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Morphological and molecular characterization of Sicilian carob (*Ceratonia siliqua* L.) accessions

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Abstract. The evergreen carob tree (*Ceratonia siliqua*) is considered one of the oldest trees in the world, cultivated since ancient times in the Mediterranean Basin, for its edible and high nutritional fruits, adapted to human and animal consumption. Spain is the main producer, followed by Italy, Portugal, Greece, Morocco, and Turkey. In Italy, the cultivation of carob is concentrated in a few provinces and insists on an area of more than 5,500 hectares. In this work 19 accessions, showing interesting fruit traits were analysed morphologically and genetically. Overall, 13 quantitative characters were considered regarding leaf (5 characters), pod (5) and seed (3). To investigate the genetic diversity 8 fluorescently labelled SSR primers were used, indicated as polymorphic in the literature. A UPGMA dendrogram was constructed to depict identity cases and relationships among the accessions. Clustering showed discrimination among accessions from Eastern and Western Sicily. The morphological characterisation does not clearly discriminate any of the cultivars recognized by the growers, similarly, the molecular analysis showed a reduced level of diversity. Since most of these local accessions are of unknown origin and that they are representative of the local germplasm they still warrant protection for their economic and environmental value.

Keywords: carob tree, morphological analysis, microsatellites or SSR markers, genetic diversity, conservation.

INTRODUCTION

The evergreen and rustic carob tree, *Ceratonia siliqua* L., a diploid species ($2n = 48$) belonging to the Fabaceae family, is grown since ancient times in most countries of the Mediterranean basin for economic and environmental value.

This tree characterizes the Mediterranean vegetation and landscape. In the Mediterranean area, pastures with carob trees were traditional agro-sil-

vo-pastoral systems, representing multifunctional systems that can contribute to the preservation of agrobiodiversity and traditional knowledge (Venturi et al. 2022). Carob trees planted with other trees such as olive, fig, grapevines, or almonds and with annual perennial crops between the rows represents an agroforestry practice aiming at integrating woody perennials and an agricultural crop growing as part of the understory.

The carob tree can thrive in calcareous and dry soils (Batlle 1997; Tous et al. 2013), making the species suitable for the valorization of marginal areas for agriculture. Its fruits (pods) are legumes and can have elongated, compressed, or curved shapes (Batlle 1997), they are rich in nutritional values and bioactive molecules properties, showing a wide range of biological properties with important health-promoting effects (prevention of cancers, lowering of LDL cholesterol, antidiabetic effects) (Avallone et al. 1997; Zunft et al. 2003; Goulas et al. 2016; Theophilou et al. 2017). Carob seeds can be used to produce carob bean gum, a food-thickening agent (Bouzouita et al. 2007; Kyratzis et al. 2019).

The carob is considered native to the Eastern Mediterranean and it was mainly spread in cultivation in the Western Mediterranean countries by Greeks, and Romans who selected and propagated genotypes by scion grafting (Baumel et al. 2022). Anyway, multiple origins of domestication were identified both in the Eastern and Western Mediterranean Basin (Baumel et al. 2022). The differences between cultivated varieties and wild progenitors are relative to the size and position of fruits (La Malfa et al. 2007). The cultivation in specialized orchards has taken place only recently, because in the past carob cultivation essentially comprised wild rootstocks distributed naturally and randomly grafted with phenotypes of selected scions (La Malfa et al. 2014).

Spain is the main producer of Carob followed by Italy, Portugal, Greece, Morocco, and Turkey (Batlle 1997; Bulca 2016).

Nowadays in Italy, carob cultivation is prevalent in South-eastern Sicily, in the provinces of Syracuse and Ragusa, where it has represented an important economic resource for centuries. In the Monti Iblei (South-eastern Sicily) the enclosed fields with carob trees are included in the "National Catalogue of Historical Rural Landscapes" where a dense network of low walls marks out plots where carob trees provide food and shade to livestock left grazing there after the wheat harvest (Agnoletti, 2011; 2013).

Carob pods in Sicily are directly marketed or transformed into derivatives for animal or human feed (Caruso et al. 2008; La Malfa et al. 2012). In the last twenty years, on the island, the demand for carob has undergone a transformation moving from products exclusively for animal feed and widespread culinary traditions towards a sought-after product (chocolate, liqueurs, and cough sweets) thanks to local companies that improved the processing of carob. According to the latest published statistical data, carob cultivation in Sicily concerns about 5,415 ha (ISTAT 2022).

In Sicily, the carob genotypes were introduced at different times by Phoenicians, before the Greek and Roman dominations and later during the Arab domination (Ramón-Laca and Mabberley 2004; Baumel et al. 2022). The Sicilian carob germplasm includes peculiar cultivars multiplicated by seeds or grafted. In the field farmers in general grow few phenotypes.

Different studies have been performed on carob genetic diversity by using different molecular markers (Barracosa et al. 2008; Caruso et al. 2008; La Malfa et al. 2014; Viruel et al. 2018; Di Guardo et al. 2019; Kyratzis et al. 2019; Baumel et al. 2022). Similarly, many studies have been conducted to check the nutritional composition of carob products (Avallone et al. 1997; Zunft et al. 2003; Bouzouita et al. 2007; Bulca, 2016; Goulas et al. 2016; Theophilou et al. 2017).

Although among the molecular markers, microsatellite or SSR (Simple Sequence Repeats) represent the marker of choice for fingerprinting study and a powerful instrument for germplasm management, allowing diversity assessment among standardized databases (e.g. peach – Marchese et al. 2005; palm – Giovino et al. 2021, 2023; fig – Costa et al. 2017; apple – Venison et al. 2022; sweet cherry – Ordidge et al. 2021; Trifonova et al. 2021; almond – Dangl et al. 2009; Cimò et al. 2017; hazelnut – Fiore et al. 2022; olive – Atrouz et al. 2021; Marchese et al. 2023), in the carob species La Malfa et al. (2014) found low genetic diversity by using a set of EST-SSR. Viruel et al. (2018) tried to improve the detection of diversity by using next-generation sequencing of SSR loci, screening populations throughout the Mediterranean Basin – from Spain, Greece, Lebanon, and Morocco. They found hidden SNP mutations in the SSR amplicons which can be considered an additional source of genetic variation, however, the differences among populations were not so large.

The purpose of the current study was to characterize carob accessions from Sicilian farms using morphological and SSR markers to detect the level of diversity and for conservation purposes.

MATERIALS AND METHODS

Plant material

The specimens used for morphological statistical analysis and molecular analysis were collected in the private farms and the regional collection field and voucher specimens were deposited in herbarium SAAF-University of Palermo. A total of 19 accessions were georeferenced and collected from local farms (Table 1, Figure 1).

Morphological characterization

Overall, 14 accessions were used for morphological analysis (Table 1). For each accession, 10 measurements were taken for each quantitative character on 3 different samples. Overall, 15 descriptors were considered regarding leaf (6 characters), pod (5), and seed (4) based on carob descriptors developed by Batlle and Tous (1997) (Table 2).

A principal component analysis (PCA) and a discriminant analysis (DA) were performed, following Boyd (2002), Giovino et al. (2015), and Domina et al. (2017; 2022). The PCA was based on logarithmic values of continuous quantitative characters, using PAST version 4.11 (Hammer et al. 2001; Hammer et al. 2022). The DA, with the individuals a priori assigned to the postulated cultivars, was performed on continuous and discrete numerical characters. Each of the 13 continuous characters was also subjected to univariate analysis.

Table 1. Carob trees accessions analyzed in this study.

Code	Accession	Country	Geographic coordinates	Morphological analysis	Molecular analysis	Voucher
1	Cicero	Italy	36° 51' 09" N 14° 55' 05" E	X	X	SAF100086
2	Fratantonio_E	Italy	36° 53' 10" N 14° 54' 25" E	X	X	SAF100087
3	Fratantonio_G	Italy	36° 48' 24" N 14° 54' 20" E	X	X	SAF100088
4	Fratantonio_S	Italy	36° 53' 33" N 14° 54' 14" E	X	X	SAF100089
5	Iacono	Italy	36° 50' 42" N 14° 34' 07" E	X	X	SAF100090
6	Licitra	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100091
7	Maltese	Italy	36° 46' 49" N 14° 42' 54" E	X	X	SAF100092
8	Racemosa	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100093
9	Scrofani	Italy	36° 51' 10" N 14° 41' 40" E	X	X	SAF100094
10	Tantillo	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100095
11	Tenuta Chiaramonte	Italy	36° 50' 00" N 14° 38' 27" E	X	X	SAF100096
12	Latinissima	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100097
13	Pasta	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100098
14	Saccarata	Italy	36° 52' 49" N 14° 54' 17" E (collection field)	X	X	SAF100099
15	Torretta	Italy	36°86'56.17"N 14°83'70.71"E		X	SAF100100
16	Terrasini	Italy	36°86'55.48"N 14°83'75.43"E		X	SAF100101
17	Cinisi	Italy	36°86'55.48"N 14°83'75.43"E		X	SAF100102
18	NA CAR	Israel			X	SAF100103
19	Israel CAR	Israel			X	SAF100104

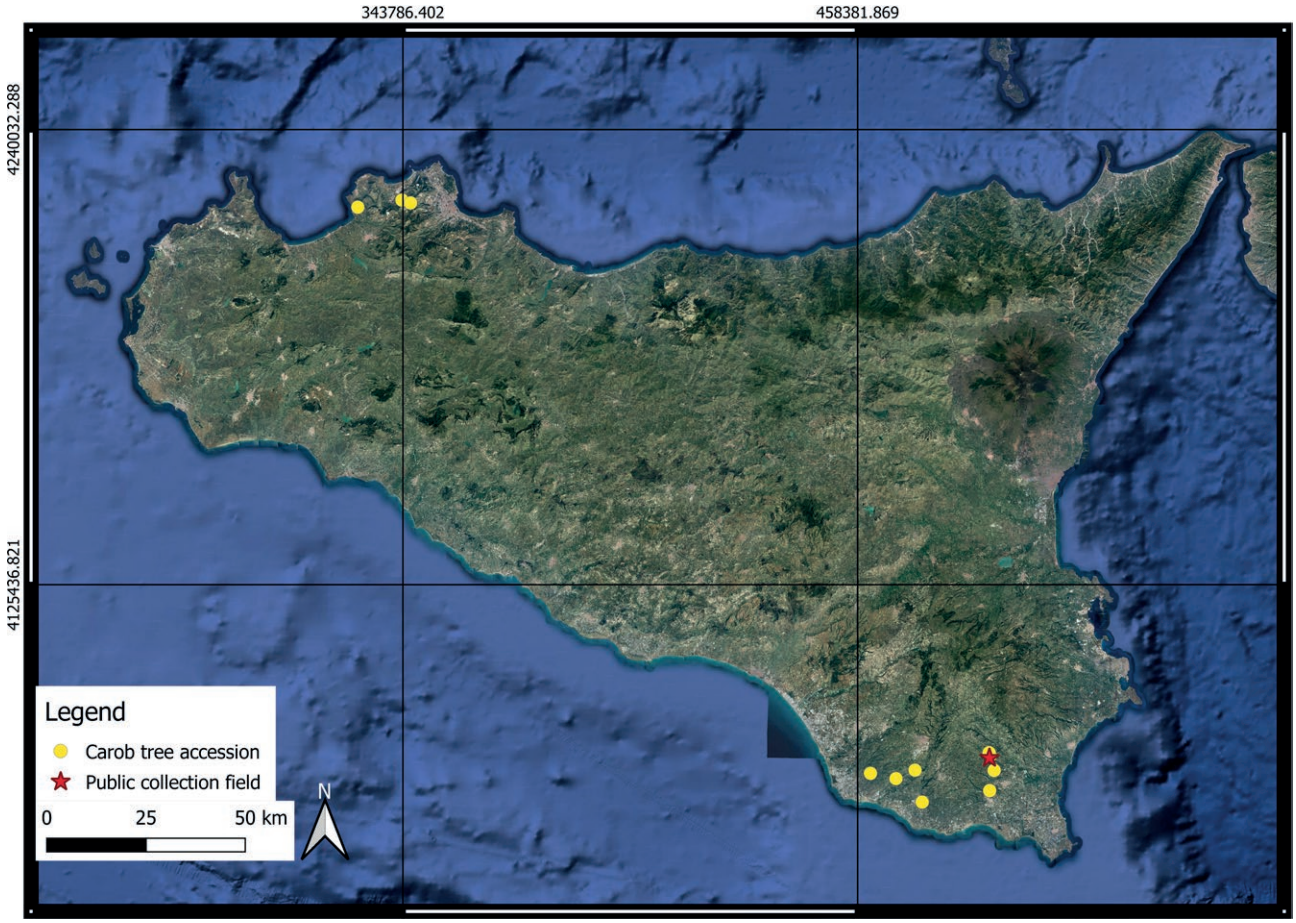

Figure 1. Location sites of carob trees where accessions were sampled (yellow pointer) and of regional collection field (red star).

Genomic DNA Extraction and SSR genotyping

Overall, 19 accessions were used for molecular analysis (Table 1). Genomic DNA extraction was performed from young leaves by using the Doyle and Doyle protocol (1987). DNA quantifications were carried out with NanoDrop 1000 Spectrophotometer and dilution were made to the final concentration of 150 ng/µl. Eight Single Sequence Repeats (SSRs) loci were chosen: Cesi_98_gct6; Cesi_187_at15; Cesi_673_ct9; Cesi_722_ag9; Cesi_976_ta5tg6 and Cesi_1187_at9 reported by La Malfa et al. (2014), and two C09 and C24 developed by Viruel et al. (2018) following number and range of expected alleles, reported heterozygosity in the literature, to set multiplexes PCR reactions. For multiplexing, the SSR loci were used for the amplification of eight genotypes and once detected their expected allele size range in comparison with the literature. Fluorescent dyes (FAM, HEX, NED, and PET; Life Technologies, Thermo Fisher, Foster City, CA, USA) were used for each locus, and multiplexes PCR were developed, and the markers used are shown in Table 3.

Table 2. Morphological traits among studied *Ceratonia siliqua* accessions.

	Descriptor	Type
Leaf	No. leaflets	discrete
	leaflet length	mm, continuous
	leaflet width	mm, continuous
	leaf axis length	mm, continuous
	Distance of the first pair of leaflets from the base	mm, continuous
	leaflets petiole length	mm, continuous
Pod	length	mm, continuous
	width	mm, continuous
	edge thickness	mm, continuous
	groove thickness	mm, continuous
	pod weight	gr, continuous
Seed	length	mm, continuous
	width	mm, continuous
	thickness	mm, continuous
	No. seeds per Pod	discrete

Table 3. Specific biomarkers for the determination of intraspecific variability

Marker	Sequence 5'-3'
Cesi_976F	TCCTGAAGGCTGAAGATGATG
Cesi_976R	CAAACCAATGAAGGGCTCTA
Cesi_98F	GCCACCACTTTGAAGGAAGA
Cesi_98R	GCTAGAAGCAGGAGCAGGAG
Cesi_1187F	TTCTCGTCGCCCAAACTG
Cesi_1187R	CTCCCTCATCTCCTTCGTTG
Cesi_187F	ATAACTGGGCGTTCTTTGCTT
Cesi_187R	ATTATCTCTTGCTTTGTGGTCCT
Cesi_509_F	GCCACCTCTCCCTCTTCTC
Cesi_509_R	TTTTGTTCTAATTTTGCTTGCA
Cesi_673F	GAATAGGGCAGAGAGAACAGG
Cesi_673R	TCAAAGGAAGATGAGAAAGAAATCC
Cesi_722F	AGGCTCACACGAAACCCTAA
Cesi_722R	CTGCCACAAGATGATAGATTTG
VirC-09_F	AAGACTCGGCAGCATCTCCAGGCTTTGTAGCTGCCCATTG
VirC-09_R	GCGATCGTCACTGTTCTCCAGAAGGTTGGATAGCGTCCTG
VirC-24_F	AAGACTCGGCAGCATCTCCAAGCTGCAATTTGAGGAATAAAGC
VirC-24_R	GCGATCGTCACTGTTCTCCAACATCCAAAACCCTAGAGCAAG

The PCR reactions were performed in 8 µl reactions containing 1.5 ng template genomic DNA, 1 x Multiplex PCR master mix (Qiagen) and 0.2 µM of each primer on a Geneamp Pcr System 9700 Thermocycler (Thermo Fisher) using the following touch-down protocol: initial denaturation at 95°C for 5 minutes followed by 10 cycles of 95°C for 45 s, 65°C for 30 s with a reduction in temperatures of 1°C per cycle and 72°C for 45 s, then 25 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 45 s. The final extension was performed at 72°C for 30 minutes.

The SSR analysis was performed with an ABI 3135xl Genetic Analyzer (Applied Biosystems, Thermo Fisher, Foster City, CA, USA), using ABI GeneScan and Genotyper software for allele sizing and scoring.

SSR diversity analysis

By analyzing SSR in a plant's genome, researchers can create a unique fingerprint for that species and use it to identify and differentiate it from other closely related species. SSRs have been successfully used for the identification of genetic diversity between cultivars of the same species (Glynn et al. 2009; Marra et al. 2013; Fiore et al. 2022). The SSR data were analyzed using the software package Cervus 3.0 (Kalinowski et al. 2007). The total number of alleles (Na), the number of effective alleles (Ne), Polymorphic Information Content (PIC), He and Ho, and null alleles were computed.

A UPGMA dendrogram was constructed by using the software Darwin6 (Perrier and Jacquemoud-Collet 2006).

RESULTS

Morphological analysis

Leaf width and length ranged from 27.4 mm (Scrofani) to 46.3 mm (Latinissima) and from 41.5 mm (Scrofani) to 67.6 mm (Tantillo), respectively. The number of leaflets/leaves was found between 7.6 and 9.9. The average pod dimensions (width, length, and thickness) were found between 20 and 26.1 mm, 130.3 and 232 mm and 6-11 mm for the accessions. The pod weight was variable and ranged from 14.7 g (Tantillo) to 50.1 g (Saccarata) among genotypes (Table 4).

Figure 2 shows the quality of representation of the variables by cos2. The results of PCA indicated that the first two principal components explained 68% of the data variability, the plot shows the loading of each studied variable (arrows), and the arrow lengths approximate their variance, whereas the angles between them represent their correlations. The seed characters are positively correlated, they are grouped together, and on the opposite side there are the leaves descriptors. The seed length was positively correlated with pod thickness and negatively correlated with the number of leaflets. Furthermore, pod weight is negatively correlated with the leaflet

Table 4. Leaf, pod and seed descriptors of carob accessions.

	Leaflets					Pod				Seed			
	N. Leaflets	length (mm)	width (mm)	leaf axis length (mm)	distance first pair leaflets from the base	petiole length (mm)	pod weight (mm)	length (mm)	width (mm)	groove thickness 1 (mm)	groove thickness 2 (mm)	N. seeds per pod (mm)	length (mm)	width (mm)	thickness (mm)
Cicero	8.4	49.4	36.3	95.8	23.4	2.6	25.8	139.0	24.4	9.4	4.7	13.3	8.8	6.2	2.3
Fratantonio_E	8.3	52.5	39.8	112.4	26.3	2.7	32.2	141.5	26.1	11.4	5.0	12.0	9.3	6.8	2.5
Fratantonio_G	8.0	55.2	38.2	98.5	24.7	2.5	19.2	132.2	23.3	9.9	5.5	12.0	8.3	6.0	2.5
Fratantonio_S	8.9	43.0	29.4	88.6	19.5	2.5	38.0	156.7	24.7	8.3	5.7	13.0	9.7	7.0	3.0
Iacono	7.6	42.7	34.1	68.3	23.5	2.5	36.1	142.2	23.7	10.2	6.0	13.0	10.0	7.3	2.8
Licitra	7.6	42.7	34.1	68.3	23.5	2.5	36.1	142.2	23.7	10.2	6.0	13.0	10.0	7.3	2.8
Maltese	8.8	46.1	35.7	103.0	21.8	2.3	24.9	131.1	25.2	10.1	5.3	12.0	9.8	6.7	2.7
Racemosa	8.8	54.9	42.6	112.8	23.4	2.5	29.1	150.0	20.0	6.0	4.5	13.0	7.0	4.5	2.0
Scrofani	8.7	41.5	27.4	90.8	19.3	2.3	17.2	133.5	21.8	7.6	5.5	13.5	8.3	5.8	3.3
Tantillo	8.6	67.6	45.7	135.1	30.9	2.5	14.7	145.0	20.0	8.5	5.0	15.0	9.5	7.0	3.0
Tenuta Chiaramonte	7.8	48.1	33.6	97.6	24.0	2.5	27.2	130.3	23.9	9.9	6.2	12.7	9.3	7.0	2.8
Latinissima	9.4	58.9	46.3	140.5	26.5	3.1	36.6	152.5	23.0	8.3	4.8	12.0	10.5	8.0	3.3
Pasta	9.9	47.7	30.5	131.6	26.7	2.7	38.1	232.0	25.7	9.4	5.0	15.0	8.8	7.8	3.3
Saccarata	9.2	53.4	42.5	138.7	30.0	3.2	50.1	155.0	25.0	10.0	6.0	11.0	10.0	7.5	3.0

Figure 2. Biplot illustration of PCA analysis, squared cosine (cos2) shows how accurate the representation of our variables or individuals on the PC plane.

Figure 3. Correlation matrix of different descriptor in different accession of carob tree.

length and width. The trait correlations are also indicated using absolute Pearson correlation coefficients, with red shades indicating high absolute correlation and blue shades indicating low absolute correlation (Figure 3). We found a negative correlation between seed length and the number of seeds in the pod and a positive correlation between leaflets length and leaf axis length.

No continuous numerical morphological character discriminates the single cultivars since all the values overlap. Both the PCA and the DA are of little significance because they represent a small portion of the vari-

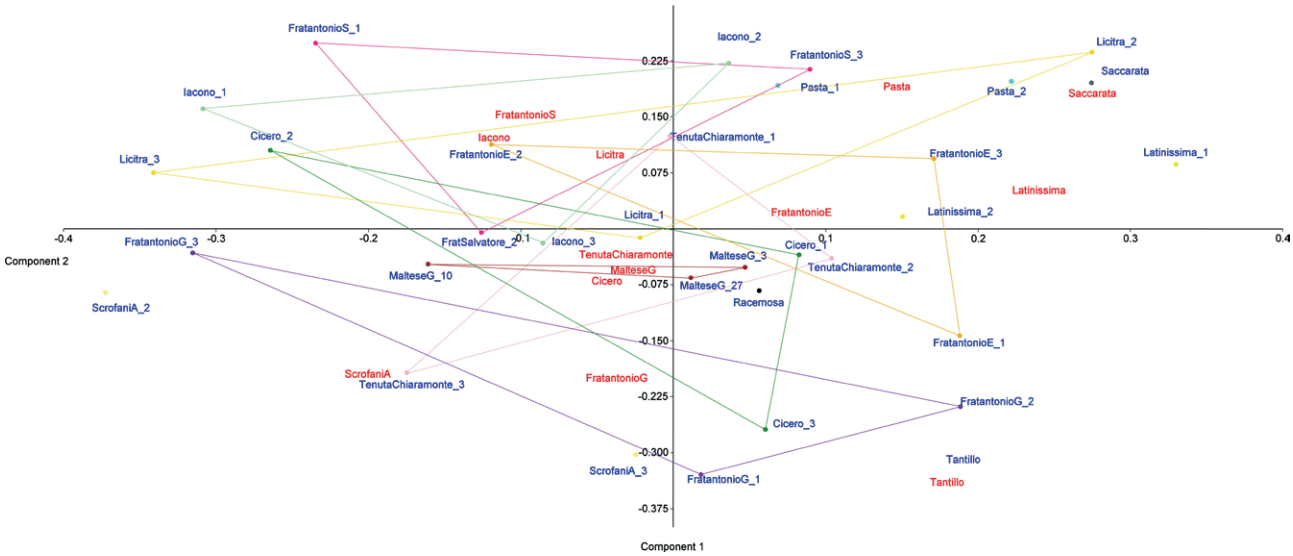

Figure 4. Principal Component Analysis based on the 13 continuous morphological characters, with groups corresponding to the 18 studied accessions. PC1: Eigenvalue 0.0367, % variance 32.969; PC2: Eigenvalue 0.0293, % variance 26.284.

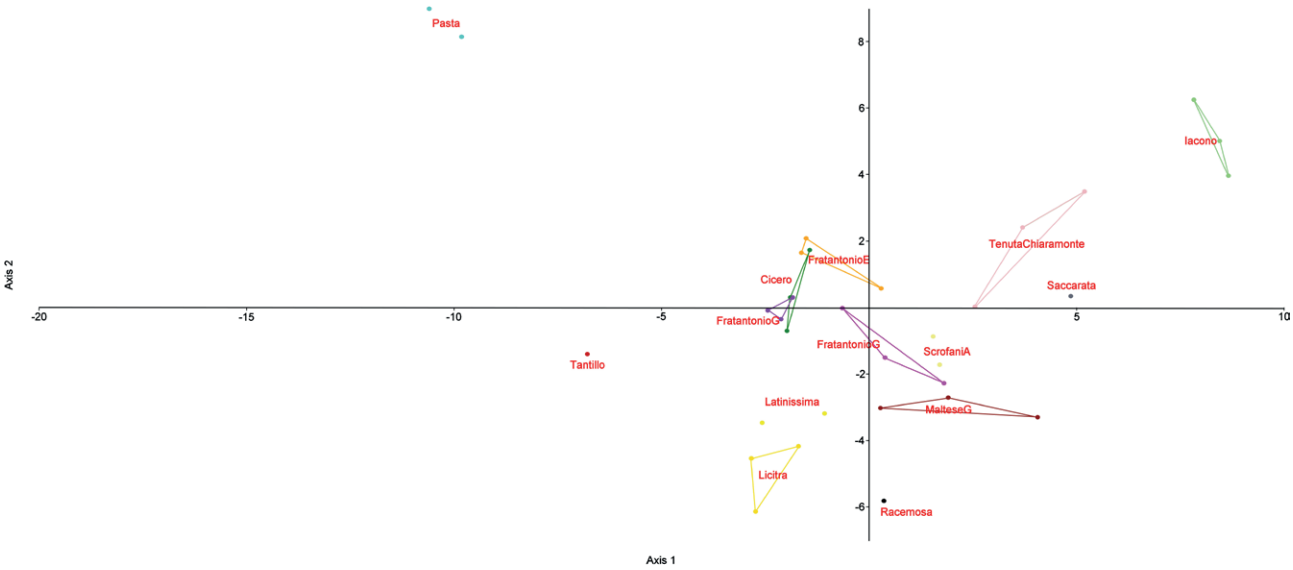

Figure 5. Discriminant analysis (DA), based on the 15 considered morphological characters with groups corresponding to the nine studied taxa. Axis 1: Eigenvalue 31.498, % variance 22.37; Axis 2 Eigenvalue 21.532, % variance 28.97.

ability. The PCA done on the complete dataset (Figure 4) shows an almost complete overlapping of all examined accessions. The DA on the complete dataset discriminates only the accessions "Iacono" and "Pasta" (Figure 5).

SSR marker diversity

The 8 SSR markers used in this analysis showed a low level of polymorphism; the mean number of alleles per locus resulted 3.875; the mean proportion of loci typed was 0.8750; the mean expected heterozygosity was 0.5029 and the mean polymorphic information content (PIC) was 0.4129. The most polymorphic primer pair was Cesi_187 which amplified 6 alleles, while Cesi_722 and Cesi_1187 only three alleles. The Primer Cesi_673 resulted monomorphic thus it was eliminated from the cluster analysis.

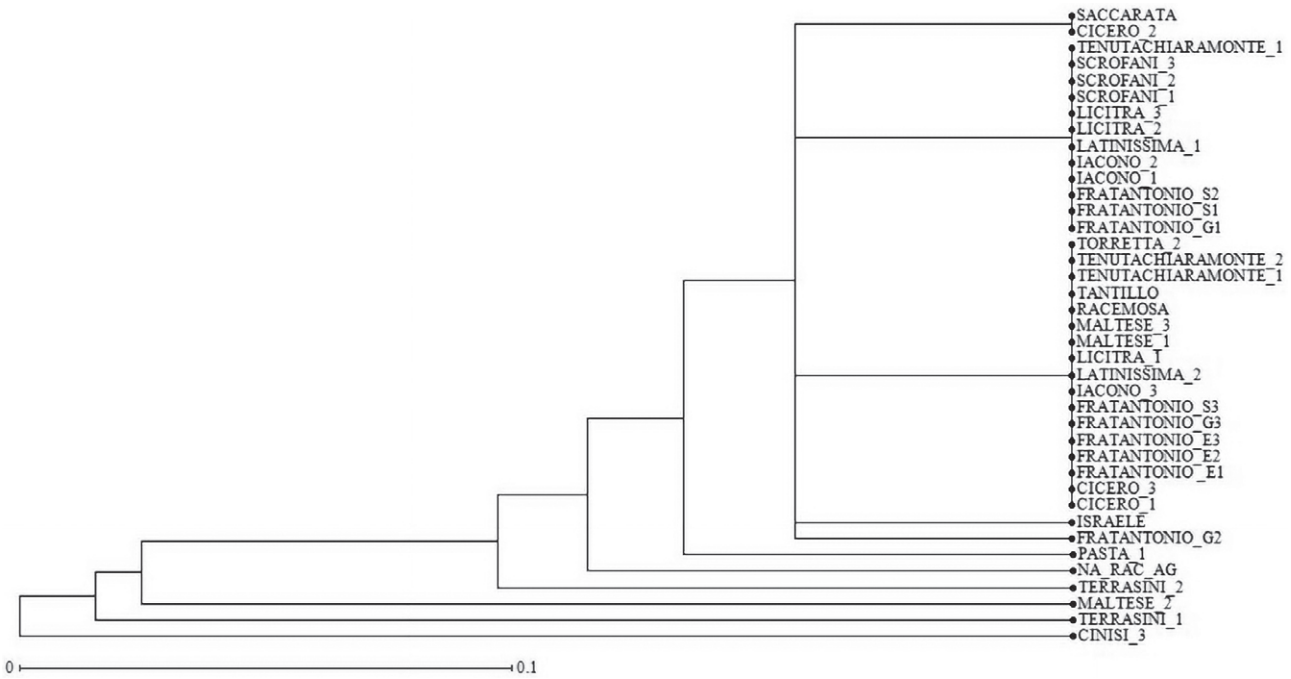

Figure 6. Dendrogram of 19 carob accessions based on simple matching coefficients.

Cluster analysis

As depicted in the UPGMA dendrogram, based on 7 SSRs, as Cesi_673 was monomorphic, constructed with the Dice dissimilarity index by using DARwin 6 software (CIRAD), only 9 accessions studied were discriminated, while most were undiscriminated (Figure 6).

DISCUSSION

The carob species represents a genetic resource of adaptation to the environmental and dry climatic conditions of Sicily, which is the leading Italian region for carob production, producing 98% of the Italian carobs, accounting for 35,0838 tons (ISTAT 2022). Carob pods are mainly produced in Ragusa areas in the south-eastern part of Sicily, where the most common varieties are "Latinissima" (or known synonyms "Giubiliana", "Cipriana", "Cipriota", "Masculina"), Racemosa (or "Moresca", "Spada", "Sciabulara"), Saccarata (or "Latina", "Fimminedda", "Milara") (Caruso et al. 2006, Blangiforti et al., 2022).

Most carob present in specialized orchards are nowadays grafted trees; the grafted carob trees improve phenotypic traits: fleshiness, size and sweetness of the pod and productivity (Batlle and Tous 1997; Tous et al. 2013). Female cultivars are the most important trees in commercial orchards in Mediterranean countries (Albanell et al. 1996) appreciated for their pods and seeds employed as raw materials in the food, pharmaceutical and cosmetic industries (Vourdoubas et al. 2002). Carob pods have been investigated as a material for bioethanol production (Biner et al. 2007).

The carob characterization can be performed at both the morphological and genetic levels. In our study, all the accessions were female, and it was evident the low phenotypical diversity among them that was almost overlapping. The DA analysis enabled the discrimination of only the accessions Iacono and Pasta. Barbagallo et al. (1997) recorded that pod size and numbers of normal and aborted seeds were the characters with a certain degree of polymorphism in analyzing a survey of sixteen Sicilian carob cultivars. They recognized cultivar groups according to a geographic criterion.

Our accessions, in general, showed many leaflets between 7.6 and 9.9 with higher values than the number reported by Korkmaz et al., (2020) analyzing Turkish genotypes where it ranged from 5.9 to 7.1. Regarding the morphological character of pod carob cultivars in Sicily, La Malfa et al. (2012) reported average pod weight, pod width, pod length, and pod thickness as 13.7–33.4 g, 19.3–26.8 mm, 14.9–22.9 cm and 6.8–14.0 mm, respectively, which is in accordance with our study.

Similarly, our dendrogram (Figure 6) showed a limited SSR variability. The most diverse accessions were Terrasini_1, Maltese_2, Cinisi_3, NA_RAC_AG, and

Pasta_1, mostly from Western Sicily. Three groups of identity were found: Saccarata/ Cicero_2; a group of Latinissima_1 accessions and a group of Latinissima_2/ Racemosa/Maltese accessions closely related to a plant derived from a seed originated in Israel and Frantantonio_G2. Clustering showed discrimination among accessions from Eastern Sicily and Western Sicily.

As observed for the whole Mediterranean area (Baumel et al. 2022), the morphological and genetic diversity recorded in the Sicilian carob accessions turned out to be very low.

Sicilian farmers can distinguish vegetatively or sexually propagated accessions to which local names are given but the analysis of this germplasm has shown that the morphological and genetic variations are minimal. The low level of diversity within a given geographic area can be explained by the asexual propagation of selected clones (La Malfa et al. 2014). However, considering that most of the local accessions are of unknown origin and that they are representative of a typical germplasm they still deserve attention and protection.

Baumel et al. (2022) also observed higher genetic variability in the eastern and western Mediterranean basin and lower in the central Mediterranean. Further molecular analysis including SNP sequencing may better shed light on the carob genetic diversity and origin.

Nonetheless, given the multiple origins of domestication and the presence of haplotypes peculiar to the central Mediterranean (Baumel et al. 2022), the knowledge and conservation of the Sicilian germplasm is found to be extremely important for the conservation of carob biodiversity to protect both the agro-forest ecosystem and landscape of Mediterranean region. The cultivation of this multi-functional tree is ideal for agricultural diversification in semi-arid areas therefore in the next coming year it is expected to increase due to climate change, considering its resistance to drought, high rusticity, low requirements for orchard management, and low environmental footprint (Zemouri et al. 2020; Tzatzani et al. 2023).

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