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Grapevine rupestris stem pitting-associated virus (GRSPaV) and vein necrosis: effect of genetic variability in symptoms expression

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INDEX

Page

Abstract 03

1. **General introduction** 05

1.1 Vein Necrosis 06

- *Diagnosis*

- *Aetiology*

1.2 Grapevine Rupestris Stem Pitting-associated Virus 09

- *Association with RSP*

- *Association with Syrah decline*

- *Association with Vein Necrosis*

1.3 Overall objectives 16

2. **Molecular detection of Grapevine Rupestris Stem Pitting-associated Virus in grapevine accessions**

2.1 Introduction 17

2.2 Materials and Methods 18

2.3 Results 23

2.4 Discussion 30

3. **Molecular and serological characterization of ORF 5 (CP gene)**

3.1 Introduction 31

3.1.1 Molecular characterization 31

3.1.2 Serological characterization 33

3.2 Materials and Methods 36

3.2.1 Molecular characterization 36

3.2.2 Serological characterization 41

3.3 Results 43

3.3.1 Molecular characterization 43

3.3.2 Serological characterization 51

3.4 Discussion 52

4. Molecular characterization of a fragment of ORF 1 (replicase gene)

4.1 Introduction 54

4.2 Materials and Methods 56

4.3 Results 60

4.4 Discussion 66

5. Biological indexing for Vein Necrosis Disease

5.1 Introduction 68

5.2 Materials and Methods 70

5.3 Results 75

5.4 Discussion 79

6. Final conclusions 80

Bibliographic references 83

Appendix 1 90

Abstract

Vein necrosis (VN) is a virus-like disease of grapevine that is latent in V. vinifera *and in most of rootstock and hybrids, with the only exception of rootstock 110 Richter (*V. berlandieri x V. rupestris). *Despite its ubiquitous presence in many grape growing regions, its economic impact on production is still largely unknown, because of its latency in* V. vinifera*. Though the causal agent of VN has not been identified, as the disease is able to be transmitted by grafting, VN is then considered a virus-like disease. Recent studies suggested a correlation between VN and the virus Grapevine rupestris stem pitting-associated virus (GRSPaV*).

In this research, the presence and molecular variability of GRSPaV in grapevine accessions from Tuscany and California was studied through RT-PCR and sequence analyses. RT-PCR analyses with group-specific primer sets distinguished three molecular groups of GRSPaV variants. All of the three groups were detected both in Tuscan and Californian grapevine accessions.

Amplification, cloning and sequencing of two distinct viral genomic regions were carried out in order to obtain a finer molecular characterization of GRSPaV isolates. Depending on the genomic region analyzed, five (ORF5) to six (ORF1) phylogenetic groups of virus variants were observed. Notably, the sixth phylogenetic group identified according to sequence analysis of a 299 nucleotides fragment of ORF1 comprises only GRSPaV variants derived from Tuscan grapevine accessions, that show low nucleotide identity with any other GRSPaV sequence deposited in GenBank. This could represent a new and possibly yet unknown phylogroup of GRSPaV variants, that has named "group 5".

Linking the results of GRSPaV molecular characterization with those of VN biological indexing previously conducted on the accessions object of the study, a strong correlation emerged between VN positive plants and infections by GRSPaV variants belonging to phylogenetic groups 2a and 2b.

In order to further assess this correlation, a biological indexing trial was performed for 37 grapevine accessions from Italy (Tuscany and Apulia), USA, Portugal and Japan. Results obtained confirmed the hypothesis that only phylogenetic groups 2a and 2b of the virus are able to induce VN symptoms on indicator host 110 Richter.

These findings show evidence of a different role of GRSPaV variants in VN determinism; only phylogenetic groups 2a and 2b appear to be able to induce symptoms of VN. This could suggest the possibility to diagnose VN by RT-PCR assays with group-specific primers, representing a much faster, cheaper and more simple way to monitor the diffusion and incidence of this disease.

1. General introduction

Grapevine (*Vitis* spp.) is the most widely cultivated fruit crop worldwide, encompassing about 8 million hectares of arable land (Vivier and Pretorius, 2002) with about 67200 kilotons produced in 2007 (FAOSTAT, 2009). Since their first appearance over 65 million years ago in Eurasia, south western Asia, Mediterranean region and central Europe (de Saporta, 1879), grapes from *V. vinifera*, have been used extensively for making wine throughout the world (This *et al.*, 2006). Other members of the family *Vitaceae* used in wine grape breeding programs, especially for breeding rootstocks and inter-specific hybrids, include *Muscadinia rotundifolia*, *V. aestivalis*, *V. amurensis*, *V. berlandieri*, *V. candicans*, *V. caribaea*, *V. champinii*, *V. cinerea*, *V. cordifolia*, *V. labrusca*, *V. longii*, *V. riparia*, *V. rupestris* and *V. simpsonii* (This *et al.*, 2006). Wine, the main product of wine-grapes, has been made for millennia. Besides their economic importance as alcoholic beverages, wines also have ancient historical connections with the development of human culture (McGovern, 2004) and, in recent years, the health benefits of wine consumption in moderate amounts have been recognized (German and Walzem, 2000).

Italy is ranked 2nd internationally in grape production behind China. In Italy, grape production is the 1st most economically important agricultural crop. (FAOSTAT, 2011).

Although the grape berry is used for multiple purposes, wine production from cultivars of *V. vinifera* have the highest economic impact (Mullins *et al.*, 1992).

In 2012, 4089000 tons of wine have been produced in Italy, 2385904 of which were destined to export, for an economic value 6075404 $ (FAOSTAT, 2012).

Tuscany has a long and well-established wine-makers tradition: its wines are known and appreciated in the whole world. In the last decades, the industry has strongly expanded in the region, significantly contributing to the regional economy. Currently, wine grapes are grown in about 60327 hectares (ha) and wine production reach 2097621 tons (ISTAT, 2012).

Because grapevines are commonly vegetatively propagated, spread of many debilitating viruses occurs through cuttings, resulting in economic losses to growers. Since virus

diseases cannot be controlled by economically feasible chemical agents similar to fungicides, strategies aimed at the management of grapevine viruses are largely directed at preventing virus spread by utilizing virus-tested planting material.

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On a worldwide basis, the grapevines appear to be infected with more viruses than any other perennial woody species. Currently, the International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG) recognized more 60 viruses reported on genera *Vitis* and *Muscadinia*, belonging to 27 genera and exhibiting the whole set of genome types: single-stranded DNA, double-stranded DNA, double-stranded RNA, single-stranded negative-sense RNA, single-stranded positive-sense RNA (Martelli, 2012).

The ‘traditional’ virus diseases such as fanleaf, leafroll, rugose wood and fleck represent a group of well-known disorders in several grape-growing countries around the world (Hewitt, 1954; Goheen *et al*., 1958; Savino *et al*., 1989; Martelli, 1993), while many of the other viral and virus viral-like disorders are of limited geographic distribution. The aetiology of many of these diseases remains largely unsolved.

1.1 Vein necrosis

Vein necrosis (VN) is a virus-like disease, reported for the first time in France in 1973 (Legin and Vuittenez), and later found in some regions of the former Soviet Union (Milkus and Schterenberg, 1978), in Italy and Bulgaria (Martelli *et al*., 1978). To date it is widespread in several European countries and the basin of the Mediterranean, where, at times, has reached high levels of incidence (Credi *et al*. 1985). Surveys conducted in Tuscany on "Sangiovese", "Aleatico”, "Mammolo", “Prugnolo gentile” and “Vermentino bianco” have confirmed the very high presence even in this region, reaching frequency percentages ranging from 61.0% to 90.0%. (Triolo and Materazzi, 2000; Triolo and Materazzi, 2004; Bouyahia *et al*., 2006).

The disease is latent in *V. vinifera* and in most of the species and hybrids rootstocks, with the exception of *V. rupestris* x *V. berlandieri* Richter 110 (110 R). On this last host, in fact, the infection causes very noticeable macroscopic symptoms, causing significant

reductions in growth accompanied by the appearance, from the late spring, of necrosis on veinlets and veins on the underside of the leaf blade (Fig. 1). With time, necrotic spots also appear on the upper side of the leaf. Symptoms generally appear first on the leaves at the base of the shoots, and then gradually affect younger leaves, causing a premature yellowing and fall. Severe strains may induce necrosis of tendrils and dieback of green shots, with an almost complete cessation of growth and, eventually, death of the 110 R host plant.

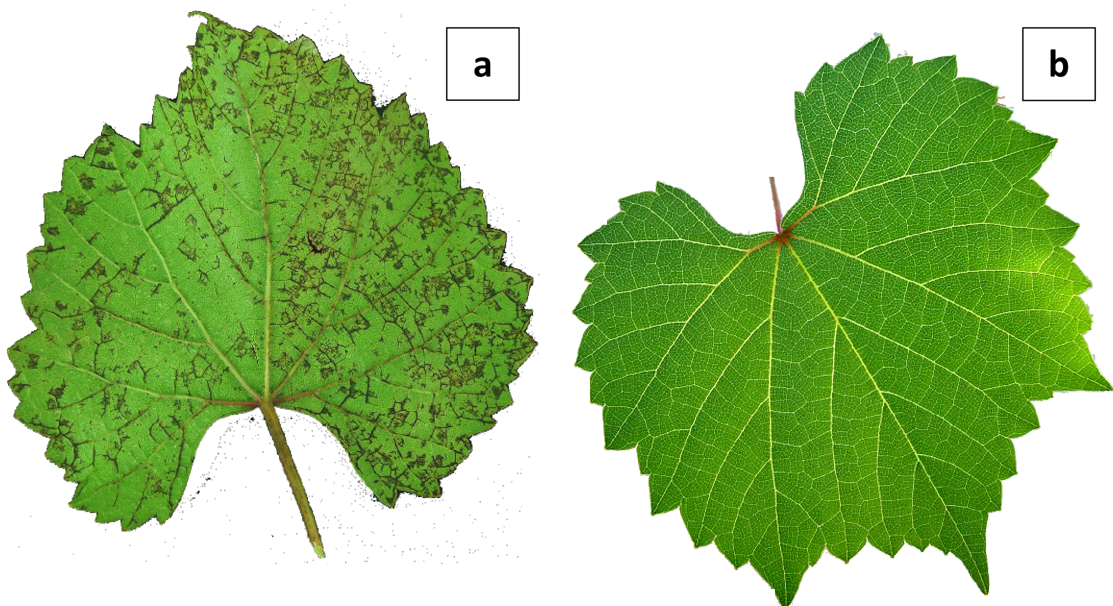

Figure 1. VN symptoms on the underside of 110R leaf blades. Pictures of symptomatic (**a**) and symptomless (**b**) 110R leaves.

In a classification concerning the economic importance of the most common systemic diseases of grapevine, vein necrosis is part of the third group (Tab. 1) (Martelli, 2002). In fact, despite its wide diffusion, its economic impact on production is still largely unknown, because of its latency in *V. vinifera* and in most rootstock hybrids.

Table. 1. Classification of the main systemic diseases of grapevine based on their economic importance. (Martelli , 2002).

Group I	- *Grapevine degeneration* (fanleaf and other nepoviruses)	
	- *Leafroll complex*	
	- *Rugose wood complex*:	
		Rupestris stem pitting
		Kober stem grooving
		Corky bark
		LN 33 stem grooving
	- *Fleck*	
	- *Phytoplasma*:	
		Flavescence Dorée
		Bois Noir
Group II	- *Enations*	
Group III	- ***Vein necrosis***	
	- *Vein mosaic*	

VN diagnosis

Since the etiology of VN is still unknown, biological assay with indicator species of the genus *Vitis* is the only diagnostic method available. The diagnosis can be easily carried out through indexing with rootstock hybrid 110 R, with symptoms appearing about 2-3 months after the graft. Diagnosis time can be reduced micrografting shoots of the indicator onto the plants to be tested by green grafting (Walter *et al*., 1990). With this technique, symptoms appear within 30-60 days.

VN aetiology

Though the causal agent VN has not been identified, as the disease is able to be transmitted by grafting, VN is then considered a virus-like disease (Martelli *et al*., 1978). In the past, within the phloem of infected vines, were observed like bodies phytoplasma (Milkus and Schterenberg, 1978), but their relationship with this disease have never been proven (Triolo *et al*., 1998).

Recent studies carried out in Apulia allowed to consider a close correlation between VN and the virus *Grapevine rupestris stem pitting-associated virus* (GRSPaV) (Bouyahia *et al*., 2005), a foveavirus initially isolated from plants affected by "*Rupestris* stem pitting", one of the syndromes of the "Rugose Wood” (RW) complex.

The spread of VN, at the moment, seems to be linked exclusively to the marketing of infected propagating material. In fact, the disease can not be transmitted by mechanical inoculation on herbaceous plants. No alternate hosts or vectors are known.

1.2 Grapevine rupestris stem pitting-associated virus

Grapevine rupestris stem pitting-associated *virus* [GRSPaV; genus *Foveavirus*; family *Betaflexiviridae* (King *et al*., 2012)] is a graft-transmissible virus (Fig, 2) widely distributed in many grape-growing regions around the world (Minafra and Boscia, 2003).

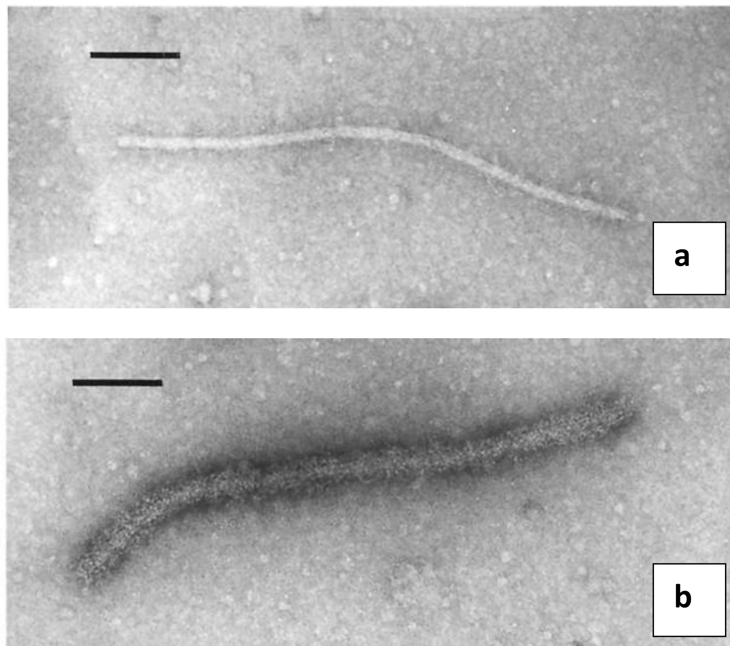

Figure 2. Particles of non-decorated (**a**) and decorated (**b**) Grapevine Rupestris Stem Pitting-associated virus observed by Immunosorbent electron microscopy (Petrovic *et al*., 2003)

The positive-sense, single-stranded RNA of GRSPaV is 8725 nucleotides (nt) in length, excluding the poly A tail, and encodes five open reading frames (ORFs) (Meng *et al.* 1998; Zhang *et al.* 1998) (Fig. 3). The 3'-most ORF (ORF 5) encodes a 28 kiloDalton (kDa) coat protein (CP), whereas the 5'-most ORF (ORF 1) encodes the viral replicase

polyprotein of 244 kDa. ORFs 2, 3 and 4 encode polypeptides of 24, 13 and 8 kDa, respectively, which together constitute the 'triple gene block' (TGB) proteins TGBp1, TGBp2 and TGBp3 (Meng *et al.*, 1998). Recent studies have shown that TGBp1 has both a cytosolic and nuclear distribution, whereas both TGBp2 and TGBp3 are associated with the endoplasmic reticulum (Rebelo *et al.*, 2008). A putative ORF6 encoding a 14kDa protein of unknown functions, partially overlapping the CP, has been reported in some variants of GRSPaV (Meng *et al.*, 1998; Zhang *et al.*, 1998; Lima *et al.*, 2006a).

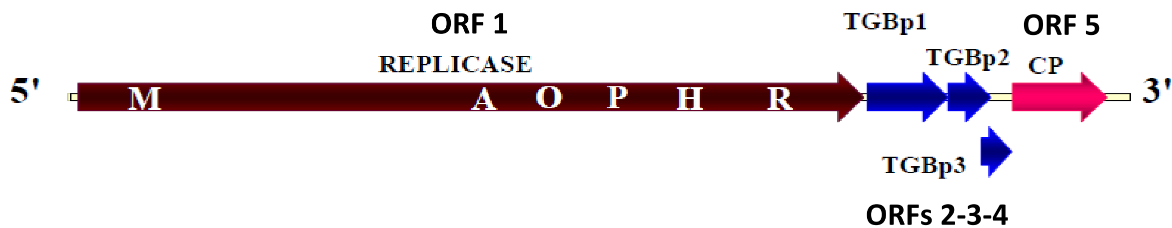

Figure 3. Genome organization of GRSPaV. Each block arrow represents an Open Reading Frame (ORF). ORFs with same colour have similar functions. Domains of the replicase (**M**= Methyltransferase, **A**= AlkB, **O**= Out-like peptidase, **P**= Papain-like protease, **H**= RNA helicase, **R**= RNA-dependent RNA polymerase) are shown. The map was drawn based on the complete genome sequence available on GenBank (AF057136).

Grapevine (*Vitis* spp.) is the only known natural host of GRSPaV. The virus can be spread via vegetative propagation and grafting (Minafra and Boscia, 2003) and possibly through seeds of infected grapevine (Lima *et al.*, 2006a). Although GRSPaV has been detected in the pollen of infected grapevines (Rowhani *et al.*, 2000), its spread through pollen is not confirmed. The virus is not transmissible by mechanical inoculations and no biological vector has been reported. Studies have indicated the biological association of GRSPaV with rupestris stem pitting (RSP), one of the 4 disorders of the rugose wood complex (Meng *et al.*, 1998; Zhang *et al.*, 1998). RSP shows pitting symptoms on the woody cylinder below the graft union in 'St. George' grapevines (*V. rupestris* Scheele).

GRSPaV occurs in nature as a family of molecular variants (Martelli and Boudon Padieu, 2006). Several studies conducted on the CP gene and on the helicase domain in ORF 1 revealed that GRSPaV comprise a family of sequence variants segregated into 3 or 4 phylogenetic groups (Meng *et al*. 1999; Rowhani *et al*. 2000; Casati *et al*. 2003; Santos *et*

al. 2003; Terlizzi and Credi 2003; Lima *et al*. 2006a; Habili *et al.*, 2005; Nolasco *et al*., 2006; Nakaune *et al.*, 2008).

Recently, new groups of GRSPaV variants have been described. Goszczynski (2010), analyzing a 199 base pairs (bp) fragment of the RdRP domain in ORF 1, clustered GRSPaV variants from South Africa in 5 groups. Terlizzi *et al*. (2011), using a new broad-spectrum primer set designed in the polymerase domain of ORF 1, described 7 groups.

Moreover, several studies of different grapevine diseases suggested that such variants might have a diverse pathological role in RSP (Meng *et al*., 2005), VN (Bouyahia *et al*., 2006) and Syrah decline (Lima *et al*., 2006b) aetiology, some of which may not elicit RSP symptoms when graft-inoculated on the indicator 'St George' (Meng *et al.*, 2005). In addition, some variants that are latent in *Vitis vinifera* cultivars and in most American *Vitis* species and hybrids can produce necrosis of the veinlets when graft-inoculated on 110R (Bouyahia *et al.*, 2005). In recent years, GRSPaV, along with a novel marafivirus and several viroids, has been documented in Californian Syrah grapevines showing decline symptoms (Al Rwahnih *et al.*, 2009), although the role of GRSPaV in Syrah decline is yet to be resolved.

<u>*Association with RSP*</u>

Rupestris stem pitting (RSP) is a syndrome of the RW complex, which causes a distinctive basipetal pitting in the wood extending downward from the graft union (visible after the removal of the cortex), with consequent reduction of vigor of the plant (Fig. 4).

Symptoms occur only on *V. rupestris* St. George and a few other rootstocks related to *V. rupestris*. Despite RSP is the most widespread RW syndrome, information about its etiology were rather scarce until a few years ago.

Although the transmissibility of RSP for grafting let assume the viral nature of the disease, only researches conducted in 1998 permitted to make a significant step forward, with the identification of a virus closely associated with RSP, called Grapevine Rupestris Stem Pitting-associated Virus (GRSPaV), described for the first time as the probable causal agent of this syndrome (Zhang *et al*., 1998).

Figure 4. Rupestris stem pitting symptoms. Healthy (left) and RSP-positive (right) trunks; bark was removed to reveal pitting and grooving symptoms (photo by University of California, Integrated Viticulture).

The cause-effect relationship between GRSPaV and RSP remains, however, still to be completely elucidated. In recent years, thanks to the development of more powerful diagnostic protocols for the detection of GRSPaV by RT-PCR and Western blot, many authors detected GRSPaV in plants that indexed negative to RSP. In detail, the use of the two diagnostic techniques, singularly or in combination, on accessions negative to the biological indexing, allowed to highlight that a significant percentage of these plants, between 22.0% and 66.0%, were infected by GRSPaV. (Meng *et al*., 1999a; Meng *et al*., 2000; Nolasco *et al*., 2000; Meng and Gonsalves, 2003; Bouyahia *et al*., 2005).

The finding, by Meng *et al*. (2005), that a variant of GRSPaV, detected on *V. rupestris* St. George, (GRSPaV-SG1), did not induce RSP symptoms on this indicator, may partially explain the low percentages of association listed above and may suggest a differential role of different GRSPaV genetic variants in RSP aetiology.

Association with "Syrah decline"

The " Syrah decline" is a disease that was firstly described in France (Renault-Spilmont *et al.*, 2002), the symptoms of which appear on vines of the variety "Syrah" or "Shiraz", causing leaf reddening, scorching, swollen graft unions, and stem necrosis symptoms (Fig. 5); generally the scion dies after one or two years, while the rootstock continues to grow.

Nowadays the aetiology of these symptoms has not yet been clarified. In the last years, a growing number of vineyards in many grape-growing countries have been reporting disorders with vines of the variety "Syrah". The symptoms exhibited by plants are similar to those of the "Syrah decline", with abnormalities at the grafting point, early reddening of the leaves and slow maturation of the fruits, which have a low concentration of sugars.

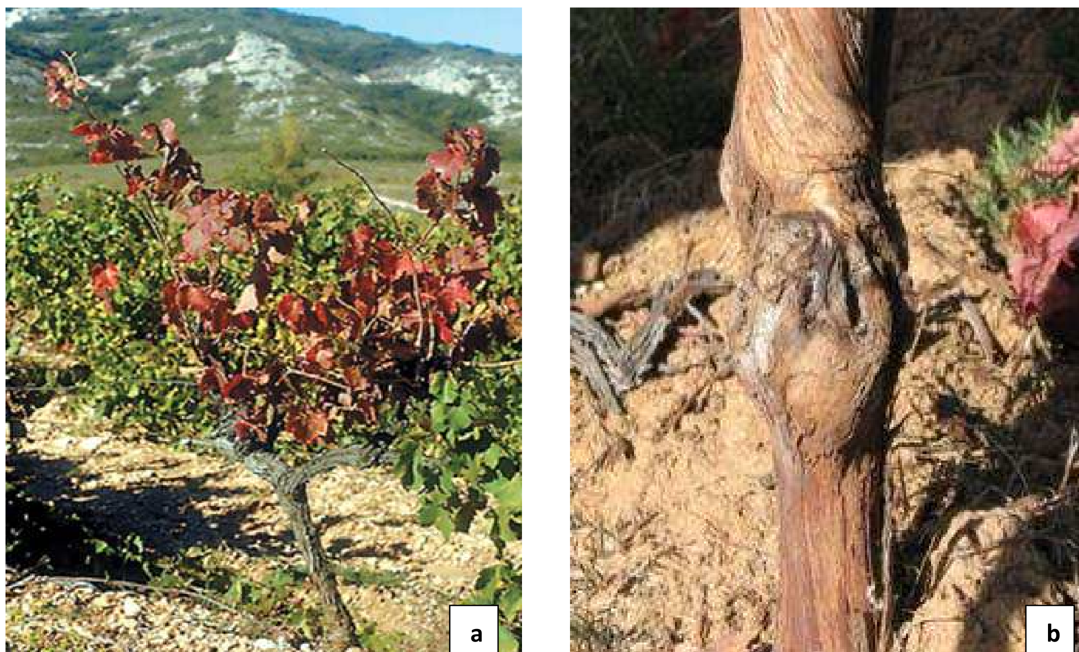

Figure 5. Syrah decline symptoms: (**a**) leaf reddening affecting the entire canopy of an infected accession and (**b**) swelling and cracking at the graft union (Battany *et al.*, 2004)

In Australia (Habili *et al.*, 2005) and California (Lima *et al.*, 2006b), GRSPaV was detected in plants of *V. vinifera* "Syrah" presenting symptoms of "Syrah Decline".

Analysis conducted by RT-PCR on extracts of leaf petioles of “Syrah” vines from California to verify the presence of all the major grapevine-infecting viruses have indicated the presence of only GRSPaV (Lima *et al*., 2006b).

The genome of this variant of GRSPaV , called GRSPaV-SY , was composed of 8725 nucleotides and organized in 6 ORFs.

The nucleotide sequence of GRSPaV-SY had a homology of 77.0% compared to the sequences of 4 reference variants already known (GRSPaV , GRSPaV-1, GRSPaV-SG1, GRSPaV-BS), and these 4 isolates are much more related to each other than with GRRPaV-SY.

The capsid protein gene (ORF 5) of GRSPaV-SY is the region of the genome that has the highest nucleotide and aminoacidic homology (83.0-84.0% and 91.0-92.0%, respectively) with the sequence of other variants of GRSPaV, while the replicase gene (ORF 1) is the more variable region (75.0-76.0% nucleotide and 85.0% aminoacidic homology).

A similar survey was conducted in Australia, on plants of *V. vinifera* ‘Syrah’ presenting symptoms similar to those of the "Syrah decline" (Habili *et al*., 2005). Even in this case, by RT-PCR was rediscounted the presence of a variant of GRSPaV in symptomatic plants. The analysis and comparison of the nucleotide sequence of a region of 628 bp of the gene for replicase indicates a low nt identity between this variant and the other GRSPaV isolates previously reported.

Nonetheless, recent papers suggest that frequent presence of this variant in Syrah decline-affected plants, reported by Habili *et al*. (2005) and Lima *et al*. (2006b), could may be only accidental and seem to reject the association of GRSPaV and GRSPaV-SY variant in the aetiology of Syrah decline (Goszczynski *,* 2010; Beuve *et al*., 2013).

Association with vein necrosis disease

Studies carried out in Apulia in 2005, showed a high presence of GRSPaV in infected plants by VN , suggesting that this foveavirus may be the correlated to the aetiology of the disease (Bouyahia *et al*., 2005).

In particular, after that 109 (52.4%) of 218 grapevine accessions tested positive to the biological indexing for VN, all these plants were investigated by Enzyme-linked

Immunosorbent Assays (ELISA tests) to check the presence of the 8 main grapevine viruses: grapevine fanleaf nepovirus (GFLV), grapevine leafroll associated ampelovirus 1 (GLRaV-1), grapevine leafroll associated closterovirus 2 (GLRaV-2), grapevine leafroll associated ampelovirus 3 (GLRaV-3), grapevine leafroll associated closteroviridae 7 (GLRaV-7), grapevine vitivirus A (GVA), grapevine vitivirus B (GVB) and grapevine fleck maculavirus (GFkV). Only 16 (7.3%) of 218 samples tested were found to be infected by at least one of the 8 viruses and, moreover, the ELISA-positive samples were equally divided between plants positive or not to VN. Further investigations, carried out by RT-PCR, allowed to detect an extremely high presence of GRSPaV. In detail, among 174 plants surveyed , 93 of which indexed positive and 81 of which indexed negative to VN , GRSPaV was founds in as many as 91 (97.8 %) of the 93 VN-positive plants and only in one of the negative ones, therefore showing a close association between the foveavirus GRSPaV and VN.

Recently, studies about the association of VN and GRSPaV were carried out in our department, conducted on grapevine accessions collected in Tuscany during clonal and sanitary improvement programs. Each of these accessions were previously tested by ELISA for the 9 main viruses infecting grapevine (GFLV, arabis mosaic nepovirus (ArMV), GFkV, GVA, GVB, GLRaV-1, GLRaV-2, GLRaV-3 and GLRaV-7) and biologically indexed on specific *Vitis* indicators for the presence of grapevine degeneration, leafroll, fleck, Kober stem grooving, LN33 stem grooving, Rupestris stem pitting, corky bark, enation, vein necrosis and vein mosaic diseases.

Molecular assay (RT-PCR) for GRSPaV detected the virus in 53 out of 62 plants, 38 (71.7%) of which presented specific VN symptoms. In order to understand the reason of the VN-negative, but GRSPaV-positive accessions, each sample was tested with PCR primers able to amplify 3 distinct molecular groups of the virus, named G I, G II, and G III (Rowhani *et al*., 2000).

The results obtained suggested a differential role of GRSPaV variants in VN aetiology, with all of the 38 plants infected by G I variants that were positive to the VN indexing (Bouyahia *et al*., 2006).

These findings, besides confirming the relationship between GRSPaV and VN, let hypothesize that only a group a GRSPaV variants could be involved in the aetiology of this disease. Since then, studies about GRSPaV molecular characterization and this virus-disease association were carried out, and are actually ongoing, on a larger sample size of different geographical origin, in order to better clarify the role of GRSPaV in the determinism of VN disease.

1.3 Overall objectives

The aims of this research comprise the detection of GRSPaV and its genetic variants in grapevine accessions from Tuscany and California, their molecular and serological characterization and the investigation of the pathogenetic role of different virus variants in VN symptoms expression.

This has been achieved through the following steps:

- Detection of different GRSPaV variants in the grapevine accessions object of the study.
- Amplification, cloning and sequencing of ORF5 and a portion of ORF1 and phylogenetic analysis.
- Serological detection of different virus variants by Western Blot.
- Biological indexing of grapevine accessions infected by different groups of GRSPaV variants with VN indicator 110 R for the diagnosis of VN.

2. Molecular detection of Grapevine Rupestris Stem Pitting-associated Virus in grapevine accessions

2.1 Introduction

GRSPaV is a member of the genus *Foveavirus* in the newly established family *Betaflexiviridae* (King *et al*., 2012). It is not mechanically transmissible (Martelli and Jelkmann, 1998) and no natural vector has been found until now. GRSPaV has been reported from almost all vine growing areas in the world, where it seems to have a high incidence. The aetiological role of GRSPaV, however, remains to be elucidated.

Data regarding the genomic variability of RSPaV can be found in several papers (Meng *et al*., 1999b; Meng *et al*., 2000; Rowhani *et al*., 2000; Soares *et al*., 2000; Casati *et al*., 2003; Lima *et al*., 2003; Santos *et al*., 2003; Terlizzi and Credi 2003). Although different primers operating in different genomic regions of the virus were adopted for these studies, many authors quite consistently clustered GRSPaV variants in 3 or 4 groups. Rowhani *et al*. (2000) designed, in the CP gene of GRSPaV, 3 sets of group-specific primers able to amplify different virus variants.

The availability, in our department, of an experimental collection field of grapevine accessions collected throughout Tuscany, made it possible to further investigate the relationship between GRSPaV and VN disease.

In the present study, we performed two-step RT-PCR diagnosis of GRSPaV on RNA extracted from phloem scrapings with both universal and group-specific primer sets.

The aims of the work were:

a) to check the efficiency of universal primers for GRSPaV detection;

b) to discover if one or more GRSPaV variants groups infect grapevine accessions in Tuscany;

c) to select “pure sources” grapevine accessions infected by only one GRSPaV group, in order to study the biological properties of single virus variants.

2.2 Materials and Methods

Plant material

Object of the study were 99 grapevine accessions, comprising both *V. vinifera* and rootstock varieties (Tab. 2).

62 of these accessions were collected in different locations of Tuscany within clonal and sanitary improvement programmes and are being held in a collection field in the Department of Food Agriculture and Environment (DAFE) at the University of Pisa. The collection comprises rootstocks *V. rupestris*, *V. berlandieri x V. riparia* Kober 5BB and *V. vinifera* varieties Sangiovese, Aleatico, Ciliegiolo, Tenerone, Mammolo, Colorino, Verdello, Prugnolo gentile, Canaiolo.

All of these grapevine plants previously tested negative to ELISA tests for the presence of 9 viruses associated with major detrimental diseases: GFLV, ArMV, GFkV, GLRaV-1, GLRaV-2, GLRaV-3, GLRaV-7, GVA and GVB. Each putative clone was also indexed on 6 to 8 replicates of each of the following 6 indicators: (**I**) *V. vinifera* cv. Cabernet franc for leafroll; (**II**) LN 33 (Couderc 1613 x *V. berlandieri*), for corky bark, LN 33 stem grooving and enation disease; (**III**) Kober 5BB (*V. berlandieri* x *V. riparia*), for Kober stem grooving; (**IV**) *V. rupestris* cv. St. George, for fanleaf, fleck and rupestris stem pitting; (**V**) 110R for VN; (**VI**) *V. riparia* Gloire de Montpellier for vein mosaic (Triolo and Materazzi, 2000; Triolo and Materazzi, 2004).

No symptomatic responses from any of the indexed accession was recorded for leafroll, grapevine degeneration, corky bark, LN33 stem grooving, Kober stem grooving, rupestris stem pitting, enation disease and vein mosaic, but when indexed on 110 Richter, 38 of the 62 accessions showed VN symptoms. (Triolo and Materazzi, 2000; Triolo and Materazzi, 2004).

The remaining 37 grapevine accessions are part of the grapevine clones collected worldwide and held at Foundation Plant Services (University of California, Davis). They all previously tested negative to PCR assays for the detection of GFLV, ArMV, GFkV, GLRaV -1, -2, -3, -4, -5, -6, -7, GVA, GVB, GVD, GVE, ToRSV and TRSV (only one accession, “Inzolia”, was positive to GVA).

Table 2. List of the grapevine accessions object of the study, species/hybrid and the place of the collection field where they have been maintained.

Accession	Species / hybrid	Collection at:
5V-20-1	*V. rupestris*	S. Piero a Grado, Pisa
5V-20-2	*V. rupestris*	S. Piero a Grado, Pisa
5V-20-3	*V. rupestris*	S. Piero a Grado, Pisa
5V-20-4	*V. rupestris*	S. Piero a Grado, Pisa
5V-20-5	*V. rupestris*	S. Piero a Grado, Pisa
RUP1	*V. rupestris*	S. Piero a Grado, Pisa
BSK3-1	*V. berlandieri x V. riparia*	S. Piero a Grado, Pisa
BSK3-2	*V. berlandieri x V. riparia*	S. Piero a Grado, Pisa
BSK3-3	*V. berlandieri x V. riparia*	S. Piero a Grado, Pisa
BSK1	*V. berlandieri x V. riparia*	S. Piero a Grado, Pisa
1/18	*V. vinifera*	S. Piero a Grado, Pisa
2/1	*V. vinifera*	S. Piero a Grado, Pisa
5/1	*V. vinifera*	S. Piero a Grado, Pisa
34/46	*V. vinifera*	S. Piero a Grado, Pisa
AL.CAV.1	*V. vinifera*	S. Piero a Grado, Pisa
AL.CAV.4	*V. vinifera*	S. Piero a Grado, Pisa
AL.CAV.5	*V. vinifera*	S. Piero a Grado, Pisa
CA	*V. vinifera*	S. Piero a Grado, Pisa
CC4	*V. vinifera*	S. Piero a Grado, Pisa
CC5	*V. vinifera*	S. Piero a Grado, Pisa
CC6	*V. vinifera*	S. Piero a Grado, Pisa
CC8	*V. vinifera*	S. Piero a Grado, Pisa
CC10	*V. vinifera*	S. Piero a Grado, Pisa
CC13	*V. vinifera*	S. Piero a Grado, Pisa
CC17	*V. vinifera*	S. Piero a Grado, Pisa
CC19	*V. vinifera*	S. Piero a Grado, Pisa
CC21	*V. vinifera*	S. Piero a Grado, Pisa
CC24	*V. vinifera*	S. Piero a Grado, Pisa
CC25	*V. vinifera*	S. Piero a Grado, Pisa
CC26	*V. vinifera*	S. Piero a Grado, Pisa
CC29	*V. vinifera*	S. Piero a Grado, Pisa
CHI8	*V. vinifera*	S. Piero a Grado, Pisa
CIL.SIRIO2	*V. vinifera*	S. Piero a Grado, Pisa
COR20	*V. vinifera*	S. Piero a Grado, Pisa
COR33	*V. vinifera*	S. Piero a Grado, Pisa
COR35	*V. vinifera*	S. Piero a Grado, Pisa
COR36	*V. vinifera*	S. Piero a Grado, Pisa
FA	*V. vinifera*	S. Piero a Grado, Pisa
GALLO5	*V. vinifera*	S. Piero a Grado, Pisa
GALLO7	*V. vinifera*	S. Piero a Grado, Pisa
GALLO15	*V. vinifera*	S. Piero a Grado, Pisa
KA	*V. vinifera*	S. Piero a Grado, Pisa
LUM01	*V. vinifera*	S. Piero a Grado, Pisa
MSAS1	*V. vinifera*	S. Piero a Grado, Pisa
MSAS3	*V. vinifera*	S. Piero a Grado, Pisa
MLOB7	*V. vinifera*	S. Piero a Grado, Pisa
MLOB9	*V. vinifera*	S. Piero a Grado, Pisa
MLOC2	*V. vinifera*	S. Piero a Grado, Pisa
MLOC15	*V. vinifera*	S. Piero a Grado, Pisa
RA	*V. vinifera*	S. Piero a Grado, Pisa

Accession	Species / hybrid	Collection at:
SGSAS2	*V. vinifera*	S. Piero a Grado, Pisa
SGSAS3	*V. vinifera*	S. Piero a Grado, Pisa
SGSAS4	*V. vinifera*	S. Piero a Grado, Pisa
SG.SIRIO1	*V. vinifera*	S. Piero a Grado, Pisa
SM6/10	*V. vinifera*	S. Piero a Grado, Pisa
SME9	*V. vinifera*	S. Piero a Grado, Pisa
SMF13	*V. vinifera*	S. Piero a Grado, Pisa
SMH22	*V. vinifera*	S. Piero a Grado, Pisa
TENSAS1	*V. vinifera*	S. Piero a Grado, Pisa
V6F12	*V. vinifera*	S. Piero a Grado, Pisa
WA	*V. vinifera*	S. Piero a Grado, Pisa
YA	*V. vinifera*	S. Piero a Grado, Pisa
Albillo Mayor 01	*V. vinifera*	FPS Davis , California
Albillo Real 01	*V. vinifera*	FPS Davis , California
Arinarnoa 01	*V. vinifera*	FPS Davis , California
B2R24P69 01	*V. vinifera*	FPS Davis , California
B2R45P72 01	*V. vinifera*	FPS Davis , California
Barbera	*V. vinifera*	FPS Davis , California
Cabernet Franc 14	*V. vinifera*	FPS Davis , California
Cabernet Sauvignon 31	*V. vinifera*	FPS Davis , California
Cabernet Sauvignon 43	*V. vinifera*	FPS Davis , California
Chardonnay 68	*V. vinifera*	FPS Davis , California
Chardonnay 72	*V. vinifera*	FPS Davis , California
Chardonnay 83	*V. vinifera*	FPS Davis , California
Diamond Muscat	*V. vinifera*	FPS Davis , California
Green Hungarian	*V. vinifera*	FPS Davis , California
Inzolia	*V. vinifera*	FPS Davis , California
Kandhara	*V. vinifera*	FPS Davis , California
Madrone Moon Farm	*V. vinifera*	FPS Davis , California
Malaga	*V. vinifera*	FPS Davis , California
Merlot 18	*V. vinifera*	FPS Davis , California
Mourvedre	*V. vinifera*	FPS Davis , California
Negrette	*V. vinifera*	FPS Davis , California
Olmo 1266	*V. vinifera*	FPS Davis , California
Pinot Noir 22	*V. vinifera*	FPS Davis , California
Pinot Noir 54	*V. vinifera*	FPS Davis , California
Pinot Noir 78	*V. vinifera*	FPS Davis , California
Pinot Noir 85	*V. vinifera*	FPS Davis , California
Refosco 03	*V. vinifera*	FPS Davis , California
Ribolla Gialla	*V. vinifera*	FPS Davis , California
Riesling	*V. vinifera*	FPS Davis , California
Sangiovese 13	*V. vinifera*	FPS Davis , California
Schioppettino	*V. vinifera*	FPS Davis , California
Thomcord 02	*V. vinifera*	FPS Davis , California
Tinta Amarella	*V. vinifera*	FPS Davis , California
Trousseau	*V. vinifera*	FPS Davis , California
Zinfandel	*V. vinifera*	FPS Davis , California
Zinfandel 09	*V. vinifera*	FPS Davis , California

Sampling, RNA extraction and cDNA synthesis

Mature canes were collected from different part of the canopy in order to minimize possible variations due to uneven distribution of the virus in infected tissues.

Total RNA was extracted according to MacKenzie *et al.* (1997), with slight modifications. Briefly, 0.5 g of plant material was macerated in a plastic bag in presence of 5 ml grinding buffer containing 4 M guanidine isothiocyanate, 0.2 M sodium acetate pH 5,2, 5 mM EDTA, 2.5% (wt/vol) PVP-40 and 1% (vol/vol) 2-mercaptoethanol (added just before use). 2 ml of the homogenate was recovered and centrifuged at full speed for 5 minutes. 1 ml of the lysate was transferred into a new tube, mixed with 100 µl of 20% (wt/wol) sarkosyl and incubated at 70 °C for 10 minutes with intermittent shaking. Approximately 600 to 650 µl of homogenate was transferred to a QIAShredder column (RNeasy Plant Mini Kit, Qiagen) and the RNA extraction procedure continued according to the kit manufacturer's protocol. Total RNA was eluted from the RNeasy column using 50 µl of RNase-free sterile water and stored at -20 °C.

For synthesis of first-strand cDNA, 10 µl of RNA extract were mixed with 2 µl of random hexamer primers (Roche) (0.5 µg/µl), denatured at 95 °C for 5 min and quickly cooled on ice for 2 min. Reverse transcription was done for 1 h at 37 °C by adding 10 µl AMV Reverse Transcriptase 5X Reaction Buffer (Promega), 2.5 µl of 10 mM dNTPs mix, and 5 units Avian Myeloblastosis Virus (AMV) reverse transcriptase (Promega, USA) in a final volume of 50 µl.

GRSPaV diagnosis with broad spectrum primers

Molecular GRSPaV detection was performed by PCR using 2 sets of broad spectrum primers, aiming to understand if they would produce identical results, or if one primer set would be able to better detect GRSPaV.

Primers RSP13/RSP14 amplify a portion of the helicase domain in ORF1 (Meng *et al.*, 1999a), while RSP 5/6 (Santos *et al.*, 2006) are designed on ORF5, encoding the CP (Tab. 3).

Table 3. Broad-spectrum primers for the detection of GRSPaV used in this study, 5'-3' sequences and amplicon length.

Primer	Sequence (5'-3')	Amplicon
RSP 13	GATGAGGTCCAGTTGTTTCC	339 bp
RSP 14	ATCCAAAGGACCTTTTGACC	
RSP 5	CGYAGATTTTGCATGTACTA	258 bp
RSP 6	GCTTKCCTTCAGCCATAG	

5 µl of cDNA were added to PCR mixture yielding final concentrations of 1x GoTaq Buffer (Promega, USA), 1.5 mM $MgCl_2$, 0.4 mM each deoxynucleotide triphosphate, 0.2 µM each primer and 1 U Taq DNA Polymerase (Promega, USA) per reaction, in a total volume of 25 µl.

PCR reactions were performed with the following parameters: 3 min at 95 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 52 °C, and 35 sec at 72 °C, followed by a final extension of 7 min at 72 °C. PCR products (10 µl) were analyzed on a 1.5% agarose gel, stained with GelRed (Biotium, USA) and visualized under UV light.

GRSPaV diagnosis with groups specific primers

Following GRSPaV diagnosis, all of the 99 accessions were tested by PCR with GRSPaV group-specific primers (Rowhani *et al*., 2000), whose sequences were kindly supplied by Prof. Rowhani (University of California - Davis, USA). These degenerated primers, designed on ORF 5, are able to specifically amplify 3 distinct groups of molecular variants.

5 µl of cDNA were used in 25 µl PCR reactions prepared as previously described, with thermal cycle as follows: 3 min at 95 °C followed by 35 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 35 sec at 72 °C, followed by a final extension of 7 min at 72 °C.

2.3 Results

GRSPaV diagnosis with broad spectrum primers

Products of the expected size, 258 bp and 339 bp, were observed in agarose gels using primers RSP 5/6 and RSP 13/14, respectively.

In total, 99 samples from *V. vinifera* varieties and/or rootstocks were assayed by two-step RT-PCR.

- *Samples from Tuscany*

Samples were tested with both primer pair RSP 13/14 and RSP 5/6. When using primers RSP 13/14, GRSPaV was detected in 38 accessions (61.2%), while primer set RSP 5/6 was able to detect the virus in 53 (85.5%) samples, including all of the 38 previously positive to RSP 13-14. The remaining 9 accessions (14.5%) provided negative results with both primer pairs (Fig. 6; Tab. 4).

Interestingly, all of the accessions previously symptomatic to VN, tested positive to GRSPaV with primers RSP 13/14. Also, negative results were obtained with primers RSP 13/14 when testing accessions that resulted negative to VN indexing.

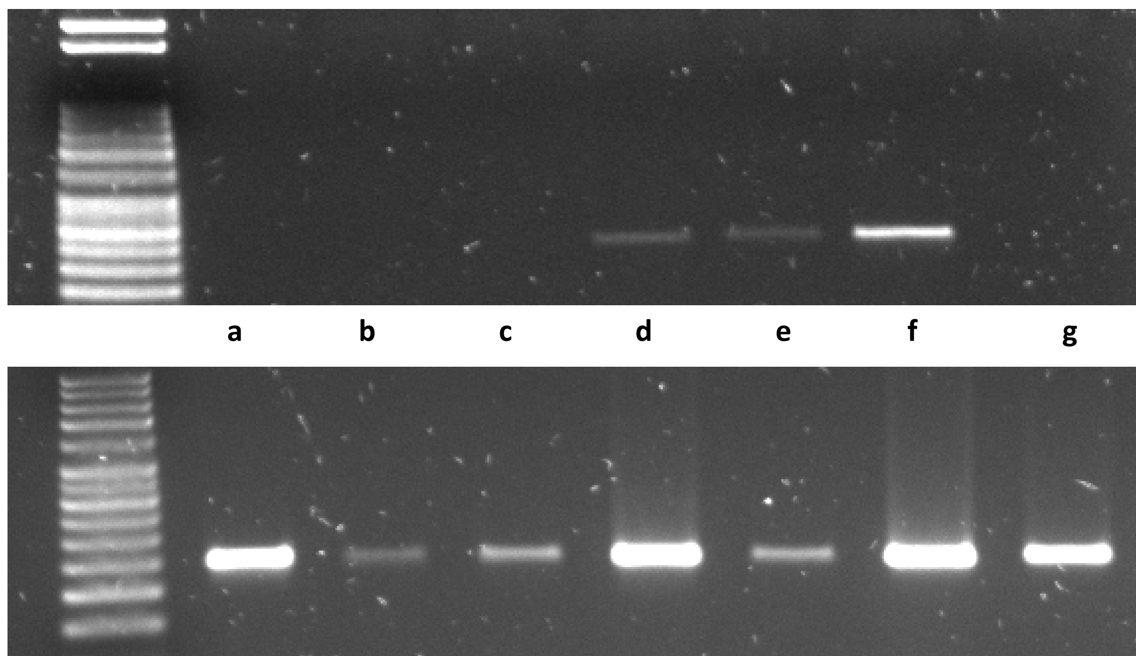

Figure 6. Results of RT-PCR with primer pairs RSP 13/14 (up) and RSP 5/6 (down) on agarose gel visualized by UV light. Samples, from left to right: **a**=Gallo7; **b**=Gallo15; **c**=SMH22; **d**=MLOB9; **e**=COR24; **f**=COR33; **g**=2/1.

Table 4. Results obtained with “broad-spectrum” primer sets RSP 5/6 and RSP 13/14 for the detection of GRSPaV

	Primer set	
	RSP 5/6	**RSP 13/14**
+	53	38
-	9	24
Total	62	62

- *Samples from California*

Accessions were tested with primer set RSP 5/6. All of the 37 accessions, as expected, showed the specific amplicon of 258 bp, resulting infected by GRSPaV.

- *Overall results of GRSPaV detection*

In total, GRSPaV was detected in 90 of the 99 tested plants, both in rootstock hybrids and *V. vinifera* varieties.

<u>*GRSPaV diagnosis with groups specific primers*</u>

Results obtained with group-specific primers matched those achieved with primer pair RSP 5/6. In fact, all of the plants tested positive with RSP 5/6 showed positive reaction with at least one of the group-specific primer pairs, while no one of the GRSPaV-negative accessions produced amplicons of the expected size.

All of the 3 different GRSPaV groups were observed in the accessions tested. GRSPaV variants detected by G I-specific primers were the most present and were detected in 53 accessions (58.9 %). Both group G II and G III variants were present in 46 (51.1%) of the 90 GRSPaV-infected plants (Fig. 7). The 3 groups, however, were quite evenly distributed (Fig. 8).

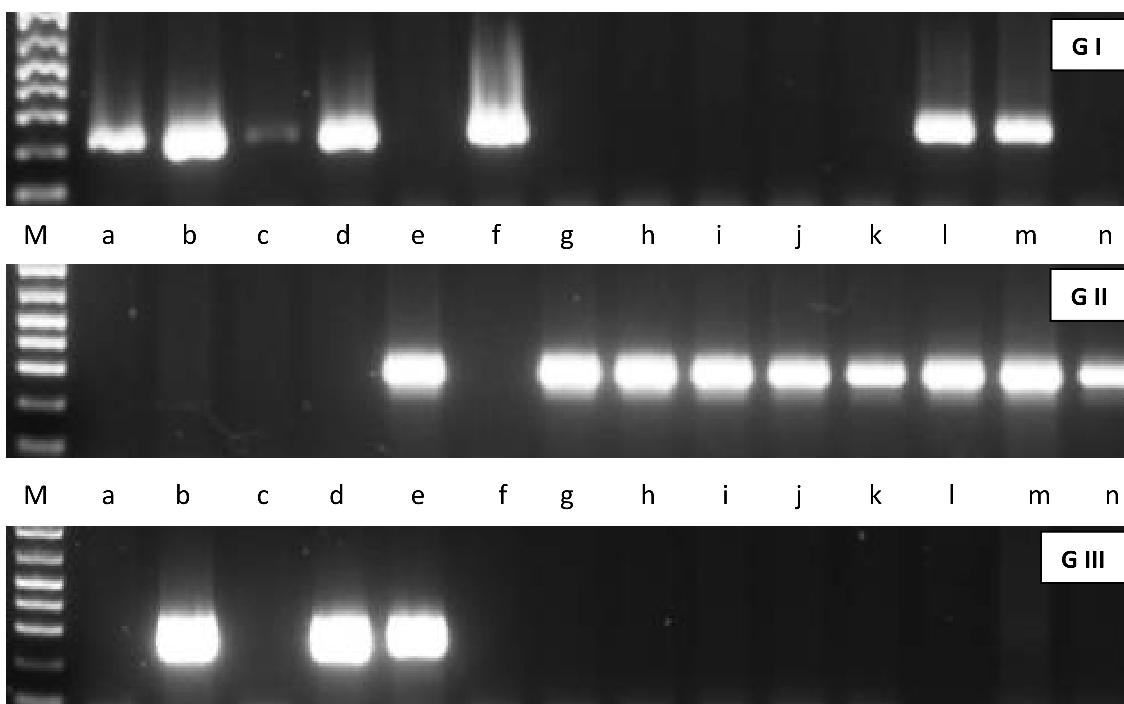

Figure 7. Results of RT-PCR with G I, G II and G III group-specific primer pairs on agarose gel visualized by UV light. Samples, from left to right: **M**=marker **a**=Pinot Noir85, **b**=Sangiovese13, **c**=Refosco03, **d**=Arinarnoa01, **e**=B2R24P69 01, **f**=Pinot Noir22, **g**=Zinfandel09, **h**=Olmo1266, **i**=Schioppettino, **j**=Thomcord02, **k**=Albillo Mayor01, **l**=Albillo Real01, **m**=Cabernet Franc14, **n**=Cabernet Sauvignon43.

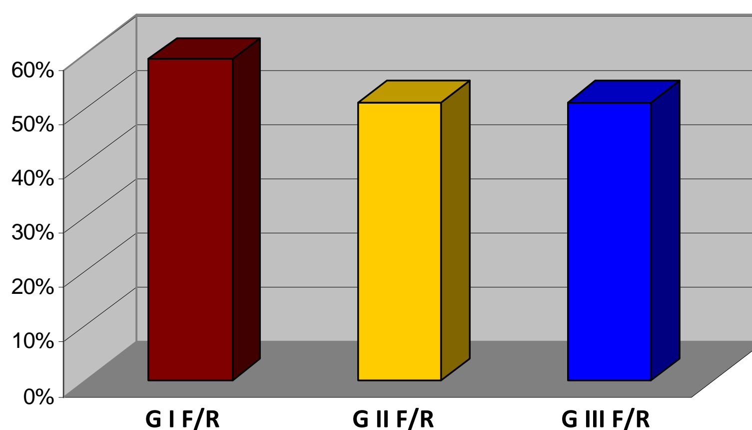

Figure 8. Incidence of the 3 groups of GRSPaV variants amplified by each group-specific primer set on the total amount of GRSPaV-infected plants

- Samples from Tuscany

Limited to the 53 GRSPaV-positive accessions from Tuscany, group G I and G III were the most widespread infecting, respectively, 38 (71.7 %) and 35 (66.0 %) plants. Group G II

variants were detected in a lower number of samples (24), representing 45.3% of the infected plants (Fig. 9).

It is interesting to observe that group G I variants were detected, singularly or in mixed infection, in all and only the 38 samples providing positive results to the biological indexing for VN and that previously tested positive to GRSPaV primer set RSP13/14.

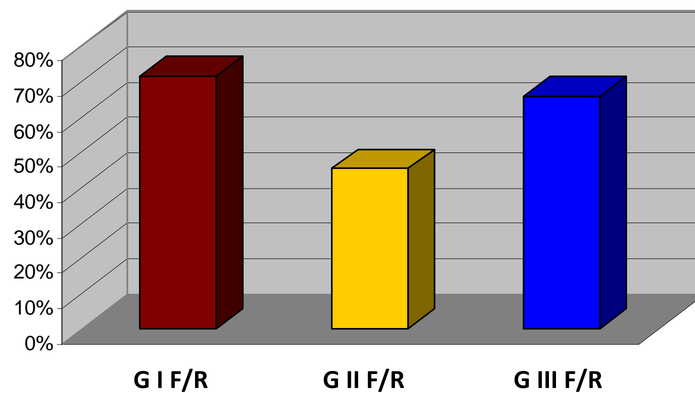

Figure 9. Incidence of group G I, G II, and G III infections on the total amount of GRSPaV infected accessions from Tuscany

- *Samples from California*

The distribution of GRSPaV variants in California differed from the situation in Tuscany, with group G II being predominant. It was detected in 21 out 37 accessions (56.8 %), while groups G I and G III were less widespread and were detected in 15 (40.5%) and 11 (29.7%) samples respectively (Fig. 10).

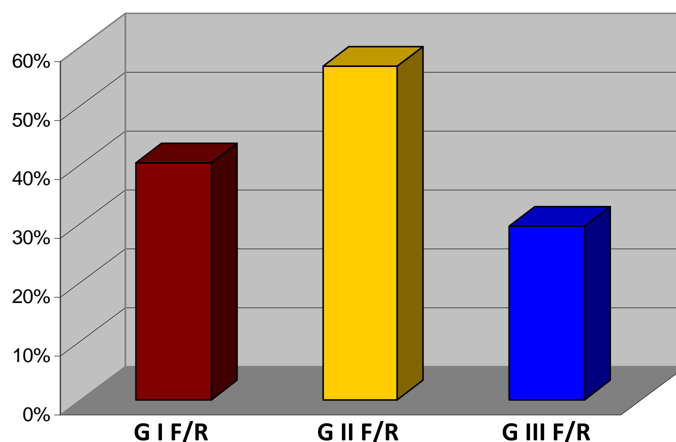

Figure 10. Incidence of group G I, G II, and G III infections on the total amount of GRSPaV infected accessions from California.

- Mixed infections

The occurrence of mixture of GRSPaV variants in the same isolate, as already described by Nolasco *et al.* (2006), was frequently observed in 44 (48.9 %) infected accessions. Mixed infection by variants of G I and G III was the most common, as it was detected in 19 (21.1%) out of 90 GRSPaV-infected accessions and a considerable amount of 11 samples (12.2 %) showed mixed infection by variants belonging to all of the 3 groups. Groups G II + G III and G I + G II infections were detected in 8 (8.9 %) and 6 (6.6 %) plants, respectively (Fig. 11).

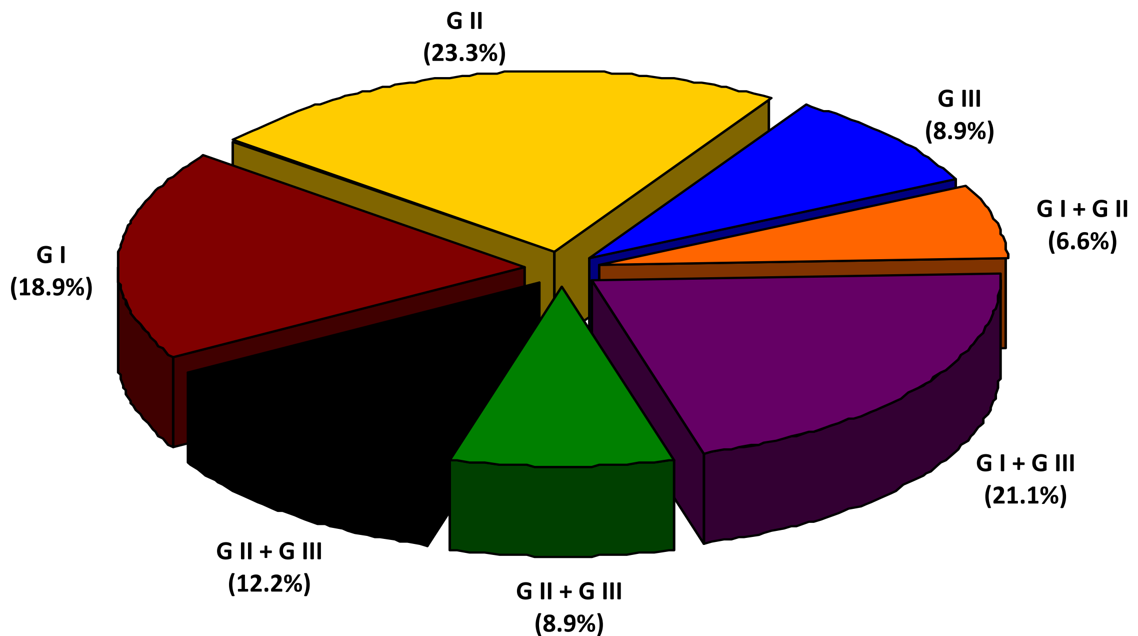

Figure 11. Incidence of different single and/or mixed infection combinations on the total amount of GRSPaV infected plants.

Details of the results obtained with each primer pair are listed in table 5.

Table 5. Results of RT-PCR with each broad-spectrum and group-specific primer pair, combined with previous results from biological indexing for VN (when available).

Accession	VN	RSP13/14	RSP 5/6	G I F/R	G II F/R	G III F/R
34/46	-	-	-	-	-	-
5V-20-1	-	-	-	-	-	-
5V-20-2	-	-	-	-	-	-
5V-20-3	-	-	-	-	-	-
5V-20-4	-	-	-	-	-	-
5V-20-5	-	-	-	-	-	-
AL.CAV.1	-	-	-	-	-	-
AL.CAV.4	-	-	-	-	-	-
GALLO5	-	-	-	-	-	-
AL.CAV.5	-	-	+	-	-	+
BSK 1	-	-	+	-	-	+
M.SAS.1	-	-	+	-	-	+
M.SAS3	-	-	+	-	-	+
TEN.SAS.1	-	-	+	-	-	+
2/1	-	-	+	-	+	-
5/1	-	-	+	-	+	-
BSK3-1	-	-	+	-	+	-
BSK3-2	-	-	+	-	+	-
BSK3-3	-	-	+	-	+	-
SMH 22	-	-	+	-	+	-
CHI8	-	-	+	-	+	+
GALLO15	-	-	+	-	+	+
GALLO7	-	-	+	-	+	+
V6F12	-	-	+	-	+	+
1/18	+	+	+	+	-	+
CC10	+	+	+	+	-	+
CC13	+	+	+	+	-	+
CC19	+	+	+	+	-	+
CC21	+	+	+	+	-	+
CC24	+	+	+	+	-	+
CC29	+	+	+	+	-	+
CC4	+	+	+	+	-	+
CC5	+	+	+	+	-	+
COR20	+	+	+	+	-	+
MLOB9	+	+	+	+	-	+
RA	+	+	+	+	-	+
SG.SAS.2	+	+	+	+	-	+
SG.SAS.4	+	+	+	+	-	+
WA	+	+	+	+	-	+
YA	+	+	+	+	-	+
CA	+	+	+	+	+	+
CC25	+	+	+	+	+	+
CC6	+	+	+	+	+	+
COR33	+	+	+	+	+	+
COR35	+	+	+	+	+	+
COR36	+	+	+	+	+	+
MLOC15	+	+	+	+	+	+
SM 6/10	+	+	+	+	+	+

Accession	VN	RSP13/14	RSP 5/6	G I F/R	G II F/R	G III F/R
SME 9	+	+	+	+	+	+
SMF 13	+	+	+	+	+	+
CC17	+	+	+	+	-	-
CC8	+	+	+	+	-	-
CIL.SIRIO2	+	+	+	+	-	-
MLOC2	+	+	+	+	-	-
LUM01	+	+	+	+	-	-
RUP 1	+	+	+	+	-	-
SG.SAS.3	+	+	+	+	-	-
SG.SIRIO1	+	+	+	+	-	-
CC26	+	+	+	+	+	-
FA	+	+	+	+	+	-
KA	+	+	+	+	+	-
MLOB7	+	+	+	+	+	-
Mourvedre	n.d.	n.d.	+	-	-	+
Diamond muscat	n.d.	n.d.	+	-	-	+
Chardonnay 72	n.d.	n.d.	+	-	-	+
Barbera	n.d.	n.d.	+	-	+	+
Trousseau Gris	n.d.	n.d.	+	-	+	+
Ribolla gialla	n.d.	n.d.	+	-	+	+
B2R24P69 01	n.d.	n.d.	+	-	+	+
Negrette	n.d.	n.d.	+	-	+	-
Tinta Amarella	n.d.	n.d.	+	-	+	-
Malaga	n.d.	n.d.	+	-	+	-
Green Hungarian	n.d.	n.d.	+	-	+	-
Kandhara	n.d.	n.d.	+	-	+	-
Madrone Moon Farm	n.d.	n.d.	+	-	+	-
Cabernet sauvignon 31	n.d.	n.d.	+	-	+	-
B2R45P72 01	n.d.	n.d.	+	-	+	-
Pinot noir 78	n.d.	n.d.	+	-	+	-
Zinfandel 09	n.d.	n.d.	+	-	+	-
Olmo 1266	n.d.	n.d.	+	-	+	-
Schioppettino	n.d.	n.d.	+	-	+	-
Thomcord 02	n.d.	n.d.	+	-	+	-
Albillo mayor 01	n.d.	n.d.	+	-	+	-
Cabernet sauvignon 43	n.d.	n.d.	+	-	+	-
Merlot 18	n.d.	n.d.	+	+	-	+
Sangiovese 13	n.d.	n.d.	+	+	-	+
Arinarnoa 01	n.d.	n.d.	+	+	-	+
Riesling	n.d.	n.d.	+	+	+	+
Inzolia	n.d.	n.d.	+	+	-	-
Zinfandel	n.d.	n.d.	+	+	-	-
Pinot noir 54	n.d.	n.d.	+	+	-	-
Chardonnay 68	n.d.	n.d.	+	+	-	-
Barbera 07	n.d.	n.d.	+	+	-	-
Chardonnay 83	n.d.	n.d.	+	+	-	-
Pinot noir 85	n.d.	n.d.	+	+	-	-
Refosco 03	n.d.	n.d.	+	+	-	-
Pinot noir 22	n.d.	n.d.	+	+	-	-
Albillo real 01	n.d.	n.d.	+	+	+	-
Cabernet franc 14	n.d.	n.d.	+	+	+	-

2.4 Discussion

Based on the results obtained by GRSPaV detection with broad-spectrum and group-specific primers, we confirm the previously reported association between VN and the foveavirus Grapevine rupestris stem pitting associated virus.

GRSPaV is very common in grapevine, and accessions from Tuscany showed an infection rate by GRSPaV of 85.5 %.

The results obtained with the group-specific primers show that all of the 3 molecular groups of the virus are present in Tuscany and in the accessions held at Foundation Plant Services (University of California – Davis).

Interestingly, combining the results previously obtained with VN indexing of the Tuscan grapevine accessions (Triolo and Materazzi, 2000; Triolo and Materazzi, 2004) with the outcome of RT-PCR analyses, only virus isolates belonging to group G I appear closely related to VN. In fact, group-specific primer set G I detected the virus in all and only the 38 VN+ accessions.

Infections with groups G II and/or G III, recorded in vein necrosis free accessions, suggest that such groups are unable to induce vein necrosis. Their presence in vein necrosis infected accessions was always associated to the presence of group G I.

About the possibility to diagnose VN by RT-PCR, it could be proposed to use the pair of primers RSP 13/14 or G I, as they were able to specifically detect the “pathological strain” of GRSPaV in VN infected accessions.

On the other side, primers RSP 5/6 were more effective in the detection of all GRSPaV molecular variants; they could be employed as universal primers for the diagnosis of all the virus variants. However, positive samples obtained with these primers will include both VN-infected samples and accessions positive to Group II and III.

Although other elements are needed to obtain further and conclusive information about the aetiology and the pathological role of the GRSPaV molecular groups, these results report indications on the biological differentiation between molecular variants of GRSPaV.

3. Molecular and serological characterization of ORF 5 (CP gene)

3.1 Introduction

3.1.1 Molecular characterization

A high degree of sequence diversity was documented since when the first GRSPaV isolates were being sequenced. Meng *et al*. (1999b) found GRSPaV to consist of a heterogeneous population of sequence variants sharing between 75-99% identities, still having identical genome structures. It was also found that this heterogeneous population separated into distinct groups of viral variants and that the incidence of mixed infection in a single vine with different viral variants of GRSPaV is high. By looking at several sources of *V. vinifera*, it was discovered that the presence of sequence variants is independent of genotype or geographic origin of the host plant, which raised further questions in terms of transmission and dissemination of this ubiquitous virus.

The findings outlined above stimulated further investigation into the genetic variability of GRSPaV from researchers around the globe.

The use of different nomenclature systems for GRSPaV molecular groups and the different genome region analyzed by independent research groups often made it difficult to compare and harmonize the phylogenetic analyses from different studies.

Using primers designed to the CP of GRSPaV, several research groups identified the existence of 3 distinct groups of viral variants obtained from different geographic regions, based on coat protein sequences alone (Rowhani *et al*., 2000; Casati *et al*., 2003; Terlizzi and Credi, 2003). More recent and extensive analyses of GRSPaV genetic variability revealed the presence of 4 groups of sequence variants (Santos *et al.*, 2003; Meng *et al*., 2006; Nolasco *et al*., 2006).

Based on the complete CP coding sequence of GRSPaV, most of the studies revealed that GRSPaV variants clustered in 4 groups, often differently named from different authors, but each of them co-incidentally comprising at least one of the full genome sequences available.

A comparison of the two most adopted nomenclatures proposed by Meng *et al*. (2006) and Nolasco *et al.* (2006) is given in Figure 12.

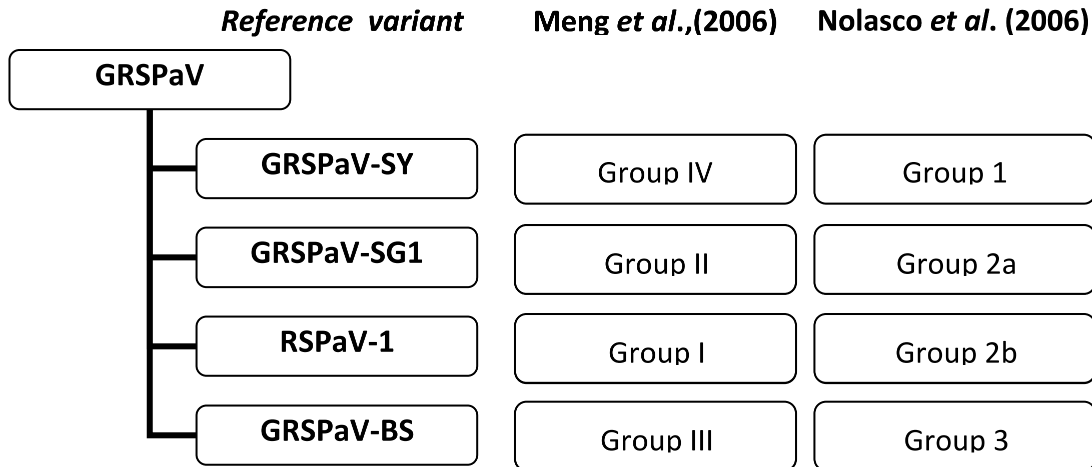

Figure 12. Comparison of the two main nomenclature systems for the classification of GRSPaV variants

In recent years, the full genome of more GRSPaV isolates have been completely sequenced. Up to now, 11 full genome GRSPaV isolates, from different geographical origin, have been fully sequenced and are available in GenBank (GenBank accession numbers: NC_001948.1; AY881626.1; AY881627.1; AF026278.1; AY368590.1; AY368172.2; KC427107.1; JQ922417.1; FR691076.1; HE591388.1; JX559646.1), including the variant named “GRSPaV-PN” (Lima *et al*., 2009), which notably presents significant variability when compared with reference variants for each of the 4 GRSPaV molecular groups. GRSPaV-PN was proposed to constitute a separate group of GRSPaV variants (Terlizzi *et al.*, 2011).

3.1.2 Serological characterization

The great value of serological methods for plant virus identification is based on the specific reaction between the viral antigens and their specific antibodies. An antigen is a molecule that, when injected into a vertebrate animal (usually a mammal or a bird), it can trigger an immune response in the animal which results in production of specific antibodies that can combine with the foreign antigen (Naidu and Hughes, 2001; Purcifull *et al*., 2001; Lima *et al*., 2005; Astier *et al*., 2007).

Virus particles themselves and their proteins have several antigenic determinants (epitopes) which vary in their amino acid sequence and have the properties of inducing the production of specific antibodies. The virus particles, their coat proteins and the other types of virus induced proteins can function as antigens (Hiebert *et al*., 1984; Naidu and Hughes, 2001; Purcifull *et al*., 2001; Lima *et al*., 2005; Astier *et al*., 2007; Lima *et al.*, 2012).

Antibodies are also proteins of the immunoglobulin group (Ig) produced against specific antigenic determinants and are present in the animal blood. The immunoglobulin G (IgG) is the most common type of Ig produced and, consequently most commonly involved in the serological tests for plant virus identification. This type of antibody is composed of four linked polypeptides with Y-shape of approximately 150 Kd, with two identical heavy chains and two identical light chains of polypeptides (Fig. 13). The IgG has two identical combining sites specific for antigenic determinants called paratopes in the NH_2 terminal regions of the heave and light chains. These two identical combining sites have highly variable aminoacid sequence, which permit the production of specific IgGs for the different virus epitopes. The C-terminal regions of the heavy chains are linked together by sulfur bridges to produce the fragment crystallizable (Fc) fraction of the antibody (Fig. 13) which links specifically with protein A or cell membranes (Almeida *et al.*, 2001; Purcifull *et al*., 2001; Lima *et al.*, 2012).

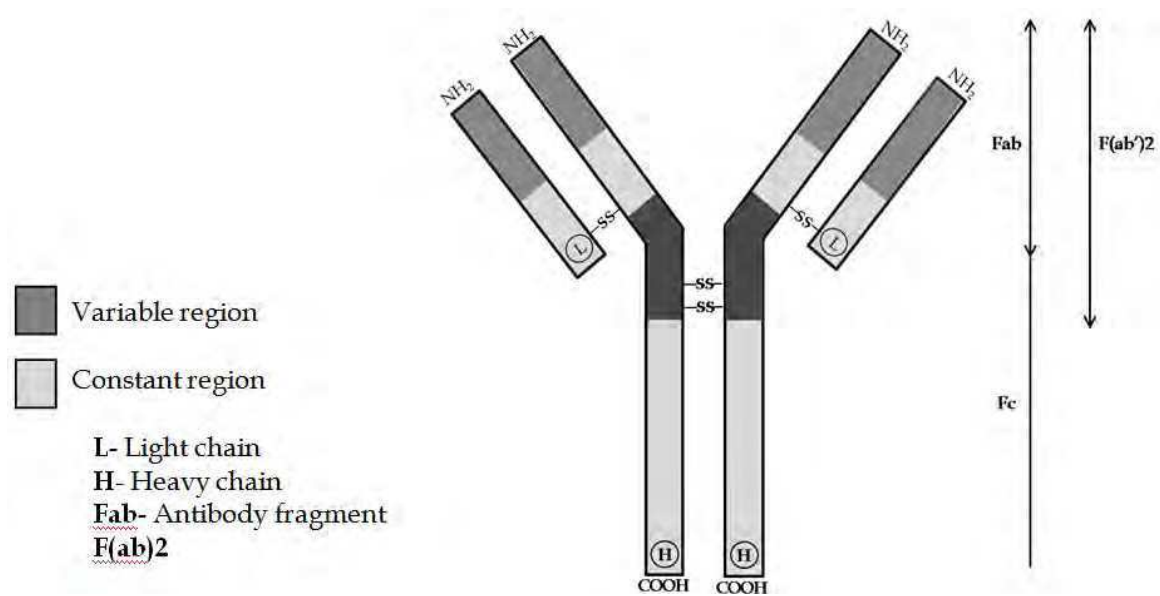

Figure 13. Diagrammatic representation of the structure of an immunoglobulin G (IgG) molecule. Fab, F(ab)2, and Fc represent fragments obtained by enzyme cleavage of IgG. (Lima *et al.*, 2012)

Generally the methods that involve the antigen antibody reactions *in vitro* are simple and do not require sophisticated and expensive apparatus. The most serious limitation to using serology for plant virus identification and detection is the difficulty in producing a good virus-specific antiserum. Most antisera used for plant virus identification and detection are usually prepared by immunizing mammals with purified plant virus or their different types of proteins. However several other methods have been used to produce very specific antibodies, including monoclonal antibody (Mab) which consists of a single type of antibody that reacts with only one specific epitope of a virus protein.

The production of antiserum for some plant viruses is, mainly, limited by the difficulty in purifying such viruses free from plant protein contaminants and in appropriate concentration to be used as antigen. Several other methods have been developed for plant virus antigen production, including the transformation of bacterial cells with the virus coat protein gene. Although not very specific for virus species/strains discrimination, the polyclonal antiserum is very useful and practical for virus surveys and indexing.

Up to date, two polyclonal antibodies to GRSPaV have been produced (Minafra *et al*., 2000; Meng *et al*., 2003), both of them against a recombinant coat protein of GRSPaV. Minafra *et al.* (2000) reported the use of the antibody to effectively detect GRSPaV by western blot, but not by ELISA, possibly because of a low efficiency of the antiserum in recognizing the native viral coat protein from tissue samples.

In our study, western blot assays were performed to determine antibody specificity to GRSPaV variants belonging to 4 different molecular groups.

In western blot the virus protein antigens are transferred from polyacrylamide gels in which they were previously separated by electrophoresis, to nitrocellulose or nylon membranes.

Several methods can be used to transfer the virus protein and the electro-blotting is the most used system. Similar to ELISA techniques, the proteins are detected in the membrane by the use of specific enzyme labelled antibodies (Almeida *et al*., 2001; Purcifull *et al*., 2001).

3.2 Materials and Methods

3.2.1 Molecular characterization

The viral sources used in this study came from grapevine plants selected through the 99 accessions from Tuscany and California (Tab. 5), listed in chapter 2. A total number of 22 accessions, representative of all the possible combinations of both single and mixed GRSPaV variants infections (Tab. 6) were investigated.

Table 6. List of the 22 grapevine accessions representative of every possible combination of GRSPaV infection.

Accession name	G I	G II	G III	Collected at
CC 8	+	-	-	San Piero a Grado – Italy
MLOC2	+	-	-	San Piero a Grado – Italy
CC17	+	-	-	San Piero a Grado – Italy
5/1	-	+	-	San Piero a Grado – Italy
SMH 22	-	+	-	San Piero a Grado – Italy
MSAS1	-	-	+	San Piero a Grado – Italy
MSAS3	-	-	+	San Piero a Grado – Italy
Barbera 07	+	-	-	FPS UCDavis – California
Pinot Noir 54	+	-	-	FPS UCDavis – California
Refosco 03	+	-	-	FPS UCDavis – California
Chardonnay 68	+	-	-	FPS UCDavis – California
Chardonnay 83	+	-	-	FPS UCDavis – California
Inzolia	+	-	-	FPS UCDavis – California
Cabernet Sauvignon 43	-	+	-	FPS UCDavis – California
Thomcord 02	-	+	-	FPS UCDavis – California
Tinta Amarella	-	+	-	FPS UCDavis – California
Mourvedre	-	-	+	FPS UCDavis – California
Diamond Muscat	-	-	+	FPS UCDavis – California
Albillo Real 01	+	+	-	FPS UCDavis – California
Arinarnoa 01	+	-	+	FPS UCDavis – California
B2R24P69	-	+	+	FPS UCDavis – California
Riesling	+	+	+	FPS UCDavis – California

RNA extraction from phloem scrapings and cDNA synthesis was performed according to the protocol previously described in Chapter 2.

Primers RSP 52/53 (Rowhani *et al.*, 2000) were used to amplify a 905 bp amplicon, containing the whole CP gene.

5 µl of cDNA were added to PCR mixture yielding final concentrations of 1x GoTaq Buffer (Promega, USA), 1,5 mM $MgCl_2$, 0.4 mM each deoxynucleotide triphosphate, 0.2 µM

each primer and 1 U Taq DNA Polymerase (Promega, USA) per reaction, in a total volume of 50 µl.

PCR reactions were performed with the following parameters: 3 min at 95 °C followed by 40 cycles of 30 sec at 94 °C, 45 sec at 52 °C and 1 min 30 sec at 72 °C, followed by a final extension of 10 min at 72 °C. 10 µl of the PCR products were analyzed on a 1.5% agarose gel, stained with GelRed (Biotium, USA) and visualized under with light.

Samples from Tuscany

Bands of the expected size (905 bp) were excised from the gel and PCR products were purified with Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's protocol.

The resulting DNA solution was used in cloning reactions, using pGEM®-T Easy vector System II (Promega, USA) (Fig. 14).

Ligation reactions were set up by mixing 3 µl of DNA purified from agarose gel, 5 µl of 2x Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol), 1 µl pGEM®-T Easy Vector (50 ng), 1 µl of T4 DNA Ligase enzyme (3 units/µl) and sterile water to a final volume of 10 µl. The reactions were incubated overnight at 4°C.

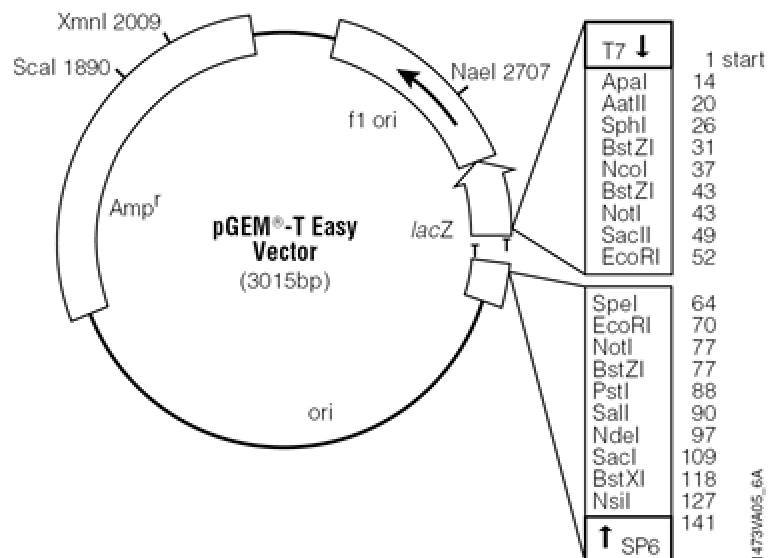

Figure 14. pGEM®-T Easy Vector.

2 µl of each ligation reaction were added into a vial containing 50 µl of thawed JM 109 High Efficiency Competent Cells (Promega, USA), gently mixed and incubated for 20 min on ice. The cells were then heat-shocked for 50 sec at 42°C and put on ice for 2 min.
950 µl of room temperature SOC medium (20 g/L Tryptone; 5 g/L Yeast Extract; 4.8 g/L $MgSO_4$; 3.603 g/L dextrose; 0.5g/L NaCl; 0.186 g/L KCl) was added, and the cells were incubated shaking (150 rpm) for 1.5 h at 37 ºC. After a brief centrifuge spin, 800 µl of the supernatant was discarded and the cells were gently resuspended in the remaining SOC medium.
100 µl of the transformed cells from each sample were plated on 2 LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C.
6 to 10 white colonies for each sample were selected from the transformants growing on the plates, picked up with a sterile toothpick, plated on a new LB/ampicillin masterplate, incubated overnight at 37 °C and stored for 4 weeks at 4 °C.
The toothpicks were then inserted in eppendorf tubes containing 50 µl of molecular biology grade water and incubated 10 minutes at 99 °C. 1 µl of this solution was used in PCR reaction with primers RSP 52/53 in order to assess the presence of the PCR product insert in the colonies.
6 to 10 clones for each GRSPaV isolate were tested by Single Strand Conformation Polymorphism (SSCP) analysis, in order to choose the clones to be sequenced, excluding the identical ones. The protocol as described by Nolasco *et al*. (2006) was adopted, with slight modifications.
Briefly, the acrylamide gel was prepared with 1 ml of 10x TBE, 7 ml of H_2O, 2.5 ml of 40% acrylamide/bis-acrylamide (29:1), 75 µl of ammonium persulfate (0.1 g/ml) and 12.5 µl of tetramethylethylenediamine (TEMED). The clones to test were amplified by PCR with primers RSP 52/53 and 5 µl of the PCR product was mixed with 15 µl of denaturing solution (95 % formamide, 0.05% xylencyanol, 0.05% bromophenol blue, 20 mM EDTA), incubated for 10' at 100 °C and placed on ice.
Samples were loaded on the gel and vertical electrophoresis was performed in 1x TBE with a voltage of 200V for 4 hours (100V for the first 15'), taking care to periodically cool the buffer.

After the electrophoresis, gels were incubated for 30’ in a 10% acetic acid solution, followed by 3 washing steps with distilled water; then incubated for 30’ in a 1% nitric acid solution and washed again 3 times with distilled water.

Silver staining was performed incubating the gel for 30’ in a the silver nitrate solution (0,1% silver nitrate, 0,0015% formaldehyde), rinsed with distilled water and immersed in a sodium carbonate solution (3% sodium carbonate, 150 µl of 37% formaldehyde, 1% sodium thiosulfate). Once the bands appeared sharply, the staining reaction was stopped incubating the gel in a 10% acetic acid solution.

Selected clones were picked up from the masterplate and placed in 3 ml of LB medium containing ampicillin (final concentration 0,05 mg/ml) and grown overnight at 37 ºC while shaking at 150 rpm.

The cultures were then centrifuged at 10.000 g for 5 m to pellet the cells, the supernatant was discarded, and plasmids were purified using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) according to manufacturer’s instructions. Plasmids were finally eluted in 100 µl of molecular biology grade water and shipped for sequencing (Eurofins MWG, Germany).

Samples from California

Bands of the expected size (905 bp) were excised from the gel and PCR products were purified with Zymoclean kit (Zymo Reasearch, USA) according to the manufacturer’s protocol.

Ligation reactions were set up by mixing 2 µl of DNA purified from agarose gel, 5 µl of 2x Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol), 0,5 µl pGEM®-T Easy Vector (50 ng), 1 µl of T4 DNA Ligase enzyme (3 units/µl) and sterile water to a final volume of 10 µl. The reactions were incubated overnight at 4 °C.

DH5α competent cells were prepared (protocol in appendix 1) and transformed by electroporation with Gene Pulser apparatus (Bio-Rad).

In a cold eppendorf tube, 100 µl of competent cells were mixed with 3 µl of ligation mixture and kept on ice for 5 minutes. The mix was then transferred in a cold

electroporation cuvette, electroporated (2.50 kV, 1 pulse; *EC2* setting on Bio-Rad Gene Pulser) and added with 900 µl of SOC medium. The cell suspension was transferred in a 12 ml tube and incubated for 1,5 h at 37 °C, shaking at 250 rpm. Two 2YT/ampicillin/IPTG/X-Gal plates were inoculated with 100ul and 200 µl of transformed cells suspension for each sample and incubated overnight at 37 °C.

6 to 12 white colonies for each sample were picked up using a sterile toothpick and transferred to a 96 wells culture plate containing 200 µl of LB medium + 0,8% glycerol + ampicillin (1 µl ampicillin/1 ml LB). Culture plates were incubated overnight at 37 °C and stored at -80 °C.

6 to 10 clones for each sample were directly sequenced from culture at UC-Davis Sequencing facility.

Contigs were created using Vector NTI 11 software (Invitrogen, USA) and alignments of nucleotide sequences were done using CLUSTALW (Thompson *et al*., 1994) with default settings.

In addition to the sequences obtained in our work, corresponding sequences of GRSPaV isolates available in GenBank (Table 7) were included in the subsequent analyses.

Nucleotide and aminoacidic identities between sequences were computed using the p-distance model included in the MEGA5 software (Tamura *et al*., 2011) and plotted using Microsoft Excel spreadsheet (Microsoft Office 2003, Microsoft Corporation, USA).

Sequences with nucleotide identity above 99% between clones of the same isolate were considered as a single sequence, while sequences derived from additional clones of an isolate sharing with nucleotide differences greater than 1% were considered as different independent clones.

Evolutionary relationships were inferred from multiple sequence alignments calculated by CLUSTALW using the Neighbour Joining (NJ) method (Saitou and Nei, 1987) with 1000 bootstrap replications. The evolutionary distances were computed using the Kimura two-parameter model (Kimura, 1980). These phylogenetic analyses tools were implemented by the molecular evolutionary genetics analysis (MEGA) software version 5.0.

Table 7. List of sequences available in GenBank included in the analysis

Accession name	GenBank accession number	Accession name	GenBank accession number
RSPaV-1	AF057136.1	ORPN21b	FJ943327.1
GRSPaV	AF026278.1	Hiz1	AB331431.1
GRSPaV-3138-07	JX559646.1	Hiz3	AB331432.1
GRSPaV-SY	AY368590.1	Ham1	AB331441.1
GRSPaV-BS	AY881627.1	OE8	AB331423.1
GRSPaV-SG1	AY881626.1	OE11	AB331424.1
GRSPaV-MG	FR691076.1	Vs284-23	AY927686.1
GRSPaV-PG	HE591388.1	B1-1	AY927682.1
GRSPaV-WA	KC427107.1	B11-2	AY927679.1
GRSPaV-GG	JQ922417.1	B1-2	AY927683.1
GRSPaV-PN	AY368172.2	B10-3	AY927681.1
EWMR1c	FJ943335.1	CNO15	DQ364982.1
NX-XDL-2	KF731986.1	TRCV4	DQ364987.1
EWMR1b	FJ943334.1	CF6	DQ364994.1
FBSB1b	FJ943343.1	BOS3	DQ364991.1
WWGB1	FJ943312.1	NE423TP	DQ364990.1
FDCL1	FJ943284.1	BL11a	DQ364980.1
EWCH5a	FJ943293.1		

3.2.2 Serological characterization

Western blot assay was performed to determine the serological properties of GRSPaV isolates that previously tested positive to the 3 group-specific primer sets against the polyclonal antibody to recombinant GRSPaV CP developed by Minafra *et al*. (2000).

7 grapevine accessions singularly infected by GRSPaV variants amplified by a single group-specific primer set were selected (Tab 8).

Table 8. List of grapevine accessions tested by Western Blot, and the results previously obtained by with group-specific primers

Accession	Positive to group-specific primers
CC8	G I
CC17	G I
MLOC2	G I
5/1	G II
SMH22	G II
MSAS1	G III
MSAS3	G III
34/46	Negative control

Total proteins were extracted from 200 mg of tissue from cortical scrapings, ground to a fine powder in liquid nitrogen and homogenized by grinding in volumes of extraction buffer (Berger *et al.*, 1989) (0.5M Tris-HCl pH 8.8, 2% SDS, 4% 2-mercaptoethanol, 40% sucrose).

The homogenate was clarified by centrifugation at 3.000 rpm for 5 min. The supernatant fraction was boiled for 5 min, then placed on ice and mixed with an equal volume of Laemmli buffer (Laemmli, 1970). 20 µl of the total protein extract (corresponding to the equivalent of 4 mg of tissue) for each sample were then loaded and separated on 10% SDS-polyacrylamide gels using a mini-protean II gel apparatus (BioRad).

Gels were electroblotted on pre-wetted polyvinyl fluoride membranes (PVDF, Immobilion-P, Millipore) and incubated overnight at 4 °C with a 1:1000 solution of the antiserum in blocking solution (1% BSA, 5% non-fat dry milk, 0.05% Tween-20 in 1x TBS buffer). After three washes of 10 minutes each in washing buffer (0.1% Tween-20 in 1x TBS buffer), the membranes were incubated, shaking for 30 minutes at room temperature with an 1:2000 dilution of anti-rabbit IgG-Alkaline Phosphatase conjugate (Sigma-Aldrich) in blocking solution. Three sequential washing steps of 15 minutes each were followed by staining in nitroblue tetrazolium-5-Bromo-4-chloro-3-indolyl phosphate solution (Sigma-Aldrich). Reaction was stopped with 1% EDTA solution.

3.3 Results

3.3.1. Molecular characterization

Amplicons of the expected size were amplified by primer pair RSP 52/53 and successfully purified from all of the 22 grapevine accessions (Fig. 15)

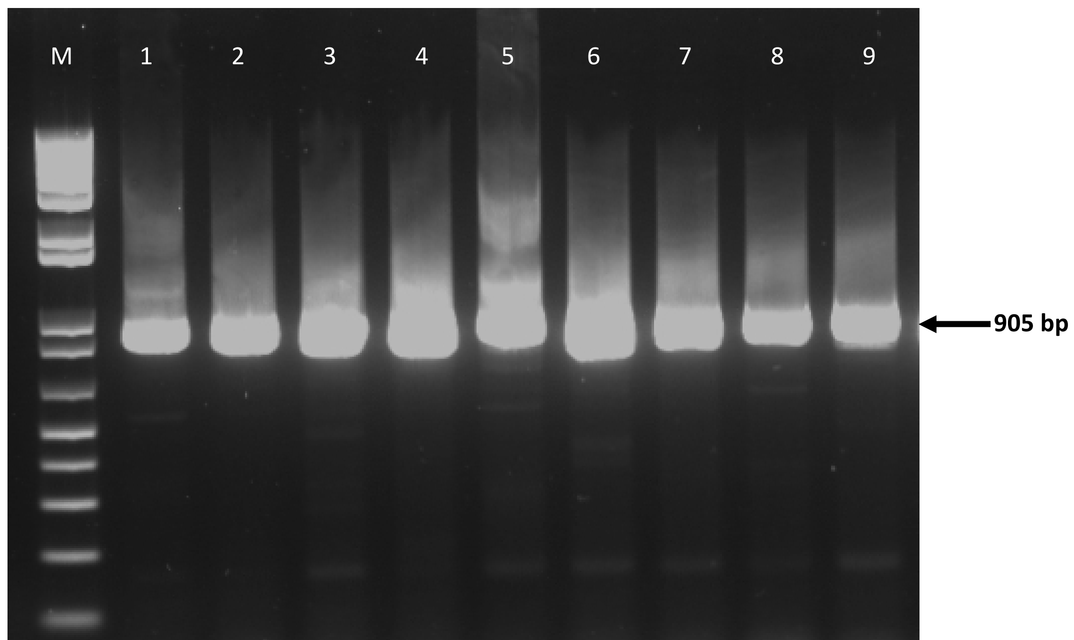

Figure 15. 905 bp amplicons obtained after PCR with primers RSP 52/53. Lanes from left to right: M= Marker; 1 to 9= Negrette, Tinta Amarella, Inzolia, Malaga, Barbera, Trousseau Gris, Green Hungarian, Kandhara, Mourvedre.

Primers RSP 52/53 amplified a DNA fragment of 905 bp, encompassing the entire CP and 62 and 63 bp upstream and downstream of the CP, respectively. However, the flanking sequences were removed and only the CP gene (780 bp) sequences were used for analyses.

Clones obtained from the 7 Tuscan GRSPaV isolates, subjected to SSCP analysis (Fig. 16), showed 23 different profiles (Tab. 9)

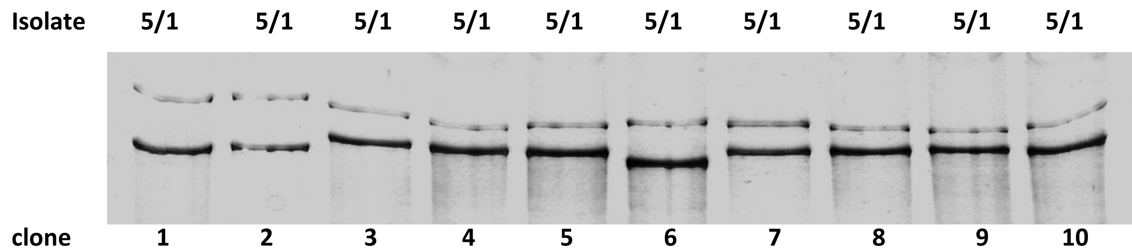

Figure 16. SSCP profiles obtained from 10 clones of the same isolate (5/1).

Table 9. Number of different SSCP profiles obtained from each single isolate.

Isolate	n. of different SSCP profiles
CC8	3
CC17	2
MLOC2	5
5/1	3
SMH22	3

In total, 27 clones from Tuscany (representative of the 22 different SSCP profiles) and 179 clones from California (8 to 16 for each isolate) were sequenced.

As previously stated, sequences sharing more than 99% nucleotide (nt) identity from the same isolate were considered as a single clone. A total of 36 different sequences (9 from Tuscany and 27 from California) were identified after the sequencing.

In pairwise comparisons, the 36 CP sequences obtained in this study showed identities ranging between 81.7 and 100.0% at the nt level and between 91.4 and 100.0% at the aminoacid (aa) level. Similar ranges of values were obtained when comparisons were made with corresponding sequences in GenBank. These values are within the limits of species demarcation criteria in the family *Betaflexiviridae,* where isolates sharing greater than 72% nt or 80% aa sequence identities between their CP or polymerase genes are considered one species (King *et al*., 2011). Based on these results, it can be concluded

that CP sequences are specific to GRSPaV and divergent variants of the virus are present in the grapevine accessions object of this work.

The CP-based phylogenetic relationships of GRSPaV clones obtained in this study were compared among themselves and with 35 corresponding sequences from other grape-growing regions available in GenBank (Tab. 7). This analysis, using the neighbor-joining (NJ) method, included a total of 71 CP sequences.

The results showed segregation of GRSPaV CP sequences into five major lineages (Fig. 17). We designated each of these lineages with a reference isolate to maintain a standardized nomenclature of GRSPaV sequence variant groups in analogy with previous reports.

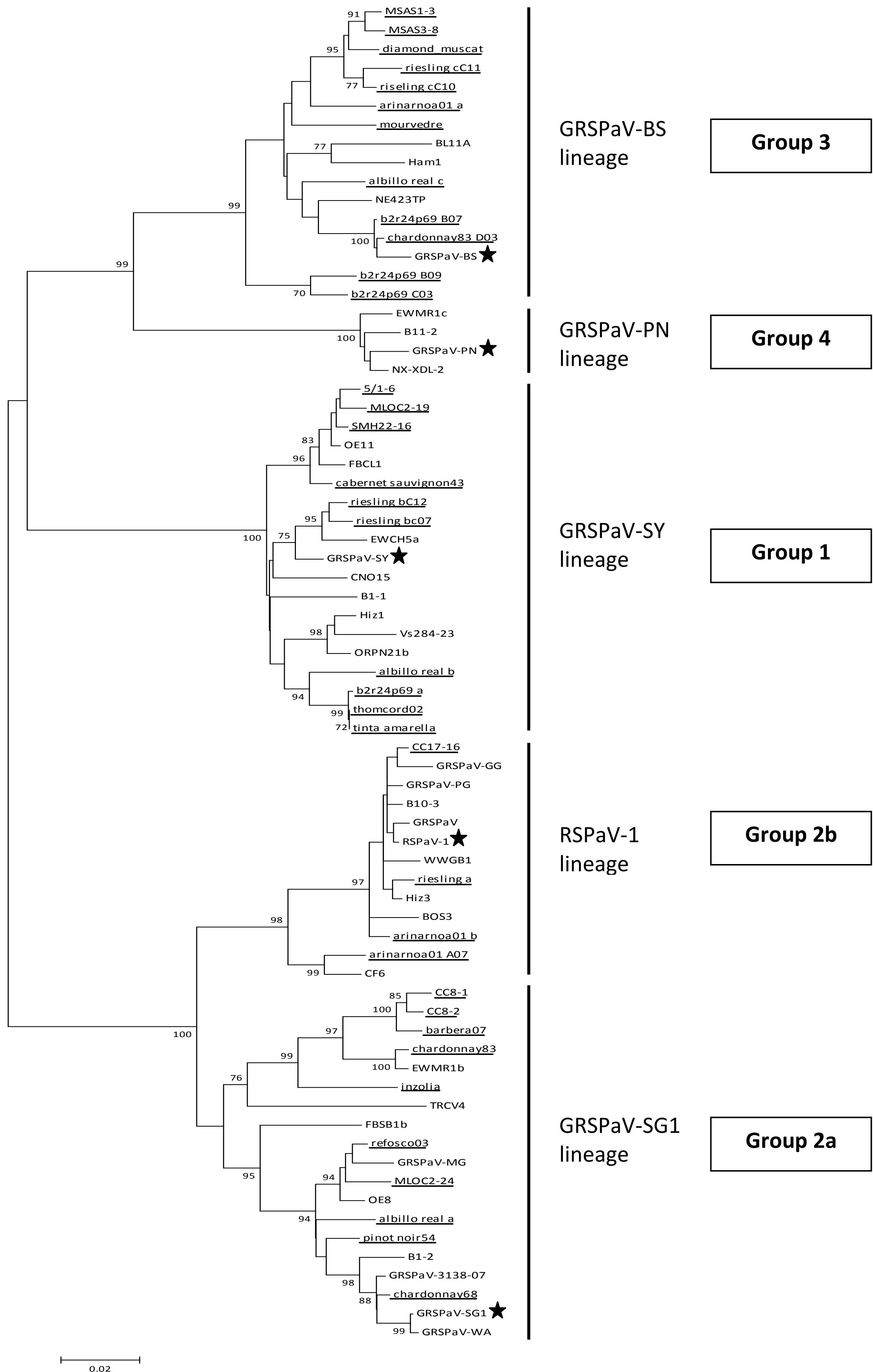

Figure 17. Phylogenetic relationship among GRSPaV isolates. Multiple alignment included complete CP gene sequences obtained in this study (underlined) and available in GenBank. Reference variants for each lineage are marked with an asterisk. Phylogenetic tree was constructed with neighbour-joining method, evolutionary distances were computed using the Kimura 2-parameter method. Only bootstrap values higher than 70% are shown

The average nt and aa identity between sequences within the same group is more than 94.0% and 97.0%, respectively.

On the contrary, average nt identity between different lineages never exceeds 90% (83.1% between RSPaV-1 and GRSPaV-BS to 88.3% between GRSPaV-BS and GRSPaV-PN), with the exception of GRSPaV-SG1 and RSPaV-1 lineages, which share 90,66% nt identity (Tab. 10).

Table 10. Average percentages of nucleotide and amino acid identity within (**a**) and between (**b** and **c**) molecular groups. The values were computed using the p-distance model included in the MEGA5 software (Tamura *et al*., 2011)

a)

	nt %	**aa %**
GRSPaV-SG1	94.0	97.8
RSPaV-1	97.7	98.5
GRSPaV-SY	96.3	98.6
GRSPaV-BS	95.3	98.8
GRSPaV-PN	98.3	98.4

b)

nt %	**GRSPaV-SG1**	**RSPaV-1**	**GRSPaV-SY**	**GRSPaV-BS**	**GRSPaV-PN**
GRSPaV-SG1	-				
RSPaV-1	90.7	-			
GRSPaV-SY	83.7	83.6	-		
GRSPaV-BS	832	83.1	84.7	-	
GRSPaV-PN	82.3	83.5	84.5	88.3	-

c)

aa %	**GRSPaV-SG1**	**RSPaV-1**	**GRSPaV-SY**	**GRSPaV-BS**	**GRSPaV-PN**
GRSPaV-SG1	-				
RSPaV-1	97.1	-			
GRSPaV-SY	93.0	93.5	-		
GRSPaV-BS	93.0	93.6	93.5	-	
GRSPaV-PN	93.3	94.0	94.1	96.1	-

Considering the nucleotide and amino acid identities between the various lineages, we named the GRSPaV molecular groups according to the nomenclature proposed by Nolasco *et al*. (2006).

Lineage GRSPaV-SY corresponds to group 1, GRSPav-SG1 and RSPaV-1 are, respectively, groups 2a and 2b, while GRSPaV-BS is group 3. The GRSPaV-PN lineage, not included in the nomenclature by Nolasco, has been named group 4 (Fig 17).

Molecular groups 1, 2a, 2b, and 3 contained 10, 10, 4 and 12 sequences from this study, respectively, and 9, 9, 9 and 4 from GenBank. None of our sequences clustered in the group 4, represented in the phylogenetic tree by the reference variant GRSPaV-PN and 3 other sequences available in GenBank.

Sequence alignment showed the presence of 253 parsimony informative sites and 56 singleton sites in the CP gene. Minimum nucleotide homology between the variants in pairwise comparisons was 81.0% and most of the sequence variants shared about 83.0% nucleotide identity (Fig. 18).

The nucleotide diversity (mean nucleotide distance between two sequences) as estimated by the Kimura 2 parameters model was 0.1462 substitutions per site. The maximum distance observed in the set of sequences reached 0.2225 substitutions per site. Nucleotide diversity decreased from the 5' toward 3' end. A clear global minimum is apparent around position 500 of ORF5 sequence (Fig. 19).

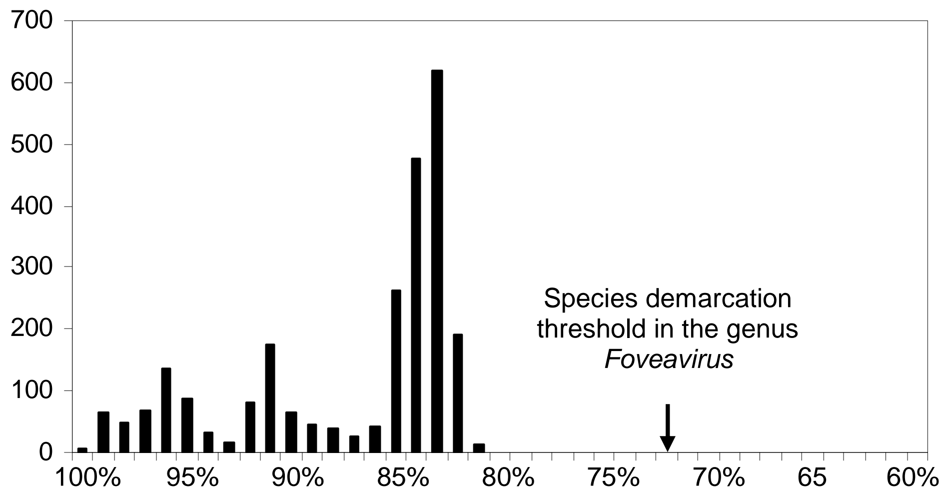

Figure 18. Distribution of the percentages of pairwise identities among 71 CP sequences of GRSPaV isolates. Each bar represents the total number of pairwise nucleotide comparisons sharing the same percentage of nucleotide sequence identity. The species demarcation threshold is according to criteria established by the International Committee on Taxonomy of Viruses (King *et al*., 2011).

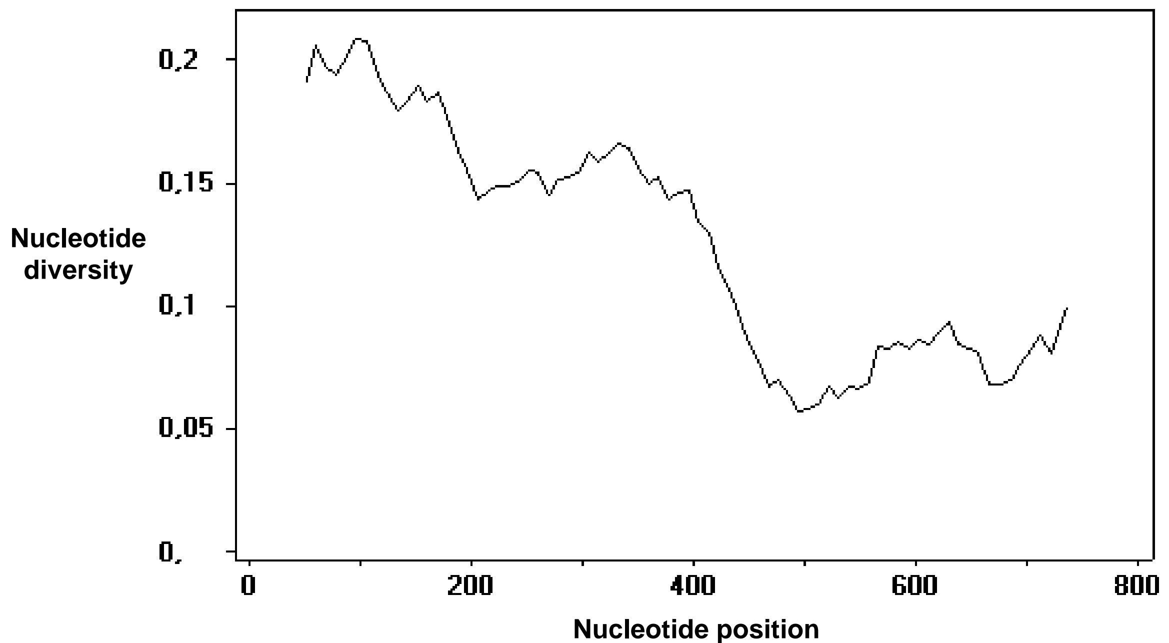

Figure 19. Nucleotide diversity along the GRSPaV CP gene. Nucleotide diversity was obtained in successive windows of 99 nt (step 9 nucleotides).

The nucleotide composition was in average 24.2% T, 19.7% C, 29.5% A, and 26.6% G, and did not differ significantly within the different groups. There was no relationship with geographic origin.

The coat protein amino-acid sequence was deduced from the CP gene sequence by translation using the universal code. It consists of 259 aminoacids from which 183 are conserved among the variants included in the analysis; the global diversity (p-distance) was 0.0511.

The phylogenetic analysis also allowed to understand which molecular groups of GRSPaV variants are amplified by each of the group-specific primer sets designed by Rowhani *et al*. (2000), since the different groups of GRSPaV variants amplified by each primer set were never compared to the molecular groups defined in phylogenetic analyses (Tab. 11).

The variants amplified by G I primer set correspond the lineages RSPaV-1 and GRSPaV-SG1, variants amplified by G II primer set correspond to the GRSPaV-SY lineage, and those amplified by primer pair G III correspond to GRSPaV lineage. We could not

determine if the 3 group-specific primer sets are able to amplify also molecular variants belonging to the GRSPaV-PN lineage, due to the lack of such GRSPaV variants in the grapevine accessions object of this study.

Table 11. Correspondence of GRSPaV phylogroups amplified by each group-specific primer set (Rowhani *et al*., 2000).

	Reference variant lineage	Nolasco *et al*, 2006	Meng *et al*., 2006
G I	GRSPaV-SG1	2a	II
	RSPaV-1	2b	I
G II	GRSPaV-SY	1	IV
G III	GRSPaV-BS	3	III

The group-specific primers were efficient to discriminate the different GRSPaV lineages. Out of the 22 GRSPaV isolates that were sequenced, representative of the different single and mixed GRSPaV infections, only 3 showed infection by GRSPaV variants slightly different than that expected by the results of group-specific primers. (Tab. 12).

Table 12. Comparison of GRSPaV variants characterization obtained with RT-PCR group-specific primers and with cloning and sequencing of ORF5.

GRSPaV-isolate	**Group-specific primers**	**GRSPaV group**
CC8	G I	2a
CC17	G I	2b
MLOC2	G I	2a + 1
5/1	G II	1
SMH22	G II	1
MSAS1	G III	3
MSAS3	G II	3
Barbera07	G I	2a
Pinot noir 54	G I	2a
Refosco03	G I	2a
Chardonnay68	G I	2a
Chardonnay83	G I	2a + 3
Cabernet sauvignon 43	G II	1
Thomcord02	GII	1
Tinta amarella	G II	1
Mourvedre	G III	3
Diamond muscat	G III	3
Albillo real 01	G I + G II	2a+ 1 + 3
Arinarnoa01	G I + G III	2b + 3
B2R24P69	G II + G III	1 + 3
Riesling	G I + G II + G III	2b + 1 + 3
Inzolia	G I	2a

The infection status detected by RT-PCR with group-specific primers was confirmed by cloning and sequencing for 19 out of 22 isolates (86.4%). Infection by group 1 was not detected by RT-PCR in accession MLOC2, and molecular variants belonging to group 3 were not detected in accessions Chardonnay 83 and Albillo Real 01.

3.3.2 Serological characterization

Bands of ca. 28 kDa, the expected size of the GRSPaV coat protein were recognized by the antiserum to recombinant GRSPaV CP and detected in preparations from all of the seven selected grapevine accessions. No comparable bands were visible in the healthy grapevine control (Fig. 20)

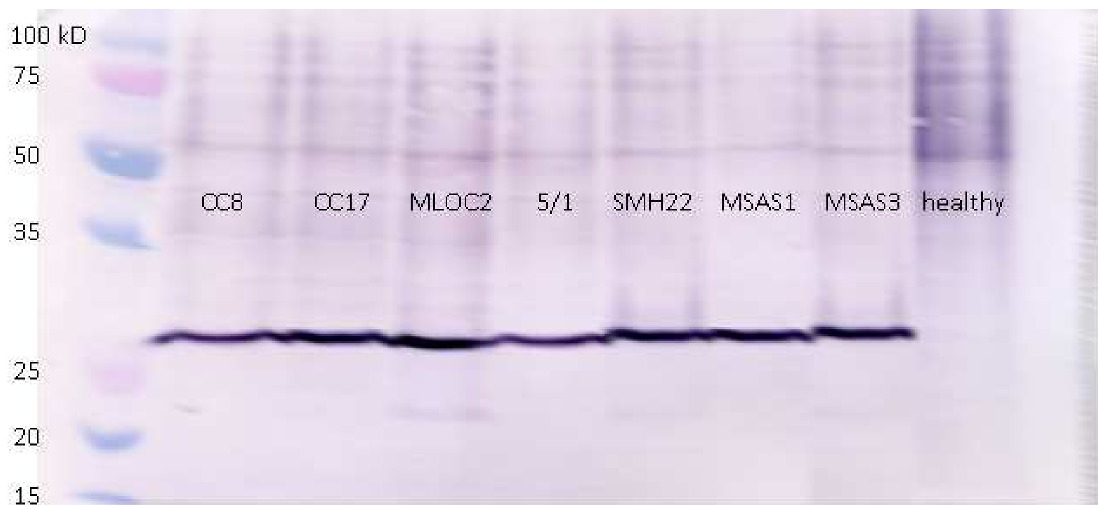

Figure 20. Western blot analysis for detection of GRSPaV in cortical scrapings from grapevine accessions infected by different GRSPaV variants: molecular groups 2a (CC8 and MLOC2), 2b (CC17), 1 (5/1 and SMH22) and 3 (MSAS1 and MSAS3).

These results shows that the antibody to the recombinant GRSPaV coat protein produced by Minafra *et al*. (2000) is able to detect virus variants from all of the 4 molecular groups tested (groups 1, 2a, 2b and 3).

3.4 Discussion

In this study, the molecular diversity of field isolates of GRSPaV in grapevine accessions from Tuscany and California was assessed. A phylogenetic approach was used to analyze a total of 71 full-length CP sequences (36 from this study and 35 from GenBank). As only a few isolates from Italy and none from Tuscany were included in previous studies, the work represents an analysis of GRSPaV isolates at a global level, extending previous investigations conducted in other grape-growing regions of the world (Habili *et al*. 2005; Lima *et al*. 2006; Meng *et al*. 2006; Nolasco *et al.* 2006; Nakaune *et al*. 2008; Alabi *et al*., 2010; Terlizzi *et al*., 2011).

The results reinforce that the global GRSPaV population is very diverse, with numerous, disparate strains segregating into five distinct lineages (Fig. 17). Several factors could contribute to the perpetuation of such a complex and dynamic population structure. The perennial and clonal propagation of grapevines, chronic infection for many years without host mortality, and absence of a biological vector provide a conducive environment for the evolution of a population of genetically related variants.

The fact that grapevines often remain in the field for several years or decades, coupled with cultural practices such as grafting onto rootstocks and topworking to new cultivars, could mean that new variants may be introduced into a plant providing additional opportunities for changes in the viral population within individual grapevines. The presence of genetically distinct variants within a single infected plant validate this argument.

Given the high degree of the global genetic variation among GRSPaV sequences, it is unlikely that the estimated error rate of 0.27×10^{-4} per base pair per cycle for Taq polymerase (Bracho *et al*., 1998) would have contributed to the apparent nucleotide divergence obtained in this study and previous reports (Habili *et al*. 2005; Lima *et al*. 2006; Meng *et al*. 2006; Nolasco *et al.* 2006; Nakaune *et al*. 2008; Alabi *et al*., 2010).

Phylogenetic analysis of CP GRSPaV sequences obtained in this study and retrieved from GenBank supports the existence of 5 phylogroups, comprising the 4 groups described, even if with a different nomenclature system, by several authors (Meng *et al*., 2006;

Nolasco *et al*. 2006; Nakaune *et al*., 2008) and the fifth group representing the GRSPaV-PN lineage (Lima *et al*., 2009).

Both isolates from Tuscany and California were distributed in 4 groups, confirming a lack of clustering by geographical origin.

The GRSPaV variants distinctively amplified by the group-specific primers designed by Rowhani *et al.* (2000), that were never been correlated with the GRSPaV phylogenetic groups, were compared with molecular groups identified by phylogenetic analysis of the CP sequences. It emerged that the different primer sets are able to discriminate and singularly amplify GRSPaV variants from groups 2a and 2b (primer set G I), group 1 (primer set G II) and group 3 (primer set G III).

Considering the average nucleotide and aminoacidic identities between different groups and also the biological properties of GRSPaV variants with regard to VN symptoms expression, the nomenclature of GRSPaV molecular groups adopted by Nolasco *et al.* (2006) appear more fitting, and was adopted in this study. In fact, groups 2a and 2b share between them higher nt and aa % identities than with any other groups.

Also, they are both amplified by group-specific primer set G I that, as described in chapter 2, showed a correlation with VN symptoms expression.

With regard to the serological properties of the coat proteins expressed by 4 different GRSPaV phylogenetic groups, the use of the anti-GRSPaV antibody produced by Minafra *et al.*, (2000) in western blot effectively detected a single protein band of the size expected for GRSPaV CP. This suggests a basic homogeneity of the viral coat protein, regardless the reported genetic variability of GRSPaV. The use of western blot as a method for the detection of the virus provided results comparable to those achieved by RT-PCR with GRSPaV-specific “broad-spectrum” primers RSP 5/6.

4. Molecular characterization of a fragment of ORF 1 (replicase gene)

4.1 Introduction

Most of the studies on the genetic diversity of GRSPaV conducted so far have involved only genomic regions of ORF5, encoding the capsid protein (CP).

Studies conducted on GRSPaV genomic regions other than CP focused on the helicase domain in ORF1 (encoding the viral replicase).

Meng *et al.* (2006) reported the existence of 4 phylogenetic groups of GRSPaV variants, based on sequence of a 339 bp fragment amplified by primer set RSP 13/14 (Meng *et al.*, 1999a). The 4 groups identified corresponded to the same groups outlined by phylogenetic analysis of ORF5 sequences.

A recent study by Alabi *et al.* (2010), revealed the same 4 groups by analysis of sequences obtained with primer set RSP 13/14.

In 2011, Terlizzi *et al.*, based on GRSPaV sequences obtained by cloning of RSP 13/14 PCR products, outlined the existence of a fifth molecular group including the recently sequenced GRSPaV-PN variant (Lima *et al.*, 2009) and identified a new sixth group of molecular variants named “group VI” comprising GRSPaV sequences obtained from a “Muller Thurgau” grapevine. In the same paper, they reported the use of a new set of degenerate primers (RSP 35/36) amplifying a 478 bp fragment in the polymerase domain of ORF1. With this new set of primer, they were able to detect virus variants clustering in an additional new seventh group, named “group VII” (Fig. 21).

Unfortunately, sequences obtained in their study were not submitted to GenBank.

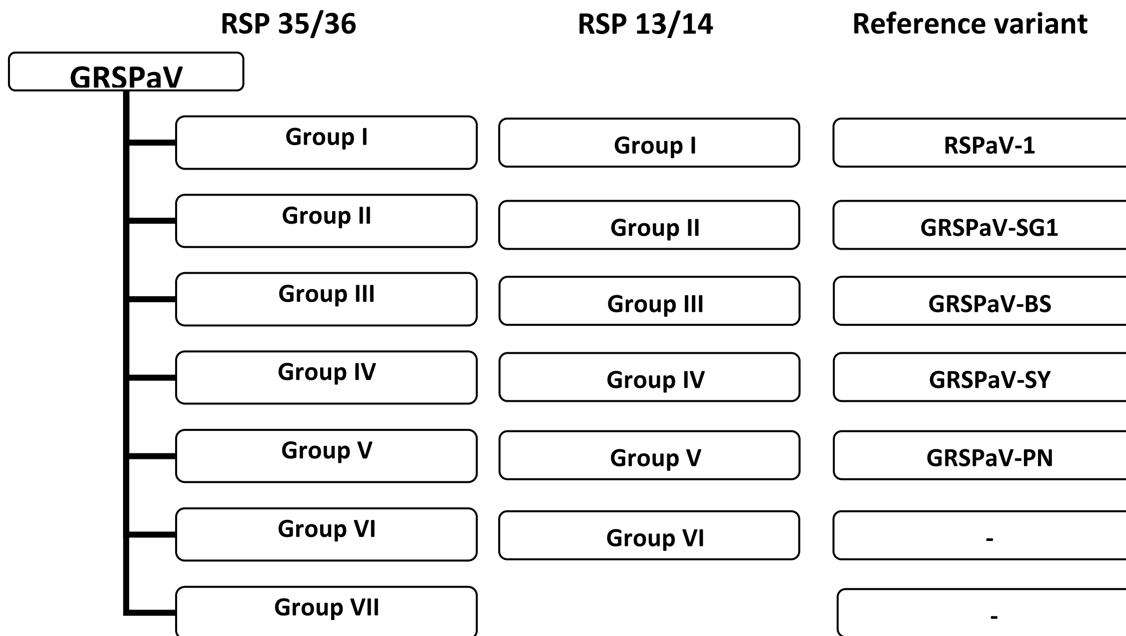

Figure 21. Phylogenetic groups of GRSaV variants identified by Terlizzi *et al*. (2011) with different primer pairs and reference variant for each group (when available)

In this study, we aim to compare the results of the phylogenetic analysis performed on the basis of the ORF5 sequence to the one based on sequences from ORF1, in order to check the similar or different clustering of our accessions in phylogroups depending on the genomic region analyzed.

4.2 Materials and Methods

The viral sources came from a selection of the 99 accession tested by RT-PCR with GRSPaV-specific primers in Chapter 2. 7 accessions, singularly infected by different groups of GRSPaV variants and already characterized on the basis of their ORF5 sequences were chosen (Tab. 13).

Table 13. List of accessions object of this study

Accession	RT-PCR with group specific primers	GRSPaV molecular group
CC8	G I	2a
CC17	G I	2b
MLOC2	G I	2a
5/1	G II	1
SMH22	G II	1
MSAS1	G II	3
MSAS3	G III	3

RNA extraction from phloem scrapings and cDNA synthesis was performed according to the protocol previously described in Chapter 2.

Primers RSP 13/14 (Meng *et al.*, 1999a) were used to amplify a 339 bp fragment in the helicase domain of ORF1. Also, a new set of degenerate primers external to RSP 13/14, named RSP d1 F/R, was designed in this study on the consensus sequences of 6 GRSPaV isolates whose full genome sequence is available in GenBank (Fig. 22) (RSPaV-1: accession number AF057136.1; GRSPaV-SG1: accession number AY881626.1; GRSPaV-MG: accession number FR691076.1; GRSPaV-SY: accession number AY368590.1; GRSPaV-BS: accession number AY881627.1; GRSPaV-PN: accession number AY368172.2).

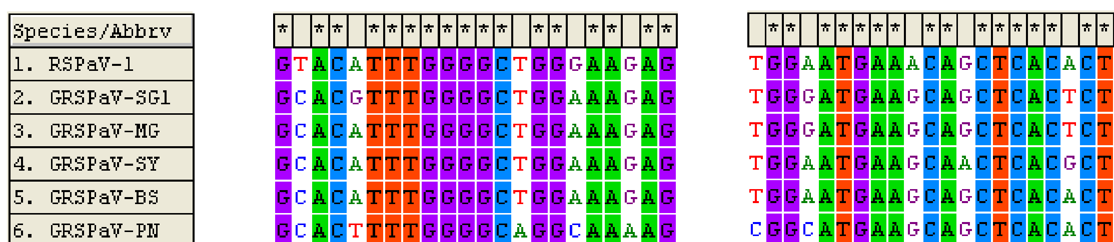

Figure 22. Alignment of GRSPaV sequences in order to design degenerate primers RSP d1 F and RSP d1 R. Sequences used in this alignment were from six reference isolates of *Grapevine rupestris stem pitting-associated virus*, RSPaV-1 (AF057136.1), GRSPaV-SG1 (AY881626.1), GRSPaV-MG (FR691076.1), GRSPaV-BS (AY881627.1), GRSPaV-SY (AY368590.1) and GRSPaV-PN (AY368172.2).

5 µl of cDNA were added to PCR mixture yielding final concentrations of 1x GoTaq Buffer (Promega, USA), 1.5 mM $MgCl_2$, 0.4 mM each deoxynucleotide triphosphate, 0.2 µM each primer and 1 U Taq DNA Polymerase (Promega, USA) per reaction, in a total volume of 50 µl.

PCR reactions were performed with the parameters reported in Table 14.

10 µl of the PCR products were analyzed on a 1.5% agarose gel, stained with GelRed (Biotium, USA) and visualized under with light.

Table 14. PCR Cycling conditions for each primer set

Primer set	Initial denaturation	Denaturation	annealing	extension	Final extension
RSP 13/14	95°C x 3' 1 x	94°C x 45''	52°C x 45'' 40 x	72°C x 45''	72°C x 1' 1 x
RSP d1 F/R	95°C x 3' 1 x	94°C x 45''	50°C x 45'' 40 x	72°C x 1'	72°C x 45''

Bands of the expected size (655 bp) obtained after PCR reactions with primer set RSP d1 F/R were excised from the gel and PCR products were purified with Wizard SV Gel and PCR Clean-Up System kit (Promega, USA) according to the manufacturer's protocol.

The resulting DNA solution was used in cloning reactions, using pGEM®-T Easy vector System II (Promega, USA).

Ligation reactions were set up by mixing 3 µl of DNA purified from agarose gel, 5 µl of 2x Ligation Buffer (60 mM Tris-HCl, pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10% polyethylene glycol), 1 µl pGEM®-T Easy Vector (50 ng), 1 µl of T4 DNA Ligase enzyme (3 units/µl) and sterile water to a final volume of 10 µl. The reactions were incubated overnight at 4°C.

2 µl of each ligation reaction were added into a vial containing 50 µl of thawed JM 109 High Efficiency Competent Cells (Promega, USA), gently mixed and incubated for 20 min on ice. The cells were then heat-shocked for 50 sec at 42 °C and put on ice for 2 min.

950 µl of room temperature SOC medium was added, and the cells were incubated shaking (150 rpm) for 1.5 h at 37 °C. After a brief centrifuge spin, 800 µl of the

supernatant was discarded and the cells were gently resuspended in the remaining SOC medium.

100 µl of the transformed cells from each sample were plated on 2 LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C.

10 white colonies for each sample were selected from the transformants growing on the plates, picked up with a sterile toothpick, plated on a new LB/ampicillin masterplate, incubated overnight at 37 °C and stored for 4 weeks at 4 °C.

The toothpick was then inserted in eppendorf tubes containing 50 µl of molecular biology grade water and incubated 10 minutes at 99 °C. 1 µl of this solution was used in PCR reaction with primers RSP d1 F/R in order to assess the presence of the PCR product insert in the colonies.

7 to 10 clones from each accession were picked up from the masterplate and placed in 3 ml of LB medium containing ampicillin (final concentration 0.05 mg/ml) and grown overnight at 37 °C while shaking at 150 rpm.

The cultures were then centrifuged at 10.000 g for 5 m to pellet the cells, the supernatant was discarded, and plasmids were purified using Wizard Plus SV Minipreps DNA Purification System (Promega, USA) according to manufacturer's instructions. Plasmids were finally eluted in 100 µl of molecular biology grade water and shipped for sequencing (Macrogen Europe, The Nethelands).

Contigs were created using Vector NTI 11 software (Invitrogen, USA) and alignments of nucleotide sequences were done using CLUSTALW (Thompson *et al*., 1994) with default settings.

In addition to the sequences obtained in our work, corresponding sequences of GRSPaV isolates available in GenBank (Tab. 15) were included in the subsequent analyses.

Nucleotide and aminoacidic identities between sequences were computed using the p-distance model included in the MEGA5 software (Tamura *et al*., 2011) and plotted using Microsoft Excel spreadsheet (Microsoft Office 2003, Microsoft Corporation, USA).

Sequences with nucleotide identity above 99% between clones of the same isolate were considered as a single sequence, while sequences derived from additional clones of an

isolate sharing with nucleotide differences greater than 1% were considered as different independent clones.

Evolutionary relationships were inferred from multiple sequence alignments calculated by CLUSTALW using the Neighbour Joining (NJ) method (Saitou and Nei, 1987) with 1000 bootstrap replications. The evolutionary distances were computed using the Kimura two-parameter model (Kimura, 1980). These phylogenetic analyses tools were implemented by the molecular evolutionary genetics analysis (MEGA) software version 5.0.

Table 15. List of sequences available in GenBank included in the analysis

Accession name	GenBank accession number	Accession name	GenBank accession number
RSPaV-1	AF057136.1	EWCH9	FJ943371.1
GRSPaV	AF026278.1	EWCS3	FJ943407.1
GRSPaV-3138-07	JX559646.1	Paulsen 1103	DQ278620.1
GRSPaV-SY	AY368590.1	Pinot Noir 8	DQ278636.1
GRSPaV-BS	AY881627.1	Ravat 34-6-4	DQ278641.1
GRSPaV-SG1	AY881626.1	Ravat 34-7-6	DQ278643.1
GRSPaV-MG	FR691076.1	Seyval 7	DQ278646.1
GRSPaV-PG	HE591388.1	WWDC1	FJ943372.1
GRSPaV-WA	KC427107.1	WWVD1	FJ943404.1
GRSPaV-GG	JQ922417.1	BCZR1	FJ943358.1
GRSPaV-PN	AY368172.2		

4.3 Results

Based on the alignment of 6 different GRSPaV variants, degenerate primers RSP d1 F and RSP d1 R were designed (Tab. 16). They amplify a DNA fragment of 655 bp corresponding to the genomic region comprised between nt positions 4128 and 4782 of reference variant RSPaV-1.

Table 16. Broad-spectrum primers RSP d1 F/R, 5'-3' sequences and amplicon length.
W= A + T; M= A + C; R= A + G; Y= C + T ; K= G + T

Primer	Sequence (5'-3')	Amplicon
RSP d1 F	GCACWTTTGGGGCWGGMAARAG	655 bp
RSP d1 R	AGYGTGAGYTGCTTCATKCCR	

Amplicons of the expected size were amplified by primer pairs RSP 13/14 and RSP d1 F/R. PCR with primer set RSP 13/14 produced amplicons only for 3 out of the 7 tested accessions. On the contrary, the newly designed primer set RSP d1 F/R was able to amplify the 655 bp fragment of GRSPaV from all of the 7 accessions, irrespective of the different phylogenetic groups of virus variants (Fig. 23)

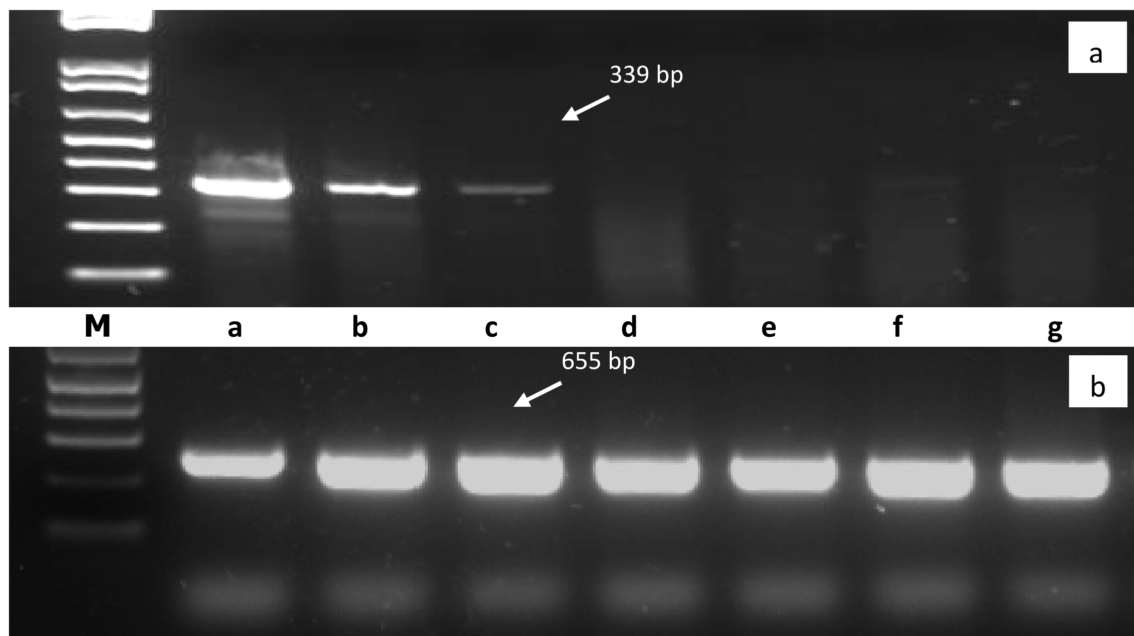

Figure 23. Results of RT-PCR with primer pairs RSP 13/14(a) and RSP 5/6(b) on agarose gel visualized by UV light. Samples (from left to right): **M**= marker; **a**= CC8; **b**= CC17; **c**= MLOC2; **d**= 5/1; **e**= SMH22; **f**= MSAS1; **g**= MSAS3.

PCR products obtained with primer set RSP d1 F/R were extracted from the gel, purified and cloned; 63 clones were obtained and sequenced.
As previously stated, sequences sharing more than 99% nucleotide (nt) identity from the same isolate were considered as a single clone. A total of 17 different sequences were identified after the sequencing.

In pairwise comparisons, the 17 CP sequences obtained in this study showed identities ranging between 78.5 and 99.1% at the nt level and between 91.2 and 100.0 % at the aminoacid (aa) level. Similar ranges of values were obtained when comparisons were made with corresponding sequences in GenBank. The phylogenetic relationships of clones obtained in this study were compared among themselves and with 21 corresponding sequences from other grape-growing regions available in GenBank (Tab. 15). Due to different primer sets used in several other studies, the analysis was conducted on a 299 bp fragment comprised in the 655 bp sequences obtained in this research.
This analysis, using the neighbor-joining (NJ) method, included a total of 38 sequences.
The results showed segregation of GRSPaV CP sequences into 6 major lineages (Fig. 24).
In order to maintain a standardized nomenclature of GRSPaV sequence variant groups, we named each group in continuity with the classification proposed by Nolasco *et al.* (2006), according to the reference isolates present in each phylogroup.

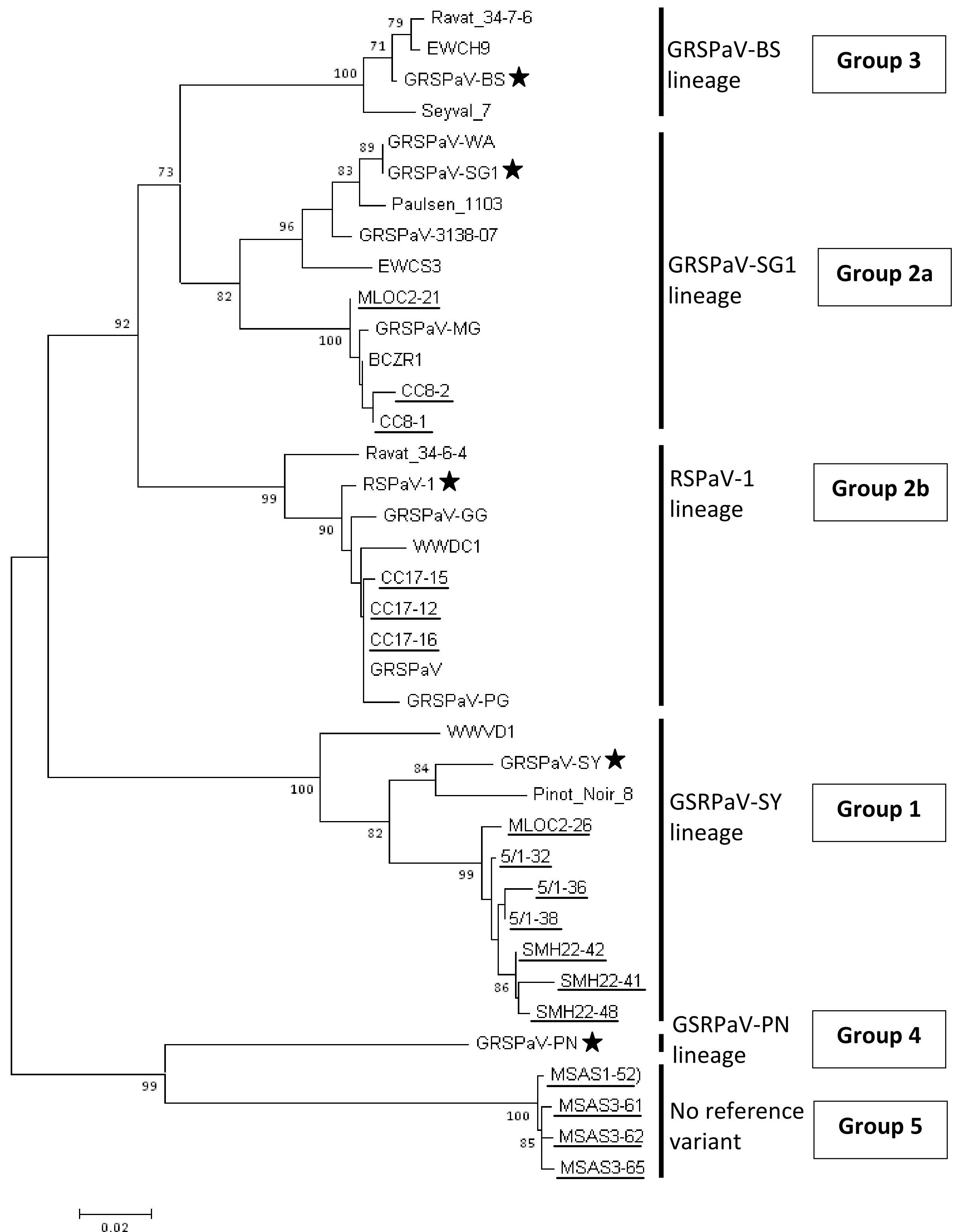

Figure 24. Phylogenetic relationship among GRSPaV isolates. Multiple alignment 299 nt fragment in the helicase domain of ORF1. Sequences obtained in this study are underlined. Reference variants for each lineage are marked with an asterisk. Phylogenetic tree was constructed with neighbour-joining method, evolutionary distances were computed using the Kimura 2-parameter method. Only bootstrap values higher than 70% are shown

Average nt and aa identity within groups 4 and 5 were not calculated, as they only contain one sequence (group 4) or sequences from the same isolates (group 5).

The average nt and aa identity between sequences within the same group is more than 95.4% and 98.5%, respectively.

On the contrary, average nt identity between different lineages never exceeds 90% (77.4% between group 1 and group 4 to 89.5% between group 3 and group 2a), while average aa identity ranges from 91.3% (between group 1 and group 4) to 97.9 (between group 3 and groups 2a/2b) (Tab. 17).

a)

	nt %	**aa %**
Group 1	95.6	98.8
Group 2b	98.1	99.3
Group 2a	95.4	98.5
Group 3	98.3	100
Group 4	n. c.	n. c.
Group 5	n. c.	n. c.

b)

nt %	**Group 1**	**Group 2b**	**Group 2a**	**Group 3**	**Group 4**	**Group 5**
Group 1	-					
Group 2b	81,6	-				
Group 2a	80.8	88.3	-			
Group 3	80.9	87.3	89.5	-		
Group 4	77.4	81.1	79.8	81.1	-	
Group 5	78.2	78.8	78.6	79.4	83.8	-

c)

aa %	**Group 1**	**Group 2b**	**Group 2a**	**Group 3**	**Group 4**	**Group 5**
Group 1	-					
Group 2b	94.2	-				
Group 2a	94.7	97.8	-			
Group 3	94.3	97.9	97.9	-		
Group 4	91.3	93.8	92.8	95.0	-	
Group 5	93.8	96.2	95.4	95.5	94.4	-

Table 17. Average percentages of nucleotide and amino acid identity within (**a**) and between (**b** and **c**) molecular groups. The values were computed using the p-distance model included in the MEGA5 software (Tamura *et al*., 2011)

Molecular groups 1, 2a, 2b, and 5 contained 7, 3, 3 and 4 sequences from this study, respectively, and 3, 7, 6 and 0 from GenBank. None of our sequences clustered in the group 4 (represented in the phylogenetic tree only by the reference variant GRSPaV-PN) and in group 3.

Group 5 comprises only variants obtained in this study, and no other GRSPaV sequence available in GenBank shows more than 85% nt identity with them. The closest match is GRSPaV-PN (accession number AY368172.2), which shares about 84% nt identity.

Sequence alignment showed the presence of 103 parsimony informative sites and 17 singleton sites in the 299 bp fragment object of this analysis. Minimum nucleotide identity between the variants in pairwise comparisons was 76.3% and most of the sequence variants shared about 82% to 79% nucleotide identity (Fig. 25).

The nucleotide diversity (mean nucleotide distance between two sequences) as estimated by the Kimura 2 parameters model was 0,171 substitutions per site.

These values are within the limits of species demarcation criteria in the family *Betaflexiviridae,* where isolates sharing greater than 72 % nt or 80 % aa sequence identities between their CP or polymerase genes are considered one species (King *et al*., 2011). Based on these results, it can be concluded that sequences are specific to GRSPaV and that divergent variants of the virus are present in the grapevine accessions object of this work.

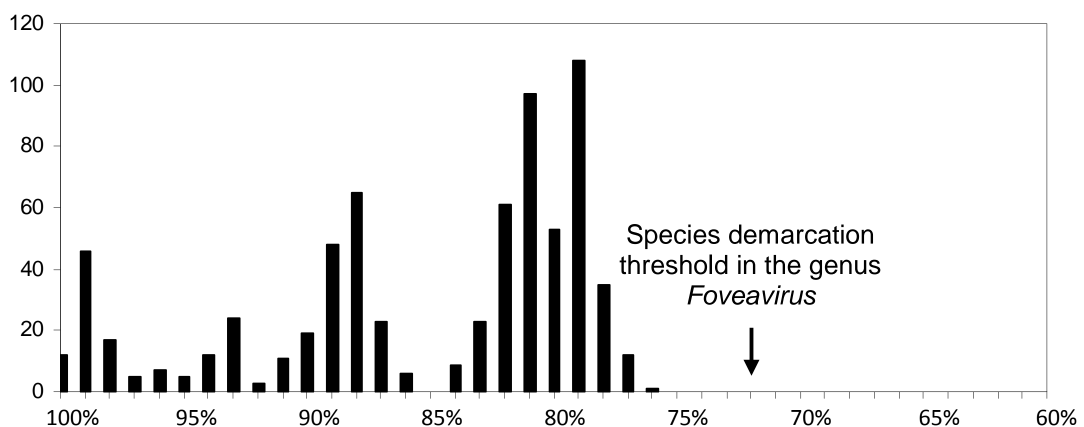

Figure 25. Distribution of the percentages of pairwise identities among 38 partial ORF1 sequences of GRSPaV isolates. Each bar represents the total number of pairwise nucleotide comparisons sharing the same percentage of nucleotide sequence identity. The species demarcation threshold is according to criteria established by the International Committee on Taxonomy of Viruses (King *et al*., 2011).

The nucleotide composition was in average 30.1% T, 21.0% C, 27.0% A, and 21.8% G, and did not differ significantly within the different groups.

The partial replicase aminoacid sequence encoded by the 299 bp fragment was deduced by translation using the universal code. It consists of 99 aminoacids of which 77 are conserved among the variants included in the analysis.

4.4 Discussion

In this study, we further assessed the molecular diversity of field isolates of GRSPaV in grapevine accessions from Tuscany. A phylogenetic approach was used to analyze a total of 38 partial ORF1 coding sequences (17 from this study and 21 from GenBank).

The results confirm the clustering of GRSPaV variants in several phylogenetic groups. The groups identified on the basis of a 299 nt sequence in the helicase domain of ORF1 (nt 4373 to 4711 of reference variant RSPaV-1) are 6, in contrast with the 5 obtained according to the ORF5 sequences.

Notably, the new phylogroup identified consists only of GRSPaV sequences obtained from our Tuscan grapevine accessions MSAS1 and MSAS3. The virus variants infecting these plants clustered in group 3, together with reference variant GRSPaV-BS, according to their ORF5 sequence, but they now form a distinct and possibly yet unknown group. No GRSPaV sequences available in GenBank is part of this cluster, the most similar sequence appear to be GRSPaV-PN (accession number AY368172.2), sharing about 84% of nucleotide identity.

Adopting the nomenclature system proposed by Nolasco *et al.* (2006), we named the groups with numbers from 1 to 4 (groups 1, 2a, 2b, 3 ad 4) according to the reference variants present in each cluster, as previously described in Chapter 3. The sixth, new group identified was named “group 5”.

Recently, a study by Terlizzi *et al.* (2011) reported the existence of two new groups of GRSPaV variants, based on 478 nt sequences in the polymerase domain of ORF1. Unfortunately, a comparison of sequences from “group 5” and these two additional groups could not have been made because of the different primers used to amplify a different portion of ORF1. Also, the sequences obtained by Terlizzi *et al.* (2011) are not available in GenBank.

In addition to the different clustering of GRSPaV variants from grapevine accessions MSAS1 and MSAS3 based on ORF1 and ORF5 sequences and the identification of a new “group 5”, it is also interesting to note that all virus variants from accessions previously indexed positive to VN, amplified by group-specific primers G I and classified in

molecular groups 2a and 2b according to their ORF5 sequence, confirm to be part of groups 2a and 2b, together with reference variants GRSPaV-SG1 and RSPaV-1.

5. Biological indexing for vein necrosis disease

5.1 Introduction

In order to define the actual role in causing vein necrosis symptoms by the above described genetic variants of GRSPaV , a biological indexing test was conducted in our Department and in collaboration with the University of Bari and the University of Milan in the PRIN project by Ministero dell'Università e della Ricerca in the frame of "Programmi di Ricerca scientifica di Rilevante Interesse Nazionale" (PRIN 2007) under the project prot.2007RHMMJH "Biological and molecular characterization of Grapevine rupestris stem pitting associated virus (GRSPaV)".

Biological indexing may be defined as any test that reproducibly assess the presence or absence of specific pathogens, or identify a disease on the basis of the reactions induced on specific indicator plants (Di Terlizzi, 1998). The recognition that symptoms caused by virus or virus-like diseases could be reproduced from one plant to another by the transfer of a "*contagium vivum fluidum*" (Beijerick, 1898) was significant for the birth of plant virology as a science. Until the late 1950s, virus diagnosis was dominated by field symptomatology and field tests. Early virologists learned that not only symptoms could be transferred from one plant to a similar one, but also that other genera of plants were susceptibly infected and, moreover, that the symptoms on these plants were characteristic for specific viruses.

Notwithstanding the increasingly development of serological and especially molecular assays, biological indexing with indicator species still remains a valuable and often irreplaceable method for the detection of viral plant diseases.

In fact, serological and molecular techniques are limited to known viruses for which antibodies and genome sequences are available, moreover molecular methods are costly and require specific laboratory equipment and well trained personnel. In addition, the targets of these techniques, nucleic acids and proteins of viruses, makes them unsuitable for the diagnosis of diseases whose causal agent is still unknown.

VN is considered as a virus-like disease, that means its causal agent is still unknown. Thus, grafting on *Vitis* indicators is the only viable indexing method. Rootstock hybrid 110 Richter (*V. berlandieri* x *V. rupestris*) is the only susceptible host for VN and must be used as indicator.

Although reliable, biological indexing by grafting is time-consuming, labour-intensive and requires availability of indexing fields where to conduct the trial.

A relatively new technique, green-grafting, has been developed for the vegetative propagation of grapevines. It has been successfully adopted in several grape-growing countries to rapidly diagnose viral disease (Walter *et al.*, 1990; Lahogue *et al.*, 1995; Kassemeyer *et al.*, 1997; Kaserer *et al.*, 2003).

This grafting technique was used for the indexing of GRSPaV-infected grapevine accessions, in order to assess the role of different GRSPaV variants groups in the expression of VN disease.

5.2 Materials and Methods

Grapevine material (woody canes) was collected in the experimental vineyard located in San Piero a Grado (Pisa, Italy) during February 2010. Woody cuttings from additional Italian grapevine accessions to be included in the indexing trial were provided by University of Bari and University of California-Davis.

Also, six grapevine accessions infected with different GRSPaV variants already described in literature were kindly provided by Foundation Plant Services (University of California – Davis, USA), Estacao Agronomica Nacional, (INRB Instituto Nacional de Recursos Biológicos, Portugal) and Grape and Persimmon Research Station (National Institute of Fruit Tree Science, Japan). In total, 37 accessions, representative of every possible combination of single and mixed GRSPaV infection, were part of the biological indexing trial (Tab. 18).

All of these accessions were previously tested by RT-PCR with GRSPaV group-specific primers (Rowhani *et al.*, 2000).

Table 18. List of the 37 grapevine accessions indexed for VN, their species, origin and results of GRSPaV detection with group-specific primers

Code	Species / hybrid	Accession	Origin	Group-speific primers
BA02	*V. vinifera*	C5G2	Bari	G I
BA03	*V. vinifera*	C20N4	Bari	G I
BA04	*V. vinifera*	C30O4	Bari	G I
BA05	*V. vinifera*	C43H4	Bari	G I
BA06	*V. vinifera*	L32M	Bari	G I
BA07	*V. vinifera*	C23A2	Bari	G I
PI01	*V. vinifera*	CC8	Pisa	G I
PI02	*V. vinifera*	CC17	Pisa	G I
PI03	*V. rupestris*	RUP1	Pisa	G I
CA01	*V. vinifera*	Inzolia	Davis	G I
J2	*V. labruscana*	Hiz3	Japan	G I
J3	*V. labruscana*	Hiz5	Japan	G I
PI04	*V. vinifera*	5/1	Pisa	G II
PI05	Kober5BB	BSK3	Pisa	G II
CA02	*V. vinifera*	Zinfandel09	Davis	G II
CA03	*V. vinifera*	Thomcord02	Davis	G II
U1	*V. vinifera*	Syrah8	California	G II
P1	*V. sylvestris*	110395	Portugal	G II
P2	*V. sylvestris*	110403	Portugal	G II
J1	*V. labruscana*	Hiz1	Japan	G II
PI06	Kober5BB	BSK1	Pisa	G III
CA04	*V. vinifera*	Diamond Muscat	Davis	G III
BA08	*V. vinifera*	L48E	Bari	G I + G II
PI07	*V. vinifera*	MLOB7	Pisa	G I + G II
PI08	*V. vinifera*	CC26	Bari	G I + G II
CA05	*V. vinifera*	Cabernet Franc 14	Davis	G I + G II
PI09	*V. vinifera*	1/18	Pisa	G I + G III
PI10	*V. vinifera*	COR20	Pisa	G I + G III
PI11	*V. vinifera*	Gallo15	Pisa	G II + G III
PI12	*V. vinifera*	V16F12	Pisa	G II + G III
PI13	*V. vinifera*	Gallo7	Pisa	G II + G III
PI14	*V. vinifera*	COR16	Pisa	G II + G III
BA11	*V. vinifera*	C20H3	Bari	G I + G II + G III
BA12	*V. vinifera*	C51D3	Bari	G I + G II + G III
PI15	*V. vinifera*	SM6/10	Pisa	G I + G II + G III
PI16	*V. vinifera*	CC6	Pisa	G I + G II + G III
PI17	*V. vinifera*	34/46	Pisa	Negative

Cuttings of 110R mother plants negative to GRSPaV and to VN, used as biological indicator in the Department of Agriculture, Food and Environment (DAFE) at University of Pisa, were collected from the experimental vineyard of DAFE located in San Piero a Grado (PI).

Woody cuttings were trimmed to lengths with two buds each and planted bud-side up in perlite under a mist bed in greenhouses boxes. Cuttings that produced shoots by 3–8

weeks were removed from perlite substrate, planted in plastic pots containing potting mix and maintained in greenhouse in order to obtain own rooted cuttings (Fig. 26).

Figure 26. Propagation of plant material for biological indexing. (**a**) production of own rooted cuttings on perlite substrate; (**b**) production of own rooted cuttings of biological indicator 110 R; (**c**) transfer of own rooted cuttings to pots and (**d**) conservation of the plants in greenhouse

When the shoots reached an adequate size (diameter of about 4 to 6 mm), shoots of the indicator 110R were grafted onto the accessions to be indexed.

Portion of 110R shoots of the adequate diameter were cut to lengths of one bud each and a long tapered wedge was shaped with a bistoury blade. Meanwhile, the shoot of grapevine accession to be tested, that acts as rootstock, was cut after 3-4 nodes and a slit was made down the centre of the shoot (Fig. 27).

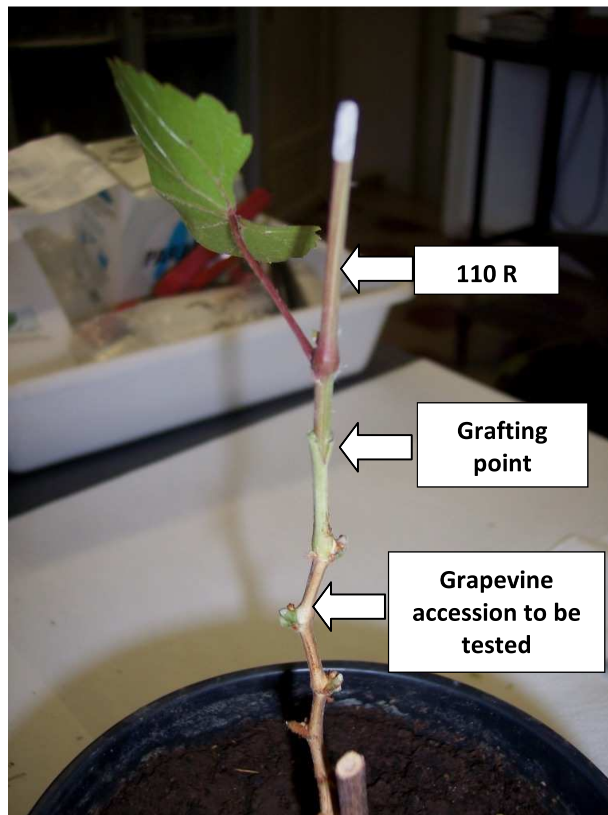

Figure 27. Example of green-grafting technique. The biological indicator 110R is grafted onto the grapevine accession to be tested.

After the insertion of the scion in the slit of the rootstock, the grafting point was taped with parafilm to keep the union point tightened; the rootstock and the scion had the same diameter at the point of contact, so that the cambium layers of the union matched.

Buds and leaves from the rootstock shoot were removed in order to force the growth of the scion bud; the leaf of the scion was cut to half of the original size to reduce transpiration. (Martelli *et al.*, 1993).

Grafting with 110R was successfully performed during springtime and early summer (from April to July) and repeated each year for the unsuccessfully grafted accessions or to index more replicates of each accession.

Grafted plants were covered with a plastic bag to maintain humidity and then maintained in greenhouse with average temperatures of 18-25°C. After 3-4 days some cuts were made on plastic bags in order to avoid condensation. Plastic bags were then removed after the 110R shoot was developed. The greenhouse was ventilated and lightly shaded from May to September.

Leaves and petioles of the 110R shoot were periodically controlled for the expression of specific VN symptoms and their development. The intensity of the symptoms was recorded using a pathometric scale from + (mild symptoms) to +++ (severe symptoms) (Tab. 19)

Table 19. Pathometric scale used to determine symptoms intensity and description of corresponding symptoms

Symptoms intensity	Symptoms Description
-	Healthy 110R leaves, no VN-specific symptoms.
+	Isolated necrotic areas on leaf veinlets visible the underside leaf of leaf blade
++	Vein necrosis diffused on an extended part of the leaf blade. Leaf begins to roll.
+++	Most of the leaf surface shows vein necrosis, with necrotic areas forming a dense network clearly visible also on the upper side of the leaf blade. Leaf is rolled down and necrotic reactions appear also on leaf petioles.

5.3 Results

Vegetative propagation and biological indexing was successfully concluded for all of the 37 grapevine accessions.

Results of the biological indexing could be recorded 30-40 days after the grafting date. On symptomatic accessions, the first VN symptoms appeared on the abaxial side of 110R leaves after 20-30 days from the grafting, consisting of small necrotic areas on the veins of leaves at the base of the shoot. With time, symptoms develop to veinlets and cover a bigger surface of the leaf blade; necrotic areas appear also on the veins of apical leaves.

Symptomatic leaves tend to roll down, as a consequence of necrosis on vein tissues and exhibit vitreous consistency.

Severe symptomatic manifestations also induce necrotic striations on leaf petioles and green canes.

A selection of the symptoms observed is reported in Figure 28.

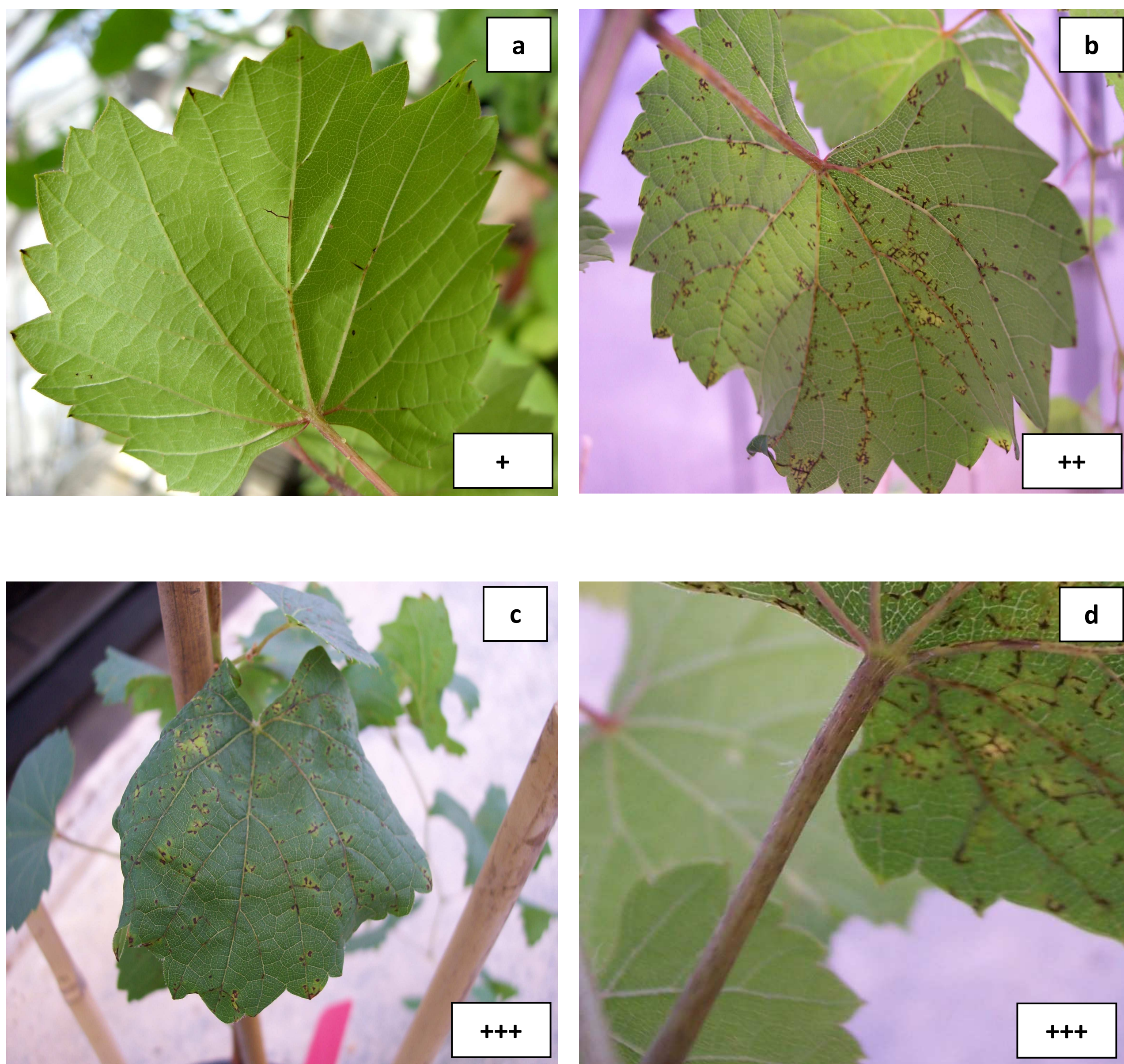

Figure 28. Selection of the VN-specific symptoms observed on 110R and their intensity level. (**a**) mild VN symptoms appearing on the abaxial side of 110 R leaves (+); (**b**) macroscopic VN symptomatology (++) ; (**c**) VN symptoms appearing on the upper side of 110 R leaves and downward rolling of the leaf blade (+++); (**d**) details of symptoms appearing on leaf petioles (+++).

In total, out of the 37 accessions indexed, 22 tested positive, inducing VN-specific symptoms on the indicator 110 R, while the remaining 15 (including the negative control not infected by GRSPaV) did not show any VN symptoms (Tab. 20).

Table 20. Results of the biological indexing for the 37 grapevine accessions. Chronological evolvement of each accession in the indexing steps. ✓: positive result; x: negative result; **+:** mild symptoms; **++:** medium intensity symptoms; **+++:** severe symptoms.

Code	Accession name	Species/ hybrid	Origin	GRSPaV molecular group	Rooted on perlite	Maintained in pots	Indexing (top grafting)	Symptoms development	Symptoms intensity
BA 02	C5G2	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	+++
BA 03	C20N4	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	++
BA 04	C30O4	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	+++
BA 05	C43H4	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	+
BA 06	L32M	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	++
BA 07	C23A2	*V. vinifera*	Bari	**G I**	✓	✓	✓	✓	++
PI 01	CC8	*V. vinifera*	Pisa	**G I**	✓	✓	✓	✓	+++
PI 02	CC17	*V. vinifera*	Pisa	**G I**	✓	✓	✓	✓	++
PI 03	RUP1	*V. rupestris*	Pisa	**G I**	✓	✓	✓	✓	++
CA01	Inzolia	*V. vinifera*	California	**G I**	✓	✓	✓	✓	+
J2	Hiz 3	*V. labruscana*	Japan	**G I**	✓	✓	✓	✓	++
J3	Hiz 5	*V. labruscana*	Japan	**G I**	✓	✓	✓	✓	++
PI 04	5/1	*V. vinifera*	Pisa	**G II**	✓	✓	✓	x	-
PI 05	BSK3	Kober 5BB	Pisa	**G II**	✓	✓	✓	x	-
CA02	Zinfandel09	*V. vinifera*	California	**G II**	✓	✓	✓	x	-
CA03	Thmocord02	*V. vinifera*	California	**G II**	✓	✓	✓	x	-
J1	Hiz 1	*V. labruscana*	Japan	**G II**	✓	✓	✓	x	-
P1	110395	*V. sylvestris*	Portugal	**G II**	✓	✓	✓	x	-
P2	110403	*V. sylvestris*	Portugal	**G II**	✓	✓	✓	x	-
U1	Syrah 8	*V. vinifera*	USA	**G II**	✓	✓	✓	x	-
PI 06	BSK1	Kober 5BB	Pisa	**G III**	✓	✓	✓	x	-
CA04	Diam. Muscat	*V. vinifera*	California	**G III**	✓	✓	✓	x	-
BA 08	L48E	*V. vinifera*	Bari	**G I + G II**	✓	✓	✓	✓	+++
PI 07	MLOB7	*V. vinifera*	Pisa	**G I + G II**	✓	✓	✓	✓	++
PI 08	CC26	*V. vinifera*	Pisa	**G I + G II**	✓	✓	✓	✓	++
CA05	Cab. Franc14	*V. vinifera*	California	**G I + G II**	✓	✓	✓	✓	+
PI 09	1/18	*V. vinifera*	Pisa	**G I + G III**	✓	✓	✓	✓	++
PI 10	COR 20	*V. vinifera*	Pisa	**G I + G III**	✓	✓	✓	✓	+++
PI 11	Gallo15	*V. vinifera*	Pisa	**G II + G III**	✓	✓	✓	x	-
PI 12	V16F12	*V. vinifera*	Pisa	**G II + G III**	✓	✓	✓	x	-
PI 13	Gallo 7	*V. vinifera*	Pisa	**G II + G III**	✓	✓	✓	x	-
PI 14	COR 16	*V. vinifera*	Pisa	**G II + G III**	✓	✓	✓	x	-
BA 11	C20H3	*V. vinifera*	Bari	**G I + G II + G III**	✓	✓	✓	✓	+
BA 12	C51D3	*V. vinifera*	Bari	**G I + G II + G III**	✓	✓	✓	✓	+++
PI 15	SM6/10	*V. vinifera*	Pisa	**G I + G II + G III**	✓	✓	✓	✓	++
PI 16	CC6	*V. vinifera*	Pisa	**G I + G II + G III**	✓	✓	✓	✓	++
PI 17	34/46	*V. vinifera*	Pisa	**Negative control**	✓	✓	✓	x	-

Total: **37 accessions**

It is interesting to note that all and only the 22 accessions infected by GRSPaV variants amplified by G I group-specific primers (corresponding to phylogenetic groups 2a and 2b), in single (12) or mixed (10) infection with other groups, resulted positive to the indexing. On the contrary, 14/14 accessions infected by virus variants amplified by group-specific primers G II and/or G III (corresponding to phylogenetic groups 1 and 3) never induced VN symptoms. (Tab. 21)

Table 21. Number of VN+ and VN- accessions for each combination of GRSPaV infection

	RT-PCR with GRSPaV group-specific primers							
	G I	**G I + G II**	**G I + G III**	**G I + G II + G III**	**G II**	**G III**	**G II + G III**	**Neg. control**
VN indexing +	12	4	2	4	0	0	0	0
VN indexing -	0	0	0	0	8	2	4	1
Total: 37	12	4	2	4	8	2	4	1

5.4 Discussion

Considering the relationship reported in Chapter 2, where all and only grapevine accessions positive to the biological indexing for VN were positive to GRSPaV group-specific primer set G I, a biological indexing trial was conducted for 37 grapevine accessions collected from Italy (Tuscany and Apulia), USA, Portugal and Japan.

The results of the biological indexing confirm and support the hypothesis that only GRSPaV variants amplified by group-specific primers G I (corresponding to phylogenetic groups 2a and 2b) are able to induce VN-specific symptoms expression.

This remarks a different role of GRSPaV variants in VN aetiology. Variants amplified by group-specific primers G II and G III (corresponding to GRSPaV phylogroups 1 and 3), did not appear correlated with VN disease, while only virus variants amplified by group specific primers G I showed a strong association with this disease.

This study allowed to better investigate the association between VN disease and GRSPaV reported by Bouyahia *et al.*, (2005). Considering the results obtained we should consider only phylogenetic groups 2a and 2b of the virus as causal agents of VN, while groups 1 and 3 did not appear to have any correlation with this disease.

6. Final conclusions

The results obtained in this study allowed to get a clear picture of the different variants of GRSPaV infecting grapevine accessions and to investigate the different role that different molecular variants of the virus assume in the determinism of grapevine vein necrosis disease (VN).

A first screening of different virus variants was performed by RT-PCR amplification with "broad spectrum" primers RSP 5/6 and RSP13/14 and "group-specific" primers (G I; G III; G III) for GRSPaV.

RT-PCR with primers RSP 13/14 detected GRSPaV from grapevine accessions positive to VN and showed negative results when testing VN- accessions. On the other side, primers RSP 5/6 amplified a wider range a GRSPaV variants, also from VN- plants.

"Group specific" primers were able to discriminate 3 groups of virus variants, named G I, G III and G III. Interestingly, primers G I detected the virus from VN+ accessions, exactly matching the results obtained with RSP 13/14.

Therefore, an evident correlation appeared between infection by GRSPaV variants amplified by primers RSP 13/14 and G I, and VN symptoms expression.

The molecular characterization of different GRSPaV isolates, performed by amplification, cloning and sequencing of GRSPaV ORF5 (encoding the coat protein gene) and a portion of ORF1 (encoding the viral replicase) allowed us to compare the GRSPaV variants present in our plant material with GRSPaV isolates from other grape-growing regions already deposited in GenBank, and to conduct phylogenetic analyses.

A new set of degenerate primers, named RSP d1 F/R, was designed and proved to be highly effective in detecting different GRSPaV variants and possibly superior to GRSPaV "broad spectrum" primers RSP 13/14. RSP d1 F/R amplify a 655 bp fragment in the helicase domain of ORF1.

Depending on the genomic region considered, GRSPaV variants were clustered in 5 (ORF5) or 6 (ORF1) phylogenetic groups. The sixth phylogroup, identified according to a fragment of ORF1 sequence, was composed only by sequences obtained in this study that show low identity (lower than 85%) with any other GRSPaV sequence available in

GenBank. This could represent a new, distinct group of GRSPaV variants that has been named “Group 6”.

Regarding the association between VN and GRSPaV, the molecular characterization revealed that accessions previously indexed positive for the disease always were infected by variants belonging to groups 2a and 2b, in single or mixed infection with other virus variants. Comparing the results of the molecular characterization with those obtained by RT-PCR with “group specific” primers, we could note that virus variants from phylogenetic groups 2a and 2b were always amplified by primer set G I.

Grapevine accessions infected by different groups of virus variants were also serologically tested by Western Blot, using a polyclonal antibody kindly provided by Dr. A. Minafra (Istituto di Virologia Vegetale – CNR di Bari). The antibody was able to detect virus variants of the different phylogenetic groups, suggesting a basic homogeneity of the viral coat protein, regardless the reported genetic variability of GRSPaV ORF5.

In order to further assess and confirm the relationship between GRSPaV groups 2a-2b and VN disease, a biological indexing trial for the diagnosis of VN was conducted involving grapevine accessions from Italy (Tuscany, Apulia), USA, Portugal and Japan. Each of these accessions were previously tested with group-specific primers.

The results of the biological indexing for VN further confirmed the hypothesis that only GRSPaV variants amplified by “group specific” primers G I (belonging to phylogenetic groups 2a and 2b) are involved in VN symptoms expression. In fact 100% of the accessions infected, singularly or in mixed infection, by variants detected by primers G I always induced VN symptoms on indicator 110R. On the contrary, all of the accessions positive to primer pairs G II and/or G III never induced symptoms of VN on 110R leaves.

These findings support the hypothesis of a differential role of GRSPaV variants in VN aetiology, which appear to be associated only GRSPaV variants belonging to phylogenetic groups 2a and 2b (reference isolates GRSPaV-SG1 and RSPaV-1). This could suggest the possibility to diagnose VN by RT-PCR assays with GRSPaV G I primers.

Until now, the only way to identify plants affected by VN was the long and laborious biological indexing method with indicator 110R.

The chance to diagnose VN by PCR would allow a much faster, cheaper and more simple way to monitor the diffusion and incidence of this disease, whose economic impact on the production is still largely unknown, because of its latency in *V. vinifera* and in most rootstock hybrids.

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Appendix. 1

<u>Competent cells preparation</u>

1. The day before: prepare a new culture of DH5α cells. Streak them on new LB plates with no ampicillin and incubate overnight at 37°C

Day 1:

2. LB without NaCl preparation:

   - 10g of Tryptone,

   - 5g of yeast extract

   - add enough milliQ water to homogenize. Then transfer to a cylinder and bring volume up to 1L. Dispense 250mL in four 1L flasks. Autoclave.

3. Autoclave 2 L of MilliQ water and put in -20C to cool for day 2.

4. Pick one colony from the DH5α cells and place in a falcon tube with 15 mL of LB (no ampicillin) media. Shake overnight at 37°C.

Day 2:

5. Add 2.5mL of the DH5α cells to each of the 1L flasks w/LB and put in shaker.

6. Check the O.D after 3-4 hrs. When the O.D is about 0.6, transfer all contents in flask to the 4 plastic bottles for bacterial use and immediately chill on ice for 15mins.

7. After time has passed centrifuge at 6000g for 15 mins.

8. Remove supernatant and resuspend pellets in 3mL of cold MilliQ water, vortex until homogenized, then add 200 ml water to each bottle and centrifuge as previous.

9. Repeat step 8 again.
10. Add 20mL of cold 10% glycerol to 1 of the bottles, resuspend the pellet and transfer the contents to the next bottle, and resuspend by vortex. Repeat this process until all four bottles have been consolidated into one, keeping everything on ice.
11. Centrifuge this bottle as previous, making sure to balance the centrifuge.
12. Remove supernatant (pellet should be bigger), and resuspend in a final volume of 3mL of 10% glycerol by vortexing.
13. Aliquot in 100µL into eppendorfs and store at -80C.