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Molecular characterization and differentiation of cucumber mosaic virus subgroups in Serbia by RT-PCR-RFLP

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Abstract: Samples of various host plants from different locations in Serbia showing foliar symptoms resembling those caused by the cucumber mosaic virus (CMV) were collected. Samples were considered CMV-infected after detection by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Fifteen CMV isolates were selected for developing a reverse transcription-polymerase chain reaction (RT-PCR) restriction fragment length polymorphism (RFLP) protocol and a more detailed molecular characterization of all five genes. Based on *in silico* RFLP, the following restriction enzymes were selected: *Hind*III, *Sac*II (1a gene), *Mlu*I (2a gene), *Stu*I, *Sal*I (2b gene), *Bae*I (movement protein (MP) gene of CMV), *Sfc*I, and *Hae*III (capsid protein (CP) gene), which were capable of distinguishing between subgroups of CMV based on the obtained characteristic restriction patterns. According to *in situ* RFLP, the predominant haplotype IA; IA, IA; IA, IA was determined in 14, while the distinct haplotype II; II, II; II, II was found in only one isolate. Sequence analyses of two selected Serbian CMV isolates with different restriction patterns and haplotype profiles confirmed the RFLP results, showing that isolates 674-11 and 137-13 belong to subgroups IA and II, respectively. Different restriction patterns after digestion of all five CMV genomic regions proved to be a simple way to investigate the natural population of CMV. This study provides insight into the genetic structure of the CMV population in Serbia.

Keywords: cucumber mosaic virus, RFLP analysis, sequencing, population structure

INTRODUCTION

Cucumber mosaic virus (CMV, *Cucumovirus*, Bromoviridae) was first discovered in cucumber and muskmelon in Michigan and in cucumber in New York states in 1916 [1,2]. CMV has been found in all parts of the world, affecting more than 1,300 species of agricultural and horticultural crops. It is primarily transmitted by over 80 species of aphids in a non-persistent manner, but also through the seeds of some plant species, parasitic plants, and mechanically [3,4].

The genome of CMV is composed of three ss (+) RNAs denoted RNA1, RNA2, and RNA3. RNA1 and RNA2 encode proteins 1a and 2a, respectively, which together form the RNA-dependent RNA polymerase viral component of the replicase complex [5]. RNA2 additionally encodes protein 2b, which is involved in host-specific long-distance movement, symptom severity, and suppression of RNA silencing [6]. RNA3 is bicistronic and encodes the movement protein (MP) and coat protein (CP). MP is coded by an open reading frame (ORF) located in the 5'-half of RNA3 and is involved in the cell-to-cell and vascular movement of the virus, as well as aphid-mediated transmission [3]. The second ORF, located in the 3'-half, is expressed through subgenomic RNA4 and encodes for CP. The CP is engaged in encapsidation, systemic movement within plants, host range, and aphid transmission [7]. Some isolates can encapsidate small satellite RNAs (satRNAs), which can cause modification of symptoms induced by infection [3].

Currently, it is broadly accepted that CMV isolates around the world can be classified into two major groups, called subgroup I and II, based on serological features, peptide mapping of the coat protein, nucleic

acid hybridization, RT-PCR combined with RFLP, and nucleotide sequence similarities [3,8-10]. The isolates of subgroup I can be further divided into subgroups IA and IB based on phylogenetic analyses [11]. Isolates in subgroups IA and II are distributed worldwide, while subgroup IB consists essentially of East Asian isolates and several isolates from the Mediterranean region, California, Brazil, and Australia [4,12-17]. High-temperature tolerance is greater in subgroup I than subgroup II, while symptoms in subgroup II are less severe [18].

In Serbia, CMV has a destructive impact on the production of several economically important crops, such as tomato [19-20], pepper [21], tobacco [22], and cucurbits [23-25]. All Serbian CMV isolates from field and vegetable crops recorded in 2015 were characterized as belonging to subgroup IA [21-25]. However, a report on the ornamental pot plant marigold (*Calendula officinalis*) and a more recent report on tomato point to the occurrence of subgroup II in Serbia [20,26]. This study's main objective was to develop and validate a reverse transcription polymerase chain reaction followed by enzyme digestion (RT-PCR-RFLP) as a fast, reliable, and less expensive method than sequencing for differentiating between CMV isolates in subgroups IA, IB, and I. Also, this method is a simple way to characterize many CMV isolates and their rapid selection for further molecular study to obtain more reliable data on the genetic structure and variability of natural CMV populations in Serbia.

MATERIALS AND METHODS

Virus isolates

Fifteen CMV isolates were obtained from various hosts in several locations in Serbia (Supplementary Table S1). They were derived from plant samples showing symptoms resembling those caused by CMV collected between 2007 and 2013 (Supplementary Fig. S1). The samples were considered CMV-infected after serological detection. Serological testing was performed using the DAS-ELISA kit with commercial antiserum specific for the detection of CMV (Bioreba AG, Switzerland), following the manufacturer's instructions. Additionally, a Tfn isolate, kindly provided by Dr. D. Gallitelli (University of Ceará, Brazi Università degli Studi di Bari Aldo Moro, Italy), was used for *in situ* digestion with restriction enzymes and served as a control for CMV isolates of subgroup IB.

Reverse transcription polymerase chain reaction (RT-PCR)

To amplify genomic regions of all five CMV genes, total RNAs of 15 Serbian isolates were extracted from 100 mg of freeze-dried leaves of serologically positive field host plants using the RNeasy Plant Mini Kit (Qiagen GmbH, Germany). RT-PCR was performed with the One-Step RT-PCR kit (Qiagen) using five pairs of primers specific for different CMV regions (Supplementary Table S2). Three pairs of primers were designed during this study based on multiple alignments of deposited CMV sequences in GenBank for each region using the Clustal W program [27]. One-step RT-PCR was performed with 2 µL extracted RNA in a 25 µL reaction mixture consisting of 5 µL 5× Qiagen OneStep RT-PCR buffer (containing 12.5 mM $MgCl_2$), 400 µM each of the four dNTPs, and 1 µL Enzyme Mix (Omniscript Reverse Transcriptase, Sensiscript Reverse Transcriptase and HotStarTaq DNA Polymerase). Final primer concentrations were as follows: 1.5 µM RNA1a-fwd, RV11 and CMVCPrev, 3 µM 2brev and CMV3a-rev, 4.5 µM RNA1a-rev and CMVCPfwd, 6 µM RW8, 2bfwd and CMVMP3. Reverse transcription was performed at 50°C for 30 min, followed by a PCR denaturation step at 95°C for 15 min, and final extension at 72°C for 10 min. The cycling conditions in a thermal cycler (Applied Biosystem 2720 Thermal Cycler) and the number of cycles were specific for each primer pair (Supplementary Table S2). The Serbian CMV isolate from tomato (GenBank Accession Number KT270490) was used as the positive control in RT-PCR assays, and the PCR mix with RN*ase*-free water served as the negative control in each RT-PCR reaction. Amplification products were separated on a 1% agarose gel in tris-borate EDTA (TBE) buffer and visualized on a UV transilluminator after staining in ethidium bromide (EB) solutions.

In silico restriction enzyme analysis

To find the best restriction enzyme or combination of restriction enzymes for all five genomic regions, nine sequences of reference CMV isolates representing three CMV subgroups [subgroup IA: Fny respective

accession numbers for RNA1, RNA2 and RNA3: D00356, D00355, D10538, I17F (HE793683, HE793684, Y18137) and Y (D12537, D12538, D12499); subgroup IB: Tfn (Y16924, Y16925, Y16926), Vir (HE962478, HE962479, HE962480) and CTL (EF213023, EF213024, EF213025); subgroup II: LY (AF198101, AF198102, AF198103), R (HE793685, HE793686, Y18138) and Q (X02733, X00985, M21464)] were trimmed to a size corresponding to the length of the amplified fragments for each gene and exported to the pDRAW32 program (http://www.acaclone.com/) for *in silico* digestion with all available restriction enzymes. After the analysis, eight restriction enzymes were selected to distinguish the different subgroups of five CMV regions. Virtual restriction profiles were generated in a 1.5% agarose gel, and the sizes of restriction fragments were estimated by comparison against an Invitrogen 1 kb DNA ladder.

In situ restriction enzyme analysis

The PCR amplified fragments were digested by the eight restriction enzymes (Thermo Scientific: *Hae*III, *Hind*III, *Mlu*I, *Sal*I, *Sac*II, *Sfc*I, and *Stu*I, as well as New England BioLabs, Inc: *Bae*I), which were selected based on the results of *in silico* sequence analyses of reference CMV isolates. All enzymatic digestions were performed according to the manufacturers' protocol. The digestion mixture included 10 µL of the amplified PCR products diluted in RN*ase*-free water in ratios from 1:9 to 3:7 (depending on the yield of PCR product), 1.5 µL of reaction buffer (10×), 0.1 µL of restriction enzyme (10 U/µL), and 0.1 µL of S-adenosyl methionine (SAM), used only for the restriction enzyme *Bae*I. The volume of the mixture was adjusted to 15 µL using RN*ase*-free water. According to the manufacturer's instructions, different reaction buffers were used to prepare the RFLP reaction mixture, depending on the enzyme. Tango buffer was used for *Sfc*I, R buffer for *Hae*III, *Mlu*I, and *Hind*III, B buffer for *Sac*II, and *Stu*I, O buffer for *Sal*I, and CutSmart buffer for *Bae*I restriction enzymes. Buffers were selected based on the maximum activity for each enzyme. The mixture was incubated at 37°C for 3 h, and the reaction was stopped by heating at 65°C for 20 min for *Sfc*I, *Mlu*I, *Sac*II, and *Sal*I or by heating at 80°C for 20 min for *Hae*III, *Hind*III, and *Stu*I. The *Bae*I enzyme was incubated at 25°C for 15 min and inactivated by heating at 65°C for 20 min. The digestion results were analyzed by electrophoresis on 1.2% agarose gel in TBE buffer, stained with ethidium bromide, and visualized under a UV transilluminator.

Sequence analysis

Two Serbian CMV isolates (674-11 and 137-13) with different restriction patterns after *in situ* digestion were selected for sequencing to validate and confirm the RFLP analysis. RT-PCR products for each genomic region of the selected isolates were purified using the QIAquick PCR Purification Kit (Qiagen) and bidirectionally sequenced on an automated equipment (Macrogen-Europe BV) using the same primer pair as in RT-PCR. All sequences generated in this study were deposited in the National Center of Biotechnology Information (NCBI) GenBank database and assigned accession numbers (Data availability). The sequences of all five genomic regions of the selected Serbian CMV isolates were compared with the corresponding region of nine reference CMV isolates previously used for *in silico* restriction enzyme analyses. Divergence in partial or complete coding sequences (cds) of the selected isolates was calculated using the Clustal W program [27] and MEGA7 software [28]. A P-distance model was applied to analyze nucleotide (nt) and deduced amino acid (aa) sequences, and the divergence of selected virus isolate sequences was calculated using sequences trimmed to the length of the shortest fragment.

RESULTS

In silico restriction enzyme analysis

RFLP *in silico* analyses using sequences of reference CMV isolates proved specific and accurate for classifying CMV isolates into the three CMV subgroups. Restriction analyses of 2a and MP gene sequences successfully grouped CMV isolates by using only one enzyme, while the analyses of 1a, 2b, and CP gene sequences required the use of two enzymes (Table 1).

A combination of two restriction enzymes was used for the 1a gene. The restriction enzyme *Sac*II digested sequences of subgroup I (subgroups IA and IB) isolates into two fragments, whereas sequences of isolates belonging to subgroup II were not digested. Digestion with the restriction enzyme *Hind*III allowed subgroup I to be split into IA and IB by cutting

Table 1. Restriction patterns of CMV isolates belonging to different subgroups

Target RNA (gene)	Primer pair	Restriction enzyme	Length of the RT-PCR products after digestion (bp)[a]		
			Subgroup IA (Fny)	Subgroup IB (Tfn)	Subgroup II (LY)
RNA 1 (1a)	RNA1a-fwd/ RNA1a-rev	*Sac*II	335, 863	335, 863	1198
		*Hind*III	720, 478	1198	1198
RNA 2 (2a)	RV11/RW8	*Mlu*I	637	159, 470	173, 149, 312
RNA 2 (2b)	2bfwd/2brev	*Sal*I	805	806	280, 508
		*Stu*I	503, 302	806	788
RNA 3 (MP)	CMV MP3/ CMV3a-rev	*Bae*I	165, 564	730	387, 342
RNA 3 (CP)	CMVCPfwd/ CMVCPrev	*Hae*III	873	872	559, 310
		*Sfc*I	282, 591	872	869

[a]The lengths of fragments obtained by digesting the sequences of representative isolates Fny, Tfn, and LY for IA, IB, and II subgroups, respectively.

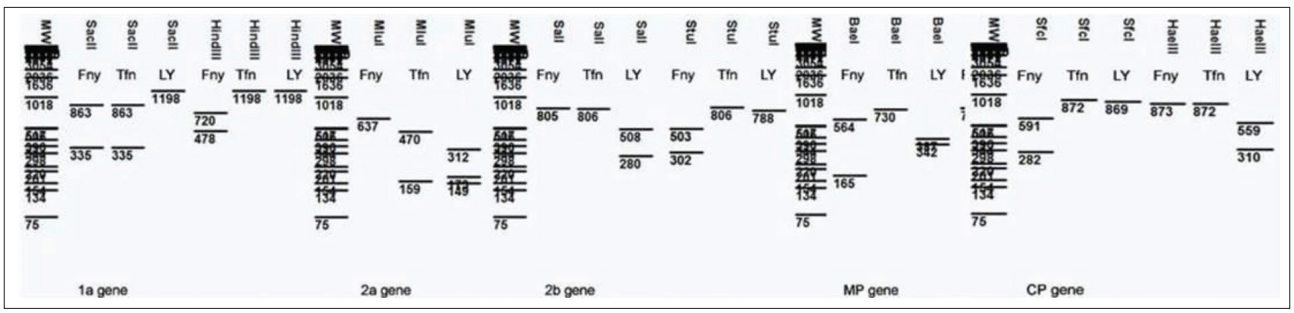

Fig. 1. *In silico* RFLP analysis of the sequences for all genes of representative isolates Fny, Tfn, and LY belonging to different CMV subgroups.

sequences of subgroup IA isolates into two fragments (720, 478 bp). Sequences of subgroup IB isolates were not digested (Fig. 1).

The restriction fragments obtained from *Mlu*I-digested PCR products of the 2a gene revealed three types of patterns. The enzyme did not digest the sequences of isolates belonging to subgroup IA, while sequences of the isolates of IB and II subgroups were digested into two (159, 470 bp) and three fragments (173, 149, 312 bp), respectively (Fig. 1).

A combination of two restriction enzymes was necessary to determine the subdivision of sequences of CMV isolates based on the 2b gene. The restriction enzyme *Sal*I digested the sequences of subgroup II isolates into two fragments (280, 508 bp), whereas the sequences of subgroup I isolates were not digested. Digestion with the restriction enzyme *Stu*I allowed the splitting of subgroup I into IA and IB by cutting sequences of subgroup IA isolates into two fragments (503, 302 bp). Sequences of subgroup IB isolates were not digested (Fig. 1).

Isolates of all three CMV subgroups can be clearly distinguished after digestion of the MP gene sequences with the restriction enzyme *Bae*I. The enzyme digested the sequences of IA and II subgroup isolates into two fragments that yielded different patterns for each subgroup (165, 564 bp and 387, 342 bp, respectively), while no restriction sites were recorded for the sequences of IB subgroup isolates (Fig. 1).

The combination of two restriction enzymes was also required to divide the CMV isolates into subgroups based on sequences of the CP gene. Separation of the CMV isolates into subgroups I and II can be achieved using the enzyme *Hae*III, which, in our study, digested the sequences of subgroup II isolates into two fragments (559, 310 bp) and did not digest the sequence of subgroup I isolates. Digestion with the restriction enzyme *Sfc*I allowed the division of the sequences of subgroup IA isolates into two fragments (282, 591 bp). Sequences of subgroup IB isolates were not digested (Fig. 1).

Table 2. Number and lengths of DNA fragments of Serbian CMV isolates generated by digestion with the indicated restriction enzymes.

Isolate	Number and lengths of DNA fragments							
	***Sac*II**	***Hind*III**	***Mlu*I**	***Sal*I**	***Stu*I**	***Bae*I**	***Hae*III**	***Sfc*I**
171-08	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
201-11	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
514-11	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
610-11	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
674-11	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
449-12	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
477-07	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
542-08	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
471-09	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
207-09	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
647-10	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
702-07	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
202-08	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
191-13	2 335,863 bp	2 720, 478 bp	1 637 bp	1 805 bp	2 503, 202 bp	2 165, 564 bp	1 873 bp	2 282, 591 bp
137-13	1 1198 bp	1 1198 bp	3 173, 149, 312 bp	2 280, 508 bp	1 788 bp	2 342, 387 bp	2 559, 310 bp	1 869 bp

Molecular amplification

RT-PCR successfully amplified PCR products for all 15 CMV DAS-ELISA-positive isolates from Serbia and a Tfn isolate from Italy. Primer pairs used in this study for the 1a gene (RNA1a-fwd/ RNA1a-rev), 2a gene (RV11/RW8), 2b gene (2bfwd/2brev), MP gene (CMVMP3/CMV3a-rev), and CP gene (CMVCPfwd/ CMVCPrev) amplified specific target cDNA fragments of 1198, 637, 805, 729, and 873 bp, respectively. Also, the positive control yielded fragments of expected sizes, while no amplification products were recorded in negative controls.

In situ restriction enzyme analysis

After enzyme digestion of RT-PCR 1a fragments with *Sac*II, most of the isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10, 702-07, 202-08, and 191-13) showed restriction patterns typical of subgroup I isolates, while digestion with the enzyme *Hind*III caused all isolates to belong to subgroup IA. However, isolate 137-13 showed restriction patterns that differed from those of isolates in the subgroup IA, and its RT-PCR product was not digested by either enzyme, indicating it belongs to subgroup II (Table 2, Figs. 2A and 2B). A

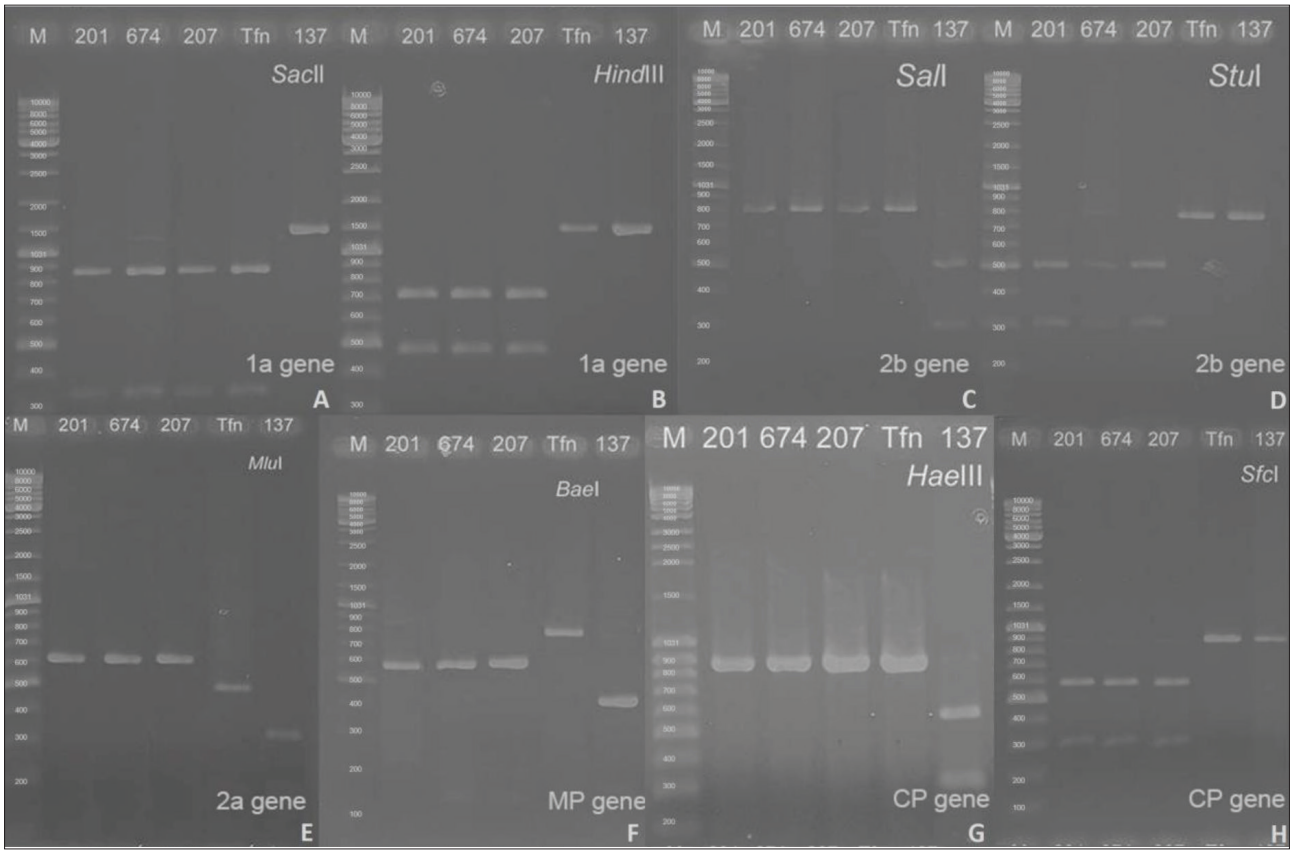

Fig. 2. *In situ* RFLP analysis of RT-PCR amplified products of isolates from different CMV subgroups separated on a 1.2% agarose gel and stained with ethidium bromide using **A** -*Sac*II, **B** – *Hind*III, **C** – *Sal*I, **D** – *Stu*I, **E** – *Mlu*I, **F** – *Bae*I, **G** – *Hae*III, and **H** – *Sfc*I enzymes. Lane: M-MassRuler^{TM}DNA ladder, Mix (Fermentas), 201-Serbian isolate from CMV subgroup IA, 674-Serbian isolate from CMV subgroup IA, 207-Serbian isolate from CMV subgroup IA, Tfn-Italian isolate from CMV subgroup IB, 137-Serbian isolate from CMV subgroup II.

small amount of unexpected patterns similar in size to isolate 137-13 was also observed in isolate 674-11. This pattern persisted when the duration of digestion and the amount of *Sac*II used were increased, and we speculate that this band appears due to the star activity of the enzyme used, since this pattern did not occur when the *Hind*III enzyme was used.

The RT-PCR products of the 2a gene of Serbian isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10, 702-07, 202-08, and 191-13) were not cut by the enzyme *Mlu*I, indicating that they belong to subgroup IA. In contrast, the fragment of isolate 137-13 was cut into three fragments, producing a pattern typical of subgroup II (Table 2, Fig. 2E).

*Sal*I digestion of the amplified 2b gene fragment revealed that isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10, 702-07, 202-08, and 191-13) shared a pattern that is typical of subgroup I isolates, but the pattern obtained with the enzyme *Stu*I was the expected restriction pattern of subgroup IA strains. However, for the 2b gene fragment, digestion results showed that isolate 137-13 produced a restriction pattern that is representative of subgroup II (Table 2, Figs. 2C and 2D).

The restriction enzyme *Bae*I successfully digested amplification products of the MP gene fragments of our 15 selected isolates. According to digestion results, 14 Serbian isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10, 702-07, 202-08, and 191-13) were grouped into subgroup IA, while the Serbian isolate 137-13 was grouped into subgroup II (Table 2, Fig. 2F).

After *Hae*III digestion of amplified CP gene fragments, the isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10,

Table 3. Nucleotide/amino acid identity of Serbian CMV isolates with reference isolates.

Serbian isolate	674-11					137-13				
Reference isolate	1a gene	2a gene	2b gene	MP gene	CP gene	1a gene	2a gene	2b gene	MP gene	CP gene
Fny	95.9/99.4	98.1/99.4	97.4/97.9	99.3/98.7	99.7/100	81.5/88.3	71.5/67.5	63.4/52.3	79.0/53.2	76.8/81.9
117F	96.1/99.4	98.1/99.4	97.4/97.9	99.3/98.3	99.2/99.5	81.4/88.3	71.5/67.5	63.7/52.3	79.1/53.2	76.8/81.5
Y	94.8/98.3	98.1/99.4	97.0/96.9	98.2/95.7	97.9/98.1	81.4/87.4	71.5/67.5	63.7/51.5	79.4/54.1	76.9/81.9
Tfn	93.3/98.0	94.2/92.2	90.1/89.7	93.9/85.3	95.6/99.1	81.8/87.1	71.2/66.9	65.7/58.8	79.7/55.8	78.0/81.9
Vir	90.9/94.3	93.1/90.9	88.1/84.5	93.0/82.9	94.2/98.6	81.0/86.9	72.7/68.8	66.3/57.7	79.4/55.4	75.5/80.0
CTL	92.8/96.6	94.6/92.2	89.4/86.6	94.3/87.9	93.4/98.1	82.6/87.1	72.7/68.2	66.0/57.7	78.6/53.2	76.3/81.9
LY	81.5/87.7	71.7/67.5	64.0/51.5	78.9/52.8	76.6/82.4	98.8/98.9	99.1/99.4	98.3/97.9	98.7/96.1	98.9/98.6
R	82.0/88.9	72.3/68.2	64.0/51.5	78.6/51.9	77.2/82.9	99.4/99.4	98.9/98.7	99.0/97.9	98.7/96.1	99.4/99.1
Q	81.5/87.7	71.2/67.5	63.4/52.6	79.0/53.2	77.2/81.9	99.0/98.9	97.4/95.3	99.3/99.0	98.0/95.2	99.4/99.5

702-07, 202-08, and 191-13) presented restriction patterns typical of subgroup I, and after digestion with the enzyme *Sfc*I they showed the restriction pattern of subgroup IA. *Hae*III digestion of the amplified CP gene fragment of isolate 137-13 revealed the expected restriction pattern of subgroup II (Table 2, Figs. 2G and 2H).

For each Serbian isolate, a haplotype was defined by the subgroup to which each of its genes belonged. According to *in situ* restriction enzyme analyses, 14 out of the 15 CMV isolates (171-08, 201-11, 514-11, 610-11, 674-11, 449-12, 477-07, 542-08, 471-09, 207-09, 647-10, 702-07, 202-08, and 191-13) have the haplotype IA; IA, IA; IA, IA. Serbian isolate 137-13 has the haplotype II; II, II; II, II. CMV subgroup IA was detected in cucurbits, pepper, tomato, tobacco, and impatiens isolates, while subgroup II was detected only in one isolate originating from tomato. A mixed reaction pattern was not found for any of the five analyzed fragments of Serbian CMV isolates. All tests were performed in 5 repetitions to avoid misinterpretation of results as an indication of mixed infection.

Sequence analysis

To confirm the subgrouping of Serbian CMV isolates revealed by RT-PCR-RFLP, two isolates with distinct restriction patterns were selected for all five genomic regions. PCR products of the partial 1a, 2a, and MP genes and complete 2b and CP genes of CMV isolate 674-11, as well as of isolate 137-13, with restriction patterns expected for subgroups IA and II, respectively, were sequenced. A comparison of the nucleotide and deduced amino acid sequence of the partial 1a gene showed that the Serbian CMV isolates shared 81.7% nt identities (88.3% aa identities). Serbian isolates 674-11 and 137-13 had nt identities ranging from 81% to 99.4% (86.9% to 99.4% aa identities) with nine reference isolates (Table 3). Based on nt and aa identities, isolate 674-11 was most like isolates in subgroup IA, whereas isolate 137-13 was most similar to isolates belonging to subgroup II.

A comparison of the partial 2a gene sequences of these two Serbian isolates showed that they shared 72.1% (68.2% aa) nt identities. Comparison of the nt and aa sequences also showed that the Serbian CMV isolates had 71.2% to 99.1% (66.9% to 99.4% aa identities) similarity to nine reference CMV isolates. Isolate 674-11 showed the highest percent nt identity with isolates in the subgroup IA and isolate 137-13 showed the highest percent nt identity with isolates from subgroup II.

The nt identity between the two Serbian isolates' complete 2b gene sequences was 64% (52.6% aa). Comparison of the nt and aa sequences showed that the Serbian CMV isolates were 63.4% to 99.3% (51.5% to 99% aa identity) like nine reference CMV isolates. Isolate 674-11 had the highest percentage of nt identity with isolates in subgroup IA, while isolate 137-13 showed the highest similarity with isolates belonging to subgroup II.

A comparison of the nt and aa sequences of the partial MP gene showed that the Serbian CMV isolates shared 79.3% nt identity (53.2% aa identity). Serbian isolates 674-11 and 137-13 shared nt identities ranging from 78.6% to 99.3% (51.5% to 99.3% aa identities) with nine reference isolates (Table 3). Based on nt and

aa identities, isolates 674-11 and 137-13 were most similar to isolates that belong to IA and II subgroups, respectively. The nt and aa identities for complete CP sequences of the two isolates from Serbia were 76.8% and 81.9%, respectively. A comparison of the two Serbian and nine reference isolates of CMV showed that they had 76.6% to 99.7% nt identity (80% to 100% aa identity). Isolate 674-11 had the highest percent nt identity with isolates in subgroup IA, while isolate 137-13 had the highest identity with isolates belonging to subgroup II.

DISCUSSION

In this study, three of five primer pairs were designed to be used for amplification of partial or complete CMV genes for further analysis of the genetic structure of the virus. Previous studies of CMV genetic population structure had also used primers covering either partial [29-32] or whole parts [33,34] of all five genes of this virus. The length of the analyzed gene sequences did not influence the differentiation of the Serbian CMV isolates into subgroups IA, IB, and II. Moreover, the primers designed in this research allowed amplification of all subgroups of all three segments of CMV, while primers used previously [31] were mostly designed for amplification of subgroups IA/IB strains and had no amplification for RNA1 or a faint amplification for the RNA2 segment of isolates belonging to subgroup II.

Genetic characterization of CMV isolates originating from Serbia was mainly carried out based on specific restriction patterns of the amplified sequences of all five genes of this virus. Different restriction patterns, based on analyses of the RNA3 fragment containing the entire CP gene using the restriction enzymes *Sfc*I and *Hae*III, allowed differentiation of all three subgroups. Many other studies used restriction enzymes such as *Alu*I, *Bam*HI, *Bsu*RI, *Eco*RI, *Eco*RV, *Hha*I, *Hind*III, *Hinc*II, *Hinf*I, *Hpa*II, *Msp*I, *Rsa*I, *Sac*I, *Sal*I, *Sau*3AI, and *Xho*I [8,14,31,33,35-44] for CP gene digestion, but in most cases these restriction enzymes only allowed differentiation between subgroups I and II. Sequence analyses of the CP gene, the most conservative and informative region, which has been studied most frequently because of its role in encapsulation, systemic movement within plants, host range, and transmission by aphids [5,45-47], proved to be helpful in grouping isolates into subgroups and for preliminary studies of population composition, but not for detecting genetic changes in the virus population. To study CMV population structure in Serbia, other genes were also included in the analysis. The patterns obtained in RFLP analyses after digestion with the restriction enzymes *Sal*I and *Stu*I, and *Hind*III and *Sac*II, allowed for the classification of the CMV isolates in subgroups IA, IB, and II based on the 2b and 1a genes, respectively. Unlike the previous CMV regions, 2a and MP regions were characterized based only on RFLP analysis with one restriction enzyme. Other authors [41,48] have also shown that a single enzyme is sufficient for these regions to distinguish all three subgroups.

Most of the Serbian isolates selected for this study belonged to subgroup IA (14 isolates) with haplotype IA; IA, IA; IA, IA, while isolate 137-13 had restriction patterns corresponding to the CMV subgroup II with haplotype II; II, II; II, II. The RT-PCR-RFLP method was successful, considering that each pattern is specific for CMV isolates of each group, which enabled the identification and classification of CMV isolates into three subgroups. The fact that each of the three RNAs of CMV evolved separately [5] and that recombinant and reassortant isolates represent over 11% of the population of this virus [4] shows that it is very important to develop rapid and reliable methods that allow the differentiation of isolates of IA, IB, and II subgroups not only on the CP gene but also on other coding and non-coding regions of the genome of this virus [31,48]. Although the presence of recombinants and reassortants was not detected in this study, RFLP analysis of the sequences of all five genes would be a useful method for determining changes in the genetic structure of the CMV population, such as the occurrence of isolates belonging to different CMV subgroups, as well as new genotypes resulting from genetic recombination and reassortment, which is very important for developing appropriate and efficient control strategies. RT-PCR-RFLP has been shown to be less expensive than sequencing, and it can provide reliable results by using multiple restriction enzymes for a given amplification product, thus avoiding errors that can lead to misinterpretation and invalid results [14,31].

Sequencing two selected Serbian isolates with different restriction patterns confirmed that the RFLP method can be used for appropriate subgrouping of

CMV isolates. A comparison of nucleotide and deduced amino acids of the different ORFs of isolates 674-11 and 137-13 showed they belong to subgroups IA and II, respectively. The similarity percentage varied depending on the analyzed RNA and gene segment, consistent with previously published data [5,7,49].

Although genotyping of RT-PCR products by restriction fragment length polymorphism (RFLP) has shown to be a handy tool for selecting an isolate for further characterization, previous results [31] also indicate the importance of selecting the restriction enzymes and test region of each CMV gene. In the case of some selected restriction enzymes, it was pointed out that during the research, the restriction site of some enzymes was identified in most but not all sequences retrieved from the databanks and was not present in the amplified region of their isolates [31]. The absence of a restriction site of the selected enzyme is possible, but errors can be avoided by selecting an appropriate restriction enzyme or performing analyses with more than one restriction enzyme, making this method reliable.

This study confirmed the prevalence of subgroup IA in Serbia and the presence of subgroup II, based on 15 analyzed CMV isolates, without the presence of recombinant and reassortant isolates. Previous studies on the CMV population in Serbia [19-22,24,26,50,51], based on analysis of the CP gene of CMV, focused on its presence and the damage that it causes. As shown by the results of previous studies, we found that different isolates of CMV subgroups originated from various host plants. It is known that there is no correlation between the detected subgroups and the host plant [3,7,46]. Because recombination and reassortment play a vital role in the evolution of viruses with multiple genomes, data based on the analysis of a single gene cannot provide precise phylogenetic results [5,13,52]. This study presents the development of a reliable and less costly protocol that allows the grouping of CMV isolates into appropriate subgroups based on different genes and a preliminary insight into the natural genetic population of this virus in Serbia.

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Conflict of interest disclosure: The authors declare no conflict of interest.

Data availability: Data underlying the reported findings are deposited in the NCBI dataset at: GenBank https://www.ncbi.nlm.nih.gov/genbank/ as follows: KT270554.1 (1a gene), KT270523.1 (2a gene), KT270542.1 (2b gene), KT270504.1 (MP gene) and JX127305.1 (CP gene) – isolate 674-11; OR257419.1 (1a gene), OR257420.1 (2a gene), OR257421.1 (2b gene), OR257422.1 (MP gene) and MH032570.1 (CP gene) – isolate 137-13. The data are also available at: https://www.serbiosoc.org.rs/NewUploads/Uploads/Zecevic%20et%20al_Dataset.pdf

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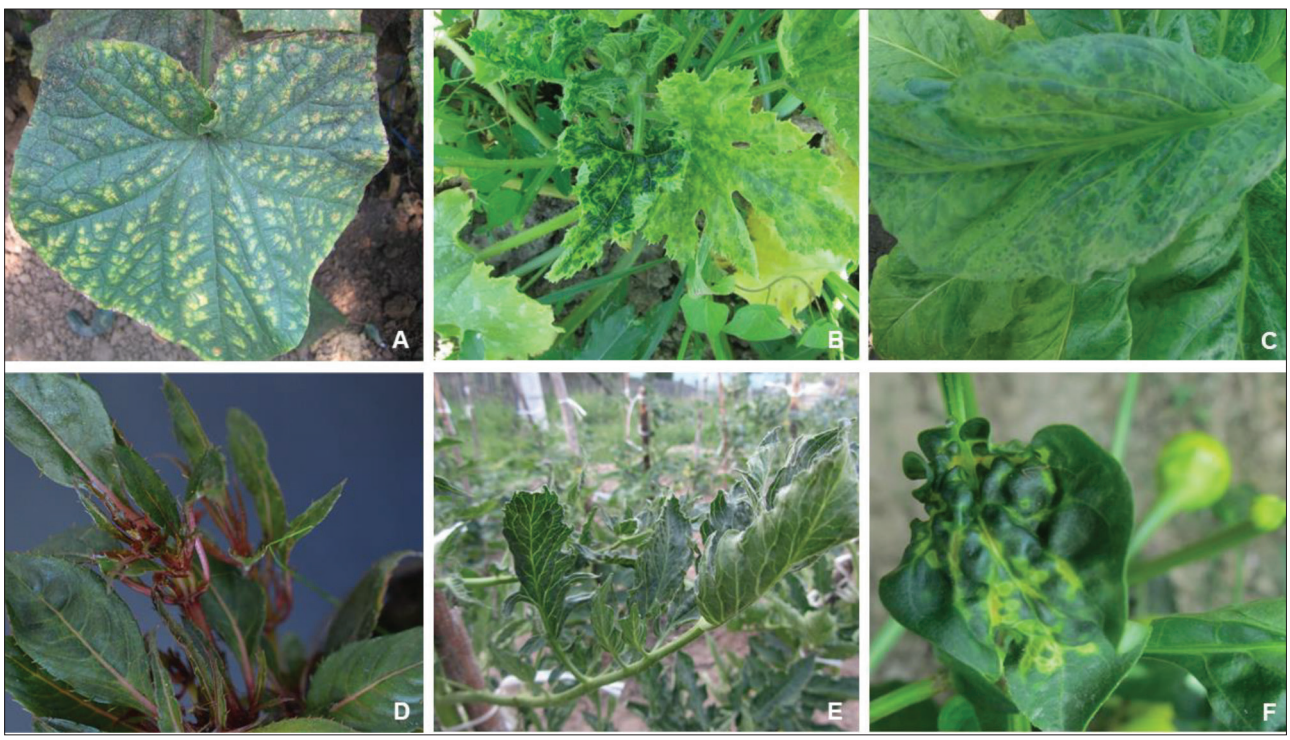
SUPPLEMENTARY MATERIAL

Supplementary Table S1. Cucumber mosaic virus (CMV) isolates originating from cucurbits, pepper, tomato, tobacco, and impatiens from Serbia.

Isolate	Year of isolation	Host	Locality
171-08	2008	*Cucurbita pepo* 'Olinka'	Staro Selo
201-11	2011	*Cucurbita pepo* 'Beogradska'	Mačkovac
514-11	2011	*Cucumis sativus*	Kupusina
610-11	2011	*Cucumis melo*	Togočevce
674-11	2011	*Lagenaria siceraria*	Porodin
449-12	2012	*Citrullus lanatus*	Silbaš
477-07	2007	*Capsicum annuum*	Apatin
542-08	2008	*Capsicum annuum*	Donji Tavankut
471-09	2009	*Capsicum annuum*	Prigrevica
207-09	2009	*Solanum lycopersicum*	Mačkovac
647-10	2010	*Solanum lycopersicum*	Togočevce
137-13	2013	*Solanum lycopersicum*	Ub
702-07	2007	*Nicotiana tabacum*	Futog
202-08	2008	*Nicotiana tabacum*	Lapotince
191-13	2013	*Impatiens hawkeri*	Rača Kragujevačka

Supplementary Table S2. Primers used for RT-PCR amplification of different genes of CMV

Target gene	Primer name	Primer sequence (5' to 3')	Cycling (temperature/time)			No. of cycles	Amplicon size (bp)	Reference
			Denaturation	Annealing	Extension			
1a	RNA1a-fwd	TGGTAGCCTCCCACGGCGATA	94°C/60 s	51°C/60 s	72°C/60 s	35	1198	This study
	RNA1a-rev	GAYTGCATRGACATACCATT						
2a	RV11	GTTTATTTACAAGAGCGTACGG	94°C/30 s	53°C/60 s	72°C/60 s	35	650	Finetti Sialer et al., 1999.
	RW8	GGTTCGAARRWATAACCGGG						
2b	2bfwd	TTTGTTGAYMGRYTGAAGTTT	94°C/60 s	46°C/60 s 50°C/60 s	72°C/60 s	5	804	This study
	2brev	CCTTCCGAAGAAAYCYAGGA				30		
MP	CMVMP3	GAGTGYGACCTAGGYCGRCATCA	94°C/60 s	60°C/60 s	72°C/60 s	35	728	This study
	CMV3a-rev	CTAARGACCGTTAACCACCTGC						
CP	CMVCPfwd	TGCTTCTCCRCGARWTTGCGT	94°C/60 s	52°C/60 s	72°C/60 s	35	871	Milojević et al., 2012.
	CMVCPrev	CGTAGCTGGATGGACAACCCG						



Supplementary Fig. S1. Symptoms of CMV infection: **A** – interveinal yellowing on *Cucumis sativus*; **B** – severe mosaic and blistering on *Cucurbita pepo* 'Beogradska'; **C** – severe mosaic and puckering on *Nicotiana tabacum*; **D** – mosaic and leaf narrowing with reduced leaf size on *Impatiens hawkeri*; **E** – mosaic, leaf curling and deformation on *Solanum lycopersicum*; **F** – severe mosaic and leaf deformation on *Capsicum annuum*.