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Genetic Diversity of *Didymella bryoniae* for RAPD Profiles Substantiated by SCAR Marker in Korea

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Twenty isolates of *Didymella bryoniae* were isolated from infected cucurbit plants in various growing areas of southern Korea in 2001 and 2002. Random Amplified Polymorphic DNA (RAPD) group [RG] I of *D. bryoniae* was more virulent than RG IV to watermelon. Virulence of the RG I isolate was strong to moderate to cucumber, whereas that of the RG IV varied from strong, moderate to weak. Two hundred seventy-three amplified fragments were produced with 40 primers, and were analyzed by a cluster analysis using UPGMA method with an arithmetic average program of NTSYS-PC. At the distance level of 0.7, two major genomic DNA RAPD groups were differentiated among 20 isolates. The RG I included 7 isolates from watermelon and one isolate from melon, whereas the RG IV included 12 isolates from squash, cucumber, watermelon and melon. Amplification of internal transcribed spacer (ITS) region and small subunit rRNA region from the 20 isolates yielded respectively a single fragment. Restriction pattern with 12 restriction enzymes was identical for all isolates tested, suggesting that variation in the ITS and small subunit within the *D. bryoniae* were low. Amplification of the genomic DNAs of the tested isolates with the sequence characterized amplified regions (SCAR) primer RG IF-RG IR specific for RG I group resulted in a single band of 650bp fragment for 8 isolates out of the 20 isolates. Therefore, these 8 isolates could be assigned into RG I. The same experiments done with RG IIF-RG IIR resulted in no amplified PCR product for the 20 isolates tested. An about 1.4 kb-fragment amplified from the RG IV isolates was specifically hybridized with PCR fragments amplified from genomic DNAs of the RG IV isolates only, suggesting that this PCR product could be used for discriminating the RG IV isolates from the RG I isolates as well other fungal species.

Keywords : 18S rDNA, *Didymella bryoniae*, ITS, RAPD group, SCAR

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Didymella bryoniae (Auersw.) Rehm (anamorph: *Phoma cucurbitacearum* (Fr.:Fr.) Sacc.), which causes gummy stem blight (foliar) and black rot (fruit phase), of cucurbits, occurs throughout the southern Korea. Gummy stem blight (GSB) occurred more frequently in greenhouse culture than in field culture, in Korea, which it is most destructive disease of melon and watermelon (Kwon et al., 1997).

The amplification of specific regions of the fungal genome by polymerase chain reaction (PCR) may be a sensitive technique in detecting the length polymorphisms without preliminary work, such as isolation of cloned DNA probes, preparation of filters for Southern hybridization, or nucleotide sequencing necessary in RFLP technique (Keinath et al., 2001; Lévesque. C.A., 1995).

Recently, Random Amplified Polymorphic DNA (RAPD) technique has been applied to elucidate the genetic diversity of the genomic DNA of *Pyricularia oryzae* (Hong et al., 1996), *Xanthomonas campertris* pv. *vesicatoria* (Chung et al., 1997), and *Phomopsis citri* (Koh et al., 1998). This technique was used to generate specific, diagnostic DNA probe useful for identification and detec- tion of *Erwinia carotovora* subsp. *carotovora* (Kang et al., 1998), diagnosis of fastidious *Xylella fastidiosa* from asymtomatic pierce's disease of grape (Schaad et al., 2002), and also for quantification of biomass of *Magnaporthe grisea* by real-time PCR (Qi & Yang, 2002).

Keinath et al. (1995) attempted to distinguish *D. bryoniae* from *Phoma* spp, and Somai et al. (2002b) devloped RAPD technique to distinguish *D. bryoniae* from *Phoma* spp. with a microtiter-based PCR-enzyme linked immunosorbent assay (ELISA) technique.

The sequence characterized DNA markers have been developed for the PCR-based detection system as a specific primer to identify and differentiate target fungal species from others. Vandemark et al. (2000) and Larsen et al. (2002) investigated a PCR assay based on sequence characterized amplified regions (SCARs) for detecting the presence of *Aphanomyces euteiches* and *A. cochliodes* in

both the roots of peas grown in natural field soil and in naturally infested soil and identification of alfalfa brown rot pathogen, *Phoma sclerotioids*, respectively

The internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) evolve more quickly than the genes themselves and may vary among species within a genus (White et al., 1990), allowing the development of PCR primers that quickly amplify a given species. Many plant pathogens have been detected with PCR primers that are specific for the ITS regions. Hong et al. (1999) designed four PCR primers based on ITS sequences that could be used to unambiguously discriminate between *Phytophthora capsici* (Leonian), *P. cinnamomi* (Rands), *P. megakarya* (Brasier and Griffin), and *P. palmivora* (Butler).

The purpose of this study was to investigate the differentiation of genomic DNA profiles in *D. bryoniae* isolates, from the intensive cucurbits-growing areas in the southern Korea, using RAPD and RFLP of ITS regions of ribosomal DNA. We also analyzed the genomic DNA of *D. bryoniae* to develop the specific molecular marker for identifying of Gummy stem blight (GSB) isolates.

Materials and Methods

Fungal isolates. The sources and origins of the 20 isolates of *D. bryoniae* used in this study are shown in Table 1. Previously we reported that a total of 220 isolates was obtained in 2001 and 2002 from infected cucurbit plant growing in various locations of the southern Korea. Based on our observation on the symptoms and the morphological characteristics, the fungal species associated with the symptoms was identified as *D. bryoniae*, and confirmed to be pathogenic on cucurbit leaves (Fig. 1). Fungal isolation carried out on water agar (WA). Two isolates were provided by Department of Agricultural Biology, Chonnam National University, Gwangju, Korea. All of the *D. bryoniae* isolates were maintained on potato dextrose agar (PDA) (Difco Laboratories, USA) at 27°C, for long-term preservation, also isolates were grown on PDA in sealed tubes and stored at 4°C.

Virulence test. Watermelon (Wonsegae, Dongbu Hannong Chemical, Korea) and cucumber (Indong-chengjang, Joongang, Korea) were seeded in 60% vermiculite-40% peat (Tosili, Shinan growth, Korea). Two-week-old plants were inoculated by spraying approximately 2 ml of pycnidiospores suspension of *D. broyniae*. Inoculated plants were held in a mist chamber at 100% relative humidity for 3 days to promote infection and lesion expansion (Arny and Rowe, 1991).

Disease severity on individual plants was rated 5 days after inoculation on a scale of leaf blight degree; +++: strong (> 50% leaf area diseased), ++: moderate (26-50% leaf area diseased), +: weakly (1-10% leaf area diseased), – : healthy (0% leaf area diseased). Disease leaf tissue from one plant per replicate was cultured as described previously to reisolate pathogens. The individual tests were completely randomized designs with ten replicate pots for each host and isolate. Each tests included control sprayed with distilled water.

Table 1. Virulence of the 20 isolates of *Didymella bryoniae* isolated from infected cucurbit plant in various areas of southern Korea in 2001 and 2002

Isolates	Origin		Pathogenicity[b] to	
	Host	Region	Watermelon	Cucumber
Didymella bryoniae RG I[a]				
GS01-05	Watermelon	Hadong	+++	++
GS01-06	Watermelon	Hadong	+++	+++
GS01-07	Watermelon	Hamyang	+++	++
GS01-09	Oriental melon	Hamyang	+++	+++
DW96-88	Watermelon	Haman	+++	+++
DW96-123	Watermelon	Haman	+++	++
GS01-19	Watermelon	Jinju	+++	++
GS01-20	Watermelon	Jinju	+++	++
Didymella bryoniae RG IV				
GS01-01	Squash	Namhae	ND	ND
GS01-02	Cucumber	Jinju	++	+++
GS01-03	Squash	Uireyeong	+	+++
GS01-04	Oriental melon	Uireyeong	+	+
GS01-08	Watermelon	Hamyang	++	++
GS01-10	Watermelon	Hamyang	++	++
GS01-11	Cucumber	Jinju	++	+
GS01-12	Cucumber	Jinju	++	++
GS01-13	Cucumber	Jinju	+	++
GS01-14	Cucumber	Jinju	++	+++
GS01-15	Cucumber	Jinju	+	++
GS01-16	Watermelon	Jinju	++	++

[a]RAPD group (RG) I and IV are distinguished by RAPD analysis.
[b]Degree of leaf blight: +++, strong (> 50% leaf area diseased); ++, moderate (26-50% leaf area diseased); +, weakly (1-10% leaf area diseased); – , healthy (0% leaf area diseased); ND, not determined since pycnidia or pseudothesia were not available. DW96-88W and DW96-123W were provided by Department of Agricultural Biology, Chonnam National University, Korea.

Genomic DNA extraction. Fungal genomic DNA was extracted from the dried mycelia using a modification of method described by Vandemark et al. (2000). The mycelia germinated from pycnidiospores of *D. bryoniae* were collected, lyophilized, and were ground with liquid nitrogen in a pre-cooled mortar and a pestle. The ground mycelia were hydrated for 10 min at room temperature in 3 ml of extraction buffer [200 mM Tris-HCl. pH 8.5, 250 mM

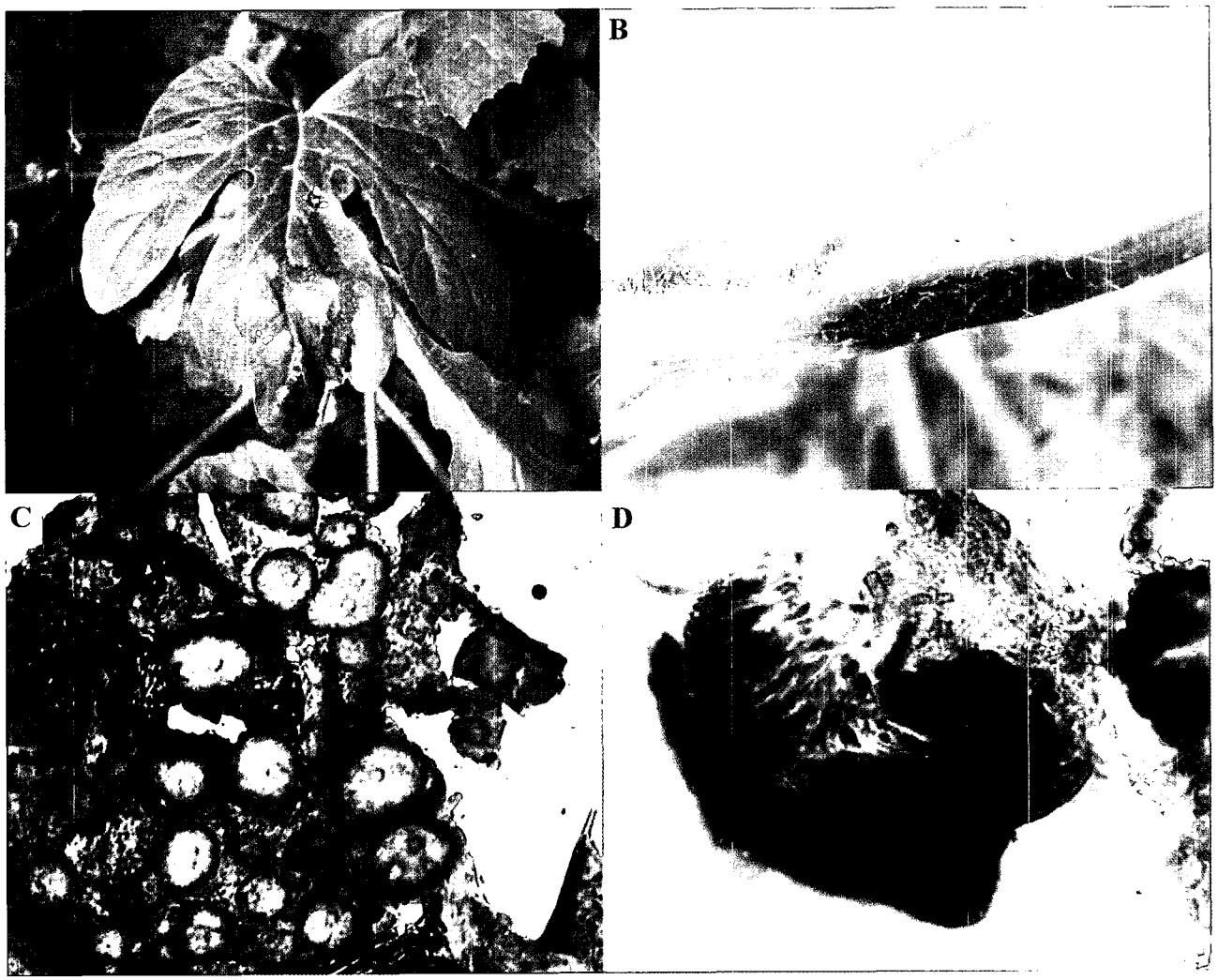

Fig. 1. Symptoms on the leaf (A) and stem (B) of watermelon plants caused by the gummy stem blight (GSB) pathogen, *Didymella bryoniae.* Pycnidia (C, 50X), asci and ascospores (D, 200X) produced on the surface of infected plants.

NaCl, 25 mM EDTA., and 0.5% Sodium dodecyl sulfate (SDS)]. The DNA was purified with phenol/chloroform/isoamyl alcohol (25:24:1) and was precipitated by ethanol in the presence of sodium acetate.

Amplification conditions for RAPD. Oligonucleotide decamers were obtained from United British Colombia University (UBC). PCR amplification was performed in a total reaction volume of 25 μl. Reaction mixture contained; 100 ng of genomic DNA, 18 ng of primer, 0.6 U of *Taq* DNA polymerase (Bioneer, Korea), 10 × reaction buffer, and 10 mM dNTP (dCTP, dGTP, dATP, and dTTP). 35 amplification cycles were carried out in a GeneAmp PCR System 9600 (Perkin-Elmer, USA) according to the following program: 1 min of denaturation at 94°C, 1 min of annealing at 35°C, and 1 min 30 s of extension at 72°C; the initial denaturation step was 3 min at 94°C, and last extension step was extended to 4 min. The PCR products were electrophoresed on a 1.0% agarose gel in 1 × TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.2). The gels were stained with 0.5 μg of ethidium bromide (EtBr) per ml for 40 min and then destained in 1 mM $MgSO_4$ for 2 min.

Analysis of RAPD data. RAPD patterns with each of the random primers used were compared for the 20 isolates of *D. bryoniae.* Presence (coded 1) or absence (coded 0) of each fragment was recorded. A dendrogram was constructed based on the binomial data matrix of RAPD and RFLP analysis, using an unweighted pair group method with an arithmetic average (UPGMA) option in the Numerical Taxonomy System for Personal Computer (NTSYS-pc), version 1.80.

Amplification of the ITS and small subunit rRNA

regions. Primer pairs of ITS1 (5'-TCCGTAGGTGAACC-TGCGG-3') / ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and NS1 (5'-GTAGTCATATATGCTTGTCTC-3') / NS8 (5'-TCCGCAGGTTCACCTACGGA-3') were used for amplification of ITS and small subunit (18s rDNA) of rDNA, respectively (Hong et al., 1998b; White et al., 1990). PCR was conducted in 25 μl reaction volumes. Each reaction tubes contained 100 ng of template DNA solution prepared above, 10X buffer, 25 mM $MgCl_2$, 1.25 mM (each) dNTP, 0.8pM primer, 1unit of *Taq* DNA polymerase (5 unit/μl) and massed up with ddH_2O. The thermal cycling parameters were denaturation, 1 min at 95°C, annealing, 1 min at 58°C, and polymerization, 2 min at 72°C. 35 cycles were conducted and the first denaturation and the last polymerization time were extended to 4 min and 8 min, respectively. The success of amplification was monitored by 1% agarose gel electrophoresis.

Digestion of rDNA by restriction enzymes. The PCR-amplified small subunit and ITS regions were digested with 12 restriction enzymes (those recognizing four nucleotides: *Acc*II, *Alu*I, *Hae*III, *Hha*I, *Hinf*I, *Msp*I, *Mbo*I, *Rsa*I, *Taq*I; five cut: *Ava*II, *Cfr13*I; six cut: *Eco*RI) according to the manufacture's instruction. The digested fragments were separated on 2% MetaPhoreTM Agarose (FMC Bio-product, USA) with 1X TAE buffer.

Screening for RAPD markers and SCAR primer reaction. Genomic DNA of each fungal isolates were screened for developing of RAPD markers generated by random decamer primers from UBC. DNA Markers that were selectively amplified from the 20 isolates of *D. bryoniae* but not amplified from the other pathogen for specific detection of *D. bryoniae.* Two sequence characterized amplified region (SCAR) primer pair, RG IF (5'-TGTCGTTGACATCATTCCAGC-3'), RG IR (5'-ACCACTCTGCTTAGTATCTGC-3'), RG IIF (5'-GCTAAGCCTTAATCTAGCTGC-3'), and RG IIR (5'-GAGAGTAAGCTAACCTAAAGG-3'), were published by Keinath et al. (2001) as a probe for detection of *D. bryoniae.*

SCAR reactions, approximately 100 ng samples of DNA were used in 25 μl amplification reactions containing 15 mM Tris, pH 8.0, 2.5 mM $MgCl_2$, 100 mM each of dCTP, dGTP, dATP, and dTTP, 200 mM of each of the two respective SCAR primer, and 2.5 units of *Taq* DNA polymerase (Bioneer, Korea). Thermo cycling parameters were optimized and a final profile was employed that consisted of single cycle of 3 min at 95°C; 1-step: 10 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min 30s at 72°C, 2-step: 10 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min 30s at 72°C, 3-step: 15 cycles of 1 min at 94°C, 1min at 64°C, and 1 min 30s at 72°C; and a final extension for 10 min at 72°C. SCAR reactions using DNA extracted from isolated samples were carried out as described above PCR reaction condition. Entire PCR reactions were resolved by electrophoresis as described above.

DNA gel blot analysis. DNA gel blot hybridization was done as previously described by Hong et al. (1996). Ten micrograms of the PCR fragemtents of *D. bryoniae* (RGI, GS01-05, GS01-06; RG IV,GS01-01, GS01-02) and the other fungal species (*Alternaria alternata, Cladosporium* sp, *Colletotrichum* sp., *Corynespora* sp., *Trichoderma* sp, and *Fusarium oxysporum*), which were amplified by the UBC primer 258 (5'-CAGGATACCA-3'), were transferred into Nitrocellulose membrane and hybridized with a probe originated from a RG IV isolate. A probe for DNA gel blot hybridization was labeled with the chemilumines using ECL (Enhanced Chemiluminescence) direct nucleic acid kit (Amersham, UK). The probe DNA was diluted to a concentration of 10 ng/μl using the sterile distilled water. The hybridized DNA was detected using the ECL Luminescent Detection Kit following the manufacturer's instructions (Amersham, UK).

Results

Fungal isolates and virulence test. The 18 fungal isolates used in this study were obtained in 2001 and 2002 from various cucurbit-growing areas of the Southern Korea, identified as *D. bryoniae*, and confirmed to be pathogenic on cucurbit leaves. The *D. bryoniae* isolates originally derived from watermelon and melon were subsequently identified as RAPD group (RG) I by RAPD analysis and were confirmed to be more virulent to watermelon than were the RG IV isolates from squash, cucumber, melon, and watermelon (Table 1). Virulence of the RG I isolates were strong to moderate to cucumber, whereas that of RG IV varies from strong, moderate to weak. Watermelon and cucumber isolates were more virulent than squash isolates (Table 1).

PCR amplification. Genomic DNA of the gummy blight pathogen was amplified with UBC random primers to yield polymorphic fragments (Table 2). The 40 primers gave consistently clear bands with genomic DNAs of twenty isolates of *D. bryoniae*, of which the polymorphisms were useful for classifying isolates of *D. bryoniae*. Two hundred seventy-three amplified fragments were produced with 40 primers (Table 2). Representative eight primers, UBC104, 116, 191, 121, 125, 237, 243, and 261 generated PCR products shown in Fig. 2.

Cluster Analysis of RAPD Data. By combining results

Table 2. List of decamer oligonucleotides used as random primers

Primer number	Sequence (5'-3')	Number of polymorphic fragments	Primer number	Sequence (5'-3')	Number of polymorphic fragments
103	GTGACGCCGC	8	178	CCGTCATTGG	5
104	GGGCAATGAT	8	191	CGATGGCTTT	7
105	CTCGGGTGGG	5	199	GCTCCCCCAC	6
106	CGTCTGCCCG	9	203	CACGGCGAGT	8
109	TGTACGTGAC	3	205	CGGTTTGGAA	8
110	TAGCCCGCTT	6	210	GCACCGAGAG	5
111	AGTAGACGGG	8	211	GAAGCGCGAT	9
112	GCTTGTGAAC	6	212	GCTGCGTGAC	7
115	TTCCGCGGGC	7	213	AAGCCTCCCC	9
116	TACGATGACG	7	222	AAGCCTCCCC	11
119	ATTGGGCGAT	11	227	GGGTGAACCG	6
121	ATACAGGGAG	5	228	GCTGGGCCGA	7
125	GCGGTTGAGG	9	230	CGTCGCCCAT	6
133	GGAAACCTCT	7	232	CGGTGACATC	5
134	AACACACGAG	7	237	CGACCAGAGC	5
146	ATGTGTTGCG	2	243	GGGTGAACCG	9
149	AGCAGCGTGG	6	258	CAGGATACCA	4
164	GAAGGCTCTG	6	261	CTGGCGTGAC	9
172	ACCGTCGTAG	6	272	AGCGGGCCAA	8
177	TCAGGCAGTC	8	273	AATGTCGCCA	5
Subtotal among array		134			139
	Total among			273	

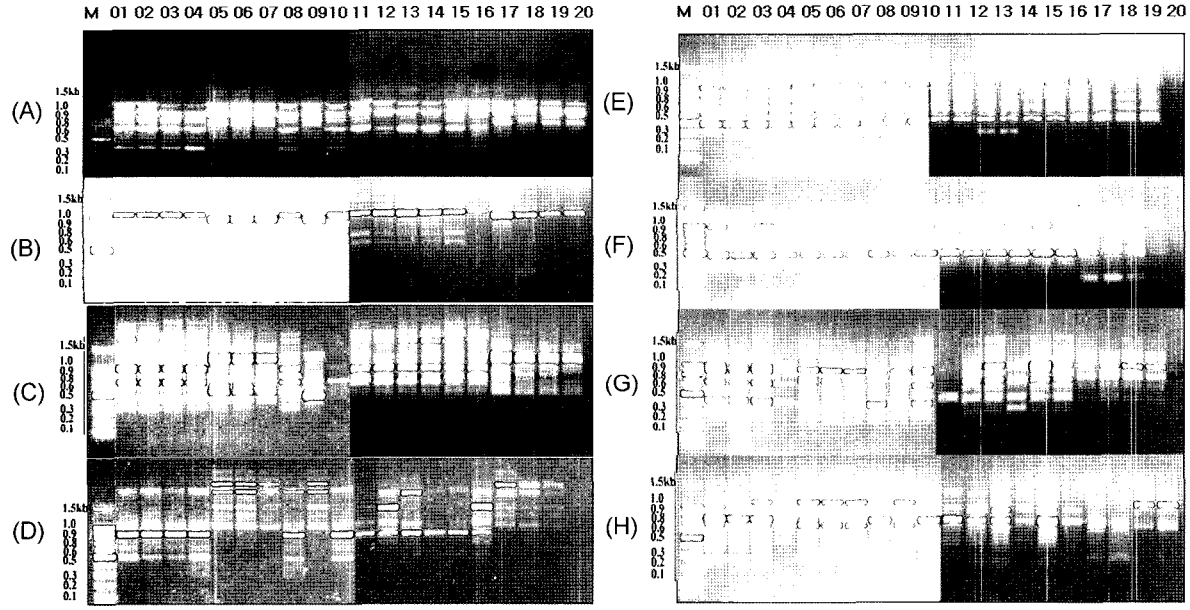

Fig. 2. RAPD patterns on 1.2% agarose gels from the 20 isolates of *Didymella bryoniae* with UBC random decamer primers (A) 104, (B) 116, (C) 191, (D) 121, (E) 125, (F) 237, (G) 243, and (H) 261 . Lane M, DNA marker (1 kb DNA ladder); lanes 1-20, refer to the 20 isolates in Table 1.

obtained by the 40 primers, 273 fragments positions were scored for presence versus absence (1/0) for all of the isolates tested. The combined data from the 273 bands were analyzed by a cluster analysis using UPGMA method with

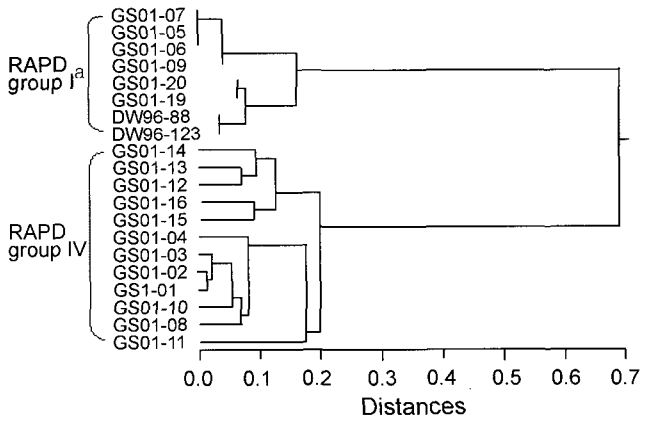

Fig. 3. Dendrogram derived from the RAPD profiles of the 20 isolates of *D. bryoniae*, constructed by Unweighted Pair Group (UPGMA) method with an arithmetic average program of NTSYS-pc (Version 1.80). a: Two groups were subsequently determined as RG I and IV by the selected SCAR primers (Fig. 5). Refer to the isolates designations in Table 1 for each RAPD group.

Table 3. PCR identification of the *D. bryoniae* isolates by with SCAR primers

Isolate	Origin		PCR products[b]	
	Host	Location	RG IF-RG IR	RG IIF-RG IIR
Didymella bryoniae RG I[a]				
GS01-05	Watermelon	Hadong	+	−
GS01-06	Watermelon	Hadong	+	−
GS01-07	Watermelon	Hamyang	+	−
GS01-09	Melon	Hamyang	+	−
DW96-88	Watermelon	Haman	+	−
DW96-123	Watermelon	Haman	+	−
GS01-19	Watermelon	Jinju	+	−
GS01-20	Watermelon	Jinju	+	−
Didymella bryoniae RG IV				
GS01-01	Squash	Namhae	−	−
GS01-02	Cucumber	Jinju	−	−
GS01-03	Squash	Uiryeong	−	−
GS01-04	Squash	Uiryeong	−	−
GS01-08	Melon	Hamyang	−	−
GS01-10	Watermelon	Hamyang	−	−
GS01-11	Watermelon	Jinju	−	−
GS01-12	Cucumber	Jinju	−	−
GS01-13	Cucumber	Jinju	−	−
GS01-14	Cucumber	Jinju	−	−
GS01-15	Cucumber	Jinju	−	−
GS01-16	Watermelon	Jinju	−	−

[a]RG I and IV are distinguished by RAPD analysis with 40 UBC random primers.
[b]+ and − indicate the presence or absence of PCR fragment that were produced by SCAR reaction.

an arithmetic average program of NTSYS-PC (Version 1.80) to generate a dendrogram (Fig. 3). At the distance level of 0.7, two major genomic DNA RAPD groups were differentiated among 20 isolates. RG I included 8 isolates from watermelon except one isolate from melon. RG IV included 12 isolates from squash, cucumber, watermelon and melon (Table 3).

Amplification of ITS region and RFLP analysis. Both ITS and 18S rDNA regions were amplified by primer pairs ITS1/ITS4 (data not shown) and NS1/NS8 to detect intra-specific diversity of *D. bryoniae*. Amplification of ITS region and 18S rDNA region from the 20 isolates always resulted respectively in a single 550bp fragment (data not shown) and a single 2.1 kb fragment, respectively (Fig. 4A). Restriction pattern of 12 restriction enzymes were identical for all isolates tested, suggesting that variation in the ITS region within the *D. bryoniae* was low (Fig. 4B and 4C).

SCAR primer reaction. Amplification of the genomic DNA of the tested isolates with SCAR primer RG1F-RG1R specific for RG I group resulted in a single band of 650bp fragment only for 8 isolates out of the 20 isolates (Fig. 5), which precisely corresponded to the eight isolates clustered in the upper portion of Fig. 3. Therefore, these 8 isolates could be assigned into the RG I (Table 3). The same experiments done with RGIIF-RGIIR resulted in no amplified PCR product for the 20 isolates tested (data not shown). This result suggested that the other 12 isolates would be neither RG I nor RG II.

Screening of RAPD markers for RG IV specific primer. In order to develop RG IV specific primer, genomic DNAs of *D. bryoniae* RG IV (lane 1, GS01-01; lane 2, GS01-02), RG I (lane 3, GS01-05; lane 4, GS01-06) and other fungi species, such as *A. alternata*, *Cladosporium* sp., *Colletotrichum* sp., *Corynespora* sp., *Trichoderma* sp., and *F. oxysporum*, were subjected to PCR amplification with UBC primer 258 (5'-CAGGATACCA-3') (Fig. 6A). An about 1.4 kb-PCR fragment amplified from a RG IV isolate, when probed with all of the amplified DNAs from the fungal isolates, hybridized with only those of the RG IV isolates (Fig. 6B), suggesting that this PCR product could be used for differentiating the RGIV isolates from either RGI isolates or the other fungal species.

Discussion

Gummy stem blight pathogen, *D. bryoniae*, is very difficult not only to monitor the inoculum levels prior to host

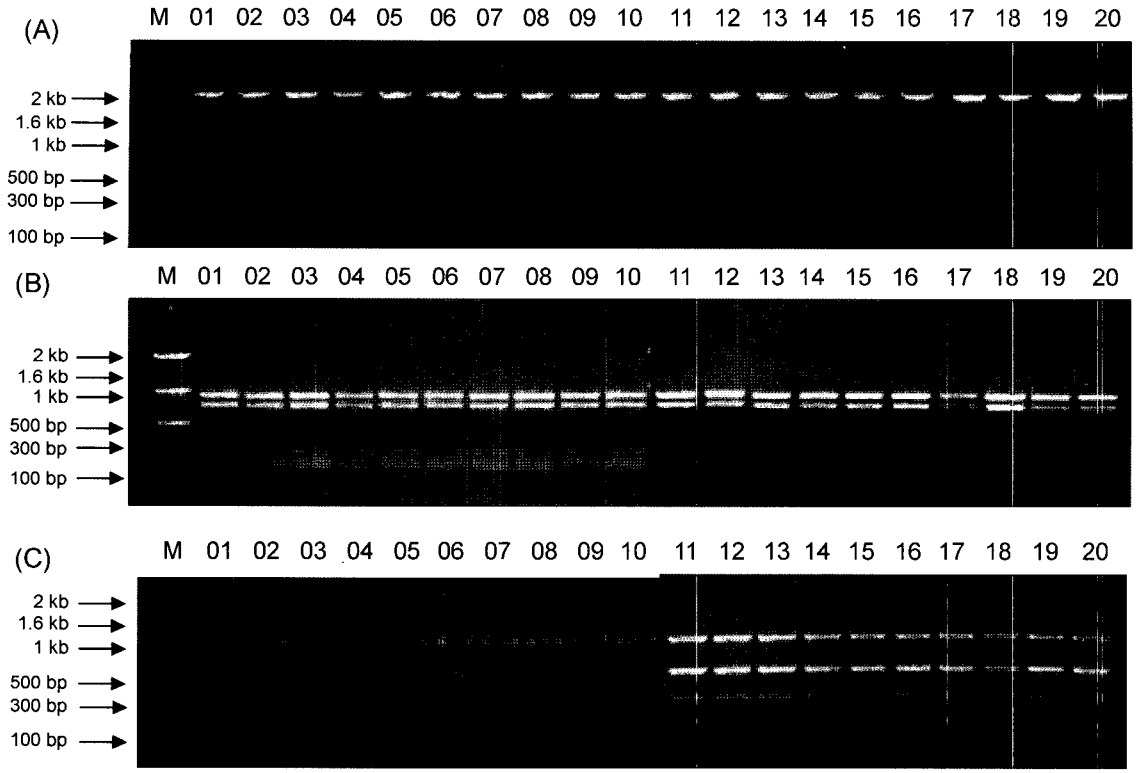

Fig. 4. Amplification and RFLP patterns on 1% agarose gels of the amplified 18S rDNA region from the 20 isolates of *D. bryoniae*. Amplified DNA fragments (A) were digested with endonuclease *Hinf*I (B) and *Ava*II (C), respectively. Lane M, molecular markers (1 kb DNA ladder); lane 1-20, Refer to the isolate designations in Table 1.

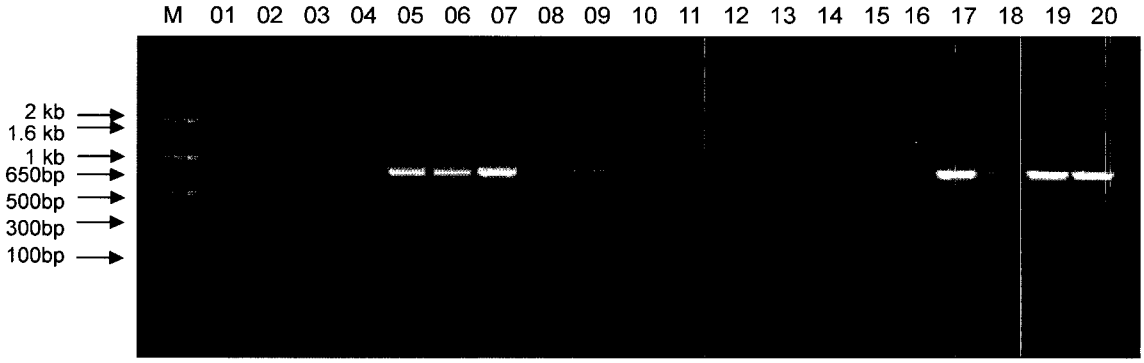

Fig. 5. PCR amplification pattern of the 20 isolates of *D. bryoniae* with SCARs specific primer RG IF and RG IR (Somai et al., 2002b). Refer to the isolates designations in Table 1 for each RAPD group.

infection, but also to detect rapidly using standard method of culturing infected tissue on agar media. Furthermore, it is destructive and hard to control, once the epidemic has been established in field condition. We have applied RAPD technique to elucidate the genetic diversity of the genomic DNA of *D. bryoniae* and also to generate specific diagnostic DNA probe useful for identification and detection.

RG I isolates were more virulent on watermelon than RG IV isolates were. One RG IV isolate GS01-01 from squash did not sporulate *in vitro*. The least virulent isolates on both watermelon and cucumber were isolated from squash. Those originated from cucumber GS01-02 and GS01-14 were more virulent to cucumber, which may indicate that some degree of host preference may exist in isolates of *D. bryoniae*. Again, such a generalization should be made with caution; other cucumber isolates being only of moderate virulence to cucumber. Similar observation was also made by St. Amand and Wehner (1995) who reported variation in virulence for cucumber isolates not host specificity from geographically diverse areas.

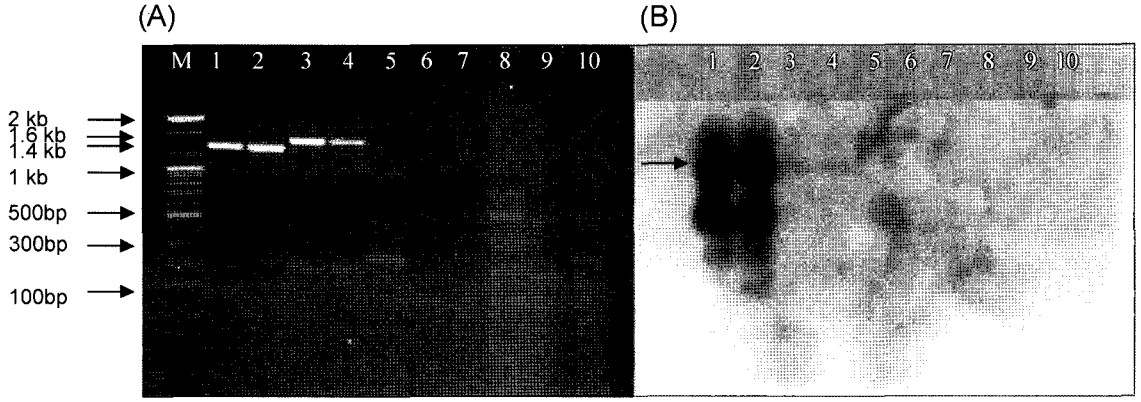

Fig. 6. PCR amplification (A) and Southern hybridization (B) of the PCR products amplified from 4 isolates of *D. bryoniae* and the other plant pathogenic fungi using a candidate SCAR primer 258. The 1.4 kb of SCAR product amplified from *D. bryoniae* was used as detection probe. Lane M, molecular marker (100bp DNA ladder); lane 1-2, *D. bryoniae* GS01-01, GS01-02 (RG IV); lane 3-4, *D. bryoniae* GS01-05, GS01-06 (RG I); lane 5, *Alternaria* sp.; lane 6, *Cladosporium* sp.; lane 7, *Colletotrichum* sp.; lane 8, *Corynespora* sp.; lane 9, *Trichoderma* sp.; lane 10, *Fusarium* sp..

The isolates of *D. bryoniae*, we have studied, did exhibit the differences in virulence certainly, but no specificity toward any host plant species among cucurbitaceae.

We investigated the differentiation of genomic DNA in *D. bryoniae* isolates from the intensive cucurbits-growing areas in the southern Korea using RAPD. The genomic DNA of the GSB pathogens was amplified with UBC random primers to yield polymorphic fragments (Fig. 2, Table 2). Two hundred seventy-three amplified fragments were produced with 40 primers, which were analyzed 273 polymorphic DNA bands by a cluster analysis using UPGMA method with an arithmetic average program of NTSYS-PC (Version 1.80) to generate a dendrogram (Fig.3). This dendrogram of RAPD profiles reveled two distinct groups; one group included the 8 isolates from watermelon except one isolate from melon and the other group included 12 isolates from squash, cucumber, watermelon and melon. These two groups were subsequently determined to be RG I and RG IV by SCAR primer reaction, respectively.

Using RAPD fingerprinting, Somai et al. (2002a) previously differentiated *D. bryoniae* into three phylogenic groups, *i.e.*, RG I, represent predominate isolates; RG II, dominate and RG IV, only one isolate in USA.

Our attempts to detect intraspecific diversity of ITS region and 18s rDNA region for 20 isolates and restriction digestion of amplified fragment with 12 enzymes did not reveal polymorphism (Fig 4). This result suggests that variation in ITS region and 18s rDNA region within the *D. bryoniae* was very low, and thus not suitable for designing species-specific primers. Nielson et al. (2002) also reported similar results for *Botrytis* spp. These results would indicate that rDNA of this fungus may be less heterogeneous compared to other chromistan pseudo-fungi or *Rhizoctonia solani* representing various anastomosis groups (Hong et al., 1998a).

The ITS region of nuclear rDNA have been amplified and used to develop a species-specific probe for *Pythium ultimum* (Lévesque et al., 1994), *Phytophthora capsici*, *P. cinnamomi*, *P. megakarya*, *P. palmacera* respectively and genus Phytophthora probe (Lee and Taylor, 1992) and *Phytophthora cryptogea-P. drechsleri* complex group (Hong et al., 1999). Hong et al. (1998b) also investigated intraspecific genetic diversity of Korean isolates of *P. drechsleri* and suggested three distinct groups: PdG1, PdG2 and PdG3.

Vandemark et al. (2000) developed a PCR assay system based on SCARs. Pairs of extended PCR primers were designed to amplify sequence characterized DNA markers for the identification and detection of *Aphanomyces euteichs*. Similar approach was made by Larsen et al. (2002) who developed a rapid method using PCR-based SCARs markers for *Phoma sclerotioides* from brown rotted alfalfa root as well as soil sample in a single day. Nielsen et al. (2002) designed SCARs primers (BA29/BA12) for direct amplification of isolates of *Botrytis* spp. associated with neck rot of onions. They were able to distinguish the five groups: *Botrytis alada* AI and AII (*B. allii*); *B. byssoides*; *B. squamosa*; and *B. cinerea*, through the digestion of PCR amplification product with the restriction enzyme *Apo*I.

As shown in Figure 5, amplification experiment with SCAR specific primer RG1F-RG1R resulted in a single band of 650bp fragment only for 8 isolates out of 20 isolates, which precisely corresponded to eight isolates clustered in upper portion of Fig. 3. Therefore, it is

suggested that these isolates GS01-05, 06, 07, 09, 17, 18, 19 and 20 should be designated as RG I. However, the same set of experiment done with RGIIF-RGIIR (Table 3) did not result in any amplified product. This result suggested the rest 12 isolates should be different from RG II reported in US by Somai et al. (2002a). Therefore, diversity of Korean isolates of *D. bryoniae* and their distribution were determined to be different from that of the US isolates: predominating RG IV isolates in Korea, which is rarely occur in USA.

Recently, Somai et al. (2002b) grouped *D. broyniae* isolates to RG I, RG II or RG IV, whereas *Phoma* isolates clustered to RG III. Therefore, we would designate second RAPD group isolates as RG IV, based on the RAPD profile of genomic DNA, for Korean isolate of gummy stem blight pathogen. We noticed the variation in virulence between two RAPD group isolate inoculated to watermelon and cucumber (Table 1).

In order to develop RAPD markers for RG IV specific primer, a candidate PCR fragment (1.4 kb) was purified and Southern hybridized to the amplified fragment RG IV isolates (Fig. 6). This candidate probe recognized only RG IV isolates. This promising candidate probe is being cloned subsequently for further study toward RG IV-specific forward and reverses primer set, which is not available elsewhere in the world.

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