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Reclassification of *Xanthomonas campestris* pv. *citri* (ex Hasse 1915) Dye 1978 forms A, B/C/D, and E as *X. smithii* subsp. *citri* (ex Hasse) sp. nov. nom. rev. comb. nov., *X. fuscans* subsp. *aurantifolii* (ex Gabriel 1989) sp. nov. nom. rev. comb. nov., and *X. alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov.; *X. campestris* pv *malvacearum* (ex Smith 1901) Dye 1978 as *X. smithii* subsp. *smithii* nov. comb. nov. nom. nov.; *X. campestris* pv. *alfalfae* (ex Riker and Jones, 1935) Dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker et al., 1935) sp. nov. nom. rev.; and "var. fuscans" of *X. campestris* pv. *phaseoli* (ex Smith, 1987) Dye 1978 as *X. fuscans* subsp. *fuscans* sp. nov.

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Abstract

Bacterial canker of citrus is a serious disease of citrus worldwide. Five forms of the disease have been described, cankers "A", "B", "C", "D", and "E". Although considerable genetic diversity has been described among the causal agents of the five forms of citrus canker and supports multiple taxons, the causal agents currently are classified as pathovars *citri* ("A"), *aurantifolii* ("B/C/D") and *citrumelo* ("E") of a single species, *Xanthomonas campestris* pv. *citri* (or *X. axonopodis* pv. *citri*). To determine the taxonomic relatedness among strains of *X. campestris* pv. *citri*, we conducted DNA–DNA relatedness assays, sequenced the 16S-23S intergenic spacer (ITS) regions, and performed amplified fragment length polymorphism (AFLP) analysis, using 44 strains representative of the five recognized forms of citrus canker. Under stringent DNA reassociation conditions (T_m−15 °C), three distinct genotypes of citrus pathogens were revealed: taxon I included all "A" strains; taxon II contained all "B", "C", and "D" strains; and taxon III contained all "E" strains. The three citrus taxa showed less than 50% (mean) DNA–DNA relatedness to each other and less than 30% (mean) to *X. campestris* pv. *campestris* and *X. axonopodis* pv. *axonopodis*. Taxa I and II strains share over 70% DNA relatedness to *X. campestris* pv. *malvacearum* and *X. campestris* pv. *phaseoli* var. fuscans, respectively (at T_m−15 °C). Taxon III strains share 70% relatedness to *X. campestris* pv. *alfalfae*. Previous

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and present phenotypic data support these DNA reassociation data. Taxon II strains grow more slowly on agar media than taxa I and III strains. Taxa I and III strains utilize maltose, and liquefy gelatin whereas taxon II strains do not. Taxon I strains hydrolyze pectate (pH 7.0) whereas Taxon II strains do not. Taxon III strains utilize raffinose whereas Taxon I strains do not. Each taxon can be differentiated by serology and pathogenicity. We propose taxa I, II, and III citrus strains be named, respectively, *Xanthomonas smithii* subsp. *citri* (ex Hasse, 1915) sp. nov. nom. rev. comb. nov., *Xanthomonas fuscans* subsp. *aurantifolii* (ex Gabriel et al., 1989) sp. nov. nom. rev. comb. nov., and *Xanthomonas alfalfae* subsp. *citrumelo* (ex Riker and Jones) Gabriel et al., 1989 nov. rev. comb. nov. Furthermore, based on the analysis of 40 strains of 19 other xanthomonads, we propose to reclassify *X. campestris pv. malvacearum* (ex Smith, 1901) Dye 1978 as *X. smithii* subsp. *smithii* sp. nov. comb. nov. nom. nov.; *X. campestris* pv. *alfalfae* (ex Riker and Jones) Dye 1978 as *X. alfalfae* subsp. *alfalfae* (ex Riker et al., 1935) sp. nov. nov. rev.; and "var. fuscans" (ex Burkholder 1930) of *X. campestris* pv. *phaseoli* (ex Smith, 1897) as *X. fuscans* subsp. *fuscans* sp. nov.

Keywords: DNA–DNA hybridization; 16S-23S ITS sequencing; AFLP analysis; Citrus xanthomonads

Introduction

Xanthomonas campestris pv. *citri* [Hasse 1915] Dye 1978, the causal agent of citrus canker, was first described by Hasse [36] in the USA, although the disease was most likely known in India some years earlier [21]. Citrus canker causes severe damage to both trees and fruit of many *Citrus* species grown under tropical and sub-tropical conditions [24,49,62]. However, the disease also has been observed under more arid conditions in the Middle East [1]. Most citrus-producing countries free of the disease have strict regulations (zero tolerance) and do not allow importation of fruit or plant materials unless they have passed inspection. Considerable effort has been made to eradicate citrus canker from Florida and success has been declared three times, in 1933, 1947, and 1994 [58]. The current eradication effort began in 1998 at a current cost of over 100 million US dollars [28].

Xanthomonas citri, like most xanthomonads, was reclassified in 1980 [18] as *X. campestris* pv. *citri* due to inadequate phenotypic data [74]. Other forms of citrus canker attributed to *X. campestris* pv. *citri* have been described [35,61,62]. The original Asiatic canker (canker "A") disease affects many *Rutaceae* species, including sweet orange (*Citrus sinensis* (L.) Osbeck), tangerine (*C. reticulata* Blanco), sweet lime (*C. limetta*), pummelo (*C. maxima*) (Burm. f.) Merr., and grapefruit [*Citrus* x *paradisi* Macfad. (pro sp.)]; it characteristically produces hyperplasia-type lesions on leaves, fruit, and stems, thus the name "citrus canker". A form of canker affecting primarily lemon (*C. limon* (L.) Burm. f.) in Argentina, Paraguay, and Uruguay has been referred to as cancrosis "B" [7]. The "B" strains cause lesions on Mexican lime (*C. aurantifolia* (Christm.) Swingle), sour orange (*C. aurantium* L.), Rangpur lime (*Citrus* x *limonia*) (Osbeck), a cross between mandarin orange (*C. reticulata*) and lime, sweet lime, citron (*C. medica* L.), and occasionally orange, mandarin, and pummelo [58]. A form of canker affecting only Mexican lime was described in Brazil in 1971 [47]. Namekata and Oliveira [48] proposed the causal organism be named *X. citri* f. sp. *aurantifolia*, based upon serological, physiological, and pathological differences [47] between the newly described lime pathogen and *X. campestris* pv. *citri*. The disease later was referred to as canker "C" [53] and the name *X. aurantifolii* proposed for the causal organism [23]. Other hosts for these strains include sour orange (*C. aurantium* L.) and lemon [58]. Cancrosis "D" was described on Mexican lime in Mexico; the organism was reported to be serologically different from the "A", "B", and "C" strains and differed pathologically by failing to cause symptoms on fruit [52]. This disease has not been reported since the original description, however. Cancrosis "E" was originally described in Florida as citrus canker, based primarily on serological identity of the causal organism with *X. campestris* pv. *citri* [57]; however, the disease later was referred to as citrus bacterial spot [29]. The causal organism was shown to be genetically very different by restriction fragment length polymorphism (RFLP) analyses from *X. campestris* pv. *citri* resulting in the proposed name *X. campestris* pv. *citrumelo* [23]. Bacterial spot occurs only in nursery stocks and the causal bacterium produces flat, sometimes sunken, water-soaked chlorotic lesions which become black, not the erupted canker lesions typical of *X. campestris* pv. *citri* [23,29,30].

Gabriel et al. [23] proposed re-elevating *X. campestris pv. citri* to species rank on the basis of unique RFLP patterns; however, this proposal failed to gain support due to a lack of DNA–DNA reassociation and phenotypic data [73]. A short time later DNA–DNA reassociation assays using the S_1 nuclease method revealed that the "A" strains were only 30% related to *X. campestris* pv. *campestris* and that citrus strains "A" and "B" shared only 62–63% relatedness [20].

In contrast, the "A" strains shared 90% relatedness to *X. campestris* pv. *malvacearum* [20]. Genomic analysis, including RFLP and amplified fragment length polymorphism (AFLP)-based techniques [17,22,23,27,32,35,39,41,42], and pulse field gel electrophoresis [20], have all shown considerable differences among the various citrus pathogens. More recent DNA–DNA reassociation assays using S_1 nuclease method have shown a relatedness of 50% or less among the citrus pathogen groups "A", "B/C/D/" and "E" [63]. Unlike the results of Egel et al. [20] and Sun et al. [63], Vauterin et al. [67], using a spectrophotometric assay for DNA–DNA relatedness reported that all the citrus pathogens belonged to the same species-level DNA homology group. Additionally, Vauterin et al. [65] showed that each of the five groups of citrus xanthomonads shared less than 70% DNA relatedness to *X. campestris* pv. *campestris* but greater than 70% relatedness with *X. axonopodis pv. axonopodis* (*X. axonopodis*). Vauterin et al. [65], therefore, proposed placing all of the citrus bacteria into *X. axonopodis* as *X. axonopodis* pvs. *citri* ("A" strains), *aurantifolii* ("B/C/D" strains), and *citrumelo* ("E" strains). Using nucleotide sequence analysis of the highly conserved leucine-response regulatory protein (*lrp*) gene, Cubero and Graham [15] showed that the three citrus pathogens segregated separately (within their Cluster 1). Although Cubero and Graham [15] unfortunately did not include *X. axonopodis* pv. *axonopodis* among the strains assayed for *lrp* nucleotide sequences, they described their *lrp* Cluster 1 as the "*X. axonopodis* cluster 1" confirming support for the reclassification of the genus proposed by Vauterin et al. [65]. Based on numerous published genetic differences, Schaad et al. [56] rejected the proposal to place *X. campestris* pv. *citri* and some 33 other pathovars of *X. campestris* into *X. axonopodis* [65]. To avoid further confusion, Schaad et al. [56] proposed maintaining the citrus xanthomonads as *X. campestris* pv. *citri* until additional DNA–DNA reassociation and phenotypic data became available, as first suggested by Egel et al. [20].

In this study, we examine the genetic and phylogenetic relatedness of 44 strains of citrus xanthomonads to *X. axonopodis* pv. *axonopodis* and 40 strains of 19 other xanthomonads, including the type strain, *X. campestris* pv. *campestris*, using the S_1 nuclease DNA–DNA relatedness assays, 16S-23S intergenic spacer (ITS) sequence assays, AFLP analysis, and phenotypic tests. Results showed that none of the citrus xanthomonads were related at the species-level to *X. axonopodis* pv. *axonopodis* or *X. campestris* pv. *campestris*. On the basis of these data and previously published genetic [3,15,20,23,26,34,35,40,41,65,66] and phenotypic data [24,47,68], we propose that the citrus strains representing groups "A"; "B/C/D"; and "E "be classified into three separate taxa, *X. smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii*, and *X. alfalfae* subsp. *citrumelo*, respectively. Furthermore, we propose that the cotton (*Gossypium hirsutum* L.) pathogen, *X. campestris* pv. *malvacearum,* be classified as *X. smithii* subsp. *smithii*. Additionally, we propose the bean (*Phaseolus vulgaris* L.) pathogen, *X. campestris* pv. *phaseoli* var. *fuscans,* be classified as *X. fuscans* subsp. *fuscans* and the alfalfa (*Medicago sativa* L.) pathogen, *X. campestris* pv. *alfalfae,* be classified as *X. alfalfae* subsp. *alfalfae*.

Materials and methods

Bacterial strains and pathogenicity

Strains of *Xanthomonas* were obtained from the American Type Culture Collection (ATCC), Manassas, VA, International Collection of Phytopathogenic Bacteria (ICPB), Ft. Detrick, MD and several other sources, including several original "A" group strains isolated from dried citrus leaves intercepted at San Francisco International Airport (Table 1). Cultures were maintained for routine use by monthly transfer on YDC slants [54,72] and, except for the yellow, non-mucoid *X. axonopodis*, only typical, yellow mucoid colonies were included. All labeled strains for DNA–DNA relatedness assays were checked for pathogenicity, as described below. Cells were grown in liquid NBY [69] and the resulting log phase suspensions adjusted in 0.85% saline to contain 10^5 cfu/ml [54]. Newly unfolded leaves of Mexican lime, Mandarin orange, Duncan grapefruit, or lemon seedlings and first true leaves of cotton seedlings were infiltrated with an inoculum using the blunt end of a 1.0 ml syringe. For cabbage (*Brassica oleraceae* L.) and sugarcane (*Saccharum officinarum* L.), the leaf mid vein and stem (growing point), respectively, of 2–3 leaf-stage plants were injected with a suspension of 10^5 cfu/ml using a 26-gauge needle and syringe. Bean, alfalfa, and cotton leaves were atomized with similar prepared inocula. Control inoculations were made with 0.85% saline. After 10–14 days at 30 °C in a lighted (14 h) dew chamber (Percival model E-54U-DL, Boone, Iowa), results were recorded. All cultures were grown and plants inoculated under containment conditions. All strains were tested for pathogenicity except two (F-57 and F-79) of the eight additional strains of group "E" citrus strains used for phentypic characterization (see below).

DNA–DNA relatedness

DNA was extracted as described [55]. DNA–DNA relatedness assays were performed using a modified S_1 nuclease technique [37] and a stringent temperature of $T_m - 15$ °C [10], as described [55] unless stated otherwise.

Table 1. Source of strains of citrus and other xanthomonads used in this study

	Strain[a]	Source[b]	Origin
Citrus			
A group	10415 (T6-1)	1	Thailand
	10518^{T}* (3213 = ATCC 49118 = LMG 9322)	2	Florida
	10469, 10481 (XC 322),(XC 328)	3	Saudi Arabia
	10609 (1723)	4	Brazil
	10660, 10661-67 (1003), (1105-08 and 1011-13)	5	Florida
	10680,10692	6	Japan
	10678,10691	6	India
	10688	6	Cambodia
	10645	6	Indonesia
	10690	6	Afghanistan
	10679	6	Sri Lanka
	10681	6	Iran
	10682	6	Thailand
	10697	6	Korea
	10693	6	Laos
	10476 (Xc 62 = NCPPB 3234 = LMG 9177)	7	Japan
B group	10470^{T}* (Xc 64 = NCPPB 3236 = LMG 9179)	7	Argentina
	10475 (Xc 69 = NCPPB 3237 = ATCC 51301 = CFBP 2868)	8	Argentina
	10618*, 10620 (IBSBF 392), (IBSBF 1583)	4	Brazil
C group	10471* (Xc 70 = NCPPB 3233 = CFBP 2866)	9	Brazil
	10519 (51302 = Xc 340 = IBSBF 417)	10	Brazil
	10621,10623 (IBSBF 380 = CFBP 2905), (1473)	4	Brazil
	10622, (IBSBF 434 = ICMP 8435)	4	Brazil
	10624 (IBSBF 1495)	4	Brazil
D group	10472* (Xc 90 = LMG 9182)	7	Mexico
E group	10599, 10473*, 10478 (F-6 = LMG 9163),	7	Florida
	(F-5 = LMG 9162), (F-100 = LMG 9169)	8	Florida
	10482, (4600 = LMG 9323)	8	Florida
	10483^{T}* (ATCC 49120 = LMG 9325)	2	Florida
	10587 (F 258)	7	Florida
	10480 (F1 = LMG 160)	7	Florida
Xanthomonas species and pathovars			
campestris	10419 (43304)	10	Oregon
	10434^{T}* (NCPPB 528 = ICMP 13, = ATCC 33913)	11	England
	10322	12	Georgia
axonopodis	10375*(19312 = ICMP 50 = ICPB XA 103)	10	Columbia
	10687 (ICMP 8681 = ICPB XA 115)	13	Columbia
malvacearum	10446*,10447* (H),(N)	2	Florida
	10335* (CFBP 2350 = NCPPB 528)[c]	14	N. Zealand
	10528^{T} (ATCC 9924 = ICMP 217 = ICPB XM 13)	10	South Carolina
	10522 (14928)	10	
	10531 (2b)	15	Uzbekistan
glycines	10900 (XP21 = ICMP 244)	12	Oklahoma
	10912 (XP 22)	12	Oklahoma
	10913 (XP 23)	12	Wisconsin
fuscans	10351 (XCPF)	16	Turkey
	10520^{T}* (ATCC 19315, = NCPPB 381 = ICMP 239)	10	
	10535 (95-06)	17	Honduras
	10917 (= ATCC 13464 = ICMP 242)	12	Tanzania
	10963 (XP 201)	12	
	10969 (XP 207)	12	Brazil

Table 1. (*continued*)

	Strain[a]	Source[b]	Origin
dieffenbachiae	10785, 10788 (XD 114,118)	12	Hawaii, Florida
alfalfae	10701^{T}* (XA 121 = ATCC 11765 = LMG495)	12	India
	10704 (XA 129)	12	Egypt
poinsettiicola	10979 (XP 220)	12	USA
physalidicola	10941 (XP 172)	12	Japan
juglandis	10341 (LMG 747) = ATCC 49083 = ICMP 35 = ICPB XJ 123 = NCPPB 411 = ICMP 11304	18	New Zealand
vitians	10371 (XV 171)	12	
	10668, 10669 (9805,9812)	19	California
phaseoli	10338 (XP 20 = LMG 7455 = NCPPB 3035)	11	USA
	10350 (XCP-B1)	16	Turkey
	10943[d] (XP 175 = NCPPB 554)	12	Sudan
cassavae	10452 (LMG 673)	20	Malawi
hyacinthi	10456 (LMG 739)	20	Netherlands
cucurbitae	10352	6	Seed, unknown
pisi	10458 (LMG 847)	20	Japan
incanae	10514 (13462)	10	USA
codiaei	10453 (LMG 8678)	20	USA
begoniae	10517 (49082 = VPI-21)	10	New Zealand
translucens	VPI-32 (ATCC 19319)	21	USA
fragariae	10454	20	USA
vignicola	10523 (ATCC 11649)	10	USA

*Denotes labelled strain.

[a]ICPB numbers; numbers in parenthesis are the original source codes of bacteria as received.

[b]1, N. Thaveechai, Kasetsart University, Bangkok, Thailand; 2, D. Gabriel, Univ. FL, Gainesville, FL; 3, J. Hartung, USDA, Beltsville, MD; 4, J. Rodrigues Neto, Instituto Biologicó, Sao Paulo Dept. Agric., Campinas Brazil.; 5, Schubert, Florida Dept. Plant Industries (FDPI), Gainesville, FL; 6, Original isolations this study; 7, E. Civerolo, USDA, Fresno, CA; 8, J. Miller, FDPI, Gainesville, FL; 9, Rosetti, Sao Paulo, Brazil; 10, American Type Culture Collection, Manassas, VA; 11, National Collection of Plant Pathogenic Bacteria, England; 12, International Collection of Phytopathogenic Bacteria, USDA, Ft. Detrick, MD; 13, International Collection of Micro-organisms from Plants, Auckland, New Zealand; 14, Collection Francaise de Bacteries Phytopathogenes, Angers, France; 15, L. Glukhova, Tashkent, Uzbekistan; 16, M. Ozakman, Ankara, Turkey; 17, Anne Vidaver, Univ. Nebraska, Lincoln, NE; 18, Laboratorium Microbiologie Gent, Belgium; 19, R. Gilbertson, U.C. Davis, Davis, CA; 20, J. Jones, Univ. Fla, Gainesville, FL; 21, G. Lacy, VPI, Blacksburg, VA.

[c]Strain received as *X. campestris* pv. *campestris* type strain.

[d]Strain deposited as *X. sojense* (*glycines*) type strain.

This procedure was modified so that prior to use in reassociation assays, all stock solutions of target single-stranded (ss-) DNAs (200 ng/ml of 400–600 bp fragments stored frozen (−20 °C) in TE buffer [1 mM EDTA in 10 mM Tris at pH 8.0]) were thawed at 68 °C for 5 min. To determine if genetic subgroups existed with the species-level clusters (⩾70% relatedness), some closely related strains were examined at higher stringency (T_m−8 °C), as described for definition of the subspecies of *Xylella fastidiosa* [55].

Intergenic spacer region (ITS)

Direct PCR amplification of the ITS fragment between the 16S and 23S r RNA genes was carried out using universal *Escherichia coli* primers 1493f and 23r and a 9700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA) as described [5,43,55]. The products were purified and sequenced using an ABI 310 Capillary Sequencing Apparatus according to the manufacturers instructions (Applied Biosystems).

AFLP analysis

The preparation of template DNA for PCR was performed according to Vos et al. [70]. Genomic DNA (100 ng) of each strain was digested with *Eco*RI/ *Mse*I restriction endonucleases. Corresponding adaptors were ligated to the restriction fragments with T_4DNA ligase. For selective amplification 1 µl of a 10-fold diluted ligation mixture was amplified with *Eco*RI +*0* (5′-GAC TGC GTA CCA ATT C-3′) and *Mse*I + C (5′- GAT GAG TCC TGA GTA AC-3′) primers in a GeneAmp PCR System 2700 (Applied Biosystems). *Eco*RI + 0 primer was labeled with infrared fluorescent dye IRDye™ 700 (Li-Cor Inc, Lincoln, NE). The temperature profile was as follows: 94 °C denaturation for 2 min followed by 10 cycles of 94 °C for 20 s, 66 °C for 30 s (annealing temperature reduced 1 °C at each cycle), and 72 °C for 1 min; then 20 cycles of 94 °C for 20 s; 56 °C for 30 s, and 72 °C for 1 min; and finally a 5 min extension at 72 °C. The amplified products were separated on a 6.5% polyacrylamide gel. Electrophoresis was performed on a LI-COR Long ReadIR™ DNA Sequencer (model 4200) and the image data were automatically collected and simultaneously recorded during electrophoresis.

The GelCompar version 4.2 software (Applied Maths, Kortrijk, Belgium) was used to analyze the data. The dendrogram obtained was based on the unweighted pair group method with averages (UPGMA).

Phenotypic characters

For growth on YDC, FS and mSX agars [54], cultures were streaked onto plates with a loop and the plates incubated at 28 °C. SX agar was modified by reducing the amount of methyl violet 2b by 50% (mSX). Starch hydrolysis was determined by growth on NSCA [50] and brown pigment production was determined on NBY and YDC agars. Utilization of carbohydrates was tested as described [54] except that 0.5% agar was used in a 24 well tissue culture plate. After autoclaving, 2.0 ml of the soft agar medium was added to each well. The media were inoculated by adding 10 µl of a 10^{-2} dilution of a 0.1 OD suspension of an overnight liquid NBY shake culture of the appropriate strain to be tested. Saccharic acid and aspartic acid were tested for alkaline production. The basal agar medium without any carbohydrate was included as a negative control. Gelatin hydrolysis, and litmus milk test (casein digestion) were determined, as described [54]. Pectate degradation was determined at neutral (7.0) pH according to Hildebrand [8]. Results were recorded after 7 days, except when stated otherwise, at 28 °C. Eight additional strains of "E" group citrus bacteria (F-54, F-57, F-77, F-78, F-79, F-81, F-92, and F-306) received from E. Civerolo were used for carbohydrate utilization, pectate hydrolysis, and litmus milk.

Results

Pathogenicity

All group "A" strains produced lesions on leaves of all *Citrus* species tested with considerable hyperplasia and chlorosis. Group "B" and "C" strains produced similar lesions with hyperplasia on lime and lemon leaves but not on orange and the hyperplasia was less pronounced than those caused by the "A" strains. Group "C" strains produced a chlorosis on grapefruit and orange leaves. The single group "D" strain (ICPB 10472) caused similar symptoms to those caused by the "B" and "C" strains. Strains of group "E" caused some yellowing and slight watersoaking on all citrus species tested but little or no hyperplasia. All strains of *X. campestris* pv. *malvacearum*, *X. campestris* pv. *phaseoli* var. fuscans, *X. campestris* pv. *campestris*, *X. campestris* pv. *alfalfae* caused disease when inoculated into their respective hosts: cotton (angular water soaked lesions), bean (water soaked spots), cabbage (black veins and chlororis), and alfalfa (yellow water-soaked spots). None of these strains caused disease in citrus and none of the 26 citrus strains caused disease in cotton, alfalfa or beans. Both strains of *X. axonopodis* pv. *axonopodis* produced narrow (0.1-0.2 mm wide) linear (2-3 cm long) chlorotic lesions in sugarcane leaves.

DNA–DNA relatedness

Based upon DNA-reassociation assays at high stringency (T_m−15 °C) and a recommended value of 70% DNA relatedness for establishing species [71], the 44 *Xanthomonas* strains from citrus can be grouped into three discrete taxa (Table 2); taxon I contains all "A" strains (including A^* and A^w), taxon II contains all "B/C/D" strains, and taxon III contains all "E" strains.

The mean internal DNA relatedness value among strains within taxa I–III was 82%, 81%, and 76%, respectively (Table 2). Taxon I showed a mean reciprocal relatedness of 45% to taxon II and 38% to taxon III. The mean reciprocal relatedness of taxa I–III to *X. campestris* pv. *campestris* and *X. axonopodis* pv. *axonopodis* was 11% and 24%, respectively. Of the 18 other known *Xanthomonas campestris* pathovars tested, only six showed greater than 70% relatedness to a citrus taxon strain. Taxon I citrus strains showed a mean relatedness of over 72% to strains of pathovars *malvacearum* and *glycines* and reciprocal results with pv. *malvacearum* were 78% (Table 2). At higher stringency (T_m−8 °C), the mean reciprocal reassociation assay values between six strains each of pathovars *citri* and *malvacearum* was 62% (Table 3). Taxon II citrus strains showed a mean relatedness of 79% to *X. campestris* pv. *phaseoli* var. fuscans and pv. *vignicola*

and reciprocal results with var. fuscans were 75% (Table 2). We failed to distinguish among taxon II strains at higher stringency (T_m−8 °C); the mean reciprocal similarity among citrus strains "B, C, and D" and var. *fuscans* strains was 80%. Strains of Taxon III showed a mean relatedness of 70% to *X. campestris* pv. *alfalfae* and pv. *dieffenbachiae* and reciprocal results with labeled pv. *alfalfae* ICPB 10701 were 75%. Higher stringency (T_m−8 °C) assays between pv. *alfalfae* and six taxon III strains showed a mean reciprocal relatedness value of 71% (Table 3).

ITS sequence comparisons

A total of 538 nucleotides (nt) were sequenced for 49 xanthomonads. *X. axonopodis* pv. *axonopodis* and *X. campesris* pv. *campestris* were very different from the citrus xanthomonads (Table 4) Among the strains of group "A" and those of "B/C" all shared 100% relatedness (Table 5). Strains of group "B/C" differed from the single "D" strain by a single nt (99.8% similar) (Table 5). *X. campestris* pv. *phaseoli* var. fuscans strains shared 99.6% relatedness (2 nt difference) and 99.8% (1 nt difference) relatedness with strains of group "B/ C" and "D", respectively. "E" strains differed by 1 nt among themselevs and by 1 or 2 nucleotides from *X. campestris* pv. *alfalfae* and *X. campestris* pv. *dieffenbachiae* (Table 5). Strains of group "A", "B/C"and "D" and "E" differed by 5 or more nts from *X. campestris* pv. *campestris* and *X. axonopodis* (Table 4). Of the other pathovars, *X. campestris*. pv. *malvacearum* shared 99.8% (1 nt difference) relatedness with the group "A" strains but only 98.7–98.9% (6–7 nt difference), and 98.3–98.5% (8–9 nts difference), with groups "B/C", "D", and "E", respectively. Strains of *X. campestris* pv. *phaseoli* var. fuscans shared 99.6% or greater relatedness with groups "B, C, and D", but 99.1% or less with groups "A" and "E". Strains of *X. campestris* pv. *malvacearum* and *X. campestris* pv. *glycines* were identical. Strains of pv. *alfalfae* shared 99.6% or greater relatedness with "E" strains, 99.1% or less to groups "A", "B/C", and "D" (Table 5), and 99.4% (3 nt difference) with *X. campestris* pv. *dieffenbachiae*.

AFLP analysis

The AFLP analysis of 27 citrus strains and ten outgroup strains generated up to 53–65 distinct fragments. Each of the three DNA–DNA groups "A", "B/ C/ D", and "E" strains had distinct patterns (Fig. 1) which were clearly different from patterns of *X. axonopodis* pv. *axonopodis* or *X. campestris* pv. *campestris*. The cluster analysis of AFLP fingerprints showed a high level of correlation with DNA–DNA reassociation results. All citrus strains fell into three distinct clusters using a minimum value of 46% similarity. *X. axonopodis* pv. *axonopodis* and *X. campestris* pv. *campestris* strains clustered separately from all citrus strains with similarity coefficients to the citrus pathogens below 25%. All the "A" group strains, including "A^w" strains (RFLP varient strains 10661 and 10662 from Wellington, FL. [63] and an "A^*" strain (RFLP varient strain 10469 from Southwest Asia [34,68]) had similar patterns with a similarity coefficient above 70%. *X. campestris* pv. *malvacearum* clustered with "A" group citrus strains and *X. campestris* pv. *glycines* at 46% and 56%, respectively. The "B/C/D" strains clustered with var. fuscan*s* at a linkage of 45% (Fig. 1).

Phenotypic characters

All xanthomonads tested, except *X. axonopodis* pv. *axonopodis*, produced 1 mm, yellow, convex, round, mucoid colonies on YDC within 3 days and were able to hydrolyze starch on NSCA. *X. axonopodis* colonies reached 1 mm after 7 days on YDC. All citrus strains grew well with single 1 mm colonies visible on FS and mSX agars after 2–3 days whereas *X. axonopodis* pv. *axonopodis* required more than 10 days. Phenotypically, *X. axonopodis* pv. *axonopodis* can easily be distinguished from all xanthomonads we included (Table 6). All strains of *X. campestris* pv. *phaseoli* var. fuscans as well as 3 of 10 strains of citrus group "B/C/D" produced a soluble brown pigment on NBY and YDC agars. All citrus strains produced acid from fucose and glucose (data not shown), produced an alkaline reaction in litmus milk, reduced aspartic acid and utilized cellobiose for growth. "E" strains were easily distinguished from other citrus strains by their rapid growth; single 1 mm sized colonies within 30–32 h on YDC and 40–44 h on FS. In contrast "A" and "B/C/D" groups required 40–44 h on YDC and 48–52 h on FS and 56–60 h on YDC and 70–76 h on FS, respectively (Table 6). "B/C/ D" strains grew much slower on YDC, FS, and mSX than did the "A" or "E" group. Group "A" and "E" strains utilized maltose and hydrolyzed pectate, while group "B/C/D" did not. Group "E" strains produce acid from cellobiose and utilize raffinose whereas group "A" did not (Table 6). Group "A" strains hydrolyzed pectate and caused an alkaline hydrolysis in litmus milk; *X. campestris* pv. *malvacearum* did not hydrolyze pectate and caused an alkaline reacton in litmus milk without hydrolysis. *X. campestris* pv. *campestris* produced an alkaline reaction on saccharic acid and an acidic reaction on cellobiose while the "A" group did not. The "A" strains reduced aspartic acid while *X. c*ampestris pv. *campestris* did not (Table 6). Group "B/C/D" strains were differentiated from *X. campestris* pv. *phaseoli* var. fuscans strains as well as all other tested xanthomonads by precipitating casein in the litmus milk

Table 2. Percent DNA relatedness among strains of citrus xanthomonads causing type "A, B/C/D, and E" lesions on citrus and *X. campestris* pv. *campestris* (XCC), *X. axonopodis* pv. *axonopodis* (XAA), and other *Xanthomonas* species and pathovars as determined by the S_1 nuclease method between single-stranded target DNAs and ^{33}P-labeled single-stranded probe DNAs reassociated at $T_m - 15\,^{\circ}C$

Testor DNAs	**Probe DNAs**															
		XCC	XAA	"A"	"A"	*X. malvacearum*			"B"	"C"	"C"	"D"	X. *fuscans*	"E"	"E"	*X. alfalfae*
	Strain	10434	10375	10415	10518	10446	10447	10335	10470	10618	10471	10472	10520	10473	10483	10701
	***Xanthomonas campestris* pv. *campestris*, relatedness to X. *axonopodis* pv. *axonopodis*, 11%[a] (reciprocal 11%)**															
campestris	10434	**100**[b]	7[c]	12	9	18	12	13	7	20	—	—	12	16	16	17
campestris	10419	91	—[d]	10	7	14	—	3	6	12	—	6	—	10	11	—
campestris	10322	91	—	9	7	14	—	5	7	5	8	—	12	14	7	—
	***Xanthomonas axonopodis* pv. *axonopodis mean* relatedness to Taxons I, II, III 23%[a] (reciprocal 25%)**															
axonopodis	10375	13	**100**	22	18	34	23	27	38	32	35	—	35	30	20	22
axonopodis	10687	—	90	18	12	21	—	—	22	—	26	—	—	—	21	22
	Taxon I, mean internal relatedness: 82%[e]															
citri "A"	10415	12	24	**100**	80	70	72	80	44	42	28	—	59	27	39	30
citri "A"	10518	9	14	73	**100**	75	—	79	32	33	41	—	—	—	37	48
citri "A"	10678	—	—	93	84	86	—	—	55	43	—	—	—	—	—	—
citri "A"	10476	—	—	73	84	68	—	—	47	31	43	—	—	—	—	50
citri "A"	10679	—	—	89	86	82	—	—	55	40	—	—	—	—	—	—
citri "A"	10680	—	—	100	91	77	—	—	48	41	—	—	59	—	—	—
citri "A"	10681	—	—	94	80	86	—	—	—	—	—	—	54	—	—	—
citri "A"	10682	—	—	83	99	84	—	—	32	—	—	—	—	—	—	—
citri "A"	10688	—	—	87	91	85	—	—	—	—	—	—	—	—	—	—
citri "A"	10690	—	—	97	80	76	—	—	—	—	—	—	55	—	—	—
citri "A"	10691	—	—	100	81	75	—	—	—	—	—	—	—	—	—	—
citri "A"	10692	—	—	100	—	72	—	—	48	—	—	—	54	—	—	—
citri "A"	10693	—	—	89	82	81	—	—	45	—	—	—	—	—	—	—
citri "A"	10697	—	—	77	90	76	—	—	44	—	—	—	—	—	—	—
citri "A"	10545	—	23	84	84	—	—	—	39	38	—	54	50	32	32	52
citri "A"	10609	—	—	83	76	74	—	—	42	—	57	49	—	—	—	—
citri "A"*	10469	13	41	88	83	70	—	90	55	42	36	48	48	29	41	29
citri "A^{w}"	10660	—	22	84	73	84	—	—	43	48	45	—	51	28	28	—
citri "A^{w}"	10665	—	22	89	77	87	—	—	55	36	30	58	54	—	22	35
citri "A^{w}"	10666	—	—	81	84	90	—	—	55	40	47	49	43	29	24	—
citri "A^{w}"	10667	—	—	92	—	82	—	—	—	—	39	—	46	—	41	—
malvacearum	10446	18	26	67	68	**100**	—	95	57	39	34	47	38	26	36	44
malvacearum	10447	23	15	82	67	98	**100**	82	45	41	40	33	44	32	37	—
malvacearum	10335	15	—	76	63	98	85	**100**	31	36	30	34	58	—	31	—
malvacearum	10522	—	—	72	69	83	94	99	48	52	28	—	34	—	25	—
malvacearum	10528	—	23	89	63	98	—	—	—	36	—	30	57	—	28	—
malvacearum	10531	—	14	87	65	95	—	—	—	33	—	36	56	—	32	—
glycines	10900	—	20	89	64	74	—	—	53	36	—	—	45	—	38	52
glycines	10912	—	—	—	65	79	—	—	49	55	—	—	—	—	43	—
glycines	10913	—	—	—	62	75	—	—	53	30	—	—	—	—	22	—

Table 2 (continued)

									Taxon II, mean internal relatedness: 81%							
aurantifolii "B"	10470	8	35	50	20	48	33	51	**100**	84	74	84	77	32	49	—
aurantifolii "B"	10618	—	25	54	23	55	—	—	90	**100**	85	76	74	38	37	34
aurantifolii "B"	10620	—	28	50	46	55	—	—	100	73	85	89	75	38	29	24
aurantifolii "C"	10471	7	25	46	30	48	48	43	82	89	**100**	81	78	39	43	22
aurantifolii "C"	10519	5	29	51	44	56	—	40	95	92	76	90	80	44	40	—
aurantifolii "C"	10621	—	28	56	45	49	—	—	100	91	—	88	78	38	31	23
aurantifolii "C"	10622	—	22	52	24	60	—	—	81	80	87	—	71	40	28	35
aurantifolii "C"	10623	—	19	50	42	50	—	—	81	82	—	70	70	40	36	46
aurantifolii "C"	10624	—	24	52	40	52	—	—	78	76	—	—	77	29	—	—
aurantifolii "D"	10472	10	20	47	—	41	46	56	92	83	76	**100**	71	27	41	44
fuscans	10520	—	21	49	40	38	—	59	76	75	75	65	**100**	—	26	43
fuscans	10351	16	24	30	37	51	—	—	89	78	85	84	100	40	26	—
fuscans	10969	—	—	—	38	56	—	—	84	72	81	—	93	24	—	—
fuscans	10917	—	—	31	26	61	—	—	81	69	97	73	82	25	27	—
fuscans	10963	—	—	30	—	49	—	—	75	68	88	—	86	—	—	—
vignicola	10523	—	20	56	46	61	—	—	82	73	94	83	72	35	31	—
														Taxon III, mean internal relatedness: 76%		
citrumelo "E"	10473	5	21	47	30	50	—	38	35	33	42	—	21	**100**	91	77
citrumelo "E"	10483	—	——	30	21	49	—	37	32	32	38	—	47	94	**100**	73
citrumelo "E"	10599	—	22	37	30	38	—	—	38	27	—	—	—	67	74	73
citrumelo "E"	10478	14	22	28	33	42	—	33	36	30	—	36	34	75	78	66
citrumelo "E"	10480	—	22	—	20	43	—	—	37	34	37	—	37	70	90	73
citrumelo "E"	10482	4	23	27	—	35	—	36	26	22	33	35	20	81	71	72
citrumelo "E"	10587[f]	—	27	39	35	47	—	—	37	26	—	36	43	75	86	—
alfalfae	10701	—	24	45	40	43	—	—	37	37	27	28	28	72	77	**100**
alfalfae	10704	—	28	50	40	38	—	—	40	32	29	31	23	68	72	98
dieffenbachiae	10788	—	22	27	31	47	—	—	35	33	33	—	31	—	66	77
dieffenbachiae	10785	—	—	23	—	37	—	—	—	—	—	—	45	—	70	77
Other pathovars of *Xanthomonas campestris*, relatedness to Taxa I, II, & III																
vitians	10371	28	—	19	9	20	—	—	13	10	9	17	17	—	22	—
vitians	10668	—	7	13	8	20	—	—	10	10	—	18	9	—	13	22
vitians	10669	—	7	19	7	22	—	—	7	13	—	—	13	—	15	20
phaseoli	10338	8	24	28	36	36	35	40	52	45	33	40	28	37	36	36
phaseoli	10350	17	21	30	—	27	—	47	41	43	30	—	34	—	34	—
phaseoli	10943[f]	13	27	28	32	36	—	—	—	37	41	29	31	—	21	36
begoniae	10517	4	44	37	39	28	—	20	27	31	21	—	41	43	50	—
codiae	10453	15	8	6	8	12	—	—	18	15	7	—	15	14	5	9
cassavae	10452	15	16	13	3	14	15	5	—	1	10	0	18	19	8	15
hyacinthi	10456	7	7	3	6	13	—	—	5	8	3	—	9	7	4	6
translucens	VPI32	9	3	6	4	—	—	7	9	3	—	—	5	3	5	—
fragariae	10454	14	5	6	7	3	—	5	10	9	6	—	7	6	0	—
cucurbitae	10352	0	9	5	3	7	6	2	—	8	4	—	2	3	0	—
pisi	10458	12	16	18	13	19	7	9	14	12	4	1	11	10	8	6

Table 2 (continued)

Testor DNAs	Probe DNAs														
		XCC	XAA	"A"	"A"	*X. malvacearum*			"B"	"C"	"C"	"D"	X. *fuscans*	"E"	"E"	*X. alfalfae*
	Strain	10434	10375	10415	10518	10446	10447	10335	10470	10618	10471	10472	10520	10473	10483	10701
juglandis	10341	21	20	13	17	—	—	5	1	1	—	—	18	13	11	19
incanae	10514	54	—	10	16	20	11	—	—	14	4	—	9	9	15	—

[a]Mean reciprocal % DNA relatedness calculated from pair-wise, heterologous tests between two taxa.
[b]Controls: Homologous reassociations (bolded figures) between probe and testor single-stranded DNAs from the same strain are set to 100% DNA relatedness; heterologous reassociations between probe and salmon sperm single-stranded DNAs (not shown) are set to 0% DNA relatedness.
[c]Heterologous pairwise reassociations (non-bolded figures) between ^{33}P-labeled and testor single-stranded DNAs; average of at least two determinations presented.
[d]—, Pair-wise test not performed.
[e]Internal % DNA relatedness: mean calculated from heterologous pairwise tests (non-bolded figures) within the taxon (boxed figures) but excluding 100% homologous values.
[f]Previously identified as *Xanthomonas campestris* pv. *aurantifolii* "B" and pv. *glycines,* respectively.

Table 3. Percent DNA relatedness among strains of xanthomonads causing type "A, B/C, and E" lesions on citrus and *Xanthomonas campestris* pv. *malvacearum* (XM), pv. *phaseoli* var. *fuscans* (XF), and other closely related xanthomonads as determined by the S_1 nuclease method between single-stranded testor DNAs and ^{33}P-labeled single-stranded probe DNAs reassociated at T_m-8 °C

Target DNAs		Probe DNAs[a]								
	Strain	"A" 10415	"A" 10518	XM 10446	"B" 10618	"B" 10470	"C" 10471	XF 10520	"E" 10483	*alfalfae* 10701
campestris	10434	17[b]	—[c]	12	22	—	19	23	26	—
	10322	9	—	—	—	—	—	—	—	—
axonopodis	10375	24	27	26	44	—	38	33	29	21
		Taxon I, internal relatedness 73%[e]								
citri "A"	10415	**100**[d]	—	68	—	—	—	—	—	—
	10518	—	**100**	69	—	—	—	—	—	—
	10609	82	90	59	—	—	—	—	—	—
	10476	—	—	55	—	—	—	—	—	—
	10545	—	—	64	—	—	—	—	—	—
	10678	—	—	57	—	—	—	—	—	—
citri"A*"'	10469	91	73	43	—	—	—	43	—	—
citri "A[w]"	10660	89	84	59	—	—	—	59	—	—
	10665	100	88	69	—	—	—	52	—	—
	10667	100	—	59	—	—	—	—	—	—
malvacearum	10522	63	65	89	—	—	—	—	—	—
	10446	70	69	**100**	—	—	—	—	—	—
	10447	59	60	81	—	—	—	—	—	—
	10531	63	60	99	—	—	—	33	—	—
	10528	52	—	100	—	—	—	38	—	—
	10335	58	—	92	—	—	—		—	—
					Taxon II, internal relatedness 86%					
aurantifolii "B"	10470	26	—	—	—	**100**	86	83	30	—
	10618	33	—	—	**100**	78	**100**	76	27	—
	10620	—	—	—	94	100	100	85	—	—
aurantifolii "C"	10471	—	—	—	72	100	**100**	77	32	—
	10519	21	—	—	100	100	100	99	32	—
	10621	—	—	—	—	—	—	92	—	—
	10622	—	—	—	99	100	100	66	—	—
	10623	—	—	—	—	—	—	75	—	—
	10624	—	—	—	—	—	—	69	—	—
aurantifolii "D"	10472	43	—	—	82	100	81	87	33	—
fuscans	10520	—	—	—	**62**	70	70	**100**	—	—
	10351	—	—	—	80	84	81	100	—	—
	10969	—	—	—	79	68	86	100	—	—
	10917	—	—	—	92	64	92	100	—	—
vignicola	10523	—	—	—	—	—	—	100	—	—

Table 3 (continued)

Target DNAs		Probe DNAs[a]								
	Strain	"A" 10415	"A" 10518	XM 10446	"B" 10618	"B" 10470	"C" 10471	XF 10520	"E" 10483	*alfalfae* 10701
									Taxon III, internal relatedness 74%	
citrumelo "E"	10473	—	—	—	—	—	—	—	83	76
	10599	—	—	—	—	—	—	—	71	70
	10478	—	—	—	—	—	—	—	76	65
	10480	—	—	—	—	—	—	—	87	71
	10482	—	—	—	—	—	—	—	70	71
	10483	—	—	—	—	—	—	—	**100**	70
dieffenbachiae	10788								70	69
alfalfae	10701								73	**100**
	10704								—	86

[a]Mean reciprocal % DNA relatedness calculated from pair-wise, heterologous tests between two taxa.
[b]Heterologous pairwise reassociations (non-bolded figures) between ^{33}P-labeled and testor single-stranded DNAs; average of at least two determinations presented.
[c]—, Pair-wise test not performed.
[d]Controls: Homologous reassociations (bolded figures) between probe and testor single-stranded DNAs from the same strain are set to 100% DNA relatedness; heterologous reassociations between probe and salmon sperm single-stranded DNAs (not shown) are set to 0% DNA relatedness.
[e]Internal % DNA relatedness: mean calculated from heterologous pairwise tests (non-bolded figures) within the taxon (boxed figures) but excluding 100% homologous values.

test. Group "B/C/D" strains utilized mannitol but not maltose while *X. campestris* pv. *phaseoli* var. fuscans utilized maltose but not mannitol. *X. campestris* pv. *campestris* utilized maltose and hydrolyzed pectate whereas group "B/C/D" strains did not. Group "E" strains were differentiated from *X. campestris* pv. *alfalfae* strains by utilization of raffinose, reduction of aspartic acid and non-reduction of saccharic acid. *X. campestris* pv. *campestris* reduced saccharic acid but not aspartic acid while group "E" strains reduced aspartic acid but not saccharic acid.

Discussion

These results show that the five recognized pathogenic groups ("A", "B", "C", "D", and "E") of citrus-pathogenic xanthomonads can be classified into three separate taxa. We confirm results of earlier S_1 nuclease DNA–DNA reassociation studies [19,20] showing that strains of the citrus groups "A", "B", and "E" share less than 70% homology. We extend these results and show that strains of group B share 70% or more relatedness with strains of group "C" and "D". Egel et al. [19] further reported that citrus groups "A", "B", and "E" shared less than 70% relatedness to several other xanthomonads, including *X. campestris* pv. *campestris*, *X. campestris* pv. *vesicatoria*, and *X. campestris* pv. *phaseoli*. The "A" strains did, however, share 70% or greater relatedness with *X. campestris* pv. *malvacearum*. In contrast, Vauterin et al. [65], using a spectrophotometric DNA–DNA reassociation assay reported that all the citrus strains ("A", "B", "C", "D", and "E" groups) shared 70% or more relatedness among themselves and with *X. axonopodis* pv. *axonopodis* and proposed placing all the citrus pathogens into *X. axonopodis* as pathovars *citri*, *aurantifolii*, and *citrumelo*. Nucleotide sequence analyses of the leucine responsive regulatory (*lrp*) gene has been cited in support of maintaining the three citrus pathogen groups as pathovars of *X. axonopodis;* unfortunately *X. axonopodis* pv. *axonopodis lrp* sequences were not included among those bacteria analyzed [15]. However, our reciprocal DNA reassociation tests between *X. axonopodis* pv. *axonopodis* and the citrus strains showed mean reciprocal relatedness values of less than 30%, an amount considerably less than the 70% reported by Vauterin et al. [65]. To verify that our labelled strain of *X. axonopodis* was typical and authentic, an additional strain obtained from the International Collection of Microorganisms from Plants (ICMP, Auckland, New Zealand) which we confirmed to be phenotypically representative and pathogenic, was also included among the strains in our assays.

The large differences between Vauterin et al. [65] and our results were unexpected. One explanation could be in the differences in methods used for reassociation; Vautein et al. [65] used a spectrophotometric technique to measure reassociation and did not include reciprocal results. The spectrophotmetric method is known to result in values that can be 15–25% higher than those obtained by the S_1 procedure used for this work [33,38]. With the spectrophotometric method, homologous as well as non-homologous annealing occurs, often resulting in higher DNA reassociation values [37,38]. Because the conversion of single-stranded DNA (ssDNA) to double-stranded DNA (dsDNA) cannot be directly observed spectrophotometrically, an algorithm is used to determine the amount of heterologous annealing among ssDNA and dsDNA molecules resulting from homologous reassociations. In contrast, we used the more conservative S_1 nuclease technique specifically recommended for phylogenetic analyses [59,60]. In the S_1 nuclease method, labelled probe ssDNA is only a small fraction (1:500–1:700) of the concentration of the target ssDNA, practically eliminating homologous re-annealed DNA. Following S_1 nuclease digestion, all ssDNAs are removed insuring that only heterologous annealed probe DNA is estimated directly from beta emissions [37,38]. Our reciprocal results agreed closely at both $T_m-15\,^{\circ}C$, the usual highly stringent temperature of reassociation in phylogenic studies [9,10], and at $T_m-8\,^{\circ}C$, which provides even higher stringency and is used to discern intra-specific relationships [55].

Another explanation for inflated reassociation values with the spectrophotometric technique is possible. A long-term storage problem with high mole % G+C ssDNAs, such as those of xanthomonads, has been observed (J.L. Johnson and G. H. Lacy, unpublished). For instance, in 1994, heterologous reassociations were performed as described by Johnson [38] using freshly prepared, frozen (−20 °C) stock solutions of ssDNAs. In three heterologous reassociations using ^{125}I-labeled ssDNA from *X. campestris* pv. *citri* strain ATCC 49118 as probe to target ssDNAs from pv. *phaseoli* strain ATCC 9563 and *X. campestris* pv. *vignicola* strain ATCC 11646, and *X. campestris* pv. *campestris* strain ATCC 33913 ssDNA as probe to *X. campestris* pv. *phaseoli* ATCC 9563, reassociation values with freshly prepared target ssDNAs were 33%, 51%, and 10%, respectively. In 1995, after many reuses of the target ssDNAs including repetitive freezing (−20 °C) and thawing (at 15–18 °C), reassociation values using the same stock solutions unexpectedly rose to 59%, 62%, and 27%, respectively. J.L. Johnson had not observed this phenomenon of increased reassociation values with stored target ssDNA during his many experiments with DNA of bacteria with lower mole % G+C (G. H. Lacy, personal communication). Later in 1995, these same stock solutions of ssDNAs were again thawed (15–18 °C) but additionally heated for 5 min at 68 °C before use. Reassociation values returned to levels

Table 4. Comparison of ITS sequences based on primers 1493F and 23R for strains of *Xanthomonas campestris* pv. *campestris* and citrus xanthomonad groups "A, B, C, D, and E", and *X. axonopodis* pv. *axonopodis* and several other xanthomonads

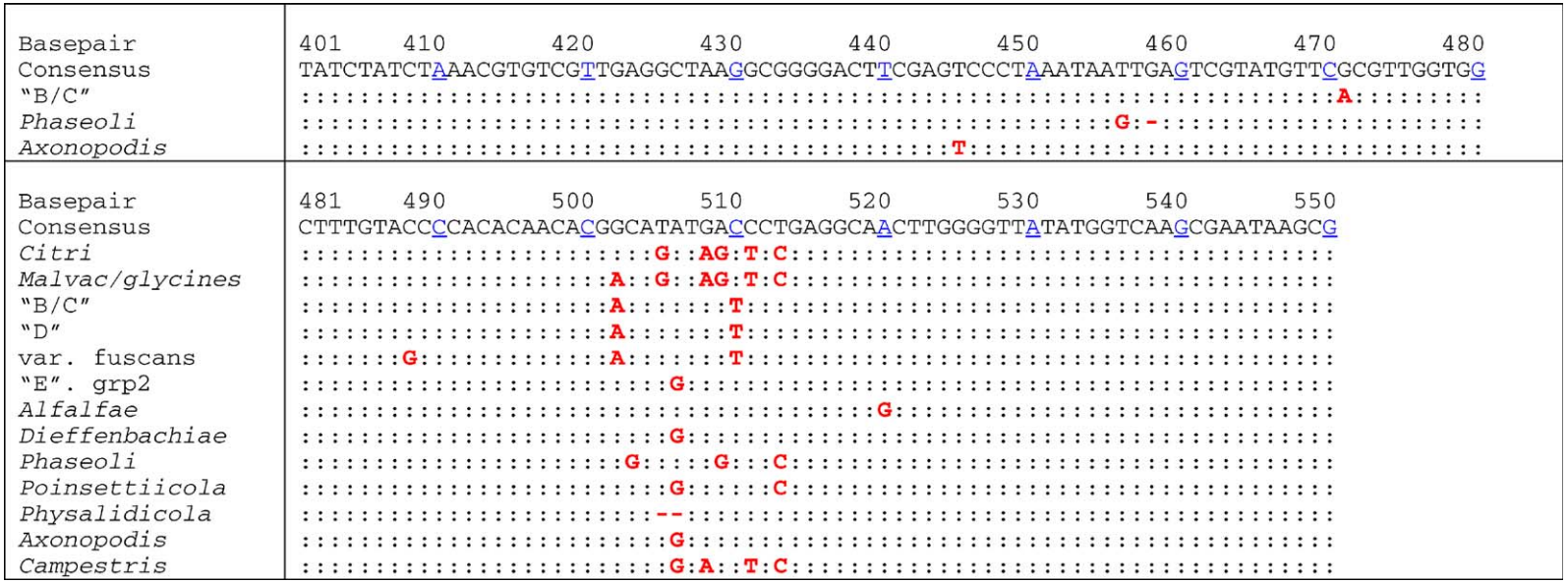
```
Basepair        1       10        20        30        40        50        60        70        80
Consensus       GTAGCCGTATCGGAAGGTGCGGCTGGATCACCTCCTTTTGAGCATGACGTCATC-GTCCTGTCGGGCGTCCTCACAAATT
Axonopodis      ::::::::::::::::::::::::::::::::::::::::::::::::::::::-::::::::::::::::::::::C:
Campestris      ::::::::::::::::::::::::::::::::::::::::::::::::::::T::TC:::::::::::::::::::::

Basepair        81      90        100       110       120       130       140       150       160
Consensus       ACCTGCATTCAGAGATGCGTATCGGCACAGGCCGGTATGCGAAAGTCCCATCATGGGGCCTTAGCTCAGCTGGGAGAGCA
"E", grp1       ::::::::::::::::::::::::::::::::::A:::::::::::::::::::::::::::::::::::::::::::::
"E", grp2       ::::::::::::::::::::::::::::::::::A:::::::::::::::::::::::::::::::::::::::::::::
Alfalfae        ::::::::::::::::::::::::::::::::::A:::::::::::::::::::::::::::::::::::::::::::::
Physalidicola   ::::::::::::::::::::::::::::::::::A:::::::::::::::::::::::::::::::::::::::::::::
Campestris      ::::-::::::::::::T:A::C:::::::::T::::::::::-::::::-:TT::::::::::::::::::::::::::

Basepair        161     170       180       190       200       210       220       230       240
Consensus       CCTGCTTTGCAAGCAGGGGGTCGTCGGTTCGATCCCGACAGGCTCCACCAT-TTGAGTGAAACGACTTTGGGTCTGTAGC
Campestris      ::::::::::::::::::::::::::::::::::::::::::::::::::::A::::::::::A:::::C::::::::::

Basepair        241     250       260       270       280       290       300       310       320
Consensus       TCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCGGTGGTTCGAGTCCTCCCAGACCCACCACTCTGAATGTAGTG
Campestris      ::::::::::::::::::::::::::::::::::::::::::A::::::::::TA::::::::::::::::::::::::::

Basepair        321     330       340       350       360       370       380       390       400
Consensus       CACACTTAAGAATTTATATGGATCAGCGTTGAGGCTGAGACATGTTCTTTTATAACTTGTGACGTAGCGAGCGTTTGAGA
"E", grp1       ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
"E", grp2       ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Alfalfae        ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Dieffenbachiae  ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Phaseoli        ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Poinsettiicola  ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Physalidicola   ::::::::::::::::::::C::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
Axonopodis      ::::::::::::C:::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
```



The consensus sequence ($\geqslant$57%) was estimated from ITS sequences from 19 pathovars of *X. campestris*. *X. campestris* pv. *campestris* strain 10434 was included as an outgroup and is not included in the consensus. The entire consensus sequence is presented. For brevity, other ITS sequence segments identical to the consensus sequence are not presented. For unique ITS patterns, only those bases differing from the consensus sequence are presented. Fourteen distinct ITS patterns were found. All strains included in each ITS group had identical ITS sequences. Strains are: *Xanthomonas campestris* pv. *citri* (10415, 10481, 10660, 10662, 10666, 10667), *X. campestris* pv. *malvacearum*/glycines (10446, 10335, 10447, 10528, 10531/ 10900,10912,10913), citrus xanthomonad groups "B/C" (10470, 10618, 10621, 10471, 10620, 10622, 10624), and *X. campestris* pv. *phaseoli* (10338, 10943, 10350), *X. campestris* pv. *phaseoli* var. fuscans (10917, 10351,10520, Xp207), citrus "E", group 1 (10483, 10473,10480), citrus "E", group 2 (10482, 10478), *X. camestris* pv. *alfalfae* (10701, 10704), *X. campestris* pv. *dieffenbachiae* (10785, 10788), *X. axonopodis pv. axonopodis* (10375), *X. campestris* pv. *campestris* (10434, 10322, 10419).

Individual strains with distinct ITS patterns are citrus "D" (10472), *X. campestris* pv. *poinsettiicola* (10979), *X. physalidicola* (10941), *X. axonopodis* pv. *axonopodis* (10375). *X. campestris* pv. *phaseoli* strain 10943 was originally deposited in the ICPB as *X. campestris* pv. *sojense* (*glycines*).

Table 5. Differences in number of nucleotides (lower left) (one nt = 0.2% difference) and mean percent relatedness (upper right) of Intergenic spacer region (ITS) sequences of strains of *Xanthomonas campestris* pv. *campestris*, *X. axonopodis* pv. *axonopodis*, citrus xanthomonad groups "A, B, C, D, and E", and several other pathovars of *X. campestris*

XANTHOMONAD (ITS groups)	*X. campestris* pv. *campestris*	*X. axonopodis*	"A"	*X. c.* pv. *malvacearum*, pv. *glycines*	"B" & "C"	"D"	*X. c.* pv. *phaseoli* var. fuscans	"E" group 1	"E" group 2	*X. c.* pv. *dieffenbachiae*	*X. c.* pv. *alfalfae*
X.campestris pv. *campestris* (3)[a]	**100**	95.9	96.5	96.3	95.7	95.9	95.7	96.1	95.9	96.3	95.7
X. axonopodis pv. *axonopodis* (1)	22	**100**	98.3	98.1	98.7	98.9	98.7	99.1	98.9	99.3	98.7
Citrus group "A" (6)	19	9	**100**	99.8	98.5	98.7	98.5	98.5	98.7	98.7	98.5
Pathovar *malvacearum* (5), pv. *glycines* (3)	20	10	1	**100**	98.7	98.9	98.7	98.3	98.5	98.5	98.3
Citrus group "B" & "C" (7)	23	7	8	7	**100**	99.8	99.6	98.9	99.1	99.1	98.9
Citrus group "D" (1)	22	6	7	6	1	**100**	99.8	99.1	99.3	99.3	99.1
Pathovar *phaseoli* var. *fuscans* (4)	23	7	8	7	2	1	**100**	98.9	99.1	99.1	98.9
Citrus group"E" 1 (2)	21	5	8	9	6	5	6	**100**	99.8	99.8	99.6
Citrus group "E" 2 (3)	22	6	7	8	5	4	5	1	**100**	99.6	99.8
Pathovar *dieffenbachiae* (3)	20	4	7	8	5	4	5	1	2	**100**	99.4
Pathovar *alfalfae* (2)	23	7	8	9	6	5	6	2	1	3	**100**

[a]Numbers in paranthesis are the number of strains sequenced; sequence base on primers 1493F and 23R.

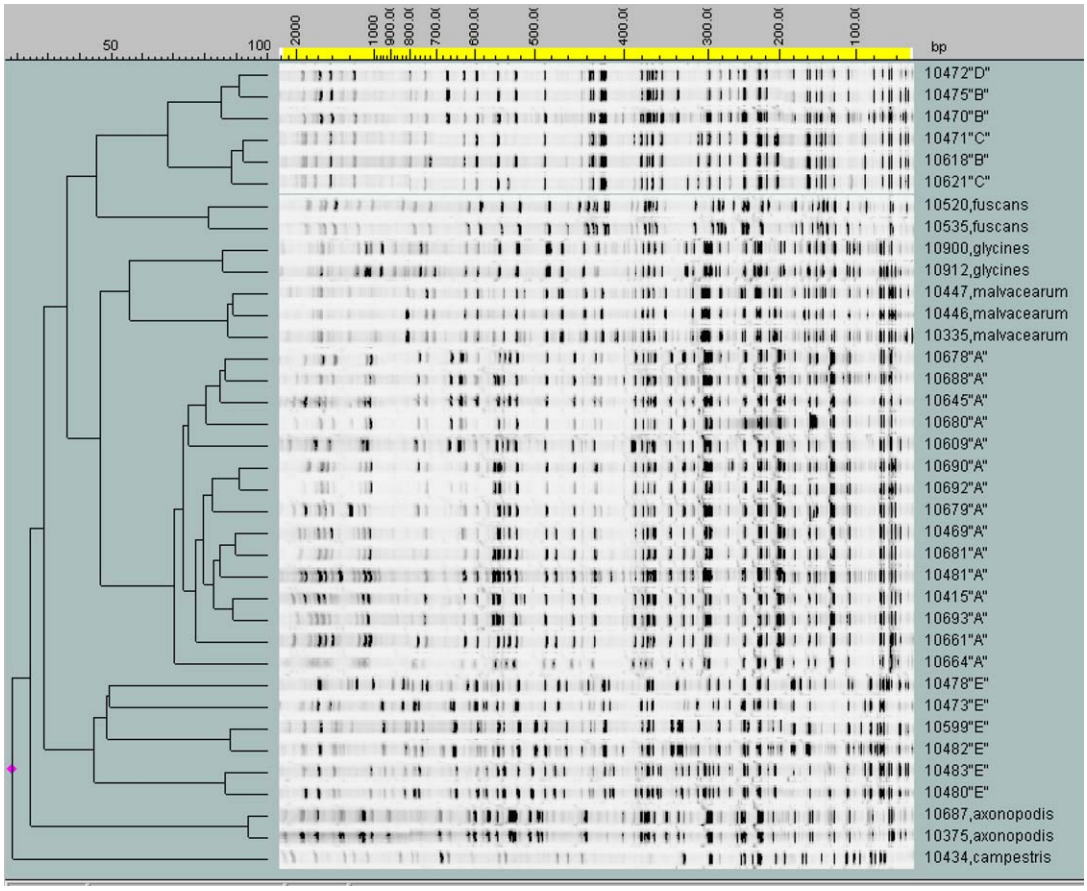


Fig. 1. AFLP patterns of *Xanthomonas campestris* pv. *citri groups* "A, (10469 is A^* and 10661 & 64 are A^w) B, C, D, and E"; *X. campestris* pv. *malvacearum*, X. *axonopodis* pv. *axonopodis*, and *X. campestris* pv. *campestris*. Dendrogram based on the unweighted pair group method with averages (UPGMA).

(39%, 48%, and 10%, respectively) similar to those obtained in 1994 with freshly prepared target ssDNAs. Comparing this apparent increase in DNA relatedness associated with long-term storage with the initial % DNA relatedness between freshly prepared probe and target DNAs, we found that the increase in reassociation values among organisms sharing 25–35% relatedness (similar to the relatedness of *X. axonopodis* pv. *axonopodis* with the citrus pathogens shown in Table 2) could be greater than 30% (Fig. 2). Adding the expected increase associated with using the spectrophotometric method [33,37] with the unexpected increase associated with this "cyclical-freezing and thawing phenomenon" may explain how reassociation values as high as those reported by Vauterin et al. [65] among the "axonopodis" groups of xanthomonads may have occurred. Since this study was conducted over a 12-month period, all ssDNAs used as targets were heated to 68 °C for 5 min prior to use in DNA reassociation assays.

Using the S_1 nuclease method for DNA relatedness, we confirm previous results showing a 70% or greater DNA relatedness at T_m−15 °C between the "A" strains and *X. campestris* pv. *malvacearum* [19]. Also, we confirm the results of Vauterin et al. [66] showing a 70% or greater relatedness between the "A" strains and *X. campestris* pv. *glycines.* Vauterin et al. [65] included several additional xanthomonads, including *X. campestris* pathovars *malvacearum*, *begoniae, alfalfae*, *phaseoli*, *phaseoli* var. fuscans, *dieffenbachiae*, *cassavae, vesicatoria, vitians, vignicola*, and *vasculorum* in the same homology group (*X. axonopodis*) as the five groups of citrus pathogens. We show that none of these bacteria share species-level DNA relatedness (70% or greater) with *X. axonopodis* pv. *axonopodis*. However, we agree with Egel and Stall [20] that pv. *malvacearum* shares over 70% relatedness to the "A" citrus strains (T_m−15 °C). Additionally, nucleotide sequence analysis of the *lrp* gene show a high relatedness between the "A" strains and *X. campestris* pv. *malvacearum* [15]. *X. campestris* pv. *phaseoli* var. fuscans shares over 70% (mean) with the "B/C/D" strains, at both T_m−15 °C and T_m−8 °C. Furthermore, our results showed that *X. campestris* pv. *phaseoli* var. fuscans and "A" strains shared a mean relatedness of 44 and 51% at T_m−15 °C

Table 6. Characters useful for differentiating among citrus xanthomonads, *Xanthomonas smithii* subsp. *citri* (Xsc), *X. fuscans* subsp. aurantifolii (Xfa), *X. alfalfae* subsp. *citrumelo* (Xac), and from xanthomonads *X. smithii subsp. smithii (Xss*), *X. fuscans subsp. fuscans* (Xff), *X. alfalfae* subsp. *alfalfae* (Xaa*)*, *X. campestris* pv. *campestris* (Xcc*)*, and *X. axonopodis* pv. *axonopodis* (Xaax)

Character	Xsc (2)[a]	Xss (3)	Xfa (4)	Xff (1)	Xac (2)	Xaa (1)	*X*cc (1)	*Xaax (1)*
DNA/DNA relatedness to:[b]								
Xsc (21)	86	79	44	52	31	41	11	24
Xss (6)	72	93	39	48	31	44	19	20
Xfa (10)	43	49	84	75	37	33	8	26
Xff (5)	35	52	79	90	28	43	16	23
Xac (7)	31	41	33	34	79	72	8	23
Xaa (2)	44	41	33	26	72	98	ND	26
Xcc (3)	9	11	9	12	12	17	91	7
Xaax (2)	18	26	31	35	24	22	13	90
ITS dissimilarity:[c]	(6)	(5)	(8)	(4)	(5)	(2)	(3)	(2)
Xsc	0	1	7	8	7	8	19	9
Xfa	7	6	0	1	4	5	22	6
Xac	7	8	4	5	0	1	21	5
Xcc	19	20	22	23	21	23	0	22
Xaax	9	10	6	7	5	7	22	0
Growth on:[d]	(14)	(6)	(10)	(5)	(6)	(2)	(6)	(2)
YDC agar	40–44 brn−	40–44 brn−	56–60 brn +	46–50 brn +	30–34 brn−	30–34 brn−	40–44 brn−	150–170 brn−
FS agar	48–52	48–52	70–76	48–52	40–44	40–44	46–50	1 week +
mSX	56–60	56–60	80–84	56–60	48–52	48–52	56–60	1week +
Utilization of:[e]								
Arabinose	13/14	2/5	6/10	1/3	a15/15	a2/2	3/3	0/2
Maltose	14/14	3/5	1/10	3/3	a13/15	a2/2	3/3	0/2
Lactose	14/14	2/5	8/10	1/3	a15/15	a2/2	3/3	0/2
Mannitol	10/14	0/5	9/10	1/3	a15/15	a2/2	3/3	0/2
Melizitose	0/14	0/5	3/10	0/3	5/15	2/2	2/3	0/2
Cellobiose	7/7	nd	8/8	nd	a15/15	a2/2	a2/2	0/1
Raffinose	0/14	0/5	3/10	0/3	15/15	0/2	2/2	0/1
Saccharic acid[f]	0/7	nd	4/8	nd	0/15	2/2	2/2	0/1
Asparatic acid[f]	7/7	nd	6/8	nd	13/15	0/2	0/2	0/2
Pectate hydrolysis[g]	14/14	0/5	1/10	2/3	10/15	2/2	3/3	0/2
Litmus milk[h]	ah13/14	a4/5	ap8/10	ah3/3	ah5/15	ah2/2	ah1/3	0/2
Gelatin liquified	14/14	5/5	3/10	3/3	14/15	2/2	3/3	0/2
Pathogenicity[i]	Citrus	Gos	Citrus	Pv	Citrus	Ms	Br	As,So

[a]Numbers in paranthesis are numbers of strains tested.
[b]Reassociations done at T_m−15 °C; figures are mean percent.
[c]Number of nucleotides different.
[d]Time (hours) for colonies to reach 1 mm at 28 °C; m, mucoid; nm, non- mucoid;—, no growth. Brown pigment (brn) produced (+) or not produced (−) on YDC.
[e]Number of strains positive/numberof strains tested; a, acid; nd, not determined.
[f]Alkaline production with medium adjusted to pH 6; nd, not determined.
[g]Number of strains positive/number of strains tested under neutral pH; pits present after 7 days at 28 °C.
[h]Number of strains positive/number tested; a, alkaline; h, hydrolized; p, precipitated; after 7 days at 28 °C.
[i]Gos, *Gossypium*; Pv, *Phaseolus vulgaris*; Ms, *Medicago sativa*; Br, *Brassica*; As, *Axonopus scoparius*.; So, *Saccharum officinarum*.

and T_m−8 °C, respectively. With regard to the "E" strains, our results showed that they shared over 70% in average relatedness with pathovars *alfalfae* and *dieffenbachiae* at T_m−15 °C and T_m−8 °C. Our results showing *X. axonopodis* (sensu Vauterin [65]) pathovars *begoniae*, *phaseoli*, *phaseoli* var. fuscans, *cassavae*, and *vitians* only sharing 44%, 21–27%, 21–24%, 16%, and 7% relatedness, respectively, with *X. axonopodis* pv. *axonopodis* do not support reclassifying them as *X. axonopodis* [65]. Our results agree that *X. campestris* pv. *vitians* should not be reclassified as *X. axonopodis* pv. *vitians* [4,54] but, like the others, left as a pathovar of *X. campestris* until additional DNA–DNA relatedness and ITS sequencing assays have been completed.

Gabriel et al. [23] proposed reinstating the "A" group strains of *X. campestris* pv. *citri* to species status (*X.*

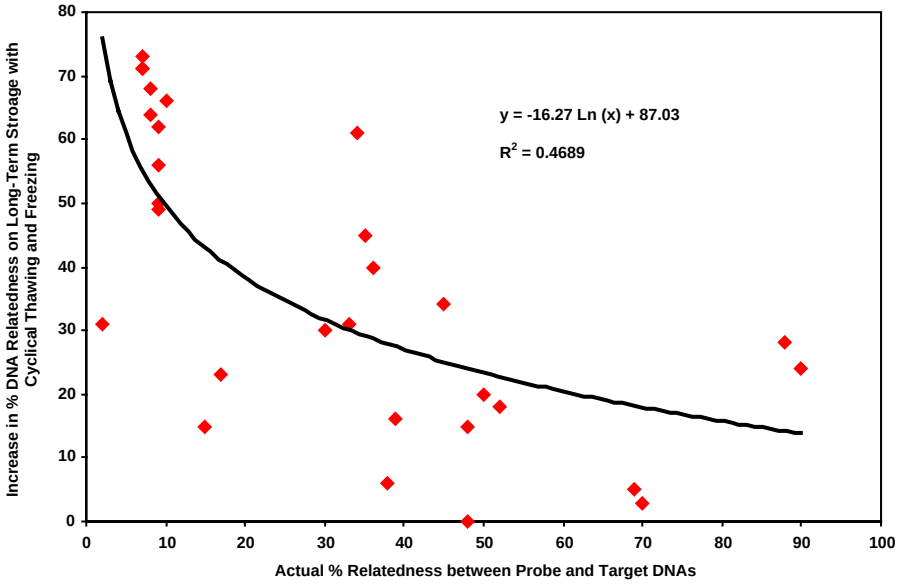


Fig. 2. Effect of cyclical freezing and thawing of stored target ssDNAs on % DNA relatedness. The logarithmic trendline compares the apparent increase in % DNA relatedness (*X*-axis) with cyclical freezing (−20 °C) and thawing (12–17 °C) of the target ssDNAs (*Y*-axis) with the actual % relatedness of the probe to target DNAs after heating to 68 °C for 5 min (*X*-axis). Target ssDNAs were stored in buffer (1 mM EDTA in 10 mM TrisHCl at pH 8.0) at −20 °C for 6 months to over one year and were cyclically thawed and re-frozen during multiple uses. Probe ssDNAs were labeled with ^{125}I and the % DNA relatedness was determined by the methods of Johnson [38]. The apparent the increase (*Y*-axis) was determined subtracting the actual % DNA relatedness after heating the target ssDNAs to 68 C for 5 min from the apparent % relatedness with thawing in cold tap water (12–17 °C). ssDNAs used as probes and/or targets were obtained from *Xanthomonas* species and/or pathovars *albilineans* (ATCC33915), *alfalfae* (ATCC11648) *axonopodis* (ATCC19312), *campestris* (ATCC33913), *citri* (ATCC49118), *citrumelo* (ATCC49120), diffenbachiae (ATCC23379), *gardneri* (ATCC19865), *malvaceraum* (ATCC9924 and ATCC49290), *pelargonii* (ATCC8721), *phaseoli* (ATCC9563), *vesicatoria* (ATCC11551, ATCC11633, ATCC35937), and *vignicola* (ATCC11648).

citri) based upon RFLP analysis, however, that proposal has not been accepted due to a lack of DNA-reassociation data [73]. When we compared our AFLP data with the DNA-DNA reassociation assay results we found the two methods agreed very closely when an AFLP similarity coefficient value of about 45% or greater was used for establishing a sub-species or species. The AFLP data were very useful in confirming the close relatedness between any two groups with a 60–69% DNA relatedness.

The ITS sequencing results correlated highly with the DNA/DNA reassociation results when using a sequence similarity value above 99.6% as belonging to the same subspecies. Strains among citrus group "B/C/D" shared 99.8% ITS-ITS similarity (1 nt different). Our results and those of Egel and Stall [20] showed a mean DNA relatedness of 70% or greater between the "A" group strains and *X. campestris* pv. *malvacearum*. When a higher stringency of T_m−8 °C was used the mean percent relatedness remained above 60%. Also, the ITS region of the two bacteria are nearly identical, differing by only one nucleotide. Furthermore, analysis of the *lrp* gene of several xanthomonads, including citrus groups "A, B, C, and D" strains, showed that the "A" strains were highly related to pv. *malvacearum* [15,31].

Based on genetic and phenotypic analysis, we propose that pv. *malvacearum* and the citrus "A" strains be classified as subspecies of the same species. To avoid confusion between these two important pathogens, we propose a new species, *X. smithii* in honor of the pioneer American phytobacteriologist Erwin F. Smith, who described *X. malvacearum* in 1901, to include both organisms. We propose *X. campestris pv. malvacearum* and the "A" strains be named *X. smithii* subsp. *smithii* and *X. smithii* subsp. *citri*, respectively. Our DNA reassociation assays, ITS sequencing, and AFLP results also showed a close similarity between pv. *glycines* and the "A" strains (Tables 2 and 3). However, we suggest that pv. *glycines* remain as *X. campestris* pv. *glycines* until additional data are available. Several variants (based on genetic analysis and host specificities) of the "A" strains of citrus canker bacterium have been described, including the "*A**" strain from Southwest Asia and "A^{w}" (Wellington) strain from Florida [63,68]. Based on our DNA–DNA reassociation assays and phenotypic tests, these "A^{w}" and "A*" strains are typical strains of *X. smithii* subsp. *citri*.

We confirm results of earlier phenotypic studies showing that group "A" strains can be differentiated from group "B" and "C" strains [24,47]. We extend

these results by showing that the "A" strains utilize maltose, and hydrolyze pectate whereas "B/C/D" strains do not. The "B/C/D" strains cause an acid precipitation of litmus milk whereas the"A" strains produce an acid hydrolysis. Gabriel et al. [23] proposed reclassifying the "B/C/D" group of citrus xanthomonads as *X. campestrs* pv. *aurantifolii*. Our results agree that these three groups of bacteria are highly related. However, our results also showed a mean reciprocal DNA relatedness of 77% between the "B/C/D" bacteria and *X. campestris* pv. *phaseoli* var. fuscans. That high level of reassociation was supported by additional reassociations at T_m-8 °C showing a mean reciprocal DNA reassociation value of 80% for var. fuscans and the "B/C/D" strains. Also, the ITS sequence of the two organisms differed by only one or two nucleotides. These results support classifying these organisms as the same species. However, we propose that these bacteria be classified as subspecies since they can be differentiated phenotypically (Table 6). The epithets "fuscans" and "aurantifolii" are not considered valid names [75], however, both are widely used by plant pathologists for describing a bean and citrus pathogen, respectively. Since "fuscans" was described prior to "*aurantifolii*", we propose that var. fuscans be elevated to species status and contain *X. fuscans* subsp. *fuscans* and *X. fuscans* subsp. *aurantifolii*. Although var. fuscans was previously designated as a variety of the bean pathogen *X. campestris* pv. *phaseoli*, genetic analysis shows a wide divergence between *X. campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. fuscans [6,13; G.H. Lacy, unpublished]. Additionally, our DNA reassociation results showed a high relatedness between var. fuscans and *X. campestris* pv. *vignicola* (Table 2). Since we have only limited data with *X. campestris* pv. *vignicola* strains, we suggest that pv. *vignicola* remain as *X. campestris* pv. *vignicola* until additional data are available. All strains of var. fuscans and 30% of pv. *aurantifollii* strains produced a brown pigment on NBY and YDC agar. These results agree with the reported production of a brown pigment on common agar media by "C" strains [17], *X. campestris* pv. *phaseoli* var. fuscans [12,46] and *X. campestris* pv. *vignicola* [12].

Based upon current and previous results [19,23,68], the citrus group "E" pathogens should be classified as *X. alfalfae* subsp. *citrumelo*. The "E" group bacteria showed a mean reciprocal relatedness of more than 70% at both T_m-15 °C and T_m-8 °C with the *X. campestris* pv. *alfalfae* strains and the ITS region differed by only one or two nucleotides. We prefer the epithet *alfalfae* over *citrumelo* because *X. alfalfae* was described in 1935 [51] whereas pv. *citrumelo* was described in 1987 [57] and is of little economic importance. Previous RFLP results suggested a close relationship between group "E" strains and *X. campestris* pv. *alfalfae* [23,30,35]. Gabriel et al. [23] showed positive cross-species pathogenicity between some "E" strains and most *X. campestris* pv. *alfalfae* strains with Duncan grapefruit (*C. paradisi*) or Swingle citrumelo (*C. paradisi* x *Poncirus trifoliiata*) and alfalfa and concluded the symptoms were indistinguishable from the control homologous strains. Although we failed to observe symptoms on alfalfa plants when inoculated with the "E" strains, our negative results could have been due to the cultivar differences in alfalfa. Since these strains are genetically highly related but distinguishable phenotypically (Table 6), we propose they be classified as subspecies. Our AFLP results agreed with the RFLP results [35] and *lrp* analysis [15] that Florida citrus nursery strains ("E") are very different from any of the canker strains (groups "A" and "B/C/D"). We agree that the strains of *X. campestris* pv. *dieffenbachiae* included in this study share 70% or more relatedness with "E" strains [65] and has a highly related *lrp* gene [15]. However, since we have limited data with strains of pv. *dieffenbachiae*, we suggest it remain as *X. campestris* pv. *dieffenbachiae* until additional DNA–DNA relatedness data are available.

Summary of characters. Table 6 summarizes some of the most important characters for distinguishing among the three citrus pathogens, X. *smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii* and *X. alfalfae* subsp. *citrumelo*, and between these three citrus pathogens and *X. campestris* pv. *campestris* and *X. axonopodis* pv. *axonopodis*. DNA relatedness assays and ITS sequence assays separate *X. smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii,* and *X. alfalfae* subsp. *citrumelo* from *X. campestris* pv. *campestris, X. axonopodis* pv. *axonopodis* and 19 other xanthomonads. All three citrus pathogens are differentiated from each other and from *X. axonopodis* pv. *axonopodis* and *X. campestrris* pv. *campestris* by phenotypic analysis. Furthermore, the three citrus xanthomonds can be easily differentiated from each other by several simple phenotypic tests (Table 7).

Species descriptions

The descriptions of the species *X. smithii*, *X. fuscans*, and *X. alfalfae* are the same as that of the genus *Xanthomonas* Dowson 1939. *X. smithii* can be subdivided into two subspecies, *smithii* and *citri*; *X. fuscans* can be divided into subspecies *fuscans* and *aurantifolii*; and *X. alfalfae* can be divided into subspecies *alfalfae* and *citrumelo*.

Xanthomonas smithii subsp. smithii sp. nov. comb. nov. nom. nov.; smi' thi.i. N.L. gen. masc. n. smithii of Smith, in honor of Erwin F. Smith who first described *X. malvacearum* in 1901. *X. smithii* subsp. *smithii* replaces the former taxon *X. campestris* pv. *malvacearum* (Smith, 1901) Dye 1978. The bacterium causes angular

Table 7. Simplified phenotypic differentiation among citrus xanthomonads, *Xanthomonas smithii* subsp. *citri*, *X. fuscans* subsp. *aurantifolii*, and *X. alfalfae* subsp. *citrumelo*

Character	*X. smithii sbp.citri*	*X. fuscans sbp.aurantifolii*	*X .alfalfae sbp. citrumelo*
Utilization of:[a]			
Maltose	+	−	+
Raffinose	−	V−	+
Saccharic acid	−	V+	−
Pectate hydrolysis	+	−	V+
Litmus milk; alk, ppt[b]	−	+	−
Gelatin liquefied	+	V−	+
Growth on:[c]			
YDC agar (Brown pigment)	40–44 h (−)	56–60 h (+)	30–34 h (−)
FS agar	48–52 h	70–76 h	40–44 h
mSX agar	56–60 h	80–84 h	48–52 h
Hyperplastic leaf lesions[d]	+	+	−

[a] +, 80% or greater positive; −, 80% or greater negative; V^+, 50–79% positive (= 21–49% negative); V^-, 50–79% negative (= 21–49% positive).
[b] Alk = alkaline reaction; ppt = precipitated.
[c] Time (h) for colonies to reach 0.8–1.0 mm in diameter at 28 °C on yeast dextrose-calcium carbonate agar, Field-Sasser agar, and modified selective xanthomonas agar. Brown water soluble pigment produced (+) or not produced (−) on YDC.
[d] Results of pathogenicity tests on lime (*Citrus limetta*); leaf lesion showing excessive growth of cells; raised and erumpent.

leaf spot and black arm of cotton (*G. hirsutum L.*). *X. smithii* subsp. *smithii* is differentiated from *X. campestris* pv. *campestris* and most other pathovars by DNA reassociation assays [65 and this study] and by serology [64] and SDS-PAGE patterns of membrane proteins [67]. *X. campestris* pv. *campestris* utilizes melizitose and hydrolyzes pectate whereas *X. smithii* subsp. *smithii* does not. *X. smithii* subsp. *smithii* is distinguished from *X. smithii* subsp. *citri*, *X. fuscans* subsp. *fuscans* and subsp. *aurantifoli*, and *X. alfalfae* subsp. *alfalfae* and subsp. *citrumelo* by DNA reassociation assays, ITS sequencing, and phenotypic characters (Table 6). *X. smithii* subsp. *smithii* produces an alkaline reaction without hydrolysis in litmus milk whereas *X. smithii* subsp. *citri* causes an alkaline reacton with hydrolysis. *X. smithii* subsp. *smithii* grows on FS and mSX agars, liquifies gelatin, and most strains (60%) utililize maltose. The pathotype strain of *X. smithii* subsp. *smithii* is ICPB $10528^{\mathbf{T}}$ = ATCC $9924^{\mathbf{T}}$ = ICMP $217^{\mathbf{T}}$ = LMG $785^{\mathbf{T}}$ = NCPPB $2005^{\mathbf{T}}$ = PDDCC $2870^{\mathbf{T}}$.

X. smithii subsp. *citri* (ex Hasse 1915) sp. nov. nom. rev. comb. nov. *X. smithii* subspecies. *citri* causes bacterial canker of citrus. *X. smithii* subsp. *citri* may be distinguished from *X. smithii* subsp. *smithii*, *X. fuscans* subsp. *aurantifoli* and *X. alfalfae* subsp. *citrumelo* strains by DNA/DNA reassociation assays, ITS sequencing, and phenotypic traits. *X. smithii* subsp. *citri* utilizes arabinose and lactose and hydrolyzes pectate whereas *X. smithii* subsp. *smithii* does not. *X. smithii* subsp. *citri* reduces aspartic acid whereas *X. campestris* pv. *campestris* does not. The latter utilizes raffinose and reduces saccharic acid whereas the former does not. Both bacteria are easily differentiated by host pathogenicity assays and by serology [2,3,11,14,64], and membrane protein analysis [44,67]. Serology differentiates *X. smithii* subsp. *citri* from *X. fuscans* subsp. *aurantifolii* [24,25,47]. Strains of *X. smithii* subsp. *citri* are susceptible to bacteriophage CP1 and CP2 whereas those of *X. fuscans* subsp. *aurantifolii* are not [47]. *X. smithii* subsp. *citri* grows on FS and mSX agars, utilizes arabinose, maltose, lactose, mannitol, cellobiose, and asparatic acid; hydrolyzes pectate, liquifies gelatin, and results in an alkaline hydrolysis of litmus milk. The pathotype strain of *X. smithii* subsp. *citri* is ICPB $10518^{\mathbf{T}}$ = ATCC $49118^{\mathbf{T}}$ = Gabriel $3213^{\mathbf{T}}$ = LMG)$9322^{\mathbf{T}}$.

X. fuscans subsp. *fuscans* sp. nov.; fus'cans L. part. adj. fuscans, browning/darkening. *X. fuscans* subsp. *fuscans* [*X. campestris* pv. *phaseoli* var. fuscans (ex Burkholder, 1930)] causes bacterial blight of beans. *X. fuscans* subsp. *fuscans* is differentiated from *X. campestris* pv. *campestris* by serology [63] and membrane protein analysis [45,64]. *X. fuscans* subsp. *fuscans* is differentiated from other xanthomonads by DNA/DNA reassociation assays, ITS sequences, and phenotypic traits. Strains of *X. fuscans* subsp. *fuscans* grow on FS and mSX agars, utilizes maltose, hydrolyzes pectin, and produces an alkaline hydrolysis of litmus milk. All strains of *X. fuscans* subsp. *fuscans* produce a soluble brown pigment on several common agar media including YDC [12,16,46, this study]. Except for some strains of *X. fuscans* subsp. *aurantifolii*, and *X. campestris* pv. *vignicola,* no other xanthomonad is known to produce such a brown pigment. The pathotype strain of *X. fuscans* subsp. *fuscans* is ICPB $10520^{\mathbf{T}}$ = ATCC $19315^{\mathbf{T}}$ = NCPPB $381^{\mathbf{T}}$ = ICMP $239^{\mathbf{T}}$ = LMG $826^{\mathbf{T}}$.

X. fuscans subsp. *aurantifolii* (ex Gabriel et al., 1989) sp. nov. nom. rev. comb. nov.; au. ran. ti.fo'li.i. N.L. neut. n. *Aurantium*, a genus of citrus plants, gen. neut. n. folii of/from leaf, L. gen. neut. n. aurantifolli from citrus leaves. *X. fuscans* subsp. *aurantifolii* causes canker on Mexican lime and occasionally on lemon, orange, and grapefruit. *X. fuscans* subsp. *aurantifolii* is differentiated from other xanthomonads by DNA/DNA reassociation assays and phenotypic traits. X. *fuscans* subsp. *aurantifolii* is distinguished from *X. smithii* subsp. *citri* and *X. alfalfae* subsp. *citrumelo* by precipitating litmus milk and failing to hydrolyze gelatin. *X. fuscans* subsp. *aurantifolii* does not utilize maltose or hydrolyze pectate whereas *X. smithii* subsp. *citri* and *X. fuscans* subsp. *fuscans* do. *X. fuscans* subsp. *aurantifolii* precipitates litmus milk, whereas subsp. *fuscans* does not. *X. fuscans* subsp. *fuscans* is distinguished from *X. smithii* subsp. *citri* and *X. campestris* pv. *campestris* by failing to utilize arabinose and lactose. Serology differentiates *X. smithii* subsp. *citri from X. fuscans* subsp. *aurantifolii* [24,25,47]. Strains of *X. smithii* subsp. *citri* are susceptible to bacteriophage CP1 and CP2 whereas those of *X. fuscans* subsp. *aurantifolii* are not [47]. Strains of *X. fuscans* subsp. *aurantifolii* utilize lactose, mannitol, and cellobiose and precipitate litmus milk. Strains of *X. fuscans* subsp. *aurantifolii* produce single colonies on YDC and FS agar after 56–60 and 70–76 h, respectively at 28–30 °C. In contrast, *X. smithii* subsp. citri produces single colonies in only 40–44 h and 56–60 h, respectively, and *X. alfalfae* subsp. *alfalfa*e and subsp. *citrumello* grow in only 30–34 and 40–44, respectively. The pathotype strain of *X. fuscans* subsp. *aurantifolii* is ICPB 10470^{T} = NCPPB 3236^{T} = CFBP 2901^{T} = LMG 9179^{T}.

X. alfalfae subsp. *alfalfae* (ex. Riker et al., 1935) sp. nov. nom. rev. *X. alfalfae* subsp. *alfalfae* causes leaf spot of alfalfa. *X. alfalfae* subsp. *alfalfae* is distinguished from *X. campestris* pv. *campestris* and other xanthomonads by DNA/DNA reassociation assays and ITS sequencing and by producing acid from most carbon sources whereas *X. campestris* pv. *campestris* does not. *X. campestris* pv. *campestris* utilizes raffinose whereas *X. alfalfae* subsp. *alfalfae* does not. *X. alfalfae* subsp. *alfalfae* grows faster on YDC agar than do most other xanthomonads. Strains of *X. alfalfae* subsp. *alfalfae* produce an alkaline reaction on saccharic acid whereas strains of *X. alfalfae* subsp. *citrumelo* do not. *X. alfalfae* subsp. *alfalfae* utilizes arabinose, maltose, lactose, mannitol, melizitose, and cellobiose, liquifies gelatin, and produces an alkaline hydrolysis of litmus milk. The neotype strain of *X. alfalfae* subsp. *alfalfae* is ICPB 10701^{T} = ATCC 11765^{T} = LMG 495^{T}.

X. alfalfae subsp. *citrumelo* (ex. Riker et al., 1935) Gabriel et al., 1989 sp. nov. nom. rev. comb. nov. X. *alfalfae* subsp. *citrumelo* causes leaf spot of citrus. *X. alfalfae* subsp *alfalfae* is distinguished from *X. campestris* pv. *campestris* and other xanthomonads by DNA/DNA reassociation assays and ITS sequencing. *X. alfalfae* subsp. *citrumelo* strains are differentiated from *X. smithii* subsp *smithii* and *X. fuscans* subsp. *aurantifolii* by serological assays [3,27,40]. *X. alfalfae* subsp. *citrumelo* utilizes raffinose whereas *X. alfalfae* subsp. *alfalfae, X. smithii* subsp. *citri,* and *X. smithii* subsp. *smithii* strains do not. *X. alfalfae* subsp. *alfalfae* and subsp. *citrumelo* can be differentiated from *X. fuscans* subsp. *aurantifolii* on their more rapid growth on agar media, liquefaction of gelatin, and utilization of maltose. *X. alfalfae* subsp. *citrumelo* is distinguished from *X. smithii* subsp. *citri* by utilizing raffinose, producing acid from cellobiose and mannitol, and growing faster on YDC and FS agars. All strains of *X. alfalfae* subsp. *citrumelo* utilize mannitol and raffinose whereas strains of *X. smithii* subsp. *smithii* do not. *X. alfalfae* subsp. *citrumelo* utilizes arabinose, maltose, lactose, mannitol, melizitose, and cellobiose, liquifies gelatin, and produces an alkaline hydrolysis of litmus milk. The holotype strain of *X. alfalfae* subsp. *citrumelo* is ICPB 10483^{T} = ATCC 49120^{T} = LMG 9325^{T}.

All strains are available in the International Collection of Phytopathogenic Bacteria (ICPB) maintained by USDA/ARS, Ft Detrick.

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