Raspberry bushy dwarf virus in Slovenia - geographic distribution, genetic diversity and population structure

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Abstract Raspberry bushy dwarf virus (RBDV) is a long-known virus naturally infecting *Rubus* and grapevine. It is also one of the economically most important viruses of raspberries, but there are only a limited number of sequences covering a substantial part of the genome available in the databases. The aim of this study was: i) to study the geographic distribution of RBDV in Slovenia, and ii) to sequence RNA2 of several red raspberry and grapevine RBDV isolates and study their phylogeny and population structure. Geographic distribution studies were performed over a period of 13 years in three wine-growing regions of Slovenia (Primorska, Podravje and Posavje). The highest incidence of RBDV was found in Podravje (58.8%) and the lowest in Primorska (5.1%). Big differences were observed between Vipavska dolina (10.2%) and three other wine-growing districts of Primorska region (0.4–1.2%). Almost complete RNA2 sequences were obtained for four red raspberry isolates and seven grapevine isolates. Additionally, only coat protein sequences were obtained for three red raspberry isolates. Phylogenetic and population diversity analyses were performed on all available RBDV sequences. Phylogenetic analysis has shown clear differences in sequences from *Rubus* and grapevine that form two highly supported clades. In RNA2

analysis additional two sub-clades were found in grapevine clade. Two major subclades were identified also in the *Rubus* clade with further differentiation within these subclades. Purifying or stabilizing selection was found to be acting on both, CP and MP genes while few codons were found to be under positive selection.

Keywords RBDV · Population structure · Grapevine · *Rubus* · Genetic diversity · Selection pressure

Raspberry bushy dwarf virus (RBDV) is a well-studied virus found naturally in raspberries, blackberries and grapevines (reviewed in Martin et al. 2013). In red raspberries, RBDV has long been implicated in crumbly fruit disease, which has been shown to be more severe in mixed infections with one or more aphid transmitted viruses (Martin et al. 2013; Quito-Avila et al. 2014).

RBDV, a sole member of the genus *Idaeovirus*, is pollen transmitted. Natural infection with RBDV has long been confined to *Rubus* spp. while experimental host range is wider (Jones et al. 1982). The RBDV presence in *Rubus* spp. has been reported from Europe, North America, South America, South Africa, Japan and New Zealand (Barbara et al. 2001; Dal Zotto et al. 2017; Dulić-Marković and Ranković 1992; Isogai et al. 2012; Kooyman et al. 1982; Matus et al. 2008; Mavrič et al. 2003; Quito-Avila et al. 2013; Spak and Kubelkova 2000; Strik and Martin 2003; Valasevich et al. 2011; Wall and Shamoun 1990; Wood 1995). It was found in different wild and cultivated *Rubus* plants (red and black raspberry, blackberry, blackberry-

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raspberry hybrids, wild *R. idaeus* var. *strigosus*, *R. occidentalis*, *R. parviflorus*, *R. leucodermis*, *R. multibracteatus*, *R. glaucus*, *R. arcticus*) (Chamberlain et al. 2003; Kokko et al. 1996; Martin et al. 2013; Quito-Avila et al. 2013; Wall and Shamoun 1990). In 2003 the first natural RBDV infection of non-*Rubus* host was reported for grapevine from Slovenia (Mavrič et al. 2003) and later also from Serbia and Hungary (Jevremović and Paunović 2011; Mavrič Pleško et al. 2012; Czotter et al. 2018). Additionally, a virus showing high sequence similarity to RBDV was found in citrus plants in USA (Derrick et al. 2006) and two new idaeoviruses were found in privet (Navarro et al. 2016) and in black currant (James and Phelan 2017; Thekke-Veetil et al. 2017).

The genome of RBDV is bipartite single-stranded RNA encapsidated in quasi-isometric particles. RNA1 contains one large open reading frame (ORF) encoding a 188 kDa protein with motifs of viral RNA helicases and polymerases (Ziegler et al. 1992). RNA2 has two ORFs, the one at the 5'-end encodes a 39 kDa movement protein (MP) and the one at the 3'-end a 30 kDa coat protein (CP) (Natsuaki et al. 1991). Parts of the genome were sequenced for RBDV isolates originating from different countries but there is still a very limited number of sequences available in the database.

Studies of RBDV in Slovenia started in 2002 when the virus was first detected in red raspberries from collection plantation of Agricultural Institute of Slovenia. In 2003, the RBDV infection was confirmed in grapevine grafts of cvs. Laški Rizling (Italian Riesling) and Štajerska Belina by DAS-ELISA and immunocapture RT-PCR and a few grapevine and red raspberry isolates were further characterized (Mavrič et al. 2003; Mavrič et al. 2004; Mavrič Pleško et al. 2009). RNA2 sequences used for molecular characterization of these isolates are some of the few RBDV RNA2 sequences available.

In this study the presence of RBDV was surveyed in grapevine in three wine-growing regions of Slovenia during the period of 13 years. Selected grapevine and red raspberry samples were used for molecular characterization and phylogenetic analysis of RBDV. Additionally, the population structure of RBDV was studied using available CP, MP and RNA2 sequences.

Over a period of 13 years (2003–2015), 2505 grapevine samples were collected from vineyards in all three wine-growing regions of Slovenia (Primorska, Podravje, Posavje). For geographical distribution

studies, 1393 samples (55.6%) originated from Primorska, 730 (29.15%) from Podravje and 382 (15.25%) from Posavje wine-growing regions. All samples were tested for RBDV by DAS-ELISA using Bioreba reagents according to manufacturer's instructions. The highest incidence of RBDV was determined in Podravje region (58.8%) and the lowest in Primorska region (5.1%) The results are presented in Table 1. In Primorska region where 4 winegrowing districts are defined, considerable differences in the RBDV incidence were observed between Vipavska dolina (10.2%) and other wine-growing districts of this region (0.4–1.2%) (Table 1, Fig. 1).

Over 30 grapevine cultivars collected in all wine-growing regions were analyzed. The largest number of samples was collected in Italian Riesling, Refošk, Merlot, Malvazija, Chardonnay and Pinot Gris. The highest infection rate was detected in Chardonnay, Rizvanec, Šipon, and Zweigelt (80% or higher) followed by Rhein Riesling (72.3%) and Italian Riesling (53.5%). Most of the RBDV positive samples from Primorska were collected in Vipavska dolina (Table 1) where the infection was confirmed in Italian Riesling (59%), Sauvignon (38.7%) and Malvazija (2.6%). In Posavje, the infected cultivars were Italian Riesling and Rhein Riesling (27.1% and 59.1% respectively) while in Podravje the majority of tested cultivars were found to be infected. The widespread infections with RBDV in Podravje indicate that the infection in Slovenia might originate in this region and had spread to other regions through infected planting material of cultivars grown in all regions. This would explain the high infection rate in

Table 1 Results of serological detection of RBDV in grapevine samples from different wine-growing regions of Slovenia

Wine-growing region/ district	positive	negative	all	positive (%)
Primorska	71	1322	1393	5.1
Vipavska dolina	64	566	630	10.2
Kras	1	252	253	0.4
Goriška Brda	2	187	189	1.1
Slovenska Istra	4	317	321	1.2
Podravje	429	301	730	58.8
Štajerska Slovenija	429	301	730	58.8
Posavje	51	331	382	13.35
Dolenjska	33	140	173	19.1
Bela Krajina	18	191	209	8.6

Fig. 1 Geographical presentation of grapevine sampling locations and the incidence of RBDV in analyzed samples in wine-growing regions of Slovenia

Vipavska dolina in comparison with the rest of Primorska since Italian Riesling is a predominant cultivar of Slovenia but in the Primorska region it is almost exclusively grown in Vipavska dolina.

In spite of importance of RBDV in raspberry production, there are only eight whole genome sequences and an additional seven sequences of complete or almost complete RNA2 available in the databases. Obtaining additional RNA2 sequences of RBDV from grapevine and red raspberry was the main aim of this study that allowed for better understanding of its diversity. Several grapevine and red raspberry samples, confirmed to be infected with RBDV by DAS-ELISA, were selected for diversity study. The selected grapevine samples were from different wine-growing regions while raspberry samples all originated from collection plantation of Agricultural Institute of Slovenia at Brdo pri Lukovici in central Slovenia. For the purpose of this study, all samples used for diversity study are referred to as isolates. The IC RT-PCR or total RNA extraction and RT-PCR were used to confirm RBDV infection of seven grapevine and seven red raspberry samples and to amplify two PCR products covering almost complete RNA2 of

RBDV. The IC RT-PCR, RT-PCR, cloning and sequencing were performed as previously described (Mavrič Pleško et al. 2009). Sequences were analysed using Geneious version 8.1.8 and 11.1.4 (<http://www.geneious.com>, Kearse et al. 2012) and assembled into contigs. Almost complete RNA2 sequences (including complete ORFs for CP and MP) were obtained for seven grapevine and four red raspberry isolates and only CP sequences were obtained for three red raspberry isolates. Sequences were deposited in the GenBank under Accession Numbers KY417868 KY417881.

Phylogenetic relationships between the isolates were inferred from UPGMA clustering method. Trees were constructed from (1) near complete RNA2 alignment of 27 sequences with 2124 nucleotides (Fig. 2), and (2) CP gene alignment from 41 RBDV sequences with 822 nucleotides (Fig. 3); 14 obtained from this study and 27 from Genbank database (Table 2). Data were bootstrapped with 1000 re-samplings to test the robustness of the lineages in the trees. Analyses were performed using Tamura 3-parameter method implemented in MEGAX software (Kumar et al. 2018). Four sequences were used as an outgroup: DQ120126 (RBDV

Fig. 2 Evolutionary analysis of RBDV sequences originating from *Rubus* spp. and grapevine (*Vitis* sp.), based on the nucleotide sequences of the RNA2 (2124 nt, 2248 positions). Horizontal lines are in proportion to the number of substitutions per site. Numbers at branch nodes represent bootstrap support of 1000 replicates, but only values higher than 50% are shown. Sequences in bold were obtained during this study

RNA2 sequence from *Rubus multibracteatus*, China, Chamberlain et al. 2003), DQ100358 (CP gene of Citrus idaeovirus, USA, Derrick et al. 2006), KY399999 (RNA2 sequence from blackcurrant leaf chlorosis associated virus, USA, Thekke-Veetil et al. 2017), and KX838924 (RNA2 from blackcurrant leaf chlorosis associated virus, Canada, James and Phelan 2017). Sequences from raspberry and grapevine formed two distinct and highly supported clades (> 92%) in RNA2-based and CP-based phylogenetic trees (Figs. 2 and 3). All grapevine sequences clustered together in one clade. Lower isolate diversity was observed within grapevine clade of CP gene. Overall, no correlation between white and red cultivar isolates was noticed. The clade of RNA2 sequences from raspberry showed higher internal clustering with two major sub-clades, one with Slovenian and Ecuador sequences (93%), and the other with Belarus and UK sequences (100%). Further differentiation was observed also within these sub-clades. Higher diversity was also observed in phylogenetic tree of raspberry CP gene.

It has already been demonstrated that grapevine and raspberry RBDV sequences differ genetically and phylogenetically from each other. The distinction was first

observed after the discovery of RBDV in grapevine (Mavric Plesko et al. 2009) and also by Valasevich et al. (2011) when RBDV raspberry sequences from Belarus and Sweden were compared to all available RBDV sequences. Phylogenetic analysis of RNA2 sequences performed in the study of Valasevich et al. (2011) confirmed clear differentiation between grapevine and raspberry sequences. Further differentiation was observed within raspberry clade. Analysis performed in our study confirmed this differentiation with additional sequences in each of the two clades. No obvious geographical differentiation could be observed from phylogenetic analysis, however, considering the low coverage of countries or geographical regions, this was understandable.

The selection pressure working on CP and MP genes from the RBDV populations was studied based on the d_N/d_S ratio calculated from the average number of non-synonymous substitutions per non-synonymous site (d_N), and the average number of synonymous substitutions per synonymous site (d_S) using algorithms implemented in DnaSP v6 (Rozas et al. 2017). Nucleotide diversity of CP and MP sequences was similar, but in both cases lower in grapevine (GR) (Table 3). The ratio

Fig. 3 Predicted relationships between RBDV sequences originating from *Rubus* spp. and grapevine (*Vitis* sp.), based on the nucleotide sequences of CP gene (822 nt). Horizontal lines are in proportion to the number of nucleotide differences between branch

nodes. Numbers at branch nodes represent bootstrap support of 1000 replicates, but only values higher than 50% are shown. Sequences in bold were obtained during this study

d_N/d_S was significantly below 1.0 indicating a strong purifying (stabilizing) selection acting on both CP and MP genes.

Cumulative behaviour plots (Fig. 4) for synonymous and non-synonymous substitutions along the coding regions were constructed using the ‘xyplot’ option of

Table 2 List of RBDV sequences used in this study

GenBank Accession Number	Isolate name (RNA/gene)	Country	Host	Reference
FR687358	SE3 (RNA2)	Sweden	red raspberry	Valasevich et al. 2011
FR687357	BY22 (RNA2)	Belarus	red raspberry	Valasevich et al. 2011
FR687356	BY8 (RNA2)	Belarus	red raspberry	Valasevich et al. 2011
FR687355	BY3 (RNA2)	Belarus	red raspberry	Valasevich et al. 2011
FR687354	BY1 (RNA2)	Belarus	red raspberry	Valasevich et al. 2011
NC_003740	R15 (RNA2)	UK	red raspberry	Natsuaki et al. 1991
KJ007640	Ec_Az (RNA2)	Ecuador	<i>R. glaucus</i>	Quito-Avila et al. 2014
AB948215	J1 (RNA2)	Japan	Red raspberry	Isogai et al. 2014 (unpublished)
EU796090	CmGR-2 (RNA2)	Slovenia	<i>C. murale</i>	Mavrič Pleško et al. 2009
EU796089	CmRR-1 (RNA2)	Slovenia	<i>C. murale</i>	Mavrič Pleško et al. 2009
EU796088	RR-1 (RNA2)	Slovenia	red raspberry	Mavrič Pleško et al. 2009
EU796087	GR-2 (RNA2)	Slovenia	grapevine	Mavrič Pleško et al. 2009
EU796086	GR-4 (RNA2)	Slovenia	grapevine	Mavrič Pleško et al. 2009
EU796085	GR-6 (RNA2)	Slovenia	grapevine	Mavrič Pleško et al. 2009
DQ120126	R.multi. (RNA2)	China	<i>R. multibracteatus</i>	Chamberlain et al. 2003
KY417868	RR-2 (RNA2)	Slovenia	red raspberry	This study
KY417869	RR-3 (RNA2)	Slovenia	red raspberry	This study
KY417870	RR-5 (RNA2)	Slovenia	red raspberry	This study
KY417871	RR-8 (RNA2)	Slovenia	red raspberry	This study
KY417872	GR-7 (RNA2)	Slovenia	grapevine	This study
KY417880	GR-8 (RNA2)	Slovenia	grapevine	This study
KY417881	GR-9 (RNA2)	Slovenia	grapevine	This study
KY417873	GR-10 (RNA2)	Slovenia	grapevine	This study
KY417874	GR-11 (RNA2)	Slovenia	grapevine	This study
KY417875	GR-12 (RNA2)	Slovenia	grapevine	This study
KY417876	GR-13 (RNA2)	Slovenia	grapevine	This study
D01052	R15 (CP)	UK	<i>C. quinoa</i>	Mayo et al. 1991
AB698501	J3 (CP)	Japan	red raspberry	Isogai et al. 2012
AB698500	J2 (CP)	Japan	red raspberry	Isogai et al. 2012
AB698499	J1 (CP)	Japan	red raspberry	Isogai et al. 2012
AY894679	CP Z13-b (CP)	Finland	red raspberry	Wang et al. 2008
AY894678	CP Z13-a (CP)	Finland	red raspberry	Wang et al. 2008
AF259798	Can-S (CP)	Canada	<i>N. benthamiana</i>	Jones et al. 2000
AF259796	D1 (CP)	Scotland	<i>C. quinoa</i>	Jones et al. 2000
AF259795	D200 (CP)	Scotland	red raspberry	Jones et al. 2000
KY417877	RR-4 (CP)	Slovenia	red raspberry	This study
KY417878	RR-6 (CP)	Slovenia	red raspberry	This study
KY417879	RR-7 (CP)	Slovenia	red raspberry	This study
MF446640	RBDV-HUSZHU (CP)	Hungary	grapevine	Czotter et al. 2018
KY308191	EB-42 (CP)	Argentina	red raspberry	Dal Zotto et al. 2017
KX838924	3124-03D1 (RNA2)	Canada	blackcurrant	James and Phelan 2017
KY399999	Oregon (RNA2)	USA	blackcurrant	Thekke-Veetil et al. 2017

Table 3 Estimates of mean codon-based evolutionary diversity

Gene	Group	<i>n</i>	No. codons	<i>Pi(s)</i>	<i>Pi(a)</i>	<i>d_N/d_S</i>
MP	All	26	358	0.097	0.017	0.193
	RU	15		0.097	0.013	
	GR	11		0.021	0.007	
CP	All	39	274	0.128	0.012	0.086
	RU	27		0.115	0.010	
	GR	12		0.029	0.005	

RU *Rubus* isolates, GR grapevine isolates, *n* - number of analyzed sequences in the group, *Pi(s)* - number of synonymous substitutions per site, *Pi(a)* - number of non-synonymous substitutions per site

SNAP available on the HIV database website (www.hiv.lanl.gov; Korber 2000). They indicated that non-synonymous substitutions (red line) are distributed equally throughout the length of the MP (Fig. 4a) and CP (Fig. 4b) coding region. Generally, the number of

synonymous substitutions over non-synonymous substitutions is greater in protein-coding regions. However, in the case of MP (Fig. 4a), the synonymous substitutions showed a biphasic distribution with its rate being lower than the rate of non-synonymous in the codon region of 1 to 106 codons. Both GR and RU sequences contributed to observed biphasic distribution/phenomenon (data not shown). However, a few codons were found to be under positive selection.

Testing for selective pressures operating on MP and CP genes was performed using an online tool Datamonkey Adaptive Evolution Server (Delpert et al. 2010). The analyses showed that 0.16–0.21% of codons were under a negative (purifying) selection. The FEL analysis (Fixed Effects Likelihood) revealed a very small proportion of codons under positive selection in MP gene (0.006%) and none in CP gene (Table 4). In addition, we used MEME (Mixed Effects Model of Evolution) as it was shown to have greater resolving

Fig. 4 Cumulative behaviour of synonymous (green line) and non-synonymous (red line) substitutions along the coding region of (A) MP and (B) CP gene in RBDV isolates. Accumulation of amino acid substitutions within the MP (*n* = 25) and CP (*n* = 38) protein sequences of RBDV isolates, as determined by SNAP program, is plotted along the coding region. The analysed coding region consisted of 358 codons for MP and 274 codons for CP gene sequences. Outgroup sequence DQ120126 was not included in the analysis

Table 4 Positively selected sites in MP and CP genes estimated by FEL and MEME models

RNA2		Fixed effects likelihood model (FEL)		Mixed effects model of evolution (MEME)	
ORF	No. codons	No. negatively selected sites	No. positively selected sites	Sites with episodic diversifying selection	Lineage specific codon diversification
MP	358	59 (0.16%)	2 (0.006%)	5 (0.014%)	12 (RR2) (DQ120126); 17 (FR687357); 32 (GR9); 257 (DQ120126) (FR687358); 333 (EU796086) (NC_003740);
CP	274	58 (0.21%)	0	2 (0.007%)	63 (RR2, RR5, RR6, AF259798, KJ007640); 78 (AF259796, D01052, FR687355)

power than FEL (Murrell et al. 2012). Test revealed that five codons for MP and two for CP gene could be under a positive selection, which represents 0.014–0.007% of codons in total. The majority of codons are therefore under neutral selection, which agrees with the calculated d_N/d_S ratio (Table 3). The sites of episodic diversifying selection that could be mapped to specific lineages are summarized in Table 4. The positively selected sites were found to be present in RBDV lineages originating from *Rubus*, and less from grapevine. A rapid screening for recombination events was performed using GARD (Kosakovsky Pond et al. 2006), a recombination detection method implemented in Datamonkey server (Delpert et al. 2010) and RDP4 program using default parameters (Martin et al. 2015). We found no evidence for recombination events.

Sequence divergence, genetic differentiation and gene flow were estimated by algorithms implemented in DnaSP v6 (Rozas et al. 2017). To estimate genetic differentiation, four subpopulation groups were created: (1) GR group with sequences from grapevine, (2) RU group with sequences from *Rubus*, (3) RU-SLO with Slovenian *Rubus* sequences only, and (4) RU-OTH

group with *Rubus* sequences not originating from Slovenia. Genetic differentiation between groups was estimated with statistics K_{st}^* , Z^* , H_{st} , and S_{nn} subjected to permutation tests (Hudson et al. 1992; Hudson 2000). The DnaSP tool was used also for estimating gene flow (F_{ST}) between populations (Hudson et al. 1992). The comparison of GR and RU groups revealed strong genetic differences in both CP and MP genes, as evidenced by statistically significant values of all statistics for detecting genetic differentiation (Table 5). Hudson's S_{nn} indicated evidence for differentiation between GR and RU, and also between Slovenian *Rubus* and other *Rubus* isolates. Hudson's F_{ST} also indicated the presence of inter-population diversity between above-mentioned groups. The F_{ST} value of 0.371 for CP group RU-SLO vs. RU-OTH is close to the threshold value of 0.33 (Rozas et al. 2003). This might indicate more frequent gene flow between population groups RU-SLO and RU-OTH as opposed to infrequent gene flow among all other groups. However, similar situation was not detected for MP gene.

In conclusion, our survey confirmed the presence of RBDV in grapevine in all wine-growing regions of

Table 5 Genetic differentiation and gene flow estimation between subgroups of RBDV, based on MP and CP gene sequences

Gene	Comparison/sub-population	^a Pi (tot)	^b dN/dS	^c K _{st} *	^c Z*	^c H _{st}	^c S _{nn}	^d F _{ST}
MP	GR vs. RU	0.028	0.193	0.204***	3.995***	0.019*	1.000***	0.657
	RU-SLO vs. RU-OTH	0.018	0.123	0.276**	3.092***	0.077**	0.857*	0.434
CP	GR vs. RU	0.039	0.086	0.131***	4.968***	0.007*	1.000***	0.538
	RU-SLO vs. RU-OTH	0.028	0.077	0.109***	4.370***	0.024**	0.808**	0.371

^aPi(tot): total estimate of nucleotide diversity between subgroups; ^bdN/dS: indicator of selective pressure operating on protein-coding genes (1 – neutral; >1 – positive; <1 – purifying); ^cK_{st}*, Z*, H_{st}, S_{nn} – statistics with permutation tests for detecting genetic differentiation between subpopulations (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$); ^dF_{ST} – Wright's fixation index for quantification of the genetic differentiation ($F_{ST} > 0.33$ suggests infrequent gene flow)

Slovenia with the highest incidence in Podravje and the lowest in Primorska. Several economically important cultivars were found to be infected. The knowledge about RBDV infection in grapevine is still very limited. It has been reported from Slovenia, Hungary and Serbia (Mavrič Pleško et al. 2009; Mavrič Pleško et al. 2012; Czotter et al. 2018; Jevremović and Paunović 2011). However, we would expect that its distribution is wider than currently known. The present study is an important contribution to the knowledge about RBDV variability, especially for grapevine isolates. The study considerably raised the number of available sequences in the databases which may help to improve the available molecular tests for RBDV detection in research and diagnostics. The phylogenetic analysis of RBDV sequences confirmed the host-based differentiation of RBDV isolates. Further differentiation into subclades was observed within grapevine and within *Rubus* isolates. The data indicate the possible greater variability of RBDV than previously thought. No recombination events were detected within CP and MP genes.

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Compliance with ethical standards

The authors declare that no human participants or animals were involved in this research.

Conflict of interest The authors declare that they have no conflict of interest.

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