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Short Communication

The genetic diversity of *Citrus dwarfing viroid* populations is mainly dependent on the infected host species

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As with viruses, viroids infect their hosts as polymorphic populations of variants. Identifying possible sources of genetic variability is significant in the case of the species *Citrus dwarfing viroid* (CDVd) which has been proposed as a dwarfing agent for high-density citrus plantings. Here, a natural CDVd isolate (CMC) was used as an inoculum source for long-term (25 years) and short-term (1 year) bioassays in different citrus host species. Characterization of progenies indicated that the genetic stability of CDVd populations was high in certain hosts (trifoliate orange, Troyer citrange, Etrog citron, Navelina sweet orange), which preserve viroid populations similar to the original CMC isolate even after 25 years. By contrast, CDVd variant populations in Interdonato lemon and Volkamer lemon were completely different to those in the inoculated sources, highlighting how influential the host is on the genetic variability of CDVd populations. Implications for risk assessment of CDVd as a dwarfing agent are discussed.

Viroids, single-stranded, small (246–401 nt) and circular RNAs lacking protein-coding ability, replicate and move systemically in susceptible plants, frequently causing diseases (Ding, 2009; Flores *et al.*, 2005; Tsagris *et al.*, 2008). Viroid species are classified into the families *Avsunviroidae* (type species *Avocado sunblotch viroid*) and *Pospiviroidae* (type species *Potato spindle tuber viroid*, PSTVd), grouping viroids replicating and accumulating within the chloroplast and the nucleus, respectively (Owens *et al.*, 2012). In early studies, a single pathogenic (P) domain was identified as the molecular determinant of symptoms elicited by PSTVd (Schnölzer *et al.*, 1985; Keese & Symons, 1985; Owens *et al.*, 1996). More complex scenarios were shown later for this and other members of the family *Pospiviroidae* (Sano *et al.*, 1992; Qi & Ding 2003; Flores *et al.*, 2012). In several viroid/host combinations even one nucleotide change, mapping outside the P domain, may largely modify viroid pathogenicity (Qi & Ding 2003; Serra *et al.*, 2008).

Like viruses (Domingo *et al.*, 1978), viroids form polymorphic populations of variants in their hosts, which differ slightly to each other and are generally distributed around a predominant sequence (the master sequence) (Codoñer *et al.*, 2006), thus showing the features typical of a quasi-species (Eigen, 1993). Sequence diversity within viroid populations results from two divergent forces: high mutation rates during viroid replication (Gago *et al.*, 2009), catalysed by a host DNA-dependent RNA polymerase forced to use an RNA template without proof-reading ability, and adaptive constraints, which include certain selective pressures imposed by the host species (Flores *et al.*, 2011). The first evidence of host plant involvement in determining genetic diversity in viroid populations was provided by the identification of specific mutations, designated the 'tomato signature', in variants of the species *Citrus exocortis viroid* (CEVd) (family *Pospiviroidae*, genus *Pospiviroid*) recovered from hybrid tomato tissues (Semancik *et al.*, 1993). Additional studies confirmed the great genetic flexibility of CEVd in several herbaceous and woody hosts, in some cases associated with evident changes in symptom expression (Szychowski *et al.*, 2005; Gandía *et al.*, 2007; Bernad *et al.*, 2009; Hajeri *et al.*, 2011). Phylogenetic analyses indirectly support a similar conclusion for the species *Hop stunt viroid* (Kofalvi *et al.*, 1997;

The GenBank/EMBL/DDBJ accession numbers for the complete sequences of the *Citrus dwarfing viroid* variants are JF970266.1 for H2-2, JF970267.1 for H2-7, EU938647.1 for H6-2, EU938651.1 for H6-10, JF970268.1 for H10-7, EU938652.1 for H14-13, EU938653.1 for H14-14, JF970269.1 for H14-16, EU938648.1 for H15-9, EU938649.1 for H16-2, JF970265.1 for H16-9, EU938654.1 for H16-13, EU938650.1 for H20-3, JF970270.1 for H20-7, EU938641.1 for PR-1, EU938642.1 for PR-3, EU938643.1 for PR-7, EU938644.1 for CR-1, EU938639.1 for VR-4, JF12070.1 for VR-15, JF812069.1 LS-4, EU938640.1 for LS-10 and JF970264.1 for LS-11.

A supplementary figure and two supplementary tables are available with the online version of this paper.

Table 1. CDVd inocula sources, inoculated citrus species and variant composition of each isolate as found in long-term (25 years; inoculation source CMC) and short-term (1 year; inoculation source either H6 or ER) bioassays

Inoculation source	Inoculated species	Isolate	Sequenced variant	Frequency of CVd-IIIb (%)*	Frequency of other variants (%)
CMC	Trifoliate orange	H6	9	78	22
CMC	Troyer citrange	H15	9	78	22
CMC	Trifoliate orange	H16	9	56	44
CMC	Trifoliate orange	H2	9	70	30
CMC	Trifoliate orange	H10	9	89	11
CMC	Trifoliate orange	H17	9	78	22
CMC	Trifoliate orange	H20	10	70	30
CMC	Trifoliate orange	H14	10	70	30
H6	Trifoliate orange	PR	10	70	30
H6	Sour orange	SR	10	100	0
H6	Troyer citrange	CR	10	90	10
H6	Volkamer lemon	VR	10	0	100
H6	Etrog citron	ER	10	100	0
ER	Navelina sweet orange	NS	0	100	0
ER	Interdonato lemon	LS	10	0	100

*CVd-IIIb: type sequence GenBank no. AF184147 (Rakowski *et al.*, 1994).

Amari *et al.*, 2001), another member of the family *Pospiviroidae* (genus *Hostuviroid*), which infects citrus and many other botanical species (Hadidi *et al.*, 2003).

The identification of potential sources of genetic diversity is of significant interest in the case of the species *Citrus dwarfing viroid* (CDVd) (formerly Citrus viroid III) (Rakowski *et al.*, 1994; Vernière *et al.*, 2004), another member of the family *Pospiviroidae* (genus *Apscaviroids)* with a host range restricted to citrus. Notwithstanding, CDVd causes petiole bending and necrosis accompanied by general leaf dropping in Etrog citron (ER) and it induces dwarfing in citrus plants grafted on *Poncirus trifoliata* and its hybrids without detrimental effects on canopy, yield or fruit quality (Hutton *et al.*, 2000; Vidalakis *et al.*, 2011). For this reason, the use of this viroid as a dwarfing agent in high-density citrus plantings has been encouraged in different citrus-growing countries (Semancik *et al.*, 1997; Tessitori *et al.*, 2002; Hardy *et al.*, 2004). Although available data support the high genetic stability of CDVd populations in some hosts, such as Valencia orange (Semancik *et al.*, 1997) and ER (Owens *et al.*, 1999), CDVd sequence variants differing in biological properties and pathogenicity have been reported (Owens *et al.*, 2000; Murcia *et al.*, 2009). This genetic variability is particularly interesting in risk assessing CDVd as a dwarfing agent in high-density citrus plantings (Semancik, 2003), calling for further studies to identify possible sources of genetic instability for this viroid.

Here, the issue of whether the inoculated host species may influence CDVd sequence stability is specifically addressed. To this aim, we used the previously reported Italian CDVd isolate, named CMC (Tessitori *et al.*, 2002), as the source of a natural viroid population for investigating its evolution in several citrus species.

Previous molecular characterization of the CMC source showed a CDVd population with a master sequence of 294 nt in length (Tessitori *et al.*, 2002), identical to the formerly known CVd-IIIb (Rakowski *et al.*, 1994), a CDVd variant used recently to identify plant genes involved in host–viroid interaction (Tessitori *et al.*, 2007) and already reported as a dwarfing agent (Albanese *et al.*, 1996; Duran-Vila & Semancik, 2003). Due to the absence of other viroids, the CMC isolate has been used as a source of the dwarfing agent in different experimental and commercial citrus orchards in South Italy (Albanese *et al.*, 1996; Rizza *et al.*, 2010).

In the first step of our study, CDVd genetic stability was investigated over time in two different hosts: trifoliate orange [*P. trifoliata* (L.) Raf] and Troyer citrange (*Citrus sinensis* × *P. trifoliata*). To this end, we took advantage of seven trifoliate orange seedlings and one Troyer citrange seedling bark inoculated with the same original CMC source in 1984 and grown in natural field conditions to study agronomic effects of the dwarfing agent on these rootstocks. As expected, 25 years post-inoculation (p.i.) CDVd-infected hosts showed a significant reduction in the canopy volume with respect to that of the uninoculated controls, confirming data already observed in the same plants 11 years p.i. (canopy reduction of 20–26 %) (Albanese *et al.*, 1996). No specific symptoms of chronic infections by other pathogens were observed (fungi, bacteria and viruses). CDVd infection was confirmed by testing nucleic acid preparations obtained as reported previously (Flores & Llàcer, 1988) using sequential PAGE (Rivera-Bustamante *et al.*, 1986), thus excluding infections by other viroid species and ascertaining similar CDVd accumulation levels in the tested samples. Subsequently,

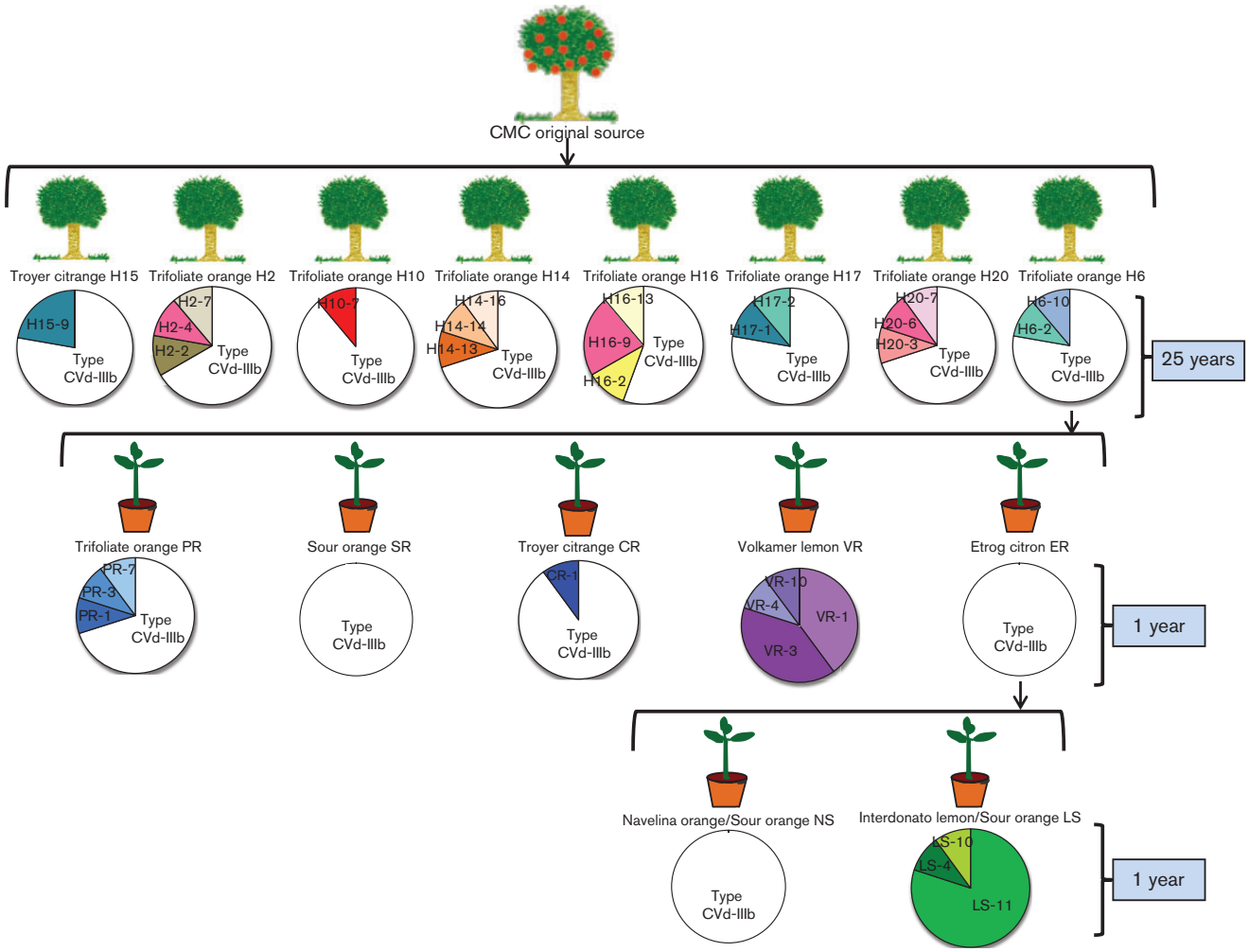

Fig. 1. CDVd transmission chart with progeny viroid populations. Arrows mark the inoculation source of each bioassay. Years elapsed from bark inoculation to progeny characterization in each bioassay are indicated in the boxes on the right. With the exclusion of the reference CVd-IIIb, the name of each variant is composed of the name of the isolate in which it was found and the variant number.

full-length viroid cDNA from each inoculated tree was generated by RT-PCR using the specific primer pair CMC2 (5′-GACGACGACAGGTAAGTTTCCCT-3′) and CMC1 (5′-GACGAAGGCAGCTAAGTTGGT-3′) (complementary and homologous to residues 67–89 and 90–110, respectively) and *Taq* DNA polymerase (Promega), and then it was cloned and sequenced as previously described (Tessitori *et al.*, 2002). In line with former studies on CDVd and other members of the family *Pospiviroidae* (Góra *et al.*, 1994; Góra-Sochaka *et al.*, 1997; Owens *et al.*, 2000), nine or ten clones were sequenced for each inoculated tree, totalling 74 full-length CDVd nucleotide sequences.

Multiple sequence alignments showed no major differences in CDVd populations isolated from trifoliate orange or Troyer citrange hosts (Table 1 and Fig. S1, available in JGV Online). With the exclusion of the H6-2 and H17-2 variants, which were 293 nt, all the other CDVd variants showed a length of 294 nt. Moreover, 73 % of the sequences (54 out of the 74 variant totals) corresponded to the previously reported CV-IIIb (Rakowski *et al.*, 1994), which was also identified as the master sequence in the CDVd populations from all the inoculated trees. Interestingly, CVd-IIIb had also been identified as the master sequence in the inoculation source CMC (Tessitori *et al.*, 2002), thus supporting the high genetic stability over time (25 years) of the CMC dwarfing isolate in trifoliate orange and Troyer citrange grown in natural field conditions.

In the second step, the sequence stability of CDVd in hosts/rootstocks with different grades of susceptibility to citrus viroids was tested in short-term greenhouse assays. In this study, trifoliate orange H6, a CDVd isolate representative of the low-genetic diversity of the viroid populations observed in the previous experiment, was the source for bark inoculation of 1-year seedlings of the indicator ER and the following citrus

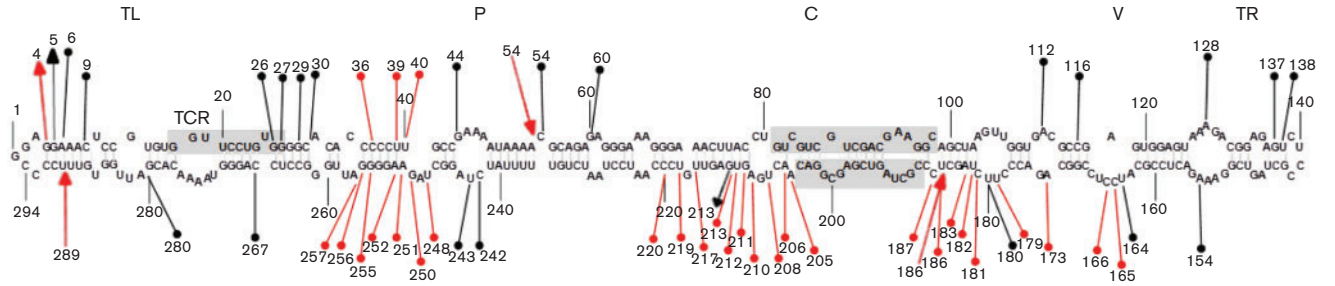

Fig. 2. Secondary structure proposed for the CVd-IIIb type variant (GenBank no. AF184147, Rakowski *et al.*, 1994), with the five structural domains identified within members of the family *Pospiviroidae* (Keese & Symons, 1985). The polymorphic positions identified in the variants from VR and LS are marked in red, while polymorphic positions in progeny variants from Troyer citrange, Trifoliate orange, SR and NS isolates are marked in black with respect to CVd-IIIb (see also Fig. S1). Circles (on the end of lines), arrowheads pointing to, and arrowheads pointing away from the sequence indicate nucleotide changes, insertions and deletions, respectively. Domains: TL, terminal left; P, pathogenic; C, central; V, variable; TR, terminal right.

rootstocks: sour orange (SR), trifoliate orange (PR), Carrizo citrange (CR) and Volkamer lemon (VR). Ten viroid cDNA clones for each inoculated seedling were sequenced 1 year p.i. Except for the expected moderate leaf dropping on ER, no other phenotype alterations were observed. Interestingly, clones from ER and SR seedlings corresponded to the CVd-IIIb variant, and a large prevalence of this variant (70 and 90 %, respectively) was also registered in the progeny of PR and CR isolates (Table 1 and Fig. 1), with diverging variants showing generally only one or few point mutations compared with the master sequence (Fig. S1).

By contrast, the composition of the CDVd population in VR isolates was extremely different to that of the original source: none of the ten sequenced clones matched CVd-IIIb or any other variants previously reported in the inoculated source. Furthermore, a new master sequence (VR-1) showing five nucleotide changes compared with CVd-IIIb was identified, thus suggesting a possible role of the inoculated host in amplifying genetic diversity in viroid progeny. Additional analyses by multiple sequence alignments showed that the four variants VR1, VR6, VR8 and VR9, and the VR3, VR5, VR7 and VR10 variants (Fig. S1) are identical to each other. The BLAST program (Altschul *et al.*, 1990) revealed that these variants were also identical to the TS1 (GenBank no. AB054627.1) and TS2 variants (AB054628.1), respectively, both sequenced by Ito *et al.* (2002) from the Shiranui citrus cultivar [(*Citrus reticulata* × *C. sinensis*) × *C. reticulata*]. The VR4 clone showed two additional changes (C_{165}→U and C_{166}→U) compared with those of the previous two groups of variants (Fig. S1), while the VR15 clone had a higher number of nucleotide changes compared with those of the CVd-IIIb and the TS1 and TS2 variants (Table S1, Fig. S1).

Altogether, these data confirm the stability of CDVd populations in certain hosts (SR, trifoliate orange, Troyer citrange and ER), but at the same time they highlight the role of the rootstock VR as a potential source of fast (only 1 year) and significant genetic diversity in the viroid population.

Finally, two commercial citrus varieties, the Interdonato lemon (LS) and Navelina sweet orange (NS), both grafted on viroid-free SR rootstock, were tested for their ability to influence the genetic stability of CDVd. In this experiment, the ER isolate from the previous trial was used as the viroid source for the bark inoculation of 2-year-old LS and NS scions. We selected this inoculation source because of its homogeneous viroid population, prevalently composed of the variant CVd-IIIb (Fig. 1). The prevalence of CVd-IIIb in this isolate was further confirmed by extending sequencing to seven additional full-length CDVd cDNA clones and finding that all the new sequenced variants corresponded to CVd-IIIb. One year p.i., sequencing of progeny variants showed that most cloned variants were 294 nt in length, irrespective of the inoculated host species, with only two variants, LS-10 and LS-4, out of the 20 sequenced with a length of 295 and 293 nt, respectively (Table 1). However, while all sequenced clones from inoculated NS corresponded to the variant CV-IIIb (Table 1, Fig. 1), substantially reflecting the variant composition in the original inoculation source, the CDVd population in the LS differed greatly from the latter in a period of just 1 year. In fact, the master sequence in the viroid population from LS diverged at nine positions from the CVd-IIIb variant and no clone similar to CVd-IIIb or to any other variant previously reported in the original CMC source was detected, thus confirming the role of the LS in amplifying sequence diversity in CDVd progeny.

Phylogenetic analysis of the CDVd variants sequenced in this study did support a possible response of the CDVd progenies to selective pressure imposed by the host. In the phylogram generated by the Unweighted Pair Group Method with Arithmetic Mean method (Sneath & Sokal, 1973) using the MEGA version 5 program (Tamura *et al.*, 2011) (Fig. S1), all the variants differing from CVd-IIIb and recovered from trifoliate orange (H and PR) and Troyer citrange (H15 and CR) plants are clustered in a single group, close to CVd-IIIb (defined as the root in the

tree), regardless of the time elapsed since the inoculation (20 years or 1 year), thus supporting a minimal influence of time on CDVd variability in those hosts. Moreover, LS and VR progeny variants, recovered just 1 year p.i., cluster close to each other and distant to CVd-IIIb and the most related variants, highlighting the role of the host species as a source of genetic variability. The two LS4 and VR15 variants, probably due to several additional nucleotide changes, are found in a separate branch but close to the LS and VR clusters (Fig. S1).

The genetic diversity of CDVd was also quantified by calculating the Shannon entropy (H) mean, which provides an estimation of mutation probability at each sequence position within the viroid populations (Schneider & Stephens, 1990). These values were calculated for all the tree isolates characterized in this study by considering the alignments of the variants sequenced in each progeny. As shown in Table S2, from which the isolates with H mean values lower than 0.02 (corresponding to a single mutation in a single variant) were removed, CDVd populations from LS and NS had much higher H mean values than those of other hosts, thus confirming the higher polymorphism of viroid populations in the former isolates.

As shown in other viroids (Flores *et al.*, 2012), the nucleotide changes identified in the CDVd variants sequenced in this study do not induce major modifications in the rod-like secondary structure of CDVd because they mainly map at loops or are compensatory mutations (Fig. 2). However, while changes with respect to the reference variant (CVd-IIIb) were mostly distributed throughout the molecule in progeny variants from trifoliate orange and Troyer citrange, they were prevalently concentrated in two regions in the lemon isolate variants, corresponding to the lower strand of the central and P domains. In the context of this study, it is noteworthy that point mutations with respect to CVd-IIIb in the P domain of CDVd variants had been previously associated with the modification of pathogenic severity in ER (Owens *et al.*, 1999), thus supporting possible involvement in pathogenicity of a structural region that is particularly variable in the CDVd lemon isolates.

In conclusion, our study highlights the influence of the host in determining the genetic stability of CDVd populations. This contributes significantly to the risk analysis of CDVd as a dwarfing agent. In line with previous results (Semancik *et al.*, 1997; Owens *et al.*, 1999), CDVd genetic stability is high in certain rootstocks and varieties (Semancik, 2003); however, we have shown that CDVd populations selected in other hosts, such as LS and VR, accumulate high levels of sequence heterogeneity in just 1 year. The reasons for this high CDVd genetic variability remain unknown; however, since in our experiments all inoculated seedlings were grown in the same conditions, we can exclude the involvement of environmental conditions, which have been shown to play a role in viroid genetic stability in other viroid/host combinations (Matoušek *et al.*, 2001).

Although pathogenic properties of these progeny populations are still unknown, these findings suggest using CDVd as a dwarfing agent in lemon cultivars and their hybrids with particular caution. These considerations are consistent with conclusions reported by Broadbent *et al.* (1988), exclusively based on phenotypic effects in Eureka lemon inoculated with a dwarfing agent (probably CDVd) on Eureka lemon.

As a general conclusion, our study suggests the need for in-depth studies on CDVd genetic stability in citrus scions and rootstocks before proposing them as possible hosts of this viroid in high-density citrus plantings. In addition, it cannot be excluded that both the high level of inoculum present in high-density plantings could favour the natural transmission of CDVd to lemon cultivars, and new pathogenic variants could be generated in this host.

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