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REVIEW

Believing is seeing: lessons from emerging viruses in grapevine

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Abstract

Among the grapevine viruses recently identified, grapevine vein clearing virus (GVCV), grapevine Pinot gris virus (GPGV) and grapevine red blotch virus (GRBV) have emerged in the last decade as new threats to the grape and wine industry. Initially reported in Italy (GPGV) and the USA (GVCV and GRBV), GPGV and GRBV have a wider distribution at present, likely as a result of an extensive exchange of infected, propagative material, while GVCV seems to remain restricted to the mid-western regions in the USA. Much progress has been made on the ecology of these three emerging viruses since the last reviews were published in 2017 (Cieniewicz et al. 2017a; Qiu and Schoelz 2017; Saldarelli et al. 2017). Here we compile and critically analyze the latest information on these three viruses with a special emphasis on the (i) association between the genetic make-up of GPGV isolates and chlorotic mottling and leaf deformation symptoms, (ii) epidemiological and ecological attributes of GVCV and GRBV, and (iii) impacts of GRBV, particularly on molecular underpinnings of fruit ripening physiological pathways. Common trends among these three emerging grapevine viruses but also some unique characteristics are highlighted. Finally, we conclude on how critical it is to embrace 'believing is seeing' in the case of emerging grapevine viruses to remain relevant and impactful in providing research-based management solutions, while leading a sustained dialogue with grape grower's communities, extension educators, policy makers and regulatory authorities.

Keywords Ecology · Grapevine red blotch virus · Grapevine pinot gris virus · Grapevine vein clearing virus · Impacts · Management · Pathogenicity

Introduction

With more than 80 different viruses identified so far, *Vitis* spp. host the most viruses among cultivated crop species (Martelli 2018). The occurrence of a multitude of viruses in this widely-grown fruit crop is likely explained by (i) a very long history of domestication and coexistence, (ii) a sparsity of resistance sources in *Vitis* spp., and (iii) an extensive exchange of germplasm on a global scale. Among the numerous viruses recently identified in grapevines, essentially through the application of high throughput sequencing technologies, are grapevine vein clearing virus (GVCV), grapevine Pinot gris virus (GPGV), and grapevine red blotch virus (GRBV). These three viruses have emerged in the past decade as serious threats to the wine and grape industry. Although they have likely been present in grapevines for a long time, their detrimental effect on vine growth and production only became noticeable once introduced into the plant propagation material, and subsequently into commercial vineyards. Early on, symptoms associated with GVCV, GPGV and GRBV (Fig. 1) were deceiving due to similarities with symptoms caused by well-known viruses such as those involved in fanleaf degeneration disease (in the case of GPGV and GVCV) or associated with leafroll disease (in the case of GRBV). This clearly delayed their identification and the development of specific diagnostic tools. Eventually, efforts to characterize GVCV, GPGV and GRBV provided insights into their genome organization and expression, population structure, distribution, and unique epidemiological features. Common attributes among these three emerging viruses and some of their unique characteristics will be presented and discussed, and lessons learned during

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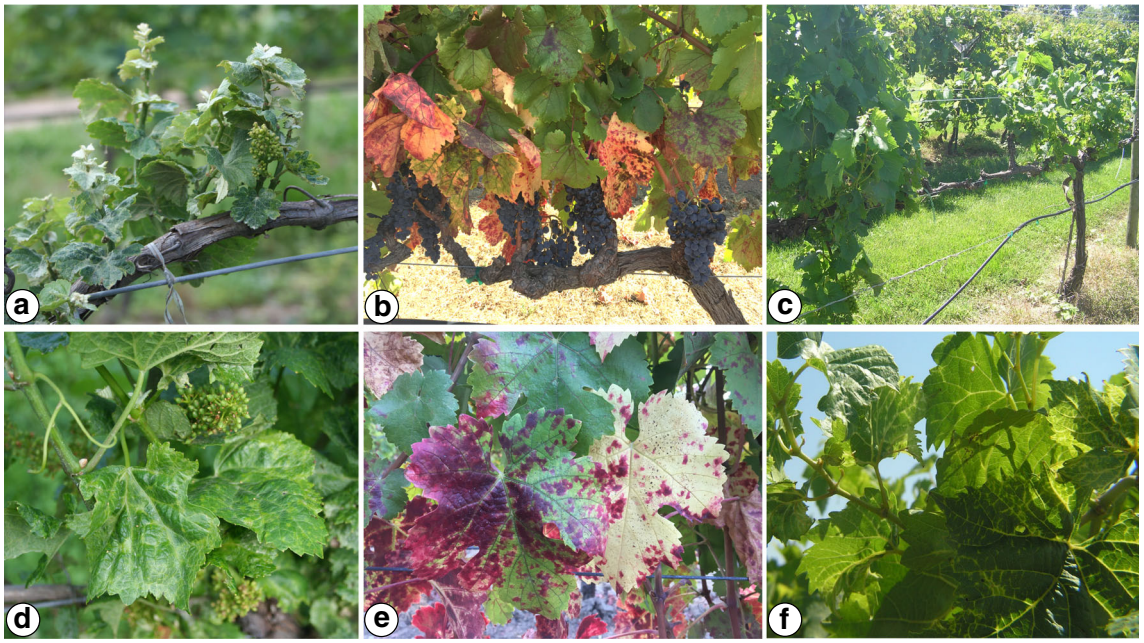

Fig. 1 Typical symptoms of grapevine chlorotic mottling and leaf deformation disease on a GPGV-infected (A) 'Traminer' and (D) 'Pinot gris'; of grapevine red blotch disease on a GRBV-infected (B) 'Cabernet Sauvignon' and (E) 'Cabernet franc'; and of vein clearing disease on a GVCV-infected (C, right) compared to a healthy (C, left) 'Chardonel' and close-up of leaves and shoots of a (F) GVCV-infected 'Chardonnel'. GPGV: grapevine Pinot gris virus; GRBV: grapevine red blotch virus; and GVCV: grapevine vein clearing virus

their discovery will be reviewed, as they elegantly underscore how believing is seeing.

Grapevine Pinot gris virus and chlorotic mottling and leaf deformation disease: Still a puzzling relationship

Grapevine Pinot gris virus (GPGV) represents a puzzling but stimulating subject of research with respect to its association with grapevine chlorotic mottling and leaf deformation disease (GLMD) symptoms (Fig. 1). This unresolved association likely slowed down research on this emerging virus and is delaying its inclusion in official regulatory protocols that govern the production of clean grapevine propagation material. Such an uncertainty was recently considered in an exhaustive report on GPGV to evaluate risks to the Australian viticulture (Constable et al. 2019). This pest risk analysis was provisional, as assessing the possible consequences of GPGV spread, establishment and impact in Australia is challenging because of the existing gaps in knowledge about this virus.

A major step forward to investigate the link between GPGV and GLMD comes from the development of infectious full-length GPGV cDNA clones that cause symptoms in *Nicotiana benthamiana* and reproduce disease symptoms in *V. vinifera* after agroinoculation (Tarquini et al. 2019). Interestingly, two infectious clones, one originating from a "virulent" GPGV variant and the other from an "asymptomatic" virus variant, induced GLMD symptoms in *V. vinifera* and reproduced vineyard-observed symptom recovery five months after agroinoculation. High expectations are stemming from the use of this system to evaluate cultivar susceptibility and better characterize pathogenicity features of this virus by reverse genetics experiments.

Since the last review (Saldarelli et al. 2017) GPGV has been reported in Brazil (Fajardo et al. 2017), Chile (Medina et al. 2018), USA (Al Rwahnih 2018), Pakistan (Rasool et al. 2017), Croatia (Vončina et al. 2017), France (Spilmont et al. 2018), Greece (Zikou 2018), Hungary (Czotter et al. 2018), Italy (Gentili et al. 2017), Poland (Eichmeier et al. 2017), Spain (Ruiz-García and Olmos 2017; Morán et al. 2018), Turkey (Elçi et al. 2018; Ulubaş Serçe et al. 2018), Ukraine (Eichmeier et al. 2018), United Kingdom (Silva et al. 2018), Australia (Wu and Habili 2017) and Iran (Tokhmechi and Koolivand 2019), Moldavia (Abou Kubaa et al. 2019a) and Lebanon (Abou Kubaa et al. 2019b) (Fig. 2). Noteworthy, the record from Brazil was from a *Vitis* germplasm repository (Fajardo et al. 2017). The occurrence of GPGV on a global scale illustrates how fast a virus of grapevine can be disseminated to new geographic areas. This is not too surprising given the intensive exchange of propagative material at a global scale. Since the initial studies of Bertazzon et al. (2016) that suggested eastern European countries as a potential origin of GPGV and indicated the years after 2005 as the likely date of introduction in the Veneto region (Italy), other reports documented earlier introductions of GPGV to new areas. A Brazilian survey (Fajardo et al. 2017) showed the highest rate of GPGV infections (78.3%) in grapevine

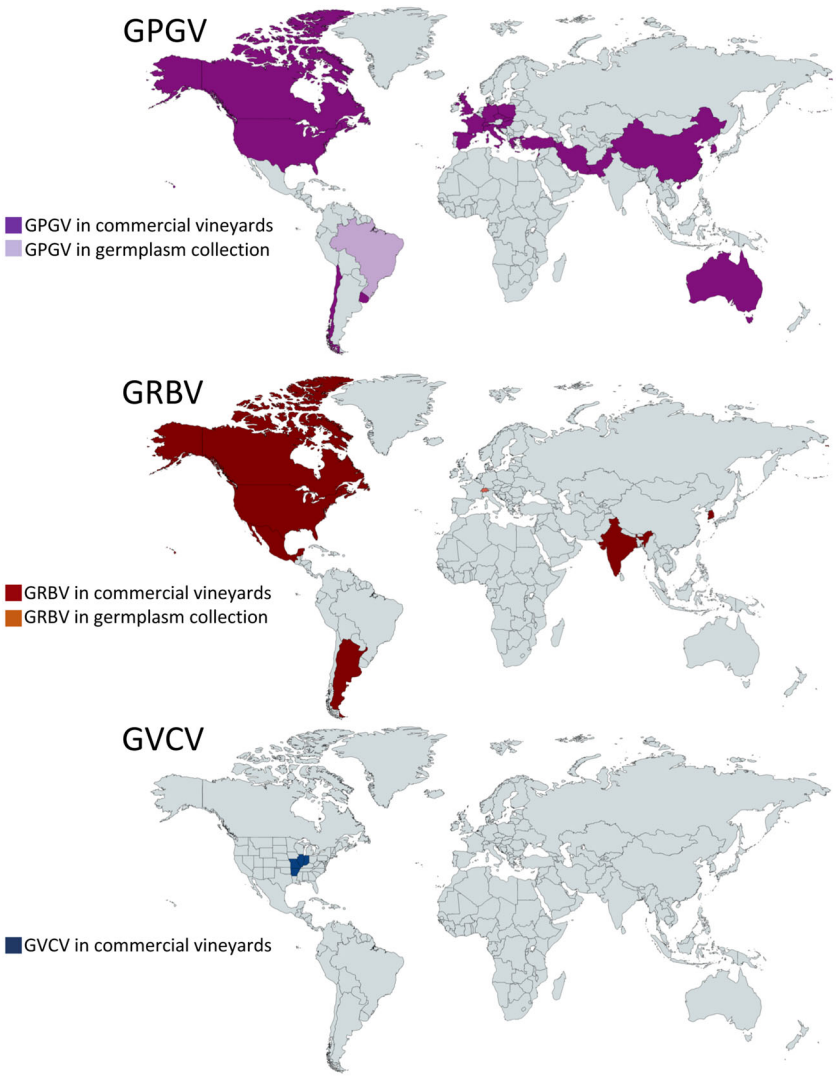

Fig. 2 Distribution of grapevine Pinot gris virus (GPGV, top panel), grapevine red blotch virus (GRBV, middle panel) and grapevine vein clearing virus (GVCV, bottom panel) in vineyards at a global scale. The presence of the three emerging viruses in commercial vineyards (dark color) and grape germplasm repositories (pale color) is indicated. Maps were created with mapchart.net©

cultivars imported starting in 2015. Similarly, GPGV introduction occurred around 2011 in Chile (Nicola Fiore, personal information) and in 1998–2011 in Australia (Wu and Habili 2017). In two Italian Regions, Sardinia and Lazio, the virus was not found in vineyards older than 10 years and/or planted with autochthonous cultivars (Gentili et al. 2017). Additionally, GPGV is widespread in Napa County in California, USA with varied infection rates (8.7%–100%) across several cultivars (Al Rwahnih 2018), although no spread was documented from the GPGV-infected 'Touriga National' that was imported in 1981 and maintained at UC-Davis (Al Rwahnih et al. 2015, 2016). Notwithstanding, the People's Republic of China was recently identified as the probable area of origin of GPGV based on high throughput sequence analyses and phylogeographic reconstructions of a profuse collection of variants (Hily et al. 2019).

There is evidence for local spread of GPGV following introduction in a vineyard. Sentinel grapevines placed close to symptomatic vines in infected vineyards in Trentino became infected after one year of exposure to natural infection (Malagnini et al. 2018). In addition, eriophyid mites (*Colomerus vitis*) collected from the same bait plants tested positive for the virus. Moreover, an aggregated distribution of GLMD-exhibiting vines associated with GPGV presence was found over three-years in two vineyards in Trentino, although disease symptoms were correlated with mite infestations in only one vineyard. Similarly, Bertazzon et al. (2018) described an increase of symptomatic plants in eight vineyards in Veneto and an increase (from 20% to 77%) of GPGV infection of healthy vines planted in two vineyards during three years of observations. Among these newly planted vines, nine out of 66 showed GLMD symptoms while 53 out of 66 were found infected by GPGV at the end of the trial. Noteworthy, GPGV variants from symptomatic vines clustered in clade B/C of "virulent" GPGV variants (Bertazzon et al. 2017).

The existence of phylogenetically distinguishable "virulent" and "asymptomatic" GPGV variants (Saldarelli et al. 2015) was confirmed by Bertazzon et al. (2017). The latter

authors further separated "virulent" GPGV variants in clade B (i.e. those present in vineyards with less than 1% of disease incidence) and clade C (i.e. those present in vineyards with higher rates of disease incidence) based on the analysis of a genome region spanning the movement protein (MP) and coat protein (CP) genes. Such a distinction was also reported by Tarquini et al. (2019) by analyzing the full-length genome sequence of 20 GPGV isolates.

In addition to phylogenetic differences, the MP of GPGV "virulent" strains is six amino acids shorter compared to asymptomatic virus strains. This truncation is caused by a specific T/C polymorphism in the stop codon (Saldarelli et al. 2015). This association between polymorphism and symptomatology was partially confirmed by analyzing vines from 289 vineyards of mainly *V. vinifera* 'Glera' and 'Pinot gris' in Veneto (Bertazzon et al. 2017). However, such association was not found by Moran et al. (2018) during a survey of Spanish vineyards in which a new polymorphism in the MP gene was found to result in a five amino acid shorter MP. Both T/C polymorphism and phylogenetic analysis of the MP/CP genome region (Saldarelli et al. 2015) hold true for asymptomatic Ukrainian (Eichmeier et al. 2018) and Polish (Eichmeier et al. 2017) virus isolates. These results are consistent with reports of other isolates from Eastern Europe (Glasa et al. 2014), suggesting the recent divergence of a local clade that hypothetically led to GPGV isolates eliciting symptoms in Veneto, Friuli Venezia Giulia and Trentino. Accordingly, Tarquini et al. (2019) documented genomic recombination in nine GPGV isolates from northeastern Italy and described additional polymorphisms in the MP and RNA-dependent RNA polymerase (RdRp) genes. Specifically, these authors identified five polymorphisms in the MP gene that caused amino acid changes and a premature stop codon. The five polymorphisms are associated with a cluster of "virulent" GPGV isolates from the C clade (Bertazzon et al. 2017). Curiously, all these polymorphisms lie in the MP gene that can also occur as a shorter protein. In this view, asymptomatic GPGV isolates reported outside Europe can be hypothetically considered remnants of the original progenitors from Eastern Europe that, for an unknown reason, remain stable from an evolutionary perspective, or, alternatively, do not elicit symptoms in particular grapevine cultivars. Further studies are necessary to validate this hypothesis. Another explanation of the ability of some GPGV strains to induce symptoms was ascribed to the presence of higher virus titers in symptomatic vines (Bertazzon et al. 2017), a finding that was not confirmed by a Spanish study (Moran et Al 2018). Additionally, the role of nutritional deficiency, particularly boron deficiency, should be considered in elucidating the nature of chlorotic mottling and leaf deformation symptoms (Ermacora et al. 2018). In any event, the relationship between GPGV and its natural host in terms of disease symptom development remains enigmatic.

Advances in understanding the transmission mode of GPGV by *C. vitis* showed virus acquisition and transmission to healthy vines after a minimum of four hours (Malagnini et al. 2016) while Moran et al. (2018) found that this mite species acquired up to 161 ± 15 GPGV genome copies after an acquisition time of five days. GPGV epidemiology, which assumes *C. vitis* monophagy, is complicated by the existence of potential alternate hosts of the virus (Demián et al. 2018; Gualandri et al. 2017). After a first find in *Chenopodium album* L. (Fat Hen) and *Silene latifolia subsp. alba* (Mill.) (Gualandri et al. 2017), GPGV was successively detected in *Ascelpias syricaca* (common milkweed), *Rubus* (e.g. raspberries and blackberries), *Rosa* (e.g. rose), and even in *Fraxinus* (Ash species) (Demián et al. 2018). These results suggested that other vectors could transmit the virus and that GPGV can potentially be endemic in eastern Europe. Interestingly, all the GPGV isolates retrieved in these hosts in Hungary belong to the asymptomatic clade based on phylogenetic analyses and MP/CP polymorphism.

GPGV sequence variability incited the development of robust molecular detection by RT-PCR (in either endpoint or real time format). An exhaustive list of primers and assays is reported by Constable et al. (2019). Recently published primers and protocols target the RdRp and CP genes (Bertazzon et al. 2017), the MP/CP regions (Moran et al. 2018), and the 5′ untranslated region of the genomic RNA and the MP/CP regions (Demián et al. 2018). A LAMP protocol was also developed (Zikou et al. 2018). In addition, a large proficiency test involving 19 Italian laboratories (Gentili et al. 2018) validated two endpoint and two real time RT-PCR assays using 25 GPGV-infected (target) and 19 GPGV-free but virus-infected (nontarget) *V. vinifera* and rootstock accessions (http://sito.entecra.it/portale/cramanualidettaglio.php?idmanuale=23504&lingua=IT). All diagnostic methods were effective in terms of sensitivity, specificity, accuracy, repeatability and reproducibility, although analytical sensitivity was higher in real time assays. Phloem tissue from woody canes was used to harmonize the assays. Noteworthy, detection of grapevine viruses other than GPGV and different methods of sample preparation and nucleic acid extraction did not affect the efficiency of the tests.

An ELISA kit for GPGV was developed in 2018 by Bioreba AG (Switzerland). Both rabbit and alpaca immunizations were done with an *Escherichia coli* expressed protein (Poignavent et al. 2018). Different GPGV isolates were detected in phloem scrapings from dormant canes and in leaves from young shoot. To the best of our knowledge, no information is available on the performance of this serological kit on a large scale.

Histological analyses of field-grown GPGV-infected vines (Tarquini et al. 2019) showed flexuous filamentous viral particles in bundle-sheath cells of phloem parenchyma from leaf tissue. Particles were immuno-labelled with a GPGV-specific

antiserum (Gualandri et al. 2015). Altered bundle sheath cells contain membrane-bound structures and viral particles which are reminiscent of viral replication complexes described in other plant virus systems (Hyodo et al. 2014).

Studies evaluating the impact of GPGV reported up to 85% decrease of fruit production of *V. vinifera* 'Pinot gris' vines showing diverse levels of symptoms severity in two vineyards (Bertazzon et al. 2015). The impact of GPGV was more variable in 'Glera' with a 66% decrease in fruit production and a lack of differences in vines with diverse symptom severity in three different vineyards. The average weight of fruit cluster was reduced in symptomatic vines of all but one 'Glera' vineyard, while alteration of qualitative fruit juice parameters (acidity, sugars) were not consistently observed in all the five vineyards surveyed. Similarly, Malossini et al. (2015) observed a significantly lower number and weight of clusters in symptomatic vines of 'Traminer' (−60%) and 'Pinot gris' (−50%) and a limited growth in both cultivars, as illustrated by a reduced pruning weight.

Coordinated multidisciplinary efforts are now necessary to fill gaps in knowledge as biological tools and basic information on GPGV are available. Research should aim to establish a clear association between GPGV and GLMD, and address disease epidemiology and the impact of the virus. Additionally, studies on genomic traits of virulence, cultivar susceptibility and evolutionary history of GPGV with particular emphasis on factors inducing the generation of "virulent" strains are desirable to better understand the virus biology.

Grapevine red blotch virus: Impact and ecology

A sub-optimal performance of certain vineyards of red-berried *V. vinifera* cultivars that manifested some type of leaf reddening was noticed by growers in California, USA in the early to mid 2000's. However, the corresponding red blotch disease was described only in 2008 (Calvi 2011) and grapevine red blotch virus (GRBV) was not discovered until 2011 (Cieniewicz et al. 2017a; Krenz et al. 2012; Al Rwahnih et al. 2013). Foliar disease symptoms, i.e. red blotches, in red-fruited cultivars are usually localized to older leaves, typically appear around véraison, and become more pronounced later in the season (Fig. 1). White-fruited cultivars exhibit more discrete foliar symptomatology, i.e. interveinal chlorosis and necrosis (Cieniewicz et al. 2017a). Red blotch disease has emerged as one of the most important virus diseases of grapevine in North America (Fig. 2), and therefore has stimulated research efforts aimed at developing management strategies to limit its impact on vineyard profitability.

Grapevine red blotch virus was ratified as a member of the family *Geminiviridae*, serving as the type member of a new genus, *Grablovirus* (Varsani et al. 2017). The assignment of GRBV to a new genus in the family *Geminiviridae* is essentially based on its genome organization and sequence information. To date, 120 full-length GRBV genome sequences have been deposited in NCBI GenBank. As previously reported, GRBV variants group into two major phylogenetic clades (Krenz et al. 2014). The majority of sequences (77 of 120) are in phylogenetic clade 2, which has lower intra-clade variability (up to 4.6% divergence), and 43 sequences are in phylogenetic clade 1, in which the intra-clade variability is up to 6.1% (Fig. 3). Interclade variability ranges from 3.7–9.2% divergence (not accounting for recombination). No geographic or cultivar specificity is assigned to these two groups of genetic variants, and no biological difference between the two types of variants is known.

Since knowledge on GRBV biology and management was last reviewed (Cieniewicz et al. 2017a), several important advancements have been made toward understanding the impacts of GRBV on fruit quality and vine health. The first study on the impact of red blotch disease, which was then referred to as "red-leaf disease" and predated the discovery of GRBV, was documented in a Master's thesis (Calvi 2011). This was the first report of reduced sugar level (°Brix) and other negative impacts on fruit quality in GRBV-infected vines. This study suggested that delayed harvest and early crop thinning could help mitigate the impact of the disease. Later a study on the impact of GRBV on foliar physiology revealed higher glucose and fructose, higher phenolics and terpenoids, and an altered amino acid profile in the leaves of GRBV-infected *V. vinifera* 'Cabernet franc' (Wallis and Sudarshana 2016). Reduced photosynthesis and vegetative growth were observed in graft-inoculated *V. vinifera* 'Gamay,' accompanied by reduced total soluble solids, 50% lower anthocyanin concentration, and higher pH (Reynard et al. 2018). Primary metabolic pathways typically associated with early berry development were upregulated in infected post-véraison *V. vinifera* 'Zinfandel' berries, whereas pathways associated with ripening were downregulated in infected berries collected post-véraison (Blanco-Ulate et al. 2017). Consistent with studies on 'Cabernet franc' (Calvi 2011; Wallis and Sudarshana 2016), 'Cabernet Sauvignon' and 'Merlot' (Girardello et al. 2019), a negative impact of GRBV on ripening of 'Zinfandel' was observed (Blanco-Ulate et al. 2017). In 'Chardonnay', GRBV infection results in slightly reduced Brix at harvest (1–2°) and higher flavanols. However, site- and season-specific differences in the impacts of GRBV on fruit quality and ripening were observed for 'Chardonnay', 'Merlot', and 'Cabernet Sauvignon' (Girardello et al. 2019). These observations are consistent with similar specific differences reported for other grapevine viruses (Mannini and Digiaro 2017).

Hormonal networks associated with ripening and stress response (abscisic acid, ethylene, and auxin) were disrupted in GRBV-infected 'Zinfandel' berries (Blanco-Ulate et al. 2017). Enzymes involved with the flavonoid biosynthesis

pathway were upregulated in GRBV-infected 'Gamay', suggesting activation of defense mechanisms against GRBV (Buchs et al. 2018). Physiological characterization of GRBV-infected 'Cabernet Sauvignon' on two different rootstocks also demonstrated reduced total soluble solids in berries from GRBV-infected vines, as well as delayed anthocyanin accumulation and lower titratable acidity (Martínez-Lüscher et al. 2019). This study revealed a desynchronization among ripening processes, rather than just a delay, suggesting that the impacts of GRBV cannot be adequately remedied by sequential harvesting (Martínez-Lüscher et al. 2019). Studies on the physiology of GRBV-infected vines have provided important insights into the impact of GRBV on vineyard productivity, but more work is needed to better understand how the virus affects fruit quality. For instance, with the exception of a single study on the impact of GRBV on 'Chardonnay' (Girardello et al. 2019), the effects of red blotch disease on other white-fruited cultivars are unknown. Understanding environmental or region-specific impact of GRBV on grapevine physiology and berry quality may lead to improved strategies for mitigating the disease.

GRBV is distributed in most major viticulture regions throughout the United States (Krenz et al. 2014; Brannen et al. 2018; Yao et al. 2018; Thompson et al. 2019; Schoelz et al. 2019; Jones and Nita 2019). It has also been detected in Canada (Poojari et al. 2017; Xiao et al. 2015), Argentina (Luna et al. 2019), Mexico (Gasperin-Bulbarela et al. 2018), Switzerland (Reynard et al. 2018), India (Marwal et al. 2019), and South Korea (Lim et al. 2016) (Fig. 2). It should be noted that GRBV occur in a virus collection vineyard but not in production vineyards in Switzerland (Reynard et al. 2018).

The three-cornered alfalfa hopper (*Spissistilus festinus* [Say], Membracidae) has been reported as a vector under greenhouse conditions (Bahder et al. 2016a) and is associated with GRBV spread in a diseased vineyard in California (Cieniewicz et al. 2018a). In the latter study, spread occurred predominantly within the study vineyard, suggesting that the planting material served as an essential source of virus inoculum for secondary spread (Cieniewicz et al. 2018a). A recent study suggested that secondary spread of GRBV is occurring in at least two locations in Oregon, though by an unknown vector (Dalton et al. 2019). The discovery of *S. festinus* as a vector of GRBV of epidemiological importance spurred a need for research on the phenology and behavior of this GRBV vector in vineyards. Thus far, *S. festinus* is known to prefer leguminous hosts over *Vitis* spp. for both feeding and reproduction, and is an occasional pest of legumes in the southern USA (Beyer et al. 2017; Preto et al. 2018a). Although *S. festinus* will feed on grapevine and ingest GRBV (Bahder et al. 2016a; Cieniewicz et al. 2018a) and will even oviposite in grapevine (Preto et al. 2018b), it does not seem to colonize grapevine, but rather stays near vineyard edges and overwinters on vineyard groundcover (Preto et al. 2019).

Studies on red blotch epidemiology in vineyards in Napa County, California revealed differential GRBV spread rates associated with relative abundance of *S. festinus* (Cieniewicz et al. 2017b, 2018a, 2019). In a 'Cabernet franc' vineyard planted in 2008 with an estimated initial GRBV incidence of less than 2%, disease increased by 10% (4% to 14%) in a five-year period. In contrast, incidence in an adjacent 'Cabernet Sauvignon' vineyard, also planted in 2008 with an estimated 40% GRBV incidence at planting, increased by less than 1% in the same time period. Insect surveys conducted in 2015–2016 in the 'Cabernet franc' vineyard and in 2017–2018 in the 'Cabernet Sauvignon' vineyard revealed a 10-fold higher *S. festinus* capture rate on sticky cards in the 'Cabernet franc' compared to the 'Cabernet Sauvignon' vineyards. These spread dynamics are in stark contrast to a study in New York over the same time frame (2014–2018), in which a 'Merlot' vineyard with 40% GRBV incidence revealed no indication of secondary spread, nor presence of *S. festinus* (Cieniewicz et al. 2019). These findings clearly indicated that local and regional environments impact the epidemiology of red blotch disease and the potential for secondary spread.

Currently the only known hosts of GRBV are *Vitis* species. In addition to production vineyards, GRBV was also detected in free-living vines in Napa County, California, USA (Perry et al. 2016), a finding which was confirmed in an independent study (Bahder et al. 2016b). A follow-up study on the distribution and diversity of GRBV in wild grapevine populations in northern California suggested that the direction of virus spread is predominantly from commercial vineyards to wild vines, rather than wild vines serving as a substantial inoculum source (Cieniewicz et al. 2018b). In contrast, GRBV was not detected in any wild *Vitis* spp. in New York (Cieniewicz et al. 2018b). Though, free-living grapevines should still be considered as potential sources of GRBV inoculum near newly planted vineyards and nursery operations in areas where GRBV is spreading.

Spread of GRBV occurs at a faster rate (5–10% annually) when initial disease incidence is high (greater than 25%), but slower spread (1–2% annually) occurs if initial disease incidence is low (less than 10%) (Cieniewicz et al. 2017b, 2019). These vineyard studies have provided biological context for the recommendation to rogue and remove infected vines if disease incidence is less than 30%, but to remove the entire vineyard and re-plant with vines derived from virus-tested nursery stock if red blotch disease incidence is greater than 30% (Ricketts et al. 2017). The scarcity of *S. festinus* in vineyards, despite the occurrence of GRBV spread, strengthens the rationale behind recommendations to focus on removal of GRBV inoculum sources, rather than reducing *S. festinus* abundance, for disease management (Cieniewicz et al. 2019). Regional environmental factors and differences

in viticulture practices likely impact GRBV epidemiology. Therefore, studies on secondary spread and *S. festinus* ecology in vineyards are needed in other viticulture regions than California to devise optimal management strategies to mitigate GRBV spread. For instance, other areas to consider for studies on secondary spread of GRBV might be more southern regions in the USA, where *S. festinus* is known to be abundant in legume crops (Beyer et al. 2017).

A major limiting factor in large-scale epidemiology studies and diagnostics is that detection assays for GRBV can be cost-prohibitive and not adapted to high throughput testing. A high throughput and less expensive method of indexing vines for GRBV would be useful for growers and for researchers. Unfortunately, thus far, attempts to visualize virus particles and develop a serological test for GRBV have not been successful. However, transcriptomic (Vargas-Ascencio et al. 2019) and proteomic studies (Buchs et al. 2018) may provide new opportunities for producing diagnostic serological tests for GRBV. Detection of GRBV proteins by mass spectrometry revealed the products of the V1 (coat protein) and V2 (unknown function) open reading frames in infected leaves (Buchs et al. 2018). This was the first time GRBV proteins have been detected *in planta*. The protein load of the V1 product was six times higher in petioles compared to leaves (Buchs et al. 2018), which may support the hypothesis that GRBV is phloem-restricted, or at least phloem-preferred. A higher GRBV detection rate in vascular tissues was also demonstrated by qPCR (Setiono et al. 2018). Petioles are therefore a richer source of tissue for GRBV proteome studies (Buchs et al. 2018) and diagnostics.

GRBV has seven putative open reading frames (ORFs), of which four are in the viral (v) sense and three are in the complementary (c) sense. The four v-sense ORFs overlap, as do the three c-sense ORFs. The v-sense and c-sense ORF clusters are separated by a long intergenic and short intergenic region, respectively (Krenz et al. 2014; Vargas-Asencio et al. 2019). Based on sequence homology to members of the genus *Mastrevirus*, a splicing event of a c-sense transcript to produce a replicase-associated protein was predicted in silico (Krenz et al. 2014). This c-sense splicing event and formation of a C1-C2 intron was confirmed by RT-PCR (Yepes et al. 2018) and by RNAseq of GRBV-infected *V. vinifera* (Vargas-Asencio et al. 2019). In addition, evidence for another intron in the v-sense intron spanning the V2 ORF was found by RNAseq. Splicing of the viral-sense intron is predicted to delete the N-terminus of the encoded V2 protein (Vargas-Asencio et al. 2019). These authors present several hypotheses for the regulatory role of these introns in the GRBV infection cycle and suggest that grabloviruses have a novel genome expression strategy compared to other geminiviruses (Vargas-Asencio et al. 2019). Further studies into the genomic regulation of the GRBV infection cycle may provide insight into the reasons for the repeated failures to produce antisera and visualize particles.

Red blotch disease management is predicated on an understanding of the infection biology and the ecological factors influencing spread. Several important advances have been made in GRBV diagnostics and regulation. There are routinely used, robust PCR-based assays, including a multiplex PCR and qPCR assay for GRBV detection (Al Rwahnih et al. 2013; Krenz et al. 2014, Setiono et al. 2018). The optimal tissue sampling strategy for accurate detection is to sample older leaves, collect composites (multiple leaves), and test petioles (Setiono et al. 2018). Recently a LAMP assay was also developed. The major advantages of a LAMP assay are the high specificity, high sensitivity, low cost, and elimination of the need for isolation of nucleic acids (Romero Romero et al. 2019). A recombinase polymerase amplification (RPA) AmplifyRP® Acceler8® test was developed by Agdia Inc., which also eliminates the need for nucleic acid purification, has higher sensitivity than PCR, and is user-friendly (Li et al. 2017). Another colorimetric detection assay based on CRISPR Cas12a was recently developed (Li et al. 2019). Improvements in diagnostic technology will streamline the testing required for grapevine certification programs, increase the accessibility to growers and consultants, and allow for the testing of increased sample numbers for epidemiology studies and other research applications.

Important advances have been made in recent years on the biology, ecology, epidemiology, physiological impact, and diagnostic technology for GRBV. However, more work is needed in several areas. Though *S. festinus* is a vector of GRBV under greenhouse conditions (Bahder et al. 2016a) and is associated with GRBV spread in vineyards (Cieniewicz et al. 2018a, 2019), the transmission mode and tritrophic virus-vector-host interactions are poorly understood. GRBV detection in new areas or new cultivars is frequent, with new reports every year. However, with the exception of studies in California, New York, and Oregon, the epidemiology of GRBV has not been studied in other regions. Additionally, further research on the genomic expression strategies of GRBV and other grabloviruses will lend important insights into GRBV biology. Moreover, further study on the host range of this virus may ameliorate experimental difficulties encountered with grapevine, and also provide insight into potential environmental reservoirs of GRBV.

Grapevine vein clearing virus: Ecology and epidemiology

Grapevine vein clearing virus (GVCV) was first reported in *V. vinifera* 'Chardonnay' exhibiting short internodes, abnormal shoots, and deformed leaves with translucent veins (Fig. 1) (Zhang et al. 2011a). Cordons of infected vines died back

and the vines declined (Fig. 1). The disease caused the removal of several vineyards, and is a major threat to the grape industry in the Midwest region of the USA (Fig. 2) (Qiu and Schoelz 2017; Qiu et al. 2007).

GVCV belongs to the genus *Badnavirus* in the family *Caulimoviridae*. Its genome has a double-stranded, circular DNA molecule of 7725–7765 bp in length (Beach et al. 2017; Petersen et al. 2019; Zhang et al. 2011b). The plus-strand genome encodes three ORFs. The genome of GVCV variants shares 92 to 99% identical nucleotides. ORF II, which has an 9-nt indel, is the most variable ORF, sharing as low as 82% identical nucleotides among GVCV variants (Beach et al. 2017). Even within a single population, GVCV genome variability is in a range of 0.7 to 1.5% (Howard and Qiu 2017) and diverse GVCV variants exist not only in vineyards (Guo et al. 2014) but also in native plants (Petersen et al. 2019).

Several free-living *Vitis* species are native to the Midwest region, USA. They grow along riverbanks and forest edges, and among agroecological interfaces. Initially, surveying wild *V. rupestris* populations led to the identification of two distinct variants, GVCV-VRU1 and -VRU2 (Beach et al. 2017). The *V. rupestris* vines infected with the two GVCV variants were located 200 km apart. In a more recent survey, GVCV was also found in 10% of 186 wild *V. cinerea, V. palmata,* and *V. vulpina* collected from a range of native sites. In addition, GVCV was found in 134 out of 399 (34%) wild *Ampelopsis cordata* collected across the Midwest region (Petersen et al. 2019). *A. cordata* is a vine that belongs to the same Vitaceae family as *Vitis* species. Some of these *A. cordata* show mild vein clearing and mottle symptoms in their native habitats (Petersen et al. 2019). In one case, the same GVCV variant was found in a native *A. cordata* and a proximal vineyard, suggesting a close relationship between GVCV variants in native plants and a production vineyard.

The fact that GVCV is present in wild *Vitis* species and *A. cordata* across a wide geographic area suggested the existence of an insect vector. Similarly, the occurrence of the same GVCV variant in wild *A. cordata* and a nearby cultivated grapevine supported virus transmission by a vector. The grape aphid (*Aphis illinoisensis*) is native to the Midwest and infests wild *A. cordata* and *Vitis* species. Thus, it was selected as a candidate vector for transmission experiments. Results revealed transmission of GVCV from *A. cordata* to 'Chardonel' by the grape aphid in greenhouse experiments (Petersen et al. 2019). Therefore, the grape aphid is a vector of GVCV. In addition, the same GVCV variant was detected in *A. cordata* and grape aphids infesting *A. cordata* in riparian areas, suggesting that the grape aphid is a vector of epidemiological significance (Petersen et al. 2019).

Recent surveys of grape aphids for GVCV revealed a 41% virus incidence in 443 single aphids collected in the summers of 2018 and 2019 (Qiu, unpublished results). Grape aphids are prolific and carried by wind over long distance. It is plausible that viruliferous aphids carry GVCV and scatter the virus across native habitats and viticultural areas. Therefore, there could be a constant exchange of GVCV variants among vectors and hosts in cultivated and unmanaged ecosystems. Dynamic dispersal and parthenogenesis in aphids might accelerate evolution of GVCV populations. A similar case was documented for an emerging potyvirus in legume plants in Australia (Webster et al. 2007).

In the historical context of viticulture, thousands of cuttings were collected from native wild *Vitis* species in the Midwest region of the USA during the phylloxera epidemics in 1860–1890s and shipped to France for use as rootstocks (Campbell 2004). If GVCV was present in native wild *Vitis* species during that period, the virus would have been transmitted to cultivated grapevines by grafting, infected vines would have been distributed in many viticultural areas in France and elsewhere, and GVCV-infected vines would have served as virus reservoirs for secondary spread in vineyards. These hypotheses are not very plausible because, to date, GVCV has only been found in wild hosts and cultivated grapevines in the Midwest of the USA. Therefore, the emergence of GVCV in wild *Vitis* plants likely occurred after the phylloxera crisis. Furthermore, a total of 380 samples of 31 different *Vitis* species, including *V. rupestris, V. cinerea, V. palmata,* and *V. vulpina*, at the two *Vitis* national germplasm repositories maintained by the US Department of Agriculture in Geneva, New York and Davis, California, were screened for GVCV; none of these accessions tested positive for GVCV (Qiu, unpublished results). The 380 germplasm samples tested originated primarily from native *Vitis* habitats of Eurasian, East Asian, and North American regions that were collected from 1893 to 2000. Thus, it is reasonable to assume that spread of GVCV occurred in wild *Vitis* species and cultivated grapevines in the Midwest of the USA fairly recently, likely from *A. cordata* or other unidentified wild hosts.

Virus diseases are among the highest number of emerging diseases (Anderson et al. 2004). This is largely a result of expansion and growth of agricultural crops that are introduced to new areas (Fargette et al., 2006). Cultivated grapevines were introduced to the Midwest region of the USA in the 1830s (Ambers 2012), and thus became new hosts to indigenous viruses. Introduction of new hosts to a native landscape frequently results in accidental dispersal and stochastic transmission of preexisting viruses, and subsequent emergence of a new virus disease in a crop (Elena et al. 2014; Jones 2009; Jones and Coutts 2015; Lefeuvre et al. 2019). Expansion of viticulture areas from the 1980s to today in the Midwest has created more connections and developed more intricate interfaces in diverse viticulture ecosystems. Vineyards are typically embedded in a vast wild plant landscape in the Midwest region. Remaining habitats of wild *Vitis* and *Ampelopsis* plants are natural reservoirs of GVCV (Petersen et al. 2019). A permanent population of a pathogen in wild hosts clearly

creates an opportunity for a new disease to emerge on cultivated hosts (Jones 2009; Papaïx et al. 2015; Roossinck 2019). Ecological structure, composition and configuration of natural vegetation and adjacent agricultural settings influence pathogen epidemics and evolution (Lefeuvre et al. 2019; Papaïx et al. 2015). These agroecological interfaces provide a good model for studying the epidemiology, ecology and evolution of plant viruses (Shates et al. 2019), particularly of GVCV.

Conclusions

Newly emerging virus disease epidemics often begin in vineyards with numerous uncertainties such as limited, if any, understanding of the causative agent, scarcity of knowledge of its mode of infection and dispersal, and lack of biological tools to study the virus. Growers and scientists respond to these new, undesired situations quickly and with coordinated, multidisciplinary efforts. In the early to mid 2000's, grape growers brought to the attention of the research community a number of undesired vineyard conditions in terms of vine growth, and fruit production and quality for which the occurrence of viruses was suspected. Best judgment was exercised early on to identify potential culprits, although the identification of viruses in association with the conditions reported by growers was somewhat delayed because of some observational biases such as anchoring bias, i.e., relying heavily on an initial impression (nutritional deficiency and leafroll-like viruses in the case of GRBV or the occurrence of a nepovirus in the case of GPGV and GVCV), and confirmation bias, i.e., unconsciously attending to evidence that confirms our existing beliefs or expectations (a leafroll-associated virus rather than a new virus species in the case of GRBV, and a nepovirus rather than a new virus species in the case of GPGV and GVCV). Some lessons are to be learned from these experiences. First, initial work on these three viruses reminded us that vineyard observations require seeing beyond what is in front of our eyes. Such an approach is critical to (i) avoid creating or reinforcing initial assumptions and beliefs that are likely deceiving, and (ii) recognize how observations are influenced by what we are familiar with and what we feel comfortable with. In other words, trusting what is in front of our eyes and what our mind wants to see can prevent sound and timely responses to emerging virus disease. Therefore, the ability to regularly question our own assumptions is critical because appearances can be deceptive.

Following the identification of GVCV (Zhang et al. 2011b), GPGV (Giampetruzzi et al. 2012) and GRBV (Al Rwahnih et al. 2013; Krenz et al. 2012), reliable diagnostic tools and methodologies were developed. This led to the establishment of a strong association between symptomatic vines and virus presence (Al Rwahnih et al. 2013; Saldarelli et al. 2015; Zhang et al. 2011a). However, this association did not prove causality. Therefore, infectious full-length genomic clones were engineered and successfully used to demonstrate the causative role of GRBV and GPGV in red blotch disease (Yepes et al. 2018) and chlorotic mottling and leaf deformation disease (Tarquini et al. 2019), respectively. Similar efforts are underway for GVCV. Noteworthy, similar to other grapevine viruses, infection of GRBV in rootstocks is latent (Yepes et al. 2018). The use of an infectious clone from an asymptomatic variant of GPGV did not validate vineyard observations as agroinoculated vines manifested typical chlorotic mottling and leaf deformation symptoms (Tarquini et al. 2019). Therefore, more work is needed to elucidate the association of GPGV with disease symptoms, particularly in light of nutritional deficiencies in vineyards (Ermacora et al. 2018).

First reported in Italy (GPGV) and the USA (GRBV), GPGV and GRBV have a wider distribution nowadays, likely as a result of the extensive exchange of infected, propagative material (Fig. 2). To the contrary, GVCV seems to remain restricted to the mid-western regions in the USA, suggesting that aphid-mediated transmission might a predominant mode of dispersal rather than the distribution of infected propagation material (Fig. 2).

GPGV, GVCV and GRBV have unique epidemiological attributes compared to other insect-transmitted grapevine viruses. GPGV is transmitted by the eriophyid mite *C. vitis*, GRBV is transmitted by the treehopper *S. festinus*, and GVCV is transmitted by the grape aphid *A. illinoisensis*, while some grapevine virus species of the family *Secoviridae* are transmitted by dagger nematodes, and other grapevine virus species of the families *Closteroviridae* and *Betaflexiviridae* are transmitted by mealybugs or soft scale insects (Martelli 2018). Although progress has been made on the identification of vectors, there are still epidemiological unknowns. For example, free-living grapes have been identified as sources of GRBV (Cieniewicz et al. 2018a) and GVCV (Petersen et al. 2019). Other native hosts have also been identified for GVCV (Petersen et al. 2019). Similarly, some annual herbaceous and perennial plants have been identified as sources of GPGV (Demián et al. 2018; Gualandri et al. 2017). What is the role of these alternative hosts in virus spread? Can they serve as virus reservoirs and contribute to secondary spread in vineyards? More work is needed to address these important epidemiological issues although transmission of GVCV from infected *A. cordata* to the interspecific hybrid 'Chardonel' by the grape aphid was documented (Petersen et al. 2019). Additionally, limited information is available on the transmission modality and efficiency of GPGV, GRBV and GVCV by their respective vectors. This information is important to devise optimal disease managements strategies.

The origin of the three emerging viruses and their evolutionary history has been partially addressed but remains essentially unsolved, except for GPGV for which the People's Republic of China was suggested as the center of origin

(Hily et al. 2019). Could GVCV have originated from free-living populations of *A. cordata* or other native hosts in mid-western regions in the USA? Similarly, could GRBV have originated from free-living populations of *V. californica* and hybrids derived thereof in California, USA? Although these hypotheses seem plausible, more investigations are needed to validate them.

For disease management, the selection of clean planting material remains the most efficient approach to limit the presence of emerging viruses in vineyards. The production of clean vines is realized through certification and the use of cuttings and buds from extensively tested stocks in foundation vineyards. In the USA, due to the severity of GRBV impacts, the most prominent grape-producing States (California, Washington, New York and Oregon) have changed their certification standards to include GRBV in their routine testing of foundation stocks. In addition, nurseries have destroyed their G2 increase blocks and established new ones with clean, virus-tested material in isolated sites. For GPGV, uncertainties about the relationship between the virus and chlorotic mottling and leaf deformation symptoms undermine discussions on a resolution to modify existing, regional regulatory frameworks or the European framework for the inclusion GPGV. In the case of GVCV, preventing virus spread is very challenging, given that grapevines are permanent in a vineyard ecosystem and grape aphids reproduce prolifically. Removing all wild host reservoirs is almost impossible, and applying insecticides to control grape aphids is not practical. One effective management strategy could be to grow GVCV-resistant interspecific hybrid cultivars such as 'Norton' (Qiu, unpublished) and 'Chambourcin' (Guo et al. 2014), but other grape cultivars, although susceptible to GVCV, are still in high demand. One scheme of curbing GVCV spread might be to grow resistant grape cultivars along the edge of the forest as a resistant host buffer zone to shield susceptible cultivars. More work is needed to validate this hypothesis.

At the level of infected vineyards, economic studies on GRBV suggested rogueing as an optimal strategy for disease management if disease incidence is less than 30%, and entire vineyard removal and replanting with vines derived from virus-tested foundation stocks if disease incidence if more than 30% (Ricketts et al. 2017). These disease management recommendations are only guidelines that shall be customized for actions by vineyard managers. This is because singularities exist among wine estates and grape-growing regions in terms of vineyard management practices and level of tolerance to a disease. Similar studies on the economic impact of GPGV and GCVC would be of interest to facilitate the adoption of strategies to reduce the virus inoculum in diseased vineyards through rogueing or vineyard removal.

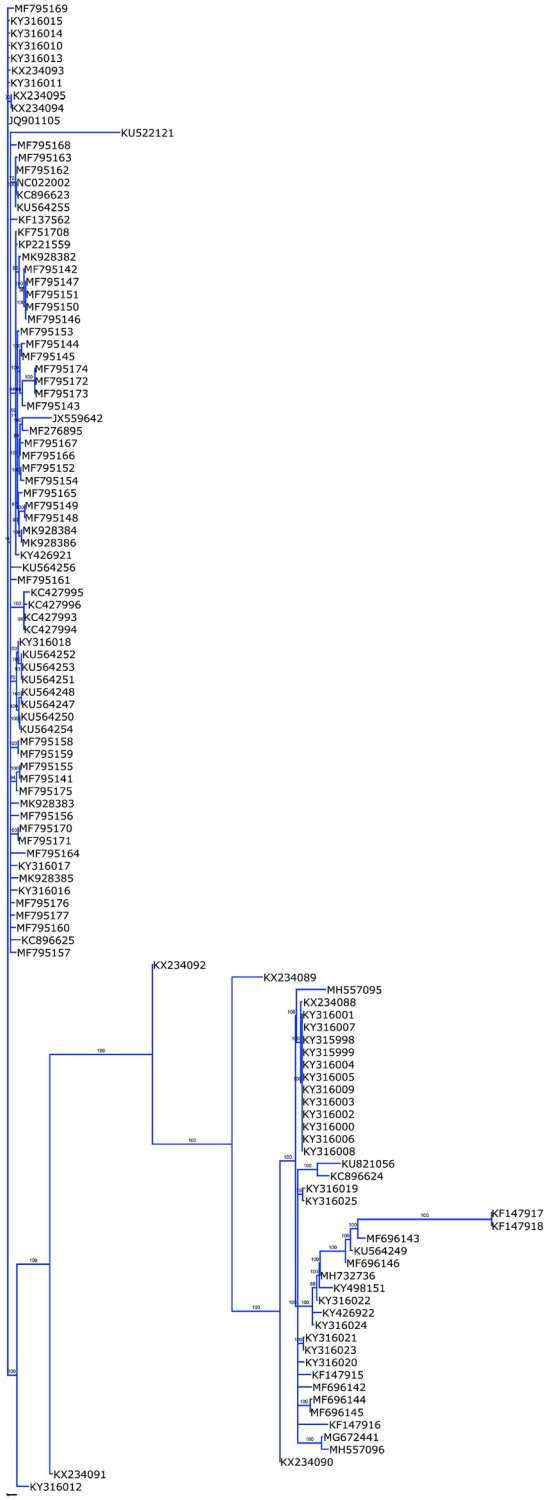

Fig. 3 The full-length genome of more than 120 grapevine red blotch virus isolates group into two major clades, in which higher intra-clade diversity is observed within clade 1 compared to clade 2. Phylogeny was constructed using MUSCLE alignment and maximum likelihood (RaxML) tree inference. Branches with less than 80% bootstrap support (100 replicates) were collapsed

In conclusion, believing is seeing. This philosophy is critical when addressing emerging virus diseases in vineyards in order to avoid disseminating false information, abstain from reaching hasty conclusions, and steer clear of impatient decisions. Embracing 'believing is seeing' is decisive for scientists to remain relevant and impactful. Otherwise, growers and vineyard managers might be exposed to careless considerations that can lead to the adoption and implementation of suboptimal or even inefficient disease management strategies. This can potentially have dramatic consequences regionally or at a larger scale when dealing not only with emerging virus diseases in grapevine but also emerging diseases in other fruit crops, as sadly experienced recently with *Xylella fastidiosa* subsp. *pauca* in olives in Southern Italy (Saponari et al. 2019). Coordinated, multidisciplinary approaches in conjunction with a constant dialogue between the research and grower communities, as well as extension educators, policy makers and regulatory authorities, are key to address emerging viruses in grapevines and curb their socioeconomic impacts.

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Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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