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Date July 28, 1993

DNA AMPLIFICATION FINGERPRINTING OF CENTIPEDEGRASS

A Thesis

Presented for the

Master of Science Degree

The University of Tennessee, Knoxville

Kristal Rebecca Weaver

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DEDICATION

This thesis is dedicated to my grandparents

Mr. James 'Pap' Webb

and

Mrs. Anthony 'Mamaw' Webb

they gave me the love, support and the confidence in my own abilities to achieve anything that made this possible.

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ABSTRACT

The DNA amplification fingerprinting (DAF) method was used to characterize five cultivars of centipedegrass. The technology was optimized for centipedegrass through experiments testing the parameters for each reagent . The optimal reaction mixture was found to be: 200 μM each dNTP, 1x buffer stock, 3 μM primer (octomer), 0.05-0.15 ng/μl volume template, 1.5 mM $MgCl_2$, and 0.3 units/μl reaction enzyme (Ampli*Taq* Stoffel fragment polymerase).

After optimization, five centipedegrass cultivars ('Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial' and 'Tifton common') were separated using DAF with primers 8.6d (GTAACGCC) and 8.6i (GTTACGCC). Studies of endonuclease digestion prior to amplification found the following endonucease-primer combinations were successful in separating centipedegrass cultivars: *Msp*I-8.6i, *Hinf*I, *Msp*I-8.6h and *Hinf*I, *Alu*I, *Rsa*I-8.6h.

Amplification patterns were scored and a phylogenetic analysis was conducted to explore relationships of the five cultivars. The analysis found that the cultivar 'Tennessee Hardy' is significantly different from the other four cultivars, which have several potential relationships.

A technique to isolate products from silver-stained polyacrylamide gels was developed and 5 products were isolated. Restriction fragment length polymorphism (RFLP) analysis was done on the centipedegrass cultivars using two of these isolation products as probes without further purification by subcloning. One was subcloned and the results compared. The results were similiar enough to indicate that further purification by subcloning was not necessary in order to make probes by the method used.

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CHAPTER 1

INTRODUCTION

DNA fingerprinting has increased in popularity as a diagnostic tool for genetic typing. Various methods of DNA analysis are currently being used in the investigation of capital crimes by law enforcement agencies for fingerprinting of a wide range of biological organisms, including plants, and in gene mapping applications.

There are many methods of DNA fingerprinting, each having its own unique advantages and disadvantages. Amplification techniques appear suited to strict identification or separation of two or more closely related individuals, especially when there are small DNA quantities available and/or when the DNA is degraded. DNA hybridization techniques appear suited to situations where a large amount of good quality DNA is available. Hybridization protocols (Botstein et al, 1980; Southern 1975; Jeffreys et al, 1985) have been in use much longer than the amplification techniques, but neither system is completely understood. The application of the amplification techniques (Mullis et al, 1986; Williams et al, 1990; Welsh and McClelland 1990; Caetano-Anollés et al, 1991c) will increase as more information is gained on the mechanisms involved.

Turfgrass DNA fingerprinting is still in its infant stages, but is rapidly catching up with popularity of application in other plants. Proprietary rights to certain grass cultivars for use on golf courses, athletic fields, and even on home lawns

are of great importance in the United States and other countries. Long standing grass cultivar identification methods are based on morphological description of characteristics which can be altered by climatic, nutritional, mechanical and pathological influences. DNA analysis is the "state-of-the-art" technology that identifies an organism regardless of the variations induced in its morphology.

DNA analysis of turfgrasses can not only provide protection of proprietary rights, but has applications as a breeding tool, in disease and insect diagnosis, and eventually in gene transfer of unique characteristics of economic importance, such as herbicide and disease resistance, or cold hardiness. By studying the DNA of plants possessing certain preferred traits, the genes which induce those traits can be identified, isolated and transferred to a desirable cultivar which is already established in specific use situations, for example, introducing selected herbicide resistance into non-herbicide resistant cultivars used on golf greens. The applications of DNA typing are limited only by the knowledge of the people using them and as more knowledge is gained more applications will be discovered and used.

CHAPTER 2

LITERATURE REVIEW

Since the discovery during the mid-twentieth century that genetic information is encoded and transferred in DNA, scientists have been developing methods to quantify, qualify, differentiate, transfer and replicate DNA in vitro. New methods of DNA analysis are being developed at an incredible rate and researchers are still trying to understand the mechanisms by which these techniques are driven. The first step in DNA analysis is the isolation of DNA from the cells of the organism being studied. Several DNA isolation procedures have been reported for use with various organisms (Apuya et al, 1988; Beckman and Soller, 1986; Culpepper et al, 1990; Dellaporta et al, 1983; Helentjaris et al, 1986; Sayavedra-Soto et al, 1993). These methods can be adapted at least to a specific, optimized genus and may use different reagents with varying results when applied to different organisms. Large amounts of good quality DNA are necessary for traditional fingerprinting which is difficult to obtain from some organisms (Kirby, 1990). This may be due to many factors including difficulty in separating the DNA from certain organic compounds.

Restriction Fragment Length Polymorphism

One of the first methods of DNA analysis developed at the molecular level is termed restriction fragment length polymorphism (RFLP) analysis (Botstein et al, 1980). This method identifies and separates individuals or groups of individuals

based on the presence or absence of specific DNA sequences. This was an improvement over traditional morphological and biochemical assays because it allowed more closely related individuals to be separated with greater accuracy.

The RFLP technology involved the combination of several important discoveries. Certain natural enzymes of bacterial origin known as restriction endonucleases recognize specific DNA base pair sequences and cut DNA at these sites (Zabeau and Roberts, 1979). This leaves the DNA cut released into specific size fragments that are easily separated by electrophoresis in an agarose gel medium. Separation occurs because DNA has an overall negative charge and when an electrical current is passed through a semi-solid medium containing DNA fragments, these fragments are pushed and pulled by the negatively and positively charged currents, respectively. As the fragments migrate through the gel, they separate by size because the smaller fragments are able to travel faster through the gel matrix and migrate farther than the larger fragments which stay closer to the top of the gel. The DNA is stained with ethidium bromide (EtBr), an intercolating agent which fluoresces under ultra-violet light. The DNA is then transferred from the gel to a stable Nylon membrane support (Southern, 1975), where it cannot elute or diffuse as with agarose gels. The DNA on this membrane could then be screened with cloned DNA from the same or different genomes. Clones can be made by ligating DNA into a suitable bacterial vector, and transforming *Escherichia coli* cells. The transformed *E. coli* cells are grown, harvested, the plasmid DNA isolated, and the insert purified and labeled (Morelle, 1988; Sambrook et al, 1989). Once the restricted, genomic DNA has been transferred to the membrane, the radioactively or colorimetrically labeled DNA probe is applied to the membrane under conditions which facilitate the binding of the radioactive DNA to its complementary sequence. Membrane-bound radioactive

DNA then exposes an X-ray film producing dark bands (Beckman and Soller, 1986).

Variations of the standard RFLP analysis have been developed to adapt it for more specific uses. Variable number of tandem repeats (VNTR) is a popular method of DNA typing which relies on the number and presence of tandemly-repeated minisatellite regions (Jeffreys et al., 1985; Nakamura et al., 1987). Minisatellites are regions of the genome where a sequence of DNA is repeated end to end, multiple times. The number of repeats is highly variable from individual to individual constituting a useful diagnostic tool.

RFLP technology has been used to ascertain information on many animals such as monkey (Arnemann et al, 1989), mouse (Epplen et al , 1988), birds (Burke and Brufoord, 1987) and fish (Nanda et al, 1990) with possible applications in breeding and control of wildlife poaching. Diseases such as sickle cell anemia (Kan et al, 1976), muscular dystrophy (Witkowski and Caskey, 1988), Huntington (Meissen et al, 1988) and cancer (Vogelstein et al, 1989) have been studied using RFLP technology. Other major applications involve human paternity testing (Jeffreys et al, 1986) and chromosome mapping (Watkins, 1988). RFLPs have been used in the study of plant genomes for breeding, progeny analysis, and gene transfer applications. DNA studies in rice (Dallas, 1988), apples (Nybom and Schaal, 1990), corn (Evola et al, 1986), lettuce (Landry et al, 1987), potato (Gebhart et al, 1989), soybean (Keim et al, 1990; Landau- Ellis and Gresshoff, 1992; Landau-Ellis et al, 1991) and centipedegrass (Sayavedra-Soto et al, 1993) have all used RFLP markers.

While the RFLP method has proved useful in a wide variety of situations and for DNA of many organisms, the technique has several disadvantages. The large

amount of clean, non-degraded DNA needed for RFLP analysis is not always obtainable in forensic cases or from certain plant parts at particular times of the season. For example, restrictable dogwood DNA was only isolated from young leaves in spring. Late season growth was full of phenols and polysaccharides (Culpepper et al, 1991). The time needed to obtain a DNA profile (fingerprint) can be relatively long often giving non-informative results. Radioactivity and EtBr can be hazardous to the health of the technicians working with the samples. Informative probes may not be available for a particular species being studied; their generation may take considerable time.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was first introduced in the late 1980s (Mullis et al, 1986; Mullis and Faloona, 1987; Saiki et al, 1988). This technique utilizes a DNA polymerase to make multiple copies of targeted areas of the genome which alleviates the problem of requiring a large amount of DNA. PCR relies on the ability to mimic, *in vitro*, the process whereby the cell replicates its own DNA. A DNA sample is combined with two oligonucleotide primers (20-30 nucleotides in length), free nucleotides, a buffer solution and a thermostable DNA polymerase. The reaction takes place during a series of temperature cycles which promote, in order, denaturing of the sample DNA (usually 94°-96°C), annealing of the primers (55°C), and extension of the targeted DNA (72°C). Since the replication doubles in each cycle, many copies of the targeted area are made in a relatively short time. This procedure was greatly enhanced with the use of thermostable DNA polymerases which do not have to be replenished after each temperature cycle. These are predominantly isolated from thermophilic bacteria

like *Thermus aquaticus* , a bacterium that grows in hot springs (Gelfand, 1988; Saiki et al, 1988; Dickson, 1993) or similar thermophilic bacteria.

A system that allows RFLPs to be amplified from small amounts of DNA termed AMP-FLP is one application of PCR (Allen, 1989). This allows the analysis of DNA from minute or degraded samples. The presence or absence of the product is then easily determined in a fraction of the time it would take to complete the standard RFLP screen. This also eliminates the need for radiolabeling because the product can be visualized on a silver-stained polyacrylamide gel (Bassam et al, 1991). PCR has also been applied to cloning and DNA sequencing, making these methodologies easier and less time consuming (Cooper and Isola, 1990; Herrmann et al, 1990; Kovalik et al, 1991; Parks et al, 1991).

Some advantages of PCR over the standard RFLP analysis are that it is faster, works with slightly degraded DNA, needs only small amounts of sample, and does not require radioactivity. However, there are some disadvantages. PCR requires sequence information to synthesize primers, and the acquisition of this information can be expensive and time-consuming.

Random Amplified Polymorphic DNA

In late 1990 a technology termed randomly amplified polymorphic DNA (RAPD) analysis was introduced (Williams et al, 1990). This technique utilizes one arbitrarily chosen primer which is usually ten nucleotides in length. The reaction mixture consists of 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM of each deoxynucleoside triphosphate (dNTP), 0.2 μM primer, 5 ng of genomic DNA, and 0.5 unit *Thermus aquaticus* (*Taq*) DNA polymerase. This reaction mixture is cycled for amplification (usually at 94°C for 1 min, 36°C for 1

min, and 72°C for 2 min). The products are visualized by agarose gel electrophoresis and EtBr staining. RAPD products have been used as RFLP probes to identify molecular markers (Klein-Lankhorst et al, 1991; Michelmore et al, 1991; Wiliams et al, 1990). Studies of many organisms including bacteria (Akopyanz et al, 1992), *Discula* (Haemmerli et al, 1992), soybean (Tingey et al, 1991), wheat (Devos and Gale, 1992; He et al, 1992; Vierling and Nguyen, 1992), broccoli and cauliflower (Hu and Quiros, 1991) and chickens (Owen and Uyeda, 1991) have been done using RAPD. The purpose of these studies was to determine genetic diversity and further understand genetic relationships between organisms.

Some advantages of this method are the shorter time frame involved in running the reaction as compared to the RFLP method, lack of radioactivity required in standard RAPD analysis (radioactivity becomes necessary for applications in molecular mapping), and no prior sequence knowledge of the primer is needed for primer synthesis. Drawbacks of the method are the irreproducible appearance of certain products in the reactions, only a few bands produced compared to other methods, and low product resolution due to agars gel visualization with Et BR staining.

Arbitrarily Primed Polymerase Chain Reaction

The arbitrarily primed polymerase chain reaction (AP-PCR) technique was first introduced by Welsh and McClelland (1990). This technique uses a single arbitrary primer (usually 20-34 nucleotides long). The reaction mixture contains 0.025 units of *Taq* polymerase, Stratagene Inc. buffer at 1x stock adjusted to 4 mM with $MgCl_2$, 0.2 mM of each dNTP, 10 μM primer and DNA (various concentrations). AP-PCR differs from PCR and RAPD in the cycling of the

reaction by using two low stringency cycles of 94°C for 5 min, 40°C for 5 min, 72°C for 5 min, followed by 10 high stringency cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. Next 90 µl of solution (2.25 units of *Taq* polyermase in 1x buffer, 0.2 mM dNTPs and 50 µCi α-[^{32}P] dCTP) was added to the reaction and 20-30 more rounds of high stringency cycles followed.

AP-PCR has been used to study relatedness of *Leptospira* (Ralph et al, 1993), determine parentage in maize (Welsh et al, 1991), and isolate and characterize alleles of colorectal tumors (Peinado et al, 1992). While AP-PCR has a time advantage over RFLP technology, and unlike standard PCR requires no prior knowledge of the genome, AP-PCR does require the use of radioactivity which limits its use to larger labs equipped for this type of work.

DNA Amplification Fingerprinting

DNA Amplification Fingerprinting (DAF) was introduced by Caetano-Anollés et al (1991c). This technology relies on the use of one or more short arbitrary primers of 5 or more nucleotides (usually 8) in length. The DAF reaction mixture consists of 10-20 pg of DNA, 3 µM primer, 2.5 units of DNA polymerase (either *Taq* or Ampli-*Taq* polymerase), reaction buffer [10 mM Tris·HCl (pH 8.3), 50 mM KCl, and 2.5 mM $MgCl_2$], and 200 µM of each dNTP. The reaction is cycled 30-40 times at 96°C for 1 sec, 30°C for 10 sec, 72°C for 10 sec). DAF has the advantages of being fast, not using radioactivity, rendering many products relative to other amplification methods (10-100), excellent visualization by use of polyacrylamide gels and silver-staining (Bassam et al, 1991), and requiring no prior knowledge of the genome in study . The major drawback is the time necessary to optimize the reaction for genomes of different complexity, a common feature of all the amplification technologies. Preliminary work had been done to isolate DAF

products directly from silver-stained polyacrylamide gels (Caetano-Anollés et al, 1992a). DAF has been used in typing bacteria (Bassam et al, 1992b), *Azolla-Anabaena* (Eskew et al, 1992), *Streptococcus uberis* (Jayarao et al, 1992), fungi (Trigiano et al, 1992), soybean (Caetano-Anollés et al, 1991a) and turfgrass (Caetano-Anollés et al, 1991b).

Rationale

Molecular genetics has only recently been applied to the field of turfgrass science. Applications of molecular genetics include variety protection, seed certification, gene mapping, and eventually gene transfer. Turfgrass is a multi-billion dollar industry in the United States alone (Kidd, 1993), but the industry is way behind in methods of certifying seed lots and in varietal protection rights. Centipedegrass was chosen for study because it is a single species with cross-fertilization potential, but featuring a wide diversity in cold tolerance which is a unique characteristic uncommon to this species (Sayavedra-Soto et al, 1993).

Of the variety of methods of amplification fingerprinting, DAF was chosen for centipedegrass analysis due to the advantages already discussed. Preliminary work had been done using DAF to analyze turfgrasses (Caetano-Anollés et al, 1991b) which gave a starting point for further optimization studies. Several other studies utilizing DAF were available, which further simplified and explained the technique (Bassam and Caetano-Anollés, 1992; Bassam et al, 1992 a,b; Caetano-Anollés et al, 1992 a,b).

Optimization studies were done on each reagent and on some combinations of reagents to determine the "window of operation" allowed by the technique when used on centipedegrass. These studies also allowed the technique to be directed

toward specific goals and a better understanding of the involvement of reaction components on the amplification process.

DAF bands were scored in a system where 1 = present and 0 = absent. This information was then entered into an appropriate computer program (PAUP, phylogenetic analysis using parsimony) for phylogenetic analysis of the centipedegrass cultivars. Phylogenetic trees generated this way were useful in understanding the relatedness and origins of certain centipedegrass cultivars.

In the analysis of centipedegrass, DAF generated bands were used as RFLP probes to assess what kinds of sites were targeted during the amplification procedure. RFLP methodology allowed for the determination of band content by probing genomic DNA with isolated DAF products. By quantifying many of these products, a search for single copy sequences of DNA which may constitute genetic markers could be undertaken.

CHAPTER 3

MATERIALS AND METHODS

The following five genotypes of centipedegrass [*Eremochloa ophiuroides* (Munro) Hack.] were investigated: the cultivars 'Tennessee Hardy' (L.M. Callahan, The University of Tennessee), 'Tennessee Tuff' (R. Jensen, Tifton, Georgia), 'Oklawn' (W. W. Huffins, Oklahoma State University), 'Centennial' (R. Dickens, Auburn University) and the common type 'Tifton common' (W. Hanna, Tifton Georgia Agricultural Experiment Station, University of Georgia). DNA was isolated for each and compared using DNA amplification fingerprinting (DAF) and Southern hybridization techniques. Probes for Southern hybridizations were obtained by picking and isolating DAF products and then cloning the isolated product.

Tissue Collection

Newly emerged leaves (<2 inches) were collected from plants propagated in the greenhouse with available light year-round or from field plots. Tissue collections were done in the early morning to minimize polysaccharide content and maximize DNA yield. Plant material was harvested using scissors washed in 70% ethanol, immediately wrapped in aluminum foil, and frozen in liquid nitrogen. Materials were stored at -70°C until processed.

DNA Isolation

DNA was isolated using the two-day procedure of Dellaporta et al. (1983) as modified by Sayavedra-Soto et al (1993).

Leaf blades frozen in liquid nitrogen were placed in a pre-chilled mortar and coarsely ground with a pestle. Tissue was then ground to a fine powder with 0.25 g of polyvinylpolypirrolidone (PVPP) and suspended in 15 ml of extraction buffer (0.1 M Tris·HCl, 0.2 M Na_2EDTA, 0.5 M NaCl, pH 8) with 10 µl of β-mercaptoethanol. The extraction buffer prevented the interference of oxidizers during the extraction process. The sample was thawed at room temperature and transferred to a 50 ml Oak Ridge centrifuge tube containing 1 ml of 20% sodium dodecyl sulfate (SDS) which favors the release of DNA by breaking open cell membranes. The contents of the tube were gently, but thoroughly, mixed and incubated at 65°C for 5 min, gently mixed again and incubated for an additional 15 min.

Following incubation, 5 ml of 5 M potassium acetate was added and mixed by gentle rotation until homogeneity was achieved, to help eliminate proteins and polysaccharides by complexing with the insoluble potassium dodecyl sulfate precipitate. The sample was placed on ice for 20 min and centrifuged at 18,000 rpm (JA20 Beckman rotor) at 4°C for 20 min. The supernatant was filtered using a pleated filter (#588, Schleicher & Schuell) into a 50 ml centrifuge tube containing 10 ml of isopropanol pre-chilled to -70°C. After gentle mixing, the sample was incubated at -20°C for 30 min.

The precipitated nucleic acids were collected by centrifugation at 16,000 rpm (JA20 Beckman rotor) at 4°C for 20 min. The supernatant was discarded, the

tubes inverted in a slanted centrifuge rack and air-dried for 10 min. Residual isopropanol was removed by blotting with a paper tissue. The nucleic acids were redissolved in 0.7 ml of TE buffer (10 mM Tris·HCl, 1 mM Na_2EDTA, pH 8) and incubated overnight at 4°C.

The next day, the tube was incubated at 65°C for 10 min to ensure the DNA pellet was dissolved completely and centrifuged at 5,000 rpm in a Labfuge-B (1 second). The supernatant was transferred to a 1.5 ml Eppendorf tube using a wide bore 1 ml pipette tip, centrifuged 10 min at 14,000 rpm in a bench-top centrifuge (at 4°C), and the supernatant transferred again. The nucleic acids were precipitated with 75 µl of 3 M sodium acetate (pH 7.0). The tube was gently shaken to mix the salt before adding 500 µl of isopropanol chilled to -70°C. Samples were incubated for 2 hours at -20°C, and centrifuged at 14,000 rpm at 4°C for 20 min. The pellet was washed twice with 70% ethanol pre-chilled to -20°C, air-dried or spun in a preheated Eppendorf Speed Vac until the residual alcohol evaporated. DNA was resuspended in 100 µl of deionized water and stored at 4°C until analysis.

DNA Quantification

DNA concentrations were measured using a dedicated mini-fluorimeter (TKO 100, Hoeffer Scientific). A 2 ml aliquot of a dye solution [prepared by mixing 10 µl of stock Hoechst 33258 (1 mg/ml in H_2O) and 100 ml of 1x TNE buffer (100 mM Tris, 1.0 M NaCl, 10 mM EDTA, pH 7.4)] was mixed with 2 µl of the standard DNA of calf thymus to calibrate the fluorimeter, or with sample DNA to determine the DNA concentration.

DNA Amplification

The samples were amplified by the DNA Amplification Fingerprinting technique developed by (Caetano-Anollés et al, 1991b). Prior to amplification, the template DNA was diluted to 0.5-1.5 ng/µl with sterile deionized water. The amplification components were added in the following order for a final 25 µl reaction volume: 12.75 µl sterile deionized H_2O, 2.5 µl deoxyribonucleosides (200 µM of each dNTP), 1.5 µl $MgCl_2$ (25 mM stock), 2.5 µl Reaction Buffer [100 mM Tris, 100 mM KCl (pH 8.3)], 2.5 µl Primer (30 µM octomer stock), 2.5 µl Template, 0.75 µl DNA polymerase [10 units/µl, Ampli-*Taq* Stoffel fragment (Perkin-Elmer /Cetus)].

The mixture was vortexed for 1 sec, centrifuged, overlaid with 2 drops of heavy white mineral oil (Mallinckrodt; McGaw Park, IL) and amplified (usually overnight) for 35 cycles using either a two-step cycle of 1 sec at 96°C and 1 sec at 30°C in an twin block thermocycler (Ericomp Inc.; San Diego, CA) or a three step cycle of 30 sec at 96°C, 30 sec at 30°C and 30 sec at 72°C with a Biosycler oven thermocycler (Bios Corporation; New Haven, CT).

The mineral oil was separated from the sample by adding 150 µl of chloroform. The amplified DNA (layer floating near the top) was retrieved, placed in a fresh tube and diluted if necessary.

DNA Separation by Polyacrylamide Gel Electrophoresis

Separation of DNA after amplification was done by polyacrylamide gel electrophoresis (PAGE) using a Bio-Rad Mini-Protean II gel apparatus. The gel clamp assemblies were set up in the following order: clean large glass plate, polyester backing film (GelBond PAG; FMC, Rockland, ME), 0.45 mm spacers, and small glass plate assembled under running, deionized water. All

components were pushed flush at the bottom, and locked into the assembly. These were air-dried overnight in the dark (the backing film is light sensitive). The next day the gel clamp assemblies were placed into the casting tray and clean, dry 0.45 mm ten lane Teflon combs were laid out. A gel mix was made up as follows: 4.2 g urea, 1.0 ml of TBE buffer stock [10 times stock: 1 M Tris·HCl (121.1 g/l Trizma base), 0.83 M boric acid (51.35 g/l), 10 mM $Na_2EDTA·H_2O$ (3.72 g/l)], and 1.35 ml acrylamide/cross-linker stock solution (38% acrylamide and 2% piperazine diacrylamide).

This solution was brought to a final volume of 10 ml with deionized water. While this solution was mixing, a solution of 10% ammonium persulfate was prepared. The ammonium persulfate solution (150 µl) and TEMED (15 µl) were added to the gel mix to induce polymerization. The mixture was delivered to the clamp assemblies by a 10 ml syringe attached to a 0.45 µm-pore-size membrane filter to remove any dust particles or solids present in the liquid. The Teflon combs were then put in place, and the gels set aside to polymerize.

After polymerization (usually 20-30 min), the gels were assembled on the electrode core and placed in the buffer tank. The outer and inner buffer chambers were filled with running buffer (TBE, 1 times stock), the combs were removed, wells cleaned with a 1 ml syringe by injecting buffer into the wells until clean, and the gels were pre-run for 5-10 min at 110V.

While the gels were pre-running, 3 µl of DNA sample and 3 µl of loading buffer (6 g of urea and 4 mg of xylene cyanol FF in 5 ml of deionized H_2O) were mixed in a microtiter plate. After cleaning the gel wells again, the samples were loaded using small-bore flat pipette tips. The gel was electrophoresed at 110 volts for 1-2

hours at which time the gels were removed from the rigs and placed in 5-10% acetic acid to fix the DNA.

Silver Staining of Polyacrylamide Gels

Silver staining was done according to the methodology outlined by Bassam et al. (1991). While the gels were soaking in acetic acid, solutions of silver nitrate and sodium carbonate were prepared as follows:

<u>silver nitrate solution</u>

1 g/l silver nitrate (Mallinckrodt; McGaw Park, IL)

1.5 ml/l 38% formaldehyde in solution (Mallinckrodt; McGaw Park, IL)

<u>sodium carbonate solution</u>

30 g/l sodium carbonate (Kodak; Rochester, NY)

3 ml/l 38% formaldehyde in solution (Mallinckrodt; McGaw Park, IL)

2 mg/l sodium thiosulfate (Sigma; St. Louis, MO)

Each gel requires about 100 ml of each solution. The sodium carbonate solution was placed on ice to lower the temperature to 8-10°C (necessary for optimum development). The gels were washed three times (2 min each) with 100 ml of deionized H_2O. After the last wash, the gels were impregnated with silver nitrate solution for a minimum of 20 min. The gels were quickly washed with deionized H_2O, and developed in 8-10°C sodium carbonate solution until satisfactory image contrast occurred. The developer was poured off quickly and cold 5-10% acetic acid immediately added to halt image development. After 2-5 min in acetic acid, the gels were soaked in deionized H_2O for 15 min and then allowed to air-dry. The next day the gels were photographed and stored for future reference.

Analysis of Phylogeny

After DNA amplification and separation by PAGE, the relationships of the cultivars were explored by the phylogenetic analysis using parsimony (PAUP, version 3.0s) developed by D. L. Swofford. This is a microcomputer program designed to find and calculate the relatedness of individuals based on presence and absence of bands. Bands were scored as 1 = present and 0 = absent. The data were analyzed by a heuristic search using the branch-swapping/tree bisection option to yield a dendogram.

Isolation of Amplification Products

Isolation of DAF products directly from a silver-stained gel allowed for an interesting choice of probes for use in Southern hybridization experiments. Polymorphic or monomorphic amplification products (bands) were selected, isolated and used as probes for amplification patterns and genomic DNA. A method to excise and purify a single molecular weight amplification band from a DAF gel after silver-staining was developed to determine if: a) the band was a single copy piece of genomic DNA that amplifies very efficiently; b) a sequence of DNA that has many repeats in the genome; or c) many sequences of DNA that were all approximately the same size. This information would determine if band isolation is sufficient or if classical cloning is needed to make useful probes from DAF products. The purified product(s) was used directly as a probe, or cloned to determine if the isolated product(s) from the band and a cloned product from the band hybridized to the same sites.

A "pure" amplification band was obtained directly from a silver-stained DAF gel by the following steps:

1. The desired band was chosen from the dry gel and was moistened with a drop of deionized H_2O.
2. The band was excised using a flamed, but cooled dissection probe and placed in an amplification reaction mix (see previous description).
3. The DNA product(s) contained in the band was reamplified with the original primer and visualized by PAGE and silver staining.
4. The gel was allowed to dry and the process repeated until only one strong band was visible in the gel.

The process usually involved 3-5 rounds of picking and amplification. If at any round no band(s) were seen, a reaction was set up using the failed reaction mixture as template. This never failed to yield bands in the next round.

Restriction of Genomic DNA

DNA was restricted as a pre-treatment prior to DNA amplification and electrophoresis of gels to be used in Southern hybridization. Restriction of DNA for DNA amplification was done under the following conditions: 50-150 ng of DNA template, an appropriate buffer for the enzyme (1x stock), 1 µl of each enzyme used (1-3), and deionized water to bring the final total volume to 10 µl. This reaction was incubated at 37°C for 1 hour and diluted to the appropriate template concentration for the amplification reaction.

Genomic DNA was restricted with various enzymes before separation by agarose gel electrophoresis and Southern hybridization. About 10 µg of DNA was added to universal buffer (1x stock), 1 µl of enzyme, 1 µl of RNAse A, and deionized water to bring the final volume to 25 µl which was incubated (at 37°C for most enzymes) for 1 hour. At the end of this time, the restricted DNA was run on a

0.9% agarose mini-gel for 75 min at 120 V to determine if the DNA was sufficiently digested for membrane transfer.

DNA Separation by Agarose Gel Electrophoresis

For Southern transfer and vacuum blotting, agarose gels were used to separate DNA products from DAF and restricted genomic DNA . Agarose is used commonly for DNA separation, and the DNA is stained with EtBr . DNA obtained from DAF reactions was separated in 2% agarose-TBE gels whereas genomic DNA was separated in 0.9% agarose-TBE gels. The agarose solution was microwaved until no solids were visible, cooled to 50°C and poured into a large gel casting tray containing a 10 or 15 tooth comb. After the gel set, the comb was removed and the gel placed in a running tray filled with TBE running buffer, a few millimeters above the gel. The gels were loaded with 24 µl of the DAF reaction and 5 µl of standard 6x tracking buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30 % glycerol in ddH_2O) or 20 µl of restricted genomic DNA and 6x tracking buffer. DNA preparations were equilibrated to 50°C for 5 min. DAF gel were electrophoresed for 2-3 hours at 120 V while genomic DNA gels for 16 hours (overnight) at 30 V.

Ethidium Bromide Staining of Agarose Gels

Gels were removed from the electrophoretic apparatus, placed in a pouring tray, and stained with a 10 µM solution of EtBr. The gel was soaked 10 min, the EtBr solution was poured into a waste container, and the gel was destained for 20 min under a stream of slowly running water (which was not allowed to run directly onto the gel surface). The gel was then placed on a UV light source for visualization and photography.

Southern Transfer and Vacuum Blotting of Agarose Gels

Transfer of DNA from agarose gel to Zeta-Probe membrane was done according to the protocol outlined in Sambrook et al (1989). A large gel was run for three hours at 120 volts the morning of the blotting for amplified DNA from a DAF reaction. For restricted genomic DNA, a large gel was run the night before for 16 hours at 30 volts. Solutions were made up one day in advance, and the membrane was cut to the appropriate size (12.1 cm x 15.3 cm). Plastic sheets with "windows" measuring 12.7 cm x 16.0 cm were set aside.

The vacuum blotting unit was set up as follows: bottom attached to the vacuum hose, white mat with the slick side up, clear plastic sheet with the "window". The pre-measured membrane was dampened by dragging back and forth in a pan of deionized water. The membrane was placed inside the "window", the gel was carefully laid on top with care taken to insure there were no bubbles that would prevent the vacuum sealing and the wells were at the top of the membrane to use as distance markers after hybridization with the radio-labeled probe. The thick plastic top was placed on the apparatus, tightly screwed down and the vacuum pump was turned on and adjusted to 40 cm of water.

To cut the genomic DNA and "drive" it through the gel, 0.25 M HCl was mounded on top of the gel . This solution was left on until the lower dye level turned yellow (which usually took 60-90 min). The solution was kept on top of the gel and recycled over the gel during this period. When the dye was completely yellow, the solution was pipetted off and discarded. The gel was gently wiped with a gloved finger to remove the excess HCl.

Because of the small size of the DAF products, 60 -90 min in HCl rendered the blots useless. To help cut and transfer the DAF generated DNA, the gel was placed in 0.25 M HCl for 5 min and blotted with NaOH only.

The 0.4 M NaOH was pippeted on top of the gel to denature the DNA and to aid in the transfer to the membrane. This solution was left in place until the dye front turned a blue-green which usually took about 30 min. During this stage, the solution was recycled back to the top of the gel as overflow occurred. After the complete color change occurred, the solution was removed and discarded. The 0.4 M NaOH was then added to twice the depth of the gel and allowed to sit covered in Saran Wrap with space for air to circulate for an hour. After the hour, the solution was pipetted off and saved.

After releasing the vacuum, the gel was carefully removed from the apparatus and discarded. The membrane was carefully lifted out and gently washed back and forth several times in a pan containing 2x SSC (3 M NaCl, 0.3 M $Na_3C_6H_5O_7 \cdot 2H_2O$) to remove any residual agarose. The membrane was placed on a sheet of filter paper to absorb most of the moisture, then placed on a dry piece of filter paper and into the UV Stratalinker 1800 for automatic cross-linking. After cross-linking, the membrane was stored between two new sheets of filter paper in a sealable plastic freezer-bag to await pre-hybridization.

Cloning

Cloning was done using the TA Cloning™ kit (Invitrogen Inc.) following the accompanying procedure. First, the vector DNA was spun briefly in a bench-top centrifuge to collect the material at the bottom of the supplied vial, and was resuspended in 8.8 µl of TE buffer (final concentration of 25 ng/µl). Then

reagents were added in the following concentrations: 7 µl sterile water, 1 µl 10x ligation buffer, 2 µl resuspended vector DNA , and 1 µl T4 DNA Ligase. This mixture was incubated overnight at 12°C.

The next day, the tubes containing the reaction were taken from the 12°C water bath, briefly spun in a bench-top centrifuge and placed on ice. A supply of 10 cm LB (Luria Broth) agar plates was made containing amplicillin at a concentration of 50 µg/ml. The LB agar medium was made, sterilized, and poured in a sterile environment. These plates were allowed to set for about 15 min with the lids ajar, and placed upside down in a 37°C incubator to facilitate drying.

While the plates were drying, 0.5M β-mercaptoethanol was allowed to thaw and 2 µl was added to a tube of provided competent cells. The ligation mixture was added in the amount of 1 µl to the competent cells and mixed by gentle tapping. This reaction was placed on ice for 30 min. The reaction was incubated at 42°C for exactly 60 seconds, placed on ice for 2 min, and finally 450 µl of the provided SOC (2% Bacto tryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM Glucose) medium was added and incubated for 1 hour at 37°C at 225 rpm in a gyrator shaker.

The sterile, dry LB agar plates were coated with 20 µl of X-Gal (50 mg/ml stock; spread on the agar surface by using an L-shaped glass rod), which was then allowed to diffuse into the agar for an hour, after which the reaction was placed on ice. Quantities of 25 µl and 100 µl were each spread using an L-shaped glass rod onto separate agar plates. These plates were inverted and incubated at 37°C overnight. White colonies that developed were chosen, picked, and spread onto fresh LB agar plates which were allowed to grow in at 37°C 12-48 hours.

Insert Isolation by Polymerase Chain Reaction

Single colonies from these plates were picked and placed in a reaction mixture containing the following: 5.0 µl deionized H_2O, 1 µl deoxyribonucleoside triphosphates (200 µM of each dNTP), 1 µl reaction buffer [10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$], 1.0 µl each PCR primer (T3-CGCAATTAACCC TCACTAAAGGG and T7-GTAATACGACTCACTATAGGGCG). The T3 and T7 primers amplify approximately 50 bp of the vector on either side of the insertion site.

The reaction was placed in the Ericomp block thermocycler and run on the following program overnight: 1 cycle of 95°C for 3 min and 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 2 min with a final "soak" cycle of 72°C for 3 min.

Once in the thermocycler block, the reaction was started by adding enzyme (Ampli-*Taq* DNA polymerase) at a concentration of 1 unit/reaction, the so-called "hot start" (Erlich et al, 1991). This was done 1 minute into the first cycle of 95°C.

Labeling of Membrane Bound DNA

DNA on the membranes generated by Southern transfer of both restricted genomic DNA and DAF products was radiolabeled with ^{32}P. The isolated products from the DAF reactions were used as template in a standard DAF reaction with these changes: $MgCl_2$ concentration of 3 mM and the replacement of the (dATP) with ^{32}P α-dATP.

At least 60 ml of pre-hybridization solution (1 mM Na_2EDTA, 0.5 M $NaHPO_4$, 7% SDS) was heated to 58°C. A Quick Spin™ Column (Boehringer Mannheim)

was mixed, opened and placed in a test tube rack to drip for about 10 min. Then 100 µl of 1x SSC was added and the column was spun in the Labfuge centrifuge for 2 min at 2,200 rpm. The liquid was then removed from the collection tube and disposed in a sink. The column was ready to receive probes.

The radio-labeled probe was removed from the thermocycler, spun down, brought to 75 µl total volume with the addition of 50 µl of 1x SSC, pipetted carefully into the column, which was loaded into a 15 ml Corning tube and placed in the centrifuge. The column was spun on 2,400 rpm for 4 min. The probe was pipetted out of the collection tube and placed in an Eppendorf tube. The volume was drawn up into a 200 µl Pipetteman and "racked" down to measure the volume of the liquid. This amount was recorded, and 1 µl of the probe was pipetted into a LSC (Beckman Liquid Scintilation Counter) vial containing filter paper. After adding 3.5 ml of scintillation fluid, the vial was placed in the LSC Scintiliator and the number of counts for the probe was measured. This number was multiplied by the total volume of the probe and this gave the total count of the probe, which was divided by the total area of the membrane to give the counts/cm^2.

The membranes containing the DNA were then placed in a plastic bag as far down and to the side as possible, and 30 ml of pre-hybridization solution was added. The bag was incubated at 58°C for 15 min to 2 hours. At the same time, the probe was heated to 95°C for 10 min. Then the probe was spun down. The membranes were removed from the oven and the pre-hybridization solution was poured off into the sink. A fresh 30 ml of pre-hybridization solution was added to the bag and the probe was pipetted into the bag. The bubbles were rolled up with a glass pipette, and the bag was heat-sealed above and below the bubbles.

The bag was then sealed on the side and as close to the top of the membrane as possible. The bag containing the membrane was then placed in a pan in the 60°C waterbath and allowed to shake gently overnight.

The labeled membrane was removed from the waterbath and the plastic bag (the radioactive liquid containing the probe was disposed of in the radioactive liquid waste container) and placed in a pan containing a 58°C 5% SDS wash solution (5% SDS, 1 mM Na_2EDTA, 0.04 M $NaHPO_4$). The membrane was then moved into a small pan containing 58°C 5% SDS wash solution and placed in the 60°C shaker bath for 30 min. This liquid was also disposed of in the radioactive liquid waste container, and the membrane placed in another 58°C 5% SDS wash for 30 min and then washed again with a 1% SDS wash. This final liquid was disposed of down the radioactive sink and the membrane was placed on filter paper to dry. The membrane was dried on two sheets of filter paper and was placed in a plastic bag, which was sealed on all four sides and the excess cut away.

The membrane sealed in the bag was then taped onto a sheet of fast blue X-Ray film (Cronex) in a Kodak X-omatic X-ray cassette, and another sheet was placed on top of the bag. The film cassette was placed in the -70°C freezer for 24 hours to allow for exposure to occur on the DAF profiles and 5 days exposure for the genomic profiles.

The X-ray cassette was removed from the freezer and allowed to come to room temperature. The cassette was taken into a dark room, where the film was removed and placed in developer (Kodak GBX developer and replenisher)) for 6 min, given a quick wash in water, placed in setting solution (Kodak GBX fixer and replenisher) for 3 min, and rinsed in running water for 5 min. At this time the film was hung up to dry and then analyzed.

CHAPTER 4

OPTIMIZATION OF TURFGRASS DNA AMPLIFICATION

I have investigated and optimized many important parameters affecting amplification and experimental reproducibility of DAF. This constitutes an essential step before widespread use and adoption of this technology for the identification of turfgrass DNA. DAF relies on the amplification of arbitrary target sites in the genome under non-stringent reaction conditions, a process that is conceptually and mechanistically distinct from the PCR. Therefore, assumptions derived from the PCR need not necessarily be extended to DAF, both for the purposes of specifying amplification reaction conditions and for understanding the basic mechanisms involved. Using the centipedegrass cultivar 'Tennessee Hardy' and the octomer primer AACGGGTG as an example primer-template combination, I optimized amplification parameters using a truncated derivative of a DNA polymerase isolated from *Thermus aquaticus* (the so-called Stoffel fragment) (Perkin-Elmer/Cetus). Amplification parameters were examined following a process of analysis where fixed parameters were kept within an optimal range. The parameters examined condition the chemical environment and the extent of the amplification reaction. They were crucial for the generation of reproducible fingerprints.

DAF profiles contain amplification products (bands) that vary in intensity (Fig 4.1). Products have been classified into primary, secondary and tertiary categories by visual examination (Bassam et al. 1991). Primary products were those most efficiently amplified, secondary products are those of intermediate intensity, and tertiary products are weak intensity products only appropriately visualized when amplification product concentration is increased. To reveal experimental consistency at the level of tertiary products, all amplification samples were electrophoresed undiluted.

Magnesium

Magnesium plays an important role in the DAF reaction because the interactions of nucleotide, primer, template and enzyme with magnesium determine the outcome of amplification. Template, primer and nucleotides are negatively charged molecules that can sequester magnesium making it unavailable to the enzyme. The recommended amount of $MgCl_2$ lies between 2.5 and 5.0 mM as listed by the manufacturer of the enzyme. The effect of magnesium concentration on the number, consistency and distribution of DAF products was tested over a range of 0 to 9 mM. Turfgrass DNA was amplified most efficiently with 1.5 to 3.0 mM magnesium (Figure 4.1). Later experiments demonstrated that 1.5 mM worked more effectively, probably because lower magnesium concentrations decreased primer mismatching, an effect which should be avoided when analyzing high complexity genomes such as centipedegrass.

Variations in enzyme, nucleotides (Figure 4.2 and 4.3) or template (Figure 4.4 and 4.5) concentrations changed their interaction with the magnesium affecting the reaction by either altering fingerprints or inhibiting amplification.

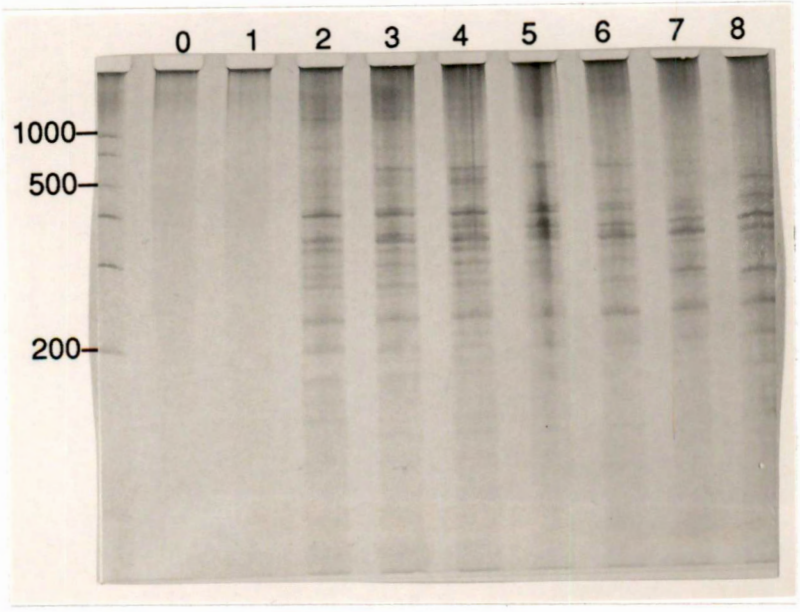

Figure 4.1. Effect of magnesium chloride concentration on DAF of 'Tennessee Hardy' centipedegrass . This gel shows the effect of magnesium chloride from 0-8 mM on a DAF reaction. The primer was 8.6c (AACGGGTG), 3.0 ng/25 µl reaction volume of DNA was amplified using the Stoffel fragment polymerase, and the amplification was viewed by 5% PAGE .

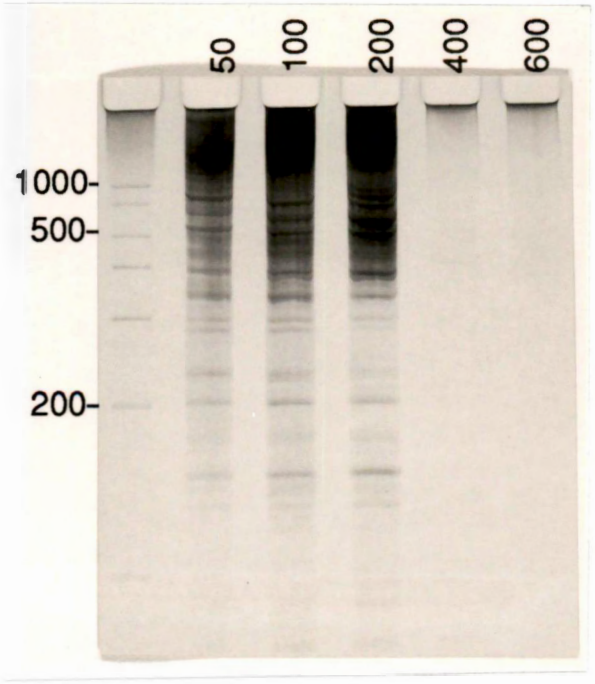

Figure 4.2. Effect of nucleotide concentrations on DAF of 'Tennessee Hardy' centipedegrass at 1.5 mM magnesium chloride concentration. Nucleotide concentration ranged from 0-600 µM of each dNTP. Primer 8.6c (AACGGGTG) was used to with the Stoffel fragment polymerase and 3 ng of DNA in the 25 µl reaction. The amplification was viewed by 5% PAGE.

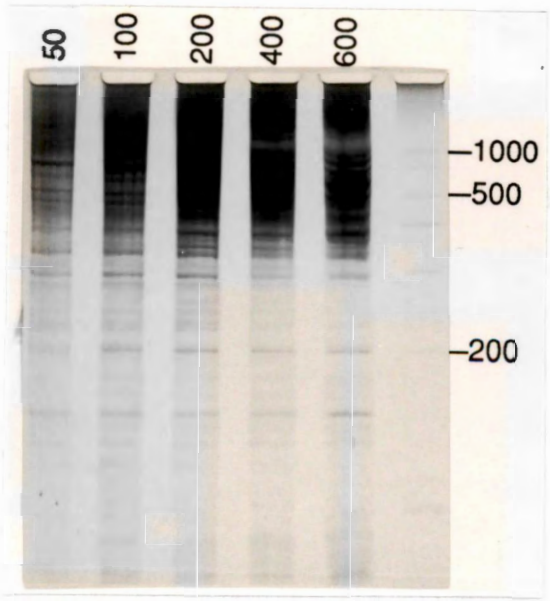

Figure 4.3. Effect of nucleotide concentrations on DAF of 'Tennessee Hardy' centipedegrass using 6.0 mM magnesium chloride.. Nucleotide concentration ranged from 0-600 µM of each dNTP. Primer 8.6c (AACGGGTG) was used with the Stoffel fragment polymerase and 3 ng of DNA in the 25 µl reaction. The amplification was viewed by 5% PAGE.

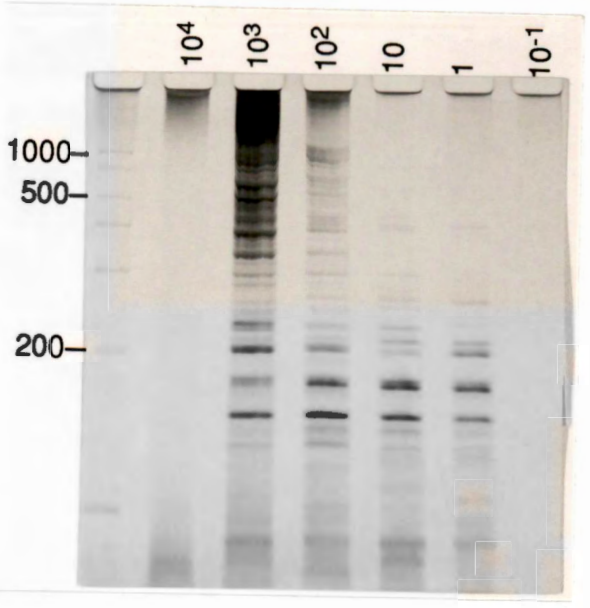

Figure 4.4. Effect of template concentrations on DAF profiles of 'Tennessee Hardy' centipedegrass using 1.5 mM magnesium chloride. The amount of template needed to react efficiently with the other components was studied using a range of 1×10^4 to 1×10^{-1} pg/µl. Primer 8.6c (AACGGGTG) was used with the Stoffel fragment polymerase and visualized by 5% PAGE.

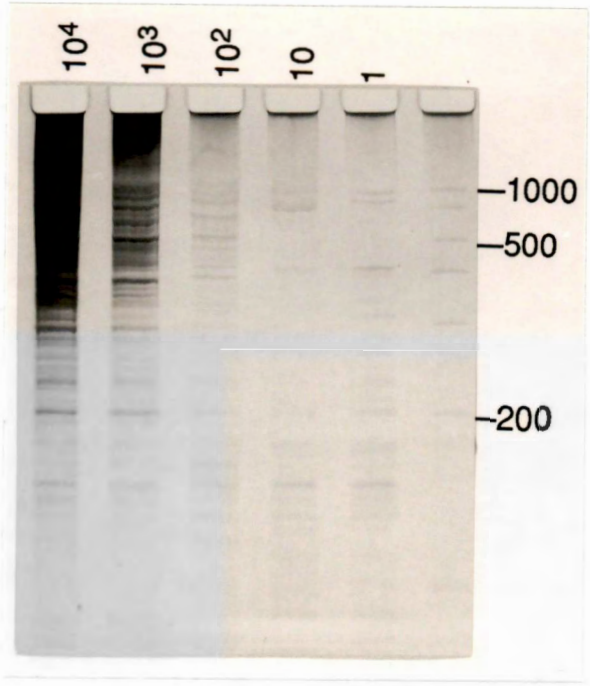

Figure 4.5. Effect of template DNA concentrations on DAF profiles of 'Tennessee Hardy' centipedegrass using 6.0 mM magnesium chloride. The amount of template needed to react efficiently with the other components was studied using a range of 1×10^{4} to 1×10^{-1} pg/µl. Primer 8.6c (AACGGGTG) was used with the Stoffel fragment polymerase and visualized by 5% PAGE.

T. aquaticus DNA polymerase requires magnesium for DNA strand extension. However, it was not known if other divalent cations could replace magnesium in the amplification reaction. DNA amplifications using calcium, barium or strontium chlorides at 1.5, 3, and 6 mM each in place of $MgCl_2$ yielded no apparent amplification products. The effect of these compounds appeared to be inhibitory. When amplifications were done in the presence of both $MgCl_2$ and either $BaCl_2$ or $SrCl_2$ at 1.5 mM, no amplification products were observed. If done in the presence of $CaCl_2$ (1.5 mM), amplification occurred but with greatly reduced efficiency (Figure 4.6). These results suggested that divalent cations blocked either primer extension, enzyme anchoring to primer-template duplexes, or both. Of these three explanations, the second appears more likely in light of the existence of amplification at reduced efficiency in the presence of added calcium. Alternatively, the small size of calcium ions may preclude a complete blockage of primer extension, allowing magnesium binding to the enzyme at only some amplification sites.

Template

Fingerprints can be produced from very small amounts of template DNA. Between 10 and 20 pg of template can be used, and sometimes less than 1 pg is sufficient (Caetano-Anollés et al. 1991c). However, very low amounts of template may not be adequate for reproducible amplification.

I examined a range of template DNA concentrations from 0.0012 (1.2 pg) to 12 ng/µl in the presence of two levels of $MgCl_2$ (1.5 mM and 6.0 mM). In all experiments, DNA was diluted to the desired concentration in deionized water using fresh dilutions every three months due to possible DNA breakdown at low concentrations in water. Amplifications with low $MgCl_2$ levels (Figure 4.4)

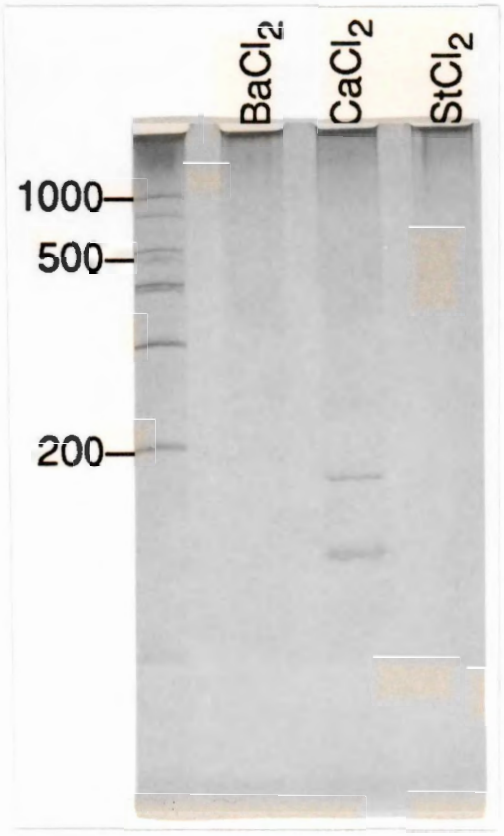

Figure 4.6. The amplification of 'Tennessee Hardy' centipedegrass was greatly affected by the addition of calcium chloride, barium chloride and strontium chloride. Primer 8.6c (AACGGGTG) was used with the Stoffel fragment polymerase, 3 ng of DNA, 200 of µM each dNTP, 1x buffer (manufacturer) and 1.5 mM magnesium chloride in a 25 µl reaction volume. One divalent cation was added to each reaction at a concentration of 1.5 mM. The reaction was visualized by 5% PAGE and preceded by a molecular weight marker.

produced consistent DNA profiles within the range 0.012-1.2 ng/μl. Amplification with 0.0012 ng/μl produced consistent major products in the lower molecular weight ranges. However, some secondary and tertiary products were lost. No amplification was observed with 12 ng/μl DNA, probably due to inhibitors left from DNA isolation or template-magnesium interaction. When high levels of $MgCl_2$ were used (Figure 4.5), consistent DNA profiles were only found within the range 1.2-12 ng/μl DNA. Lower template concentrations produced inconsistent bands, usually weaker secondary or tertiary products. The high DNA concentration in this case was not inhibitory, however, the overall range of amplification products was reduced. This smaller range of template concentrations able to support amplification may result from an increase in primer-template mismatching known to occur when high levels of magnesium are used (Bassam et al. 1992b).

Enzyme

Previous studies have shown that the AmpliTaq Stoffel fragment enzyme produced consistent amplification products from bacterial DNA over a wider enzyme concentration range when compared to the AmpliTaq enzyme (Bassam et al. 1991). I found that 0.2 units/μl of the Stoffel enzyme produced clear and consistent results. Amplification with enzyme concentrations as low as 0.1 units/μl reaction still rendered adequate fingerprints although showing the effects of overloading due to no dilution of the final amplification reaction (Figure 4.7). It should be noted that enzyme is the most expensive and important component of the amplification reaction, and it is always of advantage to decrease its concentration to a minimum. However, to avoid inconsistencies due

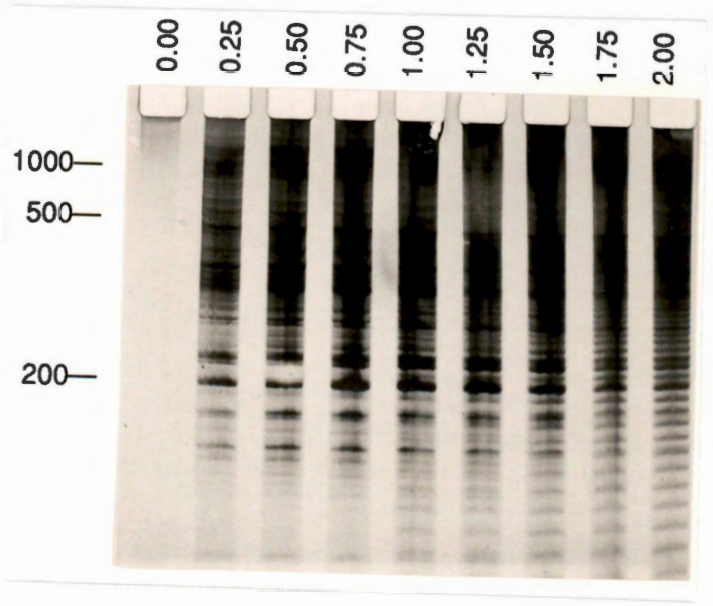

Figure 4.7. Effects of enzyme concentrations on 'Tennessee Hardy' centipedegrass DAF profiles. Enzyme concentrations of the Stoffel fragment polymerase from 0-2 units/µl of total reaction volume were used. Primer 8.6c (AACGGGTG) was used with 3 ng DNA, 1.5 mM magnesium chloride and 200 µM of each dNTP.

to batch-to-batch variations in the enzyme, low concentrations should not be used routinely. In particular, reliable results using small volume reactions (10 µl) are only achieved if the concentration of enzyme was increased (data not shown).

More than 10 units/µl of enzyme produced bands appearing at regular intervals resembling a ladder pattern. These "ladder bands" obliterate the DAF pattern rendering it uninformative. These ladder bands could result from primer-primer amplifications, from "slippage of the polymerase" (polymerase jumping during amplification), or from "out-of-register" (incorrect base pair addition during amplification) annealing of repeat unit sequences at high product concentrations (Erlich et al. 1991), and have been observed in DAF fingerprints generated using high primer and enzyme concentrations (Bassam et al. 1991).

I have encountered enzyme lots that did not amplify at all or did so with greatly reduced efficiency. This occurred infrequently. When amplifications failed for no apparent reason, the enzyme should be tested with known reagents to determine its reaction efficiency. Recently, enzyme batch-to-batch variation seems to be increasing, and it has become necessary to increase the amount of enzyme to achieve reproducible fingerprints.

Primer

To test the effect of primer levels on the amplification reaction, I tested concentrations varying from 0 to 9.6 µM (Figure 4.8). Optimal amplification profiles were obtained consistently with primer concentrations between 2.4-7.2 µM. I chose the concentration of 3.0 µM for subsequent optimizations as it was the amount of primer recommended by Bassam et al (1992b) and fell comfortably

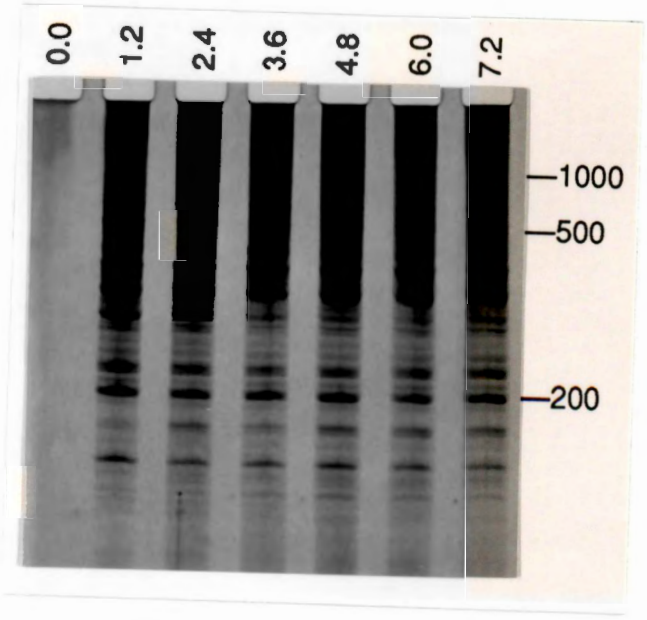

Figure 4.8. Effect of primer concentrations on 'Tennessee Hardy' centipedegrass DAF profiles. The concentration of primer was from 0-7.2 µM. Primer 8.6c (AACGGGTG) using the Stoffel fragment polymerase, 1.5 mM magnesium chloride, 1x buffer (manufacturer), 3 ng DNA and 200 µM of each dNTP in a 25 µl reaction volume.

within the "window of consistency". At concentrations lower than 2.4 μM and higher than 9.6 μM, bands started to disappear.

Nucleotides

I found that concentrations of each individual nucleotide in the range 40-320 μM rendered adequate amplifications when using 1.5 mM $MgCl_2$, the primer 8.6c (AACGGGTG) and 3 units of enzyme/25 μl reaction volume (Figure 4.2). DNA amplifications using 6.0 mM magnesium extended the optimal range to 600 μM (Figure 4.3). Control reactions without nucleotides produced no amplification products. High nucleotide levels sequestered magnesium making it unavailable to the reaction. Increasing magnesium levels then allowed the use of higher nucleotide levels. An optimized concentration of 200 μM for each nucleotide was chosen for subsequent experiments.

Buffer

I found that the amplification reaction was quite tolerant of the buffer concentration used. The recommended buffer contained 10 mM Tris-HCl (pH 8.3) and 10 mM KCl (usually a tenth of buffer stock provided by the enzyme manufacturer). The critical reagent in the buffer is the KCl as increasing and decreasing KCl effects ionic strength in the reaction. This shift in ionic strength is known to effect amplification reactions. Concentrations of both reagents within the range of 4 -32 mM sustained adequate amplifications (Figure 4.9). If no buffer was added amplification occurred in some cases, but rendered inconsistent banding patterns, sometimes unreadable on silver-stained polyacrylamide gels. If more than 16 mM buffer was used, the pattern developed extra bands in the low molecular weight regions (usually below 200 bp). Increasing buffer concentration

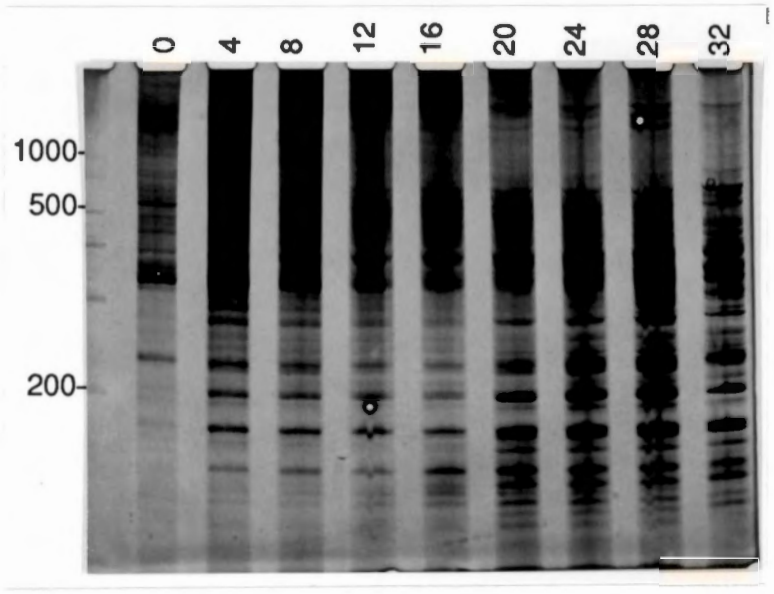

Figure 4.9. Effect of buffer concentrations on DAF profiles of 'Tennessee Hardy' centipedegrass. Buffer ranging from 0-32 mM (Tris-HCl and KCl) was examined. Primer (3 μM) 8.6c (AACGGGTG) was used with Stoffel fragment polymerase, 200μM of each dNTP, 3 ng DNA, and 1.5 mM magnesium chloride in a 25 μl reaction.

appeared to shift pattern intensity, as patterns appeared to increase in intensity below 300 bp and decreased in intensity above 400 bp.

Conclusions

I demonstrated that DAF produced reliable DNA fingerprints provided amplification conditions were carefully optimized. While fingerprints were produced without day-to-day experimental variability, even at the highest level of resolution, many factors of the amplification reaction had tremendous impact on their outcome. I examined the effects of magnesium, template, enzyme, primer, nucleotide and buffer concentration, and found that the effect of these factors was usually interrelated.

In all subsequent experiments the concentrations of reagents were as follows:

Nucleotides	200 μM of each dNTP
Buffer	1x stock
Primer	3 μM
Template	0.05-0.15 ng/μl reaction volume
$MgCl_2$	1.5 mM
Enzyme	0.3 units/μl reaction volume

The reaction was scaled down to a 10 μl total reaction volume and diluted 1:1 after amplification to enhance clarity when running the amplification on PAGE.

Magnesium, besides being a co-factor for DNA polymerase, interacts with nucleotides, primer and template. These negatively charged molecules appear to quantitatively bind magnesium, decreasing the availability of free cations and the efficiency of the amplification reaction. Therefore, any change in the concentration of nucleotides, primer or template requires a compensatory change

in the magnesium concentration. Similar phenomena have been suggested for the PCR (Saiki, 1988).

Compensatory changes in magnesium concentration may affect the specificity of the amplification reaction, generally by increasing the chances of primer-template mismatching. Therefore, these reagents must be carefully optimized by testing reproducibility of DNA fingerprint patterns. For example, high template concentrations inhibited amplification product formation, probably by sequestering free magnesium. The addition of higher magnesium levels to compensate for this effect, can in turn compromise the specificity of the reaction causing faint irreproducible patterns. Dilution of the template DNA was the best solution. If an amplification did not work when using a new template, it was usually beneficial to try reducing the concentration of the template DNA especially when using magnesium at the lower ranges and complex genomes like centipedegrass.

High magnesium concentrations were used for optimal fingerprinting of prokaryotic genomes with arbitrary primers (Bassam et al. 1992b; Jayarao et al. 1992). The number of expected amplification products (visualized as bands) obtained from bacterial genomes of about 1×10^6 bp in size when using octomer primers was almost zero (G. Caetano-Anollés, personal communication). High magnesium levels favor primer-template mismatching, increasing the number of amplification products attainable from these low complexity genomes (Bassam et al, 1992c). DNA fingerprinting of high complexity genomes, like turfgrasses, does not require high magnesium levels, since almost all amplification sites resulted from the annealing of the primer to perfectly complementary sites in the template.

The optimized amplification conditions described here for centipedegrass DNA worked well also with a variety of other turfgrass species like bermudagrass, bluegrass, and *Zoysia*.

CHAPTER 5

DNA AMPLIFICATION FINGERPRINTING OF CENTIPEDEGRASS

Five centipedegrass cultivars were fingerprinted and their phylogeny analyzed. These cultivars included one with very high cold tolerance ('Tennessee Hardy') and one with moderate cold tolerance ('Oklawn'), determined by their survival ability at 610 m (2,000 ft) elevation in outdoor experimental plots. The other three cultivars ('Tennessee Tuff', 'Centennial' and 'Tifton common') exhibited no cold-tolerance at 610 m. Centipedegrass was first introduced into the United States in the early 1900s from Southeast Asia. Until 1983 only one improved cultivar, 'Oklawn' from the Oklahoma State University Agricultural Experiment Station, had been released (Bouton et al, 1983). Centipedegrass in general is very tolerant of poor soil conditions, needs minimum upkeep, and is comparatively more resistant to disease and insect attacks than most grasses (Beard, 1973). Centipedegrass cultivars typically are not cold tolerant. They are commonly used for Deep South lawn situations. Thus, a cultivar expressing cold tolerance is considered very unique. It can be propagated either by seed but more commonly by vegetative propagules.

Preliminary identification studies of these cultivars using RFLP analysis with various probes allowed distinction of several of them (Sayavedra-Soto et al. 1993). For further cultivar identification and to establish phylogenetic

relationships, I used the optimized DAF protocol (described in Chapter 4) to amplify centipedegrass DNA. Amplification of template DNA with or without prior endonuclease cleavage, using single arbitrary primers, produced sets of discrete diagnostic markers appropriate for phylogenetic analysis (Appendix A). The result of these studies establish genetic relationships between centipedegrass cultivars which may be of importance in determining their origin and securing their identification for varietal protection.

Genomic DNA Amplification using Arbitrary Primers

The five centipedegrass cultivars were fingerprinted using a set of fourteen primers (their sequences are described in Appendix B), which produced readable and reproducible profiles (Figure 5.1). The GC contents of these primers were 50% (1 primer), 62.5% (7 primers) and 75% (6 primers). Figure 5.1 shows fingerprints generated with some of these primers. Some primers failed to produce readable fingerprints (containing too few or too many bands) and were not included in this analysis. Most of these primers had a 70-100% GC content. For clarity, only bands smaller than 500 bp in length were scored and the gels were loaded with amplification products diluted to a fifth of the original concentration. Only four of the primers produced polymorphic DNA bands. From a total of 221 bands scored that correspond to primary and secondary bands, 30 were polymorphic (ie. the band was missing in at least one of the cultivars). Polymorphic DNA then represents 13.6% of the portion of the genome sampled using this set of primers. Two of the informative primers separated all five cultivars. The other two primers, while unable to separate 'Centennial' from 'Tifton Common', produced unique fingerprints for the remaining centipedegrass cultivars.

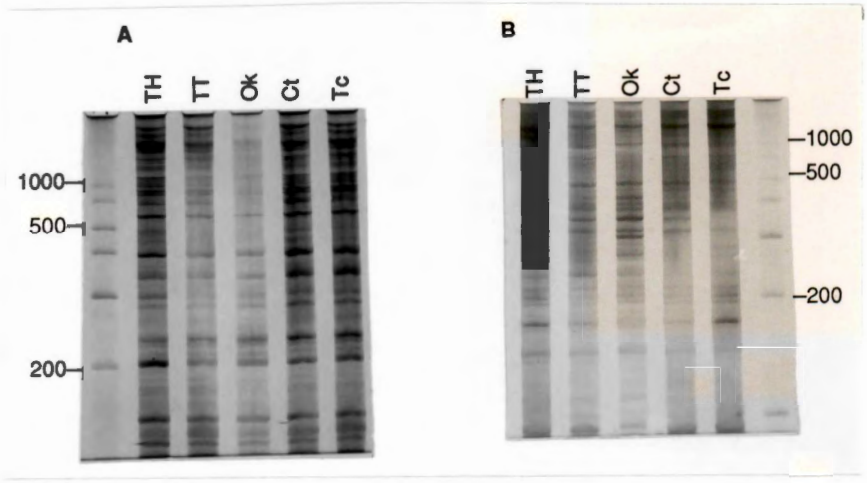

Figure 5.1. Differentiation of centipedegrass cultivars using DAF. The two panels show the amplification patterns of the five centipedegrass cultivars 'Tennessee Hardy' (TH), 'Tennessee Tuff' (TT), 'Oklawn' (Ok), 'Centennial' (Ct), and 'Tifton common' (Tc) using two different primers. Each profile was generated using 2.5-3.0 ng DNA /25 µl reaction , 200 µM of each dNTP, 1.5 mM $MgCl_2$ and 1x buffer (Manufacturer's stock). The gel in panel A is of a non-diagnostic profile where each band is found in each cultivar and was generated using the primer 8.6c (AACGGGTG). The gel in panel B is a diagnostic profile generated using the primer 8.6h (GAAACGCC).

Amplification using Arbitrary Primers following Pre-digestion of Genomic DNA

Digestion of template DNA or amplification products with several restriction endonucleases (restriction sites are listed in Appendix C) was used to increase polymorphic DNA (Caetano-Anollés et al. 1993). DAF of previously cleaved DNA allowed identification of closely related fungal isolates, plant cultivars and near-isogenic lines. I used this strategy as an additional strategy to separate the five centipedegrass cultivars.

Pre-digestion of template DNA with a single endonuclease yielded somewhat different results from amplifications of non-digested templates using the same primers (Figure 5.2). Template cleavage affected primer-template relationships by destroying amplicons (a segment of DNA flanked by primer recognition sites that can be amplified) and allowing the amplification of different genomic sites. Therefore, each endonuclease-primer combination was expected to produce a different profile.

I examined the amplification of template DNA restricted with single endonucleases in 12 primer-endonuclease combinations. Polymorphisms were detected in 11 cases. From a total of 237 bands scored (less than 500 bp in length), 42 were polymorphic. Polymorphic DNA then represented 17.7% of the portion of the genome sampled using this set of primers and cleaved template combinations.

The cultivar 'Tennessee Hardy' was separated from all of the other cultivars in four separate analysis using four endonuclease-primer combinations (Appendix A). The cultivar 'Tennessee Tuff' was separated using five endonuclease-primer

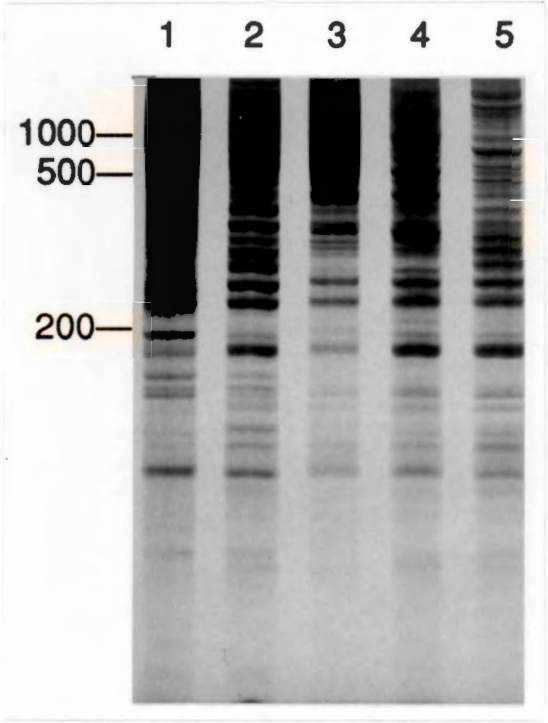

Figure 5.2. Profiles of 'Tennessee Hardy' produced using the same primer (GTTACGCC). The differences seen in the profiles are attributed to endonuclease digestion of the template prior to amplification with combinations of 1, 2, and 3 endonucleases used to digest the template DNA. Endonucleases used are *Taq*I (Lane 1), *Alu*I and *Rsa*I (Lane 2), *Bst*UI and *Msp*I (Lane 3), *Hinf*I, *Msp*I and *Bst*UI (Lane 4) and *Hinf*I, *Rsa*I and *Alu*I (Lane 5).

combinations, 'Oklawn' using three, 'Centennial' using five, and 'Tifton common' using four. Out of 12 endonuclease-primer combination analysis, 10 showed some degree of polymorphic separation between at least one cultivar and the others. The 10 endonuclease- primer combinations (*Alu*I-8.6i, *Alu*I-8.6j, *Bst*UI-8.6h, *Msp*I-8.6e, *Msp*I- 8.6i, *Taq*I-8.6c, *Taq*I-8.6d, *Taq*I-8.6i, *Taq*I-8.6h, and *Taq*I-8.7j) left 0 to 4 cultivars indistinguishable. One endonuclease-primer combination (*Taq*I-8.6h) separated 'Tennessee Hardy', 'Tennessee Tuff' and 'Tifton common' from 'Oklawn' and 'Centennial', but no individual cultivar could be identified. One endonuclease-primer combination (*Msp*I-8.6e) exhibited no polymorphisms.

Template DNA restricted with two or three endonucleases was examined in four and two primer-endonuclease combinations, respectively (Appendix A). DAF profiles generated from template digested with two endonucleases (*Bst*UI, *Msp*I-8.6i; *Alu*I, *Rsa*I-8.6i; *Hin*fI, *Msp*I-8.6h; and *Hin*fI, *Msp*I-8.6i) produced 65 bands (less than 500 bp in length), eight of which were polymorphic (12.3%). DAF profiles generated from template digested with three endonucleases (*Hin*fI, *Alu*I, *Rsa*I-8.6h and *Hin*fI, *Msp*I, *Bst*UI-8.6i) produced 35 bands (less than 500 bp in length), 6 of which were polymorphic (17.1%). One of these profiles differentiated only 'Tennessee Hardy' and 'Tennessee Tuff', while the other was additionally able to differentiate the cultivar 'Oklawn'.

Phylogenetic Analysis

The results of the primer screen and band scoring were entered into the PAUP computer program for phylogenetic analysis (Appendix A). These were entered as three data sets, each producing a phylogram portraying the probable phylogeny. The three data sets were: non-digested, pre-digested (endonuclease-

primer combinations), and a combination of both. The calculations were based on the presence and absence of specific DAF markers among the different cultivars. Three phylogenetic "trees" were generated by the heuristic search using the branch swapping/tree bisection option using the PAUP program developed by D. L. Swofford (PAUP, version 3.0s).

The first phylogram produced represented all of the data generated by DAF of undigested templates (Appendix A). There were 221 bands scored and subsequently analyzed by PAUP. The analysis linked 'Tennessee Tuff' and 'Oklawn' together and placed 'Tennessee Hardy' as having the most distant relationship to the other four cultivars. (Figure 5.3).

Analysis of data generated by endonuclease-primer combinations produced another tree slightly different from the tree generated by the analysis of undigested template analysis (Figure 5.4). This set of data is, unlike the previous set with no endonuclease digestion, not arbitrary. Primers chosen for use in most cases were ones which showed polymorphisms with the undigested template amplifications. This would tend to shift the data and would explain the major differences in the branching patterns with the exception of 'Tennessee Hardy' which remains distant from the other cultivars. In this tree 'Oklawn' is most tightly linked to 'Tifton common', and 'Tennessee Tuff' the most distant relative to the other four cultivars. The cultivar 'Tennessee Hardy' remained significantly distant from the other four cultivars.

When both sets of the data (Figure 5.3 and 5.4) were analyzed together, there was a total of 458 bands scored (Appendix A) and the phylogentic analysis generated two possible trees. A search for a consensus tree was carried out and one tree

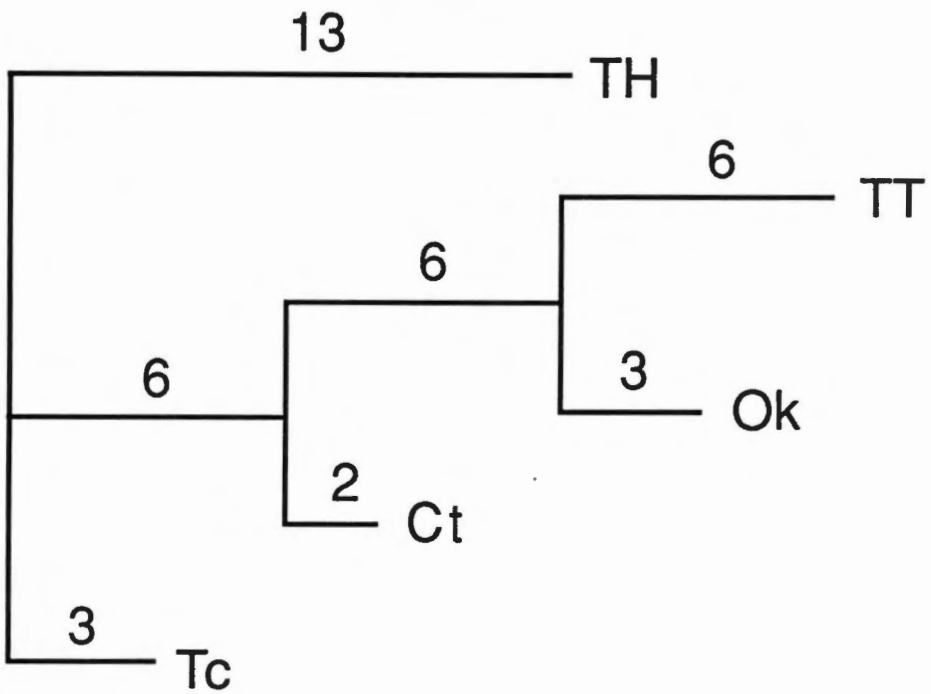

Figure 5.3. Phylogenetic tree generated by PAUP for five cultivars of centipedegrass. The PAUP computer program produced one possible unrooted tree from markers generated by amplification of five centipedegrass cultivars 'Tennessee Hardy' (TH), 'Tennessee Tuff' (TT), 'Oklawn' (Ok), 'Centennial' (Ct), and 'Tifton common' (Tc) with 14 primers. The templates were not digested prior to amplification and each set of less than 500 bp bands was scored.

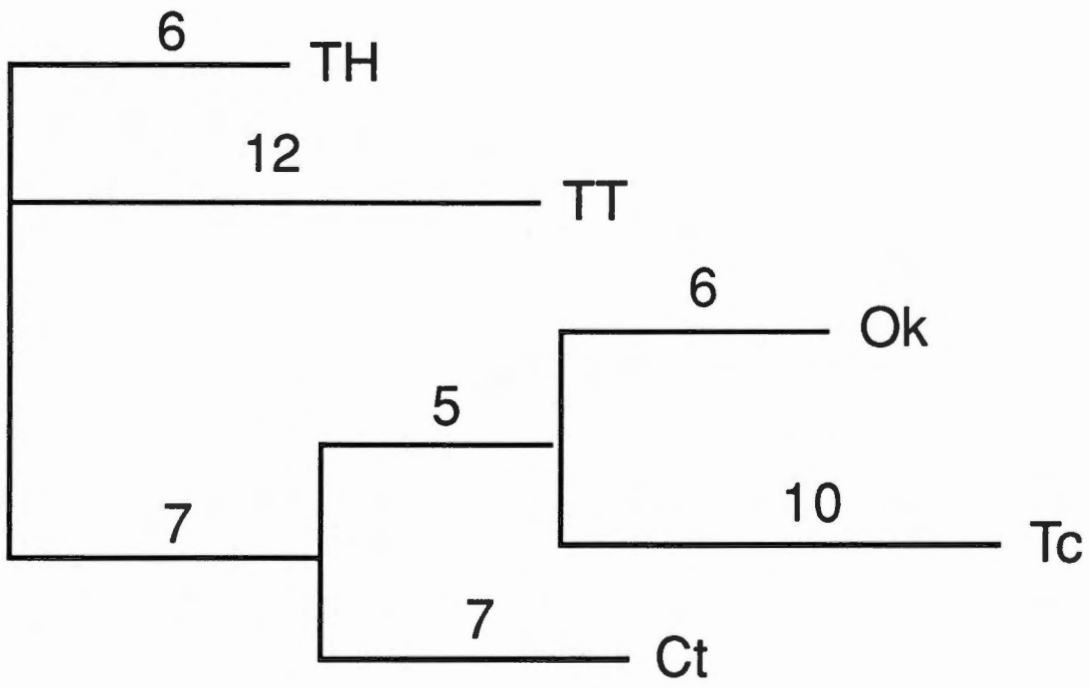

Figure 5.4. PAUP tree generated using data from centipedegrass templates digested prior to amplification. The templates of the five centipedegrass cultivars 'Tennessee Hardy' (TH), 'Tennessee Tuff' (TT), 'Oklawn' (Ok), 'Centennial' (Ct), and 'Tifton common' (Tc) cultivars were digested using 1, 2, or 3 endonucleases prior to amplification.

was produced (Figure 5.5). This tree points toward a close relationship between the cultivars 'Centennial' and 'Tifton common' with the other three cultivars being some distance away. The phylogenetic analysis of this information indicates that in all cases the cultivar 'Tennessee Hardy' is never linked (figure 5.3, 5.4, and 5.5) to any of the other four with various relationships seen between these other cultivars.

Conclusions

DNA amplification fingerprinting using single arbitrary octomer primers permitted the separation of the five centipedegrass cultivars. The elevated number of monomorphic bands generated with 14 primers suggested that the five cultivars are closely related and probably have a common origin. However, the cultivar 'Tennessee Hardy' demonstrated significant genetic differences from the other four cultivars.

To more effectively separate the centipedegrass cultivars, I used template digestion with one or more restriction endonucleases prior to DNA amplification as a strategy to increase the level of polymorphism. However, the cultivar 'Tennessee Hardy' was still significantly different from the other four cultivars whose relationships to one another changed somewhat using this strategy. Digesting DNA with one enzyme increased polymorphic DNA only moderately. Digesting DNA with more than one enzyme had been shown to dramatically increase polymorphic DNA in other plants (Caetano-Anollés et al, 1993). However, due to the relatively small sample size of multiple endonuclease digestions, no trend can be predicted for centipedegrass using this study.

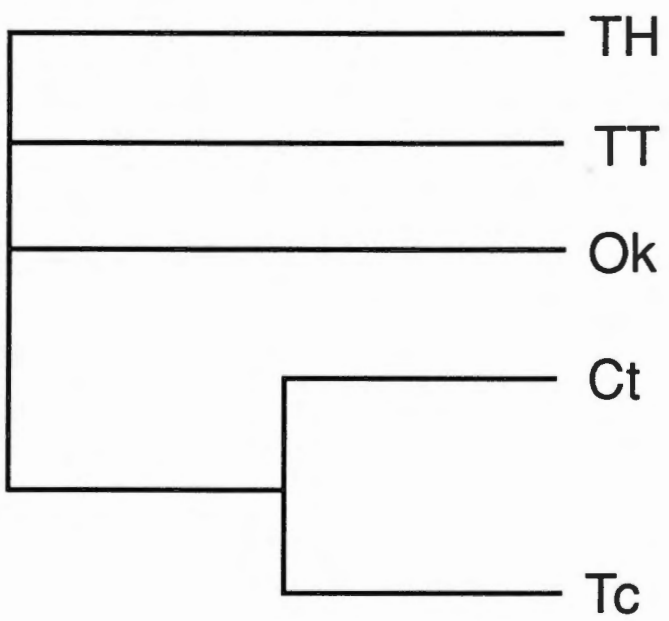

Figure 5.5. PAUP consensus tree produced using all available centipedegrass data. This tree represents the combination of all centipedegrass information from DAF profiles of the five centipedegrass cultivars 'Tennessee Hardy' (TH), 'Tennessee Tuff' (TT), 'Oklawn' (Ok), 'Centennial' (Ct), and 'Tifton common' (Tc). This combines the non-digested template information and the 1, 2, and 3 endonuclease digestions. PAUP produces two possible trees for this information and the consensus is shown above.

The combination of all available data still maintained 'Tennessee Hardy' as significantly different from the other four cultivars. The relationships of the other four cultivars was slightly shifted from the previous data analysis.

CHAPTER 6

DAF GENERATED PROBES FOR RFLP ANALYSIS

DAF identifies differences in DNA of closely related cultivars, as shown for centipedegrass in Chapter 5. These differences were seen as variations in banding patterns or fingerprints of the different cultivars. While differences were informative in separating and identifying specific cultivars, little is known of what causes these differences. Furthermore, the nature of amplified products within the entire genome of the plant must be explored for markers to be reliably used in gene mapping applications.

DAF products were used as probes in RFLP analysis of centipedegrass genomic DNA to understand better the nature of sequences being amplified. Interesting monomorphic and polymorphic bands were isolated from silver-stained gels and used as probes directly or after being cloned. The comparison of the isolated and cloned products was useful in determining whether the target sequence defined by the amplification product was a single or multiple copy in the genome.

Isolation of DNA Amplification Bands

The isolation of individual DNA fragments from a gel matrix, while the simplest approach to subcloning, required an effective procedure to elute the DNA molecules. Nucleic acids can be eluted into liquid or solid supports by blotting (Southern 1975), gel compression by freeze-squeeze (Thuring et al. 1975; Tautz

and Renz 1983) or flush (Grey and Brendel 1992) DNA extraction, centrifugal filtration through filter membranes (Zhu et al. 1985) or siliconized sterile glass wool (Heery et al. 1990), electroelution into dialysis bags (McDonell et al. 1977) or DEAE cellulose (Dretzen et al. 1981; Sylvers and Beresten 1993), use of low-melting point agarose (Wieslander 1979; Ausubel et al. 1992; Zintz and Beebe 1991), and even passive overnight diffusion into buffer. These methods usually require further purification by phenol and chloroform extraction and/or concentration before subcloning. I used a simple procedure to isolate DNA amplification bands from silver-stained polyacrylamide gels that used subsequent rounds of amplification of DNA diffusing passively from gel segments during thermal cycling. The method was particularly suited to the isolation of products from complex DNA profiles, like those generated using DAF, without needing further purification.

Using the direct "picking" procedure, I isolated five bands of different lengths from centipedegrass DNA for further use in Southern hybridization experiments (Table 6.1). These bands were obtained from profiles generated by amplification using different primers, directly from silver-stained 5% polyacrylamide gels. The high intensity bands chosen for isolation were primary products expected to contain elevated levels of DNA and show the least amplification interference from silver-staining and separation components of the gel. The desired bands, usually interesting monomorphisms or polymorphisms, were used directly as templates for further amplification. Subsequent rounds of amplification, DNA separation, and band isolation produced in each case a single strong band on the gel. Product isolation usually involved three to five rounds of isolation by amplification. Visualization of DNA by silver-staining using a technique that detects DNA at the picogram level (Bassam et al. 1991) allowed for sensitive

Table 6.1. Summary of isolated band information. Five bands were isolated from silver-stained polyacrylamide gels using 3 primers and 3-5 cycles of amplification.

Band	Primer Code	Band Size	# of Cycles to isolate	Polymorphic/ Monomorphic
#1	8.6c	200 bp	5	Monomorphic
#2	8.6c	150 bp	3	Monomorphic
#3	8.6h	175 bp	3	Monomorphic
#4	8.6i	105 bp	5	Monomorphic
#5	8.6i	230 bp	5	Polymorphic

visualization of contaminating products in each isolation round. In this study, an amplification appeared to fail, however, subsequent amplification using the failed reaction mixture as template always produced the expected bands. This could be due to inhibition of amplification by metallic silver, the formation of very stable "hairpin loops" during amplification (phenomena where terminal sequences of products are complementary and anneal to one another forming a stable loop structure), or the annealing to products with complementary sequences enriched during purification.

As an example, Figure 6.1 shows the isolation of a 200 bp band from a DAF fingerprint obtained from 'Tennessee Hardy' centipedegrass using the octomer AACGGGTG. This band was chosen because its size was easily followed during isolation (the band migrated with one of the molecular weight standards) and because it was monomorphic between all five centipedegrass cultivars studied. The band was isolated after three rounds of amplification, band excision, and separation in 5% polyacrylamide gels, and was subsequently used as a probe for Southern hybridization to centipedegrass DAF patterns transferred to Nylon membranes. Only a single band was observed in autoradiograms validating the isolation procedure.

Figure 6.2 shows how polyacrylamide gel concentration influenced DNA fragment isolation. After the isolation procedure was successfully used to obtain centipedegrass products, I wanted to apply the technique to a similar sized plant genome to determine if the procedure would work with other plants giving it broad applications in plant genome analysis. When working with soybean amplifications, I ran 4.5% polyacrylamide gels which were being used by other scientists exploring the soybean genome with DAF. I immediately noticed that it

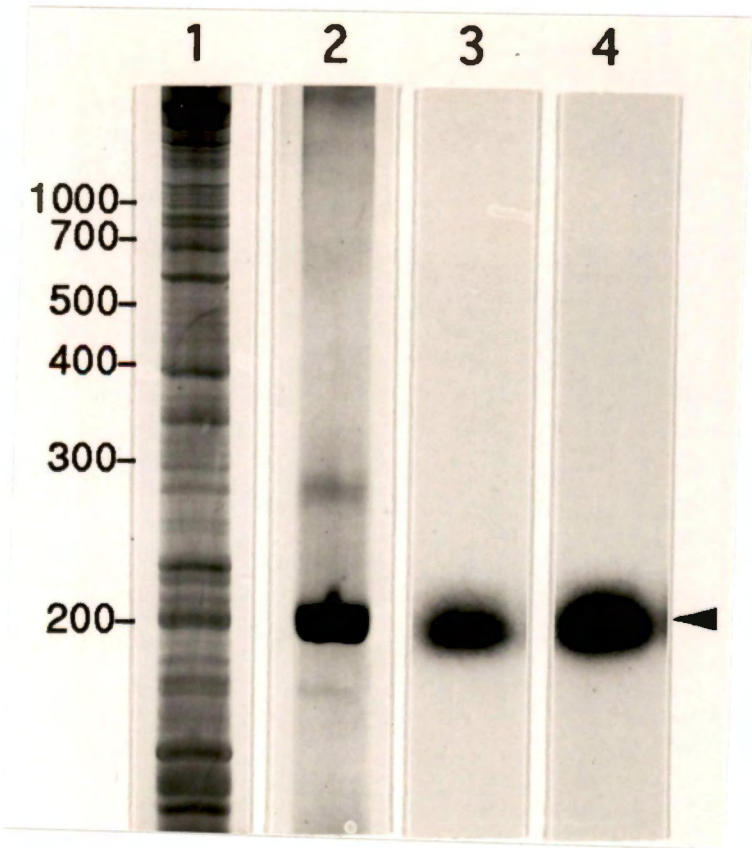

Figure 6.1. Isolation of a 200 bp DNA fragment from centipedegrass DNA amplified with an arbitrary octomer primer. A monomorphic fragment (arrowhead) was isolated (as described in Materials and Methods) from a DNA profile obtained from 'Tennessee Hardy' centipedegrass by DAF using primer AACGGGTG . This fragment was present in four other centipedegrass cultivars. Lane 1, DAF profile; lane 2, isolated DNA fragment (arrowhead); lanes 3 and 4, confirmation of isolation by Southern hybridization of the isolated 200 bp (arrowhead) fragment to the profile of lane 1 transferred to a Nylon membrane. In lane 4 the blot was probed with a subcloned isolated fragment.

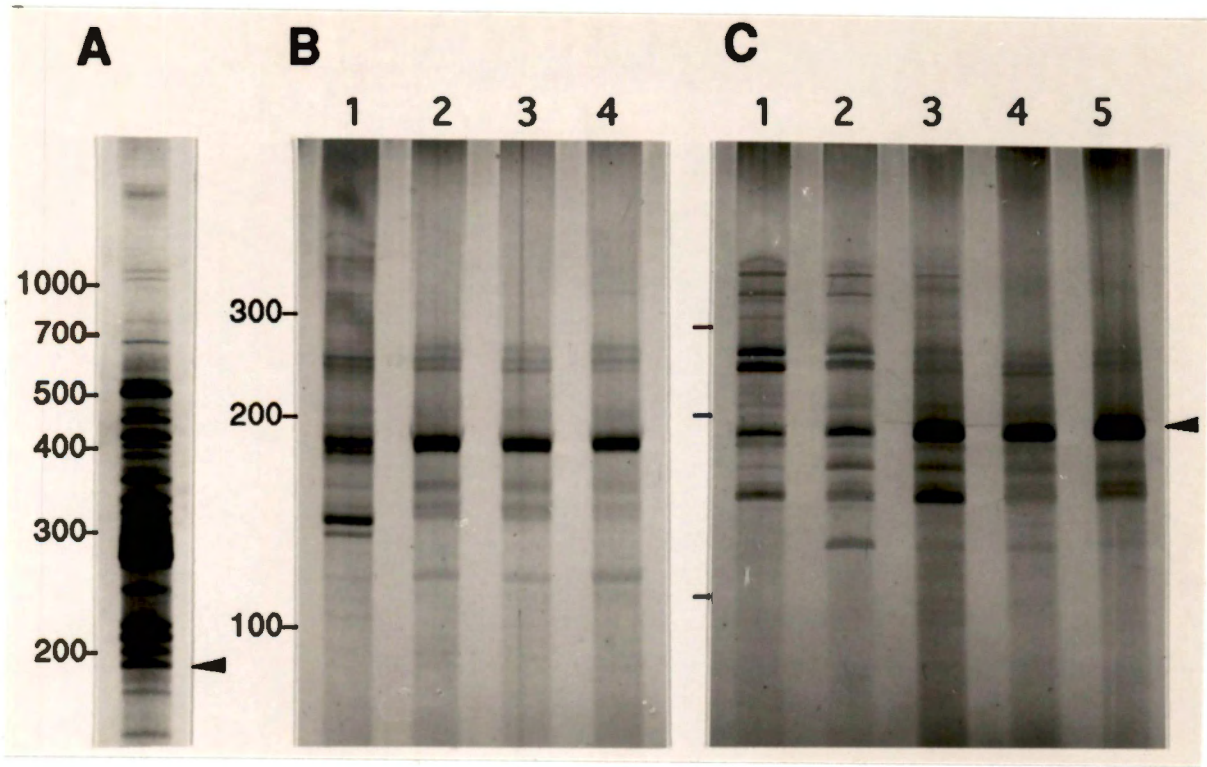

Figure 6.2. Effect of polyacrylamide concentration on the isolation of a 180 bp DNA fragment from soybean. A polymorphic fragment (arrowhead) was isolated from a DAF profile (panel A) obtained from cultivar 'Bragg' using the arbitrary primer GATCGCAG and template predigestion with 3 restriction endonucleases (Caetano-Anollés et al. 1993). During isolation, amplification products were obtained following subsequent rounds of band excision, amplification, and DNA separation in 6% (panel B) and 4.5% (panel C) polyacrylamide gels. The number of amplification rounds is indicated in each lane.

was more difficult to isolate a band, and in trying to solve this problem determined that the concentration of polyacrylamide influenced the procedure. Isolation of a 180 bp-fragment amplified from soybean (*Glycine max* L. Merr.) cultivar Bragg using the octomer GATCGCAG and 4.5% polyacrylamide gels required five rounds of amplification. This fragment was one of several DNA markers that were found tightly linked to a developmental locus controlling nodulation in soybean (Caetano-Anollés et al. 1993). In contrast, only three isolation rounds were needed when 6% gels were used. Loading the gels with lower amounts of template did not alter the efficiency of band isolation. Higher polyacrylamide concentrations most probably increased DNA isolation efficiency by decreasing product carryover during electrophoresis, although other explanations were possible. Isolation of DNA fragments using other variants of *Taq* polymerase (like Klentaq LA, AB Peptides, St. Louis, MO) proved very difficult. Successive isolation rounds were unable to eliminate contaminating products. This could result from very efficient amplification of low level contaminants, or low susceptibility of the enzyme to traces of metallic silver present in the reaction.

Southern Hybridization of Products with and without Cloning

It was assumed that the isolated bands could be further separated into sub-products, and this was determined by sub-cloning. To determine if further cloning of the isolates was necessary for reliable use as a Southern hybridization probe, the 200 bp and 175 bp isolates from 'Tennessee Hardy" DAF profiles using primer GAAACGCC were subcloned in plasmid pCRII. The cloned inserts were recovered directly by the PCR of resuspended colonies from transformed bacteria. About 50 colonies were screened to recover one with the 200 bp insert.

After confirming the expected size of the band, the PCR product was used as template in a DAF reaction using the original amplification conditions, and the cloned fragment was stored for radiolabeling. Despite screening 25 colonies, the cloned 175 bp product did not yield the expected 175 bp band. This may have been caused by the primer stock becoming contaminated causing the product to be lost in amplification with other products.

The products contained in isolated bands were used as probes in Southern hybridization experiments both with and without a further subcloning step. The 200 bp product(s) was radiolabeled and hybridized to DAF profiles of all five centipedegrass cultivars and transferred to Nylon membranes, obtained using a homologous (Figure 6.3) and a heterologous primer (Figure 6.4). The homologous primer was the same use to originate the probe. The blots showed one hybridization signal in DAF blots obtained using the homologous primer (Figure 6.3). The five centipedegrass cultivars yielded the same hybridization pattern, confirming the monomorphic nature of the 200 bp product(s). As expected, there was no observable hybridization to DAF patterns produced with the heterologous primer (Figure 6.4).

Similarly, the isolated 175 bp product(s) produced only one hybridization band in all five of the centipedegrass cultivars (Figure 6.5). This product(s) did not produce bands in Southern hybridizations produced from DAF patterns amplified with heterologous primers.

Southern hybridization of the 200 bp product(s) to genomic centipedegrass DNA from the five centipedegrass cultivars, and the experimental cultivar 'Tifton TC-238', is also shown (Figure 6.6). In these experiments, DNA was restricted with endonucleases *Hin*dIII and *Eco*RI. In both cases, the probe hybridization

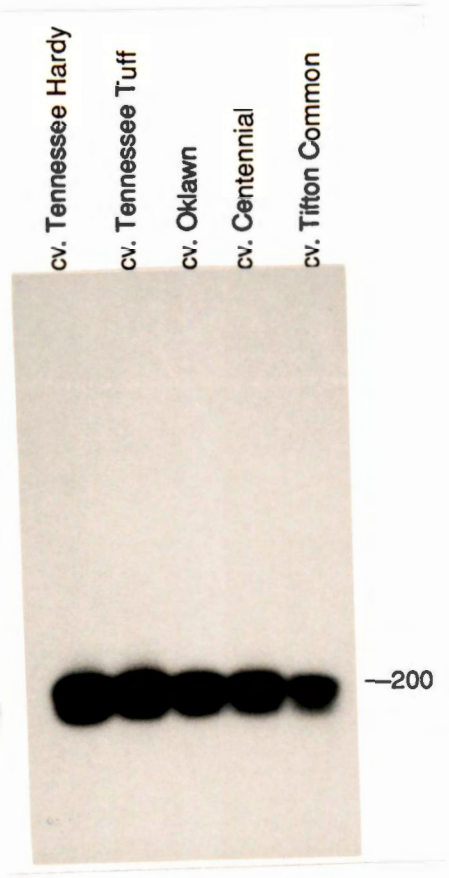

Figure 6.3. Audioradiogram of 5 centipedegrass cultivars probed with an isolated 200 bp band from 'Tennessee Hardy'. DNA amplification profiles of the five centipedegrass cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial' and 'Tifton common' created by the same primer 8.6c (AACGGGTG) were blotted onto Nylon membrane and probed with the isolated 200 bp 'Tennessee Hardy' product.

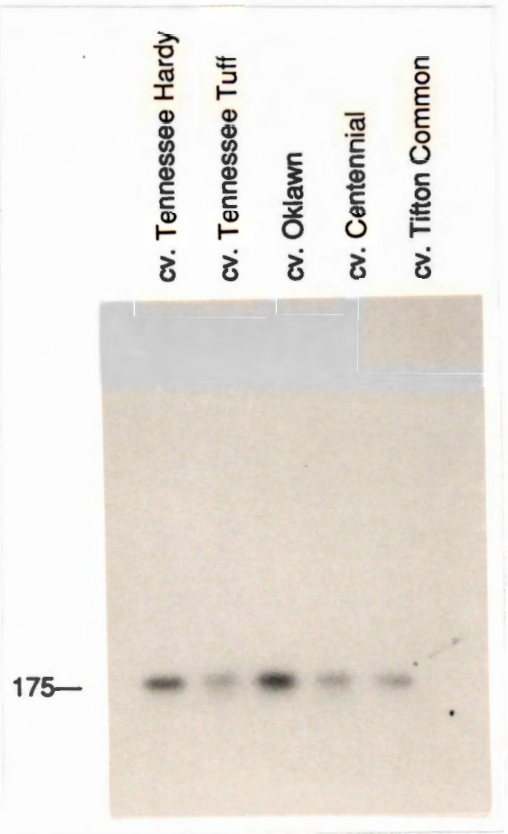

Figure 6.5. Five cultivars of centipedegrass probed with a 175 bp band isolated from an amplification profile of 'Tennessee Hardy'. DNA amplification profiles of the five centipedegrass cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial' and 'Tifton common' created by the same primer 8.6h (GAAACGCC) as the probe were blotted onto Nylon membrane and probed with the isolated 175 bp 'Tennessee Hardy' product.

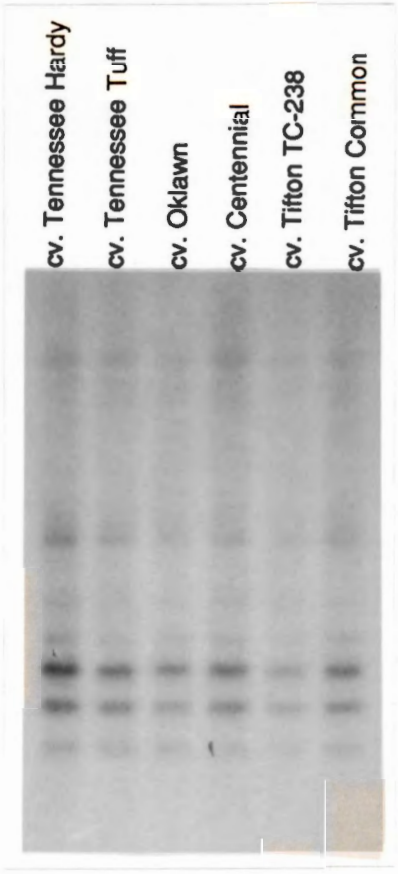

Figure 6.6. A blot of restricted genomic DNA probed with a 200 bp 'Tennessee Hardy' amplification product. The five centipedegrass cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial' and 'Tifton common' and an experimental cultivar 'Tifton TC-238' were restricted with *Hin*dIII (lanes 1-6). This blot was then probed with the 200 bp isolated DAF product which hybridized to the same molecular weight area in all six lanes.

produced the same band in all six cultivars. The number of bands seen in the *Hin*dIII digested DNA was eight after a 5 day exposure. The *Eco*RI digested DNA showed no bands after a five day exposure.

In a final experiment, the isolated 200 bp and 175 bp products were hybridized to DAF profiles of different turfgrass species (bermudagrass, Kentucky bluegrass, *Zoysia*, with 'Tennessee Hardy' centipedegrass as the positive control) produced using the homologous primers (Figure 6.7). The 'Tennessee Hardy' control showed the expected hybridization signal at 200 bp and 175 bp, respectively. Surprisingly, hybridization to the distant turfgrass species produced, in some cases, not only these signals but also smears from hybridization to high molecular weight fragments.

To confirm the above experiments, the isolated products were subcloned and hybridized to the same blots described above. Identical results were obtained in all instances, when the cloned probes were hybridized to DAF blots of centipedegrass varieties and distant grass species. Results confirmed the smearing and banding pattern in the distant grass species. The genomic hybridization to the five centipedegrass cultivars also yielded virtually identical banding patterns with differences in intensity of the bands with the *Hin*dIII digest. However, the subcloned probe took 3 weeks to show the expected signals instead of 5 days even though the radioactive counts for the probe were higher—the 5 day exposure showed one intense band that was present in the isolated probes pattern only faintly. The *Eco*RI digest yielded two bands in all cultivars after 3 weeks, but like the isolated probe, hybridization showed no bands after five days. These differences in band intensity may be due to variations in the amount of probe and radioactive count differences during hybridization.

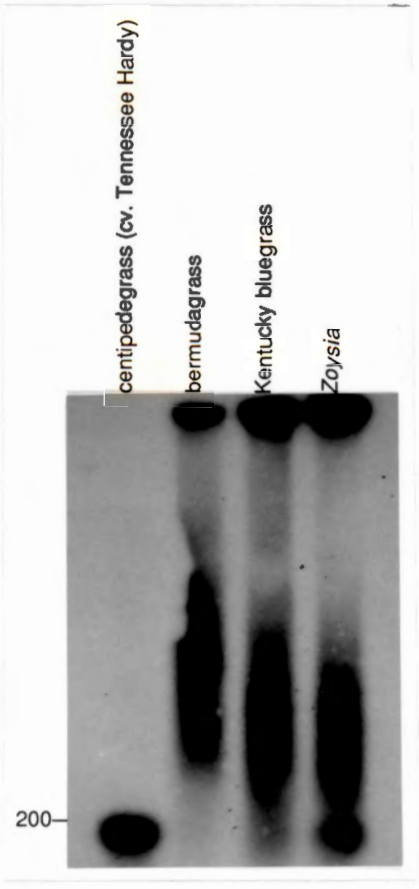

Figure 6.7. Hybridization of a 200 bp 'Tennessee Hardy' product to DAF profiles of distant turfgrass species. DAF profiles of the control 'Tennessee Hardy' (lane 1) and 3 other turfgrass species (lane 2 bermudagrass, lane 3 Kentucky bluegrass, and lane 4 *Zoysia*) which were generated using the same primer 8.6c (AACGGGTG) that generated the probe. the arrowhead points to the 200 bp area of the audioradiogram and the hybridization present in lanes 1, 3, and 4.

Conclusions

The PCR can reproducibly amplify picogram quantities of DNA (Mullis and Faloona 1987; Erlich et al. 1991) without requiring the time and labor involved in cloning, subcloning and plasmid amplification (Sambrook et al. 1989). The sensitivity and specificity of PCR can be increased by re-amplification of amplified DNA fragments using "nested primers" (Zintz and Beebe 1991), "hot start" PCR (D'Aquila et al. 1991; Erlich et al. 1991; Mullis 1991), or subsequent subcloning. These procedures ensure the purity of the amplification product by avoiding products arising from false priming events. The generation of complex profiles by DAF makes DNA fragment isolation physically more difficult. This fingerprinting technique produces a collection of amplification products representing discrete portions of a genome, with many products sharing a potentially similar molecular size. Confirmation of interesting monomorphic or polymorphic products by Southern hybridization often requires their isolation for subsequent use as DNA probes.

I have isolated DAF bands directly from silver-stained gels bound to polyester backing films that have been stored dried for as long as 2 years in our laboratory. The procedure of DNA isolation from preserved fingerprints may be especially important when genetic evidence is entered in a court of law, for plant variety rights enforcement and related deposition requirements, or for retrospective examination and extended study of interesting genetic material.

The product isolation procedure was surprisingly effective. Originally, it was thought that metallic silver deposited during DNA staining would interfere in the amplification reaction. However, this did not occur. One limitation, however, was that band isolation was restricted to those of less than 350 bp in length.

bands of higher molecular weight were not easily amplified as single bands and could be lost in the first or second round of amplification. This phenomenon could be explained by the interference of silver in the amplification of the larger products in these bands because of increased silver deposition, or alternatively because they are held more tightly in the gel matrix and are not made available for amplification. I have not attempted to solve this limitation. However, isolation was shown to be affected by polyacrylamide concentration and the DNA polymerase used during isolation. Low percentage polyacrylamide gels required an extended number of amplification rounds for band purification, and enzymes like Klentaq LA made isolation very difficult.

In one particular case, a 230 bp band was unable to be separated from another amplification product about 180 bp. This band retained its relative intensity no matter how many times the purification amplification procedure was conducted. This may result either because the contaminant product was identical in sequence but had a 50 bp deletion, or because it was able to amplify so efficiently that it could not be eliminated.

I have shown that the direct isolation of DAF products from silver-stained gels could easily generate probes for Southern hybridization. These probes could be used to determine if the amplification product represented a single or multi-locus site in the genome, or to confirm its absence from other DAF patterns. In similar experiments and with this same purpose, DAF products generated from bacteria (Caetano-Anollés et al. 1992b) and soybean (unpublished) have been isolated. Isolated DAF products that have and have not been subsequently cloned gave virtually the same results when hybridized to DAF profiles of centipedegrass cultivars and distant grass species, and to centipedegrass genomic DNA,

transferred to Nylon membranes. Therefore, DNA fragments isolated by amplification can be used directly as probes without further subcloning, or can be subcloned for DNA sequencing without further purification.

Developing probes in this manner is a fairly quick way to obtain informative and indicative probes for turfgrass cultivars. While there is not enough information to know if the isolated product without further subcloning can be used in gene mapping and gene transfer technologies later, there is evidence that these isolated products make informative probes for identification purposes.

More work needs to be done on developing probes in this manner to gain insight on the character of the amplification products. While this data set is informative it alone cannot explain what is happening during amplification. More products need to be isolated and cloned, and the results compared to explore any trends in amplification.

The results of these hybridization experiments suggest something interesting about the 200 bp isolated amplification band. The product is only present within one size amplicon in centipedegrass as demonstrated by the very specific hybridization of the probe to the five centipedegrass cultivars. However, when hybridized to more distant grass species (bermudagrass, Kentucky bluegrass, and *Zoysia*) the result is a smearing pattern. This indicates that the sequence(s) is present in multiple copies throughout the amplification profile and therefore is very abundant in these genomes. One possible explanation for this phenomena is different evolutionary paths in genome structure which might be similar to the one hypothesized by Moore et al (1993).

CHAPTER 7

CONCLUSIONS

The results of my investigations showed that the optimized DAF process gives reproducible and diagnostic fingerprints for the five centipedegrass cultivars studied. The cultivar 'Tennessee Hardy' was shown to be significantly different with the endonuclease and primer combinations used in these studies from the other four centipedegrass cultivars 'Tennessee Tuff', 'Oklawn', 'Centennial', and 'Tifton common'. DAF products can be successfully isolated from silver-stained polyacrylamide gels and used as hybridization probes with or without further subcloning.

Each reactant in the DAF reaction was optimized for centipedegrass. The primer 8.6c (AACGGGTG) was used in all experiments (at the optimal recommended 3 μM concentration) with the enzyme AmpliTaq Stoffel fragment polymerase (0.3 units/μl) and the cultivar 'Tennessee Hardy'. The optimal concentration of magnesium chloride was 1.5 mM which allowed for reproducible amplification and appears to keep primer-template mismatching to a minimum. I found 2.5 ng/25 μl reaction volume was the optimum amount of template DNA for each cultivar of centipedegrass. Enzyme levels were optimal at 0.3 units/μl reaction volume in most instances. However, frequent batch-to-batch enzyme variations produced experimental failures with enzyme levels of 0.1 units/μl reaction volume . Some batches produced perfect amplifications at these levels. Each new

enzyme batch needed to be tested with an amplification reaction which was shown to be successful. The optimal nucleotide concentration for centipedegrass was the recommended 200 µM each dNTP. Reaction buffer was optimal at the 1x stock recommended by the manufacturer. Reactions using these conditions were reproducible.

The five centipedegrass cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial', and 'Tifton common' were successfully fingerprinted using DAF. All of the primers used were octomers with 14 tested with undigested centipedegrass DNA. Of these fourteen primers four were informative: 8.6d, 8.6h, 8.6i and 8.7h. Of these four only two distinguished all five centipedegrass cultivars from each other (8.6d and 8.6i).

When centipedegrass DNA was amplified using 12 single endonuclease-primer combinations, 11 demonstrated some degree of polymorphic character. Centipedegrass DNA was amplified using four double endonuclease-primer combinations and two triple endonuclease-primer combinations. Of the four double endonuclease-primer combinations, three showed some degree of polymorphic character with the *HinfI*, *MspI*-8.6h amplification being the most informative. The most informative of the two triple endonuclease-primer combinations was *HinfI AluI*, *Rsa*I-8.6h.

The data from these amplifications were scored (band present on the gel = 1 and band absent on the gel = 0) and analyzed using PAUP. Fingerprints generated with the many primer and endonuclease-primer combinations indicate that the five cultivars are closely related, with genomes that appear highly conserved. Phylogenetic "trees" generated from PAUP had one thing in common. The cultivar 'Tennessee Hardy' was shown to be significantly different from the other

four cultivars studied using each of the two data sets (DNA-primer and DNA, endonuclease-primer) and a combination of the two. Relationships of the other four cultivars 'Tennessee Tuff', 'Oklawn', 'Centennial', and 'Tifton common' to each other and 'Tennessee Hardy' varied, but 'Tennessee Hardy' grouped separately from the other four cultivars.

Products from DAF gels can be isolated and used as probes for RFLP analysis. A total of five amplification products were isolated from silver-stained polyacrylamide gels. I demonstrated that products silver stained in polyacrylamide gels can be isolated for use as probes using a variety of primers (three total in these experiments) and band intensities. I also found that gels up to two years old can be rehydrated and bands isolated from them.

Using an isolated 200 bp product (with and without subcloning) as a probe, the isolation procedure did not require further subcloning to produce usable probes for RFLP analysis. The comparison of the DNA amplification product with and without cloning also provided information on the character of this 200 bp band from 'Tennessee Hardy'. This band did not contain a single copy sequence (at least two products within the isolated band by Southern Hybridization results of the cloned product) or single copy product in the genome (eight hybridization bands were revealed with restricted genomic centipedegrass DNA).

This study represents the first significant step of applying molecular amplification techniques to the analysis of turfgrass DNA. Although much ground was traversed, numerous new problems and challenges were detected, which will require extensive future research.

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APPENDICES

APPENDIX A

DAF Generated Data

DAF data from the five centipedegrass cultivars 'Tennessee Hardy', 'Tennessee Tuff', 'Oklawn', 'Centennial', and 'Tifton common' which were amplified using a number of primers and endonucleases. Their amplification patterns were scored on a basis of 1 = band present and 0 = band absent.

Template	8.5b	8.6c	8.6d	8.6e	8.6f	8.6g	8.6h
TN Hardy	1111111	1111111111111111	11101111001111111011	111111111111	1111111111111111	1111111111111111	1101101111111101
TN Tuff	1111111	1111111111111111	11011101110111110101	111111111111	1111111111111111	1111111111111111	1111011111111011
Oklawn	1111111	1111111111111111	11011101110111110010	111111111111	1111111111111111	1111111111111111	1111001111111001
Centennial	1111111	1111111111111111	11111100111011011100	111111111111	1111111111111111	1111111111111111	1111001111111101
Tifton Common	1111111	1111111111111111	11111111011111001010	111111111111	1111111111111111	1111111111111111	1111001111111101

	8.6i	8.7c	8.7e	8.7h	8.7i	8.7k	8.7l
TN Hardy	11101101111101111	11111111111111111111111111111	111111111	11111110111011111111	111111111111	1111111111111	1111111111111111
TN Tuff	11111101111011111	11111111111111111111111111111	111111111	11111111010111111111	111111111111	1111111111111	1111111111111111
Oklawn	11011101111011111	11111111111111111111111111111	111111111	11110110010111111111	111111111111	1111111111111	1111111111111111
Centennial	11011101110000111	11111111111111111111111111111	111111111	11110111010111111111	111111111111	1111111111111	1111111111111111
Tifton Common	11100111110001111	11111111111111111111111111111	111111111	11110111010111111111	111111111111	1111111111111	1111111111111111

	8.6i (*Alu*I)	8.6j (*Alu*I)	8.6h (*Bst*UI)	8.6 c (*Msp*I)	8.6e (*Msp*I)	8.6i (*Msp*I)
TN Hardy	11111101111111111	11111111111111111	01111111111011111	11111111111111111111100111111	1111111	11110111101011111111101111
TN Tuff	11111101111111111	11111111111111111	11111111111111111	11111111111111111111100111111	1111111	11110111011011111101101111
Oklawn	11111101111111111	11111111111111111	11111111111011111	11111111111111111111001111	1111111	11110111101011110101101111
Centennial	11111101111111011	11111111110111111	11111111111011111	11111111111111111111011111	1111111	11110111101011111111101111
Tifton Common	11111111111111111	11111111111111111	11111111111011111	11111111111111111111001111	1111111	11111111111011110101111111

	8.6c (*Taq*I)	8.6d (*Taq*I)	8.6h (*Taq*I)	8.6i (*Taq*I)	8.6j (*Taq*I)	8.7j (*Taq*I)
TN Hardy	11111111111111111111101110	11111101111011111	11111111111111111011	11010000111011111111111	11111111111011111111111	11111111011111101111
TN Tuff	11111111111111111101101111	11111101111111111	11111111111111111011	11111100111011111111111	11111111111111111111111	11111110101010111111
Oklawn	11111111111111111111011111	11111101111011111	11111111111111111111	11111011111011111111111	11111111111111111111111	11111111011111101111
Centennial	11111111111111111101000010	11111011111011111	11111111111111111111	11111100111011111111111	11111111111011101111111	11111111011011011111
Tifton Common	11111111111111111101100110	11111011111011111	11111111111111111011	11111010111111111111111	11111111111111111111111	11111111011111101111

	8.6 i (*Bst*UI, *Msp*I)	8.6i (*Alu*I, *Rsa*I)	8.6 h (*Hinf*I, *Msp*I)	8.6i (*Hinf*I, *Msp*I)	8.6h (*Hinf*I, *Alu*I, *Rsa*I)	8.6i (*Hinf*I, *Msp*I, *Bst*UI)
TN Hardy	11111111111111	01111111111111111111	11110101111111	11111111111111111	11110111111111111011111	110101111111
TN Tuff	11111111111111	11111111111111111111	11101101111111	11111111110111111	11011111101111111011111	110111111111
Oklawn	11111111111111	11111101111111111111	11101011111111	11111101110111111	11011111101111111111111	111111111111
Centennial	11111111111111	11111111111111111111	11101001111111	11111101110111111	11111111101111111011111	111111111111
Tifton Common	11111111111111	11111111111111111111	11101001111111	11111101110111111	11111111101111111011111	111111111111

APPENDIX B

Primer Codes and Sequences

Primer code	Sequence
8.5b	
8.6c	AA CGG GTG
8.6d	GT AAC GCC
8.6e	GA CGT AGG
8.6f	GA TCG CAG
8.6g	CT AAC GCC
8.6h	GA AAC GCC
8.6i	GT TAC GCC
8.6j	GT ATC GCC
8.7c	GA GGG TGG
8.7e	CC TGG TGG
8.7g	CC AGG TGG
8.7h	CC TCG TGG
8.7i	CC TGC TGG
8.7j	CC TGG AGG
8.7k	CC TGG TCG
8.7l	CC TGG TGC

APPENDIX C

Endonuclease Recognition Sites

Endonuclease	Recognition site
*Alu*I	AGCT TCGA
*Bst*UI	CGCG GCGC
*Hinf*I	GANTC CTNAG
*Msp*I	CCGG GGCC
*Rsa*I	GTAC CATG
*Taq*I	TCGA AGCT

VITA

Kristal Rebecca Weaver was born in Knoxville, Tennessee on December 3, 1968. She graduated from Central High School in 1987 . In July of 1987 she entered The University of Tennessee as an undergraduate majoring in Anthropology (field of interest was forensic identification) and in December of 1989 she graduated with a Bachelor of Arts degree with honors. During the summer of 1989 she began independent research in the Zoology department studying *Drosophilia* mutations and continued this research until the summer of 1990. In August, 1990 she accepted a Graduate Research Assistantship with Dr. Lloyd Callahan in the Department of Ornamental Horticulture and Landscape Design doing DNA fingerprinting of turfgrass. She is a member of the academic fraternities Pi Alpha Xi (serving as Vice- President during the 1992-1993 academic year) and Gamma Sigma Delta.