SHORT COMMUNICATION

CHARACTERIZATION OF RUSSIAN *PLUM POX VIRUS* ISOLATES PROVIDES FURTHER EVIDENCE OF A LOW MOLECULAR HETEROGENEITY WITHIN THE PPV-C STRAIN

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SUMMARY

Plum pox virus (PPV)-positive samples from one sweet (*Prunus avium*) and four sour cherry (*P. cerasus*) trees from three geographically distinct regions of Russia (Moscow, Samara and Volgograd) were analysed. The average genetic distance among the five Russian PPV-C isolates in the region spanning the 3′terminal part of the nuclear inclusion b gene (NIb) and the hypervariable 5′terminal part of the coat protein gene reached 1.6%. To widen the information on PPV-C diversity, the full-length genome sequence of an isolate (Volk143) was determined directly from the original host tissues. The analysis of the complete genome sequences showed a high identity of Volk143 and the previously characterised PPV-C isolates with only 1.4-1.8% divergence. Co-infection with the recently identified PPV-CR strain was not observed, even though the PPV-CR is known to occur in the regions of origin of the isolates studied here. The present results unambiguously confirm the natural infection of sweet and sour cherry trees by PPV-C in different Russian regions and further contribute to our understanding of the poorly known PPV-C diversity.

Key words: cherry, sharka, PPV-C strain, Russia.

Plum pox virus (PPV), genus *Potyvirus*, is the causal agent of Sharka, the most important viral disease of *Prunus* crops (Cambra *et al*., 2006; Barba *et al*., 2011). On the basis of molecular, serological and biological differences, eight PPV strains are now recognised worldwide (referred to as D, M, EA, C, Rec, W, T and CR in the order of their discovery). The PPV-M, D and Rec strains are widely distributed in Europe and are considered as the three major strains (Šubr and Glasa, 2013; Garcia *et al*., 2014). Sweet cherry trees are immune to infection by PPV-D and -M strains, which remain restricted to the inoculation point (Dosba *et al*., 1987). Reports of natural PPV infection of cherry date back to the early 1990's (Bilkey, 1992) and further molecular characterization efforts have shown that cherry-adapted isolates represent a distinct strain denoted PPV-C (Nemchinov and Hadidi, 1996; Nemchinov *et al*., 1996). Recently, a second cherry-adapted PPV strain (PPV-CR) has been described (Glasa *et al*., 2013, Chirkov *et al*., 2013). PPV-C isolates have not been reported so far to have a major epidemiological impact on cherry trees in the countries in which they have occasionally been found, i.e. Moldova (Nemchinov and Hadidi, 1996), Italy (Crescenzi *et al*., 1997), Bulgaria (Topchiiska, 1997), Syria (Al-Chaabi *et al*., 1997) and Hungary (Nemchinov *et al*., 1998). Unfortunately, with the exception of isolates from Moldova and Italy, there has been no adequate molecular characterization or sequencing of the isolates from other countries, so that some of these reports can be questioned, and information on PPV-C diversity remains scarce. More recently, cherry trees naturally infected with PPV-C have been found in Belarus (Malinowski *et al*., 2012) and Croatia (Kajic *et al*., 2012), suggesting that this PPV strain may be more widespread than originally thought. In the present work, we obtained the partial genomic sequences of five PPV-C isolates from sour cherry (*Prunus cerasus*) and sweet cherry (*P. avium*) trees from Russia and determined the complete genome sequence for one of them. These results extend the information available on PPV-C distribution and genetic diversity.

Five PPV isolates were collected from sour and sweet cherry trees displaying a range of symptoms (Fig. 1) in three geographically distinct regions of Russia [Moscow, Samara and Volgograd (Table 1)]. The symptoms observed are similar to those reported previously for PPV-C or PPV-CR infection but are also reminiscent of those caused by other cherry-infecting viruses. The presence of three of these viruses, *Prunus necrotic ringspot virus* (PNRSV), *Cherry leafroll virus* (CLRV) and *Prune dwarf virus* (PDV) was therefore assessed in the collected samples by DAS-ELISA using a commercial kit (Neogen Europe, UK). PDV was found in one of the five tested samples (SamSad-14), while PNRSV and CLRV were not detected. A RT-PCR assay specific for PPV-CR (Glasa *et al*., 2013) was also used on all five samples but failed to identify this second cherry-adapted strain of PPV.

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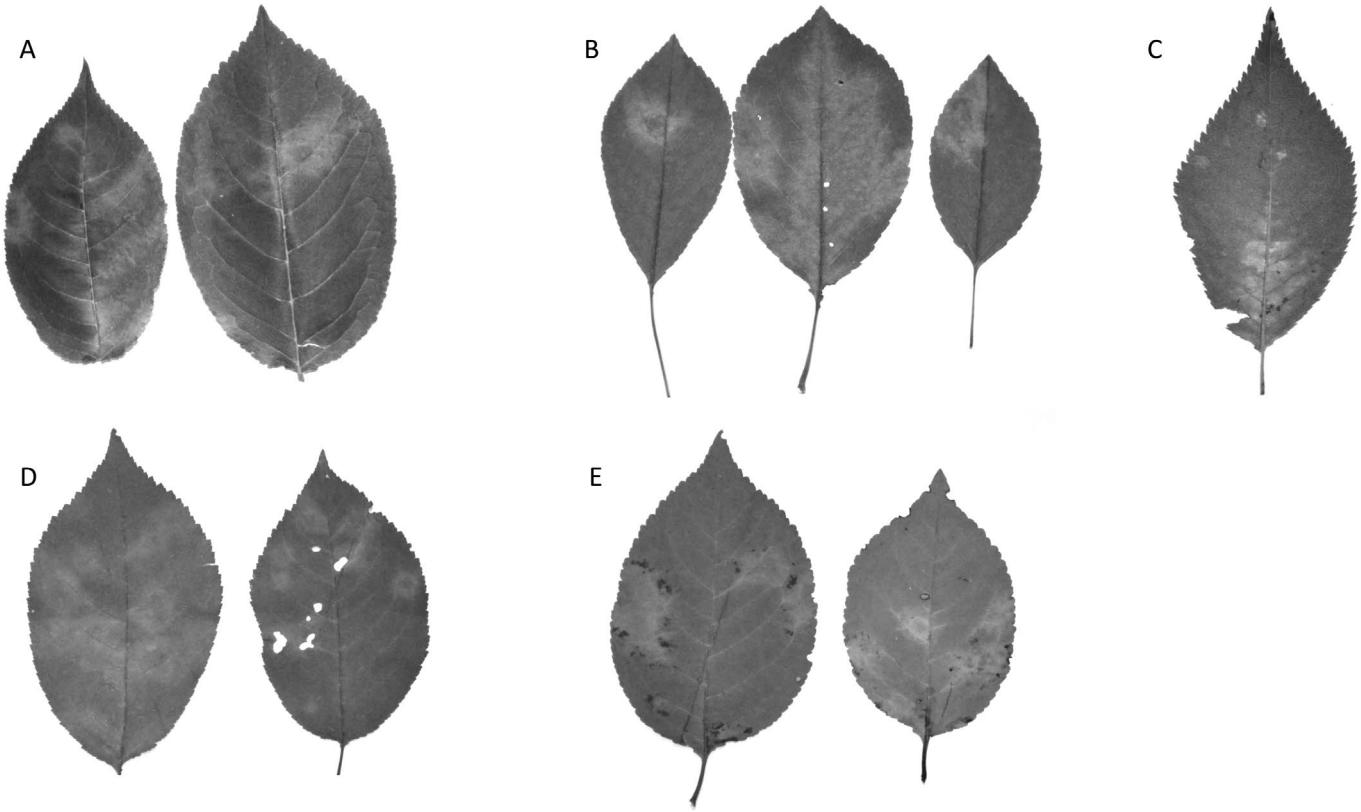

Fig. 1. Symptoms observed on the leaves of the PPV-C-infected cherry trees. A. Volk-143; B. ILM-1; C. MSXA-63; D. SamSad-9; E. SamSad-14.

Table 1. List of Russian PPV-C isolates characterised in this study

Isolate	Locality	Type of plantation	Host	GenBank accession No.
Volk143	Volgograd	Private garden	Sour cherry	KJ787006[a]
ILM-1	Moscow region, Ramenskoe district	Private garden	Sour cherry	KJ787007[b]
SamSad-9	Samara region, Sizran district	Orchard (cultivar collection)	Sour cherry	KJ787010[b]
SamSad-14	Samara region, Sizran district	Orchard (cultivar collection)	Sour cherry	KJ787009[b]
MSXA-63	Moscow	Orchard (cultivar collection)	Sweet cherry, cv. Urodgajnala Susova	KJ787008[b]

[a]complete genome, [b]NIb-CP region.

Based on the use of a RT-PCR assay reported to be PPV-C-specific (Nemchinov and Hadidi, 1998) at the time when PPV-CR was unknown, a survey conducted in 2008-2012 in several regions of the Russian Federation had disclosed the presence of PPV-C in both sweet and sour cherry trees (Prikhodko *et al*., 2013). These PPV-positive samples were analysed further in the present work (Table 1). Total RNAs were extracted from infected leaves using the NucleoSpin RNA Plant kit (Macherey-Nagel, Germany). A two-step RT-PCR protocol was consistently used. First-strand cDNA was synthesized by reverse transcription of total RNA using random hexamer primers and the *Avian myeloblastosis virus* (AMV) reverse transcriptase (both from Promega, USA) and used in subsequent PCRs. Initial detection was performed using the polyvalent PPV-specific P1/P2 primers (Wetzel *et al*., 1991), which amplified a product of the expected size from all five isolates. The sequences of this short region were identical for all five isolate (data not shown). As this genome fragment is not adequate for phylogenetic analysis due to its high level of conservation, a 746 bp product spanning the 3´ terminal part of the nuclear inclusion b gene (NIb) and the hypervariable 5´ terminal part of the CP gene was amplified as described (Predajna *et al*., 2012) and sequenced. Sequence analyses were performed using the MEGA v.5 software (Tamura *et al*., 2011) and online tools (http://expasy.org/tools/protparam.html). The obtained nucleotide sequences were deposited in GenBank under accession Nos. KJ787006-KJ787010. Pairwise comparisons with available PPV sequences and phylogenetic analyses assigned unambiguously the five Russian isolates to the PPV-C strain (Fig. 2). The average genetic distance between the five Russian PPV-C isolates reached 1.6% (±0.003). In comparison, the divergence in this region, calculated among four previously characterised PPV-C isolates (HQ840517, HQ840518, AY184478, Y09851) reached 2.0% (±0.4%). The DAG motif, essential for aphid transmission, was conserved in the polyprotein of all Russian isolates.

Table 2. Comparison of the Volk143 genome (polyprotein) sequence with that of other fully sequenced PPV-C isolates.

	BY101		BY 181		SoC		SwC	
	Genome	Polyprotein	Genome	Polyprotein	Genome	Polyprotein	Genome	Polyprotein
5´NCR	2[a] 0.014 (±0.009)[b]		2 0.014 (±0.009)		3 0.021 (±0.011)		3 0.021 (±0.011)	
P1	10 0.011 (±0.003)	2 0.007 (±0.004)	8 0.009 (±0.003)	3 0.010 (±0.006)	13 0.014 (±0.004)	2 0.006 (±0.004)	12 0.013 (±0.004)	3 0.010 (±0.005)
HC	14 0.010 (±0.002)	4 0.004 (±0.002)	14 0.010 (±0.003)	4 0.004 (±0.003)	12 0.009 (±0.003)	5 0.011 (±0.005)	9 0.007 (±0.002)	4 0.009 (±0.004)
P3	16 0.015 (±0.004)	6 0.017 (±0.007)	15 0.014 (±0.003)	4 0.011 (±0.005)	15 0.014 (±0.004)	7 0.020 (±0.007)	19 0.018 (±0.004)	9 0.026 (±0.008)
PIPO	3 0.009 (±0.005)	1 0.009 (±0.009)	3 0.009 (±0.005)	1 0.009 (±0.009)	5 0.016 (±0.007)	1 0.009 (±0.009)	3 0.009 (±0.005)	1 0.009 (±0.009)
6K1	3 0.019 (±0.010)	0	3 0.019 (±0.010)	0	3 0.019 (±0.010)	1 0.019 (±0.019)	3 0.019 (±0.010)	1 0.019 (±0.019)
CI	35 0.018 (±0.003)	5 0.008 (±0.003)	36 0.019 (±0.003)	5 0.008 (±0.003)	46 0.024 (±0.004)	12 0.019 (±0.005)	43 0.023 (±0.004)	9 0.014 (±0.005)
6K2	3 0.019 (±0.011)	0	2 0.013 (±0.009)	0	2 0.013 (±0.009)	0	2 0.013 (±0.009)	0
NIa	12 0.009 (±0.003)	1 0.002 (±0.002)	11 0.008 (±0.002)	1 0.002 (±0.002)	20 0.015 (±0.003)	4 0.009 (±0.004)	18 0.014 (±0.003)	4 0.009 (±0.004)
NIb	25 0.016 (±0.003)	7 0.014 (±0.004)	27 0.017 (±0.003)	7 0.014 (±0.004)	29 0.019 (±0.003)	9 0.017 (±0.005)	34 0.022 (±0.004)	17 0.033 (±0.007)
CP	16 0.016 (±0.004)	8 0.024 (±0.008)	16 0.016 (±0.004)	8 0.024 (±0.008)	20 0.020 (±0.004)	8 0.024 (±0.008)	29 0.029 (±0.005)	19 0.057 (±0.013)
3´NCR	4 0.018 (±0.009)		5 0.023 (±0.009)		4 0.018 (±0.009)		12 0.055 (±0.014)	

[a]number of differences, [b]mean genetic distance (standard error)

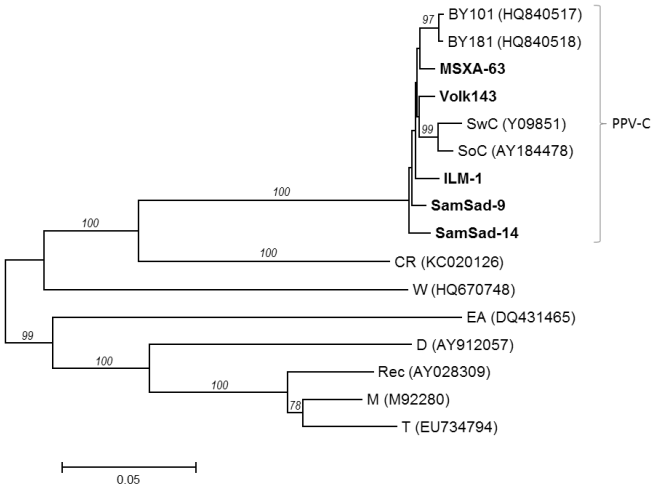

Fig. 2. Phylogenetic tree of the partial NIb-CP sequences of the Russian PPV-C isolates determined in this study (highlighted in bold) and selected PPV sequences retrieved from GenBank (the isolate name is followed by accession number). The tree was constructed by the neighbour-joining method, using the p-distance model.

Four complete genomes of PPV-C isolates are available in GenBank (accessed on February 2014). Two of these isolates, SoC (AY184478, Moldova) and SwC (Y09851, Italy) had been maintained on herbaceous host prior to sequencing, whereas the genomes of the two Belarus isolates [BY101 (HQ840517) and BY181 (HQ840518)], were determined directly from the original cherry host material (Malinowski *et al*., 2012). To widen the information on PPV-C diversity which, contrary to the other PPV strains has not been investigated, the full-length genome sequence of the isolate Volk143 was determined directly from the original host tissues, by sequencing overlapping PCR fragments amplified using the TaKaRa LA Taq polymerase (TaKaRa, Japan) (primer sequences are available from the corresponding author). The RT-PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) and directly sequenced using an automated DNA sequencer (ABI 3130xl Genetic Analyser; Applied Biosystems, USA). The complete genome of Volk143 is 9,795 nt in length and is fully co-linear with that of the isolates Soc, SwC, BY101 and BY181. Comparisons showed an overall 139, 140, 166 and 181 nucleotide differences between Volk143 and the BY101, BY181, SoC and SwC isolates, respectively [mean nucleotide divergence reached 1.3% (±0.1%)]. No substantial peaks of divergence between Volk143 and the other PPV-C isolates could be detected using a sliding window analysis of nucleotide diversity (data not shown), and the comparison of sequences showed rather a random pattern of nucleotide/amino acid substitutions (Table 2).

All motifs characteristic of potyviral proteins were conserved in the genome of Volk143. Interestingly from 75 aa positions conserved among cherry-adapted isolates (strains -C and -CR) reported earlier (Glasa *et al*., 2013), 72 aa remain unchanged also in the Volk143 polyprotein (except for substitutions $S_{150}F$, $G_{1636}S$ and $F_{2882}L$), strengthening the likelihood that these aa are potential candidates as genetic determinants of the ability to infect cherry species and/or of adaptation to cherry hosts. The presence of the overlapping *Potyviridae* PIPO ORF (Chung *et al*., 2008)

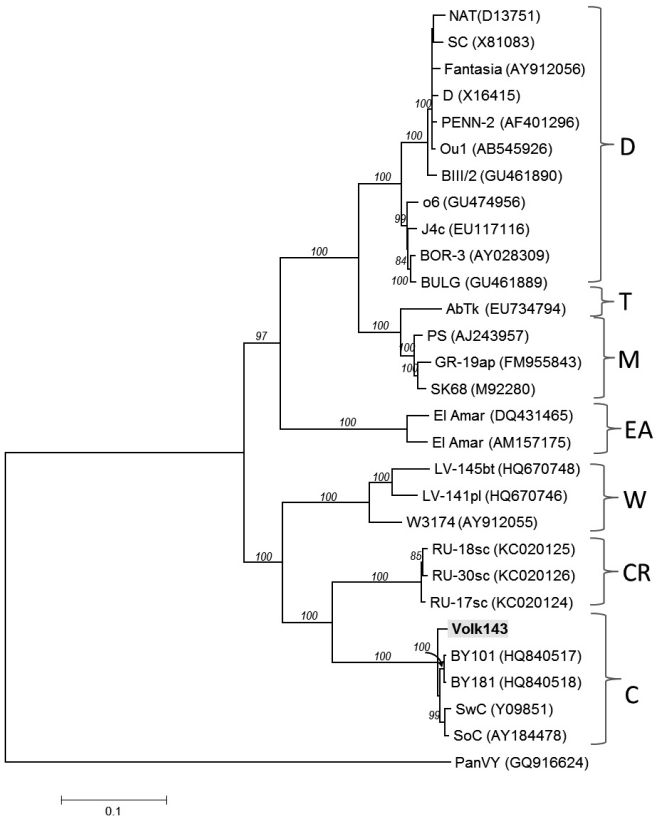

Fig. 3. Phylogenetic tree generated from complete nucleotide PPV genome sequences retrieved from GenBank and the Volk143 isolate sequence determined in the present study (highlighted in bold). The isolates are identified by their names and accession numbers. Scale bar indicates a genetic distance of 0.1. Bootstrap values >70 (1000 bootstrap re-samplings) are indicated on the branches as percentages. The affiliation of the isolates to PPV strains is indicated to the right of the tree. *Panax virus Y* was used as an outgroup.

was also identified in the Volk143 genome at nt positions 2906-3223, starting with an initiation AGA_6 frameshift site. The theoretical molecular weight and isoelectric point of the deduced 106 aa long PIPO peptide were predicted to be 12.140 kDa and 10.74, respectively. The Volk143 PIPO is virtually identical to PIPOs of other PPV-C isolates (with only 1 aa difference) but, despite a similar length, there are 23 aa differences with the PIPO of PPV-CR isolates (KC020124-KC020126).

The present results unambiguously confirm the natural infection of sweet and sour cherry trees in different regions in Russia (Moscow, Samara, Volgograd) by PPV-C isolates and further contribute to our understanding of PPV-C diversity. The molecular data show that notwithstanding the different geographic origin of the PPV-C isolates characterised to date, the diversity within the PPV-C strain remain low (1.3% based on the five complete genome sequences available, including that of isolate Volk143). This value is comparable to that of PPV-Rec (1.5%, seven sequences), PPV-D (1.2%, 72 sequences) and PPV-CR (0.9%, three sequences) (Glasa *et al*., 2012). Such low divergence might suggest a later evolutionary history of PPV-C and a recent transfer of PPV to cherry hosts. In contrast, both cherry-infecting strains are distantly phylogenetically related to PPV-W, the most variable PPV strain so far known (Glasa *et al*., 2011; Sheveleva *et al*., 2012).

Interestingly, Russia is up to now the only country where both PPV-C and PPV-CR have been reported. Co-infection of these two strains were not observed in the samples analysed in the present investigation, even though PPV-CR is reported to occur in the regions of origin of the isolates studied here (Glasa *et al*., 2013; Chirkov *et al*., 2013). Similarly, up to now, no natural infection of non-cherry *Prunus* hosts has been observed with either the PPV-C or PPV-CR, although such hosts are susceptible to both strains under experimental conditions (Bodin *et al*., 2003; Boeglin *et al.* 2004). These results suggest that barriers may exist, agronomical or otherwise, to the spread of cherry-adapted PPV isolates to non-cherry hosts, in a fashion reminiscent of the extremely infrequent observation of infections to peach of PPV-Rec. Further studies will be needed to evaluate the possibility of natural PPV-C infection to non-cherry hosts or the potential effects of co-infections of cherry by PPV-C and PPV-CR and their consequences.

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