Progress report for the research project entitled:

Identification of creeping bentgrass (*Agrostis palustris* Huds.) cultivars using simple sequence repeats (SSRs)

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EXECUTIVE SUMMARY

Title: Identification of creeping bentgrass (*Agrostis palustris* Huds.) cultivars using simple sequence repeats (SSRs)

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Project Description: Over a two year period we have proposed to isolate genetic markers called simple sequence repeats (SSRs) for cultivar identification in creeping bentgrass. Once isolated, the SSRs will be used to study diversity within and among creeping bentgrass cultivars. We also propose to test the utility of the SSRs across *Agrostis* species.

How Ours is Different: SSRs have not been used for distinguishing among creeping bentgrass cultivars. Cultivar identification has been done using isozymes (Yammamato and Duich 1994, Warnke et al. 1997) and RAPDs (Golembiewski et al. 1997) but both methods fail to deiscriminate with certainty among closely related individuals. In addition, isozymes, RAPDs, and RFLPs often lack enough alleles at a given locus to be used as linkage triats for mapping (Lin et al. 1996). We have already studied the utility of SSRs for cultivar identification in perennial ryegrass (Kubik et al. 1999) and have found them useful for establishing genetic relationships and for distinguishing closely related perennial ryegrass clones. We have also determined that the optimum sample size needed to represent a perennial ryegrass cultivar is at least 20 individuals and the optimum number of SSR loci necessary to represent a cultivar is 15. This work should be applicable to our studies in creeping bentgrass.

Potential benefits: We hope this research will result in tools that will help with the following: 1) maintenance of varietal purity 2) protection of breeder and consumer rights 3) assessment of diversity within and among creeping bentgrass cultivars as well as other *Agrostis* species, 4) identification of persisting clones in an overseeded green. This might give insight into which clones are superior competitors and are most adapted to their surroundings and 5) while not proposed as an objective in this study, once initially isolated, SSRs will also be useful for linkage mapping which will make marker assisted breeding possible.

Deliverables: Markers that can be used by the entire turfgrass community, peer reviewed journal articles, data interpretation and data analysis regarding the cultivars used in this study

Budget Summary: Year 1: $24,940 Year 2: $24,940 **Total**: $49,880

Introduction

Current methods of cultivar identification (RAPDs, RFLPs, isozymes, and morphological characteristics) within creeping bentgrass have failed to discriminate with certainty among closely related individuals (Golembiewski et al. 1997, Warnke et al. 1997, Yamamoto and Duich 1994). There is a need for more sensitive methods to distinguish creeping bentgrass cultivars and assess genetic relationships. Simple sequence repeats (SSRs) are a class of molecular marker that are useful genetic tools in plants (Weising et al. 1989, Condit and Hubbell 1991, Akkaya et al. 1992, Morganti and Oliveri 1993, Zhao and Kochert 1993, Guilford et al. 1997, Kubik et al. 1999). SSRs are tandemly repeated DNA sequences (e.g. GAGAGAGAGA) found at many locations within the genomes of eukaryotic organisms. SSRs consist of a core repeating unit (e.g. GA) that can range from one (e.g. A...) to 6 base pairs long (e.g. GACTGT...) (Lui et al. 1995). SSRs are usually referred to in the notation (AA)n, where the nucleotides in parenthesis represent the unit that is repeating and n is the number of times the unit is repeated in a row (n=5 in the above example). One of the most unique properties of SSRs is, at a given genomic location (i.e. locus) they differ in total length (n) from one plant to another. The difference in length among different plants is referred to as a length polymorphism. It is important to mention however, that not every SSR locus in an organism's genome is polymorphic. Some SSRs do not differ in length among individuals. Their length actually remains fixed over time. These SSRs are referred to as monomorphic loci. The challenge facing researchers is to screen through an organism's genome to locate and characterize only polymorphic SSR loci. Only polymorphic loci are useful for cultivar identification and genetic profiling. It has been suggested that polymorphisms may arise from genetic mutations due to unequal crossing over or replication slippage during meiosis (Goldstein and Pollock 1997). SSR polymorphism can be easily monitored in plants using a technique called the Polymerase Chain

Reaction (PCR). PCR utilizes short sequences or primers that flank the SSR. The repeat region amplified by these primer pairs is referred to as an SSR locus.

Four general features make SSRs useful genetic markers. First, they are widely dispersed throughout eukaryotic genomes, making them useful for linkage mapping (Weissenbach et al. 1992, Bell and Ecker 1994, Senior and Heun 1993, Broun and Tanksley 1996). Second, they can be assayed on automated DNA sequencers, making them relatively easy to score. Third, unlike other PCR based markers SSRs are co-dominant (i.e. both maternal and paternal contribution can be scored). Co-dominance is useful for identifying heterozygous individuals, making SSRs the tool of choice for genetic analysis and marker assisted selection (Hearne et al. 1992, Powell et al. 1996). Finally, some SSR loci can be highly polymorphic. Other molecular marker based fingerprinting techniques (RAPDs, RFLPs, isozymes) have been criticized for their lack of polymorphism among closely related germplasm. However, the polymorphism exhibited at SSR loci has proven useful for distinguishing among even the most closely related cultivars (Olufowote et al. 1997, Rongwen et al. 1995, Thomas and Scott 1993). Over a two year period, we propose to isolate SSRs from the creeping bentgrass genome and use them for both cultivar identification and to assess genetic diversity within and among cultivars.

Objectives

In this study we propose to: 1) construct a DNA library enriched for selected SSRs 2) isolate and characterize approximately 30 loci from the SSR enriched creeping bentgrass DNA library 3) use SSRs to explore diversity within closely and distantly related creeping bentgrass cultivars (G-1, A-2, A-4, G-6, SR1020, Crenshaw, Pennlinks, Penneagle, Seaside and L-93, and Penncross). 4) explore genetic relationships among creeping bentgrass cultivars 5) determine the

optimum sample size needed to represent a cross pollinated cultivar and 6) assess the utility of isolated SSR loci for use within other *Agrostis* species.

Since the onset of this research project on 2/1/00, progress has been made towards points one and two of the proposed objectives. The majority of this progress report will consist of procedures and results concerning objectives one and two.

Materials and Methods

A. Construction of SSR enriched DNA library

1. DNA isolation and digestion

Total genomic DNA was extracted from MCB 17, a single creeping bentgrass clone collected at Piping Rock Golf Course in Long Island, NY using Qiagen Dneasy Plant Mini Kit. DNA extracted from 10 Dneasy columns was combined, ethanol precipitated, and re-suspended in enough water to get a final concentration of 1ug/ul. 50ug of total genomic DNA was digested with Sau 3AI for 2hrs. at 37° C. DNA was size fractionated on a 1% agarose gel. DNA fragments ranging between 200- 800 bp were excised from the gel and purified using GeneClean II kit. A ligation was performed using the DNA (200-800bp) and two adaptor sequences (long adaptor – 5' phosphate-gATCCCAAgCTTCCCgggTACCgC, short adaptor – gCggTACCCgggAAgCTTgg). Prior to ligation with the creeping bentgrass DNA, the adaptor sequences were annealed to each other by heating to 95°C for 5 min. and then allowing them to reach room temperature.

2. Preparation of Dynabeads M –280 with streptavidin

Removed 200ul of magnetic Dynabeads M-280 (Dynal) and washed once with 1X BW buffer (2 X BW Buffer – 10mM Tris-Hcl pH 7.5, 1mM EDTA, 2M NaCl). The beads were re-suspended in 200ul 2X BW Buffer and combined with 400pmol 3' biotinylated oligo $(CA)_8$ that had been suspended in 200ul water. Beads and oligonucleotide were incubated at room

temperature for 1 hr. to get attachment of the oligo to the streptavidin- coated beads. The biotinyalted $(CA)_8$ oligonucleotide will capture creeping bentgrass DNA fragments containing GT repeats of varying lengths during subsequent hybridization.

3. Hybridization of target DNA to oligo- bound magnetic beads

Dynabeads were washed twice in 1X BW buffer and then suspended in 400ul 5X SSPE + .1% SDS. The supernatant was removed and the beads were re-suspended in 300ul 10X SSPE + .2% SDS prewarmed to 65° C. The bead suspension was then split into two aliquots of 150 ul. Meanwhile, 4ug of DNA from step 1 was suspended in a final volume of 150ul of water and denatured (makes double stranded DNA single stranded) by heating the sample to 95° C for 5 min. The beads (150ul) + the DNA (150ul) were mixed and allowed to hybridize for 3hrs. at 65°C with mixing every 20 min. After incubation, the beads were rinsed 2 X at room temperature for 5 min. with 400ul SSPE + .1% SDS. The beads were then washed once at the initial hybridization temperature (65°) in 2X SSPE (hot wash), once at room temperature with 200ul TE + 50 mM NaCl, and finally re-suspended in 200 ul TE.

4. Amplify enriched fraction of ssDNA (PCR)

The magnetic beads from the previous step should have ssDNA fragments bound to them containing the SSR of interest. In order to use these DNA fragments for future steps, the bond between the DNA and the beads needs to be severed. The physical bond between the two is so strong, it cannot even be broken by extreme heat (which can also damage DNA). Since the DNA cannot be physically severed from the beads, a copy of the ssDNA bound to the beads is made using one of the adaptor sequences, mentioned earlier, as a PCR primer. The PCR reaction contained 10ul of magnetic beads with bound DNA, 10uM (10ul) short adaptor, 10ul 10X PCR buffer, 2mM (10ul) dNTP, 6ul 25mM $MgCl_2$, 53ul water, and 1ul Taq (5U). Amplification was performed in a Perkin Elmer DNA Thermal Cycler 480. The parameter for amplification

consisted of a 5min. incubation at 94°C followed by a single thermal cycle consisting of 1min. at 94°C, 1 min. at 55°C, and 1 min. at 72°C. The single thermal cycle parameter was repeated 30 times followed by a final elongation at 72°C for 10 min.

5. Southern blot

To be sure the DNA fragments captured on the magnetic beads contained predominantly GT SSRs, a portion(10ul) of the PCR reaction was run on a 1% TAE agarose gel along with a 1:2000 dilution of the initial ligation reaction and 2ul of the "hot wash". The DNA on the agarose gel was transferred to a Hybond N+ membrane (Amersham) by gravity flow and then fixed to the membrane using a UV Stratlinker. Hybridization was performed at 42°C using a DIG labeled $(GT)_{10}$ oligonucleotide probe. The membrane was developed using DIG non-radioactive detection kit. The signal from the PCR reaction should be much greater than that from either the "hot wash" or the ligation if the enrichment was successful.

6. Clean- up of PCR and removal of adaptors

The remainder of the PCR reaction (90ul) was ethanol precipitated and dried to remove any excess adaptors, dNTP and Taq. The DNA was re-suspended in 45 ul of water and re-digested with Sau3AI to remove the adaptor sequences and thereby restoring the sticky ends. The restriction digest was performed at 37°C for 2 hrs. The reaction was then heated to 65°C for 20 min. to inactivate Sau3AI.

7. Ligation, packaging and plating

15 ng of DNA (with an average insert size of 450bp), from the previous step, was ligated to 1 ug of lambda ZapExpress vector pre-cut with BamHI (Stratagene) using 2 U T4 DNA ligase and 1ul of 10X ligase buffer. The total reaction was 10 ul. The reaction was incubated overnight at 4°C. 1 ul of this ligation reaction was packaged, according to manufactures instructions (Stratagene), using 6ul of Gigapack III Gold Packaging Extract (Stratagene). 1 ul of the

packaged reaction was combined with 200ul XL1–Blue plating cells (OD_{600} of .5) and incubated at 37°C for 15min. The entire contents of this reaction was mixed with 6ml of NZY Top Agar (pre-warmed to 48°C) and plated on a NZY agar plate. After the top agar set, the plates were inverted and incubated overnight at 37°C.

8. Choosing plaques and sequencing

Several hundred well separated plaques were picked off NZY plates using sterile, 1000ul pipet tips that have had the ends cut to increase the 'bore" diameter. Plaques were placed into polypropylene tubes containing 500ul SM Buffer and a few drops of chloroform. Individual phage clones were stored at 4°C. In order to sequence individual plaques, a single clone excision protocol was performed using a protocol from Stratagene (Instruction Manual ZAP Express predigested vector kit). Once colonies for each plaque were generated, individual colonies were grown in LB broth containing Kanamycin (50ug/ml). DNA was isolated from the cultures using Qiagen Miniprep Spin Kit. Samples were then sequenced using either the ALFexpress or ABI 373 automated DNA sequencers

9. PCR primer selection and testing for SSR polymorphism

Sequence data from clones containing SSRs were analyzed for primer selection, and PCR primers were chosen to flank the SSR. The PCR primers designed to flank the SSR, were first tested on DNA from MCB 17, the original creeping bentgrass clone from which the library was made. Polymerase chain reactions contained 10mM Tris-HCl pH 8.3, 50mM KCl, .25mM each dNTP, 12.5 pmol each oligonucleotide primer, .5 units of Amplitaq DNA polymerase, 50ng template DNA, and between 1mM and 4mM $MgCl_2$, depending on the primer combination, in a total volume of 25ul. 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. After PCR conditions were optimized on MCB 17, we assessed SSR variation in a

preliminary creeping bentgrass population consisting of 4 single plants (MCB17, a single clone from the cultivar Penncross, a single clone from the cultivar Crenshaw, and a single clone from the cultivar L-93.) DNA was extracted from each clone using .1g of leaf tissue and the Qiagen Dneasy Plant Mini Kit. PCR was performed with annealing temperatures and $MgCl_2$ concentrations found to be optimal in MCB 17. The PCR products were visualized on a 4% agarose gel and the level of polymorphism (if any) was assessed.

Results

A. Constructing an SSR enriched library

The library enrichment protocol described in the Materials and Methods section has been attempted nine times. Thus far, library construction has been considered unsuccessful. Currently there is a DNA library enriched to some extent for (GT) SSRs, however, it has three major problems. First, the level of enrichment for (GT) SSRS is very low (<10%). The authors of the enrichment protocol suggest the library should be between 30 and 70% enriched for the desired SSR. A library with a low percentage of enrichment requires researchers to take an additional library- screening step which is quite laborious and expensive. Secondly, 50% of the clones in this library contain inserts < 200bp. This would suggest that the creeping bentgrass genome might have frequent Sau3AI restriction sites in its genome. Different adaptors containing internal BamHI sites (long adaptor 5' P-GATCGGTGAATTCGGCTCCTAGGCTG, short adaptor – CCACTTAAGCCGAGGATCCGA) were substituted for the adaptor sequences described in the Materials and Methods section of this report. This was supposed to alleviate the Sau3AI problem, but success has not yet been attained with these adaptors. They seem to have their own set of different problems. Thirdly, many of the clones in the library are redundant. That is to say, the same few SSRs are being over-represented in the library making it difficult to isolate any unique SSR loci to use for fingerprinting.

In the many attempts to construct an enriched library, sequence data was generated for many plaques. Plaques were sequenced to understand the dynamics of each library in hopes of trouble shooting some of the many problems. During these attempts a total of 139 clones were sequenced. 15 of these 139 clones happened to contain (GT) SSRs and PCR pimers were designed for 12 of these SSR loci. Also, an initial attempt was made at screening the most recent GT "enriched" library constructed. 1,188 colonies from this library were screened using a $(GT)_{10}$ probe. 47 positive clones were identified, isolated and sequenced, however only 10 unique SSRs were identified. The remainder of the sequenced clones contained redundant SSRs. Due to the redundancy of the library, additional library screening was abandoned due in large to the time and money screening and sequencing would waste. Despite the lack of success to date, PCR primers were designed for 22 unique SSR loci (12 from failed library construction attempts and 10 from library screening). The primer sequences are listed in Table 1.

It is important to remember that not all SSR loci isolated are useful for DNA fingerprinting. Some loci show no differences among different plants in a species and are therefore monomorphic and not at all informative for our purposes. In order to test the utility of isolated SSR loci for fingerprinting, they must be amplified, using PCR, on a test population of creeping bentgrass individuals. The 22 loci in Table 1 were tested on 4 individual clones (mentioned in M&M section) and their polymorphism was assessed (Table 2). Of the 22 primer pairs designed to flank SSR loci, only 2 (CB 9 and CB 18) proved to be polymorphic or show differences among the individuals in the preliminary screening population. 10 of the 22 loci were monomorphic (the same size in all individuals) in the test population. 2 primer pairs exhibited complex banding patterns, 4 primer pairs did not amplify an SSR locus and 4 primer pairs are still questionable and will require repeat testing.

Discussion

Currently, an SSR enriched library has not been successfully constructed. Attempts are being made to troubleshoot, however, if the problems are not resolved shortly, Dr. Bill Meyer and myself will send MCB 17 DNA to GIS Inc. (Genetic Identification Services). This is a commercial corporation that specializes in making SSR enriched DNA libraries in a vast number of plant and animal species. If it is necessary to do this, Dr. Meyer will pay for these services out of his budget. Meanwhile, we have successfully isolated and characterized two SSR loci (CB 9 and CB 18) from the creeping bentgrass genome that can be used for cultivar identification. Since our goal is to isolate 30 polymorphic SSR loci, there is still a tremendous amount of work to be done. Future efforts will concentrate on isolating the additional SSR loci necessary to start profiling creeping bentgrass cultivars.

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Table 1: Sequences of 22 primer pairs from creeping bentgrass

Locus	5' primer	Microsatellite	3' primer
CB1	TCgTCAATCTTgAAACTAC	$(GT)_3G(GT)gAT(GT)_3gC(GT)_2$	CTCCgAgTAAgCCACAT
CB3	gAATCCTCgACCgATATC	$(CA)_5$	ACCAAAAggCgCTCCAgCT
CB4	TACggTAAACATCTgATTC	$(GT)_6$	gACATgCACgACCACATC
CB5	gAACCCATgACCTgCCgCTg	$(CT)_5(CA)_5CT(CA)_4CT(CA)_3AgATAT(CT)_7$	gTTATCCCTgCggCAAACggTg
CB6	TgACTCTCgCTTgggCTTC	$(GT)_2g(GT)_3g(GT)_2ggTA$	TCAAACCCTTACATTCCACAg
CB7	CAgCATgCATggACTg	$(CA)_4CTTAA(CA)_2TACTACACg(CA)_7$	AgggTCCATATgCCATTg
CB8	TCAgTTTgTTTTggTTAAg	$(GT)_6gC(GT)_2(GA)_3AA(GA)_4$	CTCTAgAAgTACTCTCgAg
CB9	TaggCTCgACATCACCgCgT	$(CA)_4CgT(CA)_4$	gTTCCCAAgCTCATCgTTCATC
CB10	gTTggACCggTTAgTCAC	$(GT)_{16}$	gAACgAATgAggAAACgA
CB11	CTgCATgTATAgTCgTCgTAT	$(CA)_{10}$	ACgCACTCgCATgTgTATTC
CB12	ATCTTgAAACTACgTgAC	$(GT)_3ggTgAT(GT)_3gC(GT)_2$	AgTggTCTgACTAATgTAg
CB14	gAATgATgCCCCTAgTgTA	$(GT)_{19}$	TCATCATCgTCATTACAggT
CB15	ACATAgTggTCTCAgCAg	$(CA)_6Cg(CA)_7TA(CA)_2$	gTAgTCTTACggAATTgggCT
CB16	TACCggAAgCTTgggATCAgA	$(CA)_9$	ATgTAgCATTCgTggCAgC
CB17	TCTTCTTCTCTCTCAATCT	$(CA)_{12}$	ACAATgTAgTCTCTgTggTA
CB18	gATAgTCTACTTAgTTCA	$(GT)_{11}TTTA(GT)_{13}$	CTCATgATggTgCgTgAAg
CB19	ACCATTggTTCAAgAAC	$(CA)_4$	gATAgTCTACTTAgTTCA
CB20	gTAgggTTggACCggTTAgT	$(GT)_{16}$	TAAgAgAgggCgAACgAATg
CB21	AgTTCATCgAgCCAgTCggA	$(CT)_{28}CA(CT)_2(CA)_8TT(CT)(CA)_4TT(CA)_2$	CATATCATTTgAgCggA
CB22	CAgCTTATTTggAgAACCT	$(CA)_{12}CT(CA)_6gA(CA)_9CT(CA)$	TgCAgACgATggAATATgAATTAg
CB23	gTCgTCgTATAgTAgTgTgCAT	$(CA)_{10}$	CACTCgCATgTgTATTCA
CB24	CATTgAgTTgTTgTTTgTTTA	$(GT)_9gC(GT)_5$	ATTCTATgATAggAAggTAg

Table 2 : Screening 22 primer pairs for polymorphism on a preliminary creeping bentgrass test population

Locus	Size(bp) MCB 17*	PCR status
CB1	169	monomorphic
CB3	124	monomorphic
CB4	134	momomorphic
CB5	187	no amplification
CB6	128	monomorphic
CB7	143	no amplification
CB8	141	no amplification
CB9	127	polymorphic
CB10	134	no amplification
CB11	198	monomorphic
CB12	139	monomorphic
CB14	137	questionable
CB15	164	complex pattern
CB16	152	complex pattern
CB17	161	questionable
CB18	126	polymorphic
CB19	96	questionable
CB20	120	questionable
CB21	179	monomorphic
CB22	153	monomorphic
CB23	182	monomorphic
CB24	125	monomorphic

* size in bp was determine br looking at sequnce data from MCB 17