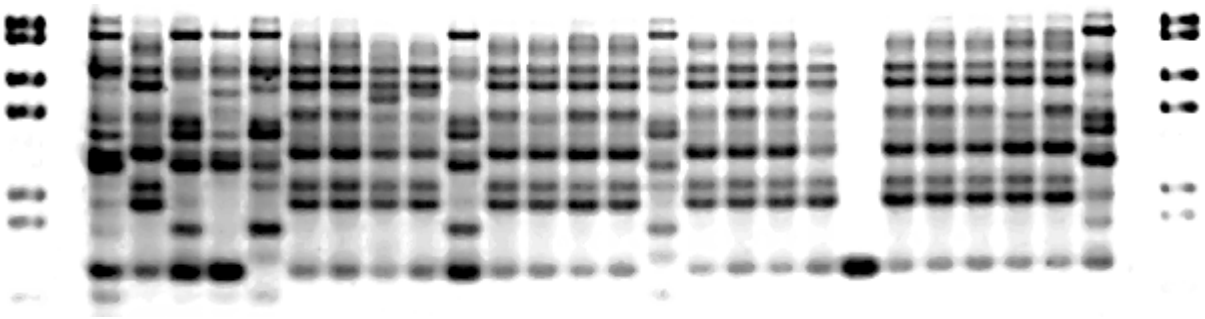
Distinguishing off-types in Tifway and Tifdwarf bermudagrass



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The problem

Improved hybrid bermudagrasses such as Tifway and Tifdwarf are supposed to provide consistent golf playing surfaces, but they often exhibit a mosaic of patches of different kinds of bermudagrass. This is especially severe on greens, but Florida golf courses also show the problem in fairways. To understand the problem, we completed a genetic fingerprinting study, "Distinguishing off-types in Tifway and Tifdwarf bermudagrasses."

The 2-year, $66,000 project was supported cooperatively by the Florida Golf Course Superintendents Association and the Florida Turfgrass Research Foundation. Matching funds were provided by the Golf Course Superintendents Association of America to the Florida Golf Course Superintendents Association. Using multiple techniques, we identified bermudagrass patches as genetic off-types, and formulated prevention guidelines.

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Patches in the fairways

Fig. 1. Golf Course X, January 1994.

While greens are most seriously affected by genetic patches, fairways sometimes show the problem as well, for example, Golf Course X, Palm Beach County, Florida (Fig. 1). This was an excellent example for the objective of our study: to determine the identity and origin of unknown bermudagrasses. Golf Course X had been fumigated with methyl bromide and was planted with what was believed to be Tifway (=T-419) bermudagrass.

First, let us analyze the distribution of the patches from a

digitally enhanced image of a fairway from Golf Course X (Fig. 2). On this and other fairways, the bermudagrass patches occurred as distinctive variants, "Blue green" and "Dark green." There was a repeating pattern, Blue green here, Dark green there, throughout this and other fairways. The patches were surrounded by a yellowish matrix grass, which may be described by analogy to Swiss cheese. The surrounding "matrix" grass looks like the cheese part of Swiss cheese, while the patches are like the holes.

Our evidence will show that the patches were different grasses, i.e., off-types, thus were not caused by fertilization, acid injection, rainy weather, or any other known environmental factor. The roundish, convex-margined shape of the patches shows they were probably increasing in size from a point source in the center of the patch. DNA evidence will show that the matrix grass was Tifway bermudagrass, while the patches were not Tifway, but were contaminants.

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Chromosomes and morphology

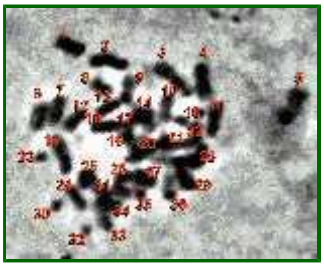

Fig. 3. Chromosomes (2n=36) from patch bermudagrass at Golf Course X.

Fig. 4. Leaf hairs on Tifway 419.

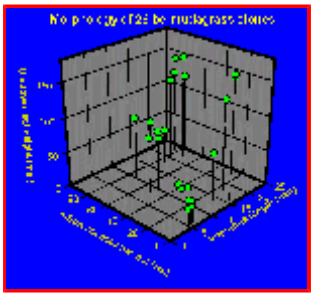

Fig. 5. Morphology profiles of 26 grasses.

Besides DNA fingerprinting, traditional methods were helpful in bermudagrass identification. The mitotic chromosomes of bermudagrass are extremely small and difficult to distinguish, yet their number distinguished the patch bermudagrasses from the surrounding matrix grass. The chromosome number of the patch bermudagrasses was about 36 (Fig. 3), thus they were common bermudagrass, not Tifway. The chromosome number of the matrix grass was about 27, the same as reported for Tifway.

Morphologic traits also distinguished the patches from Tifway. A replicated grow-out was performed on 26 grasses in six replicated pots. The patch grasses were very seedy, while Tifway and matrix grasses produced few seedheads. The patch grasses had normal anthers and shed pollen, as expected for 36 chromosome common bermuda. Tifway and the matrix grasses were sterile. Tifway and the matrix grasses had very many (100+), fine, leaf blade hairs (Fig. 4), while the patch grasses that we studied had longer, less abundant hairs. (Some other bermudagrasses, such as Ormond, have few or no leaf blade hairs.)

A combination of traits, for example, unmown leaf height, inflorescences per pot, and internode length, revealed a cluster of Tifway-like plants that differed from the Blue green and Dark green patch grasses (Fig. 5). Bermudagrass samples from different clusters differed statistically (probability of false differences < 5%) in most cases.

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DNA fingerprinting

Fig. 6. Trimming bermudagrass leaves for DNA extraction.

Fig. 7. PCR is done in a thermal cycler.

DNA fingerprinting is one of the most precise techniques available for comparing biological samples, such as unknown bermudagrasses. The method requires explanation. We meticulously extracted DNA from 29 unknown plants plus the Georgia standards for Tifway, Tifdwarf, and Tifgreen (Fig 6).

We then used a chemical reaction called "PCR" (polymerase chain reaction) to produce millions of copies of the tiny amount of starter DNA (Fig. 7). PCR jump-starts the copying by using a "primer" fragment, a chemical which we introduced. There are many kinds of primers, and each acts as a key to identify one specific kind of recognition site in the grass DNA. Regions of grass DNA lying between pairs of recognition sites get copied. So the primer acts like a bookend -- wherever primers bind reasonably closely at two nearby sites on the grass DNA, the region between the sites is copied. Not all grasses have exactly the same sequences in the same places, thus copying with PCR yields somewhat different DNA fragments for different grasses, depending upon the location of the recognition sites. The primers that we used were "random" -- we had no idea ahead of time which kinds of fragments would show up in which grasses.

By this method, a DNA fingerprint was based on the varying sizes of DNA fragments copied from different grasses, determined by our choice of primer and the underlying DNA differences (presence, absence, and location of recognition sites). Using carefully defined PCR conditions, two samples having identical DNA must provide the same range of DNA fragment sizes, thus each sample has the same fingerprint. Grasses with different DNA may show differences in DNA fragment sizes, for the same reasons. So amplified fragments are the first step to producing a DNA fingerprint.

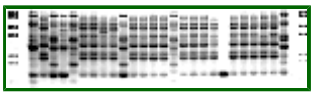

Fig. 8. DNA fingerprints (reverse image) of 26 bermudagrasses.

Because the copied DNA fragments differ from one another in molecular size (measured in "base-pairs"), they can be spread out (Fig. 8) to produce a distinctive banding pattern like the UPC (universal price code) bars which are scanned at the supermarket counter. To create separate bands out of the DNA fragments, the amplified mixture of each bermudagrass DNA was separated by fragment size by being pulled along a gradient of electrical charge, a technique known as electrophoresis. Parallel lanes containing DNA from different samples were run in tandem, for the same length of time. The DNA was then stained, to reveal separated bands, and photographed using UV fluorescence. Some bands represented large, slow moving DNA molecular fragments, over a thousand base-pairs long. Other bands represented small, quick-moving DNA fragments. We found only 3 to 8 useable bands per primer, but by using a choice of several good primers we found over 50 strong, distinctive bands, any one of which was clearly present or absent for a particular grass (Fig. 9).

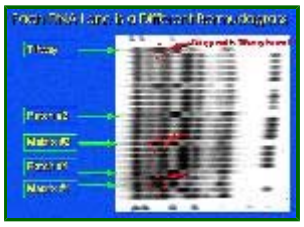

Fig. 9. A labeled series of electrophoresis lanes..

The scanned image of the bands (Fig. 10) revealed distinctive peaks (each band representing DNA of a particular molecular base-pair size) and valleys (regions with no DNA). The presence or absence of a peak, or diagnostic fragment, distinguished some bermudagrasses as different.

How repeatable was DNA fingerprinting? After exhaustively seeking the best "primers," the procedures were repeated at both Fort Lauderdale and Gainesville. Over 80% of the bands found at one laboratory were found at the other, while repetitions within the same laboratory were 100% consistent. Small bands were frequently inconsistent, and were not used.

Fig. 10. Digitally-scanned bands are represented by peaks.

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Patch grasses have a common clonal origin

Fig. 11. Golf Course Y, July 1994.

Fairways at Golf Course Y, Palm Beach County, Florida (January 1994) showed the same visual pattern as Golf Course X: Blue green and Dark green patches in a yellowish matrix (Fig. 11).

DNA evidence confirmed that the visual impression had a genetic basis -- the Blue green and Dark green patch grasses on Golf Course Y had the same DNA fingerprint as similar patches on Golf Course X (Fig. 12). The DNA profile of the matrix bermudagrass samples from both Golf Course X and Golf Course Y matched one another precisely, and also matched the Georgia standard of Tifway. About 50 DNA bands matched precisely across samples from different golf courses, a coincidence that could occur by chance only once in two billion times. The coincidence was beyond normal chance occurrences.

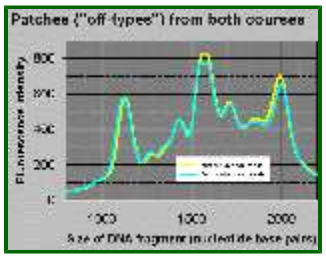

Fig. 12. DNA fingerprints of patch grasses matched exactly across golf courses.

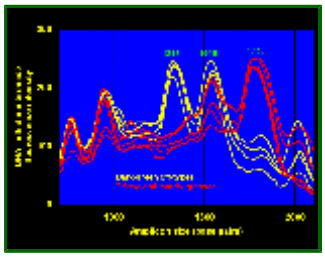

Fig. 13. DNA fingerprints from Tifway matched the matrix fairway grass on four golf courses. Dark green off-types matched across four golf courses.

Did these off-types arise on the golf courses as mutations or seedlings? Neither. Somatic mutations and seedlings are known in bermudagrass and other plants. They generate a medley of variation, not the repeating pattern we observed. At some point, the patch grasses may have arisen as seedlings, but before they were planted on the golf courses. There were other golf courses involved. Not only was the Blue green grass the same on Golf Course X and Y, but the Dark green grass was the same grass on Golf Course X, Y, Z, and W. Each type patch must have had a single clonal origin (barring one-in-two billion probabilities), thus they had to have been planted (Fig. 13). For example, a 1750 base-pair peak present on Tifway, and four unknown grasses from different golf courses, was absent in the Dark green variant (yellow line). In contrast, a 1315 base-pair was present on Dark green bermudagrasses from four different golf courses, but was absent from Tifway and matrix grasses. So DNA fingerprinting repeatedly matched unknown matrix grasses to the known Tifway, distinguished among knowns and unknowns, and matched unknown patch grasses across four golf courses. The evidence does not say when or how the patch grasses were planted, just that they were the same grasses.

Thus the first accomplishments of the study were:

1. **We proved for the first time, by the combined evidence of morphology and DNA that some off-types are contaminants that were planted, not mutations.**
2. **It was proven that fairway off-types can be readily distinguished by DNA fingerprinting, with 80%+ repeatability between our separate laboratories, in Fort Lauderdale and Gainesville.**

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Greens bermudagrass variations

Fig. 14. Patches of off-type bermudagrass on a golf course green..

Golf course greens are under considerable stress from close mowing and play, thus off-types are a greater problem there than on fairways (Fig. 14).

We studied 18 greens bermudagrasses; most represented trade samples, but a few were off-types from golf course greens. Tifgreen had taller unmown height and a greater number of seedheads than Tifdwarf (please go back to Fig. 5). Differences were apparent visually, once the samples were grown out (Fig. 15).

Fig. 15. Greens bermudagrasses were classified morphologically into three groups: Ultradwarf, Tifdwarf, and Tifgreen.

Morphology data showed that the distinction among greens bermudagrasses was highly significant statistically. There were three distinctive groups, Tifdwarf, Tifgreen, and an ultradwarf group. Three out of three non-certified trade samples of Tifdwarf were statistically different morphologically from Tifdwarf, but indistinguishable from Tifgreen, thus another accomplishment was:

3. **The study proved from morphology that some non-certified material sold as 'Tifdwarf' is not 'Tifdwarf,' rather it is probably Tifgreen..**

Could this be verified by DNA fingerprints? No. Early in the study, some DNA preparations showed apparent differences among greens bermudagrasses, but this was based on weak bands. Upon reextraction of more DNA from the same plants, purportedly diagnostic bands disappeared. For example, going back to Fig. 8, notice that 18 of the lanes have an identical profile-these were all greens samples. What do we make of this? The PCR procedure for copying DNA is sensitive to many factors, some of which can be reasonably controlled, but others cannot. In the case of the fairway grasses, there were sufficient strong bands for diagnostic comparisons; we did not have to resort to weak bands. In the case of greens bermudagrasses, there were no strong diagnostic bands, and the weak bands we attempted to use were probably derived from weak chemical reactions.

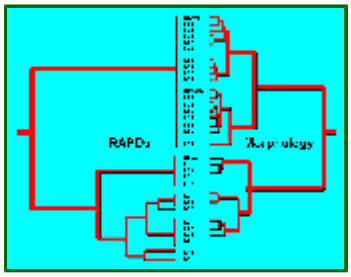

Fig. 16. Comparison of the tree of relatedness of bermudagrasses by DNA-RAPDS and morphology. The deeper the cleft between two branches, the more distantly related are the grasses.

The "tree of relatedness" of all the 32 grasses, for both DNA and morphology, tells a similar story (Fig. 16). The known and unknown grasses separated into distinctive branches based on morphology, representing clusters of similarity. Although DNA fingerprinting yielded the same branches as morphology for fairway grasses, DNA fingerprinting could not separate the greens grasses the way that morphology could.

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Why are greens grasses difficult to distinguish?

Greens bermudagrasses may represent mutations, a sudden genetic change affecting only a single piece of DNA. Tifdwarf is believed to have arisen naturally by mutation from Tifgreen. Bermudagrass has thousands of genes, each associated with thousands of DNA subunits, so a mutation affecting only one of those subunits within one gene would be a very small part of the organism's total DNA. Happening onto such a genetic change using PCR amplification would require great good fortune, like finding a needle in a haystack. Therefore, the chance of copying DNA from the small region where the mutation had occurred would have been unlikely. And we conclude,

4. **Greens off-types, while distinguishable by morphology, were not distinguishable by DNA, therefore they are probably point mutations or limited to a single gene.**

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Do greens mutate after planting?

Fig. 17. Genetic off-types on the greens often appear to recur in the same form on both the same and different greens.

Did the probable mutations originate on the greens or before the golf course was planted? Here there was no direct evidence, only circumstantial evidence. Looking at greens off-types over the years, one of us (PB) has noticed what appear to be repeating patterns of similar-looking patches, across individual greens, and across different greens on the same golf course (Fig. 17). A "mutation" which recurs in different places was probably planted, not a series of mutations, because the latter would be different from one another.

Fig. 18. Mid-Pacific Golf Club, Kailua, Hawaii, showing one of 15 original greens which were planted to Tifdwarf in the early 1960's.

Other indirect evidence helps refute the recurring mutation theory as an important source of the variation on greens. Greens on some courses, and occasionally greens on individual courses, have remained apparently pure genetically for many years. Examples include the Mid Pacific Golf Club, Kailua, Hawaii (Fig. 18), and Winter Pines Golf Club, in the Orlando, Florida area. On both courses, the original greens are almost completely pure. While off-typing is generally progressive, getting worse over the years, it doesn't always occur, even on relatively old courses.

In the late 1970's, Boca Greens, a Palm Beach County, Florida golf course, had off-type variations on all 18 greens, except the practice green near the clubhouse was pure. This was puzzling at first, because the practice green had been planted at the same time by the same contractor as the 18 greens on the golf course. On checking the source of plant material, the golf course superintendent learned that the grassing contractor had run out of sprigs after planting 18 holes, and had to go back for another load to do the practice green. With these anecdotal observations, we conclude:

5. **Circumstantial evidence suggests that greens off-types are sometimes planted.**

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Is DNA testing the answer?

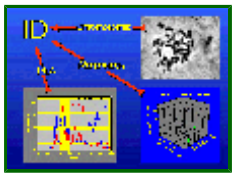

Fig. 19. Bermudagrasses can be identified by a combination of chromosomes, morphology, and DNA fingerprinting.

No, not for routine screening. While DNA testing is a powerful tool for confirming visual clues, it is our opinion that its best use is as a research tool and for ensuring the integrity of foundation sprig stock. Common bermudagrass contaminants of Tifway were proven to be contaminants, but the visual evidence was already overwhelming. DNA typing only proved what we already knew. DNA testing can help ensure cultivar purity, but only if there is there is diligent field sanitation, record-keeping, and maintenance of ditches.

Because contaminant off-types in greens appear to arise from mutation, it will be difficult to identify a diagnostic fingerprint. Each potential new mutation could affect a different part of the DNA, thus even if there were reliable banding differences, new research would be required for each new mutation. Any new mutation that was not already detectable visually would have an extremely rare chance of being sampled. On the other hand if, there were any question, based on visual evidence, that the wrong plant was growing, it would be cheaper and faster to just kill it.

DNA testing may also give the buyer of grass a false sense of security that the plant material is clean, when

in fact that could only be determined if every leaf blade in the field were DNA-tested. Therefore, we conclude:

6. **With present technology, DNA testing of greens grasses was ineffective. Fairways are a different matter, but even there, visual clues are most powerful in first detecting variants, and DNA is a secondary confirming tool. An example of the effective use of DNA fingerprinting would be where a field is visually uniform, but it needs to be determined that it is Tifway.**

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Prevention guidelines

Off-type bermudagrasses can arise from several sources, but the movement of contaminated planting sources is the first place to stop it.

The golf course superintendent can seek assurances that the sprig vendor is aware of the potential problem of contamination and has made efforts to prevent it. While the recurrence of new mutations is a possible source of contamination, it appears that many instances of off-types are due to contamination. Therefore, the golf course superintendent can:

1. Request certified plant material be used when it is available. The Southern Seed Certification Association (334-821-7400, FAX 334-844-4901, Box 2619, Auburn, AL 36831) currently certifies golf course bermudagrasses in Florida. The Association inspects fields in Florida.
2. Request a list of 1-4 year-old plantings from prospective grassing contractors.
3. Request written documentation on where the source grass originated.
4. Personally inspect prospective source fields, hopefully having the opportunity to look at areas where the grass has been mown closely over several months. But don't expect to see too much!
5. Include appropriate performance specifications in the bid, with a timeline for inspection of quality and consistency, and an appropriate remedy (e.g., a performance bond). Most users are very concerned over consistency.
6. Warn the greens committee that genetic purity does not exist, certainly not beyond the first few years, but that off-typing can be reduced for a while with considerable effort and expense.
7. Walk newly sprigged areas on a daily basis as they are growing in, and use nonselective herbicide (e.g., glyphosate) to kill any questionable patches.

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Acknowledgements

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