Phenotypic assessment and quantitative trait locus (QTL) analysis of herbage and seed production traits in perennial ryegrass (*Lolium perenne* L.)

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Abstract

The aims of this study were to develop a genetic linkage map of perennial ryegrass, identify quantitative trait loci (QTL) for herbage and seed production traits, and to identify DNA markers associated with QTL for use in marker-assisted selection (MAS).

Major traits identified for herbage production were leaf elongation rate (LER), leaf lamina length (LL), tiller number (TN) and tiller weight (TW), and for increased seed production were seed yield per head (SdYH), reproductive tiller number (RT), reproductive tillers with matured heads (TMH), florets per head (FH), spikelets per head (SH), florets per spikelet (FS), floret site utilization (FSU) and seed weight (TSW).

A genetic linkage map spanning 582 centimorgans (cM) was constructed with EST-SSR (simple sequence repeat markers derived from expressed sequence tags) and used to identify QTL for herbage dry weight (DW) and seed yield per plant (SdYP), and their key component traits. Significant genotype by environment effects were encountered for herbage yield, with fewer QTL identified in spring than in autumn. For some traits, ranking of genotypes differed greatly between seasons and different QTL were identified.

QTL for DW were identified on linkage groups (Lg) 1 and 6. The QTL on Lg 6 co-located with QTL for TN, while that on Lg 1 co-located with LER and LL. Markers at Lg 1 QTL (qDW-03-1.1) may be more useful for increasing herbage production by MAS because selection for high LER and long LL has been suggested to increase herbage production in perennial ryegrass. QTL for SdYP were identified on Lg 2 and Lg 6. The QTL on Lg 6 co-located with QTL for SdYH, FSU and TSW, while that on Lg 2 co-located with FH, SH and FS. For seed production, markers at Lg 6 QTL (qSdYP-03-6) may be very useful because this QTL co-located with QTL for SdYH, FSU and TSW, and SdYH has been identified previously as a key selection criterion for increasing seed yield.

Marker-trait validation confirmed markers pps0495 and pps0698 identified by QTL analysis to be potentially useful for selecting for fast leaf appearance and long LL, respectively, in perennial ryegrass.

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Dedication

This thesis is dedicated to my father, Mr Moinina Sartie, mother, Mrs Mattu Jeneba Sartie and my children, Samuel Gbormuma Sartie, Cornelia Mattu Jeneba Sartie and Lucy Mubao Sartie.

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List of Abbreviations

ALf	leaf appearance interval (days)
ALg	ligule appearance interval (days)
ANOVA	analysis of variance
AFLP	amplified fragment length polymorphism
ATi	tiller appearance interval
BC	backcross
BLUP	best linear unbiased predictor
CAPs	cleaved amplified polymorphic sequence
CIM	composite interval mapping
cM	centiMorgan
DNA	deoxyribonucleic acid
DH	days to heading
EST-SSR	SSR markers that are derived from expressed sequence tags
DW	herbage dry weight (g)
F_1	first filial generation
F_2	second filial generation
FH	florets per head
FS	florets per spikelet
Fst	tiller site filling
FSU	floret site utilisation
Ha	hectare
H_b	broad sense heritability
I	Grasslands Impact cultivar
KgN/ha	kilograms nitrogen per hectare
L	litre
LAI	Leaf area index
LED	leaf elongation duration (days)
LER	leaf elongation rate (cm/day)
LL	leaf lamina length (cm)
Lg	linkage group
LOD	logarithm-of-odds ratio

LRS	likelihood ratio statistic
LS	leaf sheath length (mm)
LSD	least significant difference
LW	leaf lamina width (mm)
MAS	marker-assisted selection
$MJ/m^2/day$	micro joules per metre square per day
MQM	multiple QTL Mapping
NaCl	sodium chloride
n_r	number of replications
ns	number of seasons
PCA	principal component analysis
PC1	principal component 1
PC2	principal component 2
PC3	principal component 3
PCR	polymerase chain reaction
PGH	plant growth habit
PI	plant productivity index
QTL	quantitative trait locus
RAPD	randomly amplified polymorphic DNA
RCB	randomised complete block
RFLP	restriction fragment length polymorphisms
REML	residual maximum likelihood
Rpm	revolutions per minute
RT	number of reproductive tillers
S	Grasslands Samson cultivar
SCAR	sequence characterised amplified region
SdYH	seed yield per head (mg)
SDC	size-density compensation
SdYP	seed yield per plant (g)
SE	standard error of the mean
SH	spikelets per head
SIM	simple interval mapping
SIL	stem internode length (cm)
SL	spike length (cm)

SMC	soil moisture content
SNP	single nucleotide polymorphism
SOH	spread of heading
SSR	simple sequence repeat
STN	September tiller number
t DM/ha	tonnes dry matter per hectare
TN	tiller number
TSW	1000 seed weight (g)
TMH	% tillers with matured head
TW	tiller weight (g)
VT	vegetative tiller number
v/v	volume per volume
wt	weight
w/v	weight per volume
σ^2_g	genotypic component of variance
σ^2_ε	residual variance of means of genotypes
σ^2_{gs}	genotypic component of variance across season
λ	lambda

1

Overview

1.1 General introduction

Genetic improvement in crop or forage production, involves selection and crossing of the selected parents to produce progeny with desirable phenotypes. Phenotype expression is affected by genes of the progeny and the environment in which the progeny live. Environmental conditions are variable, with each affecting the expression of a trait. Most traits of agronomic importance in forage species, such as herbage and seed yield and their components are determined by a complicated interaction between numbers of genes (Elgersma 1990; Stuber *et al.* 1999) and genotype-by-environment interactions are also important. The location on a chromosome of genes controlling particular traits is referred to as a quantitative trait locus (QTL). Conventional plant breeding is based on visual assessment of phenotype, evaluation and recurrent selection. Breeding for improvement in quantitative traits using this methodology is laborious and involves testing progeny for a long period before the product is delivered to the market. In New Zealand, it may take up to 15 years and cost up to one million dollars to breed a perennial ryegrass cultivar (Agriseeds 2004). Conventional plant breeding has enabled a range of improvements in perennial ryegrass performance, such as increase in herbage yield and quality and manipulation of flowering date, albeit with a requirement for high nitrogen input (Humphreys 2005). However, the use of nitrogen fertiliser is now increasingly considered to be environmentally unfriendly. Regulation of nitrogen use on farms is being introduced progressively throughout Western Europe (De Clercq *et al.* 2001) and is now under discussion in some regions of New Zealand.

A recent development in plant breeding is the emergence of marker-assisted selection (MAS) technology to select plants with desirable traits, using molecular (DNA) markers linked to QTL associated with a trait of interest. MAS will potentially expedite the breeding process. It reduces population generation time and cost, and enables the selection of desirable plants even before they are field tested. MAS does not involve the insertion of foreign DNA, and so is much less controversial than some other

emerging technologies. Efforts are underway to apply MAS in forage crop breeding and, in perennial ryegrass, successes have been reported in relation to selection for nitrogen use efficiency (Van Loo *et al.* 2003) and nutritional value (Humphreys and Turner 2001). MAS requires a 'library' of DNA markers and a precise genetic linkage map indicating their position in the genome, QTL analysis to assess correlation between traits of interest and particular markers, and confirmation (often referred to as validation) of the marker-QTL/trait linkage in more than one population.

1.2 Objectives

Broadly, this research was part of a larger programme to establish a New Zealand capability in MAS for perennial ryegrass, and the components of the programme that formed part of this thesis were:

1. Establishment and phenotypic characterisation of an F_1 mapping population from perennial ryegrass parent plants of divergent plant type;
2. Development of a genetic linkage map indicating marker positions within the genome;
3. Identification of QTL for herbage yield, seed production and other traits (Table 1.1), in the mapping population, and of the associated DNA markers;
4. Validation of the marker-QTL linkages so identified in another ryegrass plant population

1.3 Thesis structure

This thesis consists of eight chapters. Following this overview, Chapter 2 provides a literature review relevant to this study. Chapter 3 assesses the morphological differences between the two perennial ryegrass cultivars, Grasslands Samson and Grasslands Impact, from which the parent plants for the mapping population in this study were chosen. Chapter 4 examines the phenotypic variability within the mapping population for herbage yield and related traits in two experiments conducted in autumn and spring in a glasshouse; while Chapter 5 assesses seed yield and contributing traits in a spaced plant field experiment. Chapter 6 describes QTL discovery for herbage and seed yield and related traits, and Chapter 7 outlines the QTL validation for leaf appearance interval and leaf length in a different population of perennial ryegrass. Chapter 8 provides general discussion and conclusions.

Trait abbreviation	Trait description
DW	Herbage dry weight (g)
ALf	Leaf appearance interval (days)
ALg	Ligule appearance interval (days)
LED	Leaf elongation duration (days)
LER	Leaf elongation rate (cm/day)
LL	Leaf lamina length (cm)
LW	Leaf lamina width (mm)
LS	Leaf sheath length (mm)
TN	Tiller number
TW	Tiller weight (g)
PI	Plant productivity index
SIL	Stem internode length (cm)
ATi	Tiller appearance interval
Fst	Tiller site filling
SdYP	Seed yield per plant (g)
SdYH	Seed yield per head (mg)
FS	Florets per spikelet
FH	Florets per head
SH	Spikelets per head
STN	September tiller number
RT	Number of reproductive tillers
VT	Vegetative tiller number
TMH	% tillers with matured head
SL	Spike length (cm)
DH	Heading date
TSW	1000 Seed weight (g)
PGH	Plant growth habit
SOH	Spread of heading
FSU	Floret site utilisation

Table 1.1: Definition of abbreviations used for the assessment of the I×S perennial ryegrass mapping population.

2

LITERATURE REVIEW

2.1 Genetic improvement in perennial ryegrass

The ultimate (broad) goal of forage grass breeding in most temperate countries is to reduce the economic and environmental costs of animal production from grassland (Wilkins and Humphreys 2003). Varietal improvement in forage grass breeding programmes started about 1930 and has resulted in improvement in crop productivity through the use of improved varieties, leading to reduced costs of fresh milk, milk products and meat (Wilkins and Humphreys 2003). Varietal improvement is an attempt to produce novel cultivars that have high economic and environmental values, such as high yield and resistance to environmental stresses. Varietal improvement is mainly based on selection, and the choice of a particular selection method is influenced by the ease with which the trait can be measured. Genetic improvement in breeding programmes depends on the ability of breeders to identify and select plants carrying desirable genes and cross them to produce hybrids. In forage species, improvement is aimed at major areas such as dry matter yield, seed yield, nutritional value for animals, persistency, tolerance to environmental stress, and resistance to pests and diseases (Wilkins and Humphreys 2003). Conventional breeding involves generation of plants having desirable traits, evaluation and selection of superior individuals based on phenotype, and recombination of the superior individuals to generate a new population for subsequent cycles of selection and improvement (Allard 1960; Simmonds 1979). It is the tool generally used in crop improvement.

Perennial ryegrass (Figure 2.1) is an out-breeder (cross-pollinated). Population improvement based on some form of recurrent selection of individual spaced plants or their half-/full-sib families is the main means for its improvement (Humphreys *et al.* 2006). This process relies on observable phenotypes which are in themselves affected by the environment. Genetic improvement of forage grasses by conventional breeding *per se* is very slow and laborious due to the need to repeat evaluation of breeding populations over time and across environments. Molecular breeding approaches

involving the use of DNA markers to assist selection can complement and enhance conventional plant breeding programmes (Wilkins and Humphreys 2003; Yamada *et al.* 2005). DNA markers have been successfully used in marker-assisted selection (MAS) for some economic traits in perennial ryegrass, for example, nutrient quality (Humphreys and Turner 2003) and nitrogen use efficiency (Dolstra *et al.* 2003; Van Loo *et al.* 2003). The objective of future forage breeding is to exploit the opportunities provided by new technologies to assess and manipulate genotype and phenotype more precisely. MAS helps to develop more targeted breeding strategies for a wider range of traits serving a broader range of objectives (Humphreys 2005).

Figure 2.1: A perennial ryegrass plant

2.2 General background of Perennial ryegrass

Perennial ryegrass, *Lolium perenne* L. is diploid (2n = 14), and belongs to the Poaceae family. It has a genome size of 2.3 Gbp (Giga basepair), similar to that of humans, and

composed of a relatively small number of genes and a large fraction of repetitive DNA (Sugiyama *et al.* 2002). It is native to Europe, temperate Asia, and North Africa but is now cultivated in many other parts of the world, including North and South America, Europe, New Zealand, and Australia (Walton 1983; Balasko *et al.* 1995; Jung *et al.* 1996). It is used as a forage crop and as an amenity grass or turf. It is a very useful pasture grass species because of its high digestibility and palatability, and is widely used in temperate regions. It is the principal feed source for dairy cows in New Zealand but is also suitable for other classes of livestock, especially those with high nutrient requirements such as young growing animals (Evers *et al.* 1996). There are several reasons for the popularity of perennial ryegrass including forage quality, ease of establishment and performance on a variety of soils, good tolerance to grazing and adequate seed production (Wilkins 1991).

2.3 Production environment

Perennial ryegrass can behave as an annual, short-lived perennial, or perennial, depending on geographic origin of the material and environmental conditions (Balasko *et al.* 1995). It is best adapted to cool, moist climates, with a minimum precipitation range of 450 to 650 mm. It can grow on a variety of soils but thrives best on a fertile, well-drained soil with a pH between 5.5 and 7.5 (Hannaway *et al.* 1999), and under an optimum temperature of 20°C with adequate light, water and nutrient supply (Mitchell 1954). Temperature and light affect both the rate of plant growth and timing of plant developmental stages like flowering (McKenzie *et al.* 1999). Generally, grass growth starts when the mean temperature rises above 10°C and is then largely independent of temperature, the rate of growth under optimal nutritional conditions being mainly determined by light intensity (Alberda 1966). Temperature remains the main climatic factor to characterise grass growth potential when solar radiation is adequate and fairly stable (Thomas 1975). The appearance rate of perennial ryegrass organs, such as leaves and tillers, increases with increase in light intensity at a constant temperature of 20°C. Above 20°C, organ appearance rate is not dependent on temperature, but as organ weights decline, relative growth rate decreases with increase in temperature (Hunt and Field 1978).

2.4 Growth and reproduction

The life cycle of perennial ryegrass consists of vegetative and reproductive phases.

2.4.1 Vegetative phase

The vegetative phase starts when the germinated seed produces a coleoptile through which the first leaf emerges. This phase comprises root, shoot, leaf and tiller development. During this phase, there is very little above-ground internode elongation. It used to be commonly stated that leaves and adventitious roots arise from a compact stem (Hunt and Field 1978), but it is now recognised that soil turnover processes such as earthworm activity lead to significant underground internode elongation in the vegetative phase as a mechanism for growing points to recover from burial (Matthew *et al.* 1989; Brock and Fletcher 1993).

2.4.2 Reproductive phase

This phase comprises flower and seed production. For perennial ryegrass, floral initiation requires a vernalization period of 6 weeks of cold temperatures (below about 10°C) and a photoperiod (day-length) greater than 13 hours (Evans 1964; Balasko *et al.* 1995). Changes in day-length and low temperature cause the stem apex to change from vegetative to reproductive stage (Langer 1990). Reproductive tillers are incapable of further leaf or tiller initiation because floral parts are formed at the apex. The previously compact stem elongates during head emergence from the growth of internodes to project the spike above the leaf canopy. Maximum above-ground growth rates occurs during the reproductive stage (Hunt and Field 1978) and this occurs in the spring period (Hunt and Field, 1978). Perennial ryegrass is self-incompatible and reproduction is by seed through cross pollination. The perennial ryegrass seed head (or inflorescence) is technically described as a spike and carries spikelets that contain florets (flowers) which develop into seeds after fertilisation (Langer 1990).

2.5 Herbage yield traits in perennial ryegrass

Herbage yield in perennial ryegrass ultimately obeys the yield component equation:

Herbage mass = tiller population density x mean tiller weight (1)

Tiller density and tiller weight, in turn, are both the end result of growth processes subject to intra- and inter-plant competition. Processes that interact in determining them include leaf elongation rate (LER), leaf elongation duration (LED), leaf appearance rate, tiller appearance rate, tiller number (TN), tiller site filling (Fs), leaf length (LL) and leaf

area index (LAI) as shown in Figure 2.2. Leaf appearance rate controls both numbers of tiller buds produced and LED. As leaf appearance rate decreases, LED increases and the number of tiller bud produced decreases, since there is one tiller bud in the axil of each leaf (Robson 1967). The expression of these traits is influenced by the genetic constitution of the plants and the environment in which the plants grow (van Loo 1992; Skinner and Nelson 1994; McKenzie *et al.* 1999).

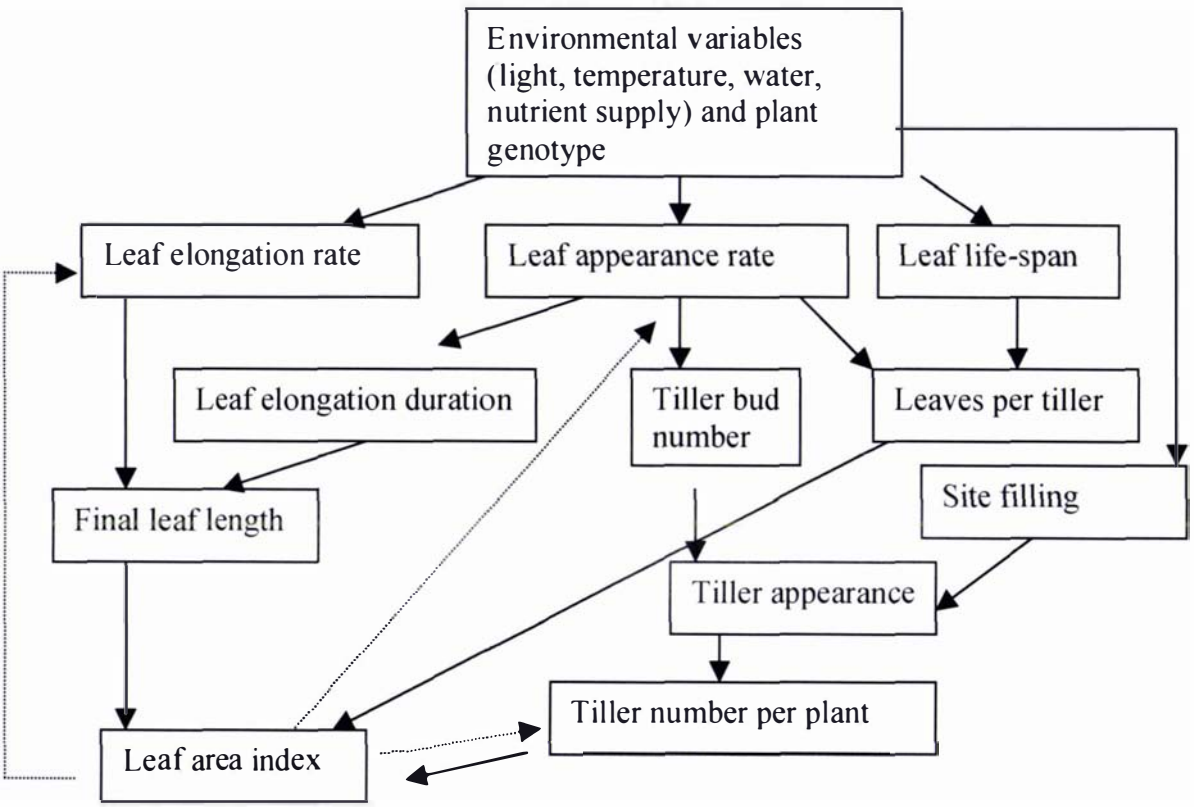


Figure 2.2: Relationship between leaf growth components, sward leaf area index, and tillering (after Chapman and Lemaire 1993 and Bahmani et al. 2000). The dotted lines refer to light quality.

2.5.1 Leaf Production

Generally, leaf development in grasses goes through four stages: (1) establishment of the cell division zone, (2) establishment of the cell elongation zone, (3) linear growth, and (4) cessation of cell division and gradual decrease in leaf elongation rate until elongation ceases (Skinner and Nelson 1994). The number of healthy leaves on a ryegrass tiller at any one time is typically three (but ranging from two to four in different growth environments). When the fourth leaf emerges, the first becomes yellow and dies, even when that leaf is fully illuminated (Milthorpe and Ivins 1966).

2.5.1.1 Leaf appearance rate

Leaf appearance rate influences tiller production through determining the number of tiller sites available. Fast leaf appearance is often associated with the production of many small tillers, while a low leaf appearance reduces the number of available buds and tends to result in the production of fewer large tillers (Lemaire and Chapman 1996). ALf is determined by temperature and light which affect the rate of assimilate supply and utilization at the stem apex (Hunt and Thomas 1985). ALf increases with increasing irradiance at a constant temperature of 20°C (Hunt and Field 1978), and remains constant when light and temperature conditions are constant (Mitchell 1954), but any substantial changes in either variable changes the rate accordingly (Hunt and Thomas 1985). ALf has been found to be comparatively unaffected by compensatory adjustments in other parameters and is therefore useful in selection programmes aimed at increasing productivity (van Loo 1992).

2.5.1.2 Leaf elongation rate

Leaf elongation rate is an important trait in herbage production, as it determines the availability of herbage between cutting or grazing intervals. The faster the LER, the more herbage will be produced (Bahmani *et al.* 2000). The initial elongation from a resting primordium is characterized by production of new, uniformly sized cells which continue to divide until an epidermal cell division zone has been established. Relative leaf elongation rate is comparatively slow during this period. Once the division zone is established, epidermal cells at the distal end of the leaf begin rapid elongation without further division, while epidermal cell production continues near the leaf base. An increase in elongation rate is observed and the leaf reaches its maximum relative leaf elongation rate. The final stage of elongation occurs when cell division ceases at the

leaf base. As the supply of new cells for leaf growth stops and older cells reach their final length the elongation zone shrinks, causing leaf elongation rate to decrease until all cells reach their final length and elongation ceases (Skinner and Nelson 1995).

2.5.1.3 Leaf elongation duration

In ryegrass there is typically one leaf elongating at any one time and so LED is approximately the reciprocal of ALf or ligule appearance interval (ALg). LED varies markedly with temperature and increases slightly with more severe defoliation, but otherwise tends to be very stable for a given genotype. Between genotypes, variation in LED can be responsible for variation in leaf length and mean tiller size (Robson 1967; Bahmani *et al.* 2000).

2.5.1.4 Leaf lamina length

Long leaf lamina length is needed for high herbage yield. As indicated above, this could arise from increased LER, LED or a combination of both. Leaf blade length varies with time of the year. In Britain, LL increases progressively from January to July and then decreases through to December, and leaves produced in June/July have the longest blades (Davies 1977). This pattern can be attributed to seasonal increase in LER in summer. The physiological rules governing commencement and cessation of cell division in leaf meristems were proposed by Durand (Durand *et al.* 1999) and mean that successive leaves in a regrowth cycle will be longer than the last.

2.5.2 Tillering

The tiller is the primary structural unit of a mature plant, and has the same terminal apex structure as the main stem or primary tiller. Second-order tillers behave in the same way as primary tillers (Milthorpe and Ivins 1966). Activation of tiller buds, or senescence of smaller tillers is the mechanism whereby grass swards adjust and optimise their leaf area in response to changes in light or grazing regime. Hence, in a study of New Zealand perennial ryegrass cultivars, Grasslands Ruanui and Ellett, tiller production was lower in autumn than in other seasons (Bahmani *et al.* 2003). In the field, tiller densities often peak in late winter-early spring when herbage height is lowest and are lowest at the end of summer after accumulation of surplus growth (Hunt and Field 1978; Donaghy 1999). The application of nitrogen fertilizer increased tillering, but irrigation had no effect on tillering (Bahmani *et al.* 2003) although it was earlier

reported that water stress reduces tiller site filling and hence final tiller number (van Loo 1992). Tiller density is affected by tiller appearance rate and tiller death. Tiller appearance rate is affected by light and temperature levels and also by the ability of tiller bud to develop into tillers. Low irradiance combined with high temperature can reduce tillering in perennial ryegrass (Hunt and Thomas 1985). Also, low red/far-red (R/FR) light ratios reduce tillering in forage grasses (Deregibus *et al.* 1983; Casal *et al.* 1985). Red light increases tillering while far-red light decreases tillering, and this effect is greater in secondary and tertiary tillers than primary tillers, and can vary with plant genotype (Casal 1988). The proportion of available tiller buds that develop into tillers is measured in different ways; tiller site filling (Davies 1974), tiller site usage (Skinner and Nelson 1992), or nodal probability (Matthew *et al.* 1998; Bos and Neuteboom 1998). The proportion of tiller buds that eventually form new tillers has by definition a theoretical maximum of 1 (Bahmani *et al.* 2000). Tiller site filling has a theoretical maximum of 0.69 ($\log_n 2$), denoting doubling of tiller population with each leaf appearance interval (Neuteboom and Lantinga 1989). Tiller death is largely influenced by light regime (Hunt and Field, 1978). Shading increases leaf length but decreases tillering (Kays and Harper, 1974, Bahmani *et al.* 2000), and this effect is due to low red/far red ratio associated with shading (Casal *et al.* 1985; Casal *et al.* 1987). This situation also triggers an increase in leaf area, through increased leaf elongation rate and leaf elongation duration (Allard *et al.* 1991; Bahmani *et al.* 2000), resulting in compensation for the lower light interception (Bahmani *et al.* 2000).

Active production of new tillers in swards slows down as the percentage light intercepted approaches a maximum (Davies 1971; Robson 1973), and increasing shade at the base of the sward reduces tillering (Mitchell and Coles 1955; Davies *et al.* 1983). No association has been found between cessation of tillering and changes in the accumulated carbohydrate in the tiller bases, and this indicates no direct effect of availability of photosynthate (Davies and Thomas, 1983), but there are suggestions that this reduced tillering is mainly due to a regulatory effect of light quality on tillering rate and not an indirect consequence of enhanced assimilatory capacity (Deregibus *et al.* 1983). The reason for reduced tillering at maximum light interception or at increased shading at tiller bases was suggested to be photomorphogenic (Davies *et al.* 1983), independently of the elucidation of the mechanism of this response by Deregibus (1983) and Casal (1985). A low rate of tillering in larger plants or in swards must be ascribed

to the changes brought about by competition for light reaching individual tillers (Davies and Thomas 1983).

The rate of production of tiller in relation to the rate of leaf appearance appeared to be virtually independent of weather conditions. The relative rate of tiller production is controlled by rate of leaf appearance. Davies and Thomas (1983) reported low site filling in larger plants and suggested that the site redundancies were probably caused by within-plant competition for light at the tiller bases. In terms of plant growth stage, tiller density declines during reproductive growth, but can increase rapidly after interruption of reproductive growth. The proportion of tillers that go reproductive varies with the cultivar and growth conditions. The proportion of tillers turning reproductive has been reported to vary from 4% (Gangi *et al.* 1981) to 58% (Wilkins 1997) in the UK, and in New Zealand perennial ryegrass swards in spring varied from as little as 5 – 10%, to over 30% depending on variety and conditions (Bahmani *et al.* 2003). Tillers that are formed before winter provide the majority of flowering tillers, while those that are formed during winter are important in providing a dense sward for the main spring growth period. Secondary reproductive tillers elongate after the main group reproductive tillers are decapitated, and these late developing tillers include tillers that appear in winter and spring (Korte 1986). Frequent defoliation induces rapid tillering (Korte 1986), but tiller survival is less affected by defoliation. Tiller density, if too low, could limit pasture growth potential by limiting the number of growing leaves. Where tiller densities are sufficient to induce tiller competition, differences in tiller density tend to be compensated for by differences in growth per tiller (Hunt and Field 1978). There is a negative relationship at canopy level between tiller size and tiller density (Hernandez Garay *et al.* 1999), sometimes called size/density compensation (SDC), in undefoliated (Kays and Harper 1974; Lonsdsale and Watkinson 1982) and in defoliated grass swards (Matthew *et al.* 1995). High sward leaf area index (LAI) inhibits tillering (Simon and Lemaire 1987), and this is an important mechanism in the negative correlation between tiller density and leaf length which in turn is a major component of sward LAI (Bahmani *et al.* 2000). The SDC relationship between tiller density and tiller size means that a tiller size increase may be observed in the case of tiller population decrease with increasing herbage, without any change in sward productivity or a tiller population increase may be a simple reflection of reduced herbage mass, again without necessarily indicating a change in sward productivity

(Hernandez Garay *et al.* 1999). Matthew *et al.* (1995) proposed a productivity index (PI) which could be obtained by calculating the movement of tiller size/density coordinates perpendicular to a size/density compensation line. PI thus takes account of both tiller size and tiller number simultaneously. PI provides a comparative measure of leaf area index and productivity of a sward (Matthew *et al.* 1995), and can be used to predict herbage yield (Hernandez Garay *et al.* 1999).

2.6 Seed yield and component traits in perennial ryegrass

Seed yield is an important factor in the commercial success of a forage grass cultivar but breeding for seed yield has received less attention (Marshall and Wilkins 2003), generally because forage production is the primary interest of these plants, and in addition, breeding for seed yield is a laborious process and often does not produce the expected results. For instance, seed yield of perennial ryegrass plants in a spaced-plant experiment was found (Elgersma 1990b), to be poorly correlated with seed yield in drilled plots. This is because seed yield is a complex quantitative trait that is affected by several component traits and the environment. Perennial ryegrass seed yield is the outcome of several component traits including number of reproductive tillers or spikes per plant, number of florets or seeds per spike, floret site utilisation and seed weight (Elgersma 1990a; Marshall and Wilkins 2003). Floret site utilisation is otherwise referred to as seed set or floret fertility. Florets per spike can be further broken down to spikelets per spike and florets per spikelet. However, the contribution of each component trait to seed yield varies in different studies. Some found that seed yield in ryegrass and cereals is primarily dependent on the number of spikes per unit area (Langer 1979), while others identified floret site utilisation (Fang *et al.* 2004) and seed number per spike (Bugge 1987) as the major seed yield component traits.

Ryle (1966) reported that floret development in ryegrass is affected by the date of origin of the tiller, temperature and daylength. Tillers arising in early autumn are more likely to have more florets per spikelet than those tillers arising in spring. The total number of florets in ryegrass decreases as daylength or temperature increases and this decrease in total floret numbers is a consequence of fewer spikelets and fewer florets per spikelet. The total number of florets on a spike depends on the number of spikelets and the number of florets developing from each spikelet. Spikelet number varies from 20-30,

and each spikelet consists of 4-14 florets with the basal spikelets developing more florets than the terminal ones (Ryle 1966).

Floret site utilisation is influenced by the time of spike emergence, environment and the genotype of the plant (Knowles and Baenziger 1962). Late emerging spikes of ryegrass generally developed fewer fertile florets than the early emerging spikes (Anslow 1963), and the application of nitrogen fertilizer and irrigation also affect floret site utilisation (Ryle 1966). When soil moisture is limiting during the critical development phase between head emergence and seed formation, seed yields can be drastically reduced owing to the abortion of some fertile florets (Hebblethwaite 1977).

Seed weight varies with the time of spike emergence, and the position of spikelet and floret in the spike. Early emerging spikes produce the heaviest seeds. Within the spike of ryegrass, the basal spikelets develop slightly heavier seeds than the terminal ones and, within spikelets, seeds from the basal florets are heavier than those in terminal florets (Anslow 1964). Seed weight in grasses also varies with crop management factors such as nitrogen supply (Lambert 1956) and seed rates at planting or sowing.

Seed-shedding, which is the loss of seed from over-ripe spikes in both standing crop and during harvest, is a further factor that influences the amount of harvested seed. However, the problem of uneven ripening of seed heads in crops of many perennial grasses remains to be overcome. Although Ryle (1966) suggested that selection and breeding for seed retention appears to be the most promising approach to solving this problem, breeding for uniform heading within a plant will also be a good option.

In New Zealand, perennial ryegrass seed constitutes the largest component of forage seed production (Pyke *et al.* 2004). Seed yields of 3000 kg/ha or more are being achieved due to the adoption of better management techniques such as the use of plant growth regulators and modern fungicides (Rolston *et al.* 2004), compared with yields of 750 – 1170 kg/ha common 20 years ago (Rolston and McCloy 1997).

2.7 Molecular breeding

Molecular breeding is a new approach towards the improvement of genetic traits (traits that can be inherited from parents to off-spring), especially quantitative traits that are affected by a number of genes and the environment. Molecular breeding is based upon the use of molecular (DNA) markers to identify the precise location of a gene (or genes) affecting a trait in an organism. Regions of the plant chromosomes that contain important genetic information governing interesting and heritable phenotypic traits are identified by co-inheritance with DNA markers. The linkage of particular DNA marker variants (alleles) with a heritable phenotypic trait enables the identification of the region of a particular chromosome that is important for the phenotype. This is important for plant variety development because it means that plant breeders can use markers to test for the presence of important traits rather than testing for the traits themselves. This is called marker-assisted selection (MAS).

MAS technology is being rapidly adopted in breeding programmes for the improvement of complex (quantitative) traits in plants and animals (Asins 2002; Buckler and Thornsberry 2002; Wilkins and Humphreys 2003; Humphreys *et al.* 2006). Major elements of the development of MAS tools include genetic linkage mapping, quantitative trait loci (QTL) analysis and marker-trait association validation. Genetic variation in mapping population(s) is fundamental to the success of this technology.

2.7.1 Genetic variation of traits in perennial ryegrass

Genetic variation may be described as the variation in DNA sequence amongst individuals within a population. The primary sources of genetic variation include mutations (changes in DNA), gene flow and sexual reproduction. Genetic variation occurs when a gene appears in alternative forms known as alleles, and the formation of a new allele combination is through the process of recombination. The alleles of a gene are located at the same position or locus on parental chromosomes (homologous chromosomes), with each allele contributing differently to the gene function. In a population, for any given gene there can be from one to many alleles; however, because most organisms carry only one or two chromosome sets per cell, any individual organism carry only one or two alleles per gene (Griffiths *et al.* 2005). In a population derived from many unrelated founder individuals (e.g. natural population), there are

more alleles than in an artificial population derived from a restricted founder set (mostly F_1 and F_2).

In perennial ryegrass, genetic variation has been reported for many agronomically-significant traits including leaf lamina length (Cooper and Edwards 1961; Edwards 1970; Ghesquiere *et al.* 1994; Hazard *et al.* 1996; Barre *et al.* 2002), leaf elongation rate (Edwards 1970; Hazard *et al.* 1996), leaf width (Edwards 1970), leaf elongation duration (Edwards 1970), leaf appearance rate (Edwards 1970), tiller number (Cooper and Edwards 1961; Hayward and Breese 1966), tiller size (Hazard *et al.* 1994), dry matter yield and chemical composition (Sokolovic *et al.* 2002), heading date (Hayward 1967; Charmet and Balfourier 1994) and seed yield and its component traits (Bugge 1987; Elgersma 1990; Elgersma 1990a). Genetic variation also exists for seed quality in terms of 1000 seed weight (Elgersma 1990; Sugiyama *et al.* 2002) and in seed germination capacity (Hayward and Breese 1966).

2.7.2 Recombination

Recombination is the production of new allele combinations in an organism through the process of meiosis. Meiosis involves two successive nuclear divisions, with only one round of DNA replication, that produce gametes or sexual spores that have one-half of the genetic material of the original cell (Griffiths *et al.* 2005). Recombination comes about in two ways: (1) crossover events between homologous chromosomes, which occur between linked genes on the same chromosome and (2) independent assortment, which occurs between genes on separate chromosomes or on the same chromosome but far apart. With respect to two genes under consideration, a maximum value recombinant frequency (50%) suggests that the two genes assort independently. Generally, for genes close together on the same chromosome pair, recombinant frequencies are significantly lower than 50%. The farther apart genes are, the closer they approach a 50% recombinant frequency (Griffiths *et al.* 2005). Recombination frequency reflects the physical distance between two loci on a chromosome on the assumption that the probability of crossing over is proportional to the distance between the loci (Jones *et al.* 1997). Recombination frequency is calculated as number of recombinants divided by total number of progeny, and is usually expressed as a percent recombination or it may be converted to map units measured in centiMorgans (cM) (Haldane 1919; Kosambi 1944), equivalent to one percent recombination frequency. In

grasses and many other plants, recombination does not occur with equal frequency across the genome (Jones *et al.* 1997), and there are regions of recombination 'hot spots' where recombination occurs at a higher rate.

2.7.3 Gene mapping

A gene is a genetic factor controlling a trait that is inherited from generation to generation. In a more precise sense, a gene is the fundamental physical and functional unit of heredity, which carries information from one generation to the next. Physically, it is a segment of DNA composed of a transcribed region and a regulatory sequence that makes transcription possible (Griffiths *et al.* 2005). Genes are located linearly on chromosomes in the nucleus of a cell. The location of a gene on a chromosome is called a gene locus. Genes can interact in different ways to affect their phenotype (s). The interaction can be in the form of epistasis, where the action of one gene is affected by the state of the other (Carlborg and Haley 2004), pleiotropy, where one gene leads to many different phenotypic expressions (Zhuang *et al.* 1997; Wilkins and Humphreys 2003; Yamada *et al.* 2004; Dudley *et al.* 2005). A QTL may also be referred to as a gene.

Gene (QTL) mapping describes the identification of genes, or QTL, with regards to their location in a genome, using DNA markers. It facilitates isolation of genes based simply on measurement of their effect(s) on phenotype - requiring no information about the specific functions performed by the gene(s) (Paterson 1996b). It utilises the concept of classical genetics with tools from molecular biology. Methods in gene mapping include genetic linkage mapping (see below), QTL analysis and marker-trait association validation.

2.7.3.1 Linkage mapping

Genetic linkage mapping is the identification of the linear location and order of individual genes or DNA markers in the genome. It is based on the principle of genetic linkage, that is, genes or markers close together on same chromosome will be inherited together at a relatively higher frequency from parent to progeny than those physically more separated on the chromosome. Genetic linkage mapping requires a mapping population, and a DNA marker resource.

2.7.3.1.1 Mapping population

A mapping population is a population in which recombination of parental alleles can be traced. The type and size of mapping population are very important for QTL analysis but their choice largely depends on the goal of the mapping project, the species concerned, type of marker system, traits to be mapped and the availability of time and resources. In choosing the crossing parents, it is recommended that they should be different in traits of interest in order to facilitate linkage mapping and segregation analysis (Young 1994). However, when using mapping populations based on highly heterozygous parents, differences in the trait of interest between the parents is not essential, because many of the genes underpinning the trait still segregate in the population (e.g. Barrett *et al.* 2004). There are several methods of generating a mapping population, but the choice depends on the pollination pattern of the plant species. In open pollinated species like perennial ryegrass, populations suitable for genome mapping can be generated via a cross between two heterozygous genotypes (Grattapaglia and Sederoff 1994) or between one heterozygous parent and a doubled haploid parent (Bert *et al.* 1999; Jones *et al.* 2000). One advantage of the former breeding method is that it minimises the risk of inbreeding depression and segregation distortion in the mapping population, both of which can significantly reduce the power and utility of a genome map (Faville *et al.* 2003). The successful use of a double heterozygous (or double-pseudotestcross) strategy has been reported in linkage mapping for perennial ryegrass (Faville *et al.* 2004), white clover (Barrett *et al.* 2004) *Eucalyptus* (Grattapaglia and Sederoff 1994) and asparagus (Lewis and Sink 1996).

The size of the mapping population is important because it determines the resolution of a map and the precision in marker ordering (Young 1994). The larger the mapping population is, the higher the chance of seeing more recombinants in the study and hence the better the map resolution. However, population size may be limited by several factors including the number of seeds available and the amount of time and resource available for data collection. Populations less than 50 individuals provide too little mapping resolution to be useful (Young 1994). If the goal is high resolution mapping or QTL mapping, much larger populations will be required (Young 1994; Beavis 1998). Different population sizes have been reported suitable for gene or QTL mapping for forage species: 183 progeny for QTL analysis of morphological, developmental, winter hardiness and herbage quality traits in perennial ryegrass (Yamada *et al.* 2004; Cogan *et*

al. 2005); 182 plants for QTL analysis of white clover seed production (Barrett *et al.* 2005); and 137 plants for QTL analysis for vernalisation requirement, heading time and number of panicles in meadow fescue (Ergon *et al.* 2006), and for other species: 1000 F_2 plants to construct a high resolution map for tomato (Messeguer *et al.* 1990); and 1800 F_2 plants for QTL mapping in maize (Stuber *et al.* 1987).

2.7.3.1.2 DNA markers

Genetic variation can be detected using visible mutations (morphological markers), isozyme markers and DNA markers. However, visible mutation and isozyme marker methods have become less popular because they are prone to environmental effects and also they can not be produced on a large scale. They have generally been superseded by DNA markers (Paterson 1996a).

DNA markers are most frequently different length DNA fragments that are all inherited in a simple Mendelian manner (Skot 2003). They are derived from a DNA region that shows sequence polymorphism between individuals within a species (Andersen and Luuerstedt 2003). DNA markers of most types (section 2.7.3.1.2.1) are available in large numbers (thousands) for any species and are independent of environmental conditions (Huff 2001).

2.7.3.1.2.1 Types of DNA markers

There are various types of DNA markers, but the choice for use is affected by several factors, including the amount of genetic variation they can generate (that is, the number of alleles they can detect), the size of DNA fragment they can detect, the amount of DNA they will require, the ease of their use with automation, the breeding objectives, population structure, genomic diversity of the species under investigation, marker system availability, time required for analysis, and the cost per unit information (Staub *et al.* 1996).

The most commonly used classes of DNA markers used in genetic mapping in plants are restriction fragment length polymorphisms (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and microsatellites or simple sequence repeats (SSR) (Paterson 1996a; Toureaand *et al.* 2002). Other, less frequently-used DNA marker types include single nucleotide

polymorphism (SNPs), cleaved amplified polymorphism sequence (CAPs) and sequence characterised amplified regions (SCARs). SSRs have evolved as the method of choice in genome mapping because they are highly polymorphic, co-dominant (they allow the expression of all alleles at a locus), they can detect length differences in DNA sequence that are as small as one nucleotide, the procedures relating to their use are semi-automated, allowing for high-throughput processing, and they can be synthetically produced in large quantities (Paterson 1996a; Faville *et al.* 2003). They are highly reproducible and transferable between laboratories and/or experiments and are particularly useful for whole genome mapping to dissect the genetic basis of quantitative traits, followed by MAS (Forster 1998).

SSR markers that are derived from expressed sequence tags (EST-SSR) are generally less polymorphic than SSRs developed from enriched libraries, but they tend to be transportable across species boundaries. Additionally, because they are derived from genes, they facilitate the location of specific genes on a chromosome (Faville *et al.* 2003).

2.7.3.1.3 A genetic linkage map

A genetic linkage map is a genomic map of a species, indicating the chromosome number, location and order of genes or DNA markers on the chromosomes of the species. It is composed of linkage groups (LG) that represent the haploid number of chromosomes present in the genome of that species. The genes or marker loci on the chromosomes are represented by marker positions on the LG. The distances between the genes or marker loci are measured in cM (Haldane 1919; Kosambi 1944) based on the recombination frequencies between genes or markers. A genetic linkage map is useful for several purposes including QTL mapping, MAS and gene isolation and cloning.

To be useful for QTL analysis, a genetic linkage map needs to contain sufficient number of markers, and to be 'saturated'; that is without large regions where no markers are found. Hence, the number of DNA markers needed to make a complete genetic map increases with the genome size of the species (Lander and Schork 1994). For most species, an adequate coverage of the genome can be achieved with approximately 100-150 marker loci evenly spaced along the chromosomes (Tuberosa *et al.* 2002).

Generally a map is complete when markers are evenly placed along the chromosomes at an average spacing of about 5 cM (Paterson 1996b). Other criteria used to determine the completeness status of a genetic map include: (1) when the number of linkage groups is equal to the haploid number of chromosomes in the organism, (2) when the newly mapped markers invariably show genetic linkage to existing markers, suggesting that all regions of the chromosomes are covered by the genetic map and (3) statistical analysis by comparing current map length with estimated genome length based on recombination fractions (Lange and Boehnke 1982; Chakravarti *et al.* 1991).

2.7.3.1.3.1 Genetic map construction

There are several recommended procedures for constructing genetic maps (Staub *et al.* 1996; Liu 1998). The first step in linkage mapping is the screening of DNA markers. Marker screening is necessary to identify those that are polymorphic or monomorphic, and to determine their segregation pattern (e.g., 1:1, 1:2:1, 3:1 or 1:1:1:1) in the mapping population. Polymorphism screening is usually done by assaying a large number of possible DNA markers in a small set of progeny randomly sampled from the mapping population. If a marker does not show polymorphism for the set of progeny, the marker will be a non-informative monomorphic marker and will not be used in the data analysis (Liu 1998). The size of the screening sample depends on the available equipment for polymerase chain reaction (PCR) reactions. For example, screening sample sizes 6, 8, or 12 may be convenient for an 8 x 12 = 96 well PCR reaction plate. The segregation pattern of the markers in the progeny will be tested to know if they follow the expected ratio (a test for goodness of fit). Mapping software like JoinMap® 3.0 can analyse segregation patterns using chi-square and log likelihood ratio test statistics.

When polymorphic markers are identified, they will be used next to genotype the whole mapping population. The genotypic data will then be used for linkage analysis using mapping software such as JoinMap® 3.0, MapMaker, MapManager, and CriMap. Before data is submitted for linkage analysis, it should be checked for missing data of individual markers and genotypes so that those with high missing values will be discarded in order to reduce the risk of erroneous linkage, inter-locus map distances and marker order.

The next step involves linkage analysis of marker loci to (a) place them into groups and (b) order them in their respective groups. It involves pair-wise linkage analysis for all possible two-locus combinations based on homologous recombination between non-sister chromatids during meiosis. Linkage analysis for a two-locus combination is based on comparison of observed and expected frequencies of possible genotypic classes (Liu 1998). This is done using declared threshold values for the existence of linkage between marker loci, for both grouping and ordering marker loci. A recombination value of less than 0.5 is used to declare linkage between loci.

Threshold values for grouping and ordering are established based on a test for independence, referred to as the logarithmic-of-odds (LOD), which expresses the level of certainty of the occurrence of the groups and the order of loci. The LOD score is the $\log_{10}$ of the ratio of the likelihood under the estimated recombination frequency ($r = \acute{r}$) to the likelihood under the null hypothesis of unlinked loci ($r = 0.5$): $LOD = \log_{10} (L(r = \acute{r}) / L(r = 0.5))$ (Maliepaard *et al.* 1997; Liu 1998). A high LOD score represents a high level of stringency. Linkage grouping criteria need to be more restrictive to limit the occurrence of false linkage - unexpectedly large linkage groups are a sign of false linkage - hence LOD thresholds for grouping are generally in the region of 6. The occurrence of a large number of unlinked genetic markers may be a sign of low quality data, small population size and/or a small number of DNA markers. If many markers are used, a relatively high genome coverage is achieved, the data are highly informative, the genetic model for the data analysis is adequate and the criteria for the grouping are reasonable, then the number of linkage groups should be equal to the haploid number of chromosomes for the organism (Liu 1998). Ordering of markers within linkage groups generally use thresholds of LOD 2.0 and maximum recombination value, $r = 0.40$. Finally, map distances are estimated in cM using either the Kosambi mapping function (Kosambi 1944), which compensates for any external interference from adjacent recombination events, or the Haldane function which nullifies the existence of external interference (Liu 1998).

2.7.3.1.3.2 Genetic linkage mapping using a pseudo-testcross strategy

The construction of a genetic linkage map using a pseudo-testcross F_1 mapping population structure derived from a cross between a pair of heterozygous parents is commonly employed in outcrossing species like perennial ryegrass (Grattapaglia and

Sederoff 1994; Porceddu *et al.* 2002; Shepherd *et al.* 2003; Carlier *et al.* 2004). This entails the construction of individual parental maps using dominant marker types (Table 6.1: segregation types 1-4) in the first instance to enable the comparison of recombination frequencies and marker order in both parents. The next stage involves joining together the parental maps as a consensus map, using markers that are heterozygous in both parents (Table 6.1: segregation types 5-7). Both the parental and consensus maps may be used for genetic mapping, but the latter will have a higher level of marker saturation than either of the parental maps.

2.8 Quantitative Trait Loci (QTL) mapping

A quantitative trait locus (QTL) is a chromosomal region containing gene(s) responsible for variation of complex, or quantitative, genetic traits (e.g. plant yield, height, disease resistance) in plants and animals. A quantitative trait is referred to as a polygenic trait because its expression probably involves more than one gene, and phenotype results from the combined action of the genes, and the interactions between genes and the environment (Paterson 1996b; Stuber *et al.* 1999). The procedure for finding and locating QTL is called QTL mapping or QTL analysis. QTL analysis provides a means of identifying the genomic location of genes responsible for the genetic variation of quantitative traits and opens new opportunities in genetic research and genetic improvement.

QTL analysis is useful for several applications including: (1) MAS as a marker associated with a QTL for a trait of interest may be used for indirect selection in breeding selection programmes, (2) gene isolation and cloning, and (3) dissecting complex traits into component traits to ease the identification of individual gene effects involved in a trait (Humphreys *et al.* 2002).

QTL analysis involves bringing together mapping population phenotypic data collected from glasshouse or field experiments, and DNA marker data developed for the mapping population in laboratory. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups at marker loci and to determine whether significant differences exist between groups with respect to the trait

being measured (Tanksley 1993; Young 1996). A significant difference between phenotypic means of the groups indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard *et al.* 2005). Methods used to analyse QTL include single marker analysis or point analysis (based on a simple t-test or ANOVA or linear regression), simple interval mapping (SIM) and multiple or composite interval mapping (CIM, combining SIM with linear regression) (Liu 1998; Toureaand *et al.* 2002). The last two methods are commonly used because they are considered statistically more powerful. SIM analyses intervals between adjacent pairs of linked markers along chromosome simultaneously instead of analysing single markers, while CIM combines interval mapping with linear regression and includes additional DNA markers in the statistical model in addition to an adjacent pair of linked markers for interval mapping (Zeng 1994). Multiple QTL Mapping (MQM) is a type of CIM specific to MapQTL software (Van Ooijen *et al.* 1996). The most likely position of a QTL can be determined by using LOD or likelihood ratio statistic (LRS) which can be converted as LRS = 4.6 x LOD (Liu 1998). The LOD threshold for declaring a significant QTL may be determined by permutation testing (Churchill and Doerge 1994). QTL positions are defined by the map position of the peak LOD score, and confidence interval (~95%) is commonly defined by the peak ±2 LOD (Van Ooijen 1992).

QTL analysis may be performed using several computer programmes, including MapQTL, MapMarker/QTL, QTLSTAT, PGRI, QTL Cartographer, and QGENE (Liu. 1998).

When more than one QTL are identified affecting a trait, non-additive genetic relationships may exist among them, including epistasis, pleiotropy or linkage (Fulton *et al.* 1977; Tanksley and Nelson 1996; Zhuang *et al.* 1997; Doerge 2002). Complex genetic interactions amongst QTL, such as epistasis, are of particular interest as they indicate regions of the genome that might not otherwise be associated with the quantitative trait using a one-dimensional search (Doerge 2002). QTL discovery for a trait may be affected by the environment, which may result in changes in QTL number, location and effect between experiments (Paterson *et al.* 1991; Zhuang *et al.* 1997; Fry *et al.* 1998; Jiang *et al.* 1998; Vieira *et al.* 2000; Teulat *et al.* 2001; Jarso and Keneni 2003; Ungerer *et al.* 2003) and this necessitates QTL validation.

Efforts are in progress for QTL analysis in forage species (Inoue *et al.* 2004; Yamada *et al.* 2004; Barrett *et al.* 2005; Cogan *et al.* 2005; Ergon *et al.* 2006). QTL are being identified for a range of traits in perennial ryegrass: resistance to crown rust (Dumsday *et al.* 2003; Muylle *et al.* 2005), flowering time (Armstead *et al.* 2004; Yamada *et al.* 2004), digestibility (Cogan *et al.* 2005), water soluble carbohydrates (Humphreys and Turner 2001; Humphreys and Turner 2003; Turner *et al.* 2006) and winter survival (Yamada *et al.* 2004; Yamada and Forster 2005).

2.8.1 QTL and DNA marker-trait verification

Verification of QTL and DNA marker-trait association is necessary to substantiate a biological basis for observed marker-trait associations, to provide precise estimates of the magnitude of QTL effects, and to predict QTL expression at a given age or in a particular environment (Brown *et al.* 2003). Verification and confirmation of QTL are important for the selection of those QTL associated markers to be used in a practical breeding programme (Boerma and Mian 1999). QTL for a given trait may be verified by repeated detection across genetic backgrounds and/or environments, as indicated by co-location of QTL (Brown *et al.* 2003).

2.8.2 DNA marker-trait verification

QTL mapping, based on genetic linkage analysis, identifies markers that are linked to gene(s) associated with observed variation in a trait. The identification of simply inherited markers in close proximity to a QTL forms the basis of MAS. For the application of MAS, one downfall of using linked markers for selection is that linkage maps in general are of relatively low resolution and therefore there is a good chance that recombination will occur between the marker and the QTL during the breeding process. If that happens, the marker allele used for selection may no longer be in phase with the selected QTL in subsequent generations. Clearly, MAS relies on a tight linkage between a marker and a QTL and there is a significant risk that recombination between marker and QTL will render MAS unreliable. Therefore, the association between a marker and a trait requires confirmation before using the marker in MAS. One approach for determining marker-trait association is by screening populations of different genetic backgrounds with markers that were previously identified to be linked to QTL within a mapping population (Li *et al.* 2001; Fasoula *et al.* 2003; Skot 2003).

As differences in trait expression by parents of a mapping population are fundamental to QTL discovery, it is necessary to determine morphological differences between cultivars Grasslands Samson and Grasslands Impact from which parents of the mapping population used for this study were derived. This led to the conduct of Experiment 1.

3

Morphological differences between Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.1. Introduction

Grasslands Samson and Grasslands Impact are cultivars of perennial ryegrass (*Lolium perenne* L.), and were bred by AgResearch Grasslands. These cultivars are among the more successful New Zealand-bred perennial ryegrass cultivars. Grasslands Samson was bred from a breeding pool based on founder plants taken from old pastures throughout New Zealand; Grasslands Impact was based on crosses between the cultivar 'Grasslands Nui' and an ecotype from Galicia, Spain (H.S. Easton, personal communication).

A segregating population constructed by AgResearch Grasslands using an individual from Grasslands Samson and Grasslands Impact as parents was to be used for genetic linkage mapping and QTL analysis of herbage and seed production traits in perennial ryegrass. QTL mapping populations are derived from a cross between two parent genotypes with known phenotypic differences, which implies genetic differences, in specific traits of interest. For example, a mapping population for QTL analysis of lodging resistance and related traits in Italian ryegrass, known as NDxNW, was derived from Nioudachi as the resistant parent and Nigatawase as the susceptible parent (Inoue *et al.* 2004). With respect to Grasslands Samson and Grasslands Impact, the former tends to be erect and to have larger tillers, while the latter is more prostrate with a later flowering date, though there are little or no quantitative data available. Similarly, there is no published information as to whether or not herbage and seed production traits differed between these cultivars. To better understand the similarities and differences of the two cultivars from which the parent plants of the mapping population had been chosen, Experiment 1 was set up to determine, for plants grown from seed in a glasshouse, the mean and variance for the more important vegetative growth traits for Grasslands Samson and Grasslands Impact perennial ryegrass cultivars.

3.2. Traits to be measured:

Given the anecdotal information on difference between Grasslands Samson and Grasslands Impact perennial ryegrass cultivars in tiller size and growth habit, and the interest in obtaining information on these and other traits, the following measurements were included in Experiment 1:

i. Leaf appearance interval;
ii. Ligule appearance interval;
iii. Leaf elongation rate;
iv. Leaf elongation duration;
v. Leaf blade length;
vi. Leaf blade width;
vii. Leaf sheath length;
viii. Tiller appearance interval;
ix. Tiller site filling.

3.3. Materials and Methods

3.3.1. Experimental set up

Seeds of Grasslands Samson and Grasslands Impact (both infected with wild-type endophyte) were obtained from the Margot Forde Germplasm Centre at the AgResearch Grasslands Research Centre, Palmertson North, New Zealand. The seeds were germinated by placing on moist Whatman Filter paper for 5 days at 20°C, in Petri-dishes in an incubator. Seedlings were then transplanted into plastic 'Planter bag' pots (i.e polythene plastic bags, as commonly used in plant nurseries for seed raising) (64 x 64 x 150 mm), filled with a commercial potting mix. Fifty seedlings of each cultivar were transplanted into pots in this way, which were grown in a glasshouse set to run at 21°C and watered daily. Twenty plants; 10 plants of each cultivar, were laid out on tables in five block-replicates of a randomised complete block (RCB) design. The plants were re-randomised within blocks once per week to neutralise any environmental effect within the blocks. The experiment ran for 10 weeks in spring 2002.

3.3.2. Data collection

Data collection started when seedlings were 14 days old and continued every other day until every seedling had produced 10 leaves. Data were collected for six leaves (leaf 4 to leaf 10) as the first three leaves had already appeared in most plants before the start of data collection. Ligule appearance interval was measured for seven leaves (leaf 3 to leaf 10) as only the first two leaves had already produced ligules prior to the start of the measurement. Methodology for measuring each trait was as follows:

i) **Leaf appearance interval** (ALf) – The date a leaf tip became visible was recorded, and appearance interval was determined as the number of days between the appearance of successive leaves.

ii) **Ligule appearance interval** (ALg) - The date the ligule of a leaf became visible was also recorded, and ligule appearance interval was calculated as the number of days between appearance of ligules of successive leaves. Clearly Alf and Alg would be closely related.

iii) **Leaf elongation duration** (LED) - the difference in time (days) between leaf tip appearance and ligule appearance of a given leaf.

iv) **Leaf lamina length** (LL) - A leaf lamina was considered to have stopped growing when its ligule was visible. LL was measured as the distance (cm) from the ligule to the leaf tip.

v) **Leaf elongation rate** (LER) - This was calculated by dividing LL by LED, with units of cm/day.

vi) **Leaf lamina width** (LW) - LW was measured to the nearest 0.5 mm, using a ruler, with the measurement taken mid-way between the ligule and the apex of a mature leaf (usually the leaf immediately below the emerging leaf).

vii) **Leaf sheath length** (LS) - LS was measured in cm from the soil surface level to the ligule, using a ruler.

ix) **Tiller appearance interval** (ATi) –Tiller appearance was recorded only for primary tillers. ATi was calculated as the number of days between appearances of successive primary tiller buds.

x) **Tiller number** (TN) - TN was a direct count of total number of tillers per plant and was carried out at the end of the experiment.

xi) **Tiller site filling** (Fst) - Tiller site filling was calculated by dividing the number of developed primary tiller buds by the number of fully developed leaves (10 leaves / plant for all plants) on the main shoot of each plant (Skinner and Nelson 1992).

xii) **Effect of coleoptile tiller on tiller production** - In addition, since the presence of absence of the coleoptile tiller has been proposed as a possible reason for variation in plant tiller number (Bahmani *et al.* 2000), total tiller number for plants with or without the coleoptile tiller was counted at the end of the experiment. In total, 15 Grasslands Impact plants and 20 Grasslands Samson plants out of 50 produced coleoptile tillers. Fifteen of the 20 plants in Grasslands Samson were randomly selected across the five replications, and 15 plants without coleoptile tillers were also randomly selected across replications in both cultivars.

3.3.3. Data analysis

For variables other than ALf, ALg, and ATi, means of the two cultivars were compared using analysis of variance (ANOVA) in GenStat version 8.0 (Genstat 2005). Data for TN was log-transformed prior to analysis to improve normality. For data on ALf, ALg, and ATi, mean appearance intervals were calculated for individual leaf positions (see Figures 3.1, 3.2 and 3.6 below), and then averaged for each plant, by regressing days elapsed at appearance against position for each leaf, ligule, or primary tiller. Cultivar means and their standard errors for these individual plant averages were then compared (Table 3.1).

3.4. Results

For many traits, the two cultivars were remarkably similar, although at least one of the expected morphological differences between the cultivars was confirmed with Grasslands Samson having fewer tillers per plant than Grasslands Impact (Table 3.1).

3.4.1 Leaf appearance interval

In both cultivars, a new leaf appeared every week, but Grasslands Samson produced new leaves faster ($p \leq 0.05$) than Grasslands Impact (Table 3.1). ALf in both cultivars increased with time (Figure 3.1).

Traits	Grasslands Samson	Grasslands Impact
Leaf appearance interval (days)	6.22 ± 0.08	6.54 ± 0.08
Ligule appearance interval (days)	6.45 ± 0.08	6.78 ± 0.07
Leaf elongation duration (days)	8.3 ± 0.1	8.4 ± 0.1
Leaf elongation rate (cm /day)	3.2 ± 0.5	3.3 ± 0.6
Leaf lamina length (cm)	20.8 ± 3.6	21.6 ± 4.4
Leaf lamina width (cm)	0.75 ± 0.11	0.68 ± 0.09
Leaf sheath length (cm)	4.25 ± 0.83	5.26 ± 1.42
Tiller appearance interval* (days)	6.89 ± 0.14	7.10 ± 0.12
Tiller site filling*	0.726 ± 0.007	0.722 ± 0.009
Total tiller number (log_{10})	1.82 ± 0.01	1.87 ± 0.01
Tillers per plant for a random sample of 15 plants with coleoptile tiller present	44.2 ± 1.1	59.8 ± 2.2
Tillers per plant for a random sample of 15 plants with coleoptile tiller absent	44.8 ± 1.7	58.4 ± 1.6

* Measured for the seedling main stem in each pot.

Table 3.1: Morphological trait means (± SEM) for perennial ryegrass cultivars Grasslands Samson and Grasslands Impact grown in a glasshouse in spring (October to November) 2002.

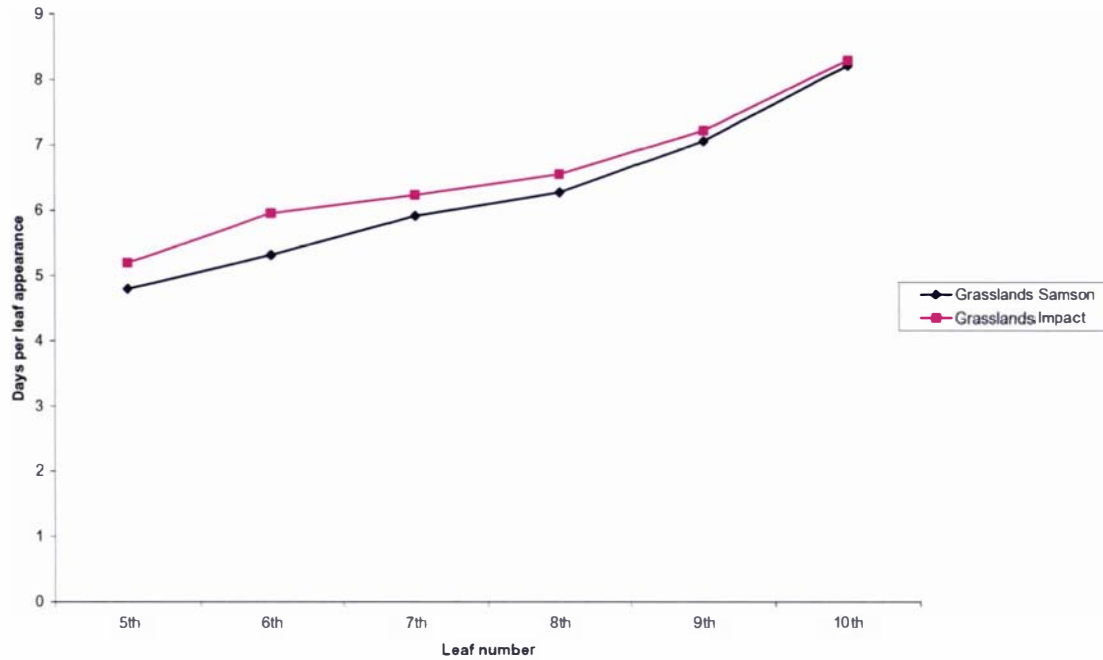


Figure 3.1: Leaf appearance in Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.4.2 Ligule appearance interval

Ligule appearance of leaf 10 was slowed and had not occurred by the end of the experiment in seven plants of Grasslands Impact and in one plant of Grasslands Samson. ANOVA for ligule appearance rate was performed on slope calculated for appearance of seven ligules (leaves 3 to 9) and on eight ligules (leaves 3 to 10), but only the former indicated significant difference between the two cultivars. ALg was faster ($p \leq 0.05$) in Grasslands Samson than in Grasslands Impact (Table 3.1). However, ALg increased with time in both cultivars (Figure 3.2).

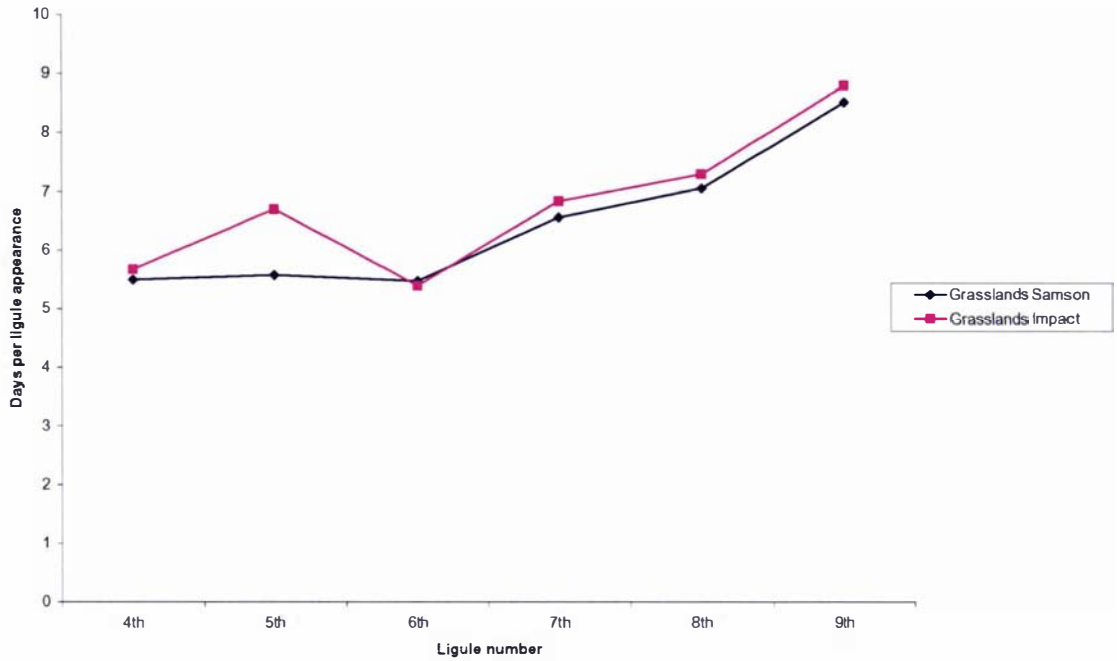


Figure 3.2: Ligule appearance interval (days) in Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.4.3 Leaf lamina length

Analysis of variance on plant means for leaf length indicated no significant difference ($p \leq 0.05$) between cultivars. However, when analysis separated cultivar and leaf effects in a fixed model (GENSTAT 8), the difference between the two cultivars was significant ($p < 0.01$). In both cultivars, the leaf lamina progressively increased, increase slowed after the fifth leaf, and after the seventh leaf, LL declined (Figure 3.3).

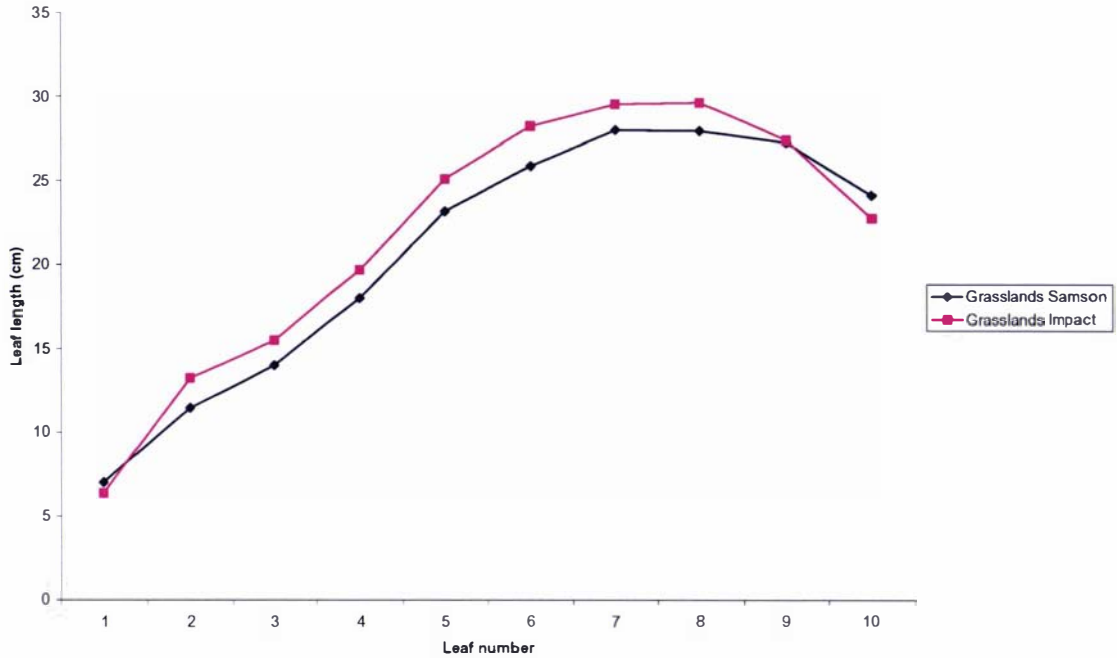


Figure 3.3: Leaf lamina length of Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.4.4 Leaf lamina width

Leaf laminae of Grasslands Samson were significantly wider (0.75 cm, $p \leq 0.01$) than those of Grasslands Impact (0.68 cm). The leaf lamina width progressively increased with the age of plant up to a point and then started to decrease (Figure 3.4). This phenomenon applied to both cultivars.

3.4.5 Leaf elongation rate and duration

There were no significant differences ($p \leq 0.05$) in leaf elongation rate or leaf elongation duration between the two cultivars. The leaf elongation rate and duration were respectively 3.2 cm/day and 8.3 days for Grasslands Samson and 3.3 cm/day and 8.3 days for Grasslands Impact.

3.4.6 Leaf sheath length

The average leaf sheath length of Grasslands Impact (5.3 cm) was significantly greater ($p \leq 0.01$) than that of Grasslands Samson (4.2 cm). This difference in leaf sheath length increased with the age of plants (Figure 3.5). The increase in sheath length of

Grasslands Impact over that of Grasslands Samson varied from 2% for leaf one to 19% for leaf nine.

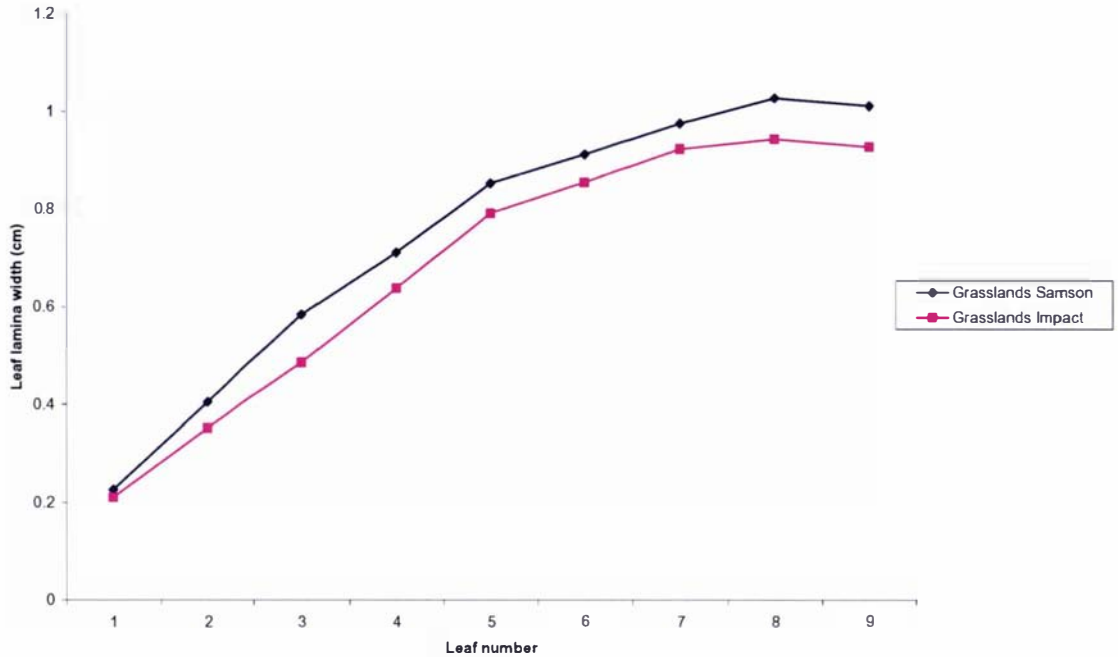


Figure 3.4: Leaf lamina width of Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

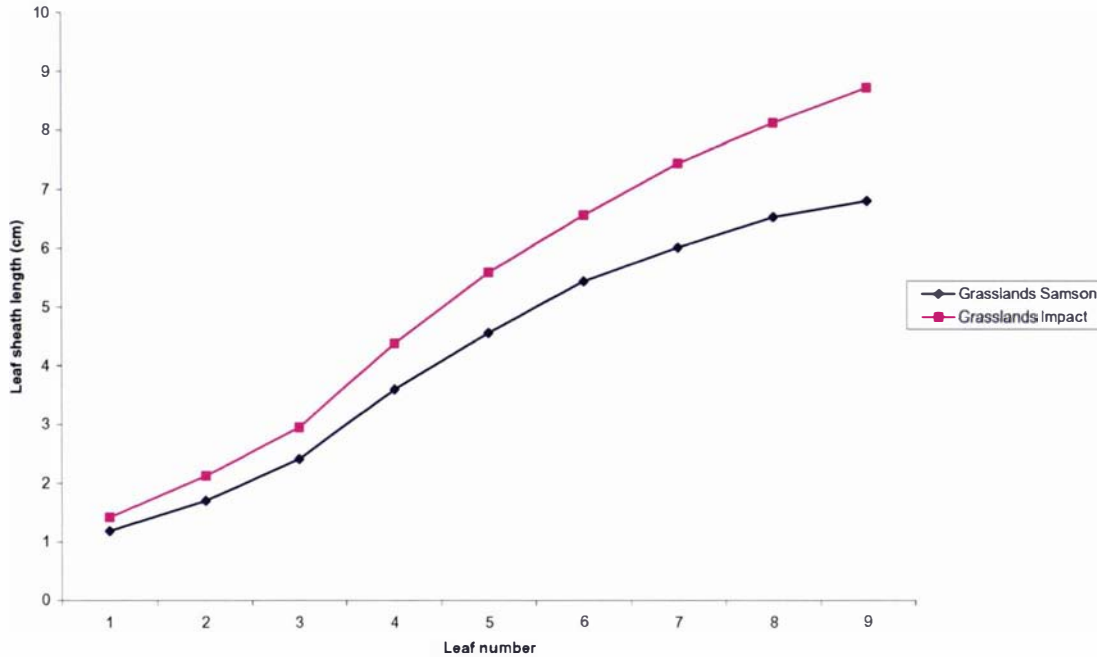


Figure 3.5: Leaf sheath length of Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.4.7 Tiller appearance interval and total tiller number

There was no difference in tiller appearance interval in Grasslands Samson (6.8 days) and Grasslands Impact (7.1 days). Both cultivars produced tillers more slowly towards the end of the experiment (Figure 3.6). Total tiller number, however, was significantly higher ($p \leq 0.05$) in Grasslands Impact (77.5) than in Grasslands Samson (67.8) (Table 3.1).

3.4.8 Tiller site filling

Tiller site filling was similar ($p \leq 0.05$) in both cultivars, and very high (Table 3.1).

3.4.9 Appearance of first primary tiller

There was no difference ($p \leq 0.05$) in time of primary tiller appearance in both cultivars. The first tiller in Grasslands Samson and Grasslands Impact appeared after 15 and 16 days, respectively, from the date the seedlings were transplanted into pots in the glasshouse.

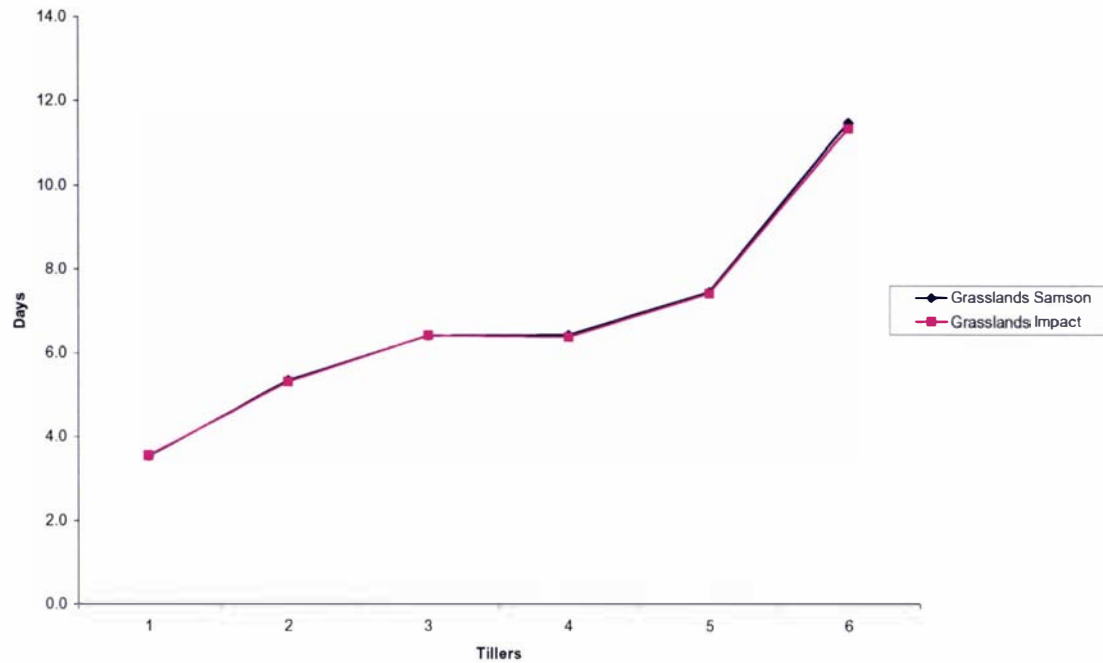


Figure 3.6: Interval between successive primary tillers (days) in Grasslands Samson and Grasslands Impact perennial ryegrass cultivars

3.4.10 Effect of coleoptile tiller on plant tillering behaviour

The production of a coleoptile tiller did not significantly ($p \leq 0.05$) increase tiller production in either cultivar (Table 3.1). None of the coleoptile tillers appeared before the first primary tiller. They either appeared together with the first primary tiller or later.

3.5. Discussion

Of all the seven leaf traits measured (ALf, LED, LER, LW, LS, LL and ALg) Grasslands Samson and Grasslands Impact were different ($p \leq 0.05$) in only four (ALf, LW, LS and ALg). Grasslands Samson had broader leaves and both ALf and ALg were slightly faster for Grasslands Samson than for Grasslands Impact. Interestingly, ALg was slightly greater than ALf for both cultivars, and this was likely linked to each leaf being slightly longer than the previous one (Figure 3.3). Leaf appearance rate in ryegrass was reported to vary from 14 to 26 days in spring (Davies 1977), but in this study both cultivars produced a new leaf every week, probably reflecting warmer temperatures (21°C) in the glasshouse where the plants were kept, than in a British

spring. Temperature is a major determinant of leaf appearance rate and elongation in perennial ryegrass (Parsons 1988), and an increase in temperature was associated with increases in these traits in several grass species including ryegrass (Agnusdei 1999). Leaf sheath length, according to a model of Durand (Durand *et al.* 1999) influences timing of cessation of cell division in emerging laminae, and the longer LL for Grasslands Impact may well explain the longer ALf and ALg (Table 3.1). Increased tiller production in grasses is normally associated with increased leaf appearance rate (i.e. shorter ALf) and higher tiller site filling (Davies 1974; Bahmani *et al.* 2000). However, in this study Grasslands Impact with the higher TN exhibited longer ALf than Grassland Samson, and Fst was not higher. This suggests that the pattern of daughter tiller production by the primary tiller is not indicative of the whole plant daughter tiller production. Bos and Neuteboom (1998) reported that tillers at different hierarchical positions on a plant show different rates of daughter tiller development. This suggests that the secondary or higher order tillers in Grasslands Impact produced daughter tillers faster or in more sites than those in Grasslands Samson.

Fst has been reported to have a theoretical maximum of 0.693 tillers/tiller/leaf appearance interval (Neuteboom and Lantinga 1989). In this study, Fst was slightly higher than the theoretical maximum (0.726 for Grasslands Samson and 0.722 for Grasslands Impact). This indicates firstly 100% site filling over the whole plant, meaning that in this experiment tillering was not suppressed by lower light levels at the base of the plants. A higher Fst than the theoretical maximum is difficult to explain, but could occur where secondary tillers had a slightly faster ALf than the main stem tiller for which Fst was measured. Tiller appearance declined in both cultivars as they advanced in age possibly due to self-shading of tiller bases within a dense canopy (Mitchell and Coles 1955; Kays and Harper 1974; Davies *et al.* 1983).

The date of the appearance of the first primary tiller was similar in the two cultivars (15 days from sowing for Grasslands Samson and 16 days for Grasslands Impact). It has been suggested that differences in timing of the appearance of the first primary tiller may partly explain different tiller number per plant in ryegrass (Bahmani *et al.* 2000), but this was not the case in this experiment. Similarly, the expression of the coleoptile tiller should theoretically lead to higher tiller production in grasses (Lewis and Garcia

1979), but presence of the coleoptile tiller was not associated with increased tiller number per plant in either cultivar (Table 3.1).

3.6. Conclusion

Grasslands Samson and Grasslands Impact perennial ryegrass cultivars were used as parents to establish a mapping population for QTL analysis of eco-physiological traits in perennial ryegrass. These cultivars are different in leaf appearance interval, leaf lamina width, leaf sheath length, ligule appearance interval and tiller number per plant. Grasslands Samson has broader leaves and shorter leaf sheath, and also produces leaf and ligule faster than Grasslands Impact. In terms of whole plant performance, Grasslands Impact produces more tillers than Grasslands Samson.

4

Phenotypic assessment of herbage production traits in a population of perennial ryegrass

4.1. Introduction

Development of cultivars with increased herbage yield is a major aim in forage grass improvement (Wilkins and Humphreys 2003). Perennial ryegrass (*Lolium perenne* L.) is the most widely grown forage grass in the temperate regions of the world. Its maximum yield potential has been estimated to be 29 t DM/ha over a growing season (around 100kg/ha/day) (Humphreys 2005). Conventional plant breeding may have the potential to increase herbage yield to 25 t/year, though cultivars currently available, for instance in UK, can produce annual yields of 17 t DM/ha, and a high nitrogen input is required to obtain this yield (Humphreys 2005).

Herbage yield in perennial ryegrass is influenced by several interacting morphological characteristics (Figure 2.2, chapter 2) which include leaf appearance interval (ALf), leaf elongation rate (LER), leaf length (LL), tiller weight (TW) and tiller number (TN) (Chapman and Lemaire 1993; Bahmani *et al.* 2000). All these characters are quantitative traits with their expression influenced by the environment, and are interdependent in their control of forage growth (Sleper *et al.* 1977; Chapman and Lemaire 1993). In a more absolute sense, increased herbage production of a sward has to arise from a change in one of two fundamental yield components either TN (Nelson and Zarrough 1981; Hernandez Garay *et al.* 1993; Hernandez Garay *et al.* 1999) or TW (Volenec and Nelson 1983; Bahmani *et al.* 2000). At the plant level, TN and TW are negatively correlated and are subject to size-density compensation (SDC) (Chapman *i* and Lemaire 1993). Hence, a tiller size increase with increasing herbage mass is often associated with a tiller population density decrease, and size-density compensation in these circumstances does not necessarily imply any change in sward productivity. Similarly, a tiller population increase may be a simple reflection of reduced herbage mass, again without necessarily indicating a change in sward productivity (Hernandez Garay *et al.* 1999). To differentiate between productivity-related change in TW or TN

and productivity-neutral negative correlation between TW and TN, Matthew *et al.* (1995) proposed a productivity index (PI) based on the -3/2 self thinning relationship (Yoda *et al.* 1963). The fundamental assumption in calculating the PI, supported by experimental observation, is that -3/2 compensation between TN and TW values over time would represent a constant sward leaf area index, whereas movement above or below a -3/2 trajectory would indicate increase or decrease in leaf area index, respectively. Thus, PI provides a theoretical basis for taking account of both TW and TN simultaneously, and provides a comparative estimate of leaf area index and productivity of a sward (Matthew *et al.* 1995; Hernandez Garay *et al.* 1999). It has also been reported (van Loo 1992; Bahmani *et al.* 2001) that LER increase is not generally offset by changes in other traits, and so is another potentially useful measure of plant yield potential.

In the present study the interest is to make use of the agronomic parameters just described, within the emerging discipline of QTL mapping. QTL mapping identifies genetic loci responsible for variation in measured phenotypic traits by means of molecular markers which can then be used in MAS to accelerate the genetic improvement of those traits. In respect of requirements imposed on a phenotype assessment exercise when the objective is QTL discovery, forage grasses including perennial ryegrass, exhibit wide seasonal variation in plant yield and constituent traits, and often display genotype-by-season interaction. QTL discovery for a trait in one season therefore needs to be confirmed in other seasons in order to identify a robust QTL, and to have a better understanding of the genetic nature of the trait. As a further requirement for QTL discovery, the mapping population should exhibit significant variation for the traits under investigation.

4.2. Objectives

The overall objective of this study was to assess phenotypic variation of a perennial ryegrass mapping population and its two parents during a vegetative growth period for herbage yield and related traits, with a view to QTL analysis of those traits. More specifically, this experiment aimed to:

1. Assess phenotype of the mapping population and its parents for herbage yield and component or related traits (such as leaf appearance interval, leaf elongation rate, tiller number and tiller weight) in autumn.
2. Repeat the assessment in spring

4.3. Materials and Methods

4.3.1 Mapping population

A full-sib F_1 mapping population (n = 200), 'I×S' of perennial ryegrass was constructed from a pair cross between one plant of Grasslands Impact (I) as seed parent (infected with wild-type endophyte) and one plant of Grasslands Samson (S) as pollen parent (infected with AR6 endophyte) in 2002 at AgResearch Grasslands Research Centre, New Zealand. The parents were about 2 years older than progeny. The parent cultivars have contrasting growth habit and flowering dates. Grasslands Samson is erect and vigorous with typical mid-season reproductive characteristics. Grasslands Impact is a dense, fine-leaved, persistent ryegrass with late reproductive development. These cultivars were compared in Experiment 1 (Chapter 3). Two hundred progeny of the pair cross were randomly selected and constituted the "I×S" perennial ryegrass mapping population. The plants were clonally divided for field and glasshouse experiments and for DNA extraction and genetic analysis after they had been growing for about 3 months. Sixteen clones (2-3 tillers/clone) of each progeny and the two parents were established during January and February 2003, in 'Planter' bags (64 x 64 x 150 mm), containing commercial bulk potting mix, and were maintained outdoors on concrete areas until they were needed.

4.3.2 Experimental setup and design

4.3.2.1 Autumn assessment

This experiment was designated Experiment 2 (Figure 4.1) and was conducted in autumn (April to July) 2003. Four clones of each of the 200 plants and their two parents were transferred from the concrete area into a glasshouse in the first week of March 2003. Three clones (replicates) were arranged on tables in a randomised complete block design (RCBD) with one clone per block. The table top comprised a woven steel grill which was covered with felty material, overlaid by black nylon mat. Watering of plants was by capillary system. Matting beneath the plants was kept moist

through a network of capillary tubes attached to a main tube connected to a water tap, allowing the Planter bags to absorb water from the bottom. The experiment was watered automatically for 10 minutes in every 24 hours. Plants in each block (1.1m x 2.4m) were spaced in rows at 78 plants per m^2. Border rows around and between blocks were established with the fourth clone of the plants. Initially, there was evidence of stem rust (*Puccinia graminis*) infestation on some plants, and all plants were sprayed with Folicur (Triazole) (430g/L of Tebucorazole suspension concentrate) for treatment against the disease. One week later, the plants were cut 6 cm above soil level to allow fresh re-growth. Another week later, dominant tillers of similar age were identified (one tiller/clone) and tagged with a split ring made from 3 mm diameter plastic tube. All leaf measurements were performed on the tagged tillers.

Figure 4.1: Layout of Experiment 2 of the I×S perennial ryegrass mapping population assessed in glasshouse in autumn 2003

4.3.2.2 Spring assessment

This experiment was designated Experiment 5. One plant of each of the 200 individuals in the mapping population and of its two parents was collected from the concrete area, and sub-divided to obtain three equal-sized ramets of three tillers, as replicates of each

genotype in the current experiment. The ramets were established in new Planter bags (64 x 64 x150 mm) and kept in a glasshouse for 3 weeks before the start of experiment. The experiment was conducted in spring (September to October) 2004 using the same methodology and in the same glasshouse where Experiment 2 was conducted in autumn.

4.4 Data collection

4.4.1 Assessment of growth environment

4.4.1.1 Light and temperature

A data logger (LI-1000 Data logger, LI-COR, inc., Lincoln, NE 68504, USA) linked to two temperature sensors and two light sensors was used to record temperature and solar radiation in the glasshouse for both experiments. Readings were taken across the experiments (front and back of table) during the first and last weeks of the experiments. Readings of solar radiation were also taken from above and below the leaf canopy in the last week of the experiments.

4.4.1.2 Water holding capacity of soil mix

In order to know if the mix could retain enough water for the plants during the periods between successive irrigation (i.e., once every 24 hours), the water holding capacity (WHC) of the commercial potting mix was determined using methods described by Gardner (Klute 1986). Three Planter bags containing the soil mix were water-saturated from the bottom, excess free water was allowed to drain for 24 hours, and the bags were weighed. The soil mix was then dried at 105°C for 16 hours and reweighed. The water holding capacity was calculated as follows;

WHC = ((Wt. of wet soil mix – Wt. of dry soil mix)/Wt. of dry soil mix) (1).

4.4.1.3 Uniform availability of water to plants

In order to know if after irrigation, water was uniformly available to all experimental plants, a test was conducted to measure the amount of water in Planter bags of potting mix at different table positions of every part in the experiment. Eighteen Planter bags were filled each with an equal weight of potting mix. Six were placed in each of the three experimental blocks; two at the front, middle and back of the table on which the experiment was laid. They were irrigated for 10 minutes, and then weighed 24 hours later. The soil was oven dried at 105°C for 24 hours and weighed again. Soil moisture content (SMC) was calculated as follows;

SMC % = 100 x (Wt. of soil after irrigation – Wt. of oven dried soil)/ Wt. of oven dried soil. (2)

4.4.2 Phenotype assessment

Data were collected on the following traits: ALf, ligule appearance interval (ALg), LL, LER, leaf elongation duration (LED), herbage dry weight (DW), TN, TW and PI from all 200 plants and their two parents in both autumn and spring experiments, while stem internodes length (SIL) was measured only in the autumn experiment. Leaf data (ALf, ALg, LL, LER and LED) were collected on four consecutive fully developed leaves on each tagged tiller. Methodology followed that of Bahmani *et al.* (2000) with modifications as in section 3.3.2 of Chapter 3. TN was counted as total tillers per plant at the start and end of experiment. To measure DW, herbage was harvested from each plant at 6 cm above soil level and placed in a paper bag. All plants in an experiment were harvested on the same day, dried together in the same oven for 12 hours at 90°C and weighed. During weighing, five to 10 samples were drawn from the oven at any one time to prevent them absorbing moisture from the outside air. The oven was also reheated once or twice over the course of a weighing session because its frequent (intermittent) opening and closing would expose samples to moist air. DW samples were weighed on a 4 decimal point Balance (Mettler PM6100, Watson Victor Ltd., Wellington, New Zealand).

Data collection for the autumn experiment was initiated 6 weeks after plants were potted and 7 days after plants were trimmed to allow fresh re-growth. For the spring experiment, data collection started 4 weeks after plants were potted and 7 days after they were trimmed.

TW and PI were derived from DW and TN for each plant using the formulae:

$$TW = DW / TN \quad (3)$$

and

$$PI = Log(TW)-(-1.5 \times Log(TN) / A) \quad (4)$$

Where: -1.5 = constant assumed for SDC; Log(TN) = log of TN; Log(TW) = log of TW; A = surface area of Planter bag (m^2).

SIL was measured 1 to 2 weeks following herbage DW harvesting. Each plant with all tillers intact was removed from its Planter bag and roots washed. Clusters of tillers were separated according to hierarchical position within the plant, and clusters then further separated into single tillers. Three tillers were selected from each clone in every block: the tiller with the longest basal vegetative true stem and two other tillers selected randomly. Internode length (mm) (Figure 4.2) was measured on the first six internodes (i.e. between nodes 1 to 7) from the base of the lowest leaf using a low power (14x) binocular microscope and a ruler.

4.5. Data analysis

Data were analysed for differences among genotypes and among replicates using the residual maximum likelihood (REML) analysis option in GenStat (Genstat 2005). The analysis resulted in the generation of best linear unbiased predictor (BLUP) values for the genotypes, based on traits measured in autumn and spring. Broad sense heritability estimates were calculated separately for both experiments as

$$H_b = \sigma^2_g/(\sigma^2_g+(\sigma^2_\varepsilon/r)) \quad (5)$$

Where, σ^2_g = genotypic component of variance, σ^2_ε = residual variance of genotypes and r = number of replications (Burton and De Vane 1953).

Figure 4.2: Three tillers of Grasslands Impact perennial ryegrass plant showing stem internode length (SIL).

Heritability estimates across seasons (autumn and spring) were calculated for all measured traits as

$$H_b = \sigma^2_g/(\sigma^2_g+(\sigma^2_{gs}/n_s + \sigma^2_\varepsilon/n_r n_s)) \quad (6)$$

Where, σ^2_{gs} = genotypic component of variance across season, n_s = number of season and n_r = number of replications.

Multivariate analysis was also performed using Principal Component Analysis (PCA) in MINITAB to identify patterns of correlation between measured variables and plants with a favourable combination of herbage yield related traits. PCA was chosen as this multivariate approach is considered to be particularly suitable for datasets with possible correlations among attributes (Manly 1994). Also as the data matrix comprised 202 genotypes (the 200 full-sib progeny plus I and S parents), other forms of multivariate analysis would have been unwieldy with a data matrix of this size. PCA results

presented are for data averaged over the three replicates. However, to confirm the results presented were biologically meaningful, an expanded PCA (not reported) was performed with replicated data for each variable (three replicates) entered as separate columns in the data matrix. For the first three PCs (i.e., the three PCs presented for the PCA) from data averaged over replicates, coefficients of a particular variable in each PC varied little across replicates, indicating that these three PCs reflected genuine genotypic variation and not random data 'noise' associated with differences between replicates.

4.6. Results

4.6.1 Glasshouse environment

Temperatures were similar in autumn and spring experiments, but light levels and day-lengths were much higher in spring (Table 4.1).

The water holding capacity of the potting mix was 620 g water/kg mix, which shows that an adequate amount of water would be retained in the soil to sustain plant growth between successive irrigation times. Water availability to every plant was judged sufficiently uniform not to affect plant growth, although plants at the middle of each replicate received slightly more water than those in the front or back (Table 4.2).

The plant nutrient status of the potting mix used as growth medium was not measured, but the same mixture has been used regularly and found to sustain optimum growth of ryegrass for over 2 years (John Ford, personal communication). No indication of moisture or nutrient deficit was observed at any time in either experiment.

Season	Temperature (°C)		Solar radiation ($MJ/m^2/day$)			Daylength (hours)
	Range	Mean	Glasshouse	Above canopy	Below canopy	
Autumn (2003)	17-28	21	2.6	2.4	0.45	10.2
Spring (2004)	16-28	20	8.5	8.4	0.85	13.1

Table 4.1: Mean temperature and solar radiation (above and below leaf canopy) in the glasshouse, and mean day-length recorded during the assessment of the I×S mapping population and its parents for herbage yield and related traits in autumn 2003 and spring 2004

	Table part		
Replication	Front	Middle	Back
1	14 %	18 %	16 %
2	14 %	18 %	16 %
3	16 %	20 %	17 %

Table 4.2: Soil moisture content of potting mix in Planter bags in each replication at different positions on the table 24 hours after 10 minutes irrigation. Front: towards centre of glasshouse; Back: near the glasshouse wall.

4.6.2 Phenotypic assessment of herbage yield traits

Mean values and ranges of the traits studied in autumn and spring are shown in Table 4.3. TN was static in autumn, but in spring there was more than a twofold increase in TN even though the autumn experiment ran for a longer period (April to July) than that of spring (September to October). However, the spring experiment was initiated with fewer tillers (13 tillers/plant) as compared to the autumn (35 tillers/plant), so that tiller production, TW, DW and PI (derived using TN) were not compared between the two seasons. Mean values were significantly different between seasons for ALf, LER, LED, LL and ALg.

There was significant ($p \leq 0.01$) variation within the plant population for all the measured traits in both autumn and spring. Normality tests (Appendix 4.1) indicated non normality for TN, DW, ALf, ALg, LED and LER in autumn, and PI, TN and LL in spring, but the histograms did not show an obvious skew for the traits (Figure 4.3).

Significant ($p \leq 0.05$) replicate effects were observed in both seasons with higher values of ALg, LER and SIL in replicate three, and TW in replicates one and two in the autumn assessment. In the spring assessment, DW and PI were higher in replicate one and TW was higher in replicate three.

	Autumn (2003)		Spring (2004)		T-test
Trait	Mean	Range	Mean	Range	(season)
DW	3.2 ± 0.0	2.2 - 4.9	1.9 ± 0.0	0.9 - 2.8	--
ALf	13.3 ± 0.8	9.0 - 17.0	11.2 ± 0.1	8.5 - 14.5	**
ALg	12.5 ± 0.1	7.8 - 17.2	11.3 ± 0.1	8.2 - 15.2	**
LED	15.2 ± 0.1	10.0 - 19.0	13.3 ± 0.1	9.3 - 18.3	**
LER	1.2 ± 0.0	0.6 - 1.9	2.0 ± 0.0	1.15 - 3.1	**
LL	17.6 ± 0.1	11.6 - 25.8	26.5 ± 0.2	18 - 37	**
TN (start)	34.7 ± 3.7	20 - 69.9	13.4 ± 2.2	5.0 - 27.7	--
TN (end)	34.2 ± 3.2	21 - 61	31.3 ± 3.7	14.3 – 63.7	--
TW	0.09 ± 0.00	0.05 - 0.14	0.05 ± 0.00	0.03 - 0.1	--
PI	5.3 ± 0.0	5.5 - 6.6	3.6 ± 0.0	3.1 - 3.8	--
SIL	2.8±0.5	0.6 - 5.1			

** Differences between seasons significant at $p < 0.001$

Table 4.3: Mean (±SE) and range of herbage production traits of the I×S perennial ryegrass mapping population and their parents measured in a glasshouse during autumn 2003 and spring 2004. DW: herbage dry weight (g); ALf: leaf appearance interval (days); LER: leaf elongation rate (cm/day); LED: leaf elongation duration (days); LL: leaf lamina length (cm); ALg: ligule appearance interval (days); TN: tiller number; TW: tiller weight (g); PI: productivity index and SIL: stem internode length (cm).

The means of the three clonal replicates for S and I parent plants differed for ALf, ALg, TN, TW, PI and SIL in autumn, but in spring, they were different only in LL and TW (Table 4.4). SIL was only measured in autumn, and was longer in I than in S.

	Autumn (2003)			Spring (2004)		
Trait	S	I	$LSD_{0.05}$	S	I	$LSD_{0.05}$
DW	3.2	2.7	0.7	1.4	1.4	0.7
ALf	9	13.5	2.2	8.8	9.7	2.0
ALg	9.2	13.2	2.5	7.8	10.7	2.9
LED	11.4	14.6	3.1	10.7	11.3	2.7
LER	1.6	1	0.4	2.3	2.9	0.7
LL	17.8	14.8	3.8	24	32.6	7.0
TN	1.4	1.7	0.1	0.1	0.6	0.7
TW	0.12	0.06	0.02	0.08	0.04	0.02
PI	5.8	6.3	0.1	3.3	3.5	0.2
SIL	1.2	3.5	1.5			

Table 4.4: Mean values from three clonal replicates for parents of the I×S perennial ryegrass mapping population assessed together with the population in a glasshouse during autumn 2003 and spring 2004. LSD values are for cultivar effect in each environment. DW: herbage dry weight (g); ALf: leaf appearance interval (days); LER: leaf elongation rate (cm/day); LED: leaf elongation duration (days); LL: leaf lamina length (cm); ALg: ligule appearance interval (days); TN: tiller number; TW: tiller weight (g); PI: productivity index and SIL: stem internode length (cm); S: Grasslands Samson parent; I: Grasslands Impact parent

The estimates of broad sense heritability (H_b) were relatively high in both seasons (Table 4.5). In general, the heritabilities were similar in both seasons for LER and LED, but were a little higher in autumn for all the other traits.

The correlation coefficients among herbage yield and related traits in autumn and in spring and combined over seasons are shown in Table 4.6. DW correlated significantly ($p<0.01$) with TN and TW in autumn, but only with TW in spring, indicating that conditions in spring predispose to TW being the irnportant yield component, rather than TN. PI, incorporating information on TN and TW, was a better predictor of DW in both seasons than either TN or TW alone, and irrespective of whether TN (autumn) or TW (spring) was more closely correlated with DW. PI correlated highly with TN and

negatively (-0.65) with TW in autumn, but did not correlate ($p > 0.01$) with either of the traits in spring. There was no significant ($p \leq 0.05$) correlation between DW and LER. DW correlated sizeably with ALf and LL but only in autumn. LL significantly correlated with TN and TW but the magnitude of correlation with TN was relatively higher in spring while that of TW was higher in autumn. In autumn TN negatively correlated with TW and positively with PI. LER correlated negatively with ALf, LED, and PI, but positively with LL and TW in both seasons. The correlation between LER and TN was negative in autumn but positive in spring. High correlations between some variables, for instance, ALf and ALg would mean that they measure the same trait in different ways.

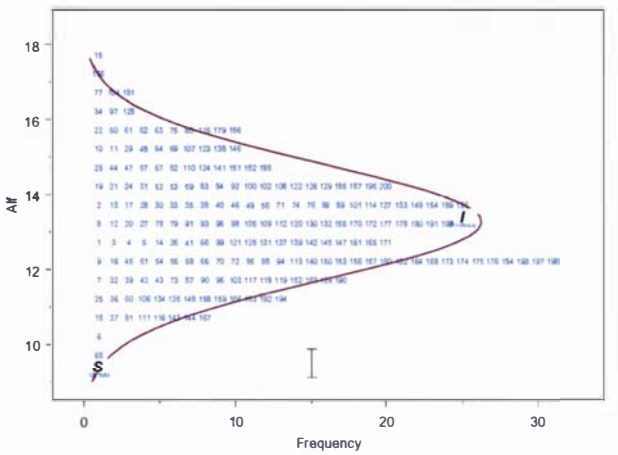
Trait	σ^2_g			σ^2_{ge}	H_b		
	Autumn	Spring	Combined		Autumn	Spring	Combined
DW	0.11 ±0.02	$6.2x10^{-2}$ ±$1.3x10^{-2}$	0.03 ± 0.01	0.06± 0.01	0.62	0.52	0.31
ALf	1.7 ±0.2	0.9 ± 0.1	0.48 ± 0.13	0.8± 0.1	0.74	0.63	0.42
ALg	1.6 ±0.3	0.9 ± 0.2	0.9 ± 0.2	0.3 ± 0.1	0.67	0.45	0.61
LED	1.2 ±0.3	1.2 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.51	0.57	0.44
LER	$13.7x10^{-3}$ ±$3.3x10^{-3}$	0.05 ± 0.01	0.005 ± 0.005	0.025 ± 0.006	0.44	0.46	0.15
LL	2.9 ±0.5	4.7 ±1.2	2.3 ± 0.6	1.4 ± 0.6	0.61	0.43	0.46
TN (end)	$4.4x10^{-3}$ ±$0.6x10^{-3}$	$9.5x10^{-3}$ ±$1.6x10^{-3}$	0.0 ± 0.0	0.008 ± 0.001	0.74	0.63	0
TW	$18.1x10^{-5}$ ±$2.5x10^{-5}$	$71.8x10^{-5}$ ±$1.2x10^{-5}$	$3.4x10^{-5}$ ± $1.3x10^{-5}$	$9.2x10^{-5}$ ± $1.5x10^{-5}$	0.75	0.62	0.32
PI	0.0047 ±0.0007	$7.9x10^{-3}$ ±$1.5x10^{-3}$	0.002 ± 0.001	0.004 ± 0.001	0.63	0.56	0.31
SIL	0.4±0.0				0.59		

Table 4.5: Genotype variance component (σ^2_g), genotype x season variance component (σ^2_{ge}) and broad sense heritability value (H_b) of herbage production traits of the 1×S mapping population of perennial ryegrass assessed in a glasshouse during autumn 2003 and spring 2004. DW: herbage dry weight (g); ALf: leaf appearance interval (days); LER: leaf elongation rate (cm/day); LED: leaf elongation duration (days); LL: leaf lamina length (cm); ALg: ligule appearance interval (days); TN: tiller number; TW: tiller weight (g); PI: productivity index and SIL: stem internodes length (cm)

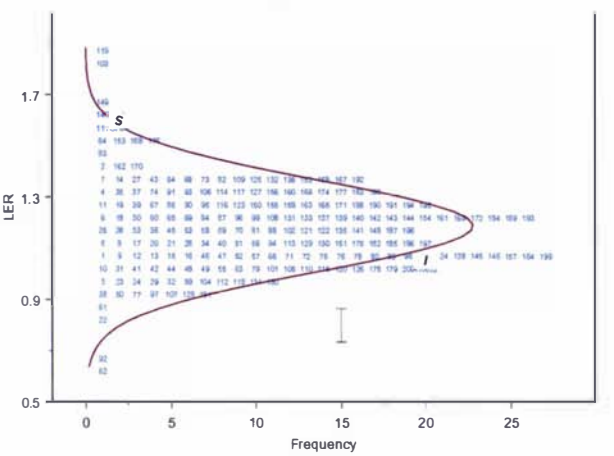
		DW	ALf	ALg	LED	LER	LL	TN	TW
ALf	Autumn	0.15							
	Spring	0.04							
	Combined	0.12							
ALg	Autumn	0.06	0.86						
	Spring	0.07	0.68						
	Combined	0.11	0.85						
LED	Autumn	0.18	0.77	0.67					
	Spring	0.08	0.82	0.57					
	Combined	0.16	0.83	0.70					
LER	Autumn	0.08	-0.44	-0.33	-0.41				
	Spring	-0.01	-0.49	-0.27	-0.59				
	Combined	0.01	-0.39	-0.26	-0.47				
LL	Autumn	0.22	0.28	0.30	0.22	0.62			
	Spring	0.09	0.11	0.15	0.16	0.68			
	Combined	0.13	0.26	0.27	0.27	0.65			
TN	Autumn	0.44	0.23	0.21	0.26	-0.24	-0.18		
	Spring	0.17	-0.25	-0.04	-0.19	0.40	0.30		
	Combined	0.40	0.07	0.17	0.14	0.17	0.18		
TW	Autumn	0.28	-0.14	-0.20	-0.15	0.32	0.36	-0.73	
	Spring	0.38	-0.11	-0.19	-0.10	0.19	0.18	-0.07	
	Combined	0.26	-0.11	-0.11	-0.10	0.21	0.20	-0.18	
PI	Autumn	0.53	0.23	0.20	0.26	-0.22	-0.15	0.99	-0.65
	Spring	0.92	0.09	0.14	0.14	-0.08	0.05	0.18	0.05
	Combined	0.88	0.19	0.19	0.24	-0.13	0.04	0.45	-0.08

Table 4.6: Coefficients of correlations between herbage yield related traits assessed in autumn, spring and across seasons ($p<0.05$ if $r\geq0.14$; $p<0.01$ if $r \geq0.18$). DW: herbage dry weight (g); ALf: leaf appearance interval (days); LER: leaf elongation rate (cm/day); LED: leaf elongation duration (days); LL: leaf lamina length (cm); ALg: ligule appearance interval (days); TN: tiller number; TW: tiller weight (g); PI: productivity index.

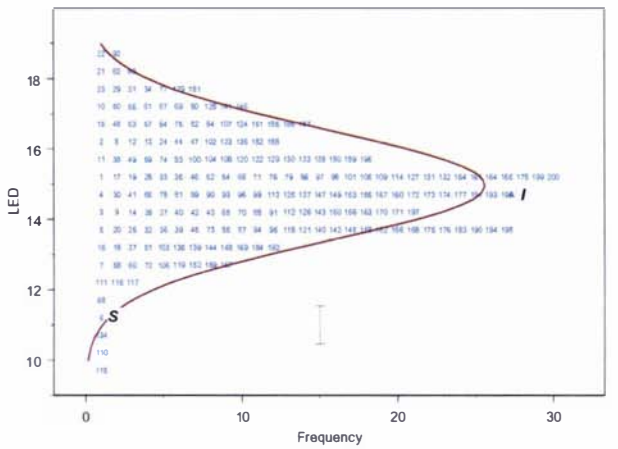
Leaf appearance interval (days)



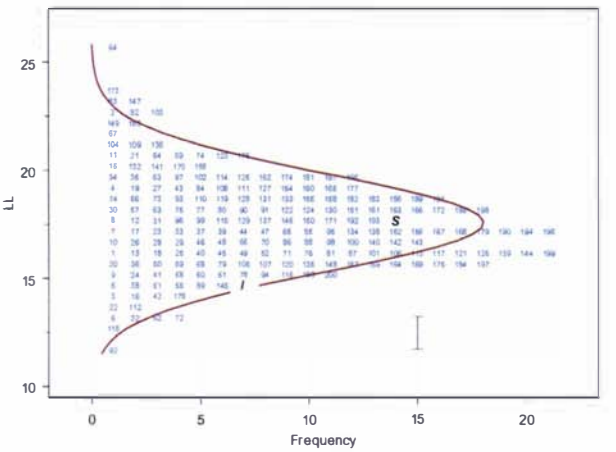
Leaf elongation rate (cm/day)



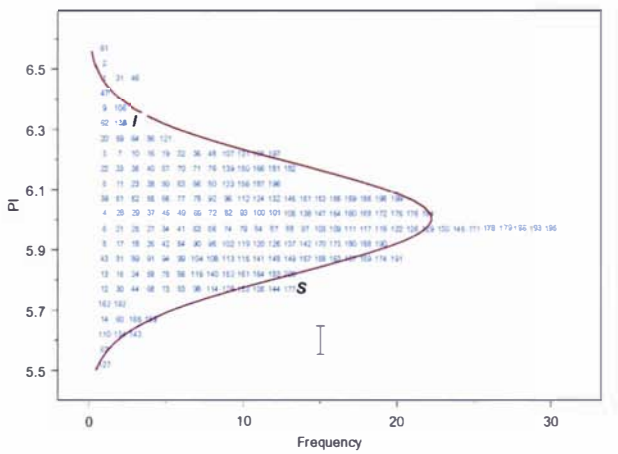
Leaf elongation duration (days)



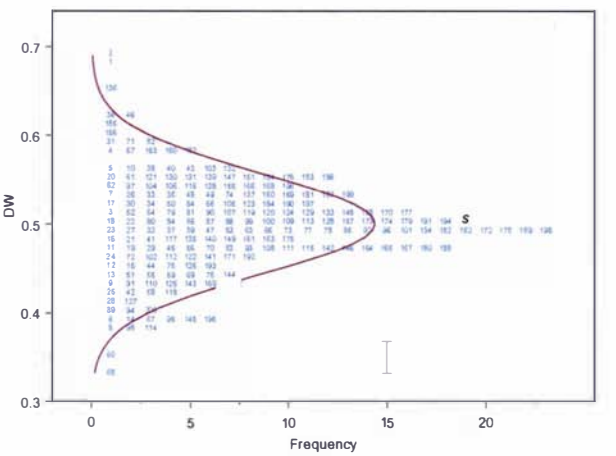
Leaf lamina length (cm)



Productivity Index



Herbage dry weight (g)



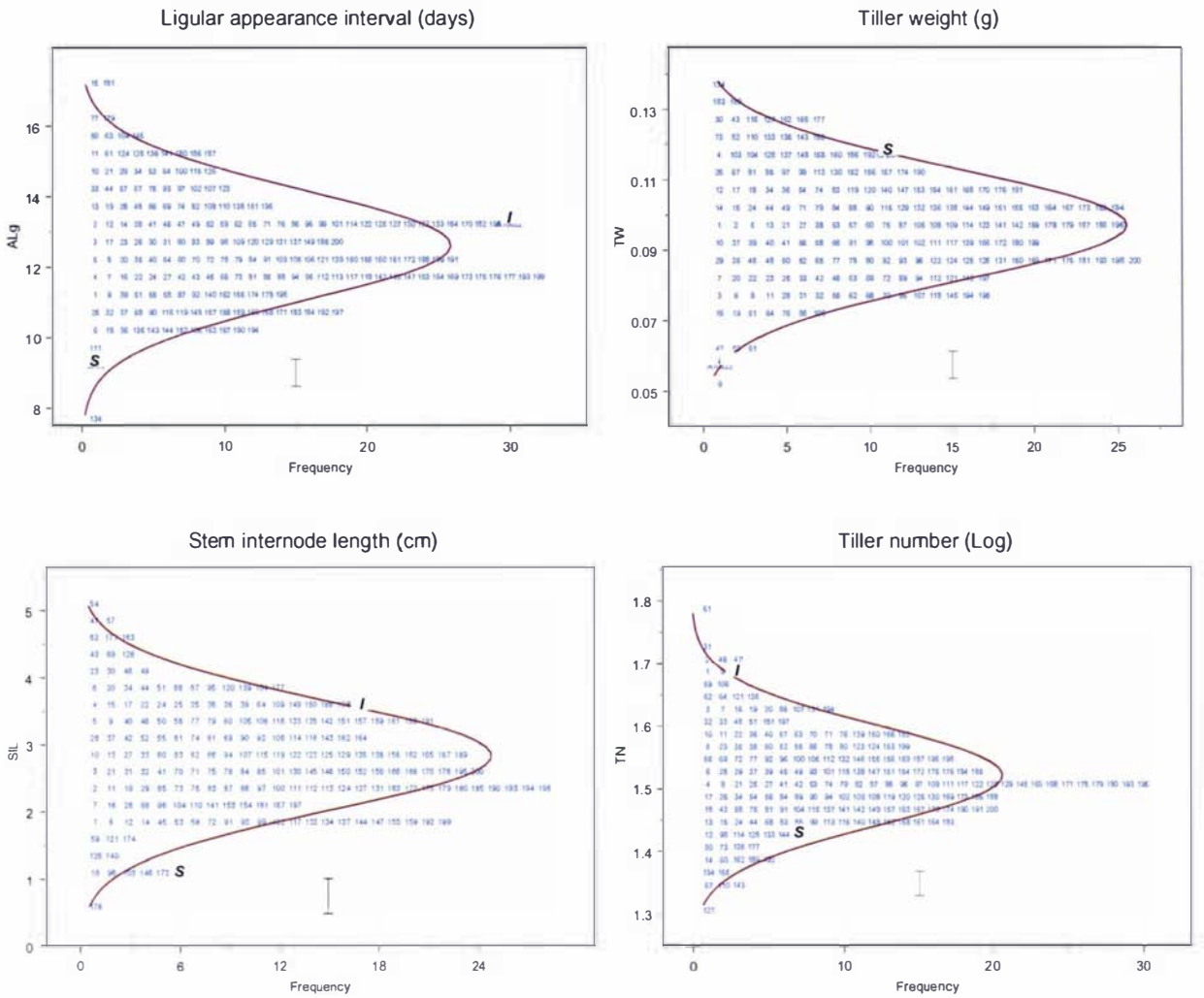


Figure 4.3: Frequency distribution of phenotypes for herbage yield and related traits in the I×S perennial ryegrass mapping population assessed in the glasshouse in 2003 (means of three replicates). I: Grasslands Impact plant; S: Grasslands Samson plant, and the error bar indicates least significant difference of means.

PCA was used to collectively examine the pattern of associations between traits across the mapping population in autumn and in spring. The first three of the 13 available PCs had eigenvalue >1, between them accounting for 76 % and 74 % of the observed variation between genotypes in autumn and spring respectively, and are reported here (Table 4.7 and Appendices 4.2.1 and 4.2.3). As stated in the methods section, these three PCs also yielded similar coefficients for each replicate when replicate data for autumn were included in an expanded PCA (Appendix 4.2.2).

In comparing PCs for autumn and spring, spring PC1 reflects autumn PC1, spring PC2 reflects autumn PC3 and spring PC3 reflects autumn PC2. In both seasons, PC appears to be ordinating plants on essentially the same criteria, though with different priority (spring PC2 and autumn PC3 reversed). Spring PC1 and autumn PC1 have in common a high LED and low LER relationship. PC1 and PC2 in both autumn and spring have a size/density component which appears to be weaker in spring, when TW is the dominant determinant of DW. Autumn PC3 associates increased DW with increased LER and long LL, while spring PC2 identifies TW as the main component for DW in spring. PC3 in both seasons separated the two parent plants which did also PC1 in autumn (see Appendix 4.2.4 for PC scores). Spring PC2 and autumn PC3 both identified PI as a good predictor for DW.

	Autumn 2003			Spring 2004		
Traits	PC1 (36%)	PC2 (23%)	PC3 (17%)	PC1 (32%)	PC2 (24%)	PC3 (18%)
DW	0.175	0.052	-0.578	0.069	-0.658	-0.211
ALf	0.393	0.382	0.134	0.494	0.032	0.126
ALg	0.363	0.369	0.133	0.397	-0.013	0.179
LED	0.382	0.329	0.100	0.469	0.004	0.061
LER	-0.300	0.111	-0.493	-0.330	-0.181	0.549
LL	-0.038	0.488	-0.397	0.007	-0.239	0.722
TN	0.412	-0.327	-0.283	0.495	0.024	0.125
TW	-0.309	0.387	-0.143	-0.082	-0.364	0.053
PI	0.405	-0.305	-0.339	0.115	-0.586	-0.251

Table 4.7: Principal component analysis of herbage yield and related traits of the 1×S perennial ryegrass mapping population assessed in a glasshouse in autumn 2003 and in spring 2004. DW: herbage dry weight (g); ALf: leaf appearance interval (days); LER: leaf elongation rate (cm/day); LED: leaf elongation duration (days); LL: leaf lamina length; ALg: ligule appearance interval (days); TN: tiller number; TW: tiller weight (g); PI: productivity index.

4.7 Discussion

4.7.1 Growth environment

During each assessment season (autumn or spring), all experimental plants were exposed to similar levels of temperature, sunlight, water and nutrient, and the same management. Uniformity of conditions in growth environment is basic to an accurate assessment of phenotypes within a population of plants, so that any variation in the phenotype would be a result of genotypic differences among plants in the population.

The mean temperature of the glasshouse during the autumn and spring experiments was within the temperature range (20-25°C) for normal growth of perennial ryegrass (Mitchell 1954; Alberda 1966; Hunt and Field 1978; White and Hodgson 1999). Solar radiation was dramatically less in autumn than in spring (Table 4.1), while daylength and light intensity were decreasing during the autumn experiment and

increasing in the spring experiment, possibly changing plant responses. In addition plants would have been in the early stages of reproductive growth by the end of the spring experiment and this too may have changed plant behaviour.

Sunlight reaching the plant base was about 10 % and 17 % of the incident radiation in the glasshouse (Table 4.1), and this indicates that neighbouring pots essentially formed a canopy with reasonably complete closure. The amount of solar radiation for optimum performance of perennial ryegrass is not clear, but some reports suggest that grass growth occurs in temperate areas with solar radiation ranging from 1 $MJ/m^2/day$ in midwinter to around 9 $MJ/m^2/day$ in midsummer (Woodward and Sheehy1983). In this study, solar radiation varied from 2.6 $MJ/m^2/day$ in autumn to 8.5 $MJ/m^2/day$ in spring. The combination in autumn of low light levels and high temperatures would have resulted in some stress on plants in Experiment 2.

4.7.2 Phenotypic variation

The I×S perennial ryegrass mapping population was significantly ($p<0.01$) variable in herbage yield and related traits. Heritability estimates were similar for LER and LED in both seasons, but were higher in autumn for all the other traits. However, these estimates correspond with those reported elsewhere in spaced-plants of perennial ryegrass (Yamada *et al.* 2004).

The separation of the parents within a trait differed seasonally, with variation in more traits during autumn than in spring (Table 4.4). Parents were significantly ($p\leq 0.05$) different in LL and TW in spring, but in the autumn they were different in all the traits except in DW, LER, LED and LL. In Experiment 1 (Chapter 3) where 50 plants of each parent cultivar were assessed in spring, Grasslands Samson and Grasslands Impact were different in ALf, ALg and TN, with Grasslands Samson producing leaf and ligule faster while Grasslands Impact produced more tillers.

In comparing trait means of the parents, ALf, ALg and LED were shorter and LER was lower in Experiment 1 (Table 3.1, Chapter 3) than either in Experiment 2 or 5 (Table 4.4). Plants studied in Experiment 1 were raised from seedlings, and faster growth rates in Experiment 1 may imply greater vigour in seedlings than in cuttings

during the early establishment phase of a plant. However, assessments in Experiments 2 and 5 were based only on three replicates of one parent plant which may not be representative of the cultivar from which the parent was selected. The parental phenotypes were faster than those of the progeny in ALf and LED in spring, but were equal to or very close to progeny mean for all other traits in both seasons. This suggests there was no ageing effect of the parents even though they were about two years older than their progeny.

Stem internodes were longer in I than in S. This confirms the earlier report of extensive stolon formation in Grasslands Impact (Donaghy 1999), and it is a feature of persistence in perennial ryegrass.

4.7.3 Identification of herbage yield traits

In this study, PCA identified LER, LL, TW, TN and ALf, as traits associated with herbage yield, and this supports findings reported earlier (Chapman and Lemaire 1993; Hernandez Garay *et al.* 1999; Bahmani *et al.* 2000). However, the magnitude of contribution of each trait toward herbage yield varies with season. LER and LL were identified as major related traits in autumn, but with the onset of reproductive growth in spring, TW was identified as the main trait for increased herbage production.

4.7.4 Individual trait performance

Individual trait performance varied with season. Spring production was associated with longer leaves, faster appearance of leaves, and faster leaf elongation than autumn production, and spring production has been associated with faster growth rates (Smetham 1990) mainly as a result of the transition from vegetative to reproductive development which is associated with changes in several major physiological processes that alter the response of grass crop to its environment (Parsons 1988). TN significantly increased in spring but was static in autumn. PI, accounting for TN and TW in estimating herbage yield correlated positively with DW in both seasons, and with TN in autumn. TW had a moderate but stable contribution to herbage yield in both seasons, and was associated with LER and LL. Tiller size/density effect was

only pronounced in autumn. This indicates that the importance of TN and TW as yield determinants in herbage production is affected by the environment.

Despite high LER having been identified as an important indicator of herbage yield in perennial ryegrass (van Loo 1992; Chapman and Lemaire 1993; Bahmani *et al.* 2000), there was no correlation between LER and herbage yield (DW) in this study (Table 4.6). The only variable in the data set that did correlate with DW was PI, a derived variable using information about both TN and TW. However, on PCA of the autumn data set, it was found that DW featured most strongly in PC3, explaining just 17% of the overall variation in the data set, and that plants with a high score for this PC did indeed tend to have high LER as well as high PI. This would indicate that there are plants in the mapping population which exhibit high LER without achieving high DW. If this is correct, then selection gains might be negated if such plants were chosen for breeding purposes as a result of using LER as an indicator of yield potential. This point requires further more detailed study. It is possible that lack of correlation between LER and DW might be attributed to the methods used in this study to assess LER relative to DW. LER was measured as LL divided by LED, and DW was measured only once at the end of experiment, whereas in other studies (Chapman and Lemaire 1993; Bahmani *et al.* 2000), LER and DW were measured several times as regrowth between two successive defoliations.

4.8 Conclusion

This study identified statistically significant, heritable variation within the mapping population for almost every measured and derived variable describing plant sward-forming traits (TN, TW, PI, ALf, ALg, LER, LED, DW and SIL). As such all the variables are candidates for QTL discovery. With respect to prediction of yield potential, only PI consistently correlated with DW, although PC3 of the autumn study did also involve LER. PCA identified major components for herbage yield as LER, and LL in autumn and TW in spring. One question that now arises is whether QTL for traits such as DW will be more strongly associated with the measured variables, or with more complex relationships such as that represented by scores for autumn PC3.

5

Phenotypic assessment for variation in seed yield and yield component traits in a perennial ryegrass population

5.1 Introduction

Seed yield, measured as the total saleable seeds recovered from harvesting and processing a seed crop, is an important factor in the commercial success of a forage grass cultivar. A grass cultivar that has the combined ability for efficient seed and herbage production is an indispensable requirement in the pastoral industry, and will have preference in the commercial market. In some cases, however, a cultivar may have good forage production ability and forage quality, but with lower seed yielding ability, and this will reduce the market value of the cultivar. One such case is the perennial ryegrass cultivar Tolosa (Agriseeds 2003), which was recently withdrawn from the market, despite excellent forage production capacity, apparently because seed producers were consistently obtaining lower yields than with other cultivars.

The complex genetic nature of seed yield mitigates against the development of a cultivar with combined seed and forage production ability, and there is also a perception that seed production and forage production are negatively correlated in forage grass species (van Wijk 1980; Bugge 1987). Seed yield is a quantitative trait, and its expression is influenced by several genes and the environment. In perennial ryegrass, seed yield per plant (SdYP) is the product of the number of reproductive tillers per plant (RT) and seed yield per head (SdYH), where

SdYH = Spikelets per head (SH) x florets per spikelet (FS) x floret site utilisation (FSU) x 1000-seed weight (TSW) (1)

In contributing toward seed yield, RT determines the number of spikes, while SH, FS and FSU are co-determinants of the potential number of seeds per spike. 1000 seed weight (TSW) is a measure of seed size (which influences seed vigour). In New Zealand, to achieve acceptable germination, a minimum perennial ryegrass seed size is 1.5 g per 1000 seeds (P. Rolston personal communication, 2003). Seed yield can be assessed based on whole plant or per spike basis.

Seed yield in forage grasses has received less attention from plant breeders than forage production (Marshall and Wilkins 2003), because feed is the primary interest of these plants, and in addition, breeding for seed yield is a laborious process and often does not produce the expected results. For instance, seed yield of perennial ryegrass plants in a spaced-plant experiment (a common design in breeding experiments for cultivar improvement) was found (Elgersma 1990b), to be poorly correlated with seed yield in drilled plots (the normal practice for commercial seed production). This highlights the complex genetic nature of yield and the sensitivity of seed yield to the plant environment. As seed yield represents the combined effect of several component traits, the identification of major component traits and their genetic loci for MAS may accelerate the improvement of seed yield in perennial ryegrass. In parallel with the investigations reported in Chapter 4, it was therefore decided to conduct a study to determine genotypic variation in seed yield within the same mapping population, and to use QTL analysis to identify markers or alleles associated with seed yield and its component traits.

5.2. Objectives

The objectives of this experiment (Experiment 3) were:

1. To assess the I×S mapping population for variation in seed yield and yield related traits.
2. To identify major component traits for seed yield in perennial ryegrass.
3. To provide a data set for QTL analysis.

5.3 Materials and methods

5.3.1 Plant material and experimental design

The experiment was carried out from July 2003 to February 2004 at the AgResearch Grasslands site in Palmerston North, New Zealand. Four cloned replicates of the I×S mapping population plants (n=200) and of the two parents were transplanted to the field in a randomised complete block (RCB) design, one copy of each plant per replicate. At transplanting in August 2003, plants comprised 10-15 tillers and a recommended spacing of 60 cm between plants was used (Bajwa *et al.* 1996). The area occupied by the experiment was 302.4 m^2.

5.3.2 Agronomic practices

5.3.2.1 Soil analysis

The soil at the experimental site is alluvial Kairanga silt loam (Cowie 1978), and analysis by E-lab. Limited, Hamilton, New Zealand, indicated the following nutrient levels: Calcium, 8 (MAF QT); Olsen Phosphate, 44 (µg/ml); Potassium, 8 (MAF QT); Sulphate Sulphur, 7(ppm); Magnesium, 22 (MAF QT) and Sodium, 5 (MAF QT). The pH was 5.5. Based on these results, lime and superphosphate fertiliser were applied at 3 tonnes/ha and 300kg/ha, respectively, 2 weeks after plants were transplanted to the field.

5.3.2.2 Cultural practices and technologies

Nitrogen application from 150 kg/ha to 250 kg/ha has been reported to increase ryegrass seed yield to over 2000 kg/ha (Rolston and McCloy 1997; Cookson *et al.* 2000). In this experiment, a total nitrogen application of 200 kgN/ha was applied as urea in three equal splits on 1st September, 1st October and 15th October.

Weeding was carried out in October, 8 weeks after transplanting. The base (18 cm radius) of each clone was first weeded manually using a hand hoe, and the following herbicides were then applied; 2,4-D Ester (3 L/ha) to kill broad weeds and Dicamba (2 L/ha) to kill clover (Figure 5.1). Buster (glufosinate ammonia) (3 L/ha) was applied with a roller between plants to kill other weed species. These herbicides provide effective control of weeds, but have no effect on ryegrass seed yield or seed quality (Ivany *et al.* 2002).

The plant growth regulator Moddus® trinexapacethyl (TE) was applied at 800mL/ha at first head emergence (5th November) and then 3 weeks later to prevent plants from lodging. Moddus retards stem elongation by inhibiting hydroxylation of the growth inactive form of gibberellic acid to the active form (King *et al.* 1997). This results in stem shortening of reproductive tillers and increases their efficiency through increased number of seeds per spikelet and improved floret site utilization (Chastain *et al.* 2003).

Stem rust (*Puccinia graminis*) infestation was observed on some plants after heading and Systhane 125 was applied at 20mls/100L of water/ha in December 2003 and January 2004 to control rust.

Figure 5.1: Weed control in I×S perennial ryegrass mapping population plants grown as spaced-plants in the field in 2003.

5.3.3 Data collection

5.3.3.1 Plant development:

To monitor plant development, the following data were recorded:

Tiller number (STN): During September 2003, tiller numbers per plant were estimated by dividing the base of a plant into four equal portions and counting tiller number for one portion (selected at random), and multiplying by 4.

Heading date (DH)): This was recorded as the number of days from transplanting, to when three heads were visible on each plant. This measurement was repeated in 2004 and expressed as days after 31st October.

Spread of heading (SOH): The number of days between the emergence of the first and the thirtieth head of each plant.

Plant growth habit (PGH): This was recorded after heading using a subjective scale of 1 to 9 (1=erect, 3= semi erect, 5=medium, 7= semi-prostrate and 9=prostrate).

5.3.3.2 Spike characteristics:

Three spikes were harvested randomly from each plant 7 weeks after heading, placed in sealed plastic bags and kept in a refrigerator. The following were later recorded and averaged for the three selected spikes:

Spike length (SL): Average length (cm) measured from the first node to the tip.

Spikelets per head (SH): All spikelets on each spike were counted.

Florets per spikelet (FS): The number of florets on the bottom, middle and top spikelets of each spike were counted and averaged.

Florets per head (FH): estimated by multiplying values obtained for spikelets per head and florets per spikelet, as described above.

5.3.3.3 Seed yield per plant:

Plants were harvested with hand-shears to about 10 cm above ground level between 6 and 26 January 2004 as seed heads ripened. All harvested tillers from a plant were put into an opened paper bag and stored in a field laboratory on tables for drying. After 3 weeks at ambient temperature, the following data were collected:

Reproductive tiller number (RT): All harvested tillers that produced heads.

Tillers with matured heads (TMH): Tillers with matured and ripened seed heads, indicated by brown or gold coloured florets and expressed as percentage of total reproductive tiller.

Seed yield per plant (SdYP): Weight of seed (g) per plant measured after drying, threshing and cleaning of seeds. Seed cleaning was done mechanically using a South Dakota Seed Blower. The scale on the machine was set at 4.5 to clean seed to a TSW of over 1.3g. Setting above 4.5 cleaned seeds to a TSW above 1.6g which would have resulted in losing saleable seeds. The air flow rate of the machine at the 4.5 scale was measured with a LCA 6000 Airflow anemometer (Airflow Developments Ltd., England). The cross sectional area of the air stream with average velocity of 3.86m/s was calculated to $0.00053m^2$ and the air flow rate was calculated to $0.002m^3/s$. Seed moisture content was determined (ISTA 2004) after seed cleaning on 100 seeds per

genotype for four genotypes using the high constant oven method at 130°C (±2°C) for one hour. The average moisture content was 11%.

Floret site utilization (FSU): Calculated as the number of saleable seeds per plant divided by the number of florets counted per plant post anthesis (Elgersma 1985).

This was determined by the formula:

FSU = (SdYP (g) x 1000 / TSW (g))/ (number of spike per plant x FH) (2)

Seed yield per head (mg): This was calculated as follows:

Seed yield per head (SdYH) = (1000 x SdYP)/ number of reproductive tillers with mature heads per plant.

Seed weight (TSW): This was measured as 1000 seed weight. Two samples of 100 filled seeds were randomly selected from each plant from three replicates, weighed, and the average weight multiplied by 10 to calculate TSW.

Figure 5.2: Processing seed samples of the I×S perennial ryegrass mapping population plants grown as spaced plants in the field in 2003.

5.3.3.4 Climatic factors

Data on temperature, rainfall and sunshine during the period of experimentation were recorded at a weather station 600 m distant from the experimental plots (Table 5.1).

Month	Air Temp (°C) (max)	Air Temp (°C) (min)	Soil Temp (°C) (10cm)	Rainfall (mm/month)	Sun (hr)/day
July (2003)	12.7	1.6	5.9	83.7	5.5
August (2003)	14.8	4.7	7.9	27.9	5.5
September (2003)	15.3	6.3	10.3	15.3	4.3
October (2003)	16.4	6.2	-	89.9	-
November (2003)	17.1	8.8	13.2	81.0	4.3
December (2003)	20.3	11.6	16.1	80.6	4.4
January (2004)	20.5	8.4	15.8	27.9	6.9
Mean	16.7	6.8	11.5	58.0	5.2

Table 5.1: Mean monthly temperature, rainfall and sunshine during July 2003 to January 2004

5.3.4 Data analysis

Analysis of variation was performed using GenStat version 8.1 (Genstat 2005) to identify significant differences between genotypes and replicate structure for all analysed traits. Broad sense heritability estimates were calculated for each trait as

$$H_b = \sigma^2_g/(\sigma^2_g+(\sigma^2_\varepsilon/r)) \quad (3)$$

Where σ^2_g = genotypic component of variance, σ^2_ε = residual variance of genotypes and r = number of replications (Burton and De Vane 1953). Principal Component Analysis (PCA) in MINITAB was also performed to identify patterns of correlation between measured variables.

5.4. Results and discussion

The soil nutrient and temperature conditions (Table 5.1) were within ranges for optimum growth and should have provided good growth conditions for perennial ryegrass.

Within the I×S mapping population, plants were significantly ($p<0.01$) different for all traits, and there were significant differences between parents also ($p<0.05$, Table 5.3).

Broad sense heritability values ranged from 0.27 for SH, up to 0.94 for DH and FSU (Table 5.3). These data confirm genetic variation in all of the assessed traits, and the values observed are comparable with earlier heritability estimates for reproductive traits in perennial ryegrass (Elgersma 1990c; Yamada *et al.* 2004). Floret number was higher on the bottom and middle spikelets than on those at the top of spike (data not shown). There was variation between plants of the same genotype, but there was no significant difference ($p \leq 0.05$) among replications in the estimated variance components. The frequency distribution in the population for all traits was normal except for PGH and SH that indicated non normality (Appendix 5.1), but their histograms did not show any obvious skewness (Figure 5.3). The range of progeny values extended beyond the range of the parents in some of the traits (SL, SH, TMH, SdYH and FSU) and this represents a potential source of novel genetic variation which may be significant for crop improvement (de Vicente and Tanksley 1993).

The parents, I and S were significantly ($p \leq 0.05$) different in all the traits except in FH, RT, SL and TMH. The I parent plants had significantly lower values than the mean of the progeny in TSW, RT, FH, SdYH and SdYP, but had higher FSU and longer DH, whereas the S parent plants headed earlier and had high FSU, but greater spread of heading within plant than most of the progeny. FSU was higher in both parents than in most of the genotypes. Tiller number counted in September correlated better with vegetative tiller number counted in glasshouse (Experiment 2, chapter 4) than with reproductive tiller number (Table 5.2). The correlation between reproductive tiller number and vegetative tiller number counted in the glasshouse was not significant ($p<0.01$). There was good correlation (0.66) between heading date assessed in 2003 and 2004.

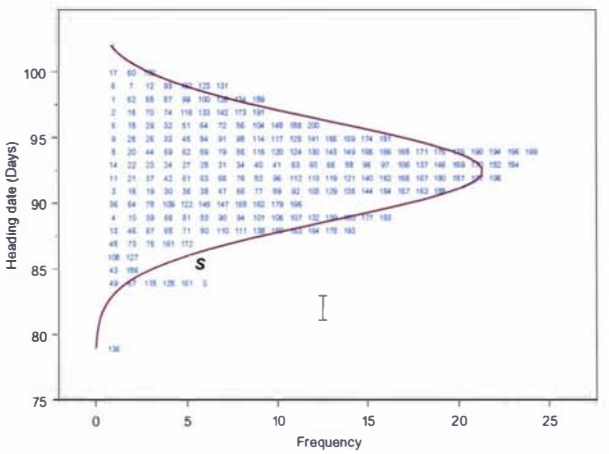
	STN	RT	VT
RT	0.29		
VT	0.36	0.14	

Table 5.2: Coefficient of correlation among September tiller number (STN), reproductive tiller number (RT) and vegetative tiller number (VT) ($p<0.05$ if $r \geq 0.14$; $p<0.01$ if $r \geq 0.18$).

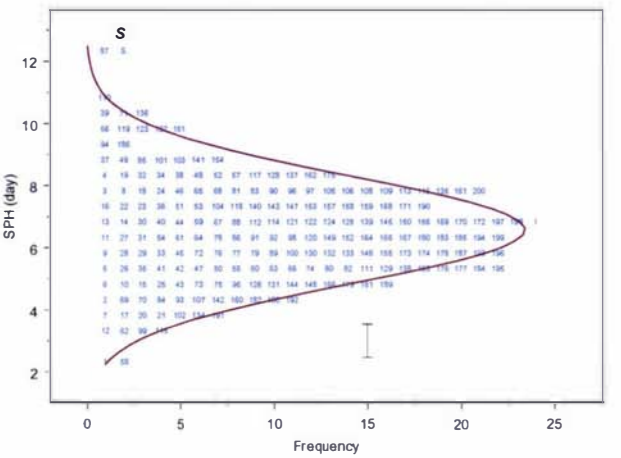
Trait	Mean	Range	S	I	σ^2_g	H_b	SE	$LSD_{0.05}$
SdYP	35.3	11.9 - 64.5	27.7	11.9	77.9±10.5	0.75	5.1	14.5
SdYH	91.9	46.7 - 169.6	103.6	65.1	358.9±48.2	0.75	10.9	30.6
FS	8.4	7.0 - 11.0	8	7	0.17±0.06	0.33	0.6	1.7
FH	226	162 - 298	224	162	230±75	0.33	22	61
SH	27	21 - 31	29	23	0.7±0.3	0.27	1	4
RT	392	184 - 592	272	184	3922±674	0.59	51	145
TMH	83	65 - 96	81	90	14.9±2.9	0.62	4	11
SL	19.6	16.1 - 23.6	19	19.77	0.57±0.16	0.39	0.9	2.7
DH (2003)	92	79 - 102	84	102	12.9±1.4	0.94	1	3
DH (2004)	20	10 – 35	18	25	0.41±0.05	0.77	1	1
TSW	1.9	1.35 - 2.48	2.08	1.35	0.028±0.004	0.76	0.1	0.3
PGH	3	1 -7	1	7	0.8±0.1	0.77	1	1
SOH	6.6	2.0 - 13.0	13	7	1.6±0.3	0.58	1.1	3.1
FSU	0.22	0.09 - 0.42	0.26	0.31	0.0017±0.0003	0.94	0.0	0.0

Table 5.3: Mean, range, genotypic variance (σ^2_g), broad sense heritability (H_b), SE and LSD of means of genotypes for seed-yield-related traits of the I×S perennial ryegrass mapping population genotypes and the two parents assessed in the field as spaced plants in 2003. SdYP: seed yield per plant (g); DH (2003): days to heading from time of transplanting in the field; DH (2004): days to heading from 1 November; FH: floret per head, FS: floret per spikelet; FSU: floret site utilization; PGH: plant growth habit; RT: reproductive tiller number; SL: spike length (cm); SOH: spread of heading (days); SH: spikelets per head; SdYH: seed yield per head (mg); TMH: % tillers with matured heads; TSW: 1000 seed weight (g); S: Grasslands Samson parent; I: Grasslands Impact parent.

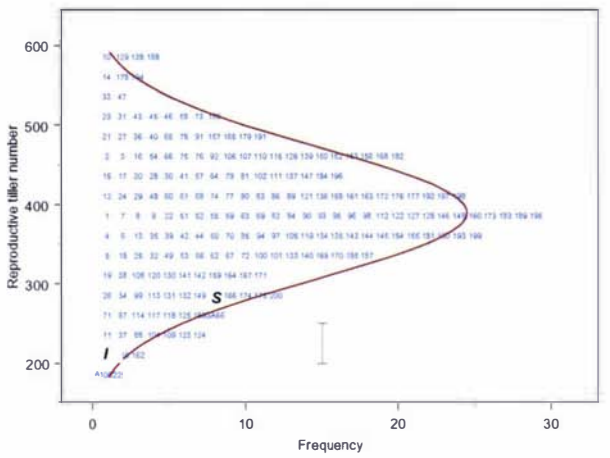
Heading date (Days from planting)



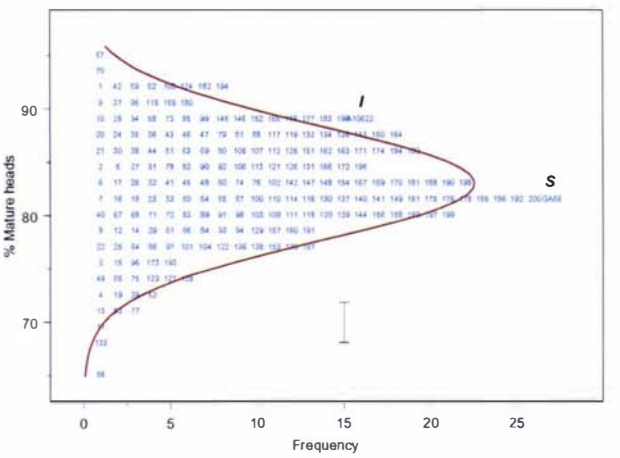
Spread of heading (day)



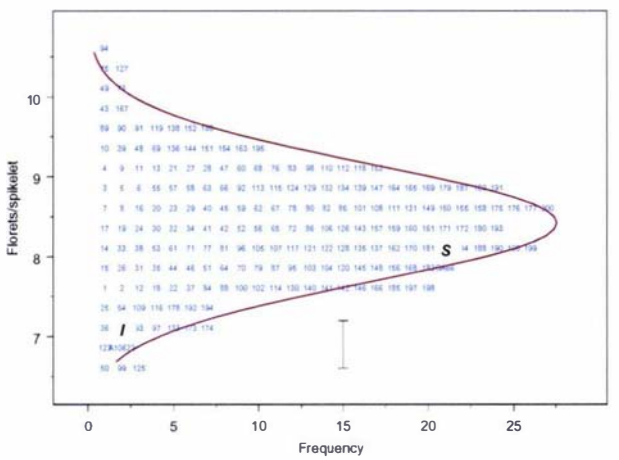
Reproductive tiller number



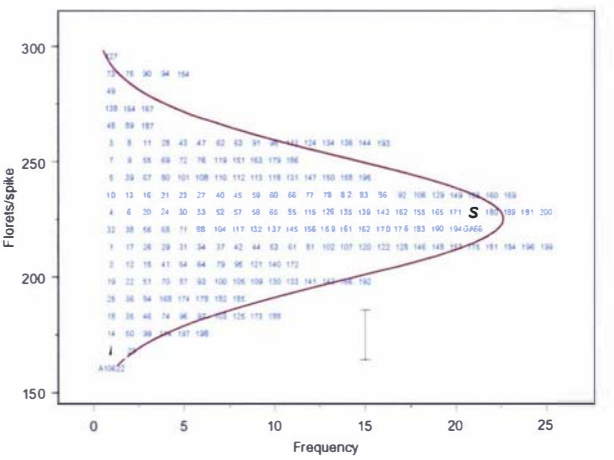
Tillers with matured heads (%)



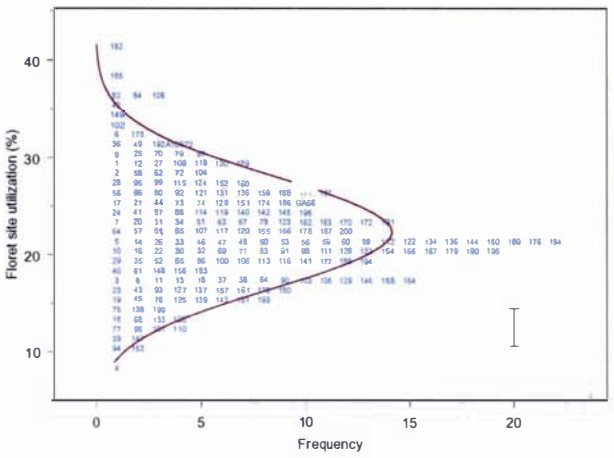
Florets per spikelet



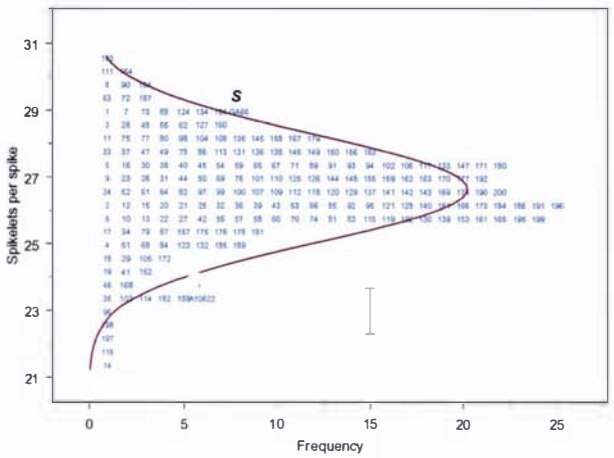
Florets per head



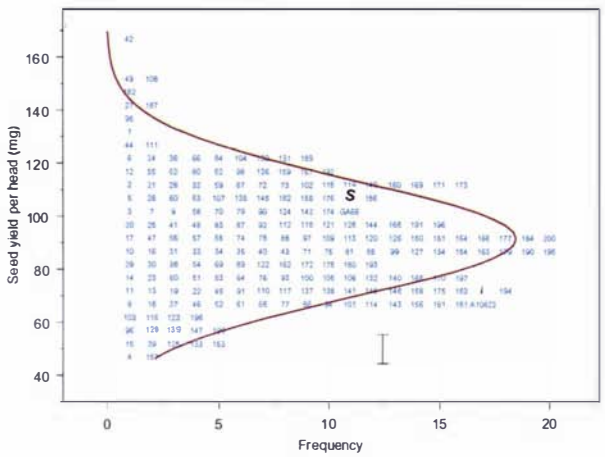
Floret site utilization (%)



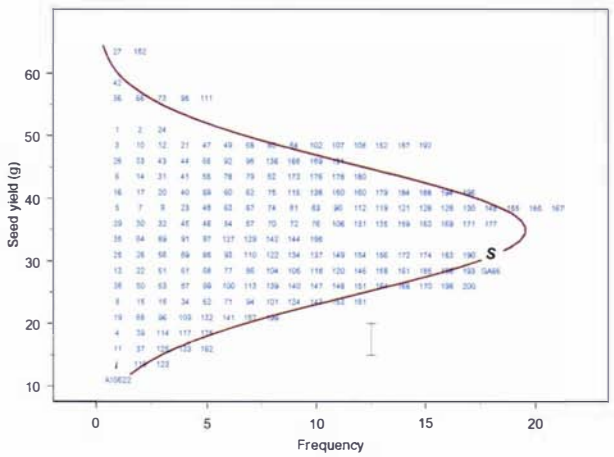
Spikelets per head



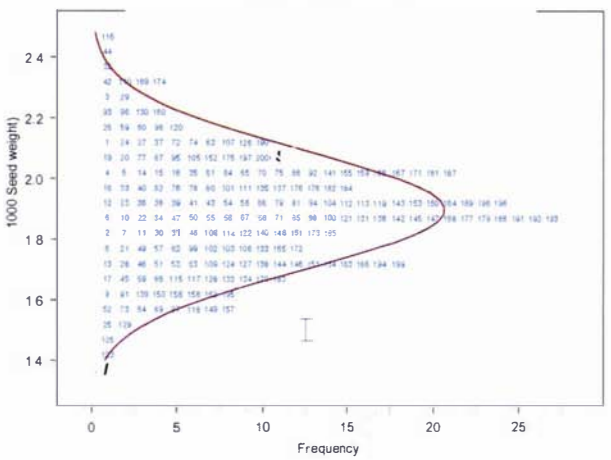
Seed yield per head (mg)



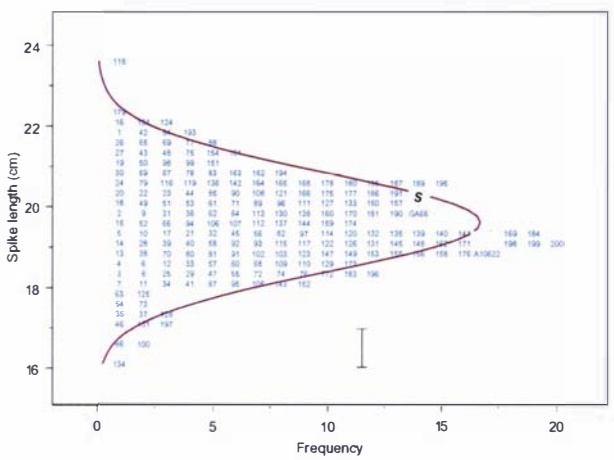
Seed yield per plant (g)



1000 Seed weight



Spike length (cm)



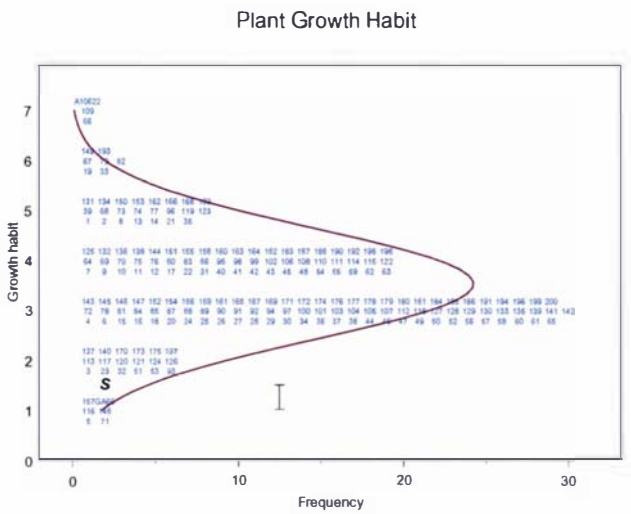


Figure 5.3: Frequency distribution of phenotypes for seed yield and related traits in the I×S perennial ryegrass mapping population assessed as spaced plants in the field in 2003 (means of four replicates). I: Grasslands Impact plant; S: Grasslands Samson plant, and error bar indicates least significant difference of means.

	SdYP	SdYH	FS	FH	SH	RT	TMH	SL	DH	TSW	PGH	SOH
SdYH	0.74											
FS	0.23	0.14										
FH	0.24	0.22	0.87									
SH	0.14	0.23	0.27	0.70								
RT	0.62	-0.05	0.23	0.15	-0.04							
TMH	0.66	0.04	0.20	0.12	-0.04	0.96						
SL	0.15	0.23	0.11	0.15	0.15	-0.02	-0.02					
DH	-0.07	-0.01	-0.47	-0.43	-0.16	-0.12	-0.09	-0.12				
TSW	0.19	0.23	-0.08	-0.11	-0.10	0.00	0.01	0.07	0.08			
PGH	0.15	0.11	0.07	0.05	-0.01	0.06	0.06	-0.04	-0.04	-0.19		
SOH	-0.44	-0.25	0.09	0.05	-0.03	-0.35	-0.40	-0.02	-0.41	0.03	-0.21	
FSU	0.48	0.75	-0.20	-0.17	-0.05	-0.14	-0.03	0.12	0.21	-0.13	0.13	-0.28

Table 5.4: Coefficients of correlations between seed yield component traits ($p<0.05$ if $r \geq 0.14$; $p<0.01$ if $r \geq 0.18$). SdYP: seed yield per plant (g); DH: days to heading from time of transplanting in the field; FH: floret per head, FS: floret per spikelet; FSU: floret site utilization; PGH: plant growth habit; RT: reproductive tiller number; SL: spike length (cm); SOH: spread of heading (days); SH: spikelets per head; SdYH: seed yield per head (mg); TMH: % tiller with matured heads; TSW: seed weight (g).

Of the 78 pairwise correlations amongst the 13 traits, only 38 (49 %) were significant ($p<0.05$) (Table 5.4). SdYP correlated strongly with SdYH (0.74), TMH (0.66) and RT (0.62), moderately with FSU (0.48), FH (0.24) and FS (0.23), and minimally with TSW (0.19) and SH (0.14), but negatively (-0.44) with SOH. SdYH correlated strongly with FSU (0.75) and moderately with TSW (0.23), SH (0.23), SL (0.23) and FH (0.22). RT and FSU were negatively correlated. Seed yield in this experiment was determined at a minimum weight of 1.3 g /TSW. The strong correlation between SdYH and SdYP indicates that the former can be used to give a good estimate of the latter. In other cases correlations arise because two variables essentially measure the same thing (e.g. RT and TMH) or because a measured variable is ambiguous (e.g. time to produce 30 heads is not a 'pure' measure of SOH as the values would also be shorter where TMH was higher).

However, when associations between traits across the mapping population are examined collectively by PCA (Table 5.5 and Appendix 5.2.1), the understanding that emerges is somewhat different from that obtained from comparing pairwise correlations (Table 5.4). The first three of the 13 available PCs had eigenvalue >1, between them accounting for 60 % of the observed variation between genotypes, and are reported here (Table 5.5). The pattern of coefficients for PC1 indicates that this PC is useful to identify plants with low (or high) SdYP, and that plants with low SdYP were also likely to have low TMH and RT (and hence high SOH), and to a lesser extent likely to have high FS and high FH. Notably FSU and TSW did not contribute strongly to PC1. PC2 indicates early heading plants which had high FH, FS and SH, but low FSU; plants in this category did not have a strong tendency to either high or low seed yield. PC3 indicates that plants with low tiller number (RT and TMH) tended to have high FSU and SdYH, but not necessarily high SdYP. This suggests that the correlation between SdYH and FSU and between SdYH and SdYP in fact represent different subsets of plants in the population, and that selection for high SdYH based on simple correlations in Table 5.4 may be detrimental rather than beneficial to SdYP.

Traits	PC1 (25%)	PC2 (19%)	PC3 (16%)
SDYP	-0.501	-0.187	0.047
SDYH	-0.331	-0.179	0.483
FS	-0.279	0.41	0.017
FH	-0.295	0.477	0.145
SH	-0.183	0.313	0.266
RT	-0.379	-0.046	-0.484
TMH	-0.4	-0.092	-0.441
SL	-0.116	0.045	0.241
DH	0.115	-0.41	0.013
TSW	-0.024	-0.101	0.034
PGH	-0.109	-0.047	0.042
SOH	0.266	0.337	0.078
FSU	-0.165	-0.365	0.419

Table 5.5: PCA of seed yield components showing the first three PCs (with eigenvalue >1) accounting for 60% of the variation. SdYP: seed yield per plant (g); DH: days to heading from time of transplanting in the field; FH: floret per head, FS: floret per spikelet; FSU: floret site utilization; PGH: plant growth habit; RT: reproductive tiller number; SL: spike length (cm); SOH: spread of heading (days); SH: spikelets per head; SdYH: seed yield per head (mg); TMH: % tiller with matured heads; TSW: seed weight (g).

The major traits associated with increased SdYP were identified as RT, TMH, SdYH, FS and FH (Tables 5.4 and 5.5). These traits, except TMH have all been identified as seed yield components in perennial ryegrass (Bugge 1987; White 1990; Elgersma 1990a; Marshall and Wilkins 2003). The number of reproductive tillers effectively contributing to yield was identified in this study as the most critical determinant trait for seed production in perennial ryegrass. Differences in tiller production may reflect the poor correlation between spaced and drilled plants (Elgersma, 1990). Spaced plants are more likely to produce more tillers than drilled ones due to reduced competition for nutrients and other growth factors. However, Elgersma (1990) identified number of

spikelets as the main difference between spaced and drilled plants, but the ranking was different from that of seed yield.

Increased production of reproductive tillers increases plant seed yield but this may be detrimental to forage quality. The implication of the PCA results is that selecting for SdYH would be useful, providing plants with low RT (and high SdYH as a result of this) were rejected first. This indicates that selection for increased SdYH would be effective in improving plant seed yield, as suggested earlier (Bugge 1987; Marshall and Wilkins 2003).

TSW and seed yield had weak positive correlation (0.19), suggesting that TSW is more a determinant of seed quality than of yield in perennial ryegrass (Cookson *et al.* 2000; Rowarth *et al.* 2000).

Heading date affects FSU and consequently SdYH (PC2), and late heading in perennial ryegrass had been associated with poor seed production probably due to reduced floret fertility (Anslow 1963). However, late heading is associated with increased quality of herbage and is favoured in herbage production. SOH had a negative effect on SdYP and component traits (PC1 and Table 5.3). This illustrates the importance of developing a cultivar with uniform heading time of tillers within a plant if yield losses in grass seed production are to be minimised by reducing the production of infertile florets or under-sized seeds.

5.5. Conclusions

The IxS mapping population indicated significant variation for all the assessed seed yield and related traits. The parents were different in all other traits, but similar in the production of florets per spikelet, reproductive tillers, matured harvestable spike and spike length.

Of the seed yield components in perennial ryegrass, number of reproductive tillers was identified as the most critical component for seed production. An increased seed yield can be achieved by increased tiller production. The number of reproductive tillers, especially those with matured heads at time of harvest, and the seed yield per head are

both major determinants of seed yield per plant, and FSU is the major component for seed yield of an individual tiller in a plant. Late heading was associated with decreased seed yield.

It should be noted these conclusions are specific to the I×S perennial ryegrass mapping population in a spaced plant experiment, and a confirmation of these results for other environments or populations of perennial ryegrass will be required.

6

Quantitative trait loci (QTL) for herbage and seed production traits in perennial ryegrass

6. 1 Introduction

Molecular marker technology offers a means to dissect and characterise complex traits and to identify beneficial allelic variants. Once markers associated with the trait of interest have been identified, marker assisted selection (MAS) can be used in breeding programmes to more efficiently and effectively identify desirable plants from a population, and manipulate genes responsible for a favourable performance of a trait.

One approach to identifying such markers is QTL mapping, which is the identification and location of genetic loci that are associated with a trait. QTL analysis is based on the principle of detecting an association between phenotype and the genotype of markers. Markers are used to partition the mapping population into different genotypic groups at marker loci and to determine whether significant differences exist between groups with respect to the trait being measured (Tanksley 1993; Young 1996). A significant difference between phenotypic means of the groups indicates that the marker locus being used to partition the mapping population is linked to a QTL controlling the trait (Collard *et al.* 2005).

QTL analyses are generally performed using Simple Interval Mapping (SIM) or the more sophisticated Composite Interval Mapping (CIM), both of which are available in several software packages, including MapQTL® (http://www.kyazma.nl/). SIM is based on the joint frequencies of a pair of adjacent markers and a putative QTL flanked by the two markers, and it searches for QTL between pairs of adjacent loci along a linkage group. However, because SIM is based on the assumption that a single segregating QTL influences the trait, it is unable to resolve the number of QTL influencing a trait, the precise locations of QTL, and it is associated with a low statistical power (Liu 1998). CIM is a combination of SIM and multiple linear regression (Zeng 1994), and is more informative and efficient. CIM searches for QTL between loci on all linkage groups simultaneously and it identifies the most likely QTL position. As forage crop

production is affected by environment (geographic location, season, year, biotic and abiotic challenges), QTL discovery needs to be replicated across environments in order to substantiate the robustness of a QTL identified for a trait.

QTL mapping using SIM or CIM requires a genetic linkage map constructed with molecular markers. Molecular markers, also known as DNA markers, reveal sites of variation in the DNA of a species (Jones *et al.* 1997), and they are the most widely used type of marker, because they are easy to obtain in large quantities (thousands) for any species and are independent of environmental conditions (Huff 2001) as compared to morphological or isozyme markers. There are several types of DNA markers, including restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellites, also known as simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP) and single nucleotide polymorphism (SNP). The choice of a marker system for genetic mapping is based on several factors, including: the amount of genetic variation they can detect, the ability to detect all alleles present at a locus, the amount of genomic DNA required for analysis, compatibility with automation, breeding objectives, population structure, genomic diversity of the species under investigation, marker system availability, time required for analysis, and the cost per unit information (Staub *et al.* 1996). Perennial ryegrass is an obligate outcrossing species, and its breeding generally involves crossing pairs of heterozygous parents, which results in the segregation of up to four alleles at a single locus (Maliepaard *et al.* 1997; Lespinasse *et al.* 2000) as shown in Table 6.1. Therefore, a marker system that can distinguish or detect all alleles at a locus will be the one of choice.

SSR loci arise as a result of local repetition of small sequence motifs in short tandem arrays, with the length of a basic repeat varying from 1 to 6 bp (Tautz 1989). Genetic polymorphism arises due to variation in the number of repeated units, probably as a consequence of slippage during DNA replication (Levinson and Gutman 1987). SSR markers are co-dominant and polymorphic and are capable of identifying two or more alleles at a locus (Jones *et al.* 1997). EST-SSR markers (simple sequence repeat markers derived from expressed sequence tags) are SSRs that exist within a gene sequence in both coding and non-coding regions (Scott *et al.* 2000; Cordeiro *et al.* 2001). EST-SSR markers can be developed at no cost from EST databases, and once

developed, these markers, unlike genomic SSRs, may be used across a number of related species (Gupta *et al.* 2003). Although EST-SSRs may show lower levels of intraspecific polymorphism than those derived from non-coding genomic regions (Cordeiro *et al.* 2001), they provide the potential advantage of close linkage to a significant gene variant, and they have been used to successfully construct genetic linkage maps for a number of plant species, including perennial ryegrass (Faville *et al.* 2004).

To construct a genetic linkage map in outcrossing species like perennial ryegrass, a pseudo-testcross F1 mapping population structure derived from a cross between a pair of heterozygous parents is commonly employed (Grattapaglia and Sederoff 1994; Porceddu *et al.* 2002; Shepherd *et al.* 2003; Carlier *et al.* 2004). This entails the construction of individual parental maps using dominant marker types (Table 6.1: segregation types 1-4) in the first instance to enable the comparison of recombination frequencies and marker order in both parents. The second stage involves joining together the parental maps as a consensus map, using markers that are heterozygous in both parents as 'bridging loci' (Table 6.1: segregation types 5-7). While parental or consensus maps may be used for genetic mapping, the consensus map will have a higher level of marker saturation than either of the parental maps.

In ryegrass, QTL have been reported for morphological and physiological traits such as heading date and vernalisation response (Armstead *et al.* 2004; Yamada *et al.* 2004; Jensen *et al.* 2005), vegetative traits (Yamada *et al.* 2004), lodging (Inoue *et al.* 2004), nitrogen use efficiency (Van Loo *et al.* 2003), winter hardiness, water soluble carbohydrate and other forage quality characteristics (Humphreys and Turner 2003; Cogan *et al.* 2005; Turner *et al.* 2006).

The main objective of this research was to identify QTL associated with herbage and seed production traits in the I×S perennial ryegrass mapping population. The specific objectives were:

1. To construct a precise genetic linkage map of perennial ryegrass
2. To conduct QTL analysis of herbage production traits measured in autumn and in spring glasshouse environments

3. To conduct QTL analysis of seed yield and related traits measured in a field environment.
4. To identify DNA markers associated with significant QTL as potential MAS tools.

Genotypic analysis of EST-SSR markers for the construction of the I×S genetic linkage map, reported in this study, was carried out as part of a wider programme within the AgResearch Forage Genomes mapping group, with the author having responsibility for developing approximately 15% of the marker data.

Segregation type	Genotypes (parent 1 x parent 2)	Description	JoinMap code
1	AA x AB	BC type, 2 alleles	nnxnp
2	AB x AA	BC type, 2 alleles	lmxll
3	AA x BC	BC type, 3 alleles	nnxnp
4	BC x AA	BC type, 3 alleles	lmxll
5	AB x AB	F2 type	hkxhk
6	AB x AC	3 alleles	efxeg
7	AB x CD	4 alleles	abxcd

Table 6.1: Marker segregation types in a full sib F_1 family from a pair cross between heterozygous individuals. BC type refers to a first generation backcross.

6.2 Materials and methods

6.2.1 Plant material and phenotype assessment

Phenotyping of the I×S mapping population is described in Chapters 4 and 5. Assessment of herbage yield and related traits was replicated in autumn 2003 and spring 2004 as described in Chapter 4 (experiments 2 and 5) and heading date was measured in spring 2003 and 2004 as described in Chapter 5.

6.2.2 EST-SSR marker analysis

6.2.2.1 DNA extraction and quantification

For DNA extraction leaf tissue was harvested from one clonal replicate of each mapping population plant and both parent plants, which at the time of harvest were growing outside in Planter bags (64 x 64 x 150 mm). Ten to 15 cm of fresh leaf was harvested from each plant, labelled and wrapped in aluminium foil, immediately snap-frozen in liquid nitrogen and taken to the laboratory for storage in a -80°C freezer. Young leaves were used in order to maximise yields of intact DNA (Kidwell and Osborn 1992). The samples were subsequently taken from the freezer and genomic DNA was extracted using a modified DNA extraction micro-preparation method (Fulton *et al.* 1995) as follows:

Micro preparation buffer (2.5 parts DNA extraction buffer, 2.5 parts nuclei lysis buffer, 1.0 parts sarcosine solution and 0.005 g sodium bisulfite/mL) was first prepared and kept at room temperature. For each sample, 750 μL was transferred into a 1.5mL microcentrifuge tube and about 100 mg of frozen leaf sample added. Microcentrifuge tubes containing the leaf tissue, two inert beads and buffer were capped tightly and agitated in a Tissue Disruptor (Savant Fastprep™ FP 120) for 2 to 4 minutes. The tubes were mixed briefly, and then incubated in 65°C in a water bath for 30-45 minutes, with the tubes being gently mixed periodically. Tubes were filled with cold (-20°C) chloroform solution (24:1(v/v) chloroform:octanol)) (~700 μL), and mixed by sandwiching tubes between two racks and vigorously inverting 50-100 times. They were centrifuged (Eppendorf centrifuge 5810R) at 13000 rpm (34000G) for 7 minutes, and the aqueous phase was transferred (~ 600 μL) to a new tube. Cold isopropanol (2/3 volume of the aqueous solution) was added and tubes inverted gently until DNA precipitated. Tubes were again centrifuged at 13000 rpm for 10 minutes, isopropanol poured off and 200 μL of 75% ethanol added. Tubes were then flicked to dislodge the pellet and then spun briefly to resettle the pellet. Ethanol was poured off and the pellet dried by placing tubes inverted on paper tower at 37°C for 10 minutes or at room temperature for 30 minutes. DNA was resuspended in 100 μL of TE buffer (10 mM Tris and 1 mM EDTA at pH 8.0) containing 0.1 μL RNase A (10 mM Tris at pH 7.5 and 15 mM NaCl) (bulk solution of TE plus RNase A prepared for all samples, just prior to use) at 37°C for 30 minutes. The tubes were centrifuged again at 13000rpm for 2 minutes, and the DNA samples were kept in labelled 1.5 mL microcentrifuge tubes

and stored in a freezer at -20°C. DNA quantity was subsequently estimated by visual comparison against Lambda (λ) DNA standards (10 – 250ng).

The 100-well agarose gels were prepared using 180ml of 0.5xTBE buffer (Tris Borate-EDTA, pH 8.0), 2% (w/v) agarose and 7 µL of ethidium bromide solution (10mg/mL). DNA samples were mixed briefly by flicking the tube, and then centrifuged at 13000rpm for 1 minute. 2 µL of DNA solution plus 3 µL of 6x loading dye (15% ficoll (w/v); 0.25% bromophenol blue; 0.25% xylene cyanol FF) were loaded on to the gel and run at 80V for 2-3 hours. Quantities were estimated by eye against the λ DNA standards, and based on these estimations, DNA solutions of 5ng/µL were made for each genotype.

6.2.2.2 Marker analysis

The markers used predominantly in this study were perennial ryegrass EST-SSR markers (identified in this thesis by prefix pps-). Their discovery and primer design were carried out as described in Faville *et al.* (2004). Briefly, dinucleotide to pentanucleotide-based SSRs were identified in EST unigenes using the SSR detection programme Sputnik (http://rast.abajian.com/sputnik/).

Primers for the identified SSRs were designed using Primer 1.0 (http://www genome.wi.mit.edu/ftp/distribution/software/), to meet the following criteria: 18–26 bp in length, optimal melting temperature 60°C, G+C% of 40–60%, PCR product length 100–250 bp. Primer design excluded sections of ambiguous sequence, and SSRs located less than 30 nucleotides from one or the other ends of a unigene were also eliminated due to difficulty in the design of suitable primer pairs. Primers were synthesised by Invitrogen (Carlsbad, Calif., USA) or Integrated DNA Technologies, Inc. (Coralville, Iowa, USA). Forward primers were synthesised with a 21 nucleotide M13 tail sequence at the 5′-terminus (5′-TGTAAAACGACGGCCAGT-3′), to facilitate the universal labelling of PCR products by a fluorophore-labelled M13 primer (Schuelke 2000). Reverse primers were synthesised with the sequence 5′-GTTTCTT-3′ at the 5′ end to promote non-templated adenylation at the 3′-terminus of the PCR product (Brownstein *et al.* 1996).

A set of 863 primer pairs was selected, primarily on the basis of SSR array length, for screening in the IxS mapping population. Additional markers were also screened. STS (Sequence-tagged site) markers (prefix ppt-) were developed using the same principles as SSR markers, that is, two primers amplifying a genomic region that differs in length between the two parents, except that instead of the length difference being due to the SSR repeat sequence, it is due to an anonymous insertion or deletion event (e.g an intron). A number of tall fescue (*Festuca arundinacea*) EST-SSR markers (prefix nfa-) were also used (Saha *et al.* 2004). For all marker types, primer pairs were initially evaluated for amplification efficiency and polymorphism using only the parental genotypes. In instances of indeterminate polymorphism type (e.g. one primer pair detecting a single locus of segregation type ABxAB, which could also be two monomorphic loci with the allelic structures AAxAA and BBxBB), further evaluation of segregation was performed using a set of six progeny genotypes plus the two parents. Polymorphic markers were used for genetic linkage map construction. PCR amplifications were conducted in a 10 μL reaction volume (96-well plate format) for polymorphism screening, and in an 8 μL reaction volume (384-well plate format) for genetic mapping. A Biomek 2000 liquid handling robot (Beckman Coulter Instruments, Fullerton, Calif., USA) was used to set up the PCR reactions. A 10 μL reaction volume contained 10 ng (2 μL) of genomic DNA, 2.5 mM magnesium chloride, 1x PCR buffer (Invitrogen), 0.2 mM of each dNTP, 0.0375 μM forward primer, 0.15 μM reverse primer, 0.15 μM of fluorescent-labelled M13 primer and 0.06 U of Platinum Taq DNA polymerase (Invitrogen). An 8 μL reaction volume contained the same components with volumes scaled by a factor of 0.8. The fluorophores used were 6-FAM, NED, VIC and PET (Applied Biosystems, Foster City, Calif., USA). PCR was carried out using iCyclers (Bio-Rad, Hercules, Calif., USA) with the following profile:(1) 94°C for 4 min, (2) 30 cycles of: 94°C for 30s, 55°C for 30s and 72°C for 30s, (3) 8 cycles of: 94°C for 30s, 53°C for 30 s and 72°C for 30s, (4) 72°C for 30 min (after Schuelke 2000).

The PCR products were analysed on an ABI 3100 Genetic Analyser using a 22 cm capillary array with POP-7 polymer (Applied Biosystems). In the screening phase, only one fluorophore was used for labelling PCR products and no pooling of products was conducted. In the mapping phase, PCR products labelled with each of the four fluorophores were quadruplexed using a Quadra 96 SV liquid handling system (Tomtec,

Hamden, Conn., USA). Electropherograms were analysed using ABI Prism GeneScan (v 3.7, Applied Biosystems), and genotype data was scored using ABI Prism Genotyper (v 3.7, Applied Biosystems).

6.2.3 Linkage analysis and genetic map construction

Polymorphic primer pairs detected in screening were used to generate genotypic data for 188 F_1 individuals of the I×S mapping population. Linkage analysis and map construction were performed using JoinMap® 3.0 software (http://www.kyazma.nl/) following recommended procedures for a cross pollinating 'CP' population type. The I×S population was analysed as a two-way pseudo-testcross (Grattapaglia and Sederoff 1994). Genetic linkage maps were first established for each of the parents ('I' and 'S') to compare locus order and recombination frequency between loci in both parental meioses. For these analyses 221 polymorphic markers of dominant segregation types 1-4 (Table 6.1) were used. Markers of segregation types 6 and 7 (Table 6.1) were also included, after recoding as dominant types 1 and 2. Markers and individual progeny (plants) with missing values greater than 20% and 10%, respectively, were excluded from mapping. JoinMap® 3.0 was used to estimate segregation distortion, determine linkage groups, order loci within linkage groups and construct linkage maps. Grouping of markers was performed with a logarithm-of-odds (LOD) score threshold of 6.0. Ordering of markers within groups used thresholds of LOD 2.0 and maximum recombination value, r = 0.40. Map distances in centimorgans (cM) were calculated using the Kosambi mapping function (Kosambi 1944).

Following comparison of the parental maps, a consensus map was constructed with JoinMap® 3.0 using a combined marker dataset from the two parents (I and S). This is made possible by the presence of 'bridging' loci, polymorphic loci detected by the same SSR primer pair in both parents (segregation types 5-7, Table 6.1). Grouping of markers was performed with LOD score threshold of 7.0. Ordering of markers within groups used a LOD threshold of 2.0, and maximum r = 0.40.

6.2.4 QTL Analysis

QTL analysis was performed using SIM and Multiple QTL Mapping (MQM), implemented in MapQTL® 4.0 software (http://www.kyazma.nl/). MQM is a CIM

module that is specific to MapQTL®. For each trait, the phenotypic mean value for each genotype was used for QTL analysis. Principal component analysis (PCA) scores for the first three components (PC1, PC2 and PC3) of herbage production traits assessed in the glasshouse in autumn 2003 (Appendix 4.2.4) and seed production traits assessed in the field in 2004 (Appendix 5.2.2) were also used for QTL analysis. SIM was performed first (using the JoinMap default 5 cM interval) and, subsequently, cofactors for MQM were selected using the automatic cofactor selection option based on a combination of forward selection and backward elimination (Van Ooijen *et al.* 2002). Cofactors were identified by firstly selecting six to eight markers at approximately 20cM intervals, (including QTL markers identified via SIM) on a linkage group. Automatic cofactor selection was then executed. This process was repeated on a linkage group-by-linkage group basis, with identified co-factors from previous linkage groups included when computational limitations allowed (using this procedure, no more than nine cofactors could be included in the automatic cofactor selection process at one time). MQM was then run on a trait by trait basis using all of the identified cofactors for each trait. Significant ($p \leq 0.05$) QTL for each trait were declared at a genome-wide LOD threshold level determined by permutation testing (Churchill and Doerge 1994) of the dataset (n=1000 permutations) using the MapQTL software. Map positions were defined by the peak LOD score obtained in MQM and confidence intervals were defined by the peak ± 2 LOD (Van Ooijen 1992). The name of a QTL consisted of a trait it associated with followed by year of trait assessment and linkage group.

In the CP population type used, up to four alleles (*a*, *b*, *c*, and *d*) may be segregating at a given marker locus or QTL in the mapping population. Here *ab* represented the genotype of parent 'I' and *cd* for parent 'S'. These alleles assorted into genotype classes *ac*, *ad*, *bc*, and *bd* in the F_1 progeny. In MapQTL 4.0, genotype class phenotype means were automatically calculated for all four combinations at each marker locus and for inferred genotypes at 5 cM intervals if an interlocus gap exceeded 5 cM. These data were used to infer the direction of alleleic effect at each QTL locus.

6.3 Results

6.3.1 EST SSR markers

A total of 863 EST-SSR primer pairs, ranking highest in terms of SSR array length were screened for amplification efficiency and polymorphism in the F_1 I×S mapping population, and of these 692 (80%) obtained clear and efficient PCR amplification profiles. Of the 692 EST–SSR primer pairs that showed efficient amplification, 241 (35%) detected polymorphic loci (heterozygous in one or both parents) in the F_1. Of these, 16 were of segregation type 5 (Table 6.1), which cannot be represented as dominant markers, and therefore were not used for genetic linkage analysis of the individual parental datasets, but were included for the consensus map. In addition to the remaining 225 polymorphic primer pairs (EST-SSR markers), seven other polymorphic markers, five EST-SSRs from tall fescue (Saha *et al.* 2004) and two sequence-tagged site (STS) markers developed from perennial ryegrass ESTs were also used. A mixture of segregation types was observed across all polymorphic markers tested in this population, but most common were segregation types 3 and 4 (Table 6.1). The number of alleles per locus varied from two to four.

6.3.2 Construction of genetic linkage maps

Maps were constructed using 188 progeny and 228 markers (226 EST-SSR markers and two STS markers). Separate genetic linkage maps were first constructed for each parental genotype, 'I' and 'S', excluding 16 markers of segregation type 5 (Table 6.1). Maps for both parents were obtained at LOD 2.0, with the 'I' map containing 99 loci assigned to seven linkage groups and the 'S' map comprising 100 loci across the seven linkage groups (Table 6.2), indicating a similar level of heterozygosity in both genotypes. The average length of a linkage group was 70.3 cM in 'I', and in the 'S' map the average linkage group length was 61.7 cM. Irregular distribution of marker loci was observed within and between linkage groups in each parental map, and between parental homologues (Table 6.2). Four marker loci in 'I' (pps0029, pps0108, pps0760, nfa041) and five in 'S' (pps0008, pps0483, pps0490, pps0600, nfa041) failed to fall into any of the seven linkage groups as they all fell out at a LOD threshold lower than the LOD 6 grouping threshold and were not mapped. Three primer pairs (pps0502, pps0698, pp0766) identified more than one locus. Total map length was higher in 'I' (492 cM) than in 'S' (432 cM), with a mean marker density of 5.2 cM/locus in 'I' and

4.3 cM/locus in 'S' (Table 6.2). Homologous linkage groups from 'I' and 'S' were identified and aligned using marker loci that were heterozygous in both parental genotypes (segregation types 6 and 7, Table 6.1) (Table 6.2). Twenty-five of these heterozygous, or bridging, markers detected loci across the seven linkage groups (Table 6.2). A consensus map (Figure 6.1) was constructed using the individual parental polymorphic markers and the 25 bridging markers, plus the 16 markers of segregation type 5 (Table 6.1). Of 228 markers, 64 were not mapped - five markers did not group at the LOD threshold of 6.0 or 5.0 (pps0008, pps0029, pps0108, pps0760, nfa041), and the remaining 59 were excluded during ordering of the linkage groups. They did not map during this process because they failed to meet the default goodness-of-fit criteria set by the JoinMap software. The markers pps0483 and pps0490 failed to group on a parental map, but were successfully grouped on the consensus map.

This map contains 163 loci and spans 582.2 cM with a mean locus density of 3.6 cM/locus (Table 6.2, Figure 6.1). As with the parental maps, the distribution of marker loci on the consensus map is uneven. Eight gaps greater than 10 cM exist: one gap on each of linkage groups (Lg) 2 and 5, and two on each of Lg 3, 4 and 7. The number of marker loci per linkage group ranged from 14 to 30 (mean = 23), with Lg 1, 5, 6 and 7 having fewer marker loci than average (Table 6.2). Individual linkage groups were between 41 cM and 114 cM in length (mean = 83.2 cM), with Lg 1 and 6 being shorter than the mean.

Segregation distortion (deviation from expected Mendelian 1:1 ratio) of markers can trigger problems in mapping analysis since map distances may be over-estimated or skewed (Cogan *et al.* 2005). There was significant segregation distortion ($P \leq 0.05$) observed at 21 marker loci (17 % of total loci) on the consensus map with distorted loci present on all linkage groups except for Lg 6 (Figure 6.1). Of the 27 loci showing significant segregation distortion, 16 affected loci (10 %) were from the 'I' map and 11 (7 %) from the 'S' map. Some grouping of distorted loci was observed; in particular six marker loci (all from the 'S' parent) showing significant segregation distortion were clustered on the proximal half of Lg 7.

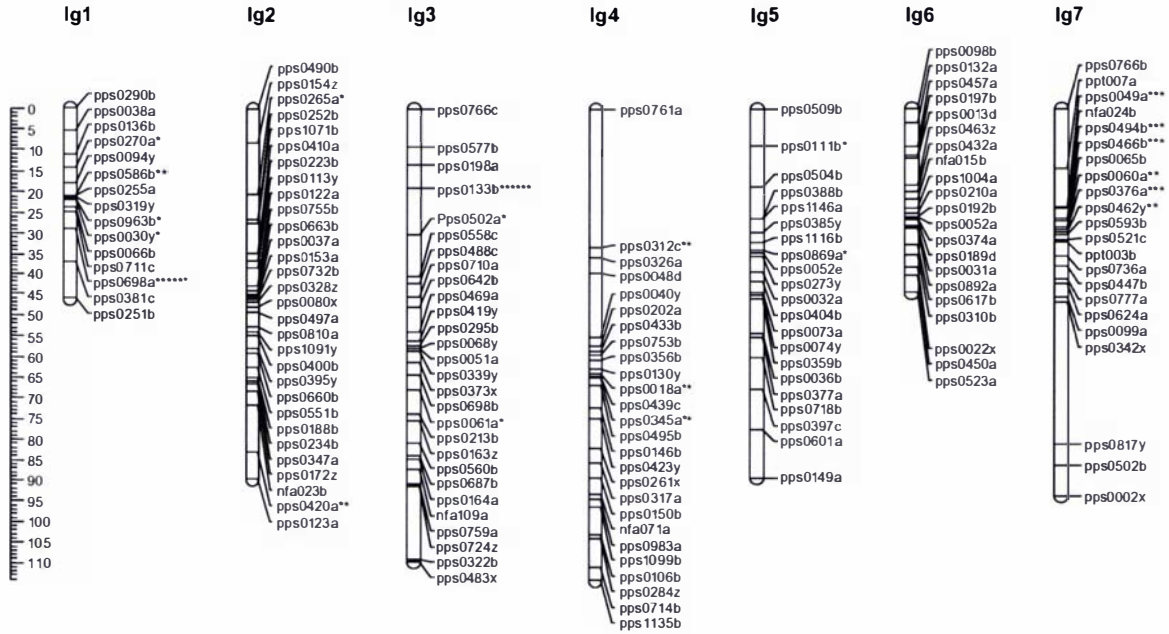


Figure 6.1: Consensus genetic linkage map of the I×S perennial ryegrass population. Linkage groups (Lg 1-7) are numbered in accordance with the International Lolium Genome Initiative (ILGI) reference map nomenclature through comparison of conserved SSR locus location (Faville et al., 2004). Marker loci are indicated at the right of linkage groups, and map distance is shown by the cM scale at the left of the figure. Distorted segregation ratios for individual markers: *p≤0.1, **p≤0.05, *** p≤0.01, ****** p≤0.0001. Marker suffixes (letters) refer to the marker allele. Suffixes 'a', 'b', 'c' and 'd' indicate a marker of segregation type 1, 2, 3 or 4; 'z" is a marker of segregation type 5; 'y' is a marker of segregation type 6; 'x' indicates a marker of segregation type 7.

Linkage	Length (cM)			Number of loci mapped				Mean locus density (cM/locus)		
group	I	S	C	I	S	Bridging	C	I	S	C
Lg1	44.3	25.2	40.8	10	9	3	14	4.4	2.8	2.9
Lg2	70.3	47.9	89.8	15	15	4	30	4.7	3.2	2.9
Lg3	92.1	92.2	109.7	22	15	6	28	4.2	6.1	3.9
Lg4	77	79.9	114.1	9	22	4	26	8.6	3.6	4.4
Lgg	88.3	68.6	89.3	15	12	3	21	5.9	5.7	4.3
Lg6	54.2	45.1	44.6	12	11	1	22	4.5	4.1	2
Lg7	65.8	72.8	93.9	16	16	4	22	4.1	4.6	4.3
Total	492	431.7	582.2	99	100	25	163	36.4	30.1	24.7
Mean	70.3	61.7	83.2	14.1	14.3	3.1	23.3	5.2	4.3	3.6

Table 6.2: Genetic linkage map length, number of loci mapped and locus density in parental and consensus genetic linkage maps of I×S perennial ryegrass population (I= one plant of Grasslands Impact parental map, S= one plant of Grasslands Samson parental map, C=consensus map).

6.3.3 QTL discovery

Generally, MQM identified more QTL than did SIM for all the traits assessed (Tables 6.3, 6.4 and 6.5). Although many of the QTL identified by SIM were also identified by MQM, only a smaller subset (i.e. 14 out of 30 and 10 out of 17 for herbage production traits in autumn and spring respectively, and 27 out of 37 for seed production traits) were significant (maximum LOD value greater than the genome-wide LOD threshold for a trait at $p<0.05$) in MQM. A QTL with a LOD score lower than the genome-wide LOD threshold was referred to as suggestive.

6.3.3.1 QTL for herbage yield and related traits in autumn

A total of 41 significant QTL were identified in the autumn dataset: 30 for herbage yield and related traits, and 11 for the three PC scores (Table 6.3, Figure 6.2). QTL were identified for all herbage yield and related traits assessed, and these occurred on all linkage groups except Lg 5. The total number of significant QTL identified for a trait ranged from two (for LED, ALf, TN and SIL) to seven (for LL) (Table 6.3, Figure 6.2). Three significant QTL were identified for DW, two on Lg1 (qDW-03-1.1 and qDW-03-1.2) and one on Lg 6 (qDW-03-6). The total phenotypic variation explained (PVE) in the population for DW was largely accounted for by the QTL qDW-03-6 (PVE 13.4 %) which mapped closely to QTL for TN (qTN-03-6) and PI (qPI-03-6). For the two DW QTL on Lg 1, qDW-03-1.1 co-located with QTL for LER (qLER-03-1) and LL (qLL-03-1), while qDW-03-1.2 co-located with qTN-03-1. Both of these positions were also occupied by QTL for PC3 (qPC3-03-1.1 and qPC3-03-1.2). The QTL on Lg 6 for DW and TN shared the same allelic effect, both showing increased phenotypic performance with an allele from the S parent, as indicated by genotypic sub-class means generated by the QTL analysis (Table 6.6). Alignment of the direction of allelic effect, although less straightforward, was also indicated at both Lg 1 QTL positions for DW and its related traits QTL, LER/LL and TN (Table 6.6). The co-location of QTL for DW with QTL for TN and LL was supported by significant correlation coefficients ($r = 0.44$ with TN; $r = 0.22$ with LL) (Table 4.6).

QTL for LED (qLED-03-4), ALg (qALg-03-4) and ALf (qALf-03-4) co-located to the same position, at marker locus pps0423, on Lg 4 (Table 6.3). In all cases, the highest

genotypic subclass means were for an allele from the I parent. QTL for TW and LL co-located at the lower end (qTW-03-3.1 and qLL-03-1) and upper end (qTW-03-3.2 and qLL-03-3.2) of Lg 3, and at both locations, they also co-located with qPC2-03-3.1 and qPC2-03-3.2, respectively. On Lg 7, two QTL for LER (qLER-03-7.1 and qLER-03-7.2) co-located with qLL-03-7 and qPC3-03-7.1.

Stem internodes length (SIL) has been associated with persistence in perennial ryegrass (Donaghy 1999), and its QTL were identified on Lg 2, 3 and 4. The major QTL (qSIL-03-4) accounting for 19.7 % of the trait variation was located on Lg 4. Amongst all traits assessed, SIL correlated significantly (0.24) only with LED.

The genotypic class means and sources of alleles affecting phenotypic performance are shown in Table 6.6.

			SIM					MQM				
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
DW	qDW-03-1.1*	1						21.3 (16.9-21.8)	3.75	5.14	10.5	pps0711c
	qDW-03-1.2*	1						35.0 (32.5-40.8)	3.75	4.03	8.2	pps0136b
	qDW-03-2.1	2						8.4 (0.0-18.6)	3.75	3.48	5.2	pps0154z
	qDW-03-2.2	2						57.9 (54.2-58.9)	3.75	3.10	4.7	pps0395y
	qDW-03-6*	6	18.5 (9.3-23.9)	2.65	3.28	8.5	pps0463z	38.1 (35.5-38.7)	3.75	7.66	13.4	pps0022x
	qDW-03-7.1	7						42.5 (38.6-44.8)	3.75	3.34	5.7	pps0624a
	qDW-03-7.2	7						93.9 (81.2-93.9)	3.75	3.37	4.9	pps0002x
	Total variation explained (%) by all QTL					8.5					52.6	
	Total variation explained (%) by genome-wide significant QTL										32.1	
ALf	qALf-03-1	1						0.0 (0.0-8.0)	3.95	3.29	5.0	pps0251b
	qALf-03-3	3	40.5 (0.0-73.8)	2.95	3.20	8.2	pps0558c	40.5 (29.1-42.2)	3.95	2.76	4.3	pps0558c
	qALf-03-4*	4	74.8 (61.7-79.8)	3.05	10.37	24.0	pps0423y	74.8 (72.2-80.1)	3.95	11.40	16.9	pps0423y
	qALf-03-5	5	8.8 (0.0-24.7)	2.75	4.91	16.8	pps0111b	8.8 (0.0-17.4)	3.95	3.72	7.8	pps0111b
	qALf-03-6*	6	25.2 (13.9-44.6	2.55	4.29	11.2	pps0210a	25.2 (23.2-25.6)	3.95	5.02	6.9	pps0210a
	Total variation explained (%) by all QTL					60.2					40.9	
	Total variation explained (%) by genome-wide significant QTL										23.8	
ALg	qALg-03-1*	1	9.3 (0.0-40.8)	2.45	3.24	8.3	pps0381c	0.0 (0.0-7.1)	3.75	4.49	8.2	pps0251b
	qALg-03-3	3	40.5 (0.0-69.0)	2.85	2.98	7.8	pps0558c	40.5 (29.2-41.6)	3.75	3.44	4.7	pps0558c
	qALg-03-4*	4	74.8 (57.4-89.3)	2.85	5.68	13.9	pps0423y	74.8 (72.2-80.4)	3.75	7.63	12.2	pps0423y
	qALg-03-5	5	8.8 (0.0-32.1)	2.65	3.65	12.3	pps0111b	8.8 (0.0-16.7)	3.75	3.37	8.9	pps0111b
	qALg-03-6.1*	6	25.2 (14.9-44.6)	2.65	4.88	13.0	pps0210a	19.9 (18.5-20.7)	3.75	5.78	10.2	pps0432a
	qALg-03-6.2	6	35.2 (12.9-44.6)	2.65	4.07	10.3	pps0310b					
	Total variation explained (%) by all QTL					65.6					44.2	
	Total variation explained (%) by genome-wide significant QTL										30.6	
LED	qLED-03-4*	4	66.8 (57.4-86.6)	3.10	6.91	18.5	pps0495b	74.8 (72.3-80.2)	3.85	9.40	16.2	pps0423y
	qLED-03-4.2	4						111.1 (106.5-114.1)	3.85	3.40	5.8	pps0714b
	qLED-03-5	5	0 (0.0-16.3)	2.75	3.48	9.3	pps0509b					
	qLED-03-7*	7						41.2 (37.2-42.3)	3.85	3.93	7.0	pps0777a

Table 6.3 (Cont...)

			SIM					MQM				
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
	Total variation explained (%) by all QTL					27.8					29	
	Total variation explained (%) by genome-wide significant QTL										23.2	
LER	qLER-03-1*	1	21.3 (13.3-32.8)	2.35	3.76	9.60	pps0711c	21.3(15.4-22.0)	3.75	4.86	8.5	pps0711c
	qLER-03-2	2	62.6 (21.6-89.8)	2.75	3.01	7.7	pps0551b	62.6 (58.2-64.6)	3.75	3.03	5.5	pps0551b
	qLER-03-3.1*	3						73.8 (67.3-74.3)	3.75	7.44	26.2	pps0213b
	qLER-03-3.2*	3						81.0 (78.7-81.8)	3.75	7.55	27.4	pps0560b
	qLER-03-4	4						89.3 (82.3-102.6)	3.75	3.47	8.1	pps0150b
	qLER-03-7*	7	35.5 (10.0-56.0)	2.85	3.80	10.4	pps0736a	31.4 (30.2-31.6)	3.75	8.05	7.8	pps0521c
	qLER-03-7.2*	7						29.0 (28.1-29.1)	3.75	6.72	4.8	pps0060a
	Total variation explained (%) by all QTL					27.7					88.3	
	Total variation explained (%) by genome-wide significant QTL										74.7	
LL	qLL-03-1*	1	17.3 (11.3-23.5)	2.45	8.15	22.0	pps0698a	17.3 (12.8-18.5)	3.75	8.60	13.0	pps0698a
	qLL-03-2*	2						38.6 (35.4-41.0)	3.75	3.85	5.4	pps0113y
	qLL-03-3.1*	3						0.0 (0.0-11.8)	3.75	3.84	14.1	pps0766c
	qLL-03-3.2	3						73.8 (66.2-83.7)	3.75	3.26	5.2	pps0213b
	qLL-03-3.3*	3						109.1 (91.5-109.7)	3.75	6.66	10.0	pps0322b
	qLL-03-4.1*	4						64.2 (62.2-64.4)	3.75	5.72	14.4	pps0018a
	qLL-03-4.2*	4						66.8 (65.0-70.2)	3.75	5.22	13.6	pps0495b
	qLL-03-5	5	35.5 (8.8-51.2)	2.75	3.08	7.8	pps0273y					
	qLL-03-7*	7						35.5 (31.8-36.5)	3.75	4.66	6.1	pps0736a
	Total variation explained (%) by all QTL					29.8					81.8	
	Total variation explained (%) by genome-wide significant QTL										76.6	
TN	qTN-03-1*	1	35.0 (21.3-40.8)	2.45	4.23	12.4	pps0136b	35 (30.0-40.8)	3.85	3.85	8.9	pps0136b
	qTN-03-2	2						8.4 (0.0-18.2)	3.85	3.81	8.1	pps0154z
	qTN-03-4	4						57.4 (54.9-58.5)	3.85	2.71	5.3	pps0202a
	qTN-03-6*	6						40.4 (36.6-44.6)	3.85	4.24	9.2	pps0450a
	Total variation explained (%) by all QTL					12.4					31.5	
	Total variation explained (%) by genome-wide significant QTL										18.1	
TW	qTW-03-1	1	31.8 (9.3-40.0)	2.45	3.44	10.2	pps0270a					

Table 6.3 (Cont...)

			SIM					MQM				
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
	qTW-03-2	2						0.0 (0.0-8.0)	3.75	3.39	6.7	pps0490b
	qTW-03-3.1*	3						30.2 (18.8-40.2)	3.75	5.17	12.0	pps0502a
	qTW-03-3*	3	91.5 (89.2-109.7)	3.05	6.46	15.8	pps0724z	91.5 (91.1-106.8)	3.75	8.33	12.6	pps0724z
	qTW-03-4.1*	4						57.4 (55.3-58.5)	3.75	4.05	7.7	pps0202a
	qTW-03-4.2	4						114.1 (106.1-114.1)	3.75	3.18	8.1	pps1135b
	qTW-03-7	7	14.4 (0.0-44.5)	2.85	3.24	10.8	ppt007a	28.4 (20.0-28.9)	3.75	2.68	3.0	pps0065b
	Total variation explained (%) by all QTL					36.8					50.1	
	Total variation explained (%) by genome-wide significant QTL										32.3	
	qPI-03-1	1	35 (20.3-40.8)	2.45	3.92	11.6	pps0136b	35.0 (30.0-40.8)	3.75	3.57	8.3	pps0136b
	qPI-03-2	2						8.4 (0.0-17.7)	3.75	3.68	7.6	pps0154z
PI	qPI-03-6*	6						40.4 (36.9-44.6)	3.75	4.79	10.6	pps0450a
	Total variation explained (%) by all QTL					11.6					26.5	
	Total variation explained (%) by genome-wide significant QTL										10.6	
	qSIL-03-2*	2	68.5 (46.3-78.8)	2.85	3.08	7.9	pps0172z	68.5 (66.1-70.3)	3.75	3.77	6.9	pps0172z
	qSIL-03-3	3	30.2 (0.0-84.0)	2.95	3.28	10.8	pps0502a	48.2 (41.7-53.9)	3.75	2.74	4.9	pps0642b
SIL	qSIL-03-4*	4	74.8 (63.9-96.4)	2.95	6.43	15.6	pps0423y	85.6 (81.8-86.4)	3.75	9.93	19.7	pps0317a
	Total variation explained (%) by all QTL					34.3					31.5	
	Total variation explained (%) by genome-wide significant QTL										26.6	
	qPC1-03-1	1	35.0 (20.3-40.8)	2.45	3.92	11.6	pps0136b					
	qPC1-03-2	2						8.4 (0.0-18.6)	3.70	3.55	7.3	pps0154z
	qPC1-03-4*	4						74.8 (72.0-80.6)	3.70	5.40	9.0	pps0423y
PC1	qPC1-03-5	5						0.0 (0.0-8.1)	3.70	3.60	7.5	pps0509b
	qPC1-03-6*	6						40.4 (39.3-44.6	3.70	4.57	8.2	pps0450a
	Total variation explained (%) by all QTL					11.6					32.0	
	Total variation explained (%) by genome-wide significant QTL										17.2	
PC2	qPC2-03-1*	1	17.3 (9.3-24.3)	2.35	6.36	19.0	pps0698a	17.3 (9.9-18.6)	3.80	6.65	13.7	pps0698a
	qPC2-03-3.1*	3						30.2 (18.8-40.2)	3.80	4.65	9.9	pps0502a
	qPC2-03-3.2*	3	109.7 (90.2-109.7)	3.05	5.47	13.7	pps0483x	109.7 (108.2-109.7)	3.80	6.47	11.1	pps0483x
	qPC2-03-4	4	66.8 (56.2-82.1)	2.95	4.60	10.5	pps0495b	66.8 (64.8-72.0)	3.80	3.41	4.6	pps0495b

Table 6.3 (Cont...)

Trait	QTL	Linkage group	SIM					MQM				
			Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
	qPC2-03-6	6	18.5 (0.0-26.0)	2.65	3.76	9.9	pps0463z	0.0 (0.0-2.4)	3.80	3.36	5.6	pps0098b
	Total variation explained (%) by all QTL					53.1					44.9	
	Total variation explained (%) by genome-wide significant QTL										34.7	
PC3	qPC3-03-1.1*	1						21.3 (17.0-21.7)	3.85	6.89	17.4	pps0711c
	qPC3-03-1.2*	1						35.0 (31.8-40.8)	3.85	5.49	15.5	pps0136b
	qPC3-03-3.1*	3						54.0 (47.7-54.9)	3.85	5.17	13.5	pps0469a
	qPC3-03-3.2*	3						84.9 (82.7-86.1)	3.85	4.37	12.6	pps0164a
	qPC3-03-7.1*	7	35.5 (23.6-46.9)	2.85	3.88	10.4	pps0736a	37.8 (34.8-38.6)	3.85	8.69	18.1	pps0447b
	qPC3-03-7.2*	7						81.2 (57.0-86.3)	3.85	4.41	8.7	pps0817y
	Total variation explained (%) by all QTL					10.4					85.8	
	Total variation explained (%) by genome-wide significant QTL										85.8	

*Genome-wide significant QTL at p≤0.05

Table 6.3: QTL identified by Simple interval mapping (SIM) and Multiple QTL mapping (MQM) for herbage yield and related traits of the I×S perennial ryegrass population assessed in a glasshouse in autumn 2003. DW: herbage dry weight; ALf: leaf appearance interval; ALg: ligule appearance interval; LED: leaf elongation duration; LER: leaf elongation rate; LL: leaf lamina length; TN: tiller number; TW: tiller weight; PI: productivity index; SIL: stem internodes length; PC1: principal component 1; PC2: principal component 2 and PC3: principal component 3. Name of a QTL consists of a trait followed by year of trait assessment and linkage group. LOD: logarithm of odds ratio.

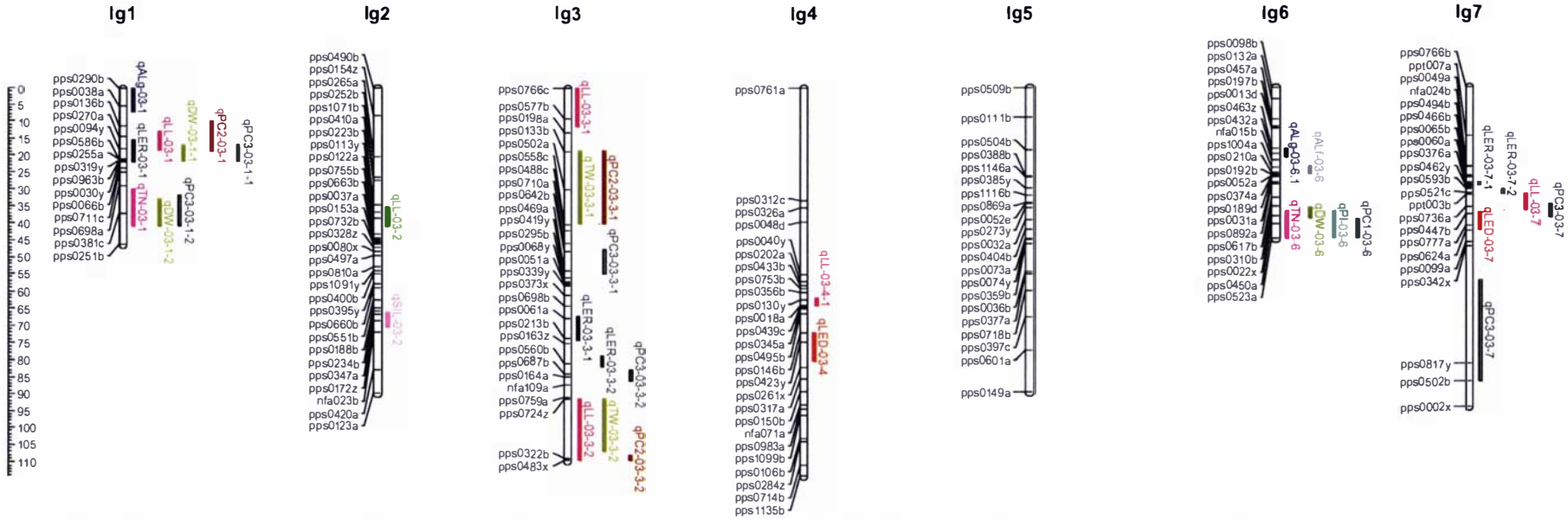


Figure 6.2: Ryegrass genetic linkage map, showing seven linkage groups (Lg) and QTL discovered for herbage yield and related traits of the I×S perennial ryegrass mapping population assessed in autumn 2003. Molecular markers are indicated on the left of each Lg, and QTL are indicated as filled blocks on the right (with 2 LOD support interval). Length of Lg is indicated by the centimorgan (cM) scale at the left of the figure.

6.3.3.2 QTL for herbage yield and related traits in spring

A total of 17 significant QTL were identified in the spring dataset, and were discovered on all linkage groups except Lg 7 (Table 6.4, Figure 6.3). The number of significant QTL identified per trait ranged from one (for LER and DW) to five (for LED) in spring (Table 6.4, Figure 6.2). No significant QTL were identified for TW, TN, PI and LL. In comparison with autumn, there were fewer significant QTL for every trait assessed in spring except for LED and ALf, both of which had more QTL in spring than were detected in autumn. Additionally, on a trait-by-trait basis there was little congruence between the location of QTL identified in the autumn and spring datasets. Only QTL for ALg on Lg 1, 4 and 6, LED on Lg 4 and ALf on Lg 4 appeared in both seasons.

Only one significant QTL, qDW-04-2.2 (accounting for 9.2% PVE in the population) was identified for DW, and this occurred on Lg 2. This QTL co-located with QTL for LED (qLED-04-2.2) and PI (qPI-04-2), and was in close proximity to suggestive QTL (present but not significant at the assigned LOD threshold) for TN (qTN-04-2) and LL (qLL-04-2) (Table 6.4). A suggestive QTL (qDW-03-2.2) was also indicated within 3cM of this location in autumn, (LOD score of 3.10 and PVE of 4.7%). A second suggestive QTL (LOD 3.43) for DW (qDW-04-2.1) was also identified on Lg 2 and co-located with a suggestive QTL for TW (qTW-04-2). Again, a suggestive DW QTL (qDW-03-2.1) also mapped to a similar position on Lg 2 in the autumn dataset. Co-located QTL were also discovered for ALf, ALg and LED on Lg 4 (qALf-04-4.1, qALg-04-4 and qLED-04-4), ALf and LED (qALf-04-3, qLED-04-3) on Lg 3 with ALg QTL (qALg-04-3) mapped to a neighbouring position, and for qLED-04-2.2 and qDW-04-2.2 on Lg 2 (Table 6.4). On Lg 3 increase in phenotypic values of all three QTL was associated with allele from I parent but on Lg 4 they were associated with allele from S parent. On Lg 2, increase in phenotypic value for qLED-04-2.2 and qDW-04-2.2 was associated with alleles from the S parent (Table 6.6).

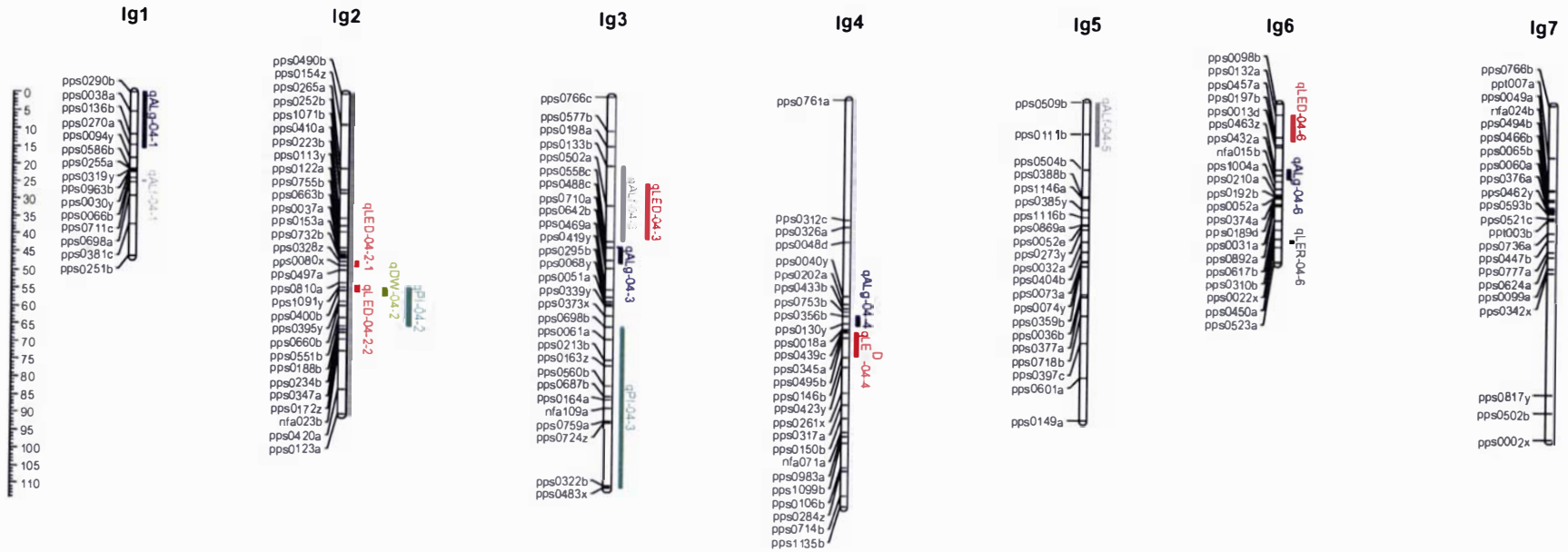


Figure 6.3: Ryegrass genetic linkage map, showing seven linkage groups (Lg) and QTL discovered for herbage yield and related traits of the I×S perennial ryegrass mapping population assessed in spring 2004. Molecular markers are indicated on the left of each Lg, and QTL are indicated as filled blocks on the right (with 2 LOD support interval). Length of Lg is indicated by the centimorgan (cM) scale at the left of the figure.

			SIM					MQM				
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
DW	qDW-04-2.1	2						0 (0.0-20.6)	3.95	3.43	8.10	pps0490b
	qDW-04-2.2*	2	54.9 (35.8-79.8)	2.65	3.46	8.90	pps0400b	54.9 (54.0-56.5)	3.95	4.11	9.20	pps0400b
	Total variation explained (%) by all QTL					8.9					17.30	
	Total variation explained (%) by genome-wide significant QTL										9.20	
ALf	qALf-04-1*	1	24.6 (2.0-40 8)	2.45	3.92	9.80	pps0963b	24.6 (24.3-24.7)	3.85	5.39	6.90	pps0963b
	qALf-04-3*	3						30.2 (19.0-40.3)	3.85	6.30	11.10	pps0502a
	qALf-04-4.1*	4	66.8 (56.2-88.6)	2.95	4.68	12.50	pps0495b	64.2 (62.9-64.3)	3.85	7.81	10.70	pps0018a
	qALf-04-4.2	4	103.2 (90.3-104.1)	2.95	4.83	13.70	pps0106b	103.2 (93.4-103.8)	3.85	3.19	4.90	pps0106b
	qALf-04-5*	5						0 (0.0-12.0)	3.85	4.08	5.20	pps0509b
	Total variation explained (%) by all QTL					36.0					38.80	
	Total variation explained (%) by genome-wide significant QTL										33.90	
ALg	qALg-04-1*	1	9.3 (0.0-28.3)	2.45	4.84	12.30	pps0381c	9.3 (0.0-15.3)	3.75	6.29	9.00	pps0381c
	qALg-04-3*	3	54.0 (9.2-70.0)	3.15	3.25	8.60	pps0469a	45.9 (42.3-46.8)	3.75	5.38	7.00	pps0710a
	qALg-04-4*	4	62.9 (56.2-80.0)	2.95	5.83	14.20	pps0130y	62.9 (60.7-63.1)	3.75	9.42	13.10	pps0130y
	qALg-04-5	5	8.8 (0.0-49.2)	2.75	3.03	9.10	pps011b	8.8 (0.0-17.3)	3.75	3.30	5.60	pps0111b
	qALg-04-6*	6						19.9 (18.5-21.0)	3.75	4.85	7.00	pps0432a
	Total variation explained (%) by all QTL					44.20					41.70	
	Total variation explained (%) by genome-wide significant QTL										36.10	
LED	qLED-04-2.1*	2	43.2 (22.6-62.2)	2.65	3.43	8.90	pps0122a	48.3 (47.1-48.7)	3.75	4.96	8.60	pps0080x
	qLED-04-2.2*	2						54.9 (54.0-56.3)	3.75	4.35	7.50	pps0400b
	qLED-04-3*	3	30.2 (0.0-56.2)	3.15	3.14	8.60	pps0502a	30.2 (24.0-40.0)	3.75	5.01	10.00	pps0502a
	qLED-04-4*	4	66.8 (56.2-88.6)	2.95	5.2	14.30	pps0495b	66.8 (65.1-71.8)	3.75	6.76	10.60	pps0495b
	qLED-04-6*	6						9.3 (3.4-10.5)	3.75	3.76	6.90	pps0457a
	Total variation explained (%) by all QTL					31.80					43.60	
	Total variation explained (%) by genome-wide significant QTL										43.60	
LER	qLER-04-5	5						89.3 (77.3-89.3)	3.85	3.08	10.40	pps0149a
	qLER-04-6*	6	38.1 (35.3-44.4)	2.65	5.27	12.90	pps0022x	38.1 (38.1-38.9)	3.85	6.01	13.40	pps0022x
	qLER-04-7	7						32.0 (29.0-35.2)	3.85	3.08	6.80	ppt003b

			SIM				MQM					
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
	Total variation explained (%) by all QTL					12.90					30.20	
	Total variation explained (%) by genome-wide significant QTL										13.40	
LL	qLL-04-2	2						48.3 (45.1-87.9)	3.75	2.35	5.40	pps0080x
	qLL-04-4	4						103.2 (74.8-103.9)	3.75	2.86	6.70	pps0106b
	Total variation explained (%) by all QTL					0.0					12.10	
	Total variation explained (%) by genome-wide significant QTL										0.00	
TN	qTN-04-2	2						47.1 (0.0-55.4)	3.85	2.18	5.10	pps0328z
	qTN-04-6	6	26.6 (11.5-49.6)	2.65	3.09	7.80	pps0189d	26.6 (26.2-27.6)	3.85	3.28	7.80	pps0189d
	Total variation explained (%) by all QTL					7.8					12.90	
	Total variation explained (%) by genome-wide significant QTL										0.00	
TW	qTW-04-1	1						0 (0.0-25.2)	4.00	2.47	6.30	pps0251b
	qTW-04-2	2						0 (0.0-45.1)	4.00	2.41	5.90	pps0490b
	qTW-04-7.1	7						26.2 (14.4-35.5)	4.00	2.75	7.20	pps0494b
	qTW-04-7.2	7						93.9 (72.0-93.9)	4.00	2.39	5.80	pps0002x
	Total variation explained (%) by all QTL					0					25.20	
	Total variation explained (%) by genome-wide significant QTL										0.00	
PI	qPI-04-2*	2						54.9 (54.0-65.0)	3.85	6.66	16.80	pps0400b
	qPI-04-3*	3						109.7 (64.3-109.7)	3.85	3.85	12.90	pps0483x
	qPI-04-4	4						114.1 (96.4-114.1)	3.85	2.48	14.10	pps1135b
	Total variation explained (%) by all QTL					0.0					43.80	
	Total variation explained (%) by genome-wide significant QTL										29.70	
PC1	qPC1-04-1	1	24.6 (2.0-40.8)	2.50	2.65	6.30	pps0963b	24.6 (2.0-40.8)	3.21	3.80	6.00	pps0963b
	qPC1-04-3	3	30.2 (0.0-56.2)	3.10	3.07	10.30	pps0502a	30.2 (0.0-56.2)	2.79	3.80	7.10	pps0502a
	qPC1-04-4	4	103.2 (90.3-104.1)	2.95	4.55	12.50	pps0106b	103.2 (90.3-104.1)	3.04	3.80	5.50	pps0106b
	qPC1-04-5	5	0 (0.0-12.0)	2.80	2.37	6.50	pps0509b	0 (0.0-12.0)	2.64	3.80	6.10	pps0509b
	Total variation explained (%) by all QTL					35.60					24.70	
	Total variation explained (%) by genome-wide significant QTL										0.00	
PC2	qPC2-04-2.1*	2	0 (0.0-45.1)	2.90	3.80	10.30	pps0490b	0 (0.0-45.1)	4.35	3.80	10.60	pps0490b
	qPC2-04-2.2	2	54.9 (54.0-65.0)	2.90	3.28	8.40	pps0400b	54.9 (54.0-65.0)	3.70	3.80	8.10	pps0400b
	Total variation explained (%) by all QTL					18.70					18.70	

			SIM				MQM					
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
	Total variation explained (%) by genome-wide significant QTL										10.60	
PC3	qPC3-04-1*	1	17.3 (11.3-23.5)	2.95	3.99	12.10	pps0698a	17.3 (12.8-18.5)	3.99	3.80	12.10	pps0698a
	Total variation explained (%) by all QTL					12.10					12.10	
	Total variation explained (%) by genome-wide significant QTL										0.00	

*genome wide significant QTL ($p \leq 0.05$)

Table 6.4 QTL identified by Simple interval mapping (SIM) and Multiple QTL mapping (MQM) for herbage yield and related traits of the I×S perennial ryegrass population assessed in a glasshouse in spring 2004. DW: herbage dry weight; ALf: leaf appearance interval; ALg: ligule appearance interval; LED: leaf elongation duration; LER: leaf elongation rate; LL: leaf lamina length; TN: tiller number; TW: tiller weight and PI: productivity index. Name of a QTL consists of a trait followed by year of trait assessment and linkage group. LOD: logarithm of odds ratio.

6.3.3.3 Seed production and related traits

A total of 44 significant ($p \leq 0.05$) QTL were identified for seed yield and related traits, and the PC scores (Table 6.5, Figure 6.4). QTL were identified for all traits except for RT and TMH. The number of significant QTL identified for a trait ranged from one (for SdYH) to four (for SH and DH) (Table 6.5, Figure 6.4). Three QTL for SdYP were identified on Lg 1, 2 and 6 but only those on Lg 2 and 6 were significant, and together these accounted for 21.4% of the trait variation (PVE) in the population. The QTL on Lg 6 had the highest LOD score (LOD 6.4) and PVE (14%). Co-located QTL for component traits were also identified at the SdYP QTL positions on Lg 2 and Lg 6 (Table 6.5). On Lg 2 SdYP QTL (qSdYP-03-2) co-located with QTL for PC1 (qPC1-03-2), FH (qFH-03-2) and PGH (qPGH-03-2), and was mapped close to QTL for SH (qSH-03-2) and FS (qFS-03-2). The increase in phenotypic mean from qSdYP-03-2, qFH-03-2 and qFS-03-2 on Lg 2 was associated with an allele from the I parent while that of qPGH-03-2 and qSH-03-2 was associated with an allele from the S parent (Table 6.6).

On Lg 6 qSdYP-03-6 co-located with qSdYH-03-6, qTSW-03-6, qPC1-03-6, and qPC3-03-6. Increase in phenotypic performance of qSdYP-03-6 and qSdYH-03-6 was associated with an allele from the I parent, while that of qTSW-03-6 was associated with an allele from the S parent (Table 6.6). QTL for FSU (qFSU-03-1) and SL (qSL-03-1) mapped close to each other on Lg 1, and the increase in their phenotypic values may be associated with an allele from the I parent.

QTL for DH were identified on the same linkage groups (Lg 2, 4 and 7) and at the same positions (those on Lg 2 and 4 only) in 2003 and 2004. The QTL on Lg 2 (qDH-03-2 and qDH-04-2) accounted for the largest PVE, at 29.7 % and 28.4 % in 2003 and 2004 respectively (Table 6.5). In both years, QTL on Lg 4 (qDH-03-4 and qDH-04-4) accounted for 14 % PVE in the population, while those on Lg 7 (qDH-03-7 and qDH-04-7) had the smallest effects (5-7%). QTL for DH (qDH-03-7) co-located with qLED-03-7 on Lg 7 and with qSOH-03-2.1 on Lg 2. Correlation between DH and all other traits was negative but was only significant with SOH (-0.41). At qDH-03-7, late heading and prolonged leaf elongation duration were associated with an allele from the

S parent, while at qSOH-03-2.1, prolong SOH and late heading were associated with different alleles but also from the S parent (Table 6.6).

Co-located QTL were identified for herbage and seed production traits on Lg 1(qFH-03-1, qSH-03-1 and qLL-03-1), Lg 2 (qSdYP-03-2, qFH-03-2 and qLL-.3-2) and Lg 6 (qDW-03-6 and qFH-03-6). On Lg 2 and 6, an increase in phenotypic values for SdYP and FH were associated with an allele from the I parent, while that for DW was associated with an allele from the S parent. On Lg I, an increase in phenotypic values for FH and SH were associated with an allele from the S parent, while LL was associated with an allele from the I parent (Table 6.6). Correlations between the seed production traits (SdYP, FH and SH) and herbage production traits (LL and DW) were negative (Table 6.7) but not significant ($p<0.01$).

In breeding programmes, a QTL that accounts for the largest phenotypic variation of a trait in a population or has a high LOD score may be the choice of breeders. A QTL can be classified as minor or major depending on the amount of phenotypic variation it explains. By one standard, a minor QTL explains less than 10 % of PVE while a major QTL explains more than 10 % PVE (Collard *et al.* 2005). On this basis, 32 major and 27 minor QTL were identified in this study of QTL affecting herbage yield and seed yield, and their related traits, and the PVE by QTL in the I×S population was skewed towards QTL of smaller effect (Figure 6.5).

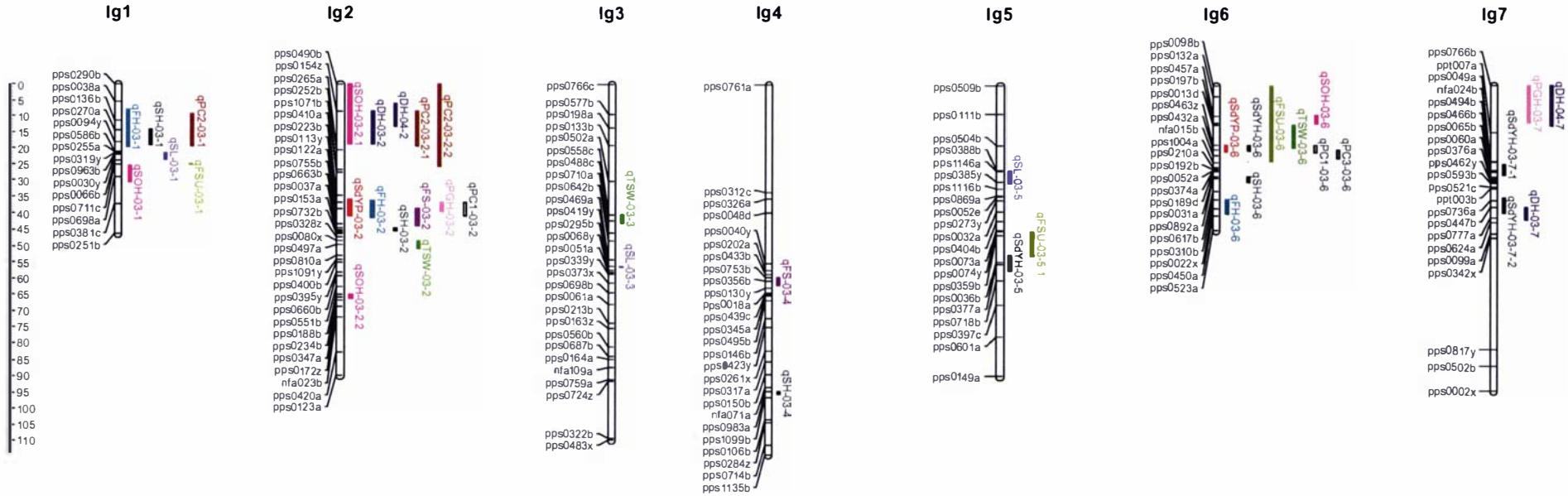


Figure 6.4 Ryegrass genetic linkage map, showing seven linkage groups (Lg) and QTL for seed yield, seed yield related traits and PC scores in the I×S perennial ryegrass population assessed as spaced plants in the field. Molecular markers are indicated on the left of each Lg, and QTL are indicated as filled blocks on the right (with 2 LOD support interval). Length of Lg is indicated by the centimorgan (cM) scale at the left of the figure.

			SIM					MQM				
Trait	QTL	Linkage group	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker	Position (cM ±2LOD)	LOD threshold	Maximum LOD value	Variation explained (%)	Marker
SdYP	qSdYP-03-1	1	28.2 (21.3-31.8)	2.63	3.44	8.70	pps0094y	28.2 (25.3-30.6)	3.75	3.3	6.30	pps0094y
	qSdYP-03-2*	2	38.6 (9.4-44.2)	2.77	3.88	9.60	pps0113y	38.6 (36.7-71.9)	3.75	3.85	7.40	pps0113y
	qSdYP-03-6*	6	19.9 (9.0-26.0)	2.70	6.49	16.80	pps0432a	19.9 (18.5-20.5)	3.75	6.41	14.00	pps0432a
	Total variation explained (%) by all QTL					35.10					27.70	
	Total variation explained (%) by genome-wide significant QTL										21.40	
SdYH	qSdYH-03-1	1	24.9 (21.3-31.8)	2.48	3.01	7.60	pps319y	24.9 (24.5-25.2)	3.75	3.27	4.60	pps0319y
	qSdYH-03-2	2	38.6 (9.4-44.2)	2.95	3.57	8.90	pps0113y	38.6 (35.2-42.3)	3.75	2.99	5.10	pps0113y
	qSdYH-03-5.1	5	54.4 (36.0-87.3)	2.85	3.68	9.50	pps0036b	46.2 (44.9-54.8)	3.75	3.11	5.70	pps0359b
	qSdYH-03-5.2*	5						55.1 (52.3-57.1)	3.75	5.06	9.40	pps0377
	qSdYH-03-6*	6	19.9 (19.0-23.0)	2.70	10.25	25.30	pps0432a	19.9 (18.3-20.3)	3.75	10.36	18.00	pps0432a
	qSdYH-03-7.1*	7						26.8 (24.3-27.8)	3.75	4.04	9.80	pps0466b
	qSdYH-03-7.2*	7						37.8 (34.6-39.6)	3.75	4.42	10.90	pps0447b
	Total variation explained (%) by all QTL					51.30					63.50	
	Total variation explained (%) by genome-wide significant QTL										48.10	
FH	qFH-03-1*	1	17.3 (0.0-36.0)	2.39	3.97	10.50	pps0698a	17.3 (7.7-19.5)	3.85	3.9	8.00	pps0698a
	qFH-03-2.1	2	8.4 (0.0-70.5)	2.82	3.45	10.40	pps0154z					
	qFH-03-2.2*	2	43.2 (17.4-65.0)	2.82	3.92	10.30	pps0122a	38.6 (36.2-41.3)	3.85	4.18	8.10	pps0113y
	qFH-03-4	4	39.8 (15.4-72.0)	2.81	3.51	9.00	pps0048d	55.2 (37.1-60.9)	3.85	3.46	6.60	pps0040y
	qFH-03-6*	6						38.1 (35.3-39.9)	3.85	3.85	7.70	pps0022x
	Total variation explained (%) by all QTL					40.20					30.30	
	Total variation explained (%) by genome-wide significant QTL										23.80	
SH	qSH-03-1*	1	17.3 (0.0-30.2)	2.58	3.95	10.60	pps0698a	17.3 (14.3-19.0)	3.70	5.06	4.00	pps0698a
	qSH-03-2*	2						45.1 (44.4-45.3)	3.70	4.84	7.60	pps0663b
	qSH-03-4.1*	4						94.6 (94.6-95.6)	3.70	3.87	22.80	pps0983a
	qSH-03-4.2	4						114.1 (111.1-114.1)	3.70	2.68	20.20	pps1135b

Table 6.5 (Cont…)

Trait	QTL	Linkage group	SIM Position (cM ±2LOD)	SIM LOD threshold	SIM Maximum LOD value	SIM Variation explained (%)	SIM Marker	MQM Position (cM ±2LOD)	MQM LOD threshold	MQM Maximum LOD value	MQM Variation explained (%)	MQM Marker
	qSH-03-6.1	6	0.0 (0.0-19.9)	2.61	3.78	15.30	pps0098b					
	qSH-03-6.2*	6	32.7 (19.9-44.6)	2.61	3.75	9.60	pps0617b	28.7 (28.2-30.0)	3.70	6.29	5.30	pps0892a
	qSH-03-7	7						35.5 (30.3-81.2)	3.70	2.32	2.50	pps0736a
	Total variation explained (%) by all QTL					35.50					62.40	
	Total variation explained (%) by genome-wide significant QTL										59.90	
FS	qFS-03-2*	2	43.2 (0.0-79.8)	2.94	3.61	9.50	pps0122a	43.2 (38.6-43.8)	3.87	4.38	10.30	pps0122a
	qFS-03-4*	4	60.7 (16.0-76.8)	3.00	4.12	11.00	pps0356b	60.7 (59.5-61.9)	3.87	4.89	11.80	pps0356b
	Total variation explained (%) by all QTL					20.50					22.10	
	Total variation explained (%) by genome-wide significant QTL										22.10	
SL	qSL-03-1*	1	22.5 (0.0-40.8)	2.43	3.31	8.30	pps0066b	22.5 (21.3-23.5)	3.75	4.47	6.40	pps0066b
	qSL-03-2	2						54 (43.2-64.0)	3.75	2.14	3.10	pps1091y
	qSL-03-3*	3	56.2 (53.2-58.1)	2.90	8.99	21.00	pps0419y	56.2 (56.2-56.4)	3.75	12.18	19.70	pps0419y
	qSL-03-4	4	94.6 (66.4-114.1)	2.92	3.17	9.70	pps0983a	94.6 (93.4-95.8)	3.75	3.34	6.20	pps0983a
	qSL-03-5*	5						29.8 (26.4-30.6)	3.75	5.86	9.00	pps0385y
	Total variation explained (%) by all QTL					39.00					44.40	
	Total variation explained (%) by genome-wide significant QTL										35.10	
FSU	qFSU-03-1*	1	24.9 (21.3-31.8)	2.40	3.36	8.40	pps0319y	24.9 (24.6-25.1)	3.85	4.21	7.00	pps0319y
	qFSU-03-3	3						57.9 (0.0-109.7)	3.85	2.12	3.30	pps0068y
	qFSU-03-5.1*	5	46.2 (37.5-58.1)	2.71	4.17	11.40	pps0359b	46.2 (45.1-52.6)	3.85	4.99	9.80	pps0359b
	qFSU-03-5.2	5	89.3 (24.7-89.3)	2.71	2.99	9.30	pps0149a					
	qFSU-03-6.1*	6	11.5 (1.0-22.7)	2.78	5.23	12.90	pps0197b	11.5 (0.0-23.4)	3.85	6.01	9.20	pps0197b
	qFSU-03-6.2	6						18.5 (0.0-25.4)	3.85	2.28	4.40	pps0463z
	Total variation explained (%) by all QTL					42.00					33.70	
	Total variation explained (%) by genome-wide significant QTL										16.80	
SOH	qSOH-03-1*	1						28.2 (25.3-30.5)	3.75	4.39	9.50	pps0094y

Table 6.5 (Cont...)

Trait	QTL	Linkage group	SIM Position (cM ±2LOD)	SIM LOD threshold	SIM Maximum LOD value	SIM Variation explained (%)	SIM Marker	MQM Position (cM ±2LOD)	MQM LOD threshold	MQM Maximum LOD value	MQM Variation explained (%)	MQM Marker
	qSOH-03-2.1*	2	8.4 (0.0-79.5)	2.79	3.51	8.80	pps0154z	8.4 (0.0-18.7)	3.75	4.96	10.00	pps0154z
	qSOH-03-2.2*	2	65.8 (52.8-79.8)	2.79	3.63	9.40	pps0234b	65.8 (65.0-66.0)	3.75	6.92	15.70	pps0234b
	qSOH-03-4.1	4						89.3 (74.8-92.7)	3.75	2.58	16.00	pps0150b
	qSOH-03-4.2	4						114.1 (101.4-114.1)	3.75	2.58	15.30	pps1135b
	qSOH-03-6*	6						11.5 (8.9-11.7)	3.75	4.28	5.60	pps0197b
	Total variation explained (%) by all QTL					18.20					72.10	
	Total variation explained (%) by genome-wide significant QTL										40.80	
DH 2003	qDH-03-2*	2	8.4 (0.0-79.5)	2.9	11.46	28.2	pps0154z	8.4 (8.4-18.7)	3.75	18.89	29.7	pps0154z
	qDH-03-4*	4	60.7 (16.0-76.8)	2.74	6.94	17.50	pps0356b	66.8 (65.1-68.3)	3.75	15.5	13.80	pps0495b
	qDH-03-5	5						89.3 (60.0-89.3)	3.75	2.98	3.70	pps0149a
	qDH-03-6	6						21.7 (21.7-25.2)	3.75	3.63	4.40	nfa015b
	qDH-03-7*	7	42.5 (7.0-93.9)	2.74	3.18	8.40	pps0624a	41.2 (37.2-41.7)	3.75	5.79	5.00	pps0777a
	Total variation explained (%) by all QTL					54.10					56.60	
	Total variation explained (%) by genome-wide significant QTL										48.50	
DH 2004	qDH-04-2*	2	8.4 (0.0-13.8)	2.90	9.37	24.40	pps0154z	8.4 (6.0-13.4)	3.75	13.49	28.40	pps0154z
	qDH-04-3	3	45.9 (38.7-57.2)	2.95	3.50	10.00	pps0710	81.0 (74.0-83.2)	3.75	3.32	5.00	pps0560b
	qDH-04-4*	4	60.5 (43.3-93.5)	2.74	3.70	9.60	pps0356b	60.5 (59.3-61.4)	3.75	7.97	13.70	pps0356b
	qDH-04-7*	7	86.5 (47.1-93.9)	2.74	3.96	8.60	pps0817	0.0 (0.0-12.2)	3.75	4.44	7.30	pps0766b
	Total variation explained (%) by all QTL					52.60					54.00	
	Total variation explained (%) by genome-wide significant QTL										49.40	
TSW	qTSW-03-2.1	2	20.6 (9.4-66.6)	3.00	4.12	15.20	pps0256a					
	qTSW-03-2.2*	2	49.4 (10.4-73.8)	3.00	4.25	11.30	pps0497a	49.4 (48.3-50.6)	3.80	4.26	7.10	pps0497a
	qTSW-03-3*	3	42.3 (29.3-53.7)	2.92	7.20	18.70	pps0488c	42.3 (40.5-43.2)	3.80	8.87	15.60	pps0488c
	qTSW-03-5	5						18.7 (8.8-26.2)	3.80	2.62	4.00	pps0504b
	qTSW-03-6*	6	18.5 (3.4-28.7)	3.80	3.93	9.90	pps0463z	18.5 (11.9-19.4)	3.80	3.93	6.00	pps0463z
	qTSW-03-7	7						14.4 (0.0-22.7)	3.80	2.81	4.70	ppt007a

Table 6.5 (Cont...)

Trait	QTL	Linkage group	SIM Position (cM ±2LOD)	SIM LOD threshold	SIM Maximum LOD value	SIM Variation explained (%)	SIM Marker	MQM Position (cM ±2LOD)	MQM LOD threshold	MQM Maximum LOD value	MQM Variation explained (%)	MQM Marker
	Total variation explained (%) by all QTL					55.10					37.40	
	Total variation explained (%) by genome-wide significant QTL										28.70	
PGH	qPGH-03-2*	2	38.6 (9.4-44.2)	3.04	5.58	13.60	pps0113y	38.6 (36.7-39.5)	3.85	10.93	16.20	pps0113y
	qPGH-03-3	3						81 (75.2-83.3)	3.85	3.51	3.90	pps0560b
	qPGH-03-4*	4	60.7 (16.0-76.8)	2.96	5.99	15.20	pps0356b	60.7 (59.5-61.2)	3.85	10.07	16.60	pps0356b
	qPGH-03-5	5						39.3 (35.5-41.7)	3.85	3.76	5.30	pps0032a
	qPGH-03-7.1*	7						0 (0.0-11.3)	3.85	6.88	12.90	pps0766b
	qPGH-03-7.2	7						93.9 (86.5-93.9)	3.85	3.83	6.80	pps0002x
	Total variation explained (%) by all QTL					28.80					61.70	
	Total variation explained (%) by genome-wide significant QTL										45.70	
RT	qRT-03-2	2	71.8 (46.3-79.7)	2.30	3.09	7.80	nfa023b	71.8 (68.5-81.8)	3.75	2.78	6.60	nfa023b
	Total variation explained (%) by all QTL						7.8				6.60	
	Total variation explained (%) by genome-wide significant QTL										0.00	
TMH	qTMH-03-5	5						35.5 (13.8-82.6)	3.95	2.45	6.10	pps0273y
	qTMH-03-7	7						0.0 (0.0-23.6)	3.95	2.51	13.20	pps0766b
	Total variation explained (%) by all QTL					0.00					19.30	
	Total variation explained (%) by genome-wide significant QTL										0.00	
PC1	qPC1-03-1	1	28.2 (10.1-40.8)		3.79	9.50	pps0094y	25.3 (21.3-28.9)	3.75	2.85	5.70	pps0586b
	qPC1-03-2*	2	38.6 (21.9-45.0)		4.75	11.70	pps0113y	38.6 (36.7-40.7)	3.75	4.43	9.10	pps0113y
	qPC1-03-6*	6	19.9 (0.0-32.1)		4.62	12.60	pps0432a	19.9 (18.5-20.9)	3.75	4.22	9.70	pps0432a
	Total variation explained (%) by all QTL					33.80					24.50	
	Total variation explained (%) by genome-wide significant QTL										18.80	
PC2	qPC2-03-1*	1	17.3 (0.0-34.7)		3.63	9.30	pps0698a	17.3 (9.3-19.3)	3.75	4.07	7.80	pps0698a
	qPC2-03-2.1*	2	8.4 (0.0-25.0)		6.36	17.00	pps0154z	8.4 (8.4-19.3)	3.75	7.29	15.30	pps0154z
	qPC2-03-2.2*	2						20.6 (0.0-25.8)	3.75	3.74	12.80	pps0265a
	qPC2-03-4*	4	60.7 (44.8-62.9)		6.99	17.90	pps0356b	60.7 (59.5-61.2)	3.75	8.98	16.30	pps0356b

Table 6.5 (Cont…)

Trait	QTL	Linkage group	SIM Position (cM ±2LOD)	SIM LOD threshold	SIM Maximum LOD value	SIM Variation explained (%)	SIM Marker	MQM Position (cM ±2LOD)	MQM LOD threshold	MQM Maximum LOD value	MQM Variation explained (%)	MQM Marker
	Total variation explained (%) by all QTL					44.20					52.20	
	Total variation explained (%) by genome-wide significant QTL										39.40	
PC3	qPC3-03-4	4	94.6 (52.2-114.1)		3.07	10.50	pps0983a					
	qPC3-03-5	5	44.7 (35.5-59.1)		4.18	10.40	pps0073a	44.7 (42.0-87.3)	3.80	3.26	6.40	pps0073a
	qPC3-03-6.1*	6	21.7 (0.8-26.0)		5.53	15.60	nfa015b	21.7 (19.9-22.7)	3.80	4.6	11.60	nfa015b
	qPC3-03-6.2	6	40.4 (0.0-44.6)		4.72	11.90	pps0450a					
	Total variation explained (%) by all QTL					48.40					18.00	
	Total variation explained (%) by genome-wide significant QTL										11.60	

*genome wide significant QTL ($p \leq 0.05$)

Table 6.5: QTL identified by Simple interval mapping (SIM) and Multiple QTL mapping (MQM) for seed production and related traits assessed in the I×S perennial ryegrass population assessed in spaced-plants in the field in 2003/2004. SdYP: seed yield per plant; SdYH: seed yield per head; FH; florets per head; SH: spikelets per head; FS: florets per spikelet; SL: spike length; FSU; floret site utilisation; SOH: spread of heading; DH: days to heading; TSW: 1000 seed weight; PGH: plant growth habit; RT: reproductive tiller number; TMH: reproductive tillers with matured heads at time of harvest; PC1: principal component 1; PC2: principal component 2 and PC3: principal component 3. Name of a QTL consists of a trait followed by year of trait assessment and linkage group. LOD: logarithm of odds ratio.

Marker	Trait QTL	QTL Position (cM)	Genotype class means				parent
			ac	ad	bc	bd	
	qLER-03-1	21.3	1.304	1.217	1.168	1.223	I
pps0711c	qDW-03-1.1	21.3	0.507	0.461	0.453	0.503	I
	qLED-03-4.1	74.8	15.510	16.575	14.649	15.999	S
	qALf-03-4	74.8	13.645	14.802	12.894	13.821	S
pps0423y	qALg-03-4	74.8	12.923	13.892	12.319	12.959	S
	qLED-03-7	41.2	15.510	15.771	14.657	15.535	S
pps0777a	qDH-03-7	41.2	128.192	130.289	128.678	130.073	S
	qALg-03-6	19.9	12.965	11.708	12.415	11.852	S
	qSdYP-03-6	19.9	34.6397	38.7424	28.2651	31.1151	I
pps0432a	qSdYH-03-6	19.9	97.3077	105.42	78.6969	90.2373	I
	qTN-03-1	35	1.558	1.571	1.616	1.570	I
pps0136b	qDW-03-1.2	35	0.507	0.545	0.569	0.540	I
	qTN-03-6	40.4	1.558	1.517	1.551	1.498	S
pps0450a	qPI-03-6	40.4	6.077	5.970	6.060	5.928	S
	qLL-03-1	17.3	20.665	19.694	18.425	18.798	I
	qFH-03-1	17.3	226.246	248.225	238.051	242.684	S
pps0698a	qSH-03-1	17.3	26.4554	27.7204	27.6712	27.6961	S
	qLL-03-2	38.6	20.665	19.598	20.752	19.874	S
	qSdYP-03-2	38.6	34.4934	36.6898	30.4992	29.6663	I
	qFH-03-2	38.6	226.247	215.937	215.428	205.345	I
pps0113y	qPGH-03-2	38.6	4.44194	3.91914	3.98265	3.20635	S
	qLL-03-4.2	66.8	20.664	20.862	24.190	25.455	I
pps0495b	qDH-03-4	66.8	128.192	125.315	126.938	123.753	S
	qDW-03-6	38.1	0.507	0.480	0.512	0.455	S
pps0022x	qFH-03-6	38.1	226.246	224.414	207.961	214.586	I
	qDH-03-2.1	8.4	127.473	131.841	126.973	131.056	S
pps0154z	qSOH-03-2.1	8.4	5.68436	4.21488	5.61271	5.05219	S
	qFS-03-4	60.7	8.49881	8.76355	8.79161	9.23173	I
pps0356b	qPGH-03-4	60.7	4.44196	5.26297	4.09925	4.32602	I
	qALf-04-3	30.2	12.9445	12.9484	12.3911	12.0448	I
	qLED-04-3	30.2	15.5518	14.4916	14.2877	14.2072	I
pps0502	qTW-03-3.1	30.2	0.097	0.090	0.085	0.082	I
	qALf-04-4.1	64.2	12.944	13.691	12.654	13.262	S
pps0018	qLL-03-4.1	64.2	20.664	21.470	17.092	16.379	I
	qLED-04-2.2	54.9	15.552	15.725	14.155	15.802	S
	qPI-04-2	54.9	3.619	3.512	3.639	3.645	I
pps0400	*qDW-04-2.2*	54.2	1.1932	1.07714	1.18244	1.24462	S
pps0130	*qALg-04-4*	62.9	12.919	13.535	12.298	13.393	S
pps0495	*qLED-04-4*	66.8	15.552	16.315	15.010	16.031	S
pps0710	*qALg-04-3*	45.9	12.9178	13.1694	12.2737	12.3994	I
pps0022x	*qLER-04-6*	38.1	2.29143	2.1278	2.37264	2.43131	I
pps0213	qLER-03-3.1	73.8	1.304	1.524	1.165	1.469	S
pps0560	qLER-03-3.2	81.0	1.304	1.123	1.533	1.184	S
pps0521	qLER-03-7.1	31.4	1.304	0.985	1.315	0.491	S
pps0060	qLER-03-7.2	29.0	1.304	1.533	1.340	2.032	S
pps0210	qALf-03-6	25.2	13.645	12.682	13.361	12.645	S

Table 6.6 (Cont...)

Marker	Trait QTL	QTL Position (cM)	Genotype class means				parent
			ac	ad	bc	bd	
pps0251	qALg-03-1	0.0	12.923	13.716	12.822	12.379	I
pps0322	qLL-03-3.3	109.1	20.665	19.536	21.706	20.564	S
pps0736	qLL-03-7	35.5	20.665	19.662	20.424	19.276	S
pps0172	qSIL-03-2	68.5	2.272	1.976	2.538	2.561	I
pps0317	qSIL-03-4	85.6	2.279	3.056	2.365	3.062	S
pps0724	qTW-03-3.2	91.5	0.097	0.085	0.106	0.100	I
pps0202	qTW-03-4.1	57.4	0.097	0.098	0.107	0.103	I
pps0319	qFSU-03-1	24.9	0.246309	0.237652	0.276564	0.257648	I
pps0359	qFSU-03-5	46.2	0.24631	0.226668	0.210234	0.255946	I
Pps0197	qFSU-03-6	11.5	0.24631	0.296629	0.258611	0.28891	S
pps0122	qFS-03-2	43.2	8.49881	8.2755	8.20571	7.85502	I
pps0356	qDH-04-4	60.5	19.7438	18.4484	18.7979	17.4339	S
Pps0766	qDH-04-7	0.0	19.7438	20.2918	19.9015	21.3441	S
pps0419	qSL-03-3	56.2	20.4251	19.9711	18.9806	19.1257	I
pps0066	qSL-03-1	22.5	20.4251	20.4913	21.0501	20.2337	I
pps0385	qSL-03-5	29.8	20.4251	20.3483	19.9991	19.4581	I
pps0497	qTSW-03-2	49.4	1.73232	1.87588	1.81561	1.85941	S
pps0488	qTSW-03-3	42.3	1.73227	1.92362	1.74133	1.84721	S
pps0463	qTSW-03-6	18.5	1.73232	1.80126	1.66817	1.76497	S
pps0983	qSH-03-4-1	94.6	26.4554	27.2235	28.0167	28.5487	I
pps0892	qSH-03-6	28.7	26.4554	25.9445	24.8816	25.6219	I
pps0663	qSH-03-2	45.1	26.4554	25.6907	26.0229	25.2721	S
pps0377	qSdYH-03-5	55.1	95.932	93.749	79.805	92.833	I
pps0466	qSdYH-03-7.1	26.8	95.932	95.967	121.322	120.376	I
pps0447	qSdYH-03-7.2	37.8	95.932	102.783	76.197	69.564	I
pps0234	qSOH-03-2.2	65.8	5.68433	7.0366	6.78706	7.45714	S
pps0094	qSOH-03-1	28.2	6.26	5.036	4.283	4.948	I
pps0197	qSOH-03-6	11.5	6.26	5.785	6.765	7.003	I

Table 6.6: SSR markers, genotype class means and alleles associated with significant ($p<0.05$) QTL for herbage yield and seed yield and their related traits assessed in the I×S perennial ryegrass mapping population. Parental alleles were *a* and *b* for parent 'I', and *c* and *d* for parent 'S'. QTL for herbage production traits in spring are distinguished from those in autumn by italicisation. DW: herbage dry weight; ALf: leaf appearance interval; ALg: ligule appearance interval; LED: leaf elongation duration; LER: leaf elongation rate; LL: leaf lamina length; TN: tiller number; TW: tiller weight; PI: productivity index; SIL: stem internodes length; SdYP: seed yield per plant; SdYH: seed yield per head; FH; florets per head; SH: spikelets per head; FS: florets per spikelet; SL: spike length; FSU; floret site utilisation; SOH: spread of heading; DH: days to heading; TSW: 1000 seed weight and PGH: plant growth habit.

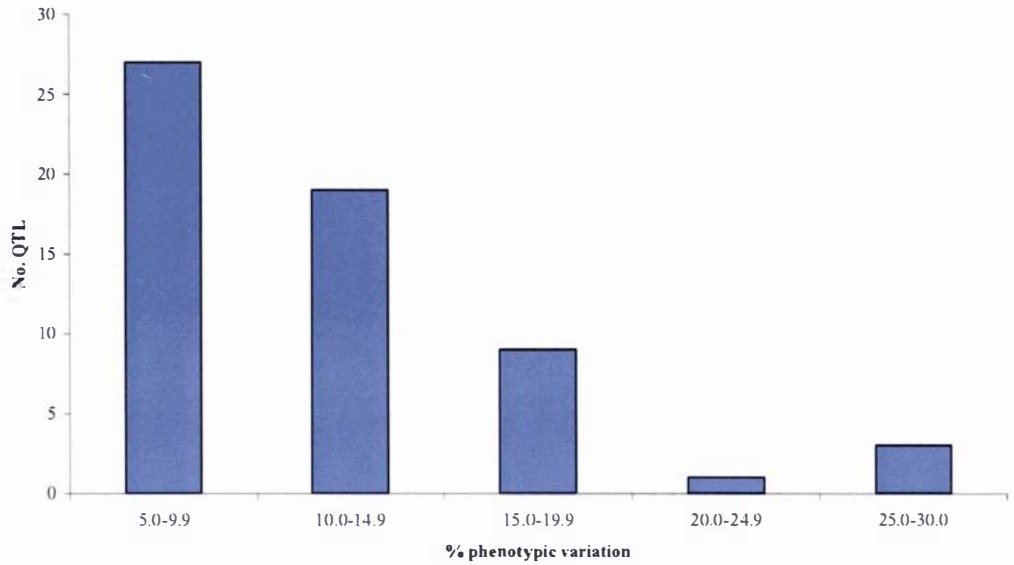


Figure 6.5: Percent phenotypic variation attributed to individual significant QTL for herbage and seed yield related traits in I×S perennial ryegrass population.

6.4 Discussion

6.4.1 Genetic map

A double pseudo-testcross strategy (Grattapaglia and Sederoff 1994) was used to develop a genetic linkage map for the I×S perennial ryegrass mapping population, using EST-SSR markers. This map represents the second map developed using this proprietary EST-SSR resource (Faville *et al.* 2004). The double pseudo-testcross method initially identifies individual polymorphism in each parent allowing the construction of two individual specific maps, and is a common approach used to map outcrossing plant species (Grattapaglia and Sederoff 1994; Porceddu *et al.* 2002; Shepherd *et al.* 2003; Carlier *et al.* 2004). Separate parental maps (constructed at a LOD 2.0 threshold) identified 99 parent 'I' and 100 parent 'S' loci, indicating a similar level of heterozygosity in both genotypes. An integrated, or 'consensus', map of length 582 cM was subsequently developed, expressly to facilitate QTL analysis (although either or both the parental and consensus maps may be used, with the individual analyses providing a degree of internal validation). Compared with the parental maps, the consensus map provides a greater degree of locus saturation, extending the

characterisation of polymorphic variation throughout the entire genome, which facilitates the location of QTL (Maliepaard *et al.* 1997). QTL discovery is facilitated if a linkage map contains a sufficient number of markers (>100 depending on species), spaced not more than 10 cM apart to reduce the likelihood of double cross-over occurring either side of the QTL (Darvasi *et al.* 1993; Hayward *et al.* 1994). The mean marker interval on this map is 3.6 cM, and pairwise distances between loci were all less than 10 cM, except for nine intervals, one on Lg 2, and two each on Lgs 3, 4, 5 and 7 that had intervals ranging from 11cM to 34 cM; these data confirm the suitability of this map for QTL analysis. There were very few unlinked markers, signifying the high quality of data used for constructing this map (Liu 1998).

There was segregation distortion at 21 marker loci with Lg 7 being particularly affected, as observed also by (Armstead *et al.* 2004). Segregation distortion can be caused by several factors including: (a) genotyping errors (Knox and Ellis 2002; Hackett and Broadfoot 2003), (b) the presence of a gene influencing embryo viability or seedling survival within a genomic region, resulting in selection against linked marker genotypes, or (c) the presence of self-incompatible genes, as a result of partial identity between the genotypes of the male and female parents at the S and Z loci (Jones *et al.* 2002). The clustering of distorted markers on Lg 7 is unlikely to be due to either genotyping error (confirmed by checking of genotypic data), or self-incompatibility loci, because the S and Z loci have been mapped on Lg 1 and Lg 2, respectively in ryegrass (Lewis *et al.* 1980; Thorogood and Hayward 1991). This suggests that the distorted region on Lg 7 may be associated with a gene influencing embryo viability or seedling survival, but this cannot be confirmed here.

Of the remaining loci demonstrating segregation distortion, there was no significant clustering detected, so it is possible that distortion at these loci was due to genotyping error (Knox and Ellis 2002; Hackett and Broadfoot. 2003). These markers were, however, retained on the map after checking that they had no altering effect upon marker order or map distances on their respective linkage groups.

6.4.2 Major traits identified for herbage production

Significant ($p \leq 0.05$) QTL for herbage yield (DW) were identified on Lg 1 and Lg 6 in autumn and on Lg 2 in spring (Tables 6.3 and 6.4; Figures 6.2 and 6.3). The DW QTL portrays an interesting association with its related traits on Lg 1. It co-located with QTL for LER and LL on one hand and with TN on the other hand. On Lg 2, one DW QTL co-located with QTL for TN and PI and another with QTL for LL and LER, while on Lg 6, it co-located with QTL for TN and PI (Table 6.3, Figure 6.2). Suggestive QTL for DW were also identified on Lg 2 (qDW-03-2.1 and qDW-03-2.2) and Lg 7 (qDW-03-7.1 and qDW-03-7.2) in autumn (Table 6.3). In spring, other suggestive QTL for DW (qDW-04-2.1) and TW (qTW-04-2) were co-located on the proximal end of Lg 2 (Table 6.4). This indicates that LER, LL, TN, TW and PI are important components for herbage production in perennial ryegrass.

Assessing the potential usefulness of the different QTL identified for increase in herbage production involves several considerations. Although DW QTL on Lg 6 had the largest PVE (13.4%), it is linked only to QTL for TN and PI, while that on Lg 1 (qDW-03-1.1) with PVE of 10.5% is linked to both LER and LL. It is likely that qDW-03-1.1 may be more useful for increased herbage production, because LER has been identified as a primary trait for productivity, and selection for high LER and long LL has been suggested to increase herbage production in perennial ryegrass (van Loo 1992; Bahmani *et al.* 2000). It has been previously understood that long LL is associated with increased leaf elongation duration (LED) and long leaf appearance interval (ALf) resulting in low TN per plant (Lemaire and Chapman, 1996). In the current study, LL correlated more strongly with LER, especially in spring, and did so with LED only in autumn. Additionally, the relationship between TN and LER was direct in spring but inverse in autumn (Table 4.6). This suggests that these relationships may be affected by season or environment. High LER had been associated with the production of long LL without necessarily reducing tiller number per plant (Bahmani *et al.* 2000).

TN and TW are both associated with increased productivity, but they have a negative relationship at a canopy level (Table 4.6) and are subject to size-density compensation (Chapman and Lemaire 1993), so that use of either alone in selection will not necessarily increase productivity. Selection for larger tillers is reported to be associated

with poor persistence, partly due to whole plant removal by grazing cattle (Thom *et al.* 1998). However, productivity index (PI) provides a theoretical basis for taking account of both TN and TW simultaneously, and provides a comparative estimate of leaf area index and productivity of a sward (Matthew *et al.* 1995; Hernandez Garay *et al.* 1999). In this study, PI was associated with increased DW, and QTL for PI and DW were mapped at similar positions on Lg 2 in spring and on Lg 6 in autumn, but the direction of allelic effect was the same in autumn and different in spring (Table 6.6).

QTL for herbage production traits in perennial ryegrass have also been reported by other researchers, but only for few traits; leaf lamina length QTL were identified on Lg 1, 2, 3, 4 and 7 (Yamada *et al.* 2004) and for tiller size on Lg 3 (Yamada *et al.* 2004), and there is a likelihood that these QTL are equivalent to some of the QTL identified in the current study.

6.4.3 Major traits for seed production

Important traits underpinning increased seed production were identified in this study (Chapter 5) as number of reproductive tillers (RT), especially those with matured heads (TMH) at time of harvest, seed yield per head (spike) (SdYH), florets per head (FH), florets per spikelet (FS), spikelet per head (SH), floret site utilization (FSU) and 1000 seed weight (TSW). All of these traits, except TMH, have been reported previously as being components of seed yield in ryegrass (Bugge 1987; White 1990; Elgersma 1990a; Marshall and Wilkins 2003). The proportion of RT that contains mature florets or seeds at time of harvest had been overlooked in seed production studies, but was identified in this study (Chapter 5) as a major contributor to plant seed yield (SdYP).

Significant QTL for SdYP were identified on Lg 2 and Lg 6, with that on Lg 6 accounting for the largest PVE (14%). SdYP QTL co-located with QTL for FH, SH, FS, PGH and PC1 on Lg 2, and with QTL for SdYH, TSW, FSU and PC1 on Lg 6. Traits for which QTL co-located with SdYP QTL, showed a positive correlation with SdYP (Table 5.4) indicating that these traits may be under similar genetic influence. SdYP QTL on Lg 6 may be very useful for increasing seed production in perennial ryegrass, because in addition to its accounting for the largest PVE, it is also linked to QTL for SdYH, FSU and TSW (Table 6.5). High yield of quality seed is the main goal

of a viable seed production system. Seed vigour is a major quality standard in the assessment of grass seed stock for planting, and seed size (TSW) is used as an important criterion to predict the quality of seed for planting. An increase in SdYH was associated with increase in FSU and TSW (Table 5.5), both of which are associated with seed quality. SdYH correlated strongly (0.74) with SdYP (Table 5.4), and their QTL were associated with the same allele (Table 6.6). SdYH has been identified by other authors as a key selection criterion for increasing seed yield (Bugge 1987; Marshall and Wilkins 2003). Results here therefore indicate that selecting for SdYP QTL on Lg 6 is selecting for increased production of quality seeds. Alleles for increased TSW and FSU were from the S parent while that for increased SdYP was from the I parent, and this indicates the possibility of developing a cultivar with increased production of quality seeds. A mapping analysis of a seed quality test, e.g. standard germination and seedling vigour, or accelerated ageing tests, would assess whether there is any genetic relationship between TSW and seed vigour.

Although reproductive tiller number was identified in this study as the most critical trait in seed production, there was no significant QTL discovered for RT and TMH. The reason for not discovering QTL for RT and TMH is not clear, because QTL discovery is dependent on the heritability of a trait and the occurrence of adequate variation in the population for the trait. Heritability estimates were moderately high for these traits (Table 5.3, chapter 5), and their data fit a normal distribution (Figure 5.1, chapter 5), but the parents did not differ significantly for them (Table 5.3, chapter 5). However, elsewhere in this dataset QTL were identified for traits where heritability estimates were low and the parents were not significantly different for the trait (e.g. FS (0.33) and SL (0.39)) or where heritability estimates were high and the parents were not significantly different for the trait (e.g., LL (0.61), TN (0.74) and DW (0.62)). It is possible that QTL governing these traits occur in a region not covered by the genetic linkage map used here for QTL analysis. Alternatively, the complexity of the RT and TMH traits (integrating tiller number, proportion of tillers developing spikes and timing of this process) could mean that many loci are likely to be involved in their genetic control, and in this data set no one locus assumed statistical significance. There were suggestive QTL, for instance, qRT-03-2, qTMH-03-5 and qTMH-03-7 (Table 6.5). Finally, epistasis may also be a factor in the genetics underlying the phenotypic variation

observed in the population, but this was not assessed in the current study. The elucidation of epistatic effects, or the use of a different population structure and/or a population of a larger size may enable the identification of significant QTL for these traits.

6.4.4 Heading date

Head emergence in grass swards is associated with a decrease in digestibility of herbage (Parsons 1988), and modern cultivars are carefully characterised for heading date (DH). Since this trait may be associated with FSU and seed yield (Anslow 1963), independent QTL for DH will be valuable if they enable selection for seed production within specific heading date groups. QTL for DH were identified on Lg 2, 4 and 7 across two consecutive years, and major QTL on Lg 2 and 4 mapped to similar positions in both years (Table 6.5). In each year, DH QTL accounted for 49% total PVE. QTL for DH on Lg 4 and Lg 7 have been reported elsewhere (Armstead *et al.* 2004; Yamada *et al.* 2004). Phenotypic assessment (Chapter 5) associated the S parent with early heading and the I parent with late heading, but the QTL analysis indicated that the QTL alleles causing separation in DH appear to come from the S parent.

6.4.5 Comparison of PCA and QTL analysis

The results of QTL analysis and PCA indicated a high degree of similarity in identifying traits that are important for increase in DW and increase in SdYP in perennial ryegrass. For instance, PC3 of autumn herbage production data (Table 4.7) identified LER, TN, LL, TW and PI as important for increase in DW, and QTL for PC3 scores were identified at similar positions with QTL for DW, LER, TN, LL and TW (Table 6.3). For the seed production traits, PC1 of Table 5.5 identified SdYH, RT, TMH, FH, and FS as important traits for increase in SdYP, and PC1 QTL were also identified at similar position with QTL for SdYP, SdYH, FH, SH and FS (Table 6.5). Moreover, PC2 of Table 5.5 was associated with early heading plants and its QTL co-located with heading date (DH) QTL on Lg 2 and 4 (Tables 5.6 and 6.5). This shows that PCA and QTL results are coherent.

The origin of alleles affecting some of the traits assessed in this study, as derived from genotypic subclass means by MQM analysis in MapQTL, indicates that favourable QTL

alleles can be derived from the parent that showed poor phenotypic performance for the trait. For instance, the S parent showed increased SdYP, SdYH and TSW while the I parent showed increased TN and late DH, but alleles for high SdYP, SdYH and TSW appear to come from the I parent while those for increased TN and late DH were from the S parent. This means that for this population, where the two parents are from cultivars that differ considerably in terms of DH, the QTL identified in this study of an F_1 population are most likely not those that distinguish the parents. In other words, in the F_1 progeny, the loci that distinguish the parents do not segregate and therefore their QTL were not identified in this population. By using an advanced generation population, such as an F_2 type population, the alleles would segregate and there would be an opportunity to successfully identify a QTL associated with this putative major gene.

6.4.6 Nomination of candidate SSR markers associated with herbage and seed production traits

The usefulness of a marker for MAS depends in part on the amount of phenotypic variation the marker explains for the trait in the population. It has been recommended (Li *et al.* 2001) that phenotypic selection for a trait will be more effective if alleles at the major QTL are selected rather than those at minor QTL, because minor QTL have small effects on the phenotype. Fifty-two EST-SSR markers associated with significant QTL for herbage and seed production traits and their related traits in perennial ryegrass were identified, and fifteen were associated with QTL affecting more than one trait (Table 6.6). Markers linked to QTL affecting more than one trait (co-located QTL) may be used to identify plants with an allele or a suite of alleles for several economic traits simultaneously. However, the use of such markers in MAS requires some caution as there may be a possibility of selecting a plant with an undesirable trait. For instance, if a marker is linked to a co-located QTL (Table 6.6) and the traits are associated with the same QTL allele, (e.g. pps0711 linked to QTL for DW and LER and both affected by an allele from the I parent) it would mean that the QTL are closely linked and they can be inherited together, and as such, marker pps0711 may be used to select a plant with increased DW and high LER.

If co-located QTL are associated with different alleles (e.g. marker pps0022 linked to QTL for DW and FH with increased DW associated with allele from one parent and

increased FH associated with allele from the other parent), it would suggest that the expression of one trait may affect the expression of the other, as in the case of dominance versus recessive. If that is the case, marker pps0022 will not select plants for both increased DW and FH, rather, selecting for increased DW will mean selecting against increased FH and verse versa. The identification of a QTL allele that is associated with a DNA marker forms the basis of MAS, and currently, MAS based on single QTL allele is perhaps the most powerful approach that uses DNA markers effectively (Ribaut and Hoisington 1998). The number of alleles of DNA markers used in this study varied from two to four (Table 6.1), and the identification of a marker allele that is associated with QTL allele for trait performance is also important so that MAS will be based on the presence of specific alleles of a marker rather than just the marker. However, gene interactions (e.g. epistasis) and the environment may affect trait expression. Validation of DNA marker-trait associations will confirm the marker allele associated with the QTL allele underpinning trait performance, so that the marker may be useful to identify plants that carry an allele for desired trait performance.

Markers identified for herbage yield and related traits were: DW (pps0711, pps0136, pps0022 and pps0400); LER (pps0213, pps0560, pps0321, pps0060 and pps0711); TN (pps0450 and pps0136); TW (pps0724 and pps0202); LL (pps0698, pps0113, pps0495, pps0018, pps0322 and pps0736). Markers pps0136, pps0450, pps0711 and pps0777 may be used to respectively select plants with combined ability for increased DW and TN, increased TN and PI, increased DW and LER and long LED and late DH because each pair of traits is associated with the same allele (Table 6.6).

Markers identified for seed yield and component traits were: SdYP (pps0432 and pps0113); SdYH (pps0432, pps0377, pps0466 and pps0447); SH (pps0892, pps0983, pps0698 and pps0663); FS (pps0122 and pps0356); FSU (pps0359, pps0197 and pps0319); and TSW (pps0463, pps0497 and pps0488). Marker pps0432 may be important for selecting a plant for increased SdYP as a result of increased SdYH because the two traits are associated with the same allele (Table 6.6).

Heading date markers identified were: pp0777, pps0495, pps0154, pps0766 and pps0356. Markers associated with QTL for other traits assessed in this study are shown in Table 6.6.

It is recommended that all markers identified in this study to be linked to significant QTL be validated in other populations of perennial ryegrass before they are used in MAS.

6.5 Conclusion

An EST-SSR-based genetic linkage map spanning 582cM was used successfully for QTL analysis of traits associated with herbage and seed production, and may be used to further characterise genes involved in perennial ryegrass productivity. Reports on QTL discovery for herbage and seed production and their related traits in perennial ryegrass are few, and this limits the possibilities for comparing results obtained from this study with those of other researchers. However, in instances where QTL discovery had been reported in this species, the results are largely in agreement with findings in this study. QTL were identified for herbage yield and its related traits, and qDW-03-1.1 may be more useful for increased herbage production, because it is associated with high LER and long LL, which are major components for increased DW. For increasing seed production, qSdYP-03-6 may be very useful, because in addition to its accounting for the largest PVE, it is also linked to QTL for SdYH, FSU and TSW. QTL were identified for vegetative tiller number (TN), but not for reproductive tiller number, indicating the complex nature of reproductive tiller production in perennial ryegrass. Markers were identified that may be potentially useful in MAS after they are validated to improve forage and seed production in perennial ryegrass.

7

Validation of the association of SSR markers to leaf appearance interval and leaf lamina length in an independent perennial ryegrass population

7.1 Introduction

The ultimate aim of QTL analysis is to identify molecular markers linked to QTL for a trait of interest and to apply those markers in MAS strategies within breeding programmes. Before using molecular markers in MAS, Dudley (1993) suggested first identifying associations between marker alleles and QTL, then using those associations to develop a selection scheme. The association between a QTL effect and a marker allele identified in a population can be lost in subsequent generations if further crossovers (during meiosis) occur between the marker and the QTL, resulting in the two becoming separated. In order to confirm the genetic linkage between a marker allele and QTL allele, a validation population needs to have undergone a series of meioses since its constitution so that any linkage between QTL allele and a marker allele is a true linkage. Validation refers to (a) experimental confirmation that the marker in question is associated with the trait of interest, and (b) identification of marker alleles associated with desirable trait performance, in a population other than the mapping population. A population derived from a different genetic background of the same or a related species can be ideal for trait (QTL)-marker association validation (Lander and Kruglyak 1995; Brown *et al.* 2003) because it has developed independently of the QTL discovery population.

Leaf appearance interval (ALf) and leaf lamina length (LL) are important traits determining sward behaviour in perennial ryegrass (Chapman and Lemaire 1993; Bahmani *et al.* 2000). Independently located QTL with large effects were identified for ALf (on Lg 4) and LL (on Lg 1) by SIM QTL analysis when the full sibling I×S perennial ryegrass mapping population was assessed in autumn 2003 for herbage yield and related traits (Chapter 4), and these QTL were later confirmed by MQM analysis in 2005 (Table 6.3, Chapter 6). In the intervening period, the SIM information served as the basis for undertaking an assessment of phenotypic performance for these traits in

relation to the QTL-linked markers in perennial ryegrass, as a validation of their utility prior to the use of the markers in MAS breeding.

7.2 Objectives

The objective of this experiment (Experiment 4) was to validate in another population of perennial ryegrass the association between trait performance and SSR markers that were linked to QTL for the ALf and LL traits in the I×S mapping population.

7. 3 Materials and methods

7.3.1 Plant material and experimental design

A population of half-sib families (n=100) with two plants per family was derived by pair crossing the perennial ryegrass cultivars Grasslands Samson and Grasslands Impact, and designated population RC1. Seeds were harvested and the resulting seedling population was grown in an isolation house to freely inter-pollinate. Seeds were harvested from each plant, and two plants (half-sibs) were further selected from the seeds harvested from each mother plant. The plants were first established in a spaced plant experiment in a field. For the glasshouse experiment described here, a section of each plant was dug out and divided into three clones, and each clone, consisting of three tillers was planted in 'Planter' bag (64 x 64 x 150 mm) containing commercial potting mix. The experiment was conducted side by side with Experiment 5 (Chapter 4) in a glasshouse in spring 2004. The experimental design used was a randomised complete block of three replications with each block containing one clonal replicate of each of the 200 plants. The experiment was designated as Experiment 4.

7.3.2 Phenotype data collection and analysis

Data were collected from RC1 for tiller number (TN) at the start and end of experiment, tiller weight (TW), ALf, ligule appearance interval (ALg), leaf elongation rate (LER), leaf elongation duration (LED), LL, herbage dry weight (DW) and productivity index (PI) using methods described in Experiment 2 (Chapter 3). Those data for traits other than the targeted ALf and LL were collected as a means of undertaking similar trait: marker validations for those traits at some time in the future. Data were analysed for

differences between families, between plants within a family, and between clones using analysis of variance in GenStat (Genstat 2005).

7.3.3 Genotype data

DNA from the 200 RC1 plants was extracted and purified using methods described in Chapter 6, section 6.2.2.1. EST-SSR markers linked to QTL for LL (Lg 1) and ALf, (Lg 4) in the 1×S population were selected on the basis of underlying or closely flanking the SIM QTL interval. A total of seven markers, three for the ALf QTL and four for the LL QTL (Table 7.1) were assayed for association with these traits in the RC1 population using the following procedures: (1) DNA from each of the 200 plants was screened against the seven markers and the presence (1) or absence (0) of the all marker peaks in each plant was recorded. Unreliable marker peaks were considered as missing data. (2) Tests for association between marker alleles and mean LL and ALf values (n=3) was undertaken using binomial logistic regression analyses implemented in GenStat version 8.1 (Genstat 2005), for which significance was declared at $p<0.01$. Criteria set for identifying potentially useful marker alleles associated with a trait were: (a) allele occurs at between 30% and 70% frequency in the RC1 population, (b) allele presence is associated with higher trait performance than allele absence, (c) performance difference for a trait authenticated at < 1% error rate.

Trait	QTL (LOD, %PVE)	EST-SSRs tested	Number of alleles
ALf	qALf-03-4 (10.4, 24.0)	pps0146	2
		pps0423	4
		pps0495	3
LL	qLL-03-1(8.2, 22.0)	pps0066	2
		pps0030	4
		pps0698	4
		pps0711	2
	Total	7	21

Table 7.1: Allele numbers identified for QTL-associated EST-SSR markers tested for association with phenotypic performance for traits ALf and LL in perennial ryegrass validation population RC1. Logarithm of odds (LOD) score and phenotypic variation explained (%PVE) for the ALf and LL QTL associated with these markers in mapping population 1×S are also shown.

7.4 Results and discussion

7.4.1 Phenotype assessment

There was significant ($p \leq 0.01$) variation in the RC1 population for all the traits assessed. Families were similar for most of the traits, but significantly different ($p \leq 0.05$) only for LER and LL, and marginally ($p=0.06$) in TN (Table 7.2). Significant differences between plants in a family were found ($p \leq 0.01$) in all traits except in LER, TW and DW. There was a significant ($p \leq 0.05$) replicate effect for all traits except for LL, TN, TW and DM indicating the difficulty of synchronising the environment even under glasshouse conditions.

Trait	Population mean (mean ± SEM)	P-value		
		Family	Plants/family	Reps
ALf	9.86 ± 0.07	0.44 ^{ns}	0.001 **	0.004*
ALg	10.66 ± 0.93	0.15 ^{ns}	<0.001 **	0.02*
LED	12.45 ± 1.01	0.53 ^{ns}	<0.001**	0.004*
LER	2.48 ± 0.30	0.03 *	0.08 ^{ns}	0.04*
LL	28.61 ± 2.83	0.03 *	<0.001 **	0.76 ^{ns}
TN	0.65 ± 0.08	0.06 ^{ns}	<0.001**	0.19 ^{ns}
TW	0.05 ± 0.10	0.39 ^{ns}	0.53 ^{ns}	0.51 ^{ns}
DW	1.77 ± 1.13	0.33 ^{ns}	0.71 ^{ns}	0.22 ^{ns}
PI	3.53 ± 0.11	0.11 ^{ns}	<0.001 **	<0.001**

Table 7.2: Population (n = 100 half-sib families) mean of trait, standard error of means and p-value of family, plant within family and replicate effects of perennial ryegrass QTL validation population of RC1 assessed in a glasshouse in spring 2004. ALf = leaf appearance interval (days); ALg = ligule appearance interval (days); LER = leaf elongation rate (cm/day); LED = leaf elongation duration (days); LL = leaf lamina length (cm); TN = tiller number; TW = tiller weight (g); DW = herbage dry weight (g); PI = productivity index.

7.4.2 QTL-marker validation of genetic effects

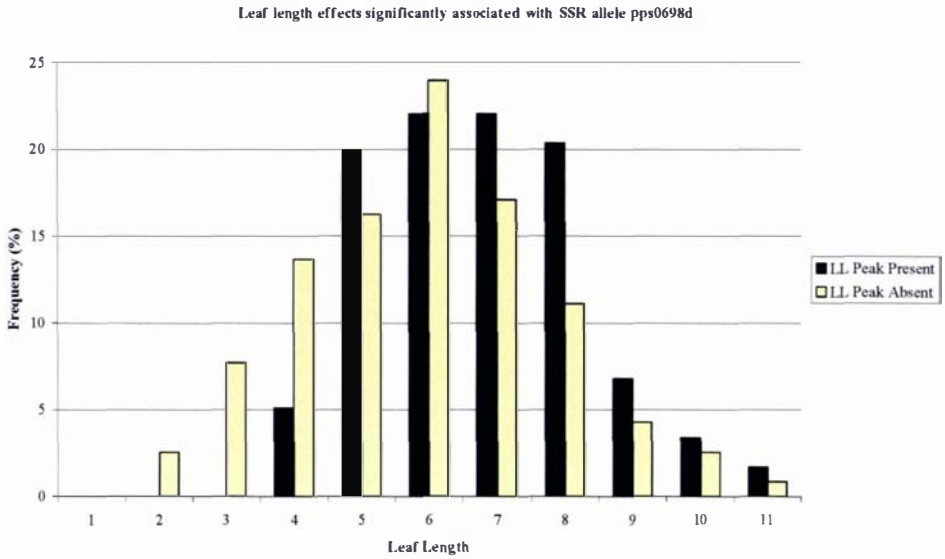
Associations identified in the mapping population between SSR markers and QTL for ALf and LL were tested in this population of perennial ryegrass. A total of 21 alleles were detected for all seven markers with number of alleles per marker ranging from two to four (Table 7.1). Seven alleles were eliminated because they had missing data greater than 20 % and eight further alleles were eliminated because they were rare in the population and the statistical analysis used could not be reliably applied. Four alleles were eliminated from the remaining six because they were not significantly ($p \leq 0.01$) associated with the trait performance. Of the remaining two alleles, allele 'd' (145 bp) of marker pps0698 was significantly ($p \leq 0.01$) associated with increasing LL, and allele 'c' (190bp) of pps0495 was significantly ($p \leq 0.01$) associated with decreasing ALf. In the RC1 population, plants with these marker alleles exhibited on average a 5.9 % increase and 4.6 % decrease in LL and ALf respectively (Table 7.3, Figure 7.1).

However, this test needs to be extended to populations derived from a wider genetic background (involving other perennial ryegrass cultivars) for a robust confirmation of marker-trait association, and for comparison of the percent change in trait performance. Analysis of MQM was later performed, and it also identified the same markers as did SIM to be associated with LL and ALf (Table 6.3, chapter 6), but in addition, the former identified other significant QTL on other linkage groups to be associated with these traits. Markers associated with those other QTL would also be worth testing together with pps0698 and pps0495 in order to identify the marker that has the strongest association with the trait.

Allele frequency			Marker	Trait	Percent Change	Performance Mean Value		Error Probability
1	0	missing				Marker Absent	Marker Present	
66	80	17	pps0698	LL	5.9 %	27.91	29.56	0.0036
73	99	18	pps0495	ALf	4.6 %	10.07	9.63	0.0095

Table 7.3: Association between DNA marker profile and plant performance for LL and ALf in the RC1 perennial ryegrass population.

(a)



(b)

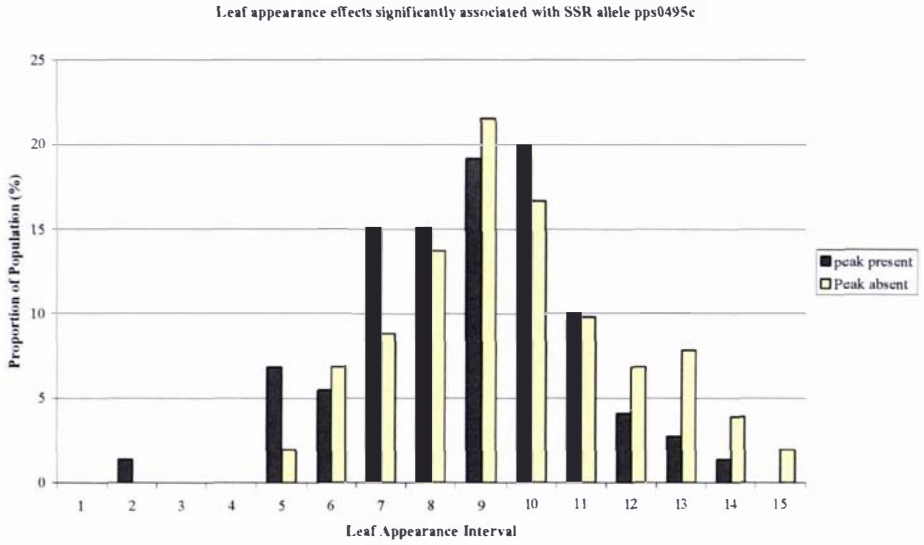


Figure 7.1: (a) Marker associated with LL increase, and (b) marker associated with ALf decrease among individuals from the perennial ryegrass population of half-sib families in Experiment 4. In Fig. 7.1a the dark bars represent plants carrying allele 'd' of the marker pps0698, while the yellow bars indicate plants without the allele. In Fig. 7.1b the dark bars represent performance of plants carrying allele 'c' of the marker pps0495, and the yellow bars indicate distribution of ALf values for the population without the allele.

7.5 Conclusion

Alleles at markers loci pps0495 and pps0698 were shown to be associated with phenotypic performance for traits ALf and LL, providing validation of the QTL identified in the population I×S, and highlighting the potential of these markers for selection for enhanced performance of ALf and LL in perennial ryegrass breeding populations.

8

General Discussion

8.1 Development of molecular selection tools for perennial ryegrass forage improvement

The development of a cultivar for increased productivity is a key objective in forage improvement (Wilkins and Humphreys 2003). MAS technology may be potentially useful in forage breeding, and successes have been reported in relation to selection for nitrogen use efficiency (Van Loo *et al.* 2003) and nutritional value (Humphreys and Turner 2001). This technique improves selection efficiency and allows desirable plants to be selected even before they are field tested, and it is environmentally friendly. The successful development of MAS tools involves the identification of trait to be improved, QTL discovery for the trait, identification of DNA marker(s) linked to the QTL and the validation of marker-trait association.

8.2 QTL of major traits for herbage and seed production

Phenotypic assessment involving herbage and seed yield and related traits identified major component/related traits for increased herbage production (DW) as LER, LL, TN and TW, and those for increased seed production (SdYP) as RT, TMH, SdYH, FSU, FH, SH, FS and TSW. Yield is a complex trait, and the identification of its component traits, and breeding for individual component traits has been proposed to enhance yield (Donald 1968; Sparnaaij and Bos 1993). This is because component traits analysis dissects complex traits into various components that may be analysed as single gene or oligogenic traits, and that instead of looking for QTL for yield, the determination of QTL for yield components should provide more useful information (Backes *et al.* 1995; Yin *et al.* 2002). This study discovered QTL for DW and SdYP, and all their component traits except for RT and TMH. QTL associated markers may be used in MAS to improve forage and seed production either through the use of markers associated with QTL for the main traits (DW and SdYP) or those markers associated with individual component traits.

If selection for increased herbage production is based on QTL associated with DW, two QTL, one on Lg 1 and the other on Lg 6 may be useful. QTL on Lg 6 had the larger PVE (13.4%) and appeared to be linked to QTL for TN and PI, while the QTL on Lg 1 (PVE of 10.5%) was linked to LER and LL. However, although QTL on Lg 6 had a bigger PVE, Lg 1 QTL may be more useful because it is associated with LER and LL, and selection for high LER has been suggested to increase herbage production in perennial ryegrass (Bahmani *et al.* 2000).

If selection for increase in seed production is based on QTL associated with SdYP, two QTL, one on Lg 2 and the other on Lg 6 may be useful. The QTL on Lg 6 had a larger PVE (14.0%) and was linked to QTL for SdYH, FSU and TSW, while the QTL on Lg 2 had PVE of 7.4% and was linked to FH, SH and FS. SdYP QTL on Lg 6 is not only the major QTL but it is also associated with increased seed quality (TSW) as well as increased FSU and seed yield per head (SdYH), and selection for increased SdYH had been suggested to increase seed production in ryegrass (Bugge 1987; Marshall and Wilkins 2003).

Markers from these QTL (including, pps0711 for DW QTL on Lg 1, pps0022 for DW QTL on Lg 6, pps0113 for SdYP QTL on Lg 2 and pps0432 for SdYP QTL on Lg 6) represent robust candidates for MAS for the improvement of herbage and seed production in perennial ryegrass.

8.3 Combined ability for seed and herbage production

The development of a forage grass cultivar that has a combined ability for increased seed and herbage production is uncommon because these two traits are previously believed to be negatively correlated (van Wijk 1980; Bugge 1987). In this study, no significant ($p<0.05$) correlation was observed between DW and SdYP, but the important traits identified for increased production of DW correlated negatively with those identified for increased SdYP. Co-location of suggestive QTL for SdYP and DW was identified within 7cM of each other on Lg 1 indicating that the two traits might be under similar genetic control. In this study, alleles associated with QTL for increased DW and increased SdYP generally appeared to come from different parents, which indicates a possibility of breeding a cultivar with combined ability for herbage and seed production.

However, if selection for increased seed yield is based on Lg 6 QTL which also co-located with QTL for SdYH, FSU, TSW and ALf, it may be possible to identify a plant with reasonable seed production ability without necessarily reducing herbage production because such a plant may produce fewer reproductive tillers with large seed heads (see PC3 Table 5.5) and more vegetative tillers for herbage production.

8.4 Environmental effects on QTL

There was significant genotype x environment effect on QTL discovery which suggested that gene expression of traits was affected by environment. The number, genomic location and magnitude of effects of QTL varied with season or year. For instance, LER was affected by five QTL in autumn, but only by one in spring (Tables 6.3 and 6.4). Amongst those QTL discovered for LER in autumn but not discovered in spring was a major QTL on Lg 3 with a high LOD of 7.6 and explaining 27.4 % of the total variation for LER in the mapping population. This indicates that the expression and number of genes involved in regulating a quantitative trait is affected by the environment. In other instances, however, a particular gene contributing to the regulation of a trait appeared to be significant irrespective of season, but with a variation in the magnitude of its contribution, for example, QTL for ALg at 19.9 cM on Lg 6 (Tables 6.3 and 6.4) and QTL for DH at 8.4 cM on Lg 1 (Table 6.5). QTL were identified for all the traits in autumn, but there were none for LL, TN, TW and PI in spring, although there were suggestive QTL for these traits. Those QTL that were stable across environments will be very useful in MAS for selecting genotypes with plastic performance irrespective of season or year. The majority of the QTL were environmentally sensitive which may be used in MAS to select genotypes adapted to a specific environment (Asins 2002). However, the biological basis of these QTL needs to be confirmed through verification in populations of different genetic backgrounds, and the environmental impact on QTL expression also requires further study of the current mapping population.

8.5 QTL validation

Validation of QTL is a pre-requisite for MAS in order to confirm (a) the existence of the QTL irrespective of environment and plant population, (b) the true linkage between a QTL allele and the QTL associated DNA marker, and (c) the association between the

marker allele and the trait performance. Again, a single segregating population provides only partial information, and a more complete picture only comes through analysing several populations, allowing for QTL diversity to show up. QTL discovered for a subset of the traits assessed in this study were validated in several ways below:

8.5.1 Validation in another population

EST-SSR markers that were identified to be linked to QTL for leaf appearance interval and leaf length in the I×S mapping population were validated in a population of several half-sibling families of perennial ryegrass (Chapter 7). Markers pps0698 and pps0495 were confirmed to be linked to leaf lamina length and leaf appearance interval respectively, and their alleles, 'd' of marker pps0698, and 'c' of pps0495 were associated with 6% and 5% increase in plant performance respectively. Markers associated with QTL for traits of interest are required to be validated using procedures described in chapter seven or by other means to confirm their linkage with the traits before implementation in MAS.

8.5.2 Validation by comparative study

8.5.2.1 Other studies with ryegrass

QTL discovered for several traits in this study have also been identified by other researchers. For instance, some of the QTL identified in this study for tiller number (Lg 1), plant type (Lgs 4 and 7), spikelets per head (Lg 1), spike length (Lgs 1, 3 and 5) and heading date (Lg 4) have been previously identified (Yamada *et al.* 2004). Alignment of maps used in both studies to a sufficient resolution, for example by marker exchange using markers associated with QTL in the respective studies, would allow more stringent comparison of the relative chromosomal location of genes influencing these traits and the markers associated with those loci would be useful in MAS for perennial ryegrass improvement.

8.5.2.2 Other related species

Conservation of genetic synteny exists among related grass species which allows the transfer of information across those species (Devos and Gale 2000), and may be used to validate genetic information. Comparative studies using ryegrass and other related species have confirmed the existence and the genomic location of some of the QTL reported here. For instance, Lgs 4 and 7 where QTL for heading date were discovered,

are syntenic to chromosomal regions affecting this trait in other grass species including rice, barley and meadow fescue (Armstead *et al.* 2004; Armstead *et al.* 2005; Ergon *et al.* 2006). The possibility of identifying DNA markers that are linked to those syntenic regions across related grass species will facilitate MAS in those species.

8.6 Conclusion

This study has established the fundamentals for MAS development in perennial ryegrass. A perennial ryegrass genetic linkage map was constructed with EST-SSR markers for QTL analysis, and may be used to characterise genes involved in perennial ryegrass production and other related species. Component traits with major effects on target traits for herbage and seed production were identified, and QTL were discovered for all investigated traits except for reproductive tiller production. Markers were identified that may be used in MAS for improvement in herbage and seed production in perennial ryegrass. Two markers, pps0495 and pps0698 were validated and confirmed to be potentially useful for selecting for fast leaf appearance and long leaf length in perennial ryegrass. There was a significant GxE effect associated with QTL discovery, and plant growth performances were different between autumn and spring.

8.7 Implications and future research

This study not only has commercial implications, through the identification of markers for use in MAS, but also provides a basis for improved understanding of the genetic basis, and inter-relationships, among vegetative as well as reproductive traits in perennial ryegrass. However, to better understand these processes, future work addressing the following observations would be necessary:

1. Co-located QTL were identified for several traits, and the effect of pleiotropy and the mode of gene interactions (e.g. epistasis) among QTL requires investigation.

2. In most cases multiple QTL (loci) were associated with a trait, and it can be difficult to decide which marker to select for use in MAS. The following options are worth considering in order to decide which of the markers may be most useful: (i) select the marker linked to the QTL that has the highest LOD score or the largest PVE and, (ii)

screen the markers in another population to identify the one with strongest association with the trait. I suggest the second option be carried out.

3. All QTL markers to be validated across populations and environments for confirmation of association with trait performance.

4. Results reported here are based on glasshouse conditions for herbage production assessment and on spaced plant for seed production assessment. Future research should include application of these results to grazed pasture conditions.

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Appendices

Appendix 4.1 Normality Test for herbage production traits

Experiment 2
Critical values of test statistics (marginal tests)

Test statistic	15%	10%	5%	2.5%	1%
Anderson-Darling	0.576	0.656	0.787	0.918	1.092
Cramer-von Mises	0.091	0.104	0.126	0.148	0.178
Watson	0.085	0.096	0.116	0.136	0.163

Stem internode length

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2770	0.0397	0.0360

Significance $p>0.15$

Herbage dry weight

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.2926	0.2068	0.1852

$p<0.01$ strong evidence of non-normality

Tiller number

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	4.3922	0.8134	0.6720

$p<0.01$ strong evidence of non-normality

Leaf appearance interval

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.9019	0.1397	0.1058

p<0.05 evidence of non-normality

Ligule appearance interval

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.3979	0.0693	0.0603

Significance p>0.01

Leaf lamina length

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.5609	0.0818	0.0667

Significance p>0.01

Leaf elongation duration

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.0479	0.1808	0.1445

p<0.01 strong evidence of non-normality

Leaf elongation rate

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.4789	0.1821	0.1536

p<0.01 strong evidence of non-normality

Tiller weight

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2694	0.0309	0.0301

Significance p>0.01

Experiment 5

Critical values of test statistics (marginal tests)

Test statistic	15%	10%	5%	2.5%	1%
Anderson-Darling	0.576	0.656	0.787	0.918	1.092
Cramer-von Mises	0.091	0.104	0.126	0.148	0.178
Watson	0.085	0.096	0.116	0.136	0.163

Productivity index

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	2.2624	0.3819	0.3025

$p<0.01$ strong evidence of non-normality

Tiller number

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.3182	0.1803	0.1408

$p<0.03$ evidence of non-normality

Herbage dry weight

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2237	0.0308	0.0270

Significance $p>0.01$

Leaf lamina length

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.7040	0.1360	0.1360

$p<0.01$ strong evidence of non-normality

Tiller weight

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.5628	0.0789	0.0596

Significance p>0.01

Leaf elongation duration

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.7707	0.1257	0.1055

p<0.01 strong evidence of non-normality

Leaf appearance interval

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.6738	0.1104	0.0917

Significance p>0.01

Leaf elongation rate

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.4154	0.0706	0.0648

Significance p>0.01

Appendix 4.2 Principal component analysis of herbage production traits

4.2.1 PCA for Autumn Experiment

Principal Component Analysis: LER, LED, ALg, LL, ALf, TN, SIL, DW, TW, PI
Eigenanalysis of the Correlation Matrix

Eigenvalue	3.5745	2.2600	1.7194	1.0833	0.8203	0.3110	0.1296	0.0755
Proportion	0.357	0.226	0.172	0.108	0.082	0.031	0.013	0.008
Cumulative	0.357	0.583	0.755	0.864	0.946	0.977	0.990	0.997

Eigenvalue	0.0213	0.0050
Proportion	0.002	0.001
Cumulative	0.999	1.000

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
LER	-0.300	0.111	-0.493	-0.268	0.380	-0.040	0.301	0.587
LED	0.382	0.329	0.100	0.049	-0.032	-0.812	0.260	0.071
Alg	0.363	0.369	0.133	-0.236	-0.014	0.506	0.615	-0.153
LL	-0.038	0.488	-0.397	-0.272	0.274	-0.064	-0.369	-0.557
Alf	0.393	0.382	0.134	-0.061	-0.064	0.224	-0.561	0.555
TN	0.412	-0.327	-0.283	-0.071	0.065	0.012	-0.029	-0.068
SIL	0.132	0.080	0.066	0.697	0.684	0.126	0.034	-0.025
DW	0.175	0.052	-0.578	0.390	-0.407	0.094	0.076	0.044
TW	-0.309	0.387	-0.143	0.386	-0.370	0.057	0.058	-0.015
PI	0.405	-0.305	-0.339	-0.027	0.015	0.010	-0.033	-0.038

Variable	PC9	PC10
LER	-0.047	-0.013
LED	-0.010	-0.004
Alg	-0.011	0.014
LL	0.052	0.005
Alf	-0.031	-0.012
TN	-0.367	-0.703
SIL	0.008	0.005
DW	0.544	-0.057
TW	0.664	0.030
PI	-0.351	0.708

4.2.2 Extended PCA for Autumn Experiment

Eigenvalue	3.2285	2.1292	1.2722	1.0667	0.9153	0.8475
Proportion	0.269	0.177	0.106	0.089	0.076	0.071
Cumulative	0.269	0.446	0.552	0.641	0.718	0.788

Eigenvalue	0.7689	0.6560	0.3799	0.3272	0.2252	0.1835
Proportion	0.064	0.055	0.032	0.027	0.019	0.015
Cumulative	0.852	0.907	0.939	0.966	0.985	1.000

Variable	PC1	PC2	PC3	PC4	PC5	PC6
ALf1	-0.316	-0.336	-0.106	-0.280	-0.208	0.249
ALf2	-0.356	-0.283	-0.200	0.006	-0.104	-0.392
ALf3	-0.290	-0.281	-0.515	-0.007	0.212	-0.052
LER1	0.204	0.347	-0.286	0.275	0.144	-0.269
LER2	0.162	0.233	-0.308	-0.542	0.185	0.541
LER3	0.249	0.240	0.021	-0.332	-0.592	-0.281
TN1	-0.384	0.110	0.333	-0.181	-0.194	0.083
TN2	-0.351	0.250	0.157	0.398	-0.201	0.305
TN3	-0.350	0.266	0.369	-0.115	0.390	-0.048
DW1	-0.281	0.286	-0.251	-0.198	-0.315	-0.205
DW2	-0.186	0.366	-0.408	0.347	-0.181	0.282
DW3	-0.231	0.368	-0.066	-0.281	0.364	-0.338

4.2.3 PCA for Spring Experiment

Principal Component Analysis: LER, LED, ALg, LL, ALf, TN, DM, TW, PI

Eigenanalysis of the Correlation Matrix

Eigenvalue	2.9071	2.1810	1.6110	1.0777	0.5536	0.4626	0.1729	0.0212
Proportion	0.323	0.242	0.179	0.120	0.062	0.051	0.019	0.002
Cumulative	0.323	0.565	0.744	0.864	0.926	0.977	0.996	0.999

Eigenvalue	0.0129
Proportion	0.001
Cumulative	1.000

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
LER	0.446	-0.196	-0.402	-0.093	0.279	-0.162	-0.208	-0.096
LED	-0.518	-0.067	-0.172	-0.086	-0.163	0.486	0.396	-0.054
Alg	-0.422	-0.105	-0.301	0.107	0.013	-0.783	0.309	-0.006
LL	0.089	-0.312	-0.627	-0.225	0.232	0.289	0.181	0.082
Alf	-0.521	-0.039	-0.214	-0.126	-0.041	0.030	-0.813	-0.002
TN	0.211	-0.278	-0.268	0.524	-0.721	0.050	-0.093	-0.016
DM	-0.062	-0.611	0.314	0.007	0.088	-0.021	-0.026	0.710
TW	0.110	-0.282	0.167	-0.749	-0.477	-0.180	0.034	-0.240
PI	-0.120	-0.563	0.286	0.264	0.293	0.071	-0.019	-0.647

Variable	PC9
LER	-0.660
LED	-0.518
Alg	0.016
LL	0.524
Alf	0.039
TN	0.029
DM	-0.105
TW	0.028
PI	0.081

4.2.4 PC Scores for Autumn and Spring herbage production

	Autumn 2003			Spring 2004		
Plant	APC1	APC2	APC3	SPC1	SPC2	SPC3
1	2.46328	3.33515	-3.13125	-1.61265	-1.53001	-0.51623
2	3.43067	2.93654	-3.75215	-1.13615	-2.28564	0.49996
3	1.68975	1.51202	0.8355	-3.14136	-0.23683	-0.44762
4	-0.86963	0.40955	-2.14093	2.68189	-0.94659	1.31188
5	0.38449	1.34811	-0.51811	-1.98669	-1.2551	-0.97614
6	-2.2857	2.14207	2.52852	-2.97096	-0.55034	-1.87382
7	0.06629	2.95398	-0.20786	-0.5124	-1.26714	-0.37335
8	0.08428	-0.7223	2.04212	1.07236	0.93199	-0.05396
9	2.17824	3.23588	2.45954	0.28626	-0.16819	0.47504
10	3.00184	-0.38031	-0.88621	-1.05306	0.82621	0.5343
11	2.15584	-0.45632	0.56932	-0.95704	-0.66599	0.20881
12	-0.70854	-1.97357	0.42335	-1.31141	-0.39656	0.06093
13	-0.24149	-1.77835	0.83146	-3.71327	2.03588	1.191
14	-2.24929	-1.58796	0.98022	1.21855	-0.73185	2.38081
15	1.79822	-3.91052	0.29903	-1.02394	-2.07281	2.16852
16	0.64321	2.25613	1.44877	-0.9885	0.20887	0.40728
17	-0.22656	-0.66793	-0.47398	-1.14252	0.57985	1.62452
18	-2.41154	0.94916	-0.08234	-2.41928	1.29793	1.27689
19	1.90376	1.0137	0.88935	1.63748	-0.92411	0.46875

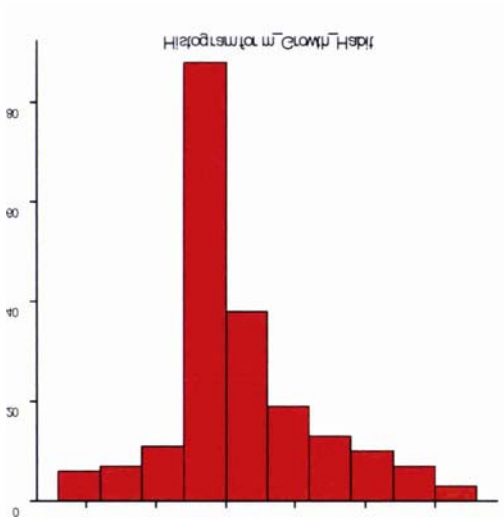
20	1.33376	2.15459	-0.10012	1.39535	-0.04069	0.17661
21	1.43527	-1.72511	-0.02807	3.18828	-1.27549	2.58586
22	2.96457	-0.55747	0.6481	2.15991	-2.78326	0.75098
23	2.16169	-0.55367	0.66309	0.86323	-0.66377	-0.31049
24	-0.2753	-1.67927	0.41637	-0.93581	-0.93432	-0.74851
25	-1.38209	0.88729	1.57655	-1.06826	0.21245	-1.29186
26	-0.62727	-0.19756	-0.6379	-2.66142	0.01344	-1.06983
27	-0.73592	0.29364	-0.16983	-2.94914	1.86201	0.4171
28	0.82685	-0.32657	1.95522	0.86806	-1.29534	-0.72611
29	2.30177	-1.90135	0.64878	0.62048	0.73399	-0.24044
30	-1.43656	-1.60923	-1.47275	-2.32992	-2.64213	0.34434
31	4.3756	2.29389	-0.86818	-1.17209	-2.51737	0.23545
32	0.50622	2.16663	1.43069	-0.72311	0.73635	-3.14336
33	1.72076	0.71726	-0.02698	0.80061	-0.22265	1.97752
34	1.70833	-2.28356	-1.03906	1.34908	0.62544	-1.78558
35	0.82288	0.80942	-0.45353	0.36151	-0.0186	-0.00654
36	-0.41177	2.58637	-1.94209	-0.32443	-0.7742	-1.58036
37	-1.60349	1.69768	0.44713	-0.71879	1.10715	-2.97511
38	1.66273	0.15129	-0.52478	-0.65588	-0.66938	-1.42946
39	-0.91724	1.3379	0.40282	-1.19813	2.09182	1.34422
40	0.7518	1.04591	-0.70781	-1.39034	-2.93095	-0.08815
41	0.22953	-0.22207	0.88153	-1.14518	-0.33224	1.02354
42	-0.61298	0.29284	1.96422	1.36198	0.92481	-1.35522
43	-2.03522	-0.02529	-1.89287	1.39917	-2.41686	1.06514
44	0.17258	-2.49844	0.51983	0.70175	0.45601	0.24353
45	-0.56581	0.89428	0.9565	-0.03079	-1.50342	1.45233
46	3.40087	2.46593	-1.52892	-1.02427	0.36374	-0.26393
47	3.97248	1.90137	0.99157	1.57811	0.26467	0.37941
48	2.69021	-0.15865	-0.06115	3.44827	2.44198	-0.38506
49	0.72653	-0.57449	-0.61391	-0.67011	-1.55846	-2.55421
50	3.10858	-1.37056	0.03267	2.25918	-0.995	-0.68108
51	0.18707	1.77382	1.74663	-0.8665	-2.68334	-1.23801
52	1.14392	0.04726	0.21556	1.39216	0.98635	-1.46779
53	1.08971	-1.32693	-0.3318	0.87105	-0.92293	2.75943
54	-1.19931	-0.02515	-0.97031	0.92882	-2.47291	2.05345
55	0.09691	0.91964	1.86256	-0.81161	0.85751	-0.44085
56	0.72051	-1.35685	0.31421	3.50875	1.96855	1.85394
57	2.41513	-0.21241	-1.43232	-0.92702	-1.88475	-0.58061
58	-1.85155	-0.03271	1.46817	-0.73912	-1.68053	0.05307
59	2.67571	1.36363	1.83868	3.67432	-1.14796	0.28321
60	-2.8737	-0.70864	2.08562	-2.14377	3.24511	-2.00872
61	6.25664	1.83648	0.35774	0.46367	-0.05204	-0.70088
62	4.62512	-0.06835	0.37872	-0.92128	0.05962	-0.40668
63	3.40694	-1.40119	0.36767	1.29519	-0.93046	-0.87342
64	2.90734	0.70646	-0.1909	4.07131	1.41026	0.81204
65	0.17548	0.6468	0.52001	-1.9285	0.31649	0.00983
66	0.61589	0.65019	0.00539	-0.69064	2.21209	0.45875
67	-0.97748	-3.93647	0.43396	1.55589	-0.43967	2.49605
68	-3.51074	0.89502	2.8035	0.66327	1.57714	1.81385
69	1.5368	-1.07431	0.89499	4.66115	-0.97971	-0.28974
70	0.48905	1.53527	1.05959	-0.72751	0.26968	-0.97663
71	1.52435	0.52725	-1.45149	0.88669	2.54484	-0.8312
72	-0.35793	0.90731	1.46314	-1.99305	-0.11087	-1.0506
73	-2.72076	-0.45366	-0.74085	1.52082	0.24413	0.55834

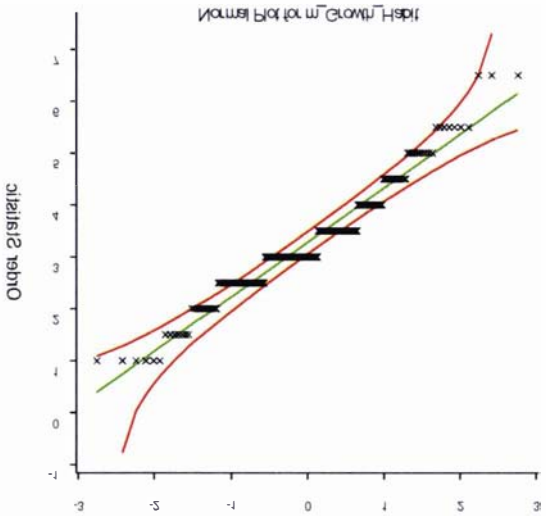
74	0.24936	-0.82664	-1.04072	-0.02333	1.8917	-0.43423
75	0.84456	-2.55033	0.3854	1.53147	4.95005	2.2933
76	1.57052	0.57259	1.67322	6.05423	0.38439	-2.75177
77	3.25601	-2.38428	0.31409	1.55042	-0.33249	0.05485
78	0.57562	0.67221	0.72911	-1.07294	-0.53638	-1.76027
79	0.0478	-0.27754	0.0054	2.33733	-1.63052	0.89193
80	2.02457	-0.67205	0.16198	2.65837	-0.64837	-1.36836
81	-1.76831	0.06012	-0.44433	-0.70019	-0.66793	-0.9292
82	0.48443	-1.08529	-2.7971	2.86648	-0.99282	0.73434
83	-1.16468	-1.48234	-0.60305	0.84269	0.30322	0.41595
84	0.20746	-1.00156	-0.49257	0.00164	-1.29638	1.38359
85	-2.03518	-0.31669	-0.24057	0.12626	-0.29726	-2.11006
86	2.19634	1.32996	0.8757	-0.41594	-1.61079	-0.48226
87	-1.36153	0.97255	0.06174	-1.80048	1.21956	-1.21896
88	-0.8164	0.17471	-0.2014	3.3191	2.46783	-0.56789
89	-0.14123	-0.9426	2.05123	0.16074	1.17263	0.1904
90	-1.47911	0.48755	-0.43199	1.30142	-0.49089	-0.58055
91	-1.30612	-0.54893	0.77971	-0.9164	-0.3638	0.17516
92	2.18489	-0.81816	1.01262	0.19683	-0.99004	0.25849
93	0.3999	0.04072	0.40671	2.2817	-0.07101	0.23701
94	-1.05118	0.23018	2.02374	-1.37476	1.05122	-1.78187
95	-1.32898	0.73391	2.13683	0.70433	0.26817	-0.08059
96	0.38723	0.218	0.33161	1.41332	1.79778	0.33148
97	1.35504	-1.88159	-0.93008	3.82299	1.2515	0.76003
98	-1.08371	-1.6892	1.82974	-0.68458	0.59111	-0.57504
99	-1.13765	-0.95467	-0.48176	3.45877	0.11344	-0.06297
100	1.37899	-0.89864	-0.00623	-2.53103	2.99875	0.13107
101	0.75365	-0.43753	0.55933	1.05782	1.93907	-1.08261
102	0.67054	-1.3175	0.31307	-0.09089	0.62697	0.38549
103	-2.57662	1.17691	-2.56588	-1.54914	-0.58668	-2.72972
104	1.37898	-2.86807	-1.24539	0.70805	-0.88901	1.81703
105	0.54262	0.1149	-0.14184	0.57746	1.76562	1.49816
106	0.9726	3.65115	0.17183	0.70935	-0.89088	3.12569
107	3.32113	-0.13984	0.65098	-0.445	-0.36095	0.1614
108	0.06613	-1.41809	0.10181	-0.19616	-0.23921	-0.39632
109	-0.29689	-0.0798	-0.49537	5.42938	0.71218	1.15409
110	-2.56084	-2.2349	0.67961	-1.08262	1.58545	0.46153
111	-2.85011	2.0885	0.34905	-1.94514	-1.2077	0.51996
112	0.37115	0.42658	1.49398	-2.16159	0.71014	0.97078
113	-1.31245	-0.41113	-0.1581	-1.79412	1.90484	-0.50329
114	-1.0527	-1.4864	1.09681	-3.04503	-2.68629	1.28968
115	-0.53642	-1.13253	0.63805	1.4939	0.73756	0.38305
116	-3.25578	0.59451	-1.34736	0.32698	-1.33145	-1.38418
117	-1.67819	1.11447	0.30193	0.41851	0.09083	-0.82428
118	-0.32436	0.55632	2.34032	3.39565	0.45178	-1.58175
119	-3.00609	1.31758	-1.50467	1.88103	0.83128	0.13124
120	0.02406	-0.99647	-0.27162	-0.20199	-0.03968	-0.80839
121	1.2791	2.55449	-0.08442	-0.83166	1.06906	-1.01537
122	0.63643	-0.6911	0.77587	1.05838	-0.63654	-1.26079
123	1.59026	-0.42175	-0.77249	2.05077	-1.78091	-1.36947
124	2.28291	-1.00924	-0.05962	0.9452	-0.797	1.22405
125	-0.48367	-0.19012	1.16147	-1.50951	-0.98722	0.00739
126	0.72812	-0.98282	1.28116	0.79736	3.80147	-0.02611
127	-2.78091	-3.14481	0.0712	0.66235	0.7504	-1.28605

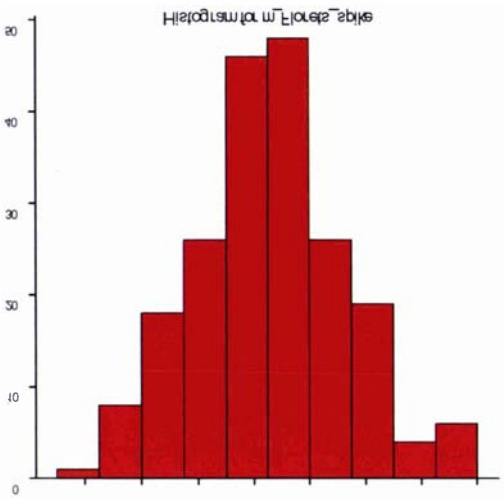
128	1.05912	-3.68427	-0.6574	-1.55242	0.45774	0.26707
129	0.35823	-0.70616	-0.28732	-0.27644	-1.37496	-2.60263
130	0.08577	-0.67076	-1.3085	-0.66693	-0.86211	0.14776
131	1.27976	1.68308	-0.31147	-1.46678	1.88098	0.20358
132	0.36267	0.38946	-1.61824	0.5856	3.80601	0.91321
133	-0.94147	-1.77565	-0.95293	1.18045	0.06559	1.1439
134	-4.66499	0.00786	-0.34314	-0.56827	-1.32716	0.98654
135	-3.05803	-0.1379	-0.19537	-2.03166	1.10739	-3.28698
136	3.40079	0.23657	-3.3688	0.83638	0.13155	-0.92643
137	-1.11045	-0.44872	-0.9944	-1.89914	-3.01792	-0.90857
138	1.25626	-0.95121	-0.75584	1.15291	-1.77108	-0.112
139	0.39818	1.60364	-0.40192	1.11312	-0.79124	-0.62507
140	-1.86557	-0.23035	0.00535	-1.55738	-0.56886	-1.48675
141	0.84612	-2.37833	0.14481	-1.03267	0.16727	2.05444
142	-1.36268	0.0388	0.37942	-0.1807	-0.2506	-0.95548
143	-3.76908	-1.0074	0.32842	-3.37757	-1.22332	0.34555
144	-2.84034	0.25622	0.92589	-0.93755	0.12858	2.5681
145	-0.53083	0.23897	2.32614	0.45405	0.4289	-1.67791
146	2.54602	-1.52979	0.57703	2.65516	0.98034	1.0297
147	-1.05172	0.82027	-1.64242	0.7739	0.91993	0.75331
148	-2.24643	0.29175	-0.33771	-1.41842	0.92689	-2.23295
149	-1.49824	-0.38867	-0.89089	2.60994	1.59029	0.50999
150	0.28599	1.53712	-0.15574	0.30916	1.04498	-0.0575
151	1.24191	-0.78894	-1.05531	3.01662	4.03021	-0.1015
152	-2.8209	0.48008	-0.19226	-0.18542	1.28837	0.47603
153	-0.47928	1.34747	-2.08618	-0.49165	-0.417	0.7497
154	0.4987	-0.20886	-1.05617	2.02137	-1.92552	0.8815
155	-0.0046	0.47177	-0.73032	-2.00393	3.21362	-2.18387
156	-0.81831	1.65392	-2.11311	2.32931	2.72934	-0.09269
157	-1.45585	-0.28749	-0.17652	-2.38343	2.15417	-1.21418
158	-2.34992	0.51805	-0.76732	1.0881	0.73934	-0.38694
159	-1.30122	2.09084	-0.12045	0.61002	-0.22738	0.21062
160	-0.98011	0.47797	-2.05587	0.32549	-1.23783	-0.43178
161	-1.35094	-0.98059	-0.27606	-4.66728	1.99178	2.65925
162	-2.98089	-0.70069	-0.86493	-3.5394	0.60222	0.89186
163	-2.06963	0.44444	0.14414	-2.35634	1.76334	2.12117
164	-1.16122	-0.68836	0.56285	-0.28701	-1.38528	0.57016
165	-2.71248	-1.80885	-0.7426	-1.66722	0.21742	-2.05354
166	-0.43309	2.54308	-0.39288	-1.06147	-1.00548	-0.28495
167	-2.79129	1.08768	0.25254	-0.45648	1.57438	-1.46343
168	-1.65653	1.38438	-1.49524	0.99478	-1.14449	1.20098
169	-1.47565	0.54837	1.46072	3.52209	-0.13066	-0.62043
170	-1.12679	-0.49589	-0.8309	-1.3405	0.45639	1.40575
171	-0.77289	0.71985	0.63083	-0.99901	0.41079	-0.60524
172	-0.17772	0.25502	0.22484	-4.43335	0.06671	0.81958
173	-1.69317	0.61399	-0.87028	0.79473	-0.32586	1.63234
174	-1.75386	-0.14506	-0.49737	-0.80655	-0.50243	-0.66261
175	-0.42283	0.66158	0.8145	-1.5686	-1.09244	-0.74029
176	-1.46599	1.10837	-1.53582	0.93878	0.18455	-0.01493
177	-2.0698	-1.11694	-1.12282	-4.05888	0.36193	2.38911
178	-0.05972	-0.02938	0.68887	-0.47236	0.46863	0.75458
179	2.3612	-2.50246	-0.2602	2.1277	-1.56589	0.50883
180	0.88979	-1.50367	0.75837	2.89785	-0.6913	-1.25067
181	4.31706	-1.76887	-0.30101	1.50004	-1.89189	0.21771

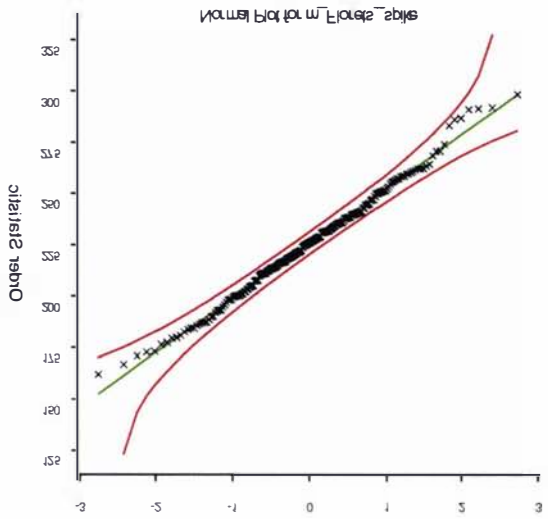
182	1.95185	0.17475	-1.44847	-1.63219	-2.21041	-0.32768
183	-2.80533	0.27536	-1.76474	-2.59028	0.25119	0.70681
184	-1.30529	1.08809	-0.22895	-3.96503	-0.77941	0.18007
185	0.84085	-0.55781	-2.64084	-0.35327	-2.67529	-0.20372
186	1.4176	-2.38738	-1.62191	0.69327	-0.07641	0.12218
187	1.84747	-0.66937	-0.54223	0.1114	-0.53891	1.37791
188	0.00405	-1.02261	-0.01373	0.92149	-1.81795	-0.44659
189	-1.87688	-2.27387	-1.15727	-0.63525	-1.51248	0.86952
190	-1.90538	0.65868	-0.59178	-2.77764	1.27722	2.35698
191	-1.09051	-0.52384	-0.60009	2.39453	-1.21873	-0.12412
192	-3.36885	-0.2327	-0.2464	-2.87041	0.01344	0.86024
193	-0.95179	0.35356	0.96147	3.29626	1.71063	-0.76617
194	-0.06408	2.88283	0.84055	2.56591	-0.97468	-2.28946
195	0.49289	-0.74252	1.85395	0.25058	-0.55732	2.33161
196	0.85334	0.23175	-0.46873	-1.57129	-0.9425	-0.60432
197	0.48648	2.30205	0.50054	-0.5142	-2.68639	-0.83814
198	-0.53393	1.34388	0.46545	0.32487	-1.72674	-0.7508
199	0.53478	0.75412	-0.0944	-1.6819	1.29579	0.55961
200	-0.03494	-1.52168	1.86942	-0.68332	0.86539	-0.69616
201	-4.81524	1.70239	-0.6942	-2.76158	1.02	2.54995
202	2.61691	1.95317	2.43464	-4.41112	1.55285	-0.33064

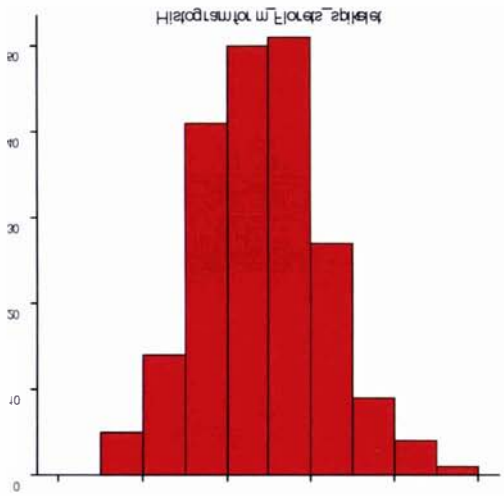
Appendix 5.1 Normality tests and checks for seed production traits

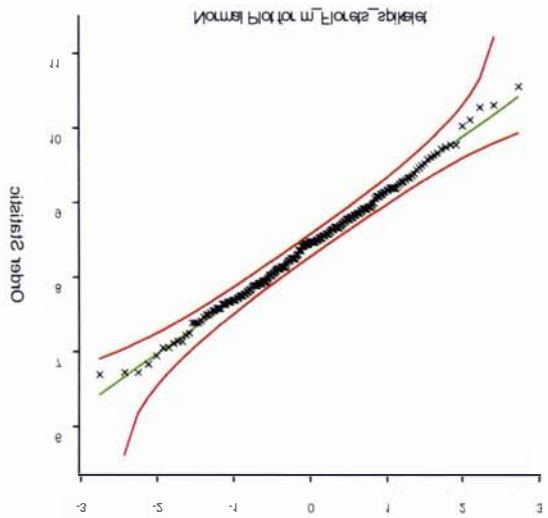


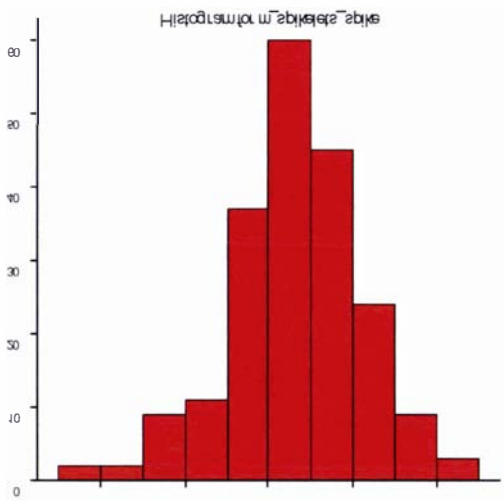


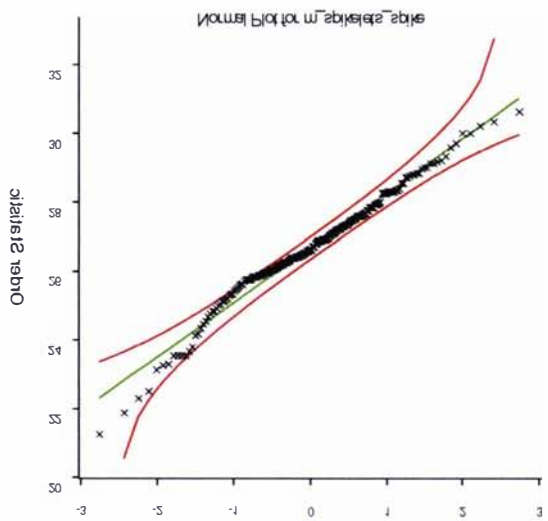

Histogram for Forest_abyss


Normal Q-Q plot of Forest_abyss
Order Statistic


Histogram for Forest_abyssal


Normal Q-Q plot of Forest_abyssal
Order Statistic


Histogram for abyssal_abyss


Normal Q-Q plot of abyssal_abyss
Order Statistic

Histogram for m_brkfength

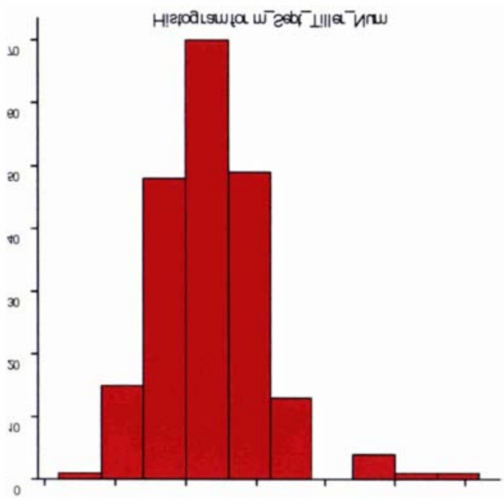
Normal Plot for m_brkfength
Order Statistic

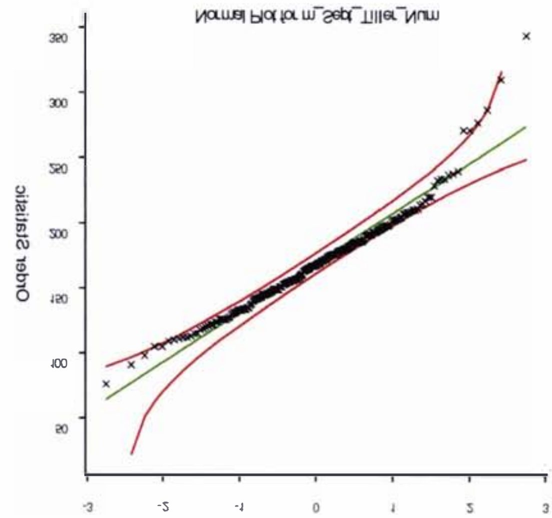
Histogram for m_speed_of_impact

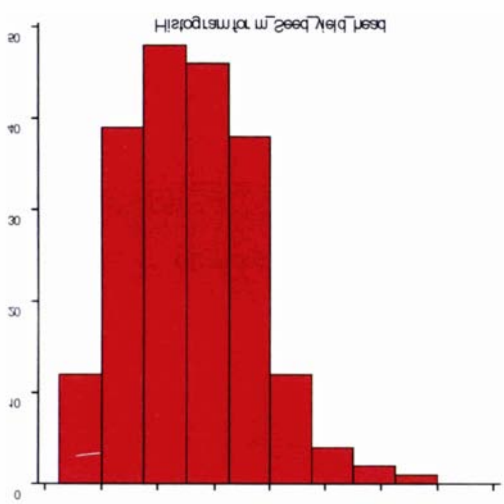
Normal Plot for m_speed_of_impact
Order Statistic

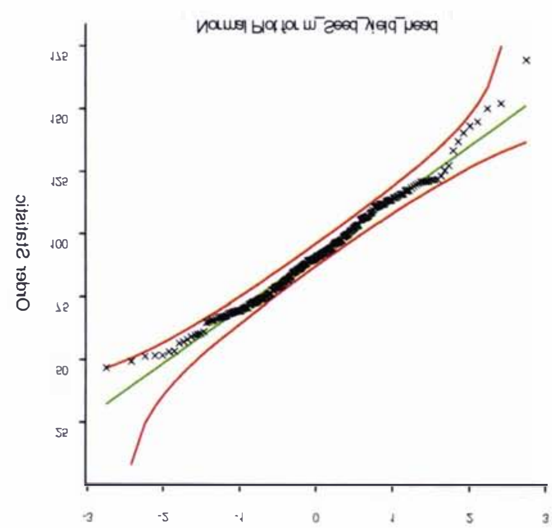
Histogram for m_Date_from_birthing_of_impact

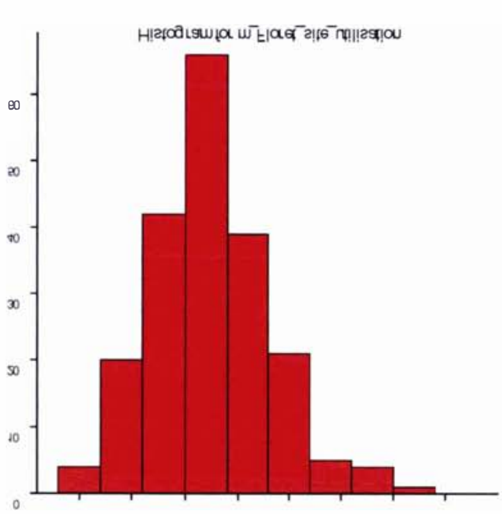
Normal Plot for m_Date_from_birthing_of_impact
Order Statistic

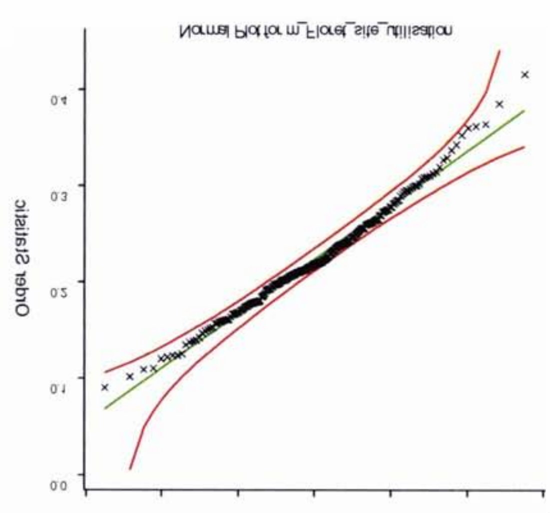

Histogram for m_Sept_Tiller_Num


Normal Plot for m_Sept_Tiller_Num
Order Statistic

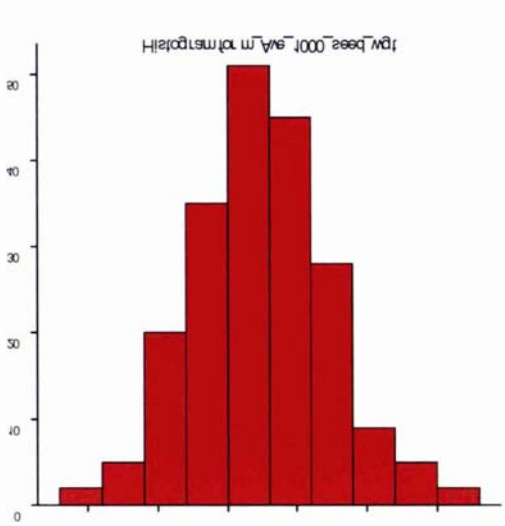

Histogram for m_Seed_yield_head

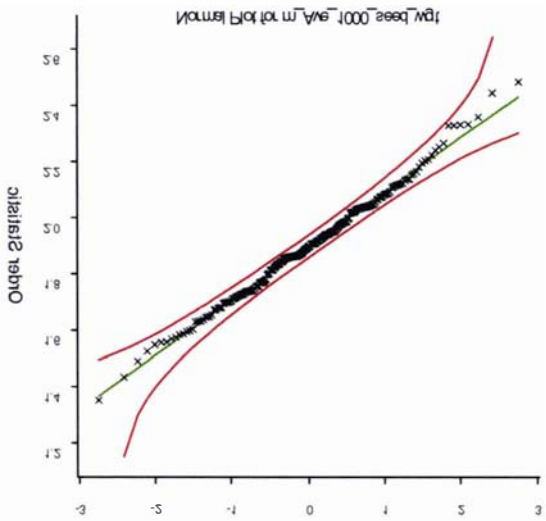

Normal Plot for m_Seed_yield_head
Order Statistic


Histogram for m_Floret_site_utilisation


Normal Plot for m_Floret_site_utilisation
Order Statistic

Histogram for m_Seed_Yield_Plant
Normal Prob for m_Seed_Yield_Plant
Order Statistic
Histogram for m_Matured_Tillers
Normal Prob for m_Matured_Tillers
Order Statistic
Histogram for m_Reproductive_Tillers
Normal Prob for m_Reproductive_Tillers
Order Statistic





Critical values of test statistics (marginal tests)

Test statistic	15%	10%	5%	2.5%	1%
Anderson-Darling	0.576	0.656	0.787	0.918	1.092
Cramer-von Mises	0.091	0.104	0.126	0.148	0.178
Watson	0.085	0.096	0.116	0.136	0.163

Plant growth habit

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	3.9992	0.7830	0.7500

$p<0.01$ strong evidence of non-normality

Floret per head

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.1988	0.0202	0.0185

Significance $p>0.01$

Floret per spikelet

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.1858	0.0286	0.0277

Significance p>0.01

Spikelet per head

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.0750	0.1630	0.1531

p<0.01 strong evidence of non-normality

Spike length

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.3057	0.0502	0.0487

Significance p>0.01

Spread of heading

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.5607	0.0808	0.0712

Significance p>0.01

Days to heading

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.5400	0.0852	0.0743

Significance p>0.01

September tiller number

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	1.3666	0.1677	0.1273

$p<0.05$ evidence of non-normality

Seed yield per head

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.6489	0.0877	0.0700

Floret site utilisation

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.6803	0.1208	0.0999

Significance $p>0.01$

Seed yield per plant

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.3773	0.0620	0.0599

Significance $p>0.01$

Reproductive tiller with matured heads

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2459	0.0359	0.0337

Significance $p>0.01$

Reproductive tiller number

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2624	0.0394	0.0394

Significance $p>0.01$

1000 seed weight

Marginal tests

Variate	Anderson-Darling	Cramer-von Mises	Watson
1	0.2674	0.0361	0.0333

Summary statistics for Plant growth habit

==========

Number of values = 202

Mean = 3.272

Minimum = 1.000

Maximum = 6.500

Lower quartile = 2.500

Upper quartile = 4.000

Skewness = 0.483

Standard Error of Skewness = 0.171

Kurtosis = 0.640

Standard Error of Kurtosis = 0.341

Summary statistics for Florets_per head

==========

Number of values = 202

Mean = 225.5

Minimum = 161.9

Maximum = 298.4

Lower quartile = 207.6

Upper quartile = 240.9

Skewness = 0.2

Standard Error of Skewness = 0.2

Kurtosis = -0.1

Standard Error of Kurtosis = 0.3

Summary statistics for Florets_per spikelet

==========

Number of values = 202

Mean = 8.426

Minimum = 6.694

Maximum = 10.556

Lower quartile = 7.917

Upper quartile = 8.889

Skewness = 0.116

Standard Error of Skewness = 0.171

Kurtosis = -0.042

Standard Error of Kurtosis = 0.341

Summary statistics for Spikelets_per spike

==========

Number of values = 202

Mean = 26.67

Minimum = 21.25

Maximum = 30.62

Lower quartile = 25.88

Upper quartile = 27.62

Skewness = -0.38

Standard Error of Skewness = 0.17

Kurtosis = 0.78

Standard Error of Kurtosis = 0.34

Summary statistics for spike length

==========

Number of values = 202

Mean = 19.62

Minimum = 16.12

Maximum = 23.60

Lower quartile = 18.85

Upper quartile = 20.43

Skewness = 0.08

Standard Error of Skewness = 0.17

Kurtosis = 0.15

Standard Error of Kurtosis = 0.34

Summary statistics for Spread_of heading

==========

Number of values = 202

Mean = 6.587

Minimum = 2.250

Maximum = 12.500

Lower quartile = 5.250

Upper quartile = 7.750

Skewness = 0.376

Standard Error of Skewness = 0.171

Kurtosis = 0.490

Standard Error of Kurtosis = 0.341

Summary statistics for Days_to_heading

==========

Number of values = 202

Mean = 126.33

Minimum = 113.00

Maximum = 135.50

Lower quartile = 123.75

Upper quartile = 128.75

Skewness = -0.34

Standard Error of Skewness = 0.17

Kurtosis = 0.23

Standard Error of Kurtosis = 0.34

Summary statistics for Sept_tiller_number

==========

Number of values = 202

Mean = 169.3

Minimum = 76.0

Maximum = 344.0

Lower quartile = 145.0

Upper quartile = 190.0

Skewness = 0.9

Standard Error of Skewness = 0.2

Kurtosis = 2.6

Standard Error of Kurtosis = 0.3

Summary statistics for Seed_yield_per head

==========

Number of values = 202

Mean = 91.88

Minimum = 46.74

Maximum = 169.62

Lower quartile = 74.48

Upper quartile = 107.14

Skewness = 0.45

Standard Error of Skewness = 0.17

Kurtosis = 0.24

Standard Error of Kurtosis = 0.34

Summary statistics for Floret_site_utilisation

==========

Number of values = 202

Mean = 0.2235

Minimum = 0.0900

Maximum = 0.4161

Lower quartile = 0.1850

Upper quartile = 0.2587

Skewness = 0.4286

Standard Error of Skewness = 0.1711

Kurtosis = 0.3814

Standard Error of Kurtosis = 0.3405

Summary statistics for Seed_yield_per plant

Number of values = 202
Mean = 35.09
Minimum = 11.95
Maximum = 64.47
Lower quartile = 27.23
Upper quartile = 42.00
Skewness = 0.16
Standard Error of Skewness = 0.17
Kurtosis = -0.32
Standard Error of Kurtosis = 0.34

Summary statistics for Reproductive tiller with matured_heads

Number of values = 202
Mean = 324.3
Minimum = 165.0
Maximum = 528.0
Lower quartile = 270.0
Upper quartile = 373.0
Skewness = 0.1
Standard Error of Skewness = 0.2
Kurtosis = -0.2
Standard Error of Kurtosis = 0.3

Summary statistics for _Reproductive_tiller number

Number of values = 202
Mean = 390.0
Minimum = 184.0
Maximum = 592.0
Lower quartile = 339.0

Upper quartile = 448.0

Skewness = 0.0

Standard Error of Skewness = 0.2

Kurtosis = -0.2

Standard Error of Kurtosis = 0.3

Summary statistics for 1000 seed wgt

Number of values = 202

Mean = 1.898

Minimum = 1.352

Maximum = 2.483

Lower quartile = 1.754

Upper quartile = 2.033

Skewness = 0.180

Standard Error of Skewness = 0.171

Kurtosis = 0.134

Standard Error of Kurtosis = 0.341

Appendix 5.2

5.2.1 PCA for Seed production experiment

Principal Component Analysis: SdYH, FH, SdYP, RT, TMH, SL, SH, FS, TSW, PGH, SOH, DH, FSU

Eigenanalysis of the Correlation Matrix

Eigenvalue	3.2854	2.5280	2.0236	1.3137	1.0159	0.8927	0.8187	0.5989
Proportion	0.253	0.194	0.156	0.101	0.078	0.069	0.063	0.046
Cumulative	0.253	0.447	0.603	0.704	0.782	0.851	0.914	0.960

Eigenvalue	0.3230	0.1039	0.0505	0.0340	0.0118
Proportion	0.025	0.008	0.004	0.003	0.001
Cumulative	0.985	0.993	0.996	0.999	1.000

Variable	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
SdYH	-0.331	-0.179	0.483	0.130	-0.104	0.190	-0.037	-0.018
FH	-0.295	0.477	0.145	-0.072	0.213	0.084	-0.006	0.113
SdYP	-0.501	-0.187	0.047	0.132	-0.103	0.091	-0.046	-0.105
RT	-0.379	-0.046	-0.484	0.053	-0.035	-0.102	-0.058	-0.111
TMH	-0.400	-0.092	-0.441	0.050	-0.043	-0.079	-0.094	-0.113
SL	-0.116	0.045	0.241	0.252	-0.062	-0.871	0.228	0.199
SH	-0.183	0.313	0.266	-0.110	0.505	-0.079	-0.022	-0.589
FS	-0.279	0.410	0.017	-0.066	-0.023	0.222	0.024	0.648

TSW	-0.024	-0.101	0.034	0.694	0.130	0.321	0.513	-0.040
PGH	-0.109	-0.047	0.042	-0.546	-0.299	0.062	0.736	-0.151
SOH	0.266	0.337	0.078	0.248	-0.415	0.095	-0.134	-0.223
DH	0.115	-0.410	0.013	-0.118	0.581	0.032	0.058	0.264
FSU	-0.165	-0.365	0.419	-0.143	-0.235	0.030	-0.325	0.029

Variable	PC9	PC10	PC11	PC12	PC13
SdYH	-0.019	-0.348	0.137	0.251	-0.597
FH	0.071	0.011	-0.733	0.225	0.026
SdYP	0.097	-0.458	0.003	-0.204	0.633
RT	0.163	0.038	-0.185	-0.548	-0.476
TMH	0.151	0.282	0.199	0.678	0.056
SL	0.094	0.011	0.020	0.003	0.010
SH	0.042	0.178	0.349	-0.149	0.017
FS	0.088	0.154	0.464	-0.178	0.024
TSW	-0.052	0.332	-0.069	-0.056	0.040
PGH	0.161	0.043	-0.014	0.013	-0.008
SOH	0.705	-0.034	0.031	0.012	-0.004
DH	0.620	-0.089	-0.019	0.029	-0.018
FSU	0.087	0.641	-0.160	-0.163	0.097

5.2.2 PC scores

Plants	PC1	PC2	PC3
1	2.92076	-2.67729	1.79171
2	2.12946	-2.71964	-0.45187
3	1.57425	1.47448	-0.19499
4	-2.68225	2.20748	-1.9872
5	0.00428	0.33685	0.59787
6	0.75748	-1.74454	0.72271
7	1.24385	-0.23638	0.3748
8	-0.35187	2.17688	-0.03476
9	1.24153	-0.20522	0.93235
10	3.25453	0.0181	-2.64445
11	-2.23751	2.22187	1.33458
12	1.42242	-3.25965	0.22666
13	-1.11354	1.68016	-0.68026
14	0.00111	-2.37552	-3.70525
15	-1.45839	-0.57721	-2.05959
16	1.21669	0.91	-0.28724
17	0.38413	-2.29505	-1.457
18	-2.52898	-0.68402	-0.36141
19	-2.48029	-0.0077	0.24024
20	1.64816	-0.90922	-0.49852
21	2.74365	-0.75171	-0.59236
22	-1.23911	-0.35496	-0.29855
23	0.61402	1.29687	-2.07837
24	1.81415	-0.90316	1.22459
25	-1.65393	-2.24511	0.26368
26	-1.00579	-0.72262	1.31203
27	3.7223	-1.16086	0.80763
28	2.40351	0.4897	0.60346
29	-0.2602	-0.78307	-1.07683
30	0.67061	0.49714	-0.40227
31	1.51782	-0.8869	-1.29016
32	-0.88403	-0.85625	1.06796
33	2.00529	-0.77214	-1.55191
34	-2.35081	-0.24732	0.61307
35	-1.43807	-1.2614	-1.50506
36	1.73037	-2.44001	-0.01322
37	-3.53069	1.15314	0.20079
38	-1.68504	1.10959	0.34927
39	-2.3786	2.89264	-0.63331
40	1.13699	0.55713	-1.16823
41	0.89466	-1.34626	-1.26565
42	2.94718	-2.8893	3.09834
43	3.2491	1.84077	-1.66668
44	1.01354	-1.54871	1.47217
45	1.12571	0.62853	-2.20329
46	-0.34472	-0.78864	-3.12001
47	2.89682	0.6113	-1.71153
48	1.52809	2.39829	0.91403
49	2.38943	2.35892	3.49082
50	-1.9092	-2.04053	0.12976
51	-1.60376	-0.95634	-1.0417
52	-1.77497	0.23364	-0.16082
53	-2.26628	0.22622	0.03901
54	0.22676	-0.58084	-1.20779
55	1.84985	0.86939	1.17602
56	-1.75476	0.41652	1.06055
57	0.22285	1.19515	-1.38873
58	2.9287	-1.90296	-1.40399
59	1.69618	0.07924	1.48188
60	0.61901	-0.15607	-0.5549
61	-1.00855	0.29272	-0.54991
62	1.94816	-0.12849	1.75949
63	1.2813	0.98692	0.72773
64	-0.4848	-0.48197	-0.83082
65	1.1063	0.04707	-0.49688
66	3.00648	-1.03998	0.20741

67	0.90519	1.60042	1.31629	121	0.02545	-0.89151	0.20823
68	-1.22806	1.6535	-1.90408	122	-0.74646	-0.03439	-0.44046
69	0.58588	0.76355	0.18821	123	-4.77993	-13.8578	0.73567
70	0.40962	-2.71324	0.23219	124	-0.57127	1.39457	3.18771
71	-2.69634	1.49538	1.67288	125	-4.03672	-0.45611	-0.35617
72	0.49928	-0.5461	1.71638	126	0.67795	-1.13436	-1.07367
73	4.49255	1.84997	-0.41623	127	0.72812	4.53436	0.80178
74	-0.3669	-3.06009	-0.5117	128	-0.81218	0.69044	-0.63978
75	2.5669	2.89376	-0.18776	129	0.90218	1.27466	-3.67309
76	0.87713	1.1069	-1.53479	130	-0.06255	-2.03121	1.83944
77	-0.02604	0.99351	-0.01451	131	0.5961	-0.70868	2.11971
78	2.09941	0.66246	-0.42692	132	-1.67193	0.73273	0.37915
79	1.41264	-1.99537	0.14578	133	-3.47401	-0.13008	-0.71713
80	2.34844	0.27491	0.63137	134	0.35412	0.29092	-0.24462
81	0.20244	-0.24267	-0.92579	135	-0.08372	0.41153	0.93192
82	1.86703	-0.86086	1.65664	136	1.84683	2.55498	1.69341
83	0.67893	0.93191	0.31461	137	-0.7587	0.16128	-1.33803
84	1.31433	-3.05763	1.38977	138	2.70347	2.65861	-2.92767
85	-0.47775	0.58434	0.28335	139	-0.4551	1.49009	-1.93073
86	-0.51835	0.74581	-1.18788	140	-2.00861	-0.21071	0.41304
87	-3.15684	-0.75698	1.59793	141	-2.9715	0.19379	0.14411
88	-1.52895	0.27132	2.61877	142	-0.47735	-1.73706	1.10537
89	0.72142	2.06284	-0.60989	143	-1.92853	0.60432	-1.26104
90	1.74403	3.45454	1.18221	144	0.69673	1.53663	0.26749
91	0.74449	1.67998	-1.71922	145	0.33878	-0.10624	0.89768
92	1.5573	-0.09946	-0.43003	146	-1.11891	0.4245	-1.12652
93	-1.26153	-2.07575	-0.93228	147	-0.45172	2.01097	-1.80292
94	-0.49338	4.75405	-0.1158	148	-1.53991	-0.34867	-1.37892
95	1.91799	-2.40825	0.56743	149	0.09629	-0.09157	1.88892
96	-2.51756	-0.44532	-1.68219	150	1.59865	1.45271	-0.95914
97	-1.59147	-1.6924	0.66583	151	-1.03574	2.3427	1.4265
98	1.88354	0.41492	1.29356	152	2.20429	-0.55109	-1.09286
99	-1.98935	-2.79966	0.68004	153	-0.67238	2.09045	-2.26252
100	-2.06315	-1.10629	-0.86027	154	0.607	2.94355	1.43967
101	-2.11945	2.54997	-1.12674	155	0.64394	-0.22993	0.46955
102	1.70517	-2.87107	0.44557	156	-0.17958	0.5032	-1.57107
103	-4.45344	0.15584	0.43252	157	-1.80361	0.76672	-2.88829
104	-0.89552	-0.78541	3.57262	158	-0.45612	2.02627	-0.9724
105	-1.77506	-0.05209	-0.73739	159	-0.41334	-0.86388	1.91045
106	0.36705	1.231	-1.31007	160	1.33112	-0.11765	1.5353
107	1.67008	-1.01568	-0.78752	161	-0.85744	1.13944	-0.926
108	2.35518	0.32039	3.36086	162	-3.01899	0.93928	2.05617
109	-2.8436	-0.80784	1.82997	163	1.1696	0.79966	0.14536
110	-0.14859	2.441	-1.18785	164	0.13606	2.76295	1.65725
111	3.20442	1.34227	1.01942	165	1.17785	0.03209	-1.52566
112	0.2495	0.68197	-0.40005	166	-1.27254	-1.17473	1.11098
113	-1.47139	1.79064	1.06091	167	1.14356	1.91256	1.93544
114	-3.28632	-1.34521	0.42506	168	1.08487	-2.23676	-0.98907
115	1.89531	-1.06577	-0.88576	169	0.25902	0.16954	1.2825
116	-5.41568	-2.12666	-0.01847	170	-1.48677	0.15073	0.43587
117	-2.85264	0.58683	0.95711	171	-0.59318	-0.52248	1.14094
118	0.00436	1.7789	3.21534	172	-0.8358	-0.37166	-1.05303
119	0.98247	1.35919	1.39021	173	-0.22481	-3.2028	0.71355
120	-1.70398	-0.60609	0.88833	174	-1.36674	-2.09741	1.37465

175	-2.62226	0.75953	0.50167
176	0.88268	-0.8388	-0.50255
177	0.52652	1.03084	-0.85056
178	1.55328	-0.87899	-2.1666
179	2.24094	0.96797	-0.36371
180	1.5073	-0.24466	-1.53933
181	-1.60667	-0.40609	-0.84635
182	3.53634	-4.3791	0.81382
183	-0.36926	0.71578	-0.76814
184	0.06795	-0.76844	-1.10706
185	-0.5099	-2.69072	1.71856
186	-0.31625	2.42601	2.15096
187	2.5822	0.69206	3.36799
188	1.23216	-2.06305	-3.77596
189	1.6546	-1.945	1.01537
190	-0.93983	-0.05581	0.51107
191	1.89101	-1.02489	-0.76635
192	1.01278	-3.56034	1.00509
193	0.4047	2.16865	1.39895
194	1.49323	-0.56954	-2.35853
195	0.86914	0.4341	-0.01233
196	0.4497	-1.15672	-0.95676
197	-2.19579	-2.01957	-2.18636
198	-1.99559	-0.95782	-2.4329
199	-2.52992	0.5303	-1.71877
200	-1.43717	0.42259	1.46334
201	-4.70896	-3.2596	1.14964
202	-1.87081	2.14404	2.67574