Restriction Fragment Length Polymorphisms Distinguish *Xanthomonas campestris* Strains Isolated from Florida Citrus Nurseries from *X. c.* pv. *citri*

John S. Hartung and E. L. Civerolo

U.S. Department of Agriculture, Agricultural Research Service, Plant Science Institute, Fruit Laboratory, Beltsville, MD 20705.

We gratefully acknowledge the technical assistance of A. S. O'Conner.
Accepted for publication 24 February 1989.

ABSTRACT

Hartung, J. S., and Civerolo, E. L. 1989. Restriction fragment length polymorphisms distinguish *Xanthomonas campestris* strains isolated from Florida citrus nurseries from *X. c.* pv. *citri*. Phytopathology 79:793-799.

Genomic DNA prepared from 21 strains of *Xanthomonas campestris* pv. *citri*, 14 strains of *X. campestris* isolated from Florida citrus nurseries, and 10 strains of five other pathovars of *X. campestris* was used for restriction fragment length polymorphism (RFLP) analysis. The *X. c. citri* strains included 14 strains from group A, five strains from group B, and single strains from groups C and D. Seven cosmid clones selected from a library of strain XC62 of *X. c. citri* (group A) were used to screen for *Eco*RI and *Pvu*II polymorphisms, which were quantified by calculating coefficients of similarity (*F*). Statistical analysis of the RFLP data distinguished the group A strains from the other strains of *X. c. citri*, which formed a second distinct group (group B/C/D). On the basis of these analyses, a clonal population structure is evident for this globally distributed pathogen, consistent with previous groupings of the strains based on other criteria. A relatively heterogeneous group of related strains of *X. campestris* has been isolated from Florida citrus nurseries. Statistical analysis of the RFLP data revealed a significant separation between the 14 strains of *X. campestris* isolated from Florida citrus nurseries and all strains of *X. c. citri*. Thus, this collection of strains is not closely related to previously recognized strains of *X. c. citri*. This is consistent with disease symptomology, epidemiology, and distribution. We conclude that the disease associated with *X. campestris* in Florida citrus nurseries is not a form of citrus bacterial canker and that the pathogen is not a strain of *X. c. citri*.

Citrus bacterial canker (CBC), caused by strains of *Xanthomonas campestris* pv. *citri*, is endemic in many regions of the world where citrus is grown. Typical symptoms include raised necrotic lesions on fruit, leaves, and stems. Severe infestations can cause defoliation and premature fruit drop (1,26). CBC was introduced into Florida in 1910, probably from Japan (2). After a massive eradication campaign, Florida was declared free of CBC in 1933 (1).

Four forms of CBC have been described, on the basis of host range, geographic distribution, serology, phage typing, and plasmid analyses of bacterial strains (5,6,13,26): the A form, or Asiatic CBC, which occurs worldwide; the B form, or cancrosis B, which occurs primarily in Argentina; the C form, or Mexican lime cancrosis, which occurs in Brazil; and the D form, or Mexican bacteriosis, which occurs in Mexico. The A form is the most widespread variant of the disease, and group A strains of *X. c. citri* are also the most virulent. Although the symptoms of the four forms of CBC on susceptible hosts are superficially and ultrastructurally similar, the relationship among the four groups of strains (groups A–D) is largely unknown.

In 1984, the discovery of leaf and stem lesions caused by *X.*

campestris in Florida citrus nurseries aroused great consternation and prompted extensive eradication efforts (23). In spite of these efforts, similar lesions have been found in nurseries in each succeeding summer and fall at locations throughout Florida. Strains of *X. campestris* isolated from citrus nurseries do not cause raised lesions on diseased plants, as do all strains belonging to groups A–D of *X. c. citri*. Instead the Florida nursery strains produce flat, water-soaked lesions of various sizes. They are also generally found only on nursery stock, and not on mature trees in commercial groves.

In 1986, the A form of CBC was found on a large number of "backyard" trees in the area of Tampa, Florida, and in one commercial grove (23). The relationship, if any, of the Florida citrus nursery strains of *X. campestris* to *X. c. citri* is not understood. We have been interested in developing rapid and reliable methods for distinguishing between the strains of bacteria causing the various forms of CBC as well as the new leaf-spotting disease of Florida citrus nurseries. The relationship of the A strains of *X. c. citri* in Florida to other A strains has also been of interest.

On the basis of genomic fingerprinting (14) and restriction fragment length polymorphism (RFLP) analysis (12), groups A and B of *X. c. citri* strains are distinct. The collection of strains from Florida nurseries did not form a single group, and many different genomic fingerprints and RFLP patterns were observed.

Although genomic fingerprinting is relatively easy to perform, the extremely large number of bands in a lane makes comparisons between gels and between lanes within a gel difficult. If the genomic fingerprint is transferred from an agarose gel to a nitrocellulose filter and fragments of labeled DNA are used as hybridization probes, a subset of bands from the fingerprint can be visualized (25). The resulting hybridization patterns reveal the presence or absence of RFLP. This technique has been used to study the relatedness of plasmid DNAs in pathovars of *X. campestris* (17); to distinguish pathovars of *X. campestris* (18) and of *Pseudomonas syringae* (8); to study the population structure of pathovars of *X. campestris*, including *X. c. citri* (12); to distinguish between strains of *Agaricus* (4); and to distinguish species of *Meloidogyne* (21). We now report a comparative RFLP analysis of *X. c. citri* strains isolated worldwide as well as *X. campestris* strains isolated from Florida citrus nurseries.

MATERIALS AND METHODS

Bacterial strains and extraction of genomic DNA. RFLP patterns of 21 strains of *X. c. citri* were compared. These included 14 group A strains, isolated in five countries and Florida; five group B strains, isolated in Argentina; one group C strain, isolated in Brazil; and one group D strain, isolated in Mexico (Table 1). Fourteen strains of *X. campestris* isolated from Florida citrus nurseries were also analyzed. Ten strains of five other pathovars of *X. campestris* were also analyzed, for comparative purposes. Genomic DNA was prepared and digested to completion with the restriction endonucleases *Eco*RI and *Pvu*II as described previously (14).

Construction of RFLP probes. A complete genomic library (2,000 CFU) of the group A strain XC62 was prepared by standard methods (19). The restriction endonuclease *Sau*3A was used to partially digest XC62 DNA. Size-selected fragments (more than 20 kb) were ligated with T4 DNA ligase into the cosmid cloning vector pCP13 (7), a derivative of pLAFR1 (10). The vector had been digested with the restriction endonuclease *Bam*H1 and treated with calf intestinal alkaline phosphatase. The ligation mixture was then packaged into lambda phage particles in vitro. The phage particles were used to transfect *Escherichia coli* DH1 to tetracycline resistance. Individual clones from this library were arbitrarily selected as the source of the RFLP probes. The cosmids were purified from bacterial strains grown in LB broth by the alkaline lysis technique and isopycnic centrifugation in cesium chloride/ethidium bromide density gradients (19). The purified cosmids were labeled with biotin-11-dUTP by nick translation. The cosmid probes used in this study were designated pXC621, pXC622, pXC626, pXC627, pXC628, pXC6210, and pXC6211 and had inserts of 26, 22, 30, 18, 22, 28, and 27 kb, respectively. They were independent clones, which did not cross-hybridize.

Sources of reagents and supplies. Enzymes and biotinylation kits were from BRL, Gaithersburg, MD. Lambda packaging extracts were from Promega, Madison, WI. Chemicals were from Sigma, St. Louis, MO.

Electrophoresis and blotting conditions. Digested DNA (7 μg) was separated, per lane, in 14-cm 0.8% agarose gels with TPE buffer (0.08 M Tris phosphate and 0.002 M EDTA) (18) at 5 V/cm until the bromphenol blue tracking dye was 2 cm from the end of the gel. The gels were stained with ethidium bromide, and the DNA was transferred to nitrocellulose (24). All blotting experiments were carried out at least twice.

Hybridization and detection of RFLP. Standard stringent hybridization conditions were used without formamide (19). Incubations were at 70 C, and probe concentrations were 400 ng/ml. After the series of posthybridization washes, the filters were rinsed with three changes of buffer I (0.1 M Tris HCl, pH 7.5, and 0.15 M NaCl) and blocked with bovine serum albumin.

TABLE 1. Strains of *Xanthomonas campestris*

Strain	Pathovar[a,b]	Comments[c]
XC62	*citri* A	Japan
XC63	*citri* A	Japan
XC91	*citri* A	Argentina, D. Zagory
XC92	*citri* A	Argentina, D. Zagory
XC101	*citri* A	Guam
XC102	*citri* A	Guam
XC100	*citri* A	Pakistan
XC98	*citri* A	Yemen
XC103	*citri* A	Florida, J. Miller
F134	*citri* A	Florida, J. Miller
F198	*citri* A	Florida, J. Miller
F200	*citri* A	Florida, J. Miller
XC115	*citri* A	NCPPB
XC118	*citri* A	PDDCC
XC64	*citri* B	Argentina, J. Miller
XC69	*citri* B	Argentina, J. Miller
XC93	*citri* B	Argentina, D. Zagory
XC94	*citri* B	Argentina, D. Zagory
XC95	*citri* B	Argentina, D. Zagory
XC70	*citri* C	Brazil, V. Rosetti
XC90	*citri* D	Mexico
F1	FCN-1	J. Miller
F2	FCN-1	J. Miller
F3	FCN-1	J. Miller
F4	FCN-1	J. Miller
F5	FCN-2	J. Miller
F6	FCN-3	J. Miller
F29	FCN-4	J. Miller
F30	FCN-4	J. Miller
F49	FCN-4	J. Miller
F51	FCN-5	J. Miller
F54	FCN-6	J. Miller
F94	FCN-7	J. Miller
F95	FCN-7	J. Miller
F97	FCN-7	J. Miller
X7	*campestris*	Cabbage, J. Miller
X8	*campestris*	Broccoli, J. Miller
X11	*dieffenbachiae*	Anthurium, J. Miller
X12	*dieffenbachiae*	Philodendron, J. Miller
X35	*phaseoli*	NCPPB
X36	*phaseoli*	D. Gabriel
X45	*phaseoli*	ATCC
X40	*manihotis*	L. Sequeira
X60	*alfalfae*	D. Gabriel
X61	*alfalfae*	D. Gabriel

[a]The letters A–D with *X. c. citri* refer to groups A–D of strains of that pathovar (5,26).

[b]The pathovar status of the Florida citrus nursery (FCN) strains is undetermined; the numerals 1–7 with FCN strains refer to different nursery sites.

[c]ATCC = American Type Culture Collection; NCPPB = National Collection of Plant Pathogenic Bacteria, England; PDDCC = Plant Diseases Division Culture Collection, New Zealand.

The streptavidin/alkaline phosphatase detection system was used, following the manufacturer's protocol.

Similarity coefficients. Coefficients of similarity (20) between pairs of strains were calculated with the following formula:

$$F = 2n_{xy}/(n_x + n_y)$$

where n_x and n_y are the number of fragments in strains X and Y, respectively, and n_{xy} is the number of fragments in strain X that match fragments in strain Y. "Band matches," which determined the coefficients of similarity, were observed by direct comparison of hybridization patterns within the same blot. When strains that were to be compared were located on separate blots, preliminary band matches were determined after estimation of the molecular weights of the hybridizing bands by comparison with lambda/*Hin*dIII size standards. These preliminary band matches were then confirmed by samples tested in the same gel.

Statistical analysis. The values of F, the coefficient of similarity between strains, are the means of seven observations (seven probes with one endonuclease) in comparisons of strains of different pathovars of *X. campestris*; they are the means of 14 observations (seven probes with two endonucleases) in comparisons of different strains of *X. c. citri* and Florida citrus nursery strains. Analysis of variance was performed separately on the data summarized in Tables 3 and 5 with the SAS/STAT system (22). For this analysis the variable "type" was created to describe strain comparisons in terms of the groups to which the strains belong. For example, the comparison of the group A strain XC62 and the group A strain XC63 was a type AA comparison; that of XC62 and the group B strain XC64 was a type AB comparison. The strains of *X. c. citri* (Table 1) were from groups A–D. The strains of *X. campestris* isolated from Florida citrus nurseries were assigned to group U and its subgroups E–G on the basis of genomic fingerprinting (14) and by inspection of Table 5. Data from the same sorts of comparisons were collected in the same type variable. The analysis was blocked for the effect of endonuclease choice on the data, and the different probes were treated as replicates to test for significant effects due to the type of comparison.

Because the F value is only an estimate of the true mean relatedness (μ) of two strains or groups of strains, 95% confidence intervals containing μ were calculated (27). If groups A and B of *X. c. citri* were members of a common population, the mean value of F in AA comparisons would not be significantly different from the mean value in AB comparisons. If, however, groups A and B were truly distinct, these mean values of F would be significantly different. After analysis of variance demonstrated statistically significant differences between types, Bonferroni T-tests ($p = 0.05$) were performed to determine if the values of F for particular comparisons were significantly different. The Bonferroni method keeps the experiment-wide error rate fixed at the level of α. This allowed statistical inferences to be drawn regarding the relatedness of groups of strains.

RESULTS

For closely related strains the value of F should approach 1.0; for unrelated strains the value should approach 0. This assumption was validated by calculating values of F for 10 strains of five different pathovars of *X. campestris* (Table 2). As expected, the F values for between-pathovar comparisons (e.g., *X. c. campestris* strain X7 vs. *X. c. dieffenbachiae* strain X11) were lower than those for within-pathovar comparisons (e.g., *X. c. campestris* strain X7 vs. strain X8 and *X. c. phaseoli* strain X35 vs. strain X36 or X45), although there was some overlap in the range of these values (e.g., *X. c. phaseoli* strain X35 vs. *X. c. manihotis* strain X40). The values of F ranged from 0.07 to 0.99 for these comparisons, which gives an indication of the sensitivity of the

TABLE 2. Coefficients of similarity[a] based on *Eco*RI restriction fragment length polymorphisms for strains of *Xanthomonas campestris*

		X. c. campestris		*X. c. dieffenbachiae*		*X. c. phaseoli*			*X. c. manihotis*	*X. c. citri* A	*X. c. citri* B
	Strain	X7	X8	X11	X12	X35	X36	X45	X40	XC62	XC64
X. c. campestris	X7	—	0.76	0.09	0.10	0.15	0.09	0.12	0.07	0.15	0.12
	X8		—	0.12	0.15	0.15	0.19	0.21	0.21	0.17	0.08
X. c. dieffenbachiae	X11			—	0.49	0.65	0.51	0.55	0.58	0.33	0.35
	X12				—	0.40	0.41	0.47	0.43	0.33	0.36
X. c. phaseoli	X35					—	0.71	0.79	0.68	0.30	0.42
	X36						—	0.99	0.51	0.21	0.34
	X45							—	0.52	0.23	0.27
X. c. manihotis	X40								—	0.38	0.45
X. c. citri A	XC62									—	0.51
X. c. citri B	XC64										—

[a]The coefficients (calculated as described in reference 20) are averages of seven observations (analysis with the seven probes described in the text).

TABLE 3. Coefficients of similarity[a] based on restriction fragment length polymorphism (RFLP) for strains of *Xanthomonas campestris* pv. *citri*

Strain	Group	XC62[b] A	XC100 A	XC98 A	XC64 B	XC69 B	XC93 B	XC94 B	XC95 B	XC70 C	XC90 D
XC62[b]	A	—	0.87	0.97	0.62	0.61	0.61	0.61	0.61	0.62	0.61
XC100	A		—	0.82	0.61	0.59	0.60	0.60	0.60	0.64	0.60
XC98	A			—	0.60	0.59	0.60	0.60	0.60	0.60	0.60
XC64	B				—	0.89	0.89	0.89	0.89	0.82	0.89
XC69	B					—	0.96	0.96	0.96	0.85	0.96
XC93	B						—	1.00	1.00	0.84	1.00
XC94	B							—	1.00	0.84	1.00
XC95	B								—	0.84	1.00
XC70	C									—	0.89
XC90	D										—

[a]The coefficients (calculated as described in reference 20) are averages of 14 observations (analysis with the seven probes described in the text and the restriction endonucleases *Eco*RI and *Pvu*II).
[b]Strains XC62, XC63, XC91, XC92, XC101, XC102, XC103, XC115, XC118, F134, F198, and F200 were indistinguishable by RFLP analysis ($F = 1.00$). The F values for those strains were also identical to those presented for XC62.

panel of probes used and the range of variation extant in *X. campestris*. However, because the number of strains was limited, with only one to three strains of each pathovar, these data are not intended to quantitatively reflect the relationships among these strains.

Only very limited polymorphisms were detected among the 14 group A strains tested, which formed a very homogeneous group ($F = 0.98$) (Table 3). The strains in group B/C/D (XC64, XC69, XC93, XC94, XC95, XC70, and XC90) were also closely related ($F > 0.82$). However, the group A strains were clearly distinct from the group B/C/D strains (e.g., for XC62 vs. any of the strains in group B/C/D, $F = 0.6$). The F values were substantially the same when calculated for either the *Eco*RI or the *Pvu*II digest (not shown). The F value in type AA comparisons was significantly different at the 5% level from the F values in type AB, AC, and AD comparisons (seeTable 6, below). The F value in type BB comparisons was significantly different from the value in type BC comparisons but not significantly different from that in type BD comparisons.

Considerable polymorphism was observed in the collection of Florida nursery strains. Four probes detected polymorphism in the group, including pXC628, which did not detect any polymorphism in group A of *X. c. citri* but produced as many as six hybridization patterns with both *Eco*RI (Fig. 1) and *Pvu*II (not shown) digests of DNA from the Florida nursery strains. Some probes that did not reveal polymorphism in these strains are also of interest. Probes pXC626 (Fig. 2A) and pXC6211 (not shown) did not detect *Eco*RI polymorphism in the Florida nursery strains, but the common hybridization pattern was clearly different from that of group A and group B/C/D of *X. c. citri*. Probe pXC6210 failed to detect *Eco*RI polymorphisms in any strain of *X. c. citri*; however, with this probe all Florida citrus nursery strains showed patterns different from that shared by all strains of *X. c. citri* (Fig. 2B). Probe pXC622 was the only probe that did not distinguish the Florida citrus nursery strains from group A of *X. c. citri*.

The relationships between the representative strains of *X. campestris* isolated from Florida citrus nurseries and strains of other pathovars of *X. campestris* were quantified by calculating the coefficient of similarity (Table 4). The citrus nursery strains (F1, F5, F6, F49, F94, F95, and F97) were more closely related to each other ($F > 0.7$) than they were to *X. c. campestris* strain X7 ($F = 0.1$), *X. c. dieffenbachiae* strain X11 ($F = 0.51$), *X. c. phaseoli* strain X35 ($F = 0.47$), and *X. c. manihotis* strain X40 ($F = 0.40$). *X. c. alfalfae* strains X60 and X61 were related to the nursery strains ($F = 0.75$).

The Florida citrus nursery strains showed only a limited relationship to any strain of *X. c. citri* ($F = 0.3$) (Table 5), with no greater relationship to either the A or the B/C/D strains. These differences (for example, AA vs. AU or BB vs. BU) were significant at the 5% level (Table 6). Apparent subgroups were also detected. Strains F1–F5 and F54 formed one subgroup ($F > 0.9$); F6, F29, F30, F49, and F51 formed a second subgroup ($F > 0.8$); strains F94, F95, and F97 formed a third subgroup ($F > 0.7$). These apparent subgroups were designated E, F, and G, respectively. They were detected when either *Eco*RI or *Pvu*II polymorphisms were examined. The differences between them were statistically significant at the 5% level.

DISCUSSION

The results of this study confirm and extend the conclusions of our earlier study (14). In that study no *Eco*RI polymorphisms were detected in groups A and B of *X. c. citri*. In the present study, two of the Asiatic strains could be distinguished from the other Asiatic strains by RFLP analysis of *Eco*RI digests, and limited polymorphism was detected within group B. These polymorphisms were not detected in the earlier study because the fragments involved were too large to be resolved in the 5% polyacrylamide gels of that study. Although some polymorphisms were detected within the collection of A and B strains of *X. c. citri*, the overall variability within each of these groups is small, consistent with a clonal population structure for this pathogen, which is generally the case with pathovars of *X. campestris* (12). This uniformity is perhaps surprising for the group A strains, because they were isolated in six geographically separate locations, but it is consistent with the dissemination of the pathogen in infected plant material. The RFLP variants detected within group A, XC98 and XC100, were isolated in Yemen and Pakistan. We have examined a large number of additional A strains from other parts of the Indian Ocean basin but have not found any other RFLP variants (not shown). None of the four A strains isolated at separate sites in Florida could be distinguished from one another, from the type strains of *X. c. citri*, or from the other Asiatic strains of *X. c. citri* except for strains XC98 and XC100, from Yemen and Pakistan. The original source of the Asiatic strains recently found in Florida remains unknown and will not be easily traced by this type of analysis, because of the clonal population structure of this pathogen worldwide. It is unfortunate that no strains isolated in the original CBC outbreak in Florida (1910) are available for analysis. However, those strains were probably of the Asiatic group, on the basis of the historical record (1).

Taken together, the RFLP analysis summarized in Tables 3 and 6 suggests two clonal groups within *X. c. citri*: the Asiatic group (group A) and the cancrosis B group, which includes the single available C and D strains (group B/C/D). Although variation was small within groups, variation between groups was large and statistically significant ($p = 0.05$). The single available C strain remains in this group in spite of being significantly different (Table 6), because determinations based on such limited data must be used cautiously. The single available D strain from

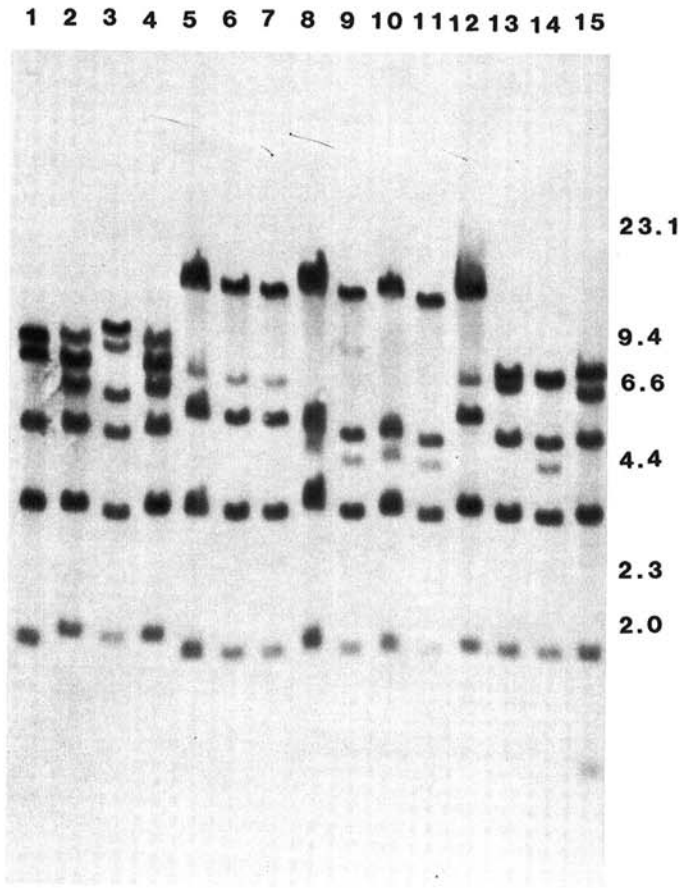

Fig. 1. Southern blot of genomic DNA from strains of *Xanthomonas campestris* digested with *Eco*RI. Lane 1, XC62; lane 2, XC64; lane 3, XC70; lane 4, XC90; lane 5, F2; lane 6, F4; lane 7, F5; lane 8, F6; lane 9, F29; lane 10, F49; lane 11, F51; lane 12, F54; lane 13, F94; lane 14, F95; lane 15, F97. The hybridization probe was pXC628. The positions of size markers in kilobase pairs are shown in the margin.

Mexico (XC90) could not be distinguished from strains XC93–XC95 of group B ($F = 1.0$) (Table 3). The available data suggest that Mexican bacteriosis (group D) could be an outbreak of cancrosis B (group B). This would be the first occurrence of cancrosis B outside Argentina. This conclusion must be viewed as tentative, however, because only one Mexican bacteriosis strain has been available for analysis. Although the B form of CBC is associated with lemon in Argentina, whereas the D form is associated with Mexican lime in Mexico, lemon and lime are closely related botanically. This apparent difference in host range may only reflect the varieties of citrus grown in different regions.

The clonal population structure evident in *X. c. citri* is consistent with the clonal population structure revealed by isozyme analysis of other bacterial taxa (24). Isozyme analysis of this collection of strains at 14 loci detected only limited variation within strains of *X. c. citri* (15).

In a recent RFLP analysis of *X. campestris*, which included strains of *X. c. citri*, Gabriel et al (12) also found distinct clonal groups within *X. c. citri*. It is of interest that these workers found almost no relationship ($F = 0.15$) between groups A and B, whereas we find a much closer relationship ($F = 0.6$) between them. The discrepancy may be due to their more limited strain selection and the use of different probes. With a panel of seven probes and a large selection of strains of a single pathovar, RFLP

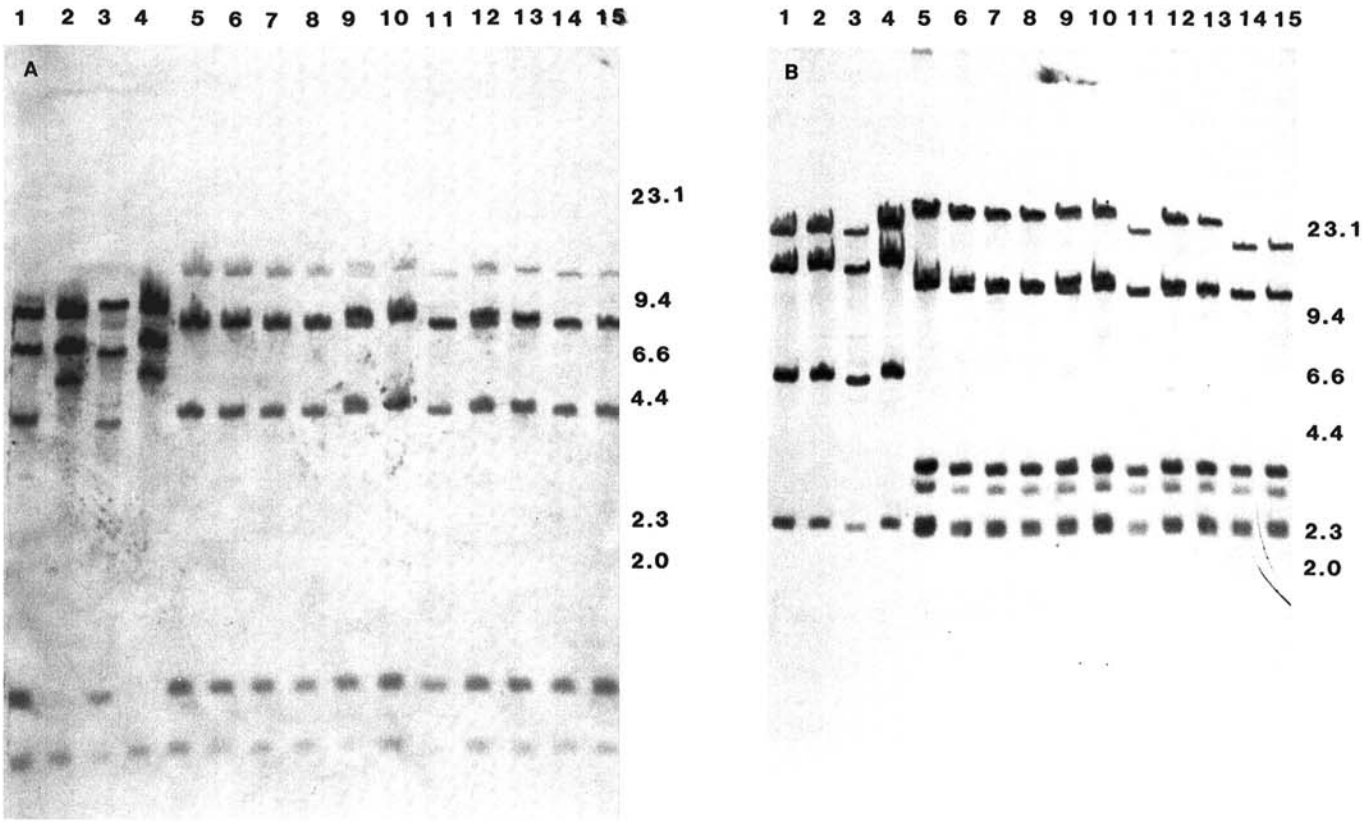

Fig. 2. Southern blot of genomic DNA from strains of *Xanthomonas campestris* digested with *Eco*RI and probed with **A,** pXC626 and **B,** pXC6210. Lane 1, XC62; lane 2, XC64; lane 3, XC70; lane 4, XC90; lane 5, F2; lane 6, F3; lane 7, F4; lane 8, F5; lane 9, F6; lane 10, F49; lane 11, F51; lane 12, F54; lane 13, F94; lane 14, F95; lane 15, F97. The positions of size markers in kilobase pairs are shown in the margin.

TABLE 4. Coefficients of similarity[a] based on *Eco*RI restriction fragment length polymorphisms for strains of *Xanthomonas campestris* isolated from Florida citrus nurseries and other pathovars of *X. campestris*

		FCN strains[b]							*X. c. alfalfae*		*X. c. campestris*	*X. c. dieffenbachiae*	*X. c. phaseoli*	*X. c. manihotis*
	Strain	F1	F5	F6	F49	F94	F95	F97	X60	X61	X7	X11	X35	X40
FCN strains[b]	F1	—	1.00	0.88	0.88	0.74	0.69	0.71	0.70	0.78	0.10	0.51	0.47	0.40
	F5		—	0.88	0.88	0.74	0.69	0.71	0.70	0.78	0.10	0.51	0.47	0.40
	F6			—	1.00	0.85	0.82	0.82	0.79	0.83	0.10	0.52	0.47	0.43
	F49				—	0.85	0.82	0.82	0.79	0.83	0.10	0.52	0.47	0.43
	F94					—	0.83	0.83	0.76	0.78	0.10	0.52	0.43	0.43
	F95						—	0.83	0.71	0.73	0.09	0.53	0.47	0.42
	F97							—	0.70	0.79	0.10	0.54	0.47	0.42
X. c. alfalfae	X60								—	0.73	0.10	0.52	0.40	0.43
	X61									—	0.12	0.50	0.43	0.42
X. c. campestris	X7										—	0.08	0.06	0.06
X. c. dieffenbachiae	X11											—	0.63	0.60
X. c. phaseoli	X35												—	0.76
X. c. manihotis	X40													—

[a]The coefficients (calculated as described in reference 20) are averages of seven observations (analysis with the seven probes described in the text).
[b]Strains of *X. campestris* isolated from Florida citrus nurseries, pathovar undetermined.

TABLE 5. Coefficients of similarity[a] based on restriction fragment length polymorphisms for strains of *Xanthomonas campestris* isolated from Florida citrus nurseries and *X. c.* pv. *citri*

Strain	Group[b]	XC62 A	XC64 B	XC70 C	XC90 D	F1 U	F2 U	F3 U	F4 U	F5 U	F6 U	F29 U	F30 U	F49 U	F51 U	F54 U	F94 U	F95 U	F97 U
XC62	A	—	0.62	0.62	0.61	0.35	0.35	0.34	0.35	0.35	0.35	0.36	0.36	0.36	0.36	0.35	0.30	0.32	0.29
XC64	B		—	0.82	0.89	0.29	0.29	0.29	0.29	0.29	0.28	0.28	0.28	0.28	0.29	0.30	0.34	0.34	0.33
XC70	C			—	0.89	0.30	0.30	0.31	0.31	0.31	0.33	0.33	0.33	0.33	0.39	0.31	0.38	0.37	0.34
XC90	D				—	0.29	0.29	0.29	0.29	0.29	0.30	0.30	0.30	0.30	0.33	0.30	0.33	0.32	0.32
F1	U					—	0.95	0.93	0.93	0.92	0.78	0.76	0.78	0.78	0.66	0.93	0.65	0.62	0.68
F2	U						—	0.97	0.97	0.97	0.82	0.81	0.82	0.82	0.71	0.98	0.69	0.66	0.73
F3	U							—	1.00	1.00	0.83	0.81	0.83	0.83	0.73	0.99	0.70	0.69	0.73
F4	U								—	1.00	0.83	0.81	0.83	0.83	0.73	0.99	0.70	0.69	0.73
F5	U									—	0.83	0.82	0.83	0.83	0.73	0.99	0.70	0.69	0.73
F6	U										—	0.98	1.00	1.00	0.88	0.83	0.73	0.73	0.78
F29	U											—	0.98	0.98	0.90	0.82	0.72	0.73	0.77
F30	U												—	1.00	0.88	0.83	0.74	0.71	0.78
F49	U													—	0.90	0.83	0.73	0.73	0.78
F51	U														—	0.72	0.69	0.70	0.76
F54	U															—	0.70	0.68	0.74
F94	U																—	0.76	0.79
F95	U																	—	0.77
F97	U																		—

[a]The coefficients (calculated as described in reference 20) are averages of 14 observations (analysis with the seven probes described in the text and the restriction endonucleases *Eco*RI and *Pvu*II).
[b]A–D = groups A–D of *X. c. citri* (5,26); U = undetermined pathovar, designating strains isolated from Florida citrus nurseries.

TABLE 6. Confidence intervals for selected mean comparisons

Type comparison[a]	Simultaneous lower confidence limit[b]	Difference between means[c]	Simultaneous upper confidence limit
AA − AB	0.341	0.366*	0.391
AA − AC	0.313	0.360*	0.407
AA − AD	0.324	0.369*	0.414
BB − BC	0.014	0.103*	0.191
BB − BD	−0.113	−0.027	0.060
BB − AB	0.280	0.333*	0.386
UU − AU	0.416	0.468*	0.519
UU − BU	0.462	0.514*	0.565
UU − CU	0.426	0.479*	0.532
UU − DU	0.456	0.507*	0.559
EE − EF	0.113	0.173*	0.234
EE − EG	0.202	0.267*	0.331
FF − FG	0.133	0.214*	0.296
EE − EL	−0.068	0.059	0.186

[a]Each pair of letters represents a "type," designating strain comparisons in terms of the groups to which the strains belong: A–D = groups A–D of *Xanthomonas campestris* pv. *citri* (5,26); U = undetermined pathovar of *X. campestris*, designating all strains isolated from Florida citrus nurseries; E–G = subgroups E–G of group U, consistent with previously reported results (14) and as described in the text; L = strains of *X. c. alfalfae*.
[b]Limits of 95% confidence intervals calculated with Bonferroni *T*-tests (22).
[c]Based on data presented in Tables 3–5. Values followed by an asterisk are significant differences ($\alpha = 0.05$).

analysis can statistically distinguish related subgroups within a single pathovar. Alternatively, the significantly different F values in the type AA and type AB comparisons in our study ($F = 0.98$ and $F = 0.60$, respectively) could reasonably be used as an argument for the existence of two pathovars.

RFLP analysis detected extensive variation within a group of 14 strains of *X. campestris* isolated from Florida citrus nurseries. Eight of the 14 strains could be uniquely identified by at least one probe. Thus, the collection of 14 Florida citrus nursery strains is clearly more heterogeneous than the collection of 14 Asiatic strains of *X. c. citri* isolated worldwide (Tables 3 and 5). Previous work (14) and inspection of Table 5 reveal three possible subgroups within the Florida citrus nursery strains (F1–F5 and F54 in subgroup E; F6, F29, F30, F49, and F51 in subgroup F; and F94, F95, and F97 in subgroup G). Although these subgroups were significantly different on the basis of our *T*-tests, this result should be interpreted cautiously because of the limited number of strains in each. The diversity of the RFLP patterns within the Florida citrus nursery strains revealed with probes should not obscure the fact that the coefficients of similarity between these strains were always high (Table 5), suggesting that they are in fact a group of related strains. An RFLP analysis of a smaller number of strains of *X. campestris* isolated from Florida citrus nurseries also detected variation between strains (12). The diversity of RFLP observed with the group of Florida citrus nursery strains is consistent with the previously observed wide variation in electromorphs detected during multilocus isozyme analysis (15) and variations in the plasmid DNA content of these strains (11).

The RFLP analyses conducted in this study indicate that the Florida citrus nursery strains are not derived from any recognized group of *X. c. citri* (e.g., by the loss of a gene or genes required for the raised-lesion symptom typical of CBC), as the coefficients of similarity between these strains and *X. c. citri* are low and significantly different from those of strains within groups of *X. c. citri*. This work confirms the observation that the nursery strains are somewhat related to strains of *X. c. alfalfae* (12). The coefficient of similarity between *X. c. alfalfae* (strains X60 and X61) and the nursery strains of *X. campestris* ($F = 0.75$) is in fact much higher than the value for *X. c. citri* vs. the nursery strains ($F = 0.3$) (Tables 4 and 5). The difference between the nursery strains and two strains of *X. c. alfalfae* (UU vs. UL in Table 6) was not statistically significant, although the groups were not identical. The biological significance of this observation is not clear. If larger numbers of strains of other pathovars were examined, similar relationships might be found. The observed genomic diversity in the Florida citrus nursery strains is inconsistent with a single clonal origin by introduction from elsewhere or by a mutational event in another pathovar. Therefore, the origin of this group of related yet diverse strains remains unresolved.

Strains of *X. campestris* have been named on the basis of the plant from which they were first isolated (3). Clearly distinguishable forms of *X. c. citri* have been given letter designations A–D (5,26). Following these conventions we provisionally placed the Florida nursery strains of *X. campestris* in group E of *X. c. citri* (14). This is no longer tenable. Although the present study demonstrates a complex population structure for the Florida nursery pathogen or pathogens, these strains share common traits that distinguish them from all known strains of *X. c. citri*. These include significant differences in host-pathogen

interactions (9,16), symptomology, and epidemiology (23). The strains can also be differentiated by serology, phage typing (Civerolo, *unpublished*), and multilocus isozyme analysis (15). In this context the significantly different coefficients of similarity between strains of *X. c. citri* and the strains from Florida citrus nurseries ($F = 0.3$, Table 5) is compelling. We propose that the disease associated with the Florida nursery strains is not CBC and that it be called citrus bacterial spot disease. We consider the pathovar status of the group of strains causing the disease to be unresolved but suggest that *X. c. citri* not be used in connection with citrus bacterial spot disease, because the pathogen or pathogens are sufficiently distinct from strains in groups A–D of *X. c. citri* that the use of the pathovar designation for this organism or these organisms is misleading.

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