

The question of *Citrus viroid IV* as a *Cocadviroid**

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Summary. It has been suggested that *Citrus viroid IV* (CVd-IV) be classified as a species within the genus *Cocadviroid*. This relationship was based on the presence of a terminal conserved hairpin (TCH) and absence of a terminal conserved region (TCR) as specific structural motifs in common with isolates of *Coconut cadang-cadang viroid* (CCCVd) as well as phylogenetic relationships with members of the genus *Cocadviroid*. Evidence is presented for a “vestigial” TCR in CVd-IV as well as the introduction of the terminal repeat region (TRR) motif and an alternative sequence analysis that suggests a closer phylogenetic relationship of CVd-IV to isolates of *Citrus exocortis viroid* (CEVd), a species within the genus *Pospiviroid* than to CCCVd. This position is further supported by the striking similarity of biological properties between CVd-IV and CEVd with the suggestion offered that biological evidence be considered for specific adjustments to any overall classification scheme for viroids.

Introduction

The scheme proposed by Flores et al. [6] to include *Citrus viroid IV* (CVd-IV) [1, 15] in the genus *Cocadviroid*, family *Pospiviroidae*, implied a phylogenetic relationship to the type species, *Coconut cadang-cadang viroid* (CCCVd). While welcoming discussion and suggestions for this relationship as a “proposal”, this scheme was adopted for taxonomic classification [5] based on conserved sequences and sites of particular specific structural motifs as identified within viroid genomes. These sequences comprised the well accepted central conserved region (CCR) of the central domain [10] as well as two additional cassettes designated as the terminal conserved region (TCR) and the terminal conserved hairpin (TCH) [4].

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More recently, this scheme was supported by phylogenetic analysis [2] in which the same local structural similarities were again considered as most significant parameters and preserved by manual manipulation prior to sequence alignment and phylogenetic tree reconstruction. The uniquely distinct biological characteristics of CVd-IV and CCCVd were never considered in formulating the original proposal of a phylogenetic relationship for CVd-IV with CCCVd nor the taxonomic designation. Both structural and biological evidence is offered here for discussion of CVd-IV as a *Cocadviroid*.

Consideration of specific structural motifs

A case may be made for a more general occurrence of permutations or vestigial sequences related to the TCH and TCR motifs than were previously recognized by Flores et al. [4] (Fig. 1A). In addition to these, evidence for a decided relationship between CVd-IV and *Citrus exocortis viroid* (CEVd) can be drawn from the reported identification of a terminal repeat region (TRR) specific to CEVd-related variants [17–19].

The terminal conserved hairpin (TCH)

In the presentation of structural motifs by Flores et al. [4], *Potato spindle tuber viroid* (PSTVd) as the type species in the genus *Pospiviroid* is represented as being devoid of a TCH in noting a distinction with species of the genera *Hostuviroid* and *Cocadviroid* of the family *Pospiviroidae*. However, within the left terminal (T_L) domain of CEVd, also classified as a *Pospiviroid*, can be found a cassette of 10 nucleotides common to the 13 designated to represent the TCH of *Hop stunt viroid* (HSVd) and CCCVd. The following comparison of similar regions found in CEVd with that of CVd-IV and CCCVd indicates a core of about 80% of a TCH in CEVd. A similar “core” TCH of 7–9/13 nucleotides can be found in eight additional isolates in the genus *Pospiviroid*.

CCCVd: C.C.C.C.U.C.U.G.G.G.G.A.A.
CVd-IV: C.C.C.C.U.C.U.G.G.G.G.A.A.
CEVd: –.C.C.C.U.C.–.G.G.G.–.A.A.

The terminal conserved region (TCR)

The 16 nucleotide TCR is indicated as a prominent feature of the *Pospiviroid*, PSTVd, the *Apscaviroid*, *Apple scar skin* (ASSVd), and the *Coleviroid*, *Coleus blumei* 1 (CbVd 1) but absent in members of the *Hostuviroid* and *Cocadviroid* genera. Although, according to the phylogenetic presentation in Flores et al. [4], a TCR should not be characteristic of CVd-IV as a *Cocadviroid*, in a manner similar to the evidence for an incomplete TCH in CEVd, a case can be constructed for a core of a “TCR” in the CVd-IV genome.

From the description of the model TCR, it can be suggested that the sequence proximal to the 3' end of the motif is highly conserved and that considerable

variability is to be found in the nucleotides near the 5' terminus as evidenced in the three "N" nts in the five terminal positions. A comparison of a comparative locus in CVd-IV with the TCR of CEVd provides evidence for a "vestigial TCR". This locus is characterized by identity with a 5 nucleotide cluster of the conserved 3' terminal end of the model TCR. The same 5 nucleotide cluster can be found in seven additional members of the genus *Pospiviroid*. In addition to this conserved cluster, a total of 7/16 nucleotides are common to CVd-IV and CEVd and a total homology of 9/16 nucleotides can be extrapolated from the other variable sites as presented in the model.

TCR model:	5'	C.N.N.G.N.G.G.U.U.C.C.U.G.U.G.G.	3'
CVd-IV:		U.A.A.A.A.A.C.A.G.C.U.U.G.U.G.G.	
CEVd:		C.U.U.G.A.G.G.U.U.C.C.U.G.U.G.G.	

Considering this striking similarity in the highly conserved portion of the TCR and an overall homology of about 56%, it seems reasonable to suggest that CVd-IV is not totally "without" a TCR. It further might be conjectured that the abbreviated form of a TCR found in CVd-IV may be a reflection of the considerably reduced size of the CVd-IV genome (284 nt) when compared with that of CEVd (371 nt) as the *Pospiviroid* used for this comparison.

The terminal repeat region (TRR)

Since the taxonomic relationship of CVd-IV as a *Cocadviroid* has been promulgated on the application of proposed "... conserved sequence and structural motifs..." [6], it seems reasonable that additional structural features shared by viroids should also be considered for definition of a phylogenetic context.

Variants derived from infection with citrus exocortis viroid have been identified from tomato, CEVd-D92 [18] and CEVd-D104 [17], as well as from eggplant, CEVd-D96 [3], that contain repeated sequences specific to loci of the variable (V) and right terminal (T_r) domains. Variations of this structural motif as a terminal repeat region (TRR) can also be observed in two additional CEVd variants and three asymmetric but biologically active CEVd "forms" [19]. A significant portion of sequence homology of the TRR in CEVd variants can be found in the CVd-IV genome (Fig. 1B) as two large cassettes of 37 nucleotides in the V-T_r region and 21 nucleotides in the T_r. With this, CVd-IV reflects a 63% identity of 58/92 nucleotides with the TRR of CEVd.

The structural motifs as introduced by Flores et al. [4] are presented in Fig. 1A with modifications introduced to include the broader occurrence of a "core" TCH, a "vestigial" TCR, and a TRR illustrated in Fig. 1B.

The viroid variants represented by a TRR may not represent artifacts or curiosities unique to CEVd infection as similar enlarged forms also accompany late infection by CCCVd [7, 8, 12]. It can be conjectured that the pathway for synthesis of these structures may be perhaps more common to viroid replication but only more apparent with CEVd and CCCVd infection by accumulating to a detectable titer.

Consideration of homology among complete viroid genomes

From the comparative features displayed by specific regions within the viroid genomes, an analysis was made of the sequence homology of complete viroid genomes in the absence of manual manipulations driven by conserved sequences and structural motifs (Table 1). Agreement of values can be seen among viroids accepted as having either TCH or TCR motifs. However, CEVd presumably lacking a TCH displays a higher homology (76.4%) with CVd-IV than the other viroids containing a TCH. This value is within the range comparable to the relationship of CEVd with PSTVd (77.4%) as *Pospiviroids*. This analysis of broad based homology suggests a closer relationship of CVd-IV to CEVd and related viroids than to CCCVd (68.6%).

Phylogenetic relationships by sequences alignment

Coupled with the expanded view of discrete structural motifs as presented here, it prompts the question whether another protocol for phylogenetic analysis might support a closer relationship of CVd-IV with CEVd as a *Pospiviroid* than with CCCVd as a *Cocadviroid*.

Table 1. Sequence homology among viroids with representative terminal conserved hairpin (TCH), terminal conserved region (TCR), and terminal repeat region (TRR) structural motifs

	Viroid pair	Similarity or identity ^a (%)
A. Viroids with a TCH:	CVd-IV ^b : HSVd	71.8
	CVd-IV : CCCVd	68.6
	HSVd : CCCVd	69.0
	+TCR:	
	CEVd : CVd-IV	76.4
	CEVd : HSVd	63.6
	CEVd : CCCVd	69.9
B. Viroids with a TCR:	PSTVd : ASSVd	62.1
	+“TCH”:	
	CEVd : PSTVd	77.4
	CEVd : ASSVd	61.9
C. Viroids with a TRR:	CEVd : CEVd-D92	97.0 ^c
	CEVd : CVd-IV	76.4
	CEVd-D92 : CVd-IV	78.6

^a% similarity and % identity of complete genome sequence calculated by GCG-GAP according to the settings of [5] (gap opening and gap extension penalties 15 and 1, respectively) with the selection “end gaps penalized like other gaps” and without manual adjustments between the conserved regions

^bCVd-IV; *Citrus viroid IV*, HSVd; *Hop stunt viroid*, CCCVd *Coconut cadang-cadang viroid*, CEVd; *Citrus exocortis viroid*, PSTVd; *Potato spindle tuber viroid*, ASSVd; *Apple scar skin viroid*

^cRepresents % homology of the sequence without consideration of the size of the molecules

Phylogenetic analyses of molecular data can be made by a number of statistical based methods as well as selected parameters within these that govern the relationships generated. The approach employed by Flores et al. [5] for viroids and later expanded by Elena et al. [2] for viroids, satellite RNAs, and hepatitis delta virus (HDV) supported the relationship of CVd-IV with CCCVd as two species of the genus *Cocadviroid*. However, it should be acknowledged that variations in phylogenetic patterns can be generated by strict molecular modeling as the "... the predictive power of a model in biology is quite low." [13].

These differing relationships need to be subject to review and interpretation utilizing the complete fund of information available in defining the properties of the contributing components. A test for the sequence similarity among viroid-like molecules by Jenkins et al. [9] indicated that viroids, satellite RNAs, and HDV "... cannot be aligned in a statistically significant way." and Monte Carlo analysis rates sequence similarity of CVd-IV to CEVd or CCCVd as equivalent. With these uncertainties, a relevant comment of Nei and Kumar [13] that the "... phylogenetic tree produced should be interpreted with caution and common sense" seems appropriate.

From this perspective, a minor modification in the process of phylogenetic analysis made by Flores et al. [5] and Elena et al. [2] was explored for defining the relationship among CCCVd, CVd-IV, and CEVd. A significant distinction however was the absence of "... manually editing the result to preserve all the local similarities ..." This exercise was not intended as a comprehensive accounting for all viroids and viroid related molecules but simply as a focused definition framed with six viroid sequences representative of the three genera of the family *Pospiviroidae*.

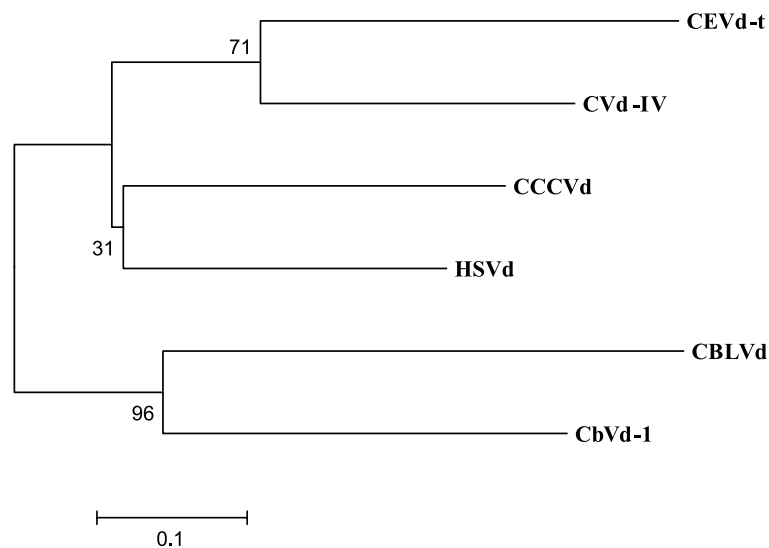


Fig. 2. Neighbor-joining consensus phylogenetic tree (10,000 bootstrap replicates, numbers on the tree nodes % values). Evolutionary distances were estimated according to the model of Jukes and Cantor. The phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1. Sequence alignment by T-Coffee software

The minor modification was introduced in sequence alignment using the T-Coffee program that is proposed to produce more accurate multiple alignments than CLUSTAL [14]. The consensus phylogenetic tree was constructed from 10,000 bootstrap replicates using the minimum evolution distance method Neighbor-Joining with evolutionary distances estimated according to the model of Jukes and Cantor. The described analysis was conducted using MEGA version 2.1 [11]. This analysis constituted only an alteration of the alignment algorithm used by Flores et al. [5], however resulted in a distinctly different phylogenetic relationship for CVd-IV as more closely related to CEVd than to CCCVd (Fig. 2).

Biological properties as an indicator of phylogenetic relationships

With this demonstration that phylogenetic modeling can be a subjective and variable statistical art, biological relationships gain importance and accepted patterns of phylogeny need to be reinforced by or compatible with biological properties. A highly apparent biological similarity for CVd-IV with CEVd and distinct from CCCVd can be drawn from the broad range of hosts presented in Table 2. In spite of a high occurrence in the field, CCCVd displays a very restrictive host range limited to a few species of palm. It has been reported that 44 species in 12 families other than *Palmae* have been inoculated but not infected by CCCVd [16]. In contrast, although CVd-IV is limited in nature even in citrus growing regions, a broad host range can be demonstrated. Specificity between CVd-IV and CEVd is supported by transmission to *Gynura aurantiaca* limited to CEVd and related members of the genus *Pospiviroid*.

It is important to reiterate that the specific focus of this study has been toward a clearly defined phylogenetic relationship of *Citrus viroid IV* and not any broader context of phylogeny among viroids and viroid-like molecules. Data presented from comparative features of localized structural motifs, complete genome, and biological properties support a close relationship between *Citrus viroid IV* and *Citrus exocortis viroid*. Some choices of purely statistical analysis of nucleotide sequences may indicate a relationship between CVd-IV and CCCVd. However, it might be conjectured that these relationships may have been driven by genome size as a form of selection pressure that factored significantly in maintaining a structure for survival. Might there also be a case for convergent evolution among like sized transmissible molecular species that is exposed in the art of statistical modeling?

From the data and propositions introduced here, the proposal is offered that in the definition of phylogenetic relationship among viroids:

1. A review of biological properties in the relationship be considered.
2. That the biological evidence be evaluated in concert with sequence homology and structural motifs and accepted as a valid parameter for defining phylogeny.
3. That phylogenetic relationships suggested by statistical analysis be subject to review by compelling biological relationships.

Table 2. Biological relationship among *Citrus exocortis viroid* (CEVd), *Citrus viroid IV* (CVd-IV) and *Coconut cadang-cadang viroid* (CCCVd) from host range and symptom severity

Test plant	Viroid inoculum		
	CEVd	CVd-IV	CCCVd ^a
Citron			
<i>Citrus medica</i>	++++	+++	NT
Gynura			
<i>Gynura aurantica</i>	+++	++ ^b	NT
Tomato			
<i>Lycopersicon esculentum</i>	++++	++++	–
<i>L. esculentum</i> × <i>L. Peruvianum</i>	+++	++	NT
Cucumber			
<i>Cucumis sativus</i>	+	+	–
Chrysanthemum			
<i>Chrysanthemum morifolium</i>	++++	++	–
Eggplant			
<i>Solanum melongena</i>	+	+	NT
Datura			
<i>Datura stramonium</i>	+	+	–
Coconut			
<i>Cocos nucifera</i>	NT	NT	++++
<i>Palmae</i> species	NT	NT	+

^aData kindly provided by Dr. J. Randles

^bInitial report made here

– = No transmission

+ = Evidence of viroid replication

++ to ++++ = Symptom severity

NT = Not tested

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