Characterization of Sicilian rosemary (*Rosmarinus officinalis* L.) germplasm through a multidisciplinary approach

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Abstract

A germplasm collection of *Rosmarinus officinalis* was harvested from 15 locations in Sicily. Eleven wild and 4 cultivated populations were collected and, due to the surveyed area covered, they can be considered as a representative panel of Sicilian genetic background of the species. *Ex situ* plant collection was transferred to the field cultivation in homogeneous conditions for characterizing through a multidisciplinary approach. The study included morphological traits observations (growth habitus, flower color, number and size of leaves, length and number of internodes), VOC profiles using HS-SPME, genome size by flow cytometry analysis, and genetic characterization by means of DNA and nuclear microsatellite (nSSR) investigation. To detect any pattern within- and among-populations variability, all morphological and chemical data were submitted to ANOVA, while clustering and structure population analysis were carried out using genetic profiles. The present work allowed us to distinguish rather well between wild and cultivated genotypes and to underline the biodiversity richness among rosemary Sicilian germplasm, never highlighted, useful for future breeding programs addressed to exploit this important resource.

Key words

Medicinal and aromatic plants; volatile organic compounds; wild populations; genetic diversity; simple sequence repeat.

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Introduction

Rosemary (*Rosmarinus officinalis)* is a xeromorphic, evergreen shrub belonging to *Lamiaceae*, including wild and cultivated forms distributed throughout the Mediterranean area, classified in three subspecies: *R. officinalis* subsp. *officinalis*, *R. officinalis* subsp. *palaui*, native to Maiorca and Minorca, and *R. officinalis* subsp. *valentinus*, recently described in the coastal area around Valencia, in South-Eastern Spain (Ferrer-Gallego et al. 2014). Rosemary is commonly used for culinary and ornamental purposes since ancient times (Mateu-Andrés et al. 2013), and being rich in bioactive compounds, it has many important medicinal and functional properties, ranging from antibacterial to antidiabetic, anti-inflammatory, antitumor and antioxidant (Sánchez-Camargo and Herrero 2017; Andrade et al. 2018). Moreover, rosemary is also a source of natural compounds with allelopathic potential (Alipour and Saharkhiz 2016; Atak et al. 2016) as many other Mediterranean species (Mamoci et al. 2011; Araniti et al. 2013, 2014, Mercati et al. 2019). Three *Rosmarinus* species grow wild in the Mediterranean area: *i) R. officinalis*, widespread throughout the Basin; *ii) R. eriocalix*, present in the South-Eastern of Spain, Morocco, Algeria and Libya; and *iii) R. tomentosus*, native to the coastal area between Granada and Malaga, in Southern Spain. Several hybrids were also found, including *Rosmarinus x lavandulaceus* (*R. eriocalix* x *R. officinalis*) and *R. x mendizabalii* (*R. officinalis* x *R. tomentosus*) (Rosúa 1981, Morales 2010, Euro+Med 2018). More recently, a new classification included the three species within the genus *Salvia*, with the denominations *Salvia rosmarinus*, *Salvia jordanii*, and *Salvia granatensis*, respectively (Drew et al. 2017).

In Italy, *R. officinalis* is the only native plant of the genus (Pignatti 1982), occurring with a variety of growth habits, morphological traits, flower colors, and aromatic features (Nunziata et al. 2019). In Sicily, wild populations of *R. officinalis* may be found in a specific phytocoenosis *(Rosmarinetea officinalis)* located in rocky ridges and eroded slopes of carbonate nature mostly along the North-Eastern sea coast, from which they sometimes extend into the inland (Gianguzzi et al. 2015). The interested area is one of the 52 glacial refugia identified within the Mediterranean basin, and, together with Sardinia, Corsica and Balearic Islands, represents one of the 10 regional hotspots of plant biodiversity (Tyrrhenian islands; Médail and Quézel 1999; Médail and Diadema 2009).

The need to favor the safeguard and the crop exploitation of wild Sicilian rosemary is a critical point, due to two major aspects. The first is related to the concrete risk that wild Sicilian populations may be further reduced due to the increased harvesting for domestic self-supply, addressed to food or self-medical purposes. Under ecological balance conditions, the collection from wild or semi-wild populations is usually able to cope with the demand from market, provided it is limited and steady. However, the increase in demand, due to the enhancement of researches that enlarge the exploitation opportunities for the species, often leads to the impossibility to cope with it by means of a simple

increase of collection from wild populations. The increasing interest of industry towards wild plants has in some cases contributed to a decline in natural populations, and many species all around the world are presently at risk of extinction. Such depletion model, described in the early 90's (Homma 1992, 1996), has been extensively validated for many spontaneous populations belonging to different species. In such conditions, especially for slowly growing species and in the absence of specialized cultivations, wild populations may severely decline (Lamrani Alaoui and Hassikou 2018). This issue has a great importance for many species native to the rainy forests of Amazonia, but it is also relevant for many Mediterranean plants, since depletion in natural stands was claimed already for some wild population of Spanish Arnica, Gentian, and others (Schippmann et al. 2002). Indeed, an extensive decrease of rosemary wild populations due to the excessive pressure of gathering practices has been already described in Sardinia (Mulas and Mulas 2005), and could become a concrete possibility also in Sicily. A medium-large scale cultivation of the plants that bear a major interest for industrial purposes, such as rosemary, could be an important step in order to safeguard their natural populations.
The second reason for addressing efforts in the exploitation of Sicilian rosemary germplasm is due to a lack of homogeneity in the marketed material. Even when plant material is supplied by means of nurseries and multiplication centers, limited attention is paid to its genetic characterization with the aim to avoid a large heterogeneity. The lack of genetic knowledge about rosemary germplasm hampers breeding programs for an efficient exploitation of this species.
The available literature offers a great deal of references about rosemary's morphological variability. Notwithstanding, in contrast to other medicinal and aromatic plants, an official descriptors list for rosemary is not available as far, making difficult to compare literature data collected from different environments. To date, two different descriptor lists were proposed by the Italian Council for Research in Agriculture (CREA 2013) and the International Union for the Protection of new Varieties of Plants (UPOV 2000). Although they are substantially different in the approach to data measurements and in the importance assigned to each character, both proposals discriminate varieties mainly for ornamental purposes, insofar as the UPOV list sets as reference varieties the two ornamental Barbecue and Blue Lagoon (Hatch 2013).
In addition to morphological and agronomic traits, several efforts were addressed to explore rosemary chemical variability. Based on their essential oil profile, three main chemotypes of rosemary were identified: *cineoliferum* (with a high occurrence of 1,8-cineole), *verbenoniferum* (with verbenone >18%) and *camphoripherum* (> 20% camphor) (Pintore et al. 2002; Napoli et al. 2010). Many other chemotypes were further defined, but a large part of this variability appeared to be related to harvest season, geographic origin, and climatic pattern (Salido et al. 2003; Zaouali et al. 2005; Varela et al. 2009; Napoli et al. 2010; Jordán et al. 2011). By combining chemical and agro-morphological data

from a wild rosemary collection from southern Italy, three biotypes were also classified (De Mastro et al. 2004): *i*) long shoots, high number of axillary shoots, small-sized leaves and a high yield of camphor-rich (>40%) essential oils; *ii)* medium-sized shoots and leaves, low number of small-sized axillary shoots, low essential oil yield with the predominance of α-pinene/verbenone; and *iii)* low number of large-sized leaves, a fair number of axillary shoots and quite small shoots, intermediate essential oil yield, with a predominance of α-pinene (>20 %), verbenone, and 1,8-cineole. However, due to the polygenic fashion and the environment effects on many agro-morphological and chemical traits, they cannot be easily used to distinguish closely related samples (Zaouali et al. 2012). Therefore, a more robust and stable characterization of rosemary germplasm might include more reliable plant descriptors and markers, such as floral morphology, genome size and molecular profiles.

Nuclear DNA content showed a key role in systematics and a useful tool in biodiversity estimation (Kellogg 1998; Leitch et al. 2005). Flow cytometry is an effective and fast approach to assess the amount of nuclear DNA and relative genome size in all biological species (Dolezel and Bartos 2005; Dolezel et al. 2007). Genome variation could be an indicator of genetic divergence and speciation process (Murray 2005; Garnatje et al. 2007), highlighting possible molecular mechanisms involved in these processes (Petrov et al. 2000; Bennetzen et al. 2005; Harkess et al. 2016).

Among molecular markers, microsatellites (SSRs - Simple Sequence Repeats) are co-dominant and highly informative markers, abundant and uniformly distributed throughout plant genomes, and broadly used to genotype a wide range of plant species (Carimi et al. 2011; Jiao et al. 2012; Mercati et al. 2015; Fu et al. 2017). Until now, studies on *R. officinalis* genetic diversity are limited, both for wild germplasm and cultivated varieties. Currently, only few works report the characterization of limited collection by using different types of molecular markers, such as Random Amplified Polymorphic DNA (RAPD) (Angioni et al. 2004; Zaouali et al. 2012), nuclear ribosomal sequences (ITS) (Rosselló et al. 2006), allozymes (Zaouali and Boussaid 2008; Zaouali et al. 2012), nuclear (nSSR) and plastidial (cpSSR) Simple Sequence Repeat (Segarra-Moragues and Gleiser 2009; Mateu-Andrés et al. 2013). Preliminary information available about the genetic variability of rosemary in western Mediterranean basin support the hypothesis that this area could be a diversification center of *R. officinalis* (Mateu-Andrés et al. 2013). More recently, High Resolution Melting (HRM) approach was also proposed as a cost and time effective system to characterize rosemary populations (Nunziata et al. 2018; Nunziata et al. 2019). The system is an alternative method to capillary electrophoresis, providing percentage of HRM curves confidence for each locus, named GCP (Genotype confidence percentage), as a direct measure of the genetic similarities, but HRM method is not able to furnish “true” genetic profiles. Indeed, HRM approach assumes that

melting curves should be as different as fragments are diverse. As well known, the system shows many sources of error, and GCP, based on a Euclidean and non-genetic distance, is not linearly proportional to similarity of sequences (Hewson et al. 2009; Chagné 2015). As a consequence, many common statistical analyses adopted in population genetics, based on allele frequency, cannot be developed (e.g. expected and observed heterozygosity, fixation index, genetic differentiation, structure analysis etc.). Finally, unlike to more common capillary electrophoresis approach and the widespread PCR instruments, easily available in all molecular biology laboratories, the HRM system requires specific qPCR equipment and software.

To our knowledge, a comprehensive characterization of rosemary, including morphological, chemical and genetic analyses is missing. In the present work, a *R. officinalis* collection, counting wild and cultivated genotypes, representing the whole Sicilian genetic background for this species, has been characterized by means of a multidisciplinary approach. With this purpose, morphological traits and VOCs patterns were evaluated, flow cytofluorimetric analysis was performed, and the entire collection was genotyped by a panel of nuclear SSRs. These are still the most accessible, fast and low-cost system (being able to work in multiplex) currently available. This technique is able to furnish unique and repeatable profiles for each genotype and population, useful also to build a reference datasets in rosemary.

Methods

Arrangement of plants collection and sampling for morphological observations

With the aim to cover the lack of knowledge about wild and cultivated rosemary from Sicily, a collection activity started in the 2013 winter season. Vegetative parts of both wild and cultivated plants were collected, mostly growing in the Northern coastal area of Sicily (Figure 1; Table 1). Since the surveyed area covered most of the basiphilous rocky substrates where native *R. officinalis* populations may be retrieved (*Rosmarinetea officinalis* class), the collected samples may be considered representative of the genetic background of *R. officinalis* from Sicily. To sample a representative collection, according to plant density, almost three to 15 plants for each population were collected. As suggested by Zaouali et al. (2005), since *R. officinalis* propagates vegetatively, plants were considered different when growing at a distance >20 m; from each mother plant, 5 to 10 stem cuttings were picked up and soon inserted into 104-cells polystyrene trays filled with a mixed soil:peat (70:30 v:v) substrate. The trays were constantly surveyed to evaluate the survival and establishment of plants. After plant rooting, they were transplanted into a collection field in the experimental farm “Sparacia” (Department of Agricultural, Food and Forest Sciences, University of Palermo, Cammarata, Agrigento, Italy, 37°38°06” N; 13°45’47” E), with the aim to preserve the

genetic collection of rosemary. In the field site, both climatic pattern and soil conditions are typical of the Mediterranean dry environments, with 350-600 mm average annual rainfall, mainly distributed throughout the fall-winter period, dry and hot summers, and typically clayey soils. Prior to transplant, 1 t ha^{-1} organic pelletized fertilizer was spread and buried by soil work; transplant was done arranging plants at a 1 x 1 m distance.

Growth and development of established plants were periodically surveyed. In December 2017, representative samples for each population (one to 9 plants each) were harvested (Table 1). Fresh young herbaceous twigs were used for genome size, flow cytometry and morphological traits evaluation, using the most important traits: number of nodes within 10 cm, mean internode length (cm), number of leaves for whorl, average dimensions (length and width in mm) of leaves (Table 2). The same leaf samples were furthermore collected for molecular analysis, directly frozen in liquid nitrogen and then stored at -80° C until use.

Analysis of VOCs

In late spring 2017, when plants were at a vegetative stasis after blooming, samples from young herbaceous twigs (2-3 for each individual, amounting about 20 g of fresh material) were collected to perform VOCs (volatile organic compounds) analyses. They were identified through the HS-SPME (Head Space-Solid Phase MicroExtraction) coupled with GC-MS. This technique, already successfully used to analyze volatiles in many medicinal and aromatic plants (Carrillo and Tena 2006; Carrubba et al. 2009, 2011; D'Auria and Racioppi 2015; Sgorbini et al. 2015), may allow a quick and effective qualitative screening among individuals based on major VOCs emitted by plants. Since no solvent is required, this procedure may allow reducing the size of sample and its manipulation. With this purpose, leaves were separated from the collected twigs and put (approx. 0,5 g for each sample) in a 5 mL vial, immediately sealed with a silicon septum and left for at least 24 h at 25 °C for stabilization and achievement of equilibrium conditions, and thereafter inserted in the injector port of the GC. A GC-MS Thermo with autosampler was used for the chromatographic analyses. A capillary column Carbowax Supelco (60 m × 250 μm × 0.25 μm film thickness) was used as stationary phase under the following experimental chromatographic conditions: while desorption for one minute in the injector in split mode for SPME fiber, using He as carrier gas at flow rate of 1 ml/min, the injector temperature was held at 250°C. Oven temperature program: an initial 5 min isotherm at 40°C followed by a linear temperature increase of 4°C min^{-1} up to 250°C. MS scan conditions were the following: source temperature 230°C, interface temperature 280°C, E energy 70 eV, mass scan range 33-300 amu. A commercial library (NIST 05) provided with Linear Retention Indices, was used interactively with MS data for compounds identification. Standards, required to confirm some

assignments, were obtained from Honeywell Fluka™, Thermo Fisher Scientific Inc. (Germany) and used without further purification.

Genome size and flow cytometry evaluation

One hundred mg of fresh leaf tissue was used to determine the ploidy level, while 150 mg of the same tissue were collected to determine DNA content per nucleus, using 50 mg of fresh pea (*Pisum sativum* L.) leaf tissue as internal standard (2C=9.07 pg DNA). The legume was chosen from a list of recommended plants as excellent standard for DNA content evaluation (Johnston et al. 1999, Dolezel et al. 2007). To separate nuclei from rosemary cells, leaf tissues were chopped and dispersed into the nuclei extraction buffer (Partec solution CyStain® UV Precise P, 250 tests) added with one drop of Tween 20 and 1% w/v PVP, which was subsequently filtered (30-µm Cell-Trics filter). In order to reduce mechanical damage, the scalpel blades used for chopping were replaced every three samples. The nuclei were stained in 4,6-diamidino-2-phenylindole (DAPI) staining buffer (Partec Cystain UV precise P). Routinely, 3000–4000 nuclei were measured per sample and histograms of DNA content were generated using Partec software package (Partec-FlowMax®). The 2C DNA content was calculated based on the fluorescence intensity of the G1 peaks of both the internal standard and rosemary samples. The same operator on the same machine, adopting three biological replicates for each sample, performed the analyses.

DNA extraction and microsatellite analysis

Genomic DNA was extracted and purified from leaves (100 mg) by using DNeasy Plant Mini Kit (Qiagen, Milan, Italy). Stock solutions of DNA were resuspended in 70 µL Nuclease-free water (Merk Millipore Corporation). DNA quantity and quality were measured using Biophotometer® D30 (Eppendorf, Hamburg, Germany) and stored at -20°C. Molecular investigations were carried out by amplifying 7 nuclear microsatellites (nSSR) Roff101, Roff135, Roff246, Roff424, Roff438, Roff515 and Roff850, from Segarra-Moragues and Gleiser (2009). PCRs were performed in 20 µl reaction mixtures starting from 50 ng DNA as described in Mercati et al. (2013a), using different annealing temperatures (Ta), depending on primer pairs used. The fragments were analyzed on an ABI PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Data analysis

All quantitative data, including morphological traits and VOCs, were submitted to statistical analysis by means of the statistical package Minitab® v 17.1.0. A preliminary univariate ANOVA by location was carried out, and whenever the ANOVA showed a significant result, mean differences were

validated through Tukey's test. The differences between wild and cultivated populations were detected by calculating a single DF contrast within the factor "locations" (Gomez and Gomez 1984). The alleles were sized by Gene Mapper v. 4.1 software (Table S1). The main genetic parameters, including the number of alleles per locus (N), number of effective alleles (N_e), major allele frequency (M), observed (H_o) and expected heterozygosity (H_e), Inbreeding coefficient (F), Polymorphism Information Content (PIC), were evaluated for each SSR used using GenAlEx6 (Peakall and Smouse 2006) and PowerMarker (Liu and Muse 2005) software.

Principal Component Analysis (PCA) of both morphological traits and VOCs was carried out using R/FactoMiner (Le et al. 2008).

A Pearson's correlation analysis ($p<0.05$) was also carried out by Hmisc R/package (https://cran.r-project.org/web/packages/Hmisc/index.html) to confirm PCA results. A scatter plot showing correlation coefficients between traits and their significance was developed by R/Performance Analytic (https://cran.r-project.org/web/packages/PerformanceAnalytics/index.html).

To study the genetic relationships among rosemary populations, cluster analysis based on UPGMA (Unweighted Pair Group Method with Arithmetic Mean) algorithm was performed. The phylogenetic tree was developed by R/poppr (Kamvar et al. 2014) with Bruvo's distance (Bruvo et al. 2004). The bootstrap analysis was performed based on 1000 re-samplings.

A model-based (Bayesian) clustering was performed to estimate genetic relationship among samples and the population structure by STRUCTURE software (Pritchard and Wen 2003). The program was set as previous reported in Mercati et al. (2013b) and twenty independent runs for *K* ranging from 1 to 10 were carried out. An *ad hoc* statistic, proposed by Evanno et al. (2005), was used to determine the most probable *K* value, to compensate for overestimation of subgroup number by STRUCTURE. Samples with membership probabilities ≥ 0.8 were assigned to the corresponding subgroups and lines with membership < 0.8 were assigned to a mixed subgroup.

Finally, a Discriminant Analysis of Principal Components (DAPC), implemented in the R/adegenet (Jombart and Ahmed 2011), was also carried out to validate and confirm cluster and STRUCTURE results. The number of PCs (principal components) retained was evaluated using the cross-validation approach. To verify the assignment of individuals to clusters, the K-means algorithm, 'find.clusters', was used.

Results

Morphological traits and volatile organic compounds analysis

Three years after transplanting, many plants showed an erect growth habitus (Table 2). All exhibited a pale violet corolla ground color (except MAR population, whose corolla was mainly light blue).

Analysis of variance (ANOVA) highlighted significant differences among populations for only two morphological traits (length of leaves – LL, and number of leaves per whorl – NL), while no significant difference was observed between wild and cultivated plants. The cultivated population named CAS showed the longest leaves, with a mean leaf length of 18.5 mm, whereas the cultivated population PA exhibited the shortest (11.8 mm) leaves arranged in dense whorls (Table 2). The means for each VOC detected by HS-SPME and the related univariate ANOVA are reported in Table 3. Seven volatiles out of twelve showed significant differences among populations; α–pinene showed the largest differences, averaging 20.4% and 40.2% in wild and cultivated populations, respectively. Many compounds that were showing significant differences among populations, also highlighted significant differences between groups ("W *vs.* C"). By contrast, 1,8-cineole did not show significant differences among populations at univariate ANOVA, but a significant differentiation between wild and cultivated plants was detected by single DF contrast (Table 3). In detail, rather all wild populations exhibited a 1,8-cineole content higher than 40% (on average 46.2 %) with an outstanding higher value in plants from L7 population, whereas cultivated plants showed a 30.5% average content of the same compound (Table 3).

PCA on morphological traits did not allow us to define distinct clusters for wild and cultivated populations, although about 70% variability was explained (Figure S1). As a whole, the first axis seemed to be more related with leaves width, whereas the second PC with their length. As expected, mean length of internodes and number of nodes per 10 cm, being inversely correlated, were located on opposite quadrants of the PCA score plot; number of leaves per whorl followed the same trend of number of nodes (Figure S1).

By contrast, although the multivariate analysis on VOCs explained a lower value of total variability (49%), PCA results allowed us to distinguish wild from cultivated rosemary populations (Figure 2A). Indeed, six out of seven samples, belonging to the cultivated populations, were clearly separated by PCA first component (Dim1). In addition, 62% of samples collected in Torrenova (ME) (all TOR samples, and one plant each belonging to L1 and L2 populations), were separated by the second component (Dim2) from the others. Limonene, α-pinene, and α-terpinene were most weighing for Dim1 able to separate wild and cultivated populations. Sabinene, camphene, 1,8-cineole and ocimene mainly contributed to the variability explained by Dim2 (Figure 2A). These evidences were confirmed by Pearson's correlation analysis (Figure 2B), showing positive and negative significant correlations ($p<0.05$). Among these, 1,8-cineole *vs* α-pinene and limonene showed the higher (negative) correlation coefficients (Figure 3B).

Flow cytometry and genome size evaluation

To evaluate the genome size and ploidy level/genetic stability among accessions, belonging to Sicilian *R. officinalis* germplasm, flow cytometry approach was used. No significant differences in the ploidy level estimation were detected in our collection. In all plants studied, the genome size recorded was 2C values ± 2.50 pg (1227 Mbp/C) (Figure S2).

Genetic diversity of rosemary Sicilian germplasm

Variation at seven nuclear SSR loci was evaluated on rosemary collection from Sicily. All the loci were polymorphic scoring a high mean PIC value (0.701) with an allele number ranging from 5 to 14 alleles per locus (Table 4) and a mean of major allele frequency of 0.427. Overall, genetic diversity, measured as expected heterozygosity, appeared high (H_e = 0.731) with an observed heterozygosity (H_o) ranging from 0.511 to 0.956 (Table 4). The inbreeding coefficient (F = -0.070) was negative, but could be considered in equilibrium.

A phylogenetic tree was defined based on genetic distances, cluster analysis and UPGMA algorithm (Figure 3). Five main clusters were defined (I, II, III, IV and V), and the accessions were clustered based on their geographic origins (Figure 3). Interestingly, all cultivated samples were grouped in cluster I, assembled in two private sub-clusters. The remaining four plants, belonging to cluster I, were from AL population. In cluster II, three private sub-clusters were found including all samples from Levanzo (LEV), Cefalù (L7) and two accessions from Castel di Tusa (ME) (L6). Clusters III and IV grouped plants from L3 and L5 populations, respectively. Finally, the largest numbers of samples (42%) were grouped in cluster V, divided into two smaller sub-clusters: the first one included all samples (9) from L5 population, while the second included the samples belonging to L5 population and all the samples from Torrenova (ME) (L1, L2 and TOR populations) and S. Stefano di Camastra (ME).

To infer population structure by determining the number of groups in the germplasm collection, STRUCTURE analysis was performed. Following the Evanno et al. (2005) statistic, *K*=7 was identified as the optimum number of genetic groups (*K*). Using the admixture coefficient (Q) ≥ 0.8 as cut-off of probability to assign each sample to a group identified, 33 out of 45 samples (73%) were assigned to a specific group (Table S2). In detail, all plants collected in Levanzo (LEV population) were assigned to group 1 (pink); L5 and L6 populations belonged to group 4 (orange) and group 5 (light red), respectively; four out of 5 plants from L4 population were assigned to group 6 (dark red); and finally, seven out of 8 plants collected in Torrenova (ME) and STEF population from S. Stefano di Camastra (ME) belonged to group 7 (light blue) (Table S2; Figure 4). The other samples showed an admixture genetic structure. Although samples from cultivated plants have an admixture profile

(blue and green groups), they showed a typical shape, that is very similar to samples belonging to AL population, in agreement to cluster analysis.

In the DAPC analysis, cross-validation indicated that 7 PCs and 5 DAs were useful to describe the genetic diversity of rosemary collection. These results agreed with both phylogenetic and STRUCTURE analysis. The samples were clustered based on their origin. In particular samples showing the admixture profiles K2/K3 (all cultivated genotypes and AL wild population; Figure 4; Table S1), belonging to cluster I (Figure 3), were separated from the other groups (Figure 5). Similarly, LEV, L6, and L7 populations, belonging to K1, K5 and K1/K5 (Figure 4; Table S1), respectively, and grouped in the cluster II (Figure 3), were more genetically different than the other wild populations (Figure 5). Finally, although the samples belonging to L1, L2, L4, L5, STEF and TOR showed different genetic pools (Figure 4; Table S1), they were very closely related (Figure 5). DAPC analysis allowed us to split the Sicilian germplasm in three main groups, separated in the different quadrants (Figure 5): group I, represented by cultivated genotypes and AL wild population; group II, contained LEV, L6, and L7 population; and group III with samples belonging to L1, L2, L3, L4, L5, STEF and TOR populations. Interestingly, based on Fst and Nei genetic distance (Nei 1978), the differences between group I and group II were similar to the values obtained comparing group II and III, both represented by wild populations. In addition, group I was closer to group III (Nei = 0.383) than II to III (Nei = 0.628) (Table 5).

Discussion

A significant number of papers were addressed to explore many aspects of morphological, phytochemical and genetic variability of *R. officinalis*. To our knowledge, few efforts were devoted as far to characterize this species through a multidisciplinary approach.

In Sicily, rosemary is used since ancient times, for both medicinal and food purposes (Lentini and Venza 2007). The main sources for local supply are the collection from wild populations and cultivated individuals. However, most of the traditional rosemary cultivations are represented by single individuals, mostly grown in gardens and orchards in the close surroundings of human settlements, whereas specialized and intensive cultivations are only limited to a few hectares (Migliore and Saggio Scaffidi 2007).

Our results allowed arguing that most of cultivated plants/populations derived from native wild mother plants. Since most of the wild biotypes are widespread in hardly accessible mountainous and steeply sloping areas, it is possible that a number of valuable individuals were brought to cultivation with the purpose to have more easy-to-use available plant material (Burkhart and Jacobson 2009). It seems likely that the choice was concerned mainly with leaves size (the major source of aromatic

stuff), and this hypothesis may probably explain the larger size of the leaves in the cultivated individuals, and the extensive homogeneity for this trait of the cultivated populations. Otherwise, since limited interest was paid to other aspects, the other morphological traits, such as the colour of corolla, showed homogeneity across all samples. At the same time, it would be not surprising that some individuals, classified among the "wild" biotypes, would otherwise belong to formerly cultivated ("escaped to cultivation" and naturalized) plants.

Although some distinction could be made at population level based on plant leaves size, morphological traits were not able to achieve a satisfactory discrimination among groups. This lack of discrimination among populations suggests that, once brought to cultivation in homogeneous conditions (hence, once minimized the variability due to the environment), the remaining fluctuations among the major morphological traits are not high enough to discriminate genotypes. Most variations in such traits seem to be due to the environment (as expected), rather than under genetic control. Thus, the perplexity expressed by Zaouali et al. (2012) as concerns the utility of morphological traits for assessing differences among populations sounds reasonable.

The VOC content seems more able to discriminate among populations. Of course, the available data did not allow us to distinguish among chemotypes, whose proper determination in rosemary requires a different experimental procedure (Napoli et al. 2010). Notwithstanding, VOCs obtained by HS-SPME showed a sharp separation among groups of populations, mainly noticeable in the relative content in α-pinene (on average, 40.7% in cultivated biotypes and 20.4 in wild ones) and 1,8-cineole (46.2 in wild biotypes and 30.5 in cultivated ones). Therefore, they can be classified as *cineoliferum* (or A) chemotype, as reported in previous studies (Li et al. 2016, Nunziata et al. 2019).

Flow cytometry revealed stable genome size in our collection, both in wild and cultivated populations. The genome size recorded (±2.50 pg) was in agreement to the values available in the literature for the species (Pellicer et al. 2010). However, the procedure adopted in this study could be used as a reference for all species experiencing separation difficulties, including many medicinal plants (Greilhuber et al. 2007). Indeed, this procedure allowed to isolate the nuclei coping with the complexity of the substances contained in rosemary cells.

Microsatellite analysis underlined a suitable and significant biodiversity among Sicilian germplasm. Comparing the genetic variability of our collection to that reported by Segarra-Moragues and Gleiser (2009), the unique available report utilizing nSSR in rosemary, number of alleles per locus, observed and expected heterozygosity agreed. A more recent study based on cpSSR markers identified ten haplotypes among a widespread germplasm collection belonging to whole Mediterranean basin (Mateu-Andrés et al. 2013), but biased towards populations from Spain (23 out of 47). Samples collected from different Italian regions, including plants from Agrigento and Messina (Sicily),

belonged to the two most common haplotypes (H2 and H4) and clustered in two main branches, together with Algerian, French, Moroccan and Spanish genotypes (Mateu-Andrés et al. 2013), highlighting a close genetic background. These results were confirmed by Nunziata et al. (2019) using HRM technique. However, due to the limits of this last approach, the genetic background of Sicilian populations included in that study could be partially misclassified. Indeed, genotypes from Torrenova (TOR) and S. Stefano di Camastra (STEF), two very close locations, showed high genetic diversity able to classify these genotypes in different clusters, while STEF population appeared very close to samples belonging to AL population from Vittoria (RG), a location on the other side of Sicily (Nunziata et al. 2019). Our molecular analysis, through "standard" genotyping by SSRs, supported for the first time the evidences of well distinguished genetic profiles belonging respectively to wild and cultivated populations. In addition, clustering and the identification of genetic pools (K=7) are correlated to geographic origins of populations. Therefore, they seem somehow dependent upon the anthropization (disturbance level) of the original collection site. Hence, the AL population, although belonging to the wild collection, lies close to the cultivated groups, probably due to the high level of disturbance of the original AL grown area. DAPC analysis confirmed previous results, highlighting a clear genetic diversity that allowed us to distinguish three main groups in the collection. In particular, group I represented by cultivated genotypes and AL wild population, with K2/K3 admixture profile, showed a major similarity to group II (K1, K5, and the admixture K1/K5) than what emerged from the comparison between the two wild population groups (II and III). To note, within group III (K4, K6, K7, admixture K4/K6 and K5/K7) L3 individuals, collected from a high and hardly accessible calcareous rock, were distinguished from all the other populations. In summary, the genetic analysis underlined an interestingly richness of biodiversity among Sicilian germplasm, so far never highlighted, that can be useful to plan future breeding programs in order to exploit this important resource.

Conclusions

The multidisciplinary approach applied in this work has been able to full characterize the Sicilian germplasm collection, covering the lack of knowledge about its genome size and stable SSR genetic profiles. Morphological, chemical and genetic observations, offered distinct points of view of rosemary's diversity; however, taking into account all data together allowed us to depict the relationships among populations that would have not been possible otherwise. The Sicilian rosemary has been confirmed as an important component of plant biodiversity in the Tyrrhenian region, whose conservation has been possible due to the limited and - by far - sustainable use by local populations. The new inputs from R&D sector have however opened an impressive series of new opportunities for

rosemary utilization, and it is easy to foresee that, as soon as requirements become higher, this equilibrium condition will soon show its weakness. Until now, the local germplasm did not seem to be mixed with genetic material from outside. However, further studies through nSSR genotyping of a wider rosemary germplasm collection will support the preservation that will probably become necessary in a near future.

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660

661 **Table 1** List of rosemary (*Rosmarinus officinalis*) populations collected.

662

ID population	N.[#]	W/C*	Origin	Coordinates	Collection date	Transplant in field date
L1	1	W	Torrenova (ME)	38°05'14"N; 14°40'42"E	30/12/2013	03/06/2014
L2	4	W	Torrenova (ME)	38°05'09"N; 14°39'39"E	30/12/2013	03/06/2014
L3	3	W	Motta d'Affermo (ME)	38°01'15"N; 14°28'59"E	30/12/2013	03/06/2014
L4	5	W	Castel di Tusa (ME)	38°00'21"N; 14°16'18"E	30/12/2013	03/06/2014
L5	9	W	Castel di Tusa (ME)	38°00'34"N; 14°16'14"E	30/12/2013	03/06/2014
L6	2	W	Castel di Tusa (ME)	38°00'28"N; 14°15'52"E	30/12/2013	03/06/2014
L7	2	W	Cefalù (PA)	38°01'34"N; 14°03'06"E	30/12/2013	03/06/2014
AL	4	W	Vittoria (RG)	36°35'28"N; 14°31'54"E	05/03/2014	30/10/2014
CAS	3	C	Castelvetrano (TP)	37°34'55"N; 12°47'10"E	05/08/2014	30/10/2014
FIP	1	C	Ficuzza (PA)	37°51°13"N; 13°25'37"E	21/11/2014	05/12/2014
LEV	3	W	Levanzo (TP)	37°59'18"N; 12°20'34"E	24/02/2014	30/10/2014
MAR	2	C	Marineo (PA)	37°57'18"N; 13°25'41"E	20/12/2014	22/12/2014
PA	1	C	Palermo (PA)	38°05'46"N; 13°20'53"E	24/02/2014	14/09/2014
STEF	2	W	S. Stefano di Camastra (ME)	38°00'54"N; 14°22'10"E	24/02/2014	30/10/2014
TOR	3	W	Torrenova (ME)	38°05'31"N; 14°41'47"E	24/02/2014	30/10/2014
15	45	11W 4C	-	-	-	-

663

664 [#] number of plants analysed in the present study

665 * w: wild; c: cultivated

Table 2 Morphological traits recorded in the rosemary germplasm collection. GH: growth habit; FC: ground color of the corolla; LL: leaf length (mm); LW: leaf width (mm); L/W: leaf length/width ratio; NL: number of leaves per whorl (n.); IL: length of internode (cm); NN: number of nodes/10 cm twig. For the quantitative traits, the *F* values obtained both from univariate ANOVA and from the single DF contrast "wild vs. cultivated" are indicated; when reported, means in each column followed by the same letter are significantly not different at $P \leq 0.05$ (Tukey's test)

Loc	**GH**	**FC**	**LL**	**LW**	**L/W**	**NL**	**IL**	**NN**
L1	erect	pale violet	13.7 ab	1.55	8.67	2.0 b	1.67	6.0
L2	erect	pale violet	15.8 ab	1.40	11.43	6.0 ab	1.45	7.1
L3	semi-erect	pale violet	17.8 ab	1.72	10.71	4.3 ab	1.56	6.5
L4	semi-erect	pale violet	16.5 ab	1.71	9.93	6.2 ab	1.18	8.9
L5	erect	pale violet	17.5 ab	1.62	11.23	5.3 ab	1.67	6.7
L6	erect	pale violet	13.1 ab	1.43	9.35	4.4 ab	1.12	9.0
L7	erect	pale violet	12.1 b	1.40	8.62	3.3 ab	1.83	5.5
AL	semi-erect	pale violet	13.5 ab	1.43	9.89	6.5 ab	1.03	10.1
LEV	semi-erect	pale violet	18.2 ab	1.62	11.61	5.6 ab	1.41	7.2
STEF	erect	pale violet	15.4 ab	1.60	10.22	5.9 ab	1.33	7.6
TOR	semi-erect	pale violet	14.9 ab	1.33	11.63	7.1 ab	1.31	7.7
Mean Wild (n=38)			**15,9**	**1,54**	**10.60**	**5.5**	**1.42**	**7.6**
CAS	erect	pale violet	18.5 a	1.70	11.86	5.8 ab	1.38	7.5
FIP	erect	pale violet	16.2 ab	1.55	10.65	3.0 ab	1.38	7.3
MAR	erect	light blue	17.7 ab	1.73	10.19	3.9 ab	2.04	5.3
PA	erect	pale violet	11.8 b	1.75	7.10	8.6 a	2.20	4.5
Mean Cultivated (n=7)			**17,0**	**1,69**	**10.53**	**5.3**	**1.68**	**6.4**
$F_{(14,30)}$			3.48**	<1 n.s.	1.82 n.s.	2.92**	1.50 n.s.	1.69 n.s.
W vs C $F_{(1,30)}$			1.97 n.s.	3.53 n.s.	<1 n.s.	<1 n.s.	<1 n.s.	2.40 n.s.

*=0.01<p<0.05; **=0.001<p<0.01; ***=p<0.001

675 **Table 3** Relative content (%), retention time (RT; min) and experimental retention indices (RI) of
676 VOCs detected by HS-SPME in the rosemary germplasm collection. 1: α-pinene; 2: Camphene; 3:
677 Sabinene; 4: α-Phellandrene; 5: Limonene; 6: 1,8-Cineole; 7: δ-Terpinene; 8: γ-Terpinene; 9:
678 Linalool; 10: Camphor; 11: Borneol; 12: Isobornyl-acetate. For each compound, the *F* values obtained
679 both from univariate ANOVA and from the single DF contrast "wild *vs*. cultivated" ("W *vs*. C") are
680 indicated; when reported, means in each column followed by the same letter are significantly not
681 different at $P \leq 0.05$ (Tukey's test).
682

Compound	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
RT(min)	11.41	12.01	13.2	15.27	15.42	15.51	16.67	17.86	18.36	19.97	20.81	25.18
RI	939	953	976	1005	1031	1040	1059	1062	1085	1140	1165	1280
Loc												
L1	28.4 ac	16.2	14.6 ab	1.9 b	3.1 b	29.1	0.09	0.10 b	0.04 b	5.97	0.26 ab	0.16
L2	21.3 bc	11.0	10.1 ab	2.1 b	3.2 b	44.8	0.33	0.31 b	0.14 b	6.12	0.37 ab	0.30
L3	21.0 bc	9.1	10.6 ab	3.1 b	3.0 b	47.5	0.39	0.19 b	0.14 b	4.52	0.35 ab	0.25
L4	23.7 bc	9.5	11.8 ab	2.3 b	3.1 b	43.6	0.36	0.21 b	0.13 b	4.60	0.22 ab	0.53
L5	21.0 bc	8.8	11.4 ab	2.0 b	2.7 b	49.4	0.34	0.17 b	0.10 b	3.62	0.14 b	0.33
L6	18.4 bc	10.9	8.9 ab	2.6 b	2.3 b	53.0	0.29	0.23 b	0.20 b	2.47	0.69 ab	0.12
L7	14.4 bc	4.9	7.2 ab	2.8 b	2.3 b	59.7	0.29	0.16 b	0.16 b	7.55	0.27 ab	0.20
AL	14.6 c	8.8	11.0 ab	2.1 b	2.8 b	52.9	0.38	0.18 b	0.12 b	6.06	0.65 ab	0.43
LEV	22.3 bc	12.5	12.5 ab	7.0 a	3.2 b	37.7	0.15	0.24 b	0.11 b	3.85	0.33 ab	0.11
STEF	22.9 bc	8.1	15.3 a	2.0 b	3.6 b	41.9	0.48	0.33 b	0.07 b	4.86	0.12 ab	0.27
TOR	18.6 bc	16.7	13.8 ab	1.9 b	4.3 ab	36.8	0.35	0.14 b	0.01 b	6.07	0.42 ab	0.87
Mean Wild (n=38)												
	20.42	**10.13**	**11.43**	**2.61**	**3.00**	**46.24**	**0.33**	**0.20**	**0.12**	**4.82**	**0.32**	**0.36**
CAS	50.7 a	7.2	3.9 b	2.6 b	4.1 ab	28.7	0.29	0.25 b	0.34 b	1.37	0.32 ab	0.24
FIP	46.0 ab	12.5	5.0 ab	2.9 b	7.3 a	14.4	0.51	1.08 a	1.04 a	8.03	1.08 a	0.26
MAR	32.6 ac	12.5	8.2 ab	2.9 b	4.9 ab	30.4	0.28	0.16 b	0.37 b	6.64	0.66 ab	0.32
PA	17.9 bc	8.6	15.0 ab	2.1 b	2.6 b	52.4	0.25	0.15 b	0.01 b	0.56	0.12 ab	0.29
Mean Cultivated (n=7)												
	40.17	**9.66**	**6.91**	**2.68**	**4.58**	**30.50**	**0.31**	**0.33**	**0.40**	**3.71**	**0.49**	**0.27**
$F_{(14,30)}$	4,83***	1.06 $^{n.s}$	2.74*	6.56***	4.19***	1.57 $^{n.s}$	<1 $^{n.s.}$	6.14***	4,29 ***	<1 $^{n.s}$.	2.46 *	<1 $^{n.s}$.
W vs C $F_{(1,30)}$	42.71***	<1 $^{n.s.}$	13.68***	<1 $^{n.s.}$	24.03***	8.46**	<1 $^{n.s.}$	8.71**	26.18***	<1 $^{n.s.}$	3.13 $^{n.s.}$	<1 $^{n.s.}$

*=0.01<p<0.05; **=0.001<p<0.01; ***=p<0.001
683

684 **Table 4** Main genetic parameters from the 7 polymorphic SSR loci used.

685

Locus	N	Ne	M	H_o	H_e	F	PIC
Roff_101	12	4.438	0.278	0.800	0.843	0.003	0.826
Roff_135	14	5.159	0.200	0.956	0.896	-0.184	0.887
Roff_246	7	3.029	0.533	0.689	0.660	-0.026	0.627
Roff_424	7	2.548	0.544	0.556	0.646	0.050	0.611
Roff_438	6	2.395	0.467	0.600	0.686	-0.017	0.640
Roff_515	5	2.159	0.533	0.511	0.657	-0.016	0.621
Roff_850	7	3.246	0.433	0.867	0.729	-0.297	0.695
Mean	*8*	*3.282*	*0.427*	*0.711*	*0.731*	*-0.070*	*0.701*

686

687 Number of alleles per locus (N), number of effective alleles (Ne), major allele frequency (M), observed (Ho) and expected
688 heterozygosity (He), Inbreeding coefficient (F), Polymorphic Information Content (PIC)

689

690

691 **Table 5** Fst (below diagonal) and Nei (1978) genetic distance (above diagonal) evaluated among
692 groups identified by DAPC analysis.
693

	Group I	**Group II**	**Group III**
Group I	-	1.242	0.383
Group II	0.176	-	0.628
Group III	0.069	0.131	-

694
695

Figure 1 Collection sites of the wild (yellow pins) and cultivated (red pins) samples of *R. officinalis* studied in this work.

Figure 2 A) Principal Component Analysis (PCA) referred to main VOCs detected on wild (blue triangles) and cultivated (red circles) populations of *R. officinalis.* VOCs associated to samples separation were indicated (green arrows) in the plot, underlining their significance values (0.2<cos2<0.8). **B)** Pearson's correlation matrix of selected VOCs. Positive and negative correlations are displayed in blue and red color, respectively. Size and color intensity are proportional to the correlation coefficients. The significant correlations ($p<0.05$) were highlighted.

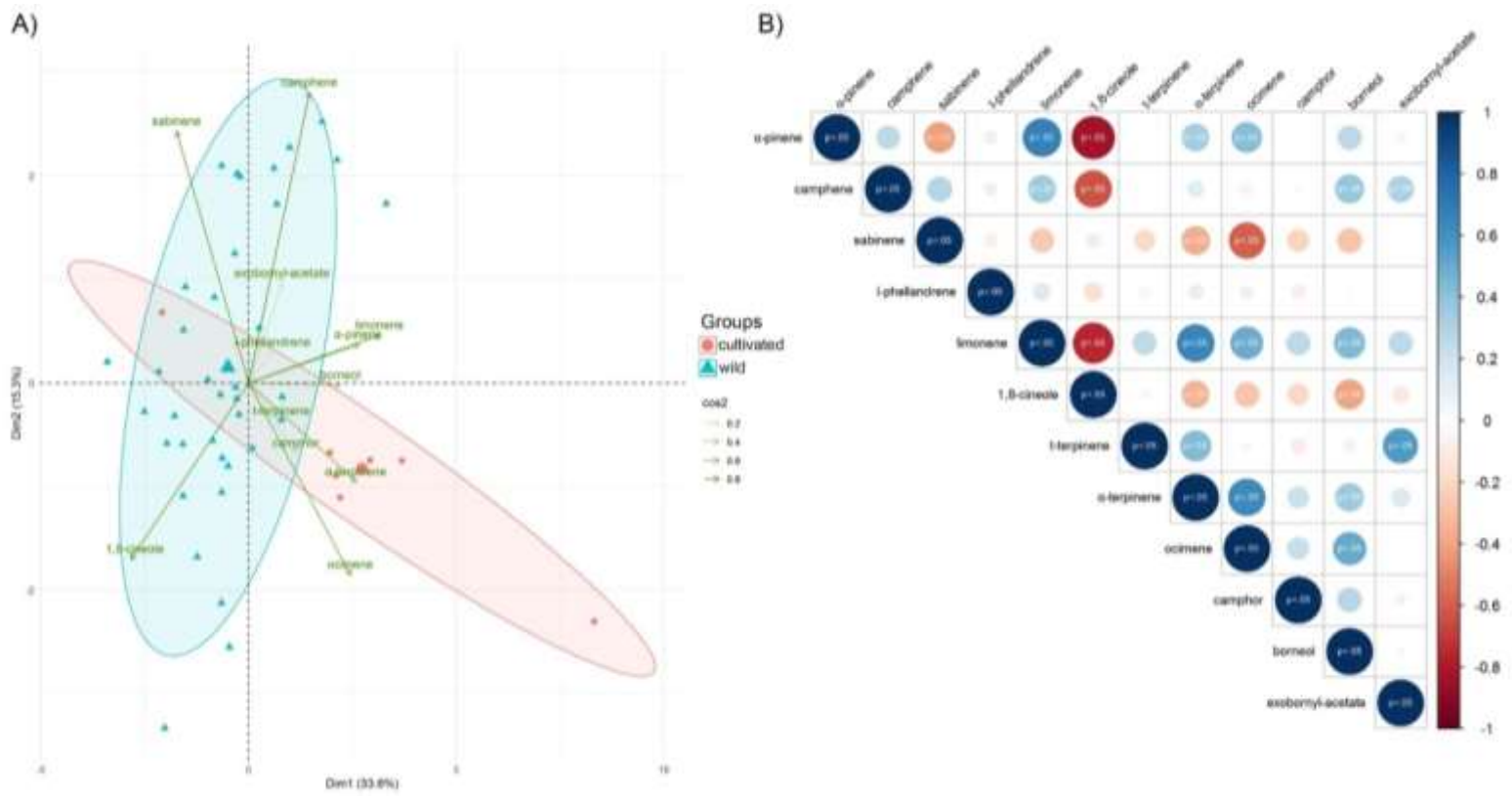


Figure 3 Genetic relationships among wild and cultivated plants belonging to Sicilian *R. officinalis* germplasm. In the figure five main clusters were highlighted.

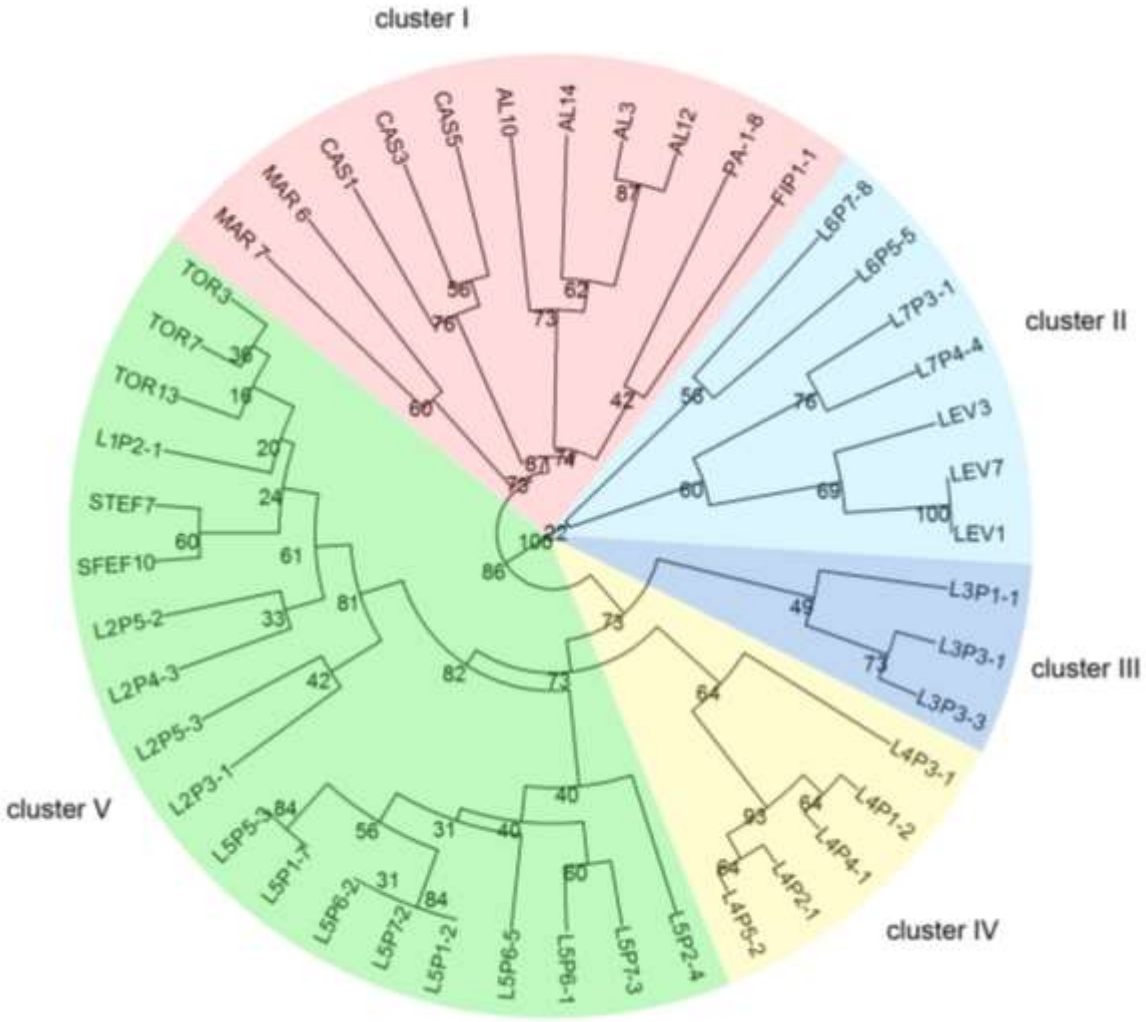


Figure 4 Admixture proportions of wild and cultivated plants belonging to Sicilian *R. officinalis* germplasm. Each vertical bar represents a sample and the color proportion for each bar represents the posterior probability of assignment of each individual to one of seven groups identified. The range of assignment probability varies from 0 to 100 %.

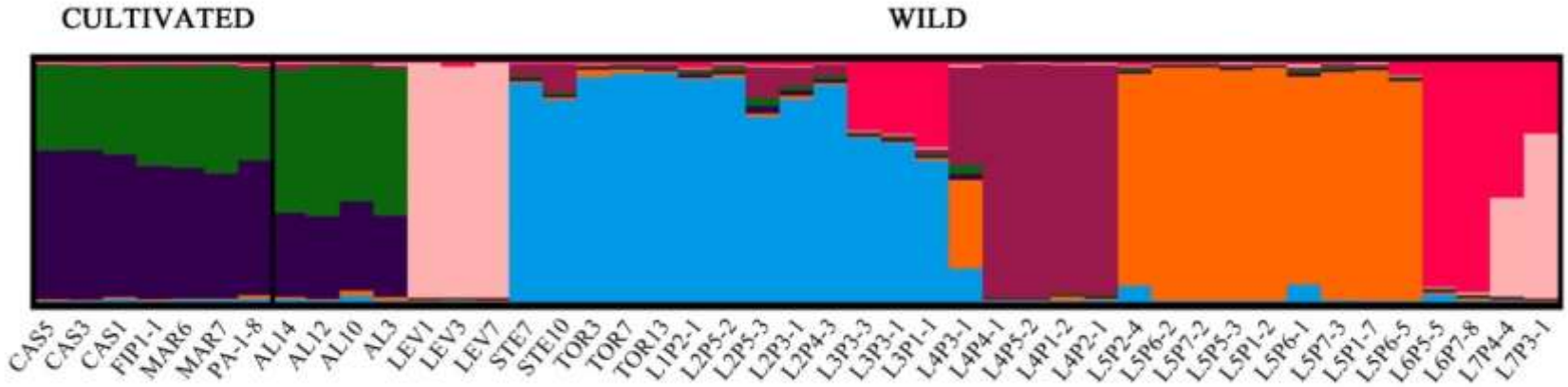
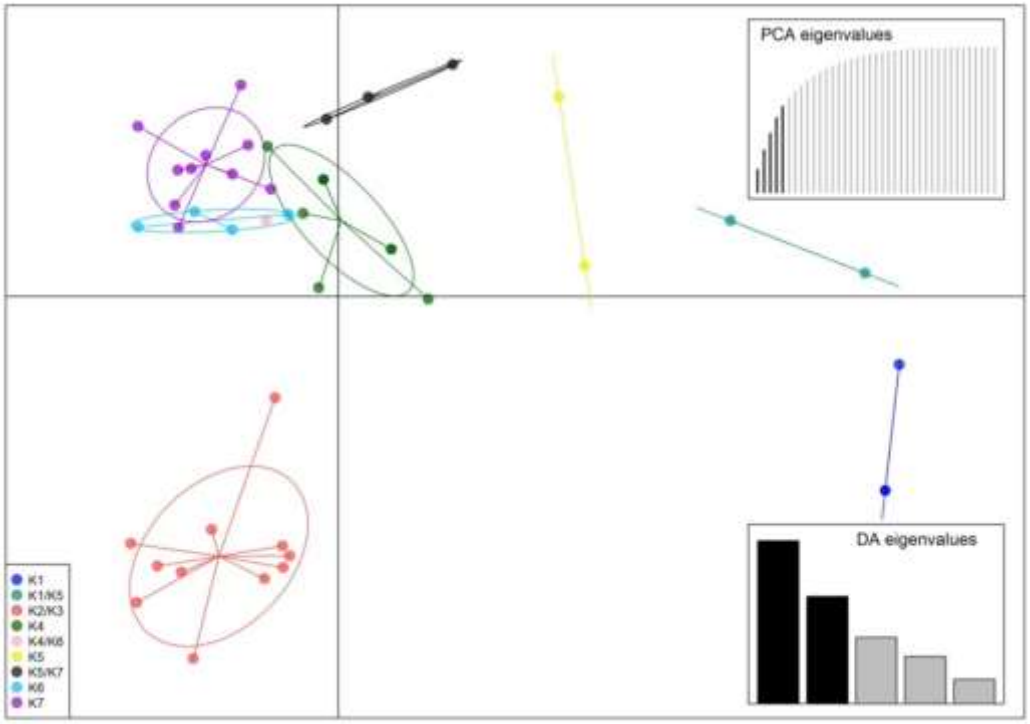


Figure 5 DAPC scatter plot for the rosemary collection studied. Different colors represent the genetic pools identified in the STRUCTURE analysis. The samples showing admixture profiles) were grouped in specific panels representing the main pools (K1/K5, K2/K3, K4/K6, and K5/K7; see Table S2).



Supplementary material

Table S1. Genetic profiles of samples belonging to the rosemary collection studied, genotyped by 7 nSSR

Sample	Roff101	Roff135	Roff246	Roff424	Roff438	Roff515	Roff850
L1P2-1	171/189	224/240	129/145	140/142	109/109	178/186	141/153
L2P3-1	144/147	224/232	129/145	138/146	101/109	166/182	141/153
L2P4-3	144/147	216/240	129/145	138/144	101/109	178/182	141/147
L2P5-2	144/147	216/232	129/145	140/142	109/109	178/182	141/147
L2P5-3	144/147	216/232	129/145	144/146	109/109	166/182	144/153
L3P1-1	168/189	224/240	129/145	146/146	101/109	182/182	141/153
L3P3-1	168/189	216/240	129/129	146/146	109/109	178/182	141/153
L3P3-3	168/189	216/240	129/145	146/146	109/109	178/182	141/153
L4P1-2	144/144	228/232	129/141	142/146	101/101	182/182	141/144
L4P2-1	144/144	228/232	129/141	142/146	101/101	182/186	141/153
L4P3-1	144/147	224/232	129/129	142/146	101/113	182/186	141/147
L4P4-1	144/144	228/232	129/141	144/146	101/101	182/186	141/144
L4P5-2	144/144	228/232	129/141	144/146	101/101	186/186	141/153
L5P1-2	144/147	224/242	129/129	142/142	109/113	182/182	141/141
L5P1-7	144/147	224/242	129/141	142/142	109/113	182/182	141/144
L5P2-4	144/144	224/240	125/125	142/142	109/109	182/182	141/141
L5P5-3	144/147	224/242	129/141	142/142	109/113	182/182	141/144
L5P6-1	141/147	224/242	129/145	142/142	101/109	182/182	141/141
L5P6-2	144/147	224/242	129/129	142/142	109/113	182/182	141/141
L5P6-5	141/147	224/242	129/129	142/142	109/113	182/182	144/159
L5P7-2	144/147	224/242	129/129	142/142	109/113	182/182	141/141
L5P7-3	141/147	224/242	125/129	142/142	101/109	182/182	141/144
L6P5-5	189/189	224/240	129/129	146/146	127/127	182/182	141/159
L6P7-8	183/183	212/224	129/129	142/146	127/127	182/182	159/159
L7P3-1	183/189	212/218	129/145	146/148	121/127	182/182	153/180
L7P4-4	183/189	202/218	133/145	146/148	113/127	182/182	153/180
AL3	168/174	202/210	129/141	142/142	101/109	166/182	141/147
AL10	147/171	200/210	129/129	142/142	101/109	166/166	144/147
AL12	168/174	202/210	129/129	142/142	101/109	166/186	141/147
AL14	144/174	202/216	121/129	142/142	101/109	166/186	144/147
LEV1	159/159	202/218	133/141	142/142	113/113	182/182	153/180
LEV3	159/189	202/218	133/145	142/148	113/113	182/182	153/180
LEV7	159/159	202/218	133/141	142/142	113/113	182/182	153/180
STEF7	141/147	228/240	129/145	140/142	101/109	178/186	141/153
STEF10	144/147	228/240	129/145	140/142	101/109	178/186	144/153
TOR3	144/147	224/240	129/145	138/142	109/109	178/182	141/153
TOR7	141/147	224/240	129/145	140/142	109/109	178/186	141/153
TOR13	144/147	224/232	129/145	140/142	109/109	178/186	141/153
CAS1	144/150	192/200	129/149	138/142	101/109	144/166	141/147
CAS3	141/150	192/200	125/149	128/146	97/109	144/166	141/150
CAS5	141/150	192/200	129/149	128/142	97/109	166/166	141/144
FIP1-1	168/174	222/236	129/141	138/142	101/109	144/144	141/150
MAR6	141/195	216/216	141/141	128/142	97/109	166/186	141/153
MAR7	168/195	216/216	121/121	142/142	97/109	144/186	141/153
PA1-8	141/186	222/236	129/129	128/142	101/109	144/182	141/153

Table S2 Posterior membership coefficients following a STRUCTURE analysis and *K* = 7.

Samples	Type	K1	K2	K3	K4	K5	K6	K7	K assigned*
CAS5	Cultivated	0.003	0.397	0.579	0.008	0.004	0.005	0.005	K2/K3
CAS3	Cultivated	0.003	0.39	0.584	0.007	0.006	0.005	0.005	K2/K3
CAS1	Cultivated	0.003	0.395	0.561	0.009	0.004	0.016	0.013	K2/K3
FIP1-1	Cultivated	0.003	0.427	0.546	0.005	0.004	0.007	0.007	K2/K3
MAR6	Cultivated	0.005	0.45	0.516	0.006	0.004	0.009	0.01	K2/K3
MAR7	Cultivated	0.004	0.466	0.508	0.004	0.004	0.005	0.01	K2/K3
PA-1-8	Cultivated	0.006	0.382	0.558	0.018	0.01	0.013	0.013	K2/K3
AL14	Wild	0.004	0.536	0.403	0.01	0.004	0.03	0.013	K2/K3
AL12	Wild	0.004	0.585	0.385	0.006	0.004	0.008	0.007	K2/K3
AL10	Wild	0.003	0.53	0.404	0.024	0.004	0.007	0.028	K2/K3
AL3	Wild	0.009	0.573	0.378	0.016	0.006	0.012	0.007	K2/K3
LEV1	Wild	0.978	0.004	0.004	0.005	0.003	0.004	0.003	K1
LEV3	Wild	0.96	0.003	0.003	0.004	0.019	0.003	0.007	K1
LEV7	Wild	0.978	0.004	0.004	0.005	0.003	0.004	0.003	K1
STEF7	Wild	0.004	0.011	0.012	0.01	0.004	0.043	0.917	K7
STEF10	Wild	0.004	0.01	0.01	0.011	0.005	0.107	0.853	K7
TOR3	Wild	0.005	0.006	0.006	0.031	0.008	0.006	0.937	K7
TOR7	Wild	0.004	0.007	0.008	0.017	0.005	0.005	0.955	K7
TOR13	Wild	0.004	0.006	0.006	0.014	0.004	0.015	0.951	K7
L1P2-1	Wild	0.006	0.014	0.013	0.008	0.027	0.005	0.925	K7
L2P5-2	Wild	0.004	0.014	0.012	0.017	0.006	0.016	0.931	K7
L2P5-3	Wild	0.004	0.031	0.036	0.014	0.015	0.131	0.769	K7
L2P3-1	Wild	0.004	0.016	0.026	0.02	0.019	0.085	0.83	K7
L2P4-3	Wild	0.004	0.015	0.013	0.01	0.008	0.045	0.905	K7
L3P3-3	Wild	0.005	0.009	0.008	0.005	0.336	0.006	0.632	K5/K7
L3P3-1	Wild	0.004	0.01	0.01	0.006	0.354	0.007	0.609	K5/K7
L3P1-3	Wild	0.007	0.011	0.01	0.012	0.415	0.017	0.528	K5/K7
L4P3-1	Wild	0.009	0.037	0.024	0.374	0.013	0.41	0.133	K4/K6
L4P4-1	Wild	0.003	0.004	0.004	0.006	0.005	0.973	0.005	K6
L4P5-2	Wild	0.004	0.004	0.004	0.004	0.004	0.974	0.006	K6
L4P1-2	Wild	0.006	0.006	0.006	0.019	0.007	0.95	0.006	K6
L4P2-1	Wild	0.006	0.006	0.006	0.008	0.006	0.959	0.008	K6
L5P5-4	Wild	0.004	0.009	0.009	0.893	0.014	0.008	0.063	K4
L5P6-2	Wild	0.004	0.004	0.004	0.973	0.005	0.005	0.006	K4
L5P7-2	Wild	0.004	0.004	0.004	0.972	0.004	0.005	0.006	K4
L5P5-3	Wild	0.007	0.006	0.006	0.961	0.004	0.01	0.005	K4
L5P1-2	Wild	0.004	0.004	0.005	0.972	0.005	0.005	0.006	K4
L5P6-1	Wild	0.01	0.014	0.014	0.872	0.01	0.011	0.069	K4
L5P7-3	Wild	0.004	0.01	0.01	0.95	0.005	0.012	0.009	K4
L5P1-7	Wild	0.007	0.006	0.006	0.962	0.004	0.01	0.005	K4
L5P6-5	Wild	0.005	0.008	0.007	0.915	0.054	0.004	0.007	K4
L6P5-5	Wild	0.005	0.005	0.005	0.01	0.944	0.005	0.026	K5
L6P7-8	Wild	0.012	0.006	0.005	0.013	0.954	0.004	0.005	K5
L7P4-4	Wild	0.409	0.005	0.005	0.004	0.567	0.004	0.006	K1/K5
L7P3-1	Wild	0.688	0.003	0.003	0.003	0.294	0.004	0.004	K1/K5

* The assigned K ($Q \geq 0.8$) was highlighted; for the samples with admixture profile, the two main pools, covering $Q \geq 0.5$, were indicated

Figure S1 PCA-biplot referred to the quantitative morphological traits in wild and cultivated plants belonging to Sicilian *R. officinalis* germplasm.

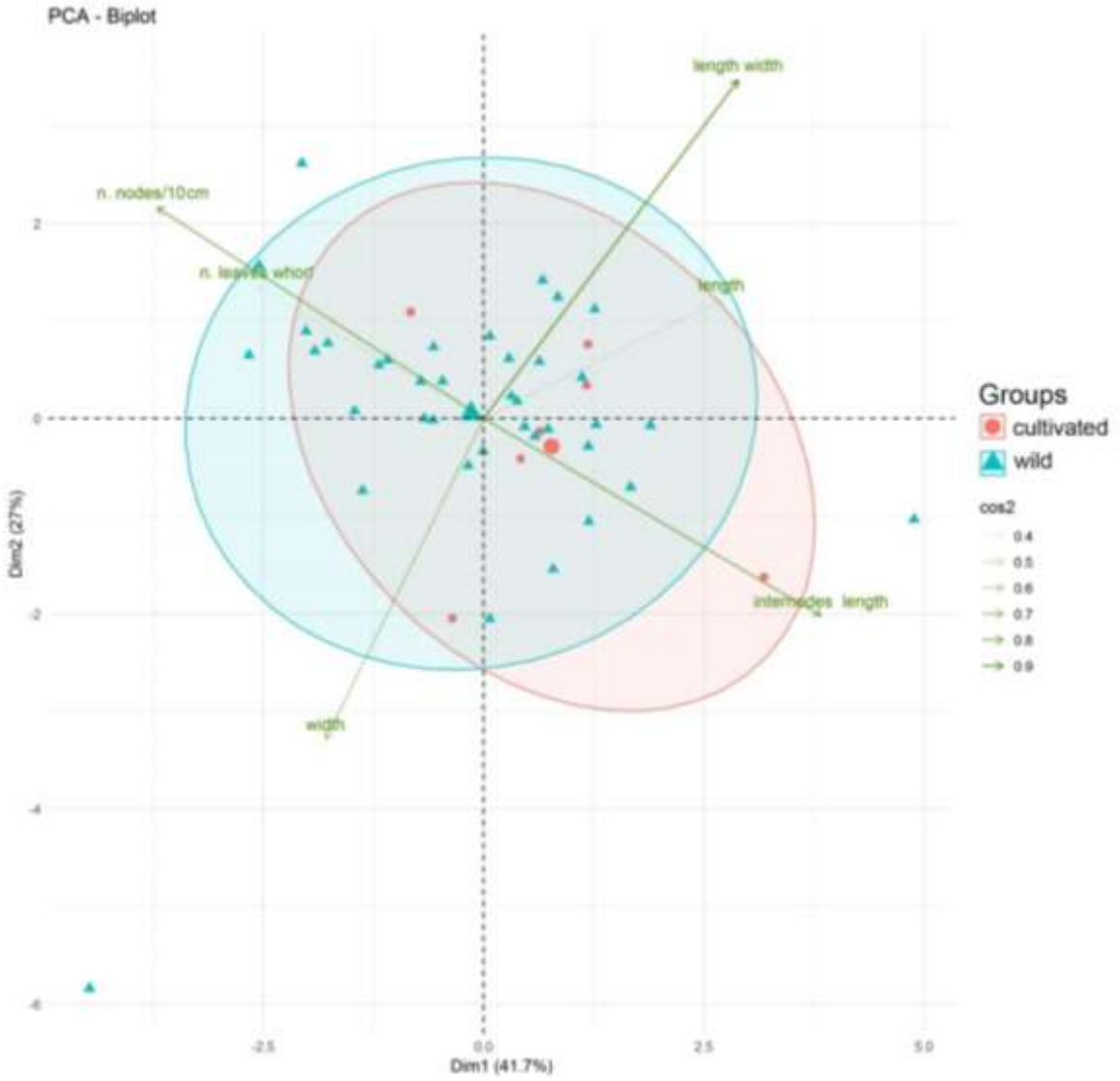


Figure S2 Genome size estimation in *R. officinalis*. Relative nuclear DNA content obtained by flow cytometric analysis from *Rosmarinus officinalis* and *Pisum sativum* (used as standard) was showed. The gain of flow cytometer was positioned on channel 600 for nuclei *Pisum*, while *Rosmarinus* nuclei peak appearing on channels 200.