± 0.54	5.74 ± 0.38	1.30 ±0.10	0.27 ± 0.05	0.10 ± 0.01
R. Zingaro	9.03 ± 0.39	8.22 ± 0.41	1.10 ± 0.01	0.34 ± 0.04	0.13 ± 0.02
Scopello	8.22 ± 0.77	7.18 ± 0.97	1.16 ± 0.10	0.29 ± 0.05	0.10 ± 0.02
Sciacca	7.96 ± 0.50	6.08 ± 0.32	1.31 ± 0.10	0.27 ± 0.04	0.09 ± 0.01
M. Pellegrino	7.18 ± 0.70	5.86 ± 0.45	1.22 ± 0.08	0.25 ± 0.04	0.09 ± 0.02

b.

Population	Seed number per fruit	Seed weight per fruit	Weight of 1000 seeds (g)	Pulp weight (g)	Pulp/seed ratio	Leaf length (mm)	Leaf width (mm)	Leaf length/ width ratio
Ribera	11.87 ± 2.53	0.05 ± 0.01	4.53 ± 0.46	0.20 ± 0.02	3.71 ± 0.12	31.42 ± 3.11	13.00 ± 1.21	2.42 ± 0.18
Ispica	12.47 ± 4.39	0.05 ± 0.02	4.47 ± 1.04	0.25 ± 0.04	4.90 ± 0.92	26.67 ± 1.80	10.22 ± 0.69	2.61 ± 0.18
Misilmeri	12.48 ± 2.64	0.05 ± 0.01	4.50 ±0.40	0.22 ± 0.05	3.97 ± 0.53	26.77 ± 1.98	11.23 ± 1.77	2.41 ± 0.30
R. Zingaro	18.43 ±4.06	0.05 ± 0.01	2.62 ± 0.24	0.29 ± 0.04	5.88 ± 0.38	26.42 ± 1.52	9.88 ± 0.55	2.68 ± 0.07
Scopello	12.66 ± 2.67	0.04 ± 0.01	4.27 ± 0.62	0.25 ± 0.05	5.79 ± 1.00	33.46 ± 4.90	13.08 ± 2.34	2.57 ± 0.22
Sciacca	15.23 ± 2.46	0.05 ± 0.01	4.50 ± 0.62	0.22 ± 0.04	4.52 ± 0.93	34.50 ± 3.47	14.08 ± 1.45	2.45 ± 0.13
M. Pellegrino	10.34 ± 2.30	0.05 ± 0.01	5.33 ± 0.59	0.20 ± 0.03	3.81 ± 0.46	27.83 ± 2.73	12.50 ± 1.97	2.25 ± 0.21

similarity of the morphological data recorded. A general lower variability of the fruit and leaf biometric characters was detected in this study compared to previously studied populations of Sardinia (Mulas and Cani, 1999). The dendrogram based on molecular markers (Figure 1) and biometric traits (Figure 3) showed genetic variation among the cultivars. The Mantel test revealed no correlation between molecular and morphological trait matrices (data not shown). However, those two dendrograms presented some accessions grouped in the same cluster in both dendrograms, such as Scopello 2 plants (Figures 2 and 3). The differences between AFLP and the biometric dendrogram could be mainly due to the morphological traits, which could be influenced by many parameters, such as the sample size, environmental conditions, and time of recording measurements.

Although the biometric data revealed no significant correlation with the molecular markers profiles, a different trend was observed with the morphological traits. Significant correlation between bush shape/plant growth behavior and the genetic cluster was found (χ^2 = 15.83, P = 0.0003; χ^2 =25.28, P = 0.0033). Figures 4a and 4b present the morphotypes' distribution based on the genetic cluster assigned. Individuals assigned to Cluster A have a flat or round bush shape, open or intermediate bush with upright basal shoots; Cluster B instead presents all bush shapes and almost all types of plant growth behavior with the exception of tree type; finally, Cluster C presented elongated bush and tree/bushy upright growth behavior. In order to explore the distribution of biometric characters among the identified genetic clusters, simple pairwise correlations were tested among these data (Table 4). Morphologic traits were associated only with Clusters B and C. Myrtle accessions belonging to these groups presented opposite trends in term of leaf morphology: Cluster B was negatively correlated with leaf length and width, while Cluster C showed a positive correlation. Based on fruit characters, most of the significant positive

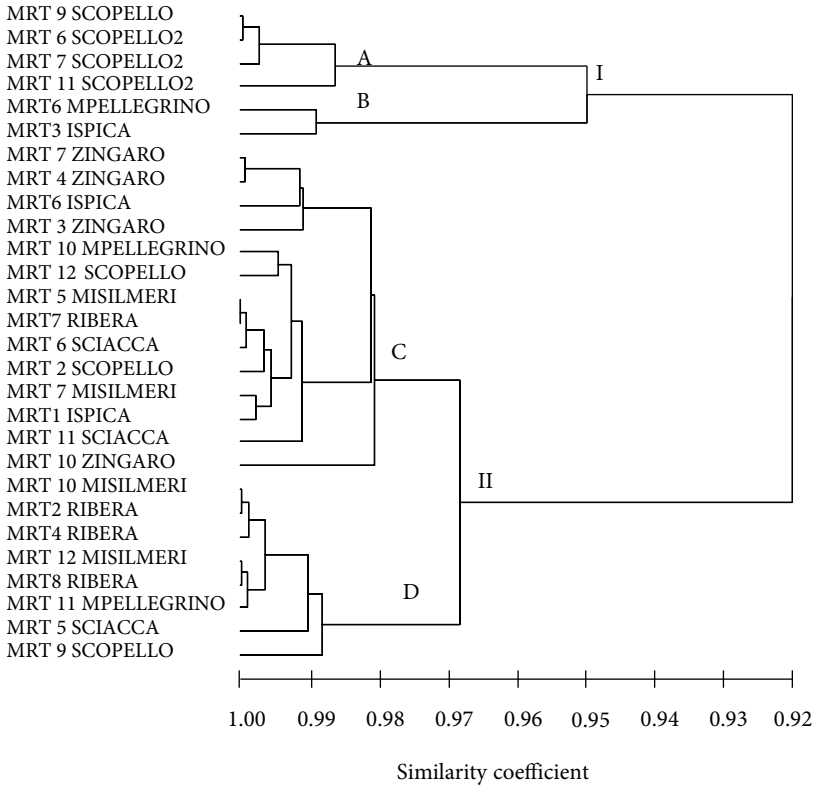

Figure 3. Hierarchical cluster dendrogram of Sicilian *M. communis* accessions based on biometric data (Ward method).

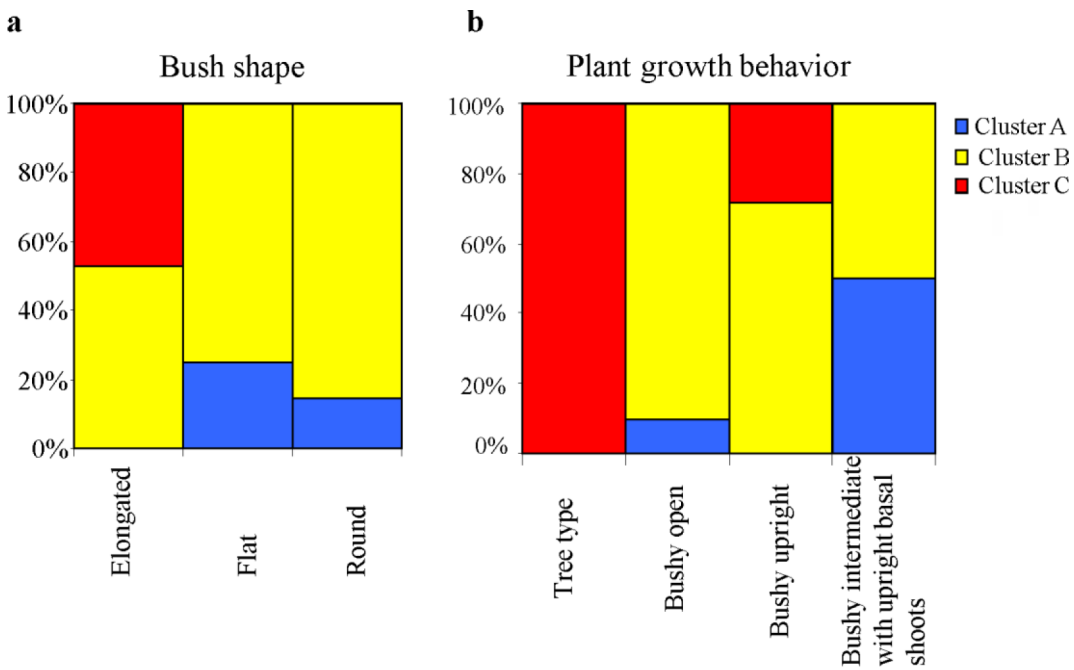

Figure 4. Morphotype distributions in the three genetic clusters identified by Bayesian clustering model analysis. Significant correlation between genetic cluster and bush shape (P = 0.0003) or plant grow behavior (P = 0.0033) was found.

Table 4. Pairwise correlation results between the genetic clusters' coefficient of membership (*Q*) and morphological traits. Significantly correlated variables are reported ($P < 0.05$).

Variable	By variable	Correlation	Signif. prob.
Leaf length (mm)	Cluster B	–0.448	0.017
	Cluster C	0.561	0.002
Leaf width (mm)	Cluster B	–0.546	0.003
	Cluster C	0.565	0.002
Fruit length (mm)	Cluster B	0.462	0.013
Fresh fruit weight (g)	Cluster B	0.441	0.020
Pulp weight	Cluster B	0.448	0.017
Dry fruit weight (g)	Cluster B	0.593	0.001
	Cluster C	–0.490	0.008
1000-seed weight (g)	Cluster B	–0.537	0.003
	Cluster C	0.465	0.013

correlations were found with Cluster B. No association between Cluster A and leaf and fruit morphological data was found.

3.4. Chemical composition

The leaf content of total phenols and tannins in Sicilian myrtle populations is reported in Table 5. Total phenol content ranged from 2466 to 3800 mg/100 g of dry weight, measured in plants from M. Pellegrino and Misilmeri, respectively. These results are lower than the findings of Mulas and Melis (2008) in Sardinian populations. No significant differences were found among populations for total phenol contents; however, the high standard deviation measured in Ispica and M. Pellegrino populations reveals a high variability of myrtle accessions belonging to these populations. Significant differences were observed among the seven population regarding tannin content, which ranged from 93.9 to 262.3 mg/100 g of leaf dry weight, measured in R. Zingaro and Misilmeri populations, respectively. These data agreed with previous data reported by Mulas and Melis (2008). The antioxidant capacity, measured as radical scavenging activity against DPPH and ABTS radicals, is also shown in Table 5. DPPH and ABTS radical quenching ranged from 21.4 to 33.5 (DPPH) and from 24.2 to 39.5 (ABTS) mmol Trolox/100 g of leaf dry weight, measured in Monte Pellegrino and Misilmeri populations, respectively. Little differences were found for the ability to quench DPPH and ABTS radicals among the populations studied. A positive correlation was calculated between total phenol content and DPPH radical scavenging activity: a higher phenol content was positively correlated with a higher antioxidant activity. A positive correlation was also found between DPPH and ABTS scavenging results (Table S5). Multivariate analysis between leaf composition and genetic clusters was performed. The coefficient of membership of Cluster B, which included most of the myrtle samples, was the only one significantly correlated to the chemical composition. The negative ρ suggested a negative correlation between genetic and chemical profiles (Spearman, $P < 0.001$) (Table S5).

Within the framework of the domestication process, previous studies were conducted on wild myrtle accessions in order to evaluate phenotypic variability (Mulas and Cani, 1999). Based on these studies a few crucial phenotypic traits, such as fruit shape and color, as well as the plant vigor, bush habitus, and the relationship between the spring shoot length and the flower/fruit quantity, were recognized as part of the ideal plant type suitable for myrtle cultivation.

Advanced selections were further studied for chemical composition of biomasses and the value of aromatic and phenolic compounds for the processing industry was clearly demonstrated (Mulas and Melis, 2008; Fadda and Mulas, 2010). Only in recent times has the genetic variability of wild and candidate cultivar selections in Sardinia been deeply explored (Melito et al., 2013a, 2014). This genetic approach highlighted the importance of the molecular markers in assessing the genetic diversity in wild accession and candidate cultivar selections.

This study represents the first exploration of the morphological, genetic, and chemical diversity of natural myrtle populations in Sicily. Based on the previous experiences of Sardinian myrtle domestication

Table 5. Tannins, phenols, and antioxidant activity (DPPH and ABTS) evaluated for each myrtle population. Tannins and phenols were measured as mg CE/100 g DW, while DPPH and ABTS were estimated as mmol Trolox/100 g DW.

Population	Tannins*	Phenols*	Antioxidant activity	
			DPPH*	ABTS*
Ispica	210.4 ± 29.2 (ab)	2788.2 ± 371.3 *	25.2 ± 5.6 (ab)	29.8 ± 6.0 (abc)
M. Pellegrino	136.9 ± 45.5 (bc)	2466.2 ±636.3	21.4 ± 3.4 (b)	24.19 ± 5.0 (c)
Misilmeri	262.3 ± 68.4 (a)	3800.1 ± 5.8	33.5 ± 3.6 (a)	39.5 ± 5.2 (a)
Ribera	162.9 ± 66.6 (bc)	2830.9 ± 11.5	25.9 ± 12.3 (ab)	29.4 ± 11.5 (abc)
R. Zingaro	93.9 ± 19.1 (c)	2762.3 ± 8.4	25.8 ± 8.1 (ab)	28.9 ± 8.5 (bc)
Sciacca	190.5 ± 29.8 (b)	3042.2 ± 7.1	26.4 ± 6.8 (ab)	30.6 ± 7.1 (abc)
Scopello	184.7 ± 49.1 (b)	3511.7 ± 10.9	32.4 ± 9.3 (a)	36.9 ± 10.9 (ab)

*Each value is the mean of the accessions belonging to each population. Means followed by a common letter are not significantly different by Duncan's multiple range test, $P \leq 0.05$.

programs, the novelty of this research is in the attempt to simultaneously use information derived from morphological, chemical, and genetic analysis to assist in cultivar selection. Some preliminary results in this direction seem to support our objective.

M. communis is an aromatic and ornamental plant used for essential oil extraction and liqueur production by berry infusion. Most of the plant uses are related to the harvest of fruits and leaves from natural populations; indiscriminate overexploitation induced a strong reduction of wild myrtle populations, which are probably not sufficient for the growing demand for liqueur production. The present study allowed the characterization of the germplasm variability of a core collection of Sicilian myrtle populations. The genetic analysis performed in this study revealed 3 main clusters that are statistically correlated to the bush shape and plant growth behavior. In addition, two of them are significantly correlated to useful biometric traits, which could be used as morphological markers for fruit and biomass production in selection and breeding programs. Finally, tannin and phenol contents, as well as antioxidant activity, revealed a level of variability moderate among the different populations but high in the whole studied population. The multidisciplinary approach allowed us to record for the first time interesting genetic, chemical, and morphological traits that could be used to select candidate clones for future domestication programs.

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Table S1. Characteristics of myrtle sampling localities. For each accession, sampling location with geographical coordinates and altitude (Alt), identity code, bush shape/growth behavior, and altitude are reported. Bush shapes (BS) were divided into elongated (E), flat (F), round (R), and tree type (TT). Plant growth behaviors (PGB) were identified as bushy upright (BU), bushy intermediate with upright basal shoots (BIUBS), bushy open (BO), and tree type (TT).

Locality	Code	BS	PGB	Alt (m)	Coordinates	
					Latitude	Longitude
Ispica (Ragusa)	MRT1 Ispica	E	BU	130	36°43′47.64″N	14°59′36.66″E
	MRT3 Ispica	E	BU			
	MRT4 Ispica	E	BU			
	MRT6 Ispica	E	BU			
Monte Pellegrino (Palermo)	MRT6 M. Pellegrino	E	BU	76	38°07′51.48″N	13°19′40.33″E
	MRT7 M. Pellegrino	E	BU			
	MRT10 M. Pellegrino	E	BU			
	MRT11 M. Pellegrino	E	BU			
Misilmeri (Palermo)	MRT2 Misilmeri 2	F	BIUBS	107	38°01′54.90″N	13°26′35.63″E
	MRT3 Misilmeri	F	BIUBS			
	MRT5 Misilmeri	F	BO			
	MRT5 Misilmeri 2	F	BIUBS			
	MRT6 Misilmeri 2	F	BIUBS			
	MRT7 Misilmeri	R	BO			
	MRT10 Misilmeri	R	BO			
	MRT12 Misilmeri	R	BO			
Riserva Zingaro (Trapani)	MRT3 R. Zingaro	E	TT	171	38°00′52.37″N	12°53′22.36″E
	MRT4 R. Zingaro	E	TT			
	MRT7 R. Zingaro	TT	TT			
	MRT10 R. Zingaro	E	TT			
Scopello (Trapani)	MRT2 Scopello	E	BU	77	37°51′24.85″N	12°52′55.98µE
	MRT5 Scopello	E	BU			
	MRT6 Scopello 2	E	BU			
	MRT7 Scopello 2	E	BU			
	MRT9 Scopello	E	BU			
	MRT9 Scopello 2	E	BU			
	MRT11 Scopello 2	E	BU			
	MRT12 Scopello	F	BU			
Ribera (Agrigento)	MRT2 Ribera	R	BO	236	37°26′19.31″N	13°15′59.18″E
	MRT4 Ribera	R	BO			
	MRT7 Ribera	R	BO			
	MRT8 Ribera	R	BO			
Sciacca (Agrigento)	MRT5 Sciacca	F	BO	44	37°35′30.86″N	13°02′23.09″E
	MRT6 Sciacca	F	BO			
	MRT7 Sciacca	F	BO			
	MRT11 Sciacca	F	BO			

Table S2. Meteorological stations' coordinates (latitude, longitude, altitude, and sea distance). Climatic data of each meteorological station were used to describe each population.

Station	Meteorological station	Latitude (N)	Longitude (E)	Altitude (m a.s.l.)	Sea distance (m)
Ribera	Giardinello	37°26′19.31″	13°15′59.18″	30	1991.37
Ispica	Cancaleo	36°43′47.64″	14°59′36.66″	30	4325.36
Misilmeri	Marraffa	38°01′54.90″	13°26′35.63″	160	7602.08
R. Zingaro	Crociferi	38°00′52.37″	12°53′22.36″	90	1158.00
Scopello	Eredità Forni	37°51′24.85″	12°52′55.98″	310	19,468.94
Sciacca	Molino Nuovo	37°35′30.86″	13°02′23.09″	90	7212.39
M. Pellegrino	Uditore	38°07′51.48″	13°19′40.33″	50	4003.41

Table S3. Monthly average meteorological data of Sicilian myrtle sampling sites: a) average temperature (Av. Tem.); b) average maximum temperature (Av. Max. Tem.); c) average minimum temperature (Av. Min. Tem.); d) average millimeters of rain (Av. Rain).

a

Collection site	Av. Tem. Jan	Av. Tem. Feb	Av. Tem. Mar	Av. Tem. Apr	Av. Tem. May	Av. Tem. Jun	Av. Tem. Jul	Av. Tem. Aug	Av. Tem. Sep	Av. Tem. Oct	Av. Tem. Nov	Av. Tem. Dec
Ispica	11.5	10.8	12.7	15.4	18.5	22.3	25.4	26.1	23.5	20.3	16.3	13.1
M. Pellegrino	20.0	19.2	22.7	27.1	32.1	37.7	42.2	42.8	38.1	33.0	27.1	22.3
Misilmeri	18.0	17.4	21.2	25.9	31.4	37.4	41.7	42.4	37.2	32.2	25.5	20.1
R. Zingaro	18.6	17.9	21.3	25.7	30.6	36.0	40.3	41.4	36.9	32.1	25.8	21.0
Scopello	17.8	16.9	20.6	25.3	30.8	37.4	41.8	42.1	36.5	31.7	25.0	20.0
Ribera	18.7	17.7	21.0	25.5	30.8	36.2	40.3	40.6	36.5	32.2	25.8	20.7
Sciacca	19.1	18.2	21.5	26.4	32.1	38.2	43.0	43.3	37.5	32.5	26.1	21.1

b

Collection site	Av. Max. Tem. Jan	Av. Max. Tem. Feb	Av. Max. Tem. Mar	Av. Max. Tem. Apr	Av. Max. Tem. May	Av. Max. Tem. Jun	Av. Max. Tem. Jul	Av. Max. Tem. Aug	Av. Max. Tem. Sep	Av. Max. Tem. Oct	Av. Max. Tem. Nov	Av. Max. Tem. Dec
Ispica	16.1	15.7	17.6	20.6	24.2	28.4	31.7	31.9	28.6	24.9	20.9	17.6
M. Pellegrino	16.0	15.5	18.1	21.1	24.6	28.3	31.2	31.6	28.2	25.0	21.1	17.5
Misilmeri	15.0	14.7	17.5	20.9	25.2	29.3	32.2	32.7	28.7	25.2	20.3	16.3
R. Zingaro	14.9	14.5	16.9	20.2	23.8	27.5	30.3	31.0	27.8	24.6	20.1	16.4
Scopello	14.0	13.6	16.2	19.8	24.0	28.7	31.6	31.7	27.4	23.9	19.0	15.4
Ribera	16.1	15.6	17.6	20.9	24.9	28.6	31.4	31.4	28.1	25.1	20.7	17.3
Sciacca	15.4	15.0	17.4	20.9	25.3	29.5	32.9	33.0	28.4	24.8	20.2	16.7

c

Collection site	Av. Min. Tem. Jan	Av. Min. Tem. Feb	Av. Min. Tem. Mar	Av. Min. Tem. Apr	Av. Min. Tem. May	Av. Min. Tem. Jun	Av. Min. Tem. Jul	Av. Min. Tem. Aug	Av. Min. Tem. Sep	Av. Min. Tem. Oct	Av. Min. Tem. Nov	Av. Min. Tem. Dec
Ispica	6.9	5.9	7.9	10.3	12.7	16.2	19.2	20.2	18.4	15.7	11.7	8.6
M. Pellegrino	8.0	7.4	9.2	11.9	14.9	18.8	22.0	22.3	19.7	16.1	12.1	9.6
Misilmeri	6.0	5.2	7.3	9.9	12.5	16.3	19.0	19.5	17.1	14.0	10.2	7.6
R. Zingaro	7.3	6.8	8.6	11.0	13.6	17.1	20.1	20.9	18.3	15.0	11.4	9.1
Scopello	7.7	6.7	8.7	11.1	13.7	17.4	20.3	20.8	18.2	15.6	11.9	9.2
Ribera	5.3	4.3	6.7	9.2	11.9	15.2	17.8	18.5	16.7	14.3	10.3	6.9
Sciacca	7.3	6.4	8.3	10.9	13.7	17.3	20.2	20.8	18.3	15.5	11.8	8.8

d

Collection site	Av. Rain Jan	Av. Rain Feb	Av. Rain Mar	Av. Rain Apr	Av. Rain May	Av. Rain Jun	Av. Rain Jul	Av. Rain Aug	Av. Rain Sep	Av. Rain Oct	Av. Rain Nov	Av. Rain Dec
Ispica	24.2	9.2	32.6	98.8	5.2	8.0	0.0	0.4	56.4	47.2	128.2	137.6
M. Pellegrino	88.7	95.4	82.2	56.7	14.0	14.0	5.7	4.8	83.6	108.1	77.5	113.2
Misilmeri	90.1	92.1	92.0	53.0	13.0	10.4	4.1	4.3	73.0	89.9	61.1	96.4
R. Zingaro	106.6	112.1	104.5	68.6	16.8	16.4	4.0	4.9	80.5	101.6	96.7	133.3
Scopello	96.5	86.6	96.9	69.4	23.1	11.9	9.7	6.0	77.2	103.2	82.1	109.6
Ribera	73.2	67.1	67.2	34.2	6.9	6.1	2.6	6.6	61.7	79.2	72.4	85.3
Sciacca	73.9	67.2	70.5	54.2	15.5	13.8	1.6	8.7	60.5	90.9	63.9	93.8

Table S4. Nonparametric pairwise correlation results between climate variables and coefficient of membership (*Q*) of the tree genetic cluster identified by STRUCTURE. In the table the variables that are significantly correlated are reported (P < 0.05).

Variable	By variable	Spearman ρ	Prob. > |ρ|
Av. Max. Tem. Dec	Cluster A	–0.89	0.01
Av. Rain Jan	Cluster A	0.79	0.04
Av. Rain March	Cluster A	0.79	0.04
Av. Max. Tem. Feb	Cluster A	–0.82	0.02
Av. Tem. Aug	Cluster B	0.79	0.04
Av. Tem. July	Cluster B	0.93	0.00
Av. Tem. Jun	Cluster B	0.86	0.01
Av. Tem. May	Cluster B	0.86	0.01
Av. Tem. Oct	Cluster B	0.79	0.04
Av. Rain Oct	Cluster C	–0.79	0.04
Av. Tem. Aug	Cluster C	–0.79	0.04
Av. Tem. July	Cluster C	–0.89	0.01
Av. Tem. Jun	Cluster C	–0.82	0.02
Av. Tem. May	Cluster C	–0.82	0.02
Av. Min. Tem. Jun	Cluster C	–0.82	0.02

Table S5. Pairwise nonparametric correlations between tannins, total polyphenols, ABTS and DPPH radical scavenging activities, and the three genetic clusters (A, B, C) identified by the STRUCTURE tool.

Variable	By variable	Spearman ρ	Prob. > |ρ|
Tannins	DPPH	0.527	0.001
	ABTS	0.556	0.0004
	Tot. polyphenols	0.662	<0.0001
Tot. polyphenols	ABTS	0.931	<0.0001
	DPPH	0.937	<0.0001
ABTS	DPPH	0.959	<0.0001
Cluster B	DPPH	–0.394	0.0175
	ABTS	–0.443	0.0068
	Tot. polyphenols	–0.517	0.0013
	Tannins	–0.489	0.0025

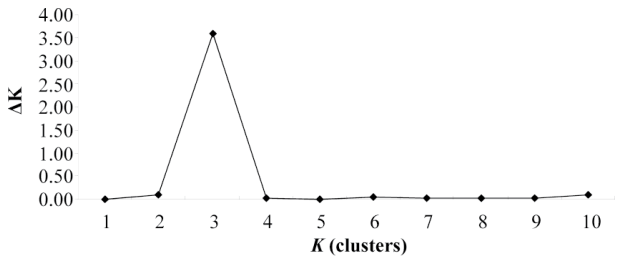

Figure S1. Estimation of the most likely number of genetic clusters (*K*) based on the method of Evanno et al. (2005). The highest ΔK was found at $K = 3$.