Characterization of Sour Cherry Isolates of *Plum pox virus* from the Volga Basin in Russia Reveals a New Cherry Strain of the Virus

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ABSTRACT

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Plum pox virus (PPV) is the causal agent of sharka, the most detrimental virus disease of stone fruit trees worldwide. PPV isolates have been assigned into seven distinct strains, of which PPV-C regroups the genetically distinct isolates detected in several European countries on cherry hosts. Here, three complete and several partial genomic sequences of PPV isolates from sour cherry trees in the Volga River basin of Russia have been determined. The comparison of complete genome sequences has shown that the nucleotide identity values with other PPV isolates reached only 77.5 to 83.5%. Phylogenetic analyses clearly assigned the RU-17sc, RU-18sc, and RU-30sc isolates from cherry to a distinct cluster, most closely related to PPV-C and, to a lesser extent, PPV-W. Based on their natural infection of sour cherry trees and genomic characterization, the PPV isolates reported here represent a new strain of PPV, for which the name PPV-CR (Cherry Russia) is proposed. The unique amino acids conserved among PPV-CR and PPV-C cherry-infecting isolates (75 in total) are mostly distributed within the central part of P1, NIa, and the N terminus of the coat protein (CP), making them potential candidates for genetic determinants of the ability to infect cherry species or of adaptation to these hosts. The variability observed within 14 PPV-CR isolates analyzed in this study (0 to 2.6% nucleotide divergence in partial CP sequences) and the identification of these isolates in different localities and cultivation conditions suggest the efficient establishment and competitiveness of the PPV-CR in the environment. A specific primer pair has been developed, allowing the specific reverse-transcription polymerase chain reaction detection of PPV-CR isolates.

Additional keywords: diversity, potyvirus.

Plum pox virus (PPV) is the only potyvirus (genus: *Potyvirus*, family: *Potyviridae*) known to infect stone fruit trees. PPV causes sharka disease in peach, plum, apricot, almond, Japanese plum, and a wide range of ornamental and wild *Prunus* spp. (2,18). In addition, although the majority of PPV isolates do not infect cherry trees, a few isolates naturally infect sweet cherry (*Prunus avium* L.) and sour cherry (*P. cerasus* L.) (13,16,24,25,31,38,40).

PPV has a single-stranded, positive-sense RNA genome of ≈9.7 kb. The genome, like that of any other potyvirus, has untranslated terminal regions (UTRs) flanking a single large open reading frame (ORF), and the corresponding viral polyprotein is cleaved by three virus-encoded proteases (P1, HC-Pro, and NIa-Pro) into 10 products (P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa-Pro, NIb, and coat protein [CP]) (28). An additional ORF (PIPO) embedded within the P3-coding region is translated as a fusion protein after +2 frameshifting (12).

Understanding the molecular variation of plant viruses is an essential step to designing good management strategies. On the basis of serological and molecular differences, seven PPV strains are now recognized (2). Among those, PPV-M, -D, and -Rec are widespread, while PPV-EA, -C, -T, and -W have lesser prevalence or geographical distribution (2,8,47).

Cherry trees were considered immune to PPV infection, because Dosba et al. demonstrated in 1987 (15) the failure of PPV-M and -D isolates to infect cherry tree genotypes after chip inoculation. Viruliferous aphid inoculation resulted in localized infection that rapidly became undetectable.

The first published reports of natural infection of sour cherry trees by PPV date back to the 1990s from Moldova (25,38,42). It has been reported that the isolates involved had been introduced into Moldova with infected sour cherry cultivars coming from Russia in the early 1970s (25,38,42). Molecular characterization of PPV isolates that naturally infect sour cherry trees indicated that they form a new strain of PPV, which was termed PPV-cherry (PPV-C) (37,38). PPV infecting sour or sweet cherry trees were later reported in Italy (13), Hungary (40), Belarus (31), and Croatia (24) although, in some cases, only as occasional findings. Thus far, all PPV isolates adapted to cherry hosts have been found to be the PPV-C strain (17,37,38). PPV-C isolates have been shown experimentally to be transmitted systemically to seedlings of sour and sweet cherry cultivars and hybrids as well as to other stone fruit species (2–4,13,16,25,38,41) Thus, the experimental woody host range of PPV-C appears to be wider than that of conventional PPV isolates, which do not infect cherry species. More information may be needed on the cherry-adapted isolates of PPV in

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains two supplementary tables and two supplementary figures.

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order to have an understanding of their effect on the cherry industry. Given the major impact of conventional PPV strains on stone fruit trees other than cherry (2,7,46), the need to further study cherry-adapted isolates seems clear.

In this study, PPV isolates originally found naturally infecting sour cherry trees in the Samara and Saratov regions of the Volga River basin, Russia, were characterized by partial and complete genome sequencing. Based on their high sequence divergence from other known PPV strains, including PPV-C, we propose that these isolates represent a second, cherry-infecting strain of PPV, termed PPV-Cherry Russia (PPV-CR).

MATERIALS AND METHODS

Viral isolates. The isolates used in the present study were obtained in 2010, 2011, and 2012 from leaves or winter shoots of symptomatic sour cherry trees (Supplementary Figure 1). The trees were grown in private gardens; commercial, experimental, or collection orchards; or, in one case, as a solitary wild tree. The isolates were obtained from different localities of the Samara and Saratov regions in southern Russia (the Volga River basin) (Table 1). One isolate was transferred to *Nicotiana benthamiana* plants by mechanical inoculation from forced winter buds. In an effort to insure that the symptoms observed were not caused by *Prune dwarf virus* or *Prunus necrotic ringspot virus*, samples were tested by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and found negative for the two viruses.

The samples were first analyzed by DAS-ELISA using polyclonal antibodies (Agden, Ayr, Scotland, UK) and by routine reverse-transcription polymerase chain reaction (RT-PCR) using polyvalent, PPV-specific P1/P2 primers (51). For further analysis, the samples were sent to Institute of Virology, Department of Plant Virology, Slovak Academy of Sciences (IV SAS), Bratislava as either dried leaves, winter budwood, or total RNAs adsorbed on FTA Whatman membranes (GE Healthcare, Buckinghamshire, UK) (1,36).

RT-PCR amplification of PPV isolates. Total RNAs were extracted from leaves or buds using the NucleoSpin RNA Plant kit (Macherey-Nagel, Düren, Germany). Alternatively, total RNAs were recovered from FTA cards as recommended by the supplier. A two-step RT-PCR protocol was used. The first-strand cDNA was synthesized using random hexamer primers, and the PrimeScript (MMLV) reverse transcriptase (TaKaRa Bio Inc., Shiga, Japan) was used as recommended by the supplier. A broad-spectrum PPV primer pair, NcuniFor/NcuniRev, amplifying a 746-bp fragment spanning the C-ter NIb/N-ter CP region (nucleotide positions 8,316 to 9,061 of isolate BOR-3, AY028309) was used together with the TaKaRa Ex Taq polymerase (TaKaRa Bio Inc.) as described (43). The RT-PCR products were gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI), and directly sequenced in both directions by priming the sequencing reactions with the same oligonucleotides as used for PCR amplification.

Full-length sequencing of PPV isolates. Complete genome sequences of the RU-17sc, RU-18sc, and RU-30sc isolates were obtained by the primer walking method (Supplementary Table 1). In total, eight overlapping PCR fragments spanning the whole genome were amplified using the TaKaRa LA Taq polymerase (TaKaRa Bio Inc.). All PCR products were directly sequenced as above. When necessary, the viral sequence was verified by independent PCR amplification and sequencing using custom-made primers. The 5′ end of the genome was amplified using a 5'-Full RACE Core Set kit (TaKaRa Bio Inc.) with the 5′-end phosphorylated antisense primer CR717P (5′TCTCAACCTTC TGC3′) and four gene-specific primers: CR640A2 (5′TCATCTT TGACAAGGTCAG3′, antisense), CR316S1 (5′CAGGCACTA GCTGTTGCAC3′, sense), CR165S2 (5′GATGACAAGTAAATG AGCC3′, sense), and CR504A1 (5′CGATTCCAGTGGCTCA ATG3′, antisense), following the manufacturer's instructions.

Sequence analyses. Partial and complete sequences were compared with the PPV sequences available in the GenBank database (www.ncbi.nlm.nih.gov) and with the PPV sequences generated by the SharCo FP7 European project (http://www.sharco.eu/sharco/). Sequence analyses were performed using either MEGA v.5 (50) or DnaSP v.5 (27) as well as the Expasy online tool (http://web.expasy.org/compute_pi/). The phylogenetic trees were inferred using the neighbor-joining algorithm implemented in MEGA v.5. Strict identity distance metrics were used for both nucleotide and amino acid sequence analyses. A search for potential recombination events involving the RU-17sc, RU-18sc, and RU-30sc isolates was performed using a dataset consisting of full-length sequences of a representative member of each known PPV strain and the RDP3 program (33).

Specific RT-PCR detection of PPV-CR isolates. For the specific detection of PPV-CR isolates, a two-step RT-PCR protocol was developed. Briefly, cDNA was synthesized from a total RNA extract (NucleoSpin RNA Plant kit; Macherey-Nagel) using random hexamer primers and AMV reverse transcriptase (both from Promega Corp.). An aliquot of cDNA was added to PCR reactions containing EmeraldAmp GT PCR Master Mix (TaKaRa Bio Inc.) and PPV-CR-specific primers CR8597F (5′ATGATGTG ACGTTAGTGGAC3′, sense) and CR9023R (5′TCGTGTGTTAG ACAGGTCAAC3′, antisense) targeting the 5′ terminal CP region (nucleotide positions 8,597 to 9,023 on the RU-30sc genome). The P1 and P2 universal primers (51) were used in a parallel PCR to confirm the presence of PPV in all tested samples. For both primer sets, the same cycling conditions were used: denaturation at 98°C for 1 min, 35 cycles of amplification (98°C for 30 s, 55°C for 30 s, and 72°C for 30 s), and a final extension at 72°C for

TABLE 1. List and location of *Plum pox virus*-Cherry Russia isolates from sour cherry trees analyzed in the present study, together with the identity and accession numbers of the sequences determined

Isolate	Geographical region (oblast)	Locality (town)	Status	Sequenced region	Accession number
RU-17sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	Complete genome	KC020124
RU-18sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	Complete genome	KC020125
RU-19sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	NIb-CP	KC020127
RU-20sc	Samara	Stromilovskije Dachi	Private garden	NIb-CP	KC020128
RU-30sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	Complete genome	KC020126
RU-32sc	Saratov	Khvalynsk	Wild solitary tree	CP	KC020131
RU-34sc	Saratov	Khvalynsk	Old experimental orchard	CP	KC020132
RU-35sc	Saratov	Khvalynsk	Old experimental orchard	CP	KC020133
RU-37sc	Samara	Malaya Czarjevtchina	Production intensive orchard	CP	KC020135
RU-38sc	Samara	Stromilovskije Dachi	Private garden	CP	KC020134
RU-39sc	Samara	Stromilovskije Dachi	Private garden	NIb-CP	KC020129
RU-40sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	CP	KC020130
RU-62sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	NIb-CP	KC539101
RU-63sc	Samara	Malaya Czarjevtchina	Cultivar collection orchard	NIb-CP	KC539102

5 min. All PCR products were analyzed by nondenaturing electrophoresis in 1% (p/vol) agarose gels and GoldView staining (Guangzhou Geneshun Biotech, Guangzhou, China).

Serological detection and characterization. The RU-30sc isolate transmitted to *N. benthamiana* was tested by different DAS-ELISA commercially available kits (Bioreba AG, Reinach, Switzerland; Adgen; and MagicDAS Plant Print Diagnostics, Valencia, Spain) according the manufacturer's instructions and by polyclonal antibodies (PAbs) produced at Instituto Valenciano de Investigaciones Agrarias (IVIA, Spain), IV SAS (Slovakia), or INRA (France). Its reaction by DAS or DASI-ELISA-based assays (5) was also assayed against a panel of 12 different typing and PPV-specific monoclonal antibodies (MAbs), including 5B-IVIA/AMR (polyvalent); AL (PPV-M and -Rec specific), 4DG5 (PPV-D specific), AC and TUV (PPV-C specific) and 4CB1 (PPV-NAT specific) (6); and 05 (23), XR4, 3C6, 4DB12, 1EB6, and 4DB7 (9,29). The PAb R3-IVIA as well as the MAb 5B-IVIA/AMR were also tested by DAS-ELISA using RU-30sc-infected *N. benthamiana* leaves homogenized 1:30 (wt/vol) in a healthy *N. benthamiana* leaves homogenate prepared at pH 5, 6, 7, and 8.

Aphid transmission. Experimental aphid transmission was performed under controlled conditions using a clonal culture of the green peach aphid (*Myzus persicae* Sulzer) and a technique involving a controlled acquisition access period (26). *N. benthamiana* plants infected with the RU-30sc isolate were used as inoculum source and healthy *N. benthamiana* at the five-leaf stage as test plants. Twenty aphids were transferred to each test plant after a 2-h starvation period followed by a 5-min acquisition period on the source leaves. Transmission results analysis was carried out using DAS-ELISA after a 2-week period.

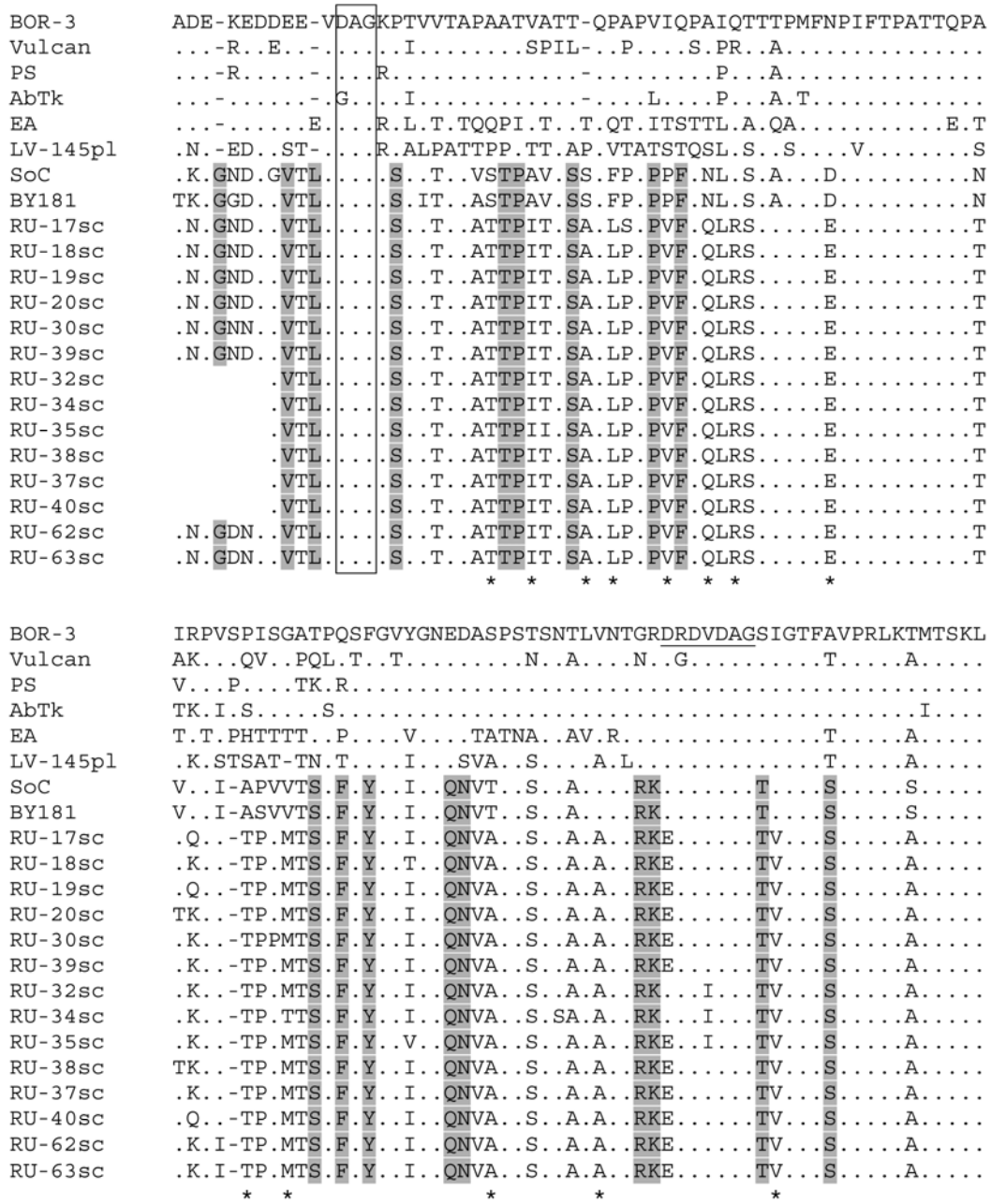

Fig. 1. Multiple alignment of the amino acid sequences of the N-terminus hypervariable region of the capsid protein of *Plum pox virus* (PPV)-Cherry Russia (CR) isolates and of representative isolates of other PPV strains. The alignment starts from the first coat protein amino acid. Amino acids identical to those of the BOR-3 (PPV-Rec) isolate are indicated by dots, while deletions are marked by a dash. The DAG motif associated with aphid transmission is boxed. Amino acids unique for PPV-CR isolates are marked by asterisks below the alignment. Amino acids shared only within cherry-adapted isolates (PPV-CR and PPV-C) are gray. The position of the 5B-IVIA-recognized epitope DRDVDAG (10) is underlined.

RESULTS

Characterization and genome organization of PPV isolates from sour cherry in the Volga River basin of Russia. PPV symptoms observed on sour cherry trees consisted of discolorations of the leaf along veins, light green bands or arabesques, and leaf distortions (sometimes in the shape of rings), as observed for PPV infection in other *Prunus* hosts. In other plants, strong yellow chlorosis along the leaf main veins was also observed. Information on symptoms of the virus on fruit is missing because sour cherry trees failed to produce fruit or fruit had dropped to the ground due to fungal infection or physiological injuries at the time of sampling. PPV infection in leaves was confirmed by DAS-ELISA with polyclonal antibodies and by RT-PCR with the polyvalent, PPV-specific P1/P2 primers but not with any strain-specific primer set (44). Moreover, an initial sequence analysis of the NIb-CP region revealed only ≈78% identity with PPV-C isolates and only ≈70% identity with PPV-M, -D, and -Rec isolates (20).

In order to determine the phylogenetic status of these cherry isolates, genome sequences were determined for three isolates (RU-17sc, RU-18sc, and RU-30sc) by sequencing eight PCR fragments spanning the full genome. The RU-17sc, RU-18sc, and RU-30sc isolates have a genome of 9,792 nucleotides, excluding the polyA tail. These sequences have been deposited in the GenBank database and the relevant accession numbers are provided in Table 1. The genomic organization is typical of PPV (2) and other potyviruses, with a large ORF and a putative P3N-PIPO ORF. The polyprotein ORF starts at nucleotide position 147 and terminates with a UAA stop codon at position 9,573 to 9,575, with a 217-nucleotide long 3′ untranslated region. This ORF encodes a polyprotein of 3,142 amino acids. The molecular weight of RU-17sc, RU-18sc, and RU-30sc polyproteins are 355.309, 355.489, and 355.609 kDa, respectively. All these elements are closely related to those observed for other PPV strains.

In particular, the RU-17sc, RU-18sc, and RU-30sc isolates exhibit a strict co-linearity to PPV-C isolates, except one triplet deletion at the end of the NIa gene, resulting in an amino acid deletion at position 2,238 compared with PPV-C polyprotein. Similar to PPV-C, the CP of PPV RU-17sc, RU-18sc, or RU-30sc is two amino acids longer than that of PPV-M, -D, or -Rec due to insertions in the CP N terminus (Fig. 1).

The small putative protein P3N-PIPO (12) is also observed overlapping with the P3 protein gene at positions 2,906 to 3,217, starting at the same position as reported for PPV-D (30). The total length of the protein is 106 amino acids, identical to those of PPV-C (BY-181) and PPV-W (LV-145bt) but 1 amino acid longer than that of PPV-M (SK-68) and PPV-T (AbTk) or 3 amino acids longer than PIPO of PPV-Rec (BOR-3) and PPV-D (Vulcan).

Nine putative protease cleavage sites were identified in the polyprotein based on the recognition motives for the three viral proteases in other PPV strains so that, as in other potyviruses, the large polyprotein is predicted to code for 10 proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, Nib, and CP (2). However, all cleavage sites contained between one and three mutations compared with at least some other PPV strains. These mutations generally affected positions showing variability between PPV strains. Except for a threonine-to-serine mutation at the P1 position of the CI/6K2 cleavage site in the RU-18sc isolate, the three Russian isolates from the Volga River basin showed identical cleavage site sequences (Supplementary Table 2). All motifs characteristic of potyviral proteins are conserved in the genomes of these three isolates, including the $FRNK_{489–492}$, $KITC_{360–363}$, and $PTK_{618–620}$ motifs in the HC-Pro and the $DAG_{2,823–2,825}$ tripeptide involved in aphid transmission in the N terminus of the CP (numbering according to the RU-30sc polyprotein sequence).

Sequence comparison with other PPV isolates. The sequence identities among RU-17sc, RU-18sc, and RU-30sc isolates were 99.1 to 99.2 and 99.2 to 99.4% at the nucleotide and amino acid level, respectively. This gives an overall within group nucleotide diversity (average pairwise divergence) of 0.8% (±0.1), showing that these isolates are very closely related, which could possibly reflect the fact that they were isolated from the same locality in the collection orchard of the Horticultural Research Institute of Malaya Czarjevtchina in the Samara region. However, from the plot of ≈10 ha, they were isolated from trees 100 to 150 m apart and of different genotypes.

The comparison of complete genome sequences has shown that the nucleotide identity values with other PPV isolates reached 77.5 to 83.5% (Table 2), the most closely related being the BY181 isolate (PPV-C strain) and most distant the ElAmar isolate (PPV-EA). The highest divergence levels between RU-17sc (and RU-18sc and RU-30sc) and BY181 were in the P1, 3′-terminal part of P3 and 5′-terminal part of the CP gene (Supplementary Figure 2), known to be the most variable parts of the PPV genome (28). The overall amino acid identity for the large genomic polyprotein was 93.5 to 88%, with, again, the highest identity level being observed with the PPV-C strain.

No evidence of a recombination signal could be identified in the genomes of RU-17sc, RU-18sc, and RU-30sc isolates as determined by the RDP3 program (33) using a data set including the complete genome sequences of representative isolates of the seven previously identified PPV strains (data not shown).

TABLE 2. Percentages of nucleotide and amino acid (in parentheses) sequence identities between various genomic regions of the RU-30sc isolate and the corresponding regions of fully sequenced *Plum pox virus* (PPV) isolates representative of known PPV strains[a]

Strain, isolate (accession number)	5′ UTR	P1	HC-Pro	P3	PIPO	6K1	CI	6K2	NIa	NIb	CP	3′ UTR	Genome[b]	Polyprotein[c]
PPV-C, BY181 (HQ840518)	82.2	85.0 (93.8)	84.9 (96.7)	81.1 (86.3)	87.9 (77.9)	82.1 (92.3)	82.9 (96.5)	79.9 (88.7)	82.3 (94.7)	82.2 (93.2)	85.6 (90.7)	95.4	83.5	93.5
PPV-W, LV-145bt (HQ670748)	68.5	77.7 (79.9)	82.0 (93.7)	80.4 (84.6)	88.2 (79.8)	84.6 (92.3)	81.7 (95.7)	71.7 (75.5)	81.9 (92.9)	83.9 (93.6)	81.9 (83.9)	93.5	81.6	90.3
PPV-M, SK68 (M92280)	60.3	76.0 (79.5)	79.5 (91.7)	75.0 (77.4)	81.4 (69.2)	82.1 (88.5)	79.2 (94.0)	76.7 (83.0)	76.6 (89.7)	81.6 (92.9)	81.4 (80.9)	92.6	78.8	88
PPV-T, AbTk (EU734794)	63	75.9 (78.9)	79.4 (91.0)	74.7 (77.1)	80.5 (67.3)	78.8 (90.4)	79.8 (94.5)	75.5 (83.0)	76.5 (90.4)	81.7 (93.4)	80.3 (79.6)	92.2	78.7	88
PPV-Rec, BOR-3 (AY028309)	63	75.1 (77.9)	79.5 (91.9)	75.6 (78.9)	81.1 (68.6)	78.2 (90.4)	79.6 (95.3)	79.9 (90.6)	77.3 (90.1)	79.6 (93.6)	81.4 (81.5)	92.2	78.6	88.7
PPV-D, Vulcan (AY912057)	61.6	75.3 (78.6)	79.3 (91.5)	76.1 (78.9)	81.7 (69.6)	76.9 (90.4)	79.3 (94.8)	79.9 (88.7)	77.6 (90.4)	78.8 (92.5)	79.3 (79.3)	94.0	78.3	88.1
PPV-EA, ElAmar (DQ431465)	62.3	73.7 (79.9)	78.6 (90.4)	74.5 (81.1)	82.4 (71.2)	75.6 (84.6)	77.8 (94.6)	80.5 (94.3)	75.9 (89.0)	80.4 (92.7)	78.7 (78.9)	93.5	77.5	88.1

[a] UTR = untranslated terminal region and CP = coat protein.
[b] Complete genome (nucleotides).
[c] Complete polyprotein (amino acids).

Phylogenetic analyses based on complete genome sequences (Fig. 2) confirmed the homology analysis results and clearly assigned the RU-17sc, RU-18sc, and RU-30sc to a distinct cluster, supported by a 100% bootstrap value. This cluster was most closely related to PPV-C and, to a lesser extent, PPV-W. PPV-C has been reported to have its origin in Russia (25,38) and, during the last several years, both PPV-C and PPV-W have been recorded from Russia or former USSR countries (19,31,34,38,48).

Overall, the multiple alignment of complete polyprotein sequences has revealed from 203 (BY181) to 378 (AbTk and SK68) amino acid substitutions between RU-30sc and representatives of other PPV strains. Interestingly, 48 of 203 amino acid substitutions (23.6%, a clear over-representation) between RU-30sc and BY181 are located within P3.

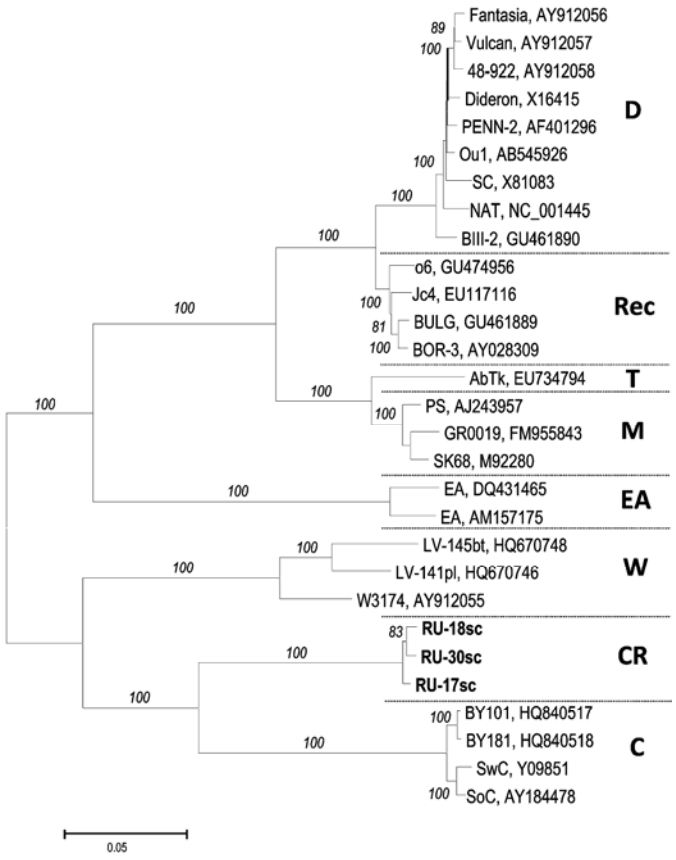

Fig. 2. Phylogenetic tree of *Plum pox virus* (PPV) isolates generated from selected complete nucleotide genome sequences retrieved from GenBank and the three sour cherry isolate sequences determined in the present study (highlighted in bold). Isolates are identified by their accession numbers. The scale bar indicates a genetic distance of 0.05. Bootstrap values >70 (1,000 bootstrap resamplings) are indicated on the branches as percentages. The phylogenetic tree was reconstructed using the neighbor-joining algorithm implemented in MEGA v.5. The affiliation of the isolates to PPV strains is indicated to the right of the tree.

In contrast, BY181 and BY101, being natural cherry-adapted isolates, the sequences of which have been determined from original cherry tissue and maintained in cherry (accession numbers HQ840517 and HQ840518) share a total of 75 unique amino acid positions with RU-17sc, RU-18sc, and RU-30sc. Although these amino acid positions shared between cherry-adapted isolates are located in all functional products (except 6K1), they are mostly distributed within the central part of P1 and NIa and the N terminus of the CP, making them potential candidates of genetic determinants of the ability to infect cherry species or of adaptation to these hosts (Table 3). Six of these amino acids positions are not conserved in PPV-C, SoC (AY184478) and PPV-C, SwC (Y09851) isolates, possibly due to the propagation history of these isolates in herbaceous plants prior the genome sequence determination (Table 3).

Aphid transmission and serological characterization of the cherry isolates from the Volga River basin. The RU-30sc isolate was successfully transmitted by mechanical inoculation from forced-infected sour cherry buds to *N. benthamiana* plants. The RU-30sc isolate was transmitted by green peach aphids from infected *N. benthamiana* to uninfected *N. benthamiana* plants with 100% efficiency in two independent experiments (10/10 plants tested positive in DAS-ELISA and RT-PCR; results not shown). The specificity of transmission was verified by direct sequencing of the CR8597/CR9023-amplified PCR product (see below).

The various isolates under study, including the RU-30sc transmitted to *N. benthamiana*, reacted positively with the tested serological PPV detection kits as well as with all tested PAbs. RU-30sc also positively reacted in DASI-ELISA with MAbs 5B-IVIA/AMR, 05, XR4, and 3C6 but did not react against any of the strain-specific MAbs tested (AL, 4DG5, AC, TUV, and 4CB1) or with the other tested PPV-specific MAbs. The positive recognition of RU-30sc by the universal 5B-IVIA/AMR MAb was substantially influenced by the pH of the plant extract, with highest optical density (OD) values recorded with an extract prepared at pH 6.0 but lower ODs observed at pH 7.0 and, particularly, at pH 8.0, at which the detection signal was still significant but ≈5 times lower than at pH 6.0 (data not shown). In parallel tests, the pH of the assay extracts did not significantly affect the ODs observed with the PAb R3-IVIA or MAb 5B-IVIA/AMR and isolates belonging to other PPV strains (result not shown).

Development of an RT-PCR based detection assay for the cherry isolates from the Volga River basin. The previously available PPV-C-specific detection tools based on the TUV- and AC-specific MAbs (35) or on CP-specific primers (39) failed to detect the sour cherry isolates analyzed in the present study (data not shown). In the case of RT-PCR, this is likely due to seven and three substitutions in the forward and reverse primer binding sites, respectively. Therefore, a multiple-sequence alignment of the NIb-CP region of the RU-17sc, RU-18sc, and RU-30sc isolates

TABLE 3. Amino acid mutations unique to the polyproteins of cherry-adapted *Plum pox virus* (PPV) isolates whose sequence was determined directly from cherry hosts, without prior multiplication in non-cherry hosts (RU-17sc, RU-18sc, RU-30sc, BY101, and BY181)[a]

Functional product	N^b	Amino acids
P1	22	V_{25}, A_{30}, I_{37}, W_{117}, E_{123}, H_{127}, T_{137}, D_{143}, P_{145}, R_{147}, S_{150}, K_{151}, H_{158}, D_{162}, N_{180}, T_{194}, H_{196}, R_{203}, Q_{206}, L_{220}, R_{266}, A_{293}
HC-Pro	7	V_{317}, Q_{342}, I_{442}, V_{457}, R_{466}, E_{640}, M_{762}
P3	7	I_{888}, N_{914}, A_{1000}, $\underline{A}_{1022}$, $\underline{Y}_{1081}$, M_{1082}, V_{1100}
6K1	0	…
CI	4	M_{1227}, Y_{1415}, G_{1636}, Q_{1779}
6K2	2	V_{1823}, A_{1854}
NIa	11	G_{1949}, I_{1957}, E_{1961}, P_{1970}, A_{1975}, L_{1979}, V_{1984}, A_{2038}, V_{2041}, $\underline{S}_{2043}$, N_{2211}
NIb	2	H_{2717}, A_{2798}
CP	20	G_{2814}, V_{2819}, L_{2821}, S_{2827}, T_{2835}, P_{2836}, S_{2840}, P_{2845}, P_{2846}, $\underline{E}_{2848}$, S_{2880}, F_{2882}, Y_{2884}, Q_{2890}, $\underline{N}_{2891}$, $\underline{R}_{2904}$, K_{2905}, T_{2913}, S_{2918}, S_{2979}

[a] Numbering is according to the RU-30sc polyprotein sequence. Amino acids not conserved in the PPV-C, SoC and PPV-C, SwC isolates are underlined.
[b] Number of conserved amino acids.

was used to design specific primers CR8597F and CR9023R, which amplify a 427-bp fragment encompassing the 5′ terminal region of the CP gene (nucleotide position 8,597 to 9,023 of the RU-30sc genome). In control assays, no amplification product was generated from PPV isolates of PPV-M, -D, -Rec, -T, -W, -EA, and -C strains whereas the expected product was readily amplified from all PPV-CR isolates assayed (Fig. 3). The identity of the amplification products was validated by their direct sequencing.

Multiple sequence alignment of the 5′-terminal CP region amplified using the CR8597F/CR9023R primers (387 bp after removing the primer sequences) showed that the identity level between the 14 PPV-CR isolates analyzed (Table 1) was 97.4 to 100% for the nucleotide sequences and 94.6 to 100% for the encoded amino acid sequences. The average pairwise genetic distances were 1.5% (±0.3%) and 2.4% (±0.7%) for the nucleotide and amino acid sequences, respectively.

DISCUSSION

Sour cherry trees displaying PPV-like symptoms on their leaves were found recently in the Samara and Saratov regions in the Volga River basin of Russia. Partial and full-length genome characterizations of several virus isolates have unambiguously confirmed that they belong to PPV but are highly divergent from any known PPV strain, including PPV-C. Indeed, the mean divergence between PPV-C isolates and the newly described sour cherry isolates reached 16.5% (Table 2), in contrast to the much lower divergence observed between PPV-D, -Rec, -T, or -M isolates (reaching 4.5 to 12.5%). In addition, these new cherry isolates could not be detected using either serological or molecular PPV-C specific detection assays (35,39). The results show that these divergent isolates should be considered as representing a new PPV strain that naturally infects sour cherry, for which the name PPV-CR is proposed. Based on their CP gene sequences, molecularly similar PPV isolates belonging to the same PPV-CR strain (sequence identity in the CP gene reaching 98.1 to 99.1 and 98.2 to 99.1% at the nucleotide and amino acid level, respectively) have recently been identified in wild sour cherry from the Moscow region (11) (accession numbers JX472432 to JX472440).

The variability observed in the CP 5′-terminal region of the PPV-CR isolates analyzed (0 to 2.6% nucleotide divergence) and the fact that these isolates were obtained from three different areas along ≈200 km of the Volga River basin (Stromilovskije Dachi, Malaya Czarjevtchina, and Khvalynsk) and in various cultivation conditions (productive orchards, abandoned old orchards, private gardens, cultivar collections, and a wild *Prunus* tree) suggest the efficient establishment and competitiveness of PPV-CR in the environment. This is also supported by the recent discovery of the same type of isolates infecting sour cherry trees in the Moscow region during an independent study (11). In this work, nine isolates from the Moscow region were investigated. Although they readily reacted against anti-PPV polyclonal reagents, these isolates failed to react against the 5B-IVIA universal MAb. Whether this might be a specificity of these isolates or whether, as reported here, ELISA conditions have to be fined tuned to reveal 5B-IVIA reactivity remains to be investigated.

The PPV-C and PPV-CR strains, although both naturally infect cherry, show quite low nucleotide identity level (83.5%) but higher amino acid identity level (93.5%) for the most closely related isolates (RU-30sc and BY181). Thus, the comparison of complete amino acid sequences of all available cherry-adapted isolates with those of non-cherry PPV isolates could be used to identify conserved mutations unique to cherry-adapted PPV. Such mutations are good candidates to be involved in the adaptation of these isolates to cherry trees (Table 3). Although the role of any specific amino acid or viral protein product in such adaptation will remain speculative, this analysis suggests that special consideration should be paid to the P1, NIa, and CP genes, in line with studies showing P1 as an important factor of potyvirus host specificity and viral adaptation to the host (32,45) and with the implication of CP in the systemic spread of PPV in the host plants (14). Indeed, it can be hypothesized that adaptation of PPV to cherry hosts could mostly reflect the capacity of the virus to move systematically in such hosts because PPV-M and -D isolates failed to infect systematically cherry trees (15).

The fact that, apart from this work, a remarkable PPV diversity has been detected in recent years from Russia (48) (PPV-W) or from adjacent, historically linked countries such as Latvia (20) (PPV-W), Belarus (31) (PPV-C), and Ukraine (34) (PPV-W) should encourage the research on PPV variability in these as-yet

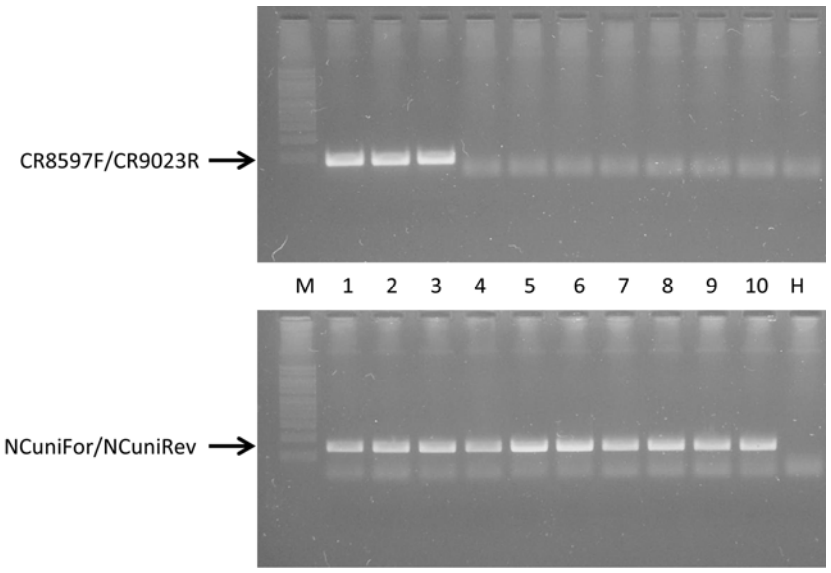

Fig. 3. Agarose gel electrophoretic analysis of reverse-transcription polymerase chain reaction amplification products from leaf samples infected with *Plum pox virus* (PPV) isolates of all known PPV strains. Upper gel: CR8597F/CR9023R primers specifically targeting the PPV-Cherry Russia (CR) isolates. Lower gel: polyvalent NcuniFor/NcuniRev primers (40). Lane M, Perfect DNA 1-kb Ladder (Novagen); lane 1, RU-17sc (PPV-CR); lane 2, RU-18sc (PPV-CR); lane 3, RU-30sc (PPV-CR); lane 4, Soc (PPV-C); lane 5, CAH-2 (PPV-M); lane 6, BOJ-1 (PPV-D); lane 7, BOR-3 (PPV-Rec); lane 8, LV-145bt (PPV-W); lane 9, El Amar (PPV-EA); lane 10, TR-289ap (PPV-T); and lane H, healthy control.

poorly explored areas, which have the potential to harbor further new, unusual, or emerging isolates of PPV.

PPV-CR isolates were detected by ELISA using commercially available kits and polyclonal antibodies or by RT-PCR using polyvalent PPV primers. The reactivity and reliability of the polyvalent 5B-IVIA/AMR MAb in DAS or DASI-ELISA was improved by controlling the pH of tested plant homogenates. The pH is a parameter that can affect the affinity and avidity of the antigen-antibody recognition (22). The epitope that is recognized by MAb 5B-IVIA/AMR (10) in the N terminus of CP (DRDVDAG) has changed to ERDVDAG in the RU-30sc and most other sour cherry isolates used in this study (Fig. 1). This fact is possibly reducing affinity in the immunological recognition and may explain the improvement in reactivity observed at a moderately acidic pH, as previously reported (49). This motif has further changed to DRDIDAG or ERDIDAG in some isolates (Fig. 1).

To facilitate the rapid and specific detection of PPV-CR isolates, the RT-PCR was optimized using primers targeting the 5′ terminal region of the CP gene. In addition to using total RNA isolated from dried leaves, successful RT-PCR amplification of PPV-CR isolates was achieved from cDNAs generated from viral RNA eluted from the cards. Detection of viral RNA was readily achieved from cards that had stayed 15 days at ambient temperature due to long postal delays, confirming the suitability of the paper-based technologies for sampling, storage, transport, and molecular detection of RNA viruses (21).

In conclusion, taking into account their genomic properties and the fact that they were recovered from naturally infected sour cherry but are highly diverged from the original cherry-infecting strain, PPV-C, the cherry PPV isolates from the Volga River basin reported here represent a new cherry PPV strain, termed PPV-CR. Also, the fact that, among all other PPV strains, the PPV-CR genomic sequence is genetically closer to that of PPV-C may suggest that some shared genetic determinants equip the virus with the ability to infect cherry species. Host range studies are necessary for PPV-CR to further delineate the differences and similarities between this strain and PPV-C. The understanding of the epidemiological properties of PPV-CR isolates (e.g., ability to infect other *Prunus* spp., competitiveness with common PPV strains, efficiency of different aphid species transmission, and so on) represents the challenge for further studies.

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