Molecular, Chemical and Morphological Tools to Explore *Vertzami* / *Marzemino* /*Barzemino* / *Balsamina* Cultivar Group

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Keywords: ampelography, anthocyanin profile, biodiversity, grapevines, SSR and AFLP markers.

Abstract

The relationships within the *Vertzami* / *Marzemino* /*Barzemino* / *Balsamina* group were investigated with the aim to recover intra-variety biodiversity. *Vertzami* is a variety grown in Lefkas (a Greek Ionian island) considered to be very close to *Marzemino*, which is grown in north-eastern Italy as well as *Barzemino* and *Balsamina*, which at the present are germplasm relics. More than fifty accessions were sampled including: *Vertzami* from different sites in Lefkas; *Marzemino* from different localities and germplasm collections; *Barzemino* from germplasm collection or from old and marginal vineyards; *Balsamina* from germplasm collections. Accessions were analysed by molecular (SSR and AFLP markers), chemotaxonomic (anthocyanin profile of berry skin) and morphological (phyllometry) methods. Results showed that *Vertzami* / *Marzemino-Barzemino* / *Balsamina* are different varieties even if phylogenetically related. *Vertzami* accessions did not show any genetic diversity and very low phenotypical differences. *Marzemino* accessions displayed a minor genetic and phenotypic diversity. Results support the idea that *Vertzami* is a distinct member of this cultivar family, which had origin in Italy and that was introduced in Lefkas by Venetians during their dominion of the Ionian islands (XIV-XVIII centuries).

INTRODUCTION

The definition of the genetic relationship among related grapevine cultivars is an intriguing question which involves several historical, biological and viticulturist aspects. The retrospection of the variety flow through the countries and the definition of the genetic structure of a cultivar group are essential to elaborate an effective programme to explore and maintain bio-diversity and to recover minor accessions as a basis for the enrichment of our cultivar assortments.

Marzemino is a variety which was more important in the past then at the present: it is one of the most ancient Italian varieties, cited as one of the best quality grapes grown in north-eastern and central Italy since the twelfth century (Scienza et al., 1997). According to the Greek ampelographers Logothetis and Vlachos (1967), *Vertzami* is a synonym or a close related variety of *Marzemino*.

Nowadays *Marzemino* is confined to small areas (less than 500 ha) in Trento and Brescia districts (north eastern Italy), while *Vertzami* is mainly grown in Lefkas (ca. 500 ha), a Greek Ionian island. Moreover it is sporadically present in western Greece and, with the synonym of *Leukas*, in Cyprus.

Marzemino suffers for a narrow intra-variety variability which seems to limit its quality potential with respect to the levels that is supposed it reached in the past. For this reason, an exploration project, to develop a strategy for the recovering of its intra-variety biodiversity was developed. To maximise the results, the possible origin centre and the

secondary diversity zones of the variety group were included in the exploration. In particular different Italian zones and the island of Lefkas were surveyed.

MATERIAL AND METHODS

Germplasm collection. More than fifty accessions were sampled including:

- different accessions of *Vertzami* from different sites in Lefkas;
- different accessions and/or putative clones or mutations of *Marzemino* from different localities and germplasm collections;
- different accessions and putative clones of *Barzemino* (a Lombard presumed synonyms of *Marzemino*) from germplasm collection or from old and marginal vineyards;
- two different *Balsamina* (another presumed synonyms of *Marzemino*) accessions from germplasm collections.

Ampelographic description. *Marzemino* and *Vertzami* accessions grown in the same field collection were comparatively described according to OIV descriptors (OIV-IPGRI-UPOV, 1983).

Phyllometric description. Leaf morphology was delineated by the method described in Schneider and Zeppa (1988).

DNA extraction. Young leaves (1-2 cm long) were harvested from rooted cuttings. These were frozen in liquid nitrogen and ground to a fine powder. Genomic DNA was extracted as described by Labra et al. (2001).

SSR analysis. DNA was analysed at the following 13 microsatellite *loci*: VVS2, VVS4, (Thomas and Scott 1993), VVMD5, VVMD6, VVMD7, (Bowers et al. 1996), VVMD17, VVMD21, VVMD24, VVMD25, VVMD27 VVMD28, VVMD31, VVMD34 (Bowers et al., 1999).

The analysis was performed by adding 15 ng of genomic DNA to a 20 µl PCR mixture containing 10 ng of the DNA primer specified for each microsatellite locus, 200 µM of each of the 4 dNTPs, 0.5 U Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. The forward primers were end-labelled with α^{33}P ATP (Amersham, Italy). PCR amplification was performed with a programmable thermal controller (PTC 100, MJ Research Inc., USA) using the following profile: 7 min at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 52 °C) and extension (1 min at 72 °C); then a final step for 7 min. at 72 °C.

AFLP analysis. AFLP was performed as described in Labra et al. (1999), except that genomic DNA (200 ng) was digested (3 h) with *Eco*RI (0.5 U) and *Mse*I (0.5 U) and ligated with EcoRI adapter (5 pmol) and MseI adapter (50 pmol). Primer pairs used in the pre-amplification reaction were M01 and E01, while the four pairs of primer E31-M32, E32-M36 and E33-M38 were used for the amplification reaction. Results were confirmed by repeating the analysis for a small number of samples.

Analysis of the DNA amplification products. In the case of the SSR analysis, 10 µl of the PCR-amplified mixture was added to 2 µl of loading buffer (80% formamide, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue, 10 M EDTA, pH 8.0) and a total of 3 µl was analysed by electrophoresis on a 4.5% sequencing polyacrylamide gel and electrophoresed in TBE electrophoresis buffer (50 mM boric acid, 1 mM EDTA, pH 8.0) for 3 h at 80 Watt. The gel was fixed in 10% acetic acid and exposed to an X-ray film. Visual inspection of the resulting autoradiograms allowed scoring of microsatellite bands. Allele sizes were determined using a Gel Doc 2000 (Biorad, USA).

In the case of AFLP, 1.5 µl of the PCR-amplified mixture was added to an equal volume of loading buffer (80% formamide, 1 mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue,10 M EDTA, pH 8.0), denatured for 5 min at 92 °C, and analysed by electrophoresis using a PAGE gel as described above.

Anthocyanins analysis. The skins from 20 frozen berries were extracted in two phases for 12 and 2 hours, with 100 and 50 ml methanol respectively. The total extract was filtered to remove plant debris, evaporated to dryness in a rotary evaporator at 37°C

and dissolved in acqueous 0,3% perchloric acid - methanol (73:27 v/v). Anthocyanin profile was determined at 520 nm using a Shimadzu HPLC LC-10 AD (Shimadzu Co., Tokyo, Japan) connected to a Shimadzu UV-VIS detector SPD-10 A according to Mattivi (1998). The extracts were analysed by HPLC under the following conditions: flow rate 0.45 ml/min; temperature 40°C; column Purospher RP18, 5 µm (250 x 4 mm) preceded by a guard column Purospher RP18, 5 µm (4 x 4 mm) (Merck, Darmstadt, Germany); solvent A: methanol; solvent B: aqueous 0,3% perchloric acid – Elution: linear gradient from 27% to 43% A in 32 minutes, from 43% to 68,5% in 13 minutes, from 68,5% to 100% in 2 minutes, then isocratically with 100% A for 3 minutes; re-equilibrating time: 5 minutes; loop: 10 µl. A calibration curve was established using malvidin 3-monoglucoside and results were expressed as "malvidin 3-monoglucoside equivalent".

The anthocyanin profiles were outlined as relative levels of Delphinidin 3-monoglucoside; Cyanidin 3-monoglucoside; Petunidin 3-monoglucoside; Peonidin 3-monoglucoside; Malvidin 3-monoglucoside; summation of their acetic and p-coumaric esters.

Statistical analysis. Each microsatellite allele or AFLP band was scored as a binary character for its absence (0) or presence (1). In the case of microsatellite analysis, presence was scored as (1) independently for the heterozygous or homozygous state. The resulting data were analysed using the software programme Genstat 5. Similarity-dissimilarity matrices were computed with the Jaccard's coefficient (JC): JC = a/(n-d) where: a = bands present in both compared genotypes; n = total number of polymorphic bands; d = bands absent in both compared genotypes. The final products were subjected to a cluster analysis using UPGMA (unweighted pair-group method with arithmetical averages) and a dendrogram was drawn.

Chemotaxonomic and phyllometric data were processed by multivariate analysis as described in the Results section. Data were analysed using SPSS (V. 11.0) statistical programme.

RESULTS

According to 96 OIV descriptors, *Marzemino* and *Vertzami* resulted very similar, even if some differences were detected. In particular *Marzemino* showed in the apical leaflets of the young shoot apex a higher density of prostrate hairs and a more intense bronze colour. The mature leaves of *Marzemino* resulted larger in size, with lobes and ondulation more evident than leaves of *Vertzami*.

The morphological differences were confirmed by a more accurate phyllometric analysis which showed that *Vertzami* and *Marzemino* leaves can be discriminated according to their size and shape. *Vertzami* had smaller leaves, more roundish and with a wider sinus angle. Also *Barzemino* leaves were discriminated from the other two groups of accessions. Its leaf characteristics resulted intermediate between *Vertzami* and *Marzemino* (Fig. 1).

The anthocyanin profiles of *Marzemino*, *Barzemino* and *Vertzami* were relatively similar. They were characterised by a prevalence of Malvidin 3-monoglucoside, but significant differences were detected with respect to all the components of the profiling (Tab 1). However the most discriminator factor among the putative varieties resulted to be the Peonidin 3-monoglucoside vs. Delphinidin 3-monoglucoside ratio (Fig. 2).

When the accessions were tested by AFPL markers, *Vertzami* and *Marzemino* were clearly separated, even if no genetic variability was detected both within *Vertzami* and *Marzemino*. *Barzemino*, the two *Balsamina* accessions and *Barzeminone* were univocally detected. *Marzemino bianco*, which was not separated by the other *Marzemino* accessions, resulted to be a point mutation of *Marzemino* (Fig. 3). These results were sensibly confirmed by microsatellite profiles (Tab. 2). Moreover they indicated that *Marzemino* and *Vertzami*, sharing 15 out of 26 alleles, are closely related varieties. No differences in SSR profiling were detected between *Marzemino* and *Barzemino*. The two *Balsamina* accessions were slightly different, in fact they differed for one allele. Nevertheless they resulted closely related to *Marzemino*. *Marzemina bianca* resulted to be

a distinct genotype even if strictly related to *Marzemino* (21 common alleles out of 26). *Barzeminone* resulted a distinct genotype, at an intermediary distance between *Marzemino* and *Vertzami*.

DISCUSSION AND CONCLUSION

On the whole, taking into account morphological characters, chemotaxonomic traits and molecular markers, it is possible to state that *Vertzami*, *Marzemino*/*Barzemino* and *Balsamina* are different varieties, even if phylogenetically related. *Vertzami* accessions did not show any genetic diversity and a very low phenotypical variability. *Marzemino*/*Barzemino* accessions displayed a few genetic variability by AFLP markers and a more marked phenotypical diversity. In fact *Barzemino* accessions were different from *Marzemino* accessions both on the basis of leaf morphology and anthocyanin profiling. Furthermore *Barzeminone*, *Balsamina* and *Marzemina bianca* have to be considered related varieties of *Marzemino*/ *Barzemino*, as well as *Vertzami* has to be considered a member of this variety-family.

These issues, the occurrence in Italy of diverse genotypes belonging to the same cultivar group, the historical relevance and extension of this variety assortment support the idea that *Vertzami*, which is a distinct member of this family, is a biotype of the ancient *Marzemino* cultivar population which, according to Logothetis and Vlachos (1967), was likely introduced in Lefkas by Venetians during their dominion of the Ionian islands (XIV-XVIII centuries).

The main conclusion arising from this survey is that to recover intra-variety variability of *Marzemino* the germplasm exploration has to be broaden in Italy.

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Tables

Table 1. Average anthocyanin profile of Barzemino, Marzemino and Vertzami.

Accession	Delphinidin 3-monoglucoside %	Cyanidin 3-monoglucoside %	Petunidin 3-monoglucoside %	Peonidin 3-monoglucoside %	Malvidin 3-monoglucoside %	summation of acetic esters %	summation of p-coumaric esters %
Barzemino	6.6 a	1.0 a	7.0 a	6.8 b	46.4 c	14.0 b	18.3 b
Marzemino	12.8 b	1.5 a	9.7 b	3.5 a	36.9 a	24.1 c	11.5 a
Vertzami	8.4 a	2.8 b	10.5 b	8.2 b	41.9 b	9.6 a	18.6 b

Means followed by the same letter are not statistically different (P=0.05).

Table 2. Microsatellite allele composition of the accession tested in the survey

Accession	VVS2		VVS4		VVMD5		VVMD6		VVMD7		VVMD17		VVMD21		VVMD24		VVMD25		VVMD27		VVMD28		VVMD31		VVMD34	
Marzemino	129	-	166	173	222	228	197	206	237	260	220	222	243	249	210	214	253	-	181	190	247	231	212	216	240	-
Barzemino	129	-	166	173	222	228	197	206	237	260	220	222	243	249	210	214	253	-	181	190	247	231	212	216	240	-
Marz. bianco	129	-	166	-	222	-	197	206	237	260	220	222	243	249	210	214	253	-	181	190	247	231	212	216	240	-
Marzemina b.	129	-	166	173	222	-	197	206	237	254	220	222	243	249	210	214	253	-	179	190	233	239	212	216	240	-
Barzemino ne	140	-	166	-	222	228	197	206	237	254	220	222	243	256	210	-	253	-	181	190	233	239	212	204	240	-
Vertzami	129	140	166	173	222	228	190	206	237	254	220	222	243	256	208	219	243	253	179	190	233	239	212	204	240	-
Balsamina E.	129	-	166	173	222	228	197	206	237	260	220	222	243	249	210	214	243	253	181	190	247	231	212	204	240	-
Balsamina R.	129	-	166	173	222	228	197	206	237	260	220	222	249	-	210	214	243	253	181	190	247	231	212	204	240	-

Figures

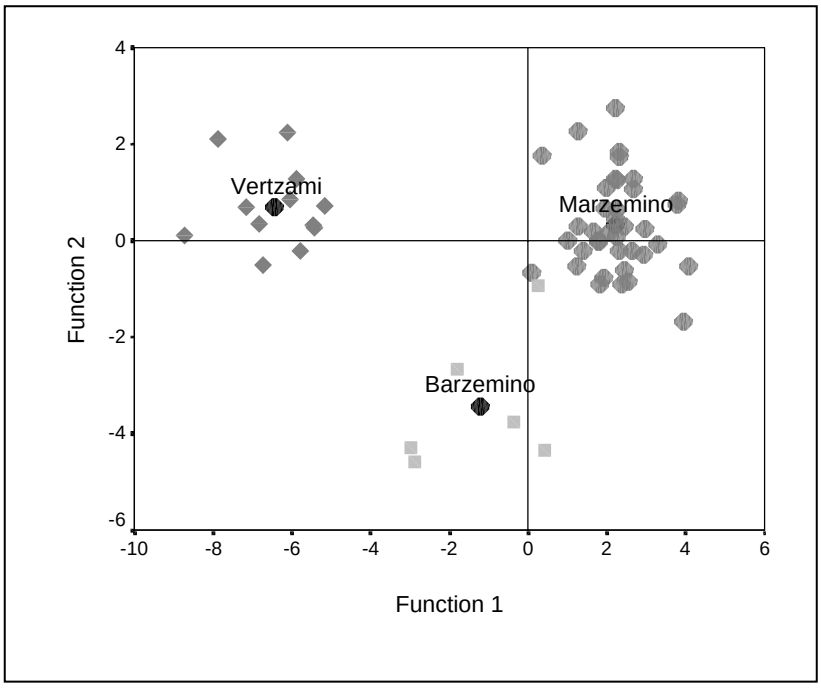

Fig. 1. Scatter plot representing the classification of the accessions according the first two discriminant functions built on the basis of 7 leaf phyllometric indexes selected out of 43 following a step wise procedures. The first function was able to explain the 89.6% of the total variability, while the second one explained the remaining 10.4%.

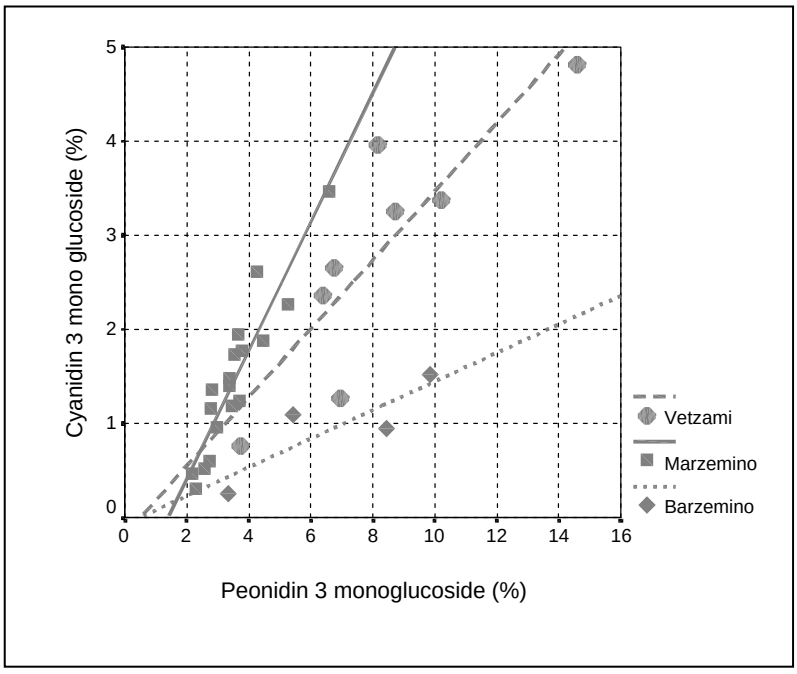

Fig. 2. Scatter plot of the accessions according to the Peonidin/Cyanidin ratio which resulted the most discriminant factors among the varieties.

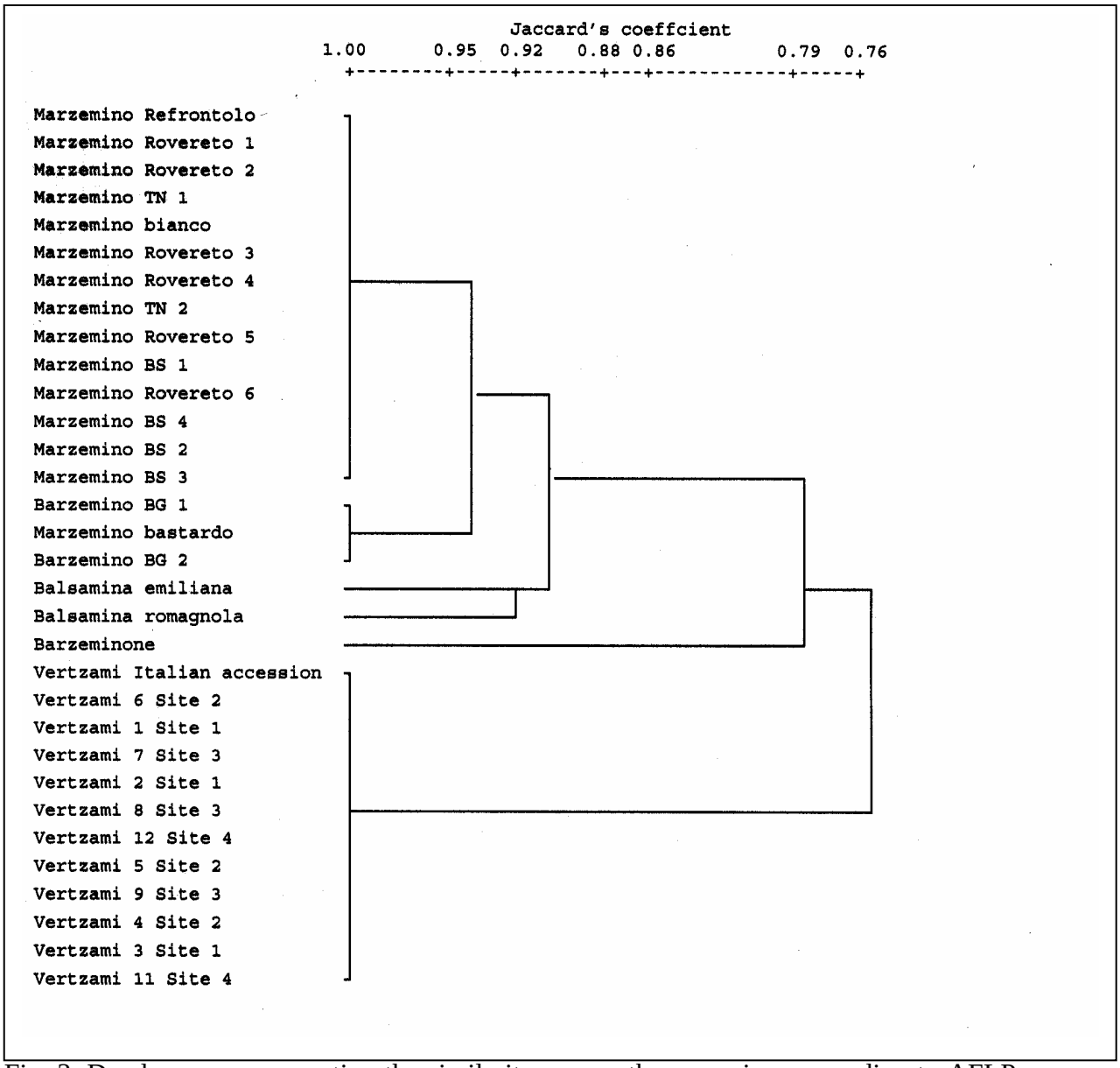

Fig. 3. Dendrogram representing the similarity among the accessions according to AFLP markers

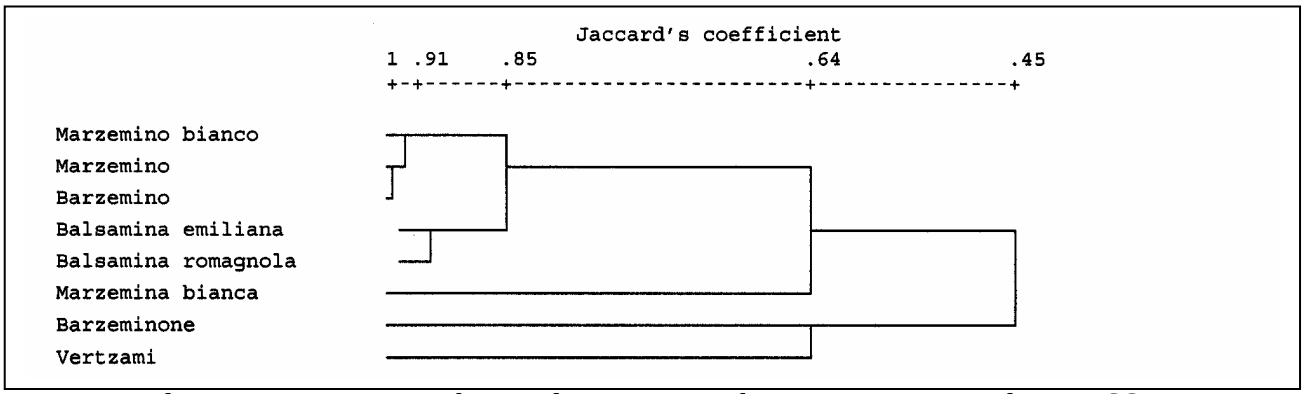

Fig. 4. Dendrogram representing the similarity among the accessions according to SSR markers