Isozyme Comparisons of *Septoria* Isolates Associated with Citrus in Australia and the United States

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We gratefully acknowledge the excellent technical assistance of Raymond Beck and the critical review of this manuscript by John Lightfield. We thank A. Sharen and T. Barksdale for providing the non-citrus *Septoria* isolates.

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Accepted for publication 29 November 1990 (submitted for electronic processing).

ABSTRACT

Bonde, M. R., Peterson, G. L., Emmett, R. W., and Menge, J. A. 1991. Isozyme comparisons of *Septoria* isolates associated with citrus in Australia and the United States. Phytopathology 81:517-521.

Septoria cultures isolated from citrus have sometimes been referred to in Australia as *S. depressa* and in the United States only as *S. citri*. However, it is not clear whether *S. depressa* and *S. citri* are the same or different species. To help determine the answer, isozymes of *Septoria* isolated from citrus in Australia and the United States were compared. Of 28 isolates studied (18 from Australia and 10 from the United States, spanning the observed morphological variation in each country) for 25 enzymes, only one isolate (AUS 335) from Australia was markedly different. The average coefficient of similarity comparing all possible pairs of isolates, except AUS 335, was 0.97, indicating little isozyme variation. Twenty-three of 25 enzymes had no detectable variation in these isolates. The average coefficient of similarity comparing AUS 335 to other isolates was 0.58. Based on the data, these isolates (except the aberrant isolate AUS 335) are of the same species.

At least 18 species of *Septoria* are reported to be associated with leaf and fruit spots of citrus (6); however, none has been extensively studied (6). The identification of species of *Septoria* on citrus depends on morphology, particularly pycnidial size, conidial length, and number of septa per conidium (6,12). These characteristics are extremely variable and, indeed, variation within a single isolate can span the reported ranges for several species (6,16). Furthermore, cultural conditions probably affect morphological characteristics and may have contributed to confusion in previous studies.

Most species of *Septoria* on citrus initially were described in the late 19th or early 20th centuries (11). Of these, only three species, *S. citri* Pass. (causal agent of Septoria spot of citrus), *S. depressa* McAlp., and *S. limonum* Pass., have appeared in the literature in the last several decades. Laundon (9) compared an herbarium specimen of *S. citri* from New Zealand with specimens from other countries and found them to be indistinguishable. He was able only to obtain one specimen of *S. limonum*, which had only a few, mostly damaged, pycnidia containing only a few conidia. The measurements of these structures did not agree with those described for *S. limonum*. (The reported conidial measurements of *S. limonum* are within the range for measurements of *S. citri*.)

Laundon (9) also examined presumptively authentic material of *S. depressa* from Australia and was unable to find structures corresponding to the description for *S. depressa*. He did, however, find that intercepted material and eight herbarium specimens from Australia labeled as *S. depressa* were identical to *S. citri*. The existence of the species *S. depressa* and *S. limonum* therefore is questionable.

Because of the uncertainty of the relationship of *Septoria* on citrus in Australia to that in the United States, and because of its importance to international trade and trade restrictions, a study was undertaken to compare isolates from the two countries.

Isozyme analysis is a useful technique presently being used by mycologists and plant pathologists to resolve taxonomic problems, identify unknown fungal isolates, "fingerprint" patentable fungal lines, analyze the extent of genetic variability in a population, trace the geographic origin of pathogens, follow the segregation of genetic loci, and determine ploidy levels at various stages in the life cycle of a fungus. This topic has been

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reviewed thoroughly (4,13). The purpose of the research described here was to compare by means of isozyme analyses isolates of *Septoria* obtained from citrus grown in Australia with isolates of *Septoria* obtained from citrus grown in the United States. Some of these isolates, including both Australian and United States, are the same as those used in a morphological comparison of *Septoria* from citrus by Emmett and Menge (6) and the isolates represent the range of variation observed. The determination of relatedness of the pathogen (or pathogens) in Australia with that in the United States by using isozyme analysis could provide critical information for evaluating isolate relationships and whether the pathogen is the same species in the two countries.

MATERIALS AND METHODS

Preparation of *Septoria citri* samples for electrophoresis. Mycelial plugs were removed from stock cultures, placed on cornmeal agar in petri plates, and incubated at 24 C. When sufficient growth was present, a 1.0-cm^2 block was cut from the culture and used to streak a plate containing potato-dextrose agar (PDA) with 0.5% (w/v) asparagine added. The plates were incubated at 24 C for 3 or 4 days in 12 h light/12 h dark. Five milliliters of sterile distilled water was added to each plate and agitated with a glass rod to dislodge mycelia and spores. Two to three milliliters of this suspension was used to seed a flask containing 50 ml of Difco Bacto yeast malt extract broth (Difco Laboratories, Detroit, MI). The flask was placed on a shaker at 100 rpm at 21 ± 1 C in a constant temperature room. After 4 or 5 days, the mycelia were collected on No. 4 Whatman filter paper (Whatman Ltd., Maidstone, England) by vacuum filtration. The mycelia were transferred to 2-ml Nunc cyotubes (NS Nunc, Kamstrup, DK-4000, Roshilde, Denmark) and stored in liquid nitrogen (−196 C) until needed for isozyme studies.

TABLE 1. Isolates of *Septoria* used in the study to compare isozymes

Designation	Taxon	Place of origin	Host
AUS 077[a]	*S. citri*	Colignan, Australia	Grapefruit
AUS 148	*S. citri*	Lock 4, Australia	Lemon
AUS 162	*S. citri*	Curlwaa, Australia	Lemon
AUS 165	*S. citri*	Iraak, Australia	Lemon
AUS 168	*S. citri*	Waikerie, Australia	Lemon
AUS 177	*S. citri*	Lyrup, Australia	Lemon
AUS 264	*S. citri*	Mildura, Australia	Navel orange
AUS 302	*S. citri*	Irymple, Australia	Lemon
AUS 303	*S. citri*	Dareton, Australia	Camellia
AUS 335	*S. citri*	Mt. Tamborine, Australia	Lime
AUS 390	*S. citri*	Winkie, Australia	Grapefruit
AUS 393	*S. citri*	Berri, Australia	Navel orange
AUS 395	*S. citri*	Paringa, Australia	Lime
AUS 436	*S. citri*	Griffith, Australia	Valencia orange
AUS 439	*S. citri*	Corbie Hill, Australia	Valencia orange
AUS 442	*S. citri*	Hanwood, Australia	Valencia orange
AUS 444	*S. citri*	Yanco, Australia	Navel orange
AUS 451	*S. citri*	Lake Wyangan, Australia	Lemon
ST3[b]	*S. tritici*	Montana	Wheat
ST4	*S. tritici*	Montana	Wheat
ST5	*S. tritici*	Oregon	Wheat
SL7[c]	*S. lycopersici*	Beltsville, MD	Tomato
090[d]	*S. citri*	Yuma, AZ	Lemon
114	*S. citri*	Yuma, AZ	Lemon
S-2	*S. citri*	Escondido, CA	Navel orange
S-3	*S. citri*	Riverside, CA	Navel orange
S-6	*S. citri*	Bakersfield, CA	Grapefruit
S-7	*S. citri*	Orange Cove, CA	Navel orange
S-9	*S. citri*	Orange Cove, CA	Navel orange
S-12	*S. citri*	Clovis, CA	Navel orange
S-19	*S. citri*	Porterville, CA	Navel orange
078	*S. citri*	Ventura, CA	Navel orange

[a]All Australian isolates of *S. citri* were provided by R. W. Emmett.
[b]All isolates of *S. tritici* were provided by A. Sharen, Montana State University, Bozeman, MT.
[c]The isolate of *S. lycopersici* was provided by T. Barksdale, USDA, Beltsville, MD.
[d]The isolates of *S. citri* from the United States were provided by R. W. Emmett and J. A. Menge.

Samples were removed from liquid nitrogen storage and allowed to thaw on ice. Two hundred fifty microliters of 0.05 M Tris/HCl buffer, pH 7.8, was added to the sample and mixed with the aid of a glass rod. Samples were refrozen in liquid nitrogen and thawed in an ice bath. Samples then were centrifuged at 2,000 *g* for 10 min in a refrigerated centrifuge at 4 C and the supernatant absorbed onto 12 × 3 mm SS 470 (Schleicher and Schuell, Inc., Keene, NH) filter paper wicks which were applied to the gels.

Gel electrophoresis. In an isozyme screen, we tested for the presence of 62 enzymes; six separate gel buffer systems were used to determine the best buffer for resolution of each enzyme present. Thirty-two enzymes were detected in sufficient concentrations for further studies.

Subsequent to the isozyme screen, 28 isolates of *Septoria* from citrus (18 from Australia, and 10 from the United States) (see Table 1) were tested for the 32 enzymes previously detected, using the best buffer for each. Horizontal starch gel electrophoresis and staining were performed as described by Micales et al (13).

Genic nomenclature and treatment of data. The genic nomenclature as described by May et al (10) was used. Abbreviations with all letters capitalized represent enzymes. Capital-lettered abbreviations with only the first letter capitalized refer to putative loci coding for the enzyme. Presumed alleles at a specific locus are designated by the relative anodal mobility of their protein products from the origin. The designation for each allele is relative to the migration of the protein product of one allele (the most common) designated 100. For example, Ak-75 is the putative allele coding for the protein product migrating 75% as far as adenylate kinase coded by allele Ak-100, the most common allele at the Ak-locus. Pgd-106 is an allele coding for phosphogluconate dehydrogenase whose molecules migrate 106% as far as molecules coded by Pgd-100, the most common allele coding for the enzyme.

Analysis of data. Isozyme data were analyzed with the computer program "Allozyme" (R. Struss, Univ. of Arizona, Tucson), which determined the degree of similarity (coefficient of similarity) according to Rogers (18).

According to this equation:

Coefficient of similarity = 1 − D

$$\text{where } D = \frac{1}{L}\sum_{i=1}^{L}\left[\frac{1}{2}\sum_{j=1}^{A_i}(Pijx - Pijy)^2\right]^{1/2}$$

and D = genetic distance, L = number of genetic loci, A_i = number of alleles at the *i*th locus, and *Pijx* and *Pijy* = frequency of the *j*th allele at the *i*th locus in population *x* and *y*, respectively.

RESULTS AND DISCUSSION

Sixty-two enzymes were screened for their detectable presence in *Septoria* isolates and 32 were present in sufficient concentrations for further work. Of these, 25 enzymes were resolved and gave interpretable results for most of the 28 isolates obtained from citrus (Table 2). Phenotypic banding patterns were identified, based on relative position of isozymes, and interpreted in terms of presumed alleles of the genetic loci coding for the isozymes. The isolates were grouped based on country of origin and coefficients of similarity (CS) determined within and between groups (Table 3). Three isolates of *Septoria tritici* Rob. in Desm. from wheat and one of *Septoria lycopersici* Speg. from tomato were included for comparisons with the citrus isolates. These could be readily differentiated from the citrus isolates by the low coefficients of similarity between the groups (Table 3).

All enzymes, except the esterases (EST) and phosphogluconate mutase (PGM), produced simple banding patterns that were interpreted as being coded by a single monomorphic (nonvariable) genetic locus in all *Septoria* isolates from citrus excluding one

TABLE 2. Twenty-five enzymes used to compare isolates of *Septoria*, best buffer for resolution, and whether variable or nonvariable in *S. citri* population

Enzyme	Abbreviation	E.C. no.	Buffer system	M or P[a]
Aspartate aminotransferase	AAT	2.6.1.1	C[b]	M
Adenylate kinase	AK	2.7.4.3	W[c]	M
Alkaline phosphatase	AKP	3.1.3.1	R[d]	M
Creatin kinase	CK	2.7.3.2	W	M
Cytochrome oxidase	CTO	1.9.3.1	R	M
Diaphorase	DIA	1.6.4.3	4[e]	M
Esterase	EST-NF	3.1.1.1	R	P
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	1.2.1.12	C	M
Glutamate dehydrogenase	GDH	1.4.1.3	R	M
β-D-Glucosidase	β-Glu	3.2.1.21	4	M
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	R	M
Glucose phosphate isomerase	GPI	5.3.1.9	C	M
Iscitrate dehydrogenase	IDH	1.1.1.42	W	M
Leucine aminopeptidase	LAP	3.4.11.1	4	M
Lactate dehydrogenase	LDH	1.1.1.27	W	M
Mannitol dehydrogenase	MADH	1.1.1.67	R	M
Malate dehydrogenase	MDH	1.1.1.37	C	M
Malic enzyme	ME	1.1.1.40	C	M
Mannose phosphate isomerase	MPI	5.3.1.8	R	M
Peptidase (leucyl-alanine as substrate)	PEP-LA	3.4.11	R	M
Peptidase (leucyl-leucyl-leucine as substrate)	PEP-LLL	or	R	M
Peptidase (phenyl-alanyl-proline as substrate)	PEP-PAP	3.4.13	R	M
Phosphogluconate dehydrogenase	PGD	1.1.1.44	C	M
Phosphoglucomutase	PGM	2.7.5.1	4	P
Triose phosphate isomerase	TPI	5.3.1.1	W	M

[a] M = Locus monomorphic, P = at least one locus polymorphic among *Septoria citri* isolates. Isolate AUS 335 is not included in this table.
[b] Buffers according to Clayton and Tretiak (5). Electrode buffer, 0.04 M citric acid adjusted to pH 6.1 with morpholine. Gel buffer, 1:9 dilution of electrode buffer. Run at 200 V for 3 h.
[c] Buffer according to Selander et al (19). Electrode buffer, 0.1 M Tris, 0.1 M maleic acid, 0.01 M EDTA, 0.1 M $MgCl_2 \cdot 6H_2O$. Adjust pH to 7.4 with NaOH. Gel buffer, 1:9 dilution of electrode buffer. Run at 100 mA for 3 h.
[d] Buffer according to Ridgeway et al (17). Electrode buffer, 0.06 M lithium hydroxide, 0.3 M boric acid, pH 8.1. Gel buffer, 0.03 M Tris base, 0.005 M citric acid, 10.0 ml of electrode buffer per liter. Adjust pH to 8.5 with NaOH. Run at 250 V for 3 h.
[e] Buffer according to Selander et al (19). Electrode buffer, 0.223 M Tris base, 0.086 M citric acid. Adjust pH to 6.3 with 1.0 M NaOH. Gel buffer, 0.008 M Tris base, 0.003 M citric acid. Adjust pH to 6.7 with 1.0 M NaOH. Run at 170 V for 2 h.

(AUS 335) from Australia (e.g., Fig. 1). PGM was interpreted as being coded by a single polymorphic (variable) locus. The esterases were interpreted as being coded by at least four loci (Fig. 1A). We were able to consistently score three zones of enzymatic activity that were interpreted as the products of three loci. Excluding isolate AUS 335, only locus Est-1 (see top band in each lane on gel) and locus Pgm were polymorphic in citrus *Septoria* (Fig. 1A).

Six isolates of *S. citri* exhibited allele Est-1-104 and nine Pgm-110. None of the isolates exhibited both. A few isolates had missing data for one or a few loci due to weak enzyme activity (resulting from poor culture growth); however, each of these had complete data for the Est-1 and Pgm loci. Interestingly, nine loci of isolate AUS 335 had no alleles in common with any other isolate of *Septoria* from citrus. These loci were Ak, Ck, Dia, Est-2, Est-3, Gpi, Lap, Mdh, and Pgd. Only two alleles were postulated in common between *S. tritici* and *S. citri* (Cto-100 and Pgd-100), and three (Gdh-100, Pep-la-100, and Tpi-100) between *S. lycopersici* and *S. citri*.

Of the 28 isolates from citrus, only AUS 335 (= SHC 335 in Emmett's collection [6]) from Australia was distinctly different (Table 3). This isolate had a coefficient of similarity (CS) value of only 0.58 (maximum value possible = 1.00) when compared with all other isolates of *Septoria* from citrus from Australia and the United States. This indicates that approximately 40% of the isozyme loci of AUS 335 were measurably different from the other citrus isolates. (AUS 335 will be omitted from the following analysis except where indicated.)

The average CS comparing each pair of isolates within the Australian population was 0.97 and the average for the U.S. population also was 0.97. These values demonstrate minimum isozyme variation within each population.

Even more important was the average CS of 0.97 when all possible pairs of each Australian with each U.S. isolate were compared. These data indicate that the two populations are of the same species.

TABLE 3. Rogers' coefficient of similarity comparing geographic groups of *Septoria citri* and *Septoria* species

Comparison	Mean similarity (Rogers')	Std. Div.	Number of Comparisons
Within:			
S. citri Australia (Aus.) (excluding Aus 335)	0.9706	0.0270	136
S. citri U.S.A.	0.9659	0.0253	45
S. citri Aus. and U.S.A. (combined)	0.9698	0.0268	351
S. tritici	1.0000	0.0000	3
Between:			
S. citri Aus. and *S. citri* U.S.A	0.9702	0.0264	170
S. citri (comb.) and *S. citri* AUS 335[a]	0.5809	0.0246	27
S. citri (comb.) and *S. lycopersici*	0.1765	0.0005	27
S. citri (comb.) and *S. tritici*	0.0952	0.0002	81
S. lycopersici and *S. tritici*	0.1250	0.0000	3

[a] *S. citri* isolate AUS 335 is from infected lime in Mount Tamborine, Australia, and is very different in isozyme phenotype from the other isolates of *Septoria* from citrus. It cannot be distinguished from other isolates of *S. citri* on the basis of morphology or pathogenicity (6).

Coefficients of similarity have not been determined for many fungal species. Micales et al (15), in a study of *Cryphonectria cubensis* (Bruner) Hodges isozymes, found the CS intraspecific values between isolates to vary from 0.84 to 1.00. Bonde et al (2) determined the average intraspecific CS value for *Tilletia indica* to be 0.83 and for *T. barclayana* 0.85. When comparing individuals of *T. indica* with those of *T. barclayana*, a morphologically very similar and closely related smut pathogen, the average interspecific CS was only 0.04, easily differentiating the two species. Bonde et al (1) also determined the intraspecific CS value for what was

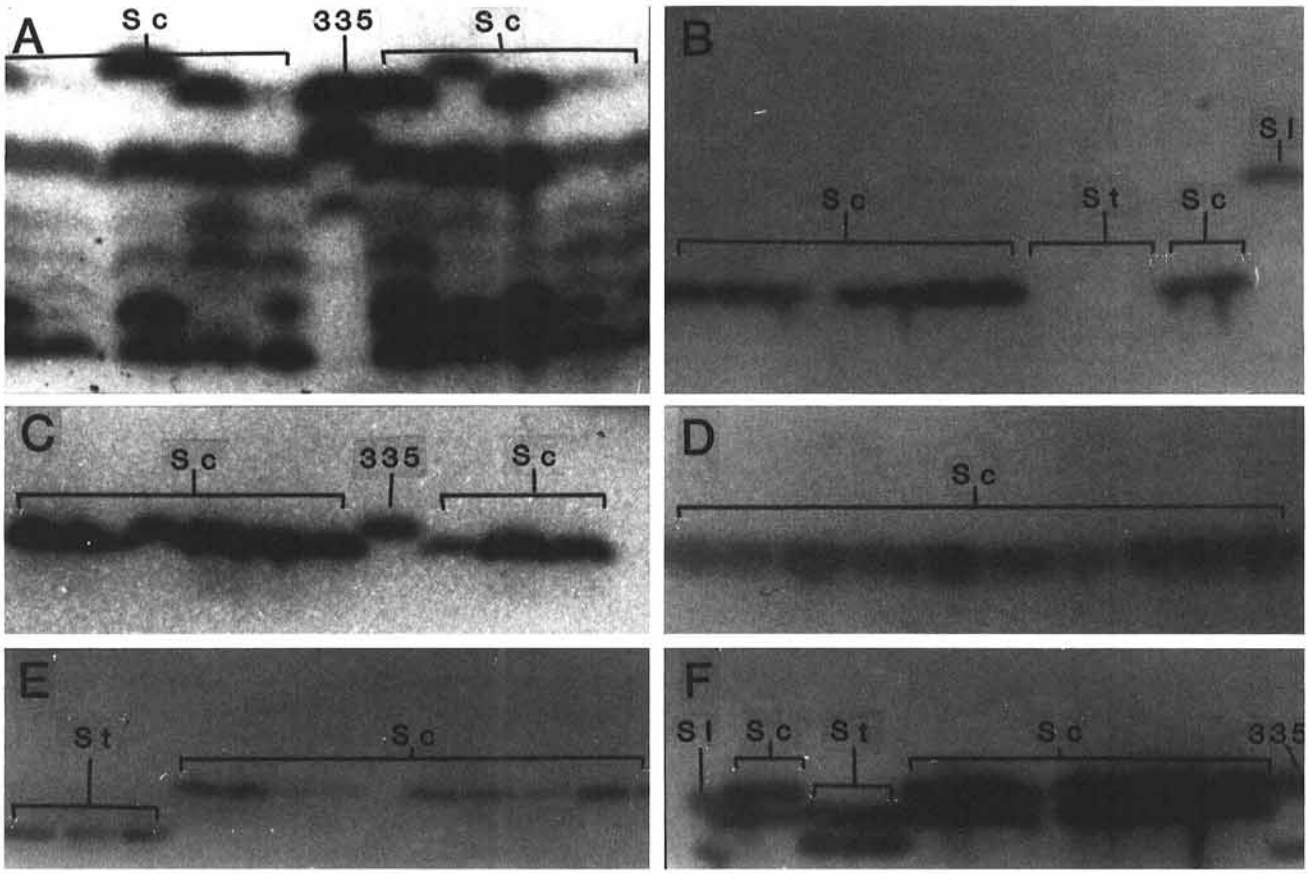

Fig. 1. Isozyme banding patterns for representative enzymes extracted from *Septoria* spp. **A,** Esterase (EST). Among *S. citri* isolates (not including AUS 335), polymorphism was detected at only one putative locus (Est-1) coding for the isozyme detected in the uppermost zone (upper band for each isolate) of enzymatic activity. Two alleles (Est-1-100, Est-1-104) were at the Est-1-locus. The second from top zone shows the same allele for each isolate except for AUS 335. All lower bands were not interpreted. **B,** Mannitol dehydrogenase (MDH). The break in the horizontal lines for Sc (left side of gel) is due to an intentional blank to aid in scoring isolates on the gel. *S. tritici* had no enzymatic activity for MDH. **C,** Phosphogluconate dehydrogenase (PGD). AUS 335 produced a slightly higher zone of enzymatic activity. All other isolates of *S. citri* are monomorphic at the Pgd-locus. **D,** Glucose-6-phosphate dehydrogenase (G6PDH). The G6pdh-locus was monomorphic for *S. citri* and AUS 335. **E,** Lactate dehydrogenase (LDH). *S. citri* and AUS 335 are monomorphic at the Ldh-locus. **F,** Malate dehydrogenase (MDH). All isolates of *S. citri*, other than AUS 335, are monomorphic at the Mdh-locus. Different banding patterns are evident for *S. lycopersici* and *S. tritici*. Sc = *S. citri*, 335 = isolate AUS 335, St = *S. tritici*, and Sl = *S. lycopersici*. When AUS 335 is not designated on gel, it is identical to all other isolates of *S. citri*.

considered to be the "true" *Phakopsora pachyrhizi* Sydow from Asia and Australia to equal 1.00, indicating no variation. Micales et al (14) calculated intraspecific CS values for four species of *Peronosclerospora* causing downy mildews on maize to vary from 0.75 to 1.00, depending on the species, and Bruckart and Peterson (3) determined CS values to be 0.72–0.75 for *Puccinia carduorum*. We recognize that one has to be careful when comparing CS values obtained in different studies. However, so as to not bias results, we have always used as wide array of enzymes as possible with the only criterion for selection being whether they can be resolved. The high average CS value comparing all possible combinations of pairs of citrus *Septoria* isolates (minus AUS 335), regardless of whether from Australia or the United States, strongly supports the hypothesis that they are of a single species.

Except for AUS 335, all isozyme variation detected in *Septoria* isolates from citrus was associated with multiple alleles coding for phosphogluconate mutase (PGM) (a single putative locus) and one of three loci (Est-1) coding for esterases. All alleles found in the Australian population, other than AUS 335, were also present in the U.S. population.

The isozymes of Australian isolate AUS 335 were significantly different from all other isolates of *Septoria* from citrus. It was the only isolate in this study isolated from lime, and the only isolate originating from Mount Tamborine, which is further north than the sources for our other Australian isolates. It produced mild symptoms on rough lemon and lime in greenhouse studies.

AUS 335 actually may be a different species of *Septoria* based on a CS of 0.58 when comparing it to the isolates of presumably *S. citri* (Table 3); however, morphologically the organism is the same based on size and shape of its conidia (6).

In studying isozyme variation in animal populations, Gottlieb (7) suggested that conspecific populations often have very high degrees of genetic similarity, often with a mean CS above 0.90, while closely related species are more variable with interspecies values around 0.50 to 0.60. Similar proposals have been made regarding plant populations (8).

In our study, the interspecies CS values comparing *S. citri* (combined) to *S. lycopersici* and *S. tritici* were 0.18 and 0.10, respectively. The CS comparing *S. citri* to AUS 335 was 0.58, suggesting a closer relationship of AUS 335 to *S. citri* than to the other two species.

The isozyme results presented here, in combination with the morphological comparison of Australian and U.S. isolates (6), suggest that the *Septoria* pathogen causing leaf and fruit spots of citrus in the major citrus-growing regions of Australia and the United States is a single species with considerable variation in size of its conidia.

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