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Analysis of Genetic Variation in *Botrytis cinerea* Isolates Using Random Amplified Polymorphic DNA Markers

CHOI, IN SIL, DAE HYUK KIM[1], CHANG WON LEE, JAE WON KIM, AND YOUNG RYUN CHUNG*

Department of Microbiology and Research Institute of Natural Science, Gyeongsang National University, Chinju 660-701, Korea
[1]Institute for Molecular Biology and Genetics, Faculty of Biological Sciences, Chonbuk National University, Chonju 561-756, Korea

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Abstract Random amplified polymorphic DNA (RAPD) markers were used to survey genetic variability among 34 *Botrytis cinerea* isolates from nine different host plants in Korea. For RAPD analysis, 115 arbitrary decamer primers were initially screened for polymorphic major DNA bands with 11 representative *B. cinerea* isolates. Eleven primers that initially detected polymorphisms were tested a second time with additional 23 isolates of *B. cinerea* as well as one isolate of *Botrytis squamosa* as an outgroup. The RAPD analyses revealed that all isolates except one showed different molecular phenotypes. Dendrograms obtained from dissimilarity matrices using the unweighted paired group method of arithmetic means (UPGMA) showed the 36.4% to 90.0% similarity among all *B. cinerea* isolates. The *B. squamosa* isolate showed the least similarity to all *B. cinerea* isolates. The cluster analyses indicated no correlation among all the characteristics examined including molecular phenotypes, host and geographic origins, year of isolation, or pathogenicity. The RAPD data suggest that a high level of genetic variation exists among Korean populations of *B. cinerea* and it seems to be caused by heterokaryosis among preexisting molecular phenotypes.

Key words: Gray mold, RAPD, molecular phenotype, *Botrytis cinerea*

Botrytis cinerea Pers.: Fr. causes gray mold on many economically important crops throughout the world. This pathogen causes heavy crop losses during both the growing seasons and storage periods, and, consequently, has been studied extensively [5, 8, 10, 15]. The fungus is known to display considerable variations in phenotype such as colony morphology, sclerotia production, pathogenicity, polygalacturonase production and fungicide resistance [4, 12, 13]. Information about the genetic variation within fungal populations is important to better understand disease outbreaks and develop new control strategies. However, estimates of genetic variability among naturally occurring populations are scarce. Field isolates and laboratory mutants of *B. cinerea* from nine different countries were crossed with reference strains of the fungus to determine the genetic bases of differences in sexual fertility and fungicide resistance [10, 11]. Crosses revealed that most field isolates were heterothallic, and carried the MAT1-1 or MAT1-2 mating-type allele. Some isolates contained both mating type alleles. Van der Vlugt-Bergmans *et al.* [27] studied genetic variation and segregation of DNA polymorphisms in eight Dutch isolates and two Italian monoascospore isolates, as well as in their progenies, using RAPD analysis. They showed that all isolates were genetically distinct and that most of the RAPD markers tested segregated in a normal Mendelian ratio of 1:1 in the progeny. A new retroelement named Boty was found in multiple copies in the genome of some *B. cinerea* isolates; it was proposed that Boty-containing and Boty-deficient groups represent two lineages in the *B. cinerea* population [7].

Molecular markers including RAPD markers are useful for analyzing genetic variation within populations of phytopathogenic fungi [6, 14, 17]. Among various molecular techniques, RAPD analysis is sensitive, quick, and simple to perform [28, 29]. This method has become popular for identifying or differentiating numerous plant pathogens including *Colletotrichum graminicola, Puccinia recondita* f. sp. *tritici* and *Fusarium solani* [6, 14, 18].

Although field isolates of *B. cinerea* exhibit considerable variations in several phenotypic characteristics, information on the genetic variability at the molecular level among isolates has been analyzed so far using only a limited

*Corresponding author
Phone: 82-591-751-5945; Fax: 82-591-759-0187;
E-mail: yrchung@nongae.gsnu.ac.kr

number of isolates from two or three host plants. In this study, we investigated the genetic variability in 34 field isolates of *B. cinerea* obtained from nine different host plants during the last 15 years in Korea.

MATERIALS AND METHODS

B. cinerea Isolates

Thirty-four isolates of *B. cinerea* were examined for RAPD patterns and pathogenicity (Table 1). Thirty-three isolates were derived from nine different hosts exhibiting symptoms of gray mold: cucumber, gerbera, ginseng, grape, kiwi, pear, tomato and strawberry. The infected samples were collected from 16 locations throughout Korea between 1980 and 1995. One isolate, KCTC 1937, obtained from butter in Japan, was acquired from the Korean Collection for Type Cultures (Taejeon, Korea) and one isolate of *B. squamosa* from a diseased onion was selected as an outgroup for molecular genetic similarity to *B. cinerea*. Most isolates obtained after 1992 were derived from a single conidium. The others were mycelial isolates derived from infected host plants. All cultures were maintained at 5°C on potato dextrose agar (PDA) by mass transfer and were also stored as mycelial discs in a 10% glycerol solution at -70°C.

Pathogenicity Test

For pathogenicity tests, each isolate was grown on PDA at 20°C for 5 days and the first true leaves of cucumber (*Cucumus sativus* L.) seedlings (two-leaf stage) were inoculated with mycelial agar plugs (5 mm in diameter). Inoculated seedlings (four replicates) were placed in a dew chamber at 20°C and the diameter of necrotic lesions was measured 3 days after inoculation.

DNA Extraction from Mycelium

Ten mycelial discs (5 mm in diameter) for each isolate were placed into 500 ml potato dextrose broth (PDB, Difco, Detroit, U.S.A.) in 2-liter Erlenmeyer flasks and cultured for 5 days at 22°C on a rotary shaker at 180 rpm. Mycelium was harvested by filtration through autoclaved cheesecloth, lyophilized and kept at -20°C until used. DNA was extracted from mycelium using a modification of the method of Lee *et al.* [19]. Approximately 1 g of mycelium was ground and suspended in 90 ml extraction buffer containing 50 mM Tris-HCl, pH 8, 5 mM EDTA, and 1% SDS. The suspensions were incubated at 65°C for 2~3 h and then centrifuged at 10,000 g for 10 min. The supernatant was treated with 100 μg/ml proteinase K and then 30% (v/v) ethanol was added for precipitation of cinerean, an extracellular polysaccharide. The cinerean was removed by filtration through cheesecloth. Following several phenol extractions, precipitation with 70% ethanol, and treatment with RNAase (50 μg/ml), the DNA was finally purified by passing through a DEAE-Sepharose column and quantified on 0.7% agarose gels containing ethidium bromide by visual comparisons with known quantities of lambda DNA.

RAPD Analysis

Primers used in PCR assays were obtained from the biotechnology laboratory at University of British Columbia (No. 301-415). One hundred and fifteen arbitrary decamer primers were initially screened for amplification of polymorphic major bands with 11 representative *B. cinerea* isolates. Eleven primers that initially detected polymorphisms were further used with 23 additional isolates; their sequences were as follows: 320, CCGG-CATAGA; 323, GACATCTCGC; 337, TCCCGAACCG; 350, TGACGCGCTC; 353, TGGGCTCGCT; 360, CTC-TCCAGGC; 366, CCTGATTGCC; 386, TGTAAGCT-CG; 396, GAATGCGAGG; 402, CCCGCCGTTG; 406, GCCACCTCCT. Amplification reactions were performed in 20 μl volumes containing 80 ng fungal DNA, 36 ng of a single primer, 250 μM of each dNTP and 0.6 units of *Taq* DNA polymerase (Korea Biotechnology, Taejeon, Korea). For amplifications, the thermocycler (Perkin-Elmer Corp.) was programmed for one cycle of 1 min at 94°C, followed by 35 cycles of 1 min at 94°C, 2 min at 35°C, 3 min at 72°C, and one cycle of 10 min at 72°C. Amplification products were visualized by agarose gel electrophoresis on 1.5% agarose gels. Only the major polymorphic bands were scored, ignoring weakly amplified bands. Each of the 11 primers amplified repeatable major polymorphic bands among the isolates. For each primer, the most common banding pattern was designated as type 1, the next most common was type 2, etc. An 11-digit molecular phenotype based on the banding pattern for each of the 11 primers was assigned to each *B. cinerea* isolate. The RAPD amplifications were repeated two times using the same DNA samples. Only replicable bands from 0.1 to 2.6 kb in size were scored for RAPD analysis. The genetic similarity among isolates was calculated as the Euclidean metric distance between all pairs of isolates. A dendrogram was constructed from the Euclidean distance matrix by the unweighted paired group method of arithmetic averages with the SAHN program of NTSYS-pc software, version 2.0 (Exeter Software, Setauket, NY, U.S.A.).

Data Analysis

The statistical analyses were performed with SAS software (SAS Institute Inc., Cary, NC, U.S.A.) to estimate any significant correlation between pathogenicity, location, host range, and RAPD group. Because sample sizes were not equal, the Scheffe's test and categorical data analysis were applied at a significance level of P=0.05.

Table 1. Pathogenicity, molecular phenotype, geographic and host origin of 34 *Botrytis cinerea* isolates and one isolate of *Botrytis squamosa*.

Isolate	Pathogenicity (diameter, cm)[a]	Molecular phenotype[b]	Host	Location[c]	Year
Botrytis cinerea					
BC-5	1.70	14121112512	Tomato	Yusong	1988
BY-2	3.10	11122111212	Cucumber	Buyoe	1994
BY-3	2.40	22121121311	Cucumber	Buyeo	1994
BY-6	3.25	11121111112	Cucumber	Buyeo	1994
BY-33	2.83	41111111212	Cucumber	Buyeo	1994
CA-36	2.25	12121135113	Ginseng	Chinahn	1980
CS-107	4.18	35134101211	Strawberry	Nonsan	1990
DJ-49	3.60	13121121111	Strawberry	Taejon	1994
DJ-71	3.25	26121111512	Strawberry	Taejon	1994
DS-522	3.80	22121121311	Strawberry	Tamyang	1990
FC-122	0.25	14131222312	Cucumber	Pyungtaek	1990
G-1	1.43	13111311611	Gerbera	Kimhae	1990
GJ-4-3	3.65	4**10**121113613	Strawberry	Gongju	1994
GJ-5-2	3.38	41232341411	Strawberry	Gongju	1994
IT-106	3.85	41113121211	Tomato	Yongin	1990
JM-1	4.05	31113132411	Tomato	Moonsan	1993
JM-2	3.48	31115132411	Tomato	Moonsan	1993
JM-3	3.65	12112132311	Tomato	Moonsan	1993
JM-4	3.58	02110020011	Tomato	Moonsan	1993
JM-42	2.23	31113132211	Tomato	Moonsan	1995
JM-43	4.68	48111122311	Tomato	Moonsan	1995
JT-101	3.70	13111111113	Tomato	Taejon	1990
JT-104	3.30	21111111113	Tomato	Taejon	1990
KC-6	4.73	24121111511	Cucumber	Yusong	1989
KCTC-1937	0.00	0**11**340526820	Butter	Japan	1985
KT-433	2.85	13211112212	Tomato	Kimhae	1990
NH-32	2.70	1913043713	Kiwi	Namhae	1992
NS-1	0.77	15212142410	Strawberry	Nonsan	1994
NS-53	2.75	13121111113	Strawberry	Nonsan	1994
NS-111	3.42	11112114112	Strawberry	Nonsan	1990
PT-33	2.60	37122242211	Pear	Pyungtaek	1992
S-1	3.48	12111112113	Cucumber	Yusong	1991
SW-31	2.20	11111122313	Grape	Suwon	1993
T91-1	4.30	12121111612	Tomato	Yusong	1991
Botrytis squamosa					
CN-25	0.44	5**12**456657930	Onion	Changnyung	1995

[a]Diameter of necrotic lesions on cucumber leaves measured 3 days after inoculation of mycelial agar discs at 20°C. Each value is a mean of four replicates.

[b]Polymorphic DNA bands randomly amplified with decamer primers 323, 350, 406, 360, 366, 386, 353, 396, 402, 320, and 337 obtained from the University of British Columbia biotechnology laboratories; 0 indicates no bands, 1 indicates the most common banding pattern, and 2 indicates the second most common banding pattern, etc. Bold digits are 10th, 11th, and 12th most common banding patterns generated by primer 350.

[c]Isolates were collected from different fields except Moonsan where all six isolates were from the same field.

RESULTS AND DISCUSSION

Pathogenicity Variation

The pathogenicity of all different test isolates collected from different host plants on cucumber seedlings was variable based on the diameter of necrotic lesions. The size of the lesions ranged from 0 to 4.73 cm (Table 1). The isolate obtained from butter, KCTC-1937, did not produce any disease symptoms on cucumbers. Isolates KC-6 and JM-43 were the most aggressive and isolates FC-122 and NS-1 were only weakly pathogenic to cucumbers. However, these isolates that differed in geographic and host origins could not be grouped on the basis of their pathogenicity reaction alone. Variation in pathogenicity and virulence has been reported among isolates of *B. cinerea* and it has been ascribed to differences in their ability to produce aspartate proteinase, β-glucosidase, pectin methylesterase, polygalacturonase, and to generate H_2O_2 or metabolize the phytoalexins [23, 24, 25, 26]. However, genetic aspects of pathogenic variation remain unresolved. In our studies, no significant correlations between pathogenicity and molecular similarity matrices could be found.

Molecular Variation

The initial screening of DNA from 11 isolates with 115 arbitrary, 10-base primers yielded eleven primers that gave reproducible polymorphic DNA patterns. Selected primers were then used to establish RAPD patterns of 34 isolates of *B. cinerea* and one isolate of *B. squamosa*. For example, Fig. 1 shows the amplification products generated with primers 337 and 353. The size of amplified DNA fragments generated with the 11 primers ranged from 0.5 to 2.6 kb. All the primers revealed polymorphisms and the diagrammatic representation of banding patterns of *B. cinerea* isolates is shown in Fig. 2. Primers 320 and 337 distinguished three molecular phenotypes and primer 406 distinguished four phenotypes. Primers 323, 353, and 360 detected five phenotypes; primers 366 and 386 detected six phenotypes; primer 396 detected seven phenotypes; and primers 402 and 350 revealed nine and 12 molecular phenotypes among *B. cinerea* isolates, respectively. The molecular phenotype of *B. squamosa* isolate CN-25 was different from those of *B. cinerea* isolates with all tested primers (Table 1). Only two isolates of *B. cinerea*, DS-522 and BY-3, which represented different hosts, locations and years,

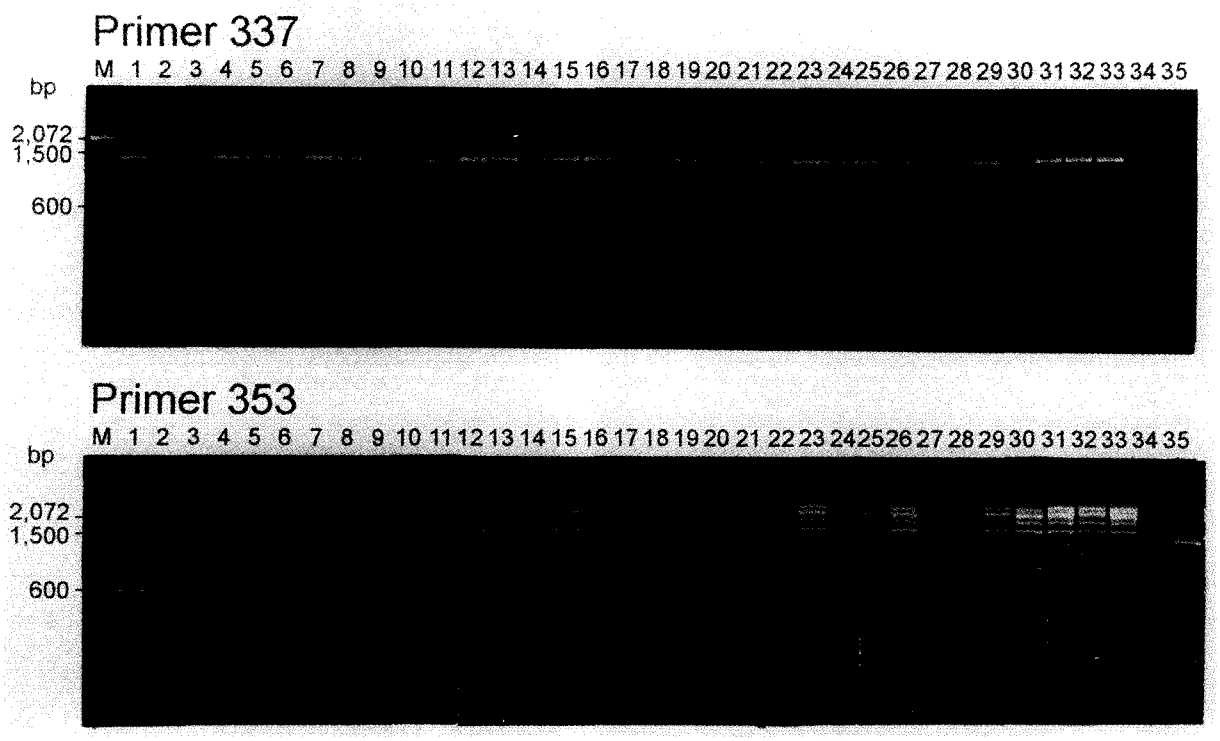

Fig. 1. Randomly amplified polymorphic DNA (RAPD) markers of *Botrytis cinerea* isolates (1-34) and *Botrytis squamosa* isolate (35) generated with University of British Columbia biotechnology laboratory primers 337 and 353.

Lanes, from left to right, show amplification products from isolates; 1, BC-5; 2, T91-1; 3, KC-6; 4, S-1; 5, G-1; 6, KT-433; 7, JT-104; 8, JT-101; 9, DJ-71; 10, DJ-49; 11, NS-111; 12, FC-122; 13, PT-33; 14, DS-522; 15, JM-1; 16, JM-2; 17, JM-3; 18, JM-4; 19, JM-42; 20, JM-43; 21, CS-107; 22, NS-1; 23, NS-53; 24, IT-106; 25, BY-33; 26, BY-6; 27, BY-3; 28, BY-2; 29, GJ-4-3; 30, GJ-5-2; 31, SW-31; 32, NH-32; 33, CA-36; 34, KCTC-1937; 35, CN-25. Selected molecular weight size markers are indicated in base pairs to the left of each RAPD group.

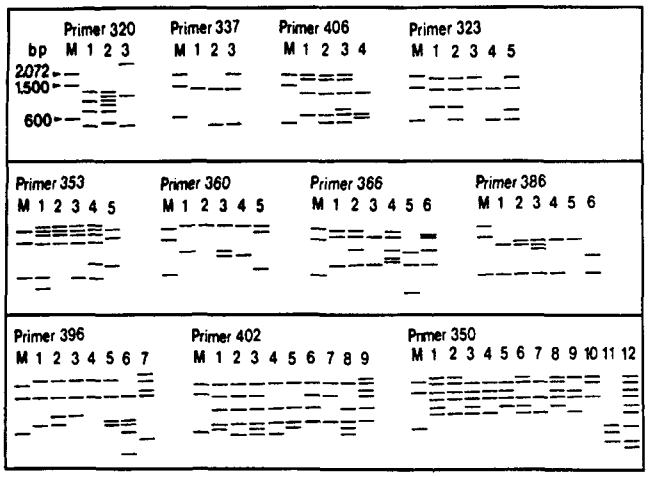

Fig. 2. Diagrammatic representation of banding patterns of 34 *Botrytis cinerea* isolates and one *Botrytis squamosa* isolate using University of British Columbia biotechnology laboratory primers 320, 323, 337, 350, 353, 360, 366, 386, 396, 402, and 406.

Each lane number represents a DNA banding pattern; 1 indicates the most common DNA banding pattern, 2 indicates the second most common banding pattern, etc. Selected molecular weight size markers (M) are indicated in base pairs to the left of each RAPD group.

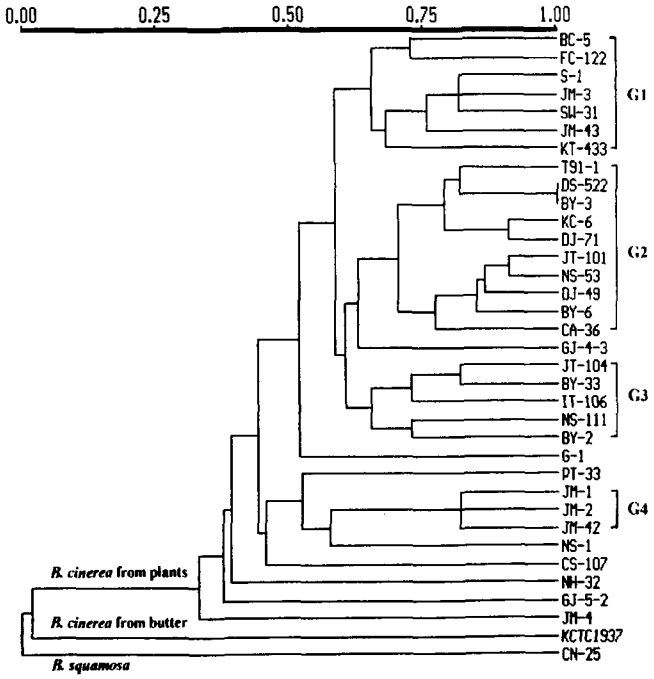

Fig. 3. Dendrogram showing relationships among 34 *Botrytis cinerea* isolates and one isolate of *Botrytis squamosa*.

The unweighted pair group arithmetic mean method in the SAHN program of NTSYS-pc, version 2.0 was used. The set of brackets on the right indicates grouping of isolates based on randomly amplified polymorphic DNA analysis with 11 primers.

showed an identical molecular phenotype (22121121311). All other isolates differed in their molecular phenotypes when RAPD profiles from eleven primers were combined. The molecular phenotypes distinguished by primer 337 varied by only one band from the most common banding pattern. Computer analysis of the RAPD molecular phenotypes resulted in a matrix with a Euclidian dissimilarity coefficient for each pair of isolates. The similarity within *B. cinerea* isolates ranged from 27% to 91%. Four clusters containing multiple isolates with more than 64% similarity were observed. RAPD group 1 (G1) contains seven isolates that differed in pathogenicity and were derived from different locations and hosts. RAPD group 2 (G2) and group 3 (G3) contained ten and five isolates, respectively, which were again from different locations, various hosts and different degrees of pathogenicity. However, RAPD group 4 (G4) contained exclusively three tomato isolates, JM1, JM2, and JM42. These isolates were collected from the same field and may have had a clonal origin and been subjected to only minor variations. Six isolates from a single field in Moonsan showed a large amount of genetic variation (36%~82% similarity) (Table 1, Fig. 3). The non-pathogenic isolate KCTC1937 showed very low similarity to all the other *B. cinerea* isolates. However, no relationship between the host range and molecular phenotypes was observed since isolates from tomatoes, cucumbers and strawberries were 27%~82%, 46%~73%, and 27%~91% similar, respectively. The statistical and cluster analyses indicated that no correlation between molecular phenotypes and host range, geographic origin and year of isolation were observed (Lambda value=0.105, P= 0.409; Lambda value=0.286, P=0.082; Lambda value= 0.200, P=0.111). In addition, means of pathogenicity in each molecular phenotype did not differ significantly (Scheffe's test, P=0.05).

Genetic exchange in fungal populations can be hampered by barriers such as geographic isolation, vegetative incompatibility and sexual incompatibility [1, 2]. Molecular phenotypes of the 34 field isolates used in this study were very heterogeneous with the exception of only two isolates. It is not uncommon for *B. cinerea* to display considerable variation in phenotypes such as growth patterns, pathogenicity, enzyme production and fungicide resistance [10, 13, 20, 21, 22]. Phenotypic diversity was observed even in asexual offspring and it was ascribed to the multinucleate and heterokaryotic nature of this fungus [12, 16]. Furthermore, in a segregation assay of RAPD markers in progeny derived from a single ascospore, several unexpected non-Mendelian phenomena were observed [27]. Sexual reproduction with meiotic recombination may be an important source of genetic variation based on recent studies showing that most field isolates were heterothallic and polyploid [3, 9]. The *B. cinerea* genome also contains a retroelement, Boty, a polymorphic dispersed repeated DNA sequence with a long terminal

repeat. This is believed to be responsible for the accumulative sequence divergency due to genetic drift and rearrangement [7].

Our data on RAPD analyses revealed that isolates collected during the last 15 years in Korea contained a great deal of genetic variation, which is in agreement with previous studies [4]. However, it was interesting that two strains, DS522 and BY3, collected from different geographic locations and different hosts in different years had identical RAPD phenotypes. This genotype might represent a stable haploid status of these isolates. The fact that six monoconidial tomato isolates, JM1, JM2, JM3, JM4, JM42, and JM43, from the same field showed high genetic diversity ranging from 36% to 82% similarity between isolates, indicated the genetic diversity in a small field was equal to that of the genetic diversity, 27% to 91%, throughout the country. This high variation within a small tomato field, in turn, might make it difficult to apply efficient disease management strategies. Three of these tomato isolates, JM1, JM2, and JM42, comprised exclusively of RAPD group 4 (G4) with only minor variations, appear to be clonal or homotype [16]. This clonal variation suggests that not only phenotypic diversity but also genetic variation in asexual progeny occurred because of the multinucleate and heterokaryotic nature of Korean isolates of *B. cinerea*. Although sample sizes are very limited, the molecular phenotypes of isolates from Moonsan, a restricted small area, seemed to have changed rapidly so that isolates of *B. cinerea* displayed an additional molecular phenotype in only two years (1993 to 1995). Further studies concerning temporal and spatial genetic variability in field isolates are currently being conducted in our laboratory.

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