Internal Transcribed Spacer Regions 1 and 2 and Random Amplified Polymorphic DNA Analysis of *Didymella bryoniae* and Related *Phoma* Species Isolated from Cucurbits

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ABSTRACT

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Didymella bryoniae (anamorph *Phoma cucurbitacearum*) is the causal agent of gummy stem blight, although other *Phoma* species are often isolated from cucurbit plants exhibiting symptoms of the disease. The molecular and phylogenetic relationships between *D. bryoniae* and these *Phoma* species are unknown. Isolates of *D. bryoniae* and *Phoma* obtained from cucurbits grown at various geographical locations in the United States were subjected to random amplified polymorphic DNA (RAPD) analysis and internal transcribed spacer (ITS) sequence analysis (ITS-1 and ITS-2) to determine the molecular and phylogenetic relationships

within and between these fungi. Using RAPD fingerprinting, 59 isolates were placed into four phylogenetic groups, designated RAPD group (RG) I, RG II, RG III, and RG IV. *D. bryoniae* isolates clustered in either RG I (33 isolates), RG II (12 isolates), or RG IV (one isolate), whereas all 13 *Phoma* isolates clustered to RG III. There was greater than 99% sequence identity in the ITS-1 and ITS-2 regions between isolates in RG I and RG II, whereas isolates in RG III, *P. medicaginis* ATCC 64481, and *P. exigua* ATCC 14728 clustered separately. On muskmelon seedlings, a subset of RG I isolates were highly virulent (mean disease severity was 71%), RG II and RG IV isolates were slightly virulent (mean disease severity was 4%), and RG III isolates were nonpathogenic (disease severity was 0% for all isolates). The ITS sequences indicate that RG I and RG II are both *D. bryoniae*, but RAPD fingerprints and pathogenicity indicate that they represent two different molecular and virulence subgroups.

Didymella bryoniae (Auersw.) Rehm, anamorph *Phoma cucurbitacearum* (Fr.:Fr.) Sacc., causes gummy stem blight (GSB) of cucurbits throughout the world (20,27). The pathogen has a wide host range within the Cucurbitaceae, which includes cucumber (*Cucumis sativus* L.), muskmelon (*Cucumis melo* L.), watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai), pumpkin and squash (*Cucurbita* spp.), and several wild cucurbits (27,33). No host specificity has been observed among isolates from different cucurbit hosts (12,13,36). Resistance to *D. bryoniae* is available in cucumber (26,30), watermelon (18), and muskmelon (17); however, resistant cultivars have not performed well in commercial production (27). GSB has become more severe as genetic resistance to other cucurbit diseases has become more widely used (26,29).

Few studies have examined the molecular and genetic characteristics of *D. bryoniae*. The pathogen is homothallic in nature (4) and produces pseudothecia and ascospores in culture (6,33) and on infected host tissues (24,33). Isolates from different cucurbit hosts exhibited few genetic differences when subjected to random amplified polymorphic DNA (RAPD) fingerprinting (12). Eight isolates of *D. bryoniae* did not show any differential virulence to a set of eight cucumber genotypes (28).

Species other than *D. bryoniae* have also been isolated from plant tissues exhibiting symptoms of GSB. *P. exigua* (*Ascochyta*

phaseolorum) has been found on cucumber, muskmelon, and watermelon in North Carolina (10), and *P. lagenariae* has been isolated from pumpkin in New York (7). However, isolates of *Phoma* sp. recovered from cucurbits in South Carolina and New York did not produce symptoms in cucurbit seedlings inoculated with them (12,36). The objective of this study was to address and clarify the genetic association between *D. bryoniae* and related *Phoma* isolates, using phylogenetic relationships based on RAPD markers and sequence information from the internal transcribed spacer (ITS) region of rDNA. RAPD markers and ITS data have proved useful for elucidating phylogenetic relationships in other fungi (9,25), including teleomorphic fungi and related species in the same anamorph genus (3,11,35).

MATERIALS AND METHODS

Fungal cultures. A total of 62 isolates of *D. bryoniae* and *Phoma* spp. were used in this study. Thirty-two isolates (with the prefix C or W) were from South Carolina, collected from three fields on one farm on Edisto Island, Charleston County, in 1994 (Table 1). *P. medicaginis* ATCC 64481 and *P. exigua* ATCC 14728 were obtained from the American Type Culture Collection, Manassas, Virginia. Twenty-eight other isolates were provided by T. A. Zitter, including isolates from seven New York counties, Florida, North Carolina, Wisconsin, and Arizona (Table 1). All isolates except *P. medicaginis* and *P. exigua* were grown on one-quarter-strength potato-dextrose agar (QPD) at 23°C with a 12-h photoperiod for 2 weeks (12). Isolates were initially identified as either *D. bryoniae* or *Phoma* sp. by their mycelial growth rate and the size and shape of their conidia and ascospores (12).

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TABLE 1. Random amplified polymorphic DNA (RAPD) groups of isolates of *Didymella bryoniae* and *Phoma* used in this study

Isolate ^a	Host	Geographic origin ^b
RAPD group I		
<i>D. bryoniae</i> C61	Cucumber	South Carolina
<i>D. bryoniae</i> C62^c	Cucumber	South Carolina
<i>D. bryoniae</i> C64	Cucumber	South Carolina
<i>D. bryoniae</i> C66	Cucumber	South Carolina
<i>D. bryoniae</i> C68	Cucumber	South Carolina
<i>D. bryoniae</i> C69	Cucumber	South Carolina
<i>D. bryoniae</i> C70	Cucumber	South Carolina
<i>D. bryoniae</i> C72	Cucumber	South Carolina
<i>D. bryoniae</i> NY6	Cucumber	New York
<i>D. bryoniae</i> C45	Muskmelon	South Carolina
<i>D. bryoniae</i> C46	Muskmelon	South Carolina
<i>D. bryoniae</i> C48	Muskmelon	South Carolina
<i>D. bryoniae</i> C49	Muskmelon	South Carolina
<i>D. bryoniae</i> C51	Muskmelon	South Carolina
<i>D. bryoniae</i> C53	Muskmelon	South Carolina
<i>D. bryoniae</i> C76	Muskmelon	South Carolina
<i>D. bryoniae</i> C79	Muskmelon	South Carolina
<i>D. bryoniae</i> C81	Muskmelon	South Carolina
<i>D. bryoniae</i> C82	Muskmelon	South Carolina
<i>D. bryoniae</i> FL1	Muskmelon	Florida
<i>D. bryoniae</i> NY1	Muskmelon	New York (4)
<i>D. bryoniae</i> NY2	Muskmelon	New York
<i>D. bryoniae</i> NY3	Muskmelon	New York (7)
<i>D. bryoniae</i> WI1	Muskmelon	Wisconsin
<i>D. bryoniae</i> FL2	Watermelon	Florida
<i>D. bryoniae</i> NY4	Watermelon	New York
<i>D. bryoniae</i> NY5	Watermelon	New York (7)
<i>D. bryoniae</i> W73	Watermelon	South Carolina
<i>D. bryoniae</i> W74	Watermelon	South Carolina
<i>D. bryoniae</i> W95	Watermelon	South Carolina
<i>D. bryoniae</i> W99	Watermelon	South Carolina
<i>D. bryoniae</i> W100	Watermelon	South Carolina
<i>D. bryoniae</i> W101	Watermelon	South Carolina
<i>D. bryoniae</i> W103	Watermelon	South Carolina
RAPD group II		
<i>D. bryoniae</i> NY8	Butternut squash	New York (2)
<i>D. bryoniae</i> NY9	Butternut squash	New York (3)
<i>D. bryoniae</i> NY15	Butternut squash	New York (7)
<i>D. bryoniae</i> NY16	Butternut squash	New York (7)
<i>D. bryoniae</i> NY10	Cucumber	New York (7)
<i>D. bryoniae</i> NY11	Cucumber	New York (7)
<i>D. bryoniae</i> AZ1	Muskmelon	Arizona
<i>D. bryoniae</i> NY13	Muskmelon	New York (7)
<i>D. bryoniae</i> NY14	Muskmelon	New York (7)
<i>D. bryoniae</i> NY7	Pumpkin	New York (1)
<i>D. bryoniae</i> NY12	Pumpkin	New York (5)
<i>D. bryoniae</i> NY17	Pumpkin	New York (7)
RAPD group III		
<i>Phoma</i> sp. NY18	Butternut squash	New York (3)
<i>Phoma</i> sp. C63	Cucumber	South Carolina
<i>Phoma</i> sp. C65	Cucumber	South Carolina
<i>Phoma</i> sp. C67	Cucumber	South Carolina
<i>Phoma</i> sp. C73	Cucumber	South Carolina
<i>Phoma</i> sp. C75	Cucumber	South Carolina
<i>Phoma</i> sp. NC1	Cucumber	North Carolina (8)
<i>Phoma</i> sp. NC2	Cucumber	North Carolina (9)
<i>Phoma</i> sp. NY19	Cucumber	New York (1)
<i>Phoma</i> sp. C55	Muskmelon	South Carolina
<i>Phoma</i> sp. NY20	Muskmelon	New York (6)
<i>Phoma</i> sp. NY21	Muskmelon	New York (4)
<i>Phoma</i> sp. W97	Watermelon	South Carolina
RAPD group IV		
<i>D. bryoniae</i> NY22	Muskmelon	New York (7)
RAPD group not tested^c		
<i>P. medicaginis</i> ATCC 64481	Unknown	ATCC
<i>P. exigua</i> ATCC 14728	Unknown	ATCC

^a Isolates in bold type were used for sequencing ITS-1 and ITS-2 regions.

^b The Florida isolates were from Collier County. The New York isolates were from (1) Albany, (2) Montgomery, (3) Oneida, (4) Onondaga, (5) Ontario, (6) Schoharie, and (7) Tompkins counties. The North Carolina isolates were from (8) Clinton and (9) Duplin counties. The South Carolina isolates were from Charleston County.

^c Isolates not included in RAPD analysis.

Genomic DNA isolation. Mycelium of all isolates grown on QPDA in the dark at 23°C was scraped from agar plates, added to sucrose-casein-salts liquid medium (15), and grown as described previously (12). Cultures were decanted through cheesecloth, and mycelium was washed with water. After as much water as possible was squeezed out, 1.0 g of mycelium was frozen in liquid nitrogen before being stored at -80°C. DNA was extracted according to a standard protocol using 5 ml of lysis buffer and corresponding amounts of other reagents (14). The DNA precipitate was spooled with a hooked glass rod, washed in 70% ethanol, and resolubilized in sterile deionized water. The DNA concentration was determined with a fluorometer (Hoefer TKO 100) and adjusted to 10 ng/μl.

RAPD assays. Commercial RAPD 10-mer oligonucleotide primers were purchased from Operon Technologies (Alameda, California). PCR amplification was performed in a Perkin-Elmer-Cetus thermal cycler (Norwalk, Connecticut) using 40 ng of genomic DNA, as described previously (12). All RAPD reactions were repeated at least once to verify reproducibility.

Fig. 1. Random amplified polymorphic DNA (RAPD) fingerprints of nine isolates of *Didymella bryoniae* from Florida, Wisconsin, and New York (lanes 1 to 9, RAPD group I), 12 isolates of *D. bryoniae* from Arizona and New York (lanes 11 to 22, RAPD group II), six isolates of *Phoma* spp. from North Carolina and New York (lanes 23 to 28, RAPD group III), and *D. bryoniae* NY22 (lane 29, RAPD group IV) amplified with Operon primer OPK-4. Lane 10 is a 100-bp marker.

Fig. 2. Random amplified polymorphic DNA (RAPD) fingerprints of isolates of *Phoma* spp. (lanes 1 to 7, RAPD group III) and *Didymella bryoniae* (lanes 9 to 25 and 27 to 33, RAPD group I) from South Carolina amplified with Operon primer OPK-4. Lanes 8, 26, and 34 are 100-bp markers.

tion on banding profiles from all primers used in this study was utilized to determine the genetic distances between isolates and to construct a dendrogram. For cluster analysis of RAPD data, owing to the difficulty in visually comparing band sizes from different gels, the South Carolina isolates were analyzed separately from the remaining isolates. However, isolates FL1, AZ1, and NC1, belonging to RAPD groups I, II, and III, respectively, were used to correlate results from the two groups of isolates. ITS sequences from individual isolates were analyzed by Geneworks (release 2.3, Intelligenetics, Mountain View, California), and a consensus sequence was obtained for each RAPD group. ITS-1 and ITS-2 consensus sequences were aligned for each RAPD group. RAPD and ITS data were analyzed by Phylogenetic Analysis Using Parsimony (PAUP, version 3.1.1, Smithsonian Institution, Washington, D.C.). Aligned ITS-1 and ITS-2 sequences were analyzed separately.

Pathogenicity tests. Three isolates chosen to represent RG I (FL1, NY3, and NY5), three from RG II (NY8, NY13, and NY14), and three from RG III (NY18, NY20, and NY21) plus the single isolate in RG IV (NY22), for a total of 10 isolates, were assayed for pathogenicity to 1-week-old seedlings of muskmelon (*Cucumis melo* cv. Classic), as described previously (12). Each test had a completely randomized design with three replicate pots, 10-cm in diameter, and three seedlings per pot for each isolate. Uninoculated control plants were sprayed with a sterile sucrose-

casein solution. Isolate W21, a *Phoma* sp. nonpathogenic to muskmelon, was included as a negative inoculation control treatment (12). The pathogenicity test was performed twice. The tests were conducted in a reach-in growth chamber maintained at 24°C. A humidifier was placed in the chamber to provide relative humidity above 90%. Disease severity was rated daily, from the day after inoculation to the sixth day after inoculation, on a scale of 1 (0% of leaf area diseased) to 12 (100% of leaf area diseased) (8). Once disease ratings were complete, diseased leaves were cultured on QPDA to reisolate the fungi with which the seedlings had been inoculated. Disease ratings were converted to percentage leaf surface area diseased using the midpoint percentage. Data were analyzed by the use of the general linear model of SAS (SAS Institute, Cary, NC), and means were compared with Fisher's protected least significant difference test.

RESULTS

RAPD analysis. Of the 38 primers from the Operon T and K kits that were initially screened, 36 primers (95%) gave polymorphic bands. For RAPD analysis, we used the 10 random primers that gave the greatest number of polymorphic bands. These 10 primers were OPK-1, OPK-4, OPK-8, OPK-9, OPT-01, OPT-07, OPT-12, OPT-13, OPT-14, and OPT-18; sequences can be obtained from Operon Technologies. On average, five to 10 RAPD

Fig. 4. Results of Phylogenetic Analysis Using Parsimony (PAUP) based on the random amplified polymorphic DNA (RAPD) fingerprints of isolates of *Didymella bryoniae* and *Phoma* from South Carolina. Reference isolates (in bold type) are NC1 in RAPD group (RG) III, FL1 in RG I, and AZ1 in RG II.

bands were generated for each of the 10 primers. Visual evaluation of RAPD profiles indicated that the 59 isolates examined could be placed in four RAPD groups. Thirty-three isolates identified as *D. bryoniae* on the basis of morphology had identical banding patterns and were placed in a distinct RAPD group, RG I (Figs. 1 and 2). RG I contained 24 isolates from South Carolina, six isolates from New York, two isolates from Florida, and one isolate from Wisconsin (Table 1). Eleven isolates from New York and one isolate from Arizona, also identified as *D. bryoniae*, had very similar banding patterns that were distinct from those of isolates in RG I, and they were placed in RG II. Thirteen isolates (seven from South Carolina, four from New York, and two from North Carolina) were placed in RG III. One isolate, NY22, always exhibited a RAPD profile unlike any others and was therefore placed in RG IV. These four distinct RAPD groups occurred with every one of the 10 primers; each group contained the same isolates, regardless of which primer was used. Isolates of *D. bryoniae* assigned to RG I, II, and IV could not be distinguished by colony morphology, production of pycnidia or pseudothecia, or conidial shape or septation.

Estimation of evolutionary distance using PAUP supported the assignment of isolates to the RAPD groups. Of the 59 isolates examined in this study, 33 *D. bryoniae* isolates clustered in RG I, 12 clustered in RG II, and 13 clustered in RG III (Figs. 3 and 4). *D. bryoniae* NY22 was the only isolate assigned to RG IV. Bootstrap analysis of the data supported the separation of *D. bryoniae* and *Phoma* isolates in the RAPD groups (bootstrap value 100%). All isolates in RG I, particularly the South Carolina isolates collected from one farm, were tightly grouped together, as indicated by the short branch lengths (Fig. 4). Isolates in RG II and RG III were less tightly grouped together.

ITS sequence analysis. The ITS-4 and ITS-5 primers uniformly amplified a fragment of approximately 500 bp from 35 representative isolates from RG I, RG II, RG III, and RG IV (Table 1). Sequence analysis revealed that the bands were 522 bp long in the RG III isolates, 526 bp in the RG II and RG IV isolates, and 527 bp in the RG I isolates. Alignment of the consensus sequences indicated that the ITS-1 region of RG I isolates (Gen-

Bank accession number AF495849), RG II isolates, and NY22 was 140 bp long, and, except at position 131, there was complete sequence identity (99.3% identical) (Fig. 5). At position 131, RG I had a purine (either an adenine or a guanine residue), whereas RG II and NY22 had an adenine residue only. The ITS-1 sequences of RG III isolates (137 bp), *P. exigua* ATCC 14728 (138 bp), and *P. medicaginis* ATCC 64481 (137 bp) were shorter, because of base deletions in the sequences. Among the ITS-1 sequences of RG I, RG II, and RG III isolates, NY22, *P. medicaginis* ATCC 64481, and *P. exigua* ATCC 14728, there were 14 base changes, which consisted of 11 substitutions and three deletions.

The ITS-2 sequence of RG I isolates (GenBank accession number AF495850) was 161 bp long, and the remaining sequences were either 159 bp (RG III isolates, *P. medicaginis* ATCC 64481, and *P. exigua* ATCC 14728) or 160 bp (RG II isolates and NY22), owing to base deletions (Fig. 6). Each isolate had a unique ITS-2 sequence, i.e., no ITS-2 sequences of any of the isolates were identical. RG I and RG II isolates had the closest sequence identity, except that the sequence from RG II isolates had a deletion at position 153 and was therefore shorter by one base. Among the RG I, RG II, and RG III isolates, NY22, *P. medicaginis* ATCC 64481, and *P. exigua* ATCC 14728, there were 12 base changes, which consisted of nine substitutions and three deletions.

Phylogenetic analysis of the ITS-1 sequences indicated that RG II isolates and NY22 were identical to RG I isolates (*D. bryoniae*) (Fig. 7). However, analysis of the ITS-2 sequences indicated that only RG I and RG II isolates were identical and that NY22 was very closely related (one "weighted" character different) (Fig. 8). Phylogenetic analysis of the ITS-1 and ITS-2 sequences indicated that the RG I, RG II, and RG III isolates and NY22 did not cluster

Fig. 5. Computer alignment of the internal transcribed spacer region ITS-1 consensus sequences. RG I = *Didymella bryoniae*, random amplified polymorphic DNA (RAPD) group I; RG II = *Didymella bryoniae*, RAPD group II; PE = *Phoma exigua* ATCC 14728; PM = *Phoma medicaginis* ATCC 64481; RG III = *Phoma*, RAPD group III. Dots indicate base similarity, and dashes indicate deletions.

Fig. 6. Computer alignment of the internal transcribed spacer region ITS-2 consensus sequences. RG I = *Didymella bryoniae*, random amplified polymorphic DNA (RAPD) group I; RG II = *Didymella bryoniae*, RAPD group II; PE = *Phoma exigua* ATCC 14728; PM = *Phoma medicaginis* ATCC 64481; RG III = *Phoma*, RAPD group III. Dots indicate base similarity, and dashes indicate deletions.

with either *P. exigua* or *P. medicaginis* (Figs. 7 and 8). Of the isolates in the four RAPD groups, those in RG III were most closely related to *P. medicaginis*. RG III isolates were separated from *P. medicaginis* ATCC 64481 by two and four base changes in the ITS-1 and ITS-2 dendrograms, respectively.

Pathogenicity. Of the 10 isolates tested for pathogenicity—three from each of RG I, RG II, and RG III plus one from RG IV (NY22)—only the three *D. bryoniae* RG I isolates produced severe disease on muskmelon leaves (Table 2). In two independent tests, the mean disease severity for the RG I *D. bryoniae* isolates was always greater than 65% (Table 2). The *Phoma* isolates in RG

III were nonpathogenic (0% disease). *Phoma* isolate W21 was also nonpathogenic. For the RG II isolates and NY22, disease severity ranged from 2.6 to 9%, which is significantly ($P = 0.05$) greater than that of the control treatment and treatment with the RG III isolates but significantly less than that of treatment with the RG I *D. bryoniae* isolates.

DISCUSSION

The purpose of this study was to elucidate the genetic relationship between *D. bryoniae* and related *Phoma* isolates that are often simultaneously isolated from plant material with symptoms of GSB. An earlier study showed the usefulness of RAPD analysis in detecting significant polymorphisms between *D. bryoniae* and *Phoma* spp. (12). The present study expands on the previous study by using RAPD analysis and ITS sequence analysis on a larger and more diverse collection of isolates to determine their phylogenetic relationships. RAPD analysis of the *D. bryoniae* and *Phoma* isolates used in this study subdivided the isolates into four unique sets of genetic fingerprints, RG I, RG II, RG III, and RG IV. The *D. bryoniae* isolates in RG I could easily be identified by comparing their profiles with those observed in a previous study (12). All of the remaining isolates except one exhibited two different yet distinct RAPD fingerprints, RG II and RG III. All of the RG II isolates except one originated from New York. In a previous study, two of three isolates from New York had a RAPD profile different from other *D. bryoniae* isolates (12). The present sampling of a larger collection of isolates from New York confirms the previous observation. It therefore appears that two genotypes of *D. bryoniae* may be associated with and isolated from plants exhibiting symptoms of GSB.

RG I isolates of *D. bryoniae* were pathogenic to muskmelon seedlings, with an average disease severity of 71%, whereas the RG III isolates were nonpathogenic (0% disease severity). This difference in pathogenicity confirms the results of our previous study using different isolates (12). The RG II isolates incited low levels of disease in seedlings, with a mean disease severity of 5%. This level of disease is lower than that recorded in another study with 20 different isolates also assigned to RG I, II, and III (36). However, in that study, RG II isolates were also intermediate in virulence between *D. bryoniae* RG I and *Phoma* isolates, not only

Fig. 7. Phylogenetic groupings of *Didymella bryoniae* and *Phoma* spp. based on internal transcribed spacer region ITS-1 sequences. RG = random amplified polymorphic DNA (RAPD) group.

Fig. 8. Phylogenetic groupings of *Didymella bryoniae* and *Phoma* spp. based on internal transcribed spacer region ITS-2 sequences. RD = random amplified polymorphic DNA (RAPD) group.

TABLE 2. Mean disease severity on muskmelon 6 days after inoculation with 11 isolates of *Didymella bryoniae* and *Phoma*

Isolate	RAPD group ^a	Disease severity ^b	
		Square root transformation	Back-transformed value (%)
<i>Phoma</i> sp. W21	...	0.12	0.1
<i>D. bryoniae</i> NY3	I	8.58	73.6
<i>D. bryoniae</i> NY5	I	8.52	72.6
<i>D. bryoniae</i> FL1	I	8.24	67.9
<i>D. bryoniae</i> NY13	II	2.97	8.8
<i>D. bryoniae</i> NY8	II	1.76	3.1
<i>D. bryoniae</i> NY14	II	1.60	3.0
<i>D. bryoniae</i> NY22	IV	1.72	2.6
<i>Phoma</i> sp. NY18	III	0.00	0.0
<i>Phoma</i> sp. NY20	III	0.00	0.0
<i>Phoma</i> sp. NY21	III	0.00	0.0
LSD _{0.05} ^d		1.42	

^a Random amplified polymorphic DNA group.
^b Mean percentage leaf surface area with symptoms of gummy stem blight, averaged across three replications in each of two experiments. For an uninoculated control, disease severity values were 0.00 (square root transformed) and 0.0% (back-transformed).
^c The RAPD group of this isolate was not determined in this study, but it is likely to be in group III (12).
^d Fisher's protected least significant difference on the square root transformed scale.

in muskmelon but also in cucumber, watermelon, and pumpkin. Segregation of the isolates into three virulence groups conforms identically to the occurrence of the three main RAPD groups. Therefore, RAPD analysis can be used to identify each virulence group. All isolates of *D. bryoniae* RG I, II and IV were pathogenic in muskmelon, regardless of their host of origin. This lack of host specificity agrees with that documented in previous studies (5, 12, 13, 36).

Isolate NY19 (representing RG III) was identified as *P. medicaginis* by G. Kinsey at the International Mycological Institute (personal communication). Isolate AZ-1 (an RG II isolate) and NY22 (the lone RG IV isolate) could not be positively identified, but potential names for both isolates included *P. exigua*, *P. cucurbitacearum*, and *P. lycopersici*. We therefore included *P. medicaginis* and *P. exigua* as reference cultures during the ITS sequence analysis. NY22 clustered very closely with *D. bryoniae*. However, the ITS sequences exhibited a significant number of base differences between RG III isolates and *P. medicaginis* ATCC 64481 and between RG II isolates and *P. exigua* ATCC 14728, suggesting that there was less sequence similarity between RG III isolates and *P. medicaginis* or between RG II isolates and *P. exigua*. Isolate NY19 has also been classified as *P. americana* (16, 21). In another phylogenetic study (21), which used only ITS-1 sequences, NY19 was distinguished from *D. bryoniae* by 10 base changes, similar to the 12 base changes we found using a consensus sequence for 10 *Phoma* isolates.

ITS sequence data indicated a closer relationship between RG I and RG II than RAPD data did. RAPD analysis includes multiple loci throughout the entire genome and generates multilocus data (31, 34). RAPD analysis is capable of distinguishing closely related fungal isolates, such as leaf- and stem-infecting forms of rust fungi on willow (19). The entire genome is subject to faster evolutionary changes than the highly conserved ITS regions, which only evolve slowly (2). Phylogenetic analysis derived from ITS-1 and ITS-2 sequence data grouped *D. bryoniae* isolates in RG I and RG II into a single, common phylogenetic cluster; thus, the two RAPD groups could not be distinguished from each other using these criteria. RG III isolates, on the other hand, clustered separately from *D. bryoniae* isolates in RG I and RG II. This suggests that, phylogenetically, RG III isolates are distantly related to *D. bryoniae* RG I isolates, while RG II isolates are very closely related to *D. bryoniae* RG I isolates. However, close examination of RG II showed that, although the RAPD banding profiles of these isolates were unique, the profiles often shared many common bands with *D. bryoniae* RG I and *Phoma* RG III isolates. Parsimony analysis of RAPD data indicated that the evolutionary distance between RG I and RG II was similar to that between RG III and RG II, since branch lengths were almost equal. The simplest explanation for this is that RG I and RG II are genetic subgroups within *D. bryoniae* that are diverging. Because of the long history of cucurbit cultivation in the eastern United States (22), it is possible that the *D. bryoniae* population in New York is diverging from the *D. bryoniae* population in other parts of the United States. A similar hypothesis of divergence from a common ancestor was proposed to explain phylogenetic relationships among the wheat bunt fungi (25).

On the evidence of several results, it is clear that *D. bryoniae* RG I, *D. bryoniae* RG II, and *Phoma* RG III represent distinct molecular subgroups. This study identified sequence differences between *D. bryoniae* and *Phoma* in the ITS-1 and ITS-2 regions. Further, ITS-1 and ITS-2 sequencing and phylogenetic analyses revealed that *D. bryoniae* RG II isolates shared greater than 99% sequence similarity with *D. bryoniae* RG I isolates. RAPD profiles, pathogenicity, and virulence data from the current study supported the results obtained in previous studies (12, 36). We therefore conclude that RG I and RG II isolates are both *D. bryoniae*, but that they represent two different molecular and virulence subgroups.

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Bruns, T. D., White, T. J., and Taylor, J. W. 1991. Fungal molecular systematics. *Annu. Rev. Ecol. Syst.* 22:525-564.
- Bryan, G. T., Daniels, M. J., and Osbourn, A. E. 1995. Comparison of fungi within the *Gaeumannomyces-Phialophora* complex by analysis of ribosomal DNA sequences. *Appl. Environ. Microbiol.* 61:681-689.
- Chiu, W. F., and Walker, J. C. 1949. Morphology and variability of the cucurbit black rot fungus. *J. Agric. Res.* 78:81-102.
- Chiu, W. F., and Walker, J. C. 1949. Physiology and pathogenicity of the cucurbit black-rot fungus. *J. Agric. Res.* 78:589-615.
- Corlett, M. 1981. A taxonomic survey of some species of *Didymella* and *Didymella*-like species. *Can. J. Bot.* 59:2016-2042.
- Farr, D. F., Bills, G. F., Chamuris, G. P., and Rossman, A. Y. 1989. *Fungi on Plants and Plant Products in the United States*. American Phytopathological Society, St. Paul, MN.
- Horsfall, J. G., and Barratt, R. W. 1945. An improved system for measuring plant diseases. (Abstr.) *Phytopathology* 35:655.
- Hseu, R. S., Wang, H. H., Wang, H. F., and Moncalvo, J. M. 1996. Differentiation and grouping of isolates of the *Ganoderma lucidum* complex by random amplified polymorphic DNA-PCR compared with grouping on the basis of internal transcribed spacer sequences. *Appl. Environ. Microbiol.* 62:1354-1363.
- Jenkins, S. F., Jr., and Person, L. H. 1973. Ascochyta blight of cucurbits caused by *Ascochyta phaseolorum*. (Abstr.) *Phytopathology* 63:444.
- Kaiser, W. J., Wang, B.-C., and Rogers, J. D. 1997. *Ascochyta fabae* and *A. lentis*: Host specificity, teleomorphs (*Didymella*), hybrid analysis, and taxonomic status. *Plant Dis.* 81:809-816.
- Keinath, A. P., Farnham, M. W., and Zitter, T. A. 1995. Morphological, pathological and genetic differentiation of *Didymella bryoniae* and *Phoma* spp. isolated from cucurbits. *Phytopathology* 85:364-369.
- Lee, D.-H., Mathur, S. B., and Neergard, P. 1984. Detection and location of seed-borne inoculum of *Didymella bryoniae* and its transmission in seedlings of cucumber and pumpkin. *Phytopathol. Z.* 109:301-308.
- Lee, S. B., Milgroom, M. G., and Taylor, J. W. 1988. A rapid, high yield mini-prep method for isolation of total genomic DNA from fungi. *Fungal Genet. Newsl.* 35:23-24.
- Maiero, M., Bean, G. A., and Ng, T. J. 1991. Toxin production by *Alternaria solani* and its related phytotoxicity to tomato breeding lines. *Phytopathology* 81:1030-1033.
- Morgan-Jones, G., and White, J. F. 1983. Studies in the genus *Phoma*. I. *Phoma americana* sp. nov. *Mycotaxon* 16:403-413.
- Norton, J. D., and Cosper, R. D. 1989. AC-70-154, a gummy stem blight resistant muskmelon breeding line. *HortScience* 24:709-711.
- Norton, J. D., Cosper, R. D., Smith, D. A., and Rymal, K. S. 1986. AU-Jubilant and AU-Producer watermelons. *HortScience* 21:1460-1461.
- Pei, M. H., Whelan, M. J., Halford, N. G., and Royle, D. J. 1997. Distinction between stem- and leaf-infecting forms of *Melampsora* rust on *Salix viminalis* using RAPD markers. *Mycol. Res.* 101:7-10.
- Punithalingam, E., and Holliday, P. 1972. *Didymella bryoniae*. No. 332 in: *Descriptions of Pathogenic Fungi and Bacteria*. Commonw. Mycol. Inst., Kew, England.
- Reddy, P. V., Patel, R., White, J. F., Jr. 1998. Phylogenetic and developmental evidence supporting reclassification of cruciferous pathogens *Phoma lingam* and *Phoma wasabiae* in *Plenodomus*. *Can. J. Bot.* 76: 1916-1922.
- Robinson, R. W., and Decker-Walters, D. S. 1997. *Cucurbits*. CAB International, Wallingford, UK.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schenck, N. C. 1968. Epidemiology of gummy stem blight (*Mycosphaerella citrullina*) on watermelon: Ascospore incidence and disease development. *Phytopathology* 58:1420-1422.
- Shi, Y. L., Loomis, P., Christian, D., Carris, L. M., and Leung, H. 1996. Analysis of the genetic relationships among the wheat bunt fungi using RAPD and ribosomal DNA markers. *Phytopathology* 86:311-318.
- Sitterly, W. R. 1972. Breeding for disease resistance in cucurbits. *Annu. Rev. Phytopathol.* 10:471-490.
- Sitterly, W. R., and Keinath, A. P. 1996. Gummy stem blight. Pages 27-28 in: *Compendium of Cucurbit Diseases*. T. A. Zitter, D. L. Hopkins, and C. E. Thomas, eds. American Phytopathological Society, St. Paul, MN.
- St. Amand, P. C., and Wehner, T. C. 1995. Eight isolates of *Didymella bryoniae* from geographically diverse areas exhibit variation in virulence

- but no isolate by cultivar interaction on *Cucumis sativus*. Plant Dis. 79:136-1139.
29. Sumner, D. R., and Phatak, S. C. 1987. Control of foliar diseases of cucumber with resistant cultivars and fungicides. Appl. Agric. Res. 2:324-329.
 30. Wehner, T. C., and St. Amand, P. C. 1993. Field tests for cucumber resistance to gummy stem blight in North Carolina. HortScience 28:327-329.
 31. Welsh, J., and McClelland, M. 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res. 18:7213-7218.
 32. White, T. J., Bruns, T., Lee, S., and Taylor, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pages 315-322 in: PCR Protocols: A Guide to Methods and Applications. M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White, eds. Academic Press, San Diego, CA.
 33. Wiant, J. S. 1945. Mycosphaerella black rot of cucurbits. J. Agric. Res. 71:193-213.
 34. Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., and Tingey, S. V. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.
 35. Zhang, A. W., Hartman, G. L., Riccioni, L., Chen, W. D., Ma, R. Z., and Pedersen, W. L. 1997. Using PCR to distinguish *Diaporthe phaseolorum* and *Phomopsis longicolla* from other soybean fungal pathogens and to detect them in soybean tissues. Plant Dis. 81:1143-1149.
 36. Zuniga, T. L. 1999. Gummy stem blight (*Didymella bryoniae*) of cucurbits: Pathogen characterization and inheritance of resistance in melon (*Cucumis melo*). Ph.D. dissertation, Cornell University, Ithaca, NY.