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Genetic variability of *Plum pox virus* isolates in the Czech Republic

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Abstract *Plum pox virus* (PPV), the causal agent of Sharka disease, is an important pathogen of stone fruit trees. In this study, 24 new Czech PPV isolates from five different orchards were collected and characterized, molecularly. PPV-D isolates were identified in all orchards studied; whereas PPV-Rec isolates were identified in only two of them. A phylogenetic analysis on (Cter) NIb-(Nter) CP was performed. Three Czech PPV-D isolates BOH11CZ, BOH12CZ, and BOH13CZ diverged into a significantly separated cluster. PPV-Rec isolates formed a fairly homogenous group. However, the Bohutice and the Lipov PPV-Rec isolates clustered in two significantly separated branches.

Keywords PPV-D · PPV-Rec · Phylogenetic analysis · Sequencing

Plum pox virus (PPV) is the causal agent of 'Sharka' disease of stone fruit trees, especially in Europe. Based on the symptoms, serological, and epidemiological data, as well as comparisons at the protein and nucleic acid levels, four groups have been identified: PPV-D, PPV-M, PPV-EA, and PPV-C (Kerlan and Dunez 1979; Candresse et al. 1998). PPV-D and PPV-M are the most important, from an economic point of view. The PPV-M strain is the most pathogenic strain, especially for peaches and apricots. The occurrence of PPV-M has mostly been reported from eastern, south-eastern, and central Europe. The PPV-D strain occurs mainly in western Europe, the Mediterranean, and the countries of central Europe (e.g. Czech Republic). PPV-EA is represented by a few isolates found in Egypt (Wetzel et al. 1991). The fourth strain, PPV-C, was described in Moldavia (Nemchinov and Hadidi 1996). An atypical PPV isolate was described from Canada, and represents the distinct strain PPV-W (James et al. 2003; James and Varga 2005). Recently, a new sixth strain, PPV-Rec, was described, resulting from the recombination of PPV-D and PPV-M (Glasa et al. 2004). The members of this strain were found in: Bosnia and Herzegovina (Matić et al. 2006), Albania, Bulgaria, Czech Republic, Germany (Glasa et al. 2004), Hungary (Salamon and Palkovics 2002), Pakistan (Kollerová et al. 2006), Slovakia (Glasa et al. 2002), and recently in Turkey (Candresse et al. 2007). The first detailed serological and molecular characterization of Czech PPV isolates showed the presence of PPV-D and PPV-M serotypes, with a massive occurrence of PPV-D (Navrátil et al. 1998). The use of M specific monoclonal antibodies could not discriminate between members of PPV-M and PPV-Rec. Also, some recombinant events between these strains have been proposed (Navrátil et al. 1998).

Plum (*Prunus domestica*) and myrobalan (*Prunus cerasifera*) trees, showing the typical PPV symptoms,

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of diffuse spots and oak mosaic, were sampled during the period from September 2005 to October 2006. Leaf samples were taken from five plum orchards within the Czech Republic (Table 1). The visual observations showed massive PPV infections in Bohutice I, Lipov and Týn nad Bečvou. Sporadic infections in Bohutice II and Brtev were also observed. The presence of PPV was confirmed by PCR in all the orchards studied. The twenty-four isolates were chosen for detailed molecular analysis.

All the isolates studied were successfully discriminated into PPV-D and PPV-Rec strains (Table 1), using a simplified method based on the analysis of the (Cter) NIb-(Nter) CP region (Šubr et al. 2004). The results were confirmed in 17 samples by specific typing, targeting CI and (Cter) CP regions (Glasa et al. 2002; Candresse et al. 1998). PPV-D isolates were identified in all orchards, whereas the PPV-Rec isolates were found only in two plum orchards, Lipov and Bohutice I. Both of these orchards were established at the beginning of the 1980s, when trees of the cvs 'Čačanska najbolja' (Cacak Best) and 'Čačanska lepotica' (Cacak Beauty) were planted from nursery stock imported from the former Yugoslavia. PPV symptoms were noticed there by growers in the first year of planting (and more recently, since 1984) by the Plant Protection Service inspectors (Dr. Ackerman, personal communication). The occurrence and spread of PPV in the Lipov locality has been studied since 1994; originally, the first isolates were identified as PPV-M (Navrátil et al. 1998). The isolate from this locality, named '302', was later determined to be PPV-Rec (Glasa et al. 2004). Recently, a significant occurrence of PPV-Rec has been confirmed in both orchards. Spread of PPV-Rec, out of the original orchard, has not been noticed in the Bohutice I locality; a sporadic outbreak, up to 0.3 km, has been observed in the Lipov locality.

Table 1 Origin of PPV isolates used in this study and their accession numbers

Isolates	Localities	Host	Target region			Accession numbers
			NIb-CP	CI	CP	
LIP7CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	–	M	EF504286
LIP10CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	–	M	EF504295
LIP14CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	D	M	EF504289
LIP20CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	D	M	EF504293
LIP23CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	D	M	EF504287
LIP49CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	Rec	–	M	EF504288
LIP22CZ	Lipov	*Prunus domestica* plum seedling	D	D	D	EF504261
LIP33CZ	Lipov	*Prunus domestica* autochthonic plum	D	D	D	EF504266
LIP47CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	D	D	D	EF504269
LIP50CZ	Lipov	*Prunus domestica* 'Domácí velkoplodá'	D	D	D	EF504274
BOH1CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	D	M	EF504280
BOH2CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	nt	M	EF504284
BOH3CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	D	–	EF504279
BOH4CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	–	–	EF504282
BOH5CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	D	M	EF504283
BOH6CZ	Bohutice I	*Prunus domestica* 'Čačanska lepotica'	Rec	D	–	EF504281
BOH11CZ	Bohutice II	*Prunus domestica* autochthonic plum	D	D	D	EF504277
BOH12CZ	Bohutice II	*Prunus domestica* autochthonic plum	D	D	D	EF504278
BOH13CZ	Bohutice I	*Prunus domestica* 'Čačanska najbolja'	D	D	D	EF504276
BOH14CZ	Bohutice II	*Prunus domestica* 'Čačanska najbolja'	D	D	–	EF504263
BRE12CZ	Brtev	*Prunus domestica* 'Čačanska lepotica'	D	D	D	EF504265
TY100CZ	Týn nad Bečvou	*Prunus domestica*	D	D	D	EF504271
TY104CZ	Týn nad Bečvou	*Prunus domestica* 'Čačanska lepotica'	D	D	D	EF504267
TY106CZ	Týn nad Bečvou	*Prunus domestica* 'Čačanska najbolja'	D	D	D	EF504272

NIb-CP (Cter)NIb-(Nter)CP (according to Šubr et al. 2004), *CI* CI (according to Glasa et al. 2002), *CP* (Cter)CP (according to Candresse et al. 1998), *D* PPV-D, *M* PPV-M, *Rec* PPV-Rec, – negative amplification, *nt* not tested

The partial (Nter) NIb-(Cter) CP genomic region was used for the phylogenetic analysis. The fragment was obtained in two steps: the first part using mD5/mD3 or mD5/mM3 primer pairs (Šubr et al. 2004), and the second part by PPV-RR primer (Varga and James 2005) in combination with the newly designed primer PPV-CP CORESENS: 5′-ACCCCATTTTCA CTCCAGC-3 (94°C for 3 min; 40 cycles of 94°C for 45 s, 55°C for 60 s, and 72°C for 60 s; then 72°C for 7 min). All of the amplicons were cloned into pGEM-T vector (Promega), and at least three colonies for each were sequenced (3130 Genetic Analyzer, Applied Biosystems). The final sequences, of 1170 bp in length, covering the (Cter) $NIb^{334\ bp}$ and (Nter) $CP^{836\ bp}$ region, were deposited within GenBank.

Comparison and phylogenetic analysis was performed by the neighbour-joining method, using ClustalW; by maximum parsimony, using PAUP version 4.0b10 (Swofford 2002); and also by maximum likelihood, using PHYML v 2.4.4 (Guindon and Gascuel 2003). The most appropriate model for DNA substitution was identified using MODELTEST v. 3.06 (Posada and Crandall 1998). The phylogenetic analysis of PPV-Rec isolates showed that they form a

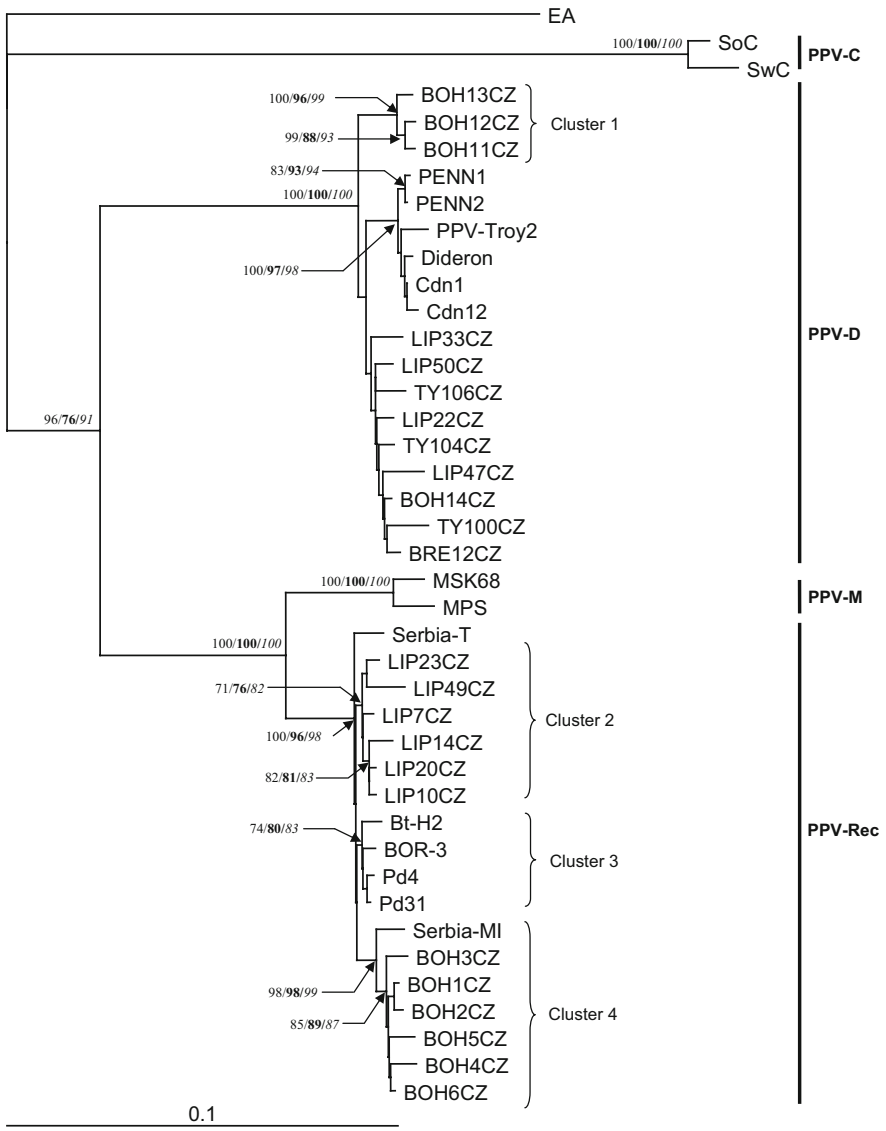

Fig. 1 Neighbour-joining phylogenetic tree of PPV isolates reconstructed from 1170 bp (Cter) NIb-(Nter) CP region (nt 8244–9413). The numbers represent the bootstrap values obtained for neighbour-joining (*first numbers*), parsimony criterion (*bold numbers*) and maximum likelihood under HRY85 evolution model (*italic numbers*). The scale represents 0.1 substitutions per site. Only bootstrap values >70% are shown. Accession number of PPV isolates used are: *Pd4* (AJ566344), *Pd31* (AJ566345), *BOR-3* (AY028309), *Bt-H2* (AJ566346), *Serbia-MI* (AY690605), *Serbia-T* (AY690609), *MPS* (AJ243957), *MSK68* (M92280), *Dideron* (X16415), *PPV-Troy2* (AM260934), *Cdn12* (AY953266), *Cdn1* (AY953261), *PENN1* (AF401295), *PENN2* (AF401296), *SoC* (AY184478), *SwC* (Y09851), *EA* (AM157175)

rather homogenous group. However, three PPV-Rec clusters could be significantly distinguished (Fig. 1). The first is represented by the very close PPV-Rec isolates from Bohutice I (BOH1CZ, BOH2CZ, BOH3CZ, BOH4CZ, BOH5CZ, BOH6CZ) and the Serbian isolate (Serbia-MI) (Cluster 4, Fig. 1). The PPV isolates from Lipov (LIP7CZ, LIP23CZ, LIP49CZ, LIP20CZ, LIP14CZ, LIP10CZ) form the second cluster (Cluster 2, Fig. 1), and the third cluster is represented by the Slovak and Hungarian isolates (BOR-3, Bt-H2, Pd4, Pd31) (Cluster 3, Fig. 1). The branching was significantly supported by neighbour-joining, maximum parsimony, and likelihood analyses. To compare more isolates from the GenBank database, the shorter segments of (Cter) $NIb^{334\ bp}$ and (Nter) $CP^{297\ bp}$ were used. The analysis showed a close relationship of the Bohutice PPV-Rec isolates with the Serbian isolates (Serbia-B, Serbia-PO2, Serbia-MI, Serbia-ST, Serbia-PO3).

Analysis of all PPV-D isolates studied, shows that they formed a monophyletic group. One cluster represented by the isolates BOH11CZ, BOH12CZ, and BOH13CZ diverged separately (Cluster 1, Fig. 1). Analysis of the shorter sequence, showed relationships of the aforementioned Bohutice isolates with the Bosnian isolates (BOS37PI, BOS49PI. One of them, Bosnian isolate BOS49Pl, was earlier characterized by Matić et al. (2006). Recently, some other divergent isolates have been described from Kazakhstan (Spiegel et al. 2004) and Pakistan (Kollerová et al. 2006).

In this study, the current state of knowledge about PPV isolate variability was broadened with the analysis of the new Czech PPV-Rec and D isolates. A substantial diversity was found among Czech PPV-D isolates. Phylogenetic analysis of Czech PPV-Rec isolates, together with knowledge about the importation of plant materials from the Serbian nurseries, supported the hypothesis of Glasa et al. (2005) that former Yugoslavia represents the centre of dispersion of the PPV-Rec isolates.

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