Research *You Can Use*

Bermudagrass DNA Fingerprinting

This powerful tool can be used to distinguish genetic differences that are important in protecting plant patents.

BY MICHAEL P. ANDERSON AND YANQI WU

The fingerprinting of plant, animal, and human DNA (deoxyribonucleic acid) has been practiced among researchers and forensic scientists for many years, especially garnering widespread attention from notorious criminal cases involving DNA evidence. DNA fingerprint analysis is powerful and capable of distinguishing one individual from another. Each of us has a unique DNA pattern, as do plant species and plant varieties.

DNA DIFFERENCES

All organisms, including grasses, have identifiable characteristics. These characteristics make an organism unique from all others. Physical characteristics in bermudagrass, such as leaf texture or leaf color, are obvious and readily discernable. However, some characteristics require detailed measurements, while others are more qualitative in nature. Some distinguishing features can be observed with little or no training, while others need close inspection by trained and experienced personnel. Many subtle differences among closely related bermudagrasses cannot be readily distinguished visually. Another method is necessary to differentiate these bermudagrasses: DNA fingerprinting.

Differences among organisms are coded by their DNA, which is a very long molecule made up of a specific sequence, in linear order, of four distinct chemicals called nucleotides. If human DNA were represented by single letters standing for each distinct nucleotide (adenine, cytosine, guanine, and thymine) on a blank page, the length of the alphabetic sequence would run at least to one million pages, enough to fill 1,000 large volumes.

The DNA sequence dictates the look of an organism and how it responds to the immediate environment, and it is different for every organism. Consequently, the DNA sequence can be used to distinguish one organism from another. DNA fingerprinting is nothing more than a sophisticated technique to sample an organism's DNA sequence, projecting the differences as a kind of *bar code* for ready identification and comparison.

Most DNA fingerprinting depends on a technique known as PCR or *polymerase chain reaction*. PCR was developed in the mid-'80s to efficiently amplify specific segments of DNA many-fold. The PCR technique uses short DNA segments composed of anywhere from 6 to 20 nucleotides known as primers, which are complementary to segments of the target DNA. The primers figuratively scan for matches in the target DNA sequences. Once a match is found, then amplification of that segment begins. If there are many matches, many segments will be amplified.

This mixture of amplified segments, known as *amplicons*, can be separated on an electrophoretic gel system, which effectively sieves amplicons based on size. The gel is stained with fluorescent dyes to reveal what looks like a banding pattern or a bar code. Multiple primers can be used to scan different portions or the total genomic DNA, revealing additional bar coding. Fingerprinting with many primers is capable of differentiating even the most closely related of all organisms. Thus, while two bermudagrasses may be physically indistinguishable from each other, the DNA fingerprinting can highlight the intrinsic differences in their DNA by using PCR-based techniques.

All organisms can be fingerprinted and their DNA patterns stored and analyzed. Analysis of the banding pattern is performed using a variety of statistical techniques known as *cluster analyses*. The data are inputted in the form of presence or absence of a particular PCR amplicon or *electrophoretic band* and cluster analysis analyzes the data and connects those organisms that show similar patterns. However, to be effective, there must be enough similarities, as well as differences, in the pattern to reveal relationships among all tested organisms.

A number of fingerprinting techniques exist. These techniques differ in the ability to differentiate organisms, the amount of labor required, the extent of automation available, the expense of use, and the nature of the specific targeted DNA segments. AFLP (Amplified Fragment Length Polymorphism), DAF (DNA Amplification Fingerprinting), SSR (Simple Sequence

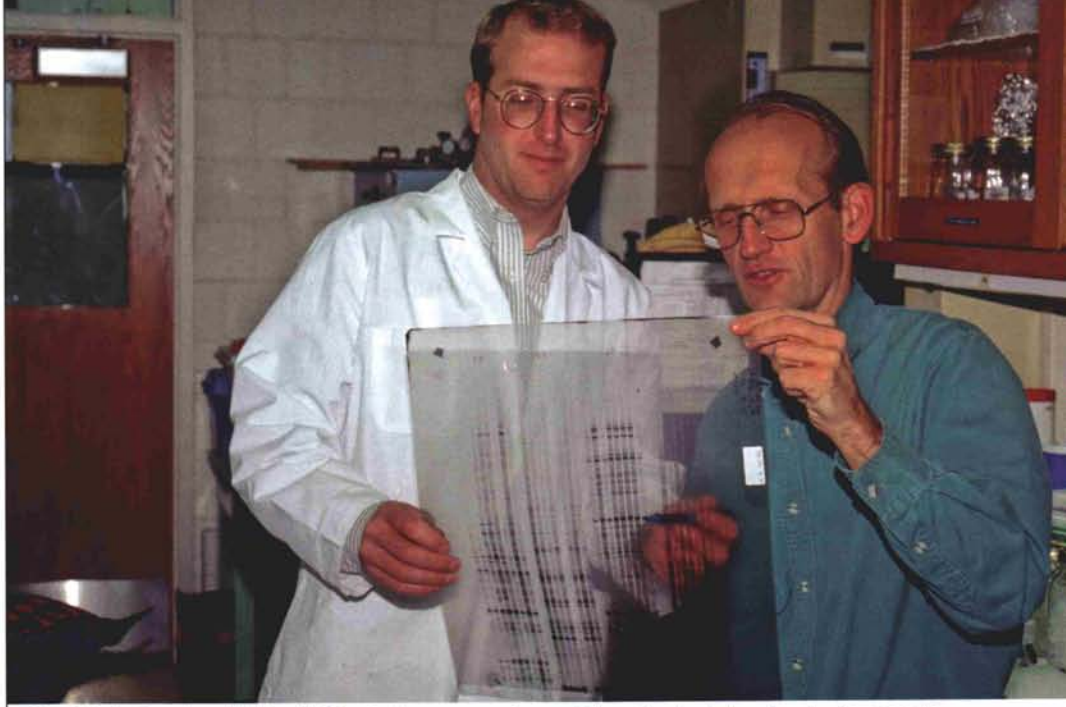
Repeats), and RAPD (Random Amplification of Polymorphic DNA) are a few of the more commonly used techniques used to fingerprint DNA. All of these utilize PCR to amplify segments of DNA based on the DNA sequence. Sophisticated and expensive commercial packages and instrumentation exist to automate and increase the resolution of the fingerprinting procedure.

HOW IS DNA FINGERPRINTING USED?

We have used DNA fingerprinting to look at the genetic relationship among a wide range of bermudagrasses. Some of the first work highlighted the differences among high-quality commercial cultivars and select bermudagrasses found in germplasm collections. Caetano Anolles et al[2] surveyed 13 bermudagrass cultivars, including African, common bermudagrass, and several interspecific hybrids for genetic relatedness using DAF. Results showed that DNA fingerprints were easily distinguishable, and the analysis showed clear genetic relationships among all bermudagrass varieties.

To probe the limits of the ability to distinguish bermudagrasses, we fingerprinted Tifway and its irradiation-induced mutant Tifway II, which presumably differed in one or a few nucleotide changes in the DNA sequence. In order to differentiate these very closely related varieties, we found it necessary to use 81 distinct primer combinations to find a one-band difference among all 81 fingerprints.[2] From this early work, it was clear that investigators can differentiate and draw genetic relationships even among the most closely related bermudagrasses.

Breeders often collect from around the world a wide range of plant introductions in the hope of finding specific genetic traits that may be put to productive use. The genus *Cynodon* (bermudagrasses) is comprised of 9 species.[4] Oklahoma State University is home to a worldwide collection of bermudagrass varieties and plant introductions that was initiated by the geneticist Jack Harlan. Charles Taliaferro, and more recently Yanqi Wu, two bermudagrass breeders at OSU, have added significantly to this collection, making it one of the most comprehensive collections of *Cynodon* germplasm in the world. Understanding the genetic relatedness among *Cynodon spp.* and varieties gives us a better understanding of the genetic makeup of the *Cynodon* genus.



Drs. Mark Gatschett (left) and Mike Anderson use advanced biotechnological and molecular genetic tools to understand the genetics of Oklahoma State University's bermudagrasses.

At times, doubts about the genetic identity of a particular variety surface. In previous work, our laboratory responded to the need to evaluate the widely used variety U3 for genetic fidelity.[1] U3 was an early success made up of bermudagrasses collected from golf courses in the southern U.S. in the 1930s. U3 showed moderate cold tolerance and fine-textured leaves and was a general improvement when compared to previous cultivars.

DNA fingerprinting was employed to distinguish the current labeled U3 from presumably authentic U3 collections assembled from around the country. Results showed that the currently labeled U3 varieties differed substantially from the presumably authentic U3 varieties. How these differences came about could not be addressed by the fingerprinting technique, but the research underscored the need for evaluating current varieties for genetic stability and purity. In addition, our research, as well as that of others,[9] has discovered a few other discrepancies between the historical pedigree claims of several varieties and their actual genetic relationships using fingerprinting techniques.

GAINING BERMUDAGRASS DIVERSITY WORLDWIDE

New bermudagrass germplasm has been and is now being collected and assembled into worldwide collections from many sources. There are areas where collections have only recently been assembled from specific locations such as southern and southeastern Asia. Recently, a number of bermudagrasses from China were added to the OSU germplasm collection. DNA fingerprinting using the AFLP technique was used to evaluate the diversity within this germplasm.

The Chinese collection seemed surprisingly diverse[7] and distinct from other bermudagrasses from other locations around the world.[6] Further work in our laboratory easily separated

the Chinese collection from all U.S. varieties tested. Overall, the work indicated a source of significant variation in the new Chinese collection, which may contain valuable genes for bermudagrass development. Additional diversity assessments need to be done on collections from India and other areas not previously surveyed.

The same techniques used for DNA fingerprinting are also used for molecular genetic analysis of specific traits. The goal here is to locate specific genetic elements or genes that contribute substantially to those traits. This is performed by first constructing populations with significant variation in a particular trait of interest, and then performing the DNA fingerprinting technique on members of the population to identify specific genetic elements that correlate with the expression of that trait. These genetic elements are visualized as unique bands on electrophoretic gels that appear to correlate with traits of interest. The bands are valuable in that they can serve as genetic markers, markers that are based on the DNA sequence rather than some physical characteristic of the plant.

Sophisticated computer software analysis can gauge the contribution of the DNA element associated with the marker to the genetic makeup of the phenotype. These markers can be used to increase the efficiency of selection in a process known as marker-assisted selection. Marker-assisted selection has been shown to be very effective in enhancing germplasm improvement in a variety of cropping systems.[3,5,8] Constructions and evaluation of mapping populations, and utilization of molecular genetic analysis, are major goals of the OSU bermudagrass team.

DNA fingerprinting of individuals within a population provides information concerning the genetic makeup of a population. The individual makeup of the population may change with time, depending on natural selection and genetic inflow from neighboring bermudagrasses. To observe these shifts, DNA fingerprinting can be used to document and track alterations in population makeup of seeded bermudagrasses under a variety of environmental conditions over time.

Oklahoma State University is home to a worldwide collection of bermudagrass varieties, much to the credit of Dr. Charles Taliaferro (pictured). Dr. Mike Anderson and his colleagues are using DNA fingerprinting techniques to understand the genetic relatedness and gene function of this important turfgrass.

PLANT PATENTING

DNA fingerprinting can have an impact in the area of patent protection. Many years of effort are expended to develop commercial varieties. Institutions have a substantial investment in developmental costs and are increasingly desirous of recovering some of those costs through plant variety protection and the collection of royalties from consumers. To support the patent application process, differences in morphology, cultural characteristics, and pedigree need to be presented in order to distinguish the proposed variety from those that are currently available. DNA fingerprinting is currently being used on a limited basis to document the genetic differences of new varieties in the patent process. Any infringement on the patent would have to use the DNA fingerprints and other characteristics to justify a patent infringement lawsuit. The process may be costly and subject to interpretation by experts, but it may be worth the effort when the stakes are large.

In summary, DNA fingerprinting is a valuable technology that is being used to assist producers, breeders, geneticists, and researchers in evaluating bermudagrass populations and germplasm for genetic diversity and background. Information from DNA fingerprinting techniques allows researchers to make informed decisions concerning progress in developing high-quality bermudagrass lines. DNA fingerprinting technology remains a powerful technique to assess the genetic diversity of bermudagrasses worldwide and to protect plant varieties from infringement. At OSU, our projects have been involved in using DNA fingerprinting to further bermudagrass improvement.

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CONNECTING THE DOTS

An interview with Drs. Michael Anderson *and* Yanqi Wu *regarding DNA fingerprinting.*

Q: *As you note, most of us are aware of DNA fingerprinting from criminal cases, but from a research perspective, how long have DNA fingerprinting techniques been available?*

A: Fingerprinting has been around for quite some time. In plants, some of the earliest DNA fingerprinting involved a technique known as RAPD, which was developed in the late 1980s. Bermudagrass fingerprinting did not take place until about the early 1990s. Advancement in fingerprinting mainly comes from the use of high-resolution instrumentation that greatly increases the accuracy and resolution of the technique, but at a cost. Instruments typically cost from $70,000 to $500,000, and the kits for doing the fingerprinting are expensive as well.

Q: *Do you think that at some time in the future, in order to receive a plant patent for a new cultivar, breeders will have to submit DNA fingerprint evidence that establishes this new cultivar as genetically unique from existing cultivars?*

A: Currently this is not a requirement, but it may be advisable. A patent contains morphological descriptions that distinguish the new cultivar from those already released. Whether it becomes a requirement depends on the decisions of the courts. Patents are granted for inventions (including new varieties) that are useful, new, and non-obvious. The DNA fingerprint establishes whether a new variety is new genetically, but it does not indicate utility. The utility factors must also be documented to distinguish the new variety.

Q: *You mentioned the use of primers to characterize specific genotypes. Is there a ballpark number of primers that are necessary to adequately characterize a genotype, or does it depend completely on the relatedness of the genotypes?*

A: It depends on how closely related your cultivars are and what technique you are using. When using AFLP, you may need from 8 to 14 primer pairs to differentiate bermudagrasses adequately. With DAF you need anywhere from 4 to 12 primer pairs. If the bermudagrasses are very divergent, 4 primers give satisfactory results. There is an additional technique known as mini-hairpin DAF or MHP-DAF, which scans the amplicons created in the first DAF reaction for additional differences. With this technique it is possible to distinguish even very closely related bermudagrasses with no more than 4 MHP-DAF primers.

Q: *You mentioned that the use of DNA fingerprinting can be used to protect plant patents. Have there been cases where DNA fingerprinting has been used and either found patent infringement or a situation where the plant cultivar was not what it was supposed to be?*

A: I am not aware of any at this time. Patent lawyers who specialize in plant variety protection would be aware of the legal history behind this particular question. In answer to your second question, yes, there are cultivars out there that claim a certain pedigree, but in reality they are not closely related to the described variety. I know of three such cases. The most obvious one is the U3 variety referred to in the article. It seems to me that if a company is selling a variety labeled as a protected variety and if the actual variety does not conform to the legal patent description, then that company's variety is open to legal challenge as far as ownership is concerned.

Q: *How important are the Chinese bermudagrass germplasm additions to the bermudagrass breeding effort at OSU? Are there specific traits in the Chinese bermudagrasses that have a high priority for introduction into new bermudagrass cultivars here in the U.S.?*

A: Currently there is great interest in screening this collection for productive traits. Some of the germplasm have desirable seed yield, seed quality, genetic color, and/or some other traits related to turf performance. Our best guess is that some of the collections will be incorporated into our existing breeding program and contribute substantially to future OSU releases.

Q: *To your knowledge, are most breeding programs using marker-assisted selection (MAS) as an integral part of cultivar development?*

A: Most breeding programs are not using marker-assisted selection for their variety development. Part of the impediment to using the molecular techniques is due to lack of training and expertise. However, experience in molecular aspects of breeding is becoming very common for the breeders coming out of graduate school, so I expect the trend towards the acceptance of molecular approaches to continue with a newer crop of breeders.

Jeff Nus, Ph.D., *manager, Green Section research.*

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Editor's Note: *This complete paper can be found at the USGA's Turfgrass and Environmental Research Online (http://usgatero.msu.edu).*

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