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Riječ gostujućeg urednika

Poštovani čitatelji Glasila Future,

pred Vama je specijalno izdanje časopisa posvećeno profesoru emeritusu Bogdanu Cvjetkoviću. Svojim znanstvenim i stručnim radom, koji traje više od pola stoljeća, prof. Cvjetković ostavio je značajan i neizbrisiv trag ne samo u Hrvatskoj nego i svjetskoj fitopatologiji i fitofarmaciji. Ostavljajući iza sebe brojne generacije diplomiranih inženjera agronomije svojim entuzijazmom i predanošću fitopatologiji uspio je „zaraziti“ te biti predani mentor 18 magistara znanosti te 8 doktora znanosti. Širokog znanja i znanstvenih interesa magistrirao je na Prirodoslovno-matematičkom fakultetu u Zagrebu iz području biljne virologije, a doktorirao na svojem *alma mater* Agronomskom fakultetu u Zagrebu kod profesora Josipa Kišpatića na području biljne mikologije. U želji da ovim brojem djelomično oslikamo široko područje interesa prof. Cvjetkovića, ovo specijalno izdanje obuhvaća tri izvorna znanstvena rada te dva prethodna priopćenja iz područja biljne mikologije, bakteriologije te virologije. Kraj ovog specijalnog izdanja posvećen je crticama iz života dr. Željka Jurjevića, jednog od doktora znanosti koji je doktorirao pod mentorstvom prof. Cvjetkovića, a trenutno s uspješnom karijerom u Sjedinjenim Američkim državama (EMSL Analytical, Inc.). Izrazito mi je drago da su se sudjelovanju u ovom broju odazvali znanstvenici koje se bave fitopatologijom na području Hrvatske, ali i kolege iz inozemstva, dajući svoj značajan doprinos kvaliteti ovog specijalnog izdanja, ali i izražavajući pijetet prof. Cvjetkoviću.

Prvi rad kolegica Dušice Kovačević, Katarine Zečević te Ivane Stanković s Poljoprivrednog fakulteta Univerziteta u Beogradu govori o djelomičnoj molekularnoj karakterizaciji izrazito polifagnog virusa mozaika krastavca izoliranoga iz dvije biljke božura sa simptomima mozaika i klorotičnih prstenova. Nakon potvrde virusa serološkim i molekularnim metodama sekvenciranjem dijela genoma proteinskog omotača utvrđeno je da izolati iz božura pripadaju u podgrupu IA. Autorice skreću pozornost da bi božur kao trajnica mogao imati značajnu epidemiološku ulogu u kontekstu značajnog izvora ovog virusa.

Rad kolega Kirila Bahcevandziewa te Antónia A. Monteiro (Research Centre for Natural Resources, Environment and Society - CERNAS, Portugal) vodi nas u područje fenotipskih i genotipskih interakcija između različitih kupusnjača te ekonomski značajnog uzročnika plamenjača kupusnjača (*Hyaloperonospora brassicae*). Kroz istraživanje je utvrđeno da izolati navedenog patogena iz različitih područja Europe pokazuju različite stupnjeve patogenosti. Analizirani model gen-za-gen otvara nove mogućnosti istraživanja rezistentnosti kod različitih kupusnjača te gena za patogenost uzročnika plamenjače.

Da su na gljivične patogene osjetljive i invazivne biljne vrste govori rad autora Darija Ivića i Adrijane Novak (Hrvatska agencija za poljoprivredu i hranu). Analizom stabala pajasena sa simptomima

sušenja i propadanja na području Nacionalnog parka Krka utvrđena je prisutnost 15 različitih vrsta polifagnih gljiva iz rodova *Diaporthe*, *Diplodia*, *Dothiorella*, *Fomitiporia*, *Fusarium*, *Paraconiothyrium*, *Peroneutypa*, *Rosellinia*, *Schizophyllum* te *Verticillium*. Autori ističu da je ulogu utvrđenih gljiva u sušenju i propadanju ove invazivne vrste potrebno utvrditi testovima patogenosti.

Prethodno priopćenje doktorice znanosti Katarine Martinko i studentice Ivone Novaković sa Sveučilišta u Zagrebu Agronomskog fakulteta donosi preliminarne rezultate *in vitro* istraživanja protugljivičnog djelovanja esencijalnih ulja timijana, divljeg mažurana i lovora na uzročnika crne truleži plodova različitih poljoprivrednih kultura (*Aspergillus niger* Tiegh.). Autorice zaključuju da prvenstveno eterična ulja timijana i divljeg mažurana imaju veliki potencijal kao fumiganti u kontroli crne truleži uskladištenih poljoprivrednih proizvoda, te kao takvi predstavljaju svojevrsnu alternativu trenutno često korištenim fungicidima.

Prethodno priopćenje doktorice znanosti Jelene Plavec (Hrvatska agencija za poljoprivredu i hranu) opisuje uzročnika bakterioznog paleža lijeske (*Xanthomonas arboricola* pv. *corylina*) utvrđenog metodom lančane reakcije polimerazom iz rasadnika i komercijalnih nasada lijeske na području Hrvatske. Imajući u vidu sve veću popularnost ove kulture u našoj zemlji autorica skreće pozornost da će u budućnosti biti potrebno povesti više pažnje u praćenju ovog ekonomski značajnog patogena svrstanog na listu reguliranih nekarantenskih štetnika ne samo lijeske, već i drugih vrsta iz roda *Corylus*.

Crtice doktora znanosti Željka Jurjevića sažimlju različite dijelove profesionalnog razvoja prof. Cvjetkovića, ali ujedno predstavljaju i jednu toplu životnu priču protkanu zajedničkim trenucima provedenima s profesorom.

Vežući se na životopisne crtice dr. Jurjevića, i osobno kao jedan od doktoranada, mogu reći da bi se o liku i djelu prof. Cvjetkovića mogla napisati ne jedna, nego više knjiga. Na kraju mogu reći da mi je bila iznimna čast i zadovoljstvo intenzivno surađivati s profesorom sve do njegovog odlaska u mirovinu. I danas, sa životopisnim pričama i neograničenim praktičnim iskustvom, dragi mi je sugovornik na Zavodu za fitopatologiju u čiji razvoj je utkao značajno razdoblje svojega života i kojem je dao svoj neprocjenjivi obol!

Prof. dr. sc. Darko Vončina



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Partial molecular characterization of cucumber mosaic virus isolate infecting garden peony (*Paeonia officinalis*) in Serbia

Dušica Kovačević^{1*}, Katarina Zečević¹, Ivana Stanković¹

izvorni znanstveni rad (original scientific paper)

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Abstract

Paeonia officinalis (family Paeoniaceae), known as the garden peony, is a very popular flowering plant with large, showy flowers grown in many gardens in Serbia. In May 2021, peony plants showing chlorotic ringspot and severe mosaic of leaves were observed in a private garden in Zemun (District of Belgrade, Serbia). Symptomatic leaves from two plants were collected and analysed for the presence of cucumber mosaic virus (CMV), tobacco rattle virus (TRV), alfalfa mosaic virus (AMV) and tomato spotted wilt virus (TSWV) using commercial ELISA kits. CMV was detected serologically in both peony samples and no other plant virus was identified. The causal agents from both ELISA-positive samples were successfully mechanically transmissible to *Chenopodium quinoa* and *Nicotiana glutinosa* plants. CMV infection in symptomatic garden peony plants was also confirmed by RT-PCR with CMV-specific primers amplifying the complete coat protein (CP) gene and parts of the 3'- and 5'-UTRs. Selected ELISA-positive sample (318-21) was Sanger sequenced using the same primer as in RT-PCR and phylogenetic tree based on complete CP sequences showed that Serbian CMV isolate from garden peony belongs to the CMV subgroup IA.

Key words: peony, viruses, *Cucumovirus*, ELISA, bioassay, RT-PCR, phylogenetic analysis.

Introduction

Species of the genus *Paeonia* are amongst the most popular garden plants in regions with a temperate climate. They have been cultivated for several thousand years in China and their cultivation spread to many countries, including Serbia. This plant genus is divided into woody and herbaceous species based on their morphological characteristics and life forms (Kamenetsky and Dole, 2012). There are numerous peony hybrids and varieties, each with their own unique characteristics. One of them is *Paeonia officinalis*, the garden peony, which attracts attention with its large flowers. It is known that

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these plants can be infected by various pathogenic microorganisms. Among the phytopathogenic fungi, *Botrytis paeoniae* and *B. cinerea*, which cause bud blast, stand out (Rogers, 1995). In addition to these fungi, phytopathogenic viruses also play an important role and represent serious constraint to garden peony production worldwide. The most common reported virus in peony species is tobacco rattle virus (TRV), which causes peony ring spot disease. Its presence has been confirmed worldwide (Europe, USA, Japan, and New Zealand) (Chang et al., 1976; Robertson et al., 2009; Samuitien et al., 2009). Additionally, alfalfa mosaic virus (AMV) was detected in plants grown in the botanical garden of the University of Parma (Bellardi et al., 2003). In France, cucumber mosaic virus (CMV) was isolated and identified on *P. lactiflora* (Cardin et al., 2010), while tomato spotted wilt virus (TSWV), the species *Orthotospovirus tomatomaculae*, can also lead to infection in plants of this genus. However, the range of viruses that peonies can harbor have been expanded considerably with the development of high-throughput sequencing technology, including grapevine leafroll-associated virus-3 (GLRaV-3), the most recently discovered virus infecting peonies (Mischenko et al., 2023).

CMV (genus *Cucumovirus*, family *Bromoviridae*) is widespread throughout the world and is particularly prevalent in regions with a temperate climate. It is one of the most economically important viruses affecting numerous cultivated plant species worldwide. CMV has a broad host range and infects more than 1200 species from at least 100 plant families. It persists in many perennial cultivated and weed plants, and its spread is facilitated by insect vectors. The transmission primarily occurs in a non-persistent manner by aphids, with over 80 aphid species involved, of which *Myzus persicae* and *Aphis gossypii* are the most efficient. Additionally, transmission via seeds of certain cultivated and weed plants and mechanical sap transmission have also been documented (Palukaitis et al., 1992; García-Arenal and Palukaitis, 2008).

The genome of CMV consists of three linear, positive sense RNAs, designated RNA 1 to RNA 3. RNA 1 and RNA 2 code for viral replicase proteins 1a and 1b, respectively. RNA 2 codes for protein 2b, which is involved in the suppression of gene silencing, expression of symptoms and the spread of pathogen. Bicistronic RNA 3 encodes protein 3a (MP - movement protein), facilitating virus movement within the plant and aphid-mediated transmission. Additionally, it encodes protein 3b, also known as coat protein (CP), which encapsulates RNAs but also enables the cell-to-cell and systemic movement, and aphid transmission (Palukaitis et al., 1992; Jacquemond, 2012).

CMV strains are divided into two subgroups: I and II. Subgroup I is further subdivided into subgroups IA and IB. Isolates of subgroups IA and II are distributed worldwide, while isolates of subgroup IB originate mainly from East Asia, although some isolates have also been found in other parts of the world (Jacquemond, 2012; Giakountis et al., 2018). The virus may also encapsidate a small linear single-stranded satellite RNAs (satRNAs), that may enhance or attenuate symptoms induced by CMV, as has been recorded in lethal necrosis syndrome in tomato plants (García-Arenal and Roossinck,

2019). So far, more than 180 sequence variants of CMV satRNAs are associated with CMV I and II subgroup isolates. They are classified in three main phylogenetic clusters: necrogenic satRNAs, non-necrogenic satRNAs, and larger satRNAs which can be either necrogenic or non-necrogenic (Palukaitis and García-Arenal, 2019).

In Serbia, CMV is one of the most frequently detected and economically most important virus of numerous vegetable and field crops (Stanković et al., 2011; Vučurović et al., 2011, 2012; Milojević et al., 2013b; Milošević et al., 2017; Nikolić et al., 2018; Milošević et al., 2020; Stanković et al., 2021), as well as in various ornamental plant species (Milojević et al., 2013a; Milojević et al., 2014; Milošević et al., 2015; Milojević et al., 2016, ; Zečević et al., 2024). In addition, CMV was recently recorded for the first time in garden peony (Zečević et al., 2023b).

The aim of this study was to determine the genetic relationship of the new Serbian CMV isolate from garden peony with isolates available from GenBank database, including previously identified Serbian isolates. This information is the first step towards a better understanding of the epidemiology of the virus and the development and implementation of appropriate control measures.

Material and methods

Samples collection and serological detection

Two peony plants with virus-like symptoms were noticed in a private garden in Zemun (District of Belgrade, Serbia) in May 2021. Two samples of symptomatic leaves (one sample per plant) were collected and transported to the Laboratory for virology and mycology at Faculty of Agriculture, University of Belgrade.

Collected samples were tested with double-antibody sandwich (DAS)-ELISA using commercially available diagnostic kits (Loewe Biochemica, Sauerlach, Germany) against the common peony viruses: CMV, TRV, AMV and TSWV. Briefly, samples were homogenized with extraction buffer at a ratio of 1:10 (weight/volume) using a cold mortar and pestle. Absorbance at 405 nm was determined using an ELISA microplate reader (DAS srl, Italy). Positive samples had absorbance value that was two times higher than the absorbance of the negative control. Commercial positive and negative controls were included in each ELISA test.

Mechanical transmission

Five plants of each of the two test species *Chenopodium quinoa* and *Nicotiana glutinosa* were mechanically inoculated with the crude sap extracted from two ELISA-positive samples (isolates 318-21 and 319-21) using 0.01 M phosphate buffer (pH 7.0) and silicon carbide abrasive. The test plants were inoculated at the 2 to 3 true leaf stage and kept in a greenhouse at 22-25°C for up to four weeks

post-inoculation. The presence of CMV in test plants was verified serologically four weeks post-inoculation.

Molecular detection

Total RNAs Serbian CMV isolates 318-21 and 319-21 were extracted using RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was carried out using the One-Step RT-PCR kit (Qiagen, Hilden, Germany) and primers, CMVCPfwd and CMVCPrev (Milojević et al., 2012), which amplifies an 871-bp fragment of the entire CP gene and parts of the 3'- and 5'-UTRs. Serbian CMV isolate from Cucurbita pepo 'Olinka' (GenBank Accession Number HM065510) was used as a positive control, while the PCR mix with RNase-free water served as a negative control.

RT-PCR was done in a total volume of 25 µl, containing 1x Qiagen OneStep RT-PCR buffer, 400 µM dNTP mix, 0.6 µM of each primer, 1 µl Qiagen OneStep RT-PCR enzyme mix, and 50-100 ng of RNA template. Amplification was performed in the Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific, USA) with the following conditions: reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min, with final extension step at 72°C for 10 min. The size of the amplified products was determined by 1% agarose gel electrophoresis in TBE buffer after staining in ethidium bromide (EB) solutions and visualization on ETX-F20.M UV transilluminator (Vilber Lourmat, France).

Detection of CMV satRNAs

Possible presence of satRNAs in two garden peony samples was tested using RT-PCR and primers CMVsat-fwd/CMVsat-rev (Škorić et al., 1996). Serbian CMV satRNAs isolate (KM358138) was used as a positive control, while RNase-free water served as a negative control. RT-PCR reactions were performed in a volume of 25 µl as described previously. PCR amplification of CMV satRNA was performed under the following conditions: 2 cycles at 94°C for 1 min, 42°C for 1 min, 72°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The size of the amplified products was determined as described above.

Sanger sequencing and phylogenetic analysis

RT-PCR product obtained from a CMV-positive sample 318-21 was directly sequenced in both directions using the same primers as in RT-PCR assay. The obtained sequence was deposited in GenBank and compared with CMV isolates available using BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>).

Further characterization was performed by reconstruction of phylogenetic tree using 29 complete CP sequences of CMV isolates retrieved from the GenBank (table 1) and the CMV CP sequence generated in this study. Phylogenetic tree was constructed using the maximum parsimony method implemented in MEGA X software (Kumar et al., 2018) with the default parameter applying 1000 rounds of bootstrapping and bootstrap values <60% were omitted. An isolate of peanut stunt virus (Acc. No. U15730) was used as the outgroup sequence. Based on MODELTEST implemented in MEGA X, Kimura 2-parameter model with Gamma distribution (K2+G) was selected for calculation of the diversity within and between subgroups.

Table 1. Coat protein (CP) gene sequences of cucumber mosaic virus (CMV) used in the phylogenetic analysis.

Isolates	Country	Host plant	GenBank Accession Number
253-15	Serbia	<i>Solanum lycopersicum</i>	KC847071
207-09		<i>S. lycopersicum</i>	MN656189
471-09		<i>Capsicum annuum</i>	KC847073
581-11		<i>C. annuum</i>	KC414926
1-12		<i>Peperonia tuisana</i>	KC505441
473-12		<i>Citrullus lanatus</i>	KC878465
79-13		<i>Tulipa sp.</i>	KJ854451
Trk7		Hungary	<i>Trifolium repens</i>
NS		<i>Nicotiana glutinosa</i>	AJ511990
I17F	France	<i>S. lycopersicum</i>	Y18137
R		<i>S. lycopersicum</i>	Y18138
Ri-8	Spain	<i>S. lycopersicum</i>	AM183119
Tfn	Italy	<i>S. lycopersicum</i>	Y16926
TN	Japan	<i>S. lycopersicum</i>	AB176847
PF		/*	AB368501
Y		<i>N. tabacum</i>	D12499
Ly2	Korea	<i>Lilium longiflorum</i>	AJ296154
NT9	Taiwan	<i>S. lycopersicum</i>	D28780
PoCMV7-7	Syria	<i>S. tuberosum</i>	AB448695
RZ	China	<i>N. tabacum</i>	EF159146
CTL		<i>Brassica chinensis</i>	EF213025
Cb7		<i>S. lycopersicum</i>	EF216867
Tsh		<i>S. lycopersicum</i>	EF202597
Q	Australia	<i>C. annuum</i>	M21464
LY		<i>Lupinus angustifolius</i>	AF198103
S	South Africa	<i>Cucurbita pepo</i>	AF063610
LS	USA	<i>Lactuca sativa</i>	AF127976
FNY		<i>Cucumis melo</i>	D10538
N1-03		<i>Vinca minor</i>	JF918966

* Unknown host plant.

Results and discussion

Symptoms and virus detection using DAS-ELISA

In May 2021, chlorotic ringspot and severe mosaic on leaves (figure 1) were observed on two peony plants grown in a private garden in Zemun (District of Belgrade, Serbia). Serological assays revealed that CMV was the only virus detected in both collected samples, the new Serbian isolates were identified as 318-21 and 319-21. No other tested viruses were detected.

CMV has a wide host range, including numerous ornamental plants (Palukaitis et al., 1992; García-Arenal and Palukaitis, 2008). The symptoms caused by CMV as well as the severity of the disease vary depending on CMV molecular characteristics, including the presence of a satellite RNA, the host genotype, the growth stage, the time of infection, and environmental factors (Mochizuki, 2012; Zhao et al., 2016). In some cases, CMV infection may occur asymptomatic. Conversely, it can lead to systemic necrosis (Jacquemond, 2012). Generally, in ornamental plants, CMV most commonly induces mosaic. Additionally, ring spots, mottling of flowers, bud necrosis, and stunted growth of plants may occur (Ashfaq et al., 2017). On *Peonia lactifera* species, CMV causes very pronounced mosaic and the appearance of chlorotic rings (Cardin et al., 2010), which were also recorded in this study.



Figure 1. Symptoms of cucumber mosaic virus (CMV) infection recorded in garden peony from Zemun (Serbia) with severe mosaic and chlorotic rings. (PHOTO: I. Stanković, 2021).

Bioassay

In order to biologically characterize the CMV ELISA-positive isolates 318-21 and 319-21, crude sap extract from symptomatic peony plants were used to mechanically inoculate five plants each of two species: *C. quinoa* and *N. glutinosa*. All inoculated plants reacted uniformly and showed characteristic symptoms of CMV infection, which is in correlation with previous reports (Milojević et al., 2013b, 2014; Choi et al., 2015). Local chlorotic spots in mechanically inoculated *C. quinoa* and severe mosaic and leaf malformations in *N. glutinosa* plants were noticed seven- and 14-days post-inoculation, respectively. CMV infection in all mechanically inoculated plants was confirmed using DAS-ELISA.

RT-PCR assay

The result of RT-PCR testing revealed that both symptomatic garden peony samples yielded an amplicon of 871 bp confirming the presence of CMV. The primer pair CMVsat-fwd/rev was unable to amplify amplicons from both garden peony CMV isolates, indicating that no satRNA sequences are associated with selected CMV isolates.

Various pathogenic microorganism can cause serious economic losses in ornamentals industry, but viruses are a major constraint for most ornamental plants, especially for species which are exclusively vegetatively propagated due to the accumulation of viruses in propagative material (Valverde et al., 2012; Mitrofanova et al., 2018). CMV is the plant virus with the broadest host range, infecting several agriculturally important crops such as tomato, tobacco, cucurbits, and legumes, as well as a variety of ornamental plants (Palukaitis et al., 1992). So far, CMV has only been detected in *P. lactiflora* in France (Cardin et al., 2010) and this study is the second detection of the virus in peony plants.

Sequence analysis and phylogeny

The RT-PCR product of the selected isolate 318-21 was purified and bi-directional sequenced as described above (PP818664). BLAST results showed that CP sequence of the new Serbian CMV isolate has the highest nt identity of 99.54% with Serbian isolate 514-11 (KT270567) from *Cucumis sativus*.

Phylogenetic tree (Figure 2) showed clustering of the selected isolates into tree subgroups IA, IB and II supported by bootstrap values of 100% and an overall level of nucleotide diversity of 0.256 ± 0.019 . Subgroup I was further subdivided into two subgroups, IA and IB. The genetic diversity among subgroups ranged from 0.0553 ± 0.0068 to 0.3470 ± 0.0370 , whereas diversity within each group was lower (IA- 0.022 ± 0.003 ; IB- 0.054 ± 0.007 ; II- 0.012 ± 0.002). The Serbian CMV isolate originating from garden peony belonged to subgroups IA. According to the previous study on the genetic population in Serbia (Milošević et al., 2017; Stanković et al., 2021; Vučurović et al., 2012; Zečević et al., 2023a), the isolates of CMV subgroup IA are widespread. Therefore, the majority of isolates worldwide belong

to subgroup IA (Roossinck, 2002; Bonnet et al., 2005; García-Arenal et al., 2008; Jacquemond, 2012). Subgroup II isolates have also been found in ornamental plants in our country (Milošević et al., 2015) and tomato (Stanković et al., 2021), but their frequency is much lower. The characterization of CMV isolates is most often based on the CP gene (Roossnick, 2002), but recent studies have shown the importance of genetic characterization based on each of the three RNAs, because genetic recombination and reassortment events are significant for evolution of viruses with multiple genomes as CMV (Lin et al., 2003; García-Arenal and Palukaitis, 2008). This is only a partial characterization of CMV isolate from garden peony. Further research should be carried out to determine the variability within the CMV population in Serbia, based on the analysis of all five genes of CMV. This will provide more precise information on its population structure in our country.

Considering the fact that garden peonies are widespread and popular garden plants in Serbia, the presence of CMV could be a limiting factor for the cultivation of these plants. In addition, this finding has significant implications for the successful production of various ornamental plants, since peonies, as perennial plants, serve as a virus reservoir and additional source of inoculum and represent an important link in the epidemiology of CMV.

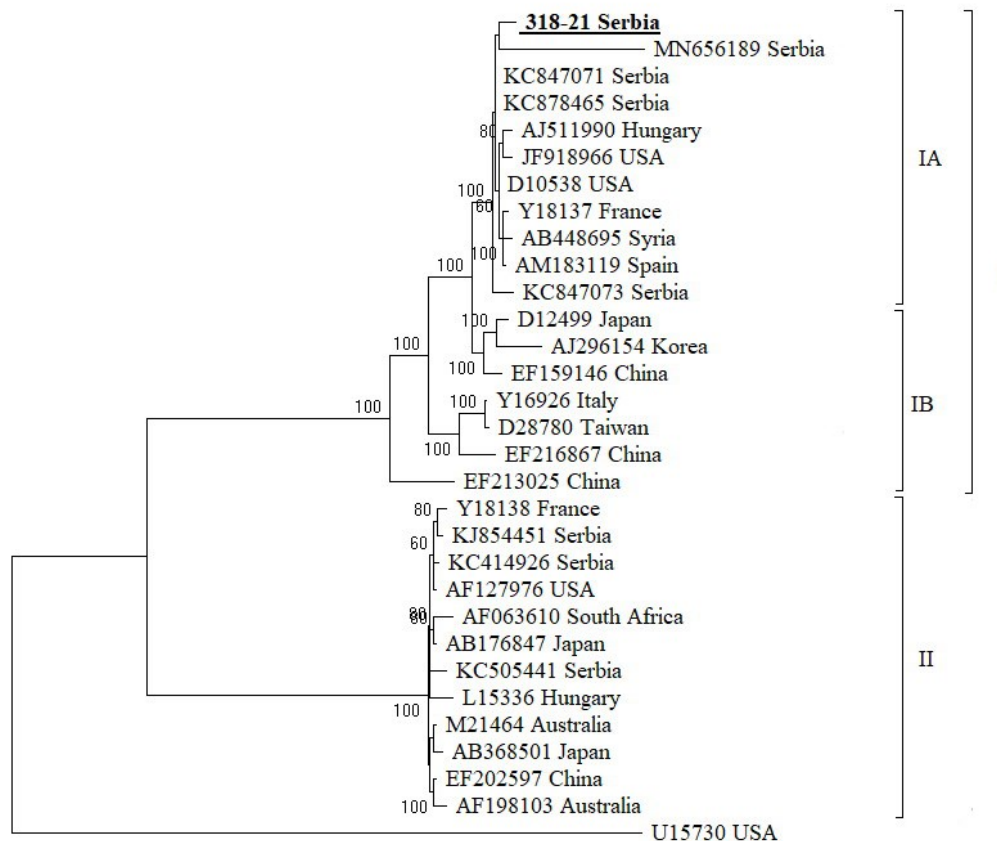


Figure 2. Phylogenetic tree comparing on the complete CP sequences of 30 cucumber mosaic virus (CMV) isolates using MEGA X and 1,000 iterations. Bootstrap values greater than 60% are indicated on the corresponding branches. The CMV isolate from this study is underlined and in bold.

Conclusion

Having previously reported the occurrence of CMV in garden peony (Zečević et al., 2023b), we have now partially characterized the virus for the first time by Sanger sequencing and phylogenetic analysis of the complete CP gene. The new Serbian isolate, which originates from the garden peony, belongs to the subgroup IA. This is epidemiologically very important because garden peony, as a new perennial host of CMV, could represent a significant reservoir of the virus and an additional source of inoculum in our country. Due to the great damage CMV causes to various hosts worldwide, including many vegetable and ornamental plants, constant control and monitoring of the virus is necessary.

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