

## Expressed sequence tag-derived microsatellite markers of perennial ryegrass (*Lolium perenne* L.)

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**Abstract** An expressed sequence tag (EST) library of the key grassland species perennial ryegrass (*Lolium perenne* L.) has been exploited as a resource for microsatellite marker development. Out of 955 simple sequence repeat (SSR) containing ESTs, 744 were used for primer design. Primer amplification was tested in eight genotypes of *L. perenne* and *L. multiflorum* representing (grand-) parents of four mapping populations and resulted in 464 successfully amplified EST-

SSRs. Three hundred and six primer pairs successfully amplified products in the mapping population VrnA derived from two of the eight genotypes included in the original screening and revealed SSR polymorphisms for 143 ESTs. Here, we report on 464 EST-derived SSR primer sequences of perennial ryegrass established in laboratory assays, providing a dedicated tool for marker assisted breeding and comparative mapping within and among forage and turf grasses.

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Simple sequence repeat (SSR) markers are widely used for genetic and breeding applications in several crop species due to the high information content, widespread distribution in plant genomes and highly reproducible analysis based on PCR. In perennial ryegrass, a major component of permanent pastures and meadows of temperate regions worldwide, SSR markers are mainly involved in cultivar identification and comparison (Kubik et al. 2001; Roldán-Ruiz et al. 2001), genetic diversity studies (Jensen et al. 2007), genetic linkage mapping and trait dissection in order to implement marker assisted breeding strategies (Yamada et al. 2005). Although several studies report on the development of SSR markers in perennial ryegrass (Faville et al. 2004; Gill et al. 2006; Jensen et al. 2007; Jones et al. 2001; Kubik et al. 2001; Lauvergeat et al. 2005), the number of publicly available microsatellite markers is still limited and has to be increased in order to ensure a reasonable genome coverage required for linkage mapping and trait dissection in *Lolium* species. Since isolation and characterisation of SSR loci from genomic libraries is labour-intensive and costly (Squirrell et al. 2003), extended genome and cDNA sequencing projects have been exploited for *in silico* gene discovery and marker development. Gene associated SSRs derived from ESTs are of particular interest because the genes may functionally determine trait variation. These SSRs are highly transferable to other pedigrees, which makes them valuable for the development of anchor markers in genetic mapping studies (Varshney et al. 2005).

Out of 25,744 ryegrass EST sequences, 1,458 microsatellite motif containing ESTs were identified using the Perl script MicroSATellite (MISA) (Thiel et al. 2003), representing 955 nonredundant SSRs (Asp et al. 2007). Of these, a subset of 211 ESTs were not used for primer design because at least one of the DNA sequences flanking the SSR was too short or inappropriate for designing high quality primer pairs (e.g., too low GC content). The remaining 744 SSR containing ESTs with a minimum of five repeats for dinucleotide motives and four repeats for

trinucleotide motives were used to design one to three primer combinations (1,221 in total) with the Primer 3 software (Rozen and Skaletsky 2000). Primer design met the following criteria: (1) primer length between 18 and 24 bp (target: 20 bp), (2)  $T_m$  between 57 and 61°C (target: 60°C;  $\Delta T_m < 1^\circ\text{C}$ ), (3) GC content between 40 and 70%, and (4) predicted PCR product length between 150 and 400 bp.

Primer amplification was tested within six *L. perenne* and two *L. multiflorum* genotypes, representing mapping (grand-) parents of the p150/112 intraspecific ILGI reference population (Bert et al. 1999), VrnA (Jensen et al. 2005), pop8490 (Barre et al. 2000) and Xtg-FAL (Studer et al. 2006). PCR reactions were conducted in a total volume of 20  $\mu\text{l}$  containing 25–50 ng of genomic DNA, 1  $\times$  PCR buffer, 0.2 mM of each dNTP, 2 mM  $\text{MgCl}_2$ , 0.375 U of Taq DNA polymerase and 0.2  $\mu\text{M}$  of the respective forward and reverse primer. PCR amplification was performed using a touchdown profile with an initial denaturation step of 5 min at 94°C followed by 12 cycles of 30 s at 94°C, 1 min at 72°C and 1 min at 72°C with a lowering of the annealing temperature of 1°C at each cycle and 30 cycles of 30 s denaturation at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C. After a final extension of 5 min at 72°C, size of amplification products was identified using fluorescent dye labelling with subsequent detection on a megabace 1000 96 capillary electrophoresis system (GE Healthcare), an ABI Prism 3100 genetic analyser (Applied Biosystems) or based on polyacrylamide gel electrophoresis.

Out of a total of 1,221 primer pairs, 618 (51%) amplified clear PCR products, representing 464 different ESTs (62% of the 744 unique ESTs). These 464 successfully amplified ESTs were blasted against the nr protein database of Genbank using the BLASTX programme (September 2007) and revealed 325 (70%) significant hits ( $E \leq 1^{-10}$ ), 184 ESTs (40%) were associated with annotated genes. One primer pair per EST amplifying one or two bands of the expected size was used to screen polymorphisms within the mapping population VrnA, whose parental genotypes have been used to establish the EST library. Within a subset of eight VrnA  $F_2$  genotypes, 306 SSRs were successfully amplified, including 143 polymorphic SSR markers. Here, we report on the

**Table 1** Sequences and characteristics of EST-derived primer pairs revealing polymorphisms in the mapping population VrnA (Jensen et al. 2005), whose parental genotypes have been used to establish the initial EST library

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	$T_m$ <sup>b</sup>	SSR motif	Expected product size	Number of VrnA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G01_002 (ES700445)	FI-CAAGACCAAACCGAGAGAGG	59.8	(AG)7	201	2	EAY87839.1	3.00E-60	
	RI-TCTCCTCTCGACTTCCAGA	60.4						
G01_007 (EY458052)	FI-CCTCAGCAGCAGTTACAGCA	60.4	(GCA)5	210	2	BAD61300.1	3.00E-19	
	RI-GGCTGGGTTGTGAATGAAGT	59.9						
G01_024 (ES699910)	FI-AAAGTTCCTCGTTGAGGATCT	59.9	(CCA)5	332	2	EAY92164.1	1.00E-37	
	RI-ATGCCGACATTAGGAACCAC	60.1						
G01_025 (ES699924)	FI-GCTTTGCAGACGAAAGGTCTC	60.1	(AAT)4	160	2			
	RI-GGCACAGAACCGTTGCTAAT	58.5						
G01_027 (ES699978)	FI-CACGTAATTCGTCGTCCAT	58.5	(AT)6	165	2			
	RI-CATGCGGTCCCGTCTTATTT	60.2						
G01_031 (ES699945)	FI-ATGAACACCCAGGATTGGAA	60.2	(ATG)6	329	2	EAY98986.1	8.00E-18	
	RI-TGATGCAAGCTCAGGGTTTG	59.6						
G01_033 (ES699868)	FI-ATTAATCGCCCTCGTTCAAA	59.6	(TGA)5	254	2	ABA91631.2	1.00E-14	Fructose-bisphosphate aldolase, [ <i>Oryza sativa</i> (japonica cultivar-group)]
	RI-AAAATCCACGGGTCACACAT	60						
G01_037 (ES699806)	FI-CAAGGCTTTGCACTCTGTGAA	60	(GA)6	226	2			
	RI-TCGGGAGAACTGGAAACAAC	60.1						
G01_043 (ES699796)	FI-AGCCGCTCAACTTTGAAGAA	60.1	(AG) <sup>9</sup> ataa (AG) <sup>10</sup>	247	2			
	RI-TTGGTGGTGGTGAAGTTGAA	60.5						
G01_044 (ES699788)	FI-ACCATTGCAGGGATCAAAGA	60.5	(GAT)5	327	3	EAY73604.1	2.00E-78	
	RI-ATTATGCCCCCTCTGAGCTT	60						
G01_045 (ES699707)	FI-TGCTAAAAACAGCAGCCAATG	60	(GAT)5	377	2	NP_001067340.1	2.00E-55	RAB5A protein [ <i>Oryza sativa</i> (indica cultivar-group)]
	RI-TTGAACATGGGAATGCCACTG	59.7						
G01_047 (ES699598)	FI-CCCCAGAGCATCCATAATA	59.7	(GT)7	150	3			
	RI-CCACGGAAAGCTAGGACTTG	59.7						

Table 1 continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G01_048 (ES699573)	FI-TGGTCATTGCCAGCATCTAC RI-TCATTAGGGCTTGGGAAC TG	59.7 60.1	(GAA) <sub>4</sub>	241	2	EAZ22176.1	1.00E-100	
G01_053 (ES699500)	FI-GAAAAAGGTTGGGGTTTGT RI-CGAAAGAGGCGTAGATCTTG	60.1 59.9	(CGG) <sub>5</sub>	230	3	NP_001056102.1	3.00E-93	
G01_054 (ES699490)	FI-AGACCAGCACGAGGTGAAAGT RI-TGCATGCTAAITCCAGGTTG	59.9 60	(TGG) <sub>5</sub>	324	2	EAY95192.1	1.00E-18	
G01_063 (EY457990)	FI-GTGTGCAGTTTGGTCAATG RI-GACGCAACAAAAC TTGTGGA	60 61	(TA) <sub>6</sub>	373	2	EAZ32936.1	6.00E-25	
G01_068 (ES699326)	FI-GCATCCATGTCTCCAAGGAA RI-TAGGCCCCCATCATCAGTTTC	61 60.2	(CAG) <sub>5</sub>	313	2	NP_001064508.1	7.00E-36	
G01_073 (ES699298)	FI-TAGCCAGACAGCCTTCGICT RI-GGAGGAGCCACAGTCACATT	60.2 60	(GGC) <sub>6</sub>	384	2			
G01_075 (ES699284)	FI-ATAGACGAGGTCGGGTTCTT RI-CGCTGATAGCACCTTCTTCC	60 60	(TGC) <sub>4</sub>	294	2	NP_001043891.1	1.00E-89	
G01_080 (ES699275)	FI-TCTGCAACTTGGGTTGTTTC RI-GCCGTA AAAAGACCGTTGTGT	60 60.4	(CTC) <sub>6</sub>	272	3			
G01_090 (ES699128)	FI-ACAACAAGAAAGAGCCGATC RI-ACTCGGGACCAATCACAA C	60.4 60.3	(CCG) <sub>5</sub>	365	2	P02275	8.00E-43	H2A1_WHEAT Histone H2A.1 (wcH2A-9)/protein H2A [ <i>Triticum aestivum</i> ]
G01_094 (ES699103)	FI-ACTCTCCCGTCTCCAC RI-TGCTTACCCCTTGCTCCACTT	60.3 61.2	(CGC) <sub>7</sub>	188	2	NP_001062500.1	1.00E-27	Putative 40S ribosomal protein S25 (RPS25B) [ <i>Oryza sativa</i> ( <i> japonica</i> cultivar-group)]
G01_095 (ES699091)	FI-CACTCTTCTCCCGGATCA RI-GGATCTCATGTGATGTCC	61.2 59.6	(GCC) <sub>5</sub>	283	2	NP_001065632.1	3.00E-74	40S ribosomal protein S16, expressed [ <i>Oryza sativa japonica</i> cultivar-group)]
G02_004 (ES700403)	FI-CCGCGAGTAGAAAAAGAGGA RI-AGTGGGGAGTTGAAAACTT	59.6 60	(CAT) <sub>5</sub>	150	2	EAZ02197.1	2.00E-77	

**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	$T_m$ <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G02_013 (EY458048)	F1-AGAGGAGGAGGCCTGAAAAG R1-CCGAAGACACGAAACAGGAT	60 60.6	(GTT)4	183	2	NP_001042157.1	2.00E-23	
G02_017 (ES700234)	F1-CCTCATCCAAACCCTAACCC R3-AGCTCCTTCTCCTCCCTGAC	60.6 59.9	(CCG)	152	2	EAY97646.1	5.00E-95	
G02_018 (EY458040)	F1-ACAATGTGTTGAGGCTGCTG R1-ATGCTGCTTTGGCAAAATCT	59.9 59.9	(GGA)5	268	2	EAY91126.1	2.00E-52	
G02_022 (ES700158)	F1-GGTGCTCCAAATGGACAAGAT R1-CGCCAACAAAGGAGCTATC	59.9 60.1	(GTG)4	174	2			
G02_025 (ES700102)	F1-GAGTTTGAAGATCCCCGTGA R1-GCCATGATGCAGAAAGAGGT	60.1 59.3	(TGG)5	223	4	AAV84884.1	1.00E-98	Alternative splicing regulator [ <i>Triticum aestivum</i> ]
G02_029 (ES700070)	F2-CTTCCAAATCCAAAGCGATCT R2-GCTGCTTCTCCTTCTTGG	59.3 59.7	(GTC)5	192	2			
G02_032 (ES699970)	F1-ATCGTGTGCCTGGAAACTG R2-CAAGCAGCAAAAGTTGCACAT	59.7 60.1	(CAT)5	195	2			
G02_033 (ES699985)	F1-CTGGGAGCAGTGGTTTTGAT R1-ATCATCCAGGTCAGCCGTAA	60.1 60	(GAT)5	235	2			
G02_035 (ES700001)	F1-GCGACTCTCGTTTCTTGTGTC R1-CGGATTCAAATGCAATCAGA	60 60.1	(TC)7	175	2			
G02_047 (ES699667)	F1-GCCTTTGTCCTTGGAAATCA R1-TCCAAAACGATTCAGAAAGCA	60.1 60	(TG)11	355	3			
G02_048 (ES699641)	F1-GTGTGTTCTCTCGTGGATG R1-TGCTATGTGTTGCTCCTTGC	60 60	(GT)6	260	1			
G02_049 (ES699689)	F1-CCGGTTGACGGAGTTGTAGT R1-GCTTACAAAGTGCCGAAAGAG	60 59.5	(TGT)5	203	2			

Table 1 continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G02_053 (ES699580)	F1-GGCGTGTGACATAGGCAAT R2-AAACACCACCATCCCTATCCCTG	59.5 (ATT)4 60.4		204	1			
G02_057 (ES699506)	F1-CTGCTATCTCGGCCAGTTCAT R1-GAAAGGGATTGCATCTCGAA	60.4 (GCG)4 59.9		232	3			
G02_069 (EY457982)	F1-GCTGCAGATGATGATGAGGA R1-TAGGGTTGAGGGAGGAGCA	59.9 (AGA)4 60		318	2	BAD09428.1	1.00E-37	Putative 3-methyladenine-DNA glycosylase [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G02_075 (ES699286)	F2-GCCGGGTGTAGGACTGTAAA R2-CTTCCCAAGATCACCCAGAT	60 (TAG)5 59.4		312	2			
G02_079 (ES699258)	F1-GGAAACCAACAAGCAAAGAA R1-GACCCGAATCGAAATTCAGGA	59.4 (AG)6 60.6		172	3	NP_001042895.1	2.00E-94	Putative Pro kinase interactor 1 [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G02_092 (ES699159)	F2-ACCAATCCCCACCTCATCT R2-AGGTCTCGACGGCTCTCTTG	60.6 (AGC)5 60.2		263	2	NP_001042044.1	3.00E-43	
G03_002 (ES699027)	F1-GCATCTCTACGGGCTCTAC R1-GAAAAAGCTGAGGCAACCAAG	60.2 (GAA)4 60		266	2			
G03_003 (ES699028)	F1-GGAGATGGCACTCTCTCGTC R2-CGATCCAACGGGAACTAA	60 (CCT)4 60.5		308	2	NP_001047493.1	9.00E-25	Putative defensin [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G03_010 (ES699101)	F1-ACTCCCCGCAAAAACCCCTA R2-GGTTGAACCCAGTCTTGACA	60.5 (CGC)4 60.3		153	2	NP_001056613.1	8.00E-87	Putative 60S ribosomal protein L13E [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G03_013 (EY457952)	F2-CAGCTGTCTCTGCTCACA R1-GCAGGTGATACATCGCACAT	60.3 (ATC)4 59.9		206	2	AAR06360.1	8.00E-21	Putative metacaspase [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G03_015 (EY457953)	F1-AGTGTGACGGCACTGAGATG R1-CCACCTAGAGGTTGGAGCAG	59.9 (ATA)4 60.2		279	2	NP_001049857.1	4.00E-49	Pathogen-related protein [ <i>Oryza sativa</i> ]/JIOsPR10 [ <i>Oryza sativa</i> ]
G03_016 (ES699142)	F1-CACCTCCCCTTCTCCTCCTC R1-GGGTAAGCATCTCTCGTTCAGC	60.2 (CGC)4... (AAG)4 60.2		175	1	AAM92710.1	1.00E-119	Putative 40S ribosomal protein S3 [ <i>Triticum aestivum</i> ]

**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G03_020 (EY457960)	F1-ACCCAACTTACGGCACTCCAG R1-TTCCATGCCAACAGGTACAGC	60.2 (TGTCG) <sub>4</sub> 60.1		276	4	CAD88266.1	5.00E-13	Metallothionein-like protein type 3 [ <i>Hordeum vulgare</i> subsp. <i>vulgare</i> ]
G03_028 (ES699288)	F1-CCCCTGTAGTCCCTCCTCTC R1-TGAGTCCGTGTGCGTGTTC	60.1 (GCC) <sub>4</sub> 60		343	2			
G03_039 (EY457986)	F1-GCTCCAGGACTTCTTCAAG R1-TTGGTGGGATGGTGTTC	60 (CTG) <sub>4</sub> 59.4		220	3	AAB99745.1	1.00E-115	HSP70 [ <i>Triticum aestivum</i> ]
G03_045 (ES699459)	F1-TACTCTCTATTCGCCGAGCTC R2-AACTGATCGTCCAGAAACC	59.4 (CTCCT) <sub>4</sub> 59.7		170	2	BAA84993.1	4.00E-49	Histidine-containing phosphotransfer protein [ <i>Zea mays</i> ]
G03_049 (EY458009)	F3-AAGCCCTTCCCTTTACCTTGC R1-ACCGACTCTGTACGAAGCAC	59.7 (TGA) <sub>4</sub> 60.1		238	2	NP_001056390.1	1.00E-110	GTP-binding nuclear protein RAN-B1 [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G03_052 (EY458016)	F1-AGTCGACTGACCCGATGAAC R1-AAAGCCGTGCTCAGGTAGAA	60.1 (CGC) <sub>4</sub> 60		278	2	EAY79872.1	8.00E-24	
G03_054 (ES699637)	F1-TGGACTTACCTGGGGTCTG R1-TTCTCTATCCAACATGCAAA	60 (GTA) <sub>4</sub> 60		352	2			
G03_055 (ES699629)	F1-CCACTGACAAACCCTCACCT R1-AATGCTGCCCTCCTCTAAAT	60 (AAGA) <sub>4</sub> 60.7		288	2	NP_001057248.1	5.00E-10	Putative pollen-specific LIM domain protein [ <i>Oryza</i> <i>sativa</i> ( <i>japonica</i> cultivar-group)]
G03_058 (ES699635)	F2-CCTGTACCAAAAGCGTCCAG R2-CATTTTACTTGGCCAGCAGCA	60.7 (GCA) <sub>4</sub> 60.1		332	2	EAZ40363.1	4.00E-17	
G03_065 (ES699742)	F1-GTCGTCTCATCATCGTCTT R1-TTACAATCGCGACGACATA	60.1 (CAG) <sub>4</sub> 59.9		194	2			
G03_069 (ES699879)	F1-GGGACTGGACGATAAAGTGA R1-GGAACAAGAGAGCTGGTGA	59.9 (AAG) <sub>4</sub> 60		316	2	EAY85268.1	4.00E-54	
G03_072 (ES700017)	F1-TCATGCCCTCATCCTTATC R1-GGGCCAGAGCACACTAAGAG	60 (CTG) <sub>4</sub> 60		179	2	NP_001059109.1	7.00E-26	Synaptobrevin-like protein [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]

Table 1 continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G03_073 (ES699987)	F1-AAACCGGCTCTACACCATCAC R1-CAAAGCTCACAACGGCTACA	60 60.1	(CGA) <sub>4</sub>	253	2	NP_001062219.1	3.00E-29	Thylakoid lumen protein, chloroplast precursor-like [ <i>Oryza sativa japonica</i> cultivar-group]]
G03_079 (EY458032)	F2-CAGAGCTGCTCCATGTTCAA R2-AAAAGTGGCCTTCAACCTCCT	60.1 59.6	(CAG) <sub>4</sub>	330	3	BAD21528.1	4.00E-75	Putative brown planthopper-induced resistance protein 1 [ <i>Oryza sativa japonica</i> cultivar-group]]
G03_089 (ES700225)	F1-TCACCAAACACCACATCTCTC R3-GCTGCTCGTACTGCTCGTAG	59.6 60.1	(CTCC) <sub>4</sub>	203	3	NP_001044293.1	1.00E-111	Katamin [ <i>Oryza sativa japonica</i> cultivar-group]]
G03_092 (ES700252)	F1-ACAAGATGAGGCTGGACACC R2-GTCCCTTCTTGTGGTTTCCAG	60.1 59.9	(CGC) <sub>4</sub>	184	2	AAU82107.1	4.00E-95	20S proteasome beta 5 subunit [ <i>Triticum aestivum</i> ]]
G03_096 (EY458049)	F2-CTATGGTGCAGCAAAGGACAA R1-CGACACAGCCGATCTCTCA	59.9 59.7	(GGT) <sub>4</sub>	237	2	NP_001062583.1	2.00E-80	Putative poly(A)-binding protein [ <i>Oryza sativa japonica</i> cultivar-group]]
G04_002 (ES699030)	F1-GACGTCCCTTTCCTCCTCTT R1-ATATATACAGGCGCGGATGC	59.7 60.2	(GGT) <sub>4</sub>	153	3			
G04_028 (EY457970)	F2-GGAAGCAAGGAAGGAGAACC R1-AGGTGGTGTGCCGTAAGAAG	60.2 61.7	(ACG) <sub>4</sub> ... (CGA) <sub>4</sub>	306	2	NP_001050127.1	1.00E-48	17.7 kDa low temperature induced protein [ <i>Oryza sativa</i> <i>japonica</i> cultivar-group]]
G04_030 (ES699331)	F1-ATGCAGCCACAGTGACCTA R1-GAAATCTGGCAGTGTGAGG	61.7 60.6	(GCC) <sub>4</sub>	281	2			
G04_034 (EY457989)	F2-TGACCTCAGCTACGACGACA R1-GCCTCTCCTCCCGTTTCCCTAT	60.6 60	(CGGA) <sub>4</sub>	283	2	AAU08786.1	4.00E-26	NAC domain transcription factor [ <i>Triticum aestivum</i> ]]
G04_043 (ES699476)	F1-TCAAACCACCACCTTTGACA R1-CAGGCTCTCCTCCTCTCTCT	60 60	(AGG) <sub>4</sub>	225	2	NP_001063563.1	1.00E-98	
G04_048 (ES699511)	F1-GAGCAAAGACACCAGCATCA R1-AGAAATCCCTTCCCTTCTCA	60 60.1	(CGG) <sub>4</sub>	248	2			
G04_052 (ES699556)	F1-ATGGGCTAGACATCGTTTCG R1-TGATGATGCGGTGGAAGATA	60.1 60	(CGC) <sub>4</sub>	274	2	NP_001062502.1	2.00E-76	Cyclophilin [ <i>Oryza sativa japonica</i> cultivar-group]]



**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G04_055 (EY458023)	F1-CACCATGATTAATGCGCAAC R2-CAGTCTCCTCCGACCTCTTG	60 60	(GGC) <sub>4</sub>	358	3			
G04_056 (ES699686)	F1-CAAGGGTGTGGCGATTAAC R1-ATCGGCATCATCATCAGACA	60 60	(GATCT) <sub>4</sub>	168	2	ABC55720.1	3.00E-32	Putative RH2 protein [ <i>Zea mays</i> ]/RNA helicase 2 [ <i>Zea mays</i> ]
G04_057 (ES699604)	F1-GTGTCTCTCCTTGTCTTTTCG R-ACATTGAGCCATCCAGCATT	60 60	(CCGA) <sub>4</sub>	327	2	P40880	1.00E-54	CAHC_HORVU Carbonic anhydrase, chloroplast precursor (carbonate dehydratase)/carbonic anhydrase
G04_059 (ES699619)	F1-GAAAGACAAAAGCGACGAAAG R1-AGTGGTCTTTCTCTGCTTCCA	60 59.8	(AGA) <sub>4</sub>	287	2			
G04_065 (ES699734)	F1-AACAAGGAATGCGGAGAAAGA R1-GCTTCCACAAGGAAGAAAGG	59.8 60.3	(GTG) <sub>4</sub>	234	2			
G04_067 (ES699747)	F1-TGGCAGTACAAATAGCTGAACG R1-TTGCAGGTTTCTGTGCACCTC	60.3 59.9	(CAC) <sub>4</sub>	180	2			
G04_092 (ES700244)	F1-GGACTTGCAAAGTCAATCAGC R1-CTCGAACTGTGTTCCCGAATA	59.9 59.1	(GTGC) <sub>4</sub>	227	2	CAA56314.1	1.00E-101	MAP KINASE [ <i>Avena sativa</i> ]
G04_098 (ES700422)	F1-GAGGAAGCAAAAACAGCACAA R1-GAGGACGAGGCTTCGAACAG	59.1 60.7	(AAG) <sub>4</sub>	207	2	AAM11916.1	6.00E-53	
G05_005 (ES699094)	F2-GGCAGGTGGACCTTGACA R2-GAGATTGACGGCGACGAG	60.7 60.8	(CGG) <sub>4</sub>	282	2	EAZ02233.1	2.00E-98	
G05_014 (ES699131)	F1-CCATCACAGATTCGGAGAGC R1-GGTAGGGGATAGGGAAG	60.8 60.1	(CGC) <sub>5</sub>	164	2	NP_001048971.1	1.00E-47	
G05_023 (EY457959)	F2-GCGAAAACCAAAAGTGATCT R1-GCATGTCAGTCAACACACC	60.1 59.7	(GAT) <sub>5</sub>	203	2	EAY76242.1	2.00E-16	
G05_024 (ES699168)	F2-GCAACCTCGTAGACCACCTC R3-CAATCTTGTGATGTCGCGGATA	59.7 59.8	(GCG) <sub>4</sub>	180	2	CAA48400.1	5.00E-41	Ribosomal protein L12 [ <i>Secale cereale</i> ]

Table 1 continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G05_028 (EY457963)	F1-GACCTCACCTCACCAGCTC R1-CTTCAAGAGCCTTGCTCACC	59.8 60.1	(GGA) <sub>4</sub>	360	2	NP_001042526.1	1.00E-20	Peptide transporter protein -like [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_030 (ES699190)	F1-GGGGAAAGGAAGTCAGTCTC R1-GCCAACAACAGAGACAGCA	60.1 59.5	(GCT) <sub>5</sub>	245	2	AAL47687.1	4.00E-82	Glutathione-S-transferase Cla47 [ <i>Triticum aestivum</i> ]
G05_033 (ES699201)	F1-GCAGCTTCTCCCAACACTT R1-GCACGCTAAGTTTCGGTGTT	59.5 59.6	(AGC) <sub>4</sub>	152	3	AAX08107.1	9.00E-65	Cinnamoyl-CoA reductase [ <i>Triticum aestivum</i> ]
G05_035 (ES699203)	F1-GCCGCAGGAAGAAGATTAGA R2-TCTTGTTCACAGGGTGAT	59.6 60.2	(GCT) <sub>4</sub>	219	2	NP_001053193.1	1.00E-42	
G05_041 (ES699231)	F1-TAAACGGCGGATAGATTGCTC R1-GTCGGAGTTCAGAGTCGAAGG	60.2 60.2	(AGG) <sub>4</sub>	208	2	NP_001047013.1	5.00E-46	Putative SNAP25 [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_044 (ES700454)	F1-GACCGATTGGAACCAACAAC R2-CGATGCTTTCAGCGGTTAAT	60.2 59.6	(CTC) <sub>4</sub>	271	2	NP_001065900.1	4.00E-58	Nitrogen fixation protein, putative, expressed [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_046 (ES699282)	F1-TACCTCCAGCAACAGCTTCA R1-TTCTGAAACTGGCTGCAATG	59.6 59.6	(GCA) <sub>5</sub>	249	2	NP_001045993.1	5.00E-61	Putative auxin response transcription factor(ARF6) [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_050 (ES699310)	F1-FGCTCAGATGGTCTGCAGTT R1-CTGGGAAGAAGCAGTTCTGG	59.6 60.1	(CAT) <sub>4</sub>	289	2	EAY94464.1	2.00E-73	
G05_056 (EY457973)	F2-ATGGTGAAGATGCCCTCAAG R2-CCAGTACCCCTGCACCTATT	60.1 59.7	(GGA) <sub>5</sub>	379	2	AAC08009.1	3.00E-77	DnaI-related protein ZMDJ1 [ <i>Zea mays</i> ]
G05_065 (ES699367)	F1-ATGAAGATCGCCACTCACCT R1-TGCTGCTGCTACCGTTCTTA	59.7 60	(TCT) <sub>6</sub>	203	2	CAE92374.1	2.00E-43	Ocs-element binding factor 1 [ <i>Secale cereale</i> ]
G05_070 (ES699441)	F1-CCCATTCTCTGATCCCTCCA R1-CATCTGCCATGTGATGCTCT	60 60.3	(CT) <sub>7</sub>	312	2	NP_001042703.1	1.00E-64	Putative cationic peroxidase isozyme 40 K precursor [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_071 (EY458003)	F1-CTGGTCCGGTCAGTCAGTCT R1-TTTTGTGGACAAACTGCAAC	60.3 60.7	(TTC) <sub>5</sub>	224	4	ABR25977.1	3.00E-27	Osiaa30-auxin-responsive aux/iaa gene family member [ <i>Oryza sativa</i> ( <i>indica</i> cultivar-group)]

**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	$T_m^b$	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G05_073 (ES699475)	F1-CTCATCGGCATCTTCCAAAAG R2-GTAGATGACCCCGTCTTGT	60.7 (TC)7 59.5	(TC)7	251	3	AAS48644.1	9.00E-80	Putative ABA-induced protein [ <i>Cynodon dactylon</i> ]
G05_076 (ES699567)	F1-TCCCGATCTCACACATTTG R2-CGTGCTCGAGGCAGTAGAG	59.5 (AG)6 59.8	(AG)6	185	2	ABD92943.1	1.00E-121	Alpha tubulin-1B [ <i>Triticum aestivum</i> ]
G05_081 (ES699712)	F1-CCAACCAAGAGTCCGGAAG R1-AGGGTCTCGGAGATGCTG	59.8 (AG)10 60	(AG)10	192	2			
G05_082 (ES699706)	F1-GACGGCAGGTACTGGACAAT R1-TTCCTAGGTCGGTAGGCCAAA	60 (TA)6 60.1	(TA)6	326	2	AAC78643.1	3.00E-32	Ribulose-1,5-bisphosphate carboxylase small subunit [ <i>Avena vaviloviana</i> ]
G05_088 (ES700025)	F1-TGGAGACATGGAGGAAACC R1-CGTGCACAAACAGAAAAACCA	60.1 (TG)6 59.8	(TG)6	243	3	AAK27373.1	4.00E-39	Triose phosphate/phosphate translocator [ <i>Oryza sativa</i> ]
G05_089 (ES699993)	F1-AAAGTCCGCTTGACACTA R1-AGGGCACAAATGCACITTA	59.8 (CTG)5 60.1	(CTG)5	323	2	CAA58110.1	4.00E-20	Jasmonate induced protein [ <i>Hordeum vulgare</i> subsp. <i>vulgare</i> ]
G05_090 (ES699930)	F2-CAGAAAGAGGCTCAAGCAACC R1-CACACAACCAGTGCTATTG	60.1 (GA)7 59.6	(GA)7	230	2	NP_001042817.1	2.00E-51	Putative ATP citrate lyase a-subunit [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_092 (ES700116)	F1-GCCTCCTCAAACCCCTAACCT R1-TGCCGAGAACTTGATCTGGTG	59.6 (GGC)8 60	(GGC)8	348	2	NP_001062338.1	1.00E-46	Nuclear transport factor 2 (NTF-2) [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_099 (EY458050)	F1-AGCCTTCTTTGGGAGAGGAG R1-TCTCTCTGGCAGTTCTGGT	60 (GGA)5 60.7	(GGA)5	260	3			
G05_108 (ES699955)	F1-GGACCCAGGGACCTTTAGTG R1-CAGGGTCAATGTCTCAGGT	60.7 (GAG)4 59.9	(GAG)4	152	2	NP_001051798.1	1.00E-15	
G05_109 (ES699919)	F1-TGCAGACGGGACTGTAAGTG R1-TTGAAAACAAGCCGAAACC	59.9 (ACC)4 59.8	(ACC)4	395	2	BAD81300.1	1.00E-59	Putative S-domain receptor-like protein kinase [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_121 (EY458034)	F1-CGTCTTCCAAAGATCGACA R1-TTGGGATCCATGCACTATAACA	59.8 (TGC)4 60	(TGC)4	226	4	O64393	4.00E-62	WHW2_WHEAT Wheatwin-2 precursor (pathogenesis- related protein 4b) [ <i>Triticum aestivum</i> ]

**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	T <sub>m</sub> <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G05_122 (ES700165)	F1-AGCACAAAGAAGCTCCAAA R1-CGACCATGCTGGTGATGTAG	60 60	(CCT) <sub>4</sub>	392	2	AAT76982.1	5.00E-46	
G05_127 (ES700228)	F2-AAAATGTTGTCCGGTGAGAGG R2-GATGAGCGGTCTCTTCTTGC	60 60	(CCA) <sub>4</sub> ... (GCA) <sub>4</sub>	257	2	EAY98743.1	1.00E-50	
G05_129 (EY458043)	F1-CTTTTGTCTTCTGCACCTTCC R1-AAACCACACAGCAGATGAAAGC	60 60.3	(TGC) <sub>4</sub>	182	2	NP_001060675.1	1.00E-18	Fibroin heavy chain precursor-like protein [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_130 (ES700253)	F2-CACCACCACCTCGCTACAG R2-GTCAAGCAAAAAATGGCAAGG	60.3 60.4	(AGC) <sub>4</sub>	154	2			
G05_132 (EY458046)	F1-CAGATGGCGATGCTCTACAG R1-CTTGTCTTGTCCGAATCGT	60.4 60.5	(CGA) <sub>4</sub>	300	2	NP_001054282.1	7.00E-51	
G05_134 (ES700272)	F1-CCTGGGTCCACCTTCTCTT R1-GCGGGCTGTAGACGAAAGTAG	60.5 60.1	(CGC) <sub>4</sub>	339	3	EAZ13733.1	9.00E-75	
G05_139 (ES700330)	F1-GGTACGGACTCTCCCTCTCC R1-AGCTTGGCTATGTTCCGATT	60.1 60.2	(CGC) <sub>4</sub>	268	2	AAR01225.1	3.00E-80	Putative aminotransferase AGD2 [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G05_144 (ES700381)	F1-AGTCACTTCTCGGCCCTCCT R3-TCAGGAGCTGCCCATTAATTC	60.2 60.1	(GCG) <sub>4</sub>	226	2	CAA63662.1	1.00E-103	Xyloglucan endotransglycosylase (XET) [ <i>Hordeum</i> <i>vulgare</i> subsp. <i>vulgare</i> ]
G06_006 (ES700027)	F1-GTGGATCCAGAGTTGGCACT R1-CTCCCTCGTCCATTGACATT	60.1 60	(GAT) <sub>4</sub>	259	2	NP_001049527.1	1.00E-43	
G06_021 (ES699358)	F1-ACGCCATTACTCTGTCCAC R3-ATCTCCTCGGAGACCCACT	60 60.1	(GGC) <sub>4</sub>	239	2	NP_001057112.1	1.00E-77	Putative transmembrane protein Tmp21 precursor [ <i>Oryza</i> <i>sativa</i> ( <i>japonica</i> cultivar-group)]
G06_028 (EY457991)	F2-TGGAGGATTGAGTGCTACCC R1-CCATGATGCCACACACAAAAT	60.1 60	(ATG) <sub>4</sub>	304	2	EAZ07690.1	9.00E-54	
G06_029 (ES699379)	F1-TTGTCGAAAGCTGGTGATCTG R2-GGCATTCGTTTGGATAATG	60 60.4	(ATA) <sub>4</sub>	260	2	NP_001044947.1	4.00E-64	Putative 5'-3' exoribonuclease (XRN3) [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]

**Table 1** continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	$T_m$ <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G06_031 (EY457992)	FI-GCCGAGTTC AACCTTTCAGA RI-GCTTCCCTGCTCTTCT	60.4 60	(GCC) <sub>4</sub>	265	2	NP_001042679.1	3.00E-70	Putative MAWD binding protein [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G06_039 (ES699418)	FI-AGAAGGCTCGTCGGGTCTA RI-CCCAACCAAGTCAAGAAAGAA	60 60.4	(TCG) <sub>4</sub>	351	3	NP_001047574.1	5.00E-39	Monothiol glutaredoxin-S6 precursor/putative glutaredoxin I [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G06_041 (ES699422)	FI-GTGGTTGGTGGCCCTCTAATG RI-GCCGACAATGGAGAATGAGT	60.4 59.9	(GCT) <sub>4</sub>	284	2	EAZ08137.1	1.00E-106	
G06_051 (EY458010)	FI-CGACATCGAGTACCACGAGA RI-GAACGGGTATGGCACCTCTA	59.9 60.1	(CCG) <sub>4</sub>	277	2	EAY91882.1	1.00E-27	
G06_079 (ES699793)	FI-GCAACGACGAGGAGATCAAT RI-ACCACCGGTGAGTACATTGC	60.2 59.9	(GCCG) <sub>4</sub>	186	2			
G06_089 (ES700020)	FI-AGATGGGAGGTGATCAGGTG RI-GAATCTTGGCAGAAAGCCCTA	59.9 59.8	(AGA) <sub>4</sub>	153	3			
G06_096 (EY458028)	F3-GATCTTGCAGGCCGTCTAAC RI-GCCAGCGTCTTTATTTAGGC	59.8 60	(TTG) <sub>4</sub>	200	2	EAZ20762.1	1.00E-51	
G07_025 (ES700366)	FI-AACTCCCTATTGTGGCGATG RI-AGCAGCAAGTGTGCAACAAC	60 59.7	(CTG) <sub>4</sub>	255	1			
G07_034 (EY457976)	FI-ACCAGCAACCCGACGAAAC RI-GCCGCTGTGATGGAAAGA	59.7 60	(CTCCAC) <sub>4</sub>	349	2	EAY76152.1	1.00E-108	
G07_037 (ES699351)	FI-CCGAGTCCAGCTCAATCTTC RI-ATCCGCCAAGAACTCGTAGTC	60 60	(CCG) <sub>4</sub>	215	3	CAJ86166.1	8.00E-38	
G07_038 (EY457979)	FI-GACGCACAACCTGCAACAAC RI-TCTTGACTTCTTCTTGCTTGA	60 60.1	(GCT) <sub>4</sub>	335	2	NP_001048488.1	1.00E-72	
G07_042 (EY457985)	FI-AATCAAAACAAGCCGAGATG RI-TGATAGTGTGCCACGGGTA	60.1 59.9	(AGA) <sub>4</sub>	331	2	EAY86454.1	9.00E-17	

Table 1 continued

Marker name (EST acc nr) <sup>a</sup>	Primer sequences 5'–3'	$T_m$ <sup>b</sup>	SSR motif	Expected product size	Number of VmA alleles	Best significant BLASTX hit	E-value	Gene annotation <sup>c</sup>
G07_056 (ES699421)	F1-CAAAGAAGTCACGCCACAAA R2-GCTGGTGTAGCAGATGAGCA	59.9 59.6	(CCGCT) <sub>4</sub>	253	2	CAD57742.1	2.00E-97	RAC-ROP-like G-protein [ <i>Hordeum vulgare</i> subsp. <i>vulgare</i> ]
G07_057 (ES699425)	F1-GGGGACAGCTGCAACAAT R1-GGGACTTCAACCAGTTGTTC	59.6 60.1	(CCT) <sub>4</sub> ... (CGC) <sub>4</sub>	167	2	CAG38125.1	4.00E-35	Cystatin Hv-CPI9 [ <i>Hordeum vulgare</i> subsp. <i>vulgare</i> ]
G07_058 (ES699433)	F1-AAAGGAGCTCCAGCAAGATGA R1-GGGGGAGAGGCTTCAATAAC	60.1 60.6	(GCC) <sub>4</sub>	201	4	NP_001042508.1	1.00E-18	Salt-stress root protein RS1/plasma membrane polypeptide-like [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G07_065 (ES699478)	F1-CTCCACTCCAGTCCACAACC R1-GAAACATGGAGAGGGTCAAGGA	60.6 59.7	(CTC) <sub>4</sub>	175	3	EAZ45248.1	6.00E-86	
G07_066 (ES699486)	F1-TTTCGGGAGGTGTAAGA R1-GTAGGTGGACCTGGTGCTGT	59.7 59.8	(GCA) <sub>4</sub>	192	2	NP_001062495.1	2.00E-28	Putative vesicle-associated membrane protein 725 (AVVAMP725) [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G07_071 (ES699543)	F1-CATCTATGCTCCTTGCCACTCT R1-AACGAATCCGATCGAAACAG	59.8 60	(TCC) <sub>4</sub>	201	2	EAZ12938.1	3.00E-49	
G07_074 (EY458019)	F1-GAAAGCACATCGACGAGATCA R1-ATGGCATTGATACCCCTCCAC	60 60.1	(AGC) <sub>4</sub>	294	2	BAB63672.1	3.00E-85	Putative cysteine protease CPI [ <i>Oryza sativa</i> ( <i>japonica</i> cultivar-group)]
G07_075 (EY458020)	F1-ATTTTGGATTCTGCCGTCCTG R1-GAGGTCGCTTGGTTGTCTGT	60.1 60	(AGGA) <sub>4</sub> ... (ACG) <sub>4</sub>	348	2	ABF95962.1	2.00E-39	
G07_083 (ES699578)	F1-ACTGTGGGAGATGACGAACC R1-CCTCAAAGCCCAATAAAGCAG	60 59.9	(AAG) <sub>4</sub>	250	2	EAY72779.1	3.00E-11	
G07_088 (ES699692)	F1-CCGGGACAGAACTGATGAT R1-CGAGCATTCTCCTTCTCC	59.9 60	(CTA) <sub>4</sub>	345	2	EAZ05534.1	5.00E-32	
G07_098 (ES699737)	F1-ACTGAGGCAGTGGAAAGAGGA R1-TGCAACCAAAAATAACCA	60 60.1	(AAG) <sub>4</sub> ... (AGA) <sub>4</sub>	301	2			

<sup>a</sup>EST acc nr: Genbank accession number of the EST used for primer design<sup>b</sup> $T_m$ : Melting temperature<sup>c</sup>Significant BLASTX hit with known functional annotation

sequences of 464 primer pairs amplifying in at least one of eight parental genotypes along with the marker name, EST accession number, melting temperature, SSR repeat motif and number, expected PCR product size as well as the gene annotation of the corresponding EST (electronic supplementary material). A subset of these markers revealing polymorphisms in *VrnA* is given in Table 1.

Although EST-derived microsatellite markers are reported to yield lower levels of intraspecific polymorphisms compared to SSRs developed from non-coding genomic regions (Saha et al. 2006), the number of 143 SSR markers, which were polymorphic in *VrnA*, was high (47% of the 306 primer pairs amplifying in *VrnA*, 19% of 744 ESTs used for primer design). This is comparable to previous findings in ryegrasses, where similar proportions (21–31%, depending on the library source) of SSRs for which primers had been tested, were polymorphic and could be mapped in a specific population (Faville et al. 2004; Gill et al. 2006; Hirata et al. 2006).

As EST-SSRs are based on transcribed genes involved in specific biochemical or physiological pathways, they may provide very close associations with loci controlling trait variation, allowing the development of functional markers, which are particularly useful for breeding applications (Andersen and Lübberstedt 2003). The percentage of ESTs with significant gene annotations (40%) is comparable to results found in previous studies, where 47% of mapped EST-SSRs were functionally annotated with BLASTN hits ( $E < 1^{-10}$ ) to other plant species (Faville et al. 2004). GeneThresher<sup>®</sup>-derived SSRs revealed 45% to be highly similar ( $E < 1^{-7}$ ) to genes expressed in other species (Gill et al. 2006). Moreover, the enhanced transferability to other pedigrees and species (almost 80% for *Festuca-Lolium* species; Saha et al. 2004) makes them useful for alignment of linkage maps and for studying interspecific synteny.

In conclusion, this research significantly contributes to the set of publicly available EST-SSR markers in the most important temperate forage grass species. The characterised primer sequences, which are particularly useful for marker assisted breeding strategies, are forming a valuable resource in the development of anchor markers for comparative mapping and evaluation of functionally associated genetic loci.

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