Genetic Analyses of *Xanthomonas axonopodis* pv. *dieffenbachiae* Strains Reveal Distinct Phylogenetic Groups

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ABSTRACT

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A comprehensive analysis of 175 *Xanthomonas axonopodis* pv. *dieffenbachiae* strains isolated from 10 Araceae hosts was done to identify pathogen variation. The strains were subjected to repetitive extragenic palindromic sequence polymerase chain reaction and four major phylogenetic clusters were generated. A subset of 40 strains isolated from *Anthurium*, *Dieffenbachia*, and *Syngonium* was further defined by amplified fragment

length polymorphism and fatty acid methyl ester analysis and the same four phylogenetic clusters were observed. Comparison of representative strains in the first three clusters using DNA-DNA hybridization and multi-locus sequence analysis supports the previous reclassification of strains in cluster I, including the *X. axonopodis* pv. *dieffenbachiae* pathovar reference strain (LMG695), to *X. citri*. Our research findings indicate that strains in cluster I, isolated primarily from anthurium, probably represent an undescribed pathovar. Other phylogenetic subclusters consisting primarily of strains isolated from xanthosoma and philodendron in clusters III and IV, respectively, may yet represent other undescribed species or pathovars of *Xanthomonas*.

Members of the plant family Araceae are grown globally as ornamental foliage crops. Species of *Aglaonema*, *Anthurium*, *Caladium*, *Dieffenbachia*, and *Syngonium* are produced intensively in glasshouse production facilities in tropical and temperate regions throughout the world. Because of its virulence and its broad host range within the Araceae family, *Xanthomonas axonopodis* pv. *dieffenbachiae* is considered to be the most serious bacterial disease causing extensive crop losses in commercial foliage plant production.

This disease, commonly known as bacterial blight, was first observed on dieffenbachia and described as *Bacterium dieffenbachiae*, then later designated *X. dieffenbachiae* (8,25). With the introduction of the pathovar system, the nomenclature was revised to *X. campestris* pv. *dieffenbachiae* (44). Through a comprehensive DNA-DNA hybridization study, *X. campestris* pv. *dieffenbachiae* was identified in DNA homology group 9, and was reclassified as *X. axonopodis* pv. *dieffenbachiae* (41).

It has been postulated (3,4,6,12) that bacterial blight strains originating from specific Araceae hosts or in specific geography may reflect genetically distinct groups. Most recently, *X. axonopodis* pv. *dieffenbachiae* has been included in the emended species description of *X. citri* (1); however, that description excludes those strains pathogenic to *Anthurium* and *Syngonium* spp. Similarly, other studies had suggested the use of pathovar *syngonii* for strains originating from syngonium (7,45).

The economic value of anthurium production combined with the threat of losses due to *X. axonopodis* pv. *dieffenbachiae* has prompted numerous studies of these bacteria using genetic, serological, and physiological methods. In a study using random

amplification of polymorphic DNA (RAPD) markers (20), strains recovered from anthurium could be distinguished from strains originating from dieffenbachia or philodendron. *Syngonium* strains were not included in that study. In a follow-up study, sequenced characterized amplified region (SCAR) markers developed from cloned RAPD fragments were capable of differentiating strains isolated from anthurium from those isolated from dieffenbachia, aglaonema, philodendron, and syngonium (21). Using nested polymerase chain reaction (PCR) markers developed for the detection of the anthurium blight pathogen, Robene-Soustrade et al. (31) found that pathogenic strains could be detected in planta. The *syngonium* strains could be distinguished from anthurium strains by restriction digestion using several restriction enzymes. Accurate identification and typing of bacteria currently relies on a number of approaches, including 16S rDNA sequence analysis, DNA genotyping methods restriction fragment length polymorphism (RFLP), RAPD, repetitive extragenic palindromic sequence (Rep)-PCR, and amplified fragment length polymorphism (AFLP), and phenotypic data fatty acid methyl ester (FAME) analysis, carbon source utilization (BIOLOG, Hayward, CA); however, species are currently defined based on DNA-DNA homology (36). Using genus *Xanthomonas* as a model, AFLP and Rep-PCR were compared with DNA-DNA homology studies (29) and a high correlation was observed between the two genotyping techniques, suggesting that these fingerprinting methods may function as core techniques in polyphasic taxonomy. One exception was *Xanthomonas* DNA homology group 9, which exhibited low DNA-DNA homology values (29,41). In a follow-up study, DNA from 339 *Xanthomonas* strains composing 80 pathovars across all 20 DNA homology groups were subjected to Rep-PCR. DNA homology group 9 was divided further into six subgroups, with strains originating from anthurium placed in subgroup 9.4 and strains from dieffenbachia in subgroup 9.6 (30).

The purpose of our study was to perform a detailed genetic characterization of heterogeneous populations composed of distinct species and pathovars of xanthomonads pathogenic on plants

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains one supplemental figure and one supplemental table.

in the Araceae family using genotyping methods Rep-PCR, AFLP, FAME, DNA-DNA homology, and multilocus sequence analysis (MLSA).

MATERIALS AND METHODS

Xanthomonas strain sources and original hosts of strains used in this study are listed in Table 1. These 175 strains were recovered over a 20-year period from 10 Araceae genera. Strains were routinely cultured on nutrient agar or in broth. Strains originating from syngonium were cultured on media amended with 0.5% sucrose. Bacteria were stored in 15% glycerol at -80°C .

Rep-PCR. DNA from all strains was isolated from overnight cultures using the Illustra GenomicPrep Kit (GE Healthcare, Buckinghamshire, UK). Genomic fingerprints were generated for all 175 strains in Table 1 using Rep-PCR. Separate reactions were run for BOX, enterobacterial repetitive intergenic consensus (ERIC), and REP using standard described methods (23). The

resulting PCR products (7 μl each) were separated on 1.5% agarose gels, stained with ethidium bromide and photographed under a UV transilluminator, with a Kodak Digital Documentation System 120 (Rochester, NY). The resulting profiles obtained from BOX, ERIC, and REP were combined and cluster analysis was performed using the unweighted pair-group method with arithmetic mean (UPGMA) implemented in BioNumerics (version 2.5; Applied Maths, St. Martens-Latem, Belgium).

AFLP. A subset of 40 strains was selected based on the four distinct Rep-PCR clusters (Fig. 1) and was further genotyped with AFLP using *EcoRI* and *MseI* restriction enzymes, adaptors, and primers (16,42). Selective amplification reactions were performed using the M00/E00 selective nucleotide primer pair with Eco primer labeled with IR700 dye. Fragments were resolved using the Global LI-COR IR2 system. Gel images of the resulting banding patterns were recorded as tiff files using the SAGA software (LI-COR, Lincoln, NE). Profiles generated with AFLP using primer pairs M00/E00 and M02/E00 were com-

TABLE 1. Sources and hosts of the 175 *Xanthomonas* strains utilized in this study

Strain	Host	Geographic origin	Source ^a
LMG695 ^b , LMG7484, LMG8664	<i>Anthurium</i>	Brazil	BCCM/LMG
1272	<i>Anthurium</i>	California	DAC
1145-87, 20-88, 21-88, 1500-89, 1501-89, 2050-89If, 2050-89st, 2050-89pt, 2129-89If, 2129-89st, 2444-89, 2912-89	<i>Anthurium</i>	Florida	DDB
2927-89, 2025-89	<i>Anthurium</i>	California	DDB
DPI 072-743, DPI 072-745, DPI P87-2307, DPI P90-5399, DPI P91-2557	<i>Anthurium</i>	Florida	JWM
X01-156, X13, X14, X585, X586, X746, X747, X788, X834, X841, X1176, X1185, X1186, X1353, X1354, X1567, X1568, X1671, X1672, X1673, X1705	<i>Anthurium</i>	Florida	MREC
A990-5, A844-1, D1.21, D17.10, D18.00, D38.10, D40.10, D45.40, D46.20, D52.00, D55.1, D61.11, D68.00, D69.10, D70.00, D71.50, D94.40, D101.00, D110.00, D120.00, D131.00, D135.00, D139.00, D145.00, D147.00, D150.00, D158.00, D160.00, D162.00, D170.00, D182.00, D183.00	<i>Anthurium</i>	Hawaii	AMA
B27, B33	<i>Anthurium</i>	Puerto Rico	MZS
1772-88, 2033-89	<i>Aglaonema</i>	Florida	DDB
X811, X1268, X1559, X1564, X1610, X1713	<i>Aglaonema</i>	Florida	MREC
D184.00, D185.00	<i>Aglaonema</i>	Hawaii	AMA
DPI P89-4526-10	<i>Caladium</i>	Florida	JWM
X807, X813, X1188, X1706	<i>Caladium</i>	Florida	MREC
DPI 89-3142-1	<i>Colocasia</i>	Florida	JWM
D122.00, D204.00, D206.00, D219.00, D227.00, D228.00	<i>Colocasia</i>	Hawaii	AMA
2507A-89	<i>Dieffenbachia</i>	Florida	DDB
DPI P87-2081, DPI P89-4119-1	<i>Dieffenbachia</i>	Florida	JWM
X175, X183, X1694, X1703, X1708, X1709, X1718	<i>Dieffenbachia</i>	Florida	MREC
2218-83	<i>Dieffenbachia</i>	Florida	GWS
LMG7399 ^c	<i>Dieffenbachia</i>	United States	ATCC
LMG7400	<i>Dieffenbachia</i>	United States	BCCM/LMG
DPI P91-3902	<i>Epipremnum</i>	Florida	JWM
X176, X1699	<i>Epipremnum</i>	Florida	MREC
D191.00	<i>Epipremnum</i>	Hawaii	AMA
9586	<i>Philodendron</i>	Florida	ICMP
DPI 076-953, DPI P88-3370, DPI P90-3705, DPI P90-3919	<i>Philodendron</i>	Florida	JWM
X170, X642, X1181, X1420, X1474	<i>Philodendron</i>	Florida	MREC
3466-91, 359A-92, 359B-92	<i>Syngonium</i>	Florida	DDB
DPI P90-3731-1	<i>Syngonium</i>	Florida	JWM
X158, X159, X161, X162, X166, X172, X173, X178, X181, X187, X191, X192, X840, X1674, X1697, X1698, X1701, X1702, X1704, X1707, X1711	<i>Syngonium</i>	Florida	MREC
D36.12, D36.10, D129.12 D129.1M, D129.1D	<i>Syngonium</i>	Hawaii	AMA
LMG9055 ^d	<i>Syngonium</i>	United States	BCCM/LMG
D30.00, D93.00	<i>Spathiphyllum</i>	Hawaii	AMA
M101, M102, M103, M105, M106, M110, M113, M114, M115, M116, M117, M123, M124, M126, M127	<i>Xanthosoma</i>	Florida	KLP

^a Strains were obtained from the following laboratories: AMA = Anne M. Alvarez, Department of Plant Pathology, University of Hawaii at Manoa, Honolulu 96822; ATCC = American Type Culture Collection, Manassas, VA 20108; BCCM/LMG = Belgian Coordinated Collections of Micro-organisms, Brussels; DAC = Donald A. Cooksey, University of California—Riverside; DDB = D. D. Brunk, Plant Disease Diagnostics, Inc., Apopka, FL 32703; GWS = Gary W. Simone, Department of Plant Pathology, University of Florida, Gainesville 32611; ICMP = International Collection of Micro-Organisms from Plants, Mt Albert, Auckland, New Zealand; JWM = J. W. Miller, Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville 32602; KLP = Kenneth L. Pernezny, Everglades Research and Education Center, Belle Glade, FL 33430; MREC = Mid-Florida Research and Education Center, University of Florida, Apopka 32703; MZS = Mildred Zapata-Serrano, University of Puerto Rico, Mayaguez, PR 00680; NCPPB = National Collection of Plant Pathogenic Bacteria, York, UK; and NZTCC = New Zealand Type Culture Collection.

^b Pathovar reference strain for *X. axonopodis* pv. *dieffenbachiae*.

^c Type strain for *X. axonopodis* pv. *dieffenbachiae*.

^d Pathovar reference strain for *X. axonopodis* pv. *syngonii*.

binned and cluster analysis was conducted using UPGMA in BioNumerics.

FAME analysis. Extraction and differentiation of FAMES was performed on the subset of 40 strains using procedures previously described (33). Strains were grown on trypticase soy broth agar and subjected to FAME analysis using the Sherlock microbial identification system (midi-inc.com).

DNA-DNA hybridization. Twelve *X. axonopodis* pv. *dieffenbachiae* strains isolated from three Araceae hosts were selected from Rep-PCR clusters I, II, and III (Fig. 1). These strains were compared with 7 other *X. axonopodis* pathovars and 14 other species of xanthomonads (Table 2). Selected strains were evaluated using the S_1 nuclease method for estimating DNA homology (11,17–19,41). DNA was extracted from strains using the Marmur procedure as described (17), adjusted to 200 ng/ μ l, and sheared using a French pressure cell as described (18). DNA probes were labeled with 33 P using a hexamer random primer DNA labeling kit (Invitrogen, Grand Island, NY). 33 P was incorporated by incubation at 25°C for 60 min. DNA probes were cleaned and desalted in 10 mM Tris containing 1 mM EDTA and 0.1% sodium dodecyl sulfate (pH 8.0) on NAP-25 columns (Pharmacia, Picataway, NJ). DNA was reassociated in 1.2 M NaCl and 0.8 mM HEPES [pH 7.0] containing 22.7% formamide (18). The long-term freeze-thaw artifact affecting reassociation values was avoided by

heating thawed target DNA for at least 5 min at 68°C (35). The melting temperature of xanthomonad DNA ($T_m = 95.8^\circ\text{C}$) was determined from the average mole percent guanine plus cytosine (%G+C) of 65.2% (2) using the formula of Marmur and Doty (24). For DNA reassociation (T_r), we used $T_m - 15^\circ\text{C}$ ($T_r = 80.8^\circ\text{C}$), a more stringent temperature recommended for reassociation and used in most DNA similarity studies of xanthomonads (14,17,34,37,41). This stringency can delineate closely related taxons (35 to 70% DNA similarity) such as those that occur among the *axonopodis* group of *Xanthomonas* (30). Because lower temperatures favor nonspecific DNA binding in organisms with G + C as high as 70%, others have used lower temperatures ($T_m - 30^\circ\text{C}$ to $T_m - 25^\circ\text{C}$) (10,44) or the stringent ΔT_m to determine the amount of nonspecific DNA binding for reassociations at lower T_m s (5,9,13,40). Because each 1% of formamide allows for a decrease in the reassociation temperature of 0.6°C , the actual temperature of reassociation used was $22.7\% \times 0.6^\circ\text{C} = 13.6^\circ\text{C}$; $T_r = 75.4 - 13.6 = 61.8^\circ\text{C}$. All DNA-DNA reassociations were carried out in a water bath at $T_r \pm 0.5^\circ\text{C}$ for 24 h. The S_1 nuclease digestion was performed and similarity values were obtained using the methods previously described, except modified to use 33 P-labeled DNA (10,17,35).

MLSA. The following three representative strains were selected for MLSA sequencing from the first three clusters (Figs. 1 to 3):

Fig. 1. Cluster analysis of 175 *Xanthomonas* strains using BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) primers. Banding patterns were compared between strains using Pearson Correlation and unweighted pair-group method with arithmetic mean with the BioNumerics program (ver. 2.1).

cluster I *X. axonopodis* pv. *dieffenbachiae* pathovar reference strain isolated from anthurium (LMG695), cluster II *syngonium* pathovar reference strain for *X. axonopodis* pv. *syngonii* (LMG9055), and cluster III *X. axonopodis* pv. *dieffenbachiae* type strain (LMG7399). Six loci were bi-directionally sequenced for use in MLSA. Primers and cycling parameters for *fusA*, *gap-1*, *gltA*, *gyrB*, *lacF*, and *lepA* have been described previously by Almeida et al. (2). Raw sequence data were assigned Phred quality scores, trimmed, and assembled using CodonCode Aligner version 4.0.3. Consensus sequences were aligned and concatenated to sequences to pathovars and species of *Xanthomonas* obtained via the Plant Associated and Environmental Microbes Database (<http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl>). Multiple representatives of *X. citri* and *X. fuscans* were included and *Stenotrophomonas maltophilia* was used as an outgroup. Sequences were aligned using CLUSTALW implemented in MEGA5 (38,39). Maximum likelihood searches were conducted using MEGA5 using the model and parameter suggested for the data matrix by Modeltest (28). Using the same matrix as in MEGA and the model suggested by MODELTEST, Bayesian Markov chain Monte Carlo phylogenetic analysis was conducted with MrBayes 3 (15,32). Each Markov chain in the Bayesian search was started from a random tree and run 2.0×10^6 cycles, sampling every 100th cycle from the chain. Four chains were run simultaneously, three hot and one cold. Each simulation was run twice and the default settings for the priors on the rate matrix

(0 to 100), branch lengths (0 to 10), and proportion of invariant sites (0 to 1) were used. Stationarity of the sum of the natural log of the likelihoods of the trees in each of the four chains weighted according to the temperatures of the chains was evaluated by monitoring likelihood values graphically. The initial 100 trees in each run were discarded as burn-in. The remaining trees were used to construct majority rule consensus trees. Bayesian posterior probabilities for each clade were derived from trees remaining after discarding the burn-in samples. For ease of visual comparison with bootstrap values, we present these probabilities as whole numbers 0 to 100. Posterior probabilities $\geq 95\%$ are generally regarded as strong support for a clade's existence (43).

RESULTS

Rep-PCR. Using the combined Rep-PCR profiles, four genetic clusters were observed. Although not exclusive, the observed genetic clusters appear to correlate with host of origin (Fig. 1). Cluster I represents strains originating from anthurium, including the *X. axonopodis* pv. *dieffenbachiae* pathovar reference strain LMG695, as well as LMG7484 and LMG8664. Cluster II was composed of strains exclusively recovered from *syngonium* and included pathovar reference strain pv. *syngonii* LMG9055. Cluster III contained *X. axonopodis* pv. *dieffenbachiae* type strain LMG7399, LMG7400, and strains originating from *aglaonema*, *caladium*, and *xanthosoma*. Cluster IV was composed of strains

TABLE 2. DNA homology (percent similarity) among 12 *Xanthomonas axonopodis* pv. *dieffenbachiae* strains isolated from three Araceae hosts compared with 7 other *X. axonopodis* pathovars and 14 other species of xanthomonads^a

DNAs from tester strains (host of origin)	DNA from probe strains labeled with ³³ P				
	LMG 7399 ^T	LMG695	LMG9055	X1674	X191
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> strains isolated from Araceae hosts					
<i>X. dieffenbachiae</i> pv. <i>dieffenbachiae</i> , syn. <i>X. citri</i> LMG 7399 ^T (<i>Dieffenbachia</i>)	100	62	64	56	61
<i>X. dieffenbachiae</i> pv. <i>dieffenbachiae</i> , syn. <i>X. citri</i> LMG7400 (<i>Dieffenbachia</i>)	97	36	51	69	69
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> LMG695 (<i>Anthurium</i>)	54	100	77	80	78
<i>X. axonopodis</i> pv. <i>syngonii</i> LMG9055 (<i>Syngonium</i>)	42	61	100	86	80
<i>X. axonopodis</i> pv. <i>syngonii</i> X1674 (<i>Syngonium</i>)	50	71	93	100	103
<i>X. axonopodis</i> pv. <i>syngonii</i> X191 (<i>Syngonium</i>)	50	58	100	96	100
<i>X. axonopodis</i> pv. <i>syngonii</i> X430 (<i>Syngonium</i>)	50	72	100	99	102
<i>X. axonopodis</i> pv. <i>syngonii</i> X1477 (<i>Syngonium</i>)	50	72	98	100	103
<i>X. axonopodis</i> pv. <i>syngonii</i> X1707 (<i>Syngonium</i>)	50	70	93	96	102
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> X476 (<i>Anthurium</i>)	52	72	101	79	77
<i>X. axonopodis</i> pv. <i>dieffenbachiae</i> LMG8664 (<i>Anthurium</i>)	43	52	89	86	78
Additional species or pathovars of xanthomonads					
<i>X. albilineans</i> LMG493	4	3	...	...	...
<i>X. graminis</i> LMG726 ^T	11	6	...	...	...
<i>X. citri</i> FB1020 (=NCPPB528; CFPB222350)	...	55	...	...	...
<i>X. fuscans</i> subsp. <i>aurantifolii</i> ATCC51301	...	56	...	...	...
<i>X. euvesicatoria</i> FB1250 (=NCPPB2968; 75-3)	...	60	...	...	...
<i>X. fragariae</i> FB1243	1	7	...	...	...
<i>X. alfalfae</i> subsp. <i>citrumelonis</i> FB1274 (=Gabriel 4600)	...	62	...	...	...
<i>X. fragariae</i> LMG710	...	13	...	...	...
<i>X. gardneri</i> LMG6962	...	18	...	...	...
<i>X. hyacinthi</i> LMG739 ^T	23	6	...	...	...
<i>X. campestris</i> pv. <i>campestris</i> LMG568 ^T	...	19	...	...	...
<i>X. campestris</i> pv. <i>campestris</i> ATCC33440	20	14	...	...	...
<i>X. eucalypti</i> LMG700 ^T	15	18	...	...	...
<i>X. pisi</i> LMG847 ^T	18	20	...	...	...
<i>X. vesicatoria</i> FB638	15	...	...	...	...
<i>X. badrii</i> LMG546 ^T	18	23	...	...	...
<i>X. cassava</i> LMG673 ^T	...	23	...	...	...
<i>X. codiae</i> LMG867 ^T	9	22	...	...	...
<i>X. oryzae</i> pv. <i>oryzicola</i> LMG6797	55	25	...	...	...
<i>X. arboricola</i> pv. <i>juglandis</i> LMG747	5	23	...	...	...
Additional axonopodis group pathovars					
<i>X. axonopodis</i> pv. <i>axonopodis</i> LMG982 ^T	39	27	...	...	...
<i>X. axonopodis</i> pv. <i>erythrinae</i> LMG698	...	56	...	...	...
<i>X. axonopodis</i> pv. <i>phaseoli</i> LMG7455 ^T	50	...	...	...	...

^a Controls: homologous tests (bold values) between probe and tester DNAs from the same strain are set to 100% DNA similarity; heterologous tests between probe and salmon sperm (not shown) are set to 0% DNA relatedness. Double lines enclose heterologous pairwise tests (nonbold values) that average within $\geq 70\%$ DNA similarity forming a species-level taxon; 100% homologous values are excluded from the average; ... indicates pairwise test not performed. T = type strain.

primarily from philodendron. A subcluster containing four strains was observed between cluster III and IV and tentatively assigned a designation of IVb. *X. campestris* pv. *campestris* pathovar reference strain LMG568 served as a control outlier separate from all strains tested. The assigned Rep-PCR cluster for each of the 175 *X. axonopodis* pv. *dieffenbachiae* strains can be found in Supplemental Table S1.

AFLP. Based on the four major clusters obtained with Rep-PCR profiles, a subset of 40 representative strains, including 3 strains from IVb, was subjected to AFLP. Using the combined M00/E00- and M02/E00-derived profiles, an UPGMA dendrogram was generated (Fig. 2). Four clusters were observed, represented by strains originating from dieffenbachia (LMG7399/7400),

anthurium (LMG695/7484/8664), syngonium (LMG9055), and philodendron (X642). These findings were consistent with the findings of the combined Rep-PCR cluster analysis (Fig. 1). The three strains included from tentative Rep-PCR subcluster IVb were no longer separated from other strains in cluster IV (Fig. 2).

FAME analysis. All 40 strains were identified as *Xanthomonas* following fatty acid extraction and analysis using MIDI's TSBA40 4.10 library. All strains exhibited levels of 15:0 iso and 15:0 anteiso fatty acids characteristic of xanthomonads (27). Relationships of 39 of the strains are presented as a dendrogram generated using the MIDI software. Results were similar to AFLP analysis, with strain 2507A-89 being an atypical outlier (Fig. 2); thus, this strain was eliminated from the final dendrogram (Fig.

Fig. 2. Cluster analysis of 40 *Xanthomonas axonopodis* pv. *dieffenbachiae* strains based on amplified fragment length polymorphism using M02/E00 and M02/E02 primers in combination. Banding patterns were compared between strains using Pearson Correlation and unweighted pair-group method with arithmetic mean with the BioNumerics program (ver. 2.1). Original host, repetitive extragenic palindromic sequence polymerase chain reaction (Rep-PCR) cluster designation, and geographic isolation location noted for each strain.

3). These strains formed four clusters similar to those observed with the Rep-PCR and AFLP analysis, although FAME analysis was not as definitive as either of the previous two methods. Again, cluster I was represented by strains predominantly isolated from anthurium, including *X. axonopodis* pv. *dieffenbachiae* pathovar reference strain LMG695 (Fig. 3). Two strains isolated from dieffenbachia (Rep-PCR cluster I) fell within this cluster. Cluster II was again composed of strains isolated from syngonium. Cluster III contained the *X. axonopodis* pv. *dieffenbachiae* strains LMG7399 and LMG7400, although these strains are more distantly related based on fatty acid profiles.

DNA-DNA hybridization. Two species-level clusters, each internally interrelated by $\geq 70\%$ DNA hybridization, occurred among the *X. axonopodis* pv. *dieffenbachiae* strains tested (Table 2). Heterologous reciprocal levels of hybridization between these two clusters averaged 58%. One cluster contained the *X. axonopodis* pv. *dieffenbachiae* strains LMG7399 (*X. axonopodis* pv. *dieffenbachiae* reference strain) and LMG7400, both isolated from dieffenbachia, with $>90\%$ internal DNA homology. The second species-level cluster was centered on *X. axonopodis* pv. *dieffenbachiae* strains LMG695, LMG8664, and LMG9055 isolated from *Anthurium* or *Syngonium* spp. and had an internal DNA homology of 97%. DNAs from *X. axonopodis* pv. *dieffenbachiae* strains LMG7399 and LMG695 were hybridized at high

levels (average = 58%) but at less than the species level ($\geq 70\%$) to three strains, representing pathovars of *X. axonopodis* other than *X. axonopodis* pv. *dieffenbachiae* and including the species type strain LMG982 of *X. axonopodis* pv. *axonopodis*. The *X. axonopodis* pv. *dieffenbachiae* strain probe DNAs LMG7399 and LMG695 shared an average of 16% hybridization with 16 other xanthomonad strains representing 19 *Xanthomonas* spp. (Table 2).

MLSA. Using the MLST scheme of Almeida et al. (2), all six loci were PCR amplified and sequenced directly. The resulting sequences were concatenated and aligned to sequences downloaded from PAMDB (2). The resulting phylogenetic tree is shown in Supplemental Figure S1. Using Bayesian and Likelihood methods, *X. axonopodis* pv. *dieffenbachiae* pathovar reference strains anthurium (LMG695) and syngonium (LMG9055) were observed in clade with *X. axonopodis* pvs. *begoniae*, *manihotis*, and *phaseoli*. High posterior probabilities and bootstrap values support this clade. The *X. axonopodis* pv. *dieffenbachiae* type strain (LMG7399) was placed in a separate clade basal to *X. citri*, *X. fuscans*, and *X. axonopodis* pvs. *glycines* and *vignicola*.

DISCUSSION

When 175 strains isolated from nine Araceae hosts were genotyped with Rep-PCR, four major genetic clusters were gen-

Fig. 3. Dendrogram cluster analysis based on fatty acid methyl ester profiles of 39 xanthomonads affecting Araceae hosts. Original host, repetitive extragenic palindromic sequence polymerase chain reaction (Rep-PCR) cluster designation, and geographic isolation location noted for each strain.

erated (Fig. 1). These clusters may represent different pathovars or, potentially, other species not previously described by Louws et al. (23). These phylogenetic clusters were reaffirmed by both AFLP and FAME analysis (Figs. 2 and 3). These clusters support previous research regarding the heterogeneity within this group of xanthomonads and support the reclassification of certain groups.

These Rep-PCR-based clusters appeared to group strains based on their host of origin. Rep-PCR clusters I and II clearly represented strains originating from anthurium and syngonium, respectively. Additionally, the DNA-DNA hybridization experiments support the separation of these two groups from cluster III, which contains *X. axonopodis* pv. *dieffenbachiae* type strain LMG7399 and LMG7400, which were placed into DNA homology group 9 by Vauterin et al. (41). These two strains were later placed into group 9.6 with *X. citri* by Rademaker et al. (30) based on Rep-PCR. It was later suggested that these strains be renamed *X. citri* based on supporting AFLP and MLSA data (1). Our DNA-DNA hybridization also supported this separation, showing <70% homology with representatives from clusters I (LMG695) and II (X191, X1674, and LMG9055). Previous MLSA data from three loci (1) and four loci (26) also support this separation, as does our MLSA comparison using six loci.

Cluster I contained strains primarily isolated from anthurium and pathovar reference strain *X. axonopodis* pv. *dieffenbachiae* (LMG695). Our Rep-PCR, AFLP, and DNA-DNA hybridization data also support the separation of these two populations in cluster I and II from other *X. axonopodis* pv. *dieffenbachia* strains.

Cluster II contained strains isolated from syngonium and includes pathovar reference strain *X. axonopodis* pv. *syngonii* (LMG9055). The pathovar *syngonii* was described based on NaCl tolerance, pH optima, gelatin hydrolysis, and growth on SX media (7). In an attempt to differentiate the two organisms with monoclonal antibodies, Lipp et al. (22) came to a similar conclusion, stating that typical syngonium strains are most similar to anthurium strains whereas the atypical syngonium strains are more similar to those strains isolated from dieffenbachia. Chase et al. (6) concluded that physiological and pathological tests and fatty acid analysis were not useful in differentiating *X. axonopodis* pv. *dieffenbachiae* from *X. campestris* pv. *syngonii*. Cluster II represents fastidious xanthomonads that are specialized in pathogenicity in syngonium. The pathovar *syngonii*, previously described by Dickey and Zumoff (7), would be an appropriate designation for these strains.

Rep-PCR cluster IV consisted of strains isolated from a number of different hosts and had a very low similarity coefficient. However, there was a subcluster made up of isolates originating from philodendron. Further research is needed to determine whether these isolates within cluster IV represent other distinct species or pathovars.

This study exemplifies the heterogenetic nature of the xanthomonads that infect plants in the family Araceae. Understanding the interrelationship among pathogenic variants of these xanthomonads is important for the development of an effective integrated pest management program. That program must include effective diagnostic protocols, breeding for host resistance, and subsequent implementation of prudent regulatory initiatives.

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