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شناسایی بیوانفورماتیک دو ویروس زعفران در ایران با استفاده از آنالیز داده‌های توالی‌یابی با راندمان بالا

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چکیده

زعفران (*Crocus sativus*) یک گیاه چند ساله با ارزش است که به‌طور وسیعی در ایران بعنوان یک گیاه ادویه‌ای و دارویی کشت می‌شود. بیمارگرهای متعددی از جمله ویروس‌ها می‌توانند زعفران را آلوده کرده و کیفیت و کمیت محصول را کاهش دهند. بدلیل دردسترس نبودن مواد شیمیایی ضد ویروسی بصورت تجاری، تشخیص دقیق و قابل اعتماد یک جنبه حیاتی در مدیریت بیماری‌های ویروسی است. امروزه توالی‌یابی با بازده بالا (high throughput sequencing) به تکنولوژی رایج تشخیص ویروس‌های شناخته شده و نیز ویروس‌های جدید در گیاهان تبدیل شده است. در این مطالعه با استفاده از آنالیز توالی‌یابی با بازده بالا، ویروس پنهان زعفران (SaLV) و ویروس تی هسته داران (PrVT) درداده‌های ترانسکریپتوم حاصل از خامه زعفران شناسایی شد و ترادف تقریبا کامل این ویروس‌ها بدست آمد. ترادف نوکلئوتیدی SaLV ۹۸,۳ درصد یکسانی ترادف با تنها جدایه موجود در ژن بانک داشت. کمترین و بیشترین میزان یکسانی ترادف نوکلئوتیدی جدایه زعفران PrVT با تپوویروس‌های موجود در ژن‌بانک بین ۴۲,۹ درصد (ویروس تی سیب زمینی جدایه پرو) و ۷۶,۹ درصد (جدایه GR168 ویروس تی زیتون(OlVT)) بود. در درخت تبارزایی، جدایه زعفران PrVT به همراه دو جدایه OlVT از یونان و یک جدایه PrVT در یک گروه قرار گرفتند. این تحقیق، اولین گزارش از وقوع PrVT بر روی زعفران در دنیا می باشد.

کلمات کلیدی: ویروس، زعفران، HTS، ایران، PrVT

In silico identification and phylogenetic analysis of two saffron infecting viruses in Iran using high throughput sequencing

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Abstract

Saffron (*Crocus sativus*) is a high-value perennial plant cultivated widely in Iran as a spice and medicinal plant. Several pathogens including viruses can infect saffron and decrease its quality and quantity. Precise and reliable detection is a critical aspect of disease management in viral diseases because of the lack of commercially available chemicals against plant viruses. Nowadays, high throughput sequencing (HTS) has been considered as a routine technology for detection of known and novel viruses in commercial plants. In this study, using HTS data, saffron latent virus (SaLV) and prunus virus T (PrVT) were identified in transcriptomic data of stigma of saffron and near complete genome of these viruses were obtained. The near complete genome of SaLV isolate had 98.3% nucleotide sequence identity with the only available isolate in GenBank. The complete genome of saffron isolate of PrVT shared 42.9 (potato virus T isolate Peru) – 76.9 % (olive virus T isolate GR168) nucleotide sequence identity with those of tepoviruses available in GenBank. In a phylogenetic tree, the PrVT isolate clustered with two Greece OlVT isolates along with an isolate of PrVT. To the best of our knowledge, this is the first report of PrVT infection in saffron worldwide.

Keywords: Saffron, Virus, HTS, Iran, PrVT

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Introduction

Saffron (*Crocus sativus* L.) is a perennial plant in the iris family (Iridaceae) which is used as a spice and medicinal plant. Because of its safranal, picrocrocin and crocin content, saffron has anti-depressant, antiviral (Soleymani *et al.* 2018) and anti-cancer (Escribano *et al.* 1996) and so many other health beneficial properties. Iran with 350 tons of saffron is known as the largest producer in the world and accounts for about 90% of its total production (Negbi 1999; Shahnoushi *et al.* 2019). Saffron is cultivated in most provinces of Iran among which Razavi Khorasan province is one of the main saffron- production regions in the country (Koocheki 2018). Despite this, the yield of saffron per hectare in Iran is too low (3.17- 4.32 kg/ha) compared to other major producers including Spain, Italy and Greece which obtain much higher yields (up to 10 kg/ha) (Gresta *et al*. 2008; Koocheki 2018; Koocheki & Seyyedi 2019). Unfavorable weather conditions, soil nutrient deficiency, pest and diseases may reduce its productivity (Koocheki & Seyyedi 2019).

Plant viruses pose great economic losses to the agricultural production. Several viruses have been reported on saffron. So far, bean yellow mosaic virus (BYMV) (Caiola & Faoro 2011), saffron latent virus (SaLV) (Parizad *et al.* 2016; Parizad *et al.* 2017; Tavakoli Bardaskan *et al.* 2023) and turnip mosaic virus (TuMV) (Heidari *et al.* 2018) all belonging to the family *Potyviridae* has been reported on saffron from Iran on mostly asymptomatic saffron samples. *Potyviridae* is the second largest family of plant viruses including 12 genera of both mono and bipartite single-stranded positive sense RNA (+ssRNA) viruses with flexuous filamentous particles. The members of *Potyvirus*, the largest genus, are transmitted non-persistently by aphids, mechanical inoculation or via seeds. SaLV has been recently approved as a new species in the genus (Inoue-Nagata *et al.* 2022). It was first identified in Iran on asymptomatic saffron samples with above 70% of the samples tested positive for the virus. Since saffron is propagated via vegetative corms, the high prevalence of SalV was not surprising (Parizad *et al.* 2017).

Tomato spotted wilt virus (TSWV; genus *Orthospovirus*, family *Tospoviridae*) also has been detected on saffron in South Khorasan province of Iran (Farokhoond *et al.* 2017). TSWV has a broad host range (more than 1000 plant species) and it can be transmitted through four genera of thrips in a persistent manner along with sap inoculation (EPPO 2022).

Narcissus mosaic virus (NMV) has been found to infect *Crocus* spp. in the Netherlands (Miglino *et al.* 2005). NMV belongs to the genus *Potexvirus* in the family *Alphaflexiviridae*. Potexviruses have flexuous filaments particles and a single linear +ssRNA genome. The members usually have limited host range and are transmitted by mechanical contact (ICTV Reports 2020).

The genus *Tepovirus* is a member of the family *Betaflexiviridae* which comprises type species potato virus T (PVT). The virions are flexuous filaments which contain a single stranded +ssRNA (Adams *et al.* 2012). By NGS analysis of dsRNAs from prunus trees, prunus virus T (PrVT) was characterized and proposed as a new member of the genus *Tepovirus* (Marais *et al*. 2015). Recently, Olive virus T (OlVT) has been proposed as a new species in Greek (Xylogianni *et al.* 2021). The virus was graft transmissible, though it was symptomless.

High throughput sequencing (HTS) has been considered as a routine and reliable technique for detection and identification of known and/or novel viruses (Çağlayan *et al.* 2019; Gazel *et al.* 2020; Çağlayan *et al.* 2020; Roumi *et al.* 2021). Contrary to the traditional methods, HTS does not need prior information about sequence of the pathogens. Hence, the number of reported known and novel viruses on different hosts is increasing rapidly (Maliogka *et al.* 2018). The discovery and characterization of new viruses in crops is crucial. Some of these new viruses can be emerged as potential pathogens threatening crops, or they can be used as biological control agents against other viruses either wild-type or recombinant viruses (Redila *et al.* 2021). Hence, the aim of this work was to study the virus/viruses infecting saffron in Iran using high throughput sequencing.

Materials and Methods

Plant material and sequencing

A saffron stigma tissue (Qaen isolate, Karaj-Iran) was subjected to RNA-seq analysis in Illumina HiSeq 2000 platform (90nt, paired reads) in order to elucidate its functional genomics (Mahmodi *et al.* 2014). The transcriptomic data was retrieved from the GenBank (SRS541821) and used for identifying viral sequences.

HTS analysis

The transcriptomic data was analyzed by Geneious Prime 2019.1.3 (https://www.geneious.com) for presence of possible virus and viroids. BBDuk 38.37 was used for trimming (adapters, low quality and short reads), SPAdes assembler 3.10.0 (Zerbino 2010) for de novo assembly and Geneious mapper for mapping the reads against references. Alignments of nucleotide (nt) and deduced amino acid (aa) sequences were carried out by MAFFT v7.450 (Katoh & Standley 2013). Contigs larger than 200 nucleotides were filtered using tBLASTx and BLASTn.

Phylogenetic analysis

Phylogenetic relationships among the isolates were analyzed using MEGAX (Kumar *et al.* 2018). The relevant sequences were retrieved from GenBank. Then multiple alignments were used to reconstruct a phylogenetic tree using neighbor-joining method (Saitou & Nei 1987) with 1000 bootstraps. RDP5 software (Martin *et al.* 2020) including RDP, Chimaera, BootScan, 3Seq, GENCONV, MaxChi and SiScan programs was used to investigate possible recombination events between isolates. Recombination signals were considered as valid if they could be detected by at least 3 methods.

Results

Data analysis

HTS resulted in 52,075,128 raw reads and, after adaptor removing and trimming steps, 41,727,310 unique reads remained which were *de novo* assembled by SPAdes into 74589 contigs ranging from 105-8722 nts in length. Contigs larger than 200 nts were screened by tblastx and blastn, resulted in identification of two viruses: PrVT and SaLV.

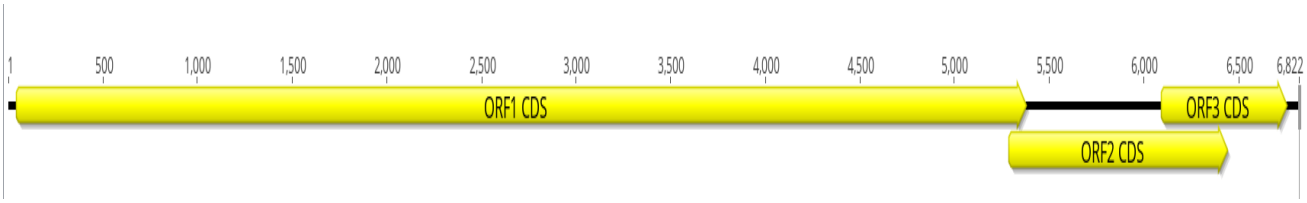


Figure 1. The genome organization of saffron isolate of PrVT from Iran. ORF1 encodes replication associated proteins (RdRp), ORF2 encodes movement protein (MP) and ORF3 encodes coat protein (CP).

Characterization of PrVT isolate of saffron

A 6822 nt long contig covering the full length of PrVT was recovered from the HTS dataset. The unique reads were mapped back to the contig under very stringent conditions in Geneious mapper (99% identity), which resulted in an identical consensus made from 29,518 reads (mean coverage = 367).

The contig was annotated using PrVT complete genome (Figure 1) and pairwise alignments were done with 17 full length *Tepovirus* genomes available in the GenBank including prunus virus T (PrVT), ficus tepovirus A (FiTA), potato virus T (PVT), trichosanthes tepovirus A (TrTA), zostera virus T (ZoVT) and cherry virus T (ChVT). The results are shown in Table 1. The complete genome of the saffron isolate of OlVT (ACC. No. BK061317) shares the highest identities with that of OlVT GR168 isolate (76.9%) while it had the lowest nt sequence identities with potato virus T isolates ranging from 42.9-43.9%. According to the species demarcation criteria in the family *Betaflexivirdae* including coat protein/polymerase nt identities less than 72% (Adams *et al.* 2012), the saffron isolate of PrVT is closely related to the two OlVT isolates and a PrVT isolate (C21). The RdRp and CP genes of saffron isolate of PrVT shared

75.2-75.8 and 80.8-82 percent nt identity, respectively, with those of OlVT isolates available in the GenBank (Table 1). It shared 73.4% and 79.4% nt identity of its RdRp and CP genes with those of PrVT C21 isolate, correspondingly.

Table 1. Pairwise percentage identities between saffron isolate of PrVT and *Tepovirus* sequences available in GenBank.

Viruses	Accession Numbers	Complete genome	RdRp		MP		CP	
			*nt	**aa	nt	aa	nt	aa
OlVT	MW582811	76.9	75.8	83.9	80.8	80.2	82	86.9
	MW582812	76.2	75.2	83.4	79.9	80.7	80.8	84.2
PrVT	MW331539	72.9	71.4	78.8	79.2	79.6	81.4	85.5
	MW331540	73	71.5	79.1	79.1	78.9	81.1	85.5
	KF700263	72.6	70.9	78.6	79.1	79.1	82	86.4
	KF700262	74.4	73.4	82.6	78.7	78.9	79.4	83.3
PVT	AB697482	43	44.3	31.3	40.9	23.1	42.1	30
	EU835937	42.9	44.1	31.7	41	23.1	42.1	30
	MH069211	43	44.1	31.7	41.1	23.1	41.6	30
	JF297562	43.1	44.1	31.6	39.9	23.1	41.6	28.6
	MH680825	43.9	45.3	31.9	39.3	23.9	40.5	29.5
FiTA	MH898491	55.1	53.7	50.1	65.1	62.4	59.8	64.3
TrTA	MH898525	54.7	53.5	49.2	60.6	52.7	60.2	58.8
ZoVT	MK514426	54.3	53.7	48.5	60.8	54.3	51.1	48.3
	MK514427	54.4	53.9	48.3	60.1	54.6	51	48.3
	MK514428	53.3	53	48	59.8	54.7	50.1	47.9
ChVT	MT090966	55.5	54	50.1	64.7	61.1	61.3	64.7

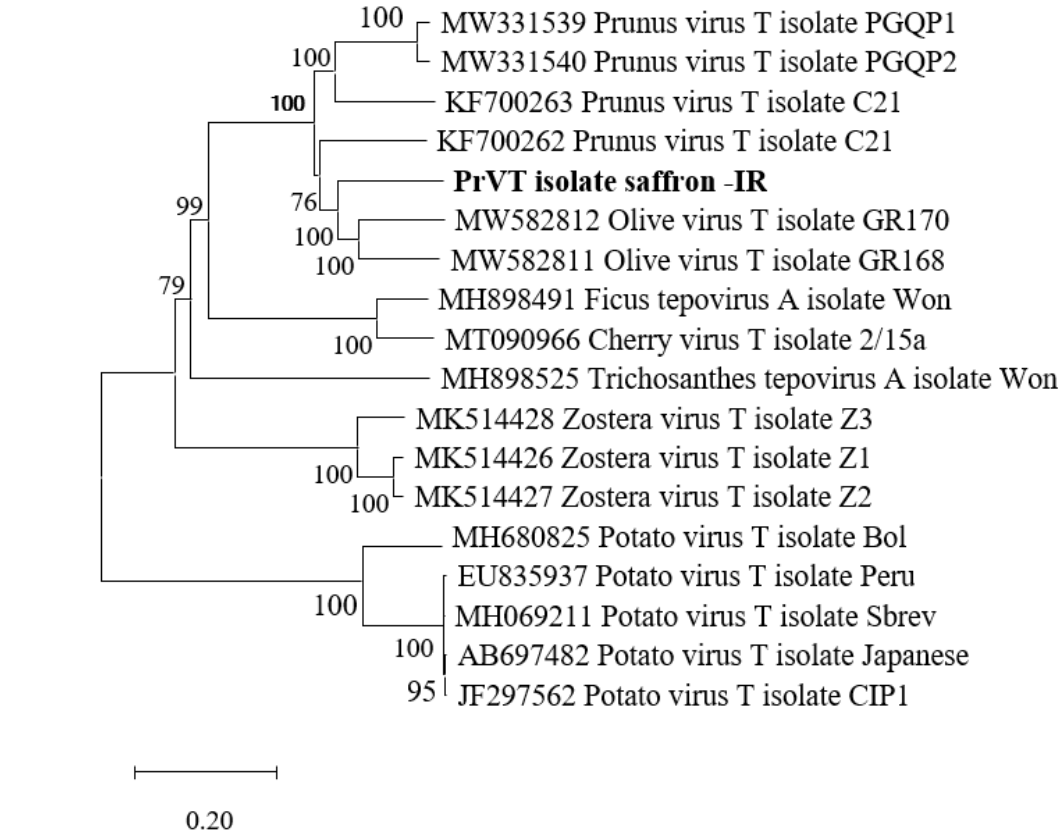
nt: nucleotide **aa: amino acid

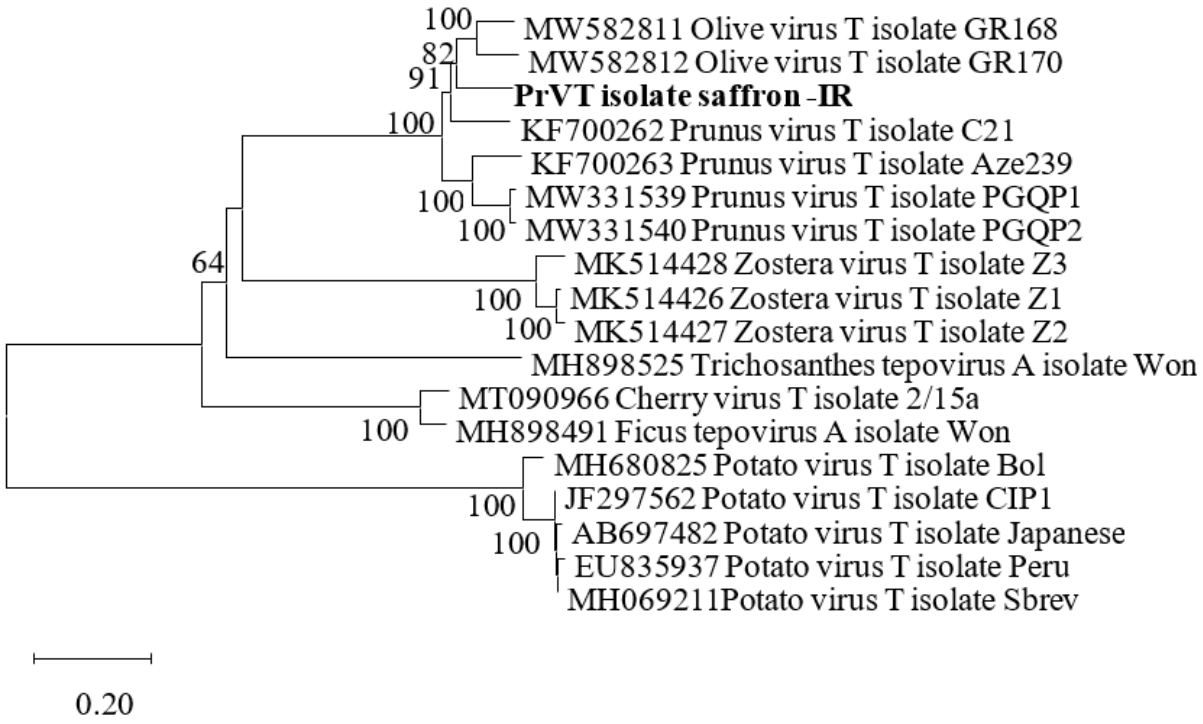
In a phylogenetic tree drawn from epoviruses full genomes available in GenBank using neighbor joining method with 1000 bootstraps (Figure 2), the saffron isolate of PrVT was clustered (100 bootstrap) with two Greece OlVT isolates found in olive and C21 isolate of PrVT, while it was distantly related to the other PrVT isolates (share 72.6-74.4% nt sequence identities). Deduced aa sequences of above-mentioned isolates for RdRp and CP (Figure 2) were used for the reconstruction of the phylogenetic tree. The tree for RdRp, resulted in the same topology as complete genome tree, while in the tree drawn from aa sequences of CP, the saffron isolate of OlVT was placed in a distinct branch of the cluster including OlVT and PrVT isolates. The RdRp and CP genes of saffron isolate of OlVT shared 83.4-83.9 and 84.2-86.9 percent aa identity, respectively, with those of OlVT isolates available in the GenBank (Table 1). Meanwhile, the RdRp and CP genes of the saffron isolate had 78.6-82.6 and 83.3-86.4 percent aa identity, respectively, with those of OlVT isolates available in the GenBank.

Recombination analysis by RDP5 using the full-length genome, RdRp, MP and CP nucleotide sequences did not detect any valid signal in the saffron isolate of OlVT.

Characterization of SaLV isolate

Blast annotation of the contigs also revealed 16 contigs (210-825 nt) resembling to SaLV. Using mapping of the contigs and trimmed unique reads against refseq of SaLV (NC_036802), near complete genome of the virus was obtained and submitted into the GenBank (Acc. No. BK061316). The genome was 9571nt in length and only 56 nts from 5ˈUTR and 65 nts from 3ˈUTR were missing compared to the reference (NC_036802). It shared 98.3% nucleotide identity with the refseq. Polyprotein of the two isolates shared 98.3 and 98.4% identity at nucleotide and amino acid levels, respectively. In the phylogenetic tree using the complete genome of related potyviruses, the SalV isolate was clustered with the only available isolate of the virus in the GenBank (Figure 3).


100
MW331539 Prunus virus T isolate PGQP1
100
MW331540 Prunus virus T isolate PGQP2
100
KF700263 Prunus virus T isolate C21
KF700262 Prunus virus T isolate C21
PrVT isolate saffron -IR
99
76
100
MW582812 Olive virus T isolate GR170
100
MW582811 Olive virus T isolate GR168
79
MH898491 Ficus tepovirus A isolate Won
100
MT090966 Cherry virus T isolate 2/15a
MH898525 Trichosanthes tepovirus A isolate Won
MK514428 Zostera virus T isolate Z3
100
MK514426 Zostera virus T isolate Z1
100
MK514427 Zostera virus T isolate Z2
MH680825 Potato virus T isolate Bol
EU835937 Potato virus T isolate Peru
100
MH069211 Potato virus T isolate Sbrev
100
AB697482 Potato virus T isolate Japanese
95
JF297562 Potato virus T isolate CIP1
0.20


100
MW582811 Olive virus T isolate GR168
82
MW582812 Olive virus T isolate GR170
91
PrVT isolate saffron -IR
100
KF700262 Prunus virus T isolate C21
KF700263 Prunus virus T isolate Aze239
100
MW331539 Prunus virus T isolate PGQP1
100
MW331540 Prunus virus T isolate PGQP2
64
MK514428 Zostera virus T isolate Z3
100
MK514426 Zostera virus T isolate Z1
100
MK514427 Zostera virus T isolate Z2
MH898525 Trichosanthes tepovirus A isolate Won
MT090966 Cherry virus T isolate 2/15a
100
MH898491 Ficus tepovirus A isolate Won
MH680825 Potato virus T isolate Bol
100
JF297562 Potato virus T isolate CIP1
100
AB697482 Potato virus T isolate Japanese
EU835937 Potato virus T isolate Peru
MH069211Potato virus T isolate Sbrev
0.20

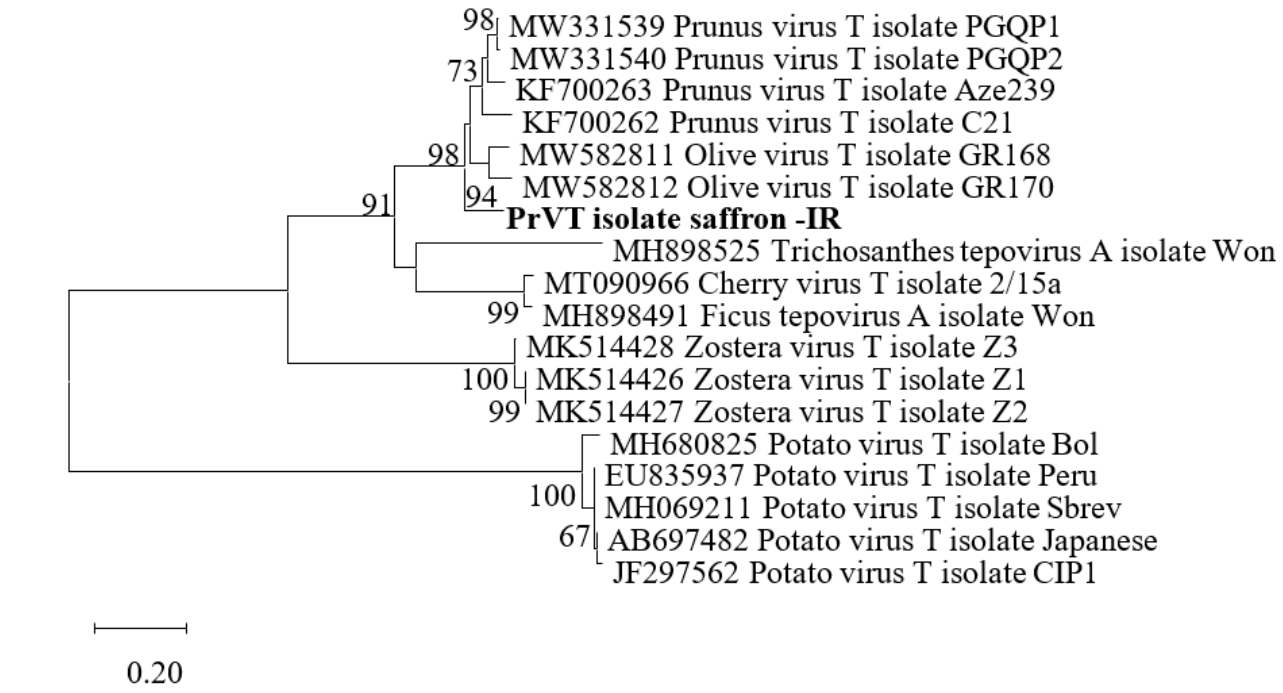


Figure 2. Phylogenetic trees using complete genomes (top), Pol gene aa sequences (middle) and CP gene aa sequences (bottom) of tepoviruses by neighbor joining method with 1000 bootstraps. The saffron isolate of PrVT is shown in bold. The bootstrap values are shown next to the branches.

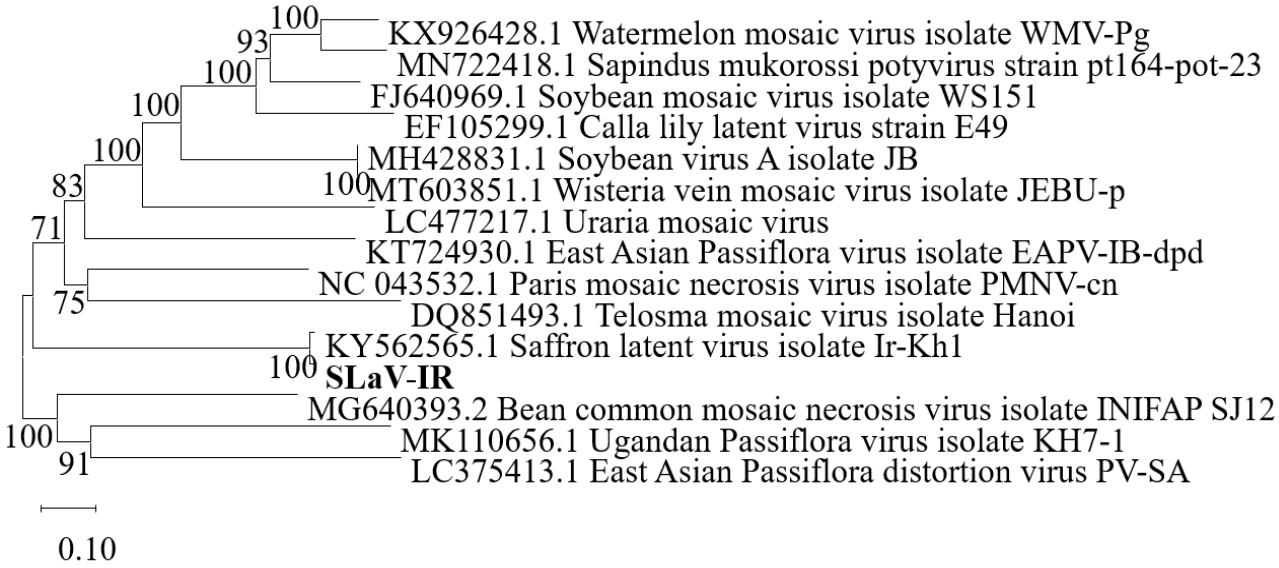


Figure 3. A phylogenetic tree reconstructed from complete genomic sequences of selected potyviruses and SaLV-IR using neighbor joining method with 1000 bootstrap. The isolate from Iran is shown in bold. The bootstrap values are shown next to the branches.

When filtering the rest of the contigs using tBLASTx, 184 contigs (201-546 nts long with 24.2-54.4% identities) were related to *Cladosporium fulvum* T-1 virus LTR-retrotransposon. Despite BLASTn analysis that showed they resemble to the plant genomes; it is not clear whether they are homologous sequences of the retroviruses integrated into the plant genome and further research is needed to investigate this issue.

Discussion

In this study, we used HTS and explored possible occurrence of virus(es) and viroids infecting saffron

led to detection of two viruses; SaLV and PrVT in Iran. The complete genome of saffron isolate of PrVT (PrVT-IR) shared the lowest nt sequence identities with potato virus T isolates ranging from 42.9-43.9 %, while it had the highest identities with those of OlVT isolates available in the GenBank (76.2-76-9%). However, the saffron isolate of PrVT was also closely related to PrVT isolates (72.6-74.4% nt sequence identities) (Table 1). In phylogenetic tree using the full genome of tepoviruses, the saffron PrVT isolate clustered with two Greece isolates of OlVT detected in olive and C21 isolate of PrVT (Figure 2).

Pairwise alignments of RdRp and MP genes of saffron isolate of OlVT with two olive isolates of OlVT at both nt and aa levels showed that they have the highest identity among the tepoviruses. However, comparing CP nt and aa sequences revealed that at least in some cases, the saffron isolate of PrVT and other PrVT isolates share higher identities than the two OlVT isolates (Table 1). In the phylogenetic tree drawn using aa sequences of CP, the saffron isolate of OlVT grouped with OlVT and PrVT isolates but in a separate clade. On the other hand, the phylogenetic tree reconstructed using aa sequences of RdRp, the saffron isolate of OlVT again was grouped with the two OlVT isolates next to an isolate of PrVT (C21). According to the updated criteria for species demarcation in the *Betaflexiviridae* family (Silva *et al*. 2022), it seems that OlVT might be considered as a strain of PrVT. OlVT is still not an accepted species for the ICTV, because of its closeness to PrVT. Taking together, the virus identified in Iranian saffron belongs to PrVT species.

SaLV was previously reported from Iran and in this study near complete genome of a SaLV (SaLV-IR) was recovered from the HTS data. The genome SaLV-IR isolate had 98.3% nucleotide sequence identity with the only available isolate in the GenBank. Furthermore, Polyprotein of the two isolates shared 98.3 and 98.4% identities at nucleotide and amino acid levels, respectively.

Plant virologists have discovered numerous viruses using high throughput sequencing (HTS), which led to the advancement of our knowledge on the diversity of viruses in nature. Previous HTS-based detection studies indicated that it may work even better than conventional detection methods in terms of sensitivity, specificity, repeatability, scalability and cost (Çağlayan *et al.* 2019, 2020; Gazel *et al.* 2020; Soltani *et al.* 2021; Roumi *et al.* 2021). For example, HTS-based diversity studies of potato virus M infection in tomato confirmed that this approach holds great potential for characterization of virus isolates escaping standard detection methods such as RT-PCR (Glasa *et al*. 2019). HTS is becoming a routine diagnostic platform for the detection of viruses/viroids on grapevine, wheat, fruit trees, banana, tomato, pepper and many other plants. Ongoing evaluation of the performance of HTS-based protocols is being accomplished to acceptance and employ implement them by as international standards for plant quarantine and certification programs (Maliogka *et al*. 2018; Soltani *et al*. 2021; Redila *et al*. 2021; Hanafi *et al*. 2022). HTS can provide fast and reliable alternative to PCR-based detection method for viruses and viroids, especially at the earliest stages of infection, which is particularly important in saving resources and quick elimination of infected and non- sanitized plants (Hanafi *et al*. 2022).

We confirmed that HTS can act as a precise identification tool for novel and symptomless viruses especially in saffron. The results of this study can pave the way for further researches in order to develop better detection and control strategies of saffron viruses both in Iran and in the world.

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