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Incidence and Coat Protein Characterization of Apple Stem Pitting Virus Isolates from Isparta Province of Turkey

Türkiye'nin Isparta İlinden Elde Edilen Apple Stem Pitting Virus İzolatlarının Yaygınlığı ve Moleküler Karakterizasyonu

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Abstract: Turkey is one of the major countries in the world in terms of fruit growing due to its climate diversity and geographical features. Apple, which is one of these fruits, is grown intensively in Turkey, and especially the Mediterranean Region is important for apple agriculture. Today, viral diseases are considered as an important yield loss factor in apple farming. This study investigated the incidence and molecular characterization of apple stem pitting virus (ASPV) in Isparta province, a significant apple-producing region in Turkey. By using the DAS-ELISA and RT-PCR methods, ASPV infection was found in 7 out of 70 collected apple leaf samples. The partial nucleotid sequences of ASPV were obtained and registered in GenBank for accession numbers. The generated similarity matrix by using the representative isolates revealed that the new ASPV isolates shared 79–93% of their nucleotide sequences with GenBank reference acessions. The isolates collected in this research were clustered in group 1 of the phylogenetic tree that was created by selecting a specific number of isolates from GenBank and thought to be reliable in the phylogenetic differentiation of ASPV. This is the first study to examine the prevalence of ASPV in the Isparta region and its phylogeny. It is possible that the results of the research will contribute to a better understanding of the situation of ASPV in Turkey.

Keywords: ASPV, phylogenetic relationship, molecular characterization

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Öz: Türkiye, sahip olduğu iklim çeşitliliği ve coğrafi özellikler nedeniyle meyvecilik açısından dünyada oldukça önemli bir ülke konumundadır. Bu meyvelerden bir tanesi olan elma ülkemizde yoğun olarak yetiştiriciliği yapılan bir meyve olup özellikle Akdeniz Bölgesi elma tarımı ile ön plana çıkmaktadır. Günümüzde viral hastalıklar elma tarımı içerisinde önemli bir verim kayıp unsuru olarak ele alınmaktadır. Bu çalışmada Türkiye'nin önemli bir elma üreticisi konumunda olan Isparta ilinde apple stem pitting virus (ASPV)'nin yaygınlık durumu belirlenmişve moleküler karakterizasyonu gerçekleştirilmiştir. Toplanan 70 adet elma yaprağı örneğinden 7'sinde ASPV enfeksiyonu DAS-ELISA ve RT-PCR yöntemi ile belirlenmiştir. Virüsün kılıf protein bölgesinin kısmi dizi bilgisi elde edilerek GenBank erişim numaraları alınmıştır. Oluşturulan benzerlik matrisinde izolatların GenBank referans izolatları ile 79-93% oranında nükleotid düzeyinde benzerlik gösterdiği tespit edilmiştir. GenBank'ta yer alan ve ASPV'nin filogenetik ayrımında güvenilir olduğu değerlendirilen belirli sayıda izolat seçilerek elde edilen filogenetik ağaç 6 gruba ayrılmış ve bu çalışma kapsamında elde edilen izolatlar filogrup 1 içerinde yer almıştır. Bu çalışma ASPV'nin Isparta bölgesinde yaygınlık durumunun incelendiği ilk çalışma özelliğindedir. Araştırma sonuçlarının ASPV'nin Türkiye'deki durumunun daha iyi anlaşılmasına katkı sunacağı düşünülmektedir.

Anahtar Kelimeler: ASPV, filogenetik ilişki, moleküler karakterizasyon

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INTRODUCTION

Apple stem pitting virus (ASPV) with a positive single-stranded RNA (ssRNA) is one of the most devastating viral pathogens of pome fruits in the world (Nemeth, 1984). The genome of ASPV consists of five open reading frames (ORFs) and has 9.200–9,300 nucleotides in the family Betaflexiviridae (Adams et al., 2004). The flexible filamentous ASPV particles may aggregate end-to-end in host cells and have dimensions of 12–15 nm in width and 800 nm in length (Ma et al., 2016). Viral replicase polyprotein and triple gene block proteins (TGBps) are encoded by ORF1, ORF2, and ORF4 respectively whereas the coat protein of virus (CP) is encoded by ORF5 (Jelkmann, 1994).

Since there is currently no identified vector, non-certified propagative material and grafting are most probable to be responsible for the virus's spread (Claude and Fauquet, 2004). ASPV affects a numerous plants and causes different types of symptoms such as leaf red mottling, fruit stony pits, xylem pits, decline, epinasty, vein yellowing or pear necrotic mark linked to the host plant, genotype, and isolate/strain of virus (Jelkmann, 1994; Mathioudakis et al., 2006, 2010; Wu et al., 2010). Some economically significant diseases, including deterioration of quince fruit, epinasty, the loss of apple Spy 227, and yellowing of pear vein are also linked to ASPV (Stouffer, 1989; Jelkmann, 1994). The infected plant variation of the ASPV is primarily limited to commercial pome fruits, but it has also recently been reported from pear- and apple-rootstocks, some ornamentals, and the members of undomesticated *Rosaceae* (Constable et al., 2007; Ma et al., 2008; Mathioudakis et al., 2010, 2012), with cherry being the one exception (Yang et al., 2017). ASPV infection causes yellowish veins (Wu et al., 2010), reddish spotting (Komorowska et al., 2011), necrotic spots on pear, or pittings in various pear cultivars (Mathioudakis et al. 2009). Infection of ASPV is often linked to the existence of other viruses such as apple stem grooving virus (ASGV), apple mosaic virus (ApMV) and, apple chlorotic leaf spot virus (ACLSV). Combination attacks of three pathogens result in severe reductions in fruit quality and quantity (Ma et al., 2016).

Holmes (2019), indicated that high rates of mutation in RNA viruses lead to the formation numerous genetic variety in pathogen genome. According to Liu et al. (2012), five of ASPV genes exhibited high variation, mainly in the ORF that encodes the CP, which made it imperative to research the ASPV variation according to the CP. Further researches have revealed that the CP is made up of a variant N-terminal and a conserved C-terminal region (Yoon et al., 2014). In fact, numerous ASPV isolates varies greatly in both length and sequence of CP that range from 1125 to 1245 nt (Komorowska et al., 2011; Yoon et al., 2014). By examining a section of the replicase-encoding ORF, a remarkable genetic variance in isolates of ASPV was also revealed (Mathioudakis et al. 2010).

Turkey is regarded as an important source of plant species' germplasm because it provides ecologically favorable circumstances for the majority of them (Nadeem et al., 2021; Tekin et al., 2022; Yeken et al., 2022). In terms of output tonnage, apples are among the most major fruits. The first studies on ASPV in Turkey started by Çağlayan et al. (2006). This research is followed only by studies conducted on the diagnosis and prevalence of the virus (Birişik et al. 2006; Birişik ve Baloğlu 2011; İlbağı vd., 2013). However, the phylogenetic structure of a ASPV population has not yet been studied in detail in Turkey. To fill this gap, an ASPV population was obtained and analysed in terms of phylogenetic structure from Isparta province, located in the Mediterranean region of Turkey, is a prominent province in terms of apple production. This papers describes the phylogenetic structure of ASPV by obtaining the gene sequences of the partial CP of virus genome. A better knowledge of the demographic structure of ASPV is likely to result from this study.

MATERIAL AND METHOD

Survey and DAS-ELISA

A total of 70 viral sypmtom showing apple leaf samples were collected from Isparta province of Turkey (Figure 1) during 2020-2021. Polyclonal antibodies were used for the Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay (DAS-ELISA) as described in Clark and Adams (1977) and in accordance with the manufacturer's instructions for ASPV detection (BIOREBA, Reinach, Switzerland). DAS-ELISA utilized controls, both positive and negative, provided by the kit. To evaluate change in color at 60 minutes following the addition of the substrate (p-nitrophenyl phosphate), a microplate reader (Thermo Scientific

Multiscan FC Microplate Photometer, USA) was used. A sample was deemed positive, if its mean absorbances were higher by at least two times than those of the negative controls.

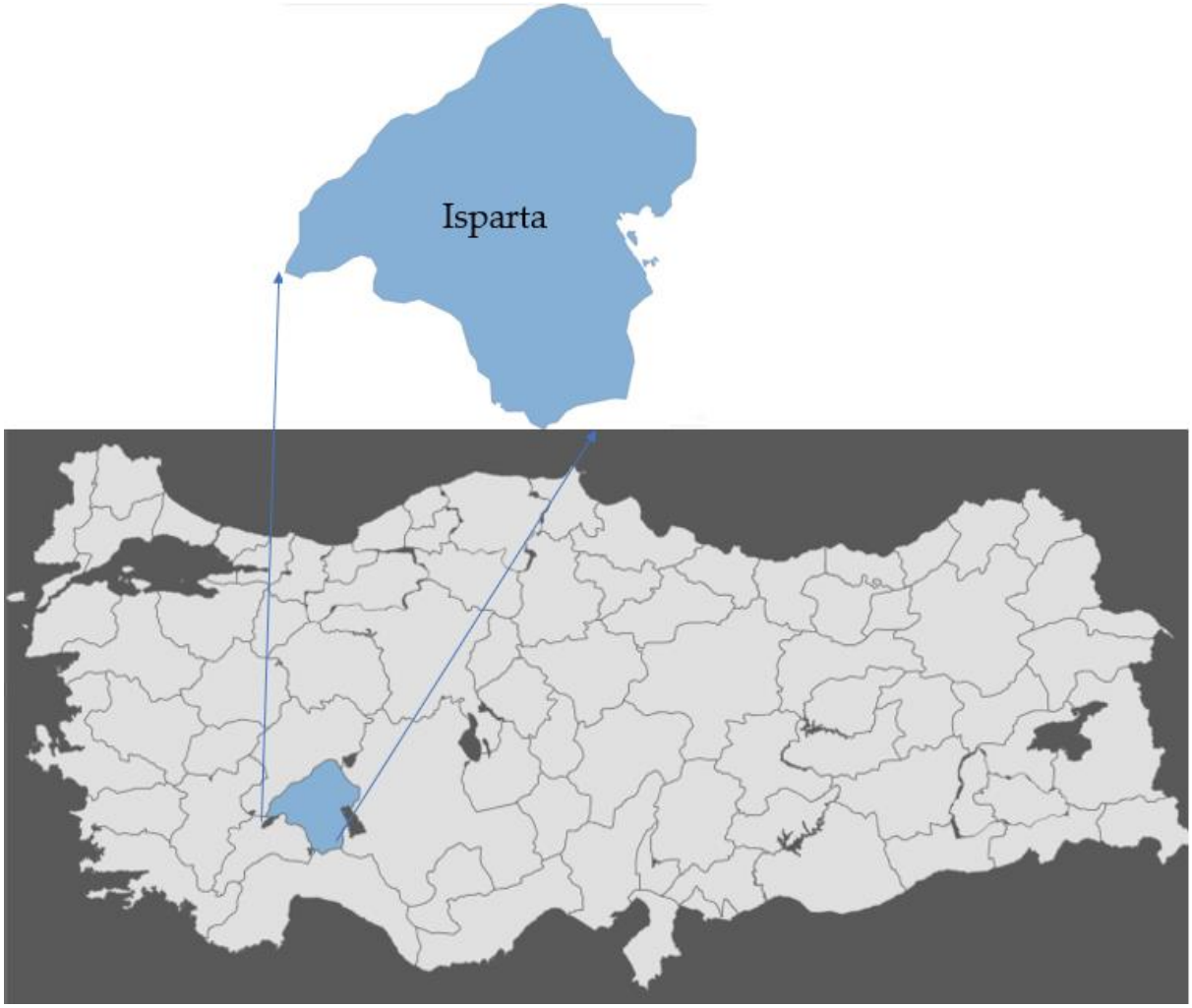

Figure 1. The location of survey area in Turkey.
Şekil 1. Survey bölgesinin Türkiye lokasyonu.

Extraction of Total RNA and Nucleotide Sequences

To verify the DAS-ELISA findings and obtain partial nucleotide sequences for the samples, seven samples that were signaled positive after the serology were also analyzed by RT-PCR. Ratio of ASPV (%) prevalence was determined by dividing the all of isolates that was positive in both RT-PCR and DAS-ELISA tests by all of the samples.

NucleoZOL RNA extraction liquid (Macherey-Nagel GmbH & Co. KG, Germany) was applied to obtain total RNA according to instructions of manufacturer from 120 mg leaf samples. The obtained RNA concentrations were adjusted to a concentration of 10 ng/µl using a Nanodrop spectrophotometer (Thermo Scientific, USA).

The 50 µl one-step RT-PCR mixture contained 2 µl RevertAid Reverse Transcriptase (Thermo scientificTM -EP0441), 1.5 µl of each primer (10 mM), 1.5 µl of RNA, 25 µl (1.5 U) *Taq* DNA Polymerase 2x Master Mix (contains 1.5 mM $MgCl_2$) (Ampliqon, Denmark), and nuclease-free water. The reaction was performed as described in Ji et al. (2013) by using ASPV-F (TGGAACCTCATGCTGCA) and ASPV-1R (TTGGGATCAACTTTACTAAAAAGCATAA) primers that produces 360 bp between 8878-9238 nt in ASPV genome. Amplified products were loaded into a 1.5% 1X TAE agarose gel and stained with

APronosafe DNA fluorescence marking (Condalab, Madrid, Spain). An electrophoresis equipment (ThermoFisher Scientific, Massachusetts, USA) was used for DNA visualization at 75 V for 60 minutes. The ampliqons were sequenced bi-directionally using the Sanger method by a commercial company (BM Laboratory Systems, Turkey).

Phylogenetic Analyses

The lowest BIC (Bayesian Information Criterion) results were obtained for the nucleotides aligned by MEGA X v.10.2.4 with Gamma Distribute Rates among Sites (K2+G) (Kumar et al., 2018) using the Kimura-2 parameter model (Kimura, 1980). As a result, the K2+G employed for Neighbor-Joining (NJ) statistical methods were applied to create the phylogenetic tree. Sequence Demarcation Tool (SDT) v1.2 software was used to figure out the percentage of amino acid (aa) and nucleotide (nt) sequence identities, and branches were supported by 1000 bootstrap replications (Muhire et al., 2014). An ACLSV isolate (KY579378) was inclued as outgroup for the phylogeny.

RESULTS AND DISCUSSION

Survey and Incidence

ASPV is widespread throughout the world, but it mostly infects members of the *Maloideae* family, which includes pears and apples. Other knowledge of ASPV infection also include grapevine from South Africa (GenBank No.: EU247940.1–EU247950.1), wild members of *Rosaceae* from Greece (Mathioudakis and Katis, 2006), and cherries from India (Dhir et al., 2010). This paper includes the ASPV infections in apple trees in Isparta, an important apple producer provinve of Turkey. In order to test for ASPV, 70 apple leaf samples were obtained from the province of Isparta by field surveys. Seven samples were shown to have ASPV infection, according to the serological identification research by DAS-ELISA. Despite the new detection assays such as high-throughput sequencing (HTS) analysis (Morán et al., 2020), are used for the ASPV detection, the DAS-ELISA is still widely used ASPV detection method in different studies (Dhir et al., 2010; Ma et al., 2019; Dhir et al., 2021). In this research, DAS-ELISA has been successfully used in the diagnosis of ASPV and its results are consistent with RT-PCR.

A total prevalence rate of 10% for ASPV in the locations studied was determined by RT-PCR testing on all of these samples. In the studies conducted in Turkey on ASPV infection, surveys have been carried out in various regions of Turkey (Birişik et al., 2006; Çağlayan et al., 2006; Birişik ve Baloğlu, 2011; İlbağı et al., 2013), and it has been observed that there is not yet a comprehensive survey related to Isparta. In studies conducted in K.Maraş, Osmaniye and Adana on prevalence studies of ASPV, biological indexing studies show a prevalence of 54.5% for ASPV (Birişik et al., 2006). Similarly, in another studies conducted by Çağlayan et al. (2006) and Birişik ve Baloğlu (2011), revealed that ASPV caused infection at different rates in different plant varieties. A recent study of the Spanish-growing regions for loquats, a 15% ASPV infection incidence rate was found (Morán et al., 2020). There are possible reasons for the variability of results incidence. For example, the use of various primer pairs that correspond to various regions of the ASPV genome or the use of various detection techniques are potential explanations for the differences in ASPV detection rates between the various studies (Komorowska et al., 2010). In addition, viral titres may vary during the year (Mathioudakis et al., 2009) or in various plant tissues (Klerks et al., 2001). Isparta is one of the provinces of Turkey that stand out with its intensive apple production. Isparta province was surveyed within the scope of surveys and ASPV infection was detected. The data produced by this study showed that ASPV maintains its importance and the virus can be detected in new regions.

Nucleotide Sequences and Phylogenetic Analyses

The deduced sequences had a length of 303 nucleotides and included the partial CP gene sequences of the ASPV. NCBI GenBank accession numbers OP476707–OP476713 were acquired for the sequences. As a consequence, the NJ phylogenetic analysis based on CP region, which is from different regions of the world (Table 1) and reliable for molecular typing of ASPV, comprised all 40 isolates (32 Genbank + 7 new + 1 out group isolate). NJ methods constructed a phylogenetic tree based on the CP region (Figure 2).

Table 1. The selected isolates for phylogeny representing the different parts of the world.
Tablo 1. Filogeni için dünyanın farklı bölgelerini temsil eden izolatlar.

Accesion	Origin	Host
KM873721	India	Apple
FR694922	India	Apple
FJ970961	Turkey	Apple
OM313368	China	*Pyrus betulifolia*
OM313375	China	*Pyrus betulifolia*
AF491930	Poland	Apple
AF491930	Poland	Apple
FJ970955	Belgium	Apple
HM125156	China	Apple
MW843002	Brazil	Apple
KC791787	South Korea	Apple
FJ970951	Ukraine	Apple
KC791786	South Korea	Apple
EU314950	China	Apple
KY429159	Poland	Pear
JX673785	China	Pear
JX673788	China	Pear
KY176828	Poland	Apple
MW810256	Greece	Quince
AF345895	Poland	*Pyrus communis*
AJ968944	Czech Republic	Apple
KY176810	Czech Republic	Pear
KY176812	Poland	Pear
KY176813	Poland	Pear
MW842992	Brazil	Apple
OM313373	China	*Pyrus betulifolia*
OM313367	China	*Pyrus betulifolia*
HM125160	China	Apple
GQ265914	China	Pear
JF946773	China	*Pyrus communis*
JF946774	China	*Pyrus communis*
FJ970960	Turkey	Apple

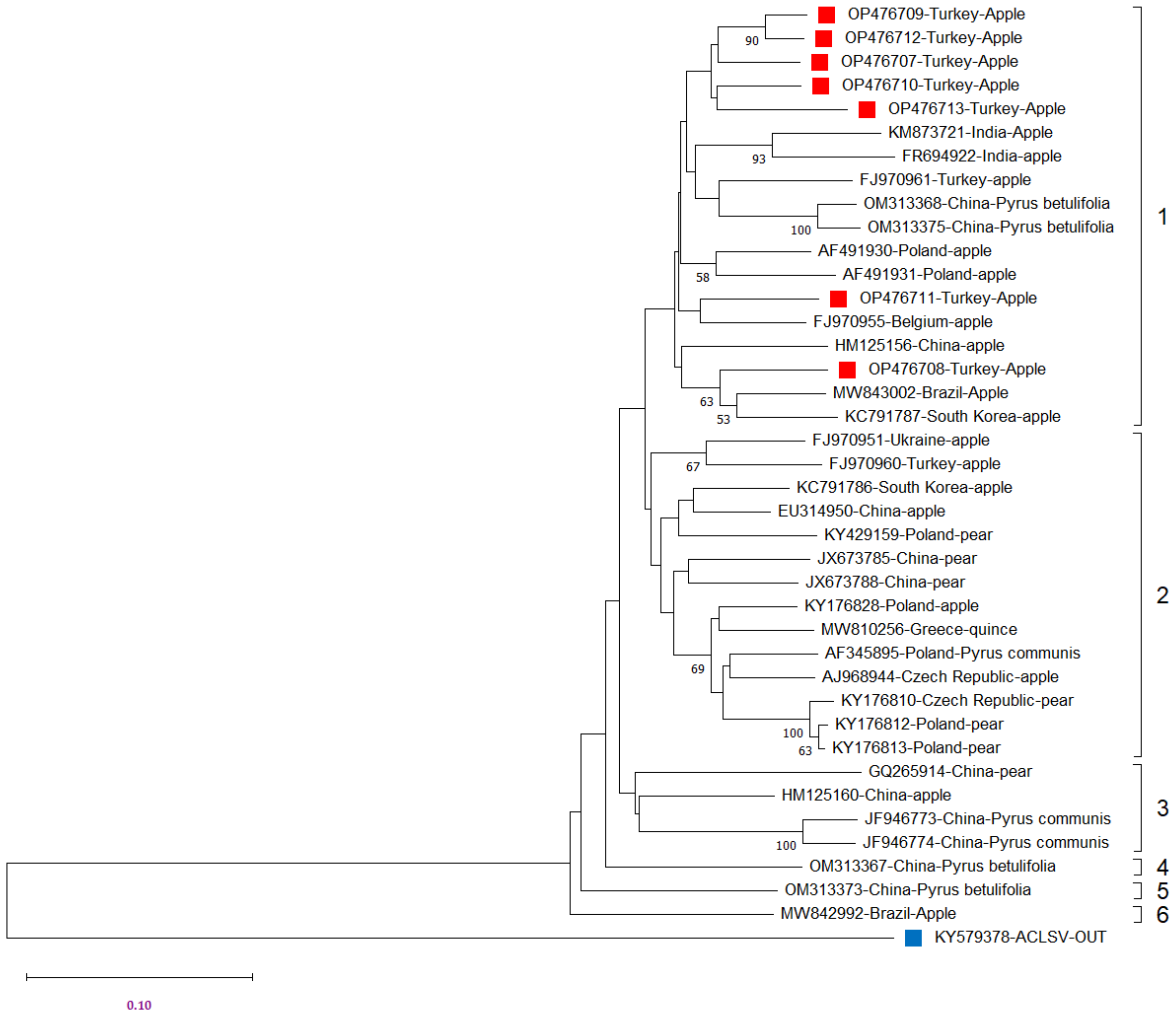

Figure 2. Phylogenetic analysis using neighbor-joining based on the nucleotide sequences of the ASPV CP region. (Red highlights indicated new isolates from this study and only >55% values were shown).
Şekil 2. ASPV CP bölgesinin nükleotid dizilerine dayalı komşu birleştirme yöntemi ile oluşturulan filogenetik ağaç. (Kırmızı işaretli izolatlar, bu çalışmadan elde edilen yeni izolatları göstermekte olup sadece % 55 üzeri değerlere yer verilmiştir).

The phylogenetic tree shows that the isolates selected from Genbank and the isolates newly sequenced in this study were clustered in six different phylogroups. Figure 2 shows that seven novel isolates sequenced by this study clustered in group 1 with other 18 isolates from Poland, Belgium, China, Brazil, South Korea, India and Turkey. The phylogrup 2 consists of 14 isolates from Turkey, Ukraine, South Korea, China, Poland, Greece and Chech Rrepublic. While the phylogroup 3 has the Chinesee isolates, the other phylogroups hosted isolates from China and Brazil. The constructed phylogenetic tree revealed that there is no gruping in terms of origin and host plant for ASPV. The coloured matrix constructed by using SDT showed that the similarity rate of new Turkish isolates mostly ranged between 79-93% (Figure 3) while the newly sequenced isolates OPF476711, OPF476713 and OPF476708 has a similarity index lower than 79 %. In a similar study, he CP gene showed considerable diversity across all sequenced viral isolates (70.7-93.5% at the nucleotide level and 77.8-98.7% at the amino acid level) (Komorowska et al., 2010). The findings obtained in the study are in agreement with our research.

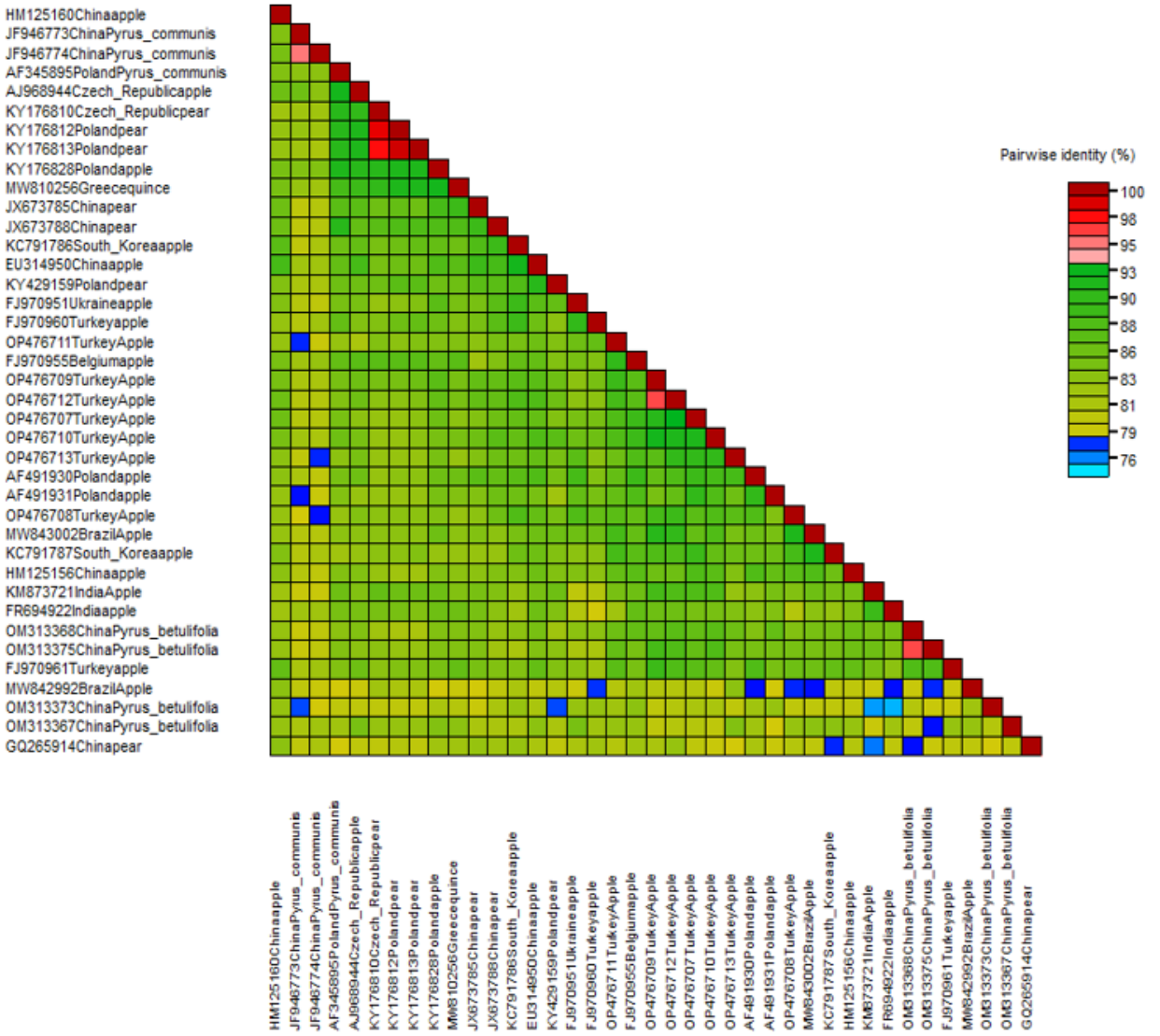

Figure 3. Coloured similarity matrix of newly sequenced isolates with GenBank reference isolates for phylogenetic tree.
Şekil 3. Filogenetik ağaç için GenBank referans izolatları ile bu çalışmada elde edilen izolatların renkli benzerlik matrisi.

A study revealed considerable inter- and intra-group variability across the samples from various nations and host plants, demonstrating the existence of ASPV based on CP sequences, host plant, and the chosen identification assay showing isolates had a propensity to cluster together in a phylogenetic analysis. (Mathioudakis et al., 2021). Another study in agreement with the findings in this article was Yoon et al. (2014), revealed that whereas the amino acid sequence similarity of eight ASPV isolates ranged from 87.7 to 98.5%, the nucleotide sequence identity ranged from 77.0 to 97.0%. In another study with different results from our study, the phylogenetic tree obtained with ASPV isolates was divided into two main groups (Hu et al., 2017). The reasons for these different findings may be the number of isolates, the number of data entered from countries, and the differences in the studied gene region. According to previous finding (Liu et al., 2012), the ASPV isolates' phylogenetic grouping seemed to match the hosts from which they were separated; however, the results of this investigation lacked sufficient sequencing data to substantiate these findings. Since prior studies (Komorowska et al., 2010; Wu et al., 2010; Liu et al., 2012; Yoon et al., 2014) mostly addressed on molecular features of CP of ASPV isolates from Korla pear and apple, we verified the connection between the apple isolates.

CONCLUSION

In conclusion, this papers includes the prevalence rate and molecular characterization of ASPV isolates obtained from Isparta, an important apple producing province of Turkey. The research describes the

sequence information for the CP gene region of ASPV isolates and reveals their phylogenetic relationships according to the CP gene region with reference isolates in GenBank. Research results contain information about the latest situation of ASPV in Turkey. In order to better understand the prevalence rate and population structure of ASPV in Turkey, it may be recommended to conduct surveys in different areas and to sequence different regions of the virus genome.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare that are relevant to the content of this article.

DECLARATION OF AUTHOR CONTRIBUTION

A.Ç: field surveys, serological and molecular detection tests, preparing sequence alignments, phylogenetic analyses, writing and editing the manuscript.

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