

Relationships Among Pathogenic and Nonpathogenic Isolates of *Fusarium oxysporum* Based on the Partial Sequence of the Intergenic Spacer Region of the Ribosomal DNA

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Using PCR, we amplified and sequenced ~1,000 bp of the 5' end of the intergenic spacer (IGS) of the rDNA in 15 isolates of *Fusarium oxysporum* and one isolate of *F. subglutinans*. Isolates were selected to represent diversity in our collection based on differences in pathogenic race, vegetative compatibility group (VCG), mitochondrial DNA (mtDNA) haplotype, IGS haplotype, and DNA fingerprint. The objective of this research was to clarify the origin of virulence within *F. oxysporum*, the relationship between pathogenic and nonpathogenic strains, and the evolution of the different races of *F. oxysporum* f. sp. *melonis*. Bootstrapped parsimony analysis of the partial IGS sequence data identified a phylogenetic tree with highly significant branches. The two *F. oxysporum* f. sp. *melonis* VCGs, 0131 and 0134, were separated into distinct lineages. Race was not distinguished by significant IGS sequence differences within the pathogen VCGs. One exception was a race 1 isolate which was associated with VCG 0131 but, based on both mtDNA and IGS haplotype, had greater affinity with VCG 0134. Two IGS sequence types were found in this race 1 isolate, one suggesting an affiliation with VCG 0131 and the other similar to isolates in VCG 0134. This may have resulted from past somatic or sexual interactions between *F. oxysporum* f. sp. *melonis*, VCGs 0131 and 0134. Nonpathogens that were vegetatively compatible with the pathogen were not closely related to the pathogen based on IGS sequence data. Thus, nonpathogens and pathogens may share common alleles at vegetative compatibility loci by coincidence rather than because of recent clonal derivation from a common ancestor.

Additional keyword: Fusarium wilt.

Fusarium oxysporum Schlechtend.:Fr is an anamorphic species which includes numerous plant pathogenic strains causing wilt diseases on a broad range of agricultural and ornamental host plant species. Strains with the same limited host range are grouped together into a forma specialis

(Armstrong and Armstrong 1981). Some formae speciales are further subdivided into two or more pathogenic races. Although pathogenic strains of *F. oxysporum* are regarded as specialized parasites (Garrett 1970), given their ability to colonize the xylem of a living plant, other strains of *F. oxysporum* are apparently incapable of causing disease (Burgess 1981; Gordon and Okamoto 1990; Gordon et al. 1992). In fact, most strains of *F. oxysporum* are demonstrably avirulent on the plant from which they were isolated (Hendrix and Nielsen 1958; Katan 1971). These nonpathogenic strains are nevertheless very aggressive colonizers of plant roots (Gordon et al. 1989), but they induce no symptoms and have no obvious detrimental effect on the colonized plant. Nonpathogenic strains are also saprophytic, competing effectively with other soilborne fungi in the exploitation of crop residue under field conditions (Gordon and Okamoto 1990). Thus, although it can never be stated conclusively that an isolate is not pathogenic to any potential host plant, *F. oxysporum* has a well-documented capacity to persist by means other than pathogenesis. Furthermore, *F. oxysporum* is a common inhabitant of native soils (Gordon et al. 1992; Smith and Snyder 1975) and yet it is virtually unknown as a pathogen of plants in native settings.

Because nonpathogenic strains of *F. oxysporum* are very abundant in temperate agricultural soils and because new pathogenic races appear in new areas with some regularity, the question arises as to whether pathogenic strains may be selected from local populations of otherwise nonpathogenic strains. Considerable evidence now indicates that for most formae speciales, a limited number of clones (or clonal lineages) have a broad distribution (Bosland and Williams 1987; Jacobson and Gordon 1991; Kistler et al. 1991; Larkin et al. 1990). Thus most new occurrences of a given Fusarium wilt disease probably reflect recent movement of a pre-existing strain rather than an independent local origin of the pathotype.

Although a few well-studied examples support the distinctiveness of pathogenic strains, most formae speciales have not been closely examined. Moreover, some nonpathogenic strains of *F. oxysporum* appear closely related to a co-occurring pathogen (Appel and Gordon 1994; Gordon and Okamoto 1992a, 1992b; Gordon et al. 1992), based on vege-

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tative compatibility, similarity in mitochondrial DNA or both. Thus, the nature of the relationships among pathogenic and nonpathogenic strains remains poorly understood and it is as yet unclear whether the wilt pathogens are confined to a unified subgrouping within *F. oxysporum* or are dispersed among an array of clonal lineages. This issue is of both theoretical and practical importance. In the first instance, this system may provide insight into the evolution of pathogenic forms within a species well adapted to a nonpathogenic existence. As a practical matter, knowledge of what populations constitute reservoirs of new pathotypes would contribute to more effective management of Fusarium wilt diseases.

A definitive understanding of the evolutionary origins of pathogenic forms in *F. oxysporum* will require a sensitive measure of interstrain relationships. Comparisons of conserved DNA sequences, the standard approach to phylogenetic questions at or above the species level, is clearly the method of choice. However, recourse to this methodology is only possible if a genomic region can be identified which is conserved, such that homologous sequences can be found in all isolates under study, and yet is sufficiently variable to reveal informative differences among individuals within a species.

The ribosomal DNA (rDNA), which includes the 18S, 5.8S, and 28S genes, provides conserved sequences which allow

taxa as diverse as plants, animals and fungi to be included in the same phylogenetic tree. Whereas the rDNA gene sequences are likely to reveal few differences within a species, the spacer regions are much more variable (Hillis and Dixon 1991) and thus may provide useful intraspecific comparisons. In particular, the intergenic spacer (IGS), which separates repeat units within tandem arrays of the rDNA genes, is known to show differences within a species (Hillis and Davis 1988).

Although multiple copies of the rDNA repeat unit are found in the same organism, they are not evolving independently (Dover 1986). This is attributed to the phenomenon termed concerted evolution which acts to homogenize the tandem arrays of rDNA within an individual and fix the rDNA repeat units within a sexually reproducing population (Dover 1986). The major mechanisms thought to account for concerted evolution are unequal crossing over and gene conversion. Variability in copy number and length of the rDNA repeats within a species and even within an individual has been attributed to duplication or deletion of complete repeats occurring during crossover events (Coen et al. 1982; Pukkila and Skrzynia 1993). Within a species, variability in the IGS region is generally believed to be short lived because the rate of homogenization is faster than the rate of speciation (Hillis and Davis 1988). Even when the rDNA tandem arrays are located on five nonhomologous chromosomes, as in man and

Table 1. Description of isolates for which partial IGS sequence was determined

Species	Isolate ^a	Geographic origin	Pathogenicity ^{b/} host	Race ^b	VCG ^c	mtDNA haplotype ^d	IGS haplotype ^e	A ^f 5' end (bp)	B ^g middle (bp)	C ^h 3' end (bp)	Sequence length ⁱ (bp)
<i>F. o. melonis</i>	B3-1S	Wicomico Cty., MD	pathogen/melon	0	0131	MO	AEEA	379 [6]	379 [2]	295 [2]	1,053
<i>F. o. melonis</i>	B9-9Sa	Wicomico Cty., MD	pathogen/melon	1	0131	MR	ACCA	379 [6]	379 [1]a	295 [6]	1,053
	B9-9Sb								380[1]b		1,054
<i>F. o. melonis</i>	B1-4S	Wicomico Cty., MD	pathogen/melon	2	0131	MO	AEEA	379 [1]	379 [1]	295 [1]	1,053
<i>F. oxysporum</i>	B10-5S	Wicomico Cty., MD	nonpathogen	-j	0131	MO	AEEA	379 [8]	316 [1]	295 [1]	990
<i>F. o. melonis</i>	MC10-9S	Caroline Cty., MD	pathogen/melon	1,2	0134	MR	ACCA	379 [8]	380 [1]	295 [2]	1,054
<i>F. o. melonis</i>	MC1-1S	Caroline Cty., MD	pathogen/melon	1	0134	MR	ACCA	379 [3]	380 [2]	295 [3]	1,054
<i>F. oxysporum</i>	C3-5R	Wicomico Cty., MD	nonpathogen	-	0134	MP	AAAA	379 [1]	380 [2]	295 [3]	1,054
<i>F. oxysporum</i>	MC7-5Sa	Caroline Cty., MD	nonpathogen	-	0134	ML	CDAB	377[14]	303[5]a	295 [3]	975
	MC7-5Sb								335[1]b		1,007
<i>F. oxysporum</i>	D9-3R	Caroline Cty., MD	nonpathogen	-	0131	MY	CDAB	380 [4]	303 [1]	295 [2]	978
<i>F. oxysporum</i>	MC10-7S	Caroline Cty., MD	nonpathogen	-	14	ME	CDAB	380 [1]	303 [1]	295 [1]	978
<i>F. oxysporum</i>	CRIII	San Joaquin Cty., CA	nonpathogen	-	k	N	AEEA	334 [1]	386 [1]	295 [2]	1,015
<i>F. oxysporum</i>	MC9-8Sa	Caroline Cty, MD	nonpathogen	-	10	MG	EBDE	538 [6]	150 [0]	320[5]a	1,008
	MC9-8Sb									295[1]b	983
<i>F. oxysporum</i>	MC4-1S	Caroline Cty, MD	nonpathogen	-	3	MC	DBBC	379 [5]	162 [4]	490 [6]	1,031
<i>F. oxysporum</i>	RA-4E	San Joaquin Cty., CA	nonpathogen	-	l	MO	ADFB	379 [1]	-	609 [1]	988
<i>F. o. cubense</i>	RPMW5	Malawi	pathogen/banana	/	/	/	/	379 [4]	380 [2]	295 [6]	1,054
<i>F. subglutinans</i>	SL1	Santa Barbara, CA	pathogen/pine	1	1	/	/	- [1]	- [1]	- [1]	1,105

^a *Fusarium oxysporum* and *F. oxysporum* f. sp. *melonis* isolates were isolated either from soil or from roots; *F. oxysporum* f. sp. *cubense* and *F. subglutinans* were isolated from diseased hosts, banana and pine, respectively. The letters 'a' and 'b' designate the two alternate forms of IGS sequence found in isolates B9-9S, MC7-5S and MC9-8S.

^b *F. oxysporum* isolates were tested using a root dip assay on differential cultivars of muskmelon to determine pathogenic race; *F. oxysporum* f. sp. *cubense* and *F. subglutinans* were isolated from diseased hosts, banana and pine, respectively.

^c Vegetative compatibility group.

^d Mitochondrial DNA haplotype.

^e Intergenic spacer (IGS) haplotype.

^f Section A is the 5' end of the ~1,000-bp IGS fragment amplified using the polymerase chain reaction (PCR).

^g Section B is in the middle of the ~1,000-bp PCR amplified IGS fragment.

^h Section C is at the 3' end of the 1,000-bp PCR amplified IGS fragment.

ⁱ Total number of base pairs in the complete sequence of the PCR amplified IGS fragment for each isolate.

^j Symbols: - = not applicable; / = no data; [] = number of clones sequenced.

^k Designation of a vegetative compatibility group to which a number has not been assigned.

^l Designation of a vegetative compatibility group to which a number has not been assigned.

apes (Arnheim et al. 1980), the rDNA is evolving in a concerted fashion (Ohta and Dover 1983).

Interspecific variation in the sequence of the IGS region has been documented in plants such as *Cucurbita* spp. (King et al. 1993), and the root rot fungus, *Armillaria* (Anderson and Stasovski 1992). Using the entire rDNA repeat as a probe, no intraspecific differences were observed in *Sclerotinia* spp. (Kohn et al. 1988) or *Candida* spp. (Magee et al. 1987). Direct PCR amplification and RFLP analysis of IGS in *Tylospora* (Erland et al. 1994) and *Saccharomyces* (Molina et al. 1993) supported the utility of the IGS region in distinguishing species with no apparent intraspecific variation. Other examples reveal high levels of intraspecific variation in the IGS region. Based on RFLP analysis using the entire rDNA repeat unit as a probe, variation was found to be localized in the IGS region in mice (Suzuki et al. 1986) and other rodents (Allard and Honeycutt 1991), humans (Seperack et al. 1988), rice (Sano and Sano 1990), *Daphnia pulex* (Crease and Lynch 1991) and in the fungi, *Aspergillus fumigatus* (Spreadbury et al. 1990), *Rhizoctonia solani* (Vilgalys and Gonzalez 1990), and *Rhynchosporium secalis* (McDermott et al. 1989). In PCR amplified IGS region, RFLP variation was also found in the fungi, *Histoplasma capsulatum* (Vincent et al. 1986), *Puccinia graminis* (Kim et al. 1992), and *Fusarium oxysporum* (Appel and Gordon 1995). Intraspecific variation in IGS may reflect a slow rate of concerted evolution in species characterized by infrequent sexual reproduction, or a predominantly clonal mode of reproduction.

Restriction digests of the IGS in *F. oxysporum* revealed variation indicative of sequence differences among isolates in this species (Appel and Gordon 1995). An examination of 56 isolates, selected to represent the genetic diversity in a larger collection, identified 13 different IGS haplotypes. Eight of these IGS haplotypes and 2 unique ones that were previously undetermined were sequenced in the present study, including several isolates of *F. oxysporum* f. sp. *melonis* W. C. Snyder & H.N. Hansen, the cause of Fusarium wilt of muskmelon. Previous work on *F. oxysporum* f. sp. *melonis* has shown that a given race is associated with more than one vegetative com-

patibility group (VCG) and that more than one race may be associated with a single VCG (Jacobson and Gordon 1991). Mitochondrial (mt) DNA haplotypes (Jacobson and Gordon 1990), IGS haplotypes (Appel and Gordon 1995) and DNA fingerprints (Schroeder and Gordon 1993) tend to correlate with VCG. This constellation of characters thus serves to define different clonal lineages within *F. oxysporum* f. sp. *melonis*. The isolates we selected for sequencing included *F. oxysporum* f. sp. *melonis* strains representing four different races and two different clonal lineages, nonpathogenic strains which are closely related to *F. oxysporum* f. sp. *melonis* based on vegetative compatibility and/or mtDNA haplotype (Appel and Gordon 1994; Gordon and Okamoto 1992a, 1992b), and nonpathogenic strains which represent extremes of mtDNA diversity in *F. oxysporum* (Appel and Gordon 1994; Gordon and Okamoto 1992a, 1992b; Gordon et al. 1992). One isolate of the forma specialis, *F. oxysporum* f. sp. *ubense* (E.F. Smith) Snyder & Hansen, was included for comparison because it causes a wilt disease on banana, a host not closely related to melon. A single isolate of *F. subglutinans* was included as a potential outgroup.

Our objective was to determine if the extent and nature of IGS sequence variation would allow us to resolve intraspecific relationships in *F. oxysporum* and thereby provide insight into the evolution of pathogenic forms in this species.

RESULTS

Location of *EcoRI* sites.

The primers, CNL12 and U:49-67, together amplified a DNA fragment ranging in size from 975 to 1,105 bp containing the 5' end of the IGS region (Table 1; Fig. 1).

Variation in size was due to single or multiple base insertions or deletions (Fig. 2). In most isolates, *EcoRI* digested the ~1,000-bp fragment into the three pieces (see Materials and Methods), designated A, B and C (Table 1; Fig. 1). Region A contained ~270 bp of the 3' end of the 28S rDNA gene and the 5' end of the IGS, extending from the CNL12 primer 377 to 538 bp into the IGS region, depending on the

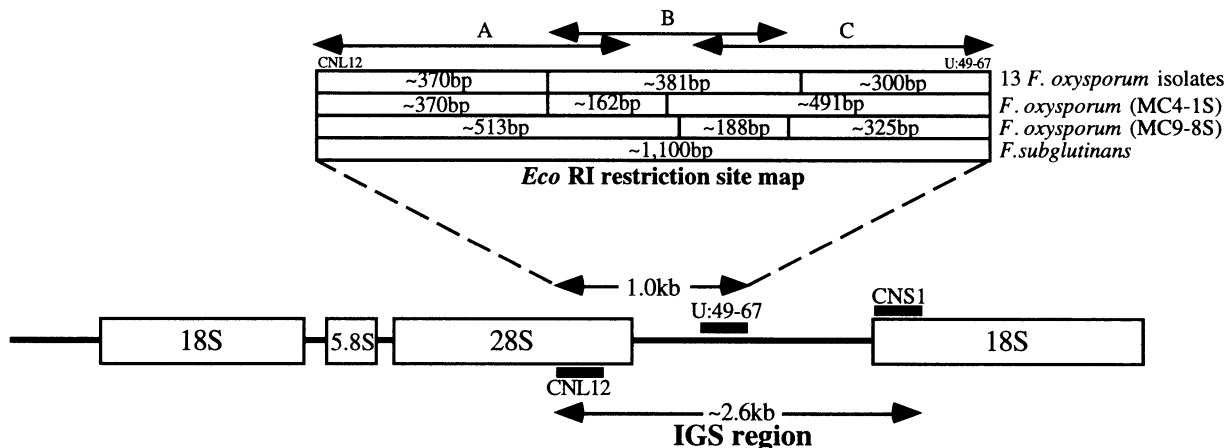


Fig. 1. Schematic diagram of the ribosomal DNA (rDNA) repeat unit showing the CNL12 and CNS1 primer sites used in PCR amplification of the intergenic spacer (IGS) region. The position of the new primer, U:49-67, is shown with the size of the resulting ~1,000-bp partial IGS amplification product when the new primer is combined with CNL12 in PCR. *EcoRI* sites were added to the 5' end of each PCR primer to create restriction sites for cloning the partial 1,000 bp IGS fragment. The location of the *EcoRI* restriction sites used for cloning are shown for the different *Fusarium oxysporum* isolates. *F. subglutinans* had no internal *EcoRI* sites.

CNL12 primer

B3-1S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 B9-9Sa CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 B9-9Sb CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 B1-4S CTGAACGCCTCTAAGTCAGNMMNNNNNGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 B10-5S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 D9-3R CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC10-9S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC1-1S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACNACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 C3-5R CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC7-5Sa CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC7-5Sb CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC10-7S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 CRII CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC9-8Sa CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC9-8Sb CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 MC4-1S CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 RA-4E CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 Foc CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA
 Fs CTGAACGCCTCTAAGTCAGAATCCATGCCAGAACCGGGTGATACCACCCGCACGTATAGATGGACAAGAAATAGGCTTCGGCTTAGCGTCTTAGCAGGCCA 100BP

Cfo I

B3-1S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 B9-9Sa TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 B9-9Sb TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 B1-4S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 B10-5S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 D9-3R TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC10-9S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC1-1S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 C3-5R TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC7-5Sa TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC7-5Sb TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC10-7S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 CRII TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC9-8Sa TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC9-8Sb TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 MC4-1S TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 RA-4E TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 Foc TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG
 Fs TTCTTCCACGCGCTCGAAGCGTCGTGGXTATTTCCGGTATTGTAATTTCAACACGAGCGGGGTCAAATCCTTTGCAGACGACTTAGCTGTGCGAAACGG 200BP

Ava II

B3-1S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 B9-9Sa TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 B9-9Sb TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 B1-4S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 B10-5S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 D9-3R TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC10-9S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC1-1S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 C3-5R TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC7-5Sa TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC7-5Sb TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC10-7S TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 CRII TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC9-8Sa TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC9-8Sb TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 MC4-1S TCCGTGTAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 RA-4E TCCGTGTAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 Foc TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA
 Fs TCCGTGAAGCAGTAGAGTAGCCTTGTGTTACGATCTGCTGAGGGTAAGCCGTCCTTCGCCTCGATTTCCCAAATGGGTTCTCCGGAXTTTCTGGAGAGA 300BP

Eco RI

B3-1S CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCTCCGACGGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT
 B9-9Sa CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCTCCGACGGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT
 B9-9Sb CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCTCCGACGGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT
 B1-4S CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCTCCGACGGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT
 B10-5S CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCTCCGACGGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT

Fig. 2. The complete sequence of the 5' end of the intergenic spacer (IGS) region is shown for *Fusarium subglutinans* and 15 *F. oxysporum* isolates. For three isolates of *F. oxysporum*, B9-9S, MC9-8S, and MC7-5S, two different sequences from each isolate are shown, designated (a) or (b). The aligned sequence is 1,223 bp in length extending from the CNL12 primer on the 5' end to the U:49-67 primer at the 3' end. The sequence was aligned to show single base substitutions and insertion/deletions, ranging from 1 to 136 bases in length. For parsimony analysis, each deletion event was counted only once regardless of its length and is designated by an (X). Missing known or unknown data are designated by (*) and (N), respectively. Restriction sites for the enzymes, *EcoRI*, *CfoI*, *AvaII*, and *Sau3A*, are shown underlined. (continued on next page)

D9-3R CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTCGATTCGGTCG*****X**
 MC10-9S CTTGTAGGGGTTGTGGGATTTTTXXGATATGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****GTGATAGGAGT
 MC1-1S CTTGTAGGGGTTGTGGGATTTTTXXGATATGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****GTGATAGGAGT
 C3-5R CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****GTGATAGGAGT
 MC7-5Sa CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTCATTCGGTCGATAGGAGT
 MC7-5Sb CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTCGATTCGGTCG*****X**
 MC10-7S CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTCGATTCGGTCG*****X**
 CR11 CTTGTAGGGGTTGTGGGATTTTTXXGATG*****GAATTC*****CGTCGATAGGAGT
 MC9-8Sa CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGTCGCGGTGAGGGTAGTCGAGTTGGACTTGGTGGAAATTTGGTTCCGTCG*****X**
 MC9-8Sb CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGTCGCGGTGAGGGTAGTCGAGTTGGACTTGGTGGAAATTTGGTTCCGTCG*****X**
 MC4-1S CTTGTAGGGGTTGTGGGTTTTTTXCGATGTGTCGCTCCGGACGTCGCGGTGAGGGTAGTCGAGTTGGACTTGGTGGAAATTCGATTCGGTCG*****X**
 RA-4E CTTGTAGGGGTTGTGGGTTTTTTXXGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTGGACTTGGTGGAAATTC*****XTCNNNNNNNN
 Foc CTTGTAGGGGTTGTGGGATTTTTXXGATGTGTCGCTCCGGACGGGCGGTGCAGGGTAGTCGAGTTAGACTTGGTGGAAATTC*****CGTCGATAGGAGT
 Fs CTTGTAGGGGTTGTGGGTTTTTTTCGATGTGTCGCTCTGGTCGGGCGGTGCAGGGTAGTCGAGTTGGACTTGGTGGAAATTCATTCGGTCG*****X**

400BP

Sau 3A

Sau 3A

B3-1S TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 B9-9Sa TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 B9-9Sb TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 B1-4S TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 B10-5S TCCGTCGAGTATGGTCGGCAGATCXTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 D9-3R *****AGTATGGCTGGCACGATCXTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC10-9S TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC1-1S TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 C3-5R TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC7-5Sa *****AGTATGGCTGGCACGATCXTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC7-5Sb *****AGTATGGCTGGCACGATCXTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC10-7S *****AGTATGGCTGGCACGATCXTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 CR11 TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC9-8Sa *****AGTCTGG**X*CATCGTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC9-8Sb *****AGTCTGG**X*CATCGTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 MC4-1S *****AGTCTGG**X*CATCGTGTGTCGGGXTTGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 RA-4E NNNNNNAXTCTGGXTGGX*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 Foc TCCGTCGAGTCTGGTCGGC*****X*****TGTGTGTTGGACGGTGCAGGGTAGGCTGCTTGGXACATGGTCGGGTCGAGGGTCGATTCGAGGG
 Fs *****AGTCTGGCGGAXXCTTTGTXXCGGXTGTGTCGTTGGACGGCAGCGTAGGCTGCTTGGXCTTGTGTCGATTCGAGGGTCGATTCGAGGG

500BP

B3-1S CC*****X*****GGCCTGTCGATGATGTGTGATGATGCGGCTCAGGGTAGGCTGGTT
 B9-9Sa CCGATTCGAGGGCC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 B9-9Sb CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 B1-4S CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 B10-5S *****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 D9-3R *****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC10-9S CCGATTCGAGGGCC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC1-1S CCGATTCGAGGGCC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 C3-5R CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC7-5Sa *****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC7-5Sb *****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC10-7S *****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 CR11 CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC9-8Sa CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC9-8Sb CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 MC4-1S CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 RA-4E CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 Foc CC*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT
 Fs CT*****X*****GGCCTGTCGATGATGTGTGATGATGATGCGGCTCAGGGTAGGCTGGTT

600BP

Eco RI Sau 3A

Ava II

Sau 3A

B3-1S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 B9-9Sa TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGG*****X*****TACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 B9-9Sb TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 B1-4S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 B10-5S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 D9-3R TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC10-9S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC1-1S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGG*****X*****TACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 C3-5R TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC7-5Sa TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC7-5Sb TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC10-7S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 CR11 TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC9-8Sa TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC9-8Sb TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 MC4-1S TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 RA-4E TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 Foc TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA
 Fs TGTCTTGGTCCAATTTGATXGTCGGCTCCCGTGCAGGCCAGAGTGAXGGGGGTTCCAGGGTAGGTCACAGGGTAGGCAGCTTAGATTTGGXCCGATCTGGA

700BP

Fig. 2. Continued from previous page.

Sau 3A **Sau 3A**

B3-1S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGGTGCCAGCGGTGTAGGGTAGGCTAGTTF
 B9-9Sa GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 B9-9Sb GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGCT
 B1-4S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGGTGCCAGCGGTGTAGGGTAGGCTAGTTF
 B10-5S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 D9-3R GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC10-9S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC1-1S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 C3-5R GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC7-5Sa GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC7-5Sb GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC10-7S GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 CR11I GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 MC9-8Sa NNN
 MC9-8Sb NNN
 MC4-1S TGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 RA-4E GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 Foc GGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF
 Fs TGTTCGATTCCTCCGCCCCCG*****X*****CGGATCTGXACACTGTCAAACAGAGATGCCAGCGGTGTAGGGTAGGCTAGTTF 800BP

Eco RI

B3-1S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 B9-9Sa TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 B9-9Sb TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 B1-4S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 B10-5S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 D9-3R TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC10-9S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC1-1S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 C3-5R TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC7-5Sa TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC7-5Sb TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC10-7S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 CR11I TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 MC9-8Sa NNN
 MC9-8Sb NNN
 MC4-1S TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 RA-4E TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 Foc TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG
 Fs TCXGTCCCT**XGCCAGGTTGCGATTTGXGACGAGATATGTGGTCTAGGGTAGGCTCTAGGGTAAAGTAGAAATTCGAGTTTCGTGCCGACAGTTTTCTXG 900BP

B3-1S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 B9-9Sa TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 B9-9Sb TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 B1-4S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 B10-5S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 D9-3R TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC10-9S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC1-1S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 C3-5R TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC7-5Sa TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC7-5Sb TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC10-7S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 CR11I TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC9-8Sa TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC9-8Sb TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 MC4-1S TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 RA-4E TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 Foc TGGGTGTCTGGTAGGTACAGGGTAGG*****X*****X*****CAAATCTCCCTCCGGCCAGTA
 Fs TAGGTGTAGGTTAGGTACAGGGTAGGATTTCTCCGGCCAGTACTGCTTATGCGTGCAGAAATGTTCTGTGTC*****X***** 1000BP

Sau 3A

B3-1S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 B9-9Sa CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 B9-9Sb CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 B1-4S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 B10-5S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 D9-3R CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC10-9S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC1-1S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 C3-5R CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC7-5Sa CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC7-5Sb CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC10-7S CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 CR11I CTGTGCTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG
 MC9-8Sa *****TGGTGGTGGTGGTCGAGTCGATTTTTTGTGTTTGGCCATACTATTGAATTTGGCGAAATTCAAAAGTGGCTCGGGAGTCCCAGCCTGGCGTGCGTCCG

Fig. 2. Continued from previous page.

isolate. The B region contained the next 162 to 386 bases downstream from A, occupying the middle of the ~1,000 bp amplified sequence. The B region was the most variable of the three, with numerous base substitutions and major insertions and deletions among the 15 isolates. The C region was approximately 295 to 490 bp in length and relatively conserved among the 15 isolates. The *F. oxysporum* isolate, RA-4E, and *F. subglutinans* had fewer *EcoRI* restriction sites than the other isolates, producing only two or one DNA fragment, respectively (Table 1; Fig. 1).

Fidelity of PCR.

The Taq DNA polymerase error rate was estimated, to assess the potential impact on our sequencing results. We repeatedly cloned from a single PCR reaction of isolate MC7-5S and sequenced 13 clones containing section A (377 bp) of this ~1,000 bp DNA fragment (Fig. 1). Among the 4,901 bases sequenced there were two base substitutions (A to G), and (T to C) (Fig. 3). Although these differences could reflect actual differences within some of the rDNA tandem repeat

units, it is unlikely given that this sequence contains ~270 bp of the 3' end of the 28S gene region and was found to be highly conserved among all *F. oxysporum* isolates examined in this study. That the differences are due to PCR mistakes is suggested not only by the consensus of the 13 clones from isolate MC7-5S, but also the other 15 *F. oxysporum* isolates which shared the consensus bases at these two sites (Fig. 2). Both were transition errors, the type commonly made by the Taq DNA polymerase. The error rate in 35 cycles of PCR was equal to 4.0×10^{-4} mutations per base duplication or 1 mutation for each 2,450 nucleotides polymerized, and is close to the rate found in other systems (Chen et al. 1991; Saiki et al. 1988; Tindall and Kunkel 1988).

Sequence data.

The complete sequence is shown in Figure 2 with the CNL12 primer at the 5' end and the U:49-67 primer at the 3' end. The actual length of the sequence, ranges from 975 to 1,105 bp, depending on the isolate. The total size, 1,223 bp shown in Figure 2, was estimated with allowances made for

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MC9-8sb CTTGTCTGGTXXGTCGTGAGTCGATTTTTTTGTTTTGCCATACTATTGAATTTTGC GGAAATTCAAAAGTGGCTCGGGAGTCCC GCCTGGCGTGCCTCCG
MC4-1S *****TGGTXXGGCCGTGAGTCGATTTTTTTGTTTTGCCATACTATTGAATTTTGC GGAAATTCAAAAGTGGCCCGGAGTCCGGTCTGGCGTGCCTCCG
RA-4E CTTGTCTGGTXXGTCGTGAGTCGATTTTTTTGTTTTGCCATACTATTGAATTTTGC GGAAATTCAAAAGTGGCTCGGGAGTCCC GCCTGGCGTGCCTCCG
Foc CTTGTCTGGTXXGTCGTGAGTCGATTTTTTTGTTTTGCCATACTATTGAATTTTGC GGAAATTCAAAAGTGGCTCGGGAGTCCC GCCTGGCGTGCCTCCG
Fs *****TGGTXXGGCCGTGAGTCGATTTTTTTGTTTTGCCATACTAAAACGAATTTTGC GGAAATTCAAAAGTGGCCACGAGTCCGGTCTGGCGTGCCTCCG 1100BP

B3-1S GCTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGANN
B9-9Sa ACTCGAACCTCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
B9-9Sb GCTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
B1-4S GCTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
B10-5S ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
D9-3R ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC10-9S ACTCGAACCTCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC1-1S ACTCGAACCTCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
C3-5R ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC7-5Sa ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC7-5Sb ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC10-7S ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
CRIII ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC9-8Sa ACTCGAATATCGTCGGTGGACATATGAGAGGGAGCAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC9-8Sb ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
MC4-1S ACTCGAACATCGTCGGTGGACATATGAGAGGGAGCAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
RA-4E ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
Foc ACTCGAACATCGTCGGTGTACATATGAGAGGGAGTAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG
Fs ACTGGAACATCGTCGGTGGACATATGAGAGGGAGCAATC NNNNNNNNNNNNCCATGCGGAGCTGCCGGGTAGGTA AAAAGTAAAGAGG 1200BP

Cfo I U:49-67 primer
B3-1S NNNNGTGTCCGCGTCTGTATT
B9-9Sa CGCGGTGTCCGCGTCTGTATT
B9-9Sb CGCGGTGTCCGCGTCTGTATT
B1-4S CGCGGTGTCCGCGTCTGTATT
B10-5S CGCGGTGTCCGCGTCTGTATT
D9-3R CGCGGTGTCCGCGTCTGTATT
MC10-9S CGCGGTGTCCGCGTCTGTATT
MC1-1S CGCGGTGTCCGCGTCTGTATT
C3-5R CGCGGTGTCCGCGTCTGTATT
MC7-5Sa CGCGGTGTCCGCGTCTGTATT
MC7-5Sb CGCGGTGTCCGCGTCTGTATT
MC10-7S CGCGGTGTCCGCGTCTGTATT
CRIII CGCGGTGTCCGCGTCTGTATT
MC9-8Sa CGCGGTGTCCGCGTCTGTATT
MC9-8Sb CGCGGTGTCCGCGTCTGTATT
MC4-1S CGCGGTGTCCGCGTCTGTATT
RA-4E CGCGGTGTCCGCGTCTGTATT
Foc CGCGGTGTCCGCGTCTGTATT
Fs CGCGGTGTCCGCGTCTGTATT 1223BP

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Fig. 2. Continued from previous page.

alignment (Fig. 4). There were 172 single base substitutions (63% were transitions), 47 single base insertions or deletions, and 17 large insertions or deletions ranging in size from 4 to 136 bp. Most of the sequence was alignable except for certain inserted or deleted regions that were present in only some isolates, especially certain regions found in *F. subglutinans* that were not found in *F. oxysporum* (Fig. 4). Restriction enzyme sites tested previously that define the IGS haplotypes (Appel and Gordon 1995) are listed with the base sequence underlined (Fig. 2).

Phylogeny.

For parsimony analysis, a difference attributable to an insertion/deletion event was counted as a single character regardless of the length of the inferred change; all base substitutions were counted as characters without weighting. A strict consensus of 404 trees of length 288, based on parsimony analysis and bootstrapping, is shown in Figure 5. Two distinct lineages of *F. oxysporum* f. sp. *melonis* correlated with VCG (0131 and 0134) and mtDNA haplotype (Jacobson and Gordon 1990), indicating that significant differences in IGS sequence can be found among isolates associated with this forma specialis. The two *F. oxysporum* f. sp. *melonis* VCGs (0131 and 0134) were separated by 21 changes. Pathogenic races in *F. oxysporum* f. sp. *melonis* were not clearly differentiated based on IGS sequence. For example, in VCG 0134, three changes separated race 1 from race 1, 2 and in VCG

0131 only two changes separated race 2 from race 0, which is nearly within the range of the PCR error rate. *F. oxysporum* f. sp. *ubense* was approximately 19 to 22 changes away from *F. oxysporum* f. sp. *melonis* VCGs 0131 and 0134, respectively, so it appeared closer to *F. oxysporum* f. sp. *melonis* than to some nonpathogenic strains. Isolate B9-9S is associated with VCG 0131 (Appel and Gordon 1994) but virulence phenotype, mtDNA haplotype, IGS haplotype (Appel and Gordon 1995), and DNA fingerprint (unpublished) suggest an affiliation with VCG 0134. Two IGS sequences were found in this isolate, one closely related to VCG 0131 and the other close to VCG 0134. Sequence B9-9S (a) differed from VCG 0134 by 6 changes and B9-9S (b) differed from VCG 0131 by 5 changes, suggesting a close relationship to each pathogen VCG IGS type (Fig. 5).

Two nonpathogens were associated with each *F. oxysporum* f. sp. *melonis* VCG, 0131, and 0134. Isolate B10-5S (VCG 0131), a nonpathogen also similar to the pathogen with respect to mtDNA haplotype, differed in IGS sequence by approximately 26 changes and did not cluster near VCG 0131 in the consensus tree (Fig. 5). Other nonpathogens, MC7-5S (VCG 0134) and D9-3R (VCG 0131), were similar to each other in IGS haplotype and sequence, but were ~25 changes away from pathogenic isolates in shared VCGs. The sequence of the remaining nonpathogen, C3-5R (VCG 0134) was closer to *F. oxysporum* f. sp. *ubense* (~10 changes) than to *F. oxysporum* f. sp. *melonis* (~28 changes). Overall, nonpatho-

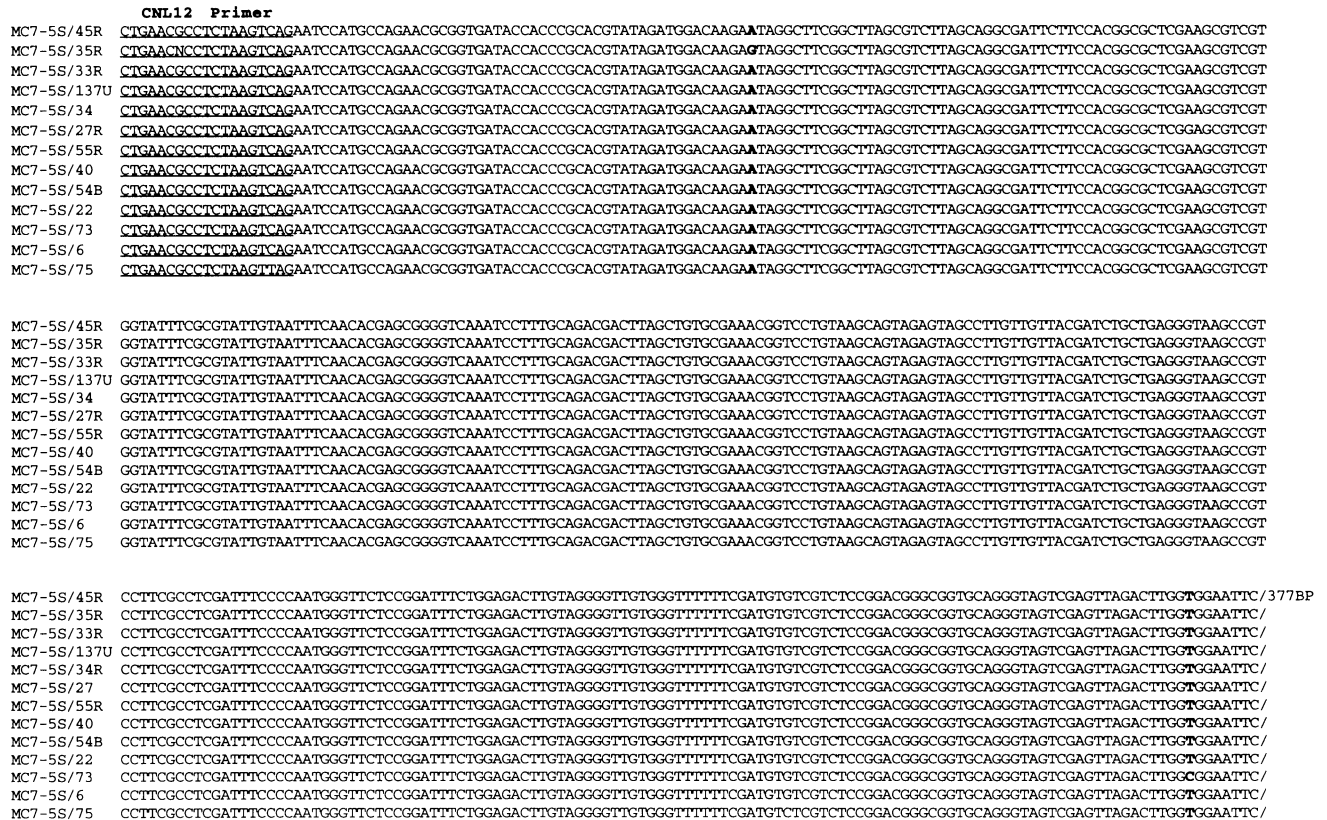


Fig. 3. The Taq DNA polymerase error rate was estimated to assess the potential impact on our sequencing results. This set of aligned sequence data is the result of sequencing 13 copies of the 377 bp region from the 5' end of the intergenic spacer (IGS) region. All of these clones were from the same PCR reaction of isolate MC7-5S and are aligned to show 2 base substitutions or possible PCR errors, which are indicated by bold type. The first ~270bp of the sequence includes the 3' end of the 28S gene and is the most conserved part of the IGS region.

gens associated with pathogen VCGs were not closely related to the pathogens, based on differences in IGS sequence (Fig. 5).

Phylogenetic trees were also made using Kimura two-parameter distance and neighbor joining analyses in the PHYLIP 3.5 program. Deletions in the data set were counted by the PHYLIP program only after the distance matrix was calculated using PAUP 3.1 and then these distances were put into the PHYLIP format for neighbor joining analysis. Alternative analyses were conducted with and without deletions as characters and +/- weighting of transversions (twice that of transitions). The tree resulting from the analysis not counting the deletions as characters and weighting transversions twice that of transitions is shown in Figure 6. Overall the trees produced in these analyses did not differ from the PAUP consensus tree with respect to the placement of the more statistically significant branches which included the *F. oxysporum* f. sp. *melonis* groups, the more distantly related nonpathogens (MC9-8S and MC4-1S) and *F. subglutinans* (Fig. 6). Clustering of the other isolates held no significant support from bootstrap in either the PAUP consensus tree (Fig. 5) nor the PHYLIP generated neighbor joining tree (Fig. 6). The main difference was that the PHYLIP-generated trees all consistently grouped isolates C3-5R and *F. oxysporum* f. sp. *cubense* together and close to the *F. oxysporum* f. sp. *melonis* groups (Fig. 6), whereas the PAUP tree leaves the relationship of these isolates unresolved (Fig. 5).

Differences within the same isolate.

For some isolates, multiple copies of the same clone were sequenced (Table 1). All homologous clones from the same isolate were identical in sequence, except for three isolates, B9-9S, MC7-5S, and MC9-8S. Parsimony and neighbor joining analyses were conducted including both sequence

types and the results are shown in the phylogenetic trees (Figs. 5 and 6). Only nine parsimony inferred changes separated the sequences MC7-5S (a) and (b). However, there were 69 and 18 changes between the cloned intra-isolate IGS fragments from MC9-8S and B9-9S, respectively.

DISCUSSION

The utility of sequence comparisons in the IGS region, for phylogenetic purposes, will depend on mechanisms by which this region evolves. The occurrence of sequences signaling both the initiation and the termination of transcription (King et al. 1993) as well as conservation of secondary structure in the IGS (Baldrige et al. 1992) should impose some constraints on the evolution of this region. We found very little length variation in the IGS of *F. oxysporum* and *F. subglutinans* (Appel and Gordon 1995 and unpublished data) which also argues for limitations on the structural changes which are tolerated. Nevertheless, length variation has been reported in many fungi including, *Cochliobolus heterostrophus* (Garber et al. 1988), *Schizophyllum commune* (Buckner et al. 1988; Specht et al. 1984), *Yarrowia lipolytica* (Van Heerikhuizen et al. 1985), *Coprinus cinereus* (Wu et al. 1983), *Pythium* (Buchko and Klassen 1990; Martin 1990), and *Phanerochaete chrysosporium* and *Sporotrichum pulverulentum* (Raeder and Broda 1984), where the differences are attributed to insertions or deletions in the arrays of subrepeats within the IGS region. These examples of structural variants are thought to result from unequal crossing over and thus might be considered intermediates in the process of concerted evolution (Coen et al. 1982; Dover 1986; Hillis and Davis 1988).

To the extent that concerted evolution leads to fixation of an IGS sequence within a species, it will facilitate inter-

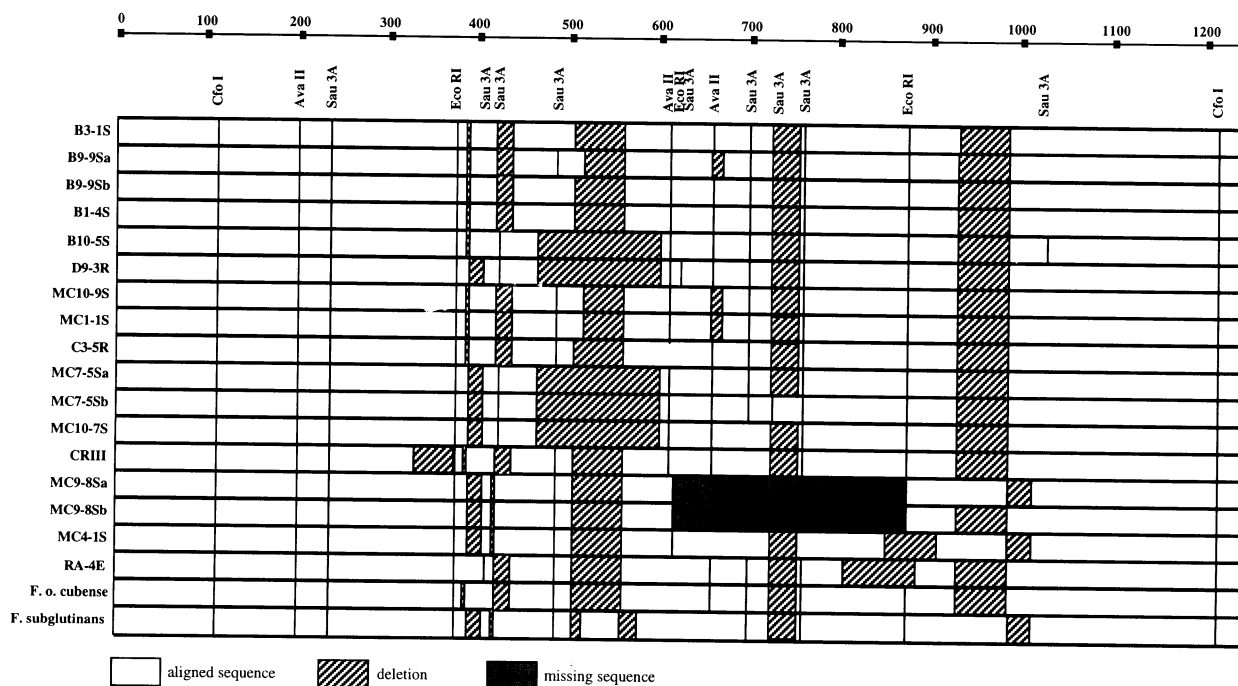


Fig. 4. A schematic diagram of the 5' partial intergenic spacer (IGS) sequence showing the alignment of major deletion/insertions for *Fusarium subglutinans* and 15 *F. oxysporum* isolates. For three isolates of *F. oxysporum*, B9-9S, MC9-8S, and MC7-5S, alignments for each of two different sequences are shown, designated (a) or (b). Single base insertion/deletions or base substitutions are not shown.

specific comparisons because, among reproductively isolated taxa, the IGS will be free to diverge, whereas within species differences will be minimized. Consistent with this expectation, the IGS sequence upstream of the 5S gene provided a useful measure of interspecific relationships in *Armillaria* spp. (Anderson and Stasovski 1992). Similar findings, based on RFLP data, have been reported for *Sclerotinia* spp. (Kohn et al. 1988), *Candida* spp. (Magee et al. 1987), *Saccharomyces* spp. (Molina et al. 1993), and *Tylospora fibrillosa* (Erland et al. 1994).

To the extent that fixation of an IGS sequence within a population is dependent on meiotic interactions, concerted evolution would not lead to a common IGS sequence within a species of isolated subpopulations or in a species which lacks sexual reproduction. Using the rDNA as a probe, RFLPs attributable to intraspecific variation in the IGS region have been reported for *Rhizoctonia solani* (Vilgalys and Gonzalez 1990), *Rhynchosporium secalis* (McDermott et al. 1989), and *Aspergillus fumigatus* (Spreadbury et al. 1990). Similarly, restriction digests of PCR amplified IGS show intraspecific variation in *Histoplasma capsulatum* (Vincent et al. 1986). The variation in IGS which we report here for *F. oxysporum* confirms the differences in sequence inferred from the recognition of different IGS haplotypes (Appel and Gordon 1995) and supports the view that concerted evolution is not operative or is occurring at a reduced rate in this species, possibly due to a predominantly clonal mode of reproduction. If so, differences in IGS sequence may provide a reasonable measure of interstrain differences in fungi such as *F. oxysporum*.

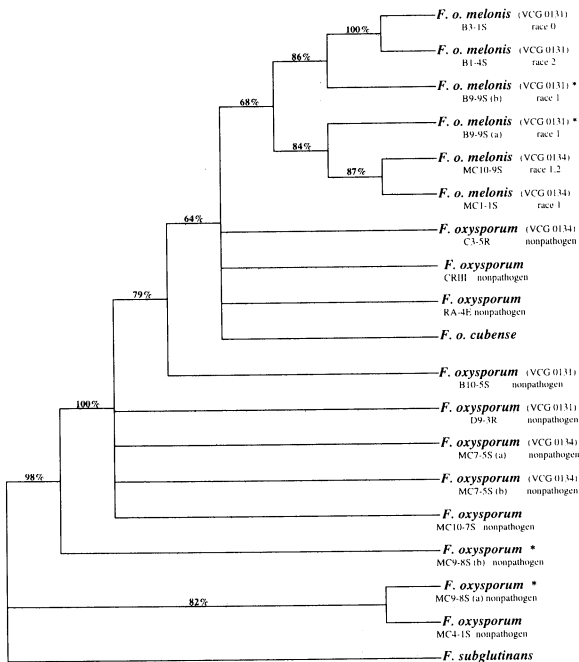


Fig. 5. Strict consensus of 404 trees of length 288 based on parsimony analysis with assigned bootstrap values. The ~1,000-bp sequence of the 5' region of the intergenic spacer (IGS) region of *Fusarium subglutinans* and 15 *F. oxysporum* isolates was analyzed using PAUP 3.1. In this analysis, deletions, regardless of size, were included as single characters and base substitutions were not weighted. (*) indicates single isolates that have 2 different IGS sequences, designated (a) or (b).

Within-strain IGS variation complicates the use of this region as a basis for establishing inter-isolate relationships because the phylogenetic placement of strains displaying this variation is rendered ambiguous. Mechanisms such as mitotic gene conversion and unequal crossing over among sister chromatids act to maintain homogeneity of the rDNA arrays within an individual (Jackson and Fink 1981; Klein and Petes 1981; Szostak and Wu 1980). However, if mutational changes accumulate substantially faster than they can be homogenized, intra-isolate IGS variants may be common. In fact, differences in rDNA within an isolate have been reported for other fungi. In the diploid fungi, *Saccharomyces* (Petes and Botstein 1977) and *Pythium* (Martin 1990), two forms of rDNA were observed to occur in a 1:1 ratio which could be due to the presence of divergent rDNA arrays on separate chromosomes. This explanation is certainly plausible for diploid organisms but may also apply to haploid fungi under some circumstances.

In some isolates of *F. oxysporum* f. sp. *cubense*, the rDNA is found on more than one chromosome, possibly due to aneuploidy (Boehm et al. 1994). The occurrence of distinct rDNA types on different chromosomes as a result of partial diploidy has also been demonstrated in *Neurospora* (Butler 1992). Similarly, partial diploidy in *F. oxysporum*, resulting from prior somatic or sexual interactions, might have resulted

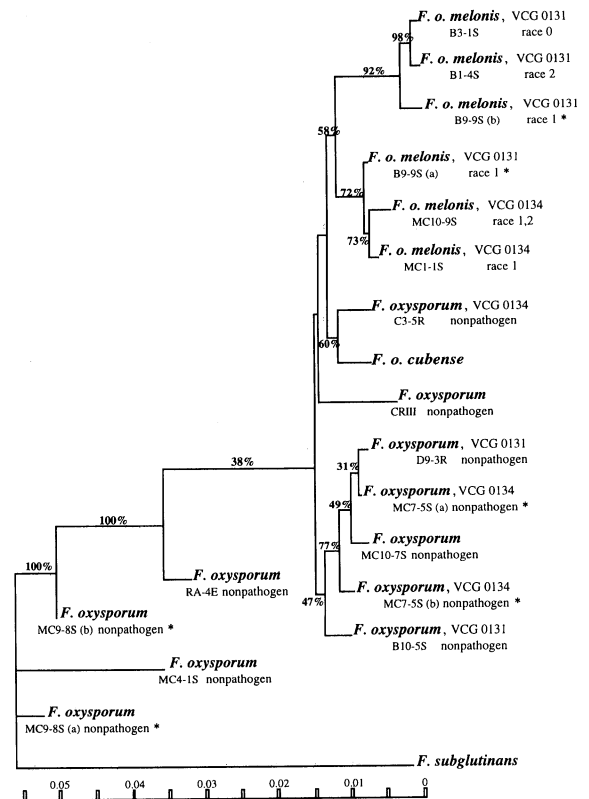


Fig. 6. A distance and neighbor joining tree with assigned bootstrap values, produced using the PHYLIP 3.5 program, based on ~1,000 bp of sequence data from the 5' end of the intergenic spacer (IGS) region from *Fusarium subglutinans* and 15 *F. oxysporum* isolates. This analysis was conducted ignoring deletions as characters and weighting base substitutions, (transversions twice transitions via Kimura two-parameter estimates). (*) indicates single isolates that have 2 different IGS sequences, designated (a) or (b).

in the occurrence of rDNA loci, on different chromosomes, which are associated with different IGS sequences. Preliminary data, based on Southern hybridizations of cloned rDNA with chromosomes separated by pulsed-field gel electrophoresis, support the occurrence of rDNA loci on two or more chromosomes in B9-9S and MC9-8S (H. C. Kistler and T. R. Gordon, unpublished).

Clearly a more complete understanding of the basis for divergent IGS sequences within an isolate will be required before a definitive phylogenetic analysis can be undertaken. Thus for any strain to be examined it must be established whether or not it is polymorphic with respect to the IGS region and all variant forms must be analyzed. If the polymorphic isolates have only two IGS variants, as would be expected for partial diploids, a systematic evaluation of IGS would be practical. If variants are more numerous than we have found, the IGS may prove to be too complex for resolving intraspecific relationships. In any case, more information is required before it can be established to what extent the phylogeny of IGS sequences mirrors the phylogeny of the organisms.

The facility with which IGS sequences were aligned bodes well for a broader application of IGS variation to phylogenetic analysis in *F. oxysporum* and perhaps other fungal pathogens as well. Furthermore, the observed differences in IGS were consistent with the development of robust phylogenies. In the partial IGS sequence, base substitutions were common with transitions occurring more often than transversions. Length differences, ranging from a single base to 79 bases, were also observed but the length of the complete sequence showed little variation, based on size estimates of PCR amplified IGS (Appel and Gordon 1995). Length differences do not pose a problem provided they are recognized and do not preclude proper sequence alignment. Although length changes were treated as characters in our analysis, excluding them did not substantially alter the topology of the phylogenetic trees.

Some aspects of the intraspecific phylogeny of *F. oxysporum* based on partial IGS sequence may be evaluated in terms of their congruence with other measures of interstrain relationships. For example, most *F. oxysporum* f. sp. *melonis* isolates cluster according to VCG (Figs. 5 and 6), which is consistent with inter-isolate affinities based on similarities in mtDNA (Appel and Gordon 1994; Jacobson and Gordon 1990) and DNA fingerprinting (Schroeder and Gordon 1993). Also, the phylogenetic placement of nonpathogenic isolates which were vegetatively compatible with the pathogen tends to support previous conclusions that the isolates in question share VC alleles by coincidence rather than clonal derivation from a recent common ancestor (Appel and Gordon 1994, 1995).

Although *F. oxysporum* f. sp. *melonis* isolates cluster together, to the exclusion of any nonpathogenic strains, bootstrap support for this grouping is not particularly strong. This cluster did appear in every consensus tree generated by parsimony analysis and in the neighbor joining trees, regardless of how base substitutions were weighted and whether or not deletions were excluded from the data set. However, that is not to suggest that all formae speciales of *F. oxysporum* are expected to be phylogenetically distinct. On the contrary, based on similarities in mtDNA (Kim et al. 1993) and DNA

fingerprints (Namiki et al. 1994; Schroeder and Gordon, unpublished), other formae speciales causing disease on cucurbits are very close to *F. oxysporum* f. sp. *melonis* and IGS sequence comparisons may suggest similarly close relationships.

The single isolate of *F. oxysporum* f. sp. *cubense* did not cluster with *F. oxysporum* f. sp. *melonis* isolates nor was it clearly distinct from several nonpathogenic strains. Thus it seems unlikely that pathogens are a monophyletic grouping within *F. oxysporum*, but the examination of additional formae speciales will be required to confirm this impression. The present analysis does document significant differences among the nonpathogenic strains. Two nonpathogens (MC9-8S and MC4-1S) were closer to *F. subglutinans* than to other isolates of *F. oxysporum*, suggesting they may be properly assigned to a taxon other than *F. oxysporum*. Thus our data offer further evidence that isolates morphologically identifiable as *F. oxysporum*, are genetically diverse (Donaldson et al. 1995). Phylogenetic analysis based on differences in mtDNA also reveals this diversity (Appel and Gordon 1994; Gordon and Okamoto 1992a, 1992b; Gordon et al. 1992). If the anamorphic species *F. oxysporum* is comprised of phylogenetically distinct groupings, it is possible that the genetic potential to induce wilt diseases is not found in all of them.

In conclusion, variation in the IGS of the rDNA appears to offer considerable potential to resolve interstrain relationships within *F. oxysporum*. Through a broader sampling of pathogenic forms in this species it should be possible to gain a much clearer picture of how pathogenicity evolved and to more accurately gauge the potential for the development of novel pathotypes in the future.

MATERIALS AND METHODS

Strains, culture conditions, and DNA extraction.

Isolates of *F. oxysporum* f. sp. *melonis* were selected to represent diversity in pathogenic race, VCG, mtDNA haplotype, IGS haplotype, and DNA fingerprint (Appel and Gordon 1994, 1995; Schroeder and Gordon 1993). Pathogenic isolates included *F. oxysporum* f. sp. *melonis* VCGs 0131 (races 0, 1 and 2), 0134 (races 1 and 1,2), and *F. oxysporum* f. sp. *cubense*. The *F. oxysporum* isolates also included nonpathogens from soil collected in Maryland (Appel and Gordon 1994) and California (Gordon and Okamoto 1992a, 1992b; Gordon et al. 1992). Nonpathogen variability within *F. oxysporum* was represented by an additional 8 VCGs, 9 mtDNA haplotypes, and 8 different IGS haplotypes from Maryland and California (Table 1). An isolate of *F. oxysporum* f. sp. *cubense*, cause of Fusarium wilt of banana, from Malawi, was included as a forma specialis that causes a wilt on a host distantly related to muskmelon. As a possible outgroup we included a *F. subglutinans* isolate from California pathogenic to Monterey pine. *F. subglutinans* was selected based on its close relationship to *F. oxysporum* (Gaudet et al. 1989). Each single spore isolate was grown on filter paper overlaid on PDA, dried and stored at 4°C. To obtain mycelium for DNA extraction, the isolates were grown in Vogel's liquid medium (Vogel 1956), filtered, and lyophilized. DNA was extracted according to the methods used by Jacobson and Gordon (1990).

Polymerase chain reaction.

The intergenic spacer (IGS) region of the ribosomal DNA was amplified using the primers CNL12 (CTGAACGCCTC-TAAGTCAG) and CNS1 (GAGACAAGCATATGACTACTG), the complement of primer NS1, with priming sites at the 3' end of the 28S gene and the 5' end of the 18S gene, respectively (Fig. 1) (Anderson and Stasovski 1992; White et al. 1990). The estimated size of the amplified IGS fragment of *Fusarium oxysporum* was ~2.6 kb based on comparison to size markers separated on a 3% agarose gel after a 3-h run at 2.9 V/cm. Amplification reactions included template DNA and the following concentrations of other constituents, 200 µM dNTPs, 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris (pH 8.3), 0.1 mg/ml gelatin, the primers CNL12 and CNS 1, each at 0.5 µM, and 1.5 units of Taq DNA polymerase per 50-µl reaction (Promega). Each reaction was overlaid with a drop of mineral oil (Sigma) (White et al. 1990). Temperature cycling was carried out with a programmable heat block (Microprocessor Controlled Temperature Incubation System, Crocodile II by Appligene, Inc.). An initial denaturation step of 94°C for 85 s was followed by 35 amplification cycles of denaturation, annealing, and extension. Temperature and times for these steps in the first 13 cycles were 95°C for 35 s, 58°C for 55 s, and 72°C for 45 s. Cycles 14 to 26 and 27 to 35 used the same parameters, except that the 72°C extension steps were lengthened to 2 and 3 min, within the two sets of cycles, respectively. After 35 cycles were completed, the samples were incubated for an additional 10 min at 72°C (White et al. 1990). We included negative controls (no DNA template) for each set of experiments to test for the presence of DNA contamination of reagents and reaction mixtures.

Identification of a new priming site between CNL12 and CNS1.

Total DNA from a 50-µl PCR reaction of the IGS region was digested with the enzyme *Sau3A* (Boehringer Mannheim) into more than 5 fragments. Sticky end ligation was used to clone these *Sau3A* fragments into pUC18 vectors cut with the complementary enzyme, *Bam*HI, according to manufacturer's instructions (Boehringer Mannheim). The cloned fragments were transformed into *E. coli* strain DH5α competent cells (Gibco). Four hundred white colonies were picked off the plates and screened using in situ colony hybridization and the PCR amplified, gel purified, and P³²-labeled (Amersham Random Primer Labeling Kit) IGS product as a probe (Maniatis et al. 1982). One 480-bp clone was chosen for sequencing using the reverse and universal primer sites on the pUC18 plasmid (Amersham/USB Sequenase Version 2.0 Sequencing Kit). Based on this sequence, two primers were designed according to criteria described by Steffan and Atlas (1991) and synthesized by the Biochemistry Dept., UC Berkeley. Amplification, using the protocol described above, was repeated with the two new primers together and in combination with CNL12 or CNS1. The combination of the new primer, U:49-67 (AATACAAGCACGCCGACAC), and CNL12 using PCR, produced an amplification product of ~1,000 to 1,100 bp beginning ~270 bp before the 3' end of the 28S gene and extending ~700 to 800 bp into the IGS region (Fig. 1).

Cloning PCR amplified IGS fragments.

*Eco*RI sites were added to the 5' ends of both primers,

CNL12 and U:49-67, and used in PCR as described above (Scharf 1990). Two 50-µl reaction products were digested with *Eco*RI (Promega) and mixed with 300 µl of sterile H₂O in a Millipore Ultrafree MC30 column and spun at low speed to remove excess primers, enzymes, and concentrate the DNA from 100 µl to a total volume of 30 µl. Five to seven microliters of the *Eco*RI cut, cleaned, and concentrated IGS DNA was cloned using sticky end ligation with *Eco*RI cut pUC18 plasmid (Boehringer Mannheim).

Screening and sequencing clones.

Possible clones were screened for inserts by making plasmid minipreps, cutting this DNA with the restriction enzyme *Eco*RI and then visualizing the DNA fragments after electrophoresis on a 0.8% agarose gel. The clones that contained inserts were cleaned using cesium chloride-ethidium bromide density centrifugation and then sequenced to identify which part of the ~1,000 bp IGS region it contained. Because most of the amplified IGS fragments had internal *Eco*RI sites, as many as three different clones were required to obtain the complete ~1,000 bp sequence. Sequencing reactions were carried out using the protocol for Amersham/USB Sequenase Version 2.0 sequencing kit. To estimate the Taq error rate, we repeatedly cloned from a single PCR reaction until there were 13 copies of the 377-bp clone containing the 5' region of this ~1,000-bp fragment.

Data analysis.

Sequences were manually aligned and analyzed using parsimony analysis (PAUP 3.1; D. L. Swofford, Illinois Natural History Museum, Champaign, IL) to describe relationships among these isolates. Bootstrap analysis, using the heuristic search, was performed to test the statistical significance of each branch. Distance and neighbor joining analysis was performed using the PHYLIP program (version 3.5).

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