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Original scientific paper

Occurrence and molecular characterization of wheat streak mosaic virus in wheat in Serbia

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SUMMARY

The wheat streak mosaic virus (WSMV), vectored by the wheat curl mite, is globally distributed and threatens wheat production worldwide. Since its first occurrence in Serbia in the 1960s, WSMV presence has not been monitored. In 2019, a total of 62 samples of five wheat cultivars from eight locations in Serbia were collected and tested for the presence of nine common wheat viruses: WSMV, barley yellow dwarf virus-PAV, -MAV, -SGV, and -RMV, cereal yellow dwarf virus-RPV, wheat spindle streak virus, brome mosaic virus, and soil-borne wheat mosaic virus, using individual or multiplex RT-PCR. WSMV was detected in 58.1% of the tested samples in seven wheat crops at five different locations. Species-specific primers failed to detect the presence of the other eight tested viruses. For further confirmation of WSMV, RT-PCR with the WS8166F/WS8909R primers covering the coat protein (CP) gene was carried out for both amplification and sequencing. The amplified product of the correct predicted size (750 bp) derived from four selected isolates, 98-19, 99-19, 102-19 and 120-19, was sequenced and deposited in GenBank (MT461299, MT461300, MT461301 and MT461302, respectively). Serbian WSMV isolates showed very high nucleotide identity (98.16-99.02%) and shared a deletion of triplet codon GCA at nucleotide position 8412-8414 resulting in deletion of glycine amino acid (Gly_{2761}). Phylogenetic analysis conducted on CP gene sequences revealed the existence of four clades, named A, B, C and D, and one recently introduced clade B1. All Serbian wheat WSMV isolates grouped into clade B together with other European isolates and one isolate from Iran. The results of this study provide the first insight into molecular characterisation of Serbian WSMV isolates, indicating their close relationship with other European isolates and existence of a single genotype in the country. Phylogenetic analysis also confirms the dispersal of WSMV isolates throughout Europe from a single locus.

Keywords: wheat streak mosaic virus, wheat, RT-PCR, molecular characterization, phylogeny, Serbia

INTRODUCTION

Wheat is the most widely grown crop in the world, providing for 20% of daily protein requirements of 4.5 billion people (Lucas, 2012). In Serbia, wheat is grown on 577.499 ha, with an average yield of 4.4 t/ha (RZS, 2019). Concerning the harvest area, wheat is the second major crop in Serbia after corn (RZS, 2019). Most of Serbian wheat is produced in the Vojvodina province (southern part of Pannonian Plane), which has a favorable climate for winter wheat cultivation (Šeremešić et al., 2017; RZS, 2019).

Viruses may become limiting factors for successful wheat production and numerous viral diseases compromise wheat production worldwide. More than 50 viruses are currently known to infect wheat (Lapierre & Signore, 2004; Ordon et al., 2009). Viruses of wheat and other cereals can be divided into two major groups, regarding their transmission: soil-borne viruses vectored by the plasmodiophorid *Polymyxa graminis,* and viruses transmitted by insects or mites (Ordon et al., 2009). Two soil-borne viruses, the soil-borne cereal mosaic virus (SBCMV) and barley yellow mosaic virus (BaYMV) (Roberts, 2014), three insect transmitted viruses, the barley yellow dwarf viruses (BYDVs), cereal yellow dwarf viruses (CYDVs) and wheat dwarf virus (WDV), and the mite-transmitted wheat streak mosaic virus (WSMV) (Ordon et al., 2009; Singh & Kundu, 2018; Mishchenko et al., 2019) are the most important viruses that cause serious wheat diseases. In recent years, there has been a significant increase in the number and prevalence of wheat viruses, but what is most threatening is the increase in their economic importance (Seifers et al., 2008; Spaar et al., 2008; Mishchenko et al., 2019). Global climate change is predicted to cause a further increase in the incidence and importance of wheat viruses, especially of viruses transmitted by aphids (BYDV and CYDV), leafhoppers (WDV) or mites (WSMV) (Ordon et al., 2009; Trębicki et al., 2015).

WSMV, the type member of the *Tritimovirus* genus in the family *Potyviridae*, is one of the most widespread and harmful viruses of cereal crops (Brunt et al., 1996; Rabenstein et al., 2002; Burrows et al., 2009; Singh & Kundu, 2018; Mishchenko et al., 2019). Almost a century ago, the disease caused by WSMV was first observed in the Central Great Plains of the USA and described as "*yellow mosaic*" of winter wheat (McKinney, 1937; Hunger, 2010). The virus is widely distributed in major wheat-growing regions of Eurasia and North America, but also in Mexico, Brazil, Argentina, Australia, and New Zealand (Stenger & French, 2009; Hadi et al., 2011; Navia et al., 2013). Under natural conditions, the virus is transmitted primarily by wheat curl mites (WCM, *Aceria toshicella* Keifer [1969]) and by seeds of infected plants to a lesser extent (Skoracka et al., 2014; Singh & Kundu, 2018). Even though seed transmission occurs at low rates from 0.2 to 0.5% (up to 1.5%) and is not important locally, it enables global spreading of the virus by international trade and exchange of germplasm (Jones et al., 2005; Singh et al., 2018). The host range of WSMV includes wheat, oat, barley and maize, but also many other wild and grown members of the *Poaceae* family (French & Stenger, 2002; Dráb et al., 2014; Chalupníková et al., 2017; Singh & Kundu, 2017). Yield losses caused by WSMV are estimated at 1-2% annually (Appel et al., 2014), but they can vary greatly, ranging from 7-13% in Kansas (Atkinson & Grant, 1967) to over 83% in Australia (Lanoiselet et al., 2008) or even cause a complete crop failure (Stenger & French, 2009). Subsequent financial losses are substantial, and only in the Kansas State (USA) in 2017 they were estimated at 76.8 billion US $ (Kansas Wheat Commission, 2017 http://kswheat.com/growers/wheat-streak-mosaic-virus). In some countries, such as the Czech Republic, WMSV is considered as a re-emerging pathogen since its significance dramatically increased after almost 30 years of absence (Chalupníková et al., 2017).

The first and most prominent disease symptoms usually appear on field margins, closest to the source of vector mites (Singh et al., 2018). Young leaves exhibit parallel pale green and yellow stripe forming mosaic patterns which, in case of early (autumn) infection, may progress over the spring to stunting, yellowing, marginal necrosis and subsequently to poor tillering (Vacke et al., 1986; Chalupníková et al., 2017; Singh & Kundu, 2017; Singh et al., 2018). Early disease symptoms can be misleading and confused with nutritional disorder, damage caused by chemicals or environmental effects (Singh et al., 2018).

In Serbia, WMSV was found for the first time in the 1960s (Šutić & Tošić, 1964, 1966). The following studies by Tošić (1971) confirmed a significant presence of WSMV in important wheat production areas, as well as the presence of mixed infections with brome mosaic virus (BMV). Apart from WSMV and BMV in Serbia, BYDV-PAV, BYDV-MAV, CYDV-RPV and WDV are also present in wheat fields (Šutić & Tošić, 1964, 1966; Krstić et al., 2018; Stanković et al., 2019). After these initial WMSV studies, no additional investigation was conducted although the results at that time suggested a significant impact and distribution of WMSV. Moreover, virus-like symptoms have been increasingly noticed in wheat crops in Serbia over the last few years. Therefore, the objectives of this study were to determine the presence of WSMV in wheat crops, evaluate its distribution in the country, and to determine the genetic relationship of Serbian WMSV isolates with those from other parts of the world.

MATERIALS AND METHODS

Field survey - collection of plant samples

In the spring of 2019, winter wheat samples showing virus-like symptoms, including pale green and yellow parallel stripes followed by mild to severe leaf rolling and stunting of plants, were randomly collected from 10 crops at eight locations: Bački Brestovac, Inđija, Dolovo, Bački Maglić, Lugovo, Gibarac, Umka and Vršac. After visual inspection, a total of 62 symptomatic plants of five wheat cultivars, including Anapurna, Apache, Salasar, Foxyl, and Sobred, were collected. The samples were transported to the laboratory in hand-handled cooler and stored at -20°C until RNA extraction and RT-PCR analyses were performed.

Molecular detection of wheat viruses

In order to determine the presence of wheat viruses in collected samples, individual or multiplex reverse transcription (RT)-PCR assays were carried out using specific primers for the detection of nine most economically important wheat viruses: barley yellow dwarf virus-PAV, -MAV, -SGV, -RMV (BYDV-PAV, -MAV, -SGV, and -RMV), cereal yellow dwarf virus-RPV (CYDV-RPV), wheat spindle streak virus (WSSMV), WSMV, BMV, and soil-borne wheat mosaic virus (SBWMV) (Deb & Anderson, 2008; Trzmiel et al., 2016). Total RNAs were extracted from 100 mg of freeze-dried leaves of the collected samples by the CTAB method (Li et al., 2008) and used as a template in individual RT-PCR for the detection of BMV or multiplex RT-PCR assay for simultaneous detection of other mentioned wheat viruses. The RT-PCRs were performed using the One-Step RT-PCR kit (Qiagen GmbH, Germany) and different sets of virus-specific primers (Table 1). RNA extracted from healthy wheat plants and RT-PCR mix with RNase free water served as negative controls in each RT-PCR reaction.

The RT-PCR reaction mixture included 5µl of 5x Qiagen OneStep RT-PCR buffer, 400 µM of each of the four dNTPs, 1 µl of RT-PCR enzyme mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase, and HotStar Taq DNA Polymerase), 0.6 µM of each viral sense and complementary sense primer, and 1 µl of extracted RNA in a final volume of 25 µl. Multiplex RT-PCR reactions were performed in a thermal cycler (Applied Biosystems 2720) under the following conditions: reverse transcription was performed at 50°C for 30 min, followed by an initial PCR denaturation step at 95°C for 15 min, and 6 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s with the annealing temperature decreasing by 1°C in each successive step and extension at 72°C for 30 s. These 6 cycles were followed by 30 cycles at 95°C for 30 s, 55°C for 1 min, 72°C for 30 s and final extension at 72°C for 10 min. For BMV, the first strand cDNAs were synthesized at 50°C for 30 min and terminated at 95°C for 15 min, and then PCR was carried out by performing 35 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 1 min, followed by final extension at 72°C for 10 min. Amplified products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under a UV transilluminator.

Table 1. Primers used for the detection of wheat viruses by individual or multiplex RT-PCR

Virus	Primer	Sequence (5' to 3')	Amplicon size (bp)
BYDV-PAV*	PAVL1	AGAGGAGGGGCAAATCCTGT	295
	PAVR1	ATTGTGAAGGAATTAATGTA	
BYDV-MAV	MAVL1	CAACGCTTAACGCAGATGAA	175
	MAVR1	AGGACTCTGCAGCACCATCT	
BYDV-SGV	SGV L2	ACCAGATCTTAGCCGGGTTT	237
	SGV R2	CTGGACGTCGACCATTTCTT	
BYDV-RMV	RMVL1	GACGAGGACGACGACCAAGTGGA	365
	RMV R	GCCATACTCCACCTCCGATT	
CYDV-RPV	RPV L	ATGTTGTACCGCTTGATCCAC	400
	RPV R	GCGAACCATTGCCATTG	
WSSMV	WSSMV L1	GCAACCCTTAGCGAAGTCAG	154
	WSSMV R1	GAGGCTCCGTGTCTCATAGC	
WSMV	WSMV L2	CGACAATCAGCAAGAGACCA	193
	WSMV R2	TGAGGATCGCTGTGTTTCAG	
SBWMV	SBMV L2	CCTATGGCGTCCTAACGTGT	219
	SBMV R2	CACAATCTGCAGGAAGACGA	
BMV	BMVcp-F	GATCTATGTCCTAATTCAGCG	626
	BMVcp-R	CCAGTCAGGGGCTCTCCGAGC	

* BYDV-PAV, -MAV, -SGV, and –RMV: *Barley yellow dwarf virus-PAV, -MAV, -SGV, -RMV*; CYDV-RPV: *Cereal yellow dwarf virus-RPV*; WSSMV-*Wheat spindle streak virus*; WSMV- *Wheat streak mosaic virus;* SBWMV-*Soil-borne wheat mosaic virus*; BMV-*Brome mosaic virus*

Sequence analysis

The identity of four selected Serbian WSMV isolates (98-19, 99-19, 102-19 and 120-19), originating from different locations, was further confirmed by amplification of the 750 bp PCR fragment containing the N-terminal and core region of the coat protein gene using the primer pair WS8166F (5′ GAG AGC AAT ACT GCG TGT ACG 3′) and WS8909R (5′ GCA TAA TGG CTC GAA GTG ATG 3′) (Kúdela et al., 2008). The components of the RT-PCR reactions were as previously described, while amplifications were performed in a thermal cycler under the following conditions: reverse transcription was performed at 50°C for 30 min, followed by an initial PCR denaturation step at 95°C for 15 min, and 30 cycles of denaturation at 94°C for 45 s, annealing at 53°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 10 min. The size of the amplified products was determined as described in the previous section.

After purification with the QIAquick PCR Purification Kit (Qiagen), RT-PCR products of four selected isolates were sequenced directly in both directions, using the same primer pair as in RT-PCR, and deposited in GenBank (Table 2). Sequences of the Serbian WSMV isolates were compared with each other and with the WSMV sequences available

in the GenBank database using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/), ClustalW (Thompson et al., 1994) and MEGAX software (Kumar et al., 2018). A p-distance model was applied for nucleotide (nt) and deduced amino acid (aa) sequence analyses and the divergence of the sequences of WSMV isolates was calculated after trimming to the length of the shortest fragment.

Phylogenetic tree

A maximum-likelihood phylogenetic tree was constructed using four Serbian WSMV isolates obtained in this study and 53 WSMV sequences retrieved from GenBank from other parts of the world (Table 2). The best-fitting model of nt substitution was investigated using MODELTEST implemented in MEGAX, and the Kimura 2-parameter model Gamma distributed (K2+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. Sequence of a closely related *Tritimovirus*, oat necrotic mottle virus (ONMV), GenBank accession number AY377938, was used as the outgroup sequence.

Table 2. Coat protein gene sequences of wheat streak mosaic virus isolates used for phylogenetic analyses

Isolate	Host plant	Country of origin	GenBank accession number
98-19	*Triticum aestivum*	Serbia	MT461299
99-19	*Triticum aestivum*	Serbia	MT461300
102-19	*Triticum aestivum*	Serbia	MT461301
120-19	*Triticum aestivum*	Serbia	MT461302
Marmagne	*Triticum aestivum*	France	HG810953
Hoym	*Triticum aestivum*	Germany	HG810954
Austria	*Triticum aestivum*	Austria	LN624217
Czech	*Triticum aestivum*	Czech Republic	AF454454
Sidney 81	*Triticum aestivum*	Nebraska, USA	AF057533
El Batan 3	*Triticum aestivum*	Mexico	AF285170
Type (PV57)	*Triticum aestivum*	USA	AF285169
ID96	*Triticum aestivum*	Idaho, USA	AF511618
ID99	/*	USA	AF511619
MON96	*Triticum aestivum*	Montana, USA	AF511630
WA99	*Zea mays*	Washington, USA	AF511643
Naghadeh	*Triticum aestivum*	Iran	EU914917
Saadat-Shahr	*Triticum aestivum*	Iran	EU914918
Turkey 1	*Triticum aestivum*	Turkey	AF454455
Ukraine-Mal-18	*Triticum aestivum*	Ukraine	MH523356
Ukraine-Ep-18	*Triticum aestivum*	Ukraine	MH523357
Russia	*Triticum aestivum*	Russia	AF454459
Hungary	*Triticum aestivum*	Hungary	AF454456
Toskana	*Triticum aestivum*	Italy	FJ606885
Burgund	*Triticum aestivum*	France	FJ606884
WSMV-1313	*Triticum aestivum*	Lithuania	KJ720819

Table 2 - continued. Coat protein gene sequences of wheat streak mosaic virus isolates used for phylogenetic analyses

SK512	*Triticum aestivum*	Slovakia	FJ613359
SK349	*Triticum aestivum*	Slovakia	EU723085
SK350	*Triticum aestivum*	Slovakia	EU723086
WSMV-Sz_	*Triticum aestivum*	Poland	KP261825
Turkei	*Triticum aestivum*	Turkey	FJ606886
TR	*Triticum aestivum*	Turkey	KC900901
KosHJR	*Triticum aestivum*	Czech Republic	FJ216409
Policko-CRI	*Triticum aestivum*	Czech Republic	FJ216412
Turondot	*Triticum aestivum*	Czech Republic	KY419568
SlastJR	*Triticum aestivum*	Czech Republic	FJ216414
WSMVcz1	*Triticum aestivum*	Czech Republic	FJ216408
Bodycek	*Triticum aestivum*	Czech Republic	KY419571
ar1	*Agropyron repens*	Czech Republic	KY419572
Strain pp1	*Phleum pratense*	Czech Republic	KY419573
Strain pp2	*Poa pratensis*	Czech Republic	KY419574
Iran	*Triticum aestivum*	Iran	AF454458
Ger	*Triticum aestivum*	Germany	AJ889242
Agdia	*Triticum aestivum*	Czech Republic	FJ695510
Arg1	*Triticum aestivum*	Argentina	FJ348356
OSU	/	/	AF511634
WO93	*Zea mays*	Ohio, USA	AF511644
PV106JM	*Zea mays*	Ohio, USA	AF511638
PV106H	*Zea mays*	Ohio, USA	AF511637
GY93	*Zea mays*	Kansas, USA	AF511607
H94PM	*Pennisetum glaucum*	Kansas, USA	AF511610
WH94S	*Sorghum bicolor*	Kansas, USA	AF511611
H95S	*Sorghum bicolor*	Kansas, USA	AF511614
H98_Kansas	*Chloris virgata*	Kansas, USA	AF511615
Tamworth 1	*Triticum aestivum*	Australia	AY327866
Bordertown	*Triticum aestivum*	Australia	AY327870
Galong	*Triticum aestivum*	Australia	DQ888804
Yerritup	*Triticum aestivum*	Australia	DQ888802

*/-data not available

RESULTS

Symptoms observed in the field

During the spring of 2019, typical virus-like symptoms were observed in winter wheat fields in Serbian most important wheat growing regions. Plants exhibited prominent yellow stripes progressing to a mosaic pattern on leaves (Figure 1), which later merged in chlorotic or yellow parts of leaves or whole leaves (Figure 2). The observed symptoms sometimes included mild to severe leaf deformations, such as leaf rolling and wilting. During the early spring, symptoms appeared on plants at field edges. At the end of the growing season, a characteristic disease gradient from very severe on field edges to decreasingly severe towards field depth was observed in some locations, while in others infected plants were randomly distributed across the field. Infected plants exhibited also stunting and reduction in tiller number.

Figure 1. Wheat streak mosaic virus: parallel mosaic and stripes on wheat leaves

Figure 2. Wheat streak mosaic virus: leaf yellowing

Molecular detection of WSMV

Molecular analysis of wheat samples revealed the presence of WSMV in 58.1% of the tested samples collected from seven commercial wheat crops at five locations: Bački Brestovac, Inđija, Bački Maglić, Lugovo, and Vršac (Table 3). Three locations, Dolovo, Gibarac and Umka, were proved to be free of either WSMV or any other tested virus. The highest incidence of WSMV (100% samples testing positive) was in the locations Inđija and Bački Maglić, while virus presence was proved in all three inspected crops at the location Bački Brestovac. At the location Vršac, WSMV was detected in 71.4%, while the virus was confirmed in 62.5% of the tested samples at the Lugovo location.

All tested samples were negative for BYDV-PAV, -MAV, -SGV, and -RMV, CYDV-RPV, WSSMV, BMV, and SBWMV. Also, no amplification products were recorded in the healthy controls.

Table 3. Number of tested and percentage of wheat streak mosaic virus positive samples in 2019

Location	Cultivar	Number of fields inspected	Number of tested/% of WSMV positive samples
Bački Brestovac	Anapurna	2	12/100
	Appache	1	4/100
Inđija	Anapurna	1	3/100
Bački Maglić	Anapurna	1	7/100
Lugovo	Apache	1	8/62.5
Dolovo	Foxyl	1	6/0
Gibarac	Salasar	1	9/0
Vršac	Sobred	1	7/71.4
Umka	Anapurna	1	6/0
Total		10	62/58.1

Molecular identification and phylogenetic analysis of WSMV

Primer pair WS8166F/WS8909R specifically amplified fragments of the expected size of 750 bp in all four selected isolates. The amplified fragments were sequenced and four sequences of Serbian WSMV isolates generated in this study were submitted to GenBank database of the NCBI and assigned with accession numbers shown in Table 2.

The CP gene sequences of the four Serbian isolates shared nt identities of 98.16% to 99.02% (99.57 to 100% aa identities). The highest percentage of nt identity was shown between the isolates 98-19 and 99-19 (99.02%), while 99-19 was the most distant from the isolate 102-19 (98.16%). BLAST search analysis revealed that the sequences of four Serbian WSMV isolates proved to be identical at the nucleotide level from 98.59 to 99.44% with those from other parts of the world. Nucleotide sequences of the isolates 98-19 and 99-19 showed the highest homology with a Polish barley isolate (MH939146) of 99.44% and 99.02%, respectively. Sequences of the isolates 102-19 and 120-19 had the highest homology with a Czech wheat isolate (FJ216409) of 98.59% and 99.28%, respectively.

All four Serbian WSMV isolates had a specific deletion of three nucleotides at the position 8412-8414 nt in the CP gene. Positions are numbered according to the positions of the WSMV reference isolate (NC_001886). This *deletion of* triplet *codon GCA* resulted in the deletion of glycine amino acid at the position 2761 (Figure 3).

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                    2735                                2761
NC_001886           AAGGSGSGSAQTQSNNVSVMAGLDTGGAKTDQGSGSKGTGGSFTSNPVRT
MT461299 98-19      AAGGSGSGSAQTQSSNVSVMAGLDTG-AKTGQGSGSKGTGGSFVSNPVRT
MT461300 99-19      AAGGSGSGSAQTQSSNVSVMAGLDTG-AKTGQGSGSKGTGGSFVSNPVRT
MT461301 102-19     AAGGSGSGSAQTQSSNVSVMAGLDTG-AKTGQGSGSKGTGGSFVSNPVRT
MT461302 120-19     AAGGSGSGSAQTQSSNVSVMAGLDTG-AKTGQGSGSKGTGGSFVSNPVRT
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Figure 3. Comparative analysis of a part of amino acid sequences of the coat protein (CP) gene of Serbian wheat streak mosaic virus isolates and referent wheat streak mosaic virus isolate (NC_001886) showing deletion of glycine aa residue at position 2761

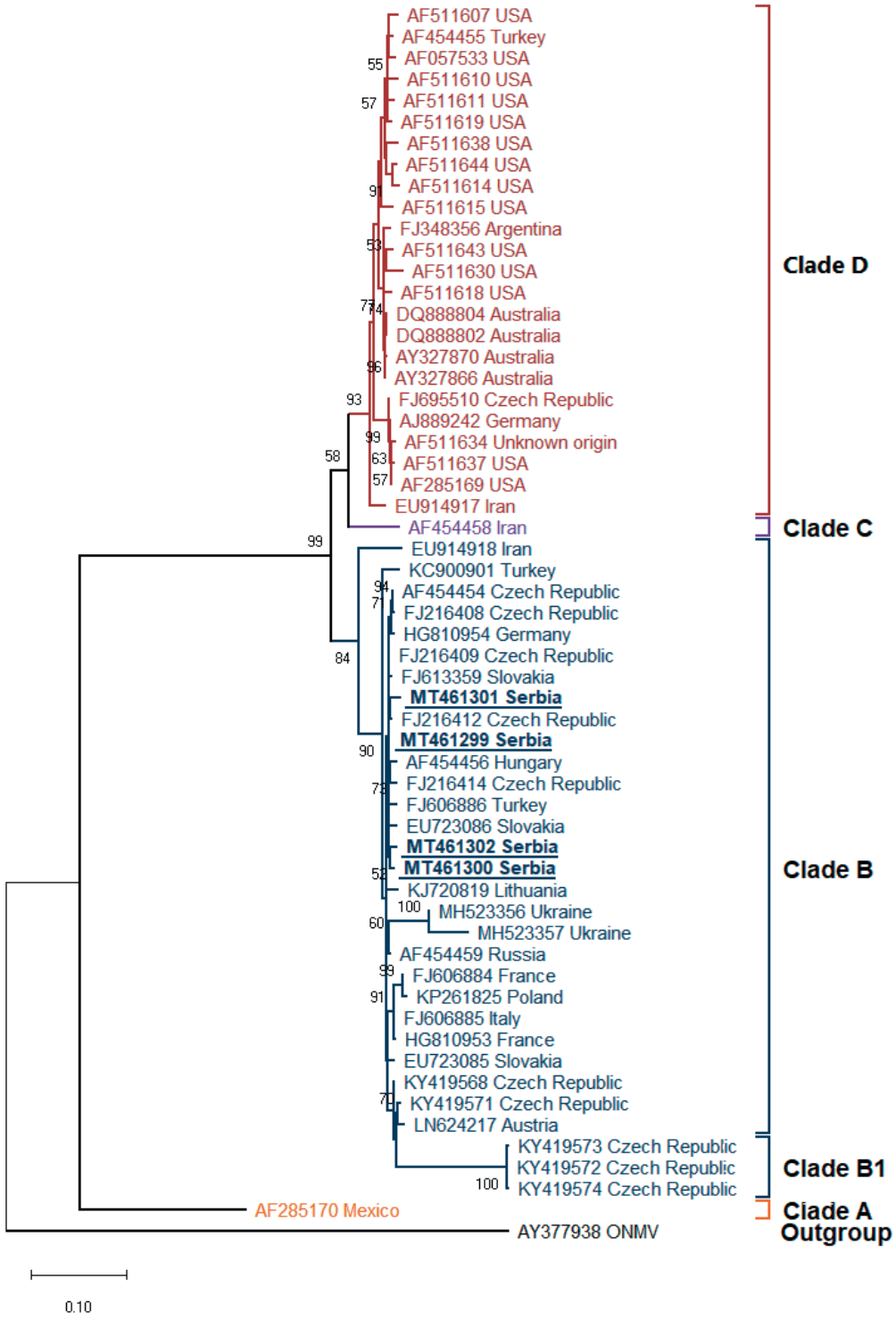

Figure 4. Maximum likelihood tree based on nucleotide sequences of the coat protein (CP) gene of 57 wheat streak mosaic virus (WSMV) isolates. Phylogram was generated with MEGAX using Kimura 2-parameter model Gamma distributed. Bootstrap analysis was performed with 1000 replicates and bootstrap values (>50%) are shown next to relevant branches. Scale bars: substitutions per site. The Serbian WSMV isolates generated in this study were bolded and underlined. The sequence of oat necrotic mottle virus (ONMV; AY377938) was used as an outgroup.

A Maximum Likelihood tree (Figure 4) was constructed using a 683 nt fragment of the CP gene from 57 WSMV isolates from all over the world. The phylogenetic tree indicated a division of WSMV population in five clades (Clades A, B, B1, C and D) with high bootstrap values for major clades containing more than one isolate B, B1 and D (84, 100 and 93, respectively). The overall genetic diversity of WSMV sequences in the reconstructed phylogenetic tree was 0.083±0.006. Genetic diversities between clades ranged from 0.085±0.010 to 0.3286±0.017. Clade A consisted of a single isolate from Mexico (AF285170), which is genetically most distant from the other WSMV. Clade B consisted of Eurasian wheat isolates from 13 different countries (Iran, Turkey, Czeck Republic, Germany, Slovakia, Serbia, Hungary, Lithuania, Ukraine, Russia, France, Poland and Austria). All four Serbian isolates were grouped together with most of the isolates within clade B originating from Europe and Asia. Serbian WSMV isolates clustered closely with isolates from the Czech Republic, Slovakia, Hungary and Turkey. Clade B showed the greatest intragroup variability in the phylogenetic tree (0.029±0.002) and isolates of this group are characterized by deletion of a triplet codon GCA (Glycine amino acid) in the CP gene sequence. Clade B1 consisted of three isolates from grasses collected in the Check Republic and was most closely related to clade B, showing 0.1134±0.011 inter-group variability. Clade C consisted of a single isolate from Iran. The most divergent clade, considering geographical distribution, was clade D, comprising isolates from five continents (Europe, Asia, Australia, North and South America), but intra-group sequence diversity in this clade was slightly lower than in clade B (0.026±0.003).

DISCUSSION

Although wheat is the second most important food crop in Serbia and despite the fact that Tošić (1971) reported the presence and significant distribution of WSMV in important wheat growing areas in Serbia, no data were available over the past decades on the presence and distribution of WSMV or other wheat infecting viruses in Serbia. Therefore, the occurrence, incidence and prevalence of wheat viruses in Serbia are unknown today. Only recently, a survey searching for wheat viruses, initiated by Stanković et al. (2019), showed that WSMV is present and widespread in the country. Today, WSMV is the most common wheat virus around the world which causes losses of up to 100% (Stenger & French, 2009; Hadi et al., 2011; Navia et al., 2013). Yield losses caused by WSMV can reach up to 464.5 US $ per hectare (Velandia et al., 2010), endangering production or even making it entirely unsustainable. WSMV affects not only yield, but also root development and water use efficiency of infected wheat plants (Price et al., 2010). In recent years, WSMV has become a re-emerging virus in cereal crops in the Czech Republic (Chalupníková et al., 2017).

This study showed that WSMV occurred as a single infection in five out of eight inspected wheat growing locations in Serbia during 2019. The presence of WSMV was detected in 58.1% of the tested samples and all samples were negative for the presence of other tested viruses. All collected samples originating from three locations (Inđija, Bački Maglić and Bački Brestovac) were WSMV positive, and a lower but significant percentage of samples from two other locations (Vršac and Lugovo) were also positive (71.4% and 62.5%, respectively). WSMV commonly occurs in complexes with other wheat viruses (Byamukama et al., 2013). The investigation carried out by Tošić (1971) revealed also the presence of mixed infection of WSMV and BMV, but in our present study WSMV was found only as single infection. Since none of the tested viruses, including WSMV, were detected in three wheat fields, further investigation will be focusing on identifying the causal agent(s).

Early-season symptoms mostly appeared on plants at field margins, and as the season progressed the infected plants developed an obvious disease gradient. In some fields, where the presence of WSMV was not proved, symptomatic plants were scattered throughout the field. Chalupníková et al. (2017) observed a similar distribution of WSMV symptoms in the Czech Republic. Symptoms of streak mosaic and yellowing decreased in severity towards field centre, as noticed also by Workneh et al. (2009). Migration of the wheat curl mite vector, *Aceria tosichella*, from grassy areas and bordering crops into wheat fields, stimulates the spreading of infection (Hunger, 2010).

A comparison of nucleotide sequences of WSMV isolates collected from different wheat-growing regions of the world has confirmed the existence of WSMV genetic diversity (Singh et al., 2018). Based on the

CP gene sequence, the WSMV population is divided into four clades, named A, B, C, and D, and a recently introduced clade B1 (Stenger & French, 2009; Robinson & Murray, 2013; Singh & Kundu, 2017; Singh et al., 2018; Mishchenko et al., 2019). Topology of the phylogenetic tree obtained in this study and nucleotide similarities between clades are in accordance with previous studies (Stenger & French, 2009; Robinson & Murray, 2013; Singh & Kundu, 2017; Singh et al., 2018; Mishchenko et al., 2019). All Serbian WSMV isolates were grouped into clade B together with other European WSMV isolates and one isolate from Iran, implying that they share a single common ancestor. The analysis also indicated the existence of a single genotype of WSMV in Serbia. Moreover, nucleotide sequences of the CP gene of Serbian WSMV isolates are characterized by a deleted triplet codon GCA at nucleotide position 8412-8414, resulting in deletion of the amino acid glycine (Gly_{2761}), as previously reported for isolates belonging to B and B1 clades (Gadiou et al., 2009; Mishchenko et al., 2019). All these results imply that European isolates are common and had been widely dispersed throughout European countries from a single focus (Gadiou et al., 2009). Unlike the genetic uniformity of WSMV isolates in Europe, considerable genetic variation in WSMV populations was found in the USA (Robinson & Murray, 2013). Reports on three genotypes of WSMV coexisting in Iran (Schubert et al., 2015), and discovery of European WSMV isolates in the U.S. Pacific Northwest region (Robinson & Murray, 2013) and Canada (Bennypaul et al., 2019), have revealed transferring and diverse distribution of WSMV globally. The observed WSMV diversity in the USA and obvious introduction by movement of viruliferous vectors or infected seed through continually expending trade in plants and plant products, indicate a constant need to study and evaluate WSMV population structure.

This study provides the first information on the presence of WSMV in Serbia after initial investigation that was carried out almost 50 years ago. WSMV continues to be a threat to wheat production in Serbia and its importance may increase in the future. Further investigation is needed to provide information on the biology, ecology and epidemiology of the disease and its vector. Considering that a substantial number (41.9%) of collected samples during the survey were negative for eight tested viruses, a further thorough survey and testing for the presence of other wheat-infecting viruses are required. In addition, the results of this work provide the first data on molecular characterisation of WSMV isolates originating from Serbia, indicating their close relationship with other European isolates and existence of a single genotype in the country. The extent of a possibly greater genetic diversity of WSMV in Serbia will be assessed when more isolates of different origin have been collected.

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Pojava i molekularna karakterizacija virusa crtičastog mozaika pšenice na pšenici u Srbiji

REZIME

Virus crtičastog mozaika pšenice (*Wheat streak mosaic virus,* WSMV) je široko rasprostranjen, prenosi se grinjom kovrdžanja pšenice i nanosi štete u proizvodnji pšenice u mnogim regionima sveta. Nakon prve detekcije u Srbiji 60-ih godina XX veka, prisustvo ovog virusa nije praćeno. Tokom 2019. godine, sakupljeno je ukupno 62 uzorka pet sorti pšenice poreklom sa osam lokaliteta u Srbiji koji su testirani na prisustvo devet najznačajnijih virusa pšenice: WSMV, virus žute patuljavosti ječma-PAV, -MAV -SGV i -RMV (barley yellow dwarf virus-PAV, -MAV, -SGV i -RMV [BYDV-PAV, -MAV, -SGV i -RMV]), virus žute patuljavosti žitarica-RPV (cereal yellow dwarf virus-RPV, CYDV-RPV), virus vretenastog crtičastog mozaika pšenice (wheat spindle streak mosaic virus, WSSMV), virus mozaika ovsika (brome mosaic virus, BMV) i virus mozaika pšenice koji se prenosi zemljišnim pseudogljivama (soil-borne wheat mosaic virus, SBWMV) primenom RT-PCR ili multiplex RT-PCR. Prisustvo WSMV dokazano je u 58,1% testiranih uzoraka pšenice sakupljenih u sedam useva na pet različitih lokaliteta, dok prisustvo drugih testiranih virusa nije dokazano ni u jednom od testiranih uzoraka. U cilju dalje identifikacije, umnožavanje i sekvenciranje gena za proteinski omotač (*coat protein*, CP gen) obavljeno je primenom RT-PCR metode uz korišćenje WS8166F/WS8909R prajmera. Amplifikovani fragmenti odgovarajuće veličine (750 bp) četiri odabrana izolata: 98-19, 99-19, 102-19 i 120-19 su poslati na sekvenciranje, a dobijene sekvence su deponovane u GenBank bazu podataka (MT461299, MT461300, MT461301 i MT461302). Izolati WSMV poreklom iz Srbije pokazuju visok stepen nukleotidne identičnosti (98,16-99,02) i deleciju GCA kodona na poziciji 8412-8414 koja dovodi do delecije aminokiseline glicin (Gly_{2761}). Filogenetska analiza, na osnovu sekvence CP gena, pokazala je grupisanje odabranih izolata u četiri podgrupe, A, B, C i D, i jedne nedavno izdvojene podgrupe B1. Izolati WSMV iz pšenice poreklom iz Srbije grupisali su se u podgrupu B zajedno sa ostalim izolatima iz Evrope i jednim izolatom iz Irana. Rezultati ovih istraživanja daju prvi uvid u molekularnu karakterizaciju izolata WSMV poreklom iz Srbije, ukazujući na blisku evolutivnu povezanost izolata iz Srbije sa drugim evropskim izolatima i postojanje jedinstvenog genotipa u našoj zemlji. Takođe, filogenetske analize ukazuju na širenje izolata WSMV u Evropi iz jednog centra porekla.

Ključne reči: virus crtičastog mozaika pšenice, pšenica, RT-PCR, molekularna karakterizacija, filogenija, Srbija