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Detection of linkage disequilibrium QTLs controlling low-temperature growth and metabolite accumulations in an admixed breeding population of *Leymus* wildryes

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Summary

Low-temperature soluble carbohydrate accumulations are commonly associated with anthocyanin coloration, attenuated growth, and cold adaptation of cool-season grasses. A total of 647 AFLP markers were tested for associations with anthocyanin coloration, tiller formation, leaf formation, cumulative leaf length, percent soluble carbohydrate, and dry matter regrowth among replicated clones of an admixed *Leymus* wildrye breeding population evaluated in low-temperature growth chambers. The admixed breeding population was derived from a heterogeneous population of *L. cinereus* × *L. triticoides* F_1 hybrids, with two additional generations of open pollination. Two AFLP linkage maps, constructed from two full-sib mapping populations derived from the same F_1 hybrid population, were integrated to produce a framework consensus map used to examine the distribution of marker-trait associations in the admixed F_1OP_2 population. Thirty-seven linkage blocks, spanning 258 cM (13.6%) of the 1895 cM consensus map, contained 119 (50%) of the 237 markers showing at least one possible trait association ($P < 0.05$). Moreover, 28 (68%) of the 41 most significant marker-trait associations ($P < 0.005$) were located in 15 QTL linkage blocks spanning 112.9 cM (6%) of the linkage map. The coincidence of these 28 significant marker-trait associations, and many less significant associations, in 15 relatively small linkage blocks (0.6 cM to 21.3 cM) provides evidence of admixture linkage disequilibrium QTLs (ALD QTLs) in this heterogeneous breeding population. At least four of the remaining 13 putative marker-trait associations ($P < 0.005$) were located in genetic map regions lacking other informative markers. The complexity of marker-trait associations results from heterogeneity within and substantial divergence among the parental accessions.

Abbreviations: ALD – admixture linkage disequilibrium; CHO – carbohydrate

Introduction

Leymus wildryes are cool-season perennial grasses, providing critical habitat and forage for livestock and wildlife throughout cold and/or arid regions of Europe, Asia, and the Americas. The most abundant North American representatives of this genus include *L. triticoides* (creeping or beardless wildrye) and *L. cinereus* (basin wildrye). Aggressive rhizomes and adaptation to poorly drained alkaline sites, primarily within the western United States, characterize *L. triticoides*. Conversely, *L. cinereus* is a tall bunchgrass and

adapted to deep well-drained soils from Saskatchewan to British Columbia, south to California, northern Arizona and New Mexico, and east to South Dakota and Minnesota (Anderson, 2002). Basin wildrye (*L. cinereus*) is the largest cool-season bunch grass native to the western United States. Interspecific hybrids of allotetraploid ($2n = 4x = 28$) *L. cinereus* and *L. triticoides* display regular meiosis (Dewey, 1972), disomic inheritance (Wu et al., 2003), and considerable breeding potential as dryland forages for the semi-arid western regions of the United States. In addition to *L. cinereus*, *L. triticoides* and other North American *Leymus* wildryes, U.S. grass breeders have also evaluated several Old World *Leymus* wildryes and the closely related *Psathyrostachys* wildryes (Jensen et al., 2002; Vogel & Jensen, 2001). Thus, high-density molecular genetic linkage maps have been constructed to facilitate *Leymus* research and improvement (Wu et al., 2003).

Most cool-season grasses reversibly synthesize and store water-soluble fructan and sucrose during the fall and spring (Chatterton et al., 1987; Chatterton et al., 1988; Olien & Clark, 1993). These soluble carbohydrates (CHO) can be rapidly metabolized to facilitate plant growth under fluctuating low temperatures or supply a perfusion of fructose through periplasmic space as a rapid response to freezing (Olien & Clark, 1995). Although fructans may not serve directly as cryoprotectants, fructan accumulation during fall may provide the primary carbon source for synthesis of sucrose during winter (Chatterton et al., 1988; Olien & Clark, 1993; Yukawa et al., 1995). Sucrose accumulates during the coldest winter months (Chatterton et al., 1988; Olien & Clark, 1993) and is the most abundant cyroprotective solute in the cytosol (Koster & Lynch, 1992).

The accumulation of nonstructural carbohydrates is thought to occur when growth is attenuated and photosynthate demand decreases (Chatterton et al., 1988). The cold hardening of winter rye is dependent on irradiance and day length, which evidently control the partitioning of photoassimilates between growth and frost tolerance (Griffith & McIntyre, 1993). Genetic studies of segregating spring- and winter-annual wheat (Galiba et al., 1997) and barley (Hayes et al., 1993) indicate that fructan accumulations are closely associated with vernalization requirement, which also controls bolting and growth habit. However, genetic differences in photosynthesis (Hurry & Huner, 1991; Kaul & Riesner, 1981; Savitch et al., 1997), enzyme activity (Tognetti et al., 1990), or translocation between shoots and roots (Equiza et al., 1997) may also contribute to variability in the accumulation of soluble CHOs. In some cases, the accumulation of soluble CHOs cannot be ascribed to anatomical or morphological differences (Tognetti et al., 1990). In any case, significant genetic variation for low-temperature soluble CHO accumulations has also been observed among winter-annual or perennial cool-season grasses (Chatterton et al., 1989; Kaul & Riesner, 1981; Tognetti et al., 1990; Sagisaka et al., 1991; Yukawa et al., 1995).

The accumulation of anthocyanin pigments is also associated with low-temperature exposure and changes in light intensity in monocots (Christie et al., 1994; Pietrini & Massacci, 1998) and dicots (Leyva et al., 1995). Anthocyanins probably modulate photosynthesis during cold acclimation (CA) (Pietrini & Massacci, 1998); however, anthocyanins *per se* do not impart freezing tolerance and their role in CA is unclear (Leyva et al., 1995). The anthocyanin synthesis pathway is well characterized and several early steps involve cold-inducible genes including phenylalanine ammonia-lyase and chalcone synthase (Christie et al., 1994; Leyva et al., 1995). Studies also suggest that anthocyanin synthesis and/or chalcone synthase gene expression are directly induced by sugars (Larronde et al., 1998; Takeuchi et al., 1994; Tsukaya et al., 1991) and are associated with the accumulation of soluble CHO (Chatterton et al., 1989; Chatterton et al., 1990). Therefore, anthocyanins are useful phenotypic markers for genetic and molecular studies of plant gene expression during cold stress and acclimation (Christie et al., 1994) and may facilitate selection of genotypes with higher concentrations of soluble CHO (Chatterton et al., 1989, 1990). However, the genetic basis for this putative association of anthocyanin coloration and soluble CHO accumulations has not been well characterized.

Hu et al. (2002) tested associations among AFLP markers and low-temperature growth and metabolite accumulations among replicated clones of an admixed breeding population derived through two generations of open pollination, beginning with a population of interspecific hybrids of *L. cinereus* and *L. triticoides*. Of the 204 AFLP markers tested, 20 showed significant associations with one or more traits related to low-temperature growth and metabolite accumulations. Marker-trait associations and correlated segregation patterns among DNA markers (including markers have significant effects) were explained by admixture linkage disequilibrium (Hu et al., 2002), but genetic maps could not be constructed from these admixed populations. More recently, Wu et al. (2003) constructed high-density molecular genetic maps for two full-sib populations derived from the same population

of interspecific hybrids used to develop the admixed breeding population evaluated by Hu et al. (2002). These maps (Wu et al., 2003) included 1583 AFLP markers and 50 anchor markers (i.e. STS, SSR, and RFLP markers from wheat barley and other cereals). The full-sib mapping populations described by Wu et al. (2003) are well-suited for their intended use of conventional QTL mapping, but may lack the genetic heterogeneity required for commercial seed production and plant vigor.

The objective of this study was to identify marker-trait associations in the admixed *L. cinereus* × *L. triticoides* breeding population (Hu et al., 2002) using population specific AFLP markers and examine the genomic distribution of corresponding markers using linkage maps constructed using full-sib populations described by by Wu et al. (2003). Classically, mapping has involved families segregating for phenotypes attributable to the loci of interest, and linkage with markers has been sought by pedigree analysis (Briscoe et al., 1994). This approach, while powerful, has several drawbacks (Briscoe et al., 1994). One drawback is that conclusions are generally based on a small number of parental individuals. In plants, genetic mapping has been simplified using large full-sib families derived from two parental individuals. This approach may be useful for autogamous plants, however most natural and cultivated allogamous plant varieties have a broader genetic base. Indeed, recent QTL analysis of rhizome proliferation, flowering, and plant height in the full-sib TTC1 and TTC2 *Leymus* populations (Wu et al., 2003) have revealed substantial similarities and dissimilarities in terms of QTL location, magnitude, and number (results unpublished), which can be attributed to genetic heterogeneity within the parental accessions. For example, rhizome spreading QTLs were detected in seemingly homologous and homoeologous regions of LG3a and LG3b in both TTC1 and TTC2 populations whereas unique QTLs were detected on LG6a and LG5Xm in TTC1 and TTC2, respectively. Likewise, relatively strong and consistent plant height and flowering QTL effects were detected in homologous regions of LG2a and LG4Xm, respectively, in both TTC1 and TTC2 populations. However a number of other unique, albeit smaller and inconsistent, plant height and flowering QTL effects were also detected in each population over the first three years of evaluation. Thus, sampling effects within heterogeneous populations and varieties may substantially influence QTL detection in full-sib families. The procedure undertaken here takes advantage of increased linkage disequilibrium that occurs when isolated races or subspecies mate and interbreed, with inferences based on a broad array of heterogeneous individuals (Briscoe et al., 1994). Thus, admixture linkage disequilibrium (ALD) can be detected among markers and/or functional alleles with sufficiently different frequency distribution in the parental strains (Briscoe et al., 1994; Collins-Schramm et al., 2002; Stephens et al., 1994). For the admixed breeding population considered here, a special (ideal) case of admixture will be considered in that no further infusion of parental strains was allowed following cross hybridization of the *L. cinereus* and *L. triticoides* accessions. Hence, ALD attributable to physical linkage should be present, but associations between unlinked loci (i.e. gametic disequilibrium among divergent lines) will be reduced to zero through independent assortment of chromosome homologues (Briscoe et al., 1994). Further random mating in subsequent generations reduces ALD as a function of map distance, thus increasing requirements for both marker saturation and sample size (Briscoe et al., 1994; Collins-Schramm et al., 2002). Association-based genome scans, without admixture, is likely to require 50,000 or more markers in humans (Collins-Schramm et al., 2002). Although resources for full genome scans may be available for some species, this is not true for the perennial grasses. In any case, ALD facilitates genetic analysis of outbred populations when pedigree analysis is not feasible (Briscoe et al., 1994; Collins-Schramm et al., 2002; Stephens et al., 1994) and has considerable relevance to allogamous plants. Our hypothesis is that admixture linkage disequilibrium will enable detection of marker-trait associations in the *L. cinereus* × *L. triticoides* breeding population. Moreover, QTL alleles that have highly divergent effects and allele frequencies between these parental species and accessions may be detectable at a preponderance of closely linked AFLP loci (i.e. clusters of marker-trait associations). Thus, one of our major objectives was to examine the genomic distribution of marker-trait associations using molecular genetic linkage maps that were constructed using the closely related TTC1 and TTC2 full-sib mapping populations (Wu et al., 2003). Although codominant DNA markers are highly favorable in well-characterized organisms, the AFLP technique (Vos et al., 1995) is a robust and highly efficient method of DNA fingerprinting that is well suited for many other large-genome species, including a wide diversity of forage and range plants, for which extensive DNA sequence information and resources are lacking. Thus, the utility of AFLPs and other DNA

markers in ALD studies is an important question in plant genetics.

Materials and methods

Plant materials

The admixed *L. cinereus* × *L. triticoides* breeding population was originally described by Hu et al. (2002). Briefly, this experimental breeding population was developed by controlled crosses between the Acc:636 (*L. cinereus*) and Acc:641 (*L. triticoides*) accessions. Approximately five plants from each parental accession (Acc:636 and Acc:641) were mated to generate the F_1 interspecific hybrid populations, however these plants were not preserved. Seeds for the heterogeneous Acc:636 plants, used as male parents, were originally received from the Agriculture Research Centre at Lethbridge, Alberta and presumably originate from Alberta or Saskatchewan. Seeds for the heterogeneous Acc:641 plants, used as female plants, were originally collected by Dr. Kay H. Asay near Jamieson, Oregon. Representative seed has been maintained for the Acc:636 and Acc:641 accessions, and representative plants from these parental accessions have been extensively genotyped. A population of 77 F_1 *L. cinereus* × *L. triticoides* F_1 hybrids was grown next to another population of 96 *L. triticoides* × *L. cinereus* F_1 hybrids. Seedlings from the 77 *L. cinereus* × *L. triticoides* F_1 hybrids, open pollinated, were transplanted to a field evaluation containing 1934 F_1OP_1 plants. The F_1OP_1 field evaluation was culled down to 115 plants with selection for plant vigor and moderate rhizome spread (intermediate spreading compared to parental species). Approximately 1560 F_1OP_2 seedlings from the 115 F_1OP_1 plants, open pollinated, were propagated in cone containers and transplanted to the Utah State University Greenville Farm (North Logan, UT) in March, 1998. During early spring growth, this F_1OP_2 population displayed wide variation for green and purple (anthocyanin) coloration and substantial variation in overall plant vigor during early spring growth. Tillers from each of 66 green and 66 purple F_1OP_2 divergent selections (anthocyanin coloration) were each cloned in two 6-cm wide cone containers, along with two clones from each of six Acc:641 plants and six Acc:636 plants taken from field plots of the original parent populations established in 1987. These clonally replicated F_1OP_2 propagules were maintained in a greenhouse between 25 °C and 15 °C for approximately three months until all plants displayed a green, healthy appearance.

Phenotypic evaluations

Phenotypic and biochemical evaluations were described by Hu et al. (2002). Briefly, all leaves and tillers were counted and measured immediately before and after the low-temperature growth chamber treatment. Clones were arranged in two randomized complete blocks (RCB design) and grown under normal nutrient fertility conditions in controlled environmental chambers for 45 d. The growth chambers were maintained at 5 °C during the night and 10 °C during the day, with 11 h of light from a mixture of fluorescent and incandescent bulbs. Subsequent anthocyanin accumulation was rated on a scale of 0–9, where 9 indicated the highest observable levels of coloration. Free and combined ketohexose was estimated by a ketose-specific modification of the anthrone method based on the method of Jermyn (1956). Experiments in our laboratory indicate this method, described as follows, will measure most of leaf fructan and one-half of the sucrose (results unpublished). Following low-temperature growth chamber treatments, green-leaf clippings were freeze-dried, ground to a fine powder, and then stored at –70 °C. Three aqueous extractions (2 ml each) of lyophilized leaf powder (20 mg) were combined and filtered through a 0.45 μm membrane to make 6-ml sample extracts. Analyses were performed by mixing 20 μl of extract plus water (the ratio depending on soluble CHO concentration in the sample) with 200 μl of anthrone reagent in a 96-well microplate and incubating for 1 h at 37 °C. Color development was measured at 620 nm in a SpectraMax Plus[1] plate reader and compared with an inulin standard. The anthrone reagent was prepared by first dissolving 1 g of anthrone in 380 ml concentrated H_2SO_4, and then slowly diluting this solution with 120 ml of water using an ice bath and stirring plate.

Map development and genotyping

The genetic maps described by Wu et al. (2003) were developed using two individual F_1 hybrids, TC1 and TC2, from the same *L. cinereus* × *L. triticoides* F_1

[1] Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

hybrid population that was intermated to develop the broad-based, admixed breeding population utilized in this study and Hu et al. (2002). The TC1 and TC2 hybrids were backcrossed to one common *L. triticoides* tester plant (T-tester), to produce the two full-sib populations (TTC1 and TTC2) used to construct two comparative maps described by Wu et al. (2003). The 164-sib TTC1 map included 1069 AFLP markers and 38 anchor loci in 14 linkage groups spanning 2001 cM. The 170-sib TTC2 map contained 1002 AFLP markers and 36 anchor loci in 14 linkage groups spanning 2006 cM. Some 488 homologous AFLP loci and 24 anchor makers were mapped in both populations, using Joinmap 3.0[1] (Van Ooijen & Voorrips, 2001). These common markers showed virtually identical linear orders among the TTC1 and TTC2 homologues (Wu et al., 2003). However these results were presented as two comparative maps (Wu et al., 2003), appropriate for their intended use in QTL mapping, rather than one integrated consensus map. Nevertheless, a total of 1583 AFLP markers, amplified using 23 *Eco*RI–*Mse*I and 17 *Pst*I–*Mse*I AFLP primer combinations, and 50 anchor loci (i.e. RFLP, SSR, and genic STS markers mapped in wheat, barley, and/or other cereal species) were mapped into 14 linkage groups corresponding to the 14 chromosomes of allotetraploid *Leymus* based on gamete segregation from the TC1 and TC2 hybrids (Wu et al., 2003). Thus, we expected the admixed breeding population (Hu et al., 2002) to segregate for many of the same AFLP markers that were mapped by Wu et al. (2003) as well as other marker alleles that were not mapped.

Hu et al. (2002) originally fractionated AFLPs using an ABI373[1] slab-gel electrophoresis instrument (PE Applied Biosystems[1]) with GS-500 size standards (PE Applied Biosystems[1]), whereas Wu et al. (2003) fractionated AFLPs using a more efficient ABI3100[1] capillary electrophoresis instrument (PE Applied Biosystems[1], Foster City, CA) with GS-400 size standards (PE Applied Biosystems[1]). Apparent interactions between DNA sequence and relative mobility between these two methods of slab-gel and capillary electrophoresis made it very difficult, sometimes impossible, to identify homologous DNA markers between these two studies. Thus, we reanalyzed a subset of 96 genotypes from the *L. cinereus* × *L. triticoides* breeding population using 18 of the 23 *Eco*RI–*Mse*I AFLP primer combinations screened by Wu et al. (2003), using the same ABI3100[1] capillary electrophoresis instrument and GS-400 size standards used by Wu et al. (2003). However, this study was pared down to 96 genotypes to facilitate efficient genotyping using 96-well sample plates. In particular, 36 plants showing a preponderance of *L. triticoides* markers alleles from a skewed distribution of genotypes were culled from the original set of 132 plants, based on data genotypic marker data described by Hu et al. (2002). Genotyping of the remaining 96 genotypes enabled reliable identification of DNA markers that are homologous to mapped AFLP markers (Wu et al., 2003) and substantially increased marker density in the admixed breeding population. A total of 18 highly informative AFLP primers were analyzed; E36M48, E36M50, E36M61, E36M62, E37M47, E37M49, E37M60, E37M62, E38M47, E38M49, E38M59, E38M60, E41M47, E41M48, E41M59, E41M60, E41M61, and E41M62 as described by Vos et al. (1995), with minor modifications (Wu et al., 2003). As described in the results below, a consensus map of corresponding AFLP markers was developed using Joinmap 3.0[1] from the TTC1 and TTC2 mapping populations described by Wu et al. (2003). Only marker fragments that were present in one parental accession (i.e. *L. cinereus* Acc:636 *or L. triticoides* Acc:641) but absent in the other were analyzed, assigning marker alleles in coupling phase among the respective founder populations. Thus, our consensus map (Results below) provides a useful framework for analyzing the genomic distribution of ALD QTL markers in the admixed breeding population.

QTL analysis

All polymorphic AFLPs were tested for possible association with average number of new tillers, average number of new leaves, average leaf growth (cm), average percent soluble carbohydrate content, average anthocyanin coloration rating (0–9), and average dry matter regrowth (mg) by the non-parametric Kruskal-Wallis rank sum test using MapQTL 4.0[1] (Van Ooijen & Maliepaard, 1996), which is equivalent to the Wilcoxon rank sum test with two genotypic classes as employed in this study. This nonparametric test does not make any assumptions about the probability distribution of the quantitative traits. This relatively simple test could be performed using a wide variety of software, however MapQTL[1] provided and efficient means of integrating this test with the new consensus maps developed using Joinmap[1] as described above. Because this association test was used with many linked and unlinked factors, a significance level less than 0.005 for QTL detection as recommended by Van Ooijen & Maliepaard (1996). Thus, we used a significance threshold of 0.005 for

identification of possible ALD QTLs in the admixed breeding population. However, we expect to observe a gradient of diminishing significance at flanking loci, as a function of genetic recombination. Therefore, all marker-trait associations ($P < 0.05$) were identified whether or not flanking markers displayed significant effects. In this context, our criteria for QTL detection includes one or more markers with significance levels less than 0.005, flanked by a preponderance of closely linked adjacent markers (approximately 20 cM or less) with significant but diminishing effects (Hu et al., 2002). Because markers alleles were assigned in coupling phase according to presence or absence of fragments in the parental accessions, we only considered linkage blocks containing synergistic marker-trait associations (i.e. positive or negative, but not mixed) as evidence of ALD QTLs. Although we used a more conservative threshold of 0.005, the overall distribution of all marker effects significant at the $P < 0.05$ or better was also investigated as part of this ALD study.

Results and discussion

Development and implementation of a consensus-framework genetic linkage map

Eighteen AFLP primer combinations amplified 647 polymorphic DNA fragments from the admixed breeding population that were also mapped in the TTC1 and TTC2 populations (Wu et al., 2003), in addition to other polymorphic markers that were not mapped. As expected, the linear order and map distances among these 647 markers, integrated using JoinMap (Figure 1), was very similar to the comparative TTC1 (2001 cM) and TTC2 (2066 cM) maps described by Wu et al. (2003). The linkage group names used in this consensus map (Figure 1) correspond to those used by (Wu et al., 2003), which were provisionally numbered according to the 7 homoeologous groups of other Triticeae species (i.e. wheat, barley, and rye). However, more anchor markers are needed to develop accurate comparative maps between *Leymus* and other Triticeae species.

Considerable variation in the relative frequency of *L. cinereus* and *L. triticoides* alleles was apparent amongst linked and unlinked markers in the admixed breeding population (Figure 1), originally described by Hu et al. (2002). Wide variability in the segregation ratios of closely linked DNA markers (Figure 1) can be attributed to heterogeneity of DNA marker alleles within parental accessions of the F_1 hybrid population and genetic recombination through two generations of admixture via open pollination. Thus, it would be virtually impossible to construct a genetic linkage map from the admixed breeding population *per se* and it should be recognized that map distances shown in (Figure 1) probably underestimate the actual amount of recombination of *L. cinereus* and *L. triticoides* chromatin in this study. It should also be noted that Wu et al. (2003) primarily mapped *L. cinereus* (Acc:636) marker alleles that were absent in the *L. triticoides* (Acc:641) tester plant. Thus, most of the markers used in this study (Figure 1) were present among samples ($n = 6$) of Acc:636 but absent among samples ($n = 6$) of Acc641. Because marker alleles were assigned in coupling phase according to their presence in one founder population or the other (but not both) these markers were in linkage disequilibrium with physically linked genes controlling divergent traits (LD QTLs) between the *L. cinereus* Acc:636 and *L. triticoides* Acc:641 accessions, as a result of admixture. However, many of these DNA markers (Figure 1) may not have been fixed in both parental accessions, even though marker alleles were discretely assigned to one parental accession or the other. In actuality, some of the trait variation and marker-trait linkage associations may result from heterogeneity within parental accessions and not all linkage phase assignments were certain in this admixed breeding population. In addition to admixture of divergent populations, genetic bottlenecks are another possible source of linkage disequilibrium (Briscoe et al., 1994). The founder effect of selecting approximately 5 Acc:636 and approximately 5 Acc:641 parents, used to generate the F_1 population, may have generated linkage disequilibrium (among heterogeneous markers and genes) within founder chromatin lineages that would persist through this admixed population, but which linkage phase assignments may be incorrect. Nevertheless, we anticipated that most of the marker-trait associations would be generated by admixture of the divergent species, resulting in correct linkage phase assignments.

Identification and map location of linkage disequilibrium QTL markers

A total of 237 markers (i.e. 37% of the 647 markers tested) displayed a total of 365 possible trait associations that were significant at the $P = 0.05$ level or better, as indicated in Figure 1 and Table 1. We identified 37 possible linkage blocks spanning 258 cM (13.6%) of the 1895 cM genome, which contain a

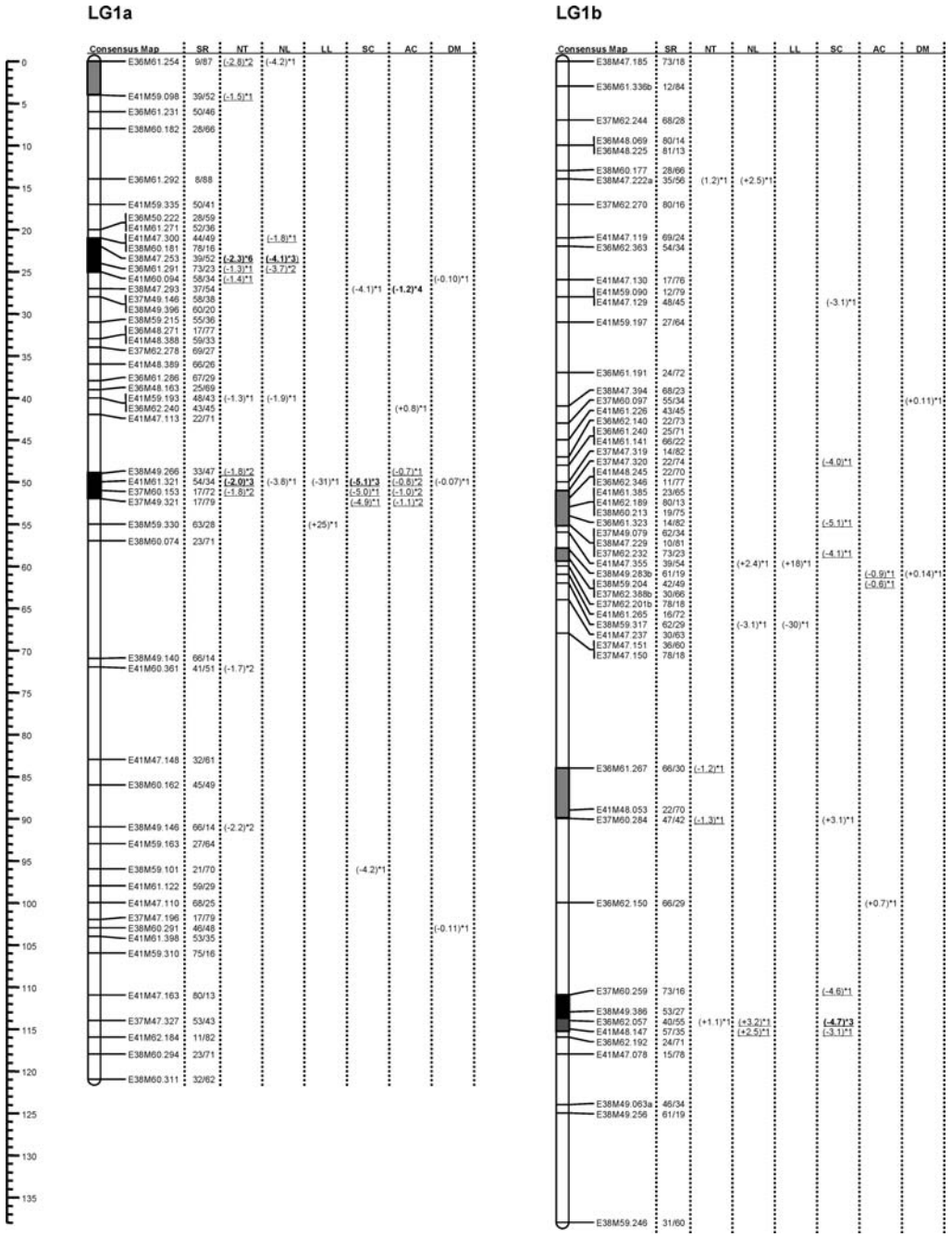

Figure 1. Consensus map for 647 AFLP markers evaluated in the admixed *Leymus cinereus* × *L. triticoides* breeding population, based on the full-sib TTC1 and/or TTC2 mapping populations originally published by Wu et al. (2003). Segregation ratios (SR) of null (*L. triticoides*)/dominant (*L. cinereus*) AFLP alleles, in the admixed *L. cinereus* × *L. triticoides* breeding population (Hu et al., 2002), are indicated for each marker. Likewise, putative effects of the *L. cinereus* marker alleles on low-temperature growth traits (NT = number of new tillers; NL = number of new leaves; LL = cumulative centimeters leaf length; DM = mg dry matter) and metabolite accumulations (SC = percent soluble carbohydrate; AC = anthocyanin coloration), in the admixed *L. cinereus* × *L. triticoides* breeding population (Hu et al., 2002), are indicated by additive effects in parenthesis and corresponding significance values (*1 = 0.05; *2 = 0.01; *3 = 0.005; *4 = 0.001; *5 = 0.0005; *6 = 0.0001). Linkage blocks containing a preponderance of marker effects significant at the 0.05 level or less (underline text) are shaded. Likewise, those linkage blocks containing at least one marker-trait association significant at the 0.005 level (bold text) are indicated by dark shaded linkage blocks, which constitute putative admixture linkage disequilibrium (ALD) QTLs. (*Continued on next page*.)

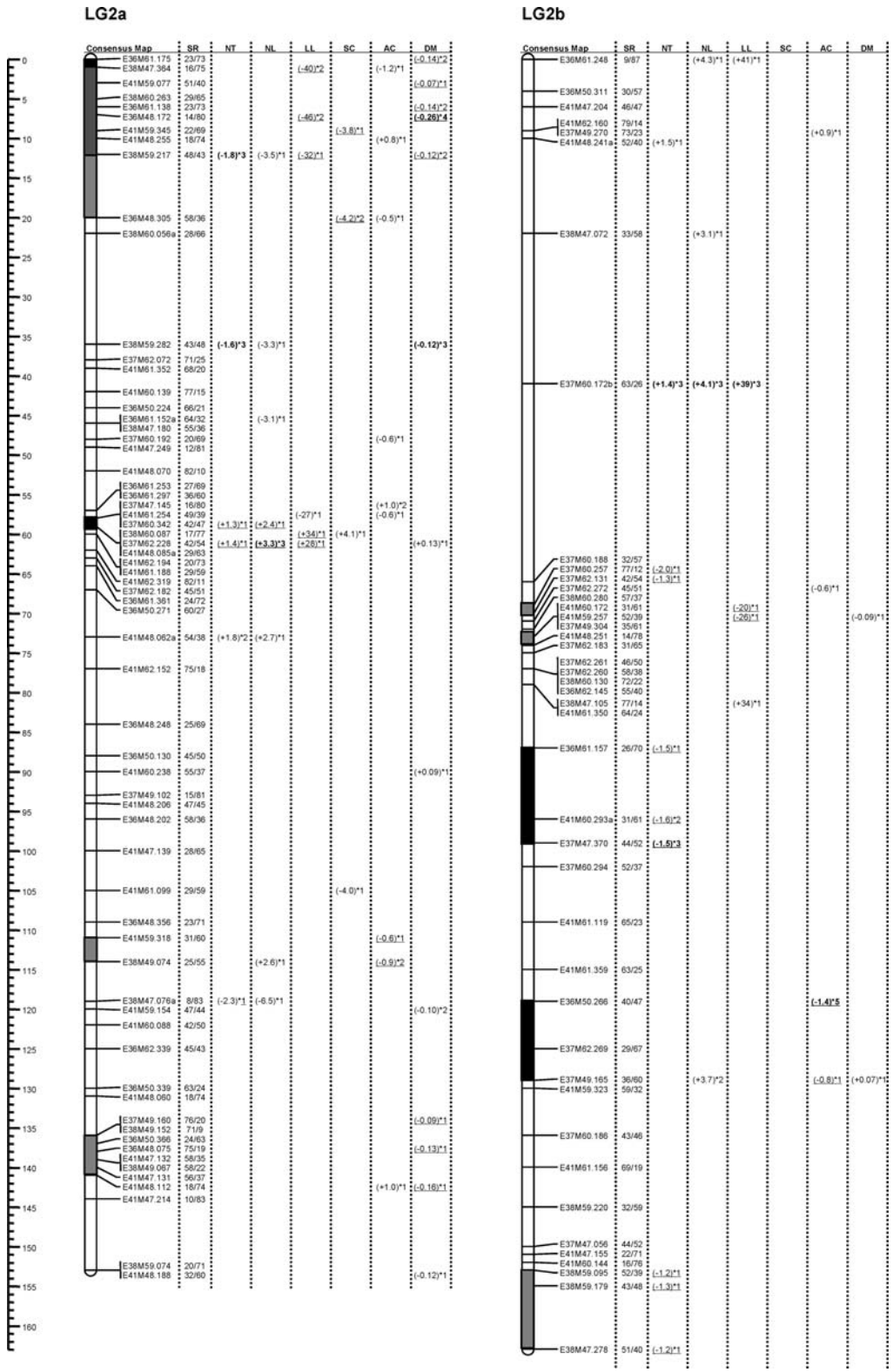

Figure 1. (*Continued*).

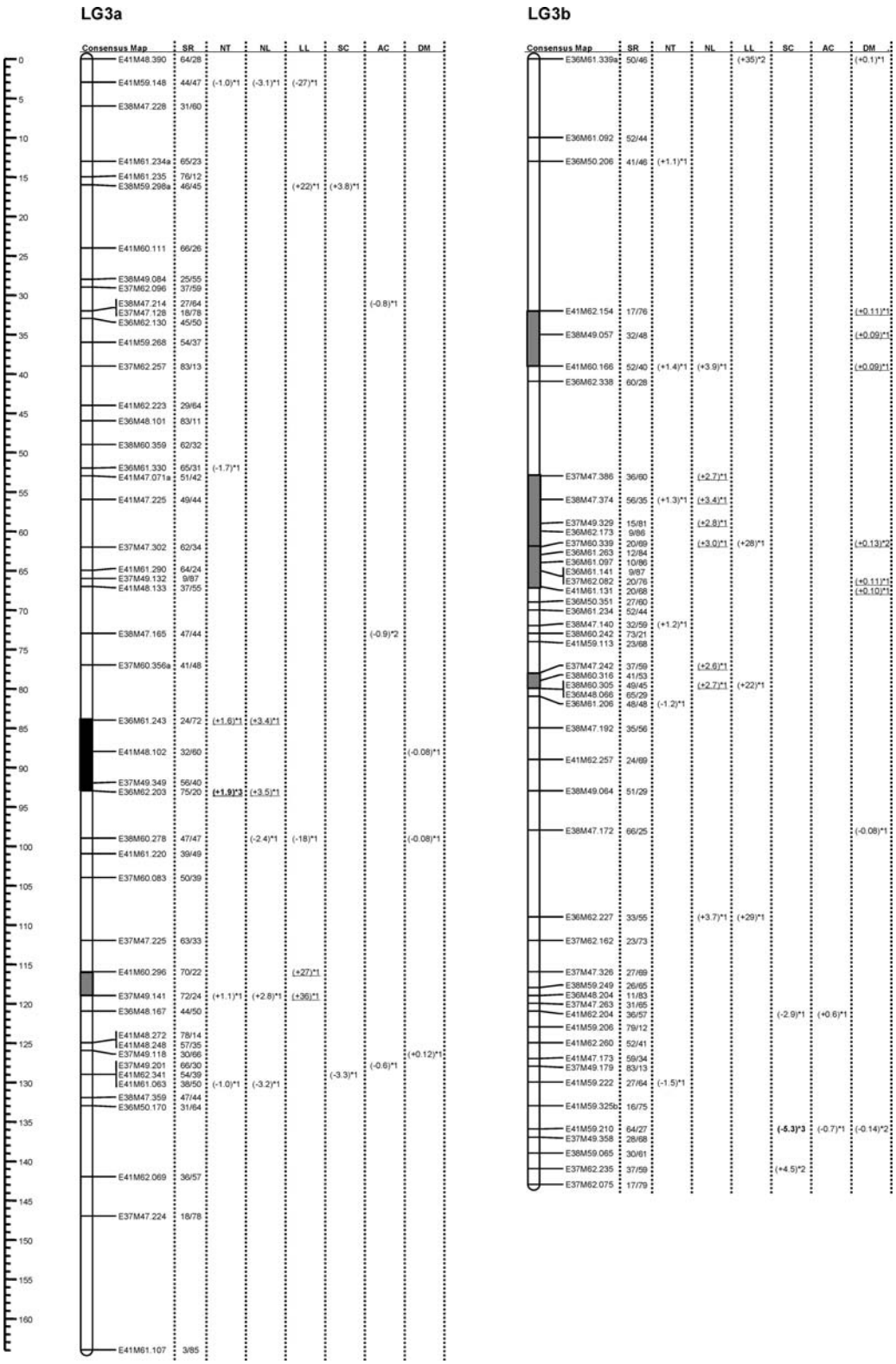

Figure 1. (*Continued*).

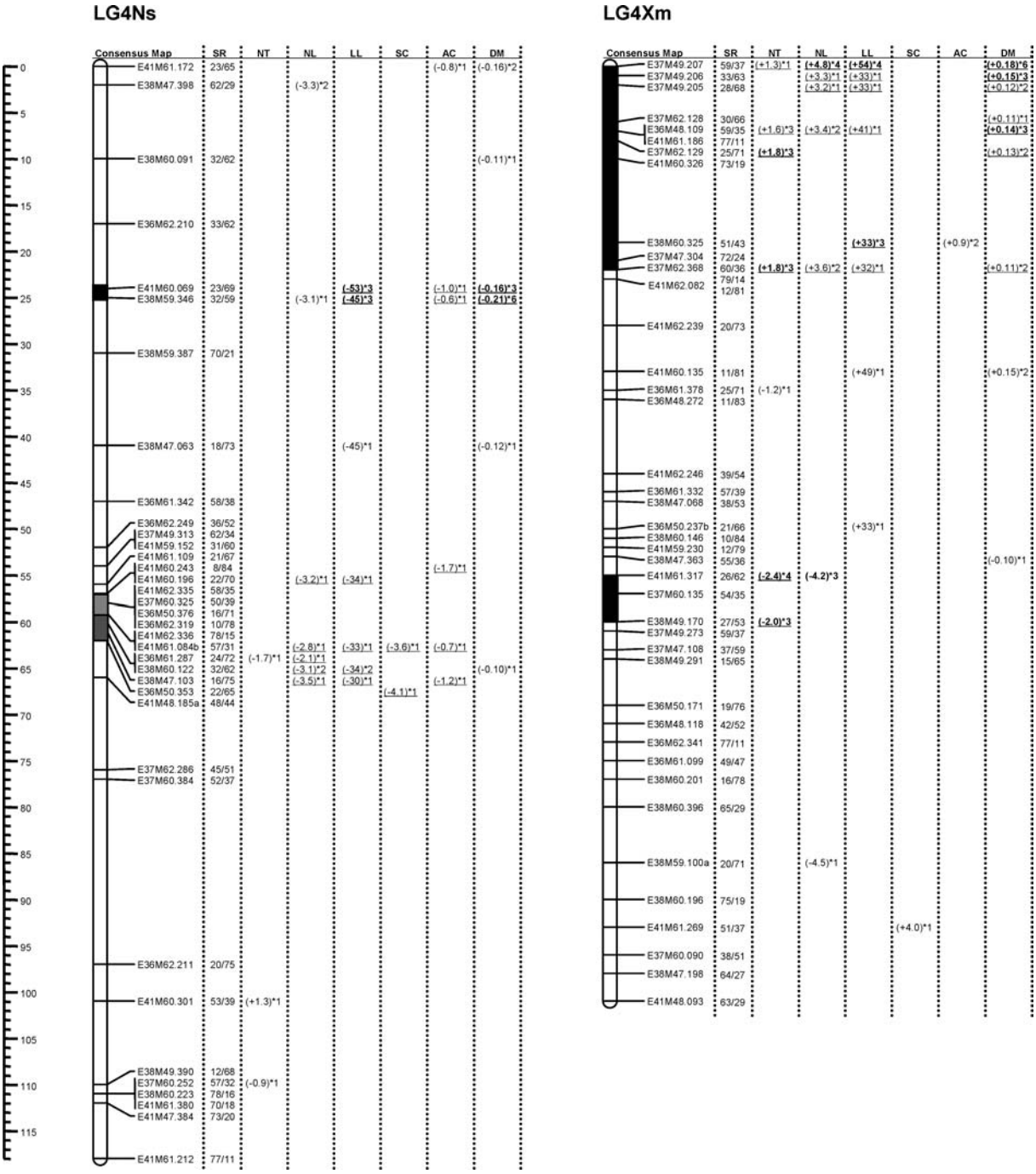

Figure 1. (*Continued*).

preponderance of adjacent, closely linked marker-trait associations (Figure 1). These linkage blocks were defined as relatively small groups of closely linked (i.e. approximately 20 cM or less) marker-trait associations within linkage groups constructed by Wu et al. (2003). These 37 linkage blocks contained 166 (45.5%) of the 365 marker-trait associations, or 119 (50.2%) of the 237 markers showing at least one trait effect (Table 1).

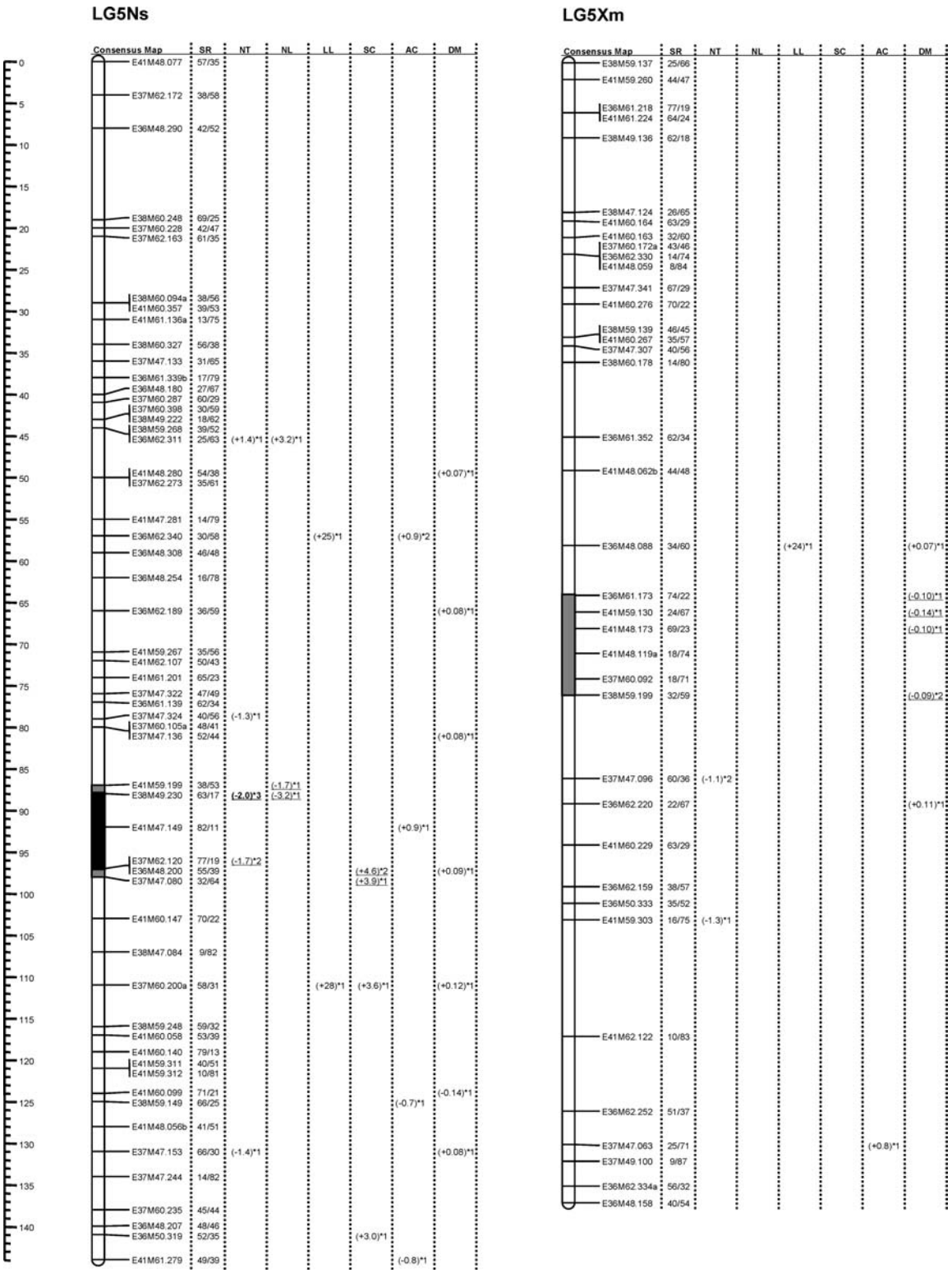

Figure 1. (*Continued*).

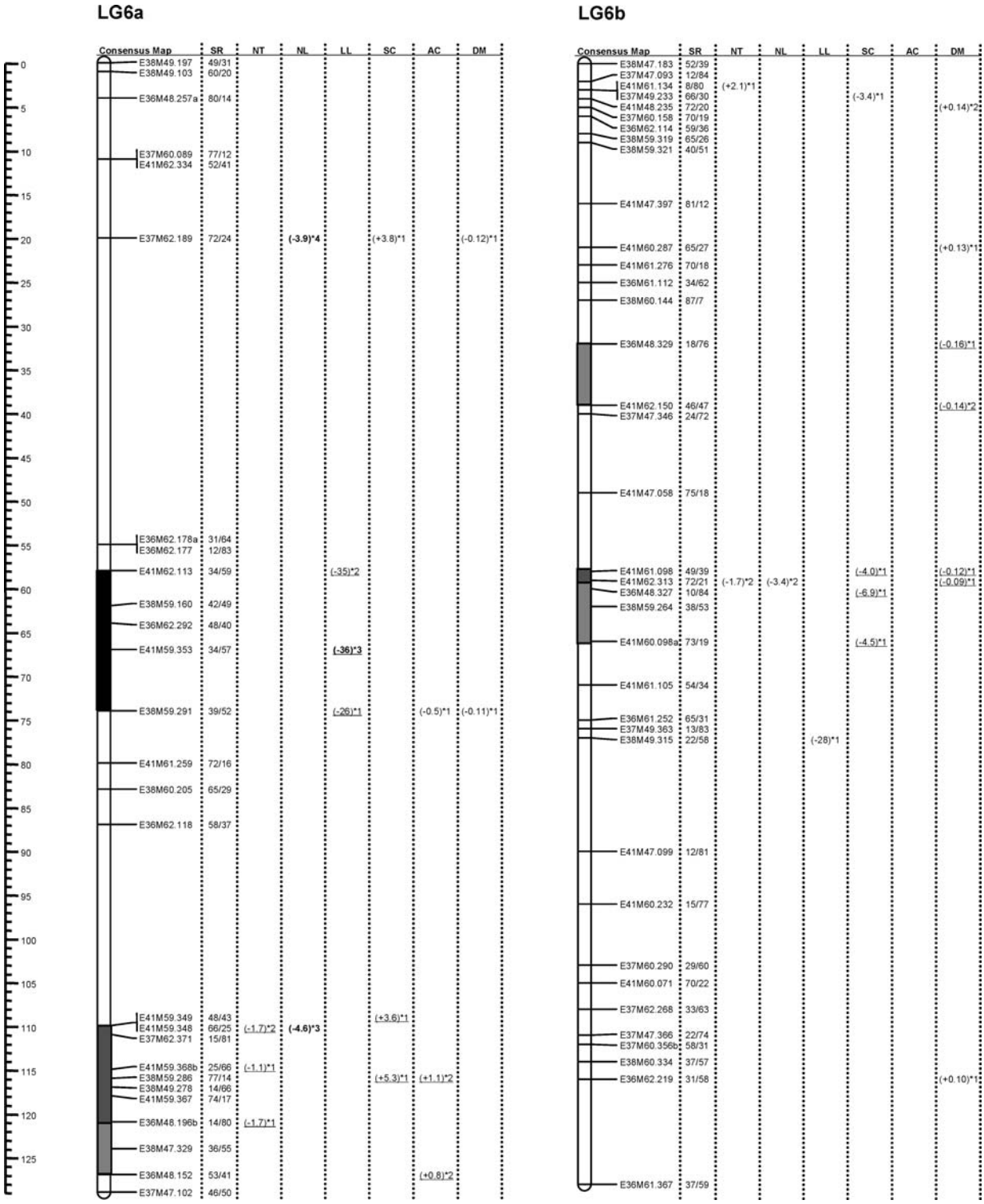

Figure 1. (*Continued*).

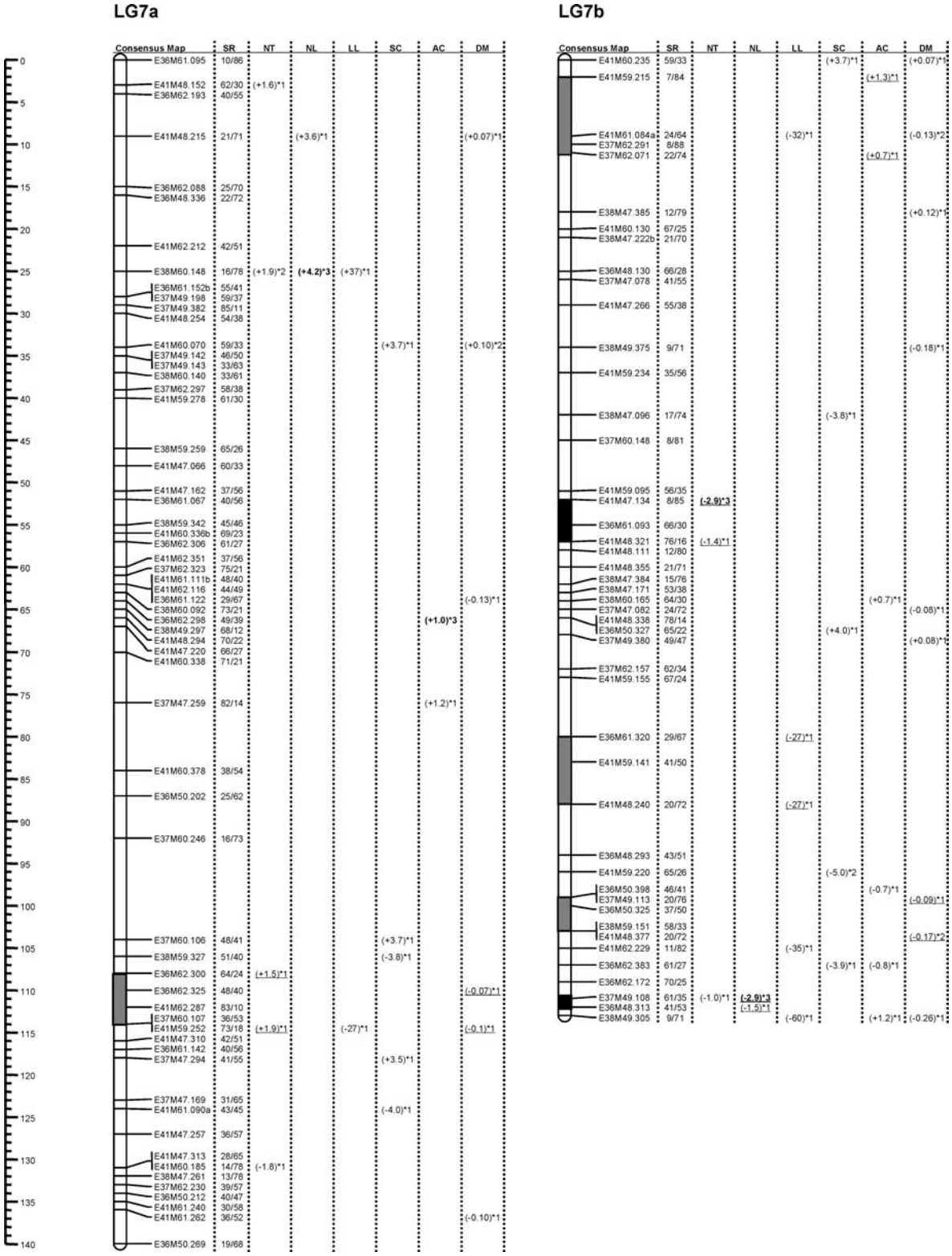

Figure 1. (*Continued*).

Table 1. Summary of genetic map distribution for all possible ALD-QTL marker effects ($P < 0.05$) in a *Leymus cinereus* × *L. triticoides* F_1OP_2 breeding population: NT = number of new tillers, NL = number of new leaves, LL = leaf length, SC = percent soluble carbohydrate, AC = anthocyanin coloration, and DM = dry matter

Group markers (distance)	**NT markers linked[a]/total** distance (% total)	**NL markers linked[a]/total** distance (% total)	**LL markers linked[a]/total** distance (% total)	**SC markers linked[a]/total** distance (% total)	**AC markers linked[a]/total** distance (% total)	**DM markers linked[a]/total** distance (% total)	**Overall traits** and markers linked[a]/total	**Overall markers linked[a]/total** distance (% total)
LG1a	**8/11 (73%)**	**3/6 (50%)**	**0/2 (0%)**	**3/5 (60%)**	**4/6 (67%)**	**0/3 (0%)**	**18/33 (55%)**	**10/18 (56%)**
50 (122 cM)	9.5 cM (8%)	3.5 (3%)	0 (0%)	2.3 (2%)	2.8 (2%)	0 (0%)		11.8 (10%)
LG1b	**2/4 (50%)**	**2/5 (40%)**	**0/2 (0%)**	**6/8 (75%)**	**2/3 (67%)**	**0/2 (0%)**	**12/24 (50%)**	**10/16 (63%)**
55 (138 cM)	6.5 (5%)	0.5 (1%)	0 (0%)	8.1 (7%)	0.8 (1%)	0 (0%)		15.4 (13%)
LG2a	**2/6 (33%)**	**2/8 (25%)**	**5/6 (83%)**	**2/4 (50%)**	**2/9 (22%)**	**8/13 (62%)**	**21/46 (46%)**	**16/28 (57%)**
65 (154 cM)	0.6 (0.4%)	0.6 (0.4%)	11.6 (8%)	11.7 (8%)	2.6 (2%)	17.3 (11%)		28.5 (19%)
LG2b	**8/10 (80%)**	**0/4 (0%)**	**2/5 (40%)**	**–**	**2/4 (50%)**	**0/2 (0%)**	**12/25 (48%)**	**12/19 (63%)**
43 (163 cM)	23.4 (14%)	0 (0%)	0.1 (0.1%)		9.5 (6%)	0 (0%)		33.0 (20%)
LG3a	**2/6 (33%)**	**2/6 (33%)**	**2/5 (40%)**	**0/2 (0%)**	**0/3 (0%)**	**0/3 (0%)**	**6/25 (24%)**	**4/15 (27%)**
48 (165 cM)	9.6 (6%)	9.6 (6%)	2.5 (2%)	0 (0%)	0 (0%)	0 (0%)		12.1 (7%)
LG3b	**0/6 (0%)**	**6/8 (75%)**	**0/3 (0%)**	**0/3 (0%)**	**0/2 (0%)**	**6/8 (75%)**	**12/30 (40%)**	**11/21 (52%)**
49 (143 cM)	0 (0%)	10.5 (7%)	0 (0%)	0 (0%)	0 (0%)	12 (8%)		22.5 (16%)
LG4Ns	**0/3 (0%)**	**5/7 (71%)**	**6/7 (86%)**	**2/2 (100%)**	**5/6 (83%)**	**2/6 (33%)**	**20/31 (65%)**	**9/15 (60%)**
36 (118 cM)	0 (0%)	3.6 (3%)	4.2 (4%)	2.7 (2%)	4.5 (4%)	0.6 (1%)		5.9 (5%)
LG4Xm	**6/7 (86%)**	**5/7 (71%)**	**6/8 (75%)**	**0/1 (0%)**	**0/1 (0%)**	**7/9 (78%)**	**24/33 (73%)**	**10/16 (63%)**
41 (101 cM)	26.3 (26%)	21.3 (21%)	21.3 (21%)	0 (0%)	0 (0%)	21.3 (21%)		26.3 (26%)
LG5Ns	**2/5 (40%)**	**2/3 (67%)**	**0/2 (0%)**	**2/4 (50%)**	**0/4 (0%)**	**0/7 (0%)**	**6/25 (24%)**	**5/18 (28%)**
56 (144 cM)	9.0 (6%)	0.7 (0.5%)	0 (0%)	0.6 (0.5%)	0 (0%)	0 (0%)		10.3 (7%)
LG5Xm	**0/2 (0%)**	**–**	**0/1 (0%)**	**–**	**0/1 (0%)**	**4/6 (67%)**	**4/10 (40%)**	**4/9 (44%)**
38 (137 cM)	0 (0%)		0 (0%)		0 (0%)	8.4 (13%)		8.4 (6%)
LG6a	**3/3 (100%)**	**0/2 (0%)**	**3/3 (100%)**	**2/3 (67%)**	**2/3 (67%)**	**0/2 (0%)**	**10/16 (63%)**	**9/10 (90%)**
27 (129 cM)	11.7 (9%)	0 (0%)	16.7 (13%)	6.6 (5%)	11.1 (9%)	0 (0%)		34.4 (27%)
LG6b	**0/2 (0%)**	**0/1 (0%)**	**0/1 (0%)**	**3/4 (75%)**	**–**	**4/7 (57%)**	**7/15 (47%)**	**6/12 (50%)**
37 (128 cM)	0 (0%)	0 (0%)	0 (0%)	8.6 (7%)		9.2 (7%)		16.0 (13%)
LG7a	**2/5 (40%)**	**0/2 (0%)**	**0/2 (0%)**	**0/5 (0%)**	**0/2 (0%)**	**2/6 (33%)**	**4/22 (18%)**	**3/16 (19%)**
61 (140 cM)	6.7 (5%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4.3 (3%)		6.7 (5%)
LG7b	**2/3 (100%)**	**2/2 (100%)**	**2/5 (40%)**	**0/5 (0%)**	**2/6 (33%)**	**2/9 (22%)**	**10/30 (33%)**	**10/24 (42%)**
48 (113 cM)	4.6 (4%)	0.8 (1%)	8.0 (7%)	0 (0%)	9.0 (8%)	3.8 (3%)		26.2 (23%)
Overall	**37/73 (51%)**	**29/61 (48%)**	**26/52 (50%)**	**20/46 (43%)**	**19/50 (38%)**	**35/83 (42%)**	**166/365 (45.5%)**	**119/237 (50.2%)**
647 (1895 cM)	107.9 (5.7%)	51.1 (2.7%)	64.4 (3.4%)	40.6 (2.1%)	40.3 (2.1%)	76.9 (4.1%)		257.5 (13.6%)

[a]Preponderance of closely linked and/or adjacent marker effects significant at 0.05 level.
[b]Pleiotropic markers counted only once.

Thus, over 50% of all possible marker-trait associations ($P < 0.05$) were located within 13.6% of the genetic recombination map (Table 1). However, the process of identifying linkage blocks containing a preponderance of significant marker effects was subjective. The distribution of significant marker effects is obviously confounded by a non-random distribution of markers. Thus, it is difficult to rigorously test for a non-random distribution of significant marker effects. Nevertheless, 28 (68%) of the 41 most significant marker effects ($P < 0.005$) were located within 15 linkage blocks spanning 112.9 cM (6%) of the 1895 cM linkage map (Figure 1, Tables 1 and 2). The coincidence of the 28 most significant marker-trait associations ($P < 0.005$),

and many less significant marker effects ($P < 0.05$), in 15 relatively small linkage blocks (0.6 cM to 21.3 cM) provides compelling evidence (Figure 1) of ALD QTLs in this admixed, heterogeneous breeding population. At least 4 of the remaining 13 putative ALD QTL marker effects ($P < 0.005$) were located in genetic map regions lacking other useable flanking markers in this population. Although the consensus map shown in Figure 1 has several regions void of DNA markers, the original maps were constructed using substantially more DNA markers (Wu et al., 2003). The maps described by Wu et al. (2003) had good marker coverage throughout most of the genome, providing a reliable and useful framework for the markers used in this admixed breeding population.

Twelve (80%) of the 15 *L. cinereus* ALD QTL alleles displayed negative effects on one or more traits, which is consistent with differences between the parental accessions and overall trait correlations reported by Hu et al. (2002). The *L. cinereus* Acc:636 accession displayed substantially less growth, less soluble carbohydrate accumulation, and less anthocyanin coloration compared to the *L. triticoides* Acc:641 accession (Hu et al., 2002). The *L. cinereus* ALD QTL marker alleles in the LG1a 21–26 cM, LG4Xm 54–59 cM, LG7b 110–112 cM intervals were associated with negative effects on growth of new tillers, new leaves, and possibly other traits. The *L. cinereus* ALD QTL marker alleles in the LG1a 49–53 cM interval were associated with negative effects on growth of new tillers, soluble carbohydrate accumulation, and possibly other traits (especially anthocyanin coloration). The latter ALD QTL probably accounts for much of the overall trait correlation ($r = 0.39$) between soluble carbohydrate accumulation and anthocyanin coloration (Hu et al., 2002). The *L. cinereus* ALD QTL marker alleles in the LG2a 0–21 cM interval were associated with negative effects on total dry matter and possibly other traits. The *L. cinereus* ALD QTL marker alleles in the LG2b 86–99 cM and LG7b 52–57 cM intervals were associated with negative effects on the development of new tillers per se. The *L. cinereus* ALD QTL marker alleles in the LG2b 119–129 cM interval were associated with the most significant (negative) effect on anthocyanin coloration per se, but had little if any other trait associations. The *L. cinereus* ALD QTL marker alleles in the LG4Ns 24–26 cM interval were associated with negative effects on total leaf length, dry matter regrowth, and possibly other traits including anthocyanin coloration. The *L. cinereus* ALD QTL marker alleles in the LG6a 57–74 cM intervals had negative associations with total leaf length per se, even though *L. cinereus* has longer leaves.

Although most of the significant markers displayed negative trait associations with the *L. cinereus* alleles, the *L. cinereus* marker alleles showed positive trait associations for three putative ALD QTLs. The *L. cinereus* ALD QTL marker alleles on LG2a (58–60 cM) were positively associated with growth of new leaves and less significant effects on growth of new tillers, total leaf length, and possibly other traits. The *L. cinereus ALD* QTL marker alleles on LG3a (84–94 cM) was positively associated with growth of new tillers and less significant effects on the number of new leaves. The *L. cinereus* ALD QTL marker alleles on LG4Xm (0–21 cM) were associated with relatively strong positive effects on the growth of new tillers, new leaves, total leaf length, and dry matter regrowth. The ALD QTL on LG4Xm (0–21 cM) was one of the most compelling ALD QTL effects detected in this study. Although *L. cinereus* displayed less growth in total leaf length, compared to *L. triticoides*, it should be noted that mature *L. cinereus* (that largest native grass of western North America) has much longer leaves. Thus, *L. cinereus* certainly has the potential to contribute positive growth alleles. We were, however, somewhat surprised to find positive tillering effects associated with *L. cinereus* DNA marker alleles on LG2a, LG3a, and LG4Xm, because *L. triticoides* (the strongly rhizomatous species) displayed a much greater profusion of new tillers compared to *L. cinereus*.

The strongest overall trait correlations ($r > 0.70$) were between the number of new tillers and the number of new leaves; the number of new leaves and leaf growth; and between leaf growth and dry matter regrowth under low temperatures (Hu et al., 2002). Formation of new tillers presumably contributed to the formation of new leaves, thus several ALD QTLs (Figure 1) evidently affect both traits and contribute to a relatively strong correlation ($r = 0.73$) between these traits (Hu et al., 2002). Likewise, formation of new leaves presumably contributed to total leaf length, thus several ALD QTLs (Figure 1) evidently affect both traits and contribute to a relatively strong correlation ($r = 0.76$) between these traits (Hu et al., 2002). Several ALD QTLs displayed synergistic effects on total leaf length growth and dry matter regrowth (Figure 1), which under the correlation ($r = 0.70$) between these traits (Hu et al., 2002). Thus, many ALD QTLs (Figure 1) displayed synergistic effects on two or more traits, including growth and/or metabolite accumulations, which were consistent with the overall

trait correlations (Hu et al., 2002). However, two ALD QTLs had antagonistic effects on growth and soluble carbohydrate accumulation. In particular, the *L. cinereus* ALD QTL marker alleles in the LG1b 111–115 cM interval were associated with strong negative effects on soluble carbohydrate accumulation and small positive effects on the development of new tillers and new leaves. Conversely, the *L. cinereus* ALD QTL marker alleles in the LG5Ns 86–97 cM interval were associated with negative effects on low-temperature growth and positive effects on low-temperature accumulations of soluble carbohydrate and anthocyanin coloration. The LG5Ns ALD QTL markers are located near the CDO504 locus (Wu et al., 2003), which is closely associated with the *vrn*–1 gene of wheat (Galiba et al., 1997) and barley (Hayes et al., 1993). Decreased low-temperature growth and increased soluble carbohydrate accumulations are also associated with the homoeologous *vrn*–1 genes of wheat (Galiba et al., 1997) and barley (Hayes et al., 1993). The accumulation of nonstructural carbohydrates generally occurs when growth is attenuated and photosynthate demand decreases (Chatterton et al., 1988). In any case, synergistic and antagonistic marker effects were detected between growth and soluble carbohydrate accumulations were detected in this study, which may explain lack of overall trait correlations between growth traits and soluble carbohydrate accumulations (Hu et al., 2002).

With most AFLP marker alleles having known and coupled linkage phase (i.e. alleles specific to one founder population), most adjacent (linked) marker effects were synergistic (i.e. positive or negative) as a result of admixture linkage disequilibrium (Figure 1). However, several chromosome regions displayed seemingly mixed gene effects among closely linked markers. For example linked E38M47.364, E41M48.255, and E36M48.305 markers on LG2a displayed positive, negative, and positive associations with anthocyanin coloration, respectively. Likewise, the adjacent linked E37M47.145 and E41M61.254 markers on LG2a also displayed positive and negative associations with anthocyanin coloration. Adjacent E38M49.074 and E38M47.076a markers on LG2a displayed positive and negative associations with growth of new tillers. The closely linked E41M59.210 and E37M62.235 markers on LG3b displayed negative and positive associations with soluble carbohydrate accumulations. Adjacent E37M60.106 and E38M59.327 markers on LG7a had positive and negative associations with soluble carbohydrate accumulations. Ordered and linked E41M60.235, E41M61.084a, and E38M47.385 markers on LG7b displayed positive, negative, and positive associations with total dry matter regrowth, respectively. Finally, closely linked E37M47.082 and E37M49.380 markers on LG7b displayed negative and positive associations with total dry matter regrowth. The vast majority of AFLP markers that were mapped in both TTC1 and TTC2 populations mapped to homologous chromosome regions (Wu et al., 2003). However, a small subset of apparently similar markers, including E38M47.076a and E41M61.084a, mapped to non-homologous chromosomes. Although the vast majority of comigrating fragments are probably allelic in this genetic background, some exceptions are likely to occur (Rouppe van der Voort et al., 1997; Wu et al., 2003). In any case, this uncertainty does not explain all of the repulsion-phase linkages between the gene effects noted above. As we suspected misinterpretation of linkage phases, we reviewed several of these repulsion-phase linkages. In all of these reviews, we did not find any instances where the allelic phases could have been misinterpreted. We speculate that seemingly opposing gene effects among closely linked markers were caused by genetic heterogeneity and linkage disequilibrium within the parental founder populations (i.e. approximately 5 *L. cinereus* and 5 *L. triticoides* plants) used to create the F_1 hybrid population. This founder effect (i.e. genetic bottleneck) may have created linkage disequilibrium between heterogeneous marker alleles and QTL alleles (Briscoe et al., 1994), within parental lineages, that persisted through two additional generations of admixture.

We deduce that most of the marker trait associations (Figure 1; Table 1) were the result of admixture between species with highly marker allele frequencies and QTL allele frequencies, evident by an unlikely preponderance of adjacent (linked) markers that have significant ($P = 0.05$ or better) unidirectional effects on the same trait(s) (Table 1). However, a substantial number of other marker-trait associations (clustered or solitary) may be the result of heterogeneity of marker alleles and heterogeneity of QTL alleles, within populations, coupled by found effects in the relatively small parental groups of this admixed breeding population. In many cases, perennial grass breeders working with allogamous species are unlikely to adopt molecular markers for QTL identification and marker assisted selection unless these procedures can be applied in broad-based, genetically heterogeneous populations. Initial studies of marker-trait associations in this admixed breeding population were very difficult to interpret (Hu et al., 2002). Molecular genetic linkage

maps, used in this study, provide essential information about the genomic distribution of these marker trait associations. Additional genotypic information from the parental source populations would further facilitate the interpretation of results from this QTL detection method. Although more advanced experimental designs and technologies may be needed to reliably detect and quantify QTL divergence among and heterogeneity within allogamous populations, these methods must also be easily accessible, robust, and relatively simple if they are to going to be widely adopted in plant breeding especially where a multitude forage species are concerned. Indeed, the availability of high-throughput capillary electorophoresis instruments would now enable us to genotypically screen larger populations. However, our ability to accurately measure many phenotypes is another important and limiting factor in QTL detection. Nevertheless, results of this study suggest that linkage disequilibrium, created by population admixture and selection of founder parents, might be effectively used to identify and select QTLs in genetically broad-based, allogamous breeding populations.

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