Cotton leafroll dwarf virus (CLRDV) Identification

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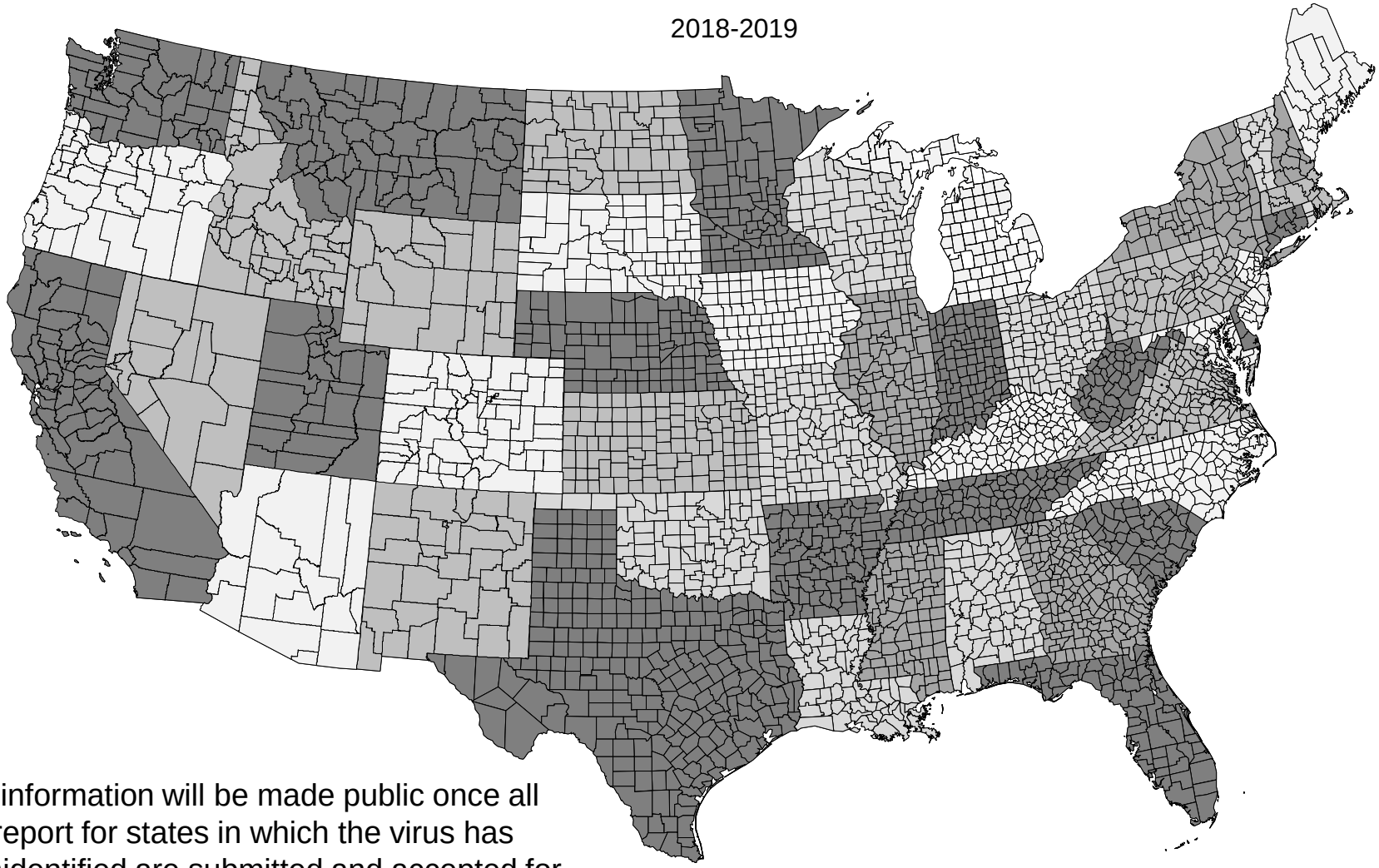
extension
ALABAMA A&M & AUBURN UNIVERSITIES

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 CP low	F R	ATGAATACGGTCGTGGGTAG CTATTTTGGATTGTGGAATT	433 bp		4	N. benth
CLRDV-For1 CLRDV-Rev1	F R	ACGACGAAGACGAGGAGGTC GAACCGGAGGATGTTGAAGAGG	249 bp	MS	1	MS primers first set
AL674F AL1407R	F R	CCGTAGCGGTCATCGTCTTT TAACCCTGACACAGTGGGGA	733 bp	AZ	2	AZ primers
AtropaNad2.1a AtropaNad2.2b	F R	GGACTCCTGACGTATACGAAGGA AGCAATGAGATTCCCCAATATCAT	850 bp 188 bp	Australia	8	Internal control
CLRDV3675F Pol3982R	F R	CCACGTAGRCGCAACAGGCGT CGAGGCCTCGGAGATGAACT	307 bp	Australia	7	Current primers Nested PCR CP – 2nd round
Pol3628F Pol4021R	F R	TAATGAATACGGYCGYGGSTAG GGRTCMAVYTCRTAAGMGATSGA	393 bp	Australia	Sharman, unpublished	Nested PCR CP – 1st round
Pol3628F Pol3982R	F R	S/A S/A	350 bp	Australia	Sharman, unpublished	General Polerovirus RT-PCR 5' end of CP gene
Pol3870F AS3	F R	ATCACBTTCGGGCCGWSTYTWTCAGA CACGCGTCIACCTATTTIGGRTTITG	370 bp	Australia	6	General Polerovirus RT-PCR 3' end of CP gene
CLRDV_ORF0F CLRDV_ORF0R	F R	GTCTCGTGTATGTTGAATTTGATCAT CTCAACTGCTYTCTCCTTCAC	790 bp	Australia	Sharman, unpublished	Nested PCR P0 gene – 1st round
CLRDV90F CLRDV794R	F R	GCAGARTYTCTTCCGCAGCTCT CGCCTTCATCGTCAAAATGGTA	705	Australia	Sharman, unpublished	Nested PCR P0 gene – 2nd round
COXf COXr	F R	GTA TGC CAC GTC GCA TTC CAG A GCC AAA ACT GCT AAG GGC ATT C		USDA/APHIS/CPHST WI-B-T-D-2	5	Internal control Cytochrome oxidase
Nad5f Nad5mr	F R	GAT GCT TCT TGG GGC TTC TTK TT ATC TCC AGT CAC CAA CAT TRG CAT AA	180	USDA/APHIS/CPHST WI-B-T-1-31		Internal control Nad5 gene
CLP0F CLP0R	F R	CAC CAT GTT GAA TTT GAT CAT CTG CAG ACT GCT TTC TCC TTC AC		Brazil	3	P0 gene
CLRDV-RdRpF2 CLRDV-RdRpR1	F R	GGA GCC GCA CAA ACA AGC TAA 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
3. 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.
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.
5. Li, W. H., Hartung, J. S., and Levy, L. 2006. Quantitative real-time PCR for detection and identification of Candidatus Liberibacter species associated with citrus huanglongbing. Journal of Microbiology Methods 66:104-115.
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7. 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.
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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		
H20	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.

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.**

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.

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.

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			
CLP0F (10 mM)	0.5	1			
CLP0R (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

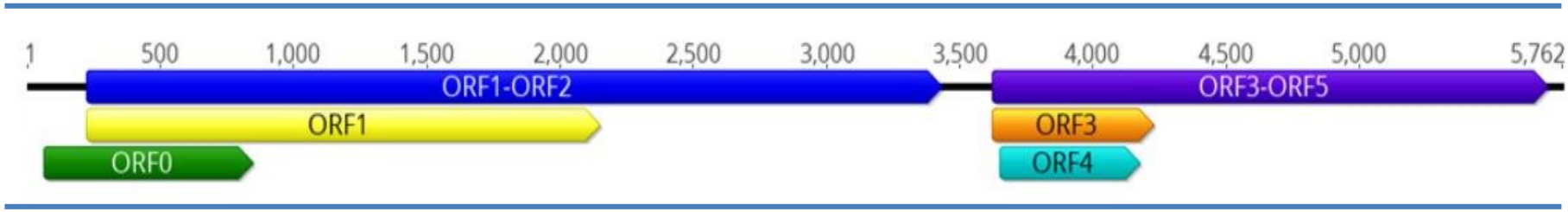
Primers

CLP0F/CLP0R
880bp

AL674F/AL1407R
733bp

RdRpF2/RdRpR1
770bp

CLRDV3675F/
Pol3982R
307bp



P0 – silencing suppressor

P1 – VPg precursor

P1-P2 – RNA dependent RNA polymerase

P3 – coat protein

P4 – movement protein

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

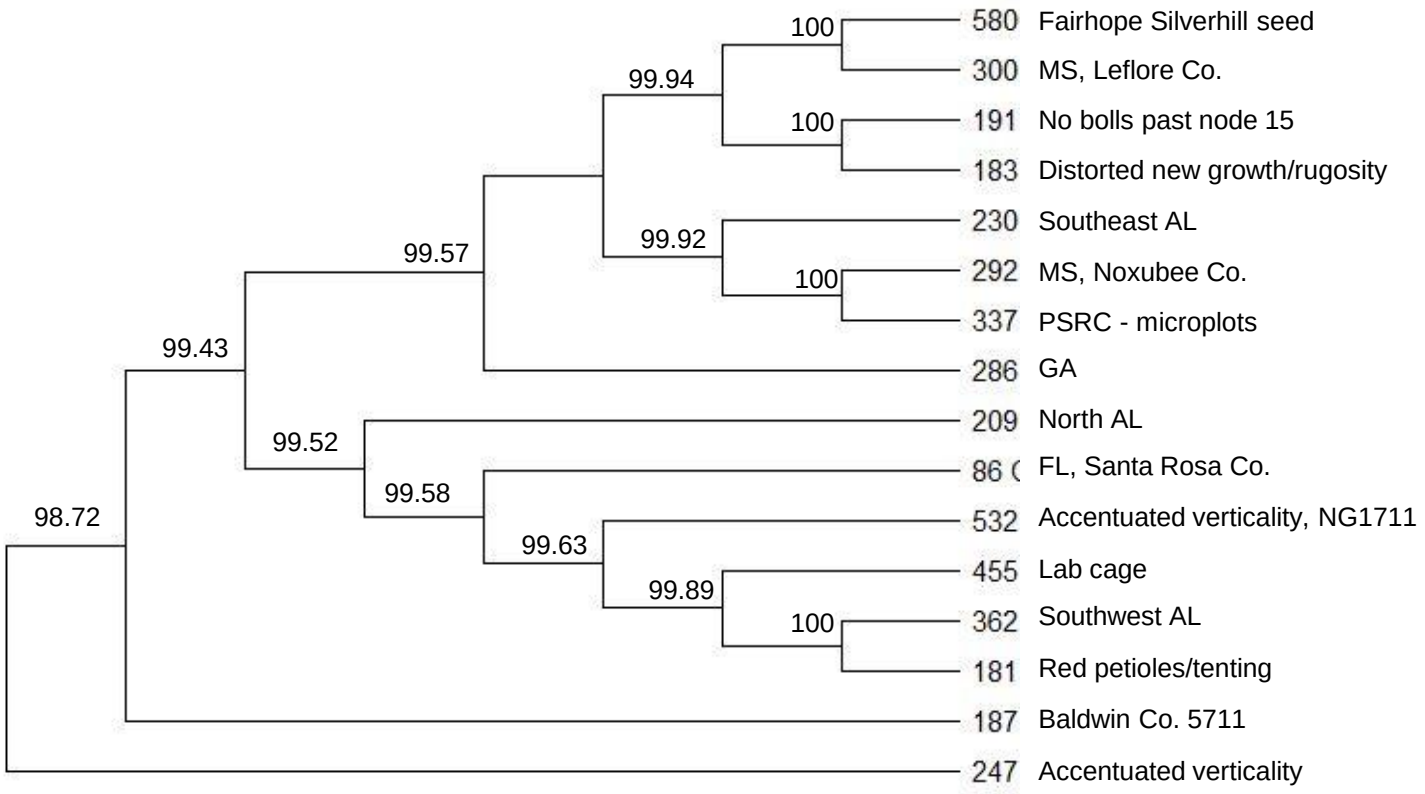
Alabama PBU – September 2, 2019

Kassie Conner, AU

Alabama Cullers Rotation – August 9, 2019

Kassie Conner, AU

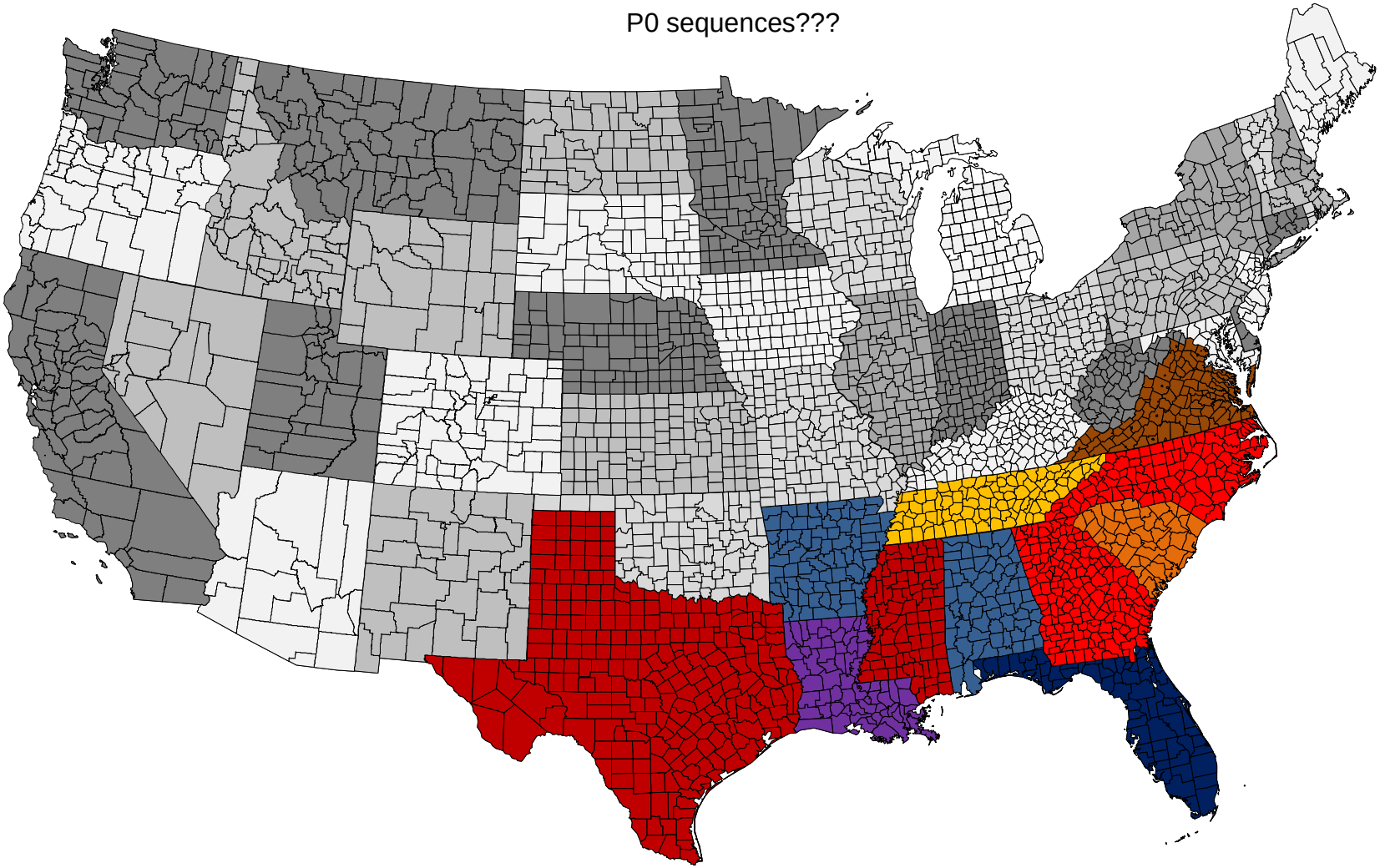
P0 Sequences



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

A. Sofia Avelar, Roberto Ramos Sobrinho, Kassie Conner, Robert L Nichols, Kathy S Lawrence, and Judith K Brown

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