Eur J Plant Pathol
https://doi.org/10.1007/s10658-020-01958-2

Identification and characterization of cardamom vein clearing virus, a novel aphid-transmitted nucleorhabdovirus

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Accepted: 10 February 2020

Abstract The vein clearing (*kokke kandu*) disease suspected to be caused by an unknown virus is an important production constraint of cardamom in India. In the present study the causal virus was transmitted from infected to healthy cardamom plants through the aphid, *Pentalonia caladii*. Small RNA (sRNA) and RNA sequencing (RNA-seq) of the aphid-inoculated plant showed several nucleorhabdovirus-like contigs. The sRNA and RNA-seq results were verified through reverse transcription polymerase chain reaction (RT-PCR) using total RNA from infected plant and primers designed from the contigs. The cloning and sequencing of RT-PCR products resulted in a sequence of 13,392 bases that showed similarities to nucleorhabdoviruses. The sequenced region contained six open reading frames in the order 3'-N-P-P3-M-G-L-5′ and showed nucleotide sequence identities ranging from 37 to 55% with nucleorhabdoviruses indicating its distinct nature for which we propose the name, cardamom vein clearing virus. A reliable RT-PCR and SYBR Green-based real-time RT-PCR assays were developed for the detection of the virus that will aid in the identification and propagation of virus-free cardamom plants.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10658-020-01958-2) contains supplementary material, which is available to authorized users.

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Keywords Aphid transmission · Small RNA sequencing · RNA sequencing · RT-PCR · Real-time RT-PCR · Detection

Introduction

Cardamom (*Elettaria cardamomum*), the 'Queen of Spices' is grown in Guatemala, India, Sri Lanka, Papua New Guinea and Tanzania. The crop is affected by three viral diseases namely, mosaic, chlorotic streak and vein clearing. Mosaic disease caused by cardamom mosaic virus (Genus: *Macluravirus*: Family: *Potyviridae*) is characterized by interveinal discontinuous yellowish stripes parallel to veins (Jacob and Usha 2001). Chlorotic streak caused by banana bract mosaic virus (BBrMV) (Genus: *Potyvirus*; Family: *Potyviridae*) is characterized by intraveinal continuous or discontinuous spindle shaped yellow or light green streaks along the veins and midrib (Siljo et al. 2012). The vein clearing disease, the causal virus of which is not known so far has become a threat to cardamom cultivation in India since 1993 (Govindaraju et al. 1994). The first visible symptoms of the disease include, chlorosis of the veins followed by rosetting, loosening of leaf sheath and shredding of leaves. Newly emerging leaves get entangled in the older leaves and form hook-like tillers and hence the disease is locally known by the name *kokke kandu*. Leaf sheaths of the infected plants exhibit mottling symptoms. Light green patches with shallow grooves are also seen on the immature capsules. Cracking of fruits and partial sterility of seeds are other

Published online: 15 February 2020

associated deformities (Venugopal 2002). Unlike mosaic disease, the plants affected with vein clearing disease decline rapidly with yield reduction up to 62–84% in the first year of peak crop. The affected plants become stunted and perish within 1–2 years of infection.

Plant infecting rhabdoviruses are classified in four genera of which those in the genera *Dichorhavirus* and *Varicosavirus* have bipartite genome while those in the genera *Cytorhabdovirus* and *Nucleorhabdovirus* have monopartite genomes (Jackson et al. 2005; Dietzgen et al. 2017). Cytorhabdoviruses and nucleorhabdoviruses are differentiated based on their site of virus replication. Nucleorhabdoviruses have bacilliform enveloped virions, 45–100 nm in diameter and 130–300 nm in length. They consist of unsegmented negative sense ssRNA genome of 12 to 14 kb coding for at least six proteins namely, N (Nucleocapsid), P (Phospho), P3 (Movement), M (Matrix), G (Glyco) and L (RNA-dependent RNA polymerase) with in some cases additional accessory genes usually located between N-P, P-M and G-L genes (Walker et al. 2011; Dietzgen et al. 2017; Liu et al. 2018). Of these, G and M proteins constitute the major structural component of the virion envelope while N, P and L proteins interact with genomic RNA to form the ribonucleoprotein core that is required for virus replication. According to the latest update from the International Committee on Taxonomy of Viruses (Amarasinghe et al. 2019), 10 species are classified in the genus and some other proposed nucleorhabdoviruses are awaiting taxonomic classification. The present study based on small RNA (sRNA) sequencing and RNA sequencing (RNA-seq) showed that a novel virus likely belonging to the genus *Nucleorhabdovirus* as the cause of the vein clearing disease of cardamom. Further, a specific and sensitive reverse transcription (RT) PCR and SYBR Green-based real-time RT-PCR assays were developed for detection of the virus.

Materials and methods

Virus transmission

Cardamom plants naturally infected with vein clearing virus (CdVCV) and showing typical disease symptoms were collected from Sakleshpur, Hassan District, Karnataka, India. Aphid species, *Pentalonia caladii* maintained on healthy cardamom plants were used for transmission studies. The identity of the aphid species was confirmed through PCR and sequencing of cytochrome oxidase subunit 1 (COI) gene (Foottit et al. 2010). Aphids were allowed to feed on infected cardamom plants for 24 h to acquire the virus. The viruliferous aphids were then transferred to healthy seedlings of cardamom raised from seeds under insect-proof glass house conditions for one week and later killed by spraying with insecticide, dimethoate @0.1%. In another approach, about 300 seeds collected from symptomatic cardamom plants were sown and plants raised under insect-proof conditions to determine if the causal virus is seed-transmitted.

Small RNA and RNA sequencing

Two strategies namely, small RNA (sRNA) sequencing and RNA sequencing (RNA-seq) were adopted to identify viral sequences associated with the disease. For sRNA sequencing, total RNA was isolated from three pooled biological samples using Xcelgen Plant RNA Mini Kit from aphid-inoculated cardamom plants that showed typical symptoms of the disease. The sRNA library was prepared using Illumina TruSeq Small RNA Prep Kit from 1 μg total RNA. The cDNA was PCR- amplified and one library was sequenced using 1 × 50 bp chemistry by Illumina Hiseq 2500 platform at the Xcelris Labs Limited, Ahmedabad, India. Total reads obtained were filtered to remove adapter sequences using Trimmomatic-v3.6 followed by size selection (16–28 bases) using cutadapt v1.9. The size selected reads were used for the identification of viruses through Velvet 1.1.07 and CLC genomics workbench v6. The de novo assembled contigs obtained from the above were subjected to BLASTN and BLASTX program using non-redundant plant virus database. For RNA-seq, library preparations were constructed using total RNA depleted of rRNA using NEBNext UltraTM RNA library preparation kit from Illumina. The cDNA was amplified and one library was sequenced using 2 × 150 bp chemistry by Illumina Hiseq 2500 platform at the Nucleome Informatics Private Limited, Hyderabad, India. The de novo assembled contigs using BinPacker and rnaSPAdes were subjected to BLAST N and BLAST X searches against non-redundant plant virus database.

RT-PCR, cloning and Sanger sequencing

In order to confirm the results of sRNA and RNA-seq, primers were designed based on the sequences of the

specific contigs that mapped to nucleorhabdovirus reference genome (Supplementary Table 1) and subjected to RT-PCR using total RNA isolated from an infected cardamom plant as template. The RT-PCR reaction was carried out in 1x *Taq* assay buffer and contained 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 400 μM dNTP mix, 10 pM each of forward and reverse primers, 1 U of RNase inhibitor, 1.25 U of MuMLV reverse transcriptase, 0.75 U of *Taq* DNA polymerase and 1 μl total RNA as template in a final volume of 50 μl. Prior to the addition of RNA to the reaction mixture, the RNA was heated to 80 °C for 10 min and rapidly cooled on ice for 10 min. Single-step RT-PCR was carried out in an Eppendorf Master Cycler Gradient system. cDNA was synthesized at 42 °C for 45 min followed by 35 cycles at 94 °C for 30 s, 50–58 °C (depending on Tm of the primer pair) for 30 s and 72 °C for 1–2 min (depending on the expected product size) and a final extension at 72 °C for 10 min. Experimental controls were set up using total RNA from virus-free plant and nuclease free water instead of template RNA. The reaction products were subjected to agarose gel electrophoresis and DNA visualized using ethidium bromide under UV light. Amplicons were cloned into pTZ57R/T (Fermentas, USA) as per manufacturer's protocol and two clones each were sequenced from both directions by Sanger dideoxy chain termination method at the automated sequencing facility at Agri Genome, Kochi, India.

Sequence analyses

The sequences were assembled with Seqaid Version 3.6 (Peltola et al. 1984). ORFs were predicted by ORF finder [www.ncbi.nlm.nih.gov/projects/gorf] and translated into amino acids using Expasy tool. Sequences of complete genome of available nucleorhabdoviruses and selected other rhabdoviruses including cytorhabdoviruses, dichorhaviruses and varicosavirus were retrieved from NCBI database and used for analysis (Supplementary Table 2). The analyses were carried out using the complete genomes in the form of nucleotide and translated amino acid sequences. Sequences were aligned using clustal X (Thompson et al. 1997) and percent identity was calculated using Clustal omega (www.ebi.ac.uk/Tools/msa/clustalo). cNLS Mapper (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi) and Net Nes1.1 (http://www.cbs.dtu.dk/services/NetNES/) were used to predict nuclear localization and nuclear export signals (Cour et al. 2004). Phylogenetic analysis was performed using Neighbor-Joining method in Mega 6 with a bootstrap analysis of 1000 replicates.

RT-PCR and SYBR Green-based real-time RT-PCR assays for virus detection

To detect CdVCV in field-infected plants, total RNA isolated using RNeasy Plant Mini kit (Qiagen, Hilden, Germany) was subjected to RT-PCR and SYBR Green-based real-time RT-PCR using primers designed to the nucleocapsid (N) gene. RT-PCR was performed using the primers AIB 395 and AIB 396 (Supplementary Table 1) as described above. For real-time RT-PCR, forward (5' ACGTGTTCACAGAGGCAGTG 3′) and reverse (5' ATACAGGGTTGCGGACATTC 3′) primers for an expected amplicon length of 168 bp were synthesized. Real-time RT-PCR reaction was carried out in a final volume of 25 μl each containing: 12.5 μl of 2x QuantiFast™ SYBR Green PCR Master mix (Qiagen, Hilden, Germany), 1.0 μl (1 μM) of each forward and reverse primer and 1 μl template (about 120 ng) and 50 U of Revert Aid reverse transcriptase (Fermentas, Maryland, USA). Thermocycling conditions consisted of 42 °C for 45 min, 95 °C for 5 min, followed by 35 cycles of 95 °C for 20 s and 60 °C for 20 s. Total RNA from healthy cardamom and a water control were used as negative controls. Amplicons were subjected to melt analysis from 60 °C to 95 °C. The specificity of products of the real-time RT-PCR was also periodically verified by electrophoresis on a 1.5% agarose gel and by directly sequencing the gel-purified PCR product. To determine sensitivity of detection, total RNA was serially diluted from 10^0 to 10^{-5} and 1 μl of each of the dilutions were subjected to both RT-PCR and real-time RT-PCR assays. Both assays were validated using 1 μl of total RNA isolated from infected cardamom samples collected from different regions along with known positive and negative controls.

Results

Virus transmission

Sequence analysis of the COI gene of aphid showed 100% identity with *Pentalonia caladii* (GenBank accession number GU140254) confirming identity of the aphid species. Of the eighteen cardamom plants

inoculated, eight plants showed typical vein chlorosis symptoms within one month after inoculation (Fig. 1). None of the 120 plants raised from seeds that were collected from the virus infected plant showed any symptoms indicating that the virus is not transmitted through seeds.

Identification of CdVCV in RNA squencing data

Initially total RNA isolated from the aphid-inoculated symptomatic plant was subjected to sRNA sequencing which generated 68,641,448 clean 16–26 bp reads. Velvet assembly of these sequences generated 27,234 and 5187 contigs ranging from 33 to 478 (average length 52 nt) and 37 to 652 (average length 67 nt) in K-mer value 17 and 19, respectively. A BLASTX search revealed many contigs with maximum amino acid (aa) sequence identity up to 50% with black currant associated nucleorhabdovirus (BCaRV) (GenBank accession MF543022), datura yellow vein virus (DYVV) (KM823531), sonchus yellow net virus (SYNV) (L32603), apple root stock virus A (ARV-A) (MF 778545) and alfalfa-associated nucleorhabdovirus (AaNV) (MG848563). A total of 575 contigs mapped to these viruses covering partial nucleocapsid (N), phosphoprotein (P), putative movement protein (P3), glycoprotein (G) and polymerase (L) genes. CLC de novo assembly also gave similar results that generated 19,797 contigs ranging from 40 to 1359 nt (average length 64 nt). Of these, 550 contigs aligned with partial sequences of BCaRV, DYVV, SYNV, ARV-A and AaNV. By aligning all contigs we did not get overlapping sequences (especially in the large region of G and L genes) covering the entire genome. Hence total RNA from the same plant was subjected to RNA-seq, where 62,205,708 bp clean reads were obtained and de novo assembly generated 72,788 contigs (average length 1065 nt). BLASTN and X searches revealed that contigs ranging from 412 to 8453 nt had highest sequence identities with BCaRV, DYVV, SYNV, ARV-A and AaNV. When contigs obtained from both sRNA and RNA-seq were aligned, overlapping contigs were obtained covering almost the entire genome.

Confirmation of CdVCV genome sequence and analyses

To confirm the NGS results, total RNA was extracted from symptomatic and healthy leaves of cardamom and subjected to RT-PCR using specific primers designed based on NGS contig sequence (Supplementary Table 1). Cloning and sequencing of RT-PCR products by the Sanger method produced a sequence of 13,392 nucleotides (from the infected cardamom plant) which was deposited in the GenBank with accession number MN273311. The BLAST N and BLASTX search of the sequence showed identities with nucleorhabdoviruses. The sequenced region contained six open reading frames (ORFs) and partial 3′ leader (185 nt) and 5′ trailer (177 nt) sequences (Fig. 2). Our efforts to determine the 3′ and 5′ end sequences using rapid amplification of cDNA ends did not give any results. The genome organization of CdVCV resembles that of other nucleorhabdoviruses containing six ORFs in order of 3'-N (nucleocapsid, 469aa) (from 186 to 1595 nt), P (phosphoprotein, 347aa) (from1651 to 2694 nt), P3 (movement protein, 322aa) (from 2768 to 3736 nt), M (matrix protein, 302aa) (from 3921 to 4829 nt), G (glycoprotein, 640aa) (from 4913 to 6835 nt) and L (polymerase, 2095 aa) (from 6928 to 13,215 nt) (Fig. 2). The ORFs are separated by untranslated region, 55 bases (from 1596 to 1650 nt between N and P), 73 bases (from 2695 to 2767 nt between P and P3), 184 bases (from 3737 to 3920 nt between P3 and M), 83 bases (from 4830 to 4912 nt between M and G) and 92 bases (from 6836 to 6927 nt between G and L.

The coding-complete genome sequence of CdVCV is 37 to 55% identical to that of different nucleorhabdoviruses, with highest nucleotide sequence identity with BCaRV followed by DYVV and SYNV (Table 1). The nt and aa sequences of different ORFs of CdVCV shared maximum identities in L (49–55% and 39–47%), N (42–55% and 35–47%), G (42–55% and 27 to 47%), M (38–50% and 21–40%), P3 (39–50% and 19–32%) and P (37–48% and 20–26%) with BCaRV, DYVV, SYNV, ARV-A and AaNV compared to other nucleorhabdoviruses such as eggplant mottled dwarf virus (EMDV), maize Iranian mosaic virus (MIMV), maize mosaic virus (MMV), maize fine streak virus (MFSV), physostegia chlorotic mottle virus (PhCMoV), potato yellow dwarf virus (PYDV), rice yellow stunt virus (RYSV) and taro vein chlorosis virus (TaVCV) (Table 1). Sequence comparison clearly showed that CdVCV shared highest identity with BCaRV for all the six ORFs both in nt and aa levels (Table 1). Comparison of the complete genome sequences between known species of nucleorhabdoviruses showed

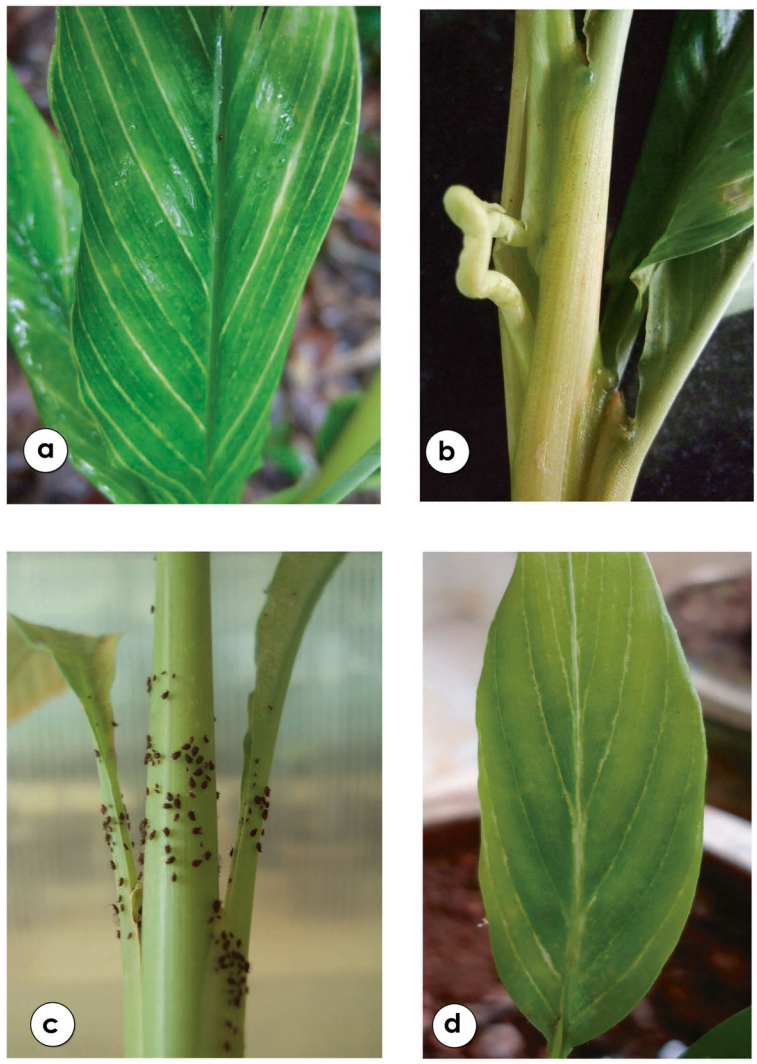

Fig. 1 Symptoms of cardamom vein clearing disease. (a) Infected leaf showing vein chlorosis symptoms (b) Emerging leaf entangled with old leaf giving hook like (locally known as *kokke kandu*) appearance (c) Rearing of aphid (*Pentalonia caladii*) in cardamom (d) Aphid-inoculated cardamom showing typical vein chlorosis symptoms four weeks after inoculation

a maximum identity of 52% while maximum identity in different ORFs was 56% and 68% at the nt and aa levels, respectively (Table 1). As CdVCV showed highest identity of 55% in the coding-complete genome and 47 to 55% in the different ORFs with known nucleorhabdoviruses, CdVCV should be considered a member of a new virus species in the genus, *Nucleorhabdovirus*.

As reported for other nucleorhabdoviruses all IGRs of CdVCV also contained conserved sequence motifs (3' AUUCUUUUUGGUUG 5′) that include nt corresponding to the 3′ terminus of the mRNA, non-transcribed intergenic fragment and a consensus region for the transcriptional start of the following gene (Table 2). All encoded proteins of CdVCV are predicted to contain mono- and bipartite importin- α dependent

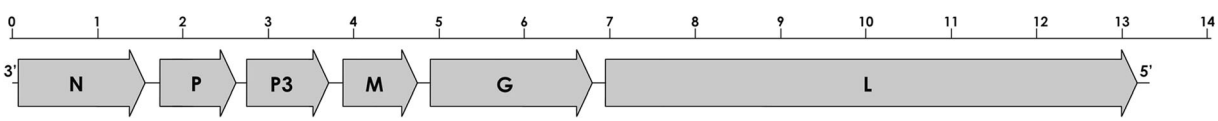

Fig. 2 Genome map of cardamom vein clearing virus showing different open reading frames (N, P, P3, M, G and L)

Table 1 Pairwise per cent identities in the nucleotide and amino acid sequences of different proteins of cardamom vein clearing virus (CdVCV) with other nucleorhabdoviruses

Nucleorhabdovirus isolates	Complete genome*	Nucleocapsid (N)		Phosphoprotein (P)		Movement protein (P3)		Matrix protein (M)		Glycoprotein (G)		Polymerase (L)	
	NT	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA	NT	AA
CdVCV-BCaRV	55	55	47	48	26	50	32	50	40	55	47	55	47
CdVCV-DYVV	52	48	41	43	26	43	31	47	33	48	39	52	47
CdVCV-SYNV	52	53	45	42	25	41	21	47	31	53	45	52	45
CdVCV-ARV-A	49	51	43	38	19	40	25	34	18	43	28	50	56
CdVCV- AaNV	47	42	35	37	20	39	19	38	21	42	27	49	39
CdVCV-others**	37–42	24–38	17–21	28–30	9–14	30–41	8–17	31–38	12–17	25–38	17–21	24–39	17–21
BCaRV-DYVV	52	56	50	44	27	46	27	46	34	49	40	53	49
BCaRV-SYNV	52	54	47	44	27	44	26	42	28	52	43	52	45
BCaRV-ARV-A	48	51	43	38	25	43	23	34	22	43	30	50	51
BCaRV-AaNV	46	48	35	38	15	37	15	40	17	41	27	49	38
DYVV-SYNV	49	55	49	41	26	47	27	42	26	49	41	50	44
DYVV-ARV-A	48	52	47	38	19	38	21	33	21	41	29	50	68
DYVV-AaNV	46	48	38	41	14	39	23	40	16	41	25	48	38
SYNV-ARV-A	47	48	41	38	20	38	18	32	22	42	29	49	50
SYNV-AaNV	46	47	36	40	14	40	19	41	17	40	25	48	38
ARV-A-AaNV	47	49	42	37	18	40	20	47	18	40	23	50	35

*13,392 nt regions of CdVCV was compared with corresponding region of other viruses

**Include EMDV, MFSV, MIMV, MMV, PYDV, RYSV, TaVCV, PhCMoV

NT, nucleotide; AA, amino acid

Designation of isolates is provided in Supplementary Table 2

nuclear localization signals (NLS) (Table 3). Highest scores of 7.5–8.0 predicted nuclear localization for CdVCV N, M and L proteins while scores of 4.3–5.9 for the P, P3 and G proteins predicted localization to both the nucleus and cytoplasm. The predicted NLS of CdVCV N was located close to the C-terminus of the protein as found for BCaRV, DYVV and SYNV. Predicted leucine rich nuclear export signal was seen in all proteins of CdVCV except the matrix protein (Table 3).

Phylogenetic analysis of the coding-complete genome, deduced aa sequence of L and N proteins of CdVCV and other rhabdoviruses revealed closest relatedness of CdVCV with BCaRV, DYVV, SYNV, ARV-A

Table 2 Conserved sequences in the cardamom vein clearing virus (CdVCV) intergenic regions

Gene junction	Polyadenylation	Intergenic spacer	Transcription start
3′ Leader-ORF 1	UUUCUUUU	GUG	UUG
N-P	AUUCUUUUU	GG	UUG
P-P3	AUUCUUUUU	GG	UUG
P3-M	AUUCUUUUU	GG	UUU
M-G	AUUCUUUUU	GG	UUG
G-L	AUCUUUUU	GG	UUA
Consensus	(A/U)UU(C/U)UUUUU	G(G/U)G	UU(G/A)

Table 3 Analysis of cardamom vein clearing virus (CdVCV) sequence for nuclear localization signals (NLS) and nuclear export signals

ORF/gene (aa)	Protein	Predicted NLS (cNLS Mapper score)	Predicted nuclear export signal site
1/N (469 aa)	Nucleocapsid	^{432}PASQKRKAPET (8)	I^{355}
2/P (347 aa)	Phosphoprotein	Bipartitie (4.3)	$L^{201, 204, 210}$, Q^{202}, K^{203}, D^{205}, I^{206}
3/P3 (350 aa)	Movement protein	Bipartite (5.9)	L^{51}, K^{52}
4/M (302 aa)	Matrix protein	^{256}PQLKRKIRGI (7.5)	nd
5/G (640 aa)	Glycoprotein	Bipartite (4.6)	I^{214}
6/L (2095 aa)	Polymerase	^{920}RPHKKTKTMTNF (8)	$L^{170, 1463, 1468, 1471, 1475}$ I^{1448}

nd, not detected

cNLS (cell nuclear localization signal) predicted using cNLS mapper

Nuclear export signals predicted by NetNES1.1

and AaNV that formed a cluster clearly separated from other nucleorhabdoviruses (Fig. 3). Cytorhabdoviruses, dichorhaviruses and varicosaviruses formed separate clusters well separated from each other and from nucleorhabdoviruses. Phylogenetic tree for all other five proteins also showed similar topology grouping CdVCV,

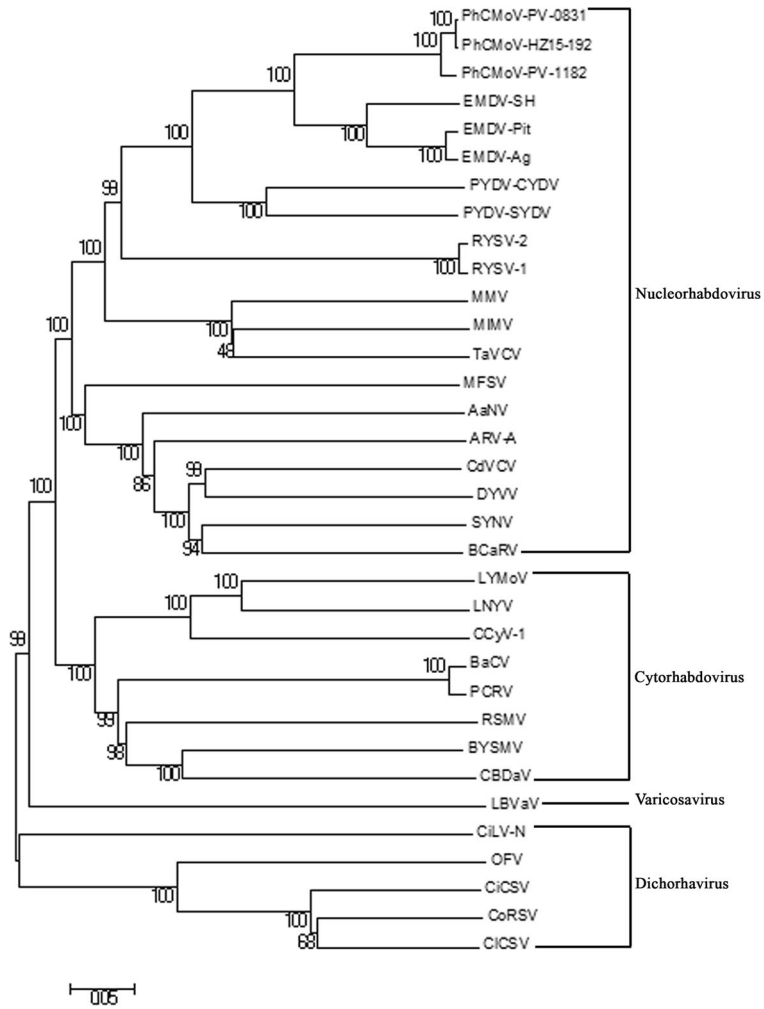

Fig. 3 Phylogenetic tree drawn using neighbor-joining method in Mega 6 with a bootstrap analysis of 1000 replicates based on the complete sequenced region of CdVCV with corresponding region of nucleorhabdoviruses, cytorhabdoviruses, dichorhaviruses and varicosaviruss. The designation given to each of the isolates and their GenBank accession numbers is provided in Supplementary Table 2

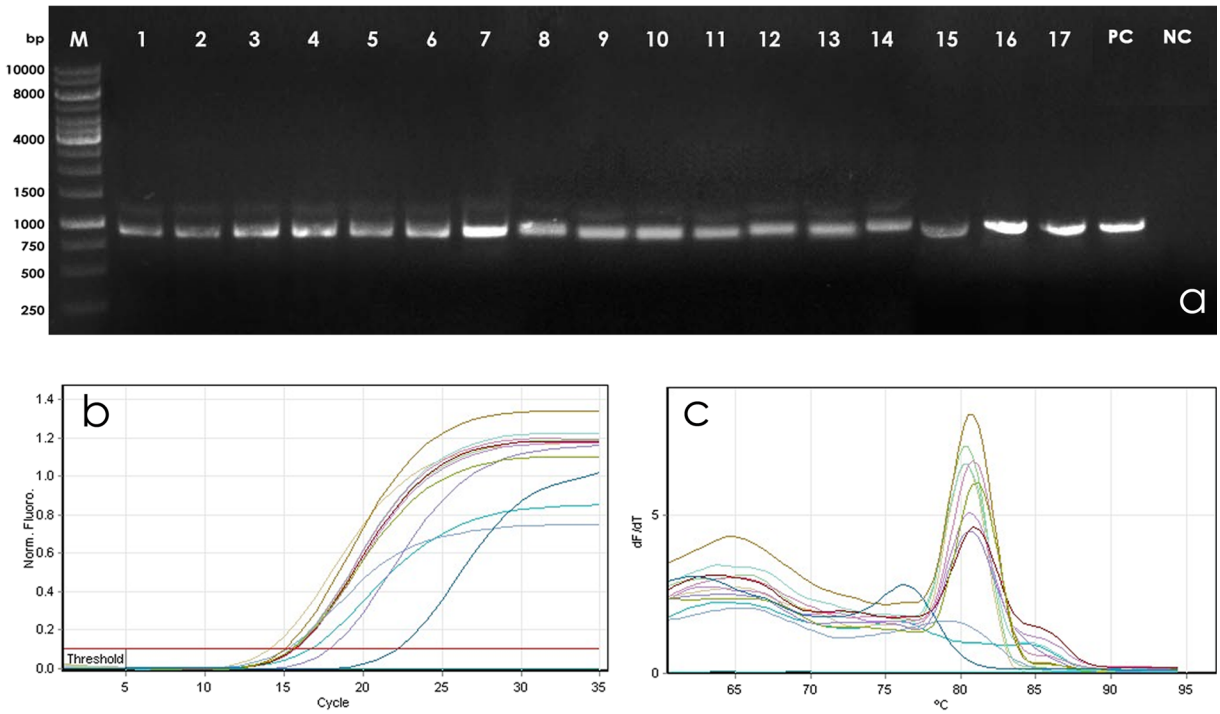

Fig. 4 Validation of RT-PCR (a) and SYBR Green-based real-time RT-PCR (b, c) for the detection of cardamom vein clearing virus in field samples of cardamom collected from different regions. (a) Lane M, molecular size markers; Lane 1–17, test samples of cardamom; Lane PC and NC, known positive and negative controls. (b) Show amplification curves and Ct value obtained for different samples. No amplification curve was seen in negative control. (c) Melt curve analysis of the same samples

BCaRV, DYVV SYNV, ARV-A and AaNV in a single cluster (not shown).

Development of diagnostic assays for CdVCV

Both RT-PCR and SYBR Green-based real-time RT-PCR enabled detection of CdVCV in infected but not in uninfected cardamom. The identity of the amplicon was confirmed by sequencing (not shown). Further, specificity of both diagnostic assays was confirmed by testing cardamom plants co-infected by CdVCV and cardamom mosaic virus (CdMV). Sensitivity of detection of CdVCV was undiluted RNA extract (10^0 dilution) for gel-based RT-PCR and 10^{-4} for real-time RT-PCR. Real-time RT-PCR was ten thousand times more sensitive than RT-PCR for the detection of CdVCV. Validation of both RT-PCR and real-time RT-PCR assays using total RNA from different cardamom cultivars and regions showed detection of CdVCV in all symptomatic samples tested except in the negative control (Fig. 4). As cardamom is propagated vegetatively through suckers, the detection assays developed in the present studies can be used to identify virus-free mother plants for propagation and also to screen germplasm accessions for any resistance source against the virus.

Discussion

Nucleorhabdoviruses are reported to infect both dicots and monocots. In the present study, we identified and characterized the complete coding region of a novel nucleorhabdovirus causing vein clearing (*kokke kandu*) disease, an important production constraint of cardamom in India (Govindaraju et al. 1994; Venugopal 2002). The causal virus was not transmitted through seeds. Following aphid inoculation, typical vein clearing symptoms appeared four weeks later and the identity of the causal virus was established by sRNA sequencing and RNA-seq followed by verification by amplicon sequencing. The sequenced region included the complete coding region of the virus that contained six ORFs, each ORFs being separated by IGR that has a conserved sequence (Fig. 2). The nucleotide sequence identities of 37 to 55% between CdVCV and other nucleorhabdoviruses fall into the range of 41 to 54% among known species of

rhabdoviruses (Heaton et al. 1989; Bejerman et al. 2015; Dietzgen et al. 2015, 2017; Menzel et al. 2018; Wu et al. 2018). Therefore, CdVCV should be considered a member of a new species in the genus, *Nucleorhabdovirus.* The sequence analysis provides evidence of the close relationship of the five nucleorhabdoviruses, (BCaRV, DYVV, SYVV, ARV-A and AaNV with CdVCV (Table 1; Fig. 3). The CdVCV genome has highly conserved regulatory regions separating the viral genes as observed in BCaRV, DYVV, SYNV and other nucleorhabdoviruses (Goodin et al. 2001; Ganesan et al., 2013; Dietzgen et al., 2015; Wu et al. 2018). Among all, CdVCV ORF sequences are most similar to their respective counterparts in BCaRV (Table 1), although sequence identity is only 47% between the N, G and L proteins of these viruses. The level of sequence diversity between known species of nucleorhabdoviruses ranges between 46 and 59% (Jackson et al. 2005; Walker et al. 2011; Dietzgen et al. 2015). Phylogenetic studies using coding-complete genome and different ORFs are in agreement with percent nt/aa identity that showed distinct nature of CdVCV and its close clustering with a group of nucleorhabdoviruses including BCaRV, DYVV, SYVV, ARV-A and AaNV (Fig. 3). Strong predicted NLS in the N, L and M proteins of CdVCV indicate that this virus replicates in the nuclei of infected cell as reported for other nucleorhabdoviruses. N protein of several nucleorhabdoviruses including BCaRV, DYVV and SYNV localizes exclusively in the nucleus and harbour NLS at the C-terminus (Dietzgen et al. 2015; Wu et al. 2018). As reported for DYVV, SYNV and BCaRV, CdVCV P protein is also predicted to localize both in the nucleus and cytoplasm (Goodin et al. 2001; Dietzgen et al. 2015; Wu et al. 2018).

This is the first report of a nucleorhabdovirus infecting cardamom. BCaRV appears to be the most closely related nucleorhabdovirus sequenced to date (Wu et al. 2018). Cardamom is a monocot belonging to the family *Zingiberaceae* while black currant (*Ribes nigrum*) is a woody shrub in the family Grossulariaceae and native to temperate parts of central and northern Europe and northern Asia. The CdVCV vector is the aphid, *Pentalonia caladii* while the vector of BCaRV is unknown. Hogenhout et al. (2003) predicted that an insect was the primary host of the rhabdovirus ancestor. The similarity of the deduced amino acid sequences of the CdVCV and other known aphid-transmitted nucleorhabdoviruses such as SYNV suggests that an ancestral aphid-transmitted virus may have been their progenitor (Bandyopadhyay et al. 2010). The novel cardamom nucleorhabdovirus, CdVCV in this report and macluravirus, cardamom mosaic virus (CdMV) are both transmitted by the aphid, *P. caladii* (Jacob and Usha 2001). Among the cardamom plants that we collected in the CdVCV-infected field, a few were co-infected by both viruses (data not shown). A similar kind of mixed infection of a mastrivirus (wheat dwarf virus) and a nucleorhabdovirus (wheat yellow striate virus) transmitted by the leaf hopper, *Psammotettix aliens* was reported in wheat by Liu et al. (2018). In the absence of a resistant variety, use of virus-free planting material is an efficient method of controlling the virus. CdVCV could be detected through gel-based RT-PCR and real-time RT-PCR assays in all symptomatic plants tested. Beyond the healthy control, the assays were specific and did not cross-react with CdMV infecting cardamom. However, nothing is known about natural variability of CdVCV. In order to have a fool-proof detection, large number of virus isolates need to be sequenced and oligonucleotides derived to the conserved region should be used in the detection.

Acknowledgements Authors are thankful to Science and Engineering Research Board (SERB), Government of India for the funding (EMR/2016/001135), Head (Division of Crop Protection), Distributed Information Sub Centre and Director, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India for facilities.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human participants and /or animals The present research did not involve any experimentation on humans or animals.

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