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Full Length Research Paper

Genetic diversity in some introduced pasture grass cultivars revealed by inter-simple sequence repeats (ISSR) markers

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This study was focused on studying the genetic diversity among pasture grass cultivars using inter-simple sequence repeats (ISSR) markers to determine their genetic background. Six grass cultivars were used in this study: two perennial ryegrass (*Lolium perenne* L.) cultivars (Aries and Quartet), two endophyte-free tall fescue (*Festuca arundinacea* Schreb.) cultivars (Fawn and K5666v) and two orchardgrass (*Dactylis glomerata* L.) cultivars (Tekapo and Niva). Analysis of the 15 selected ISSR primers among the six grass cultivars generated 77 bands, 66 (85.7%) of which were polymorphic. The two perennial ryegrasses cultivars (Aries and Quartet) were distinguished by a 600-bp amplification fragment produced by primer UBC807. The 500-bp amplification fragment was produced by primer UBC825, which was distinguished in Niva, but absent in Tekapo- orchard grass. Furthermore, Fawn-tall fescue was distinguished by a 400-bp amplification fragment produced by primer UBC825. The minimum genetic similarity (GS) value was obtained between the two orchard grass cultivars (Niva and Tekapo), while the maximum GS value was obtained between the two perennial ryegrasses cultivars (Aries and Quartet).

Key words: Genetic variation, inter-simple sequence repeats (ISSR) marker, pasture grass.

INTRODUCTION

Perennial ryegrass, orchardgrass and tall fescue are cool season plants (C3 plants), which have an optimum growing temperature of 18 to 24°C (Rohweder and Albrecht, 1995). Perennial ryegrass is a temperate species grown throughout the world including North and South America, Europe, New Zealand and Australia. Orchard grass (Cocksfoot in Europe and Australia) is commonly recommended for pastures in the North-eastern and North Central United States (Van Santen and Sleper, 1996). Throughout the world, orchard grass is found in areas of moderate to high rainfall, moderate winter and warm summer (Christie and McElroy, 1995). Also, it can be grown in low rainfall environment such as the Mediterranean and North Africa region. Tall fescue is used widely for green forage, turf and conservation purposes (Collins and Hannaway, 2003). It has high yield stability and broad adaptation to different environments, whether grown as monoculture or binary mixtures (Al-Ghumaiz, 2006). The mentioned grass species are important because of their diversity and adaptation to a wide range of environmental conditions. In addition, cultivars within species have different adaption based on their country of origin. Previous studies indicated that producers should select cultivars that originate from a location with similar climatic conditions (Warnock et al., 2005). Since the indicated species have adaptation to wide range of environmental conditions, producers in Saudi Arabia could benefit from growing such species in their agricultural systems.

Many molecular techniques are being used to distinguish cultivars in crop species. Various DNA-based

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Abbreviations: ISSR, Inter-simple sequence repeats; **GS,** genetic similarity.

marker systems have been applied to several plant groups to assess their level of relatedness, for development of marker-assisted selection and cultivar discrimination (Paplauskienė et al., 2007; Xiong et al., 2007). One of the most efficient molecular marker methods, in terms of ability to produce polymorphic markers within a comparatively short time and with a limited budget is inter-simple sequence repeats (ISSR) profiling for total genomic DNA. Inter-simple sequence repeats (ISSRs) have become widely used in many plant species and have a potential to contribute in breeding, genetics and systematics (Posselt et al., 2006). In addition, studies have indicated that ISSRs produce more reliable and reproducible bands when compared with random amplification of polymorphic DNAs (RAPDs) because of the higher annealing temperature and longer sequence of ISSR primers (Nagaoka and Ogihara, 1997; Qian et al., 2001). ISSRs have been used for cultivar identification in barley *(Hordeum vulgare)* (Hou et al., 2005), wheat *(Triticum aestivum)* (Motawei et al., 2007), perennial ryegrass (Pivoriene and Pasakinskene, 2008) and turfgrass (*Cynodon dactylon* and *Cynodon transvaalensis*) (Al-Humaid et al., 2004). Moreover, ISSR markers have potential importance in facilitating selection procedure, particularly for pyramiding two or more different genes, aiming at a more durable and broad-spectrum resistance (Motawei et al., 2007). Therefore, ISSRs have proved to be useful in population genetic studies, especially in detecting clone diversity and fingerprinting closely related individuals (Wolfe and Liston, 1998). Our study seeks to determine the genetic variation between cultivars within species using ISSR markers.

MATERIALS AND METHODS

Site description and plant materials

Field experiments were conducted under irrigation conditions during 2008 and 2009 seasons at Qassim University Agricultural Research and Experimental Station (26° 18 28 N, 43° 46 E). The site of the experiment was sandy loam soil that contained low level of soluble salts (EC = 1.5 dsm^{-1}) and low organic matter (1.3%) with pH value of 8.1. Cool season grass cultivars were established in a randomized complete block design (RCBD) with three replications using 3 m^2 plot (10 rows per plot) with seeding rate of 20 kg ha^{-1}. Each replication consisted of six grass cultivars as follows: two perennial ryegrass, two orchardgrass and two endophyte-free tall fescue cultivars. Table 1 listed the name and country of origin of species and cultivars used in this study. Planting date was December 27, 2009. Nitrogen (N) was applied to all plots following each cutting in the form of diammonium phosphate (DIP) at a rate of 50.4 kg ha^{-1}.

Genetic variation among cultivars

Bulk leaf samples of each grass cultivar were collected and ground into fine powder with liquid nitrogen. DNA was extracted in 10 ml of cetyltrimethlyammonium bromide (CTAB) buffer consisting of 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 1% CTAB.

The homogenate was incubated for 2 h at 65°C with occasional mixing. Following incubation, 5 ml of chloroform/isoamylalcohol (24:1) was added to the tubes, mixed and centrifuged at 2600 g for 10 min. The aqueous phase was removed and put in a fresh tube and an equal volume of ice-cold isopropanol was added followed by centrifugation as above to precipitate the DNA. The pellet was dissolved in Tris EDTA (TE) buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). The DNA concentration was assessed spectrophotometrically at 260 nm, and quality was assessed by the 260/280 ratio (Sambrook et al., 1989). The DNA was suspended to a final concentration of 10 ng/µl in 0.5X TE and stored at 4°C.

ISSR assay

The ISSR-PCR method was carried out according to Negaoka and Ogihara (1997). Amplification was carried out in 25 µl reaction volumes, containing 1X Taq polymerase buffer (50 mM KCl, 10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$) and 1 unit of Taq polymerase (Pharmacia Biotech, Germany) supplemented with 0.01% gelatin, 0.2 mM of each deoxynucleotide triphosphates (dNTPs) (Pharmacia Biotech, Germany), 50 ρmol of ISSR primers (Table 2), and 50 ng of total genomic DNA. Amplification was performed in a thermal cycler (Thermolyne Amplitron) programmed for one cycle of 2 min at 94°C; and 35 cycles of 30 s at 94°C, 45 s at either 42 or 50°C, depending on the melting temperature (Tm) value of the primer pair, and 1.3 min at 72°C; followed by 20 min at 72°C. After completion of PCR, samples were cooled immediately to 10°C and stored at 4°C until gel separation. A gel-loading solution (5 µl) was added, and 10 µl of the total product volume was resolved in 1.5% agarose in 1X Tris-acetate-EDTA (TAE) buffer for 2 h aside with a 100-bjp ladder (Pharmacia, Germany) as the size standard. Gels were stained in ethidium bromide and images were recorded.

Data analysis

Data of ISSR analyses were scored on the basis of the presence or absence of the amplified products for each primer. If a product was present in a cultivar, it was designated “1”, if absent it was designated “0”. Pair-wise genetic similarity matrix was generated among the six grass cultivars using Jaccard similarity coefficient (Jaccard 1908). The similarity coefficients were performed using NTSYS-PC software version 2.0 (Rohlf, 2000).

RESULTS AND DISCUSSION

Genetic variation results for grass cultivars

Analysis of the 15 selected ISSR primers among the six grass cultivars, included in this study generated 77 bands, 66 (85.7%) of which were polymorphic (Table 2). The dinucleotide repeats $(CA)_n$ primer had more bands than the other dinucleotide and trinucleotide repeats $(GAG)_n$ primers. The dinucleotide $(AC)_n$ and trinucleotide repeats $(GAG)_n$ primers generated the highest ISSR banding pattern polymorphism (10%) among grass cultivars (Table 2). This might indicate that di- and trinucleotide-based ISSR-PCR markers could provide potential marker in pasture grass genome. Primers were evaluated for their ability to identify any cultivar as illustrated by the example of Primers UBC807 and UBC-

Table 1. Description of the six grass cultivars used in this study.

Species name	Cultivar name	Country of origin
Perennial ryegrass	Aries	New Zealand
Perennial ryegrass	Quartet	New Zealand
Orchardgrasses	Tekapo	New Zealand
Orchardgrasses	Niva	Czech Republic
Tall fescues	K5666v	New Zealand
Tall fescues	Fawn	Oregon -USA

Source: AMPAC seed company-USA.

Table 2. ISSR primers used in this study and summary of ISSR markers from grass cultivars.

Primer	Primer sequence	Annealing temperature (°C)†	Amplified product	Fraction polymorphic fragment‡
Di-nucleotide repeats				
UBC807	(AG)8T	50	5	4/5
UBC810	(GA)8T	50	6	6/6
UBC811	(GA)8C	50	4	2/4
UBC825	(AC)8T	50	7	7/7
UBC826	(AC)8C	50	3	2/3
UBC827	(AC)8G	50	3	2/
D24	(CA)6CG	42	9	8/9
D12	(GA)6CG	42	5	4/5
Tri-nucleotide repeats				
D14	(CAC)3GC	42	5	4/5
G07	(GAA)5CG	50	6	6/6
G11	(CAA)5GC	50	2	1/2
HB13	(GAG)3GC	42	7	7/7
HB14	(CTC)3GC	42	6	6/6
HB15	(GTG)3GC	42	6	5/6
UBC864	(ATG)6	50	3	2/3

†Determined empirically; ‡number of polymorphic fragments/number of fragments amplified. ISSR, Inter-simple sequence repeats.

825 (Figure 1). The two perennial ryegrass cultivars (Aries and Quartet) were distinguished by a 600-bp amplification fragment produced by primer UBC807. This amplification fragment was present in Quartet, but not in Aries (Figure 1). The 500-bp amplification fragment produced by primer UBC825, which was distinguished in Niva, was absent in Tekapo- orchard grass (Figure 1). Furthermore, Fawn-tall fescue was distinguishable by a 400-bp amplification fragment produced by primer UBC825, which was absent in K5666V. Also, Fawn-tall fescue showed superiority in dry matter yield over K5666V-tall fescue and the other grass cultivars in surviving high temperatures (AL-Ghumaiz and Motawei, 2011). Therefore, cult polymorphism between various cultivars can arise through: nucleotide changes that prevent amplification by introducing a mismatch at one priming site; deletion of a priming site; insertions that render priming sites too distant to support amplification; and insertions or deletions that change the size of the amplified product (Motawei et al., 2007). The detected degree of polymorphism was high, showing that the ISSR markers were efficient in detecting genetic variability between the tested cultivars. The efficiency of ISSR markers in identifying cultivars and genotypes of perennial ryegrass have been reported (Ghariani et al., 2003; Posselt et al., 2006).

The ISSR-derived data were used to calculate the genetic similarity (ISSR-GS). The genetic similarity co-efficient varied between 0.31 and 0.92 (Table 3). The minimum GS value was derived between the two orchard grass cultivars (Niva and Tekapo), while the maximum GS value was derived between the two perennial

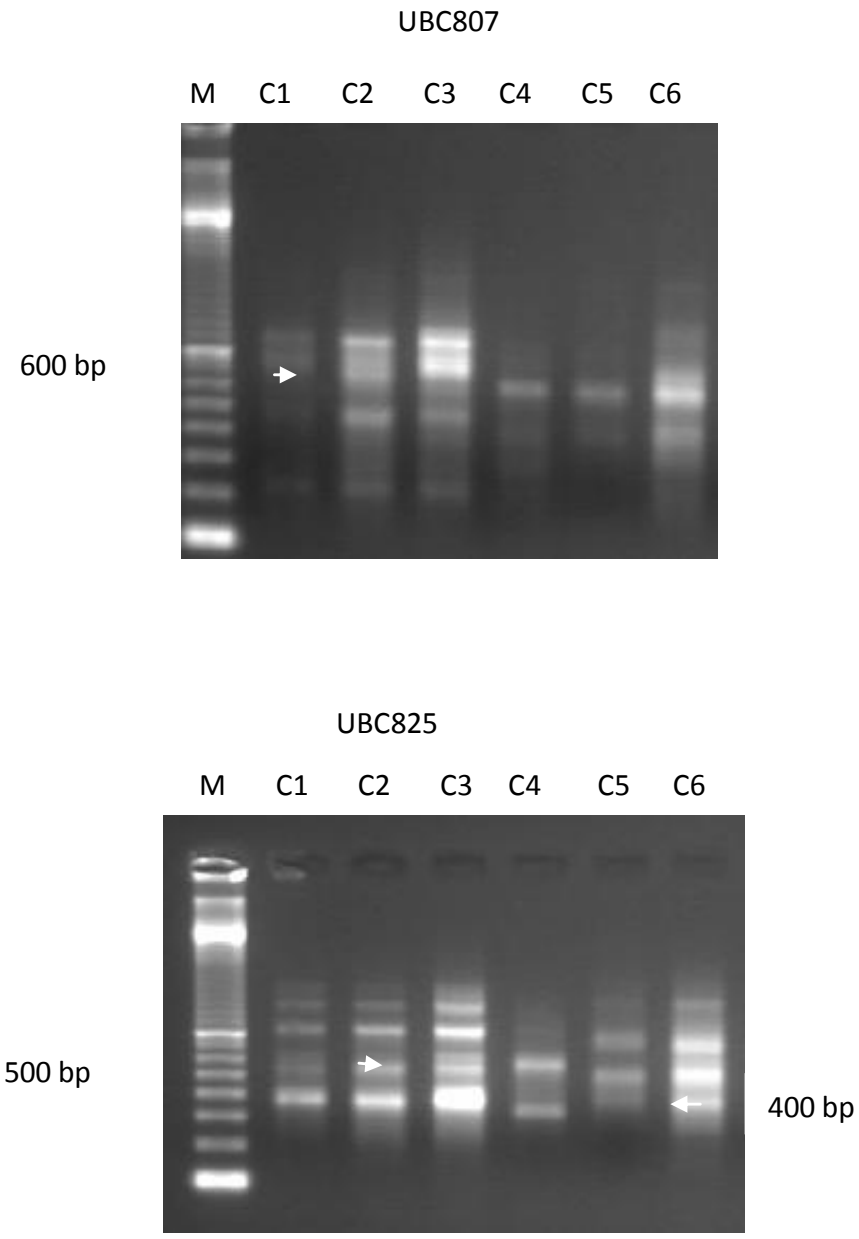

Figure 1. Comparison of ISSR banding patterns generated with primers UCB807 and UBC825 and six grass cultivars DNAs (C1, Aries; C2, Quartet; C3, Niva; C4, Tekapo; C5, K5666V; C6, Fawn). "M" indicates 100-bp size marker ladder. ISSR, Inter-simple sequence repeats.

Table 3. Genetic similarity coefficients among grass cultivars determined from analysis using 77 ISSR loci.

Grass cultivar	Aries	Quartet	Niva	Tekapo	K5666V	Fawn
Aries	1.00					
Quartet	0.92	1.00				
Niva	0.89	0.91	1.00			
Tekapo	0.41	0.38	0.31	1.00		
K5666V	0.48	0.46	0.38	0.56	1.00	
Fawn	0.43	0.46	0.38	0.61	0.79	1.00

ISSR, Inter-simple sequence repeats

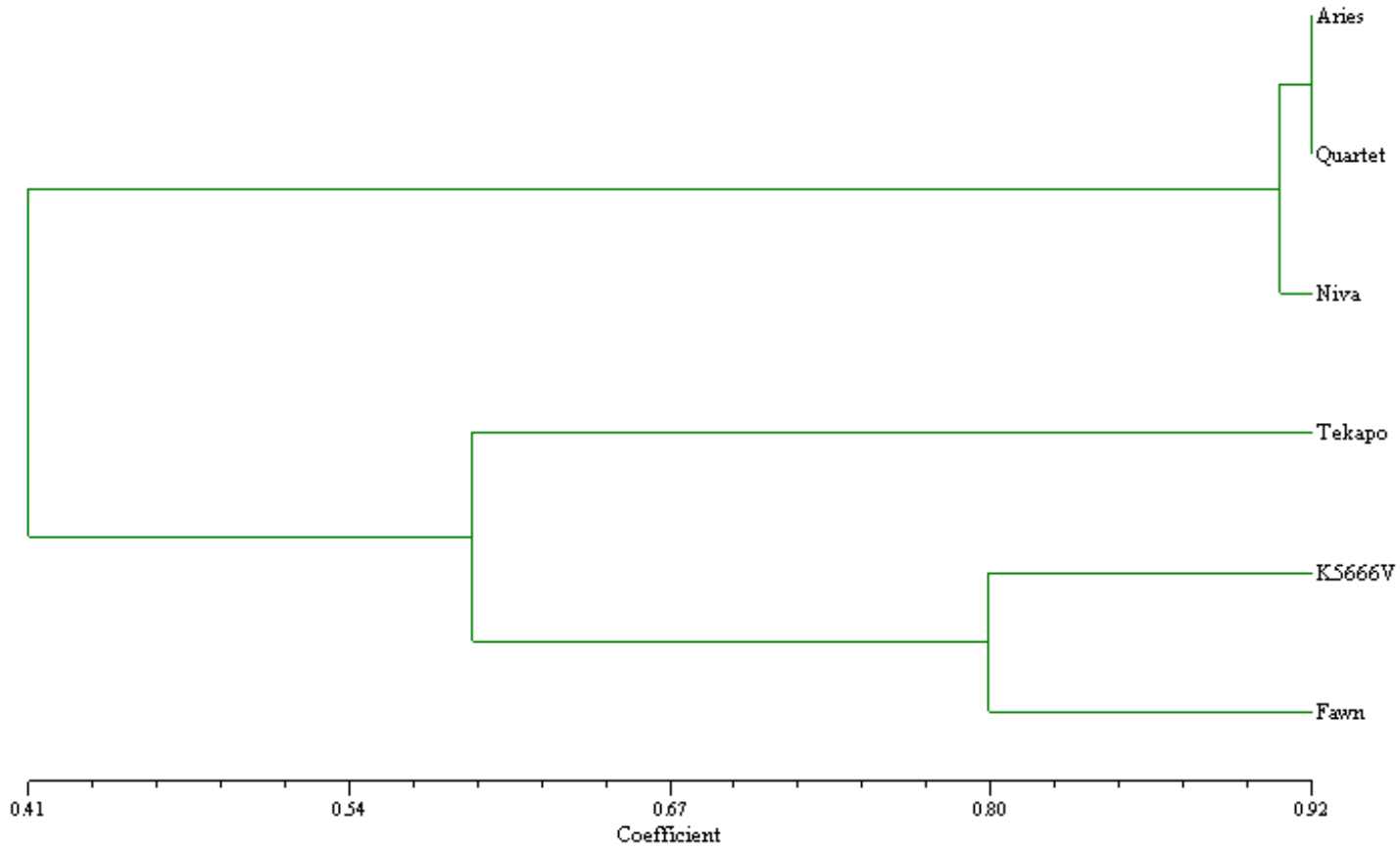

Figure 2. Dendrogram constructed from similarity coefficients and showing the clustering of the tested grass cultivars.

ryegrass cultivars (Aries and Quartet). The genetic similarity between the two tall fescue cultivars (K5666V and Fawn) was lower (0.79) than the two perennial ryegrasses cultivars (Aries and Quartet). Cluster analysis was conducted to generate unweighted pair group method with arithmetic mean (UPGMA) dendrogram elucidating for relationships among grass cultivars (Figure 2). The dendrogram generated using pooled ISSR data divided the grass cultivars into three main clusters (Figure 2). The first cluster is composed of two subclusters: the first subcluster consists of Quartet and Aries; and the second subcluster consists of Niva. The second cluster is composed of the orchard grass cultivar (Tekapo). The third cluster is composed of two cultivars of tall fescue (K5666V and Fawn). Pivorienė et al. (2008) demonstrated that most of the cultivars of perennial ryegrass can be easily distinguished using ISSR assessment. High variability of ISSR markers and high mapping density as compared with RFLP and RAPD data make these dominant, microsatellite-based mole-cular markers ideal for pro-ducing genetic maps of individual species (Nagaoka and Ogihara, 1997). These features, combined with greater robustness in repeat-ability of experiments and less prone to changing band patterns with changes in constituent or DNA template concentrations, make them superior to other readily available marker systems in investigations of genetic variation among very closely related individuals and in crop cultivar classification (Fang and Roose, 1997; Nagaoka and Ogihara, 1997). The results clearly demonstrate that a methodology based on ISSR markers can be used to identify and fingerprint grass cultivars.

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