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ORIGINAL ARTICLE

First complete sequences and genetic variation of plum pox virus T strain in *Prunus dulcis* and *Prunus cerasus*

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Abstract

The complete genome of plum pox virus strain T isolates from five different *Prunus* spp., including almond (*P. dulcis*) and sour cherry (*P. ceracus*) isolates, was fully sequenced using the primer pairs designed in this study. The five isolates were aligned with other 50 PPV-T isolates whose complete genome sequences were available in GenBank and then subjected to phylogenetic and diversity analyses. Recombination analysis showed no significant signal detected in the five newly sequenced isolates while confirming four recombinant isolates reported in a previous study. Nucleotide and amino acid phylogenetic trees clustered the tested isolates into three major groups: Balkan 1, 2, and 3. Strain T isolates shared high nucleotide and amino acid identities among them. Diversity analysis applied different parameters to found that the sequences of P3 and 6K1 genes were more conserved over other genes. In accordance, the highly variable P1 and CP genes were found to experience weaker purifying pressures, $\omega = 0.127$ and 0.219, respectively, than other genes. The three neutrality tests gave negative values to all genes, suggesting that strain T populations have expanding or bottleneck selections. Genetic make-up of the only known sour cherry isolate is highly identical to isolates from other *Prunus* spp. Therefore, this study has updated our knowledge of T strain diversity in new hosts and provided a clear picture of genetic variation and host relationships.

Keywords Diversity and polymorphism analyses · Neutrality tests · Phylogenetic analysis · Recombination analysis · RT-PCR

Introduction

The 'Sharka' disease, caused by plum pox virus (PPV) infection on stone fruits (*Prunus* spp.), often results in large yield losses. The virus is widespread and still spreading to new regions where *Prunus* spp. was cultivated (Çelik et al. 2022a). Aphids transmit the virus for short distances, and then contaminated plant materials further transport the virus over long distances from one area to another (Isac et al. 1998; Akbaş et al. 2013). Variations in virus strains, host plant species and cultivars, and environmental conditions affect how symptoms manifest in PPV-infected plants. However, its typical symptoms are generally light green rings, streaks, and dots on leaves and fruit. The infected fruits may

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also show visible anomalies, a sugar content reduction, a taste decline, and an early fruit deterioration. The disease has forced many European countries to remove infected trees on thousands of hectares of planting areas (Cambra et al. 2006). Protective measures are the mainstay of the fight against PPV, and biological, serological, and molecular techniques are used to identify the virus early on (Gildow et al. 2004).

PPV genome is typical of a member of *Potyvirus*; it has an ssRNA molecule of approximately 9700 nucleotides in length and is organized into one long open reading frame (ORF) that encodes a single polyprotein weighing 355.5 kDa (James et al. 2013). Because PPV may infect a wide range of hosts and is spread by aphids and other propagation materials, isolates may be introduced into many different geographical areas (Glasa and Candresse 2008). This made it possible for the genome of an isolate to rapidly evolve through recombination with genomic fragments from other isolates and mutations caused by local population-specific causes (Coşkan et al. 2022). Consequently, among potyviruses, PPV has the most different strains including T (Turkey), M (Marcus), D (Dideron), Rec (Recombinant), An (Ancestor), EA (El Amar), W (Winona), C (Cherry), and CR (Cherry Russian) (García et al. 2014; Morca et al. 2020). Besides that, CV (Cherry Volga) has been reported as a possible new strain (Chirkov et al. 2018).

Turkey is high in the world ranking with its approximately 3 million tons of stone fruit production (TUIK 2022). Its agroecological conditions have made the country an excellent area for cultivating many stone fruit species. Unfortunately, these conditions have also made the country a diversity center for viral species infecting stone fruits. PPV-T, a strain first described in Turkey (Ulubaş Serçe et al. 2009), is widespread in the country (Teber et al. 2019; Çelik and Ertunç 2021) but has not been reported in other countries except for Albania (Palmisano et al. 2015). Several studies have concluded that PPV-T is the dominant strain in the Turkish PPV pool infecting plum (*Prunus domestica*), peach (*P. persica*), apricot (*P. armeniaca*), and almond (*P. dulcis*) (İlbağı and Çıtır 2014; Gürcan and Ceylan 2016; Teber et al. 2019). While there is no report of PPV-T in sweet cherry (*P. avium*), a single isolate was serologically and molecularly detected naturally infecting sour cherry (*P. cerasus*) recently (Coşkan et al. 2022).

Recombination, mutation, natural selection, migration, and genetic drift are the five primary processes driving natural evolution (Rubio et al. 2013; Tokhmechi et al. 2021; Çelik et al. 2022b). Plant viruses possess enormous potential for genetic diversity, countless recombination events, high mutation rates, quick evolution, and environmental adaption (Dolan et al. 2018). Understanding numerous variants is required for research on the molecular evolutionary history of viruses, which is exceedingly difficult (Moury et al. 2004; Coşkan et al. 2022). For plant RNA viruses, particularly in the populations of potyviruses, recombination, and mutation are the most frequent evolutionary factors in genetic diversity and variation (Gibbs et al. 2020).

In this study, we provided complete genome sequences of plum, peach, apricot, and almond isolates as well as the first PPV-T infecting sour cherry isolate, which all were reported by Coşkan et al. (2022), and conducted a population study based on the full ORF and individual genes sequences with other isolates available in NCBI (National Center for Biotechnology Information) GenBank database. Previously only a short fragment of genomes of the almond and sour cherry isolates have been sequenced and studied (İlbağı and Çıtır 2014; Coşkan et al. 2022), thus this research could give a clearer picture of the current strain T diversity in Turkey. It might also close some knowledge gaps on the genetic variation and host relationships since only strains C and CR were previously associated with Sharka in sour cherry (James et al. 2013; Garcia et al. 2014; Jelkmann et al. 2018).

Materials and methods

PPV-T isolates, primer design, RT-PCR, and sequencing

In this current study, we completed the full genome sequencing of the first PPV-T sour cherry (P13 ANK) isolate as well as the plum (P81 ANK), peach (P93 ANK), apricot (P720 ANK), almond (P1086 ANK) isolates that had been partially sequenced and reported by Coşkan et al. (2022). Total RNAs extracted in the previous study (Coşkan et al. 2022) were used as templates in the RT-PCR tests using primer pairs designed to amplify different regions of the PPV-T genome. To design the new set of primers for whole genome sequencing, reference isolates of different PPV strains were downloaded from the NCBI GenBank database and then aligned using ClustalW algorithm (Thompson et al. 1994) in the Mega X program v.10.2.4 (Kumar et al. 2018). Oligonucleotides belonging to different regions of the PPV genome were generated in the Primer-BLAST software (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the aligned reference sequences. The map of primer positions and information on overlapping regions are shown in Supplementary Material-Fig. 1, and the primer sequences and references are given in Supplementary Material-Table 1.

The reactions for all primer pairs in the RT-PCR were performed in a volume of 100 µl: 24 µl 5 × GoTaq Flexi Buffer, 4 µl $MgCl_2$ (25 mM), 2.4 µl dNTPs (10 mM), 1 µl GoTaq polymerase enzyme (5U/µl), 4 µl forward primer (10 µM), 4 µl reverse primer (10 µM), 0.8 µl Reverse Transcriptase (200 U/µl, ProtoScript® II Reverse Transcriptase-M-MuLV), 0.8 µl RNase inhibitor (5000 U/ml), and 6 µl

RNA. RT-PCR amplifications were performed via a one-step protocol for all primer pairs. The thermal cycler programs were as follows: 50 min at 42 °C, 10 min at 95 °C, 35 cycles of 60 s at 95 °C, 60 s at annealing temperatures (Supplementary Material-Table 1), and 1.5 min at 72 °C, followed by a final extension for 10 min at 72 °C. PCR products were loaded into 1% 1X TAE agarose gel stained with Pronasafe nucleic acid staining solution (Conda, Madrid, Spain) for 1 h at 80 V and visualized under UV transillumination.

Bands of the expected size in all PCR products for each primer pair were purified from agarose gels and cloned into pGEM-T Easy (Promega Corp., Wisconsin, USA) as recommended by the manufacturer. Three independent clones were selected for each recombinant plasmid and then sequenced with plasmid-specific primers (T7-SP6) by a commercial firm (BM Laboratory Systems, Ankara, Turkey) using the Sanger method. Fragments of the raw sequence sequences were visualized and assembled into whole genomes using MEGA X software. The consensus sequences were subjected to BlastN analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with reference sequences in the GenBank database.

Phylogenetic analysis

The obtained full ORF sequences of five isolates were aligned with 50 PPV-T isolates in GenBank using the ClustalW algorithm (Thompson et al. 1994) implemented in MEGA X. Coat protein alignment was created by trimming the CP region according to PPV RefSeq (NC_001445). Using the lowest Bayesian Information Criterion scores, Tamura–Nei parameter model (Tamura and Nei 1993) with Uniform Rates among Sites was determined found to be the most suitable substitution model for the nucleotide (nt) analysis of ORF and CP datasets, while Jones-Taylor-Thornton (Jones et al. 1992) was for the amino acid (aa) analysis of the two datasets. Phylogenetic trees for nt and aa alignments of full ORF and CP were constructed using the Neighbor-Joining (NJ) statistical methods implemented in MEGA X, with 1000 bootstrap replications. The percentage identities of ORF and CP datasets at nt and aa levels were determined using Sequence Demarcation Tool (SDT) v1.2 (Muhire et al. 2014).

Recombination analysis

A possible recombinant event on full ORF alignment of 55 PPV-T isolates was scanned using RDP v.4.56 software with its suite options: 3Seq, Siscan, Bootscan, MaxChi, Chimaera, GENECONV, and RDP algorithms in default parameters (Martin et al. 2015). Any event confirmed by at least five algorithms (Bonferroni-corrected p value of < 0.05) was considered significant (Martin et al. 2015), and the recombinant isolate was not included in the diversity and population analyses to obtain more accurate results. The same analysis was performed on the alignment of full ORF of PPV-T sour cherry isolate (P13 ANK) with nine C and eight CR isolates to determine a possible inter-strain recombinant event.

Population structure and neutral selection analysis

The genetic variation in each PPV-T gene was reflected by different parameters: the number of haplotypes (h), haplotype diversity (Hd), the number of variable sites (S), the total number of mutations (η), the average number of nt differences between compared sequences (k), average pairwise nt diversity (per site) (π), the total number of non-synonymous sites (dN), total number of synonymous sites (dS), and transcriptional selection ($\omega = dN/dS$), was calculated using DnaSP v.6.12.03 program (Rozas et al. 2017). A gene was assessed to be experiencing positive (diversifying), neutral, and negative (purifying) selection when dN/dS is $> 1, = 1$, and < 1, respectively (Rozas et al. 2017). Neutral selection tests implemented in DnaSP v.6.12.03: Tajima's D (Tajima 1989), Fu and Li's D^* and F^* (Fu and Li 1993) were done with a window length of 100 sites and step size of 25 sites to measure genetic divergence on each PPV-T gene.

Results

Full genome sequences and phylogenetic analysis

The whole genome sequences of five PPV-T isolates were obtained and then deposited in GenBank with accession no. ON745776–ON745780. Neighbor-joining trees constructed based on nt and aa sequences of full ORF formed three major clades to cluster the tested isolates: Balkan 1, Balkan 2, and Balkan 3. All isolates were distributed precisely into the three major clades in both nt and aa trees of full ORF. The new five isolates were placed in Balkan 1 with other Ankara isolates, suggesting a more substantial relationship between diversity and location than hosts (Fig. 1). Phylogenetic trees based on nt and aa sequences of CP gene were generally clustered isolates in three groups, similar to those of full ORF trees, but some isolates formed branches outside the three major basal (Fig. 1). In line with the phylogenetic trees, Balkan 1 isolates were found by SDT analysis to have higher nt and aa identities to Balkan 2 than to Balkan 3 isolates at full ORF level (Table 1).

Recombination analysis

Significant recombination signals were detected in isolates with acc no. MF346235, MF346237, MF346250, MF346259 (Table 2). Analysis on the alignment of P13 ANK with nine

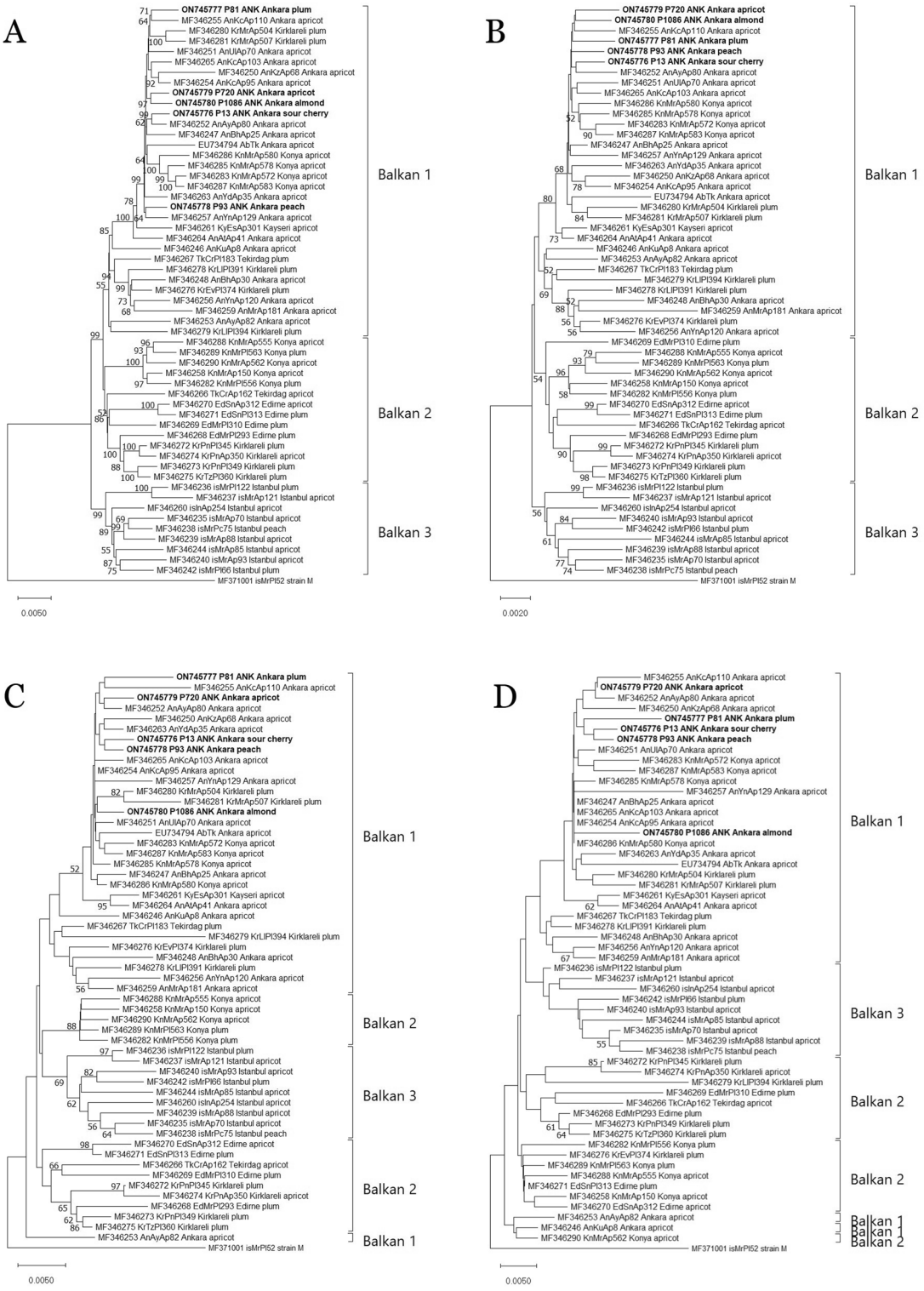

Fig. 1 Neighbor-joining phylogenetic analysis of the nucleotide (A) and amino acid (B) sequences of full ORF, and the nucleotide (C) and amino acid (D) sequences of coat protein gene of PPV strain T, generated by MEGA X software. Branches were supported with 1000 bootstrap replicates, only values of > 50% were shown. Five new isolates from different *Prunus* spp. in Ankara province were printed in bold and clustered in Balkan 1 group, showed closer relationship of geographic location than plant hosts. A strain M isolates (isMrPl52) was used as out-group

Table 1 Identity percentages at full ORF level among different phylogroups of PPV strain T tested in this study

	Balkan 1		Balkan 2		Balkan 3	
	nt (%)	aa (%)	nt (%)	aa (%)	nt (%)	aa (%)
Balkan 1	97.6–99.7	98.5–99.6	97.2–98.4	98.4–99.3	97.1–98.3	98.4–99.3
Balkan 2			97.8–99.6	98.8–99.8	96.8–98.2	98.5–99.2
Balkan 3					97.8–99.2	98.9–99.4

Table 2 Putative recombination events detected by RDP4 analysis of the full ORF sequences of five new and 50 GenBank PPV strain T isolates

No.	Recombinant	Parents: major/minor	Breakpoints[1] (begin/end)	RDP-implemented method[2] (*p* value)
1	MF346235	MF346238/unknown	5812–6590	R (7.063×10^{-4}) B (5.526×10^{-4}) M (1.876×10^{-3}) C (9.255×10^{-5}) S (6.660×10^{-4}) 3S (2.063×10^{-2})
2	MF346237	MF346244/unknown	5792–6908	R (6.729×10^{-7}) G (2.969×10^{-9}) B (1.247×10^{-7}) M (1.647×10^{-5}) C (4.771×10^{-6}) S (5.596×10^{-6}) 3S (5.314×10^{-7})
3	MF346250	MF346254/unknown	3974–4446	R (4.777×10^{-21}) G (6.657×10^{-19}) B (7.233×10^{-19}) M (1.518×10^{-11}) C (1.223×10^{-10}) S (3.142×10^{-11}) 3S (4.541×10^{-23})
4	MF346259	EU734794/unknown	5450–5948	R (3.248×10^{-2}) G (2.360×10^{-4}) B (3.591×10^{-6}) M (1.582×10^{-3}) C (4.917×10^{-2}) S (1.387×10^{-8})

[1] Position in alignment

[2] **R** RDP; **G** GENECOV; **B** BootScan; **M** MaxChi; **C** Chimaera; **S** Siscan; **3S** 3Seq

C and eight CR isolates did not observe any significant recombination event.

Population structure and neutral selection analysis

The structure of PPV-T populations according to genetic variation and polymorphism of the full ORF and each of the genes was estimated using several genetic diversity parameters. The largest variable sites ($S=351$), the average number of nt sequences differences between isolates from the same population ($k=30.805$), and mutation within the segregation sites ($\eta=366$) were assigned to Cl gene while the largest average pairwise nt diversity (per site) ($\pi=0.019$) was obtained by three coding regions: HC-Pro, 6K1, and NIa-Pro (Table 3). The ω values for full ORF and all tested genes were all found to be below 1, with the maximum and minimum were estimated for CP (0.219) and Nia-VPg (0.013), respectively (Table 3).

Neutral selection analysis by Fu and Li's *F** and Tajima's *D* tests consistently allocated negative values to all the tested genes, while Fu and Li's *D** estimated positive values only for P1 gene (Table 4). Furthermore, all of the obtained values were statistically significant except those assigned to 6K1 genes (Table 4), suggesting high confidence in the calculation.

Discussion

The nt and aa phylogenetic tree constructed in this study supported a previous report that the European part of Turkey (in the Balkan) likely originated PPV-T isolates

Table 3 Summary of genetic diversity and polymorphism analyses on full ORF and different gene regions of PPV strain T

Coding region	*N*	*h*	*Hd*	*S*	η	*k*	π	dS	dN	ω
Full ORF	51	51	1.000	1761	1862	163.673	0.017	0.061	0.004	0.066
P1	51	51	1.000	195	208	16.733	0.018	0.055	0.007	0.127
HC-Pro	51	51	1.000	279	301	25.605	0.019	0.068	0.004	0.059
P3	51	51	1.000	165	169	16.855	0.016	0.054	0.005	0.093
6K1	51	29	0.937	28	28	3.029	0.019	0.081	0.002	0.025
Cl	51	51	1.000	351	366	30.805	0.016	0.061	0.003	0.049
6K2	51	29	0.918	28	29	1.918	0.012	0.045	0.003	0.067
NIa-VPg	51	49	0.998	104	109	10.131	0.018	0.075	0.001	0.013
NIa-Pro	51	49	0.998	148	153	14.419	0.019	0.081	0.002	0.025
NIb	51	51	1.000	285	302	27.542	0.018	0.065	0.004	0.062
CP	51	51	1.000	178	197	16.637	0.017	0.041	0.009	0.219

N number of isolates, *h* number of haplotypes, *Hd* haplotype diversity, *S* number of variable sites, η total number of mutations, *k* average number of nucleotide differences between sequences, π nucleotide diversity (per site), dN non-synonymous nucleotide diversity, dS synonymous nucleotide diversity, ω = dN/dS

Table 4 Summary of demography test statistics of full ORF and different gene regions of PPV strain T

Population	Fu and Li's *D**	Fu and Li's *F**	Tajima's *D*
Full ORF	− 3.74052**	− 3.77426**	− 2.20822**
P1	4.29969**	− 4.23619**	− 2.29922**
HC-pro	− 3.82209**	− 3.85054**	− 2.23479**
P3	− 3.46658**	− 3.47393**	− 1.97960*
6K1	− 2.18366 ns	− 2.38972 ns	− 1.69219 ns
Cl	− 3.70645**	− 3.77285**	− 2.25367**
6K2	− 4.00069**	− 4.04986**	− 2.32313**
NIa-VPg	− 2.61762*	− 2.88319*	− 2.06831*
NIa-pro	− 3.03398*	− 3.19107*	− 2.06435*
NIb	− 3.50872**	− 3.57292**	− 2.13496*
CP	− 3.76540**	− 3.80962**	− 2.23238**

ns not significant

*$p < 0.05$

**$p < 0.02$

(Teber et al. 2019). Isolates clustering into smaller groups based on geographic locations and variation in complete genome sequences: Ankara-Konya1-Kayseri, Ankara-Balkan, Istanbul, Konya2, and Balkan proposed by Teber et al. (2019). However, we divided the 55 tested isolates into three major phylogroups in this study. We named them Balkan 1, 2, and 3 (Fig. 1) since rapid isolates movement among regions within Turkey will likely make the previous version of clustering obsolete quickly. The Balkan 3 consists of evolutionary isolated isolates so far were only found in Istanbul province. All five newly sequenced isolates from Ankara province were distributed in the Balkan 1 group, although they were from different host species. Meanwhile, other isolates from different *Prunus* spp. were also spread in all three phylogroups. Therefore, currently there are stronger correlations between phylogroups and geographic locations than host species. These results also showed that isolates from phylogroup potentially have a broad host range. However, complete sequences of PPV-T isolates from the Eastern part of Turkey and other countries are still needed to accurately determine the origin and diversity center of strain T.

Molecular characterization of the sour cherry isolate of PPV strain T (P13 ANK) at full genome level was crucial as previously only strains C and CR were known to cause natural infection in sour cherry (James et al. 2013; Garcia et al. 2014; Jelkmann et al. 2018). It has been observed that the shared unique aa substitutions in the middle part of P1 and NIa and the N terminus of the capsid protein might be responsible for strains C and CR molecular adaptation to cherry hosts (Glasa et al. 2013). On the other hand, P13 ANK does not have those distinctive features but shares very high nt and aa identities with other T isolates instead. These demonstrated that the numbers of nt and aa mutations in the P13 ANK genome were negligible; thus, T isolates readily infect sour cherry without specific genetic changes. Likewise, sour cherry is highly likely susceptible to other T isolates.

RDP analysis also confirmed no inter-strain recombination event between P13 ANK with known C and CR isolates. The results of intra-strain RDP analysis were in line with a previous study which deduced MF346235 (isMrAp70), MF346237 (isMrAp121), MF346250 (AnKzAp68), MF346259 (AnMrAp181) to be recombinant isolates (Teber et al. 2019). There was no significant recombinant signal found in the genome of five newly sequenced isolates in this study.

The genetic diversity on the complete CP and two other regions of the genome of T isolates had been reported before (Coşkan et al. 2022). However, such information for

the other genes was unavailable. The analysis of this study showed lower divergence in the sequences of P3 and 6K1 genes, commonly used to differentiate PPV strains (Glasa et al. 2002), compared to some other genes, including P1, HC-Pro, NIb, and CP. The N-terminal region of CP has been known to have diverse mutations that did not affect PPV fitness in *Prunus* spp. (Carbonell et al. 2013).

All tested genes received ω values < 1, indicating negative (purifying) selection pressure was at work. P1 ($\omega = 0.127$) and CP ($\omega = 0.219$) genes were observed to be under weaker constrain than other genes. Therefore, P1 and CP were more prone to diversification than other genes, in agreement with the diversity analysis results, which deduced high genetic variability in both genes. Similarly, the P1 and CP of bean common mosaic virus (BCMV), another *Potyvirus*, also obtained higher dN/dS ratios than other genes (Abadkhah et al. 2020).

The three neutrality tests assigned negative values to all genes suggested that the currently highly similar strain T populations are now evolving out of expanding or bottleneck selections. Therefore, it is likely that there will be more division of strain T into new subgroups or even distinct phylogroups in the future.

Conclusions

This study demonstrated that the only PPV-T isolated from sour cherry (P13 ANK) shared very high nt and aa identities with other T isolates. Furthermore, there was no recombination between the genome of P13 ANK with sour cherry isolate of C and CR. Coşkan et al. (2022) reported that sour cherry infection with PPV is rare, and an additional molecular identification study focused on sour cherries is needed. Therefore, more identification stud is needed to uncover the diversity of sour cherry isolates of PPV in Turkey. The knowledge obtained in this study showed the need to extend surveys into eastern region of Turkey to understand the origin of strain T thoroughly. The information presented here might also advance our control strategies for PPV.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s13205-023-03746-1.

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Author contributions All authors contributed to the study conception and design. BA: conceptualization, investigation, funding acquisition, writing, review and editing. AFM: resources, methodology, investigation, writing, funding acquisition, review and editing. SÇ: investigation, resources, writing. AIS: methodology, formal analysis, writing—original draft. HÇK: investigation, writing—review and editing AÇ: formal analysis, writing—original draft, writing—review and editing. All authors have read and approved the final manuscript.

Data availability The nucleotide sequences of present study are available in the GenBank repository (https://www.ncbi.nlm.nih.gov/genbank/) with the accession number ON745776, ON745777, ON745778, ON745779, and ON745780.

Declarations

Conflict of interest The authors declare that they have no conflict of interest in the publication.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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