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First report of *Cotton leafroll dwarf virus* in Thailand using a species-specific PCR validated with isolates from Brazil

M. Sharman[1] · S. Lapbanjob[2] · P. Sebunruang[2] · J.-L. Belot[3] · R. Galbieri[3] · M. Giband[4,5] · N. Suassuna[5]

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Abstract Partial virus genome sequence with high nucleotide identity to *Cotton leafroll dwarf virus* (CLRDV) was identified from two cotton (*Gossypium hirsutum*) samples from Thailand displaying typical cotton leaf roll disease symptoms. We developed and validated a PCR assay for the detection of CLRDV isolates from Thailand and Brazil.

Keywords Polerovirus · Luteovirus · Luteoviridae · Cotton blue disease

A serious disease of cotton (*Gossypium hirsutum*) referred to as cotton leaf roll has been reported from Thailand since at least the early 1970s with symptoms including leaf rolling, intense green foliage, vein yellowing on younger leaves, and moderate to severe stunting of affected plants (Kaowsiri 1982). It caused widespread disease outbreaks and production losses in the susceptible cotton cultivar Deltapine Smoothleaf in 1972 and was largely responsible for a dramatically reduced area of cotton production in Thailand thereafter (Kaowsiri 1982). The symptoms of cotton leaf roll disease from Thailand were very similar to those of cotton blue disease described from Africa (Cauquil 1977) and both were believed to be of viral origin.

The single stranded RNA virus, *Cotton leafroll dwarf virus* (CLRDV, Genus: *Polerovirus*, Family: Luteoviridae) has now been shown to be the causal agent for cotton blue disease from Brazil (Corrêa et al. 2005), Argentina (Distéfano et al. 2010) and India (Mukherjee et al. 2012). The transmission of cotton leaf roll disease from Thailand by *Aphis gossypii* (Michelotto and Busoli 2007; Sarindu and Siddhipongse 1980) and observed particle morphology (Sakwong 1985) are consistent with cotton leaf roll being caused by a luteoviridae species related to CLRDV. However, molecular characterisation of a viral agent associated with cotton leaf roll disease from Thailand has remained undetermined.

In this paper we test the hypothesis that cotton leaf roll disease from Thailand is associated with CLRDV. We did this using a CLRDV-specific assay which we validated with CLRDV-infected cotton samples from Brazil. Other degenerate primers were used to amplify and sequence a larger fragment of partial genome sequence from two Thailand cotton leaf roll samples.

Five samples of cotton variety Deltapine Smoothleaf displaying typical cotton leaf roll disease symptoms (Fig. 1) were collected in August 2011 from Takfa, Thailand. Symptoms included severely dwarfed plants with shortened branch internodes and small, thicker leaves with leaf edges rolled downwards. Severely affected plants sometimes displayed a prostrate growth habit with a zig-zag appearance of the stem. Samples were lyophilised and lodged in the Queensland Government plant virus collection with isolate numbers Q3513, Q3514, Q3515, Q3518 and Q3519. Samples of cotton blue disease from Brazil displayed symptoms essentially as

✉ M. Sharman
murray.sharman@qld.gov.au

[1] Queensland Department of Agriculture and Fisheries, Ecosciences Precinct, GPO Box 267, Brisbane, QLD 4001, Australia

[2] Department of Agriculture, Nakhon Sawan Field Crops Research Center, Takfa, Nakhon Sawan 60190, Thailand

[3] Instituto Mato-Grossense do Algodão, Rodovia BR 070 - KM 265, Campo Experimental Zona Rural, Primavera do Leste, MT, Brazil

[4] CIRAD, UMR AGAP, Avenue Agropolis, 34398 Montpellier Cedex 5, France

[5] Embrapa Algodão, Rodovia GO-462, km 12 Zona Rural C.P. 179, 75375-000 Santo Antônio de Goiás, GO, Brazil

Fig. 1 **a** Severe symptoms of cotton plants infected with CLRDV from Thailand showing down curling of leaves, stunted growth and zig-zag appearance of stem; **b** prostate growth of severely affected plants (shown by *arrows*)

described for cotton leaf roll from Thailand. They were collected from two locations in Brazil; from *G. hirsutum* (isolates Q3523 and Q3524) and *G. mustelinium* (isolate Q3525) from Santa Helena de Goiás, Goiás, and from *G. hirsutum* (isolates Q4625 and Q4631) from Primavera do Leste, Mato Grosso.

Table 2 PCR primer pairs used in this study showing location of amplicon on genome and approximate size

Primer pair	Target region	Approximate expected product size (bp)
Pol3167F / AS3	3′ end of RdRp + IGR + CP[a]	1070
Pol3870F / AS3	3′ end of CP	370
Pol3870F / Pol4333R	3′ end of CP	460
Pol3628F / Pol3982R	5′ end of CP	350
CLRDV3675F / Pol3982R	Middle of CP	310

[a] Abbreviations used for genome regions: *RdRp* RNA dependant RNA polymerase gene, *IGR* intergenic region, and *CP* coat protein gene

Total nucleic acid extracts were prepared using a modified CTAB method (Cordova et al. 2003; Dellaporta et al. 1983) as follows. Approximately 10 mg of desiccated symptomatic plant tissue was ground in 1 ml of 2 % CTAB buffer (2 % CTAB, 1.4 M NaCl, 100 mM Tris, 20 mM EDTA, 1 % PVP-40, 0.5 % $NaSO_3$) and incubated at 60 °C for 30 min with intermittent mixing before adding 800 µl of chloroform:isoamyl alcohol (24:1) and mixed by inversion. After centrifugation at 14,000 g for 5 min, 400 µl of aqueous phase was added to 700 µl isopropanol, mixed by inversion and spun at 14,000 g for 15 min. The resulting pellet was washed twice with 700 µl of 70 % ethanol and spun at 14,000 g for 2 min for each wash step. The pellet was dried prior to re-suspension in 100 µl of nuclease free water.

To design PCR primers (Table 1), previously published sequences for CLRDV from Argentina, Brazil and India (Genbank accessions GU167940, HQ827780 and JN033875 respectively), Chickpea stunt disease associated virus (CpSDaV, Y11530), Cotton bunchy top virus (CBTV, JF803842), *Cucurbit aphid borne yellows virus* (CABYV, JF803842), *Chickpea chlorotic stunt virus* (CpCSV, NC_008249) and *Beet western yellows virus* (BWYV, AF473561) were aligned using the MUSCLE algorithm (Edgar 2004) included in the MEGA5 software package

Table 1 PCR primers used in this study

Primer name	Sequence (5′ to 3′)	Target species[a]
Pol3167F	YTVGGTTTYAAAGTCGAGG	CLRDV, CBTV, CABYV, BWYV, PLRV
AS3[b]	CACGCGTCIACCTATTTIGGRTTITG	see Abraham et al. (2008)
Pol3870F	ATCACBTTCGGGCCGWSTYTWTCAGA	CLRDV, CpCSV, CABYV, PLRV, BWYV, TuYV, BLRV, CBTV, SbDV
Pol4333R	GGRTTKCCYTCATAACCCCA	CLRDV, CABYV
Pol3628F	TAATGAATACGGYCGYGGSTAG	CLRDV, CBTV, TuYV, BWYV, CABYV
Pol3982R	CGAGGCCTCGGAGATGAACT	CLRDV, CBTV, CABYV, CpCSV, SbDV
CLRDV3675F	CCACGTAGRCGCAACAGGCGT	CLRDV specific

[a] Virus species (in addition to CLRDV) known to work with these primers (data not shown)

[b] Primer AS3 published by Abraham et al. (2008)

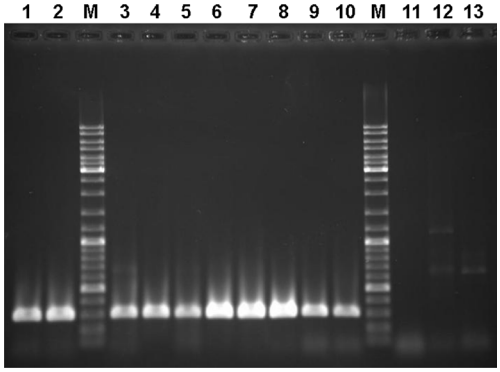

Fig. 2 TBE electrophoresis gel for CLRDV-specific PCR with primers CLRDV3675F / Pol3982R for Thailand cotton leaf roll samples Q3513, Q3514, Q3515, Q3518 and Q3519 in lanes 1 to 5 respectively, and Brazilian cotton blue disease samples Q3523, Q3524, Q3526, Q4625 and Q4631 in lanes 6 to 10 respectively. CLRDV-specific products are approximately 310 bp and marker bands (lane M) are at 100 bp intervals up to 1000 bp. Lanes 11–12 are Australian cotton samples infected with cotton bunchy top virus and lane 13 is healthy cotton. Marker lanes (M) are GeneRuler DNA ladder mix (Catalogue # SM0332, Life Technologies)

(Tamura et al. 2011). Regions that were either in common to all members or specific to CLRDV were selected by eye.

Synthesis of cDNA was done using SuperScript III reverse transcriptase (Invitrogen) as per the manufacturer's instructions with primer Pol3982R, Pol4333R or AS3. PCRs were done using various primer combinations (Table 2) with 1 unit native *Taq* DNA polymerase (Invitrogen), 1.75 mM $MgCl_2$, 200 mM dNTPs, 200 nM of each primer and 2 μl of cDNA template in a 25 μl reaction volume. Temperature cycling parameters for all PCRs consisted of an initial denaturation of 95 °C for 60 s, then 35 cycles of: 95 °C for 15 s, 62 °C for 20 s, 56 °C for 10 s and 72 °C for 40 s; followed by a final extension of 72 °C for 3 min.

The generic primer pairs, Pol3870F / AS3 and Pol3628F / Pol3982R were very useful for initial detection of the Thailand CLRDV which had some sequence differences to the Brazil CLRDV. One or both of these primer pairs also detected the Luteoviridae species CABYV, CpCSV, CBTV, *Bean leafroll virus* (BLRV), *Soybean dwarf virus* (SbDV), *Potato leaf roll virus* (PLRV), *Turnip yellows virus* (TuYV) and BWYV (data unpublished).

We used the CLRDV-specific PCR (CLRDV3675F / Pol3982R) to amplify a product of the expected size (310 bp) from reference samples of CLRDV from Brazil and the five symptomatic cotton samples from Thailand, while the CBTV samples were negative (Fig. 2). This indicated CLRDV was detected in all five symptomatic samples from Thailand and that the primer CLRDV3675F is useful for the specific detection of this virus from geographically diverse locations.

PCR products derived using primer pairs Pol3167F / AS3, Pol3870F / AS3 and Pol3870F / Pol4333R from Thailand samples Q3513 and Q3514 were sequenced directly (Australian Genome Research Facility, Brisbane) to provide at least twofold coverage of the target regions. Nucleotide and putative amino acid sequences were aligned using the MUSCLE algorithm (Edgar 2004) and MEGA5 was used to calculate pairwise distances between sequences. Phylogenetic relationships were inferred using the maximum-likelihood method with GTR-GAMMA specified as the model of evolution. After removal of the primer sequences, the resulting partial genome fragments were 1113 nt in length (Genbank accession numbers KP176643 and KP176644), consisting of 239 nt of the 3′ end of partial RdRp gene, the intergenic region, the complete coat protein gene and 81 nt of 5′ end of the partial ORF5 readthrough gene. Samples Q3513 and Q3514 shared greater than 99.5 % nt identity over the 1113 nt length. As such, further analysis is discussed for sample Q3513 only. The closest match

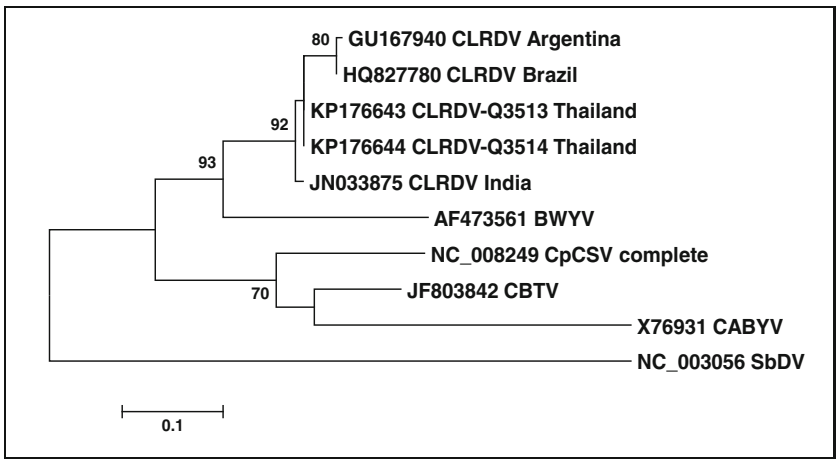

Fig. 3 Phylogram obtained from a Maximum Likelihood analysis for the partial coat protein for a 186 amino acid overlap from Thailand samples presented in this study (Q3513 and Q3514) and other previously published Luteoviridae members. The *scale bar* represents the number of amino acid substitutions per site. Maximum likelihood support values (>50 %) are shown at the nodes. Genebank accession numbers are shown on phylogram. See text for details of virus abbreviations

to isolate Q3513 was with CpSDaV and CLRDV from India (Y11530 and JN033875), both with 98 % nt identity, although this was only for the 540 bp overlap of the partial coat protein gene available for these Indian samples. As Corrêa et al. (2005) suggested, it appears that CpSDaV reported by Naidu et al. (1997) may be the same virus as CLRDV. Over a 1113 bp overlap, sample Q5313 had a 92 % nt identity with CLRDV from Argentina (GU167940) and the next closest matching species was with CBTV with only 65 % nt identity. Sample Q3513 also had a 96 % aa identity with the Argentinian CLRDV for the complete putative coat protein. Phylogenetic analysis of the partial coat protein sequence for Thailand isolates Q3513 and Q3514, clearly shows these group with other CLRDV samples from Brazil, Argentina and India (Fig. 3). The total putative coat protein of both Thailand isolates was 201 amino acids but analysis was done for an overlap of 186 amino acids to include the partial coat protein sequence available for the Indian CLRDV (JN033875).

These results confirm the symptomatic cotton samples from Thailand previously referred to as cotton leaf roll disease, were infected with CLRDV, the causal agent of cotton blue disease. A disease similar to Cotton blue disease has been reported from Vietnam (Quyen et al. 2008) but no associated virus has been described. To the best of our knowledge, our study represents the first report of CLRDV from south-east Asia and greatly extends its known global distribution. It appears likely that this virus has been present for at least several decades as the cause of the reported cotton leaf roll disease from Thailand. Management of the disease in Thailand can be benefited by the advances in genetic improvement of cotton for resistance to CLRDV conducted in other countries (Morello et al. 2012). The inheritance of resistance to cotton blue disease is conditioned by one dominant gene in some sources of genetic resistance (Pupim-Junior et al. 2008).

This report of CLRDV infecting cotton in Thailand and previous reports of infections of legume crops demonstrate further investigation is warranted to determine the distribution of CLRDV in the Asian region and its possible alternative hosts. The diagnostic assay we have developed and validated for CLRDV may assist in future host range and epidemiology studies. This will help to clarify the importance of this virus in diseases of cotton and legume crops.

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References

Abraham AD, Varrelmann M, Vetten HJ (2008) Molecular evidence for the occurrence of two new luteoviruses in cool season food legumes in Northeast Africa. Afr J Biotechnol 7:414–420

Cauquil J (1977) Estudes sur une maladie d'origine virale du cotonnier: la maladie bleue. Coton Fibres Tropicales 32:259–278

Cordova I, Jones P, Harrison NA, Oropeza C (2003) *In situ* PCR detection of phytoplasma DNA in embyros from coconut palms with lethal yellowing disease. Mol Plant Pathol 4(2):99–108

Corrêa RL, Silva TF, Simões-Araújo JL, Barroso PAV, Vidal MS, Vaslin MFS (2005) Molecular characterization of a virus from the family Luteoviridae associated with cotton blue disease. Arch Virol 150(7):1357–1367

Dellaporta S, Wood J, Hicks J (1983) A plant DNA minipreparation: version II. Plant Mol Biol Report 1(4):19–21. doi:10.1007/BF02712670

Distéfano A, Bonacic Kresic I, Hopp H (2010) The complete genome sequence of a virus associated with cotton blue disease, cotton leafroll dwarf virus, confirms that it is a new member of the genus *Polerovirus*. Arch Virol 1–6. doi:10.1007/s00705-010-0764-3

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32(5):1792–1797. doi:10.1093/nar/gkh340

Kaowsiri T (1982) Leaf roll disease, the most important disease of cotton. J Thai Phytopathol Soc 2:7–9

Michelotto MD, Busoli AC (2007) Caracterização da transmissão do virus do mosaico-das-nervuras do algodoeiro pelo pulgão *Aphis gossypii* com relação a ersistencia e ao tempo necessario para inoculação. Bragantia 66:441–447

Morello CL, Pedrosa MB, Suassuna ND, Lamas FM, Chitarra LG, Silva Filho JL, Andrade FP, Barroso PAV, Ribeiro JL, Godinho VPC, Lanza MA (2012) BRS 336: a high-quality fiber upland cotton cultivar for Brazilian savanna and semi-arid conditions. Crop Breed Appl Biotechnol 12:92–95

Mukherjee AK, Chahande PR, Meshram MK, Kranthi KR (2012) First report of *Polerovirus* of the family *Luteoviridae* infecting cotton in India. New Dis Rep 25:22

Naidu RA, Mayo MA, Reddy SV, Jolly CA, Torrance L (1997) Diversity among the coat proteins of luteoviruses associated with chickpea stunt disease in India. Ann Appl Biol 130(1):37–47. doi:10.1111/j.1744-7348.1997.tb05781.x

Pupim-Junior O, Schuster I, Pinto RB, Pires E, Belot J-L, Silvie P, Chitarra LG, Hoffmann LV, Barroso P (2008) Inheritance of resistance to cotton blue disease. Pesq Agrop Brasileira 43(5):661–665

Quyen LQ, Hai NT, Mai TAH, Hao V, Binh NTT, Bu'u DN, Dieu DX, Underwood E (2008) Cotton production in Vietnam. In: Andow DA, Hilbeck A, Nguyen VT (eds) Environmental risk assessment of genetically modified organisms: challenges and opportunities with Bt cotton in Vietnam, vol 4. CABI Publishing, United Kingdom, pp 24–63

Sakwong W (1985) Diagnosing of cotton leaf roll disease by serological and electron microscopic techniques. Kasetsart University, Bangkok

Sarindu N, Siddhipongse S (1980) Transmission of the causal agent of cotton leaf roll disease. Paper presented at the 2nd Southeast Asian Symposium on Plant Diseases in the Tropics, Bangkok (Thailand), 20–26 Oct 1980, Kasetsart University, Bangkok, Thailand, 20–26, Oct 1980

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739. doi:10.1093/molbev/msr121