GENETIC DIVERSITY OF CREEPING BENTGRASS CULTIVARS USING SSR MARKERS

Christine Kubik, Joshua Honig, William A. Meyer and Stacy A. Bonos*

ABSTRACT

There is an increasing demand for new and improved cultivars to meet environmental restrictions and challenges. This results in the development and release of many turfgrass cultivars each year. Reliable and definitive cultivar identification becomes critical to maintain varietal purity and to protect breeder and consumer rights. Simple sequence repeats (SSRs) are a class of genetic marker that are useful for cultivar identification in many plant species. The objectives of this study were to: 1) characterize 30 SSR markers in creeping bentgrass, 2) determine genetic diversity within and among 13 creeping bentgrass cultivars (L-93, Penncross, Pennlinks, Penneagle, Crenshaw, SR 1020, Penn A-4, Penn A-2, Penn G-2, Penn G-6, Southshore, Putter and Seaside), and 3) determine if SSRs can be used to identify the cultivar origin of clones sampled from a golf course green. Thirty SSRs markers were isolated from a genomic library of creeping bentgrass enriched for di and tri-nucleotide repeats. The 30 SSR markers amplified 409 different alleles in 15 bentgrass populations (13 cultivars and two golf green samples). A genetic distance matrix was created from the SSR data in Genalex 6.1. An UPGMA dendogram and principal component analysis were developed from the genetic distance matrix and used to assess the genetic relatedness between bentgrass populations. Both UPGMA and PCA analysis grouped bentgrass populations into similar clusters which corresponded well to the breeding histories. Golf green samples showed a high similarity to originally seeded cultivars and not overseeded cultivars. AMOVA results revealed higher genetic diversity within populations compared to among them. This research indicates that SSR markers are a useful tool to measure genetic diversity within creeping bentgrass and could be used to solve practical management issues and to supplement morphological and agronomic data for plant variety protection.

Abbreviations: AMOVA – Analysis of Molecular Variance; PCA – Principal Component Analysis; SSR – Simple Sequence Repeats; UPGMA – Unweighted Pair Group Method of Arithmetic Averages

Keywords: UPGMA, principal component analysis, AMOVA

C. Kubik, J. Honig, W.A. Meyer and S.A. Bonos*, Dep. of Plant Biology and Pathology, School of Environmental and Biological Sciences, Rutgers University, 59 Dudley Rd. Foran Hall, New Brunswick, NJ, 08901-8520. *Corresponding author: (bonos@aesop.rutgers.edu).
INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* L.) is an important cool-season turfgrass for temperate golf course putting greens due to its spreading growth habit and ability to tolerate low mowing heights. The demand for improved cultivars has resulted in the development and release of several new cultivars each year (Golembiewski et al., 1997). In the past 10 years, 15 new cultivars have been developed. There are currently 42 cultivars on the market. (Bonos, 2007). As a result, reliable and definitive cultivar identification is critical to maintain varietal purity and protect breeder and consumer rights (Yamamoto and Duich, 1994). Little is known about genetic relationships among and within creeping bentgrass cultivars, in part, because it is a cross pollinated species. In most cases, the exact parental clones of these synthetic cultivars are largely unknown due to the nature of bentgrass breeding techniques (Warnke et al., 1997). Breeders must understand relationships within and among cultivars in order to increase hybrid vigor and reduce re-selection within existing germplasm, which can narrow the genetic base of cultivars (Warnke et al., 1997). It is also important for turfgrass managers to understand which cultivar they are managing as the cultivars vary in pathogen susceptibility and management requirements.

Molecular markers can be used to distinguish within and among cultivars and to assess genetic relationships. Isozymes (Yamamoto and Duich, 1994 and Warnke et al., 1997), RFLPs (Restriction Fragment Length Polymorphism) (Caceres et al., 2000) RAPDs (Random Amplified Polymorphic DNA) (Golembiewski et al., 1997) and AFLPs (Amplified Fragment Length Polymorphisms) (Vergara and Bughrara, 2003; Vergara and Bughrara, 2004) have been used for both creeping bentgrass cultivar identification and to determine genetic diversity in bentgrass species. However, each of these methods has its limitations. Isozymes are proteins which can vary in expression under different environmental conditions. They also fail to discriminate closely related genotypes due to a lack of polymorphism (http://www.ndsu.nodak.edu/instruct/mcclean/plsc731/mapping/mapping1.htm). RFLPs are hybridization based markers which make them time consuming, laborious and more technically demanding than PCR-based markers (Gupta et al., 1999). While RAPDs are PCR-based markers, and provide ample polymorphism, their consistency and repeatability are questionable. (Devos and Gale, 1992; Gupta et al., 1999). Additionally, RAPDs have been reported as either uninformative or inefficient for genetic diversity studies (Behera et al., 2008; Gallego et al., 2005; Jeung et al., 2005; Mueller and Wolfenbarger, 1999; Yu et al., 2005). AFLPs are another PCR-based marker system, but require more technically demanding skills than RAPDs and often require silver staining, autoradiography or fluorescence based detection (Gupta et al., 1999).

Simple sequence repeats (SSRs) are a class of genetic marker that have proven to be useful tools in plant genetic analysis (Akkaya et al., 1992; Condit and Hubbell, 1991; Guilford et al., 1997; Kubik et al., 1999; Morganti and Oliveri, 1993; Weising et al., 1989; Zhao and Kochert, 1993) and have replaced isozymes, RAPDs and RFLPs due to their high polymorphism and versatility (Goldstein and Pollock, 1997). Several studies have compared polymorphism obtained from isozymes, RAPDs, or RFLPs to that obtained using SSRs (Innan et al., 1997; Liu et al., 1995; Terauchi and Konuma, 1991; Wu and Tanksley, 1991). In all cases, SSRs were more polymorphic per locus. This polymorphism is useful for distinguishing among closely related varieties (Olufowote
et al., 1997; Rongwen et al., 1995; Thomas and Scott, 1993) and for assessing genetic relationships among individuals (Goldstein and Pollock, 1997). In fact, several researchers have concluded that microsatellites have the greatest utility of any marker system (Akkaya et al., 1992; Morgante and Olivieri, 1992; Goldstein and Pollock, 1997; Gupta and Varshney, 2000; Ellegren, 2004; Song et al., 2005; Ellis and Burke, 2007). SSRs have been used successfully to study genetic diversity and polymorphisms in many species including corn (Zea mays L.) Lubberstedt et al., 1998), rice (Oryza sativa L.) (Olufowote et al., 1997) barley (Hordeum vulgare L.) (Struss and Plieske, 1998), and perennial ryegrass (Lolium perenne L.) (Kubik et al., 1999; Kubik et al., 2001).

Marker-assisted cultivar identification, using SSRs, can have practical implications for turfgrass breeders and managers. Because they are highly polymorphic and may identify individuals with a unique marker fingerprint, it is possible that SSRs may be used to identify parental clones for new cultivar development that maximize hybrid vigor and genetic diversity (Cho et al., 2004; Xu, 2003). In addition to their role in parental identification, SSRs can be used to study genetic relationships within and among cultivars of creeping bentgrass, thereby improving our understanding of the genetic diversity in creeping bentgrass. This information could be used to develop better cultivar blending recommendations. Molecular markers can also help golf course superintendents, athletic field managers, sod growers and other turf managers determine the varietal composition of their turfgrass areas. This is important because turfgrass cultivars have different management requirements and different responses to pathogens and pests. These characteristics will affect the overall performance and type of management that will be required to maintain a high quality turf. In this study we wanted to see if molecular markers (SSRs) could be used to determine whether overseeding practices used to transition from older cultivars to an improved cultivar were effective.

The objectives of this study were to: 1) characterize 30 SSR markers in creeping bentgrass, 2) determine genetic diversity within and among 13 creeping bentgrass cultivars (L-93, Penncross, Pennlinks, Penneagle, Crenshaw, SR 1020, Penn A-4, Penn A-2, Penn G-2, Penn G-6, Southshore, Putter and Seaside), 3) determine if SSRs can be used to identify the cultivar origin of individual clones sampled from a golf course green.

**MATERIALS AND METHODS**

**SSR Isolation and Characterization**

Total genomic DNA was extracted from MCB 17, a single creeping bentgrass clone collected from Piping Rock Golf Course in Long Island, NY, using Qiagen DNeasy Plant Mini Kit (Valencia, CA). DNA was sent to Genetic Identification Services Inc. (GIS, Chatsworth, CA) for the construction of an SSR library enriched for GA, GT and GCT SSRs. GIS Inc. delivered a DNA library 50% enriched for GA and GT repeats and a library 10% enriched for the tri-nucleotides GCT and AAT. One 100ul aliquot of each library was plated out on four separate LB agar plates containing ampicillan, IPTG and Bluo-Gal. The plates were incubated at 37°C overnight. Several hundred individual colonies were chosen from the GA and GT enriched libraries, and grown in 6ml of LB broth containing ampicillan. DNA was isolated from the cultures using Qiagen Miniprep Spin Kit. Samples were sequenced using either the ABI 373 or 377 automated DNA sequencers (Applied Biosystems, Foster City, CA). Sequence data from the clones containing SSRs were analyzed for primer selection,
and PCR primers were designed to flank regions surrounding the SSR motif. All the primer pairs were first screened on a subset of DNA from five single plants (MCB 17 and a single clone from each Penn A-2, Crenshaw, Seaside, and L-93) which represented a range in genetic diversity based on the breeding histories. The polymorphic primer pairs with non-specific amplifications and/or too faint products were discarded from the final assay.

The polymerase chain reactions contained 10mM Tris-HCl pH 8.3, 50mM KCl, 0.25 mM each dNTP, 12.5 pmol each oligonucleotide primer, 0.5 units of AmpliTaq DNA polymerase, 50 ng template DNA and between 1mM and 4mM MgCl₂ depending on the primer combination in total volume of 25μl. The parameters for the single thermocycle were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. PCR consisted of 30 cycles with a final elongation for 10 min at 72°C. The PCR products were visualized on a 4% agarose gel and the level of polymorphism (if any) was assessed.

**Plant Material**

Certified seed from 13 creeping bentgrass cultivars was germinated in small pots containing Pro-Mix BX (Premier Horticulture, Quakertown, PA). Seven cultivars (Penncross [Hein, 1958], Pennlinks, Penneagle, Penn A-2, Penn A-4, Penn G-6, and Penn G-1) were provided by Turf Seed Inc. (Canby, OR). The additional six cultivars (Crenshaw [Engelke et al., 1995], Seaside, L-93, SR 1020 [Robinson et al., 1991], Southshore [Hurley et al., 1994] and Putter [Stanton et al., 1993]) were provided by Seed Research of Oregon (Corvallis, OR). At the seedling stage, 30 individual plants per cultivar were selected and transplanted into single cell flats for subsequent DNA analysis. When the plants were fully established, 0.1 g of leaf tissue was collected from each plant and frozen at -80°C.

Additional plant material was collected from two putting greens from Stanton Ridge Country Club in Whitehouse, NJ (Greens 11 and 15) to determine if a cultivar could be identified with SSR markers and to determine if overseeding a new cultivar into an existing green is effective in transitioning from one cultivar to another. The two golf greens (11 and 15) were originally seeded to 25% Putter, 25% Southshore, 25% Cobra, and 25% Pennlinks at 0.08g m⁻² in 1993. Overseeding was done in the spring and fall of each year at 0.08 g m⁻² of seed per green (each green was approx. 929 m²). The overseeding operation was performed in conjunction with seasonal aerification. Starting in the fall of 1997, L-93 was used for overseeding. Regular trinexapac-ethyl applications were made on the golf greens but growth regulation was reduced leading up to aeration. No growth regulators were used specifically to improve overseeding practices. Topdressing was applied (22.4 Mg/ha) in conjunction with aeration events. Light topdressing (approximately 45 kg / green) was applied biweekly during the growing seasons of each year. Mowing heights were maintained at 0.32 cm (0.125 in). The putting greens received 22 kg N m⁻² per year. Granular fertilizer applications were used in the spring and fall and liquid applications were used during the growing seasons. Pesticides were applied biweekly at labeled rates for control of dollar spot (caused by the fungus Sclerotinia homoeocarpa F.T. Bennett), brown patch (caused by the fungus Rhizoctonia solani Kühn) and pythium (caused by Pythium spp.) during the growing seasons of each year. In fall of 1999, green 11 was seeded with L93 to repair vandalism in its midsection.

In the fall of 1999, six plugs were taken at random from each of the two golf greens. The repaired section was not made evident when samples were collected. The
plugs were separated into 30 individual plants and maintained in the greenhouse for subsequent DNA analysis. A total of 450 plants were evaluated in this experiment (15 populations [13 creeping bentgrass cultivar populations and two putting green populations] x 30 individuals). DNA was extracted from each plant using Qiagen DNeasy Plant DNA extraction kit and genotyped for all 30 SSR primer pairs.

Genotyping

Fifteen creeping bentgrass cultivars/populations were genotyped at 30 SSR loci for a total of 13,000 PCR reactions (30 loci x 30 individual plants per cultivar/population x 15 populations). Each polymerase chain reaction contained 10 mM Tris-HCl ph 8.3, 50 mM KCl, 0.25 mM each dNTP, 12.5 pmol each of oligonucleotide primer (the 5’ end of the forward primer was fluorescently labeled with one of three possible dyes - 6-FAM, Hex, or NED), 0.5 units of taq polymerase, 50 ng of template DNA, and between 1 mM and 4 mM MgCl₂, depending on the primer combination, in a total volume of 25 µl. The parameters for a single thermocycle were 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. PCR consisted of 30 cycles with a final elongation for 10 min at 72°C. PCR products labeled with different fluorescent dyes were pooled (1 µl of each reaction) and the volume was brought to 10 µl with sterile water. 1 µl of the pooled mixture was combined with 9 µl of Hi-Di Formamide (Applied Biosystems) and 1 µl of the Genescan – 500 ROX Size Standard (Applied Biosystems). The samples were heated at 95°C for 5 min and quenched on ice. Raw data was generated on the ABI 3100 Genetic Analyzer and genotypes were scored using Genotyper software (Applied Biosystems).

Statistical Analysis

Statistical procedures for genetic analysis were first developed for diploid organisms and are not equipped to handle organisms with higher ploidy levels that contain more than two alleles at a given locus. Creeping bentgrass is a tetraploid with four sets of chromosomes and therefore can have four possible alleles at a given locus. Because of this, the polymorphic SSR bands for each individual were scored as single dose allele markers. To do this, each allele at a given locus was scored for presence or absence. This resulted in a data set of 1’s and 0’s for each of the 450 individuals. Only bands that could be scored consistently among populations were used.

Genetic diversity parameters were calculated using Genalex 6.1 (Peakall and Smouse, 2006). Genalex is unique in that distance matrices can be generated for multiple marker types - including co-dominant, haploid and binary genetic data sets. Since the SSR markers used in this study were scored for presence/absence, the binary data set option was used for all analyses. Nei’s genetic distance matrix (Table 1) was calculated according to Nei (1972) in Genalex 6.1. To visualize differences between populations, the genetic distance matrix was used to run a Principal Component Analysis (PCA) (also in Genalex). PCA analysis was based on a correlation matrix of the SSR frequency data. Sigma plot was used to make plots of the first three eigenvalues. As a supplement to the PCA analysis, the genetic distance matrix generated in Genalex was also used to develop a dendogram using UPGMA (Unweighted Pair Group Method of Arithmetic Averages).
The UPGMA tree was developed using Proc Cluster analysis in SAS (Cary, NC). Pairwise population comparisons were analyzed with an analysis of molecular variance (AMOVA) using Genalex 6.1 based on 999 permutations. The AMOVA procedure in Genalex follows the methods of Excoffier et al. 1992; Huff et al., 1993; and Peakall et al., 1995. AMOVA estimates and partitions total molecular variance within and between populations and then tests the significance of partitioned variance components using permutational testing procedures (Excoffier et al., 1992). AMOVA calculates $\Phi_{PT}$ which is analogous to Fst when the data are haploid or binary (Peakall and Smouse, 2006). The $\Phi_{PT}$ values represent the proportion of the total variance that is partitioned between two populations (Excoffier et al., 1992; Huff, 1997; Peakall and Smouse, 2006).

**RESULTS AND DISCUSSION**

**SSR isolation and characterization**

A total of 260 primer pairs, flanking SSR sequences isolated from the genomic SSR library, where screened on five creeping bentgrass clones (MCB 17 and a single clone from each Penn A-2, Crenshaw, Seaside, and L-93) to test for polymorphisms. Forty-seven of the 260 primer pairs designed, amplified a polymorphic SSR locus. The 47 primer pairs were then tested on the 30 individual plants from each population. Thirty of the 47 primer pairs were highly informative among the creeping bentgrass populations evaluated and these 30 were chosen for genetic analysis (Table 2).

These 30 new SSR primer pairs have not been reported previously. The 30 polymorphic loci identified between eight and 20 alleles with an average of 13.7 alleles per locus in 15 populations of creeping bentgrass. The 30 SSR loci amplified a total of 409 alleles that were used for genetic analysis. Many of these successfully amplified a PCR product in colonial bentgrass (*A. tenuis* L.), dryland bentgrass (*A. castellana* L.), velvet bentgrass (*A. canina* L.) and redtop (*A. gigantean* L.) (data not shown) proving they could be useful for additional applications such as detection of hybrids between species and syntenic mapping projects.

**Genetic diversity among creeping bentgrass cultivars**

Nei’s genetic distance was used to estimate the genetic relationship between bentgrass populations with values ranging from 0.054 and 0.011, the smaller values...
indicating a closer relationship (Table 1). The highest similarity was observed between the two populations sampled from Stanton Ridge golf course while the lowest similarity (most diversity) was observed between Penn A-2 and the Stanton Ridge golf course population from Green 11. These low numbers indicate that although some visible differences obviously exist between cultivars, in general, creeping bentgrass cultivars are very closely related and share a high degree of genetic similarity. This is not surprising considering the breeding histories of these cultivars (Table 3). Many trace to similar germplasm sources. Kubik et al. (2001) and Huff (1997) also found high levels of genetic similarity between cultivars of perennial ryegrass.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Primer A</th>
<th>SSR</th>
<th>Primer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>GA 8</td>
<td>5' GTA AAG CTG CGT AGG GTC T 3'</td>
<td>(CT)17</td>
<td>5' TAA TAG TCA GGT AAC GAA ATT C 3'</td>
</tr>
<tr>
<td>GA 27</td>
<td>GGG GAT CCA TGC ACT TTC TTC AGG</td>
<td>(GA)14</td>
<td>ACA CGG GGG CCC ACT TGT C</td>
</tr>
<tr>
<td>GA 40</td>
<td>TGG GGA CGG ACC TAC T</td>
<td>(GA)30</td>
<td>GAA CAT TGC TCA CGA ACT CT</td>
</tr>
<tr>
<td>GT 4</td>
<td>TAG TAG AGT CGT ATC CAT C</td>
<td>(CA)13</td>
<td>GTG TCC ACC ATC GGC CAT CAG TCT ATA</td>
</tr>
<tr>
<td>GT 10</td>
<td>CAT TGG GCT CTT TGG GCT GCT AT</td>
<td>(GT)26</td>
<td>ACT TGG GGC GGG ATT TGA</td>
</tr>
<tr>
<td>GT 14</td>
<td>AGG ACC GAG ATT TGG ACA</td>
<td>(GT)10</td>
<td>GAA TGT AAG TAG TAA TCG AGC T</td>
</tr>
<tr>
<td>CB 9</td>
<td>TAG GCT CGA CAT CAC CGC GT</td>
<td>(CA)4CGT(CA)4</td>
<td>GTF CCC AAT TCT ATC GGT TGT CAT C</td>
</tr>
<tr>
<td>GT A</td>
<td>CAT AAG CCT GGT GCG TGG GGA</td>
<td>(GT)12</td>
<td>TGC AGC AGA CAC AAC TAC ATC</td>
</tr>
<tr>
<td>GT E</td>
<td>GTC TGA AGC AAC TTG CAG GGG GC</td>
<td>(CA)CG(CA)(2)(CA)(5)(GA)13</td>
<td>ATC TGA GCA GAG GAT TGG AAC TG</td>
</tr>
<tr>
<td>GA C</td>
<td>AGT CCG CCG CTA GCT ATG TCA</td>
<td>(CT)112CC(CT)22</td>
<td>AGC CAT TGA ATT CGT CAC A</td>
</tr>
<tr>
<td>GCT 2</td>
<td>TAC ATC GAG ATG GAA TTT GGC G</td>
<td>(GCT)7</td>
<td>TCT CCA GCC ATC GGC TAC TCC A</td>
</tr>
<tr>
<td>GA B</td>
<td>CAT CGA ACT CCC AGT GTG ACT G</td>
<td>(CT)18</td>
<td>TAG CTT TGG TTT TGT GTC GGA</td>
</tr>
<tr>
<td>GA 62</td>
<td>CAC GTC AGA GCT CAA GTA GA</td>
<td>(GA)22</td>
<td>TCG ACA CTG GGA CAC TAT TC</td>
</tr>
<tr>
<td>GA 84</td>
<td>CAT ACT AGA ACC AGG CTA CA</td>
<td>(CT)4(CT)24</td>
<td>ACG GAG GCG ACA ATG GGC AC</td>
</tr>
<tr>
<td>GA 88</td>
<td>TGA GGG GCT GGG CAA AGA G</td>
<td>(GA)28</td>
<td>AGA GTC GAC CTG CAG GCA TG</td>
</tr>
<tr>
<td>GA 67</td>
<td>GAT TGG TGC AGT CGT GTC AGT</td>
<td>(GA)17CA(GA)2</td>
<td>GAC CTC CGG CAG ATG CAT</td>
</tr>
<tr>
<td>GA 75</td>
<td>ATT CGT TCA TAT ATC ACC GCT</td>
<td>(GA)23</td>
<td>TCG ACC TGC AGG CAT GCA AG</td>
</tr>
<tr>
<td>GA 136</td>
<td>CAC ACG TCC CGG TTA CTT G</td>
<td>(CT)25</td>
<td>CAC CGC TCA AAA ATA TAG AAG AGA</td>
</tr>
<tr>
<td>GT 121</td>
<td>CAC TCG CCT GCC TGC TGA A</td>
<td>(CA)36</td>
<td>TTA AGA AAA CAC CGA CAC G</td>
</tr>
<tr>
<td>GT 141</td>
<td>ACA TGG TCG AAT TTT TAT TGT ATT</td>
<td>(GA)24G(GA)</td>
<td>GGA ATT ATC ACA TAG CCA ACC</td>
</tr>
<tr>
<td>GT 143</td>
<td>CCG CAA TGT GTC TGG GGT AC</td>
<td>(GT)29</td>
<td>CAG GAT ATG CGG GTG GTG CT</td>
</tr>
<tr>
<td>GA 147</td>
<td>ATG TGG TGG GTG TGT TAA A</td>
<td>(GA)28</td>
<td>AGC GTA TGT TGG TAT C</td>
</tr>
<tr>
<td>GT 169</td>
<td>GAA GGA AGA AAA CCC CGA TGA ACT</td>
<td>(GT)12</td>
<td>AAG CGG AGA AGC AAA AAA GCA GA</td>
</tr>
<tr>
<td>GT 105</td>
<td>GGC CTA CCA TAA CAC TAC A</td>
<td>(CA)55</td>
<td>GCC AAC GCC GCA ATG GTA AT</td>
</tr>
<tr>
<td>GA 171</td>
<td>CAT CAT GGG CTT TAG GAG CAC TT</td>
<td>(CT)4(CG)(CT)14(GG)(CT)2</td>
<td>GAG AGT CGT CCT GGG AAC ATG AGA</td>
</tr>
<tr>
<td>GA 229</td>
<td>CAC CGC TCA AAA ATA TAG AAG AGA</td>
<td>(GA)25</td>
<td>CAC ACA CTG CGG CCT TAC TG</td>
</tr>
<tr>
<td>GA 242</td>
<td>GTC GGA GGC GGC AGC AAC C</td>
<td>(CT)16(C)T</td>
<td>GTC CAG CGC CAG ATT CAA CAC C</td>
</tr>
<tr>
<td>GA 249</td>
<td>ATA TAT CAC CGG TCA AAA AT</td>
<td>(GA)20</td>
<td>GGA ACT ATC ACA TGG CCA CAC</td>
</tr>
<tr>
<td>CBP8106</td>
<td>CTC CGG ATG CGG CTT TGT TT</td>
<td>(GCT)8</td>
<td>CAC CGC GCC ATC AGC ATC AG</td>
</tr>
<tr>
<td>CBP771</td>
<td>GCC GCT TAT GTC CCA TCA G</td>
<td>(CA)8(G)(CT)G(CAA)7GAA(CAA)2</td>
<td>GCA CAG GCG TCT TAT TGA AT</td>
</tr>
</tbody>
</table>

UPGMA analysis of SSR marker variation corresponded well to the breeding histories (Table 3) for the cultivars evaluated (Figure 1) with a few exceptions. These results indicate that SSR markers were effective in distinguishing between creeping bentgrass cultivars. Additionally, this research indicates that SSR markers were useful in differentiating between closely related germplasm sources and could be used to supplement morphological and agronomic data used for plant variety protection and/or cultivar identification.

The UPGMA analysis resulted in 5 distinct clades with Penn A-2 forming its own clade (Figure 1). The first clade was made up of Crenshaw and Penncross. This grouping is expected based on the breeding history since
of Crenshaw trace to Penncross. This first clade is related to the second clade which was made up of Penneagle, Pennlinks, Penn G-6, Seaside and Penn A-4. This is also not surprising since most of these cultivars were developed by the breeding program at Penn State University and could have shared similar germplasm sources. The cultivar, Penneagle traces some of its origin to Seaside (Figure 1) which is consistent with the placing of Seaside in this grouping. Penn G-6 and Penn A-4 were selected from Augusta National Golf Course which was originally seeded to Penneagle and overseeded with Penncross. According to the breeding history of Pennlinks, the maternal clone does not trace to any other known cultivars. However, Pennlinks was developed at Penn State University and may share some common pollen sources with other ‘Penn’ varieties and therefore it is not surprising that it was also included in this clade. These results are similar to other genetic analysis.

Figure 1. An UPGMA dendogram analysis of 15 creeping bentgrass (*Agrostis stolonifera* L.) populations using 409 single dose alleles from 30 SSR primer pairs. Thirty individual plants from each of the 15 bentgrass populations were evaluated for polymorphism for all 409 alleles.
conducted with other marker types (Vergara and Buhgrara, 2004; Warnke et al., 1997) although only a few cultivars used in this experiment were similar to those used in other reports. The third clade grouped Southshore with Putter, indicating that these two cultivars are distinctly different from the other cultivars in the study.

The fourth clade grouped SR 1020, L-93, and Penn G-1 together. This clade does not come together exactly as predicted by the breeding histories for these cultivars. Based on the breeding history, SR 1020 should be related to Penncross and Crenshaw, however, this cultivar shows more genetic similarity to L-93 and Penn G-1. The similarity between SR 1020 and Penn G-1 can be explained by the fact that they both trace to the Penn State breeding program. Additionally, SR 1020 and L-93 may share some common germplasm sources that trace back to Arizona. Surprisingly, Penn G-1 and L-93 showed relatively high similarity to each other. It is possible that some of the clones used in L-93 were related to either Penncross or Penneagle, (where Penn G-1 originated from) since many of the collections used in the development of L-93 came from old golf courses in NJ, NY and PA. These golf courses could have been established with similar germplasm that was used in the development of Penneagle, since Penneagle traces to popular older cultivars such as Washington, Seaside and Cocoos bent.

The placement of Penn A-2 in its own distinct clade did not correspond as predicted from the breeding history. We would expect Penn A-2 and Penn A-4 to be similar and therefore grouped together with Penneagle and other related cultivars, based on the breeding history for these cultivars. However it formed its own clade indicating that it was genetically distinct from the other cultivars. Interestingly, the morphology of this cultivar appears to be very distinct from the other Penn varieties evaluated in this study (personal observation, William A. Meyer).

The nature of breeding cross-pollinated turfgrass cultivars may account for some of the inconsistencies between breeding histories and the UPGMA analysis. Most turfgrass cultivars are composite cultivars. In this technique, parental clones with similar characteristics are chosen to cross with one another. Although the clones are selected for similar maturity, it is difficult to predict exactly when the plants will pollinate. Assortative mating can occur which results when plants flowering at the same time, pollinate each other. This can lead to the exclusion of one or more plants that have a slightly different flowering time. This can result in an unequal contribution of parent plants in the final genetic makeup of a cultivar. In addition, the parents are not saved to reconstitute the cultivar in composite cultivar breeding. This makes it difficult to predict the genetic contribution of an individual cultivar. Breeding histories typically trace back maternal germplasm and do not always include paternal parent information. These factors could have resulted in a cultivar with slightly different genetic makeup than the breeding history predicts. Other genetic diversity studies with perennial ryegrass also did not find complete congruence between breeding histories and dendograms (Huff, 1997; Kubik et al., 2001).

Additionally, UPGMA analysis assumes neutral evolution. That is to say, it assumes equal rates of mutation among all taxa. Creeping bentgrass cultivars experience cycles of phenotypic recurrent selection. The UPGMA clustering method is very sensitive to evolutionary rates that are not neutral, and can result in an erroneous topology. (Opperdoes, www.icp.ucl.ac.be/~opperd/private/)
upgma.html). Similarly, most phylogenetic methods assume bifurcation, not reticulation of taxa, but creeping bentgrass breeding is the result of recurrent selection from a pool of similar germplasm, which do not represent bifurcating lineages, similar to perennial ryegrass breeding (Kubik et al., 2001). Another type of analysis with different assumptions may reveal different genetic relationships, however there are assumptions made with all types of genetic analyses. The comparison of the results from two different analysis methods may be a better indication of true genetic similarities and differences between populations.

The principal component analysis provides an alternative view of the genetic distances among creeping bentgrass cultivars compared to the UPGMA dendogram (Figure 2). The first three eigenvectors accounted for 69% of the variation. The PCA graph corresponded very well to the UPGMA cluster analysis. However, instead of five main groups there were four. The largest group included the cultivars, Penn A-4, Penn G-6, Penneagle, Seaside, Pennlinks, Crenshaw and Penncross which corresponded to the first two clades in the UPGMA dendogram (Figure 2) and comprised mainly cultivars developed in the Penn State breeding program. The second group included Southshore, Putter, and the two populations from Stanton Ridge Golf course (described below) while the third group contained L-93, Penn G-1 and SR 1020. The cultivars in these two groups segregated into the same two clades in the UPGMA tree (Figure 1). Additionally, Penn A-2 was very distinct from the other cultivars, as also observed with the UPGMA tree. The similarity between these two analyses supports the genetic diversity results observed in this study.

Pairwise population comparisons were examined using Analysis of Molecular Variance (AMOVA) (Table 4). The AMOVA indicated that most (73%) of the molecular variation in *A. stolonifera* populations exists among individuals within populations, with lesser amounts among populations (27%). Permutation tests (based on 999 permutations) suggest that the overall $\Phi_{PT}$ was significantly different from the null

![Figure 2. A three dimensional principal component analysis of 15 creeping bentgrass (*Agrostis stolonifera* L.) populations using 409 single dose alleles from 30 SSR primer pairs. The percentage of variation attributable to each of the first three eigenvalues is presented on each corresponding axis. The circles encompass the four major groups of creeping bentgrass populations. Cultivar names are abbreviated due to space restrictions. SR11 and SR15 refer to the two samples collected from Stanton Ridge Golf Course in 1999.](image-url)

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Est. Var.</th>
<th>%</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among Pops</td>
<td>14</td>
<td>3857.3</td>
<td>275.5</td>
<td>8.9</td>
<td>27%</td>
<td>0.001</td>
</tr>
<tr>
<td>Within Pops</td>
<td>411</td>
<td>9826.8</td>
<td>23.9</td>
<td>23.9</td>
<td>73%</td>
<td>0.001</td>
</tr>
<tr>
<td>Total</td>
<td>425</td>
<td>13684.1</td>
<td>32.8</td>
<td>100%</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Stat Value</td>
<td></td>
<td></td>
<td>P(rand &gt;= data)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Phi_{PT}$</td>
<td>0.271</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
distribution ($\Phi PT = 0.27, P = 0.0001$) (Table 4), which indicates the differences among cultivars are significant. Similar results were observed in other outcrossing species including buffalograss (*Buchloe dactyloides*) (Huff et al., 1993; Peakall et al., 1995), *Physaria bellii* (Kothera et al., 2007), and perennial ryegrass (*Lolium perenne*) (Kubik et al., 2001).

Future research should include conducting this analysis with additional cultivars. New cultivars have been developed, since this research was conducted. It will be important to understand the genetic relationships between the newly released cultivars and older cultivars to add to the database for future research, optimize blending recommendations, and assist with parent selection in breeding programs. Additional molecular markers should be developed to improve the resolution of cultivar identification so that in the future molecular markers could be used to quickly identify cultivars. It will also be important in the future to conduct additional research to determine the best analytical method for cultivar identification and breeder protection.

**Cultivar Identification**

We were interested to know if we could determine the cultivar composition of the two golf greens at Stanton Ridge golf course by using 30 SSR markers. In both the UPGMA dendogram (Figure 1) and the PCA analysis (Figure 2) the Stanton Ridge samples (SR11 and SR15) showed a high similarity to Putter and Southshore, which were two of the cultivars originally used on the golf course. Interestingly, the samples did not show a high similarity to L-93, the cultivar that was being overseeded. These results indicate that the superintendent’s overseeding program has not proven effective to date. The results of this study have an important practical application for golf course superintendents. These results indicate that SSR markers can be used to identify cultivars of unknown origin or unknown source and could be a very effective tool for turfgrass scientists and turf managers in the future.

**CONCLUSIONS**

This publication provides the first publicly available SSR primers for creeping bentgrass. These markers can be utilized by other scientists to create linkage maps and conduct additional genetic diversity studies. The SSR markers identified in this report showed a high level of polymorphism among 15 creeping bentgrass populations and were useful in discerning genetic relationships between the populations of creeping bentgrass evaluated in this study. The genetic relationships found between the 13 cultivars and the two Stanton Ridge putting green samples were the same in both the UPGMA and PCA analysis which support the results observed.

Overall, four main groups were visible. The first group consisted of cultivars developed by the Penn State University creeping bentgrass breeding program and trace to Penncross, Penneagle and/or Seaside. The second group, consisting of L-93, Penn G-1 and SR 1020 may be more genetically related than their breeding histories predict. The third group included Southshore, Putter and the two populations from Stanton Ridge Golf Course. These results indicated that the two putting green samples from Stanton Ridge seem to be closely related to Southshore and Putter. This indicates SSR markers could be useful in answering practical management issues related to cultivar identification. It is our goal to develop additional SSR markers that can be further applied to practical turfgrass management issues, and help to discern the best analytical methods for understanding
genetic relationships and identifying cultivars in cross pollinated species.

ACKNOWLEDGEMENTS

The authors would like to thank the United States Golf Association and the Rutgers Center for Turfgrass Science for funding this research. They would also like to thank Crystal Rose-Fricker and Leah Brilman for Certified seed of the creeping bentgrass cultivars used in this study. They authors would further like to thank Leah Brilman for assistance with breeding history information.

REFERENCES


