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Phylogenetic relationships of two Ukrainian tomato isolates of potato virus M and genetic variability analysis of its population

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Summary. – The aim of this study was to investigate biological and molecular properties of two Ukrainian tomato isolates of potato virus M (PVM), K-16 and Pol-14, to determine their phylogenetic relationships and the genetic variability of PVM isolates. Study of phylogenetic relationships of two Ukrainian tomato PVM isolates with 35 isolates represented in GenBank was conducted. It was found that the coat protein (CP) gene sequence identity between two Ukrainian PVM isolates is 94.3% at the nucleotide level and 100% at the amino acid level. The highest level of the sequence identity (97.0% and 96.5% nt and 100% aa) have the isolates K-16 and Pol-14 with the German potato isolate DSMZ PV0273, Indian potato isolates Del 123, Del 134, Del 147, M 34 and Chinese isolate from pepino GS-6-2 (isolate K-16), which testifies about their common origin. Ukrainian tomato isolates K-16 and Pol-14 belong together with all European, Chinese, Iranian, Indian isolates to PVM-o clade or group I. It was found that the nucleotide substitutions in the capsid protein gene of all tomato PVM isolates (except the Italian) are synonymous. Analysis showed that the global dN/dS ratio for the entire CP gene sequences used in the study was 0.041 ($p < 0.01$). Pairwise comparisons showed that the dN/dS values were also significantly lower than 1 for all of CP sequence pairs that testifies that the PVM CP gene experiences a negative (purifying) selection pressure.

Keywords: potato virus M; Solanum lycopersicum; phylogenetic analysis; genetic variability; selection pressure

Introduction

Development of modern approaches based on genome sequencing provides an opportunity to investigate the nucleotide sequences of the genomes of plant viruses and mathematically reliably establish degree of their relationship, making conclusions about their evolution (Garcıa-Arenal *et al.*, 2003). Due to high rates of replication and mutation, and large populations, RNA viruses have an abundant genetic variability in their natural populations (Lauring *et al.*, 2013). Knowledge of the population structure of viruses will help us to understand such important features of their biology as changes in geographical range, phylogenetic relationships, epidemiological routes, the underlying evolutionary mechanisms of RNA viruses, and their emergence as new epidemics (Ge *et al.*, 2014); this understanding will be essential for designing strategies for controlling viruses (Acosta-Leal *et al.,* 2011).

The demarcation criteria for species delineation in the genus *Carlavirus* includes serological relatedness, host range and sequence identity of the CP gene (Adams and Antoniw, 2004; Mahy and Van Regenmortel, 2010). Sequence analysis has shown that members of distinct species of carlaviruses share less than 72% nucleotide sequence identity (or 80% amino acid sequence identity) in their entire CP genes. Currently, no reliable criteria have been specified for strain differentiation within a single carlavirus

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Abbreviations: CP = coat protein; PLRV = potato leafroll virus; PVM = potato virus M; PVS = potato virus S; PVX = potato virus X; PVY = potato virus Y; TMV = tobacco mosaic virus

species; however, it has been suggested that different strains belonging to the same carlavirus species share 75–90% identity in the core region of their CPs (excluding the N and C termini) (Adams and Antoniw, 2004; Mahy and Van Regenmortel, 2010). So far, the population dynamics of carlaviruses has not been studied extensively. Different isolates of potato virus S (PVS), which is closely related to PVM, have been classified into two biologically different strains. Interestingly, it was found that this differentiation is reproducible by phylogenetic analysis of the PVS CP gene (Cox and Jones, 2010; Xu *et al.*, 2010; Salari *et al.*, 2011; Tabasinejad *et al.*, 2014). This finding confirmed that the CP region is a suitable genomic region to study the genetic diversity of carlaviruses.

Recent studies of PVM genetic diversity showed that PVM isolates divided into two groups: group I, Euro-Asian, which includes tomato and potato isolates from Italy, Germany, Czech Republic, Latvia, China, India, Iran, Poland and Russia and group II (potato isolates from the USA and Canada) (Xu *et al.*, 2010). Later, Tabasinejad *et al.* (2014) named these two major clades PVM-ordinary (PVM-o) and PVM-divergent (PVM-d), respectively. It was shown that potato isolates of PVM from Iran and Czech Republic are located within both clades (Tabasinejad *et al.*, 2014; Plchova *et al.*, 2015). This suggests that the geographical origin is not the only determining factor of the phylogenetic divergence of PVM isolates into two main clades. It should be noted that these studies are devoted to phylogenetic study of potato strains of PVM. Phylogenetic analysis of tomato PVM isolates was conducted only in China (Ge *et al.*, 2012). The aim of this study was to investigate phylogenetic relationships of two Ukrainian tomato PVM isolates, establish their possible origin and genetic diversity of PVM isolates.

Materials and Methods

Samples collection and virus diagnostics. Tomato leaf samples with abaxial rolling symptoms were collected during 2014–2016 from the fields of Poltava and Kyiv regions in Ukraine.

Identification of the viruses in sap of tomato leaves was performed by DAS-ELISA. Specific antibodies against potato virus M, potato virus Y (PVY), potato virus S, potato virus X (PVX), potato leafroll virus (PLRV), and tobacco mosaic virus (TMV) (Loewe, Germany) were used. Antigen samples were prepared by grinding of leaf tissue in PBS buffer, pH 7.4, at the ratio 1:2 (w/V). Leaf samples from healthy tomatoes were also included as negative controls. Positive controls were commercial (Loewe, Germany). The results were recorded on Termo Labsystems Opsis MR reader (USA) with Dynex Revelation Quicklink software at the wavelength of 405 nm. Samples were considered positive when their absorbance values at 405 nm were at least three times higher than those of negative controls (Crowther, 1995).

Viral particle morphology was studied by transmission electron microscopy. Negative staining of virions was performed with the 2% solution of phosphotungstic acid for 2 minutes and studied by electron microscope JEM 1400 (JEOL, Japan).

Molecular analyses. Total RNA was extracted from fresh leaves using Genomic DNA purification kit (Thermo Scientific, USA) following the manufacturer's instructions. Two step RT-PCR was performed. The reverse transcription was performed using RevertAid Reverse Transcriptase - genetically modified Moloney murine leukemia virus reverse transcriptase, MMuLV RT (Thermo Scientific, USA) according to the manufacturer's instructions. Amplification was performed using thermocycler (Genetic research instrumentation LTD, Great Britain). Specific oligonucleotide primers to part of PVM CP gene were used: PVM1 (5' taactgcagatgccgtcttg 3'), PVM2 (5' tgcgatgtctttgt gcgtat 3') (Antipov *et al.*, 2007). DNA product of 276 bp was amplified. Amplification was performed in 12,5 µl of Dream Taq PCR Master Mix (2x) buffer (containing Dream Taq DNA polymerase, 2x Dream Taq buffer, 0,4 mmol/l of each dNTP and 4 mmol/l of $MgCl_2$), 7,5 µl nuclease-free water, 1 µl of each primer (10 µmol/l), and 3 µl of cDNA. The temperature regime for amplification reactions was as follows: initial denaturation for 3 min at 95°C, followed by 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The final extension was at 72°C for 10 min. PCR products were separated on a 1.5% agarose gel with DNA marker MassRuler DNA Ladder Mix ready-to-use (SM 0403, Thermo Scientific, USA), stained with ethidium bromide, and visualized under UV light. The PCR products were purified from the agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Great Britain). Sequencing of the purified amplified DNA fragments was carried out with the 3130 Genetic Analyzer (Applied Biosystems, USA).

Phylogenetic analysis. Sequences of Ukrainian PVM isolates were compared with PVM sequences in the NCBI database with the BLAST program (http:// www.ncbi.nlm.him.gov). PVM isolates used in this study are listed in Table 1. Nucleotide and amino acid sequences were aligned using Clustal W in MEGA 7 (http://www.megasoftware.net/). Phylogenetic trees for the part of coat protein gene of 2 Ukrainian PVM isolates and 35 PVM isolates from different countries were constructed by the maximum-likelihood method (ML) (Huelsenbeck and Rannala, 1997) using the best-fitting models. To check the reliability of the constructed tree we used bootstrap test with 1000 bootstrap replications. Multiple alignment of the coat protein amino acid sequences of PVM isolates was performed by EBI CLUSTALW (2.1) (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Synonymous/nonsynonymous (dN/dS) mutation ratio calculations. To calculate the dN/dS ratio, an indicator of the evolutionary direction, the CP nucleotide sequences of all PVM isolates were codon-aligned. The ratio of the rate of nonsynonymous (dN) to the rate of synonymous (dS) mutations was calculated using the Nei-Gojoboori method in the SNAP program (http://www.hiv.lanl.gov/content/ sequence/SNAP/SNAP.html).

Results and Discussion

A survey of tomato plants (*Solanum lycopersicum*) in fields in Kyiv and Poltava regions of Ukraine showed that 18 to 25% of the inspected plants demonstrated abaxial leaf rolling (Fig. 1). Plant samples with such symptoms were taken to the study. It should be noted that tomato isolates of PVM from China caused mosaics on the tomato leaves and latent infection (Ge *et al*., 2012) but didn't induce abaxial leaf rolling like in Ukraine. Italian PVM-To isolate in mixed infection with PVY induced severe stunting accompanied by rolling and distortion of the leaflets, whereas infection by PVM-To alone was symptomless (Grieco *et al.*, 1997). Previously, we have found that abaxial leaf rolling symptoms on tomato in Ukraine are caused by PVM or PVY or co-infection of both viruses (Mishchenko *et al.*, 2013).

Therefore, ELISA was performed with antibodies against PVM and PVY. Antibodies against PVS, PVX and PLRV were also used. DAS-ELISA and RT-PCR showed that all tomato samples with abaxial leafrolling symptoms selected in 2014 were infected with PVM (61/152; infected/total) or PVM+PVY (91/152). In contrast, in 2016 only PVM was detected in tomato samples with abaxial leafrolling symptoms (24/24). PVY, PVS, PVX and PLRV antigens were not detected. One sample (cv. Hurma, Kyiv region, 2016) was PVM+TMV positive. It should be noted that during our long-term studies of the nature of abaxial leaf rolling symptoms on tomatoes in Ukraine (over 10 years) this is the first detection of TMV in co-infection with PVM or PVY. Two PVM-positive samples were taken for this study: tomato plants cv. Hurma, Kyiv region, 2016 and cv. Mir, Poltava region, 2014. PVM isolates from these plant samples were named K-16 and Pol-14, respectively.

To study the morphological features of tomato PVM isolates, transmission electron microscopy method was used. It was found that virions of tomato PVM isolates K-16 and Pol-14 are filamentous, 630 ± 20 × 13 nm in size. Thus, the size and morphology of virions of Ukrainian tomato PVM isolates K-16 and Pol-14 are typical for carlaviruses and do not differ from other potato and tomato PVM isolates described in the literature (Grieco *et al.*, 1997; Adams *et al*., 2012; Mishchenko *et al*., 2013).

To investigate phylogenetic relationships of two Ukrainian tomato PVM isolates K-16 and Pol-14, DNA sequences were compared with the sequences of 35 PVM isolates and strains from GenBank (Table 1). Phylogenetic analysis of the nucleotide sequences of the part (211 nt) of CP gene (positions 7822–8032 nt) of PVM isolates K-16 and Pol-14 and 35 isolates and strains of this virus showed the highest level of the pairwise sequence identity with a number of European, Indian, Iranian and Chinese PVM isolates (Table 1). The sequence identity between two Ukrainian PVM isolates was 94.3% at the nucleotide level and 100% at the amino acid level.

Fig. 1

Abaxial leaf rolling on tomato caused by PVM

The highest level of the nucleotide sequence identity (97.0% and 96.5%) and amino acid sequence (100%) had isolates K-16 and Pol-14 with the German potato isolate DSMZ PV0273 and Indian potato isolates Del 123, Del 134, Del 147 and M 34, which testifies about their common origin (Fig. 2). It should be noted that isolate K-16 had also the highest percentage of homology (97.0% nt and 100% aa) for nucleotide and amino acid sequence, respectively, with Chinese isolate from pepino GS-6-2 (Table 1). According to the two classifications of PVM strains proposed by Tabasinejad *et al*. (2014) and Xu *et al.* (2010), Ukrainian tomato isolates K-16 and Pol-14 with all European, Chinese, Iranian, Indian isolates belong to PVM-o clade or group I, respectively (Fig. 2). Canadian isolates formed a second clade (PVM-d or group II) and had a low percentage of nucleotide sequence homology with studied isolate K-16 (70.0–72.1%) and Pol-14 (70.7–73.6%) (Fig. 2, Table 1). Such indicators are lower than the demarcation threshold for species differentiation (Adams and Antoniw, 2004; Mahy and Van Regenmortel, 2010), which testifies about the high variability of PVM population. Similar data was obtained by Tabasinejad *et al*., their result was 73.3%, as a conclusion they stated: "high intrapopulation diversity of PVM CP suggests the involvement of mechanisms other than just point mutations and may have resulted from differential host or vector selection pressure" (Tabasinejad *et al.,* 2014).

Results about possible origin of Ukrainian PVM isolates coincide with historical facts about the import of potatoes to Ukraine. According to these data, potato was

Table 1. Nucleotide and amino acid sequence identity of part of the CP gene of the Ukrainian tomato PVM isolates with isolates/strains from other countries (%)

No.	Isolate/strain name	Acc. No. in GenBank	K-16		Pol-14		Country of origin	Host	Reference
			nt	aa	nt	aa			
1	K-16	MF998090	---	---	94.3	100	Ukraine	*Solanum lycopersicum*	This study
2	Pol-14	MF998089	94.3	100	---	----	Ukraine	*Solanum lycopersicum*	This study
3.	GS-T1	KF561661	93.8	100	92.2	100	China	*Solanum lycopersicum*	Ge *et al.*, 2014
4.	GS-T8	KF561662	93.8	100	92.2	100	China	*Solanum lycopersicum*	Ge *et al.*, 2014
5.	GS-T10	KF561663	93.8	100	92.2	100	China	*Solanum lycopersicum*	Ge *et al.*, 2014
6.	Gansu	JN835299	93.8	100	92.2	100	China	*Solanum lycopersicum*	Ge *et al.*, 2012
7.	Bari	X85114	95.4	98.4	94.9	98.4	Italy	*Solanum lycopersicum*	Grieco *et al.,* 1997
8.	JL-3-9	KF561612	95.4	100	93.8	100	China	*Solanum muricatum*	Ge *et al.*, 2014
9.	YN-1-7	KF561667	96.5	100	94.9	100	China	*Solanum muricatum*	Ge *et al.*, 2014
10.	YN-2-6	KF561647	96.5	100	94.9	100	China	*Solanum muricatum*	Ge *et al.*, 2014
11.	YN-1-8	KF561645	96.5	100	94.9	100	China	*Solanum muricatum*	Ge *et al.*, 2014
12	YN-1-1	KF561642	96.5	100	94.9	100	China	*Solanum muricatum*	Ge *et al.*, 2014
13	GS-6-2	KF561638	97.0	100	93.8	100	China	*Solanum muricatum*	Ge *et al.*, 2014
14	AGF-5	KJ919964	96.5	100	96.5	100	India	*Solanum tuberosum*	GeneBank
15	Del-147	KJ462137	97.0	100	96.5	100	India	*Solanum tuberosum*	GeneBank
16	Del-134	KJ462136	97.0	100	96.5	100	India	*Solanum tuberosum*	GeneBank
17	Del-123	KJ462135	97.0	100	96.5	100	India	*Solanum tuberosum*	GeneBank
18	M-34	KF471070	97.0	100	96.5	100	India	*Solanum tuberosum*	Katiyar *et al.*, 2013
19	Russian wild	D14449	95.4	98.4	94.9	98.4	Russia	*Solanum tuberosum*	Zavriev *et al.*, 1991
20	Ar-17	EF397744	94.3	96.8	93.8	96.8	Iran	*Solanum tuberosum*	Pourrahim *et al.,* 2007
21	Kh-92	EF397743	94.3	96.8	93.8	96.8	Iran	*Solanum tuberosum*	Pourrahim *et al.,* 2007
22	Ha-64	EF397747	94.9	98.4	94.3	98.4	Iran	*Solanum tuberosum*	Pourrahim *et al.,* 2007
23	Es-34	EF397746	94.9	96.8	94.3	96.8	Iran	*Solanum tuberosum*	Pourrahim *et al.,* 2007
24	TZ:PVM12U:11	KC866622	96.5	100	95.9	100	Tanzania	*Solanum tuberosum*	Chiunga *et al.,* 2013
25	DSMZ PV0273	EU604672	97.0	100	96.5	100	Germany	*Solanum tuberosum*	Flatken *et al.,* 2008
26	Chernigov-05	DQ883806	94.9	100	94.9	100	Ukraine	*Solanum tuberosum*	Antipov *et al.*, 2007
27	Priekuli	GQ496609	96.5	100	95.9	100	Latvia	*Solanum tuberosum*	GeneBank
28	VIRUBRA 4/007	HM854296	95.4	98.4	94.9	98.4	Czech Republic	*Solanum tuberosum*	Plchova *et al.,* 2015
29	VIRUBRA 4/035	HQ005276	94.3	98.4	94.9	98.4	Czech Republic	*Solanum tuberosum*	Plchova *et al.,* 2015
30	VIRUBRA 4/016	HM991708	94.9	100	94.3	100	Czech Republic	*Solanum tuberosum*	Plchova *et al.,* 2015
31	M57	AY311395	95.9	100	94.3	100	Poland	*Solanum tuberosum*	GeneBank
32	Uran	AY311394	95.9	100	94.3	100	Poland	*Solanum tuberosum*	GeneBank
33	Ca128	EF063387	71.4	95.2	72.9	95.2	Canada	*Solanum tuberosum*	Xu *et al.,* 2010
34	CL4	EF063385	71.4	95.2	72.9	95.2	Canada	*Solanum tuberosum*	Xu *et al.,* 2010
35	Ca513	EF063389	70.0	87.1	70.7	87.1	Canada	*Solanum tuberosum*	Xu *et al.,* 2010
36	CL3	EF063384	72.1	93.5	73.6	93.5	Canada	*Solanum tuberosum*	Xu *et al.,* 2010
37	Ca508	EF063388	72.1	95.2	73.6	95.2	Canada	*Solanum tuberosum*	Xu *et al.,* 2010

brought to Ukraine from Europe in 19th century. Possible origin of Ukrainian PVM isolates from India can be explained by the fact that India is one of the leading countries in potato growing in the world, from where it is transported abroad, including Ukraine. It should be noted that the some of Indian PVM isolates induced the abaxial leaf rolling symptoms on potato plants (Katiyar *et al.*, 2013) similar to those we have identified in tomatoes in Ukraine.

Question about the determining factors in the phylogenetic divergence of PVM isolates into two main clades is still open. We did not reveal clear division of PVM strains and isolates according to the host plant. Moreover, the highest percentage of identity of Ukrainian tomato isolates was found

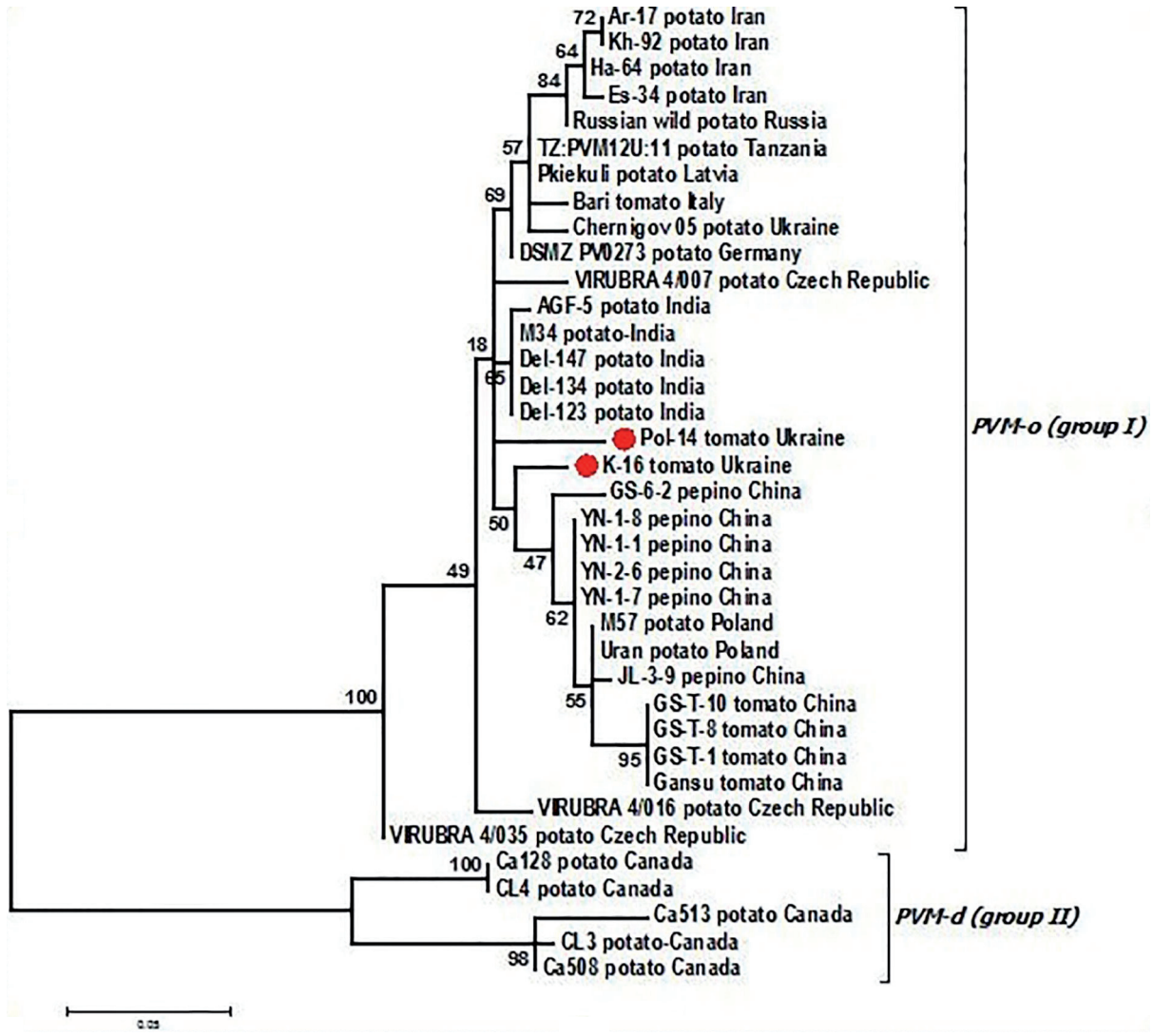

Fig. 2

Maximum likelihood (ML) tree based on nucleotide sequences of part of the CP gene of Ukrainian PVM isolates K-16 and Pol-14 and isolates from other countries (names and GenBank Acc. Nos. are given in Table 1)

The phylogenetic tree was constructed applying General Time Reversible (GTR) model. The values at the nodes indicate the percentage of replicate trees, in which associated taxa clustered together (number of bootstrap trails: 1000 replicates). The scale bar shows the number of substitutions per base.

with potato PVM strains. But on the other hand, analysis showed that isolates K-16 and Pol-14 have a high level of homology with tomato and pepino isolates (Table 1). Also, as seen from ML tree, all tomato and pepino isolates are located near each other, indicating their common origin (Fig. 2).

It was found that the nucleotide substitutions in the capsid protein gene of all tomato PVM isolates taken to this analysis (including those Ukrainian) are synonymous, i.e. those that do not lead to the amino acid substitution in the encoded protein. The exception is the Italian tomato isolate Bari, which has substitution of threonine to proline at position 250.

To explore the evolutionary forces acting on the PVM CP gene, the dN/dS values were calculated for all of the PVM CP sequences included in our study (Table 1). This ratio indicates the amount of nonsynonymous to synonymous mutations. dN/dS ratio for isolate K-16 compared to all other isolates was 0,033, for isolate Pol-14-0,029. This indicates more synonymous nucleotide substitutions in CP gene of isolate Pol-14 compared to isolate K-16.

The global dN/dS ratio for all of the sequences taken to the study was 0.041 ($p < 0,01$). This value is less than 1, showing that the PVM CP gene experiences a negative (purifying)

selection pressure. Pairwise comparisons showed that the dN/dS values were also significantly less than 1 for all of the CP sequence pairs. No CP sequence pair with significant positive selection (dN/dS>1) was found in the pairwise comparisons. The same results were obtained for complete CP gene sequences (Tabasinejad *et al.*, 2014). Thus, we suggest that genetic stability of PVM population is ensured by the effect of negative selection - selection to maintain the sustainability of the genome. Investigation of other PVM genes is necessary for more accurate conclusions and better understanding of PVM evolution.

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