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# Genetic Variation in *Fusarium oxysporum* Isolated from Wilt-infested Alfalfa in Hokkaido

**Eiichi MIYAGAWA, Motoki SATO, Kozo ASANO[1] and Eiryu OKAMOTO**

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## ABSTRACT

Genetic variation of *Fusarium oxysporum* isolated from wilt-infested alfalfa of 4 different locations (Ebetsu city, Nayoro city, Yakumo-cho, and Shimizu-cho) in Hokkaido was analyzed by random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism of the intergenic spacer (IGS) region of the ribosomal DNA (IGS-RFLP) analyses. For RAPD analyses, four 10-mer RAPD primers (OPC2, OPN4, OPP7, and OPQ5) were used. A total of 31 bands in gels separated by electrophoresis were scored (1/0) for the 4 primers tested. Genetic distances between each isolate were calculated using the program OCTAN Ver. 1.0, and a cluster analysis was used to generate a dendrogram showing the relationships between them. The isolates in Hokkaido were divided into three RAPD groups (group 2, 3, and 4) at a similarity value of more than 90%. American strains (ATCC strains) were included in a group (group 1) that was different from the Japanese strains. The IGS region was amplified using the primers of CNL 12 and CNS 1. An amplified fragment of approximately 2,600 b.p. was cut with the enzymes of *Ava* II, *Sau*3AI, *Cfo* I and *Eco*R1, revealing 4, 4, 1, and 2 restriction patterns, respectively. The isolates in Hokkaido were divided into six IGS types based on the combination of these patterns. The geographic origin was not found to be associated with the RAPD group or the IGS type. The RAPD and IGS-RFLP analyses revealed polymorphism within the isolates of *F. oxysporum* from wilt-infested alfalfa.


## INTRODUCTION

Alfalfa, protein-rich fodder legume grown worldwide, is one of the superior animal feeds especially palatable to dairy cattle. The cultivation area of alfalfa is about ten thousand hectare and shows a tendency to go up in Hokkaido[19]. However, alfalfa seldom persists beyond three or four years[15]. Wilt disease by *Fusarium oxysporum* has been regarded as one of the main causes of the phenomenon[15,17].

Assessment of genetic diversity in *Fusarium oxysporum* strains is important in epidemiology (short or long range pathogen dispersal), breeding for disease resistance, control of disease and plant quarantine. The modified polymerase chain reaction (PCR) with a single short arbitrary primer (10–16 mer), which requires no prior sequence information, has proved useful in detecting genetic polymorphisms among organisms. This amplification technique (random amplified polymorphic DNA; RAPD analysis) has been performed to investigate the genetic diversity in *F. solani forma specialis* (f. sp.) *cucurbitae*[5], *F. oxysporum* f. sp. *dianthi*[14], *F. oxysporum* f. sp. *vasinfectum*[3], *F. oxysporum* f. sp. *ciceris*[12], *F. oxysporum* f. sp. *pisi*[8], *F. oxysporum* f. sp. *cubense*[4] and *F. oxysporum*[6] associated with wilt of angsana (*Pterocarpus indicus*). This technique can also generate specific DNA fragments useful for genome mapping, identification of isolates,

酪農学園大学酪農学科、農業微生物学
Department of Dairy Science, Agricultural Microbiology, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[1] 北海道大学農学部、応用生命科学専攻
Division of Applied Bioscience, Faculty of Agriculture, Hokkaido University, Kita-9, Nisi-9 Kita-ku, Sapporo, Hokkaido 001-0009, Japan

and application in molecular ecology[9,23].

The ribosomal RNA genes (rDNA) in fungal cells are organized in a single linkage group as an array of tandemly repeated multigene units. The intergenic spacer (IGS) regions (Fig. 1), which separates rDNA repeat units, are much more variable[10] and thus may provide useful intraspecific comparisons. IGS regions that were amplified by PCR, were ingested with proper restricted enzymes, electrophoresed on gel, and detected their separated bands on the gel under ultraviolet transillumination. The IGS-RFLP analysis can inform the base arrangement of the regions indirectly, and is regarded as a useful mean to examine genetic difference, i.e. *Histoplasma capsulatum*[20], *Puccinia graminis*[13], and *Fusarium oxysporum*[2].

In this work, we used RAPD and IGS-RFLP analyses to identify genetic variation among *F. oxysporum* isolates from wilt-infested alfalfa in Hokkaido.

## MATERIALS AND METHODS

**Strains** The strains used are shown in Table 1. Six strains from Ebetsu city, five from Yakumo-cho, six from Shimizu-cho, and five from Nayoro city were isolated from alfalfa *Fusarium* wilt in August to September 1995 and July to August in 1996. When pathogenicity tests were conducted using a root dipping method[16] with alfalfa (cv. Vertus), all the strains were pathogenic against the alfalfa. Three ATCC strains, SOUCHI 18, and SA were provided from the American Type Culture Collection, the National Grassland Research Institute and Yukijirushi Shubyo Co., respectively, as the reference strains of *F oxysporum* f. sp. *medicaginis*.

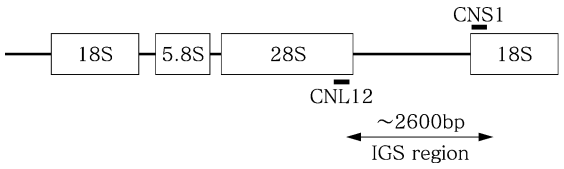


Fig. 1. The ribosomal DNA (rDNA) repeat unit and approximate locations of CNL12 and CNS1 primers.

**Cultivation of the fungus** Each of these strains was grown on a potato dextrose agar (PDA, Nissui Seiyaku Co.) at 25℃ for 4 days. A mycelial block (2-3 $mm^3$) of each strain was inoculated into 200 m1 of potato dextrose broth (PDB, Difco), and was cultivated by shaking it for 7 days. The culture was filtrated by s sterile cheese-cloth, and was centrifuged at 3,000 rpm for 10 min. The pellet was washed two times with sterile distilled water by centrifugation. The organism was freeze-dried, and stored at −20℃ until use.

**Extraction of DNA** The freeze-dried organism (0.3-0.4 g) was ground using a mortar and a pestle without thawing by cooling. Extraction of DNA from the fungus was performed by the method of Weising *et al*.[21]. The DNA pellet extracted was air dried, dissolved in 1 ml of l x TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH8), and stored at −20℃. Measurement of DNA concentration was done spectrophotometrically using a Beckman DU7400 spectrophotometer[22]. The DNA concentration was adjusted to 20 ng/$\mu$l with l x TE buffer.

**RAPD analyses** Crowhurst *et al*.[5] reported that the banding patterns obtained with the 10-mer primers were usually superior to those obtained with the longer primers. Then we used 10-mer

Table 1. List of strains used

| Locality | Strain | | | | | |
|---|---|---|---|---|---|---|
| America | ATCC 46587 | ATCC 52168 | ATCC 52169 | | | |
| Tochigi | SA | | | | | |
| Chitose | SOUCHI 18 | | | | | |
| Ebetsu | C-2-1 | D-1-2 | D-6-2 | E-4-2 | F-4-2 | P-1-1 |
| Yakumo | YKA-23 | YKA-24 | YKB-22 | YTC-11 | YTC-21 | |
| Shimizu | STA-22 | SMC-21 | SME-31 | SNA-12 | SNB-21 | SOC-11 |
| Nayoro | NHD-21 | NHE-22 | NAE-21 | NHYC-11 | NKB-11 | |

primers for RAPD analyses. Six kinds of RAPD analysis primers (Primer 1-6, Pharmacia), and 100 kinds of Random Primer (Operon) containing Kit C, Kit N, Kit O, Kit P, and Kit Q were tested. OPC2 (5'GTGAGGCGTC3'), OPN4 (5'GACCGACCCA3'), OPP7 (5'GTCCATGCCA3') and OPQ5 (5' CCGCGTCTTG3'), that were Operon-made and the clear difference observed among the strains in the preliminary experiment, were selected as the primers of this study because the bands detected were proper for analyses and reproducible.

RAPD reaction mixture contained 6.5 μl of Premix Ex Taq™ (Takara), 0.5 μl of 25 pM/μl primer, 20 ng of total DNA, and an adequate amount of sterile distilled water in a total volume of 13 μl. Amplification was performed with an automated thermal cycler (GeneAmp PCR System 2400, Perkin Elmer) programmed as follows: one cycle for 5 min at 94℃ (initial denaturing step) before the addition of the Taq polymerase, followed by 35 cycles at 30 sec at 94℃ (denaturing), 30 sec at 50℃ (annealing), and 1 min at 72℃ (elongation). One cycle at 5 min at 72℃ (final elongation step) was conducted after the 35 cycle reactions. A setting of annealing temperature of 50℃ is proposed by Inagaki *et al*.[11]. We accepted this temperature, because clearer bands were obtained at 50℃ than at 36-38℃ generally employed.

The amplified DNA fragments (5 μl) were separated by electrophoresis in 4-20% gradient polyacrylamide TBE-PAGE mini (Tefco) in 1 x TBE buffer. An electrophoretic apparatus (model STC-808, Tefco, Nagano-pref.) was used under the current of 20 mA for 50 min. DNAs having 50 to 2500 base pairs (FMC Bioproduct, Rockland, ME; 50, 100, 200, 300, 400, 500, 525, 750, 1,000, 1,250, 1,500, 2,000, and 2,500 b.p.) were used as the molecular markers. After electrophoresis, the gel was stained in 5 ppm of ethidium bromide solution (BIO-RAD) for 30 min, photographed by Polaroid camera on an UV-transilluminator (312nm).

**Phenetic analysis** The banding patterns were compared to determine the genetic relatedness among the isolates. A separate data matrix was constructed for each primer by scoring (1 or 0) each isolate for the presence or absence of major bands[4,8]. The results obtained from the 4 primers were pooled, and similarity coefficients were calculated from these matrix data by the formula of Nei and Li[18]. A dendrogram based on similarity coefficients was made using the unweighted pair-group, arithmetric average (UPGMA) method[1,5]. The software (OCTAN Ver. 1.0) for making the dendrogram was one developed by Dr. M. Okuda in National Institute of Chugoku Agriculture.

**IGS-RFLP analyses** DNA of each strain was amplified with primer CNL 12(CTGAACGCCTCTAAGTCAG) and primer CNS 1(GAGACAAGCATATGACTACTG) which Appel and Gordon[2] used to amplify the IGS region. The reaction mixture (total volume of 50 μl) of PCR was composed of 25 μl of Premix EX Taq™ (Takara), 1.6 μl of 0.5 μM primer CNL 12, 1.7 μl of 0.5 μM primer CNS 1, 20 ng of total DNA and an adequate amount of sterile distilled water. Amplification was performed in the thermal cycler programmed for one cycle of 1 min at 94℃, followed by 30 cycles of 30 sec at 94℃, 1.5 min at 58℃, 1.5 min at 72℃, and a final incubation at 72℃ for 5 min.

Whether or not the IGS region (about 2,600 b.p.) was amplified was confirmed by electrophoresis of the PCR products (0.5 μl) on 1.5% SeaKem GTG agarose (FMC) for 60 min at 50 V. Electrophoresis was performed with a submarine mini gel apparatus MUPID-2 (Kosumo Baio Co.) using an 1 x TBE buffer. The PCR products were digested with the restriction enzymes *Ava*II (*Eco*47I), *Cfo*I (*Hha*I), *Sau*3AI (each, Toyobo) and *Eco*RI (Takara) according to the manufactur's direction. Reaction mixture (total volume of 10 μl) was composed of 3 μl of the PCR products, 5U of the restriction enzyme, 1 μl of enzyme buffer and an adequate volume of sterile distilled water, then incubated at 37℃ overnight. The reaction mixture (2 μl) was electrophoresed on 2-3% NuSieve GTG agarose (FMC) for 45 min at 100V with the MUPID-2 apparatus. After electrophoresis, gels were stained with 5 ppm ethidium bromide solution, and photographed under UV transillumination (312 nm).

## RESULTS AND DISCUSSION

**RAPD analyses** RAPD patterns based on 4 kinds of primers are shown in Fig. 2. A matrix table was made by scoring the presence (1) or absence (0) of each band (Table 2). After a simi-

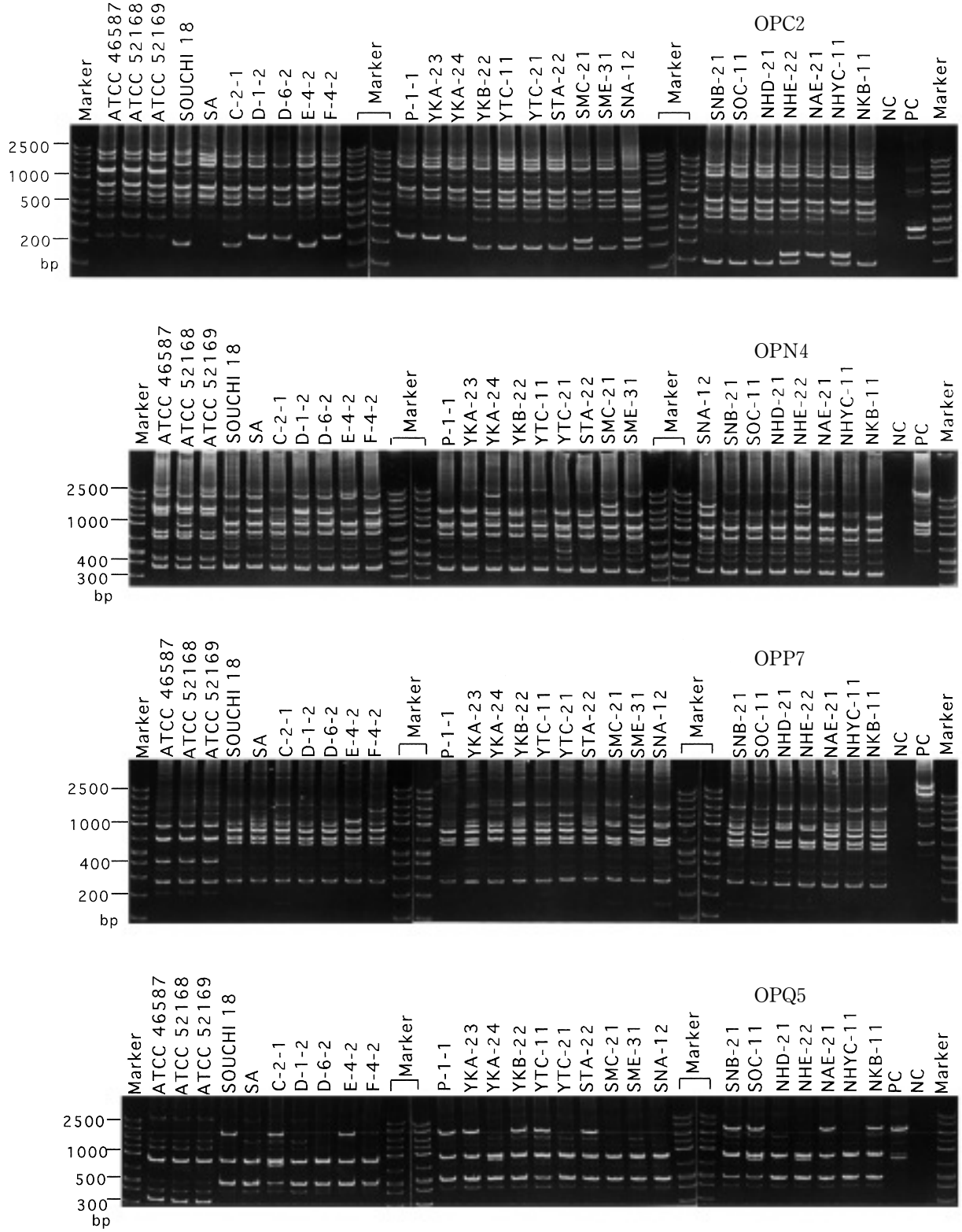


**Fig. 2.** RAPD patterns amplified with the primers (OPC2, OPN4, OPP7 and OPQ5).

**Table 2.** Data matrix of RAPD bands with 4 primers

| Strain | Primer | | | |
|---|---|---|---|---|
| | OPC2 | OPN4 | OPP7 | OPQ5 |
| ATCC 46587 | 1 0 1 1 1 0 0 | 1 0 1 1 1 0 0 0 1 1 1 1 | 0 1 0 1 0 1 1 | 0 1 0 0 1 |
| ATCC 52168 | 1 0 1 1 1 0 0 | 1 0 1 1 0 0 1 0 1 1 1 1 | 0 1 0 1 0 1 1 | 0 1 0 0 1 |
| ATCC 52169 | 1 0 1 1 1 0 0 | 1 0 1 1 1 0 0 0 1 1 1 1 | 0 1 0 1 0 1 1 | 0 1 0 0 1 |
| SOUCHI 18 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| SA | 0 1 1 1 0 0 0 | 1 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| C-2-1 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 1 1 0 |
| D-1-2 | 1 0 1 1 1 1 0 | 1 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| D-6-2 | 1 0 1 1 1 1 0 | 1 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| E-4-2 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| F-4-2 | 1 0 1 1 1 1 0 | 1 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| P-1-1 | 1 0 1 1 1 1 0 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| YKA-23 | 1 0 1 1 1 1 0 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| YKA-24 | 1 0 1 1 1 1 0 | 1 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 0 0 1 | 0 1 1 1 0 |
| YKB-22 | 1 0 1 1 1 0 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| YTC-11 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| YTC-21 | 1 0 1 1 1 0 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| STA-22 | 1 0 1 1 1 0 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| SMC-21 | 1 0 1 1 1 1 1 | 0 1 0 1 0 1 0 1 0 0 0 1 | 1 1 0 1 1 0 1 | 0 1 0 1 0 |
| SME-31 | 1 0 1 1 1 0 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| SNA-12 | 1 0 1 1 1 1 1 | 0 1 0 1 0 1 0 1 0 0 0 1 | 1 1 0 1 1 0 1 | 0 1 0 1 0 |
| SNB-21 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| SOC-11 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 1 1 0 |
| NHD-21 | 1 0 1 1 1 0 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 1 1 0 1 1 0 1 | 0 1 0 1 0 |
| NHE-22 | 1 0 1 1 1 1 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 1 1 0 1 1 0 1 | 0 1 1 1 0 |
| NAE-21 | 1 0 1 1 1 1 0 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |
| NHYC-11 | 1 0 1 1 1 1 1 | 0 0 0 0 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 0 1 0 1 0 |
| NKB-11 | 1 0 1 1 1 0 1 | 0 0 0 1 0 1 0 1 0 0 0 1 | 0 0 1 1 1 0 1 | 1 1 0 1 0 |

1 means the presence of DNA band, and 0 the absence of it

larity coefficient was calculated according to the matrix data, a dendrogram was constructed using the similarity coefficient (Fig. 3). Five groups having a similarity coefficient of more than 90% were formed. Group 1 contained three ATCC strains; Group 2 was composed of SOUCHI 18, C-2-1, E-4-2, YKB-22, YTC-11, YTC-21, STA-22, SME-31, SNB-21, SOC-11, NKB-11, and NHYC-11; group 3 was D-1-2, D-6-2, F-4-2, P-1-1, YKA-23, YKA-24, and NAE-21; and group 4 contained SMC-21, SNA-12, NHD-21, and NHE-22. Group 5 contained only SA. These results are shown in Table 4.

The isolates from Hokkaido formed three different groups (2, 3 and 4). The isolates from Ebetsu city and Yagumo-cho belonged to group 2 and 3. The isolates from Shimizu-cho belonged to group 2 and 4, and the isolates from Nayoro city belonged to group 2, 3, and 4. The similarity coefficient between the three ATCC strains (isolates in American) and all the isolates in Japan was below 52%. Therefore, ATCC strains seem to be different in lineage from those in Japan. The coefficient between SA (an isolate in Tochigi Prefecture) and the isolates in Hokkaido were low in similarity (75%). SA seems to contain a little different phylogeny from the isolates in Hokkaido. The SOUCHI 18 strain isolated from Chitose city belonged to group 2, therefore, the strains of group 2 were isolated from all areas collected in Hokkaido. It is assumed that the strains of group 2 are of wide distribution in Hokkaido.

**IGS-RFLP analyses** After the confirmation of that the band corresponds to the amplified IGS region by electrophoresis (data not shown), the PCR products were digested with 4 kinds of restriction enzymes, and then electrophoresed (Fig. 4). The used strains were divided into four patterns by both *Ava*II and *Sau*3AI. They were

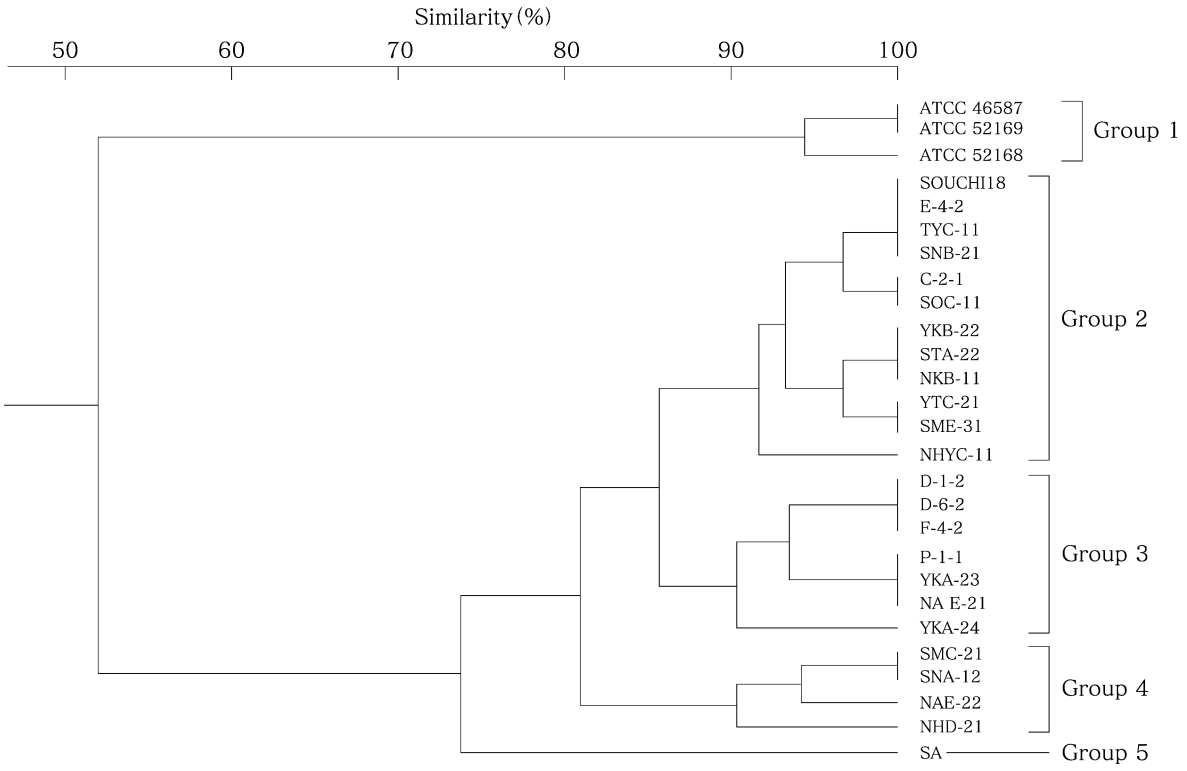


**Fig. 3.** Dendrogram showing relationships among 27 *Fusarium oxysporum* strains.

then divided into two patterns by *Eco*R1. One pattern was formed by *Cfo*I. The authors classified these strains into six IGS types based on these patterns (Table 3). The results classified by the IGS type are shown in Table 4. Type 1 included D-6-2, F-4-2, P-1-1, YKA-23, and NAE-21, and Type II included SOUCHI 18, E-4-2, YTC-21, and SME-31. Type III was composed of YKA-24, YKB-22, SMC-21, SNA-12, NHE-22, NHYC-11, and NKB-11, and Type IV was composed of three ATCC strains, C-2-1, SNB-21, and SOC-11. Type V contained SA, YTC-11, STA-22, and NHD-21, and Type VI contained only D-1-2.

**Table 3.** IGS types and restriction patterns

| IGS type[1)] | Restriction enzyme | | |
|---|---|---|---|
| | AvaII | *Sau* 3AI | *Eco*RI |
| I | A[2)] | E | I |
| II | A | E | J |
| III | A | F | I |
| IV | B | G | I |
| V | C | H | I |
| VI | D | G | I |

[1)] IGS types represent the combination of patterns obtained with three enzymes used.
[2)] Corresponds to the band in Fig. 3.

Appel and Gordon[2)] separated the strains of *F. oxysporum* f. sp. *melonis* into 13 IGS types, that were pathogen to melon, using the same enzymes as we examined. Edel *et al.*[7)] performed IGS-RFLP analyses for 400 strains of *F. oxysporum* isolated melon, tomato, wheat, flax, and soil using 7 kinds of restrict enzymes. These strains were divided into 16 IGS types. Woudt *et al.*[24)] carried out IGS-RELP analyses of pathogen and non-pathogen strains of *F. oxysporum* isolated from cyclamens. These strains were separated into six IGS-types. The isolates from alfalfa in Hokkaido were separated into 6 IGS types, and each strain in Hokkaido belonged to all of these types. In conclusion, variation in the IGS of the rDNA appears to have considerable potential to resolve interstrain relationships within *F. oxysporum*.

When RAPD groups connected with IGS-types (Table 4), RAPD group 1 contained only IGS IV which were ATCC strains. RAPD group 2 con-

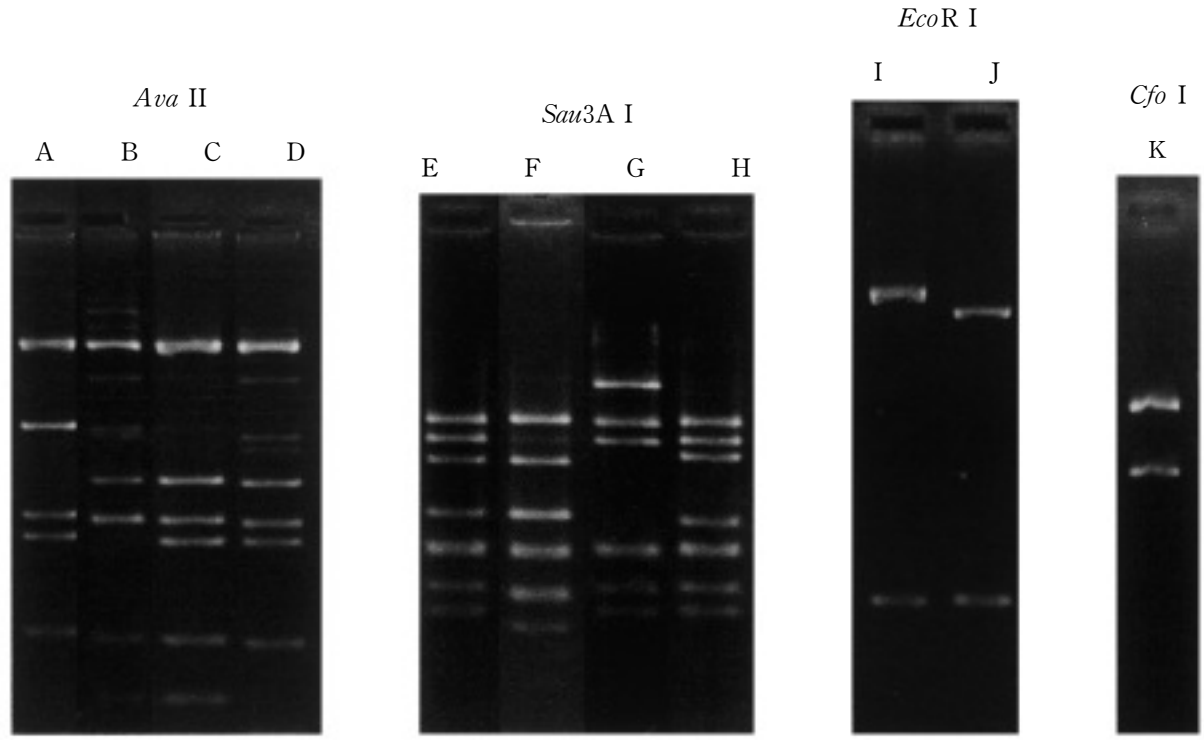


**Fig. 4.** RFLP patterns generated by digestion of the IGS region with the restriction enzymes.

sisted of IGS II, III, IV, and V; RAPD group 3 consisted of IGS I, III and VI; and RAPD group 4 consisted of IGS III and V. RAPD group 5 only contained IGS V. RAPD analysis does compare the difference of total DNA, however IGS-RFLP analysis does that of the IGS region of rDNA. It seems to be difficult to find the interrelation between RAPD groups and IGS types because they're analytical methods from a different angles. However the application of the two methods is effective in characterizing isolates and providing insight into the complex relationship of *F. oxysporum*.

The RFLP and RAPD analyses suggest that there may be genetic diversity between isolates of the infested alfalfa. It is the first report of RAPD and IGS-RELP analyses for *F. oxysporum* from alfalfa wilt. Geographic origin was not found to be associated with RAPD and IGS-RFLP analysis. Future studies should include a large sample size of isolates from the present sources, as well as from additional diverse geographic locations to better understand the biodiversity within this fungus.

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**Table 4.** RAPD groups and IGS types of *Fusarium oxysporum*

| RAPD groups | IGS types | Strains |
|---|---|---|
| 1 | IV | ATCC 46587<br>ATCC 52168<br>ATCC 52169 |
| 2 | II | SOUCHI 18<br>E-4-2<br>YTC-21<br>SME-31 |
| | III | YKB-22<br>NHYC-11<br>NKB-11 |
| | IV | C-2-1<br>SNB-21<br>SOC-11 |
| | V | YTC-11<br>STA-22 |
| 3 | I | D-6-2<br>F-4-2<br>P-1-1<br>YKA-23<br>NAE-21 |
| | III | YKA-24 |
| | VI | D-1-2 |
| 4 | III | SMC-21<br>SNA-12<br>NHE-22 |
| | V | NHD-21 |
| 5 | V | SA |


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## 要　　約

アルファルファ萎凋病から分離した *Fusarium oxysporum* の菌株間の類縁関係を明らかにするために，Random Amplified Polymorphic DNA (RAPD)解析及びリボソーム DNA スペーサー領域（Intergenic Spacer Region）の制限酵素切断フラグメント長多型（Restriction Fragment Length Polymorphisms）解析（IGS-RFLP 解析）を行った。道内 4 地域（江別市，名寄市，八雲町，清水町）から分離された 22 菌株を含め計 27 菌株を供試した。本菌株を培養し，Weising らの方法で DNA を抽出した。RAPD 解析では 4 種類のプライマーを使用して増幅し，電気泳動を行った。それぞれの増幅バンドパターンを数値化し，系統樹作成ソフトを使用して UPGMA 法でデンドログラムを作成した。IGS-RFLP 解析では，プライマーCNL12 と CNS1 で IGS 領域を増幅し，増幅断片を制限酵素 *Ava* II，*Sau* 3A1，*Cfo* 1，*Eco* R1 で消化し，電気泳動を行った。RAPD 解析では類似度 90%以上で 5 つのクラスタに分かれた。IGS-RFLP 解析では 4 種類の制限酵素パターンから 5 つの IGS タイプに分かれた。以上の結果から，本分離株は遺伝的にかなり多様性であることが示唆された。