Original Scientific paper
10.7251/AGRENG2101019P
UDC 578.5:634.711(477)

PHYLOGENETIC ANALYSIS OF UKRAINIAN ISOLATE OF *RASPBERRY LEAF BLOTCH VIRUS*

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ABSTRACT

Raspberry leaf blotch virus (RLBV) is a new member of the *Emaravirus* genus (*Fimoviridae*, *Bunyavirales*). Representatives of this family are characterized by a segmented "-" RNA genome, helical nucleocapsid and enveloped spherical or pleomorphic virions 80-120nm in diameter. Transmission of these viruses is carried out by eriophyid mites. The virus is widely distributed in Europe. In this paper, the phylogenetic relationships between the Ukrainian RLBV isolate and isolates from the other countries from the GenBank database were investigated. Samples of symptomatic raspberry plants were selected for the work. Total RNA was isolated and RT-PCR was performed using primers to the region of the nucleocapsid protein (P3) gene. The resulting amplicon with a length of about 500bp was sequenced. Sequences were analyzed using BLAST and MEGA7 programs. As a result of BLAST analysis, it was shown that the Ukrainian isolate of RLBV has a high similarity to some Finnish, British, Serbian and Slovak isolates (93-99% similarity). Nevertheless, the dendrogram constructed in MEGA7 did not distribute these isolates in a separate cluster. Interestingly, sequences of isolates from Finland, Britain, and the Balkans were segregated into different parts of the phylogenetic tree. This pattern can be explained by the low divergence of the virus population or small number of isolates in the database.

Keywords: *Raspberry leaf blotch emaravirus, raspberry, Ukraine.*

INTRODUCTION

Raspberry leaf blotch virus (RLBV) is a novel member of the *Emaravirus* genus characterized with spherical or pleomorphic enveloped virions and segmented negative-sense RNA genome. There are 8 segments: RNA1 encodes an RNA-dependent RNA polymerase (RdRP), RNA2 –glycoproteins precursor (GPs), RNA3 – a nucleocapsid protein (NC), RNA4 – movement protein (Yu *et al*., 2013) and functions of the rest 4 related segments remain unclear (Lu *et al.*, 2015). RLBV is transmitted exclusively by Eriophyid mite *Phyllocoptes gracilis* and was initially described from raspberries as the pathogen associated with leaf blotch disorder (RLBD) in Great Britain (McGavin *et al.* 2012). This virus was also found in Finland (Bi *et. al.*, 2012), Bulgaria (Mavrič *et al.*, 2014), Poland (Cieslinska *et*

al, 2014), Montenegro (Zindović *et al.*, 2015), Serbia (Jevremovic *et al.*, 2019), Bosnia and Herzegovina (Delic *et al.*, 2020), France (Marais *et. al*, unpublished). The virus is considered to be a serious threat for raspberry plantations in Europe (Delić *et al.*, 2020). RLBV is the first emaravirus detected in Ukraine (Pozhylov *et al.*, 2018). In view of the growing export of Ukrainian raspberries (Ministry of Agrarian Policy and Food of Ukraine, 2018), economical importance of this fruit rises, increasing the neediness of raspberries' viral diseases study. The development of modern virology is closely linked to the use of molecular methods. Nowadays, it is possible to predict viral characteristics on the basis of already known data, using only information about its nucleotide or amino acid sequences. Thus, it can be said that the use of molecular phylogeny can be one of the most convenient tools for studying the epidemiological and biological properties of viruses that have recently been discovered. Polymerase chain reaction (PCR) is a rapid and convenient tool for detection of plant viruses (Uyeda and Masuta, 2015). In this paper molecular methods were used to detect RLBV on raspberries in Ukraine and investigate phylogenetic relationships of its novel Ukrainian isolate.

MATERIAL AND METHODS

In summer 2017, samples were collected from symptomatic raspberries from several locations in Kyiv city and Kyiv region (Ukraine). Total RNA was extracted using Ambition PureLink™ RNA mini kit (Invitrogen, USA) following the manufacturer's instructions. Then the samples were tested for RLBV by RT-PCR using primers pair 1287 (forward):5′-ATCCAGTAGTGAACTCC-3′and 1095(reverse):5′-CACCATCAGGAACTTGTAATGTTT-3′ (Lu *et al*., 2015), which is specific to the nucleocapsid (NC) protein gene and targets a 570 bp fragment. Reverse-transcription was performed using 3 mcl of total RNA and RevertAid Reverse Transcriptase (Thermo Scientific, Lithuania) following the manufacturer's instructions. The PCR was performed using PCR Master Mix (Thermo Scientific, Lithuania) following the next procedure: denaturation at 73° for 5 min, 35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 45 s, and final extension at 72°C for 5 min. The products of total RNA extraction and PCR were checked by horizontal gel electrophoresis using 1,5% agarose (Ultrapure agarose Gibco BRL, Life Technologies, USA) in Tris-borate-EDTA buffer, and GeneRuler 1 kb DNA ladder (Thermo Scientific, Lithuania). RT-PCR products were purified using QIAquick Gel extraction kit (Qiagen, Germany), and then sequenced using Applied Biosystems 3730 x 1 DNA Analyzer. The resulting sequences were aligned using ClustalW and compared using BLAST analysis and Sequence Demarcation Tool Version 1.2 (SDTv1.2, Muhire *et al.*, 2014). Phylogenetic properties were elucidated using MEGA7 software (Kumar *et al*., 2016). Obtained plots and matrices were subsequently processed using Microsoft Windows XP Paint 6.1 (Microsoft, USA) and Origin 9 (OriginLab Corporation, USA).

RESULTS AND DISCUSSION

Several samples with symptoms of RLBD (wide chlorotic spots on the leafs, leaf deformation) were collected from Khmelnytskyi, Kyiv and Vinnytsia regions. RT-PCR using total RNA samples purified from symptomatic leafs (Figure 1A) has yielded positive results (Figure 1B). After wards the products were reamplified and prepared for the following sequencing. The product of expected size (about 500 bp) was sequenced and obtained sequence was deposited in the GenBank (MK123270.1). BLAST analysis of obtained 503 bp fragment of NC gene of RLBV showed its high identity to isolates from Bosnia and Herzegovina, Britain, Slovakia and especially identity to some isolates from Serbia and Finland (up to 99%). To compare our isolate with the others available from the GenBank, pairwise identity matrices were obtained using SDTv1.2. For higher readability and elimination of sequences with low coverage, only 18 isolates of 85 available from the GenBank are shown in the matrices. Initially, a phylogenetic tree of RLBV NC sequences available from the GenBank was built. For better topology and repeatability only 79 out of 85 were used in the mentioned tree. Sequences that represent each country in each branch of a dendrogram generated from 79 suitable for the analysis RLBV isolates were chosen for displayed phylogenetic tree. As expected, identity between amino acid sequences was much higher (Figure 2, the upper right corner) due to synonymous substitutions.

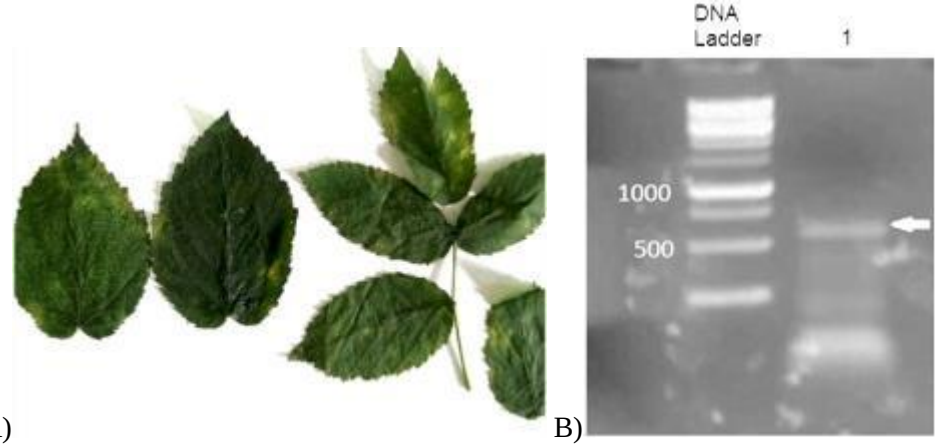

Figure 1: A) Symptomatic raspberries leafs. B) PCR product of 500+bp

The general appearance (two triangles separated with a single rectangle composed of different colors) of mentioned identity matrix (Figure 2, the lower left corner) indicated the presence of two groups of different sequences in our datasets.

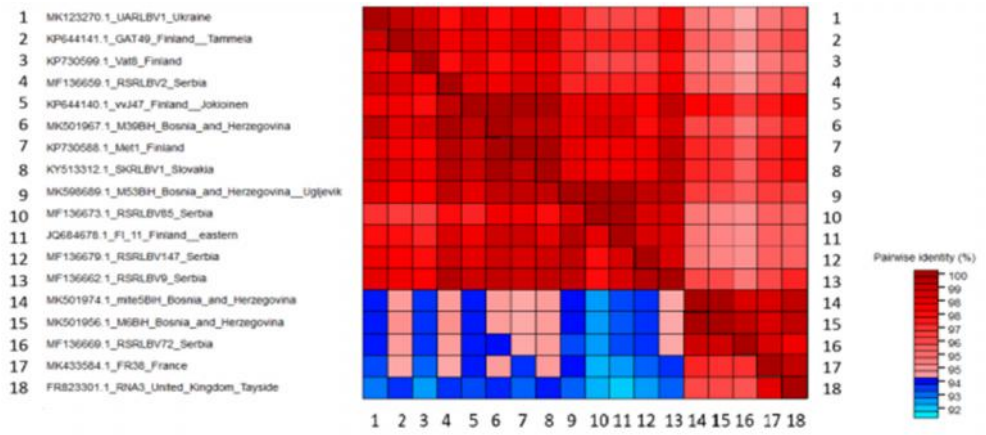

Figure 2: Color-coded pairwise identity matrices generated from 18selected RLBV sequences. Distance matrices obtained with SDTv1.2, and merged in MS Paint. The lower left corner: comparison of RLBV partial NC nucleotide sequences. The upper right corner: comparison of RLBV amino acid sequences translated from nucleotide sequences using MEGA7.

Also, pairwise identity frequency distribution plots were built to achieve a deeper insight into RLBV sequences identity. Comparison of a plot generated from 79 RLBV nucleotide sequences with a plot generated from 79 RLBV amino acid sequences showed that the first one had its main peak between 90 and 95% of pairwise identity (Figure 3, the black line), while the second one had its peaks after 95% of pairwise identity (Figure 3, the red line), which clearly proved our previous statement about higher identity of amino acid sequences. However, there were two peaks on both plots suggesting at least two clusters on phylogenetic tree generated from RLBV deduced amino acid sequences. To conclude, both plots showed high level of identity between isolate sequences.

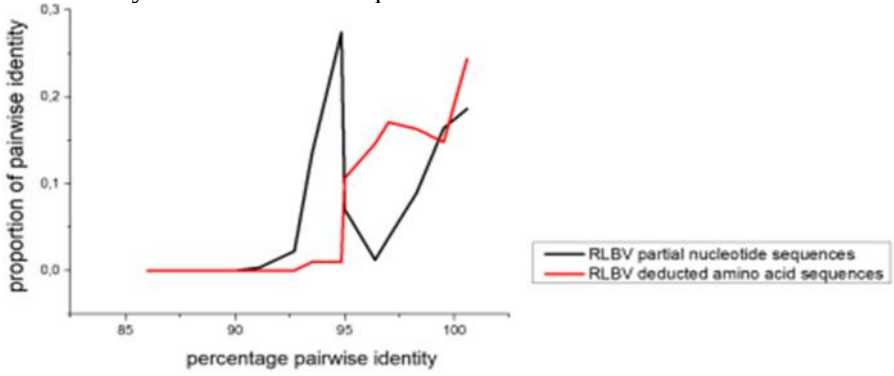

Figure 3:Pairwise identity frequency distribution plot generated from 79 RLBV partial nucleotide sequences and RLBV amino acid sequences, translated from partial NC nucleotide sequences using MEGA7. The initial data were obtained using SDTv1.2, and the plot was built using Origin 9.

Amino acid sequence-based dendrogram (Figure 4) shows division of isolates into two clusters, as predicted by the identity matrices and in accordance with previous studies (Jevremovic *et al*., 2019). Phylogenetic analysis showed the attribution of the Ukrainian RLBV isolate into cluster I. Also, there is a special clade in the cluster I formed by Ukrainian RLBV isolate and the most similar isolates from Finland, which may indicate common past events in virus evolution.

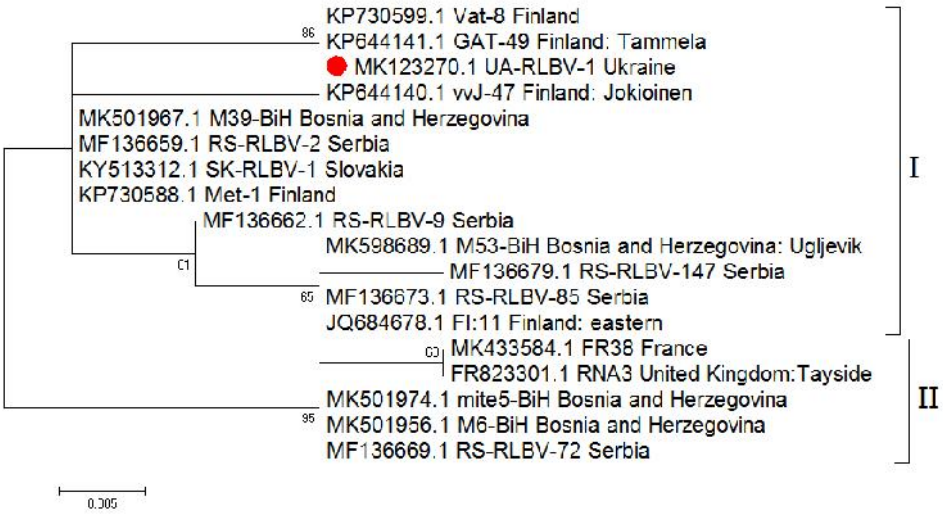

Figure 4: Phylogenetic analysis of 18 RLBV partial NC amino acid sequences, translated from partial NC nucleotide sequences using MEGA7. Ukrainian isolate is marked with a red dot. The analysis was performed with MEGA7 using Maximum Likelihood method. Bootstrap Replications – 1000. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Bar represents branch lengths measured in the number of substitutions per site.

As expected, synonymous substitutions prevailed over non synonymous ones, leading to higher identity of amino acid sequences of the isolates as compared to their nucleotide sequences, which was in line with general concepts.

Segregation of isolates in two clusters wasn't related to their country of origin. It can be explained with the exchange of planting material between countries with following propagation of the virus by eriophyid mite. This can explain the presence of isolates with high identity (98,6–99,2%) in Ukraine and Finland, Serbia, Slovakia. Also, there was no significant difference between some isolates even from different clusters. Probably, there are not enough isolates in the GenBank and the whole picture of RLBV distribution in Europe remains unclear. It would be useful to have the sequences of other fragments of viral genome to know the number of the genome fragments because not every isolate contains all of them (in particular, RNAs 5-8) (Jevremovic *et al.*, 2019). Additionally, identity of RNAs 1-3 can be used as taxa demarcation criteria for members of order *Bunyavirales* (ICTV, 2020).

Sequences of all of genome fragments can be used to study reassortments, which can occur in emaraviruses (Patil *et al.*, 2017). As for now, it is difficult to reconstruct the pattern of RLBV spread between the countries. Our further research will include more regions of Ukraine and will bring us more information about distribution and diversity of RLBV, its impact on raspberry yield and probable ways of transfer through the country and abroad.

CONCLUSIONS

Ukrainian RLBV isolate was detected on a symptomatic raspberry plant using molecular methods, partially sequenced and deposited to the GenBank (accession number: MK123270.1). The analysis of RLBV nucleocapsid gene sequences revealed little differences between them that can be visualised with a pairwise identity matrix or a phylogenetic tree, which clustered a RLBV isolate from Ukraine with isolates from Slovakia and some isolates from Bosnia and Herzegovina, Finland and Serbia. The presence of RLBV isolates sequences from different countries in one cluster and sequences from one country in different clusters can be explained with high homogeneity of viral population due to natural properties of the virus and the vector or small number of sequences in the database and their short length. Still, future research needed to accumulate more sequences of this and other fragments of RLBV genome.

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