

## MOLECULAR TYPING OF *PLUM POX VIRUS* ISOLATES IN CROATIA

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### SUMMARY

Previous studies have ascertained the presence of *Plum pox virus* strain M (PPV-M) and D (PPV-D) in various *Prunus* species all over Croatia. Susceptible plum varieties, known as particularly endangered by recombinant PPV strains (PPV-Rec), were taken into consideration for the molecular typing of PPV isolates carried out in this study. Samples were collected in commercial orchards established with imported plant material, except for one sample coming from the urban Zagreb area. Six out of seven samples tested PPV positive by DAS-ELISA. A modified IC-RT-PCR approach was used for amplifying two target sequences, the carboxy-terminus part of the capsid cistron (Cter)CP and the virus segment spanning the carboxy-terminus part of N1b (Cter)N1b to the amino-terminus part of CP [(Nter)CP]. PCR amplification based on the use of the primers targeting the (Cter)CP region revealed the presence of PPV-M in all positive samples. The presence of PPV-D was found in three samples in mixed infection with PPV-M. Two samples harbored PPV-Rec (Valjevka, wild plum). Six sequences of (Cter)CP amplicons clustered in two groups, in accordance with the strain differentiation (PPV-M, PPV-D), while two (Cter)N1b/(Nter)CP sequences clustered within the group of well known PPV-Rec isolates. Phylogenetic analyses suggest different origin of two PPV-Rec isolates in accordance with biological data. If distinct geographical location and different source plant genesis are considered, higher molecular diversity of PPV in Croatia than previously reported is predictable from this first PPV-Rec report.

*Key words:* PPV-Rec, (Cter)CP, (Cter)N1b/(Nter)CP, phylogeny, molecular diversity, strain typing.

### INTRODUCTION

Isolates of the *Plum pox virus* (PPV), the causal agent of Sharka disease, are currently grouped into six subgroups or strains (Glasa and Candresse, 2005). PPV-M and PPV-D are widespread in Europe (Candresse *et al.*, 1998), PPV-C (Nemchinov *et al.*, 1996) with natural host range restricted to cherries is only sporadically present in the area, while PPV-EA and PPV-W have only been reported from Egypt and Canada, respectively (Wetzel *et al.*, 1991a; James and Varga, 2005). The sixth PPV subgroup, represented by PPV-Rec, apparently resulted from an ancient homologous recombination event between isolates of strains D and M at a point in the 3' terminus of the N1b gene (Glasa *et al.*, 2004). PPV-Rec cannot be distinguished from PPV-M in tests routinely performed for PPV detection and typing (Cambra *et al.*, 2002) and data about its occurrence and distribution emerged only after techniques targeting the recombination region have been applied (Glasa *et al.*, 2002; 2004). So far, PPV-Rec has been detected in the countries of central and eastern Europe (Glasa *et al.*, 2004; 2005; Matic *et al.*, 2005; Dallot *et al.*, 2008), as well as in Turkey (Candresse *et al.*, 2007).

In Croatia, Sharka was reported in the eastern part of the country as early as 1949 but the virus itself was identified twenty years later (Pleše, 1969 and references therein). Even though the destructive character of the disease to the stone fruit industry and its potential for rapid spread were recognized at the time, no systemic field surveys were done. The nursery surveys encompassing visual inspections and ELISA testing of mother trees were initiated in 1988 and the situation was summarized in an EPPO Conference by V. Kajic (Roy and Smith, 1994). The survey continued in the following years but, from the 2004 onwards, it was complemented by investigations of PPV incidence and distribution in orchards, garden trees and spontaneous plants (Mikec *et al.*, 2006). ELISA screening, serological detection with PPV strain specific commercial antisera, and IC-RT-PCR/RFLP testing showed that PPV-M is widespread in the country. PPV-D foci were found in the far east and far west, together with cases of mixed infection in European plums (Mikec *et al.*, 2008). Although the

molecular typing of PPV strains in the country is in progress, the problem of PPV molecular epidemiology, including the presence of PPV-Rec, has not yet been addressed. Since the highest PPV incidence was found in plums (Mikec *et al.*, 2006) which are particularly sensitive to PPV-Rec, we focused our molecular typing efforts on a particular subset of plums displaying severe symptoms.

## MATERIALS AND METHODS

**Virus sources.** Symptomatic samples representing three varieties of European plum (*Prunus domestica* L.) were collected in May of 2006 and 2007. Commercial orchards from three different localities in the eastern part of Croatia were surveyed (Table 1). These orchards had been established exclusively with imported plant material in the last 3-7 years. Additionally, a symptomatic plum seedling of unknown variety, was sampled in the urban Zagreb area in a private garden away from any commercial orchard. It displayed severe fruit and leaf symptoms even in summer months. All samples were kept frozen (-20°C) prior to testing.

**IC-RT-PCR based PPV strain differentiation.** For both serological and PCR assays, 1 g of leaf tissue per sample was homogenized in 10 ml of extraction buffer (phosphate-buffered saline, pH 7.2, with 0.05% Tween 20, 2% PVP and 0.2% DIECA). Initial screening of the samples for PPV presence was carried out by DAS-ELISA (Clark and Adams, 1977) using polyclonal and conjugated monoclonal antibodies and the controls supplied with the kit (Bioreba, Switzerland). Samples displaying OD values twice higher than the value of the negative control, were considered positive. (Cter)CP region was amplified by IC-RT-PCR using P1/P2 primers (Wetzel *et al.*, 1991b) following the procedure by Wetzel *et al.* (1992) and using the same polyclonal antibodies and controls as above. In order to differentiate PPV strains, specific primer pairs for strains M (P1/PM) and D (P1/PD) were used (Olmos *et al.*, 1997). For PPV-Rec detection, a modified IC-RT-PCR approach was

used combining an immunocapture step (Glasa *et al.*, 2002) using Bioreba polyclonal antibodies and 1 µM mD5/mM3 primers (Šubr *et al.*, 2004) that amplify the (Cter)NIB/(Nter)CP region. PPV-Rec positive control was kindly provided by S. Dallot through M. Viršček-Marn. In every PCR assay, a reaction mix devoid of DNA template was included as control.

**Sequence analyses.** Amplification products obtained from regions (Cter)CP (229 bp) and (Cter)NIB/(Nter)CP (579 bp) were purified (QIAquick PCR Purification Kit, Qiagen, USA) and sequenced in both directions (Macrogen sequencing service, South Korea). As reference sequences, (Cter)CP and (Cter)NIB/(Nter)CP gene sequences of biologically well characterized isolates of strains M, D, and Rec were retrieved from the GenBank (www.ncbi.nlm.nih.gov): PS Serbia (AJ243957), SK68 (M92280), Serbia MI (AY690605), Troy6 (AM260937), Serbia T (AY690609), Pd31 (AJ566345), Chile20 (AF440745), Troy2 (AM260934), BRC 8 (AF421120), MYV 3 (AF450313), BOS148PI (AJ749996), BOS64PI (AJ749998), BOS157PI (AJ749997), Serbia V (AY690610), Serbia B (AY690603), Serbia ST (AY690608), Serbia PO3 (AY690607), KRN7 (AF421123), PD31 (AJ566345), BOR 3 (AY028309), BOS150PI (AJ749995), BOS49PI (AJ749999), Fantasia (AY912056), Cdn 123-1 (AY953267), and Penn4 (DQ465243).

Portions of the (Cter)CP and (Cter)NIB/(Nter)CP sequences identical to primers were excised without disruption of the reading frame and aligned by ClustalX 1.8 (Thompson *et al.*, 1997). Neighbor-joining method applying Kimura 2-parameter evolutionary model was used for phylogenetic reconstructions within the program package MEGA 3.1 (Kumar *et al.*, 2004). The tree topology was evaluated by bootstrap analysis based on 1000 iterations. The sequence diversity was calculated by using a default option within the same program package. The recombinant nature of sequences was confirmed by using the program SimPlot 3.5.1 (Lole *et al.*, 1999), where applicable.

All (Cter)CP and (Cter)NIB/(Nter)CP sequences obtained in this work were submitted to the GenBank under accession numbers EU327547-EU327554.

**Table 1.** Strain typing of PPV isolates from severely symptomatic plums.

Sample code	Plum variety	Planting year	Sampling year	Locality	ELISA	IC-RT-PCR		
						M	D	Rec
21	Cacanska Rodna	2001	2006	Vukovar	+	+	+	-
1	Cacanska Rodna	2004	2007	Tovarnik	+	+	+	-
2	Valjevka	2004	2007	Tovarnik	+	+	-	+
3	Cacanska Rodna	2005	2007	Tovarnik	-	-	-	-
4	Cacanska Rana	2005	2007	Tovarnik	+	+	-	-
5	Cacanska Rodna	2005	2007	Djakovo	+	+	+	-
6	Unknown	volunteer	2007	Zagreb	+	+	-	+
grown in 1995								

## RESULTS AND DISCUSSION

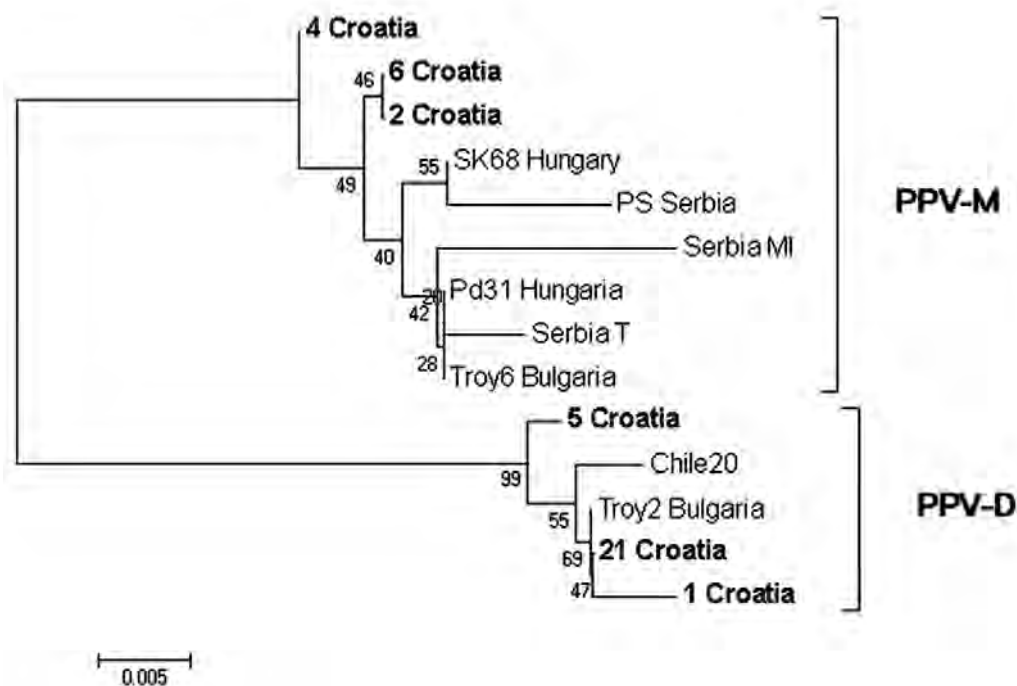
Six out of seven symptomatic plum trees were PPV-positive in ELISA (Table 1), showing OD values at last three times higher than that of the negative control (not shown). These results were corroborated by IC-RT-PCR experiments using either P1/P2 primers (not shown) or strain specific primer pairs (Table 1). Plum orchards sampled in this study are located in the eastern part of the country and were chosen for sampling because of the severe PPV-like symptoms observed during monitoring. The plum of unknown variety from Zagreb had been sampled in previous surveys, but no positive reaction had been obtained by ELISA (Kajic, unpublished results), either because of a very low relative virus concentration in the previous years and/or wrong sampling time.

IC-RT-PCR typing (not shown) confirmed the presence of PPV-D in three out of four cv. Cacanska Rodna samples (21, 1, and 5) from three different localities, that were in mixed infections with PPV-M. Only cv. Cacanska Rana from Tovarnik (sample 4) contained PPV-M alone, while PPV-M and PPV-Rec amplification signals were obtained both in (Cter)CP and (Cter)NIb/(Nter)CP regions from cv. Valjevka and the Zagreb plum (samples 2 and 6, respectively). This is the first time that the presence in Croatia of PPV-Rec is ascertained.

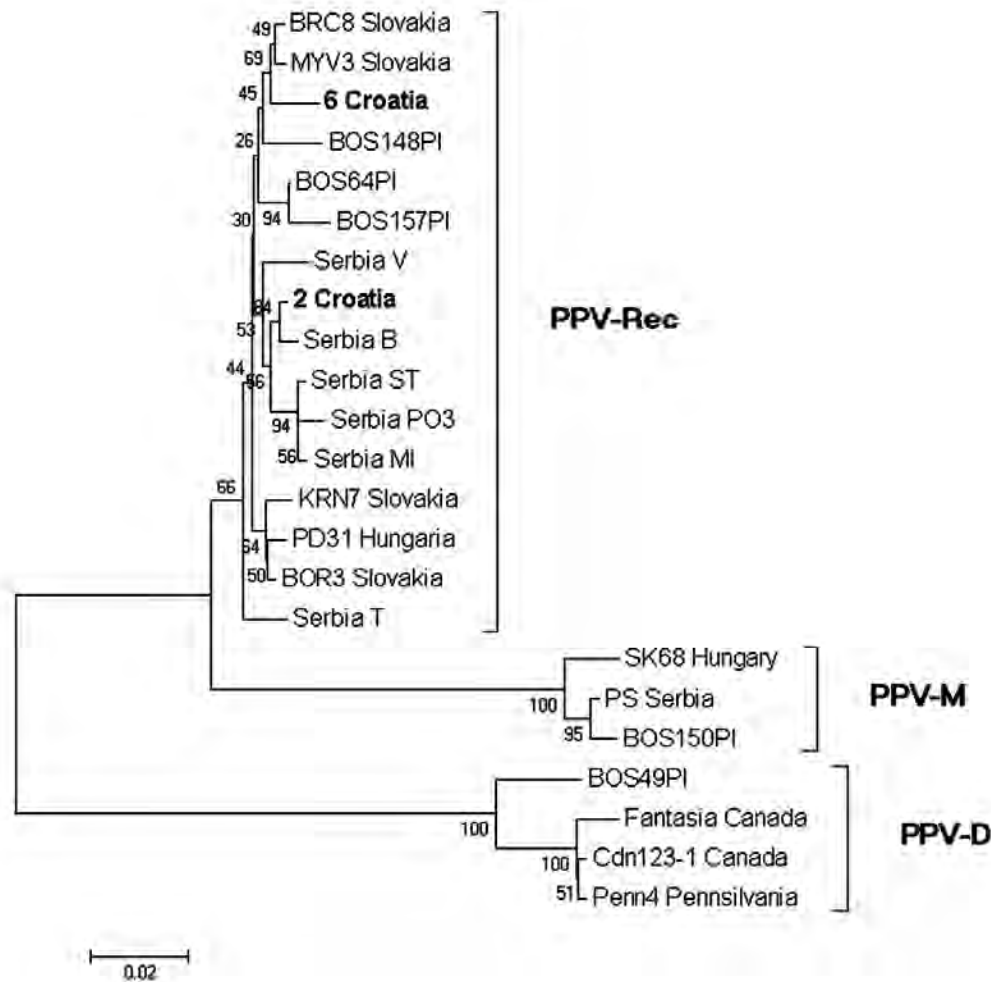
Phylogenetic analysis of the (Cter)CP region confirmed that the Croatian PPV isolates 2, 4, and 6 clustered together with reference isolates of strain M (Fig.

1). Isolates 2 and 6 had identical (Cter)CP sequences while sample 4 shared 99.56% sequence identity with them. When these isolates were compared with the M strain reference sequences, they showed the highest sequence identity (99.56%) with the isolates Bulgarian Troy6 (samples 2 and 6) and Hungarian SK (sample 4). Samples 1, 5, and 21 formed a distinct cluster with reference isolates of strain D, as expected (Table 1, Fig. 1). Croatian isolates shared sequence identities of 98.69% (5, 1), 99.13% (5, 21), and 99.56% (1, 21). Sample 21 had sequence identical to that of Bulgarian isolate Troy2. Sequence diversity within group D was 0.006. This parameter was almost twice as high within group M (0.01). Even though all D isolates were present in mixed infections with PPV-M in cv. Cacanska Rodna plants, direct sequencing of products amplified by P1/P2 general primers resulted in only strain D sequences. This may be due to the predominance of this strain in the virus populations within these samples. It would be interesting to investigate this further in the population structure analysis.

(Cter)NIb/(Nter)CP sequences of isolates 2 and 6 clustered with other reference isolates known as recombinants, and were distinct from the reference isolates of strain M and D (Fig. 2). Sequence diversity within subgroup PPV-Rec was 0.019, a value between the figures for subgroup M (0.016) and D (0.024). SimPlot analysis (not shown) of both recombinant isolates (2 and 6) confirmed that the first third of the analyzed region dis-



**Fig. 1.** Neighbor-joining phylogenetic tree constructed with PPV (Cter)CP gene sequences of Croatian samples 1, 2, 4, 6, and 21 (bold). Reference sequences of isolates with known biological characteristics are included in the tree. Bootstrap values are presented next to tree nodes. The bar represents 0.005 nucleotide substitution per site.



**Fig. 2.** Neighbor-joining phylogenetic tree constructed with PPV (Cter)NIB/(Nter)CP gene sequences of Croatian samples 2 and 6 (bold). Reference sequences of isolates with known biological characteristics are included in the tree. Bootstrap values are presented next to tree nodes. The bar represents 0.02 nucleotide substitution per site.

played a higher similarity to strain D sequences, while the rest of the region displayed higher similarity to strain M sequences, suggesting the most plausible recombination scenario.

(Cter)NIB/(Nter)CP sequence of sample 2 and isolate Serbia B had 99.46% identity, while sample 6 shared the highest nucleotide identity (98.92%) with the Slovakian isolate BRC8. Croatian samples 2 and 6 shared only 98.03% of nucleotide identity suggesting their different origin. This is in accordance with the other data available for these samples. Sample 2 is the PPV isolate from cv. Valjevka imported from Serbia (Table 1) and shares the highest identity with Serbia B, an isolate known to have the same geographical origin (Glasa *et al.*, 2005). The other recombinant isolate (sample 6), found in the wild plum from Zagreb, is highly similar to the Slovakian BRC8 isolate also found in naturally infected plum (Glasa *et al.*, 2002). Since PPV in Croatia and neighboring countries is endemic (Matic *et al.*,

2005; Mikec *et al.*, 2006) and PPV-Rec has long been present (Cervera *et al.*, 1993; Glasas *et al.*, 2005), genetically polymorphic populations probably coexist in Zagreb, as well as in the whole country. The tree infected with PPV-Rec in Zagreb grew from a seed, therefore its transmission by aphids appears most plausible. Finding such a case in this pilot study, calls for further investigations of the recombinant strain in wild plums and its role in the PPV epidemiology.

#### ACKNOWLEDGEMENTS

We are indebted to Drs. Sylvie Dallot and Mojca Viršček-Marn for providing the PPV-Rec positive control. This work was financed by the Croatian Ministry of Agriculture, Forestry and Water Management, as well as by the grant no. 119-1191192-1222 from the Croatian Ministry of Science, Education and Sports.

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