Molecular Methods Aid in the Identification of Turfgrass Pathogens

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Rapid and accurate diagnosis of turfgrass diseases is imperative for successful disease management, but this is not always easy. A good example is the confusion surrounding the various patch diseases or disease complexes. Turf managers can either look at the symptoms and make an educated guess (this is correct many times), or they can send samples to a plant pathogen detection clinic, such as the University of Wisconsin Turfgrass Disease Diagnostic Laboratory (TDDL), where trained specialists have a variety of tools for identifying pathogens.

First they carefully inspect the turfgrass with the aid of the eye and microscopes for symptoms and for various diagnostic fungal structures. In many cases, the suspected pathogens (mainly fungi) are cultured on media specially designed to aid in identification. Unfortunately some fungi grow slowly and some fungi do not readily produce unique, identifiable diagnostic structures, such as spores, in culture. So these methods do not always lead to a timely and successful identification of the pathogen. New molecular techniques give some hope for faster and more specific diagnoses.

The molecular method the TDDL staff is currently working on to aid in the identification of turfgrass fungal pathogens is the polymerase chain reaction (PCR). PCR allows amplification (multiplication) of a specific region of a pathogen's DNA so that this DNA region can be used for identification. Without this amplification, which is over a million fold, the sample of DNA would be too small to use in any identification method.

DNA is the molecular blueprint of life. It uses four "letters" called nucleotides to form "words" that make up the instructions for all the proteins necessary to build an organism. In order to better identify fungal turfgrass pathogens we are studying a region of the DNA known to be quite different in different fungal species. This region (Figure 1) contains the DNA instructions (genes) for making some of the components (such as ribosomal RNA) of ribosomes, cellular structures that function in the synthesis of proteins.

In Figure 1, the instructions (genes) for these ribosomal components are labeled small rDNA, 5.8s rDNA, and large rDNA. All organisms have these very same ribosomal genes. In between these genes, however, are areas of DNA, labeled ITS A and ITS B in Figure 1, that are quite different in different fungal species but very similar in individual cultures of the same species. We may be able to identify fungi by amplifying one of these areas (ITS A) by polymerase chain reaction and comparing the size and DNA sequence of this area with those in known fungi.

An example might help explain this variation in this ribosomal gene region. If we compare the percent DNA similarity for one variable region (ITS A) between two sorghum plants (data from the GenBank—a national library of DNA sequences), the percent DNA similarity is >95%, just what is expected for individuals of the same species. Now, if we compare

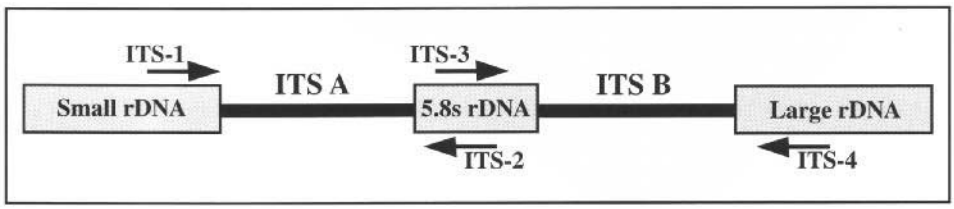

FIGURE 1. Diagram of the ribosomal gene region showing the location of the ITS polymerase chain reaction primers (e.g. arrow labeled ITS-1) used for amplification of fungal turfgrass pathogen DNA. The conserved regions, the genes, are the small, large, and 5.8s rDNAs, and the variable regions of DNA between the genes are ITS A and ITS B. The DNA fragments amplified are the regions between ITS-1 and ITS-2 (ITS-A), ITS-3 and ITS-4 (ITS-B), and ITS-1 and ITS-4 (White et al. 1990. PCR Protocols. Pp. 315-322.). (See Figure 2 for a specific example).

the DNA sequence of this region of sorghum with that from another monocot species such as bluegrass, the percent DNA similarity is only 63%. If we compare the same regions for sorghum and an even more distantly related plant such as soybean (a dicot, data from GenBank), the percent DNA similarity is even less, 46%. Thus, the more unrelated two species are, the greater the differences in the DNA sequence for this region.

When we started to study turfgrass fungi, we wanted to see if two isolates (individual cultures) of the same species would have nearly identical DNA sequences in the ITS A region. Dr. Randy T. Kane, plant pathologist with the Chicago District Golf Association, sent us two isolates of the anthracnose fungus, *Colletotrichum graminicola.* One isolate was obtained from bentgrass and one from annual bluegrass. As expected, the percent similarity was high, 98%. This fungus is a member of the Ascomycete group. Comparison of this same region in the anthracnose fungus and in another member of this same general group, the dollar spot fungus *Sclerotinia homeocarpa,* gave a DNA similarity of 56%. When we compared the anthracnose fungus to the brown patch fungus *Rhizoctonia solani,* a fungus in a different major group, the Basidiomycetes, the DNA similarity was 37%. Thus, as in the plant example, the more distantly related the fungi are, the greater the DNA sequence differences in this region are.

From these limited comparisons, it is evident that the differences between species of turfgrass fungal pathogens are very great, and this information can be used for developing new rapid diagnostic methods.

How do we obtain this DNA sequence data? First we must amplify the DNA from the ribosomal region (Figure 1) by the method mentioned earlier, polymerase chain reaction. To do this you must first know something about the nucleotide sequence, or "letter/word" order, of the region you are interested in. The polymerase chain reaction requires the help of a pair of primers, which are short DNA segments (about 20 nucleotides or letters) that will attach to either side of the region to be amplified (Figure 1). In this case the primers are identical to parts of those ribosomal genes that are the same in all species. The primers direct the amplification of the variable region (ITS A) between them.

So far we have used this new technology for two cases of diagnosis at the TDDL. The first case was that of an isolate of an unknown snow mold fungus called unknown #7 from Plum Lake Golf Club in Sayner, WI. This same fungus was observed on several courses in the Vilas County area and was also found on samples from the Fox River Valley. It had very large, dark sclerotia, four times the size of gray snow mold fungus and so we knew it was not the gray snow mold fungus or the speckled snow mold fungus. One possibility was the snow scald fungus, *Sclerotinia borealis,* which has been reported in Canada and Wisconsin.

For comparison, we needed a known culture of this fungus and it was obtained from Dr. Drew Smith, a retired plant pathologist at Saskatoon, Canada. To kill the fungus before it was sent to us from Canada, Dr. Smith microwaved the sclerotia. DNA was extracted from these *S. borealis* sclerotia and from those of the unknown #7, and polymerase chain reaction amplification was performed.

The different sizes of DNA fragments produced by polymerase chain reaction were separated by gel electrophoresis and made visible with UV light. They show up as light bands as seen in the electrophoresis gel in Figure 2. The size of each DNA fragment is determined by comparing it to DNA fragments of known size. Lane 1 (Figure 2) contained a mixture of several known sizes of DNA

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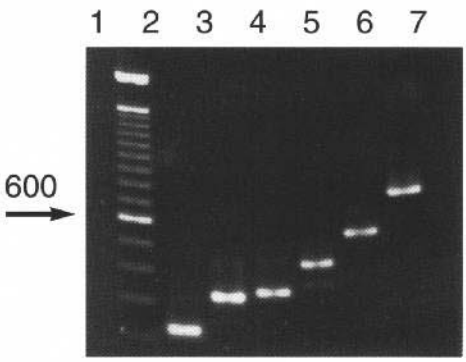

FIGURE 2. Photograph of an electrophoresis gel separating DNA fragments of different sizes. The DNA fragments are the light bands on the black background. Lanes 2-7 contain DNA from different polymerase chain reactions. Lane 1 has a mixture of DNA fragments of known size. These differ in size by 100 nucleotides or "letters" and the smallest one is 200 nucleotides. Compare the sizes of the DNA fragments in lane 2 and lane 3. They are different, so these two fungi, the unknown #7 and S. borealis, are different. Lane 1 = the known sizes of DNA used as a standard, Lane 2 = ITS A DNA from S. borealis, Lane 3 = ITS A DNA from unknown #7, Lane 4 = ITS B DNA from S. borealis, Lane 5 = ITS B DNA from unknown #7, Lane 6 = ITS1-4 DNA from S. borealis, Lane 7 = ITS1-4 from unknown #7.

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fragments. These were applied at the top edge of the gel and an electric current was turned on. The smallest fragment moved to the bottom and the larger ones were spread out in between. The DNA fragment from the ITS A region of the known *S. borealis* (lane 2) is smaller (moves farther down the gel) than that from the unknown #7 (lane 3). Since these two fungi had different sizes of DNA fragments from the ITS A region, they are not the same fungus, so we suspected that unknown #7 was a different pathogen.

The next step was to determine the sequence of nucleotides ("letters") in these two DNA fragments of the ribosomal variable region ITS A (Figure 1). This would be the final proof of the difference between unknown #7 and the standard *S. borealis.* After the DNA was purified, it was taken to the Biotechnology Center on campus where it was sequenced (nucleotide order determined). The percent DNA similarity for this region in these two fungi was only 42%, so they are very different fungi and probably belong to two different major fungal groups, the Ascomycetes for *S. borealis* and the Basidiomycetes for unknown #7.

These molecular techniques showed absolutely that unknown #7 is not the snow scald pathogen *(S. borealis)* and that it is in the major group of fungi, the Basidiomycetes, which contains the gray snow mold fungus. Currently, Steve Millett, a graduate student in Department of Plant Pathology, is determining how closely related unknown #7 fungus is to the gray snow mold fungus.

The second case in which polymerase chain reaction was helpful involved fungus isolate 96-112 from University Ridge Golf Course, Verona, WI. This fungus had hyphae characteristic of *Rhizoctonia solani,* the causal agent of Rhizoctonia brown patch, and when first grown on culture media, its growth pattern was similar to cultures of *Rhizoctonia solani.* After two weeks, however, the culture developed small orange balls (sclerotia) about the size of cabbage seeds. This is not typical for Rhizoctonia solani.

We decided to use polymerase chain reaction to help unravel this mystery. We extracted DNA from the fungus 96-112 and from a culture of *R. solani,* and used polymerase chain reaction to obtain the ITS A fragments from both fungi. These fragments were sequenced and the percent DNA similarity between the two was found to be 46%, so we knew that these two fungi are not closely related. We currently think that this fungus, isolate 96-112, is a *Rhizoctonia zeae.* Very little research has been done with *R. zeae* and it may respond differently to environmental factors than the brown patch fungus does. Several other isolates of this *R. zeae*-like fungus were isolated in July 1997 and they were not associated with typical brown patch symptoms. In one case, the symptoms looked like a necrotic spot caused by Pythium sp., and in another situation this fungus was associated with dollar spots against which the DMI fungicides had not been effective.

Polymerase chain reaction methods have assisted TDDL staff in their efforts to understand which turfgrass pathogens are present in Wisconsin. They provide a way to determine whether or not fungi that look alike really are alike by giving a view at the molecular level, the DNA sequence. We plan to expand this procedure to include a collection of standard sequences of the ITS A region for the common turfgrass pathogens found in Wisconsin. Also, it appears that because the ITS A region is very different between all the major turfgrass fungi, it will be possible to develop specific and rapid detection methods for identification of fungal pathogens in plant tissues.

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