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# Study of genetic diversity among inter-intraspesific hybrids and original grapevine varieties using AFLP molecular markers

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## Abstract

Several grapevine varieties are grown in vineyards worldwide including interspecific (*Vitis vinifera* × *Vitis* spp), intraspecific (*V. vinifera*L. × *V. vinifera* L.) and inter×intraspecific (interspecific×intraspecific) hybrids with unknown origin, ambiguous naming and genetic identity. In this study, the genetic relations among eighteen grapevine hybrids and original varieties (*Vitis vinifera* L.) which have mostly been described by ampelography data were analysed using AFLP (Amplified Fragments Length Polymorphism) molecular marker technology. AFLP polymorphic fragments generated by a combination of restriction digestion and PCR amplifications were assessed for analysis of the polymorphisms among accessions. Polymorphic bands were scored and genetic similarity (GS) was calculated by Dice coefficient. Cluster analysis and principle coordinate analyses (PCO) of the results addressed the genetic distance among inter-intraspecific hybrids and original grapevine varieties; detected the level of genetic similarity between varieties and specific mutant or clone; assessed the genetic relations among varieties involved in the same pedigree; recognized same genotypes under different names (synonymes) and proposed the genetic identity for an unknown, in ampelography data variety cultivated in UK vineyards.

***Keywords:*** fingerprinting; hybridization; pedigree; synonymes; *Vitis* spp; *Vitis vinifera*

## Introduction

According to the two Vitis databases [Vitis international variety catalogue (http://www.vivc.bafz.de/index.php) and European Vitis database (http:// www.genres.de/eccdb/vitis/)], the total number of *Vitis* species varieties and genotypes existing in the grapevine collections worldwide, has been estimated to about 40.000 variety names belonging to approximately 18.500 varieties (Dettweiler and Eibach, 2003). Although classic ampelography (Galet, 1979) and other morphometric methods (Swanepoel and deVilliers, 1987; Rubio and Yuste, 2004) have been successfully used to differentiate grapevine varieties, they are particularly complex for the identification and study of genetic relations among varieties, while they are of inadequate sensitivity for discrimination at the clonal level (Mullins et al., 2000). In order to study the diversity within grapevine germplasm collections, analyse the relatedness of varieties and to identify multiple genotypes within supposedly homogeneous clonal cultivars, molecular markers like Restriction Fragment Length Polymorphism (RFLPs; Grando et al. 1995), Random Amplified Polymorphic DNA (RAPDs; Buscher et al. 1994**;** Stavrakakis and Biniari, 1998), Amplified Fragment Length Polymorphism (AFLPs**;** Ergül et al. 2006) and microsatellites or Simple Sequence Repeat (SSR**;** Lefort and Roubelakis-Angelakis, 2001**;** This et al., 2004) have been used over the last decades.

AFLP is a molecular marker technique for fingerprinting genomic DNA based on PCR technology that can be used for DNAs of any origin and complexity (Vos et al. 1995). In grapevine, AFLPs have been widely used as a tool for determining the identity of specific grapevine varieties (Fossati et al., 2001), to assess the genetic variation among clones (Blaich et al., 2008) and to address intraspecific and interspecific genetic and phylogenetic relationships (Cervera et al., 2005; de Andrès et al., 2007); visualizing DNA polymorphism between samples without prior sequence knowledge using only a limited set of genetic primers.

Using the AFLP technique with six primers combinations, the aim of this study was to analyse the genetic diversity among eighteen grapevine accessions

**Table 1.** The eighteen grapevine varieties (name, original pedigree and species) analysed by AFLPs, according to Vitis International Variety Catalogue and European Vitis Database of Federal Centre for Breeding Research on Cultivated Plants (BAZ)-Grapevine Breeding Institute 'Geilweilerhof' of Germany.

| Varieties | Original pedigree | Species |
|---|---|---|
| Kerner | Trollinger × Riesling | Intraspecific cross |
| Kernling | Kerner Mutation | Intraspecific cross |
| Kettern | Unknown | Unknown |
| Müller-Thurgau | Riesling × Madeleine Royale | Intraspecific cross |
| Perlan | Chasselas Blanc synonym | *V. vinifera* L. |
| Chasselas Blanc | Original variety | *V. vinifera* L. |
| Phoenix | [(Silvaner × Riesling) × Müller-Thurgau] × S.V. 12-375 | Intraspecific × Intrespecific cross |
| Regent | [Silvaner × Müller-Thurgau] × Chambourcin | Intraspecific × Intrespecific cross |
| Reichensteiner | Müller-Thurgau × (Madeleine d'Angevine × Calabreser Froehlich) | Intraspecific cross |
| Riesling Weiss | Heunisch Weiss (or Traminer) × *V.* Sylvestris | Intraspecific cross |
| Scheurebe | Riesling Weiss × Unknown | Intraspecific × Unknown cross |
| Siegerrebe | Madeleine d'Angevine × Traminer Rot | Intraspecific cross |
| Madeleine d'Angevine | Malingre × Madeleine Royal | Intraspecific cross |
| Rondo | Zarya severa × Saint Laurent D'A | Intrespecific × Intraspecific cross |
| Gagarin Blue* | Saperavi Severnyi × Muscat Hamburg | Intrespecific × Intraspecific cross |
| Triomphe d'Alsace | MG 101-14 S.P. × Knipperle | Interspecific cross |
| Michurinetz | *V. amurensis* × Getsh | Interspecific cross |
| Seyval Blanc | Seibel 5656 × Seibel 4986 | Interspecific cross |

***** Skelton (2001)

in order to show the genetic distance and understand the genetic relations among three interspecific hybrids (Seyval Blanc, Michurinetz, and Triomphe d'Alsace), ten intraspecific hybrids (Kerner, Kernling, Müller-Thurgau, Reichensteiner, Riesling Weiss, Scheurebe, Siegerrebe, Madeleine d'Angevine), four inter× intra-specific hybrids (Phoenix, Regent, Rondo, Gagarin Blue), two *Vitis vinifera* varieties (Perlan and Chass-elas Blanc) and an unknown in ampelography data variety cultivated in UK vineyards called Kettern.

## Materials and methods

### *DNA extraction*

The grapevine varieties taken into consideration are listed in Table 1. Young leaf samples from eighteen cultivars were collected from Eglantine vineyard in Nottinghamshire, UK and were transported to the laboratory in liquid nitrogen. The samples were freeze-dried at -40ºC and stored at -20ºC. Total DNA was extracted from 20 mg powdered freeze-dried vine leaves using DNeasy Plant Mini Kit (Qiagen) follow-ing the manufacturer's protocol.

### *Production of AFLP amplified fragments*

AFLP analyses were performed according to Vos et al. (1995) with a few modifications. Two different restriction enzymes (*Eco*RI & *Mse*I) were used simult-aneously for the digestion of genomic DNA. The samples were placed in a 37ºC PCR machine overnight and then incubated at 70ºC to inactivate the restriction enzymes. The digestion of DNA was followed by the ligation of adapters consist of a core sequence and an enzyme-specific sequence for each of the restriction enzymes. The AFLP pre-amplification reaction was primed using primers which are complementary to the adaptors *Eco*RI and *Mse*I with an additional selective 3′ nucleotide (*Eco*RI + A/ *Mse*I +C). Pre-amplification PCR with primers having one single selective nucleotide was performed for 20 cycles with the following cycle profile: a 30 s DNA denaturation step at 94ºC, a one min annealing step at 56ºC, and one min extension step at 72ºC. For the selective amplification reactions six primers combinations were used. Selective amplification of restriction fragments was carried out using primers with 3 selective bases at their 3′ end on the *Eco*RI primers and 3 selective nucleotides on the *Mse*I primers (Table 2). The *Eco*RI primer for each combination was radioactively labelled by phosphorylation of the 5′ end using [$\gamma^{-33}$] ATP and T4 polynecluotide kinase. The selective amplification reaction was performed by touchdown PCR under the following PCR conditions for 37 cycles: 2 min for DNA denaturation step at 94ºC, a 30 s annealing step at 65ºC, and 1 min extension at 72ºC for 1 cycle. The annealing temperature in the first cycle was 66ºC, and was subsequently reduced in each cycle by 1ºC for the next 11 cycles. The PCR reaction was continued with 30 s at 94ºC, 30 s at 56ºC, 60 s at 72ºC for the 25 remaining cycles. Finally the reaction was completed at 72ºC for 2 min. At the end of PCR the samples were denatured by adding an equal volume (10 µl) of formamide dye [98% formamide, 10mM EDTA (pH 8.0), 0.05% bromophenol blue**,** 0.05% xylene cyanol] to each reaction. The mix (final volume 20 µl was heated for 3 min at 90ºC and held at 4ºC. Five µl of each sample were loaded on a 40% denaturing polyacrylamide gel. The denatured samples (5 µl of samples per lane) were loaded onto the gel and run at 65 W for 2 h. An invitrogen 330 bp marker ladder was

**Table 2.** Number of amplified fragments and polymorphic bands and the average polymorphism (%) produced by six primer combinations during the AFLP analysis of the eighteen grapevine varieties.

| Primer combinations | Number of amplified fragments | Number of polymorphic bands | Average polymorphism (%) |
|---|---|---|---|
| **1.** Eco + ACA/ Mse + CAC | 60 | 24 | 40.0 |
| **2.** Eco + ATG/ Mse + CAC | 80 | 19 | 23.75 |
| **3.** Eco + ATG/ Mse + CAA | 60 | 11 | 18.33 |
| **4.** Eco + ATC/ Mse + CAC | 55 | 10 | 18.18 |
| **5.** Eco + ACT/ Mse + CTG | 50 | 11 | 22.0 |
| **6.** Eco +ACC/ Mse + CAA | 60 | 16 | 26.66 |

also loaded. The ladder was radioactively labelled with [$\gamma^{-33}$] ATP. The gel was transferred on 3 MM paper and dried on a gel dryer for approximately 2 h. The dried gel was placed in an X-ray cassette with a Fuji medical X-ray film for the appropriate time according to the radioactivity of the samples. X-ray films were developed in an Amersham Life Science Hypoprosesor in the dark room. Polymorphic amplification products visualized by autoradiography were scored manually.

***Data analysis***

Amplified DNA fragments were scored for the presence "1" or the absence "0". Genetic similarity (GS) between two varieties was calculated by Dice coefficient, while for GS analysis among the eighteen varieties a dendrogram was constructed by cluster analysis based upon the UPGMA (Unweighted Pair Group Method with Arithmetic Averages) algorithm using PAST (Paleontological Statistics) software package (Hammer et al., 2001). Input data were also processed by Principle Coordinate Analysis (PCO) for the analysis of first two coordinates. The study of analysis was based on information from Vitis International Variety Catalogue and European Vitis Database of Federal Centre for Breeding Research on Cultivated Plants (BAZ)-Grapevine Breeding Institute 'Geilweilerhof' of Germany (Maul and Eibach, 2003) (Table 1).

**Results**

The amplified fragments produced by AFLPs were separated according to length. The resulting amplified fragments showing the same mobility were considered as identical DNA fragments. The size of amplified products ranged from 100 bp to over 300 bp (Fig 1). The six primer-pairs generated a total of 365 amplified fragments of which 89 (24.5%) were clearly polymorphic over all the genotypes, with average polymorphism between 18.1 and 40% (Table 2). According to Dice coefficient, the GS between two varieties ranged from 0.4 to 0.96 (Table 3). The highest GS was evident between Kerner and Kerling (0.961), Riesling and Kettern (0.955), and Chasselas Blanc and Perlan (0.919). On the other hand, the lowest GS was observed between Seyval Blanc, Triomphe d'Alsace and Michurinetz with the remaining varieties. According to the analysis of dendrogram, grapevine accessions were grouped in clusters as shown in Fig 2. The eighteen varieties were separated in two main groups. The first group included the hybrids of Seyval

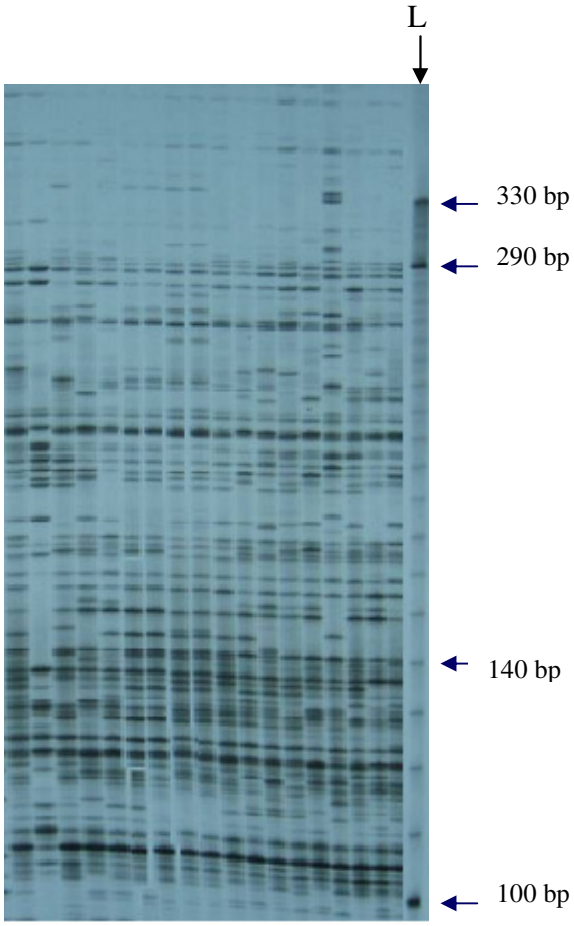


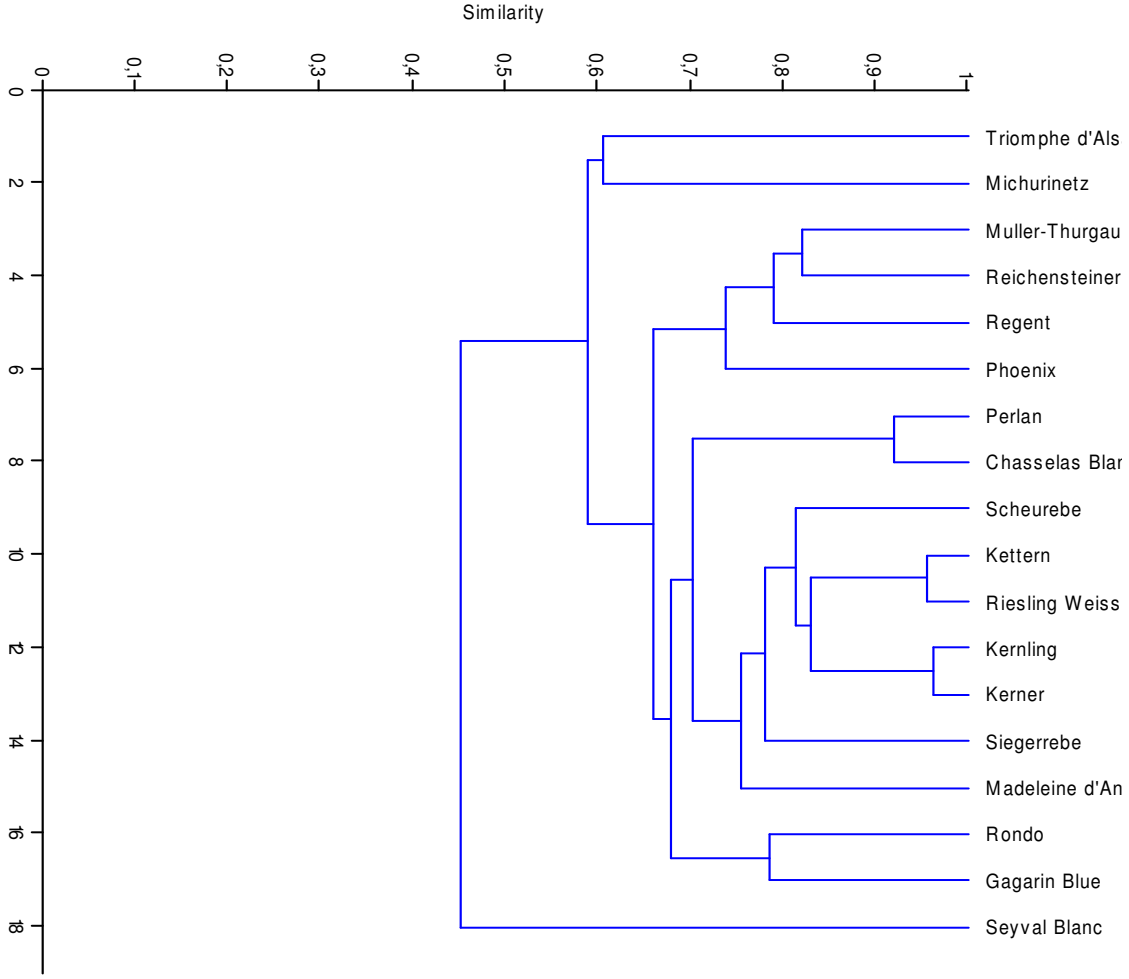

**Fig 1.** AFLPs gel analysis of eighteen grapevine varieties together with an invitrogen 330 bp marker ladder (L). A number of polymorphic bands are visible among the cultivars showing the genetic similarity of the population. The DNA fingerprints were generated using the primer combination No1 (*Eco* + ACA/ *Mse* + CAC). The arrows at the right of the gel indicate the marker size.

Blanc, Triomphe d'Alsace and Michurinetz with GS lower than 0.6, and the second group included the remaining hybrids and original varieties with GS higher than 0.6. Further- more, the second group was separated in four sub-groups and two extra independent varieties. The first sub-group included Müller-Thurgau, Phoenix, Regent, Reichensteiner varieties (Müller-Thurgau's sub-group). The second sub-group included the Perlan and Chasse- las Blanc varieties (original varieties sub-group).

**Table 3.** Genetic similarity value of eighteen grapevine varieties based on the AFLP amplified fragments obtained with six primer combinations using Dice coefficient.

| | Kerner | Kernling | Kettern | Madeleine d'Angevine | Michurinetz | Müller-Thurgau | Perlan | Chasselas Blanc | Phoenix | Regent | Reichensteiner | Riesling Weiss | Scheurebe | Seyval Blanc | Siegerrebe | Rondo | Gagarin Blue | Triomphe d'Alsace |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Kerner | 1 | | | | | | | | | | | | | | | | | |
| Kernling | 0.961 | 1 | | | | | | | | | | | | | | | | |
| Kettern | 0.844 | 0.848 | 1 | | | | | | | | | | | | | | | |
| Madeleine d'Angevine | 0.75 | 0.752 | 0.833 | 1 | | | | | | | | | | | | | | |
| Michurinetz | 0.551 | 0.526 | 0.543 | 0.442 | 1 | | | | | | | | | | | | | |
| Müller-Thurgau | 0.7 | 0.7 | 0.8 | 0.735 | 0.477 | 1 | | | | | | | | | | | | |
| Perlan | 0.682 | 0.666 | 0.752 | 0.722 | 0.561 | 0.684 | 1 | | | | | | | | | | | |
| Chasselas Blanc | 0.659 | 0.673 | 0.742 | 0.703 | 0.5 | 0.705 | 0.919 | 1 | | | | | | | | | | |
| Phoenix | 0.612 | 0.61 | 0.712 | 0.645 | 0.411 | 0.741 | 0.695 | 0.695 | 1 | | | | | | | | | |
| Regent | 0.666 | 0.666 | 0.716 | 0.672 | 0.506 | 0.78 | 0.666 | 0.635 | 0.738 | 1 | | | | | | | | |
| Reichensteiner | 0.741 | 0.727 | 0.755 | 0.75 | 0.533 | 0.821 | 0.8 | 0.758 | 0.695 | 0.788 | 1 | | | | | | | |
| Riesling Weiss | 0.83 | 0.818 | 0.955 | 0.777 | 0.536 | 0.773 | 0.71 | 0.695 | 0.687 | 0.66 | 0.721 | 1 | | | | | | |
| Scheurebe | 0.813 | 0.833 | 0.793 | 0.705 | 0.449 | 0.702 | 0.596 | 0.636 | 0.584 | 0.611 | 0.643 | 0.776 | 1 | | | | | |
| Seyval Blanc | 0.515 | 0.553 | 0.495 | 0.446 | 0.406 | 0.558 | 0.522 | 0.591 | 0.454 | 0.585 | 0.606 | 0.484 | 0.561 | 1 | | | | |
| Siegerrebe | 0.779 | 0.782 | 0.737 | 0.754 | 0.517 | 0.579 | 0.642 | 0.626 | 0.597 | 0.601 | 0.672 | 0.689 | 0.678 | 0.457 | 1 | | | |
| Rondo | 0.743 | 0.745 | 0.724 | 0.672 | 0.571 | 0.627 | 0.704 | 0.692 | 0.625 | 0.666 | 0.685 | 0.648 | 0.711 | 0.525 | 0.764 | 1 | | |
| Gagarin Blue | 0.655 | 0.655 | 0.655 | 0.678 | 0.588 | 0.654 | 0.654 | 0.65 | 0.659 | 0.615 | 0.684 | 0.581 | 0.618 | 0.452 | 0.704 | 0.784 | 1 | |
| Triomphe d'Alsace | 0.642 | 0.641 | 0.66 | 0.533 | 0.605 | 0.612 | 0.693 | 0.657 | 0.607 | 0.568 | 0.607 | 0.672 | 0.653 | 0.533 | 0.568 | 0.702 | 0.597 | 1 |

**Fig 2.** Cluster analysis of eighteen grapevine varieties representing their genetic similarity based on the AFLP amplified fragments obtained with six primer combinations (see Table 2). The dendrogram was constructed applying the UPGMA clustering method to the Dice estimates.



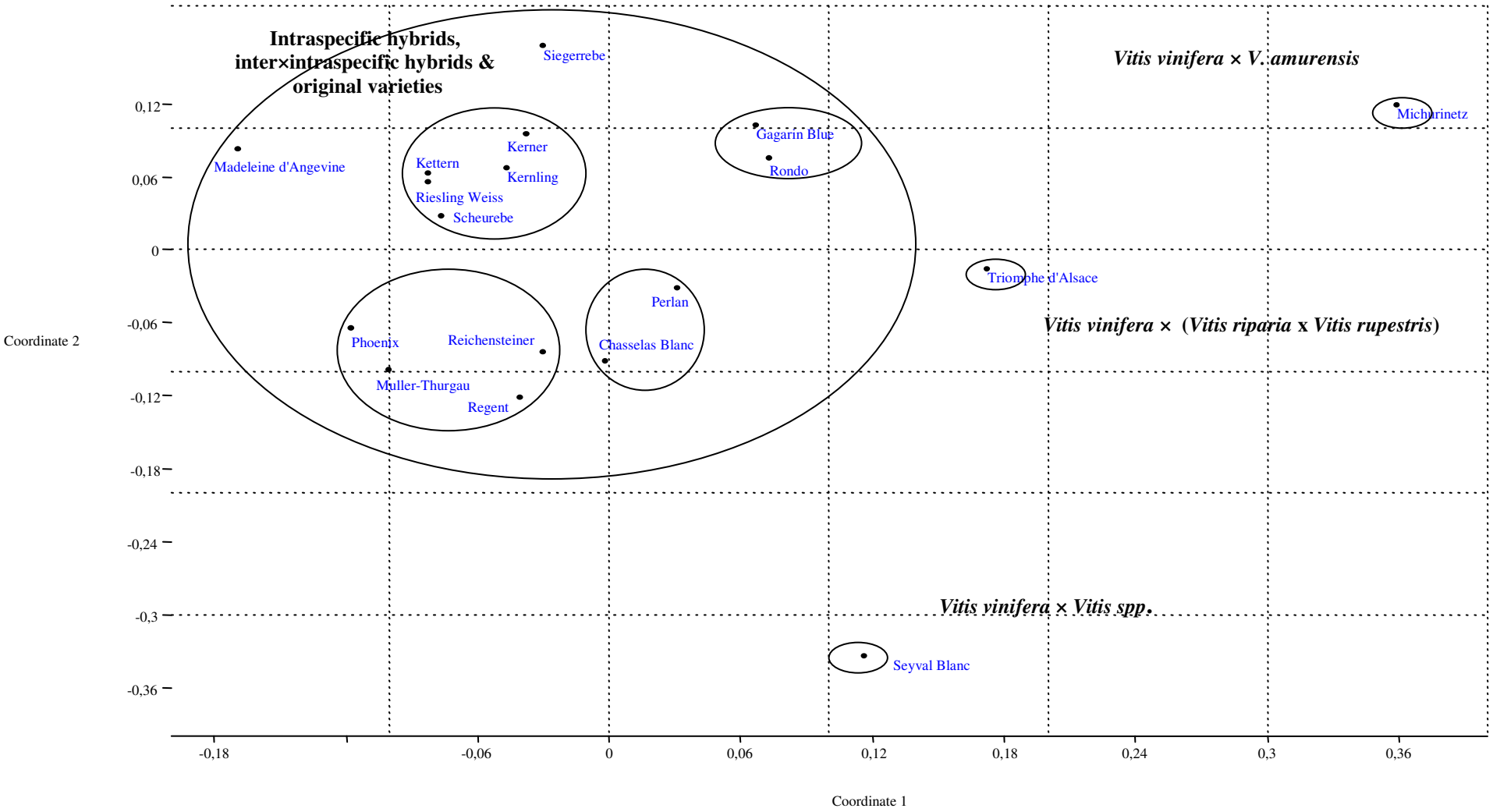

The varieties Kettern, Kernling, Riesling, Kerner, Scheurebe belonged to a third sub-group (Riesling's sub-group), while Rondo and Gagarin Blue created the forth sub-group. Siegerrebe and Madeleine d'Angevine were shown as independent varieties from the other four sub-groups.

According to PCO results (Fig 3), the majority of the varieties could be placed in a main group consisting of thirteen hybrids and two original varieties, while the Triomphe d'Alsace, Michurinetz and Seyval Blanc are autonomous. The main group could be divided in four sub-groups and two independent varieties similarly to the cluster analysis.

## Discussion

Hybridisation has been used by grape breeders to improve the productivity and resistance of varieties to environmental stress for the past 150 years. Nowadays, many interspecific hybrids offer resistance to frost, phylloxera and several fungal diseases (Korbuly, 2000**;** Pollefeys and Bousquet 2003), while breeding programs based on intraspecific hybridisation have been very successful for table and wine grapes (Mullins et al., 2000). The definition of genetic distance among grapevine varieties and inter-intraspecific hybrids by AFLP molecular markers, was firstly introduced by Cervera et al. (1998a, b) and strongly recommended by Martinez-Zapater et al. (2000). Based on the results of these principle studies, the first group of three interspecific hybrids of Seyval Blanc (*V. vinifera* × *V.* spp.), Michurinetz (*V. amurensis* × *V. vinifera*) and Triomphe d'Alsace [(*V. riparia* × *V. rupestris*) × *V. vinifera* ] were clearly differentiated from the second group of inter×intraspecific hybrids, intraspecific hybrids and original varieties, showing GS between 0.3 and 0.6 according to cluster analysis (Fig 2). On the other hand, the group of remaining hybrids and two original varieties analysed by this study showed GS from 0.6 to 1 (Fig 2) addressing the genetic distance proposed by the two principle studies. Within the same group, the relation between Kerner and Kernling with GS higher than 0.9, can be considered as relation between an original variety and a somalclonal variant (Martinez-Zapater et al., 2000), confirming the ampelography data that Kernling is a mutant of Kerner. Also, Kettern variety and Riesling showed GS higher than 0.9. There is no information concerning Kettern in ampelography data, other than an account of a variety grown in UK with the name Kettern-Riesling. These results strongly suggested that Kettern is a mutation of Riesling. In addition, a GS higher than 0.9 was also presented between Chasselas Blanc and Perlan confirming that these accessions are synonyms accord-

**Figure 3.** The scatter plot of the first and second principal coordinates analysis on eighteen grapevine varieties based on the AFLP amplified fragments obtained with six primer combinations (see Table 2).



ing to Vitis International Variety Catalogue (Table 1).

Scheurebe is another intraspecific crossing that is well established in a number of regions. Riesling has been confirmed as one parent of Scheurebe but according to Vitis International Variety Catalogue, the second parent of this cross has been questioned. Based to the cluster analysis, the GS among Scheurebe and Riesling's group (Kerner, Kernling, Kettern and Riesling) is between 0.8 and 0.9 (Fig 2). The same variability of GS was shown also between, the varieties Riesling or Kettern, with the intraspecific hybrids of Riesling, Kerner (Trollinger × Riesling) and Kernling (Kerner mutation). Similarly, the GS between the intraspecific variety Reichensteiner [Müller-Thurgau × (Madeleine d'Angevine × Calabreser Froehlich)] and Müller-Thurgau, was between 0.8 and 0.9 too (Fig 2). According to Cervera et al. (1998b), GS between 0.8 and 0.9 was shown among closely related accessions like sibling table grape cultivars known to be derived from the same cross. In this study, it is also suggested that GS between 0.8 and 0.9 could be shown between the parent and the 1st offspring generation varieties or offspring mutant, such as Riesling with Scheurebe, Riesling with Kerner, Riesling with Kernling, Müller-Thurgau with Reichensteiner. Madeleine d'Angevine and Siegerrebe (Madeleine d'Angevine × Traminer Rot), is another parent- 1st offspring relation with GS between 0.8 and 0.9 showed by cluster analysis (Fig 2).

One of the most widely grown intraspecific hybrid worldwide is Müller-Thurgau. This variety was considered a Riesling × Silvaner cross, but the involvement of Silvaner in this cross was excluded using DNA analysis (Buscher et al., 1994). However, the parentage of the variety was suggested to be the Riesling × Madeleine Royale by Dettweiler et al. (2000). The cluster analysis in this study, showed that Müller-Thurgau is not considered as first generation offspring of Riesling because they belong to different sub-groups (Fig 2). This result could be due to the selected primer pairs used in this study and further investigation is required. On the other hand, Müller-Thurgau was grouped successfully with hybrids belonging to the same pedigree as parent of cross or as ancestor (Müller-Thurgau's sub-group). According to cluster analysis, Reichensteiner [Müller-Thurgau× (Madeleine d'Angevine × Calabreser Froehlich)] and Müller-Thurgau were shown as 1st generation-offspring and parent respectively with GS higher than 0.8, while the two inter×intraspecific hybrids, Regent [Silvaner × Müller-Thurgau] × Chambourcin] and Phoenix [Bacchus (a Müller-Thurgau offspring) × Seibel] as 2nd generation-offsprings of Müller-Thurgau. Two other inter× intraspecific hybrids Rondo (Zarya Severa × Saint Laurent D'A) and Gagarin Blue (Seperavi Severnyi × Muscat Hamburg), which share the same ancestor *V. amurensis*, were also grouped successfully with GS close to 0.8 (Fig 2).

Simirar to cluster analysis, scatter plot of the first and second principal coordinates analysis addressed the genetic distance among these eighteen varieties (Fig 3). According to PCO, the interspecific hybrids of Triomphe d'Alsace, Seyval Blanc and Michurinetz were autonomous. Furthermore the genetic relations among the remaining grapevine varieties addressed the level of genetic similarity between varieties and specific mutant or clone (Kerner and Kernling; Riesling and Kettern); the synonymes (Chasselas Blanc and Perlan); the genetic relations among varieties involved in the same pedigree (Kerner, Kernling, Kettern, Riesling, Scheurebe; Müller-Thurgau, Phoenix, Regent, Reichensteiner; Rondo and Gagarin Blue; Madeleine d'Angevine and Siegerrebe) and finally the genetic identity for Kettern, an unknown in ampelography data variety cultivated in UK vineyards.

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