Isozyme Analysis of *Colletotrichum gloeosporioides* from Five Host Genera

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ABSTRACT

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Thirty-three isolates of *Colletotrichum gloeosporioides* isolated from five plant genera were characterized using isozyme electrophoresis. Using 11 selected enzymes, 148 electrophoretic phenotypes were identified among the 33 isolates examined. Nicotinamide adenine dinucleotide dehydrogenase (NADHDH) and diaphorase (DIA) yielded the greatest numbers of electrophoretic phenotypes. Three major groups (I, II, and III) and four subgroups (IA, IB, IIIA, and IIIB) were delineated within *C. gloeosporioides* among the five host genera following a cluster analysis of electrophoretic phenotype values for the 11 enzymes. With the exception of one isolate from citrus, *C. gloeosporioides* isolates clustered on the basis of host origin. This study suggests that isozyme analysis may be a useful tool for characterizing intraspecific population diversity within *C. gloeosporioides*.

Additional keywords: *Aeschynomene, Carya, Citrus, Glomerella cingulata, Malva, Stylosanthes*

The fungal plant pathogen *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (teleomorph, *Glomerella cingulata* (Stoneman) Spauld & H. Schrenk) is a genetically variable fungal species (1,21,22,26) comprising a number of host-specialized forms capable of causing disease on a wide range of hosts (2,9,20,21). Some weed pathogens have been developed as bioherbicides or are under development as weed control agents (6,19). Characterization of host-specific fungal isolates remains difficult, yet is critical for differentiating isolates from several hosts.

Several molecular and genetic methods have been used to differentiate *C. gloeosporioides* isolates pathogenic to different plant hosts (3). Electrophoresis of total soluble proteins has been used to characterize *C. gloeosporioides* isolates from five different hosts and to estimate genetic relatedness within and between groups of isolates (12). Protein profiles among diverse isolates of *C. gloeosporioides* showed limited similarity, but those within some groups obtained from specific hosts were relatively uniform (12). Trout and TeBeest (25) showed that rDNA restriction fragment length polymorphism (RFLP) analysis was useful for differentiating *C. gloeosporioides* f. sp. *aeschynomene* from *C. gloeosporioides* f. sp. *malvae*, while RFLP analysis using a glutamate dehydrogenase (GDH) probe could distinguish only groups comprising certain species and formae speciales. Isozyme electrophoresis has been used widely to assess genetic diversity in fungi (3) and could be a useful tool for differentiating intraspecies variability in *C. gloeosporioides* obtained from diverse hosts. This technique has been used by mycologists and plant pathologists to assess the amount of genetic variation within and among fungal populations, to trace the geographic origins of pathogens, and to determine ploidy levels in fungi (17,18). At this time, few isozyme studies have addressed the problem of discriminating host-specific isolates of *C. gloeosporioides* (3).

The objective of this study was to examine isozyme phenotype diversity within isolates of *C. gloeosporioides* obtained from five host genera and to determine if the information could be utilized for clarifying host relationships among isolates of *C. gloeosporioides.*

MATERIALS AND METHODS

Isolates. Thirty-three isolates of *C. gloeosporioides* from five host genera from eight geographical locations were selected for isozyme analysis (Table 1). An isolate of *C. sublineolum* Henn. (785) was selected for comparative purposes in the analysis. Single-spore cultures of all the isolates were maintained in cryogenic storage at –80°C in the Department of Plant Pathology, University of Arkansas, Fayetteville.

Enzyme extraction. All isolates were grown on YPSS medium (12) (yeast extract, 4 g; soluble starch M-100, 15 g; potassium phosphate, 1 g; magnesium sulfate, 0.5 g; and agar, 16 g per liter of distilled H_2O) in petri plates for 4 days at 25°C. Mycelial plugs were transferred to Fries nutrient broth (12) (ammonium tartrate, 5 g; ammonium nitrate, 1 g; magnesium sulfate, 0.5 g; potassium phosphate monobasic, 11.3 g; potassium phosphate dibasic, 2.6 g; glucose, 20 g; yeast extract, 5 g per liter of distilled H_2O; and 0.2 ml of mineral stock solution [lithium chloride, 167 mg/liter; copper chloride, 107 mg/liter; manganese chloride, 72 mg/liter; molybdenum tetroxide, 34 mg/liter; and cobalt chloride, 80 mg/liter]) in 250-ml Erlenmeyer flasks and incubated 5 days on a rotary shaker at 250 rpm, 24°C. The mycelium was collected on filter paper (Whatman International Limited, Springfield Mill, Maidstone, KY) by vacuum filtration, rinsed once with 1× TEN buffer (10 mM Tris-Cl; 1 mM EDTA, pH 7.4; and 100 mM NaCl) and then washed three times with distilled water.

The fungal mat was blotted with a paper towel to remove excess liquid and placed in a 50-ml conical tube containing 10 ml of liquid nitrogen. The samples were stored temporarily at –20°C and then lyophilized overnight, ground into a fine powder using a prefrozen mortar and pestle, and frozen at –80°C.

Enzymes were extracted following the methods of Bonde et al. (3). Frozen mycelial powder (30 mg) was homogenized by vortexing in 313 µl of extraction buffer (300 µl of 0.05 M Tris, pH 7.5; 10 µl of 100 mM phenylmethylsulfonyl fluoride [PMSF]; and 3 µl of 0.5 M EDTA) and stored in the dark at 4°C. After 10 h, samples were centrifuged at 14,000 × *g* for 10 min at 10°C. The supernatant was collected and placed into another microfuge tube with a 0.2-µm filter unit, and 8 µl of PMSF stock was added. The sample was centrifuged at 14,000 × *g* for 15 min at 10°C. After centrifugation, the supernatant was maintained on ice, and protein content was established by the Bradford dye-binding technique (4) using the Bio-Rad assay kit (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as a standard. Absorbance (595 nm) values were determined using a spectrophotometer, and values were converted to protein concentrations. Each sample was adjusted to 150 µg of protein per ml with Tris sample buffer (0.05 M Tris-Cl, pH

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7.5). All aliquots were stored at –80°C until electrophoresis.

Electrophoresis. Enzyme separation was conducted using discontinuous native polyacrylamide-gel electrophoresis at 4°C and a Tris-glycine (pH 8.3) buffer system following the methods of Davis (11). Electrophoresis was conducted using a 7% resolving gel (pH 8.9) and a 4% stacking gel (pH 6.8) at 10°C with a constant current of 25 mA. Two µl of glycerol was added to each sample, along with 1 µl of bromophenol blue (2.5 mg/ml) as a dye marker to denote the migration front. A protein volume of 10 µl was added to each sample lane. Isolates 1125, 1133, 1145, and 785 were used as reference standards for all electrophoretic runs.

Enzyme staining. Following electrophoresis, each gel was stained for a single enzyme as previously described (7), and relative band mobilities were measured from the 7%–4% gel interface to the final point of migration. To obtain Rm ratios, the migration of detected bands was measured and divided by the dye front mobility (5). The gels were photographed and fixed in 5% methanol:10% acetic acid (vol/vol) solution and vacuum dried between two cellulose membranes. The height of the resolving gel was recorded to account for gel-to-gel variation. At least two electrophoretic runs were performed from different protein extractions for each isolate using the 11 selected enzymes (Table 2).

Data analysis. Bands were recorded as 0 if absent or 1 if present in the data matrix for each enzyme, and each isolate was assigned to a phenotype class. The relative staining intensities of bands were disregarded. Relative relatedness of the test isolates was assessed using multivariate statistical analysis using similarity coefficients (SIMQUAL) (NTSYS-PC, 1993, Exeter Publishing Co., Setavket, NY). A matrix of similarity coefficients for each pair of isolates was constructed using a similarity program (SIMQUAL) utilizing the formula $S_m = (a + b)/n$, where a = number of isozyme bands common in the pair of isolates, b = number of bands present in one isolate but absent in the other, and n = total number of isozyme bands. To determine relative relatedness, a cluster analysis was performed for the similarity coefficients, using the unweighted pair-group method with arithmetic averages (UPGMA) in NTSYS-PC. In addition, the cophenetic (COPH) program within NTSYS-PC was used to test the goodness of fit of the cluster to the data matrix. The

Table 2. Enzymes used for determination of isozyme polymorphisms

Enzyme	Abbreviation	EC no.
Oxidoreductases		
Diaphorase	DIA	1.6.4.3
Glucose-6-phosphate dehydrogenase	G6DH	1.1.1.49
Malic enzyme	ME	1.1.1.40
Menadione reductase	MR	1.6.99.2
Nicotinamide adenine dinucleotide dehydrogenase	NADHDH	1.6.99.3
Nicotinamide adenine dinucleotide phosphate dehydrogenase	NADPHDH	1.6.99.1
6-Phosphogluconate dehydrogenase	6PGD	1.1.1.44
Superoxide dismutase	SOD	1.15.1.1
Transferases		
Aspartate aminotransferase	AAT	2.6.1.1
Phosphoglucomutase	PGM	2.7.5.1
Isomerases		
Phosphoglucoisomerase	PGI	5.3.1.

Table 1. Culture number, host, source, and location of *Colletotrichum gloeosporioides* isolates used for isozyme analysis

Culture no.	Species/subspecies	Host	Source	Location	Source ref.
785	*C. sublineolum*	*Sorghum halepense*	J. Mitchell	Arkansas	B1
1142	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD4
1143	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD5
1144	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD8
1145	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD11
1146	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD12
1147	*C. gloeosporioides*	*Citrus aurantifolia*	M. Davis	Florida	MD14
817	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	84-12
818	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	83-43
819	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	83-43-1
820	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	84-15
821	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	85-33-A
822	*C. gloeosporioides* f. sp. *malvae*	*Malva pusilla*	K. Mortenson	Canada	84-24
1125	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Alabama	CRP1
1126	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Louisiana	CRP5
1127	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Mississippi	CRP2
1128	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Georgia	CRP6
1129	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Georgia	CRP3
1130	*C. gloeosporioides*	*Carya illinoensis*	C. Reilly	Georgia	CRP4
60	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene indica*	H. Holcomb	Louisiana	IS-2
61	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	H. Holcomb	Louisiana	IS-5
62	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	H. Holcomb	Louisiana	Isol 6-1
1131	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	D. TeBeest	Arkansas	Cla2a
1133	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	D. TeBeest	Arkansas	RBES5A
1135	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	D. TeBeest	Arkansas	RBES3B
1149	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	D. TeBeest	Arkansas	Cla2C
1150	*C. gloeosporioides* f. sp. *aeschynomene*	*Aeschynomene virginica*	D. TeBeest	Arkansas	Stu5A
1348	*C. gloeosporioides*	*Stylosanthes guianensis*	M. Templeton	New Zealand	NZ7696
1353	*C. gloeosporioides*	*Stylosanthes guianensis*	M. Templeton	New Zealand	NZ7017
1177	*C. gloeosporioides* (B)[a]	*Stylosanthes guianensis* cv. *graham*	J. Manners	Australia	UQ62
1176	*C. gloeosporioides* (A)[a]	*Stylosanthes scabra* cv. *seca*	J. Manners	Australia	UQ14
388	*C. gloeosporioides*	*Stylosanthes hamata*	ATCC44228	Australia	ATCC44228
852	*C. gloeosporioides*	*Stylosanthes hamata*	R. Sonoda	Florida	S-1 7303
853	*C. gloeosporioides*	*Stylosanthes hamata*	R. Sonoda	Florida	S-2 7303

[a] (A) and (B) pathotypes, Irwin and Cameron (1978).

calculation of a correlation coefficient was performed using MXCOMP (matrix comparison) to compare the similarity matrix to the cophenetic value matrix.

RESULTS

Isozyme study. Thirty-three isolates of *C. gloeosporioides* from five host genera were characterized using the 11 selected enzymes (Table 2). *C. sublineolum* (isolate 785) was used as an outlier for comparison and could be distinguished as a unique electrophoretic phenotype (EP) for 10 of the 11 enzymes (Table 2). Based on the Rm values of the isozyme bands, electrophoretic phenotypes were assigned to a unique set of bands for each of the 11 enzymes tested (Fig. 1). A total of 148 electrophoretic phenotypes was detected among the 33 isolates examined. The number of electrophoretic phenotypes detected within host groups varied for each host group tested. The greatest number of EPs was detected in isolates from *Stylosanthes,* and the fewest were recorded for isolates from *Malva.*

Cluster analysis. A dendrogram representing 33 isolates of *C. gloeosporioides* and one isolate of *C. sublineolum* (Fig. 2) was generated from the EP matrix using NTSYS-PC. Three major groups of *C. gloeosporioides*, designated I, II, and III, were delineated by the cluster analysis. In addition, groups I and III each could be divided into two subgroups (A and B) at a relative relatedness level slightly less than 0.10. A good fit between the phenetic tree generated by UPGMA cluster analysis and the original data was indicated by a high cophenetic correlation value of 0.993. *C. sublineolum* (785) was delineated from 31 of the 33 isolates of *C. gloeosporioides.*

Group IA contained all the pecan isolates and an isolate (785) of *C. sublineolum,* whereas Group IB consisted of five of six citrus isolates. The remaining citrus isolate, 1144, clustered along with the isolates from *Stylosanthes* in Group II.

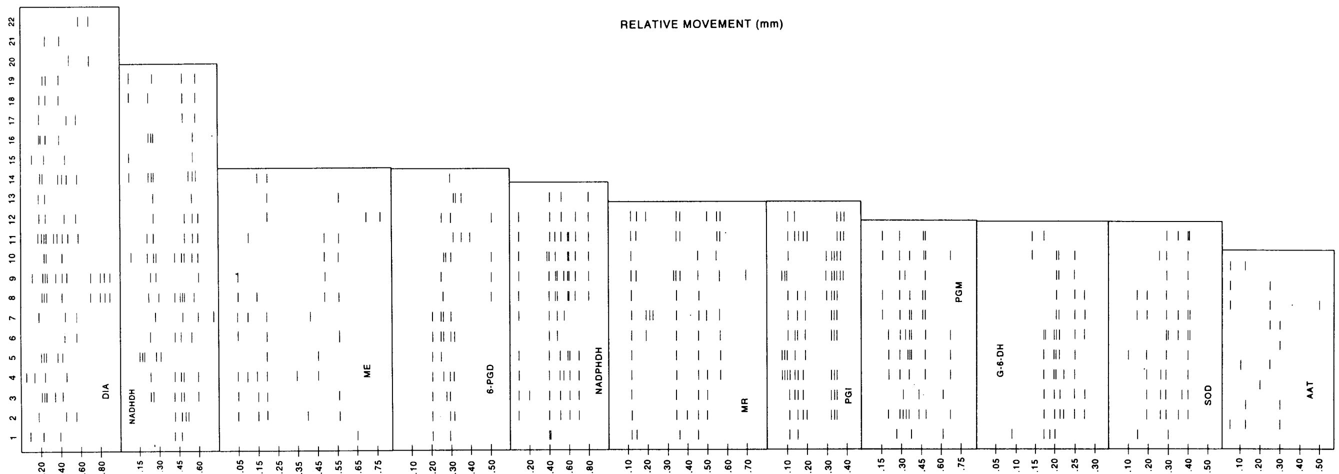

Fig. 1. Electrophoretic phenotypes assigned to 33 isolates of *Colletotrichum gloeosporioides* and one isolate of *C. sublineolum* using 11 enzymes.

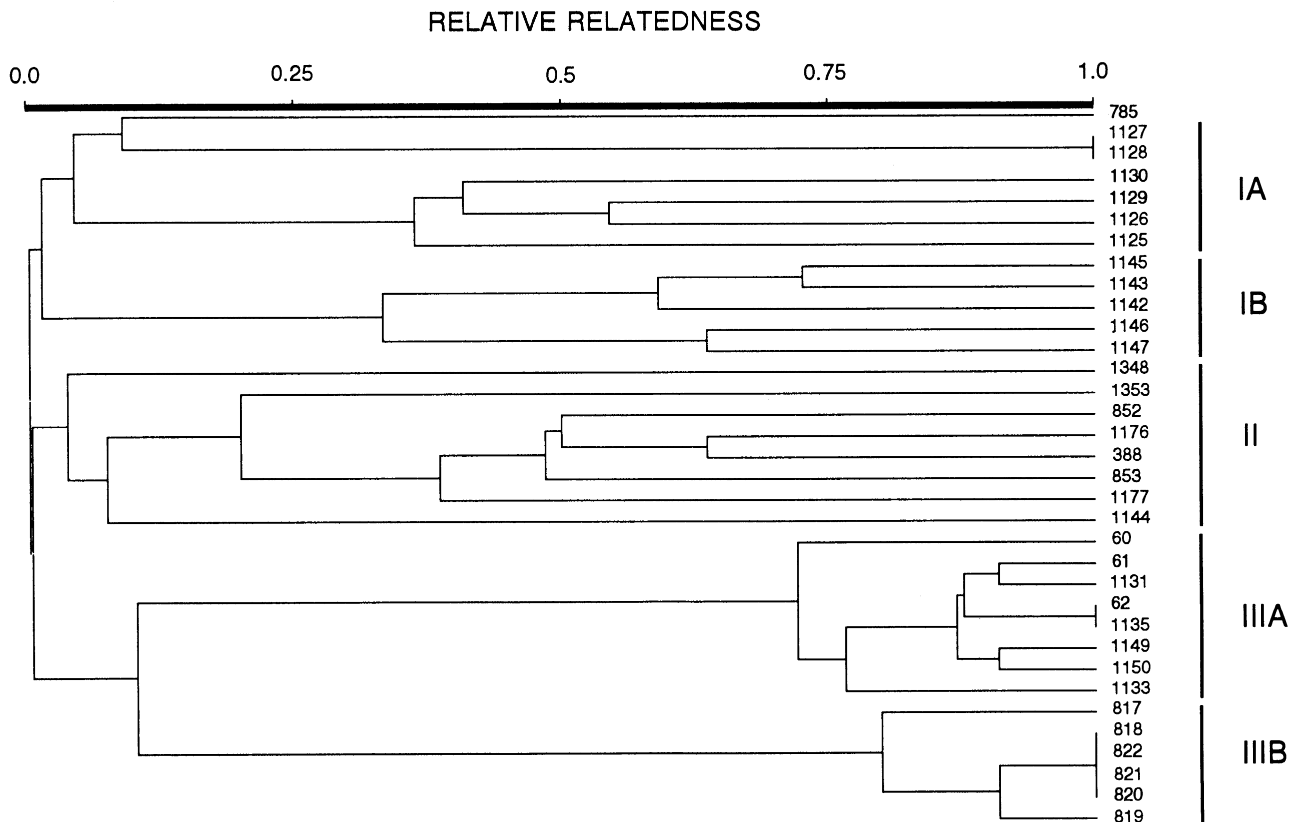

Fig. 2. Dendrogram based on 33 isolates of *Colletotrichum gloeosporioides* using the unweighted pair-group method with arithmetic averages (UPGMA).

Two distinct pathotypes of *C. gloeosporioides* (Types A and B) that cause different anthracnose diseases on *Stylosanthes* in Australia have been identified (13). Isolates 1176 and 1177 represent A and B pathotypes, respectively. The two isolates had different EPs in seven of 11 enzymes tested (Table 3). It is not known whether the remaining *Stylosanthes* isolates represent Type A or B strains.

The remaining two host groups, *Aeschynomene* and *Malva pusilla*, formed clusters that contained all of their respective isolates in Groups IIIA and IIIB, respectively. With the exception of the *C. sublineolum* outlier, these two groups contained the fewest total EPs. These results agree with other molecular analyses (10,12) conducted using these isolates.

Isolates 1127 and 1128, from pecan (*Carya*), were distinct from the other four pecan isolates. Both isolates had the same EPs for nine of the 11 enzymes tested and only one similar EP compared with the other pecan isolates. When pecan isolates were tested by RFLP analysis and a probe for glutamate dehydrogenase (GDH) (C. L. Trout, *personal communication*), isolate 1128 was distinct from all the other pecan isolates (1126, 1127, 1125, and 1129) tested. Using an rDNA probe, isolates 1125 and 1128 were the same, whereas 1126, 1127, and 1129 differed by one or two bands (C. L. Trout, *personal communication*).

DISCUSSION

EPs for *C. gloeosporioides* varied for most enzymes within and among isolate groupings based on host. However, three major groups (I, II, and III) could be delineated within *C. gloeosporioides* among the five host populations following a cluster analysis of phenotype values for all 11 enzymes (Fig. 2).

The presence of multiple bands for several enzymes was unexpected but is similar to the results obtained by Laroche et al. (14). Because *C. gloeosporioides* produces haploid, uninucleate conidia (23), and all cultures were started from single spores, it is unlikely that multiple bands could be attributed to heterokaryosis. As in Laroche et al. (14), multiple bands could be attributed to multiple loci or the production of secondary isozymes by posttranslational processes. Despite the larger than expected number of bands, results were consistent and repeatable under conditions of the study.

Electrophoresis of total soluble proteins has been used to characterize host-specific isolates of *C. gloeosporioides* and to estimate genetic relatedness within and between isolates from different hosts (12). Comparisons of protein profiles of isolates of *C. gloeosporioides* from different host species showed limited similarity, but protein profiles of isolates from the same host, even if from different geographic locations, were usually relatively uniform (12). Similar results have been obtained using RFLP analysis of mitochondrial DNA (10). In limited studies, isolates of *C. gloeosporioides* f. sp. *aeschynomene* were homogeneous and distinguishable from other host-specific isolates within *C. gloeosporioides* (12). Bonde et al. (3) used isozyme analysis of 13 enzymes to distinguish *C. fragariae* Brooks and *C. gloeosporioides*.

In our work, the greater extent of isozyme phenotypic variability discovered among some populations of *C. gloeosporioides* could be attributed to greater genetic diversity (15). Masel et al. (16) detected extensive karyotypic variation involving both chromosome number and length in isolates of *C. gloeosporioides* obtained from *Stylosanthes* spp. The authors suggest that chromosomal rearrangements may play a role in generating variation in this pathogen and that proc-

Table 3. Assigned electrophoretic phenotypes for isolates of *Colletotrichum gloeosporioides*[a]

Host	Isolate	Enzyme										
		AAT	DIA	G6DH	ME	MR	NADHDH	NADPHDH	6PGD	PGI	PGM	SOD
Citrus	785	1	1	1	1	1	1	1	1	1	1	1
	1142	6	13	7	11	9	13	9	9	10	9	9
	1143	1	14	7	0	9	12	10	9	10	9	9
	1144	7	11	6	10	8	3	8	8	9	8	8
	1145	1	12	7	10	9	12	9	9	10	9	9
	1146	7	15	8	11	9	14	11	10	10	9	9
	1147	0	16	8	11	10	15	11	10	10	9	9
Malva	817	9	22	11	13	12	18	13	11	12	11	11
	818	0	22	11	13	12	17	13	11	12	11	11
	819	0	22	11	13	12	19	13	14	12	11	11
	820	0	22	11	13	12	17	13	11	12	11	11
	821	0	22	11	13	12	17	13	11	12	11	11
	822	0	22	11	13	12	17	13	11	12	11	11
Carya	1125	4	5	3	4	3	3	2	4	4	4	3
	1126	2	4	3	4	4	4	2	4	4	3	3
	1127	1	2	2	12	2	2	2	2	3	2	2
	1128	1	2	2	12	2	2	2	2	3	2	2
	1129	3	4	3	3	4	4	2	3	2	3	4
	1130	2	3	3	2	3	3	3	3	2	3	3
Aesc	60	7	17	9	14	11	16	12	11	11	10	10
	61	7	18	10	14	11	16	12	12	11	10	10
	62	7	19	10	14	11	nd	12	nd	11	10	10
	1131	7	18	10	14	11	16	12	13	11	10	10
	1133	8	21	10	14	11	16	12	12	11	10	10
	1135	7	20	10	14	11	16	12	13	11	10	10
	1149	7	21	10	14	11	16	12	13	11	10	10
	1150	7	19	10	14	11	16	12	12	11	10	10
Stylo	1348	2	6	4	5	5	5	4	5	5	5	5
	1353	5	7	5	2	6	6	5	6	6	5	6
	1176	5	8	6	7	7	9	6	7	8	7	7
	1177	5	10	6	8	7	10	7	6	6	5	7
	388	5	9	6	9	7	11	6	7	6	7	7
	852	5	8	5	6	7	7	6	7	7	5	7
	853	5	9	6	9	7	8	6	0	7	6	7
Total		9	22	11	14	12	19	13	14	12	11	11

[a] Phenotypes based on Rm values for each enzyme band (nd = no data).

esses responsible may be active during either somatic growth or parasexual recombination in this presumably haploid organism. Isolates from *Stylosanthes* had the greatest number of EPs for three enzymes (ME, NADHDH, and PGI). These isolates also represent the greatest amount of geographic diversity and were obtained from several different *Stylosanthes* species or cultivars.

Some of the host-specific isolates tested have been reported to produce the teleomorph *Glomerella cingulata* (8,22), while others have not been reported to produce the sexual stage (23). Only recently has it been observed that some isolates of *C. gloeosporioides* that are pathogenic to different, distantly related hosts can be sexually compatible (8). Such is the case between some isolates of *C. gloeosporioides* f. sp. *aeschynomene* and several isolates of *C. gloeosporioides* from pecan in controlled studies (8). However, the teleomorph has not been reported on either host in nature. There also have not been any reports of the teleomorph occurring on *Malva pusilla*. There has been one report of the teleomorph on dead tissue of *Citrus*, but the perfect stage has not been observed on a living host and is not believed to play a major role in the disease cycle (24). High levels of variability among isolates from a given host and geographic location may indicate that sexual reproduction or some other recombination mechanism may be occurring.

Isozyme polymorphisms can be used as neutral genetic markers to assess relative genetic relatedness among fungi. Because isozyme electrophoresis is rapid and inexpensive compared with some other types of molecular analyses, a large number of enzymes can be screened rapidly. It can be used in combination with DNA methodologies, such as RFLP analysis, along with cultural characteristics and pathogenic relationships to characterize a fungal population (27). This investigation has illustrated the value of using isozyme analysis for differentiating and characterizing pathogenic isolates within *C. gloeosporioides* to gain a greater understanding of the genetic and biological relationships of this fungal species. In some cases, it may be possible to use isozyme analysis as an alternative to routine host inoculation to differentiate host-specialized isolates of *Colletotrichum.*

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