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A Multidisciplinary Approach for the Assessment of the Last Surviving 'Marrone di Chiusa Pesio' Chestnut Trees in the Piemonte Region (Italy)

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Abstract: Chestnut orchards are a multifunctional resource, providing not only fruit or wood but also playing a role in the conservation of mountain and hillside landscapes. In the Piemonte Region, Italy, a rich genetic heritage of chestnut genotypes has contributed to considerable biodiversity and environmental value. The study aimed to valorize an important example of the chestnut agrobiodiversity in the Piemonte Region by focusing on the 'Marrone di Chiusa Pesio' (MCP) cultivar (cv). A multidisciplinary approach was applied, involving genetic and morphological analyses, Visual Tree Assessment (VTA), and phytochemical and nutritional profiling. The plant census provided identification and geolocation of 187 MCP specimens; the 20 most representative trees were genetically analyzed, and then, through the VTA, their morpho-functional status was evaluated. The nutraceutical properties and phytochemical composition were assessed by measuring the total polyphenol content (TPC), antioxidant capacity (AOC), and other phytochemical classes through spectrophotometric and chromatographic methods. The results showed significantly higher TPC values (ranged from 36.51 ± 1.60 mgGAE/100 g of dried weight—DW to 103.14 ± 1.24 mgGAE/100 g DW) compared to other 'Marrone-type' cultivars, along with high levels of key phenolic markers, bioactive compounds, and nutritional substances. These included tannins (about 22–28 mg/100 g DW) and cinnamic acids (about 23–25 mg/100 g DW), followed by flavonols, benzoic acids, organic acids, monoterpenes, vitamin C, and catechins, listed in order of predominance. A Principal Component Analysis (PCA) was performed to observe the distribution of the samples and their correlations based on the chemical composition. The results confirmed the interesting phytochemical properties of the 'Marrone di Chiusa Pesio' nuts, together with their good morphological and functional properties. Given the ongoing genetic erosion of *Castanea sativa* cultivars, due to cultivation abandonment and climate change, the main factors contributing to the progressive loss of biodiversity worldwide, the presented approach aimed to provide an overview of the conservation status of the local agrobiodiversity. This study highlighted the value of a local chestnut cultivar, presenting the low conservation status of the few remaining specimens. The goal was to define the significant phenotypic variation regarding MCP in the considered area due to environmental variations, which may be of interest in its genetic adaptation to climate change. The study may potentially encourage the development of strategies for actively conserving the forest agrobiodiversity and hillside ecosystem services in the highly diverse landscapes of the Alpine valleys.

Keywords: *Castanea* spp.; UPOV descriptors; phytochemicals; antioxidants; biodiversity conservation; multivariate analysis

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1. Introduction

Chestnut orchards are a multifunctional resource, valuable not only for fruit and wood production but also for their role in conserving mountain and hillside landscapes [1]. They are included in the "habitats of community interest" as defined by Habitats Directive 92/43/EEC (habitat 9260). In Italy, chestnut trees cover more than 7% of the total area of national forests. Of the 780,000 hectares of chestnut forests, approximately 50,000 hectares are dedicated to fruit cultivation [2]. For many centuries, chestnuts served as the main source of income and subsistence for people living in the mountains and hills. However, after the Second World War, chestnut cultivation declined due to socio-economic changes due to industrialization [1,3] and phytopathogenic diseases, such as ink disease (caused by *Phytophthora cambivora* [Petri] Buism.) and chestnut blight (*Cryphonectria parasitica* [Murr.] Barr) [4]. In Italy, human activity has produced over 300 cultivars [5]; 232 are officially registered in the national register of fruit species cultivars [6,7]. As a result, specific chestnut products from the Piemonte Region, such as 'Castagna di Cuneo' e 'Marrone della Valle di Susa', were awarded the certification of Protected Geographical Indication thanks to centuries of selection in the foothills (Protected Geographical Indication (PGI) Regulation (EC) No. 1050/2007 [8]).

This study focused on the 'Marrone di Chiusa Pesio' (MCP), a *Castanea sativa* Mill. cultivar deeply connected to the Piemonte mountain community where it was selected for the first time in the late 12th century. Documents from the foundation of the "Certosa di Chiusa Pesio" [9] indicate that one-fifth of the land donated to the monastic order comprised chestnut orchards. The municipality of Chiusa di Pesio demonstrated high production in 1945, being the second-largest municipality in the Cuneo area in terms of surface dedicated to chestnut orchards, with approximately 1700 hectares, representing 18.7% of the municipal territory [10,11]. Currently, the local fruit production competes with Euro–Japanese hybrids, but it does not generate enough income to properly value the local MCP cultivar [12].

One of the key goals of the agrobiodiversity strategy (Convention on Biological Diversity, CBD, 2002) is to protect local germplasm from socio-economic changes caused by rural abandonment and specific pathogen attacks. Therefore, preserving local genotypes with their unique adaptive and technological characteristics is crucial. Additionally, the market demand is increasingly focused on typical high-quality products [13]. The present work aimed to investigate the conservation status of the MCP trees in selected areas of the Pesio Valley, its native area. Once identified, the trees were genetically analyzed and geolocated. The fruits were subjected to morphological, nutritional, and nutraceutical analyses and the results were compared to competing cultivars. Finally, a Visual Tree Assessment (VTA) was performed to describe the morpho-functional status of each tree.

2. Materials and Methods

2.1. Plant Census and Identification

A plant census was performed and 187 MCP trees were identified in a flat area of about 4 km^2, known as "Regione Vigne", in Chiusa di Pesio (Cuneo Province, Italy) (Figure S1). Soils in this area, characterized by a significant presence of skeleton, are sandy-loam (SL) and loamy sand (LS) types. Each plant was tagged with an identification number and geolocated by GPS. Additionally, detailed cartography was produced with QGIS software 3.20 [14] to facilitate data sharing for accession identification both in the field and on the map.

2.2. Genetic Analysis

Young fresh leaves of 20 representative MCP accessions (coded from 101 to 120) were collected in the area (centroid: 44°19′56″ N 7°40′34″ E) in spring–summer and stored at −80 °C. Trees were selected based on plant distribution and age, and DNA analysis of at least 1 tree per chestnut orchard was performed. According to the Doyle and Doyle protocol [15], DNA was extracted from 0.2 g of plant material and the analysis was

performed at 10 microsatellite loci (SSR): CsCAT1, CsCAT3, CsCAT6, CsCAT8, CsCAT14, CsCAT16, CsCAT17, CsCAT41 [16], QpZAG110 [17], and EMC38 [18]. PCR was performed using a PTC 100 thermal cycler (MJ Research Inc., Watertown, MA, USA) in a total volume of 20 µL containing 50 ng of DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2 µL of GeneAmp 10X PCR buffer (100 mM Tris–HCl pH 8.3, 500 mM KCl), 1 µL of 10% Bovine Albumin Serum (BSA), 0.5 µM labeled forward primer, and 0.5 µM reverse primer, 0.5 Units of AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA). In this study, markers were amplified in single PCRs. DNA amplification reactions were performed under the following conditions: an initial step of 9 min at 94 °C, followed by 28 cycles of 30 s at 94 °C, 45 s at 54 °C, 90 s at 72 °C, and a final extension step of 30 min at 72 °C. The same conditions were applied to all the used primers. The forward primers were labeled with a fluorochrome (6-FAM, HEX, or NED). Samples were then analyzed on a 3130 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). Data were analyzed using the GeneMapper Software 4.0 (Applied Biosystems), and alleles were defined by their size (in bp) compared with the size standard GeneScan™ 500 LIZ®.

2.3. Visual Tree Assessment (VTA)

The MCP trees, genetically analyzed, were then evaluated in relation to their status in accordance with the VTA method [19]. The visual analysis assessed vitality, phytosanitary status, and symptoms of mechanical flaws (such as swellings, depressions, stem inclination, and root collars) to evaluate the morpho-functional condition of the MCP trees. VTA required specific plant information to be obtained, which was acquired with various instruments: a relascope to measure height, a dendrometer for diameter at breast height (DBH, 1.30 m above the ground), and a metric wheel for the crown width. A core sample was obtained from 11 trees using Pressler's drill to determine the age of the plants. The annual growth increments of each cross-section were carefully measured, with a precision of 0.01 mm. This measurement was taken by marking the distance between the start of the early wood and the end of the latewood, which represented one year of growth for the tree. For the remaining trees, age was inferred in relation to the diameter to minimize wounds on the trunks and the risk of pathogen infections, such as chestnut blight. A validation of this method used for age estimation through diameter measurement was performed. This method was also carried out on the plants selected to determine their age using Pressler's drill, resulting in similar results.

2.4. Morphological and Qualitative Fruit Analysis

Fruits and burrs from the MCP accessions were collected and morphologically evaluated. Further, 1 kg of chestnuts for each tree was phenotyped based on several parameters, including the International Union for the Protection of New Varieties of Plants (UPOV) [20] descriptors. The biometric data were then statistically analyzed to obtain useful indices to better describe the MCP cv traits. UPOV descriptors were also used to describe burrs' features by analyzing the density of prickles and their ramifications. Table 1 shows the UPOV descriptors utilized in this study.

Table 1. UPOV descriptors used to describe MCP fruit and burr samples.

No.	Descriptor
	Burr Morphology (Exterior)
27	Burr: density of prickles
	Burr: prickle ramification
	Burr: prickle length

Table 1. *Cont.*

No.	Descriptor
Chestnut Morphology (Exterior)	
Weight: fruits per kg	
36	Fruit: size
31	Fruit: shape
	Fruit hairiness
35	Fruit: color
	Fruit: stripes
32	Fruit: size of the hilum
Chestnut Morphology (Inside)	
27	Fruit: embryony
29	Fruit: penetration of seed coat into the embryo
30	Fruit: degree of penetration of seed coat into the embryo
	% Seed-episperm detachment
37	Seed coat: adherence to the kernel (fresh fruit)
38	Kernel: color of flesh

2.5. Nutraceutical–Nutritional Analysis

2.5.1. Sample Preparation

The selected MCP samples were grouped into 6 subgroups based on pedoclimatic conditions, crop management practices (pruning, irrigation, fertilization, etc.), and the overall vegetative status of the plant. After harvesting, chestnuts were stored at 4 °C and 95% RH to preserve their original traits and chemical composition until sample preparation. Fruits were manually peeled to remove the pericarp and episperm and cut into regular cubes (5 × 5 mm). The nuts were stove-dried at 50 °C for 48 h to achieve a stable weight, then ground into flour and stored in sealed plastic bags until extraction.

2.5.2. Extraction Protocols

A solution of 25 mL of methanol, water, and hydrochloric acid (HCl) (37%) (95:4.7:0.3, *v*/*v*/*v*) was used to extract organic acids, monoterpenes, and polyphenols. These extracts were pre-injection-filtered by a PTFE membrane (0.45 µm). An 80% ethanol solution was used to macerate the samples and extract sugars. Samples were homogenized, centrifuged, and then stored at 5 °C with 95% relative humidity until analysis. Vitamin C components, namely ascorbic and dehydroascorbic acids, were extracted by using a solvent composed of ethylenediaminetetraacetic acid (EDTA, 0.05%), sodium fluoride (4 mM), and hydrochloric acid (0.1 M) in methanol and deionized water (5% and 95%). 3-(1,2-dihydroxy ethyl)furo-[3,4-b]quinoxaline-1-one (DFQ) was obtained by adding o-phenylenediamine (OPDA) solution to the extracted samples to separately identify ascorbic acid and dehydroascorbic acid (DHAA). The extracts were then stored at 4 °C and 95% RH before analysis to maintain the original levels of bioactive compounds, in particular vitamin C and other thermosensitive molecules.

2.5.3. Spectrophotometric Analysis

The Slinkard and Singleton protocol [21] with the Folin–Ciocâlteu reagent was followed to quantify total polyphenol content (TPC), expressing the results as mg of gallic acid equivalents (GAEs) per 100 g of dry weight (DW). The absorbance was recorded at a wavelength of 760 nm using ultraviolet/visible (UV–Vis) spectrophotometry. The FRAP (ferric reducing antioxidant power) assay, according to the Benzie and Strain method [22],

modified by Pellegrini et al. [23], was used to evaluate the antioxidant activity (AOC). In this case, the results were expressed in mmol of Fe^{2+} ions per kg of DW. Quantification was performed by spectrophotometry at a wavelength of 595 nm.

2.5.4. Chromatographic Analysis

Chromatographic analysis was carried out using an Agilent Technologies HPLC system (series 1200) combined with a UV–Vis diode array detector (Santa Clara, CA, USA). Six different chromatographic methods were followed (Table 2).

Table 2. Chromatographic conditions of the used HPLC methods.

Method	Compounds of Interest	Stationary Phase	Mobile Phase	Flow (mL min^{-1})	Wavelength (nm)
A [1]	cinnamic acids, flavonols	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: 10 mM KH_2PO_4/H_3PO_4 pH = 2.8 B: CH_3CN	1.5	330
B [2]	benzoic acids, catechins, tannins	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: $H_2O/CH_3OH/HCOOH$ (5:95:0.1, *v*/*v*/*v*) pH = 2.5 B: $CH_3OH/HCOOH$ (100:0.1, *v*/*v*)	0.6	280
C [3]	monoterpenes	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: H_2O B: CH_3CN	1.0	210–250
D [4]	organic acids	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: 10 mM KH_2PO_4 $/H_3PO_4$ pH = 2.8 B: CH_3CN	0.6	214
E [5]	vitamins	KINETEX—C18 column (4.6 × 150 mm, 5 µm)	A: 50 mM KH_2PO_4 B: 5 mM $C_{16}H_{33}N(CH_3)_3Br/CH_3OH/H_20$ (5:95, *v*/*v*)	0.9	261, 348
F [6]	sugars	SphereClone—NH_2 column (4.6 × 250 mm, 5 µm)	A: H_2O B: CH_3CN	0.5	267

[1] Method A: gradient analysis: 5% B to 21% B in 17 min + 21% B in 3 min. [2] Method B: gradient analysis: 3% B to 85% B in 22 min + 85% B in 1 min. [3] Method C: gradient analysis: 30% B to 56% B in 15 min + 56% B in 2 min. [4] Method D: gradient analysis: 5% B to 14% B in 10 min + 14% B in 2 min. [5] Method E: isocratic analysis: ratio of phase A and B: 95:5 in 10 min. [6] Method F: isocratic analysis: ratio of phase A and B: 5:85 in 12 min.

The analytes were quantified using calibration curves obtained by external standards; for each analyte of interest, standard solutions were prepared at known concentrations according to Donno et al. [24]. The 31 selected compounds, expressed in mg/100 g of DW or, for sugars, in g/100 g of DW, belonged to 5 classes of polyphenols (benzoic acids, cinnamic acids, catechins, tannins, and flavonols), vitamin C (ascorbic and dehydroascorbic acids), monoterpenes, organic acids, and sugars.

2.5.5. Data Analysis

Results obtained by morphological and chemical analysis were subjected to a one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) post hoc test. For statistical calculations, IBM SPSS Statistics 22.0 (IBM, Armonk, NY, USA) was used and significance was accepted at $p \leq 0.05$ level. Furthermore, a multivariate Principal Component Analysis (PCA) on the correlation matrix (Varimax rotation) was also carried out using Minitab 18.0 to observe the distribution of the considered samples and their correlations based on their chemical composition. On the same matrix, Bartlett's test of sphericity (BTS) and the Kaiser–Meyer–Olkin index (KMO) were used. The phytochemical variables (TPC, AOC, and phytochemical classes) were standardized (Z-score) and recom-

bined into principal components based on their level of correlation, corresponding to an eigenvalue greater than or equal to 1 and able to explain at least 60% of the total variance.

3. Results and Discussion

In this study, one-hundred-eighty-seven trees have been identified; four are in the municipality of Pianfei, while the remaining ones are located in the municipality of Chiusa di Pesio, along the Pesio torrent in an area of approximately 3 km^2.

3.1. Genetic Analysis

The genetic analysis of 10 SSR loci in 20 selected individuals was performed; the obtained allele profiles were compared with the reference 'Marrone' DNA profile, included in the database developed at the Department of Agricultural, Forestry and Food Sciences (DISAFA—University of Turin). The results confirmed that 19 genotypes were 'Marrone di Chiusa Pesio' (Table 3), showing the same genetic profile as reported in previous studies [25]. Accession 113 was not identified to MCP, and it presented just one allele in common with it. It can probably be considered as a seedling. The comparison was expanded to other common local cultivars. The analysis revealed that this accession was not even genetically correlated to 'Garrone Rosso', 'Garrone Nero', or 'Bracalla' (Table 3), three genotypes among the most common local cultivars. Therefore, it was not considered for subsequent analyses.

Table 3. Genetic profiles of the samples analyzed at 10 SSR loci in relation to the reference [25].

Accession	Identification	CsCAT1	QpZAG110	CsCAT3	CsCAT17	CsCAT6	CsCAT16	EMCs38	CsCAT8	CsCAT14	CsCAT41
From 101 to 112 and from 114 to 120	Marrone di Chiusa di Pesio	215–223	208–208	226–240	147–153	160–174	127–133	238–242	202–208	133–150	227–233
113	unknown	194–215	208–211	226–278	130–153	160–183	133–144	242–262	202–208	133–133	212–233
Reference	Marrone di Viterbo Marrone della Val di Susa Marrone di Chiusa di Pesio	215–223	208–208	226–240	147–153	160–174	127–133	238–242	202–208	133–150	227–233

The international strategy on agrobiodiversity, developed by the Convention on Biological Diversity (CBD) in 2002, is very important for safeguarding local germplasms from several threats, such as specific pathogen attacks and related diseases (e.g., chestnut blight, ink disease, and chestnut gall wasp), and societal changes due to rural abandonment. These topics are crucial to maintaining the specific technological and adaptive traits of local genotypes, such as the 'Marrone di Chiusa di Pesio' chestnut cultivar. By valorizing and preserving these genotypes, the genetic agrobiodiversity of crops is more than protected because they may potentially maintain the ability to adapt their traits to changing environmental conditions and provide important resources for the future [26]. For this reason, the study highlighted the importance of conservation efforts to maintain agrobiodiversity and ensure the sustainability of agri-food systems.

3.2. Visual Tree Assessment (VTA)

The VTA on the 19 MCP samples identified by the DNA characterization defined the morpho-functional conditions of the trees. Table 4 shows the main issues identified in the 19 individuals, describing their morpho-functional conditions.

The VTA showed a low presence of chestnut blight (*Cryphonectria parasitica*) on the 19 MCP trees. However, among the one-hundred-eighty-seven identified trees, ink disease (*Phytophthora cambivora*) appeared to be a major concern as it has caused significant damage both to mature and young MCP individuals, resulting in the death of three accessions in 2018. The tree cores obtained with the Pressler's drill showed that 65% of the specimens were over 50 years old, with the other specimens being around 30 years old. One accession was two years old (ID 101). Based on the data, the average growth rate during the first 10 years of their life was 0.85 mm/year, with a range of 0.50 to 1.4 mm/year. In the following

years, the average growth rate was slightly lower at 0.65 mm/year, with a wider range of 0.35 to 1.9 mm/year. These data suggest that chestnut trees have a moderate growth rate in their early years but may slow down in their adult age, similar to other studies [27–29]. The wide range in growth rates and ages also indicated that environmental conditions may influence the variations among the trees, but further research is needed to fully confirm this hypothesis. It is important to consider the correlation between the geographical distribution of the variation and environmental factors during a Visual Tree Assessment to accurately assess the health and morphological properties of the trees. The results of the present Visual Tree Assessment varied because of several environmental factors, including soil composition, microclimatic conditions, altitude, and orientation. For example, the trees at higher altitudes presented different growth patterns and traits compared to those at lower altitudes, as already reported in previous studies [30,31]. Similarly, areas with types of soil with different levels of access to water and nutrients affected the overall tree health and appearance. Moreover, the climatic conditions (e.g., sunlight, rainfall, and temperature) may have also influenced tree development and growth. The phytosanitary status and the lack of agronomic management also had significant influences, which varied from plant to plant. In general, more than 80% of the MCP chestnut trees dated back to the post-World War II period. Some accessions reached a height of 19 m with a maximum crown diameter of 16 m (Table 5).

Table 4. The main issues identified in the considered 19 samples during the Visual Tree Assessment.

Plant No.	Collar Defects	Trunk Defects	Crown Defects
101	-	-	-
102	-	-	Dieback
103	-	Bifurcation	Dieback
104	Suckers	-	Apical dieback
105	Suckers	Inclination	Reduced crown insertion height
106	-	Inclination; Vines	Dieback
107	-	Inclination	Irregular crown
			Dieback
108	-	Inclination; Healed and/or open wounds	Sparse crown
		Healed wounds	Dieback
109	Suckers	Inclination; Epicormic branches	Dieback; Poor vegetative vigour
110	-	Healed wound	Dieback
111	-	Inclination	-
112	-	-	Dieback; Sparse crown
114	-	Inclination; Healed wound; Cavity	Unbalanced crown
115	-	-	Dieback
116	-	-	Dieback
117	Suckers	Inclination	Apical dieback; Unbalanced crown
			Unbalanced crown
118	-	-	-
119	-	Vines	Dieback
120	-	-	-

In any case, the preliminary results of this study should be confirmed by further analysis. Indeed, the consistency of the VTA may be improved by using more specific criteria for the evaluation of phytosanitary status, vitality, and other tree traits. These assessments may often be subjective; the use of clearer criteria and guidelines can ensure that all trees are assessed more consistently. Further analysis could potentially lead to

more comparable and reliable data, which may be very important to better decide on tree conservation and management efforts. Moreover, more specific criteria may also identify potential areas of concern more easily, enabling effective and more timely interventions.

Table 5. Dendrometric parameters observed for the morpho-functional evaluation of the considered chestnut trees.

Plant No.	Diameter h 1.30 (cm)	Height (m)	Age (Year)	Crown Diameter (m)	Crown Insertion Height (m)
101	3	1.2	2	0.5	0.5
102			25–35		
103	60	9	38	10	180
104	37	10	32	4	185
105	35	10	32		120
106			106		180
107	81	15	74	12	180
108	67	18	57	8	230
109	59	19	63	9	240
110	62	11	67	7	210
111	82	19	97	16	330
112			50+		
114	81	11	50+	10	240
115	64	12	50+	7	280
116	94	15	50+	12	230
117	85	11	50+	8	250
118	38	7	25–30	5	220
119	107	17	76	12	230
120			50+		

Table 6 collects a comparison between the carpological traits and the overall plant characteristics derived from the morphological evaluations (Table 6).

Table 6. Irrigation, morpho-functional conditions, number of fruits per kg, fruit size, and episperm intrusion (expressed by UPOV parameters [15]) of the considered MCP trees in the studied area.

Plant No.	Irrigation	Morpho-Functional Conditions	No. Fruits per kg	Fruit Size	Episperm Intrusion
101	Present	-	-	-	-
102	Present	Good	86	Medium	Weak 1
103	Present	Good	94	Medium	Weak 3
104	Present	Good	78	Large	Weak 1
105	Present	Good	81	Medium	Weak 1
106	Present	Good	73	Large	Weak 2
107	Present	Fair	86	Medium	Weak 3
108	Present	Mediocre	83	Medium	Weak 3
109	Present	Mediocre	102	Small	Weak 4
110	Present	Fair	77	Large	Weak 3

Table 6. *Cont.*

Plant No.	Irrigation	Morpho-Functional Conditions	No. Fruits per kg	Fruit Size	Episperm Intrusion
111	Absent	Good	87	Medium	Strong
112	Present	Good	86	Medium	Weak 3
114	Absent	Fair	104	Small	Weak 1
115	Present	Good	82	Medium	Weak 1
116	Present	Good	89	Medium	Weak 2
117	Absent	Mediocre	101	Small	Weak 3
118	Present	Excellent	76	Large	Weak 1
119	Present	Good	79	Large	Weak 1
120	Present	Good	74	Large	Weak 2

The pedoclimatic and agronomic conditions in the studied area were homogeneous, except for trees 111, 114, and 117, which were not irrigated. Sample 101 did not bear any fruit because of the young age of the plant. The average fruit size was medium to large (84 fruit/kg). Only three samples presented more than 100 fruit/kg, apparently related to the possibility of emergency irrigation. Samples 114 and 117, on the other hand, experienced stress, probably due to a lack of irrigation, resulting in stunted and poor-quality production. All the other samples showed positive correlations between the overall health status indicated by the VTA and the fruit traits. These results suggest that the poor vegetative conditions of the plants accentuate the penetration of the episperm into the cotyledon, sometimes leading to a complete partition of the fruit.

3.3. Nutraceutical Analysis

Polyphenols are the main group of biologically active non-nutrients in food, characterized by antioxidant, antibacterial, and anticancer properties [32]. Various screening methods, both biological and chemical, have been validated to determine the antioxidant capacity of chestnuts. Among these, the FRAP test used in this research is one of the methods employed. The phytochemical and nutritional traits, enriched by the evaluation of the antioxidant capacity of six MCP subgroups, were established by chemical analysis. The total polyphenol content (TPC) is presented in Table 7, showing significant variability among the analyzed samples, probably due to several environmental factors that caused different stress levels among the plants considered. The values recorded ranged from 36.51 ± 1.60 mgGAE/100 gDW to 103.14 ± 1.24 mgGAE/100 gDW in samples 111 (whose trees were not irrigated) and 116, respectively.

Table 7. Total polyphenol content (TPC) and antioxidant capacity (AOC) of the analyzed samples.

ID	Total Polyphenol Content (TPC) (mgGAE/100 g DW)	Antioxidant Activity (AA) (mmol Fe2+/kg DW)
104	77.20 ± 0.69^{d}	21.41 ± 2.73^{a}
111	36.51 ± 1.60^{a}	18.15 ± 2.04^{a}
112	42.33 ± 1.18^{b}	19.14 ± 2.09^{a}
115	49.34 ± 3.35^{c}	20.70 ± 5.90^{a}
116	103.14 ± 1.24^{d}	23.88 ± 2.96^{a}
120	73.28 ± 1.24^{d}	21.40 ± 2.23^{a}

Different letters for each sample indicate statistically significant differences at $p < 0.05$.

The content of polyphenols, vitamins, monoterpenes, and organic acids contribute, among other factors, to the antioxidant capacity (AOC). These antioxidants, which elimi-

nate free radicals, prevent loss of function and damage to biological membranes, detoxify enzymes, maintain health, and reduce the risk of diseases [33]. Previous research by Neri and colleagues [34] showed that 'Marrone di Chiusa Pesio' presented significantly higher TPC amounts than other 'Marrone'-type cultivars. 'Marron Buono Di Marradi', for instance, showed TPC values of 10.1 ± 0.6 mgGAE/100 gDW, lower than the data collected for the MPC (Table 7). In this study, the MCP extracts also showed good antioxidant capacity, with a maximum value of 23.88 ± 2.69 mmol Fe^{2+}/kgDW and a minimum value of 18.15 ± 2.04 mmol Fe^{2+}/kgDW. These values were compared with those obtained by Neri and colleagues [34], which quantified antioxidant properties ranging from 4.77 ± 0.34 mmol Fe^{2+}/KgDW to 8.15 ± 0.16 mmol Fe^{2+}/KgDW. Another study obtained an AOC ranging from 9.30 ± 0.39 mmol Fe^{2+}/kg DW ('Bouche de Bètizac') to 19.96 ± 1.89 mmol Fe^{2+}/kgDW ('Garrone Rosso'). Nevertheless, establishing the contribution of each single bioactive compound to the total antioxidant activity may be difficult because of the synergistic combination and interaction between the different substances (phytocomplex). Each antioxidant compound may improve the effectiveness of the others, influencing the overall response (total antioxidant capacity) [35].

The phenolic compounds, selected for their phytochemical properties, were categorized as follows to determine their contribution to the total polyphenolic composition (Table 8): cinnamic acids (ferulic, caffeic, chlorogenic, and coumaric acids), benzoic acids (gallic and ellagic acids), catechins (catechin and epicatechin), tannins (vescalagin and castalagin), and flavonols (isoquercitrin, hyperoside, rutin, quercetin, and quercitrin).

Table 8. The phenolic fingerprint of the analyzed samples.

ID	Cinnamic Acids (mg/100 gDW)	Benzoic Acids (mg/100 gDW)	Catechins (mg/100 gDW)	Tannins (mg/100 gDW)	Flavonols (mg/100 gDW)
104	23.32 ± 1.66	2.92 ± 0.74	9.19 ± 0.48	27.62 ± 3.26	16.99 ± 1.84
111	23.86 ± 1.95	2.87 ± 0.58	9.29 ± 0.92	27.57 ± 6.55	17.34 ± 1.93
112	23.16 ± 1.42	2.73 ± 0.19	9.59 ± 0.32	26.99 ± 3.04	16.95 ± 1.90
115	23.53 ± 0.84	3.28 ± 0.09	9.94 ± 0.34	23.58 ± 1.94	17.61 ± 2.90
116	24.09 ± 1.27	3.20 ± 0.15	9.20 ± 0.73	26.89 ± 1.46	17.07 ± 1.89
120	23.67 ± 0.34	2.80 ± 0.33	8.89 ± 0.46	22.97 ± 2.53	17.41 ± 3.07

Tannins enhance the health-promoting properties of chestnuts as these molecules are effective at neutralizing free radicals [36]. Table 8 shows that tannins were the predominant class of polyphenols in the MCP chestnut extracts. Tannins are important compounds for human health due to their antioxidant, antimicrobial, chemopreventive, and cardioprotective properties [37]. Among this class, the main ones detected were hydrolysable ellagitannins: vescalagin, ranging from 17.74 ± 1.73 mg/100 gDW (ID 120) to 22.67 ± 6.29 mg/100 gDW (ID 111), and castalagin, with values between 5.30 ± 1.02 mg/100 gDW (ID 112) and 4.80 ± 1.37 mg/100 gDW (ID 116). Table S1 in the Supplementary Materials reports the values of the single compounds investigated. The study by Araújo et al. [38] confirmed the importance of these two molecules as antimicrobial compounds. The concentrations reported in this study were higher than the ones obtained in other 'Marrone'-type cultivars analyzed in previous studies, such as 'Marrone di Marradi' (3.6 ± 0.3 mg/100 gDW), 'Marrone della Valle di Susa' (19.2 ± 2.0 mg/100 gDW), and 'Marrone di Castel de Rio' (23.2 ± 1.6 mg/100 gDW) [35].

Cinnamic acids were the second predominant class, known for their anti-inflammatory, antioxidant, neuroprotective, anticancer, antimicrobial, and antidiabetic properties [39]. The results obtained in this study were slightly higher than the values reported in other studies (Table 8) [35]; in particular, chlorogenic acid reported the highest values among these phenolic acids, ranging from 14.10 ± 0.57 mg/100 gDW (ID 120) to 14.62 ± 1.17 mg/100 gDW (ID 111) (Table S1). Chlorogenic acid, in addition to the properties common to the class as a

whole, plays a crucial role in the regulation of lipid and glucose metabolism, contributing to weight loss and the prevention of obesity-related diseases [40]. Coumaric acid was the second most abundant compound in the class of cinnamic acids, with the amounts ranging from 6.64 ± 0.75 mg/100 gDW (ID 112) to 7.07 ± 0.23 mg/100 gDW (ID 115). Ferulic and caffeic acids were also quantified in all the samples. However, the concentrations were lower than <2 mg/100 gDW (Table S1).

The flavonol class, the third most abundant in MCP samples (Table 8), appears to be closely linked to the prevention of neurodegenerative diseases caused by oxidative damage in the central nervous system and cardiovascular diseases, such as atherosclerosis [41]. This class is also excellent at inhibiting in vitro low-density lipoprotein oxidation and quenching active oxygen species. The most representative flavonol was isoquercitrin, which was detected in all the samples at good levels from 12.61 ± 1.07 mg/100 gDW (ID 112) to 13.01 ± 1.78 mg/100 gDW (ID 120), followed by hyperoside, which showed values between 1.48 ± 0.41 mg/100 gDW (ID 116) and 1.87 ± 0.81 mg/100 gDW (ID 120). The values of rutin ranged from 1.22 ± 0.22 mg/100 gDW (ID 104) to 1.59 ± 0.17 mg/100 gDW (ID 111) (Table S1). Significant statistical differences were detected in quercetin among the samples, with values ranging from 0.59 ± 0.17 mg/100 gDW (ID 111) to 0.85 ± 0.07 mg/100 gDW (ID 112), and in quercitrin, with values ranging from 0.29 ± 0.09 mg/100 gDW (ID 120) to 0.78 ± 0.09 mg/100 gDW (ID 115) (Table 9). Comparing the values obtained in this study with the findings of Beccaro et al., 2020, 'Marrone di Chiusa Pesio' had a higher content of flavonols than the European–Japanese hybrids (from 4.4 ± 0.2 mg/100 gDW in 'Precoce Migoule' to 8.9 ± 0.7 mg/100 gDW in 'Bouche de Bétizac') and some *C. sativa* cultivars (0.5 ± 0.1 mg/100 gDW in 'Mansa' or 1.1 ± 0.0 mg/100 gDW in 'Bouche Rouge'). On the other hand, it showed comparable values with the cultivars 'Gabiana' (17.3 ± 1.1 mg/100 gDW), 'Marrubia' (17.1 ± 0.8 mg/100 gDW), and 'Marrone di Castel del Rio (16.3 ± 0.8 mg/100 gDW) [35].

Table 9. Main phenolic compounds in the flavonol class with the potential to discriminate the considered samples. Different letters for each sample indicate statistically significant differences at $p < 0.05$.

ID	Quercetin (mg/100 g DW)	Quercitrin (mg/100 g DW)
104	0.70 ± 0.04 ^{ab}	0.64 ± 0.04 ^{b}
111	0.59 ± 0.17 ^{a}	0.72 ± 0.16 ^{b}
112	0.85 ± 0.07 ^{b}	0.51 ± 0.17 ^{ab}
115	0.68 ± 0.10 ^{ab}	0.78 ± 0.09 ^{b}
116	0.84 ± 0.03 ^{b}	0.58 ± 0.08 ^{ab}
120	0.69 ± 0.07 ^{ab}	0.29 ± 0.09 ^{a}

Different letters for each sample indicate statistically significant differences at $p < 0.05$.

Benzoic acids have anti-inflammatory, anticancer, anti-HIV, anti-atherosclerotic, and anti-hepatotoxic properties, being important compounds in the human diet [42]. Among the benzoic acids considered, gallic acid was identified (from 1.57 ± 0.16 mg/100 gDW in sample 116 to 1.91 ± 0.30 mg/100 gDW in sample 115) and quantified at higher levels than ellagic acid (from 1.02 ± 0.15 mg/100 gDW in sample 120 to 1.63 ± 0.16 mg/100 gDW in sample 116) (Table S1). According to Beccaro et al., 2020, both molecules were more abundant in MCP than in other chestnut cultivars. Ellagic acid in particular showed higher values than other *C. sativa* cultivars (<0.9 mg/100 gDW in 'Marrone di Castel del Rio', 'Marrone di Marradi' IGP, and 'Marrone della Val di Susa', <0.9 mg/100 gDW in 'Garrone Rosso', 'Madonna', and 'Mansa'; <0.3 mg/100 gDW in 'Neirana della Val di Susa' and 'Tarvisò') and the main Euro–Japanese hybrid (<0.2 mg/100 gDW in 'Bouche de Bétizac'). In any case, some of these cultivars (e.g., 'Marrone di Castel del Rio', 'Marrone di

Marradi', 'Marrone della Val di Susa', etc.) were reported as synonymous with 'Marrone Fiorentino' [25]. Gallic acid was only slightly higher than in some varieties of sweet chestnut (<1.0 mg/100 gDW in 'Bouche Rouge', 'Garrone Rosso', 'Gentile', 'Mansa', and 'Neirana della Val di Susa') [35].

The identification of catechins, represented by epicatechin and catechin, was an important result since they inhibit the proliferation of human cancer cell lines and the enzyme cyclooxygenase, and they also inhibit lipid peroxidation [35]. Epicatechin showed the highest values, ranging from 7.90 ± 0.45 mg/100 gDW (ID 116) to 8.68 ± 0.20 mg/100 gDW (ID 115), while the catechin amounts were lower than <2 mg/100 gDW (Table S1). These low concentrations may be due to the peeling process during extract preparation [43] as catechins are mainly found in the epicarp and episperm [43]. This is confirmed by the study of Beccaro et al., 2020 [35] (catechin between 0.11 ± 0.03 mg/100 gDW and 3.32 ± 0.09 mg/100 gDW; epicatechin between 1.24 ± 1.07 mg/100 gDW and 15.32 ± 3.56 mg/100 gDW), which reported the same preparation method as in the present research.

Monoterpenes, a broad group of naturally occurring bioactive compounds, are extensively used for their aromatic properties as well as their antioxidant and anti-inflammatory benefits. Many molecules in this class exhibit antibacterial and antitumor activity [44,45]. The levels of monoterpenes were very similar among the samples (from 263.94 ± 10.23 mg/100 gDW in sample 115 to 284.00 ± 8.56 mg/100 gDW in sample 116), and no statistically significant differences were detected among the single compounds (Table 10). Terpinene was the most abundant compound, with a maximum value of 147.75 ± 1.93 mg/100 gDW (ID 120) and a minimum of 152.55 ± 5.27 mg/100 gDW (ID 112). Limonene was another monoterpene quantified in high amounts (from 78.40 ± 7.13 mg/100 gDW in sample 111 to 98.44 ± 4.09 mg/100 gDW in sample 116). Terpinolene, sabinene, and phellandrene showed lower maximum values than the other terpenes (9.21 ± 0.69 mg/100 gDW, 15.65 ± 2.04 mg/100 gDW, and 12.87 ± 1.18 mg/100 gDW, respectively) (Table S1). The amounts of monoterpenes obtained in this study were comparable with those of 'Marrone di Castel de Rio' (158.1 ± 5.4 mg/100 gDW), 'Marrone di Marradi' (119.9 ± 19.3 mg/100 gDW), and 'Marrone della Valle di Susa' (281.2 ± 45.4 mg/100 gDW). Compared to some *C. sativa* cultivars, such as 'Mansa' (45.7 ± 4.5 mg/100 gDW), 'Bouche Rouge' (64.3 ± 15.8 mg/100 gDW), and 'Tarvisò' (95.8 ± 34.3 mg/100 gDW), the MCPs showed significantly higher values [35].

Table 10. Content of other bioactive and nutritional compounds detected in the analyzed samples.

ID	Monoterpenes (mg/100 g DW)	Organic Acids (mg/100 g DW)	Vitamin C (mg/100 g DW)	Sugars (g/100 g DW)
104	269.29 ± 1.62	317.18 ± 2.32	11.46 ± 0.58	4.14 ± 0.68
111	264.92 ± 7.31	318.79 ± 7.11	11.73 ± 1.55	4.29 ± 0.49
112	282.17 ± 5.23	330.76 ± 9.94	11.77 ± 0.58	4.57 ± 0.35
115	263.94 ± 10.23	328.33 ± 4.44	11.88 ± 1.51	4.25 ± 0.06
116	284.00 ± 8.56	322.39 ± 7.65	12.21 ± 1.05	4.03 ± 0.67
120	276.82 ± 10.02	319.32 ± 4.65	11.77 ± 0.67	4.10 ± 0.54

Organic acids, ranging between 317.18 ± 2.32 mg/100 gDW (ID 114) and 330.76 ± 9.94 mg/100 gDW (ID 112) (Table 10), are antioxidants sometimes used in pharmacological experiments, as various studies have shown. These substances, together with fiber, can help to maintain a healthy digestive system [46]. They increase the bioavailability of mineral elements (e.g., iron and calcium) in the diet. In particular, high levels of quinic acid, the most prevalent in MCP samples, may be metabolized to hippuric acid, which is useful to alleviate infections in the urinary tract [47], whereas citric acid plays an important role in regulating the functioning of the urinary tract by inhibiting the adhesion of calcium oxalate crystals to renal epithelial cells [48]. The values ranged from 168.36 ± 1.08 mg/100 gDW (ID 111) to 176.07 ± 4.02 mg/100 gDW (ID 112) for quinic acid

and from 107.23 ± 10.95 mg/100 gDW (ID 112) to 95.75 ± 3.10 mg/100 gDW (ID 104) for citric acid (Table S1).

Vitamin C was assessed as the sum of dehydroascorbic and ascorbic acids because of their biological activity in human bodies. Its primary molecular role is as an antioxidant, and deficiencies in vitamin C can lead to conditions such as scurvy and anemia [49]. According to Prezzi et al. [50], the vitamin C content varied greatly depending on the species and the cultivar, with values ranging from 9.45 ± 0.03 mg/100 gDW for *Castanea pumila* to 7.40 ± 0.26 mg/100 gDW for the Japanese cultivar 'Ishizuki Precoce', and from 3.82 ± 0.06 to 12.32 ± 0.01 mg/100 gDW for the Euro–Japanese hybrid, from 1.12 ± 0.31 to 6.87 ± 0.09 mg/100 gDW for the *C. sativa* chestnut-type cultivar, and from 2.26 ± 0.32 to 7.62 ± 0.41 mg/100 gDW for the 'Marrone'-type chestnut. The analyzed MCPs had a higher range of vitamin C values, with a minimum of 11.46 ± 0.58 mg/100 gDW (ID 104) and a maximum of 12.21 ± 1.05 mg/100 gDW (ID 116) (Table 10). The results showed that MCPs are an excellent source of vitamin C, providing between 14.33% and 15.26% of the Reference Daily Intake (RDI) for women (RDI 80 mg/day) and between 12.73% and 13.57% for men (RDI 90 mg/day) [51].

The samples showed no statistically significant differences in terms of sugar content. Sucrose was the most representative sugar in the MCP samples, with values between 1.83 ± 0.13 g/100 gDW (ID 116) and 2.26 ± 0.16 g/100 gDW (ID 111), an important parameter for assessing the commercial potential [52]. Glucose and fructose were always detected in the extracts analyzed, with concentrations ranging from 0.96 ± 0.09 g/100 gDW (ID 111) to 1.43 ± 0.26 g/100 gDW (ID 112) and from 0.78 ± 0.24 g/100 gDW (ID 116) to 1.12 ± 0.36 g/100 gDW (ID 115), respectively (Table S1).

3.4. Multivariate Analysis

The therapeutic effects of consuming fresh fruit and the derived products result from the synergistic or additive interaction of multiple phytochemicals that collectively contribute to disease prevention [41].

The following multivariate analysis was therefore performed to simultaneously investigate the presence of multiple molecules and quantify their values in the analyzed extracts, providing a tool for evaluating synergistic effects rather than relying on the action of a single compound. For improved recognition of the analyzed extracts, a chemometric approach was applied, coupled with the HPLC fingerprint technique, as reported by Cirlini et al. [42]. The combination of chromatographic fingerprinting and chemometric evaluation confirmed that all the samples showed a similar phytochemical composition in relation to the same genotype described by previous genetic analyses. This combined approach may be a potential effective tool for the traceability and quality control of chestnut products, thus obtaining a label certification, to select the best raw material based on the desired characteristics and properties, valorizing the local genotypes and preserving biodiversity. The multivariate strategy enabled the collection of several variables. To obtain an overview of the nutraceutical value of the chestnuts derived from the selected groups of plants and to analyze the potential relationships between the samples, a Principal Component Analysis (PCA) was carried out. Compounds belonging to the same chemical class were grouped into bioactive classes for the multivariate analysis of the data. Significant collinearity between the variables was indicated by Bartlett's sphericity test ($p < 0.05$). In this study, the Bartlett's sphericity test value was 0.04 and the KMO index was 0.54. The four principal components (PCs) accounted for 68% of the total variance (PC1 to PC4, with contributions of 24.3%, 17.3%, 14.4%, and 12.0%, respectively), confirming what was explained in the Materials and Methods. Moreover, to obtain an easily interpretable graph, the components were simplified to the two main PCs, retaining approximately 50% of the total variance.

The score plot (Figure 1) showed the positions of the six samples in the PC plane (mean values of three replicates for each sample) in relation to their nutritional properties, nutraceutical traits, and phytochemical compositions. Looking at the graph of the PCA scores (Figure 1), the distribution of the samples was very heterogeneous and did not allow

them to be classified into statistical groups, confirming the results of the ANOVA tests performed both on the classes of compounds and the individual molecules. Therefore, a further PCA was performed by taking the mean values of the samples (N = 3). The obtained graph enabled approximating them into defined no-statistical groups (Figure 2): samples 111, 112, 116, and 120 were distributed close to the horizontal axis corresponding to PC1. In particular, samples 111 and 112 resulted on the left part of the plot, while samples 116 and 120 were on the right part. This distribution is associated with the content of catechins and flavonols (increasing to the left) and the content of monoterpenes and vitamin C (increasing to the right). Instead, samples 104 and 115 were vertically distributed (PC2).

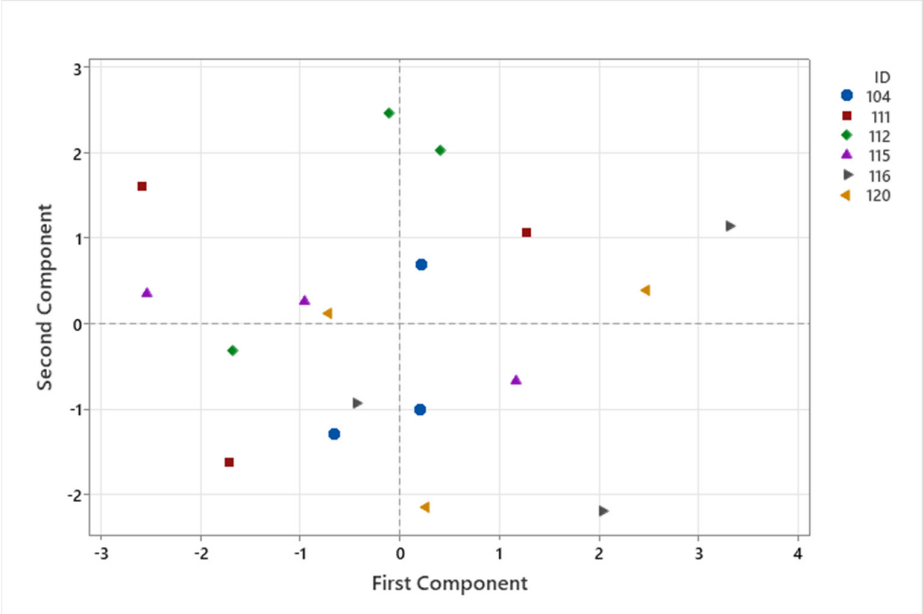

Figure 1. PCA score plot of the MCP samples.

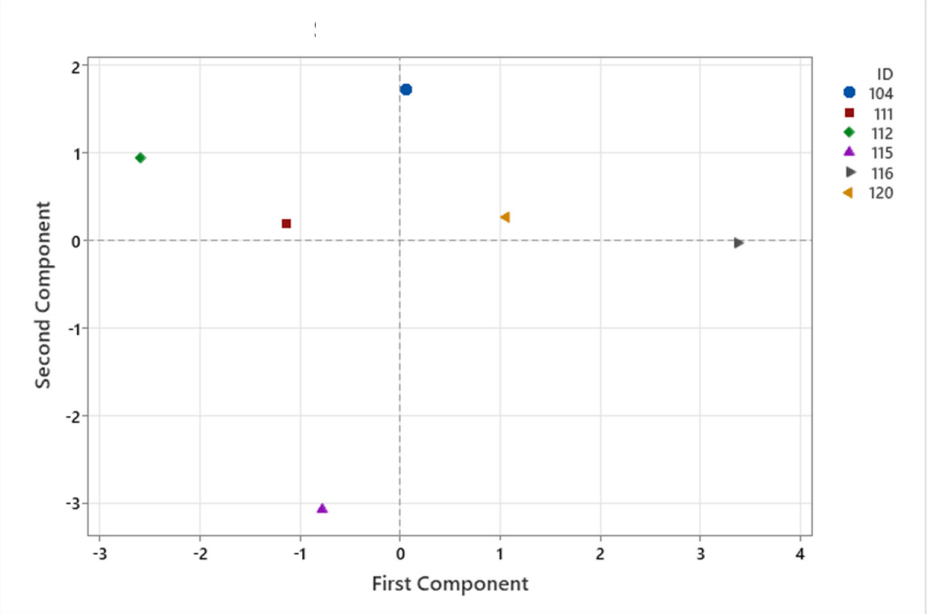

Figure 2. Mean PCA score plot of the considered MCP samples. Mean values (n = 3) were included for all the samples.

The phytochemical classes are represented as vectors in the PCA loading plot (Figure 3). PC1 showed a correlation with the content of monoterpenes, vitamin C, catechins, and flavonols, while PC2 was related to organic acids and benzoic acids.

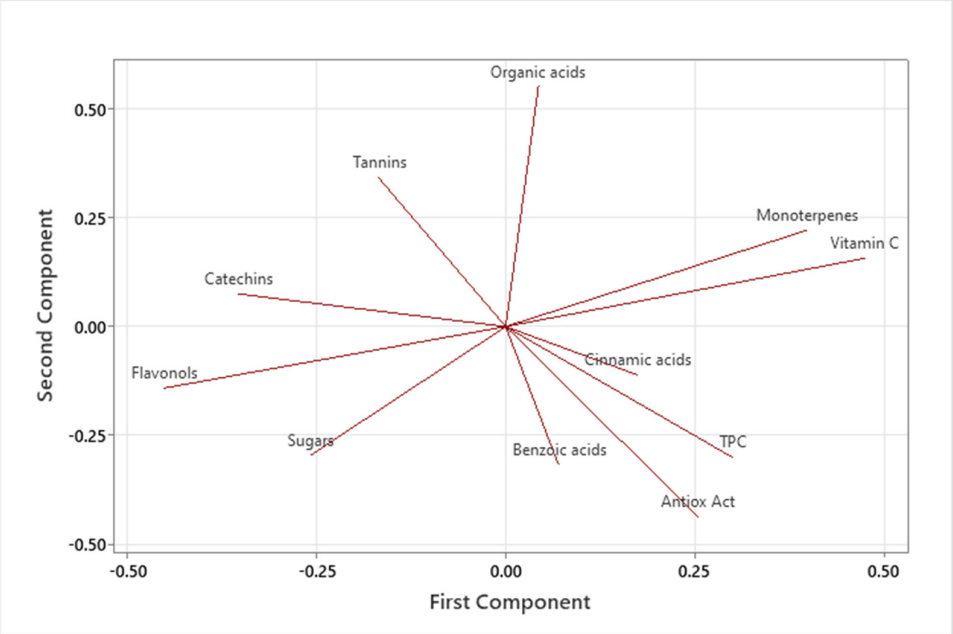

Figure 3. PCA loading plot of the considered variables.

The loading plot showed that monoterpenes, vitamin C, flavonols, and catechins are related to each other by competing in the composition of the PC1 and revealing correlations with samples 111, 112, 116, and 120, although with different amounts of each specific molecule. The same is observed for PC2, where organic and benzoic acids are each related, and with samples 104 and 115. For better 2D visualization, PC3 has been projected into the two-dimensional plot, although it represents a third dimension of variation. This action facilitated a comprehensive understanding of the data structure by enabling the contribution of the original variables to PC3 within the same plot. In this way, the correlation of the TPC and AOC with PC3 was observed. The correlation between the TPC and AOC was tested and confirmed by the application of the Pearson Correlation Coefficient (PCC). For the calculation, the TPC and antioxidant capacity values of the six samples were considered as replicates of a single sample as the genetic analysis has confirmed that the samples were genetically identical. The resulting correlation coefficient was 0.52, indicating a good correlation between the two variables. The TPC–AOC correlation was limited since antioxidant capacity depends not only on polyphenol content but also on vitamin C, monoterpenes, and organic acids. Moreover, the Folin–Ciocalteu assay is an aspecific test for polyphenols, as reported in several studies [16,19]. This result confirmed the outcome of the nutraceutical analysis.

4. Conclusions

The peculiarity of this research consists of undertaking an interdisciplinary approach to the characterization of plant accessions, from a tree assessment to a fruit chemical characterization. The multidisciplinary approach has enabled an overall assessment of the conservation status and the potential related to the valorization of the 'Marrone di Chiusa Pesio' cultivar. The genetic analysis performed in this study confirmed that the considered plants belonged to the same genotype. The identified trees were subjected to a Visual Tree Assessment (VTA) and carpological analysis to evaluate their overall vegetative status and properties. These analyses revealed that most individuals exhibited excellent

morpho-functional conditions. Moreover, the data collected regarding the fruits confirmed a medium–high quality level in most of the UPOV parameters.

The phytochemical analysis demonstrated that MCP presented significantly higher TPC values compared to other 'Marrone'-type cultivars, even if most of them were synonymous with 'Marrone Fiorentino', together with high levels of the most important phenolic markers and other bioactive compounds and nutritional substances. Moreover, a PCA on the phytochemical parameters was carried out to confirm the univariate statistical results and observe the distribution of the considered samples according to their chemical composition. PC1 showed a correlation with the content of monoterpenes, vitamin C, catechins, and flavonols, while PC2 was related to organic and benzoic acids. No statistical groups were identified, but four samples (111, 112, 116, and 120) were mainly correlated with PC1, while the other two (104 and 115) were correlated with PC2.

Given the ongoing genetic erosion of *C. sativa* cultivars, due to cultivation abandonment and climate change, the main factors contributing to the progressive loss of biodiversity worldwide, the presented approach aimed to provide an overview of the conservation status of the local agrobiodiversity. This study highlighted the value of a local chestnut cultivar with high nutritional properties and market potential, emphasizing the low conservation status of the few remaining specimens.

The goal was to define the significant phenotypic variation regarding MCP in the considered area due to environmental variations, which may be of interest in its genetic adaptation to climate change. The study may potentially encourage the development of strategies for actively conserving the forest agrobiodiversity and hillside ecosystem services in the highly diverse landscapes of the Alpine valleys.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/d16120711/s1, Table S1: Levels of bioactive and nutritional compounds in analyzed 'Marrone di Chiusa Pesio' samples. Figure S1. Geographical distribution of the analyzed trees of 'Marrone di Chiusa Pesio'.

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