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New highly divergent Plum pox virus isolates infecting sour cherry in Russia

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ABSTRACT

Unusual *Plum pox virus* (PPV) isolates (named Tat isolates) were discovered on sour cherry (*Prunus cerasus*) in Russia. They failed to be recognized by RT-PCR using commonly employed primers specific to the strains C or CR (the only ones that proved able to infect sour cherry) as well as to the strains M and W. Some of them can be detected by RT-PCR using the PPV-D-specific primers P1/PD or by TAS-ELISA with the PPV-C-specific monoclonal antibody AC. Phylogenetic analysis of the 3′-terminal genomic region assigned the Tat isolates into the cluster of cherry-adapted strains. However, they grouped separately from the C and CR strains and from each other as well. The sequence divergence of the Tat isolates is comparable to the differences between the known PPV strains. They may represent new group(s) of cherry-adapted isolates which do not seem to belong to any known strain of the virus.

1. Introduction

Plum pox virus (PPV; genus *Potyvirus*; family *Potyviridae*) is a causal agent of sharka, economically the most important viral disease of stone fruits affecting their yield and quality (Cambra et al., 2006). The PPV genome is typical of potyviruses and consists of a single stranded positive sense RNA 9.8 kb in length with a 5′-terminal viral genome-linked protein (VPg) and a 3′ poly (A) tail. A long open reading frame (ORF), flanked with the 5′- and 3′-non-coding regions (NCR), is translated into a 355 kDa polyprotein processed by virus-encoded proteases to 10 functional proteins, namely P1, HcPro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb and coat protein (CP). A short overlapping ORF, called PIPO, was predicted within the P3 cistron and can be expressed by ribosomal frameshifting as a product fused with the N-terminal region of P3. Based on genomic sequence differences and phylogenetic analysis, nine PPV strains (D, M, C, EA, W, Rec, T, CR, and An) are recognized to date (Garcia et al., 2014 and the references therein).

PPV is able to infect most, if not all, *Prunus* species (Llácer and Cambra, 2006; James and Thompson, 2006; Polak, 2006). At the same time, PPV strains may differ in their host range. Among nine known strains only two, C and CR, have been proven able to infect sour cherry (*P. cerasus*) (James et al., 2013; Garcia et al., 2014). Both strains seem to have a common origin. Their adaptation to sour cherry is probably attributed to the unique amino acids located mainly in the P1, NIa and CP and conserved among PPV-C and PPV-CR isolates (Glasa et al., 2013). Apart from sour and sweet cherries as natural hosts, PPV-C is capable of infecting a number of other *Prunus* species experimentally (Nemchinov et al., 1998; Barba et al., 2011). The host range of the recently discovered strain CR remains to be determined. The CP gene of PPV-C and PPV-CR consists of 996 nucleotides encoding the corresponding protein of 332 amino acids. The known isolates of PPV-CR and some isolates of PPV-C contain the D96E mutation in the universal epitope located within the N-terminal domain of the CP. As a result, they either fail to be detected with the monoclonal antibody 5B (Chirkov et al., 2013a) or can be still recognized by this antibody after lowering the pH of the extract (Glasa et al., 2013). This mutation has not yet been discovered in other PPV strains.

The identification of the strains C and CR is based on reverse transcription-polymerase chain reaction (RT-PCR) using primers targeting the (Cter)NIb - (Nter)CP genomic region of PPV-C (Nemchinov et al., 1998; Nemchinov and Hadidi, 1998; Szemes et al., 2001) or PPV-CR (Glasa et al., 2013). Triple antibody sandwich (TAS) ELISA with the monoclonal antibody AC was also developed for the specific detection of strain C (Myrta et al., 2000). Employing these methods, a large number of PPV-C isolates was found on sour and sweet cherries in the countries of the former USSR: Moldova (Kalashyan et al., 1994; Nemchinov and Hadidi, 1996), Belarus (Malinowski et al., 2012), and Russia (Chirkov et al., 2013b; Glasa et al., 2013, 2014). In addition, isolates belonging to the strain C were sporadically observed in Italy (Crescenzi et al., 1997; Fanigliulo et al., 2003), Romania (Isak and Zagrai, 2006), Hungary (Nemchinov et al., 2008) and Croatia (Kajić et al., 2012). PPV-CR was found only in Russia (Chirkov et al., 2013a; Glasa et al., 2013).

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Fig. 1. Symptoms on the sour cherry leaf infected with the Plum pox virus isolate Tat-2.

Sour cherry is the most common stone fruit crop that is widespread throughout the European Russia. Surveys of numerous local cultivars in orchards and private gardens as well as wild trees in many geographically distant regions revealed the high incidence of PPV on sour cherry. The Russian cherry-adapted isolates have been shown to belong to the strains C or CR (Chirkov et al., 2013b, 2015; Glasa et al., 2013, 2014).

In 2015, some unusual PPV isolates were discovered under survey of sour cherry plantings in the middle Volga river region of Russia. Typical symptoms of PPV infection were observed on the leaves (Fig. 1). Despite the fact that these isolates were found on sour cherry, they were not recognized by RT-PCR with commonly employed PPV-C or PPV-CR-specific primers. At the same time, some of them could be successfully detected by RT-PCR assay using the PPV-D-specific primers P1/PD. This work was aimed at serological and molecular characterization of these isolates. The study on the 3'-terminal genomic regions showed that they represent a new group of cherry-adapted PPV isolates which does not seem to belong to any known strain of the virus.

2. Materials and methods

2.1. Plant material

The PPV isolates were sampled in cultivar and hybrid sour cherry collections situated in the middle Volga river region, the Republic of Tatarstan, Russia. Due to their location, new cherry-adapted isolates were named here as “Tat isolates”. The isolates Tat-2, Tat-3 and Tat-4 were found in some abandoned collection plots on cherry root offshoots of unknown origin. The isolate Tat-26 was detected on a hybrid of local sour cherry cultivars. Samples of the leaves displaying typical symptoms of sharka disease were subjected to serological and molecular analyses. Fresh leaves were used for the PPV detection, initial characterization of the isolates and extraction of total RNA. Additionally, symptomatic leaves were cut into small pieces, divided randomly into portions of approximately 0.5 g weight, which were lyophilized and stored at 4 °C until use.

2.2. Detection and identification of the virus by ELISA and RT-PCR

The suspected samples were analyzed by double antibody sandwich (DAS) ELISA using a reagent set SRA 31505 (Agdia, USA) and by TAS ELISA with the universal monoclonal antibody 5B, PPV-D-specific monoclonal antibody 4DG5 (Cambra et al., 1994) and PPV-C-specific monoclonal antibody AC (Myrta et al., 2000) supplied with the K-10B, K-12B and K-14B kits (Agritest, Italy), respectively. Approximately 0.1*g* of the leaf tissue was triturated in extraction buffer (2 ml) consisting of phosphate-buffered saline (PBS), 0.02% (v/v) Tween 20, 2% (w/v) polyvinylpyrrolidone (molecular weight of about 40,000), 0.5% (v/v) Triton X-100% and 0.02% (w/v) sodium azide, as was described previously (Sheveleva et al., 2012). The extracts were clarified by low speed centrifugation, added into the microplate wells (MaxiSorp microtiter plates, Nunc) and incubated in a temperature-controlled shaker for 2 h at 37 °C (450 rpm). The subsequent steps were performed following the kit supplier's instructions. The optical densities were measured 30 min after substrate addition at the wavelength of 405 nm using a Titerteck Multiscan microplate reader (Eflab Oy, Finland).

Immunocapture RT-PCR assay was performed according to Wetzel et al. (1992) using polyclonal PPV-specific antibodies (Agdia, USA), an oligo-dT primer for the first strand cDNA synthesis and the primers P1/P2 (Wetzel et al., 1991) or primers specific to the 3′-NCR (Levy and Hadidi, 1994) for the PCR. Strain typing was done by conventional PCR using the same RT preparations and primers specific for the strains C, CR, W, D and M, according to the original protocols (Olmos et al., 1997; Nemchinov and Hadidi, 1998; Szemes et al., 2001; James and Varga, 2004; Glasa et al., 2013). Experimental conditions for the RT-PCR assays were as described originally for each primer set. PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

The same extracts obtained from fresh leaves were used for DAS and TAS ELISA as well as for immunocapture RT-PCR assays. Lyophilized leaves containing the isolates 1410 (Sheveleva et al., 2012), Fl-3 (Chirkov et al., 2013a), Bg66 (Chirkov et al., 2013b) and Pav-17 (Chirkov et al., 2016) from the collection of our laboratory were used as positive controls for the strains W, CR, C and D, respectively. The PPV-M positive control was provided with the K-11B kit (Agritest, Italy). The negative control was obtained from the PPV-free sour cherry leaves.

2.3. Sequencing and analysis of the sequences

Total RNA was purified from infected leaves using RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instruction, with the exception that another lysis buffer was used for RNA extraction (MacKenzie et al., 1997). The first strand cDNA was synthesized using the random hexamer and oligo-dT primers. The 3′-terminal genomic region of the Tat isolates spanning the entire CP gene and flanking sequences of the NIb gene and 3′-NCR ((Cter)NIb-CP-3′NCR) was amplified using three different primer sets. The PCR products of 1340 base pairs (bp) encompassing the entire CP gene and the most part of the 3′-NCR were amplified using the forward primer PD3W (5′-ACATAGCAGAGACAGGATTG-3′) (Sheveleva et al., 2011) and the reverse primer that targets the 3′-NCR (Levy and Hadidi, 1994). Amplification of the cDNA was performed using the proof-reading Encyclo DNA polymerase (Evrogen, Russia) and included the following cycles: denaturation at 94 °C for 30 s, primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min 40 s for 35 cycles with a final extension at 72 °C for 10 min. Another 3′-terminal segment of the Tat-3 and Tat-26 genomes of 1846 bp, including a part of the NIb gene and the entire CP, was amplified using the forward Potyvirid primer 2 (Gibbs and Mackenzie, 1997) and the reverse primer 4CPR1 (Matic et al., 2011), under the conditions described by Gibbs and Mackenzie (1997). The 3′-proximal third of the NIb gene of the isolates Tat-2 and Tat-4 of 987 bp was amplified using the forward potyvirid primer NIb2F (Zheng et al., 2010) and a specially designed internal reverse primer tat2cpR (5′-ACCAAAGTTACGTTATCATCA-3′) targeting positions 15–35 in the CP gene. Amplification conditions consisted of: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 45 °C for 30 s, 72 °C for 1 min 10 s, and 72 °C for 10 min. The PCR products were purified from the agarose gel using a Cleanup Standard kit (Evrogen, Russia) and sequenced in both directions using Evrogen facilities (Moscow, Russia).

The sequences of (Cter)NIb-CP-3′NCR genomic regions were assembled from the corresponding sequences of overlapping PCR fragments and trimmed by length to 1747 nucleotides (nt). They comprised 576 nt of the NIb gene, 996 nt of the CP together with 175 nt of the 3′-NCR and were deposited in the GenBank database under accession numbers KX685593 - KX685596. Multiple alignments were obtained using the ClustalW v.2.1 program available online at the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/) or an older version of the program implemented in the BioEdit sequence alignment editor v. 7.2.4 (Hall, 1999). Nucleotide and encoded amino acid (aa) sequence identities and genetic distances between isolates were calculated using the ClustalW algorithm implemented in

the MegAlign v. 7.1.0. program (DNASTAR Lasergene).

2.4. Phylogenetic analysis

Phylogenetic analysis of the (Cter)NIb-CP-3′-NCR genomic region of the Tat isolates was performed employing the PHYML 3.0 (20130219.patch) version of the maximum likelihood algorithm (Guindon and Gascuel, 2003; Néron et al., 2009). Graphical presentation was done by MEGA (v.4.0.1) program (Tamura et al., 2007). Available corresponding sequences of isolates of the C and CR strains were retrieved from GenBank: RU-30sc (KC020126), RU-17sc (KC020124), RU-18sc (KC020125), BY101 (HQ840517), BY181 (HQ840518), SwC (Y09851), SoC (AY184478), and Volk143 (KJ787006). Some Russian PPV-C (Bg6, Bg26, Bg66, Pul) and PPV-CR (Kp8-2U, Kp8-1, Pul-DS, Fl-3, Pul-1) isolates from different localities that were completely sequenced but not yet submitted to GenBank, were also included to the analysis. The isolate LV-145bt (HQ670748) was employed as a representative of the strain W that is known to be most closely related to the strains C and CR (Garcia et al., 2014). The isolate of the strain EA (AM157175) was used as the phylogenetic outgroup.

2.5. Recombination analysis

The recombination detection program RDP v. 4.69 (RDP4) (Martin et al., 2015) was used for the analysis of the (Cter)NIb-CP-3'-NCR genomic region of the Tat isolates. Corresponding sequences of representative isolates of all the known PPV strains were retrieved from GenBank and included to the alignment as follow: AbTk (EU734794, strain T), AL11pl (HF674399, An), BOR-3 (AY028309, Rec), EA (AM157175, EA), Fantasia (AY912056, D), LV-145bt (HQ670748, W), RU-17sc (KC020124, CR), SK68 (M92280, M) and Volk143 (KJ787006, C). Recombination events, likely parents of recombinants, and recombination breakpoints were inferred using the RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, and 3Seq methods implemented in the RDP4 with default settings, with the exception that “sequences are linear“ option was chosen.

2.6. Western blot analysis

Western blot analysis was carried out as described by Sheveleva et al. (2012). Leaf samples were homogenized in the 2x loading buffer using 1/15 ratio (g/ml). Crude extracts were clarified by low speed centrifugation and heated at 96 °C for 7 min. Proteins were resolved under denaturing conditions by sodium dodecyl sulfate polyacrylamide (10% w/v) gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P transfer membrane (Millipore) using a Semi-Dry Transfer Cell (Bio-Rad). Membranes were blocked with 5% (w/v) skim milk powder (Fluka), diluted in PBS, and probed with the antibody 5B from a K-10B kit (Agritest, Italy) at a 1/5000 dilution. Horseradish peroxidase-labelled anti-mouse IgG (W4021, Promega) was used as the detecting antibody at the 1/20000 dilution. All the blocking and detection steps were performed for 1 h at room temperature. PBS with 0.1% (v/v) Tween 20 was used for membrane washing and dilution of immunospecific reagents. Bound antibodies were detected using ECL Western blotting detection reagents (Promega).

3. Results

3.1. Initial characterization of the Tat isolates

All the Tat isolates induced typical symptoms of sharka disease (pale spots and rings of irregular shape) (Fig. 1). Distinct symptoms were visible on the leaves from top to bottom of the root offshoots and were also observed throughout the canopy of the tree infected with the isolate Tat-26, indicating the well established systemic PPV infection.

Table 1
Serological analysis of the Tat isolates using DAS and TAS ELISA.

Isolate	Optical density at the wavelengh of 405 nm[a] with antibody:			
	Polyclonal[b]	5B[c]	AC[c]	4DG5[c]
Tat–2	1.66	0.98	0.76	0.08
Tat–3	2.27	1.87	1.09	0.10
Tat–4	1.93	1.23	0.13	0.11
Tat–26	2.74	2.51	1.55	0.10
Bg66[d]	2.56	2.65	2.19	0.10
Fl–3[d]	2.98	0.12	0.10	0.09
Pav–17[d]	2.71	2.45	0.10	2.11
Negative control[e]	0.09	0.09	0.11	0.11

[a] Mean from duplicates.
[b] DAS ELISA using a reagent set SRA 31505 (Agdia, USA).
[c] TAS ELISA using K-10B, K-14B and K-12B kits (Agritest, Italy), respectively.
[d] Isolates belong to the strains C (Chirkov et al., 2013b), CR (JX472437) and D (KP198597), respectively.
[e] PPV-free cherry.

The Tat isolates were successfully detected in symptomatic leaves by both DAS and TAS ELISA (Table 1). The high optical densities indicated significant amount of the virus in the samples suggesting good adaptation of the Tat isolates to the host. The Tat isolates were also detected by the immunocapture RT-PCR assay with the primers P1/P2 or with those targeting the 3′-NCR. The expected PCR products of 243 and 220 bp, respectively, were amplified in all the samples, indicating that the Tat isolates indeed belong to PPV.

The results of strain typing were controversial. Tat isolates (with the exception of the Tat-4) reacted with the PPV-C-specific antibody AC in TAS ELISA, suggesting that they can belong to PPV-C (Table 1). However, no specific product of amplification was observed in RT-PCR using the PPV-C-specific primer sets HsoC-1/CsoC-1 (Nemchinov et al., 1998), SoC-2 (Nemchinov and Hadidi, 1998) or M10/M11 (Szemes et al., 2001). Moreover, these isolates failed to be detected by RT-PCR with the PPV-CR-specific primers CR8597F/CR9023R (Glasa et al., 2013). Apparently, the Tat isolates belong to neither C nor CR strains. In addition, no positive reaction was observed in RT-PCR using the primers specific to the strains M or W. At the same time, the isolates Tat-2, Tat-4 and Tat-26 were recognized by RT-PCR assay using the PPV-D-specific primers P1/PD (Fig. 2). In contrast, none of the Tat isolates reacted with the PPV-D-specific antibody 4DG5 (Table 1). Thus, in according with the TAS ELISA results, the isolates Tat-2, Tat-3 and Tat-26 probably belong to the strain C, while the results of RT-PCR indicated that the Tat-2, Tat-4 and Tat-26 may be related to the strain D.

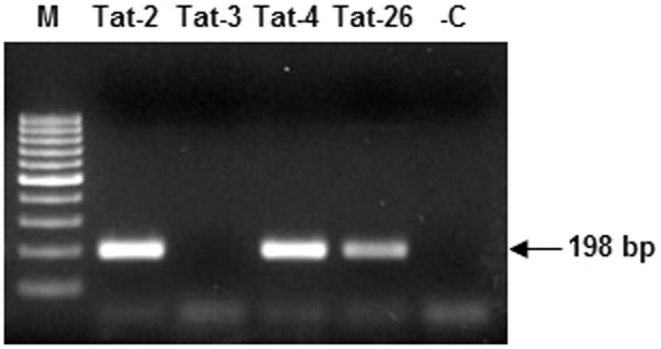

Fig. 2. Agarose gel electrophoresis analysis of reverse transcription – polymerase chain reaction amplification products from Tat isolates using PPV-D-specific primers P1/PD. M - GeneRuler 100 bp DNA ladder (Thermo Scientific). (–C) – negative control. The arrow to the right of the picture shows the PCR products of 198 base pairs.

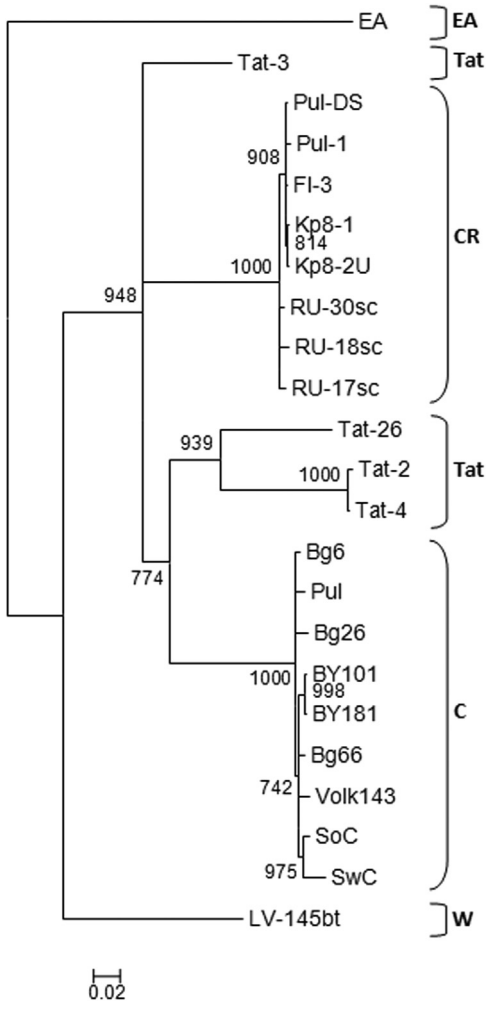

Fig. 3. Phylogenetic analysis of the 1747 nt long (Cter)NIb-CP-3′-NCR sequences of cherry-adapted *Plum pox virus* isolates. The names of isolates are indicated on the branches; their affiliation to the PPV strain or group is shown on the right of the tree. Accession numbers of the isolates included in the analysis are as follows: RU-30sc (KC020126), RU-17sc (KC020124), RU-18sc (KC020125), BY101 (HQ840517), BY181 (HQ840518), SwC (Y09851), SoC (AY184478), Volk143 (KJ787006), Tat-2 (KX685593), Tat-3 (KX685594), Tat-4 (KX685595), Tat-26 (KX685596). The corresponding 3′-terminal genomic sequences of the Russian PPV-C (Bg6, Bg10, Bg26, Bg60, Bg66) and PPV-CR (Kp8-2U, Kp8-1, Pul-DS, Fl-3, Pul-1) are not yet deposited in GenBank. The isolate LV-145bt (HQ670748) represents the strain Winona. EA sequence (AM157175) representing the strain El Amar was used as the phylogenetic outgroup. The tree was reconstructed with the maximum likelihood algorithm. Analysis was performed by the ClustalW v.2.1 and PHYML v.3.0 software. The scale bar indicates the number of substitutions per nucleotide. Bootstrap values (1000 replicates) are displayed next to the nodes. Nodes with less than 70% support were collapsed.

3.2. Identification of the strain of the new isolates

To identify the strain of the Tat isolates, the (Cter)NIb-CP-3′-NCR segment of their genomes (1747 nt) was sequenced. Phylogenetic analysis allowed assigning all the Tat isolates into the group of the cherry-adapted strains as supported by high bootstrap values (Fig. 3). The closely related Tat-2 and Tat-4 as well as the isolate Tat-26 formed a distinct cluster clearly separated from the C and CR strains. This cluster may have a common ancestor with PPV-C as supported by a 77.4% bootstrap value. Tat-3 seems to cluster separately from the rest of Tat isolates. The corresponding node on the Fig. 3 was collapsed but its bootstrap value was higher than 50% (521 of 1000 replicates).

Percentages of identity among cherry-adapted isolates based on pairwise comparison of the nt sequences of the four segments within the 3′-terminal genomic region are presented in Table 2. The C-terminal segment of the NIb gene ((Cter)NIb) and the N-terminal segment of the CP gene ((Nter)CP) were the most variable in contrast to the rest of the genome that is more conserved. The mean values of the sequence identity between the Tat isolates and the representatives of PPV-C and PPV-CR were 80.6, 80.7, 89.1, 95.3% and 80.3, 76.5, 91.3, 96.4% for the (Cter)NIb, (Nter)CP, (Cter)CP and 3′-NCR, respectively. On the other hand, the mean values of the sequence identity between the isolates belonging to the strains C and CR in the corresponding genomic segments were 80.8%, 74.8%, 90.8% and 95.7% (not shown in the Table 2), which agrees well with the data obtained from the analysis of other isolates belonging to these strains (Glasa et al., 2013). Thus, the differences between the Tat isolates and the other cherry-adapted strains within each of the four genomic segments are comparable to the levels of nt identity commonly observed between the PPV strains (James et al., 2013; Garcia et al., 2014). The mean values of the sequence identity within the group of the Tat isolates (excluding those between the Tat-2 and Tat-4) were 80.9%, 81.4%, 90.4% and 96.8% for the (Cter)NIb, (Nter)CP, (Cter)CP and 3′-NCR, respectively. They were typical for the PPV interstrain diversity indicating that each of the Tat isolates by itself (with the exception of the closely related Tat-2 and Tat-4) may represent a highly divergent evolutionary lineage. In addition, among the Tat isolates, the 5′-terminal segment of the CP gene of the Tat-3 is the closest to PPV-C suggesting that the recombination took place in this genomic region.

3.3. Recombination analysis of the 3′-terminal genomic region of the Tat isolates

The RDP4 analysis supported the recombination event in 5′-terminal segment of the CP gene of the Tat-3 that was predicted on the basis of the sequence similarity analysis (Fig. 4A). This event was confirmed by seven different algorithms, implemented in the RDP4, with statistically significant P-values: RDP 9.180×10, GENECONV 3.938×10, BootScan 5.573×10, MaxChi 5.804×10, Chimaera 9.469×10, SiScan 1.461×10 and 3Seq 3.430×10. Recombination breakpoints have been identified at positions 600 and 887 in the alignment that corresponded to positions 24 and 311 in the entire CP gene. The recombined sequence was of 287 nt in length, encompassing the region coding for hypervariable N-domain almost completely. The isolate Volk143, belonging to the strain C, was inferred as a minor parent. According to the auxiliary phylogenetic analysis, the putative recombined sequence was clustered with the isolate Volk143, thus providing some additional evidence that the Tat-3 is a true recombinant (data not shown). The major parent is unknown. The MaxChi algorithm illustrated the recombination most clearly (Fig. 4B). The finding of recombination may explain the higher percentages of identity between the Tat-3 and PPV-C isolates within the N-terminal segment of the CP (Table 2). Another recombination event in the isolate BOR-3 (strain Rec), displayed in Fig. 4A, has been characterized previously (Glasa et al., 2004).

3.4. Serological and sequence analyses of the coat protein

Similar to the strains C and CR, the CP gene of the Tat isolates consists of 996 nt encoding the corresponding protein of 332 aa. Analysis of the deduced aa sequences showed that the CPs of the Tat isolates contain the DAG motif, associated with the aphid transmission, at positions 13–15 (Fig. 5). The intact universal epitope ^{96}DRDVDAG^{102} is presented on the Tat isolates that explains their reactivity with the antibody 5B in TAS ELISA. Instead of the putative PPV-D-specific epitope ^{56}PATKP^{60} (Candresse et al., 2011), the Tat isolates contain the sequence ^{59}PNVRP^{63} shared by the known PPV-C isolates that may not be recognized with the antibody 4DG5.

About 70 unique amino acids residues along the entire polyprotein were found to be conserved among PPV-C and PPV-CR isolates (Glasa et al., 2013, 2014). Probably, they distinguish the cherry-adapted

Table 2
Percentages of nucleotide sequence identities in the 3′-terminal genomic region of the natural cherry-adapted isolates of Plum pox virus.

Isolate	Strain	C-terminal segment of the NIb gene[a]				N-terminal segment of the coat protein gene[b]				C-terminal segment of the coat protein gene[c]				3'-non-coding region[d]			
		Tat-2	Tat-3	Tat-4	Tat-26	Tat-2	Tat-3	Tat-4	Tat-26	Tat-2	Tat-3	Tat-4	Tat-26	Tat-2	Tat-3	Tat-4	Tat-26
BY181	C	80.2	81.0	80.0	82.6	80.1	85.3	80.7	80.4	88.6	90.5	88.9	88.5	93.7	97.1	94.3	96.0
Volk143	C	79.5	80.2	79.3	82.3	80.4	85.9	81.0	81.7	88.6	90.5	88.9	88.8	93.7	97.1	94.3	96.0
RU–17sc	CR	80.2	81.4	80.3	79.3	75.8	76.1	75.8	78.1	91.1	91.9	90.9	91.4	95.4	97.7	96.0	97.7
Pul-DS	CR	80.0	81.6	80.2	79.1	75.5	76.5	76.1	78.4	91.1	91.6	90.9	91.5	94.9	97.1	95.4	97.1
Tat–2			79.5	99.5	82.6		81.4	98.7	80.7		89.8	99.7	91.1		95.4	99.4	97.7
Tat–3				79.7	80.7			81.4	82.0			90.1	90.1			96.0	97.7
Tat–4					82.4				81.4				91.1				97.1

[a] The last 576 nucleotides (nt).
[b] From 1–306 nt.
[c] From 307–996 nt.
[d] 175 nt.

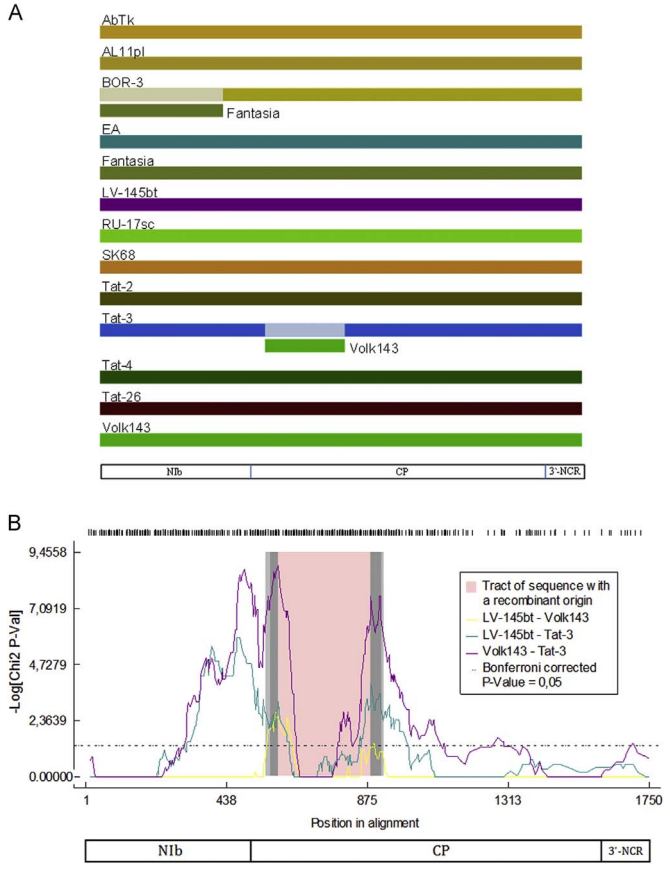

Fig. 4. Analysis of recombination events in 3′-terminal genomic region of the Tat isolates of *Plum pox virus* by the Recombinant Detection Program (RDP) v.4.69 (Martin et al., 2015). Alignment consisted of the four Tat isolates and representative isolates of all known PPV strains. (A) Summarized results of the detection of the recombination events obtained by the seven different methods implemented in the RDP4. The names of isolates are indicated above the long bars. The names of inferred minor parents (Fantasia and Volk143) are indicated next to short bars beneath the corresponding isolate bars. (B) Detection of recombination sequence in the isolate Tat-3 using MaxChi algorithm implemented in the RDP4. The map of the 3′-terminal genomic region of the virus is presented in scale below the graphs.

strains from the rest and are considered potential genetic determinants that may be relevant to the ability of PPV to infect sour and sweet cherries. Twenty of them are located in the CP, mainly in the N-terminal domain. Almost all these positions have been shown to be conserved in the N-terminus of the CP of the Tat isolates, further supporting their possible role in the host range determination (Fig. 5). In the Tat-26 two substitutions (V9I and F38L) were found. Apparently, these positions have no relevance to the virus adaptation to sour cherry. In addition, the Tat isolates have the unique motif ^{13}DxxKxTxTT^{21} at the N-terminus of the CP shared by other cherry-adapted strains (Carbonell et al., 2013).

The CP of the Tat isolates reacted with the antibody 5B in the Western blot analysis (Fig. 6). Molecular weight (MW) of the upper bands, corresponding to the full-size CP, was about 39.6 kDa in accordance with their electrophoretic mobility that exceeds about 3 kDa the MW value calculated from amino acid composition. This fact may indicate some posttranslational modification(s) of the CP and needs further investigation. In addition, the antibody 5B stained a number of lower MW bands which can represent CP degradation products.

3.5. Characterization of the sequences targeted with virus- and strain-specific primers

The primers specific to the strains C, CR and D were compared with target sequences of the Tat isolates to explain the unexpected results of the strain typing. As a whole, from 6 to 14 mismatches between the primers designed for strains C and CR identification and the corresponding sequences of the Tat isolates were found (Table S1). A large number of mismatches can apparently explain the lack of recognition of the Tat isolates using the PPV-C and PPV-CR-specific primers. In contrast, two and four mismatches between the forward primer PD and its target sequences in the isolates Tat-2/4 and Tat-26, respectively, do not affect the detection of these isolates with the PPV-D-specific primers. No amplification was observed in the case of the Tat-3, probably because of six substitutions in the primer PD binding site.

4. Discussion

Until recently, only C and CR strains have proved to be able to infect sour cherry (James et al., 2013; Garcia et al., 2014). Here, we discovered and partially characterized new cherry-adapted isolates that do not belong to these strains and seem to differ from any known strain of the virus. Furthermore, a high degree of divergence within the group of the Tat isolates was discovered, with the exception of the closely related Tat-2 and Tat-4. The interstrain nucleotide sequence identities between complete genomes of different PPV strains are ranged from 71.1% for the strains EA and C to 95.4% for the strains D and Rec (Subr and Glasa, 2008). In the 3′-terminal genomic region, the nucleotide sequence identities between the isolate RU-30sc (PPV-CR) and the other PPV strains were 78.8–83.9%, 78.7–85.6% and 92.2–95.4% for the genes NIb, CP and 3′-NCR, respectively (Glasa et al., 2013). Thus, the sequence divergence of the Tat isolates is comparable to the differences between the known PPV strains. They may represent new group(s) of cherry-adapted isolates which do not seem to belong to any

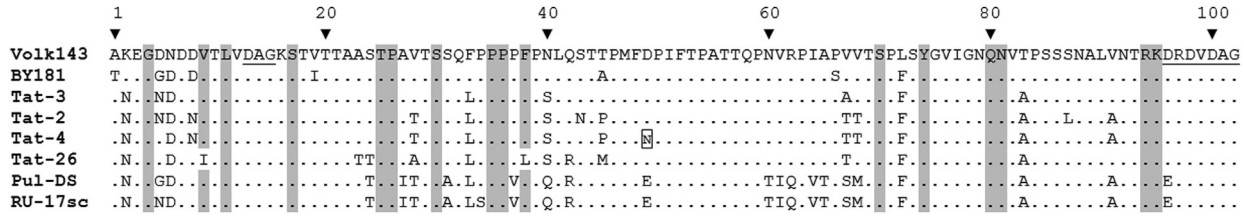

Fig. 5. Multiple alignment of the N-terminal sequences of the Plum pox virus coat protein of the Tat isolates and natural cherry-adapted isolates belonging to the strains C (Volk143, KJ787006; BY181, HQ840518) and CR (Pul-DS, JX472436; RU-17sc, KC020124). The alignment starts from the first CP amino acid. The amino acid positions shared by PPV-C and PPV-CR isolates (Glasa et al., 2013) are shaded. The DAG motif and universal epitope are underlined. The asparagine (N) residue at position 49 is boxed.

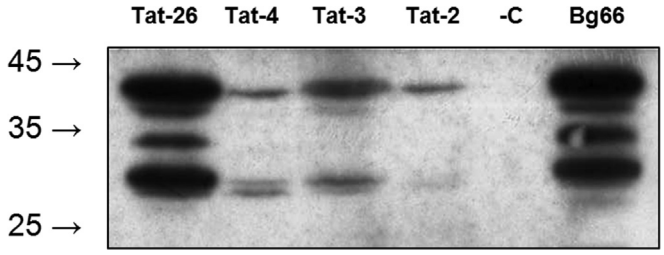

Fig. 6. Western blot analysis of the Tat isolates. The isolate Bg66 belongs to the strain C. -C - negative control from PPV-free cherry. Arrows to the left of the picture indicate the positions of the molecular weight markers (kDa) (Thermo Scientific).

known strain of the virus.

The isolates Tat-2/4, Tat-26 and PPV-C may derive from a common ancestor. The phylogenetic position of the Tat-3 remains to be determined. Full genome sequencing of the Tat-3 would contribute to establish the true taxonomic status of this isolate. These conclusions are based on the analysis of the comparatively short 3′-terminal genomic region of the new isolates. Although this region of PPV genomes is usually phylogenetically informative, whole-genome sequencing and further study on distribution of these isolates in the environment have to be performed to establish phylogenetic positions and taxonomic status of the Tat isolates. To date, it is considered that there are 9 strains of PPV, more than any other potyvirus (Garcia et al., 2014). The findings reported here show that the genetic diversity of PPV may be even higher and probably is not limited to the nine known strains of the virus.

The recombination event has been predicted in the CP gene of the Tat-3 (Fig. 4). The recombinant region was only 87.4% identical to the corresponding sequence of the minor parent probably due to accumulation of mutations in both isolates after the recombination. Alternatively, an unknown PPV-C isolate, more closely related to the Tat-3 in the recombinant region than the Volk143, might actually be a minor parent. Some uncertainty of the phylogenetic position of the Tat-3 seems to be related with recombination that is known to distort the results of the phylogenetic analysis. It should be noted that the possibility of recombination in the 5′-proximal segment of the CP gene was assumed for PPV-C (Nemchinov et al., 1998). Another recombination event between the strain C isolates SoC and SwC has been recently found in the 3'-terminal segments of their genomes (James et al., 2016). Apparently, recombination can play an important role in origin and evolution of the isolates infecting sour and sweet cherries.

The PPV-C-specific antibody AC did not react with the Tat-4 in TAS ELISA (Table 1). The epitope recognized by this antibody is not yet mapped. The antibody AC was found to compete with the polyclonal antibodies produced against a synthetic peptide corresponding to the residues 1–14 of the N-terminal region of the CP of the isolate SwC (Crescenzi et al., 1997; Myrta et al., 2000). The comparison of the first 14 aa of the CP of natural cherry-adapted isolates did not reveal any aa substitutions, which could explain the lack of the Tat-4 reactivity with the antibody AC. At the same time, asparagine acid (D) at position 49, shared by PPV-C and the isolates Tat-2, Tat-3 and Tat-26, is substituted with asparagine (N) in the CP of the Tat-4. If position 49 is part of the epitope recognized by the antibody AC, the D49N mutation can be crucial for the antibody binding due to obvious differences of the side chains of these amino acids. Four consecutive proline (P) residues at positions 34–37 may contribute to the folding of the polypeptide chain in such a way, that this putative epitope is located in close proximity to the very N-terminus of the CP.

The Tat isolates can be readily detected by serological and molecular methods using polyclonal antibodies to PPV, the monoclonal antibody 5B (Cambra et al., 1994) and either universal primers P1/P2 (Wetzel et al., 1991) or those targeting the 3'-NCR (Levy and Hadidi, 1994). However, serious discrepancies between serological and molecular methods were found while determining the strain of the new isolates. Three out of four Tat isolates (Tat-2, Tat-3, Tat-26) reacted with the PPV-C-specific antibody AC. As for the known strain-specific primers, the isolates Tat-2, Tat-4 and Tat-26 were recognized by RT-PCR using only the PPV-D-specific primers. This could potentially lead to misinterpretation of the strain typing results. The strain identification is very important to control the sharka disease. The new primers for specific detection of the Tat isolates should be apparently designed.

The strains C and CR are widespread in European Russia. They were never detected or only sporadically identified outside the former USSR until now. The prevalence of these strains in Russia is in obvious contrast with their rare findings in other regions of the world. In this work we characterized one more distinct group of isolates from sour cherry. They clustered with the other cherry-adapted strains and have a lot in common with them probably due to a common origin and adaptation to the same host. Thus, the vast majority of cherry-adapted PPV isolates were found in Russia suggesting that the strains C, CR as well as the Tat isolates originated from and evolved mainly in Russia.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2016.12.016.

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