AFLP analysis of a worldwide collection of *Didymella bryoniae*

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*Didymella bryoniae* (anamorph *Phoma cucurbitacearum*) is an ascomycete that causes gummy stem blight, a foliar disease that occurs on cucurbits in greenhouses and fields throughout the world. In a previous study using RAPD analysis, little genetic diversity was found among isolates of *D. bryoniae* from New York and South Carolina, USA. Here we report the use of amplified fragment length polymorphism (AFLP) analysis to assess the genetic variation within a worldwide collection of *D. bryoniae*. 102 field and greenhouse isolates from ten states in the USA (California, Delaware, Florida, Georgia, Indiana, Maryland, Michigan, Oklahoma, South Carolina, and Texas) and seven other countries (Australia, Canada, China, Greece, Israel, Sweden, and The Netherlands) were examined. Seven different AFLP primer-pair combinations generated 450 bands, of which 134 were polymorphic (30%). Using cluster analysis, two groups and a total of seven subgroups were delineated. Representative isolates varied in their virulence on muskmelon and watermelon seedlings, but the degree of virulence was not strongly associated with AFLP groupings. However, isolates from the northern USA grouped separately from isolates originating from the southern USA.

INTRODUCTION

The ascomycete *Didymella bryoniae* (anamorph *Phoma cucurbitacearum*) is a necrotrophic pathogen of cucurbits (*Cucumis*, *Citrullus*, *Cucurbita*, and other genera). It causes the diseases gummy stem blight and black rot, which are most devastating in warm, humid climates (Bala & Hosein 1986, McGrath, Vawdrey & Walker 1993, Sitterly & Keinath 1996) and greenhouses (Arny & Rowe 1991, van Steekelenburg 1982) worldwide. In the USA, *D. bryoniae* is prevalent in the southern states (Wiant 1945, Schenck 1968, Keinath & Duthie 1998) but also is found in northern areas (Chiu & Walker 1949, Zuniga 1999).

Previously, RAPD profiles were used to compare isolates of *D. bryoniae* from South Carolina, Florida and New York (Keinath, Farnham & Zitter 1995). The RAPD profiles differentiated *D. bryoniae* from *Phoma medicaginis* (also named as *P. americana*; Reddy, Patel & White 1998), a weak parasite on cucurbits (Keinath et al. 1995, Zuniga 1999), but there was little diversity among isolates of *D. bryoniae* collected from the eastern USA. More recently, RAPD profiles from additional isolates and sequence analysis of the ITS-1 and -2 regions of the ribosomal DNA of *D. bryoniae* demonstrated the existence of two molecular and virulence subgroups, designated RAPD Group (RG) I and II (Somai et al. 2002b). To date, RG I is more widely distributed within the USA than RG II is.

Amplified fragment length polymorphism (AFLP) analysis has been used to successfully estimate the amount of genetic variation within fungal species (Vos et al. 1995). Pongam, Osborn & Williams (1999) used AFLP to analyse the genetic variability of *Leptosphaeria maculans*, the causal agent of blackleg on crucifers. They found evidence to support the hypothesis that the disease was introduced into North Dakota from Western Canada. Majer, Lewis & Mithen (1998) used AFLP analysis to determine genetic diversity and population structure of *Pyrenopeziza brassicae*, the causal agent of light leaf spot of *Brassica* spp. Gonzalez et al. (1998) compared AFLP analysis and RAPD
analysis on 59 isolates of *Colletotrichum lindemuthianum*. Based on the AFLP data, isolates were found to group according to host cultivar, type of cultivation, and geographic location. To date, AFLP analysis has not been utilized to estimate genetic diversity in *D. bryoniae*.

The objective of this study was to compare the genetic similarity of *D. bryoniae* isolates originating from commercial cucurbit fields and greenhouses in different geographic locations within and outside the USA.

## MATERIALS AND METHODS

### Collection and preparation of isolates

102 isolates of *Didymella bryoniae* were selected from a larger set (170) obtained between 1997 and 1999. Isolates were recovered originally from individual leaves, stems or fruits. Isolates generally originated from single or contiguous fields, different counties within a state or from greenhouses (Table 1).

Isolates already in culture were recultured by removing a 1-cm$^2$ agar plug from the culture and placing it in a Petri dish containing quarter-strength potato dextrose agar (QPDA). Isolates obtained from host tissues were prepared by removing a 1 cm$^2$ piece of tissue, disinfesting with 70% ethanol for 2 min, briefly submerging in 10% sodium hypochlorite, rinsing with sterile, deionized water and then placing the tissue into Petri dishes containing QPDA. *D. bryoniae* growing from tissue pieces was transferred to fresh QPDA. Permanent filter paper cultures were revived by placing a piece of filter paper containing conidia on QPDA plates. Permanent lyophilized cultures were restored in accordance with the American Type Culture Collection (ATCC) protocol.

All cultures were grown at 23 °C with a 12 h photoperiod for 10–15 d under white fluorescent light to induce formation of pycnidia. Three millilitres of water were added to plates and conidia dislodged using a glass rod. One hundred microlitres of a suspension of 10$^4$ conidia ml$^{-1}$ was spread onto water agar plates and placed under fluorescent light. After 18–20 h, single germinating conidia were removed, transferred to QPDA plates and incubated at 23 °C under fluorescent light for 5 d. A 1 cm$^2$ agar plug was taken from each plate and placed into a separate 125 ml Erlenmeyer flask containing 50 ml of liquid medium designed for culture of *Alternaria solani* (Maiero, Bean & Ng 1991). Flasks were shaken in the dark at 23 °C at 75–100 rpm for 4–5 d.

### DNA extraction

Mycelia (10–15 g) and liquid media were separated by pouring the culture through a funnel lined with Mira Cloth® (Calbiochem, San Diego). The mycelia were then rinsed twice with distilled water, dried between paper towels, placed into a mortar and submerged in liquid nitrogen. The tissue was ground into a fine powder and extracted using a Qiagen® DNeasy Plant Mini kit (Qiagen, Santa Clarita, CA). DNA was eluted and stored in TE buffer. The concentration of the extracted DNA was determined with a Hoefer TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco).

### Specific PCR primers

Three species-specific PCR primers, developed previously in our laboratory (Somai, Keinath & Dean 2002a), were used to confirm the identity of all fungal isolates. Each of the three primers amplify a unique band within the genomes of either *Didymella bryoniae* RG I, *Phoma medicaginis*, or *D. bryoniae* RG II.

### AFLP procedure

The AFLP procedure was performed as described by Vos et al. (1995) with a few modifications. The AFLP Analysis System II (Small Genome) Kit (Life Technology, Gaithersburg, MD) was the source of reagents. *Didymella bryoniae* DNA (300 ng) was double digested with 1 µl *Eco*RI/*Mse*I restriction enzyme mix (3.2 units each) and 2.6 µl 5 × reaction buffer with the final volume brought up to 14 µl with sterile water. The reaction was incubated at 37 °C for 2 h. Adapters were then ligated onto the restricted fragments and used as priming sites for the subsequent PCR. For this ligation, a 12.5-µl adapter/ligation mixture was added directly to the completed restriction digest reaction. The ligation reaction mixture contained 5 pmol *Eco*RI and 50 pmol *Mse*I adapters; 0.5 unit T4 DNA-ligase (Life Technology, Gaithersburg, MD); 0.4 mM ATP; 10 mM Tris-HCl, pH 7.5; 10 mM MgAc; and 50 mM KAc. The ligation reaction was incubated at 20 °C for 2 h and then diluted five-fold with TE buffer.

The pre-amplification reaction was performed using the restricted fragments with ligated adapters as template. The *Eco*RI primer used was fully complimentary to the *Eco*RI adapter and contained no selective bases. The *Mse*I primer was fully complimentary to its adapter, but contained one selective nucleotide (cytosine). The PCR 20-µl mix contained 2 µl template DNA,

<table>
<thead>
<tr>
<th>Host Field Greenhouse</th>
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</thead>
<tbody>
<tr>
<td>Watermelon (Citrus lanatus)</td>
</tr>
<tr>
<td>Muskmelon (Cucumis melo)</td>
</tr>
<tr>
<td>Cucumber (Cucumis sativus)</td>
</tr>
<tr>
<td>Gourd (Cucurbita pepo)</td>
</tr>
<tr>
<td>Winter squash (Cucurbita spp.)</td>
</tr>
<tr>
<td>Total</td>
</tr>
</tbody>
</table>

Isolates were kindly provided by B. Bruton, D. Egel, K. Everts, S. Freeman, T. Isakeit, S. Koike, T. Kucharek, H. Martin, K. Mayberry, K. Ng, D. Sumner, and J. Vakalounakis. Detailed information about individual isolates is available from A.P.K.; representative isolates are preserved in ATCC.
0.4 unit Tag polymerase (PerkinElmer, Norwalk, CT), 200 mM Tris-HCl, 15 mM MgCl₂, 500 mM KCl and 16 µl of pre-amplification primer mix (0.25 mM of each dNTP, 16.5 pmol of MseI primer and sterile water). The following conditions were used for PCR: 94 °C for 30 s, 56 °C for 60 s, and 72 °C for 60 s for 20 cycles. All pre-amplification reactions were run on a PerkinElmer 480 thermal cycler and overlaid with mineral oil. The amplification product was diluted 20-fold with TE.

The selective amplification used the diluted (1:20) pre-amplification product as template. The primers used for this amplification step were more selective than the primers of the pre-amplification. The EcoRI primer was complimentary to the adapter sequence with the exception of the addition of two selective nucleotides. The chosen EcoRI primer was end-labelled with a radioactive isotope (γ-³²P ATP) to aid in the analysis. The end-labelling reaction mixture contained: 135 ng EcoRI primer, 5 units T4 kinase, 2.8 µl 5 x kinase buffer, 2.9 µl γ-³²P and was brought up to 14 µl with sterile water. The end-labelling reaction was incubated at 37 °C for 1 h. The MseI primer was also complimentary to the adapter sequence with the addition of either one or two selective nucleotides. A total of seven primer pair combinations were used for this study (Table 2).

<table>
<thead>
<tr>
<th>Primer-pairings</th>
<th>Sequences</th>
<th>Polymorphisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>MseI + C and EcoRI + AG</td>
<td>5'-GATGAGTCCTGAGTAA-C-3' + 5'-GACTGCGTACCAAATTC-AG-3'</td>
<td>27</td>
</tr>
<tr>
<td>MseI + C and EcoRI + TG</td>
<td>5'-GATGAGTCCTGAGTAA-C-3'</td>
<td>18</td>
</tr>
<tr>
<td>MseI + C and EcoRI + TT</td>
<td>5'-GATGAGTCCTGAGTAA-C-3' + 5'-GACTGCGTACCAAATTC-TG-3'</td>
<td>28</td>
</tr>
<tr>
<td>MseI + C and EcoRI + AC</td>
<td>5'-GATGAGTCCTGAGTAA-C-3'</td>
<td>20</td>
</tr>
<tr>
<td>MseI + C and EcoRI + TA</td>
<td>5'-GATGAGTCCTGAGTAA-C-3' + 5'-GACTGCGTACCAAATTC-AC-3'</td>
<td>17</td>
</tr>
<tr>
<td>MseI + C and EcoRI + TC</td>
<td>5'-GATGAGTCCTGAGTAA-C-3'</td>
<td>20</td>
</tr>
<tr>
<td>MseI + CA and EcoRI + TC</td>
<td>5'-GATGAGTCCTGAGTAA-CA-3' + 5'-GACTGCGTACCAAATTC-TC-3'</td>
<td>4</td>
</tr>
</tbody>
</table>

Data analysis

DNA fragments visualized on film were recorded in order according to their molecular weight. The highest molecular weight band-row was labelled one, with the rest following in order until the lowest molecular weight band-row was recorded. Each polymorphic AFLP marker was scored as present (1) or absent (0) across all isolates in a given row. Markers that could not be clearly scored were excluded from the analysis. To verify the reproducibility of the AFLP protocol, AFLP reactions were repeated with DNA extracted a second time from 10% of the isolates. These samples did not differ in their banding pattern from the original samples (data not shown).

The genetic similarities among isolates were examined using cluster analysis and the NTSYSpc 2.0 (Numerical Taxonomy System) software package (Rohlf 1993). The resemblance matrix used for the cluster analysis was created based on simple matching and Dice similarity coefficients using the SIMQUAL program. The SAHN clustering program was used to create dendrograms with UPGMA (Pongram et al. 1999). The TREE program was used to plot dendrograms.

Pathogenicity tests

Watermelon (Citrullus lanatus cv. ‘Jubilee II’) and muskmelon (Cucumis melo cv. ‘Classic’) were seeded in 60% vermiculite–40% peat potting mix. After 1 wk, three seedlings were transplanted per 10-cm plastic pot. Eight isolates of Didymella bryoniae were selected to represent six of the seven AFLP subgroups observed in this study (Table 3). Three isolates were chosen from

Table 2. Primer-pairings, sequences and number of polymorphisms found using seven AFLP primer pair combinations.
I-a, the largest subgroup, and one isolate was chosen from each of subgroups I-b, I-c, I-d, II-b, and II-c. Isolates were grown on QPDA plates for 2–3 wk at 23–26 °C and a 12 h photoperiod. Cultures were flooded with a 0.1% sucrose–0.05% casein solution (Bergstrom, Knavel & Kuc 1982) and gently scraped. In tests one and two, 10 ml of mycelial suspension was diluted in 90 ml sucrose–casein solution. In test 3, a suspension of 2 × 10⁵ conidia ml⁻¹ was used (also prepared with sucrose–casein solution). Two-wk-old plants were inoculated by spraying ca. 2 ml mycelial or conidial suspension per pot. Control (non-inoculated) plants were sprayed with sterile sucrose–casein solution. Plants were held in a mist chamber at 90–100% RH for 3 d to promote infection and lesion expansion (Keinath et al. 1995). The individual tests were randomized complete block designs with four replicate pots for each host and isolate arranged with two blocks in each of two mist chambers. The experiment was performed three times. Disease severity on individual plants was rated 4–5 d after inoculation on a scale of 1 (0% leaf area diseased) to 12 (100% leaf area diseased) (Horsfall & Barratt 1945). Diseased leaf tissue from selected plants was cultured on QPDA to reisolate *Didymella bryoniae* RG I using the primer pairs. There were 11 isolates of *Phoma* sp. and 12 isolates of *D. bryoniae* RG II. The 30 isolates that did not react with any of the three primer pairs were grown on QPDA plates and examined morphologically. Thirteen of these isolates (G1, G2, G4 and all 10 isolates from Canada) appeared identical to *D. bryoniae* (Corlett 1981, Keinath et al. 1995) and so were included in the AFLP analyses. Twenty-eight of these 130 isolates of *D. bryoniae* were not used in the AFLP analyses because their places of origin already were represented by at least 9 isolates.

### AFLP analysis

450 bands were amplified with the seven AFLP primer pairs and 134 (30%) were polymorphic. The number of amplified fragments ranged from 40 to 86 per gel. On average, 19 polymorphic markers were detected per primer pair combination. Fig. 1 is an example of a gel illustrating typical polymorphic bands.

The 102 isolates analyzed separated into two groups based on UPGMA and the simple matching coefficient (Fig. 2). Group I contained 83 isolates from Australia, China, Delaware, Florida, Georgia, Greece, Israel, Maryland, Oklahoma, South Carolina, Sweden, Texas, The Netherlands, four of the California isolates from one collection (CA-Q), and one of the Indiana collections (IN-T). Group II contained all isolates from Canada, Michigan, and second Indiana collection (IN-U), and the remaining six isolates from California. Group I was further separated into four subgroups based on an 82% similarity level. Subgroup I-a contained isolates from China, Florida, Israel, Oklahoma, South Carolina, Sweden, Texas, The Netherlands, four of the Georgia isolates, and one isolate from Greece (GR-V3). The two isolates from China (CH-1 and -2) were the isolates most distant from the rest of the isolates in this subgroup (87% similarity level). Subgroup

<table>
<thead>
<tr>
<th>Isolate</th>
<th>AFLP group</th>
<th>Geographic origin</th>
<th>Location</th>
<th>Host of origin</th>
<th>Disease severity (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q2</td>
<td>I c</td>
<td>California</td>
<td>Greenhouse</td>
<td>M</td>
<td>28.3, 17.4, 22.5 abc</td>
</tr>
<tr>
<td>Y34</td>
<td>I d</td>
<td>Australia</td>
<td>Field</td>
<td>M</td>
<td>41.8, 8.5, 22.0 a</td>
</tr>
<tr>
<td>D4</td>
<td>I a</td>
<td>Texas</td>
<td>Field</td>
<td>M</td>
<td>17.9, 13.0, 15.3 ab</td>
</tr>
<tr>
<td>S3</td>
<td>I a</td>
<td>South Carolina</td>
<td>Greenhouse</td>
<td>W</td>
<td>20.5, 8.0, 13.5 abc</td>
</tr>
<tr>
<td>R2</td>
<td>I a</td>
<td>Taiwan</td>
<td>Greenhouse</td>
<td>W</td>
<td>12.8, 3.9, 7.7 bcd</td>
</tr>
<tr>
<td>X21</td>
<td>II b</td>
<td>Canada</td>
<td>Greenhouse</td>
<td>C</td>
<td>12.4, 2.0, 6.1 bcd</td>
</tr>
<tr>
<td>G4</td>
<td>II c</td>
<td>California</td>
<td>Greenhouse</td>
<td>W</td>
<td>9.4, 1.5, 4.6 cd</td>
</tr>
<tr>
<td>J6</td>
<td>I b</td>
<td>Delaware</td>
<td>Field</td>
<td>W</td>
<td>4.5, 2.3, 3.3 d</td>
</tr>
<tr>
<td>Non-inoculated control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.0, 0.0, 0.0 c</td>
</tr>
</tbody>
</table>

| Mean square error | – | – | – | – | 2.96, 1.18, 2.00 |

| a | W, watermelon; M, muskmelon; C, cucumber. |
| b | Mean percentage leaf surface area diseased, averaged over three tests. Means shown are back-transformed from square-root transformed means used for analysis. |
| c | Means followed by the same letter are not significantly different, Waller–Duncan k-ratio t test, k = 100 (approximates P = 0.05). |

### RESULTS

#### Efficacy of specific primers

170 isolates were screened using the three species-specific pairs of PCR primers. One hundred and seventeen isolates (69%) were positively identified as *Didymella bryoniae* RG I using the primer pairs. There were 11 isolates of *Phoma* sp. and 12 isolates of *D. bryoniae* RG II. The 30 isolates that did not react with any of the three primer pairs were grown on QPDA plates and examined morphologically. Thirteen of these isolates (G1, G2, G4 and all 10 isolates from Canada) appeared identical to *D. bryoniae* (Corlett 1981, Keinath et al. 1995) and so were included in the AFLP analyses. Twenty-eight of these 130 isolates of *D. bryoniae* were not used in the AFLP analyses because their places of origin already were represented by at least 9 isolates.
I-b contained isolates from Delaware, Maryland and six isolates from Indiana (IN-T). Subgroup I-c contained four isolates from California (CA-Q) and four isolates from Greece. Subgroup I-d contained all isolates from Australia. Isolate GA-F2, located between subgroups I-c and I-d, was not joined to any subgroup.

Group II was separated into three subgroups also based on an 82% similarity level. Subgroup II-a contained two isolates from Indiana (IN-U), one isolate from Michigan and a single isolate from California (Q3). This isolate from California was loosely joined to the group at a similarity level below 72%. Subgroup II-b contained all isolates from Canada. Subgroup II-c contained five isolates from California (CA-G). The five California isolates were joined to the Canadian isolates at a 78% similarity level.

**Pathogenicity tests**

All eight isolates of *Didymella bryoniae* tested were pathogenic on both watermelon and muskmelon. All non-inoculated control plants were symptomless in all three tests. There was no interaction between isolate and host. The cultivar of muskmelon used was more susceptible to gummy stem blight than the cultivar of watermelon used in this experiment. Isolates differed significantly in virulence, with isolate Q2 being the most virulent over all three tests and isolate J6 being the least virulent (Table 3). The mean disease severity of the six isolates from AFLP group I was significantly greater than the mean disease severity of the two isolates from group II (single-degree-of-freedom contrast, $P = 0.01$). The three isolates that originated from fields did not differ in virulence from the five isolates that originated from greenhouses. The five isolates that originated from watermelon did not differ in virulence from the three isolates that originated from muskmelon or cucumber.

**DISCUSSION**

The reason for the separation of *Didymella bryoniae* isolates into two AFLP groups was not obvious. The majority of isolates in group II (84%) were from greenhouses, which could be a possible reason for the separation of these isolates from group I. However, 43% of the isolates in group I also originated from greenhouses. Therefore, examining the data at the subgroup level provides more insight about the overall pattern.

Isolates clustered into subgroups based on two criteria, geographical origin and source (field or greenhouse). In addition, the field isolates from Florida (I-a), Georgia (I-a), South Carolina (I-a), Indiana (I-b), and Eastern Shore Maryland–Delaware (I-b) formed sub-subgroups within their respective subgroups. Subgroup I-d contained all seven Australian isolates. These isolates clustered within the subgroup by geographic location, with the isolates from Bowen (Y34, Y35) and Gumbilu (Y41) grouping closely together, followed by the isolates from Clare (Y28, Y29) and Tully (Y26), respectively. An exception to this clustering by geography was isolates from the southwestern USA (Texas and Oklahoma) that were sporadically distributed throughout subgroup I-a. In RAPD analysis of *D. rabiei* from chickpea, Navas-Cortez and Perez-Artes (1998) found some isolates from Spain and all from Pakistan clustered together in the same RAPD
group, whereas isolates from India and the USA were found in multiple RAPD groups. Subgroups I-a and I-b separated as a function of latitude within the United States. Subgroup I-a was the largest of all subgroups, containing 53 isolates. The majority (98%) of isolates from the southern USA, including Florida, Oklahoma, South Carolina, Texas, and Georgia (four isolates), was contained within

**Fig. 2.** Dendrogram of 102 isolates of *Didymella bryoniae* based on cluster analysis with the Unweighted Paired Group Method using Arithmetic means of Amplified Fragment Length Polymorphisms obtained with seven primer pairs. AU, Australia; CA, California; CH, China; CN, Canada; DE, Delaware; FL, Florida; GA, Georgia; GR, Greece; IL, Israel; IN, Indiana; MD, Maryland; MI, Michigan; NL, The Netherlands; OK, Oklahoma; SC, South Carolina; SW, Sweden; TX, Texas. Letters in isolate designations represent different collections or donors.
subgroup I-a. This grouping confirms the results obtained with a smaller collection of *D. bryoniae* isolates, where isolates from Florida and South Carolina appeared similar based on RAPD profiles (Keinath et al. 1995). Subgroup I-b contained isolates from the northern United States, including Delaware, Maryland, and Indiana (six isolates in collection IN-T). It was joined to subgroup I-a at a similarity coefficient of 83%. The isolates from Delaware and Maryland, all collected from the Delmarva Peninsula, were separated from the Indiana isolates at an 89% similarity. The clustering differences between I-a and I-b might be attributed to environmental conditions within the different regions. Isolates from northern states are subjected to longer, colder winters than isolates originating from the south (Chiu & Walker 1949). These different environmental conditions may act to apply different selective pressures on the fungus.

Isolates originating from the field tended to cluster separately from greenhouse isolates within a group. Within group I, greenhouse isolates from California (CA-Q) and Greece clustered separately into subgroup I-c, with California isolates being more loosely associated with each other than the isolates from Greece were. Within group I-a, field (SC-L) isolates from South Carolina clustered separately from greenhouse isolates (SC-S). In group II, greenhouse isolates from Canada and from a second greenhouse in California clustered to form subgroups II-b and II-c, respectively. Most (13 of 15) isolates within these two subgroups did not react with the *D. bryoniae*-specific PCR primer, but were included because their morphology was identical to *D. bryoniae*. Subgroup II-b was comprised of the Canadian isolates that originated from one grower’s three greenhouses. Isolates X16, X19, and X20, which formed a separate sub-subgroup, originated from the second greenhouse.

Although isolates from greenhouses tended to cluster separately from field isolates, greenhouse isolates did not cluster closer to one another than field isolates did. Seed contamination has been suspected as a contributor to the occurrence of gummy stem blight in greenhouses (Brown, Howard & Knight 1970, Koike 1997, Lee, Mathur & Neergaard 1984, Richardson 1979). Within greenhouses, less diversity might be expected because seeds from the same lot may carry similar or closely related isolates of the pathogen. However, such introduced isolates may play a role in increasing the genetic variation of isolates within a region. For example, the isolates from California greenhouse collection Q originated from seed grown in Thailand (Frank V. Sances, pers. comm.).

Another reason for the genetic similarity exhibited by many isolates may be that *D. bryoniae* is a homothallic fungus (Chiu & Walker 1949). One would expect a lack of sexual recombination within a region to lead to a low level of genetic diversity (McDonald 1997). Majer et al. (1998) suggested that in regions where high levels of genetic variation existed, sexual reproduction might be occurring. In this study, isolates generally clustered based on the regions from where they were isolated, with the exception of isolates from the southwestern USA. Isolates from this region tended to possess more variation than the other groups of isolates. This may be due to some low level of sexual reproduction occurring within these locations. Schenck (1968) found that pseudothecia were present in large stem and crown lesions on watermelon, as well as on tendrils and leaf petioles, which provides direct evidence for sexual reproduction occurring in the field. He also trapped ascospores above watermelon fields throughout the growing season. Therefore, ascospore production and dispersal could play a role in the development of variation within populations of this pathogen.

The number of selective nucleotides used in the AFLP analysis in this study was reduced from that of similar AFLP studies (Majer et al., 1998, Pongam et al. 1999). Using two selective nucleotides with both the *MseI* and *EcoRI* primers provided too few bands for analysis. To gain more fragments and potentially more polymorphic markers, a single nucleotide extension was used with the *MseI* primer along with a two-nucleotide extension for the *EcoRI* primer, to provide an optimum number of bands. An average of 21.7 polymorphic markers was detected per gel using six primer pair combinations. The seventh primer pair combination utilized two nucleotides for both the *MseI* primer and the *EcoRI* primer. Only four polymorphic markers were detected using this combination. The ratio of polymorphic markers to the number of bands scored was comparable, but not equal to, similar studies examining the genetic diversity of other ascomycetes using the AFLP technique. In one study, 49 isolates of *Leptosphaeria maculans* from eight different geographic locations were compared using AFLP analysis (Pongam et al. 1999). Six primer combinations generated 400 amplified fragments, of which 10% were polymorphic.

All eight isolates in the representative group of isolates tested were pathogenic on both watermelon and muskmelon with no differential virulence between hosts regardless of the host of origin, as reported previously (Chiu & Walker 1949, Keinath et al. 1995, Lee et al. 1984). Isolates from countries other than the USA were not more virulent than isolates from the USA, as reported by St Amand & Wehner (1995). Isolates differed among AFLP groups based on the mean disease severity they incited. However, it is not yet clear to what extent the differences in virulence of isolates contribute to the differences among AFLP groups.

In conclusion, AFLP analysis revealed more genetic variation within *D. bryoniae* than previously observed with RAPD analysis (Keinath et al. 1995). Genotypic differences were observed particularly among isolates collected from different geographical locations. However, because there was no differential virulence among selected isolates, it should not be necessary to screen...
REFERENCES


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