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Phylogeny of *Prunus necrotic ringspot virus* cherry variants based on RNA 3 coding region

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Abstract

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Prunus necrotic ringspot virus (PNRSV) is an important pathogen of stone fruit species (*Prunus* sp.) including sweet (*Prunus avium*) and sour cherry (*Prunus cerasus*). In present study twenty seven cherry variants of PNRSV from three regions of Bulgaria were molecularly studied based on the nucleotide and amino acid sequences of the coat protein (CP) and the movement protein of RNA3 coding region. Fifteen more variants were studied only in the CP genome region. Phylogenetic analyses of the two genome regions revealed the formation of the four well-defined phylogroups PV32-I, PV96-II, PE5-III and CH30. Based on the nucleotide and amino acid sequences of CP genome region twenty-five and sixteen variants belonged to PV96-II and to PV32-I phylogroups, respectively. With one exclusion phylogenetic analyses based on the nucleotide and amino acid sequences of the MP genome region showed the same relationships among studied variants observed in the CP genome region. The comparison of CP and MP amino acid sequences revealed that the studied variants in each of the two groups, PV96-II and PV32-I had phylogroup-conserved amino acid residues. The results obtained showed a correlation between the phylogeny based on the nucleotide and amino acid sequences of RNA3 genomic region and the described for infected with PNRSV cherry trees pathotypes, known as rugose disease and mild mottle virosis. The specific amino acids present both in the CP and MP genome regions of all studied cherry PNRSV variants together with the symptoms on naturally infected cherry trees corresponded to the mild virosis pathotype.

Keywords: cherry; RT-PCR coat protein; movement protein; phylogenetic analyses

Introduction

Prunus necrotic ringspot virus (PNRSV) is a member of the genus *Ilarvirus* in the family *Bromoviridae* and its genome is organized into three single-stranded, plus-sense RNAs. RNA1 (3.332 nt) and RNA2 (1.943 nt) encodes for replicase proteins P1 and P2, respectively. The RNA3 (1.951 nt) encodes a 5'-proximal movement protein (MP) and a 3'-proximal coat protein (CP), which is expressed via a subgenomic RNA4 (Guo et al., 1995; Sanches-Navaro & Pallas, 1997).

PNRSV has a worldwide distribution and besides the apple, hop and rose infects also many *Prunus* spp. as the peach, nectarine, cherry, apricot, almond and plum trees (Fulton, 1983; Mink, 1992). The virus is transmitted by pollen, seed and the routine vegetative propagation methods (George & Davidson, 1963). The symptoms caused by PNRSV may depend to the virus isolate, host species and/or the cultivar and climatic conditions. PNRSV infection in cherry can be latent or symptomatic consisting of smaller leaves, diffused chlorotic rings and/or spots, necrotic lesions or spots, and the 'tattered' leaves (Uyemoto & Scott, 1992; Jones & Sutto, 1996).

PNRSV exists as many serologically and biologically distinct strains or isolates (Barbara et al., 1978; Crosslin & Mink, 1992). The cherry isolates of PNRSV were classified into the three serotypes CH3, CH9 and CH30, and the two pathotypes known as rugose mosaic disease and mild mottle virosis (Mink et al., 1987).

Numerous PNRSV isolates have been characterized by comparison of the nucleotide and amino acid sequences of the coat protein and the movement protein genes and phylogenetically grouped into the four groups named PV32-I, PV96-II, PE5-III and CH30 (Scott et al., 1998; Aparicio et al., 1999; Vaskova et al., 2000; Glasa et al., 2002). Presence of correlation between symptoms severity and the primary sequences of the MP or CP genes was observed in the case of PNRSV isolates from cherry (Hammond & Crosslin, 1998), while for PNRSV from other hosts a lack of association between molecular variability, symptomatology and geographical region was reported (Aparicio et al., 1999; Vaskova et al., 2000; Aparicio et al., 2002).

As a continuation of our work in respect to the occurrence of *Ilarvirus* infections of sweet and sour cherry in Bulgaria we investigated and characterized a number of PNRSV variants based on the sequences of CP and MP genes, thus enhancing the knowledge of their molecular variation.

Materials and Methods

Virus material and serological detection

Leaf samples from sweet and sour cherry trees showing and not showing symptoms were collected in early springs of the period of 2018-2020 from three regions of the country. In total 1917 and 972 samples from sweet and sour cherry, respectively were tested for PNRSV, *Prune dwarf virus* (PDV) and *Apple mosaic virus* (ApMV) by double antibody sandwich-immunosorbent assay (DAS-ELISA) (Clark & Adams, 1977). Standard protocol for each virus was followed as given by the manufacturer (Loewe Phytodiagnistica Gmbh). Both, specific and conjugated antibodies were used in quantity of 100 µl and incubated at 37ºC for 4h. 150 µl of diluted 1:20 extracts (w/vol) were loaded in duplicate wells of polystyrene microtiter plate and incubated overnight at 4ºC. Between each step the plate was washed 3 times with PBS-T (phosphate-buffered saline-Tween). 100 µl freshly prepared p-nitrophenylphosphate in substrate buffer (1mg/ml) were loaded to each well. The plate was incubated at room temperature and photometric measurement was done at 405 nm after 2 h. Samples were considered as positive if their absorbance values were more than three times higher of the negative control.

Total RNA extraction

Total nucleic acids were extracted from approximately 100 mg fresh leaves by the use of a commercially available RNeasy Plant Mini extraction kit (Qiagen, Germany). The RNA samples were dissolved in 50 µl of RNase-free water and used as template to generate the single stranded copy DNA (cDNA). All RNA samples were stored at -70ºC.

cDNA synthesis and PCR amplification of the coat protein and the movement protein genes

The CP and the MP genes of PNRSV variants were amplified by two-step reverse transcription-polymerase chain reaction (RT-PCR) method using Axygen Maxygene II thermal cycler. The two sets of specific primers used for the amplification of the CP were as follows: primer I (antisense): 5'-TCACTCTAGATCTCAAAAGCAG-3' / primer II (sense) 5'-GAGCTCTGGTCCCACTCAGG-3' (Spiegel et al., 1999) and R4 (antisense): 5'-AGTGTGCTTATCTCACTCTAG-3' / F3 (sense): 5'-ATGGTTTGCCGAATTTGCAATCAT-3' (Paduch-Cichal & Sala-Rejczak, 2011). The expected products for the mentioned above sets of primers were 616 bp and 700 bp amplicons, respectively. For primer set I/II the initial denaturation was performed at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 60 s. For the primer set F3/R4 the thermocycling was carried out as follows: initial denaturation at 94°C for 2 min, than 35 cycles of 94°C for 10 s, 53°C for 30 s and 72°C for 40 s. The final polymerization for the two sets of primers was for 5 min at 72°C.

The 900 bp fragment of MP gene was amplified using the sense primer: 5'-GTTGGTTGAATAGTGTTTCAGTATGGCC-3' and the antisense primer: 5'-AGCGTGGGTATGATTGCAAATTCGG-3' (Scott et al., 1998). PCR for the MP gene was performed for 2 min at 94°C, followed by 40 cycles at 94°C for 10 s, 62°C for 30 s and 72°C for 90 s and final extension for 10 min at 72°C.

Amplified products (5 µl each) were stained with GelRed dye (stock solution 10 000x) (Biotium) and electrophoresed at 80–140 V for 1–1.5 h in 1.2% agarose gels in 40 mMol/L Tris-acetate and 1 mMol/L EDTA, pH 8.0 (TAE).

Nucleotide sequencing and phylogenetic analyses

Following amplification, the PCR products were purified using IllustraTM GFXTM PCR DNA purification system (Healthcare, UK) and custom sequenced in both directions with the same oligonucleotides used for PCR (Eurofins, Germany). The resulting sequences were validated with the blastn and blastp programs of the National Center for Biotechnology Information, Bethesda, MD, USA and deposited

in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank) (accession no. in Table 1).

Multiple nucleotide and amino acid alignments were conducted using ClustalW 1.8 (Higgins et al., 1994). Phylogenetic trees were generated from nucleotide and amino acid alignments of the CP and MP sequences of PNRSV variants by the neighbor-joining (NJ) method with Kimura two parameter using Molecular Evolutionary Genetics Analysis (MEGA) software program version 6 (Tamura et al., 2013). The statistical significance of the clusters and branches of the phylogenetic tree was tested by 'bootstrap' analyses with 1000 replications. Bootstrap values below 70% were collapsed. Corresponding sequences of PNRSV representatives available from GenBank database were used as references and their accession numbers are as follows: PV0096 (S78312.1), PE5 (L38823) (Guo et al., 1995), PV32 (Y07568) (Sanchez-Navarro & Pallas, 1997), CH30 (AF034994), CH9 (AF034992), CH38 (AF034991), CH39 (AF034990), CH57 (AF034993), CH61 (F034989), CH71 (AF034995) (Hammond & Crosslin, 1998) and ChrIt.bla 1 [AJ133210 (CP); AJ306821 (MP)] (Aparicio et al., 1999).

Results and Discussion

Virus material and serological detection

DAS-ELISA results showed that from totally tested 2889 от leaf samples from sweet and sour cherry trees 710 (24.5%) reacted positive for PNRSV and PDV. ApMV was not detected confirming its absence as previously reported for several surveyed regions of the country (Kamenova et al., 2020). A higher rate of infection (27.2%) was found in sweet cherry, than in sour cherry (19.6%). PNRSV was the most prevalent in sour cherry (80.1%), while PDV was the most prevalent in sweet cherry (72.4%). The number of infected with a mix of the two viruses tested samples was higher in sour cherry (13.5%), than in sweet cherry (2.3%).

The origin and the symptoms on the trees infected with the selected for further molecular analysis PNRSV variants are described in Table 1. DAS-ELISA results of these trees showed presence of PNRSV in 34 trees and in 8 trees a mix of PNRSV and PDV was found. The symptoms caused by PNRSV and PDV are very similar and for that reason they are not counted in the determination of the trees with and without symptoms on the leaves. In the case of only PNRSV infection on the leaves of 22 trees chlorotic or necrotic spots, chlorosis around the veins and perforation were observed, while 12 trees were symptomless. A study for the occurrence of *Ilarviruses* in sweet and sour cherry in Bulgaria has shown both, symptomatic and non-symptomatic infection with PNRSV (Kamenova et al., 2020). The typical symptoms induced by PNRSV include chlorosis, necrosis, leaf deformation and growth defects (Fiore et al., 2008). Depending on the isolate the reaction of PNRSV-infected cherry trees is from symptomless to a rugose mosaic disease (Howell & Mink, 1988).

Table 1. Information for studied PNRSV variants

PNRSV variants	Origin	Symptomatology [a,b]	Accession numbers: CP[c,d,f] / MP[c,f] genes
B84	Southwest/*Pr. avium*	necrosis and perforations[a]	MK392160[c] MW296228[c]
St32	South Central/*Pr. cerasus*	necrosis and perforations[a]	MK392163[c] MW186689[c]
St37	South Central/*Pr. cerasus*	chlorosis around the central and the secondary veins[a]	MK392164[c] MW186690[c]
Iv50/8	Western/*Pr. avium*	nd [b]	MK392167[c]; MW186692[c]
K134	Western/*Pr. avium*	symptomless[a]	MK392166[c]; MW186691[c]
K139	Western/*Pr. avium*	symptomless[a]	MK392168[c]; MW296230[c]
K160	Western/*Pr. avium*	chlorotic pattern[a]	MK392169[c] MW723616[c]
Vl4	Western/*Pr. cerasus*	chlorotic and necrotic spots, perforations [a]	MN635762[c] MW186695[c]
Vl13	Western/*Pr. avium*	chlorotic and necrotic spots[a]	MN635760[c]; MW186694[c]
Kost76	Western/*Pr. avium*	chlorotic pattern[a]	MN635758[c]; MW186693[c]
EM75	South Central/*Pr. avium*	necrotic spots, perforation and yellowing around the central nerve[a]	MN635757[c]; MW296229[c]
EM80	South Central/*P. avium*	necrotic spots, perforation and yellowing around the central nerve[a]	MW296233[c] MW723617[c]

Table 1. Continued...

KM1	Western/*P. avium*	necrotic spots[a]	MN635761[c] MW296231[e]
KS3	Western/*Pr. cerasus*	symptomless[a]	MN635763[c] MW186696[e]
Kust3	Western/*Pr. avium*	symptomless[b]	MT178254[c] MW186688[e]
Kust4	Western/*Pr. avium*	light green spots[b]	MT178255[c] MW723618[c]
Vrat17	Western/*Pr. avium*	symptomless[a]	MW723610[c] MW723611[e]
Scr21	Western/*Pr. cerasus*	chlorotic and necrotic spots, perforations[a]	MW723614[c] MW723615[e]
PN1	Western/*Pr. cerasus*	chlorotic spots[a]	MT009386[c] MW186681[e]
PN2	Western/*Pr. cerasus*	necrotic spots[b]	MT009387[c] MW186682[e]
PN5	Western/*Pr. avium*	chlorotic spots[b]	MT009388[c] MW29623[e]
PN6	Western/*Pr. avium*	symptomless[a]	MT009389[c] MW186683[e]
PN7	Western/*Pr. avium*	necrotic spots[a]	MW186679[f] MW186684[f]
PV8	Western/*Pr. avium*	symptomless[a]	MT009391[f] MW186685[f]
PN10	Western/*Pr. cerasus*	necrotic spots[a]	MW18667[f] MW186686[f]
PN11	Western/*Pr. avium*	narrow leaves with chlorotic spots[a]	MW186680[f] MW186687[f]
PN15	Western/*Pr. avium*	single distorted leaves[a]	MW207218[f] MW207219[f]
Lil26	Western/*Pr. avium*	symptomless[a]	MW732613[d]/NA
Koser18	Western/ *P. avium*	symptomless[a]	MW732612[d]/NA
PA33	Southwest/*P. avium*	symptomless[a]	MK392157[c]/NA
B66	Southwest/*P. avium*	chlorotic and necrotic spots perforation[a]	MK392158[c]/NA
B80	Southwest/*P. avium*	chlorotic spots[b]	MK392159[c]/NA
St10	South Central/*P. cerasus*	symptomless[a]	MK392161[c]/NA
St60	South Central/*P. cerasus*	symptomless[a]	MK392162[c]/NA
K10	Western/ *Pr. avium*	symptomless[a]	MK392165[c]/NA
Iv2/3	Western/ *Pr. avium*	nd[b]	MK392170[c]/NA
St6	South Central/*Pr. cerasus*	chlorosis between the veins, distortion[a]	MN635759[c]/NA
Ber56	Western/*Pr. avium*	chlorosis and necrosis between the veins, perforations[a]	MN635756[c]/NA
Hr2	Western/*Pr. cerasus*	necrotic spots[a]	MN734265[c]/NA
Nef4	Western/*Pr. cerasus*	symptomless[a]	MN722633[c]/NA
Rad	Western/*Pr. cerasus*	chlorotic and necrotic spots, perforations[a]	MN722634[c]/NA
Kust1	Western/*Pr. avium*	single "tattered" leaves[a]	MT178253[c]/NA

Used abbreviated designations:
Symtomatology [a] – single PNRSV infection
Symptomatology [b] – mixed infection: PNRSV and PDV
Symptomatology [a, b] – no data (nd)
CP accession number [c] – reference: Kamenova, I. & Borisova, A. (2021). Molecular variability of the coat protein gene of *Prunus nectrotic ringspot virus* on sweet and sour cherry in Bulgaria. *Journal of Plant Pathology*, *103*: 97-104.
CP accession number [d] – this study
MP accession number [e] – this study
CP and MP accession numbers [f] – reference: Kamenova, I. & Borisova, A. (2021). Biological and molecular characterization of *Prunus necrotic ringspot virus* isolates from sweet and sour cherry. *Biotechnology & Biotechnological Equipment* 35 (1): 567–575.
NA = sequence not available

Nucleotide sequencing

In total 42 PNRSV variants were included in present work, from which twenty seven were sequenced both in the CP and MP genomic regions, and fifteen more variants were sequenced only in the CP genome region (Table 1).

Phylogenetic and sequence identity analyses of CP encoding region

The obtained sequences were compiled and trimmed to correspond to the CP gene, as the variants amplified with primer pair I/II were 596-bp long and had putative translation products of 198 amino acids. PNRSV variants amplified with F3/R4 primers were 675-681-bp-long, which corresponded to 225-227 amino acids.

A phylogenetic tree based on the CP nucleotide alignments of studied PNRSV variants and the isolates representative of the reported phylogenetic groups PV32-I, PV96-II, PE5-III and CH30 is shown in Figure 1**.** Constructed tree revealed the formation of the known four groups, which were highly supported by the bootstrap values. Twenty six PNRSV variants fall in the PV96-II group, as 15 of them formed a separate cluster (A) together with the isolate CH61, reported as a mild pathotype of PNRSV (Mink et al., 1987). The other ten variants from PV96-II group formed cluster B with isolate CH39 (USA), which was defined also as a mild pathotype (Crosslin & Mink, 1992) and the PV0096 isolate (*P. mahaleb*) from Germany. One of studied PNRSV variants, Vl13 separated from the two described clusters.

Sixteen PNRSV variants fall in PV32-I group and formed a cluster separated from the referent isolates CH9, CH38 and CH57, reported as rugose type isolates from USA (Mink et al., 1987; Crosslin & Mink, 1992). One of the analyzed variants (Scr21) was closely related to the apple isolate PV32. According to Hammond & Crosslin (1998), the CH9 serotype isolates represent the two major groups of biological variants, severe rugose disease and mild virosis. No correlation between the geographical origin of studied Bulgarian variants and their phylogenetic grouping was found, as the variants from included regions separated both in each of the two clusters in PV96-II group, as well in PV32-I group. Such lack of relation between the geographical and/or host species origin and the phylogenetic grouping of PNRSV isolates has been reported in many studies (Aparicio et al., 1999; Spiegel et al., 1999; Moury et al., 2001; Glasa et al., 2002; Yurtmen et al., 2002; Hammond 2003; Ulubaş-Serçe & Ertűng, 2004, Sala-Rejczak & Paduch-Cichal, 2013).

None of studied variants belonged to PE5-III composed from several severe cherry isolates from USA and two peach isolates (Hammond, 2003), as well to CH30 group composed of CH30 and CH71 reported as rugose and mild pathotypes,

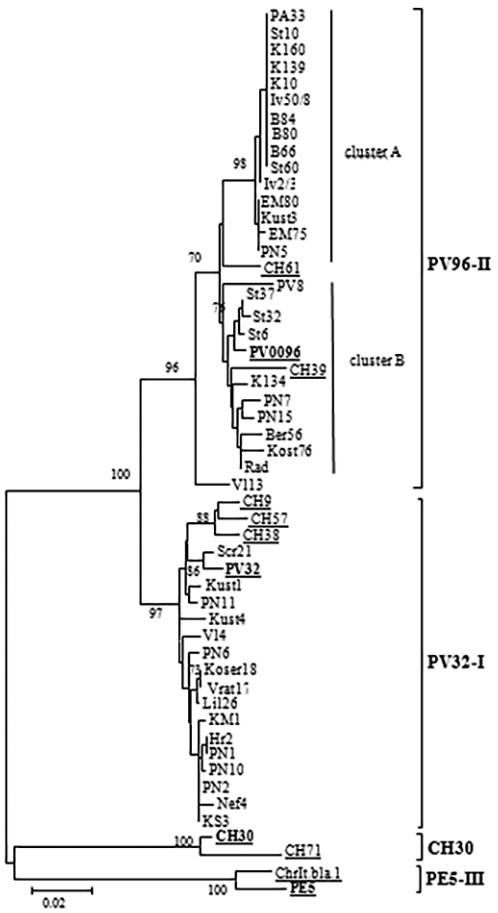

Fig. 1. Phylogenetic analyses of studied *Prunus necrotic ringspot virus* (PNRSV) variants based on the nucleotide sequences of their coat protein gene. The tree was constructed with MEGA6 using the neighbour-joining method. The numbers at the nodes indicate percentage of 1000 replicates, as the values below 70% are not shown. The underlined isolates are the representatives of the respective phylogroup. The bar represents 0.02 substitutions per site

respectively (Mink et al., 1987; Crosslin & Mink, 1992).

Comparison of the nucleotide sequences of studied PNRSV variants showed that in comparison with the variants from PV96-II group, those belonging to PV32-I phylogroup contained six more nucleotides, which were CTAGGA in the case of thirteen variants and two of them contained ATAGGA in positions 120-125. One of the examined variants (Scr21) contained at the above mentioned position the GTAGGA extra nucleotides, as the referent isolate PV32. The presence of the extra nucleotides was reported as group

specific for PNRSV isolates belonging to the PV32-I phylogroup (Aparicio et al., 1999; Hammond, 2003).

The PV96-II intra-group nucleotide identity among studied PNRSV variants was from 97% to 100% and between them and the referent isolates CH61 and CH39 from 97 to 98%. Three of the studied variants (St32, St37 and St6) originating from infected *Pr. cerasus* trees from one and same geographical location were 100% identical to PV0096 at nucleotide level.

The nucleotide identity among studied PNRSV variants belonging to PV32-I phylogroup was the same (97-100%) as those found among studied isolates from PV96-II group, while between studied PV32-I group variants and the ruse type isolates (CH9, CH57 and CH38) was 94-97%. The overall nucleotide identity of 94-100% among all studied here PNRSV variants were in the range of reported nucleotide identity of 99% for PNRSV isolates from Slovakia (Glasa et al., 2002). Nucleotide identity from 91.5% to 100% and from 97% to 100% was found for PNRSV variants from South America, North America and Iran (Fiore et al., 2008; Oliver et al., 2009; Sokhandan-Bashir et al., 2017).

Phylogenetic analysis based on the deduced amino acid sequences of CP gene showed the same four groups and topology of the main branches of the tree (data not shown). The amino acid identity among studied PNRSV variants belonging to PV96-II and between them and the referent isolates CH61 and CH39 was from 96% to 98%. The amino acid identity among studied PNRSV variants belonging to PV32-I group, and between them and the rugose type isolates CH9, CH38 and CH57 was 92-100% and 90-96%, respectively. The reported amino acid identities in CP for PNRSV isolates from different countries were in the range of 89.7-100%, 96-100% and 98.2-100% (Fiore et al., 2008; Oliver et al., 2009; Sokhandan-Bashir et al., 2017).

The comparison of CP amino acid sequences revealed that the studied variants in each of the two groups (PV96-II and PV32-I) had phylogroup-conserved amino acid residues (Figure 2). In general studied PNRSV variants belonging to PV96-II group showed lesser number common amino acid substitution with the used references from the group, in comparison with those belonging to PV32-I and the references of the group. The alignment showed four amino acid substitutions G13/S13, A36/D36, T91/M91 and R142/Q142 between studied variants from clusters A and the referent isolate CH61, respectively and the same number substitutions (G25/D25, A36/D36, L117/Q117 and E142/D142) between studied variants from cluster B and the referent CH39 isolate, respectively.

In the CP amino acid sequences of PNRSV variants from PV32-I group two more amino acids, corresponding to the observed six extra nucleotides in their composition were present. Thirteen and two variants had TR (Thr-Arg) and NR

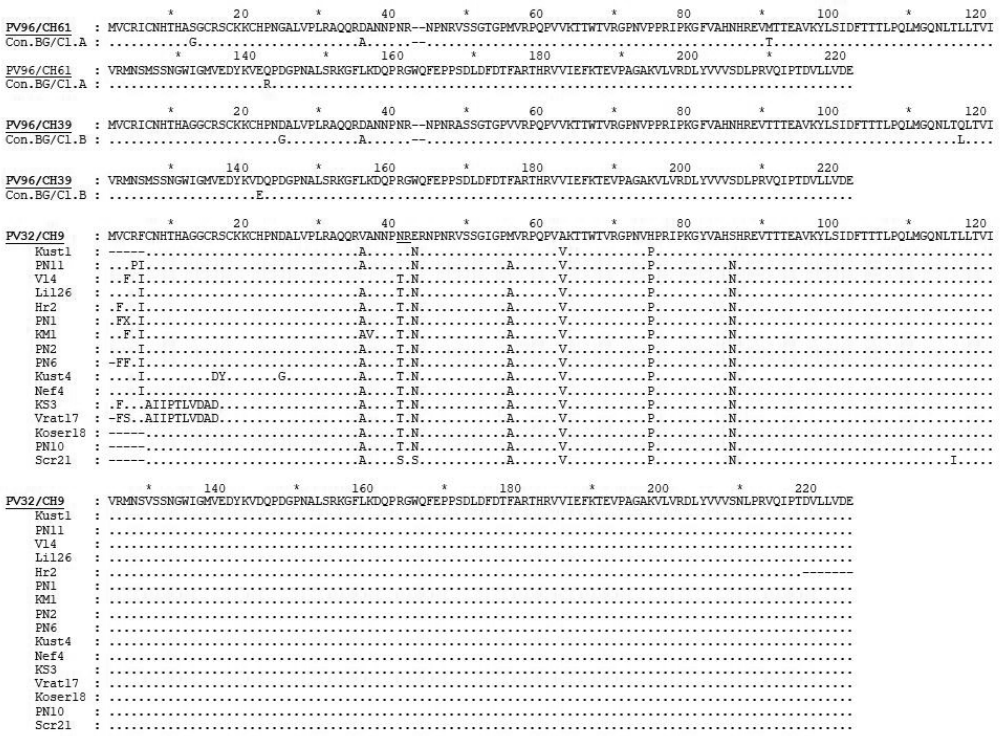

Fig. 2. Multiple alignments of amino acid sequences of the coat protein gene of studied PNRSV variants. The variants belonging to Clusters A and B from PV96-II phylogroup are presented as consensus alignments. The GenBank representative isolates of PV96-II and PV32-I groups are underlined. Dots indicated identical amino acid residues

(Asn-Arg) amino acid residues, respectively and Scr21 variant showed SR (Ser-Arg) amino acid residues. Additionally, all studied variants from PV32-I group had Y81 residue reported also as specific for PV32-I group isolates (Aparicio et al., 1999; Hammond, 2003). The alignment of amino acid sequences showed that the studied PNRSV variants differed from the rugose type isolate CH9 by the next common amino acid substitutions: A36/V36 (without Vl14), N43/E43 (without Scr21), A56/M56, V63/A63, P75/H75 and N86/S86.

Phylogenetic and sequence identity analyses of MP encoding region

The obtained nucleotide sequences of the MP gene were 849 nucleotides in length translated to 283 amino acids.

A phylogenetic analysis of the nucleotide sequences of MP gene of studied PNRSV variants (Figure 3) exhibited the formation of the same four groups PV32-I, PV96-II, PE5-III and CH30 observed in the phylogenic tree based on the nucleotide sequences of CP genome region. Fifteen PNRSV variants out of the analyzed twenty seven were grouped as PV96-II together with the references CH61 and CH39. Unlike the CP generated tree in the MP-based tree studied variants dispersed among the referent isolates without the formation of the two clusters (A and B). Eleven PNRSV variants were grouped in PV32-I, following the topology observed in the CP-based tree as they separated from the rugose type isolates CH57, CH9 and CH38.

The nucleotide identity among Bulgarian PNRSV variants in MP genomic region belonging to both PV96-II group PV32-I groups was from 97% to 100%. The nucleotide identity between studied PNRSV variants from PV96-II group and the references CH61 and CH39 was 98-99% and from 96% to 98% with the PV32-I references, CH9, CH38 and CH57. The overall nucleotide identity among all studied 27 PNRSV variants was 94-100%. Nucleotide identity of 82.9% - 99.9% in the MP gene was reported for a number of China isolates from different *Prunus* species (Cui et al., 2015).

In general the studied PNRSV variants showed a very similar topology (relationships), both in CP and MP genomic regions excluding PN5 variant, defined to belong in PV96-II group in the CP, and in CH30 group in the MP genomic region. PN5 variant shared 98-100% nucleotide identity with PV96-II variants and 89% with CH30 variants in CP genome region, while in MP region the nucleotide identity to PV96-II was 86-88% and 95% to CH30 variants.

The phylogenetic tree generated from the putative amino acids of MP gene showed essentially the same described above groups, as PN5 variant was grouped in CH30 phylogroup (data not shown) sharing 98% amino acid identity with bout CH30 and CH71. The amino acid identity among

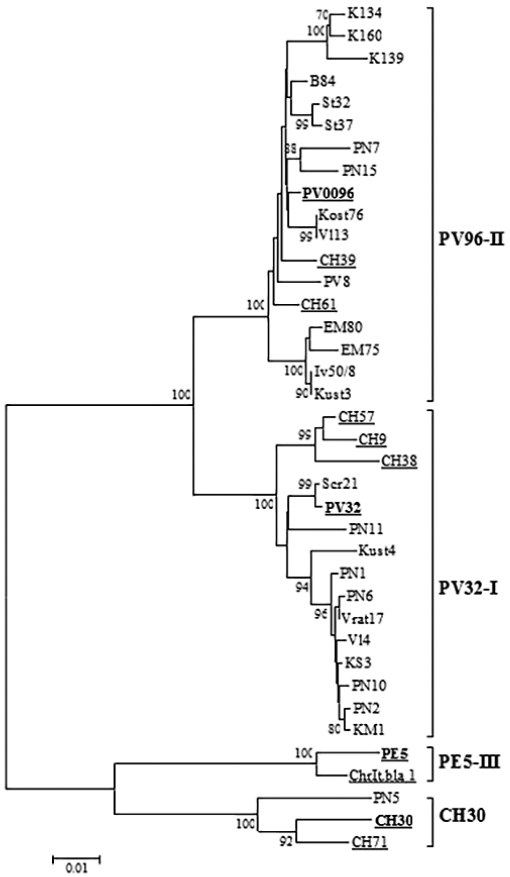

Fig. 3. Phylogenetic analyses of studied *Prunus necrotic ringspot virus* (PNRSV) variants based on the nucleotide sequences of their movement protein gene. The tree was constructed with MEGA6 using the neighbour-joining method. The numbers at the nodes indicate percentage of 1000 replicates, as the values below 70% are not shown. The underlined isolates are the representatives of the respective phyogroup. The bar represents 0.02 substitutions per site

studied PNRSV variants belonging to PV96-II was from 96% to 100% and from 97 to 99% between them and the referent isolates CH61 and CH39. The amino acid identity among studied PNRSV variants from PV32-I group, and between them and the rugose type isolates CH9, CH38 and CH57 was 97-100% and 96-98%, respectively. The intra-group amino acid identity in the MP genome region has been shown to range between 96.5-100% and 96.8-98.9% for PNRSV isolates belonging to PV96-II and PV32-I groups, respectively (Fiore et al., 2008). Amino acid identity from 90.8% to 100% in the MP genome region was reported for PNRSV from different *Prunus* spp. (Cui et al., 2015).

The alignment of amino acid sequences of PNRSV variants belonging to PV96-II group showed from none (among St37, EM80, Iv50/8 and Kust3) to single differences (from 1 to 4) between the studied and the referent isolates (CH61 and CH39) scattered throughout the whole genome region. Only one common amino acid substitution V159/A159 was observed between K134, K139, K160, B84, Kost76, Vl13, PN7 and PN15 variants and the references CH61 and CH39, respectively (data not shown).

The alignment of amino acid sequences of the rugose type isolate CH9 and studied PNRSV variants from PV32-I group (Figure 4) revealed the following common amino acid substitution: C24/S24, F55/L55, A126/E126, S156/P156, F188/I188, T215/I215 and T228/N228 (excluding variant Kust4 which at that position showed T (thr) amino acid residue as the referent isolate CH9. The amino acid residues C24/S24, F50/L50, A126/E126, T228/N228, I253/V253, D256/E256, V257/D257 and L260/T260 were reported as conservative and differentiating rugose from the mild type of the CH9 serotype isolates (Hammond & Crosslin, 1998). From the amino acid substitutions characteristic for the rugose type isolates, I253, D256, V257 and L260 were present in all studied variants, while at all other above-mentioned positions (S24, E126 and N228 (excluding Kust4), they differed from CH9.

The alignment of MP amino acid sequences of PN5 variant with those of CH30 and CH71 belonging to CH30 phylogroup, showed that PN5 and CH71 (described as mild pathotype) differed from CH30 (described as rugose pathotype) by A225/V225 and A247/V247 common amino acid substitutions, respectively (data not shown).

The results obtained here can be summarized by standing out that based on the RNA3 coding region studied cherry variants of PNRSV from several regions of the country consistently clustered phylogenetically into PV32-I and PV96-II groups. With the exclusion of PN5 variant a correlation between the phylogeny based on the nucleotide and amino acid sequences in the CP and MP genomic regions was observed. The variants belonging to PV96-II group prevailed over that belonging to PV32-I group, and confirmed the finding of many authors that more PNRSV variants from different *Prunus* species and geographical regions belong to PV96-II group. (Aparicio & Pallas, 2002; Glasa et al., 2002; Hammond, 2003; Ulubaş-Serçe & Ertung, 2004; Fiore et al., 2008; Sokhanadan-Bashir et al., 2017; Kamenova & Borisova, 2021). The majority of reported PNRSV isolates belonging to PV96-II tend to exhibit latent and/or mild symptoms (Hammond, 2003; Aparicio et al., 2003), while PV32-I group is composed predominantly of isolates inducing more severe symptoms. Indeed 12 trees infected with PNRSV (without the trees with mix infection) were symptomless, while in the other 22 trees the observed symptoms varied from chlorotic pattern to necrotic spots and perforation of the leaves. Nevertheless, part of the studied variants grouped into PV32-I they separated from the rugose type isolates, thus supporting

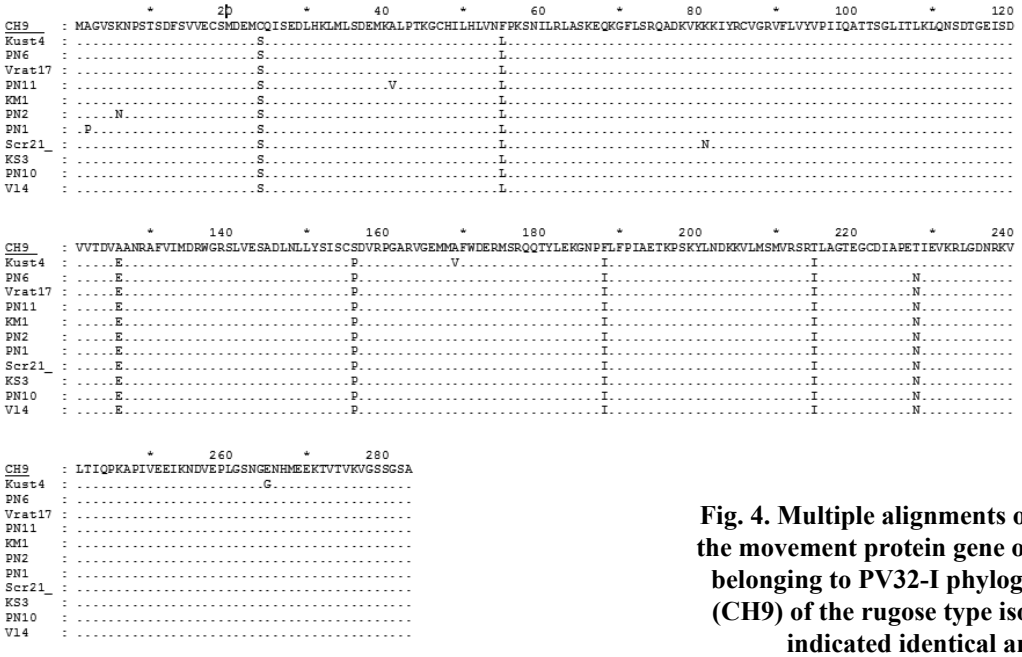

Fig. 4. Multiple alignments of amino acid sequences of the movement protein gene of studied PNRSV variants belonging to PV32-I phylogroup. The representative (CH9) of the rugose type isolates is underlined. Dots indicated identical amino acid residues

the results of Mink et al. (1987) who reported that PNRSV isolates from CH9 serotype may cause a range of symptoms, from rugose to mild mottle virosis. None of studied PNRSV variants showed the characteristic for the rugose type symptoms, as enations, distorted leaf growth with twisted leaf tips and deformed and/or uneven ripened fruits.

Conclusions

In general our results revealed a correlation between the phylogeny based on the nucleotide and amino acid sequences of RNA3 genomic region and the described pathotypes for infected with PNRSV cherry trees, known as rugose disease and mild mottle virosis. The specific amino acid composition both in the CP and MP genome regions of studied cherry PNRSV variants together with the symptoms on naturally infected cherry trees corresponded to the mild virosis pathotype.

Disclosure statement

The authors declare that they have no conflict of interest.

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