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Nucleotide Divergence Analysis of IGS Region in *Fusarium oxysporum* and its formae speciales Based on the Sequence

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The intergenic spacer (IGS) sequence of *Fusarium oxysporum* have been reported to provide reliable information concerning intraspecific variation and phylogeny of fungal species. The eleven strains of *Fusarium oxysporum* and its formae speciales belonging to section *Elegans* were compared with sequencing analysis. The direct sequencing of partial IGS was carried out using PCR with primer NIGS1 (5'-CTTCGCCTCGATTTCCCCAA-3')/NIGS2 (5'-TCGTCGCCGACAGTTTTCTG-3') and internal primer NIGS3 (5'-TCGAGGATCGATTCGAGG-3')/NIGS4 (5'-CCTCGAATCGATCCTCGA-3'). A single PCR product was found for each strain. The PCR fragments were sequenced and revealed a few within species polymorphisms at the sequence level. The size of partial IGS sequencing of *F. oxysporum* was divided into three groups; 526~527 bp including *F. o.* f. sp. *chrysanthemi*, *cucumerinum*, *cyclaminis*, *lycopersici*, and *fragariae*; 514~516 bp including *F. o.* f. sp. *lilii*, *conglutinans*, and *raphani*; 435 bp for *F. o.* f. sp. *cucumerinum* from Korea. Sequence analysis of PCR products showed that transitions were more frequent than transversions as well as the average numbers of substitution per site were range 0.41% to 3.54%.

KEYWORDS *Fusarium oxysporum*, **Intergenic spacer region (IGS), Nucleotide divergence sequencing**

In eukaryotes, rDNA is composed of tandem arrays of the basic repeat units including both highly conserved genes and more variable spacer regions. Spacer region contains both the internal transcribed spacer (ITS) and the intergenic spacer (IGS). These two spacer regions are highly polymorphic and provide useful tools for taxonomic and phylogenic studies (Chillali *et al.*, 1998). The IGS, showing considerable divergence in closely related species, often reflects both length and sequence variation (Hills and Dixon, 1991). Thus IGS has been widely investigated in many fungi including, *Armillaria* (Chillali *et al.*, 1998, Terashima *et al.*, 1998), *Pisolithus* (Gomes *et al.*, 1998), *Ascochyta* (Faris-Mokaiesh, 1996), *Paxillus involutus* (Hŏnig *et al.*, 2000), and *Bradyrhizobium* species (Doignon *et al.*, 2000), *Saccharomyces* species (Molina *et al.*, 1993) coupled with RFLP (Appel and Gordon, 1995), RAPD (Min and Ryu, 1998), and sequencing (Lee *et al.*, 2000b) analysis. In spite of the most rapidly evolving region, IGS also contains all the elements necessary for RNA transcript regulation including enhancer, promoter, terminator. The structural contradiction was explained both co-evolution of regulatory factor and co-operation of trans-acting factor. A molecular systematic approach based on the DNA sequence of several loci detects considerable variation within the *F. oxysporum*. As a preliminary report of this work, PCR-RFLP analysis of twenty-two strains in *F. oxysporum* and its formae specials showed the polymorphism in length and restriction site of IGS (Kim *et al.*, 2001). Total nine of IGS haplotypes were identified among these strains. One or two strains representing the each haplotype was compared with the partial IGS sequencing.

DNA sequence analysis was used to investigate the molecular basis of the IGS length difference and focused on identifying the region responsible for this variation. In addition to clarifying the taxonomic status and relationships among *Fusarium oxysporum* formae speciales, the purpose of this study was to assess the amount and distribution of sequence variation in a tandemly repeated IGS region.

Materials and Methods

Fungal strains, culture conditions and DNA extraction. The 11 strains of *Fusarium oxysporum* were used in this studies and listed in Table 1. Culture conditions were followed by previous paper (Kim *et al.*, 2001). Genomic DNA for PCR was extracted according to Lee *et al.* (2000a).

Partial IGS amplification and direct sequencing. The intergenic spacer region of the ribosomal DNA was amplified with using the primers CNL12 and CNS1 according to Appel and Gordon (1995). Using the PCR product as a template, partial IGS was amplified with new primers NIGS1 (5'-CTTCGCCTCGATTTCCCCAA-3') and NIGS2 (5'-TCGTCGCCGACAGTTTTCTG- 3'). Internal primers NIGS3 (5'-TCGAGGATCGATTCGAGG-3') and NIGS4

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Table 1. List of *Fusarium oxysporum* formae speciales used in this study

Strain	Isolate number	Source*	RFLP* haplotype
F. oxy. f. sp. *chrysanthemi* Luttrell *et al.*	52422	ATCC	VII
F. oxy. f. sp. *cucumerinum* Owen	16416	ATCC	I
F. oxy. f. sp. *cucumerinum* Owen		Korea	IX
F. oxy. f. sp. *cyclaminis* Gerlach	16061	ATCC	II
F. oxy. f. sp. *dianthi* (Prillieux et Delacroix) Snyder et Hansen	11939	ATCC	VI
F. oxy. f. sp. *lilii* Imle	15642	ATCC	VIII
F. oxy. f. sp. *lycopersici* (Saccardo) Snyder et Hansen	34298	ATCC	I
F. oxysporum Schlecht.	7500	Australia	IV
F. oxy. f. sp. *conglutinans* (Wollenweber) Snyder et Hansen	744001	MAFF	III
F. oxy. f. sp. *fragariae* Winks et Williams	744009	MAFF	VI
F. oxy. f. sp. *raphani* Kendrick et Snyder		Korea	III

*Source : ATCC (American type culture collection).
MAFF (Ministry of Agriculture, Forestry and Fisheries, JAPAN).
*RFLP haplotypes : Kim *et al.* (2001).

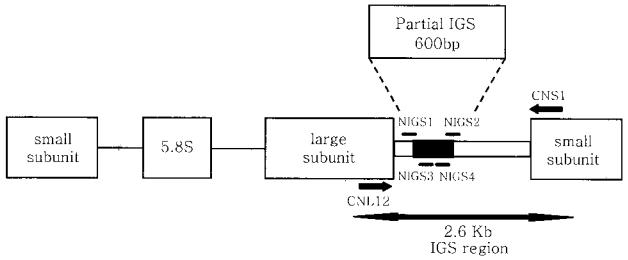

Fig. 1. Diagramatic representation of the ribosomal DNA repeat unit showing the location of the IGS amplified by primers

(5'-CCTCGAATCGATCCTCGA-3') were designed with reference to Appel and Gordon (1996) (Fig. 1). Amplification was performed in a 100 ul of reaction mixture containing 10 u*l* of 10 PCR buffer (500 uM KCl, 100 mM Tris HCl of pH 9.0, 1% Triton X-100), 2 mM $MgCl_2$, 0.4 ug of template DNA, 200 uM of each dNTPs, 25 units *Taq* DNA polymerase (BioBasic Co.) and 10 pmol of both primers. The mixture was subjected to PCR in an MWG Biotech (Germany). An initial denaturation for 5 min at 94°C was followed by 30 cycles of denaturation for 40 sec at 94°C, annealing for 40 sec at 59°C and extension for 40 sec at 72°C. A final extension of 72°C for 10 min was incorporated into the program. PCR products were separated by electrophoresis through 1.5% agarose gels, stained with ethidium bromide (EtBr) and visualized under an UV transilluminator. PCR products, confirmed on gels, were cut and purified with EASYTRAP™ DNA elusion kit. (Takara huzo Co., Ltd., Japan) PCR-direct sequencing for the partial IGS region was carried out using the PCR sequencing kit (Bioneer Co.). PCR conditions proceeded a predenaturation for 3 min at 94°C, 40 cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C and extension for 1 min at 72°C. After the thermal cycling program was completed, the reaction mixture was added to 4 u*l* of sequencing stop solution and heated for 3 min above 90°C before loading 3 u*l* of each

Table 2. The total size (bp) of IGS partial sequences in *Fusarium* strains and accession number in Genbank

	Total length	G+C content	Accession number
F. oxy. f. sp. *chrysanthemi*	526	53.6%	AF439326
F. oxy. f. sp. *cucumerinum*	527	54.6%	AF439327
F. oxy. f. sp. *cucumerinum*	435	60.1%	AF439328
F. oxy. f. sp. *cyclaminis*	527	54.8%	AF439329
F. oxy. f. sp. *dianthi*	526	55.3%	AF439336
F. oxy. f. sp. *lilii*	515	54.9%	AF439335
F. oxy. f. sp. *lycopersici*	526	53.9%	AF439330
F. oxysporum	527	55.0%	AF439331
F. oxy. f. sp. *conglutinans*	514	55.0%	AF439337
F. oxy. f. sp. *fragariae*	526	55.3%	AF439332
F. oxy. f. sp. *raphani*	516	54.1%	AF439333

reaction on a 6% polyacrylamide (acrylamide : bisacrylamide = 19 : 1) sequencing gel for approximately 4 hrs. The gel was stained using a DNA silver staining kit (Bioneer Co.) and exposed to APC film (Promega Co.).

Sequences analysis. Sequencing data, repeated several times to conform the results, were aligned manually and analyzed using program within DNASYS (version 2.1). The partial IGS sequences determined in this paper were submitted to GenBank with accession number (Table 2). Nucleotide substitution, insertion and deletion were analyzed manually, and nucleotide identity (%) was obtained using Maximum-matching program. The total number of nucleotide substitutions per site (K) was calculated with Kimura's two-parameter method $K = -0.5 \ln[(1-2P-Q)\sqrt{1-2Q}]$, where P is the proportion of transitions, and Q is the proportion of transversion (Kimura, 1980).

Results and Discussion

Length variation within partial IGS. Amplification of partial IGS rDNA with primers NIGS1 and NIGS2

yielded a single DNA fragment and resulted in the length variation among formae speciales. Those were classified with three groups into 526~527 bp, 514~516 bp, and 435 bp, respectively (Table 2). Variation in size was due to single or multiple base insertions or deletions and these results consisted with Appel and Gordon (1996). Especially, *F. oxysporum* f. sp. *cucumerinum* Korean isolate had smallest 435 bp, which showed above 90 bases deletion of the nucleotide at position 106~193, 333~344, and had the highest GC content, 60.1%. Depending on the preliminary study (Kim *et al.*, 2001) about PCR-RFLP analysis of IGS, the 11 strains were chosen as representatives of nine haplotypes for subsequent sequence analysis.

According to these analyses, we certified the intraspecific length variation of IGS in *Fusarium oxysporum*. These results were consistent with the report of Mishra *et al.* (2002). They previously performed digestion of IGS with five restriction endonucleases and revealed that RFLPs reflected intraspecific variation (47.69%) in this rDNA region of *F. culmorum* and total of 29 IGS different haplotypes were identified among 75 isolates including length variation. They concluded that there was a pronounced length variation in IGS region of *F.culmorum* that may perhaps be due to insertion/deletion events. In sequence comparison of the IGS region, length variation has been reported in many fungi including *Fusarium* species. The length variation among *F. oxysporum* (Appel and Gordon, 1996) and other strains were above 10 bp, caused by the base insertion of the nucleotide at position 44~45 (GA), 129~133 (AATTC), 396~397 (TC), and 496 (G). Those differences were attributed to insertions or deletions in the arrays of subrepeats within the IGS region (Appel and Gordon, 1996). At the level of the IGS subrepeat arrays, those structural variations were caused by unequal crossing over and gene conversion (Teresa and Crease, 1995).

Variability analysis within the partial IGS sequences. Using DNASYS program, IGS nucleotide of *F. oxyporum*

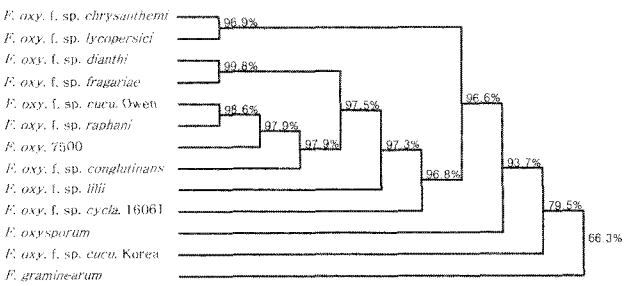

Fig. 2. Phylogenetic relationship based on the nucleotide sequence multi-alignment of the IGS region of the *Fusarium oxysporum* and its formae speciales. Numbers on the tree indicate the percent of the similarity. *F. graminearum* was referred to Min and Lee (Min and Lee, 2003).

and its formae speciales was alignments compared with *F. graminearum* referred to Min and Lee (2003). As outgroup sequencing data were aligned with multiple-alignment plot and phylogenetic relationship (Fig. 2). Through the sequence alignment, there were 100 base substitutions (68% were transitions), 2 site insertions and 5 site deletions ranging in size from 1 to 88 bp (data not shown). Base substitutions were common with transition occurring usually more frequent than transversion. It was coincided with those of Appel and Gordon (1996). According to Appel's, we detected the restriction sites of two *Ava*II, three *Sau*3A, and two *Eco*RI site, which was agreed with IGS-RFLP results among *F. oxysporum* and its formae speciales (Kim *et al.*, 2001). The average number of nucleotide substitutions per site for the partial IGS was calculated by using the method of Kimura (1980) and the respective distance matrix was shown in Table 3. The average numbers of substitution per site were range of 0.41%~3.54%. Base substitution was highest at *F. oxysporum* f. sp. *dianthi* and *lycopersici*, above 3.54%. *F. oxysporum* f. sp. *cucumerinum* Korea isolate, which was shown base deletion, was caused lower substitution. In *Drosophila melanogaster* species group, the average num-

Table 3. Matrix for the average numbers of substitutions per site for the partial IGS sequence comparisons

	1	2	3	4	5	6	7	8	9	10	11
1	0.0000	0.0254	0.0121	0.0155	0.0215	0.0229	0.0314	0.0293	0.0209	0.0294	0.0249
2		0.0000	0.0162	0.0155	0.0214	0.0166	0.0253	0.0154	0.0103	0.0155	0.0041
3			0.0000	0.0122	0.0040	0.0122	0.0203	0.0203	0.0040	0.0040	0.0162
4				0.0000	0.0215	0.0103	0.0254	0.0194	0.0125	0.0155	0.0165
5					0.0000	0.0124	0.0354	0.0254	0.0083	0.0058	0.0186
6						0.0000	0.0293	0.0187	0.0104	0.0103	0.0166
7							0.0000	0.0273	0.0230	0.0293	0.0270
8								0.0000	0.0124	0.0194	0.0164
9									0.0000	0.0062	0.0103
10										0.0000	0.0144

Number : 1. *F. oxy.* f. sp. *chrysanthemi*, 2. *F. oxy.* f. sp. *cucumerinum* Owen, 3. *F. oxy.* f. sp. *cucumerinum* Korea, 4. *F. oxy.* f. sp. *cyclaminis* 16061, 5. *F. oxy.* f. sp. *dianthi*, 6. *F. oxy.* f. sp. *lilii*, 7. *F. oxy.* f. sp. *lycopersici*, 8. *F. oxysporum* 7500, 9. *F. oxy.* f. sp. *conglutinans*, 10. *F. oxy.* f. sp. *fragariae*, 11. *F. oxy.* f. sp. *raphani*.

ber of substitutions is apparently correlated with the evolutionary distance (Schlötterer *et al.*, 1994). But it must remain, at this point, whether this is a siginificant difference or whether this is simple due to the different procedures taken to estimate these rates, especially, in this species.

The percent of the nucleotide identities was analysed with Maximum-matching program (data not shown). All of strains showed highly nucleotide similarity, above 94%. However, *F. oxysporum* f. sp. *cucumerinum* Korean isolate was shown the lowest similarity, 79~81% compared with other strains. Such a result was caused by multiple deletion in the position 106~193 (88 bp), 333~344 (12 bp) and 3 single base transitions in their sequence. *F. graminearum* was shown 63~77% similarity as compared with *F. oxysporum*. *F. graminearum* belonging to section *Discolor* was known distantly relative species to *F. oxysporum*. There was interspecies variation as well as intraspecies in IGS region (Min and Lee, 2003).

Based on the sequence analysis, the dendrogram obtained from multi-alignment plot was shown in Fig. 2. In this tree, all of the *F. oxysporum* formae speciales were clustered above 93.7% similarity except *F. oxysporum* f. sp. *cucumerinum* Korean isolate, in which it was clustered with 79.5%. The intergenic spacer region (IGS) appeared to be more rapidly evolving than any region in the rDNA repeat units. And closely related species may show considerable divergence in IGS, often reflecting both length and sequence variation (Hills and Dixon, 1991). According to Guidot *et al.* (1999), previously published data for different fungal species indicated that intraspecific variablility within the IGS is commonplace.

These results of the nucleotide divergence of partial IGS in *Fusarium oxysporum* and its formae speciales conformed that their IGS sequence analysis was in agreement with IGS-RFLP pattern and gave an useful phylogenetic information.

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