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Phylogenetic analysis of grapevine cv. Ansonica growing on the island of Giglio, Italy, by AFLP and SSR markers

by

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Summary: The geographic origin of the grapevine cultivar Ansonica (*Vitis vinifera* L.) grown on the island of Giglio, Tuscany, Italy, was investigated with molecular tools, *i.e.* AFLP and SSR (microsatellite) analysis. The scored polymorphic DNA bands were statistically analysed and the results were expressed in dendrograms showing the degree of genomic similarity among the tested cultivars: three Ansonica vines sampled in some of the oldest vineyards of the island, one Ansonica specimen grown in Tuscany, one Inzolia specimen grown in Sicily, 23 cultivars grown in different Mediterranean regions and 40 Greek cultivars.

The results obtained from AFLP or SSR approaches led to equivalent conclusions: the three grapevines sampled in Giglio were indistinguishable and showed genetic similarity with cv. Ansonica grown in the Tuscan mainland and with cv. Inzolia from Sicily, Airen from Spain, Clairette from France and Roditis from Greece. The SSR analysis showed that Sideritis and Roditis have the highest genomic similarity with Ansonica among the 40 tested Greek cultivars. The molecular analysis gives conclusive evidence for the Greek origin of Ansonica grown on the island, as previously proposed on the basis of morphological and historical studies.

Key words: SSR, AFLP, Vitis vinifera L., phylogeny, Ansonica, ampelography.

Abbreviations: SSR = Simple Sequence Repeat; AFLP = Amplified Fragment Length Polymorphism, JC = Jaccard's coefficient.

Introduction

The origin of numerous grapevine cultivars (*Vitis vinifera* L.) grown in Western Europe is highly dubious. Attempts to identify today's cultivars with varieties described in ancient Greek and Latin writings frequently led to modest and controversial conclusions. On the other hand, recently developed approaches to DNA analysis, such as RAPD, AFLP and SSR (Karp *et al.* 1998) are now, for the first time, offering an appropriate molecular support to characterise and compare genotypes independently from the phenotype. The use of these PCR-based analytical tools allows detection of DNA polymorphism at random (RAPD, AFLP) or specific SSR loci in the genome. By identifying polymorphic sequences in the genomic DNA, these tools allow phylogenetic (Heun *et al.* 1997) and taxonomic (Backman 1992; Williams and Clair 1993) studies, as well as cultivar identification (Mulkahy *et al.* 1995; Xu and Bakalinsky 1996).

The AFLP approach has already been used for phylogenetic studies on grapevine (Sensi *et al.* 1996), while the SSR (also known as microsatellite) analysis has proven useful to study the parentage of grapevine cultivars used in Australia (Thomas *et al.* 1994) and to define the origin of Cabernet Sauvignon (Regner *et al.* 1996; Bowers and Meredith 1997). In the present study these tools are applied to define the origin of cv. Ansonica grown since ancient times on the island of Giglio, Tuscany (Italy).

This small granitic island (2.2 km^2), located in the Tyrrhenian sea, is part of the Tuscanian archipelago. In its long history, it was under Etruscan, Greek, Roman and Florentine domination. Viticulture has been reported since the 6th century B.C. At different times the island was abandoned following invasions and deportation of the whole population. After repopulation viticulture resumed.

Much less is known about the origin and history of the island's grapevine cultivars. In the present study we investigated the geographic origin of cv. Ansonica, at present the most widely cultivated grapevine on the island. Morphological and historical evidence suggested a Greek origin. We present molecular evidence verifying this hypothesis and suggest its introduction from Greece *via* Sicily during the Greek civilisation.

Material and Methods

Biological material: Grapevine (*Vitis vinifera* L.) samples used in this study are listed in Tabs. 1 and 2; they can be classified in the following groups: (a) three Ansonica plants from a locality named Capel Rosso on the Giglio island (Tuscany, Italy) collected from traditional vineyards in August 1998; (b) 25 south European cultivars from the grapevine collection of C.I.VI.FRU.CE. (Regional Centre for Agriculture), Riccagioia, Pavia, Italy, and from that of IASMA (Agricultural Research Institute), S. Michele all'Adige, Trento, Italy; (c) 40 Greek cultivars from the collection of the National Agriculture Research Foundation of Lycovrissi (Athens, Greece) and from the Laboratory of Viticulture, University of Thessaloniki, Greece.

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Table 1

Reference cultivars used for the SSR and AFLP analysis, area of cultivation and site of germplasm collection

Cultivar	Area of cultivation	Germplasm collection
Airen	Spain	CIVIFRUCE
Ansonica (Giglio 1)	Giglio island	
Ansonica (Giglio 2)	Giglio island	
Ansonica (Giglio 3)	Giglio island	
Ansonica	Tuscany	CIVIFRUCE
Bianco Alessano	Southern Italy	CIVIFRUCE
Bobal	Croatia	CIVIFRUCE
Clairette	Southern France	CIVIFRUCE
Erbaluce	Northern Italy	CIVIFRUCE
Erbamatt	Northern Italy	CIVIFRUCE
Favorita	Northern Italy	CIVIFRUCE
Folle B.	France	CIVIFRUCE
Garganega	Northern Italy	CIVIFRUCE
Greco B.	Southern Italy	CIVIFRUCE
Humagne	Switzerland	IASMA
Inzolia	Sicily– Italy	IASMA
Lagarino	Northern Italy	IASMA
Malvasia B. Candia	Italy	IASMA
Malvasia Istria	Northern Italy	IASMA
Montuni	Northern Italy	IASMA
Nasco	Sardinia– Italy	IASMA
Piquepoul	Southern France	IASMA
Plavina	Dalmatia	IASMA
Roditis	Greece	IASMA
Ribolla B.	Northern Italy and Greece	IASMA
Timorasso	Northern Italy	IASMA
Trebbiano Soave	Northern Italy	IASMA
Xarello	Spain	IASMA

CIVIFRUCE (Regional Centre of Agricolture, Riccagioia), Pavia, Italy.
IASMA (Agricultural Research Institute), S. Michele all'Adige, Trento, Italy.

Table 2

Greek cultivars used for the SSR analysis and site of germplasm collection

Cultivar	Germplasm collection
Agianniotiko	LYK 1997
Agiorgitico	LYK 1997
Ampelakiotiko Mavro	LYK 1997
Amfioni	LYK 1997
Asprouda Patron	LYK 1997
Bakouri	LYK 1997
Begleri	LYK 1997
Coarna negra	LYK 1997
Fileri	LYK 1997
Fokiano	LYK 1997
Iatrou	LYK 1997
Kakotrygis	LYK 1997
Kervouniaris	LYK 1997
Kokkinorobola	LYK 1997
Korfiatis	TESS 1997
Kotselina	LYK 1997
Liatiko	LYK 1997
Mandilaria	LYK 1997
Mavro Boulgarias	LYK 1997
Mavro Kalavriton	LYK 1997
Mavrokosmas	LYK 1997
Mavro Messanikola	LYK 1997
Mavrostyfo	LYK 1997
Mavrodafni	TESS 1997
Mavrotragano	LYK 1997
Mavroudi Arachovis	TESS 1997
Moscato d'Alessandria	LYK 1997
Nerostafylo	LYK 1997
Pavlos	LYK 1997
Prekniariko	LYK 1997
Psilomavro Kalavriton	LYK 1997
Roditis	LYK 1997
Rkatsiteli	LYK 1997
Robola	LYK 1997
Savatiano	TESS 1997
Sefka	TESS 1997
Sideritis	TESS 1997
Votsichi	LYK 1997
Xinomavro	LYK 1997
Vossos	LYK 1997

LYK 1997: Collection of National Agriculture Research Foundation of Lycovrissi, Athens.
TESS 1997: Lab. of Viticulture, Department of Agriculture, Thessaloniki.

D N A e x t r a c t i o n : Young leaflets were harvested from rooted cuttings, frozen in liquid nitrogen and ground to fine powder. Genomic DNA was extracted in 5 ml of "CTAB buffer" (2 % CTAB, 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1 % w/v polyvinylpyrrolidone, 0.1 % v/v β-mercaptoethanol) as described by DOYLE and DOYLE (1990).

S S R a n a l y s i s : DNA was analysed at the following 8 microsatellite loci: VVS1, VVS2, VVS3, VVS4, VVS5 (THOMAS and SCOTT 1993), VVMD5, VVMD6, VVMD7 and VVMD8 (BOWERS *et al.* 1996). In the case of the samples of Tab. 2, the locus VVS5 was not analysed. The analysis was performed by adding 100 ng of genomic DNA to a 25 µl PCR mixture containing 0.25 µM of the DNA primer specified for each microsatellite locus by THOMAS and SCOTT (1993) or BOWERS *et al.* (1996), 200 µM of each of the 4 dNTPs, 0.5 U Dynazyme (Celbio, Italy) and Dynazyme buffer as specified by the supplier. PCR amplification was performed with a programmable thermal controller (PTC 100, MJ Research Inc., USA) with the following thermal cycles: 7 min at 94 °C; 35 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C) and extension (1 min at 72 °C); then a final step for 7 min at 72 °C.

A F L P a n a l y s i s : AFLP was based on the principles described by VOS *et al.* (1995) and performed precisely as described in the European Patent 0534858 (Keygene, Belgium). In particular, genomic DNA (1 µg) was digested (3 h)

with the restriction enzymes *Eco*RI (6-base cutter) and *Mse*I (4-base cutter). DNA fragments were ligated (with T4 DNA ligase) to 5 pmol of a biotinylated *Eco*RI adapter and 50 pmol of a non-biotinylated *Mse*I adapter (Vos *et al.* 1995). Biotinylated fragments were bound to paramagnetic Streptavidine Dynabeads M-280 (Dynal, Oslo, Norway) and washed 3 times with STEX buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8, 1 mM EDTA and 0.1 % TritonX-100). The beads were suspended in 200 µl of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA (pH 8.0) and used as template in a pre-amplification mixture containing DNA primers (E01 and M01 of Tab. 3) complementary to the core of the *Eco*RI and *Mse*I adapter, respectively. The 50 µl amplification mixture contained 5 µl of DNA-adsorbed beads, 75 ng of the selected primer, 200 µM of each dNTP, 0.5 U Dynazyme II and 5 µl

Table 3

DNA primers for AFLP analysis

Name	DNA sequence	Variable extension
M01	5'-GATGAGTCCTGAGTAA	A
E01	5'-GACTGCGTACCAATTC	A
M32	5'-GATGAGTCCTGAGTAA	AAC
M36	5'-GATGAGTCCTGAGTAA	ACC
M38	5'-GATGAGTCCTGAGTAA	ACT
E32	5'-GACTGCGTACCAATTC	AAC
E33	5'-GACTGCGTACCAATTC	AAG

Dynazyme buffer. After 2 min at 94 °C, amplification was carried out for 20 cycles of denaturation (45 s at 94 °C), annealing (30 s at 50 °C) and extension (1 min at 72 °C). After a final elongation step (7 min at 72 °C) the pre-amplification product was diluted 1:50 with water and used for selective amplification. This was carried out using one of the selective primers (E32 or E33 of Tab. 3) complementary to the *Eco*RI adapter and one of the primers (M32, M36 or M38 of Tab. 3) complementary to the *Mse*I adapter. The *Eco*RI-primer was end-labelled with α^{33}P ATP (Amersham, Italy). The amplification mixture (20 µl, final volume) contained 5 µl of the pre-amplification mixture, 5 ng of labelled *Eco*RI primer, 30 ng of *Mse*I primer, 200 µM of each dNTP, 0.5 U Dynazyme II and 5 µl Dynazyme buffer. After 2 min at 94 °C (denaturation), amplification was for 36 cycles under the following conditions: denaturation for 30 s at 94 °C; annealing for 30 s at 65 °C for the first cycle, followed by a lowering of temperature (0.7 °C) in the next 12 cycles, then at 56 °C for the remaining 23 cycles; extension for 60 s at 72 °C.

Analysis of the DNA amplification products: In the case of the SSR analysis, 10 µl of the PCR-amplified mixture were analysed by electrophoresis on 10 % polyacrylamide gel in TBE buffer (50 mM boric acid, 1 mM EDTA, pH 8.0) for 16 h at 100 mV. After staining in a 5 % ethidium bromide solution, the gel was recorded and analysed in 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^{-1} xylene cyanol FF, 1 mg·ml^{-1} bromophenol blue, 10 M EDTA, pH 8.0), denatured for 5 min at 92 °C, loaded onto a 4.5 % sequencing polyacrylamide gel and electrophoresed in TBE electrophoresis buffer for 3 h at 80 W. Then, the gel was fixed in 10 % acetic acid and exposed to an X-ray film. Visual inspection of the resulting autoradiograms allowed scoring of polymorphic bands.

Statistical analysis: Each microsatellite allele or AFLP band detected after electrophoresis of the amplification DNA products 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 of the heterozygous or homozygous state. The resulting data were analysed using the software programme SPSS (V. 8.5). Similarity-dissimilarity matrices were computed with the Jaccard's coefficient (JC) (Sneath and Sokal 1973),

$$JC = a/(n-d),$$

where a = band present in the two compared genotypes; n = total number of polymorphic bands; d = band absent in both compared genotypes. The final products of the analysis were dendrograms constructed by cluster analysis based upon UPGMA (unweighed pair-group method with arithmetical averages).

In the case of SSR, gene diversity was calculated as $1-\Sigma p^2_{ij}$ (Rongwer *et al.* 1995), where p_{ij} is the frequency of the j allele for the i microsatellite.

Results

Leaves from three grapevine plants, cv. Ansonica, from the Giglio island, as well as from the 25 south European cultivars listed in Tab. 1 were used for DNA extraction. The sampling strategy for the reference cultivars was based upon morphological similarities, geographical relationships as well as on historical remarks on the colonisation of the island in ancient times.

At first purified DNA was used to analyse SSR polymorphism by amplification at 8 loci. After PCR amplification in the presence of the specific DNA primer pair, DNA fragments were analysed in polyacrylamide gel. Tab. 4 summarizes the number of alleles and their size range; on this basis, values of gene diversity have been calculated for each microsatellite locus. They were high, ranging from 0.671 to 0.998, thus substantiating their usefulness as genomic markers. Polymorphic bands allowed the construction of a dendrogram that illustrates the genetic relationship among the analysed cultivars (Fig. 1).

The same DNA samples were analysed with the AFLP approach. This tool detects polymorphism at random DNA loci. Altogether, 268 bands were detected, 103 of these were polymorphic. The resulting dendrogram (Fig. 2) showed high correlation with that of Fig. 1.

In both dendrograms the three Ansonica plants from Giglio showed a nearly identical genomic constitution; their closest relatives were Ansonica from Tuscany, Inzolia from Sicily, Airen from Spain and Roditis from Greece.

The three Giglio Ansonica vines were then confronted with a large array of Greek grapevines: 39 cultivars randomly sampled from the Greek germplasm (Tab. 2). Samples were analysed for SSR polymorphism by PCR amplification at the

Table 4

Number of alleles, range of allele size, and gene diversity of 8 microsatellite loci assayed in two different groups of samples

	Ansonica and south European grape samples			Ansonica and Greek grape samples		
	Range of allele sizes (bp)	Number of alleles	Gene diversity	Range of allele sizes (bp)	Number of alleles	Gene diversity
VVS1	185-227	7	0.998	213-260	10	0.999
VVS2	137-160	5	0.950	137-153	7	0.957
VVS3	218-266	7	0.998	210-236	13	0.999
VVS4	165-186	7	0.965	174-194	12	0.999
VVS5	88-121	9	0.988	-	-	-
VVMD5	242-264	5	0.824	233-254	11	0.982
VVMD6	197-213	4	0.671	110-213	8	0.945
VVMD7	237-253	5	0.896	237-263	11	0.934

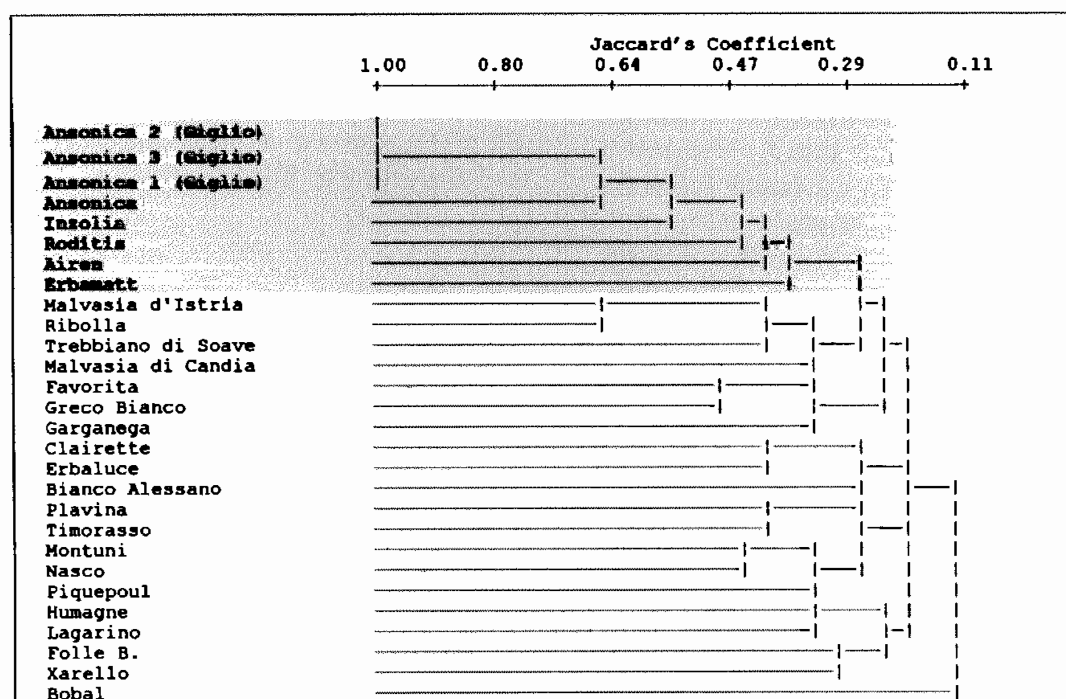

Fig. 1: Dendrogram showing the genetic relationship among samples of Ansonica grape from Giglio and 25 cultivars from southern Europe, as derived from an SSR analysis. The greyish part encloses clusters with the highest similarity to Ansonica.

7 loci shown in Tab. 4. Gene diversity was high, ranging from 0.945 to 0.999. Dendrograms confirmed the clear differentiation between Giglio and Tuscany and showed Roditis as the closest Greek cultivar to Ansonica (Fig. 3). Sideritis was the only Greek cultivar included in the Roditis dendrogram branch.

Discussion

Information on the origin and relationship among plant cultivars is of great interest both for germplasm preservation and cultivar improvement through breeding and biotechnology. In the case of grapevine, this information might be obtained joining ampelometric and chemotaxonomic (protein polymorphism, anthocyanin profile) approaches to historical, cultural and mythological data. It is conceivable that definitive answers to the numerous grapevine phylogenetic puzzles can now be given with data produced by molecular analytical approaches (Karp *et al.* 1998).

In the case of cv. Ansonica, it is well documented that this grapevine has been grown in Tuscany and on its islands since ancient times. However, the cultivar is of uncertain geographical origin. It is also conceivable that cv. Ansonica grown at different sites of the mainland of Tuscany and on its islands may have different origin and history. In the specific case of the Island of Giglio, different putative places of origin have been proposed, based on morphological analysis and historical information. A first hypothesis proposes Ansonica as a cultivar that was al-

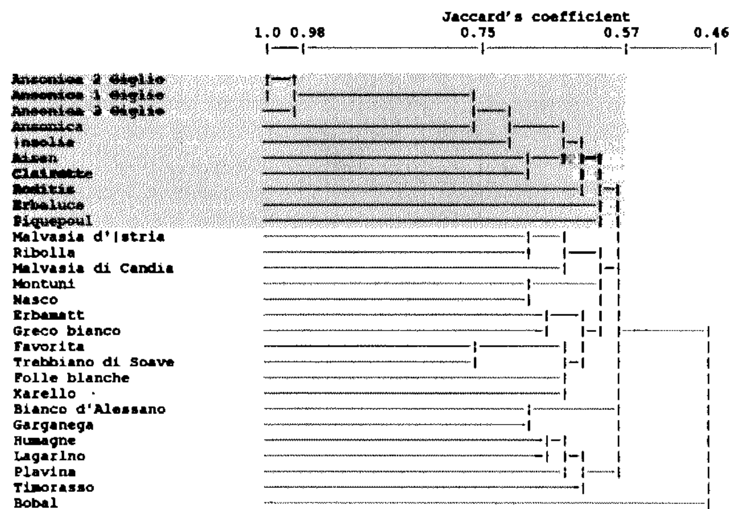

Fig. 2: Dendrogram showing the genetic relationship among samples of Ansonica from Giglio and 25 cultivars from southern Europe as derived from an AFLP analysis. For details see Fig. 1.

ready cultivated in Tuscany in the Etruscan period and was introduced to the island by V. MAGGI in 1670-1671; in that year V. MAGGI was put in charge of the agricultural and economic resurrection of the island under the Medici rulers (BRANDAGLIA 1997). A second hypothesis sees Ansonica as a cultivar introduced by Greek colonists to Sicily in the 4th century B.C. and from here brought to Giglio. A third hypothesis based on etymological studies of the term Ansonica, sees it as a French cultivar brought to Sicily by the Normans and from here to Giglio in approximately 1040 A.D. (ALESSIO and DALMASSO 1938).

We have now produced molecular evidence that strongly supports the second hypothesis, *i.e.* Greece as the place of origin of the grapevine population from which cv. Ansonica of the Island of Giglio has derived in ancient times. The ancestral of cv. Ansonica was most likely cv. Roditis (or better, the progenitor of the present Greek cultivar). This ancestral population spread out into the Tyrrhenean region giving rise to Ansonica, Inzolia and possibly other cultivars such as Clairette in France and Airen in Spain. The Ansonica/Inzolia sub-population then reached Giglio *via* Sicily. This is supported by the following consideration, based on the molecular analysis of this work:

(1) The closest varieties to cv. Ansonica from Giglio turned out to be Ansonica from Tuscany and Inzolia from Sicily. The latter two cultivars are so similar from a phenotypic point of view that the Italian official grapevine catalogue considers them synonymous. Ansonica from Giglio had already been shown to differ from Ansonica from Tus-

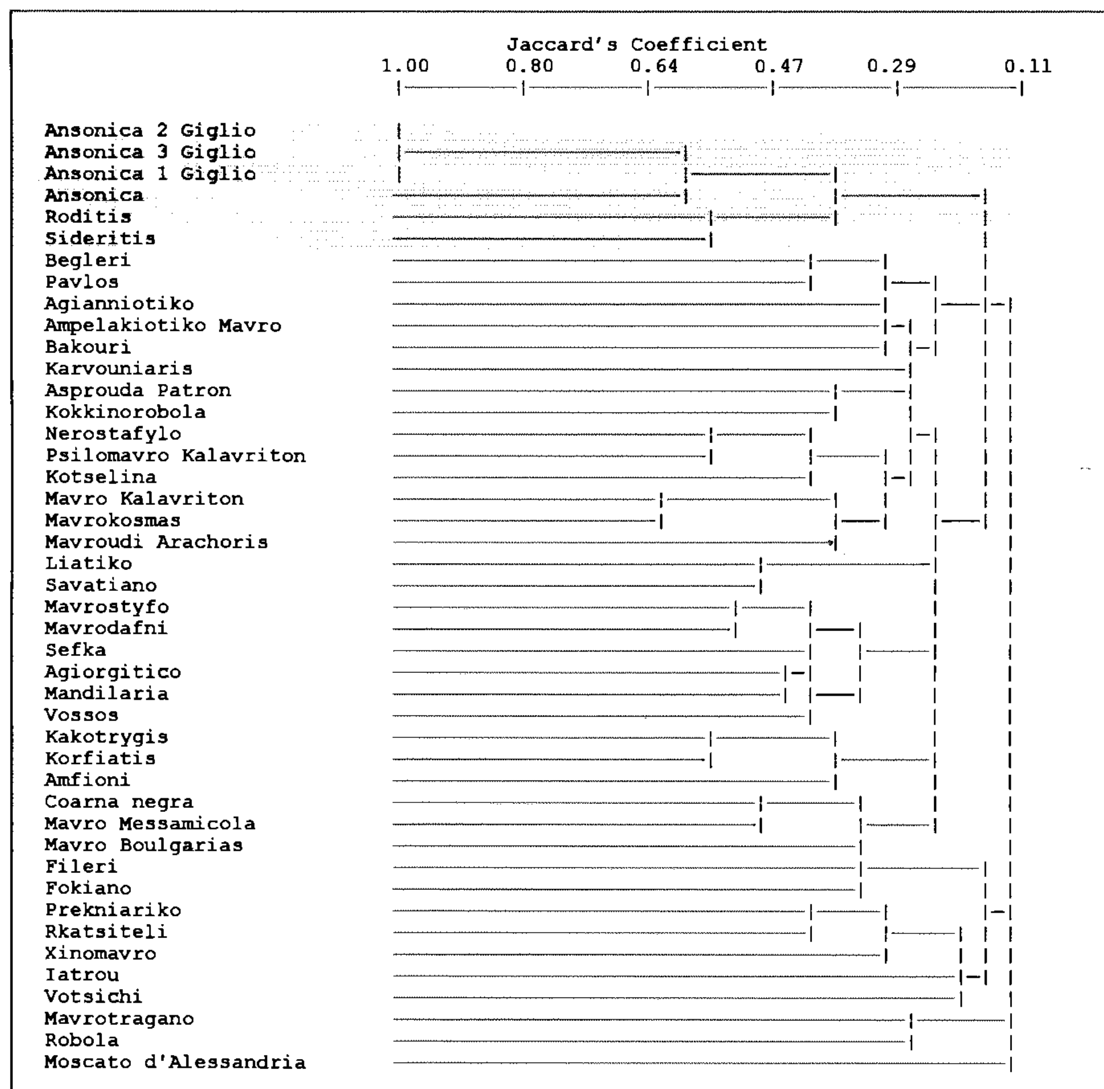

Fig. 3: Dendrogram showing the genetic relationship among Greek cultivars and Ansonica samples derived from an SSR analysis. For details see Fig. 1.

cany on the basis of some morphological characters, including shoot apex, leaf and cluster. This suggests an ancient differentiation between the Ansonica grapevines from Giglio and Tuscany.

(2) The AFLP dendrogram branch that combines Ansonica and Inzolia also includes the three cultivars Roditis, Airen and Clairette. This was similar for the dendrogram established on the SSR analysis, except that Clairette was set apart. Roditis is an ancient Greek variety. Its first citation dates back to the first century (NIKOLAU 1999, pers. comm.). At present it is widely known and grown throughout Greece where it shows wide phenotypic variability: *e.g.* in addition to typical rose coloured berries, it shows biotypes with uncoloured or small sized berries. Airen is the most frequently cultivated white variety in Spain while Clairette is an antique variety of Southern France.

(3) All other analysed cultivars, which had been selected, showed higher genomic dissimilarities from those of section (2).

(4) Comparisons on the basis of SSR analyses among the Ansonica specimen and the 40 Greek cultivars did not indicate any accession with a higher genomic similarity to Ansonica than shown for Roditis.

The presence of cultivated grapevines in the Eastern Mediterranean area is dated back to the third millennium B.C. (ZOHARY and HOPF 1993). In this work we propose that Roditis, which is one of the most ancient Greek grapevine varieties, is the most likely ancestral of the Italian cv. Ansonica.

Further investigation of the Greek grapevine germplasm and the analysis of the genetic variability within the Ansonica/Inzolia populations will definitely clear this phylogenetic question. Moreover, results of this work confirm that PCR-based DNA marker techniques provide a powerful tool for phylogenetic studies within *Vitis vinifera* L. cultivars.

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