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Molecular detection and characterization of *Hop stunt viroid* sequence variants from naturally infected pomegranate (*Punica granatum* L.) in Tunisia

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Summary. Tunisian pomegranate *Hop stunt viroid* (HSVd) variants are described. Dot-blot hybridization, S-Page, and reverse transcription polymerase chain reaction (RT-PCR) of RNA extracts from infected tissues were carried out. Results obtained by these techniques were confirmed by cDNA sequencing. The genetic diversity among the Tunisian variants was investigated, which also involved analysis of sequences of previously described HSVd variants from Tunisian citrus var. clementine and fig, and from fruit trees from other Mediterranean countries. Phylogenetic analysis showed that Tunisian pomegranate HSVd variants were clustered into two groups: a cachexia strain within the citrus type group and a recombinant citrus-plum type group. Results also showed a high haplotype diversity which was not related either to the host or to the geographical origin. Selective neutrality and genetic network tests suggest that the HSVd isolates have spread rapidly.

Key words: HSVd, sequence variability, phylogeny, Tunisia.

Introduction

Viroids are infectious, naked, small, circular, single-stranded RNA molecules of 246 to 401 nucleotides (Diener, 1991; Flores *et al.,* 1998, Hadidi *et al*., 2003). They replicate autonomously in their host plants and may cause disease or latent infection (Astruc *et al.,* 1996). Their genomes may contain a central conserved region (CCR) or a hammerhead self-cleavage structure. Viroids with the CCR replicate in the nuclei (family *Pospiviroidae*) and viroids with the hammer self-cleavage structure replicate in the chloroplasts (family *Avsunviroidae*) (Flores *et al.,* 1998; 2005; Hadidi *et al.*, 2003).

Hop stunt viroid (HSVd) is a member of the genus *Hostuviroid* and the family *Pospiviroidae.* It infects several hosts such as hop, cucumber, grapevine, citrus, plum, peach, pear (Shikata, 1990; Levy and Hadidi, 1993), apricot and almond (Astruc *et al.,* 1996; Cañizares *et al.,* 1999, Amari *et al.,* 2007). Pomegranate is also reported to be susceptible to infection by either circular satellite viral RNA (Gomez and Pallás, 2001) or HSVd in the Mediterranean region (Astruc *et al.,* 1996).

In Tunisia, previous attempts to detect viroids in fruit trees have revealed HSVd in both citrus (Elleuch *et al.,* 2003) and fig trees (Yakoubi *et al.,* 2007). HSVd also occurs in pear, peach and almond trees (Fekih-Hassen *et al.,* 2006). Nevertheless, symptom expression and the loss in fruit yield of pomegranate cultivars as a result of viroid

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infection and spread have not yet been explored. In this work, we report the molecular detection of HSVd in naturally infected pomegranate trees. We also investigate the genetic diversity among Tunisian HSVd isolates from different fruit trees (clementine, fig and pomegranate) to establish their relationship with those characterized previously from other Mediterranean countries (Amari *et al.,* 2001). Phylogenetic analysis (which established a haplotype pattern) and neutrality tests suggest that the HSVd population is spreading rapidly.

Materials and methods

Plant and viroid sources

Forty-six samples were collected from the main pomegranate growing areas in Tunisia, which include Gabes region in the south (16 samples), Testour region in the north-west (15) and Beni Khalled region in the north-east (15). Leaf samples were collected from the main cultivated varieties gabsi and zehri. Sampled trees were chosen on the basis of symptoms on the leaves and bark, consisting of general stunting, stem pitting or discoloration. A clementine HSVd/Cl1 clone (Elleuch *et al.,* 2006) was used as a positive control during the investigation.

RNA extraction for dot-blot hybridization and RT-PCR amplification

Two-hundred mg of leaf samples was powdered in liquid nitrogen and homogenized with 1 mL of extraction buffer (1.5 M NaCl, 0.15 M trisodium citrate and 1% β-mercaptoethanol). After centrifugation at 16,000 *g* for 5 min, the supernatant was transferred to 1 mL of 35% non ionic CF-11 cellulose. The mixture was then vortexed for 1 h. The cellulose was collected by centrifugation, washed three times with 1 mL of STE buffer (5 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 7.2) containing 35% of ethanol. RNA elution was performed with 300 μL of STE buffer and precipitated with 1 volume of isopropanol and 1/10 volume of sodium acetate (5M, pH 5.2). The pellet was dried in a speed-vac, washed with 70% ethanol and resuspended in 30 μL sterile water. All the RNA preparations were quantified by spectrometry (Beckman Coulter DU 730, Fullerton CA, USA) and their concentrations made equal, then kept at -80°C until use for dot-blot hybridization or the RT-PCR reactions.

Dot-blot hybridization

Two-hundred ng of the RNA preparation of each sample was combined with an equal volume of formaldehyde (15%) and heated to 65°C for 20 min. Using a dot-blot apparatus (Biorad, Paris, France), the RNA was blotted under vacuum to a nylon filter previously washed with 20× SSC buffer (150 mM NaCl, 15 mM sodium citrate). The membrane was then air-dried and kept at room temperature. A random primed-digoxygenin spanning the HSVd-clementine genome was used under high-stringency hybridization conditions (60°C). The labelling reaction and immunological revelation were performed according to the manufacturer's instructions (Boehringer-Roche Mannheim, Germany).

RNA extraction and analysis by PAGE and S-PAGE

Leaves (5 g) stripped from young shoots were reduced to powder in liquid nitrogen and homogenized in 5 mL of extraction buffer [0.4 M Tris-HCl pH 8.9; 1% (w:v) SDS; 5 mM EDTA pH 7; 4% (v:v) 2-mercaptoethanol] and 15 mL of water-saturated phenol. The total nucleic acids were precipitated in 2 M LiCl, then the soluble fraction was concentrated by ethanol precipitation and resuspended in 300 mL of TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM $MgCl_2$ pH 7.4) (Semancik *et al.,* 1975). Aliquots of these nucleic acid preparations were kept for use in the analysis below.

Aliquots (20 μL) of the nucleic acid preparations described above were subjected to 5% polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions 1× TAE (40 mM Tris-acetate, 1 mM EDTA), for 2 h at 70 mA, then stained with ethidium bromide (Morris and Wright, 1975). For S-PAGE, a segment of the ethidium bromide stained gel containing viroid RNA was subjected to a second PAGE using a denaturing buffer 0.25× TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and 8 M urea, at 18 mA for 3 h (Rivera *et al.,* 1986). The viroid pattern was revealed by silver staining (Igloi *et al.,* 1983). Two controls, corresponding to *Citrus exocortis viroid* (CEVd) and *Avocado subbloch viriod* (ASBVd), were used as molecular weight markers.

RT-PCR assay, cloning and sequencing

Two μL of the extracted RNA was used in a first-strand cDNA synthesis with 100 ng of the

26-mer reverse primer VP-19 (Kofalvi *et al.,* 1997) 5'GCCCCGGGGCTCCTTTCTCAGGTAAG3' (complementary to HSVd residues 60–85). The mixture was heated to 95°C for 5 min and immediately chilled on ice. Reverse transcription (RT) was performed at 37°C for 1 h with 6 μL of 5× buffer, 1 μL of dNTP (10 mM each), 1 μL of 100 mM DTT and 0.2 μL of MMLV reverse transcriptase (200 Units μL^{-1}, Life Technologies, Paris, France), for a final volume of 30 μL. An aliquot of the cDNA was amplified using 1 U of p*fu* DNA polymerase (Roche, Paris, France), 100 ng of each primer [the antisense VP-19 and the sense 27-mer VP-20 5'CGCCCGGGGCAACTCTTCTCAGAATCC3' (Kofalvi *et al.,* 1997), homologous to HSVd residues 78–102], 5 μL of 10× buffer, 1 μL of dNTP (10 mM each), 1.5 μL $MgCl_2$ and sterile water to a final volume of 50 μL. The PCR mixture was subjected to a first denaturation step for 2 min at 95°C, followed by 25 amplification cycles (1 min at 95°C, 1 min at 55°C, 1 min at 72°C). A final extension step was done at 72°C for 7 min.

PCR products corresponding to HSVd full-size cDNA were column-purified (QiaquicK PCR purification Kit, Qiagen, Paris, France) and cloned into pGMT-Easy plasmid (Promega, Charbonnières-les-bains, France). Two or three recombinant clones of each isolate were sequenced using universal primers with an automated DNA sequencer ABI Prism DNA-377 apparatus (Applied Biosystems, Paris, France).

Computer analysis

Multiple alignment of HSVd sequences was obtained using Mega version 3.1 (Kumar *et al.,* 2004) to be analyzed with the DnaSP program version 4.0 (Rozas *et al.,* 2003). The phylogenetic tree was constructed using the Neighbor joining methods (Saitou and Nei, 1987). To estimate nucleotide polymorphism among HSVd variants and the other reported isolates, several parameters were taken into account as indices of haplotype diversity (Hd) (Nei and Tajima, 1983), pairwise estimates of nucleotide divergence (Pi) (Jukes and Cantor, 1996), average of nucleotide differences (k) and the minimum number of recombination events (Rm). Selection neutrality for mutations in whole sequences was tested by method D of Tajima (Tajima, 1989) and methods D and F of Fu and Li (Fu and Li, 1993). The genetic relationship of the inferred haplotypes was graphically displayed by the NETWORK program version 4.5.0.0 (Bandelet *et al.,* 1999).

Results

HSVd detection

Of the 46 samples tested by dot-blot hybridization, only three were positive for HSVd: one from Cap-Bon, one from Testour and one from Gabes (Figure 1A).

Samples testing positive by dot-blot hybridization were then analyzed by S-PAGE. Faint bands

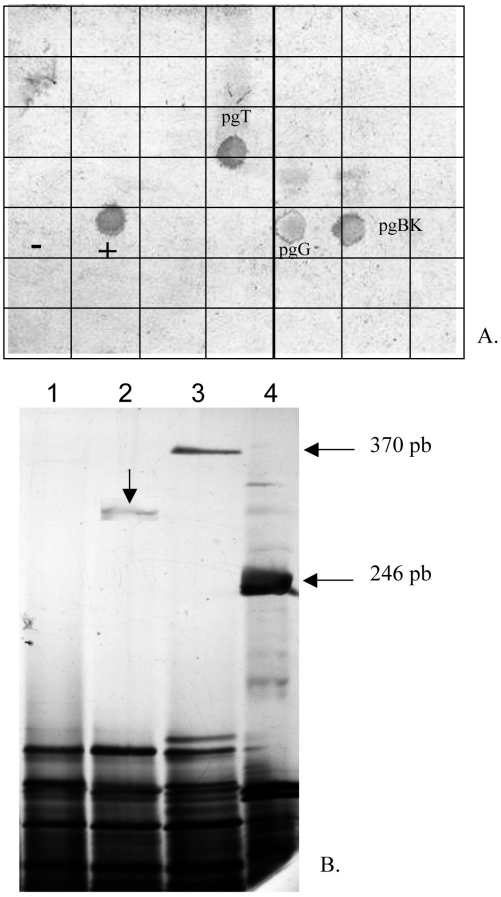

Figure 1. A. Nitrocellulose membrane showing dot-blot hybridization of citrus *Hop stunt viroid* (HSVd) DIG-labeled cDNA probe to total RNA of pgT, pgG and pgBK samples naturally infected with HSVd. The healthy control (-) and the pGEM-T-HSVd cDNA control (+) are also shown. B. HSVd from total RNA of infected pomegranate sample detected by S-PAGE and silver staining. Lane 1, healthy control; lane 2, HSVd-infected sample (indicated by an arrow); lane 3, CEVd (370 pb); lane 4, ASBVd (246 pb).

with the characteristic mobility of HSVd (about 300 pb) were seen in the corresponding sample (Figure 1B, lane 2).

Results obtained by dot-blot hybridization and S-PAGE were confirmed by RT-PCR amplification (data not shown). Results were consistent with the occurrence of three positive samples in the collection. An analogous amplicon was obtained with HSVd clone Cl1, used as the positive control. For each isolate, the corresponding PCR amplicon was cloned and two or three independent clones were sequenced.

Sequence characterization and phylogenetic analysis

The sequences obtained were submitted to GenBank and their analyses as related to those of other HSVd variants were carried (Table 1). The HSVd-pomegranate sequences ranged in size from 297 to 300 nucleotides. Each variant displayed mutations consisting in additions, deletions or substitutions (Table 2). Sequence homologies between variants of the same isolate or variants of different isolates ranged from 97 to 100% indicating that HSVd-pomegranate consisted of a

Table 1. *Hop stunt viroid* (HSVd) haplotypes deduced from sequence variants. The Tunisian HSVd-pomegranate isolates mentioned in this work are: pgBK, pomegranate isolates from the Beni Khalled region; pgG, pomegranate isolates from the Gabes region; pgT, pomegranate isolates from the Testour region. HSVd isolates included in sequence comparisons and in phylogenetic analysis were retrieved from data bases (http://www.callisto.si.usherb.ca~jpperra/viroids/hsv/html; Bussière *et al*. (1996).

Haplotype	Corresponding HSVd sequence	Reference	Haplotype	Corresponding HSVd sequence	Reference
H1	AB211242/ pgBKa/pgBKb	Ito *et al.,* 2006; this work	H27	p2	Bussière *et al.,* 1996
H2	EF523826 /APR18	Bussière *et al.,* 1996; Amari *et al.,* 2001	H28	Alm1	Kofalvi *et al.,* 1997
H3	pgGa	This work	H29	apr4	Kofalvi *et al.,* 1997
H4	pgGb	This work	H30	apr6	Kofalvi *et al.,* 1997
H5	pgGc	This work	H31	apr7	Kofalvi *et al.,* 1997
H6	AY460201	Bussière *et al.,* 1996	H32	apr8/ apr2	Kofalvi *et al.,* 1997
H7	pgTa	This work	H33	apr5	Kofalvi *et al.,* 1997
H8	pgTb	This work	H34	g3	Bussière *et al.,* 1996
H9	pgTc	This work	H35	g2	Bussière *et al.,* 1996
H10	fig1	Yakoubi *et al.,* 2007	H36	apr1/ g1/p1/g4/g6/g7	Kofalvi *et al.,* 1997; Bussière *et al.,* 1996
H11	fig2	Yakoubi *et al.,* 2007	H37	t19.4	Bussière *et al.,* 1996
H12	cl1/cl4	Elleuch *et al.,* 2006	H38	g9	Bussière *et al.,* 1996
H13	cl2	Elleuch *et al.,* 2006	H39	g5	Bussière *et al.,* 1996
H14	cl3	Elleuch *et al.,* 2006	H40	g8	Bussière *et al.,* 1996
H15	apr17	Amari *et al.,* 2001	H41	Pear	Shikata, 1990
H16	apr19	Amari *et al.,* 2001	H42	h1/h2	Bussière *et al.,* 1996
H17	apr13	Amari *et al.,* 2001	H43	h3	Bussière *et al.,* 1996
H18	apr15	Amari *et al.,* 2001	H44	cit3	Bussière *et al.,* 1996
H19	apr16	Amari *et al.,* 2001	H45	c1	Bussière *et al.,* 1996
H20	apr20	Amari *et al.,* 2001	H46	c2	Bussière *et al.,* 1996
H21	apr9/ alm2/ apr3	Amari *et al.,* 2001; Kofalvi *et al.,* 1997	H47	citIIa	Levy and Hadidi, 1992
H22	apr10	Amari *et al.,* 2001	H48	citIIb	Levy and Hadidi, 1992
H23	apr14	Amari *et al.,* 2001	H49	cit1	Bussière *et al.,* 1996
H24	apr24	Amari *et al.,* 2001	H50	cit2	Bussière *et al.,* 1996
H25	apr23	Amari *et al.,* 2001	H51	cit5	Bussière *et al.,* 1996
H26	apr22/p3	Amari *et al.,* 2001	H52	cit6	Bussière *et al.,* 1996

mixture of closely related molecules. Two similar variants from the Beni Khalled region (pgBKa/pgBKb) isolated from the same cultivar were found to be identical to the previously described cachexia-inducing HSVd variants (AB 211242) identified in citrus orchards in Japan (Ito *et al.*, 2006), indicating that this sequence was not exclusive to a specific host. The closest HSVd sequence for variants

Table 2. The sources and sequence variants of *Hop stunt viroid* (HSVd) from Tunisian pomegranate analyzed in this work. Nucleotide differences with the closest sequences are indicated. + = insertion, Δ = deletion.

Province	Pome granate variety	Sequenced clones	Size (nt)	Closest HSVd variant	Nucleotide differences with closest sequence
Gabes	Zehri	pgGa	298	HSVd.ap1 EF523826	C^{33}, U^{279}, $+A^{292}$
		pgGb	300		G^{13}, $+A^{114}$, $+G^{115}$, A^{147}, G^{179}, C^{198}, U^{279}, $+A^{292}$
		pgGc	298		G^{21}, C^{33}, U^{279}, $+A^{292}$
Testour	Gabsi	pgTa	297	HSVd Apricot AY460201	G^{26}, G^{27}, C^{33}, C^{108}, A^{143}, A^{157}, $+C^{252}$, ΔU^{276}, A^{293}, G^{295}
		pgTb	297		G^{26}, G^{27}, C^{33}, G^{110}, A^{143}, A^{157}, ΔG^{231}, A^{293}, G^{295}
		pgTc	297		G^{26}, G^{27}, G^{110}, A^{143}, A^{157}, $+C^{252}$, ΔU^{276}, A^{293}, G^{295}
Beni Khalled	Gabsi	pgBKa	300	HSVd.ex1 AB211242	
		pgBKb	300		

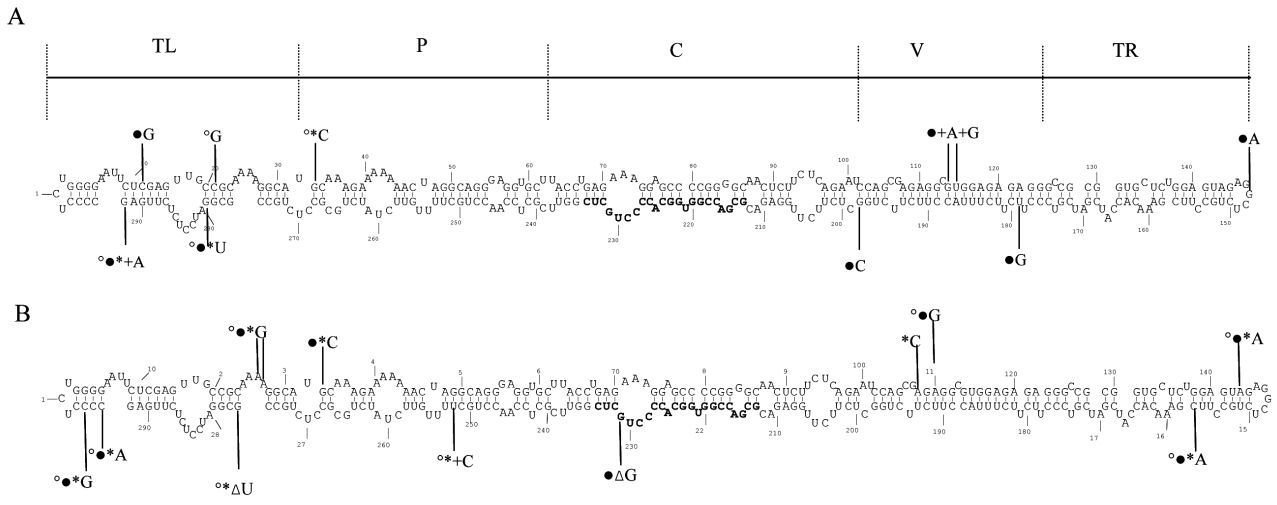

Figure 2. Most stable predicted secondary structure of *Hop stunt viroid* (HSVd) using the mfold program showing nucleotide changes within the Tunisian pomegranate HSVd variants, compared to the reference isolate HSVdh1 (Ohno *et al.*, 1983). (A). HSVd-pgG variants corresponding to pomegranate isolates from the Gabes region; (B), HSVd-pgT variants corresponding to pomegranate isolates from the Testour region. The secondary structure is shown with the boundaries of the terminal left (TL), pathogenicity (P), central conserved (C), variable (V) and terminal right (TR) domains.
* Variant a, ● Variant b, ° Variant c. Nucleotide substitutions are indicated: + insertion, Δ deletion.

(pgGa/pgGb/pgGc) isolated from southern Tunisia (Gabes region) was HSVd.AP1 (EF523826) reported previously from apricot in Turkey. All variants from the Testour region (pgTa/pgTb/pgTc) were similar to apricot HSVd (AY60201) isolated from stone fruits in Italy (Ragozzino *et al.,* 2004). These findings indicate that these two sets of variants may correspond to a new HSVd population.

All the mutations of the Tunisian isolates were shown on the most stable secondary structure predicted by the mfold program (Figure 2). The mutations were randomly distributed on the genome.

To classify the Tunisian pomegranate isolates, the sequences of the pomegranate HSVd variants characterized in this work as well as of the isolates of Tunisian clementine and fig from Tunisia and other Mediterranean countries (66 sequences) were aligned. Kovalfi *et al.,* 1997 previously reported that HSVd isolates clustered into three main groups: citrus, hop and plum-types, and into two other groups as a result of recombination events between the plum and the citrus-types, named the P-C group, and between the hop and the plum-type or the cit3 variant, giving the P-H/cit3 group (Figure 3).

As regards the Tunisian pomegranate HSVd isolates, two variants from Cap-Bon were identical to a previously described cachexia strain and clustered in the citrus-type group. This is the first report of the occurrence of a cachexia strain on a fruit tree other than citrus in Tunisia. Moreover, to the best of our knowledge, the cachexia variant has so far been reported only in citrus. The occurrence of this strain on pomegranate can be explained by the fact that the pomegranate-growing areas in Cap-bon are adjacent to fields where citrus are intensively cultivated. The variants from Gabes and Testour characterized so far were closely related to HSVd.ap1 (EF523826) and HSVd apricot (AY460201) respectively. They are consequently assigned to the recombinant P-H/cit3 type group. It should be noted that in the Gabes region, where fruit cultivation tends to centre in oases, many crops, such as pomegranate, lemon and apricot, are cultivated under date palm trees. These findings taken together suggest that the pomegranate variants identified in this work have a homogeneous origin and are related to citrus isolates.

Sequence polymorphism

After excluding insertions/deletions (indels), the number of variant sites reached 27, with 9 parsimony informative sites among the HSVd-

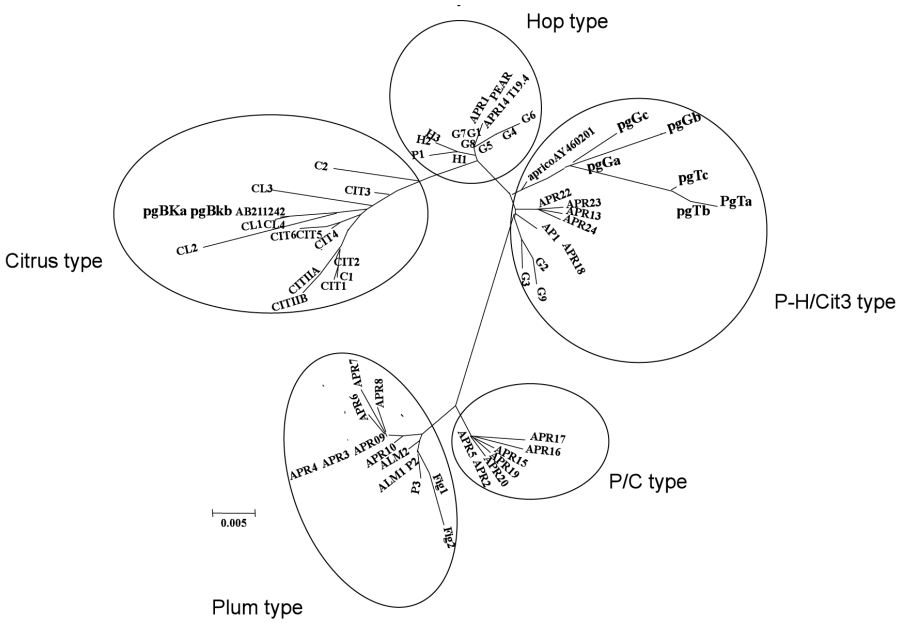

Figure 3. Neighbor-joining tree of *Hop stunt viroid* (HSVd) sequence variants from published sequences. Variant sequences determined in this work are indicated in bold type. The five phylogenetic groups identified previously by Kovalfi *et al.,* (1997) are circled

Table 3. Sequence polymorphism and divergence among *Hop stunt viroid* (HSVd) isolates from different hosts. Asterisks indicate that the test of Tajima and those of Fu and Li were negative and significant when all groups were considered.

Parameters	HSVd-Pomegranate	HSVd-Clementine Elleuch *et al.*, 2006	HSVd-Fig Yakoubi *et al.*, 2007	HSVd-Mediterranean group Amari *et al.*, 2001	All
No. of analyzed sequences	8	4	2	52	66
%G+C	0.572	0.574	0.561	0.574	0.570
S, variable polymorphic sites	27	13	02	48	29
P_{is}, parsimony informative sites	9	3	0	28	29
Svs, Singleton variable sites	18	10	02	20	30
H, haplotypes	7	4	2	44	52
P_tJC, nucleotide diversity [a]	0.03813	0.02608	0.00747	0.04188	0.4319
H_d, haplotype diversity	1.00±0.076	1.00±0.177	1.00±0.500	0.980±0.008	0.989±0.007
K, average number of nucleotide differences	10.762	7.33	2.00	11.635	10.542
R_m, minimum recombination events	0	1 (151/131)	0	3 (48/56,131/217, 255/271)	3 (48/56, 131/217, 255/271)
Tajma's D[b]	-0.69099 *P*>0.10	-0.40253 *P*>0.10	-	-0.33827 *P*>0.01	-1.01487 *P*>0.01
Fu and Li's D[b]	-0.77657 *P*>0.10	-0.18114 *P*>0.10	-	-1.51280 *P*>0.01	-2.77819* *P*<0.05
Fu and Li's F[b]	-0.83669 *P*>0.10	-0.22576 *P*>0.10	-	-1.28994 *P*>0.01	-2.51029* *P*<0.05
Fu's F[b]	-1.432 *P*=0.193 (>0.01)	0.0037 *P*=0.491 (>0.01)	0.693 *P*=0.667 (>0.01)	-29.737** *P*=0.000	-39.247** *P*=0.000

[a] (Juke and Cantor, 1996).
[b] Neutrality tests.

pomegranate sequences. The Mediterranean HSVd sequences, described previously, carried the greatest number of variable and parsimony-informative sites: 48 and 28 respectively. As regards the pomegranate HSVd variants, their sequence variations were high, with 7 haplotypes detected out of 8 analyzed sequences, yielding a haplotype diversity of 1.00. When sequence variation was determined between and within viroid groups, the Tunisian HSVd isolates, infecting pomegranate, fig and Clementine, displayed the greatest haplotype diversity.

A high level of nucleotide diversity (0.03813) (Jukes and Cantor, 1996) was detected in the HSVd-pomegranate group, as well as in the Mediterranean viroid group (0.04188). When both groups of viroids were taken together, the nucleotide diversity was 0.4319. The average number of nucleotide differences was estimated to be 10.542, that for the pomegranate viroid was 10.762 (Table 3). The high viroid sequence polymorphism may be associated with the evolutionary history of HSVd RNA, which allowed mutations to accumulate within the lineage. Viroids do not replicate as uniform populations, but as a mixture of closely related variants fitting the quasispecies model proposed for RNA replicons (Flores *et al.,* 2001).

Test of selective neutrality

Selective neutrality tests show that the test of Tajima as well as the tests of Fu and Li were not significant in each separate viroid group, but were negative and significant when all groups were taken into account (Table 3). While rejecting the neutrality assumption in all isolates, these tests suggest an excess of singleton. The negative D-value (e.g., ≤-1.5) and the pattern variations provided evidence that the viroid sequences have been expanding rapidly.

Haplotypic network

To improve the assessment of genealogical relationships among HSVd isolates, a minimum spanning network was constructed. A total of 52 haplotypes were deduced from the sequences overall. Figure 4 shows the genetic network of the inferred haplotypes. Putative haplotypes, corresponding to an intermediate evolutionary step, were not detected in the data (empty circles). As can be seen, some reticulations placed in the network are lozenge-shaped reflecting possible recombination events separate from the corresponding viroid sequence. The haplotype patterns suggest the signature of a relatively recent expansion of viroid sequences as star-like groups emerged. In the main group, several haplotypes were directly linked to the most frequent haplotype, H36, which occupied a central position in the network, with

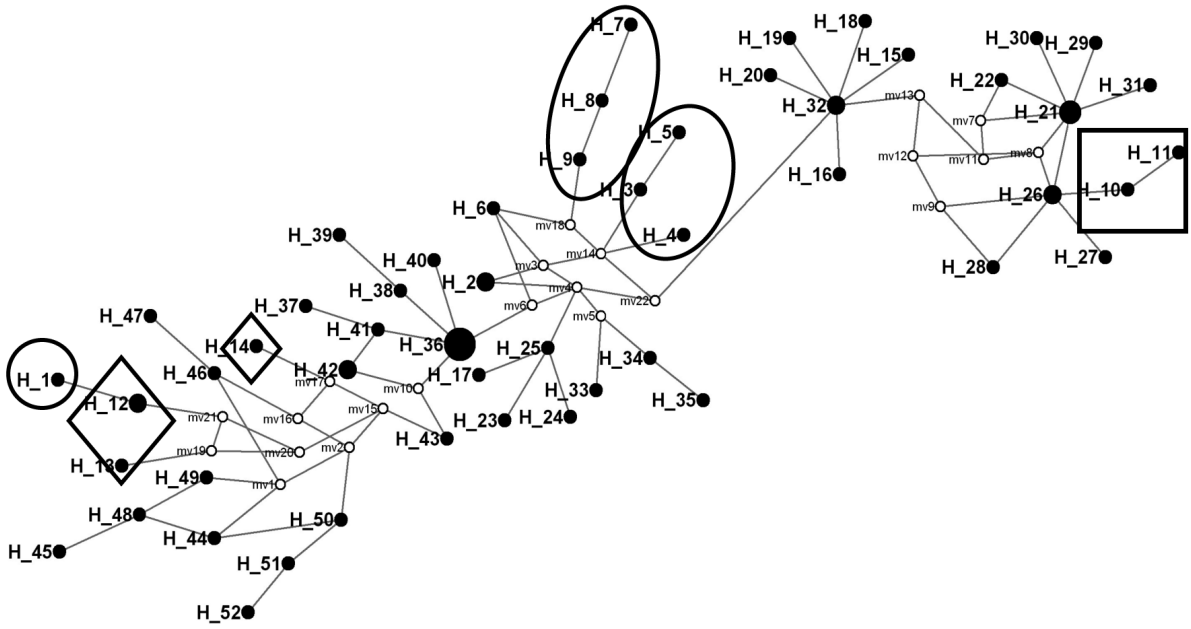

Figure 4. Network of inferred *Hop stunt viroid* (HSVd) haplotypes. Nodes are proportional to haplotype frequencies; Branch's length is proportional to the number of mutations. Tunisian pomegranate HSVd variant: ◊Tunisian clementine • HSVd variants. □ Tunisian fig HSVd variants.

the highest number of connections. This haplotype corresponded to a set of sequences within the hop type group and was most likely the common ancestor of the connected isolates, including Tunisian HSVd-pomegranate and clementine. In the second group the haplotype H32 had a central position connected to haplotypes from Cyprus. The third and the fourth minor groups included haplotypes from Morocco, Tunisia (H10/H11), Spain and Greece. The lack of a geographical structure and the star-like topology depicted by the viroid haplotypes indicate a pattern of HSVd global expansion that was due to the pathogenicity of the haplotypes and host-related evolution. The most frequent haplotype is probably the most stable within a heterogeneous population of closely related variants.

Discussion

HSVd causes a number of fruit diseases: hop stunt (Shikata, 1990), citrus cachexia (Diener *et al.,* 1988), apricot disorder (Amari *et al.,* 2007) and the recently reported jujube disease (Zhang *et al.,* 2009). Although a high incidence of HSVd has been found in some Tunisian fruit trees, this viroid is reported in naturally-infected pomegranate trees for the first time in this country. Previous work, using only the hybridization technique, suggested that HSVd occurred in pomegranate (Astruc *et al.,* 1996), but this technique may give false positive results, as is found in almond samples (Cañizares *et al.,* 1999). In our work, dot-blot hybridization, gel electrophoresis and RT-PCR revealed that HSVd occurred in the leaf samples of three pomegranates out of 46 analyzed. Since these samples were harvested from affected trees, the HSVd incidence seems not to be associated with symptomatology. However, a correlation between disease symptoms and the occurrence of HSVd was clearly demonstrated in apricot trees (Amari *et al.,* 2007). This is also the case with *Peach latent mosaic viroid* (PLMVd) (family: *Avsunviroidae*) the biological properties of which seem to be correlated with the occurrence of specific sequence variants (Rodio *et al.,* 2006; Xu *et al.,* 2008). Whether variation must occur in the viroid before it can cause disease in pomegranate is not yet clear and needs further investigation.

Sequence characterization of the Tunisian HSVd-pomegranate isolates and phylogenetic analysis placed the HSVd variants into two groups, the citrus type and the P-H/Cit 3 type. Since all isolates in this work came from pomegranate, this clustering did not correlate with the source from which the variant was sampled. These findings indicate that the Tunisian pomegranate HSVd variants represent a new population not reported before. Despite an accumulation of nucleotide changes, mainly in the TL and V domains, the secondary structure of the viroid molecule was conserved. The RNA structure is essential for survival and pathogenicity. Viroid fitness depends critically on the correct folding of the genomes (Flores *et al.* 2005). When RNA molecules fold into a functionally correct structure, they are positively filtered by natural selection, whereas mutant sequences, which fall out of the neutral neighborhood, are eliminated from the population due to their low fitness (Sanjuán *et al.,* 2006).

Characterization of sequence variations among pomegranate HSVd variants revealed a large number of polymorphic positions. Most sequences displayed several nucleotide changes from the closest HSVd in GenBank, except for two variants obtained from the north-western part of the country which were identical to a citrus strain reported previously. Of a special interest is the spread of this HSVd-cachexia inducing strain in pomegranate-growing areas. This is the first report of such a case on pomegranate trees.

Sequence heterogeneity among viroid variants has been reported before. The generation of genetic diversity in PLMVd variants resulted from fluctuations in the sequence spectrum due mainly to an accumulation of point mutations (Flores *et al.,* 2006).

The polymorphism pattern revealed differences at the sequence level within the Tunisian HSVd isolates and with isolates from other Mediterranean areas and different hosts, suggesting once again that the viroid populations consist of a pool of closely related molecules. Most of the observed variations resulted from an accumulation of mutants emerging *de novo* during viroid replication. The irregular distribution patterns of both DNA variation and haplotypes suggest that HSVd viroids have spread rapidly. Such a rapid spread does not seem to be connected with any biogeographical origin or host type.

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