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RAPD Analysis for the Evaluation of Genetic Diversity Among the *Fusarium* Species from Various Sources

Hei-Sun Choi[1], Kyong-Soo Kim[1], Myong-Jo Kim[1], Jae-Ouk Shim[2], Byong-Sup Kim[3], Min-Woong Lee[2] and Youn-Su Lee[1]*

[1]Div. of Applied Plant Sciences, Kangwon National University, Chuncheon 200-701
[2]Dongguk University, Seoul 100-715, Korea
[3]Korea Research Institute of Chemical Technology, Taejon 305-606, Korea

각종 작물로 부터 분리한 *Fusarium*속 균의 RAPD 기법을 이용한 유전분석

최혜선[1] · 김경수[1] · 김명조[1] · 심재욱[2] · 김병섭[3] · 이민웅[2] · 이윤수[1]*
[1]강원대학교 농업생명과학대학 식물응용과학부, [2]동국대학교 응용생물학과
[3]한국화학연구소 스크리닝연구센타

ABSTRACT: In this study, we evaluated the use of RAPD method to discriminate among strains of *Fusarium* species including *F. oxysporum* and f. sp. of *F. oxysporum*. As a result of the amplication, fifteen primers showed total 180 bands ranging from 0.2 to 3 Kb. Among those 180 bands, 126 polymorphic bands were used for bionominal matrix code (0, 1), and UPGMA dendrogram analysis. *Fusarium oxysporum* isolate 355 showed high similarity with *F. oxysporum* isolate 358 at 0.9603. *Fusarium roseum* isolate 87 and *F. oxysporum* isolate 358, *F. o.* f. sp. *lycopersici* isolate 69 and *F. o.* f. sp. *melongena* 68 showed low similarity of 0.3809. *Fusarium oxysporum* isolate 361 and *F. o.* f. sp. *raphani* isolate 218 showed similarity of 0.8730, *F. oxysoprum* isolate 354 and unidentified *Fusarium* sp. isolate 228 showed similarity matrix of 0.7936, and *F. roseum* isolate 87 and *F. o.* f. sp. *raphani* isolate 57 showed similarity matrix of 0.5873.

KEYWORDS: *Fusarium* sp., *F. oxysporum*, PCR, RAPD

Complex communities of fungal pathogens are involved in various diseases of many crops. In temperate regions, *Fusarium* species, including *F. oxysporum* and f. sp. of *F. oxysporum*, are the most abundant and aggressive pathogens. Infections of seedlings, roots and basal stems of plants are initiated by seed- or soilborne-inoculum, and major yield losses result from damaged plants.

The asexual fungus *Fusarium oxysporum* is a common inhabitant of soil and has a worldwide distribution. All strains of *F. oxysporum* are successful as saprophytes and are able to grow and survive for long periods on organic matter in soil. However, some strains are responsible for vascular wilt disease on many plants of economical importance (Armstrong and Armstong, 1981). These pathogenic strains show a high level of host specificity and are classified on this basis into more than 120 formae speciales and races. These pathogenic strains are not identifiable using phenotypic characters. Molecular tools have been used to characterize the diversity among pathogenic strains of *F. oxysporum* with the aim of determining genetic relatedness among formae speciales (Kim *et al.*, 1993; Kistler *et al.*, 1991; Namiki, 1994).

Pathogenic and nonpathogenic strains of *F. oxysporum* played an important role in the

*Corresponding author

ecology of *Fusarium* disease. Therefore, it would be interesting to be able to characterize the diversity among populations of nonpathogenic and pathogenic *F. oxysporum*.

Genetic variation within populations of *F. oxysporum* was assessed by vegetative compatibility analysis (Correll *et al.*, 1986; Elias *et al.*, 1991). These studies were based on the selection of nitrate non-utilizing mutants for each isolate and performing complementary pairing of the deficient mutants. This is a time consuming and not always compatible with broad application in ecological studies that require screening of large populations. In addition, this method could not provide information about genetic relatedness between strains that were not vegetatively compatible.

Direct analysis of DNA polymorphism is a more general approach to establishing genetic variation in organisms. Molecular methods involving the use of the polymerase chain reaction (PCR) have been recently described to resolve genetic variation between strains. DNA amplification by PCR with primers directed against repetitive elements, arbitrarily chosen or defined, is another strategy that can be called interrepeat PCR. The analysis of random amplified polymorphic DNA (RAPD) was proposed for discrimination between isolates of *F. oxysporum* within a particular forma specialis (Assigbetse *et al.*, 1994; Grajal-Martin *et al.*, 1993; Manulis *et al.*, 1993). In this study, we evaluated the use of RAPD method to discriminate among strains of *Fusarium* species including *F. oxysporum* and f. sp. of *F. oxysporum*.

Materials and Methods

Fungal strains

A collection of strains of *F. oxysporum* was made by either direct isolation from soils cultivated with various crops or from plant parts showing symptoms of disease (Table 1). Isolates were collected by single spore transfer and were stored by cryopreservation at −

Table 1. Isolates of *Fusarium* spp. from various locations and sources

Isolate No.	*Fusarium* species	Sources	Locations
80	*F. oxysporum*	carnation	Suwon
214	*F. oxysporum*	cucumber	Japan
354	*F. oxysporum*	gladiolus	Suwon
355	*F. oxysporum*	gladiolus	Kimhae
358	*F. oxysporum*	spinach	Whasung
361	*F. oxysporum*	−	Unknown
218	*F. o.* f. sp. *raphani*	turnip	Haenam
69	*F. o.* f. sp. *lycopersici*	tomato	Japan
68	*F. o.* f. sp. *melongena*	egg plant	Japan
64	*F. o.* f. sp. *melonis*	melon	Japan
57	*F. o.* f. sp. *raphani*	turnip	Taejon
72	*F. o.* f. sp. *cucumerinum*	cucumber	Chungyang
87	*F. roseum*	pepper	Suwon
223	*Fusarium sp.*	−	−
212	*Fusárium sp.*	pepper	Taejon
228	*Fusarium sp.*	strawberry	Gongju
369	*Fusarium sp.*	arrowhead	Taejon
372	*Fusarium sp.*	arrowhead	Taejon
492	*Fusarium sp.*	egg plant	Taejon
224	*Fusarium sp.*	−	−

70°C. The strains were characterized morphologically as *F. oxysporum* and other unidentified *Fusarium* species. Their pathogenicity was not investigated on those host plants.

DNA extraction

For large-scale preparations of pure DNA, fungal strains were cultivated at 25°C for seven days with shaking (150 rpm) in 500 m*l* of potato-dextrose broth inoculated with a spore suspension. The mycelium was harvested by filtration, washed, lyophilized, and ground with liquid nitrogen. A 1.5 m*l* microtube was filled halfway up to the conical portion with ground lyophilized mycelium, and 750 m*l* of extraction buffer (500 m*l* Nacl, 100 mM Tris-Hcl pH 8.0, 50 mM EDTA, 1.25% SDS) was added. The extraction then was performed by following Rogers and Bendich's procedure (Roges and Bendich, 1988). Finally, DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) with a final concentration of 2 μg/μ*l* and stored at 4°C until use.

PCR reaction

For PCR amplication, 10 ng of genomic DNA was added into 20 mM dNTP and 0.5 μM of random primers (Operon Tech., Inc., USA) (Table 2). One unit of Taq polymerase (Dynazyme^{TM}) was then mixed and the final volum of 20 μ*l* was used for the reaction. Reaction conditions were consisted of 5 min at 95°C (preheating), 1 min at 94°C, 1 min at 35°C, 2 min at 72°C for 45 cycles followed by storage at 4°C. Ten or fifteen μ*l* of the amplified reaction products were run in 1.5% agarose gel containging ethidium bromide (0.5 μg/m*l*) in 0.5×TBE buffer for two hours and the results were viewed on UV transilluminator.

Data analysis

A computer program, NTSYS-pc, Version 1.61 (Rohlf, 1990), was used for analysis of the electrophoretic data. Simple matching coefficients (Ssm) for each pair of isolates were calculated as described by Sneath and Sokal (1973) by formular: $S_{sm}=m/(m+u)$ where m=the number of bands found in common between two isolates, and u=the total number of bands unique to each sample. The matrix of similarity coefficients were then subjected to a cluster algorithm, the unweighted pair-group method with an arithmatic average (UPGMA), that was then used to generate a dendrogram (Romesburg, 1984; Sneath and Sokal, 1973).

Table 2. List of primers (10-mer) and their base sequences used for RAPD analysis

Primer No.	Base sequences	No. of bands
OPD-1	CAGGCCCTTC	11(7)[a]
OPD-2	TGCCGAGCTG	10(7)
OPD-3	AGTCAGCCAC	10(5)
OPD-5	AGGGGTCTTG	11(8)
OPD-7	GAAACGGGTG	13(9)
OPD-8	GTGACGTAGG	12(8)
OPD-9	GGGTAACGCC	12(6)
OPD-10	GTGATCGCAG	14(9)
OPD-11	CAATCGCCGT	14(10)
OPD-12	TCGGCGATAG	12(10)
OPD-13	CAGCACCCAC	10(4)
OPD-14	TCTGTGCTGG	10(9)
OPD-15	TTCCGAACCC	12(12)
OPD-16	AGCCAGCGAA	16(11)
OPD-17	GACCGCTTGT	13(11)
Total		180(126)

[a]The number in the parentheses are the numbers of polymorphic bands for each.

Results and Discussion

Genomic DNA was extracted from the mycelium of the total twenty isolates of *Fusarium* species, and the amplified genomic DNA showed bands with different molecular weights. As a result of the PCR reaction, 15 OPD primers produced total 180 bands. Among these 180 bands, 126 polymorphic bands were observed and their sizes ranged form 0.2 Kb to 3 Kb. Bionominal matrix code (1; presence of

Table 3. Similarity matrix based on the number of shared bands by the compared *Fusarium* species on 1.5% agarose gel in RAPD analysis[a]

	72	87	80	214	354	355	358	361	218	69	68	64	57	223	212	228	369	372	492	224[b]
87[b]	0.5238																			
80	0.6587	0.4682																		
214	0.7460	0.5555	0.5000																	
354	0.5873	0.5396	0.5634	0.5396																
355	0.6111	0.3888	0.5396	0.5634	0.5634															
358	0.5714	0.3809	0.5000	0.5714	0.5238	0.9603														
361	0.7063	0.5158	0.5396	0.8650	0.5476	0.6190	0.6269													
218	0.7222	0.5158	0.5714	0.7698	0.5952	0.6349	0.6428	0.8730												
69	0.6349	0.4444	0.5158	0.6190	0.5714	0.7222	0.7301	0.6746	0.6904											
68	0.5079	0.5555	0.5000	0.5079	0.5396	0.4365	0.4285	0.4682	0.4523	0.3809										
64	0.6666	0.4920	0.5000	0.7777	0.5396	0.5634	0.5714	0.7857	0.6904	0.6031	0.4761									
57	0.4920	0.5873	0.5476	0.4761	0.5079	0.5000	0.4603	0.4523	0.4523	0.5079	0.5396	0.4920								
223	0.4523	0.4365	0.4603	0.4841	0.5952	0.4603	0.4682	0.5238	0.5396	0.5634	0.4523	0.4365	0.5000							
221	0.7063	0.4682	0.5714	0.7222	0.5952	0.6190	0.6269	0.7619	0.7142	0.6587	0.4523	0.7222	0.5158	0.5396						
228	0.5873	0.4920	0.5634	0.5555	0.7936	0.5634	0.5396	0.5793	0.5793	0.6031	0.4920	0.5238	0.5238	0.5793	0.5952					
369	0.4841	0.5793	0.5238	0.5158	0.4841	0.4920	0.4682	0.5238	0.5396	0.4523	0.5634	0.5476	0.4682	0.4603	0.5079	0.5000				
372	0.5238	0.5079	0.5317	0.4761	0.5714	0.4682	0.4444	0.4523	0.4841	0.4761	0.5714	0.4126	0.5079	0.5317	0.5158	0.5714	0.4841			
492	0.5317	0.5634	0.5396	0.4841	0.5634	0.4603	0.4523	0.4920	0.4920	0.5000	0.5793	0.4365	0.4841	0.5714	0.5714	0.5952	0.5714	0.6269		
224	0.4444	0.5238	0.5000	0.5079	0.6190	0.4682	0.4603	0.5000	0.5158	0.5079	0.5396	0.4761	0.5555	0.6746	0.5158	0.5555	0.3730	0.5714	0.5476	

[a]Data matrix was made by scoring the presence or absence of the bands as 1 and 0, respectively. Similarity coefficients between two isolates were then calculated with modified formular of Sneath and Sokal (1973).

[b]Isolate numbers.

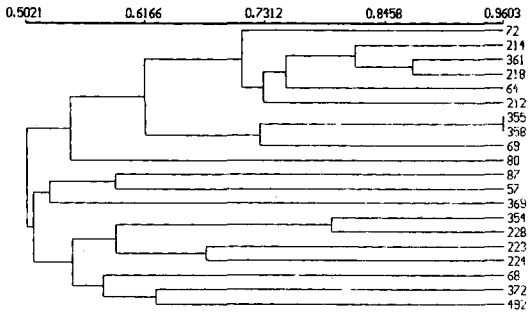

Fig. 1. UPGMA dendrogram showing the relationship among the *Fusarium* species based on the bands on 1.5% agarose gel in RAPD.

band, 0; absence of band) was constructed with 180 bands reacted with 15 primers. Based on the bionominal matrix code constructed, similarity matrix was made (Table 3) with NTSYS-Pc (Rohlf, 1990). *F. oxysporum* isoaltes 355 and 358 showed the highest similarity of 0.9603 among the compaired isolates. *Fusarium* sp. isolates 369 and 224 showed the lowest similarity of 0.3730. *F. oxysporum* isolate 80 showed the similarity of 0.5714 with unidentified *Fusarium* sp. isolates 228 and 212, and showed low similarity of 0.4603 with *Fusarium* sp. isolate 223. *F. oxysporum* isolates 214 and 361 showed high similarity of 0.8650, and isoalte 214 showed low similarity of 0.4761 with isolate 372. *F. oxysporum* isolate 354 showed similarity of 0.7936 with *Fusarium* sp. isolate 228 and of 0.4841 with *Fusarium* sp. isolate 369. *Fusarium* sp. isolate 355 showed similarity of 0.4603 both with the isolates 223 and 492. *F. oxysporum* isolate 358 showed similarities of 0.7301 and 0.4285 with *F. o.* f. sp. *lycopersici* isolate 69 and *F. o.* f. sp. *melongena* isolate 68, respectively. *F. oxysporum* isolate 361 showed similarity of 0.8730

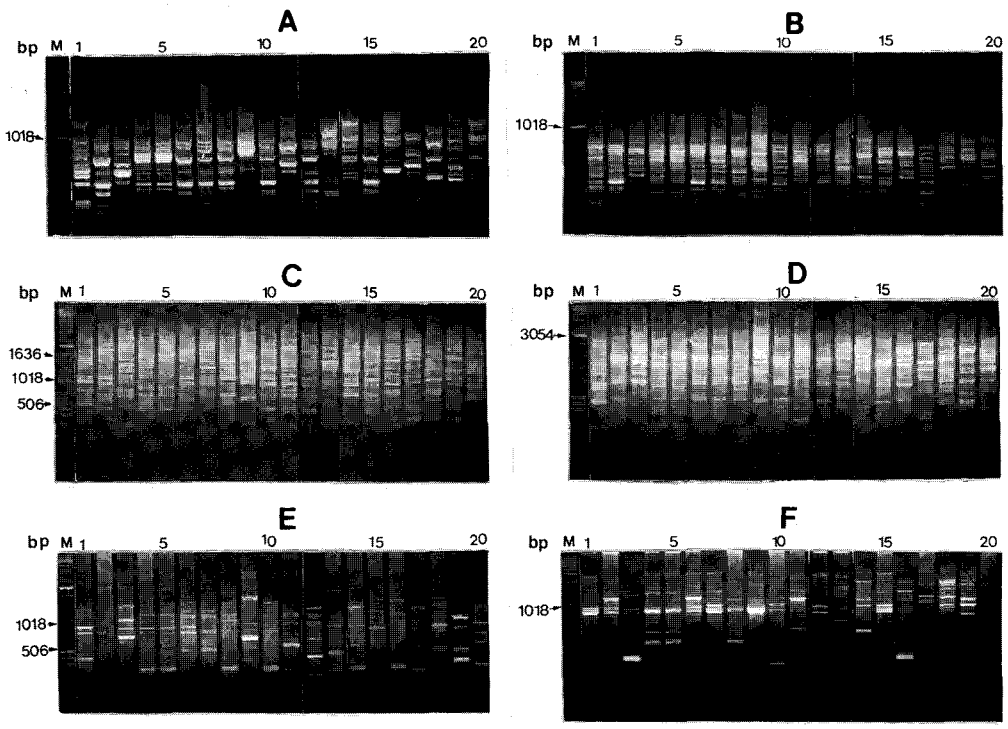

Fig. 2. PCR amplified DNA fragments using the primers listed in Table 2. A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O indicate the primer number OPD-1, -2, -3, -5, -7, -8, -9, -10, -11, -12, -13, -14, -15, -16, and -17, respectively. The numbers on top of the lane indicate the *Fusarium* isolates shown in Table 1 in order.

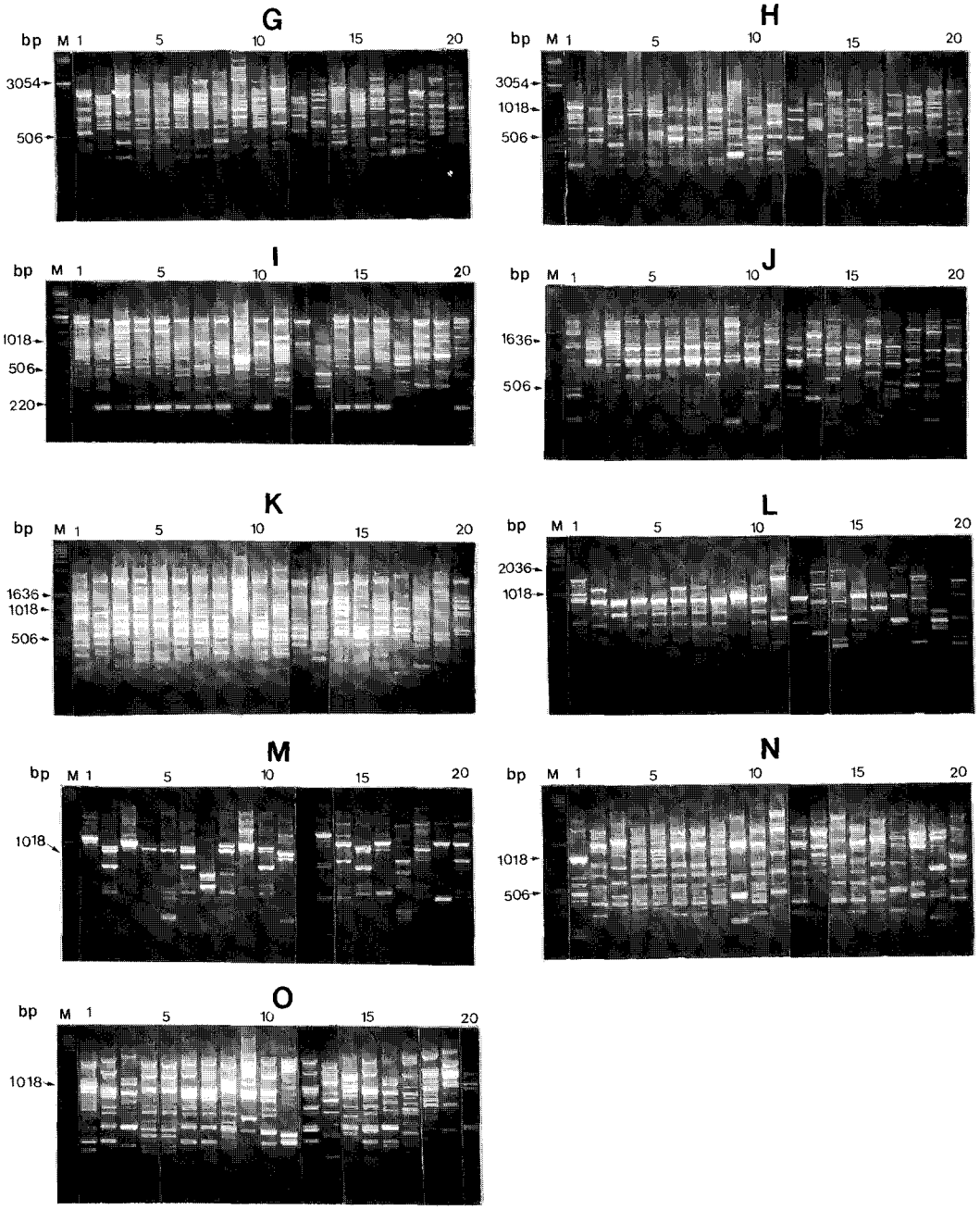

Fig. 2. continued.

with *F. o.* f. sp. *raphani* isolate 218 and of 0.4523 with isolates 57 (*F. o.* f. sp. *raphani*) and 372 (*Fusarium* sp.). *F. roseum* isolate 87 and *F. oxysporum* showed low similarity of 0.3809. *F. o.* f. sp. *cucumerinum* isolate 72 showed similarity of 0.7460 with *F. oxysporum* isolate 214, and showed similarity of 0.444 with *Fusarium* sp. isolate 224. *F. o.* f. sp. *melonis* isolate 64 showed similarity of 0.7222 with 212, and showed similarity of

0.4126 with *Fusarium* sp. isolate 372. Based on these results, UPGMA dendrogram was constructed to show the relationships among the compaired isolates (Fig. 1). The UPGMA dendrogram showed the compaired *Fusarium* isolates could be divided into 2 different groups. The first group (from isolate 73 [*F. o.* f. sp. *cucumerinum*] through isolate 80 [*F. oxysporum*]) can be divided into three sub-groups. The second group (from isolate 87 [*F. roseum*] through isolate 492 [*Fusarium* sp.] can be divided into four sub-groups.

적 요

*Fusarium*을 간단한 방법으로 DNA를 추출한 후, 유전적 다형성을 검출하는 방법인 RAPD(Randomly Amplified Polymorphic DNA)를 이용하여 *Fusarium*균의 유전적 다형성과 계통분류학적 분석을 실시하였다. 20개의 분리균을 배양하여 얻어진 균사체로부터 genomic DNA를 추출하여 PCR을 실시 15개의 primer에서 0.2~3 Kb 크기의 band를 총 180개 얻었다. 180개의 band 중 다형화 현상을 보이는 band는 126개로 이를 이용하여 bionominal matrix code(0,1)을 작성한 후 UPGMA법을 이용하여 각 균주간의 관계를 분석하였다. *Fusarium oxysporum* 균주중 355번(*F. oxysporum*)과 358번(*F. oxysporum*)은 0.9603의 높은 상동성을 보인 반면, *F. roseum* (87번)과 *F. oxysporum* (358번), 69번 (*F. o.* f. sp. *lycopersici*)과 68번(*F. o.* f. sp. *melongena*)은 0.3809의 낮은 상동성을 보였다. 361번 (*F. oxysporum*)과 218번 (*F. o.* f. sp. *raphani*)은 0.8730의 유사도를 354번(*F. oxysporum*), 228번(*Fusarium* sp.)은 0.7936의 유사도, 87번(*F. roseum*)과 57번(*F. o.* f. sp. *raphani*)은 0.5873의 유사도를 가진다.

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