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Estimation of Genetic Variation of Korean Isolates of *Phytophthora capsici* by Using Molecular Markers

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Genetic diversity of 21 Korean *Phytophthora capsici* isolates was analyzed by using several molecular markers such as random amplified polymorphic DNA (RAPD), M-13, microsatellite and random amplified microsatellite sequences (RAMS). The overall average similarity coefficient among the isolates was 86% based on the combined data obtained by the molecular markers. No molecular markers were found to be associated with hosts or geographic regions. In addition to RAPD, analysis based on repeated sequences such as $(GTG)_5$, M-13 and RAMS could be used to assess population structure of *P. capsici*.

KEYWORDS: *Phytophthora capsici*, Genetic diversity, Molecular marker, RAPD, RAMS, Microsatellite, M-13

Phytophthora blight caused by *Phytophthora capsici* is a serious disease on solanaceous species including pepper, cucumber, watermelon, tomato and melon. The phytophthora blight is a major limiting factor on the crop productions. *P. capsici* is a heterothallic oomyceteous fungus with two mating types and produces oospores by sexual reproduction. Although morphological and physiological features of this fungus have been well studied, studies on population structure of *P. capsici* are limited. In epidemiology, it is important to know the population structure of pathogens which is able to provide critical informations about disease control regarding the potential for the development of pathogenic specialization and fungicide resistance.

In 1991, Hwang *et al.* found significant variation in restriction fragment length polymorphism (RFLP) with mitochodrial DNA of *P. capsici* isolates from various pepper-growing countries. Oh and Kim (1992) reported that the sensitivity of *P. capsici* to metalaxyl varied among Korean isolates. Godwin and Coffey (1995) demonstrated considerable morphological and physiological diversity among *P. capsici* isolates and revealed two subgroups within a species on the basis of isozyme analysis.

In recent, several molecular biological methods based on polymerase chain reaction (PCR) have been succesfully used to estimate genetic variations in fungal population. Random amplified polymorphic DNA (RAPD) markers were used to differentiate among isolates of *Colletotrichum graminicola* (Guthrie *et al.*, 1992). Wolfe *et al.* (1992) observed high levels of genetic variation with three RAPD primers in 600 isolates of *Blumeria graminis f. sp hordei* from Europe. Repetitive DNA markers with tandem repeats of a consensus sequence have also been used to study intraspecific genetic variations in fungi. PCR amplification with the M-13 forward sequencing primer allowed for strain typing in *Lentinula edodes* (Kwan *et al.*, 1992). Intraspecific genetic variation in *Heterobasidion annosum* was revealed by M-13 marker (Stenlid *et al.*, 1994). Freeman and Rodriguez (1997) used microsatellites such as $(CAG)_5$ and $(GACA)_4$ to estimate genetic variation among strawberry isolates of *Colletotrichum acutatum*. Hantula *et al.* (1997) revealed a considerable genetic variation among *Phytophthora cactorum* isolates using random amplified microsatellite sequence (RAMS) technique which combines characters of RAPD and microsatellite analysis.

In this study, we examined genetic variations among Korean *P. capsici* isolates from various hosts and locations by using several molecular markers. Also we showed usefulness of these molecular markers for population genetic studies of *Phytophthora* species.

Materials and Methods

Isolates. *P. capsici* isolates used in this study are listed in Table 1. The isolates were collected from various hosts including pepper, watermelon, melon, squash and tomato. The isolates were collected from 20 different locations between 1996 to 1997. Isolates were grown in potato dextrose broth in 250 ml conical flask on an orbital shaker at 25°C. After 6 day-incubation, mycelia were harvested by vacuum filtration, frozen overnight at −20°C. The mycelia were lyophilized and stored at room temperature (RT).

DNA preparation. Lyophilized samples were ground in liquid nitrogen with mortar and pestle. Mycelial powder was suspended in 1 ml of extraction buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 100 mM EDTA pH 8.0)

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Table 1. List of *Phytophthora capsici* isolates used in this study

I.D. No.	Isolate	Host	Location	Province
1	Pa-011	Pepper	Cheongsong	Kyungpook
2	Pa-014	Pepper	Youngyang	Kyungpook
3	Pa-028	Pepper	Jincheon	Chungbuk
4	Pa-061	Pepper	Kwesan	Chungbuk
5	Pa-094	Pepper	Youngam	Chonnam
6	Pa-107	Pepper	Mooju	Chonbuk
7	Pa-138	Pepper	Youngwall	Kangwon
8	Pa-139	Pepper	Seongju	Kyungpook
9	Pa-144	Pepper	Yesan	Kyungpook
10	Pa-146	Pepper	Chongyang	Chungbuk
11	Pa-160	Pepper	Changyoung	Kyungnam
12	Pa-165	Pepper	Hapcheon	Kyungnam
13	P-9506	Tomato	Haenam	Chonnam
14	P-9512	Tomato	Kimcheon	Kyungpook
15	P-9540	Squash	Suwon	Kyonggi
16	P-9542	Squash	Youngdong	Kyungpook
17	P-9639	Watermelon	Youngam	Chonnam
18	P-9650	Watermelon	Naju	Chonnam
19	P-97131	Watermelon	Yesan	Chungnam
20	P-9649	Watermelon	Youngam	Chonnam
21	P-9633	Melon	Gongju	Chungnam

by vortexing. Twenty microliters of 20% sodium dodecyl sulfate (SDS) was added, mixed, and the tubes were incubated at RT with gently shaking for 1 h. Seventy-five microliters of 5 M NaCl and 65 μl of 10% CTAB/NaCl were added, and incubated at 65°C for 20 min. An equal volume of phenol/chloroform/isoamylalcohol (25 : 24 : 1) was added to denature proteins, and then centrifuged at 12,000 rpm at RT for 10 min. The upper aquaeous phase was transfered to a new tube, and DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 0.6 volume of cold isopropanol. Then the tubes were centrifuged at RT for 5 min. The pellet was rinsed twice with 70% ethanol and dried in clean chamber for 1 h. The pellet was dissolved in 70 μl of Tris-EDTA (TE : 10 mM Tris-HCl and 1 mM EDTA pH 8.0) plus 3 μl of ribonuclease (10 mg/m*l*) and incubated at 37°C for 1 h. The DNA was reprecipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume of ethanol at 4°C for 20 min, centrifuged, and dried. The DNA was resuspended in 70 μl of TE buffer. The amount of DNA was measured by mini-gel method.

Random amplified polymorphic DNA (RAPD). Amplification of DNA was carried out in volumes of 50 μl containing 5 μl of 10X Taq DNA polymerase reaction buffer, 2 mM $MgCl_2$, 200 uM of each dNTP, 0.5 uM random primer, 20 ng of template DNA, and 4 unit of *Taq* polymerase (Promega, U.S.A.). In preliminary tests, 35 Operon primers were screened with 5 isolates to select primers which exhibit good amplification and consistent band pattern. Finally, 8 primers were selected for estimation of genetic variation with all the isolates (Table 1). Amplification was performed in a Ericomp thermocycler (Ericomp, U.S.A.) programmed for 40 cycles of 20 sec at 94°C, 30 sec at 36°C and 45 sec at 72°C. A final extension step of 72°C for 5 min was included in the program.

Arbitrarily primed PCR (AP-PCR). For AP-PCR, primers were derived from microsatellites and minisatellites such as $(GTG)_5$, $(GACA)_4$, $(CAT)_5$ and M-13 core sequence. All primers were synthesized form Bioneer Inc., Korea. PCR reactions were performed in a total of 25 μl containing 10 ng template DNA, 2.5 μl of 10X reaction buffer, 5 mM $MgCl_2$, 200 uM dNTP, 4 unit of *Taq* DNA polymerase and 2 uM primer. DNA was amplified in the thermocycler for 30 cycles of 30 sec at 94°C, 30 sec at either 52°C for M-13 or 48°C for $(GTG)_5$, $(CAT)_5$ and $(GACA)_4$ and 60 sec at 72°C. Final extension was carried out for 7 min at 72°C.

RAMS PCR. Three primers were synthesized from Bioneer Inc., Korea (Table 2). The amplification was carried out in 40 μl reaction mixture containing 10 ng of template DNA, 4 μl of *Taq* DNA polymerase reaction buffer, 1.5 mM $MgCl_2$, 200 uM dNTP, 4 unit of *Taq* polymerase and 1 uM primer. DNA was amplified in a thermocycler for 35 cycles of 30 at 94°C, 45 sec either at 61°C for CCA-primer and CGA-primer or 58°C for GT-primer and 2 min extension at 72°C, followed by 5 min final extension at 72°C.

Electrophoresis. Amplification products were separated by electrophoresis in 1.5% agarose gel. The gel was run in TBE-buffer and the amplification products were visualized by ethidium bromide staining under UV light.

Data analysis. Genetic variation among isolates was assessed

Table 2. List of DNA primers

	Primer	DNA sequences (5'-3')
RAPD primer	OPA-01	CAGGCCCTTC
	OPA-03	AGTCAGCCAC
	OPA-10	GTGATCGCAG
	OPB-04	GGACTGGAGT
	OPB-08	GTCCACACGG
	OPB-14	TCCGCTCTGG
	OPC-05	GATGACCGCC
	OPF-13	GGCTGCAGAA
Micro-satellite	(GTG)5	GTGGTGGTGGTGGTG
	(GACA)4	GACAGACAGACAGACA
	(CAT)5	CATCATCATCATCAT
RAMS primer	CGA-RAM	DHBCGACGACGACGACGA
	GT-RAM	YHYGTGTGTGTGTGTGTG
	CCA-RAM	DDBCCACCACCACCACCA
Mini-satellite	M-13	CGCCAGGGTTTTCCCAGTCACGAC

*The following designations are used for degenerate sites: H used in this study (A, T, or C), B used in this study (G, T, or C), Y used in this study (G, A, or C) and D used in this study (G, A, or T).

by the PCR-amplified band profiles generated by primer-isolate combinations. Amplified DNA fragments were scored for all isolates based on two possible character state 0 (fragment absent) and 1 (fragment present). Unique bands or band profile associated with host or geographic region were screened by comparing all primer-isolates PCR products. NTSYS-PC software (Version 1.8) was used to compute the matrix of similarity coefficients among isolates (Rohlf, 1993). Unweighted pair-group arithmetic average (UPGMA) analysis was used to examine genetic relatedness among isolates.

Results

Twenty-one isolates of *P. capsici* were analyzed to estimate the level of genetic diversity by using several molecular markers such as RAPD, M-13, microsatellite and RAMS. In RAPD analysis, 12 out of 35 primers produced amplification products. Eight primers which consistently generated reproducible bands were selected for future studies. The eight random primers (Table 2) produced a total of 67 amplification products in 21 isolates. Among 67 RAPD markers, 50 monomorphic and 17 polymorphic bands were observed. The number of amplified products generated by each primer varied from 4 to 9. Variation in the specific pattern of one isolate compared to others was inconsistent in that it could be observed with one primer but not with another. Most primers produced polymorphic bands (Fig. 1) whereas C05 and F13 primers produced identical bands for all isolates (data not shown). No primer amplified a band and band pattern specific to the host or geographic region. Similarity coefficients among isolates ranged from 78 to 92%.

P. capsici DNA was successfully amplified with the M-13 primers. Fifteen bands were amplified with 5 polymorphic bands (Fig. 2). In DNA amplification with microsatellite primers, only $(GTG)_5$ primer produced amplified products. No amplification products were obtained with $(GACA)_4$ and $(CAT)_5$ primers. Amplification of DNA with $(GTG)_5$ produced 11 bands, 3 out of 11 were polymorphic.

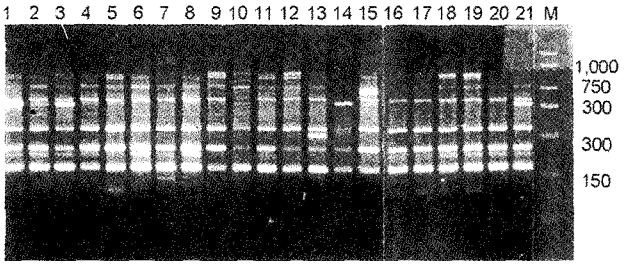

Fig. 1. Agarose gel electrophoresis of PCR products with OPB-08 primer. M. Molecular marker, Lanes 1 to 21 are isolates with I.D. number 1 to 21.

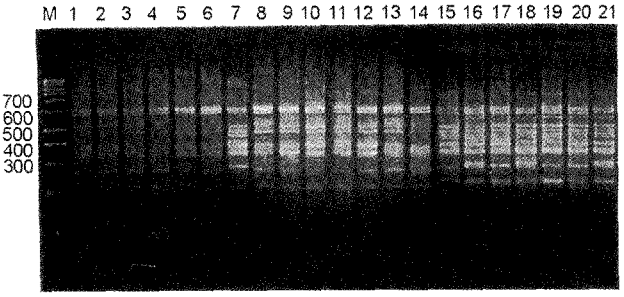

Fig. 2. Agarose gel electrophoresis of PCR products with M-13 primer. M. Molecular marker, Lanes 1 to 21 are isolates with I.D. number 1 to 21.

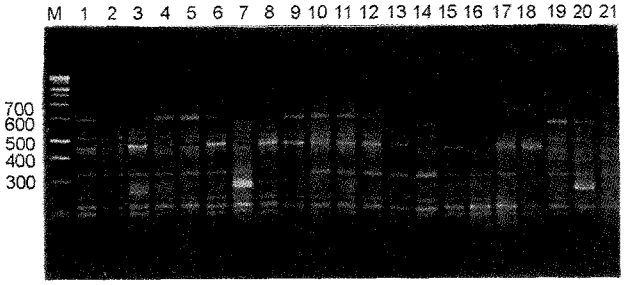

Fig. 3. Agarose gel electrophoresis of PCR products with CGA-RAM primer. M. Molecular marker, Lanes 1 to 21 are isolates with I.D. number 1 to 21.

In RAMS analysis, 12 and 7 detectable bands were obtained with CGA-primer and GT-primer, respectively (Fig. 3). Total 8 out of 19 bands amplified with CGA and GT-primers were polymorphic. Amplification products with CCA-primer was not scored in the analysis due to poor

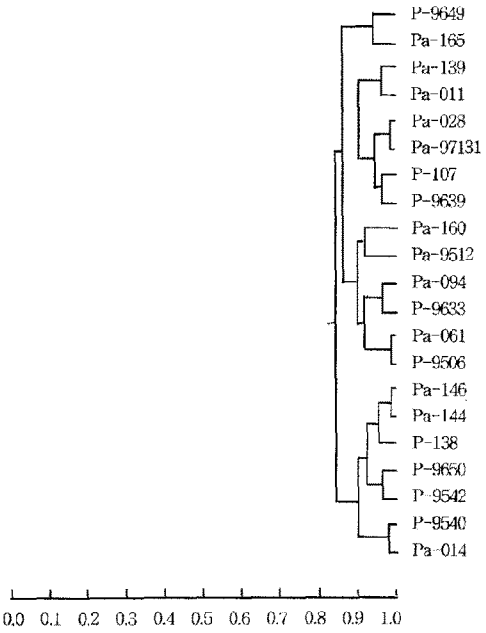

Fig. 4. Dendogram showing relationships among the 21 *Phytophthora capsici* isolates.

amplification. Similarity matrices based on RAMS markers showed similarity coefficients ranged from 75 to 100% among isolates. Band and band pattern specific for host or geographic region was not observed (Fig. 4). In order to assess overall genetic diversity of the population, all the data by molecular markers based on RAPD, minisatellite, microsatellite and RAMS were combined and estimated. Overall average similarity coefficient was 86% ranging from 78 to 93%.

Discussion

Twenty-one Korean isolates of *P. capsici* were analyzed to estimate the level of genetic diversity. Although PCR is widely used as a molecular tool in population genetics, population analysis of plant pathogens which rely only on one class of PCR-based DNA marker especially based on arbitrary primer may result in an over- or underestimation of genetic diversity of the population. A drawback of PCR markers based on arbitrary primer is that a similar band in different isolates may be generated by different DNA fragment of the same size. Also, the absence of a particular band is no guarantee that corresponding fragment is not present in genome, but may simply reflect the fact that the fragment has not been amplified. In order to overcome these limitations, population analysis with several classes of molecular markers may provide more accurate assessment of population structure of plant pathogens.

In this study, repetitive DNA markers including minisatellite, microsatellite, RAMS as well as RAPD were used to assess the genetic diversity of Korean *P. capsici* isolates. Our results showed that all these markers provided a suitable tool for the detection of genetic diversity among *P. capsici* isolates. RAPD markers produced high DNA polymorphism. This is not surprising considering that RAPD examines whole genomic DNA rather than specific region.

PCR with M-13 and $(GTG)_5$ primer produced reliable and sensitive genetic markers for assessing genetic diversity in *P. capsici*. Failure of amplification with $(GACA)_4$ and $(CAT)_5$ primer may suggest that the corresponding sites for each primer are absent in genome or distance between microsatellite sequence are too far to be amplified. Although the presence of microsatellite in *P. capsici* has not been widely investigated, amplification with $(GTG)_5$ primer may reflect that the sequence is dispersed through the genome of *P. capsici*.

The RAMS technique originally described by Zietkiewicz *et al.* combines the benefits of RAPD and microsatellite analysis. Hantula *et al.* have demonstrated it to be applicable for detecting genetic variation among *P. cactorum* isolates. Considerable amount of genetic variation of *Phlebiopsis gigantia* was revealed by RAMS analysis. In our experiment, two RAMS primers successfully produced DNA polymorphism among isolates of *P. capsici*.

In our preliminary studies, we observed that all the markers successfully amplified in this study were able to use to assess the genetic diversity of several other *Phytophthora* species including *P. cactorum, P. nicotianae, P. drechsleri,* and *P. cinnamomi* (data not shown). Therefore, these molecular markers are considered to be useful for population genetic analysis in *Phytophthora* species.

For fungus like *P. capsici* with a sexual mating system, genetic variation among isolates might be expected. Hwang *et al.* (1991) examined the genetic variation of *P. capsici* isolates of world-wide collections from pepper using mt-DNA RFLP and divided isolates into 4 groups. They reported that Korean isolates belong to group I and II. Oh and Kim (1992) reported that sensitivity to metalaxyl varied significantly with Korean *P. capsici* isolates. These previous reports represent a possible presence of genetic variation among Korean *P. capsici* isolates. Although the level of genetic variation is not high, our results showing the average of 86% similarity coefficient also demonstrated the presence of genetic variation in Korean *P. capsici* population. For oomyceteous fungi which produce sexual oospore, relatively not high level of genetic variation might be due to inbreeding or the dominance of asexual reproduction with little activity of oospore during life cycle. Soil-inhabiting fungus like *P. capsici* is prone to inbreeding because of limited dispersal capability. Future studies on the genetic variation of isolates within population in defined area and the field observation of the frequency of oospores will be needed to give some insight into this explanation.

In our study, no molecular markers were found to be associated with hosts or geographic regions. In several fungi, molecular population analysis showed that the level of genetic diversity among isolates was considerable and that no groupings associated with host and geographic region could be identified (Gosselin *et al.*, 1995; Peltonen *et al.*, 1996). There might be two possible explanations for this phenomenon. One is that the primers used were neutral markers for these characters. The other is that no host and geographic specificity exist among isolates. Peltonen *et al.* (1996) assessed the genetic variation of *Drechslera teres* population showing high genetic diversity with RAPD markers. However, the isolates could not be grouped according to geographic origin and host. Since no host specificity of *P. capsici* on the basis of biological test has been reported, the absence of molecular marker associated with a host in *P. capsici* isolates may suggest that biotypes or geographic races do not exist in *P. capsici*. Forster *et al* (1990) observed high degree of mt DNA polymorphism in *P. capsici* isolates from several different hosts. But they concluded that no RFLP group of mitochondrial DNA correlated with either host or geographic region. Our results are consistent with this previous examination.

Although our studies give basic informations about the level of genetic variation in population of *P. capsici,* population studies using RFLP of mitochondrial DNA will be useful to reveal the population structure of this fungus. The presence of genetic diversity of *P. capsici* population may indicate that pathogen population has a genetic potential for the development of fungicide-resistant population or pathogenic specialization. Continuous monitoring for population structure of *P. capsici* will be important for epidemiology and plant disease control of the pathogen.

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