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Coleus blumei viroid 8: identification and characterization of a novel coleviroid harboring a unique terminal conserved hairpin

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Research Article

Keywords: coleus, coleviroid, Coleus blumei viroid 8, terminal conserved hairpin

Posted Date: October 30th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11374496/v1

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Additional Declarations: No competing interests reported.

Abstract

Six viroids belonging to the genus *Coleviroid* (family *Pospiviroidae*) are currently reported all of which naturally infect coleus (*Coleus scutellarioides*). In 2025, a novel viroid-like circular RNA was identified in a vegetatively propagated coleus plant along with Coleus blumei viroid 1 (CbVd-1). Sequence analysis showed that the RNA is 268 nucleotides (nt) long and folds into a rod-like secondary structure. Although the sequence of its central region is identical to that of CbVd-5, its right- and left-hand regions differ significantly from those of all previously described coleviroids. The RNA also contains the central conserved region characteristic of the genus *Coleviroid* and a unique terminal conserved hairpin in its left-terminal region, comprising a 3-nt terminal loop shared with small-genome coleviroids and a 6-bp stem. Maximum likelihood phylogenetic analysis further showed that this RNA is genetically distant from known coleviroids, forming a separate clade. Bioassays with dimeric transcripts confirmed that it is infectious and replicates autonomously in coleus. Collectively, these findings demonstrate that this 268-nt infectious circular RNA represents a novel coleviroid, for which we propose the name Coleus blumei viroid 8 (CbVd-8).

1. Introduction

Viroids are the smallest known plant pathogens, consisting of non-coding circular RNAs of 234–434 nucleotides (nt) that do not encode proteins. Viroids that replicate autonomously in the nucleus via a rolling-circle mechanism are classified in the family *Pospiviroidae*, whose members adopt a rod-like or quasi-rod-like secondary structure. The family is further classified into five genera–*Pospiviroid*, *Hostuviroid*, *Cocadviroid*, *Coleviroid*, and *Apscaviroid*–based on several criteria, including the sequence of the central conserved region (CCR) located in the center of the secondary structure, the presence or absence of a terminal conserved region (TCR) or terminal conserved hairpin (TCH), and overall genomic sequence similarity.

The genus *Coleviroid* currently comprises six viroids: Coleus blumei viroid 1 (CbVd-1) (*Coleviroid alphacolei*), CbVd-2 (*Coleviroid betacolei*), CbVd-3 (*Coleviroid epsiloncolei*), CbVd-5 (*Coleviroid gammacolei*, unclassified viroid-like RNA), CbVd-6 (*Coleviroid zetacolei*, unclassified viroid-like RNA), and CbVd-7 (tentative species), all of which naturally infect coleus (*Coleus scutellarioides*, synonym *C. blumei*). Among these coleviroids, only CbVd-1, CbVd-2, and CbVd-3 are currently recognized as species by the International Committee on Taxonomy of Viruses (ICTV) [1]. Members of the genus are defined by a rod-like secondary structure, a CCR identical to that of the type species (CbVd-1), and either a TCH or a TCR, with TCH present in small-genome species and TCR in larger-genome species. Species are traditionally distinguished by less than 90% sequence identity and distinct biological properties [1, 2]. More recently, a pairwise identity score (PWIS)-based classification system proposed a threshold identity score (TIS) of 91% for species demarcation within the genus *Coleviroid* [3].

CbVd-1 was the first coleviroid to be identified and was initially discovered in a yellow commercial coleus cultivar (cv. Amarelo) in Brazil [4]. It has since been detected in diverse coleus cultivars worldwide [5–10]. CbVd-1 is seed-transmitted [8, 11, 12], with the nucleotides of the loop on left-hand region in secondary structure playing a key role in this process [12]. Although most infected coleus plants remain

asymptomatic [10, 13, 14], some cultivars develop dwarfing and mild chlorosis [4, 8, 15]. CbVd-1 does not infect tomato (*Solanum lycopersicum*) or potato (*S. tuberosum*); however, it infects several other members of the family *Lamiaceae*, including spearmint (*Mentha spicata*), Japanese mint (*Mentha canadensis* var. *piperascens*), basil (*Ocimum basilicum*), and lemon balm (*Melissa officinalis*), without causing symptoms [6, 11]. CbVd-2 and CbVd-3 were first identified in Germany [16] and were subsequently identified in China [17, 18], with CbVd-3 also reported in Croatia [19]. CbVd-5 and CbVd-6 were initially described in China [20, 21]. Since then, CbVd-5 has been detected in Japan, India, Indonesia, Canada, and Malaysia [15, 22–24], whereas CbVd-6 has been reported in Canada and Japan [25, 26]. Similar to CbVd-1, both viroids are seed-transmitted in coleus [27], and infection can induce albinism or chlorosis in some cultivars [23, 25]. CbVd-7, recently reported in Canada, has the smallest viroid genome known to date, comprising 234 nt [28].

The currently known coleviroids are thought to have originated through natural recombination, likely among members of the genus *Coleviroid*. Each coleviroid contains distinct regions of high sequence homology, reflecting exchanges of the right- and left-hand regions flanking the central region of the secondary structure (Fig. 1). Specifically, the right-hand regions (RA) of CbVd-1, CbVd-2, and CbVd-7; the left-hand regions (LB) of CbVd-2, CbVd-3, and CbVd-6; the left-hand regions (LC) of CbVd-5 and CbVd-7; and the right-hand regions (RC) of CbVd-5 and CbVd-6 are highly conserved within each group. In addition, CbVd-4, an artificial chimera comprising the left-hand region of CbVd-1 and the right-hand region of CbVd-3, is infectious in coleus [16]. These findings suggest that each species may have evolved through recombination events of the right- or left-hand regions.

In this study, we identified a novel 268-nt viroid-like circular RNA in a vegetatively propagated commercial coleus plant. This RNA contains a CCR identical to that of CbVd-1, the type species of the genus *Coleviroid*, as well as a coleviroid-specific TCH. Although its central region is identical to that of CbVd-5, its left- and right-hand regions are distinct from those of all previously described coleviroids. The RNA shares less than 70% overall sequence identity with known coleviroid, and inoculation assays using viroid-free coleus seedlings confirmed its infectivity and autonomous replication. Together, these findings indicate that this circular RNA represents a novel species within the genus *Coleviroid*, for which we propose the name Coleus blumei viroid 8 (CbVd-8).

2. Materials and Methods

Plant materials

Vegetatively propagated coleus (cultivar unknown) and seed-propagated coleus (Wizard mix—a seed mix comprising various coleus cultivars with different leaf colors; Takii & Co., Ltd., Kyoto, Japan), both commonly available in Japan, were used in this study (Supplementary Fig. S1). After sowing, Wizard mix seedlings were classified into three phenotypes based on leaf color, crimson-red (phenotype 1), green (phenotype 2), and red (phenotype 3). The coleviroid infection status was determined by RT-PCR and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (data not shown). Seedlings confirmed to be free of coleviroid infection were used for inoculation assays with CbVd-8 transcripts, whereas vegetatively

propagated clones derived from the coleviroid-free red seedlings (phenotype 3) were used for graft inoculation assays. All plants were maintained in a greenhouse.

Extraction of total RNA and removal of polysaccharides

Total RNA, including viroids, was extracted from the upper leaves of coleus plants using the 2× CTAB extraction method [29]. To remove polysaccharides, equal volumes of 2.5 M K_2HPO_4 (pH 8.3) and 2-methoxyethanol were added to the RNA solution, which was mixed vigorously and centrifuged to recover the upper aqueous phase. The aqueous phase was diluted 12.5-fold with sterile distilled water, followed by the addition of 2% CTAB solution (1/25 of the total volume), gentle mixing, and overnight incubation at 4°C. The resulting precipitate was collected by centrifugation, dissolved in 70% ethanol containing 0.5 M NaCl, pelleted by centrifugation, and finally dissolved in sterile distilled water for CbVd detection.

Detection of circular RNA by 2D-PAGE

Circular RNAs were detected by 2D-PAGE using total RNA extracted by the CTAB method. A 40 µL aliquot of the CTAB extract was mixed with 10 µL of loading dye containing bromophenol blue and xylene cyanol FF and loaded onto the first-dimension gel (7.5% polyacrylamide, acrylamide:bisacrylamide = 39:1). Electrophoresis was performed in 1× Tris-borate-EDTA (TBE) buffer for 105 min at 250 V and 25 mA. The RNA-containing lane was excised longitudinally and placed horizontally at the bottom of a glass plate to prepare the second-dimension gel. The second-dimension gel (7.5% polyacrylamide containing 8 M urea, acrylamide:bisacrylamide = 39:1) was cast over the first gel strip, and electrophoresis was performed in 1× TBE buffer with the direction of current reversed for 75 min at 450 V and 45 mA. Finally, the gel was silver-stained to visualize bands corresponding to circular RNA molecules.

Detection and identification of coleviroids using RT-PCR and sequence analysis

First-strand cDNA was synthesized in a 20 µL reaction mixture using M-MLV Reverse Transcriptase (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. PCR amplification was performed in a 25 µL reaction mixture containing 0.25 µL of LA Taq (5 U/µL, Takara Bio, Inc., Kusatsu, Shiga, Japan), 1× LA PCR buffer (Mg^{2+}-free), 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.8 µM of each primer, and 2.0 µL of the RT product. The thermal cycling conditions were as follows: initial denaturation at 94°C for 1 min; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; followed by a final extension at 72°C for 7 min.

The RT-PCR products were separated by 7.5% PAGE in 1× TBE buffer at 250 V and 25 mA for 80 min, stained with ethidium bromide (EtBr), and visualized under UV light (312 nm). Amplified fragments were excised from the gel, cloned into the pMD19 T-Vector (TaKaRa Bio), and subjected to Sanger sequencing. The resulting sequences were trimmed and analyzed using the Nucleotide BLAST tool (NCBI; https://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify coleviroid species.

Analysis of mutations in the CbVd-8 population and progeny

First-strand cDNA was synthesized in a 20 µL reaction mixture using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. PCR amplification was performed in a 25 µL reaction mixture containing 0.25 µL of Q5 DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1× Q5 Reaction Buffer, 0.2 mM dNTPs, 0.5 µM each primer (CbVd-CCR-P and CbVd-CCR-M), and 1.25 µL of the RT product. The thermal cycling conditions were as follows: initial denaturation at 98°C for 30 s; 35 cycles of denaturation at 98°C for 10 s, annealing at 68°C for 20 s, and extension at 72°C for 20 s; followed by a final extension at 72°C for 1 min.

The RT-PCR products were phosphorylated, and separated by agarose gel electrophoresis in 0.5× TAE buffer at 100 V for 40 min using a 2.0% agarose gel containing Midori Green Advance DNA Stain (Nippon Gene Co., Ltd., Tokyo, Japan). The separated fragments were visualized under UV light (312 nm), excised from the gel, and cloned into the SmaI site of the pUC19 vector. Insert sequences from individual clones were determined by Sanger sequencing. The resulting sequences were trimmed and subjected to multiple sequence alignment and mutation analysis using the ClustalW algorithm implemented in MEGA11.

Calculation of sequence identity and pairwise identity scores

Sequence identity between CbVd-8 and other coleviroids was calculated as follows. Sequences of CbVd-8 and each reference coleviroid were aligned using the MUSCLE algorithm implemented in MEGA11 with default parameters. The numbers of matching bases were determined for the full-length sequence and for each genomic region, i.e., the left-hand, central, and right-hand regions. To avoid inflation of alignment length due to gap insertions, sequence identity was calculated using Dice's coefficient (Sørensen–Dice index) according to the following formula: sequence identity (%) = (number of matching bases × 2 / total number of bases in both sequences) × 100 [30–32]. The reference sequences used for comparison were as follows: CbVd-1, X52960; CbVd-2, X95365; CbVd-3, X95290; CbVd-5, FJ151370; CbVd-6, FJ615418; CbVd-7, MW883069; and CbVd-8, PZ737693 (the major sequence identified in this study).

The PWIS was calculated according to a previously described method [3]. Full-length sequences of each coleviroid were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and aligned with the major CbVd-8 sequence using the MUSCLE algorithm implemented in MEGA11, with gap opening and extension penalties set to − 15.00 and − 6.66, respectively. The resulting alignment was saved in FASTA format and submitted to Clustal Omega (https://www.ebi.ac.uk/jdispatcher/msa/clustalo) for analysis with the following parameters: sequence type, RNA; mBed-like clustering guide-tree, no; mBed-like clustering iterations, no; distance matrix, yes; and default settings for all other parameters. The percentage identity values calculated between the major CbVd-8 sequence and each coleviroid sequence were used as the PWIS.

Molecular phylogenetic analysis

Full-length coleviroid sequences were retrieved from GenBank and aligned using the Clustal Omega. The maximum likelihood phylogenetic tree was constructed using IQ-TREE ver. 2.4.0 via the CIPRES Science Gateway [33, 34]. The sequence type was set to DNA, and the best-fit nucleotide substitution model was automatically selected using ModelFinder [35] integrated within IQ-TREE. Branch support was evaluated

with the ultrafast bootstrap method using 5,000 replicates. The resulting tree was visualized and rooted by midpoint rooting using FigTree (version 1.4.4).

Prediction of secondary structure

The secondary structures of CbVd-8 and other coleviroids were predicted using the UNAFold Web Server (mfold, RNA Folding Form version 2.3; http://www.unafold.org/mfold/applications/rna-folding-form-v2.php). Predictions were performed using the “circular” and “25°C” settings, and the structure with the lowest free energy was selected as the predicted secondary structure for each coleviroid.

Construction of dimeric CbVd-8 clone

The dimeric (2U) CbVd-8 clone was constructed as previously described [36]. CbVd-8 clone 3, which represents the major sequence identified in this study and was cloned into the pUC19 vector, was used as the template. Two fragments were amplified by PCR using different primer sets containing restriction enzyme recognition sequences at their 5' termini (Supplementary Table S1). PCR was performed under the same conditions as those used for mutation analysis of CbVd-8 sequences described above. The two amplified fragments were digested with SmaI and BsaI, or HindIII and BsaI, respectively, and ligated into the SmaI–HindIII sites of the pTAC2 vector (BioDynamics Laboratory Inc., Tokyo, Japan). The sequence of the resulting 2U CbVd-8 clone was confirmed, and the construct was subsequently used as a template for in vitro transcription.

In vitro transcription of dimeric CbVd-8 RNA

The plasmid containing the 2U CbVd-8 cDNA was linearized with HindIII and used as a template for in vitro synthesis of plus-polarity 2U CbVd-8 RNA using T7 RNA polymerase (Invitrogen). In vitro transcription and purification of the resulting transcripts were performed as previously described [37]. The purified plus-polarity 2U CbVd-8 RNA was used for inoculation assays in coleus seedlings.

For synthesis of minus-polarity DIG-labeled cRNA probes to detect plus-polarity CbVd-8 RNA, the plasmid was linearized with SmaI and used as a template for in vitro transcription with SP6 RNA polymerase (Roche Diagnostics, Basel, Switzerland). A 40 µL reaction mixture containing 1× Reaction Buffer (Roche Diagnostics), 0.5 mmol/L RNase inhibitor (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 1× DIG RNA Labeling Mix (Roche Diagnostics), 40 U of SP6 RNA polymerase (Roche Diagnostics), and 3 µg of linearized plasmid DNA was incubated at 37°C for 16 h. The template DNA was removed by treatment with RQ1 DNase (Promega Corporation, Madison, WI, USA), and the DNase was removed by sequential phenol:chloroform (1:1) and chloroform extractions. The RNA transcript was precipitated overnight with sodium acetate and ethanol and resuspended in 100 µL of ultrapure water.

To check the quality, 2 or 5 µL of the cRNA probe was mixed with an equal volume of 2× RNA sample loading buffer (Norgen Biotek Corp., Thorold, ON, Canada) containing EtBr, followed by denaturation at 80°C for 10 min. Samples were electrophoresed on a 1.2% denaturing agarose gel containing 2.2 M formaldehyde in 1× 3-(N-morpholino)propanesulfonic acid buffer and visualized under UV light (312 nm). Prior to the first use only, the cRNA probe was denatured at 95°C for 5 min and immediately chilled on ice.

Inoculation of CbVd-8 into coleus seedlings

Coleus seedlings (green-, red-, and crimson-leaf phenotypes from Wizard mix) confirmed to be free of CbVd infection were inoculated with 10 µg of plus-polarity 2U CbVd-8 transcripts per plant. Multiple incisions were made in the main stem of each seedling using a cutter coated with the transcript inoculum. The inoculated sites were covered with Parafilm, and the seedlings were maintained under greenhouse conditions.

Northern hybridization for detection of CbVd-8

CbVd-8 was detected by Northern hybridization using total RNA extracted by the CTAB method from coleus plants at 1, 3, and 5 months post-inoculation (mpi) with CbVd-8 transcripts. Hybridization was performed according to a previously described method [38] with minor modifications. Prehybridization and hybridization was conducted in 0.05 mL/cm^2 of a solution containing 50% formamide, 10% dextran sulfate, 0.18 M NaCl, 20 mM sodium phosphate (pH 7.0), 0.1% SDS, 500 µg/mL yeast tRNA, and 25 µg/mL sonicated salmon sperm DNA. Following prehybridization at 55^{o}C for 1 h, the DIG-labeled minus-polarity cRNA probe for detection of the plus-polarity CbVd-8 strand was added to the hybridization solution (2 µL probe/5 mL hybridization solution), and hybridization was performed at 55°C for 16 h. The membranes were washed twice with 2× SSC at 25°C for 15 min, once with 2× SSC containing 1 µg/mL RNase A at 25°C for 30 min, and twice with 0.1× SSC containing 0.1% SDS at 68^{o}C for 15 min. The membranes were incubated at room temperature for 30 min in 0.1 mL/cm² of blocking solution, followed by incubation with an equal volume of anti-DIG solution containing 1 µL of anti-DIG Fab fragment (Roche Diagnostics) for 30 min. After two washes with 75 mL of washing buffer (0.1 M maleic acid–0.15 M NaCl, pH 7.5, containing 0.3% Tween 20) for 10 min each at 25°C, the membranes were equilibrated in 30 mL of Buffer 3 (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 0.05 M $MgCl_2$) at 25°C for 5 min. Chemiluminescent detection was performed by sealing the membranes in plastic bags containing 10 µL/cm² CSPD (Roche Diagnostics) in Buffer 3 and imaging the signals using a ChemiDoc imaging system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

3. Results

Detection of a novel coleviroid from vegetatively propagated coleus

To investigate the diversity of coleviroids in vegetatively propagated coleus, we performed 2D-PAGE to detect circular RNAs in total RNA samples extracted from the upper leaves (Supplementary Fig. S1). Two overlapping circular RNA bands were detected (Fig. 2A). Therefore, we amplified full-length coleviroid sequences by RT-PCR using a universal primer set for coleviroids. Electrophoresis of the PCR products revealed two coleviroid-derived fragments of approximately 250–300 base pairs (bp) (Fig. 2B), suggesting co-infection of two coleviroid species, likely CbVd-1 and CbVd-5, based on their expected sizes. The amplified cDNAs were extracted from the gel, cloned, and sequenced. BLAST analysis showed that the smaller fragment was 249 bp in length and corresponded to CbVd-1, as expected. In contrast, the larger fragment exhibited only local similarity to CbVd-2, CbVd-3, CbVd-5, and CbVd-6 within the central region, and no closely related sequence was identified. To determine its complete sequence, we designed a primer

set specific to the unique region and performed PCR followed by direct sequencing. No polymorphisms were detected within the region spanning the CCR targeted by the initial universal primer set, allowing the complete circular RNA genome to be determined at 268 nt. Full-length sequences obtained from nine independent cDNA clones ranged from 267 to 269 nt. Three clones were 268 nt long and shared an identical sequence—designated the major sequence—whereas the remaining six contained a total of seven mutations, with one to two mutations per clone. The genome did not match any previously reported coleviroid species. Secondary-structure prediction of the major sequence using mfold revealed the characteristic unbranched rod-like conformation of members of the family *Pospiviroidae*, including the CCR typical of the genus *Coleviroid* (Fig. 3). Five of the seven mutations were located in the right-hand region of the predicted secondary structure (Fig. 3; Supplementary Table S2). Northern hybridization using a cRNA probe derived from the major sequence detected both monomeric and dimeric RNA forms in total RNA extracted from the original coleus plant (Fig. 2C), providing strong evidence that this RNA replicates autonomously via a rolling-circle mechanism. Collectively, these findings indicate that the 268-nt circular RNA identified in vegetatively propagated coleus represents a previously undescribed coleviroid, for which we tentatively propose the name Coleus blumei viroid 8 (CbVd-8).

Analysis of sequence homology and phylogenetic relationships of CbVd-8 with other coleviroids

To determine the evolutionary relationship of CbVd-8 with other coleviroids, we analyzed full-length genome sequence similarity using representative coleviroid sequences (Table 1). The major sequence of CbVd-8 shared 51.9–65.7% nucleotide identity with previously reported coleviroids, well below the 90% species demarcation threshold for the family *Pospiviroidae*. Analysis of the individual genomic regions showed that the upper strand of the central region of CbVd-8 shared the highest sequence identity (95–100%) with other coleviroids, whereas the lower strand exhibited 79–100% identity. Notably, CbVd-8 was identical to CbVd-5 across the entire central region. In contrast, the left- and right-hand regions shared less than 60% and 50% sequence identity, respectively, with all other coleviroids. PWIS analysis using either available sequences from each coleviroid species or all coleviroids yielded minimum and maximum PWIS values of 58.93%–68.80% and 62.50%–70.63%, respectively. In all comparisons, these values were well below the proposed 91% species demarcation threshold (TIS) for the genus *Coleviroid* [3] (Table 2, Supplementary Table S3).

Next, we performed a maximum-likelihood phylogenetic analysis using full-length CbVd-8 sequences together with all available coleviroid genomes retrieved from GenBank. All CbVd-8 clones formed a distinct, well-supported clade (100% bootstrap support), clearly separated from the other coleviroid species (Fig. 4). Together, these results provide strong evidence that CbVd-8 represents a novel species within the genus *Coleviroid*.

Table 1
Sequence identity of Coleus blumei viroid 8 (CbVd-8) with other coleviroids.

Viroid[1]	Full length		Left-hand region		Central region				Right-hand region	
					upper strand		lower strand			
size (nt)	identity (%)[2]	size (nt)	identity (%)	size (nt)	identity (%)	size (nt)	identity (%)	size (nt)	identity (%)	
CbVd-1	248	66.7	99	60.3	40	95.0	38	82.7	71	53.1
CbVd-2	301	60.5	151	43.0	40	95.0	38	80.0	72	54.0
CbVd-3	364	59.8	154	44.1	40	95.0	37	94.6	133	50.0
CbVd-5	274	66.4	84	55.4	40	100.0	37	100.0	113	50.0
CbVd-6	343	60.6	153	43.5	40	97.5	37	91.9	113	50.0
CbVd-7	234	64.9	84	55.4	40	95.0	38	82.7	72	52.8
CbVd-8	268	-	100	-	40	-	37	-	91	-

[1]Viroid sequences used in this analysis correspond to the following the GenBank accession numbers: CbVd-1, X52960; CbVd-2, X95365; CbVd-3, X95290; CbVd-5, FJ151370; CbVd-6, FJ615418; CbVd-7, MW883069; and CbVd-8, PZ737693 (the major sequence obtained in this study).

[2]Sequence identity was calculated using the Dice's coefficient (Sørensen–Dice index) according to the following formula: sequence identity (%) = (number of matching bases × 2 / total number of bases in both sequences) × 100.

Table 2
Minimum and maximum pairwise identity scores (PWISs) of Coleus blumei viroid (CbVd-8) relative to available sequences of each coleviroid species and all coleviroids.

Minimum PWIS	CbVd-1 (24)[1]	CbVd-2 (9)	CbVd-3 (8)	CbVd-5 (10)	CbVd-6 (5)	CbVd-7 (1)	coleviroids (57)
	65.15	**66.38**	**68.80**	**61.69**	**66.00**	**67.26**	**58.93**
Maximum PWIS	66.25	68.10	70.63	62.50	66.27	67.26	69.29

[1]The numbers in parentheses indicate the number of full-length coleviroid sequences retrieved from GenBank and included in the PWIS calculation.

Analysis and comparison of conserved sequence motifs in CbVd-8 and other coleviroids

According to the current ICTV definition, members of the genus *Coleviroid* adopt a rod-like secondary structure and possess a CCR identical to that of CbVd-1. Additionally, coleviroids with relatively large genomes contain a TCR, whereas those with smaller genomes possess a TCH [1, 2]. To determine whether CbVd-8 conforms to these characteristics, we analyzed its conserved sequence motifs and predicted secondary structure. Alignment of the central region of CbVd-8 with those of other coleviroids confirmed that all species contain a CCR identical to that of CbVd-1 (Fig. 5). Comparison of hairpin I—a structural element considered important for pospiviroid replication—revealed zero to two nucleotide substitutions relative to CbVd-1. CbVd-5 and CbVd-8 each contained two substitutions (G→C and C→U) within the basal stem of hairpin I (Fig. 5).

Next, we examined the presence of TCR and TCH motifs. The relatively large coleviroids—CbVd-2, CbVd-3, and CbVd-6—contained TCRs similar to those of pospiviroids. In contrast, the smaller coleviroids—CbVd-1, CbVd-5, CbVd-7, and CbVd-8—lacked both the previously described conserved TCR and canonical TCH sequences. However, analysis of their predicted secondary structure revealed a stem-loop motif at the left-hand end of the genome that resembles the TCH of hostuviroids and cocadviroids. Although this motif shows moderate sequence conservation among the smaller coleviroids, both its nucleotide sequence and stem length vary among species. Based on these structural differences, the TCH motifs can be classified into two types: type I, conserved in hostuviroids and cocadviroids; and type II, characteristic of coleviroids (Fig. 6). Type I TCH comprises a 3-nt terminal loop and a 4-bp stem. In contrast, type II TCH exhibits variation in stem length. The variants identified in CbVd-1, CbVd-5, and CbVd-7 share the same conserved 3-nt terminal loop and a 5-bp stem and were designated types II-1 and II-2, whereas the variant in CbVd-8 contains the conserved 3-nt terminal loop shared by all type II TCHs and a 6-bp stem and was designated type II-3.

Confirmation of CbVd-8 infectivity in coleus seedlings and analysis of progeny sequences

To confirm the infectivity of CbVd-8, vegetatively propagated coleus plants co-infected with CbVd-1 and CbVd-8 were grafted onto CbVd-free coleus clones (Supplementary Fig. S1, derived from phenotype 1). One month after grafting, RNA extracted from newly developed leaves of both the rootstocks and scions was analyzed by Northern hybridization. CbVd-8 was detected in the newly developed shoots of the rootstocks (Supplementary Fig. S5), confirming successful graft transmission. The infectivity of CbVd-8 was further assessed by mechanically inoculating CbVd-free coleus seedlings representing different leaf-color phenotypes with in vitro-transcribed dimeric CbVd-8 RNA (Supplementary Fig. S1). At 3 mpi, Northern hybridization of RNA extracted from the upper leaves detected both monomeric and dimeric forms in all phenotypes, confirming successful infection (Fig. 7). The progeny sequences were subsequently analyzed at 5 mpi. Sequencing of 32 independent cDNA clones recovered from CbVd-8-infected coleus plants revealed extremely limited sequence variation—only 10 clones contained a single nucleotide substitution, with each substitution occurring at a unique position, indicating that the major sequence of CbVd-8 is genetically stable (Supplementary Table S4). The inoculated plants were maintained for up to 6 mpi, however, no obvious disease symptoms were observed in any infected plant.

4. Discussion

Coleus is a natural host of coleviroids, and individual plants are frequently co-infected with multiple coleviroid species [14, 27, 39]. Vegetatively propagated coleus plants are particularly prone to harboring multiple coleviroids because, unlike seed-propagated plants, they do not undergo a reproductive phase through seed that may reduce or eliminate viroid populations. In this study, we detected a novel viroid-like circular RNA of 268 nt from vegetatively propagated coleus. This RNA adopted a rod-like secondary structure containing a CbVd-1-type CCR, a characteristic feature of the genus Coleviroid. Detection of both monomeric and dimeric forms in infected plants provided evidence of autonomous replication in planta. Furthermore, the sequence variation observed among multiple cDNA clones supported the existence of replicating variants. Collectively, these findings demonstrate that this RNA represents a novel viroid species within the genus *Coleviroid*.

Comparative analysis of the full-length sequence of the newly identified coleviroid, tentatively named Coleus blumei viroid 8 (CbVd-8), with those of other members of the genus *Coleviroid* revealed 51.9%–65.7% sequence identity, which is well below the 90% species demarcation threshold currently accepted for the genus. Although CbVd-8 shares an identical sequence with CbVd-5 throughout the central region, its left- and right-hand regions are highly divergent from those of previously reported coleviroids, showing less than 60% and 50% sequence identity, respectively. Furthermore, according to the species demarcation criteria recently proposed by Chiumenti et al. (2021), which define the TIS based on the minimum PWIS observed between species within the genus, the PWIS value of CbVd-8 was substantially below the proposed 91% TIS for the genus *Coleviroid* (Table 2, Supplementary Table S3). In addition, molecular phylogenetic analysis demonstrated that CbVd-8 variants formed a distinct clade separate from previously reported coleviroids, further supporting the classification of CbVd-8 as a novel species within the genus *Coleviroid*.

Analyses of conserved sequences revealed distinct molecular features that distinguish CbVd-8 from other coleviroids. CbVd-8 possessed a CCR identical to that of CbVd-1; however, similar to CbVd-5, it contained two nucleotide substitutions in the stem of hairpin I relative to CbVd-1. These substitutions converted a G–U pair and a G–C pair into a CU loop and a G–U pair, respectively. In particular, conversion of the G–U pair into a CU loop may substantially affect the structural stability of hairpin I, which is considered an important structural element for the infectivity and replication of pospiviroids [40, 41]. In PSTVd (isolate intermediate), substitution of the G–C pair in the hairpin I stem (positions 80 and 109) with an AC loop, a G–U pair, or an A–U pair resulted in the loss of infectivity in tomato plants [42]. Moreover, mutations within hairpin I of PSTVd have been reported to cause either loss of infectivity or reversion to the wild-type sequence [43]. Although the effects of these substitutions on coleviroid replication remain unknown, the efficient replication of both CbVd-1 and CbVd-8 in the same plant suggests that these structural differences do not prevent successful infection (Fig. 2A, B). Furthermore, our results showed that small-genomes coleviroids, including CbVd-8, lack the highly conserved TCR and TCH sequences previously described in other members of the family *Pospiviroidae*. Instead, these coleviroids possess an alternative TCH consisting of a 3-nt terminal loop shared among small-genome coleviroids and a variable 5- or 6-bp stem. Although this TCH differs in sequence from the highly conserved TCHs of hostuviroids and

cocadviroids, it maintains a similar secondary structure. Notably, CbVd-8 possesses a unique TCH with a 6-bp stem, distinguishing it from other small-genome coleviroids. Similar to the TCR, TCH is thought to play an important role in viroid replication. However, the biological significance of the TCH diversity observed among coleviroids remains unclear.

Nucleotide sequence diversity across the genome has been characterized in several coleviroid species. For example, comparison of CbVd-1 isolates from different coleus cultivars revealed that sequence variation is concentrated in the right-hand region of the predicted secondary structure [13], and that a point mutation in the left-hand region affects seed transmission efficiency [12]. In contrast, mutations in CbVd-3 are distributed throughout the genome, with relatively high frequencies observed in both termini [44]. Furthermore, coleus cultivars co-infected with CbVd-1 and CbVd-3 exhibit greater diversity in genome length and nucleotide sequence than those with single infections [19]. Similarly, sequence variation was detected among CbVd-8 isolates from the original coleus plant, with mutations predominantly occurring in the right-hand region of the genome. However, the extent and biological significance of nucleotide sequence diversity among coleviroids remain largely unknown.

Several important questions regarding coleviroid biology remain unresolved. First, with respect to pathogenicity, no adverse effects of CbVd-8 infection on the vegetative growth of coleus were observed in this study. Coleviroid infections are generally asymptomatic in coleus and other members of the family *Lamiaceae* [6, 10, 11, 13, 14]; however, CbVd-1, CbVd-5, and CbVd-6 have been reported to induce symptoms in certain coleus cultivars [4, 8, 15, 23, 25]. Despite the absence of visible symptoms, it will be important to determine whether coleviroid infection affects the production of aromatic compounds in *Lamiaceae* species, including coleus, given the economic and horticultural importance of many aromatic herbs in this family. A second unresolved question is why are such diverse species, including CbVd-8 identified here, successively detected from coleus, and why many of these viroids possess recombinant genome structures [14, 16, 20, 21, 39]. CbVd-8 is composed of novel sequences throughout the genome except for the central region and was detected in vegetatively propagated coleus plant as a co-infection with CbVd-1. However, our preliminary PCR and sequencing analyses using universal coleviroid primers did not detect any recombinant molecules between these two viroids. Nevertheless, previous studies showed that CbVd-4, an artificial chimera in which the right-hand region of CbVd-1 was replaced with the corresponding region of CbVd-2, is infectious in coleus [16]. These findings raise the possibility that naturally occurring recombinant coleviroids containing either the left- or right-hand region of CbVd-8 may exist, and we are currently investigating this hypothesis. In this study, we established a reliable CbVd-8 infection system using three different phenotypes of coleus seedlings. Furthermore, an infectious cDNA clone derived from the major sequence was stably maintained in the inoculated plants. This viroid–host system provides a valuable experimental platform for investigating nucleotide sequence diversity and its biological significance in coleviroids, as well as the interactions among different coleviroids during mixed infections.

In conclusion, we propose that the 268-nt infectious circular RNA identified in vegetatively propagated coleus is a novel coleviroid species, Coleus blumei viroid 8 (CbVd-8). CbVd-8 shares the CCR with all coleviroids and possesses the same central region as CbVd-5; however, its left- and right-hand regions

comprise novel sequences, and it possesses a unique TCH consisting of a 3-nt terminal loop conserved among small-genome coleviroids and a distinct 6-bp stem. CbVd-8 is expected to serve as a valuable model for advancing our understanding of coleviroid evolution and biological properties.

Declarations

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Funding

This work was supported by the Japanese Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (KAKENHI, Grant Number JP25K18237), and by JSPS Program for Forming Japan's Peak Research Universities (J-PEAKS) Grant Number JPJS00420240013.

Author Contribution

This study was conceived, designed, and supervised by Takashi Naoi and Teruo Sano. All authors conducted experiments, and data were analyzed by Takashi Naoi, Rino Hashimoto, and Teruo Sano. The manuscript was written by Takashi Naoi, and reviewed and edited by Takashi Naoi and Teruo Sano. All authors read and approved the manuscript.

Acknowledgement

The authors would like to thank Editage (https://www.editage.jp/) for the English language review.

Data Availability

The full-length sequence of CbVd-8 is available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under the accession numbers: clone 1, PZ737691; clone 2, PZ737692; clone 3, PZ737693; clone 4, PZ737694; clone 5, PZ737695; clone 6, PZ737696; clone 7, PZ737697.

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Figures

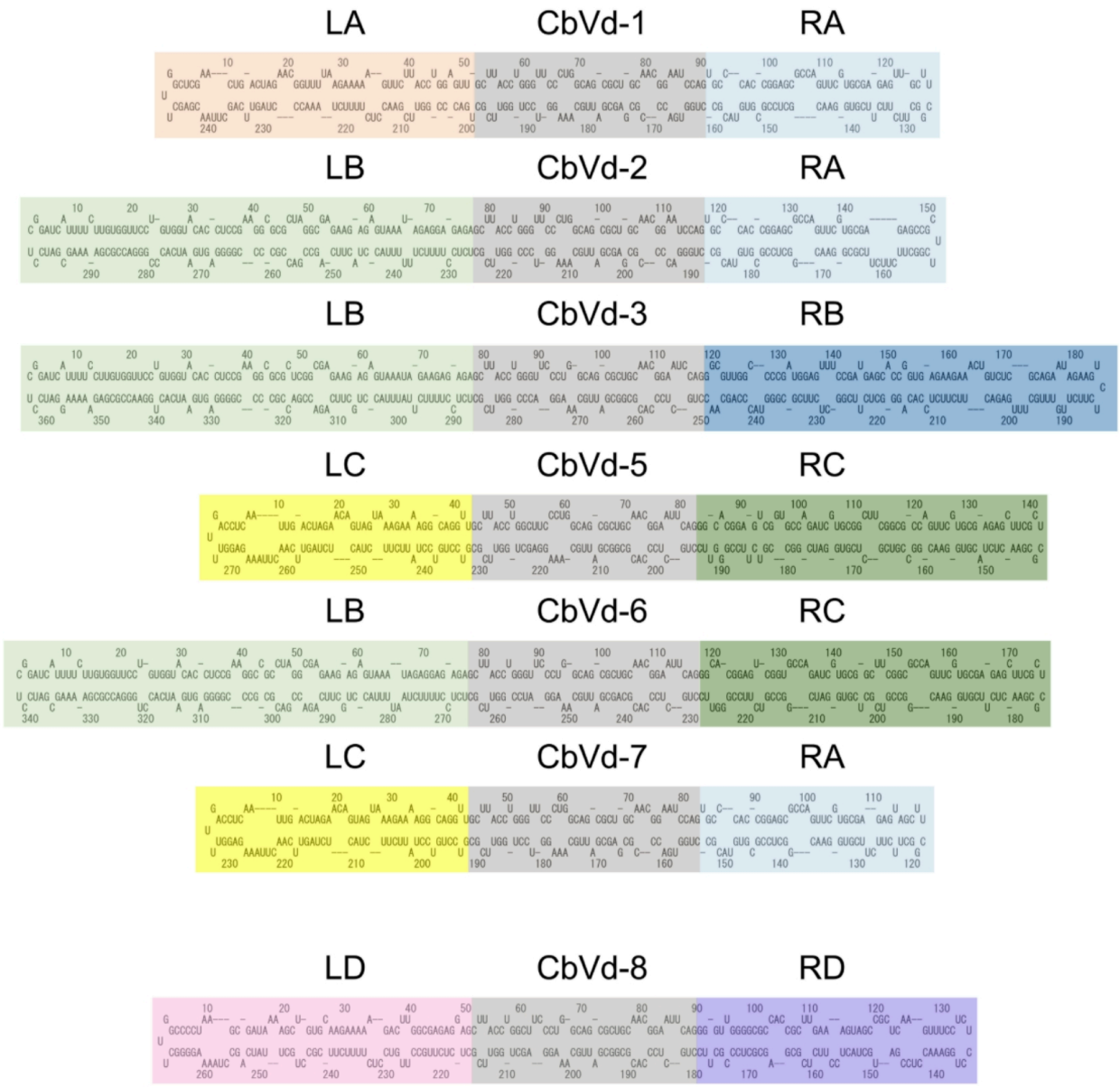


Figure 1

Predicted secondary structures of coleviroids. The left- and right-hand regions, highlighted with the same color, share highly homologous sequences. L: Left-hand region, R: Right-hand region.

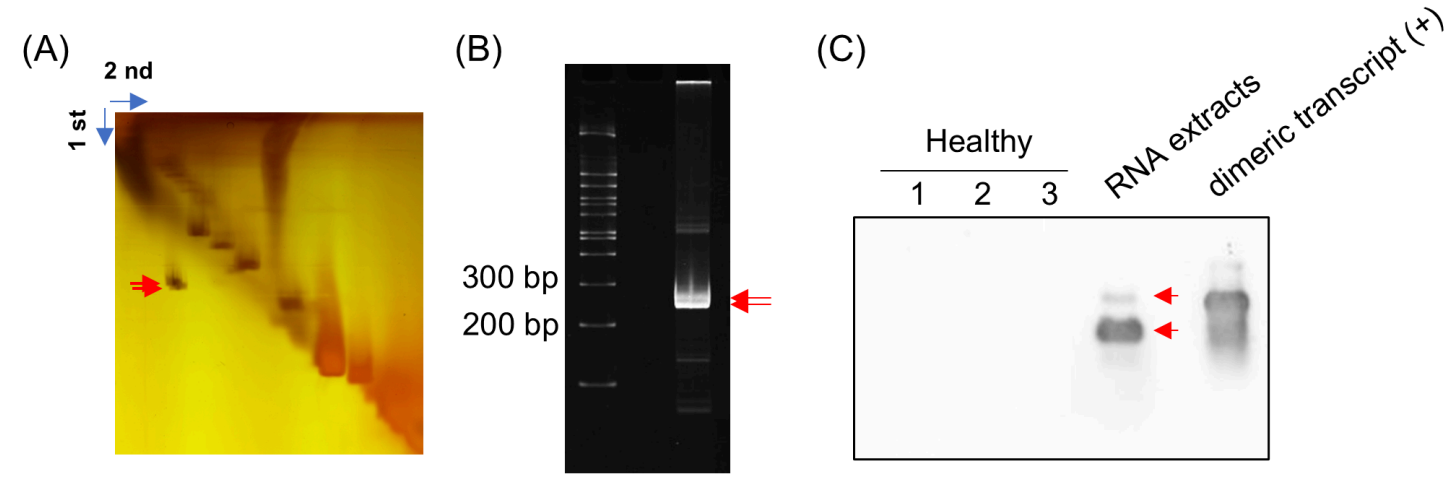


Figure 2

Detection of coleviroids in vegetatively propagated coleus. (A) 2D-PAGE detected two partially overlapping circular RNA bands migrating off the diagonal in the second-dimension gel (red arrows). (B) RT-PCR using a universal primer set for coleviroids amplified two coleviroid-derived fragments of approximately 250–300 bp (red arrows). (C) Northern hybridization using a probe specific for the 268-nt circular RNA detected both monomeric and dimeric RNA forms in vegetatively propagated coleus plants (lower and upper red arrows, respectively). Healthy-1, -2, and -3 denote phenotypes 1, 2, and 3, respectively, derived from the Wizard mix. RNA extracted from vegetatively propagated coleus plants co-infected with CbVd-1 and CbVd-8, as well as in vitro-transcribed dimeric CbVd-8 RNA used for inoculation, were included as positive controls.

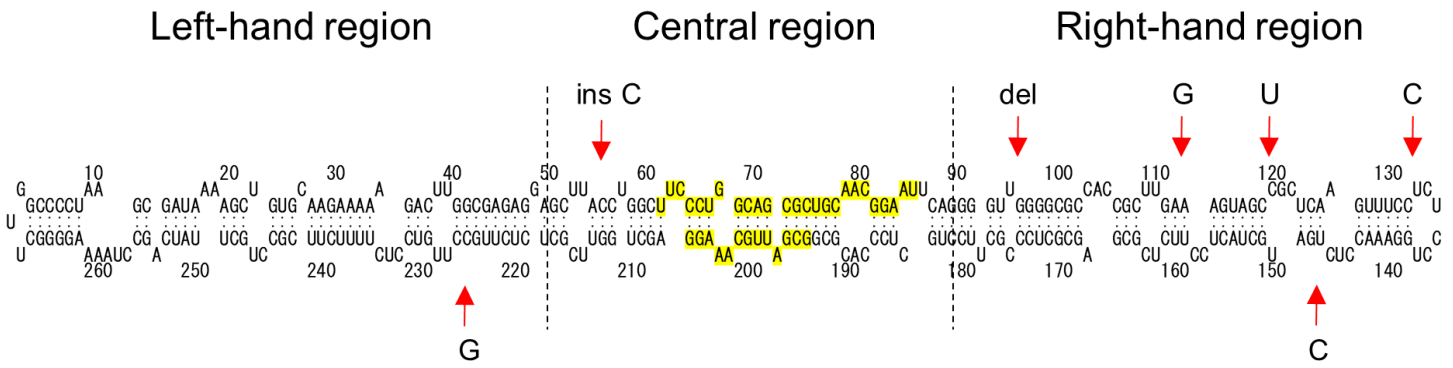


Figure 3

Predicted secondary structure of the novel coleviroid, Coleus blumei viroid 8 (CbVd-8). The yellow-highlighted region denotes the central conserved region (CCR) characteristic of the genus *Coleviroid*. Mutations identified among isolates from vegetatively propagated coleus plants are mapped onto the predicted secondary structure.

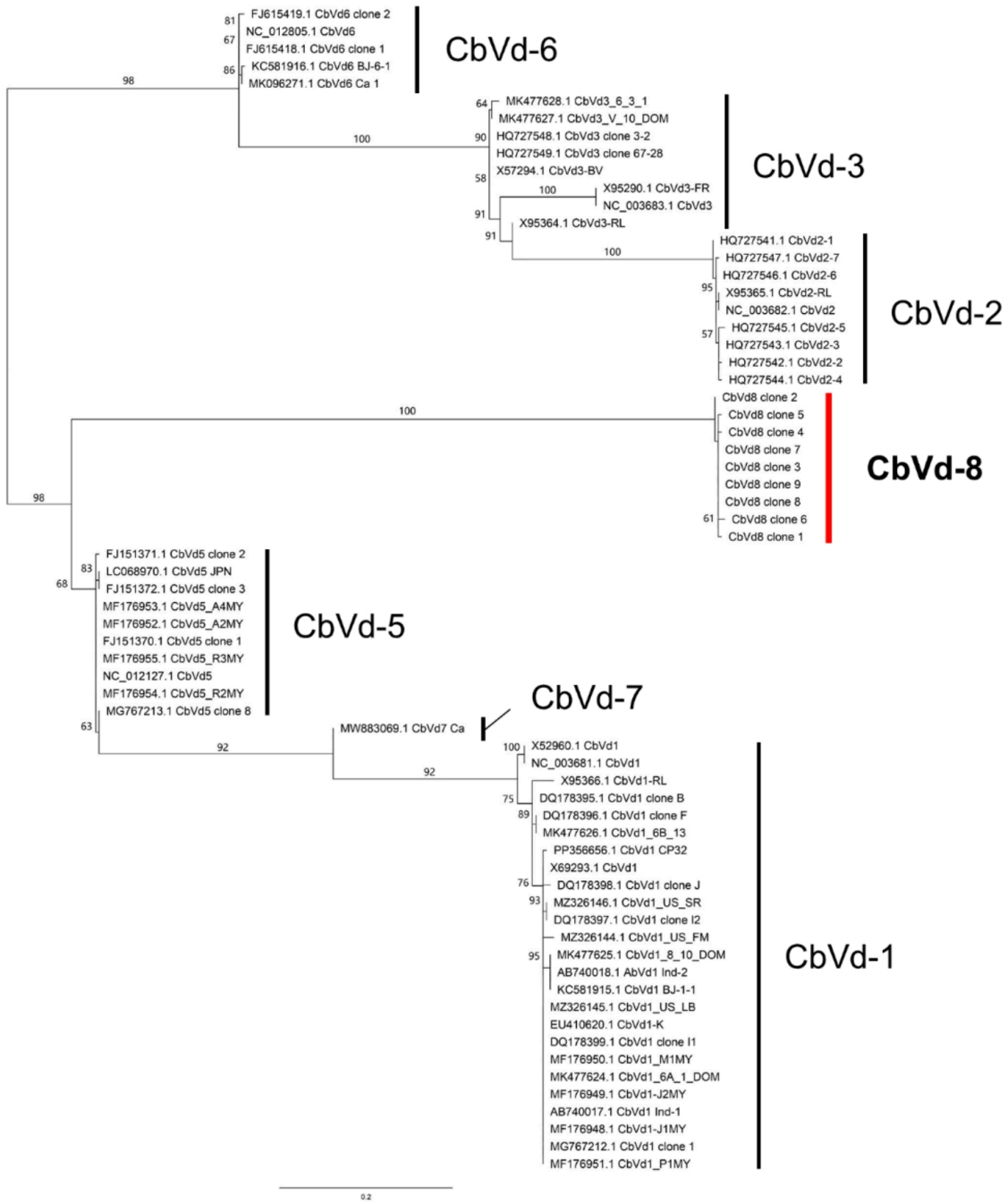


Figure 4

Molecular phylogenetic analysis of coleviroids. Full-length coleviroid sequences were retrieved from GenBank and aligned using Clustal Omega. A maximum-likelihood phylogenetic tree was constructed from the aligned sequences. The scale bar represents genetic distance. Numbers at the nodes indicate bootstrap support values, and only values ≥60% are shown. Each clone of CbVd-8 corresponds to the

following accession: clone 1, PZ737691; clone 2, PZ737692; clone 3, PZ737693; clone 4, PZ737694; clone 5, PZ737695; clone 6, PZ737696; clone 7, PZ737697; clones 8 and 9, the same sequence as clone 3.

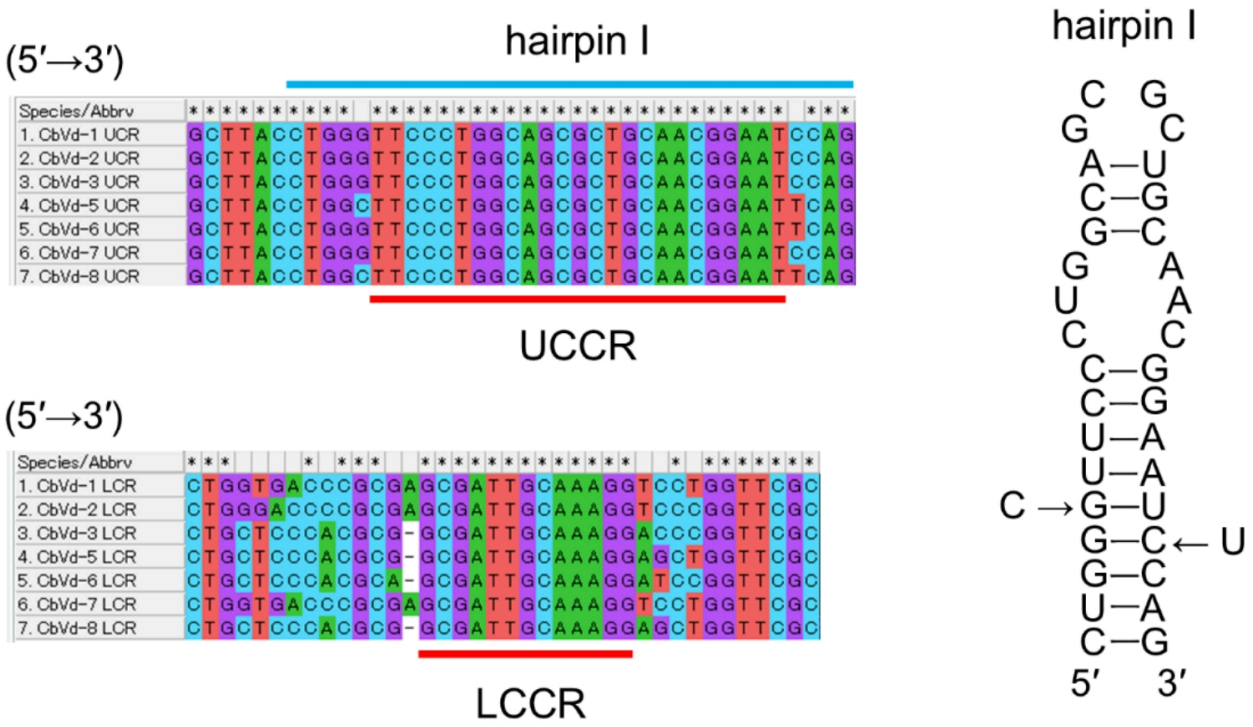


Figure 5

Alignment of the central region of coleviroids, including the central conserved region (CCR) and hairpin I. Abbreviations: UCCR, upper strand of the CCR; LCCR, lower strand of the CCR.

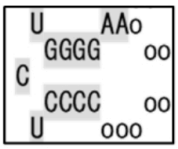
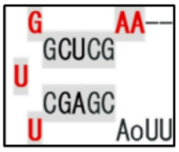
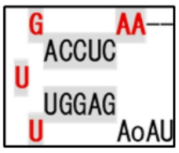
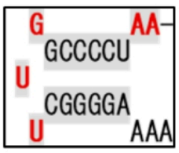
Type	Secondary Structure	Genus	Viroid name
I		*Hostuviroid* *Cocadviroid*	hop stunt viroid coconut cadang-cadang viroid coconut tinanangaja viroid hop latent viroid citrus bark cracking viroid
II-1		*Coleviroid*	Coleus blumei viroid 1
II-2		*Coleviroid*	Coleus blumei viroid 5 Coleus blumei viroid 7
II-3		*Coleviroid*	Coleus blumei viroid 8

Figure 6

Structural types of terminal conserved hairpins (TCHs) in the left-hand region of the family *Pospiviroidae*. Gray shading indicates the conserved TCH stem-loop structures, whereas red letters denote nucleotides conserved among type II TCHs. Open circles indicate variable nucleotide positions that differ among species and variants.

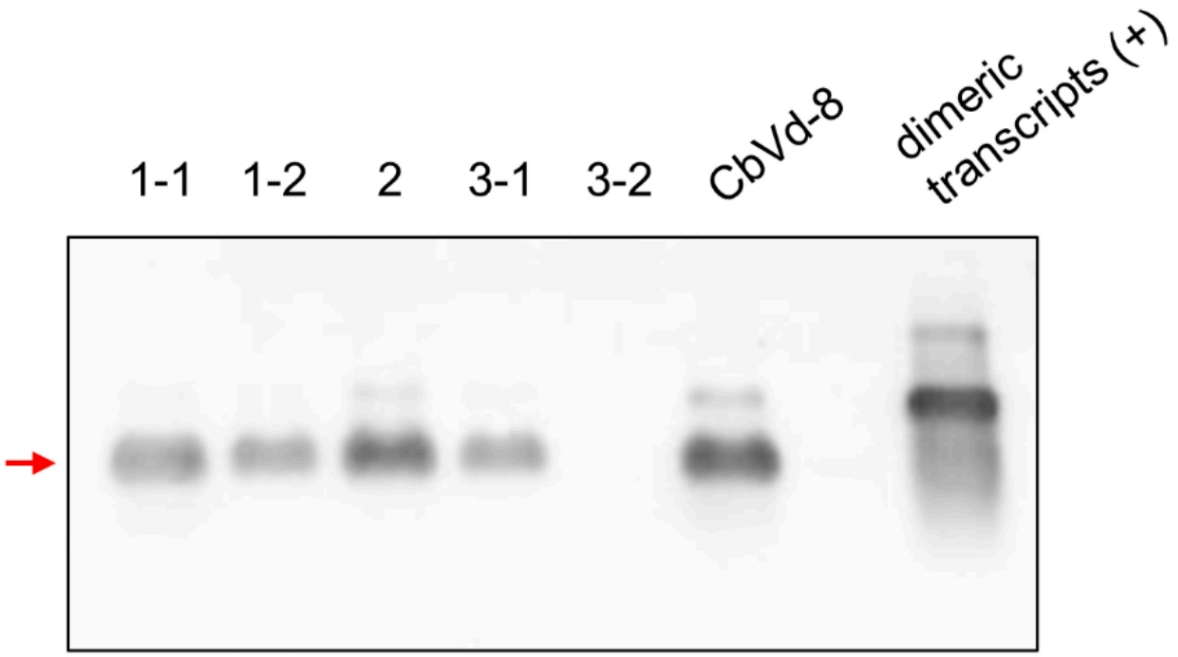


Figure 7

Detection of Coleus blumei viroid 8 (CbVd-8) in coleus seedlings inoculated with dimeric transcripts. Three months after inoculation with CbVd-8 transcripts, RNA was extracted from the upper leaves and analyzed by Northern hybridization. Monomeric and dimeric CbVd-8 RNA forms were detected in all examined coleus phenotypes, confirming infection in 2/2 plants of phenotype 1, 1/1 plant of phenotype 2, and 1/2 plants of phenotype 3. RNA extracted from vegetatively propagated coleus plants co-infected with CbVd-1 and CbVd-8, as well as in vitro-transcribed dimeric CbVd-8 RNA used for inoculation, were included as positive controls.

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