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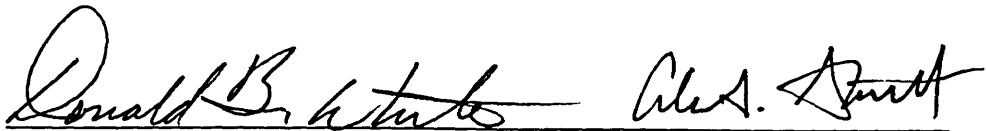

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August 1, 2003

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GRADUATE SCHOOL

The Application of ISSR PCR for Distinguishing Creeping Bluegrass (*Poa annua* var. *reptans*) Genotypes

A THESIS
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FOR THE DEGREE OF
MASTER OF SCIENCE

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DEDICATION

I dedicate this degree to my wife Sara. Her endless love and encouragement, as well as her numerous sacrifices, allowed me to finish this degree.

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The section titled "The Application of ISSR PCR for Distinguishing Creeping Bluegrass (*Poa annua* var. *reptans*) Genotypes" is written for publication in the journal *Crop Science.*

"All flesh is like grass,

and all its glory like the flower of grass.

The grass withers, and the flower falls off,

but the word of the Lord stands forever."

1 Peter 1:24-25

REVIEW OF THE LITERATURE

Poa annua L. is a ubiquitous cool season turfgrass that is reported to have originated on the European continent (Tutin, 1957). It is an allotetraploid (2n=4x=28) thought to be derived from a chance cross between *Poa infirma* Kunth and *Poa supina* Schrader (Nannfeldt, 1937; Tutin, 1952 and 1957; Darmency and Gasquez, 1997). *P. infirma* is an erect, bunch type diploid (2n=2x=14) annual that is found on the Mediterranean and Atlantic borders of Europe and has the characteristics typical of a cosmopolitan weed whereas *P. supina* is a prostrate, stoloniferous diploid (2n=2x=14) perennial that is typically found in the mountains of central Europe (Tutin, 1957). *P. annua* has a continuum of life cycle types that include the extremes of its putative parents from true annual to long-lived perennial as well as numerous variants between the two (Timm, 1965; Gibeault, 1971; Johnson et al., 1993 and Heide, 2001). The life cycle forms of *P. annua* at its extremes have been divided into two types. The annual form, designated *P. annua* var. *annua* (L.) Timm, exhibits an erect, bunch type growth habit, early and continual flowering and a short life cycle. The perennial form, *P. annua* var. *reptans* (Hauskn.), has a prostrate growth habit, nodal rooting and at its extreme a photoperiodic and vernalization requirement to flower (Timm, 1965; Johnson and White, 1997a and 1997b).

The common name for *P. annua*, annual bluegrass or annual meadowgrass, is a misnomer for the perennial form since it is not an annual. To help alleviate the confusion, the perennial form, *P. annua* var. *reptans*, has been assigned the common name creeping

bluegrass (Beard, 1999) because *reptans* means "creeping" (Bailey, 1948) while annual bluegrass remains the common name for *P. annua* var. *annua*.

In addition to the tetraploid form, *P. annua* is also found as a sterile diploid generally only on putting greens. Velguth and White (1993), in Minnesota, discovered that 24% of randomly collected *P. annua* plants from selected putting greens were diploid.

Ellis (1973) reported that *P. annua* sexually reproduces primarily through self pollination with less than 15% outcrossing. The degree of outcrossing is related to plant density and decreases with decreasing plant density (Darmency and Gasquez, 1983). In addition, Naylor et al. (1983) suggested that outcrossing was a rare event.

The range of life cycles within *P. annua* and the presence of some degree of outcrossing have made it a successful, opportunistic species, allowing it to expand its range to all continents of the world and many varying environments (Warwick, 1979; Hutchinson and Seymour, 1982; Scott, 1989). Although *P. annua* can be found in nearly all climatic conditions, creeping bluegrass, the perennial form, thrives in the maritime regions of the world where temperatures are generally moderate and moisture is abundant. Creeping bluegrass is also found in other temperate regions where irrigation is practiced and drought stress is avoided.

Although it has commonly been thought that the annual varieties are more prevalent in less intensively managed areas and the perennial varieties under more highly maintained

conditions (Law et al., 1977; Wu et al., 1987; Till-Bottraud et al., 1990; Warwick and Briggs, 1978a and 1978b), Cline (2001) concluded that the more annual types tend to be found in more stressful situations, even if intensively managed, whereas the more perennial types tend to exist where conditions are more moderate or favorable for long term survival. This can be explained by the fact that seed needs to be produced for the annual types survival. Annual bluegrass may expend many resources producing an abundance of seed that assures its survival during adverse conditions. On the other hand, creeping bluegrass partitions its resources to tillers and stolons and thereby maintains its space and expands vegetatively while growing conditions are favorable.

P. annua is an economically important turfgrass that has often been ignored even though it is the predominate turfgrass on many of the most prestigious golf courses around the world, including the majority of the courses that have hosted recent U.S. Open golf tournaments (Huff, 1999). It is particularly well adapted to extremely low heights of cut (3-4 mm), compacted soil and shade; conditions common to golf course greens and tees. *P. annua* flowers at mowing heights as low as 3 mm and can even develop germinable seed in florets removed the same day that pollination occurs (Koshy, 1969). *P. annua* has long been considered an unwanted or problem weed in highly maintained turfgrass surfaces causing disruption of the uniform surface, showing susceptibility to summer decline and diseases, sometimes flowering heavily and is occasionally undependable (Sprague and Burton, 1937; Beard, 1970; Kamp, 1981). Others look at *P. annua* as a cultivated invader that has performed as well as, if not better than, the planted species. While the former have long held the goal of ridding the turf of this so called weed, the

latter have believed it best to manage whichever species is most favored in a particular ecosystem (Beard et al., 1978; White, personal communication). It is accepted that the individual that is best adapted to a particular environment will predominate. So, if the limits of tolerance of the planted species are exceeded, a better adapted individual will take its place. *P. annua* is an opportunistic species with high plasticity that takes advantage of such situations and adapts to a wide variety of environments.

Sprague and Burton (1937) and Youngner (1959) both discovered that *P. annua* only encroached into turf areas where the growth of the desired grasses was poor. Baker et al. (1995) found that of the 148 British golf greens they examined, 75% had a *P. annua* content greater than 50% and concluded that unless a chemical control is developed for *P. annua*, it will generally be a large component of golf greens in Great Britain. This statement probably holds true for any area growing a cool-season grass on a green. Wu et al. (1987) stated that California golf greens become largely *P. annua* within as few as five to six years after establishment. Lush's (1988) succinct statement, "Once present, *P. annua* is self-perpetuating.", summarizes *P. annua's* tenacious survivability very well.

Much effort and many resources have been expended in attempts to prevent, reduce or control *P. annua* in fine turfgrass all with little success. Much of the research conducted on *P. annua* has followed the same avenue, however, little has been written about how to effectively grow and maintain high quality *P. annua* turfgrass and some of the control strategies employed have led to the development of populations that are resistant to a

number of herbicides (Yelverton and Isgrig, 1998; Fuks et al., 1992; Barros and Dyer, 1988).

On-the-other-hand, others have noticed the beneficial attributes of creeping bluegrass that make it a desirable turfgrass surface: perennial, stoloniferous, high tiller density, shade tolerant, rapid germination, dark color, and limited flowering period (Timm, 1965; Sprague and Burton, 1937; Adams and Bryan, 1980; Johnson et al., 1993).

Some plant breeders are now accumulating breeding populations, making crosses and developing cultivars for commercial release. This recent work to develop improved cultivars of creeping bluegrass has generated an increased interest by others in selection and breeding of this species. In order to improve breeding and selection of desirable traits, a technique that would accurately differentiate and identify closely related genotypes would be useful and could be used along with phenotypic data for identifying and legally protecting each variety. We have observed that the environment can significantly modify morphological characters among some creeping bluegrass genotypes (unpublished data).

P. annua has been the subject of very few genetic studies. Johnson and White (1997b) found that *P. annua* is not just a day neutral species, but also consists of individuals that are facultative long day, facultative short day, and obligate vernalization requiring. In addition, Heide (2001) discovered that variation in time to flowering is genetically determined. Johnson and White (1998) also found that the flowering types, continual

versus seasonal, are inherited in simple Mendelian patterns that are controlled by a single loci. Continual flowering is completely dominant to seasonal flowering.

Some studies have looked at the biological, morphological and ecological reasons why each type exists where it does (Law et al., 1977; Warwick and Briggs, 1978a and 1978b) and others have begun to exam the genetic relatedness of various populations in differing environments (Sweeney and Danneberger, 1995; Cline, 2001). Darmency et al. (1992) and Darmency and Gasquez (1997) looked at the inheritance of isozyme patterns while Sweeney and Danneberger (1995), Cline (2001) and Mengistu et al. (2000) used random amplified polymorphic DNA (RAPD) markers to study the relationship of wild *P. annua* collections. These studies examined diversity or population relationships within a given environment and different environments on one golf course; however, no one has studied the genetics of closely related genotypes.

Several different methods for molecular identification of cultivars exist. Restriction fragment length polymorphism (RFLP) was long the standard, but now various polymerase chain reaction (PCR) based methods are more widely used. The PCR method utilizing arbitrary primers, mainly RAPDs, has seen considerable use over the last decade. Amplified fragment length polymorphism (AFLP), which uses restriction enzymes and nonarbitrary primers, and simple sequence repeats (SSRs), which makes use of primers based upon known sequences within the genome, are other PCR based methods that are being widely used. RAPDs have been used for cultivar identification of many crops. More recently, PCR analysis using anchored simple sequence repeats or

inter-simple sequence repeats (ISSR) has been reported to be a useful and reliable new method of detecting genetic polymorphisms between accessions (Zietkiewicz et. al., 1994) and has been used in cultivar identification in many crops including wheat (Nagaoka and Ogihara, 1997), maize (Kantety et al., 1995), potatoes (Prevost and Wilkinson, 1999), Douglas-fir (Tsumura et al., 1996), and barley (Fernandez et al., 2002). The ISSR PCR method uses di-, tri-, tetra-, or pentanucleotide repeats with a one, two or three base tag attached to the 3′ or 5′ end of the repeat. This tag assures that the primer anneals to the end of the simple sequence repeat and thus provides greater specificity.

The benefit of using ISSR PCR over other methods include: (1) greater specificity and repeatability than RAPDs, (2) reduction in preparation time over AFLP because there is no need to use restriction enzymes, (3) there is no need of prior knowledge of the target sequence as with SSRs, (4) the PCR products can be visualized on an agarose gel, and (5) it is relatively inexpensive. In addition, Blair et al. (1999) and Fernandez et al. (2002) reported that ISSR provided better distinction between closely related cultivars than AFLP or RAPD, respectively.

Previous work with *Poa annua* has only been done with wild plant collections, while no one has looked at closely related genotypes. This study tests the utility of ISSR PCR for creeping bluegrass genotyping.

OBJECTIVES

The objectives of this study were to: (1) test ISSR primers for production of polymorphic markers, (2) ascertain the applicability of ISSR PCR to distinguish closely related genotypes, and (3) use ISSR PCR for genome analysis and genotyping.

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The Application of ISSR PCR for Distinguishing Creeping Bluegrass (*Poa annua* var. *reptans*) Genotypes

INTRODUCTION

Poa annua L. is a ubiquitous cool season turfgrass that is reported to have originated on the European continent (Tutin, 1957). It is an allotetraploid (2n=4x=28) thought to be derived from a chance cross between *Poa infirma* Kunth and *Poa supina* Schrader (Nannfeldt, 1937; Tutin, 1952 and 1957; Darmency and Gasquez, 1997). *Poa infirma* is an erect, bunch type, diploid (2n=2x=14) annual whereas *P. supina* is a prostrate, stoloniferous, diploid (2n=2x=14) perennial (Tutin, 1957). *P. annua* has a continuum of life cycle types that include true annuals to long-lived perennials as well as numerous variants between the two (Timm, 1965; Gibeault, 1971; Johnson et al., 1993; Heide, 2001). The life cycle forms of *P. annua* at its extremes have been divided into two types: the annual, designated *P. annua* var. *annua* (L.) Timm and the perennial, *P. annua* var. *reptans* (Hauskn.) (Timm, 1965). The common name for *P. annua*, annual bluegrass or annual meadowgrass, is a misnomer for the perennial type considering it is not an annual. To alleviate the confusion, the perennial type, *P. annua* var. *reptans*, was assigned the common name creeping bluegrass (Beard, 1999), as *reptans* means "creeping" (Bailey, 1948), while annual bluegrass remains the common name for *P. annua* var. *annua*.

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Baker et al. (1995) found that of the 148 British golf greens they examined, 75% had a *P. annua* content greater than 50%. Likewise, Wu et al. (1987) stated that California golf greens become largely *P. annua* within as few as five to six years after establishment.

Some have recognized the attributes of creeping bluegrass that make it a desirable turfgrass surface: perennial, stoloniferous, high tiller density, shade tolerant, rapid germination, dark color, and limited flowering period (Timm, 1965; Sprague and Burton, 1937; Adams and Bryan, 1980; Johnson et al., 1993). Plant breeders are now accumulating breeding populations, making crosses and developing cultivars for commercial release. In order to improve breeding and selection for desirable traits, a technique that would accurately differentiate and identify closely related genotypes would be useful. Such a technique could also be useful for legal protection of cultivars along with the usual phenotypic data which can be affected by environmental factors.

Research has begun on the biological, morphological and ecological characteristics that influence the distribution of different *P. annua* types (Law et al., 1977; Warwick and Briggs, 1978a and 1978b). Other researchers have examined the genetic relatedness of various populations in differing environments through the use of molecular markers (Sweeney and Danneberger, 1995; Cline, 2001). Darmency et al. (1992) and Darmency and Gasquez (1997) investigated the inheritance of isozyme patterns and Sweeney and Danneberger (1995), Cline (2001) and Mengistu et al. (2000) used random amplified polymorphic DNA (RAPD) markers to study the relationship of wild *P. annua* collections. These studies looked at diversity or population relationships for wild *P. annua* collections within a given environment and different environments on one golf course; however, no one has studied the genetics of closely related genotypes within a breeding program.

Several different methods for molecular identification of cultivars exist. Restriction fragment length polymorphism (RFLP) was long the standard, but now various polymerase chain reaction (PCR) based methods are more widely used. The PCR method utilizing arbitrary primers, mainly RAPDs, has seen considerable use over the last decade. Amplified fragment length polymorphism (AFLP), which uses restriction enzymes and nonarbitrary primers, and simple sequence repeats (SSRs), which makes use of primers based upon known sequences within the genome, are other PCR based methods that are being widely used.

More recently, PCR analysis using anchored simple sequence repeats or inter-simple sequence repeats (ISSR) has been reported to be a useful and reliable new method of detecting genetic polymorphisms between accessions (Zietkiewicz et. al., 1994) and has been used in cultivar identification in many crops including wheat (Nagaoka and Ogihara, 1997), maize (Kantety et al., 1995), potatoes (Prevost and Wilkinson, 1999), Douglas-fir (Tsumura et al., 1996), and barley (Fernandez et al., 2002). The ISSR PCR method uses di-, tri-, tetra-, or pentanucleotide repeats with a one, two or three base tag attached to the 3′ or 5′ end of the repeat. The tag assures that the primer anneals to the end of the simple sequence repeat and thus provides greater specificity.

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relatively inexpensive. In addition, Blair et al. (1999) and Fernandez et al. (2002) reported that ISSR provided better distinction between closely related cultivars than AFLP or RAPD, respectively.

Previous molecular marker work with *Poa annua* has only been done with wild plant collections, while no one has looked at closely related genotypes. This study examines the utility of ISSR PCR for genotyping creeping bluegrass accessions within a breeding program.

The objectives of this study were to: test ISSR primers for production of polymorphic markers, ascertain the applicability of ISSR PCR to distinguish closely related genotypes, and use ISSR PCR for genome analysis and genotyping.

MATERIALS AND METHODS

Plant Material

The twenty clonal accessions used in this study were selected from the creeping bluegrass breeding collection at the University of Minnesota. They were chosen so as to include both closely related individuals and individuals that were of diverse backgrounds. Included among the chosen accessions were 17 *P. annua* var. *reptans* (13 tetraploids and four diploids), one *P. annua* var. *annua* (tetraploid) and two outliers: one *P. supina*, and one *P. trivialis* (Table 1). All of the *P. annua* var. *reptans* clones were F_5, F_6, or F_7 selections.

DNA Isolation

DNA was extracted from not fully expanded leaf and sheath tissue using the Qiagen Plant DNeasy Mini Kit (Qiagen Inc., Valencia, California) with minor modifications to the manufacturer's suggested protocol. Immediately following collection, 150 mg of plant tissue was placed in a 2 ml microcentrifuge tube and stored at -80°C. The tissue was ground in the same tube that it was stored. Buffer AP1, Rnase A and a small amount of carborundum (used to aid in the disruption of cells during the grinding process) were added to the tissue and ground manually for two minutes using a glass tipped micropestle. The tube was vortexed and then placed on ice until a number of samples had been ground and then the Qiagen protocol was followed. The extracted DNA was quantified using the Hoefer DyNA Quant 200 Fluorometer and all samples diluted to the same concentration.

Primers

Primers 801-850, from primer set #9, obtained from the University of British Columbia (UBC, Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada) were screened to determine potential usefulness. A total of twelve primers (Table 2) were selected from the initial screening, eleven were chosen based upon the number of bands, band intensity and band separation and the twelfth primer (UMN001) had the tag on the 5′ side of the repeat. Primers were newly synthesized by Integrated DNA Technologies (IDT) (Coralville, Iowa) for use in the study.

PCR

Two independent DNA samples from each clone, extracted at different times, were used in separate PCR reactions. The reaction mixture (25 µl total volume) consisted of one unit AmpliTaq DNA polymerase (Perkin Elmer), 1X ThermoPol PCR Reaction Buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$, 0.1% Triton X-100) (New England BioLabs Inc.), 0.2 mM of each dNTP, 0.3 µM of a single primer, and 15 ng of DNA. PCR amplifications were performed on a Hybaid Omnigene Thermocycler (Hybaid Inc.) or a MJ Research PTC-150 Minicycler Thermocycler (Waltham, MA) using the following program: initial denature for 7 min. at 94°C, 35 cycles of 1 min. at 94°C, 2 min. at 52°C, 2 min. at 72°C, and a final extension of 7 min. at 72°C.

Electrophoresis and Data analysis

Five µl of 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyonol FF, 30% glycerol) was added to each PCR reaction, 20 µl of the diluted reaction was loaded on a 2% agarose gel, and samples were electrophoresed at 6.5 volts/cm for 4 hours with TAE buffer. Amplified products were detected by staining with ethidium bromide and gels were photographed using a Bio-Rad Gel Doc 2000 digital imaging system and Quantity One version 4.0.3 software (Bio-Rad Laboratories, Hercules, California). Gel images were printed with a thermal printer and scored visually for the presence or absence of amplified fragments in the range of 300-1300 bp. The ISSR bands were considered to be dominant markers and were scored as 1 (present) or 0 (absent). A similarity matrix was calculated using the correlation coefficient and used to construct a dendrogram by the

unweighted pair group method (UPGMA) using the SAHN-clustering and TREE programs from NTSys version 2.02 (Exeter Software, Setauket, NY).

RESULTS AND DISCUSSION

The initial ISSR primer screening investigated 50 primers with varying results. No amplified fragments were produced by eighteen of the primers and 12 of these were the primers that contained AT repeats. Nagaoka and Ogihara (1997) found a similar result and concluded that at the high annealing temperature used (52°C), primers with AT repeats were probably unable to anneal to the DNA. Faint fragments were produced by six primers and deemed unreliable. The remaining 26 primers produced at least one robust fragment with some also producing faint fragments. Eleven primers, of these 26, were selected for use in the full study (Table 2). These eleven were chosen because they had the greatest number of fragments and good separation between the fragments. In addition, a twelfth primer (UMN001) was designed and used to provide the study with one primer that had the tag on the 5′ side of the repeat.

The use of the twelve selected primers in the full study found that four primers produced bands that had inconsistent amplification while the remaining eight produced robust reproducible bands and were used to create the similarity matrix (Table 3) and the dendrogram (Figure 1) among the 20 clones.

The eight most useful primers produced a total of 38 bands of which 16 were exclusive to *P. supina* or *P. trivialis* (Table 2). The remaining 22 were used in differentiating the

creeping bluegrass accessions. From these 22 bands, seventeen, or 77.3%, were found to be polymorphic. Three primers that contained GT repeats produced 14 bands of which 13 were polymorphic and made up over 75% of the total polymorphic bands and had sufficient resolution to distinguish all but two of the creeping bluegrasses (Table 2 and Figure 1). Figure 2 displays the polymorphic banding pattern obtained from one of the GT repeat primers, UBC850.

The dendrogram showing the relatedness of the 20 clones (Figure 1) was constructed from the matrix in Table 3 using a similarity coefficient. The two species used as outliers were both grouped away from the creeping bluegrasses. *Poa supina* was somewhat similar to the creeping bluegrasses with a similarity coefficient of 0.20; however, *P. trivialis* showed little similarity and actually had a similarity coefficient of zero. The clustering within the dendrogram agreed with the known genealogy (Table 1) of the creeping bluegrass plants used in this study and was divided into two main clusters (A and B).

Cluster A (Figure 1) consists of four subclusters that have a high degree of relatedness within each cluster (see Table 1 for pedigrees). In subcluster A_1 three of the four accessions are closely related; UMN14745 and UMN14838 were sibling seedling selections from an earlier generation UMN10184. However, the fourth accession, UMN11930, was an unexpected addition to this subcluster. Its origin and unique phenotypic characteristics of non-flowering, and thick, somewhat curled leaves are different from UMN10184.

Subcluster A_2 confirms our known pedigrees; UMN14199 and UMN13979 are very closely related, originating from the same cross of unidentified parents. Although closely related, these two accessions are distinguishable through the use of ISSR PCR. These two accessions branch from a higher subbranch along with the subcluster that contains UMN10184, a parent commonly used in crosses. This close relationship may indicate that one of the parents of these two accessions was UMN10184.

UMN14045, UMN14524, and their common paternal parent UMN10234 comprised subcluster A_3. Phenotypically, the two progeny are quite different from their paternal parent UMN10234 (Figure 3). This genetic similarity coupled with phenotypic dissimilarity may represent the potential for particular paternal alleles, which are not necessarily exhibited phenotypically, to accumulate during the process of advancement through multiple generations of selfing and selection.

Subcluster A_4 is comprised of two plants from different geographic origins. However, they have two significant phenotypic characteristics in common; they are both ultra-dwarf and diploid (Figure 4). The grouping of these two plants is quite remarkable and indicates the possibility that two accessions collected from distant geographic regions may possess genetic similarities.

In contrast to cluster A, cluster B is a group of plants that possess little or no pedigree commonality, but can be divided into subclusters B_1 and B_2. Subcluster B_1 contains five

unrelated plants including two diploid plants that were genotypically indistinguishable (subbranch B_{1a}) with the ISSR primers that were used in this study. One possible reason for the difficulty in differentiating these two individuals may be their similar origin (Table 1); UMN14383 and UMN14833 were collected from different research greens, but because of their close proximity to one another, it is possible that they are the same clone.

Subcluster B_2 consists of two plants that originated from very different sources (Table 1) yet have some similar phenotypic characteristics; UMN12283 is an annual and UMN13861 has some annual-like characteristics; more bunch type growth habit, shorter prereproductive period, and decreased disease resistance.

Based upon subclusters A_4, B_{1a} and B_2, it appears that there may be an association between the ISSR markers and morphological or life history traits. Many more loci would be necessary to verify this association. However, an association such as this was seen with barley (Fernandez et al., 2002) where the winter and spring habit types clustered independently of each of other even when from diverse genetic backgrounds.

Ideally, the optimum result would be to find novel bands for each individual using one primer. Novel bands allow one to identify a specific variety quickly using a minimum number of primers. The reported number of novel bands in other studies using ISSR PCR has been highly variable. Mattioni (2002) found nine novel bands for 124 cultivars and Fernandez et al. (2002) found 34 novel bands for 14 cultivars. Although novel bands are desirable, there can be limitations to their usefulness. Most studies investigate only a

limited number of cultivars and as other cultivars are investigated, the novel bands may be found to occur in other genotypes. Among the amplified PCR products in the current study, two novel bands were found for two of the more unique individuals. One for UMN12283 using primer 001 (Figure 5a) and another for UMN14577 using primer 841 (Figure 5b).

Except for the single novel band mentioned previously for primer 841, primers 841 and 848 provided only interspecies and no intraspecies differences. In addition, primer 841 produced what appears to be a species specific marker for *P. annua*, however, further investigation would be necessary for verification. A species specific marker could be used to develop species specific primers.

In contrast to the potential species specific marker, was the presence of some markers common to all *P. annua* accessions and *P. supina*, and one marker present in all three species (*P. annua*, *P. supina* and *P. trivialis*). The presence of common markers across species could be explained in one of two ways. Either different markers of the same size are being independently amplified and appear to be the same product, or some regions of the genome have been highly conserved across different species of the same genera. If the latter were true, it could mean that ISSR PCR sometimes amplifies across important genomic coding regions. The amplification of such regions could aid in the discovery of the purpose for the coding region. Pasakinskiene et al. (2000) however found no evidence that functional coding regions were amplified, but Kojima et al. (1998) state that ISSR markers may mark gene-rich regions.

Ethidium bromide stained agarose gels were utilized for this study because others had reported finding approximately 10 bands/primer using this technology (Arcade et al., 2000; Nagaoka and Ogihara, 1997) and because of their ease of use. Studies employing silver stained polyacrylamide gels or fluorescence-labeled primers obtained many more scoreable bands ranging from 20 to 50 bands/primer (Fernandez et al., 2002; Nagaraju et al., 2002; Kantety et al., 1995). This study found 4.75 bands/primer and 2.75 bands/primer for all three species and *P. annua* alone, respectively. The use of silver stained polyacrylamide may have provided better visualization and reproducibility of the many faint bands that could not be consistently scored from gel to gel with agarose.

Marker similarities and differences between *P. annua* and *P. supina*, may be useful in furthering the study of the origin of *P. annua* and its relationship to its purported parents. Determining whether or not the more annual-like and perennial-type *P. annua* plants are more closely related to the annual *P. infirma* and the perennial *P. supina*, respectively, would be a fascinating extension of the current work.

Since there are no published or known microsatellite markers for *P. annua*, and they are rarely transferable from other species, the amplified fragments from this study could be sequenced and used to develop microsatellite markers just as van der Nest et al. (2000) successfully did with *Eucalyptus*. Unlike ISSR markers, microsatellites are codominant markers, which can be extremely valuable for heritability and QTL studies.

Arcade et al. (2000) and Kojima et al. (1998) both found ISSR markers that exhibited normal expected segregation ratios in the populations of larch and wheat, respectively. In addition, ISSR markers have been found to be linked to disease resistance genes in chickpea (Ratnarparkhe et al., 1998; Santra et al., 2000) and major and minor genes for seed weight in wheat (Ammiraju et al., 2001).

Important traits that could be mapped or are linked to ISSR markers, or SSR markers developed from them, which could be studied include seed dormancy along a moisture gradient (Wu et al., 1987); life history traits as related to stress (Cline, 2001); earliness of flowering (Heide, 2001); seasonal vs. continual flowering (Johnson and White, 1998); vernalization and photoperiodic requirements for flowering (Johnson and White, 1997a and 1997b); dwarfness; herbicide resistance (Mengistu et al., 2000); and disease resistance.

SUMMARY AND CONCLUSIONS

ISSR PCR produced highly polymorphic amplification products that allowed for the differentiation of all but two diploid accessions using as few as three primers. Parents, progeny, and closely related breeding lines were easily distinguishable from one another. The ISSR data also clearly illustrated relationships among the accessions, grouping *P. trivialis* and *P. supina* away from the creeping bluegrasses and forming clusters that generally agreed with known genealogies.

ISSR PCR can be used as a simple, fast and relatively inexpensive method to produce useful DNA markers in creeping bluegrass. It could be useful for cultivar identification, seed certification, and identification of turfgrass species. It also offers the possibility of developing species specific primers and microsatellite markers. Finally, it is a robust method for generating polymorphic loci that could be utilized in the study of genetic relatedness, heritability or linkage to important traits, development of linkage maps, and marker assisted breeding.

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Table 1. *Poa* accessions, chromosome number, and origin.

Accession	Chromosome number	Origin/Lineage	Notes
P. annua var. *reptans*			
UMN10184	2n=4x=28	New York	
UMN14080	2n=4x=28	New York	
UMN10234	2n=4x=28	Minnesota	
UMN11930	2n=2x=14	Minnesota	
UMN13820	2n=2x=14	Maryland	ultra-dwarf
UMN13834	2n=4x=28	Maryland	dwarf
UMN13861	2n=4x=28	12167x10042	
UMN13979	2n=4x=28	unknown cross	
UMN14045	2n=4x=28	10184x10234	
UMN14199	2n=4x=28	seedling from 13979	
UMN14745	2n=4x=28	seedling from 10184	
UMN14232	2n=4x=28	13979x10042	
UMN14838	2n=4x=28	seedling from 10184	
UMN14383	2n=2x=14	research green K-11, Minnesota	
UMN14833	2n=2x=14	research green K-3, Minnesota	
UMN14524	2n=4x=28	10042x10234	
UMN14577	2n=2x=14	Minnesota	ultra-dwarf
P. annua var. *annua*			
UMN12283	2n=4x=28	Southern US	
P. supina			
UMN13859	2n=2x=14	unknown	
P. trivialis			
UMN14404	2n=2x=14	Minnesota	

Table 2. ISSR primers used in performing PCR. Total reproducible bands (TB), total polymorphic bands (TP), proportion of polymorphic bands (PP) for all accessions and *P. annua* accessions alone and notes about amplification results.

		All accessions			*P. annua* accessions			
Primer	Sequence 5′→ 3′	TB	TP	PP	TB	TP	PP	Amplification results
UBC809	$(AG)_8G$							Inconsistent
UBC823	$(TC)_8C$							Inconsistent
UBC825	$(AC)_8T$	3	3	1.00	1	1	1.00	Good
UBC827	$(AC)_8G$	4	4	1.00	2	1	0.50	Good
UBC829	$(TG)_8C$							Inconsistent
UBC834	$(AG)_8YT$							Inconsistent
UBC836	$(AG)_8YA$	3	2	0.67	2	1	0.50	Good
UBC841	$(GA)_8YC$	5	5	1.00	2	1	0.50	Good
UBC848	$(CA)_8RG$	3	3	1.00	1	0	0.00	Good
UBC849	$(GT)_8YA$	3	3	1.00	2	2	1.00	Good
UBC850	$(GT)_8YC$	10	10	1.00	7	6	0.86	Good
UMN001	$CA(GT)_8$	7	7	1.00	5	5	1.00	Good
Total		38	37	0.96	22	17	0.77	

Y = C or T
R = A or G

Table 3. Similarity matrix among all *Poa* accessions using 38 ISSR fragments.

Access.	10184	14080	10234	11930	12283	13820	13834	13859	13861	13979	14045	14199	14745	14232	14838	14383	14404	14833	14524	14577
10184	1.00																			
14080	0.85	1.00																		
10234	0.81	0.72	1.00																	
11930	0.95	0.79	0.85	1.00																
12283	0.64	0.67	0.61	0.57	1.00															
13820	0.70	0.50	0.66	0.74	0.50	1.00														
13834	0.85	0.78	0.72	0.79	0.67	0.72	1.00													
13859	0.20	0.01	0.16	0.25	0.12	0.28	0.23	1.00												
13861	0.76	0.78	0.71	0.69	0.78	0.60	0.78	0.10	1.00											
13979	0.74	0.56	0.72	0.79	0.56	0.72	0.67	0.45	0.67	1.00										
14045	0.76	0.67	0.94	0.80	0.55	0.60	0.67	0.21	0.65	0.67	1.00									
14199	0.79	0.62	0.79	0.84	0.62	0.67	0.73	0.40	0.73	0.95	0.73	1.00								
14745	0.81	0.61	0.66	0.85	0.50	0.77	0.61	0.28	0.71	0.83	0.60	0.79	1.00							
14232	0.70	0.83	0.66	0.63	0.61	0.43	0.72	0.16	0.71	0.61	0.71	0.67	0.43	1.00						
14838	0.85	0.67	0.61	0.79	0.56	0.72	0.67	0.23	0.78	0.78	0.55	0.73	0.95	0.50	1.00					
14383	0.81	0.95	0.66	0.74	0.61	0.43	0.72	0.05	0.71	0.50	0.71	0.56	0.55	0.89	0.61	1.00				
14404	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00			
14833	0.81	0.95	0.66	0.74	0.61	0.43	0.72	0.05	0.71	0.50	0.71	0.56	0.55	0.89	0.61	1.00	0.00	1.00		
14524	0.81	0.61	0.89	0.85	0.50	0.66	0.72	0.28	0.60	0.72	0.94	0.79	0.66	0.66	0.61	0.66	0.00	0.66	1.00	
14577	0.64	0.56	0.72	0.68	0.56	0.72	0.56	0.12	0.67	0.56	0.67	0.62	0.61	0.50	0.56	0.50	0.00	0.50	0.61	1.00

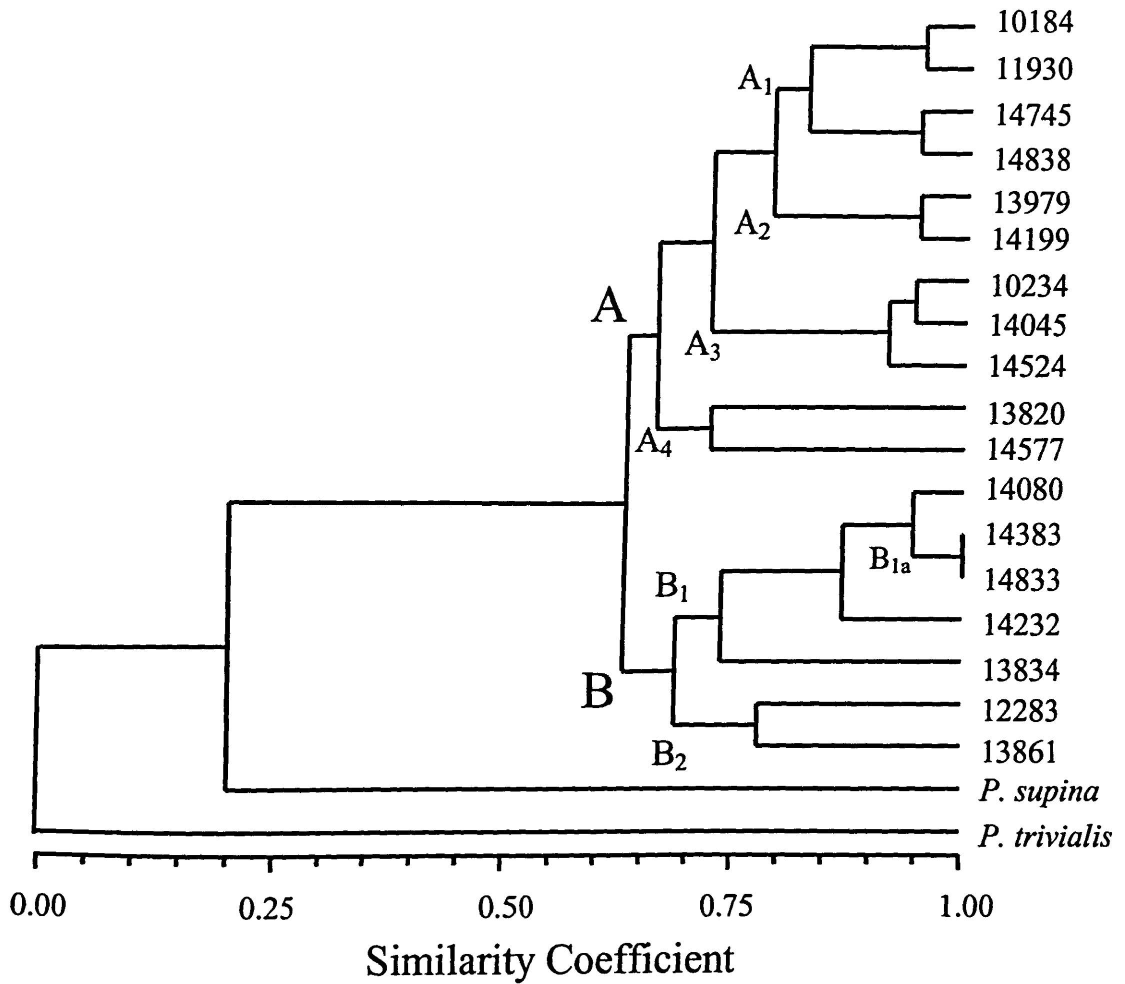

Fig. 1. Dendrogram derived from a UPGMA cluster analysis of 38 ISSR fragments showing the relatedness among 20 *Poa* accessions.

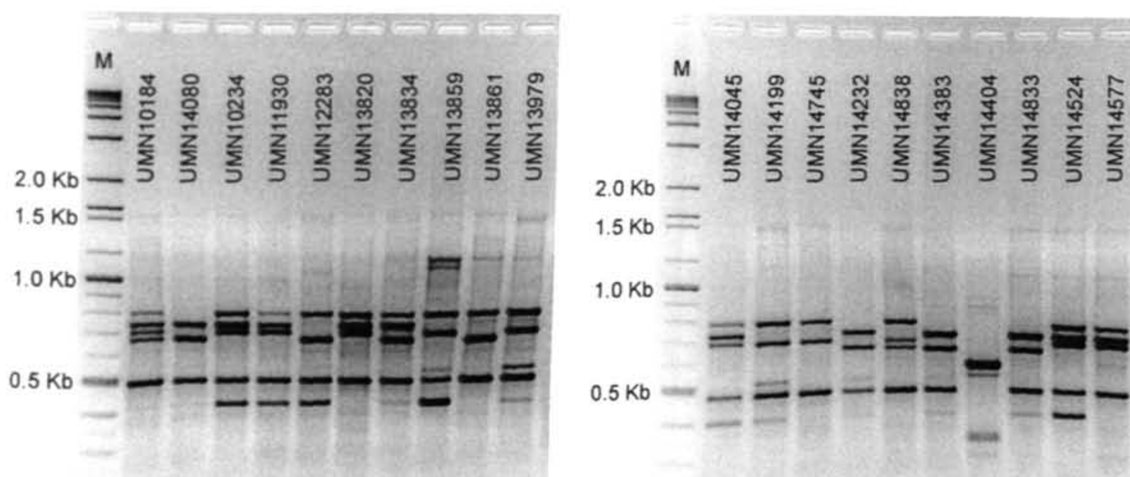

Fig. 2. Negative images of ISSR agarose gels stained with ethidium bromide showing the banding patterns obtained for all accessions using primer UBC850.

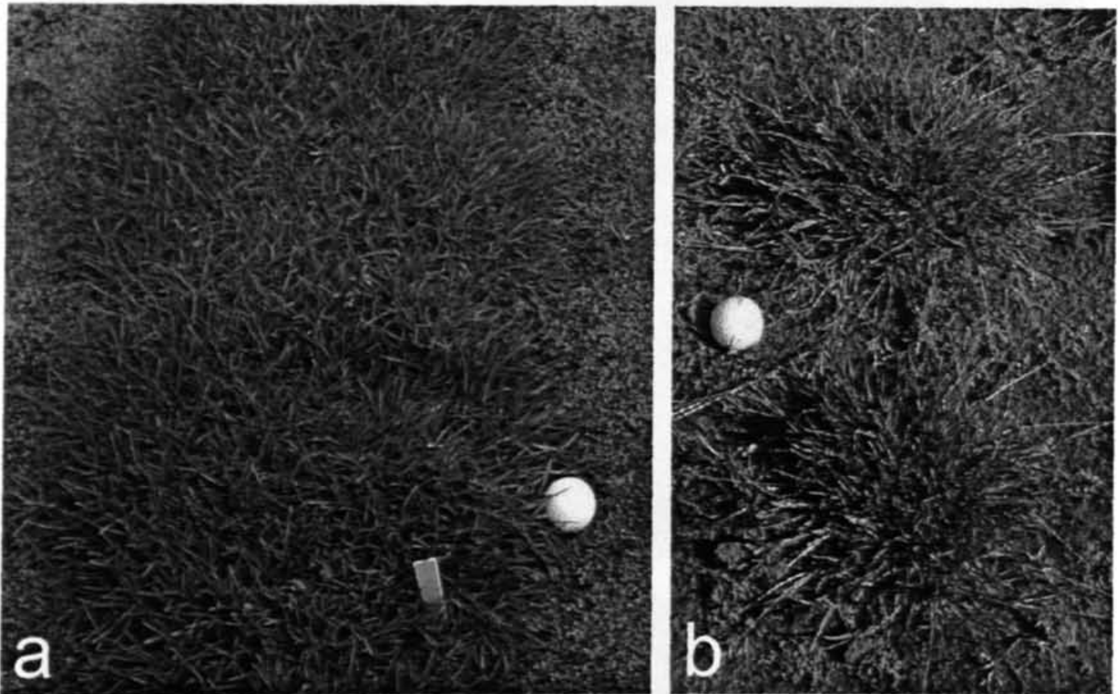

Fig. 3. Two members of subcluster A_3 showing some of the phenotypic differences between (a) UMN14524 and its paternal parent (b) UMN10234. Each plant was derived from a single seed and was approximately 120 days old. Plants are unmowed and golf ball is pictured for scale.

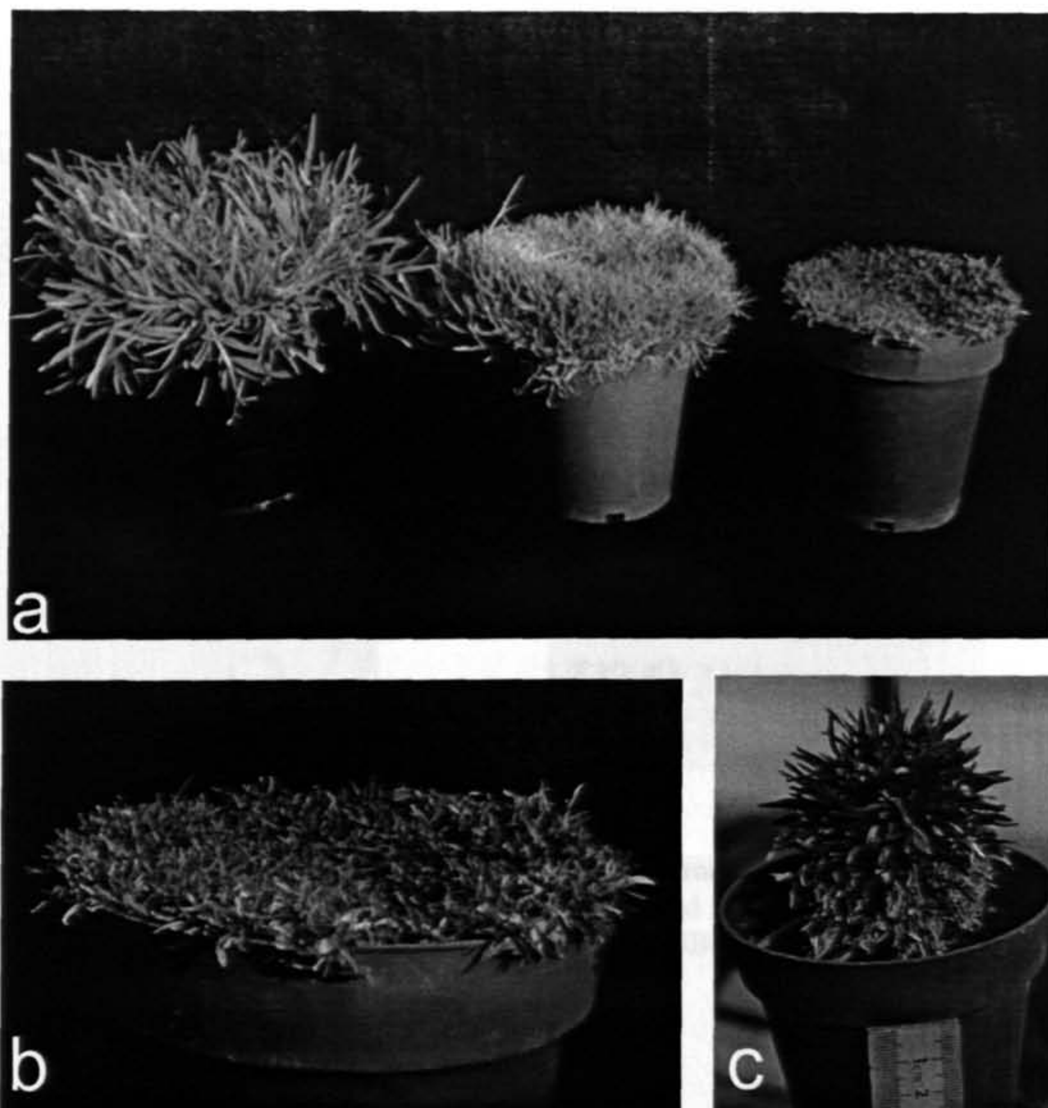

Fig. 4. Visual comparison of typical, dwarf, and ultra-dwarf forms of creeping bluegrass. (a) Left to right: typical perennial tetraploid; dwarf tetraploid, UMN13834; and ultra-dwarf diploid, UMN13820. All in standard 15 cm pots. (b) Close-up of ultra-dwarf diploid, UMN13820 in a standard 15 cm pot. (c) Ultra-dwarf diploid, UMN14577 in a standard 7.6 cm pot.

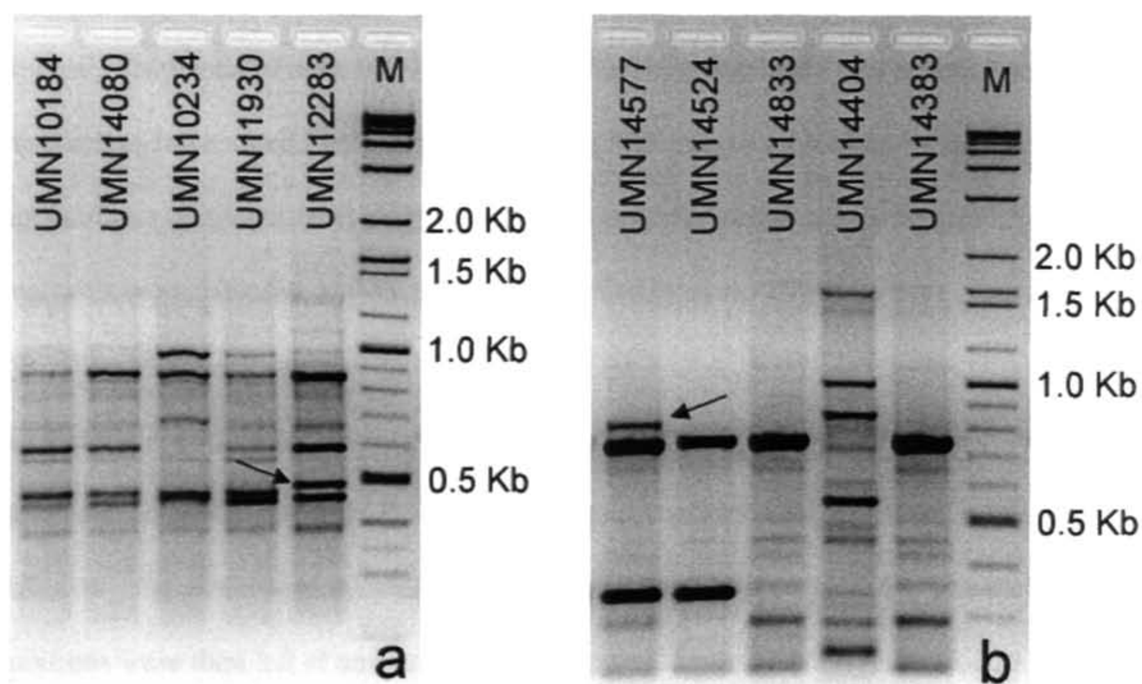

Fig. 5. Negative images of ethidium bromide stained agarose gels showing novel fragments found for two of the accessions. (a) Novel band for UMN12283 using primer UMN001 (b) Novel band for UMN14577 using primer UBC841.

Appendix 1. The effect of delayed PCR reaction start time on amplified products.

The hypothesis: that delays during reaction mixture setup would lead to inconsistent band intensity from lane to lane, blank lanes, and poor reproducibility was tested. Some researchers have noted that mispriming during PCR can be a problem in certain applications (Kaijalainen et al., 1993) and non-specific products may be amplified if reactants were mixed at ambient temperature (Oakey et al., 1999).

To test the necessity of rapidly getting the reaction mixture into the thermal cycler, accessions UMN10234 and UMN14080 along with primer UBC825 were employed following the protocol in the materials and methods section on page 18. The reaction mixtures were then left at ambient temperature for 25 min., placed on ice for 25 min., or placed immediately in a preheated block at 94°C. There was also speculation that there may be amplification differences between the different thermal cycler blocks attached to the Hybaid Omnigene Thermocycler (Hybaid Inc.), so in addition to the timing treatments, the products produced in two separate blocks were also compared. The test was performed only once and not repeated.

The results are displayed in Figure 1. Although one lane had reduced band intensities, all bands with high intensity, with the exception of one, were highly reproducible between the two thermal cycler blocks and across all three treatments even when the reaction mixture was left standing at ambient temperature for 25 minutes. This indicates that ISSR primers provide very consistent results using this method of assembling reaction

mixtures. Further investigation found that thorough mixing of the reactions eliminated many of the variations that were occurring.

REFERENCES

Kaijalainen, S., P.J. Karhunen, K. Lalu, and K. Lindstrom. 1993. An alternative hot start technique for PCR in small volumes using beads of wax-embedded reaction components dried in trehalose. Nucleic Acids Res. 21:2959-2960.

Oakey, H.J., L.F. Gibson, and A.M. George. 1999. DNA probes specific for Aeromonas hydrophila (HG1). J. Appl. Microbiol. 86:187-193.

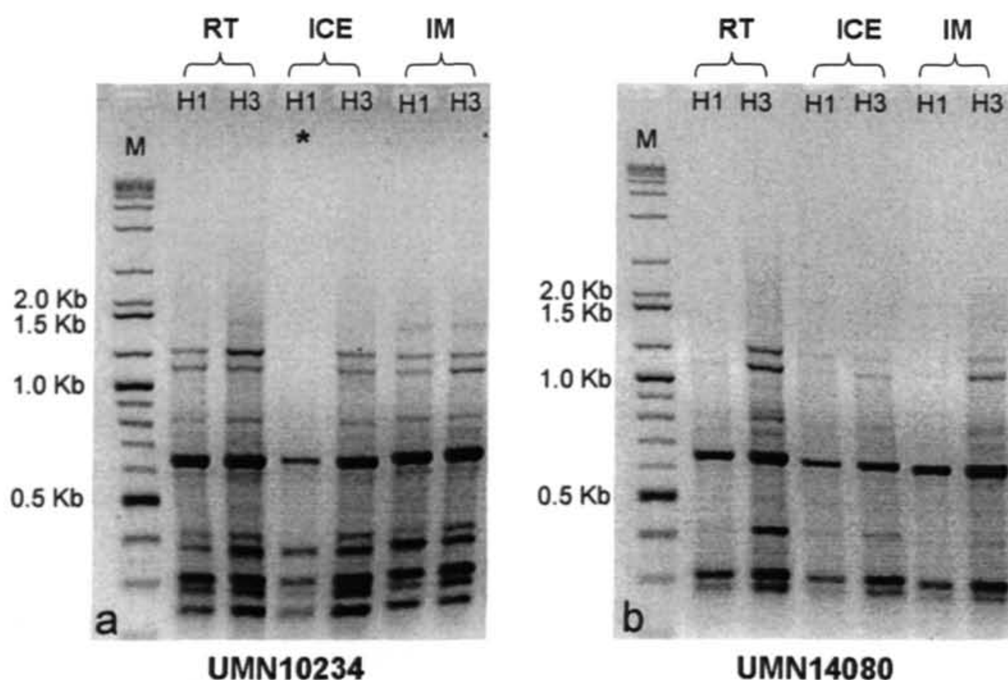

Fig. 1. Test for delays in reactions mixture setup. Negative images of ISSR agarose gels stained with ethidium bromide showing the results from two concurrent tests. One compared the two Hybaid Omnigene Thermocycler blocks 1 (H1) and 3 (H3). The other studied the results of leaving the reaction mixtures at ambient temperature (RT) for 25 min., placing them on ice (ICE) for 25 min., or placing them immediately (IM) in a preheated block at 94°C. Except for the one lane with lower band intensities (*), which was probably a random variation, and the lone high intensity band at 400 bp in H3 of RT on gel b, the robust bands were present across all treatments. (a) Study conducted with DNA from UMN10234 and primer UBC825. (b) Study conducted with DNA from UMN14080 and primer UBC825.