**Chemotaxonomic approaches to nivalenol-producing
Fusarium species Fn 2B by an application of restriction
fragment analysis of DNA**

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(Received May 30, 1997; Accepted Aug. 11, 1997)

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DNA フラグメント解析からみた nivalenol 産生性 *Fusarium* sp. Fn 2B の化学分類

Summary

A trial for chemotaxonomic analysis of toxigenic *Fusarium* species was performed by an introduction of restriction fragment length polymorphisms based on the selected DNA probes of *Fusarium* sp. Fn 2B strain, a well-known producer of trichothecene mycotoxins nivalenol and fusarenon-X. A total DNA of *F. Fn 2B* was prepared from the protoplast suspension of mycelia, digested with *Bam*HI, and ligated into *Bam*HI site of plasmid vector pUC19. Two clones harboring inserts in sizes of approximately 1.1 and 1.7 kb were randomly selected for probes. Hybridization patterns of ³²P-labeled 1.1 and 1.7 kb fragments of Fn 2B with *Bam*HI, *Eco*RI or *Hind*III-digested DNAs from *F. sporotrichioides* (4 isolates), *F. graminearum* (6 isolates), *F. tricinctum* (4 isolates), *F. poae* (4 isolates), *F. crookwellense* (3 isolates) and others, were compared by Southern blots. The data obtained with the 1.1 kb fragment have revealed a similar pattern of restriction fragment profiles among the isolates of same *Fusarium* species such as *F. sporotrichioides*, *F. tricinctum* or *F. graminearum*. While, the fragment profile of Fn 2B strain did not correspond to that of all the other *Fusarium* spp. tested. This suggested that *F. sp. Fn 2B*, originally reported as *F. nivale* by Dr. Tsunoda and later cited as *F. tricinctum* at NRRL by J. J. Ellis and to *F. sporotrichioides* at the Medical Research Council Collection (MRC, Tygerberg, South Africa) by Marasas *et al.*, may be unidentified *Fusarium* species.

Key words: nivalenol, trichothecenes, *Fusarium* species, restriction fragment length polymorphism

Introduction

Head blight diseases (scab, Akakabi-byo in Japanese) of wheat, barley and other cereal crops sporadically developed by infection of pathogenic *Fusarium* species in the world, and the consumption of these *Fusarium*-molded cereals by man and farm animals causes several food-borne diseases such as scabby wheat toxicosis in Japan, Korea and China, scabby corn intoxication and hyperestrogenism in the U. S., alimentary toxic aleukia (ATA) in Russia, and equine leukoencephalomalacia in South Africa. Extensive researches have identified numerous *Fusarium* mycotoxins such as the trichothecenes, zearalenone, moniliformin, fumonisins and others, as

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reviewed by the author^{1,2,3}), Nelson *et al.*⁴), Marasas *et al.*⁵) and Lacey⁶).

Historically speaking, Tsunoda *et al.*⁷), isolated first several toxigenic *Fusarium* species in 1963 from scabby wheat harvested in Kumamoto Prefecture, a southern part of Japan, where serious scab developed after heavy precipitation, and he identified one of these strains *F. sp.* Fn 2 (deposited as NRRL 9509) to *F. nivale* by comparison with the reference strain *F. nivale* NRRL 3249 identified by J. J. Ellis. Later, this strain was cited as *F. tricinatum* (Corda) Sacc. at NRRL and to *F. sporotrichioides* Sherb by W. F. O. Marasas *et al.*⁵). From the following evidences that (1) the trichothecene mycotoxins and their producers were grouped to the type (A) (T-2 toxin, diacetoxyscirpenol DAS *et al.* from *F. sporotrichioides*, *F. poae* etc) and the type (B) (nivalenol NIV, deoxynivalenol DON *et al.* from *F. graminearum* etc)⁸), (2) Fn 2B is well known as a potent producer of NIV⁹) and fusarenon-X (FX, 4-acetyl-NIV)¹⁰) which contaminates cereals and food worldwide¹¹), and (3) the taxonomic situation of Fn 2B based on classical morphology is still confusing, we have tried to clarify the taxonomic character by an introduction of chemotaxonomic approaches. At present, several methods such as soluble protein electrophoretic pattern, zymograms, monoclonal antibody reaction, immunoelectrogram and ribosomal proteins analysis are reported. In this experiment, we have adopted restriction fragment analysis of DNA. The data revealed that the isolate *F. sp.* Fn 2B is different from the toxigenic *Fusarium* species examined, suggesting an undescribed *Fusarium* species.

A part of present data was reported as a preliminary form¹²).

Materials and Methods

Fungi : *Fusarium* isolates used in this experiment were obtained from our stock cultures, except *F. nivale* ARC 1035 and 2051 provided by Dr. Koizumi (Agricultural Research Center, the Ministry of Agriculture, Forestry and Fisheries, Tsukuba). The isolates were cultured on potato-dextrose agar (PDA) slants at 27°C for 7-10 days before use. Species and strains numbers

Table 1 Test strains of *Fusarium* species

*1Section	Species	Major trichothecenes	Strains		
Arachnites Sporotrichiella	<i>F. nivale</i>		ARC 1035	ARC 2051	
	<i>F. poae</i>	T-2	KF 1103 R 2356	R 2145	R 2344
	<i>F. tricinatum</i>	T-2	R 2000	R 2010	R 2071
	<i>F. sporotrichioides</i>	T-2	M-1-1 R 2301	R 2017	R 2153
Discolor	<i>F. fusarioides</i>	T-2	R 2127		
	<i>F. crookwellense</i>	NIV	KF 233	KF 871	R 2166
	<i>F. culmorum</i>	DON	KF 98	R 2150	
	<i>F. graminearum</i>	DON/NIV	KF 208 OK-1-1	KF 205 TH-5-1	R 2118 TY-1-1
	<i>F. sambucinum</i>	DAS	R 2289	R 2291	
unidentified	<i>F. sp.</i>	NIV	Fn 2 B		

*1 This section is based on the system of Booth (1971).

were listed in Table 1, along with their major trichothecenes reported.

Production of trichothecenes : Each isolates were cultured in 100 ml Erlenmeyer flasks, each containing 10 g of polished rice grains previously immersed in tap water for one hr and supplemented with 5 ml of 3% peptone solution, at 25°C for one week. The moldy rice cultures were homogenized in acetonitrile-water (3 : 1), and the contents of trichothecenes in the filtrate were estimated by gas chromatography (GC) after clean-up over Florisil column¹³⁾.

Preparation of fungal DNA and hybridization : The test strains were cultured by reciprocal shaking at 25°C for 3-4 days in Sakaguchi flasks each containing 100 ml of Czapek Dox medium supplemented with 0.2% yeast extract. The mycelial mass suspended in 1.2M KCl solution was digested with 0.5% Novozyme 234 (Novo BioLabs, Bagsvaad, Denmark) at 25°C for 2-3 hr. The protoplasts harvested were incubated in PK buffer (100 mM Tris HCl buffer, pH 8.0, 12.5 mM EDTA, 150 mM NaCl, 1.0% SDS, 1mg/ml pronase) at 37°C for 3 hr, and DNA was extracted.

Construction of DNA library, Southern blots and hybridization were performed according to standard protocols, briefly as follows: the genomic DNA of Fn 2B was digested with *Bam*HI, and the fragments were ligated into the *Bam*HI sites of plasmid vector pUC19 by use of ligation kits containing T4 DNA ligase (Takara Shuzo, Kyoto). After transforming *E. coli* JN109 by calcium chloride procedure, plasmid DNA was isolated, digested by *Bam*HI, and after electrophoresis in 1.5% agarose gel, inserted DNA fragments were prepared by the Gene clean™ Kit (Bio 101, C. A.) and labeled with ³²P using multiprime DNA labeling system (Amersham, U. A.). Each 2 µg of genomic DNA were digested with *Bam*HI, *Eco*RI, or *Hind*III (Nippon Gene, Tokyo), and separat-

Table 2 Production of trichothecenes by *Fusarium*

Species	Strain	Production of trichothecenes (ppm)		
		NIV type ^{a)}	DON type ^{b)}	T-2 type ^{c)}
<i>F. poae</i>	KF 1103	n. d. ^{d)}	n. d.	4.17
	R 2356	n. t. ^{e)}	n. t.	n. t.
<i>F. sporotrichioides</i>	R 2017	n. d.	n. d.	3.57
	R 2301	n. d.	n. d.	292.2
<i>F. fusarioides</i>	R 2127	n. d.	n. d.	8.86
<i>F. tricinctum</i>	R 2000	n. d.	n. d.	5.83
	R 2010	n. d.	n. d.	299.7
<i>F. crookwellense</i>	KF 233	0.52	n. d.	n. d.
	KF 871	n. t.	n. t.	n. t.
<i>F. culmorum</i>	KF 98	n. d.	8.01	n. d.
	R 2150	n. d.	0.10	n. d.
<i>F. graminearum</i>	KF 250	n. d.	30.2	n. d.
	KF 208	n. d.	6.86	n. d.
	TY-1-1	0.14	2.05	n. d.
<i>F. sp.</i>	Fn 2B	194	n. d.	n. d.

^{a)} NIV type : NIV and 4-AcNIV

^{b)} DON type : DON, 3-AcDON or 15-AcDON

^{c)} T-2 type : T-2 or DAS

^{d)} n. d. : not detected (below 10 ppb)

^{e)} n. t. : not tested

ed by electrophoresis in 0.7% agarose gel. DNA was transferred to nylon membranes (Hybond-N, Amersham). The blots were hybridized to ³²P-labeled probes at 42°C under stringent condition (50% formamide, 6×SSC) and washed. Autoradiography on X-OMAT films was performed at -70°C with intensifying screens.

Results and Discussion

In agreement with the previous findings^{8,14,15),} *F. sporotrichioides*, *F. poae*, *F. fusarioides* and *F. tricinctum* produced the type (A) trichothecenes, and *F. crookwellence*, *F. sp.* Fn 2B and *F.*

F: Fn-2B 1: KF 1103 2: R 2145 3: R 2344 4: R 2356

(A) *F. poae*

F: Fn-2B 1: R 2000 2: R 2071 3: R 2010

(B) *F. tricinctum*

Fig. 1. Hybridization patterns of fungal DNA with 1.1 kb fragment of Fn 2B.

graminearum produced the type (B) (Table 2).

For the isolation of genomic DNA from *Fusarium* spp., the present method is simple and rapid, and yielded 60 (R2334)–900 (R2071) μg of DNA per gram of the wet mass of protoplasts, with a size greater than 100 kb and $A_{260/280}$ ratio of 1.6–2.0. Upon insertion of *Bam*HI fragments of the total DNA genome of Fn 2B into *Bam*HI sites of *E. coli* plasmid vector pUC19, followed by an introduction of the plasmids into *E. coli* JM109, two colonies were selected from the transformants. The *Bam*HI digestion of plasmids revealed two fragments with 1.1 and 1.7 kb.

Southern blot analysis on the selected *Fusarium* spp. with 1.1 kb fragment of Fn 2B as a probe was shown in Fig. 1 (A)–(D), and the detailed results on each isolates were schematically

Fig. 1. Hybridization patterns of fungal DNA with 1.1 kb fragment of Fn 2B.

presented in Fig. 2. The data on *F. nivale* and *F. sambucinum* were not presented since no notable bands were detected. The results showed that the restriction fragment patterns of Fn 2B were markedly different from those of the *Fusarium* spp. tested.

However, there are common patterns among the same species of selected *Fusarium* species, as follows ; In all 4 isolates of *F. poae*, *Bam*HI yielded the band at 0.9 kb, along with an additional band at 7 kb in KF 1103 and R 2356, and the band at 2.5 kb was common after the digestion with *Hind*III. In the two isolates (R2000 and R2071) of *F. tricinctum*, the fragments with 0.9, 8 or 2.5 kb were detected after *Bam*HI, *Eco*RI and *Hind*III, respectively. In *F. sporotrichioides*, these three restriction enzymes resulted in 8, 2.5 and 10.3 kb, and in *F. graminearum* 0.9, 8.5 and 2.5 kb were the common bands, respectively.

Since the Southern blots with the 1.1 kb fragment revealed various hybridization patterns among the DNA derived from the *Fusarium* species, it was presumed that this fragment may be highly conserved and code some common genes essential for the fungal growth. However, the sequence analysis of this 1.1 kb fragment estimated to compose 1,149 bp in length, and computer search resulted in no information about already known-genes and proteins.

Southern blot analysis with the 1.7 kb fragment revealed the bands at 1.7 kb, 9.4 kb and 4.5 kb in Fn 2B after the digestion with *Bam*HI, *Eco*RI and *Hind*III, respectively. But no significant hybridization pattern was observed with the DNA fragments derived from the selected *Fusarium*

Fig. 2. Schematic presentation of hybridization patterns of fungal DNA with 1.1 kb fragment of Fn 2B.

spp. (data not presented).

From the present data shown in Fig. 2 with 1.1 kb fragment of Fn 2B, it was demonstrated that there are common restriction fragment length patterns among the same species, and no such similar patterns were detected among the different species. It means that this 1.1 kb fragment is expected as an excellent tool for the classification of trichothecene-producing fungi. An important finding is that the hybridization pattern observed with Fn 2B is unique among the toxigenic *Fusarium* species tested. It suggested that Fn 2B is unique among the toxigenic *Fusarium* species tested. It means that Fn 2B is an undescribed *Fusarium* species.

Recently, we have determined the primary structure of peptidyl-isomerase a (PPIase) in *F. sporotrichioides*^{16,17,18}. This enzyme, known as a cyclophilin, plays an important role for *cis-trans* isomerization of prolyl peptide bonds in polypeptides and the folding process of proteins. Further, PPIase constitutes a highly conserved family of abundant proteins in eukaryotes. In this respect, such information on the amino acid sequence of selected fungal proteins is expected to give a tool for chemotaxonomy of *Fusarium* species in future.

Acknowledgements A part of the present experiments was aided by the Ministry of Education, Science and Culture and the Ministry of Agriculture, Forestry and Fisheries (Japan). The strains *F. nivale* ARC 1035 and 2051 were kindly provided by Dr. Koizumi (Agricultural Research Center, the Ministry of Agriculture, Forestry and Fisheries, Tsukuba).

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Addendum

By an introduction of multifocus analysis, phylogenic approach on toxigenic *Fusarium* spp. carried out by K. O'Donnell demonstrated that the isolate of Fn series (Fn 2, Fn 2B, Fn M) is undescribed species, and given a novel name *Fusarium kyushuiense* D'Donnell & Ueno *sp. nov.*¹⁹⁾. This finding supported our present finding obtained by DNA fragment hybridization.