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Inter-simple sequence repeat (ISSR) loci mapping in the genome of perennial ryegrass

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The aim of this study was to identify and characterize new ISSR markers and their loci in the genome of perennial ryegrass. A subsample of the VrnA F2 mapping family of perennial ryegrass comprising 92 individuals was used to develop a linkage map including inter-simple sequence repeat markers (ISSR). Twelve ISSR fragments out of 29 were mapped to the linkage groups (LG) LG1, LG2, LG4, LG6 and LG7. The total map length was 250.7 cM. Selected ISSR fragments were cloned by transformation into plasmid pTZ57R and sequenced. For four ISSRs, BLAST search revealed a significant similarity to coding regions of known sequences within the current DNA databases. An ISSR fragment of 580 bp, produced by the $(GACA)_4TC$ primer present on LG6, showed a 95% identity to the *Avena sativa* L. transposon and repetitive DNA linked to the receptor kinase gene. A 780 bp fragment generated by $(TG)_8RT$ primer demonstrated a 70% similarity to the *Hordeum vulgare* germin gene *GerA*. Inter-SSR mapping will provide useful information for gene targeting, quantitative trait loci mapping and marker-assisted selection in perennial ryegrass.

Key words. *Lolium perenne,* inter-simple sequence repeats (ISSR), DNA sequence data, mapping

INTRODUCTION

L. perenne belongs to the same Poaceae family as do wheat, barley, maize, oat, and rice. This perennial grass is widely used for livestock forage and also as amenity grass in lawns. Genome mapping data for perennial ryegrass have been accumulated rapidly over the recent years [1]. The construction of molecular marker-based genetic linkage maps, based on one-way pseudo-testcross and two-way pseudo-testcross F2 populations, has provided the basis for trait-dissection in perennial ryegrass. In addition to studies of disease resistance, flowering time variation and morphogenetic traits, a QTL for electrical conductivity correlating to frost tolerance has been located on LG4, close to a heading date QTL [2–4].

To improve the mapping data on perennial ryegrass, we employed the Inter-simple Sequence Repeat (ISSR) marker system which allows detecting polymorphisms in inter-microsatellite loci without previous knowledge of a DNA sequence. ISSRs are informative regarding numerous loci and are suitable to discriminate closely related genotype variants. ISSR markers constitute discrete markers suitable for DNA fingerprinting as a simple and quick PCR-based method [5]. ISSRs have been successfully used to estimate the extent of genetic diversity at the inter- and intra-specific level in a wide range of crop species such as rice, wheat, fingermillet, *Vigna*, sweet potato, and *Plantago* [6]. ISSR fingerprinting was useful for developing DNA markers within the *Lolium* / *Festuca* complex as a tool for marker-assisted selection and for genotype identification [7].

The present work describes the identification and characterization of new ISSR markers and their loci in the genome of perennial ryegrass. The obtained information will increase the density of the recent *L. perenne* linkage map produced by Jensen et. al. [3].

MATERIALS AND METHODS

Development of the mapping population and plant material. The mapping population of the perennial ryegrass produced at the Danish Institute of Agricultural Science (now part of University of Århus) was used in the ISSR mapping experiment. Genotypes of the diploid perennial ryegrass variety 'Veyo' and the diploid ecotype 'Falster' were selected for developing F_1 and F_2 populations because of their contrasting vernalisation requirements. The F_2 population was called VrnA mapping population and consisted of 184 genotypes [3].

Firstly, screening for the polymorphic markers was conducted using four genotypes: Falster and VEYO9 (grandparents), as well

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as F1-30 and F1-39 (F1 parents). Secondly, the polymorphic markers were scored in a F2 population sample comprising 92 genotypes.

ISSR PCR analyses. Twenty one primers, 14 di-nucleotide repeats, 2 tri-nucleotide repeats and 5 tetra-nucleotide repeats were used for ISSR amplification (Table 1). PCR reactions were carried out in the GenAmp PCR System 2700 Thermocycler (Applied Biosystems, USA). Each 20 µl of the reaction contained 50 ng of genomic DNA, 10 × PCR reaction buffer, 50 mM $MgCl_2$, 10 mM dNTP, 2.5 µM of each primer and 2 units of DyNAzyme™ II DNA Polymerase (FINNZYMES, Finland). The thermal profile for ISSR PCR was as follows: 95 °C initial denaturation for 2 min, then 40 cycles of 95 °C for 30 s, 50 °C for 1 min and 72 °C for 1 min. A final extension step of 6 min at 72 °C was followed by 10 °C. ISSR amplification products were separated by gel electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

Linkage map construction. Map construction was carried out using the Haldane mapping function within the JoinMap 3.0 software [8]. The map consists of linkage groups (LG) that are named according to the chromosome assignment found in the ILGI perennial ryegrass population (http:\\ukcrop.net\perl\ace\search\FoggDB).

Cloning and sequencing. Amplified inter-SSR fragments were excised and purified using the QIAquick gel extraction kit (QIAGEN, Germany). Fragments were cloned using the pTZ57R vector and the Inst\Aclone™ PCR Product Cloning Kit (Fermentas, Lithuania) and transformed into *E. coli* DH5ά ultracompetent cells. Plasmid DNA was extracted from overnight cultures using the QIAprep Spin Miniprep kit (QIAGEN). The nucleotide sequence was determined using the ABI PRISM 377 Applied Biosystems at the Sequencing Center (Vilnius).

Computer analysis of sequencing. DNA sequence alignment was performed using BLAST applications at the National Centre of Biotechnology Information (NCBI) web site (http:\\www.ncbi.nlm.nih.gov\).

Table 1. **ISSR marker polymorphisms in the sampled VrnA population of *L. perenne* (92 individuals)**

Primer and attribute	Fragment size (bp)	No. of scorable bands	No. of polymorphic bands	Polymorphism (%)
Di-nucleotide repeats				
G10 $(AG)_{12}$	310–790	9	4	44
155 H $(CA)_7GA$	400–1030	8	5	63
UBC810 $(GA)_8T$	300–900	12	11	92
UBC822 $(TC)_8A$	500–1200	7	7	100
UBC 823 $(TC)_8C$	480–1200	8	3	75
UBC 827 $(AC)_8G$	500–1300	12	12	100
UBC 826 $(AC)_8C$	320–650	9	7	77
UBC 834 $(AG)_8YT$	320–800	8	6	75
UBC 847 $(CA)_8RC$	390–1030	4	3	75
UBC 848 $(CA)_8RG$	350–550	4	3	75
UBC 852 $(TC)_8RA$	400–950	5	4	80
UBC 857 $(AC)_8YG$	550–1650	10	8	80
UBC 860 $(TG)_8RA$	380–990	7	3	43
UBC 858 $(TG)_8RT$	350–1030	7	5	71
Tri-nucleotide repeats				
UBC 866 $(CTC)_6$	500–1500	9	7	77
Tetra-nucleotide repeats				
G08 $(ATG)_5GA$	500–1300	11	11	100
77 H $(AGAC)_4GC$	400–1500	12	9	75
G02 $(ACTG)_4GA$	700–2000	7	5	71
G04 $(GACA)_4TC$	550–2200	11	6	55
104 H $(GACA)_4GT$	700–1700	7	5	75
105 H $(GAGA)_4GA$	400–1140	11	8	73
IN TOTAL	**310–2200**	**153**	**132**	
MEAN		**7.3**	**6.3**	**75**

RESULTS AND DISCUSSION

Our goal in the present work was to produce mapped ISSR molecular markers, to identify their sequences and to expand the existing *L. perenne* genetic map of the VrnA mapping population.

ISSR fragments were evaluated for their potential use in the molecular mapping of *L. perenne*. We tested di-, tri- and tetramer repeats designed with a single or two-base anchor. In total, 21 primers were screened for their ability to generate ISSR fingerprinting patterns, and an assessment was carried out for their polymorphism in the tested VrnA population (Table 1). The highest total number of bands was 12, amplified by the $(AC)_8G$ primer (Fig. 1b). The lowest total number of bands was 4 with the $(CA)_8RC$ and $(CA)_8RG$ primers (Fig. 1a). All primers produced polymorphic fragments with a degree of polymorphism ranging from 43% to 100%. The four most useful primers, $(GA)_8T$, $(TC)_8A$, $(AC)_8G$, and $(ATG)_5GA$, gave 42 polymorphic fragments in total, ranging from 300 bp to 1300 bp in size, and most of them (>90%) were polymorphic. Earlier, Tuvesson et al. had selected seven highly polymorphic ISSR primers for *L. perenne*: UBC 807, 823, 834, 840, 742, 888, 891 [9]. From these we only used the UBC 823 $(TC)_8C$ and UBC 834 $(AG)_8YT$ primers which gave 75% polymorphic bands between 48 bp and 1200 bp in size. The primers, $(AC)_8YG$ and $(TC)_8RA$, produced 80% of polymorphic bands. The lowest percentage of polymorphic bands was 43% and 44% in the amplicon obtained by $(AG)_{12}$ and $(ATG)_5GA$ primers. The largest fragments of 1650–2200 bp in size were generated using primers $(AC)_8YG$, $(ACTG)_4GA$ and $(GACA)_4TC$. Two primers, $(AC)_8C$ and $(CA)_8RG$, generated only very short fragments between 320 bp and 650 bp. As we expected, ISSR markers have provided a high polymorphism level at 75% on average for 21 primers used in this study. ISSRs have been generated in several crops and are known as highly polymorphic and suitable for DNA fingerprinting [10, 11].

Twelve ISSRs out of the 29 submitted were aligned into 5 linked groups (LG) (Fig. 2). For LG4, the grouping was formed at LOD (logarithm of the odds) 5, and for LG6 at LOD 4. For LG1, LG2, and LG7, linkage is supported at LOD 3. There were no ISSR markers mapped to LG3 and LG5.

Our total map length is 250.7 cM with an average distance of 13.5 cM between markers. The largest distance between markers was found on LG7 with an interval of 40.3 cM. Previously, in the same VrnA mapping family, Jensen et al. mapped quantitative trait loci (QTL) for vernalisation response in perennial ryegrass. The total map length was 250.7 cM with an average distance between markers 5 cM [3].

For the sequenced ISSRs fragments, BLAST homology search was performed in the current sequence data of the National Centre of Biotechnology Information (NCBI) (Table 2). For four of our ISSR sequences, 10 matching DNA sequences from other species were detected, showing a 66% to 100% identity. We selected sequences of a high homology to coding regions covering the sequence intervals from 50 to over 200 bp (Table 2). A high similarity was found with DNA sequences from *H. vulgare*, *L. multiflorum*, *Festuca glaucescens* × *L. multiflorum* and *A. sativa.* One of our ISSRs, a 780 bp fragment amplified with $(TG)_8RT$ showed identity to the *H. vulgare* subsp. *vulgare* germin-like protein gene 4c (*GER4c*) and to the germin A (*GerA*) gene present within 190 bp and 140 bp segments, respectively. Another 868 bp fragment, produced by the $(GAGA)_4CT$ primer, showed

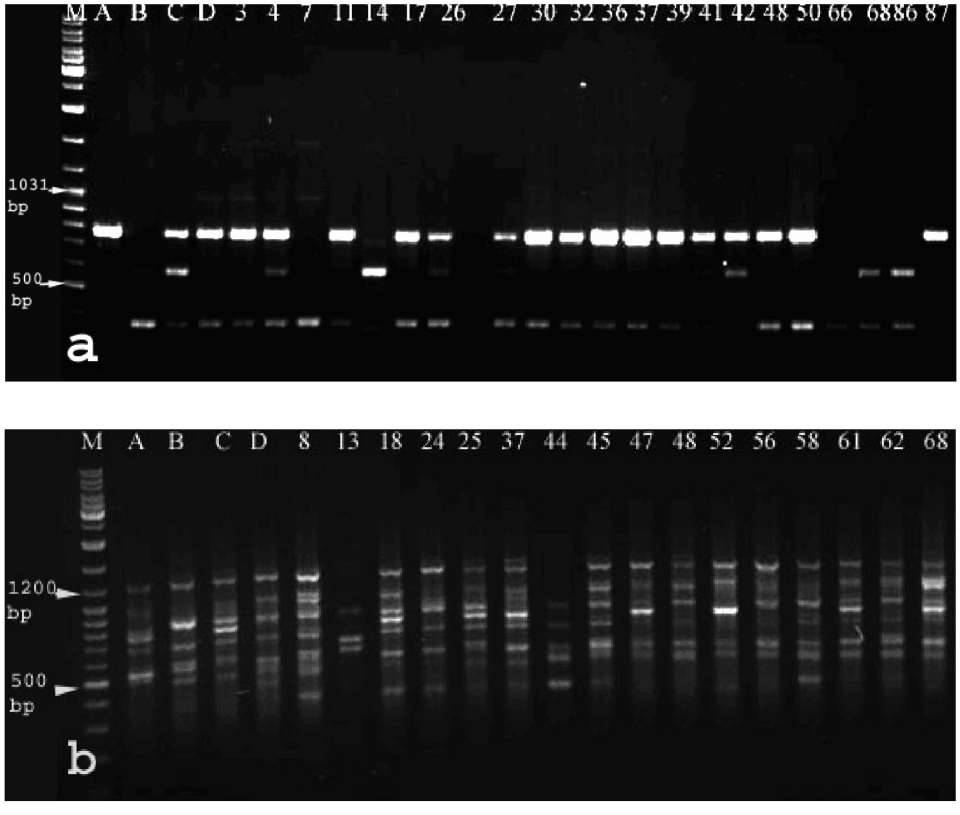

Fig. 1. ISSR profiles obtained by UBC 847 primer (a) and by UBC 827 primer (b); lanes A and B – initial parental genotypes, C and D – F_1 genotypes, other lanes – F_2 genotypes from VrNA population

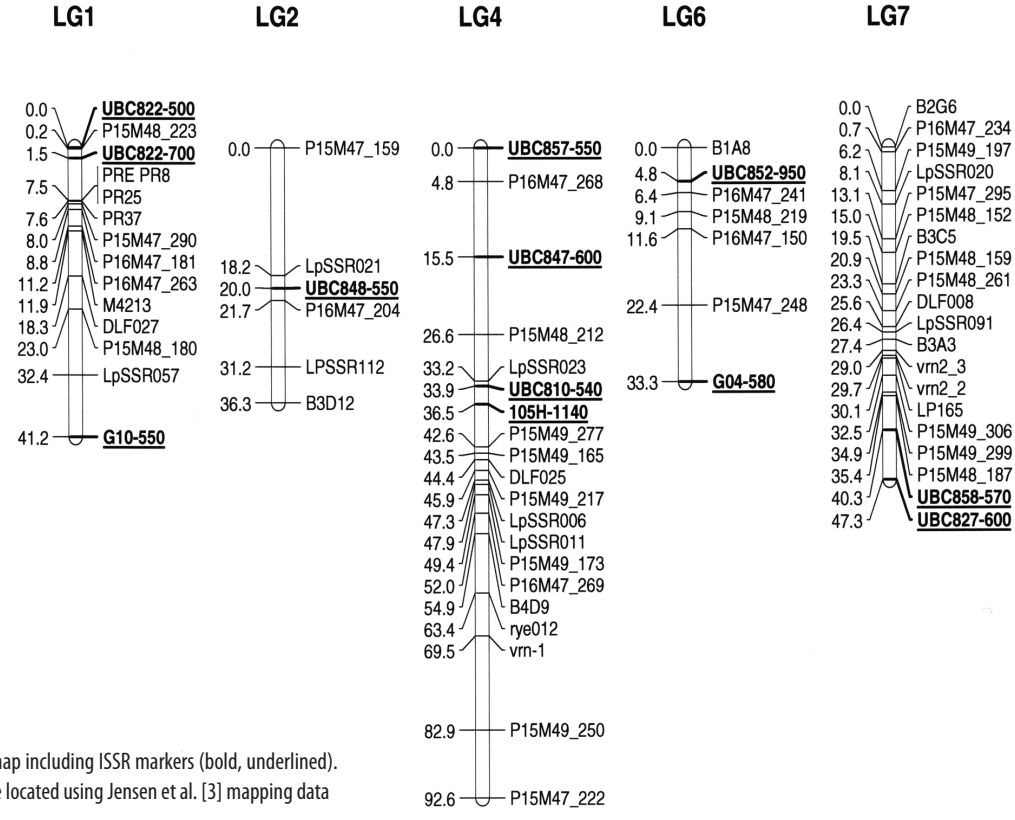

Fig. 2. Linkage map including ISSR markers (bold, underlined). ISSR positions are located using Jensen et al. [3] mapping data

Table 2. **Homologies of sequenced ISSR fragments (NCBI data bases)**

Primer	Length of PCR product (bp)	Genebank accession number	Similarity to coding regions (BLAST search)
UBC 858 $(TG)_8$RT	780	DQ647622	*Hordeum vulgare* subsp. vulgare germin-like protein 4c (GER4c) mRNA, complete cds, E = 6e–14, I = 154/190
		AF250933	*Hordeum vulgare* germin A (GerA) gene, complete cds, E = 4e–15, I = 117/140
105 H $(GAGA)_4$CT	868	AY485643	*Hordeum vulgare* subsp. vulgare BAC 615K1, complete sequence, E = 7e–14, I = 193/236
		AF254799	*Hordeum vulgare* tonoplast intrinsic protein 1 (TIP1), tonoplast intrinsic protein 2 (TIP2), and Rar1 (Rar1) genes, complete cds, E = 2e–07, I = 177/221
		Y14573	*Hordeum vulgare* DNA for chromosome 4H, E = 3e–16, I = 179/220
G10 $(AG)_{12}$	705	DQ403852	*Lolium multiflorum* clone lm28 microsatellite sequence, E = 1e–14, I = 92/105
		AJ872229	*Festuca glaucescens* x *Lolium multiflorum* microsatellite DNA, clone B2–G9, E = 5e–11, I = 42/43
G04 $(GACA)_4$TC	580	AY038013 AY038007 AY038004	*Avena sativa* isolates Pc68LrkC5, Pc68LrkB5, Pc68LrkB2 sequence containing retrotransposon, transposon and repetitive DNA linked to receptor kinase gene, E = 3e–12, I = 72–77

a high similarity with genes putatively coding for *H. vulgare* subsp. *vulgare* tonoplast intrinsic protein 1 (*TIP1*), tonoplast intrinsic protein 2 (*TIP2*), and *Rar1*, as well as to a sequence of *H. vulgare* chromosome 4H covering the interval between 220–236 bp. A 705 bp fragment, generated by the $(AG)_{12}$ primer, displayed homology to the *L. multiflorum* microsatellite sequence and *F. Glaucescens* × *L. multiflorum* microsatellite DNA within short intervals of 105 bp and 43 bp, respectively. A 580 bp fragment generated by $(GACA)_4$TC primer present on LG6 had identity to the *A. sativa* DNA sequence containing retrotransposon and repetitive DNA linked to the receptor kinase gene within a short interval of 77 bp.

Other ISSR fragments (500–1000 bp) that were present between $(TC)_8$RA, $(TC)_8$A, $(AG)_{12}$, $(CA)_8$RG, $(CA)_8$RC, $(TG)_8$RT, and $(AC)_8$G repeats (used as primers) displayed a low homology (about 30%) within small intervals (20–50 bp) to DNA from other living organisms when aligned to current NCBI data using the BLAST search (data not shown). Therefore, the BLAST analysis of our mapped ISSRs sequences indicates that the majority (7 out of 11) of them derive from non-genic sequences.

The ISSR marker-based genetic map of perennial ryegrass (*L. perenne*) reveals comparative relationships with other Poaceae genomes. Establishing syntetic relations between *L. perenne* and other cereals helps to accumulate information for comparative map alignment [12]. Enriched genetic maps are valuable resources for molecular genetic research and molecular breeding in perennial ryegrass.

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O. Pivorienė, I. Pašakinskienė, G. Brazauskas, L. Lideikytė, L. B. Jensen, T. Lübberstedt

ISSR ŽYMENŲ IDENTIFIKAVIMAS IR CHARAKTERIZAVIMAS DAUGIAMETĖS SVIDRĖS GENOME

S a n t r a u k a

Šiame darbe siekėme nustatyti paprastų pasikartojančių sekų intarpų (ISSR) lokusus daugiametės svidrės genome ir charakterizuoti jų sekas. 92 individų ėminys iš VrnA F2 šeimos buvo naudojamas ISSR sankibos genolapiui sukurti. Iš 29 tirtų ISSR fragmentų 12 buvo priskirti penkioms sankibos grupėms (*linkage groups*, LG) LG1, LG2, LG4, LG6 ir LG7. Dviejose sankibos grupėse, LG3 and LG5, žymenų negauta. Bendras genolapio ilgis – 250.7 cM. Pasirinkti ISSR fragmentai buvo klonuoti transformacijos būdu plazmidėje pTZ57R ir sekvenuoti. Keturiems ISSR fragmentams BLAST paieška DNR duomenų bazėse rado reikšmingus panašumus žinomų sekų koduojančiose srityse. 580 bp ISSR fragmentas, priklausantis LG6 grupei ir pagamintas $(GACA)_4TC$ pradmeniu, rodo 95% atitikimą su *Avena sativa* L. transpozonine bei pasikartojančia DNR seka, kurios siejasi su receptorinės kinazės genu. 780 bp fragmentas, pagamintas $(TG)_8RT$ pradmeniu, 70% atitiko *Hordeum vulgare* germino geno *GerA* seką. ISSR genolapiai suteikia svarbios informacijos, kuri padeda nustatyti daugiametės svidrės genų bei kiekybinių lokusų vietą ir gali būti panaudota atliekant kiekybinių lokusų kartografavimą bei kuriant žymenis selekcijos reikmėms.