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# Restriction fragment length polymorphisms in *Septoria tritici* occur at a high frequency

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**Summary.** A set of probes that detect restriction fragment length polymorphisms (RFLPs) in nuclear DNA has been developed for genetic studies of the phytopathogenic fungus *Septoria tritici.* Two plasmid libraries containing 0.5–1.3 or 1.3–2.4 kb fragments of *S. tritici* nuclear DNA were constructed. Seventeen random clones from each library were used as probes to screen for RFLP variation among a geographically-diverse group of six *S. tritici* isolates. Among the 196 probe-enzyme combinations tested, 145 detected RFLPs among the six isolates. The restriction enzymes *Eco*RV and *Pst*I detected RFLPs most efficiently. Three probes detected deletions. A ribosomal DNA probe from yeast did not detect a significant amount of variation. These probes will be useful for studying genetic variation, population genetics, and genome organization of *S. tritici.*

**Key words:** RFLPs – *Septoria tritici* – DNA fingerprinting – Genetic variation

## Introduction

The genetics of plant pathogenic fungi generally has been difficult to study because of a lack of easily assayed genetic markers. Most species of agricultural importance have been examined using virulence characters as the principal genetic markers. Unfortunately, many phytopathogenic fungi do not possess clearly defined gene-for-gene interactions that make them amenable to genetic analysis using virulence markers, and other genetic markers need to be developed for these fungi. Even where gene-for-gene relationships occur, virulence markers assess only variability for virulence, are slow, labor intensive, expensive, and greatly restrict sample size. Restriction fragment length polymorphisms (RFLPs) are rapidly becoming the tools of choice for studying fungal genetics because they are precise, selectively neutral, relatively easy to assay, and can provide a virtually unlimited supply of genetic markers (Michelmore and Hulbert 1987). RFLPs will be especially useful for genetic studies of pathogenic fungi that do not possess clearly defined virulence or physiological markers. RFLPs can be used to create a molecular "fingerprint" of specific individuals in a population, and hence provide powerful tools for studying populations of fungi that usually reproduce clonally. Most fungal studies to date have utilized RFLP variation in mitochondrial DNA (review in Taylor 1986; Forster et al. 1988; Kistler et al. 1987) to measure genetic distance between closely-related species. RFLPs in nuclear DNA have not been reported widely in fungi. Nuclear RFLPs in pathogenic fungi have been used to develop linkage maps (Hulbert et al. 1988) and more recently to study pathogen population genetics (McDermott et al. 1989). This paper reports the development of a set of RFLP markers that can be used for genetic studies in the Ascomycete fungus *Septoria tritici* Rob. ex Desm. [teleomorph *Mycosphaerella graminicola* (Fuckel) Schroeter], which causes septoria tritici blotch of wheat.

*S. tritici* is a haploid fungus that has no naturally occurring morphological or known physiological markers such as specific virulence genes, vegetative incompatibility groups or auxotrophic markers, so it has been difficult to study genetically. Pathogenic specialization is thought to exist,but clearly defined virulence genes have not been identified (review in Eyal et al. 1987). The sexual stage of the fungus, *M. graminicola,* has been identified in several countries (review in King et al. 1983). Recent evidence shows that

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the sexual stage may be more widespread than previously thought (Shaw and Royle 1989), but the importance of this stage in the population genetics of the fungus is not known. The goal of our research was to develop a set of genetic markers based on RFLPs that could be used to estimate the amount and distribution of genetic variability in *S. tritici* populations. We intend to use RFLP markers to achieve a better understanding of the genetics of *S. tritici* as a first step toward developing *S. tritici* as a model system in which to study plant pathogen population genetics.

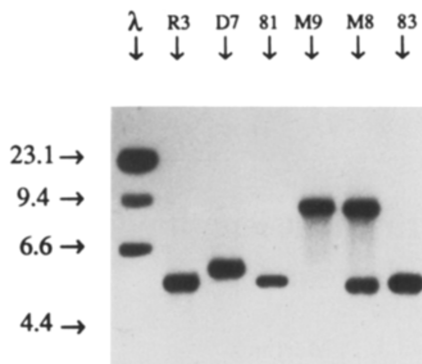


**Fig. 1.** Southern blot showing RFLPs corresponding to putative single-copy sequence in *Septoria tritici.* Probe pSTL42 hybridized to three length variants in *Eco*RI digestions. Isolate abbreviations are as follows: *R3* = Rusa3; *D7* = D7; *81* = 81.192; *M9* = MT9; *M8* = MT8; *83* = 83.20. Isolate MT8 was a mixed culture. *Size markers on the left* correspond to lambda DNA digested with *Hind*III

## Materials and methods

*Isolates of S. tritici.* The six *S. tritici* isolates used in this study originated from an international collection maintained by A. L. Scharen (transferred under APHIS permit #4504-05). Isolates 81.192, Rusa3, MT8, and MT9 originated from Montana; D7 originated from Texas; and isolate 83.20 originated from Israel. These isolates were chosen because their different geographical origins increased the likelihood of their having different nucleotide sequences, thus making them likely candidates for detecting RFLPs.

*DNA isolation.* DNA was extracted using a minipreparation procedure modified from Zolan and Pukkila (1986). *S. tritici* isolates were grown in 50 ml of yeast sucrose broth for six days at 21 °C on a shaker rotating at 150 rpm. Most isolates produced large quantities of spores by budding under these conditions, but some isolates grew as a mixture of mycelium and spores. Approximately 1–2 grams (fresh weight) of tissue was harvested by centrifugation at 1,900 *g* (15 min, top speed, IEC clinical centrifuge). Tissue was frozen with liquid nitrogen and crushed into a fine powder with a mortar and pestle. Frozen powder was dispersed in 2 ml of CTAB extraction buffer [700 mM NaCl, 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% 2-mercaptoethanol (v:v), and 1% hexadecyltrimethylammonium bromide (CTAB) (w:v)] in a 4.5 ml centrifuge tube and placed in a 60 °C heating block for 45 min, with gentle mixing every 15 min. An equal volume of chloroform:isoamyl alcohol (24:1, v:v) was added and the tube mixed by inversion to form an emulsion. The emulsion was spun in a centrifuge at 1,900 *g* for 30 min and the upper aqueous phase was transferred to a fresh 4.5 ml tube. Nucleic acids were precipitated by adding an equal volume of isopropanol and centrifuging at 1,900 *g* for 10 min. The nucleic acid pellet was dissolved in 300 µl TE (10 mM Tris, 1 mM EDTA, pH 8.0), treated with RNAse, and then phenol-extracted as described in Maniatis et al. (1982). DNA was precipitated by adding two volumes of 95% ethanol and centrifuging at 1,900 *g* for 10 min. DNA pellets were dried in a desiccator and then dissolved in 40–80 µl of TE. Average yield was 30 µg DNA per extraction.

*Probes.* Two plasmid libraries containing random *S. tritici* DNA fragments were constructed as follows. Total DNA was extracted from *S. tritici* using the minipreparation method described above. Three µg of DNA were partially-digested with *Sau*3A (BRL, Gaithersburg, MD) as described in Maniatis et al. (1982). Restriction fragments were separated by electrophoresis on a preparative 1% agarose gel. Two size classes of fragments (500–1,300 bp and 1,300–2,400 bp) were transferred from the gel onto DE81 paper by electrophoresis. Eluted fragments were ligated into the *Bam*HI site of the vector pGEM4 (Promega), which had been treated with calf intestinal alkaline phosphatase. Recombinant plasmids were used to transform *E. coli* strain HB101 as described in Maniatis et al. (1982). Two hundred colonies from each transformation were saved to use as sources of plasmid probes. Recombinant plasmids were extracted using the boiling lysis method of Holmes and Quigley (1981). Plasmid pBD4, which contains a complete ribosomal DNA (rDNA) repeating unit from *Saccharomyces cerevisiae* (Bell et al. 1977), was used as a heterologous probe. pBD4 was used previously to study RFLP variation in the pathogenic fungus *Rhynchosporium secalis* (McDermott et al. 1989).

*DNA digestion, electrophoresis, blotting, nick-translation, and hybridization.* Total DNA from each *S. tritici* isolate was digested with the restriction enzymes *Eco*RI, *Eco*RV, *XhoI*, *Pst*I, *Bam*HI, and *Dra*I according to the manufacturer's instructions (BRL). Digested DNA (3 µg per lane) was separated in 1.0% agarose TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) gels for 16–18 h at 2.4 V/cm. Capillary transfer of DNA to Zetaprobe membrane (Bio-Rad, Richmond, CA) was conducted overnight without using transfer buffer. Prehybridization and hybridization reactions were performed according to the manufacturer's instructions in an air shaker at 65°C in sealed plastic bags (2 membranes/bag) containing 5% dextran sulfate. Plasmids were labeled with $^{32}$P by nick translation using DNA polymerase I following the manufacturer's instructions (BRL). Unincorporated nucleotides were removed by passage through a Schleicher and Schuell elutip-d minicolumn. Hybridization reactions were allowed to proceed for 16 h. Membranes were washed at 60°C in 2×SSC (0.3 M NaCl, 0.03 M Na citrate), 0.1% SDS for 25 min followed by 0.5×SSC, 0.1% SDS for 25 min, and 0.1×SSC, 0.5% SDS for 35 min. Membranes were placed on X-ray film (Kodak) with intensifier screens for 12–72 h at −80°C. Labelled probes were stripped from membranes by boiling in 0.1×SSC, 0.5% SDS for 30 min before reprobing. Each membrane was probed up to ten times, allowing a large number of probe-enzyme combinations to be screened with each blot.

## Results

A total of 196 probe-enzyme combinations were tested among the 210 combinations possible using 35 probes and six restriction enzymes. Degradation of nylon membranes following repeated probing accounted for the majority of the missing probe-enzyme combina-

**Table 1.** Probe-enzyme data for RFLPs in *Septoria tritici* showing size of inserts in plasmid probes, presence or absence of length variants, and number of variants detected among six isolates

| Probe | Insert size (kb) | Enzyme | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Eco*R1 | *Eco*RV | *Xho*1 | *Pst*1 | *Bam*H1 | *Dra*1 |
| pBD4 | 9.3 | NP | NP | P(2) | NP | NP | NP |
| pSTL1 | 1.6 | R | – | R | R | R | – |
| pSTL2 | 1.3 | NP | P(2) | P(2) | P(2) | P(2) | NP |
| pSTL3 | 1.3 | R | R | R | R | R | R |
| pSTL6 | 0.5 | R | R | R | R | R | R |
| pSTL10[a] | 1.1 | P(2) | P(3) | P(3) | P(3) | P(3) | P(3) |
| pSTL12 | 1.4 | R | R | R | R | R | R |
| pSTL22 | 0.8 | P(2) | P(2) | NP | P(3) | NP | P(2) |
| pSTL28 | 1.8 | P(2) | P(2) | – | NP | NP | NP |
| pSTL29 | 2.7 | R | R | R | R | R | R |
| pSTL40 | 1.7 | R | R | R | R | R | R |
| pSTL42 | 3.1 | P(4) | P(4) | P(2) | P(2) | P(3) | NP |
| pSTL43 | 1.6 | R | R | R | R | R | R |
| pSTL46[a] | 2.5 | – | P(2) | P(2) | P(2) | – | – |
| pSTL48 | 1.6 | P(2) | P(2) | NP | P(2) | NP | NP |
| pSTL49 | 2.9 | NP | P(2) | NP | P(2) | P(4) | NP |
| pSTL53 | 1.9 | – | P(5) | P(5) | P(2) | – | – |
| pSTL56 | 2.4 | R | R | R | R | R | R |
| pSTS2 | 0.7 | P(2) | NP | NP | P(4) | P(3) | NP |
| pSTS3 | 1.0 | P(2) | P(2) | – | NP | NP | NP |
| pSTS4 | 0.9 | R | R | R | R | R | R |
| pSTS13 | 0.7 | NP | NP | NP | NP | P(2) | NP |
| pSTS14 | 0.6 | P(2) | P(2) | NP | P(2) | NP | P(2) |
| pSTS16 | 0.6 | P(3) | P(4) | NP | P(2) | P(2) | NP |
| pSTS17 | 0.8 | NP | NP | NP | NP | NP | NP |
| pSTS32 | 0.9 | NP | P(3) | P(3) | P(3) | NP | P(2) |
| pSTS40 | 1.2 | P(3) | P(3) | P(3) | P(2) | NP | P(2) |
| pSTS189 | 0.5 | NP | P(3) | P(2) | P(4) | P(2) | P(3) |
| pSTS190 | 2.3 | P(3) | P(4) | P(4) | P(3) | P(3) | NP |
| pSTS192 | 2.0 | P(2) | P(5) | P(2) | P(3) | P(4) | NP |
| pSTS194 | 0.8 | P(2) | – | P(2) | NP | P(2) | – |
| pSTS196 | 0.5 | P(2) | – | P(2) | P(2) | P(2) | – |
| pSTS197[a] | 0.5 | P(2) | P(2) | P(3) | P(3) | P(2) | P(2) |
| pSTS199 | 1.1 | P(4) | P(4) | P(3) | P(4) | P(2) | P(2) |
| pSTS200 | 1.2 | P(2) | NP | NP | NP | NP | NP |

Key to abbreviations: *P* polymorphic; *NP* not polymorphic; *R* repetitive hybridization pattern; – no data; (n) number of variants; [a] detected deletion

tions. RFLPs were detected for 74% (145 of 196) of the probe-enzyme combinations (Table 1). Altogether, 34 of the 35 probes detected RFLPs among the six isolates. Twenty-six of the 35 probes hybridized to 1–3 fragments per isolate and presumably correspond to single-copy sequences (example in Fig. 1). Eight probes hybridized to 5–30 fragments per isolate, indicating that several copies of these sequences exist per genome. Three of these repetitive probes (pSTL29, pSTL40, pSTL43) produced unique, easily scored, hybridization patterns for each isolate, suggesting that these probes may be useful for DNA "fingerprinting" of *S. tritici* isolates (example in Fig. 2). One probe, pSTS4, hybridized to 30–100 fragments in each isolate and appears to be a dispersed highly repetitive sequence. The rDNA probe pBD4 detected two variants using the enzyme *Xho*1.

On average, 2.8 electrophoretic variants were found among the six isolates for each polymorphic probe-enzyme combination using the 26 probes that produced simple banding patterns. RFLPs result from a variety of DNA sequence changes, including restriction site mutations and rearrangements caused by deletions, insertions, or inversions. It was difficult to determine the types of changes that caused the RFLPs we observed. We found no clear evidence among these isolates of an RFLP caused by an insertion. However, it appears that three of the probes detected different

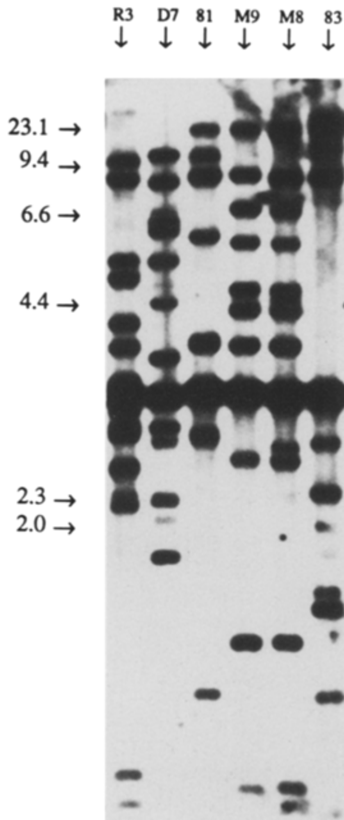


**Fig. 2.** Southern blot showing RFLP fingerprint pattern in *Septoria tritici* isolates produced by the probe-enzyme combination pSTL40-*Bam*HI. Size markers and isolate abbreviations are as described in Fig. 1

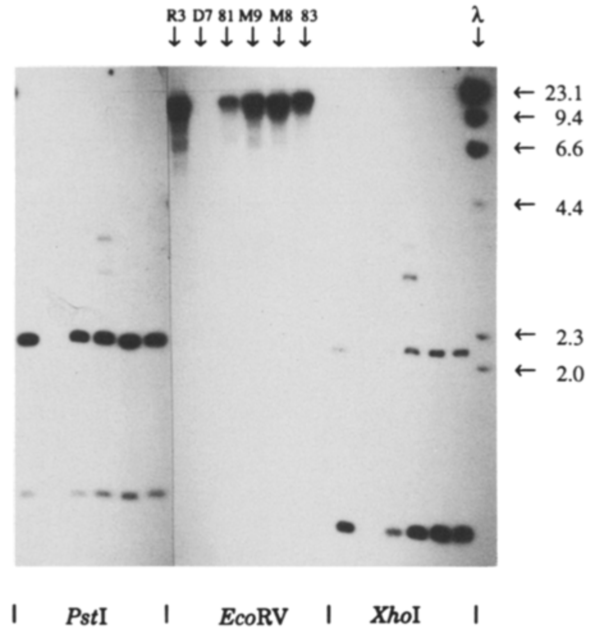


**Fig. 3.** Identification of a deletion among *Septoria tritici* isolates with probe pSTL46. Southern blots were exposed for 48 h with intensifying screens. Size markers and isolate abbreviations are as described in Fig. 1. No hybridization was detected to DNA from isolate D7 for any of the enzymes tested

deletions among the six isolates. The probes pSTL10, pSTL46, and pSTS197 did not hybridize to different subsets of isolates with any of the six enzymes (example in Fig. 3). pSTL10 did not hybridize to isolates Rusa3 or MT9; pSTL46 did not hybridize to isolate D7; and pSTL197 did not hybridize to isolates MT8, MT9, Rusa3 or D7.

Although each restriction endonuclease possessed a six-base recognition sequence, the frequency of polymorphic probe-enzyme combinations was not evenly distributed among the six enzymes. *Eco*RV and *Pst*1 were significantly ($\chi^2 = 5.62$ and 4.09, respectively; $p < 0.05$) more efficient at detecting RFLPs than *Dra*1. *Eco*RV detected the most polymorphisms (84% of probes polymorphic), followed by *Pst*1 (80%), *Eco*R1 (78%), *Xho*1 (73%), *Bam*H1 (70%), and *Dra*1 (53%). The size of *S. tritici* inserts in the plasmid probes ranged from 0.5 kb to 3.1 kb. The average insert sizes for probes used from the 0.5–1.3 kb and 1.3–2.4 kb fragment libraries were 960 bp and 1,780 bp, respectively. Eleven percent (2/18) of clones containing inserts less than 1.3 kb in length hybridized to repetitive sequences, and 44% (7/16) of clones containing inserts between 1.3–3.1 kb hybridized to repetitive sequences (Table 2). Most of the probes that hybridized to repetitive sequences were clustered in the 1,250–

**Table 2.** Frequency distribution of probes that detected repetitive DNA sequences in *Septoria tritici*. Probes were plasmids with inserts of random cloned fragments of nuclear DNA from *S. tritici*

| | Insert size (kb) | | | |
|---|---|---|---|---|
| | 0.25–0.75 | 0.76–1.25 | 1.26–1.75 | 1.76–3.25 |
| No. repetitive | 1 | 1 | 5 | 2 |
| Total | 8 | 10 | 7 | 9 |
| Frequency | 0.13 | 0.10 | 0.71 | 0.22 |

1,750 bp insert group, in which 71% (5/7) of the probes hybridized to repetitive sequences.

## Discussion

The major goal of this study was to develop a set of DNA probes that could be used to study genetic variation in *S. tritici* and to identify individual pathotypes by DNA fingerprinting. We identified two classes of probes that will be useful for these purposes. The 26 probes that hybridized to unique sequences will be useful for measuring linkage disequilibrium, comparing allele frequencies, and estimating rates of recombination among different isolates and populations. We have chosen a subset of ten of these probes, in which polymorphic fragments are between 0.4–10 kb in size, that will be used to estimate population parameters in *S. tritici* populations. The DNA fingerprinting probes will provide useful tools to study gene flow, genetic relatedness, and the geographical distribution of genetic variation in *S. tritici* populations. DNA fingerprinting is of interest to plant pathologists because it allows accurate monitoring of the movement of particular pathotypes over large geographical areas in field experiments. Fingerprinting also will allow measurement of the rate of spread of particular virulent pathotypes in natural pathogen populations, and thus will be useful in studies of host-pathogen coevolution. Using the probe pSTL29, we found length variation between *S. tritici* isolates collected from different lesions on the same leaf (manuscript in preparation). This is evidence that genetic variation in *S. tritici* populations may be distributed on a very fine scale, as was found in the plant pathogen *Rhynchosporium secalis* (McDonald et al. 1989).

RFLPs based on random, genomic probes have not been studied extensively in fungi, so it is difficult to predict a "typical" level of polymorphism. However, data from the present study suggest that *S. tritici* has a higher level of sequence variation than other fungi studied to date. Our collection of random, genomic probes detected RFLPs with a 97% efficiency (33/34 probes polymorphic with at least one enzyme). In the plant pathogenic fungus *Bremia lactucae*, 38% (76/201) of genomic probes were polymorphic (Hulbert et al. 1988). In the mushrooms *Agaricus brunnescens* and *A. bitorquis*, 70% (7/10) of random nuclear probes detected intraspecies variation with a single restriction enzyme (Castle et al. 1987). RFLPs were found for 60% (6/10) of nuclear probes used to compare seven strains of *Agaricus bisporus* (Loftus et al. 1988). R. Vilgalys (personal communication) found that 63% (5/8) of genomic probes detected length variation in *Pleurotus*. RFLP variation in other eucaryotes has been studied more extensively, but the amount of variation detected appears to be species-specific. A collection of random human genomic probes detected RFLPs with an efficiency of 32% (Schumm et al. 1988). Among *Brassica oleracea* accessions, approximately 65% of random clones were polymorphic (Figdore et al. 1988) while in lettuce 21% of genomic probes were polymorphic (Landry et al. 1987).

Our results suggest that a great deal of genetic variation exists among a small sample of isolates collected mostly from a single geographical area. The sexual state of *S. tritici* is now thought to be important for long-distance dispersal of the pathogen (Shaw and Royle 1989). The sexual cycle may also be an important source of genetic variation in *S. tritici* populations. Other *Septoria* spp. have shown high levels of genetic instability in culture which have usually been ascribed to parasexual recombination between unlike nuclei (Hooker 1957; Griffiths and Ao 1980). Newton (1988) suggested that a "balanced forward and backward mutation-induction system" could account for the instability observed in *Septoria nodorum*. Our investigation did not attempt to determine the mechanisms that produce and maintain genetic variation in *S. tritici*, but research now in progress will use RFLP probes to estimate rates of recombination in natural populations and to detect possible DNA rearrangements that can cause RFLP variation.

The data indicate that *S. tritici* contains fewer repetitive sequences than *B. lactucae*. Approximately 26% (9/34) of our genomic clones (ranging in size from 0.5–3.1 kb) contained repetitive DNA sequences. Hulbert et al. (1988) found that 30% of small genomic clones (0.4–1.0 kb inserts) and 90% of larger genomic clones (1.0–5.0 kb inserts) contained repetitive sequences. We did not find that the frequency of repetitive sequences increased proportionally with insert size. Instead, most of our repetitive probes were clustered in the 1.25–1.75 kb insert size range. Probes containing inserts larger than 1.75 kb hybridized to repetitive sequences at a lower frequency. The cause of this unequal distribution is unclear, though we suspect it may be due to sampling error. As data are collected from a larger number of probes, the proportion of large probes containing repetitive sequences may increase. The rDNA probe pBD4 detected only one polymorphism among the six isolates and six restriction enzymes tested. We consider this unusual given the high degree of variability found at other loci in *S. tritici* and the high levels of rDNA RFLP variability found in other eucaryotes (Arnheim 1983; Cluster et al. 1987; Jorgensen et al. 1987; McDermott et al. 1989). In a previous study using pBD4 as a heterologous probe in a pathogenic fungus, McDermott et al. (1989) found 20 fragment length variants among a sample of 163 *R.*

*secalis* isolates collected from a single field. A larger sample of isolates from several different countries currently is being screened for rDNA spacer variation.

Most RFLPs detected by our sample of probes appear to result from restriction site mutations or deletions. It is possible that the clones pSTS197, pSTL10, and pSTL46, which did not hybridize to different subsets of isolates, are homologous to sequences that have undergone extreme divergence in different *S. tritici* populations. We consider this unlikely, however, because we have already detected these non-hybridization polymorphisms among isolates collected from a single wheat field (manuscript in preparation). It is more likely that these probes correspond to sequences that have been deleted in different isolates. On the assumption that this is indeed so, and that the probes are a representative sample of genomic variation, it appears that deletions are a common source of genetic variability in *S. tritici.* Approximately 9% (3/34) of our clones detected deletions. Landry et al. (1987) found that 0.8% (1/123) of lettuce genomic clones detected deletions. Wijsman (1984) noted that 2.5% (4/159) of human RFLPs resulted from insertions or deletions. RFLPs caused by small deletions and insertions are common in fungal mitochondrial DNA (Taylor 1986). Our data provide evidence that deletions may be a common source of fragment length variation in nuclear DNA of some fungi.

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