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1 **QTL analysis of morphological, developmental and**
2 **winter hardiness-associated traits in perennial ryegrass**
3 **(*Lolium perenne* L.)**

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1 **ABSTRACT**

2 Quantitative trait loci (QTLs) for a number of agronomically important traits of
3 perennial ryegrass (*Lolium perenne* L.) were identified using a reference
4 molecular marker-based genetic map. Replicated phenotypic data was obtained
5 for a number of field-assessed morphological and developmental traits as well as
6 the winter hardiness-associated characters of winter survival and electrical
7 conductivity. Marker-trait association analysis was performed using a number of
8 methods, and a high degree of congruence was observed between the
9 respective results. QTLs were detected for morphological traits such as plant
10 height, tiller size, leaf length, leaf width, fresh weight at harvest, plant type,
11 spikelet number per spike and spike length, as well as the developmental traits of
12 heading date and degree of aftermath heading. A number of traits were
13 significantly correlated, and coincident QTL locations were identified. No
14 significant QTLs for winter survival in the field were identified. However, a QTL
15 for electrical conductivity corresponding to frost tolerance was located close to a
16 heading date QTL in a region that may show conserved synteny with
17 chromosomal regions associated with both winter hardiness and flowering time
18 variation in cereals. The QTL analysis of multiple phenotypic traits provides the
19 basis for marker assisted selection (MAS) of important agronomic characters,
20 allowing genetic improvement of yield, quality and adaptation in perennial
21 ryegrass breeding.

22

1 **KEYWORDS**

- 2 *Lolium perenne*, QTL analysis, morphological traits, heading date, winter
3 hardiness, conserved synteny, molecular breeding.

1 INTRODUCTION

2 Perennial ryegrass (*Lolium perenne* L.) is the most widely sown perennial forage
3 grass in temperate regions of the world. The popularity of perennial ryegrass in
4 pastoral agriculture is largely due to high yield of digestible nutrients combined
5 with good tolerance to grazing and adequate seed production (Wilkins, 1991).

6 The morphogenesis of individual grass plants within a grazed sward plays a
7 key role in determining herbage yield, persistence and recovery from grazing. In
8 vegetative plants, plant morphogenesis is described by three key variables: leaf
9 appearance rate, leaf elongation rate and leaf lifespan. The expression of each of
10 these traits is under both genetic and environmental control (Lemaire and
11 Chapman, 1996), and leaf development in *Lolium* has been demonstrated to be
12 under genetic control in a number of studies (Edwards and Cooper, 1963;
13 Rhodes, 1973; Hazard et al., 1996). Structural characteristics of plants such as
14 tiller number, leaf number and leaf size are the result of these morphogenetic
15 traits, and their measurement in breeding programs allows a dissection of the
16 complex herbage yield trait as well as predictions of the response to grazing.

17 Different ecoclimatic regions, or pastures under different grazing regimes,
18 may provide alternative selection goals for these traits. For instance, cool season
19 growth is an important breeding objective in climates with mild winters.
20 Mediterranean genotypes of perennial ryegrass have more rapid rates of leaf
21 appearance and elongation in winter than genotypes from Northern Europe and
22 hence better cool season herbage yield (Cooper, 1964). However, this trait may
23 be related to the onset of reproductive development (Kemp et al., 1989) and be

1 negatively correlated with survival during harsh winters. In addition, selection for
2 large leaves has been shown to increase yield under infrequent grazing (Rhodes
3 and Mee, 1980; Hazard and Ghesquière, 1997). However, interactions between
4 grazing management and optimal leaf size have been detected, with short-leaved
5 selections being better adapted to frequent cutting (Hazard and Ghesquière,
6 1997).

7 Genetic variation in morphogenetic traits associated with reproductive
8 development is of practical importance in perennial ryegrass breeding not only
9 due to the potential correlation between these traits and vegetative production,
10 but also to the association of these traits with seed yield (Elgersma, 1990a).
11 Traits such as spike numbers, spikelets per spike and florets per spikelet have
12 been shown to be heritable (Elgersma, 1990b).

13 The manipulation of these morphological and developmental traits in a
14 breeding program can be improved by knowledge of the underlying genetic
15 control mechanisms. The detection of QTLs associated with these traits in
16 perennial ryegrass is consequently likely to provide breeders with enhanced
17 possibilities for the development of highly adapted germplasm.

18 Perennial ryegrass is susceptible to winter stresses caused by both snow
19 moulds and low temperatures (Jamalainen, 1974; Jönsson and Nilsson, 1985;
20 Nakayama et al., 2001). In addition to the improvement of growth characteristics,
21 increased winter hardiness of perennial ryegrass is an important breeding
22 objective in northern cold climate regions. Genetic analysis of the components of
23 winter hardiness has been carried out in cereals such as wheat (*Triticum*

1 *aestivum* L.) and barley (*Hordeum vulgare* L.) (Cahalan and Law, 1979;
2 Brule-Babel and Fowler, 1988; Doll et al., 1989), allowing the chromosomal
3 location of the genes controlling these characters to be determined. A similar
4 approach may be employed for pasture grasses.

5 Genetic map-based analysis permits the dissection of complex phenotypes
6 by resolving the locations of interacting and pleiotropic genetic factors. There
7 have been relatively few reports to date of QTL analysis for agronomic traits in
8 perennial ryegrass, due to the absence of a sufficiently well developed genetic
9 map. An enhanced molecular marker-based genetic linkage map of perennial
10 ryegrass has recently been constructed through the activities of the International
11 *Lolium* Genome Initiative (ILGI) (Forster et al., 2001), using the p150/112
12 one-way pseudo-testcross mapping population. The current map contains 109
13 restriction fragment length polymorphism (RFLP) loci detected by heterologous
14 probes from wheat, barley, oat and rice. Comparative genetic mapping has
15 allowed the alignment of the perennial ryegrass genetic map with those of wheat,
16 rice and oat, revealing substantial conserved synteny with the genomes of
17 Triticeae species (Jones et al., 2002a). The p150/112 genetic map has been
18 further enhanced by the assignment of nearly 100 polymorphic perennial
19 ryegrass simple sequence repeat (LPSSR) loci (Jones et al., 2001) to locations
20 on each of the seven linkage groups (Jones et al., 2002b). As a consequence, a
21 total of more than 200 co-dominant genetic markers have been mapped in the
22 p150/112 family, along with about 200 amplified fragment length polymorphism
23 (AFLP) loci, permitting detailed genetic analysis of traits that vary within this

1 population. The SSR loci provide the means to align genetic maps between
2 different mapping families and extrapolate QTL locations between divergent
3 germplasm sources.

4 Our objective in this study was to use the enhanced genetic map of perennial
5 ryegrass to locate QTLs for a range of agronomic phenotypic traits in the
6 p150/112 population. The emphasis was on morphological and developmental
7 traits associated with pasture productivity, reproductive development traits and
8 winter hardiness characters that may influence survival and subsequent
9 performance in cold climate environments.

MATERIALS AND METHODS

Genetic Mapping Family

The p150/112 reference genetic mapping population was derived from a pair-cross between a multiply heterozygous plant as pollinator and a doubled haploid (DH) as the female parent (Bert et al., 1999; Jones et al., 2002a). The cross was generated at the Institute of Grassland and Environmental Research (IGER), Aberystwyth, UK, and clonal replicates of up to 183 progeny individuals and the multiply heterozygous parent were distributed to ILGI participant laboratories for genotypic and phenotypic analysis. The doubled haploid genotype (DH290) did not survive and was consequently not available for phenotypic analysis.

Phenotypic Assessment of Morphological and Developmental Characters

Individual plants from the p150/112 mapping family were transplanted at Nagasaka, Japan (35°49' N, 138°22' E) in the field nursery of the Yamanashi Prefectural Dairy Experiment Station (YPDES) in July 1996 with five replicates of each genotype in a randomised complete block design. Morphological characters (plant height, tiller number, tiller size [diameter of the stem of the reproductive tiller], leaf length, leaf width, fresh weight at second harvest, plant type, number of spikelets per spike and spike length) and developmental characters (heading date and aftermath heading) were measured in the subsequent year. Two cutting regimes were performed in 1997, the first at the

1 heading date for each genotype and the second on July 14th for all genotypes.
2 Plant height (maximum length in cm from the base to the top of the plant) and tiller
3 number were measured in cm on 30th June 1997, while spike length, leaf length
4 and leaf width (in cm) and tiller size (in mm) were measured at the heading date
5 for each genotype. Leaf length and leaf width were measured using the flag
6 leaves from single tillers, and spike length, number of spikelets per spike and tiller
7 size were measured on the same tillers. Fresh weight at second harvest (14th July,
8 1997) was measured in grams, plant type was measured (on 30th June, 1997) on
9 a scale from 1-9 with 1 being most erect and 9 being most prostrate, heading date
10 (ear emergence) was measured in days after May 1st 1997 and the degree of
11 aftermath heading (on 14th July 1997) was measured on a scale from 0-9 with 0
12 corresponding to no heads and 9 corresponding to many heads. Measurements
13 were made on one plant per genotype from each of the five replicates for tiller
14 number, fresh weight, plant type, plant height, heading date and aftermath
15 heading. Measurements were made on 5 leaves from two replicates (10 leaves in
16 total) for leaf length, leaf width, number of spikelets, spike length and tiller size at
17 the time of heading.

18

19 **Phenotypic Assessment of Winter Hardiness Characters**

20 One plant of each p150/112 mapping family genotype was grown outside in pots
21 at the National Agricultural Research Centre for Hokkaido Region (NARCH)
22 located at Sapporo, Japan (43°00' N, 141°25' E). Electrical conductivity was
23 measured on 3 leaves from each plant. Leaves were excised in 5 cm long

1 sections at a 10-15 cm height from the base in December 1999, and were
2 shredded, placed into culture dishes and transferred to a temperature of -2°C.
3 After a 12 hr equilibration period, the temperature was lowered manually in 1°C
4 decrements every one hour to a final value of -6°C and samples were then held
5 for 8 hrs before being placed in microtubes. Distilled water (1.0 ml) was added to
6 each tube, and samples were held at 5°C for 12 hrs. The conductivity of the
7 resulting solution was measured using a conductance meter. A comparative
8 value for 100% leakage was obtained by freezing replicate samples from each
9 genotype at -80°C for 4 hrs.

10 Individual plants from the p150/112 mapping family were also grown at the
11 field premises of NARCH, with four replications in replicated block design from
12 1999 onwards. Survival in the field at Sapporo following the winter of 1999-2000
13 was measured in April 2000 using a visual assessment score (from 1 to 5) of plant
14 recovery on all 4 replicates.

15

16 **Statistical Analysis**

17 Data analyses were carried out in SAS (SAS Institute Inc.). The significance of
18 progeny and replicate effects were analysed using general linear modelling (Proc
19 GLM), Broad sense heritabilities were calculated according to Wricke and Weber
20 (1986). The Shapiro-Wilk statistic (W-test) was used to assess the normality of
21 averaged data (normal option in Proc UNIVARIATE). Spearman's rank-order
22 correlation coefficients were determined for pair-wise comparisons of averaged
23 trait data (spearman option in Proc CORR).

1

2 **QTL Analysis**

3 A framework set of genetic markers from the p150/112-based reference map
4 (Jones et al., 2002a), including the majority of the heterologous RFLP loci, was
5 combined with the perennial ryegrass SSR locus data (Jones et al., 2002b) to
6 produce a composite dataset for QTL analysis of the phenotypic data. Following
7 genetic map construction using MAPMAKER 3.0, a sub-set of marker loci was
8 selected to provide even coverage of the genome with marker intervals of close
9 to 5 cM, and consensus map distances were subsequently used. Simple linear
10 regression (SMR) was initially employed to identify significant variation with
11 selected genetic markers to provide approximate locations for QTLs. The
12 log-of-odds (LOD) score of association between the genotype and trait data was
13 calculated using interval mapping (IM) in MAPMAKER/QTL (Lander et al., 1987)
14 with the free model of QTL effect. A minimum LOD threshold of 2.0 was selected
15 for significance of location of the QTL for IM. Composite interval mapping (CIM:
16 Zeng, 1994) was performed using the Windows QTL Cartographer 2.0
17 application (Basten et al., 1994). Permutation analysis (1000 iterations) was used
18 to establish an experiment-wise significance value at the 0.05 confidence level
19 defined as a minimum LOD threshold for each trait in CIM (Churchill and Doerge,
20 1994; Doerge and Churchill, 1996). For each form of interval analysis, the
21 maximum LOD value associated with the most closely linked marker, the weight
22 value associated with additive marker allele effects and the proportion of the
23 phenotypic variance attributable to the QTL were tabulated.

1 **RESULTS AND DISCUSSION**

3 ***Analysis of phenotypic variation for agronomic traits***

4 The distribution data for morphological and developmental traits are shown in
5 Figures 1(A) and (B). Substantial variation is observed for the majority of these
6 characters, with a range of 30.8 cm for plant height, 194.3 g for fresh weight at
7 second harvest, 24 days for variation in heading date and 4 units for plant growth
8 type from moderately erect to moderately prostrate. The corresponding data for
9 the winter hardiness characters are shown in Figure 1(C). The average
10 phenotypic score for the heterozygous parent was located towards one tail of the
11 progeny distribution range for the majority of traits. The parental phenotype was
12 very close to the progeny mean for the spike length, number of spikelets per spike
13 and winter survival traits, providing evidence for transgressive segregation.

14 Significant phenotypic variation was detected between progeny
15 individuals for all traits except for leaf width (Table 1). Broad sense heritability
16 values for significantly variable traits ranged from 0.46 for leaf length to 0.9 for
17 heading date. Significant replicate effects were observed for tiller number, plant
18 type and winter survival. The distribution of averaged data deviated significantly
19 from normality for tiller number and aftermath heading traits (skewed towards
20 high numerical values) and for the leaf length, fresh weight, plant type, heading
21 date and electrical conductivity traits (skewed towards low values).

22 Coefficients of phenotypic correlations between traits ranged from
23 non-significant to a maximum value of 0.68 (Table 2). A number of the

1 morphological and developmental traits showed highly significant positive
2 correlations, such as plant height with spike length, tiller size and leaf length, and
3 plant type with heading date. The positive correlations between shoot growth
4 characteristics reflect the overall developmental pattern of a large, vigorous plant.
5 The reproductive morphogenetic trait of number of spikelets per spike is
6 positively correlated with these characteristics, and may also reflect general plant
7 vigour. The positive correlation between heading date and plant type has been
8 previously described for populations such as the Australian variety 'Kangaroo
9 Valley' (Shah et al., 1990), in which erect growth habit is associated with early
10 flowering and prostrate growth habit is associated with later flowering. These
11 effects are also consistent with the observed positive correlation in this study
12 between later heading date and reduced plant height. However, the correlation
13 between later flowering and decreased tiller number is not general and may be an
14 artefact due to the complex history of the heterozygous parent in this cross.
15 Significant negative correlation between heading date and plant height was also
16 anticipated in this study, as each genotype was cut at the heading date and
17 showed variable rates of regrowth at the time of data collection. However, the
18 variation of plant type and tiller number is likely to be largely independent of the
19 rate of regrowth. Variation for tiller number will include the large number of tillers
20 formed during growth prior to flowering, as well as the relatively small number
21 formed during regrowth.

22 There were no significant correlations between winter survival and any of the
23 morphological or developmental traits measured.

1

2 ***Comparison of analytical techniques for marker-trait association***

3 Significant associations between marker and trait data were established for the
4 majority of traits using single marker regression (SMR), based on analysis of
5 variance (ANOVA) at the $p < 0.01$ significance level. Interval mapping (IM)
6 subsequently identified 17 significant QTLs (maximum LOD ≥ 2) for 11 of the 13
7 measured traits. Composite interval mapping (CIM) was also performed on the
8 dataset following determination of empirical LOD thresholds, allowing the
9 identification of 20 QTLs for the 11 traits (Table 3). A large proportion of QTL
10 locations were identified by both IM and CIM. For example, for the heading date
11 trait, genetic markers in the interval from 39.9 to 72.3 cM on linkage group (LG) 4
12 were significantly associated with the trait data by SMR, while IM detected a QTL
13 with maximum LOD value of 4.0 close to the xlpssrh01h06 locus at 53.5 cM, and
14 CIM detected a QTL with a maximum LOD value of 3.6 at 56.3 cM with an
15 empirical LOD threshold of 2.9.

16 A number of discrepancies in QTL detection were identified through
17 comparison of the different analytical techniques. In several instances, SMR
18 analysis revealed significant marker-trait associations and IM detected QTLs
19 above the LOD threshold, but the trait-specific threshold value determined for
20 CIM was not exceeded. For instance, for the spike length trait, genetic markers in
21 the interval from 25.5 to 84.1 cM on LG1 were significantly associated with the
22 trait data, while IM detected a QTL with maximum LOD value of 4.7 close to the
23 e33t50175 locus at 53.9 cM, but CIM detected a QTL with a maximum LOD value

1 of 2.7 at 53.9 cM with an empirical LOD threshold of 2.8. In this case, the
2 identification of a significant region of the genome controlling the trait by SMR
3 and IM and the closeness of the maximum and threshold values determined by
4 CIM would tend to support the inference of a genuine genetic effect in this region.
5 Other examples are less obvious: for the trait of plant height, SMR analysis
6 revealed significant associations for markers on LG1 and IM identified a QTL with
7 a maximum LOD value of 3.6, but the maximum LOD value determined by CIM
8 was 1.3, considerably lower than the threshold value of 2.8. In addition, for both
9 the leaf length and leaf width traits, only single markers were significantly
10 associated with the respective trait data. IM identified significant QTLs with
11 maximum LOD values of 2.1 for both traits, but no significant QTLs were
12 identified by CIM. Clearly, QTL detection in such circumstances should be treated
13 as indicative rather than definitive.

14 The different results obtained by different forms of analysis are also shown for
15 the trait of electrical conductivity. The terminal marker on LG4 (xr2702Bb) was
16 identified as significantly associated with the trait and IM detected a QTL with
17 maximum LOD value of 2.0 close to this marker. However, no significant QTLs
18 were detected on this LG by CIM. In contrast, two significant QTLs on LG6 were
19 detected by CIM, with maximum LOD values greater than the threshold value of
20 2.6. No supporting evidence is available from SMR or IM analysis for these QTL.
21 The LG6 electrical conductivity QTLs are in close linkage and in repulsion phase,
22 as indicated by weight values of -2.99 and 3.7 respectively, as well as accounting
23 for similar proportions of the phenotypic variance. The similar but opposing

- 1 effects of these regions may account for the failure of detection by SMR and IM.
- 2 For both LGs (4 and 6), but on different criteria, the QTL effects for electrical
- 3 conductivity are suggestive, but require further validation.

1 ***Genetic control of morphological and developmental traits***

2 Between 1 and 3 QTLs were detected for each of the morphological and
3 developmental characters with either IM or CIM (Table 3, Figure 2). QTLs for
4 different traits were frequently located in the same chromosomal region, with
5 coincident groups on LGs 1, 3, 4 and 5. The coincident QTLs corresponded to
6 traits that were significantly correlated, and the directions of the QTL effects were
7 in agreement with the sign of the correlations. For instance, coincident QTLs for
8 plant height, tiller size, number of spikelets per spike and spike length were
9 identified by IM on LG1, and these traits show significant positive phenotypic
10 correlations. The QTL weight values determined by both types of mapping
11 analysis (Table 3) were also all positive.

12 Coincident QTLs for traits associated with plant size have been identified in
13 three regions, most strikingly on LGs 1 and 3. The positive weight values of the
14 co-locating QTLs suggest two possible interpretations. Allelic variation at a single
15 pleiotropic locus could be responsible for the concurrent increase (or decrease)
16 of phenotypic values for the relevant traits. In barley, large QTL effects on plant
17 height are associated with variation at the *denso* dwarfing gene (Bezant et al.
18 1996), which maps to 3H, the syntenic counterpart of perennial ryegrass LG3
19 (Jones et al., 2002a). It is possible that single underlying scale-determining loci
20 may have been detected in this study. Alternatively, a number of *cis*-linked alleles
21 with similar directions of effect may be present at different loci. In either case,
22 selection for a single set of linked markers would lead to an increase for each
23 trait.

1 Despite the highly significant correlations between heading date and the
2 morphogenetic traits of plant type, tiller number and plant height, only one QTL
3 (for plant type) co-locates with the heading date QTL. This observation supports
4 the previous inference that plant type and tiller number are largely independent of
5 the period of regrowth after cutting, and that the harvesting regime following the
6 heading date did not impair the ability to detect QTLs for these traits. If the QTLs
7 resulting in phenotypic variation for these traits had been a simple reflection of
8 genetic factors segregating for heading date, a large number of coincident QTLs
9 would have been expected.

10 The only traits in this study that reveal non-coincident QTLs were plant type
11 (LG7) and aftermath heading (LG6). These QTLs offer the potential to select for
12 the corresponding traits without correlated effects on other traits. Plant type is of
13 significance in the breeding of perennial ryegrass for turf quality, which is
14 associated with a more prostrate growth habit. The large plant type QTL on LG7
15 provides a good target for marker-assisted selection of growth habit. Aftermath
16 heading is usually associated with early flowering perennial ryegrass varieties
17 suitable for hay making, which tend to show reduced perenniality and persistence.
18 For this reason, a high degree of aftermath heading is likely to be disfavoured as
19 a breeding objective but could provide a target for counter-selection based on
20 linked marker analysis.

21 A single QTL for heading date was observed in the current study. However, a
22 number of QTL positions for this trait have been reported from the analysis of
23 single mapping populations in other Poaceae species. Previous mapping studies

1 in perennial ryegrass have produced more complex results (Hayward et al., 1994).
2 The absence of common markers between the population used in this and the
3 present study prevents any inference of common location. The number of QTLs
4 and their relative importance may vary according to the origin of the genotypes
5 used to construct mapping families. Studies on geographical populations of
6 *Lolium* species covering the climatic range from the Mediterranean region to
7 northern and central Europe revealed a regular cline in flowering responses to
8 temperature and photoperiod (Cooper, 1960). The heterozygous parent of the
9 p150/112 mapping population was derived from a cross between eastern
10 European (Romanian), southern European (north Italian ecotypes) and northern
11 European ('Melle' or 'S23') genotypes, and might be expected to represent a
12 variety of response genes. It is also likely that small QTLs for heading date have
13 not been detected in this analysis. The single QTL for heading date on LG4
14 accounts for about 20% of the phenotypic variation based on IM, but the
15 character shows a high broad sense heritability (0.90), suggesting that a
16 substantial proportion of the heritable variation has not been attributed to specific
17 genomic regions.

18

19 ***Genetic control of winter-hardiness traits***

20 No significant QTLs were detected for winter survival. It is known that field
21 assessment of winter survival may be confounded by experimental errors (Fowler,
22 1979). The electrical conductivity method, also known as the ion leakage method,
23 has been used extensively for the evaluation of freezing tolerance through

1 measurement of the release of cellular electrolytes after freezing (Dexter et al.,
2 1930; Dexter et al., 1932). Previous studies of the winter survival of barley, wheat,
3 rye and triticale cultivars in Finland, where frost was considered to be the more
4 important stress factor than snow (Hömmö, 1994), showed good correlations with
5 hardening ability as assessed by the electrical conductivity method. A single QTL
6 for electrical conductivity was detected in the upper part of LG4 by SMR and IM,
7 but not CIM, accounting for 11.8% of the total phenotypic variation and adjacent
8 to the heading date QTL. Late flowering and reduced freezing tolerance (as
9 indicated by high electrical conductivity) is not significantly correlated in this cross,
10 but the weight values of the linked QTLs were positive, suggesting that *cis*-linked
11 alleles were causing an increase in phenotypic values of both traits. This is
12 inconsistent with previously identified negative correlations between freezing
13 tolerance and heading date (Humphreys and Eagles, 1988) and may be due to
14 the complex nature of the cross.

15 Winter hardiness in wheat (Sutka, 1994; Galiba et al., 1995, 1997) and barley
16 (Pan et al., 1994) is associated with QTLs on the homoeologous group 5
17 chromosomes in the same region as the vernalisation response genes that
18 control heading date. In this study, winter hardiness-associated and heading date
19 QTLs were located on LG4 in perennial ryegrass. Perennial ryegrass LG4 has
20 been proposed to correspond predominantly to the homeologous group 4
21 chromosomes of the Triticeae cereals (Jones et al., 2002a). However, the upper
22 part of LG4 has been shown to contain heterologous RFLP markers that map to
23 the wheat homeologous group 5 chromosomes and its syntenic counterpart, rice

1 chromosome 3. Evolutionary translocations between group 4 and group 5
2 homoeologous chromosomes have been observed for several Triticeae genomes
3 (Devos et al., 1995). A comparative map has been constructed for meadow
4 fescue (*Festuca pratensis* Huds.) using heterologous anchor RFLPs, many of
5 which are common with the perennial ryegrass study (Alm, 2001, 2003). A region
6 syntenic with the portion of the Triticeae 5L chromosomes that corresponds to
7 rice chromosome 3 is present on the upper part of meadow fescue LG4. The
8 *Lolium* and *Festuca* genera are closely related, and the high level of
9 recombination observed in triploid F₁ hybrids between *L. perenne* and *F.*
10 *pratensis* suggests conservation of gene order (King et al., 1998). An
11 evolutionary translocation between linkage groups corresponding to groups 4
12 and 5 of the consensus wheat map may have occurred before the divergence of
13 the Poaceae grasses. Alm (2001) also performed QTL analysis of frost and drought
14 tolerance in meadow fescue and detected a small QTL for frost tolerance on LG4.
15 This provides further evidence for the presence of genes for winter hardiness on
16 the group 4 chromosomes of the *Lolium* and *Festuca* genera as compared to the
17 group 5 homoeologous chromosomes of the Triticeae.

18

19 **Conclusions**

20 The analysis presented here provides an insight into the genetic control of a
21 number of important growth and adaptation characters in current perennial
22 ryegrass breeding programs, along with an interpretation of their
23 interdependence in genetic and developmental terms. However, it should be

1 noted that although substantial replication was performed within the experimental
2 design, the study is restricted to a particular locality and time. For this reason, it is
3 conceivable that genotype x environment (G x E) interactions could lead to the
4 detection of different QTL locations for equivalent traits in other studies. The
5 establishment of the p150/112 mapping family at a number of ILGi-participant
6 laboratories now provides the basis for such comparative QTL mapping studies.

7 The use of several marker-trait association analysis techniques such as SMR,
8 IM and CIM provides the means to assess the robustness of QTL detection in a
9 single experimental design. A high degree of congruence between the results of
10 different methods was observed in the present study, with the majority of QTLs
11 detected by the standard IM analysis confirmed by SMR and CIM. However, CIM
12 detected a number of extra QTLs for several traits, and estimates of maximum
13 LOD value with CIM showed both increases and decreases of significance
14 compared to IM. These results have provided evidence to support the inference
15 of real genetic effects associated with particular genomic regions, and may be
16 used to prioritise the selection of putative marker-QTL combinations for
17 implementation studies.

18 The identification of QTL locations for selected agronomic traits by
19 molecular-marker based mapping will allow the design of appropriate
20 marker-assisted selection strategies. This will include co-selection for desirable
21 characters with coincident QTL locations and counter-selection to break potential
22 unfavourable linkages between negatively correlated traits. The linked marker
23 haplotypes associated with favourable QTL alleles in the current family are

1 available for marker assisted selection. The use of a reference genetic map
2 containing readily transferable SSR and RFLP markers will permit the inference
3 of common QTL locations for the same traits in other experimental pair-crosses,
4 as well as comparative genetic analysis of homologous characters in other
5 Poaceae species.

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1 **FIGURE LEGENDS**

2

3 **Fig. 1**

4 Frequency distribution bar-charts for traits associated with (A) vegetative
5 morphogenesis, (B) reproductive morphogenesis and development and (C)
6 winter hardiness measured in the progeny set of the p150/112 reference genetic
7 mapping population in perennial ryegrass. Phenotypic scores have been
8 assigned to intervals on the y=0 axis up to the maximum value indicated for each
9 interval. The mean score of the heterozygous p150/112 parent is indicated in the
10 relevant interval by an arrow, with the appropriate numerical value. The doubled
11 haploid parent was not available for phenotypic analysis.

12

13 **Fig. 2**

14 Location of QTLs for morphological traits, developmental traits and electrical
15 conductivity on the SSRP, RFLP and AFLP-based reference genetic map of
16 perennial ryegrass, based on the results of interval mapping (IM). The maximum
17 likelihood position of the QTL is indicated with an arrow. Bar and line lengths
18 indicate a LOD drop of 1.0 and 2.0 respectively on either side of the maximum
19 likelihood position.

Table 1. General statistics for phenotypic traits measured in this study

	Reps	W ^a	Skew ^b	Significance ^c		
				Progeny	Rep	H ^{2d}
Plant Height (cm)	5	0.98 ^{ns}	-0.39	***	ns	0.74
Tiller Number	5	0.93 ^{**}	-0.42	***	***	0.81
Tiller Size (mm)	2	0.97 ^{ns}	-0.39	***	ns	0.59
Leaf Length (cm)	2	0.74 ^{***}	3.79	**	ns	0.46
Leaf Width (cm)	2	0.97 ^{ns}	0.03	ns	ns	0.30
Fresh Weight	5	0.96 [*]	0.57	***	*	0.73
Plant Type (1-9)	5	0.91 ^{***}	0.84	***	***	0.86
Number of Spikelets per Spike	2	0.96 ^{ns}	-0.69	***	ns	0.55
Spike Length (cm)	2	0.98 ^{ns}	-0.49	***	ns	0.85
Heading date (days after 1.05.97)	5	0.96 [*]	0.67	***	ns	0.90
Aftermath heading (0-5)	5	0.95 ^{**}	-0.41	***	ns	0.73
Electrical Conductivity	1	0.91 ^{***}	1.35	-	-	-
Winter survival (0-5)	4	0.97 ^{ns}	0.10	***	**	0.48

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = $P > 0.05$.

^a Normality of averaged data tested using the Shapiro-Wilk statistic.

^b Skewness of averaged data. Negative deviations indicate skewness towards high numerical values.

^c Significance of progeny and replicate effects analysed using general linear modeling.

^d Broad sense heritabilities.

Table 2. Correlation coefficients between traits estimated using Spearman's rank correlation analysis

	Plant Height	Tiller Number	Tiller Size	Leaf Length	Leaf Width	Fresh Weight	Plant Type	Number of Spikelets per Spike	Spike Length	Heading Date	Aftermath Heading	Electrical Conductivity
Tiller Number	0.63 ^{***}											
Tiller Size	0.45 ^{***}	0.25 [*]										
Leaf Length	0.61 ^{***}	0.43 ^{***}	0.55 ^{***}									
Leaf Width	0.18 ^{ns}	0.11 ^{ns}	0.5 ^{***}	0.39 ^{**}								
Fresh Weight	0.56 ^{***}	0.75 ^{***}	0.34 ^{**}	0.48 ^{***}	0.30 ^{**}							
Plant Type	-0.41 ^{***}	-0.32 ^{**}	0.05 ^{ns}	0.10 ^{ns}	0.17 ^{ns}	-0.04 ^{ns}						
Number of Spikelets per Spike	0.36 ^{**}	0.19 ^{ns}	0.46 ^{***}	0.36 ^{***}	0.31 ^{**}	0.34 ^{ns}	-0.19 ^{ns}					
Spike Length	0.68 ^{***}	0.38 ^{***}	0.67 ^{***}	0.68 ^{***}	0.35 [*]	0.51 ^{***}	-0.01 ^{ns}	0.63 ^{***}				
Heading Date	-0.63 ^{***}	-0.58 ^{***}	-0.27 [*]	-0.18 ^{ns}	-0.01 ^{ns}	-0.51 ^{***}	0.60 ^{***}	-0.20 ^{ns}	-0.33 ^{**}			
Aftermath Heading	-0.04 ^{ns}	-0.3 ^{**}	0.10 ^{ns}	0.15 ^{ns}	0.15 ^{ns}	-0.14 ^{ns}	0.07 ^{ns}	0.22 [*]	0.05 ^{ns}	0.06 ^{ns}		
Electrical Conductivity	0.05 ^{ns}	0.15 ^{ns}	-0.08 ^{ns}	0.08 ^{ns}	0.08 ^{ns}	-0.24 [*]	-0.07 ^{ns}	0.12 ^{ns}	0.09 ^{ns}	-0.03 ^{ns}	-0.22 ^{ns}	
Winter Survival	-0.02 ^{ns}	-0.10 ^{ns}	0.14 ^{ns}	0.03 ^{ns}	0.03 ^{ns}	0.07 ^{ns}	0.19 ^{ns}	-0.01 ^{ns}	0.07 ^{ns}	0.01 ^{ns}	0.14 ^{ns}	0.02 ^{ns}

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ns = $P > 0.05$.

Table 3. Summary data for marker-trait association analysis in the perennial ryegrass p150/112 reference genetic mapping family

Trait	Linkage group in p150/112 reference genetic map	Single marker regression cM region pr(f) <0.01	Interval mapping			Composite interval mapping					
			Maximum LOD Value	Weight ^a	Phenotypic variance attributable to QTL (%)	LOD threshold ^b	Maximum LOD Value	Weight ^a	Phenotypic variance attributable to QTL (%)	Maximum LOD position (cM) ^c	Maximum LOD position (cM) ^c
Plant Height (cm)	1	25.6-84.1	3.6	6.0	22.0	2.8	1.3	3.65	7.5	65.9	43.1
	3	31.5-72.5	5.4	7.0	29.0		2.7	5.16	12.6	52.4	44.1
Tiller Size (mm)	1	25.6-92.3	4.8	0.28	32.7	2.7	2.6	0.20	12.0	55.9	43.1
	3	22.6-90.7	5.1	0.29	28.0		4.3	0.26	16.1	39.1	31.1
Leaf Length (cm)	5	16.6	2.1	2.1	11.6	5.5	1.0	3.26	6.1	15.3	
Leaf Width (cm)	3	39.1	2.1	0.04	11.3	2.8	2.3	0.06	10.4	39.1	31.1
Fresh Weight (g)	4	-				2.8	3.3	-32.50	13.2	19.8	10.1
Plant Type (0-9)	5	0-30.4	4.2	39.3	23.0		5.6	39.30	22.8	18.6	1.1
	4	52.1-112.2	3.0	0.78	16.8	2.8	4.0	1.56	13.9	52.1	51.1
	4	52.1-112.2	3.4	0.83	18.1		2.2	0.50	5.9	100.7	90.1
Number of Spikelets per Spike	7	35.9-88.5	6.5	-1.1	31.4		8.5	-1.01	26.8	59.0	4.1
	1	84.1-92.3	2.5	1.8	14.5	2.8	3.4	2.04	16.5	79.9	53.1
Spike length (cm)	1	25.6-84.1	4.7	3.8	29.9	2.8	2.7	2.70	11.8	53.9	43.1
	3	31.5-87.2	4.3	3.5	23.4		2.6	2.59	11.2	58.2	51.1
	5	0-44.6	3.3	3.0	18.8		3.6	2.68	13.8	16.6	1.1
Heading Date (d)	4	39.9-72.3	4.0	4.7	20.9	2.9	3.6	4.64	14.2	56.3	51.1
Aftermath heading (0-5)	6	61.2-69.1	2.3	-0.4	12.7	2.9	2.6	-0.42	10.7	64.9	56.1
Electrical conductivity	4	0	2.0	1.9	11.8	2.6	1.6	1.71	8.8	6.0	
	6	-					2.7	-2.99	11.8	50.0	4.1
	6	-					3.3	3.70	12.6	58.9	56.1

^aDefined as the additive effect on average phenotype of substituting a single B allele for an A allele at the relevant marker locus in an AB x AA backcross mapping structure.

^bBased on the results of permutation analysis with 1000 iterations.

^cBased on the genetic map coordinates of Jones et al. (2002b).

Fig. 1(A)

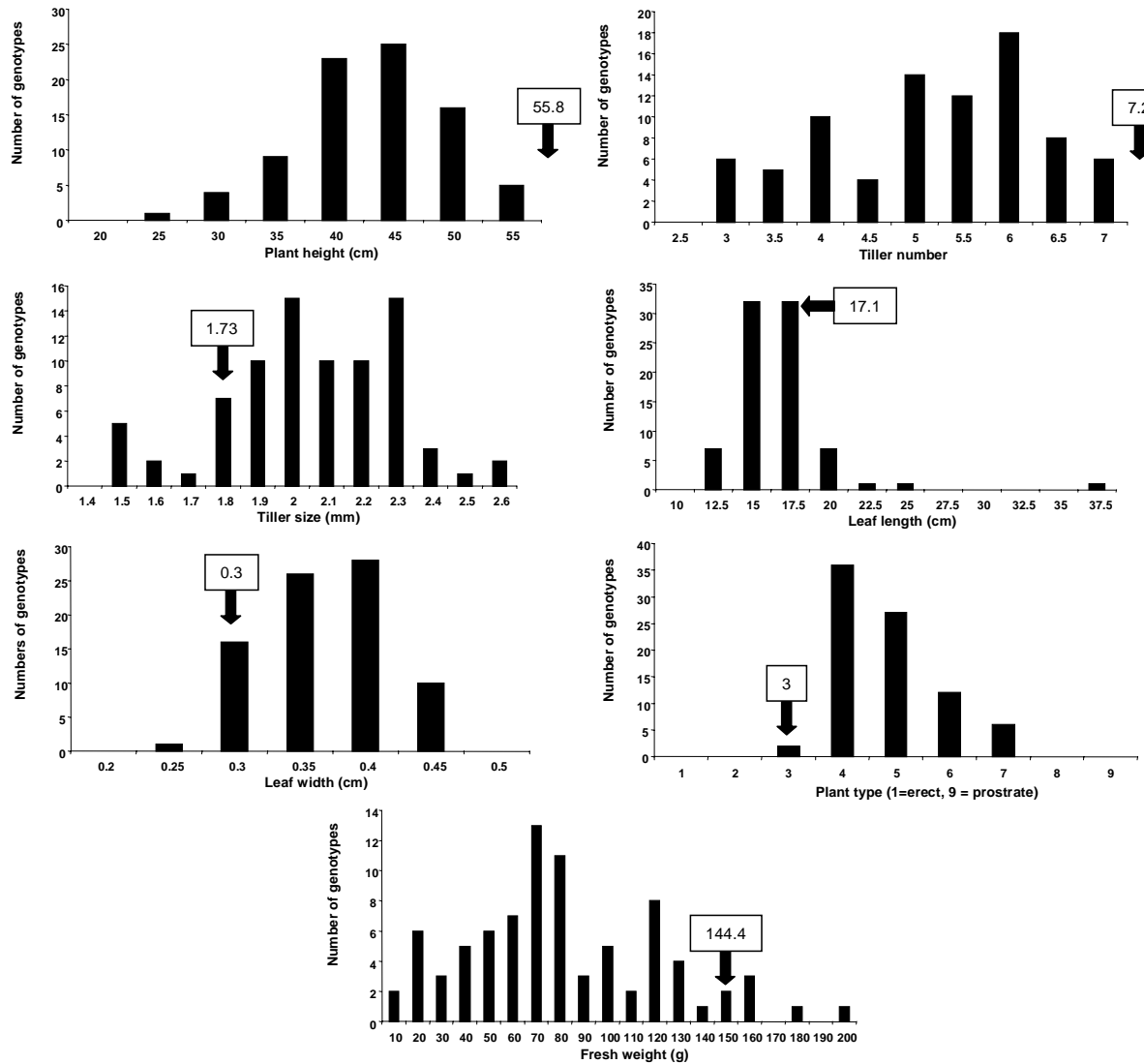
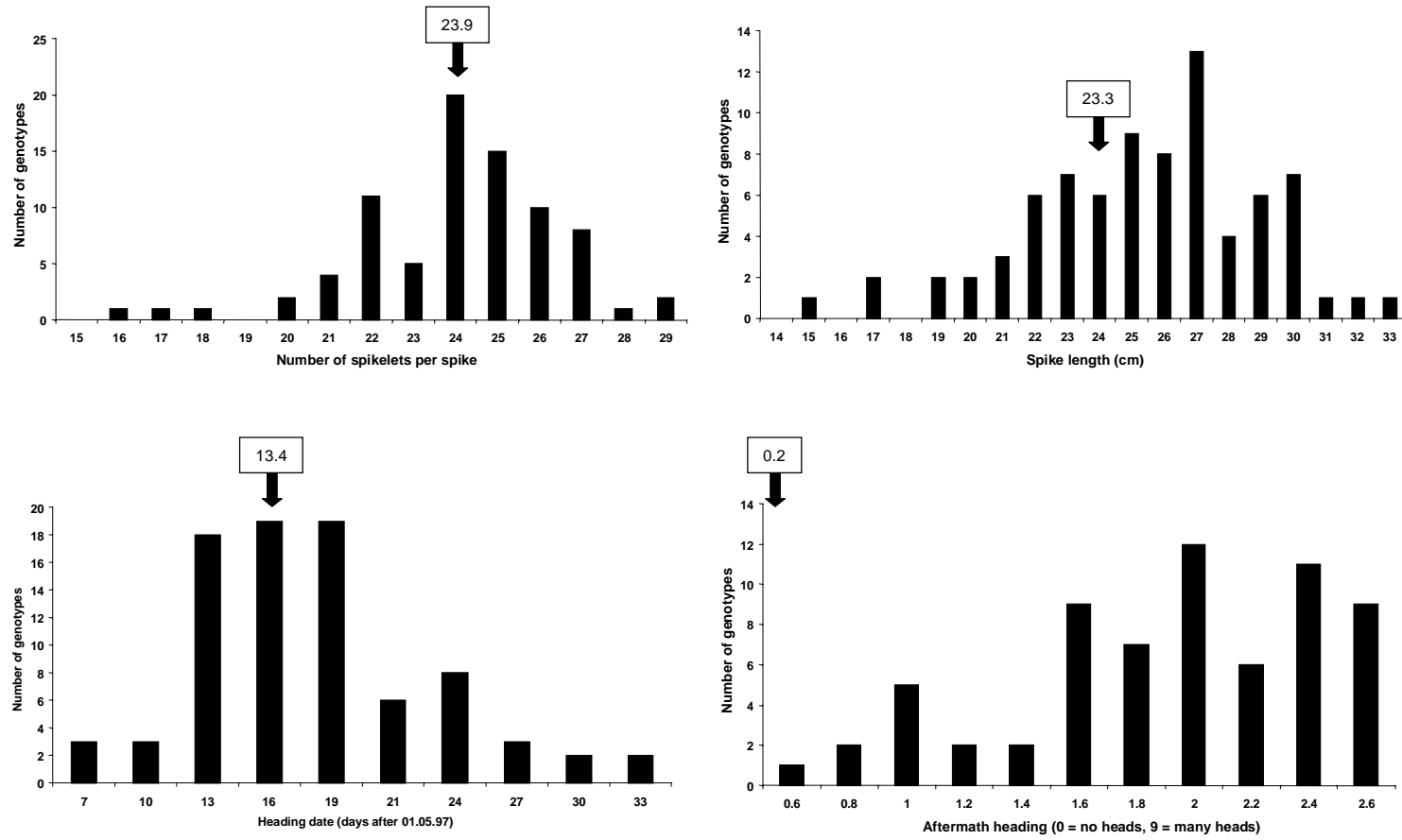


Fig. 1 (B)



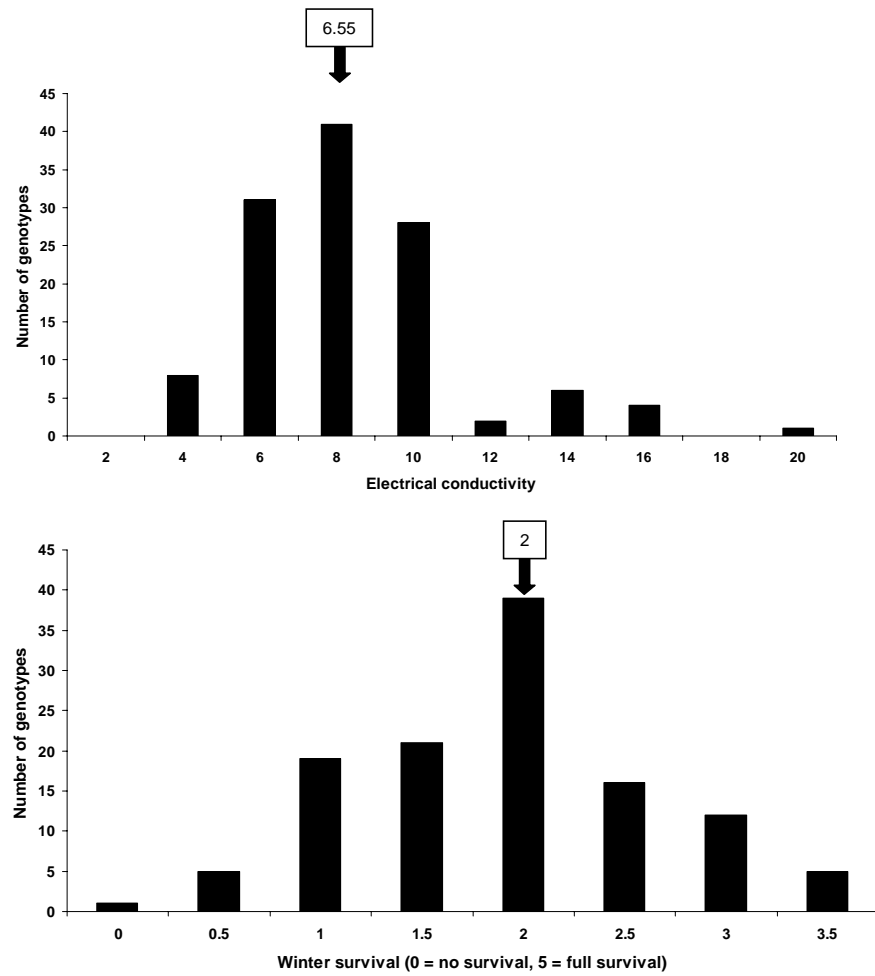


Fig. 1 (C)

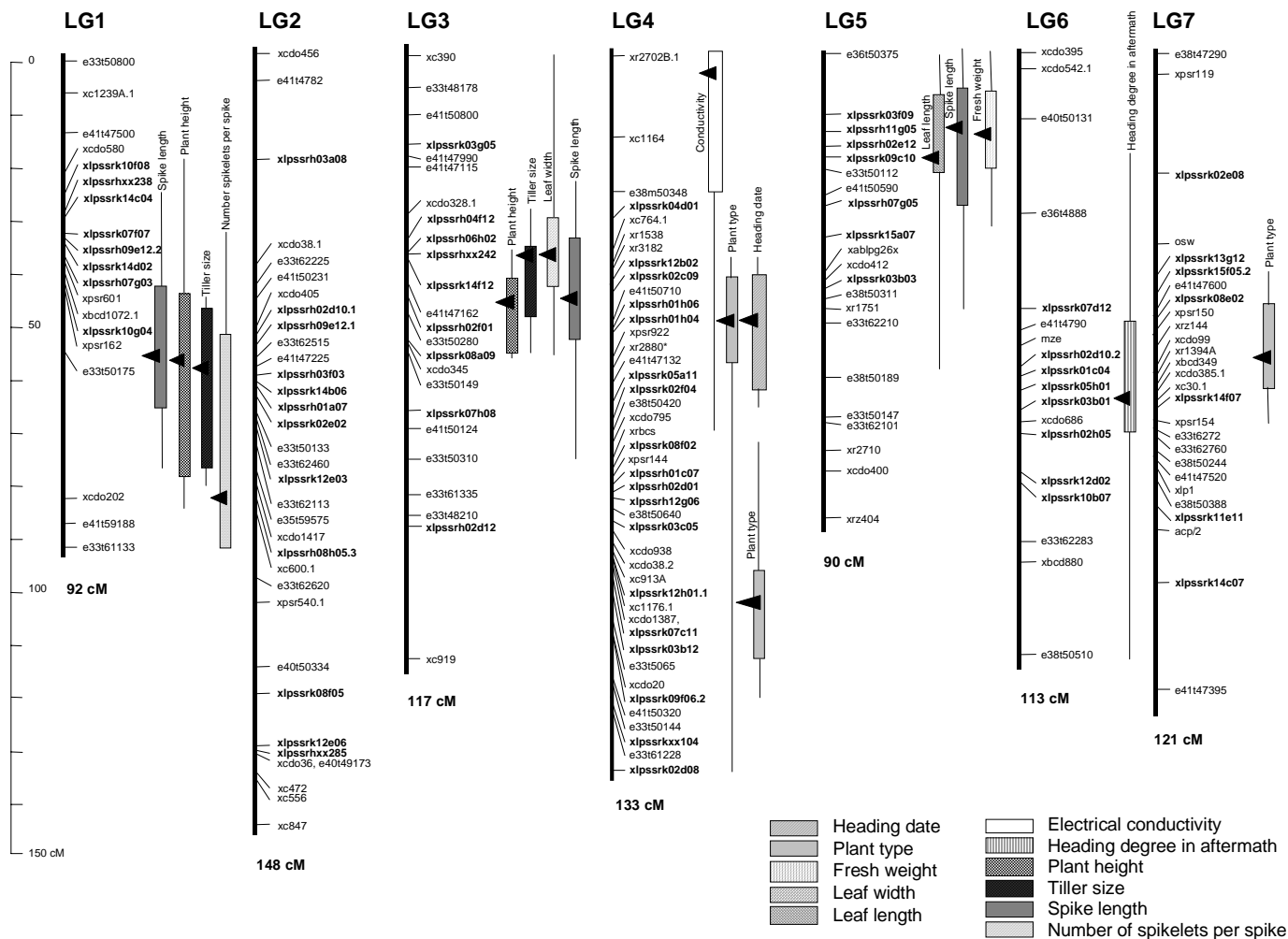


Fig. 2