

High-throughput sequencing of *Potato virus M* from tomato in Slovakia reveals a divergent variant of the virus

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Citation: Glasa M., Šoltys K., Predajňa L., Sihelská N., Budiš J., Mrkvoová M., Kraic J., Mihálik D., Ruiz-García A.B. (2019): High-throughput sequencing of *Potato virus M* from tomato in Slovakia reveals a divergent variant of the virus. *Plant Protect. Sci.*, 55: 159–166.

Abstract: High-throughput sequencing (HTS) analysis of tomato (*Solanum lycopersicum*) samples revealed the presence of *Potato virus M* (PVM) in this crop in Slovakia. Full-length genomes of three PVM isolates were obtained using both HTS and Sanger sequencing validation. While two isolates (T40 and T50) were shown to belong to major Group I, a divergent T20 isolate was phylogenetically unrelated to any known PVM variant, potentially representing a new phylogenetic group. Despite a relatively high intraspecies diversity ($17.3 \pm 0.3\%$), no evidence of recombination was detected in the dataset of available complete PVM sequences. Conventional screening of tomato plants in Slovakia using ELISA and RT-PCR further confirmed a frequent occurrence of PVM in this host. Developed RT-PCR showed its polyvalence to detect the PVM Group I isolates, however, in silico analysis of primer binding sites indicated its compromised use for Group II isolates. Our results further pinpoint the significance of HTS for unbiased unveiling of virus diversity and a need for continual optimisation of molecular detection tools.

Keywords: PVM; *Solanum lycopersicum* L.; *Carlavirus*; full-length genome; RT-PCR; phylogenetical diversity

Tomato (*Solanum lycopersicum* L.) represents an economically attractive agricultural crop in Slovakia due to its nutritional properties and usefulness for industrial processing. However, viral pathogens remain an important factor potentially limiting to-

matto yield and quality (HANSEN *et al.* 2010; JONES *et al.* 2014).

Knowledge of viral genetic diversity and the understanding of its complexity are crucial for efficient management of viral disease, i.e. through development

Supported by the Slovak Research and Development Agency, Grants No. APVV-16-0026 and No. APVV-14-0055, and by the Research & Development Operational Programme funded by the ERDF, Grant No. ITMS313021D075 (K. Šoltys).

<https://doi.org/10.17221/144/2018-PPS>

of sensitive and specific diagnostic tools (LÓPEZ *et al.* 2009; BOONHAM *et al.* 2014; PALLÁS *et al.* 2018). The application of high-throughput sequencing (HTS) has profoundly altered our understanding of the comprehensiveness of plant virome and virus variability as it provides a generic approach to identify or characterise the viral agent(s) present in a plant without previous knowledge of the virus structure or sequence (HADIDI *et al.* 2016; ROOSSINCK 2017). HTS is being extensively used in plant virology, resulting in the identification of several novel viruses, including those infecting tomato (LI *et al.* 2012; SAQIB *et al.* 2015; FONTENELE *et al.* 2017; XU *et al.* 2017; VAGHI MEDINA *et al.* 2018). Moreover, HTS-based efforts have largely contributed to extend the knowledge of the variability of already described viruses, showing real diversity of plant viruses often underestimated (POLSTON *et al.* 2015; XU *et al.* 2017).

Potato virus M (PVM) belongs to the genus *Carlavirus* (family Betaflexiviridae). PVM represents a worldwide distributed and economically important virus, affecting mainly potato crops (BRUNT *et al.* 2001). In this work, ribosomal-depleted total RNAs from PVM-infected tomato leaves were analysed using HTS, enabling to obtain complete genome sequences of PVM and further analyse its diversity in tomato in Slovakia.

MATERIAL AND METHODS

Determination of complete genome sequences of PVM isolates. Total RNAs from tomato leaves were extracted using the NucleoSpin RNA Plant kit (Macherey-Nagel, Duren, Germany) and ribosomal RNA was removed using the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, USA). The rRNA-depleted total RNAs sample was used for double-stranded cDNA synthesis using the SuperScript II kit (Thermo Fisher Scientific, Waltham, USA). The column-purified cDNA (DNA Clean & Concentrator™-5 – DNA kit; Zymo Research, Irvine, USA) was then processed with the transposon-based chemistry library preparation kit (Nextera XT; Illumina, USA). Low-cycle PCR and mutual indexing of the fragments were carried out. Fragments were purified with 1.8× AMPure XP beads (BeckmanCoulter, Indianapolis, USA) without size selection. The fragment size structure of the DNA library was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). The equimolar pool of 4 nM DNA libraries

was denatured, diluted to 13 pM and sequenced (200-bp paired-end sequencing) on the Illumina MiSeq platform (Illumina, USA).

High-quality trimmed reads were used for *de novo* assembly and contigs blasted to the viral genome database (<ftp://ftp.ncbi.nih.gov/genomes/Viruses/all.fna.tar.gz>) using CLC Genomics Workbench 7.5 and Geneious v.8.1.9. Alternatively, the reads were mapped against the available complete PVM genome sequences.

The 5'-end of the PVM genomes was determined using total RNA as a template for 5' Rapid amplification of cDNA ends experiment (SMARTer® RACE 5'/3' Kit; TaKaRa, Bio Inc., Shiga, Japan) with a gene-specific primer rPVM_509R (5' GCTCGTAGGAAC-CATACGGCAC 3'), following the manufacturer's instructions. The 3'-end of the genome was RT-PCR-amplified using the oligo(dT) primer in combination with an internal specific primer PVM_8367F (5' GGTATCTCTTACAATGTGCG 3'). In both 5'- and 3'-termini, the overlapping sequences (up to 300 bp) matched unambiguously the HTS-based sequences, confirming their accuracy.

In the case of the divergent isolate T20, Sanger sequencing of PCR products obtained using specific primers designed from the HTS-based sequences was used to close an internal gap in the genome (ca. 2.3 kb between nt 1 500 and 3 800).

Conventional serological and molecular detection. Leaf samples were obtained during July–September 2016 and 2017 from tomato (*Solanum lycopersicum* L.) plants growing in private gardens in three geographically distant localities in Slovakia.

Total RNAs were extracted from tomato leaves using the NucleoSpin RNA Plant kit (Macherey-Nagel, Germany). For RT-PCR detection, a two-step protocol was systematically used. The first-strand cDNA was synthesised using random hexamer primers and AMV reverse transcriptase (Promega Corp., Madison, USA). An aliquot of RT reaction was added to PCR carried out with TaKaRa Ex Taq polymerase (TaKaRa, Bio Inc., Japan). A primer pair PVM_d8065F (5'-CTCAATGC-CGAGGTCAGT-3') and PVM_d8445R (5'-TGACT-GAAGGTATCACCTC-3'), was designed from PVM sequences available in the Genbank database (www.ncbi.nlm.nih.gov; accessed on July 2016) in order to match the actual virus diversity.

The RT-PCR products were gel-purified using the Wizard SV Gel and PCR Clean-Up System (Promega Corp., USA), and directly sequenced in both directions by priming the sequencing reactions with the same oligonucleotides used for PCR amplification.

Table 1. List of the tomato *Potato virus M* isolates characterised in this work and overview of the detection performed on the original tomato samples

Isolate	Locality	PVM accession number	Viral pathogens identified by HTS	Standard detection (ELISA/RT-PCR)
T20	Paňa	MH558035	PVM, PVY	PVM, PVY
T40	Plavecký Mikuláš	MH558037	PVM, PVY, PVS	PVM, PVY, PVS
T50	Plavecký Mikuláš	MH558036	PVM, PVY, CMV	PVM, PVY, CMV
T16	Paňa	MH558038		PVM, PVY
T18	Paňa	MH558039		PVM, PVY, CMV, PVS
T32	Plavecký Mikuláš	MH558047		PVM, PVY, CMV
T48	Plavecký Mikuláš	MH558044		PVM, PVY
T49	Plavecký Mikuláš	MH558041		PVM, PVY
T53	Plavecký Mikuláš	MH558043		PVM, PVY
T57	Plavecký Mikuláš	MH558042		PVM, PVY
T58	Plavecký Mikuláš	MH558040		PVM, PVY
T62	Sološnica	MH558046		PVM, CMV, PVS
T65	Sološnica	MH558045		PVM, PVY

PVM – *Potato virus M*; PVY – *Potato virus Y*; PVS – *Potato virus S*; CMV – *Cucumber mosaic virus*

The presence of PVM and additional viral pathogens in the tomato samples, namely *Potato virus Y* (PVY), *Cucumber mosaic virus* (CMV), *Potato virus S* (PVS), and *Tomato mosaic virus* (ToMV) was checked by DAS-ELISA using a commercially available kit (Bioreba AG, Reinach, Switzerland). In parallel, RT-PCR was performed on the template of total RNAs using protocols for detection of PVY (PVY-9063F/PVY-9316R; SIHELSKÁ *et al.* 2016), PVS (PVS_7833F/PVS_8386R; PREDAJŇA *et al.* 2017), ToMV (ToMV-F/ToMV-R; SIHELSKÁ *et al.* 2017) and CMV (CMV_CPF 5'-TYT-CATGGATGCTTCTCCRC-3'/ CMV_CPr 5'-CTG-GATGGACAACCCGTTTC-3'; Glasa M., unpublished).

Sequence analyses. Nucleotide PVM sequences reported in this paper have been deposited in the GenBank database and have the following accession numbers: MH558035–MH558037 (full-length genomes), MH558038–MH558047 (partial genomes).

Sequence analyses and comparisons were done using the MEGA v7 program (KUMAR *et al.* 2016). The alignments were used as input data to construct phylogenetic trees by the neighbour-joining distance

method implemented in MEGA; each time the bootstrap analysis with 1 000 replicates was performed to assess the robustness of the branches. DnaSP v.5 (LIBRADO & ROZAS 2009) was used to estimate the average intra-group divergence values.

Searches for potential recombination events and identification of recombination breakpoints employed seven methods, including RDP, GENECONV, Bootscan, Maxchi, Chimaera, SiScan and 3SEQ, implemented in the RDP4 v.4.1 software (MARTIN *et al.* 2015), using default settings. Recombination events were noted if supported by at least four different methods (P -values $< 1.0 \times 10^{-6}$).

RESULTS

Mapping the individual reads (2.1–3.5 millions) obtained from T20, T40, and T50 samples against reference genomes available in the databases (NC_001361) enabled recovery of the nearly complete PVM genome of T40 and T50 isolates (Table 2). In the case of T20,

Table 2. Analysis of the ribosomal-depleted total RNA-derived high-throughput sequencing reads from the tomato samples

Isolate	Total reads	PVM mapped reads ^a	Mapped reads (%)	Average coverage	Reference genome covered ^a (%)
T20	3 585 846	259 664	7.2	3 866.4 x	73 ^b
T40	2 162 142	384 465	17.7	5 512.1 x	99.8
T50	2 466 092	299 219	12.1	4 198.6 x	99.9

^amapped to the PVM NC_001361 genome; ^bdue to an unmapped gap between nt 1 500–3 800

<https://doi.org/10.17221/144/2018-PPS>

the percentage of the reference genome covered by the reads was much lower (73%), due to its sequence divergence respect to the PVM isolate used as reference. In order to recover the full-length sequence of this putative divergent isolate, Sanger sequencing was used to fill a 2.3 kb gap in the open reading frame 1 (ORF1) region of the genome. A subsequent mapping against such completed T20 sequence showed a full-length coverage of almost whole genome (data not shown), confirming a high variability of the T20 isolate in this region of the genome. For all 3 isolates, the 5' and 3' extremities were determined by 5' RACE and oligo d(T)-anchored RT-PCR.

The genomic organisation was fully conserved between all Slovak PVM isolates and was typical of members of the genus *Carlavirus* (Figure 1). The genome was either 8 523 nt (T40 and T50) or 8 524 nt

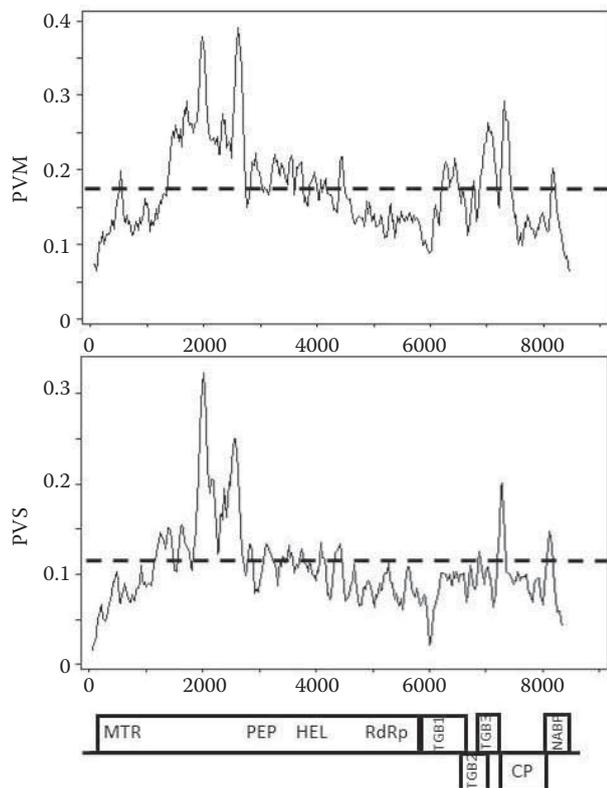


Figure 1. Nucleotide diversity (π) of *Potato virus M* (PVM) and *Potato virus S* (PVS) plotted along the full-length genomes with a window of 100 nt and a step of 25 nt. Open reading frames are indicated by boxes

MT – viral methyltransferase; PEP – peptidase; HEL – helicase; RdRp – RNA dependent RNA polymerase; TGB1, 2, 3 – Triple gene block protein 1, 2, 3; CP – coat protein; NABP – nucleic acid binding protein; the overall average diversity is indicated by a dashed line; scale schematic representation of the PVM genome is illustrated at the bottom

long and contained 6 ORFs. From 5' to 3' these correspond to a large replication-associated protein (ORF1, translated to 1964 aa), followed by three ORFs encoding the proteins recognised as triple gene block (TGB, length of 229 aa, 109 aa, and 63 aa, respectively). ORF5 is translated to the coat protein (304 aa) with calculated mass of 33.589, 33.897, and 33.917 kDa for T20, T40, and T50, respectively. The last ORF encoded a nucleic acid binding protein (NABP, 108 aa).

The genome was highly collinear between three isolates, with only one insertion in the T20 genome (7 202 nt) occurring in the intergenic region between ORF4 and ORF5. 5' non-coding region (NCR) was determined by 5' RACE and was 76 nt long in all 3 Slovak isolates, while the length of 3' NCR was 73 nt.

The PVM diversity was analysed using the available dataset of 16 full-length PVM sequences (13 available in the databases and 3 determined in this work). At the whole genome level, the average pairwise divergence among PVM isolates reached $17.3 \pm 0.3\%$. This value is higher in comparison with the known intraspecies variability of a related *carlavirus*, PVS. Computed for a dataset composed of 33 full-length PVS sequences available actually (Sept 2018) in the Genbank database, it shows an $11.4 \pm 0.2\%$ divergence. In both viruses, the highest diversity was observed in ORF1 around nt positions 1 500–3 000 (Figure 1).

Figure 2 shows a clear separation of PVM isolates into two major phylogenetic clusters. T40 and T50 isolates, originally collected from unrelated private gardens located in the same village (Plavecký Mikuláš), are phylogenetically related and fell into the major phylogenetic PVM group, together with potato and tomato isolates from Europe and Asia. Isolate YN (KY364848), reported recently as a divergent PVM variant (Su *et al.* 2017) is included in a well-supported phylogenetic group with Bangladesh isolates (MF133528–MF133530). Interestingly, the Slovak T20 isolate did not cluster together with any of the isolates analysed, either with the major group including T40 and T50 or with the one represented by Chinese and Bangladesh isolates. Thus, T20 represents a distinct lineage in the phylogenetic tree that potentially corresponds to a new phylogenetic group.

The detection of PVM in tomato has prompted further screening of its occurrence on a larger set of 61 samples. Using DAS-ELISA and RT-PCR, 10 out of 61 tomato samples were tested positive for PVM. All of these samples were included in a phylogenetic analysis based on the partial sequences spanning

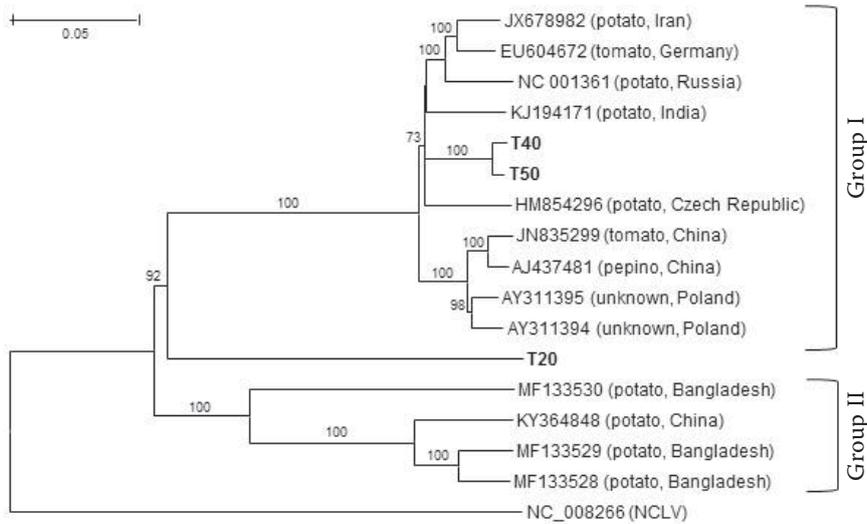


Figure 2. Phylogenetic tree generated on complete nucleotide genome sequences of PVM isolates. Isolates are identified by their GenBank accession number, host and country of origin. The Slovak isolates sequenced in the present study are highlighted in bold. An isolate of *Narcissus common latent virus* was used as an outgroup. The scale bar indicates a genetic distance of 0.05. Bootstrap values higher than 70% (1 000 bootstrap re-samplings) are indicated. The phylogenetic groups are indicated on the right margin

the 3' end of the genome (340 bp, 8 086–8 425 nt). This phylogenetic study involving 109 PVM isolates further confirmed the clustering of PVM isolates

in a major group and a separate position for PVM T20. With the exception of T20 isolate, all partially sequenced Slovak PVM isolates belong to major Group I, although divided into two subclusters (Figure 3). No evidence of recombination was identified in the dataset of 16 complete PVM genomes using RDP analysis.

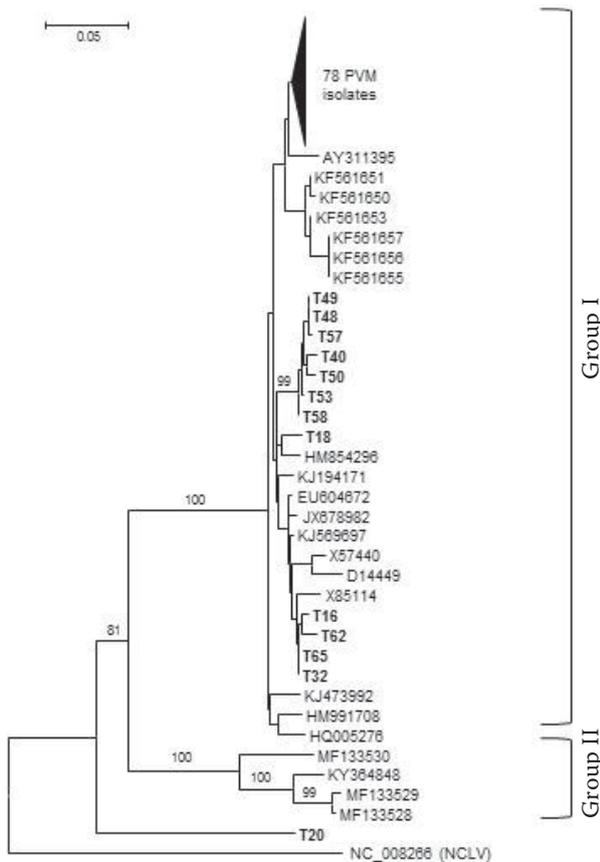


Figure 3. Neighbour-joining phylogenetic tree generated from 340 bp portion of PVM (8 086 to 8 425 nt on the NC_001361 genome). Condensed part of the tree corresponds mostly to the Chinese PVM isolates from pepino (Ge *et al.* 2014). An isolate of *Narcissus common latent virus* was used as an outgroup

The primers used for the molecular detection of the Slovak PVM isolates studied in this work (PVM_d8065F/PVM_d8445R) were designed based on the sequence dataset available in the Genbank (accessed on July 2016), targeting the highly conservative genome portions spanning the 3' end of the CP gene and 3' end of the NABP gene, respectively (Figure 4). Despite 3 and 2 mutations, respectively, in the primer sequences, the divergent T20 was amplified by RT-PCR, providing the evidence of polyvalence of these primers for Slovak PVM isolates, and likely for all PVM isolates belonging to Group I.

DISCUSSION

The reports of new virus species recovered from tomato plants are increasing (Li *et al.* 2012; SAQIB *et al.* 2015; FONTENELE *et al.* 2017; XU *et al.* 2017; VAGHI MEDINA *et al.* 2018). However, attention has also to be paid to cover the real diversity of already established and “common” tomato viruses, which could be still overlooked.

As part of the characterisation of the virome present in cultivated tomato plants in Slovakia, ribosomal-depleted total RNAs from three tomato plants grown in private gardens in the open air (labelled as T20, T40, and T50) were subjected to HTS analysis. Leaf

<https://doi.org/10.17221/144/2018-PPS>

primer (5'→3')	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
NC_001361	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
JX678982	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
JN835299	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
KJ194171	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
HM854296	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
AY311395	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
AY311394	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
EU604672	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
AJ437481	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
T40	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
T50	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
T20	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
KY364848	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
MF133528	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
MF133529	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
MF133530	CTCAATGCCGAGGTCAGT	TGACTGAAGGTATCACCTC
	8065 8083 8445 8427	

Figure 4. Alignment of the sequences corresponding to the binding sites of the detection primer pair used in this work (PVM_d8065F/PVM_d8445R). Mutations in the binding sites are grey-shaded. The primer sequences are shown at the top of the alignment and their position on the NC_001361 genome is indicated below the alignment

samples were collected during the vegetation period from plants showing either virus-like symptoms (mild leaf distortions in T20 and leaf deformations and growth reduction in T50) or having a symptomless behaviour (T40).

Blast analysis of *de novo* assembled contigs revealed the presence of PVM in all 3 tomato plants, in a complex infection involving PVY, CMV and/or PVS (Table 1). To our knowledge this is the first report and characterisation of PVM in tomato in Slovakia.

The relatively high intraspecies variability detected for PVM (17.3%) is mainly due to the recent characterisation of a divergent Chinese isolate YN (KY364848; Su *et al.* 2017) and isolates from Bangladesh (MF133528–MF133530; Xu H., direct submission). Interestingly, these divergent PVM isolates were characterised using HTS, further confirming the potential of this approach for characterisation of isolates escaping standard detection methods. In this line, the identification of Slovak T20 isolate and determination of its complete genome sequence would be highly challenging using standard detection and sequencing methods, due to its molecular distinctness from other Slovak PVM isolates.

Recombination represents one of the key evolutionary processes underlying the high genetic variability of plant RNA viruses, contributing to the macro-evolution of viruses and to the extent of their intraspecific diversity (SZTUBA-SOLIŃSKA *et al.* 2011). Within the genus *Carlavirus*, recombination was reported to occur in the evolutionary history of *Cowpea mild mottle virus* (ZANARDO *et al.* 2014) and *Chrysanthemum virus B* (SINGH *et al.* 2012). Similarly, using partial sequence data, a recombination event was identified in the TGB2-TGB3-CP

region of the Chinese PVM isolate from pepino (GE *et al.* 2014) and in the CP gene of *Blueberry scorch virus* (KALINOWSKA *et al.* 2015) and PVS (LIN *et al.* 2014). The lack of the recombination event detected within PVM in this work should thus reflect a still insufficient number of complete genome sequences available, not representative of all genetic variants identified previously by partial sequencing.

Conventional detection techniques based on RT-PCR have increased the sensitivity and accuracy of viral diagnostics, however, they only allow the detection of known viruses, and their development and need of optimisation depend on the availability of sequence data. The current knowledge and complexity of molecular data for a given virus can still be biased or incomplete not only in recently identified virus pathogens, but also in common and long-time established viruses. Consequently, developed detection techniques need not polyvalent enough, resulting in the false negative diagnostic results for divergent variants or strains.

As shown before, recently reported PVM isolates from China and Bangladesh represent a distinct genetic group (Figures 2 and 3). *In silico* analysis of their sequences revealed several mutations in the binding site of the forward primer (Figure 4). As two of these mutations affect positions close to the primer 3' end, the suitability of this primer for detection of Group II isolates might be negatively affected. Therefore, a reliable RT-PCR-based detection of Group II PVM isolates should be developed, however, probably only when more sequence data are available.

PVM is considered mostly latent or causing only moderate symptoms in infected potatoes (BRUNT *et*

al. 2001), however, less data are available for PVM infection in tomato. Although some of the tomato plants tested in this work did not show clear virus-like symptoms, several of them exhibited leaf mosaics, yellowing, and apical leaf deformation (data not shown). As the PVM-positive tomato plants were found to be also infected with CMV, PVS or PVY in double or triple infection (Table 1), it is not possible, however, to assess the potential contribution of PVM to the symptoms observed.

PVM is one of the “common” pathogens that have been recognised to infect plants for a long time (BAGNALL & LARSON 1957; BRUNT *et al.* 2001). Despite of this fact, the knowledge of its variability was limited, mainly due to the reduced availability of full-length genomes.

In this work, three Slovak PVM isolates infecting tomato have been sequenced and characterised. A phylogenetic analysis of these three full-length sequences together with other sequences available in the databases has revealed the existence of a new phylogenetic divergent group of this virus. Our results further pinpoint the significance of high-throughput sequencing approaches providing an unbiased access to viral diversity studies, with the potential consequences for the development/optimisation of routine pathogen detection.

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Received: October 11, 2018

Accepted: December 10, 2018

Published online: January 08, 2019