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Genetic variation and population structure of an isolate of *Citrus exocortis viroid* (CEVd) and of the progenies of two infectious sequence variants

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Summary. The population structure and diversity within a *Citrus exocortis viroid* (CEVd) isolate was estimated by single strand conformation polymorphism (SSCP) and sequence analysis. A predominant sequence variant (V1) representing 52.8% of the overall population was identified. V1 and other additional variants presented a composition of the P domain characteristic of severe strains of CEVd. The nucleotide diversity of this CEVd population was lower than expected according to a model of neutral evolution, suggesting a strong negative selection. Citron plants were inoculated with dimeric clones of nine sequence variants and two resulted infectious inducing the severe symptoms characteristic of the original isolate. *De novo* populations were generated from these infectious variants and like in the original CEVd isolate, both populations presented V1 as the predominant variant but they evolved to a higher nucleotide diversity.

Introduction

Viroids are small (246–401 nucleotides) covalently closed single-stranded RNAs. Like viruses, viroids replicate in their host plants in which they may act as phytopathogenic agents, however they do not code for any protein. Viroids are classified into two families, *Pospiviroidae*, composed by species with a central conserved region (CCR) and without hammerhead ribozymes, and *Avsunviroidae*, composed by species lacking CCR but able to self-cleave in both polarity strands through hammerhead ribozymes [12]. The *Citrus exocortis viroid* (CEVd) is the casual agent of exocortis disease [38] characterised by bark scaling and dwarfing

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accesion numbers AJ54795 to AJ54803.

symptoms in sensitive hosts commonly used as rootstocks, and severe stunting and leaf epinasty in indicator hosts. The predicted secondary structure of CEVd like most species of the family *Pospiviroidae* is a highly base paired rod-like structure which follows the five structural domain model proposed by Keese and Symons [23]: Terminal Left (T_L), Pathogenicity (P), Central (C), Variable (V) and Terminal Right (T_R). In contrast, Bussière et al. [5] and Gast et al. [16] described that CEVd have a cruciform branched structure involving 80 nt located in the T_l domain, which was not supported by further structural characterization by NMR and thermodynamic analysis [7].

Viroids, like other RNA genomes, are believed to have a potential for high genetic diversity due to the absence of proofreading activity of the RNA polymerases, their large populations and rapid replication [8, 15, 21]. Based on this, a model in which the replication of RNA genomes would generate a population of mutant sequences (termed quasispecies) that vary around a consensus sequence has been proposed [9, 10, 21]. Several sequence variants within a single infected plant had been identified in isolates of *Citrus exocortis viroid* (CEVd) [44], *Potato spindle tuber viroid* (PSTVd) [17], *Pear blister canker viroid* (PBCVd) [1], *Grapevine yellow speckle viroid* 1 (GYSVd-1) [34], *Peach latent mosaic viroid* (PLMVd) [2], *Citrus viroid III* (CVd-III) [29] and *Citrus bent leaf viroid* (CBLVd) [13].

To understand viroid evolution is essential to characterise the genetic structure and diversity of within-plant viroid populations and the *de novo* generation of a population from a single sequence variant. The development of tools for identification of sequence variants, like single-stranded conformation polymorphism (SSCP) analysis [31] facilitates the study of the population structure of viroids. The present work describes the characterisation of the population structure of a natural isolate of CEVd and the *de novo* generation and evolution of populations derived from inoculating individual CEVd cDNA clones.

Materials and methods

Viroid source and extraction procedure

The CEVd strain (CEVd-117) recovered from the field isolate E-117 was inoculated on the selection 861-S1 Etrog citron grafted onto rough lemon rootstock. CEVd-117 induces in trees grafted on trifoliate orange the characteristic bark scaling symptoms of the exocortis disease [42]. Inoculated citrons were kept in a greenhouse at 28 °C to 32 °C for at least 6 months before being used as a source of tissue.

Samples (5 g) of young leaves were homogenised in 5 ml of extraction buffer (0.4M Tris-HCl pH = 8.9; 1% (w/v) SDS; 5 mM EDTA; 4% (v/v) 2-mercaptoethanol) and water saturated phenol, and the total nucleic acids were partitioned in 2M LiCl [39]. 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). Aliquots of nucleic acid preparations from inoculated and non-inoculated citrons were analysed by slot-blot hybridisation to confirm CEVd infection [32] and were used for cDNA synthesis.

cDNA synthesis and cloning

First-strand synthesis was performed using the CEVd-specific synthetic oligonucleotide CEVd-1, complementary to bases 81 to 98 of CEVd-A [43] and the reverse transcriptase

(Superscript) (Gibco). Second-strand synthesis and amplification of dsDNA was performed with synthetic oligonucleotides CEVd-1 and CEVd-2 homologous to bases 99 to 117 of CEVd-A [43], in buffer containing 1 mM $MgCl_2$, 0.25 mM dNTPs, 0.5 μM of each primer and 1 U of *Taq* DNA polymerase. PCR parameters consisted of 30 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min, with a final extension of 72 °C for 5 min. Primer-directed selection was minimised as both primers correspond to a conserved sequence in the upper strand of the CCR. The size of the DNA product was determined by electrophoresis in 2% agarose gels and its relationship with CEVd was confirmed by slot-blot hybridisation. The purified DNA was ligated into pT7-Blue vector (Novagen) and recombinant plasmids from transformed cells were subjected to restriction analysis to verify the presence of an insert of the expected size.

SSCP analysis

Cloned viroid dsDNA was recovered from the plasmids by PCR amplification using the same conditions described above. Two μl of the PCR products were mixed with 20 μl of the 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% PAGE ($14 \times 11.5 \times 0.075$ cm gels) in TBE buffer (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) at 200 V constant voltage for 16 h. The DNA bands were visualised by silver staining [22].

Molecular hybridisation

Full length monomeric RNA probes complementary (cRNA) and homologous (hRNA) to the CEVd sequence were synthesised by transcription with T7 and T3 RNA-polymerases respectively, using DIG-labeled UTP (Roche) and the pCEVd plasmid as template.

Aliquots of nucleic acid preparations from inoculated citron and RT-PCR products were denatured in 20% formaldehyde and transferred to positively charged Nylon membranes (Roche) using a Hybri-slot filtration manifold (BRL), fixed by UV cross-linking and hybridised against a DIG labelled cRNA probe.

Samples subjected to SSCP analysis were electroblotted to positively charged Nylon membranes (Roche) at 200 mA for 1 h, directly from the SSCP gels, using a modified TBE buffer (40 mM Tris, 40 mM Boric acid, 1 mM EDTA, pH 8.3). The membranes were UV cross-linked and hybridised with DIG labeled cRNA and hRNA probes.

Prehybridisation and hybridisation were performed in 50% formamide and 6× SSPE as described by Sambrook et al. [37]. The membranes were prehybridised at 42 °C for 2 to 4 h and hybridised overnight at 65 °C. After hybridisation, they were washed twice in 2× SSC, 0.1% SDS at room temperature for 15 min, followed by another wash in 0.1× SSC, 0.1% SDS for 60 min at 68 °C. DIG-labelled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments) and visualised with the chemiluminiscence substrate CSPD (Roche).

Nucleotide sequence and statistical analysis

Cloned viroid cDNAs were sequenced with an ABI PRISM DNA sequencer 377 (Perkin-Elmer). Alignments of multiple sequences were performed with the program Clustal W [20] and prediction of RNA secondary structure with the program MFOLD (circular version) from the GCG package [46] and RNAviz [6].

Nucleotide distances were estimated considering alignment gaps and using the Jukes and Cantor's method for correction of superimposed substitutions with the program Haplo [26]. Nucleotide diversity was estimated using the formula $D = 2/(n(n-1))\ \Sigma n_i n_j d_{ij}$, where n is the number of clones analyzed per isolate, n_i and n_j are the number of clones of sequence

variant i and j respectively, and d_{ij} is the nucleotide distance between sequence variants i and j.

Tajima's D test [41] of the program DNASP [35] was used to test if sequence variant distribution followed the model of neutral evolution. The rationale of the test is that in a panmictic population, under the neutral mutation model, no difference would be expected between the average number of nucleotide differences and the number of segregating sites. Negative and positive values significantly different from 0 indicate the presence of negative and positive selection respectively.

Synthesis of infectious dimeric viroid sequence variants and infectivity assays

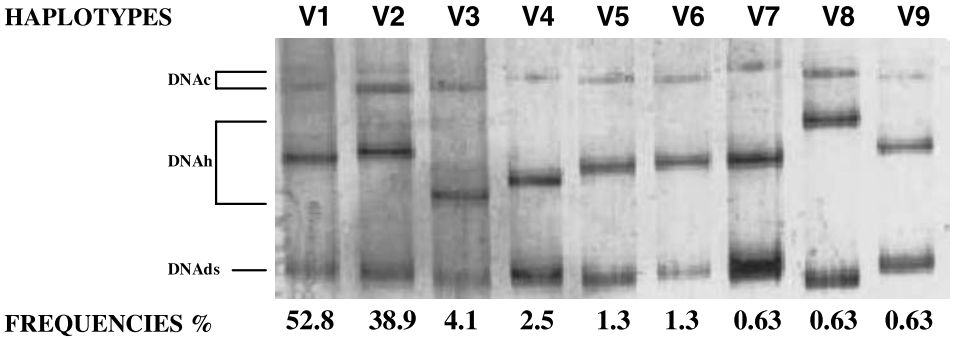
Plasmids containing monomeric cDNAs were used to generate infectious dimeric molecules. Monomeric viroid-DNA inserts were recovered as blunt-end PCR products, using the CEVd-specific synthetic oligonucleotides CEVd-1 and CEVd-2, in a buffer containing 2 mM $MgSO_4$, 0.13 mM dNTPs, 0.5 μM of each primer and 1 U of *Pwo* DNA polymerase (Roche). PCR parameters consisted of 35 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, with a final extension of 72 °C for 5 min. The size of the DNA product was determined by electrophoresis in 2% agarose gels. The DNA products were phosphorylated using 10 mM ATP and 0.3 U of T4 polynucleotide Kinase (Pharmacia), and subjected to a ligation with 2 U of T4 DNA ligase (Gibco) for 16 h at 14 °C. The dimeric molecules were purified by electrophoresis 2% agarose gels and ligated into the Eco RV site of the pBS vector and transformed into *Escherichia coli* competent cells. Plasmids from transformed cells were subjected to restriction and sequence analysis to verify the correct tandem orientation of the dimeric inserts.

The selection 861-S1 of Etrog citron was propagated by grafting onto the rough lemon rootstock. Plants were slash-inoculated with plasmid preparations (4 μg per plant) containing dimeric inserts. The inoculated plants were kept in the greenhouse at 28°–32 °C, observed for symptom expression and analysed every 3 months.

Results

Identification of CEVd variants and characterisation of a within-isolate population

CEVd inoculated citron plants developed severe symptoms of stunting and epinasty 3 months after inoculation. When nucleic acid preparations from a CEVd-infected citron plant were subjected to retrotranscription and PCR amplification using CEVd-1 and CEVd-2 primers, a monomeric CEVd-DNA product of the expected size was recovered. SSCP analysis of 316 clones revealed the existence of 44 different electrophoretic patterns. Molecular hybridisation of electroblotted samples using cRNA and hRNA probes (data not shown) identified the rapidly migrating band as dsDNA and at least two stable conformations of the cDNA and hDNA strands (Fig. 1). The cDNA and hDNA strands were chosen to discriminate CEVd sequence variants. Out of the 44 electrophoretic patterns (haplotypes) identified, two (V1, V2) were predominant (Fig. 1) and seven (V3 to V9) were identified more than once. The nucleotide sequence of 92 clones including all 44 haplotypes, was determined. From those haplotypes identified more than once, several clones were sequenced (19 for V1, 13 for V2, 4 for V3 and V5, 8 for V4, and 2 for V6, V7, V8 and V9) and showed an identical sequence, which confirms that SSCP analysis



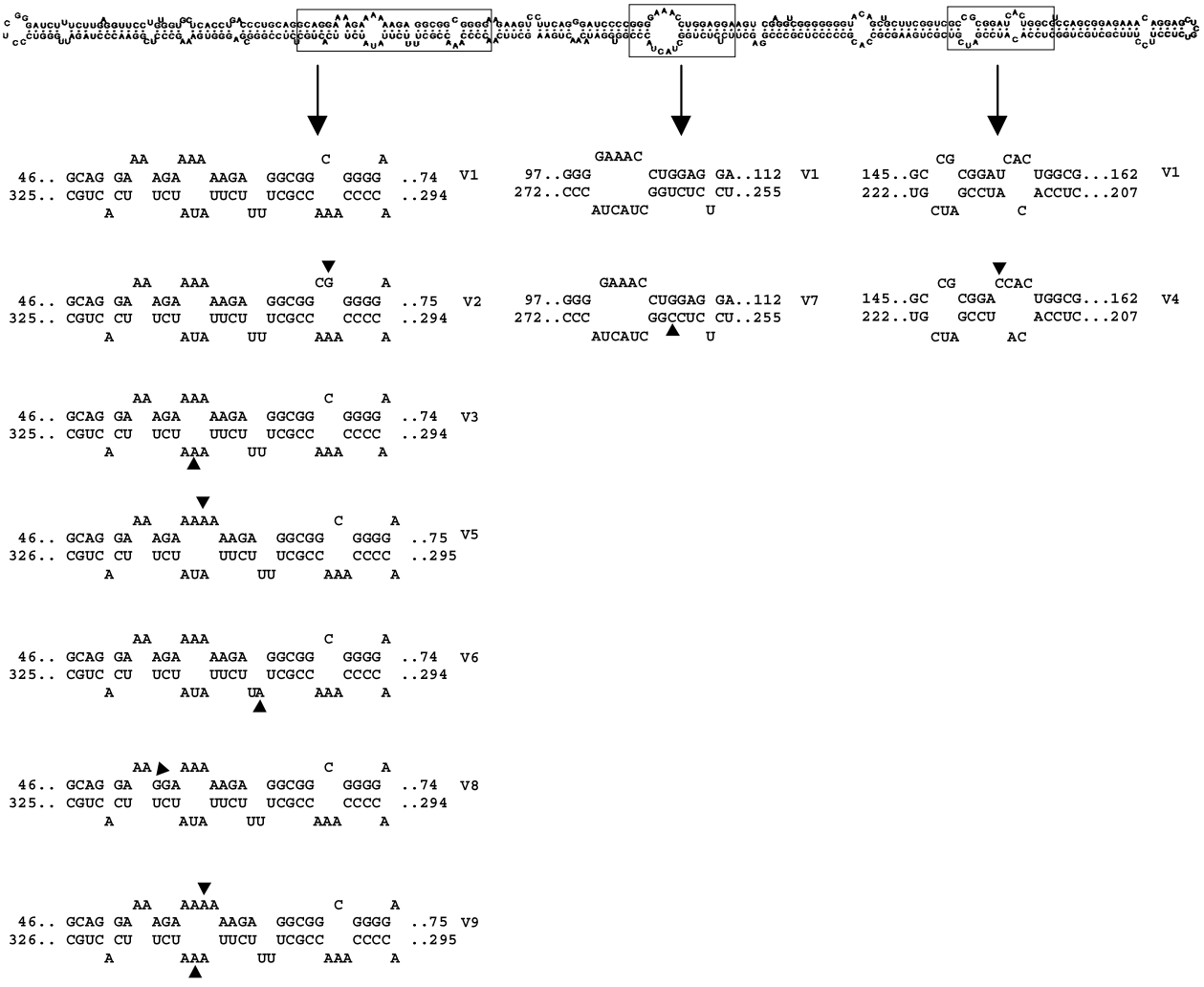


Fig. 1. Top: SSCP profiles and frequencies of sequence variants V1 to V9. Middle: Secondary structure of sequence variant V1 (boxes show the regions in which changes of sequence variants V2–V9 are located). Bottom: Effect of changes of sequence variants V2 to V9 in its secondary structure (▲, ▼ indicates the nucleotide change)

is an accurate tool to identify CEVd sequence variants. Sequencing of an RT-PCR product obtained with same nucleic acid preparations using oligonucleotides CEVd-3 complementary to bases 252 to 271 and CEVd-4 homologous to bases 272 to 291 of CEVd-A [43], indicated that no variability existed in the region of the upper strand of the C domain region in which primers CEVd-1 and CEVd-2 had been designed.

The nucleotide diversity estimated from the frequencies of sequence variants and nucleotide distances of this CEVd population was 0.0024 ± 0.0012 which is close to the diversity generated by errors of *Taq* polymerase in RT-PCR reactions [4]. Two additional RT-PCR reactions were performed using the same RNA preparation and following the same cloning strategy. SSCP analysis and sequencing of 30 clones from each, showed that in both instances, V1, V2 and V6 were found in frequencies comparable to those of the first analysis, suggesting that they are not RT-PCR artefacts and that the impact of RT-PCR errors is minimal.

To compare the nucleotide diversity of this CEVd isolate with those of other viroids, the nucleotide diversity from published data was estimated (Fig. 2),

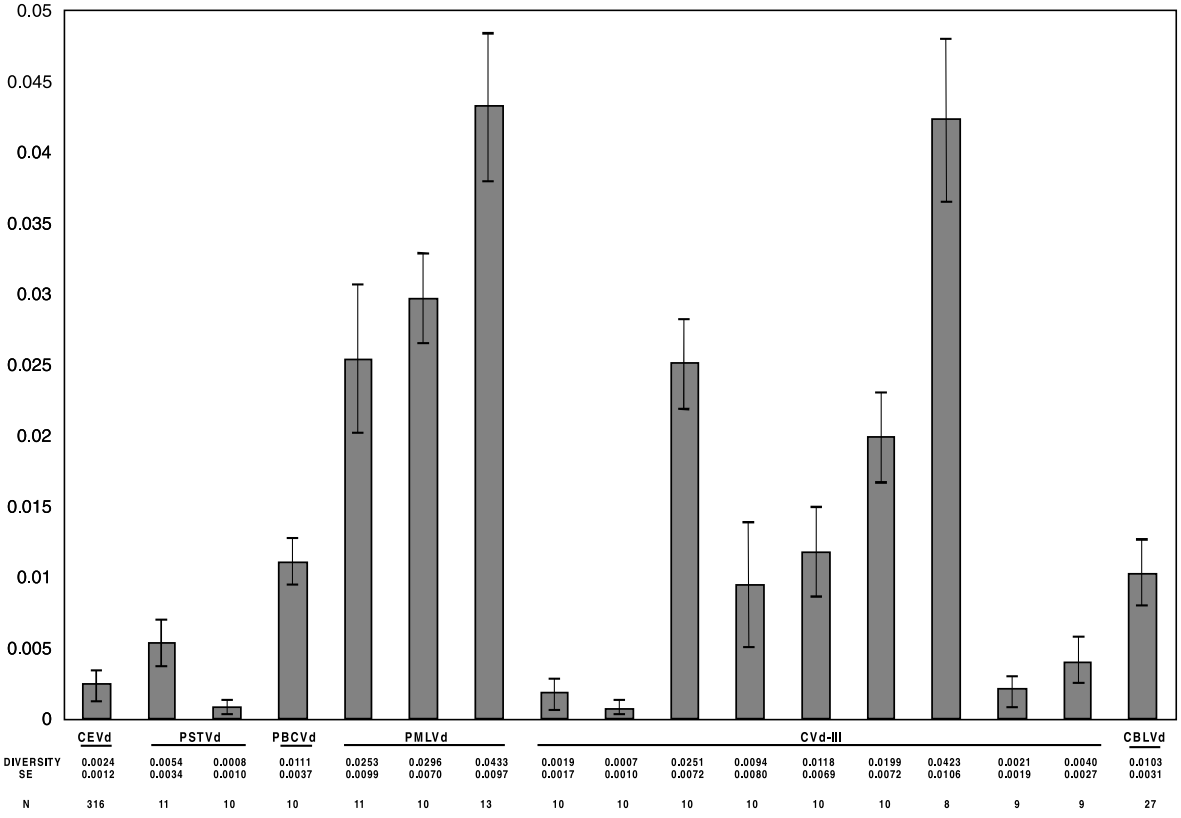


Fig. 2. Within-plant nucleotide diversities and their standard errors (SE) calculated form published data of several viroid isolates: CEVd-117 (this study); *Potato spindle tuber viroid* (PSTVd) [17]; *Pear blister canker viroid* (PBCVd) [1]; *Peach latent mosaic viroid* (PMLVd) [2, 3]; *Citrus viroid III* (CVd-III) [29] and *Citrus bent leaf viroid* (CBLVd) [13]. Each bar represents a single isolate and N indicates the number of clones of each isolate

although the number of clones available from these studies was considerably smaller. These diversities, in spite of the differences among viroids and viroid isolates, were in the same range as those estimated for plant RNA viruses [15, 25, 36, 45]. Application of Tajima's D test [41] showed that the population of this CEVd isolate had a significantly lower genetic variation than expected according to a model of neutral evolution, therefore suggesting a strong negative selection.

To compare the selection pressure in different genome regions, the average nucleotide distances were calculate separately for each of the structural domains (T_L, P, C, V, T_R) proposed by Keese and Symons [23]. The values obtained were 0.0024 ± 0.0013 for C, 0.0062 ± 0.0028 for T_R, 0.0086 ± 0.0027 for T_L, 0.0149 ± 0.0047 for V and 0.0164 ± 0.0065 for P, which is in agreement with early reports suggesting that variability was mainly found in the V and P domains [23].

Primary and predicted secondary structures

The most frequent sequence variant (V1) showed a high nucleotide identity (98.65% and 98.12%) to CEVd-C [19] and CEVd-A [43] respectively. The rest of sequence variants showed one or two nucleotide substitutions or insertions with respect to V1 (Fig. 1). Most of the changes affected the pathogenicity (P) and variable (V) domains (found in 29 and 13 of the sequence variants, respectively). Changes in the C, T_R and T_L domains were infrequent. Alignment of the 44-sequence variant sequences with CEVd-J and CEVd-30 (class A and B respectively) [44], showed that all were highly homologous to class A. Figure 1 shows the frequency and characteristics of the nine most frequent sequence variants that represent altogether 89% of the population. These sequence variants presented 18 changes characteristic of class A and 5 changes characteristic of class B. Seven additional changes were also identified (data not shown). It must be noted that the only change discriminating V2, with a frequency 38.9%, from the most frequent sequence variant V1 is a G addition in position 70, however none of the other 42 sequence variants contained this change.

The predicted secondary structure of minimum free energy of these nine sequence variants was a highly base paired rod-like structure at 25 °C and 30 °C (Fig. 1), but at 37 °C was a branched structure resulting from rearrangements within T_L domain (data not shown). The changes identified in sequence variants V2, V3, V5, V6, V8 and V9 are located in the P domain (Fig. 1). These in V3, V6 and V8 do not have effect on the secondary structure of the domain, whereas the changes in V2, V5 and V9 result in an increase of the size of the loops as a result of a single nucleotide insertion. Similarly, a single nucleotide insertion in one of the loops in the T_R domain also results in an increase of its size. The change $U \rightarrow C$ in the lower strand of the C domain do not affect the secondary structure of sequence variant V7.

Infectivity of CEVd sequence variants and characterisation of their progenies

To study the infectivity of individual sequence variants, dimeric cDNA clones of sequence variants V1 to V9 were obtained and each was slash-inoculated

in four citron plants. Every 3 months, over a 9-month-period, inoculated plants were evaluated for symptoms and CEVd infection was determined by molecular hybridisation. CEVd was detected 3 months post inoculation (m.p.i.) in three citron plants inoculated with sequence variant V1, and 9 m.p.i. in one plant inoculated with V6, whereas no infection was detected in the plants inoculated with the remaining clones (V2 to V5 and V7 to V9). All CEVd-infected citrons showed symptoms characteristic of CEVd infection and they were subjected to SSCP and sequence analysis of 21–30 clones. Each revealed various SSCP profiles demonstrating that a population of variants had been generated *de novo* from a single sequence variant.

V1 progeny 3 m.p.i. was composed predominantly of V1 and four *de novo* generated sequence variants. The most abundant sequence variants (V1, V2 and V3) were also present in the original CEVd isolate, with similar frequencies and nucleotide diversities (Table 1). This suggests that the CEVd-117 population can be generated *de novo* from its most frequent sequence variant V1. However, 6 m.p.i., the frequency of sequence variant V1 decreased and additional sequence variants were identified, causing a drastic increase of the nucleotide diversity (0.0142). Nine m.p.i., most of these sequence variants (including sequence variant V3) were not detected and diversity increased slightly (0.0169). Diversity results and Tajima's D statistics indicated a strong negative selection in V1 progeny 3 m.p.i., but 6 m.p.i its intensity decreased and 9 m.p.i. the population had a structure expected under a model of neutral evolution.

The progeny generated from V6 (detected only 9 m.p.i.) was composed of 13 sequence variants, with V1 being also clearly predominant (Table 1). Unexpectedly, the inoculated variant V6 and V2 (which were frequent in CEVd-117 and in

Table 1. Population structure and nucleotide diversity of the progeny populations generated *de novo* after inoculation with cDNA clones corresponding to sequence variants V1 and V6

Inoculated sequence variant	m.p.i[a]	N. clones[b]	N. progeny sequence variants[c]	Frequency of progeny sequence variants[d]				Nucleotide diversity[e]
				V1	V2	V3	X	
V1	3	27	5	52.0	37.0	3.7	7.3	0.0023 ± 0.0015
	6	27	12	26.0	37.0	3.7	33.3	0.0142 ± 0.0064
	9	21	5	36.8	30.6	0.0	32.6	0.0169 ± 0.0077
V6	9	30	13	46.7	0.0	0.0	53.3	0.0093 ± 0.0031

[a]m.p.i. indicate the time (months post inoculation) when the analysis was performed

[b]Number of clones analysed by SSCP

[c]Number of sequence variants identified by SSCP analysis

[d]V1, V2 and V3 refer to the most frequent sequence variants found in CEVd-117. X refers to the frequency of the rest of sequence variants taken together

[e]Nucleotide diversity was calculated as $D = 2/(n(n-1))\ \Sigma n_i n_j d_{ij}$, where n is the number of clones analyzed, n_i and n_j are the number of clones of sequence variant i and j respectively, and d_{ij} is the nucleotide distance between sequence variants i and j

the V1 progeny) were undetected. The nucleotide diversity of V6 progeny (0.0093) was greater than that of the original isolate CEVd-117 (0.0024) and showed slight negative selection.

To compare these diversities with those of the progenies generated after inoculation with of sequence variants of other viroids, the nucleotide diversities from the limited number of sequences available were calculated. These were 0.0013 to 0.0038 for PSTVd (18), 0.0132 to 0.0280 for PMLVd (3) and 0.0116 ± 0.0036 for CBLVd (14), which were in the same range as CEVd.

Discussion

The characterisation of the genetic structure of viroid populations is important to understand their evolution. With the advent of molecular virology, several reports describe the identification of sequence variants in viroid populations, however in these studies only the number of sequence variants present in the population but not their frequency were taken into consideration [1, 13, 34, 44]. This is the first study in which the structure and diversity of a large population (316 full length clones) has been characterised in terms of the number of sequence variants present, frequency of each variant and genetic nucleotide distances between variants. The CEVd population examined contained two predominant variants (V1 and V2) representing respectively, 52.8% and 38.9% of the population and other 42 sequence variants of significantly small frequencies. Some of the observed changes may have resulted from errors produced during PCR amplification using *Taq* DNA polymerase. However, the results of two independent cloning assays in which V1, V2 and V6 were found in frequencies comparable to those of the first analysis, suggest that they are not RT-PCR artifacts and that the impact of RT-PCR errors is minimal. Additionally, the observation that most changes are located in the P domain and never in strictly conserved regions (CCR, TCR) indicates that they are naturally occurring mutations. This is also supported by previous studies that used the same approach: i) No mutants were found in 30 clones obtained after PCR using as a template a single cDNA-CEVd clone [31]; ii) All the clones obtained from a single *Hop stunt viroid* (HSVd) isolate were identical [33].

In spite of the large number of CEVd variants identified, the nucleotide diversity of this population (0.0024 ± 0.0012) was lower than expected according to a model of neutral evolution, suggesting, like in the case of plant viruses, a strong negative selection. The neutral theory proposes that the majority of molecular variation has no significant adaptative or deleterious effect (nor subjected to selection). The available data on plant viruses indicate that their populations are genetically stable and their diversities generally low. However, since most of these studies focus on coding sequences, the low diversities found are probably due to the need to preserve function of viral proteins [15]. Since viroids do not code for proteins, the negative selection found in this CEVd isolate, which present an even lower diversity than viruses, is probably related to the need to maintain its primary and secondary structures.

CEVd, like other viroids of the family *Pospiviroidae*, present a highly base paired rod-like secondary structure that denatures into branched conformations in a highly cooperative way through the formation of stable hairpins that are not part of the rod-like structure [40]. In the dominant sequence variant (V1) of CEVd, the primary structures of the upper ($_{81}$-UCCUUCAGGG + 14 nt + CCUGGAGGA-$_{112}$) and lower ($_{234}$-CCUCGCCC + 91 nt + GGGCGAGG-$_{336}$) strands of the C domain are able to form the thermodynamically stable hairpins I and II respectively. None of the changes identified in the other sequence variants disturbs these sequences and therefore their ability to form these hairpins. The diversity value calculated for the C domain (0.0024 ± 0.0013) illustrates the low variability of this domain. Bussière et al. [5] and Gast et al. [16] found that CEVd may present a cruciform t-RNA-like structure involving 80 nucleotides at the T_L domain similar to the branched minimum free energy structure adopted at 37 °C by the CEVd isolate characterised in the present study. Although more recent studies based on NMR and thermodynamic analysis do not support the existence of a branched conformation, at least one of the CEVd variants tested contained mutation that appeared to stabilize the branched conformation [7]. In addition, the T_L domain presents a conserved sequence motif termed Terminal Conserved Region (TCR) [11, 24]. These observations support the low genetic diversity found in the T_L domain. The diversity of the V and P domains is in agreement with early reports suggesting that variability of viroids of the family *Pospiviroidae* is mainly found in these two domains [23]. Most changes are located in the P domain, without any apparent effect (sequence variants V3, V6 and V8) or resulting in an increase of the size of the loops (V2, V5 and V9). As reported in the case of *Potato spindle tuber viroid* (PSTVd) [28], the size and symmetry of these loops may determine the relative orientation of the flanking portions of the rod-like structure and therefore its ability to interact with putative host proteins. Surprisingly, in spite of the high frequency of V2 with a G insertion in position 70, this change is not present in any of the other sequence variants.

Previous work conducted with viroids, showed that several sequence variants could be recovered *de novo* from a single sequence variant [3, 14, 18, 27, 28, 30]. The present is the first study showing the temporal evolution of the population generated *de novo* upon infection with a single sequence variant. The predominant sequence variant V1 was infectious showing detectable replication/accumulation levels and reproducing the symptoms of the original isolate. Sequence variant V6 was also infectious reproducing the symptoms of the original isolate after a longer incubation period. The lack of infectivity by some clones could be due to the inoculation procedure used, however differences in infectivity were found using the same procedure. This suggests than the sequence variant V1 is more infectious in citron, requiring for detection and symptom expression a shorter incubation period than V6 and the rest of sequence variants.

Inoculation of CEVd sequence variants V1 and V6 generated a heterogeneous population of very closely related sequence variants, demonstrating that CEVd indeed, follows the "quasispecies model" proposed by Eigen [9]. The composition of V1 progeny 3 m.p.i, was similar to the original isolate, with V1, V2 and

V3 variants being predominant. It is difficult to explain why, after 3 additional months its genetic diversity increased considerably. Apparently the V1 progeny 3 m.p.i. and the isolate CEVd-117 had a similar population composition, but undetected differences in low frequent sequence variants might be important for the population evolution.

Surprisingly, V6 progeny contained V1 as the predominant sequence variant whereas V6 was undetected. This suggests that V1 has a higher fitness than V6, and the 6 month delay for detectable infection might be related to the time required for V1 to be generated by mutation, to accumulate and to displace V6. The displacement of the population towards V1 as the predominant sequence variant can be regarded as a reversion to the "wild type", demonstrating that populations of CEVd are stable.

It is intriguing the apparent lack of infectivity of sequence variant V2 (derived from a G insertion in V1) that is very frequent in the original isolate and in the progeny generated *de novo* after V1 infection. This might be due to a higher tendency of V1 to mutate to V2, but this is not supported by the composition of the V6 progeny in which V1 was highly frequent whereas V2 was undetected. However, the observation that none of the other sequence variants identified in the V1 progeny contained the G insertion characteristic of V2 might be an indirect evidence supporting that V2 does not replicate in spite of being found frequently in the V1 progeny, as well as in the original CEVd population. Another explanation could be that V2 might have a high fitness in some infection aspects such as replication, but low fitness in other aspects (for example cell-to-cell movement).

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