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Comparative mapping of fiber, protein, and mineral content QTLs in two interspecific *Leymus* wildrye full-sib families

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Abstract Crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), and mineral content are important components of forage quality in grasses. Elevated [K]/([Ca] + [Mg]) ratios (KRAT) substantially increase the risk of grass tetany (hypomagnesemia) in grazing animals, which is a serious problem associated with some cool-season grasses. The objectives of this study were to map and compare QTLs controlling concentrations of CP, NDF, ADF, Al, B, Ca, Cl, Cu, Fe, K, Mg, Mn, Na, P, S, Si, Zn, and KRAT in two full-sib *Leymus triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 families. Significant genetic variation and QTLs were detected for all traits, with evidence of conserved QTLs for ADF (LG1a, LG5Xm, LG7a), NDF (LG7a), Ca (LG1b), CP, (LG5Xm), KRAT (LG3b, LG6b, LG7a, LG7b), Mn (LG2b, LG3b, LG4Xm), and S (LG3a) content in both TTC1 and TTC2 families. Moreover, the direction of QTL effects was the same for 13 of the 14 homologous QTLs in both families. The TTC1 and TTC2 KRAT QTLs on LG7a and LG7b were located near markers defining homoeologous relationships between the sub-genomes of allotetraploid *Leymus*, suggesting possible QTL homoeology. Another 88 QTLs were unique to one family or the other, but many of these clustered in genome regions common between the two families. These results will support development of new *Leymus* wildrye forages and help characterize genes controlling mineral uptake and fiber synthesis.

Keywords Forage · Fiber · Grass · Mineral · Protein · QTL

Abbreviations

ADF	acid detergent fiber
CP	crude protein
KRAT	K/(Ca + Mg) ratio
NDF	neutral detergent fiber

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Introduction

Fiber, crude protein, and mineral content are important criteria in forage grass breeding (Casler and Vogel 1999; Casler 2001). Fiber content is often measured by acid detergent fiber (ADF) and neutral detergent fiber (NDF), which can limit the digestibility, palatablity, and intake of grass forages (Van Soest 1994; Falkner and Casler 1998). The NDF

fraction includes cellulose, lignin, and hemicellulose; whereas the ADF fraction is composed mainly of cellulose and lignin without hemicellulose (Van Soest 1994). The genetic control of ADF and/or NDF content has been examined by QTL analysis in perennial ryegrass (Cogan et al. 2005), maize (Krakowsky et al. 2005, 2006; Lübberstedt et al. 1997; Méchin et al. 2001) and *Arabidopsis* (Barrière et al. 2005). Crude protein and mineral content receive less attention by forage grass breeders, in part because soil fertility can have relatively large effect on these traits, because protein and mineral supplements are commonly used, and also because crude protein is often negatively correlated with yield (Casler and Vogel 1999; Casler 2001). Although much of the soluble protein is degraded to ammonia in the rumen and eventually excreted as urea, crude protein is highly digestible and non-degradable protein that passes through the rumen can be efficiently utilized in the lower digestive tract (Casler 2001). Like ADF and NDF, crude protein concentration is relatively easy to measure by near infrared reflectance spectroscopy (NIRS) analysis. Thus, these traits are often evaluated together and QTLs controlling crude protein concentration were also detected in perennial ryegrass (Cogan et al. 2005) and maize (Lübberstedt et al. 1997; Méchin et al. 2001). An inverse relationship between crude protein and fiber content can be expected due to phenological variation in reproductive development and the ratio of stems, leaf sheaths, and lamina. Thus, dissection of protein and fiber QTLs may provide some insight into the possible ontology of the underlying genes.

Although mineral supplements are relatively inexpensive and effective when properly administered, significant problems are still associated with forage mineral deficiencies or imbalance. Forage $[K^+]/([Ca^{++}] + [Mg^{++}])$ molar charge ratios (KRAT) greater than approximately 2.2 substantially increase the risk of grass tetany (hypomagnesemia) in grazing animals, which is a serious problem associated with many cool-season grasses (Mayland 1988; Sleper et al. 1989). Grass tetany occurs in about 1% of grazing livestock, one-third of which likely die (Mayland 1988). Experimental grass varieties with high magnesium content were developed to reduce KRAT and evaluate grass tetany potential in tall fescue (*Festuca arundinceae*) (Sleper et al. 2002), Italian ryegrass (*Lolium multiflorum*) (Hides and Thomas 1981; Mosely and Baker 1991), and orchardgrass (Saiga et al. 1992; Saiga and Izumi 1997). Nutritionally relevant variation in mineral concentrations and KRAT have been also documented in crested wheatgrass (*Agropyron cristatum* and *A. desertorum*) (Asay et al. 1996; Vogel et al. 1989) and Russian wildrye (*Psathyrostachys juncea*) (Asay et al. 2001; Asay and Mayland 1990; Jefferson et al. 2001; Karn et al. 2005). In comparisons with crested wheatgrass and Russian wildrye, Altai wildrye (*Leymus angustus*) had especially high KRAT values (Lawrence et al. 1982). Despite the large number of studies of KRAT variation there has not been any effort to dissect the genetic control of various forage mineral concentrations at the genome level of grasses.

The genus *Leymus* includes about 30 long-lived perennial grass species distributed throughout temperate regions of Europe, Asia, and the Americas. *Leymus* wildryes are perhaps most abundant in the mountains of central Asia and western North America. These species display remarkable variation in stature and adaptation to harsh cold, dry, and saline environments. Basin wildrye (*Leymus cinereus*) and several other large-stature *Leymus* species including Altai wildrye *(L. angustus*) and mammoth wildrye (*L. racemosus*) have high biomass accumulation potential across a wide range of high-elevation or high-latitude growing environments of western North America (Jefferson et al. 2002; Jensen et al. 2002; Lauriault et al. 2005), ideal for stockpiling fall and winter forage or biofuel feedstocks. *Leymus cinereus* is the largest (up to 2 m tall) native grass and most abundant *Leymus* species in the Great Basin, Rocky Mountain, and Intermountain regions of the western North America, where grazing livestock provide major agricultural commodities and heavily rely on natural or low-maintenance forage production. Large stature *Leymus* wildryes capable of producing abundant and valuable forage on many saline/alkaline sites where few other species are adapted. However, caespitose *L. cinereus* is susceptible to damage by intense grazing of early season and fall regrowth. Once abundant on the floodplains of major rivers, alluvial gullies, and other watered areas with deep, well drained soils in the Great Basin and Intermountain regions, *L. cinereus* has been eliminated from much of its former range due to grazing, harvesting, and

cultivation of field crops. Cultivars of *L. cinereus* are commonly used in rangeland seed mixtures in western North America, but have limited use in pastures or hay crops. The second most common *Leymus* species in western North American is creeping wildrye (*L. triticoides*). *Leymus triticoides* is a shorter (0.3–0.7 m), but highly rhizomatous grass specifically adapted to poorly drained alkaline sites in the Great Basin, California, and other regions of western North America. Creeping wildrye (*L. triticoides*) is cultivated using vegetative propagules as a saline biomass crop in California, but poor seed production limits widespread use of this species.

Although *L. triticoides* and *L. cinereus* are morphologically divergent, both species are highly self-sterile and hybridize with each other in nature. Experimental families, breeding populations, and molecular genetic maps derived from interspecific hybrids of *Leymus* species *L. cinereus* and *L. triticoides* have been developed for plant improvement and genetic investigations of functionally important traits in perennial forage grasses (Wu et al. 2003; Hu et al. 2005; Larson et al. 2006). The F1 hybrids are very robust plants showing a heterotic combination of tall plant height, large stems and leaves, prolific seed production, and relatively good seed germination from *L. cinereus* with vigorous proliferation of tillers, rhizomes, relatively good establishment (after seed germination), regrowth potential, and plant resiliency from *L. triticoides*. In terms of applied breeding, admixed breeding populations derived from interspecific hybrids of *L. cinereus* an *L. triticoides* show excellent potential for high biomass production, reduced susceptibility to grazing or harvest, and better regrowth potential. The linkage maps include 67 cross-species anchor markers (i.e. markers mapped in other grass species) used to identify and compare the 14 linkage groups of allotetraploid *Leymus* ($2n = 4x = 28$) based on synteny of corresponding markers in closely related wheat (*Triticum* spp.), barley (*Hordeum vulgare*), and cereal rye (*Secale cereale*) Triticeae cereals (Wu et al. 2003; Larson et al. 2006). Moreover, genome specific markers have been used to distinguish several homoeologous linkage groups corresponding to the Ns (*Psathytrostachys*) and Xm genomes of *Leymus* (Wu et al. 2003). The Ns genome originates from *Psathyrostachys* (Dewey 1984; Zhang and Dvořák 1991), whereas the origin of the Xm genome (Wang et al. 1994) is less certain but seems to share significant homology to the E genome of *Lophypryum* and *Thinopryum* (Löve 1984; Sun et al. 1995; Zhang et al. 2006).

Our objective here was to (1) compare plant concentrations of CP, NDF, ADF, Al, B, Ca, Cl, Cu, Fe, K, Mg, Mn, Na, P, S, Si, Zn, and KRAT between *L. cinereus* and *L. triticoides*, and (2) identify and compare QTLs controlling fiber, protein, and mineral concentrations in two full-sib mapping families, TTC1 and TTC2, derived from interspecific hybrids of *L. cinereus* and *L. triticoides*, and (3) identify strategies to improve forage quality of interspecific breeding populations that will overcome limitations of the parental species. Another objective was to compare the location of these *Leymus* QTLs with genomic regions controlling related traits in other cereal and grass species and identify possible opportunities gene discovery research in *Leymus*.

Materials and methods

Plant materials and genetic maps

The pedigree and construction of molecular genetic maps for the TTC1 and TTC2 families were originally described by Wu et al. (2003) and updated with additional markers by Larson et al. (2006). Briefly, the TTC1 and TTC2 families were derived from one *L. triticoides* Acc641 plant (*T*-tester) pollinated by two different *L. triticoides* Acc:641 × *L. cinereus* Acc:636 hybrid plants (TC1 and TC2) under separate pollen exclosures producing two distinct full-sib families (TTC1 and TTC2). The TTC1 and TTC2 families include 164 and 170 full-sib individuals, respectively, which can also be considered half-sibs of the *T*-tester plant between families. The molecular genetic maps for the TTC1 and TTC2 families were constructed using DNA markers that were present in one or both hybrids, absent in the tester, and segregating with an expected ratio of 1:1 among the full-sib progeny (Wu et al. 2003). The TTC1 map includes 1069 AFLP markers and 53 anchor loci in 14 linkage groups spanning 2001 cM. The TTC2 map contained 1002 AFLP markers and 45 anchor loci in

14 linkage groups spanning 2066 cM. Some 488 homologous AFLP loci (i.e. AFLPs of the same size) and 31 anchor markers have been mapped in both families, showing similar map order. Thus, 1583 AFLP markers and 67 different anchor loci have been mapped into 14 linkage groups, which evidently correspond to the 14 chromosome pairs of allotetraploid *Leymus*.

Forage quality evaluations

Ramets from each of the two mapping families (TTC1 and TTC2) were space planted in a randomized complete block (RCB) design with two replicates (blocks) per family at the Utah Agriculture Experiment Station Richmond Farm (Cache Co., UT). Each block contained 164 TTC1 or 170 TTC2 clones plus several parental genotypes (i.e. TC1, TC2 and *T*-tester clones) and single-plant representatives of the heterogeneous *L. cinereus* Acc:636 and *L. triticoides* Acc:641 source accessions. Individual ramets were transplanted from soil containers (4-cm diameter) in the spring of 2001 to field plots with 2-m row spacing and 2-m spacing within rows (2-m centers).

Forage samples were harvested using a hand-held sickle knife, into perforated paper bags, and subsequently transferred to forced-air ovens (60°C) on May 28, 2003 and May 5, 2004. Dried forage samples were ground to pass a 1 mm screen in a Cyclotec 1093 abrasion sample mill (FOSS Tecator, Hoganas, Sweden). For elemental analyses, the milled samples were dry washed at 640°C, dissolved with nitric acid, diluted with water, and analyzed using a Model 4300-DV inductively-coupled plasma optical emission spectrometer (ICP-OES) (PerkinElmer Life and Analytical Sciences, Inc., Wellesley, MA). The molar ion charge ratio of K–Ca and Mg (KRAT) was calculated as (%K)(0.0257)/[(%Ca)(0.0499) + (%Mg)(0.0823)]. Fiber (ADF and NDF) and crude protein (CP) concentraions were estimated by scanning milled samples using a NIRS Model 6500 (Pacific Sci. Instruments, Silver Spring, MD). Estimates of ADF, NDF, and CP concentrations based on NIRS scans were calibrated using NIRSystems software with ISI Forage Equation IS-0122FS (Infrasoft Int. LLC, Port Matilda, PA) based on tall fescue samples as described by Asay et al. (2002). Validation of this NIR-based equation was performed using laboratory assays for CP (139 assays), ADF (138 assays), and NDF (63 assays) using *Leymus* samples from the 2003 and 2004 forage harvests. The LECO CHN-2000 Series Elemental Analyzer (LECO Corp., St. Joseph, MI) was used as a laboratory assay of N (%), which was then multiplied by 6.25 to estimate CP (%). The R^2 values for CP validation were 0.91 in 2003, 0.90 in 2004, and 0.98 overall. The ANKOM-200 Fiber Analyzer (ANKOM Technol. Corp., Fairport, NY) was used for laboratory assays of NDF and ADF concentration according to manufacturer protocols. The R^2 values for ADF validation were 0.85 in 2003, 0.66 in 2004, and 0.83 overall. The R^2 values for NDF validation were 0.74 in 2003, 0.77 in 2004, and 0.92 overall.

Data analysis

Broad-sense heritabilities were determined using SAS code (Statistical Analysis Systems Institue Inc., Cary, N.C.) for estimating heritability from lines evaluated in RCB designs in multiple environments (Holland et al. 2003), modified to account for repeated measurements on perennial plants over years substituted for environments. All class variables (i.e. rep, entry, and year) were treated as random effects. Genotypic and phenotypic correlations were determined using SAS code for estimating correlations from RCB designs in multiple environments (Holland et al. 2003), also modified to account for repeated measurements over years substituted for environments. The basic SAS code for estimating heritabilities, genotypic correlations, and phenotypic correlations is available at http://www4.ncsu.edu/~jholland/heritability.html (verified 19 June, 2006).

QTL analyses were based on trait averages over the two reps and two years determined by the LSMEANS procedure of SAS. A sequential and reiterative procedure of QTL detection was performed using the MAPQTL 5 package (Van Ooijen 2004). Genome-wide interval mapping (IM) (Lander and Botstein 1989; Van Ooijen 1992) was performed in 1-cM increments to identify putative QTLs and possible cofactors used in a multiple-QTL model (MQM) (Jansen 1993, 1994; Jansen and Stam 1994). A log-likelihood ratio (LOD) threshold of 3.3 was used to identify MQM cofactors (also referred to as primary QTLs below), whereas a LOD threshold of 2.3 was used to identify other possible

secondary QTLs in the final MQM scan. The LOD threshold of 3.3 is a close approximation of a genome-wide 5% significance based on simulation tables (Van Ooijen 1999) and permutation analyses (Churchill and Doerge 1994), which seems appropriate for selecting cofactors used in genome-wide MQM scans. The more relaxed LOD threshold of 2.3 corresponds to the chromosome-wide 5% significance level as determined from permutation analyses of each linkage group (Churchill and Doerge 1994), which seemed reasonable for identification of other possible QTLs not used as cofactors in the genome-wide MQM scans. A backward elimination procedure was applied to this initial set of cofactors using a conservative significance level of 0.001 to ensure the independence of each cofactor. A reiterative process of using any new QTLs detected using MQM scans as additional cofactors was used until no additional primary QTLs (LOD $\geq$ 3.3) were found. Where possible, all 1583 AFLP markers and 67 different anchor loci were used for QTL analyses of the TTC1 and/or TTC2 families. However, for simplicity and comparative purposes only the 488 homologous AFLP markers mapped in both TTC1 and TTC2 families and 67 anchor markers are shown in the QTL graphs (Fig. 1), which were generated by MapChart version 2.1 (Voorrips 2002).

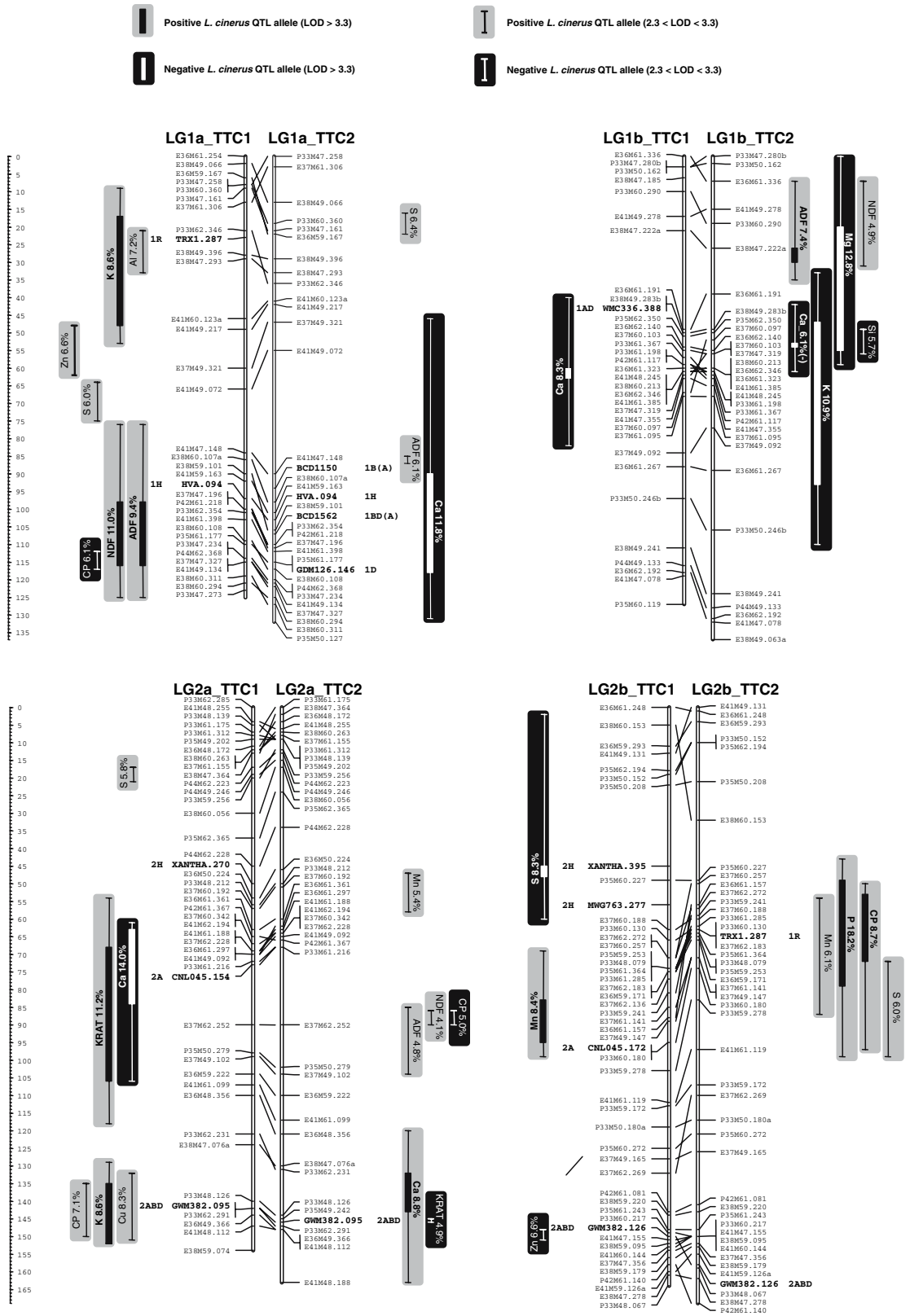
Results and discussion

Compared with the *L. triticoides* Acc:641 source accession, *L. cinereus* Acc:636 source accession showed significantly greater ADF, Cu, K, and KRAT contents and significantly lower Ca, Fe, Mg, Mn, S, and Zn contents (Table 1). Thus, significant divergence among the heterogeneous source accessions (original source of TC1 hybrid, TC2 hybrid, and *T*-tester parental clones) was apparent for these nine traits. Elevated K, depressed Ca, and depressed Mg concentrations all contributed to highly elevated KRAT values in *L. cinereus* and relatively high KRAT divergence between source accessions of the interspecific TC1 and TC2 hybrid clones (Table 1). However, the lack of divergence in other traits does not preclude genetic variation within the heterogeneous source accessions, but this could not be directly tested because the Acc:636 and Acc:641 reference plants were not clonally replicated. It should also be noted that the *L. triticoides T*-tester genotype showed significantly less Al, Fe, and NDF content and significantly greater K, Mg, Mn, P, S, and Si content compared to the *L. triticoides* Acc:641 accession (Table 1). The *T*-tester was a rogue genotype originating from spreading rhizomes or seed of open pollinated plants of several possible *L. triticoides* accessions, including Acc:641, which may account for its phenotypic differences from the Acc:641 accession.

Fig. 1 Comparison of forage quality QTLs detected in the full-sib *Leymus triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 families, exceeding 2.3 LOD (lines) and 3.3 LOD (boxes) significance levels as indicated in the legend. The updated molecular genetic linkage maps include 488 homologous AFLP markers as mapped in both TTC1 and TTC2 families and 67 anchor markers (larger bold marker text) mapped in TTC1 and/or TTC2 families (Wu et al. 2003; Larson et al. 2006). Annotation next to each anchor marker indicate homoeologous groups of barley (H), wheat (ABD), cereal rye (R), and in parentheses oat chromosome designations

Compared with the *L. triticoides T*-tester, the interspecific TC1 and TC2 hybrids both showed significantly greater ADF and KRAT contents and significantly lower Ca, S, and Zn concentrations (Table 1). Compared to the *T*-tester, the TC1 hybrid also showed significantly greater Mn content and significantly less Mg and P contents. The TC2 hybrid also showed significantly less CP, B, K, Mn, Na and Si content and significantly greater NDF and KRAT content compared to the *T*-tester genotype (Table 1). However, traits that do not show significant differences between the TC1, TC2, and *T*-tester parental genotypes may still show significant genetic variation within the TTC1 and TTC2 progeny resulting from transgressive segregation.

Significant heritabilities and QTLs were observed in the clonally replicated TTC1 and/or TTC2 populations for all traits (Table 2). Significant heritability but no significant QTLs were detected for Al and B content in the TTC2 family or Na and Si content in the TTC1 family (Table 2). Likewise, we observed relatively strong heritability with relatively weak QTL effects for B and Na content in the TTC1 family and for K content in the TTC2 family (Table 2). However, our analysis could only detect QTLs that were heterozygous in the TC1 and/or TC2 hybrids. Because of the way the maps were constructed


Positive L. cinerus QTL allele (LOD > 3.3)
Negative L. cinerus QTL allele (LOD > 3.3)
Positive L. cinerus QTL allele (2.3 < LOD < 3.3)
Negative L. cinerus QTL allele (2.3 < LOD < 3.3)
LG1a_TTC1 LG1a_TTC2
LG1b_TTC1 LG1b_TTC2
LG2a_TTC1 LG2a_TTC2
LG2b_TTC1 LG2b_TTC2
1R TRX1.287
1H HVA.094
2H XANTHA.270
2A CNL045.154
2ABD GWM382.095
1AD WMC336.388
1B(A) BCD1150
1H HVA.094
1BD(A) BCD1562
1D GDM126.146
2H XANTHA.395
2H MWG763.277
2A CNL045.172
2ABD GWM382.126
K 8.6%
Al 7.2%
Zn 6.8%
S 6.0%
NDF 11.0%
ADF 9.4%
CP 6.1%
S 6.4%
Ca 11.8%
ADF 6.1%
Ca 8.3%
ADF 7.4%
Mg 12.8%
NDF 4.9%
Ca 6.1%(-)
S 5.7%
K 10.9%
S 5.8%
KRAT 11.2%
Ca 14.0%
CP 7.1%
K 8.6%
Cu 8.3%
Mn 5.4%
CP 5.0%
NDF 4.1%
ADF 4.8%
Ca 8.8%
KRAT 4.9%
S 8.9%
Mn 8.4%
Zn 6.6%
Mn 6.1%
P 18.2%
CP 8.7%
S 6.0%

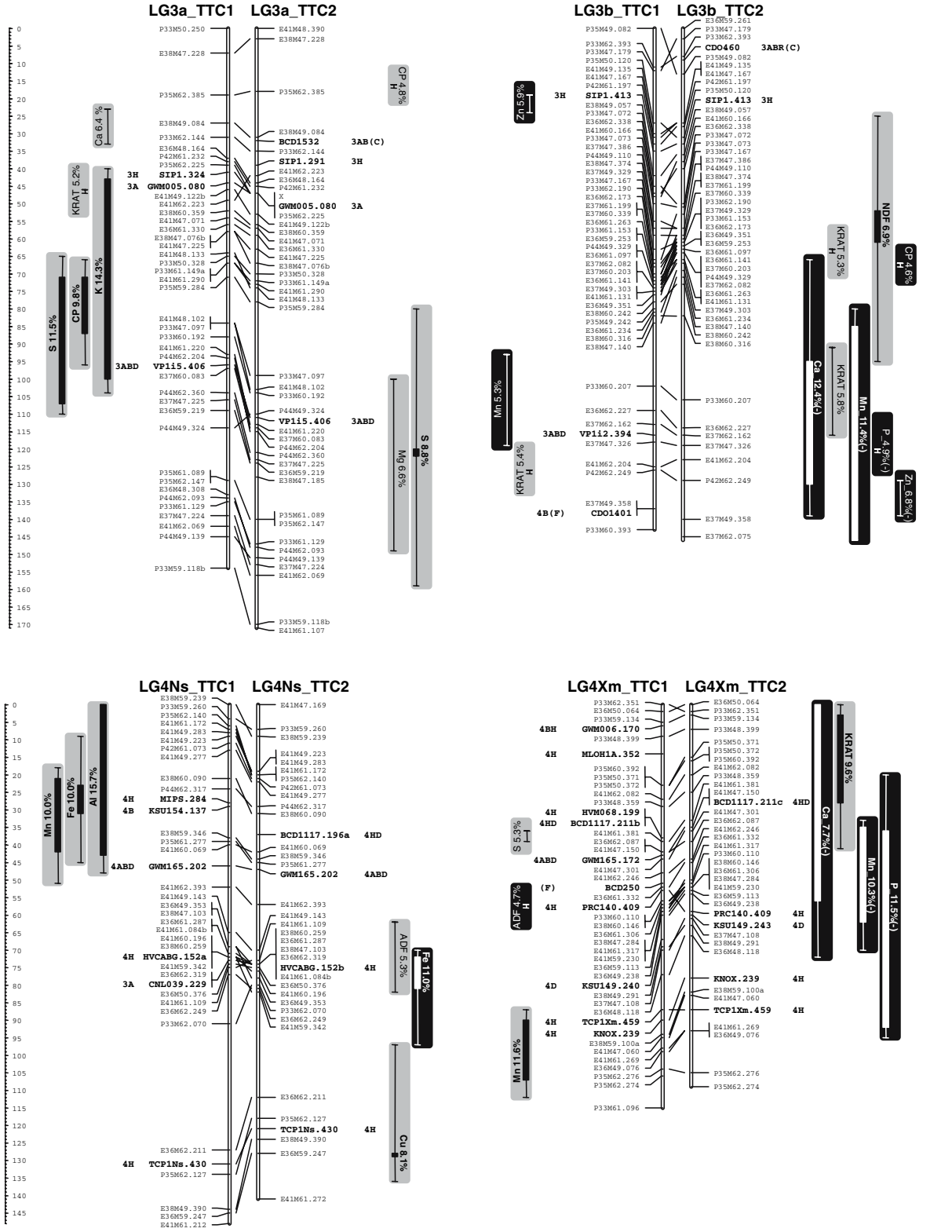


Fig. 1 continued

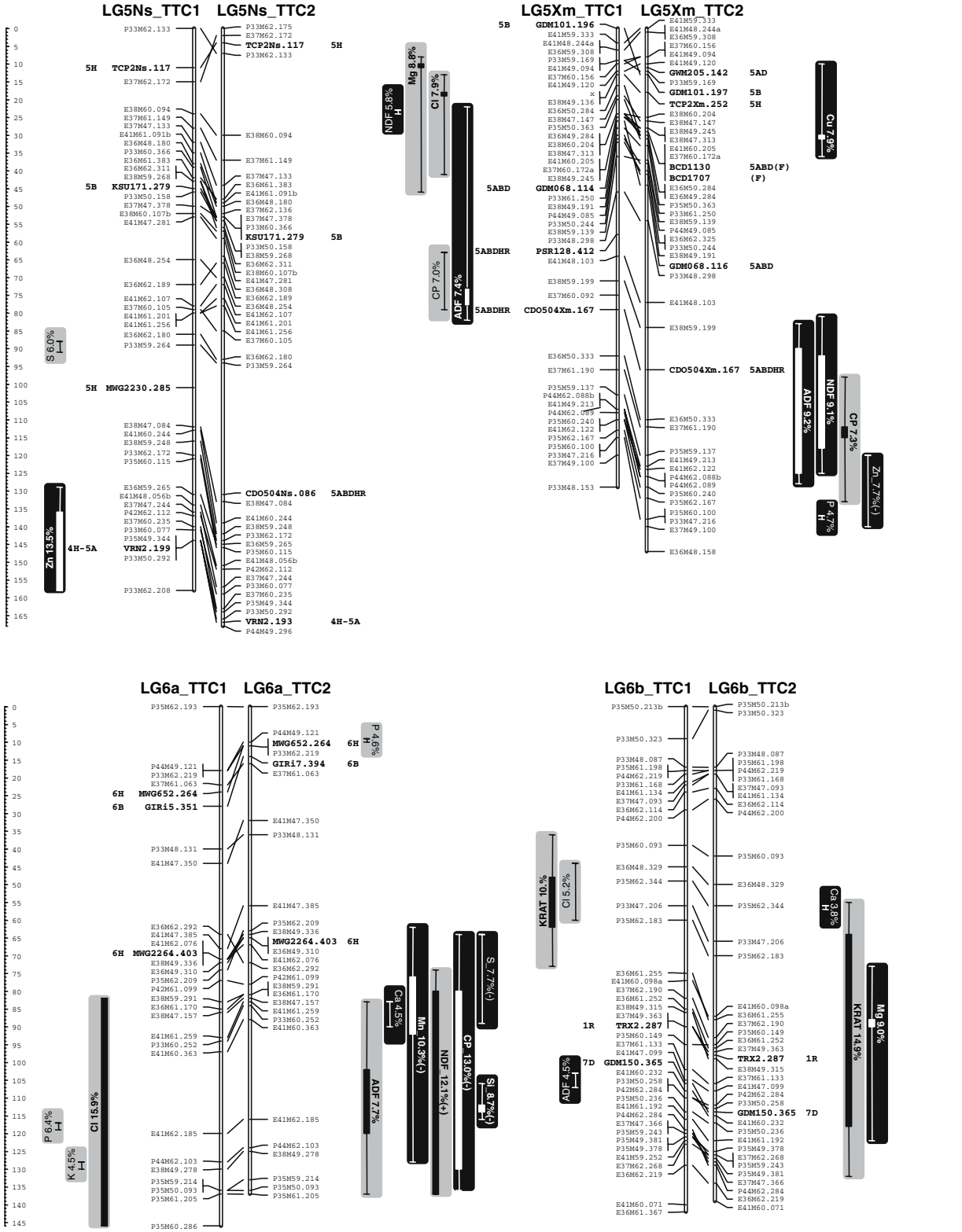


Fig. 1 continued

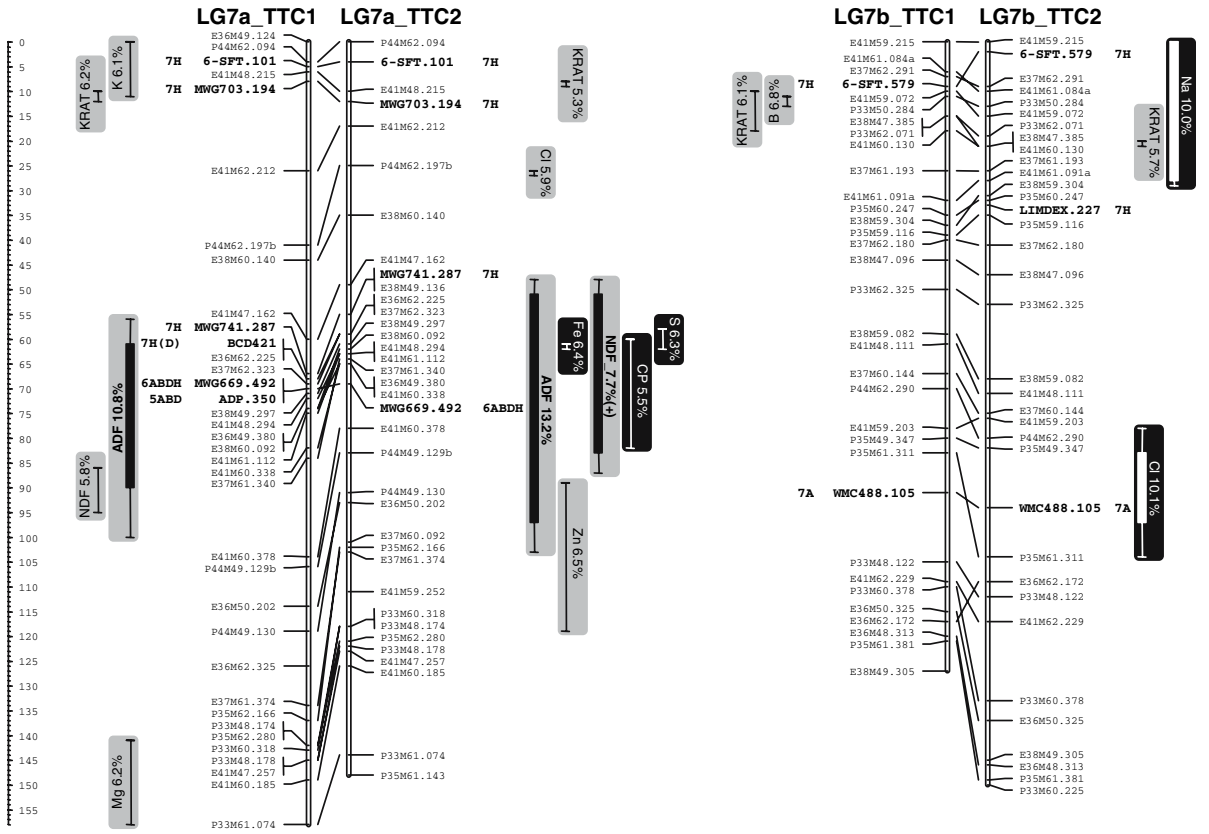


Fig. 1 continued

(Wu et al. 2003) we did not expect to detect variation caused by heterozygosity in the *T*-tester, which may explain why some traits showed significant genetic variation with weak or insignificant QTL effects. Conversely, two Fe content QTLs and one Na content QTL detected in TTC2 should be viewed skeptically since the corresponding heritabilities were not significant (Table 2). It is possible that experimental noise or measurement error in one rep may have diminished heritabilities, whereas significant QTLs were detected on the basis of significant gene effects in the other rep. In any case, most of the QTLs were supported by significant heritabilities (Table 2).

We detected 83 and 89 significant genotypic correlations among all 153 pair-wise comparisons of the 18 traits evaluated in the TTC1 and TTC2 families, respectively (Table 3). A total of 58 pair-wise comparisons showed significant genotypic trait correlation in both TTC1 and TTC2 families, including 46 comparisons that had the same sign (positive or negative) in both families. Thus, there was considerable overall correspondence of genetic variation between the TTC1 and TTC2 families, which could be attributed to correspondence of QTLs of correlated traits in one or both families (Fig. 1).

We detected 23 and 33 primary QTLs (LOD $\geq$ 3.3) used as cofactors (Table 4) for the final MQM scans of the TTC1 and TTC2 families, respectively. The final MQM scans detected 28 and 32 secondary QTLs (2.3 $\leq$ LOD $\leq$ 3.3) in the TTC1 and TTC2 families, respectively. Overall, 51 and 65 QTLs were detected in the TTC1 and TTC2 families, respectively (Fig. 1). Moreover, seemingly homologous TTC1 and TTC2 QTLs (i.e. QTLs that are overlapping by comparison of homologous markers) were evident on LG1a (ADF), LG1b (Ca), LG2b (Mn), LG3a (S), LG3b (Mn and KRAT), LG4Xm (Mn), LG5Xm (ADF and CP), LG6b (KRAT), LG7a (KRAT, ADF, and NDF), and LG7b (KRAT) (Fig. 1). Although another 88 QTLS were unique to one family or the other, many of these were

Table 1 Trait means ± SD for *Leymus cinereus* Acc:636, *L. triticoides* Acc:641, *L. triticoides* T parental genotype, interspecific TC1 and TC2 parental hybrids, and full-sib *L. triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 mapping families

Trait	Acc:636 (g = 18)[a]	Acc:641 (g = 15)[a]	*T*-tester (g = 13)[b]	TC1 (r = 14)[b]	TC2 (r = 14)[b]	TTC1 (c = 164)[c]	TTC2 (c = 168)[c]
CP (%)	19.9 ± 2.1	19.8 ± 1.7	20.7 ± 1.2	19.9 ± 1.0	20.8 ± 1.1	21.2 ± 1.4	21.8 ± 1.5
ADF (%)	25.4 ± 1.8	24.1 ± 1.2	24.7 ± 1.2	26.4 ± 1.1	25.8 ± 0.6	24.4 ± 1.2	24.1 ± 1.4
NDF (%)	51.3 ± 1.9	51.3 ± 1.2	50.2 ± 1.6	51.2 ± 1.3	51.7 ± 1.0	49.7 ± 1.5	50.0 ± 1.5
Al (ppm)	90 ± 24	113 ± 55	77 ± 18	81 ± 21	74 ± 12	101 ± 25	80 ± 17
B (ppm)	8.5 ± 3.6	7.7 ± 3.7	9.8 ± 2.0	9.6 ± 3.6	6.8 ± 2.4	8.5 ± 3.2	9.4 ± 2.6
Ca (%)	0.235 ± 0.040	0.332 ± 0.076	0.340 ± 0.044	0.298 ± 0.022	0.267 ± 0.035	0.354 ± 0.050	0.347 ± 0.043
Cl (%)	0.75 ± 0.17	0.49 ± 0.12	0.43 ± 0.07	0.72 ± 0.17	0.44 ± 0.05	0.70 ± 0.14	0.54 ± 0.10
Cu (ppm)	22.0 ± 2.4	15.2 ± 2.8	16.7 ± 3.1	18.1 ± 2.2	18.4 ± 3.2	18.1 ± 2.5	19.7 ± 3.2
Fe (ppm)	64 ± 15	92 ± 41	68 ± 10	62 ± 16	60 ± 7	84 ± 20	72 ± 13
K (%)	3.74 ± 0.38	3.14 ± 0.37	3.53 ± 0.11	3.49 ± 0.24	3.44 ± 0.13	3.72 ± 0.28	3.65 ± 0.24
Mg (%)	0.160 ± 0.014	0.181 ± 0.025	0.215 ± 0.020	0.206 ± 0.029	0.212 ± 0.017	0.192 ± 0.024	0.198 ± 0.022
Mn (ppm)	28.4 ± 5.6	36.5 ± 8.0	45.6 ± 2.7	51.7 ± 8.5	41.1 ± 4.7	40.5 ± 6.3	40.1 ± 6.8
Na (ppm)	475 ± 189	477 ± 171	495 ± 162	532 ± 188	379 ± 118	484 ± 126	460 ± 103
P (%)	0.250 ± 0.024	0.239 ± 0.030	0.262 ± 0.012	0.240 ± 0.012	0.259 ± 0.012	0.247 ± 0.023	0.259 ± 0.022
S (%)	0.139 ± 0.016	0.181 ± 0.028	0.202 ± 0.011	0.163 ± 0.009	0.171 ± 0.009	0.194 ± 0.021	0.186 ± 0.016
Si (%)	0.054 ± 0.017	0.055 ± 0.014	0.068 ± 0.015	0.057 ± 0.014	0.049 ± 0.010	0.063 ± 0.009	0.051 ± 0.008
Zn (%)	13.4 ± 2.5	18.1 ± 3.2	17.5 ± 1.6	14.1 ± 1.8	15.4 ± 1.6	16.8 ± 1.8	17.5 ± 2.7
KRAT	3.89 ± 0.38	2.63 ± 0.45	2.66 ± 0.18	2.84 ± 0.29	2.91 ± 0.23	2.92 ± 0.38	2.85 ± 0.30

[a] Sample size based on measurements number of different genotypes (g) without clonal replication

[b] Sample size based on number of different ramets (r) for each parental genotype

[c] Sample size based on means of two ramets from a number of different clones (c)

coincident (clustered) to specific regions of the TTC1 or TTC2 linkage maps (Fig. 1).

Relatively strong positive genotypic correlations were observed between ADF and NDF concentrations (Table 3) and coincident ADF and NDF QTLs were detected on LG1a, LG1b, LG2a, LG5Xm, LG6a, and LG7a (Fig. 1). This correlation is expected and observed because ADF (cellulose and hemicellulose) is a sub-fraction of NDF (cellulose, lignin, and hemicellulose). However, unique ADF or NDF content QTLs were detected on LG3b, LG4Ns, LG4Xm, and LG6b (Fig. 1), which might be attributable to effects related to the ratio of hemicellulose to cellulose and lignin since ADF essentially equals NDF plus hemicelluose. Seemingly homologous TTC1 and TTC2 ADF and/or NDF content QTLs were detected on LG1a, LG5Xm, and LG7a (Fig. 1) but the majority of ADF and NDF QTLs were unique to one family. The coincidence of relatively large ADF and NDF content QTL effects in the centromere region of LG7a (Fig. 1), in both families, was particularly interesting because NDF, in vivo dry-matter digestibility, and a cluster of lignin biosynthesis genes also co-localized in the centromeric region of LG7 in perennial ryegrass (Cogan et al. 2005). *Leymus* LG7 is predicted be the conserved syntenic counterpart to perennial ryegrass LG7, which share the telomeric 6-SFT locus (Wei et al. 2000; Lidgett et al. 2002), and other syntenic cross-species markers (Jones et al. 2002; Wu et al. 2003). But additional cross-species reference markers including genetic map assignment of the lignin biosynthesis gene ortholoci in *Leymus* would certainly help investigate this putative correspondence. Both ADF and NDF content also showed negative genotypic correlations with CP, K, S, and Zn concentrations in both families (Table 3), which can also be attributed to coincident QTLs in one or both families.

Relatively strong positive correlations were detected between KRAT and K content, in both TTC1 and TTC2 families (Table 3). Conversely, relatively

Table 2 Number of QTLs detected using a restricted multiple QTL model, % variation explained by QTLs, and broad-sense heritabilities (H) in the full-sib *Leymus triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 families

Trait	Number of TTC1 QTLs (variation explained)	TTC1 H^2 ± SE	Number of TTC2 QTLs (variation explained)	TTC2 H^2 ± SE
CP	4 (29.3%)	0.390 ± 11	7 (41.4%)	0.61 ± 0.07
ADF	5 (39.6%)	0.59 ± 0.08	7 (45.0%)	0.67 ± 0.06
NDF	3 (21.6%)	0.57 ± 0.06	6 (44.4%)	0.52 ± 0.09
Al	2 (22.9%)	0.47 ± 0.11	0.(0.0%)	0.35 ± 0.09
B	1 (6.8%)	0.57 ± 0.07	0.(0.0%)	0.32 ± 0.08
Ca	3 (28.4%)	0.78 ± 0.04	7 (50.5%)	0.53 ± 0.09
Cl	3 (30.8%)	0.44 ± 0.09	2 (14.4%)	0.43 ± 0.09
Cu	1 (8.3%)	0.32 ± 0.06	2 (16.4%)	0.55 ± 0.05
Fe	1 (10.0%)	0.42 ± 0.08	2 (17.3%)	0.24 ± 0.16
K	5 (38.9%)	0.57 ± 0.08	1 (10.9%)	0.55 ± 0.08
Mg	2 (15.0%)	0.67 ± 0.06	3 (25.4%)	0.64 ± 0.06
Mn	4 (35.0%)	0.55 ± 0.09	5 (38.0%)	0.62 ± 0.06
Na	0 (0.0%)	0.59 ± 0.07	1 (10.0%)	0.06 ± 0.25
P	1 (6.4%)	0.50 ± 0.10	5 (37.3%)	0.65 ± 0.07
S	6 (40.6%)	0.76 ± 0.05	5 (27.9%)	0.62 ± 0.06
Si	0 (0.0%)	0.22 ± 0.08	2 (14.4%)	0.30 ± 0.08
Zn	4 (28.1%)	0.56 ± 0.08	3 (19.5%)	0.52 ± 0.09
KRAT	6 (36.5%)	0.76 ± 0.04	7 (40.8%)	0.43 ± 0.10

strong negative correlations were detected between KRAT and Ca content and between KRAT and Mg content, in both TTC1 and TTC2 families (Table 3). These correlations are not surprising since KRAT is a simple ratio of K–Ca and Mg concentrations. Thus, variation in K, Ca, and Mg content should also affect KRAT. Otherwise, K, Ca, and Mg concentrations were largely independent of each other and KRAT did not show genotypic correlations with any other traits. There were a total of six and seven KRAT QTLs, five and one K content QTLs, three and seven Ca content QTLs, and two and three Mg content QTLs detected in the TTC1 and TTC2 families, respectively (Table 2). As expected from differences between parental species (Table 1), *L. cinereus* contributed all but one (TTC2 LG2a) of the positive KRAT QTLs (positive in the sense of elevated, but undesirable effect) (Fig. 1). Seemingly homologous TTC1 and TTC2 QTLs were detected for KRAT on LG3b, LG6b, LG7a, and LG7b and for Ca content on LG1b (Fig. 1). Coincident KRAT and Ca content QTLs on LG2a may also show homology between the TTC1 and TTC2 families, but they show different of effect and somewhat misaligned locations compared across these families (Fig. 1). A number of K, Ca, and Mg content QTLs had no significant QTL effect on KRAT, which suggest that these effects were counter balanced by other components of KRAT. Counter balancing effects on KRAT components were apparently significant on LG1b where increased K content effects associated with the *L. cinereus* allele were also associate with increased Ca and Mg concentrations (Fig. 1). Presumably, this also explains why the homologous Ca content QTL on TTC1 LG1b did not have significant effects on KRAT, even though no significant Mg and K content QTLs were detected on TTC1 LG1b (Fig. 1). Conversely, some KRAT QTLs (TTC1 LG3b, TTC1 LG6b, TTC2 LG7a, TTC1 LG7b, and TTC2 LG7b) evidently have significant effects on the ratio of K–Ca and Mg concentrations but had no major (significant) affect on these mineral concentrations per se (Fig. 1). Interestingly, homologous TTC1 and TTC2 KRAT QTLs on LG7a and LG7b are all located near 6-SFT marker loci and generally seem to involve the KRAT more than K, Ca, and Mg content per se (Fig. 1). Thus, LG7a and LG7b KRAT QTLs may be homoeologous. In any case, compared to other traits, KRAT showed substantially more evidence of homology between the TTC1 and TTC2 families. Taken together, these

Table 3 Genotypic correlations in the full-sib *L. triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 (below diagonal) and TTC2 (above diagonal) mapping families

	CP	ADF	NDF	Al	B	Ca	Cl	Cu	Fe	K	Mg	Mn	Na	P	S	Si	Zn	KRAT
CP		-0.81^{++}	-0.82^{++}	-0.48^{+}	0.50^{++}	NS	NS	0.26^{+}	0.44^{++}	0.50^{++}	0.37^{++}	0.37^{++}	0.65^{++}	0.58^{++}	0.69^{++}	0.90^{++}	0.50^{++}	NS
ADF	-0.73^{++}		0.82^{++}	NS	-0.35^{++}	NS	-0.23^{+}	NS	-0.57^{++}	-0.53^{++}	-0.24^{++}	NS	-0.43^{+}	-0.38^{++}	-0.62^{++}	-0.98^{++}	-0.35^{++}	NS
NDF	-0.71^{++}	0.71^{++}		NS	-0.36^{+}	-0.28^{+}	NS	NS	-0.27^{+}	-0.49^{++}	-0.41^{++}	$-0.30+$	$-0.55+$	-0.47^{++}	-0.61^{++}	-1.04^{++}	-0.46^{++}	NS
Al	-0.32^{+}	NS	0.38^{++}		NS	NS	NS	NS	NS	NS	NS	-0.79^{+}	NS	-0.88^{+}	NS	0.61^{+}	-0.86^{+}	NS
B	-0.20^{+}	NS	0.19^{+}	NS		NS	NS	0.59^{++}	NS	0.21^{+}	NS	-0.19^{+}	0.75^{++}	NS	0.15^{+}	0.74^{++}	NS	0.22^{+}
Ca	NS	-0.22^{+}	-0.41^{++}	0.19^{+}	-0.29^{++}		NS	NS	NS	0.15^{+}	NS	0.17^{+}	0.42^{+}	NS	NS	0.70^{++}	0.42^{++}	-0.53^{++}
Cl	0.26^{+}	NS	-0.23^{+}	NS	NS	NS		NS	1^{+}	0.66^{++}	0.20^{+}	-0.40^{++}	NS	NS	0.44^{++}	0.63^{+}	NS	
Cu	0.32^{+}	NS	-0.23^{+}	NS	-0.26^{+}	0.16^{+}	0.73^{++}		-0.32^{+}	0.42^{++}	NS	0.14^{+}	0.59^{+}	0.34^{++}	0.17^{+}	0.32^{+}	NS	0.31^{+}
Fe	-0.43^{+}	NS	0.41^{+}	0.99^{++}	NS	NS	NS	-0.40^{+}		NS	NS	NS	0.86^{+}	NS	NS	0.65^{+}	NS	NS
K	0.63^{++}	-0.46^{++}	-0.37^{++}	NS	NS	-0.13^{+}	0.58^{++}	0.50^{++}	NS		NS	-0.15^{+}	NS	0.18^{+}	0.49^{++}	0.61^{++}	0.27^{++}	0.49^{++}
Mg	NS	NS	NS	NS	NS	NS	0.58^{++}	NS	NS	-0.19^{++}		NS	0.25^{+}	0.44^{++}	0.30^{+}	0.34^{+}	NS	-0.56^{++}
Mn	NS	NS	NS	NS	NS	NS	-0.44^{+}	0.46	NS	NS	0.15^{+}		0.30^{+}	0.46^{++}	0.26^{++}	NS	0.29^{++}	-0.25^{++}
Na	NS	NS	NS	NS	0.84^{++}	-0.27^{++}	NS	NS	NS	NS	NS	0.17^{+}		NS	NS	0.84^{++}	NS	NS
P	0.52^{++}	NS	-0.25^{+}	-0.32^{+}	-0.29^{+}	-0.23^{+}	NS	0.58^{++}	-0.42^{+}	0.68^{++}	NS	NS	NS		0.45^{++}	NS	0.41^{++}	NS
S	0.76^{++}	-0.51^{++}	-0.46^{++}	NS	-0.35^{++}	0.11^{+}	0.22^{+}	0.43^{++}	NS	0.51^{++}	0.13^{+}	NS	-0.32^{++}	0.61^{++}		0.33^{+}	0.38^{++}	NS
Si	-0.28^{+}	NS	0.18^{+}	0.43^{++}	0.89^{++}	-0.20^{++}	0.80^{+}	NS	0.41^{+}	0.31^{++}	NS	NS	0.98^{++}	-0.25^{+}	-0.35^{++}		NS	NS
Zn	0.25^{+}	-0.16^{+}	-0.23^{+}	0.28^{+}	-0.30^{++}	0.39^{++}	NS	0.48^{++}	NS	0.46^{++}	-0.30^{++}	NS	NS	0.49^{++}	0.43^{++}	NS		NS
KRAT	0.21^{+}	NS	NS	NS	NS	-0.66^{++}	NS	NS	NS	0.64^{++}	-0.61^{++}	NS	NS	0.50^{++}	0.14^{+}	0.26^{+}	0.16^{+}	

$^{+,\ ++}$ Exceed one and two times its standard error, respectively

Table 4 Description of primary QTLs (LOD $\geq$ 3.3) used as cofactors for restricted multiple QTL model (rMQM) scans of the *L. triticoides* × (*L. triticoides* × *L. cinereus*) TTC1 and TTC2 mapping families

Trait	Family-linkage group	Position	LOD	% Explained	Additive effect of *L. cinerus* QTL allele
ADF	TTC1-LG1a	103	4.5	9.4	0.7
ADF	TTC1-LG5Xm	75	3.6	7.4	−0.9
ADF	TTC1-LG7a	77	5.1	10.8	0.8
ADF	TTC2-LG1b	29	4.0	8.2	0.8
ADF	TTC2-LG5Xm	112	4.8	9.2	−0.8
ADF	TTC2-LG6a	113	4.0	7.7	0.8
ADF	TTC2-LG7a	61	6.6	13.2	1.0
AL	TTC1-LG4Ns	24	6.1	15.7	18
Ca	TTC1-LG1b	65	3.6	8.3	−0.029
Ca	TTC1-LG2a	72	5.9	14	−0.037
Ca	TTC2-LG1a	92	6.8	11.8	−0.029
Ca	TTC2-LG1b	54	3.7	6.1	−0.021
Ca	TTC2-LG2a	140	5.2	8.8	0.027
Ca	TTC2-LG3b	106	7.1	12.4	−0.030
Ca	TTC2-LG4Xm	7	4.6	7.7	−0.024
Cl	TTC1-LG5Xm	19	3.6	7.9	0.08
Cl	TTC1-LG6a	119	6.9	15.9	0.11
Cl	TTC2-LG7b	90	3.5	10.1	−0.08
CP	TTC1-LG3a	77	3.7	9.8	0.9
CP	TTC2-LG2b	66	4.0	8.7	1.0
CP	TTC2-LG5Xm	112	3.4	7.3	0.8
CP	TTC2-LG6a	115	5.5	13	−1.1
Cu	TTC2-LG4Ns	128	3.4	8.1	1.5
Cu	TTC2-LG5Xm	30	3.3	7.9	−1.5
Fe	TTC1-LG4Ns	24	3.8	10	11
Fe	TTC2-LG4Ns	80	4.2	11	-6
K	TTC1-LG1a	37	4.1	8.6	0.16
K	TTC1-LG2a	142	4.1	8.6	0.17
K	TTC1-LG3a	75	6.5	14.3	0.21
K	TTC2-LG1b	67	4.2	10.9	−0.15
KRAT	TTC1-LG2a	104	4.7	11.2	0.25
KRAT	TTC1-LG6b	59	4.6	10.9	0.26
KRAT	TTC2-LG4Xm	6	4.3	9.6	0.19
KRAT	TTC2-LG6b	73	6.1	14.9	0.23
Mg	TTC1-LG5Xm	10	3.3	8.8	0.015
Mg	TTC2-LG1b	34	5.0	12.8	−0.016
Mg	TTC2-LG6b	89	3.5	9	−0.013
Mn	TTC1-LG2b	88	4.0	8.4	3.7
Mn	TTC1-LG4Ns	38	4.7	10	4.0
Mn	TTC1-LG4Xm	104	5.4	11.6	4.3
Mn	TTC2-LG3b	106	5.3	11.4	−4.4
Mn	TTC2-LG4Xm	56	4.5	10.3	−4.1
Mn	TTC2-LG6a	84	4.6	10.3	−4.1

Table 4 continued

Trait	Family-linkage group	Position	LOD	% Explained	Additive effect of *L. cinerus* QTL allele
Na	TTC2-LG7b	0	3.9	10	−52
NDF	TTC1-LG1a	100	4.1	11	1.0
NDF	TTC2-LG3b	59	3.8	6.9	0.8
NDF	TTC2-LG5Xm	112	4.9	9.1	−0.9
NDF	TTC2-LG6a	110	5.6	12.1	1.0
NDF	TTC2-LG7a	61	4.2	7.7	0.8
P	TTC2-LG2b	70	8.0	18.2	0.019
P	TTC2-LG4Xm	85	4.8	11.5	−0.014
S	TTC1-LG2b	48	3.5	8.3	−0.012
S	TTC1-LG3a	98	4.7	11.5	0.014
S	TTC2-LG3a	121	3.4	8.8	0.009
Si	TTC2-LG6a	113	3.3	8.7	−0.003
Zn	TTC1-LG5Ns	154	4.4	13.5	−1.3

KRAT, K, Ca, and Mg data reveal excellent opportunities to modify KRAT levels in *Leymus* although some component QTLs may have counter-acting effects.

A major macronutrient, plant K concentration showed positive genotypic correlations with CP, Cl, Cu, P, S, Si, and Zn contents in both families and negative genotypic correlations with NDF and ADF content in both families (Table 3). All pair-wise tests of genotypic correlation among ADF, NDF, CP (an effective measure of N), P, and K concentrations were significant (positive) in both families (Table 3). Likewise, coincident QTL were detected among ADF, NDF, CP, P, and K concentrations in TTC1 and/or TTC2 families (Fig. 1), but no genomic regions had significant effects on all of these traits. Thus, plant concentrations of the three major macronutrients N, P, and K were intercorrelated traits and negatively correlated with ADF and NDF content in both families (Table 3). However, weak or mostly non-significant correlations were detected among plant concentrations of the Na, Ca, K, Mg, P, and S micronutrients.

The coincidence QTLs controlling plant Al, Fe, and Mn concentrations on TTC1 LG4Ns was intriguing because these QTL peaks evidently correspond with the BCD117 locus mapped in the TTC2 family (Fig. 1). The BCD1117 marker is closely associated with a major Al tolerance gene of barley (Tang et al. 2000) and other divergent Poaceae (Magalhaes et al. 2004). The mechanism of this Al tolerance gene appears to be related to citrate secretion by roots (Zhao et al. 2003; Ma et al. 2004), which affects mobility and uptake of soil cations including Al, Fe, and Mn. There is an interesting disparity in our comparison of *Leymus*, wheat, and barley Al tolerance loci in that, like BCD117, the HVCABG marker is also closely linked to the Alt gene in barley (Raman et al. 2002), but these markers are 37 cM apart in *Leymus* (Fig. 1). In any case, the BCD1117 and HVCABG markers, linked to Al tolerance in other Poaceae species, account for the only primary QTLs (i.e. LOD $\geq$ 3.3) controlling plant Al and Fe content detected in the TTC1 and TTC2 families (Table 4; Fig. 1).

Mineral, protein, and fiber content was largely unaffected by QTLs controlling variation in plant height, rhizome spreading, proportion of bolting culms (i.e. some clones show little or no reproductive bolting), or anthesis date (Larson et al. 2006). Major plant height QTLs were detected on homologous regions of LG2a in both *Leymus* TTC1 and TTC2 families (Larson et al. 2006), but this chromosome region does not seem to have any consistent fiber, protein, or mineral concentrations in either family (Fig. 1). Rhizome spreading QTLs were detected on homologous regions of TTC1 and TTC2 LG3a and homologous regions of TTC1 and TTC1 LG3b (Larson et al. 2006), which approximately correspond with homologous S content QTLs on LG3a and

homologous Mn content QTLs on LG3b (Fig. 1). Likewise, other rhizome spreading QTLs on TTC1 LG6a and TTC2 LG5Xm were coincident with relatively minor mineral content QTLs (Fig. 1), but otherwise had no major affect on mineral, protein, or fiber content. Relatively major QTLs for anthesis date and proportion of bolting culms were detected on corresponding regions of the TTC2 LG4Ns linkage group (Larson et al. 2006), which also coincide with the Cu content QTL on LG4Ns (Fig. 1). Relatively strong bolting and anthesis date QTLs were detected on homologous regions of the TTC1 and TTC2 LG4Xm linkage group (Larson et al. 2006), but this region did not show any consistent affect on fiber, protein, or mineral concentration in both families. Relatively minor bolting QTLs were detected in homologous regions of TTC1 and TTC2 LG6a, but again we did not see corresponding homologies of mineral or fiber content QTLs in both families. Basically, most of the fiber and mineral QTLs were independent of plant height, growth habit, and flowering QTLs detected in the same field evaluations (Larson et al. 2006) and may relate to fundamentally important regulatory steps and processes of mineral uptake, mineral transport, and fiber synthesis. Fundamental components of forage quality that are excellent applications for gene discovery research in grasses. However, the *Leymus* TTC1 and TTC2 families also segregate stem diameter, leaf width, leaf length, leaf angle, and leaf texture and other traits that may affect forage quality. Thus, additional forage quality and agronomic evaluations are needed to fully ascertain the best strategies for improvement. Nevertheless, results so far indicate that it should be possible to select for reduced KRAT, improved forage quality, and other desirable yield and agronomic characteristics in heterotic breeding populations derived from interspecific hybrids of *L. cinereus* and *L. triticoides*. *Leymus* wildryes with improved grazing tolerance, high biomass production, and reduced KRAT would be of great value as a low-input feedstock for the Great Basin and Intermountain regions of western North America and similar ecoregions of the World.

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