Bacterial Gall of *Loropetalum chinense* caused by *Pseudomonas amygdali* pv. *loropetali* pv. nov.

Carrie Lapaire Harmon,[†] Sujan Timilsina, and John Bonkowski, University of Florida Department of Plant Pathology, Gainesville, FL; Debra D. Jones and Xiaolan Sun, Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, FL; Gary E. Vallad, University of Florida Department of Plant Pathology, GREC, Wimauma, FL; Laura Ramos Sepulveda and Carolee Bull, The Pennsylvania State University, University Park, PA; and Jeffrey B. Jones,[†] University of Florida Department of Plant Pathology, Gainesville, FL

Abstract

In 2012, stem gall samples on *Loropetalum chinense* were sent to Florida diagnostic labs from Alabama and Florida nurseries. A fluorescent pseudomonad was consistently isolated from the galls. The organism was originally identified in Alabama based on 16S rRNA sequencing as *Pseudomonas savastanoi*, which causes a production-limiting disease of olive. The loropetalum strains and reference strains were compared using LOPAT, Biolog, fatty acid analysis, multilocus sequence analysis (MLSA), and pathogenicity tests. The LOPAT tests placed the loropetalum strains within *Pseudomonas syringae*. Biolog and fatty acid analysis placed the strains in various pathovars of *P. syringae* and *P. savastanoi*,

respectively. MLSA of a set of housekeeping genes separated the loropetalum strains from the olive knot-inducing strains. Our work indicates there is a need to use more tests than 16S rRNA to accurately diagnose new bacterial diseases. In pathogenicity tests, the loropetalum strains produced galls only on loropetalum, but not on olive, mandevilla, or almond, indicating this strain is not a threat to the olive industry. Based on the pathogenicity assays and molecular tests, loropetalum strains represent a distinct and new pathovar, *P. amygdali* pv. *loropetali* pv. nov., for which the strain PDC13-208 (= DSMZ 105780^{PT}) has been designated as the pathotype strain.

The *Pseudomonas syringae* complex includes 10 *Pseudomonas* species and at least 60 pathovars (Young 2010). There is some debate regarding the classification of *Pseudomonas* species and the subspecies/pathovars, including the olive strain of the *P. syringae/savastanoi* bacterium (Gardan et al. 1992; Hwang et al. 2005; Penyalver et al. 2000; Ramos et al. 2012; Sarkar and Guttman 2004). Historically, nomenclature has been confused by the use of synonyms for the same pathogen found in different locations and classified as different species (Bull and Koike 2015; Young 2010). Differentiation of *Pseudomonas* strains is further complicated by a lack of understanding of fundamental physiology and metabolism (Young 2010). Additionally, sequencing of 16S rRNA, commonly used to differentiate bacteria in diagnostic laboratories, cannot resolve differences between many *Pseudomonas* species (Janda and Abbott 2007; Young 2010). This inability to easily differentiate taxa despite the genomic diversity has led to confusion concerning the taxonomy of the *P. syringae* complex (Young 2010).

P. syringae sensu lato, based on genomic comparisons using DNA-DNA hybridization, consists of nine distinct genomospecies within this pathogen complex (Gardan et al. 1999). Bull et al. (2011) determined that phylogenies based on four housekeeping genes, including *rpoD*, were useful for delineating eight genomospecies. Gardan et al. (1999) placed *P. savastanoi* in genomospecies 2, indicating that it is a synonym of *P. amygdali*, as are *P. ficuserectae* and *P. meliae*. Additionally, Marques et al. (2008), using rep-PCR for comparisons, demonstrated that *P. tremae* was also a synonym of *P. amygdali*. Likewise, analysis with *rpoD* confirmed that *P. savastanoi*, *P. ficuserectae*, *P. meliae*, and *P. tremae* are members of genomospecies 2 (also called phylogroup 3) and are thus synonyms

of *P. amygdali* and distinct from *P. syringae sensu stricto* and the other genomospecies (Parkinson et al. 2011).

Loropetalum chinense, commonly known as loropetalum or fringe flower, is a common woody ornamental in the Mid- and Deep South that is propagated by cuttings. In 2012, we received loropetalum samples from Alabama and Florida in diagnostic laboratories in Florida and isolated a fluorescent pseudomonad. In 2013, Conner et al. (2013) reported on a bacterial gall on loropetalum that was identified as *P. savastanoi* based on phenotypic characteristics along with sequence analysis of partial sequences of the 16S rRNA gene. As a result of the concern of *P. savastanoi* potentially entering the fledgling olive industry in Florida, these fluorescent pseudomonads isolated from galls on loropetalum plants were analyzed using a polyphasic approach to characterize the loropetalum strains from both Alabama and Florida. *P. savastanoi* pv. *savastanoi*, the causal agent of olive knot disease, is a member of the *P. syringae* complex. *P. savastanoi* pv. *savastanoi* exhibits variation in host range and virulence and is both phenotypically and genotypically heterogeneous (Ramos et al. 2012). As a result of the lower sequence identity with the partial sequence of the *P. savastanoi* type strain than with *P. syringae* pv. *eriobotryae*, it was necessary to more precisely define the taxonomic position of the strains. The loropetalum and reference strains were compared using LOPAT, Biolog, fatty acid analysis, multilocus sequence analysis (MLSA), and pathogenicity tests. Results from the LOPAT tests placed the loropetalum strains in *P. syringae*, while Biolog and fatty acid analysis tests placed the strains in various pathovars of *P. syringae* and *P. savastanoi*, respectively. Based on partial sequence analysis of the 16S rRNA, a representative loropetalum strain had 100% sequence identity with *P. syringae* pv. *eriobotryae* and 99.9% sequence identity with *P. meliae* (a synonym of *P. amygdali*), *P. savastanoi* pv. *savastanoi*, and *P. amygdali*. MLSAs of a set of housekeeping genes placed the loropetalum strains in genomospecies 2-Group A, which also contains all the species and pathovar *eriobotryae* mentioned above. In pathogenicity tests in which all the pathotypes in genomospecies 2-Group A and four pathotypes in Group B were compared, the loropetalum strains produced galls only on loropetalum, but not on olive, mandevilla, or almond. Sequence analysis of *iaaL* placed the loropetalum strains separate from *P. savastanoi* pv. *savastanoi* and other gall-forming pseudomonads including the *P. amygdali* type strain on almond, LMG 13184^T.

We determined that the loropetalum strains are members of genomospecies 2 and cluster more closely with the type strain of

[†]Corresponding authors: Carrie Lapaire Harmon, E-mail: clharmon@ufl.edu; and Jeffrey B. Jones, E-mail: jbjones@ufl.edu

This research was supported in part by the University of Florida IFAS Plant Diagnostic Center and projects funded by the USDA, including the USDA-NIFA Southern Plant Diagnostic Network and the USDA Agriculture and Food Research Initiative Plant Biosecurity Program.

Accepted for publication 12 October 2017.

P. amygdali (LMG 13184^T) than with *P. savastanoi* pv. *savastanoi* (LMG 2209^{PT}) or *P. syringae* pv. *erobotryae* (LMG 2184^{PT}). Here we propose a new pathovar, *P. amygdali* pv. *loropetalii* pv. nov. (DSMZ XXXX^{PT}), as the agent causing galls on loropetalum.

Materials and Methods

Bacterial strains. Bacterial strains analyzed included the following: 1) type and pathotype strains (Table 1) obtained from BCCM/LMG (Belgian Coordinated Collection of Microorganisms/Laboratory for Microbiology, Ghent University, Belgium), NCPPB (National Collection of Plant Pathogenic Bacteria), GRBio (The Global Registry of Biodiversity Repositories), and the ICMP (International Collection of Microorganisms from Plants)/Landcare Research - Manaaki Whenua; 2) 32 strains isolated from loropetalum obtained from the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (FDACS, DPI); 3) one strain isolated from a routine diagnostic sample submitted to the UF/IFAS Plant Diagnostic Center from a nursery in Alabama; and 4) one strain provided by K. Conner

(Plant Disease Clinic, Auburn University, Auburn, AL) to represent the strains studied in 2012 (Conner et al. 2013). All strains were stored at -80°C, and were grown on nutrient agar (BBL, Becton Dickinson and Co., Cockeysville, MD) at 27°C, except *P. meliae* (LMG 02220^T), *P. tremae* (LMG 22121^T), and *P. amygdali* (LMG 13184^T), which were grown at 27°C on medium 51 as described and recommended by BCCM (<http://bccm.belspo.be/>).

Biochemical and physiological tests. All strains were tested for fluorescence on King's medium B (KMB) (Schaad et al. 2001). Each strain was streaked onto a KMB agar plate and incubated at 27°C for up to 72 h. Fluorescence was tested by exposing the plate to long wavelength UV light. All strains were analyzed with Biolog 96-well plates using a plate reader (Emax Endpoint microplate reader, Molecular Devices LLC, Sunnyvale, CA) and the Biolog Microbial ID GenIII Microlog plates, software package, and library (Biolog, Hayward, CA). Fatty acid analysis of these strains was performed using the MIDI Sherlock Microbial Identification System. Dendrograms were developed with the strains including several library

Table 1. Bacterial strains used in this study

Strain designation ^a	Bacterial species	Host	Source	Biolog results
LMG 2184 ^{PT}	<i>Pseudomonas syringae</i> pv. <i>erobotryae</i>	<i>Eriobotrya japonica</i>	BCCM/LMG	<i>P. syringae</i> pv. <i>syringae</i>
LMG 2209 ^{PT}	<i>P. savastanoi</i> pv. <i>savastanoi</i>	<i>Olea europaea</i>	BCCM/LMG	<i>P. savastanoi</i> pv. <i>nerii</i>
LMG 2245 ^{PT}	<i>P. savastanoi</i> pv. <i>phaseolicola</i>	<i>Phaseolus vulgaris</i>	BCCM/LMG	<i>P. syringae</i> pv. <i>phaseolicola</i>
LMG 2220 ^T	<i>P. meliae</i>	<i>Melia azedarach</i>	BCCM/LMG	<i>P. syringae</i> pv. <i>phaseolicola</i>
LMG 5385	<i>P. savastanoi</i> pv. <i>nerii</i>	<i>Nerium oleander</i>	BCCM/LMG	<i>P. savastanoi</i> pv. <i>nerii</i>
LMG 13184 ^T (= CFBP 3205 ^T)	<i>P. amygdali</i>	<i>Prunus dulcis</i>	BCCM/LMG	<i>P. syringae</i> pv. <i>delphinii</i>
LMG 22121 ^T	<i>P. tremae</i>	<i>Trema orientalis</i>	BCCM/LMG	<i>P. syringae</i> pv. <i>glycinea</i>
LMG5066 ^{PT}	<i>P. savastanoi</i> pv. <i>glycinea</i>	<i>Glycine max</i>	BCCM/LMG	ND ^b
LMG5694 ^T	<i>P. ficuserectae</i>	<i>Ficus erecta</i>	BCCM/LMG	ND
LMG5541 ^{PT}	<i>P. syringae</i> pv. <i>ciccaronei</i>	<i>Ceratonia siliqua</i>	BCCM/LMG	ND
LMG5668 ^{PT}	<i>P. syringae</i> pv. <i>myricae</i>	<i>Myrica rubra</i>	BCCM/LMG	ND
LMG2349 ^{PT}	<i>P. syringae</i> pv. <i>ulmi</i>	<i>Ulmus</i> sp.	BCCM/LMG	ND
NCPBP3681 ^{PT}	<i>P. syringae</i> pv. <i>aesculi</i>	<i>Aesculus indica</i>	NCPBP	ND
NCPBP3682 ^{PT}	<i>P. syringae</i> pv. <i>hibisci</i>	<i>Hibiscus rosa sinensis</i>	NCPBP	ND
NCPBP3618 ^{PT}	<i>P. syringae</i> pv. <i>rhapiolepidis</i>	<i>Rhapiolepis umbellata</i>	NCPBP	ND
ICMP11894 ^{PT}	<i>P. syringae</i> pv. <i>cunninghamiae</i>	<i>Cunninghamia lanceolata</i>	ICMP	ND
MD340a	<i>P. syringae</i> pv. <i>tabaci</i>	<i>Nicotiana tabacum</i>		ND
PDC13-198, 199, 200, 201, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 227, 228		<i>Loropetalum chinense</i>	FDACS, DPI; Florida nurseries	<i>P. savastanoi</i> pv. <i>nerii</i> (15); <i>P. syringae</i> pv. <i>phaseolicola</i> (2); pv. <i>fraxini</i> (7); <i>P. savastanoi</i> ; <i>P. agaraci</i> (3); <i>P. syringae</i> pv. <i>antirrhini</i> (1); <i>P. syringae</i> pv. <i>delphinii</i> (1)
PDC12254		<i>Loropetalum chinense</i>	UF/IFAS PDC; Alabama nursery	<i>P. savastanoi</i> pv. <i>nerii</i>
PDL384		<i>Loropetalum chinense</i>	Auburn PDL; Alabama nursery	ND

^a T = Type strain, ^{PT} = pathotype strain.

^b Not determined.

Table 2. GenBank accession numbers for multilocus sequence alignment study

Strains	16s ^a	gyrB	gapI	gltA	rpoD	iaaL
PDC13-206	NA	KM593940	KM593956	KM593966	KM593986	KM593976
PDC13-208 ^{PT}	KM593950	KM593941	KM593957	KM593967	KM593987	KM593977
PDC13-219	NS	KM593942	KM593958	KM593968	KM593988	KM593978
PDC12-254	NA	KM593943	KM593959	KM593969	KM593989	KM593979
PDL474	KM593952	KM593944	KM593960	KM593970	KM593990	KM593980
LMG2184	KM593951	KM593945	KM593961	KM593971	KM593991	KM593981
LMG2209	KM593953	KM593946	KM593962	KM593972	KM593992	KM593982
LMG2245	NA	KM593947	KM593963	KM593973	KM593993	KM593983
LMG2220	KM593954	KM593948	KM593964	KM593974	KM593994	KM593984
LMG5385	KM593955	KM593949	KM593965	KM593975	KM593995	KM593985

^a NA = Strain was not sequenced for 16s gene, NS = sequence was not submitted to NCBI.

references (Sherlock version 6.1, TSBA6 database version 6.10, MIDI Labs, Inc., Newark, DE). All strains were subjected to the LOPAT tests, which included assaying for levan production, oxidase reaction, potato rot, arginine dihydrolase activity, and hypersensitive response on tobacco (Schaad et al. 2001). To determine hypersensitivity response, strains were inoculated by infiltrating bacterial suspensions adjusted to 1×10^8 CFU suspension into 'Hicks' tobacco

leaves and 'Bonnie Best' tomato leaves. Inoculations were checked daily for confluent necrosis for up to 3 days.

Pathogenicity tests. Stem inoculations of the Florida strains (PDC13-198 to PDC13-228) onto host plants were conducted by two methods, five to 10 times on a stem of each host type: 1) syringe injection of bacterial suspensions adjusted to 1×10^8 CFU/ml; or 2) insertion of a sterile toothpick dipped in a 24-h culture from a

Fig. 1. Dendrogram showing relationships of fluorescent strains isolated from loropetalum and other fluorescent pseudomonads based on fatty acid analysis.

Table 3. Comparison of fatty acid profiles of loropetalum strains with select pseudomonads^a

Fatty acid	LMG 2220 ^T	<i>P. amygdali</i> ^b	LMG 2245 ^{PT}	LMG 2184 ^{PT}	LMG 5385	LMG 2209 ^T	Loropetalum strains
10:0	0.12	TR ^c	0.07	0.07	0.11	0.09	0.07 (0.03)
10:0 3OH	3.96	14.3 (4.5)	2.78	2.76	3.36	3.17	3.25 (0.20)
12:0	5.24	5.1 (0.4)	4.78	4.42	4.14	4.23	4.40 (0.25)
11:0 iso 3OH			0.48		0.07		
13:0 iso			0.12				
13:1 at 12-13					0.07		
12:0 2OH	3.46	2.0 (0.6)	2.89	3.04	3.06	3.06	3.07 (0.15)
12:1 3OH		TR					
12:0 3OH	5.02	4.4 (1.0)	4.24	4.06	4.23	3.94	4.04 (0.13)
14:0	0.30	0.2 (0.1)	0.25	0.22	0.21	0.27	0.66 (0.13)
Sum In Feature 3	33.77	14.1	29.09	35.10	28.81	33.40	37.03 (1.29)
16:0	29.30	17.2 (4.3)	28.48	27.89	25.44	29.39	29.44 (0.75)
Sum In Feature 4	0.17						
17:0 iso			1.95	0.23	0.29	0.21	
17:0 cyclo		2.6 (0.6)	6.51	1.11	0.50	0.60	0.45 (0.32)
17:00				0.17			
16:0 3OH	0.58	7.9 (2.6)			0.08	0.16	
Sum In Feature 8	16.70	22.7 (2.8)	16.78	19.07	25.24	18.98	14.75 (1.41)
18:1 w5c					0.11		0.09 (0.03)
18:0	0.80	0.5 (0.1)	0.83	1.35	3.06	1.43	1.58 (0.29)
18:1 w7c 11-methyl	0.27		0.18	0.40	1.02	0.75	0.63 (0.15)
Sum Feature 7		0.4 (0.3)	0.34	0.11		0.20	0.34 (0.11)
19:0 10-methyl	0.30	4.7 (2.4)				0.14	0.04 (0.02)
19:0 cyclo w8c		0.2 (0.2)	0.22				
19:0 CYCIO 11-12							
19:0 cyclo w8c		0.2 (0.2)					
20:1 w7c					0.21		0.16 (0.05)

^a Strains: *P. meliae* LMG 2220; *P. amygdali*; *P. savastanoi* pv. *phaseolicola* LMG 2245; *P. syringae* pv. *erobotryae* LMG 2184; *P. savastanoi* pv. *savastanoi* LMG 5385; *P. savastanoi* pv. *savastanoi* LMG 2209; Loropetalum strains (14).

^b Fatty acid data for *P. amygdali* were taken from Table 5 in Stead (1992).

^c TR = Trace.

nutrient agar plate into young stems. Host plants included for this study were almond (*Prunus dulcis*), loropetalum (*L. chinense*), mandevilla (*Mandevilla sanderi*), oleander (*Nerium oleander*), and olive (*Olea europaea*). Plants were maintained in a greenhouse at the University of Florida. Inoculations of the two Alabama strains (G12-254 and PDL384) and the six LMG strains (LMG 2184^{PT}, 2209^{PT}, 2245^{PT}, 2220^T, 5385^{PT}, 13184^T, and 22121^T) were conducted on almond, loropetalum, oleander, and olive in a containment greenhouse at FDACS-DPI, Gainesville, FL. Each strain was inoculated as above. An additional set of pathogenicity experiments were conducted to determine pathogenicity on loropetalum by types and pathotypes within in *P. amygdali*. In these experiments, five loropetalum strains (DSMZ 105780^{PT}, PDC13-254, PDC13-672, PDC13-206, and PDC13-219) and 13 types and pathotypes from genomospecies 2 were evaluated for gall formation: *P. savastanoi* pv. *glycinea* (LMG 5066^{PT}), *P. amygdali* (LMG 13184^T), *P. ficuserectae* (LMG5694^T), *P. meliae* (LMG 2220^T), *P. syringae* pv. *ciccaronei* (LMG 5541^{PT}), *P. syringae* pv. *myricae* (LMG5668^{PT}), *P. syringae* pv. *erobotryae* (LMG 2184^{PT}), *P. syringae* pv. *ulmi* (LMG 2349^{PT}), *P. syringae* pv. *aesculi* (NCPBPB 3681^{PT}), *P. syringae* pv. *hibisci* (NCPBPB 3682^{PT}), *P. syringae* pv. *tabaci* (NCPBPB 1427^{PT}), *P. syringae* pv. *cunninghamiae* (ICMP 11894^{PT}), and *P. syringae* pv. *rhapiolepidis* (NCPBPB 3618^{PT}). Multiple inoculations (at least three) were made on a single stem by

inserting a sterile toothpick coated with a test strain into young stems and then covering the wounds with Parafilm to keep them moist. The assay was carried out in a growth chamber set to 80% relative humidity and at 27°C during 14 h of light and 25°C when the chamber was dark. The experiment was repeated twice with three replicate stems inoculated per strain in each experiment. The results were identical in both experiments. The bacteria were reisolated from galled tissue and a rep-PCR using BOX1A primers was performed to confirm that the strain was the same as originally inoculated. Plants used in this study were grown using clonal propagation of each host type in order to ensure differences in pathogenic effects were a direct result of the strain. Syringe injection of sterile tap water and sterile toothpicks dipped in sterile water served as negative controls when inoculated onto each of the host plant types. Plants were checked weekly for symptom development.

Phylogenetic analysis. Genomic DNA from loropetalum strains PDL384A, PDC13208, and PDC13219 and LMG strains listed in Table 1 was extracted using the Qiagen DNeasy plant mini kit (cat. 69104, Qiagen, Germantown, MD). For 16S rRNA sequencing, approximately 1.4 kb of the 16S rRNA sequence was amplified from DNA of each strain using the universal primers: 63f (5'-CAGGCCTAACACATGCAAGTC-3') and reverse primer 1387r (5'-GGGCGGWTGTACAAGGC-3') (Marchesi et al. 1998). Given that multiple 16S gene copies are common in *Pseudomonas* spp.

Fig. 2. Phylogenetic distribution of *Pseudomonas* strains isolated from loropetalum using 16S rRNA sequences. Maximum likelihood phylogenetic tree was constructed based on the sequence available using gamma distributed general time reversible with invariant sites model.

Fig. 3. Maximum likelihood phylogenetic distribution based on *iaa* genes for *Pseudomonas* strains isolated from loropetalum. The trees were constructed based on the model described previously.

Fig. 4. Phylogenetic distribution of *Pseudomonas* strains isolated from loropetalum using concatenated housekeeping gene sequences. Four conserved housekeeping genes were used, namely *gap1*, *gyrB*, *gltA*, and *rpoD*. Maximum likelihood phylogenetic distribution tree was constructed based on GTR + G1 model. The housekeeping genes for the loropetalum isolates were found identical to each other and were closely related to group A of genomospecies 2, *P. amygdali* strains.

(Coenye and Vandamme 2003), following PCR, the products were cloned into pGEM-T vector and transformed into *Escherichia coli* before sequencing. The cloned 16S PCR products were sequenced at the University of Florida ICBR core facility.

MLSA for the loropetalum strains and selected strains listed in Table 1 was performed by sequencing four housekeeping genes: *gyrB* (gyrase B, 507 bp), *rpoD* (RNA polymerase sigma⁷⁰ factor, 498 bp), *gap1* (glyceraldehyde-3-phosphate dehydrogenase A, 476 bp), and *gltA* (citrate synthase, 529 bp), using the MLST schema as described by Hwang et al. (2005). Amplicons were sequenced bidirectionally (Sanger sequencing, Eurofins MWG Operon, Huntsville, AL), aligned, and edited using the MUSCLE tool available within MEGA 6 (Tamura et al. 2013). Edited sequences were combined to obtain a concatenated sequence for each strain. Phylogenetic analysis was performed on concatenated sequences along with *P. syringae* pathovars (Bull et al. 2011) and other selected sequences of *P. syringae* strains from various hosts and locations displaying at most a 0.002 maximum composite pairwise distance between the strains PDL384, PDC13208, and PDC13219 and LMG strains listed in Table 1. Sequences were obtained from the Plant Associated Microbes Database (PAMDB) (<http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>) and whole genome shotgun alignments from GenBank.

The indoleacetic acid gene (*iaaL*), which is associated with knot and gall development in host plants (Penyalver et al. 2000), is widely distributed within the *P. syringae* complex (Glickmann et al. 1998), and according to Ramos et al. (2012), its phylogeny is generally congruent with the phylogeny determined using housekeeping genes. In order to amplify *iaaL*, boiled DNA from selected loropetalum strains and LMG strains listed in Table 1 was subjected to PCR using primers IAALF (GGCACCAGCGCAACATCAA) and IAALR (CGCCCTCGGAAGTCCATAC) (Penyalver et al. 2000). Following amplification, the PCR products of approximately 294 bp were

sequenced (Sanger sequencing, Eurofins MWG Operon, Huntsville, AL) and aligned with reference sequences obtained from NCBI.

For 16S rRNA, MLSA and *iaaL* sequences obtained via Sanger sequencing, alignment was done using MUSCLE alignment method (Edgar 2004) with the reference sequence available in the NCBI database. Alignments were trimmed to the same size, and sequences were deposited into the GenBank database (<https://www.ncbi.nlm.nih.gov>) for the following genes: *iaa*, 16S, *gyrB*, *rpoD*, *gap1*, and *gltA* (accession nos. KM593940 to KM593995) (Table 2). Maximum likelihood phylogenetic trees with 1,000 bootstrap samples were constructed using MEGA (Tamura et al. 2013) and RAXML (Stamatakis 2014). The general time reversible with unequal rates of nucleotide substitution model was used for the analysis.

Results

Phenotypic characterization of strains. The galls began as cankers at wounds on loropetalum and were dark with rough edges, progressing laterally until they girdled stems, causing branch dieback. The infection did not progress longitudinally or develop systemically, even when the gall was on the main stem or crown, although girdling of the stem at the crown caused death of the plant. Galls were not observed on roots or leaves. Galls were dry, and bacterial streaming was noted rarely, and only from young galled tissue. Colonies that produced a fluorescent pigment were routinely isolated on King's medium B from loropetalum stem galls. The loropetalum strains produced positive reactions for levan production and elicited HRs in tobacco and tomato. All strains were negative for oxidase, potato rot, and arginine dihydrolase activity. On nutrient agar, all the strains produced cream-colored, smooth, round, slightly undulate colonies.

Using BIOLOG, the strains from loropetalum were identified primarily as pathovars of *P. syringae* and *P. savastanoi* and in some cases other pseudomonads (Table 1). Fatty acid analysis of the Florida strains, the Alabama strains, and the reference strains indicated that the Florida strains were nearly identical (2 or less Euclidean distance [E.D.]) to each other and to the Alabama strains 12-254 and 13-474 (Fig. 1), but were less than 6 E.D. from *P. syringae* strains including *P. syringae* pv. *erobotryae*. The loropetalum strains were more distant from *P. savastanoi* oleander strain (LMG 5385) and olive strain (LMG 2209^T). However, these strains were different from the oleander, olive, *P. syringae* pv. *erobotryae* (LMG 2184^{PT}), and *P. meliae* (LMG 2220^T) strains. Fatty acid composition of the strains analyzed in this study were compared and the fatty acid composition of *P. amygdali* from a previously published article by Stead (1992) was used in this study. It is clear that the major fatty acids were present in all strains with similar percentages in all strains with the exception of *P. amygdali* (Table 3).

Sequence analyses. Sequence analysis of a 16S rRNA for a subset of loropetalum strains revealed high sequence identity with *P. syringae* pv. *erobotryae*, *P. amygdali*, *P. meliae*, and *P. savastanoi* pv. *savastanoi*. Phylogenetic analysis of 16S rRNA sequences of *Pseudomonas* strains isolated from loropetalum and other fluorescent pseudomonads showed that the representative loropetalum strains were identical to each other and to the pathotype strain of *P. syringae* pv. *erobotryae* (Fig. 2). The strains grouped with *P. syringae* pv. *erobotryae* and were slightly different from other members of genomospecies 2 identified by Gardan et al. (1999) including *P. amygdali* and *P. savastanoi* pv. *savastanoi* strains isolated from olive and oleander. Based on the *iaa* gene sequences, the loropetalum strains were identical to each other and were different from the *P. syringae* pv. *erobotryae* LMG 2184^{PT} (Fig. 3).

For MLSA, phylogenetic analysis using concatenated nucleotide sequences of four housekeeping genes (*gap1*, *gyrB*, *gltA*, and *rpoD*) of representative loropetalum strains and representative strains of all genomospecies resulted in a tree derived from the concatenated housekeeping gene sequences. The loropetalum strains had identical sequences and grouped with genomospecies 2 strains and were very close to *P. amygdali*, *P. meliae*, and *P. syringae* pv. *erobotryae* and *P. syringae* pv. *myricae* (Fig. 4). More distant from the loropetalum strains in genomospecies 2 were strains more closely related to *P. savastanoi* including *P. savastanoi* pv. *phaseolicola*.

Fig. 5. Injection inoculations of bacterial suspension of the Florida strains resulted in galls, tip dieback, and leaf drop after 4 weeks.

Pathogenicity tests. In inoculation studies, the loropetalum strains elicited galls as shown in Figures 5 and 6 only in loropetalum (Table 4). No galls were observed on wounds from the water inoculations on any of the hosts tested. Inoculations of all the strains isolated from loropetalum in Table 1 resulted in galls on loropetalum only (Table 4). Inoculations of the following pathotype and type strains listed in Table 1, which included *P. meliae*, *P. syringae* pv. *erobotryae*, *P. savastanoi* pv. *savastanoi*, *P. tremae*, and *P. amygdali*, did not produce galls on loropetalum (Table 4 and Fig. 6). Inoculation of the type or pathotype strains for the almond, oleander, and olive pathogens onto their respective hosts resulted in galls only on their respective hosts (Table 4). Additional strains listed in Table 1, which included *P. savastanoi* pv. *glycinea*, *P. savastanoi* pv. *nerii*, *P. ficuserectae*, *P. syringae* pv. *ciccaronei*, *P. syringae* pv. *myricae*, *P. syringae* pv. *ulmi*, *P. syringae* pv. *esculi*, *P. syringae* pv. *hibisci*, *P. syringae* pv. *tabaci*,

P. syringae pv. *cunninghamiae*, and *P. syringae* pv. *rhapiolepidis*, were only inoculated on loropetalum and did not produce any visible galls. The strains isolated from loropetalum produced average sized galls of 12 mm and the bacterial strains were reisolated from galls and verified by rep-PCR. On the other hand, the wounds on stems inoculated with all other strains didn't extend beyond 4 mm. Thus, the only strains causing galls on loropetalum were originally isolated from loropetalum (Fig. 6).

Discussion

We consistently isolated a fluorescent pseudomonad from galls associated with loropetalum. The bacterium was typical of the *P. syringae* complex (Lelliott et al. 1966) based on LOPAT tests. Fatty acid and Biolog analyses also placed the loropetalum strains in the *P. syringae* complex. Both techniques provided more

Fig. 6. Pathogenicity on *Loropetalum chinense* 6 weeks after inoculation. Only the loropetalum strains (upper panels) caused galls on the loropetalum. Pathotype strains of *P. amygdali* did not cause galls on this host (lower panels).

Table 4. Pathogenicity of select strains on almond, loropetalum, mandevilla, oleander, and olive^a

Strain	Almond	Loropetalum	Mandevilla	Oleander	Olive
PDC12 254A	–	+	–	–	–
PDC13-198 to PDC13-228 ^b	–	+	–	–	–
PDL384A	–	+	–	–	–
LMG 2184	–	+/-	–	–	–
LMG 2209	–	–	–	–	+
LMG 2220	–	–	–	–	–
LMG 5385	–	–	–	+	–
LMG 22121	–	–	N/A	–	–
LMG 13184	+	–	N/A	–	–

^a + = Positive reaction; – = no disease reaction; +/- = slight reaction; N/A = not tested.

^b Includes the pathotype strain PDC13-208^{PT} (DSMZ 105780^{PT}).

comprehensive phenotypic information on the strains. Pathogenicity tests confirmed that the loropetalum strains are specific to loropetalum and not pathogenic on almond, mandevilla, oleander, or olive. Furthermore, none of the other gall-producing species or pathovars produced galls on loropetalum although *P. syringae* pv. *erobotryae* produced a slight reaction on loropetalum.

Sequence analysis of the 16S rRNA placed the selected strains most closely with *P. syringae* pv. *erobotryae* and slightly more distant from the validly published species *P. savastanoi*, *P. meliae*, and the type strain of *P. amygdali*. In MLSA using the four housekeeping genes, the loropetalum strains clustered most closely with *P. amygdali*, were slightly different from *P. meliae*, and more distant from *P. savastanoi* strains. Phylogenetic analysis of the *iaa* target of the Florida strains, the published Alabama strain (strain PDL384), and the LMG type strains indicated that the loropetalum strains are different from the *P. savastanoi iaa* gene.

Gardan et al. (1999) identified nine genomospecies of which genomospecies 2 consisted of *syringae* pathovars including *lachrymans*, *erobotryae*, *morsprunorum*, and non-species *P. amygdali*, *P. meliae*, *P. savastanoi*, and *P. ficuserectae*. Of those, *P. amygdali* is the earliest valid name for genomospecies 2. Given the DNA-DNA hybridization results of Gardan et al. (1999), the species listed above along with those pathovars should be considered synonymous and the correct name for the species should be *P. amygdali* due to priority. Although *P. amygdali* could not be differentiated based on phenotypic tests, it is a validly published name and thus the new pathovar we are describing as well as other organisms in genomospecies 2 should be referred to as *P. amygdali*.

Therefore, based on physiological and biochemical tests including fatty acid analysis, BIOLOG, 16S rRNA sequencing, and more importantly MLSA and pathogenic specificity to infect loropetalum, we have identified a homogeneous group of strains that based on molecular approaches are more closely related to *P. amygdali* than to *P. savastanoi* as previously published (Conner et al. 2013). As such we, propose that this organism be identified as a new pathovar of *P. amygdali* and propose the name *P. amygdali* pv. *loropetali* pv. nov. and PDC13-208 (change to: DSM 105780) as the pathotype strain. The fatty acid profiles for the loropetalum strains were very distinct from those of *P. amygdali*, as indicated by Stead (1992). However, since none of the strains within *P. amygdali* have the same values as *P. amygdali* in Table 3, the loropetalum values are not out of the range of all *P. amygdali* strains. Stead (1992) noted large quantitative differences in certain fatty acids between *P. amygdali* and *P. syringae* and close relatives. Although qualitatively they contained similar fatty acids with minor exceptions, these differences in composition observed in *P. amygdali* may result from its slow growth (Stead 1992).

Although the loropetalum pathogens were first isolated in diagnostic laboratories, the efforts required to clarify the identity of this pathogen of loropetalum are far beyond the normal range of work involved in a diagnostic laboratory's determination; this work is more along the lines of confirmatory identification. However, it was important to determine that the host range does not include olive since Florida is a state with a growing olive industry. For now, recognizing that *P. amygdali* pv. *loropetali* pv. nov. is not a pathogen of olive nor oleander will help define its management in the ornamental trade in Florida and likely other states. The distinctive host range of the loropetalum strains, supported by the molecular assays, indicates that these strains represent a new pathovar, *P. amygdali* pv. *loropetali* pv. nov., which is proposed below.

Description of *Pseudomonas amygdali* pv. *loropetali* pv. nov. (loropetali pertaining to *Loropetalum*, the generic name of the host plant, *Loropetalum chinense*). Description as for *P. amygdali*. On nutrient agar as well as on KMB, the colonies were color cream, smooth, and round but slightly undulate. Strains fluoresced on KMB. In LOPAT tests, strains were positive for levan production and tobacco hypersensitive reaction, and negative for oxidase arginine dihydrolase activity and ability to rot potato, which identifies the strains as *P. syringae*. MLSA of concatenated genes *gyrB*, *rpoD*, *gapI*, and *gltA* place the strains in genomospecies 2-Group A of

P. amygdali. Differentiated from other pathovars by gall formation on loropetalum (*L. chinense*; natural host) and because it is not pathogenic on almond, mandevilla (*Mandevilla sanderi*), oleander (*Nerium oleander*), and olive (*Olea europaea*). The pathotype strain is designated as PDC13-208 (= DSMZ 105780^{PT}).

Acknowledgments

The authors thank E. Dickstein, A. Jeyaprakash, G. Minsavage, A. Ryan, V. Soltanov, A. Vitoreli, and J. Yarbrough for their valuable assistance with the technical aspects of this research, and Professor Nancy de Grummond (Florida State University) for her expert advice on the Latinization of the specific epithet for the organism.

Literature Cited

- Bull, C. T., Clarke, C. R., Cai, R., Vinatzer, B. A., Jardini, T. M., and Koike, S. T. 2011. Multilocus sequence typing of *Pseudomonas syringae sensu lato* confirms previously described genomospecies and permits rapid identification of *P. syringae* pv. *coriandricola* and *P. syringae* pv. *apii* causing bacterial leaf spot on parsley. *Phytopathol.* 101:847-858.
- Bull, C. T., and Koike, S. T. 2015. Practical benefits of knowing the enemy: modern molecular tools for diagnosing the etiology of bacterial diseases and understanding the taxonomy and diversity of plant-pathogenic bacteria. *Annu. Rev. Phytopathol.* 53:157-180.
- Coenye, T., and Vandamme, P. 2003. Intragenomic heterogeneity between multiple 16S ribosomal RNA operons in sequenced bacterial genomes. *FEMS Microbiol. Lett.* 228:45-49.
- Conner, K. O., Olive, J., Zhang, L., Jacobi, J., and Putnam, M. L. 2013. First report of bacterial gall on *Loropetalum chinense* caused by *Pseudomonas savastanoi* in the United States. *Plant Dis.* 97:835.
- Edgar, R. C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32:1792-1797.
- Gardan, L., Bollet, C., Abu Ghorrah, M., Grimont, F., and Grimont, P. A. D. 1992. DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and the proposal of *Pseudomonas savastanoi* sp. nov. *Int. J. Syst. Bacteriol.* 42:606-612.
- Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F., and Grimont, P. A. D. 1999. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremiae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex. Satic and Dowson 1959). *Int. J. Syst. Bacteriol.* 49:469-478.
- Glickmann, E., Gardan, L., Jacquet, S., Hussain, S., Elasri, M., Petit, A., and Dessaux, Y. 1998. Auxin production is a common feature of most pathovars of *Pseudomonas syringae*. *Mol. Plant-Microbe Interact.* 11:156-162.
- Hwang, M. S. H., Morgan, R. L., Sarkar, S. F., Wang, P. W., and Guttman, D. S. 2005. Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Appl. Environ. Microbiol.* 71:5182-5191.
- Janda, M. J., and Abbott, S. L. 2007. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. *J. Clin. Microbiol.* 45:2761-2764.
- Lelliott, R. A., Billing, E., and Hayward, A. C. 1966. A determinative scheme for the fluorescent plant pathogenic *Pseudomonads*. *J. Appl. Bacteriol.* 29:470-489.
- Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., and Wade, W. G. 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* 64:795-799.
- Marques, A. S. A., Marchaisson, A., Gardan, L., and Samson, R. 2008. BOX-PCR-based identification of bacterial species belonging to *Pseudomonas syringae*-*P. viridiflava* group. *Genet. Mol. Biol.* 31:106-115.
- Parkinson, N., Bryant, R., Bew, J., and Elphinstone, J. 2011. Rapid phylogenetic identification of members of the *Pseudomonas syringae* species complex using the *rpoD* locus. *Plant Pathol.* 60:338-344.
- Penyalver, R. G., Garica, A., Ferrer, A., Bertolini, E., and Lopez, M. M. 2000. Detection of *Pseudomonas savastanoi* pv. *savastanoi* in olive plants by enrichment and PCR. *Appl. Environ. Microbiol.* 66:2673-2677.
- Ramos, C., Matas, I. M., Bardaji, L., Aragon, I. M., and Murillo, J. 2012. *Pseudomonas savastanoi* pv. *savastanoi*: some like it knot. *Mol. Plant Pathol.* 13:998-1009.
- Sarkar, S. F., and Guttman, D. S. 2004. Evolution of the core genome of *Pseudomonas syringae*, a highly clonal, endemic plant pathogen. *Appl. Environ. Microbiol.* 70:1999-2012.
- Schaad, N., Jones, J., and Chun, W. (eds.). 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria (3rd ed.). APS Press, St. Paul, MN.
- Stamatakis, A. 2014. RAxML Version 8: A tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30:1312-1313.
- Stead, D. E. 1992. Grouping of plant-pathogenic and some other *Pseudomonas* spp. by using cellular fatty acid profiles. *Int. J. Syst. Bact.* 42:281-295.
- Tamura, K., Stecher, G., Peterson, D., Filipowski, A., and Kumar, S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol. Biol. Evol.* 30:2725-2729.
- Young, J. M. 2010. Taxonomy of *Pseudomonas syringae*. *J. Plant Pathol.* 92: S5-S14.