Morphological, Pathological, and Genetic Differentiation of *Didymella bryoniae* and *Phoma* spp. Isolated from Cucurbits

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ABSTRACT

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Didymella bryoniae (anamorph *Phoma cucurbitacearum*), which causes gummy stem blight of cucurbits, occurs throughout the eastern United States. Other *Phoma* spp., such as *P. exigua,* also have been reported to cause symptoms of gummy stem blight. Twenty-seven isolates provisionally identified as *D. bryoniae* or *Phoma* spp. were obtained from diseased watermelon, cantaloupe, cucumber, pumpkin, and squash grown in South Carolina, New York, and Florida. *D. bryoniae* was clearly distinguished from *Phoma* after 7 days of growth on quarter-strength potato-dextrose agar at 24 C with a 12-h photoperiod. *D. bryoniae* produced white aerial mycelium, olivaceous green substrate mycelium, and few pycnidia; *Phoma* produced sparse aerial mycelium and numerous pycnidia, sometimes in concentric zones. The percent monoseptate conidia for *D. bryoniae* isolates ranged from 0 to 18%, whereas no *Phoma* isolate produced any septate conidia. Seventeen of 19 *D. bryoniae* isolates were pathogenic on watermelon cv. Charleston Gray and cantaloupe cv. Classic; all eight isolates of *Phoma* and two isolates of *D. bryoniae* were nonpathogenic. Genomic DNA was extracted from all 27 isolates described above plus two additional isolates of *D. bryoniae* from New York and one from Florida. DNA was amplified using PCR primed with random oligonucleotide decamers. RAPD amplification patterns clearly differentiated *D. bryoniae* from *Phoma.* Each of five primers used produced two to four amplified fragments that were unique either to all *D. bryoniae* or to all *Phoma* isolates. Thirteen additional fragments were present in all *D. bryoniae* isolates except two of the three isolates from New York.

Additional keyword: Ascochyta cucumis.

Didymella bryoniae (Auersw.) Rehm (=*Mycosphaerella citrullina* (C.O. Sm.) Gross.), anamorph *Phoma cucurbitacearum* (Fr.:Fr.) Sacc. (=*Ascochyta cucumis* Fautrey & Roum.), causes gummy stem blight (foliar phase) and black rot (fruit phase) of cucurbits (species of *Citrullus, Cucumis, Cucurbita,* and other genera). In the southeastern United States, gummy stem blight is the most destructive foliar disease of cucumber (24) and watermelon. *Cucurbita* spp. (pumpkin and winter squash) are especially susceptible to black rot, which directly reduces both pre- and postharvest yields (28). As resistance to other foliar diseases (e.g., downy mildew and anthracnose) has been incorporated into

cucumbers and other cucurbits, losses due to gummy stem blight have increased (22,25).

Phoma species in addition to *P. cucurbitacearum* have been associated with symptoms of gummy stem blight. *Phoma lagenariae* (Thuem.) Sacc. has been isolated from pumpkin in New York (10). *Phoma exigua* Desmaz. (=*Ascochyta phaseolorum* Sacc.) was reported to be widespread on cucumber, cantaloupe, and watermelon in North Carolina (12). However, *P. exigua* is considered a weak parasite on cucurbits and other plants (3,8). Consequently, the role of other *Phoma* spp. in the epidemiology of gummy stem blight remains unclear. In the field, it is difficult to visually distinguish pycnidia of *Didymella* from those of *Phoma* (12; T. Zitter, personal observation), which makes rapid, accurate identification and diagnosis difficult. Although pseudothecia, asci, and ascospores of *D. bryoniae* can be used to positively identify this pathogen, these structures may not be present on infected tissue when a diagnosis is required, since pseudothecia develop on infected tissue later than pycnidia (27). To confirm a provisional diagnosis, isolates must be obtained in pure culture and compared with known specimens or tested for pathogenicity. Some isolates of *D. bryoniae* fail to sporulate in culture (27). In addition, Wiant (27) observed mostly nonseptate conidia, typical of *Phoma,* in his isolates of *D. bryoniae*; whereas Punithalingam and Holliday (21) described mostly one-septate conidia, typical of *A. cucumis.* According to Dorenbosch (8), conidia of *P. exigua* are "frequently" one-septate, but the percent septate conidia was not reported. Presence of septae may not be a useful character to distinguish the anamorph of *D. bryoniae* from *Phoma* spp. Additional cultural characters and more sensitive methods are needed to differentiate between these closely related species.

Traditionally, fungal plant pathogens were characterized based on morphology and pathogenicity (4,5,27). More recently, molecular markers have provided an additional tool to characterize fungal genotypes. Random amplified polymorphic DNA (RAPD) analysis of fungal genomes has been very useful for corroborating, at a genetic level, previously observed differences in morphology and pathogenicity. For example, virulent and avirulent pathotypes of *Leptosphaeria maculans* each have distinct RAPD amplification patterns (11). Isolates within the virulent pathotype were more closely related than isolates within the avirulent group, which was further separated into two geographically isolated subpopulations. A PCR assay has been developed to distinguish *Gaeumannomyces graminis* var. *graminis* from the morphologically similar but nonpathogenic *G. incrustans* isolated from turfgrasses (9). In another study, RAPD markers associated with differences in mating type of *Fusarium solani* f. sp. *cucurbitae* differentiated isolates that failed to cross with tester isolates (7). Both RAPD analysis with random primers and PCR with specific primers were used to differentiate races of *Cochliobolus carbonum* (13) and species of *Fusarium* (20).

Our long-term goal is to elucidate the genetic diversity within the *D. bryoniae* population in the eastern United States. A first step is to clarify the role of *Phoma* spp. in the epidemiology of gummy stem blight. Our working hypothesis was that *Phoma* spp. isolated from cucurbits are distinct and ecologically different from *D. bryoniae.* The objectives of this study were to compare morphological characteristics of *D. bryoniae* isolates to those of *Phoma* spp., to determine the pathogenicity and relative virulence of *D. bryoniae* and *Phoma* isolates on representative cucurbits, and to identify RAPD markers that could be used to differentiate *D. bryoniae* and *P. cucurbitacearum* from other *Phoma* spp. Preliminary reports have been published (14,15).

MATERIALS AND METHODS

Isolate collection. Leaf, stem, or fruit tissue with symptoms of gummy stem blight or black rot was collected from cucurbits grown in commercial fields and experimental plots in South

TABLE 1. *Didymella bryoniae* and *Phoma* spp. isolated from diseased cucurbit foliage or fruit in South Carolina, New York, California, and Florida

Isolate	Genus	Host	Origin	Mean disease severity[a]	Standard error
1216	*Phoma*	Cantaloupe	Horry Co., SC	0.32	0.19
C2	*Phoma*	Cantaloupe (breeding line)	Bamberg Co., SC	0.33	0.19
C3	*Didymella*	Cantaloupe	Wadmalaw Is., Charleston Co., SC	1.35**[b]	0.075
C4	*Didymella*	Cantaloupe	Wadmalaw Is., Charleston Co., SC	1.41**	0.065
C5	*Phoma*	Cantaloupe	CREC, Charleston Co., SC	0.34	0.20
C6	*Didymella*	Cantaloupe (internal fruit rot)	Colleton Co., SC	2.11***	0.10
DBA1	*Didymella*	Watermelon (pseudothecia on stem)	CREC, Charleston Co., SC	1.19*	0.065
DBA2	*Didymella*	Watermelon (pseudothecia on fruit)	CREC, Charleston Co., SC	1.48**	0.10
P3	*Didymella*	Pumpkin cv. Big Autumn	Wadmalaw Is., Charleston Co., SC	1.32**	0.065
P6	*Didymella*	Pumpkin cv. Big Autumn	Wadmalaw Is., Charleston Co., SC	1.30**	0.30
SSQ1	*Didymella*	Summer squash cv. Multipik	Lexington Co., SC	1.29**	0.27
W1	*Phoma*	Watermelon cv. Charleston Gray	Colleton Co., SC	0.33	0.19
W3	*Didymella*	Watermelon	Colleton Co., SC	1.76***	0.40
W4	*Didymella*	Watermelon cv. Sangria	Colleton Co., SC	1.42**	0.085
W5	*Phoma*	Watermelon cv. Jubilee II	Bamberg Co., SC	0.51	0.18
W6B	*Didymella*	Watermelon cv. Crimson Trio	Allendale Co., SC	1.50**	0.32
W14	*Didymella*	Watermelon	South Carolina	1.72***	0.33
W16	*Phoma*	Watermelon	South Carolina	0.47	0.27
W20	*Didymella*	Watermelon cv. Fiesta (seedling)	Calhoun Co., SC	1.33**	0.26
W21	*Phoma*	Watermelon cv. Tri-X 313 (seedling)	Calhoun Co., SC	0.76	0.14
W22B	*Didymella*	Watermelon	Edisto Is., Charleston Co., SC	1.02	0.32
W23	*Didymella*	Watermelon	South Carolina	1.56**	0.33
W24	*Didymella*	Cantaloupe (seed)	Florida	1.00	0.41
W25	*Didymella*	Cantaloupe	USDA, Charleston Co., SC	1.57***	0.41
W26	*Didymella*	Cantaloupe (seed)	Florida	1.21*	0.23
W27	*Phoma*	Cucumber cv. Calypso	CREC, Charleston Co., SC	0.15	0.15
NY1	*Didymella*	Cantaloupe	Onondaga Co., NY	1.76***	0.075
NY2	*Didymella*	Cucumber	Tompkins Co., NY	NT[c]	NT
NY3	*Didymella*	Butternut squash cv. Waltham	Oneida Co., NY	ND[d]	ND
FL1	*Didymella*	Cantaloupe	Collier Co., FL	NT	NT

[a] Percent leaf surface area diseased transformed with $(x)^{0.2}$ to stabilize variances among isolates.

[b] Means with asterisks are significantly different from the control (0.42 ± 0.146 [standard error]) according to t tests, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$.

[c] Not tested in this study. NY2 is pathogenic on cucumber and cantaloupe, and FL1 is pathogenic on cantaloupe (T. A. Zitter, *unpublished*).

[d] Not determined.

Carolina during 1991–92 (Table 1). Tissue pieces were surface disinfested in 0.05% sodium hypochlorite and plated on one-quarter-strength potato-dextrose agar (QPDA). Presumptive colonies of *D. bryoniae* and *Phoma* were single-spored and stored at 4 C. Additional isolates of *D. bryoniae* were supplied by T. A. Zitter (NY1, NY2, NY3, and FL1) and Claude E. Thomas, USDA-ARS, U.S. Vegetable Laboratory, Charleston, SC (W24, W25, and W26).

Morphological characterization of isolates. Isolates of *D. bryoniae* and *Phoma* spp. were grown on malt agar (MA) at 21 C in the dark for 3 days, then transferred to MA and grown for 7 days at 21 C in the dark (8). Two colony diameters were measured at right angles on two replicate plates for each isolate. The experiment was performed twice. Cultures then were incubated with a 12-h photoperiod for an additional week to induce sporulation (8). Cultures also were grown on QPDA and V8 agar (V8A) at ambient temperature (22–24 C) and 12-h photoperiod for 2 wk or until pycnidia were produced. Macroscopic appearance of the isolates was recorded. The length and width of 10 randomly selected conidia from QPDA cultures were measured at 1,000× magnification. An additional 100 conidia were examined for the presence of septae. For some isolates, ascospores and conidia also were measured on cultures growing from watermelon tissue pieces onto QPDA. Isolates of *Phoma* spp. also were grown on oatmeal agar (OMA) in the dark for 1 wk and then under a 12-h photoperiod (8). Conidia were measured and examined for septae as described above. A drop of 1 N NaOH was added to colony margins on OMA to check for the presence of blue-green pigment, which is diagnostic for *P. exigua* (3,8).

Pathogenicity tests. Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai cv . Charleston Gray) and cantaloupe (*Cucumis melo* L. cv. Classic) were seeded in 60% vermiculite–40% peat (Fafard No. 2 potting mix, Piedmont Nursery Supply, Spartanburg, SC). After 1 wk, seedlings were transplanted, three seedlings per 10-cm plastic pot. Isolates of *D. bryoniae* and *Phoma* were grown on QPDA plates for 2–3 wk at ambient temperature (23–26 C) and 12-h photoperiod. Cultures were flooded with 0.1% sucrose–0.05% casein solution (2) and gently scraped to release conidia from pycnidia. The suspension was filtered through four layers of sterile cheesecloth. Spore concentration was determined by counting in a hemacytometer and adjusted to 10^5 conidia per milliliter. Two-week-old plants were inoculated by spraying approximately 2 ml of spore suspension per pot. Plants were held in a mist chamber at 100% relative humidity for 3 days to promote infection and lesion expansion (1). Disease severity on individual plants was rated 4 days after inoculation on a scale of 1 (0% leaf area diseased) to 5 (>75–100%) (23). Isolates with a mean disease severity significantly ($P < 0.05$) greater than that of control (noninoculated) plants were considered pathogenic. Diseased leaf tissue from one plant per replicate was cultured as described previously to reisolate pathogens. The individual tests were completely randomized designs with four replicate pots for each host and isolate. Each test included control plants sprayed with sterile sucrose-casein solution. Isolates were assigned randomly to pathogenicity tests. All isolates were included in at least two tests.

PCR-based RAPD analysis. *Didymella* and *Phoma* were grown on QPDA in the dark for 3 days at ambient temperature. Cultures were flooded with 5 ml of sterile distilled water and scraped to remove mycelium, which was used to seed liquid cultures. Fungi were grown in sucrose-casein-salts liquid medium (18), 50 ml per 250-ml flask, for 2–3 days at ambient temperature (22–24 C) in the dark. Cultures were decanted through four layers of sterile cheesecloth, and mycelium was washed with sterile distilled water. After removing as much liquid as possible, mycelium was frozen and stored at −20 C.

Genomic DNA was extracted according to a standard mini-prep protocol (17), except that approximately fivefold greater volumes of reagents were used in 50-ml centrifuge tubes. Briefly, 1.0 g of frozen mycelium was ground in liquid nitrogen and mixed with 3.5 ml of lysis buffer (50 mM TRIS-HCl, pH 7.5; 50 mM EDTA, pH 8.0; 3% SDS) and 3.5 μl of 2-mercaptoethanol. The suspension was incubated at 65 C for 1 h, then extracted with chloroform-phenol-isoamyl alcohol (25:24:1) and chloroform-isoamyl alcohol (24:1). The DNA was precipitated with 0.1 volume of 3 M sodium acetate plus 1.5 volumes of cold isopropanol, resolubilized in TE, and reprecipitated with sodium acetate and 3 volumes of cold 100% ethanol. Purified DNA was washed with 75% ethanol, dried under vacuum, and dissolved in sterile deionized water. DNA concentration was determined with a fluorometer (Hoeffer TKO 100) and adjusted to 10 ng/μl.

Oligonucleotide decamers obtained from Operon (Alameda, CA) were used to prime PCR. The PCR reaction mixture contained 40 ng of genomic DNA, 2.5 μl of 10× buffer, 1.9 mM $MgCl_2$, 25 μg of nonacetylated bovine serum albumin (New England Biolabs, Beverly, MA), 0.1 mM deoxynucleotide triphosphates, 0.32 μM primer, and 1.5 units of Taq DNA polymerase (Promega Corp., Madison, WI) in a total volume of 25 μl. A drop of mineral oil was layered on top of all samples. PCR amplification was performed in a Perkin-Elmer-Cetus (Norwalk, CT) Thermal Cycler 480. Reaction conditions were initial denaturation at 94 C for 2 min, followed by 45 cycles of denaturation at 94 C for 1 min, annealing at 36 C for 1 min, and extension at 72 C for 2 min. The entire 25-μl sample volume was loaded onto a 1.5% agarose gel for electrophoresis at 7 V/cm with 1× TBE buffer. Products were visualized by staining gels in ethidium bromide at 0.5 μg/ml.

DNA from duplicate extractions for each isolate was run in duplicate amplification cycles. Relative molecular sizes (in base pairs) of DNA fragments clearly present on duplicate gels were determined by comparing migration distances with a 100-bp DNA ladder (Gibco BRL, Gaithersburg, MD) run on each gel. A negative control without DNA was included in all thermal cycle runs. Random oligonucleotide decamer primers were tested for amplification in preliminary reactions with a subset of the isolates. Five Operon primers, A05 (AGGGGTCTTG), K14 (CCCGCTACAC), T01 (GGGCCACTCA), T08 (AACGGCGACA), and T16 (GGTG AACGCT), that amplified sample DNA reproducibly were chosen to evaluate all fungal isolates.

Data analysis. Conidial dimensions and percent septate conidia of *D. bryoniae* and *Phoma* spp. were compared by *t* tests. Colony diameter and disease severity were subjected to analysis of variance (PROC GLM, SAS Release 6.04, SAS Institute, Inc., Cary, NC) before treatment means were compared. Disease severities were transformed before analysis by Taylor's power law ($b = 1.6$) to stabilize the variances (19).

RESULTS

Morphological characterization of isolates. Twenty-three *Phoma*-like fungi were isolated from cucurbits grown in South Carolina during 1991–92 (Table 1). These isolates, plus four additional isolates from South Carolina, New York, and Florida, could be separated readily into two groups based on the morphology of 2-wk-old cultures. On QPDA, one group produced olive to dark green or black substrate mycelium in the agar and white, hairy aerial mycelium. Pycnidia and pseudothecia, present in 13 isolates, were distributed relatively uniformly throughout the colony or, rarely, concentrated in the center of the colony. Bitunicate asci in the pseudothecia contained ascospores slightly constricted at the septum. Mean dimensions of ascospores were 13.1 (±0.85, standard deviation) × 5.45 (±0.23) μm. Cultures of the second type also produced black to dark green substrate mycelium but only sparse aerial mycelium. Pycnidia were produced abundantly over the surface of the colony; most isolates produced pycnidia in concentric rings radiating from the colony center. Cultures grown on V8A and MA generally appeared similar to those on QPDA, but differences between the two groups were less distinct, especially on V8A. Growth on V8A tended to occur more frequently in concentric zones for both groups than on the other two media. On MA, isolates of the second type had beige to reddish brown colony undersides. Based on the presence of pseudothecia containing bitunicate asci with ascospores resembling those of *D. bryoniae* (6), isolates in the first group were

considered to be *D. bryoniae,* and isolates in the second group were considered to be other *Phoma* spp. Colony margins of *Phoma* isolates were regular, not scalloped or lobed as reported for *P. exigua* (8). No pigments were produced by *Phoma* isolates grown on OMA either before or after treatment with NaOH.

Diameters of all *D. bryoniae* colonies on MA after 7 days were significantly greater than all diameters of *Phoma* colonies (Waller-Duncan *k*-ratio *t* test, $k = 500$, minimum significant difference = 0.67 cm). Diameters of *D. bryoniae* colonies ranged from 8.7 to 7.2 cm, while diameters of *Phoma* colonies ranged from 6.5 to 5.1 cm (pooled standard deviation = 0.12). Most *D. bryoniae* isolates had reached the edge of the petri plate by 7 days.

Mean length and width of *D. bryoniae* conidia, 8.0 ($\pm$0.71) $\times$ 3.3 ($\pm$0.36) μm, was greater than dimensions of *Phoma* conidia, 6.4 ($\pm$0.61) $\times$ 2.7 ($\pm$0.21) μm, when isolates were grown on QPDA (*t* test, $P < 0.0001$ and $P = 0.0011$ for length and width, and N = 14 and 7 for *D. bryoniae* and *Phoma,* respectively). Conidia of both genera did not differ significantly in size on QPDA with or without watermelon tissue (paired *t* test). Similarly, conidia of *Phoma* did not differ in length or width between QPDA and OMA. All 16 *D. bryoniae* isolates examined, technically all except C4, produced some septate conidia, but no septate conidia were observed in any *Phoma* isolate. Mean percent septate conidia ranged from 0 to 18% for individual *D. bryoniae* isolates. Mean percent septate conidia for *D. bryoniae* isolates was significantly greater than 0 on QPDA with (9.7% $\pm$ 13.2) or without (5.9% $\pm$ 6.8) watermelon leaf tissue (one-tailed *t* tests, $P < 0.05$). There was no significant difference between these two media (paired *t* test, $P > 0.20$).

Pathogenicity tests. Data from all pathogenicity tests in both experiments were combined for analysis of variance after it was determined that variances were homogenous (two-tailed *F* test, $P \geq 0.05$). Seventeen of 19 *D. bryoniae* isolates tested were pathogenic on both watermelon and cantaloupe, i.e., they had disease severities significantly greater than the control (noninoculated) treatment (*t* tests, $P \leq 0.05$) (Table 1). All eight *Phoma* isolates and two *D. bryoniae* isolates (W22B from watermelon and W24 from cantaloupe) were nonpathogenic when compared to the control treatment, although isolates W22B and W24 had disease severities greater than all *Phoma* isolates. There was no host $\times$ isolate interaction, and the cantaloupe and watermelon cultivars used were equally susceptible (*F* values not significant in analysis of variance).

All *D. bryoniae* isolates except C3 were recovered from at least seven of the eight inoculated watermelon and cantaloupe plants sampled; 14 isolates were recovered from all plants sampled. *Phoma* isolates were recovered from 17 to 63% of inoculated plants sampled, even though plants were asymptomatic or had less than 5% leaf surface area diseased.

PCR-based RAPD analysis. *D. bryoniae* and *Phoma* isolates could be distinguished with each primer based on their complete RAPD amplification patterns (Fig. 1). A total of 14 DNA fragments were found that distinguished the two groups of isolates (Table 2). Eight fragments were present in all 22 *D. bryoniae* isolates and no *Phoma* isolates, and six other fragments were present in all eight *Phoma* isolates but none of the *D. bryoniae* isolates (Table 3). In addition, 14 other fragments distinguished a subgroup within the *D. bryoniae* isolates. Thirteen of these fragments, generated by four primers, were present in all *D. bryoniae* isolates except two isolates from New York, NY2 and NY3 (Table 2). An additional 1,000-bp fragment generated with primer A05 was present in all eight *Phoma* isolates plus *D. bryoniae* NY2 (Table 3).

All primers except K14 generated one to three fragments unique to *D. bryoniae* isolates (Table 3). Primer K14 generated two fragments unique to *Phoma* isolates, whereas the other four primers each generated one fragment. All primers except T16 generated one to five fragments that separated *D. bryoniae* isolates into two groups, with NY2 and NY3 in one subgroup and the remaining 20 isolates in another.

DISCUSSION

Based on appearance in culture, production of ascospores in pseudothecia, and pathogenicity to watermelon and cantaloupe,

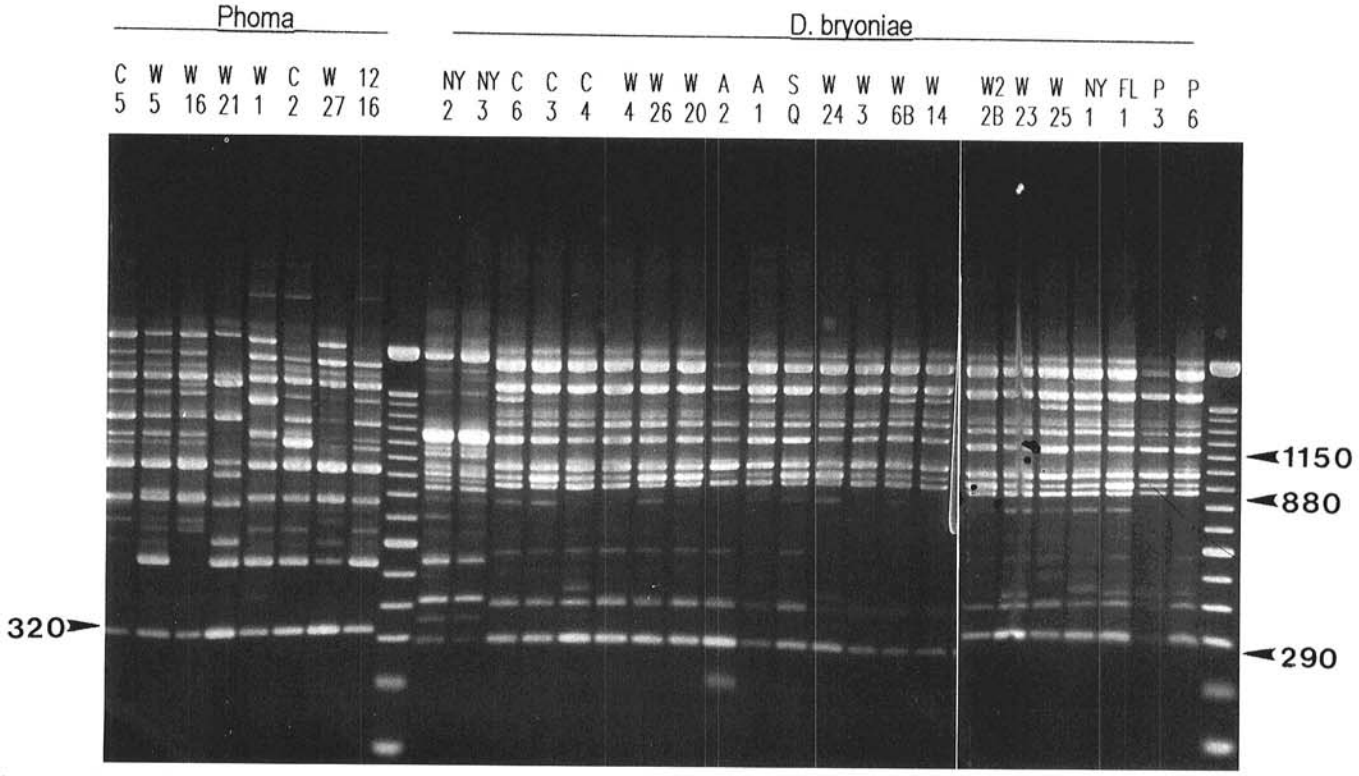

Fig. 1. RAPD amplification patterns generated with random oligonucleotide primer T08, illustrating markers specific for *Phoma* (320 bp) or *Didymella bryoniae* (290, 880, and 1,150 bp). Lanes 1–8 are *Phoma* spp., lanes 9 and 32 are a 100-bp ladder (100–2,100 bp), and lanes 10–31 are *D. bryoniae.* Isolates are in the same order as listed in Table 3. Lanes 25–32 were run on a separate gel from lanes 1–24.

19 isolates were classified as *D. bryoniae* and eight as *Phoma* spp. *D. bryoniae* isolates grew more rapidly, produced fewer pycnidia, and had longer and wider conidia than *Phoma* isolates. Ascospores of *D. bryoniae* isolates were 2–3 μm shorter but as wide as published dimensions (6,21). None of the *Phoma* isolates displayed two characteristic features of *P. exigua,* scalloped colony margins and blue-green pigment in the presence of alkali (8).

Most *D. bryoniae* isolates recovered from watermelon, cantaloupe, pumpkin, or summer squash were pathogenic to watermelon and cantaloupe under conditions conducive to development of gummy stem blight. Therefore, our results indicate that *D. bryoniae* is the pathogen that causes gummy stem blight of cucurbits. Contrary to results with *D. bryoniae, Phoma* isolates evaluated in this study were nonpathogenic or very weakly virulent. This confirms other observations which indicate *Phoma* spp. are infrequently pathogenic on cucurbits but are occasionally isolated alone (3,10) or in combination with D. bryoniae (12; A. P. Keinath and T. A. Zitter, *unpublished*).

TABLE 2. Sizes of RAPD fragments (in base pairs) which distinguished between *Didymella bryoniae* and *Phoma* and among *D. bryoniae* isolates from cucurbits

Operon primer	All *D. bryoniae*	All *Phoma*	*D. bryoniae* except NY2 and NY3
A05	900[a]	1,200	350
			410
			1,050
K14		1,800	1,100
		1,900	
T01	680	1,100	390
	850		1,350
			1,450
			1,600
T08	290	320	400
	880		580
	1,150		980
			1,600
			1,900
T16	720	1,300	
	1,100		
Totals	8	6	13

[a]Sizes of RAPD fragments were determined by comparing migration distances in duplicate 1.5% agarose gels with migration distances of 100- to 2,100-bp size markers.

RAPD amplification patterns generated with random oligonucleotide primers easily distinguished isolates of *D. bryoniae* from *Phoma.* Both products unique to *D. bryoniae* and products unique to nonpathogenic *Phoma* spp. were amplified. A similar separation was made between virulent and avirulent pathotypes of *Leptosphaeria maculans* (anamorph *P. lingam*), which may be separate species (11). Two *D. bryoniae* isolates that were avirulent were indistinguishable from virulent isolates with the RAPD markers examined in this study. However, these two isolates were clearly differentiated from nonpathogenic *Phoma* isolates by morphological and genetic characters.

Isolates DBA1 and DBA2 of *D. bryoniae,* which originated from single ascospores, were indistinguishable from the other 20 isolates which originated from single conidia. Because *D. bryoniae* is homothallic (4), the likelihood of outcrossing is low. None of our eight *Phoma* isolates produced pseudothecia, so they are presumed to lack a sexual state, as do *P. exigua* and *P. laginariae* (10). Consequently, we hypothesize that the clear distinction observed between the RAPD amplification patterns for *D. bryoniae* and *Phoma* isolates used in this study will hold true for other collections.

Two *D. bryoniae* isolates from New York, NY2 from cucumber (*Cucumis sativus* L.) and NY3 from butternut squash (*Cucurbita moschata* (Duchesne) Duchesne ex Poir.), lacked 13 DNA fragments present in all other *D. bryoniae* isolates. Four of the five primers utilized yielded fragments that identified this subgroup. This is the first evidence for genotypic variation within the *D. bryoniae* population in the eastern United States. *D. bryoniae*

TABLE 3. Distribution of RAPD markers generated by five random oligonucleotide primers among 30 isolates of *Phoma* spp. and *Didymella bryoniae* isolated from cucurbits

Isolate[a]	C	W	W	W	W	C	W	I	N	N	C	C	C	W	W	W	D	D	A	W	W	W	W	W	W	N	F	P	P
	2	1	1	2	1	2	2	1	Y	Y	6	3	4	4	2	2	B	B	S	2	B	6	1	2	2	Y	L	3	6
RAPD marker[b]	5	5	6	1	1	2	7	6	2	3	6	3	4	4	6	0	A	A	Q	4	B	B	4	B	3	1	1	5	1	1	3	6
A05-350	–[c]	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A05-410	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A05-900	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A05-1050	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
A05-1200	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
K14-1100	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
K14-1800	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
K14-1900	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
T01-390	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T01-680	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T01-850	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T01-1100	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
T01-1350	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T01-1450	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T01-1600	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-290	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-320	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
T08-400	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-580	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-880	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-980	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-1150	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-1600	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T08-1900	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T16-720	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T16-1100	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
T16-1300	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]First eight isolates are *Phoma* spp. and the remaining 22 are *D. bryoniae.*
[b]RAPD markers are identified by Operon oligonucleotide primer designation followed by DNA fragment size in bp.
[c]+ = fragment present; – = fragment absent.

NY2 is pathogenic on cantaloupe and cucumber (T. A. Zitter, *unpublished*). However, it has a 1,000-bp fragment amplified with primer A05 that is present in all eight nonpathogenic *Phoma* isolates. Additional New York isolates from a variety of cucurbits are being analyzed currently to determine the frequency of the two groups.

D. bryoniae infects all cucurbits tested (10,21), but differences in susceptibility have been reported. Chiu and Walker (5) and Lee et al (16) considered cucumber and squash to be more resistant than other cucurbits. Although we found no evidence of differential virulence, in one study disease developed more rapidly when isolates of *D. bryoniae* were inoculated onto their hosts of origin than onto other seedling cucurbits (16). Our isolates of *D. bryoniae* differed in virulence, but there was no association with the original host. Variation in virulence (disease incidence ranging from 0 to 67%) was reported previously among 10 isolates of *D. bryoniae* inoculated onto squash (5). On cucumber fruit, lesion expansion rates of 11 *D. bryoniae* isolates varied from 1 to 6 mm per day (26). The occurrence and epidemiological significance of differences in virulence and susceptibility need to be examined further, especially on cucumber and squash.

Our results provide two avenues other than pathogenicity tests to identify *Phoma*-like fungi isolated from cucurbit foliage, fruit, or seeds. For morphological identification of *D. bryoniae,* cultures may be grown on QPDA under a 12-h photoperiod. *D. bryoniae* will produce white aerial mycelium, olivaceous green to black substrate mycelium, and pycnidia distributed relatively uniformly throughout the colony, although the number of pycnidia will vary among isolates. For RAPD identification, most decamers should distinguish *D. bryoniae* from *Phoma* spp. However, differentiation can be optimized by using a primer (e.g., T08) that produces a greater number of unique bands, some specific for all *D. bryoniae* isolates and others for all *Phoma* isolates.

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