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Electrophoretic Karyotyping by PFGE in the Genus *Fusarium*

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*Fusarium*속에서 PFGE를 이용한 Electrophoretic Karyotyping

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ABSTRACT: Contour-clamped homogeneous electric field gel electrophoresis was used to establish electrophoretic karyotype for 10 species of *Fusarium* sections *Sporotrichiella, Liseola, Gibbosum, Discolor* and *Martiella*. Intact chromosomal DNA was isolated from fungal protoplast and separated under various conditions according to their size in order to improve DNA separation. The numbers of chromosome-sized DNA molecules for individual species ranged from 5-13, with individual chromosomes ranging from 0.78 Mb to 7.20 Mb in size. The total genome DNA size of each species was estimated at about 18.32 Mb to 48.20 Mb. Comparison of karyotype profiles following Southern hybridization analysis with a randomly selected genomic probe of *F. oxysporum* formae speciales *lilii* was carried out.

KEYWORDS: CHEF, Electrophoretic karyotype, *Fusarium*, Southern hybridization analysis

Members of the genus *Fusarium* can be pathogenic on a variety of commerically important plants, and produce significant quantities of mycotoxins, which, if injected, may adversely affect human and animal health. Fungal chromosomes are, in general, too small to be counted using traditional microscopic techniques; our cytogenic knowledge of fungi is therefore extremely limited.

Significant progress in the karyotype analysis of filamentous fungi has been made by the introduction of pulsed field gel electrophoresis (PFGE) techniques. These techniques have made it possible to resolve chromosomes in a size range from 0.1 to 10.0 Mb, depending on electrophoretic parameters (Mills and McClusky, 1990; Skinner *et al.*, 1991). PFGE has been successfully used to separate the chromosomes of a wide range of filamentous fungi (Fekete *et al.*, 1993) and the electrophoretic karyotypes of plant pathogenic fungi representing more than 10 genera have been described (Kim *et al.*, 1993). But very little is known about the genus *Fusarium*, except for some isolates of *F. oxysporum* (Kim *et al.*, 1993; Migheli *et al.*, 1993), *F. poae* and *F. sporotrichioides* (Fekete *et al.*, 1993).

The purpose of this investigation was to clarify the chromosome number, sizes and estimated genome sizes in different *Fusarium* species by PFGE for the first time in some species from this genus. Additionally, Southern hybridization was performed with a randomly selected genomic DNA.

Materials and Methods

Fungal strains

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Table 1. List of Fusarium spp. used in the experiment

Species	Source	Section
Fusarium poae Wollenw	NRRL 3287, USA	*Sporotrchiella*
Fusarium sporotrichioides Sherb.	NRRL 3510, USA	*Sporotrchiella*
Fusarium sporotrichioides Sherb.	C Jungsun 1, Seoul National Univ., Korea	*Sporotrchiella*
Fusarium tricinctum (Corda) Sacc.	NRRL 3299, USA	*Sporotrchiella*
Fusarium anthophilum Wollenw.	7481, Sydney Univ., Australia	*Liseola*
Fusarium proliferaum Nirenberg.	7459, Sydney Univ., Australia	*Liseola*
Fusarium equiseti (Corda) Sacc.	C. Kosung 6, Seoul National Univ., Korea	*Gibbosum*
Fusarium acuminatum ELL. & Ev.	NRRL 13552, USA	*Gibbosum*
Fusarium sambucium Fuckel.	NRRL 13500. USA	*Discolor*
Fusarium caucasium Letov.	ATCC 18791, USA	*Martiella*

The strain number, source and section of the *Fusarium* spp. investigated in these experiments were given in Table 1.

Preparation of chromosomal DNA

Intact chromosomes were obtained from fungal protoplasts using the procedure of Ha *et al.* (1991). The protoplast suspension was poured into a plug mold, solidified at 4°C for 10 min and the agarose plugs were then incubated for 24 hr at 50°C in NDS buffer (0.5 M EDTA, 10 mM Tris-HCl, pH 8.0 and 1% N-lauryl sarcosine) containing 20 mg/mL proteinase K. Subsequently, the plugs were washed three times with TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and were stored at 4°C in TE buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA).

CHEF gel electrophoresis

Electrophoresis was performed using the CHEF system (CHEF Mapper, Bio-Rad, Richmond, CA) at 12°C in circulated 1×TBE buffer (0.09 M Tris-borate, 0.002 M EDTA). The agarose concentrations, field strengths, switching intervals and running times were described in the figure legends. Gels were stained with 2 μg/mL of ethidium bromide (Sigma, USA) in 1.0×TBE buffer, visualized and photographed under a UV transilluminator (UVP ultraviolet transilluminator). *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosome size standards (Bio-Rad) served as marker.

Preparation of probe and Southern blotting

For the preparation of randomly selected genomic DNA probe, total genomic DNA was extracted from *F. oxysporum* f. sp. *lilii* as described by Zolan and Pukkila (1986). Amplification reaction was carried out in total volume of 50 μL in the presence of 10 mM Tirs-HCl (pH 9.0), 50 mM KCl, 2 mM $MgCl_2$, 10% Triton X-100, 0.2 mM each of dNTP and 2 units of Taq polymerase. Genomic DNA from *F. oxysporum* f. sp. *lilii* served as an amplification template and OPA 05 (5'-AGG GGT CTT G-3') (Operon) was used as pimer. The PCR process was performed in an Perkin Elmer DNA Thermal Cycle. 3 kb fragment among several amplified products was extracted from the gel and purified with a procedure according to Vogelstein and Gillespie (1979). This product was labelled with an ECL kit (Amersham, USA) and used as a probe in Southern blotting experiments. The DNA was blotted to Hybond-N^+-nylon membrane (Amersham) by capillary transfer according to Birren and Lai (1993). Hybridization used an ECL direct nucleic labelling and detection system (Amersham, USA).

Result analysis by image analyzer

Gel photographs were scanned with a den-

sitometer to measure the relative intensity of each stained band and were analyzed with a Cream-Image Analyzer (Cream, Kem-En-Tec. Software system, USA).

Results

The majority of the *Fusarium* strains involved in the study produced sufficient numbers of protoplast (10^9/mL), when mycelium younger than 18 hr old was digested with a mixture of 1.5% driselase (Sigma) and 0.5% *Trichoderma* lysing enzyme (Sigma).

After testing variety of running conditions for CHEF gels, we selected two sets of conditions to determine more accurate chromosome sizes and numbers. For the resolution of large- (>4.0 Mb), and medium-sized (2.0~4.0 Mb) chromosomes, 0.55% agarose concentration was used, and the field strength increased from 1.3 V/cm to 3.0 V/cm with switching intervals gradually decreasing from 4500 s to 300 s over a 136 hr running period (Fig. 1A). In order to improve the separation of small-sized chromosomes (<2.0 Mb), the agarose concentration was increased to 0.7% and the running time was decreased to 72 hr (Fig. 1B). The estimated chromosome sizes, numbers and derived genome sizes of *Fusarium* spp. were obtained by comparing and analysing the results shown in Fig. 1A and B, and were listed in Table 2. The chromosomes were numbered according to size, starting with the largest. Size estimation of the each chromosomal DNA band was based on the relative mobility of the chromosome size standards from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. The sizes of larger chromosomal bands than the largest *S. pombe* chromosome (5.7 Mb) were only approximations based on extrapolation from the standard curve, and these chromosomes could actually be much larger than we extimated. Furthermore, the sizes of these largest bands, 7.10~7.20 Mb, were reported for the first report in the genus *Fusarium* electrophoretic karyotype. The genome of *Fusarium* spp. consisted of 5~13 chromosome-sized molecules with some of them migrationg as doublets which ranged in size from 0.78~7.20 Mb. The total genome size was estimated to range from 18.32 Mb to 48.20 Mb.

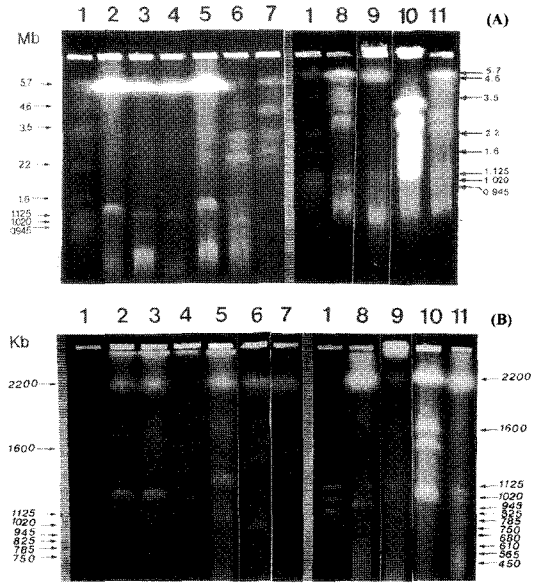

Fig. 1. Electrophoretic karyotypes for *Fusarium*. Lane: (1) size marker (2) *F. poae* NRRL 3287 (3) *F. sporotrichioides* NRRL 3510 (4) *F. sporotrichioides* C Jungsun 1 (5) *F. tricinctum* NRRL 3299 (6) *F. anthophilum* 7481 from Sydney (7) *F. proliferatum* 7459 from Sydney (8) *F. equiseti* C Kosung 6 (9) *F. acuminatum* NRRL 13552 (10) *F. sambucinum* NRRL 13500 (11) *F. caucasium* ATCC 18791. (A) Lane (1) *S. pomb, S. cerevisiae*, 0.55% agarose gel, switch time; 4,500 s decreasing to 300 s during 136 hr, field strength; 1.3 V/cm increasing to 3 V/cm. (B) Lane (1) *S. cerevisiae*, 0.7% agarose gel, 1.3 V/cm, 1,200 s during 48 hr and 5.5 V/cm, 120 s during 24 hr.

F. poae (Fig. 1, Lane 2), *F. sporotrichioides* (Fig. 1 Lane 3, 4), and *F. tricinctum* (Fig. 1 Lane 5) in the section *Sporotrichiella* showed very similar separation patterns consisting of 7~12 chromosome-sized molecules. The sizes

Table 2. Estimated chromosome numbers, chromosomes size and genome size for ten *Fusarium* spp.

Chromosome Number[c]	*Fusarium* species[a] and their chromosome size[b] (Mb)									
	POA	SP1	SP2	TRI	ANT	PRO	EQU	ACU	SAM	CAU
1	7.10	7.10	7.10	7.20	5.85	5.95[b]	5.85[b]	5.70	3.25[b]	5.80[b]
2	6.45	6.32	6.25	6.45	4.81	4.95	4.48	4.60[b]	2.72[b]	4.10
3	5.70[b]	5.60[b]	5.60[b]	5.70[b]	4.38	4.30[b]	3.80	2.25	2.40	3.50
4	4.70[b]	4.60	4.60	4.70	3.90	3.35	3.50[b]	1.17	1.65	2.85
5	3.50[b]	3.25	3.25	4.10[b]	3.20	3.05	3.05		1.50[b]	2.35[b]
6	2.40	2.25	1.25	3.40	2.76	2.60	2.55		1.35[b]	1.10
7	1.28	1.28		2.48	2.40[b]	2.25	2.10		1.12	1.06
8				1.75	1.47	0.94	0.98		1.06	0.79
9				1.47	1.02				1.02	
10				1.15	0.78					
Total	45.03	36.00	33.65	48.20	26.34	37.64	35.66	18.32	23.87	29.70

[a]Species abbreviations; POA: *F. poae* NRRL 3287, SP1: *F. sporotrichiodes* NRRL 3510, SP2: *F. sporotrichioides* C Jungsun 1, TRI: *F. tricintum* NRRL 3299, ANT: *F. anthophilum* 7481 from Sydney, PRO: F. *proliferatum* 7459 from Sydney, EQU: *F. equiseti* C Kosung 6, ACU: *F. acuminatum* NRRL 13552, SAM: *F. sambucinum* NRRL 13500, CAU: *F. caucasium* ATCC 18791. [b]Indicates doublets unresolved chromosomes. [c]Chromosomes were numbered according to their size, starting with the largest.

of chromosomes ranged from 1.15~7.20 Mb which gave an estimated total genome size between 33.65 Mb and 48.20 Mb. The staining intensity of the 4th and 5th bands of these species indicated that they might be doublets.

The *F. anthophilum* (Fig. 1 Lane 6) and *F. proliferatum* (Fig. 1 Lane 7) in section *Liseola* showed very similar DNA separation patterns consisted of 10~11 chromosome-sized DNAs with 26.34~37.64 Mb total genome size.

The karyotypes of *F. equiseti* (Fig. 1 Lane 8), *F. acuminatum* (Fig. 1 Lane 9) in the section *Gibbosum* consisted of 5~10 chromosome-sized molecules and the total genome size was estimated between 18.32 and 35.66 Mb. The chromosomes of *F. acuminatum* were poorly separated due to difficulties in obtaining large numbers of protoplasts. 1st and 4th bands of *F. equiseti* and 2nd band of *F. acuminatum* might be doublets, because of their staining intensity.

The total genome size of *F. sambucinum* (Fig. 1 Lane 10) in the section *Discolor* was 23.87 Mb consisting of 13 chromosome-sized molecules. Based on staining intensity, 1st, 2nd, 5th and 6th DNA bands of this species might be on doublet, and a large number of small size chromosomal bands were shown in *F. sambucinum*.

The karyotype of *F. caucasium* (Fig. 1 Lane 11) in the section *Martiella* consisted of 10 chromosome-sized DNA, 29.70 Mb of the total genome size. Based on staining intensity, 1st and 5th DNA band of this species might be on doublet.

Evidence for the presence of chromosomal length polymorphisms was verified by Southern blot hybridization of CHEF PAGE-fractionated chromosomes with randomly selected genomic DNA probe. The gel shown in Fig. 1A. containing chromosomal DNA was blotted to nylon membrane and probed with a randomly amplified genomic DNA band from *F. oxysporum* f. sp. *lilii* (Fig. 2). The hybridization occured mainly at the 5.80±0.15 Mb region in all species examined except *F. sambucinum* and at the 2.60±0.10 Mb region in some species. The genomic DNA probe hybridized only to a single band in each of the

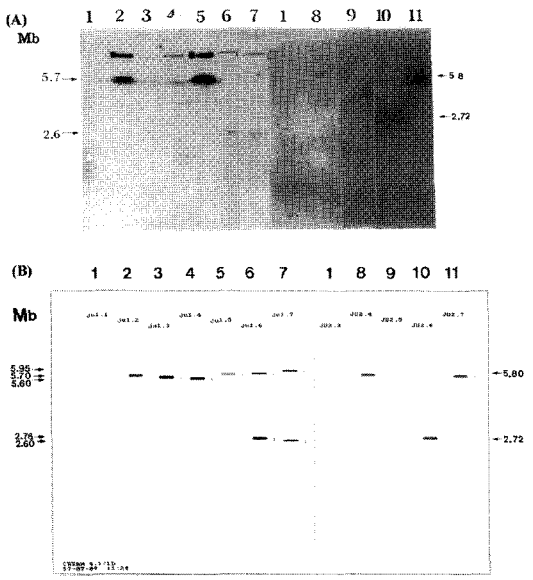

Fig. 2. Southern blot of chromosome-sized DNA molecules of *Fusarium* spp. fractionated by PFGE (Fig. 1A) and probed with random genomic DNA (A) and was analyzed by Image Analyzer (B)

species belonging to the section *Sporotrichiella* that ranged in length from approximately 5.60~5.70 Mb. Hybridization bands of 5.80~5.95 Mb region were observed in *F. anthophilum* and *F. proliferatum* (section *Liseola*), in *F. equiseti* (section *Gibbosum*) and in *F. caucasium* (section *Martiella*). Also, hybridization bands of 2.60~2.76 Mb region were observed in *F. anthophilum, F. proliferatum* (section *Liseola*) and *F. sambucinum*. Unfortunately, none of hybridization bands were observed in *F. acuminatum*. It was not certained that the lack of hybridization of probes in this species due to the lack of a sufficient quantity of DNA or to degradation of DNA on the gels.

Discussion

Several electrophoretic conditions for the CHEF gel such as agarose concentration, field strength, switching intervals, and running times were tested before a satisfactory resolution of chromosomal DNA was achieved. A major source of difficulty in achieving a precise separation was the overlapping of chromosomal band.

In general, karyotypes of the *Fusarium* spp. analyzed ranged from 18.32 Mb to 48.20 Mb consisting of 5~13 chromosome-sized molecules, with some bands migrating as doublets.

Compared with some other fungi, these genome sizes fell within the estimated 19.8~46.5 Mb as estimated the genus *Pythium* (Martin, 1995) but larger than the similar size ranges, 11.5~33.9 Mb for *Candida* species (Matsuko *et al.*, 1992).

The species belonging to the section *Sporotrichiella* contained a large number of large-sized chromosomes and more than one-half chromosome number were larger than 4.10 Mb in their karyotype. The karyotypes we resolved in this study differed significantly from those described by Fekete *et al.* (1993), in which they identified 6~9 chromosomes ranging from 1.00 Mb to 6.5 Mb in size with total genome size of 27.5~29.2 Mb. The total genome size was significantly less than we proposed for these same species. The largest chromosome they resolved was 6.5 Mb which was probably not separated. In fact, they suggested that the largest chromosome might contain more than one chromosome. To improve the separation of these clustered chromosomes, we tried various modified analytical conditions. We lowered the agarose concentration and the largest chromosomes were separated into two bands. The 1st band was the largest examined in all *Fusarium* species. When the agarose concentration was 0.6%, 1.2 V/cm with switching intervals, increasing from 3 s to 2,220 s over 228 hr running period, other remaining comigrating chromosomes were separated into two chromosomes, respectively, although these bands were shown very faint (data not shown). Electro-

phoretic karyotypes in *F. sporotrichioides* NRRL 3510 and C Jungsun 1 (from Korea) produced very similar patterns indicating that a low degree of karyotype variation existed within this species, although these two isolates were different in geographic origin.

The species in section *Liseola* contained 10~11 chromosome-sized DNAs with nearly even distributions in the large-, medium- and small-sized chromosome bands. *F. proliferatum* karyotypes resolved in this study differed from the results of Migheli *et al.* (1993) in which they identified 7 chromosomal bands consisting of one large size (6.5 Mb) and six small sized chromosomes and estimated that the total genome size was 18.1 Mb. In the same section, *F. moniliforme* was reported to have a chromosomal pattern consisting of 10~11 bands and a genome size ranging from 38.0~38.8 Mb by Migheli *et al.* (1993). Min (1995) described that the karyotype of *F. moniliforme* contained 8 bands with total genome size of 28.3 Mb. Thirty four strains of *Gibberella fujikuroi* (anarmorph *Fusarium* section *Liseola*) were known to contain 12 haploid chromosomes with a total genome size of 45.0~55.0 Mb (Xu *et al.*, 1995). These results indicated that the species belonging to section *Liseola* and the strains belonging to same species had distinct electrophoretic karyotypes.

DNA bands of *F. equiseti* in the section Gibbosum separated into large-, medium-, and small size chromosomes with even distributions. The karyotype of *F. acuminatum* belonging to the same section *Gibbosum* was different from that of *F. equiseti* and a significant variability was observed between these two species. To verify difference between these two species, another strains of *F. acuminatum* (ATCC 16560) was examined. The karyotype of *F. acuminatum* (ATCC 16560) ranged from 1.15 to 5.70 Mb was very similar with those of *F. acuminatum* (NRRL 13552) (data not shown). But the chromosomal DNA bands of *F. acuminatum* (ATCC 16560) were very fainter than for bands of other sample due to the difficulties of protoplast formation, although several agarose sample blocks were inserted in sample well. The karyotype of *F. sambucinum* in the section *Discolor* showed medium- and small sized chromosomes without large sized chromosomes. The largest chromosome in this species was 3.25 Mb and eight of twelve chromosomes were smaller than 1.65 Mb. But *F. graminearum* in the same section *Discolor* was reported to have a chromosomal patterns consisting of 6 bands with 23.2 Mb genome size and the largest chromosome was above 6.0 Mb and five of six chromosome were larger than 2.3 Mb (Min, 1995). The section *Discolor* was divided into two subsection, *Eudiscolor* and *Saubinetti* according to the shape of the macroconidium and in particular the shape of the apical cell as suggested by Raillo (Raillo, 1950). *F. sambucinum* were belonged to subsection *Eudiscolor* and *F. graminearum* to subesction *Saubinetii*. The great difference of electrophoretic karyotypes between these two species might be caused by the difference of morphological character. Waalwijk (1996) described that *F. graminearum* and *F. sambucinum* in section *Discolor* were belonged to different RFLP group of ITS (Internal Transcribed Spacer region).

Since we obtained consistent, species-specific DNA profiles from our various preparations, we are confident that our DNA bands represnt full length DNA molecules. Although not all chromosomes appeared as discrete bands, the chromosome numbers of these *Fusarium* species were determined based on comparison of results obtained from several different running conditions.

In hybridization experiments with randomly selective genomic DNA from *F. oxysporum* f. sp. *lilii*, there were hybridizing bands with

slightly different mobility among species at 5.80±0.15 Mb region in all species and this result indicated that selective genomic DNA probe had homology with chromosomal DNA bands. Differences in chromosomal DNA size in hybridization might be explained as chromosomal length polymorphisms of as much as 200~300 Kb. The genomic DNA probe also hybridized to chromosomal bands of 2.60~2.76 Mb size in the species belonging to section *Liseola*, suggesting that the probe had homology with these bands. Apparent hybridization with more than one chromosomal band suggested the probe sequence might be on more than one chromosome, or this smaller bands might contain a portion of the larger one. In our laboratory, when same random genomic DNA from *F. oxysporum* f. sp. *lilii* was used as a probe, hybridization occurred at two different regions (6.1 Mb and 3.0 Mb region) in all different *F. oxysporum* f. sp. examined, belonging to section *Elegans* (unpublished). The hybridization band patterns of the species belonging to section *Liseola* showed similar to the patterns of the species belonging to section *Elegans*. Considering the fact that section *Elegans* have been most closely related to section *Liseola*, genetic duplication of chromosome might be occurred in the species belonging to section *Liseola*. Kistler *et al.* (1995) described that hybridizing bands frequently differed in size, reflecting the polymorphic nature of presumed homologous chromosomes among the 14 isolates of *F. oxysporum* f. sp. *cubense* and that hybridizing to two different sized chromosome bands might imply an unexceptedly high level of duplication.

Interestingly, the genomic DNA probe hybridized to 2.72 Mb sized chromosomal DNA in *F. sambucinum* belonging to section *Discolor*. The hybridization band suggested that the genomic DNA probe had homology with chromosomal DNA in *F. sambucinum*. The similarity dendrogram based on ITS (Internal Transcribed Spacer) region sequence of rDNA suggested that *F. oxysporum* (section *Elegans*) would be more closely related to *F. sambucinum* than to *F. relodens* belonging to same section *Elegans* (Waalwijk *et al.* 1996). However, when nuclear DNA of *F. oxysporum* interacted with that of *F. graminearum* (section *Discolor*), as would be excepted from similarities in their morphological characteristics, *F. oxysporum* appears to be more closely related in its genetic make up to section *Liseola* than does *F. graminearum* (Ellis, 1988).

Considering the differences of electrophoretic karyotype between *F. sambucinum* and *F. graminearum* described as previously, the hybridization patterns of *F. sambucinum* with randomly selected genomic DNA from *F. oxysporum* f. sp. *lilii*, the RFLP grouping and sequencing of ITS region in *F. sambucinum* (Waalwijk, 1996), and nuclear DNA interrelatedness between *F. oxysporum* and *F. graminearum* (Ellis, 1988), further study will be required in order to perform sectional classification of *Fusarium* species.

Due to a lack of a chromosome-specific probe, it is not possible to analyze the specific content of each chromosome and identify homologous chromosomes among the species. Hybridization experiments with species-specific probes or chromosome-specific probes will be carried out to investigate whether such differences are consequences of species differentiation or chromosomal rearrangement. Although a large number of DNA probe will be required to establish the precise number of linkage group, the CHEF PFGE analysis of these species allowed an estimate of the maximum number of chromosomes and the size of the haploid genome of *Fusarium* species.

Considering previous works with *Fusarium* spp. (Boehm *et al.*, 1994; Fekete *et al.*, 1993; Kim *et al.*, 1993; Migheli *et al.*, 1993; Min, 1995; Nagy and Hornok, 1994; Park and Min.,

1995; Xu and Leslie, 1993), variation of electrophoretic karyotype was observed between and within species. The differences observed in chromosome number and length in strains belonging to differnet species of *Fusarium* or to different formae speciales of *F. oxysporum*, and different strains belonging to the same formae specials do not consititute *per se* a surprising result, as the same variability has been observed in a number of phytopathogenic fungal species, even within local population (Migheli *et al.*, 1993). Variability in chromosome number and size has been reported for several fungal species. Explanations or mechanisms responsible for such genome rearrangments include insertion, duplications, deletions, and translocations (Xu *et al.*, 1995). Aneuploidy is one cause of variable chromosome number, though it is possible that comigration of similar sized chromosomes is responsible for some of the observed differences in chromosome number (McDonald and Martinez, 1991). To draw further conclusions, additional investigations are needed.

In terms of future molecular studies, the technique we have described should prove useful in genetic mapping and in the assignment of additional genes of interest to their chromosomal locations and for the construction of chromosome-specific libraries.

적 요

CHEF (Contour-Clamped homogeneous electric field) gel electrophoresis를 이용하여 *Fusarium* section *Sporotrichiella, Liseola, Gibbosum, Discolor*와 *Martiella*에 속하는 10종의 electrophoretic karyotype을 비교하였다. Intact chromosomal DNA는 균류의 원형질체로부터 추출하였으며, 크기에 따라 다양한 조건을 주어 DNA 분자를 분리시켰다. *Fusarium*속에 속하는 종의 염색체는 0.78 Mb에서 7.20 Mb의 크기를 가진 염색체가 종에 따라 5~13개였다. 각 종의 total genome 크기는 18.32 Mb에서 48.20 Mb였다. Electrophoretic karyotype을 비교한 후 *F. oxysporum* formae speciales *lilii*로부터 무작위로 선택하여 만든 genomic DNA를 probe로 하여 Southern hybridization 분석을 수행하였다.

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