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Ribosomal DNA internal transcribed spacers are highly divergent in the phytopathogenic ascomycete *Fusarium sambucinum* (*Gibberella pulicaris*)

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Summary. Variation within the internal transcribed spacers (ITS1 and ITS2) and 5.8s ribosomal DNA gene of the heterothallic phytopathogenic filamentous fungus, *Fusarium sambucinum* (teleomorph = *Gibberella pulicaris*), was examined in 86 strains from diverse geographical locations by PCR amplification and direct sequencing in order to measure intraspecific divergence within the ITS region. Sequence analysis revealed three ITS types (A, B, C), within which divergence was extremely low (0–2.3%). Surprisingly, the level of intraspecific divergence observed between ITS types, A→B = 14.3%, A→C = 15%, and B→C = 4.6%, is much greater than that reported for any other species. The degree to which transition/transversions and insertion/deletions make up the pattern of ITS sequence evolution both within and between types was analyzed. The sequences of the ITS types exhibit a C-T transition bias together with a GC insertion/deletion bias. In comparison, the genic flanking sequences, including the 5.8s rDNA gene and 5′ end of the 28s large nuclear rDNA, are highly conserved. By the criteria of mating and DNA-DNA hybridization, all the strains examined represent a single species. Discordance between the ITS sequence data and other molecular and genetic data on *F. sambucinum* is discussed.

Key words: rDNA-molecular evolution – Internal transcribed spacer – Ascomycete fungus

Introduction

Ribosomal DNA genes are tandemly repeated multigene families containing both genic and nongenic, or spacer, regions. Each repeat unit contains a copy of the 18*s*-, 5.8*s*, and 28*s*-like rDNA (see Fig. 1) and two spacers, the internal transcribed spacer (ITS) and an intergenic spacer (IGS). The 5.8*s* rDNA gene is typically flanked by a bipartite ITS, the ITS1 and ITS2, which separates the 5.8*s* rDNA from the 18*s* and 28*s* genes, respectively (reviewed for fungi by Garber et al. 1988). Numerous studies have demonstrated that variable numbers of tandemly repeated sequences, or subrepeats, within the IGS account for the bulk of the length variation within the rDNA repeat unit (reviewed by Rogers and Bendich 1987). However, relatively little is known about the rate and pattern of intraspecific evolutionary substitution within spacers (Hillis and Davis 1986; Hancock and Dover 1988; Schaal and Learn 1988). This is in large part due to the paucity of DNA sequence data (Bruns et al. 1991).

With the relatively recent advent of polymerase chain reaction (PCR) techniques (Mullis and Faloona 1987), it is now possible to amplify, and sequence, specific regions of the genome from a large number of individuals in order to examine the pattern of intraspecific evolutionary substitution (Kocher et al. 1989). Because a high rate of intraspecific DNA divergence can affect measurements of molecular evolution between species (Wilson et al. 1985), the primary objective of this study was to measure the pattern and rate of DNA sequence divergence within the ITS region and flanking genic sequences of a genetically-defined biological species of *Fusarium* in order to determine the feasibility of using the ITS as a genetic marker in molecular systematic and phylogenetic studies of this genus. The experimental organism chosen for study was *Fusarium sambucinum* Fckl. [teleomorph = *Gibberella pulicaris* (Fr.) Sacc.], a heterothallic filamentous fungus with two mating types (Gordon 1954; Beremand et al. 1991). In this paper, sequence evolution within the ITS region of 86 isolates of *F. sambucinum* from diverse geographical locations was measured by DNA sequence analysis. Surprisingly, three divergent ITS types were discovered. The application of ITS-type-specific probes is discussed together with a comparison of the ITS sequence data with other molecular and genetic data on *F. sambucinum*.

Materials and methods

Strains and culture conditions. The 86 single-spore isolates of *Fusarium sambucinum* studied (Table 1) are stored by lyophilization in the

Table 1. Strains of *Fusarium sambucinum* studied

ITS rDNA type[a]	NRRL strain number	Geographical origin	Collector/depositor (isolate no.)[b]	Substrate	Received as[c]	MAT[d]
A1	20766	Thailand	Nelson (R-7738)	Soil	Fs	–
A1	20767	Australia, NSW	Nelson (R-4237)	Grass	Fs	–
A1	13503	Canada, PEI	Neish (DAOM 192963)	Potato	Fs	1
A1	20660	Great Britain	Ram (IMI 105488)	Stika spruce	Fsc	
A1	20477	Egypt	Booth (CBS 135.73)	Tomato	Fsul	
A1	20808	South Africa	Nelson (R-8411)	Soil	Fs	–
A1	20768	Australia, NSW	Nelson (R-5684)	Soil	Fs	–
A1	20769	Great Britain	Nelson (R-5214)	Soil	Fs	–
A1	20770	England	Nelson (R-583)	Knotweed	Fs	1
A1	13703	Iran	Nelson (R-5390)	Potato	Fs	1
A1	20771	Australia	Nelson (R-5920)	Pine	Fs	1
A1	13708	Germany	Nelson (R-6380)	Potato	Fs	1
A1	13525	Alaska	Nelson (R-7847)	Tundra	Fs	
A1	20772	Australia, NSW	Nelson (R-3248)	Grass	Fs	–
A1	20773	Australia, NSW	Nelson (R-4187)	Grass	Fs	–
A1	20774	Australia, NSW	Nelson (R-4263)	Grass	Fs	–
A1	20775	Australia, NSW	Nelson (R-4268)	Grass	Fs	–
A1	20776	Australia, NSW	Nelson (R-4272)	Grass	Fs	–
A1	20777	–	Nelson (R-5185)	–	Fs	–
A1	20778	Australia, NSW	Nelson (R-5344)	Pine	Fs	–
A1	20779	Australia, NSW	Nelson (R-5683)	Soil	Fs	–
A1	20704	Germany	Wollenweber (CBS 136.24)	Rye	Fs	
A1	20706	Germany	Wollenweber (CBS 185.29)	*Ulmus* sp.	Fs	
A1	13701	Idaho	Nelson (R-2633)	Potato	Fs	1
A1	13929	Poland	Chelkowski (KF-710)	Potato	F	1
A1	13709	Canada, NB	Neish (DAOM 196035)	Potato	F	1
B1	13504	Canada, PEI	Neish (DAOM 192966)	Potato	Fs	1
B1	13915	Netherlands	Nirenberg (BBA 64995)	Cabbage	Fs	
B1	20666	Great Britain	Benda (IMI 295478)	Potato	Ftri	
B1	20665	Great Britain	Young (IMI 264092)	Potato	Ftri	
B1	20656	Australia, NSW	Conroy (IMI 082762)	Potato	Ftri	
B1	20659	Great Britain	Spooner (IMI 273414)	*Ulmus* sp.	Fs	
B1	13916	Germany	Nirenberg (BBA 65009)	Potato	Fs	
B1	20667	Switzerland	Nelson (R-4712)	Potato	Fs	
B1	13917	France	Nirenberg (BBA 64996)	Potato	Fs	
B1	20679	New York	Nelson (R-738)	Potato	Fs	
B1	20671	Maryland	Nelson (R-6039)	Potato	Fs	
B1	20670	Egypt	Nelson (R-6686)	Potato	Fs	
B1	20681	–	Wollenweber (CBS 185.35)	–	Fsul	
B1	20782	Alaska	Nelson (R-7852)	Soil	Fs	–
B1	20783	South Africa	Nelson (R-8135)	Soil	Fs	–
B1	20784	South Africa	Nelson (R-8177)	Debris	Fs	–
B1	20785	South Africa	Nelson (R-8178)	Debris	Fs	–
B1	20786	South Africa	Nelson (R-8179)	Debris	Fs	–
B1	20787	South Africa	Nelson (R-8182)	Debris	Fs	–
B1	20788	South Africa	Nelson (R-8430)	Debris	Fs	–
B1	20789	South Africa	Nelson (R-8438)	Debris	Fs	–
B1	13932	Poland	Chelkowski (KF-366)	Potato	Fs	–
B1	13930	Poland	Chelkowski (KF-725)	Hops	Fs	–
B1	20790	South Africa	Nelson (R-7570)	Soil	Fs	–
B1	13502	Alaska	Abbas	Grass	F	–
B1	20791	Australia, NSW	Nelson (R-3032)	Grass	Fs	–
B1	20792	Australia, NSW	Nelson (R-3245)	Grass	Fs	–
B1	20793	Alaska	Nelson (R-7853)	Soil	Fs	–
B1	20794	Australia	Nelson (R-5867)	Soil	Fs	–
B1	20703	–	van Beverwijk (CBS 263.50)	Potato	Fsc	
B1	20705	Australia	Booth (CBS 136.73)	Potato	Ftri	
B1	20707	Canada, MAN	Gordon (CBS 161.57)	Potato	Fsul	
B1	13704	Poland	Golinski (KF-728)	Potato	F	1
B1	13705	Poland	Golinski (KF-735)	Potato	F	1
B1	20795	Nebraska	Leach (13707)	Potato	Fs	1
B1	20796	Colorado	Desjardins (13711)	Potato	Fs	1
B1	13495	–	Nelson	–	Fs	1
B1	20797	Argentina	Nelson (R-7715)	Cactus	Fs	–
B1	13501	Alaska	Abbas	Grass	F	–

Table 1. (Continued)

ITS rDNA type[a]	NRRL strain number	Geographical origin	Collector/depositor (isolate no.)[b]	Substrate	Received as[c]	MAT[d]
B1	20798	Minnesota	Nelson (R-110)	Pine	Fs	–
B1	13706	Iran	Nelson (R-5389)	Potato	Fs	2
B1	20799	Chile	Nelson (R-7843)	Carnation	Fs	2
B1	13700	Canada, NB	Murphy (# 2)	Potato	F	2
B1	13798	South Africa	Nelson (R-8575)	Soil	Fs	
B1	20448	South Africa	Gerlach (BBA 62443)	Soil	Fs	
B1	20451	Spain	Gerlach (BBA 62433)	Beet	Fs	
B2	20765	Minnesota	Nelson (R-5455)	Corn	Fs	2
B2	20800	Australia	Nelson (R-2155)	Pine	Fs	–
B3	20444	Iran	Gerlach (BBA 62719)	Caucasian wing-nut	Fs	
B3	20663	Germany	Nirenberg (IMI 266242)	–	Fsul	
C1	13712	Canada, NB	Murphy (# 1)	Potato	F	–
C1	20801	Azores	Rossman (AR-1753)	Wood	Gp	
C1	20781	Australia	Nelson (R-5928)	Soil	Fs	1
C1	20802	Madeira	Rossman (AR-1766)	Wood	Gp	
C1	20803	Australia	Nelson (R-5749)	Soil	Fs	–
C1	20804	Australia, NSW	Nelson (R-4170)	Grass	Fs	–
C1	20805	Canada, NB	Murphy (# 3)	Potato	F	2
C1	20806	–	Crawford	–	F	2
C1	20807	Canada	Nelson (R-6354)	Corn	Fs	2
C2	13710	Australia	Nelson (R-2882)	Potato	Fs	2

[a] See Fig. 1 for DNA sequence comparison of ITS types in *Fusarium sambucinum*

[b] R = P. E. Nelson, Department of Plant Pathology, Pennsylvania State University, University Park, Pa.; DAOM = National Mycological Herbarium, Biosystematics Research Institute, Ottawa, Ontario, Canada; IMI = CAB International Mycological Institute, Kew, Surrey, England; CBS = Centraalbureau voor Schummelcultures, Baarn, The Netherlands; BBA = H. Nirenberg, Biologische Bundesanstalt für Land- und Forstwirtschaft, Berlin, Germany; KF = Department of Plant Pathology, Agricultural University of Warsaw, Poznan, Poland; H. K., Abbas, Department of Plant Pathology, University of Minnesota, St. Paul, Minn.; A. M. Murphy, Research Station, Fredericton, NB, Canada; AR = A. Y. Rossman, Systematic Botany and Mycology Laboratory, USDA, Beltsville, Md.

[c] Fs = *Fusarium sambucinum* Fckl.; Fsc = *Fusarium sambucinum Fckl. var coeruleum* Wollenw.; F = *Fusarium* sp.; Ftri = *Fusarium trichothecioides* Wollenw.; Fsul = *Fusarium sulphureum* Schlecht.; Gp = *Gibberella pulicaris* (Fr.) Sacc

[d] MAT = alleles for mating type, data of Beremand et al. (1991); (–) = infertile; blank = not tested

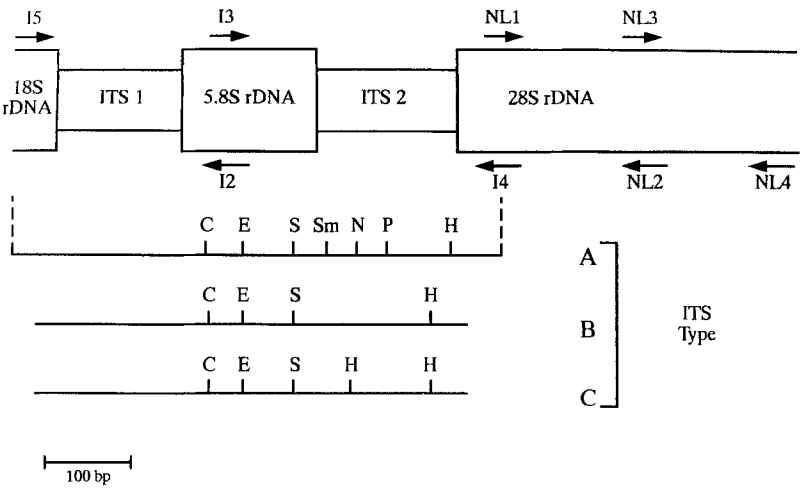

Fig. 1. Map of the rDNA region amplified and sequenced. Ribosomal RNA coding and spacer regions are indicated, as are PCR and sequencing primers (*I* = ITS). Restriction maps of the three ITS types (A, B, and C) are aligned directly below the ITS region delimited by PCR primers ITS-4 and ITS-5. *C*, *Cla*I; *E*, *Eco*RI; *S*, *Sph*I; *Sm*, *Sma*I; *N*, *Nar*I; *H*, *Hin*cII. The 5.8 *s* rDNA gene is 158 bp long in all types. The length of ITS1 and ITS2, respectively, in bp for each type is: *A* = 150, 165; *B* = 139, 147; *C1* = 142, 150; and *C2* = 141, 150

ARS Culture Collection (NCAUR, Peoria, Ill.). The distribution data listed in Table 1 should be viewed cautiously, however, since, as a potato pathogen, agricultural practices over the last 300 years may have contributed to the cosmopolitan distribution of this saprophyte/plant pathogen. Nevertheless, strains were selected to obtain the maximal range in geographical and substrate diversity, with emphasis being placed on strains that have been tested for fertility (Beremand et al. 1991). To obtain mycelium for DNA extraction, the contents of a lyophile ampule were inoculated directly into 100 ml of YM broth (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 2% dextrose) and grown on a rotary shaker at 200 rev. min^{-1} at 25 °C for 1–5 days. Mycelium was harvested by filtration, washed once with distilled H_2O, blotted briefly between paper towels and lyophilized 8 h-to-overnight.

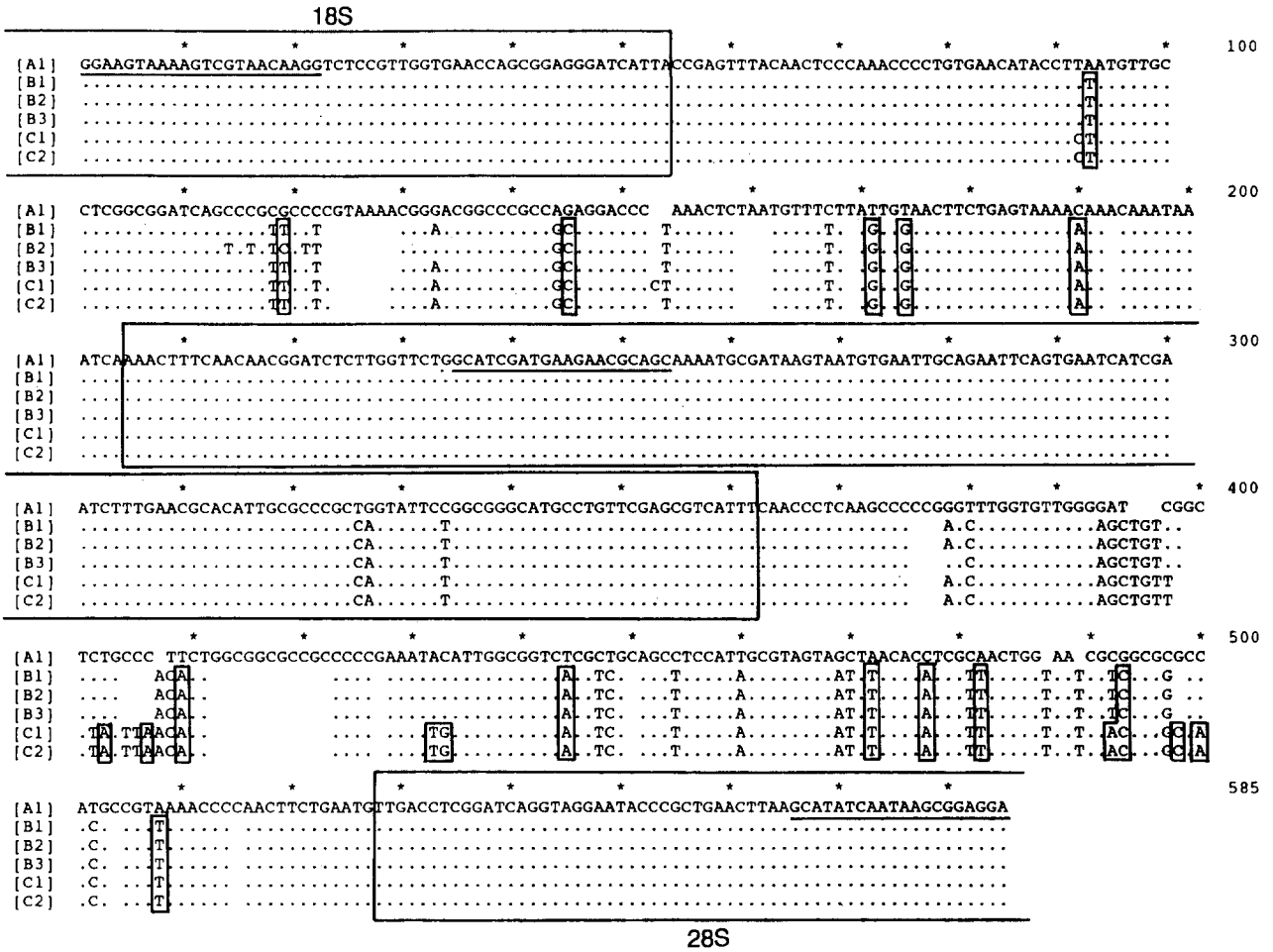

Fig. 2. Nucleotide comparison of the antisense strand of the ITS types of *F. sambucinum* amplified with primer pair ITS-4 and ITS-5. The longest ITS sequence, A1, was used as the reference sequence. Identical bases are indicated by a *dot*. Numbering of the *F. sambucinum* sequence begins with the 5′-most nucleotide sequenced. Large blocks delineate the three genic regions; the central one is the 5.8 *s* gene. Oligonucleotide priming sites are indicated by *underlines*. Transversions are distinguished from transitions by their enclosure in *small blocks*. The nucleotide position of gene-spacer junctions in the ITS region of *F. sambucinum* (*lowercase number*), aligned with *Saccharomyces cerevisiae* (*superscript number*), are as follows: 3′-18 *s* rDNA = 54^{1786}, 5′-5.8 *s* rDNA = 205^{2150}, 3′-5.8 *s* rDNA = 362^{2305}, and 5′-28 *s* = 528^{1}. Numbering in *S. cerevisiae* begins with the first nucleotide in the 18 *s* (Mankin et al. 1986) and 28 *s* (Georgiev et al. 1981) transcript. Nucleotides 394–409 and 488–500 in the *F. sambucinum* sequence are too divergent to align with confidence. Compared with ITS1, ITS2 contains twice as many substitutions and insertion/deletions. EMBL accession numbers for the sequences are X65477 through X65482

DNA extraction. DNA for PCR was extracted using a modified version of the SDS protocol of Raeder and Broda (1985). Lyophilized mycelium (approximately 50 mg) was pulverized in a 1.5 ml microfuge tube with a pipette tip, resuspended in 600 µl extraction buffer (200 mM Tris-HCl pH 8.4, 200 mM NaCl, 25 mM EDTA pH 8.0, and 0.5% SDS) and extracted with phenol/chloroform and chloroform. DNA was precipitated from the aqueous layer by the addition of 0.54 volumes of isopropanol and pelleted for 10 s in a microfuge at 10 000 rpm. The pellet was washed gently with 70% ethanol, resuspended in 100 µl of TE buffer pH 8.0 (10 mM Tris-HCl, 1 mM EDTA pH 8.0) and dissolved by incubating at 55 °C for 1 h-to-overnight. Dilute DNA samples for PCR were prepared by adding 1–4 µl of the total genomic DNA stocks to 1 ml TE/10 buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0).

Polymerase chain reaction. The priming sites of all PCR and sequencing oligonucleotide primers are indicated in Fig. 1. The internal transcribed spacer (556-to-585 base pairs in *F. sambucinum* as determined by DNA sequencing) was amplified with ITS-4 and ITS-5 primers (White et al. 1990; labeled I4 and I5 in Fig. 2). A variable region at the 5′ end of the 28 *s* large nuclear rDNA gene, incompassing domains D1 and D2 (Guadet et al. 1989; Peterson and Kurtzman 1991), was amplified with NL-1 and NL-4 (underlined in Fig. 3). The size of this PCR product in *F. sambucinum* is 605 base pairs. NL-1 is the reverse complement of ITS-4. A variable region in the mitochondrial small rDNA was amplified with the primer pair MS-1 and MS-2 (White et al. 1990; this fragment is approximately 700 base pairs in *F. sambucinum*). Single-stranded DNA templates were prepared by the asymmetric primer ratio method (Gyllensten and Erlich 1988) as modified by Bruns et al. (1989, 1990). Templates of both strands were generated using primer ratios of 1 : 50 or 2 : 50 pmoles in separate reactions in a total volume of 50 µl.

Sequencing. Following asymmetric amplification, excess primers and deoxynucleotide triphosphates were removed from samples containing sufficient single-stranded DNA by filtration in Ultrafree-MC 30 000 NMWL low-binding cellulose filter units (Millipore, Bedford, Mass.) according to the manufacturer's instructions. Single-stranded DNA templates were sequenced completely on both strands using the chain-terminating dideoxynucleotide Sequenase 2.0 kit (United States Biochemical Corporation, Cleveland, Ohio) and ^{35}S-labeled dATP. Two sequencing reactions were run on

each template, one in which the template was primed with 5 pmol of the external limiting primer and the second with 5 pmol of an internal primer (Fig. 1). Two internal sequencing primers were used for the ITS region (White et al. 1990), ITS-3 and its reverse complement ITS-2 (underlined in Fig. 2). Also, two internal sequencing primers were used for the 28 *s* large nuclear rDNA base pair NL-1-to-NL-4 fragment, NL-3 and its reverse complement NL-2 (underlined in Fig. 3). Sequencing reactions were run on 5% polyacrylamide wedge gels in a modified 'kilobase' TBE buffer (Bethesda Research Laboratories, Gaithersburg, Md.) for approximately 3 h. Gels were fixed, vacuum dried, and exposed overnight to Kodak SB film. Sequences were visually aligned with published sequences of the ITS region of *Saccharomyces cerevisiae*, *Neurospora crassa* (Chambers et al. 1986) and other fungi (Tsuge et al. 1989) in order to define the gene-spacer junctions (Fig. 1).

DNA hybridization. Strains were grown in 1 l YMG broth (0.5% yeast extract, 0.5% malt extract, 0.5% glycerol) in 2.8 l Fernbach flasks on a rotary shaker at 200 rev. min^{-1} at 25 °C for 1–3 days. Subsequent DNA extraction, purification, and shearing were as described previously (Kurtzman et al. 1980) except that DNA was purified by CsCl centrifugation (Maniatis et al. 1982). DNA complementarity was assessed by hybridization in a Gilford Response II spectrophotometer (Peterson and Logrieco 1991).

Results

PCR experiments carried out on the ITS region of *Fusarium sambucinum* genomic DNA reproducibly amplified a single double-stranded product of approximately 550–600 base pairs, using primer pairs ITS-4 and ITS-5 (labeled I4 and I5 in Fig. 1). Direct sequencing of the asymmetrically-amplified PCR products revealed that each of the 86 strains analyzed possessed one of three divergent ITS types, designated A, B, C (Fig. 1), whose complete sequence is shown in Fig. 2. Of the 86 isolates sequenced, 26 (30.2%) had the A ITS type, 50 (58.2%) the B, and 10 (11.6%) the C.

Restriction maps of the three types are shown in Fig. 1. Type A contains three unique hexanucleotide restriction sites (*Sma*I, *Nar*I, and *Pst*I) missing from types B and C while B and C are polymorphic for *Hin*cII. Sequence divergence within the ITS1 + ITS2 region is remarkably low (Table 2): A = 0% ($N = 26$), B = 2.3% ($N = 50$), and C = 0.4% ($N = 10$). Virtually all within-type divergence is in the form of single point mutations with a bias towards C-T transitions. Transversion mutations are absent within types. Conversely, sequence divergence between types within the ITS-1 + ITS2 region is surprisingly high (Table 2): A → B = 14.3%, A → C = 15%, and B → C = 4.6%. Evolutionary divergence between type A, B, and C sequences is biased towards transition/transversion point mutations. Transitions account for all of the intra-type divergence (0–1.8%) observed within the 5.8 *s* rDNA gene (Fig. 2, Table 2). When compared with

Table 2. Nucleotide divergence within and between ITS types

ITS type	Regions compared [a]								
	ITS1 + ITS2			5.8 *s* rDNA			ITS1 + ITS2 + 5.8 *s* rDNA		
	TS + TV [b]	I\D [c]	% divergence [d]	TS + TV	I\D	% divergence	TS + TV	I\D	% divergence
A	0	0	0%	0	0	0%	0	0	0%
B	6	0	2.3%	0	0	0%	6	0	1.4%
C	0	1	0.4%	0	0	0%	0	1	0.2%
A → B	29	12	14.3%	3	0	1.8%	32	12	9.9%
A → C	29	14	15%	3	0	1.8%	32	14	10.4%
B → C	9	3	4.6%	0	0	0%	9	3	2.9%

[a] The region between nucleotides 394–409 and 488–500 in the ITS sequence (see Fig. 2) was excluded from the analysis due to alignment ambiguity

[b] TS = transition, TV = transversion. Note transversions are absent within types

[c] I\D = insertion/deletion; multi-base length differences are scored as 1

[d] Percent divergence = # TS + TV + I\D/sequence length × 100

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      *         *         *         *         *         *         *         *         *         *
GCATATCAATAAGCGGAGGAAAAGAAACCAACAGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCTCTCGGGCCCG   100
AGTTGTAATTTGTAGAGGATGACTTTGATGCGGTGCCTTCCGAGTTCCCTGGAACGGGACGCCATAGAGGGTGAGAGCCCCGTCTGGTTGGATGCCAAAT   200
CTCTGTAAGTCTCCTTCGACGAGTCGAGTAGTTTGGGAATGCTGCTCTAAATGGGAGGTATATGTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGC   300
GCACAAGTAGAGTGATCGAAAGATGAAAAGCACTTTGAAAAGAGAGTTAAAAAGTACGTGAAATTGTTGAAAGGGAAGCGTTTATGACCAGACTTGGGCT   400
TGGTTAATCATCTGGGGTTCTCCCCAGTGCACTTTTCCAGTCCAGGCCAGCATCAGTTTTCGCCGGGGGACAAAGCCTTCGGGAATGTGGCTCCCTCGGG   500
GAGTGTTATAGCCCGTTGTGTAATGCCCTGGCGGGGACTGAGGTTCGCGCTTCTGCAAGGATGCTGGCGTAATGGTCATCAACGACCCGTCTTGAAACAC   600
GGACC                                                                                                   605
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Fig. 3. Sequence of the antisense strand of the 5′-end of the 28 *s* nuclear large rDNA of *F. sambucinum* for strains carrying the following ITS types: *A1* (13708), *B1* (13700), and *C1* (20805). Strains carrying ITS types *B1* (13504), *B2* (20765), *B3* (20444, 20663), and *C2* (13710) have the identical sequence except that *B1* (13504), *B3* and *C2* have an A transition substitution at nucleotide position 476 while *B2* has a T transition substitution at nucleotide position 91. Nucleotides at the two variable positions are enclosed by *blocks*. Numbering begins with the 5′-most nucleotide sequenced. Oligonucleotide priming sites are indicated by *underlines*. Since NL-1 is the reverse complement of ITS-4, the 19 5′-most nucleotides in Fig. 3 are identical to the 19 3′-terminal bases in Fig. 2. Nucleotide position 1 in the *F. sambucinum* sequence corresponds to nucleotide 39 in the *S. cerevisiae* 28 *s* rDNA sequence (Georgiev et al. 1981) and 528 in the *F. sambucinum* ITS region sequence (Fig. 2). EMBL accession numbers for the sequences are X65474 through X65476

transversions, a transition bias was observed between all types: A → B (1.9–2.4), A → C (2.2), and B → C (1–4).

While AT insertion/deletion events predominate within ITS1, overall there is a strong bias towards GC insertion/deletions within the ITS2 region. As a result, ITS2 is 60.6% G+C rich (cf., ITS1 GC content of 48.3%) with some GC rich insertion segments having the potential to form stem-loop secondary structures (e.g., nucleotides 412–423). There is a significant difference in nucleotide distribution between ITS1 and ITS2 ($P<0.01$ by χ^2 and by the z-test). Furthermore, the rate of sequence divergence within ITS2 is two-fold that of ITS1. Adjusting for differences in spacer length, the percentage of variable positions for ITS1 and ITS2 is 19% and 38%, respectively. Lastly, the mating type alleles are not randomly distributed among all of the ITS types (see Fig. 4). Using binomial probabilities, the results have probabilities of 0.04 (A), 0.79 (B), and 0.04 (C) that the underlying distribution is a 2:1 ratio as expected (Beremand et al. 1991).

Three other types of molecular data were obtained on a subset of the genetically fertile strains of *F. sambucinum* (Table 1). First, a 605-base pair variable region at the 5′ end of the 28 *s* nuclear large rDNA, amplified with primer pairs NL-1 and NL-4, was sequenced completely on both strands, revealing 0–0.16% divergence within this region among strains carrying each of the ITS types (Table 1, Fig. 3). Secondly, sequence analysis of a 400-base pair variable region at the 3′ end of the mitochondrial small rDNA, amplified with primer pairs MS-1 and MS-2, revealed zero divergence within this region among strains carrying ITS type A (13 708), B (20 765), and C (13 710) (data not shown). Thirdly, results of DNA-DNA hybridization experiments between strains carrying the two most divergent ITS types, A (NRRL 13 703) and B (NRRL 13 504), and A (13 708) and C (20 805) showed greater than 93% genetic relatedness, indicating that these strains are conspecific (Peterson and Logrieco 1991).

Discussion

The primary focus of this study was to measure the pattern and rate of DNA sequence divergence within the ITS region of genetically fertile strains of *Fusarium sambucinum* in order to determine the feasibility of using this region as a genetic marker in molecular systematic and phylogenetic studies of *Fusarium*. The PCR primer pairs used in this study (ITS-4 × ITS-5, and NL-1 × NL-4) successfully amplified PCR products of the predicted size (Guadet et al. 1989; Vilgalys and Hester 1990; White et al. 1990). As expected, DNA sequence analysis of these products revealed that the rDNA genic regions are highly conserved within *F. sambucinum*. This result, taken together with the DNA-DNA complementary data reported here and by Peterson and Logrieco (1991) and the mating type data of Beremand et al. (1991), support the view that the isolates examined in this study represent a morphologically diverse biological species. Furthermore, Peterson and Logrieco (1991) have demonstrated in DNA-DNA hybridization experiments that mating competent strains of *F. sambucinum* possess high (>90%) DNA relatedness.

Although the genetic data mentioned above indicate that *F. sambucinum* is a single species, the ITS sequence data do not support this interpretation. The startling discovery that three divergent ITS types have become fixed in the nuclear rDNA of *F. sambucinum* contrasts sharply with available data on the ITS from other fungal species where ten-fold lower intraspecific variation has been reported (Taylor et al. 1990; Baura et al. 1991; Gardes et al. 1991). The simplest interpretation of the incongruity of the ITS sequence data and other molecular and genetic data may be explained by the predominantly asexual life history of *F. sambucinum* in nature. The ITS types may define three morphologically cryptic cosmopolitan asexual populations among which sexual reproduction rarely occurs. This view is consistent with the following three observations. First, the teleomorph, *Gibberella pulicaris*, is rarely observed in nature (Gordon 1954; Booth 1971; only two of the 86 strains examined here were obtained from teleomorph collections). Secondly, the apparent imbalance of mating-type alleles in nature (2:1 MAT 1 to MAT 2, Gordon 1954; Beremand et al. 1991) argues that genetic exchange via sexual reproduction may be limited. This point is further emphasized by the discovery that the mating-type alleles are not randomly distributed among strains carrying the A and C ITS types (summarized in Fig. 4). Lastly, and perhaps most significantly, concerted evolution within the rDNA multigene family should have homogenized the ITS region during meiotic recombination (Dover 1982) but this clearly has not occurred. However, the extremely low level of nucleotide divergence observed within each of the three ITS types may indicate that either concerted evolution is operating within types or that the ITS have only recently diverged. Intrachromosomal gene conversion and unequal chromatid exchange are the primary genetic mechanisms used to explain concerted evolution within multigene families and the latter

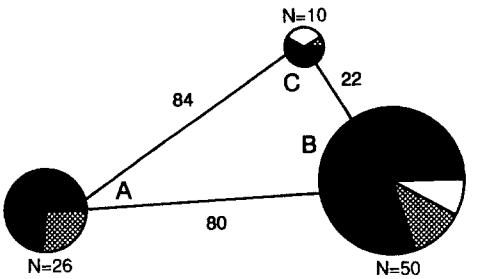

Fig. 4. Network of ITS types. Each of the three types (*A*, *B*, *C*) is indicated by a *circle* with the diameter being proportional to its "frequency" (N=number of isolates sequenced/type). *Numbers* along the network indicate the total number of variable nucleotide sites between types within the ITS1 + ITS2 region. The size of this region in base pairs (bp) including gaps is: ITS1 = 151 bp, ITS2 = 170 bp. Mating-type alleles (data of Beremand et al. 1991) associated with each ITS type are indicated: *MAT1*, *shaded*; *MAT2*, *unshaded*; Unknown, *solid black*. *A* = 8 MAT-1\18 unknown, *B* = 6 MAT-1\4, MAT-2\40 unknown, *C* = 1 MAT-1\4, MAT-2\5 unknown. These results have probabilities of 0.04 (*A*), 0.79 (*B*), and 0.4 (*C*) that the underlying distribution is a 2:1 ratio (binomial probabilities) ▨ = MAT1, □ = MAT2, ■ = Unknown

has been verified experimentally within the rDNA genes of *S. cerevisiae* (Petes 1980).

The pattern of sequence evolution within the ITS region in *F. sambucinum* and other eukaryotes (Goldman et al. 1983, Chambers et al. 1986; Tautz et al. 1987; Gonzalez et al. 1990) is a mosaic of base substitutions and insertion/deletion events that are not uniformly distributed throughout the spacer. Given that the ITS2 in *F. sambucinum* is two-fold more divergent than the ITS1, it may be under less functional constraint. However, the ITS2 is not indispensable since analysis of yeast rRNA mutants has demonstrated that it is required for correct processing of the rRNA primary transcript (reviewed by Raue et al. 1988). Furthermore, functional analysis of the central region of the ITS1 in *S. cerevisiae* has demonstrated that it is essential for the production of 18 *s* rRNA (Musters et al. 1990).

The strong C-T substitution bias observed between the sequences of the ITS types in the present study has been reported for both nuclear and mitochondrial genes (Kocher et al. 1989; Bruns and Szaro 1992), suggesting that the ITS sequences in *F. sambucinum* are young enough so that multiple substitutions have not obscured the record of a transition bias (Brown et al. 1982). Insertional events within the ITS have been shown to exhibit either an AT or GC bias (Goldman et al. 1983) and, though largely speculative, sequences with GC motifs such as those reported here for *F. sambucinum* are theorized to compensate for replication slippage (Hancock and Dover 1990) by forming stable hairpin/loop secondary structures.

Although ITS heterogeneity was not observed directly within any strain in this study, it could have gone undetected due to the PCR techniques employed, especially if one ITS type was always dominant. In this connection, divergent ITS sequences flank the two distinct 18 *s* rDNA genes that are stage-specifically expressed in *Plasmodium berghei* (reviewed by Gunderson et al. 1987). Given the within-type homogeneity and the relatively high degree of nucleotide divergence observed between the three ITS types, it should be possible to test strains for ITS heterogeneity using ITS-type-specific oligonucleotides as hybridization probes or as PCR primers (Saiki et al. 1986; Gardes et al. 1991). Also, these oligonucleotides should provide valuable tools for the investigation of additional problems. It should be possible, for example, to introduce ITS heterogeneity into the rDNA repeat unit (Petes and Botstein 1977) and then measure the effects of concerted evolution at this locus during successive generations. Furthermore, ITS-type-specific primers should provide useful genetic markers for investigating the composition of natural populations of *F. sambucinum*, about which virtually nothing is currently known.

One expectation of this investigation was that the rDNA ITS region would be useful in molecular systematic studies of *Fusarium*; however, incongruity between the ITS sequence data and other molecular and genetic data suggests that its value as a phylogenetic tool may be limited within this genus, especially until the source of intraspecific variability is fully understood. Experiments are in progress to determine whether other species of *Fusarium* possess multiple ITS types. Independent genetic markers will be required in order to accurately construct a phylogeny of *Fusarium* and unravel the molecular evolution of the ITS region within this genus.

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