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BIOLOGICAL AND MOLECULAR GENETIC IDENTIFICATION OF SOME VIRUSES THAT INFECT PLUM PLANTS

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SUMMARY

Many types of viruses exist in nature that affect plum plants. This article will discuss the most important ones. In 2020, the plum pox virus (PPV) discovery, identification, and eradication succeeded in Uzbekistan. Moreover, these investigations have also provided powerful tools for detailed analysis of the evolutionary processes and spread dynamics of PPV. These also assessed biological identification via mechanical inoculation of indicator plants and molecular characterization using RT-PCR with strain-specific primers followed by agarose gel electrophoresis, partial coat protein gene sequencing, and phylogenetic analysis. PPV reached biological identification upon mechanical inoculation of most test indicator plants, where symptoms of the virus appeared in ring mosaics on leaves of the family *Chenopodiaceae* members, such as *Chenopodium quinoa.* Molecular-genetic identification of the isolated PPV isolates confirmed they belonged to the D strain. However, the analysis of the nucleotide and amino acid sequences of the protein coat gene showed that isolates MT038048.1 (Uz1), MT038050.1 (Uz2), and MT038049.1 (Uz3) were different but closely related, derived from the same ancestor found on the closely related phylogenetic branches. Currently, this virus is absent from Uzbekistan; it has been eradicated using methods applied by the quarantine agency of the Republic of Uzbekistan.

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Keywords: Plum pox virus (PPV), isolates, RT-PCR, coat protein (CP) gene, primer design, *Chenopodium quinoa,* phylogenetic analysis

Key findings: Molecular characterization confirmed the PPV in studied samples of the apricot, sweet cherry, and plum cultivars and their hybrids in Uzbekistan.

INTRODUCTION

Apricot, sweet cherry, plum, and peach are economically important stone fruit crops in Uzbekistan. Plum pox virus (PPV) belongs to the genus *Potyvirus* and family Potyviridae, which is the causal agent of the sharka disease (Sattorov *et al.,* 2020). Plum pox virus (PPV) is the harmful pathogen behind the sharka disease, regarded as one of the most destructive viral agents affecting stone fruit crops globally. PPV severely impacts various fruit species, including plums, peaches, apricots, and other *Prunus* species, due to its efficient transmission and extensive host range, causing substantial losses (Cambra *et al.,* 2006).

PPV-infected fruit plants exhibit a wide spectrum of symptoms, including chlorotic ring patterns, distorted foliage and fruits, early fruit abscission, and diminished fruit quality. However, the symptom expression receives influence from host genotypes, environmental variables, and virus strains, and in some cases, may remain latent or barely visible. Viruses cause symptoms that have devastating consequences for various crop plants (Gao *et al.,* 2025). With the PPV complex epidemiology, long incubation period, and transmission via aphid vectors in a non-persistent manner, timely and precise detection plays a critical role in controlling the spread of the sharka disease (Navarro *et al.,* 1997).

Genome organization

The PPV genome is a positive-sense, single-stranded RNA approximately 9.8 kb in length, encoding a single large polyprotein that is further cleaved into functional proteins. Genetic variability among PPV strains, such as PPV-D, PPV-M, PPV-Rec, PPV-T, and PPV-C, complicates their detection, epidemiological tracking, and resistance breeding strategies (Wetzel *et al.,* 1991; Glasa *et al.,* 2004). Each strain exhibits distinct geographical distribution, symptoms' expression, and molecular characteristics, requiring a comprehensive molecular-genetic and biological knowledge of these virus strains (Šubr *et al.,* 2004).

Over the past two decades, considerable advancements have progressed in molecular detection techniques (reverse transcription polymerase chain reaction or RT-PCR, enzyme-linked immunosorbent assay or ELISA, and real-time PCR), genomic sequencing, and phylogenetic analysis of PPV strains. These tools have potentially enhanced the accuracy of diagnostics and helped trace the evolution and spread of the virus (Olmos *et al*., 2000; Rubio *et al.,* 2006). Meanwhile, the management approaches have evolved from simple eradication to integrated pest and virus management strategies that combine sanitation, resistant cultivar deployment, vector control, and quarantine measures (Jelkmann, 2011).

Genetic diversity and strain classification

The PPV possesses multiple genetically diverse strains, including PPV-D, PPV-M, PPV-Rec, PPV-T, and PPV-C, causing challenges for accurate detection, epidemiological monitoring, and breeding for resistance (Wetzel *et al.,* 1991; Glasa *et al.,* 2004). One of the most complex and potential aspects of PPV biology is its strain variability. Several strains of PPV have succeeded in their identification, primarily through nucleotide sequencing and serological assays. The well-characterized strains include:

PPV-D (Dideron type): One of the most widespread strains, initially detected in Western Europe, has a relatively narrow host

range and is most efficiently transmitted by vector aphids.

PPV-M (Marcus type): Common in Central and Eastern Europe, revealed broader host adaptability and faster aphid transmission rates than PPV-D.

PPV-Rec (Recombinant type): Resulting from natural recombination between PPV-D and PPV-M, showed intermediate properties and was more complicated for molecular diagnostics (Glasa *et al.,* 2004).

PPV-C (Cherry type): Found primarily in *Prunus cerasus* and *Prunus avium*, less common and not efficiently transmitted by aphids.

PPV-T (Turkey type): A more recently identified strain with unique genetic markers, first isolated in Turkey (Šubr *et al.,* 2004).

Among these PPV strains, the genetic differences significantly affect virus detection, symptom development, host range, and their geographic distribution. For example, PPV-M shows an association with more severe symptoms in apricot and peach, while PPV-D causes milder symptoms in plum (Hunter *et al.,* 1997; Navarro *et al.,* 1997). Each PPV strain has characteristics of unique molecular traits, regional prevalence, and symptomatology, making it essential to deepen our molecular and biological understanding of the said virus (Šubr *et al.,* 2004).

MATERIALS AND METHODS

Sample collection

Virus-infected samples obtained isolation from orchards located at 41°23′ N, 69°28′ E, approximately 320 m above sea level, in the Chirchik River Valley, Tashkent Region, Uzbekistan. Leaf samples came from 47 symptomatic plum trees (*Prunus domestica* L., cv. Ispolniskaya) distributed across three orchards in the Kibray District.

Selection of primers specific for PPV:

NIbF:CTTGAATGGGACAGATCAAATGA;
4CPR1:GAGAAAAGGATGCTAACAGGAATC
(Wallis *et al*., 2007; Matic *et al*., 2011, Chirkov *et al*., 2016; Sheveleva, 2020).

Obtaining cDNA by reverse transcription

In the process of obtaining cDNA by the reverse transcription (RT) method from the total RNA of the viral matrix genome, the following materials used by the study were dN6 (manufactured by Eurogen, Russia), as well as oligo-dT20-Prime. It is specific for the poly-α-sequence located at the 3'-end of the viral RNA, or its reverse (antisense) version, Prime. The mixture remained in a thermostat 'Gnome' at 65 °C for 5 min to bind the primer. At the end of incubation, the test tubes received an ice bath using a centrifuge of polished liquid into the test tube walls, with a TT mixture added.

The reaction, carried out in a thermostat 'Thermite,' was for 60 min at 37 °C (and 42 °C for other reverses). When the reaction ended, placing the test tubes for 10 min at 70 °C continued for enzyme inactivation in the thermostat 'Gnome,' before placing in an ice bath.

PCR diagnosis of PPV

The cDNA obtained by reverse transcription using the TT reaction served as the starting material. Then, for PCR amplification in a volume of 25 µl, preparing a reaction mixture included 16.5% water, 2.5 µl of 10x Taq DNA polymerase buffer (Eurogen), 0.5 µl of a 10 mM dNTP mixture, and 2 µl of 25 mM $MgCl_2$ solution. Likewise, the reaction mixture contained forward and reverse primers (concentration 10 p.m./µl) and 0.5 µl of Taq CR. The use of this procedure aided the diagnosis of PPV.

The required components entailed calculations and mixes in the same quantities as described above. The ZR sample underwent the following process: denaturation (94 °C, 3 min), amplification for 30–35 cycles, with the

polynucleotide chain restored for 5–10 min at 72 °C and 4 °C. The above procedure obtained the PZR product required for sequencing, but performing the reaction ensued in a volume of 50 µl, with the DNA polymerase (Evrogen) used for synthesis. PZR product analysis employed the ethyldibromide in agarose gel prepared in 1x Tris-acetate buffer (TAE, Thermo Scientific) using electrophoresis.

Electrophoresis, as performed, was at 80V for 40–60 min using the horizontal electrophoresis SE-1 (Chelicon, Russia). The gel transilluminator analysis had a wavelength of 312 nm at TFP-M/WL (Vilber Lourmat, France), before being photographed using the gel registrar Sistema Multi Doc-it (UVP, UK). The PPV's first identification in Uzbekistan was by Sattorov *et al.* (2023) in 2020 in the Kibray District of Tashkent Region. Its complete eradication relied on control measures taken in collaboration with the Republican Organization for Quarantine and Plant Protection.

RESULTS AND DISCUSSION

Several strains and isolates of the Plum pox virus (PPV) have reached successful identification to date. These strains differed based on plant cultivars, viral properties, and ecological conditions. In identifying diverse virus strains and isolates, the study of their biological properties, particularly their infectivity and disease symptoms induced, as well as the use of test indicator plants for virus diagnostics, has proven an effective approach for such studies. The healthy plant propagation material exploration is one of the most effective approaches for farmers' adoption. For successful certification programs and the production of such propagation materials, the availability of sensitive diagnostic methods appeared to be crucial (Makkouk *et al*., 1993).

A profound understanding of viral characteristics makes it possible to distinguish different viruses and their strains by using indicator plants that happen to be susceptible to one virus but not to another. This method serves as the basis for obtaining 'virologically pure' genetic materials and biologically purified viral inoculums free from other contaminating viruses. Therefore, the need to use more generic techniques that can be taught quickly and easily to relatively unskilled staff is vital (Ward *et al*., 2004).

The presented study aimed to investigate the symptomatic manifestation of plum rings mosaic virus (PPV) on indicator plants. To this end, at the initial stage of the experiments, the collection of plum leaf samples exhibiting ring mosaic symptoms transpired, placing them in polyethylene bags. Infectious sap's preparation as described earlier had the indicator plants mechanically inoculated (Table 1).

From the viral sap, the infectious extract sustained centrifugation at 5000 rpm for 10 min to eliminate bacterial and fungal contaminants, after which obtaining a clarified viral sap (CVS) followed. Using this preparation subsequently served as the inoculum for mechanical inoculation of virus-free indicator plants grown under controlled environmental conditions. Each inoculated plant received labels based on the virus type and date of inoculation, after which regular monitoring of the disease symptom development continued. These observations appear in Table 1. Upon visual inspection of indicator plants after inoculation, various mosaic lesions (spots, yellowing, and loss of greenery), deformations, and formation of circles, virus-like lines, spots on fruits, hardening of fruits, and premature ripening entailed recording (Sattorov *et al*., 2023).

The results further revealed the majority of mechanically inoculated test-indicator plants exhibited ring-shaped spotting symptoms characteristic of the virus. Specifically, among members of the family *Chenopodiaceae*, three *Chenopodium* species showed typical reactions: *Chenopodium quinoa* developed yellow ring spots within 7–9 days, the species *Ch. amaranticolor* and *Ch. album* displayed yellow ring-shaped spots on the leaf surface after 10–11 days, while the species *Ch. Murale,* unlike the other species, showed yellow chlorotic spots on the leaves within 6–7 days. Similarly, in the family *Solanaceae* species, *Datura stramonium* and *D. metel*

Table 1. Disease symptoms of PPV isolates collected on test-indicator plants under Uzbekistan conditions.

Family / Species of indicator plants		Disease symptoms	Symptom appearance period (days)
Chenopodiaceae			
1	*Chenopodium quinoa*	SHD	7-9
2	*Ch. amaranticolor*	–	10-11
3	*Ch. murale* L.	–	6-7
4	*Ch. album* L.	–	10-11
Solanaceae			
5	*Datura stramonium*	SYoM	10-12
6	*D. metel*	–	18-20
7	*Nicotiana barley*	M	20-22
8	*Nicotiana glutinosa*	SXD va SM	14-16
9	*Nicotiana benthamiana*	SHM	16-18
10	*Petunia hybrida*	–	–
11	*Solanum melongena*	–	–
12	*Capsicum annuum*	–	–
13	*Nicandra physalodes*	ND	14-16
Leguminosae			
14	*Pisum sativum*	YaAM	18-20
15	*Phaseolus vulgaris*	–	–
16	*Vigna sinensis*	–	–

Note: The symbols used in the table denote the following meanings: SHD - Yellow ring spot, SXD - Yellow chlorotic spot, SYoM - Yellow diffuse mosaic, YaAM - Green ring mosaic, SXM - Yellow ring mosaic, M - Mosaic, N - Necrosis, YaN - Large ring necrosis, QJND - Dark brown necrotic spot. ND = necrotic distortion; SM = systemic mosaic; SXM = systemic yellow mosaic. All were verified against the original data.

Figure 1. Plants tested for PPV using the TT-PCR method: A–*Datura stramonium* with systemic ring mosaic symptoms; B–*Chenopodium quinoa* with ring mosaic symptoms; V–*Chenopodium quinoa* with red mosaic symptoms (obtained from the experimental site of the Kibray District of the Tashkent Region), and G–plum leaf (*P. domestica* L.) and Ispolniskaya variety with ring mosaic symptoms.

developed diffuse yellow mosaic symptoms, which became visible 10–12 days after inoculation (Figure 1).

The symptoms were less visible on thicker leaves. A panel of indicator plants, including *Chenopodium quinoa*, *C. amaranticolor*, *Cucumis sativus*, *Datura metel*, *D. stramonium*, *Nicotiana benthamiana*, *N. glutinosa*, *N. rustica*, *N. tabacum* cv. White Burley, and *Vigna unguiculata*, underwent sapinoculation. Only *C. amaranticolor* plants developed symptoms in the form of pinpoint chlorotic local lesions, 7 to 10 days after the inoculation (Sorrentino *et al*., 2017).

In other host plants belonging to this family, symptoms such as systemic yellow mosaic and yellow mosaic were visible. The disease manifestations recorded in the remaining test-indicator species are available in Table 1. In subsequent experiments of the test-indicator plants, determining the dynamics of virus symptom development succeeded. The results further established that PPV did not induce any visible disease symptoms in members of the family Leguminosae, including *Vigna sinensis* and *Phaseolus vulgaris*. Likewise, it had no influence on representatives of the family Solanaceae, such as *Petunia hybrida* and *Solanum melongena* (Table 1). Potyviruses can cause a wide range of diseases. Symptoms on infected plants include mosaic (potato), spot (chili pepper), ringspot (papaya), necrotic or chlorotic lesions (cowpea and beans), scale brittleness (lily), necrosis, streaking, stunted growth, and wilting (Sharma *et al*., 2014).

The results revealed the symptom development dynamics of PPV infection manifested in three distinct forms primarily based on the indicator plant species. Specifically, the species *Chenopodium album* exhibited yellow chlorotic spotting on the leaf lamina and systemic mosaic accompanied by chlorosis of young leaves, and the species *Ch. amaranticolor* developed characteristic yellow ring spots. Species *Ch. quinoa* showed dark-centered yellow rings along with systemic mosaic, while the species *Datura stramonium* and other related species displayed clear mosaic symptoms. The symptom patterns provide robust evidence of PPV infection in the studied plants. Therefore, subsequent research emphasizes the necessity of applying immunological and molecular-genetic approaches for reliable virus diagnostics. Existing research on shark disease includes three main themes: rapid and accurate diagnosis, identification of sources of resistance, and development of new PPV-resistant cultivars using classical or biotechnological methods (María *et al*., 2015).

In summary, the findings disclosed that PPV isolates observed under the climatic conditions of Uzbekistan induced distinct and reproducible symptom patterns on a wide range of test-indicator plants. Most hosts developed ring mosaic, yellow chlorotic spots, and, in some cases, green ring-shaped lesions, which can serve as diagnostic markers for biological identification of PPV.

Immunological methods showed distinction as it was possible to determine several samples simultaneously with extreme sensitivity, requiring horses and prepared conjugates and substrates that recognize the AG to be studied. However, the molecular biological methods relied on PZR, distinguishable from immunological methods by their highest sensitivity. Through the PZR method, many variants have reached successful development to date, using the reverse transcriptase enzyme to identify the RNA-catching plant viruses and using the TT-PZR method based on cDNA extraction and its amplification. PZR is distinct with its highest sensitivity (at the expense of exponential reproduction of the DNA fragment) and its specificity (micro and macro) focused on determining the unique part of the genetic material of organisms. The examination rate—the time of obtaining the results—gave a calculation to be less than 24 hours for some infections. From a technical viewpoint, the employment of sequential quantitative PCR assays in virus monitoring helps identify false positive results caused by inadvertent contamination of samples with traces of viral nucleic acids or PCR products (Watzinger *et al*., 2006).

Therefore, in this research, it is important to identify the virus, whose symptoms have undergone studies using indicator plants in an immunological and molecular-genetic way. It is also vital to carry out phylogenetic analysis of isolated isolates, which have reached isolation from the plum plants with infectious properties.

As a result of the IFA (immunofluorescent antibody and microscopy assay), all the tested plants using the IFA method, with disease symptoms ranging from yellow chlorotic spots to green systemic mosaic, acquired infections of AHV, and all of them had high virus concentrations (1.789 to 2.873 and above) (Table 2). The IFA tests conducted on strain diagnostics revealed that

Table 2. ELISA diagnostics of PPV and its strains.

Name of the plant from which the sample was taken for testing	Symptoms of disease in the sample	Common antibody for all strains of PPV	Strains			
			M	W	D	S
			IFA reaction indicators			
Prunus domestica, Ispolniyskaya variety Prunus	SHD*	2.985	0.254	0.156	OUT	0.243
Variety *Prunus domestica*, Ispolniyskiety	SXD	2.985	0.167	0.187	1.534	0.278
Chenopodium quinoa	SHD	1.563	0.267	0.300	2.432	*0.243
Dourmon (*Datura stramonium*)	SYoM	OUT	0.187	0.276	2.783	0.083
Tobacco (*Nicotiana glutinosa*)	SXD+SM	1.843	0.104	0.207	0.982	0.290
Tamaki (*Nicotiana benthamiana*)	SXM + YaAM	2.245	0.211	0.184	1.258	0.291
Physalis (*Nicandra physalodes*)	QJND	1.789	0.231	0.243	1.945	0.188
Green peas (*Pisum sativum*)	YaAM +SXD	2.873	0.156	0.278	OUT	0.173

Note**:** The symbols used in the table denote the following meanings: SHD - Yellow ring spot, SXD - Yellow chlorotic spot, SYoM - Yellow diffuse mosaic, YaAM - Green ring mosaic, SXM - Yellow ring mosaic, YaN - Large ring necrosis, QJND - Dark brown necrotic spot.*** - IFA when examined using an analyzer the highest indicator was recorded "out" in a position above 3,000 indicators. Recording a pointer below 0.300 indicates that the virus has not been detected. ND = necrotic distortion; SM = systemic mosaic; SXM = systemic yellow mosaic. All were verified against the original data.

the identified isolate from the climatic conditions of Uzbekistan was specific to AHV strain D, while the remaining strains were not detected (Table 1). The RT-PCR was according to the amplification of a specific fragment of an RNA molecule. Under the action of reverse transcriptase (TT, RT, and reverse transcription), complementary DNA (cDNA) succeeded in its synthesis from a single-stranded RNA molecule, from which a single-stranded DNA attained amplification before being used in a simple PCR process. In the near future, one can expect that diagnostic laboratories will have to continue to develop their own methods for quantifying many important viral targets (Watzinger *et al*., 2006).

In Uzbekistan, the IFA analysis method has been effective in diagnosing phytoviruses to date, with their sensitivity reported to be $\sim10^{-3}$–10^{-5} units/ml of pathogen, while the sensitivity of polymerase chain reaction was $\sim10^{-1}$–10^{-2} units/ml of pathogen. Therefore, in subsequent studies, the PCR method was applicable for the molecular identification of PPV, considering its high sensitivity. For molecular genetic identification, the necessary primers and PCR conditions using the TT-PCR method proceeded as described by Sheveleva *et al.* (2020). For this purpose, the collected samples came from plants with yellow chlorotic and ring spot symptoms, performing molecular identification using the RT-PCR method (Figure 1).

The samples collected from the infected plants incurred freeze-drying and diagnosis in the scientific laboratory 'Biochemistry of Plant Viruses' at the Department of Virology, Moscow State University, Moscow, Russia. Prof. Chirkov S.N. supervised the process based on the presented sequence by the RT-PCR method. For molecular diagnostics, the two types of primers used were P1/P2 for PPV diagnostics (PCR product 243 bp) and P1/PD primer (PCR product 198 bp) for strain D diagnostics. The RT-PCR results visualization continued on a 2% agarose gel in an electrophoresis device; the electrophoregram obtained as a result of the examination appears in Figure 1. Virus-containing specimens can remain at ambient temperature or be stored at +4 °C for several days without the loss of yield and integrity because the protection of viruses from nuclease attack prevails as long as no disruption occurs on the protein (+/− lipid) coat (Anderson *et al*., 2003).

Electrophoresis proceeded on a 2% agarose gel. The strain diagnostics through molecular analysis, as performed, used specific primers P1/P2 (Wetzel *et al*., 1991) for PPV and P1/PD (Olmos *et al*., 1997). The PCR conditions took place as described by Sheveleva *et al*. (2020). The preparation of

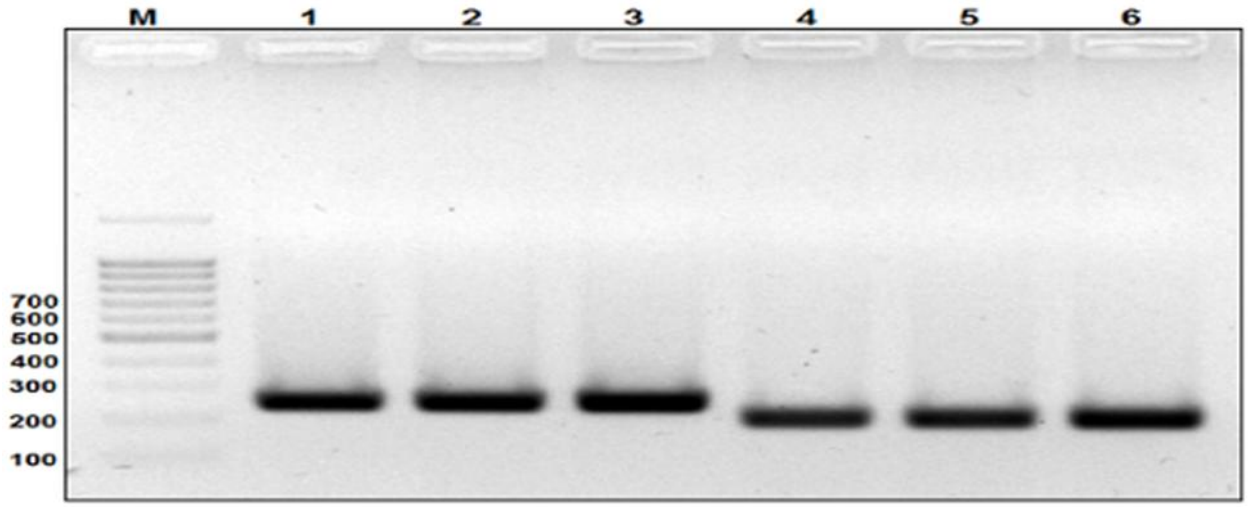

Figure 2. Molecular identification of PPV by RT-PCR. Electrophoresis performed on a 2% agarose gel. M - 100 bp DNA ladder Plus. Samples from plum plants with yellow chlorotic and ring-shaped spots on leaves 1-3, samples 4-6 (1,2,3) are the results of diagnostics performed to identify the strain. The study used Primers: P1/P2 (Wetzel *et al*., 1991) for PPV diagnostics and P1/PD (Olmos *et al*., 1997) for strain diagnostics. PCR conditions performed as described by Sheveleva *et al*. (2020).

primers P1/P2 was for the most common virus strains, and as a result, all three samples emerged to be infected with PPV, and the PCR product size was 243 bp (Figures 1 and 2).

Molecular diagnostic research to determine which identified strain virus belongs to where also continued using a special primer P1/PD. The PCR product was 198 bp, and as a result, the study confirmed the identified virus isolate was specific to the D strain. Most isolates belonged to the D strain. The prevalence of PPV-D was understandable since this strain was the most common worldwide. In each country where PPV existed and strains' type entailed detection, PPV-D isolates accounted for a significant population of this virus (Sheveleva *et al*., 2020).

Based on the results, one can conclude the diagnosis of PPV spread in the environmental conditions of the Tashkent Region proceeded using the TT-PCR method with special primers P1/P2. Given this virus was specific to strain D of PPV, the molecular genetic analysis using special primers P1/PD developed for strain diagnosis was valid. The results further revealed that PPV entered the NBG with a contaminated stone fruit material resulting from several independent introductions from different regions worldwide before spreading over plantations by aphids (Sheveleva *et al*., 2020).

The PPV-D with 12 reference samples (Europe, Middle East, and Central Asia) has a nucleotide identity of 96.4%–99.1%, the highest similarity with Turkish isolates (98.7%–99.1%). Nowadays, various strains and isolates of phytopathogenic viruses entail identification globally, with their comprehensive study considered crucial in the development of countermeasures. In the territory of Uzbekistan, determining the degree of virus spread has been successful in several plants based on past research. In particular, the resistance levels of various cultivars and samples of barley, millet, wheat, and corn to barley yellow dwarf virus and maize mosaic virus, which infect these cereal crops, have also had studies (Makhmudov *et al.,* 2023; Sobirova *et al.,* 2025a and 2025b). Various features of S, A, M, L, Y, and X viruses from the phytopathogenic viruses infecting potato plants, including the spread of a single potato virus X, and the biological features such as disease symptoms were evident in indicator-test plants (Fayziev and Vakhabov*,* 2019). In particular, plum pox virus, which mostly infects

fruit trees, first attained detection in plum plants under the climatic conditions of Uzbekistan (Sattorov and Fayziev, 2024 and 2025).

CONCLUSIONS

The isolate of this virus that has spread in Uzbekistan has achieved a successful transfer to the gene pool "Collection of unique scientific objects of phytopathogens and other microorganisms" at the Institute of Genetics and Experimental Biology of Plants of the Academy of Sciences of the Republic of Uzbekistan. It is also being used for scientific research purposes (Reference of the Academy of Sciences of the Republic of Uzbekistan dated March 15, 2024, No. 4/1255-610). Molecular genetic identification of the isolated PPV isolates confirmed their belonging to the D strain.

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