DIFFERENTIAL PROPERTIES OF *GRAPEVINE VIRUS B* ISOLATES FROM CROATIAN AUTOCHTHONOUS GRAPEVINE CULTIVARS

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SUMMARY

A total of 1116 grapevine accessions from 14 different Croatian domestic cultivars were tested for the presence of *Grapevine virus B* (GVB) by DASI-ELISA. Samples were taken from 51 commercial vineyards in the Dalmatian region. GVB was detected in 35 samples (3.14%) of nine cultivars namely: Dobricic, Mladenka Vlaška, Nincuša, Babic, Plavac mali, Maraština, Pošip and Vugava. The presence of GVB was further confirmed by molecular and biological assays. RT-PCR conducted on 14 ELISA-positive samples using the primer pair H28/C410 confirmed the serological data. Partial nucleotide sequences of the coat protein and putative RNA binding protein cistrons were generated for the virus isolates from the nine cultivars and compared with each other and other sequenced GVB. Isolates. Analyses showed sequence variation among the Croatian GVB isolates: they shared 81.3-99.5% similarity or 79.8-83.9% similarity with the GVB reference isolate deposited in GenBank. Deduced amino acid sequences of the partial putative RNA-binding protein gene shared between 80.5 and 100% identity within Croatian isolates and 83.2-86.7% with reference isolate. Fourteen GVB-positive samples were selected for further biological characterization by mechanical inoculation onto a range of herbaceous hosts. Symptoms induced on herbaceous hosts varied according to the isolate. In most cases, GVB isolates from domestic Croatian cultivars induced chlorotic spots and/or vein banding in *Nicotiana occidentalis* and *N. cavicola*. However, in some cases, symptoms observed in these two hosts included also necrotic local lesions followed by yellowing, deformation and rolling/curling. To the best of our knowledge this is the first report of GVB in Croatia.

Key words: DASI-ELISA, RT-PCR, herbaceous hosts, sequencing.

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INTRODUCTION

Vineyards in Croatia cover more than 40,000 ha, representing one of the most extensively grown woody crop, especially along the Dalmatian coast. Croatian vine-growing districts can be divided into two distinct climatic regions, i.e. "Continental" and "Coastal". According to the official varietal list, a total of 197 *Vitis vinifera* cultivars are currently grown in the country (Maletic *et al.*, 2007), 70 of which are considered autochthonous, their importance varying according to the region.

In the "Continental" region the autochthonous cultivars are grown on 5% of the total vineyard area and are of minor importance, Moslavac bijeli being the prevailing cultivar. A completely different situation occurs in the "Coastal" region. Native cultivars are grown on more than 80% of the vineyard area, while in the southern part (Dalmatia) they make up more than 90% of the vineyards (Pejic *et al.*, 2000). According to the "Main register of grape, wines and fruit wines producers" the most important red-berried cultivars Plavac mali, Plavina and Babic are grown on *ca* 28%, 9% and 5% of Dalmatians vineyards, respectively. Trbljan is the dominant (495 ha, 9.5%), white-berried cultivar grown in Dalmatia, followed by Kujunduša (328 ha, 6.3%), Maraština (242 ha, 4.6%) and Pošip (227 ha, 4.3%). Other domestic cultivars are of local significance and grown on a small scale.

Viral diseases are one of the main threats to grapevine production worldwide and require particular management strategies like compulsory use of healthy ("virus-free") planting material and vector control. Croatia has recently started clonal and sanitary selection programs aimed at the identification of the best clones of autochthonous grapevine cultivars to be introduced into a future certification program. This initiative raised renewed interest for the study of viruses present in the native grape germplasm.

Recent investigations revealed high infection rates with *Grapevine leafroll-associated virus 1, 2* and *3* (GLRaV- 1, 2 and 3), especially in some autochthonous cultivars and grape-growing areas (Karoglan Kontic *et al.,* 2009; Voncina *et al.*, 2010). However, there are no records of the presence and distribution of *Grapevine*

virus B (GVB), the putative causal agent of corky bark disease, which is widespread in most grape-growing regions of the world (Martelli and Boudon-Padieu, 2006), and is included in the OEPP/EPPO certification scheme for grapevine (Anonymous, 2008). This virus has filamentous particles *ca.* 800 nm in length, containing a single-stranded RNA *ca.* 7,600 nucleotides in size (Boscia *et al.*, 1993). Long distance transmission is ensured by infected planting material while short distance transmission is mediated by several species of pseudococcid mealybugs from the genera *Pseudococcus* and *Planococcus* (Boscia *et al.*, 1993; Garau *et al.*, 1995; Golino *et al.*, 1995). GVB incidence has recently been the objective of studies conducted on local grape germplasm in Italy (Credi *et al.*, 2003) and in Slovenia (Tomazic *et al.*, 2005) but not in Croatia, a lack of knowledge that prompted the present investigation.

MATERIALS AND METHODS

Survey and plant material. Surveys were carried out during autumn 2006 in Dalmatia (southern coastal region of Croatia), the main growing area of 14 autochthonous cultivars. Samples were collected from 51 commercial vineyards included in the clonal selection scheme (Maletic *et al.,* 2008). Each sample was represented by at least 3 lignified cuttings taken from the basal part of the plant. Samples were labeled, placed in plastic bags and stored at 4°C until testing, which was done within the following 60 days. The number of samples/cultivar roughly mirrored their importance: i.e. the most important domestic red-berried cv. Plavac mali was represented with the greatest number of samples (284) taken from 17 different vineyards. A total of 1116 samples were collected and analysed.

Serological tests. Serological detection of GVB was done by DASI-ELISA using a commercial kit (Agritest, Italy). Cortical shavings taken from each cutting from one grapevine accession were mixed together to a total weight of 0.2 g and used as antigen source. Shavings were pulverized in a mortar with the addition of liquid nitrogen and mixed with 3 ml of grapevine extraction buffer (Agritest) All steps of DASI-ELISA were carried out according to manufacturer's instructions and readings were made with an EL800 spectrophotometer (BioTek, USA) at a wavelength of 405 nm 2 h after adding p-nitrophenylphosphate (Sigma, USA). Samples with absorbance greater than three times the average value of the negative controls were considered positive. Cuttings from some GVB-positive plants or from vines with questionable ELISA results were self rooted and tested again during June 2008 using young leaf petioles as the source of antigen.

RNA extraction and RT-PCR. Fourteen randomly selected samples positve in DASI-ELISA were also tested by RT-PCR to confirm the serological results. Total RNA was extracted from 100 mg of cortical shavings using an RNeasy Plant Mini Kit (Qiagen, Germany) following the manufacturer's instructions. Concentrations of extracted RNA were measured with a BioPhotometer (Eppendorf, Germany) at a wavelength of 260 nm after 1:50 dilution with 10 mM Tris-HCl buffer (pH 7.0). Absorbance was measured 5 times per sample and the average value was calculated. According to the absorbance values obtained, 1 to 3 µl of RNA (approximately 100 ng) were used as a template for RT-PCR. The reaction was conducted using a Qiagen OneStep RT-PCR kit in a 25 µl PCR-mixture consisting of: 5 µl Qiagen OneStep RT-PCR buffer, 1 µl dNTP mix, 1 µl of Qiagen OneStep RT-PCR enzyme mix, 5 µl of Q-solution and 0.6 µM of each primer pair H28/C410 (Minafra and Hadidi, 1994). Cycling conditions were as follows: reverse transcription at 47°C for 50 min followed by the initial denaturation step at 95°C for 15 min and 35 cycles of DNA amplification (94°C for 30 sec, 50°C for 1 min, 72°C for 90 sec). Final extension was at 72°C for 10 min. Reaction products were analyzed by electrophoresis in 1.5% agarose gel buffered in 1X TBE, stained in ethidium bromide and visualized under UV light.

Sequencing and phylogenetic analysis. RT-PCR products obtained from the nine grapevine accessions were purified using a GenElute PCR clean-up kit (Sigma, USA) according to the manufacturer's instructions and directly sequenced by the dideoxynucleotide termination cycle method using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequencing mix was prepared by diluting BigDye Terminator v3.1 Ready Reaction Mix with BigDye terminator sequencing buffer (5X) at 1:4 ratio. Cycle sequencing was performed as follows: 2 µl of cDNA was mixed with 2 µl of sequencing mix, 1 µl of primer (3.2 pmol) and brought to a final volume of 7 µl with ultra pure water. The PCR cycle was as follows: initial denaturation at 96°C for 1 min followed by 35 cycles of DNA amplification (denaturation at 95°C for 20 sec, annealing of primer at 55°C for 15 sec and elongation at 60°C for 4 min). Each amplified product was brought to a final volume of 10 µl with ultra pure water, then purified by a BigDye XTerminator purification kit (Applied BioSystems, USA) according to the manufacturer's instructions. Purified fragments were sequenced in an ABI 3130 Genetic Analyzer (Applied Biosystems, USA) in both directions. Sequences were edited using BioEdit Sequence Alignment Editor ver. 7.0.9.0 (Hall, 1999). Pairwise sequence comparisons of Croatian GVB isolates and GVB sequences available in GenBank were done with ClustalW program (Higgins *et al.,* 1994).

Fragments of each sequence coding for the partial putative RNA binding protein were aligned and imported in the ModelTest (Posada and Crandall, 1998) for calculation of likelihood scores using 11 schemes and based on Akaike information criterion values (Akaike, 1974). The best model was chosen (GTR+G) and Bayesian analysis was performed using MrBayes 3.1 program (Ronquist and Helsenbeck, 2003) with four chains and 500,000 generations. The consensus phylogram and clade credibility values were visualized using TreeView program (Page, 1996) and GVA P163M5 isolate (accession No. DQ855082) as an outgroup.

Biological assays. Dormant cuttings from 14 DASI-ELISA- and RT-PCR-positive grapevine accessions were self rooted under greenhouse conditions to obtain growth for mechanical transmission to *Nicotiana occidentalis* and *Nicotiana cavicola.* To increase susceptibility, 24 h before inoculation test plants were placed in darkness (Albrechsten, 2006). As a source of inoculum 1 g of petioles from young leaves was ground in a mortar in the presence of 5 ml of 3% nicotine in 0.01 M potassium phosphate and 0.01 M cysteine HCl (Boscia *et al.*, 1993). Each extract was rubbed on three plants of *N. occidentalis* and *N. cavicola* at the 4-6 fully developed leaf stage which were previously dusted with carborundum. Negative controls were inoculated in the same way using plant material from a GVB-negative vine. Before and after inoculation plants were kept under conditions of 12 h lighting at an average temperature of 22°C and 12 h of darkness at an average temperature of 20°C. Plants were observed daily for a period of one month for symptom development to be then tested for GVB, *Arabis mosaic virus* (ArMV), *Grapevine fanleaf virus* (GFLV), *Grapevine virus A* (GVA) and GLRaV-2 by ELISA according to the protocols provided by the manufacturer of the reagents (Agritest, Italy).

RESULTS AND DISCUSSION

DASI-ELISA revealed that of the 1116 tested grapevine accessions 35 (3.14%) were infected by GVB. A similarly low infection rate (2.4%) was found in Italy during clonal selection of *V. vinifera* cultivars (Credi *et al.*, 2003). Considering that our survey dealt with plants with apparently good agronomical traits and without visible symptoms on the foliage, it is likely that GVB infection rate is somewhat greater than determined in this study.

GVB-positive samples were checked for the presence of symptoms on the woody cylinder. After bark removal the majority, but not all, of samples showed abnormalities of the woody cylinder resembling those of the rugose wood complex disease (Martelli and Boudon-Padieu, 2006). However, none of the GVB-infected plants died two years post-ELISA tests, although some appeared less vigorous in comparison with GVB-free surrounding plants of the same variety.

To compare the suitability of different tissues for GVB detection, ELISA was done on a limited number of positive samples using cortical tissue from dormant cuttings or leaf petioles from vegetating self-rooted cuttings. Ten of 19 tested samples were positive in both tests regardless of the type of tissue used for virus extraction. Additionally, two accessions (BAB-100 and PMC-093) that in assays from cortical shavings were scored as being of uncertain infection status (absorbance values between two and three times the average value of negative controls), were positive in tests performed during vegetation. The presence of GVB in both samples was confirmed by RT-PCR and successful transmission to herbaceous hosts. The opposite results were obtained for six accessions (BAB-054, VM-148, VN-108, PMC-009, VVL-137 and VVL-143) that tested negative during vegetation, but not when dormant cuttings were used as virus source. ELISA results were confirmed via RT-PCR assays for all samples except for VM-148 and PMC-009 that were not subjected to amplification. Interestingly, one accession (MAR-101) was negative in ELISA tests but positive in RT-PCR and biological assays.

For the majority of samples, cortical shavings taken from dormant cuttings were the best source for GVB detection by ELISA, in agreement with the results by Bonavia *et al.* (1996). Nevertheless, in our experience, 7 out of 19 tested grapevine accessions gave the highest ELISA response when green tissue was used as antigen source (Table 1).

The highest infection rate (30.61%) was observed in a cv. Mladenka vineyard in the Kaštela district. The reason for such a high infection rate may be the use of buds from infected mother plants for establishing the new vineyard or because of efficient dissemination of the virus by mealybug vectors. The presence of vectors in the sampled vineyards was not checked. Nevertheless, during summer and autumn of 2006, *Planococcus ficus*, a vector of GVB, was frequently found in Dalmatian vineyards (Masten Milek, 2007) and *Pseudococcus longispinus* is commonly present on *Carissa* sp. in the coastal Croatian region (Masten Milek, 2007). Thus, mealybugs may disseminate GVB in vineyards of this area.

Some ELISA-positive samples (including some uncertain ones) were further tested by RT-PCR using the primer set H28/C410 designed to amplify a genomic portion including a dozen of the 3'-terminal codons of the viral CP gene, the short intergenic region (IR) and almost the entire ORF5 (113 out of 124 codons) coding for a putative RNA-binding protein (Saldarelli *et al.,* 1996; Shi *et al.*, 2004). Amplicons of the expected size (460 bp) were obtained from all tested samples includ-

Table 1. Detection of GVB by ELISA and RT-PCR in infected Croatian native grapevine cultivars.

Cultivar	Grapevine accession	Location	ELISA readings *	ELISA readings **	RT-PCR	Mechanical transmission	Sequencing
Babic	BAB-051	Primosten, vineyard 1	**1.231**	**0.843**	nt	nt	nt
	BAB-054	Primosten, vineyard 2	**0.387**	0.269	+	unsuccessful	nt
	BAB-063	Primosten, vineyard 2	**0.841**	**1.481**	+	positive	nt
	BAB-100	Primosten, vineyard 1	0.247	**0.567**	+	positive	done
Dobricic	VD-101	Kastela, vineyard 1	**2.423**	**0.953**	+	positive	done
	VD-102	Kastela, vineyard 1	**1.067**	**0.640**	+	positive	done
Marastina	MAR-101	Danilo Biranj	0.217	0.241	+	positive	nt
	MAR-103	Danilo Biranj	**0.451**	**0.712**	nt	nt	nt
Mladenka	VM-102	Kastela, vineyard 2	**1.151**	nt	nt	nt	nt
	VM-104	Kastela, vineyard 2	**0.435**	nt	nt	nt	nt
	VM-113	Kastela, vineyard 2	**0.767**	nt	nt	nt	n
	VM-114	Kastela, vineyard 2	**1.235**	nt	nt	nt	nt
	VM-118	Kastela, vineyard 2	**2.284**	nt	nt	nt	nt
	VM-119	Kastela, vineyard 2	**0.342**	nt	nt	nt	nt
	VM-120	Kastela, vineyard 2	**1.01**	nt	nt	nt	nt
	VM-121	Kastela, vineyard 2	**3.823**	nt	nt	nt	nt
	VM-123	Kastela, vineyard 2	**0.457**	nt	nt	nt	nt
	VM-132	Kastela, vineyard 2	**3.636**	**0.911**	+	unsuccessful	done
	VM-133	Kastela, vineyard 2	**0.745**	nt	nt	nt	nt
	VM-134	Kastela, vineyard 2	**0.745**	nt	nt	nt	nt
	VM-135	Kastela, vineyard 2	**1.428**	nt	nt	nt	nt
	VM-148	Kastela, vineyard 2	**0.661**	0.377	nt	nt	nt
	VM-149	Kastela, vineyard 2	**0.487**	nt	nt	nt	nt
Nincusa	VN-108	Kastela, vineyard 3	**0.447**	0.517	+	unsuccessful	done
Plavac mali	PMC-009	Island Vis, vineyard 1	**0.760**	0.487	nt	nt	nt
	PMC-010	Island Vis, vineyard 1	**2.765**	**1.701**	+	positive	nt
	PMC-011	Island Vis, vineyard 1	**0.316**	**0.753**	+	positive	done
	PMC-085	Island Korcula, vineyard 1	**1.087**	**0.888**	+	positive	done
	PMC-093	Island Korcula, vineyard 1	0.267	**1.197**	+	positive	nt
	PMC-169	Peninsula Peljesac	**0.313**	nt	nt	nt	nt
Posip	PO_-047	Island Korcula, vineyard 2	**0.967**	nt	nt	nt	nt
Vlaska	VVL-101	Kastela, vineyard 4	**0.847**	**0.687**	nt	nt	nt
	VVL-137	Kastela, vineyard 5	**0.800**	0.404	+	positive	done
	VVL-143	Kastela, vineyard 5	**1.099**	0.387	+	positive	done
Vugava	VUG-115	Island Vis, vineyard 2	**0.313**	nt	nt	nt	nt
ELISA positive controls (average value)			2.910	3.951	-	-	-
ELISA negative controls (average value)			0.103	0.176	-	-	-

* ELISA made from cortical shavings during the dormant period. ** ELISA made from petioles of young leavesin June. nt - not tested. ELISA readings in bold indicate positive grapevine accessions with absorbance greater than three times the average value of the negative controls.

ing those were serologically doubtful (BAB-054, BAB-100, MAR-101, VN-108, PMC-093, VVL-137 and VVL-143) (Fig. 1).

Nucleotide sequences of the GVB isolates determined in this study have been deposited in GenBank under the following accession Nos: HM042895 (BAB-100); HM042896 (PMC-011); HM042897 (PMC-085); HM042898 (VD-101), HM042899 (VD-102); HM042900 (VM-132); HM042901 (VN-108); HM042902 (VVL-137); HM042903. (VVL-143).

Sequence analyses showed that nucleotide (nt) identity among Croatian GVB isolates ranged between 81.3 and 99.5%. Grapevine accessions taken from the same vineyard, such as VVL-137/VVL-143 and VD-101/VD-102 shared the highest nt identity (99.0 and 99.5% respectively), suggesting infection with the same strain of GVB. Comparison analyses with the GVB reference isolate (NC_003602) showed between 79.8 and 83.9%

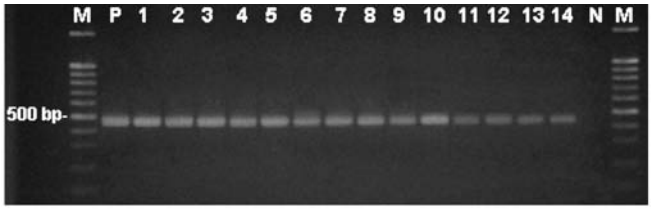

Fig. 1. Ethidium bromide-stained agarose gel of RT-PCR products generated from grapevine accessions using primers H28/C410. Lanes 1-14 contain different grapevine accessions, negative control is in lane N and positive control in lane P. Reference markers (100 bp ladder, Intron Biotechnology, Korea) are in lanes M.

identity at the nt level. Comparison of the amino acid sequences of part of the putative RNA-binding protein gene showed between 81.4 and 100% identity among the Croatian isolates and between 83.2% and 86.7% with GVB reference isolate (Table 2). Our results are similar to those published by Shi *et al.* (2004), who reported identities varying between 76% and 100% in the same genomic region among 20 GVB isolates from three different countries.

From the phylogenetic tree (Fig. 2) it is evident that the isolates PMC-085, VN-108 and VM-132 are the most distant and cluster with isolate GVB 17NM1 from Japan. Sample PMC-011 clustered with Murcia M5 and Murcia M9 sample. Isolates from grapevine accessions from the same vineyard clustered closely (VD-101 and VD-102; VVL-137 and VVL-143). The explanation for such high sequence variation in Croatian isolates may be due to vegetative propagation of grapevine which can lead to mixing (and possible recombination) of different GVB strains from different sources, i.e. buds taken from mother plants of unknown sanitary status and rootstocks from nurseries multiplying foreign, sometimes untested planting material.

In contrast to results reported by Bonavia *et al.* (1996), GVB was successfully transmitted through mechanical inoculation to *N. cavicola* and *N. occidentalis* from 11 out of 14 grapevine accessions (78.6%). Symptoms observed were similar to those originally described by Boscia *et al.* (1993), and in all 11 cases infection was systemic. The first symptoms appeared 10-15 days after inoculation as polygonal chlorotic spots and vein banding/clearing, followed by necrotic local lesions, yellowing, deformation or puckering, downward or upward rolling of the leaves and rosette-like growth (Table 3). The type and timing of appearance of these symptoms depended on the specific GVB strain. The necrotic areas on stems described by Nickel *et al.* (2002) were not observed. There was not any correlation between induced symptoms and origin of the virus except in the case of VD-101 and VD-102 which produced very similar responses in theses hosts. Interestingly, in the case of BAB-100, we successfully transmitted two nepoviruses

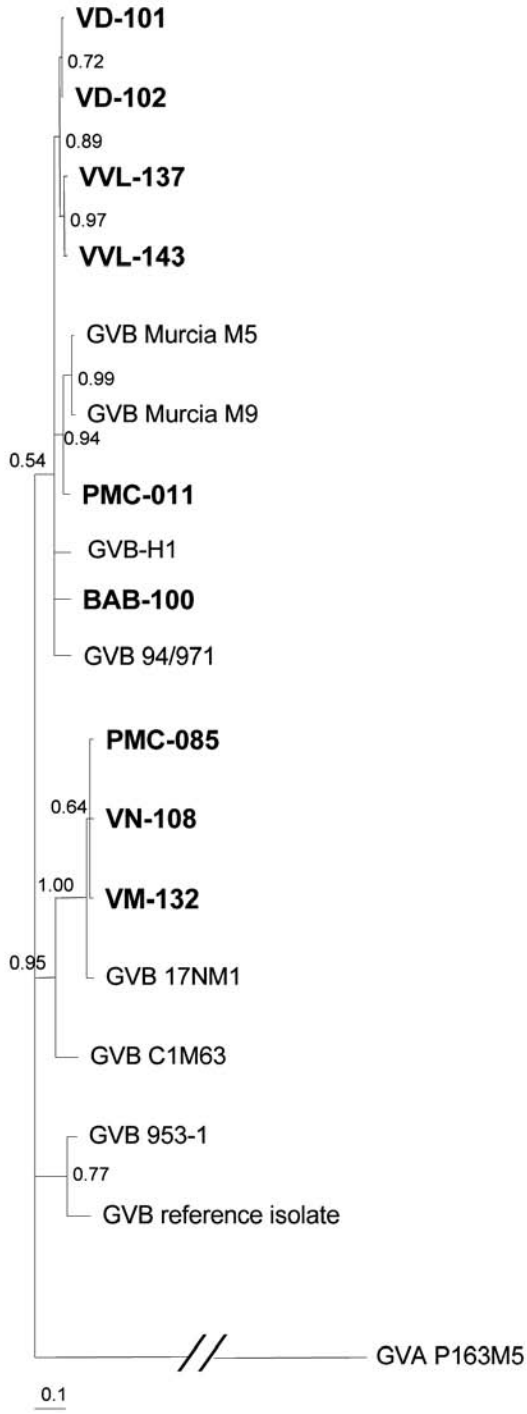

Fig. 2. Phylogenetic tree constructed with partial amino acid sequences of the nine Croatian GVB isolates (BAB-100, PMC-011, PMC-085, VD-101, VD-102, VN-108, VM-132, VVL-137, VVL-143), and with GVB sequences from GenBank: Murcia M5 (AJ748850), Murcia M9 (AJ748851), H1 (GU733707), 94/971 (EF583906), 17NM1 (AB222857), C1M63 (GQ415407), 953-1 (GU984638), reference isolate (NC_003602). GVA isolate P163-M5 (DQ855082) was used as an outgroup. Bayesian analysis was done using the MrBayes 3.1 program (Ronquist and Helsenbeck, 2003). Consensus phylogram and clade credibility values were visualized using TreeView program. Croatian isolates are highlighted in bold.

Table 2. Percent (%) identities between nucleotide and amino acid sequences of Croatian GVB isolates and the GVB reference isolate (accession No. NC_003602). Data above the diagonal correspond to nucleotide sequence identities of the entire PCR product (416 nts). Data below the diagonal report identities of 113 amino acid sequences of the putative RNA binding protein.

Isolates	BAB-100	PMC-011	PMC-085	VD-101	VD-102	VM-132	VN-108	VVL-137	VVL-143	Reference isolate
BAB-100	■	91.6	82.5	93.5	93.8	82.5	82.2	92.8	92.6	81.5
PMC-011	96.5	■	81.9	91.4	91.6	81.9	81.5	91.4	91.1	82.2
PMC-085	84.1	84.1	■	81.9	82.5	98.3	98.3	81.5	81.7	79.8
VD-101	95.6	95.6	81.4	■	99.5	81.9	81.7	97.6	97.4	83.7
VD-102	95.6	95.6	81.4	100	■	82.5	82.2	98.1	97.8	83.9
VM-132	84.1	81.4	99.1	81.4	81.4	■	98.1	81.5	81.7	79.8
VN-108	83.2	83.2	98.2	80.5	80.5	97.4	■	81.3	81.5	79.8
VVL-137	95.6	95.6	81.4	99.1	99.1	81.4	80.5	■	99.0	83.9
VVL-143	96.5	96.5	82.3	98.2	98.2	82.3	81.4	99.1	■	83.7
Reference isolate	85.8	86.7	84.1	86.7	86.7	84.1	83.2	86.7	86.7	■

Table 3. Symptoms of GVB on herbaceous hosts observed 30 days post inoculation. The presence of GVB was confirmed by DASI-ELISA. All plants were also tested for the presence of ArMV, GFLV, GLRaV-2 and GVA.

Grapevine accession	Organ of herbaceous hosts	Symptoms on *Nicotiana cavicola*	Symptoms on *Nicotiana occidentalis*
BAB-100*	YL	UR, PCS, D	PCS, VB
	OL	Y, PCS, VB of basal part, NLL on top	PCS, VB
VD-101	YL	PCS	PCS
	OL	PCS	PCS,
VD-102	YL	PCS	PCS
	OL	PCS, NLL on top	PCS
PMC-011	YL	UR, PCS, P, RG	DR, PCS; NLL, P
	OL	VB, Y, NLL	VB, PCS, NLL on top, P
PMC-085	YL	VB, P, DR	PCS, P
	OL	VB	VB on basal and middle part
VVL-137	YL	PCS, UR	PCS
	OL	VB, PCS	VB, NLL on top, PCS
VVL-143	YL	PCS, UR	PCS, Y, P
	OL	VB, D, PCS	PCS
Negative control	YL	NS	NS
	OL	NS	NS

YL, young leaves; OL, old leaves; Y, yellowing; PCS, polygonal chlorotic spots; UR,upward rolling of the leaves; DR, downward rollingof the leaves; VB, vein banding; D, deformation; NLL, necrotic local lesions; P - puckered, RG - rosette-like growth, NS - no symptoms.
BAB-100* - ELISA positive for GVB, ArMV and GFLV.

(ArMV and GFLV) along with GVB. The presence of these additional viruses in both herbaceous plants did not affect significantly the symptomatology as induced by GVB only.

In conclusion, in several native grapevine cultivars from Dalmatia, we have identified and partially characterized a number of isolates of GVB, a virus that had not been previously recorded from Croatia. In the majority of the cases virus presence was accompanied by symptoms on the woody cylinder. Several randomly chosen GVB isolates were characterized biologically and molecularly. Both analyses showed variations, either in symptomatology induced in herbaceous indicator plants or at the nucleotide level. Taking into account that our survey targeted cultivars with relatively good agronomic traits (vigor, productivity, absence of obvious foliar symptoms) and that the ELISA kit employed was based on monoclonal antibodies it is possible that GVB is more widespread in Dalmatian vineyards than estimated in this study (*ca* 3%).

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