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Length heterogeneity in ITS 2 and the methylation status of CCGG and GCGC sites in the rRNA genes of the genus *Peronosclerospora*

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Summary. The polymerase chain reaction (PCR) was used with primers complementary to conserved flanking sequences to amplify the internal transcribed spacer 2 (ITS 2) of the rDNA repeat units of five *Peronoscleropora* isolates, one each of *P. sorghi, P. maydis, P. sacchari* and two of *P. zeae*. In contrast to the situation found in most fungi that have been examined, length heterogeneity was evident in each sample. The rDNA composition of the amplified bands was confirmed by Southern hybridizations using an ITS 2 amplified from *P. sorghi* and cloned rDNA from *Neurospora crassa* as probes. Length heterogeneity was also detected in genomic DNA digests using the same probes. In addition to one dominant fragment for each isolate, there were several less frequent fragments of different sizes, and the isolate(s) for each species had a unique banding pattern for ITS 2. The absence of 5-methylcytosine residues in CCGG and GCGC sequences in the ribosomal genes of these four *Peronosclerospora* species was demonstrated by the production of identical banding patterns with ribosomal DNA probes following digestion of genomic DNA with *Msp*I and *Hpa*II, and by complete digestion with *Cfo*I.

Key words: *Peronosclerospora* – ITS 2 length heterogeneity – rDNA methylation

Introduction

The genes coding for 5.8 *s*, 17 *s* and 26 *s* ribosomal RNA in fungi are present as tandemly repeated units organized in a head to tail array (17 *s*–5.8 *s*–28 *s*) with 60 to 200 copies per haploid genome (Cassidy et al. 1984; Russell et al. 1984; Garber et al. 1988). The repeat unit is transcribed as a single 35 *s* rRNA precursor that is subsequently cleaved into its component rRNA molecules and internal transcribed spacer sequeces (Russell et al. 1976). Some species of fungi also include the 5 *s* rRNA within the rDNA repeat unit (Cassidy et al. 1984; Buckner et al. 1988), whereas other fungi code the 5 *s* rRNA elsewhere in the genome (Selker et al. 1981). A high level of conservation in the restriction endonuclease recognition sites has been observed in the coding (functional) region of rRNA genes for different species of fungi within one genus (Verbeet et al. 1983; 1984; Chambers et al. 1986). Intervening between the rRNA segments of the rDNA repeat are internal transcribed spacers (ITS) and between the transcriptional units there are non-transcribed spacer (NTS) sequences. It is in these spacer sequences where most variation exists when comparing different isolates, species, or genera of fungi.

Several members in the genus *Peronosclerospora* are very important plant pathogens. They can cause extremely destructive diseases on maize, sorghum and sugarcane, particularly in the tropical and subtropical regions of the world (Frederiksen and Renfro 1977). The identification of members of this group of fungi can be very difficult because the primary characteristics currently used to define *Peronosclerospora* species are the size and shape of the conidia and conidiophore, which are highly variable and, significantly, dependent on the host species or cultivar from which the pathogen was collected, the environmental conditions at the time of sporulation, the time of collection, the mounting fluid used during microscopic examination and the person making the observation (Williams 1984). In this paper, we report the presence of length heterogeneity of ITS 2 in the rDNA repeat units of representative isolates of four species of *Peronosclerospora*, and discuss the potential for using this heterogeneity in the identification of members of this group of biotrophic fungi. The absence of detectable methylation of cytosine residues occurring in CCGG or GCGC sequences in the rRNA genes is also reported.

Materials and methods

Sample collection. Samples of *P. sacchari, P. maydis,* and *P. zeae* were provided by Dr. M. R. Bonde, from cultures maintained in the

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USDA-ARS Foreign Disease-Weed Science Research Unit at Fort Detrick in Frederick, Maryland. The two *P. zeae* isolates were formerly designated *P. sorghi*-Thailand isolates, but on the basis of isozyme (Micales et al. 1988), epidemiology (Bonde et al. 1985), and DNA banding patterns (Yao 1991) have been reclassified as *P. zeae* (Yao 1991). A true *P. sorghi* isolate, pathotype 1, is from a local collection and is maintained in a greenhouse of the Department of Plant Pathology, Texas A and M University. The collection of fungal conidia and conidiophores followed methods described previously (Micales et al. 1988; Yao et al. 1990). Cultures of five other fungi, *Curvularia lunata, Rhizoctonia* sp., *Penicillium* sp., *Trichoderma* sp., and *Alternaria* sp., that had previously been isolated from sorghum and maize seeds were also available, and were used for comparison. The fungi were cultured on potato dextrose agar (PDA) medium for 8 days and the mycelium from four PDA plates provided the sample for DNA isolation.

DNA isolation and manipulation. Total genomic DNA of fungi was isolated as previously described (Yao et al. 1991). Standard DNA manipulations were performed according to Sambrook et al. (1989), unless otherwise stated. Plasmid pRW614a, containing an entire 6.3 kb ribosomal DNA repeat unit from *Neurospora crassa*, was kindly provided by Dr. Peter Russell. A 1.3 kb fragment that includes the 5.8 *s* rDNA and ITS 2 of *P. sorghi* was produced by using the polymerase chain reaction. Both the 6.3 kb insert of pRW614a and the 1.3 kb fragment of 5.8 *s* rDNA and ITS 2 were collected in low melting point agarose gels following electrophoresis and used as probes in Southern hybridizations. Plasmid DNA isolation followed the method of Zhou et al. (1990).

DNA blots and hybridization. Restriction enzyme digestions were carried out according to the recommendations of the supplier (Promega, Madison, Wis.). Following digestion, total genomic DNA of *Peronosclerospora* species was subjected to electrophoresis in a 0.9% agarose gel in TBE buffer at 3 V/cm overnight. Alkaline blotting of DNA from the gel to a GeneScreen Plus hybridization membrane (Dupont) utilized the method developed by Reed and Mann (1985). After transfer, the membranes were rinsed in SSC for 5 min, then dried in a vacuum oven at 80 °C for 2 h. DNA hybridization procedures were the same as previously described (Yao et al. 1991). The 6.3 kb ribosomal DNA clone of *N. crassa* and the 1.3 kb fragment which includes the 5.8 *s* and ITS 2 sequences of *P. sorghi* rDNA were labeled with ^{32}P-dATP in low melting gel using random primers, following the protocol recommended by the supplier (United States Biochemical, Cleveland, Ohio). After hybridization, membrane strips were exposed to Kodak Blue Brand X-ray film at −70 °C with an intensifying screen. The exposure time was 30 min to 12 h.

Polymerase chain reaction. All reagents for PCR except primers were purchased from Promega. A 100 µl PCR mixture contained reaction buffer, 200 µM of each deoxynucleotide triphosphate (dNTP), 1.0 µM of each primer, 25 ng of template DNA, and 2.5 units of *Taq* DNA polymerase, and was overlaid with 100 µl of mineral oil. The primers for amplification of internal transcribed spacers (ITS), as described by White et al. (1990), were synthesized in the Advanced DNA Technology Laboratory in the Biology Department, Texas A and M University. The sequences and locations of the four ITS primers used are diagrammed in Fig. 1. A negative control containing all reagents except for the template was run with each primer set. The mixtures were subjected to PCR amplification using a Perkin-Elmer Cetus Thermal Cycler for 25 cycles. The temperatures and times used for PCR were as follows: in the first cycle, the mixtures were denatured at 94 °C for 4 min, followed by 1 min at 50 °C and in separate experiments at 37 °C for primer annealing, and 1 min at 72 °C for primer extension; the conditions for the following 24 cycles were the same as the first, except that the time for DNA denaturation was reduced to 1 min. The final extension time for PCR amplification was 7 min at 72 °C.

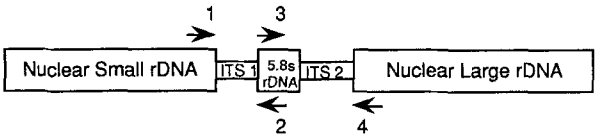


Fig. 1. Locations of PCR primers for internal transcribed spacers on the nuclear rDNA map. The *arrowheads* represent the 3′ end of each primer. The sequence for each primer is written in the 5′–3′ direction. Primer 1, TCCGTAGGTGAACCTGCGG; primer 2, GCTGCGTTCTTCATCGATGC; primer 3, GCATCGATGAAGAACGCAGC; primer 4, TCCTCCGCTTATTGATATGC

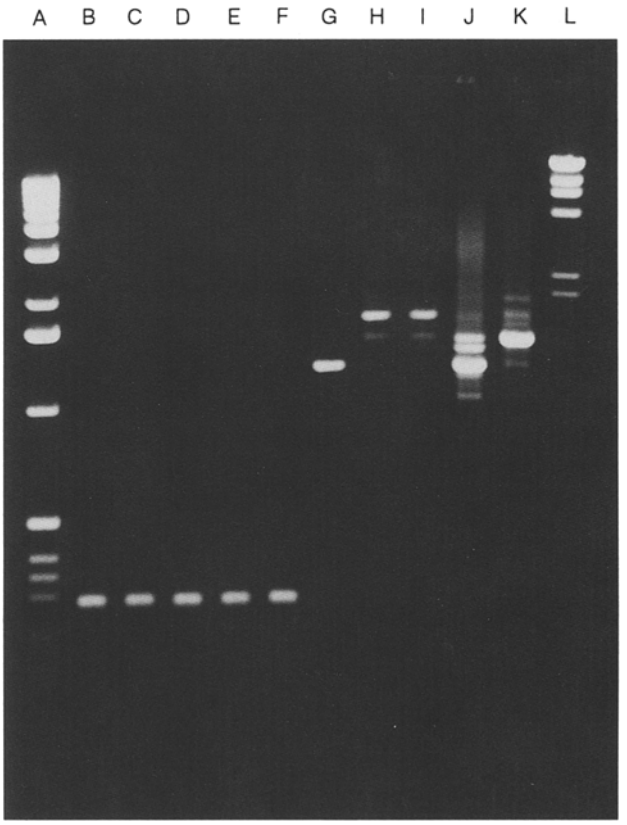


Fig. 2. PCR amplification of ITS 1, ITS 2 and the 5.8*s* rDNA sequences of five isolates of *Peronoscleorspora*. *Lane A*, 1 kb ladder; *lanes B–F*, ITS 1, amplified using primers 1 and 2; *lanes G–K*, ITS 2 and the 5.8*s* rDNA, amplified with primers 3 and 4. For each of the primer sets, the lanes contain from left to right *P. sorghi* pathotype 1, *P. zeae* Thai isolate 1, *P. zeae* Thai isolate 2, *P. maydis* Malang isolate and *P. sacchari* Guangxi isolate, respectively. *Lane L* is a lambda/*Hin*dIII digest

Results

The results of the polymerase chain reaction amplification of the internal transcribed spacers for single isolates of *P. sorghi*, *P. sacchari*, *P. maydis* and two of *P. zeae*, along with the five comparison fungi, *C. lunata*, *Rhizoctonia* sp., *Penicillium* sp., *Trichoderm* sp. and *Alternaria* sp., are shown in Fig. 2 and 3. Identical amplification products were visible whether the primer annealing temperature was 50 °C or 37 °C. It is obvious that the internal transcribed spacer 2 of *Peronosclerospora* species is very different from that of the other fungi. Compared to control fungi for which PCR products were visible, the ITS

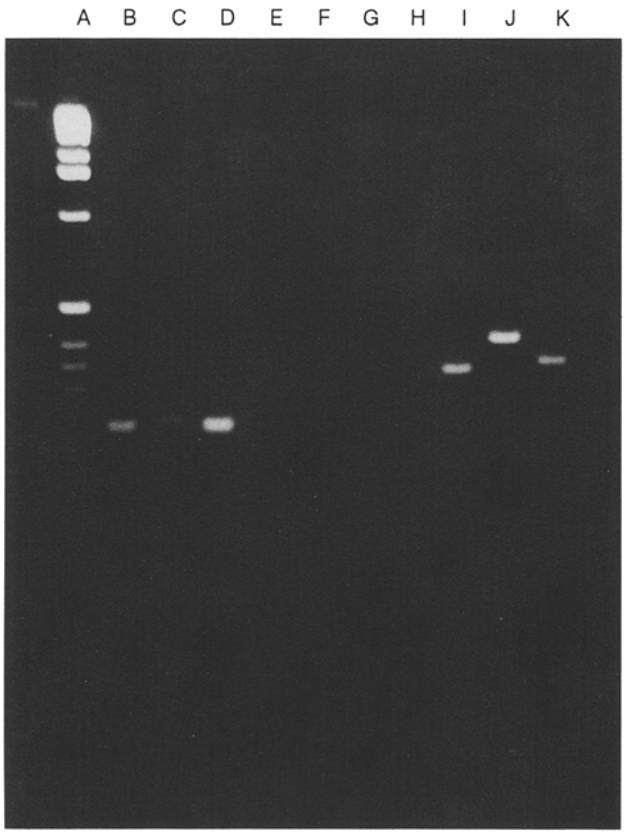


Fig. 3. PCR amplification of ITS 1, ITS 2 and 5.8*s* rDNA sequences of five control fungi. *Lane A*, 1 kb ladder; *lanes B–F*, ITS 1, amplified using primers 1 and 2; *lanes G–K*, ITS 2 and 5.8*s* rDNA, amplified with primers 3 and 4. For each of the primer sets, the sources of DNA for the five lanes are *C. lunata, Alternaria* sp., *Penicillium* sp., *Rhizoctonia* sp., and *Trichoderma* sp., respectively

2 of *Peronosclerospora* species is much longer; in addition to one dominant fragment for each isolate, there are several fragments of different sizes that amplify to a lesser extent. The isolates for each species have a unique banding pattern, and four banding patterns were identified. (The polymorphic ITS 2 bands for *P. sorghi* are visible after hybridization to the major band, Fig. 4a, lane B).

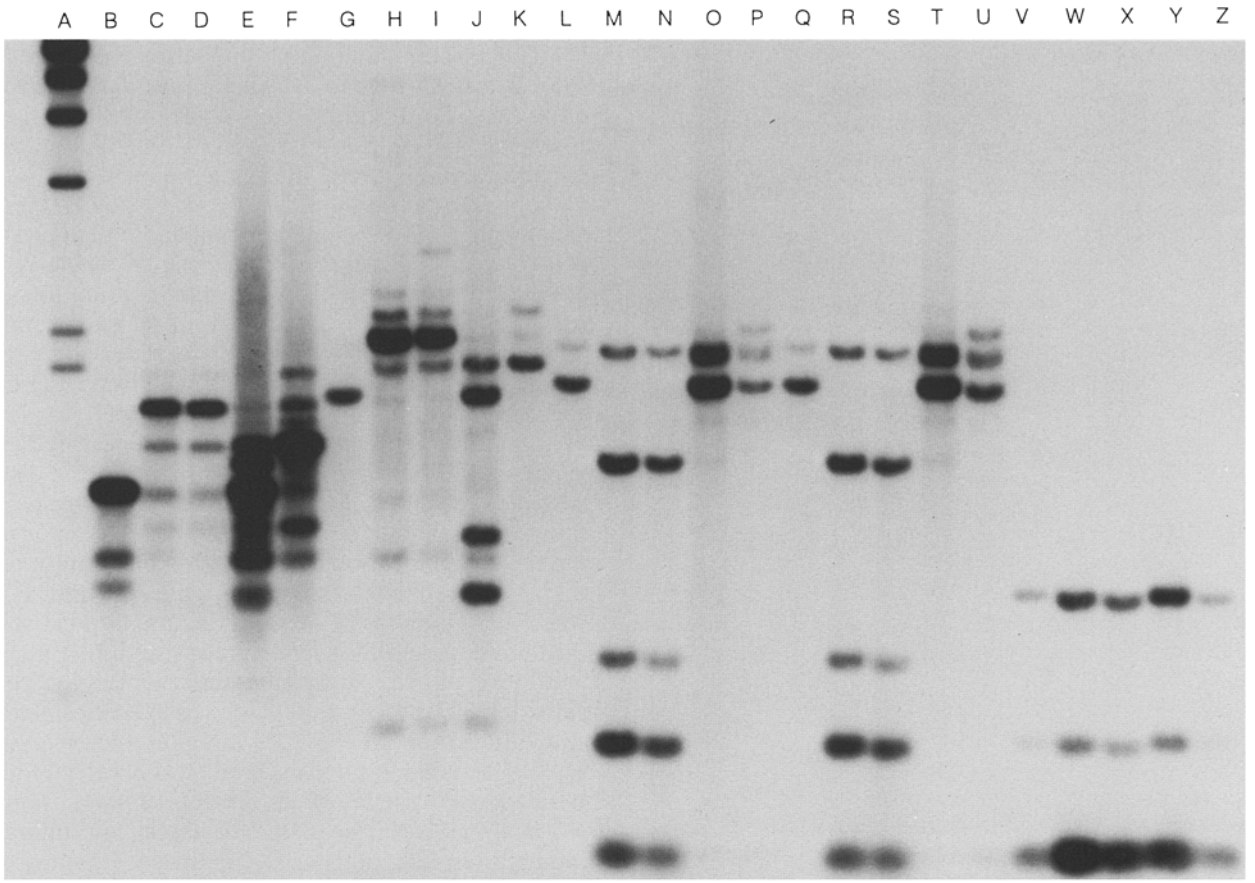
The dominant 1.3 kb fragment produced by PCR amplification of *P. sorghi* DNA was used to probe a Southern blot membrane containing 0.25 µl of the PCR products from each of five *Peronosclerospora* isolates and *Sau*3AI-, *Msp*I-, *Hpa*II-, or *Cfo*I-digested total genomic DNA of each isolate. The results show that all fragments amplified using ITS 2 primers share sufficient sequence homology to hybridize to the probe, supporting the contention that they reveal heterogeneity in ITS 2. The strengths of the hybridization signals also confirm that the ITS 2 bands are all amplified from *Peronosclerospora*, and not from DNA of any contaminating species that may have been present.

Length heterogeneity of *Peronosclerospora* ITS 2 sequences was also supported by Southern hybridization to genomic digests. As would be expected, the size and number of hybridizing fragments varied for the restriction enzymes used, depending on the location of restriction sites in or on either side of ITS 2. For example, *Sau*3AI cut outside this region so that the corresponding bands are larger but the banding patterns are compatible with the PCR results (Fig. 4, A). Where the 1.3 kb probe hybridized to a larger restriction fragment, such as the upper fragments detected in lanes M, N, and O after digestion with *Msp*I (Fig. 4a), the same fragments hybridized to a complete ribosomal DNA probe (Fig. 4b). Restriction enzyme *Cfo*I gave an identical banding pattern with three fragments for every isolate, one of which is very faint and most likely has only a small region of overlap with the probe. The presence of identical bands and the strong hybridization signal for the smaller band suggest that the recognition sites for *Cfo*I are conserved in ITS 2 and 5.8 *s* rDNA, and imply that the size variability in ITS 2 results from different numbers of subrepeat units with specific sizes. The other three enzymes produced distinct patterns for each species. Size heterogeneity in restriction fragments that include ITS 2 is most clearly demonstrated in the genomic DNA blots by the hybridization of the 1.3 kb ITS 2 probe to three bands, all larger than 1.3 kb, in the *Sau*3AI digests of *P. zeae* (Fig. 4a, lanes H and I). When the same blot was hybridized with the 6.3 kb ribosomal gene of *N. crassa*, all four enzymes resulted in unique RFLP banding patterns for each species of fungi, but hybridization to the ITS 2 region was very weak (Fig. 4b, lanes B-F). The Southern blots obtained following restriction with enzymes *Eco*RI, *Hin*dIII, *Bam*HI, *Pvu*I and *Pst*I revealed only one large, non-migrating band for all isolates in the trial. This implies that there are no restriction sites for these five enzymes in the ribosomal genes of these four *Peronosclerospora* species (data not shown). All five isolates of *Peronosclerospora* species produced a 300 bp-long ITS 1, which is comparable in size to that of the control fungi (Figs. 2 and 3).

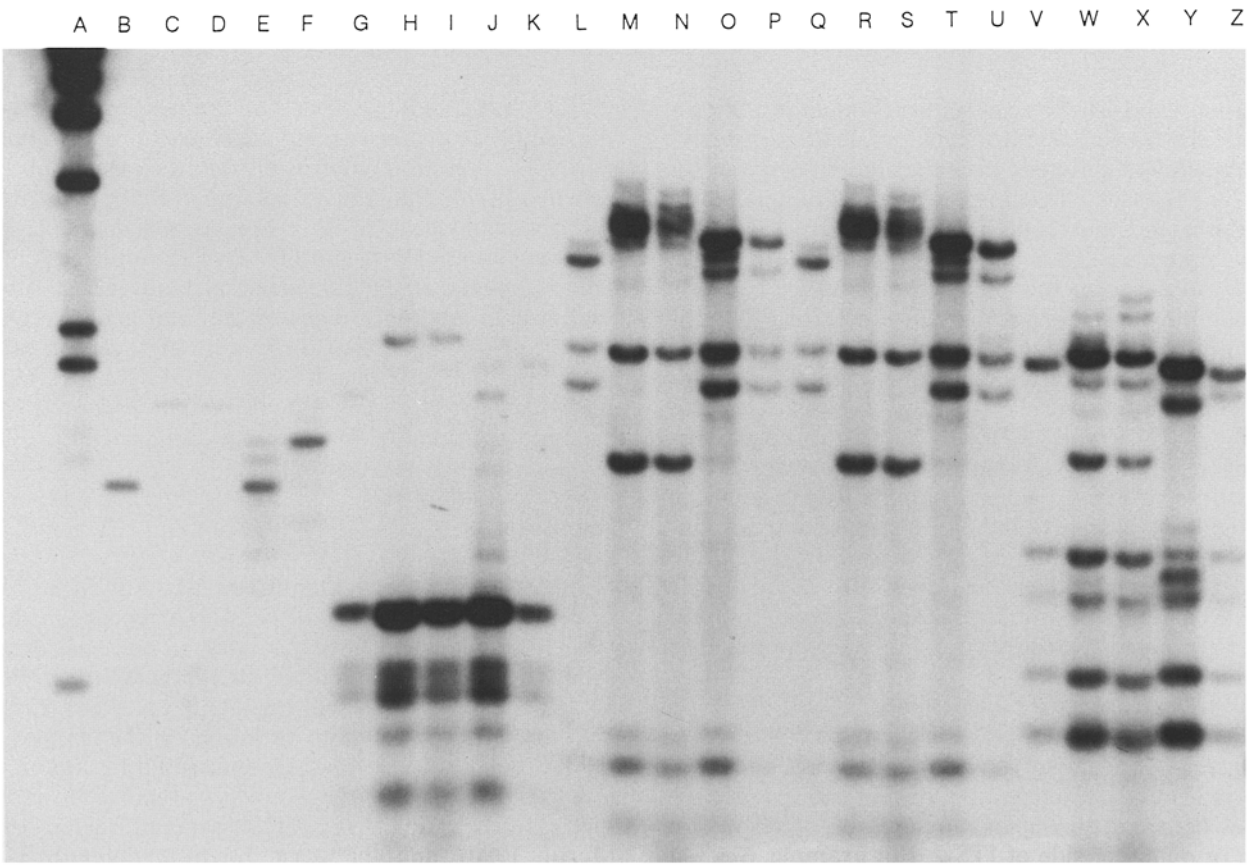
The methylation of cytosine residues occurring in CCGG and GCGC sequences in the ribosomal genes of the five *Peronosclerospora* isolates was examined by comparing the digestion patterns produced by the restriction enzymes *Msp*I, *Hpa*II and *Cfo*I on Southern blots, using the ribosomal gene of *N. crassa* as well as the 1.3 kb fragment (5.8 *s* rDNA and ITS 2) of *P. sorghi* as probes. Since *Msp*I and *Hpa*II gave identical banding patterns for each isolate, and *Cfo*I digested the total genomic DNA completely, it seems that there is no methylation of cytosine residues in either CCGG or GCGC sites in the ribosomal genes of the five *Peronosclerospora* isolates used in the trial (Fig. 4).

Discussion

Changes in restriction endonuclease recognition sites and in the length of the NTS of the rDNA repeat unit have been observed in different isolates of one species and different species of one genus for a number of fungi (Petes and Botstein 1977; Russell 1984; Garber 1988). Length heterogeneity attributed to variations in the NTS of the rDNA repeat unit in the same individual isolate of one fungus is rarely seen, having only been reported in several species of the genus *Pythium* (Klassen and Buchko 1990; Martin 1990), and the yeast *Yarrowia lipolytica* (Van


A B C D E F G H I J K L M N O P Q R S T U V W X Y Z

A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B

Heerihuizen et al. 1985), though it is common in plants and animals (Bach et al. 1981; Rogers et al. 1986). Specht et al. (1984) found that the specific differences in the length of the rDNA repeat unit in different strains of the fungus *Schizophyllum commune* are due to insertions of 200 or 400 bp of DNA at a single site, and Buckner et al. (1988) further demonstrated that these strain-specific length polymorphisms in rDNA are most likely located in the ITS of the rDNA repeat unit. However, the possibility of one or more introns within the 18 *s* coding region has not been excluded.

In the present study, the universally conserved flanking sequences within the ribosomal genes were used as primers for PCR amplification of the ITS regions and the 5.8 *s* rDNA of isolates representing four *Peronosclerospora* species. Confirmation that ITS 2 was amplified was obtained when it was demonstrated that both a presumed ITS 2 probe and a known rDNA probe hybridized to the same restriction fragments in Southern blots of genomic DNA. In each isolate, length heterogeneity was revealed by the presence of multiple bands following amplification and the pattern was different except for the two *P. zeae* isolates. Since the possibility of length polymorphisms in the 200 bp of the 5.8 *s* rDNA fragment in fungi is remote, this observation can almost certainly be attributed to length variations in the ITS 2 of the rDNA repeat units in the genus *Peronosclerospora*. Even the shortest ITS 2 sequences amplified for all four *Peronosclerospora* species are considerably longer than have been demonstrated for other species. However, observations reported in an abstract (Wiglesworth et al. 1991), stating that the 1.5 to 2 kb length of the ITS 2 of *P. maydis* and *P. sacchari* limits the ability to directly compare their sequences to those of other oomycetes, agrees quite well with the results of the present study.

The two isolates of *P. zeae* from Thailand produced identical banding patterns after PCR amplification of their ITS 2 and 5.8 *s* rDNA. DNA from six more isolates of *P. sorghi* collected in Texas (pathotype 3), and in Africa (North Nigeria, South Nigeria, Zimbabwe, Zambia and Mozambique) also showed identical banding patterns to the *P. sorghi* pathotype 1 isolate on an ethidium bromide-stained gel after PCR amplification with primers 3 and 4 (data not shown). This was unexpected because DNA was extracted from pooled conidia and coniodiophores produced on the infected leaves collected in the field for each isolate in the trial. This suggests that the banding pattern of ITS 2 and 5.8 *s* rDNA is unique for each species. If verified, this will be very useful for identification of this group of important plant pathogenic fungi.

Russell et al. (1985) showed that methylated cytosine residues were concentrated in the rDNA region of *N. crassa* and that 5 mC residues were commonly found in the non-transcribed spacer. In the present study, the restriction enzyme *Cfo*I and isoschizomers *Msp*I and *Hpa*II were used to examine the distribution of 5 mC in the rDNA regions of isolates of four *Peronosclerospora* species. Restriction fragments that include rDNA were detected on Southern blots using a radioactively labeled 6.3 kb fragment that includes the entire ribosomal repeat unit of *N. crassa* as a probe, and with a 1.3 kb probe that includes the 5.8 *s* rDNA and ITS 2 of *P. sorghi*. No evidence for methylation of 5 mC in CCGG or GCGC sequences was found. Another plasmid, which only has the insert of the NTS of *N. crassa*, did not show any hybridization with the DNA of *Peronosclerospora* species, which means that the methylation status of cytosine residues in the CCGG and GCGC sequences of the NTS in the ribosomal genes of *Peronosclerospora* species would not be revealed by the probes used in this study.

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Fig. 4 A, B. Southern-blot autoradiographs of DNA from: *lane A*, lambda/*Hin*dIII; *lanes B–F*, PCR-amplified ITS 2 and 5.8*s* rDNA; *lanes G–K*, *Sau*3AI digests of genomic DNA; *lanes L–P*, *Msp*I digests of genomic DNA; *lanes Q–U*, *Hpa*II digests of genomic DNA; *lanes V–Z*, *Cfo*I digests of genomic DNA. Lanes left to right in every group contain DNA from *P. sorghi* pathotype 1, *P. zeae* Thai isolate 1, *P. zeae* Thai isolate 2, *P. maydis* Malang isolate and *P. sacchari* Guangxi isolate, respectively, hybridized to the radioactively labeled 1.3 kb fragment of 5.8*s* rDNA and ITS 2 of *P. sorghi* (A), and the 6.3 kb fragment of the rRNA gene of *N. crassa* (B)

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