© 1996 Oxford University Press Nucleic Acids Research, 1996, Vol. 24, No. 10 **1793–1798**

Compilation and analysis of viroid and viroid-like RNA sequences

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Received February 6, 1996; Revised and Accepted March 29, 1996

ABSTRACT

We have created a catalogue comprising all viroid and viroid-like RNA sequences which to our knowledge have been either published or were available from on-line sequence libraries as of October 1, 1995. In the development of this catalogue nomenclature ambiguities were removed, the likely ancestral sequence of most species was determined and the most stable secondary structures of these sequences were predicted using the MulFold package. Only viroids of PSTVd-type possessed a rod-like secondary structure, while most other viroids adopted branched secondary structures. Several viroids have predicted secondary structures that include either a Y or cruciform structure reminiscent of the tRNA-like end of virus genomes at an extremity. However, it remains unknown whether or not these predicted structures are adopted in solution, and if they serve a particular function *in vivo*. Additional information such as the position of the self-catalytic domains are included in the catalogue. An analysis of the data compilated in the catalogue is included. The catalogue will be available on the world wide web (http://www.callisto.si.usherb.ca/~jpperra), on computer disk and in printed form. It should provide an excellent reference point for further studies.

INTRODUCTION

Viroids are small single-stranded circular RNA molecules (246–463 nt) that infect higher plants, causing diseases in crop species and resulting in important economic losses in the agricultural industry (1). It has been proposed that viroids replicate in a DNA-independent manner via a rolling circle mechanism involving the synthesis of multimeric strands which are then cleaved into monomeric fragments and circularized producing the progeny viroids (1,2). We have developed a catalogue in order to facilitate viroid research by presenting a large amount of viroid sequence and related data in a comprehensive and user-friendly format. We compiled a total of 182 sequences from 21 viroids (3–70), eight plant satellite viroid-like RNAs (71–79) and the viroid-like domain of the human hepatitis δ virus RNA (80). In addition, we have included a transcript from the mitochondrial DNA satellite II from both *newt* (81) and carnation (82) which have been proposed to be retroviroid-like elements evolving from viroids by retroposition into host DNA (83). Table 1 is a summary of these sequences. Here, we describe the catalogue and present an analysis of its content.

THE CATALOGUE

This compilation comprises all sequences that to our knowledge had been published or were available from the sequence library file servers (NCBI sequence librairies) as of October 1, 1995. Only complete sequences were retained because it appeared difficult to establish a criteria on how large a sequence fraction should be in order for it to be listed in the compilation. With the heterogeneity that occurs in viroids, the inclusion of sequences from partial cDNA clones could result in erroneous conclusions in subsequent studies, and therefore they were omitted.

Among viroids, the classification system proposed by Koltunow and Rezaian has been adopted (84). Viroids are divided in two types, the ASBVd-type (also named group A) whose members possess the capacity to self-cleave, but do not possess a conserved core region (CCR); and the PSTVd-type (or group B) whose members possess a CCR, but have no known self-cleaving properties. Viroids of PSTVd-type are subdivided in two groups: the PSTVd group (or subgroup B1) and the ASSVd group (or subgroup B2). The viroids of these two groups are easily distinguished by the sequence forming their respective CCR (84). Only the sequences of HSVd, which is a member of PSTVd group, were subdivided according to their initial isolation host.

In the catalogue each species is listed by its complete name and number of sequence variants (see Fig. 1). This is followed by, for each species, a listing of the sequence variants with their new identifications (see below), accession numbers for sequence library file server, bank loci (when available), number of nucleotides, numbers of each type of nucleotide, complete publication information, and the complete nucleotide sequence in 10 nt blocks in order to facilitate further analyses. For species which possess known self-catalytic domains (hammerhead, hairpin and delta), the localization of the conserved sequences required for cleavage to occur are reported. In addition, a secondary structure prediction of the most likely ancestral variant was derived using the MulFold structure prediction package (see below). The chosen most likely ancestral variants are reported in Table 1, while their predicted secondary structures are appended to the catalogue.

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Table 1. Summary of the viroid and viroid-like RNA sequences included in the catalogue

RNA species	Abbreviation	number of compilated sequences	size (nt)	selected variants	size of selected variant (nt)	catalytic motifs
Viroids						
ASBVd-type (group A)						
avocado sunblotch viroid	ASBVd	19	246-251	ASBVd.2	247	hh+ / hh-
peach latent mosaic viroid	PLMVd	2	337-338	PLMVd.1/.2	337-338	hh+ / hh-
PSTVd-type (group B)						
PSTVd group (subgroup B1)						
coconut cadang-cadang viroid	CCCVd	7	246-301	CCCVd.2	246	
citrus exocortis viroid	CEVd	31	368-463	CEVd.16	371	
columnea latent viroid	CLVd	2	370-372	CLVd.1	370	
chrysanthemum stunt viroid	CSVd	2	354-356	CSVd.2	354	
coconut tinangaja viroid	CTiVd	2	254	CTiVd.1/.2	254	
citrus viroid species IV	CVd IV	1	284	CVd IV.1	284	
hop latent viroid	HLVd	1	256	HLVd.1	256	
hop stunt viroid	HSVd	23	294-303	HSVh.1	297	
potato spindle tuber viroid	PSTVd	27	341-361	PSTVd.1	359	
tomato apical stunt viroid	TASVd	2	360-363	TASVd.1/.2	360	
tomato planta macho viroid	TPMVd	1	360	TPMVd.1	360	
ASSVd group (subgroup B2)						
australian grapevine viroid	AGVd	1	369	AGVd.1	369	
apple scar skin viroid	ASSVd	4	329-331	ASSVd.3	329	
citrus bent leaf viroid	CBLVd	2	315-318	CBLVd.2	315	
citrus viroid species III	CVd-III	2	294-297	CVd-III.2	294	
coleus viroid	CoVd	2	248	CoVd.1/.2	248	
grapevine yellow speckle viroid	GYSVd	33	363-369	GYSVd.27	368	
grapevine 1B viroid	G1BVd	1	363	GV1Bd.1	363	
pear blister canker viroid	PBCVd	1	315	PBCVd.1	315	
Satellite RNA						
Luteovirus						
barley yellow dwarf virus satellite RNA	vBYDV	1	322	vBYDVd.1	322	hh+ modified / hh-
Nepovirus						
arabis mosaic virus satellite RNA	sARMV	1	300	sARMV.1	300	hh+ / hp-
chicory yellow mottle virus satellite RNA S1	sCYMV-S1	1	457	sCYMV-S1.1	457	hh+ / hp-
lucerne transient streak virus satellite RNA	vLTSV	3	322-324	vLTSV.3	322	hh+ / hh-
tobacco ringspot virus satellite RNA	sTobRV	2	359-360	sTobRV.1	359	hh+ / hp-
Sobemovirus						
subterranean clover mottle virus satellite RNA	vSCMoV	2	332-338	vSCMoV.2	332	hh+
solanum nodiflorum mottle virus satellite RNA	vSNMV	1	377	vSNMV.1	377	hh+
velvet tobacco mottle virus satellite RNA	vVTMoV	2	365-366	vVTMoV.1	365	hh+
Other related RNA						
carnation stunt associated viroid	rCarSV	2	275	CarSV.1/.2	275	hh+ / hh-
Hepatitis delta virus*	vHDV	1	367	vHDV	367	delta+ / delta-
Newt satellite 2 transcript	rNS2T	1	281	Newt.1	281	hh+

The selected variants are the likely ancestral ones determined as described in the text. The presence of known catalytic motifs are identified by hh for hammerhead, hp for hairpin and delta for self-catalytic domains found in the hepatitus delta virus. '+' and '–' indicate the polarity of the RNA which possesses the self-catalytic domain. 'd' indicates a viroid, 'v' a virusoid (i.e. a plant viroid-like satellite circular RNA), 's' a plant viroid-like linear satellite RNA and 'r' a viroid-like retroelement. nt is for nucleotide.
*Because it is only a domain of HDV that is related to the viroid, only one variant sequence has been included even though several other sequences have been determined.

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ASSVd-type  (subgroup  B2)

-  Australian  grapevine  viroid  (AGVd),  1  variant

>AGVd.1
accession numbers:X17101(embl), 58574(gi)
Locus Agvccs
369 nucleotides (76A, 103C, 111G, 79T)
Rezaian,M.A.Australian grapevine viroid-evidence for extensive
recombination.Nucleic Acids Res. 18, 1813-1818 (1990)

TGGGCACCAA CTAGAGGTTC CTGTGGTACT CACCGAAGGC CGCGAACGTA GGAAAGAAAA AGATAGAAAA
GCTGGGTAAG ACTCACCTGG CGACTCGTCG TCGACGAAGG GTCCTCAGCA GAGCACCGGC AGGAGGCGCT
ATGCAGGAAC GCTAGGGGTC CTCCAGCGGA GGACTGAAGA AACTCCGGTT TCTTCTTTCA CTCTGTAGCT
GGAATCCCTG TTGCGCTTGC TGGCGAAACC TGCAGGGAAG CTAGCTGGGT CCCGCTAGTC GAGCGGACTC
GTCCCAGCGG TCCCAACCAG TTTTCTTTAT CCTATTTTTC CTGCGGGCGC CCGGTCGTGG TTACCCTGGA
GCTCCCTGTT TGGAGGCCC
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Figure 1. Example of the AGVd sequence catalogue entry.

PROPOSITION OF AN ACCURATE IDENTIFICATION SCHEME

In order to simplify the nomenclature of the included sequences, we used an identification scheme based on the usual abbreviation of an RNA species followed by a number. For example, the original PSTVd sequence (30) is identified as PSTVd.1. The number is a function of the date of report. For sequences already published or reported in on-line libraries, priority was given to publication date over library submission date. When more than one sequence was reported simultaneously, we attributed arbitrary numbers to the entries. Exclusively for HSVd variants, a letter according to the initial isolate host precedes the number. For example, HSVd.h1 refers to a species isolate from the hop. We believe that the proposed nomenclature will facilitate further sequence identification.

In creating this catalogue we clarified some identification ambiguities. Our proposed clarifications are listed below:
(i) Previously, two sequences have been identified as grapevine viroid, GVVd (7,25). Phylogenetic analysis of these sequences (data not shown) clearly demonstrated that one is a variant of CEVd (7), which we identified as CEVd.22; while the other is a HSVd variant infecting the grapevine (25), which we identified as HSVd.g5;
(ii) The second sequence reported as a GYSVd variant has been reported elsewhere as a distinct species, GVd 1B (59). We used the latter identification as it is supported by phylogenetic analysis which assigned 52 substitutions between the GVd 1B and the likely ancestral GYSVd.27 variant (data not shown);
(iii) Two sequence variants of CPFVd have been reported (28,29). They belong to HSVd species isolated from cucumber. We have identified these two sequences as HSVd.c1 and .c2.
(iv) Dapple apple viroid (DAVd; EMBL acession number X71599) appeared as the likely ancestral variant of apple scar skin viroid (ASSVd.4), and not as a different species.
(v) We used the nomenclature CoVd (*Coleus viroids*) which included the *Coleus blumei viroid species I* (CoVd.1, ref. 55) and the *Coleus yellow viroid* (CoVd.2, ref. 56). This is in agreement with the suggestion of Fonseca *et al*. (56).

ANALYSIS OF THE CATALOGUE

We analyzed the compilated sequences for the general characteristics of viroids and viroid-like RNAs taking for account the phylogenetic reconstruction reported by Elena *et al*. (85), as well as an updated reconstruction confirming the previous one (F. Bussière, D. Lafontaine and J.-P. Perreault, unpublished data). No relationship can be discerned between the nucleotide percentages, sequence length and phylogenetic clustering. The nucleotide differences between variants of the same species are located primarily in the pathogenesis (P) and the variable (V) domains of the viroids, however they are not restricted to these two domains as concluded previously (86). Furthermore, no relationship has been established between viroids and either their host or their worldwide geographic distribution, nor have any specific signature sequences been identified for viroids which infect the same host. In contrast, some interesting features were pointed out by phylogenetic reconstructions and secondary structure predictions.

Prediction of the most likely ancestral variants

In order to study the relationship between variants belonging to the same species and to identify the most likely ancestral sequence, we performed either direct inferences or phylogenetic reconstructions. The identification of the probable ancestral variants will be useful in further phylogenetic reconstructions between species. When two sequences were known, we initially looked to see if one could be derived from the other, and then used the ancestral one if this was indeed the case. If they did not fulfill this requirement, the sequences were considered as belonging to different taxa. If more than two variants existed, the ancestral state was either directly inferred, or obtained by phylogenetic analysis. In the latter cases the sequence alignments were carried out by means of a multiple sequence algorithm with hierchical clustering (Multalin package, ref. 87) followed by minor sequence rearrangements, and then the phylogenetic analysis was performed using maximum parsimony method (PAUP package, ref. 88). We deduced the current probable ancestral variant among the sequences available in Table 1, and appended this list to the compilation. The addition of more sequences may facilitate the identification of ancestral variants.

Exhaustive phylogenetic reconstitutions of the species that have large numbers of variants (ASBVd, CCCVd, CEVd, GYSVd, HSVd and PSTVd) have been performed. The case of CCCVd was the simplest among these viroids. Five CCCVd variants resulted from a duplication of a region of CCCVd.2 and then subsequent substitutions occurred (CCCVd.3–CCCVd.7), as suggested previously (86). CCCVd.1 has a one nucleotide change as compared to CCCVd.2, and this substitution is not found in the

other five larger variants; therefore, we infered CCCVd.2 as the oldest variant based on the available data. In contrast, the other viroids with several variants required phylogenetic reconstructions to identify the likely oldest sequence. Most of the variants of these viroids differ only by a very small number of substitutions (~1–10 mutations and/or deletions and/or insertions). When all variants of one of these species were considered for a phylogenetic reconstruction, several sequences could have evolved in different manners from either the ancestral variants or other variants. Thus, several trees with the same total length were inferred for a specific species (also observed by Dr Robert Owens for HSVd and PSTVd, personal communication). Because no biological data permits validation of these phylogenetic trees (for example, replicational data), a consensus tree was deduced and the likely oldest sequence defined as the ancestral one.

Possibly, the identity of the host infected by a viroid may be useful for the validation of phylogenetic trees. For example HSVd variants infect a wide range of hosts including hop, grapevine, plum, pear, peach, citrus and cucumber. Taking into account the host specificity of HSVd variants, we analyzed the trees derived in order to verify whether variant clusters reflected their host specificity. As previously reported (26), the HSVd variants clustered mainly in three variant types, (i) the hop (hop, grapevine, peach and pear isolates), (ii) the plum (grapevine, peach and plum isolates) and (iii) the citrus (citrus and cucumber isolates). The HSVd phylogenetic tree did not strictly reflect the host specificity; hence this did not allow validation of the phylogenetic data.

For viroids that have only two known variants, the sequences are nearly perfectly identical, and therefore did not allow identification of the ancestor. One exception was TASVd in which TASVd.1 and .2 show a sequence homology of 91.5% (50) and appear the most distant variants of the same species with two known sequences. A phylogenetic reconstruction of some PSTVd-type viroids shows that the two TASVd sequences branched onto the main lineage (Fig. 2). These two branches were separated by an average of 16 substitutions. These results may support two different possibilities, either the two TASVd sequences belong to two different species which infect the same host, or they are two variants of a same species. The latter case suggests that CEVd has evolved directly from TASVd and not from a common ancestor. Further characterization should determine which of these two scenarios is the correct one.

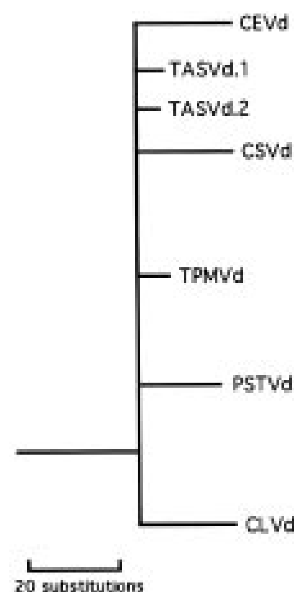

Figure 2. Phylogenetic tree of some PSTVd group viroids.

Secondary structure predictions

In order to allow comparison between the most stable secondary structures of the ancestral species, we performed computer analysis on the previously selected sequences. We used the MulFold structure prediction package (89) of GCG (Genetic Computing Group) version 8.0 installed on a UNIX system (at the Institut de Recherche Clinique de Montréal). For each selected sequence, a prediction of the secondary structure was obtained, and the resulting structures transformed into 'connect' files using the plotfold-H software. The connect file of each predicted structure follows the sequence in the website, thereby allowing any investigator to work within it using his own graphics package. For the printed version of the catalogue, the secondary structure connect files have been displayed using the RNADrawn package and are appended.

Most of the published viroid secondary structures were predicted with the original RNAfold package. These original predictions led to the proposal that the most stable secondary structure of the classical viroids of the PSTVd-type (i.e. PSTVd and ASSVd groups) are rod-like structures composed of alternating single- and double-stranded regions (1,86). With time, the rod-like secondary structure almost became an identification criterion for viroids. Analysis of our predicted secondary structures led to unexpected results that are summarized in Figure 3. To facilitate the analysis, the results are presented based on the phylogenetic tree of viroids and related RNAs.

Excluding CEVd and CSVd, all members of the PSTVd group (including HSVd, CCCVd and related viroids) adopted a rod-like most stable secondary structure. In contrast, CEVd and CSVd have, at one end of their rod-like structures, a cruciform tRNA-like structure. The CEVd cruciform structure involves 80 nt at the left extremity, while the CSVd version is composed of 73 nt and is located at the right end of the rod. These cruciform structures resemble the tRNA-like structure of RNA virus genomes. However, the tRNA-like structure predicted in these two species have not been demonstrated to occur in solution, nor have they had a function attributed to them.

The structure predictions of the viroids from ASSVd group show a gradual evolution from rod-like secondary structures to more and more branched structures. AGVd is a perfect rod-like shape, ASSVd adopts a rod-like secondary structure with a small Y at the left end, while GYSVd has several branches leading to the absence of the initial basic rod-like structure. Other viroids of the ASSVd group follow the same scheme as a function of their phylogenetic position.

ASBVd-type viroids are ASBVd and PLMVd. ASBVd adopts a mostly rod-like secondary structure with a small Y at the left end. In contrast, both PLMVd variants adopt branched secondary structures. Similarly, most of the viroid-like satellite RNAs adopted branched secondary structures which included the basic rod-like shape and a tRNA-like structure at one extremity. Finally, the two transcripts related to viroids, rCarSV and rNS2T, and the only luteovirus (vBYDV) adopted branched secondary structures, while the viroid-like domain vHDV formed a long rod-like structure (data not shown).

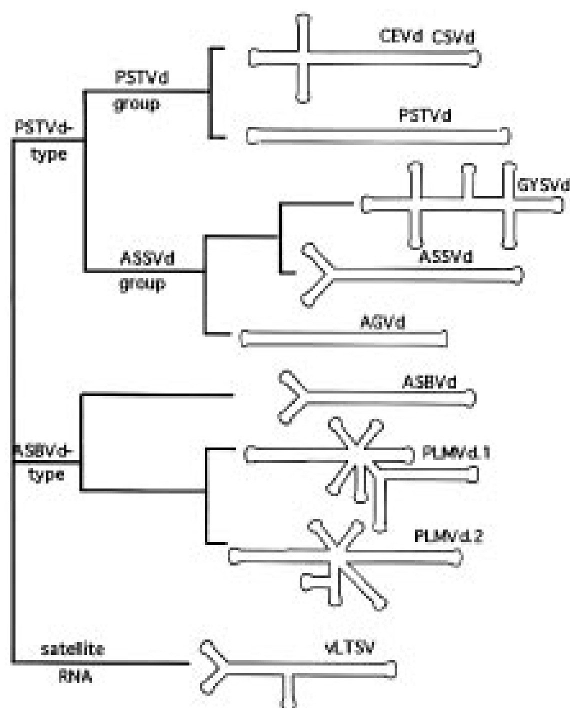

Figure 3. Schematic representation of the predicted secondary structure derived using the MulFold structure prediction package and considering the phylogenetic relationship. The length of the lineages are not proportional to the number of substitutions between the species.

The case of the two PLMVd sequence variants established by Hernandez and Flores (69), led to some interesting observations. The most stable structure of PLMVd.1 was originally predicted using the RNAfold package (69), and has recently been revised by them (personal communication) using the MulFold package resulting in several minor differences. We have confirmed their latest prediction. The left hand domain, which is composed of the sequences involved in the formation of the hammerhead motifs of both polarities, is rod-like in structure (69). Eleven of the 15 nt differences between the two PLMVd variants are in the left hand region, which is the most stable region of PLMVd (70). These nucleotide differences affect the native secondary structure only locally. In contrast, the central and right hand regions are composed of several hairpins and four of the 15 nt differences are sufficient to yield relatively different structures between the variants. These results clearly show that minimal nucleotide differences may drastically affect the predicted most stable secondary structure; hence, this may be important for the biological activity associated with these regions. Analysis of the computer predicted secondary structures is instructive for the formulation of hypotheses on structure–function relationships. However, *in vitro* as well as *in vivo* characterization of biological structures is obviously more accurate for the structure–function relationship.

Analysis of both the free energy values and the proportion of GC, AU and GU base-paired regions of the predicted secondary structures as a function of either clustering or placing in a phylogenetic tree did not lead to any conclusions. Moreover, analysis of the position of the nucleotide differences does not indicate that covariations are frequently observed within viroid sequences. However, this may due to our lack of knowledge of viroid biological secondary structures. This affirmation is supported by the analysis of the hammerhead sequence present in PLMVd. As mentioned previously, the 11 nt differences between the two PLMVd variants in the left hand region affect the most stable secondary structure only locally. However, these nucleotide differences do not affect the hammerhead secondary structures (70). As a result covariation of base paired nucleotides is observed, suggesting a selective pressure in favor of the self-cleavage activity (70). This example shows that the covariation is associated with the secondary structures that have biological importance. Clearly, the identification of the viroid biological secondary and tertiary structures is an important research avenue.

COMPLETLENESS, ACCURACY AND AVAILABILITY OF THE DATA

We have attempted to compile a catalogue of all viroid and viroid-like sequences published in journals or available from the GenBank and EMBL nucleotide sequence libraries. To this end, the EMBL and NCBI library file servers were scanned using several queries for new sequences. Sequences obtained were added to the catalogue as well as any pertinent, useful information. The authors would appreciate being informed of any omitted sequences or errors in the data set. We intend to correct any such errors in the future. Furthermore, the catalogue will be updated a few times per year. In future catalogue updates, no priority will be attributed to publication. This compilation will be available on the world wide web (http://www.callisto.si.usherb.ca/~jpperra). In this manner all viroid researchers will have the possiblity of, as well as some responsibility for, updating this compilation by electronic mail submission (jp.perre@courrier.usherb.ca) following the example in Figure 1. The viroid and viroid-like RNA catalogue will be also available on floppy disks, readable on microcomputers operating under MS-DOS, or in hard copy form.

ACKNOWLEDGEMENTS

The authors thank Drs Rudra P. Singh and Robert A. Owens for critical review of sections of the catalogue. This work was supported in part by grants from the Natural Sciences and Engineering Research Council (NSERC) and by the Medical Research Council (MRC) of Canada to J.-P.P, and in part by a scientific team grant from Fonds pour la Formation des Chercheurs et l'Avancement de la Recherche du Québec (FCAR). F.B. and D.L. are recipients of predoctoral fellowships from FCAR and Fonds de la Recherche en Santé du Québec (FRSQ), respectively. J.-P.P. holds a scholarship from the Medical Research Council of Canada.

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