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# Development and annotation of perennial Triticeae ESTs and SSR markers

**B. Shaun Bushman, Steve R. Larson, Ivan W. Mott, Paul F. Cliften, Richard R.-C. Wang, N. Jerry Chatterton, Alvaro G. Hernandez, Shahjahan Ali, Ryan W. Kim, Jyothi Thimmapuram, George Gong, Lei Liu, and Mark A. Mikel**

**Abstract:** Triticeae contains hundreds of species of both annual and perennial types. Although substantial genomic tools are available for annual Triticeae cereals such as wheat and barley, the perennial Triticeae lack sufficient genomic resources for genetic mapping or diversity research. To increase the amount of sequence information available in the perennial Triticeae, three expressed sequence tag (EST) libraries were developed and annotated for *Pseudoroegneria spicata*, a mixture of both *Elymus wawawaiensis* and *E. lanceolatus*, and a *Leymus cinereus* × *L. triticoides* interspecific hybrid. The ESTs were combined into unigene sets of 8780 unigenes for *P. spicata*, 11281 unigenes for *Leymus*, and 7212 unigenes for *Elymus*. Unigenes were annotated based on putative orthology to genes from rice, wheat, barley, other Poaceae, *Arabidopsis*, and the non-redundant database of the NCBI. Simple sequence repeat (SSR) markers were developed, tested for amplification and polymorphism, and aligned to the rice genome. *Leymus* EST markers homologous to rice chromosome 2 genes were syntenous on *Leymus* homeologous groups 6a and 6b (previously 1b), demonstrating promise for *in silico* comparative mapping. All ESTs and SSR markers are available on an EST information management and annotation database (<http://titan.biotech.uiuc.edu/triticeae/>).

**Key words:** Triticeae, *Leymus*, *Elymus*, *Pseudoroegneria spicata*, expressed sequence tag, simple sequence repeat, microsatellite, comparative genomics.

**Résumé :** Les hordées comprennent des centaines d'espèces tant du type annuel que pérenne. Bien qu'il existe pour les céréales annuelles comme le blé et l'orge de nombreux outils génomiques, les hordées pérennes sont dépourvues des ressources génomiques pour des travaux de cartographie ou des études de diversité génétique. Afin d'augmenter l'information nucléotidique disponible pour les hordées pérennes, trois banques d'EST ont été produites et annotées pour le *Pseudoroegneria spicata*, pour un mélange de l'*Elymus wawawaiensis* et de l'*E. lanceolatus*, ainsi que pour un hybride interspécifique entre le *Leymus cinereus* et le *L. triticoides*. Les EST ont été combinés en collections d'unigènes au nombre de 8780 chez le *P. spicata*, de 11281 chez les *Leymus* et de 7212 chez les *Elymus*. Les unigènes ont été annotés en fonction de l'orthologie putative avec les gènes du riz, du blé, de l'orge, d'autres graminées, d'*Arabidopsis* ou de la banque de séquences non-redondantes du NCBI. Des marqueurs microsatellites (SSR) ont été développés, testés pour leur amplification et polymorphisme, et alignés sur le génome du riz. Les marqueurs EST du *Leymus* homologues aux gènes situés sur le chromosome 2 du riz étaient synténiques aux groupes homéologues 6a et 6b (1b) chez *Leymus*, ce qui illustre le potentiel de la cartographie génétique *in silico*. Tous les EST et marqueurs microsatellites sont disponibles au sein d'une base de données permettant la gestion et l'annotation des EST (<http://titan.biotech.uiuc.edu/Triticeae/>).

**Mots-clés :** hordées, *Leymus*, *Elymus*, *Pseudoroegneria spicata*, étiquette de séquence exprimée, microsatellite, génomique comparée.

[Traduit par la Rédaction]

## Introduction

Triticeae contains over 400 species housed in 14 genera (Barkworth 2007). Both annual and perennial species are

present; the annual species include cereals such as wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.), while the perennial species include a wide diversity of forage and range grasses. Perennial Triti-

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ceae are circumboreal in distribution, grow predominantly in the Northern hemisphere, and are characterized by varying ploidy levels and introgression (Barkworth 2007). They are well adapted to abiotic stresses and provide an important source of forage and habitat for livestock and wildlife (Asay and Jensen 1996a, 1996b), and can be used for re-seeding and stabilization in semi-arid wildland restoration efforts (Jones and Larson 2005).

Genome relationships within Triticeae have been characterized by analyses of meiotic pairing, and genomes have been given alphabetic designations (Dewey 1984; Love 1984). Of the approximately 50 Triticeae species native to North America, 43 are classified as *Elymus* or *Leymus* species (Barkworth 2007). Worldwide, *Elymus* and *Leymus* are polyploid genera that contain approximately 200 of the 400 Triticeae species (Barkworth 2007). North American *Elymus* species contain the **St** and **H** genomes (Mason-Gamer et al. 2002), with the **St** genome derived from a perennial diploid, *Pseudoroegneria spicata* (Pursh) A. Löve, and the **H** genome derived from an unknown *Hordeum* species (Dewey 1971). *Leymus* species contain the **Ns** and **Xm** genomes, where the **Ns** genome is derived from the genus *Psathyrostachys* and the **Xm** genome remains uncharacterized (Zhang and Dvorak 1991; Wang et al. 2006).

Although there is a wealth of cytogenetic information among the perennial Triticeae, very little DNA sequence information is available. Previously available expressed sequence tag (EST) resources of perennial Triticeae grasses comprise only 1884 ESTs from *Leymus chinensis* (Jin et al. 2006). Molecular mapping efforts in perennial Triticeae species rely on heterologous markers developed for the annual cereals, with a correspondingly lower number of amplified and polymorphic loci (Wu et al. 2003; Larson et al. 2006). Heterologous SSR markers have also been used in genetic diversity studies in more recalcitrant species (MacRitchie and Sun 2004; Fu and Thompson 2006). However, the transferability of heterologous markers decreases as species diverge (Sun et al. 1998; Thiel et al. 2003; Zhang et al. 2005), which is accompanied by a higher likelihood of homoplasy or polymorphism due to complex mutational events among the species (Thiel et al. 2003; Saha et al. 2004; Fu and Thompson 2006; Zhang et al. 2006).

Development of codominant markers among more closely related species can therefore improve the robustness and accuracy of comparative mapping and genetic diversity research. Expressed sequence tags are fundamental for the dissection of complex traits, for estimations of molecular diversity and population structure, or for comparative mapping (Varshney et al. 2005). As EST-SSR markers are derived from transcribed regions, they have high rates of successful amplification and associated gene annotations (Varshney et al. 2005). Genetic diversity studies using codominant EST markers can be more powerful than those using dominant markers, as codominant markers are able to accurately test heterozygosity.

As sequence information is lacking in perennial Triticeae species, we report the development of EST libraries for *P. spicata* and *Elymus* and *Leymus* species. These species are crucial in rangeland forage production and restoration, contain genomes present in the majority of perennial Triticeae species, and have the potential for comparative map-

ping with annual Triticeae and other grasses. Our objectives were to develop and annotate EST sequences and SSR markers for the libraries, provide predicted genetic map locations of the ESTs based on alignments with rice (*Oryza sativa* L.), assess the SSR marker amplification efficiencies, and test the synteny of *Leymus* EST markers aligned to rice chromosome 2 genes using a *Leymus* genetic mapping population described by Wu et al. (2003).

## Materials and methods

### Tissue preparation for construction of EST libraries

Construction of the *P. spicata* EST library used plants from the cultivar 'Anatone' (United States Department of Agriculture Forest Service 2004) and a wildland collection made near Midas, Nevada, USA (B.K. Waldron; 41°12.068'N, 116°23.180'W, elevation 1788 m). RNA was obtained from etiolated seedlings, salt- and drought-stressed shoots and crowns, and salt- and drought-stressed roots (Table 1). For the etiolated seedlings, seeds were combined and placed in germination pouches, watered with deionized water, and germinated in the dark at 25 °C for 10 days. For the salt- and drought-stressed tissues, seedlings were germinated in a greenhouse in Logan, Utah, in 16 oz plastic cups in a sterilized 80:20 mix of sand-clay and subjected to repeated cyclical drought similar to that described in Sack and Grumm (2002). Tissue harvest occurred 10 weeks after emergence, when water levels were approximately 10% of soil holding capacity and when leaves had visible loss of turgor. For collection, soil was loosened and seedlings were washed quickly in tap water and frozen in liquid nitrogen. Shoots (including the crowns) were separated from roots for subsequent RNA extraction and library tagging.

*Elymus wawawaiensis* J.R. Carlson & Barkworth and *E. lanceolatus* (Scribn. & J.G. Sm.) Gould were used for the *Elymus* library. Subterranean tiller meristems were collected from clones of one caespitose *E. wawawaiensis* plant, and tiller and rhizome meristems were collected from clones of one rhizomatous *E. lanceolatus* plant. Both collections were from field plants growing at Wellsville, Utah, and harvested in September 2006. The meristems were briefly washed in deionized water and frozen in liquid nitrogen. Collections of the two species were kept separate for subsequent library tagging.

The *Leymus* library comprised two tissues from interspecific hybrid progeny of one *L. cinereus* (Scribn. & Merr.) A. Löve plant and one *L. triticoides* (Buckl.) Pilger plant. Subterranean rhizome or tiller meristems were collected from greenhouse-grown clones, briefly washed in deionized water, and frozen in liquid nitrogen. Spring tillers were collected at a farm near Richmond, Utah, during March 2005. Young tillers at a height of approximately 8–10 cm from the soil surface were cut at the soil-surface level, and above-ground material was placed in tubes and immediately placed in liquid nitrogen.

### Library construction, sequencing, and analysis

Total RNA was extracted from tissue using TRIzol reagent (Invitrogen, Carlsbad, California) and purified through QIAGEN RNeasy Midi columns (QIAGEN, Valencia, California). Integrity of RNA was validated with denaturing

**Table 1.** Number of EST clones sequenced from both directions for each tagged tissue in three perennial Triticeae species libraries.

Library	Species	Tagged tissue	No. of ESTs
<i>P. spicata</i>	<i>P. spicata</i>	Salt- and drought-stressed shoots	4 616
	<i>P. spicata</i>	Salt- and drought-stressed roots	4 135
	<i>P. spicata</i>	Etiolated seedlings	6 529
	<i>P. spicata</i>	Tag unidentified	848
		Total	16 128
<i>Leymus</i>	<i>L. cinereus</i> × <i>L. triticoides</i> F <sub>1</sub>	Spring tillers above ground	5 053
	<i>L. cinereus</i> × <i>L. triticoides</i> F <sub>1</sub>	Tiller and rhizome meristems	7 963
	<i>L. cinereus</i> × <i>L. triticoides</i> F <sub>1</sub>	Tag unidentified	2 344
		Total	15 360
<i>Elymus</i>	<i>E. lanceolatus</i>	Tiller and rhizome meristems	7 797
	<i>E. wawawaiensis</i>	Tiller meristems	1 048
	Unknown	Tag unidentified	1 523
		Total	10 368

agarose gels or a Bioanalyzer (Agilent, Santa Clara, California). Poly(A)<sup>+</sup> mRNA was isolated from total RNA using the Oligotex Direct mRNA kit (QIAGEN). Double-stranded cDNAs were synthesized using the SuperScript double-stranded cDNA synthesis kit (Invitrogen). For the *P. spicata* and *Leymus* libraries, mRNAs were converted to cDNAs by priming with modified *NotI*-oligo(dT) primers that included a unique 5 bp DNA sequence inserted after the *NotI* cloning site for each of the separate tissues in each library, thus three tags for *P. spicata* (shoots and crowns, *NotI*-ACGCA(T)<sub>18</sub>; roots, *NotI*-ACCGA(T)<sub>18</sub>; seedlings, *NotI*-TCGCA(T)<sub>18</sub>) and two tags for *Leymus* (rhizomes, *NotI*-TCGCA(T)<sub>18</sub>; tiller meristems, *NotI*-TCCGA(T)<sub>18</sub>). Equal amounts of double-stranded cDNAs from each tissue source were pooled, ligated to *EcoRI* adaptors (5'-AATTCGGTGTGCTGTCG-3'; Promega, Madison, Wisconsin), and digested with *NotI*. Double-stranded cDNAs greater than 600 bp were selected and directionally cloned into *EcoRI*-*NotI* digested pBlue-script II SK+ phagemid vector (Stratagene, La Jolla, California). Library normalization for the *P. spicata* and *Leymus* cDNAs followed the protocol of Bonaldo et al. (1996).

For the *Elymus* library, 1 µg of poly(A)<sup>+</sup> mRNA from each sample was converted to double-stranded cDNA using the Creator SMART cDNA library construction kit (Clontech, Mountain View, California). Briefly, mRNA was primed with an oligo(dT)<sub>30</sub> primer containing an *SfiI*B restriction site and reverse-transcribed using SuperScript II (Invitrogen). The first-strand cDNAs for the two samples were differentially tagged at the 5' end with adaptors (*E. lanceolatus*, 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'; *E. wawawaiensis*, 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGAGGG-3') and converted to double-stranded cDNA by primer extension using these adaptors, as described in the manual. Double-stranded cDNAs from both samples were pooled in equal amounts, normalized using the Trimmer-Direct kit (Evrogen, Moscow, Russia), and cloned into the pDNR-LIB vector (Clontech). All three directionally cloned libraries were transformed into electrocompetent DH10B cells (Invitrogen).

The *P. spicata* and *Leymus* libraries were sequenced with

standard T7 (5' end) and M13rev(-24) (3' end) primers. The *Elymus* library was sequenced with a custom primer for the 5' end of the inserts (5'-CGAGCGCAGCGAGTCAGT-3') and an anchor-(T)<sub>18</sub> primer for the 3' end of the inserts (5'-TTTTTTTTTTTTTTTTTTT-3'). Base calling used a Phred quality score cutoff of 20 for a threshold length of at least 200 bp. Vector sequences were detected and masked using the Cross\_Match program (<http://bozeman.mbt.washington.edu/phrap.docs/phrap.html>), and repeat and low-complexity sequences were removed using RepeatMasker (Smit et al. 1996–2004). Bacterial, mitochondrial, ribosomal, viral, and other unwanted sequences were filtered out based on BLASTN searches. Resulting filtered, high-quality sequences were assembled into contigs using Paracel TranscriptAssembler (PTA; [www.paracel.com](http://www.paracel.com)). The average number of ESTs per contig was calculated by subtracting singletons from the total number of clean sequences in the forward and reverse directions and then dividing the resulting number of sequences by the number of contigs. Additionally, all contigs were manually inspected for false contigs using the TranscriptView program of PTA. The EST information management application (ESTIMA) database (Kumar et al. 2004) was used for curation and dissemination of the ESTs (<http://titan.biotech.uiuc.edu/ESTIMA/>).

Annotation for the ESTs was obtained by BLASTX or tBLASTX queries against grass, non-redundant protein, and *Arabidopsis* protein databases with a cutoff expectation value (*E* value) of 10<sup>-5</sup>. The databases examined were non-redundant protein sequences, rice sequences, and *Festuca* sequences from the National Center for Biotechnology Information (NCBI); the Poaceae protein database of Gramene ([ftp://ftp.gramene.org/pub/gramene/release26/data/protein/sequence/poaceae\\_sptr.fa](ftp://ftp.gramene.org/pub/gramene/release26/data/protein/sequence/poaceae_sptr.fa); February 2007); the barley, maize, wheat, and rye Gene Indices databases of The Institute for Genomic Research (<http://www.tigr.org>); and *Arabidopsis* proteins from The Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org>).

### SSR marker development and mapping

Unigene sets of the three libraries were queried for SSRs using SSRFinder (<http://www.maizemap.org/bioinformatics/SSRFINDER/>; October 2007). The SSRFinder parameters

were as follows: amplicon lengths between 80 and 250 bp, primer annealing temperatures of 60 °C, and minimum repeat lengths of 12 bp and 4 repeat motifs. To identify which SSRs resided within protein-coding regions of the unigenes, the NCBI non-redundant database was downloaded locally (4 January 2008) and entries from *Arabidopsis* and Poaceae grasses were extracted to create a plant-specific database. BLASTX was performed with the default scoring matrix and the following parameters: filter = seg+xnu and score = 100. Then, the BLASTX output was parsed to identify protein-coding regions of the EST sequences and these were compared with positions of the SSRs. An *E* value less than 10<sup>-5</sup> was used as a cutoff for significance of the sequence alignments.

Amplification success and projected polymorphism of SSR primers were tested on parental plants of existing or incipient genetic mapping populations for each of the three libraries. The *P. spicata* SSRs were tested on a hybrid progeny of Anatone × T1442 and a hybrid progeny of Goldar (Gibbs et al. 1991) × P22 (an experimental line). *Leymus* SSRs were tested on the parents of the mapping populations reported in Wu et al. (2003). *Elymus* markers were tested on three *E. wawawaiensis* × *E. lanceolatus* F<sub>1</sub> hybrids and an *E. lanceolatus* plant, used as backcross parents for a mapping population. Standard hot-start PCR amplification procedures were used, except that 1 μmol/L R110-ddCTP (PerkinElmer, Waltham, Massachusetts) was spiked into each reaction, and amplification products were resolved on an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, California) at the Utah State University Center for Integrated Biosystems (Logan, Utah).

All *Leymus* SSR markers homologous to rice chromosome 2 genes were genotyped in the *Leymus* mapping population (Wu et al. 2003) using JoinMap 4.0 (Van Ooijen 2004). Polymorphic markers were initially assigned to one of 14 possible linkage groups using the “Create Groups Using a Map Node” and “Assign Ungrouped Loci to SCL-Groups” functions. A strongest cross link (SCL) threshold of 20 LOD was used to assign new markers to the linkage groups (LGs), and LGs were joined using the “Combine Groups for Map Integration” function. Consensus maps for LG6a and LG1b (renamed 6b herein) were calculated by regression mapping using only linkages with a recombination frequency smaller than 0.4, linkage LOD greater than 1.0, goodness-of-fit jump threshold of 5.0, ripples after each added locus, and Haldane’s mapping function with a third round to force any remaining markers on the map. However, only those AFLP markers that were previously mapped in both *Leymus* populations, fitted in second-round regression mapping, and required to fill gaps between new ESTs and other previously mapped anchor markers were used for further analyses and presentation in this report. *Leymus* EST sequences putatively orthologous to rice chromosome 2 genes were aligned to the NCBI rice genome assembly at ftp.ncbi.nih.gov/genomes/Oryza\_sativa/ on 7 December 2006. Rice BAC clones containing the Cent0 (RCS2) centromere-specific retrotransposon sequences from GenBank accession AF058902 (Dong et al. 1998; Cheng et al. 2002) were identified by BLAST search against the NCBI rice genome database. The resulting rice BAC clones were also aligned to the rice chromosome 2 physical map.

## Results

Three normalized EST libraries were constructed from (1) bluebunch wheatgrass (*P. spicata*), (2) one plant each of Snake River (*E. wawawaiensis*) and thickspike (*E. lanceolatus*) wheatgrasses, and (3) an interspecific hybrid between basin wildrye (*L. cinereus*) and beardless wildrye (*L. triticoides*). Each of the tissues in each of the libraries was tagged, and each tissue was represented by several thousand ESTs, except the subterranean tiller meristems of *E. wawawaiensis* in the *Elymus* library (Table 1). The low number of sequences for that species is likely due to a low amount of initial RNA. Sequences were obtained in both directions for 16 128 clones of the *P. spicata* library, 15 360 clones of the *Leymus* library, and 10 368 clones of the *Elymus* library (Table 1).

The ESTs were assembled into unigene sets of 8 780 unigenes for *P. spicata*, 11 281 unigenes for *Leymus*, and 7 212 unigenes for *Elymus* (Table 2). Across all three libraries, over 55% of unigenes were contigs, with an average length of 1017 nucleotides per contig. Among singletons the average length was 670 nucleotides. The *P. spicata* and *Elymus* libraries had an approximate 2:1 ratio of contigs to singletons, while the *Leymus* ratio was closer to 1:1. As forward and reverse sequences of the same clone that did not overlap were considered separate singletons, the larger number of *Leymus* singletons mainly resulted from larger clone insert sizes and non-overlapping sequence reads. The redundancy of the *P. spicata* library was 32%, with an average of 3.9 sequences per contig. The redundancy of the *Elymus* library was 41% with an average of 3.2 sequences per contig, and the redundancy of the *Leymus* library was 39% with an average of 3.8 sequences per contig. All three library databases were curated and are searchable on the ESTIMA Web site (<http://titan.biotech.uiuc.edu/triticeae/>).

The three libraries were compared by BLASTX or tBLASTX searches against the NCBI non-redundant protein database, the rice database of the NCBI, The Arabidopsis Information Resource database, the Poaceae database of Gramene, and several other grass sequence databases (Table 2). Comparisons of the *P. spicata* library with cereal crop sequences yielded homology hits to annual Triticeae sequences for 81%–93% of the unigenes, with the exception of rye at 42%. The low number of hits with rye was probably due to the low number of ESTs for rye relative to the other grass libraries. Comparisons with *Arabidopsis*, the NCBI non-redundant database, and *Festuca* yielded hits for more than 66% of the unigenes. The *Elymus* and *Leymus* libraries exhibited similar results, although with slightly fewer hits (Table 2). The number of unigenes with no match to any of the databases tested was 331 for *P. spicata*, 324 for *Elymus*, and 592 for *Leymus* (Table 2).

Expressed sequence tags from each library were again compared with rice genomic sequences, but using a BLASTN search with an *E* value cutoff of 10<sup>-5</sup>. The resulting hits were aligned with the physical map of rice provided by NCBI ([ftp.ncbi.nih.gov/genomes/Oryza\\_sativa/](ftp.ncbi.nih.gov/genomes/Oryza_sativa/); February 2007). The numbers of ESTs with associated rice genes and map positions are listed by rice chromosome in Table 3, and correspond with 93% to 98% of the ESTs with BLASTX hits to rice in Table 2. The number of hits decreased gener-

**Table 2.** Numbers of unigenes within three Triticeae EST libraries, and the numbers of unigenes with significant BLAST hits ( $E < 10^{-5}$ ) to other grass databases.

	<i>P. spicata</i>	<i>Leymus</i>	<i>Elymus</i>
Number of contigs	6450	6217	4702
Number of singletons	2330	5064	2510
Number of unigenes	8780	11281	7212
Number of BLAST hits			
Nr.aa	7591 (86%)	9755 (86%)	6023 (84%)
TAIR Arabidopsis.aa	6880 (78%)	8787 (78%)	5315 (74%)
NCBI_Rice.aa	7537 (86%)	9671 (86%)	5972 (83%)
Barley_TC	7653 (87%)	9617 (85%)	6161 (85%)
Maize_TC	7072 (81%)	8994 (80%)	5602 (78%)
Rye TCs	3669 (42%)	4388 (39%)	2335 (32%)
Wheat TCs	8191 (93%)	10223 (91%)	6565 (91%)
NCBI_Festuca	5790 (66%)	7178 (64%)	4390 (61%)
Poaceae	7378 (84%)	9487 (84%)	5977 (83%)
No hit	331 (4%)	592 (5%)	324 (4%)

**Table 3.** Triticeae ESTs with rice similarity hits, arranged by rice chromosome.

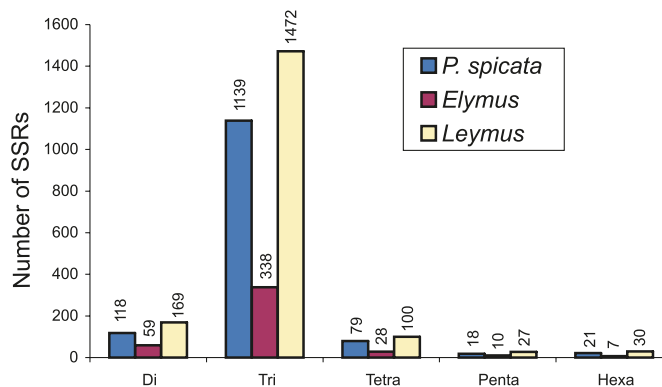
Rice chromosome	No. of Triticeae unigenes with hits		
	<i>P. spicata</i>	<i>Leymus</i>	<i>Elymus</i>
1	1071	1370	760
2	930	1173	667
3	1052	1395	780
4	639	842	490
5	681	825	499
6	539	761	430
7	552	682	418
8	494	581	373
9	407	507	277
10	352	428	262
11	362	426	265
12	336	399	313
Total	7415	9389	5534

ally in proportion to the rice chromosome size, with rice chromosomes 1 and 3 containing the largest numbers of hits (Table 3).

Among the three libraries, SSRs were detected with the minimum SSR length set to 12 bp and the minimum number of repeats set at four. Approximately 16% of the *P. spicata* and *Leymus* unigenes contained SSRs. The *Elymus* library, however, contained SSRs in only 6% of its unigenes. Over 75% of the SSRs in each library were trinucleotide repeats (Fig. 1). Unigenes with SSRs were compared with the NCBI non-redundant database to determine the location of the repeat, and 482 (35%) *P. spicata* SSRs, 123 (28%) *Elymus* SSRs, and 713 (40%) *Leymus* SSRs resided in coding regions. The remaining SSRs were located in coding regions of genes with no annotation, in regions of ambiguity with regards to coding, or in 3' untranslated regions. Over 95% of SSRs that were in coding regions were trinucleotide repeats.

For each EST library, SSR amplifications and polymorphisms were tested on parents of existing or potential genetic mapping populations. Of the 1375 *P. spicata* SSRs, 1083 (79%) amplified in two diverse plants and 451 (33%)

**Fig. 1.** SSR types in the *P. spicata*, *Elymus*, and *Leymus* EST libraries.



were polymorphic. Of the 442 *Elymus* SSRs, 331 (75%) amplified and 133 (30%) were polymorphic. Among the 1798 *Leymus* SSRs, 1620 (90%) amplified and 777 (43%) were polymorphic. The majority of SSR-containing sequences also had predicted map positions based on homology with rice sequences; for *P. spicata* there were 958 (70% of SSRs), for *Elymus* 263 (60% of SSRs), and for *Leymus* 1576 (88% of SSRs). The distribution of SSRs among the rice chromosomes (data not shown) reflected the distribution of ESTs in Table 3.

To test the accuracy of the comparative genetic map locations, we selected all *Leymus* SSR markers with homology hits to rice chromosome 2 genes that are predicted to reside on LG6 of Triticeae (La Rota and Sorrells 2004). We selected LG6 markers for two reasons: (1) LG6 of Triticeae is homologous from end to end with rice chromosome 2 (La Rota and Sorrells 2004), and (2) LG1 and LG6 of the *Leymus* genetic map were given tenuous assignments owing to a paucity of anchor markers. Twenty-seven markers were polymorphic in a *Leymus* mapping population (Wu et al. 2003) and 24 mapped to chromosome 6a or 1b (renamed 6b) in *Leymus* (Table 4; Fig. 2), indicating that the LG1b reported in Wu et al. (2003) is rather LG6b. One marker mapped to both LG6b and LG2a, while the remaining 3

**Table 4.** Map positions and sequences of *Leymus* EST-SSR primer sets predicted to map to chromosome 6.

<i>Leymus</i> marker ID	<i>Leymus</i> EST or contig ID	Expected amplicon size (bp)	Mapped <i>Leymus</i> LG	BLASTN <i>E</i> value	Forward primer
Ltc0002	BG01024A2G01.f1	155	6a and 6b	2.70E-44	AGGAGCGCAGCTTCATCATC
Ltc0014	BG01_2.526.C5.Contig868	158	6a	5.70E-212	GATCTTCTCCAGCAGGTGGTAGTC
Ltc0015	BG01043A2D10.r1	153	6b	4.40E-27	AGGAACCTACACATATCCGCTGAA
Ltc0030	BG01_2.554.C2.Contig912	155	6a	4.20E-19	TGTTGGATTAAGCGTTGCTGTCTA
Ltc0044	BG01009A2E11.f1	140	6b	8.00E-29	CTCCCTGAGCCTGGACCTG
Ltc0049	BG01024A1G10.r1	140	6b	1.20E-49	GGAGTTGATTTTCAGGATCAGTGG
Ltc0055	BG01_2.1738.C1.Contig2467	197	6a	5.10E-78	AAGAAGAAGAGGCCGAGGAATAAA
Ltc0092	BG01_2.1259.C1.Contig1852	138	6a and 6b	5.10E-31	GAGTGGGGTGGTGAAGATGG
Ltc0096	BG01006B2D04.f1	159	6a	1.10E-200	GCGCACTACCGCTCTTAGTT
Ltc0103	BG01_2.2280.C1.Contig3105	156	6a	8.20E-101	GTGTGCTGCTGCTGTTACAG
Ltc0104	BG01_2.4068.C1.Contig5011	158	6a	6.90E-33	CACCTCTCTCGCTTCTCCTCC
Ltc0118	BG01_2.547.C1.Contig900	103	6a	7.20E-83	GTTCTGAATCTTGAGAGGCGAAA
Ltc0121	BG01_2.412.C2.Contig707	113	6a	1.90E-33	ATGATGATCGGAAACCATCAATCT
Ltc0122	BG01_2.4084.C1.Contig5027	154	6a	3.90E-134	AAGTTTCTGATGATGCAGAGGAGG
Ltc0129	BG01_2.3289.C1.Contig4213	93	6a	2.10E-55	CTAGCTAGCCAGCCGAACGAC
Ltc0142	BG01_2.3674.C1.Contig4608	152	6b	1.10E-31	CGAGTCTCTGTCTGGGAGTTAG
Ltc0145	BG01_2.5053.C1.Contig5969	174	6b	4.00E-157	CCTTCGTC AACACTGAGTACAACC
Ltc0157	BG01_2.1475.C1.Contig2135	116	6a	1.40E-31	GCAATGAACACTGAATCAATCGAG
Ltc0164	BG01_2.695.C1.Contig1093	97	6b	2.40E-70	CGAACCGAAAAGCAGAGCAA
Ltc0174	BG01_2.2173.C1.Contig2980	137	6b	2.70E-193	CAGATTCAGGGAAGGAGAAGGAG
Ltc0182	BG01028B2D03.f1	150	6a and 6b	1.30E-61	ATGGTACAAGACTTACCCACAGG
Ltc0191	BG01_2.1660.C1.Contig2365	81	6b and 2a	1.60E-46	AGAACCCAACCGCACCAACT
Ltc0192	BG01_2.1982.C1.Contig2765	158	6b	2.00E-06	GCTTCTCTACCGCTACCCCTAC
Ltc0252	BG01043A1B06.r1	160	6a and 6b	5.80E-08	GGTTACAAGGTTCCGGTGGTTGTT
Ltc0084	BG01018B1E07.f1	159	7a	3.60E-74	GCTGTGCGACTACCTGAACTCC
Ltc0085	BG01026A1D12.f1	154	2b	9.20E-66	CAATACCTAGACGCTCCAGCTT
Ltc0007	BG01_2.2630.C1.Contig3494	156	7b	6.90E-12	GCGACTTGCTTACTTCACTTCATT

markers mapped to *Leymus* LGs 2b, 7a, and 7b (Table 4). The LG6 markers were generally colinear with putative rice orthologs, with a few instances of apparent genome rearrangements (Fig. 2). The SSR primer sequences, associated unigene IDs, accession numbers for original sequences, repeat types, repeat lengths, amplification success, and predicted rice physical map positions are freely available on the ESTIMA database (<http://titan.biotec.uiuc.edu/triticeae/>) or can be obtained upon request from the corresponding author.

## Discussion

This study substantially increases the amount of sequence information available in the perennial Triticeae, allowing for more extensive use of genomic methods within Triticeae and closely related grasses. The plant species used to develop these resources contain the **St**, **H**, and **Ns** genomes, which are the most widespread and abundant perennial Triticeae genomes (Dewey 1971, 1984; Love 1984; Barkworth 2007). The main venue for these EST resources will likely be within the perennial Triticeae for mapping and genetic diversity studies, and these resources allow for more robust DNA markers and sequences to be used within the tribe. Of the SSR markers detected in the respective species used to develop the EST libraries, between 78% and 90% amplified in parents of existing or potential mapping populations, and between 30% and 43% of were polymorphic among the plants tested. These levels of amplification and polymorphism are consistent with rates observed in other species

(Varshney et al. 2005). The higher level of amplification success in the *Leymus* library probably occurred because the plants used for the library were the same as the mapping population parents. The higher level of polymorphism in *Leymus* may result from interspecific hybridization of the divergent species used in the mapping population.

The majority of these ESTs also have putative orthologs in annual Triticeae, other Poaceae grasses, rice, and *Arabidopsis* (Table 2), indicating that robust comparative maps and candidate gene discovery can occur therein as well. From the ESTs, SSR markers were developed and also aligned with rice physical map positions. Additionally, because 3' untranslated regions were sequenced from the EST database, we anticipate that further markers in genes that contained no SSRs can be constructed and will likely have an increased level of polymorphism. Reports of the number of genes present in rice have ranged from 31 439 (Tanaka et al. 2008) to 49 472 (Ouyang et al. 2007). Thus, the approximate 85% of perennial Triticeae ESTs with hits to rice genes (Table 2) would represent 25% to 16% of rice genes. Despite the low coverage, several hundred perennial Triticeae ESTs are putatively orthologous to rice genes for every rice chromosome (Table 3).

Currently, rice remains the only sequenced grass genome (Dickson and Cyranoski 2001; Goff et al. 2002; Yu et al. 2002). Nevertheless, its colinearity with other cereal grasses (Gale and Devos 1998) has assisted in the detection and positional cloning of candidate genes and QTLs in other grasses (e.g., Mammadov et al. 2005; Tamura and Yamada



Reverse primer	Annotation
GTAGGAGTTGGGCTGGCTGTC	Amino acid transport protein
AAAGGATGGATCCCTGCAAGTT	Catalase
GCAGCGTCATTGATATAGAAGGCT	Glutamate receptor
CGCGTCCAAAAGATATTCTAGTGAGA	Hypothetical protein
GAGTGGCATCATGAAGTTCTCCTC	WRKY transcription factor
ATCCTTTCGTGAAGTTGTCACACA	KH domain containing protein
CGTGGATGTGCTGCAGGTAGTA	26S proteasome RPT6a subunit
AGGGTCTTAATCTTCTGCTCCCTG	Rhodanese-like domain containing protein
GTCCAGGTAGCACACCTCCG	Putative Tab2 protein
CTTTCTCGCAAAGGACGCT	AP2 domain transcription factor
GGAGGTGAGCGGTGATTCAATAGT	Plastid ribosomal protein L19
GAGCAAGAACCTGGCCTTCATT	Xyloglucan endo-1,4-beta-D-glucanase
GACATGGCATATACGGAAGGAAAA	Inorganic pyrophosphatase
ACGTCCAGAAGCTGTACCCATC	Hydrolase
GAGTAGGCCATGTACCGTCCC	RNA binding protein
AGCCACTCCACTTGCTTTGAGTAA	Ring finger family protein
AGGTCTCCGTGGTGGACTTG	Predicted gene product
CGTGTGAGACTCATCGATGTTACC	Hypothetical protein
CTCGGAATTTTCGCAACGTGA	Hypothetical protein
CGAGGAAGAAGTTGTCCTGAGGT	Hypothetical protein
GCTCATCAAGAGCAACTGAACAGA	Putative transmembrane protein
GCAGCCTCAGAGTAGAGGAGGAG	Seed maturation like protein
CATTTCGATCGTGCATTGTGT	Cell division inhibitor-1 like
AACTAATATTGTTAGGGTGCGCCA	26S proteasome subunit 7
CAGAGGAAGTGGAAACAAGCACTC	8-Oxoguanine DNA glycosylase
GGTAGTCCAGAAGCCTGTGGTAGA	RNA methylase-like protein
ATGCAATTTTAATCCCCAACAAA	Sucrose:sucrose 1-fructosyltransferase

2007; Kuraparthy et al. 2008) and has been helpful for inferring evolutionary relationships of grass genomes (Salse et al. 2008). In this study we show that the majority of perennial Triticeae ESTs have predicted map positions based on orthology to rice across a broad distribution of genes on all of the rice chromosomes (Table 3).

Twenty-four (of 27) *Leymus* SSR markers whose ESTs were similar and putatively orthologous to rice chromosome 2 genes mapped on *Leymus* LG 6a or 1b. Rice chromosome 2 and Triticeae LG6 are syntenic and colinear (La Rota and Sorrells 2004; Salse et al. 2008), and thus our mapped *Leymus* LG6 SSRs were predictably colinear to wheat. The 14 *Leymus* LGs were previously and tentatively identified using 50 heterologous anchor markers (Wu et al. 2003). However, LG1b and LG6b were not positively identified. Based on the results of this study, the original LG1b is renamed LG6b (Fig. 2).

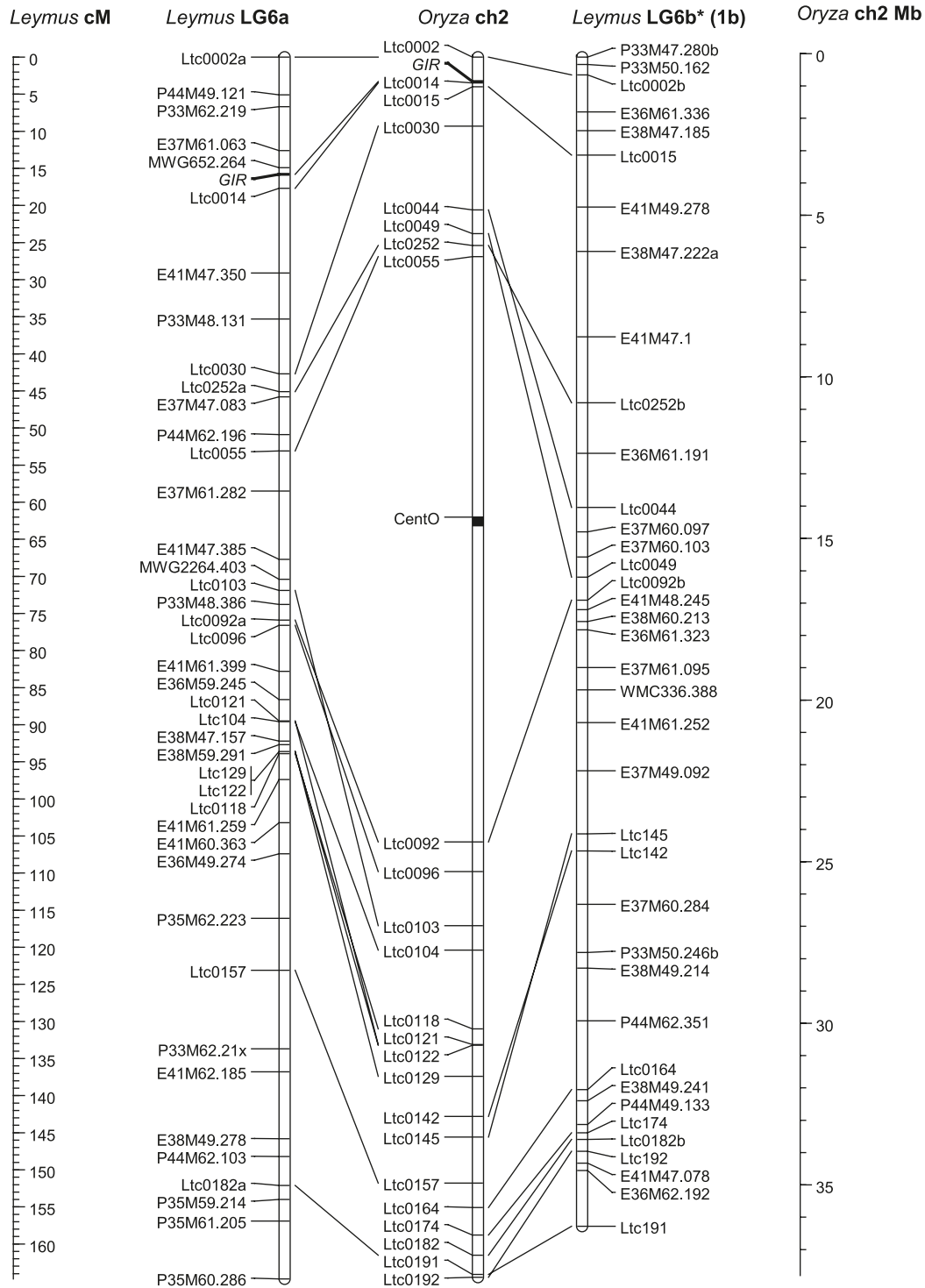
Of the three SSRs that were predicted to map to LG6 but did not, the marker located on *Leymus* LG2 could have resulted from paralogous sequences or rearrangements of genes outside of its expected homeologous locations. Indeed, previous comparative mapping between wheat and rice found wheat LG2 markers that mapped onto rice chromosome 2 (La Rota and Sorrells 2004). Two other *Leymus* markers mapped to *Leymus* LG7. Recently, duplicated regions between wheat chromosomes 6 and 7 were defined (Salse et al. 2008), and as *Leymus* and wheat likely share similar base chromosome numbers and synteny, the two markers mapping to LG7 of *Leymus* may be part of a dupli-

cated region similar to that found in wheat. Thus we confirm the colinearity of the *Leymus* markers with wheat and rice, and suggest that functional and comparative genomics tools in those model grasses will translate to the perennial Triticeae.

SSR mutations result from replication slippage, and as trinucleotide indels would not result in frame-shift mutations their presence in coding regions would not be subjected to purifying selection to the same extent as other types of SSRs (Metzgar et al. 2000). Accordingly, the abundance of trinucleotide SSRs was shown to be equivalent in both coding and non-coding regions, while non-trinucleotide SSRs occurred less frequently in coding regions relative to non-coding regions (Metzgar et al. 2000). Our results showed a consistent trend in that trinucleotide repeats comprised over 95% of the SSRs confirmed to exist in coding regions and were still the majority of SSRs that were not confirmed to lie in coding regions. Our results also agree with the paucity of non-trinucleotide SSRs in the EST libraries shown in other studies (La Rota et al. 2005; Varshney et al. 2005).

Unlike previous SSR reports for rice (Temnykh et al. 2000) and barley (Thiel et al. 2003), the perennial Triticeae trinucleotide SSRs had no specific repeat type that accounted for more than 10% of the total trinucleotide repeats. In the *P. spicata* and *Leymus* libraries, trinucleotide repeats containing C and G were more abundant than those containing AT, similar to tall fescue ESTs (Saha et al. 2004), whereas the *Elymus* library showed a more even distribution of all repeat types. Sharopova et al. (2002) predicted that the

**Fig. 2.** Genetic map of LG6 in the allotetraploid full-sib *Leymus* population, aligned to rice chromosome 2. *Leymus* SSR markers begin with "Ltc", while remaining AFLP and anchor markers were previously reported in Wu et al. (2003). Genetic map distances for *Leymus* are in centimorgans (cM), while physical map positions for rice are reported as megabases (Mb). Cent0 corresponds to a rice centromere sequence (AF058902). \*Chromosome 6b was initially misidentified as 1b in Wu et al. (2003).



polymorphism rate of SSRs was correlated to the total length of the repeat motif, with trinucleotide repeats being the least polymorphic. The majority of SSRs generated for the three Triticeae libraries would therefore be predicted to show relatively low polymorphism, yet between 30% and

43% were polymorphic among the plants we tested. This is possibly a result of the obligate outcrossing of these grasses. Additionally, the SSR abundance in coding regions may result in greater conservation and thus translation across similar species.

Using appropriate DNA markers for population genetic and evolutionary studies among perennial grasses can be challenging as a result of polyploidy, obligate outcrossing, and reticulate evolutionary relationships. Markers such as AFLP and RAPD suffer from the restrictions of dominant band patterns or closed-platform systems (Nybohm 2004). The SSRs derived from genomic libraries can have low transferability (Varshney et al. 2005). Although EST-derived SSRs and other sequence tagged site markers have higher transferability than genomic-library SSRs (Varshney et al. 2005), the use of markers from closely related species is still essential for accurate estimates of some population genetic parameters. As the relatedness of species diverges, the transferability of markers decreases and the likelihood of null alleles, homoplasy, or varying band sizes due to mutational events increases (Thiel et al. 2003; Nybohm 2004; Saha et al. 2004; Zhang et al. 2005, 2006). The diversity and structure of perennial Triticeae species have been assessed using heterologous SSR markers from wheat and barley (MacRitchie and Sun 2004; Fu and Thompson 2006), with reports of 12 and 4 polymorphic loci, respectively. The SSR and other EST-derived markers developed in this study can substantially expand the numbers of markers available and allow more robust tests of genetic diversity and comparative mapping.

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