ACTA MICROBIOLOGICA BULGARICA

Phylogenetic Analysis of Ukrainian Isolates of RNA Viruses of Plants

Irena G. Budzanivska, Tetiana P. Shevchenko, Oleksii V. Shevchenko, Valerii P. Polishchuk
Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

Abstract

The availability of analytical approaches allowing fast deciphering of nucleotide sequences enables phylogenetic analysis of viruses and establishing homology among their strains/isolates for unveiling the history of virus evolution.

This work was focused on phylogenetic analysis of the most important viruses infecting vegetable crops in Ukraine. 'Field' isolates of different plant RNA viruses were collected and analyzed from various agroecosystems. These included Potato virus Y (PVY), Cucumber mosaic virus (CMV), Watermelon mosaic virus 2 (WMV-2), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV). Using MEGA6 software, phylogenetic trees were constructed, based on sequenced viral cDNAs corresponding to parts of the coat protein genes of the studied pathogens. Differing evolutionary trajectories were shown for some of the Ukrainian virus isolates.

The study of the molecular properties of viruses, and especially those of viral genomes, has fundamental importance (tracing phylogenetic relationships of a given virus with other members of the kingdom, its evolutionary history and trends), and is also of practical significance, as such data may further be used both for developing highly specific PCR-based diagnostic techniques as well as for forecasting possible alterations and novel properties of circulating viruses for prevention of disease spread and minimizing the aggressiveness of viruses.

Key words: ToMV, PMMoV, CMV, PVY, WMV2, coat protein gene, phylogenetic analysis

Резюме

Възможността за бързо разчитане на нуклеотидните последователности позволява филогенетичен анализ на вирусите и установяване на хомоложност между техните щамове и изолати с цел разкриване на вирусната еволюция. Дейността в представения материал е фокусирана към филогенетичния анализ на важни вируси, заразяващи овощни, зеленчукови и житни култури. Изследвани са изолати на вируси, съдържащи РНК, които са събрани в различни агроекосистеми. Изолатите са на причинителите на: зелената краставична мозайка (cucumber green mottle mosaic virus - CGMMV), сливовата шарка (plum pox virus - PPV), ипсилон вирозата по картофа (potato virus – Y, PVY), Х-вирозата по картофа (potato virus X, PVX), ябълковата мозайка (apple mosaic virus - ApMV), ябълковите стъблени вдлъбнатини (apple stem pitting), ябълковото стъблено набраздяване (apple stem grooving virus), ябълковите хлоротични листни петна (apple chlorotic leaf spot virus - ACLSV), краставичната мозайка (cucumber mosaic virus - CMV), динената мозайка – 2 (watermelon mosaic virus – 2, WMV - 2), жълтата мозайката по тиквичките - зучини (zucchini yellow mosaic virus - ZYMV), доматената мозайка (tomato mosaic virus - ToMV), пиперовото умерено прошарване (paper mild mottle virus - PepMMV) и пшениченото вджуджаване (wheat dwarf virus - WDV).

Чрез MEGA5 софтуер са съставени филогенетичните дървета на основата на секвенирани кДНК-и към части на кодиращите обвивния протеин гени на изучаваните патогени. Посочени са различни еволюционни траектории за някои украински вирусни изолати. Проучването на вирусите на молекулно ниво и по-специално на вирусните геноми (проследяването на родството на даден вирус с други представители на вирусното царство, неговата еволюционна история и насока на развитие) има и практическо значение. Например за диагностиката могат да бъдат развити високоспецифични PCR–базирани техники. Също така могат да бъдат предсказани изменения и нови свойства в циркулиращите вируси, за да бъде предотвратено разпространяването на болестите и намаляване на агресивността на вирусите.

Introduction

During recent years, plant virologists have witnessed an increased interest in the advance of knowledge at the level of population and similar ecology-oriented research. This trend is common for many traditional molecular biology studies. The development of a new generation of diagnostic methods for plant viruses (such as ELISA, RIA, RIPA, PCR, RT-PCR, etc.) during the last decade enabled a new level of studying the spread of plant viruses in the environment.

The relevance of this question lies in both its fundamental and practical significance. The determination of virus spread, mechanisms of virus transmission, natural range of host plants, and research on virus response to environmental changes makes it possible not only to more fully characterize a given representative of the Vira kingdom, but also to predict the emergence and development of viral diseases for developing sound strategies of combating viral infections. This includes search for resistant varieties, control of virus reservoirs and carriers, obtaining virus-free planting material, etc.

In Ukraine, major vegetable crops (such as tomato, cucumber, sweet pepper, pumpkin, squash, etc.) are mainly suffering from viral infections induced by Potato virus Y (PVY), Cucumber mosaic virus (CMV), Watermelon mosaic virus 2 (WMV-2), Tomato mosaic virus (ToMV), Pepper mild mottle virus (PMMoV), and these viruses were studied in the context of spread, relevance, biological and molecular properties, as well as phylogenetic relationships.

At present, ToMV is widespread in Ukraine and remains the point of interest due to the harm it causes to crops, particularly tomato and some other plants of the *Solanaceae* family (Silva *et al*., 2011; Virus taxonomy, 2012). Until now, there has been no data on the phylogeny of ToMV isolates circulating in Ukraine.

The same is true for PVY and CMV infecting a wide range of cultivated and wild growing plants, both in open field and glasshouse conditions. Previously, CMV isolate of subgroup II was detected in infected pumpkin plants from Ukraine (Zitikaitė *et al*., 2011). Subgroup attribution has been established based on the RT-PCR product size. However, sequences of these isolates have not been obtained and their comparison with other known strains and isolates has not been performed. In the current work, we also proceeded with strain attribution of Ukrainian isolates of CMV based on phylogenetic analysis of the partial sequences of the coat protein gene.

In Ukraine, WMV-2 has earlier been found on plants (mostly marrow, pumpkin, zucchini, melon and cucumber) cultivated in open field conditions only. In addition to monoinfection, Watermelon mosaic virus 2 often circulates in mixed infections, typically induced by Cucumber mosaic virus (CMV), Zucchini yellow mosaic virus (ZYMV) and Cucumber aphid-born yellows virus (CABYV). Our previous results indicated that WMV-2 circulation in Ukraine showed some sort of cyclicity and irregularity. As Watermelon mosaic virus 2 is spread worldwide, it is of importance to analyze the homology of its Ukrainian isolates with reported WMV-2 isolates and strains, and to elucidate the possible ways of appearance of the Ukrainian isolates.

Pepper mild mottle virus (PMMoV) has only been discovered in Ukraine three years ago and was probably introduced with plant/seed material. Therefore, its phylogenetic properties have remained unknown but are highly relevant due to the epidemiological significance of the pathogen.

Considering all these issues, in the present study we focused on the phylogenetic analysis of the most important viruses infecting vegetable crops in Ukraine, which have still remained unexplored in evolutionary terms.

Materials and Methods

Plant samples were collected from different regions of Ukraine. The symptomatic samples were screened for the presence of viral antigens. Double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) was conducted using commercial test systems of Loewe (Germany). Plant material was homogenized in 0.1M phosphate buffered saline (PBS), pH 7.4, 1:2 (m/v). Plant components were removed by centrifugation at 5.000 g for 20 minutes at +4˚C using centrifuge PC-6. The supernatant was used for further ELISA. DAS-ELISA was performed according to the manufacturer's recommendations. The results were checked at the wavelength of 405/630 nm using Termo Labsystems Opsis MR microplate reader (USA) with Dynex Revelation Quicklink software (Crowther, 1995).

Total RNA was extracted from naturally infected plant samples using RNeasy Plant Mini kit (Qiagen, UK). The results were confirmed by electrophoresis of nucleic acids in 1.5% agarose gel.

For CMV, a two-step reverse transcription reaction (RT-PCR) was accomplished using two specific primers complementary to the coat protein gene and producing the amplicon with expected

size of 500 bp (Bariana *et al*., 1994):
forward primer –
5' TATGATAAGAAGCTTGTTTCGCGCA-3´;
reverse primer –
5' TTTTAGCCGTAAGCTGGATGGACAACCC-3´.

For ToMV, specific primers covering the coat protein (CP) gene were used (product size – 700bp) (Letschert *et al.*, 2002):
forward primer –
5' CGGAAGGCCTAAACCAAAAAG-3´;
Tob-Uni1 primer –
5' ATTTAAGTGGAGGGAAAAACACT-3´.

For PMMoV, we used primers specific to a part of the coat protein gene (product size of 387 bp) (Jarret *et al.*, 2008):
forward primer -
5' TAC TTC GGC GTT AGG CAA TC-3´,
reverse primer –
5' GGA GTT GTA GCC CAG GTG AG-3´.

PVY-specific primers covering the coat protein (CP) gene were used (product size – 569 bp) (Budzanivska *et al.*, 2014):
(As) 5'- CAAACCATAAGCCCATTCATC-3'
(S) 5'- GCACCA AATCAG GAG ATT CTA CT-3'.

For WMV2, primers specific to the CP gene of genomic RNA of French strain of WMV2 (Zohren, 2011) were used (product size – 825bp):
forward primer –
5´GAATCAGTGTCTCTGCAATCAGG-3´;
reverse primer –
5´ATTCACGTCCCTTGCAGTGTG-3´.

Purified amplicons were sequenced using Applied Biosystems 3730x1 DNA Analyzer with Big Dye terminators, version 3.1 (Applied Biosystems, USA).

The aligned sequences of the parts of the coat protein gene of the respective virus isolates were compared with published sequences of virus strains available in the GenBank database using NCBI/BLAST (http://www.ncbi.nlm.nih.gov/). The phylogenetic analysis was conducted using MEGA 5 (Tamura *et al.,* 2011). The phylogenetic trees were constructed using the Neighborhood Joining and Maximum Likelihood methods (Kimura, 1980; Felsenstein, 1982).

Results and Discussions

ToMV

Comparison of 480bp-length CP sequences using NCBI/BLAST (http://www.ncbi.nlm.nih.gov) showed that the obtained isolate (ToMV-ukr3) shares the highest identity with the group of tobamoviruses mainly infecting solanaceous plants (Tomato mosaic virus, Tobacco mosaic virus (TMV), Tomato mottle mosaic virus (ToMMV), Pepper mild mottle virus (PMMoV)). The criteria for *Tobamovirus* species differentiation determine that less than 90% nucleotide sequence identity is considered a new species (Virus taxonomy, 2012). The Ukrainian isolate showed minor differences and revealed 96-99% nucleotide identity with different ToMV strains, whereas ToMV-ukr3 shared much less identity with TMV (74-79%), PMMoV (71-73%), and ToMMV (85%). These results confirmed the attribution of the obtained isolate to ToMV.

A phylogenetic tree was built using Maximum Likelihood method Mega 6 software (Fig. 1).

On the ML tree, the Ukrainian isolate and all strains under study excepting strain ToMV-A356 were clustered together. They shared a common ancestor and close phylogenetic relationships. High homology percentage values were determined between these sequences: 98-99%. The Ukrainian isolate grouped with strains ToMV-1-2, ToMV-G26 and ToMV-G6, homology values with them were approximately 99% at nucleotide level. Strain ToMV-A356 represents the lowest homology percentage (96%) with ToMV-ukr3. Tobamoviruses demonstrate strong correlation with their angiosperm hosts (Stobbe *et al.,* 2012) supporting the suggestion that this low rate of homology could be explained by the fact that strain ToMV-A356 was extracted from *Centaurea sp*., member of the *Asteraceae* family.

PMMV

Until today, 13 strains of Pepper mild mottle virus, a novel and emerging pathogen in Ukraine, have been found to infect different hosts in various countries (Li *et al.,* 2013). Here, we phylogenetically analyzed two isolates of PMMoV found in sweet pepper and tomato based on their CP gene sequence. We obtained cDNA of expected size of 387 bp, which was further used for sequencing and construction of a phylogenetic tree (Fig. 2). The phylogenetic tree was constructed using the Neighbor-Joining method.

Obviously, the Ukrainian isolates of PMMoV collected from sweet pepper and tomato belong to the cluster composed of viruses found in Spain and Japan. Both Ukrainian isolates of PMMoV were highly homologous to each other (>99%) and thus belonged to the same strain.

CMV

A phylogenetic tree was constructed using aligned nucleotide sequences of the CP gene of various CMV strains isolated from different coun-

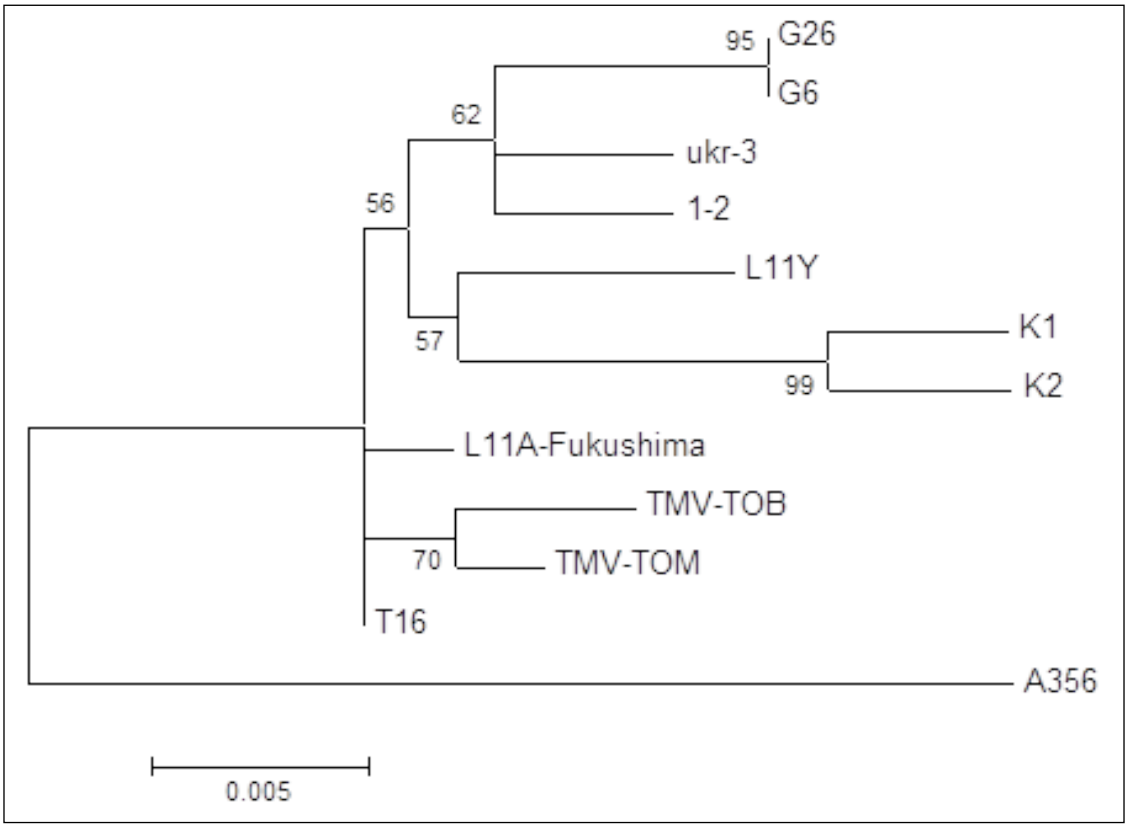

Fig. 1. Phylogenetic tree based on entire coat protein gene sequence of selected ToMV strains and Ukrainian isolate

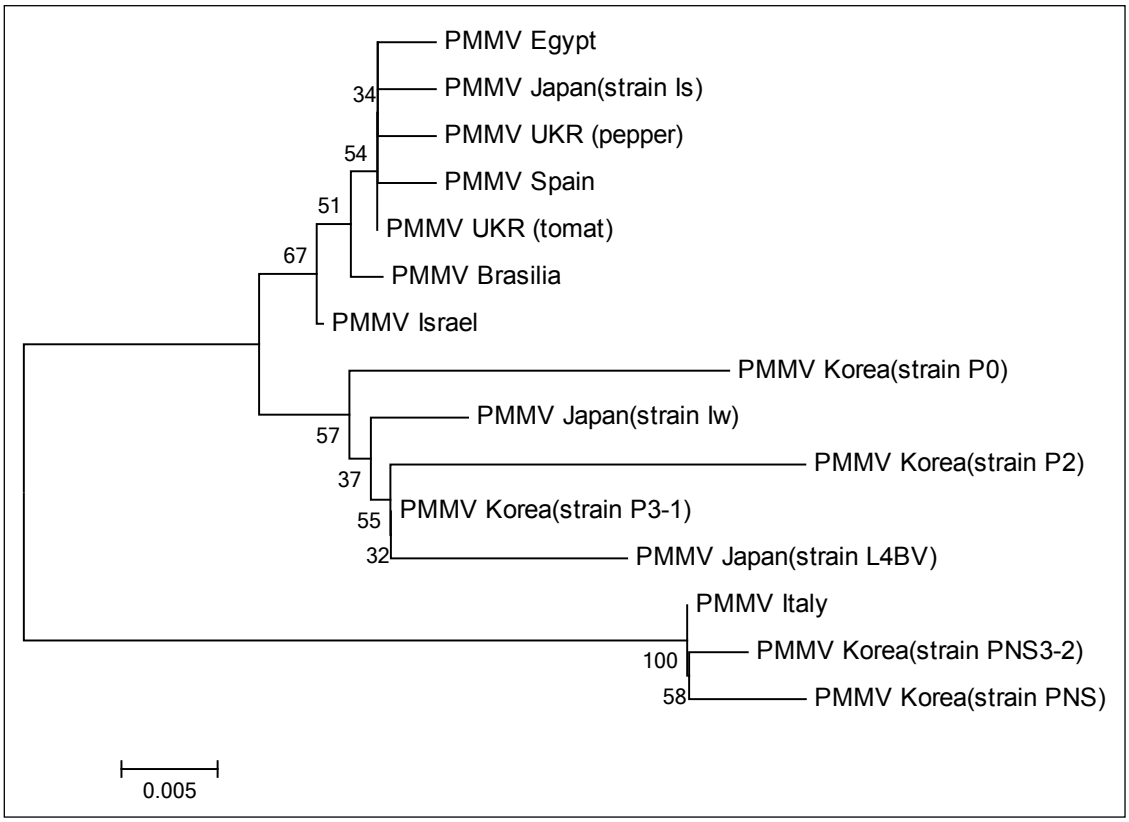

Fig. 2. Phylogenetic tree (NJ) showing evolutionary relations of Ukrainian isolate of PMMoV with published virus sequences (based on the coat protein gene)

tries and Ukrainian isolates. Phylogenetic analysis indicated three distinct clusters corresponding to subgroups IA, IB and II (Fig. 3).

CMV strains belonging to subgroup II formed a different cluster on the phylogenetic tree, well separated from the members of subgroup I. Within subgroup I, Ukrainian CMV isolates showed close phylogenetic relationships rather with the members of subgroup IB than IA, which formed a separate cluster.

Ukrainian isolates shared 79-99% nucleotide homology with the strains reported from all over the world (Table 1). According to literature data, members of the same group share more than 90% homology. The homology between strains from I and II subgroups was approximately 69-77% (Palukaitis and García-Arenal, 2003). The nucleotide sequences shared 92-94% similarity among IA and IB subgroup strains (Kumari *et al.,* 2013). The Ukrainian isolates were phylogenetically most related to each other and to the members of subgroup IB (>90% nucleotide homology). However, they were distinct from the subgroup IA and subgroup II strains. The members of subgroup II showed the lowest nucleotide homology (79-82%) with the studied Ukrainian isolates of CMV.

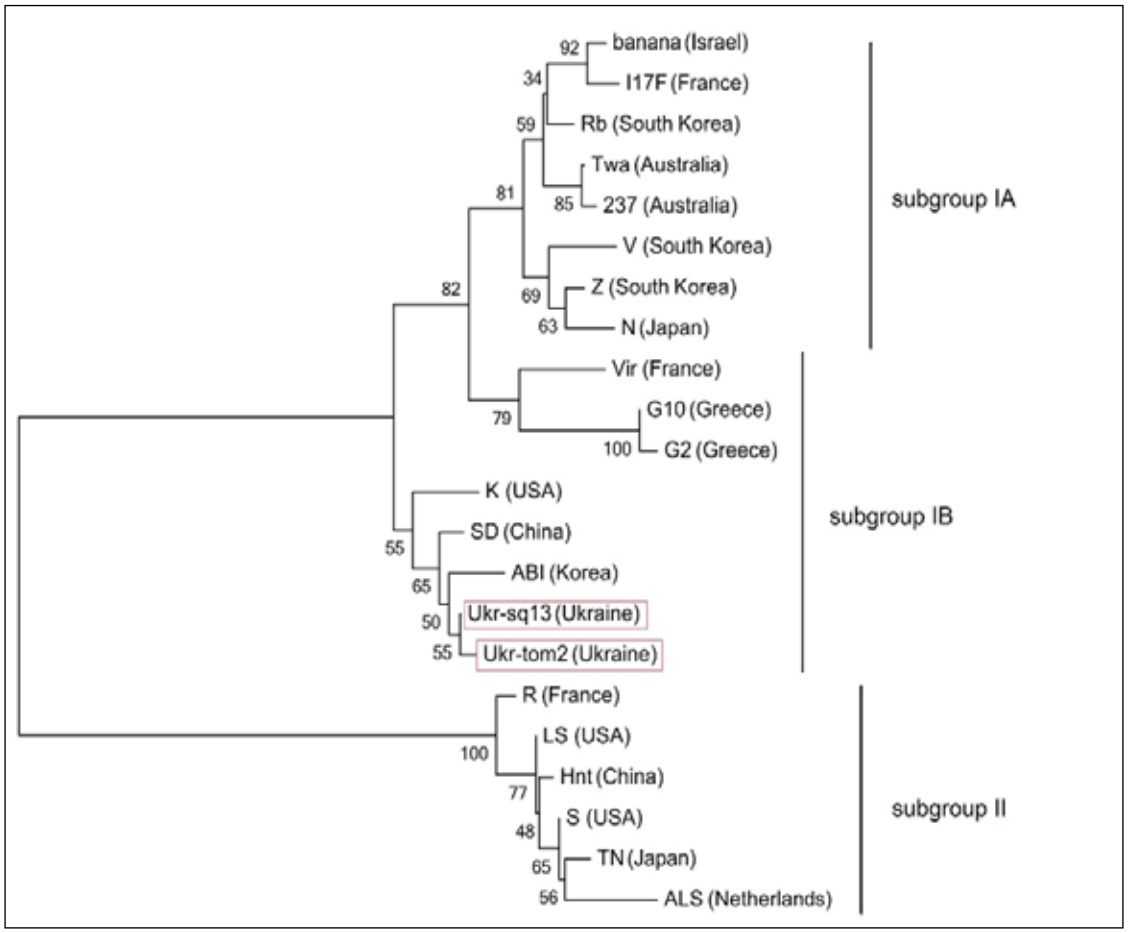

Fig. 3. Phylogenetic tree based on partial coat protein gene sequence of selected strains and Ukrainian isolates of CMV. Bootstrap values are shown above branches

The phylogenetic analysis of partial sequences of the CP gene of Ukrainian isolates of CMV revealed their highest homology and close relationships with strains ABI and SD from Korea and China. They shared approximately 98-99% homology. The infrequent amino acid substitutions revealed the high similarity in this gene region. The protein sequence of ABI strain is different from those of Ukr-sq13, Ukr-tom2, and SD, and has two amino acid substitutions (YA – IT corresponding to 601, 602 positions). Sequence comparison of Ukranian isolates showed their high similarity. Nucleotide analysis confirmed two synonymous nucleotide substitutions in positions 1612 (T (Ukr-sq13) – C (Ukr-tom2)) and 1946 (G (Ukr-sq13) – A (Ukr-tom2)). However, we were unable to identify any amino acid substitutions when comparing two Ukrainian isolates of CMV. Based on the obtained

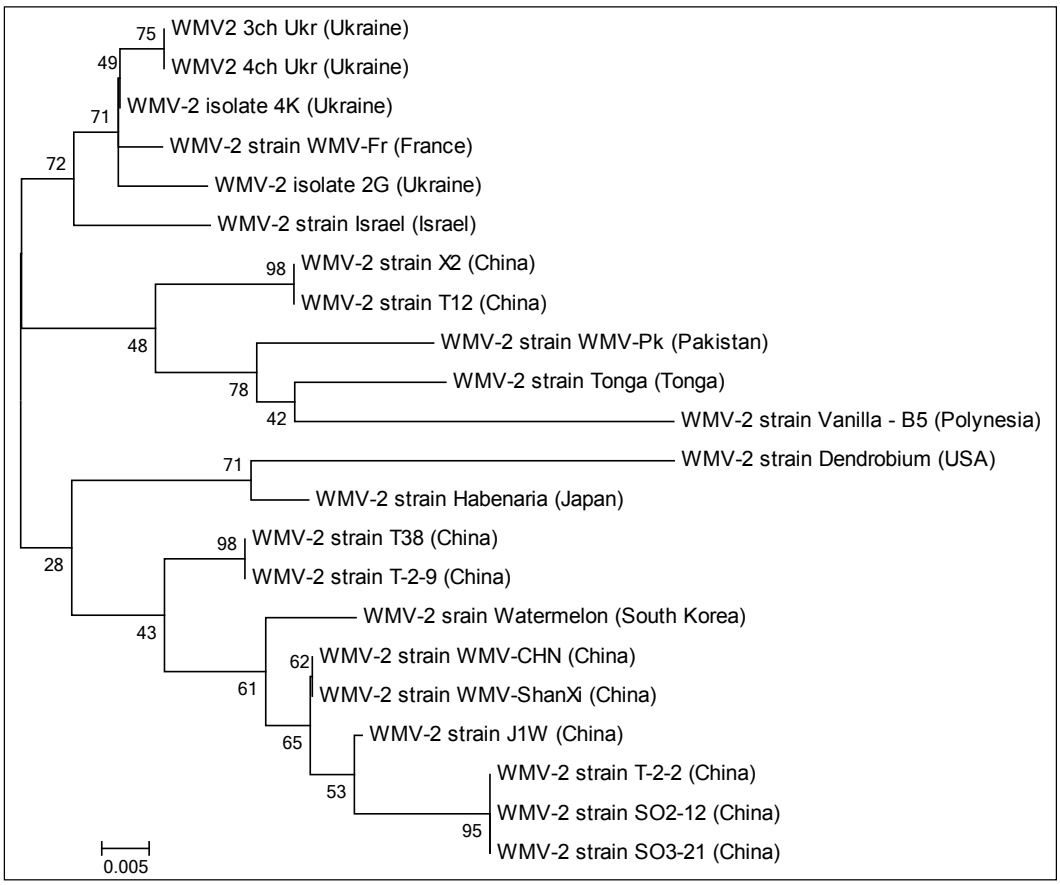

Fig. 4. Phylogenetic tree based on coat protein gene sequence of selected strains and Ukrainian isolates of WMV-2 (Maximum Likelihood, Jukes-Cantor model, 1000 bootstrap replications). Bootstrap values are shown above branches

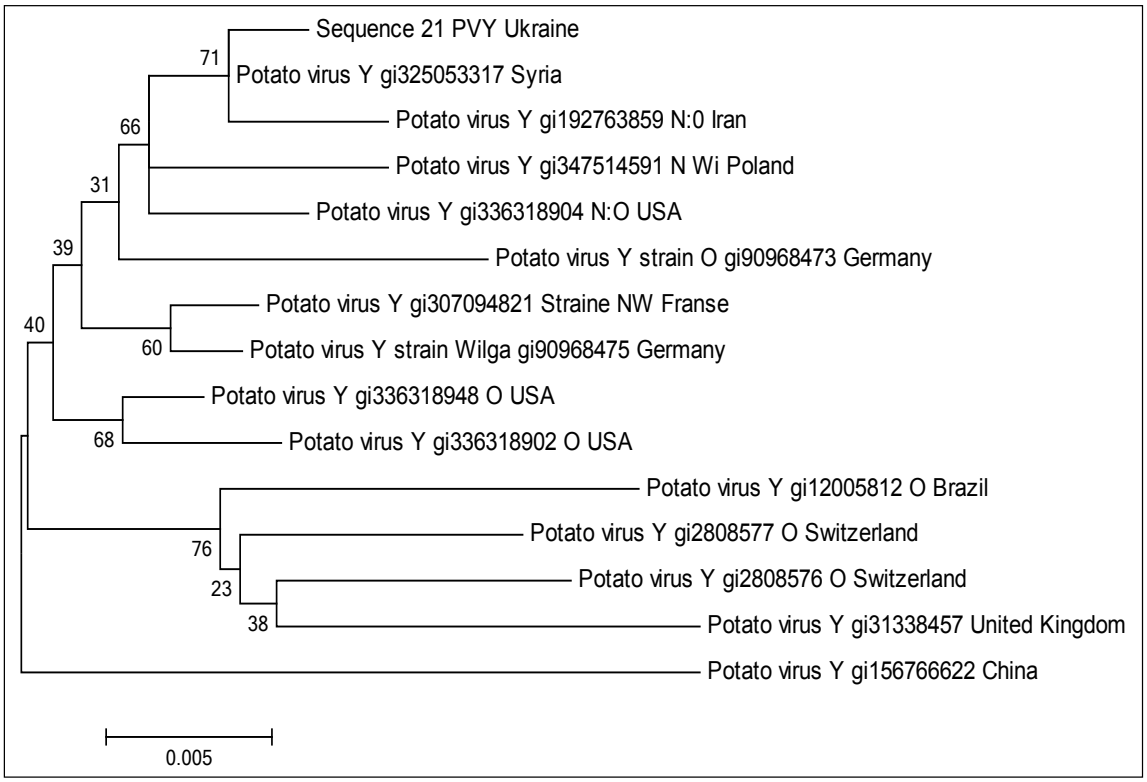

Fig. 5. Evolutionary relationships of the Ukrainian PVY isolates with PVY isolates/strains deposited in the GenBank (NCBI, USA). Evolutionary history has been reconstructed, using Neighbour Joining method (Saitou and Nei, 1987) for nucleotide sequence of the coat protein gene. Evolutionary distances were calculated using p-distance method (Tamura *et al.,* 2011) for 1000 bootstrap replications (number of base changes per site)

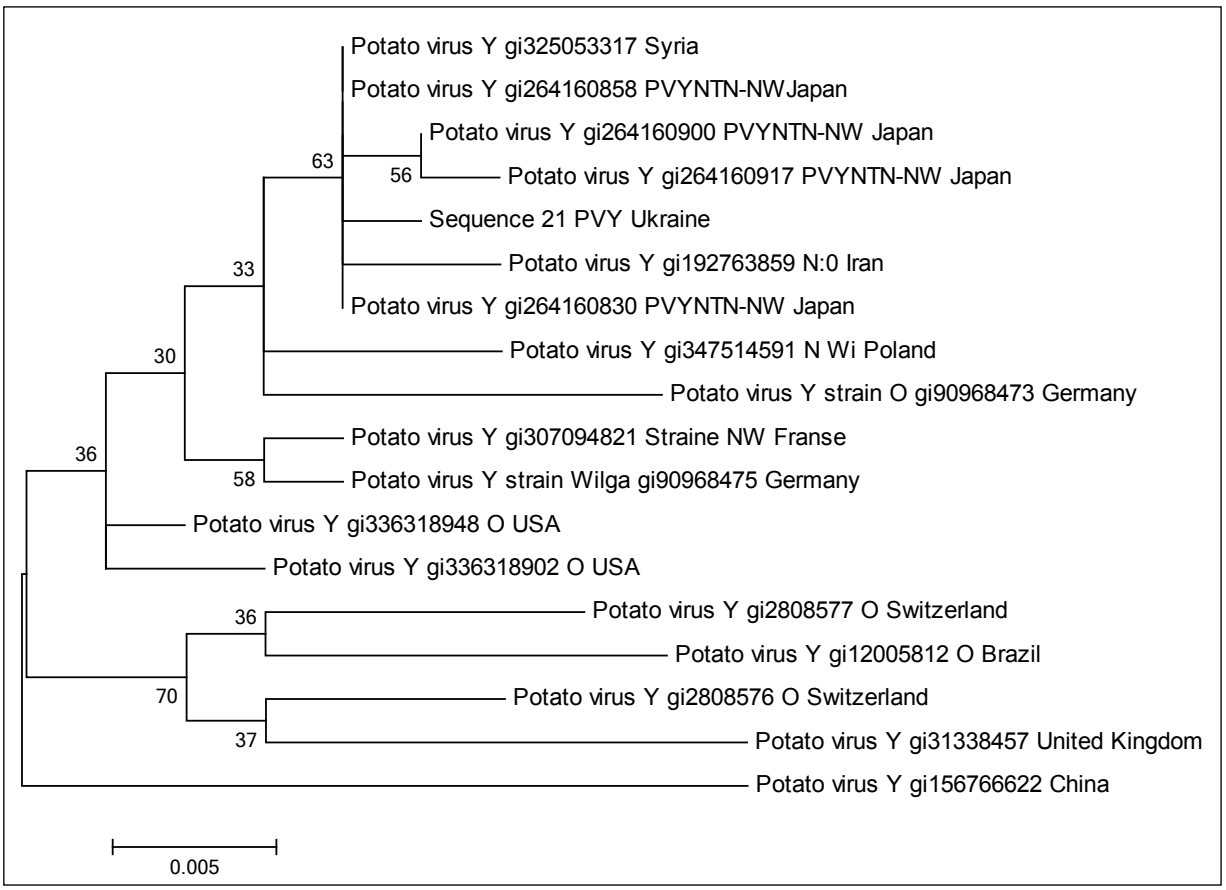

Fig. 6. Molecular phylogenetic analysis of nucleotide sequences of the PVY coat protein gene using Maximum Likelihood method (Felsenstein, 1982).

results, these Ukrainian isolates of CMV were identified as belonging to the subgroup IB. Sequence data have been submitted to NCBI, accession numbers KJ921838 and KJ921837 for Ukr-tom2 and Ukr-sq13 isolates, respectively.

WMV2

After sequencing the cDNAs of Ukrainian WMV-2 isolates, the achieved nucleotide sequences (designated WMV 2-2g-Ukr and WMV 2-2k-Ukr) were compared both among themselves and with other known strains and isolates of WMV-2.

A phylogenetic tree was built using Maximum Likelihood method Mega 6 software and Jukes-Cantor model (Fig. 4). The sequences were grouped into three separate clusters.

Evidently, Ukrainian isolates WMV2–3ch–Ukr, WMV2–4ch–Ukr, 2G, and 4K were grouped in a single cluster with a homology of 97.8-98.9%, confirming low level of variability of Ukrainian isolates of Watermelon mosaic virus 2. The Israeli strain 'Israel' and the French strain WMV-Fr belonged to the same cluster and showed a high level of homology (96.6–97.8%) with the Ukranian isolates.

The other two clusters contained WMV strains from Pakistan and the Pacific Ocean region (87.6-92.1% homology to Ukrainian isolates), and from USA, Japan, China and South Korea (89,9-93,3% homology with the Ukrainian isolates).

Therefore, the Ukrainian population of the virus is highly homologous and Ukrainian isolates are related to strains from France and Israel, which may well reflect their origin. Thus, WMV-2 may have been brought to Ukraine from the Mediterranean or Middle East regions.

PVY

RT-PCR yielded cDNA corresponding to the part of the CP gene of Ukrainian isolate of PVY. This cDNA was further sequenced and compared with known sequences of PVY isolates and strains published in the GenBank (with some of them more than 600 of such sequences are available).

These relationships are even more obvious when analyzed using phylogenetic trees constructed for nucleotide sequences of the CP employing NJ method (Fig. 5).

Interestingly, both nucleotide sequence- and amino acid sequence-based phylogenetic trees for PVY CP demonstrate higly similar (though not 100% identical) position of the Ukrainian isolate in relation to others. In both cases we can see a separate cluster, including recombinant isolates (strains)

from Syria and Iran; at the same time, PVY USA N:O and Poland N-Wi strains form another closely related small cluster.

To make a conclusion on the evolutionary history of these isolates, an ML approach was used on the basis of Tamura-Nei model. A tree with high logarithm of likelihood was constructed for 1000 bootstrap replications. Branch length corresponds to the number of changes per site.

As shown on Fig. 6, the phylogenetic tree constructed using ML method underlines that the main tendencies remain the same. It can be concluded that the Ukrainian PVY isolates and PVY isolates from Syria and Iran descended from a single ancestor.

The diffuculties with interpretation of the obtained results for Ukrainian PVY isolates had not been anticipated and thus were rather unexpected. A state-of-the-art study (2006-2013) and previous (1992-2005) literature sources confirm the absence of unanimous opinion among authors regarding the unified nomenclature for strains and isolates of PVY (in particular, those found in potato). The comparison of phylogenetic trees published by different researchers is not an easy task as (most often) different 'source' virus sequences (both nucleotide and amino acid) have been used for analysis. Apparently, the recombination among the PVY genomes may lead to the development of novel variants of the virus, which may have differing phenotype (without any significant correlation with the coding sequences). In turn, phenotypic appearances will depend on the specific species and cultivar of the virus-infected plant.

As far as our work has not been aimed at studying phenotypic variations of Ukrainian isolates of PVY, our conclusions are based exclusively on the comparative analysis of nucleotide sequences in the virus genome (the coat protein gene of PVY). Phylogenetic relationships based on the CP gene of PVY and explored using the ML method may be used as an indirect index of relationships for full-size genomes of potyviruses (Roossinck, 1997) and for establishing species of viruses for which only CP gene sequences are available.

Our conclusions regarding close relationships between the Ukrainian PVY isolate and isolates from Syria and Iran, and about their putative common origin is strongly supported by the fact that these isolates are closely positioned in the other phylogenetic trees (PVY_Siria, PVY_Iran, PVY_China 2011). Following this lead and according to Chikh-Ali *et al.* (2007), it is viable to suggest that the Ukrainian isolate of PVY belongs to the strain group O, subclade N:O (using another isolate classification proposed by Saitou and Nei, 1987) – to the subclade O N-Wilga N:O.

Conclusion

From the current work it can be inferred that the Ukrainian isolate of ToMV is not an emergent strain able to break the resistance in plants (Yamamoto *et al.,* 2002). Thus, known strategies for control of tomato diseases caused by ToMV may be applied. Phylogenetic analysis of cDNA corresponding to the CP gene of PMMoV indicates that it belongs to clusters composed of viruses found in Spain and Japan.

Despite the supposed high rate of CMV evolution and adaptation (Roossinck, 2002), phylogenetic analysis of two Ukrainian isolates of CMV reveals that their homology exceeds 95%, i.e. that these isolates belong to the same strain. In our opinion, it is of special interest as these isolates have been found in totally different host plants, tomato and squash, which belong to distant families.

Another important phylogenetic finding is that Ukrainian isolates of CMV are attributed to the subgroup IB of CMV strains. The Ukrainian isolates are mostly related to strains ABI and SD from Korea and China, respectively. Initially, the members of subgroup IB were found in (and thought to be restricted to) East Asia. Later on, subgroup IB strains of CMV were shown as widespread in Iran (Arafati, *et al.,*2013), although subgroup IA isolates were also detected (Nematollahi *et al.,* 2012). The occurrence of subgroup IB isolates of CMV suggests that they may have been introduced in Ukraine either by seed material or through exported fresh food products. In addition, we cannot reject the possibility of virus 'naturally migrating' from Iran (or other neighboring regions) into Ukraine as transported by aphids or birds.

Strangely, we have not found subgroup IA isolates of CMV in Ukraine. These are more virulent strains and isolates, which are considered to be common worldwide, including Europe. According to previous research data (Zitikaitė *et al*., 2011) and our findings, the CMV population consists of isolates of II and IB subgroups in Ukraine. Having registered severe symptoms on collected plants, we expected that they were induced by a virulent 'form' of the virus. Surprisingly, both isolates fall into subgroup IB strains of CMV. In the view of aforesaid, we deem that the obtained results rather reflect the lack of CMV monitoring in Ukraine than

reveal atypical virus spread in the region. The data also indicate that the severity of the virus-specific symptoms in field conditions may not be directly related to the degree of virulence of a given virus isolate, requiring more research of CMV biology, epidemiology and evolution.

Phylogenetic analysis of WMV-2 based on its CP gene indicates that the Ukrainian population of the virus is highly homologous and that Ukrainian isolates are related to strains from France and Israel, which may well reflect their origin. Thus, WMV-2 may have been brought to Ukraine from Mediterranean or Middle East regions (or vice versa).

Phylogenetic analysis of nucleotide sequence of the CP gene of the Ukrainian PVY isolate demonstrates that it is a recombinant virus capable of phenotypic appearances typical of both ordinary (O) strain and necrotic (N) strain, depending on the specific conditions of a plant virus infection development. The Ukrainian isolate of PVY has a significant mutational (recombinational) potential and in the future may therefore change its biological properties (in terms of the host range, for instance). This information may be of use for potato breeders as employment of wild potato varieties and its relatives in selection programs may induce unexpected 'response' of different PVY strains in new cultivars.

The established trend of low genetic variability of viruses, despite the high mutational ability of RNA viruses, indicates the impact of negative selection to maintain the stability of viral nucleotide sequences. Analysis of the phylogenetic trees topology shows the direction of isolation and low probability for the formation of a Ukrainian strain with new properties. Our data have shown high genetic conservatism for most populations of plant viruses that favors the modern hypothesis of evolution for plant RNA viruses. A high relationship level between all Ukrainian isolates has been revealed, indicating their high homogeny.

Acknowledgements

The authors are grateful to Dr. I.I. Boubriak for his invaluable support and technical assistance.

References

Arafati, N., S. Farzadfar, R. Pourrahim (2013). Characterization of coat protein gene of Cucumber mosaic virus isolates in Iran. *Iran. J. Biotech*. **11**: 109-114.

Bariana, H. S, A. L. Shannon, P. W. Chu, P. M. Waterhouse (1994). Detection of five seedborne legume viruses in one sensitive Multiplex polymerase chain reaction test. *Phytopathol.* **84**: 1201-1205.

Budzanivska, I. G., L. P. Ovcharenko, A. V. Kharina, I. I. Boubriak, V. P. Polischuk (2014). Nucleotide and amino acid sequences of a coat protein of an Ukrainian isolate of *Potato virus Y*: comparison with homologous sequences of other isolates and phylogenetic analysis. *Biopolym. Cell.* **30**: 141-148.

Chikh-Ali, M., T. Maoka, K. T. Natsuaki (2007). The occurrence and characterization of new recombinant isolates of PVY displaying shared properties of PVYNW and PVYNTN. *J. Phytopathol.* **155**: 409-415.

Crowther, J. R. (1995). ELISA. Theory and practice. Humana Press, p. 223.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**: 368-376.

Jarret, R. L., A. G. Gillaspie, D. L. Pinnow, N. A. Barkley (2008). The occurrence and control pepper mild mottle virus (PMMoV) in the USDA/ARS *Capsicum* germplasm Collection. *Seed Technol.* **30:** 27-36.

Kimura, M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol*. **16**: 111-120.

Kumari, R., P. Bhardwaj, L. Singh, A. Zaidi, V. Hallan (2013). Biological and molecular characterization of Cucumber mosaic virus subgroup II isolate causing severe mosaic in cucumber. *Indian J. Virol*. **24**: 27-34.

Letschert, B., G. Adam, D. E. Lesemann, P. Willingmann, C. Heinze (2002). Detection and differentiation of serologically cross-reacting tobamoviruses of economical importance by RT-PCR and RT-PCR-RFLP. *J. Virol. Methods.* **106**: 1-10.

Li, R., S. Gao, Z. Fei, K. S. Ling (2013). Complete genome sequence of a new tobamovirus naturally infecting tomatoes in Mexico. *Genome Announc.* **1**: 1-2.

Moradi, Z. (2011). Diagnosis and molecular variability of Watermelon mosaic virus isolates from North, East, North-East, and North-West regions of Iran. *Asian J. Plant Pathol.* **5**: 115-125.

Nematollahi, S., N. Sokhandan-Bashir, F. Rakhshandehroo, H. R. Zamanizadeh (2012). Phylogenetic analysis of new isolates of Cucumber mosaic virus from Iran on the basis of different genomic regions. *Plant Pathol. J*. **28**: 381-389.

Palukaitis, P., F. García-Arenal (2003). Cucumoviruses. *Adv. Vir. Res*. **62**: 242-323.

Roossinck, M. J. (2002). Evolutionary history of cucumber mosaic virus deduced by phylogenetic analyses. *J Virol*. **76**: 3382-3387.

Roossinck M. J.(1997). Mechanisms of plant virus evolution. *Annu. Rev. Phytopathol*. **35**: 191-209.

Saitou, N., M. Nei (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406-425.

Silva, P. P., R. A. Freitas, W. M. Nascimento (2011). Detection of tomato mosaic virus in tomato seed and treatment by thermotherapy. *Acta Hortic.* **917**: 303-308.

Stobbe, A. H., U. Melcher, M. W. Palmer, M. J. Roossinck, G. Shen (2012). Co-divergence and host-switching in the evolution of tobamoviruses. *J. Gen. Virol.* **93**: 408-418.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**: 2731-2739.

Virus taxonomy: Classification and Nomenclature of Viruses. (2012).. In: *King, A. M. Q,. E. Lefkowitz, M. J. Adams, E. B. Carstens* (eds) Ninth Report of the International Committee on Taxonomy of Viruses. London: Academic Press, pp. 1327.

Yamamoto, H. A., T. Abe, K. Ueda, M. Inoue, T. Matsumoto (2002). Missense mutation in tomato mosaic virus L11A-Fukushima genome determines its symptomless systemic infection of tomato. *J. Gen. Plant Pathol.* **68**: 385-389.

Zitikaitė, I., J. Staniulis, L. Urbanavičienė, M. *Žižytė (*2011). Cucumber mosaic virus identification in pumpkin plants. Žemdirbystė-*Agriculture*. **98**: 421-426.