Analysis of Genetic Diversity of Russian Sour Cherry *Plum pox virus* Isolates Provides Evidence of a New Strain

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Abstract

Plum pox virus (PPV) exists as a complex of nine strains adapted to different *Prunus* hosts. Unusual PPV isolates that do not belong to the known cherry-adapted strains were discovered on sour cherry in Russia. Here, two complete genomes of isolates Tat-2 and Tat-4 were determined by sequencing on the Illumina HiSeq 2500 platform. Both were composed of 9,792 nucleotides, excluding the poly(A) tail, with the organization typical of PPV and had 99.4 and 99.7% identity between each other at the nucleotide and amino acid levels. The sequence identities between Tat-2/Tat-4

and known PPV strains ranged from 77.6 to 83.3% for genomic RNA and from 80.0 to 93.8% for polyprotein. Phylogenetic analysis placed Tat-2 and Tat-4 in a separate clade, distinct from the C and CR strains. Three more Tat-2/Tat-4-like isolates were detected in local cherry plantings using the newly developed, specific RT-PCR assay. Based on the phylogenetic analysis, sequence identities, and environmental distribution, Tat-2, Tat-4, and related isolates represent a new cherry-adapted PPV strain for which the name PPV-CV (Cherry Volga) is proposed.

Plum pox virus (PPV) is the causal agent of sharka, the most important viral disease of stone fruit crops. The global economic losses are estimated at hundreds of millions of euros annually, and millions of PPV-infected peach, apricot, and plum trees have been destroyed to prevent the spread of the infection (Cambra et al. 2006). Typically, PPV induces characteristic symptoms on the leaves of infected *Prunus* plants that look like irregular pale green rings, bands, spots, or arabesques (Barba et al. 2011). The virus can be transmitted from plant to plant by aphids in a nonpersistent manner and through vegetative propagation (Rimbaud et al. 2015).

PPV is a member of the genus *Potyvirus*, family *Potyviridae*. The PPV genome is a single stranded positive sense RNA about 10 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'-noncoding regions (NCR), encodes a 355-kDa polyprotein processed by virus-specific proteases to 10 functional proteins (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb, and coat protein [CP]). A short overlapping ORF, called PIPO, was predicted within the P3 cistron and can be expressed as a P3N-PIPO fusion product via transcriptional slippage of viral RNA-dependent RNA polymerase (Rodamilans et al. 2015). Based on complete genome sequence differences and phylogenetic analysis, nine PPV strains (D, M, Rec, T, An, EA, W, C, and CR) are recognized to date (García et al. 2014).

Strains may differ in host preferences. Among known PPV strains, only two, C and CR, were believed able to infect sour and sweet cherries (Chirkov et al. 2013a; Glasa et al. 2013; James et al. 2013). However, four unusual PPV isolates (Tat-2, Tat-3, Tat-4, and Tat-26) were recently discovered on sour cherry in the Republic of Tatarstan, Middle Volga region of Russia. Phylogenetic analysis of the 3'-terminal sequences encompassing (Cter)NIb-CP-3'-NCR genomic region, allowed assigning the Tat isolates to the group of cherry-adapted strains. Except for Tat-4, they could be detected by triple antibody sandwich (TAS)-ELISA with the PPV-C-specific monoclonal antibody AC, suggesting that the Tat isolates may belong to strain C of PPV. However, all four clustered separately from the PPV-C and PPV-CR isolates and were not

recognized by reverse transcription (RT)-PCR using primers specific to the strains C or CR and to the strains M and W as well. At the same time, isolates Tat-2, Tat-4, and Tat-26 could be detected by RT-PCR using the PPV-D-specific primers P1/PD but were not identified by TAS-ELISA using PPV-D-specific monoclonal antibody 4DG5 (Chirkov et al. 2017). Thus, no correlation between the serological and molecular strain typing tests was observed.

Altogether, these results suggested that the new cherry-adapted isolates do not seem to belong to any known strain of the virus. However, analysis of partial genomic sequences may lead to a misinterpretation of strain typing data. For example, PPV-Rec and PPV-T isolates were for a long time considered as belonging to the strain M because their 3'-terminal genomic regions are very similar. The main differences between these strains are known to be associated with the 5'-proximal part of the genome due to some interstrain recombination (Glasa and Candresse 2005; Glasa et al. 2004; Ulubas Serçe et al. 2009). Hence, the affiliation of the isolate to a specific PPV strain can only be established on the basis of its complete genome sequence.

This study was aimed at sequencing and characterization of complete genomes of two distinct but closely related isolates Tat-2 and Tat-4 as well as studying their environmental dissemination. Analysis of their full-length genome sequences showed that they represent a new strain of PPV, for which we propose the name PPV-CV (Cherry Volga).

Materials and Methods

Sources of PPV genomic RNA. Sour cherry (*Prunus cerasus*) leaves proven to be infected with the isolates Tat-2 or Tat-4 (Chirkov et al. 2017) were used as a source of the virus. Immunocapture of PPV was performed according to Wetzel et al. (1992) with some modifications. Polyclonal PPV-specific antibodies from a reagent set SRA 31505/0500 (Agdia) were diluted 1/200 in 0.05 M carbonate buffer, pH 9.6, and incubated in disposable micro centrifuge tubes (Multiply-Pro cup 0.5 ml, Sarstedt) at 4°C overnight. Leaf extracts (100 µl), prepared as described previously (Chirkov et al. 2017), were incubated with the precoated antibodies 1 h at 37°C. The tubes were washed three times with phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 before and after incubation of plant sap. Genomic RNA from captured virus particles was released by treatment of tubes with 1% (v/v) Triton X-100 at 70°C for 10 min.

Complete genome sequencing. Genomic RNAs were employed for synthesis and amplification of the cDNA libraries using complete whole transcriptome amplification kit (WTA2, Sigma-Aldrich) as described previously (Sheveleva et al. 2013). The resulting libraries were subjected to high-throughput sequencing on the Illumina HiSeq 2500 platform. The contigs were assembled de novo using the

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SPAdes v.3.10.1 program (Bankevich et al. 2012). The PPV-relevant contigs were identified by a BLAST search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and aligned on the PPV genome that showed the greatest BLAST score.

To complete the sequencing of the genomes, appropriate primers were designed to fill internal gaps employing genomic RNAs as templates for RT and Encyclo DNA polymerase (Evrogen, Russia) for PCR. The 5'-terminal region of the Tat-2 and Tat-4 genomes was amplified using 5'RACE kit (Invitrogen, U.S.A.), following the manufacturer's protocol. The corresponding PCR products were purified from agarose gel with cleanup standard kit (Evrogen) and sequenced in both directions using Evrogen facilities (<http://evrogen.ru/>).

Sequence analyses. To compare Tat-2 and Tat-4 with other PPV isolates, the known complete genome sequences of PPV-C, PPV-CR, and representatives of other PPV strains were retrieved from GenBank (Table 1). In addition, the complete genomes of the Russian PPV-C (Bg6, Bg10, Bg26, Bg60, Bg66, Ka7, Ka15, Ka23) and PPV-CR (Kp8-2U, Kp8-1, Pul-DS, Fl-3, Pul-1) isolates, which are not yet deposited in GenBank, were used in the analyses.

Complete or partial nucleotide and amino acid sequences were aligned using the ClustalW v.2.1 program available online (<http://clustalw.ddbj.nig.ac.jp/>) or an older version of this program implemented in the BioEdit sequence alignment editor v.7.2.4 (Hall 1999). The sequence identity/divergence values were calculated by the ClustalW algorithm implemented in the MegAlign program v.7.1.0 (DNASTAR Lasergene). Analysis of the sequence variability along the genomes was performed using DNA Sequence Polymorphism (DnaSP v.5) software (Librado and Rozas 2009). The PhyML

3.0 version of maximum likelihood algorithm (Guindon et al. 2010) was used for phylogenetic analysis. Graphical tree presentation was performed by MEGA (v.4.0.1) program (Tamura et al. 2007). The recombination detection program v.4.69 (RDP4) (Martin et al. 2015) was employed to search for recombination in the aligned complete genome sequences listed above using default settings, with the exception that "sequences are linear" and "list events detected by >5 methods" options were chosen.

Specific RT-PCR detection of Tat-2, Tat-4, and related isolates.

Genomic RNA released from captured PPV particles was employed for the first strand cDNA synthesis using random primers and MuMLV reverse transcription (Evrogen). Two primer pairs were designed for the specific detection of Tat-2 and Tat-4 by PCR. The sense primer CV-P1F (5'-ACTGCAAGCGCATGGAAGGGGTTGGATGA-3') and antisense primer CV-P1R1 (5'-TCAGAAATGTTGCGTGGATTGAGGACGA-3') targeted Tat-2 complete genome at positions 321 to 349 and 928 to 955, respectively, within the P1 gene. The primers CV-N1bF (5'-ACGTGGATATATATGATGATCT-3') and CV-CPR1 (5'-TTGGTGGCGTATTCTGCAGGCT-3') corresponded to positions 8,107 to 8,128 and 8,694 to 8,715 in the N1b-CP region. The PCR included denaturation at 94°C for 1 min and the following cycles: denaturation at 94°C for 30 s, primer annealing at 60°C (CV-P1F/CV-P1R1) or 54°C (CV-N1bF/CV-CPR1) for 30 s and extension at 72°C for 40 s (35 cycles) with a final extension at 72°C for 7 min. The PCR products were analyzed by 2% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

PPV isolates 1410 (Sheveleva et al. 2012), Fl-3 (Chirkov et al. 2013a), Bg66 (Chirkov et al. 2013b), and Pav-17 (Chirkov et al.

Table 1. List of *Plum pox virus* isolates selected for full-size genome analyses

Isolate	Accession number ^a	Strain	Sampling location	<i>Prunus</i> host ^b
RU-17sc	KC020124	CR	Russia	<i>P. cerasus</i>
RU-18sc	KC020125	CR	Russia	<i>P. cerasus</i>
RU-30sc	KC020126	CR	Russia	<i>P. cerasus</i>
Kp8-2U	nd	CR	Russia	<i>P. cerasus</i>
Kp8-1	nd	CR	Russia	<i>P. cerasus</i>
Pul-DS	nd	CR	Russia	<i>P. cerasus</i>
Fl-3	nd	CR	Russia	<i>P. cerasus</i>
Pul-1	nd	CR	Russia	<i>P. cerasus</i>
BY101	HQ840517	C	Belarus	<i>P. cerasus</i>
BY181	HQ840518	C	Belarus	<i>P. cerasus</i>
Volk143	KJ787006	C	Russia	<i>P. cerasus</i>
SwC	Y09851	C	Italy	<i>P. avium</i>
SoC	AY184478	C	Moldova	<i>P. cerasus</i>
Bg6	nd	C	Russia	<i>P. cerasus</i>
Bg10	nd	C	Russia	<i>P. cerasus</i>
Bg26	nd	C	Russia	<i>P. cerasus</i>
Bg60	nd	C	Russia	<i>P. cerasus</i>
Bg66	nd	C	Russia	<i>P. cerasus</i>
Ka7	nd	C	Russia	<i>P. cerasus</i>
Ka15	nd	C	Russia	<i>P. cerasus</i>
Ka23	nd	C	Russia	<i>P. cerasus</i>
Tat-2	MF447179	CV	Russia	<i>P. cerasus</i>
Tat-4	MF447180	CV	Russia	<i>P. cerasus</i>
Fantasia	AY912056	D	Canada	<i>P. persica</i> var. <i>nectarina</i>
Ou1	AB545926	D	Japan	<i>P. mume</i>
Valjevka	JX013532	Rec	Croatia	<i>P. domestica</i>
BOR-3	AY028309	Rec	Slovakia	<i>P. armeniaca</i>
SK68	M92280	M	Hungary	<i>P. domestica</i>
GR0019	FM955843	M	Greece	<i>P. armeniaca</i>
AbTk	EU734794	T	Turkey	<i>P. armeniaca</i>
AL11pl	HF674399	An	Albania	<i>P. domestica</i>
LV-145bt	HQ670748	W	Latvia	<i>P. spinosa</i>
W3174	AY912055	W	Canada	<i>P. domestica</i>
EA-2005	AM157175	EA	Egypt	<i>P. armeniaca</i>
EA-2006	DQ431465	EA	Egypt	<i>P. armeniaca</i>

^a nd = Sequences not deposited in GenBank.

^b Complete genome sequencing of the following isolates was performed after their propagation in *Nicotiana benthamiana* (SwC, SoC [Fanigliulo et al. 2003], EA-2005 [Myrta et al. 2006], EA-2006 [Glasa et al. 2006]) or *N. clelandii* (SK68 [Palkovics et al. 1993]) plants.

Fig. 1. Phylogenetic analysis of the complete genome sequences of the *Plum pox virus* (PPV) isolates. The names of isolates are indicated on the branches; their affiliation to the PPV strain is shown on the right of the tree. Accession numbers of the cherry-adapted isolates are as follows: RU-17sc (KC020124), RU-18sc (KC020125), RU-30sc (KC020126), Tat-2 (MF447179), Tat-4 (MF447180), BY101 (HQ840517), BY181 (HQ840518), Volk143 (KJ787006), SwC (Y09851), SoC (AY184478). The complete genomes of the Russian PPV-C (Bg6, Bg10, Bg26, Bg60, Bg66, Ka7, Ka15, Ka23, Pul) and PPV-CR (Kp8-2U, Kp8-1, Pul-DS, FI-3, Pul-1) isolates are not yet deposited in GenBank. Representative isolates of other PPV strains included in the analysis: Fantasia (AY912056), Ou1 (AB545926), Valjevka (JX013532), BOR-3 (AY028309), SK68 (M92280), GR0019 (FM955843), AbTk (EU734794), AL11pl (HF674399), LV-145bt (HQ670748), W3174 (AY912055); strain El Amar (EA) was represented by two isolates (AM157175, DQ431465). 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 results were visualized by MEGA v.4.0.1 program. The scale bar indicates the number of substitutions per nucleotide. Bootstrap values (from 1,000 replicates) are displayed next to the relevant nodes. Nodes with less than 70% support were collapsed.

2016), belonging to the strains W, CR, C, and D, respectively, were used as controls to verify the specificity of the Tat-2/Tat-4 detection. PPV-M was provided with a K-11B kit (AgriTest, Italy). The negative control was prepared from PPV-free sour cherry leaves.

Study on Tat-2 and Tat-4 distribution in sour cherry plantings. The dissemination of Tat-2, Tat-4, and related isolates in the local sour cherry plantings was investigated using both primer pairs for the developed RT-PCR assay. Existing and abandoned collections, variety test plots, private gardens, and solitary wild trees, located at a distance of 0.1 to 1 km from the place of the initial Tat-2 or Tat-4 findings, were surveyed. Samples displaying typical symptoms of sharka disease were first analyzed by immunocapture RT-PCR as described above, with the exception that the universal primers P1 and P2 (Wetzel et al. 1991) were used for the PCR. PPV-positive samples were then retested by PCR for the presence of the strains D, M, C, W, CR according to the original protocols (Glasa et al. 2013; James and Varga 2004; Nemchinov and Hadidi 1998; Olmos et al. 1997) and also analyzed using the primers specific to Tat-2/Tat-4. Positive and negative controls are described above. The CP sequences of new isolates were amplified using the forward primer CVseq (5'-AAGCTGTACACCGATGAA GAGGCA-3') and 4CPR1 (Matic et al. 2011) targeting the C-terminal part of the NIb and 3'-NCR, respectively, followed by conventional Sanger sequencing. The CP sequences were deposited in GenBank under accession numbers MF447181 to MF447183.

Results

Genome assembly and characterization. The nearly complete genome sequences of Tat-2 and Tat-4, based on the de novo assembly, were generated. For the Tat-2 sample, five contigs (lengths from 562 to 2,839 nt) covered 91.6% of the most closely related genome (isolate BY181, HQ840518, PPV-C), with the exception of three internal gaps and 312 nt at the 5'-terminus of the genome. For Tat-4, one large contig of 9,725 nt was generated (14-fold average coverage), except for the 67 5'-terminal nucleotides. The remaining gaps were addressed by Sanger sequencing. No sequence differences were observed between the initial assembly and gap-covering PCR products in overlapping regions. It should be noted that the 3'-terminal genomic sequences (1,747 nt) of Tat-2 (KX685593) and Tat-4 (KX685595), determined previously by the conventional Sanger method (Chirkov et al. 2017), were identical to those obtained in this study by high-throughput sequencing. The full-size genome sequences of Tat-2 and Tat-4 were deposited in GenBank under accession numbers MF447179 and MF447180.

Genomes of Tat-2 and Tat-4 are composed of 9,792 nt, excluding the poly(A) tail, with the organization typical of PPV. The 5' and 3' NCRs are 146 and 217 nt in length. The large ORF of 9,426 nt starts with an AUG codon (positions 147 to 149) and terminates with UAG codon (positions 9,573 to 9,575). This ORF encodes a polyprotein of 3,142 aa with the molecular mass of 355.7 kDa. The PIPO +2 ORF

starts within a conserved G₁A₆ motif in the P3 cistron (position 2,909) and terminates at the nearest in-frame UGA stop codon (positions 3,224 to 3,226). Tat-2 and Tat-4 have 99.4 and 99.7% identity at the nt and aa levels, respectively. No recombination event was detected in the Tat-2/Tat-4 genomes (data not shown). Conserved KITC, PTK, and DAG motifs, associated with aphid transmission, were discovered in the HcPro and CP of both isolates. As for PPV-CR (Glasa et al. 2013), one triplet deletion at positions 6,865 to 6,867 compared with the PPV-C genomes was detected in Tat-2 and Tat-4, resulting in the deletion of aspartic acid residue at position 2,238 near the C-terminus of the NIa protein.

Sequence analysis and comparison with known PPV strains. The phylogenetic analysis included 34 full-length PPV sequences. It demonstrated that Tat-2/Tat-4 isolates are closely related to each other and form the cluster belonging to the group of cherry-adapted strains but clearly distinct from either C or CR strains as supported by high bootstrap value (999 of 1,000 replicates) (Fig. 1). It is similar to the values at the nodes separating acknowledged strains C/CR/W located in the same area of the tree. The tree also suggests that Tat-2/Tat-4 share a common ancestor with PPV-C. This result is in good agreement with the analysis performed for partial sequences of Tat-2/Tat-4 (Chirkov et al. 2017). The isolates Ka7, Ka15, and Ka23, belonging to strain C, were collected in the same geographic location as the Tat isolates.

Nucleotide and amino acid sequence similarities between individual genes of Tat-2 and representatives of different PPV strains are displayed in Table 2. Tat-2 is noticeably divergent from other PPV strains at the nucleotide level, regardless of the region of comparison. However, Tat-2/BY181 (PPV-C) analysis resulted in 96 to 99% identity within the 6K1, CI, and VPg proteins. The higher levels of identity were observed for the amino acid sequences indicating that many of the nucleotide substitutions in Tat-2 are silent. On the contrary, in PIPO, the values of the amino acid sequence similarity were lower than those estimated from nucleotide sequences, suggesting a prevalence of nonsynonymous mutations in this ORF. Similar results were obtained when comparing the PIPO of PPV-CR (isolate RU-30sc) with other PPV strains (Glasa et al. 2013). The high variability of the 5'-NCR is largely due to the striking differences in the sequences between nucleotides 65 to 146, whose similarities ranged from 39.0% for the pair Tat-2/Fantasia to 68.3% (Tat-2/BY181) and 73.2% between Tat-2 and RU-17sc. In contrast, 35 5'-terminal nucleotides implicated in RNA replication (Simón-Buela et al. 1997) were almost identical to other cherry-adapted isolates. However, thymidine and guanine at positions 24 and 28, respectively, were replaced with adenosine in the Tat-2 and Tat-4 genomes. Interestingly, position 24 is also occupied with adenosine in the isolates SoC and SwC (PPV-C), whose genomes were sequenced after long propagation of these isolates in herbaceous hosts (Fanigliulo et al. 2003).

The values of the sequence identity between Tat-2/Tat-4 and other cherry-adapted strains ranged from 82.6 to 83.3% for the genomic

Table 2. Percentages of nucleotide (upper row) and amino acid (lower row) sequence identities between different genomic regions of the isolate Tat-2 and representatives of the PPV strains C, CR, W, D, M, Rec, and EA

Isolate (accession number)	Strain	5' NCR ^a	P1	HcPro	P3	PIPO	6K1	CI	6K2	VPg	NIa	NIb	CP	3' NCR ^a	Genome	Polyprotein
BY181 (HQ840518)	C	81	81	80	86	91	88	86	82	84	79	80	86	95	83	
			90	93	93	82	98	99	91	96	90	92	93			93
RU-17sc (KC020124)	CR	83	81	82	81	87	83	84	81	84	80	80	86	95	83	
			90	95	88	73	92	96	87	95	94	91	92			93
LV-145bt (HQ670748)	W	70	75	81	80	87	81	81	74	81	78	80	82	93	80	
			79	91	87	78	92	95	77	91	90	91	83			89
Fantasia (AY912056)	D	62	72	79	76	82	80	78	80	78	77	77	79	94	76	
			78	91	81	72	90	95	85	88	91	89	82			88
SK68 (M92280)	M	62	72	79	76	81	76	80	79	78	77	78	80	93	78	
			79	91	81	69	90	94	85	89	89	89	84			88
BOR-3 (AY028309)	Rec	64	72	79	77	82	81	78	81	77	78	78	79	92	78	
			77	91	82	73	90	95	85	89	91	91	84			89
EA-2005 (AM157175)	El Amar	66	73	80	77	84	78	78	72	75	77	76	78	93	77	
			80	90	83	72	94	95	81	87	91	90	82			88

^a Noncoding region.

RNA and from 92.6 to 93.8% for the polyprotein. The variability between Tat-2 and PPV-CR is rather uniformly distributed along the genome with the exception of the peak at the N-terminus of the CP; the lowest level of polymorphism was detected at the C-terminus of the CP and 3'-NCR (Fig. 2). Unlike this, the diversity between Tat-2 and PPV-C is not evenly distributed across the genome. The high variability, observed in the P1, HC-Pro, NIa, and NIb genes and N-terminal segment of the CP, is in contrast with more conserved central and 3'-terminal parts of the genome. It is important to note that positions of the least polymorphic sites in the PPV-C, PPV-CR, and Tat2 genomes are almost identical, suggesting that some of them can be related to the adaptation of PPV to sour cherry.

Nine potential cleavage sites were identified in the Tat-2/Tat-4 polyproteins. Some variability was observed in their sequences when compared with other cherry-adapted isolates. A threonine is present

at position +1 in the CI/6K2 cleavage site of PPV-C and Tat-2/Tat-4, whereas the PPV-CR isolates (with the exception of RU-17sc) have a serine at this position. The cleavage site at the C-terminus of the VPg proteins of PPV-C and Tat-2/Tat-4 has an aspartic acid at position -3 instead of a glycine in PPV-CR. In the NIb-CP junction, PPV-C and Tat-2/Tat-4 have a histidine at position -2, whereas a threonine is present at this position in PPV-CR. The cleavage site between the NIa and NIb proteins seems to be the most polymorphic among the cherry-adapted strains. It consists of the heptapeptides DTVYTQ/S, ETVYNQ/S, and EVVYNQ/A in PPV-C, PPV-CR, and Tat-2/Tat-4, respectively. A valine at position -5 is unique for Tat-2/Tat-4, whereas an alanine at position +1 is shared by the isolates of the strain EA.

Seventy-five unique amino acid mutations in the polyproteins of natural cherry-adapted PPV-C and PPV-CR isolates, which may be

Fig. 2. Nucleotide diversity index between Tat-2 and representative isolates of known cherry-adapted strains CR (RU-17sc) and C (BY181) along the entire genome calculated using DnaSP v.5 software (Librado and Rozas 2009). Window of 100 nucleotides, step of 25 nucleotides. The red circles indicate the least polymorphic sites. The genome map of *Plum pox virus* (PPV) is presented in scale under the plot.

relevant to the adaptation of PPV to sour cherry, were previously identified (Glasa et al. 2013). Their comparison with the polyproteins of Tat-2/Tat-4 reduced the list to 57 amino acid positions (Table 3). It is worth noting that the significant part of the conserved amino acids (56%) is associated with the most variable genomic regions (P1 and N-terminus of the CP). A relatively large number of conserved amino acids in the VPg may reflect its important role in either virus infectivity (Jiang and Laliberté 2011) or systemic movement of the virus in sour cherry plants (Fanigliulo et al. 2003). Conserved amino acids were not found in the 6K1 and Nib proteins.

Development and application of the RT-PCR assay for the specific detection of the Tat-2/Tat-4 isolates. The immunocapture RT-PCR assay for the specific detection of Tat-2, Tat-4, and related isolates has been developed. Two primer pairs targeting the most variable regions of the virus genome (P1 and Nib-CP) were designed. The primers CV-P1F/CV-P1R and CV-NibF/CV-CPR1 amplified the expected PCR products of 645 and 617 bp, respectively. No reaction was observed with the isolates belonging to the strains D, M, C, CR, and W, indicating that both primer pairs seem to detect Tat-2 and Tat-4 specifically (data not shown).

Tat-2 and Tat-4 were initially found on cherry root offshoots in different abandoned collection plots (Chirkov et al. 2017). In this study, we tested >70 samples, randomly collected from surrounding symptomatic sour cherry plants, using RT-PCR with the universal and strain-specific primers. The vast majority of the PPV isolates found under this field survey belonged to PPV-C. In addition, three new Tat-2/Tat-4-like isolates were detected hundreds of meters from each other on cherry root offshoots (Tat-39, Tat-49) and a solitary tree of the old local cultivar Morel Rannyaya (Tat-36). They were successfully recognized by both Tat-2/Tat-4-specific primer pairs and, in agreement with the previous results (Chirkov et al. 2017), PPV-D-specific primers P1/PD (data not shown). No mixed infection with other PPV strains was detected in the plants infected with Tat-2/Tat-4-like isolates. The average CP sequence identities between the new isolates and Tat-2/Tat-4 were 99.5 and 99.1% at the nt and aa levels, indicating their very close genetic relationship.

Discussion

Unusual PPV isolates were recently discovered on sour cherry in Russia. Their 3'-terminal genomic sequences were highly divergent from the known strains of the virus (Chirkov et al. 2017). In this study, we sequenced and analyzed the full-size genomes of two closely related isolates Tat-2 and Tat-4 to determine their taxonomic status.

The phylogenetic analysis showed that Tat-2/Tat-4 formed a separate clade, distinct from C and CR strains and supported by the 100% bootstrap value. The divergence between the full-length genomes of Tat-2/Tat-4 and other PPV strains ranged from 19.5 to 27.7%, which agrees well with the values of the interstrain diversity calculated for known strains of the virus (4.4 to 22.8%) (García et al. 2014). On the other hand, the species demarcating criteria for the genera in the family *Potyviridae* are considered to be 74 to 78% for individual genes with the exception of the P1, which is characterized by a more pronounced genetic variability (Adams et al. 2005).

The values of the nucleotide sequence identity between Tat-2 and other PPV strains ranged from 71.7 to 90.5% for individual genes (Table 2), which allows allocating these isolates to a separate strain of the virus. In addition to Tat-2 and Tat-4, three more distinct Tat-2/Tat-4-related isolates were detected by the specific RT-PCR assay.

Based on the results of phylogenetic analysis, sequence identities, and environmental distribution, the isolates Tat-2, Tat-4, and three new ones reported in this study seem to represent a new PPV strain for which the name PPV-CV (Cherry Volga) is proposed due to its host and locality. This is the third cherry-adapted strain of the virus.

To ensure the rapid and specific detection of PPV-CV, the RT-PCR assay was developed using primers targeting the most variable regions of the virus genome (P1 and Nib-CP). Both primer pairs demonstrated consistent results, suggesting that the assay is suitable for the specific detection of the new strain CV. The PPV-D-specific primers P1/PD may be also used in RT-PCR as an additional tool to identify PPV-CV. Interestingly, both CV-specific primer pairs failed to recognize the isolates Tat-3 and Tat-26 supporting the idea that those two may belong to a yet unrecognized PPV strain (Chirkov et al. 2017).

A study on the prevalence of PPV-CV in the local sour cherry plantings using the strain-specific RT-PCR showed its rather limited spread. Among more than 70 samples tested, PPV-CV was revealed in only five. Remarkably, four of the five CV-like isolates were detected on root offshoots and one of them was found on the own-rooted (nongrafted) cherry tree. These data make virus spread through vegetative propagation of infected plants highly improbable and, instead, strongly suggest a crucial role of vector transmission in virus dissemination among the local plantings. *Myzus cerasi* was the only abundant aphid species in the surveyed plantings. However, this colonizing species is not considered to be a PPV transmitter (Labonne et al. 1995). Possibly, the limited PPV-CV spread may be explained by the low number and/or low efficiency of a yet unidentified visiting vector suitable for this new strain. Further analysis of numerous symptomatic root offshoots may contribute to more accurate estimation of the PPV-CV distribution.

The plantings surveyed in this study were established in the late 1950s by the introduction of a multitude of sour cherry cultivars from all over the former Soviet Union. In particular, many cultivars originated in the Urals and Siberia regions of Russia in which the PPV incidence or diversity had never been explored. The fact that the PPV-CV isolates were largely detected on root offshoots in long abandoned plantings may argue in favor of the introduction of this strain in Tatarstan. Nevertheless, we cannot exclude the possibility that the strain CV has emerged in the place where it was discovered. Apart from PPV-CV, many PPV-C isolates and the highly divergent isolates Tat-3 and Tat-26, whose phylogenetic status is yet to be determined, were found in these plantings as well (Chirkov et al. 2017). In any case, the results show that old stone fruit plantings can be a valuable source of rare and atypical PPV isolates.

The discovery of the third cherry-adapted PPV strain can contribute to a better understanding of the origin, genetic diversity, and evolution of the virus and facilitate the identification of virus-specific determinants responsible for adaptation of PPV to sour cherry.

Table 3. Unique amino acid positions conserved in the polyproteins of natural isolates of the cherry-adapted strains C, CR, and CV

Protein	Number of conserved amino acids	Positions of conserved amino acids ^a
P1	18	V ²⁵ , A ³⁰ , I ³⁷ , W ¹¹⁷ , E ¹²³ , H ¹²⁷ , T ¹³⁷ , D ¹⁴³ , P ¹⁴⁵ , R ¹⁴⁷ , K ¹⁵¹ , D ¹⁶² , N ¹⁸⁰ , T ¹⁹⁴ , H ¹⁹⁶ , R ²⁰³ , Q ²⁰⁶ , R ²⁶⁶
HcPro	3	R ⁴⁶⁶ , E ⁶⁴⁰ , M ⁷⁶²
P3	6	I ⁸⁸⁸ , N ⁹¹⁴ , A ¹⁰⁰⁰ , A ¹⁰²² , Y ¹⁰⁸¹ , M ¹⁰⁸²
CI	3	M ¹²²⁷ , Y ¹⁴¹⁵ , Q ¹⁷⁷⁹
6K2	1	A ¹⁸⁵⁴
VPg	9	G ¹⁹⁴⁹ , I ¹⁹⁵⁷ , E ¹⁹⁶¹ , P ¹⁹⁷⁰ , A ¹⁹⁷⁵ , L ¹⁹⁷⁹ , V ¹⁹⁸⁴ , A ²⁰³⁸ , S ²⁰⁴³ ,
NIa	1	N ²²¹¹
(N-ter)CP ^b	14	G ²⁸¹⁴ , L ²⁸²¹ , S ²⁸²⁷ , T ²⁸³⁵ , P ²⁸³⁶ , S ²⁸⁴⁰ , P ²⁸⁴⁵ , P ²⁸⁴⁶ , S ²⁸⁸⁰ , Y ²⁸⁸⁴ , Q ²⁸⁹⁰ , N ²⁸⁹¹ , R ²⁹⁰⁴ , K ²⁹⁰⁵
(C-ter)CP	2	S ²⁹¹⁸ , S ²⁹⁷⁹

^a Numbering according to the RU-30sc polyprotein sequence (Glasa et al. 2013).

^b Amino acids 1 to 102 of the coat protein.

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