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Scientists at Oklahoma State University have developed SSR (simple sequence repeat) genetic markers for the accurate identification of clonal bermudagrass varieties. The set of 11 microsatellite markers are highly discriminatory and have great potential to protect plant patents and ensure quality control of clonal bermudagrass.

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## PURPOSE

The purpose of *USGA Turfgrass and Environmental Research Online* is to effectively communicate the results of research projects funded under USGA's Turfgrass and Environmental Research Program to all who can benefit from such knowledge. Since 1983, the USGA has funded more than 400 projects at a cost of $31 million. The private, non-profit research program provides funding opportunities to university faculty interested in working on environmental and turf management problems affecting golf courses. The outstanding playing conditions of today's golf courses are a direct result of ***using science to benefit golf***.


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# Microsatellite Molecular Markers Accurately Identify Clonal Turf Bermudagrass Cultivars

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## SUMMARY

Scientists at Oklahoma State University have developed molecular genetics methods to identify and differentiate clonal bermudagrass varieties. The study's accomplishments include:

- We demonstrated EST sequences are a good source to develop SSR markers, and we developed 230 EST SSRs for genetic research in bermudagrass.
- A total of 32 clonal turf bermudagrass genotypes, including 31 released varieties representing most of the commercially available clonal bermudagrasses in the USA and one OSU experimental clone (OKC 70-18) were used to screen 53 EST SSRs and 79 genomic SSRs.
- Eleven highly polymorphic SSR markers were selected for varietal identification.
- From a total of 141 bands amplified by the 11 SSRs, a varietal identity tree for the 32 cultivars was generated.
- The set of 11 microsatellite markers are highly discriminatory in clonal bermudagrass identification. The work represents the first rapid and robust application of SSR markers for identifying clonal turf bermudagrass.
- The markers may be used in the application for plant patent of clonal bermudagrass, in tracing infringements on plant breeders' rights, and for quality control of bermudagrass turf management and development.


Among the major grasses currently in commercial turf use, bermudagrass is widely adapted and extensively cultivated world-wide. Bermudagrass use in the USA extends from the states of the Gulf coastal plain and the arid southwest northward into the transition zone. Most of the commercialized turf bermudagrass varieties have been vegetatively propagated, so they are often times called "clonal turf bermudagrass".

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The stolons, shoots, rhizomes, and crown buds of bermudagrass enable reproduction of the same plant. This vegetative reproductive feature has been extensively utilized in the turf trade to massively multiply clonal varieties through the production of sod, plugs, and sprigs. As the progeny plants from the clonal reproduction are the same genotypes as the parent plants, they can form a beautiful and uniform turf. Clonal turf bermudagrass varieties are primarily grown on golf courses, sports fields, home lawns, and various commercial grounds.

### Clonal Turf Bermudagrass Varieties Developed through Hybridization, Mutation, and Selection

Most clonal turf bermudagrass varieties are developed from interspecific crosses involving two species: common bermudagrass (*Cynodon dactylon*) and African bermudagrass (*C. transvaalensis*). Some commercial clonal cultivars are selections from common bermudagrass or African bermudagrass.

The late Dr. Glenn Burton, USDA ARS geneticist at the Coastal Plains Experiment Station in Tifton, Georgia pioneered the breeding of these clonal turf bermudagrasses. He crossed selected tetraploid (2n=4x=36 chromosomes) common bermudagrass plants with diploid (2n=2x=18 chromosomes) African bermudagrass plants to produce triploid (2n=3x=27 chromosomes) hybrids (1). The work was initiated in the 1940s (1). He released numerous clonally propagated turf bermudagrass varieties, named as 'Tif' series bermudagrasses (2). 'Tifway' bermudagrass is a clonal variety selected and released from the Tifton breeding program (2). Although the cultivar was commercialized in the 1960s, 'Tifway' remains a national standard cultivar used in the


**Figure 1.** Thirty-two clonal turf bermudagrasses grown in a greenhouse at Oklahoma State University in preparation for SSR marker analysis. Their varietal names are 'Baby', 'Celebration', 'FloraTex', 'Midfield', 'Midlawn', 'Midway', 'MS-Choice', 'MS-Pride', 'MS-Express', OKC-70-18, 'Latitude 36' (OKC 1119), 'Northbridge' (OKC 1134), 'Patriot', 'Premier', 'Quickstand', 'Sunturf', 'Texturf 10', 'Tifton 10', 'TifGrand', U-3-SIU, 'Vamont', 'Midiron', 'TifSpor't, 'Tifway', 'Tifway II', 'TifEagle', 'Tifgreen', 'Champion', 'Floradwarf', 'Mini Verde', 'MS-Supreme', and 'Tifdwarf'.

current (2007-2012) National Turfgrass Evaluation Program (NTEP) National Bermudagrass Test (http://www.ntep.org/bg.htm).

More recently, Dr. Charles Taliaferro and his colleagues at Oklahoma State University (OSU) developed and released a tetraploid (2n=4x=36) interspecific hybrid, named 'Patriot' (3). 'Patriot' clonal bermudagrass is a vegetatively propagated superior progeny from crossing a hexaploid (2n=6x=54 chromosomes) common bermudagrass 'Tifton 10' and a selected African bermudagrass genotype. 'Tifton 10' bermudagrass is a clonal common bermudagrass originated in Shanghai, China (4).

Some clonal bermudagrass varieties are selections from natural mutations identified in the established turf or artificially induced mutations of released cultivars. 'Tifdwarf' is an early identified mutation of a 'Tifgreen' turf planting (5). 'Tifgreen' was a clonal triploid turf variety developed and released by Dr. Burton in the 1960s (6). More cultivars including 'Pee Dee 102', 'Floradwarf', 'MS-Supreme', 'Champion', and 'Mini Verde' are selections of naturally occurring mutations of 'Tifgreen' or 'Tifdwarf' (2, 7, 8, 9). Using gamma rays of Cobalt 60 to irradiate dormant propagules of selected turf bermudagrass genotypes, scientists can produce artificially induced mutations (10). 'Tifgreen II' is an induced mutation cultivar of 'Tifgreen' (1). 'Tifway II' is a selection of artificial mutants of 'Tifway' (11).

## The Need to Establish Identity Among Clonal Turf Bermudagrass Varieties

Clonal turf bermudagrass cultivars can be different in turf quality, adaptation, management requirements, and cost of production. Each clonal cultivar has certain different traits. Therefore, selection of the best cultivar for a specific need is a very important decision for the end user to obtain suitable turf performance. However, identification of clonal bermudagrass cultivars using morphological, developmental, and physiological traits is subjective in its practice and depends on the evaluator's experience.

Many differences in traits among cultivars are quantitative in nature and modified by environmental factors, as well as cultural practices. In practical situations, uncertainty may arise regarding the genetic identity of specific plants of a particular named cultivar. Especially in intensively managed turf areas on golf courses and on sod farms, some plants don't appear to be identical. It is challenging to make a sound judgment on the identity using observable traits. Development of a reliable and easy technique is needed to facilitate the correct identification of clonal turf bermudagrass cultivars (12).

Accurate cultivar identification coupled with existing appropriate regulatory processes, such as those used in certification programs, and diligent commitment from breeders to commercial producers to installers provides improved assurance that the cultivar installed on a site is actually what is selected (13). This rigorous process protects consumers from the use of unintended cultivars, ensures the purity of the cultivars by sod producers, and protects the intellectual property of the cultivar developers.

## Microsatellite Markers and Their Attributes with Respect to Bermudagrass

Microsatellite molecular markers, often called "Simple Sequence Repeats" (SSRs), is a new marker system in bermudagrass, although this marker system has been developed and deployed in model plants and major agricultural crops for over two decades. A major reason for the lag in application of SSR technology in bermudagrass identification is the high cost associated in library construction, sequencing, and testing.

The SSRs are tandemly repeated units of short nucleotide core sequences including di-

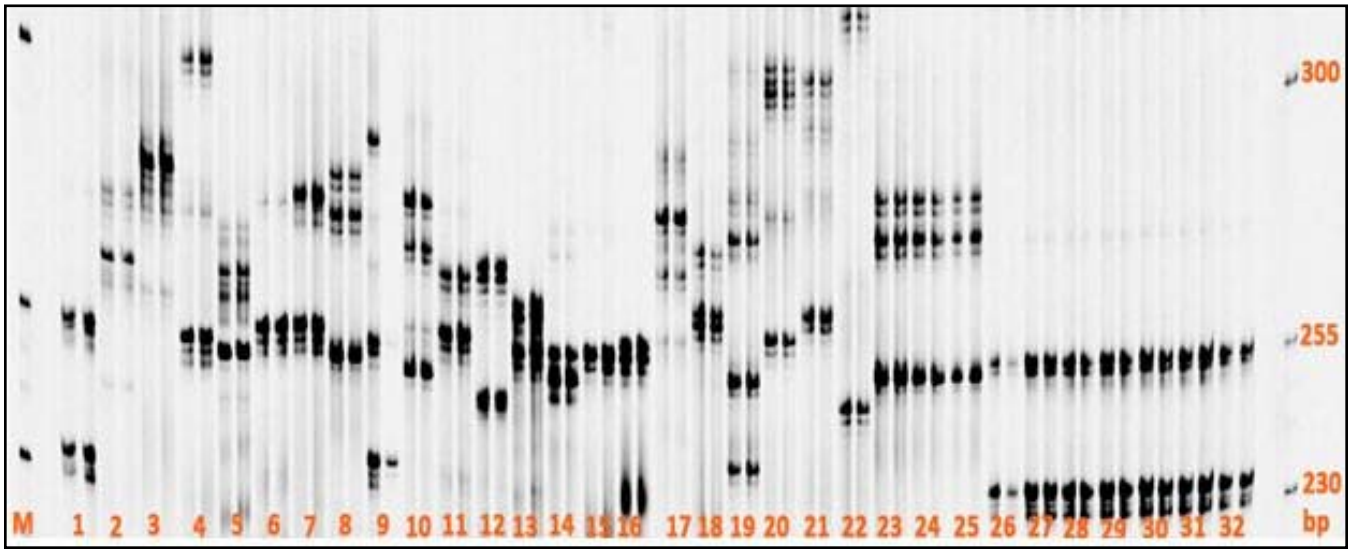


**Figure 2.** DNA profiles of 32 clonal turf bermudagrass cultivars amplified with an SSR marker CDCA 379-380. Sample ID: 1 = 'Baby', 2 = 'Celebration', 3 = 'FloraTex', 4 = 'Midfield', 5 = 'Midlawn', 6 = 'Midway', 7 = 'MS-Choice', 8 = 'MS-Pride', 9 = 'MS-Express', 10 = OKC-70-18, 11 = 'Latitude 36" (OKC 1119), 12 = 'Northbridge' (OKC 1134), 13 = 'Patriot', 14 = 'Premier', 15 = 'Quickstand', 16 = 'Sunturf', 17 = 'Texturf 10', 18 = 'Tifton 10', 19 = 'TifGrand', 20 = U-3-SIU, 21 = 'Vamont', 22 = 'Midiron', 23 = 'TifSport', 24 = 'Tifway', 25 = 'Tifway II', 26 = 'TifEagle', 27 = 'Tifgreen', 28 = 'Champion', 29 = 'Floradwarf', 30 = 'Mini Verde', 31 = 'MS-Supreme', and 32 = 'Tifdwarf'.

nucleotides, tri-nucleotides, tetra-nucleotides, penta-nucleotides, and hexa-nucleotides. DNA molecules are made up of four nucleotide bricks: A (adenine), T (thymine), G (guanine), and C (cytosine). For example, di-nucleotides include CACACACA…$CA_n$, or GAGAGA…$GA_n$, or other combinations of two among four nucleotides (A, C, G, and T), where n is any number from single digits to several hundred. Tri-nucleotide core sequences include AGT, AAG, and other combinations of three nucleotides in any number of repeats.

What scientists are interested in concerning SSRs are their polymorphisms. For example, one bermudagrass genotype may have one SSR with 10 repeats while another plant may have 20 repeats of the same SSR, which is a polymorphism. Scientists can use a lab technique called Polymerase Chain Reaction or PCR and a gel electrophoresis technique to study polymorphisms.

Another attribute that SSR markers have is their codominance, which means at each genetic locus, all DNA alleles will be sampled and amplified in the PCR reactions. In contrast, dominant markers of AFLP (Amplified Fragment Length Polymorphism), RAPD (Random Amplified Polymorphism DNA), and DAF (DNA Amplification Fingerprinting) just show a single allele on each locus. Bermudagrass primarily outcrosses because it is self-incompatible. This feature results in alleles at each of many loci of bermudagrass plants being different (heterozygous). Codominant markers such as SSRs are the best fit to reveal the heterozygous alleles of bermudagrass. For genetic studies of inheritance, SSRs are an excellent choice as they are highly reliable and reproducible across different labs and heritable across generations of the same species.

The objectives of our recent work were to develop SSR markers in bermudagrass and to use SSR markers for accurate identification of commercially available clonal turf bermudagrass cultivars. Scientists at OSU reported a first set of SSR markers developed in bermudagrass. They also reported a set of highly polymorphic SSRs to accurately identify clonal turf bermudagrass.

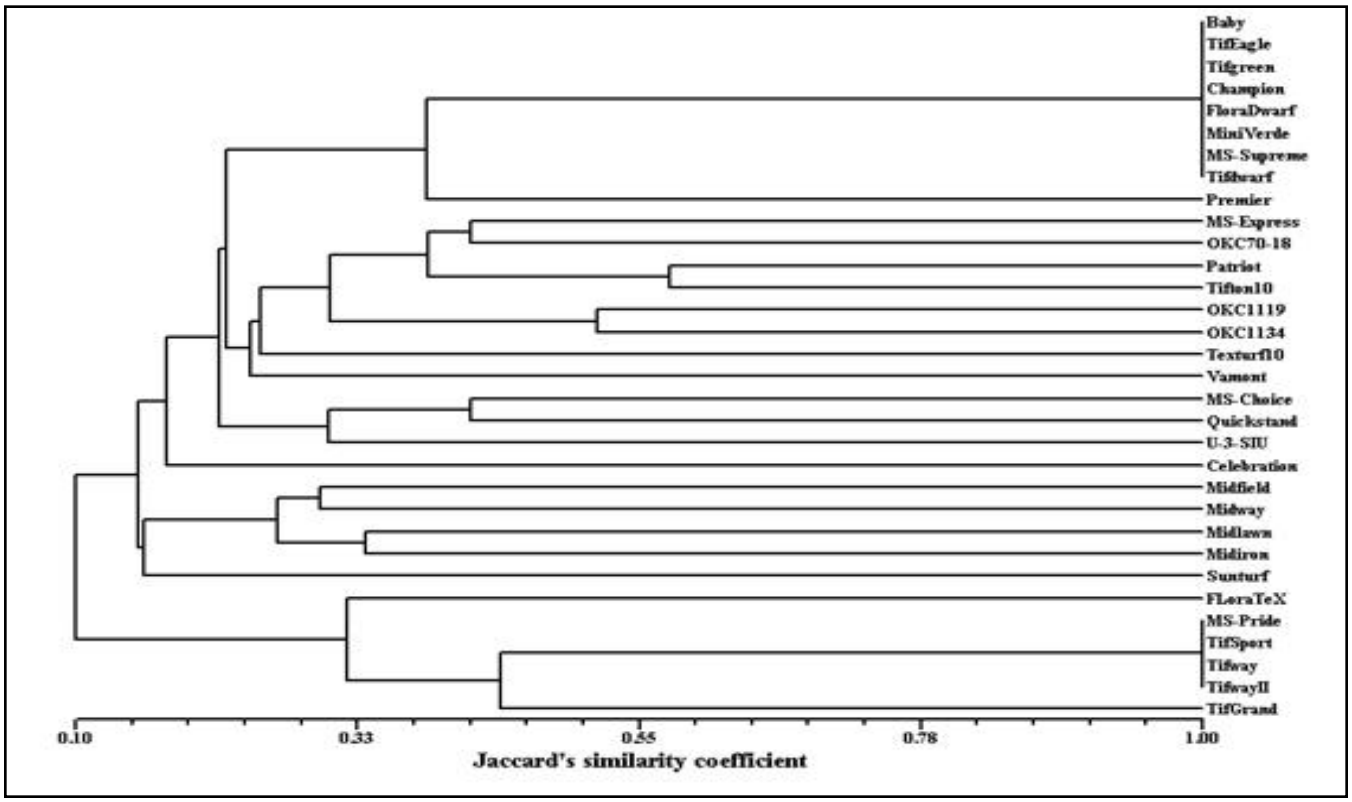


**Figure 3.** Identity tree of 32 clonal turf bermudagrass cultivars based on DNA profiles generated by 11 SSR markers. The tree was adapted from Wang at el. (13).

## Bermudagrass SSR Marker Development at OSU

EST (Expressed Sequence Tag) sequences are short fragments of genes and derived from the gene transcription process and captured by gene expression and discovery analysis. A large set of 20,237 EST sequences derived from bermudagrass has been deposited at the National Center for Biotechnology Information (NBCI). We mined the EST sequences to identify SSRs and designed SSR primers and tested them for reliable polymorphic amplification of a panel of bermudagrass DNA samples. From this work, we demonstrated EST sequences are a good source to develop SSR markers, and we developed 230 EST SSRs for genetic research in bermudagrass (14). Although it is relatively inexpensive, EST SSRs development is limited by the availability of EST sequences.

The second approach we used is more expensive and complex, but can be used to develop more SSR markers. Major steps and procedures of this method included construction of SSR-enriched genomic DNA libraries using the pUC 19 plasmid system in *Escherichia coli* DH 5α, sequencing the SSR-enriched clones at the Oklahoma State University Core Facility, and designing and testing SSR primers for their effectiveness, reliability, and polymorphism (15). Five small insert plasmid libraries enriched in SSR core sequences of $(CA)_n$, $(GA)_n$, $(ATG)_n$, $(AAC)_n$ and $(CAG)_n$, where n is the repeat number, were constructed. Those designed SSR primers are being tested. We expect a larger set of genomic SSR markers will be developed from this effort in the near future.

## SSR Identification of Clonal Turf Bermudagrass Cultivars

A total of 32 clonal turf bermudagrass genotypes, including 31 released varieties representing most of the commercially available clonal bermudagrasses in the USA and one OSU experimental clone (OKC 70-18) were used in the study (Figure 1). 'Latitude 36' (OKC 1119) and 'Northbridge" (OKC 1134) are two new clonal turf bermudagrass cultivars developed at OSU and currently being released. Respective genomic DNA samples of the selected varieties were extracted for PCR reactions.

Following the screening of 53 EST SSRs and 79 genomic SSRs, we selected 11 highly polymorphic SSR markers for varietal identification. The selected SSRs encompass seven genomic SSRs, CDCA 31-32, CDCA 55-56, CDCA 77-78, CDCA 133-134, CDCA 155-156, CDCA 379-380, CDCA 747-748, and four EST SSRs, CDE 89-90, CDE 127-128, CDE 215-216 and CDE 375-376 (13). One gel image of the 32 clonal bermudagrass genotypes amplified by an SSR marker CDCA 379-380 is given in Figure 2. Each of the 11 SSRs amplified 6 (CDE 375-376) to 25 bands (CDCA 31-32) for the 32 clonal varieties. CDCA 31-32 SSR marker is the most powerful in discriminating the bermudagrass cultivars.

From a total of 141 bands amplified by the 11 SSRs, we generated a varietal identity tree for the 32 cultivars (Figure 3). The tree indicates the SSR markers identified 20 varieties and two mutational families. One of the mutational families includes the members 'Tifgreen', 'Baby', 'TifEagle', 'Champion', 'Floradwarf', 'Mini Verde', 'MS-Supreme', and 'Tifdwarf'. Another mutational family included the members 'Tifway', 'Tifway II', 'TifSport', and 'MS-Pride'. The 20 varieties successfully identified by SSR markers all are nonmutational clonal bermudagrass cultivars. The SSR markers failed to identify mutation cultivars from each other and their respective parents.

## Summary

In summary, clonal turf bermudagrass varieties have been widely used in the USA and many other countries in the world. Accurate identification among many cultivars is important for turf growers, breeders, scientists, and informed end users. We developed the first set of EST SSR and genomic SSR markers in bermudagrass. The SSRs provide novel, accurate, and user-friendly

approaches to the molecular identification of cultivars, offering much advantage over morphological comparisons.

The set of 11 microsatellite markers are highly discriminatory in clonal bermudagrass identification. The work represents the first rapid and robust application of SSR markers for identifying clonal turf bermudagrass. The markers may be used in the application for plant patent of clonal bermudagrass, in tracing infringements on plant breeder's rights, and for quality control of bermudagrass turf management and development.


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