Genetics

Random Amplified Polymorphic DNA Markers: A System for Identifying and Differentiating Isolates of *Colletotrichum graminicola*

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ABSTRACT

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Colletotrichum graminicola, the causal agent of anthracnose of sorghum, is a highly variable fungal pathogen. The high degree of variability hinders breeding for resistance and optimal deployment of cultivars. The extent and nature of this variability can now be characterized by random amplified polymorphic DNA marker analysis, a simple and fast technique that permits differentiation among isolates.

Anthracnose of sorghum, caused by *Colletotrichum graminicola* (Ces.) G. W. Wils., is often the major constraint to production in regions where the crop is grown extensively, notably the tropics and subtropics (5). The genetic variability of the pathogen (1,2) increases the difficulty of breeding for resistance and deploying available cultivars effectively. A quick method for characterizing genotypes within the pathogen population would aid sorghum research not only by providing up-to-date information on the pathogen's genetic diversity, but also by allowing one to follow the effects of various cultivars on the genetic structure of the pathogen population. By regularly sampling diseased tissue at a number of fixed sites, researchers could follow the shifts in the genetic makeup of the pathogen population, which would provide a dynamic picture of the interactions between host and pathogen genotypes.

Random amplified polymorphic DNA (RAPD) markers (6)

are discrete fragments of DNA defined in the genome by the annealing of 10-base primers at either end and amplified exponentially by the polymerase chain reaction (PCR) (3). The primers must be fewer than 2,500 bases apart to produce visible products under the conditions used for PCR. These markers provide a mechanism for swiftly and easily characterizing differences between isolates in terms of polymorphisms of primer-defined DNA fragments. Ideally, each band amplified by the use of random primers would represent a specific locus in the genome, and alleles would produce easily identified bands of a different size. In that case, the RAPDs could readily be used as linked tags for other genes that are more difficult to screen. In fact, "null" alleles (i.e., the failure to detect a band at all) may be more common for changes at a specific locus. As long as a PCR-amplified segment of a defined size produced by a particular primer represents a specific locus in the genome of the test species, RAPD markers have great potential for use in population analysis, even when alleles cannot be identified. Evidence of this was provided by Williams et al (6), who excised amplified DNA fragments from a gel, labeled them with ^{32}P, and used them as hybridization probes to detect segregation of restriction fragment length polymorphisms. Their results demonstrated that high confidence levels could be assigned to RAPDs as genetic markers.

Polymorphisms may be of three types: fragment length, presence-absence of a band, and band intensity. Length polymorphisms are the result of insertion into or deletion from the amplified fragment between the primer-binding sites, whereas presence-absence polymorphisms are caused by the destruction or creation of one of a pair of the primer-binding sites. Many areas in the genome contain a nonbinding sequence that would, with a little alteration, serve as a primer-binding site. Mutation of, insertion into, or deletion from this site may convert the sequence into a valid primer-binding site. In this case, a new band would appear (if the criteria of proximity and suitable orientation of the corresponding primer-binding site were satisfied). Conversely, changes of sufficient magnitude to a functioning primer-binding site would lead to the disappearance of a band, because the fragment that has that site as one of its ends would no longer be amplified exponentially. The possibility of a third type of polymorphism, band intensity, arises through the variation in brightness of bands observed within a gel. Possible causes include differences in template sequence copy number and varying degrees of mismatch between primer and binding site. A technique that uses image processing to reject bands below a minimum brightness threshold (see Materials and Methods) has been developed, and it further differentiates among isolates by using the band intensity polymorphism.

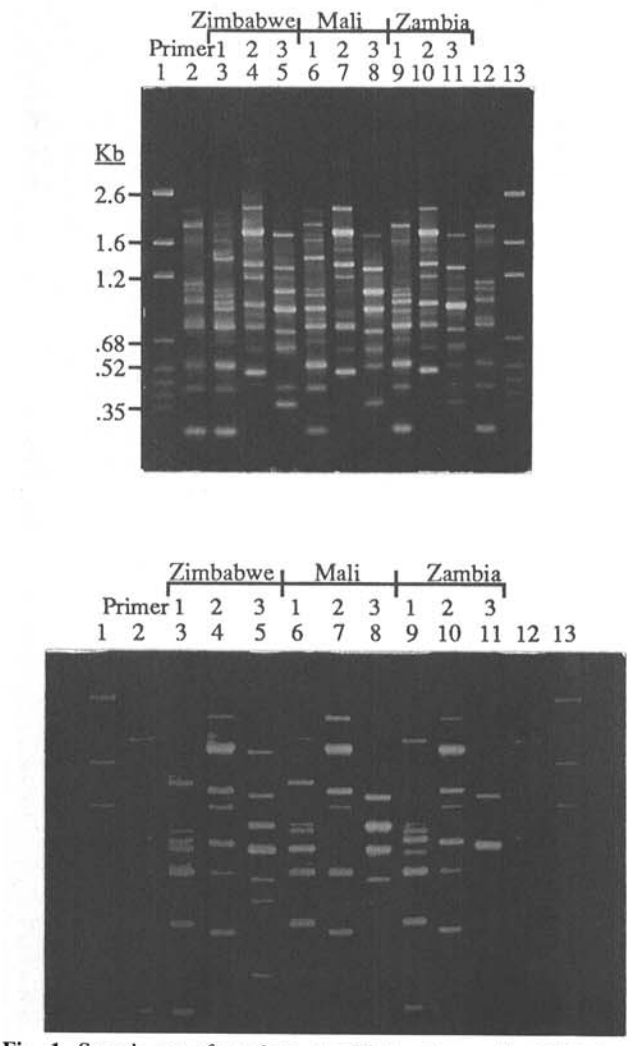

Fig. 1. Sample set of random amplified polymorphic DNA banding patterns for three African isolates of *Colletotrichum graminicola* that shows the effect of image processing. Top panel, before image processing; bottom panel, after image processing. Lanes 1 and 13, size marker (pGEM). 2 and 12, Standard primer-isolate pair; lowest molecular weight band provides brightness threshold. 3–5, Zimbabwean isolate (primers 1–3). 6–8, Malian isolate (primers 1–3). 9–11, Zambian isolate (primers 1–3).

MATERIALS AND METHODS

Three sorghum isolates of *C. graminicola* were collected from different regions of Africa, the United States, India, and Puerto Rico; three Johnson grass (*Sorghum halepense*) isolates were also collected from Texas. After single sporing, each isolate was grown in a shake culture medium (sucrose, yeast extract, and casein hydrolysate) for 4 days at about 25 C. The mycelium was harvested, and excess water was removed. Then, the mycelium was ground under liquid nitrogen in a mortar and pestle. Genomic DNA was extracted by a modified phenol extraction protocol (A. B. Livore, C. A. Blakey, and C. W. Magill, *unpublished*). Final concentration of the DNA solution (in distilled, deionized water) was adjusted to 50 ng/μl.

Each reaction volume, determined empirically at 23.0 μl, was composed of 0.1 mM each dNTPs, 2.0 μM primer, 50 ng of template, 1 unit of *Taq* polymerase (Promega, Madison, WI), 2.3 μl of *Taq* buffer (10×), and water to 23.0 μl.

The thermal cycler (COY Tempcycler; COY Corporation, Ann Arbor, MI) was programmed for an initial melt for 4 min at

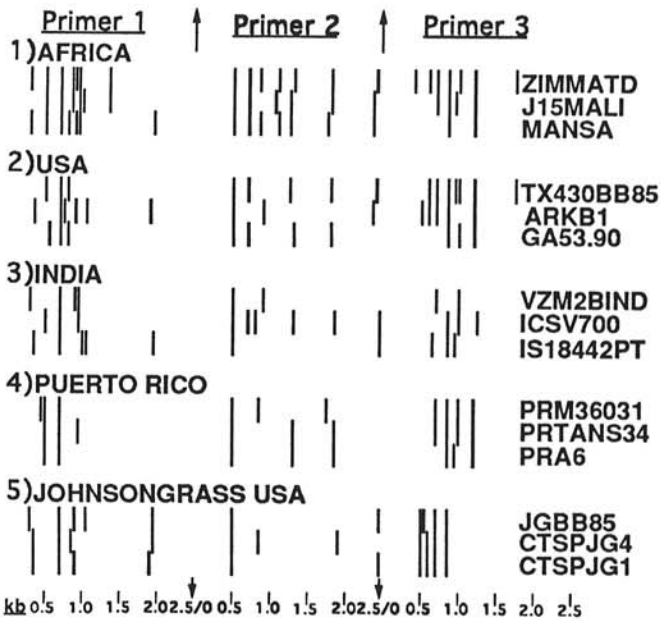

Fig. 2. Random amplified polymorphic DNA signatures obtained by using three different primers for 15 isolates of *Colletotrichum graminicola* (divided into five groups of three). The usual electrophoresis orientation of top to bottom has been rotated 90 degrees for ease of comparison.

94 C, followed by 40 cycles of 1 min at 94 C; fast ramp to 43 C for 1 min; slow ramp (8 seconds per Celsius degree) to 72 C for 2 min.

Each isolate was amplified by using three different 10-base primers in three separate reactions; the primers were designed and synthesized in-house. The sequences were 5′TCGGGAGGGT; 5′ACGCTCAAAC; and 5′AGAATCGGGG.

The amplification product (18 μl) was injected into the wells of a 2% agarose gel and electrophoresed at 50 V for 8 h, along with a molecular size marker (pGEM). The gel was stained with ethidium bromide, washed for 30 min in distilled water, and photographed on a transilluminator.

Although many bands were clear, some had intensities that were borderline, which led to difficulties in deciding whether to record them. To resolve this problem, we included a standard isolate-primer combination (replicated twice) in each amplification run. The products were electrophoresed in two lanes of the same gel used for the other isolates. The standard isolate-primer pair resulted in a bright band of about 330 bases. Using an image processing system (SEMIPS, Texas A&M University, Electron Microscopy Center), we measured the mean intensity of this band in each of the two lanes and used this measurement as the threshold value below which bands would be rejected. The image of the gel was then redefined in terms of this threshold, resulting in a new image in which bands bright or brighter than the standard remained, whereas those that were dimmer disappeared (Fig. 1). For each band, we then measured migration distances (from the mean of two readings) by using the same image processing system and converted the distances into fragment sizes.

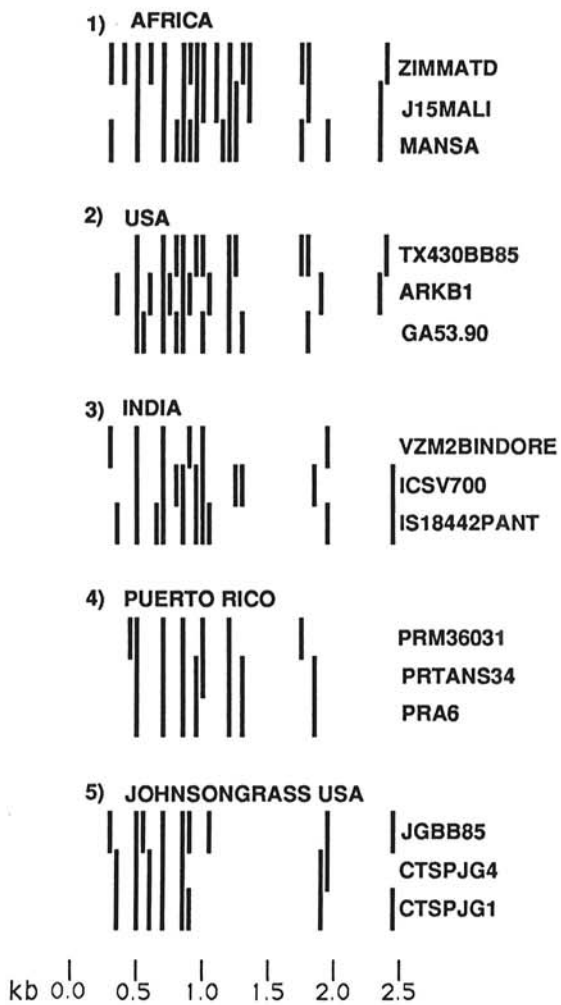

Fig. 3. Composite random amplified polymorphic DNA signatures for the same set of isolates in Figure 2. Signatures were obtained by pooling fragment size data from primers 1–3.

Each amplification reaction was performed on two separate occasions (at least one day apart) to verify consistency of the products. The regime of temperature cycles outlined above provided a high degree of consistency, and although the intensity of some bands varied from run to run, this variation was not significant after image processing.

RESULTS AND DISCUSSION

The processed images yielded fragment size data that were used to make a barcode chart; each isolate had a visual "signature" that allowed ready comparison among isolates (Fig. 2). Combining the data from each of the three primers (fragments with the same length that were products of different primers were counted only once) gave a composite signature for each isolate (Fig. 3). Comparison of signatures shows that each isolate is different in length of fragments amplified (position of each band) and in numbers of each type of fragment (number of bands per primer per isolate), indicating a high degree of variability in the fungus. The data used to generate the composite chart (Fig. 3) show that the groups all have a different mean number of fragments that can be amplified by using this set of three primers. They are, in descending order, Africa (13.3); United States (10.3); India (8.7); Johnson grass USA (8.0); Puerto Rico (7.3). Although there are no obvious differences in patterns among isolates from Africa, the United States, and India, all the signatures from Puerto Rico lack fragments at the long end of the size range, and the Johnson grass isolates show a large gap between 1.1 and 1.9 kb. This indicates that some isolates have characteristic signatures based on geographical origin. This technology may, therefore, have potential applications in quarantine and related fields.

We obtained an estimate of intragroup diversity for each primer by determining the percentage of all loci that were polymorphic

TABLE 1. Percentage of polymorphic loci within groups of isolates

	Primers			
Group	1	2	3	All
1[a]	44	56	50	53
2	50	44	50	53
3	50	37.5	50	43
4	12.5	25	25	20
5	44	19	17	30

[a] 1, Africa; 2, United States; 3, India; 4, Puerto Rico; 5, Johnson grass USA.

TABLE 2. Percentage of loci shared between groups of isolates for each primer

	Primer 1				Primer 2				Primer 3			
Group	1[a]	2	3	4	1	2	3	4	1	2	3	4
2	19				44				58			
3	44	31			19	19			33	33		
4	19	12.5	19		25	12.5	19		42	42	33	
5	31	31	37.5	6	12.5	6	12.5	12.5	25	33	17	17

[a] Group 1, Africa; 2, United States; 3, India; 4, Puerto Rico, 5, Johnson grass USA.

TABLE 3. Percentage of loci shared between groups of isolates[a]

	Group			
Group	1[b]	2	3	4
2	50			
3	37	37		
4	27	27	23	
5	27	30	30	10

[a] Composite data from primers 1–3 were used.
[b] Group 1, Africa; 2, United States; 3, India; 4, Puerto Rico; 5, Johnson grass USA.

(within each group) for that primer ("locus" is synonymous with the presence of a band in one or more isolates). We did the same analysis with pooled data from all three primers (Table 1). Although some of the data from individual primers lack consistency, the conclusion from composite data of all three primers (confirmed by Fig. 3) is that the Puerto Rican population is the most homogeneous; heterogeneity increases in the following order: Puerto Rico, Johnson grass USA, India, Africa, and United States. The homogeneity of the Puerto Rican isolates may reflect a geographically isolated gene pool, whereas the limited diversity of isolates from Johnson grass suggests that the ubiquitous nature of this weed favors a more clonal pathogen genotype.

We investigated the potential importance of geographical origin by determining what proportion of loci for each primer separately (Table 2) and for a composite of pooled primer data (Table 3) was shared between groups. Table 2, like Table 1, shows that drawing conclusions from the data generated by any one primer, although certain trends are consistent across all three, would be unwise. The composite data of Table 3 show that the Puerto Rican isolates share the fewest loci with any other group, indicating the greater genetic distance of this population. The Johnson grass USA and sorghum USA isolates share fewer loci (30%) than would be expected if the hypothesis that Johnson grass acts as a reservoir of inoculum for sorghum was true (4). The greatest separation of all in terms of shared loci is, perhaps not surprisingly, between Puerto Rican *S. bicolor* isolates and Johnson grass USA (*S. halepense*) isolates.

In conclusion, we have shown the potential of RAPDs for identifying and differentiating isolates and have demonstrated the benefits of applying image processing techniques that use a band intensity standard for generating barcode signatures. Pooled, rather than single, primer data may be useful for certain applications.

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