

**Molecular characterization of a virus from the family
Luteoviridae associated with cotton blue disease***

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Summary. Cotton blue disease is an aphid-transmitted cotton disease described in Brazil in 1962 as Vein Mosaic “var. Ribeirão Bonito”. At present it causes economically important losses in cotton crops if control measures are not implemented. The observed symptoms and mode of transmission have prompted researchers to speculate that cotton blue disease could be attributed to a member of the family *Luteoviridae*, but there was no molecular evidence supporting this hypothesis. We have amplified part of the genome of a virus associated with this disease using degenerate primers for members of the family *Luteoviridae*. Sequence analysis of the entire capsid and a partial RdRp revealed a virus probably belonging to the genus *Polerovirus*. Based on our results we propose that cotton blue disease is associated with a virus with the putative name Cotton leafroll dwarf virus (CLRDV).

Introduction

Cotton (*Gossypium spp.*) is one of the earliest cultivated and most economically important crops of the world. Cotton blue disease was first described in the Central African Republic in 1949 and since then was also reported in several regions of

*Nucleotide sequence data reported are available in the GenBank databases under the accession numbers AY758560 and AY758561.

Africa, Asia and the Americas [2]. The disease is transmitted by *Aphis gossypii* in the persistent circulative manner and symptoms are characterized by stunting due to shortening of internodes, leaf rolling, an intensive green color of the foliage and vein yellowing, suggesting a virus as the causal agent [3, 21]. Symptoms are more pronounced when plants are infected at an early stage of growth and sap inoculation is not able to transmit the disease [3]. In Brazil, a cotton disease named vein mosaic (“mosaico das nervuras”) was described in 1938 [7]. A more severe occurrence of supposedly the same disease was observed in 1962 [6] and was named vein mosaic “var. Ribeirão Bonito”. Symptomatology and aphid transmission strongly suggested that vein mosaic and cotton blue disease have the same etiology.

In Brazil, several cotton varieties are cultivated, including genotypes bred at Brazilian public and private institutions and introduced from the United States of America and Australia by cotton seed companies. Some of these varieties have been shown to be highly susceptible to cotton blue disease, with losses reaching up to 1500 kg · ha⁻¹ of seed cotton in some states [9]. The observed symptoms and aphid transmission have prompted researchers to speculate that cotton blue disease could be attributed to a member of the family *Luteoviridae*. Serological tests of infected plants performed to identify its causal agent revealed a tentative relationship with *Barley yellow dwarf virus serotypes RPV* and *PAV* [12] and *Beet western yellows virus* [21], three well characterized members of the family *Luteoviridae*. However, until now no molecular evidence supporting this hypothesis existed. Luteoviruses are grouped in three genera, based on genomic organization, replication strategies and expression mechanisms: *Luteovirus*, *Polerovirus* and *Enamovirus*. All genera share a conserved 3' portion of the genome, including the coat protein (CP) and CP-readthrough open reading frames. Consistent differences are observed in the 5' portion of the genome. Both the *Polerovirus* and *Enamovirus* genera have polymerase genes resembling those from sobemoviruses. On the other hand, the genus *Luteovirus* has similarities in this same region with carmoviruses [14]. Some viruses like *Bean leafroll virus* and *Soybean dwarf virus* have been shown to be recombinants between viruses from the genera *Polerovirus* and *Luteovirus*, since they present polerovirus-like capsids but luteovirus-like polymerase genes [8, 18]. Recently it was shown that *Sugarcane yellow leaf virus* is also a recombinant virus, but with a luteovirus-like capsid and a polerovirus-like polymerase sequence [13, 15].

Here we present the molecular characterization of a virus associated with cotton blue disease, confirming that a virus from the family *Luteoviridae* is present in this pathology. Using an RT-PCR-based assay, we were able to amplify and sequence 1405 nt from the virus genome, corresponding to part of the RNA dependent RNA-polymerase (RdRP) ORF, the full intergenic region and the coat protein (CP) ORF. Analysis of deduced amino acid sequences clearly positioned this virus as a member of the genus *Polerovirus*, family *Luteoviridae*. Both the capsid and RdRp sequences were related to those of species from the genus *Polerovirus*, ruling out the possibility of a recombination between a luteovirus and polerovirus ancestor.

Materials and methods

Virus propagation

Cotton plants from cv. CNPA ITA 90 showing severe blue disease symptoms were collected from a field plantation at the state of Mato Grosso, west-central region of Brazil, and used to feed non-viruliferous aphids under greenhouse conditions. Viruliferous aphids were then transferred to healthy young cotton plants cv. CNPA ITA 90 in the greenhouse. For infection assays, non-viruliferous *Aphis gossypii* were transferred to infected plants and maintained for two days for virus acquisition. Ten viruliferous aphids were then transferred to each healthy plant and maintained for two days for the inoculation period.

cDNA cloning and sequencing

Total RNA from infected and healthy cotton plants were obtained by phenol extraction followed by lithium precipitation, as described in [17]. 2.5 µg of total RNA were used for cDNA synthesis using degenerated primers PLF (5' ACDGAYTGYTCYGGTTTTYGACTGG 3') and PLR (5' TCTGAWARASWCGGCCCGAASGTGA 3'), resulting in the amplification of a 1058 nt fragment. Internal primers PL2F (5' AACAAATTAGTTTTAAAGTCGAGG 3') and PL2R (5' TTCTACCCACGACCGTATTCAT 3') were used for complete fragment sequencing. The complete CP fragment was obtained with primers PL4F (5' TGCGACAAATAGT TAATGAATACGGT 3') and o3R (5' GTCTACCTATTTBGGRTTNTGGAA 3'). Annealing positions for each primer are schematically represented in Fig. 2. Reverse transcription was carried out with Superscript II (Gibco BRL) for 2 hours at 45 °C. PCR reactions were carried out with a denaturation step at 95 °C for five minutes followed by forty cycles at 95 °C for 1 min, 50 °C (for primers PLF, PLR, PL2F, PL2R) or 45 °C (for primers PL4F, o3R) for 1 min, and 72 °C for 1 min (PL2F, PL2R, PL4F, o3R) or 2 min (PLF, PLR), with a final extension step at 72 °C for 10 min. All amplified fragments were cloned into pGem-T Easy (Promega) and three to six clones were sequenced with T7 and M13 universal primers using a MegaBACE automated sequencer.

Sequence analysis

Sequences from species of the family *Luteoviridae* were obtained from the GenBank database under the following accession numbers: *Chickpea stunt disease associated virus* (CpSDaV), Y11530; *Turnip yellows virus* (TYV), NC_003743; *Beet mild yellowing virus* (BMV), NC_003491; *Beet chlorosis virus* (BChV), NC_002766; *Beet western yellows virus* (BWYV), NC_004756; *Groundnut rosette assistor virus* (GRAV), AF195828; *Cucurbit aphid-borne yellows virus* (CAbYV), NC_003688; *Potato leafroll virus* (PLRV), NC_001747; *Cereal yellow dwarf virus-RPV* (CYDV-RPV), NC_004751; *Cereal yellow dwarf virus-RPS* (CYDV-RPS), NC_002198; *Soybean dwarf virus* (SbDV), NC_003056; *Tobacco vein distorting virus* (TVDV), AF402621; *Bean leafroll virus* (BLRV), NC_003369; *Barley yellow dwarf virus-PAV* (BYDV-PAV), NC_004750; *Barley yellow dwarf virus-PAS* (BYDV-PAS), NC_002160; *Barley yellow dwarf virus-GAV* (BYDV-GAV), NC_004666; *Barley yellow dwarf virus-MAV* (BYDV-MAV), NC_003680; *Sugarcane yellow leaf virus* (ScYLV), NC_000874; *Pea enation mosaic virus-1* (PEMV), NC_003629.

Amino acid sequences were analyzed with the Multalign interface [5] and phylogenetic analysis were performed using the MEGA 2.1 software [11]. The pairwise deletion option was adopted, excluding amino acid gaps from the sequence alignment. Trees were obtained with the Neighbor-joining method [20] derived from a p-distance matrix.

Northern blot

Total RNA was obtained as described in [17]. Samples (15 µg) were fractionated on a 1.2% agarose gel containing 1× MOPS and 3% formaldehyde, blotted onto a nylon membrane (Hybond-N+, Amersham) and hybridized according to [4] with the CP homologous fragment radioactively labeled with α -[32 P]-dCTP. 32 P-labeled probes were prepared by random priming and hybridization signals were visualized by autoradiography.

Results*Reproduction of cotton blue disease symptoms after aphid transmission*

Plants with typical symptoms associated of cotton blue disease were collected from the central region of Brazil and transferred to a greenhouse. The tentatively named Cotton leafroll dwarf virus (CLR DV) was transmitted to healthy young cotton plants using *Aphis gossypii* and field symptoms were successfully reproduced for five successive passages (Fig. 1). At early stages of virus infection, approximately fifteen days after inoculation, symptoms of leaf rolling were clearly observed (Fig. 1B). Newly developed leaves emerged with an obvious rolled up phenotype as well as an intense vein yellowing (Fig. 1D). Later phases of virus infection were characterized by an intensive green foliage and severe plant stunting due to internode reduction (Fig. 1E).

Partial genome amplification associates a member of the genus Polerovirus with cotton blue disease

In order to determine if the causal agent of cotton blue disease was a virus from the family *Luteoviridae*, we designed pairs of degenerate primers specific for the genera *Luteovirus* and *Polerovirus*. RT-PCR was performed from healthy and infected plants approximately thirty days post-inoculation (dpi). Agarose gel electrophoresis showed the amplification of a 1058 nt long cDNA using the *Polerovirus*-specific primers PLF and PLR. No DNA fragments were amplified using *Luteovirus*-specific primers (data not shown). The amplified fragment is predicted to comprise part of the RdRp and CP sequences. Sequence analysis from different clones confirmed similarities between the amplified sequence and ORF2, ORF3 and the corresponding intergenic region from members of the genus *Polerovirus*.

Capsid protein sequence

A consensus nucleotide sequence was constructed using three independent clones for the amplified cDNA. Sequence analysis was performed and new primers (PL4F and o3R) were designed to amplify the entire capsid protein sequence (Fig. 2). Electrophoresis analysis of RT-PCR products showed the amplification of the predicted 606 nt long cDNA only from infected plants.

The deduced amino acid sequence of the CLR DV CP gene, constructed using six independent clones, predicted a protein with 201 amino acid residues and an expected molecular mass of 22.3 kDa. Sequence analysis indicated 92% identity with the partial CP sequence from *Chickpea stunt disease associated virus* (CpSDaV), a non-classified member of the family *Luteoviridae* (Table 1).

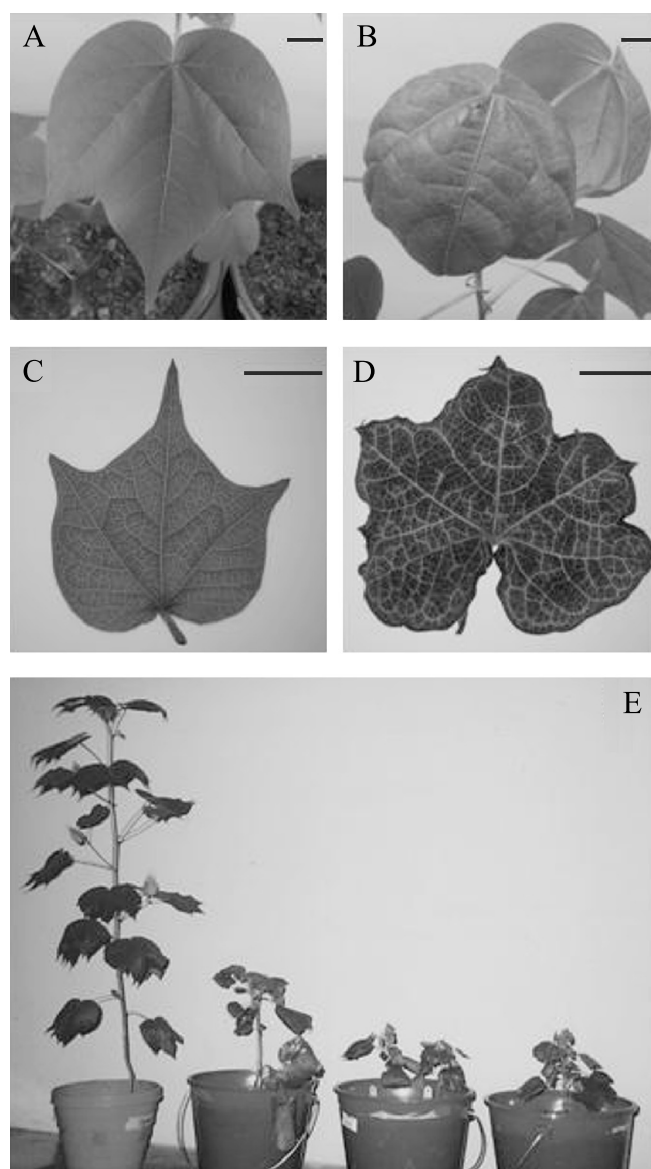


Fig. 1. Symptoms of cotton blue disease. **A** Expanded leaf from a healthy control plant. **B** Leaf roll symptoms fifteen days after inoculation. **C** Newly developed leaf from a non-inoculated plant. **D** Vein yellowing symptoms observed on newly developed leaves from inoculated plants. **E** Stunt symptoms in three inoculated plants (right) compared to a non-inoculated plant (left) of the same age. Bars represent 1 cm

Significant identities were also found with members of the genus *Polerovirus*, such as TYV (79%), BMYV (78%), BChV (78%) and BWYV (77%). Sequence alignments with members of the family *Luteoviridae* showed that CpSDaV and CLRDV had only nine amino acid substitutions (Fig. 3). The five conserved acidic residues E₁₀₉, E₁₇₀, D₁₇₃, E₁₇₆ and D₁₇₇, predicted to be on the surface of the PLRV [22] and BWYV [1] particles at the trimer axis, are indicated (Fig. 3).

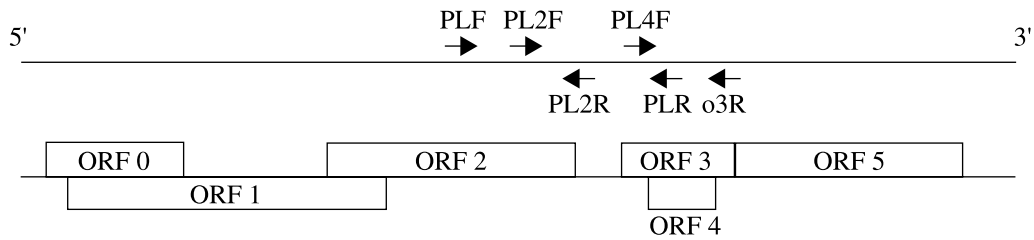


Fig. 2. Schematic representation of the polerovirus genome structure. Positions of primers PLF, PLR, PL2F, PL2R, PL4F and o3R used to amplify a 1405 nt fragment from CLR DV are indicated by arrows. *ORF0* – Silencing suppressor protein; *ORF1 + ORF2* – RNA dependent RNA polymerase; *ORF3* – Capsid protein; *ORF4* – Movement protein; *ORF3 + ORF5* – Readthrough domain of the capsid protein

Table 1. Percentage identity between the amino acid sequences of coat protein (*CP*), movement protein (*MP*) and polymerase (*RdRp*) of CLR DV and the corresponding regions of other luteoviruses

Virus	CP	MP	RdRp
CpSDaV	92%	89%	nd
TYV	79%	63%	78%
BMYV	78%	63%	76%
BChV	78%	62%	72%
BWYV	77%	60%	75%
GRAV	67%	41%	nd
CabYV	65%	45%	74%
CYDV-RPV	62%	35%	71%
CYDV-RPS	60%	34%	73%
PLRV	59%	36%	73%
SbDV	56%	38%	12%
TVDV	56%	34%	nd
BLRV	53%	30%	11%
BYDV-PAV	44%	23%	13%
BYDV-PAS	44%	22%	12%
BYDV-GAV	42%	22%	14%
BYDV-MAV	42%	21%	13%
ScYLV	43%	26%	66%
PeMV	28%	nd	49%

nd – not determined

Fig. 3. Alignment of the CP amino acid sequences from CLR DV and other virus from family *Luteoviridae* (see Material and methods for virus names). Black boxes represent residues that are present in all viruses in the alignment. Columns in the alignment with less than 100% conservation but more than 60% are shaded in gray. Shading was performed by GeneDoc program (<http://www.psc.edu/biomed/genedoc/>). Residues in the trimer axis E₁₀₉, E₁₇₀, D₁₇₃, E₁₇₆ and D₁₇₇ are indicated

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CLR DV      1 : MNTVVGRTING----RRRPRRRNRRRQ-NQPVVVVQAPRNTORRRRRRRRGG-RNR TGGRIPGGPGASS E
CpSDaV     1 : ----GRTING----RRRPRRRNRRRQ-NQPVVVVQTPRNTORRRRRRRRGG-RNR TGGRIPSGPGASS E
TYV        1 : MNTVVGRIING----RRRPRRQT RRAQRQPQVVVVQTSRATORRRPRRRRRG-NNR TGRTVPTRGAGSS E
BWYV       1 : MNTVVGRTING----RRRPRRQAARRAQRSQPVVVVQTSRTTORRRPRRRRRG-NNR TRGAVSTRGASS E
BMYV       1 : MNTVVGRTING----RRRPRRQT RRAQRSQPVVVVQASRTTORRRPRRRRRG-NNR TRRTVSTRGTGSS E
BChV       1 : MNTVVGRTING----RRRPRRQT RRAQRNQPVVVVQTSRRTORRRPRRRRRG-NNR AGRTVSTRGTGQS E
PLRV       1 : MSTVVVGNVNGGVQPPMRRRQSLRRRANRVQPVVVVTPAGQP RRRRRRRRGG-NRR SRRTGVPRGRGSS E
CYDV-RPS   1 : MSTVVLRSNGNG---SRRRRQRVARRRPAVRTQPVVVVTPNGPARRRGRRRGP A-RPRRRRTPGLGGGGRGE
CYDV-RPV   1 : MSTVVLRSNGNG---SRRRRQRVARRRPAARTQPVVVVASNGPARRRGRRR RPV-GPRRGRTPRSGGGRGE
CAbYV      1 : MNTVVAARNQONAG----RR--RRRNQR RARRDVVVVVNP IGGPP E G-RPQRN-RRR PNRGGRARRGSG E
GRAV       1 : MNTVVVRRPFGN----RAN--RRRNERTPRRNPVVVVQTPRQENS G-RRRRRN-RRR SNRGSRRNRGSG-GE
ScYLV      1 : MNTGANRSRRNV----R----RRANRRRQTRPVVVVRAP-PGPRFVRRRRR AR-VGG-NAVRGPGGRSNRD
TVDV       1 : MNTGGARRNNNG---NGGSRSTRRRRRPRQVRPVVVVAPSGTARR GGRRRRS GGRNR RSRDGVGGRS SNS E
BLRV       1 : MVARGKRVVV-----RQLQTRARRRLP----VVIATA PVRPQR RRRCGRN-NKSRGGNGFARRS SQVH
SbdV       1 : MVAVSNVAIQ-----RRRTRAAARRAPRVLMAPTVT SRPQRRCGR RRR-RNNRGGSEFISGSGSKAH
BYDV-GAV   1 : MNTVGRNNRRR-----N--GPRRARRVSAVRMVVVQPNRAGPKRRTRRRTR-GGGANLISGPAGRT--E
BYDV-MAV   1 : MNTVGRNNRRR-----N--GPRRARRVSAVRMVVVQPNRAGPKRRTRRRTR-GGGANLISGPAGRT--E
BYDV-PAV   1 : MNTVGRNGPERRA-----NQNGTRRRRTR-TVRPVVVVQPNRAGPRRRNRKRK-GGGANFVTRPTGCT--E
BYDV-PAS   1 : MNTVGRNGPERRA-----NQNGPRRRSTR-TIRPVVVVQPNRAGPRRRNRKRSG-RRGPNISPGSTGRT--E
PEMV       1 : MPTRRS-----SKANQRRRRPRRVVVVAP SMAQPTQSRRRPR-----RNKRGGGLNGSHTV

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CLR DV      65 : TEVFSKDNLSGSSSGSITFGP SLSDCPAF SNGMLKAYHEYKI SMVILBEFISEASSTSSGSI SEVDP-HCK
CpSDaV     60 : TEVFSKDNLAGSSTGIVTFG PSLSDCPAF SNGMLKAYHEYKI SMVILBEFISEASSTSSGSIAYEVDP-HCK
TYV        66 : TEVFSKDNLAGS SSGAITFGP SLSDCPAF SNGMLKAYHEYKI SMVILBEFVSEAS SQNSGSIAYELDP-HCK
BWYV       66 : TEVFSKDNLAGS SSGAITFGP SLSDCPAF SNGILKAYHEYKI SMVILBEVSEAS SQNSGSIAYELDP-HCK
BMYV       66 : TEVFSKDNLAGS SSGAITFGP SLSDCPAFADGMLKAYHEYKI SMVILBEVSEAS SQNSGSIAYELDP-HCK
BChV       66 : TEVFSKDNLAGS SSGAITFGP SLSDCPAF SNGMLKAYHEYKI SMVILBEVSEAS SQNSGSIAYELDP-HCK
PLRV       71 : TEVFTKDNLVGNIQGSFTFGP SLSDCPAFKD GILKAYHEYKITSILLQEVSEASSTSSGSIAYELDP-HCK
CYDV-RPS   68 : TEVFTKDNLAGN SSGSITFGP SLSDYPAFQNGVLKAYHEYKITGCILQEVSEASSTAAGSIAYELDP-HCK
CYDV-RPV   68 : TEVFSKDNLAGN SSGSITFGP SLS EYPAFQNGVLKAYHEYKITNCVLEQVSEASSTAAGSIAYELDP-HCK
CAbYV      63 : TEVFSKDNLTGSSTG SITFGP SLS E S PAF SNGILKAYHEYKITIMVLEFISEASSTSSGSIAYELDP-HCK
GRAV       63 : TEVFSKDNLTGS SSGSITFGP SLSDCPAF SSGILKAYHEYKI SMVKVEVSEASSTSSGSIAYELDP-HCK
ScYLV      60 : VLTFTVDDIKANSTGILKFGENLSQYAAENNGLLKAYHEYKITSITIQYNSCS SDATPGATALEVDT-SCS
TVDV       69 : TFIKNDSTKDGSSGAITFGP SLS E SVALSGV LKAYHEYKITMNIREFISESSTABGSIAYELDP-HCK
BLRV       60 : EEFVSKDNLNGN SSGSITFGP SLS ECKPLADGILKAYHEYKITMVELAKITEASSTSSGSIAYELDP-HLK
SbdV       64 : TEVFSKDGNGS SSGSITFGP SLS ECKPFSDGILKAYHEYKITSILLQEVTEASSTSSGSIAYELDP-HCK
BYDV-GAV   62 : VEFVSVNDLKAN SSGITKFGEDLSQCPALSGGILKSYHRYKITNWKVEKSHASASTVGCAMPTELDT-SCS
BYDV-MAV   62 : VEFVSVNDLKAN SSGITKFGEDLSQCPALSGGILKSYHLYKITNWKVEKSHASASTVGCAMPTELDT-WCS
BYDV-PAV   63 : VEFVSVNDLKAN SSGAITKFGP SLSQCPALSDGILKSYHRYKITSRVBEKSHASANTAGATELDT-ACK
BYDV-PAS   63 : VEFVSVNDLKAN SSGITKFGP SLSQCPALSDGILKSYHRYKITSRVBEKSHASAAATSCATEVELDT-ACK
PEMV       53 : DEFMVHGFENGNATGTVKFGP S-SDQCIKG-NLAAYQYRIVVLLKVVVQSEAAATDRGCTIAYHVDTSITK

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CLR DV      135 : LSTLSSTINKEGITKNGRKOBAASPTNGQEBWHDTS E D Q E R I L Y K N G S S S - I A G S F R V T I R C Q F H N P K -
CpSDaV     130 : LSTLSSTINKEGITKNGRKOBAASPTNGQEBWHDTS E D Q E R I L Y K N G S S S - I A G S F R I T I R C Q F H N - - -
TYV        136 : LNSLSSTINKEGITKPGKRAFTASYINCTBWH DVAEDQERILLYKNGSSS-IAGSFRITTIKCFHNP K-
BWYV       136 : LDALSSTINKEGITKPGRRABTASYINCTDWH DVAKDQERILLYKNGSSS-IAGSFRITIMKCFHNP K-
BMYV       136 : LALSSTINKEGITKPGRRABAASYINCA DWH DVAKDQERILLYKNGSSS-IAGSFRITIMKCFHNP K-
BChV       136 : LNSLSSTINKEGITKPGRRABTASYINCTBWH DVAKDQERILLYKNGSSS-IAGSFRITTIKCFHNP K-
PLRV       141 : VLSLQSYVNFKEGITKGGAKTYOARMINGV BWH D S E D Q C R I L W K N G S S S A - I A G S F R V T I K V A L Q N P K -
CYDV-RPS   138 : ISSLASTINKEITTKTGARSEPAKMINGLEBWHPSDE D Q E R I L Y K N G S S S - V A G S F K I T L R V Q L Q N P K -
CYDV-RPV   138 : ASSLASTINKEITTKTGARSEPAKMINGLEBWHPSDE D Q E R I L Y K N G S S S - V A G S F K I T L R V Q L Q N P K -
CAbYV      133 : LSLQSTINKEGITKGLRRMTAKOINCM E B W H D A T E D Q E R I L Y K N G S S S - V A G S F R I T T I K C Q V Q N P K -
GRAV       133 : SSSLQSYVNFKEGITRNGQRSMMGRYINGV BWH D A T E D Q E R F L Y K N G S S A - I A G S F R F T T I K C Q V Q N P K -
ScYLV      130 : QTTGSKITSEPMKRNAAKVEPAPFTRKDEMTT S A D Q E W L L Y K N G D S S - I A G Q F V C R F E C L F Q N P K -
TVDV       139 : LSTLQSTLRKEPVTKGQATBRAAQINGV BWH D A E D Q E R L L Y K N G S S K N V A A G F F Q H R Y T V L Q H N P K -
BLRV       130 : NNTIQSKINKE S I T K S E K K K P S R K A I N Q A M H D T S E D Q E R I L Y K N G D A K - I A G S F R V T I K V L T Q N P K -
SbdV       134 : YSEIQSLNKE S I T K S G S K R E P T R A I N C L B W H D T S E D Q E K I H Y K N G S S K - I A G S F K I S I N V L T Q N A K -
BYDV-GAV   132 : QSLGYSINSETLSK SATK N P T A Q Q I D C K B E R E S V N Q B Y M L Y K A N G S T S D T A G O F I I T I R V A N M T P K -
BYDV-MAV   132 : QSLGYSINSETLSK SATK N P T A Q Q I D C K B E R E S T V N Q B Y M L Y K A N G S T S D T A G O F I I T I R V A N M T P K -
BYDV-PAV   133 : QSALGYSINSETLSK T A S K T R S E A I N K B E F Q E S T I D Q E W L L Y K A N G T T D T A G O F I I T T M S V S I M T A K -
BYDV-PAS   133 : QSALGYSINSETLSK T A S K S R A E A I N K B E F Q E S T I D Q E W L L Y K A N G T T D T A G O F I I T I N V S M L T P K -
PEMV       122 : KAADVLLDTMNR SNGSATBGREITGDQBYE S N K D Q E F F L Y R G T G G I D - V A G H Y R I S G R I Q I M N A S L

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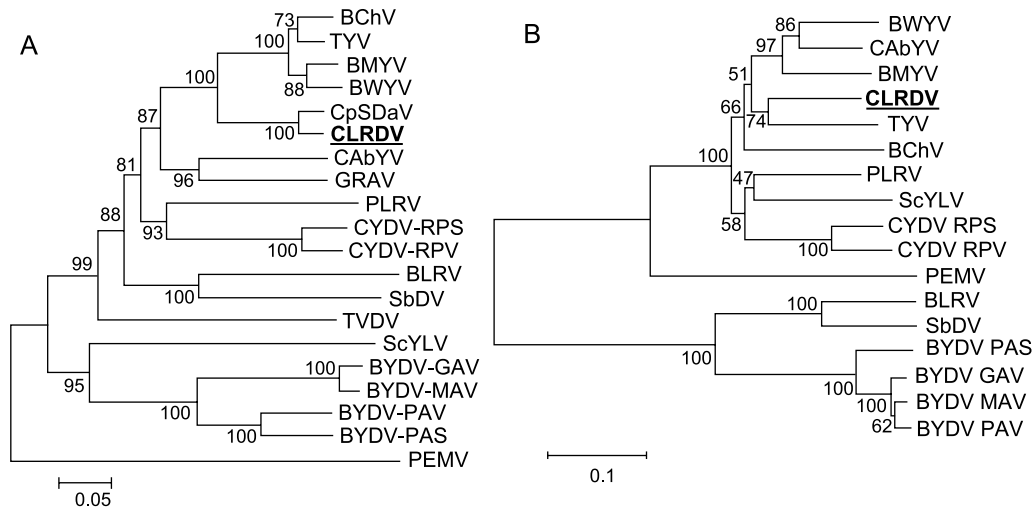


Fig. 4. Phylogenetic analysis of CLRDV and other virus from family *Luteoviridae*. The predicted amino acid sequences of the coat protein (A) and the C-terminal region of the RdRp (B) were aligned with Multalign and the tree was constructed with MEGA2. Bootstrap values are indicated at each node

E₁₇₆ is highly conserved in the genus *Polerovirus*, but hydrophobic or polar substitutions have occurred in the genus *Luteovirus* lineage at this residue. Hydrophobic substitution at E₁₇₀ was also linked to recombinant luteoviruses [8]. The alignment was used for neighbor joining phylogenetic reconstruction and showed that CLRDV clearly grouped with CpSDaV in the polerovirus branch of the tree (Fig. 4A).

Putative movement protein, RdRp and intergenic region sequences

The sequence of the putative movement protein (ORF4), which is expressed by an alternative AUG codon within the CP sequence, was also obtained. This ORF encodes a protein of 174 amino acid residues in CLRDV with an expected molecular mass of 19.8 kDa. Molecular mass and percentage identities of CLRDV ORF4 resembles those from CpSDaV, TYV, BMYV, BChV and BWYV, which were also the closest related viruses in the CP phylogeny (Table 1).

The 612 nucleotides obtained from the CLRDV RdRp encode 203 amino acid residues of the C-terminal region of the polymerase. Percent identities with other known viruses from the family *Luteoviridae* demonstrated a closer relationship with polerovirus polymerases than with luteovirus polymerases (Table 1). Phylogeny obtained from the amino acid alignment confirmed this result by grouping CLRDV with TYV in the polerovirus branch (Fig. 4B). Unfortunately no CpSDaV RdRp sequence is available for comparative analysis.

The intergenic region of 187 nucleotides between the CLRDV RdRp and CP sequences was also obtained. The CLRDV intergenic region also resembles that of members in the genus *Polerovirus*, which are usually around 200 nt long in

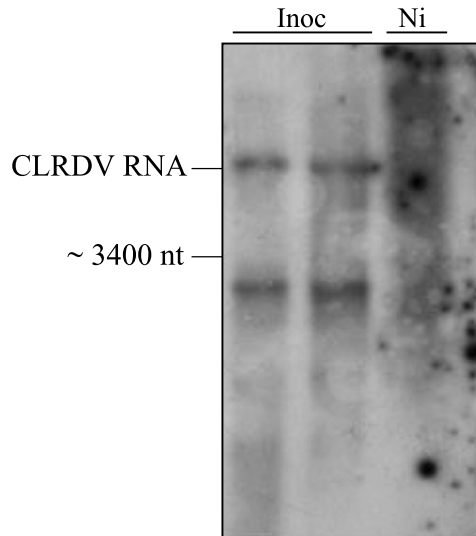


Fig. 5. Detection of CLRDV RNA by northern blot hybridization in two cotton plants showing typical blue disease symptoms. *Inoc.* – Inoculated plants at 30 dpi. *Ni* – Non-inoculated plant. 15 μ g of total RNA were loaded per lane and hybridized with a radioactively labeled CLRDV CP fragment

this part of genome, contrasting with luteoviruses which have intergenic regions around 100 nt long.

*Northern blot assay demonstrates virus presence
in infected cotton plants*

Northern blot was performed using infected and healthy plants at 30 dpi. Hybridization with the complete CP gene confirmed the viral origin of the amplified sequence. Two bands were observed in the RNA extracts of infected plants, probably corresponding to virus genomic and subgenomic RNAs (Fig. 5). No hybridization was observed in the non-inoculated plants.

Discussion

Here we show for the first time molecular data of a virus associated with cotton blue disease, an economically important disease of cotton in Brazil. We have tentatively named this virus Cotton leafroll dwarf virus (CLRDV). The percent identities and phylogenies of the CLRDV CP and partial RdRp sequences strongly suggest that the virus belongs to the family *Luteoviridae*. Also, both the CP and RdRp sequences favor classification of the virus in the genus *Polerovirus*, arguing against the possibility of it being a recombinant between a luteovirus and a polerovirus.

During our cloning process we have sequenced both DNA strands of three clones derived from the initial amplification with primers PLF and PLR and six clones containing the entire CP sequence. The same nucleotide sequence was observed in all nine clones. These results suggest the existence of a single polerovirus in infected plants since the designed primers would be able to amplify all previously characterized members of this genus. In a previous work, antiserum raised against viruses in three species of the family *Potyviridae*, one species of

the genus *Carlavirus* and four species of the family *Luteoviridae* (BWYV, PLRV, ScYLV and BYDV) were used for virus detection in cotton plants with typical blue disease symptoms [21]. ELISA results were negative for all viruses, with the exception of a weak positive signal observed with the BWYV antiserum. These data, together with our inability to amplify any DNA fragments using luteovirus-specific primers, suggest that cotton blue disease is probably associated with a single virus from the genus *Polerovirus*, family *Luteoviridae*.

The CLRDV CP sequence obtained displays 92% identity with CpSDaV, suggesting that both viruses could be strains of the same species. Species definition in the family *Luteoviridae* is consistently based on CP and RdRp sequence identities. Viruses with percent identities greater than 90% in the CP region are considered to be in the same species [23]. However, there are cases such as BWYV and BMV, which share a strong identity in the 3' part of the genome but little similarity in the 5' region. Comparative analysis of complete genome sequences from BWYV and BMV showed that BMV is a recombinant between two poleroviruses and for this reason it is not considered as a strain of BWYV [10].

The partial CP sequence of CpSDaV was obtained from chickpea plants showing stunt disease in India, but, to date, there is no available CpSDaV RdRp sequence [15]. Thus, with the current data, we cannot determine if CLRDV and CpSDaV are actually different viruses or strains of the same virus. Therefore, the complete genome sequencing of both CpSDaV and CLRDV will be of great interest to solve this taxonomic question.

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