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Restriction fragment length polymorphisms of mitochondrial DNAs from seven Fusarium species causing fusarium head blight

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ABSTRACT

Mitochondrial DNAs (mtDNAs) were purified by CsCl/bisbenzimide density-gradient ultracentrifugation from 21 strains of seven *Fusarium* species that cause fusarium head blight and mycotoxin contamination in wheat and other cereals. A partial *Pst*I clone bank, from which one of twelve *Pst*I fragments (14.7 kb) is missing, was constructed using mtDNA from strain KU-1615 of *F. graminearum*. Molecular sizes of mtDNAs of single representative strains from the seven species were determined after single-, double- and triple-digestion by four or five restriction enzymes (*Bam*HI, *Mlu*I, *Pst*I, *Pvu*II and *Xho*I), while those of others were after single-digestion by *Bam*HI and/or *Pst*I. MtDNA size varied from the smallest 49 kb in one strain of *F. avenaceum* to the largest 116 kb in one strain of *F. culmorum*. Restriction fragment length polymorphism (RFLP) analysis revealed a large interspecific variation, thus all the species were identified by their restriction fragment patterns and assigned to individual clusters except for *F. tricinctum* in that a strain studied showed identical patterns to one of two strains of *F. sporotrichioides*. Considerable intraspecific variation including size variation was also detected. These results indicated a high incidence of insertions/deletions both between and within species. On the basis of results obtained by the cluster analysis, some aspects of taxonomy in these *Fusarium* species were discussed.

1. INTRODUCTION

A number of *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. avenaceum*, *F. nivale*, *F. acuminatum*, *F. sporotrichioides* and *F. tricinctum* cause a severe disease called fusarium head blight (scab or red-mold disease) in wheat and other cereals (Nelson et al., 1981; Koizumi et al., 1991). These *Fusarium* species are important plant pathogens not only because they cause significant yield losses but also because they produce highly toxic secondary

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metabolites, i. e., trichothecene mycotoxins, including deoxynivalenol, nivalenol, T-2 toxin and their derivatives, that are harmful to livestock and human health (Marasas et al., 1984). An estrogenic toxin, zearalenone, is also produced by some of these species (Neish et al., 1983). Many incidents of human and animal toxicoses after consumption of mycotoxin-contaminated plant products have been reported, e. g., alimentary toxic aleukia in Russia and similar toxicoses in northern China, red-mold toxicoses in Japan and Korea, moldy corn toxicosis in US and Canada, and farm animal stachybotryotoxicoses in Europe (Ueno, 1980; Yoshizawa, 1983). Therefore, understanding of the relationship between causal *Fusarium* and mycotoxins produced by them are quite important in diagnosis of fusarium head blight. In this respect, reliable methods for identification of species and strains of *Fusarium* need to be established.

Mitochondrial DNA (mtDNA) has provided a useful taxonomic tool in fungi (Clark-Walker, 1992). The small size of mtDNA makes it suitable for restriction enzyme analysis, and because of its rapid rates of nucleotide substitution mtDNA has generally been useful in studying genetic differentiation within species and between closely related species (Brown, 1985; Avise, 1989). Restriction fragment length polymorphisms (RFLPs) in mtDNA have been detected in many groups of fungi. Furthermore, in an ascomycete *Podospora anserina*, a complete nucleotide sequence has been reported (Cummings et al., 1990). In *Fusarium*, an extra-chromosomal, circular DNA molecule was first detected in the post-mitochondrial supernatant fraction prepared from *F. oxysporum* f. sp. *lycopersici* (Guardiola et al., 1982). Later, this 46.5 kb circular DNA molecule was proven to be mtDNA and a restriction map was constructed (Marriot et al., 1984). Thereafter, mtDNAs of several formae speciales have been studied in *F. oxysporum*, to which mtDNA haplotypes have been assigned (Kistler et al., 1987; Jacobson and Gordon, 1990; Gordon and Okamoto, 1992; Kim et al., 1991, 1992, 1993). However, no information has yet been available on mtDNA in the important group of *Fusarium* species causing fusarium head blight in cereals.

In this study we examined RFLPs of mtDNAs from 21 strains of seven *Fusarium* species that cause fusarium head blight in wheat and barley in Japan, Bulgaria and Hungary. We constructed a partial clone bank, determined mitochondrial genome sizes of all the strains, and constructed a phylogenetic tree based on the RFLPs of mtDNA.

2. MATERIALS AND METHODS

Fusarium *strains and culture conditions*

Twenty one strains isolated from seven *Fusarium* species including *F. graminearum* Schwabe, *F. culmorum* (W. G. Smith), *F. avenaceum* (Fr.) Sacc., *F. acuminatum* (Ell et Ev.), *F. sporotrichioides* (Sherb.), *F. nivale* (Fr.) Ces., and *F. tricinctum* (Corda) Sacc. were used (Table 1). The above nomenclature was

according to the classification by Booth (1971). Mycotoxin production of all the strains were determined previously (Atanassov et al., 1994) and shown in Table 1. *F. culmoum* BUL-C-21 originated from Bulgaria, HUNGARIAN was from Hungary, and all others were from Japan.

Fusarium mycelia were grown in liquid medium containing 0.1% yeast extract (Difco), 0.1% Bacto peptone (Difco) and 1% sucrose, or in potato sucrose medium (Booth, 1971), both containing 10 μg/ml of ampicillin. Cultures were grown on a shaker at 25°C for 70 to 80 h under 16 h photoperiod at an intensity of 55–65 $\mu Em^{-2}s^{-1}$. The mycelia were harvested, washed with sterilized water, placed on a filter paper and vacuum-dried for 30 min using Buchner funnel. The mycelia were weighed, frozen with liquid nitrogen, and stored at −80°C until use.

A strain of *Saccharomyces cerevisiae* DC5 was used as an outgroup species. The strain was cultured in liquid medium containing 1% yeast extract, 2% Bacto peptone, 2% dextrose and 10 μg/ml of ampicillin at 30°C for 24 h in the dark. Cells were collected by centrifugation at 2,500 rpm for 10 min at 4°C and treated in the same way as for *Fusarium* mycelia.

Purification of mtDNA

Isolation of a crude mitochondrial fraction and purification of mtDNA were basically according to the method described by Garber and Yoder (1983). Mycelia (10–20 g fresh weight) were ground into powder using a mortar with pestle in liquid nitrogen and suspended in 120 ml of buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 0.1% bovine serum albumin, 4 mM 2-mercaptoethanol and 0.44 M mannitol. A crude mitochondrial pellet obtained after centrifugation at 28,000 rpm for 30 min (Beckman XL-90, SW-28 rotor) was suspended in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0) and 150 mM NaCl using a glass-Teflon Potter Elvehjem homogenizer. After adding sodium dodecyl sulfate to a final concentration of 2% (w/v), the homogenate was incubated at 65°C for 30 min, placed on ice for 10 min, and centrifuged at 7,000 rpm for 10 min at 4°C. Crude mtDNA was extracted twice from the supernatant with phenol-chloroform-isoamylalcohol (25: 24: 1; v/v/v).

For the purification of mtDNA, CsCl (to a final concentration of 1 g/ml) and bisbenzimide (0.4 mg/ml) were added to mtDNA dissolved in 10 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the mixture was ultra-centrifuged at 37,000 rpm at 18°C for 48 or 60 h (Hitachi 55P-72, RP50-2 rotor). The uppermost band corresponding to mtDNA was collected by syringe with 23-gauge needle under UV illumination. Bisbenzimide was removed by repeated extractions with 2-propanol. Purified mtDNA was ethanol-precipitated, dried in aspirator and dissolved in TE buffer. By this method, mtDNAs essentially free from nuclear DNA (1 to 4 μg from 1 g fresh weight of myceia) were purified without repeated ultracentrifugations that were required in the original method developed by Garber and Yoder (1983). The better resolution was obtained when the gradient

ultracentrifugation was run for 60 h than for 48 h.

Construction of a mtDNA clone bank

MtDNA from strain KU-1615 of *F. graminearum* (Table 1) was digested with *Pst*I and ligated into *Pst*I site of the dephosphorylated pUC119 with T4 DNA ligase (Takara Shuzo Co. Ltd.). *Escherichia coli* cells (Library Efficiency DH5α Competent Cells, Gibco BRL) were transformed with the recombinant plasmids and a clone bank was constructed. Briefly, individual mtDNA inserts from the recombinants were extracted after 0.8% agarose gel electrophoresis (Gray et al., 1992). The inserts were labelled by incorporation of digoxygenin (Dig) according to the manufacturer's protocol (Boehringer). The inserts of individual clones were determined by Southern hybridization of the mtDNA probed with the Dig-labelled inserts.

Estimation of mtDNA sizes

MtDNAs of single representative strains from each one of the seven species (Table 1) were single-, double- or triple-digested with either four or five restriction enzymes (*Bam*HI, *Mlu*I, *Pst*I, *Pvu*II, and *Xho*I). Digested mtDNAs were electrophoresed in agarose gel at concentrations ranging from 0.5% (for large fragments) to 2.0% (for small fragments) to estimate molecular sizes of individual fragments. For fragments larger than 20 kb, molecular sizes were determined after homology assignment of double- and triple-digested fragments by Southern hybridization with Dig-labelled inserts of the partial *Pst*I clone bank made from mtDNA of strain KU-1615 of *F. graminearum*. *Hin*dIII-digested λDNA was used as a size marker. MtDNA sizes of all other strains were estimated by comparing the sizes of the restriction fragments generated by single-digestion with *Pst*I and/or *Bam*HI with those from the corresponding single representative strains.

RFLP and cluster analyses

MtDNAs from all 21 strains were digested with *Pst*I, *Bam*HI, *Hae*III and *Hha*I. Based on the restriction fragment patterns, dissimilarity indices were calculated as percentages of differential fragments within and between the species. Homology of the corresponding *Pst*I fragments within and between species was partly determined by Southern hybridization using the partial *Pst*I clone bank. A dendrogram was constructed by unweighted pair-group method with arithmatic averaging (UPGMA) according to Sneath and Sokal (1973).

3. RESULTS

Construction of a partial mtDNA clone bank

A partial clone bank of mtDNA was constructed using strain KU-1615 of *F. graminearum* that was highly pathogenic and toxigenic to wheat (Atanassov et

Table 1. *Fusarium* strains, their mycotoxin production and mtDNA sizes

Species	Strain*	Mycotoxin production*	MtDNA size (Kb)
F. graminearum	KU-1367	NIV, F-X	91.1
	KU-1368	DON	91.1
	KU-1369	nd	92.5
	KU-1615	NIV, F-X	92.5**
	ARC-13	DON	91.1
F. culmorum	ARC-2125-1	NIV, F-X	115.5
	ARC-784 (NHL-HW-12)	NIV, F-X	109.3
	SHIN-996 (SUF-996)	ZEA	101.3**
	BUL-C-21	DON, ZEA	103.2
	HUNGARIAN	ZEA	103.9
F. avenaceum	ARC-1016-1	nd	50.8
	ARC-1048-1	nd	51.1
	SUF-1121	nd	49.0**
F. nivale	ARC-1035	nd	104.9**
	ARC-1010	nd	94.6
F. acuminatum	ARC-780 (NHL-F-1150)	nd	59.6
	ARC-2003-1	nd	53.4
	ARC-2050-2	NEOS, T-2, TOL	54.1**
F. sporotrichioides	NHL-F-985	NEOS, T-2	60.3**
	NHL-F-998	NEOS, T-2	59.0
F. tricinctum	SUF-1139	nd	59.0**

* Strains and mycotoxin production are after Atanassov et al. (1994). Abbreviations: DON, deoxynivalenol and its derivatives; NIV, nivalenol and its derivatives; F-X, fusarenone-X; ZEA, zearalenone; NEOS, neosolaniol; T-2, T-2 toxin; TOL, tetraol; nd, not detected.

** MtDNA sizes were estimated based on restriction fragments after digestion with *Mlu*I, *Pst*I, *Pvu*II, *Xho*I and *Bam*HI for these strains, while those of others were estimated after digestion with *Pst*I and/or *Bam*HI.

al., 1994). Eighty two recombinant clones were examined for their inserts after transformation of DH5α cells with pUC119 ligated to the *Pst*I fragments of mtDNA. For the initial identification of the clones, the recombinant plasmids were purified, digested with *Pst*I and gel electrophoresed through 0.8% agarose together with *Pst*I-digested mtDNA of KU-1615. By comparing molecular sizes of the inserts, eleven out of twelve independent clones were selected (pFGM-1, 3 ~12; Fig. 1). Among them three clones (pFGM-3, 4, 5) with inserts of similar sizes (12.0, 11.8 and 11.3 kb, respectively) were further identified by digestion of each fragment with *Bam*HI and gel electrophoresis. A clone containing 14.7-kb fragment (FGM-2) could not be obtained. The final identification of the individual

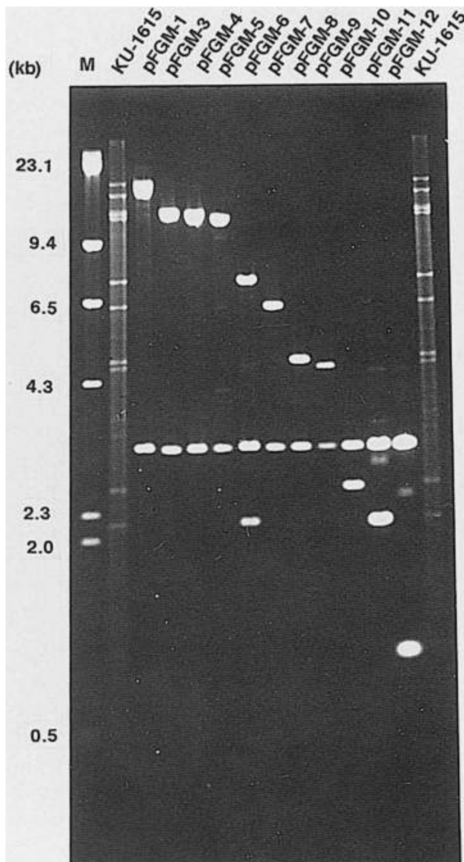


Fig. 1. A clone bank of mtDNA from strain KU-1615 of *F. graminearum*. One fragmenmt of 14.7 kb (FGM-2) is missing from the bank. A fragment with 2.2 kb seen in the lane for the clone pFGM6 is a contamination of the clone pFGM11. These clones were later separated by single colony isolation.

clones was made by Southern hybridization of mtDNA from strain KU-1615 probed with the Dig-labelled inserts.

RFLP analysis and size determination of mtDNAs

MtDNAs of single representative strains, each from the seven species, were single-, double- or triple-digested with *Bam*HI, *Mlu*I, *Pst*I, *Pvu*II and *Xho*I. MtDNA restriction fragment patterns generated from strain KU-1615 of *F. graminearum*, strain SHIN-096 of *F. culmorum* and strain SUF-1121 of *F. avenaceum* are shown in Fig. 2. Estimated molecular sizes of individual fragments and the total molecular sizes of mtDNAs of these strains are shown in Tables 2, 3 and 4, respectively. MtDNA sizes of the remaining 14 strains were estimated by comparing the *Bam*HI and/or *Pst*I restriction fragments with those of the corresponding single representative strains from the seven species (Table 1). Significant size variations were found between and within species. The *Fusarium* species were grouped into two classes based on their mtDNA sizes: one group (*F. graminearum*, *F. culmorum* and *F. nivale*) had larger mtDNAs (91–

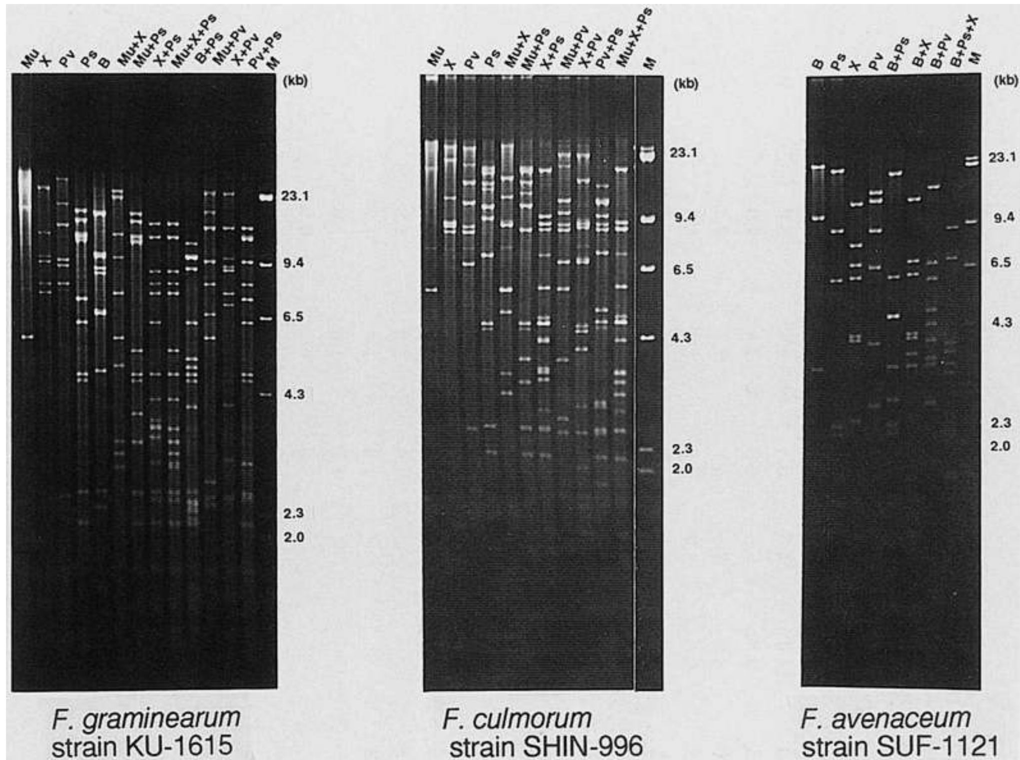


Fig. 2. Restriction fragment patterns of mtDNAs. MtDNAs from strain KU-1615 of *F. graminearum*, strain SHIN-996 of *F. culmorum* and strain SUF-1121 of *F. avenaceum* were single-, double- and triple-digested with five restriction enzymes and separated on 0.8% agarose gel. M: λ *Hin*dIII size markers; B: *Bam*HI; Mu: *Mlu*I; Ps: *Pst*I; Pv: *Pvu*II; X: *Xho*I.

116 kb) and the other group (*F. avenaceum*, *F. acuminatum*, *F. sporotrichioides* and *F. tricinctum*) had much smaller mtDNAs (49–60 kb). The largest mtDNA of 116 kb occurred in strain ARC-2125-1 of *F. culmorum* and the smallest mtDNA of 49.0 kb in strain SUF-1121 of *F. avenaceum*.

The restriction fragment patterns clearly distinguished six species (Fig. 3). However, one of the two strains of *F. sporotrichioides* (NHL-F-998) showed identical restriction patterns to one strain of *F. tricinctum* (SUF-1139). The other strain (NHL-F-985) from *F. sporotrichioides* showed restriction patterns similar to that of *F. tricinctum*.

Construction of a dendrogram by UPGMA

Using restriction fragments generated after digestion of mtDNAs from all strains with *Bam*HI (Fig. 3A), *Pst*I, *Hae*III (Fig. 3B) and *Hha*I, inter- and intraspecific genetic distances were estimated based on the dissimilarity indices calculated as percentages of differential fragments (Table 5). A large inters-

Table 2. Estimated molecular sizes (kb) of individual mtDNA restriction fragments of strain KU-1615 of *F. graminearum*

Frag. No.	*Bam*HI (B)	*Mlu*I (M)	*Pst*I* (Ps)	*Pvu*II (Pv)	*Xho*I (X)	M+Ps	M+Pv	M+X	Ps+B	Ps+X	Ps+M+X	Pv+X
1	15.0 (×2)	44.0	15.3	31.6	23.1 (×2)	15.3	23.6	23.1	11.8	14.7	14.7	23.1
2	9.8 (×2)	28.1	14.7	16.4	11.8	14.7	16.4	20.0	9.8 (×2)	12.0	12.0	13.0
3	8.8	15.8	12.0	13.0	9.5	12.0	13.0	11.8	8.8	8.6	8.6	9.5
4	8.6	5.7	11.8	9.4	9.2	11.3	9.3	9.5	6.3	7.9	7.9	9.2
5	6.7 (×2)		11.3	9.0	7.9	11.0	8.0	7.5	4.9	7.4	7.4	9.0
6	4.7		7.3	8.0	7.4	5.5	6.6	5.7	4.8	6.3	5.5	7.4
7	2.6		6.3	2.6		4.5	5.7	4.8	4.5	4.5	4.5	6.8
8	2.4		4.5	1.6		4.4	2.7	3.4	4.4	4.1	4.1	4.0
9	1.4		4.4			3.8	2.5	3.2	3.9	3.7	3.6	3.0
10			2.6			3.3	2.4	3.0	2.6	3.6 (×2)	3.3	2.6
11			2.2			2.6	1.6		2.5	3.4	3.2	1.6
12			1.0			2.2	1.1		2.4 (×2)	3.0	3.0 (×2)	1.4
13						1.0			2.3	2.6	2.6	1.0
14						0.9			2.2 (×2)	2.5	2.5	0.5
15						0.8			1.4	2.2	2.2	
16						0.3			1.3	1.7	1.7	
17									1.2	1.0	1.0	
18									1.0	0.3	0.9	
19									0.9		0.8	
20											0.3	
Total	91.5	93.6	93.4	91.6	92.0	93.6	92.9	92.0	91.4	93.1	92.8	92.1
Mean	92.5±0.8											

* *Pst*I clones are designated as pFGM-1 to -12. pFGM-2 (14.7 kb) could not be cloned.

(×2) indicates that the fragment of the same size is present in two copies.

Table 3. Estimated molecular sizes (kb) of individual mtDNA restriction fragments of strain SHIN-996 of *F. culmorum*

Frag. No.	*Mlu*I (M)	*Pst*I (Ps)	*Pvu*II (Pv)	*Xho*I (X)	M+Ps	M+Pv	M+X	Ps+Pv	Ps+X	Pv+X
1	45.7	16.6	31.0	33.1	16.6	21.6	33.1	12.8	15.9	21.1
2	32.9	15.9	17.8	19.8	15.9	17.2	14.5	11.1	9.6	13.8
3	17.2	13.6	13.8	11.6	13.6	11.1	11.6	10.8	9.0	9.3
4	5.7	12.8	11.1	9.3 (×2)	12.0	10.0	9.3	9.6	8.6	9.0
5		10.8	9.0	9.0	10.8	9.0	9.0	9.0	7.1	8.9
6		9.6	8.6	8.6	8.6	8.6	5.7 (×2)	8.6	5.7	6.9
7		7.2	6.8	1.2	4.6	6.8	5.0	7.2	4.8	6.8
8		4.6	2.5		4.5	5.7	3.4	5.0	4.6 (×2)	4.6
9		4.5	1.6		3.7	3.7	3.0	4.6	4.2 (×2)	4.4
10		2.6			3.2	2.7	1.2	4.5	3.5	4.0
11		2.1			2.6	2.5		3.0	3.4	2.9
12		1.0			2.1	1.6		2.9	3.3	2.5
13					1.1	1.2		2.6	2.8	2.0
14					1.0			2.5	2.6	1.6
15					0.9			2.1	2.1	1.5
16								1.8	1.6	1.2
17								1.6	1.2	0.5
18								1.0	1.0	
Total	101.5	101.3	102.2	101.9	101.2	101.7	101.5	100.7	99.8	101.0
Mean	101.3 ± 0.7									

(×2) indicates that the fragment of the same size is present in two copies.

Table 4. Estimated molecular sizes (kb) of individual mtDNA restriction fragments of strain SUF-1121 of *F. avenaceum*

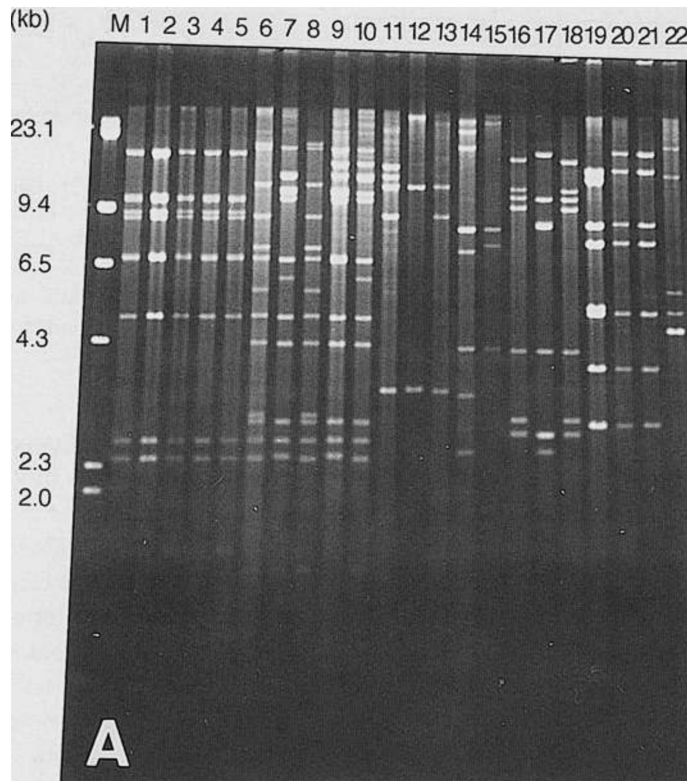
Frag. No.	*Bam*HI (B)	*Pst*I (Ps)	*Pvu*II (Pv)	*Xho*I (X)	B+Ps	B+Pv	B+X	B+Ps+X	Ps+Pv	Ps+X	Pv+X
1	35.0	26.5	14.1	12.5	20.1	14.1	12.5	8.8	12.0	8.8	8.0
2	10.8	9.4	12.9	8.0	6.1	6.6	6.9	6.9	9.1	8.0	7.8
3	3.4	6.4	9.1	6.9	4.7 (×2)	5.9	6.1	3.9	6.6	6.9	4.5
4		2.3	6.6	6.1	3.4	4.9	4.2	3.8	6.1	3.8	4.2
5		2.2	4.0	4.2	2.7	4.5	4.0	3.4	4.3	3.1	4.0
6		1.3	2.6	4.0	2.3	4.0	3.8	3.2	4.0	2.5	3.2
7		0.7		2.4	2.2	3.5	3.4	2.4	2.2	2.4	2.4 (×2)
8		0.5		1.4	1.3	3.4	1.6	2.3	1.5	2.3	2.1 (×2)
9				1.0	0.7	2.6	1.4	2.2	1.3	2.2	1.7
10				0.8	0.5		1.2	1.6 (×2)	0.8	1.6	1.4
11				0.7			1.0	1.4	0.7	1.4	1.0 (×2)
12				0.6			0.9	1.2	0.5	1.0 (×2)	0.9
13							0.8	1.1		0.8	0.8
14							0.7	1.0		0.7 (×2)	0.7
15							0.6	0.9		0.6	0.6
16								0.8		0.5	
17								0.7 (×2)			
18								0.6			
19								0.5			
Total	49.2	49.3	49.3	48.6	48.7	49.5	49.1	49.0	49.1	48.3	48.8
Mean	49.0±0.4										

(×2) indicates that the fragment of the same size is present in two copies.

pecific variation was evident: the largest distance was between *F. nivale* and *F. avenaceum* and between *F. nivale* and *F. acuminatum*, while the smallest distance was between *F. graminearum* and *F. culmorum*, when *F. sporotrichioides* and *F. tricinctum* were excluded from the comparison. A significant intraspecific variation was also observed: the largest distance was within *F. avenaceum*, while the smallest distance was within *F. graminearum*. All the strains except for a strain of *F. tricinctum* were clustered into six corresponding species (Fig. 4). A dendrogram also showed two major clusters among the seven species: one cluster consisted of *F. graminearum*, *F. culmorum*, *F. sporotrichioides*, *F. tricinctum* and *F. nivale*, and the other cluster consisted of *F. avenaceum* and *F. acuminatum*. In *F. graminearum*, two haplotypes were detected among the five strains.

4. DISCUSSION

We found a large interspecific variability in mtDNA size among the seven *Fusarium* species causing fusarium head blight (Table 1). The largest mtDNA size was ca. 116 kb in strain ARC-2125-1 of *F. culmorum* that is eqivalent to the



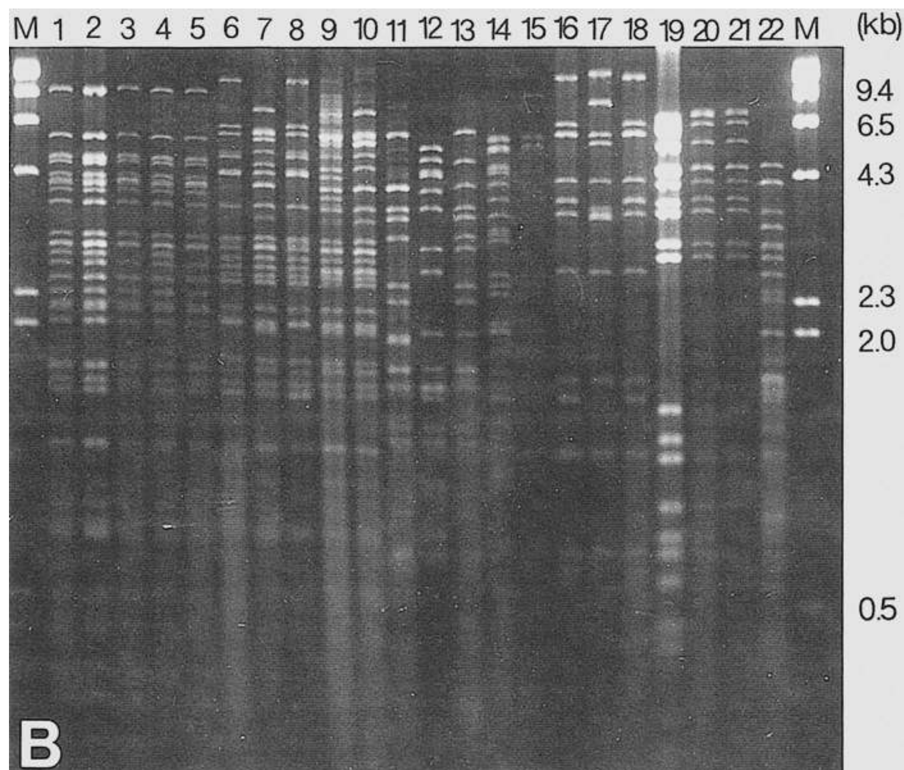


Fig. 3. RFLPs of mDNAs from 21 strains of seven *Fusarium* species. MtDNAs were digested with *Bam*HI (A) and *Hae*III (B), and separated on 0.8% and 2.0% agarose gel, respectively. The numerical numbers 1 to 22 indicate, respectively, *F. graminearum* strains KU-1367, ARC-13, KU-1615, KU-1368 and KU-1369, *F. culmorum* strains ARC-2125-1, SHIN-996, ARC-784, BUL-C-21 and HUNGARIAN, *F. avenaceum* stains ARC-1016-1, SUF-1121 and ARC-1048-1, *F. nivale* strains ARC-1035 and ARC-1010, *F. acuminatum* strains ARC-780, ARC-2003-1 and ARC-2050-2, *F. sporotrichioides* strains NHL-F-985 and NHL-F-998, *F. tricinctum* strain SUF-1139, and *S. cerevisiae* strain DC5. *S. cerevisiae* was included as an outgroup species.

116 kb mtDNA of *Cochliobolus heterostrophus* (Garber and Yoder, 1984), the largest mtDNA so far reported in fungi, while the smallest was 49.0 kb in strain SUF-1121 of *F. avenaceum*, that is close to 46.5 kb mtDNA of *F. oxysporum* (Marriot et al., 1984). A considerable size variation was also detected in mtDNAs within species (Table 1). These results suggested that the interspecific as well as intraspecific variations are due mainly to insertions/deletions but not to nucleotide substitutions. This agrees with the earlier findings that in fungi main sources of mtDNA variations are insertions/deletions and the presence of optional introns (Clark-Walker, 1992). All *Fusarium* species showed characteristic RFLPs after digestion by all enzymes studied, except for one strain of *F. sporotrichioides* that showed identical patterns with one strain of *F. tricinctum*

Table 5. A matrix of dissimilarity indices calculated as percentages of differential fragments of mtDNAs within and between seven *Fusarium* species based on restriction digests with *Pst*I, *Bam*HI, *Hae*III and *Hha*I

	grm	clm	avn	nvl	acm	spr	trc
grm	2.2						
clm	48.3	14.3					
avn	80.6	82.0	19.8				
nvl	74.9	76.5	89.3	7.7			
acm	82.4	80.5	79.3	87.1	11.8		
spr	71.9	69.8	81.6	81.5	79.5	2.6	
trc	71.6	68.6	81.6	81.7	79.2	3.8	0

Abbreviations: grm, *F. graminearum*; clm, *F. culmorum*; avn, *F. avenaceum*; nvl, *F. nivale*; acm, *F. acuminatum*; spr, *F. sporotrichioides*; trc, *F. tricinctum*.

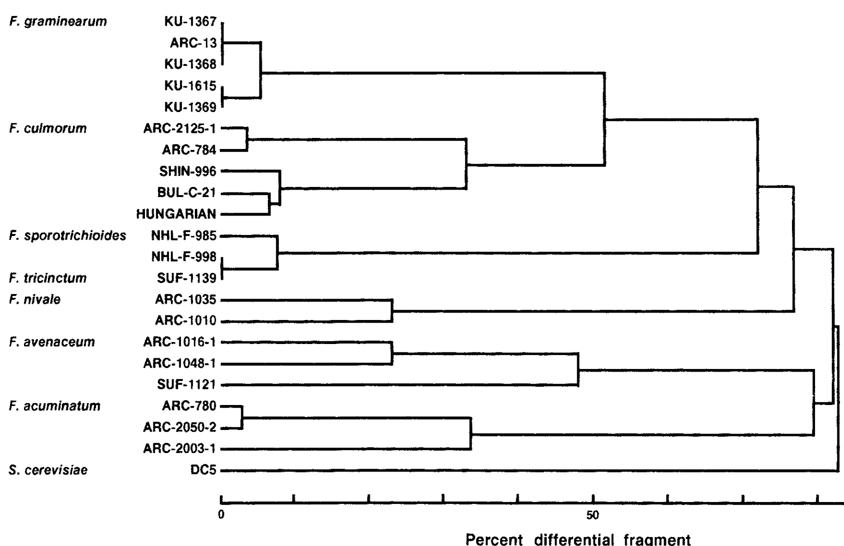


Fig. 4. A dendrogram constructed by UPGMA based on the dissimilarity indices of mtDNAs from 21 strains of seven *Fusarium* species. Data were obtained using restriction digests with *Pst*I, *Bam*HI, *Hae*III and *Hha*I. *S. cerevisiae* was included as an outgroup species.

(Fig. 3). Based on RFLPs of mtDNA, therefore, six species were clearly identified. In addition, all strains were grouped into six separated species by the cluster analysis (Fig. 4). Although the similarity index we used to evaluate the genetic relatedness likely gave an underestimation because of the high incidence of insertions/deletions, the results at least demonstrate the usefulness of mtDNA RFLPs in identifying species and strains of *Fusarium* causing fusarium head

blight in cereals. To our knowledge, this is the first report on mtDNAs in *Fusarium* species other than *F. oxysporum*. In addition, we constructed a mtDNA clone bank from strain KU-1615, that was highly pathogenic to wheat and produced high amounts of trichothecene mycotoxins both under artificial culture conditions and in infected wheat grain (Atanassov et al., 1994). Though the bank is still incomplete, it will facilitate mtDNA study in the *Fusarium* species by providing useful probes to disect mtDNA polymorphisms.

Taxonomy of the genus *Fusarium* is still ambiguous due to the large heterogeneity in their morphological and cultural characteristics even within the same species (Booth, 1984). Only the asexual state (anamorph) has been known in many species, thus they have been classified as fungi imperfecti. The sexual state (teleomorph) has been described in about a half of the genus and assigned to the three genera, *Gibberella*, *Nectria*, and *Calonectria* (Booth, 1981). Sexuality, however, occurs infrequently even in such species. Wollenweber and Reinking (1935) first established the taxonomy of *Fusarium* species based on characteristics such as morphology of macroconidia and ability to produce chlamydospores and their morphology. They described 16 sections including 65 species, 55 varieties and 22 forms. Snyder and Hansen (1945) simplified the classification by combining the above sections into nine species, and introduced the concept of forma specialis that is defined on the basis of pathogenicity against a specific host. Booth (1971) assigned 12 sections including the corresponding teleomorphs, and recognized 45 species, seven varieties and 103 formae speciales. A recent classification by Gerlach and Nirenberg (1982) describes more than 70 species and 55 varieties in the genus.

Our results presented some bases for discussing the classification systems in the genus *Fusarium*. In *Fusarium*, the classification systems developed by Snyder and Hansen (1945, hereafter referred to S.-H.) and Booth (1971) have been most widely accepted. S.-H. classified *F. graminearum*, *F. culmorum*, *F. avenaceum* and *F. acuminatum* into corresponding cultivars belonging to a single species, *F. roseum* f. sp. *cereales*, while Booth recognized all of these as independent species. We observed large interspecific mtDNA diversity among these four groups (Table 5). *F. graminearum* and *F. culmorum* formed one cluster and *F. avenaceum* and *F. acuminatum* formed another cluster (Fig. 4). The assignment of all of these into one single species, therefore, seems to be unrealistic. Our result thus supports the validity of the classification system by Booth. Wollenweber and Reinking (1935, hereafter referred to W.-R.) assigned *F. sporotrichioides* and *F. tricinctum* in the section Sporotrichiella, while Booth assigned *F. sporotrichioides* in the section Arthrosporiella because of its ability to produce polyphialidic or polyblastic conidiospores. Booth also classified *F. avenaceum* into the section Arthrosporiella, based on the same reasoning. Our study showed that *F. sporotrichioides* is distantly related to *F. avenaceum*. Moreover, one of the two strains of *F. sporotrichioides* showed the identical mtDNA restriction patterns to one

strain of *F. tricinctum* (Fig. 3). This implies either that the strain of *F. sporotrichioides* used in the present study was misclassified or that at least one group of *F. sporotrichioides* might have the same mtDNA as that of *F. tricinctum*. Some researchers disagreed with the classification of *F. sporotrichioides* by Booth because of the observation that this species seldom produce polyphialidic conidiospores (Nelson et al., 1983). S.-H. classified both *F. sporotrichioides* and *F. tricinctum* in a single species, *F. tricinctum*. Although our result appears to agree with the classification by S.-H., a further study is necessary using more strains of these species.

Two distinct haplotypes were recognized in *F. graminearum*, and in *F. culmorum* two groups consisting of five haplotypes were recognized based on mtDNA RFLPs (Fig. 4). In *F. culmorum* one group consisted of strains that possess ability to produce an estrogenic mycotoxin, zearalenone, while the other group consisted of strains that do not have this ability (Table 1). Strains that produce trichothecene mycotoxins are widely distributed in the genus *Fusarium*. On the other hand, strains producing zearalenone are restricted to the species that form macroconidia including *F. culmorum*, *F. graminearum* and *F. moniliforme* (Ichinoe et al., 1977). W.-R. described one variety cereale in *F. culmorum* based on its morphological characteristics of macroconidia. The correlation between mtDNA type and the ability of zearalenone production in this species needs to be further studied.

F. nivale has been known as a morphologically anomalous species among the genus *Fusarium* (Parkinson et al., 1981). Several researchers proposed the removal of this species from members of the genus (Gams and Muller, 1980; Samuels and Hallet, 1983). Sequence comparison of large-subunit ribosomal RNA showed that this species is equally divergent from all other *Fusarium* species (Guadet et al., 1989), supporting its reassignment to the different genus, either *Gerlachia* (Gams and Muller, 1980) or *Microdochium* (Sugiura et al., 1994). In our study, however, *F. nivale* was clustered with four other *Fusarium* species, i. e., *F. graminearum*, *F. culmorum*, *F. sporotrichioides* and *F. tricinctum* (Fig. 4). Detailed restriction maps, gene maps and/or sequence comparison of mtDNAs are required to find clues to solve this and other problems already discussed.

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