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Monitoring and phylogenetic analysis of Cucumber mosaic virus isolates from *Cucurbitaceae* plants in Ukraine

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Aim. To conduct monitoring and phylogenetic analysis of the cucumber mosaic virus isolates from agrocenoses of Ukraine. **Methods.** Enzyme-linked immunosorbent assay, RT-PCR, sequencing and phylogenetic analysis. **Results.** The agrocenoses of different regions of Ukraine were examined and the plants of *Cucurbitaceae* with the manifestations of viral diseases were selected. Using an enzyme-linked immunosorbent assay, 131 samples of *Cucurbitaceae* plants were tested and the antigens of cucumber mosaic virus (CMV) were detected. The RT-PCR resulted in amplification products of 500 bp in size, which corresponds to the sequence of the capsid protein gene of CMV. Based on the sequence data, a phylogenetic tree of the selected CMV isolates was constructed. **Conclusions.** It was found that Ukrainian isolates of CMV obtained from the plants of *Cucurbitaceae* are represented only in the cluster of group I of subgroups IA and IB.The most related to the Ukrainian isolates were strains T35 and T19 (China), strain I17F (France) and banana (Israel).

Keywords: Bromoviridae, cucumber mosaic virus (CMV), capsid protein gene, phylogenetic analysis.

Introduction

Cucumber mosaic virus (CMV) belongs to the Cucumovirus genus in the Bromoviridae family. It is present in different parts of the world and has a large number of strains. Its virion has icosahedral symmetry. The diameter of the viral particle is 29 nm [1]. CMV is transmitted non-persistently by 75 aphid species, as well as by parasitic plants, such as dodder (Cuscuta sp.), by seeds and mechanically [2, 3]. CMV can infect a wide spectrum of plants: 1300 species representing about 500 genera from 100 families [4]. The hosts of CMV are important vegetable and ornamental crops. Many plant species, including many weeds, can be reservoirs for the virus. The virus quickly and successfully adapts to new hosts and environmental conditions, so new hosts are described every year. CMV is particularly pathogenic to the members of *Cucurbitaceae*, *Solanaceae* and *Fabaceae* families, leading to 30–50% of plants

being affected during epiphytotic years [5]. The virus genome is represented by a single-stranded, positive-sense RNA that includes three segments. Five genes encode the following proteins: 1a, 2a, 2b, 3a and a capsid protein [6].

Based on the comparison of nucleotide sequences, all CMV strains and isolates are divided into groups IA, IB, II (corresponding to the serological groups DTL, Co and ToRS). To determine the group affiliation, the indicator plants, serological methods,RT-PCR, restriction fragment length polymorphism, and phylogenetic analysis can be used [7].

The CMV groups are characterised by a heterogeneous spread. Strains and isolates belonging to the groups IA and II are distributed evenly throughout the world, with the group I being more common and sometimes representing up to 80% of all isolates [1]. Representatives of the group IB are found mainly in East Asia, California, Australia and Greece. It is believed that the group I strains were introduced to these areas from East Asia [8].

Representatives of the groups IA and IB are characterised by a wide temperature optimum, found in the tropics and subtropics, where they cause large losses and epidemics. The isolates of group II cause weaker symptoms in plants and are usually found in the countries with a temperate climate [5].

The aim of the study was to conduct monitoring and phylogenetic analysis of the isolates of cucumber mosaic virus from agrocenoses of Ukraine.

Materials and Methods

The plant samples of the *Cucurbitaceae* family were collected using a visual analysis of plants for the presence of virus-like symptoms during the growing seasons of 2021–2023 from agrocenoses of 8 Ukrainian regions (Vinnytsia 18 samples, Kyiv 16 samples, Odesa 18 samples, Poltava 16 samples, Rivne 16 samples, Kherson 15 samples, Cherkasy 16 samples, and Chernihiv regions 16 samples). In each region, 16 samples of the *Cucurbitaceae* family plants were collected, with an average of 33 samples of each type of *Cucumis sativum* L., *Cucurbita pepo* L., *etc.*

Further, the material was homogenised in 0.1 M phosphate-salt buffer, pH 7.4 in a 1:2 ratio. To separate the material from plant components, the resulting homogenate was centrifuged at 5000 rpm for 20 min at + 4 °C using a PC-6 centrifuge [4]. The collected supernatant was used for the diagnosis of viral pathogens in an enzyme-linked immunosorbent assay (ELISA) in the «sandwich» modification using a test system manufactured by Loewe (Germany). The assay was performed in accordance with the recommendations of the test system manufacturer. The results were recorded on a Termo Labsystems Opsis MR reader (USA) with Dynex Revelation Quicklink software at 405/630 nm [9]. The experiments for each sample were performed in a threefold repetition [10].

Total RNA was extracted from the samples using RNeasy Plant Minikit (Qiagen, UK) [11]. The specific primers for the capsid protein region of cucumber mosaic virus were used for RT-PCR:

Forward primer — 5’TATGATAAGAAGCT TGTTTCGCGCA-3’

reverse primer — 5’TTTTAGCCGTAAGC TGGATGGACAACCC-3’

These primers are complementary to the genomic nucleotide regions and amplify a

500 bp fragment encoding a region of the CMV capsid protein.

RT-PCR was performed in the following sequence: 1 cycle for 30 s at 60 °C, 1 cycle at 95 °C for 2 s, 30 cycles at 94 °C for 15 s, 55 °C for 30 s, and 1 cycle at 68 °C for 30 s. The amplification products were analysed by electrophoresis in a 1.5% agarose gel using standard Gene Ruller 100 bp RNA Ladderplus markers (Fermentas, Lithuania) [12]. The amplification products (cDNA) were isolated from the gel and cleaned using a Thermo Scientific Gene JET Extraction Kit (USA). Sequencing of purified amplified fragments was performed on an Applied Biosystems 3730 ×1 DNA Analyzer using Big Dye terminators, version 3.1 (USA).

The following Ukrainian isolates of cucumber mosaic virus were sequenced: CMV-3/21 (*Cucurbita pepo* L. from Rivne region), CMV-34/21 (*Cucumis sativum* L. from Vinnytsia region), CMV-8/22 (*Cucurbita citrullus* L. from Odesa region), CMV-9/21 (*Cucurbita pepo* L. from Poltava region) and CMV-13/23 (*Cucumis melo* L. from Cherkasy region). The aligned cDNA sequences of Ukrainian CMV isolates were compared with the nucleotide sequences of the CMV strains and isolates belonging to the subgroups I and II, publicly available from the GenBank database (http://www. ncbi.nlm.nih.gov). The phylogenetic analysis was conducted using MEGA 6 software

The CLUSTAL W method was used for nucleotide sequence alignment. The bootstrap analysis (500 replicates) was used to test the validity of the trees [13].

Results and Discussion

To check for the presence of viral antigens, 131 samples of plants of the *Cucurbitaceae* family were selected and tested. The plant samples were collected from agrocenoses of the following regions of Ukraine: Vinnytsia, Kyiv, Odesa, Poltava, Rivne, Kherson, Cherkasy and Chernihiv regions. The following symptoms were observed on plants of cucumbers, courgettes, pumpkins, zucchini: wrinkling, deformation, corrugation and yellowing of leaf blades, necrosis, chlorosis, yellow-green mosaic, filamentous leaf blades,

Fig. 1. Symptomatic signs of viral diseases on representatives of the *Cucurbitaceae* selected from Vinnytsia, Odesa, Cherkasy agrocenoses of Ukraine:
A — tuberous growths and bordered spots on the skin of *Cucurbita pepo* L. fruit;
B — yellow-green tuberous mosaic on the skin of the *Cucumis sativum* L. fruit;
C — yellow-green mosaic on the *Cucurbita pepo* L. leaves.

dark green spots of various sizes, tuberous growths on fruits and their deformation (Fig. 1).

Subsequently, the detection of cucumber mosaic virus was carried out by ELISA in the «sandwich» modification using commercial test systems. The antigens of CMV were detected in 51 samples of plants of the *Cucurbitaceae* family. The highest percentage of infected plants was found in Vinnytsia, Odesa, Poltava, Rivne and Cherkasy regions. As shown in Fig. 2 in Vinnytsia region, 11 samples were found affected by CMV. Among the affected plants there were 5 of *Cucumis sativum* L., 4 of *Cucurbita pepo* L., 2 of *Cucurbita citrullus* L. In Odesa region, the CMV antigens were detected in 12 samples, including 4 of *Cucumis sativum* L, 3 of *Cucurbita pero* L., 3 of *Cucurbita citrullus* L. and 2 of *Cucumis melo* L. Among the selected plant samples of Rivne region, the CMV antigens were found in 10 samples, particularly in 4 of *Cucumis sativum* L., 4 of *Cucurbita pepo* L., 2 of *Cucurbita citrullus* L. In Poltava region, 9 samples of CMV-infected plants were detected, including *Cucumis sativum* L. (4), *Cucurbita pepo* L. (3), *Cucurbita citrullus* L. (2). Eight samples affected by CMV were found in Cherkasy region including 3 of *Cucumis sativum* L., 3 of *Cucurbita pepo* L., and 2 of *Cucumis melo* L. As for Kherson region, only one positive sample of CMV was found among plants of *Cucumis sativum* L. In Kyiv and Chernihiv region, no plants infected with CMV were found (Fig. 2).

Thus, based on the results of the enzyme immunoassay, a conclusion can be drawn

that cucumber mosaic virus is widespread in agrocenoses of Ukraine.

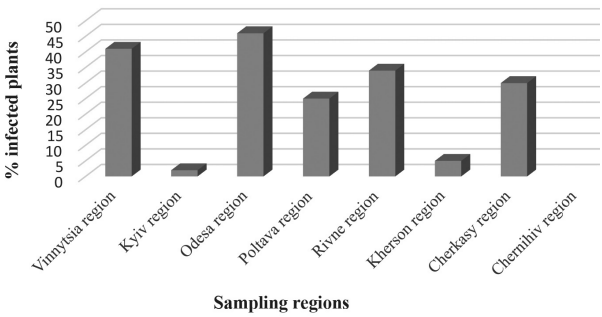

Fig. 2. The number of *Cucurbitaceae* plants infected with CMV found in different regions of Ukraine

The analysis of the data showed that the CMV antigens were not detected among the selected samples with classical symptoms of viral etiology. Therefore, these plant samples had symptoms of either a different nature (bacterial, fungal) or of an unknown viral nature which poses the challenge for researchers to conduct broader and deeper research of these symptoms, in particular using molecular genetic methods.

To genetically characterise the CMV population and to determine the group and strain of circulating isolates, nucleotide sequences of the capsid protein gene region of CMV isolates from different regions of Ukraine were obtained and analysed.

To achieve this goal, total nucleic acid was first isolated from the plant samples that showed a positive result in ELISA. After that, RT-PCR was performed using total RNA as a matrix and primers specific for the CMV capsid protein gene. As a result, a 500 bp cDNA was detected (Fig. 3).

The sequencing of the purified amplified fragments was performed. The nucleotide sequences of 449 bp for isolates CMV-9/21 and CMV-8/22, 445 bp for CMV-34/21, and 452 bp

for CMV-3/21 were obtained. The sequences of the capsid protein gene of the Ukrainian isolate CMV-13/23 (432 bp) were also used for further studies.

Identification of the CMV isolates and assessment of their genetic variability are the important steps for controlling viral diseases, especially if genetic engineering approaches are preferred.

Phylogenetic analysis was performed on the basis of nucleotide sequences of the capsid protein gene of the CMV isolates detected during the growing seasons of 2021–2023.

These isolates were obtained from *Cucurbitaceae* plant samples (*Cucumis sativum* L., *Cucurbita pepo* L., Cucurbita citrullus L., *Cucumis melo* L.) from Vinnytsia, Odesa, Poltava, Rivne and Cherkasy regions. The presence of virus antigens in these samples was previously confirmed by ELISA.

According to the degree of homology of capsid protein nucleotide sequences, Ukrainian CMV isolates can be divided into 2 groups, with intragroup homology greater than 99%. The nucleotide sequence homology of representatives of different groups was approximately 92–93%.

The obtained results indicate that these isolates are representatives of different CMV groups, so the next stage of research to establish group and strain affiliation was to compare Ukrainian isolates with known GenBank sequences of the capsid protein gene of different strains using NCBI/BLAST. For comparison and construction of the phylogenetic tree, we selected the CMV strains from different countries that differed in their biological, serological properties and group association (Table 1).

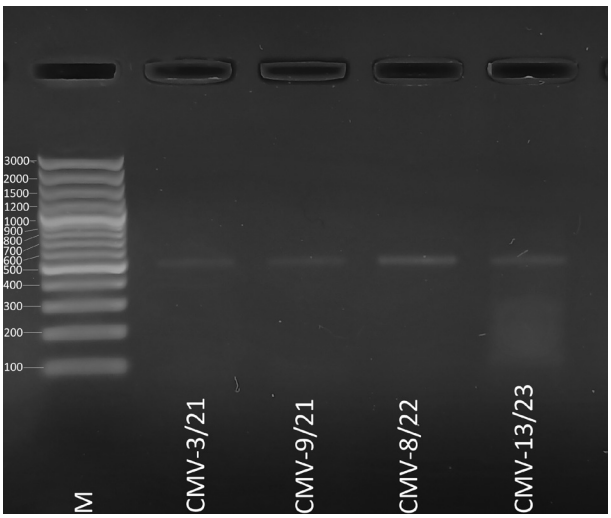

Fig. 3. Electrophoresis of samples after RT-PCR: *M* – markers (100 bp, Fermentas); CMV-8/22, CMV-13/23, CMV-9/21 – products of 500 bp in the size.

The phylogenetic tree was constructed using the nearest neighbours method in MEGA 6 using the Jukes-Cantor model (Fig. 4).

The phylogenetic tree clearly shows two clusters belonging to the CMV groups I and II. The Ukrainian isolates were represented only in the cluster of group I, but were divided between subclusters associated with the groups IA and IB.

The CMV group I is more divergent than the group II. On the phylogenetic tree, the cluster relevant to this group includes two subclusters consisting of strains of the groups IA and IB. Ukrainian isolates CMV-9/21 Ukr (*Cucurbita pepo* L. from Poltava region), CMV-3/21 Ukr (*Cucurbita pepo* L. from Rivne region) were located in the IA cluster. Isolates CMV-34/21 Ukr (*Cucumis sativum* L. from Vinnytsia region), CMV-8/22 Ukr (*Cucurbita citrullus* L. from Odesa region) and CMV-13/23 Ukr (*Cucumis melo* L. from

Cherkasy region) were located in the subcluster of the IB group.

The nucleotide sequence homology of Ukrainian isolates and strains of the group IA varied in the range of 96–99% for CMV-9/21 Ukr, CMV-3/21 Ukr, and 92–95% for strains of the group IB - CMV-34/21 Ukr, CMV-8/22 Ukr, and CMV-13/23 Ukr. In the subcluster of the group IB representatives, strains from Taiwan (243, 16, 22 strains) and China (T19, T35 strains) were observed. The homology of nucleotide sequences of isolates CMV-34/21 Ukr, CMV-8/22 Ukr, CMV-13/23 Ukr and representatives of group IB was > 95%, which once again confirms their belonging to the group IB. The group II strains formed a separate cluster.

In summary, the Ukrainian CMV isolates from plants of the *Cucurbitaceae* are represented only in the cluster of group I of subgroups IA and IB. The most commonly related

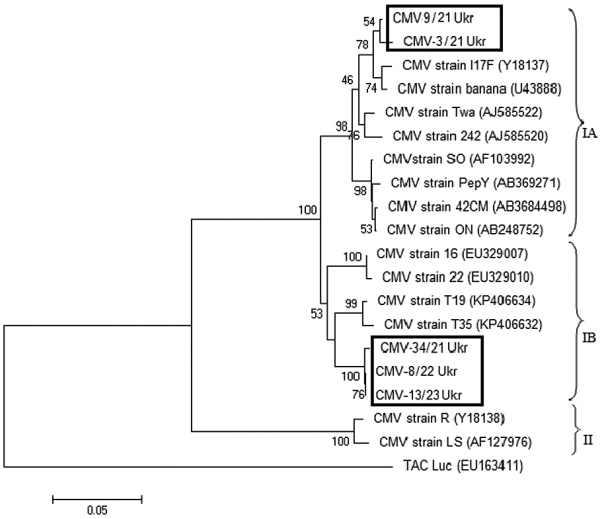

Fig. 4. The phylogenetic tree of the capsid protein gene region of the CMV group I, represented by the subgroups IA and IB, and group II was constructed using the nearest neighbours method in MEGA 6. Evolutionary distances were calculated using the Jukes-Cantor model. Ukrainian isolates are marked with rectangles, bootstrap support is indicated above the branches.

Table 1. **The list of CMV strains selected for phylogenetic analysis**

Strain	Group	Host	Number	Country
Twa	IA	*Cucumis melo* L.	JA585522	Korea
banana	IA	*Musa acuminata*	U43888	Israel
42 CM	IA	*Cucumis sativum* L.	AB3684498	Japan
I17F	IA	*Cucumis sativum* L.	Y18137	France
ON	IA	*Momordica charantia* L.	AB248752	Japan
PepY	IA	*Nicotiana benthamiana*	AB369271	South Korea
R	II	not given	Y18138	France
SO	IA	not given	AF103992	Japan
Twa	IA	*Capsicum sp.*	AJ585522	Australia
T19	IB	(*Cucumis melo* L. var. *Inodorus*)	KP406634	China
T35	IB	(*Cucumis melo* L. var. *Inodorus*)	KP406632	China
16	IB	*Cucurbita pepo* L.	EU329007	Taiwan
22	IB	*Cucurbita pepo* L.	EU329010	Taiwan
242	IA	*Cucurbita pepo* L.	AJ585520	Australia

to Ukrainian isolates were strains T35 and T19 (China), strain I17F (France) and banana (Israel).

Based on the above, it can be concluded that the CMV population in Ukraine includes representatives of the groups IA and IB, and a high degree of genetic homogeneity is observed in the isolates of both groups IA and IB (Table. 2).

It therefore follows that CMV-9/21 and CMV-3/21 are isolates of the group IA. The group II strains formed a separate cluster. No representatives of the group II were found among the analysed isolates: the homology of nucleotide sequences of Ukrainian isolates with them is approximately 73–79%.

It can be assumed that the representatives of group II were not detected in this study due to the following reasons: first, the group II strains are present in infected plants in low concentrations, too low to be easily detected by ELISA. Compared to the serological methods, RT-PCR is more sensitive, but in our case it was used only for the samples that showed a high titer of the CMV viral antigens in ELISA; secondly, it is believed that the group I strains are more numerous than the group II representatives. About 80% of the CMV population is represented by the group I strains [4]. However, Chinese scientists questioned this statement and in 2008 conducted a comparative study of the competitive ability of the group I and group II isolates in affected tobacco plants using the ELISA method. The study took into account the frequency of infection and the ratio between the number of plants with mono- and mixed infection. It turned out that isolate ZL of the group II was more com-

Table 2. **Homology of nucleotide sequences of Ukrainian CMV isolates and known strains from GenBank**

Strain	CMV-9/21	CMV-3/21	CMV-34/21	CMV-8/22	CMV-13/23
I **group**					
banana	98.4%	98.7%	92.2%	93.3%	93.3%
I17F	99%	99.4%	92.9%	94%	94%
ON	97.1%	96.1%	92.9%	94%	94%
PepY	96.7%	95.7%	93.3%	94.3%	94.3%
42 CM	98%	97.7%	93.3%	93.6%	93.6%
242	97%	96.7%	92.2%	93.3%	93.3%
Twa	97.7%	97.4%	93.3%	94.3%	94.3%
SO	94.7%	93.6%	98%	99%	99%
T19	95%	94%	95.7%	96.7%	96.7%
T35	94.7%	93.6%	95.4%	96.4%	96.4%
16	95.4%	94.3%	95.4%	96.4%	96.4%
22	95%	94%	95%	96%	96%
II **group**					
R	75.6%	74.3%	77.3%	78.6%	78.6%
LS	74.7%	73.3%	76.5%	77.8%	77.8%

petitive than isolate YQ of the group I, however, the symptoms caused by it were weaker in mono-infected tobacco plants [14]. At the same time, the group I isolates NX and YQ were stronger than the group I isolate AG. The authors point out that the recent spread of group II strains in China is the result of directed competition between representatives of different groups in mixed infections [15].

Thirdly, it was traditionally believed that the group II strains were more common in tropical regions. Although no representatives of the group II were detected among Brazilian CMV isolates, even though the study was conducted in a tropical area, the virus is represented by strains and isolates of the group IA and group IB subgroups [17]. Moreover, the strains of group IB are closely related to the strains from East Asia and originated from this area. In Iran, strains of the IB group have been detected relatively recently, and the probable reasons for their appearance in this country may be: the introduction of new varieties of cultivated plants, changes in the CMV population due to natural causes, and the use of contaminated seeds [1].

When comparing the Ukrainian isolates belonging to the group IA, the percentage of nucleotide sequence homology was 99–99.5%. Several positions with nucleotide substitutions were identified. The CMV-9/21 isolate showed a C→T transition at positions 429 and 703. The substitution at position 703 was detected only in CMV-9/21. Regarding position 429, among the strains of group IA, the nucleotides are represented by both cytosine and thymine. All Ukrainian isolates, except for CMV-3/21, show a T→C transition. In other strains, including the typical strain of this group, Fny, cytosine is present at this position. The CMV-3/21 isolate has a unique A→G transition at position 624. No substitution at this position was observed in other strains. A 2 nucleotide deletion was observed at position 751–752 in the CMV-3/21 and CMV-9/21 isolates.

Among the Ukrainian isolates of group IB, the highest nucleotide sequence homology was observed between CMV-13/23 and CMV-8/22 — 100%. Other isolates of this group differed by single nucleotide substitutions. In particular, C was present at position 351 of the CMV-34/21 isolates, and T was present in the CMV-13/23 and CMV-8/22 isolates. Among the analysed isolates, the strains with thymine in this position predominate, cytosine is present only in the representative of group IA strain CMV-legume (Japan). An A→T transversion was observed at position 713 of isolate 34/21. This substitution is unique, since all other strains and isolates have adenine at this position. CMV-34/21 isolates have A at position 685, while CMV-13/23 and CMV-8/22 isolates have G. In other strains of this group, both nucleotides are found at this position, in particular, in closely associated with Ukrainian isolates of the strains ABI, SD and K — A is located here, but in the strains Vir, 16, T35 — G.

We also compared the amino acid sequences of the Ukrainian isolates. The amino acid sequences do not differ between representatives of the same group and members of different groups, i.e. all substitutions in the nucleotide sequences of the capsid protein gene are synonymous.

Based on the above, it can be concluded that the CMV population in Ukraine includes representatives of the groups IA and IB, and the isolates of both groups IA and IB that we

have identified show a high degree of genetic homogeneity.

It is possible that CMV from the above-mentioned countries entered our country with virus-infected seeds. Therefore, it is necessary to carry out careful control and certification of seeds when importing them to Ukraine.

Conclusions

As a result of the study, the antigens of cucumber mosaic virus were detected in the plants of *Cucurbitaceae* family in Ukraine. The virus-infected plants were found in agrocenoses of Vinnytsia, Odesa, Poltava, Rivne and Cherkasy regions. The amplicons of the capsid protein gene region of CMV isolates were obtained. It was found that the obtainted isolates of CMV from the plants of family *Cucurbitaceae* are represented in the cluster of group I of subgroups IA and IB. The most similar to these Ukrainian isolates were: strains T35 and T19 (China), strain I17F (France) and banana (Israel).

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Моніторинг та філогенетичний аналіз ізолятів Cucumber mosaic virus, виділених з рослин родини C*ucurbitaceae* в Україні

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Мета. Провести моніторинг та філогенетичний аналіз виділених ізолятів вірусу огіркової мозаїки з агроценозів України. **Методи.** Імуноферментний аналіз, ЗТ-ПЛР, сиквенування та філогенетичний аналіз. **Результати.** Було обстежено агроценози різних регіонів України та відібрано рослини родини *Cucurbitaceae* із наявними проявами вірусних хвороб. За допомогою імуноферментного аналізу перевірено 131 зразок рослин родини *Cucurbitaceae* та виявлено антигени вірусу огіркової мозаїки (CMV). У результаті проведення ЗТ-ПЛР було отримано продукти ампліфікації розміром 500 п. о., що відповідає за розміром послідовності гену капсидного білка вірусу огіркової мозаїки. За результатами даних сиквенсу побудовано філогенетичне дерево виділених ізолятів CMV. **Висновки.** Встановлено, що українські ізоляти CMV, виділені з рослин родини *Cucurbitaceae,* представлені лише у кластері групи I підгрупи IA і IB. Найбільш спорідненими до українських ізолятів виявилися штами T35 і T19 (Китай), штам I17F (Франція) та banana (Ізраїль).

Ключові слова: Bromoviridae, вірус огіркової мозаїки, *Cucumber mosaic virus* (CMV), ген капсидного білку, філогенетичний аналіз.

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