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RESEARCH ARTICLE

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# Genetic diversity of *Raspberry leaf blotch emaravirus* in red raspberries from Serbia

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## Abstract

*Raspberry leaf blotch emaravirus* (RLBV) is a recently characterised virus infecting raspberries reported in several European countries. RLBV causes yellow blotching, the distortion of leaf margins, and the twisting of raspberry leaves. For a long time, similar symptoms were attributed to the feeding damage caused by raspberry leaf and bud mite (*Phyllocoptes gracilis*). From 2014−2017, a large-scale survey was conducted in Serbia to investigate the degree of association of the observed symptoms with the RLBV infection. A total of 98 symptomatic and asymptomatic samples were collected from 30 locations. All collected samples were tested on the RLBV presence by reverse transcription and polymerase chain reaction (RT-PCR) using three sets of RNA-specific primers targeting RNA-1, RNA-3, and RNA-5 of the RLBV genome. RT-PCR analysis revealed high incidence of RLBV in tested samples (68.7%). RLBV was confirmed in raspberries 'Fertödi Zamatos', 'Glen Ample', 'Meeker', 'Polana', 'Tulameen' and 'Willamette'. Twenty-one isolates were selected for sequencing the portion of the nucleocapsid (NC) gene. The nucleotide sequences of the isolates showed 93.2−100% identity. Phylogenetic analysis confirmed significant genetic variability of the Serbian RLBV isolates based on the nucleocapsid-encoding sequences and revealed the existence of two main clusters. Phylogenetic analysis of the 45 RLBV sequences from Finland, Slovakia, Scotland, and this study also confirmed the existence of two main clusters of isolates.

**Additional keywords:** *Rubus idaeus* L.; RLBV; RT-PCR; molecular diversity, phylogenetic analysis.

**Abbreviations used:** ML (maximum likelihood); NC (nucleocapsid); RdRp (RNA-dependent RNA polymerase); RLBV (*Raspberry leaf blotch emaravirus*); RT-PCR (reverse transcription polymerase chain reaction); TNA (total nucleic acids).

**Authors' contributions:** DJ, SAP and AL collected samples and carried out the experiments. DJ and SAP analyzed the data and wrote the paper.

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## Introduction

Red raspberry (*Rubus idaeus* L.) and other *Rubus* species are hosts of more than 30 viruses and virus-like agents (Martin *et al*., 2013). Most of the viruses infecting raspberries do not cause symptoms on economically important cultivars, and only a few cause visible symptoms on different plant parts. Different types of symptoms may be expressed on the leaves and fruits of sensitive cultivars, particularly in mixed virus infections. Symptoms induced by viruses can be easily misinterpreted as symptoms caused by insects or other pathogens. Yellow patches and blotches on leaves were commonly found in red raspberry orchards and for a long time were described as infestation by the raspberry leaf and bud mite (*Phyllocoptes gracillis* Nal.) (Gratwick, 1992). *P. gracillis* feeds on raspberries, causing pale green and yellow patches and blotches, twisting and distortion of the leaf margins. The fruits or individual drupelets may also be affected. McGavin *et al*. (2012) reported the presence of a new negative-strand RNA virus in the red raspberry plants with leaf blotch symptoms. The virus was also detected in the raspberry leaf and bud mite, suggesting its vector role, and was named *Raspberry leaf blotch virus* (RLBV). RLBV belongs to the genus *Emaravirus* with a genome that is 17,410 nucleotides (nt) long and consists of eight segmented negative sense RNAs, each encoding a single open reading frame (McGavin *et al*., 2012; Lu *et al*., 2015). RLBV has been confirmed only in European countries: Great Britain, Serbia, Finland, Bulgaria, Montenegro and Poland (Jevremović *et al*., 2016).

Red raspberry is economically the most important small fruit in Serbian agriculture, ranking Serbia among the World's leading producers and exporters (Petrović *et al*., 2017). Raspberry viral diseases were not studied extensively in the past decades in Serbia and were not considered a significant threat to red raspberry production. Most of the detected viruses were causing latent infections or yellow mosaic symptoms (Dulić-Marković & Ranković, 1997). In the past decade, a mass occurrence of symptoms that include yellow blotching and twisting of raspberry leaves was noticed in orchards throughout Serbia. These symptoms were primarily attributed to infestation by the raspberry leaf and bud mite. After the detection of RLBV in symptomatic red raspberry samples from Serbia (McGavin *et al*., 2012) we started an intensive survey of the presence of RLBV in the country to determine the association between the expressed symptoms and viral infection.

The aim of our study was to evaluate the reliability of previously reported primer pairs for RLBV detection and to access the genetic diversity of the selected RLBV isolates from Serbia.

## Material and methods

### Plant material

Material for the study was collected from 2014 to 2017 from commercial red raspberry orchards and spontaneous flora at 30 localities in Serbia (Fig. 1). The majority of samples originate from orchards in Western Serbia, which is the most important production region in the country. In total, 94 samples from eight red raspberry cultivars ('Fertödi Zamatos', 'Glen Ample', 'Heritage', 'Meeker', 'Polana', 'Polka', 'Tulameen', and 'Willamette') and 2 samples of wild raspberry were collected (Table 1). Samples were stored at +4°C (1−2 day storage) and/or at -80°C (longer storage).


**Figure 1.** Map of Serbia showing sampling locations (indicated by black dots).

### TNA extraction

Total nucleic acids (TNA) were extracted from fresh or frozen leaves with a modified CTAB protocol described by Li *et al*. (2008). Leaf tissue (200 mg) was put in extraction bags and ground in 2 mL of 2% cetyltrimethylammonium-bromide (CTAB) buffer (2% CTAB, 2% PVP-40, 20 mM EDTA, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0 and 0.2% 2-mercaptoethanol prior to use). One mL of the extract was transferred to 2 mL tubes, incubated at 65°C for 15 min and centrifuged at 10,400 rpm for 5 min (Eppendorf 5415 R centrifuge, Germany). The supernatant (650 µL) was transferred to a new 1.5 mL tube and vortexed with the equal volume of 24:1 chloroform/isoamyl alcohol. The mixture was centrifuged at 12,800 rpm for 10 min. The obtained supernatant (500 µL) was transferred to a new 1.5 mL tube with 350 µL ice-cold isopropanol, mixed by pipetting and centrifuged at 12,800 rpm for 10 min. The aqueous phase was decanted and the remaining TNA pellet was washed with 1 mL ice-cold 70% ethanol by centrifugation at 12,800 rpm for 5 min, dried at room temperature and dissolved in 100 µL Tris-EDTA (TE) buffer (20 mM TRIS-HCl pH 8.0).

### Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription (RT) was performed with random hexamers and Maxima Reverse Transcriptase (Thermo Scientific, USA). The obtained cDNA was used as a template in separate PCR reactions with three different primer pairs specific to three virus RNAs (RNA-1, RNA-3, and RNA-5) designed by McGavin *et al*. (2012). Primer set 1499/1500 was used to amplify a 557 nt fragment of the RNA-dependent RNA polymerase (RdRp) region of RNA1. To amplify a fragment of 567 nt, primer set 1287/1095 of the nucleocapsid (NC) of RNA3 was used, while primer set 1571/1286 was used for the amplification of a 377 nt fragment of P5 of RNA5. PCR reactions were carried out in TPersonal thermal cycler (Biometra, Germany) using Taq DNA Polymerase (recombinant) (Thermo Scientific, USA). Amplified PCR products were analyzed by electrophoresis in 1.5% agarose gel and stained by ethidium bromide. Visualisation using

**Table 1.** Detection of RLBV using RT-PCR in red raspberry samples from Serbia.

| Sample | Isolate name[a] | Cultivar | Symptoms[b] | Locality | Year of collection | RT-PCR[c] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | RNA1 | RNA3 | RNA5 |
| 1 | RS-RLBV-2 | Willamette | YLB, LD | Radobuđa | 2014 | + | + | + |
| 2 | | Willamette | YLB | Trešnjevica | 2014 | - | - | - |
| 3 | | Willamette | YLB | Trešnjevica | 2014 | - | + | - |
| 4 | RS-RLBV-4 | Willamette | YLB | Trešnjevica | 2014 | + | + | + |
| 5 | | Willamette | VC | Trešnjevica | 2014 | - | - | - |
| 6 | RS-RLBV-7 | Willamette | YLB | Latvica | 2014 | - | + | + |
| 7 | | Willamette | YLB | Stupčevići | 2014 | + | + | + |
| 8 | | Willamette | VC | Vigošte | 2014 | - | - | - |
| 9 | RS-RLBV-9 | Meeker | CP | Ivanjica | 2014 | + | + | + |
| 10 | | Willamette | YLB | Ivanjica | 2014 | + | + | + |
| 11 | | Willamette | YLB | Ivanjica | 2014 | + | + | + |
| 12 | | Willamette | ns | Ivanjica | 2014 | - | - | - |
| 13 | | Wild raspberry | LC | Ivanjica | 2014 | + | + | + |
| 14 | RS-RLBV-14 | Willamette | YLB | Kotraža | 2014 | + | + | - |
| 15 | | Willamette | YLB | Trešnjevica | 2014 | - | + | - |
| 16 | | Willamette | CP | Zlodol | 2014 | - | + | - |
| 17 | | Willamette | LC | Ljubovija | 2014 | - | - | - |
| 18 | RS-RLBV-24 | Willamette | LC | Ljubovija | 2014 | + | + | + |
| 19 | | Willamette | LC | Kadinjača | 2014 | - | - | - |
| 20 | | Willamette | LC | Kadinjača | 2014 | - | - | - |
| 21 | | Willamette | LC | Stupčevići | 2014 | - | - | - |
| 22 | | Meeker | ns | Čačak | 2014 | - | - | - |
| 23 | | Willamette | ns | Stupčevići | 2014 | - | - | - |
| 24 | | Willamette | ns | Stupčevići | 2014 | - | - | - |
| 25 | | Polka | ns | Arilje | 2014 | - | - | - |
| 26 | | Meeker | ns | Gleđica | 2014 | - | - | - |
| 27 | | Willamette | ns | Arilje | 2014 | - | - | - |
| 28 | | Willamette | YLB | Gleđica | 2014 | - | + | - |
| 29 | | Willamette | YLB | Erčege | 2014 | + | + | - |
| 30 | RS-RLBV-38 | Meeker | YLB | Bratljevo | 2014 | - | + | - |
| 31 | | Meeker | ns | Bratljevo | 2014 | - | - | - |
| 32 | | Willamette | YLB | Rudno | 2014 | + | + | + |
| 33 | RS-RLBV-41 | Willamette | YLB | Rudno | 2014 | - | + | - |
| 34 | | Meeker | YLB | Kosjerić | 2014 | + | + | - |
| 35 | | Meeker | ns | Kosjerić | 2014 | - | - | - |
| 36 | | Willamette | YLB | Dučalovići | 2015 | + | + | - |
| 37 | | Willamette | YLB | Ivanjica | 2015 | + | + | - |
| 38 | | Willamette | YLB | Ivanjica | 2015 | + | + | - |
| 39 | | Willamette | YLB | Ivanjica | 2015 | + | + | - |
| 40 | | Willamette | ns | Ivanjica | 2015 | - | - | - |
| 41 | | Meeker | ns | Ivanjica | 2015 | - | - | - |
| 42 | | Meeker | YLB | Kriva Reka | 2015 | - | + | + |
| 43 | | Willamette | YLB | Kriva Reka | 2015 | + | + | + |

**Table 1.** Continued.

| Sample | Isolate name[a] | Cultivar | Symptoms[b] | Locality | Year of collection | RT-PCR[c] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | RNA1 | RNA3 | RNA5 |
| 44 | | Willamette | YLB | Prizren | 2015 | + | + | + |
| 45 | RS-RLBV-57 | Willamette | YLB | Prizren | 2015 | + | + | + |
| 46 | | Willamette | YLB | Prizren | 2015 | - | + | - |
| 47 | | Willamette | LY | Zaglavak | 2015 | - | - | - |
| 48 | | Willamette | YLB | Zaglavak | 2015 | + | + | - |
| 49 | RS-RLBV-61 | Willamette | YLB | Kadinjača | 2015 | + | + | - |
| 50 | | Willamette | YLB | Priboj | 2015 | + | + | - |
| 51 | | Willamette | YLB | Buar | 2015 | + | + | + |
| 52 | | Willamette | YLB | Hrta | 2015 | + | + | + |
| 53 | | Willamette | YLB | Hrta | 2015 | + | + | + |
| 54 | | Willamette | YLB | Hrta | 2015 | + | + | - |
| 55 | | Willamette | YLB | Hrta | 2015 | + | + | - |
| 56 | | Willamette | YLB | Hrta | 2015 | + | + | - |
| 57 | | Willamette | YLB | Stapari | 2015 | + | + | + |
| 58 | | Willamette | YLB | Stapari | 2015 | + | + | + |
| 59 | | Willamette | YLB | Gornja Lisina | 2015 | - | + | + |
| 60 | RS-RLBV-72 | Meeker | YLB | Vlasina | 2015 | + | + | + |
| 61 | | Wild raspberry | LC | Gornja Lisina | 2015 | - | - | - |
| 62 | RS-RLBV-74 | Glen Ample | YLB | Kostojevići | 2015 | + | + | + |
| 63 | | Glen Ample | YLB | Kostojevići | 2015 | + | + | + |
| 64 | RS-RLBV-76 | Meeker | YLB | Kostojevići | 2015 | + | + | - |
| 65 | | Polka | LY | Kostojevići | 2015 | - | - | - |
| 66 | | Polka | LY | Kostojevići | 2015 | - | - | - |
| 67 | RS-RLBV-81 | Willamette | YLB | Zarožje | 2015 | + | + | + |
| 68 | | Willamette | YLB | Zarožje | 2015 | + | + | + |
| 69 | | Polka | LY | Zarožje | 2015 | - | - | - |
| 70 | | Polka | LY | Zarožje | 2015 | - | - | - |
| 71 | | Meeker | YLB | Jošanička Banja | 2015 | + | + | + |
| 72 | RS-RLBV-85 | Fertödi Zamatos | YLB | Gliječa | 2015 | + | + | - |
| 73 | | Fertödi Zamatos | LC | Gliječa | 2015 | - | - | - |
| 74 | | Willamette | YLB | Radmanovo | 2015 | + | + | - |
| 75 | | Willamette | YLB | Bresnik | 2015 | + | + | - |
| 76 | | Willamette | ns | Rudno | 2015 | + | + | + |
| 77 | RS-RLBV-104 | Willamette | YLB | Rudno | 2015 | + | + | + |
| 78 | | Willamette | ns | Sevojno | 2015 | - | - | - |
| 79 | | Willamette | ns | Sevojno | 2015 | - | - | - |
| 80 | | Willamette | YLB | Milatovići | 2015 | + | + | + |
| 81 | | Willamette | YLB | Jošanička Banja | 2015 | + | + | + |
| 82 | | Willamette | YLB | Jošanička Banja | 2015 | + | + | + |
| 83 | | Fertodi Zamatos | ns | Ivanjica | 2015 | - | - | - |
| 84 | | Fertodi Zamatos | ns | Ivanjica | 2015 | - | - | - |
| 85 | | Heritage | ns | Ivanjica | 2016 | - | - | - |
| 86 | | Polana | YLB | Bačko Dobro Polje | 2016 | + | + | + |
| 87 | | Willamette | YLB | Kriva Reka | 2016 | + | + | + |
| 88 | RS-RLBV-132 | Willamette | YLB | Kriva Reka | 2016 | + | + | + |
| 89 | | Willamette | YLB | Kriva Reka | 2016 | + | + | + |
| 90 | RS-RLBV-134 | Willamette | YLB | Jelakci | 2016 | + | + | + |

**Table 1.** Continued.

| Sample | Isolate name[a] | Cultivar | Symptoms[b] | Locality | Year of collection | RT-PCR[c] | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | RNA1 | RNA3 | RNA5 |
| 91 | RS-RLBV-138 | Willamette | YLB | Brus | 2016 | + | + | + |
| 92 | RS-RLBV-146 | Willamette | CP | Kraljevo | 2016 | + | + | + |
| 93 | RS-RLBV-147 | Willamette | YLB | Kraljevo | 2016 | + | + | + |
| 94 | | Willamette | YLB | Jelakci | 2017 | + | + | + |
| 95 | | Willamette | YLB | Jelakci | 2017 | + | + | + |
| 96 | | Willamette | YLB | Brus | 2017 | + | + | + |

[a]Only names of the sequenced isolates are given. NCBI accession numbers are given in Fig. 3. [b]YLB: yellow leaf blotch; LD: leaf distortion; VC: vein chlorosis; LC: leaf chlorosis; LY: leaf yellows; CP: chlorotic patches; ns: no symptoms. [c] +: positive PCR result; - negative PCR result.

a UV tray and imaging was performed with the Gel Doc EZ System (Biorad, USA). The presence of a fragment of the expected size in each PCR reaction was considered as a positive reaction.

### Sequence analysis

PCR products obtained with primer pair 1287/1095 specific to RNA3 of 21 isolates were custom sequenced with an ABI3730XL sequencer by Macrogen Europe (The Netherlands). The isolates for sequencing were selected based on the host cultivar, locality, and the type of the symptoms. Raw sequences were assembled with BioEdit 7.0.5.3 software (Hall, 1999). Phylogenetic relationships were reconstructed by a Maximum Likelihood (ML) tree. The Tamura 3-parameter model of nucleotide substitution with gamma-distributed rate variation across sites (T92+G) was selected as a best-fit model based on the Bayesian Information Criterion (BIC) with MEGA7 software (Kumar *et al*., 2016). The phylogenetic trees were generated using ML method with 1,000 bootstrap replications.

## Results

A total of 96 samples of raspberry leaves were analysed for the presence of RLBV using three recommended RNA-specific RT-PCR primer pairs for RLBV detection targeting RNA1, RNA3, and RNA5 (McGavin *et al*., 2012). RT-PCR results are presented in Table 1. Significant differences were shown in the efficiency of the used primer pairs. RLBV RNA 1 was detected in 56 samples (58.3%), RNA 3 in 66 (68.7%), and RNA5 in 41 (43.7%). Primer pair 1287/1095, targeting RNA3, proved to be the most efficient for RLBV detection in red raspberry samples from Serbia. Primer pairs targeting RNA1 and RNA5 failed to detect RLBV in 10 and 24 samples, respectively. RLBV was confirmed in 65 samples with leaf blotch symptoms, and only in 1 sample from the asymptomatic raspberry 'Willamette'. RLBV was confirmed in raspberries 'Fertödi Zamatos', 'Glen Ample', 'Meeker', 'Polana', 'Tulameen', and 'Willamette', but not in 'Heritage', 'Polka', and in two analysed wild raspberries with leaf chlorosis. There was no specific association between the type and intensity of the symptoms with the host cultivar. Mild or severe mottling and yellow blotches are the symptoms that were commonly found in all infected cultivars. RLBV presence was not confirmed in 31 samples, of which 16 were symptomless, and in 15 samples showing leaf yellows, leaf chlorosis, and vein chlorosis.

Nucleotide sequences of the 567 bp fragment of the nucleocapsid were determined for 21 selected isolates from 4 cultivars: 'Fertödi Zamatos' (1), 'Glen Ample' (1), 'Meeker' (4), and 'Willamette' (15). Nucleotide sequences were deposited in the GenBank sequence database (https://www.ncbi.nlm.nih.gov/) and assigned the accession numbers MF136659−MF136679. The nucleotide and amino acid sequences of the analysed Serbian isolates were 93.2−100% and 94.1−100% identical, respectively. Three sequenced isolates (RS-RLBV-4, RS-RLBV-104, and RS-RLBV-146) showed identical nt sequences. Isolates RS-RLBV-138 and RS-RLBV-147 showed the lowest percentage of nucleotide identity (93.2%). Phylogenetic analysis of the sequences of the Serbian isolates revealed the existence of two groups of isolates (Fig. 2).

Serbian isolates were then aligned with the available RLBV sequences retrieved from the GenBank sequence database including 21 isolates from Finland (Acc. JQ684678, KP644139−45 and KP730587−99), two from Slovakia (Acc. KY513312−13) and one from Scotland (Acc. FR730598). In total, 45 nt sequences were analysed. A reconstructed phylogenetic tree using the Maximum Likelihood method based on the

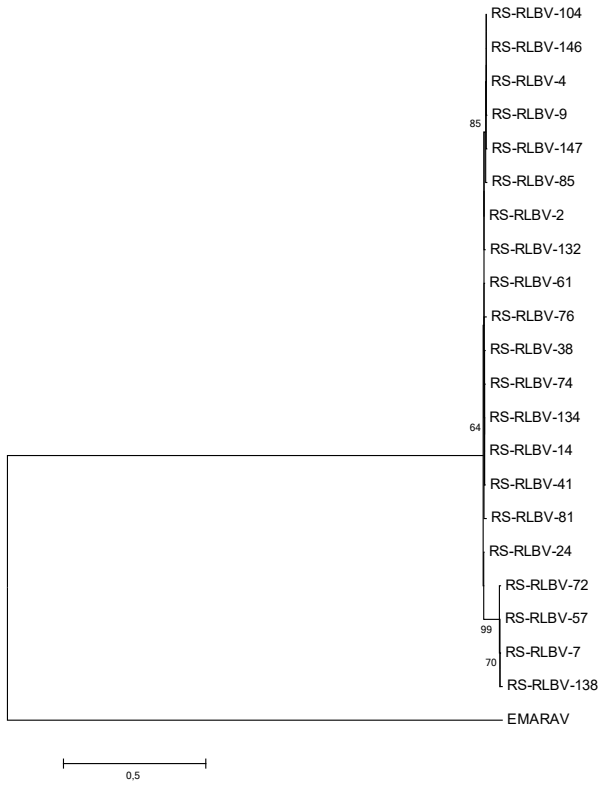


**Figure 2.** Phylogenetic analysis of partial nucleocapsid protein gene sequences of 21 Serbian RLBV isolates. Bootstrapped Maximum likelihood (ML) tree using Tamura 3-parameter method. Only bootstrap values of 50% and above calculated from 1,000 replications are shown at nodes. The tree was rooted with *European mountain ash ringspot-associated virus* (EMARAV) [GenBank accession NC_013108] as an outgroup.

Tamura 3-parameter model with gamma distributed sites revealed the existence of two clusters of closely related isolates: cluster I included 17 isolates from Serbia, 19 from Finland and 2 from Slovakia, while cluster II included 4 isolates from Serbia, 2 from Finland and 1 from Scotland (Fig. 3).

Using the sequences of 21 Serbian and 21 Finnish isolates retrieved from the GenBank database, genetic distances were estimated by implementing the Tamura 3-parameter model with MEGA7 software. Standard error estimates were obtained by a bootstrap procedure (1,000 replicates). The results showed that the overall mean value of nucleotide diversity was 0.026±0.004 (the number of base substitutions per site from averaging the overall sequence pairs±standard error estimate). The value of nucleotide diversity of 21 Serbian RLBV isolates was 0.026±0.004, and was 0.024±0.004 for 21 Finish isolates. The value of nucleotide diversity between groups of Serbian and Finnish RLBV isolates was 0.027±0.004.

# Discussion

The presence of RLBV was confirmed in several European countries with more or less significant raspberry production (Jevremović *et al*., 2016). Raspberry leaf blotch symptoms were noticed in raspberry orchards in Serbia more than five decades ago and were in general attributed to the feeding damages caused by the raspberry leaf and bud mite (Dobrivojević & Petanović, 1985; Milenković & Marčić, 2011). The first RLBV-infected red raspberry samples in Serbia were reported by McGavin *et al*. (2012), but with no further details on the analysed samples. After this report, a large-scale study on the RLBV presence and distribution in Serbia has begun. The first analysis for RLBV presence in Serbia was performed by RT-PCR using specific primers for RNA5 (McGavin *et al*., 2012). These primers failed to detect RLBV in numerous samples with typical raspberry leaf blotch disease symptoms. Therefore, a part of this study was to evaluate the reliability of the different primer pairs for RLBV detection. Primer pair 1287/1095 targeting RNA3 proved to be the most efficient among the three primer pairs used for RLBV detection in red raspberry samples from Serbia and should be further used as the preferred primer pair for RLBV detection in the country. RNA3 was also recommended as the preferred target for RT-PCR diagnostics in the study of Dong *et al*. (2016). The RT-PCR detection with other primer pairs used in our study was less reliable, especially with the primer pair used for the detection of RNA5 (Table 1). RNA5 specific primers failed in the RLBV detection in 24 samples that were positive with RNA3 primers. The high diversity of Serbian isolates in the region targeted by RNA5 primers may provide an explanation for the obtained results. Also, another possibility is that some RLBV isolates do not contain RNA5 (Dong *et al*., 2016). The RNA 5 and its encoded protein P5 are unique to RLBV and some other *Emaraviruses* (McGavin *et al*., 2012; Kumar *et al*., 2017). Possible functional role(s) of this viral protein are still unknown.

Our results confirmed the presence of RLBV in six cultivars in all surveyed localities and the strong association of leaf blotch symptoms with RLBV. The red raspberry 'Willamette' is the predominant floricane cultivar grown in Serbia for decades, with a 90% share of production. 'Meeker' accounts for 5%, with all other cultivars combined. In the past several years, many growers reported a high incidence of severe leaf blotch symptoms and significant yield decrease in 'Willamette' and 'Meeker' orchards grown according to organic farming. RLBV was confirmed in all analysed symptomatic samples from these orchards (Jevremović,

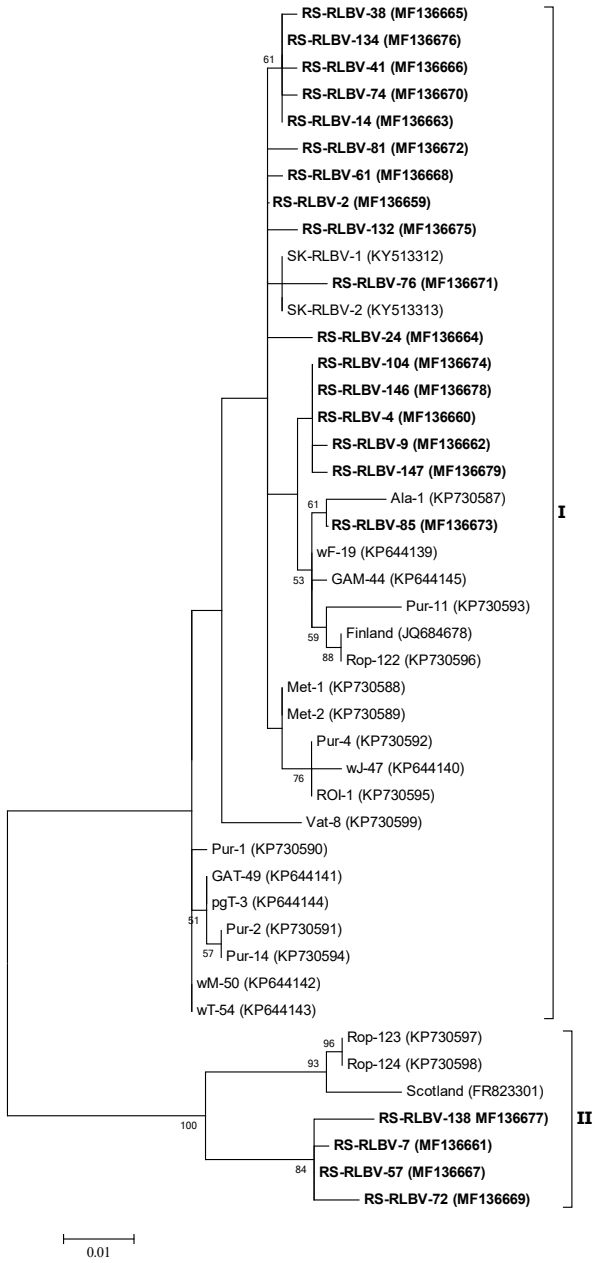


**Figure 3.** Phylogenetic analysis of 45 RLBV sequences of isolates from Serbia, Finland, Slovakia and Scotland reconstructed from nucleocapsid gene sequences. Serbian isolates are presented in bold. The analysis was performed with MEGA7 software using Maximum Likelihood (ML) method based on the Tamura 3-parameter model with gamma distributed sites (T92+G). Phylogeny was inferred after 1,000 bootstrap replications, and bootstrap support of 50% and above are shown at nodes. The NCBI accession numbers are in parentheses.

unpublished results). Due to the forbidden use of common acaricides, the control of raspberry leaf and bud mite in organic farming is difficult.

Nucleotide sequence analysis of the selected isolates from different cultivars and locations revealed considerable nucleotide diversity among the isolates. The high value of genetic diversity may suggest the long-term presence of the RLBV in Serbia. Compared to the RLBV sequences from other countries, Serbian isolates showed the highest nucleotide identity with the Slovakian isolates. Isolate RS-RLBV-76 share 99.5% of the nt identity with the Slovakian isolates SK-RLBV-1 and SK-RLBV-2.

Phylogenetic analysis of the partial nucleocapsid protein gene sequences of 45 RLBV isolates from Serbia, Finland, Slovakia, and Scotland confirmed the existence of two clusters of isolates. The reconstructed ML tree did not provide strong evidence for a specific clustering of RLBV isolates in clusters I and II according to geographical origin (Fig. 3). However, some clustering at a smaller spatial scale was detected in some cases, but not supported by bootstrap analysis. There are no further specific clustering of the Serbian isolates based on the local geographic origin or host cultivar. This may indicate the intense gene flow within the country through the latent infections of the planting material, and further virus transmission by the raspberry leaf and bud mite that was described as the most important secondary pest in Serbian commercial raspberry orchards (Milenković & Marčić, 2011). Three isolates (RS-RLBV-4, RS-RLBV-104, and RS-RLBV-146) were with identical nt sequences. These isolates were sampled from locations that are 40−50 km from each other. On the contrary, the most divergent isolates (RS-RLBV-138 and RS-RLBV-147) were located at approximately the same distance (50 km) in the same region. The practice of using the planting material from commercial orchards may contribute to the dispersal of RLBV isolates to close and distant areas.

RLBV is a pathogen that is not monitored during the production of the planting material, and not listed in the recommendations for the production of healthy planting material given by the EPPO (OEPP/EPPO, 2009). Therefore, it may be distributed via the infected material within and between countries. After RLBV discovery and its confirmed presence in several European countries, it should be expected that RLBV will be incorporated in the next revised version of the EPPO certification scheme for *Rubus* species.

The molecular analysis and characterisation done in this study provides information regarding the reliable detection of *Raspberry leaf blotch emaravirus*, and confirmed its wide distribution and significant genetic diversity in Serbia. Research into the impact of RLBV infection on vegetative growth and yield is underway.

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