Host Effect on the Genetic Variability of *Citrus exocortis viroid* (CEVd)

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ABSTRACT. Natural infections of *Citrus exocortis viroid* (CEVd) have been found in both citrus and non-citrus hosts. Field isolates of CEVd are complex populations of sequence variants (haplotypes) that fit a quasispecies model. We have previously shown that a CEVd isolate initially recovered from citrus and maintained in Etrog citron contained two predominant haplotypes with low nucleotide diversity. This CEVd infected citron was used as the inoculum source for viroid transmission to trifoliate orange and sour orange seedlings that have been growing in the field since their inoculation in 1993. CEVd recovered from trifoliate orange showing the characteristic symptoms of stunting, bark scaling and stem blotching was also characterized as a population of variants with low nucleotide diversity and containing a clearly predominant haplotype distinct from those identified in the inoculum source. CEVd recovered from sour orange, which remained symptomless, was characterized as a population with a higher nucleotide diversity in which no dominant haplotype could be identified. The tolerant viroid-host combination appeared to act as permissive host and maintained a greater CEVd variability than sensitive hosts. Similarly, a CEVd isolate recovered from a symptomless broad bean plant was characterized as a diverse population that lacked a predominant haplotype. When this CEVd source was mechanically transmitted to tomato and from tomato back to broad bean, the plants displayed stunting symptoms, and the CEVd population exhibited a predominant haplotype and low nucleotide diversity.

Index words. Quasispecies, variants, haplotyopes.

Citrus exocortis viroid (CEVd) is a species of the family *Pospiviroidae* and, like the other members of this family, has a highly base paired rod-like secondary structure which follows the five structural domains proposed by Keese and Symons (14): Terminal Left (T_L), Pathogenicity (P), Central (C), Variable (V) and Terminal Right (T_R) (Fig. 1). CEVd has a Central Conserved Region (CCR) and a Terminal Conserved Region (TCR) characteristic of members of the *Pospiviroid* genus. Like the other species of the family *Pospiviroidae,* CEVd replicates and accumulates in the nucleus and lacks RNA self-cleavage mediated by hammerhead ribozymes which is characteristic of the viroids of the *Avsunviroidae* family (8).

CEVd is the causal agent of exocortis, a bark shelling or scaling disorder affecting trifoliate orange (7). This disease affects trees grafted on trifoliate orange, citrange hybrids and Rangpur lime, which are stunted and have bark scaling symptoms in the rootstock. In the indicator Etrog citron, CEVd induces severe stunting, leaf epinasty and vein necrosis (3). The characterization of a severe CEVd isolate (CEVd-117) maintained in Etrog citron demonstrated that it was a population of closely related sequence variants (haplotypes) (9). Furthermore, infection with a single haplotype resulted in the generation of *de novo* populations of haplotypes (10) conforming to the quasispecies model proposed by Eigen for RNA replicons (4). Trifoliate orange seedling trees that had been graft inoculated with CEVd-117 were severely stunted and developed the characteristic bark scaling symptoms whereas sour orange seedling trees inoculated with the same CEVd source were symptomless (23). In the present work we describe the composition of the CEVd population recovered from infected trifoliate orange and sour orange seedlings with the aim of establishing the host effect on the composition of CEVd quasispecies.

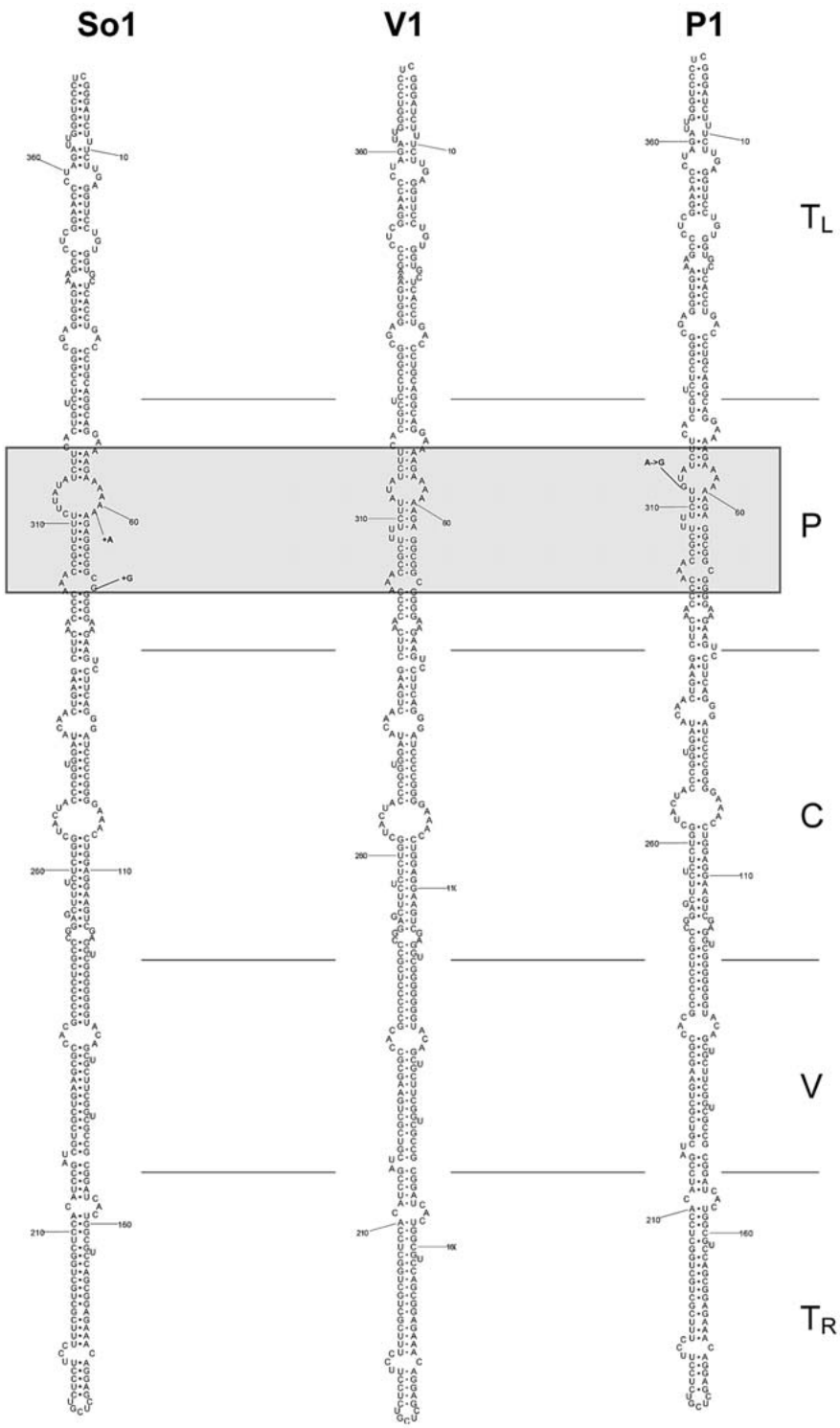

Fig. 1. Secondary structure of the dominant haplotypes recovered from infected sour orange (So1), trifoliate orange (P1) and the citron inoculum source (V1). The region in which the secondary structure is affected and the result of changes identified in So1 is shaded.

CEVd has a broad experimental host range (20), and has been reported to naturally infect other crops (5, 6, 11). Very low titers of CEVd were detected in field grown symptomless broad bean plants, but after a serial inoculation to tomato and back to broad bean, major changes were observed in the response of inoculated broad bean plants. The broad bean plants inoculated with CEVd passaged through tomato (CEVd-f) contained high CEVd titers and developed stunting symptoms (6). The original CEVd isolate (CEVd-i) and CEVd-f retained their properties after several passages to broad bean. Broad bean plants inoculated with CEVd-i always accumulated low viroid titers and remained symptomless, whereas plants inoculated with CEVd-f accumulated high viroid titers and developed stunting symptoms. These observations suggested that passage through tomato resulted in major changes in the composition of the CEVd population. The present work describes the composition of CEVd-i and CEVd-f.

MATERIALS AND METHODS

Plant materials and viroid sources. The population structure of a CEVd isolate (CEV-117) maintained in Etrog citron was characterized previously (9). In 1992, trifoliate orange and sour orange seedlings were grafted inoculated with CEVd-117 and transplanted to the field 1 yr later. In 2003, tissue was collected from these infected trees and used to characterize their respective CEVd populations.

Nucleic acid preparations containing CEVd-i and CEVd-f were used to mechanically inoculate broad bean seedlings. CEVd-i was recovered from naturally infected (and symptomless) broad bean plants and CEVd-f was recovered from stunted broad bean plants inoculated with preparations of CEVd-f that had been passed through tomato (6).

Nucleic acid extraction, cDNA synthesis and cloning. Samples (0.5 g) of trifoliate orange and sour orange bark and leaf tissue were gently homogenized in sealed plastic bags containing 5 ml of TE buffer (0.1 M Tris-HCl, pH 8.5; 50 mM EDTA; 0.5 M NaCl; 10 mM β-mercaptoethanol) and the homogenate was subjected to alkaline denaturation as described by Astruc et al. (1) and Pallás et al. (18). cDNA synthesis and amplification of full length CEVd DNA was performed as described by Bernad and Duran-Vila (these proceedings).

Samples (5.0 g) of broad bean leaves and stems were homogenized in 5 ml of extraction buffer (0.4M Tris-HCl, pH 8.9; 1% (w/v) SDS; 4% (v/v) β-mercaptoethanol; 5 mM EDTA, pH 7) containing water-saturated phenol and the total nucleic acids were partitioned in 2 M LiCl (21). The soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl; 10 mM KCl; 0.1 mM $MgCl_2$, pH 7.4). cDNA synthesis and amplification of full length CEVd DNA were performed as described by Gandía et al. (9).

The size of the DNA amplicons was estimated by electrophoresis in 2% agarose gels containing TBE (90 mM Tris-Borate, 2 mM EDTA, pH 8) buffer, and the purified DNA was ligated into the pGem-T (Promega®) vector.

SSCP analysis. cDNA clones were subject to single-stranded conformational polymorphism (SSCP) analysis. CEVd cDNA inserts were recovered from the plasmids by polymerase chain reaction (PCR) amplification (17). Aliquots (2 µl) of each PCR product were mixed with 20 µl of denaturing solution (90% formamide, 25 mM EDTA, 0.05% xylene-cyanole and 0.05% bromophenol blue), heated for 10 min at 100°C and chilled on ice. Denatured DNA was subjected to 14% polyacrylamide gel electrophoresis (PAGE) (14 × 11.5 × 0.075 cm gels)

in TBE buffer at 200V constant voltage for 16 hr. The DNA bands were visualized by silver staining (12).

Nucleotide sequence and statistical analysis. Cloned viroid cDNAs were sequenced with an ABI PRISM DNA 377 sequencer (Perkin-Elmer). Multiple sequence alignments were performed with the program Clustal W (22). Secondary structure analyses were performed with the program MFOLD (circular version) from the GCG package (25) and RNAviz (2).

Nucleotide distances were estimated considering alignment gaps and using the Jukes-Cantor (13) correction for superimposed substitutions with the Haplo program (16). Nucleotide diversity was estimated using the formula $D = 2/(n(n-1)) \sum n_i n_j d_{ij}$, where n is the number of clones analyzed per isolate, n_i and n_j are the number of clones of haplotype i and j respectively, and d_{ij} is the nucleotide distance between haplotypes i and j.

Phylogenetic analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (15). The CEVd sequences recovered from trifoliate orange, sour orange and broad bean were aligned using Clustal W (22). A phylogenetic tree was constructed using neighbour-joining clustering method (19) and input distances were estimated by the Jukes-Cantor model (13). A bootstrap analysis was conducted with 5000 pseudoreplicates.

RESULTS

CEVd populations in trifoliate orange and sour orange. A DNA product of the expected size for CEVd was recovered when nucleic acid preparations from infected trifoliate orange and sour orange were subjected to reverse transcription and PCR amplification (RT-PCR) as described.

SSCP analysis of 30 clones recovered from trifoliate orange, showed 9 different electrophoretic profiles (data not shown). As shown in Table 1, sequence analysis demonstrated the existence of at least 9 different haplotypes (P1 to P9) with P1 being the dominant haplotype representing 60% (18 clones) of the population. Comparison of the CEVd sequences recovered from trifoliate orange with those previously identified in the citron inoculum source (9) showed that these two populations were distinct. The dominant haplotype P1 differed from the dominant haplotype V1 (DDBJ/EMBL/GenBank accesion AJ54795) in CEVd-

TABLE 1
CEVd HAPLOTYPES RECOVERED FROM INOCULATED TRIFOLIATE ORANGE

Haplotype	Frequency	Changes[z]	Position	Domain	Haplotype[y] in citron
P1	18/30 (60%)				
P2	5/30 (16.6%)	U→A	185	T_R	
P3	1/30 (3.3%)	+G	73	P	V2 (25%)
		G→A	313		
P4	1/30 (3.3%)	G→C	124	V	
P5	1/30 (3.3%)	U→A	129	V	
		C→U	232	V	
P6	1/30 (3.3%)	U→C	207	T_R	
P7	1/30 (3.3%)	U→C	256	C	
P8	1/30 (3.3%)	-GGU	127-129	V	
P9	1/30 (3.3%)	G→A	26	T_L	

[z]Changes in P2 to P9 as compared to the sequence of P1. P1 differs from the dominant haplotype of citron (V1, DDBJ/EMBL/GenBank accession AJ54795) in a single A_G change in position 313 (see Fig. 1).
[y]Haplotypes identified in the citron inoculum source and their frequency in that population.

117 in a single A→G change in position 313 located in a loop of the P domain (see Fig. 1) that does not seem to affect the base pairing of the P domain. This change A→G was present in all haplotypes except P3. The nucleotide changes characteristic of this population and their positions in the secondary structure of CEVd are summarized in Table 1, and this data shows the nucleotide changes of P2 to P9 with respect to the sequence of P1. P3 was the only haplotype in trifoliate orange also found among the 44 haplotypes in CEVd-117 (Table 1). The nucleotide diversity estimated from the haplotype frequencies and nucleotide distances was 0.0018 ± 0.0008.

SSCP analysis of 30 clones recovered from sour orange yielded 21 different SSCP electrophoretic profiles (data not shown). Sequence analysis demonstrated the existence of at least 21 different haplotypes with So1 and So2, representing 16.6% (5 clones) and 13.3% (4 clones) of the population, respectively (Table 2). The remaining haplotypes (So3 to So21) were represented by only one (So5 to So21) or two clones (So3 and So4), totalling 60% of the population. Although some of the single nucleotide substitutions recovered may be due to polymerase errors, differences in the composition of the CEVd populations are unquestionable. Comparison of the CEVd sequences recovered from sour orange with those identified in the citron inoculum source (9) showed that both populations were distinct. The dominant haplotypes So1 and So2 differed from the dominant haplotype (V1) of CEVd-117 by two changes (+A and +G) located at positions 62 and 71, respectively. As shown in Figure 1, these insertions modify base pairing within the V domain. The nucleotide changes characteristic of this population and their position in the secondary structure of CEVd are summarized in Table 2 and show the nucleotide changes of So2 to So21 with respect to the sequence of So1. The nucleotide diversity estimated from the haplotype frequencies and nucleotide distances was 0.0066 ± 0.0018.

CEVd populations in broad bean. DNA products of the size expected for CEVd were recovered when preparations from bead bean infected with CEVd-i and CEVd-f were used as template for RT-PCR.

The electrophoretic SSCP profiles of 35 cDNA clones recovered from CEVd-i were all different, suggesting a very heterogeneous population in which no dominant profile was identified (data not shown). Sequence analysis of 20 clones confirmed that all the sequences were different. Alignment with the class A and class B reference sequences defined by Visvader and Symons (24) showed that three haplotypes were homologous (97.9-99.2% identity) to class B and presented the signature characteristic of this class in the P and V domains. The other seventeen were homologous to class A (96.5%-97.6% identity). The Neighbor-joining phylogenetic tree produced by the MEGA 2.1 program (15, 19) confirmed that three sequences were closely related to class B, two were related to Class A whereas the rest were separated from both (data not shown).

Conversely, SSCP analysis of the clones recovered from CEVd-f showed that 28 out 40 had the same electrophoretic profile, suggesting that the population was highly homogeneous. Sequence analysis confirmed the presence of a dominant haplotype highly homologous to CEVd class A (97.6-97.9% identity). Seven additional haplotypes were also identified and each of them presented the signature characteristic of class A in the P and V domains. Work is in progress to fully characterize this population.

Phylogenetic relationships. The phylogenetic tree constructed with the CEVd variants reported in this study shows five groups of sequences (Fig. 2). The Class B

TABLE 2
CEVd HAPLOTYPES RECOVERED FROM INOCULATED SOUR ORANGE

Haplotype	Frequency	Changes[z]	Position	Domain	Haplotype[y] in citron
So1	5/30 (16.6%)				V41 (0.3%)
So2	4/30 (13.3%)	G→A	50	P	
		A→U	302	P	
So3	2/30 (6.6%)	-A	62	P	V1(60%)
		-G	75	P	
So4	2/30 (6.6%)	-G	75	P	V9 (0.6%)
So5	1/30 (3.3%)	-A	62	P	
		-G	75	P	
		A→G	303	P	
So6	1/30 (3.3%)	G→A	50	P	
		-A	62	P	
		-G	75	P	
So7	1/30 (3.3%)	U_A	280	C	
		A→U	302	P	
So8	1/30 (3.3%)	-A	62	P	V2 (25%)
So9	1/30 (3.3%)	-A	62	P	
		A→G	314	P	
So10	1/30 (3.3%)	-G	75	P	
		UA→C	130-131	V	
		A→U	133	V	
		C→G	135	V	
		C→A	138	V	
		C→T	141	V	
So11	1/30 (3.3%)	-A	62	P	
		-G	130	V	
		A→U	302	P	
So12	1/30 (3.3%)	U→C	308	P	
So13	1/30 (3.3%)	A→U	302	P	
So14	1/30 (3.3%)	-A	62	P	
		-G	75	P	
		A→G	317	P	
So15	1/30 (3.3%)	-A	62	P	
		-G	75	P	
		U→C	137	V	
		U→C	286	C	
So16	1/30 (3.3%)	G→A	50	P	

[z]Changes in So2 to So21 as compared to the sequence of So1. So1 differs from the dominant haplotype of citron (V1, DDBJ/EMBL/GenBank accesion AJ54795) in two changes (+A and +G) located at positions 62 and 71, respectively (Fig. 1).
[y]Haplotypes identified in the citron inoculum source and their frequency in that population.

TABLE 2 (CONTINUED)
CEVd HAPLOTYPES RECOVERED FROM INOCULATED SOUR ORANGE

Haplotype	Frequency	Changes[z]	Position	Domain	Haplotype[y] in citron
So17	1/30 (3.3%)	-A	62	P	
		-G	75	P	
		A→U	236	V	
		AA→UU	302-303	C	
		U→C	328	T_L	
So18	1/30 (3.3%)	U→A	280	C	
		A→U	302	P	
So19	1/30 (3.3%)	U→C	30	T_L	
So20	1/30 (3.3%)	-A	62	P	
		-G	76	P	
		G_C	126	V	
		-U	269	C	
		A_C	302	P	
So21	1/30 (3.3%)	G→C	125	V	
		C→G	136	V	

[z]Changes in So2 to So21 as compared to the sequence of So1. So1 differs from the dominant haplotype of citron (V1, DDBJ/EMBL/GenBank accesion AJ54795) in two changes (+A and +G) located at positions 62 and 71, respectively (Fig. 1).
[y]Haplotypes identified in the citron inoculum source and their frequency in that population.

group is phylogenetically distant from the rest and contains the three variants from broad bean (CEVd-i) that are close to the consensus sequence of Class B (24). The other variants are clustered into four groups. The Class A group contains two variants of broad bean (CEVd-i) that are close to the consensus sequence of Class A (24). The variants of broad bean after passage through tomato (CEVd-f) and the variants from trifoliate orange (E-117) are clustered in two separated groups, whereas a fourth group contains mostly additional variants from broad bean (CEVd-i), sour orange, citron, and the trifoliate orange variant also found in citron.

DISCUSSION

Graft transmission to trifoliate orange and sour orange of a CEVd isolate maintained in citron resulted in major changes in the composition and sequence diversity of the viroid population. The inoculum source contained two dominant haplotypes (V1 and V2) and a low sequence diversity (0.0024 ± 0.0012) (9). Transmission to trifoliate orange resulted in changes of the structure of the population with the emergence a dominant haplotype (P1) that was not detectable in the inoculum source. Trifoliate orange may favor the accumulation of haplotypes containing a (A→G) change at position 313 located in a loop of the P domain. The sequence diversity (0.0018 ± 0.0008) was even lower than in citron. Additional work is in progress to confirm if trifoliate orange favors the accumulation of haplotypes containing this change. Transmission to sour orange resulted in a CEVd population with higher sequence diversity (0.0066 ± 0.0018) with a larger number of haplotyes. These results suggest that sour orange acts as a permissive host sustaining the replication of many different, competing haplo-

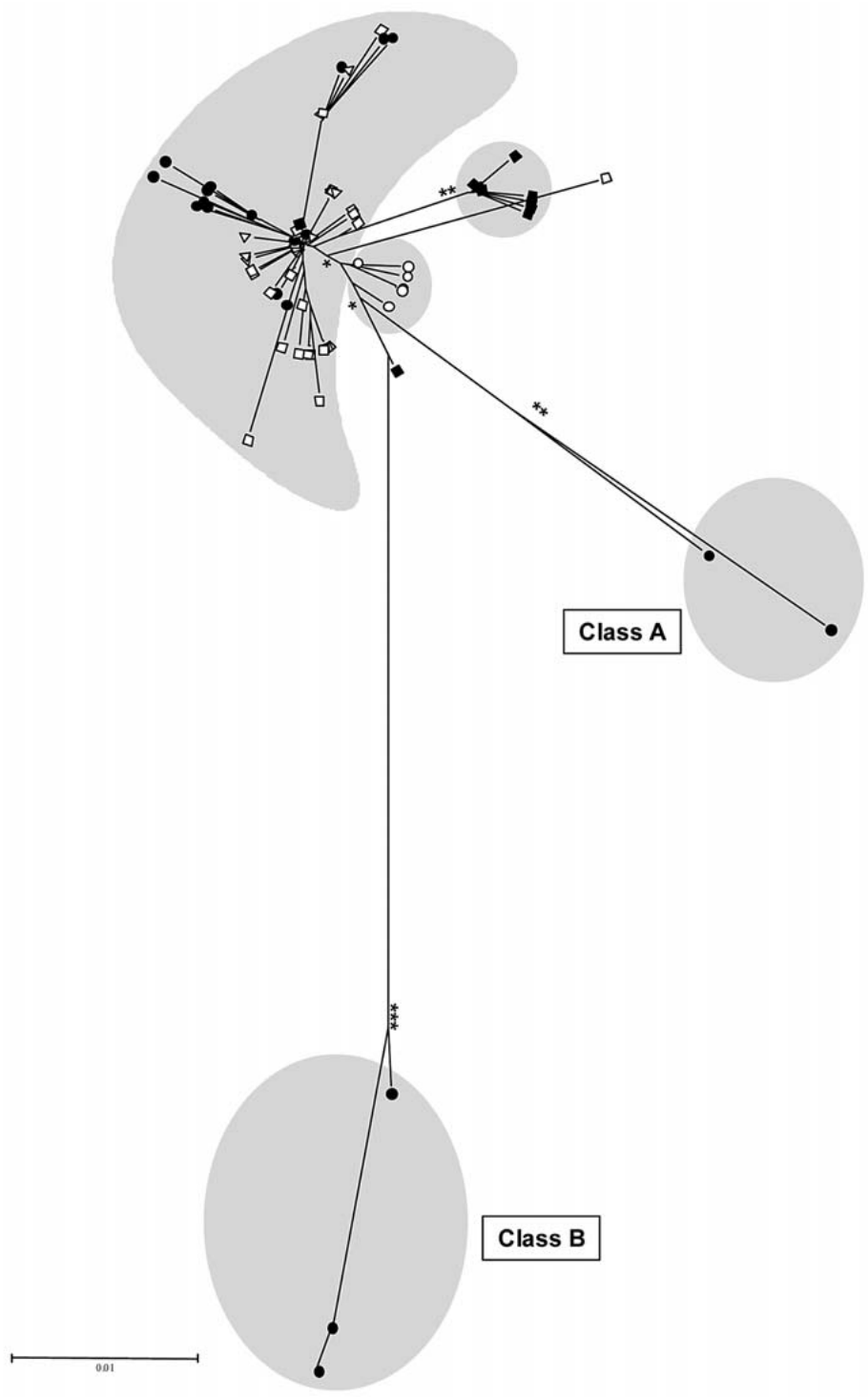

Fig. 2. Phylogenetic tree of CEVd sequence variants. The five phylogenetic groups identified are shaded. CEVd variants of broad bean (CEVd-i) (●), broad bean (CEVd-f) (○), citron (V1-V9) (△), trifoliate orange (P1-P9) (■) and sour orange (So1-So21) (□). Bootstrap values of the nodes are indicated: * = node detected in 100% of replicates; ** = node detected in 80-100% of replicates; *= node detected in >50% of replicates.**

types, whereas trifoliate orange acts as a selective host in which a dominant haplotype appears to be much more fit than the others.

A CEVd isolate (CEVd-i) recovered from symptomless broad bean plants contained a heterogeneous population of sequence variants. Transmission to tomato resulted in changes in its biological properties that were not restored by transmission back to broad bean. The CEVd recovered from these broad bean plants (CEVd-f) contained a homogeneous population of sequence variants, all of them belonging to class A.

These results demonstrate that transmission to alternate hosts results in major changes in the structure and composition of CEVd quasispecies. Symptomless viroid/host combinations (CEVd-infected sour orange and broad bean infected with CEVd-i) sustain more diverse CEVd populations than symptomatic viroid/host combinations (CEVd-infected trifoliate orange and broad bean infected with CEVd-f). Symptomless hosts act as viroid reservoirs ensuring their survival and promoting genetic diversity.

Phylogenetic analysis illustrates the relationship among the CEVd variants reported in this study. The variants identified as naturally occurring in broad bean (CEVd-i) are found in all the major groups including Class A and Class B which are the most distant from the rest. As indicated above, since CEVd populations present low sequence diversity, very little information can be obtained from phylogenetic analyses. However, the analysis indicates that the CEVd variants from broad bean and trifoliate orange are separated from the major group that includes mostly the variants identified in citron and sour orange both of which belong to the genus *Citrus* and therefore are phylogenetically more closely related than trifoliate orange and broad bean.

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