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AFLP analysis of genetic relationships among aromatic grapevines (*Vitis vinifera*)

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Abstract Genotypic diversity has been detected among aromatic grapevines (*Vitis vinifera*) by molecular markers (AFLPs). The 22 primer-pairs generated a total of 1,331 bands of which 564 (40%) were polymorphic over all the genotypes. The bootstrap analysis pointed out that a large number of polymorphic bands (200–400) has to be used for a better estimation of the genetic distances among genotypes; 383 polymorphic AFLP bands were used for the cluster and the principal coordinate analyses because they did not present missing data across all the genotypes. The cluster analysis (UPGMA), based on polymorphic AFLP markers, revealed no relationship between the Moscato and Malvasia grapevines. The Malvasias, unlike the Moscatos distinguished by their distinct muscat aroma, have to be considered a more complex group because it includes muscat and non-muscat grapevines. The principal coordinate analysis (PCO) confirmed the pattern of the cluster analysis only for those varieties which presented a low coefficient of dissimilarity, while for the other varieties there was no correspondence between the two analyses. The pattern of aggregation among aromatic grapevines in the cluster and principal coordinate analyses does not support any classification that might include an aromatic grapevine group in *V. vinifera*. Even though some synonyms and homonyms are present among aromatic grapevines (*V. vinifera*), genetic diversity exists among genotypes in AFLP markers.

Keywords Genetic diversity · Aromatic grapevines · DNA polymorphism · *Vitis vinifera*

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Introduction

Aromatic grapevines (*Vitis vinifera L.*) have been known since the Roman times. They were grown in a such great number that the first ampelographers made some attempts in the nineteenth century to group the *V. vinifera* grapevines into aromatic and non-aromatic ones; this was a difficult goal because some aromatic and non-aromatic varieties were identified with similar names. A large number of varieties under the name of Malvasia are listed in the well-known 'Ampelografia' (Molon 1906) and 'Ampélographie' (Viala and Vermorel 1909). Both authors, as well as others, were not able to differentiate the numerous grapevines under the name of Malvasia. Even if in the past several authors were inclined to consider the Malvasias as only the aromatic ones, nowadays it is difficult to exclude a large number of non-aromatic grapevines under the name of Malvasia. A less complex group of aromatic grapevines are the Moscato varieties, which are distinguished by their distinct muscat flavor. Other aromatic grapevines, not so well known as the Moscatos and the Malvasias, were also grown in limited viticultural areas under different names. The first mention of aromatic grapevines appear in ancient documents (Cato, Pliny, about 200–300 BC) but more references are reported in the literature of the twelfth century. In the latter period a large number of wines (mostly aromatic or sweet) were exported from Greece to Italy; subsequently, grapevines related to these wines might have also been introduced. Anyhow there were and there are no regions in Italy without any aromatic grapes or wines under the name of Malvasia and/or Moscato. This caused a confusion, that remains still nowadays, in the identification of aromatic grapevines especially Malvasias and Moscatos. Thus the study of the genetic relationships among aromatic varieties is important not only for propagation purposes, in order to avoid a confusion in the distribution of vegetative material of grape (*V. vinifera*), but also for germplasm maintenance, breeding programs etc.

The relationships among genotypes are evaluated through morphological characters but this requires repeated measurements because of environmental variations. Recently, molecular markers have been applied for genetic studies, variety characterization, paternity analysis, etc., because they are not affected by the environment. In *Vitis* different molecular markers have been used such as (RFLP) restriction fragment length polymorphism (Bourquin et al. 1993; Bowers et al. 1993), (RAPD) random amplified polymorphic DNA (Gogorcena et al. 1993; Buscher et al. 1994; Xiamping et al. 1996; Lodhi et al. 1997), (SSR) simple sequence repeat (Thomas et al. 1994; Bowers et al. 1996; Sefc et al. 2000; Dangl et al. 2001) and (AFLP) amplified fragment length polymorphism (Sensi et al. 1996; Cervera et al. 1998; Scott et al. 2001).

An unbiased evaluation of the genetic relationship among genotypes is obtained using a large number of morphological or molecular markers (Sneath and Sokal 1973). The AFLP approach, which enables simultaneous analysis of a large number of marker loci throughout the genome, appears to be remarkably powerful. The objective of this work is to study the genetic relationships among aromatic grapevines (*V. vinifera*) through AFLP markers.

Materials and methods

The grape varieties taken into consideration are listed in Table 1. They include autochthonous grapevines with aromatic characteristics, from a distinct muscat to a mild-neutral aroma. These ancient varieties of unknown origin were collected from different regions of Italy and maintained in the grapevine germplasm collection at CRSA 'B. Caramia' Locorotondo- Bari (Apulia), at the University of Palermo (Sicily) and at the University of Sassari (Sardinia).

Total DNA was isolated from young leaves of these grapevines as described by Bowers et al. (1993) using a CTAB buffer (3% CTAB,100 mM of Tris-HCl pH 8.0, 20 mM of EDTA pH 8.0, 1.4 M of NaCl and 0.5% v/v of β-mercaptoethanol). AFLP analysis was performed according to Vos et al. (1995) with little modification. The DNAs were digested with *Mse*I and *Eco*RI restriction enzymes, and shortly after ligated to *Eco*RI and *Mse*I adapters by adding 1 U of T4 DNA ligase and 12 nmol of ATP, in a 60-μl final volume of digestion-ligation buffer (50 mM of Tris-HCl, 50 mM of MgAc and 250 mM of KAc, pH 7.5), in 2-h incubations at 16 and 37 °C. Five microliters l of 1:10 aliquots were pre-amplified by the *Mse*I primer plus C or G and the *Eco*RI primer plus A in 25-μl reactions with 0.048 mM of each dNTP, 2.5 mM of $MgCl_2$, and 1 U of *Taq* DNA polymerase, in a 1× Magnesium-Free buffer (Promega). Then 3.2 μl of 1:45 diluted aliquots were amplified with 22 primer combinations (see Table 2). The 9.6 μl reaction contained 0.25 U of *Taq* DNA polymerase, 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 3.2 ng and 32 ng of γ-[^{33}P]-ATP-labelled *Eco*RI primer and *Mse*I primer. Amplified fragments were separated on 5% polyacrylamide denaturing gels, which were dried and laid onto X-ray films.

Positions of scorable AFLP bands were transformed into a binary character matrix ('1' for the presence and '0' for the absence of a band at a particular position); only polymorphic bands were used in this analysis and considered as a unit character. The coefficient of dissimilarity (genetic distance) between any pairs of cultivars has been determined as D = 1 – SM, the complement to the simple-matching (SM) coefficient of similarity (Sneath and Sokal 1973). To evaluate the effect of the number of bands on the estimation of the genetic distances, a bootstrap sampling procedure (Efron and Tibshirani 1991; Tivang et al. 1994) was executed through a computer program written in 'c' by the authors. The coefficient dissimilarity matrix was used for the cluster analysis (UPGMA) to study the genetic relationships among aromatic grapevines. The reliability of the cluster was assessed by applying a bootstrap procedure (WINBOOT, Yap and Nelson 1996). Some authors consider the value of 95% (or higher) obtained in bootstrap as indicating statistical support for the grouping of taxa at a branch (Felsenstein 1985). In this study the grouping of taxa is considered as being statistically significant when both cluster analysis and principal coordinate analysis (PCO) gave the same result. Thus a further evaluation of the genetic relationships among genotypes was carried out by principal coordinate analysis based on the same dissimilarity matrix. The analyses were done using different routines available on the software package NTSYS-PC version 2.0 (Rohlf 1997).

Table 1 The list of aromatic grapevines (*V. vinifera*) used for AFLP analysis

Genotypes	Code
Malvasia Bianca Furlò	Ma1
Malvasia di Candia	Ma2
Malvasia Bianca Anglani	Ma3
Malvasia Nera di Brindisi	Ma4
Malvasia Bastarda	Ma5
Malvasia Bianca Forzati	Ma6
Malvasia Coda di Pecora	Ma7
Malvasia Bianca Mancinelli	Ma8
Malvasia Bianca Yocco	Ma9
Malvasia di Sardegna S1	Ma10
Malvasia di Sardegna S2	Ma11
Malvasia di Sardegna S3	Ma12
Malvasia di Sardegna S4	Ma13
Malvasia di Lipari	Ma14
Fiano di Avellino	Fi15
Fiano Puglia Itas	Fi16
Fiano Puglia Rosato	Fi17
Moscatello	Mo18
Moscatellone Bianco	Mo19
Moscato Saraceno	Mo20
Moscato Selvatico	Mo21
Moscato Giallo	Mo22
Moscato Terracina	Mo23
Moscato Reale	Mo24
Moscato Bianco	Mo26
Moscato Nero	Mo27
Moscato Amburgo	Mo28
Moscato Canelli	Mo29
Moscatellone Nero	Mo30
Moscardella	Mo31
Marchione	Mo32
Coda di Volpe	Co25
Aleatico	Ao33

Results and discussion

The summary of AFLP markers produced by 22 primer-pairs across all genotypes is given in Table 2. The 22 primer-pairs generated a total of 1,331 bands of which 564 (40%) were polymorphic over all the genotypes. The capability of different primer-pairs to generate polymorphic AFLP markers varied significantly, ranging from 15 to 35 polymorphic bands per primer-pair over all the genotypes. Thereby it confirms the high multiplex ratio expected from the AFLP technique.

In most diversity studies, one important consideration is the evaluation of the number of markers (molecular or

Table 2 Selected primer combinations and polymorphism rates for AFLP analysis of aromatic grapevines (*V. vinifera*)

*Mse*I primers	*Eco*RI primers	Polymorphic bands	Average polymorphic bands	Average polymorphism (%)
CAC	ACA, ACC, ATA	79	26.33	47.59
CCA	ACA, ACC, ATA, ATC	92	23.00	40.53
CTG	ATA, ATG, ATT	92	30.67	39.66
CTC	ATA, ATC, ATG, ATT	112	28.00	41.64
GAA	ACA, ACT	33	16.50	32.67
GGC	ACT, ACA, ATG	56	18.67	41.79
GTC	ATC, ATG	65	32.50	34.76
GTG	ATC	35	35.00	38.46
Total		564	26.33	39.64

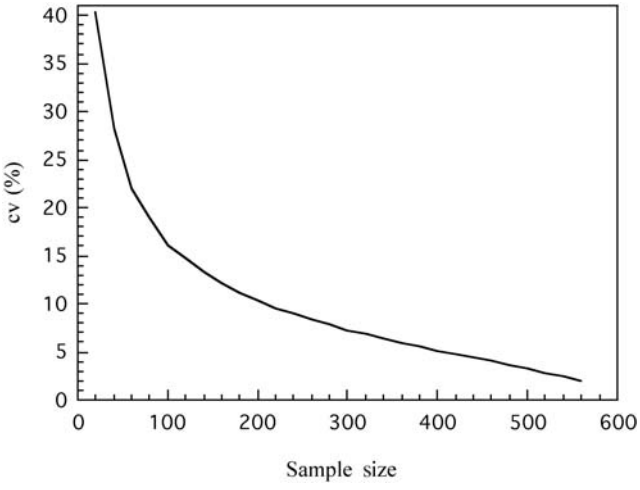

Fig. 1 Plot of the mean coefficient of variation (CV) vs AFLP band number (sample size) of the coefficients of dissimilarity among aromatic grapevines (*V. vinifera*)

morphological) for an unbiased estimation of the coefficient of similarity or dissimilarity among genotypes. The influence of the number of AFLP markers on the estimation of the coefficients of dissimilarity (genetic distance) among the aromatic grapevines was evaluated by the bootstrap sampling procedure. Figure 1 shows the distribution of the mean coefficients of variation (CV) of the dissimilarity values among the aromatic grapevines for each bootstrap sample size; it demonstrates that the mean coefficient of variation decreases as the number of bands increases. A coefficient of variation of 10% is obtained with about 200 bands while a lower CV (5%) might be obtained with a larger number of bands (about 400). This suggests the use of a large number of bands for a better estimation of the genetic distances among genotypes. Thus, in this work, 383 out of 564 polymorphic AFLP bands were used for the cluster and the principal coordinate analyses because they did not present missing data across all genotypes. It should be pointed out that the presence of missing data could cause distortions and inconsistencies in the dissimilarity coefficient calculations. Even though several AFLP markers and some genotypes were excluded from the two multivariate analyses, the number of polymorphic bands used in this analysis might be considered appropriate (Fig. 1) for an unbiased evaluation of the coefficients of dissimilarity among genotypes.

The result of the cluster analysis is reported in the dendrogram of Fig. 2. It shows that the Moscato (Mo) varieties, with a distinct muscat aroma, were separated from the Malvasia (Ma) varieties, which include both muscat and non-muscat grapevines. It indicates that no grouping of *V. vinifera* varieties into aromatic and non-aromatic grapevines can be made, as was suggested by some ampelographers in the past. The dendrogram (Fig. 2) shows also the presence of different clusters within the Moscato group as has been observed by some authors using RAPD markers (Stavrakakis and Biniari 1998; Fanizza et al. 2000). In this dendrogram only two clusters were supported by the bootstrap value (100%) for the Moscato grouping, one formed by the genotypes Mo20, Mo22, Mo26 and one by the genotypes Mo18, Mo21.

As far as the Malvasia grapevines (Fig. 2) are concerned they form different clusters. The Malvasias represent one of the most complex group because a large number of varieties, aromatic and non-aromatic, are identified under the same name (Malvasia). Several historical references, reported in an ampelographic description on the Malvasia grapevines (Dalmasso et al. 1964), connect the name of Malvasia to Monembasia, a Peloponnese (Greece) city harbor where wines were shipped from. It is known that these wines were imported to Italy in the thirteenth century, when the name of Malvasia was used in Venice to indicate the places where people used to drink these Greek wines, maybe aromatic and/or sweet; subsequently, the grapevines producing these wines were also introduced into Italy. It is likely that, exploiting the reputation of these wines, different varieties were denominated as Malvasia. Apart from the origin of these grapevines, the dendrogram (Fig. 2) shows that one cluster is formed by the grapevines Malvasia Bastarda (Ma5), Malvasia Forzati (Ma6), Malvasia Coda di Pecora (Ma7), Malvasia Mancinelli (Ma8) and Malvasia Jocco (Ma9), which are very similar at the molecular level (coefficients of dissimilarity 0.01–0.03). Even though these grapevines are grown in different regions of southern Italy, they might be only one variety or different clones of the same variety. It is frequent in *Vitis* and other fruit species that cuttings of the same variety are introduced for propagation purposes in neighbouring regions and identi-

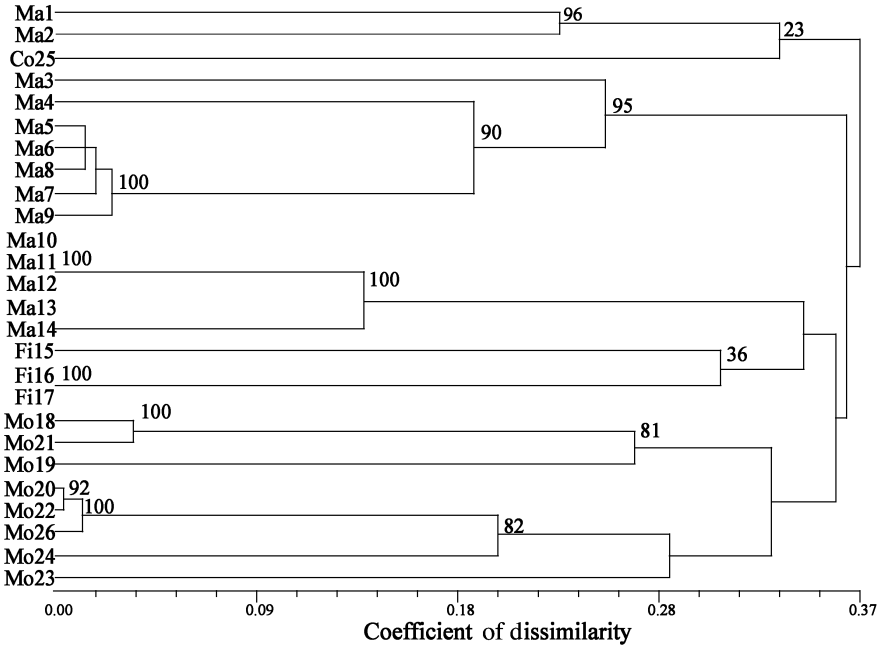

Fig. 2 Dendrogram of aromatic grapevines (*V. vinifera*) from cluster analysis based on AFLP markers

fied with different denominations; this mis-identification might be due to growers that add the name of a locality or farm to the original variety (Malvasia).

Another cluster is formed by the Malvasia grapevines grown in the main isles of Italy (Sicily and Sardinia); this includes the genotypes Ma10, Ma11, Ma12, Ma13 and Ma14. It is known that Malvasia was introduced into the isle of Sardinia from Greece during the period of the Byzantine domination as reported in old documents (Cettolini 1893). Even though the Malvasia grapevines under our observation (Ma10, Ma11, Ma12, Ma13) come from different viticulture areas of Sardinia, they are very similar at the molecular level. Thus, they might be the same variety or different clones derived from mutations on the same variety. The lack of difference among these genotypes (probable clones) at the molecular level might be due to the small fraction of the genome explored, even though a large number of AFLP markers have been used in this analysis; it is possible that a mutation might be restricted to a very small region of the genome or might involve a point mutation in a DNA regulatory sequence, which could be difficult to detect by AFLP or other similar techniques. The Malvasia of Sicily (Ma14) is different from that of Sardinia because it aggregates into the same cluster at a higher value of the genetic distance (0.15 coefficient of dissimilarity). No synonyms are reported about the Malvasia of Sicily, named Malvasia di Lipari, which is an ancient grapevine with a muscat flavor introduced from Greece into a small isle near Sicily (Nicosia and Bambara 1959).

A further cluster is formed by the Fiano grapevines (Fi5, Fi6 and Fi7). This group represents one of the so many cases of homonymy in viticulture. The name Fiano has to be attributed to the grapevine Fiano di Avellino (Fi5), which has been known since the Roman times and has

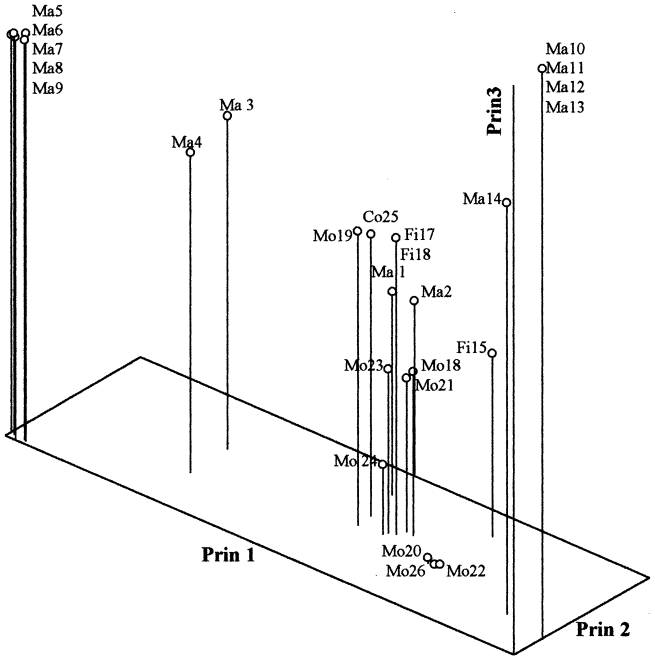

Fig. 3 Plot of aromatic grapevines (*V. vinifera*) from principal coordinate analysis based on AFLP markers

been grown in a region of southern Italy (Campania). Even though this variety was introduced into other regions, it differs from the other two Fianos (Fi16, Fi17), grown in a neighbouring region (Apulia), not only at the molecular level but also for the smooth muscat aroma; however these two Fianos (Fi16, Fi17) are very similar at the molecular level to suggest that they are the same variety. Unlike the Fiano di Avellino, the origin of the other two Fianos is not known; their denomination might have been given to a

local aromatic grapevine to exploit the reputation of the well-known variety Fiano di Avellino.

The pattern of aggregation (Fig. 2) among the other aromatic varieties does not reveal any genetic relationship among them because they aggregate at a high level of dissimilarity. Even though some of them are identified under the common name of Malvasia (Ma1, Ma2, Ma3 and Ma4), a genetic diversity exists among these genotypes. Within this group there is the aromatic grapevine Coda di Volpe (Co25), which has been grown in Campania (region of southern Italy) since the Roman times having been described in an ancient book written in Latin by Pliny (Roy-Chevrier 1900) for its peculiar cluster morphology; unlike this variety, most of the ancient grapevines cannot be recognized nowadays because, since they did not present peculiar morphological characteristics, they were not described; thus they are not identifiable among those grown now.

A further evaluation of the relationships among the aromatic grape varieties taken into consideration has been obtained through the principal coordinate analysis (PCO) based on the same dissimilarity matrix. The plot (Fig. 3) confirms the pattern of aggregation of the cluster analysis for the genotypes Ma10, Ma11, Ma12 and Ma13; these can be considered only one variety as has been supposed in the cluster analysis. The same consideration can be drawn for the group of the Fiano grapevines (Fi16, Fi17). This plot also confirms the same aggregation as in the cluster analysis for those varieties which present low dissimilarity values, such as a group of Moscatos (Mo20, Mo22, Mo26) and a group of Malvasias (Ma5, Ma6, Ma7, Ma8, Ma9). No correspondence between the two analyses were found for all the other varieties that present a high coefficient of dissimilarity.

The results of this analysis reveal genetic diversity among and within the Moscato and the Malvasia grapevines. The pattern of aggregation in the cluster and principal-coordinate analyses does not support any classification which might include an aromatic grapevine group in *V. vinifera*. Thus AFLP markers allow us to detect genetic diversity among aromatic grapevines, as well as the presence of synonyms and homonyms among some genotypes.

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