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Original scientific paper

MOLECULAR CHARACTERIZATION OF *POTATO VIRUS Y* INDUCING POTATO TUBER NECROTIC RINGSPOT DISEASE IN SERBIA

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The *Potato virus Y* (PVY) is the most important limiting factor for potato seed production in Serbia. Currently, PVY is a major concern for the potato seed growers. Initially, serological (ELISA) tests were carried out on 100 potato seed tubers from each of the seven potato cv. during 2013. The infection rates with the PVY^N was between 5 and 36%. A complete genome sequencing of the most common Serbian isolate of PVY (3D), followed by molecular characterization and phylogenetic analysis has been performed to show what group it belongs to. Our isolate's complete genome sequence (KJ946936) showed that the Serbian PVY isolate (3D) is 99.7% identical at nt level, with other tuber necrosis strain group (PVY^{NTN}) from Europe. Phylogenetic analysis revealed three consistent lineages of isolates, showing that our isolate was clustered with the isolates from Europe and North America in the PVY^N lineage which induces potato tuber necrotic ringspot disease (PTNRD). The Serbian isolate of PVY^{NTN} together with the isolates from Europe was clustered in the branch of European sublineage, with a high bootstrap support and no genetic diversity. This is the first study in Serbia demonstrating phylogenetic distinction between our isolate and other isolates of PVY.

Keywords: Potato, PVY, Genome, PTNRD, diversity

INTRODUCTION

Potato virus Y (PVY) is a type member of genus *Potyvirus* in the family *Potyviridae* with a single-stranded positive-sense genomic RNA of approximately 9.7 kb with poly (A) tail at the 3′-

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terminus and a covalently-linked VPg protein attached to the 5′-terminus (ADAMS *et al.*, 2011). The virus was discovered in the early 20th century and is transmitted in a non-persistent manner by a wide range of aphid species as well as by a vegetative propagation of potato. PVY causes significant losses in many other crops, mainly in the *Solanaceae* family: tobacco, tomato, pepper and numerous self-propagating plants (ROBERT *et al*., 2000; RAGSDALE *et al*., 2001). PVY is an economically important plant pathogen and highly associated with potato degeneration, which causes a significant loss of crops of up to 90%.

The viral RNA encodes eleven functional proteins: namely the first protein (P1), helper component proteinase (HC-Pro), third protein (P3), Pretty interesting Potyviridae ORF (PIPO) that is embedded within P3 cistron, first 6 kilodalton protein (6K1), cylindrical inclusion protein (CI), second 6 kilodalton protein (6K2), viral genome linked protein (Vpg), nuclear inclusion protein a (NIa), nuclear inclusion protein b (NIb), and coat protein (CP) (CHUNG *et al.*, 2008). PVY isolates have been categorized into several distinct groups of strains. These include the common (ordinary) group PVY^{O} (O strains), the tobacco vein necrosis group PVY^{N} (N strains), the stipple streak group PVY^{C} (C strains), PVY^{Z}, $PVY^{N\text{-}Wilga}$ and the tuber necrosis strain group PVY^{NTN} (SINGH *et al.*, 2008).

PVY^{NTN} pathotypes are the main causal agents of potato tuber necrotic ringspot disease (PTNRD). They were directly obtained from tubers exhibiting PTNRD and, apparently, originated from recombination between PVY^{O} and PVY^{N}. However, some non-recombinant isolates from North America (NA-PVY^{N} and NA-PVY^{NTN}) caused PTNRD when inoculated to potatoes (SCHUBERT *et al.*, 2007; SINGH *et al.*, 2008). More recent studies suggest that non recombinant PVY^{NTN} isolates can cause PTNRD, meaning that the recombinant structure of the genome is not necessary for the isolate to cause PTNRD (NIE and SINGH, 2003; OGAWA *et al*., 2008).

Past studies have shown that PTNRD was present in Serbia, but no detailed study of the PVY isolates found in Serbia was carried out. The PVY^{NTN} isolate has also been detected on the basis of the CP gene in Serbia (STAROVIC *et al*., 2014). The aim of this paper is the characterization and phylogenetic analysis of the detected the most prevalent PVY isolate in Serbia based on the complete genome sequence as well as a comparison to isolates from Europe and North America.

MATERIALS AND METHODS

Field surveys: collection of plant samples

A total of 100 tuber samples of seven potato cultivars with a varied degree of PTNRD symptoms were collected from the major potato producing areas in the Serbia, during 2013 (Table 1). After the harvest, the tubers were washed, examined for superficial symptoms and a total of 700 scabby tubers were selected from each site of the selected field.

Serological detection

Potato tuber samples were tested for the presence of PVY using enzyme-linked immunosorbent assay (ELISA) according to a standard DAS-ELISA protocol (CLARKS and ADAMS, 1977). Samples were analyzed using a commercial antibody specific for a detection of tobacco vein necrosis- NTN and N strains (the necrotic strain group-specific PVY reagent - «PVY necrotic», Bioreba AG, Switzerland). The top eye of the tuber extracts were bathed in the extraction buffer at a ratio of 1:20 (wt/vol). The absorbance at 405 nm (A_{405}) was recorded using a microplate reader (Titertek Uniscan II) after 1 hr of substrate incubation at room temperature

(23°C). Both commercial positive controls for the above viruses and negative controls (extracts from healthy potato leaf tissue, commercially available negative control and extraction buffer) were included in each ELISA. Samples were considered positive if the average optical density (OD) was equal to or higher than two times the average OD of the negative control.

Table 1. Collected potato cultivars and the isolates used in the PCR from different localities in Serbia during 2013

Isolates	Cultivars	Locality / altitude
1D	Caruso	Kušići / 1050 m
2D	Collete	Ivanjica / 468 m
3D	Romano	Čačak / 204 m
4D	Hermes	Guča / 450 m
5D	Volare	Golija / 1800 m
6D	Karlena	Javor / 500 m
7D	Gala	Močioci / 950 m

Molecular detection of PVY in potato tubers

RNA Extraction and first cDNA strand synthesis

Total RNAs from seven ELISA-positive potato tubers of different cultivars with PTNRD simptoms, were extracted with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Full-length cDNA was synthesized by Reverse transcription reaction. PCR was conducted using specific primer pairs for all ten genomic regions (Table 2), and they were separately amplified (GLAIS *et al*. 2002). Total RNAs obtained from the Netherlands (NAK) PVY isolate from potato and healthy potato tubers served as the positive and negative controls, respectively.

Total RNA was diluted in 50 µl of RNase free water and centrifuged for 1 min at 10000 rpm. The resulting RNA was stored at -80°C. Four microliters of concentrated total RNA extract was diluted with 6 µl of RNase free water to reduce nonspecific amplification (SINGH *et al*., 2003), incubated at 70°C for 5 min and chilled on ice for 3 min. To the denatured RNA extract, 15 µl of RT reaction mixture Oligo-dT Primer, 5x FS Buffer, 40 units of RiboLock RNase Inhibitor, 10 mM dNTP and 200 units of M-MLV reverse transcriptase (Invitrogen, CA, USA) were added to provide a final volume of 25 µl. The samples were incubated at 42°C for 1 h, followed by 75°C for 5 min to terminate the RT reaction.

The PCR mixture (50 µl) contained down-steam and upstream primers, 0.4mM dNTPs, 25 mM $MgCl_2$, 10 × PCR buffer, 5 U/µl Taq DNA polymerase (Kapa Biosystems), water and genomic DNA per reaction mixture. For all primer pairs, 30 cycles of amplification in a Thermal Cycler PCR system 2720 (Applied Biosystems) were carried out using the following program: 1 min denaturation at 94°C, 1 min annealing at 57°C and 1 min elongation at 72°C; except for CI, VPg, NIb and 3'NTR regions where the annealing temperature used was 55°C (GLAIS *et al*. 2002). Amplified products were analyzed by 1% agarose gel electrophoresis, stained with Midori Green DNA Stain (Nippon Genetics), and visualized under a UV transilluminator.

Table 2. Sequence and genomic location of primer pairs used for nucleic acid amplification

Primer	Sequence (5′ to 3′)	Genomic location
PVYc	AATTAAAACAACTCAATACA	2–21
PVYd	TG(CT)GA(CTA)CCACGCACTATGAA	955–974
HC-Pro/F	CG(AC)A(AG)GGG(CT)GATAGTGGAGT	880–899
HC-Pro/R	GTTTCTGC(CT)GCTGACACTCG	2704–2723
P3/F	CTGG(CT)ATACT(TG)ATGGCTATG	2588–2607
P3/R	CA(AG)TC(AG)CTCCTTTCAGCATC	3574–3593
CI/F	ATGGAAGAATATGATGTGCG	3478–3497
CI/R	GACACAGT(CT)TCAACTGATTG	5692–5711
VPg/F	CAATCAGTTGA(AG)ACTGTGTC	5692–5711
VPg/R	GCTTCATGCTCCAC(CT)TCCTG	6265–6284
NIa/F	ATGAAGC(CT)AAATC(AG)CTCATG	6278–6297
NIa/R	A(CT)GCAGA(AG)TG(CT)TTAGCTTGC	7011–7030
NIb/F	ATGT(AT)GT(TG)GT(AG)GAGCAAGCT	6998–7017
NIb/R	TTGTGTCATTT(CG)CTTGATGG	8565–8584
CP/F	ACCATCAAG(CG)AAATGACACA	8564–8583
CP/R	CGGAGAGACACTACATCACA	9371–9390
3′NTR$^{/F}$	AGAGAGGCACACCACCGAGG	9309–9328
3′NTR^{C}	GTCTCCTGATTGAAGTTTAC	9684–9703
Seq-5	AAAAA(CT)CGCTTAGCATGATA	1675–1694
Seq-6	AATGCAA(AG)(CT)TTCCT(CG)TGGGG	1645–1664

Sequence analyses

Sequencing in both directions of the representative isolate 3D, from the cv. Romano which showed the highest degree of infection, was performed in an automated sequencer (ABI 3730XL Automatic Sequencer Macrogen, Korea). The sequence generated in this study was deposited in the National Center of Biotechnology Information (NCBI) GenBank database. Sequence of the Serbian virus isolate was compared with the respective virus sequences available in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/) using the ClustalW program (THOMPSON *et al*., 1994) and MEGA5 software (TAMURA *et al*., 2011). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses and the divergence of selected virus isolate sequences was calculated using sequences trimmed to the length of the shortest fragment.

A phylogenetic tree was constructed using 21 full length sequences of PVY representing isolates collected worldwide which were retrieved from GenBank (Table 3) and those PVY polyprotein sequences generated in this study and using the neighbour-joining algorithm implemented in MEGA5. One sequence of *Pepper mottle virus* (PepMoV) was used as outgroup (Table 3). The best-fitting model of nt substitution was investigated using the MODELTEST implemented in MEGA5, and the Tamura 3-parameter model Gamma distributed (TN93+G) was chosen. The reliability of the obtained tree was evaluated using the bootstrap method based on

1000 replicates, and bootstrap values <50% were omitted. Intra- and inter-group diversity values were calculated as the average genetic distance using TN93+G.

Table 3. PVY isolates with complete genomic sequence from GenBank used in the phylogenetic analysis

Isolate	Origin	Location (Prefecture)	Strain or variant[a]	Accession number
NTND6	*S. tuberosum* cv. Dejima	Kyushu islands (Nagasaki)	J-NTN	AB331515
NTNHO90	*S. tuberosum* cv. Danshaku	Hokkaido islands (Hokkaido)	J-NTN	AB331517
NTNNN99	*S. tuberosum* cv. Nishiyutaka	Honshu islands (Nagano)	J-NTN	AB331518
NTNOK105	*S. tuberosum* cv. Nishiyutaka	Okinawa islands (Okinawa)	J-NTN	AB331516
NTNON92	*S. tuberosum* cv. Nishiyutaka	Tokunoshima islands (Kagoshima)	J-NTN	AB331519
Adgen-C	*S. tuberosum*	France	C	AJ890348
LYE84.2	*Lycopersicon esculentum*	Spain (Canary Islands)	C (?)	AJ439545
SASA-110	*S. tuberosum*	UK	O	AJ585195
SASA-61	*S. tuberosum*	UK	NA(O)	AJ585198
SCRI-O	*S. tuberosum*	UK	O	AJ585196
Son41	*Solanum nigrum*	France	C (?)	AJ439544
MN	*N. tabacum*	USA	(?)	AF463399
N-Jg	*S. tuberosum*	Canada	NA	AY166867
O-139	*S. tuberosum*	Canada	O	U09509
Oz	Unknown	USA	O	EF026074
RRA-1	*S. tuberosum* cv. Ranger Russet	USA	NA-NTN	AY884984
Tu660	*S. tuberosum*	Canada	NA-NTN	AY166866
11627-12	*S. tuberosum*	UK	NTN	KC634007
IUNG-4	*N. tabacum* cv. Virginia SCR	Poland	NTN	JF927752
NiBV151	*S. tuberosum* cv. Pentland Squire	Slovenia	NTN	KN396648
3D	*S. tuberosum* cv. Romano	Serbia	NTN	KJ946936

[a]Grouped according to the molecular structure, designation in brackets indicates the strain grouping based on known biological features if different from molecular; O, PVY^{O}; C, PVY^{C}; NTN, PVY^{NTN} (European type); NA, North American type of PVY^{N}; NA-NTN, North American type of PVY^{NTN}; J-NTN, Japanese type of PVY^{NTN}; ?, not sure or not defined.

RESULTS

Serological assay

From the 700 tested tubers 126 samples were ELISA-positive for the presence of PVY reacted with the necrotic strain group-specific PVY reagent - specific for PVY^N, revealing that this was a N serotype. ELISA tests performed on collected potato tuber samples resulted in the infection rates between 5- 36% for PVY^N (Table 4).

Table 4. A percentage of potato virus infections in the tubers collected from potato producing areas in Serbia, assessed by DAS-ELISA test

Cultivars	Potato virus	Percentage of virus infection
Caruso	PVY^N	15
Collete	PVY^N	23
Romano	PVY^N	36
Hermes	PVY^N	22
Volare	PVY^N	5
Karlena	PVY^N	9
Gala	PVY^N	16

Molecular detection and sequence analysis

From seven isolates coat protein (CP) regions were amplified and sequenced. Based on CP region sequences we have detected that all seven isolates belong to PVY^{NTN} strain. For further analysis and unambiguous determination of PVY^{NTN} one isolate (3D) was selected, and it has been completely sequenced. The regions encoding P1, helper component proteinase (HC-Pro) protein, P3, 6Kda 1 (6K1) protein, cylindrical inclusion (CI) protein, 6Kda 2 (6K2) protein, genome-linked viral (VPg) protein, nuclear inclusion a-proteinase (NIa-Pro) protein, nuclear inclusion b (NIb) protein and CP were 825, 1395, 1095, 156, 1902, 156, 564, 732, 1557 and 801 nucleotides long, respectively. The genome of the most common Serbian isolate of PVY^{NTN} (3D) was 9645 nucleotides long including the 5′NTR and 3′NTR regions. The complete genomic sequence of Serbian PVY^{NTN} isolate 3D (KJ946936) was 99.7% identical at nt level (99.8% aa identity), with Polish PVY^{NTN} isolates (JF927752 and JF 927761) and Slovenian PVY^{NTN} isolate (KM396648).

Phylogenetic analysis of PVY^{NTN}

A neighbour-joining tree (Fig. 1) of 21 PVY isolates was constructed using the full-length sequences of the genomic RNAs (9645 nt). Phylogenetic analysis revealed three consistent lineages of isolates (C, O and N), with high bootstrap values (100, 99 and 100%, respectively). The N lineage formed two sublineages N-Europe and N-North America. Genetic diversity among three major lineages of isolates was ranging from 0.149±0.003 to 0.228±0.005, whereas within each group and subgroup were: 0.028±0.001 (O lineage), 0.124±0.003 (C lineage), 0.004±0.000 (Europe sublineage) and 0.008±0.000 (North America sublineage). The Serbian isolate of PVY^{NTN} together with the isolates from Europe (United Kingdom, Poland and Slovenia) were clustered in the branch of European sublineage, with high bootstrap support of 100% and with no genetic diversity.

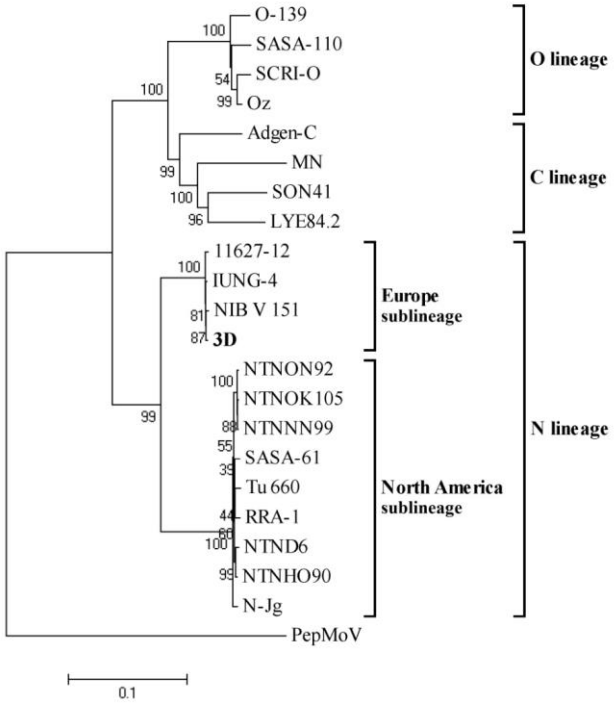

Fig. 1. Neighbour-joining tree based on nucleotide sequences of complete coat protein gene of 21 isolates of *Potato virus Y*. The tree was rooted with *Pepper mottle virus* sequence. Phylogram was generated with MEGA 5 using Tamura 3-parameter model Gamma distributed. Bootstrap analysis was performed with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. The Serbian PVY isolate is in bold.

DISCUSSION

According to the potato specialists in most parts of the world, PVY is currently considered to be economically the most harmful virus in cultivated potatoes. Serbia is extreme in that PVY has caused significant problems to potato production. The virus reduces potato yield significantly and is the most important limiting factor for seed production in Serbia and neighbouring countries (MILOSEVIC, 2009; STAROVIC *et al.*, 2014). The most dominant isolate of PVY is necrotic, able to infect more than 50% of plants in a crop, depending on location and potato cultivar. The occurrence and rate of infection with PVY has been studied over three decades and its distribution has become epidemic in Serbia and its wider region (MILOSEVIC, 2013; STAROVIC *et al.*, 2014). At the same time, previously unknown strains of PVY have evolved in or spread to the new geographical areas (KERLAN and LE ROMANCER, 1992; MCDONALD and KRISTJANSSON, 1993). With the discovery of PVY^{NTN} isolates in several European countries (KERLAN and LE ROMANCER, 1992; KUS, 1995) and the emergence of PVY^{N} strains in North America (SINGH, 1992; MCDONALD and KRISTJANSSON, 1993), the stage was set for another wave of altered nomenclature, this one based on the serological and molecular characterization of the virus. Understanding the evolutionary history of viruses and the evolutionary mechanisms driving

their selection and diversification, is an important aspect of evolutionary biology and would help us to manage viral diseases and the risk of emerging new viruses (SEO *et al.,* 2009). There have been several studies attempting to understand evolutionary history of potyviruses by analyzing genetic structures of their populations on a large geographical scales, for instance of those of *Potato virus Y* (OGAWA *et al*., 2008).

A phylogenetic tree based on sequences of the complete genome divided all 21 PVY isolates into three main lineages: PVY^{O}, PVY^{C} and PVY^{N}. The PVY^{N} lineage contained two sublineages. Nucleotide diversity of the complete genome C lineage isolates was more diverse than the O and N lineage isolates. This is the first study in Serbia to demonstrate phylogenetic distinction between our isolate (3D) and other PVY^{NTN} isolates. In addition to this, based on the full length sequence of PVY^{NTN}, two separate sublineages (Europe and North America) can be identified in the PVY^{NTN} tuber necrosis group that induces potato tuber necrotic ringspot disease. Our study with the 21 complete genome sequences, including the one from Serbia, supports the clustering of PVY isolates into Europe sublineage of the N lineage. The analysis performed in this study grouped Serbian PVY isolate with the European isolates of PVY because they show low diversity. In Europe, the non-recombinant PVY strains PVY^{O} and PVY^{N} have all but disappeared, replaced by recombinant strains PVY^{NTN} and $PVY^{N\text{-}Wi}$ (BLANCHARD *et al.,* 2008; KERLAN and MOURY, 2008). However, in the United States and Canada, PVY^{O} remains the predominant strain infecting potato, and PVY^{N} is rare, although recombinant strains are increasing in incidence and distribution (PICHE *et al.,* 2004; BALDAUF *et al.,* 2006; KARASEV *et al.,* 2008; GRAY *et al*., 2010). In New Zealand, contrary to the situation in Europe and even in North America, recombinant PVY strains have not been identified and only the PVY^{O} and PVY^{N} strains were found in potato (FOMICHEVA *et al.,* 2009). The overall shape of the phylogenetic tree reconstructed with PVY isolates was similar to those, with three consistent lineages C, O, N as previously reported (MOURY *et al*., 2002; FANIGLIULO *et al.,* 2005; GLAIS *et al*., 2002, 2005; LORENZEN *et al*., 2006).

Currently PVY is a major concern for the potato seed growers. If the PTNRD isolates of PVY continue to increase and become epidemic, PVY could become a major concern for the entire potato industry: growers, processors and consumers. In the future, we should analyze the genetic diversity and structure of the PVY population, recombination events using more diverse PVY sequence data in order to further understand its evolution and develop more efficient control measures.

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MOLEKULARNA KARAKTERIZACIJA Y VIRUSA KROMPIRA PROUZROKOVAČA PRSTENASTE NEKROZE KRTOLA KROMPIRA U SRBIJI

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Izvod

Potato virus Y (PVY) je ograničavajući faktor i jedan od najvećih problema semenske proizvodnje krompira u Srbiji. Serološkim ELISA testom analizirano je po 100 krtola sedam sorti semenskog krompira tokom 2013. godine. Procenat zaraze PVY^N kretao se od 5- 36%. U cilju određivanja molekularne karakterizacije i filogenetske analize urađena je kompletna genetska sekvenca našeg najzastupljenijeg izolata u Srbiji (3D), koja je deponovana u NCBI bazi (KJ946936), a pokazala je 99,7% nukleotidnu identičnost sa drugim evropskim nektoričnim sojevima PVY^{NTN}. Filogenetskom analizom utvrđeno je postojanje tri homogene grupe izolata, a naš izolat je svrstan u Evropsku i Severnoameričku PVY^N grupu prouzrokovača prstenaste nekroze krtola krompira (PNKK). Srpski izolat PVY^N se nalazi u evropskoj podgrupi sa visokom bootstrap podrškom, bez genetičkih razlika. Ovo je prva studija u Srbiji koja ukazuje na filogenetsku razliku našeg i drugih izolata PVY.

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