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ILLUSTRATION OF MORPHOLOGICAL FEATURES AND EARLY MOLECULAR IDENTIFICATION OF *PHYTOPHTHORA INFESTANS* IN UZBEKISTAN

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A B S T R A C T

An isolate belonging to the genus *Phytophthora* was isolated from samples of infected potato plants in Uzbekistan. The morphological characteristics of the isolate were studied and the fungus *P. infestans* belonging to the type of oomycetes was determined. The colony of *Phytophthora*. sp isolate is colorless and the mycelium appears porous when growing. When studying the microscopic appearance, it was found that the hyphae are not septated, the width of the hypha is 2.5-4.4 µm, the width of the sporangium is 12-17 µm, and the length is 18-25 µm. It was found that *P.infestans* has amphigynous antheridium with oogonium and chlamydospores. To determine the exact phylogenetic status of this isolate, rRNA gene ITS region (845 bp) and cox2 (627 bp) gene were PCR amplified. The phylogeny of both marker genes showed that the isolate was *P. infestans* and named *P. infestans* TVKT-1 due to its closeness to *P. infestans* species. The analysis of the ITS region of the rDNA gene as a barcode alone does not provide enough information for the identification of species belonging to the genus *Phytophthora*, for this, it is important to study the genetic analysis of oomycetes with the additional study of the cox2 gene. Compared to *Phytophthora infestans* species in the NCBI database, *P. infestans* TVKT-1, which we isolated, was found to have SNP mutations in the ITS region (16, 23, and 29 nucleotides) and cox2 gene (362, 364, and 365 nucleotides) nucleotide sequences.

Keywords: *Phytophthora infestans*, morphology, ITS rDNA, cox gene, molecular identification, amplification, PCR.

INTRODUCTION

Over the past four years, high volatility of potato prices has been observed in Uzbekistan, in particular, a growth of 42% at the end of 2020 (kun.uz, 2021). This is due to many factors, including the devastating effects of disease-causing phytopathogenic fungi. Late blight is the most dangerous of these diseases. In Uzbekistan, late blight disease was reported for the first time in the potato crop in 1974 (Zaprometov *et al.,* 1974). *Phytophthora* disease causes 30-40% damage to potato productivity (Khamiraev, 2018) and seriously damages tomatoes, peppers, pumpkins, citrus crops, and some trees and shrubs in the world Additionally, it is particularly harmful to plants belonging to the Solanaceae family, Additionally, it is particularly harmful to plants belonging to the Solanaceae family (Matsishina *et al.,* 2021). More than 117 species of the genus Phytophthora have been identified (Frank *et al.,* 2012). Although, there is information about the presence of *P. parasitica* and *P. infestans* species in Uzbekistan (Khamiraev, 2018; Khamiraev, 2022), molecular genetic identification studies were not conducted about these isolates yet.

It is very important to develop diagnostics for early identification of the causative agent of phytophthora disease. Of course, identification based on morpho-physiological characteristics is considered an important factor, but these characteristics may require a lot of time in the study of *P. infestans* cultures and may cause difficulties in evaluating the non-phenotypically

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manifested external characteristics of genotypically changed species. Molecular-genetic identification of *P. infestans* started 30 years ago (Moller *et al.,* 1993; Goodwin *et al.,* 1999), then identification studies were continued based on internal transcribed spacer (ITS) rRNA and cytochrome oxidase genes of the fungal genome [8]. It should be noted that comparing the ITS region of rRNA alone cannot fully analyze interspecies phylogenetic variation (Cooke *et al.,* 2000; Voglmayr *et al.,* 2014; Yang and Hong, 2018). When *Phytophthora* species were phylogenies with ITS, cox1, and cox2 genes, it was noted that the groups formed by cox1 and cox2 partial genes in the phylogenetic tree corresponded to each other.At the same time, although the main groups in the phylogenetic tree of cox2 genes with the ITS region are consistent, it has been noted that there are some variations (Martin and Tooley, 2003).

Barcoding most genera of oomycetes with ITS rRNA and cytochrome-c oxidase 1 (cox1) gene is insufficient to detect phylogenetic variation among genera. Studies have shown that the cox2 marker is easy to amplify PCR and that many higher and interspecies divergences have been observed compared to the cox1 gene region.If the cox2 gene is barcoded together with the cox1 and ITS rDNA genes, it has been noted that the phylogenetic variability can be more accurately analyzed (Choi *et al.,* 2015).

Based on the above-mentioned points, the purpose of this study is to *P. infestans* isolates, studying their morphological characteristics and molecular-genetic identification based on the ITS region of rDNA and cox2 marker genes.

MATERIALS AND METHODS

Phytophthora* sp*. Isolation: Potato tubers of the Picasso variety with dark brown spots indicating signs of *phytophthora* disease were selected and thoroughly washed in running tap water. Then, tubers were wiped with a cotton swab moistened with 80% ethanol and burned for 1-2 seconds to sterilize. Next, potato nodules were cut using a sterile scalpel. 5-6 mm pieces containing damaged and healthy tissue were placed in moist chambers and incubated at 18 °C for 16 hours, as described previously. Samples were prepared from the white powder formed on the upper part of the potato slices and examined under an NLCD-307B microscope (Wincom Company, Ltd, China) with 400x magnification. After that, agar containing oatmeal was prepared (g/L: oatmeal - 200 g; agar - 20 g; distilled water – 1L). Samples were cultivated on the agar with oatmeal and grown for 14 days at 18 °C temperature.

Morphological identification of the isolate: The morphology of the isolated fungal colony, the characteristics of its mycelium, and the morphology and size of its sporangia were determined using N-300M (UCMOSO9000KPB) (Ningbo Yongxin Optics, China) microscope.

Virulence testing: *Phytophthora* sp*.* was grown on oat agar medium for 14 days suspended in 9 ml sterile water and used to infect healthy potato tubers by 1) using a 10 µl syringe and 2) the skin of the tumor was cut and rubbed onto a healthy tissue using a sterile scalpel. Uninfected potato tuber was used as a control. Tubers were placed in wet chambers in individual cylinders and the infection rate was assessed visually. Sterile water was placed in a sterile condition to maintain the moisture of the cameras.

DNA extraction: Firstly, *Phytophthora* sp*.* was grown on an oat agar medium at the 18°C temperature for 14 days. A small piece of mycelium was picked and transferred to the 1.5 ml sterile plastic tube. After that, it was suspended in 200 µl мM LiOAc 1% SDS solution and incubated at 70 °C for 5 minutes as described previously (Marko *et al.,* 2011). After incubation, the sample was centrifuged at 15 000g for 5 minutes. The liquid form of the sample was moved to the new sterile tube. Then, 300 µl 96% ethanol was added to the tube, and the sample was mixed briefly and stored at -20 °C for two hours. Next, DNA was collected by centrifugation at 15 000 x g for 5 minutes, the supernatant was discarded and the remaining pellet was washed with 70 % ethanol. Finally, the sample was dried and precipitated DNA was eluted in 100 µl TE solution. DNA fragments were visualized using gel electrophoresis on a 0.8 % agarose gel for 30 min.

Primer designing: *NCBI database P. infestans* **(**KU992300.1) was used to design specific primers for Internal Transcribed Spacer (ITS). By using Primer3 Input software, ITS1-F, and ITS2-R specific primers were newly designed to amplify completely ITS1 and ITS2 regions of *P. infestans* and checked via the SnapGene program. Additionally, *P. infestans* fungi belong to the oomycetes class, COX2 (F) - COX2 (R), COX2RC4 primer variations were used for PCR amplification. All primers (Table 1) oligonucleotides were ordered from Integrated DNA Technologies (IDT).

Table 1

Primers name	Sequence (5′ →3′)	PCR product size (bp)	Reference
ITS1 F	GCGGAAGGATCATTACCACAC	845	This study
ITS2 R	GTTCAGCGGGTAATCTTGCC		
COX2 F	GGCAAATGGGTTTTCAAGATCC	627	(Hudspeth *et al.,* 2000)
COX2 R	CCATGATTAATACCACAAATTTCACTAC		
COX2RC4	TGATTWAYNCCACAAATTTCRCTACATTG		(Young *et al.,* 2015)

PCR and sequencing of ITS and cox2 regions: PCR amplification was conducted by using three combinations of five primers (Table 1). ITS1 F and ITS2 R specific primers (designed here) were used to completely amplify the ITS1 and ITS2 regions of *P. infestans***.** To amplify the COXII region, COX2 F - COX2 R and COX2 F - COX2RC4 primer variations were used.

PCR reactions were carried out by GenPak™ PCR Core Master Mix manual (Галарт-Диагностикум, Russian) in 20 µl and each PCR reaction contained genomic DNA up 5 ng, 10 µl dilution, 8,2 µl double distilled water, 0,4 µl forward and reverse primers. PCR conditions were followed by an initial denaturation stage of 94 °C for 3 min; 35 cycles of 94 °C for the 40s, primer annealing temperature at 55 °C for the 40s, and 70 °C extensions for 90s; and final extension of 7 min at 70 °C as described manual of GenPak ™ PCR Core. PCR products were evaluated for successful amplification using gel electrophoresis on a 2% agarose gel for 30 min, concerning a DNA marker (Evrogen), 100+ bp DNA Ladder (Figure 5. A). After that, PCR products were purified using the QIAquick® Gel Extraction Kit manual as described and the purified PCR products were sequenced.

Phylogeny: The sequencing data for the cox2 gene and ITS region of *P. infestans* was aligned with other related *Phytophthora* species via NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Its phylogenetic trees for the cox2 gene and ITS region were built using *MEGA-X* (version *10.1.8)* software.

RESULTS AND DISCUSSION

It should be noted that in Uzbekistan, the damage of phytophthora pathogen to vegetable crops such as potatoes and tomatoes has been studied, but no research work has been carried out on the isolation and molecular identification of the pure culture of this pathogen from potato tubers.The outer sides of the selected potato tubers contained sunken spots of different sizes with a brown appearance. There were brown spots along the circumference of the cuts. We observed that some infected potato pieces had white fumes that incubated at 18 °C for 16 hours in a moist chamber. It is known that the sporangia growth of *P.infestans* is closely related to external conditions, especially temperature. Zoospores were formed at low temperatures at 4-18 °C. Zoosporangia do not form zoospores, when the temperature increases to 20-27 °C, but the embryo tube grew and penetrated the plant tissue (Elansky *et al.,* 2015; Dyakov and Elansky, 2017). A preparation made from the white powders that grew on the potato slices in a humid chamber, when it was seen under a microscope, zoosporangia of the fungus *Phytophthora* sp. was found (Figure 1).

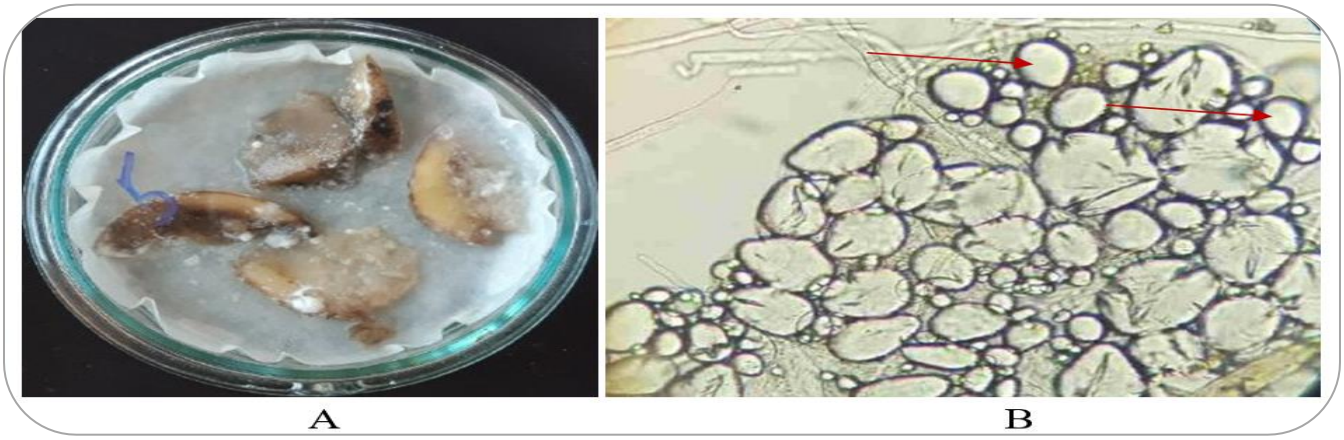

Figure 1. The appearance of white powdery mildew on potato tubers under humid chamber conditions (A) and zoosporangia of *Phytophthora* sp. in these powdery mildews (B)

Zoosporangia of the fungus *Phytophthora* sp. cultivated on agar media contained with oatmeal using a mycological hook. One isolate of *Phytophthora* sp. was isolated as a pure form. We noticed the colony of this isolate was colorless, had no clear borders, porous mycelia, and had sparse growth. When its microscopic structure was analyzed, the hyphae were not septated, the width of the hypha was 2.5-4.4 µm, the sporangium width was 12-17 µm, and the length was 18-25 µm observed (Figure 2). According to these morphological features, it was found that this isolate is similar to *P. infestans* type (Raza *et al.,* 2022).

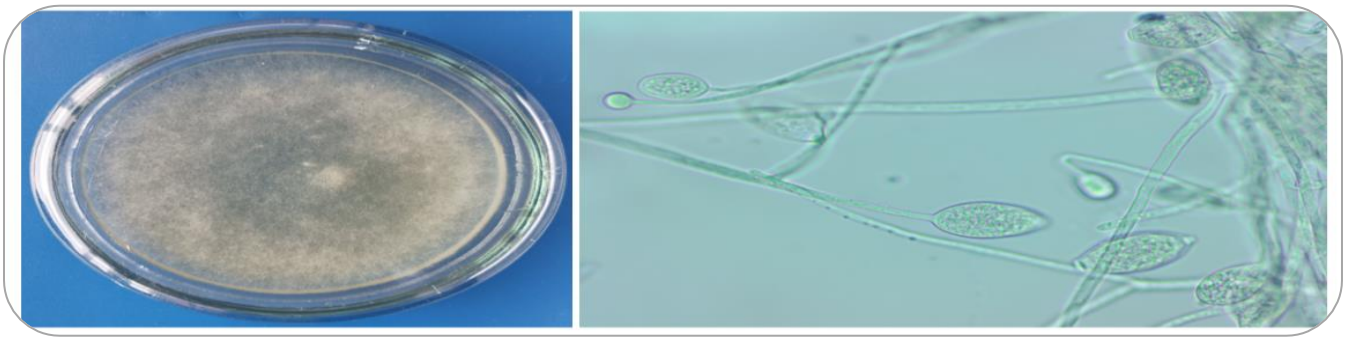

Figure 2. *Phytophthora* sp. colony and its microscopic appearance (14 days)

We found that *Phytophthora sp*. has an oogonium, chlamydospores with amphigynous antheridia, and chlamydospores (Figure 3). Our results on the morphology of *P. infestans* compared with the morphological data obtained by (Daniel and Birtukan, 2020; Gómez-Gonzàlez *et al.,* 2020) the results were compatible.

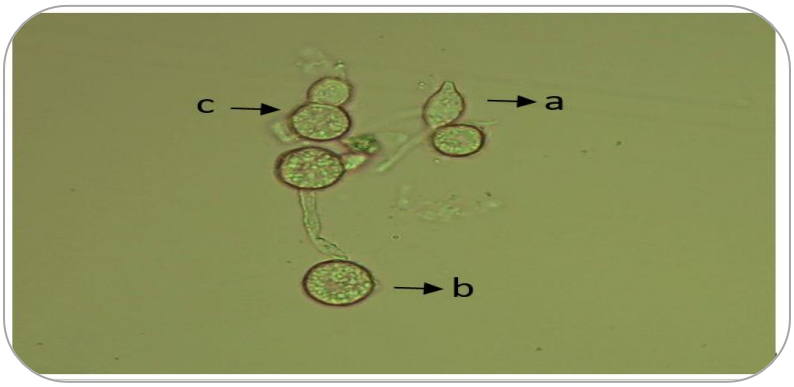

Figure 3. Microscopic analysis of the morphology of *P.infestans* isolated from potato tuber: a) lemon-shaped sporangium; b) chlamydospore; c) amphiginous antheridium and oogonium (28 days)

An artificial re-infection method was used to identify the virulence of the causative fungus [24]. Healthy potato tubers of the Picasso sort infected with the fungus *P.infestans* and incubated infected buds at 24 °C temperature for 21 days.

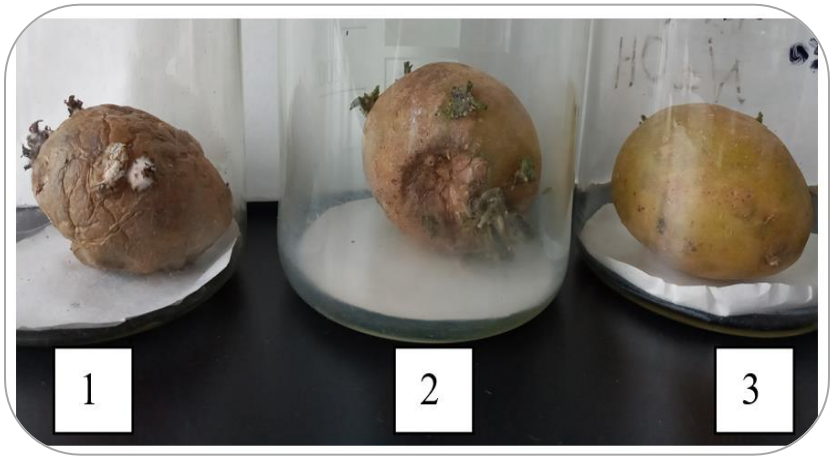

Figure 4. Potato tubers of the Picasso were artificially infected with the fungus *P. infestans* (21 days of incubation). 1- vaccination infection, 2- smear infection, 3- control

During the research, a change in the potato tubers in experimental variants from 12 days compared to the control, and the appearance of brown spots was observed in the re-infected potatoes (Figure 4). The zoosporangia appearance of *P. infestans* was confirmed by microscopic analysis, when the tubers cut and placed them in a moist chamber, and repeatedly incubated under the same conditions.

Thus, our results showed that the pathological and morphological characteristics of the fungus isolated from potatoes (infected with *P. infestans*) were confirmed to be consistent with the characteristics described in other scientific papers (Daniel and Birtukan, 2020). To find out the pathogenicity of the fungus, its virulence was checked and we found that *P. infestans* showed disease characteristics with re-infection.

PCR amplification conducted by three combinations of primers(COX2 F-COX2 R, COX2 F-COX2RC4, ва ITS1 F-ITS2 R) to identify *P. infestans* (Figure 5). As can be seen from the electrophorogram in Figure 5, as a result of the amplification of cox2 F-cox2 R and cox2 F-cox2RC4 primers, the same amplicons (627 bp) were generated in *P.infestans* isolate. It was noted that the ITS1 F and ITS2 R specific primers designed by us efficiently synthesized the 845 bp PCR product in *P. infestans* isolate (GenBank: OQ421595.1). It should be noted that primers cox2F, cox2R, cox2RC4, ITS1F, and ITS2R were shown to be specific for species belonging to the genus *Phytophthora*.

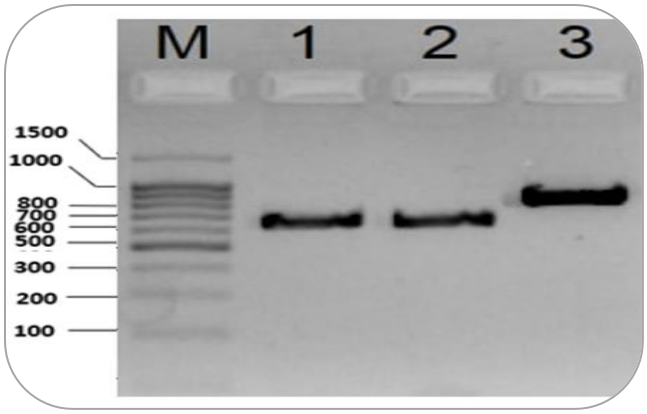

Figure 5. PCR products of *P. infestans* strain TVKT-1. M – 100 + DNA ladder, 1 – 2 cox2 gene, and 3 – ITS region.

After sequencing the PCR products, partial DNA sequences of the cox2 and ITS regions of *P. infestans* isolates were compared to *Phytophthora* species in the NCBI BLAST database. The results showed that the *P. infestans* isolate showed 99% similarity with the partial sequences of the cox2 and ITS rDNA gene of *P. infestans* in the NCBI database. According to the sequence results of cox2 and ITS regions, we named the isolate as *P. infestans* strain TVKT-1.

To construct a phylogenetic tree of the ITS region, two strains of *P. infestans* and *Phytophthora* genera *P. viticola*, *P. sojae*, *P. cinnamomi*, *P. ramorum*, *P. capsici*, *P. parasitica*, *Hyaloperonospora arabidopsidis*, *Pythium ultimum*, and *Albugo candida* such types were used.

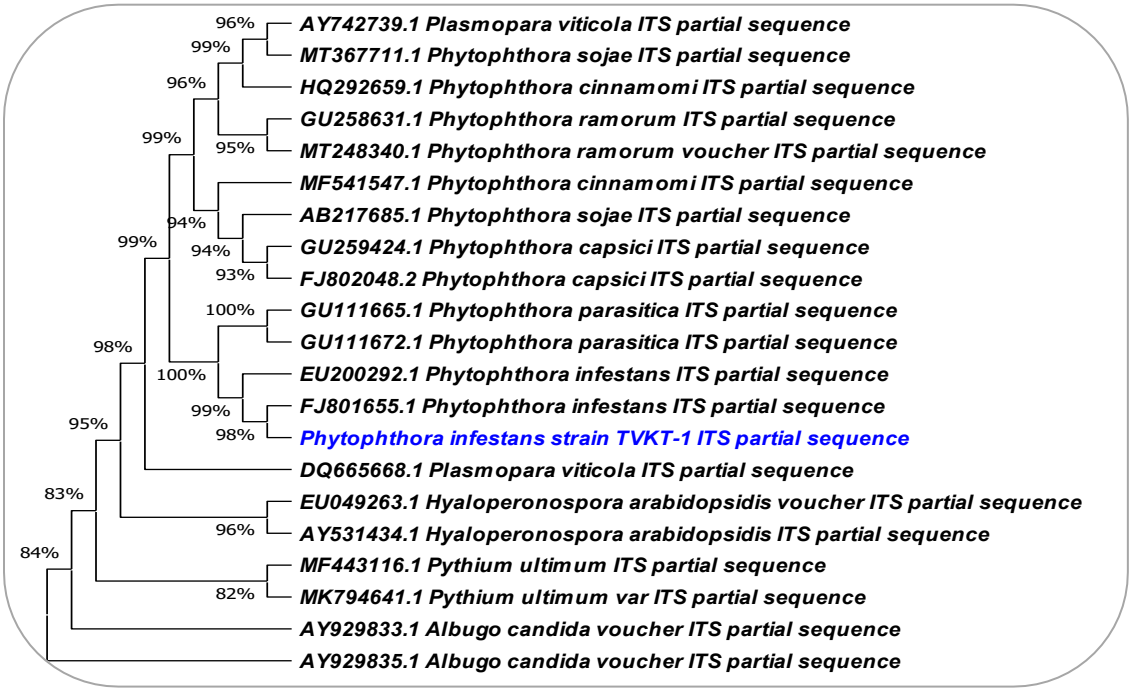

Figure 6. Phylogenetic analysis of the ITS region of *P.infestans* strain TVKT-1 and *Phytophthora* species

The phylogeny was constructed based on the Maximum likelihood (Bootstrap Replications 1000) of the MEGA X program.It was found that four clades were formed in the phylogenetic tree of genera belonging to the type of oomycetes.*Plasmospora viticola, P. ramorum, P. sojae, P. capsici*, and *P. cinnamomi* species were located in clade one, while *P. infestans* and *Phytophthora parasitica* species formed clade two. The 3rd clade contained *Hyaloperonospora* species, while the 4th clade was distinguished by the presence of *Albugo* and *Globisporangium* species.Phylogenetic analysis of the ITS region revealed that *P. infestans* strain TVKT-1 is in a different clade from most other *Phytophthora* genera and *Plasmospora genus* (AY742739.1 *Plasmospora viticola*), but it is in a subclade belonging to *P. infestans* species, which was shown by high bootstrap scores (99%).Other oomycete genera *Albugo*, *Globisporangium*, and *Hyaloperonospora* were observed to be in a different clade than *Phytophthora* when compared by the ITS region of rDNA.The phylogenetic tree data of the ITS region of rDNA showed that the genus *Plasmopara* showed genetic affinity to species belonging to the genus *Phytophthora*.

Then, when the phylogenetic analysis of *P. infestans* TVKT-1 was performed according to the mitochondrial cox2 gene, it was found that it formed 5 clades.

Compared to the ITS region, the cox2 phylogenetic tree showed that the oomycete genera *Pythium*, *Globisporangium*, and *Phytophthora* were in the first clade, showing their genetic closeness (Figure 7).Also, *P. sojae* differed from the phylogenetic tree based on ITS by being in a separate clade, different from *Phytophthora* species.Species belonging to the oomycete genus *Hyaloperonospora* were shown to be similar, with the ITS region of the rDNA gene forming a distinct clade in the phylogenetic tree, as well as the mitochondrial cox2 gene being in a distinct clade.According to cox2, *Albugo* and *Plasmospora* genera differed from genera belonging to other oomycete types, and their presence in a separate clade indicated their genetic divergence. *P. infestans* TVKT-1 strain isolated by us was placed in the same subclade with the *P. infestans* species with ID numbers DQ365743.1 and OM728533.1 and showed that it belonged to the *Phytophthora infestans* species.

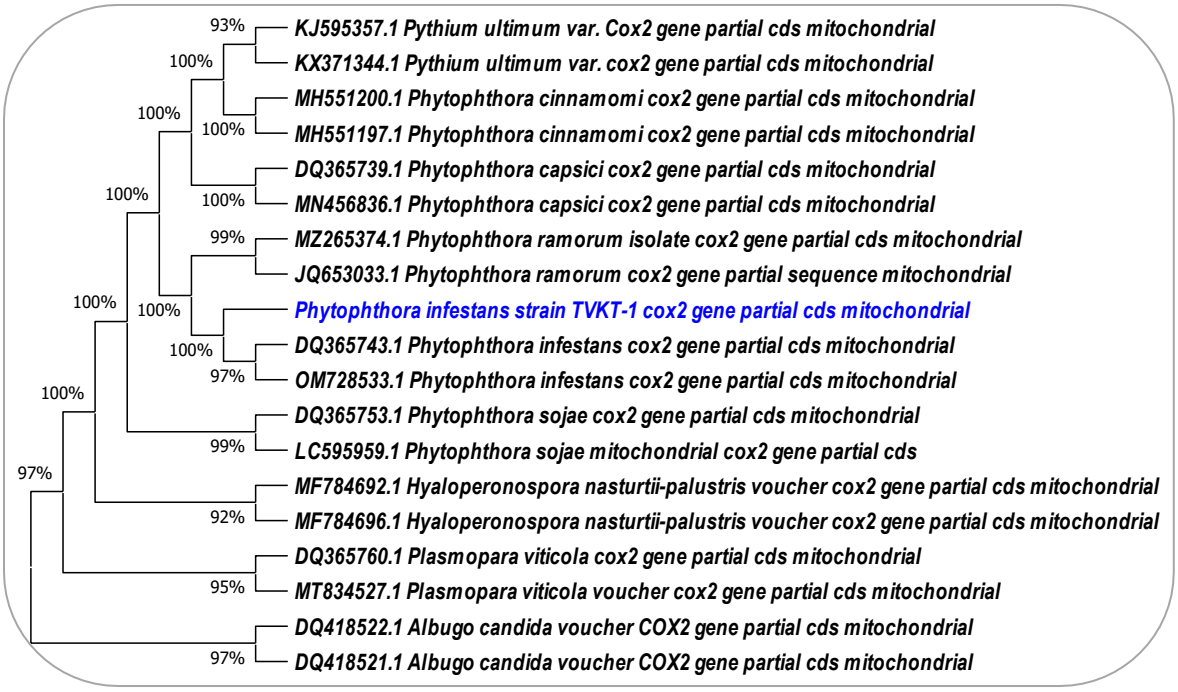

Figure 7. Phylogenetic analysis of the cox2 gene of *P. infestans* strain TVKT-1 and other *Phytophthora* species

DISCUSSION

Phytophthora infestans (Mont.) de Varu is a hemibiotrophic fungus-like organism that causes phytophthora disease in potatoes and tomatoes, which are very important crops for agriculture.*P. infestans* not only causes direct damage to the potato plant, but it has also been reported to be a carrier of potato X-virus through its zoospores (Ali *et al.,* 2010).

There are two types of *P. infestans* (A1 and A2) that reproduce sexually by producing oospores (Drenth, 1994). In recent years, new populations have been observed as a result of the crossing of A1 and A2 types,

and isolates with such genetic diversity have been reported to be more or less resistant to metalaxyl (Elansky *et al.,* 2015; El-Ganainy *et al.,* 2022). Over the past 30 years, many studies have been conducted to determine the taxonomic status of *P. infestans* by molecular genetic methods (Goodwin *et al.,* 1992; Griffith and Shaw, 1998; Li *et al.,* 2013), because it is difficult to control *P. infestans* without determining its phylogeny (Yang and Hong, 2018). There are different views on the barcoding of oomycetes through the ITS region of rDNA, and the length of the ITS1 and ITS2 regions of oomycetes and the high heterogeneity of their nucleotide sequences make it difficult to use them for general barcoding (Choi *et al.,* 2007; Garcна-Bázquez *et al.,* 2008), but ITS region has been used as a barcode for all fungi and for most *Phytophthora* taxa (Schoch *et al.,* 2012).

In addition, cox1 and cox2 genes were also used in the identification of *Phytophthora* species. These genes have been reported to provide more accurate information in oomycete barcoding when compared to the ITS region (Martin and Tooley, 2003; Robideau *et al.,* 2011). However, when the phylogeny of 31 species samples belonging to the lineage of ancient oomycetes was studied with the cox1 and cox2 genes, it was found that the cox1 genes did not amplify PCR in 3 of the 31 samples, and the cox2 primers were amplified in all 31 samples. It was noted that there are highly conserved sites in sox2 genes (Choi *et al.,* 2015).

In the identification of *Phytophthora* species, cytochrome-c oxidase 1 (cox1), internal transcribed spacer region (ITS), 60S ribosomal protein L10, beta-tubulin (β-tub), elongation factor 1 alpha, enolase, heat shock protein 90, 28S ribosomal DNA, and tigA gene fusion protein (tigA) was studied as a genetic marker.In this case, molecular-genetic identification of uncertain isolates belonging to the genus *Phytophthora* should be carried out in two stages, first, checking with the internal transcribed spacer region (ITS), and then using one or several genetic markers related to additional subclades (Yang and Hong, 2018).

In our research, the ITS region and cox2 partial genes for barcoding *P. infestans* isolated from potatoes were sequenced and compared. According to the phylogenetic result, *P. infestans* isolate TVKT-1 was found to belong to the *Phytophthora* clade.It should be noted that when the ITS region and cox2 gene nucleotide sequences of *P. infestans* isolate TVKT-1 were compared at NCBI, it was found that there was an SNP mutation. The presence of these mutations in the genes means that it is different from the species belonging to the genus *Phytophthora* that have been identified so far.The presence of genetically stimulating genetic elements in species belonging to the genus *Phytophthora* causes them to mutate rapidly (Haverkort *et al.,* 2009). Therefore, the effect of the eco-biocenosis of Uzbekistan and the rapidly changing continental climate may have caused changes in the genes of *P. infestans*.

CONCLUSION

Using the designed ITS1 and ITS2 primers, *P. infestans* isolate was amplified and phylogenetically analyzed, and it was found that it belongs to the oomycete type.PCR analysis of the cox2 gene and phylogenetic analysis confirmed that *P.* sp. TVKT-1 isolate belongs to *P. infestans* species.The use of both the ITS region and the cox2 gene in the molecular identification of *P. infestans* by barcode quality indicates genetic divergence.The presence of SNPs in the ITS region and cox2 genes of *P. infestans* TVKT-1 may have caused environmental mutations.The primers used in this study can be used as molecular genetic markers for early detection of *Phytophthora* causative agent *P. infestans* and other *Phytophthora* species.

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Nodira Azimova	:	Strain isolation, virulence testing, morpho-cultural characteristics.
Ilkhom Khalilov	:	Designing the experiments, editing the article, general design, and giving the necessary instructions.
Fazliddin Qobilov	:	Implementation of research methods and translation of the article.
Muhammad L. Nazirov	:	Primers design and searching for information about the methods used in the experiments.
Sergey Elansky	:	Partial formation of the article, some modification and analysis results.
Elena Chudinova	:	Partial formation of the article, some modification and analysis results.