# Cotton leafroll dwarf virus (CLRDV) Identification


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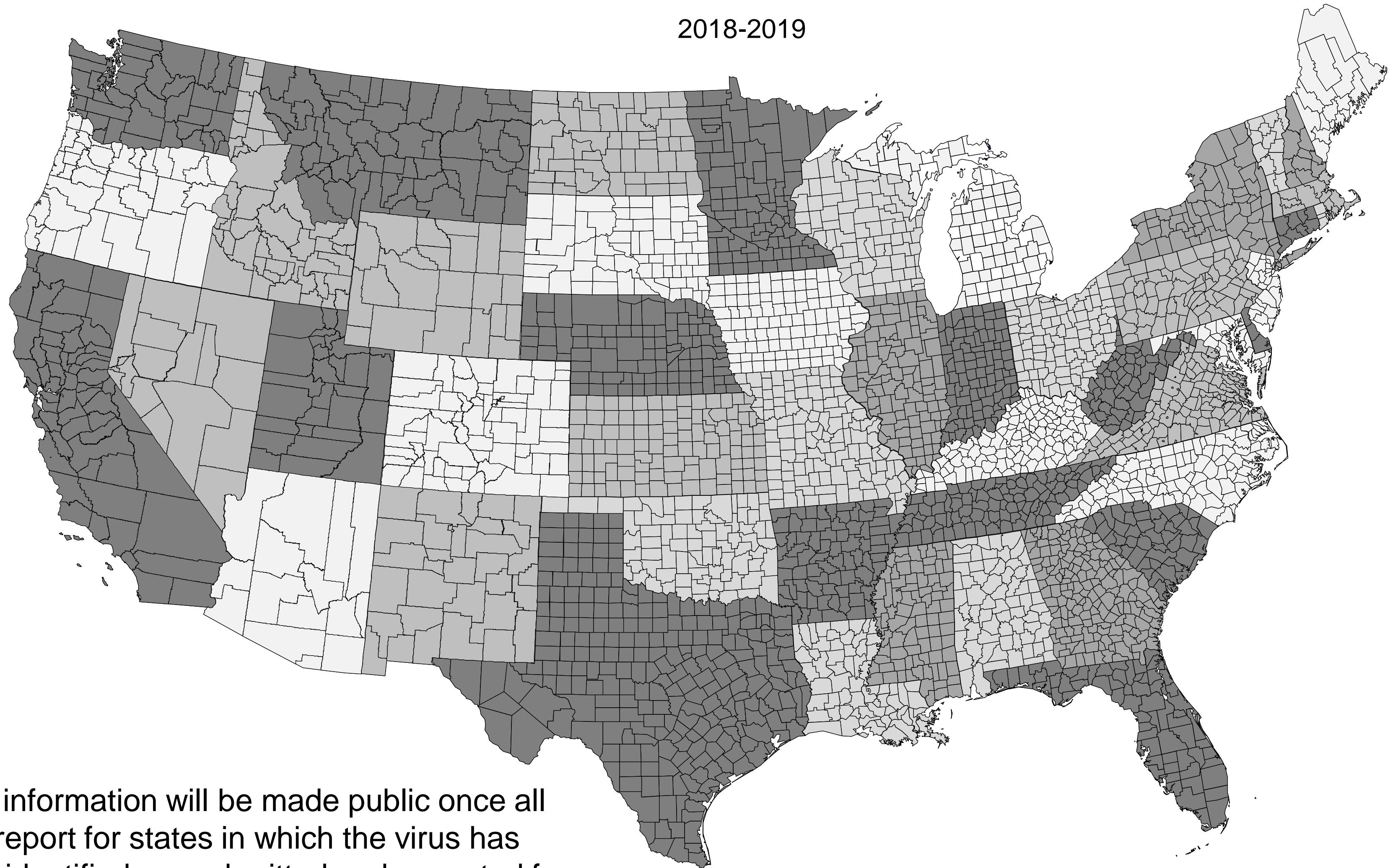
extension

ALABAMA A&M & AUBURN UNIVERSITIES

# Cotton Blue Disease Distribution

Cotton leafroll dwarf virus (CLRDV)

2018-2019



This information will be made public once all first report for states in which the virus has beenidentified are submitted and accepted for publication.

| Primer name | F/R | Primer sequence | Amplicon size | State | Source | Notes |
|---|---|---|---|---|---|---|
| CP up<br>CP low | F<br>R | ATGAATACGGTCGTGGGTAG<br>CTATTTTGGATTGTGGAATT | 433 bp | | 4 | N. benth |
| CLRDV-For1<br>CLRDV-Rev1 | F<br>R | ACGACGAAGACGAGGAGGTC<br>GAACCGGAGGATGTTGAAGAGG | 249 bp | MS | 1 | MS primers first set |
| AL674F<br>AL1407R | F<br>R | CCGTAGCGGTCATCGTCTTT<br>TAACCCTGACACAGTGGGGA | 733 bp | AZ | 2 | AZ primers |
| AtropaNad2.1a<br>AtropaNad2.2b | F<br>R | GGACTCCTGACGTATACGAAGGA<br>AGCAATGAGATTCCCCAATATCAT | 850 bp<br>188 bp | Australia | 8 | Internal control |
| CLRDV3675F<br>Pol3982R | F<br>R | CCACGTAGRCGCAACAGGCGT<br>CGAGGCCTCGGAGATGAACT | 307 bp | Australia | 7 | Current primers<br>Nested PCR CP – 2nd round |
| Pol3628F<br>Pol4021R | F<br>R | TAATGAATACGGYCGYGGSTAG<br>GGRTCMAVYTCRTAAGMGATSGA | 393 bp | Australia | Sharman, unpublished | Nested PCR CP – 1st round |
| Pol3628F<br>Pol3982R | F<br>R | S/A<br>S/A | 350 bp | Australia | Sharman, unpublished | General Polerovirus RT-PCR<br>5’ end of CP gene |
| Pol3870F<br>AS3 | F<br>R | ATCACBTTCGGGCCGWSTYTWTCAGA<br>CACGCGTCIACCTATTTIGGRTTITG | 370 bp | Australia | 6 | General Polerovirus RT-PCR<br>3’ end of CP gene |
| CLRDV_ORF0F<br>CLRDV_ORF0R | F<br>R | GTCTCGTGTATGTTGAATTTGATCAT<br>CTCAACTGCTYTCTCCTTCAC | 790 bp | Australia | Sharman, unpublished | Nested PCR P0 gene – 1st round |
| CLRDV90F<br>CLRDV794R | F<br>R | GCAGARTYTCTTCCGCAGCTCT<br>CGCCTTCATCGTCAAAATGGTA | 705 | Australia | Sharman, unpublished | Nested PCR P0 gene – 2nd round |
| COXf<br>COXr | F<br>R | GTA TGC CAC GTC GCA TTC CAG A<br>GCC AAA ACT GCT AAG GGC ATT C | | USDA/APHIS/CPHST<br>WI-B-T-D-2 | 5 | Internal control<br>Cytochrome oxidase |
| Nad5f<br>Nad5mr | F<br>R | GAT GCT TCT TGG GGC TTC TTK TT<br>ATC TCC AGT CAC CAA CAT TRG CAT AA | 180 | USDA/APHIS/CPHST<br>WI-B-T-1-31 | | Internal control<br>Nad5 gene |
| CLP0F<br>CLP0R | F<br>R | CAC CAT GTT GAA TTT GAT CAT CTG CAG<br>ACT GCT TTC TCC TTC AC | | Brazil | 3 | P0 gene |
| CLRDV-RdRpF2<br>CLRDV-RdRpR1 | F<br>R | GGA GCC GCA CAA ACA AGC TAA<br>AAC AGG CGT TCA GGT AGT TGG A | 770 | MS | 1 | MS primers second set |

# Primer Source


1. Aboughanem-Sabanadzovic, N., Allen, T. W., Wilkerson, T. H., Conner, K. N., Sikora, E. J., Nichols, R. L., and Sabanadzovic, S. 2019. First report of cotton leafroll dwarf virus in upland cotton (Gossypium hirsutum L.) in Mississippi. Plant Disease, In Press. https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-01-19-0017-PDN
2. Avelar, S.; Schrimsher, D.W.; Lawrence, K.S.; and Brown, J.K. 2018. First report of cotton leafroll dwarf virus associated with cotton blue disease in Alabama. Plant Disease. https://doi.org/10.1094/PDIS-09-18-1550-PDN
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4. Delfosse, V. C., Casse, M. F., Agrofoglio, Y. C., Kresic, I. B., Hopp, H. E., Ziegler-Graff, V., and Distefano, A. J. 2013. Agroinoculation of a full-length cDNA clone of cotton leafroll dwarf virus (CLRDV) results in systemic infection in cotton and the model plant Nicotiana benthamiana. Virus Research 175:64-70.
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9. Knierim et al. 2010; Plant Pathol. 59:991
10. Tabassum, A.; Bag, S.; Roberts, P.; Suassuna, N.; Chee, P.; Whitaker, J. R.; Conner, K. N.; Brown, J.; Nichols, R. L.; Kemerait, R. C. 2019. First report of Cotton leafroll dwarf virus infecting Cotton in Georgia, USA. Plant Disease, In Press. https://apsjournals.apsnet.org/doi/pdf/10.1094/PDIS-12-18-2197-PDN

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DNA Extraction Date________________________________

PCR Run Date______________________________________

Sample #s and location______________________________________

**Primers for amplification:**

Pol3982R: CGAGGCCTCGGAGATGAACT

CLRDV3675F: CCACCTAGRCGCAACAGGCGT

***Primer working solution (2 and 10µl)*:** Dilute 100µM stock to a **2µM** working solution by adding 2µl of the 100µM stock to 98µl H2O. Dilute 100µM stock to a **10µM** working solution by adding 10µl of the 100µM stock to 90µl $H_2O$.

**Sample Preparation:** Extract RNA from symptomatic petiole tissue using the RNeasy Plant Mini Kit (Qiagen – catalog number 74904). **Follow manufacturer's instructions.** Proceed to cDNA synthesis ASAP (same day).

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**cDNA Synthesis:** Use SuperScript® IV First-Strand cDNA Synthesis Reaction (Invitrogen- Catalog number: 18091050). **Follow manufacturer's instructions** using 2 µM gene-specific primer (Pol3982R) as shown in mastermix below.

*Master Mix for Step 1- Anneal primer to template RNA*

A. Combine following components in a reaction tube.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| 2 µM Pol3982R | 1 µl | | |
| 10 µM dNTP mix | 1 µl | | |
| H2O | 6 µl | | |
| **Total** | **8 µl** | | |

B. Add 5 µl of RNA or positive control, mix, and briefly centrifuge the components.

C. Heat RNA-primer mix at 65° for 5 minutes, then incubate at least 1 minute on ice.

*Master Mix for Step 2- Prepare RT reaction mix*

A. Vortex and briefly centrifuge 5x SSIV Buffer.

B. Combine following components in a reaction tube.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| 5xSSIV Buffer | 4 µl | | |
| 100 mM DTT | 1 µl | | |
| RNaseOUT Recombinant RNase Inhibitor | 1 µl | | |
| SuperScript® IV Reverse Transcriptase | 1 µl | | |
| Total | **7 µl** | | |

C. Cap tube, mix and briefly centrifuge the contents.

*Step 3- Combine annealed RNA and RT reaction mix for each individual sample*

*Step 4 Incubate reaction*

**PCR program:**
55°C – 10 min
80°C – 10 min

**Omit Optional Step 5 and proceed to PCR amplification.**

**PCR Amplification**

| Reagent | 1X | # Reactions | Total Volume µl | Check off |
|---|---|---|---|---|
| $H_2O$ | 17.525 µl | | | |
| 10X buffer | 2.5 µl | | | |
| $MgCl_2$ (50 mM) | 0.875 µl | | | |
| dNTP (10 mM) | 0.5 µl | | | |
| CLRDV3675F (10 mM) | 0.5 µl | | | |
| Pol3982R (10 mM) | 0.5 µl | | | |
| Platinum Taq | 0.1 µl | | | |
| **Total** | **22.5 µl** | | | |
| Template cDNA | 2.5 µl | | | |

Vortex master mix. Pipette 22.5µl of mix into each 0.2ml PCR tube. Add 2.5µl of undiluted sample cDNA, water, or positive control to the appropriate labeled tube (total volume 25µl). Close caps and place in PCR machine.

**PCR program:**
95°C – 1 min
35 cycles of:
95°C— 15 sec, 62°C – 20 sec, 56°C – 10 sec, 72°C – 40 sec
72°C— 3 min
4°C – ∞

**Prepare agarose gel:** 1% agarose gel(s) in 1x TAE buffer: 0.5g agarose → 50ml 1x TAE buffer. Add 1.25µl EtBr to the agar and pour in a clean gel cast. Insert combs as appropriate – prepare enough wells for the samples to be run. Let agar solidify.

**Load samples in gel:** Gels are run in 1X TAE buffer for 40 minutes at 70V (constant).

**Expected Results:**
Fragment/band size - 310bp

**Protocol source:**
Sharman, M.; Lapbanjob, S.; Sebunruang, P.; Belot, J. L.; Galbieri, R.; Giband, M; and Suassuna, N. 2015. First report of cotton leafroll dwarf virus in Thailand using a species-specific PCR validated with isolates from Brazil. Australian Plant Disease Notes 10:1-4.

DNA Extraction Date ___________________

PCR Run Date ___________________________

Sample #s and location _________________

**Primers for amplification:**

AL674F: CCGTAGCGGTCATCGTCTTT

Al1407R: TAACCCCTGACACAGTGGGGA

***Primer working solution:*** Dilute 100µM stock to a 2µM working solution by adding 2µl of the 100µM stock to 98µl H2O.

**Sample Preparation:** Extract RNA from sample using the RNeasy Plant Mini Kit (Qiagen – catalog number 74904). Follow manufacturer's instructions. Proceed to cDNA synthesis ASAP.

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**cDNA Synthesis:** Use SuperScript® IV First-Strand cDNA Synthesis Reaction (Invitrogen- Catalog number: 18091050). Follow manufacturer's instructions noting the primer exception as shown in master mix below.

*Master Mix for Step 1- Anneal primer to template RNA*

A. Combine following components in a reaction tube.

B. Add 5 µl of RNA/Control, mix, and briefly centrifuge the components.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| Virus Reverse Primer (2µM) AL1407R | 1 µl | | |
| 10 µM dNTP mix | 1 µl | | |
| H20 | 6 µl | | |
| **Total** | **8 µl** | | |

C. Heat RNA-primer mix at 65° for 5 minutes, then incubate at least 1 minute on ice.

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*Master Mix for Step 2- Prepare RT reaction mix*

A. Vortex and briefly centrifuge 5x SSIV Buffer.

B. Combine following components in a reaction tube.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| 5xSSIV Buffer | 4 µl | | |
| 100 mM DTT | 1 µl | | |
| RNaseOUT Recombinant RNase Inhibitor | 1 µl | | |
| SuperScript® IV Reverse Transcriptase | 1 µl | | |
| **Total RT reaction Mix** | **7 µl** | | |

C. Cap tube, mix and briefly centrifuge the contents.

*Step 3- Combine annealed RNA and RT reaction mix*

*Step 4 Incubate reaction*

**PCR program:**
55°C – 10 min
80°C – 10 min

**Omit Optional Step 5 and proceed to PCR amplification.**

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**PCR Amplification**

***Primer working solution:*** Dilute 100µM stock to a 10µM working solution by adding 10µl of the 100µM stock to 90µl $H_2O$.

| Reagent | 1X-25 µl | 1X-50 µl | RXN# | Total volume 25 µl rxn | Total volume 50 µl rxn | Check off |
|---|---|---|---|---|---|---|
| $H_2O$ | 19.05 | 38.3 | | | | |
| 10X buffer | 2.5 | 5 | | | | |
| $MgCl_2$ (50 mM) | 0.85 | 1.5 | | | | |
| dNTP (10 mM) | 0.5 | 1 | | | | |
| AL674F (10 mM) | 0.5 | 1 | | | | |
| AL1407R (10 mM) | 0.5 | 1 | | | | |
| Platinum Taq | 0.1 | 0.2 | | | | |
| **Total** | **24 µl** | **48 µl** | | | | |
| Template cDNA | 1.0 µl | 2.0 µl | | | | |

Vortex master mix. Pipette 22.5µl of mix into each 0.2ml PCR tube. Add 2.5µl of undiluted sample cDNA, water, or positive control to the appropriate labeled tube (total volume 25µl). Close caps and place in PCR machine.

**AL674 PCR program:**
94°C – 2 min
40 cycles of:
94°C— 30 sec, 55°C – 30 sec, 72°C – 30 sec
72°C— 10 min
4°C – 10s
16°C -- ∞

**Prepare agarose gel:** 1% agarose gel(s) in 1x TAE buffer: 0.5g agarose → 50ml 1x TAE buffer. Microwave the agar for 1 – 1.5 min, stirring occasionally by swirling. Let the agar cool for 3-4 minutes. Add 1.25µl EtBr to the agar and pour the agar in a clean gel cast. Insert combs as appropriate – prepare enough wells for the samples to be run. Let agar solidify.

**Load samples in gel:** Mix 20µl sample with 2µl 10X loading buffer for a total of 20µl for each sample and both controls. Mix by pipetting up and down and load mixture into the gel well. Load DNA ladder into gel. Load into the left most lane for small gels, and into the left lane and middle lane for large gels. Gels are run in 1X TAE buffer for 40 minutes at 70V (constant). View the gel on a UV illumination box and document the assay results using a digital imaging system. Save the image to the results folder as the test run and sample number (ex: ITS 867). Print a hard copy of the results to attach to this protocol. Provide a copy of the protocol and results to Dr. Conner and file a copy in the appropriate notebook in the lab.

**Expected Results:** 733bp

**Protocol Source:** Avelar, S.; Schrimsher, D.W.; Lawrence, K.S.; and Brown, J.K. 2018. First report of Cotton leafroll dwarf virus associated with cotton blue disease in Alabama. Plant Disease 103:592. https://doi.org/10.1094/PDIS-09-18-1550-PDN

DNA Extraction Date________________________________

PCR Run Date_____________________________________

Sample #s and location_____________________________________

**Primers for amplification:**

CLP0F: CACCATGTTGAATTTGATCATCTGCAG

CLP0R: ACTGCTTTCTCCTTCAC

***Primer working solution*:** Dilute 100µM stock to a 2µM working solution by adding 2µl of the 100µM stock to 98µl $H_2O$.

**Sample Preparation:** Extract RNA from sample using the RNeasy Plant Mini Kit (Qiagen – catalog number 74904). Follow manufacturer's instructions. Proceed to cDNA synthesis ASAP.

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**cDNA Synthesis:** Use SuperScript® IV First-Strand cDNA Synthesis Reaction (Invitrogen- Catalog number: 18091050). Follow manufacturer's instructions using 2 µM gene-specific primer as shown in master mix below.

*Master Mix for Step 1- Anneal primer to template RNA*

A. Combine following components in a reaction tube.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| 2 µM CLP0R | 1 µl | | |
| 10 µM dNTP mix | 1 µl | | |
| H20 | 6 µl | | |
| **Total** | **8 µl** | | |

B. Add 5 µl of RNA/Control, mix, and briefly centrifuge the components.

C. Heat RNA-primer mix at 65° for 5 minutes, then incubate at least 1 minute on ice.

*Master Mix for Step 2- Prepare RT reaction mix*

A. Vortex and briefly centrifuge 5x SSIV Buffer.

B. Combine following components in a reaction tube.

| Component | Volume for 1RXN | # Reactions | Total Volume µl |
|---|---|---|---|
| 5xSSIV Buffer | 4 µl | | |
| 100 mM DTT | 1 µl | | |
| RNaseOUT Recombinant RNase Inhibitor | 1 µl | | |
| SuperScript® IV Reverse Transcriptase | 1 µl | | |
| **Total RT reaction Mix** | **7 µl** | | |

C. Cap tube, mix and briefly centrifuge the contents.

*Step 3- Combine annealed RNA and RT reaction mix*

*Step 4 Incubate reaction*

**PCR program:**
55°C – 10 min
80°C – 10 min

**Omit Optional Step 5 and proceed to PCR amplification.**

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**PCR Amplification**

***Primer working solution*:** Dilute 100µM stock to a 10µM working solution by adding 10µl of the 100µM stock to 90µl $H_2O$.

| Reagent | 25 µl | 50 µl | # Reactions | Total Volume µl | Check off |
|---|---|---|---|---|---|
| $H_2O$ | 17.525 | 35.05 | | | |
| 10X buffer | 2.5 | 5 | | | |
| $MgCl_2$ (50 mM) | 0.875 | 1.75 | | | |
| dNTP (10 mM) | 0.5 | 1 | | | |
| CLPOF (10 mM) | 0.5 | 1 | | | |
| CLPOR (10 mM) | 0.5 | 1 | | | |
| Platinum Taq | 0.1 | 0.2 | | | |
| **Total** | **22.5 µl** | **45 µl** | | | |
| Template cDNA | 2.5 µl | 5 µl | | | |

Vortex master mix. Pipette 22.5µl of mix into each 0.2ml PCR tube. Add 2.5µl of undiluted sample cDNA, water, or positive control to the appropriate labeled tube (total volume 25µl). Close caps and place in PCR machine.

**PCR program:**
95°C – 1 min
35 cycles of:
95°C— 15 sec, 62°C – 20 sec, 56°C – 10 sec, 72°C – 40 sec
72°C— 3 min
4°C – ∞

**Prepare agarose gel:** 1% agarose gel(s) in 1x TAE buffer: 0.5g agarose → 50ml 1x TAE buffer. Microwave the agar for 1 – 1.5 min, stirring occasionally by swirling. Let the agar cool for 3-4 minutes. Add 1.25µl EtBr to the agar and pour the agar in a clean gel cast. Insert combs as appropriate – prepare enough wells for the samples to be run. Let agar solidify.

**Load samples in gel:** Mix 20µl sample with 2µl 10X loading buffer for a total of 20µl for each sample and both controls. Mix by pipetting up and down and load mixture into the gel well. Load DNA ladder into gel. Load into the left most lane for small gels, and into the left lane and middle lane for large gels. Gels are run in 1X TAE buffer for 40 minutes at 70V (constant). View the gel on a UV illumination box and document the assay results using a digital imaging system. Save the image to the results folder as the test run and sample number (ex: ITS 867). Print a hard copy of the results to attach to this protocol. Provide a copy of the protocol and results to Dr. Conner and file a copy in the appropriate notebook in the lab.

**Expected Results:**

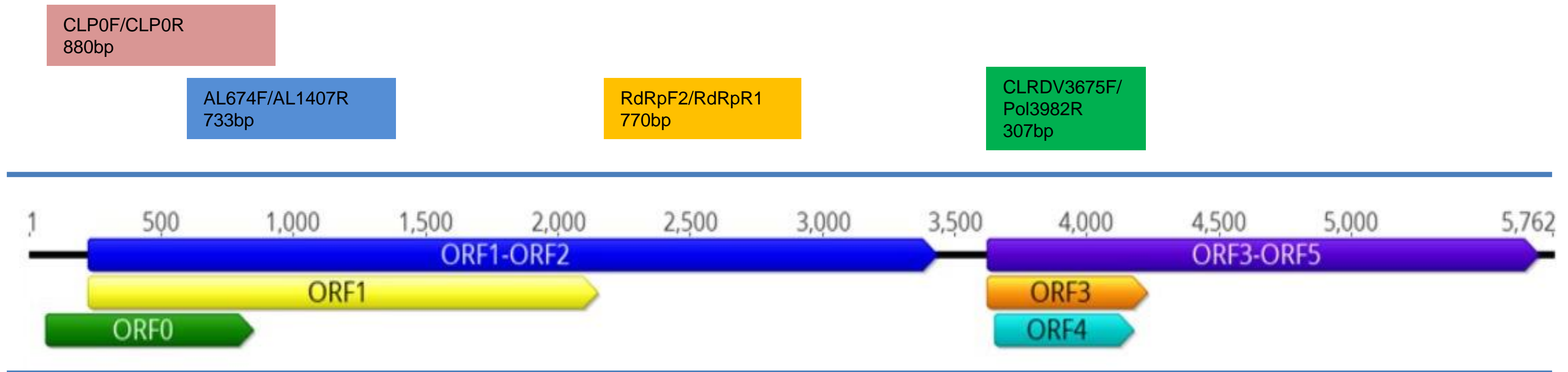
Fragment/band size: 790 bp

**Reference:**

Cascardo, R.S.; Arantes, I.L.G.; Silva, T.F., Sachetto-Martins, G., Vaslin, M.F.S., and Correa, R.L. 2015. Function and diversity of P0 proteins among cotton leafroll dwarf virus isolates. Virology Journal 12:123.

# Polerovirus Genome

## Primers




P0 – silencing suppressor

P3 – coat protein

P4 – movement protein

P1 – VPg precursor

P1-P2 – RNA dependent RNA polymerase

P3-P5 – aphid transmission protein

## Function

# Brazos Co., TX - July 24, 2019

Robert Vaughn, TAMU

# Brazos Co., TX - July 24, 2019

Robert Vaughn, TAMU

# Clemson Sentinel Plots – August 4, 2019

Jeremy Greene, Clemson

# Clemson Sentinel Plots – August 26, 2019

Jeremy Greene, Clemson

Alabama PBU – July 24, 2019

Kassie Conner, AU

Alabama GCREC – July 26, 2019

Ed Sikora, AU

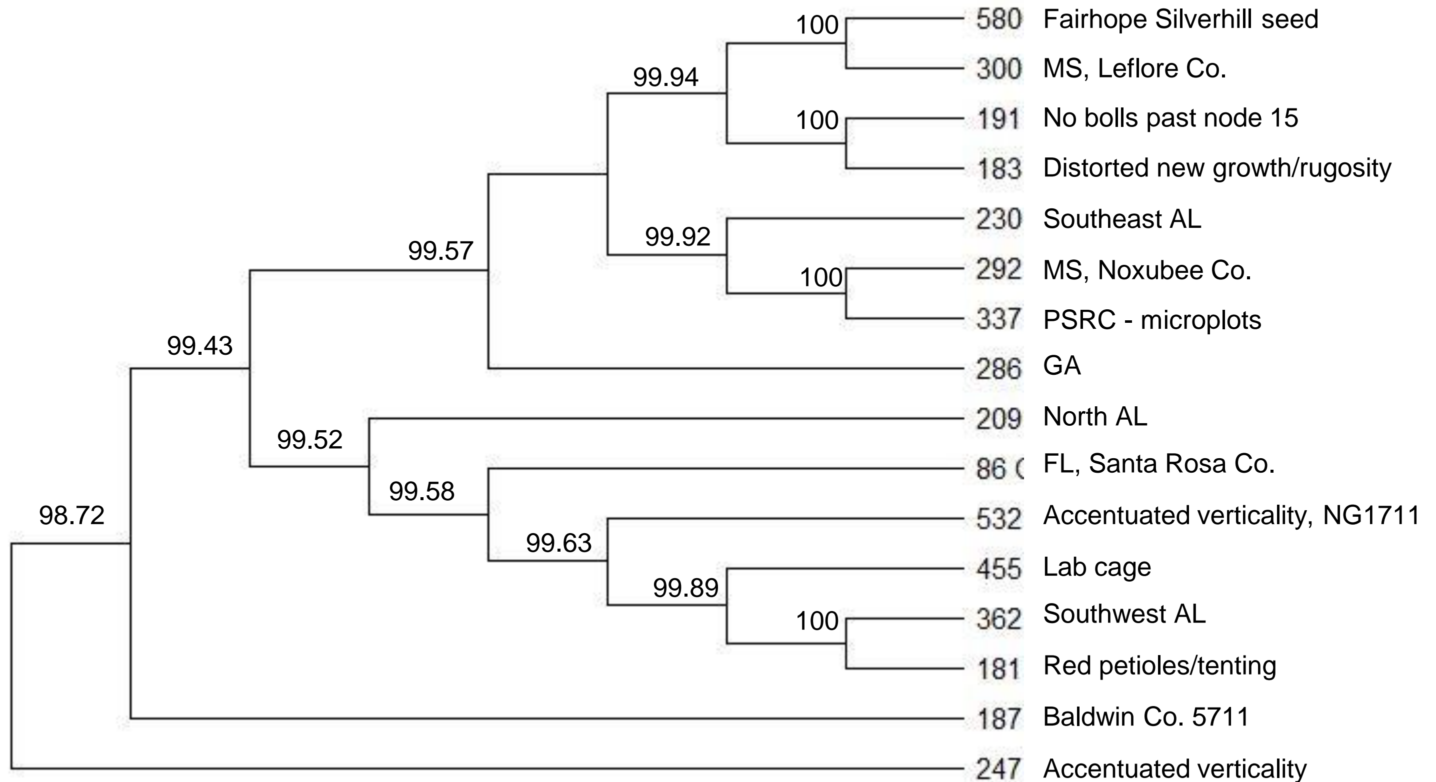
# Alabama PBU – September 2, 2019

Kassie Conner, AU

# Alabama Cullers Rotation – August 9, 2019

Kassie Conner, AU

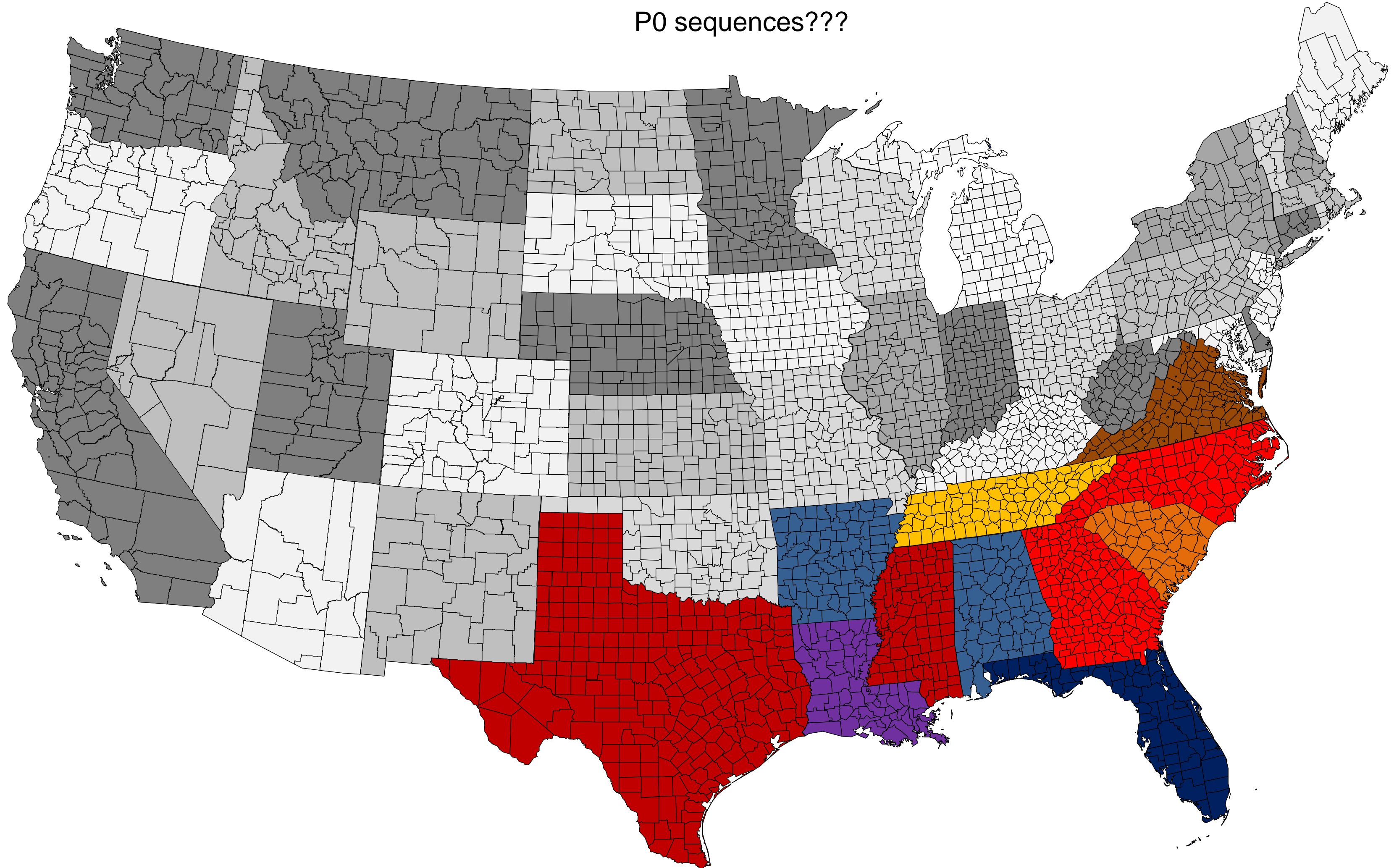
# P0 Sequences



# Sentinel Plot Cooperators

CLRDV isolates

P0 sequences???



**Characterization of the complete genome and P0 protein for a previously unreported genotype of cotton leafroll dwarf virus, an introduced polerovirus in the USA**

**Published Online:** 4 Oct 2019 | https://doi.org/10.1094/PDIS-06-19-1316-RE

- Need to compare the whole genome for isolates from different states now