Sequence of ITS-2 Amplified from Pearl Millet Downy Mildew Samples

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Introduction

Sclerospora graminicola is the causal agent of downy mildew of pearl millet (*Pennisetum glaucum*). It is an obligate oomycete that reproduces asexually to produce sporangia (that release motile zoospores) and sexually through the production of soil-borne oospores. At least 15 different pathotypes of *S. graminicola* have been defined in India based on the use of 7 host cultivar differentials (Thakur 2000). Considerable effort has been applied to the development of resistant cultivars, especially in India. Widespread use of hybrids resistant to specific pathotypes has generated changes in the pathogen population. Similarly, cultivars identified as resistant in India often turn out to be susceptible in African locations at or very soon after introduction. Consequently, studies to examine genetic variability in the pathogen have also been undertaken. In India, where samples can be collected and expanded by re-infection on greenhouse grown plants in isolation, relatively pure *S. graminicola* DNA can be extracted and used for analysis of variability. Under these conditions it has been possible to identify RFLPs that show association with unique pathotypes (Sastry et al. 1995) and to cluster isolates in groups related to mating type based on AFLP patterns (Singru et al. 2003). AFLP utilizes PCR to amplify subsets of restriction fragments, leading to highly repeatable and easily scored banding patterns that have revealed a high level of polymorphism among *S. graminicola* isolates sampled across India. DNA-based comparisons of African to Indian isolates would be of great interest for assessing the relatedness of the comparative populations and perhaps of value in predicting resistant pearl millet genotypes for local deployment. However, phytosanitary concerns and regulations prevent the international transfer of viable *S. graminicola*. Also, the limited facilities available in Africa prevent the expansion of field-collected samples in isolation under greenhouse conditions. Thus for this study, only asexual spores collected from single infected leaves and fixed in alcohol were available for DNA extraction for samples from Africa; the tiny amounts of DNA available from these samples dictated that PCR-based techniques be employed for comparisons. Here we show that targeted DNA amplification revealed contamination of some samples, but also allowed sequence and phylogenetic comparisons among others.

Materials and methods

DNA samples were provided directly for isolates from India. Sporangial samples collected on patches of cheesecloth from infected plants in Burkina Faso, Mali, Nigeria and Niger were placed in microfuge tubes filled with alcohol for shipment to the US. On arrival, the spores were collected by centrifugation, lyophilized and DNA extracted using a Phytopure® (Nucleon) kit, as directed.

Nested PCR was used to amplify internal transcribed spacer (ITS) region 2 of the ribosomal RNA encoding genes (Fig. 1). The first primers (ITS1 & 6) were in

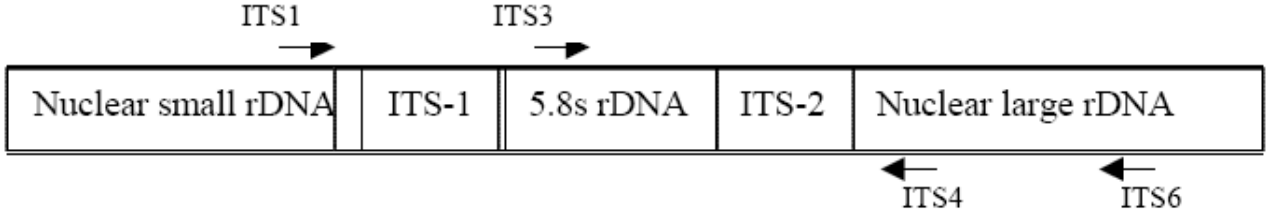

Figure 1. Locations of PCR primers for internal transcribed spacers on the nuclear ribosomal DNA map.

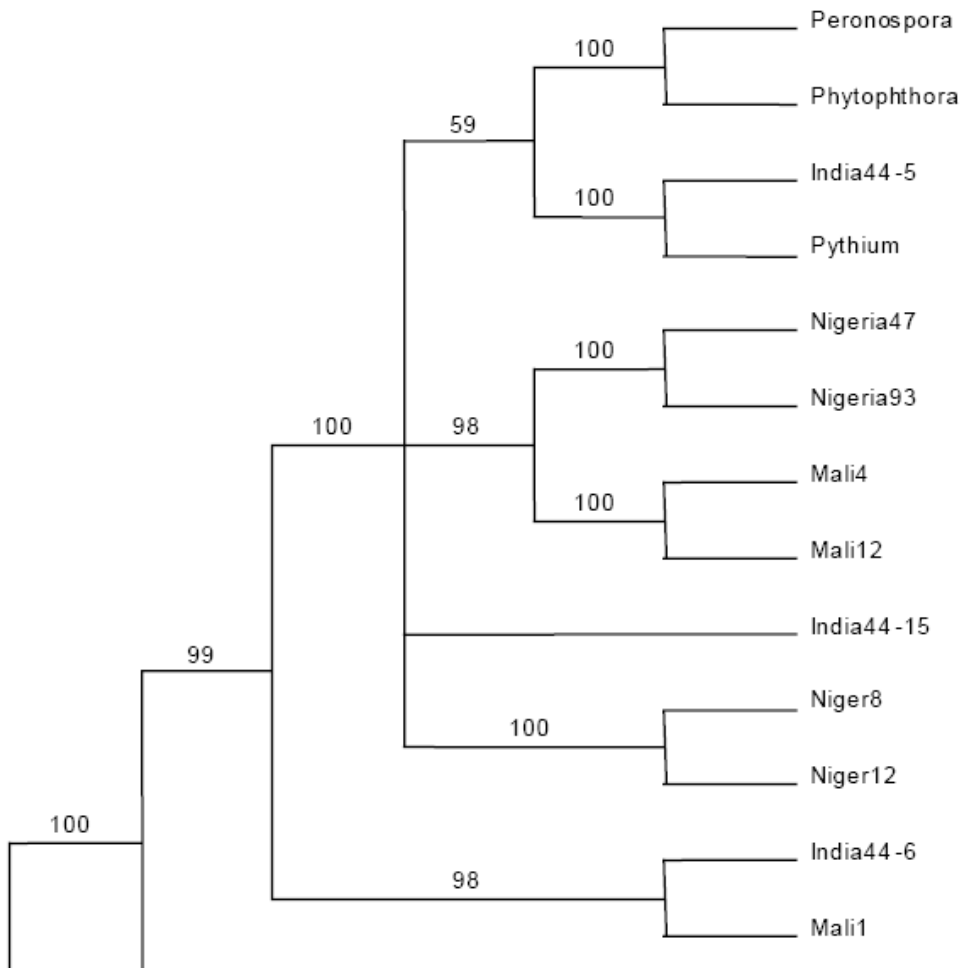

Figure 2. A bootstrap consensus of a parsimonious tree based on 400 base-pair sequences amplified from the ITS-2 region of rDNA from samples as indicated. Numbers above represent the bootstrap values. The outgroup used for constructing this tree was *Cladosporium herbarum.*

conserved regions of the large and small subunit rRNA and the second pair (ITS 3 & 4) flanked just the ITS-2 segment.

ITS-1: 5'-TCCGTAGGTGAACCTGCGG-3'
ITS-3: 5'-GCATCGATGAAGAACGCAGC-3'
ITS-4: 5'-TCCTCCGCTTATTGATATGC-3'
ITS-6: 5'-CACTTTTCAAAGTGCTTTTCATCTTTC-3'

Standard PCR reactions were used, with the following parameters: initial denaturing for 3 min at 94°C followed by 28 cycles of 1 min for denaturing at 94°C, 1 min for primer annealing at 50°C and 2 min for primer extension at 72°C. The product from the first PCR reaction was diluted 1:100 and 1 µL of it was substituted for template DNA in the second PCR reaction. The amplified ITS-2 band was cloned into using TA-cloning (Invitrogen) and sequenced at the TAMU Gene Technologies Laboratory. Phylogenetic analysis used PAUP* v4.0.

Results and discussion

DNA sequence differences can be used all the way from defining kingdoms to unique identification of individuals. When DNA amplification is involved, as is often the case when the amount of starting material is limited, either

very pure samples or primers of known specificity are essential to avoid amplifying contaminating DNA. In the case of *S. graminicola* samples from Africa neither of these conditions was met, meaning that differences detected by RAPDs or AFLP would be questionable. The transcribed spacers of rDNA genes are present in multiple copies in each genome. Their non-coding function means that changes are unlikely to have detrimental effects on survival. On the other hand, concerted evolution tends to maintain a common sequence in the multiple copies within a given species (Atkins and Clark 2003). Amplification of the ITS 2 region from presumed *S. graminicola* samples gave products of about 400 base pairs that were sequenced. BLAST searches of GenBank showed those from India and some African samples to be similar to ITS 2 from other oomycetes, including *Peronospora* spp. and *Phytophthora* spp. Other African samples were clearly derived from other species. For example, Mali sample 1 was almost identical to *Cryptococcus flavus* (P value for mismatch = 0) and Nigeria sample 69 matched ITS 2 of *Pseudozyma paraantarctica* at $P=e^{-160}$. A tree made using ITS 2 of *Cladosporium herbarum*, an ascomycete, as the "outgroup" is shown in figure 2. It shows the similarity of most isolates to other oomycetes, that in general the isolates collected from nearby locations are most similar and that isolates from India are similar to those from African countries. However, it also shows that neither of the ITS 2 sequences amplified from samples collected in Burkina Faso is from *S. graminicola* and thus serves to emphasize the caution that must be used when PCR products are the basis for measurements of diversity when pure samples are not available.

References

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