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Research Article

Occurrence of *Hop stunt viroid* in apple orchards of the Northwest Iran

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Abstract: We investigated the presence of *Hop stunt viroid* (HSVd) in apple samples, showing dappling fruit symptoms, in the Maragheh area (Northwest Iran) by means of RT-PCR. The viroid was detected only in leaves collected from symptomatic trees and a 298bp amplicon (IR-Gala) was directly sequenced in both directions. Multiple sequence alignment and Blast analyzes revealed that IR-Gala isolate shares the highest identity with grapevine isolates from Brazil and China. Amongst Iranian isolates of HSVd available in the GenBank, this isolate had the highest identity with grapevine isolate of HSVd from Maragheh region. In Phylogenetic analysis by MrBayes, IR-Gala was clustered with grapevine isolates from Brazil, China and Iran and may suggest that HSVd-apple isolate could be originated from grapevine.

Keywords: Detection, Apple, HSVd, Viroid, Iran

Introduction

Iran is the 7th largest producer of apple in the world (FAOSTAT, 2018). Numerous pests and diseases can impose serious loss to apple production. Among them, viroids can cause both qualitative and quantitative losses and hence they are taken into account in certification plans by many countries. Viroids are subviral entities and contain a circular, non-coding RNA which are considered as the smallest plant pathogens. Viroids parasitize transcription of hosts via nuclear or chloroplastic RNA polymerases which may lead to expression of the symptoms (Owens *et al*., 2012).

Several viroids have been reported to infect apple in Iran including: *Apple scar skin viroid* (ASSVd) (Yazarlou *et al*., 2012a), *Apple dimple fruit viroid* (ADFVd) (Roumi *et al*., 2017; Roumi 2018), a viroid resembling to *Hop stunt viroid* (HSVd) (Vamenani *et al.*, 2015) and *Peach latent mosaic viroid* (PLMVd) (Yazarlou *et al*., 2012b). *Apple fruit crinkle viroid* (Ito *et al*., 1993) and *Pear blister canker viroid* (Lolic *et al*., 2007) also infect apple. Recently, a new viroid named *Apple Hammerhead Viroid* has been reported from the United States, Japan, Italy, Spain, and New Zealand (Szostek *et al*., 2018).

HSVd (genus *Hostuviroid*, Family *Pospiviroidae*) which was reported for the first time in Japan (Shikata, 1987) is an important pathogen on hop fields worldwide. The viroid also infects peach, apricot, plum, almond, citrus, cucumber and cherry (Gazel *et al*., 2008; Owens *et al*., 2012; Hadidi *et al*., 2017). Furthermore, HSVd is one of the most common viroids in grapevine and it seems

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that all grapevines in Iran are infected by this viroid (Ahmadi *et al*., 2017).

Viroids are primarily transmitted by means of propagative plant materials (Owens *et al*., 2012) and they have a worldwide distribution, especially where plant certification programs are missing or ineffective. In this study, symptomatic apple samples showing dappling fruit symptoms were studied by RT-PCR and phylogenetic approaches to illuminate etiology of the disease.

Materials and Methods

Source of isolates

Two symptomatic and 2 symptomless apple samples (*Malus domestica* cv. Gala) were selected from the Maragheh region (Northwest Iran) and were subjected to RT-PCR to investigate the possible involvement of HSVd in dappling disease of apple.

Viroid detection and sequencing

For the detection of HSVd in tested samples, specific VP19/VP20 primer pairs (Astruc *et al*., 1996) were employed in RT-PCR. RNA extraction and RT-PCR were carried out as described previously (Roumi *et al*., 2017). The expected size amplicon from HSVd-positive samples was sent to sequencing company (Iontech, Turkey) and the samples were sequenced directly in both directions.

Phylogenetic analyses

The Blast search was carried out to retrieve close isolates/clones of this HSVd isolate from the GenBank. The sequences were aligned by Geneious aligner and phylogenetic tree was reconstructed in MrBayes 3.2.6 (Huelsenbeck and Ronquist, 2001) embedded in Geneious Prime software 2019.2.1 (https://www.geneious.com) using HKY model and default settings. Potato spindle tuber viroid (PSTVd, KF418767) was used as an outgroup. The 2-D structure of the isolate was drawn using MFold (Zuker, 2003).

Recombination analysis

Analysis of recombination was performed by RDP4 (Martin *et al*., 2015) using RDP, GENECONV, chimera, MaxChi, BootScan, SiScan, 3Seq and LARD methods and those recombination events confirmed by more than 3 methods were considered as true recombinants.

Results

Detection

The RT-PCR using VP19/VP20 primers, amplified the expected ~ 300bp band just from symptomatic samples. We selected an amplicon, which was later purified and sequenced in both directions. The sequences of amplicon were assembled and edited in Geneious Prime and the resulting consensus (hereafter called HSVd IR-Gala) was submitted to the GenBank (Accession number: MN695313). HSVd IR-Gala shared the highest identity (99%) to 3 Brazilian isolates (MF774873.1, MF774870.1, MF774869.1) and a Chinese isolate (AB219944.1) of HSVd detected from *Vitis vinifera*. Amongst Iranian isolates of HSVd, this isolate had highest identity (98.7%) with KF927099 accession number detected on *V. vinifera* from Maragheh, Northwestern Iran (Hajizadeh *et al*., 2012), while it shared only 92.1% identity with HSVd-like viroid reported from apple in the Northeast of Iran (Vamenani *et al*., 2015).

Phylogenetic Analysis

The multiple alignments of 39 selected isolates was adjusted manually and used for inferring a phylogenetic tree. The phylogenetic tree provided good resolution for the isolates from three main groups (citrus, hop and plum) and two recombinant groups (P-C and P-H/cit3) (Fig. 1). IR-Gala isolate was grouped with isolates from Hop group including mainly hop and grapevine isolates whereas it was distinct from those isolates and made a separate clade. In the meantime, previously described HSVd like apple isolate from Northeast of Iran was clustered in the recombinant group P-H/cit3. The grapevine isolate of HSVd from Maragheh (Northwest of Iran) did not cluster with any groups and seems to be a variant.

2-D structure of the IR-Gala isolate

The predicted secondary structure for IR-Gala formed a typical rod-like structure of pospiviroids, whereas the HSVd-like viroid reported from apple in Iran was forked between V and TR regions (Fig. 2).

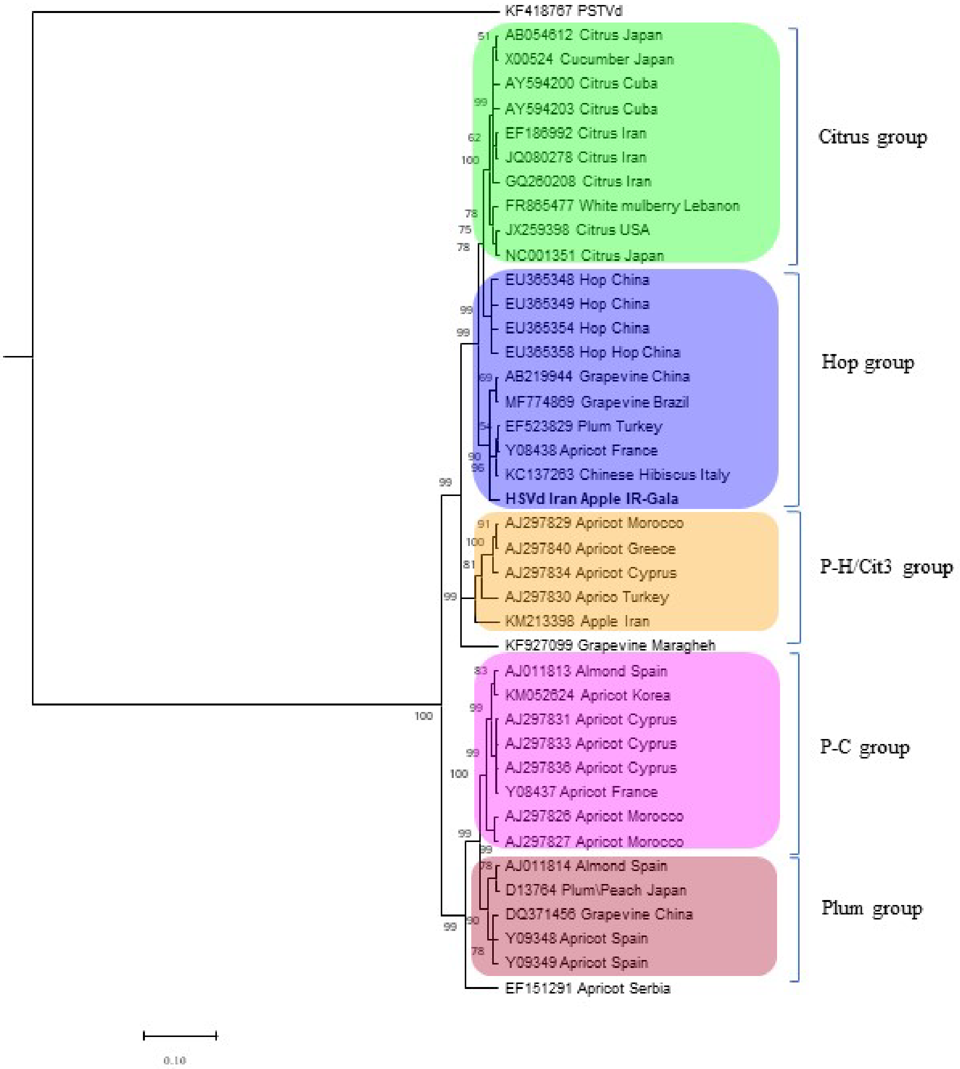

Figure 1 Phylogenetic tree reconstructed for selected HSVd isolates/clones by MrBayes. The studied isolate is in bold and bootstrap values above 50% are shown on the nodes.

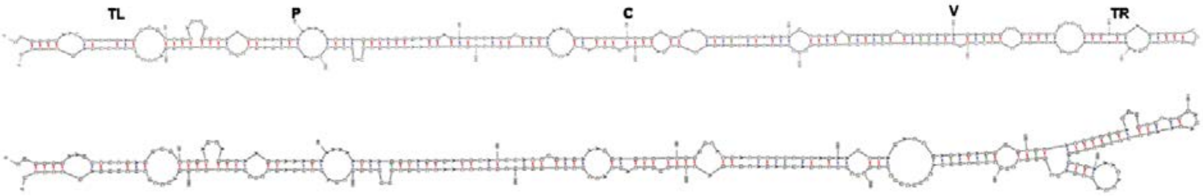

Figure 2 The 2D structure of HSVd IR-Gala (above) and HSVd like viroid reported from North-east Iran (below) predicted by mFold. TL: Terminal left; P: Pathogenic region; C: Central conserved region; V: Variable, TR: Terminal right.

Recombination Analysis

Strong signals of recombination were detected for apple HSVd-like isolate by 5 out of 8 methods. A plum isolate of HSVd from Turkey was detected as major parent and an apricot isolate from Korea was detected as a minor parent (Fig. 3). The UPGMA tree of regions derived from the major parent, placed the apple HSVd-like isolate in the Hop group, whereas it was placed at a separate branch using region from the minor parent. The grapevine HSVd Maragheh isolate was also detected as recombinant by just one method which was ignored in the analysis. However, its position as a distinct clade in the phylogenetic tree raises the possibility of recombination events in this isolate.

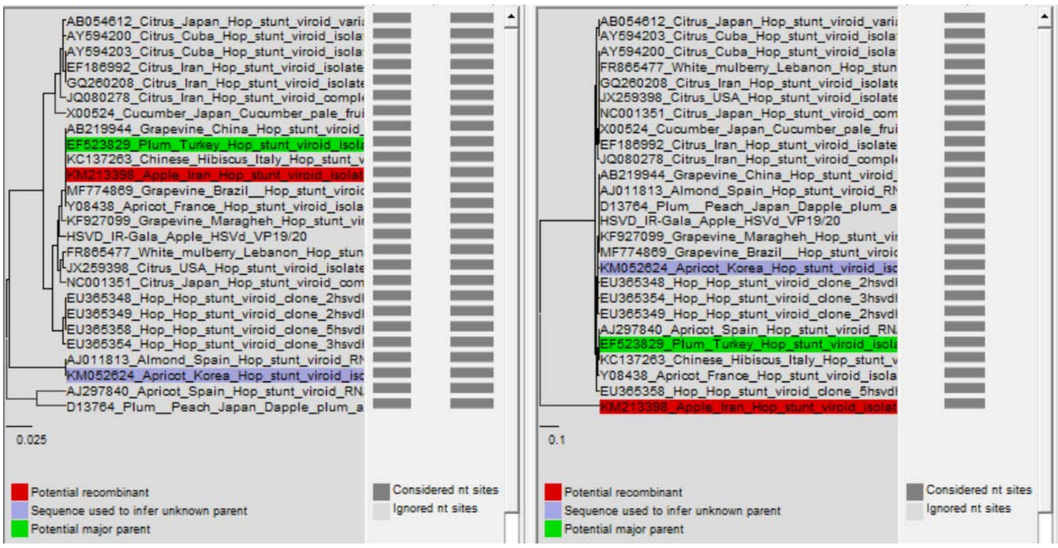

Figure 3 Recombination analysis of the dataset used for multiple alignment and phylogenetic analysis. UPGMA tree of region originated from major parent (1-111 and 231-357) (left) and regions originated from minor parent (112-230) (right).

Discussion

In this study, the presence of HSVd in apples expressing dapple fruit symptom was confirmed by RT-PCR and sequencing but no obvious relationship between viroid infection and specific symptoms was found at this stage. Multiple sequence alignment and phylogentic analysis revealed that IR-Gala isolate from apple in Maragheh region is considerably different from previously reported HSVd- like isolate and which shares only 92.1% identity. The IR-Gala isolate was closely related to grapevine isolates of the viroid from Maragheh, which is in line with Sano *et al*., (2001) findings that suggested HSVd in the hop is originated from grapevine.

The results can be worthwhile to develop sensitive and rapid detection methods for certification programs. So far, three viroids (ASSVd, ADFVd and HSVd) have been involved in the etiology of dapple fruit disease in Iran and the contribution of each viroid needs to be further investigated.

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Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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وقوع ویروئید کوتولگی رازک در باغات سیب شمال غرب ایران

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چکیده: در این پژوهش، وجود ویروئید کوتولگی رازک در سیب با علائم فرورفتگی میوه سیب در منطقه مراغه، با استفاده از روش RT-PCR مورد بررسی قرار گرفت. این ویروئید فقط در برگ‌های درختان سیب حاوی علائم ردیابی شد و یک قطعه تکثیر شده به اندازه ۲۹۸ جفت باز به‌طور مستقیم در دو جهت تعیین ترادف گردید. آنالیز هم‌ردیف‌سازی چندگانه ترادف‌ها و بلاست نشان داد که جدایه سیب ویروئید کوتولگی رازک بیش‌ترین یکسانی ترادف را با جدایه‌های برزیل و چین دارد و در بین جدایه‌های ایران به جدایه مو منطقه مراغه نزدیک‌تر بود. در آنالیز فیلوژنتیک، این جدایه به‌همراه جدایه‌های مو از برزیل، چین و ایران در یک گروه قرار گرفتند که می‌تواند نشان‌دهنده منشأ گرفتن جدایه سیب ویروئید کوتولگی رازک از مو باشد.

واژگان کلیدی: سیب، ویروئید کوتولگی رازک، ایران