Testing alternative breeding methods in white clover

A thesis
submitted in partial fulfilment
of the requirements for the Degree of
Doctor of Philosophy
at
Lincoln University
by
Richard Mark George

Lincoln University
2014

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A series of experiments were conducted to estimate the genetic parameters for an elite breeding population of white clover (*Trifolium repens* L.) in both an irrigated and dryland environment. Data collected from two environments using spaced-planted and mini-plot trials were used to demonstrate the rationale for pursing various traditional and modern marker-assisted selection breeding methods in white clover. In addition, and under the over-arching theme of investigating breeding strategies, supplementary findings are presented regarding pollination patterns within a white clover population in isolation cages.

In an experiment designed to generate half-sib and full-sib families for progeny evaluation, pollen dispersal within isolation cages using wild bumble bees (*Bombus sp*.) was monitored using simple sequence repeat molecular markers (SSR markers). Within bi-parental crosses, no detectable levels of self-fertilised germinated seedlings were detected, nor were there any detectable levels of foreign pollen sources. Within polycross isolations, no detectable levels of foreign pollen sources were detected, and self-fertilised rates were negligible (<1%). Outcross paternal progeny counts deviated significantly from random mating in two 20 parent polycrosses. Siring success of paternal genotypes decayed as the distance between them and recipient maternal parents increased. Low levels of paternal pollen dispersal at increasing distances from maternal recipients within isolation cages demonstrated the requirement for randomised multi-clonal replication in white clover polycrosses, especially when being used for the generation of half-sib families for progeny evaluation.

Development and application of a novel paternity testing method in white clover was successful at assigning paternity to known maternal half-sib progeny from a 20 parent polycross. A panel of seven pre-determined SSR markers successfully assigned paternity to 92% of the half-sib progeny. Data collected from known maternal and molecular maker determined paternal half-sib progeny demonstrated that their respective additive genetic variances were similar across a range of morphological traits and two experimental sites. Combining both known maternal and molecular marker determined paternal half-sib

selection, the rate of expected genetic gain was doubled compared to traditional half-sib family selection alone. Supplementary phenotypic selection within molecular marker determined full-sib families further improved expected genetic gain. Discrepancies in heritabilities calculated on a half-sib family means basis and that on a single plant basis support the rationale for breeders to pursue half-sib family selection methods for low heritable traits such as clover herbage yield, whereas traits with moderate heritabilities on a single plant basis, such as leaf size, support the use of phenotypic selection methods.

In a multi-site mini-plot trial conducted in two Canterbury soils which differed in soil moisture content and irrigation application, significant genetic variation for both autumn vegetative persistence and herbage yield was observed. Utilising a North Carolina I mating design, second year autumn vegetative persistence (plot coverage) ranged among full-sib families from 15 to 100% at the irrigated site and from 10 to 70% at the dryland site, clover herbage yield from 496 to 1382 kgDM/ha at the irrigated site and 93 to 326 kgDM/ha at the dryland site, and growing point density from 356 to 3111 and 115 to 867 growing points m^{-2}, at the irrigated at dryland sites, respectively. Physiological measurements indicated significant soil moisture stress at the dryland site, with water potentials up to 3.5 fold lower than the irrigated site. The extent of genetic variation observed for autumn recovery supports the notion for the use of intra-population selection for the development of improved cultivars that can persist through moderate drought conditions and demonstrate improved recovery rates following rain. Similar to the spaced-planted trials, large discrepancies in heritabilities calculated on a family means basis and that on a single plant basis support the rationale for breeders to pursue family selection methods for clover herbage yield and most vegetative stolon attributes. Heritabilities for traits such as leaf size and stolon thickness support the use of phenotypic selection methods.

Partitioning of genetic variation into both additive and non-additive genetic variation was hindered by large standard errors. Inconclusive evidence for a significant proportion of non-additive genetic variation for clover herbage yield warrants further enquiry. Significant family × replicate, family × year, family × environment and family × year × environment variance components demonstrated the requirement for breeders to include multi-site and multi-year trials with adequate plot size to identify families with broad adaptation for field application.

Keywords: *Trifolium repens*, white clover, breeding methods, genetic variances, paternity testing, half-sib families, full-sib families, spaced-planted, mini-plots, clover herbage yield, persistence, moisture stress, pollination**.**

Acknowledgements

The completion of this PhD research project and thesis was made possible by the support and advice given to me by a number of people for whom I am immensely grateful for.

I was fortunate to have an outstanding advisory and supervisory team whose wealth of both academic and practical knowledge contributed to not only this thesis but also my professional development. I would like to thank my principal supervisors, Dr Rainer Hofmann (Lincoln University) and Dr Zulfi Jahufer (AgResearch) for their guidance and support throughout my project. Their humility was inspiring. I would also like to extend this gratitude to my advisors at AgResearch, Brent Barrett and Keith Widdup.

I am exceedingly grateful for the mentoring role Dr Derek Woodfield from PGG Wrightson Seeds played throughout the course of my project, his vision, and the significant personal development opportunities he has provided me with. Likewise, I would like to thank both Dr Alan Stewart and Michael Norriss from PGG Wrightson Seeds for their practical advice and discussions regarding commercial breeding programs. I also extend this gratitude to the wider PGG Wrightson Seeds team at the Kimihia Research Centre.

I thank AgResearch staff Ivan Baird, John Ford and Greig Cousins for not only their help in the field but also their invaluable wealth of practical breeding knowledge that they have shared with me. Thanks to Dr Andrew Griffiths, Craig Anderson and Dr Benjamin Franzmayr for their assistance and guidance in the molecular biology lab and Dr Alasdair Noble and Dr John Koolard for statistical advice.

A special thanks to Anouck De Bonth and Michelle Ebbett of AgResearch for the accommodation and bike loans on my visits to Palmerston North.

I thank Stephen Stilwell, Jenny Zhao, Rosy Tung, Daniel Dash, Keith Pollock and Dave Jack for their technical assistance at Lincoln University.

I thank Emma Byrne for her support and encouragement during the project.

Finally, I wish to thank my close friends, parents, brothers and partner Colette for their love and support.

I would like to acknowledge the financial support from PGG Wrightson Seeds, MBIE (PGGS1001) and AgResearch.

Table of Contents

List of Tables

List of Figures

List of Plates

Abbreviations

HY – herbage yield

LW – leaf width

ST – stolon thickness

NN – number of nodes

RN – number of rooted nodes

BR – number of stolon branches

LS – lateral spread

GPD – growing point density

PER – vegetative persistence

HSF – half-sib family selection

♂**HS** – paternal half-sib family selection

♀**HS** – maternal half-sib family selection

PCA – principal component analysis

h_f^2 – heritability on a family means basis

h_1^2 – heritability on an individual plant basis

σ_A^2 – additive genetic variance

σ_D^2 – dominance genetic variance

σ_G^2 – genetic variance

TDR – time domain reflectometry

DNA – Deoxyribonucleic acid

PCR – polymerase chain reaction

SSR – simple sequence repeat marker

GCA – general combining ability

HSPT – half-sib progeny trialling

AWF-HS – among-and-within half-sib family selection

♀+♂**HS** – combined known maternal and molecular marker determined paternal half-sib selection

AWF-HS+MFS – among-half-sib-and-within molecular marker determined full-sib family selection

Chapter 1
Introduction

1.1 Background

White clover (*Trifolium repens* L.) is an essential component in a vast majority of New Zealand pastures (Jahufer et al., 2002; Woodfield and Caradus, 1996), Australian temperate pastures (Ayres et al., 1992), and most other temperate pastures grazed by sheep or cattle (Abberton and Marshall, 2010). The stoloniferous growth and phenotypic plasticity of this legume species make it an ideal companion in most grazed swards (Woodfield and Caradus, 1994). In New Zealand it is key to the international competitive advantage of our pastoral industries, where it provides a reliant, cheap and high quality feed source (Caradus et al., 1996). It is estimated to contribute $3 billion to New Zealand's annual economy through nitrogen fixation and forage yield alone (Caradus et al., 1996).

Both reliable seasonal herbage production and vegetative persistence have been identified as two important characteristics which are necessary for white clover to viably enhance pasture production (Gramshaw et al., 1989). However, poor vegetative persistence is considered to be a major limitation to the performance of white clover in many temperate regions of the world (Jahufer et al., 2013). This is further exacerbated in the context of summer moisture deficit (Knowles et al., 2003). To counter this limitation, improvement of vegetative persistence among a range of additional agronomic traits has been key objectives in many plant breeding programmes (Abberton and Marshall, 2010; Caradus and Williams, 1989; Taylor, 2008).

The rate at which these traits are improved in breeding programmes is dependent on a number of factors, one of which is the breeding strategy *per se*. Development of an efficient strategy hinges on the selection of an appropriate breeding method, coupled with the thoughtful allocation of resources for population development and genotype selection (Fehr, 1987). To date, the predominant breeding method in white clover has been phenotypic recurrent selection (Williams, 1987; Woodfield and Caradus, 1994). However, with both a better understanding of the quantitative genetic parameters for key white clover vegetative persistence and production traits and complementation with modern selection technologies, perhaps the current rate of genetic gain can be improved through the use of alternative efficient breeding strategies. This PhD project addresses an overarching theme of interpreting at a fundamental level the rationale for pursing alternative breeding methods in white clover.

1.2 Breeding progress in white clover

A significant proportion of the current economic value of white clover is due to 75 years of plant breeding. Conventional plant breeding has steadily delivered improved white clover performance in New Zealand pastoral systems (Woodfield and Easton, 2004). The improvement of white clover performance lies between 6% to 15 % per decade, although the extent of improvement varies among white clover leaf types (Woodfield, 1999; Woodfield and Caradus, 1994).

In comparison, the reported genetic gains in other forage species including perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum* Lam) and alfalfa (*Medicago sativa* L.) are similar or inferior (Hill et al., 1988; Holland and Bingham, 1994; Vanwijk and Reheul, 1991). The gains achieved in white clover are the result of accumulating genes with better yield potential as well as reducing the effects of yield limiting factors (Woodfield et al., 2001).

Progress in early white clover breeding programmes was achieved through improving performance within existing ecotypes (Woodfield and Caradus, 1994). Cultivars such as Grasslands Huia proved to be commercially successful for several decades. Superseding cultivars were the result of hybridising persistent ecotypes with more agronomic imported material. The extent of variation within a particular breeding pool has not been large enough to allow rapid gains under selection for desired improvements, and the use of *ex situ* genetic resources has been critical in the development of new varieties (Abberton and Thomas, 2011). The development of adapted breeding pools paved the way for phenotypic recurrent selection programmes which by in large have become the most widely adopted breeding practice (Woodfield and Caradus, 1994).

1.3 Gaps in knowledge

To increase the economic value of white clover, the attention of plant breeders has been focused towards improving its yield, vegetative persistence and forage quality attributes. However, like most forage species, agronomic improvement of white clover comparatively lags behind many grain crop species. Casler & Brummer (2008) summarised several reasons for the yield lag in forage crops relative to grain crops which included, (i) a longer breeding cycle for forage crops, most of which are perennials, (ii) lack of a “harvest index” trait to aid dry-matter partitioning into the economic product, (iii) inability to exploit heterosis in commercial cultivars, and (iv) the focus on a wide array of economically important traits of forage crops, many which are not specially correlated or may be negatively correlated with

forage yield. Furthermore, in the context of white clover, there has been slow adoption of improved breeding methods that have positively influenced the rate of genetic improvement (Woodfield and Caradus, 1994).

In an era where plant breeding is being asked to deliver results more urgently than at any time previously (Parsons et al., 2011), current bottlenecks in the rate of genetic advance will need to be resolved. While features ii) & iv) above, are unlikely to change in the near future, emphasis on more efficient breeding methods and strategies which address features i) and iii) are likely to be needed if accelerated genetic gains are to be realised. Alternative breeding methods have been compared for forage species (Burton, 1992; Casler and Brummer, 2008; Haag and Hill, 1974; Ledda et al., 2000; Riday, 2011; Vogel and Pedersen, 1993), however both empirical and theoretical studies specific to white clover are limited. In order to compare breeding methods, a comprehensive understanding of the species specific quantitative genetic parameters for key traits is necessary.

Although many studies have reported genetic parameters for white clover attributes (Annicchiarico and Piano, 1995; Annicchiarico et al., 1999; Caradus and Woodfield, 1990; Caradus and Chapman, 1996; Woodfield and Caradus, 1990), the respective studies have been conducted in single favourable environments using pot trials or mono-culture spaced-planted nurseries (Jahufer, 1998). While these studies are fundamental for providing accurate information on the genetic control of traits, the estimates are limited to uniform environments and hence heritabilities are often near their maximum potential. Such estimates of heritabilities and proportions of additive to non-additive genetic effects can therefore be misleading when compared to evaluation under different environmental conditions, such as mixed sward and grazed conditions (Hill, 1993; Hill and Michaelson-Yeates, 1987). To date, the lack of literature available on both empirical comparisons and estimation of relevant genetic parameters (both heritabilities and the relative importance of additive and non-additive genetic effects) of random mating white clover breeding populations evaluated in multiple target environments has limited the ability to compare alternative breeding methods (Jahufer et al., 2002). The genetic parameters estimated in the experiments reported in this thesis will provide breeders with improved understanding of heritabilities in actual farming systems. This will consequently allow breeders to critically evaluate their breeding methodology more realistically, if these environments are used to undertake selection.

In addition, the empirical evaluation of a promising molecular marker based selection method developed in red clover (*Trifolium pratense*) (Riday, 2011) will provide breeders with insight into its effectiveness for white clover.

1.4 Objectives

The objectives of experiments in this thesis were to compare the efficiencies of alternative breeding methods both empirically and theoretically.

Eight objectives were identified, to;

1. Generate genetic families from a random mating white clover breeding population, for evaluation in field trials.

2. Establish a multi-site spaced-planted half-sib family nursery in white clover and utilise molecular markers to evaluate paternity testing as demonstrated by Riday (2011) in red clover.

3. Evaluate the merits of paternal-based half-sib family selection methods compared to conventional maternal based half-sib family selection.

4. Identify spatial effects of pollen distribution by bumble bees within polycross isolation crossing cages.

5. Establish a multi-site transplanted mini-plot trial with propagated stolon cuttings to assess the magnitude of genetic variation for summer moisture stress.

6. Estimate the magnitude and type of genetic variation for vegetatively propagated white clover within and across dryland and irrigated experimental sites.

7. Characterise the magnitude and type of family × environment interaction across test environments.

8. Identify appropriate breeding methods which may maximise genetic gain for the traits investigated in this thesis.

1.5 Thesis structure

The structure of the thesis is depicted in Figure 1.1. Five experimental chapters are presented to address the objectives outlined above, commencing with the generation of genetic families (Chapter 3) for the subsequent field experiments in chapters 5, 6 and 7. Implications of each experimental chapter are addressed within, as they are kept as complete entities for individual publication. Key findings are pooled together in Chapter 8 and their overall implications to breeding strategies in white clover are discussed.

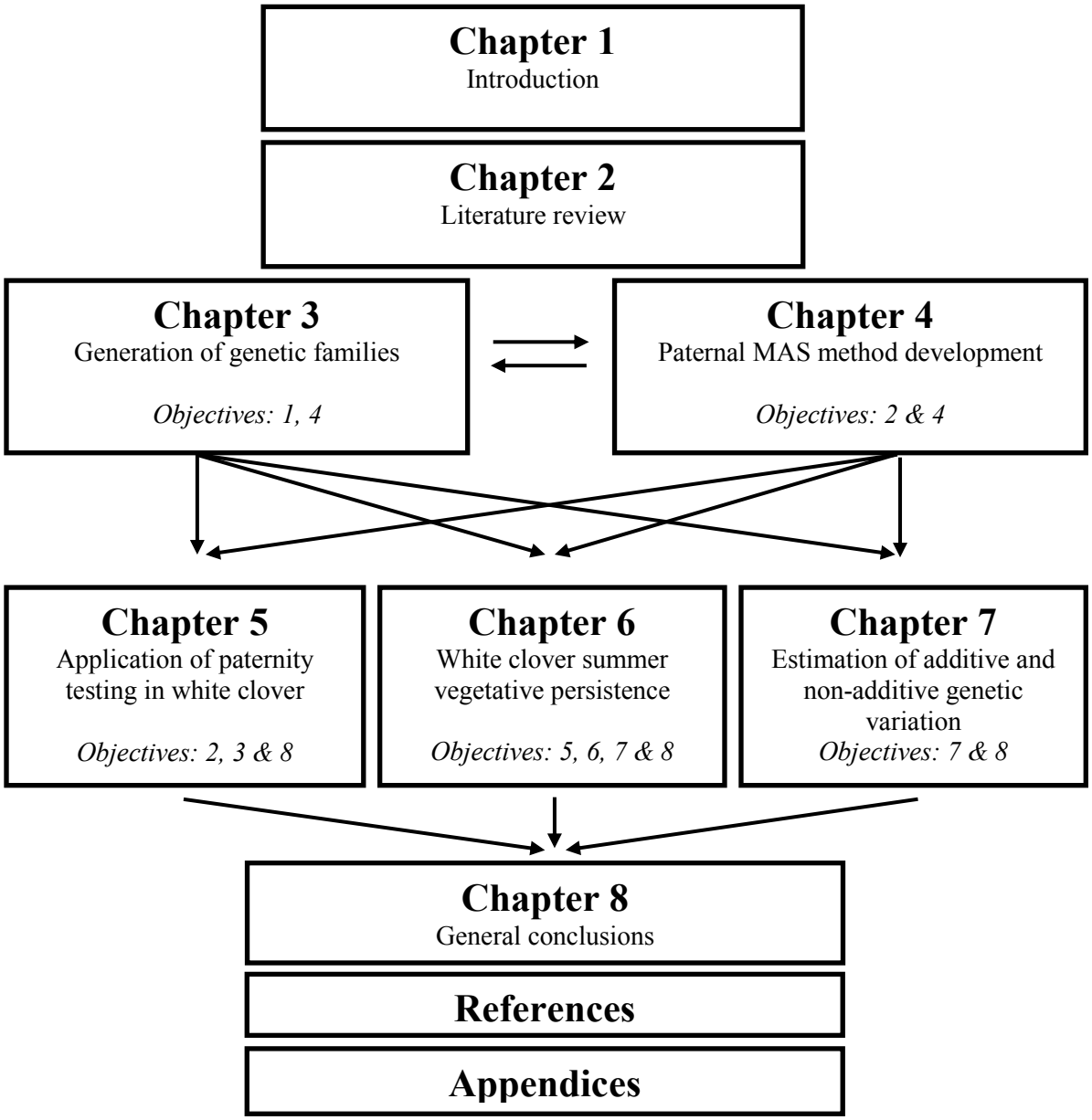


Figure 1.1 Schematic diagram of the thesis structure; depicts the relationships between thesis chapters.

Chapter 2
Literature review

2.1 Origin and systematics of white clover

White clover (*Trifolium repens* L.) is an allotetraploid (2n=4x=32) herbaceous perennial legume with a 1C genome size of 956 Mbp (Atwood and Hill, 1940; Williams et al., 1982a). Chromosome pairing is bivalent and inheritance is disomic (Atwood and Hill, 1940; Williams et al., 1982a). Allopolyploidy in this species is thought to have arisen from the hybridisation of two ancestral diploid species and subsequent chromosome doubling (Casey et al., 2010). Molecular phylogeny analysis has identified *Trifolium occidentale* and *Trifolium pallescens* as probable diploid progenitors (Ellison et al., 2006).

Sexual reproduction in white clover is regulated by a highly polymorphic, gametophytic self-incompatibility system (Casey et al., 2010). The majority of individuals are self-incompatible although a small proportion of the population have been shown to be self-compatible (Atwood, 1940; Atwood, 1941; Atwood, 1942).

The Mediterranean is considered to be the geographical centre of origin for white clover where it covers all of Europe, parts of North Africa and western Asia (Williams et al., 2010). Predominant outbreeding and disomic inheritance has resulted in white clover populations that are comprised of a heterogeneous mixture of highly heterozygous individuals. A high degree of genetic variance has been observed within and between populations in these environments (Williams et al., 2010).

2.2 White clover in New Zealand

2.2.1 Agricultural importance

Within New Zealand pastoral industries, white clover as a companion legume species in swards, is considered to be the keystone to our international competitive advantage (Caradus et al., 1996). As a forage legume, white clover possesses multiple attributes which make it the most important legume in temperate pastoral systems. These attributes include, (i) its ability to fix atmospheric nitrogen in symbiosis with the bacterium *Rhizobium leguminarosum* var. trifolli; reducing the need for nitrogenous fertiliser, (ii) its high protein content and mineral composition for increased animal performance, (iii) its stoloniferous morphology, (iv) its seasonal growth complementarity with ryegrass, and (v) its ability to improve animal feed intake and utilisation rates (Abberton and Marshall, 2005; Caradus et al., 1996).

2.2.1.1 Nitrogen fixation

Nitrogen fixation rates in New Zealand range from 17 kg N/ha/year in unimproved hill pastures to 380 kg N/ha/year in intensively managed pasture (Caradus et al., 1996). Potential nitrogen fixation rates have been illustrated to be much higher (Crush, 1987). From an economic perspective, Caradus et al. (1996) estimated the value of fixed nitrogen from white clover to be worth $1.49 billion to New Zealand's economy.

2.2.1.2 Nutritive value

White clover provides the best quality component of grazed pastures because of its high nutritive and feeding value (Caradus et al., 1996). Compared to perennial ryegrass, the other significant species in most intensive farming swards, white clover has higher concentrations of crude protein (or total N) and readily fermentable carbohydrates, but lower concentrations of lipids, water soluble carbohydrates, lignin, cellulose and fibre (Caradus et al., 1996). Live weight gains of livestock fed white clover are consistently higher than for those fed perennial ryegrass (Ulyatt, 1981). Gross milk yield and its associated components are higher from cows fed white clover than perennial ryegrass (Thomson et al., 1985). Productive advantages can be attributed to both higher voluntary intakes and higher gross efficiency (gain per unit of intake) (Caradus et al., 1996). Cosgrove (2005) simulated that milk solids/ha continue to increase until 60% of grazing swards are comprised of white clover. Current white clover percentages in dairying pastures are less than a third of this figure in New Zealand (Harris et al., 1998), with levels even lower in sheep and beef pastures.

2.2.1.3 Morphology

The stoloniferous habit and phenotypic plasticity of white clover make it an ideal companion species in most grass swards (Woodfield and Caradus, 1994). Its ability to withstand severe defoliation can be largely mitigated by clover leaf type according to the class of stock. Within cultivars a high degree of phenotypic plasticity ensures plants can adapt to environmental changes (Caradus et al., 1993).

Leaf size is highly correlated to the size of the stolons, stolon number and plant habit (Abberton and Marshall, 2010). Small leaf cultivars are considered suitable for continuous hard sheep grazing, medium types for use under rotational grazing and large or very large types for lax cattle grazing or conservation (Abberton and Marshall, 2005).

The relationship between leaf size of cultivars and their suitability for different degrees of grazing intensities are reflective of their associated stolon network. Smaller leaf types tend to

have higher stolon densities and prostrate habits making them less vulnerable to frequent grazing. In contrast, large leaf types tend to be more erect with fewer larger stolons which are susceptible to continuous defoliation (Woodfield and Caradus, 1994). Grazing tolerance, plant persistence and other stress tolerance attributes are by in large tied to the effectiveness of the stolon network, which functions as a storage reserve and anchorage structure to the soil surface (Abberton and Marshall, 2010).

2.2.1.4 Complementary growth

The growth pattern of white clover complements most temperate grasses. Within New Zealand pastoral systems, white clover complements that of perennial ryegrass, as maximum growth occurs in late spring through to summer (Harris, 1987). White clover has a wider and higher optimum temperature for growth (24^{o}C; 18-30^{o}C) than ryegrass (20^{o}C; 18-21^{o}C) (Brock, 2006) which helps it to sustain herbage production after the spring peak in ryegrass. It also maintains high quality forage over summer-autumn when perennial ryegrass losses quality after flowering in the spring (Thomson, 1984).

2.2.2 Limitations of white clover

In regions on the east coast of New Zealand such as Canterbury, where the central mountain range combined with westerly air flows create a rain shadow with <800 mm of annual rainfall (Brown and Green, 2003), white clover herbage yield and persistence is often low and variable between succeeding years (Ayres et al., 1996; Knowles et al., 2003). The low annual rainfall in these regions, combined with the exceeding summer evapotranspiration rates and variable alluvial outwash soils result in potential soil moisture deficits between 200-500mm (Salinger, 2003; Webb et al., 2000). These regions suffer from summer moisture deficits in the majority of years (Brown and Green, 2003) and poor plant survival is largely responsible for white clover herbage yield inconsistencies in these environments (Archer and Robinson, 1989).

If white clover is to make a significant contribution to pasture production systems, consistent yield performance across years and long term persistence is essential. Drought tolerance is a desirable characteristic which has long been a major objective in many arid environments and is becoming increasing important in many other temperate environments which experience periods of summer moisture stress (Abberton and Marshall, 2010). Such sensitivity is likely to be further exacerbated in the context of global climate change (Hofmann and Jahufer, 2011).

Numerous field studies have shown a sharp decline in white clover persistence under water stress (Knowles et al., 2003). In many cases, this sharp decline coincides with the loss of primary tap roots when stolons become fully reliant on their adventitious roots. The limited rooting depth of white clover makes it sensitive to water deficits (Hart, 1987).

In dryland environments where annual rainfall is below 700 mm, white clover is unlikely to survive where other dryland species including lucerne (*Medicago sativa*) and subterranean clover (*Trifolium subterraneum*) have been shown to be comparatively superior (Mills and Moot, 2010). However, scope does exist for improved cultivars that can vegetatively persist in drought conditions and demonstrate improved recovery rates following rain (Knowles et al., 2003).

2.3 Morphology and persistence mechanisms of white clover

White clover development from seed has three distinct morphological phases (Brock et al., 1988; Brock et al., 2000; Thomas, 1987b; Westbrooks and Tesar, 1955):

2.3.1 Morphological phase I

A rosette seedling phase lasting 1-3 months with minimal branching, no stem elongation and no nodal root formation.

2.3.2 Morphological phase II

A tap-rooted expansion phase lasting 1-2 years with rapid stolon elongation, extensive branching and nodal root development. Internodes emerge from the axillary buds found in the axil of leaves on the orthotrophic primary stem of the seedling (Jahufer et al., 2013). Stolons (Figure 2.1) become the basic structural unit of the plant and are characterised by a number of internodes separated by nodes which form from growth at the apical bud (Thomas, 1987a). Each node bears a trifoliate leaf with an erect petiole, two root primordia, and either an axillary bud capable of growing into a lateral stolon (plants vegetative phase) or an inflorescence (plants reproductive phase) (Thomas, 1987b). The number of lateral stolons on a plant increases with age, which in turn leads to the vegetative spread of the plant. Adventitious roots form at nodes when they come into contact with moist soil and provide a degree of nutritional dependence to each lateral stolon.

Figure 2.1 Drawing of main stolon (MS) of white clover, showing axillary buds (AB), lateral branches (LB), and a lateral stolon (LS). S = stipule; Pe = petiole; RT = nodal root primordium; I = inflorescence; P = peduncle. Emerged leaves on the main stolon, and the nodes bearing them, are numbered 1 to 8 (Figure 1.1 by Thomas (1987a)).

2.3.3 Morphological phase III

A ‘mature’ clonal phase when taproots die and large plants fragment into small self-dependent daughter clones. Loss of the primary seedling taproot commences at 12 to 18 months and stolons become dependent on nodal roots to obtain both water and nutrients from the soil (Westbrooks and Tesar, 1955) .

2.3.4 Reproduction

White clover possess two complementary mechanisms of reproduction; regeneration by seedling recruitment and regeneration via stolon propagation and vegetative persistence (Lane et al., 2000).

2.3.4.1 Asexual reproduction

Under favourable seasonal conditions, asexual reproduction in white clover is driven by the continuous process of branching and extension of stolons at their apical ends to replace the older basal sections lost due to decay and death (Jahufer et al., 2013; Lane et al., 2000). Asexual reproduction is a feature of morphological phases II and III, where the fragmentation of large plants into smaller self-dependent daughter clones propagates original mother-plants. Grazing, plant nutrition and competition can be managed to improve the rate of stolon and nodal root formation, while moisture stress, disease and insect pests have detrimental effects on asexual reproduction (Woodfield and Caradus, 1996).

2.3.4.2 Sexual reproduction

Regeneration via seedling recruitment is an important survival strategy for white clover in unfavourable environments where reliable stolon survival is marginal (Lane et al., 2000). The regeneration of seedlings is dependent on adequate seed reserves in the soil from previous years.

Flowering is influenced by a number of factors including genotype, photoperiod, temperature, nutrition, grazing management and available soil moisture (Lane et al., 2000). Inflorescences are produced from axillary buds at nodes, each consisting of 20-150 florets. Inflorescence development is primarily influenced by day length (LDP; long day plant) in germplasm originating from high altitudes, whereas germplasm from low altitudes are more dependent on temperature (Lane et al., 2000; Thomas, 1987c).

White clover is an obligate outcrossing perennial due to its gametophytic self-incompatibility (Brewbaker, 1954), and requires cross pollination via insect mediated pollinators. The presence of effective pollinators is essential for maximising seed yield (Lane et al., 2000). Honey bees (*Apis mellifera* L.) are the main pollinators of white clover, although for plant breeding purposes, breeders often use bumble bees (*Bombus* sp.) to facilitate pollination due to their ease of management in isolation cages (Williams, 1987).

2.3.5 Morphological features associated with water stress tolerance

Ayres (1996) proposed an ideotype for white clover cultivars in dryland environments. The attributes considered important for persistence included high nodal root frequency, tap rootedness, high stolon density, medium large leaves, and early flowering maturity. Jahufer, et al. (1994; 1995; 1997; 1999) demonstrated heritabilities and coefficients for a range of

morphological characters including those key traits proposed by Ayres (1996) in dryland environments.

The development of a strong network of stolons seems to be a pre-requisite of persistence and has become the fundamental feature of white clover populations that are suitable for dryland environments. However, the negative correlation between stolon density and herbage yield complicates the concurrent improvement of both vegetative persistence and yield (Abberton and Marshall, 2010; Caradus and Williams, 1989; Jahufer et al., 1999). The basis of the correlation is unknown, but possible genetic causes include pleiotrophy or linkage. The latter can be improved through selecting recombinant genotypes, while the former is unlikely to be effected by conventional breeding (Jahufer et al., 1999).

2.3.6 Physiological features associated with water stress tolerance

Woodfield and Caradus (1987) summarised three main strategies for increasing white clover survival in drought prone areas. These included; i) drought avoidance, through re-seeding prior to the onset of moisture stress, ii) improved moisture conservation through reduced leaf size, stomatal closure and high cuticular resistance, and iii) improved moisture uptake, through a more extensive, deeper or denser root system, and an increased root to shoot ratio.

Various physiological studies have investigated strategy two (Barbour et al., 1996; Brink and Pederson, 1998; Inostroza and Acuna, 2010). Barbour (1996) reported increased water use efficiency (WUE) of white clover cultivars under increasing soil moisture deficit, but failed to find significant differences among populations within various stress levels. Their results suggest that low genetic variation for WUE exists among white clover cultivars. Similar results were obtained by Brink and Pederson (1998) when they evaluated the response of white clover cultivars to different water gradients in a mixed sward trial.

Inability to control water loss through stomatal closure and cuticular resistance has been previously reported in white clover (Hart, 1987). During dry conditions, white clover leaves rapidly wilt and die (Hart, 1987). Many other plant species reduce water loss by stomatal closure. Turner (1990a) suggested the reduced stomatal closure displayed by white clover under drought may indeed be a survival strategy whereby subsequent leaf dehydration and abscission reduces leaf area and plant transpiration.

Osmotic adjustment of stolons has also been demonstrated in white clover. This physiological mechanism allows for conservation of the organ and regeneration of the plant once water availability improves (Turner, 1990b). It appears there is considerable variation in white

clover for regeneration once water availability improves (Brink and Pederson, 1998; Knowles et al., 2003).

Contrary to both Brink and Pederson (1998) and Barbour et al. (1996), Inostroza and Acuna (2010) demonstrated significant physiological differences in the water status among white clover populations. Nine naturalised populations from southern Chile and two commercial cultivars expressed different levels of stomatal regulation, dry matter partition, transpiration rate, photosynthetic capacity and consequently WUE.

The accumulation of secondary metabolites may also influence the capacity of white clover to withstand moisture stress. Hofmann and Jahufer (2011) reported the association of accumulated flavonoids with morphological types tolerant of stress. Further research by Ballizany et al. (2012b) supported previous findings where the increased induction of quercetin glycosides accumulation in response to water deficit stress was related to retaining higher levels of shoot dry matter production. Ballizany et al. (2014) reported a weak negative correlation between shoot dry matter and quercetin glycoside levels, indicating that combining high DM yield and high constitutive levels of quercetin glycosides for abiotic stress protection is possible.

2.4 Plant genetic resources

Traditionally novel material has been obtained by plant breeders through plant collection expeditions to geographical areas where germplasm with desired traits may be found (Abberton and Marshall, 2010). In addition, hundreds of accessions with and without passport data are available throughout the world in various genebank collections.

Numerous attributes such as stolon number, stolon elongation, internode length, stolon thickness, plant spread, number of rooted nodes, number of nodes, tap root diameter, leaf width, plant habit, cyanogenesis, anthocyanin leaf markings, herbage yield and many other morphological and physiological attributes vary greatly among genotypes and populations (Annicchiarico and Piano, 1995; Caradus and Woodfield, 1990; Jahufer et al., 1995; Jahufer et al., 1997; Jahufer et al., 1999; Lee et al., 1993; Rowe and Brink, 1993; Woodfield and Caradus, 1990).

2.4.1 Germplasm associated with this thesis

The random mating population used in this thesis was developed at AgResearch, Grasslands, Palmerston North, as a breeding pool targeting broad adaptation. The pedigree of this population includes commercial cultivars; Grasslands Tribute (Woodfield et al., 2003),

Grasslands Trophy (Ayres et al., 2007) and Grasslands Saracen, and also two ecotypes collected in the Waikato region of New Zealand.

Grasslands Tribute is a medium-large leaved white clover bred for tough drought tolerance and winter activity in Victoria, Australia (Woodfield et al., 2003). Its parentage includes elite genotypes from eleven half-sib families, half of which originated from a Syrian accession with the remainder from the cultivars Sustain and Crau, as well as Southern Europe germplasm in equal proportions.

Grasslands Trophy is a medium-large leaved cultivar which combines intermediate stolon density with intermediate stolon thickness, and shows high stolon survival and strong autumn regrowth following summer moisture stress (Ayres et al., 2007). Trophy's parentage includes half-sib families from Portugal, Southern France, USA and the Mediterranean. It is considered suitable for temperate dryland environments with 850-1250 mm average annual rainfall.

Grasslands Saracen is yet to be formally recognised in the literature, but it has been referred to as synthetic GC 122 by Jahufer et al. (2009). Saracen is a sister line to Trophy. It is a medium-large leaved cultivar bred from half-sib families which were derived from the best 10% of a worldwide accession breeding pool (140 world-wide accessions) after a period of 3 years of screening at Glen Innes and Armidale, NSW, Australia (Jahufer et al., 2009).

Jahufer et al. (2009) ranked all three commercial cultivars in the top performing group of a current commercial and experimental synthetics dryland trial in south-west Victoria, Australia.

2.5 Plant improvement through conventional breeding

2.5.1 Forage plant breeding methods

Plant breeding is the genetic improvement of plants through a process which is considered to be both a science and an art (Posselt, 2010). The former is a function of Mendel's laws, and the latter is governed by the breeders eye, intuition and creativity (Posselt, 2010). Plant breeding methods that can be used effectively to improve a species, are by in large determined by their mode of reproduction (Vogel and Pedersen, 1993). In forage species, given their relatively short period of domestication and outcrossing nature, most breeding methods have evolved from the breeding of field crops, particularly maize (Posselt, 2010).

Genetic gain is often the basis used to interpret the relative efficiencies of different plant breeding methodologies. The concept of genetic gain is based on the change in the mean

performance of a population that is realised with each cycle of selection (Fehr, 1987). A cycle of selection includes the establishment of a random mating population, development and evaluation of genotypes, selection of elite genotypes, and synthesis of the selected genotypes as parents to form a new population for the next cycle of selection (Fehr, 1987). Variation in the methods used at each of these steps influence the duration of the selection cycle, so breeders often compare various methods on an annual basis to mitigate cycle length bias.

The rate of genetic gain among breeding methods and their implementation in various forage species is dependent on a number of factors. These factors include the heritability of the trait(s) of interest, the biology of the species, the length of the breeding method, the correlation between spaced-plants and their performance in mixed species swards, as well as the available resources to the breeder. The basic equation used to interpret genetic gain, often presented in various forms and termed the breeders equation, is illustrated below;

$$\Delta G = \frac{kc\sigma_A^2}{\sqrt{\sigma_{ph}^2}}$$

where; ΔG= genetic gain or genetic advance, k = standardised selection differential, = σ_A^2 additive variation, c = parental control and σ_{ph}^2= phenotypic variance of the parental population.

The above equation shows that genetic gain can be improved by a number of factors, including; increasing the standardised selection differential (selection intensity), increasing the proportion of σ_A^2in the population, increasing the coefficient of σ_A^2 utilised via increasing parental control or altering family structure, and finally by reducing non-genetic effects such as environmental variance which is pooled into the phenotypic variance (Bernardo, 2010).

2.5.2 Population improvement

Similar to maize breeding (Hallauer et al., 2010a), selection procedures in forages can be divided into two distinct phases; an initial phase to select among source populations, followed by a second phase where selection is carried out within the selected populations. In the first phase of selection, breeders must decide which populations are most suitable for their purposes and allocated resources (Hallauer et al., 2010a). Multiple source populations with desirable attributes may be combined to generate a new base or breeding population. In the second phase, in an effort to maximize the genetic improvement of populations, selections among genotypes within populations are performed.

Recurrent selection methods are typically used in the second phase of breeding which are designed to increase the frequency of desired alleles for quantitatively inherited traits, while maintaining genetic variability for continued genetic improvement (Hallauer et al., 2010a). Lonnquist (1952) defined recurrent selection as "a group of breeding procedures consisting of recurrent cycles of selection for outstanding genotypes with a specific purpose in a heterozygous population and the subsequent recombination of the selected portion of the population".

Recurrent selection within closed breeding populations (intra-population improvement) are focused towards increasing the frequency of favourable alleles within the gene pool itself (Hallauer et al., 2010a), whereas recurrent selection between populations (inter-population improvement) such as reciprocal recurrent selection, are directed at changing gene frequencies of both populations in a complementary way so that a wide range of different types of gene action and interactions can be retained in the crossed population (Hallauer et al., 2010a). The former has been readily adopted in forage species, including white clover, whereas the latter has had little commercial adoption, most probably due to the lack of identified heterotic groups in forage species to capture non-additive gene action.

Selection of individual genotypes as parents for the recurrent population can be evaluated according to their phenotype (phenotypic selection) or on the basis of their progeny performance (genotypic performance). The breeder's choice between the two methods of evaluation is dependent on the trait of interest and the resources available for evaluation.

2.5.2.1 Phenotypic selection

Phenotypic selection methods are based on the performance of individual plants or on the performance of replicated clonal copies of the plant in either rows or replicates (Posselt, 2010). The phenotypes are typically evaluated using either a scored system, in which plants are denoted scores from 1-9 or 1-5 for traits of interest, or the traits are measured directly. The system allows notes to be accumulated for all individual plants, or in larger commercial breeding programmes unwanted plants can be eliminated in each round of inspection to reduce the amount of future scoring (Posselt, 2010). Various modifications to phenotypic selection are outlined below.

2.5.2.1.1 Mass selection

Mass selection is one of the more simplistic and practical breeding methods used in cross-pollinated species (Posselt, 2010). Non-replicated individual plants are evaluated and selected

on a phenotypic basis only. Phenotypic variation among plants includes genetic variation among individuals (both additive and non-additive genetic variation) within the population, as well as all types of environmental variance within and across trial sites (Hallauer et al., 2010a). The environmental effects act as a major limitation to the effectiveness of mass selection, as genotype × environment interactions cofound the genotypic value (Posselt, 2010). For traits with moderate to high heritabilities and insignificant genotype × environment interactions, mass selection has considerable advantages over other selection methods including higher selection intensities, shorter breeding cycles, and a high co-efficient of additive variation (Falconer, 1961; Posselt, 2010).

$$\Delta G_c = \frac{k\ c\ \sigma_A^2}{\sqrt{\sigma_u^2 + \sigma^2 + \sigma_{AE}^2 + \sigma_{DE}^2 + \sigma_A^2 + \sigma_D^2}}$$

where;
ΔG_c = genetic gain per cycle, k = selection intensity and c = parental control value
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_{DE}^2 – dominance × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot, σ^2 – plot-to-plot

2.5.2.1.2 Stratified mass selection

To help alleviate the environmental variance limitation alluded to in mass selection, Gardner (1961) proposed a stratified mass selection method where the use of a gridding system systematically partitions the field into sections. Each section becomes a separate unit, and the criterion for selection is the deviation of each individual phenotype from the mean phenotypic value of all plants within the same stratum (Hallauer et al., 2010a). The modified mass selection method promotes differences among plants within sections to be more likely to be due to genetic differences rather than environmental effects alone (Hallauer et al., 2010a).

Notable examples of stratified mass selection in forage species have been published by Glenn Burton at Tifton, Georgia, who illustrated phenomenal gains in Pensacola bahiagrass (*Paspalum notatum* Flugge var. saurae Parodi) (Burton, 1974; Burton, 1979; Burton, 1982; Burton, 1983; Burton, 1992).

In both phenotypic selection methods, the transfer of elite genotypes from spaced-planted nurseries to a separate isolation block improves genetic gain two fold, by doubling the parental control factor in the genetic gain equation (i.e. control over both maternal and paternal gametes).

$$\Delta G_c = \frac{k\ c\ \sigma_A^2}{\sqrt{\sigma_u^2 + \sigma_{AE}^2 + \sigma_{DE}^2 + \sigma_A^2 + \sigma_D^2}}$$

where;
ΔG_c = genetic gain per cycle, k = selection intensity and c = parental control value
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_{DE}^2 – dominance × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot

2.5.2.2 Genotypic selection

Phenotypic or individual plant selection methods are useful when traits of interest have high heritabilities, are primarily influenced by additive genetic variation and have minimal genotype × environment interactions. For low heritable traits ($h^2 < 0.20$), visual differentiation among individual plants can however become erratic and unreliable (Posselt, 2010). Genotypic or otherwise referred to as family based selection is therefore useful when traits of interest have low heritabilities and are influenced by significant genotype × environment interactions.

The primary difference between phenotypic selection and family based selection is that family selection is based on some type of progeny test (Hallauer et al., 2010a). An important feature of family selection is that selection is based on family means, which are obtained from replicated trials, that are therefore less affected by large environmental variances than individual selections (Nguyen and Sleper, 1983). Family selection is typically conducted across a set of environments using family seed, so that genotype × environment interactions have a less pronounced effect (Hallauer et al., 2010a).

Within forage species, family selection is typically carried out using either half or full-sib families.

2.5.2.2.1 Half-sib families

Two derivatives of half-sib family selection methods include (i), half-sib family selection (HSF), whereby half-sib families are evaluated, followed by the recombination of plants from remnant seed from superior families, and (ii), half-sib progeny trialling (HSPT) where again half-sib families are evaluated, but the recombination of superior parents of the best families are used instead of remnant seed (Casler and Brummer, 2008; Fehr, 1987). HSPT is theoretically twice as effective as HSF per cycle, because the selection unit and the

recombination unit are not the same (i.e. parental control value increased from 1 to 2; for further explanation see parental control in section *2.5.3.1* below).

A third derivative of half-sib evaluation, known as among-and-within-half-sib-family selection (AWF-HS) is an extension of HSF, whereby breeders implement within family selection within the best performing families to make use of the remaining ¾ of the additive variation within half-sib families. AWF-HS is carried out in spaced-planted nurseries, so that phenotypes of individuals within families can be evaluated. Unfortunately, the within family selection component does not allow for the separation between genotypic and environmental variation, unless each progeny of each family is clonally replicated (Aastveit and Aastveit, 1990).

Half-sib families can be obtained via open pollination, polycross, topcross or diallel matings (Allard, 1960). However, methods intended to generate half-sib families for half-sib progeny trialling must be designed to disperse a similar array of tester gametes among all maternal parents, so that variation in progeny performance among the parents tested is primarily due to the genetic potential of the parents and not the genetic contribution of the pollen source (Fehr, 1987). According to selection theory, the covariance among non-inbred half-sib families is ¼ of the total additive variation (σ_A^2) (Fehr, 1987; Hallauer et al., 2010a).

HSF

$$\Delta G_c = \frac{k\ c\ \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_w^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}}$$

AWF-HS

$$\Delta G_c = \frac{k\ c\ \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_w^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}} + \frac{k\ c\ \frac{3}{4}\sigma_A^2}{\sqrt{\sigma_u^2 + \frac{3}{4}\sigma_{AE}^2 + \sigma_{DE}^2 + \frac{3}{4}\sigma_A^2 + \sigma_D^2}}$$

where;
ΔG_c = genetic gain per cycle, k = selection intensity and c = parental control value
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_{DE}^2 – dominance × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot, σ^2 – plot-to-plot
r – number of reps, t – number of environments, n – number of plants per plot

2.5.2.2.2 Full-sib families

Two derivatives of full-sib family selection include; i) full-sib family selection (FSF), where full-sib families are evaluated, followed by the recombination of remnant seed from superior families, and ii) among-and-within-full-sib-family selection (AWF-FS), where again full-sib families are evaluated, followed by the recombination of the best individuals within the best families.

Full-sib families are derived directly from bi-parental matings (paircrosssing). According to selection theory, the covariance among non-inbred full-sib families is ½ of the total additive variation (σ_A^2) and ¼ of the dominance variation (σ_D^2) (Fehr, 1987; Hallauer et al., 2010a). Therefore, when selecting on a family means basis, full-sib family selection (FSF) is twice as effective as half-sib family selection (Casler and Brummer, 2008). Similarly to half-sib family selection, selection within full-sib families (AWF-FS) makes use of the remaining additive variation ($\frac{1}{2}\sigma_A^2$). A significant downfall of full-sib evaluation for intra-population improvement is the bias in family means if significant non-additive variation (dominance) is present in some families.

FSF

$$\Delta G_c = \frac{k\ c\ \frac{1}{2}\sigma_A^2}{\sqrt{\frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_D^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_w^2}{t.r.n} + \frac{\frac{1}{2}\sigma_{AE}^2 + \frac{1}{4}\sigma_{DE}^2}{t}}}$$

AWF-FS

$$\Delta G_c = \frac{k\ c\ \frac{1}{2}\sigma_A^2}{\sqrt{\frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_D^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_w^2}{t.r.n} + \frac{\frac{1}{2}\sigma_{AE}^2 + \frac{1}{4}\sigma_{DE}^2}{t}}} + \frac{k\ c\ \frac{1}{2}\sigma_A^2}{\sqrt{\sigma_u^2 + \frac{1}{2}\sigma_{AE}^2 + \frac{3}{4}\sigma_{DE}^2 + \frac{1}{2}\sigma_A^2 + \frac{3}{4}\sigma_D^2}}$$

where;
ΔG_c = genetic gain per cycle, k = selection intensity and c = parental control value
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_{DE}^2 – dominance × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot, σ^2 – plot-to-plot
r – number of reps, t – number of environments, n – number of plants per plot

2.5.2.2.3 Paternity aided half-sib family selection

Riday (2011) pioneered a novel concept to forage breeding that had only been previously utilised in tree breeding (Lambeth et al., 2001). The concept, which was demonstrated in red clover, extends the amount of information available to the breeder based on traditional half-sib family spaced-planted nurseries. The proposed concept utilises simple Mendelian inherited molecular markers, such as simple sequence repeat markers (SSR's), to identify an individual's father at a given probability when paternal identity is unknown in maternal half-sib families (Gjertson et al., 2007; Riday, 2011). Utilising individual plant phenotypes, known maternity, and molecular marker-determined paternity, maternal and paternal breeding values are calculated, and selection on both parents is achieved (Riday, 2011). Essentially the addition of paternity testing to traditional maternal half-sib family selection doubles the parental control value in the genetic gain formula and improves genetic gain twofold (i.e. breeders have control over both the maternal and paternal gametes for the recurrent population). Theoretically, combined maternal and molecular marker determined paternal selection is equally as effective as HSPT per cycle (Riday, 2011). However, in addition to HSPT, additional phenotypic selection within superior molecular marker determined full-sib families (hereafter referred to as among-half-sib-and-within molecular determined full-sib family selection [AWF-HS+MFS]) can be used to select parents for the recurrent population (see equation below); a process which usually adds an extra year to HSPT. Advantageously, parental plants do not have to be kept in mother nurseries for AWF-HS+MFS which can be problematic for annual species and species that cannot be clonally propagated in HSPT.

$$\Delta G_c = \frac{k_M \ \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_W^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}} + \frac{k_P \ \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_W^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}} + \frac{k_W \ \frac{1}{2}\sigma_A^2}{\sqrt{\sigma_u^2 + \frac{1}{2}\sigma_A^2 + \frac{1}{2}\sigma_{AE}^2 + \sigma_D^2 + \sigma_{DE}^2}}$$

where:
ΔG_c = genetic gain per cycle
k = selection intensity; k_M – maternal half-sib family, k_P – paternal half-sib family, k_w – within family
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_{DE}^2 – dominance × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot, σ^2 – plot-to-plot
r – number of reps, t – number of environments, n – number of plants per plot

2.5.3 Comparing breeding methods

When comparing breeding methods, parental control, effective population size, and genotype × environment interactions must also be considered.

2.5.3.1 Parental control

The parental control (c) factor determines the amount of additive variance that is captured in the recombination of selected individuals or families (Fehr, 1987) (Table 2.1). "Parental control in recurrent selection is the relationship between the plant or seed used for identifying superior genotypes (selection unit) and the plant or seed used for recombination (recombination unit)" (Fehr, 1987). Parental control values range from 0.5 to 2, depending on the selection method and control of the parents for the recurrent population.

Parental control is set to $c = 0.5$ when the selection unit is the same as the recombination unit and only the female parent is selected (Fehr, 1987). This is typical for phenotypic recurrent selection when female plants are selected after pollination, and the maternal seed for the recurrent population has been pollinated by selected and unselected males in the population (i.e. control of one sex only) (Fehr, 1987).

Parental control is set to $c = 1$ when the selection unit is the same as the recombination unit and both parents are selected (i.e. control of both sexes) (Fehr, 1987). This is applicable to phenotypic recurrent selection when female parents are selected before pollination and moved to a separate isolation block for half-sib selection, when remnant half-sib seed is used for recombination, for full-sib family selection, and for selfed families (Fehr, 1987).

Parental control is set to $c = 2$ when the selection and recombination units are not the same (Fehr, 1987). This is only applicable to HSPT when clones of selected genotypes (parental unit) are used for recombination after a progeny test is carried out with their respective maternal half-sib seed.

Table 2.1 Methods of intrapopulation improvement (adapted from (Fehr, 1987; Posselt, 2010)

					σ_g^2		σ_{wg}^2	
Method	**SU**	**TU**	**RU**	**c**	σ_A^2	σ_D^2	σ_A^2	σ_D^2
Mass Selection	Plant	Plant	Population	½	1	1	0	0
Mass Selection*	Plant	Plant	Plant	1	1	1	0	0
HSF	Fam	Plot	HS_{Seed}	1	¼	0	¾†	1†
HSPT	Fam	Plot	HS_{Parent}	2	¼	0	¾†	1†
AWF-HS	Plant	Plant	HS_{Plant}	1	¼	0	¾	1
AWF-HS+MFS	Plant	Plant	HS_{Plant}	1	¼ + ¼	0	½	1
FSF	Fam	Plot	FS_{Seed}	1	½	¼	½†	¾†
AWF-FS	Plant	Plant	FS_{Plant}	1	½	¼	½	¾

SU – selection unit, TU – test/evaluation unit, RU – recombination unit, c – parental control, σ_g^2- genetic variability among individuals or families, σ_{wg}^2- genetic variability within families, σ_A^2- additive genetic variance, σ_D^2- dominance genetic variance, * - recombination in a

separate isolation block (pollen control of both male and female gametes), † - within family variation cannot be utilised, HSF – half-sib family selection, HSPT – half-sib progeny trialling, AWF-HS – among-and-within-half-sib-family selection, AWF-HS+MFS – among-half-sib-and-within-molecular-marker-determined-full-sib-family selection, FSF – full-sib family selection, AWF-FS – among-and-within-full-sib-family selection.

2.5.3.2 Effective population size

Effective population size (N_e) is a function of breeding populations where inbreeding levels are applicable. Hallauer and Mirander (1981) demonstrated that comparisons among breeding methods could be done on either N_e or on the basis of equal selection intensity. Effective population size is different for various breeding methods, depending on the relationship among related individuals (for further explanation on the relative levels of inbreeding per cycle see Posselt (2010)). Short term breeding programmes favour selection intensity, whilst long term breeding programs focus on N_e (Posselt, 2010).

2.5.3.3 Genotype × environment interactions

The performance of plants reflects their genotype, their environmental conditions and the interplay between both. Problematically the contribution of genes that account for the quantitative variation of a trait change across test environments, which often causes genotypes to re-rank in their relative performance (Cooper et al., 1993; Jahufer et al., 2002). From a breeding perspective, the re-ranking of genotypes across environments is a fundamental problem when breeding for broad adaptation (Eisemann et al., 1990). The understanding of both the magnitude and consequences of G ×E interactions for selection provides breeders with a platform to ensure the most efficient breeding strategy is utilised to mitigate these limitations (Jahufer et al., 2002).

The large extent of ecological variation among forage environments requires breeders to evaluate breeding material in multiple environments (Posselt, 2010). Analyses of G × E interactions are usually confined to family based selection methods, where families are evaluated across a set of environments. Whilst clonal replication can also achieve G × E estimates in phenotypic recurrent selection methods, clonal replication is unlikely to be used on a routine basis for a large number of plants due to excessive time and expense (Casler and Brummer, 2008).

In white clover, a range of genotype × environment interactions have been reported in a relatively limited number of studies (Caradus et al., 1993; Jahufer et al., 1999; Jahufer et al.,

2009; Jahufer et al., 2013), which demonstrate the requirement for multi-season and multi-year testing to identify superior genotypes for most agronomic traits.

2.5.4 Types of cultivars

Due to both the obligate outcrossing nature of most forage species and their primarily additive gene action for most agronomic traits (Breese and Hayward, 1972), forage cultivars tend to be either open pollinated varieties or synthetic varieties. While the use of conventional synthetics capture an appreciable amount of heterosis (Allard, 1960), no major attempts have been made to capture higher proportions through the use of hybrids despite considerable attention (Brummer, 1999). The use of hybrids which capture specific combining ability as well as good general combining ability have been proposed for forage species using population hybrids (50% (Barrett et al., 2010) and 75% hybrids (Riday and Krohn, 2010)) and F_1 hybrids (Michaelson-Yeates et al., 1997).

2.5.5 Current breeding methodology in white clover

The fundamental concept of plant improvement is selecting appropriate genotypes that possess characteristics that determine adaption and agronomic performance in the target environment. Unlike crop species where the major breeding objective is to increase grain yield, the objective in white clover breeding is not to solely maximise yield in a monoculture environment but instead to produce a balanced sward with a companion grass species and maintain a reliable, consistent white clover contribution which improves economic returns from animal products (Abberton and Marshall, 2005; Jahufer et al., 2002).

Progress in early white clover breeding programmes was achieved through improving performance within existing ecotypes. Cultivars such as Grasslands Huia proved to be commercially successful for several decades. Superseding cultivars were the result of hybridising persistent ecotypes with more agronomic imported material. The extent of variation within a particular breeding pool has not been large enough to allow rapid gains under selection for desired improvements, and the use of ex *situ* genetic resources has been critical in the development of new varieties (Abberton and Thomas, 2011). The development of adapted breeding pools paved the way for phenotypic recurrent selection programmes which by in large has become the most widely adopted breeding practice (Woodfield and Caradus, 1994).

The breeding success of a white clover cultivar is dependent on the selection criteria used in the programme, while simultaneously utilising realistic evaluation systems which simulate its

intended farming environment (Evans et al., 1996). Mixed species pasture swards make sole improvement of the individual species challenging. Caradus et al. (1989) evaluated the relative merits of both mono-sward spaced-planted nurseries and small mixed species plot trials. Highly heritable traits such as leaf size showed significant correlation across a range of culture conditions due to the limited environment interaction of this trait. However, traits with low heritability such as agronomic performance showed poor correlation between spaced plant and mixed species plot trials (Caradus et al., 1989; Davies, 1970; Davies and Tyler, 1961).

Spaced planted trials have been considered to be relatively unreliable for estimates of agronomic performance (Atwood and Garber, 1942; Davies and Tyler, 1961; Gibson, 1964). However, assessment of elite individuals is the basis of plant improvement. Caradus et al. (1989) suggested that although performance of mixed species plots cannot be adequately predicted from spaced planted trials, there is reasonable correlation between top performing lines in spaced plant nurseries and top performing lines in mixed species plots to justify the use of a such a system in certain circumstances. However, undoubtedly the progressive movement in the mid to late 20th century to evaluating material in competitive swards has enhanced the ability of breeders to identify superior genotypes and populations (Woodfield and Caradus, 1994).

Variation in trial defoliation management between breeding programmes is well documented. Conclusive evidence demonstrates cultivar performance under cutting does not accurately reflect cultivar performance under intensive grazing (Dijkstra and Vos, 1972; Evans et al., 1992). Woodfield and Caradus (1994) suggested breeding programmes which continue to use mechanical defoliation may in fact degrade the agronomic performance of breeding material.

The progress made in white clover over the past century has been largely attributed to the use of progressively more realistic screening and evaluation methods and to the maintenance of a wide genetic base (Woodfield and Caradus, 1994).

2.5.6 Breeding progress in white clover

Conventional plant breeding has steadily delivered improved white clover performance in New Zealand pastoral systems (Woodfield and Easton, 2004). The improvement of white clover performance lies between 6% to 15 % per decade, although the extent of improvement varies among clover leaf types (Woodfield, 1999; Woodfield and Caradus, 1994).

In comparison, the reported genetic gains in other forage crops including perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum* Lam) and alfalfa (*Medicago sativa* L.) are similar or inferior (Hill et al., 1988; Holland and Bingham, 1994; Vanwijk and Reheul, 1991). In alfalfa, tetrasomic inheritance limits the speed of genetic gain, given the increased number of possible allelic combinations at a single heterozygous locus (Hill et al., 1988). The gains achieved in white clover are the result of accumulating genes with improved yield potential, as well as reducing the effects of yield limiting factors (Woodfield et al., 2001).

While genetic gains are superior for white clover within forage species, they are markedly lower than in many cereal species. Casler & Brummer (2008) summarised several reasons for the yield lag in forage crops relative to gain crops which included (i) a longer breeding cycle for forage crops, most of which are perennials, (ii) lack of a "harvest index" trait to aid dry-matter partitioning into the economic product, (iii) inability to exploit heterosis in commercial cultivars, and (iv) the focus on a wide array of economically important traits of forage crops, many which are not specially correlated or may even be negatively correlated with forage yield. The latter is well documented where selection response rapidly decreases as the number of uncorrelated traits increases (Fehr, 1987).

A considerable drawback with all forage species is the manipulation of harvest index. Whereas the harvest index in crop species can be subtly manipulated by partitioning more photosynthates to the grain at the expense of vegetative production, the harvest index in forage species is the total above ground biomass and only moderate changes in harvest index are possible through the redistribution of resources from the roots to shoots (Woodfield and Caradus, 1990).

While short term forage yield is important, it is not always considered first priority in white clover. White clover and perennial ryegrass swards may be used profitably for a number of years and reliability over time of the white clover contribution is more of a key concern of the farmer and, therefore, the breeder (Abberton and Marshall, 2010). Importantly, clover contribution in grazed swards is directly related to animal live weight gain (Chapman et al., 1993). Increased animal performance has been the main objective in most white clover breeding programmes and therefore its associated parameters have become important breeding selection criteria (Woodfield and Caradus, 1994). These attributes include clover content in the sward, total sward yield, persistence and forage quality.

White clover persistence is a key focus of most international breeding programmes. The development of a strong network of stolons is a pre-requisite of persistence and therefore

stolon characters have been a major focus of breeding efforts (Abberton and Marshall, 2010). The breaking of the negative correlation between leaf size and stolon density has long been a goal of breeders in an effort to increase persistence in larger more productive leaf cultivars (Woodfield and Caradus, 1994). Whilst winter hardiness has been of particular focus in UK and Scandinavia to increase persistence (Helgadottir et al., 2008), drought tolerance has long been a major objective in temperate environments with summer moisture stress. Progress has been made in both, but the latter proves somewhat more challenging.

2.6 Plant improvement through molecular breeding

2.6.1 Brief history and development of molecular markers

In the last half-century, molecular markers have evolved at a significant rate. Today in addition to morphological markers, plant breeders have a suite of molecular markers at their disposal to aid in cultivar development (Collard et al., 2005). In particular, DNA markers in plant breeding have opened a new realm in agriculture termed 'molecular breeding'.

The evolution of molecular markers in plant breeding can be traced back to the use of isozyme markers, which were an assay that was based on protein variants in enzymes. The variants could be distinguished by gel electrophoresis according to differences in size and charge caused by amino-acid substitutions (Schlotterer, 2004). Problematically, isozymes were limited by their number of polymorphic markers and their expression was influenced by both environmental and plant developmental changes (Schlotterer, 2004). A more direct molecular marker that relied on DNA variation itself, rather than variations in the electrophoretic mobility of proteins that the DNA encodes, paved way for the arrival of DNA based markers (Schlotterer, 2004).

Variation in individuals screened with DNA markers, arise from different classes of DNA mutations, such as substitution mutations (point mutations), rearrangements (insertions or deletions) or errors in replication of tandemly repeated DNA (Collard et al., 2005). Dissimilar to both morphological and biochemical markers, DNA markers are nearly unlimited in number, they are not influenced by environmental variation, and their expression is not necessary for their detection (Winter and Kahl, 1995).

The discovery and isolation of restriction endonucleases initiated the first generation of molecular markers termed restriction fragment length polymorphisms (RFLPs). RFLPs allowed DNA variation to be assayed due to single base substitutions in the recognition sequence of a restriction enzyme, resulting in unique patterns of restriction fragments

(Schlotterer, 2004). RFLP markers were highly reproducible, co-dominant and conserved across species and genera (Koelliker et al., 2010). The main breakthrough in DNA based molecular markers, however didn't occur until the development of PCR (Saiki et al., 1985). For the first time, PCR enabled any genomic region to be amplified in many individuals without the requirement for cloning and isolating large amounts of pure genomic DNA (Schlotterer, 2004).

With the innovation of PCR, a suite of PCR based markers were developed, including, SSRs, RAPDs, AFLP and SNPs to name the most popular.

SSR - simple sequence repeat markers, or otherwise known as microsatellites are DNA markers that amplify tandemly repeated sequences that are highly polymorphic, co-dominant, abundant and reasonably evenly distributed throughout genomic DNA. Because DNA sequences flanking microsatellite regions are usually conserved among individuals, primers specific for these regions are designed for use in the PCR reaction (Jiang, 2013).

RAPD - random amplified polymorphic DNA, utilise short random primer sequences that bind to and amplify multiple random sites in the genome. Advantageously, because the primers are random, no prior sequence information is required, making them useful markers in species which only primitive sequence data exists. Disadvantageously they are only scored as dominant markers (presence or absence – no information on heterozygotes) and can produce inconsistent results between populations and even laboratories (Koelliker et al., 2010).

AFLP - amplified fragment length polymorphism, are another anonymous DNA marker similar to RAPD, but relies on a more sophisticated technique of detection that has high reproducibility and better transferability (Koelliker et al., 2010). They are based on the selective PCR amplification of restricted fragments from a double digest of genomic DNA under high stringency conditions (Jiang, 2013). They tend to be difficult to score for co-dominance, so they are often treated as dominant markers (Koelliker et al., 2010). In all cases, PCR amplification yields multiple bands that show a presence/absence of variation among individuals (Schlotterer, 2004).

SNP – single nucleotide polymorphism, are markers based on single base pair changes in a DNA sequence. Their high abundance, high polymorphism rate, low cost and suitability for high throughput analyses, make them an ideal molecular marker candidate in breeding programs.

2.6.2 Application of molecular markers in forage plant breeding

The evolution of molecular markers has enabled the construction of linkage maps in many forage species. The first linkage map was published for diploid lucerne (Brummer et al., 1993), and since then, linkage maps for other major forage species including ryegrass (King et al., 2013), meadow fescue (Alm et al., 2003), tall fescue (Saha et al., 2005) and red clover (Isobe et al., 2009) have been published. In white clover, numerous genetic linkage maps have been published (Barrett et al., 2004; Cogan et al., 2006; Griffiths et al., 2013; Jones et al., 2003; Zhang et al., 2007) and an array of genetic markers are available on the public domain. Construction of linkage maps has consequently led to the identification of chromosomal regions that contain genes controlling simple (traits controlled by a single gene) and quantitative traits (QTLs - traits controlled by multiple genes) (Collard et al., 2005).

Notable examples of QTL traits in white clover include seed yield (Barrett et al., 2009; Barrett et al., 2005), forage yield (Jahufer et al., 2012) and stolon characteristics (Faville et al., 2012). Although many QTL's have been identified for numerous traits in forage species, the great majority of these have not been integrated in cultivar development programs (Brummer, 2013). Over the past decade, the adoption of QTL based breeding in forages has been hamstrung by several reasons including poor marker resources and maps in some species, implementation costs, poor transfer of application from bi-parental populations to large scale breeding populations and ultimately the difficultly in integrating markers for several QTL into synthetic cultivar development programs (Bouton, 2010; Brummer, 2013; Riday, 2011).

Recent advances in genomic tools and techniques, such as genotype-by-sequencing (GBS) and genomic selection (GS) respectively, are perhaps resolving some of the issues described above (Brummer, 2013). The adoption of GS in forages, enables focus to be broadened from pyramiding several individual QTL's, to assigning aggregate breeding values to plants based on entire genomes within elite breeding populations (Brummer, 2013). GS promises to offer improved capture of QTLs with small effects, reduce phenotyping, and perhaps the most exciting, multiple cycles of selection within the same time frame as a single phenotypic cycle of selection; improving genetic gain per unit of time (Bouton, 2010; Brummer, 2013). Whilst GS offers considerable promise in major forage species of high economic value such as perennial ryegrass, minor species, in resource limited programs are unlikely to profit from GS technology.

In such species, the roles and implementation of more simplistic marker tools are worthy. Notable examples include the characterisation and use of the self-incompatibility molecular

markers in red clover to help harness hybrid cultivar development (Riday and Krohn, 2010), the use of paternity testing in red clover to increase genetic gain in plant vigour (Riday, 2011) and seed yield (Vleugels et al., 2014), and paternity testing in lucerne to mitigate or utilise self-fertilisation and inbreeding (Riday et al., 2015; Riday et al., 2013). In addition, the use of markers to assess the level of diversity within germplasm and cultivars such as those within white clover (George et al., 2006; Kolliker et al., 2001), is an invaluable breeding tool.

2.7 Estimating genetic parameters and genetic gain computations

2.7.1 Expected gain computations

The ideal scenario to compare alternative breeding methods would be to empirically measure realised genetic gain in terms of the mean performance of a population among a range of different breeding methods. Problematically, the direct comparisons of breeding method efficiencies are time consuming, laborious and in most cases far beyond practicality for most species. Maize is one of a few exceptions, where alternative breeding methods have been compared empirically (Weyhrich et al., 1998). In forage species however, and in most other species, a common way for breeders to access the merits of alternative breeding methods is to compute the amount of genetic gain using mathematical equations listed in the previous sections (Fehr, 1987). These mathematical equations or prediction models are run using genetic parameters estimated from random mating populations to estimate their theoretical response to selection.

2.7.2 Mating designs

In order to simulate the expected genetic gain per cycle or per year for various breeding methods, accurate estimates of genetic parameters are required from a random mating population using specifically designed mating designs. Parameters of interest include those that contribute to environmental variance and those that contribute to genetic variance ($\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2$); where σ_G^2 is the genetic variance, σ_A^2 is the additive variance, σ_D^2 is the dominance variance, and σ_I^2 is the epistasis variance (Hallauer et al., 2010b).

Mating designs generate progenies that are evaluated for the estimation of components of variance. With known relationships among relatives (families), the components of variances can be translated into components of genetic variance using the known covariance's among relatives that are specific to the mating design (Hallauer et al., 2010b). These genetic parameters can then be used to predict the response to selection.

Progenies should be evaluated over a set of environments with appropriate experimental designs in order to estimate not only components of genetic variation, but also components of environmental variance (Hallauer et al., 2010b). Using appropriate experimental designs combined with multi-site trials will further improve the estimates of genetic variance by the separation of non-genetic variation such as genotype × environment interaction and sampling error.

2.7.2.1 Assumptions of mating designs

In order to adequately interpret the genetic parameters estimated from mating designs, the following assumptions about the sampled population are necessary (Hallauer et al., 2010b);

1) Normal Mendelian diploid inheritance

2) No maternal effects

3) Linkage equilibrium

4) Non-inbred relatives

5) Random selection of parents and relatives

6) No correlation of environmental effects with relatives

7) No epistasis

8) Arbitrary allelic frequencies

2.7.3 Genetic experiments in white clover

Genetic parameters such as heritability have been estimated for a number of key traits in white clover. The use of clonally replicated genotypes to estimate broad sense heritabilities of morphological attributes in white clover have been routinely practiced (Annicchiarico and Piano, 1995; Caradus and Woodfield, 1990; Lee et al., 1993; Rowe and Brink, 1993; Woodfield and Caradus, 1990). To a lesser extent, estimates of narrow sense heritabilities in white clover have also been conducted using parent offspring regression, correlation analysis and regression analysis of progeny (Annicchiarico et al., 1999; Caradus and Chapman, 1996; Woodfield and Caradus, 1990).

Despite the high frequency of studies reporting heritability estimates, relatively few estimates have been obtained from random mating populations that have been evaluated across multiple

environments using mating designs intended to partition additive and non-additive genetic effects. Subsequently, information on the relative importance of additive and non-additive genetic effects for key traits is limited in white clover (Jahufer et al., 2002). Notable exceptions in white clover include the investigation of floral attributes (Hill et al., 1989), the investigation of combining ability for response to phosphorus (Caradus et al., 1992), and the investigation of inter-specific competition on the inheritance of white clover agronomic traits (Hill, 1993; Hill and Michaelson-Yeates, 1987); all of which used the diallel mating design.

Jahufer, et al. (2002) suggested that the use of structured mating designs such as the North Carolina designs (Comstock and Robinson, 1948) will enable the estimation of the relative contributions of both additive and non-additive effects to total genetic variation for agronomic traits regulating vegetative persistence and herbage yield.

In other forage species, the relative proportion of additive to non-additive genetic variation is also limited, although crude estimates have been reported in ryegrass (Breese and Hayward, 1972), tall fescue (Piano et al., 2007), meadow fescue (Simonsen, 1977), lucerne (Riday and Brummer, 2002a; Riday and Brummer, 2002b; Riday et al., 2002) and switchgrass (Bhandari et al., 2010).

The advances in heritability parameters and analysis of genotype × environment interactions have helped to provide more accurately defined breeding objectives and strategies for white clover improvement in target environments (Ayres et al., 2007). The recent development of Grasslands Trophy for Australian dryland pasture environments is a relevant example (Ayres et al., 2007).

2.8 Summary

From the review of the literature, the following conclusions can be made:

- White clover is a significant keystone to the competitive advantage of New Zealand's pastoral industry and warrants further plant breeding attention to continue improving its economic traits.
- White clover herbage yield and vegetative persistence is often variable and limited between succeeding years, which is often exacerbated by summer moisture stress.
- An understanding of the quantity and type of genetic variation governing summer moisture stress recovery is limited in the literature.

- Phenotypic recurrent selection has been the most widely adopted breeding method for population improvement in white clover.

- In addition to phenotypic recurrent selection, there is other potentially more robust and efficient breeding methods to improve genetic gain for low heritable traits in forages.

- The integration of molecular markers into cultivar development programs has been poor despite significant research and available resources for breeders.

- The lack of genetic experiments conducted across multiple environments and random mating populations limits the ability for breeders to estimate the relative efficiencies of different breeding methods in white clover.

- The lack of genetic experiments conducted in random mating populations of white clover hinders the understanding of the relative contributions of additive and non-additive genetic variation for most agronomic traits.

In addition to the above review of the literature, supplementary reviews are also provided with each results chapter (introduction). These introductions refine the literature relevant to each chapter, and provide more detailed discussions.

Chapter 3
Generation of half-sib and full-sib families

3.1 Introduction

Plant breeders are focused on increasing the frequency of favourable alleles associated with target traits to maximize genetic gain. This is achieved through the application of a range of different breeding strategies, dependent on the mode of mating, self or cross pollination. White clover (*Trifolium repens*) is an obligate outcrossing perennial due to its gametophytic self-incompatibility mechanism (Brewbaker, 1954), and requires cross pollination via insect mediated pollinators. Honey bees (*Apis mellifera* L.) are the main pollinators of white clover, although in New Zealand, plant breeders often use bumble bees (*Bombus* sp.) to facilitate pollination due to their ease of management in isolation cages (Williams, 1987).

Controlled crosses amongst a select group or pairs of genotypes are facilitated by breeders using either hand or bee mediated techniques. Hand pollination entails the pollen of a compatible genotype(s) being deposited on the stigma of a target floret using a small brush, a toothpick or even a dehiscing anther held in tweezers (Williams, 1987). Often a time opportunity cost is associated with this method and in resource-limited programmes and in polycrosses, more effective crossing is achieved via bee pollination. Pollination using bees is usually conducted in isolated facilities to eliminate the immigration of pollen from foreign sources.

A method regularly used to inter-pollinate white clover genotypes was described by Williams (1987), whereby wild bumble bees are caught, rinsed with luke-warm water and released into isolation cages surrounded with bee-proof mesh. Rinsing the bees with luke-warm water removes any residual white clover pollen by bursting the pollen grains upon contact. The technique provides quick, cheap and readily accessible pollinators. Despite the techniques' robustness, care must still be taken to identify self-fertile or male sterile genotypes within crosses to avoid inbreeding depression. To the authors knowledge, only one study (Atwood, 1943) has investigated outcrossing and self-fertilisation rates within isolated cages for bi-parental white clover progenies pollinated by bumble bees.

Some studies (MichaelsonYeates et al., 1997; Riday et al., 2013) have also investigated the pollen distribution by bees using non-bias analysis methods within forage legume species polycrosses, including white clover. Polycross mating designs have been extensively used by

forage breeders to generate half-sib families for evaluation (Vogel and Pedersen, 1993). Evaluation of half-sib families enables identification of parental general combining ability (Fehr, 1987). In order to evaluate half-sib families, breeders assume random mating occurs among the half-sib parents, whereby the tester pollen source is an equal heterogeneous mixture of pollen from each contributing paternal genotype. Recent advances in molecular techniques now allow breeders to test this assumption.

This chapter reports on the: (i) success of using wild bumble bees (*Bombus* sp.) for generating bi-parental progenies and their associated outcrossing, self-fertilisation and contamination rates, (ii) success of using wild bumble bees for generating half-sib family progenies and their associated outcrossing, self-fertilisation and contamination rates, and (iii) the distribution of pollen within isolated polycross cages using paternity testing and the implications this has on downstream breeding applications.

3.2 Materials and methods

3.2.1 Experimental site

All crosses for the generation of progeny evaluated in the reported studies were carried out at AgResearch Grasslands, Palmerston North, New Zealand in the summer of 2011. The isolation cages for both the polycrosses and bi-parental crosses consisted of bee-proof mesh supported by aluminium frames. The cages were secured to a concrete floor and overlaid with capillary carpet to distribute moisture from a trickle-feed irrigation system which ensured a continuous supply of water to the potted flowering plants.

3.2.2 Germplasm

In 2003, ten genotypes from five germplasm accessions (cv. Tribute, cv. Saracen, cv. Trophy and two Waikato ecotypes) were polycrossed by bumble bees under isolation in cages to produce F_1 generation progeny. Equal numbers of seeds from each of the individual plants used in the polycross were mixed together to constitute a balanced bulk of the F_1 generation and the seed was stored at 4^{o}C. In 2008, 80 randomly sampled genotypes that were grown from the F_1 generation balanced bulk were subjected to a second round of random mating to reduce linkage disequilibrium and achieve Hardy-Weinberg equilibrium (Fehr, 1987). Equal seed quantities from each of the maternal F_2 generation half-sibs were combined to form an F_2 generation balanced bulk which was stored in a refrigeration unit at 4^{o}C. In June 2011, 225 seeds were randomly sampled from the F_2 generation balanced bulk, scarified using 150 grit

sandpaper, and planted at a depth of 0.5 cm into root-trainers containing a seed raising potting mix. The population was established in a glasshouse for five weeks. The glasshouse had a minimum temperature of 13^{o}C and a maximum temperature of 25^{o}C.

3.2.2.1 Polycross germplasm

Five weeks after establishment, 70 randomly selected plants from the original 225 were transplanted from root-trainers to PB 1 ½ planter bags (1 L) containing a peat and sand mix with a three month slow release Osmocote fertiliser (Everris International B.V) and maintained under glasshouse conditions. On 31/08/2011 two stolon growing points (≥25mm) from each parental genotype were cut and transplanted into 300mm × 500mm trays at AgResearch Grasslands, Palmerston North. The stolon cuttings were maintained under glasshouse conditions for a further three weeks before being transplanted into PB3 (1.7 L) planter bags and simultaneously transferred onto a well-drained concrete area outside.

3.2.2.2 Bi-parental crosses germplasm (North Carolina I mating design)

Bi-parental crosses were carried out according to the North Carolina I mating design proposed by Comstock and Robinson (1948), where ten genotypes nominated as 'males' were mated to a different set of four genotypes each nominated as 'females' (Figure 3.2). The mating design produced four full-sib families per 'male', a total of ten half-sib families (male parent in common), and 40 full-sib families among 'males'.

3.2.2.2.1 Males

The methodology as described in *3.2.2.1* was used to propagate four stolon cuttings for each of the 'male' bi-parental progeny parents on 31/08/2011.

3.2.2.2.2 Females

On the 31/08/2011, 50 random plants from the remaining 225 were transplanted from root-trainers to PB 3 (1.7 L) planter bags containing a peat and sand mix with a three month slow release Osmocote fertiliser. The plants were transferred to an outside concrete area at AgResearch Grasslands, Palmerston North.

3.2.3 Mating designs and seed harvesting

3.2.3.1 Polycross

In mid-November 2011, 20 randomly genotypes and their duplicate clone were transferred into a glasshouse at AgResearch Grasslands to encourage prolific and synchronised flowering.

On 5/12/2011, the clones from each of the 20 genotypes were placed into two separate isolation cages, measuring 1830 × 1830 × 1830 mm. Plants were placed at 30cm intervals horizontally and 40cm vertically. Each isolation cage contained one clone of each genotype in a randomised position as shown in Figure 3.1.

Isolation Cage 1				
5	1	17	19	2
4	10	11	3	12
13	8	9	18	16
15	6	20	14	7

Isolation Cage 2				
12	18	8	4	17
19	15	16	9	14
5	20	3	6	1
11	7	10	2	13

Figure 3.1 Polycross isolation cage layout. Numbers represent individual genotypes. Each genotype is represented by a single clone in each cage.

Ten wild bumble bees (*Bombus* sp.) were caught from nearby flower gardens, rinsed with clean warm water and introduced into each isolation cage. The bubble bees were monitored daily and new bees were introduced to replace any that had died. Pollination commenced on the 5/12/2011 and continued until the 13/01/12. After pollination, the clones were removed from their respective isolation cages and placed in a bee-free glasshouse. Seed heads were harvested four weeks from the last day of pollination to allow inflorescences to mature. Seed harvested from each plant was maintained separately.

Plate 3.1 White clover flowering clones inside isolation cage 1 during pollination.

3.2.3.2 Bi-parental crosses (North Carolina I mating design)

In mid-November 2011, ten randomly selected sets of 'male' clones (10 genotypes × 4 clones) were transferred into a glasshouse at AgResearch Grasslands to encourage prolific and synchronised flowering. In addition, 40 genotypes designated as 'females' were transferred into the glasshouse to synchronise flowering with the 'male' parents. In December 2011, the crosses were carried out according to the North Carolina I mating design structure (Comstock and Robinson, 1948). The 40 'male' clones were placed into separate 860 × 630 × 470 mm (height, depth, and width respectively) isolation cages with a randomly selected single 'female' plant. Four clones of each 'male' enabled four separate bi-parental crosses per 'male' genotype, in four different isolation cages (Figure 3.2).

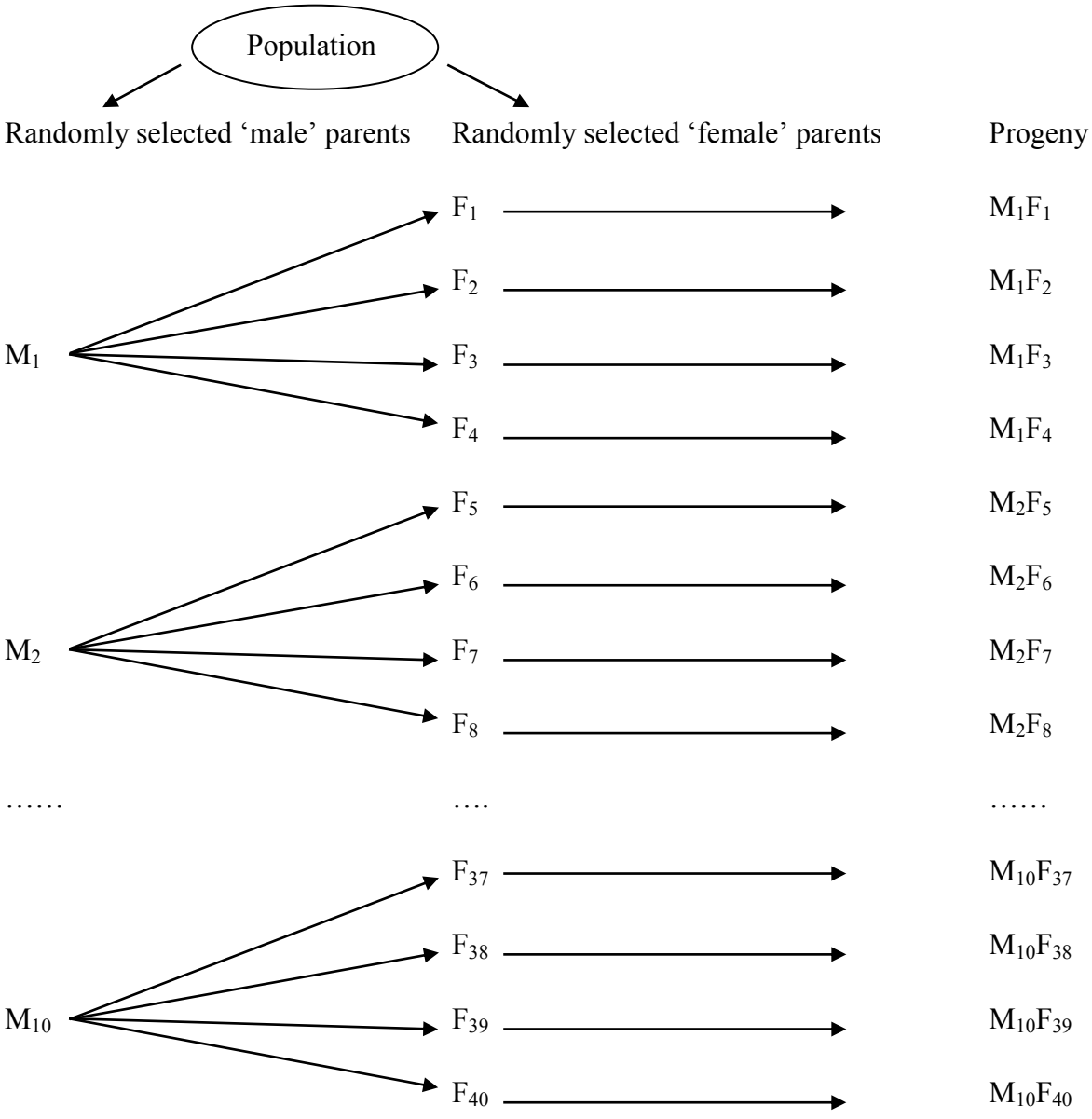


Figure 3.2 Derivation of progenies in a North Carolina I mating design. Each 'male' parent was crossed to different 'female' parents from the same population. Illustration adapted from Fehr (1987).

One wild bumble bee was caught, rinsed with clean warm water and introduced into each 'male' × 'female' isolation cage. The bumble bees were monitored daily and new bumble bees were introduced if existing bumble bees had died. Pollination continued for three weeks. The mating design produced four full-sib families per 'male', a total of ten half-sib families ('male' parent in common), and forty full-sib families. Post pollination, plants were removed from the isolation cages and transferred to a bee-proof glasshouse. Seed heads were harvested four weeks from the last day of pollination to allow inflorescences to mature.

3.2.4 Seed preparation

Harvested mature seed heads (≥ 28 days old) from both polycross and bi-parental plants were manually threshed, cleaned, their seed weight recorded and stored at 4^{o}C. Seed harvested from each full and half-sib family was maintained separately.

3.2.4.1 Polycross progeny

In April 2012 0.05g of seed per maternal clone was scarified with 150 grit sand-paper, distributed evenly into petri-dishes containing pre-moistened filter paper, and incubated at 20^{o}C for 16 hours. Forty germinated seedlings were transplanted into root-trainers at a depth of 0.5cm containing a peat and sand mix with a three month slow release Osmocote fertiliser and maintained under glasshouse conditions. Maternal half-sib families 14 and 20 had insufficient seed in one of their two maternal clones to germinate 40 seedlings per clone. To germinate a total of 80 seeds per maternal half-sib in families 14 and 20, extra seed was germinated from the highest seed yielding clone.

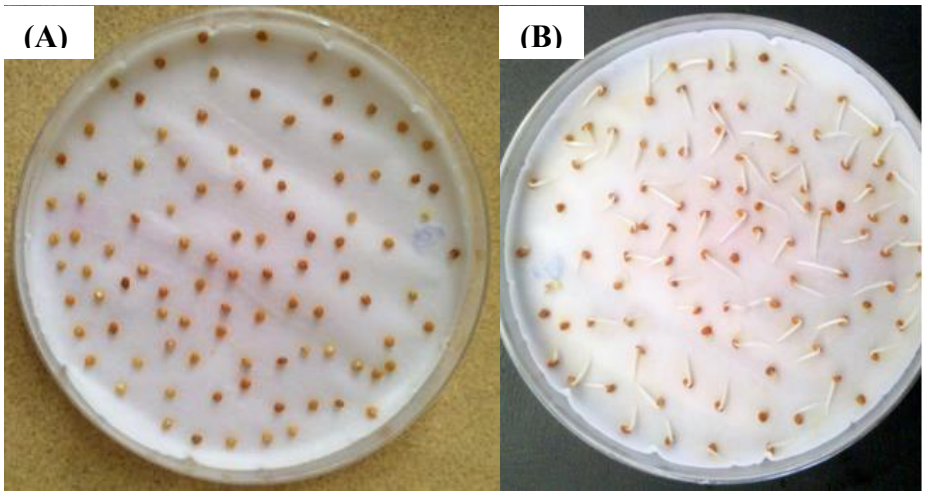


Plate 3.2 Pre (A) and post (B) incubation of white clover maternal half-sib seed.

3.2.4.2 Bi-parental crosses

In May 2013, 0.1g maternal seed from one randomly selected full-sib family per 'male' half-sib family ('male' parent in common) was scarified with 150 grit sand-paper, distributed evenly into petri-dishes containing pre-moistened filter paper, and incubated at 20^{o}C for 16 hours. After germination, 60 seedlings were immediately transplanted into 300 mm × 500 mm propagation trays containing a mix of peat and sand with a three month slow release Osmocote fertiliser and maintained under glasshouse conditions.

3.2.5 Paternity testing

DNA extractions, marker selection, PCR, paternity analysis and assignment are discussed in Chapter 4. Chapter 4 was run concurrently with this chapter, but focused on the details of the molecular biology methodologies.

3.2.6 Statistical analyses

Statistical analyses were carried out using Genstat v14 (GenStat, 2003). Cross-pollinated and self-pollinated percentages among maternal clones were calculated using proportional statistics. Goodness of fit tests were used to test whether the adjusted observed progeny counts differed significantly from the expected progeny counts among paternal half-sib families. The number of paternity determined progeny per parental genotype was plotted against their respective seed yield, and a linear regression model was used to fit a straight line to the data (Figure 3.4). Likewise, a linear regression model was used for Appendix A.2, where the number of harvested inflorescences per clone was plotted against their respective seed yield. A linear model was used for Appendix A.3, where the number of inflorescences per clone was plotted against the number of their respective paternal progeny.

To account for unequal maternal sampling and paternal assignment for each maternal half-sib clone, observed progeny counts for each paternal genotype were adjusted accordingly (Riday et al., 2013). Logarithmic models were used to fit curves (Figures 3.5 and Appendix A.1), where the distance of pollen donors from recipient maternal parents were plotted against the mean adjusted observed progeny counts per full-sib family and the adjusted observed progeny counts per full-sib family, respectively. Negative logarithmic models were used to fit curves to Figures 3.7 and 3.8 where the accumulated paternal contribution of recipient maternal parent half-sib progeny (%) was plotted against the distance of pollen donors from recipient maternal parents and number of paternal parents, respectively. Exponential models were used

to fit curves to Figure 3.6, where the distance of pollen donors from recipient maternal parents were plotted against total observed progeny counts per full-sib family.

3.3 Results

3.3.1 Polycross seed yields

Seed yields for each maternal clone ranged from 0.04 g to 6.82 g plant^{-1} in isolation cage 1 (0.18 to 30.39 % of the cages total seed yield) (Figure 3.3), with a mean of 1.12 g plant^{-1} and a median of 0.47 g plant^{-1}. In isolation cage 2, seed yields for each maternal clone ranged from 0.07 g to 2.62 g plant^{-1} (0.44 to 16.6 % of the cages total seed yield) (Figure 3.3) with a mean of 0.79 g plant^{-1} and a median of 0.71 g plant^{-1}.

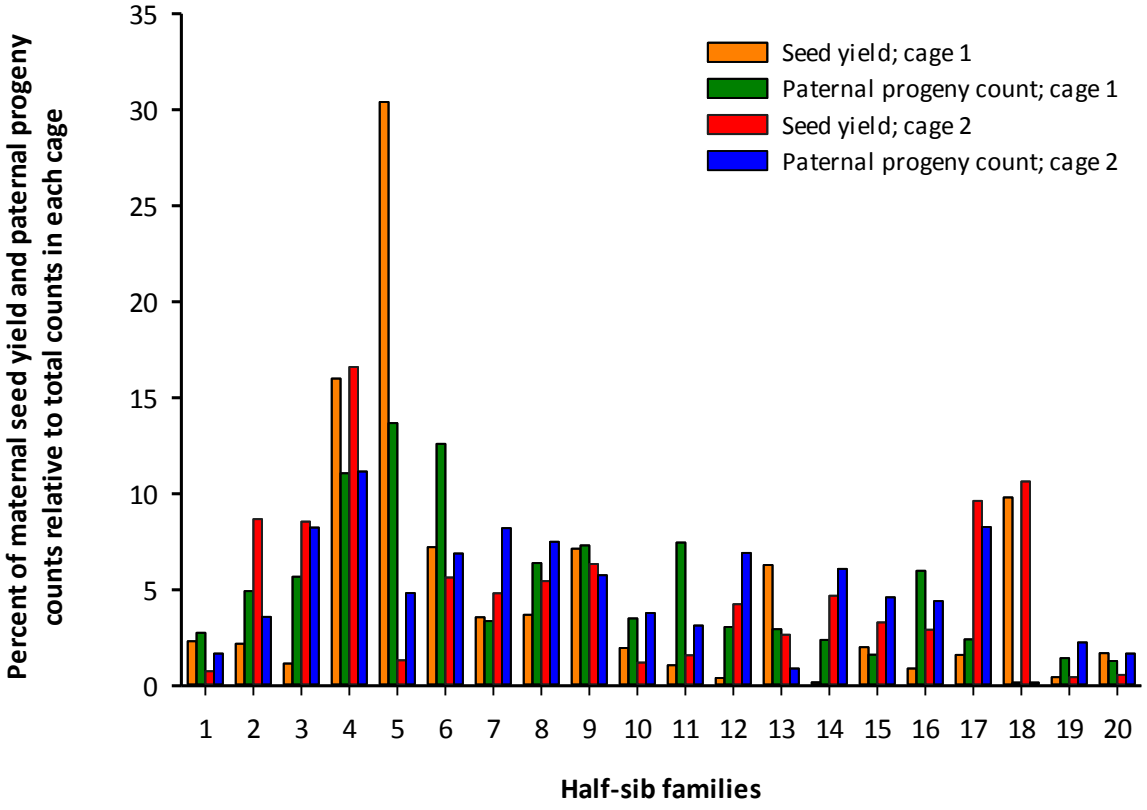


Figure 3.3 Seed yield and number of paternal progeny counts of 20 white clover half-sib families generated in two isolated polycrosses pollinated by bumble bees (*Bombus sp.*). Seed yield and adjusted observed progeny counts are presented as a percentage relative to the total seed yield and total adjusted observed progeny per isolation cage.

Observed progeny counts for each paternal genotype ranged from 1 to 84 in isolation cage 1 and 1 to 69 in isolation cage 2, although a true measurement of observed progeny counts per paternal genotype is dependent on equal sampling of all maternal genotypes (Riday et al.,

2013). Adjusted observed progeny counts for each paternal genotype ranged from 0.96 to 81.52 in isolation cage 1 (0.16 to 13.68 % of the total assigned paternal progeny) and 0.96 to 65.73 in isolation cage 2 (0.16 to 11.2 % of the total assigned paternal progeny) (Figure 3.3 and Table 3.2). In both isolation cages, paternal genotype 18 had the lowest observed progeny counts, with only one progeny per isolation cage.

Of the total progeny that had successfully assigned paternity (1185), 1176 were identified as cross-pollinated and 9 as self-pollinated (Table 3.1). No alleles other than the genotyped parental alleles were identified in either the successfully or unsuccessfully paternity assigned progeny.

Table 3.1 Maternal parents from duplicate 20 parent white clover isolated polycrosses pollinated with bumble bees (*Bombus* sp.) Per maternal genotype; bulked half-sib seed (g), number of progeny assigned paternity, number of outcrossing progeny, outcrossing pollination %, number of self-pollinated progeny and % of self-pollinated progeny.

Maternal genotype	Bulked seed yield (g)	Progeny paternity inferred	Outcrossing progeny count	Cross pollinated progeny %	Selfed progeny count	Selfed progeny %
1	0.64	61	61	100	0	0.00
2	1.86	63	63	100	0	0.00
3	1.61	55	55	100	0	0.00
4	6.21	64	63	98.4	1	1.56
5	7.03	61	60	98.4	1	1.64
6	2.51	56	55	98.2	1	1.79
7	1.56	62	62	100	0	0.00
8	1.69	55	55	100	0	0.00
9	2.60	44	41	93.2	3	6.80
10	0.63	61	61	100	0	0.00
11	0.49	62	62	100	0	0.00
12	0.76	62	62	100	0	0.00
13	1.83	63	63	100	0	0.00
14	0.78	54	54	100	0	0.00
15	0.97	59	57	96.6	2	3.39
16	0.66	60	60	100	0	0.00
17	1.88	63	63	100	0	0.00
18	3.88	59	59	100	0	0.00
19	0.17	59	59	100	0	0.00
20	0.47	62	61	98.4	1	1.61
Total	**38.23**	**1185**	**1176**	**99.24**	**9**	**0.76**

3.3.2 Maternal seed yield and pollen contribution

There was a positive linear relationship ($P < 0.0001$) between maternal seed yield and the number of adjusted observed paternal progeny counts (Figure 3.4). Excluding the low siring parent genotype 18, the regression co-efficient strengthened to 0.54. Genotypes with more flowers also tended to have higher maternal seed yields ($P < 0.0001$) (Appendix A.2). There was a linear relationship between the number of harvested inflorescences per plant (clone) and the number of paternal parents per half-sib family ($P < 0.05$) (Appendix A.3).

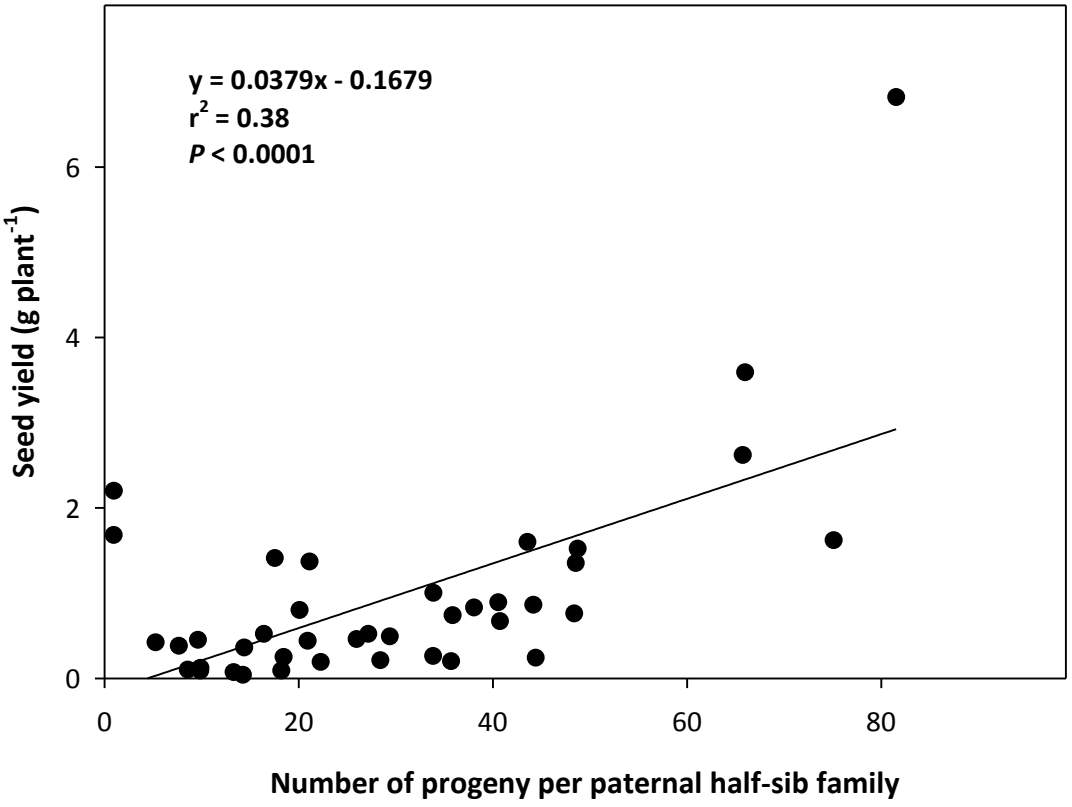


Figure 3.4 Relationship between seed yield per clone and the number of progeny sired in two 20 parent white clover polycrosses pollinated by bumble bees.

3.3.3 Paternal outcrossing counts

The adjusted observed progeny counts among paternal genotypes were not uniform in both isolation cages. Adjusted observed outcross paternal progeny in each isolation cage deviated (χ^2 test, $P < 0.001$) from expectation in a random mating population (Table 3.2).

Table 3.2 Paternal parents from duplicate 20 parent white clover isolated polycrosses pollinated by bumble bees (*Bombus* sp.). Per paternal genotype; expected progeny, observed progeny, adjusted observed progeny and % deviation from expected for all paternal assigned progeny in isolation cages one and two.

	Paternal assigned progeny (Cage 1)				Paternal assigned progeny (Cage 2)			
Paternal genotype	**Expected**	**Observed**	**Adjusted observed**	**% Deviation from expected**	**Expected**	**Observed**	**Adjusted observed**	**% Deviation from expected**
1	29.8	17	16.40	-21.60	29.45	11	9.86	-31.14
2	29.8	30	29.37	0.34	29.45	19	21.12	-17.64
3	29.8	39	33.83	15.53	29.45	51	48.52	36.37
4	29.8	63	65.97	56.03	29.45	69	65.73	66.75
5	29.8	84	81.52	91.48	29.45	23	28.40	-10.89
6	29.8	60	75.09	50.97	29.45	43	40.53	22.87
7	29.8	24	20.08	-9.79	29.45	50	48.35	34.68
8	29.8	38	38.05	13.84	29.45	46	44.17	27.93
9	29.8	47	43.54	29.03	29.45	34	33.88	7.68
10	29.8	22	20.91	-13.16	29.45	23	22.27	-10.89
11	29.8	43	44.41	22.28	29.45	16	18.45	-22.70
12	29.8	18	18.20	-19.92	29.45	42	40.71	21.18
13	29.8	17	17.53	-21.60	29.45	6	5.25	-39.58
14	29.8	15	14.24	-24.98	29.45	35	35.84	9.37
15	29.8	9	9.61	-35.11	29.45	28	27.15	-2.45
16	29.8	35	35.69	8.78	29.45	23	25.95	-10.89
17	29.8	17	14.38	-21.60	29.45	45	48.71	26.24
18	29.8	1	0.96	-48.61	29.45	1	0.95	-48.02
19	29.8	9	8.56	-35.11	29.45	14	13.30	-26.08
20	29.8	8	7.64	-36.79	29.45	10	9.85	-32.83
Total	596	596	596	-	589	589	589	-

3.3.4 Pollen distribution

The positioning of the paternal parents relative to the recipient maternal parents (within the two isolation cages) influenced outcrossing success. Paternal parents that were closer in proximity to the recipient maternal parents had higher adjusted full-sib family progeny counts than paternal parents at further distances ($P < 0.0001$) (Appendix A.1). Transforming the raw data to means, 73% and 64% of the variation among full-sib family progeny counts was explained by the distance of the paternal parent relative to the recipient maternal parent (also referred to as inter-full-sib parent pollination distance (Riday et al., 2013)) in the isolation cages, respectively (Figure 3.5).

Due to the polycross dimensions and plant arrangement within the isolation cages (rectangle), a higher frequency of full-sib combinations existed at closer inter-full-sib parent pollination

distances than at further distances within the isolation cages (see number of data points per distance in Appendix A.1). To demonstrate the pollination pattern within the isolation cages without ignoring these frequency discrepancies, total adjusted full-sib family progeny counts were plotted against inter-full-sib family distance (Figure 3.6). An exponential model fitted to the data (Figure 3.6) revealed a compounding effect where the total number of outcross full-sib progeny at each distance was a function of both the distance of paternal parents from recipient maternal parents and the number of paternal parents at each increasing distance.

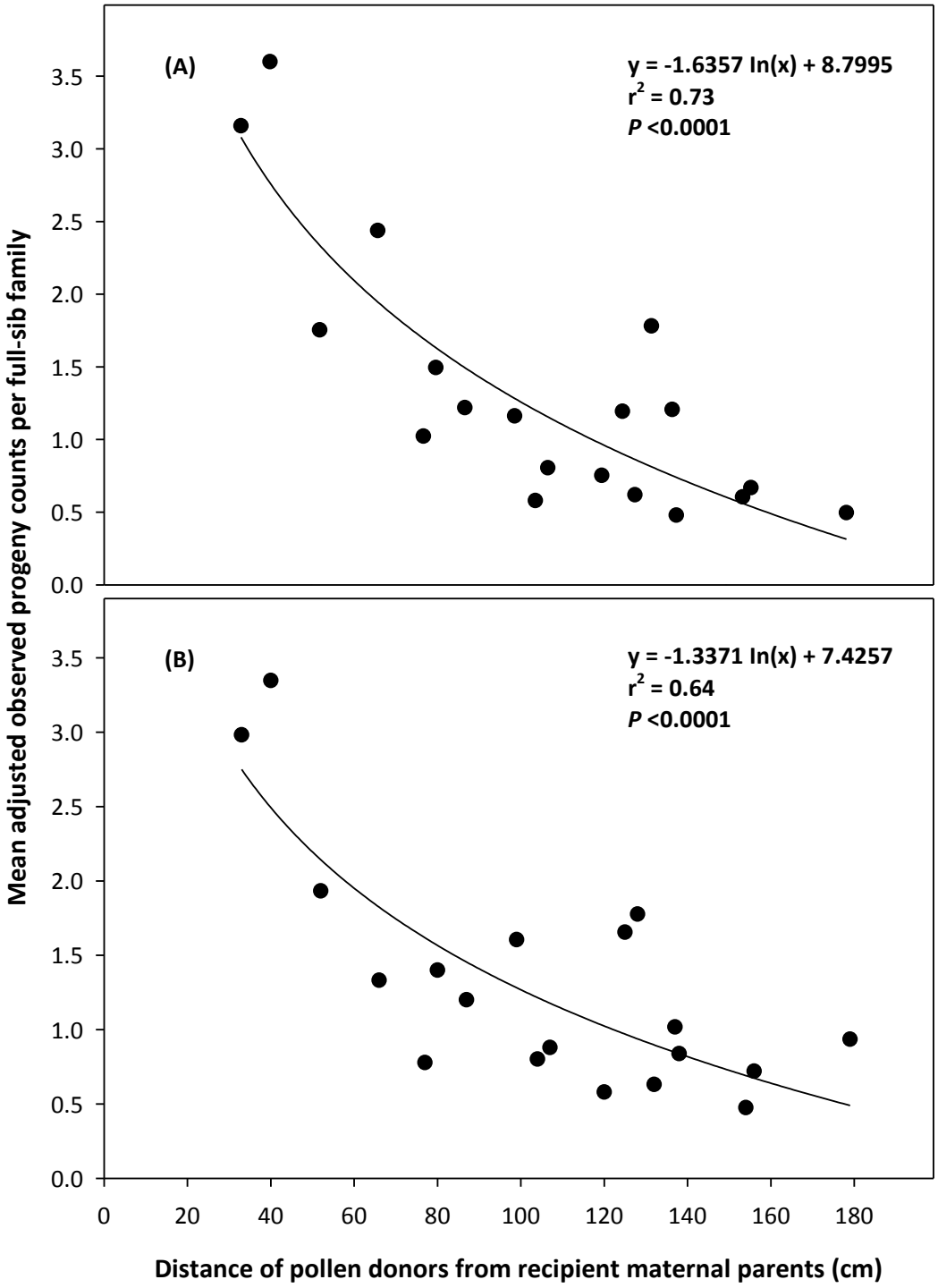


Figure 3.5 Mean adjusted (for unequal maternal sampling) observed outcross progeny counts per white clover full-sib family plotted against distance of pollen donors from recipient maternal parents in isolation cage 1 (A) and cage 2 (B).

Almost 50% of maternal half-sib seed was fertilized by paternal parents within a 50 cm radius in both isolation cages and almost 80% of maternal half-sib seed was fertilized by parental parents within a 100 cm radius in both isolation cages (Figure 3.7). A strong positive relationship between pollen donor distance from the recipient maternal parents and the

paternal contribution to maternal seed yield was evident in both isolation cages ($P < 0.0001$; $R^2 = 0.65$ and $R^2 = 0.69$) (Figure 3.7).

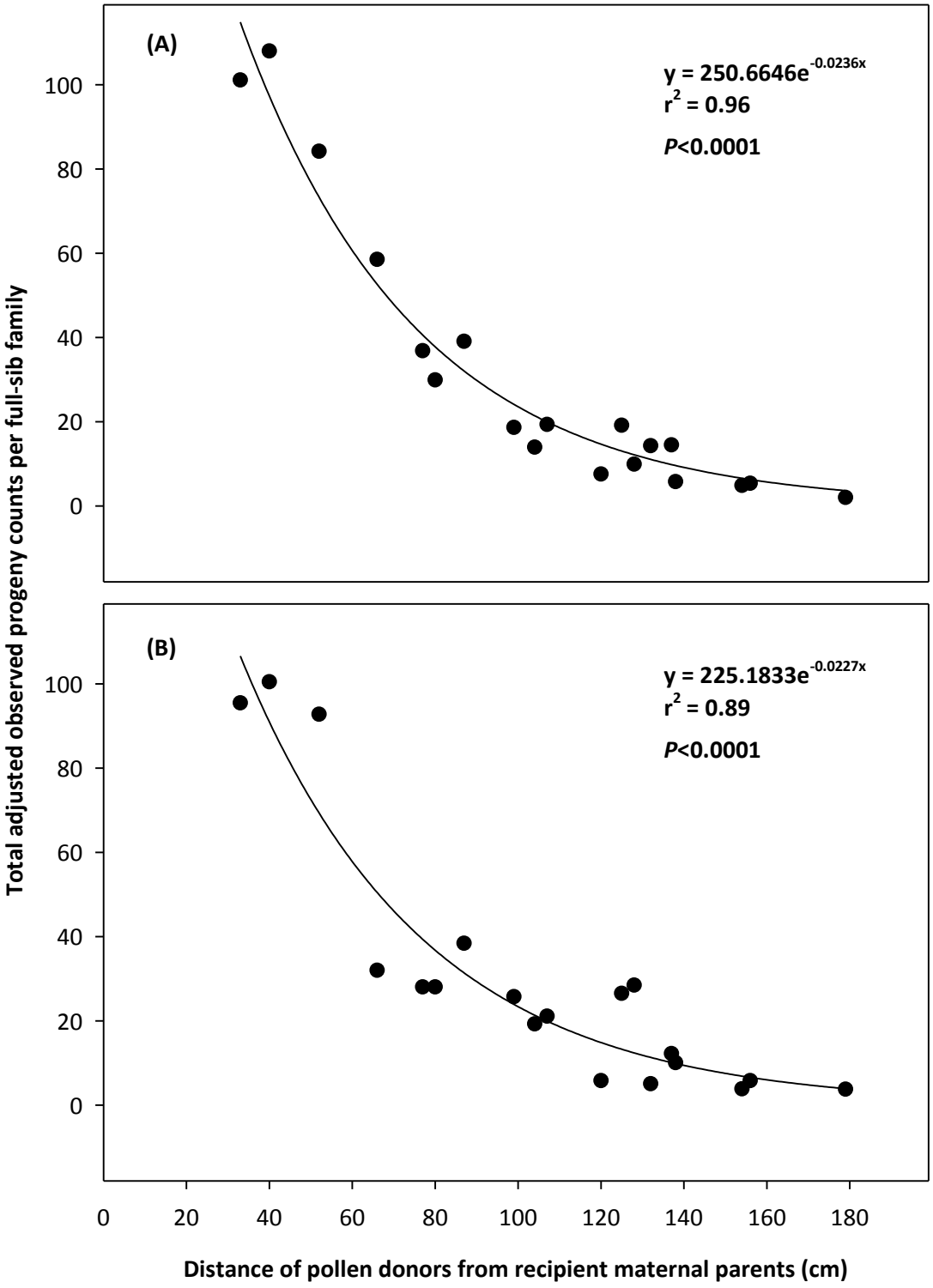


Figure 3.6 Total observed outcross progeny counts per white clover full-sib family distance plotted against distance of pollen donors from recipient maternal parents in isolation cage 1 (A) and cage 2 (B).

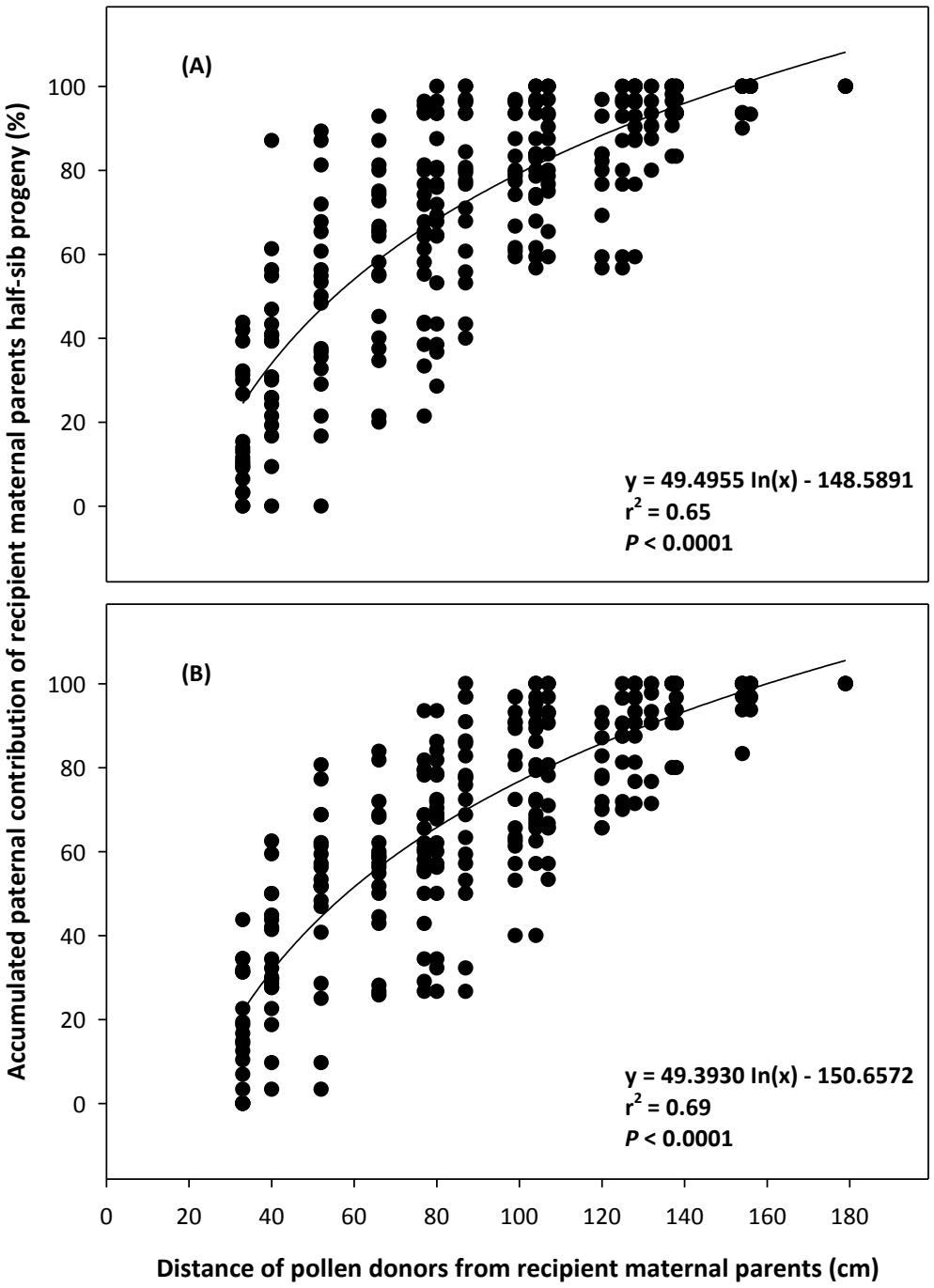


Figure 3.7 The accumulated siring contribution of white clover paternal parents at increasing pollination distances from recipient maternal parents in isolation cage 1 (A) and cage 2 (B).

3.3.5 Paternal contribution of half-sib families

The range of paternal parents contributing to a maternal half-sib family was 2 - 13 and 5 - 13 for isolation cages one and two, respectively (Figure 3.8). In both isolation cages, greater than 80% of maternal half-sib seed was pollinated by six paternal parents or less. Bulked half-sib family seed (mixed half-sib seed from both cages) failed to contain pollen sources from all

paternal genotypes (Figure 3.8). The minimum and maximum number of paternal parents contributing to bulked maternal half-sib families was 12 and 18, respectively. On average 80% of maternal half-sib seed was pollinated by nine or less paternal parents.

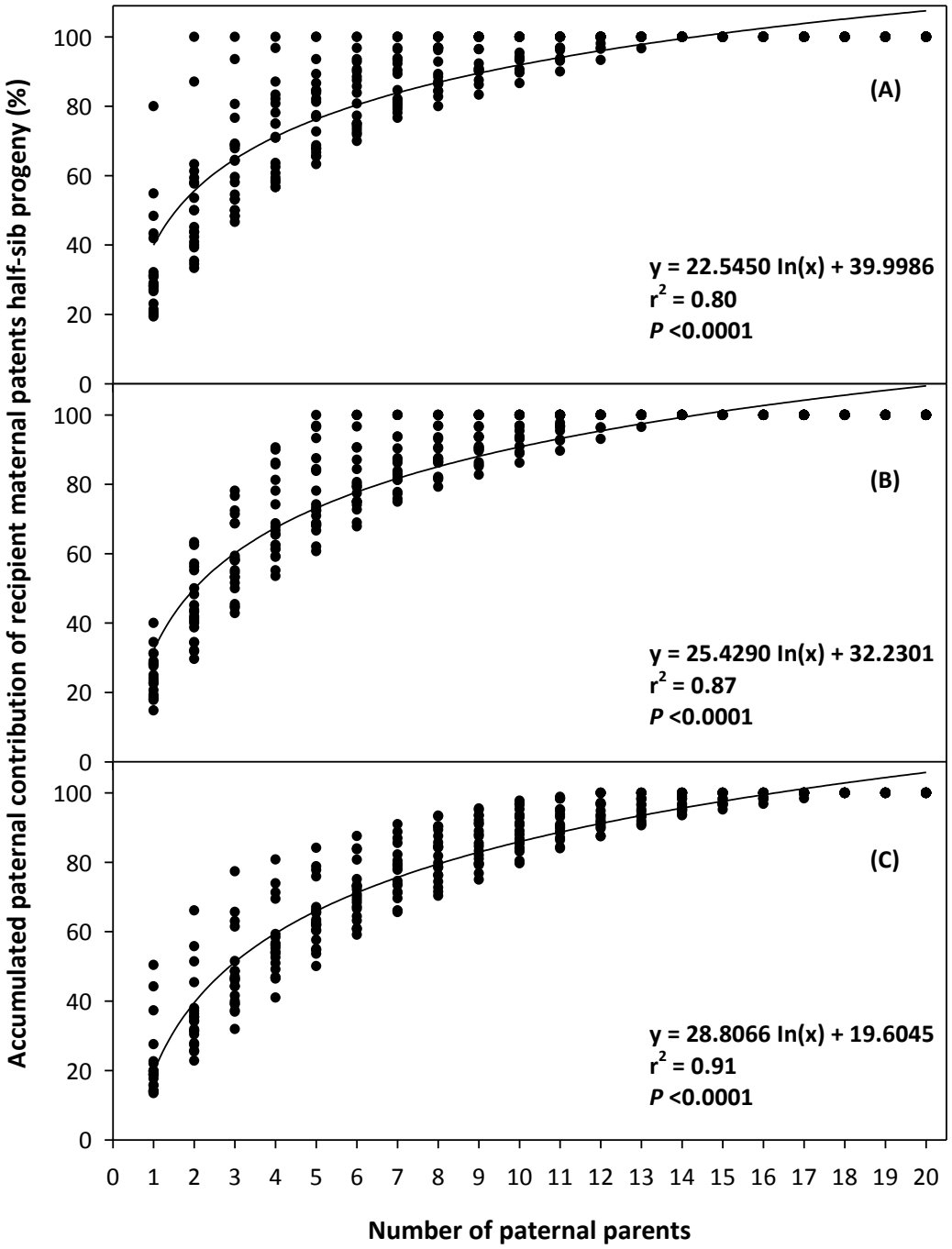


Figure 3.8 Number of paternal parents and their contribution to recipient maternal parent's seed yield in two separate white clover polycrosses containing 20 genotypes (A and B) and the bulked half-sib seed from both (C).

The number of paternal parents and their contribution to maternal half-sib progeny was only slightly improved by bulking individual isolation cage genotypes (Figure 3.8 and 3.9).

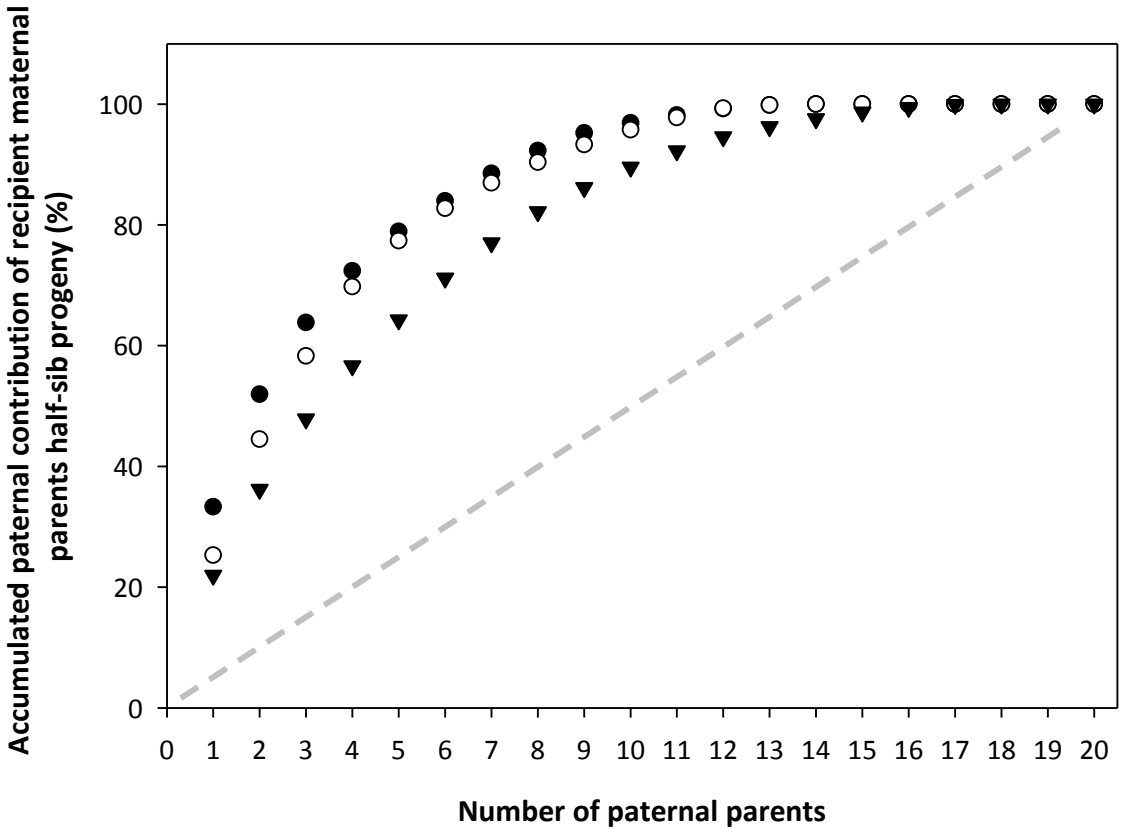


Figure 3.9 Number of paternal parents and their contribution to recipient maternal parent seed yield among two non-replicated white clover polycrosses (● isolation cage 1 and ○ isolation cage 2) and the bulked half-sib seed from both (▼). The dashed line represents perfect random mating with equal paternal contributions from all potential pollinators.

Within each isolation cage, half-sib families had different proportions of contributing paternal parents. Figure 3.10 illustrates the tester pollen source was different among maternal half-sib families.

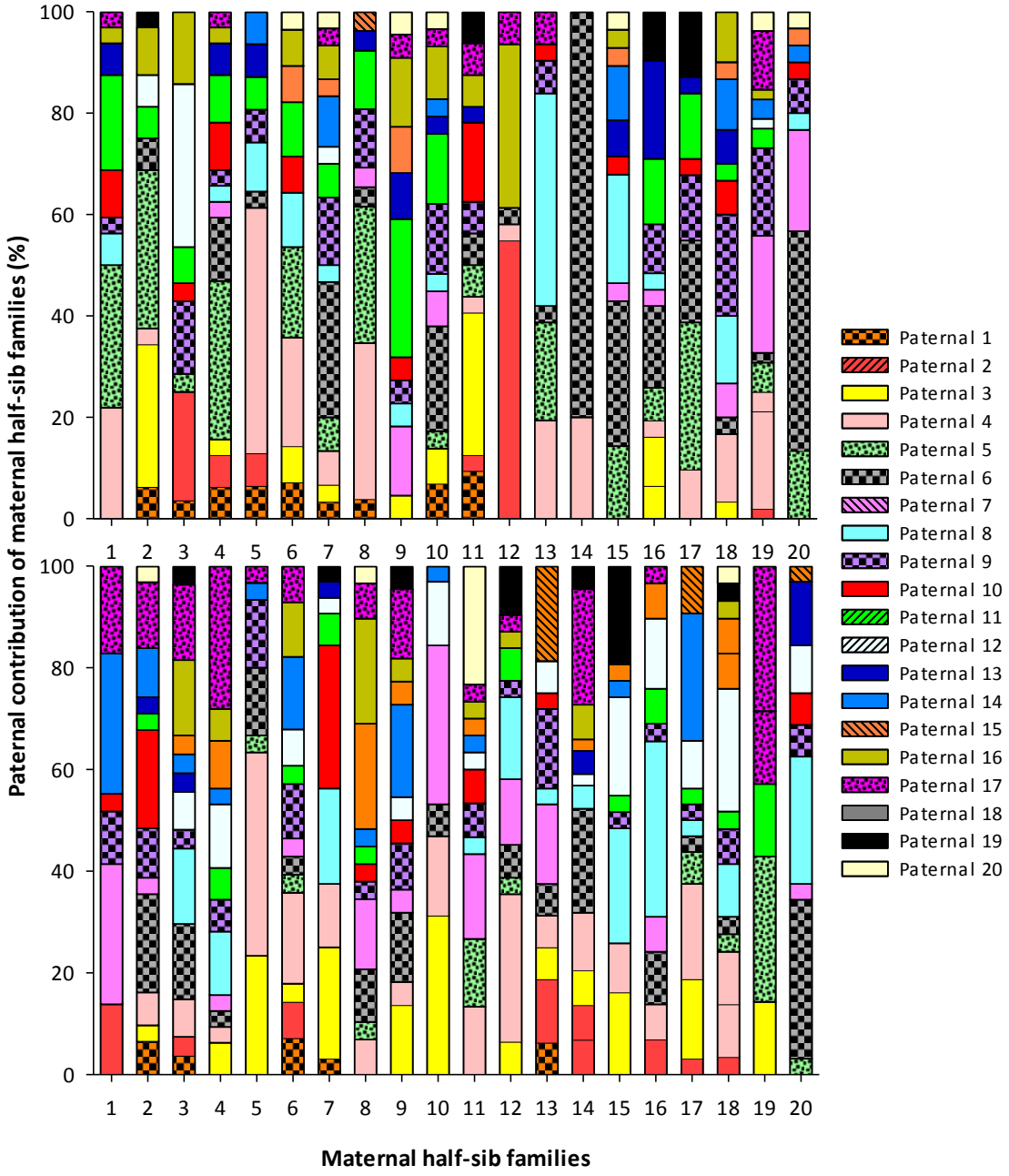


Figure 3.10Paternal contributions of 20 maternal white clover half-sib families generated in two non-replicated polycross isolations (isolated cage 1 (upper) and isolated cage 2 (lower) pollinated by bumble bees).

3.3.6 Pair-cross seed yields

Seed yields from the designated female parent of the forty bi-parental crosses ranged from 0.1g to 2.85 g plant^{-1}, with a mean of 1.04 g plant^{-1} and a median of 0.77 g plant^{-1}. From the 506 progeny sampled, representing 10 random full-sib families, 100% of progeny were cross pollinated (Table 3.3). No foreign paternal alleles or self-fertilized progeny were detected in any of the bi-parental germinated progeny.

Table 3.3 Attributes of ten randomly sampled bi-parental white clover progenies, showing per female parent; male parent genotype identity, seed yield (g $^{-1}$ plant), number of progeny sampled per family, number of successfully assigned progeny per family, outcrossing rate per family and self-fertilisation rate per family.

Female parent	Male parent	Seed yield (g)	Progeny sampled	Assigned paternity count	Cross Pollinated progeny %	Selfed Progeny %
4	**1**	1.36	51	51	100%	0%
7	**2**	2.23	51	51	100%	0%
10	**3**	2.79	51	51	100%	0%
14	**4**	0.52	47	47	100%	0%
20	**5**	0.58	51	51	100%	0%
21	**6**	0.68	51	51	100%	0%
25	**7**	0.33	51	51	100%	0%
32	**8**	0.6	51	51	100%	0%
33	**9**	1.9	51	51	100%	0%
38	**10**	0.52	51	51	100%	0%
Total	**-**	-	506	506	100%	0%

3.4 Discussion

3.4.1 Maternal seed yields and paternal progeny counts

Harvested seed yields from the designated maternal genotypes of the bi-parental crosses were in the expected range of 0.5-2 g plant^{-1} (Ford, 2013). Polycross seed yields on the other hand were lower than expected. The lower than expected seed yields may have been due to a number of contributing factors including but not limited to: higher than average rainfall and lower than average temperatures for the month of December 2011, insufficient number of pollinators, crossing duration and residue insecticides. Mortality rates for bumble bees inside the isolation cages were not recorded, although multiple bees had to be replaced over the crossing period; indicating environmental conditions were not favourable. All parental clones had ample numbers of inflorescences to reach expected seed yields (Appendix A.2), and thus inflorescence density was unlikely to be a factor.

Despite the lower than expected polycross seed yields, the technique of using wild bumble bees for pollination was successful. The bumble bees generated half and full sibling families with low and no detectable levels of self-fertilized progeny (Tables 3.1 and 3.3). The low levels of self-fertilization are consistent with the reported strong self-incompatibility of white clover (Atwood, 1940; Wright, 1939). It is important to note that no detectable levels of self-

fertilized progeny, based on SSR marker analyses, were present in any of the sampled bi-parental progenies. Selfed progeny can alter the co-variances among families and consequently bias estimated genetic variance components. Manual emasculation and hand crossing can be used instead of bees to ensure purity of full-sib families as reported by Jahufer (1999), however a drawback associated with this technique is the restriction on seed quantity and number of families generated due to its labour intensive requirements.

Due to seed availability for each clone and unsuccessful paternal assignments, there was an uneven number of progeny per maternal clone analysed. An unbalanced number of progeny per maternal clone may lead to biased results (Riday et al., 2013), and therefore progeny counts for each paternal genotype were adjusted accordingly to remove the effect of unequal maternal sampling. In the presence of random mating, progeny counts should be equal among paternal parents; however paternal counts deviated from random mating ratios in both isolation cages (Table 3.2). The fluctuating range of paternal progeny counts highlights the extent of non-random mating within the isolation cages. It is interesting to note that genotype 18 had only two paternal progeny among both isolation cages, indicating a degree of male infertility. The cause of the partial male infertility is yet to be diagnosed, but it may provide a valuable germplasm source for future white clover breeding programmes.

The linear relationship between polycross maternal seed yield and paternal progeny counts suggests high seed yielding maternal parents also tended to have higher paternal progeny counts (Figure 3.4). A similar pattern was observed by Riday (2013) in a 15 parent lucerne (*Medicago sativa L.*) polycross, although the function of that relationship was primarily due to self-fertilization. Due to the minimal self-fertilization among parents (<1%) within this experiment, a likely explanation for the positive correlation in Figure 3.4 is the effect of higher seed yielding maternal parents having more harvested inflorescences (Appendix A.2). Assuming the number of inflorescences harvested at seed maturation is representative of the actual number of inflorescences during pollination (no counts during pollination were carried out) there was a positive correlation between number of inflorescences at pollination and success of siring offspring. In other plant species, more flowers per plant have been shown to increase pollinator visits (Galloway et al., 2002; Mitchell, 1994) and in some instances, increasing their siring success (Broyles and Wyatt, 1990). It is likely that in this experiment, genotypes with more inflorescences attracted more pollinators which in turn increased the frequency of pollen transfers between donor and recipient plants, thereby increasing their paternal outcross count.

Genotypes with more harvested inflorescences did not have a higher frequency of selfed progeny. In lucerne (*Medicago sativa* L.), which is similar to white clover as an outcrossing insect-pollinated forage species (De Lucas et al., 2012), Strickler and Vinson (2000) reported that genotypes with a greater number of flowers were also inclined to have an increased level of self-fertilized progeny, as pollinators are more likely to move between flowers on the same plant. The lack of observed self-fertilized progeny in this population again highlights the strength of the self-incompatibility system in white clover. Paternity testing also confirmed the absence of any self-fertile genotypes, which have been shown to occur at low frequencies within white clover populations (Atwood, 1941).

The nil detection of foreign alleles in both the bi-parental and polycross progeny suggests the introduced bumble bees were clean of residual pollen, which is a reassuring result for field breeders using the technique described by Williams (1987). The result also confirms no handling contaminations or mistakes were made during crossing, harvest, seed cleaning and sowing. The above demonstrates the strength of paternity testing for not only increasing the efficiency of breeding methods (Chapter 5) but for also ensuring purity and diversity of cross-pollinated breeding pools.

3.4.2 Pollen distribution

The relationship between full-sib family progeny counts and distance of pollen donors from recipient maternal parents (inter-full-sib parent pollination distance) clearly indicates that positioning of pollen donor parents is imperative for successful outcrossing with recipient maternal parents (Appendix A.1, Figures 3.5 and 3.6). Similar leptokurtic patterns of pollen distribution have been described in other studies of white clover under field conditions (Clifford et al., 1996; De Lucas et al., 2012; MichaelsonYeates et al., 1997; Weaver, 1965). The exponential decay in pollinator success at increasing recipient parent distances appears to be a function of the small movements made by pollinators between succeeding inflorescences (Weaver, 1965) and a pollen dilution effect, whereby effective pollen came mostly from the last compatible inflorescence visited (MichaelsonYeates et al., 1997). Although all of these studies have investigated pollen transfer, the majority have been investigated under field conditions. Due to the requirement of isolated cages for rapid and consistent development of breeding populations, it is also imperative that pollen distribution is understood within isolation cages for white clover.

In lucerne, Riday (2013) reported a negative power function relationship between full-sib family progeny counts and inter-fullsib parent pollination distance in a 15 parent isolated cage

polycross. Michaelson-Yates et al. (1997) appears to be the sole study of pollination distribution within isolated polycross cages for white clover. Their results again confirm the significant relationship between parent distance and siring success. The results of this study, while similar to Michaelson-Yates et al. (1997), show the foraging behaviour of bumble bees in an actual white clover breeding pool with a wider range of phenotypically diverse parents as encountered in breeding programmes.

Despite previous reports in the literature, it was alarming to see such a large neighbour effect in a small 20 parent polycross, where even the furthest pollen donor plant was within a close proximity of the recipient maternal parent (179 cm). As illustrated in Figures 3.5 and 3.6, a large proportion of this nearest neighbour pollination affect was due to the unequal frequencies of potential full-sibs at closer rather than further distances. Figure 3.5 shows the likely pollination pattern in an unrestricted sized cage where full-sib frequencies are similar at various inter-full-sib parent pollination distances, whereas Figure 3.6 shows the pollination pattern in smaller cages like that used in this experiment. An unbiased relationship between full-sib progeny counts and inter-full-sib parent pollination distance could only be achieved in a wagon wheel like design, where each distance has the same number of potential outcrossing parents as presented in a field study by De Lucas et al. (2012).

It is interesting to note that despite the strong influence of inter-full-sib parent pollination distance on full-sib family progeny counts, there still seems to be variation in full-sib progeny counts between paternal parents within the same identical inter-full-sib parent pollination distance for any given maternal parent (Appendix A.1). For example, the progeny counts within a maternal half-sib family were not distributed evenly amongst all potential paternal parents at any given pollination distance. This non-uniformity warrants investigation as to whether there is a pollination pattern among paternal parents at the same distance. If a clear pattern emerges, candidate traits such as flower number, nectar flow and flower colour could be monitored, which have been shown to influence pollinators in legume species (Bosch et al., 1996; Clement, 1965).

3.4.3 Half-sib family paternal structure

Half-sib progeny trialling (HSPT) is a breeding strategy that has been extensively used in breeding perennial forages to test the performance of parental genotypes through replicated progeny trials (Vogel and Pedersen, 1993). When utilizing maternal half-sib families for HSPT, progeny are either topcrossed or polycrossed to generate half-sib families. When either approach is used, breeders often assume that each female parent (recipient parent) has the

same male tester (donor pollen source). In the case of a polycross, the male tester is made up from a heterogeneous mixture (equal proportions) of pollen from all genotypes within the polycross. Any differences among half-sib family progeny can then be primarily attributed to the genetic potential of the maternal parent and not the genetic contribution of the pollen source (Fehr, 1987).

Results obtained from this experiment show that the maternal parents in each isolation cage did not have the same male tester pollen (Figure 3.10). While this is not entirely surprising considering that there were no clonal replications per parent genotype within each isolation cage, it was surprising that the bulked maternal half-sib seed from both cages only slightly resolved the differences in paternal contributions among fathers (i.e. still very unbalanced) (Figure 3.9), and that it was far from random mating as illustrated. Perhaps if both clones of each maternal parent had been polycrossed in the same isolation cage (as opposed to the two separate cages), a better balance of paternal contributions may have be achieved as more neighbour to neighbour interactions would have existed. Nevertheless, this result highlights the importance of additional clonal replication when generating half-sib families in white clover.

Whilst the number of replicates required to achieve random mating cannot be simulated with the available data set, one could speculate that many replicates would be required judging by Figure 3.9. For a small number of entries, lattice or alpha designs are most likely required to ensure random mating as proposed by Morgan (1988) for wind pollinated species. For larger numbers of parents (≤50), Wright (1965) developed suitable polycross designs. Regardless of whether the plant species is insect or wind pollinated, polycross replication seems fundamental for the successful generation of 'true' half-sib families in forage species. Topcrossing with male rows using the base population or the previous cycle as the pollen tester is likely to be a more accurate approach for generating true half-sib families for HSPT (Posselt, 2010). At least with this approach, the tester will provide an excess of pollen, which will predominate the pollen source (Posselt, 2010) and eliminate any differences in testers between the evaluated half-sib progeny.

The low level correlation between maternal seed yield and number of fathers per half-sib family (Appendix A.4) indicates improving seed yield per clone would marginally help to reduce unbalanced male testers within non-replicated polycrosses. This tends to improve further if a component of total seed yield (number of harvested inflorescences) is increased per plant. The above is not surprising, considering genotypes with more inflorescences would

promote a greater number of pollinator visits as discussed earlier and hence interactions among more donor parents. Extremely low yielding genotypes may have been pollinated by a limited number of pollinator visits, explaining the poor number of donor parents in the progeny (Figure 3.10; genotype 14, cage 1). However, despite a slight improvement in the number of donor parents per half-sib family in higher seed yielding genotypes, even genotypes with high seed yields (i.e. Figure 3.10; genotypes 4 and 5 in isolation cage 1) still had relatively few pollen donors compared to the total available. It must be acknowledged that the number of paternal donors did not increase substantially as seed yield increased, but the contributions from the limited number of paternal donors were more uniform (Figure 3.10; genotype 4 isolation cage 1).

For practical purposes, the best polycross system would be likely to include an increase in replications, seed yield and number of inflorescences per genotype to improve random mating. This could possibly be achieved in field plots, where bigger plants can be managed more successfully. An increase in plant size and number would in turn support the requirements of a complete nucleus hive of bees. Increasing pollinator density is more manageable with complete hives and has been shown to positively influence seed yield in white clover using honey bees (Forster, 1974). Larger crosses may also facilitate the use of honey bees (*Apis mellifera*), which have been shown to not only increase seed yield per cage (Cecen et al., 2007) but also visit fewer florets per inflorescence and travel greater distances between succeeding inflorescences (MichaelsonYeates et al., 1997). If a pot system was still desired for convenience, randomizing the plants throughout the duration of crossing could help alleviate the nearest neighbour effect. Again, the effect of pollinator density and or species would also be worth investigating, as well as the duration of crossing.

In terms of other downstream applications, pollination is likely to have a pronounced influence on synthetic performance as well. First generation synthetics are generally an even blend of maternal seed yield from the inter-pollinated parents (Fehr, 1987). Equal contributions of maternal seed are bulked to limit undesirable levels of inbreeding during generation advance, often a concern of breeders in narrow based synthetics (Rumbaugh et al., 1988). However, unbeknown to the breeder, non-random mating may result in the same problem as uneven bulking of maternal half-sib seed, despite the best practices used. Perhaps equal bulking of a diallel cross between parents in the Syn0 as described by Fehr (1987) and carried out by Piano et al. (2007), is a viable method to mitigate such a problem in the first generation synthetic of non-random mating sensitive species like white clover when theoretically all of the inbreeding takes place (Busbice, 1969).

3.5 Conclusions

- The white clover inter-pollination technique described by Williams (1987) generated half-sib and full-sib progenies with low and no levels of self-fertilised progeny. No foreign alleles were detected in any of the bi-parental or polycross progeny, indicating that saturating the bees with luke-warm water was sufficient at removing residual pollen.

- The white clover inter-pollination technique described by Williams (1987) produced sufficient seed when used to generate bi-parental progenies. The technique is a sound method for producing full-sib families for evaluation in white clover breeding programmes when hand pollination is too labour intensive and expensive.

- The white clover inter-pollination technique described by Williams (1987) produced lower than expected seed yields when used for generating half-sib families in two 20 parent non-replicated polycrosses. Paternal pollen distribution within the isolation cages was by in large regulated by the distance between pollen donors and recipient maternal parents. Siring success of paternal genotypes decayed as the distance between them and recipient maternal parents increased. Breeders must acknowledge the limitations of half-sib families generated using non-replicated polycrosses in white clover.

- Polycrosses intended to generate half-sib families for half-sib progeny trialling in white clover must be designed to disperse a similar array of tester gametes among all maternal parents, so that variation in progeny performance among the tested parents is due primarily to the genetic potential/combining ability of the parents and not the genetic contribution of the pollen source (Fehr, 1987). Likely methods to obtain true half-sib families in white clover include: higher seed yields per plant, increased number of inflorescences per plant and multiple clonal replications per genotype within isolations. Alternatively, a topcross using male rows planted with seed from the base population or previous selection cycle could be used. Both of the above systems would require the use of field plots. If a pot system was still desired for convenience, re-randomizing the plants throughout the duration of crossing might alleviate the nearest neighbour effect.

Chapter 4
Methodology development: paternity testing in white clover

4.1 Introduction

Like many major perennial forage species (Riday, 2011; and references therein), the genomic tools for white clover (*Trifolium repens* L.) have reached a point where they can be incorporated into breeding programmes for targeted applications that more efficiency meet breeding objectives. Numerous genetic maps for white clover have been published (Barrett et al., 2004; Cogan et al., 2006; Griffiths et al., 2013; Jones et al., 2003; Zhang et al., 2007) and an array of genetic markers are available on the public domain. To date, a number of these markers have been developed for quantitative trait loci (QTL) in white clover (Barrett et al., 2005; Barrett et al., 2007; Faville et al., 2012; Jahufer et al., 2012), although their uptake into commercial breeding programmes have been slow. One reason for this slow uptake is the implementation costs associated with QTL-based forage breeding approaches (Riday, 2011).

Relatively recently, Riday (2011) demonstrated an alternative approach to incorporate genetic markers available in forage species to improve breeding efficiencies at a significantly reduced cost. Through the use of Mendelian inherited molecular markers, and spaced-planted nurseries, Riday (2011) demonstrated that the rate of genetic gain on a per cycle basis for half-sib family selection in red clover (*Trifolium pratense* L.) could effectively be doubled with the aid of paternity testing. Paternity testing uses Mendelian inherited molecular markers, such as simple sequence repeats (SSR's), to identify an individual's father (paternal parent) at a given probability when paternal parentage is uncertain (Gjertson et al., 2007; Riday, 2011). The simplicity of the system allows rapid uptake into field breeding programmes without the continuous capital investment required to maintain state-of-the-art genotyping facilities associated with relatively new technologies (Xu and Crouch, 2008).

No studies have investigated the breeding method demonstrated by Riday (2011) in white clover. While the method reported by Riday (2011) is not intended to be used here as a permanent substitute for QTL-based or genomic selection in white clover breeding programmes, it does effectively utilise the available resources at this point in time, in a cost effective manner.

In diploid forage species, software packages including Cervus (Kalinowski et al., 2007), FAMOZ (Gerber et al., 2003) and PATRI (Signorovitch and Nielsen, 2002) are readily

available for paternity assignment based on molecular data. In allopolyploid species, such as white clover, homologue specific molecular markers within homoeologous sets can be analysed in a similar disomic fashion using the same software. Among the software packages, the user-friendly interface of Cervus makes it an ideal candidate for white clover plant breeders. Cervus calculates likelihood ratios for each candidate parent taking account of possible typing errors (Kalinowski et al., 2007). For autopolyploid forage species, Riday (2013) developed an exclusion only analysis-based paternity testing SAS (SAS, 2008) code.

Predominant outbreeding and disomic inheritance of white clover results in breeding populations that are comprised of a heterogeneous mixture of highly heterozygous individuals. Considering this, and in addition, the abundance of available co-dominant SSR markers that are highly polymorphic, non-tightly linked and contain alleles that exist at low frequencies, white clover it an ideal candidate species for paternity testing breeding methods (Gjertson et al., 2007; Riday, 2011).

Despite the available genomic tools and software to conduct marker assisted selection (MAS) in white clover, a major keystone for the success of MAS implementation in white clover or in any forage species for that matter, relies on the successful high throughput isolation of genomic DNA. As with many marker-assisted breeding programmes, the current cost of DNA extraction is a rate limiting factor, substantially inflating the cost per data point, especially when few assays are required on each sample (Xu and Crouch, 2008). Therefore the necessity for a high throughput DNA extraction system, with consistent, cheap and robust isolated genomic DNA, is fundamental. The latter is exacerbated in species, such as white clover, that contain interfering secondary metabolites (Anderson et al., 2010; Puchooa, 2004; Stewart, 1997). Anderson et al. (2010) is the sole publication that addresses high-throughput DNA extractions in white clover, although high throughput DNA isolation methods for other recalcitrant plant species is ubiquitous in the literature (Healey et al., 2014; Puchooa, 2004; Whitlock et al., 2008). To date, no information is available on the implementation of a high throughput DNA extraction method, coupled with paternity testing in white clover, to increase the rate of genetic gain in field breeding programmes.

The objectives of this chapter were to: (i) cost effectively purify white clover DNA suitable for paternity testing, (ii) identify suitable SSR markers that distinguish paternal origin of known maternal polycross and bi-parental progeny, and (iii) assign molecular marker determined paternity to the polycross and bi-parental progeny at a given level of probability.

4.2 Materials and methods

4.2.1.1 DNA extractions

4.2.1.1.1 Polycross parents and progeny

Genomic DNA was prepared by the 96-well plate method described by Anderson et al. (2010). Duplicate leaf tissue samples from each polycross parent was collected, and single tissue samples were collected from the 1280 progeny seedlings that were establishing in root-trainers. A healthy trifoliate leaf (50-100mg fresh weight) from each plant was inserted into pre-designated wells of 96-well plates. A 4.5mm stainless steel bead was added to each well. Plates were sealed with silicone sealing mats and chilled at 4^{o}C before processing. To each well, 200 µL of extraction buffer (2M NaCl, 100mM Tris-HCl [pH 8.0], 20 mM EDTA [pH 8.0], 1.12mM sodium sulphite, 5mM ascorbic acid and 2% [wt/vol] PVP-10) was added. Leaf tissue was disrupted by grinding two times at 30 Hz for 3 minutes in a Mixer Mill MM 301 (Retsch Inc). To each well, 200 µL of extraction buffer was added, with the addition of 4 % (wt/vol) CTAB, 238 mg/L^{-1} RNase A and 1.38g/L^{-1} DIECA. An equal volume of chloroform and isoamyl alcohol (24:1, [vol/vol]) was added, followed by centrifuging for 10 minutes at 3220g. A 300 µL aliquot of supernatant was transferred to a clean 96-well plate and combined with 600 µL of absolute ethanol. Plates were centrifuged for 30 minutes at 3200g to precipitate DNA. DNA pellets were washed with 250 µL of 70% ethanol and centrifuged for 15 minutes at 3200g. DNA pellets were re-dissolved in 200 µL of $T_{0.1}E_{0.01}$. To each well, 20 µL of 7.6M ammonium acetate and 200 µL of absolute ethanol were added. Plates were centrifuged for 30 minutes at 3200g to precipitate DNA. DNA pellets were washed with 250 µL of 70% ethanol and centrifuged for 15 minutes at 3200g. DNA was dried in an incubator for 30 minutes at 37^{o}C and resuspended in 75 µl of $T_{0.1}E_{0.01}$ (pH 8.0).

Genomic DNA (gDNA) concentration was estimated by the intensity of ethidium bromide fluorescence of DNA samples compared to that of known concentrations of bacteriophage lambda DNA on 0.8% agarose gels. DNA solutions were normalized to 1-2 ng µL^{-1} on a 96 well-plate basis. In addition, a random 96-well plate was quantified using the Quant-iT PicoGreen dsDNA reagent (Applied Biosystems) and a SpectraMax Gemini XS microplate spectrofluorometer according to the manufacturer's instructions to assess the DNA quantity.

4.2.1.1.2 Bi-parental progeny

Leaf tissue was collected from 51 seedlings of 10 randomly selected full-sib families established in propagation trays. A fresh trifoliate leaf (50-100mg fresh weight) from each

plant was folded into four quarters and placed into pre-designated wells of 96-well plates. DNA extractions were carried out as described in *4.2.1.1.1*.

4.2.1.1.3 Amendments to the 96-well DNA extraction protocol

The following amendments were made to the DNA isolation protocol described by Anderson et al. (2010) to improve DNA quantity and quality (see results section 4.3.1).

1) Sampled leaflets were folded in half, and then in half again, before being placed into the wells of the 96-well plate. The folding ensured leaflets did not wrap around the outsides of the wells and promoted effective physical disruption and lysing of the tissue with the vertical action of the bead beater.

2) Plates were vigorously agitated prior to incubation at 65 ^{o}C, in order to dislodge beads and debris from well bottoms.

3) Post chloroform addition, plates were vigorously agitated followed by repeated inversion, in order to again dislodge beads and debris from well bottoms.

4) At the final precipitation step, post addition of ammonium acetate, 400 µL of ice cold absolute ethanol was added.

5) Pellets were air-dried at 65 ^{o}C for 30 minutes before being resuspended in 75 µl of hot (65 ^{o}C) $T_{0.1}E_{0.01}$ (pH 8.0).

4.2.1.2 Polymerase chain reaction (PCR) and analysis

4.2.1.2.1 Polycross parents

Twenty five single locus homoeologue-specific SSR markers and two homoeologous SSR markers were used to assay for microsatellite alleles in all polycross parents. All PCR products were considered putative alleles of the same locus, and hereafter are referred to as alleles. The duplicate DNA samples of each parental genotype were run in separate PCR reactions to obtain duplicate PCR amplifications originating from separate DNA isolations per genotype.

Primers were synthesised (Integrated DNA Technologies, Coralville, IA, USA) with modifications described by Barrett et al. (2004). Forward primers were synthesised with a 5' M13(-21) tail universal priming site modification which permitted concurrent fluorescence labelling of PCR products by a third primer with an incorporated fluorophore (Schuelke, 2000). Reverse primers had a 7-mer 5′-GTTTCTT-3′ appended to the 5′ end of each primer to promote non-templated adenylation of amplicons (Brownstein et al., 1996).

PCR reactions were performed in 10 µL volumes containing 1 x Platinum Taq buffer (Invitrogen), 0.2 m*M* of each dNTP, 2.5 m*M* of Mg, 0.4 µ*M* of forward primer, 1.6 µ*M* of reverse primer, 0.15 µ*M* of Universal FAM labelled M13(-21) primer, 0.3 U of Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California) and 4-5 ng of template DNA. Thermal cycling parameters consisted of an initial denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, with a final extension of 72°C for 30 min.

Samples were prepared for fragment size analysis by combining 5 µL of deionized formamide (HiDi) (Applied Biosystems, Inc., Foster City, California) and 0.09 µL of Gene-Scan 500 Liz size standard (Applied Biosystems) with 1.5 µL of PCR product and denatured at 94°C for 5 min on a thermal cycler. Fragment size data were collected using an ABI Prism 3130xl Genetic Analyser (Applied Biosystems) with a 36cm array. The software program GeneMarker (Soft-Genetics LLC, State College, PA) was used to visualise the electropherograms and assign alleles to the data.

4.2.1.2.2 Polycross progeny

From the original panel of 25 SSR markers, a subset of seven SSR's exhibiting single locus homoeologue-specific patterns were selected for progeny paternity testing (Table 4.1). The criteria included, in order of importance; (i) reliability of amplification, (ii) ease of allele identification, (iii) polymorphic information content (PIC), and (iv) reduced likelihood of null alleles. The latter was crudely estimated by the frequency of single allele amplifications per locus.

4.2.1.2.3 Bi-parental progeny

PCR and genetic analyses were carried out as in *4.2.1.2.1*, with the exception that only four primer pairs were used. Primers pairs; gtrs149, gtrs195, gtrs733 and gtrs965 were used due to their polymorphic information content (PIC) and robust allele amplification and identification in *4.2.1.2.2.*

4.2.1.3 Assigning paternity

4.2.1.3.1 Polycross progeny

Cervus was used for assigning paternity (Kalinowski et al., 2007). Parental genotypes were genotyped across all marker loci twice using the duplicate tissue/DNA samples, to improve confidence in the SSR scores. In cases where single alleles were present in the parental genotypes, maternal half-sib progeny confirmed either homozygosity or the presence of a null

allele. Progeny could only be scored if heterozygous genotypes were present in markers where null alleles were present in the population.

Identity of progeny from each isolation cage was kept separate, so that individual polycross analyses could be achieved. Alleles per locus and PIC were estimated using Cervus. Paternity was only assigned if at least four of the seven loci were scored. Paternity was assigned to an individual if the "trio" logarithm odd ratio (LOD) score was greater than three (Riday, 2011). Paternity was not assigned to any progeny that had any molecular marker mismatches from the parental pair, even if the LOD score was greater than three. However, records of mismatches were analysed for sources of foreign pollen.

In markers where two alleles among parental genotypes showed considerable overlap on the electropherograms, the alleles were bulked into the same BIN to facilitate distinguishing the closely sized fragments.

4.2.1.3.2 Bi-parental progeny

Cervus was used for assigning paternity (Kalinowski et al., 2007). Paternity was assigned to an individual if the candidate father and known mother had zero "trio" loci mismatches. Paternity was not assigned to any progeny that had any molecular marker mismatches from the parental pair. In all cases, an exclusion only based approach could identify self-pollinated, cross-pollinated and contaminant pollen sources due to the simplicity of the bi-parental crosses.

4.3 Results

4.3.1 DNA extractions

DNA extractions using the standard protocol presented by Anderson et al. (2010) did not consistently isolate robust genomic DNA for SSR based paternity testing. Agarose gels indicated that the isolated DNA was inconsistent in quantity and quality between samples of the same 96-well plate (Figure 4.1).

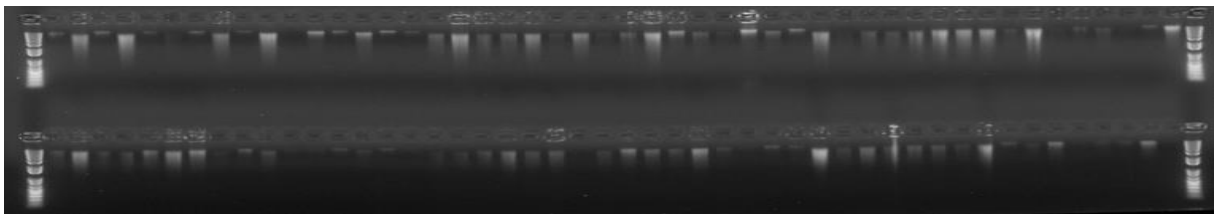


Figure 4.1 Ethidium bromide-stained agarose (0.8%) gel of genomic DNA isolated from white clover polycross progeny leaf tissue using the standard protocol presented by Anderson et al. (2010). The end wells contain 5µL of 1kb hyperladder (Applied Biosystems).

With minor modifications (referred to in section *4.2.1.1.3*) to the method presented by Anderson et al. (2010), genomic DNA was successfully isolated from 1276 of the 1277 polycross progeny. Agarose gels indicated that the isolated DNA was of high molecular weight, relatively non-degraded and free from RNA contamination (Figure 4.2). DNA extractions preformed using the modified 96 well-plate methodology yielded between 2.5-5µg of genomic DNA per sample.

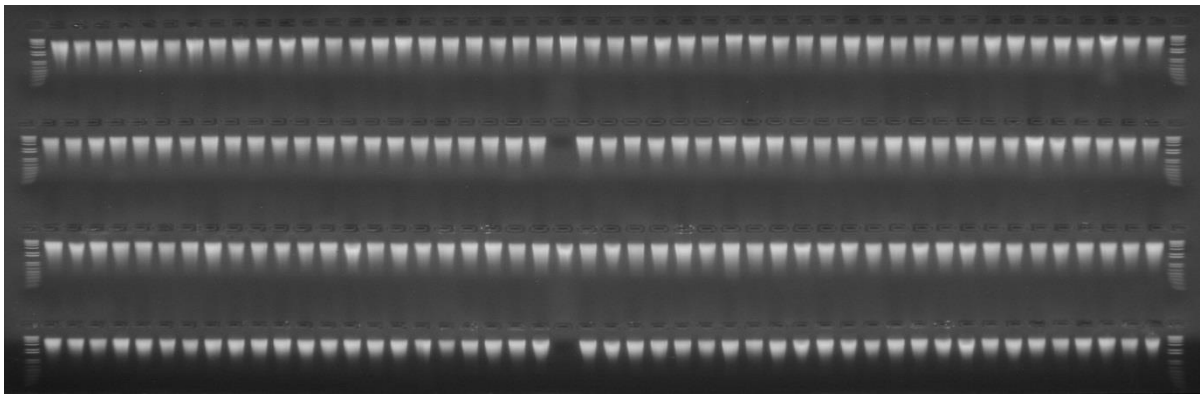


Figure 4.2 Ethidium bromide-stained agarose (0.8%) gel of genomic DNA isolated from polycross progeny leaf tissue using modifications to the Anderson et al. (2010) protocol. The empty wells in row two and four were negative controls (no plant material) and the end wells contain 5µL of 1kb hyperladder (Applied Biosystems).

4.3.2 Genotyping of polycross parents

Allelic diversity and PCR amplification across the panel of 27 microsatellite loci were variable among the polycross parents (data not shown). Repeat motifs of the microsatellite loci were two to five nucleotides long. Parental alleles amplified using dinucleotide based markers showed considerable overlap among amplified fragments (except for gtrs149), whereas markers with trinucleotide or greater sized mofits generally had greater separation among fragment sizes (see example in Figure 4.3). Among all 25 homologue specific SSR markers, amplification of a single allele per locus per genotype was common. Null allele

frequencies for all 25 single locus homoeologue-specific SSR markers and both homoeologous SSR markers were not estimated due to the lack of knowledge regarding their segregating progeny.

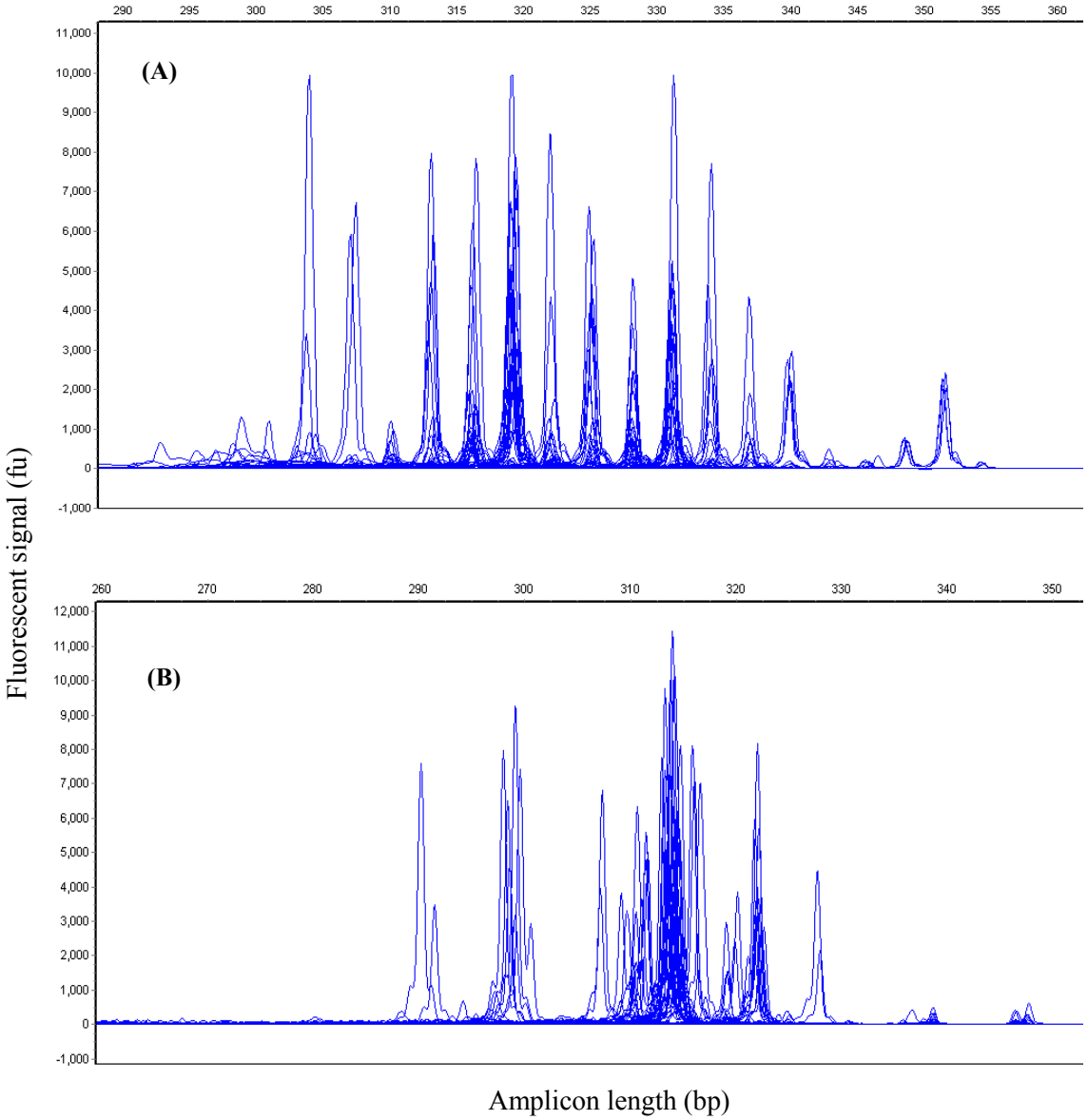


Figure 4.3 Electropherograms of white clover single locus homoeologue-specific alleles amplified using, A) a trinucleotide SSR marker and, B) a dinucleotide SSR marker.

4.3.3 Selection of SSR markers for progeny paternity testing

Using putative allele frequencies from the nominated markers, Cervus (Kalinowski et al., 2007) indicated a combination of seven single locus homoeologue-specific SSR markers would result in > 97% ($P < 0.05$) paternal assignment. Resultantly, these seven markers were selected for the progeny paternity testing panel (Table 4.1). The null allele frequencies of the

selected markers were calculated once progeny data were available. The seven markers were unlinked and randomly distributed throughout the genome (Figure 4.5).

Table 4.1 Fragment size, alleles per locus, null allele frequency, polymorphic information content (PIC) and mofit repeat for the seven single locus homoeologue-specific simple sequence repeat markers in white clover.

SSR	Fragment size (bp)	Alleles per locus	Null allele frequency	PIC	Mofit repeat
gtrs149	126-153	8	0.000	0.776	Di
gtrs195	144-167	8	0.050	0.782	Tri
gtrs366	304-352	14	0.025	0.842	Tri
gtrs635	218-235	7	0.025	0.699	Tri
gtrs733	317-349	11	0.075	0.842	Tri
gtrs789	342-365	8	0.000	0.787	Tri
gtrs965	305-323	8	0.000	0.664	Tri

The panel of seven selected single genomic SSR markers amplified consistently from all most all successfully extracted progeny DNA samples. Fluorescently labelled products consistently produced peaks of 5,000 to 10,000 fluorescent units (Figure 4.4).

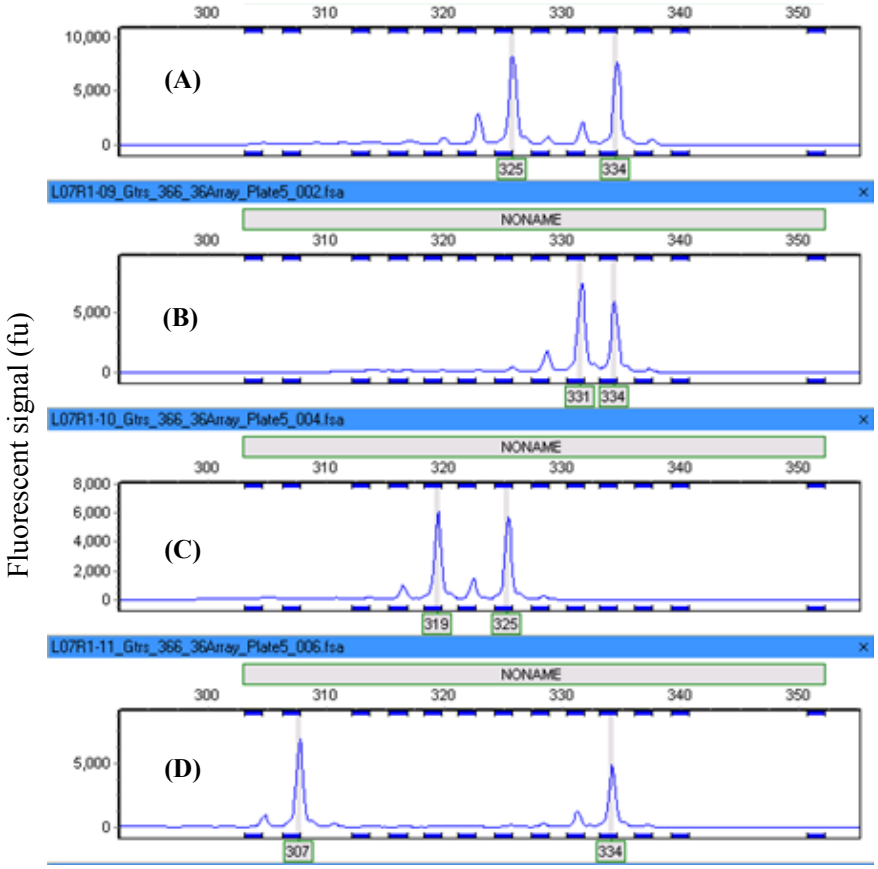


Figure 4.4 Electropherograms of white clover alleles amplified using a single locus homoeologue-specific SRR marker in polycross parent 07 (A), and three segregating half-sib progeny with different paternal alleles (B-D).

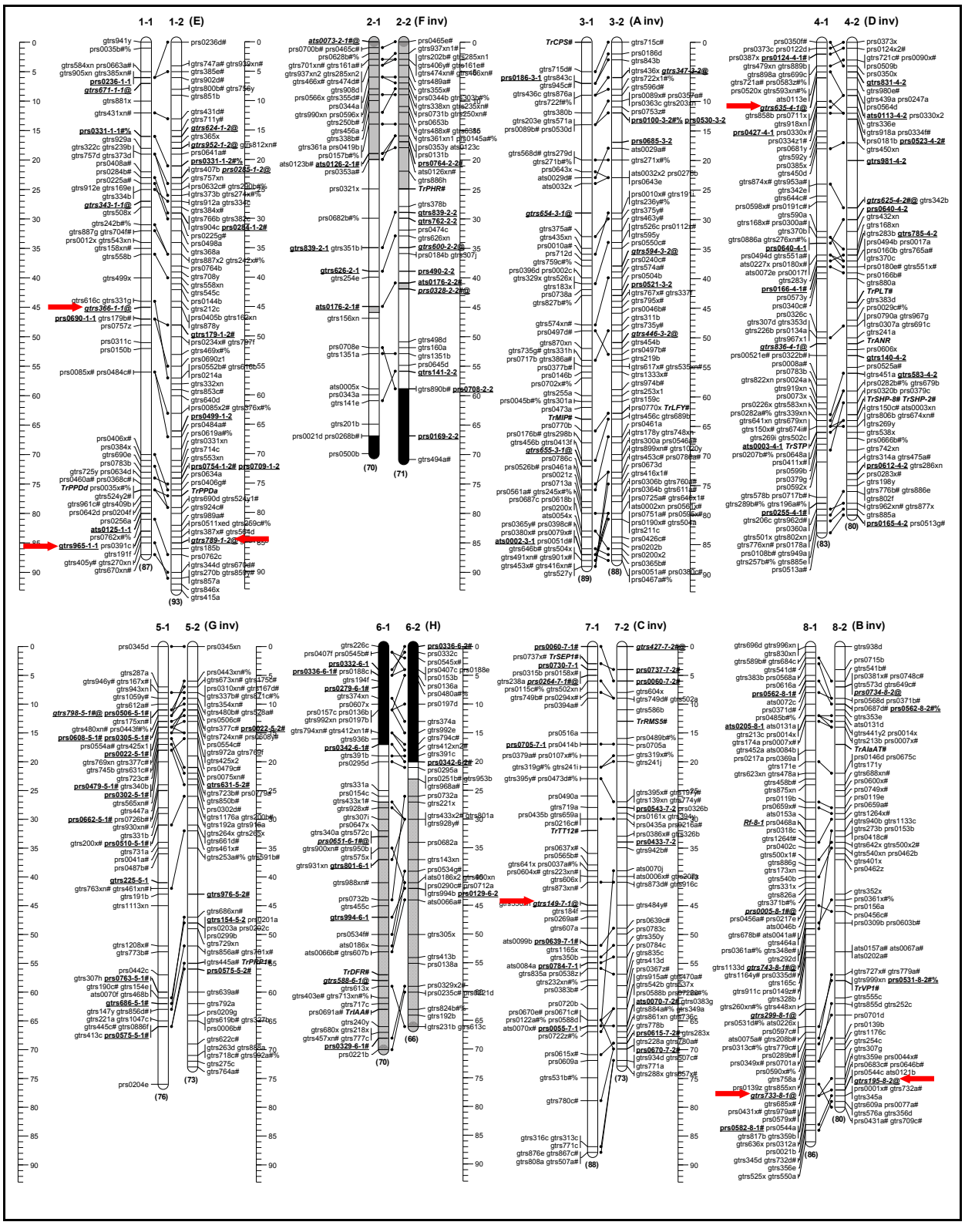


Figure 4.5 An integrated linkage map of white clover (2n=4x=32) modified from Griffiths et al. (2013). The eight homoeologous pairs of linkage groups are labelled 1-8, and homoeologues within each pair are designated 1 and 2. Estimated genetic distance (cM) is represented by the scale beside the map and the length (cM) of each homoeologue is indicated in brackets below each group. Homoeologous loci are connected by lines between homoeologue pairs. Loci labelled with bold *italics@* denote single locus homoeologue-specific loci. The red arrows identify the location of the seven single genome specific SSR markers used for paternity analysis.

4.3.4 Progeny genotyping and paternity assignment

Using the panel of seven SSR markers, the paternity of 93.13% and 92.46% of the polycross progeny were successfully determined in isolation cage one and two, respectively ($\bar{x}$ = 92.8%). Paternal assignment rate ranged from 68.75% to 100% among maternal half-sib progeny (half-sib family nine and four respectively; Table 4.2). Paternity assignment rates (%) of maternal half-sib families increased linearly with average half-sib family LOD scores (Appendix B.1), and average half-sib family LOD scores decreased with increasing number of maternal null alleles (Appendix B.2).

Table 4.2 Parental genotypes from duplicate 20 parent isolated polycrosses pollinated by bumble bees (*Bombus* sp.). Per paternal genotype the table shows combined half-sib seed (g), paternal assignment rate (%), average LOD score per half-sib family and number of null alleles per genotype among all seven SSR markers.

Parent	Seed Yield (g)	Paternal assignment rate (%)	Average LOD score	Maternal Null alleles
1	0.64	95.31	6.25	1
2	1.86	98.44	6.96	0
3	1.61	85.94	6.23	0
4	6.21	100.00	7.32	0
5	7.03	95.31	6.69	0
6	2.51	87.50	5.84	1
7	1.56	96.88	7.16	0
8	1.69	85.94	5.56	1
9	2.60	68.75	4.42	2
10	0.63	95.31	6.31	0
11	0.49	96.88	7.07	0
12	0.76	96.88	7.42	0
13	1.83	98.44	7.47	0
14	0.78	84.38	5.64	0
15	0.97	92.19	5.60	1
16	0.66	93.75	6.82	0
17	1.88	98.44	6.19	1
18	3.88	92.19	6.11	1
19	0.17	96.72	6.42	0
20	0.47	96.88	6.46	0
Mean	**-**	**92.80**	**6.40**	**-**

4.3.5 Correlations

There were no significant correlations between paternal assignment rates coupled with seed yield (g plant^{-1}), outcrossing rate %, or number of paternal parents (Figure 4.6). Genotypes with null alleles present at marker loci had lower paternal assignment rates ($P < 0.01$).

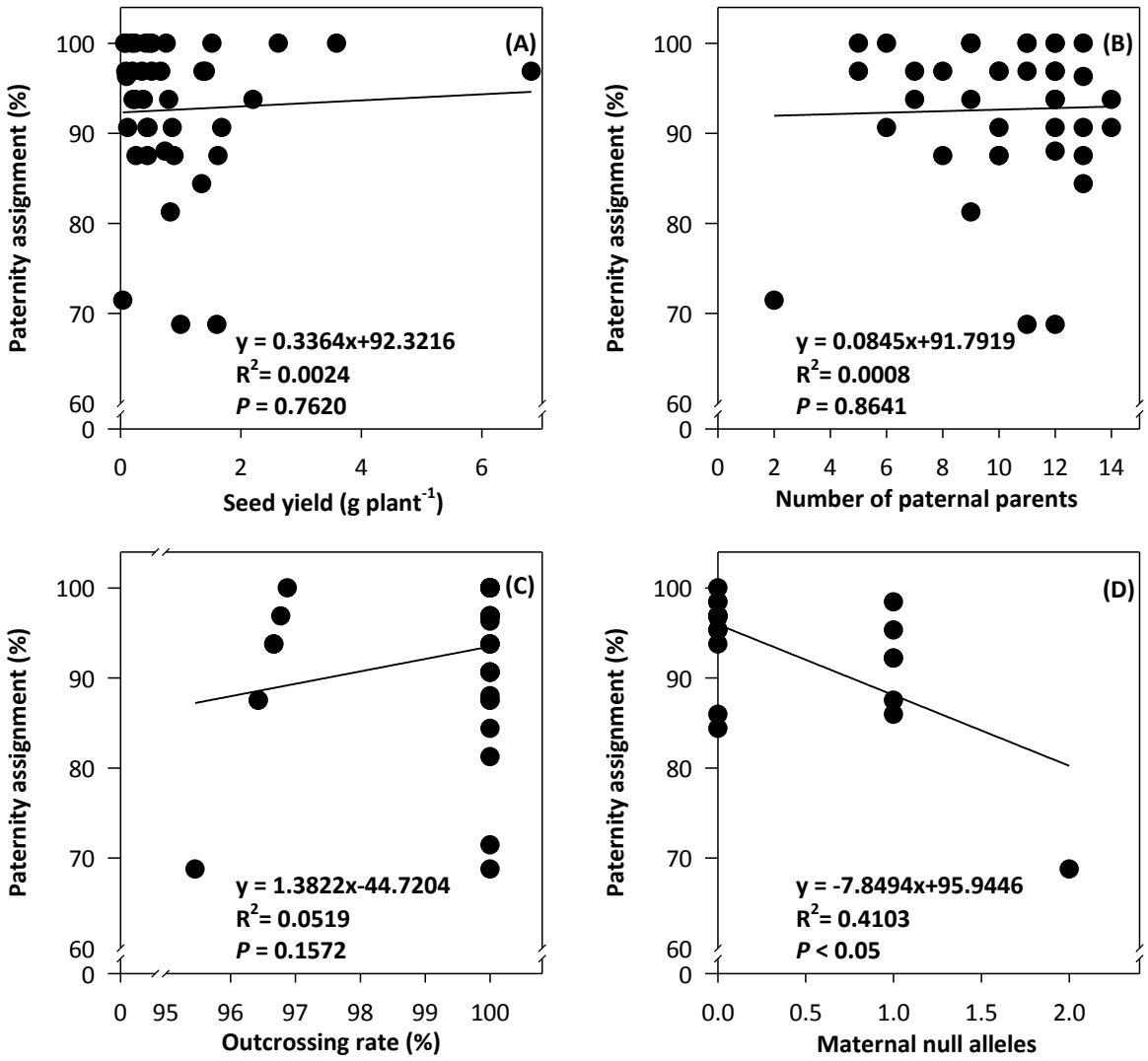


Figure 4.6 Correlations between paternity assignment rates (%) of maternal half-sib progeny coupled with: A) seed yield per plant (g plant^{-1}), B) number of paternal parents per maternal half-sib family, C) outcrossing rate of maternal half-sib families, and D) null alleles of maternal half-sib families.

4.4 Discussion

4.4.1 DNA extraction

Minor modifications to the high throughput DNA extraction method presented by Anderson et al. (2010) was sufficient to enable the isolation of genomic DNA for assigning paternity in white clover using single locus homoeologue-specific SSR markers. The methodology utilised a CTAB based extraction protocol which used cheap consumables and significantly reduced the cost per plant (Anderson et al., 2010); often a prerequisite in commercial breeding programmes. DNA working solutions were normalized to 1-2 ng/μL^{-1} on a 96 well-plate basis despite evident variation in DNA concentrations, albeit small from sample to sample. The rationale for this laxity is described by Riday (2011) and had no obvious impediments on SSR

amplification or subsequent paternal assignment. It must be acknowledged however that such tolerance can probably only be exercised when DNA extractions are relatively consistent between samples within 96-well plates, and for robust molecular markers.

These data indicate that small extra labour investment into the DNA extractions themselves pays absolute dividends for the rest of the paternity testing procedure. Essentially the DNA is the foundation for the MAS programme, and without robust and consistent DNA, the system can collapse or create problems at any time in downstream applications. In this context, it is much simpler to fix problems at the start of the procedure, than to nurse tedious problems throughout the whole process. Taking this into account, considerable care was also taken to not only extract duplicate copies of parental DNA, but also to amplify each duplicate to improve confidence in the parental SSR scores.

4.4.2 Panel of potential SSR markers

Preliminary analyses using a diverse panel of 25 single genomic and two double genomic SSR markers (Griffiths et al., 2013) among parental genotypes was useful for indicating appropriate markers for the population. Allele frequencies are population dependent and suitable markers in one population (degree of polymorphism) may not translate to another. However, irrespective of allele diversity, markers with consistent amplification in previous mapping populations *MP1* and *MP2* (Griffiths et al., 2013), generally showed similar consistency in this population.

The two homoeologous SSR markers showed considerable information per marker; however the regular overlapping of homoeologue-specific alleles made allele interpretation difficult. In addition, with the likelihood of null alleles among loci, and the lack of software analysis packages suitable for allelotetraploids, it was concluded that homoeologue-specific markers with simple disomic inheritance were more applicable for paternity testing in this white clover population, despite their lower PIC values. Within the single locus homoeologue-specific SSR markers, amplified alleles of dinucleotide repeat motif markers were the most difficult to interpret. The difficulty surrounding interpretation was due to the close proximity of the amplified fragments to one another, which was also further confounded by the one base pair array drift caused by the genetic analyser between different capillaries and runs. Due to the difficulty of setting BINs for dinucleotide based markers, they were all excluded from the potential progeny marker panel except marker gtrs149 which showed a greater spread among parental alleles.

To assist in the slight inaccuracies of the genetic analyser between different capillaries and runs (array drift ± 1bp) and especially for gtrs149, maternal half-sib progeny from each maternal parent were run in sequential order, so that the known segregating maternal alleles when compared with one another could also act as an internal control to correct for array drift.

4.4.3 Progeny genotyping and paternity assignment

The selected panel of seven SSR markers showed consistent amplification across all alleles and individuals. The presence of null alleles in four of the homoeologue-specific markers was confirmed by analysis of their segregating progeny, which consequently limited the ability to treat single allele progeny genotypes as homozygous in affected markers. The latter highlights the limitations of using co-dominant markers for paternity analysis when null alleles are present in the population.

The number of markers required to determine paternity was particularly low compared to previous studies in forage legumes (Riday, 2011; Riday et al., 2013). This was a result of having fewer polycross parents as well as investigating a breeding pool with a high level of genotypic diversity. It must be acknowledged that a higher number of parents would likely be present in commercial breeding programmes, and as a result, more markers would likely be required to differentiate paternal parents due to an increase in intra-population allelic diversity (Guthridge et al., 2001). Riday (2011) demonstrated that 11 SRR markers were required to determine 70%, 75% and 84% of paternity in three 96 parent red clover polycross populations. Species such as lucerne which display tetrasomic inheritance, require an even greater number of markers (Riday et al., 2013). Problematically, as the number of markers increases, so do the costs associated with genotyping.

Multiplexing is often utilised to improve cost efficiency by reducing the number of PCR reactions and genotyping runs (Xu and Crouch, 2008). Riday (2011) and Riday et al. (2013) successfully demonstrated paternity testing in red clover and lucerne, with just two multiplex PCR reactions containing five and six markers and nine and ten markers, respectively. Originally due to developmental time restraints and the minimal number of markers required to determine paternity in this experiment, multiplexing was not pursued. In retrospect, few difficulties would likely have been encountered with PCR optimisation for multi-plexing, considering all primer pairs required the same thermocycler conditions. Incorporating the "suitability of markers for multiplexing" into the criteria for marker selection (*4.3.3*) would have further improved the likelihood of success. With known allele ranges for each marker loci (from parental data), a primer dye-labelling strategy that utilised phosphoramidite dyes

could have easily been developed. Adoption of a two reaction assay or similar will be highly beneficial if paternity testing using SSR markers is to be adopted in white clover breeding programmes.

The degree of allelic diversity within loci also underpins the number of markers required to successfully identify paternity. Having SSR loci tightly linked to the forage species self-incompatibility locus can further compress paternity testing success into fewer SSR loci (Riday, 2011). Since the self-incompatibility locus is under negative frequency-dependent balancing selection, many alleles at equally low frequencies are expected (Riday, 2011; Wright, 1939). Unfortunately the single genomic SSR locus (gtrs952) that is close to the reported self-incompatibility locus in white clover on homoeologue 1-2 (Casey et al., 2010; Griffiths et al., 2013) amplified dimer motif repeats in this study, and consequently were difficult to segregate despite them showing the greatest allelic diversity among all 25 single locus homoeologue-specific markers investigated. Markers gtrs952 and gtrs624 would be worth investigating in future populations due to their high level of allelic diversity in the population investigated in this experiment.

4.4.4 Cost of implementation

From a cost perspective, the implementation of paternity testing in white clover breeding programmes is influenced by the number of markers required to successfully assign paternity at a certain level of probability. In this experiment, the cost of consumables per DNA extraction was approximately $0.50, and each marker was an additional $0.60 for PCR, genotyping, data collection and analysis. If labour is accounted for, it equates to approximately $1.10 per DNA extraction and $0.91 per additional SRR marker required. Multiplexing in four primer pair reactions reduces the cost of each individual marker by approximately 60% (as labour cost remains the same for fragment size analysis and interpretation). If labour is ignored; multiplexing reduces the cost of PCR and genotyping by 75%.

In addition to the cost of paternity testing parents and progeny, the cost identifying a suitable marker panel must be considered. The cost of a marker panel may be highly variable depending on the genetic characteristics of the source population. In this study, the initial panel consisted of nearly four-fold more markers than required to assign paternity at a LOD > 3 threshold. Costs for this stage can be estimated by multiplying the number of markers in the initial panel and the number of polycross parents by the costs listed above.

Plant breeders in New Zealand, and most developed countries have all the resources required to utilise paternity testing in white clover breeding programmes. The primer sequences for the molecular markers used in this study, as well as additional markers that may be required can be downloaded as a supplementary file (Griffiths et al., 2013) at the BMC genomics website. Where in-house equipment is limited or not available, outsourcing the molecular analyses to biotechnology companies is a cost effective solution. Third party contract services don't require any capital input, have fast sample turnaround, and provide molecular labour skills that breeding programmes are unlikely to economically match. Paternity assignment software such as Cervus is readily available and can be downloaded online to assign molecular marker determined paternity from molecular data.

4.4.5 Correlations

The lack of correlations in figure 4.6 led to the conclusion that paternal assignment rate was not a biasing factor in differences observed among half-sib families for seed yield, outcrossing rate, and number of paternal parents. The number of paternal progeny for genotype nine may have been biased downwards because of the lower paternal assignment rates associated with null alleles at two maternal loci.

4.5 Conclusions

- Minor modifications to the high throughput DNA extraction protocol described by Anderson et al. (2010) was suitable for providing DNA of sufficient quality to test paternity of progeny from maternal half-sib families, using single locus homoeologue-specific SSR markers in white clover.

- Seven single locus homoeologue-specific SSR markers were sufficient to identify 92.8% of the polycross progeny from a 20 parent polycross conducted in this experiment. Plant breeders however must keep in mind that the number of markers required to determine paternity of polycross progeny is dependent on the population's allelic diversity and the number of parental parents. The optimum number of markers is subject to change.

- The presence of null alleles throughout SSR loci in white clover hinders paternity assignment, and breeders should avoid using markers that amplify null alleles if possible. Screening parental material with a wider array of markers is likely to help find markers with reduced frequencies of null alleles, although actual frequencies cannot be determined until analysis of segregating maternal progeny is conducted.

Ultimately the use of more informative marker platforms instead of co-dominant markers would help alleviate the problem.

- Cervus (Kalinowski et al., 2007) was adequate for assigning paternity in white clover using single locus homoeologue-specific SSR markers. However, considering the frequencies of null alleles observed in this experiment, alternative software packages that are designed to tolerate null alleles may be advantageous.

Chapter 5
Application of paternity testing in white clover

5.1 Introduction

The rate of genetic gain is often the basis for assessment of the relative efficiencies of plant breeding methodologies. The concept of genetic gain is based on the change in the mean performance of a population that is realised with each cycle of selection (Fehr, 1987). A cycle of selection includes the establishment of a random mating population, development and evaluation of genotypes, selection of elite individuals, and mating of the selections to generate a new population for the next cycle of selection (Fehr, 1987). Variation in the methods used at each of these steps influence the duration of the selection cycle, and breeders often compare various methods in the same population on an annual basis to mitigate cycle length and improve the rate of genetic gain.

The rate of genetic gain among breeding methods and their implementation in various forage species is dependent on a number of factors. These factors include the heritability of the trait of interest, the biology of the species, the length of the breeding method, the correlation between spaced-plants and their performance in mixed species swards and the available resources to the breeder. Phenotypic or individual plant selection methods, such as mass selection, are useful when traits of interest have high narrow sense heritabilities. Family selection methods on the other hand are useful when traits of interest have low narrow sense heritabilities, due to large environmental effects (Nguyen and Sleper, 1983) and therefore require replication across space and time to partition environmental and genetic effects.

In white clover, heritabilities of important morphological traits vary significantly (Annicchiarico and Piano, 1995; Annicchiarico et al., 1999; Caradus and Woodfield, 1990; Caradus and Chapman, 1996; Jahufer et al., 2013; Lee et al., 1993; Rowe and Brink, 1993; Woodfield and Caradus, 1990), where some traits arguably favour family selection methods whilst others favour individual selection methods. A considerable drawback of the former and with reference to half-sib family selection in particular, is the lack of genetic additive variation that is utilised when remnant seed of superior half-sib families are recombined for the following cycle of selection. Breeders may capture additional additive genetic variation by carrying out phenotypic selection within the best families (among-and-within-half-sib family selection), but this technique relies on moderate within plot heritability.

Advances in molecular techniques now provide plant breeders with the opportunity to implement paternity testing within conventional half-sib selection which can effectively double its rate of genetic gain (Riday, 2011). In white clover, considering the already popular adoption of half-sib family selection at various stages in breeding programmes (Ayres et al., 2007; Bouton et al., 2005; Jahufer et al., 2009; Woodfield et al., 2003), the supplementary use of paternity testing may be an effective tool to achieve a significant jump in genetic gain per cycle of selection. No information is available on the effectiveness of paternity testing in white clover to improve the rate of genetic gain in conventional breeding methods.

The advantage of the method proposed by Riday (2011) in red clover is the ability for breeders to double the quantity of additive variation that is utilised in conventional half-sib family selection alone, with a supplementary option of phenotypic selection within the best molecular marker determined full-sib families as well. Similar gains can be achieved through the use of a variant of half-sib family selection; known as half-sib progeny trialling (HSPT), where parents of the superior half-sib families are recombined (Posselt, 2010). However, considerable drawbacks with HSPT include longer breeding cycles and the need to maintain clonal parental material throughout the duration of the progeny trials. While the latter is achievable in white clover due to its perenniality and clonal properties, breeders seldom utilise this method due to the effort required to maintain parent nurseries and the problem associated with volunteer and viral contamination; the former a problem in New Zealand, where soils have large amounts of white clover buried seed counts (Clifford et al., 1990).

A practical requirement of the breeding approach presented by Riday (2011) requires the breeder to collect information on individual plants within nurseries, and therefore spaced-planted nurseries are a necessity. A fundamental aspect of any spaced-planted forage breeding nursery is the ability of breeders to rapidly evaluate the large numbers of individual plants at a relatively low cost. Often many forage breeding programmes are resource limited, and the 'gold' standard of harvesting every individual plant above ground and determining the respective biomass per plant is impractical (Riday, 2009). Alternatively, breeders often implement visual score estimates for desirable traits which have been previously shown to have high correlation coefficients between the visual scores and actual physical units (Riday, 2009).

In forages, the efficacy of visual scoring has been explored in many species. In perennial ryegrass (*Lolium perenne* L.), visual estimates correlated with actual forage yields for plots range from 0.41 to 0.92 and for single rows from 0.48 to 0.87 (Smith et al., 2001). In spaced-

planted nurseries, Casler (2001) reported correlations of 0.64 to 0.92 in Timothy (*Phleum pratense* L.). Casler and Van Santeen (2000) also reported correlations of 0.75 to 0.94 in meadow fescue (*Festuca pratensis* Huds) and finally Riday (2009) reported correlations of 0.70 to 0.79 in red clover (*Trifolium pratense* L.). Riday (2009) also demonstrated that on an entry mean basis, which half-sib selection is conducted on, correlation between visual scores and herbage yield improve further. To the best of my knowledge, no data is available on the correlation coefficients between physical measurements of spaced-planted white clover plants established in ryegrass swards and their corresponding visual score estimates.

The objectives of this chapter were to: (i) estimate the correlation coefficients between visual scores and actual measurements of four morphological traits of white clover grown in two ryegrass spaced-planted nurseries, (ii) estimate the additive genetic variances of both maternal and paternal half-sib families, (iii) estimate heritabilities on a maternal and paternal half-sib family means basis and on an individual plant basis for four morphological traits, (iv) simulate genetic gains from a range of half-sib family selection methods based on data collected throughout the trial, and (v) discuss the efficacy of incorporating paternity testing into a white clover breeding programme.

5.2 Materials and method

5.2.1 Experimental site

The trial was conducted across two experimental sites located at the AgResearch Lincoln Research Farm in Canterbury (43^{O}37’42’’S 172 ^{O}28’3’’E) and the Ashley Dene Research Farm in Canterbury (43^{O}39’18’’S 172 ^{O}19’15’’E). At both sites the 10 year mean annual rainfall is 640 mm. At the AgResearch site, the soil is classified as a Templeton silt loam (refer to Appendix C.1 and C.2 for a soil description). At the Ashley Dene site, the soil is classified as lowcliffe stony (refer to Appendix C.1 and C.2 for a soil description).

5.2.2 Trial site preparation

The trial sites were sprayed three times over a 9 month period with the herbicide Kamba® 500 (500g L^{-1} Dicamba) at 800 mL ha^{-1} to remove resident white clover. The last application of Kamba® 500 was applied three months prior to transplanting to minimise any residue herbicide in the soil. Lime was applied across both trial sites three months prior to transplanting to raise soil pH to optimum levels of around pH 5.8-6. Superphosphate fertilizer (150 kg ha^{-1}) was applied at the AgResearch trial site and 20% Potash Superphosphate (188 kg ha^{-1}) at the Ashley Dene site in July 2012.

5.2.3 Plant material

Twenty half-sib families from the duplicate twenty parent polycrosses as described in Chapter 3 were evaluated in this experiment (see section 3.2.2). In April 2012, randomly selected samples of 0.1g of seed from each of the 20 half-sib families were scarified with 150 grit sand-paper, distributed evenly into petri-dishes containing pre-moistened filter paper, and incubated at 20^{o}C for 16 hours. Eighty germinated seedling per family were then transplanted into root-trainers at a depth of 0.5cm containing a peat and sand mix with a three month slow release Osmocote fertiliser and maintained under glasshouse conditions.

Six weeks after germination, seedlings growing in root-trainers were trimmed and placed outside onto a well-drained concrete pad. In August 2012, the established seedlings were trimmed again before being immediately transplanted into the two field nurseries.

5.2.4 Experimental design

At both trial sites, a randomised complete block experimental design with eight replicates was used (Appendix C.3 and C.4). Each replicate consisted of four progeny from each of the 20 maternal half-sib families and were spaced-planted at 1m centres (1m horizontal and 1m vertical spacing's between genotypes). The four genotypes from each maternal half-sib family were planted in a square design resembling a plot (2m × 2m). In total there were 80 genotypes per replicate, and 640 genotypes per location, with each half-sib family consisting of 32 random genotypes per location. The 32 genotypes from each maternal half-sib family for each location consisted of equal proportions of progeny from the duplicate maternal polycross clones (i.e. equal proportions of progeny from each of the two polycross isolation cages (see Chapter 3)). More specifically, each maternal half-sib plot per replicate had two progeny from isolation cage 1 and two from isolation cage 2. The half-sib family plots were completely randomised so no family was in the same trial column or row. A row of individual plants was transplanted around the outside of both nurseries (at the same 1m spacing's) to minimise border effects.

At both trial sites, the white clover plants were transplanted from root trainers into existing pure swards of perennial ryegrass (*Lolium perenne* L.). At the AgResearch site the cultivar Ceres One50 (AR37 endophyte) was sown into a cultivated seed bed at a rate of 20 kg ha^{-1} in spring 2010. At Ashley Dene, the cultivar Ceres One50 (AR1 endophyte) was sown into a cultivated seed bed at a rate of 20 kg ha^{-1} in spring 2010.

5.2.5 Trial management

Following planting, repeated hand weeding along with herbicide spot spraying was used to keep volunteer white clover seedlings to a minimum. Both nurseries were allowed to establish for three months before scoring of traits commenced. Three applications of nitrogen fertiliser (in the form of urea) were applied across both nurseries in December 2012, April 2013 and September 2013 at a rate of 20 to 25 kg ha^{-1} of N per application.

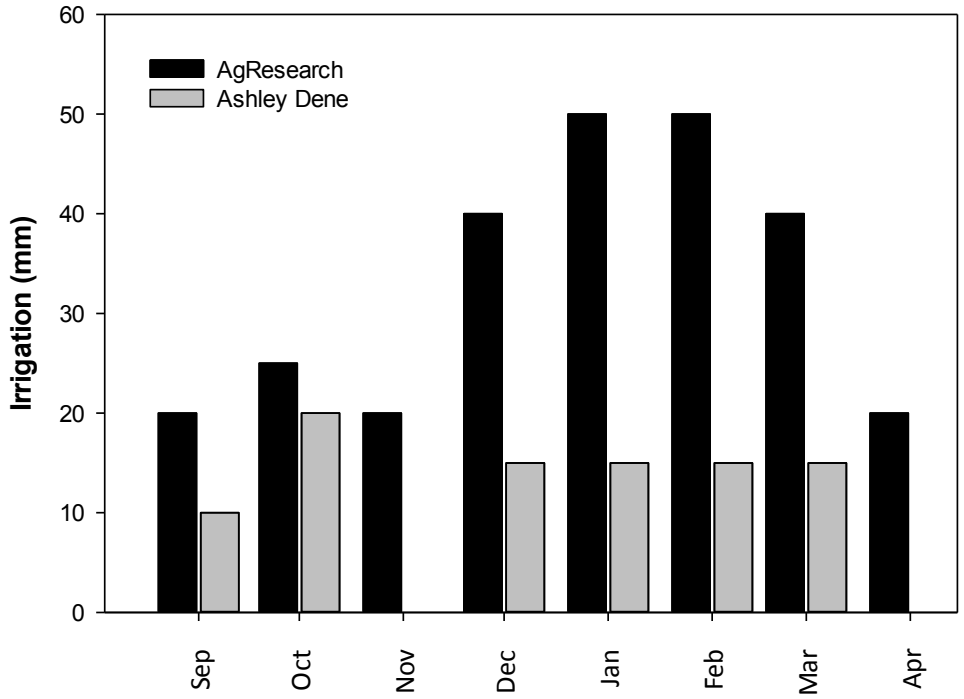


Figure 5.1 Monthly irrigation volumes at the AgResearch (■) and Ashley Dene (■) nurseries.

Both nursery sites were rotationally grazed by a mob of 70 to 100 sheep nine times over an eleven month period with time between grazings ranging from 21 days in summer to 63 days in winter. Post-grazing, nurseries were mown to a height of 4cm to homogenize the pasture cover. All herbage cut during mowing was removed off the trial sites.

The AgResearch nursery was irrigated on a fortnightly basis through November, December, January, February and March (Figure 5.1). The Ashley Dene nursery was irrigated periodically throughout the summer of 2011/2012 if monthly rainfall was below average. Irrigation volumes for both nurseries are presented in Figure 5.1. The AgResearch nursery was inter-row sprayed in late autumn 2013 with a selective herbicide (Kamba 500 at a rate of 800 mL ha^{-1}) to eliminate rapidly expanding genotypes from merging into one another.

5.2.6 Measurements

The morphological traits measured at each site were seasonal herbage yield (HY), leaf width (LW), lateral spread (LS) and number of growing points (GPD).

5.2.6.1 Herbage yield

Seasonal herbage yield was visually scored prior to each grazing at each site, except prior to grazing in both nurseries in July 2013. Visual biomass scores for each plant were recorded on a 0 to 9 scale with 0.5 unit increments allowable. Following visual scores, three randomly selected plants per denoted score (spread evenly across replicates 1, 4 and 7) per site were cut to 4cm height, the white clover foliage separated from companion sward species, dried in an air forced oven at 80^{o}C for 48 hours and the white clover dry matter weighted. At the final herbage yield score, seven randomly selected plants (10% of total plants) per denoted score (spread evenly across replicates 1, 4 and 7) per site were sampled.

5.2.6.2 Leaf width

Prior to grazing in December 2012, March 2013 and October 2013, leaf size was visually scored per plant at both sites. Visual leaf size scores for each plant were recorded on a 0 to 5 scale with 0.5 unit increments allowable. Following visual scores, the width of the centre leaflet of a first fully unfolded leaf (from the stolon apex) of six randomly selected plants per denoted score (spread evenly across replicates 1, 4 and 7) per site was recorded. At the final leaf score, the width of the centre leaflet of 13 randomly selected plants (10% of total) per denoted score (spread evenly across replicates 1, 4 and 7) per site were recorded.

5.2.6.3 Lateral spread

Prior to grazing in March 2013 and October 2013, lateral spread was visually scored per plant at both sites. Visual lateral spread scores for each plant was recorded on a 0 to 9 scale with 0.5 unit increments allowable. In March 2013 following visual scores, the spread of four randomly selected plants per denoted score (spread evenly across replicates 1, 4 and 7) per site were recorded by overlaying plants with a 1 m^2 quadrat with 5 cm cross sections and recording the number of clover occupied cells. At the final lateral spread score, the spread of seven randomly selected plants (10% of total) per denoted score (spread evenly across replicates 1, 4 and 7) per site were recorded.

5.2.6.4 Number of growing points

After grazing in March 2013 and October 2013, the number of growing points was visually scored per plant at both sites. Growing point density (GPD) scores for each plant was recorded on a 0 to 9 scale with 0.5 unit increments allowable. In March 2013 following visual scores, the number of growing points of six randomly selected plants per denoted score (spread evenly across replicates 1, 4 and 7) per site were recorded by counting the number of active stolon growing points on both the main stolons and axillary buds. At the final score, the number of growing points of seven randomly selected plants (10% of total) per denoted score (spread evenly across replicates 1, 4 and 7) per site were recorded.

5.2.7 Paternity testing

DNA extractions, DNA fragment analysis and paternity assignment were carried out as described in chapter 4.

5.2.8 Statistical analyses

The objective of the data analysis was to: (i) transform raw scores to units using calibration models, (ii) compare the estimated additive genetic variance between maternal and paternal half-sib families, (iii) estimate the magnitude of genotypic variation among maternal and paternal half-sib families, (iv) estimate their interaction with the different seasons and nursery locations, (v) estimate heritabilities on a maternal and paternal half-sib family means basis and on an individual plant basis, and (vi) simulate plant breeding methods to determine their efficiency in utilizing the estimated genetic variation.

5.2.8.1 Calibration of visual scores

Visual scores were calibrated using the sampled plants at each harvest/assessment. Due to the exponential relationship between visual scores and biomass yield (g plant^{-1}), lateral spread (m^{-2} plant^{-1}) and growing points (GPD plant^{-1}), all raw measured units (g plant^{-1}, m^{-2} plant^{-1}, and GPD plant^{-1}) at each harvest were square root transformed so that a linear model could be plotted against the denoted scores. A single linear regression was computed from the square root-transformed units at each harvest, and used to predict the square root of biomass yield ($\sqrt{}$g plant^{-1}), lateral spread ($\sqrt{}$m^2 plant^{-1}) and GPD ($\sqrt{}$GPD plant^{-1}) for all scored plants. Since the relationship between visual scores and leaf size was already linear, a single linear regression was computed from the actual raw data, and used to predict the leaf size (mm plant^{-1}) for all scored plants.

5.2.8.2 Variance component analysis

The Residual Maximum Likelihood (REML) (Harville, 1977; Patterson and Thompson, 1971; Patterson and Thompson, 1975) option in GenStat (GenStat, 2003) was used for analysis of variance of all the trait data. The REML analysis was used to obtain BLUP (Best Linear Unbiased Predictors) adjusted mean values (White and Hodge, 1989) for all four morphological traits that were used for general combining ability calculation (GCA) (Posselt, 2010).

All linear models used in the analysis of variance were assumed to be completely random except model 5.3, where environments were treated as fixed effects. Linear models used in the analyses of variance were adapted from equation 27 proposed by Nyquist (1991) for individual plants of perennial species. Within nurseries, replicate × half-sib family interactions and within-plot-variances were ignored due to high plot-to-plot variation between replicates, as a result of the low number of plants per plot and the large genetic segregation within half-sib families. The statistical significance of the variance components were calculated using deviance of log-likelihood (Galwey, 2006).

Genotypic analysis of morphological traits within environments and seasons

$$P_{ijk} = \mu + F_i + R_j + \varepsilon_{ijk} \qquad (5.1)$$

where;

P	is the phenotypic value of the k^{th} plant taken from the i^{th} family within the k^{th} replicate
μ	is the overall mean
F_i	is the effect of half-sib family i $N(0,\sigma_f^2)$,
R_j	is the effect of replicate j $N(0,\sigma_r^2)$,
ε_{ijk}	is the residual effect of plant k taken from half-sib family i in replicate j $N(0,\sigma_\varepsilon^2)$.

Genotypic analysis of morphological traits within environments but across seasons

$$P_{ijkl} = \mu + S_i + R_{(i)j} + F_k + FS_{ik} + \varepsilon_{(ijkl)} \qquad (5.2)$$

where;

P	is the phenotypic value of the l^{th} plant taken from the k^{th} family within the j^{th} replicate during the i^{th} season
μ	is the overall mean
S_i	is the effect of season i $N(0,\sigma_s^2)$,
$R_{(i)j}$	is the effect of replicate j during season i $N(0,\sigma_r^2)$,
F_k	is the effect of half-sib family k $N(0,\sigma_f^2)$,
FS_{ik}	is the effect of half-sib family k during season i $N(0,\sigma_{fs}^2)$,
$\varepsilon_{(ijkl)}$	is the residual effect of plant l taken from half-sib family k within replicate j and during season i $N(0,\sigma_\varepsilon^2)$.

Genotypic analysis of morphological traits across environments and seasons

$P_{ijklm} = \mu + E_i + R_{(i)j} + F_k + FE_{ik} + S_l + SE_{im} + b_{(ijl)} + FS_{kl} + FSE_{ikl} + \varepsilon_{(ijklm)}$ *(5.3)*

where;

P	is the phenotypic value of the l^{th} plant taken from the k^{th} family within the j^{th} replicate during the m^{th} season within the i^{th} environment
μ	is the overall mean
E_i	is the fixed effect of environment *i,*
$R_{(i)j}$	is the effect of replicate *j* within environment *i* N(0,σ_r^2),
F_k	is the effect of half-sib family *k* N(0,σ_f^2),
FE_{ik}	is the effect of half-sib family *k* within environment *i* N(0,σ_{fe}^2),
S_l	is the effect of season *l* N(0,σ_s^2),
SE_{il}	is the effect of season *l* within environment *i* N(0,σ_{se}^2),
$b_{(ijl)}$	is the effect of replicate *j* during season *l* within environment *i* N(0,σ_b^2),
FS_{kl}	is the effect of half-sib family *k* during season *l* N(0,σ_{fs}^2),
FSE_{ikl}	is the effect of half-sib family *k*, during season *l* within environment *i* N(0,σ_{fse}^2),
$\varepsilon_{(ijklm)}$	is the residual effect of plant *m* taken from half-sib family *k* within replicate *j* during season *l* and within environment *i* N(0,σ_ε^2).

5.2.9 Heritability

Heritabilities for all traits within seasons were calculated on a maternal and paternal half-sib family means basis and on a narrow sense maternal and paternal individual plant basis. Heritabilities were calculated according to models proposed by Nyquist (1991). Heritabilities on a half-sib family means basis were estimated using model 53 (Nyquist, 1991) but with the denominator replaced with the phenotypic variance among families means for perennial species (model 28). Heritabilities on an individual plant basis were estimated using model 54 (Nyquist, 1991) but with the denominator replaced with the phenotypic variance among individuals for perennial species (model 29).

Heritability on a half-sib family means basis - genotypic analysis of morphological traits within environments and seasons *(5.4)*

$$h_f^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_e^2}{rn}}$$

Narrow sense heritability on an individual plant basis - genotypic analysis of morphological traits within environments and seasons *(5.5)*

$$h_1^2 = \frac{4\sigma_f^2}{\sigma_f^2 + \sigma_e^2}$$

Heritability on a half-sib family means basis - genotypic analysis of morphological traits within environments but across seasons *(5.6)*

$$h_f^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_{fs}^2}{s} + \frac{\sigma_\varepsilon^2}{srn}}$$

Narrow sense heritability on an individual plant basis - genotypic analysis of morphological traits within environments but across seasons *(5.7)*

$$h_1^2 = \frac{4\sigma_f^2}{\sigma_f^2 + \sigma_{fs}^2 + \sigma_\varepsilon^2}$$

where;

σ_f^2 – full-sib family, σ_{fs}^2 – family × season, σ_e^2 – experimental error, σ_ε^2 – residual error,

s is number of seasons
r is number of replicates per location
n is number of plants per plot

5.2.10 Predicted genetic gain simulations

Predicted genetic gains were simulated using, i) estimated genetic parameters from analyses in this chapter (across season analyses at the AgResearch nursery [Table 5.5]) and, ii) putative parameters where estimated parameters were not available. Genetic gain equations were based on models presented by Casler and Brummer (2008), Fehr (1987), Riday (2011) and Posselt (2010) (Table 5.1).

Table 5.1 Expected genetic gains per cycle for five half-sib family selection methods and one mass selection method used in breeding white clover.

Selection method†	Family mating system	Recombination unit	Expected gain per cycle of selection‡
Mass selection	Spaced-plants	Selected plants	$\Delta G = k_I c\sigma_A^2/\sigma_{PI}$
HSF	Half-sibs	Remnant seed	$\Delta G = k_F c\frac{1}{4}\sigma_A^2/\sigma_{PFM}$
AWF-HS+MFS	Half-sibs + full-sibs	Selected plants*	$\Delta G = k_F c\frac{1}{4}\sigma_A^2/\sigma_{PFM} + k_{F!} c\frac{1}{4}\sigma_A^2/\sigma_{PFM!} + k_w c\frac{1}{2}\sigma_A^2/\sigma_{PW}$
♀+♂ HSF	Half-sibs	Random plants*	$\Delta G = k_F c\frac{1}{4}\sigma_A^2/\sigma_{PFM} + k_{F!} c\frac{1}{4}\sigma_A^2/\sigma_{PFM!}$
AWF-HS	Half-sibs	Selected plants	$\Delta G = k_F c\frac{1}{4}\sigma_A^2/\sigma_{PFM} + k_w c\frac{3}{4}\sigma_A^2/\sigma_{PW}$
HSPT	Half-sibs	Parental clones	$\Delta G = k_F c\frac{1}{4}\sigma_A^2/\sigma_{PFM}$

* Paternal parentage identified by molecular analysis
†HSF, half-sib family selection; AWF-HS+MFS, among-half-sib family and within molecular determined full-sib family selection; ♀+♂ HSF, combined maternal and paternal half-sib family selection; AWF-HS, among-and-within-family selection; HSPT, half-sib progeny trialling.
‡k_I, the standardised selection differential among individual plants; k_F, the standardised selection differential among maternal families; $k_{F!}$, the standardised selection differential among paternal families; k_w, the standardised selection differential within families; c, parental control factor; σ_A^2, additive genetic variance; σ_{PI}, the phenotypic standard deviation among individual plants; σ_{PFM}, the phenotypic standard deviation among maternal families; $\sigma_{PFM!}$, the phenotypic standard deviation among paternal families; σ_{PW}, the phenotypic standard deviation within families.

Parental control values (c) were set to one (recombination in isolated blocks with selected parents) for all methods, except half-sib progeny trialling which was set to two (Fehr, 1987; Posselt, 2010). In agreement with Casler and Brummer (2008), it was assumed that heritability on an individual-plant basis may at maximum be equal to heritability on a family mean basis, but is more likely to be smaller than heritability on a family mean basis. Putative parameters were based on fixed half-sib family mean heritabilities of 0.3, 0.5, and 0.7 by setting additive genetic variance (σ_A^2) to 0.3 and varying the among family phenotypic variance (σ_{PFM}^2) from 0.25 to 0.107 ($\frac{1}{4}\sigma_A^2/\sigma_{PFM}^2$). Individual plant heritabilities (within plot heritabilities) were varied by proportional differences between additive genetic variances and within family phenotypic variances (σ_A^2/σ_{PW}^2). In Figure 5.5, selection intensities varied (to compensate for equal effective population size; N_e) from 1% for mass selection, 10% within- and 10% among families for among-and-within half-sib family selection (AWF-HS), 10% for half-sib family selection (HSF), 10% for half-sib progeny trialling (HSPT) and 15% among-half-sib families and 50% within molecular marker determined full-sib families for among and within half-sib family selection aided with molecular marker determined full-sib family selection (AWF-HS+MFS). An equal effective population size is recommended for long-term selection programmes (Posselt, 2010). All prediction models were carried out using Microsoft Excel (Microsoft-Office, 2010).

5.2.11 Calculation of general combining ability (GCA)

General combining abilities were calculated as presented by Posselt (2010), where the mean of all half-sib family BLUPs were subtracted from individual BLUP's of each half-sib family for each trait.

5.3 Results

The polycross nursery at the Ashley Dene site had significantly lower total precipitation than the nursery at the AgResearch site from October 2012 to April 2013 (Figure 5.2). The higher precipitation at the AgResearch site was by in large the result of the fortnightly irrigation (Figure 5.1). Mean air temperatures were similar at both nursery locations over the duration of the trials.

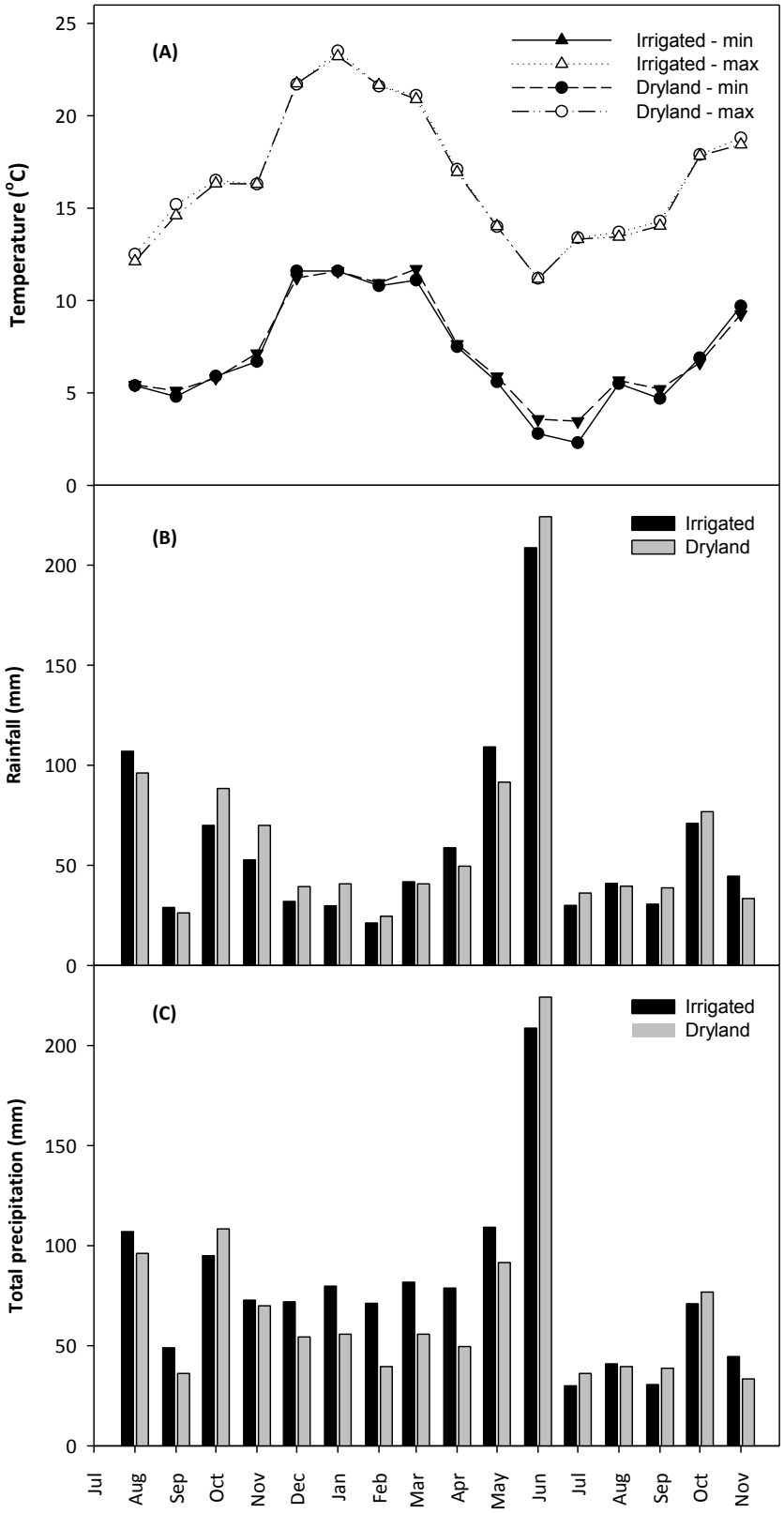


Figure 5.2 Mean monthly maximum (○, AgResearch; Δ Ashley Dene) and mean minimum (● AgResearch; ▲ Ashley Dene) air temperatures (A), monthly rainfall at AgResearch (■) and Ashley Dene (■) (B) and total monthly precipitation (included irrigation) (C) at AgResearch (■) and Ashley Dene (■) during the trial period August 2012 to November 2013.

5.3.1 Calibration curves

Among the scored morphological traits, lateral spread had the highest calibration correlation coefficient of 0.93 and 0.94 at the AgResearch and Ashley Dene nurseries, respectively (Table 5.2). Both clover herbage yield and leaf size calibration correlation coefficients ranged from 0.82 to 0.92 and 0.76 to 0.91, respectively across seasons. Growing point density correlation coefficients similarly ranged from 0.85 to 0.90 at both the AgResearch and Ashley Dene nurseries, although at Ashley Dene the GPD score in spring had a significantly lower calibration correlation coefficient of 0.57.

Table 5.2 Correlation coefficients of white clover visual score calibration curves. All visual scores were calibrated with the square root of measured units, except leaf size which was calibrated against actual measured units.

Trait	Location	Season				
		Summer	Autumn	Winter	Spring	$\bar{x}$
Herbage yield	AgResearch	0.87	0.83	0.87	0.94	0.88
	Ashley Dene	0.82	0.83	0.86	0.89	0.85
Leaf size	AgResearch	0.76	0.91	-	0.86	0.84
	Ashley Dene	0.86	0.91	-	0.83	0.87
Lateral spread	AgResearch	-	0.94	-	0.92	0.93
	Ashley Dene	-	0.95	-	0.93	0.94
Growing Point Density	AgResearch	-	0.85	-	0.87	0.86
	Ashley Dene	-	0.90	-	0.57	0.74

5.3.2 Within season analyses

There were significant ($P < 0.05$) additive genetic differences for seasonal herbage yield among the twenty white clover maternal half-sib families (Table 5.3) evaluated at the AgResearch nursery. Significant ($P < 0.05$) additive genetic variation was also estimated among the twenty maternal half-sib families for herbage yield in four out the five seasons at the Ashley Dene nursery. Narrow sense heritabilities on a maternal half-sib family means basis ranged from 0.50 to 0.80 at both nurseries, whereas narrow sense heritabilities on an individual plant basis (maternal) ranged from 0.12 to 0.44.

Clover herbage yield was significantly ($P < 0.05$) different among the twenty paternal half-sib families for 2 of the 5 seasons (Table 5.3) at both the AgResearch and Ashley Dene nurseries. Heritabilities on a paternal half-sib family means basis ranged from 0.30 to 0.76 at both nurseries, whereas narrow sense heritabilities on an individual plant basis (paternal) ranged from 0.05 to 0.37.

On average, maternal family additive genetic variances were 1.5, and 1.2 fold larger than molecular marker determined paternal family additive genetic variances at the AgResearch and Ashley Dene nurseries, respectively. The average half-sib family means heritability for clover herbage yield within seasons was 0.65, 0.49 at AgResearch and 0.61 and 0.52 at Ashley Dene for maternal and paternal half-sib families, respectively. Average narrow sense heritabilities on a single plant basis were 0.10 and 0.09 at AgResearch calculated on a maternal and paternal plant basis and 0.10 at Ashley Dene, regardless of half-sib family gender.

Table 5.3 Seasonal herbage yield variance components and their associated standard errors (± SE) for 20 white clover half-sib families based on their known maternal (♀) and molecular determined paternal (♂) half-sib families. Variance components: σ_f^2 - half-sib family variance and σ_e^2 - experimental variance. Narrow sense heritabilities were calculated on a family means basis (h_f^2) and on an individual plant basis (h_1^2).

Season	Site	Half-sib	σ_f^2	σ_e^2	h_f^2	h_1^2
Spring 2012 (× 10^2)	**AgResearch**	♀	7.58 ± 2.39*	61.9 ± 3.53	0.80 ± 0.25	0.44 ± 0.14
	AgResearch	♂	2.82 ± 1.71*	64.7 ± 3.88	0.58 ± 0.35	0.17 ± 0.10
	Ashley Dene	♀	1.09 ± 0.59*	23.0 ± 1.32	0.60 ± 0.33	0.18 ± 0.10
	Ashley Dene	♂	1.43 ± 0.80*	23.2 ± 1.41	0.66 ± 0.36	0.23 ± 0.13
Summer 2012/13 (× 10^2)	**AgResearch**	♀	6.70 ± 3.60*	138 ± 7.90	0.61 ± 0.33	0.19 ± 0.10
	AgResearch	♂	9.10 ± 4.70*	136 ± 8.10	0.68 ± 0.35	0.25 ± 0.13
	Ashley Dene	♀	1.18 ± 0.59*	20.2 ± 1.16	0.65 ± 0.33	0.22 ± 0.11
	Ashley Dene	♂	2.08 ± 0.98*	20.7 ± 1.26	0.76 ± 0.36	0.37 ± 0.17
Autumn 2013 (× 10^2)	**AgResearch**	♀	6.00 ± 3.00*	102 ± 5.80	0.65 ± 0.33	0.22 ± 0.11
	AgResearch	♂	2.90 ± 2.20	105 ± 6.30	0.47 ± 0.36	0.11 ± 0.08
	Ashley Dene	♀	2.98 ± 1.45*	47.2 ± 2.71	0.67 ± 0.33	0.24 ± 0.12
	Ashley Dene	♂	1.58 ± 1.14	51.2 ± 3.10	0.50 ± 0.36	0.12 ± 0.09
Winter 2013 (× 10^2)	**AgResearch**	♀	0.93 ± 0.51*	20.8 ± 1.19	0.59 ± 0.32	0.17 ± 0.09
	AgResearch	♂	0.39 ± 0.37	21.6 ± 1.29	0.37 ± 0.35	0.07 ± 0.07
	Ashley Dene	♀	0.86 ± 0.48*	15.1 ± 1.00	0.65 ± 0.36	0.22 ± 0.12
	Ashley Dene	♂	0.32 ± 0.35	16.1 ± 1.12	0.39 ± 0.43	0.08 ± 0.09
Spring 2013 (× 10^2)	**AgResearch**	♀	3.58 ± 1.95*	77.1 ± 4.41	0.60 ± 0.33	0.18 ± 0.10
	AgResearch	♂	1.31 ± 1.33	80.1 ± 4.80	0.34 ± 0.35	0.06 ± 0.07
	Ashley Dene	♀	0.48 ± 0.31	15.2 ± 0.87	0.50 ± 0.32	0.12 ± 0.08
	Ashley Dene	♂	0.21 ± 0.25	15.6 ± 0.94	0.30 ± 0.36	0.05 ± 0.06

*Significance at the 0.05 probability level (log-likelihood)

There were significant ($P < 0.05$) additive genetic differences for leaf size among the twenty maternal half-sib families in all three seasons (Table 5.4) at both the AgResearch and Ashley Dene nurseries. There were also significant ($P < 0.05$) additive genetic differences among the twenty paternal half-sib families in all three seasons at the AgResearch nursery, but only

during autumn at the Ashley Dene nursery. Heritabilities on a maternal half-sib family means basis ranged from 0.74 to 0.64 among nurseries, whereas on a paternal half-sib family basis they ranged from 0.79 to 0.39. Narrow sense heritabilities on a single plant basis ranged from 0.32 to 0.31 on a maternal basis and 0.41 to 0.08 on a paternal basis among nurseries.

For lateral spread, significant ($P < 0.05$) additive genetic variation was detected among the maternal half-sib families at only the AgResearch nursery in autumn. Additive genetic variation for growing point density was significant ($P < 0.05$) among maternal half-sib families in both seasons at both nurseries, whereas additive genetic variation for GPD was only significant ($P < 0.05$) among paternal half-sib families in autumn at AgResearch. Heritabilities on a maternal half-sib family means basis ranged from 0.78 to 0.70 and 0.61 to 0.38 on a paternal half-sib family basis at the AgResearch and Ashley Dene nurseries, respectively. Narrow sense heritabilities on a single plant basis were higher on a maternal basis (0.27 to 0.39) than on a paternal basis (0.07 to 0.26).

Across all three traits in Table 5.4, maternal family additive genetic variances were 1.2, and 1.9 fold larger than molecular marker determined paternal family additive genetic variances at the AgResearch and Ashley Dene nurseries, respectively.

Table 5.4 Seasonal morphological trait variance components and their associated standard errors (± SE) for 20 white clover half-sib families based on their known maternal (♀) and molecular determined paternal (♂) half-sib families. Variance components: σ_f^2 - half-sib family variance and σ_e^2 - experimental variance. Narrow sense heritabilities were calculated on a family means basis (h_f^2) and on an individual plant basis (h_1^2).

Trait	Season	Site	Half-sib	σ_f^2	σ_e^2	h_f^2	h_1^2
Leaf size	Summer 2012	AgResearch	♀	0.24 ± 0.11*	3.49 ± 0.20	0.68 ± 0.33	0.25 ± 0.12
		AgResearch	♂	0.38 ± 0.17*	3.34 ± 0.20	0.79 ± 0.35	0.41 ± 0.18
		Ashley Dene	♀	0.38 ± 0.18*	5.84 ± 0.34	0.67 ± 0.33	0.24 ± 0.12
		Ashley Dene	♂	0.13 ± 0.12	6.32 ± 0.38	0.39 ± 0.36	0.08 ± 0.07
	Autumn 2013	AgResearch	♀	0.43 ± 0.19*	4.91 ± 0.28	0.74 ± 0.33	0.32 ± 0.14
		AgResearch	♂	0.49 ± 0.23*	4.85 ± 0.29	0.76 ± 0.35	0.37 ± 0.17
		Ashley Dene	♀	0.23 ± 0.11*	3.14 ± 0.18	0.70 ± 0.33	0.27 ± 0.13
		Ashley Dene	♂	0.22 ± 0.12*	3.39 ± 0.21	0.68 ± 0.36	0.25 ± 0.13
	Spring 2013	AgResearch	♀	0.33 ± 0.17*	5.81 ± 0.34	0.64 ± 0.33	0.21 ± 0.11
		AgResearch	♂	0.26 ± 0.16*	5.93 ± 0.36	0.59 ± 0.35	0.17 ± 0.11
		Ashley Dene	♀	0.32 ± 0.16*	5.35 ± 0.32	0.66 ± 0.33	0.22 ± 0.11
		Ashley Dene	♂	0.15 ± 0.12	5.53 ± 0.35	0.46 ± 0.37	0.10 ± 0.08
Lateral Spread ($\times 10^2$)	Autumn 2013	AgResearch	♀	0.08 ± 0.04*	1.59 ± 0.09	0.63 ± 0.32	0.20 ± 0.10
		AgResearch	♂	0.04 ± 0.03	1.62 ± 0.10	0.45 ± 0.36	0.10 ± 0.08
		Ashley Dene	♀	0.02 ± 0.01	0.37 ± 0.02	0.53 ± 0.33	0.14 ± 0.08
		Ashley Dene	♂	0.00 ± 0.01	0.40 ± 0.02	0.16 ± 0.34	0.02 ± 0.05
	Spring 2013	AgResearch	♀	0.04 ± 0.03	1.26 ± 0.07	0.49 ± 0.32	0.12 ± 0.08
		AgResearch	♂	0.03 ± 0.02	1.25 ± 0.08	0.43 ± 0.35	0.09 ± 0.08
		Ashley Dene	♀	0.02 ± 0.02	1.02 ± 0.06	0.42 ± 0.33	0.09 ± 0.07
		Ashley Dene	♂	0.01 ± 0.01	1.06 ± 0.06	0.19 ± 0.34	0.03 ± 0.05
Growing point density	Autumn 2013	AgResearch	♀	0.38 ± 0.16*	3.47 ± 0.20	0.78 ± 0.32	0.39 ± 0.16
		AgResearch	♂	0.25 ± 0.13*	3.58 ± 0.22	0.69 ± 0.35	0.26 ± 0.13
		Ashley Dene	♀	0.16 ± 0.08*	2.19 ± 0.13	0.70 ± 0.33	0.27 ± 0.13
		Ashley Dene	♂	0.11 ± 0.07	2.35 ± 0.14	0.61 ± 0.36	0.18 ± 0.11
	Spring 2013	AgResearch	♀	0.65 ± 0.30*	8.91 ± 0.51	0.70 ± 0.32	0.27 ± 0.13
		AgResearch	♂	0.24 ± 0.18	9.14 ± 0.55	0.45 ± 0.35	0.10 ± 0.08
		Ashley Dene	♀	0.17 ± 0.08*	2.35 ± 0.14	0.70 ± 0.32	0.27 ± 0.13
		Ashley Dene	♂	0.05 ± 0.05	2.57 ± 0.16	0.38 ± 0.36	0.07 ± 0.07

*Significance at the 0.05 probability level (log-likelihood)

5.3.3 Across season analysis

There were significant (P<0.05) additive genetic differences among both maternal and paternal half-sib families for herbage yield across all 5 seasons at both nurseries (Table 5.5). In addition, there was a significant (P<0.05) maternal half-sib family × season interaction at the AgResearch nursery. The season variance component was the largest at both nurseries and

accounted for (averaged among maternal and paternal half-sib families) 73% and 85% of the total variation at Ashley Dene and AgResearch, respectively.

Leaf size at the AgResearch nursery was significantly (P<0.05) different among maternal and paternal half-sib families across seasons (Table 5.5). Leaf size at the Ashley Dene nursery tended to be (P<0.1) different among maternal and paternal half-sib families. There was also a significant (P<0.05) maternal half-sib family × season interaction. As with herbage yield, the largest variance component for leaf size was season. On average, the season variance component accounted for 71%, and 50% of the total variation at Ashley Dene and AgResearch, respectively.

Growing point density at the AgResearch nursery was significantly (P<0.05) different among both maternal and paternal half-sib families across all seasons (Table 5.5), but this was not evident among maternal and paternal half-sib families at the Ashley Dene nursery. Season accounted for a much greater percentage of total variation at the AgResearch nursery (31%) than at the Ashley Dene (3%) nursery.

Similarly to individual season analyses for lateral spread, only significant additive genetic differences among maternal half-sib families (P < 0.05) were present at the AgResearch nursery. The season variance component only accounted for 0 and 1% of the total variation at the AgResearch and Ashley Dene nurseries. Across all four morphological traits in Table 5.5, maternal family additive genetic variances were 0.98 and 1.4 fold larger than molecular marker determined paternal family additive genetic variances at the AgResearch and Ashley Dene nurseries, respectively, and only 1.08 fold larger over both nurseries.

Table 5.5 Morphological trait variance components and their associated standard errors (± SE) calculated across seasons within two environments (Ashley Dene and AgResearch) for 20 white clover half-sib families based on their known maternal (♀) and molecular determined paternal (♂) half-sib families. Variance components: σ_s^2 - season; σ_{sr}^2 - replicates within seasons; σ_f^2 - half-sib family; σ_{fs}^2 - half-sib × season; σ_ε^2 – residual error. Narrow sense heritabilities were calculated on a family means basis (h_f^2) and on an individual plant basis (h_1^2), and ranges are given among half-sib families on an average plant per half-sib basis.

Sources	Herbage yield (× 10^1)				Leaf size (× 10^1)			
	AgResearch		Ashley Dene		AgResearch		Ashley Dene	
	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib
σ_s^2	23.05 ± 16.35	23.38 ± 16.58	14.82 ± 10.4	14.98 ± 10.6	51.0 ± 51.22	50.25 ± 50.45	126.89 ± 127.21	125.39 ± 125.69
σ_{sr}^2	0.36 ± 0.11	0.34 ± 0.11	0.06 ± 0.02	0.05 ± 0.02	0.85 ± 0.45	0.87 ± 0.47	1.38 ± 0.62	1.48 ± 0.68
σ_f^2	0.28 ± 0.12*	0.29 ± 0.12*	0.10 ± 0.04*	0.14 ± 0.06*	2.41 ± 1.06*	4.16 ± 1.61*	1.70 ± 0.89	1.36 ± 0.75
σ_{fs}^2	0.21 ± 0.08*	0.09 ± 0.06	0.03 ± 0.02	0.01 ± 0.02	0.89 ± 0.55	0.12 ± 0.38	1.34 ± 0.66*	0.40 ± 0.49
σ_ε^2	7.99 ± 0.20	8.12 ± 0.22	2.46 ± 0.07	2.58 ± 0.07	47.27 ± 1.56	46.79 ± 1.62	47.38 ± 1.59	50.35 ± 1.79
h_1^2	0.13 ± 0.06	0.13 ± 0.06	0.15 ± 0.06	0.20 ± 0.08	0.19 ± 0.08	0.33 ± 0.13	0.13 ± 0.07	0.10 ± 0.06
h_f^2	0.75 ± 0.33	0.80 ± 0.35	0.82 ± 0.33	0.88 ± 0.35	0.75 ± 0.33	0.89 ± 0.34	0.64 ± 0.34	0.67 ± 0.37
Mean	10.1†	10.2†	4.0†	4.0†	20.6§	20.7§	14.8§	14.8§
Range	8.0 to 12.5†	8.7 to 12.6†	3.4 to 4.7†	3.0 to 4.9†	19.8 to 21.4§	19.3 to 21.9§	13.8 to 15.5§	14.1 to 15.1§
Sources	**Growing point density (× 10^1)**				**Lateral spread (× 10^4)**			
	AgResearch		Ashley Dene		AgResearch		Ashley Dene	
	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib	♀ Half-sib	♂ Half-sib
σ_s^2	33.53 ± 48.96	33.24 ± 48.44	0.68 ± 1.16	0.71 ± 1.20	0.00 ± 1.00	0.00 ± 1.00	0.99 ± 2.10	0.66 ± 1.62
σ_{sr}^2	7.82 ± 3.25	7.07 ± 3.01	0.64 ± 0.35	0.64 ± 0.38	11.90 ± 5.20	9.50 ± 4.30	3.12 ± 1.51	2.69 ± 1.40
σ_f^2	4.85 ± 1.96*	3.24 ± 1.57*	1.15 ± 0.61	0.50 ± 0.40	6.60 ± 2.80*	4.30 ± 2.30	1.84 ± 1.00	0.51 ± 0.69
σ_{fs}^2	0.27 ± 0.72	0.27 ± 0.76	0.51 ± 0.40	0.24 ± 0.35	0.00 ± 1.30	0.00 ± 1.30	0.00 ± 0.70	0.16 ± 0.70
σ_ε^2	61.88 ± 61.88	63.23 ± 2.68	22.71 ± 0.92	24.62 ± 24.62	143.00 ± 5.80	143.00 ± 6.10	69.50 ± 2.82	72.70 ± 3.10
h_1^2	0.29 ± 0.12	0.19 ± 0.09	0.19 ± 0.10	0.08 ± 0.06	0.18 ± 0.07	0.12 ± 0.06	0.10 ± 0.06	0.03 ± 0.04
h_f^2	0.92 ± 0.37	0.88 ± 0.43	0.83 ± 0.44	0.71 ± 0.57	0.88 ± 0.37	0.83 ± 0.44	0.81 ± 0.44	0.51 ± 0.69
Mean	57.6'	59.0'	29.1'	28.9'	0.21#	0.21#	0.06#	0.06#
Range	43 to 78'	46 to 78'	20 to 33'	24 to 32'	0.17 to 0.25#	0.18 to 0.24#	0.05 to 0.08#	0.04 to 0.08#

‡ Significance at the 0.1 probability level (log-likelihood) *Significance at the 0.05 probability level (log-likelihood), † g/plant, §mm/plant, 'GPD/plant, # m^2/plant

5.3.4 Across environments and seasons analysis

Leaf size was the only trait with significant (P <0.05) additive genetic differences among maternal half-sib families across both environments and seasons (Table 5.6). Clover herbage yield, GPD and lateral spread all had significant maternal half-sib family × environment interactions (P<0.05). Both GPD and lateral spread also had significant (P<0.05) maternal half-sib family × season interactions. Herbage yield also had a significant (P<0.05) maternal half-sib family × environment × season interaction.

Table 5.6 Morphological trait variance components and their associated standard errors (± SE) calculated across environments and seasons for 20 maternal white clover half-sib families. Variance components: σ_s^2 - seasons; σ_{er}^2 - replicates within environments; σ_f^2 - half-sib family; σ_{fe}^2 - half-sib × environment; σ_{es}^2 - environment × season; σ_{esr}^2 - environment × season × replicate; σ_{fs}^2 - half-sib × season; σ_{fes}^2 - half-sib family × environment × season and σ_ε^2 – residual error.

Sources	Leaf size	Herbage yield	GPD	Lateral spread (× 10^3)
σ_s^2	74.93 ± 82.11	16.02 ± 12.43	5.31 ± 18.52	0.00 ± 0.00
σ_{er}^2	0.00 ± 0.25	0.10 ± 0.05	0.00 ± 1.32	0.68 ± 0.30
σ_f^2	1.80 ± 0.82*	0.05 ± 0.06	0.00 ± 0.92	0.00 ± 0.14
σ_{fe}^2	0.26 ± 0.36	0.14 ± 0.06*	3.51 ± 1.24*	0.47 ± 0.18*
σ_{es}^2	14.66 ± 14.92	2.96 ± 2.11	11.64 ± 17.48	0.09 ± 0.11
σ_{esr}^2	1.25 ± 0.50	0.11 ± 0.04	5.50 ± 2.28	0.07 ± 0.08
σ_{fs}^2	0.47 ± 0.43	0.03 ± 0.03	1.13 ± 0.47*	0.15 ± 0.08*
σ_{fes}^2	0.63 ± 0.50	0.09 ± 0.04*	0.00 ± 0.19	0.00 ± 0.05
σ_ε^2	47.33 ± 1.12	5.31 ± 0.10	42.36 ± 1.21	10.60 ± 0.30
Mean	17.7[§]	6.65[†]	42.2'	0.13[#]
Range	16.8 to 18.5[§]	5.52 to 7.90[†]	33.6 to 53.5'	0.11 to 0.14[#]

*Significance at the 0.05 probability level (log-likelihood)
[†] g/plant, [§]mm/plant, 'GPD/plant, [#] m^2/plant

5.3.5 Comparison of maternal and paternal general combining ability (GCA)

There were significant regression coefficients (P<0.001) between maternal and parental general GCAs for seasonal herbage yield, leaf size and growing point density (Figure 5.3). Growing point density had the highest regression coefficient between maternal and paternal GCAs (0.52) and yield with the lowest (0.20). The slope of the regressions ranged from 0.51 for GPD and leaf size to 0.40 for clover herbage yield.

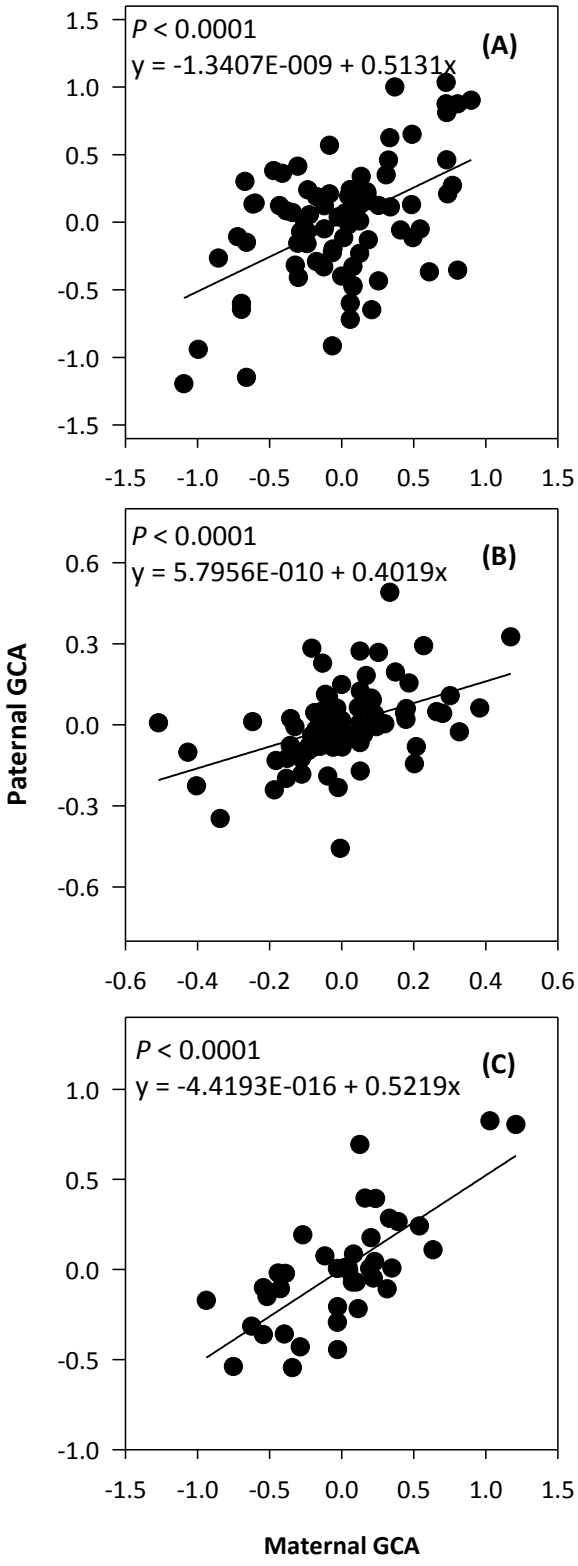


Figure 5.3 Seasonal half-sib general combining abilities (GCAs) for both environments based on known maternal and molecular marker determined paternal half-sib families for white clover leaf size (A), clover herbage yield (B) and GPD (C).

5.3.6 Simulated genetic gains using calculated genetic parameters

On a genetic gain per cycle basis, half-sib progeny trialling (HSPT) and known maternal and molecular marker determined paternal half-sib selection (♀+♂HS) were the most effective selection methods for genetic advance among all three morphological traits (HY, LS and GPD) (Figure 5.4). Known maternal and molecular marker determined paternal half-sib selection was superior to HSPT for both herbage yield (a) and leaf size (b) but not growing point density (c). Known maternal and molecular marker determined paternal half-sib selection was consistently superior to conventional maternal half-sib selection alone, across all traits, scenarios and selection intensities (Figure 5.4; a, b and c), and approximately doubled genetic gain per cycle of selection.

Paternal half-sib family (♂HS) selection alone was superior to maternal half-sib family (♀HS) selection alone for leaf size but not for GPD and was similar for herbage yield at the AgResearch nursery. Mass selection was superior to half-sib family selection methods (♀HS or ♂HS) when individual plant heritabilities were > 0.20 (Figure 5.4; c) but half-sib family selection was equal or superior to mass selection when individual plant heritabilities were < 0.20 (Figures 5.4; a) and b) respectively).

5.3.7 Simulated genetic gains using putative genetic parameters

Simulations using both putative genetic parameters, and a set time frame, AWF-HS+MFS was superior to all selection methods when individual plant heritabilities were less than 0.15 and among family heritability was 0.30 (Figure 5.5). AWF-HS+MFS was superior to all selection methods (except ♀+♂HS, when individual h^2 <0.05) when individual plant heritabilities were less than 0.25 and 0.35 and among family heritabilities were 0.50 and 0.70 respectively (Figure 5.5).

In the absence of selection within molecular determined full-sib families, ♀+♂HS was still twice as effective as HSF, but not as effective as AWF-HS+MFS. The efficiency of ♀+♂HS decreased compared to mass selection and AWF-HS as individual plant heritabilities increased. Mass selection was the best selection method when heritabilities on an individual basis were within approximately 30% of heritabilities on a family means basis (a, b and c). HSPT was only 1.5 fold more effective than HSF (instead of two fold) due to an extra year required for a second recombination (Casler and Brummer, 2008).

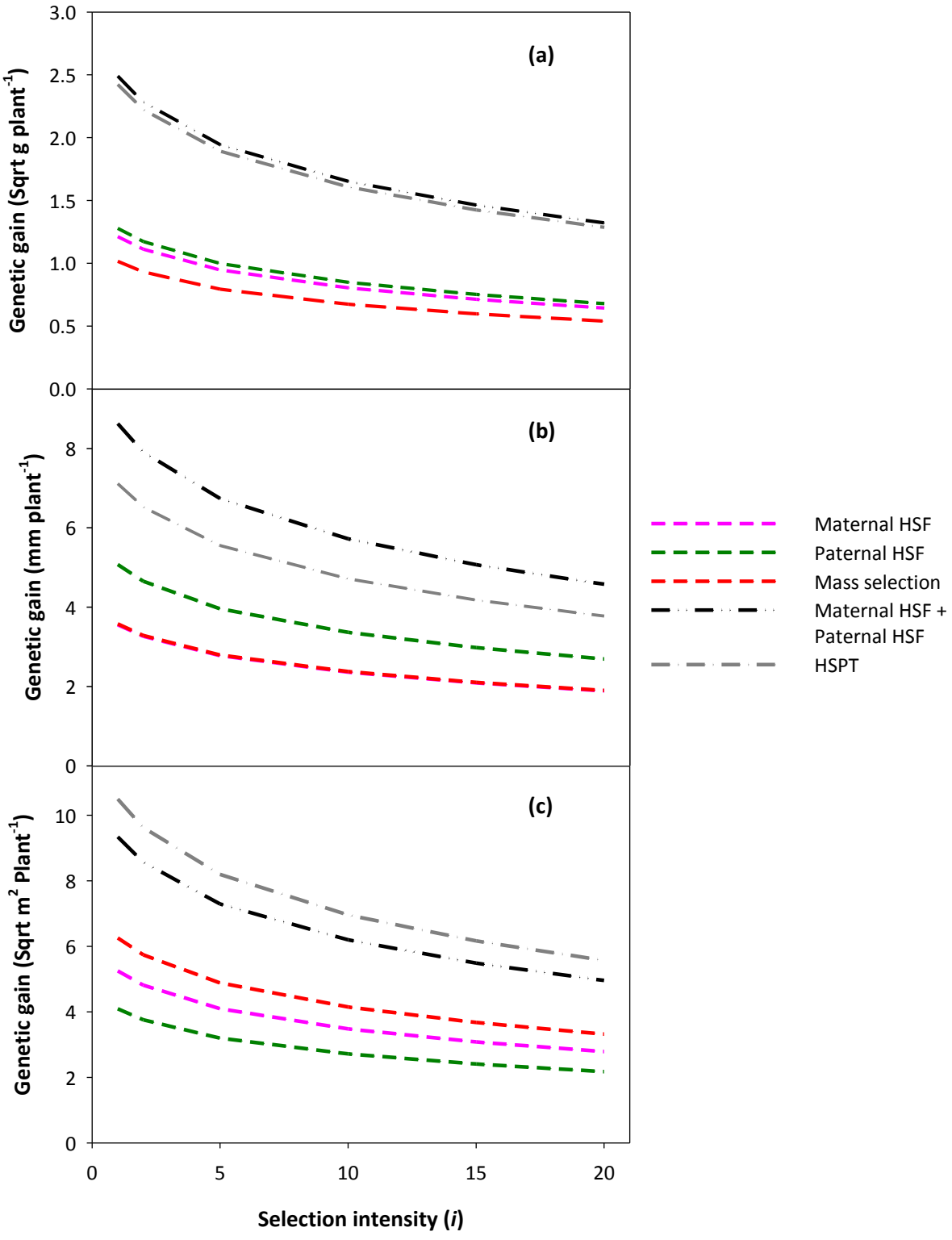


Figure 5.4 **Simulated genetic gains for three key morphological traits at the AgResearch nursery; herbage yield (a), leaf size (b) and growing point density (c) using five different breeding methods. Comparisons are made using equal selection intensities (%) and adjusted effective population size. Sqrt; square-root transformed data.**

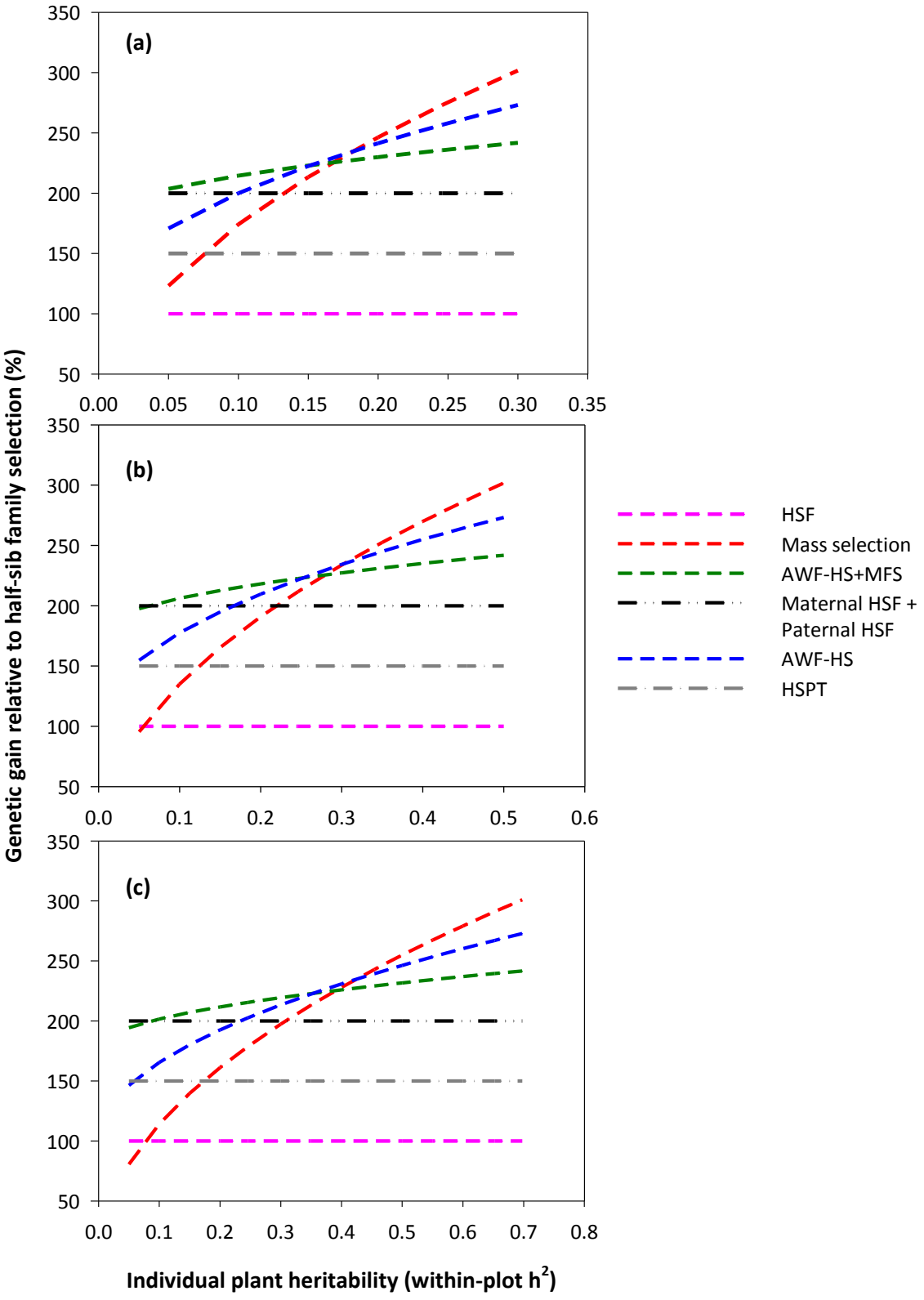


Figure 5.5 **Expected genetic gains (per every 3 years) simulated from half-sib family selection (HSF), mass selection, among-half-sib-and-within molecular marker determined full-sib family selection (AWF-HS+MFS), half-sib progeny trialling (HSPT), and among-and-within half-sib family selection (AWF-HS). All expected gains are expressed as a percentage of gains for HSF. All models are based on an equal effective population size (N_e) with adjusted selection intensities (%). Family heritabilities were fixed at, a; 0.30, b; 0.5, and c; 0.7.**

5.4 Discussion

5.4.1 Calibration curves

The correlations between visual biomass scores and above ground biomass yield in the two spaced-planted white clover nurseries, suggest that visual scores are a reasonably accurate method for measuring actual biomass yields for individual plants in white clover spaced-planted nurseries within a ryegrass sward. They are similar to previously reported biomass correlations in other perennial spaced-planted forage species, which range from 0.64 to 0.94 (Casler, 2001; Casler and van Santen, 2000; Riday, 2009). In addition, correlations between visual scores and actual measurement for leaf size, lateral spread and growing point density were high, with the exception of the GPD score at the Ashley Dene nursery in spring 2013.

Similar to Riday (2009), an exponential relationship between visual scores and biomass yield ($\sqrt{}$g plant^{-1}) was evident. This relationship was also evident for lateral spread ($\sqrt{}$m^{-2} plant^{-1}) and growing point density ($\sqrt{}$GPD plant^{-1}). As explained by Riday (2009), the above traits that were scored on a single plant basis were estimated on an order of magnitude rather than on a linear basis (i.e. this plant is X times as big as that plant, and that plant is X times as big as the next plant). Likewise to findings presented by Riday (2009), the larger the relative size of the individual plants, the more difficult it becomes to accurately score them and this was evident by the larger residuals associated with the larger score values (data not presented).

Among morphological traits, lateral spread had the highest correlation between visual scores and the transformed measured units. Using the aid of a 0.6m × 0.6m quadrat helped to visualise proportional differences between plants and most likely resulted in the high regression coefficient values. On the other hand, the poor correlation between visual score and growing point density at the Ashley Dene nursery in spring 2013 was most likely a result of the timing of the assessment. GPD scores needed to be completed within 5-8 days after intensive sheep grazing and before canopy closure of the companion sward species in order to allow estimates of visible growing points on the soil surface. In the instance of the spring assessment at the Ashley Dene nursery, the visual assessment coincided with canopy closure, and resultantly plants with higher growing point densities were difficult to distinguish.

5.4.2 Within season analyses

Seasonal analyses among half-sib families for herbage yield, leaf size and growing point density confirms that significant additive variation exists within the source breeding population for these morphological traits. The lack of significant differences among half-sib

families for lateral spread suggests that either there is little additive genetic variation among half-sib families in this population or the sampling/spaced-planted evaluation technique used was too inaccurate to quantify the variation. Considering lateral spread at the AgResearch nursery was significant among families in autumn but non-significant in spring following inter-row spraying, the latter explanation is more probable. The Ashley Dene nursery however had little additive genetic variation in either autumn or spring despite no inter-row spraying and probably reflects the little additive variation for this trait in this environment.

It is evident from Tables 5.3 and 5.4, that significant differences among half-sib families were more prevalent with maternal half-sib families than for paternal half-sib families. The higher frequency of insignificant results among paternal half-sib families compared to maternal half-sib families may have been a result of the imbalance in paternal half-sib family sizes. Due to the non-random mating of the parent polycrosses (which was discussed in Chapter 3), the number of offspring among the twenty paternal half-sib families varied greatly and in many cases, paternal half-sib families within replicates were represented by zero, one or few genotypes. Further mortalities among these initial few individuals as the trial progressed would have further upset paternal half-sib family means and increased the standard error. The latter is certainly supported by the fact that the lack of significant effects among paternal families became more pronounced as the trial duration increased.

While the theoretical ratio of maternal to paternal additive genetic variance in the absence of maternal effects should theoretically be 1:1, the ratios observed in this study considering the imbalance in paternal half-sib families were not too distant from this expected ratio. In a study by Riday (2011), red clover paternal additive genetic variances were four to five times greater than maternal family additive genetic variance and resultantly, paternity based selections alone were more than double that of maternal selection alone.

Heritabilities on a half-sib family means basis were again consistently higher for maternal half-sib families than for paternal half-sib families. However, regardless of half-sib family gender, half-sib family heritabilities were always consistently in the magnitude of two to five fold higher than heritabilities on a single plant basis, and like the findings of Riday (2011) in red clover, the family based heritabilities illustrate the theoretical rationale of pursing half-sib family selection methods in white clover. Nyquist (1991) indicated that when heritability on an individual plant basis is low due to large environmental effects, family selection should be implemented instead. An important feature of family selection is that selection is based on

family means which are obtained from replicated trials; and therefore less affected by large environmental variances than are individual selections (Nguyen and Sleper, 1983).

While more replicates help to reduce experimental error and increase heritability on a family means basis, the number of replicates used in the nurseries of this experiment did not reflect practical options in many plant breeding situations. However, considering this experiment was designed to accurately estimate genetic parameters for genetic gain simulation, extra effort was justified to improve the estimation of variance components. The rationale for eight replicates was twofold; i) to reduce experimental error (σ_e^2) via increasing the number of replicates, as replicates are a divisor for σ_e^2, and ii) to reduce plot-to-plot variation (σ^2) by reducing plot size per entry.

Plot-to-plot variation is associated with environmental differences from one plot to another, and it often increases as the amount of trial area in a replication increases because of soil heterogeneity (Fehr, 1987). Therefore σ^2can be reduced via decreasing the number of plots per replicate or decreasing the size of the individual plots. However, while decreasing plot size can help reduce σ^2 by limiting environmental variance, it is vital to have enough plants per plot to minimise the within plot variance component (σ_w^2), as σ_e^2 is influenced by both σ^2 and σ_w^2, where $\sigma_w^2 = \frac{\sigma_u^2 + \sigma_{wg}^2}{n}$ and $\sigma_e^2 = \sigma_w^2 + \sigma^2$. In this trial, since there was a fixed number of plants, increasing replicates resulted in fewer plants per replicate. Consequently by reducing the number of plants per plot (n), within-plot-variation (σ_w^2) increased significantly due to a lower divisor of genetic segregation (σ_{wg}^2) and environmental effects (σ_u^2) and ultimately experimental error (σ_e^2), as within-plot-variability is a function of the number of plants that are averaged together to determine a plot mean (Fehr, 1987). In addition, by not adequately sampling enough plants per plot, it also increased the plot-to-plot variation(σ^2), as family/plot means were highly variable within replicates, creating a family × replicate interaction. Resultantly, the σ^2 variance component was removed from analyses to minimise the replicate × half-sib family interaction and focus on family means across the trial.

To avoid genetic sampling effects in future studies, the number of plants per replicate should be chosen according to family structure (Posselt, 2010). Since genetic variability within plots is higher for half-sib families $\left(\frac{3}{4}\sigma_A^2\right)$, than for full-sib families $\left(\frac{1}{2}\sigma_A^2\right)$, more plants per plot are needed to reduce the within plot variability (σ_w^2) due to within-plot genetic segregation (σ_{wg}^2). Posselt (2010) recommended for half-sib families that15-20 plants per plot are adequate, whereas for full-sibs somewhat lower numbers might be used. This explanation of genetic

segregation (σ^2_{wg}) helps explain the magnitude of experimental error (σ^2_e) (which is a combination of within-plot-variation (σ^2_w) and plot-to-plot variation(σ^2)), compared to that of among family variation (σ^2_f) in this experiment. For future experiments/nurseries, breeders are encouraged to use fewer replicates with more plants per plot. While decreasing replicate numbers at each site would decrease family heritability via reducing the divisor of plot-to-plot variance (σ^2), this could be offset by using more plants per plot to reduce the within family variance component (increasing divisor of σ^2_w).

In regard to genetic gain, if breeders have the option of multiple trial locations, breeders would be further encouraged to use fewer replicates per environment (with adequate plants per plot as discussed above) and instead use more environments. Theory shows superior genetic gain would be realised by actually only growing one replication at many environments, because the number of environments is a divisor for both experimental error (σ^2_e) and family × environment interaction (σ^2_{fe}) in the genetic gain formula (Fehr, 1987).

5.4.3 Across season analyses

The across season analyses confirmed that significant annual additive genetic variation for herbage yield exists within the source breeding population at both environments. The maternal family × season interaction at the AgResearch nursery also indicates re-ranking of maternal half-sib families among seasons. Narrow sense heritabilities on a single plant basis were relatively consistent among maternal and paternal half-sib families and environments, ranging from 0.13 to 0.20. These heritabilities are similar to those reported on a narrow sense basis by Woodfield and Caradus (1990) (0.09 ± 0.10) but considerably lower than those reported by Annicchiarico et al. (1999) (0.52 ± 0.29). The large discrepancy in heritabilities between the experiment reported here and that of Annicchiarico et al. (1999) are likely due to either differences in source population genetic parameters or experimental circumstances. While the former cannot be disregarded, it does seem unlikely considering only 16 families were investigated in that study compared to the 20 families in the present study. A possible explanation is the trial management in which the studies were conducted, whereby the study by Annicchiarico et al. (1999) was conducted under a cutting regime whereas the present study was conducted under sheep grazing management. The difficultly of minimising heterogeneous variation in ruminant grazed trials is well documented and often results in increased residual variation which resultantly increases the denominator of the heritability formula, thereby decreasing heritability.

Similar to herbage yield, significant additive genetic variation for leaf size existed within the source breeding population at both environments across seasons. Narrow sense heritabilities on an individual plant were on average greater than the other three morphological traits and are similar to previous reports where leaf size heritability has been higher in comparison (Annicchiarico and Piano, 1995; Annicchiarico et al., 1999; Caradus and Woodfield, 1990; Caradus and Chapman, 1996; Woodfield and Caradus, 1990). It is interesting to note the large discrepancy in heritabilities between the two nurseries. The discrepancy highlights the lower heritability at the Ashley Dene, nursery which is expected under stress conditions as a consequence of the higher environmental component of the phenotypic variance (Blum, 1985).

Although additive genetic variation for growing point density was significant among maternal and paternal half-sib families at the AgResearch nursery, no additive genetic variation at the Ashley Dene nursery was detected. The additive genetic variation at the AgResearch nursery however was almost as heritable on a narrow sense basis as leaf size (average among maternal and paternal) and highlights the fact that considerable growing point density improvement can be made through field breeding practices.

The significant differences among half-sib families at the AgResearch nursery for lateral spread and the lack of significant differences among half-sib families at the Ashley Dene nursery clearly demonstrate the constraint total precipitation has on the spreading growth habit of white clover.

A common trend across all four morphological traits was the magnitude of difference between heritabilities calculated on a family means basis and those on an individual plant basis. Heritabilities on a family mean basis were consistently three to five fold larger than narrow sense heritabilities on an individual plant basis. However as pointed out earlier, it is important to note (due the high number of replicates) the heritabilities calculated on a family means basis in this study are biased upwards. These family mean heritabilities are likely to be further magnified by the fact that within-plot-variance (σ_w^2) and plot-to-plot variance (σ^2) components were pooled into the residual error term, which was divided by a bigger denominator than if the individual components were treated separately. Despite the likelihood of inflated family means heritabilities (the inflated difference is still trivial in comparison to the magnitude of difference between individual and family means heritabilities) the results demonstrate the justification of half-sib family selection methods in white clover.

5.4.4 Across seasons and environments analyses

Leaf size among both maternal and paternal half-sib families showed consistency in family ranking across both seasons and environments. The lack of family × environment interaction for this trait would suggest selection in a single environment would be adequate due to the consistent ranking of families across environments. From this standpoint, the environment with the highest heritability (AgResearch nursery) would be the most logical choice for breeders. Furthermore, due to the limited environment × family interaction in this experiment, and considering the limited grazing × genotype interactions that are reported in the literature for leaf size (Caradus et al., 1989), environments with even higher heritabilities, such as spaced-planted monoculture nurseries with the absence of animal grazing, are likely to yield even better selection gains without any detrimental effects or re-ranking of genotypes in grazed sward scenarios.

The lack of differences among maternal and paternal families for herbage yield, growing point density and lateral spread among nurseries and their significant family × environment interactions suggest half-sib family performance is largely mitigated by environmental cues under these trial circumstances. The substantial re-ranking of half-sib families for these traits across environments indicates that the families in this experiment are not broadly adapted across environments, and selection within environments for specific adaptation is required. However, these results indicate the importance of multi-site evaluation in cultivar development programmes to identify material with broad adaptation.

5.4.5 Comparison of maternal and paternal GCAs

The positive correlation coefficients demonstrate that the best performing maternal families tended to also be the best performing paternal families and vice versa (Figure 5.3). It is interesting to note that in parent offspring regression, the slope of the regression represents the heritability of the trait (Falconer, 1961). Although the slopes in this case were lower than the predicted heritabilities using REML, the traits still ranked in similar order of heritability. In other words, the higher the heritability of the trait, the better the correlation between maternal and paternal half-sib values, as the predicted GCAs are less confounded by environmental error. The positive correlations give breeders the confidence that their best maternal selections are also indirectly the best paternal selections as theory suggests.

5.4.6 Simulated genetic gains

The comparison of genetic gain between different breeding methods largely depends on the interval measured (per cycle or time), the selection intensities (%), and the effective

population sizes (N_e) at which they are compared. This discussion deals with the comparison firstly on a genetic gain per cycle basis with equal selection intensities using genetic parameters estimated in this chapter (Figure 5.4) and secondly on a more commercially representative approach taking into account equal effective population sizes and time (Figure 5.5) (further details are described in the materials and methods). The latter approach utilises artificial genetic parameters so that within-family selection methods can also be compared, since within family parameters were not accurately estimated in this experiment due to the small plot sizes and error confounded within-family variance components.

Figure 5.4 clearly demonstrates the advantage of HSPT and ♀HS +♂HS over HSF and mass selection for clover herbage yield, leaf size and growing point density at equal selection intensities (k) on a genetic gain per cycle basis. As theory suggests (Riday, 2011), known maternal and molecular marker determined paternal half-sib selection (♀HS +♂HS) (ignoring selection within full-sib families) is approximately two-fold more effective than conventional maternal half-sib family selection alone. The twofold increase in genetic gain can be explained by the genetic gain formula containing two half-sib family selection terms instead of one (maternal half-sib and paternal half-sib instead of maternal half-sib alone; see below) (Riday, 2011).

$$\Delta G = \frac{k_M \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_W^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}} + \frac{k_P \frac{1}{4}\sigma_A^2}{\sqrt{\frac{1}{4}\sigma_A^2 + \frac{\sigma^2}{t.r} + \frac{\sigma_W^2}{t.r.n} + \frac{\frac{1}{4}\sigma_{AE}^2}{t}}} + \frac{k_W \frac{1}{2}\sigma_A^2}{\sqrt{\sigma_u^2 + \frac{1}{2}\sigma_A^2 + \frac{1}{2}\sigma_{AE}^2 + \sigma_D^2}}$$

Where:
ΔG = genetic gain per cycle
k = selection intensity; k_M – maternal half-sib family, k_P – paternal half-sib family, k_w – within family
σ^2 = variance; σ_A^2 – additive, σ_D^2 – dominance, σ_{AE}^2 – additive × environment, σ_w^2 – within-plot, σ_u^2 – environmental-within-plot, σ^2 – Plot-to-plot
r – number of reps, t – number of environments, n – number of plants per plot

♀HS +♂HS selection is effectively the same as increasing the parental control value to two in the conventional maternal half-sib family selection gain formula, assuming equal selection pressure is applied to both maternal and paternal half-sib families. Alternately to ♀HS +♂HS, the parental control value in the genetic gain formula can also be doubled by recombining saved parents of the best half-sib progenies; known as half-sib progeny trialling (HSPT). Despite identical theoretical numerators between ♀HS +♂HS and HSPT, ♀HS +♂HS in Figure 5.4 was superior to HSPT when paternal half-sib family variances exceeded those of maternal half-sib variances and vice versa. However, considering that discrepancies in

maternal and paternal additive genetic variances are likely to be arbitrary, and the deviance in ratios from 1:1 are likely to be error related, genetic gains per cycle from HSPT and ♀HS +♂HS are actually likely to be equal, assuming equal selection intensities are applied for both.

In contrast to genetic gain per cycle, ♀HS +♂HS was superior to HSPT on a 3 year basis, even when maternal and paternal additive genetic variances were assumed to be equal. These findings agree with Casler and Brummer (2008). Unlike Vogel and Pedersen (1993), HSPT is not necessarily a single-cycle "dead-end" selection method, and while it can be incorporated into a recurrent scheme, it requires two recombination events per cycle to create parents for the following cycle. Consequently, an extra recombination event generally requires an extra year, and the difference in genetic gain observed between ♀HS +♂HS and HSPT in Figure 5.5 reflects the penalty associated with genetic gains over time.

In addition to superiority in time, additional phenotypic selection within the best molecular marker determined full-sib families can further advance ♀HS +♂HS in relation to HSPT. This is referred to as AWF-HS+MFS which utilises the remaining half of the additive genetic variation within molecular marker determined full-sib families; see the third term in the equation above. A significant pitfall of HSPT is not only its longer cycle time but also its lack of ability to utilise additional within family additive variation. By contrast, AWF-HS+MFS effectively utilises the within family variation, and its superiority over the other selection methods at moderate to low individual plant heritabilities are evident in Figure 5.5. AWF-HS+MFS combines genotypic and phenotypic selection simultaneously within the same nursery. Disadvantageously, it does however rely on ample genotypes within half-sib families to do so and requires a somewhat more strategic approach (due to the low probable number of individuals per optimum full-sib combination; only 50% within family selection intensity was simulated). From a pragmatic perspective, one approach would be to assess a large number of individuals per half-sib family to ensure a reasonable number of optimum full-sib combinations exist within the nursery. To save costs associated with genotyping the entire nursery, an initial 15% selection intensity could be applied across maternal families alone, followed by paternity testing within these best families to find the optimal paternal combinations. A further 50% selection intensity could then be applied on a phenotypic basis within the best full-sib combinations. Thus it can be recommended to start with a nursery of 100 half-sib families × 100 individuals per half-sib family and then select the best 15 half-sib families based on maternal data alone. Within these best 15 half-sib families, paternity testing could then be carried out on all surviving progeny (≤ 1500 progeny, of which approximately

15% (225) would have the ideal parentage), and then phenotypic selection within the best full-sib combinations could be achieved.

With regard to the other selection methods, mass selection was superior to HSF for leaf size and GPD, but not for herbage yield on a genetic gain per cycle basis when selection intensities were equal (Figure 5.4). Although the genetic gain on an individual-plant basis was lower for herbage yield than genetic gain on a family means basis, the breeder must keep in mind that this disadvantage can be offset or exceeded by the larger numbers of individual plants that can be screened, selected, and recombined in mass selection (Bernardo, 2010) (increases the numerator in the genetic gain formula by increasing *i*). This is evident in Figure 5.5, where the selection intensity of mass selection is adjusted to 1% (compared to the selection intensity of 10% between families) to compensate for an equal effective population size. Even when selection intensities were equal in Figure 5.4 and despite heritabilities on a family means basis still being higher than heritabilities on an individual plant basis for leaf size and GPD, genetic gain for HSF was lower than mass selection. A considerable drawback with half-sib family selection is the smaller fraction of additive genetic variance that is utilised among families and therefore its smaller phenotypic standard deviation, which results in the lower genetic gain per cycle. Falconer (1961) stated that family selection cannot be better than individual selection unless heritability on a family means basis is greater that the heritability on individual plant basis, by an amount that is large enough to counterbalance the lower standard deviation of the family phenotypic variance.

When heritability on a family means basis is not high enough to counterbalance its lower phenotypic standard deviation compared to mass selection, supplementary phenotypic selection within half-sib families (among-and-within half-sib family selection [AWF-HS]) can be carried out to utilise the remaining ¾ σ_A^2 within families and resultantly improve genetic gain. The benefits of AWF-HS are demonstrated in Figure 5.5, where its superiority over HSF and mass selection become evident. AWF-HS superiority over mass selection is evident when heritabilities on an individual plant basis were less than 0.17, 0.30 and 0.4 and heritabilities on a family means basis were 0.3, 0.5 and 0.7, respectively. As individual heritabilities increased from moderate to high, mass selection became the most useful selection method making use of both high additive genetic variation and high phenotypic standard deviation.

In practice, breeders must be cautious not to implement too high a selection pressure in mass selection programmes when narrow sense heritabilities are extremely low. Sleper and Poehlman (2006) argued the lower the heritability, the larger the number of plants that should

be selected and recombined to ensure that some of the selected plants are superior due to their inheritance and not due to environmental effects alone. A significant disadvantage associated with phenotypic selection methods are the environmental effects and the genotype × environment interactions that mask genotypic values. Therefore phenotypic selection methods favour traits that primarily display additive gene action, are highly heritable, have high genotypic correlations between spaced-plants and mixed swards, and can be screened in a large enough population to limit inbreeding (Posselt, 2010).

Finally, considering the similar maternal to paternal additive genetic variance ratios observed across seasons, it was not surprising that no major genetic gain advantages were observed for maternal HSF selection over paternal HSF selection and vice versa (Figure 5.4). Contrary to Riday (2011), the consistent results across morphological traits, seasons and locations in this study suggest maternal half-sib variances are at least equal if not superior to paternal half-sib variances, and certainly do not support the notion that paternal selection gains are superior to maternal selection gains under these experimental conditions. For these reasons, maternal additive genetic variance and paternal additive genetic variances were considered to be equal and only HSF selection (representing both) was simulated in Figure 5.5.

With regard to all of the above, and in accordance with Casler and Brummer (2008), the results of these formulas should only be taken as guidelines. The simulations are not intended to infer that at any particular ratio of $\sigma_A^2/\sigma_{PFM}^2$ or σ_A^2/σ_{PW}^2 there is a single value which makes one particular selection method more efficient than another under all conditions. "The choice of any one method of selection depends on the breeder, stage of the breeding programme, stage of germplasm development, stage of knowledge of the populations and objectives of the breeding program" (Hallauer and Miranda, 1981).

5.4.7 Trial design

A requirement for known maternal and molecular marker determined paternal half-sib selection is the identification and collection of phenotypic information on individual genotypes. Although maintained throughout the duration of this experiment, the horizontally creeping nature of white clover limits its effectiveness as a spaced-planted species under these experimental conditions, and requires further methodology development. Unlike red clover and lucerne, where paternal selection is suited to the growth habit of the species (Riday, 2011; Riday et al., 2013), the white clover growth habit is somewhat more challenging to maintain individualism. White clover progresses through different developmental phases, including changing from a tap rooted to a nodal rooted plant, followed by morphological phase three

where it begins to break into daughter clones (Brock and Tilbrook, 2000; Brock et al., 2000). Therefore restricting it to a given space requires the use of either physical or chemical barriers, both of which are likely to impact on measured morphological traits. This is particularly more pronounced in sward scenarios where grazing ruminants are less likely to regulate the lateral spread of the plant, due to the protection from the companion sward species. Either regular inter-row spraying or the use of physical barriers such as a dense companion species in the inter-rows (such as cocksfoot) may be a tool to mitigate spread, otherwise the application of paternal selection in white clover will have to be revised.

5.5 Conclusions

- The number of plants per plot for half-sib families of white clover should not be below recommendations by Posselt (2010). Breeders should aim for at least 15-20 genotypes per plot in order to accurately measure within-plot-variation (σ_w^2) and plot-to-plot variation (σ^2).

- Maternal family additive genetic variances were at least equal if not superior to paternal additive genetic variances, and certainly do not support the notion that paternal selection gains are superior to maternal selection gains under these experimental conditions in white clover.

- Known maternal and molecular marker determined paternal half-sib selection (♀HS +♂HS) was approximately two-fold more efficient than maternal half-sib selection alone (HSF), and conforms to theoretical expectation.

- On a predicted genetic gain per cycle basis, half-sib progeny trailing (HSPT) and known maternal and molecular marker determined paternal half-sib selection (♀HS +♂HS) were the most effective breeding methods. Re-ranking of effectiveness between HSPT and ♀ HS +♂HS breeding methods per cycle for the different traits were most likely arbitrary, and genetic gains per cycle are actually likely to be equal, assuming equal selection intensities are applied for both.

- On a predicted genetic gain per time basis, AWF-HS+MFS was the most effective breeding method when heritabilities on an individual plant basis were low. However, as heritabilities on an individual plant basis reached within 30% of heritability on a family means basis, mass selection became the most effective selection method.

- Simulation using data from this study suggests that replicated half-sib family selection methods (genotypic selection) are best for improving clover herbage yield, whereas phenotypic selection methods are similar or superior to half-sib family selection for leaf size and growing point density.

- The effectiveness of known maternal and molecular marker determined paternal half-sib selection in spaced-planted white clover nurseries is marginal due to the horizontally creeping growth habit of white clover. The breeding methodology is more likely suited for non-creeping, prostrate species such as red clover, lucerne and grass species.

- Stratification of the way in which paternity testing is utilised in white clover, such as in mixed sward plots, may significantly enhance the rate of genetic gain per year for white clover relative to conventional plant breeding methodologies.

Chapter 6

Genetic variation in a breeding population for vegetative persistence and production under summer moisture stress

6.1 Introduction

The lack of reliable seasonal herbage yield and vegetative persistence are two important constraints that have resulted in the under-utilisation of the genetic potential of white clover as a valuable forage legume (Jahufer et al., 2002; Jahufer et al., 2013). In regions on the east coast of New Zealand where the central mountain range combined with westerly air flows create a rain shadow with <800 mm of annual rainfall, these constraints are primarily governed by summer moisture stress (Brown and Green, 2003). The low annual rainfall in these regions, combined with the exceeding summer evapotranspiration rates and variable alluvial outwash soils result in potential soil moisture deficits between 200-500mm (Salinger, 2003; Webb et al., 2000). In these summer moisture stressed environments the performance and persistence of white clover is often variable and poor between succeeding years (Knowles et al., 2003).

While the improvement of vegetative persistence and herbage yield have been at the forefront of many Australasian white clover breeding programmes, historically progress has been limited (Abberton and Marshall, 2010). One rationale for the limited gain is the depletion of the genetic variation available for drought stress tolerance. Some specialised breeding programmes have targeted secondary and tertiary gene pools for acquiring novel genetic diversity (Hussain and Williams, 1997; Marshall et al., 2001; Williams et al., 2007). Despite the limited genetic variation for drought tolerance in white clover, Knowles et al. (2003) reviewed findings that suggested scope still exists within the species for development of cultivars with improved stolon vegetative persistence under moisture stress that demonstrate improved plant recovery rates following rain.

Among morphological traits deemed to be important for vegetative persistence, stolon density or growing point density ranks among the highest (Caradus and Williams, 1989). However, the negative association between stolon density and herbage yield complicates the concurrent genetic improvement of vegetative persistence and yield in this species (Jahufer et al., 1999). Leaf size, stolon thickness, number of nodes and number of rooted nodes also show strong associations with persistence and herbage yield (Annicchiarico et al., 1999; Caradus and

Williams, 1989; Jahufer et al., 1994; Jahufer et al., 1995; Jahufer et al., 1997; Jahufer et al., 1999; Jahufer et al., 2013). Physiological traits of dryland importance include increased WUE, which is often measured indirectly by ^{13}C isotopic discrimination, and the accumulation of flavonoids which confer resistance to a number of abiotic factors including soil moisture stress (Ballizany et al., 2012b; Hofmann and Jahufer, 2011).

Few studies in white clover have investigated and quantified the magnitude of intra-population genetic variation for post summer moisture stress recovery and genotype × year and genotype × environment interactions under grazing conditions. The availability of estimates of genetic parameters for key morphological/physiological attributes associated with the vegetative persistence of white clover will enable the assessment of the merits of alternative breeding strategies that could potentially improve the historic slow rate of genetic gain (Jahufer et al., 2002).

The developmental stages of white clover growth have significant implications for the evaluation of breeding material (Brock et al., 2000). White clover development from seed has three distinct morphological phases (Brock et al., 1988; Brock et al., 2000; Thomas, 1987b; Westbrooks and Tesar, 1955); i) a rosette seedling phase lasting 1-3 months with minimal branching, no stem elongation and no nodal root formation; ii) a tap-rooted expansion phase lasting 1-2 years, with extensive branching, rapid elongation of stem branches, and nodal root development; and iii) a 'mature' clonal phase, when taproots die and large plants fragment into small self-dependent daughter clones that rely solely on nodal roots.

From a plant breeding perspective, these different morphological phases present a challenge to breeders. One of the primary challenges is to identify breeding lines that are consistently superior across all morphological phases. Field evaluations of white clover have principally been conducted using trials that are either established directly from seeded plots or via transplanted established seedlings (Ayres et al., 2007; Caradus et al., 1997; Jahufer et al., 2009; Woodfield and Caradus, 1994; Woodfield et al., 2003; Woodfield et al., 2001; Woodward and Caradus, 2000). Indirect selection for high yielding genotypes in the first two morphological phases may not necessarily represent persistent and high yielding genotypes in the clonal phase (Caradus and Williams, 1989), and in some cases, selections may be counterproductive on long-term persistence and performance (Brock and Tilbrook, 2000). Therefore, due to both the trialling methodology and the distinct morphological stages of white clover, it has become a necessity for breeders to include at least a third year of

assessment to ensure that the evaluation period assesses performance of white clover breeding lines in the final clonal growth phase (Brock et al., 2000).

Although seedling regeneration occurs in perennial pastures, Archer and Robinson (1989) reported that white seedling recruitment is both unreliable between years and slow to establish through winter. The slow establishment speed of white clover provides little addition to spring and annual herbage production (Archer and Robinson, 1989). Therefore a primary mechanism for white clover perenniality that provides sufficient herbage production is vegetative persistence through stolon survival (Archer and Robinson, 1989; Hay, 1983).

Limited information is available on the genetic variation in random mating populations of white clover during their vegetative morphological phase. There is a lack of published information on these factors coupled with multi-site field evaluation under sheep grazing. The objectives of this chapter were thus to; i) establish a multi-site trial (dryland and irrigated environments) evaluating white clover families propagated from stolon cuttings in a random mating population, ii) quantify the magnitude of genetic variation among genetic families for a range of key morphological and physiological traits during the white clover vegetative clonal phase, iii) estimate heritabilities of these traits, and iv), use heritability estimates to help breeders make informed decisions regarding breeding methodology.

6.2 Materials and methods

6.2.1 Experimental site

Two trial sites were located at the AgResearch Lincoln Research Farm in Canterbury ($43^{O}37'42''S$ $172^{O}28'3''E$) and at the Ashley Dene Research Farm in Canterbury ($43^{O}39'18''S$ $172^{O}19'15''E$). Hereafter throughout this chapter, the AgResearch site is referred to as the irrigated site and the Ashley Dene site, as the dryland site. At both sites the 10 year mean annual rainfall is 640 mm. At the irrigated site, the soil is classified as a Templeton silt loam (refer to Appendix D.1 for a soil description). At the dryland site, the soil is classified as lowcliffe stony (refer to Appendix D.2 for a soil description).

6.2.2 Trial site preparation

As part of the pre-planting site preparation, both trial sites were sprayed three times over a 9 month period with the herbicide Kamba® 500 (500g L^{-1} Dicamba) at 800 mL ha^{-1} to remove resident white clover. The last application of Kamba® 500 was applied three months prior to transplanting to minimise any residue herbicide in the soil. Lime was applied across both trial sites three months prior to transplanting to raise soil pH to optimum levels of around pH 5.8-

6. Superphosphate fertilizer (150 kg ha^{-1}) was applied across both trial sites in July 2012. In November 2013, 20% Potash Superphosphate (188 kg ha^{-1}) was applied across both trial sites.

6.2.3 Plant material

In February 2012, 60 seeds were randomly sampled from two commercial cultivars (Nomad and Kopu II, to be used as controls) and 40 full-sib families (Chapter 3). The seeds were lightly scarified using 150 grit sand paper and pre-germinated on water moistened filter paper in petri-dishes incubated at 20 ^{O}C for 16 hours without illumination. After germination, seedlings were immediately transplanted into 300 mm × 500 mm propagation trays containing a mix of peat and sand with a three month slow release Osmocote fertiliser (Everris International B.V) (30 seedlings per tray) and grown under glasshouse conditions until May 2012 (min 13 $^{\circ}C$ and max 25 $^{\circ}C$). Two weeks after establishment, the seedlings were inoculated with *Rhizobium leguminarosum* var. trifolli. The plants were defoliated twice before May 2012 to encourage stolon initiation. In May 2012, two stolon cuttings per plant, for each full-sib family/control were propagated into root-trainers containing a mix of peat and sand with three month slow release Osmocote fertiliser. Of the two cuttings, one was designated for the irrigated field site and the other for the dryland field site. Each cutting consisted of a stolon tip, one expanded trifoliate leaf and 2-3cm of stolon below the node closest to the stolon apex. All other expanded trifoliate leaves were removed and the cuttings were planted so that the youngest node was horizontal to the potting mix surface. The developing stolon cuttings were inoculated with *Rhizobium leguminarosum* var. trifolli and established under glass house conditions for six weeks. Following this, the stolon cuttings were trimmed (to encourage stolon initiation) and placed outside onto a well-drained concrete pad. In August 2012, established cuttings were trimmed again before being immediately transplanted into the two field nurseries.

6.2.4 Experimental design

At both trial locations a randomised row by column design with three replicates was used. Each replicate consisted of 42 plots, containing of 40 full-sib family plots and two control cultivar plots (Nomad and Kopu II). The full-sib families and cultivar plots were completely randomised. Each plot contained 16 stolon cuttings, with each cutting representing an individual genotype. The 16 stolon cuttings were evenly transplanted inside a 600mm × 600mm plot using a 100mm soil corer (see Plate 6.1).

At both trial locations, the white clover plants were transplanted from root-trainers into existing swards of perennial ryegrass (*Lolium perenne* L.). At the irrigated site the cultivar Ceres One50 (AR37 endophyte) was sown into a cultivated seed bed at a rate of 20 kg ha^{-1} in spring 2010. At the dryland site, the cultivar Ceres One50 (AR1 endophyte) was sown into a cultivated seed bed at a rate of 20 kg ha^{-1} in spring 2010. Two weeks after transplanting, dead transplants were replaced with spare family cuttings to minimise effects due to transplanting survivorship. Plots were allowed to expand into 1m × 1m plots. Established trials are shown in Plate 6.2.

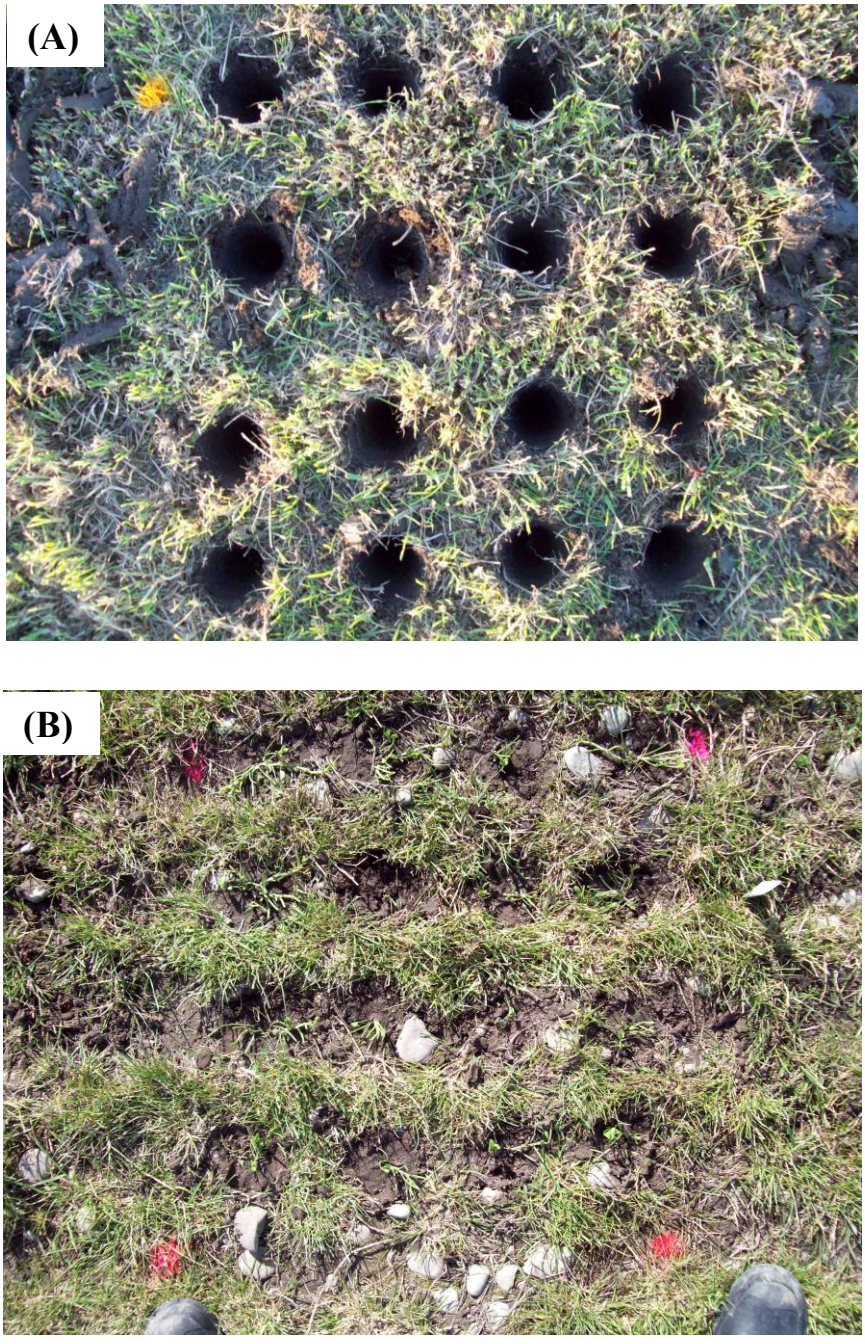


Plate 6.1 Soil coring (A) and transplanting (B) of white clover full-sib family clones at the experimental sites in August 2012.

Plate 6.2 Mini-plot white clover trials at the (A) irrigated and (B) dryland sites in December 2012.

6.2.5 Morphological measurements

6.2.5.1 Stolon traits

The morphological traits measured at each site were leaf width (LW) (mm), stolon thickness (ST) (mm), number of nodes (NN) (no. m^{-1}), number of rooted nodes (RN) (no. m^{-1}), number of stolon branches (BR) (no.m^{-1}), and growing point density (GPD) (no.m^{-2}). All morphological traits except GPD were measured on ten randomly selected stolons per plot, with the stolon apex intact, and a minimum of 5 cm stolon length sampled from plants within plots. NN, RN, and BR were counted along each piece of stolon to a maximum of 10cm (from apical end). The length of the stolon piece was recorded if it was less than 10cm long. Leaf size was measured as the width (mm) of the central leaflet of the first fully expanded leaf. Stolon thickness was measured at the midpoint between the 2nd and 3rd node from the stolon apex. All traits except GPD were measured in November 2012, April 2013, and November 2013. GPD was measured post-grazing (approximately 5-7 days after grazing) in two representative areas per plot with a 225 cm^2 quadrat in April 2013, November 2013 and April 2014.

6.2.5.2 Clover herbage yield

Herbage yield (HY) (kg DM/ha) per plot was scored on a 1-9 scale before each grazing rotation. Scores were calibrated by defoliating nine randomly selected plots to 4cm with two 450 cm^2 quadrats per replicate (each representing a 1-9 score), separating the white clover from companion sward species, drying the leaf material in air-forced ovens for 48 hours and recording their respective dry weights. White clover herbage yields were determined by taking the average from the two randomly selected samples in each plot. A single linear regression was computed from the dry matter weights at each harvest, and was used to predict the white clover biomass yield (kg DM/ha) for all scored plots.

6.2.5.3 Vegetaive persistence

Clover stolon coverage in plots was visually scored on a scale of 0-10 (representing 0 to 100% plot coverage) in April 2013, November 2013 and April 2014.

6.2.6 Physiological measurements

6.2.6.1 Water potential

Water potential was measured to assess the physiological plant water status in the irrigated and dryland environments. Plant water status was assessed on a two to three week basis at both trial sites from January 2013 to March 2013 and December 2013 to March 2014, by

measuring leaf water potential (ψ) on two randomly selected plots per replicate using a pressure chamber (Soil moisture Equipment Corp., Santa Barbara, USA). Two first fully expanded leaves per plot were excised with 4-6 cm of petiole and measured immediately. Measurements were taken approximately midway between irrigation cycles at the irrigated site. Measurements were carried out between 7am and 9am, commencing with the dryland site.

6.2.6.2 Biochemistry

Fifteen randomly selected fully expanded trifoliate leaf laminae from six top preforming, six poor performing and six average performing full-sib families as well as from both cultivars were sampled from each replicate in both trial sites in March 2013 and January 2014. Sampling took place between 11am and 1pm across both sites in both years. The samples were immediately frozen in liquid nitrogen and stored at -80 °C. The samples were subsequently freeze-dried, then finely ground in liquid nitrogen using a mortar and pestle and stored at -20 °C.

Phenolic compounds

After grinding, 50 (±1) mg of the ground material was weighed into 10mL centrifuge tubes, to which 3 mL of acidified methanol (MeOH:H_2O:HOAc at 79:20:1) was added (Hofmann and Jahufer, 2011). The samples were vortexed for 10 seconds, then extracted in the dark for 16-18 hours. On completion of extraction, the tubes were vortexed for a further 10 seconds, followed by centrifuging at 4000 rpm for 5 minutes. 1800 µL of supernatant was syringe filtered (using 0.2 µm filters) into amber HPLC vials and stored at -20 °C before being processed on an integrated HPLC machine (Agilent 1100 series, Agilent Technologies, Germany) (Hofmann and Jahufer, 2011).

Each sample was run for 47 minutes at 0.8 ml min^{-1}, using an injection volume of 10 µL. The HPLC gradient consisted of solvent A (1.5% H_3PO_4) and solvent B [HOAc:CH_3CN:H_3PO_4:H_2O (20:24:1.5:54.5)], mixed using a linear gradient starting with 80% A, decreasing to 33% A at 30 min, 10% A at 33 min and 0% at 39.3 min (Hofmann et al., 2003). Rutin standards (quercetin 3-rutinoside $C_{27}H_{16}O_{16}$ dissolved in methanol) at 0, 10, 25, 50, and 100ppm were used to calibrate readings. Quercetin glycoside and kaempferol glycoside peaks were identified from the online spectra (Markham, 1982), and total concentrations (rutin equivalents) of each flavanol were calculated in mg g^{-1} DM for each sample.

Carbon isotope discrimination

$^{13}C/^{12}C$ isotopic composition ($\delta^{13}C$) relative to the standard (V-PDB) was measured in all samples by Analytical Services, Faculty of Agriculture and Life Sciences, Lincoln University, using EA-CF-IRMS (Elemental Analyser – Continuous Flow Isotope Ratio Mass Spectrophotometry) (PDZ Europa Ltd., United Kingdom). ^{13}C discrimination (Δ) was calculated using the following equation (Farquhar et al., 1982);

$$\Delta = \frac{\delta source - \delta product}{1 + \delta source/1000}$$

where; δ_{source} = $\delta^{13}C$ of the air, assumed to be -8‰ and $\delta_{product}$ = $\delta^{13}C$ of the sample.

6.2.7 Soil measurements

6.2.7.1 Soil moisture

Soil moisture in the upper 0.2m portion of the soil profiles was monitored using time domain reflectometry (TDR). TDR rods (225 mm) were inserted into the soil and soil moisture was measured using a time domain reflectometer (Trace system, Soil Moisture Equipment, Santa Barbara, California, USA). Neutron probe access tubes (47 mm wide aluminium tubes) were installed to a depth of 1.5 m in the centre of three randomly selected plots per replicate at both trial sites in spring 2013. The access tubes allowed soil moisture measurements in 0.1 increments from 0.25 to 1.15 m using a neutron probe (Troxler Electronic Industries Inc., North Carolina, USA) in the summer of 2013/2014.

6.2.7.2 Soil surface temperature

In the 2013/2014 summer, fortnightly soil surface temperatures were recorded in three randomly selected plots per replicate at both trial sites using an infrared thermometer; Fluke 572 (Fluke Corp., Everett, WA, USA). Sward canopies were opened and three independent soil surface reading per plot were taken. The three readings per plot were averaged to determine the mean plot soil surface temperature. Where possible, soil surface readings where conducted midway between irrigation/rainfall events at the irrigated site. All readings at both sites were conducted within one hour between 1pm and 2pm.

6.2.8 Trial management

Following planting, repeated hand weeding along with herbicide spot spraying was used to keep volunteer white clover seedlings to a minimum. Both trials were allowed to establish for three months before measurements commenced. Six applications of nitrogen fertiliser (in the

form of urea) were applied across both trials in July 2012, December 2012, April 2013, August 2013, December 2013 and April 2014 at a rate of 30 to 35 kg ha^{-1} of N per application.

Both trial sites were rotationally grazed for 12-24 hours by a mob of 70 to 150 sheep (16 times at the irrigated site and 12 times at the dryland site) over an 18 month period with time between grazings ranging from 20 days in summer to 63 days in winter. Post-grazing, nurseries were mown to a height of 4cm to homogenize the pasture cover. All cut herbage was removed from the experimental areas. The irrigated nursery was watered through November, December, January, February and March in both summer seasons. The dryland trial site was irrigated periodically throughout the summer of 2012/2013 to prevent plots from reaching permanent wilting point. Irrigation volumes for both sites are presented in Figure 6.1. The irrigated trial was inter-row sprayed in autumn 2013, spring 2013 and summer 2014 with a selective herbicide (Kamba 500 at a rate of 800 mL ha^{-1}) to eliminate rapidly expanding plots from merging into one another. The dryland site was inter-row sprayed in autumn 2013 and summer 2014.

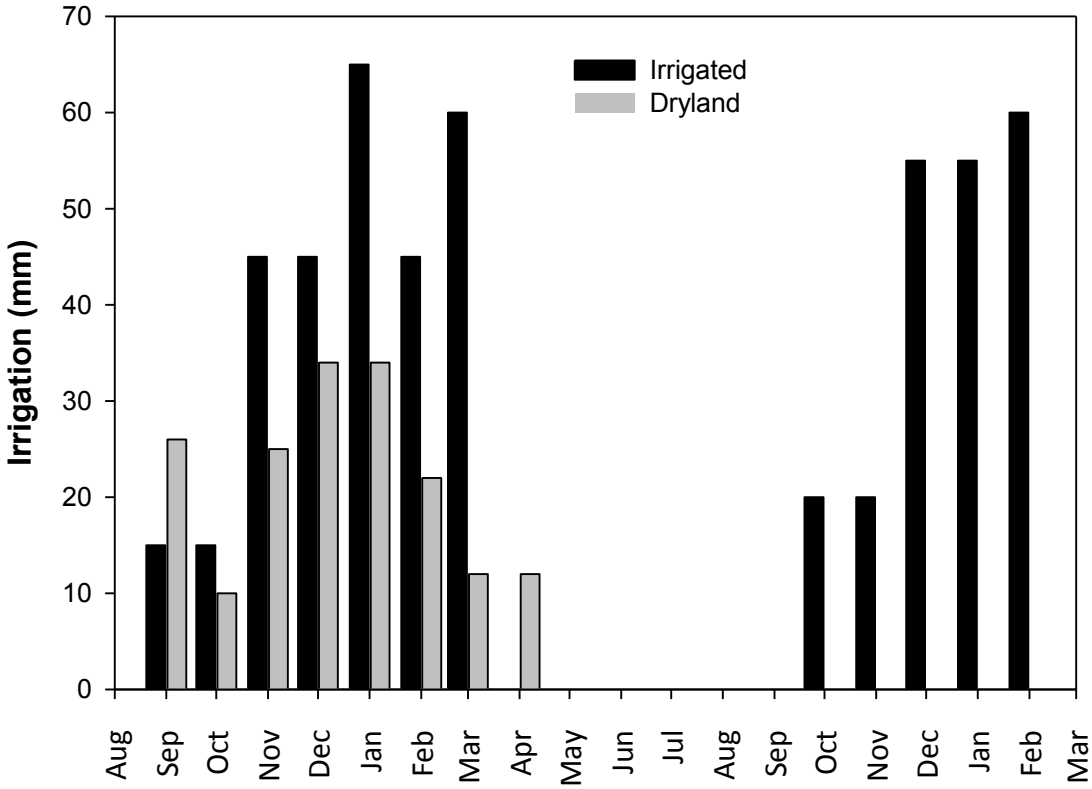


Figure 6.1 Monthly irrigation volumes at the irrigated (■) and dryland (■) sites during the trial duration (August 2012 to March 2014).

6.2.9 Statistical analyses

The objective of the data analysis was to: (i) estimate the magnitude of genotypic variation among vegetatively propagated full-sib families, (ii) estimate their interaction with the different seasons and years, (iii) estimate heritabilities on a full-sib family means basis and on an individual plant basis, and (iv) identify traits which may enhance white clover recovery post-summer moisture stress.

6.2.9.1 Variance component analyses

The Residual Maximum Likelihood (REML) (Harville, 1977; Patterson and Thompson, 1971; Patterson and Thompson, 1975) analysis option in GenStat (GenStat, 2003) was used for data analysis where only unbalanced data was available. The REML analysis was used to obtain BLUP (Best Linear Unbiased Predictors) and BLUE (Best Linear Unbiased Estimate) adjusted means (White and Hodge, 1989). These adjusted means were used in the development of genotype × trait matrices that were used in pattern analysis.

All linear models used in the analysis of variance were assumed to be completely random except, models 6.2, 6.3, 6.5 and 6.6, where environments and/or seasons were treated as fixed effects. Linear models used in the analyses of variance were adapted from equation 27 and 33 proposed by Nyquist (1991) for individual samples within plots and plot totals of perennial species, respectively. Analyses using plot totals were used for herbage yield and persistence traits, whereas all other traits were measured using multiple samples per plot. Such measurements collected on individual plants within each plot allowed the error variance to be partitioned into variance due to both random plot effects (plot-to-plot variation) and within-plot variance. Traits with no within-plot sampling resulted in plot effects and plant-within-plot effects being confounded in the residual effect, which is added as $\varepsilon_{(ijk)}$ into equation 6.4, 6.5 and 6.6 (Holland et al., 2002).

Analysis of morphological traits within environments and seasons (sampled plots)

$$P_{ijk} = \mu + F_i + R_j + a_{ij} + \varepsilon_{ijk} \qquad (6.1)$$

where;

P	is the phenotypic value of the k^{th} plant in the i^{th} family within the k^{th} replicate
μ	is the overall mean
F_i	is the effect of full-sib family i $N(0,\sigma_f^2)$,
R_j	is the effect of replicate j $N(0,\sigma_r^2)$,
a_{ij}	is the effect of half-sib family I in replicate j $N(0,\sigma_a^2)$,

ε_{ijk} is the residual effect of sample *k* taken from full-sib family *I* in replicate *j* (within-plot variation) N(0,σ_ε^2),

Analysis of morphological traits within environments but across seasons (sampled plots)

$$P_{ijkl} = \mu + S_i + R_{(i)j} + F_k + a_{jk} + W_{(jk)l} + FS_{ik} + c_{(ijk)} + d_{(ijkl)} \quad (6.2)$$

where;

μ is the overall mean
S_i is the fixed effect of season *i*,
$R_{(i)j}$ is the effect of replicate *j* within season *i* N(0,σ_r^2),
F_k is the effect of full-sib family *k* N(0,σ_f^2),
$a_{(jk)}$ is the effect of full-sib family *k* within replicate *j* N(0,σ_a^2),
$W_{(jk)l}$ is the effect of sample *l* taken from full-sib family *k* within replicate *j* N(0,σ_w^2),
FS_{ik} is the effect of full-sib family *k* within season *I* N(0,σ_{fs}^2),
$c_{(ijk)}$ is the effect of full-sib family *k* within replicate *j* within season *I* N(0,σ_c^2),
$d_{(ijkl)}$ is the effect of sample *l* within full-sib family *k* within replicate *j* within season *I* N(0,σ_d^2),

Analysis of morphological traits across environments and seasons (sampled plots)

$$P_{ijklm} = \mu + E_i + R_{(i)j} + F_k + LF_{ik} + a_{(ijk)} + W_{(ijk)l} + S_m + LS_{im} + b_{(ijm)} + FS_{km} + LFS_{ikm} + c_{(ijkm)} + d_{(ijklm)} \quad (6.3)$$

where;

μ is the overall mean
E_i is the fixed effect of environment *i*,
$R_{(i)j}$ is the effect of replicate *j* within environment *i* N(0,σ_r^2),
F_k is the effect of full-sib family *k* N(0,σ_f^2),
FE_{ik} is the effect of full-sib family *k* within environment *i* N(0,σ_{fe}^2),
$a_{(ijk)}$ is the effect of full-sib family *k* within replicate *j* within environment *i* N(0,σ_a^2),
$W_{(ijk)l}$ is the effect of sample *l* taken from full-sib family *k*, replicate *j* and environment *I* (within-plot variation) N(0,σ_w^2),
S_m is the fixed effect of season *m*,
SE_{im} is the fixed effect of season *m* within environment *j*,
$b_{(jjm)}$ is the effect of replicate *j* within season *m* within environment *i* N(0,σ_b^2),
FS_{km} is the effect of full-sib family *k* within season *m* N(0,σ_{fs}^2),
FSE_{ikm} is the effect of full-sib family *k*, within season *m* within environment *i* N(0,σ_{fse}^2),
$c_{(ijkm)}$ is the effect of full-sib family *k* within replicate *j* within season *m* within environment *i* N(0,σ_c^2),
$d_{(ijklm)}$ is the effect of sample *l* within full-sib family *k* within replicate *j* within season *m* within environment *i* N(0,σ_d^2),

Analysis of morphological traits within environments and seasons (plot totals)

$$P_{ij} = \mu + F_i + R_j + \varepsilon_{ij} \quad (6.4)$$

where;

P is the phenotypic value of the i^{th} family within the k^{th} replicate
μ is the overall mean

F_i is the effect of full-sib family *i* N(0,σ_f^2),
R_j is the effect of replicate *j* N(0,σ_r^2),
ε_{ij} is the residual effect of full-sib family *I* in replicate *j* N(0,σ_ε^2),

Analysis of morphological traits within environments but across seasons (plot totals)

$$P_{ijk} = \mu + S_i + R_{(i)j} + F_k + FS_{ik} + \varepsilon_{ijk} \quad (6.5)$$

where;

μ is the overall mean
S_i is the fixed effect of season *i*,
$R_{(i)j}$ is the effect of replicate *j* within season *I* N(0,σ_r^2),
F_k is the effect of full-sib family *k* N(0,σ_f^2),
FS_{ki} is the effect of full-sib family *k* within season *I* N(0,σ_{fs}^2),
$\varepsilon_{(ijk)}$ is the residual effect of full-sib family *k* in replicate *j* during season *i* N(0,σ_ε^2),

Analysis of morphological traits across environments and seasons (plot totals)

$$P_{ijklm} = \mu + E_i + R_{(i)j} + F_k + LF_{ik} + a_{(ijk)} + S_l + LS_{il} + b_{(ijl)} + FS_{kl} + LFS_{ikl} + \varepsilon_{(ijkl)} \quad (6.6)$$

where;

μ is the overall mean
E_i is the fixed effect of environment *i*,
$R_{(i)j}$ is the effect of replicate *j* within environment *i* N(0,σ_r^2),
F_k is the effect of full-sib family *k* N(0,σ_f^2),
FE_{ik} is the effect of full-sib family *k* within environment *i* N(0,σ_{fe}^2),
$a_{(ijk)}$ is the effect of full-sib family *k* within replicate *j* within environment *i* N(0,σ_a^2),
S_l is the fixed effect of season *l*,
SE_{il} is the fixed effect of season *l* within environment *i*,
$b_{(jjl)}$ is the effect of replicate *j* within season *l* within environment *i* N(0,σ_b^2),
FS_{kl} is the effect of full-sib family *k* within season *l* N(0,σ_{fs}^2),
FSE_{ikl} is the effect of full-sib family *k*, within season *l* within environment *i* N(0,σ_{fse}^2),
$\varepsilon_{(ijkl)}$ is the effect of full-sib family *k* within replicate *j* within season *l* within environment *i* N(0,σ_ε^2),

ANOVA in Genstat 16.0 was used for all analyses of variances where balanced data was available.

6.2.9.2 Pattern analysis

Pattern analyses techniques of principal component analysis (PCA) and cluster analysis were used to summarise information gathered from full-sib family × trait data sets (Gabriel, 1971; Kroonenberg, 1994; Watson et al., 1995). In order to remove scaling effects, the BLUP values for the respective attributes were standardised to have a mean of zero and a variance of one (Cooper and Delacy, 1994; Fox and Rosielle, 1982). In order to choose the optimum level of truncation for the resulting hierarchy from cluster analysis, the increase in the sum of squares among full-sib family groups as the number of groups increased was investigated (DeLacy,

1981). The group level selected was determined by the point where the percentage of the full-sib family sum of squares among groups did not improve substantially as the number of groups increased.

6.2.9.3 Heritability

Heritabilities for all traits within seasons were calculated on a full-sib family means basis and on an individual plant basis (broad sense heritability). Heritabilities were calculated according to models proposed by Nyquist (1991) and Holland et al. (2002). Heritabilities on a full-sib family means basis were estimated using models 53 and 57 (Nyquist, 1991), but with the denominator replaced with the phenotypic variance among families means for perennial species (models 28 and 33, respectively). Heritabilities on an individual plant basis were estimated using model 54 (Nyquist, 1991), but with the denominator replaced with the phenotypic variance among individuals for perennial species (model 29). The co-variance among full-sib families is $\frac{1}{2}\sigma_A^2 + \frac{1}{4}\sigma_D^2$ (Fehr, 1987). Thus the numerators in equations 6.8, 6.11 and 6.14 were multiplied two-fold to estimate heritabilities on an individual plant basis. Due to the confounding dominance effects in full-sib families, heritabilities on a single plant basis here are merely an upper limit to narrow sense heritabilities (Falconer, 1961) and therefore better represent broad sense heritabilities, because the full-sib family variance components comprises of both additive and non-additive genetic variation.

Heritability on a full-sib family means basis – genotypic analysis of morphological traits within environments and seasons (sampled plots)

(6.7)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_a^2}{r} + \frac{\sigma_w^2}{rn}}$$

Broad sense heritability on an individual plant basis – genotypic analysis of morphological traits within environments and seasons (sampled plots)

(6.8)

$$h_1^2 = \frac{2\sigma_F^2}{\sigma_F^2 + \sigma_a^2 + \sigma_w^2}$$

Heritability on a full-sib family means basis – genotypic analysis of morphological traits within environments and seasons (plot totals)

(6.9)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_\varepsilon^2}{r}}$$

Heritability on a full-sib family means basis – genotypic analysis of morphological traits within environments but across seasons (sampled plots)

(6.10)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_{FS}^2}{s} + \frac{\sigma_a^2}{r} + \frac{\sigma_w^2}{rn} + \frac{\sigma_c^2}{sr} + \frac{\sigma_d^2}{srn}}$$

Broad sense heritability on an individual plant basis – genotypic analysis of morphological traits within environments but across seasons (sampled plots)

(6.11)

$$h_1^2 = \frac{2\sigma_F^2}{\sigma_F^2 + \sigma_{FS}^2 + \sigma_a^2 + \sigma_w^2 + \sigma_c^2 + \sigma_d^2}$$

Heritability on a full-sib family means basis – genotypic analysis of morphological traits within environments but across seasons (plot totals)

(6.12)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_{FS}^2}{s} + \frac{\sigma_a^2}{r} + \frac{\sigma_\varepsilon^2}{sr}}$$

Heritability on a full-sib family means basis – genotypic analysis of morphological traits across environments and seasons (sampled plots)

(6.13)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_{FE}^2}{e} + \frac{\sigma_{FS}^2}{s} + \frac{\sigma_{FSE}^2}{es} + \frac{\sigma_a^2}{er} + \frac{\sigma_w^2}{ern} + \frac{\sigma_c^2}{esr} + \frac{\sigma_d^2}{esrn}}$$

Broad sense heritability on an individual plant basis – genotypic analysis of morphological traits across environments and seasons (sampled plots)

(6.14)

$$h_1^2 = \frac{2\sigma_F^2}{\sigma_F^2 + \sigma_{FE}^2 + \sigma_{FS}^2 + \sigma_{FSE}^2 + \sigma_a^2 + \sigma_W^2 + \sigma_c^2 + \sigma_d^2}$$

Heritability on a full-sib family means basis – genotypic analysis of morphological traits across environments and seasons (plot totals)

(6.15)

$$h_f^2 = \frac{\sigma_F^2}{\sigma_F^2 + \frac{\sigma_{FE}^2}{e} + \frac{\sigma_{FS}^2}{s} + \frac{\sigma_{FSE}^2}{es} + \frac{\sigma_a^2}{er} + \frac{\sigma_\varepsilon^2}{esr}}$$

***where*;**

σ_F^2 – full-sib family, σ_{FE}^2 – family × environment, σ_{FS}^2 – family × season, σ_{FSE}^2 – family × season × environment, σ_a^2 – plot to plot, σ_w^2 – within plot, σ_c^2 – plot to plot × season, σ_d^2 – within plot × season

- *e* is the number of environments
- *s* is number of seasons
- *r* is number of replicates per location
- *n* is number of plants per plot

6.3 Results

6.3.1 Environmental measurements and plant water status

Rainfall was distributed unevenly among months. Higher precipitation occurred in winter than in the summer months (Figure 6.2). Mean minimum and maximum temperatures varied with seasons (Figure 6.2). Total precipitation (which includes applied irrigation) was consistently higher at the irrigated site during the period of November to April in both successive years.

Soil moisture content in the top 20 cm of the soil profile at the irrigated site ranged between 24% and 30% before irrigation, except in mid-January 2013 when it decreased to 21% (Figure 6.3). Soil moisture content post irrigation ranged from 30% to 38% depending on the irrigation volumes applied at the irrigated site. Soil moisture content at the dryland site declined in both successive years from field capacity (or near of it), to wilting point of approximately 7-8% during the summer months. Irrigation applied at the dryland site over the summer of 2012/13 kept the soil moisture content from falling below wilting point, however in the summer of 2013/14, the lack of both rainfall and irrigation caused the soil moisture content to drop below wilting point from mid-January to the end of February (Figure 6.3).

Fortnightly soil moisture contents in the 0-115cm profile at the irrigated site on average varied little throughout the months of October 2013 to March 2014 (Figure D.3). There was some water extraction (10-15 mm^3/mm^3) in the top 40cm of the soil profile in both measurements taken in November 2013, but soil water levels were replenished by irrigation and rainfall by the next measurement in December 2013. Fortnightly soil moisture content in the 0-115cm profile at the dryland site demonstrated a declining pattern from field capacity in October 2013 to the lower limit (7-8% soil moisture content) of water extraction in soil layers down to 80cm in late January and February 2014 (Figure D.4). Soil moisture content continued to decline at that site in the lower layers of the soil horizon in the last measurements of February 2014.

In the summer of 2013/14, at the dryland site, wilting point coincided with a soil moisture content of approximately 7-8% within the top 55 cm of the soil profile (Appendix; Figure D.4) and permanent wilting point of approximately 7-8% within the top 65 cm of the soil profile (Appendix; Figure D.4).

Mean leaf water potential (MPa) was significantly (P <0.001) higher at the dryland site (180% and 236%) than the irrigated site in both successive summers (Figure 6.4). Leaf water potential was higher at the irrigated site (P<0.05) in all measurements taken in the summer of

2013/14, except for the measurement taken directly after a significant rainfall event in late December 2013. Leaf senescence at the dryland site prevented any further water potential measurements from mid-January onwards in the summer of 2014. In the summer of 2012/13, leaf water potential was higher at the irrigated site ($P<0.05$) in only half of the measurements taken.

Mean soil surface temperatures were also significantly ($P<0.001$) higher at the dryland site (32.3^{o}C) than the irrigated site (24.6^{o}C) across the summer months of 2013/14 (Figure 6.5). The high soil surface temperature recorded at the irrigated site in the first week of February 2014 (45.4^{o}C) coincided with the completion of animal grazing when ground cover/pasture cover was low. There were no significant ($P>0.05$) differences between sites in the first and fourth week of January and February 2014. These recordings were taken on overcast days.

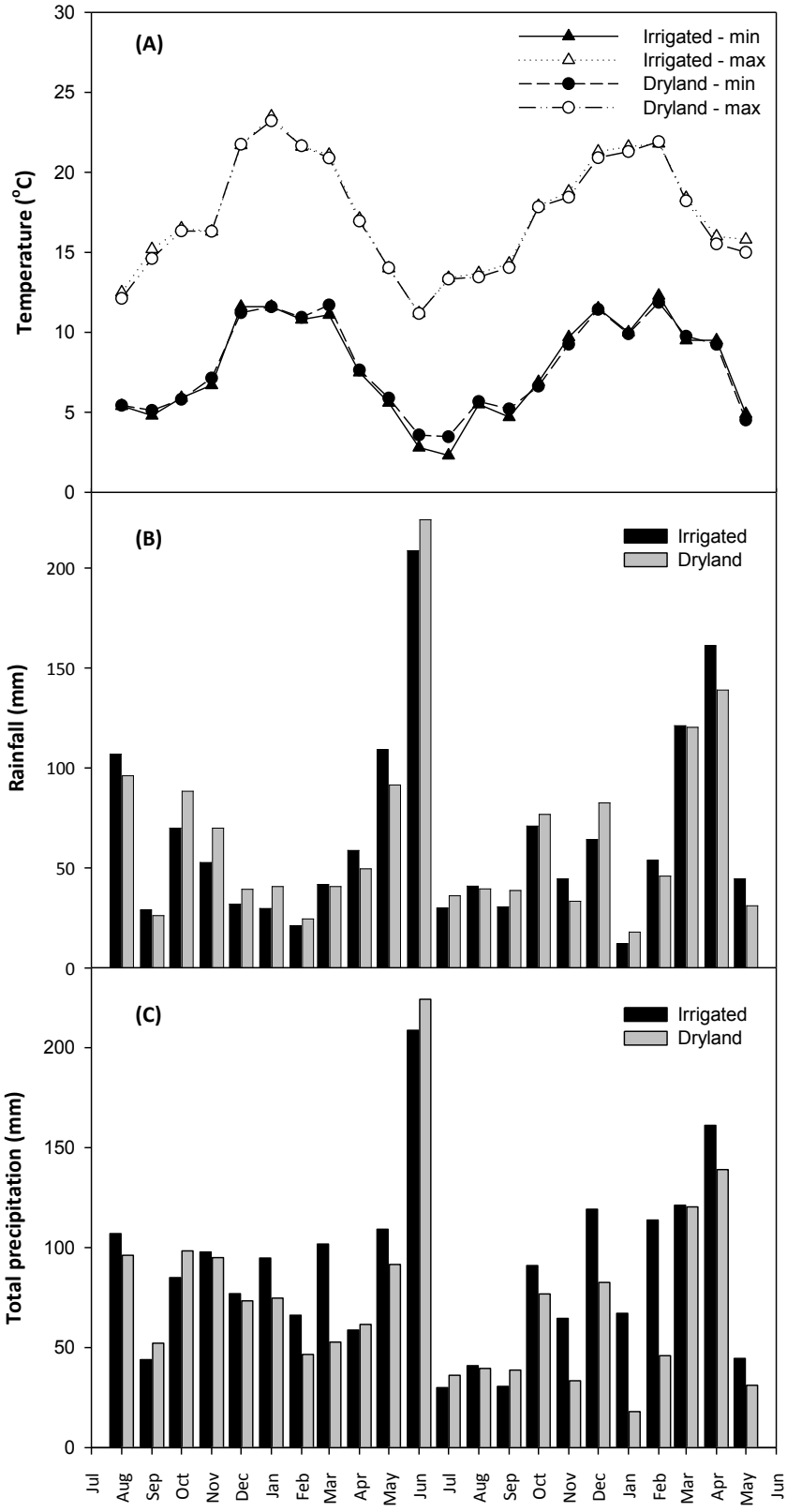


Figure 6.2 Mean monthly maximum (Δ, irrigated; ○ dryland) and mean minimum (▲ irrigated; ● dryland) air temperatures (A), monthly rainfall (B) at the irrigated site (■) and at the dryland site (■) and total monthly precipitation (including irrigation) (C) at the irrigated site (■) and at the dryland site (■) during the trial period August 2012 to May 2014.

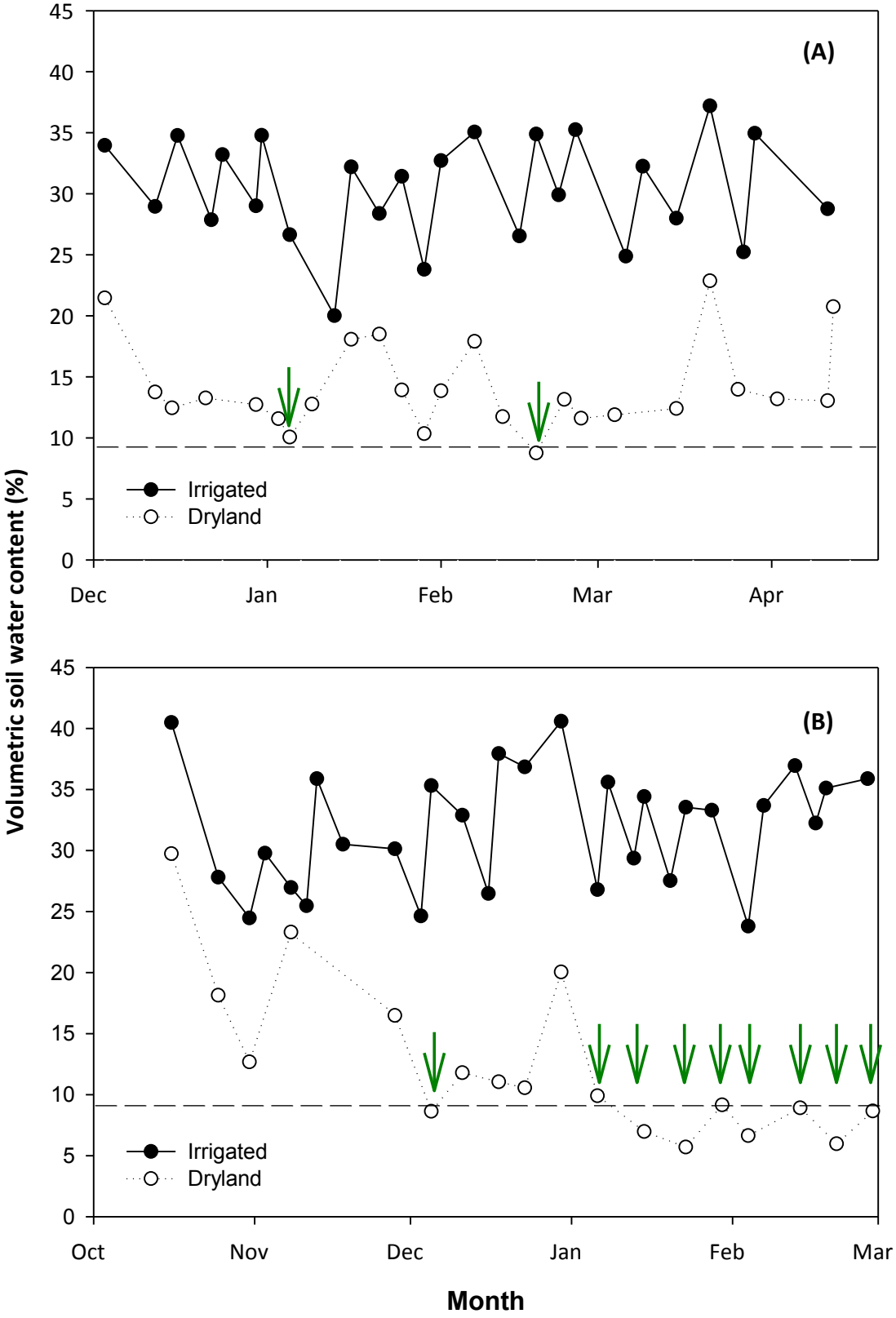


Figure 6.3 **Volumetric soil water content (%) of the irrigated (●) and the dryland (○) sites in the 0-20cm soil profile during the summer periods of (A) December 2012 to April 2013 and (B) October 2013 to March 2014. Green arrows indicate observed wilting of the white clover plants at the dryland site. The flat broken line depicts projected wilting point of the soil at the dryland site.**

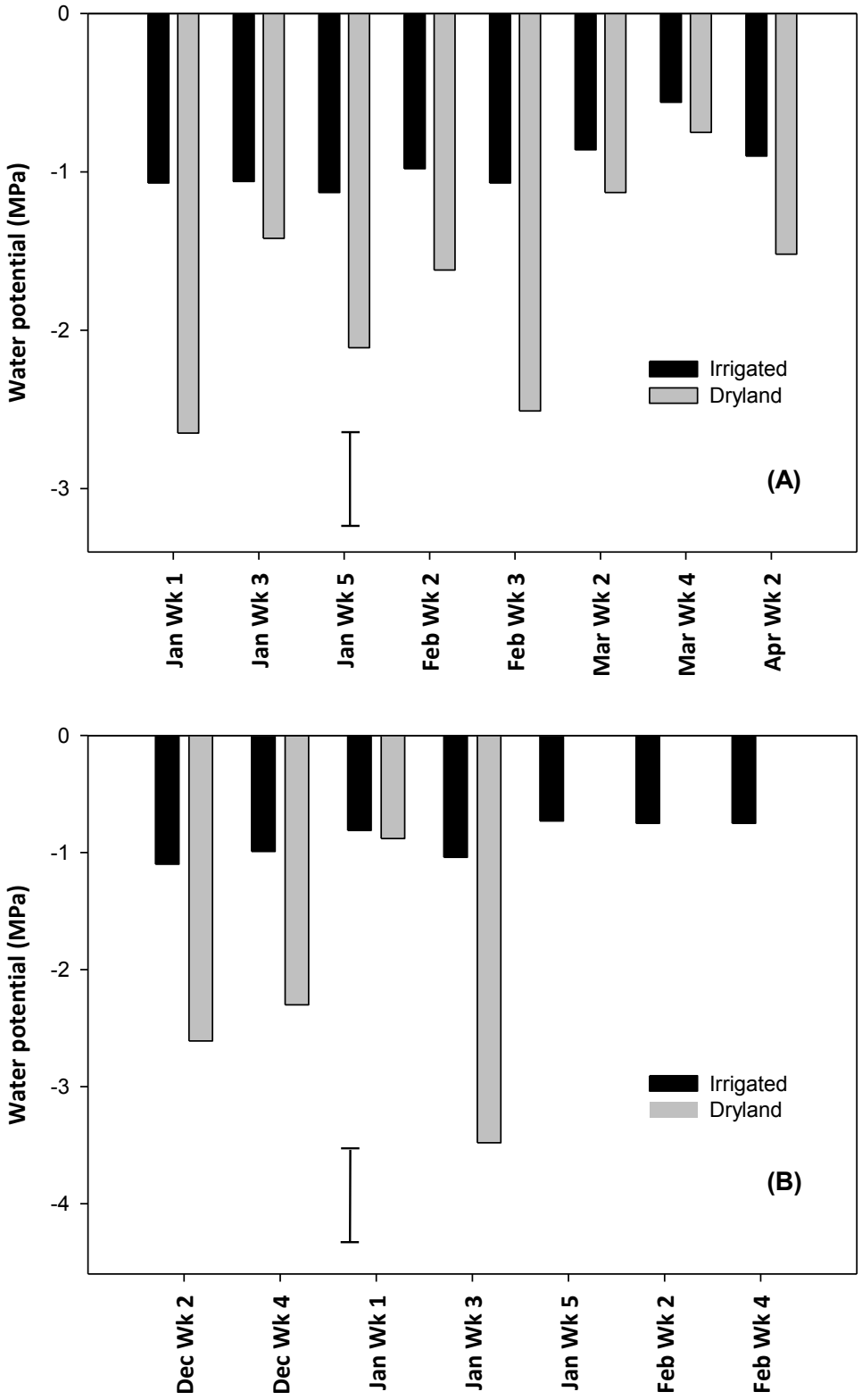


Figure 6.4 Fortnightly mean leaf water potential (MPa) of sampled white clover plants at the irrigated (■) and dryland (■) sites during the summer-moisture deficit periods (A) 2012 to 2013 and (B) 2013 to 2014. Leaf senescence at the dryland site prevented measurements from late January onwards in 2014. $LSD_{0.05}$ indicated by error bar.

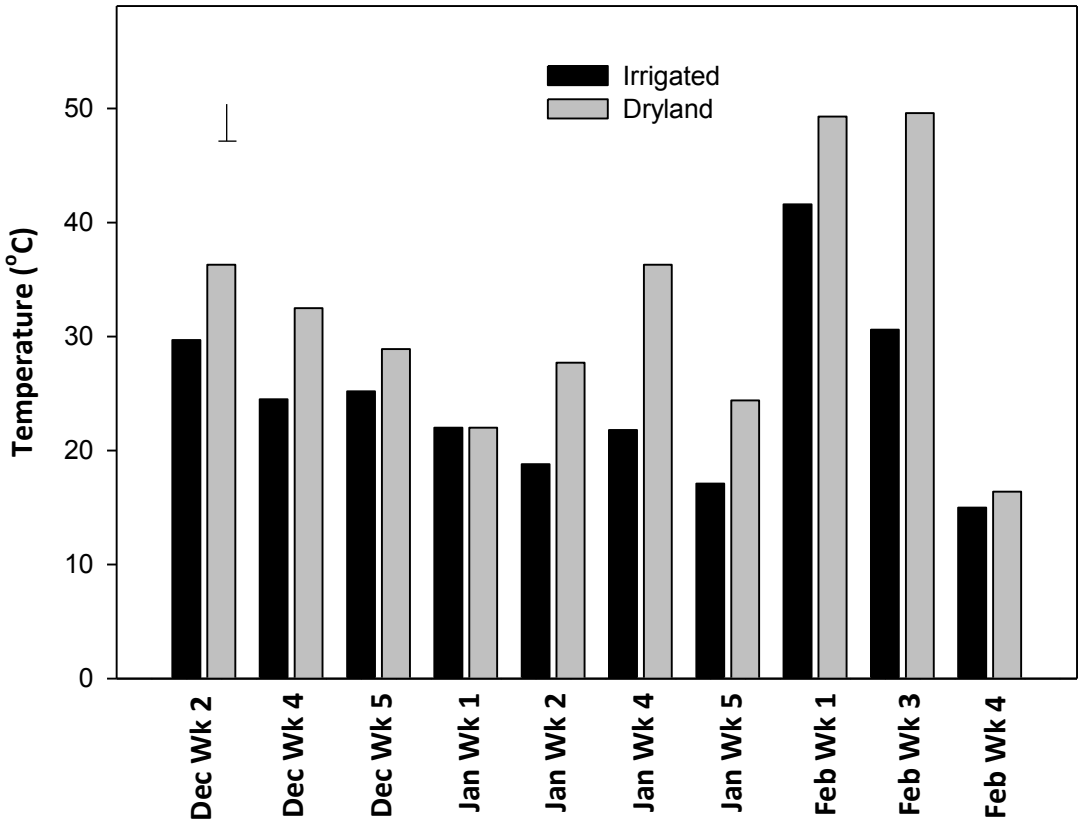


Figure 6.5 Mean soil surface temperatures measured at the irrigated (■) and dryland (■) sites during the summer 2013 to 2014. $LSD_{0.05}$ indicated by error bar.

6.3.2 Biochemistry

6.3.2.1 Carbon isotope discrimination

Results from the $\delta^{13}C$ analysis were used to calculate Δ for selected full-sib families at each site in each year. The analysis of variance indicated significant (P<0.001) genetic variation among the sampled full-sib families across sites and years. Full-sib families ranged from 20.75‰ to 21.65‰ at the irrigated site and from 19.17‰ to 20.27‰ at the dryland site. The Δ at the irrigated site was 6.6% and 9.1% higher (21.26‰ and 21.16‰) than at the dryland site (19.95‰ and 19.39‰) in 2013 and 2014, respectively (Appendix; Figure D.5). There were no significant (P>0.05) family × site, family × year and family × site × year interactions, indicating the relative performance of the sampled families did not change across environments or time. Larger leaved families demonstrated a lower Δ than smaller leaf families across both sites and years (Appendix; Figure D.6).

6.3.2.2 Phenolic compounds

Across sites and years, there was significant (P<0.001) genetic variation among selected full-sib families for accumulation of quercetin glycosides, kaempferol glycosides, and total flavonol levels (Appendix; Figures D.7 and D.8). Both site and year main effects were significant (P<0.001) for a range of flavonol components. The total flavonol accumulation at the dryland site was 120% and 92% higher than the irrigated site in 2013 and 2014, respectively (Appendix; Figure D.9). Similarly, quercetin glycoside levels at the dryland site were 158% and 83% higher than the irrigated site in 2013 and 2014. Kaempferol glycoside levels were 70% and 107% higher at the dryland site than the irrigated site in 2013 and 2014, respectively.

The ratio of quercetin to kaempferol was significantly (P<0.001) different among full-sib families across years and sites (Appendix; Figure D.7). No family × site, family × year and family × site × year interactions were identified for quercetin glycosides, total flavonols and the ratio of quercetin to kaempferol glycosides, indicating the relative performance of the selected families did not change across different seasons or environments.

Pearson correlations identified no significant (P>0.05) correlations among flavonol components with either vegetative persistence (plot coverage %) or herbage yield (kgDM/ha) within or across experimental sites (Appendix; Figures D.10 and D.11). Significant correlations among flavonol components were identified within and among sites (Appendix; Figures D.10 and D.11).

6.3.3 Summer herbage yield

The irrigated site produced 140% and 511% (P <0.001) more clover herbage yield than the dryland site in the summers of both 2012 and 2013. There was significant (P <0.001) genetic variation among families and cultivars for summer herbage yield across both experimental sites and years (families ranged from 1185 to 2000 kg DM/ha). Significant year × family (P<0.001), environment × family (P<0.05), and year × environment × family (P<0.001) interactions were identified.

At the dryland site, clover herbage yield among families varied from 1086 to 2208 kg DM/ha and from 159 to 423 kg DM/ha in summer 2013 and 2014, respectively. Variation among families at the irrigated site ranged from 1697 to 4303 kg DM/ha and from 1067 to 2409 kg DM/ha in summer 2013 and 2014, respectively.

6.3.4 Autumn recovery

The irrigated site produced 119% and 478% (P <0.001) more clover herbage yield than the dryland site in the autumn of both 2012 and 2013. There was significant (P <0.001) genetic variation among families and cultivars for autumn herbage yield across both experimental sites and years (ranged from 349 to 1000 kg DM/ha). Significant year × family (P<0.001), and environment × family (P<0.001) interactions were observed.

At the dryland site, clover herbage yield among families varied from 180 to 1180 kg DM/ha and from 93 to 326 kg DM/ha in autumn 2013 and 2014, respectively. Variation among families at the irrigated site ranged from 231 to 1390 kg DM/ha and from 496 to 1382 kg DM/ha in autumn 2013 and 2014, respectively. Across both years at the dryland site, heritability on a full-sib family means basis was 0.32 (Table 6.1).

There were no significant differences (P>0.05) between sites for white clover vegetative persistence in autumn 2013, however vegetative persistence was significantly lower (P<0.001) at the dryland site (36%) than the irrigated site (79%) in autumn 2014. There was significant (P <0.001) genetic variation among families and cultivars for autumn vegetative persistence across both experimental sites and years (ranged from 53% to 93%). Significant year × family (P<0.001), environment × family (P<0.001), and year × environment × family (P<0.001) interactions were identified.

At the dryland site, vegetative persistence among families varied from 66% to 100% and 10% to 70% in autumn 2013 and 2014, respectively. At the irrigated site, vegetative persistence among families varied from 63% to 100% and from 15% to 100% in autumn 2013 and 2014, respectively. Across both years at the dryland site, heritability on a full-sib family means basis was 0.47 (Table 6.1).

Growing point density was 172% higher at the irrigated site than the dryland site. Within years, the irrigated site had 109% and 382% (P <0.001) more growing points per m^{-2} than the dryland site in autumn 2013 and 2014, respectively. There was significant (P <0.001) genetic variation among families and cultivars for growing point density across both experimental sites and years. Significant year × family (P<0.001), environment × family (P<0.001), and year × environment × family (P<0.001) interactions were identified.

At the dryland site, growing point density among families varied from 746 to 2815 and from 115 to 867 growing points m^{-2} in autumn 2013 and 2014, respectively. At the irrigated site, growing point density among families varied from 985 to 2422 and from 356 to 3111 growing

points m^{-2} in autumn 2013 and 2014, respectively. Across both years at the dryland site, heritability on a full-sib family means basis was 0.49 (Table 6.1).

Significant positive correlation between spring growing point density and autumn herbage yield and vegetative persistence were observed at both experiment sites (Figure 6.6)

Table 6.1 White clover autumn herbage yield, vegetative persistence and growing point density for the 40 full-sib families at the dryland trial site across years. Variance components: σ_f^2 – full-sib family, σ_r^2 – replicate, σ_a^2 – plot-to-plot, σ_w^2 – within-plot, σ_{fy}^2 – family × year, σ_c^2 – year × plot-to-plot and σ_d^2 – year × within plot. Heritabilities are presented on a full-sib family means basis (h_f^2) (upper limit of narrow sense heritability).

Source	HY (kgDM/ha × 10^{-4})	Persistence* (% × 10^{-2})	GPD (m^{-2} × 10^{-5})
σ_f^2	0.455	0.420	0.429
σ_r^2	0.264	0.180	0.185
σ_a^2	0.106	0.135	0.000
σ_w^2	-	-	0.000
σ_{fy}^2	0.817	0.166	0.422
σ_c^2	3.015	2.089	0.989
σ_d^2	-	-	0.891
Mean	439 kgDM/ha	63%	1032 m^{-2}
Range	141-741 kgDM/ha	42-84%	426-1791 m^{-2}
h_f^2	0.32	0.47	0.49

*vegetative persistence

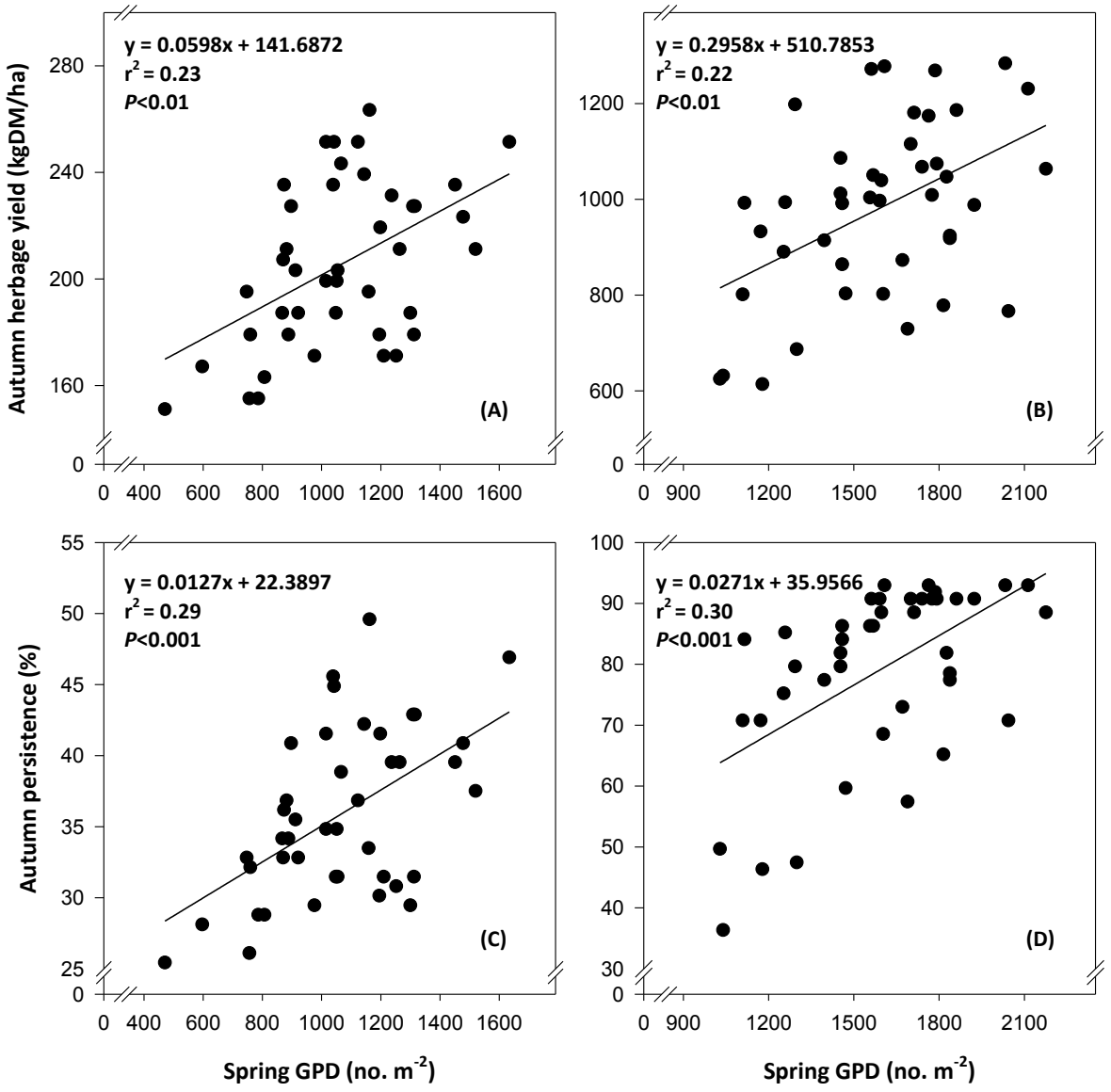


Figure 6.6 Correlations among white clover spring growing point density (GPD) and autumn herbage yield at the dryland site (A) and irrigated site (B) and spring growing point density and vegetative persistence at the dryland site (C) and irrigated site (D).

6.3.5 Multi seasonal analyses

6.3.5.1 Season fixed effects

The fixed effects analysis indicated significant differences for herbage yield (P <0.001), vegetative persistence (P <0.05), leaf width (P <0.001), number of nodes (P <0.001), number of rooted nodes (P <0.001) and stolon branching (P <0.001) at the irrigated site (Table 6.2). Growing point density was not significantly (P = 0.059) different among seasons at the irrigated site. There was significant (P <0.001) differences among seasons for the expression of all traits at the dryland site (Table 6.2).

6.3.5.2 Morphological traits (LW, ST, NN, RN, BR, GPD)

Across season analysis of variance indicated significant (P <0.05) variation among the 40 full-sib families for growing point density (GPD), leaf width (LW), stolon thickness (ST) and stolon branching (SB) at both the irrigated and dryland sites (Table 6.2). No significant (P >0.05) variation among the 40 full-sib families was identified at either environment for number of nodes (NN) or rooted nodes (RN).

Within-plot variance components over seasons were notably lower in comparison to within-plot × seasons (Table 6.2). Plot-to-plot and plot-to-plot × season variance components were similar or lower than among family variance components. Heritabilities on a full-sib family means basis varied according to the trait, but heritabilities for ST and RN were consistently the highest and lowest at both environments, respectively. Similarly, heritability on an individual basis (broad sense) was highest for ST (0.38 ± 0.11 and 0.40 ± 0.11) and lowest for RN (0.03 ± 0.03 and 0.02 ± 0.02) at the irrigated and dryland sites, respectively.

6.3.5.3 Vegetative persistence

Across seasons, no significant variation for vegetative persistence was detected at either the irrigated or dryland site for the 40 full-sib families (Table 6.2). Vegetative persistence however was significantly (P<0.05) different among entries when both control cultivars (Nomad and Kopu II) were included in the analysis (Figure 6.7). At both environments, both plot-to-plot and plot-to-plot × season variance components were significant (P<0.05) and in the magnitude of one to three fold higher than the among family variance components (Table 6.2).

6.3.5.4 Herbage yield

White clover herbage yield was significantly (P<0.05) different among the full-sib families across seasons at both the irrigated and dryland sites (Table 6.2). Seasonal full-sib herbage yield ranged from, 833-1636 kg DM/ha at the irrigated site to 410-879 kg DM/ha at the dryland site. The plot-to-plot variance component was 25.8% lower than the among family variance component at the irrigated site but 153% higher at the dryland site. The plot-to-plot × season variance component was higher than the among family variance component at both environments (236% and 458%). Heritability on a full-sib family means basis was higher at the irrigated site (0.62 ± 0.23) than at the dryland site (0.50 ± 0.24).

Table 6.2 Test of fixed effects due to season, and across season morphological trait variance components and their associated standard errors (± SE) for the 40 white clover full-sib families at both the dryland and irrigated trial sites. Variance components: σ_f^2 – full-sib family, σ_r^2 – replicate, σ_a^2 – plot-to-plot, σ_w^2 – within-plot, σ_{fs}^2 – family × season, σ_c^2 – season × plot-to-plot and σ_d^2 – season × within plot. Heritabilities were calculated on a full-sib family means basis (h_f^2) and on an individual plant basis (h_1^2) where appropriate.

Environment	Source	HY (kg/ha × 10^{-4})	Persistence (% × 10^{-1})	GPD (m^{-2} × 10^{-4})	LW (mm^{-1})	ST (mm^{-1} × 10^{2})	NN (no. m^{-1} × 10^{-2})	RN (no. m^{-1} × 10^{-2})	BR (no. m^{-1} × 10^{-2})
Irrigated site	**Season**	*P* <0.001	*P* <0.05	*P* = 0.059	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
		Variance components (± SE)							
	σ_f^2	2.45 ± 0.91	7.08 ± 3.57	9.64 ± 3.68	1.39 ± 0.44	1.12 ± 0.31	0.21 ± 0.18	0.13 ± 0.12	0.15 ± 0.08
	σ_r^2	0.91 ± 0.41	0.57 ± 0.65	0.31 ± 0.45	0.49 ± 0.31	0.13 ± 0.09	0.10 ± 0.09	0.06 ± 0.05	0.00 ± 0.01
	σ_a^2	1.82 ± 0.43	6.62 ± 2.11	3.16 ± 1.61	0.41 ± 0.15	0.27 ± 0.09	0.20 ± 0.14	0.11 ± 0.09	0.10 ± 0.07
	σ_w^2	-	-	0.51 ± 0.98	0.00 ± 0.14	0.26 ± 0.10	0.33 ± 0.23	0.00 ± 0.15	0.00 ± 0.11
	σ_{fs}^2	4.41 ± 0.60	10.94 ± 2.79	9.34 ± 2.44	0.69 ± 0.19	0.19 ± 0.07	0.75 ± 0.21	0.55 ± 0.16	0.12 ± 0.07
	σ_c^2	5.77 ± 0.38	17.86 ± 2.02	7.54 ± 2.01	0.48 ± 0.15	0.19 ± 0.08	0.30 ± 0.18	0.29 ± 0.14	0.17 ± 0.10
	σ_d^2	-	-	17.53 ± 1.60	6.09 ± 0.22	3.91 ± 0.14	9.36 ± 0.34	6.83 ± 0.25	5.37 ± 0.20
	h_f^2	0.62 ± 0.23	0.47 ± 0.26	0.61 ± 0.23	0.74 ± 0.23	0.83 ± 0.23	0.31 ± 0.27	0.29 ± 0.27	0.49 ± 0.26
	h_1^2	-	-	-	0.31 ± 0.10	0.38 ± 0.11	0.04 ± 0.03	0.03 ± 0.03	0.05 ± 0.03
	Mean	1231	86	1717	19.48	2.10	86.10	40.17	24.01
	Range	833 to 1636	56.2 to 98.1	977 to 2376	16.9 to 21.9	1.85 to 2.34	73.6 to 101.2	30.42 to 53.57	16.07 to 33.00
Dryland site	**Season**	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
		Variance components (± SE)							
	σ_f^2	0.59 ± 0.27	5.05 ± 2.56	4.62 ± 1.66	0.72 ± 0.22	1.17 ± 0.32	0.15 ± 0.12	0.05 ± 0.06	0.26 ± 0.09
	σ_r^2	0.44 ± 0.20	2.69 ± 1.91	1.39 ± 0.96	0.22 ± 0.14	0.03 ± 0.04	0.15 ± 0.11	0.05 ± 0.04	0.09 ± 0.06
	σ_a^2	0.9 ± 0.21	8.02 ± 2.34	1.68 ± 0.89	0.23 ± 0.09	0.08 ± 0.08	0.00 ± 0.00	0.00 ± 0.08	0.00 ± 0.06
	σ_w^2	-	-	0.00 ± 0.46	0.00 ± 0.09	0.02 ± 0.08	0.69 ± 0.19	0.00 ± 0.12	0.00 ± 0.12
	σ_{fs}^2	1.03 ± 0.19	2.54 ± 1.53	2.58 ± 1.01	0.15 ± 0.08	0.27 ± 0.11	0.34 ± 0.15	0.05 ± 0.09	0.06 ± 0.06
	σ_c^2	2.70 ± 0.18	17.97 ± 2.03	5.33 ± 1.18	0.37 ± 0.10	0.61 ± 0.12	0.62 ± 0.15	0.61 ± 0.14	0.25 ± 0.10
	σ_d^2	-	-	8.92 ± 0.81	4.10 ± 0.15	3.66 ± 0.13	7.33 ± 0.26	5.04 ± 0.19	4.72 ± 0.17
	h_f^2	0.50 ± 0.24	0.48 ± 0.27	0.65 ± 0.23	0.77 ± 0.23	0.84 ± 0.23	0.34 ± 0.27	0.28 ± 0.29	0.72 ± 0.25
	h_1^2	-	-	-	0.26 ± 0.08	0.40 ± 0.11	0.03 ± 0.03	0.02 ± 0.02	0.10 ± 0.03
	Mean	642	67	1039	14.92	1.98	88.21	31.11	24.17
	Range	410 to 879	46.2 to 83.0	582 to 1672	13.1 to 16.4	1.70 to 2.23	78.2 to 98.7	26.49 to 36.77	13.88 to 36.98

6.3.6 Multi seasonal and environmental analyses

6.3.6.1 Environment and season fixed effects

Test for fixed effects indicated differences between the two trial sites were significant for herbage yield (P <0.001), vegetative persistence (P <0.001), growing point density (P <0.001), leaf width (P <0.001), stolon thickness (P <0.01) and number of rooted nodes (P <0.001) (Table 6.3). No significant differences between environments were observed for number of nodes (P = 0.291) or stolon branching (P = 0.682).

Test for fixed effects also indicated significant differences among seasons (P <0.001) for all traits. Likewise, significant (P <0.001) environment × season interactions were observed for all traits except stolon branching, indicating seasonal effects varied within the different environments.

Mean full-sib family clover herbage yield across all seasons was two-fold higher at the irrigated site than at the dryland site (Table 6.4). The difference between sites was most pronounced in the summer of 2013/14, when the irrigated site produced six times more clover herbage yield than the dryland site. Similarly in autumn 2014, the irrigated site produced five-fold more clover herbage yield than the dryland site. Vegetative persistence declined at both sites over the 20 month duration of the trial, from 100% to 79% and from 100% to 36% at irrigated and dryland sites, respectively. The decline in vegetative persistence at the dryland site coincided with a declining mean growing point density from 1579 m^{-2} in autumn 2013 to only 476 m^{-2} in autumn 2014. In contrast, the GPD at the irrigated site increased from 1722 m^{-2} in autumn 2013 to 1841 m^{-2} in autumn 2014, despite a lower vegetative persistence grand mean.

Leaf width and stolon thickness were larger (31% and 6%, respectively) at the irrigated site than at the dryland site. Both leaf width and stolon thickness were considerably larger in spring 2012 and 2013 than in autumn 2013 at both sites. The number of rooted nodes was 25% higher at the irrigated site than at the dryland site. Season had a profound effect on both the number of rooted nodes and number of stolon branches, with autumn 2013 promoting substantially greater numbers than spring 2012 and 2013. The ratio of the number of rooted nodes to the number of nodes was highest in autumn 2013 with 0.65 and 0.47 rooted nodes per node at both the irrigated and dryland sites, respectively. In spring 2012 and 2013 these ratios were substantially lower with 0.39 and 0.21 and 0.27 and 0.28 at the irrigated and dryland sites, respectively.

6.3.6.2 Morphological traits (LW, ST, NN, RN, BR, GPD)

Across environments and seasons, there was significant (P <0.05) variation among the 40 full-sib families for growing point density (GPD), leaf width (LW), stolon thickness (ST), number of nodes (NN) and stolon branching (BR) (Table 6.3). No significant (P >0.05) variation was identified among the 40 full-sib families for either vegetative persistence (PER) or rooted nodes (RN) across environments.

Family × season interactions (P <0.05) were observed for herbage yield, leaf width and stolon thickness (Table 6.3). No significant (P>0.05) family × environment interactions were observed for any of the other traits. Family × season × environment interactions (P <0.05) were observed for herbage yield, vegetative persistence, growing point density, number of nodes and number of rooted nodes.

Repeated measurements for LW, ST, NN, RN, BR and GPD across seasons and environments substantially reduced within-plot variance components compared to within-plot × season × environment variance components (Table 6.3). Similarly, plot-to-plot variance components were again substantially lower than plot-to-plot × season × environment variance components.

Heritabilities on a full-sib family means basis varied according to the trait, but similar to across season analyses, showed highest values for ST (0.90 ± 0.22) and lowest for RN (0.46 ± 0.27). Heritabilities on an individual basis (broad sense) were 1.8, 2.5, 8.9, 10, and 15-fold lower than heritabilities on a family means basis for ST, LW, NN, BR and RN, respectively.

6.3.6.3 Vegetative persistence

No significant (P >0.05) differences among full-sib families were observed for vegetative persistence across all seasons and environments (Table 6.3). A significant (P <0.05) family × season × environment effect did however exist, reflecting genetic variation among families in specific environments and seasons (Tables 6.1 and 6.2).

6.3.6.4 Herbage yield

Similar to within season and across season analyses, white clover herbage yield was significantly (P < 0.05) different among the full-sib families across both environments and seasons (Table 6.3). In addition, significant (P < 0.05) family × season and family × season × environment interactions were observed for clover herbage yield. Both the plot-to-plot and plot-to-plot × season variance components were higher (134% and 433%, respectively) than

the among family variance component. Heritability on a full-sib family means basis was moderate at 0.52 ± 0.24.

Table 6.3 **Test of fixed effects due to environment, season and environment × season, and across environment morphological trait variance components and their associated standard errors (± SE) for the 40 white clover full-sib families. Variance components: σ_f^2 – full-sib family, σ_r^2 – replicate, σ_a^2- plot-to-plot, σ_w^2 – within-plot, σ_{fs}^2 – family × season, σ_{fe}^2 – family x environment, σ_{fse}^2 – family x season × environment, σ_b^2 – environment × season × replicate, σ_c^2 – environment × season × plot-to-plot and σ_d^2 – environment × season × within plot. Heritabilities were calculated on a family means basis (h_f^2) and on an individual plant basis (h_1^2) where appropriate.**

Source	HY (kg/ha × 10^{-4})	Persistence (% × 10^{-1})	GPD (m^{-2} × 10^{-4})	LW (mm^{-1})	ST (mm^{-1} × 10^{3})	NN (no. m^{-1} × 10^{-1})	RN (no. m^{-1} × 10^{-1})	BR (no. m^{-1} × 10^{-1})
	Test of fixed effects (*P* values)							
Environment	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.01	*P* = 0.291	*P* <0.001	*P* = 0.682
Season	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
Env. × Season	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* = 0.823
	Variance components (± SE)							
σ_f^2	0.98 ± 0.45	3.43 ± 2.18	4.54 ± 2.01	0.98 ± 0.29	11.84 ± 2.97	3.48 ± 1.22	1.05 ± 0.61	1.90 ± 0.66
σ_r^2	0.47 ± 0.41	0.00 ± 0.70	0.18 ± 0.46	0.19 ± 0.19	0.46 ± 0.53	0.00 ± 0.00	0.00 ± 0.22	0.00 ± 0.15
σ_a^2	1.31 ± 0.22	7.31 ± 1.58	2.35 ± 0.88	0.32 ± 0.08	1.75 ± 0.62	0.62 ± 0.88	0.19 ± 0.59	0.35 ± 0.44
σ_w^2	-	-	0.19 ± 0.51	0.02 ± 0.08	1.28 ± 0.63	5.27 ± 1.56	0.00 ± 0.96	0.00 ± 0.83
σ_{fs}^2	1.30 ± 0.28	0.60 ± 1.44	0.54 ± 1.17	0.32 ± 0.10	1.49 ± 0.62	2.03 ± 1.21	0.00 ± 0.78	0.66 ± 0.44
σ_{fe}^2	0.56 ± 0.33	2.64 ± 2.22	2.61 ± 1.63	0.08 ± 0.09	0.00 ± 0.44	0.00 ± 0.66	0.00 ± 0.73	0.06 ± 0.36
σ_{fse}^2	1.41 ± 0.27	6.14 ± 2.00	5.41 ± 1.60	0.11 ± 0.08	0.69 ± 0.65	3.30 ± 1.42	3.57 ± 1.27	0.27 ± 0.54
σ_b^2	0.38 ± 0.14	1.67 ± 1.06	0.73 ± 0.53	0.18 ± 0.11	0.42 ± 0.33	1.24 ± 0.67	0.64 ± 0.47	0.61 ± 0.41
σ_c^2	4.24 ± 0.20	17.92 ± 1.43	6.47 ± 1.13	0.43 ± 0.09	4.17 ± 0.75	4.91 ± 1.26	4.60 ± 0.98	2.05 ± 0.68
σ_d^2	-	-	13.22 ± 0.85	5.07 ± 0.13	37.8 ± 0.97	83.2 ± 2.18	59.25 ± 1.55	50.52 ± 1.31
h_f^2	0.52 ± 0.24	0.42 ± 0.27	0.56 ± 0.25	0.78 ± 0.23	0.9 ± 0.22	0.62 ± 0.22	0.46 ± 0.27	0.72 ± 0.25
h_1^2	-	-	-	0.31 ± 0.08	0.5 ± 0.1	0.07 ± 0.02	0.03 ± 0.02	0.07 ± 0.02
Mean*	936	76	1378	17.19	2.04	87.20	35.64	24.09
Range*	665 to 1268	55.2 to 91.2	854 to 1915	14.7 to 19.1	1.77 to 2.29	75.11 to 100.3	27.83 to 45.93	15.78 to 34.16

*Presented in actual units

Table 6.4 Means for white clover vegetative morphological attributes calculated from across both site and season analysis of variances.

Source	Season	HY (kgDM/ha)	Persistence (%)	GPD (m^{-2})	LW (mm^{-1})	ST (mm^{-1})	NN (no. m^{-1})	RN (no. m^{-1})	BR (no. m^{-1})
Irrigated site	**Multi-season**	1231	86	1717	19.48	2.10	86.10	40.17	24.01
Dryland site	**Multi-season**	642	67	1039	14.92	1.98	88.21	31.11	24.17
Irrigated site	**Spring 2012***	316	100	-	23.15	2.33	40.1	15.8	4.4
	Summer	2521	-	-	-	-	-	-	-
	Autumn 2013	806	91	1722	13.04	1.84	133.9	87.4	47.2
	Spring 2013	1978	87	1588	22.25	2.13	84.3	17.3	20.5
	Summer	1833	-	-	-	-	-	-	-
	Autumn 2014	979	79	1841	-	-	-	-	-
Dryland site	**Spring 2012***	496	100	-	15.48	2.13	50.7	13.5	5.0
	Summer	1566	-	-	-	-	-	-	-
	Autumn 2013	677	91	1579	11.41	1.83	104.4	48.8	46.1
	Spring 2013	989	75	1062	17.87	1.98	109.5	31.1	21.4
	Summer	305	-	-	-	-	-	-	-
	Autumn 2014	205	36	476	-	-	-	-	-

*Only one yield harvest within the spring 2012 season

6.3.7 Pattern analysis

Principal component analyses (PCA) enabled the correlation of traits for specific sites for the full-sib families and two check cultivars × trait adjusted mean matrices. Traits included HY, PER, GPD, LW, ST, and BR, generated from variance component analyses across seasons but within sites (Figure 6.7). The correlation structure among traits was indicated by the directional vectors of the biplots. The biplots indicated similar associations between HY, PER, GPD, LW, ST, and BR at both sites.

Across season analyses indicated a positive association (angles between the directional vectors were <45°) between HY, GPD and PER at both evaluation sites, as well as a positive association between ST and LW and a negative association (angles between directional vectors were equal or close to 180°) between ST and BR. Cluster analysis of the 40 full-sib families and two cultivars based on HY, PER, GPD, LW, ST and BR at the irrigated site generated five entry groups. There was separation between groups, with some degree of overlap between groups two and three. Group two displayed above population mean expression for BR and GPD, whereas group five had above average LW, HY and PER. Cultivar Nomad was clustered into group four which was below average for the bulk of morphological traits. Kopu II on the other hand was clustered into group five, with above average HY, LW and ST. Kopu II and Nomad were often on the opposite outer extremes of the white clover source population investigated.

Cluster analysis of the 40 full-sib families and two cultivars based on HY, PER, GPD, LW, ST and BR at the dryland site again generated five entry groups. Families in group four displayed above average expression for BR and GPD, whereas families clustered into group two showed above average expression for GPD, PER and HY. Group three displayed above average expression for LW and ST. Nomad was clustered into group four which was above average for BR and had a strong negative association with both ST and LW. Kopu II on the other hand was clustered into its own distinct group, with above average ST and LW and a strong negative association with BR and the other cultivar Nomad at the dryland site. Full-sib families 3, 7, 10, 23, 30, and 38 displayed above population mean expression for most significant agronomic traits at both sites. Full-sib families 7 and 10 in particular, showed outstanding performance at both sites.

Principal component analyses of the full-sib families and two cultivars × trait BLUP adjusted mean matrices enabled the correlation among traits for broad adaptation to sites. Traits included HY, GPD, LW, ST, NN, and BR, generated from variance component analyses

across both sites and seasons (Figure 6.8). The analyses of both PER and RN across both sites and seasons showed that there was no significant (P <0.05) difference in these traits among the families, and therefore they were not included in the PCA matrix. As with prior analyses, a positive association between LW and ST was evident, as well as associations between HY and GPD and GPD, NN and BR. The negative association between BR/NN and ST was still evident.

Cluster analysis of the 40 full-sib families and two cultivars based on HY, GPD, LW, ST, NN, and BR generated four entry groups (Figure 6.8). These four groups showed considerable separation with almost zero overlap between groups. Full-sib families clustered into group two displayed consistent above average expression for HY, GPD, BR and NN across both sites and seasons. The families clustered into this group were similar to those identified as outstanding families at each site and included full-sib families 3, 7, 10, 23, 25, 26, 28, 34, 38, 39 and 40. Nomad was also clustered into the above average agronomic group two. Kopu II was again clustered into its own unique group with above average HY and LW, and had a weak negative association with Nomad. Both cultivars were again at either extremes in the multivariate distribution of the white clover population investigated.

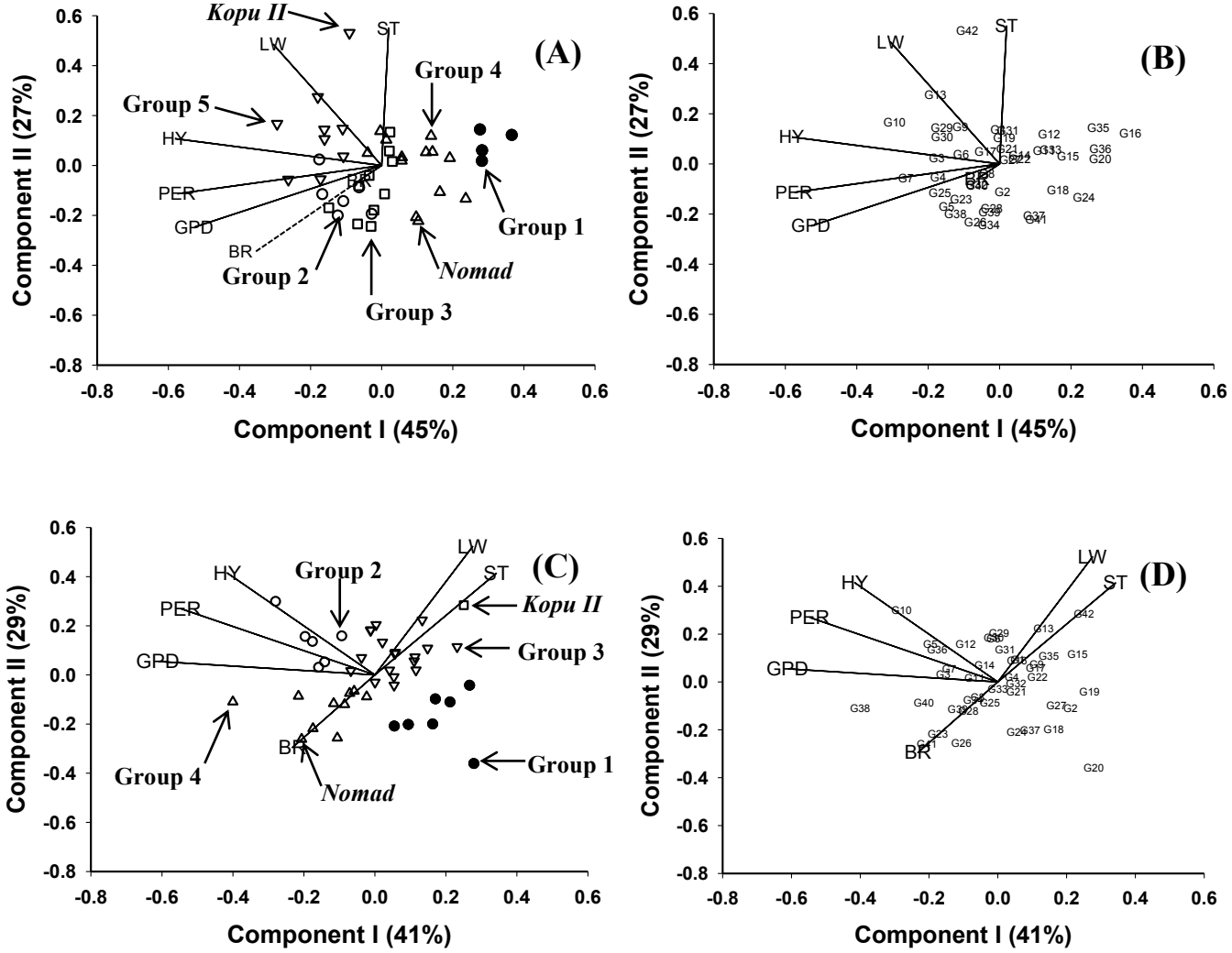


Figure 6.7 Biplots generated using adjusted means for morphological traits among the 40 white clover full-sib families and two cultivars (Nomad and Kopu II) across seasons at the irrigated (A and B) and dryland (C and D) sites. The different symbols indicate family groups 1-5 generated using cluster analysis (A and C). Family identities are labelled in B and D. The vectors represent clover herbage yield (HY), growing point density (GPD), clover sward persistence (PER), leaf width (LW), stolon thickness (ST) and number of stolon branches (BR).

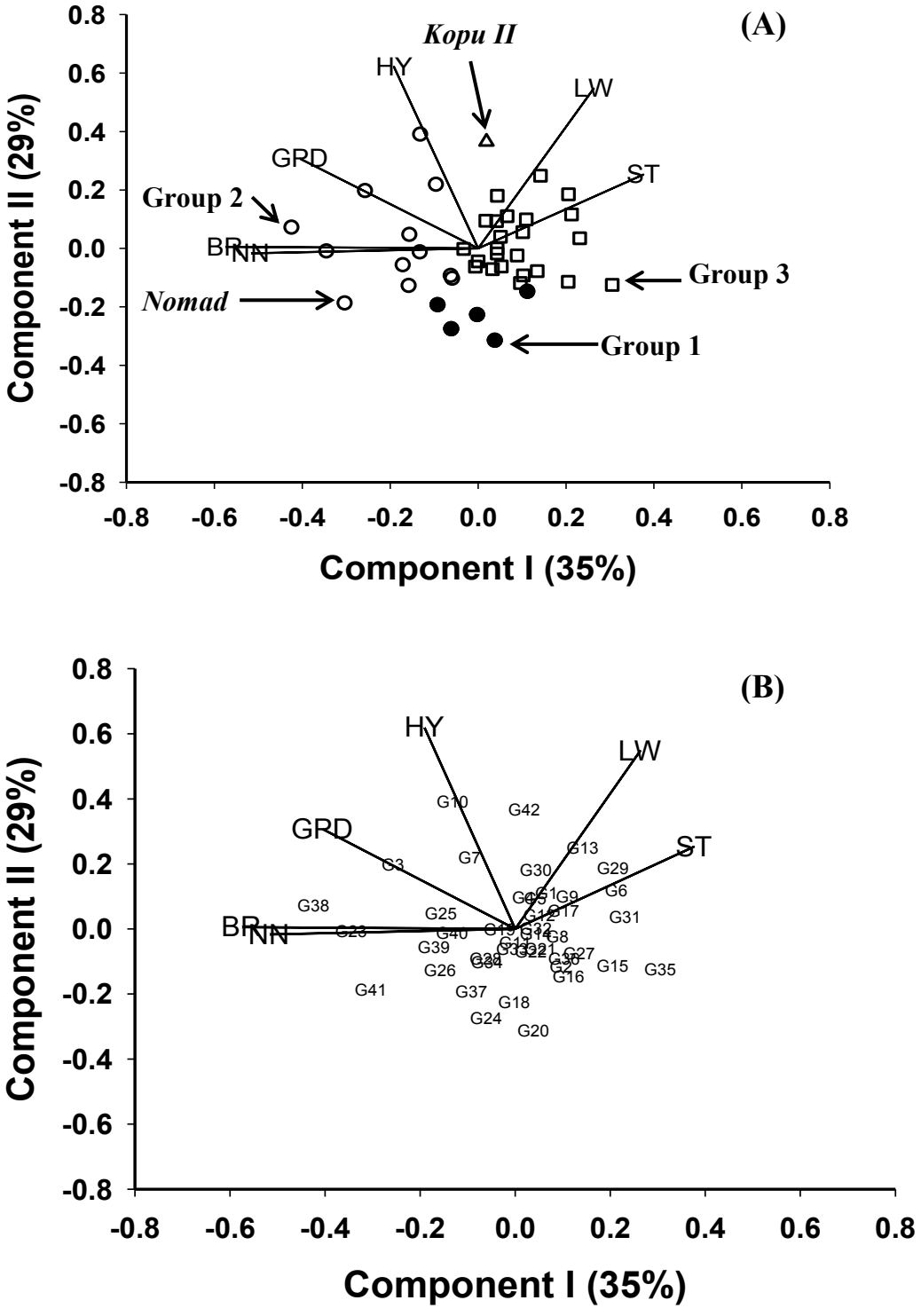


Figure 6.8 Biplot generated using adjusted means for morphological traits among the 40 white clover full-sib families and two cultivars (Nomad and Kopu II) across all seasons and both trial sites. The different symbols indicate family groups 1-4 generated using cluster analysis (A), and the numbers in (B) indicate family identity. The vectors represent clover herbage yield (HY), growing point density (GPD), leaf width (LW), stolon thickness (ST), number of nodes (NN) and number of stolon branches (BR).

6.4 Discussion

6.4.1 Fixed effects

Considerable differences between experimental sites were evident for the majority of the measured traits. The difference in summer seasonal clover herbage yield between sites was a reflection of their respective soil moisture contents. Both winter and early spring yields were comparable between sites when soil moisture levels were replenished. The discrepancies in autumn vegetative persistence between sites were also a reflection of their respective summer soil moisture contents in 2014, but not in 2013. Although plots at the dryland site experienced considerable water stress in the summer of 2012/13 (Figure 6.4), the regular application of irrigation in that season maintained plant water status above permanent wilting point and prevented detrimental effects to the white clover component of the mixed species plots. This was emphasised by the similar clover herbage yield and vegetative persistence in both sites in autumn 2013 (post-moisture stress) (Table 6.4). The irrigation volumes applied throughout the summer of 2012/2013 were similar to the minimum amounts required (40-60mm/month) for white clover growth (Brock, 2006). Contrary to the summer of 2012/13, vegetative persistence at the dryland site post-summer soil moisture stress in 2014 (autumn 2014) decreased by more than two-fold compared to the irrigated site, and clover herbage yield was 4.5 fold less. The combination of water stress (below wilting point), absence of additional irrigation, and stress duration was detrimental to white clover vegetative persistence in the summer of 2013/14. The results suggest that a large component of white clover survival between years was regulated by plant water status. This was emphasised by the differences in plant water status and autumn vegetative persistence between successive summers.

Within both sites, growing point densities were positively correlated with vegetative persistence and herbage yield. This is supported by previous studies where stolon density was a major factor governing persistence (Caradus and Williams, 1989; Woodfield and Caradus, 1996). Spring growing point densities were moderate predictors of autumn vegetative persistence and herbage yield (Figure 6.6).

Sim (2014) demonstrated in lucerne (*Medicago sativa* L.) on a similar soil type that the lower limit of soil moisture content was approximately 6-7%. It is therefore probable that the permanent wilting point seen in the summer of 2013/14 coincided with 0% plant available water content (PAWC) within the top 55-65cm of the soil profile (Appendix; Figure D.4). It is also apparent from the data that permanent wilting point also coincided at the point in time

when the soil moisture content within the top 20cm first declined below 8%. It is therefore also possible that the permanent wilting point of plants in this study coincided with depletion of PAWC in the top 20 cm of soil and not the maximum 65cm soil horizon (Figure 6.3). Due to the mixed species sward, it is difficult to differentiate the actual depth of clover soil water extraction.

Black et al. (2003) reported water extraction depths of approximately 130 to 150 cm for white clover one to three years post establishment. Their research indicated that both taproots and nodal roots were present, and thus their data was not indicative of nodal rooting depth specifically. Reports of nodal rooting depth in field experiments are limited, although Nichols (2012) reported water extraction of white clover nodal roots up to at least 95cm. The shallow depths of nodal root water extraction in this study are therefore considerably lower than both Nichols (2012) and Black et al. (2003). The comparison between this study and that of Black et al. (2003) emphasises the potential loss in plant available water (soil type dependent) between establishment (tap-rooted) and vegetative (nodal rooted) morphological phases. This finding helps to shed light on the reported sharp decline observed in white clover vegetative persistence at approximately 18 months to 24 months post establishment; when white clover plants enter their vegetative phase, their taproots die and large plants fragment into smaller daughter clones relying on tertiary stolons and adventitious roots for survival (Knowles et al., 2003; Thomas, 1987b). This decline is further exacerbated when combined with moisture stress (Knowles et al., 2003). Recognition of the positive association between tap rootedness and drought tolerance has been previously reported (Woodfield and Caradus, 1987).

Regardless of water stress, there was also a decline in vegetative persistence at the irrigated site, albeit not to the extent observed at the dryland site. A similar finding was reported by Brock and Caradus (1996) where a decline in white clover persistence initially commenced prior to any soil moisture deficit. Whilst water access seems a major factor regulating vegetative persistence at the dryland site, this was not a contributing factor at the irrigated site. From this standpoint, it also seems likely that the immediate decline in vegetative persistence and herbage yield seen at the irrigated site could have also been associated with the vegetative morphological development phase of white clover.

Purely from an observation point of view, it was interesting to note the differences in morphology between the clonal phase of white clover in the current trial, and its seedling phase which was run concurrently in the trial described in Chapter 5. It was apparent from the seedling phase in Chapter 5 that individual plants grown from seedlings (with their tap-root

still intact) seemed to have a much greater growing point density at their crown than the clonal cuttings in this experiment. This was by in large due to the seedlings still having their orthotrophic primary stem (Thomas, 1987a) which allowed multiple primary stolons to be radiated from a central growing point. Clonal fragments on the other hand could only produce an additional growing point at the original anchored node and relied on further branching from superseding tertiary stolons. Although speculative, it seemed that these smaller anchored clonal crowns not only resulted in less vigorous plants with fewer growing points, but also that they were more susceptible to damage from grazing ruminants. Brock and Caradus (1996) hypothesised that the point at which the population in general changes from dependency on seminal roots to nodal roots, may coincide with a loss in performance of the population *per se.* This statement was also supported by findings by Knowles et al. (2003).

The lower frequency of rooted nodes at the dryland site compared to the irrigated site in autumn 2013 reflected the exposure level of nodal root primordia on the existing stolons to moisture over the summer period of 2012/2013. In contrast, the minor differences between sites for RN in spring 2012 and spring 2013, likely reflects the absence in moisture deficits between both sites over early spring. Root primordia require moisture to develop into nodal roots (Thomas, 1987b) and the expectation would be that the plants at the irrigated site would have had a much greater frequency of RN in autumn, given their elevated levels of soil moisture and water application throughout the summer periods. It is likely the higher frequency of RN estimated at the irrigated site in autumn 2013 was still biased downwards compared to the actual differences, if they had been measured during the summer moisture deficit period (Table 6.4).

It could be argued that the quantity of initiated rooted nodes per unit of stolon did not accurately reflect the actual number of physically rooted nodes per unit of area. In retrospect, the former represents the response to the environment over the lifetime of a stolon segment (which may be short in comparison), whereas the latter represents an accumulation of fragmented stolons over a longer period of time (i.e. the whole summer). Both methods have been used independently to estimate the number of rooted nodes in previous studies (Jahufer et al., 1994; Jahufer et al., 1999), although the former has been previously investigated under the management of artificial grazing which would extend the life of a stolon segment and perhaps better reflect a longer proportion of the summer moisture deficit period compared to grazed stolons.

It is also important to note that the number of initiated rooted nodes may not translate into penetrating and developed permanent rooted nodes, especially at the dryland site where even actively growing nodal roots at root primordia struggled to penetrate the soil surface to anchor newly formed stolons. The seldom rainfall events and high evapotranspiration rates caused the soil to become hard and form a 'baked' like appearance, which became an impermeable layer for newly initiated nodal roots. Drying of the soil surface has been previously reported to inhibit the development of new nodal roots (Brock, 2006; Stevenson and Laidlaw, 1985). Even in the unlikely success of primordial roots penetrating the soil surface, the addition of grazing sheep further prevented nodal root establishment. Rotational grazing at three to four week intervals often ripped many newly anchored primordial roots from the soil surface, where the actively growing nodal root did not develop fast enough to anchor the stolon before grazing. This was more apparent at the dryland site, where canopy architecture was more open during the summer moisture deficit months.

Considering the above, it is not recommended to use the number of initiated rooted nodes per unit of stolon as a proxy for the number of physical rooted nodes per unit of area in future studies. Instead, the direct measurement of physically rooted nodes per unit of area is recommended as demonstrated by Jahufer et al. (1999). To an extent in this study, post grazing GPD indirectly measured the number of rooted nodes per unit of area, because only anchored axillary buds gave rise to new growing points under rotational sheep grazing. This is supported by Brock et al. (1988) where under sheep grazing, rooted stolons accounted for 85% of stolon mass. Furthermore, under drought conditions Brock (1988) also noted that stolon mass between rooted growing points withered and died, as large plants broke up into several smaller ones, leaving behind only anchored nodal roots.

The significant reduction in GPD at the dryland site between spring 2013 and autumn 2014 represents a greater ratio of stolon mortality to stolon formation. The rate of stolon mortality can be accelerated by disease, insect pests and water stress, and when this mortality exceeds stolon and nodal root formation, white clover persistence declines (Woodfield and Caradus, 1996). Archer and Robinson (1989) expanded on the effects of water stress and reported the interaction between water stress and ambient air temperature in NSW tablelands, Australia. Their results demonstrated that stolon survival began to decline when plant available water content fell below 61% and the mean weekly ambient temperatures exceeded 20°C.

In New Zealand, there is limited knowledge about the soil moisture threshold at which stolon survival begins to decline, but numerous studies have reported decreasing white clover

content in swards with increasing reductions in average summer rainfall (e.g. Knowles et al. 2003). A similar pattern was observed in this experiment. The effect of temperature on stolon persistence has been investigated at the soil surface level, and stolons with lower levels of exposure to solar radiation via shading have increased stolon survival rates (Brock, 1988; Brock and Kim, 1994).

A clear trend emerged between experimental sites for soil surface temperature in this study. Soil surface temperatures were higher at the dryland site than at the irrigated site, although there was also variation within the irrigated site, which typically reflected the stage of the defoliation rotation. The differences in soil surface temperatures between sites were at their greatest when the irrigated site was approaching defoliation, and canopy cover was high. The substantial reduction in soil surface temperatures due to pasture cover was similar to that reported by Brock and Hay (1993). It is therefore most probable that the increased soil temperatures, combined with water moisture deficits, were major contributing factors for the decline in GPD at the dryland site.

In addition to companion species cover, a degree of stolon shading has also been shown to be associated with stolon burial (Brock and Kim, 1994). Limited protection from solar radiation via stolon burial was observed at the dryland site due to the 'baked' soil surface during spring and summer, and hence recovery of stolons in autumn was primarily dependent on survival of above ground biomass. Therefore a significant difference at the irrigated site was stolon shading, due to not only the companion sward species, but also the soil interface. It seemed stolon burial at the irrigated site was influenced by a higher frequency of worm casts and animal trampling than at the dryland site and reflects the mechanism of stolon burial in previous reports where the soil interface has been soft and wet (Cresswell, 1996; Hay et al., 1987). In addition to both animal trampling and worm casts, nodal root contraction has been demonstrated to provide a third mechanism for stolon burial (Cresswell et al., 1999). However just like the former two, this mechanism is also compromised by factors increasing field soil resistance, such as lack of soil moisture/rainfall reduce. Buried stolons can account for up to 40% of stolon mass in New Zealand pastures over summer (Hay et al., 1987), and therefore potentially provide substantially more protected growing points for recovery in autumn.

Another major and often over-looked factor regulating stolon persistence is the effect of grazing animals *per se*. It was certainly obvious at the dryland site that as the summer moisture stress period commenced, the surrounding companion grass (predominantly ryegrass) became considerably weaker, resulting in an open sward that exposed the already

vulnerable white clover plants to over grazing. Sheep preferentially grazed white clover in the mixed species sward, and stripped the plants back to original anchored roots, creating a button like effect of the remaining growing points and thus preventing new nodal roots from developing. Hay et al. (1987) noted that under drought conditions where pasture growth was restricted, stolon mass was further reduced by sheep defoliation over and above losses by moisture stress alone.

Brock and Kane (2003) emphasised the important beneficial effect of ryegrass for white clover and how it helps mitigate overgrazing. They also described the problem associated with stolon stripping (overgrazing), when grazing animals have access to emerging stolons in the absence of protection from companion sward species. This effect has been emphasised in multiple studies were set-stocking mitigates drought effects through increasing not only clover density, but also that of the surrounding grass species (Brock, 1988; Brock and Hay, 1993; Brock and Caradus, 1996). The ability to restrict stolon loss via animal removal has also been demonstrated in moist and hence soft soil interfaces where stolons are tightly held to the surface (Cresswell, 1996). The reports that smaller leaf types demonstrate better overall persistence (Caradus and Mackay, 1991; Williams et al., 1982b) may therefore also be related to the ability of plants to withstand stolon mass removal among other factors.

These data suggest vegetative persistence of white clover in both water limited and water sufficient environments seem to be linked to both abiotic factors such as water stress, solar radiation and heat stress, as well as biotic factors such as disease, pests and perhaps most importantly grazing ruminants. The results presented in this study, combined with other published work (Brock and Kim, 1994; Brock and Caradus, 1996; Caradus and Williams, 1989; Woodfield and Caradus, 1996) indicate that white clover vegetative persistence is considerably determined by a moisture stress × heat × animal grazing × genotype interaction complex. The latter three factors can be largely mitigated by stock management (Brock and Kim, 1994; Brock and Caradus, 1996), which in the past and still to date is the best tool to mitigate drought effects in white clover pastures. Future studies investigating drought tolerance or resistance mechanisms in white clover must acknowledge the complexity of target environments and consider the transferability of traits improved in artificial environments to actual target environments. Improvements achieved in artificial environments maybe counterintuitive in actual farming systems. This is particularly relevant to stolon traits, where they are most likely to be removed by grazing ruminants.

Both leaf width and stolon thickness were significantly different between sites, although seasonal effects had a far greater influence on these traits. Temperature and photoperiod have been shown to largely influence organ growth in white clover (Junttila et al., 1990) and hence the extent of seasonal influence is not surprising. The similarity of NN and BR between sites was however less expected. Thompson and Harper (1988) reported significant differences in both photosynthetically active radiation (PAR) and the ratio of red to far red light between canopies present and absent with ryegrass foliage. While shading promotes larger leaf size (Caradus and Chapman, 1991) which was observed at the irrigated site, no decreases in NN or BR were observed, which could be due to lower levels of both PAR and the ratio of red to far red light (Thompson and Harper, 1988) in closed sward canopies.

6.4.2 Variance components and heritabilities

The results from this study suggest a significant amount of genetic variation for the morphological traits HY, GPD, PER, LW, ST, NN and BR within seasons and environments in the vegetatively propagated white clover population investigated. Genetic variation among full-sib families for the majority of these traits extended to seasons, years and environments. The extent of genetic variation observed in this population is similar to previous findings in other white clover populations and accessions (Caradus and Chapman, 1991; Jahufer et al., 1994; Jahufer et al., 1995; Jahufer et al., 1999; Woodfield and Caradus, 1990). Both clover vegetative persistence (PER) and the number of rooted nodes (RN) were the only traits which showed no genetic variation across environments, seasons and years. Poor genetic variation for the proportion of rooted nodes has also been reported elsewhere in low rainfall accessions by Jahufer et al. (1995).

The discrepancies in the heritabilities between the different traits reflect the proportion of environmental influence on the genetic expression of these traits. Among the traits, both stolon thickness (ST) and leaf width (LW) displayed the highest heritabilities. This is supported by previous findings where ST and LW have high heritabilities relative to other morphological traits (Annicchiarico and Piano, 1995; Annicchiarico et al., 1999; Caradus and Woodfield, 1990; Jahufer et al., 2013).

The within-plot variance components demonstrate the degree of environmental influence on the phenotypic expression of individuals within families. For NN, RN and BR, these within-plot variances were extremely large in comparison to the among families variance components. This highlights the inaccuracy of only obtaining individual plant data compared to the means of families for these traits. From a breeding perspective, these inaccuracies are

reflected in the differences between heritabilities calculated on a family means basis and on those on an individual plant basis. As discussed in Chapter 5, Nyquist (1991) recommended that when heritability on an individual plant basis is low due to large environmental effects, family selection should be implemented instead of phenotypic selection (i.e. mass selection). Taking this into account, the rationale for pursuing phenotypic selection on an individual plant basis for NN, RN and BR is questionable, and supports the rationale for family selection methods under these trial circumstances. Again as pointed out previously in Chapter 5, an important feature of family selection is that selection is based on family means which are obtained from replicated trials; and therefore are less affected by large environmental variances compared to individual selections (Nguyen and Sleper, 1983).

A smaller reduction in the difference between heritabilities calculated on a family means basis compared to an individual plant basis was observed for both ST and LW. These findings suggest that phenotypic expressions for these two traits on an individual plant level are less confounded by environmental cues than NN, RN and BR. Phenotypic selection for both LW and ST is a satisfactory and reasonably accurate selection method for intra-population improvement.

The task of drawing direct comparisons between heritabilities estimated in the present study with those estimated in other studies is somewhat challenging, despite what seems like a bulk of literature on heritabilities in white clover. Heritabilities have often been used to describe the ratio of genotypic to phenotypic variance among random genotypes that are not part of a random mating population (Fehr, 1987). In this context, the reference populations in these studies are ‘cultivars of the species’ and not a random mating population like in the current study. The phenotypic standard deviations of these reference populations are most likely to be significantly larger than those reported in closed segregating populations, and hence comparisons can be somewhat misleading. Numerous studies have also investigated genetic parameters when genotypes are a non-random sample, and estimates obtained from these studies pertain only to those genotypes and cannot be used to infer what would be expected if random genotypes were studied in a random mating breeding population (Fehr, 1987). In such cases, the term repeatability is often used instead of ‘heritability’. The comparison between this study and other studies is also confounded not only by the defoliation management of the trials, but also by the presence of companion sward species. Both factors have been shown to have large influences on white clover morphology and family ranking (Caradus et al., 1989; Evans and Williams, 1987).

To the best of my knowledge, Jahufer et al. (1999) is the sole study that has also investigated white clover breeding strategies comparable to the present study, despite significant differences in defoliation management and sward composition between studies. The study by Jahufer (1999) was conducted across both environments and years using 80 full-sib families and reported heritabilities on a full-sib family mean basis for clover herbage yield (0.38), stolon thickness (0.37), number of nodes (0.36), number of rooted nodes (0.14) and stolon branching (0.27), respectively. The heritabilities for all traits in this study compare favourably to those estimated in Jahufer et al. (1999), and likely reflect greater genotypic diversity in the population investigated for the traits tested in this experiment.

Shifting away from directly comparable studies, Jahufer et al. (1994) presented accession mean repeatability measurements for 60 white clover accessions for leaf length, stolon thickness, number of nodes, number of rooted nodes and stolon branching in a study conducted using mono-culture plots under artificial defoliation. Jahufer et al. (1995) also reported line mean repeatability measurements for 40 white clover accessions for leaf length, stolon thickness, number of nodes, number of rooted nodes and stolon branching, using the same field procedures as described by Jahufer et al. (1994). Both repeatability estimates by Jahufer et al. (1994; 1995) compare unfavourably to the findings in this experiment, despite a much larger diversity in plant material evaluated in their experiments. Perhaps the comparable estimates between data in this study and both of the above studies either illustrate the great magnitude of genotypic diversity of the population tested in this study or the better adaptation of the population in this study to the test environments which allowed it to better express its genetic potential.

Among traits with heritabilities calculated on an individual plant basis, it was interesting to note the substantially lower broad and narrow heritabilities in this study compared to those reported in the literature (Annicchiarico and Piano, 1995; Annicchiarico et al., 1999; Caradus and Woodfield, 1990; Cogan et al., 2006; Woodfield and Caradus, 1990).

It could be argued that the current study magnifies the difference between quantification of genetic components in a random mating breeding population compared to those in either a diverse panel of accessions or a non-random sample of diverse selected genotypes within a population. As explained by Jahufer et al. (2002) there is a lack of quantitative genetic parameters estimated for random mating white clover breeding populations in multiple target environments, and therefore the necessity to estimate these in order to make valid estimates of responses to selection of various breeding methods is fundamental. Inaccurate estimates of

heritabilities in non-target environments would lead to a slower response to selection in actual field breeding programmes than estimated heritabilities would lead to believe.

Lack of statistical differences among full-sib families across seasons and across environments and seasons (when check cultivars where excluded) for clover vegetative persistence was largely a result of the large plot-to-plot and season × plot-to-plot variation (Table 6.3). Plot-to-plot variation averaged across both seasons and seasons within environments was consistently higher for vegetative persistence than in all other traits. It is likely that the small 1m × 1m plots did not accurately reflect family vegetative persistence in each replicate and subsequently re-ranking among families occurred in successive replicates. Investigations into alternative effective plot sizes may be valuable from a plant breeder's perspective to accurately estimate sward persistence in future studies to reduce plot-to-plot variation.

The lack of genotype × environment interactions is an interesting discovery, and could point towards broad adaptation of the investigated clover population in both test environments. The significant genotype × season × environment interactions and genotype × season interactions are consistent with findings from previous studies (Jahufer et al., 2002; Jahufer et al., 2009; Jahufer et al., 2013) and underline the requirement for multi-season and multi-year testing to identify superior families.

6.4.3 Pattern analysis

Contrary to multiple studies where the correlations between HY and GPD or stolon density have been negative (Caradus and Williams, 1989; Jahufer et al., 1994), this study revealed a positive association between these traits. Jahufer et al. (1999) also reported a positive association between these traits in a study among 80 full-sib families. A striking similarity between the current study and that of Jahufer et al. (1999) is the identification of the breakdown of this negative association at the intra-population level. Many studies have presented data at a cultivar or accession level and hence neglected the quantitative nature of these traits within a random mating population.

6.4.4 Physiological analyses

Leaf water potential (ψ) is an indication of the water status of a plant. The findings reflected the severity of water stress at the dryland site over the summer months compared to the irrigated site. The significant drought induced reduction in white clover leaf water potential is similar to previous studies (Hofmann et al., 2007; Turner, 1991).

The discrimination against ^{13}C (Δ) was lower at the dryland site in both summers than at the irrigated site, indicating the proportion of $^{13}C/^{12}C$ assimilated in photosynthesis at the dryland site was lower, due to ribulose bisphosphate carboxylase (Rubisco) fixing proportionally more $^{13}CO_2$ (Barbour et al., 1996). Similar findings have been presented between water stressed and non-water stressed treatments in white clover (Ballizany et al., 2012a; Barbour et al., 1996). Contrary to the results of Barbour et al. (1996), significant genetic variation within sites was also observed in this field study, showing that intra-cultivar variation for Δ does exist albeit at relatively low levels (~10%). The lack of family × environment and family × year interactions for this trait demonstrate that selection for families with lower Δ in irrigated environments holds true to type in dryland environments too. Thus future breeding programmes should use the easily measurable trait ^{13}C (Δ) as a screening tool for the development of drought resistant germplasm.

The higher accumulation of flavonoids at the dryland site is consistent with previous findings where water-stress induces an increase in these secondary plant metabolites (Ballizany et al., 2012a). Contrary to previous findings in white clover (Hofmann and Jahufer, 2011), no significant negative correlations between herbage biomass and flavonol accumulation (and its components) were observed. This is therefore of significance, as the plant material in this study can be used for the development of white clover cultivars that are high in herbage yield and in protective flavonols. The concentrations of quercetin glycosides expressed among full-sib families are consistent with the range of concentrations reported in the populations' parent material (Tribute, Saracen and Trophy) (Hofmann and Jahufer, 2011). Similarly, the lower levels of quercetin glycosides expressed in the control cultivar Nomad is consistent with previous reports (Hofmann and Jahufer, 2011). Similarly to Δ, the lack of family × environment and family × year interactions demonstrate that families with higher flavonol concentrations in irrigated environments are also likely to have higher concentrations of these compounds in water limited environments.

6.5 Conclusions

- The low summer clover herbage yield among white clover families at the dryland site in 2013/14 demonstrate the poor adaptation of white clover to soils with low water holding capacity. These results agree with those presented by Mills and Moot (2010), and the use of alternative species such as sub-clover and lucerne are better suited in such environments for summer productivity.

- Genetic variation for both vegetative persistence and herbage yield post-summer moisture stress was observed, suggesting scope for developing cultivars that can vegetatively persist in moderate drought conditions and demonstrate improved recovery rates following rain.

- Considerable genetic variation for most traits among the vegetatively propagated families was evident within and across sites. The trialling of clonal material may be useful for plant breeders trying to bypass the first 1-2 years of the white clover establishment phase. However due to its time consuming preparation and lack of assessment of field seedling establishment parameters, breeders may decide to use it sparingly at key stages of a breeding programme.

- As in the previous chapter, differentials between heritabilities estimated on a family means basis and those on an individual plant basis, support the rationale for family selection methods for clover herbage yield, vegetative persistence, number of nodes, number of rooted nodes and number of branches. These data also suggest phenotypic mass selection is suitable for both leaf size and stolon thickness.

- In both the current study and a previous study by Jahufer et al. (1999), intra-population evaluation seems to identify the breakdown of the negative association between clover herbage yield and growing point density. This supports the notion by Woodfield and Caradus (1996) that it is possible to develop cultivars with increased growing point densities at a particular leaf size to improve vegetative persistence, while maintaining greater yield potential.

- The significant genotype × season × environment interactions and genotype × season interactions are consistent with previous studies and demonstrate the requirement for multi-season and multi-year testing to identify superior families.

- White clover vegetative persistence is a complex trait regulated by a number of factors including, moisture stress, heat, animal grazing, and genotype. As per previous studies, growing point density remains the most accurate predictor of autumn vegetative persistence and herbage yield.

- Investigation into alternative effective plot sizes may be valuable from a plant breeding perspective to accurately estimate vegetative persistence in future studies and to reduce plot-to-plot variation.

Chapter 7
Estimation of additive and non-additive genetic variation for traits associated with vegetative persistence and herbage yield

7.1 Introduction

The estimation of genetic parameters for key traits, associated with vegetative persistence and herbage yield in breeding populations, will enable the assessment of the merits of alternative breeding strategies to improve the rate of genetic gain (Jahufer et al., 2002). Although genetic parameters such as heritability have been estimated for a number of key traits (as discussed in Chapter 6), relatively few estimates are applicable to current random mating populations that have been evaluated across multiple environments. In addition, information on the relative importance of additive and non-additive genetic effects for key traits in white clover are limited (Jahufer et al., 2002).

In forage species, the relative proportion of additive to non-additive genetic variation has been estimated in ryegrass (Breese and Hayward, 1972), tall fescue (Piano et al., 2007), meadow fescue (Simonsen, 1977), lucerne (Riday and Brummer, 2002a; Riday and Brummer, 2002b; Riday et al., 2002) and switchgrass (Bhandari et al., 2010). Further afield, in cropping species such as maize (Hallauer et al., 2010c) more extensive research has been conducted and larger emphasis has been placed on the estimation of quantitative genetic parameters. The availability of genetic parameters for key traits provides the opportunity for the simulation of a range of breeding methods available for forages (Casler and Brummer, 2008). These estimates help determine the most efficient breeding method resulting in increasing the rate of genetic gain per selection cycle or year.

Partitioning of genetic variation into additive and non-additive genetic components is usually conducted using structured mating designs. There are a range of mating designs available for estimating genetic parameters such as; bi-parental mating, parent off-spring regression, diallel cross, North Carolina I, North Carolina II, North Carolina III, and test-crosses (Hallauer et al., 2010b). However, the adoption of these various mating designs is influenced by the biology of the investigated species and the resources available to conduct the experiments.

In white clover, the main mating design of choice has been the diallel cross design and parent offspring regression (Annicchiarico et al., 1999; Caradus and Chapman, 1996; Hill, 1993; Hill and Michaelson-Yeates, 1987; Woodfield and Caradus, 1990). The diallel mating design (Griffing, 1956) is one of the most informative methods but requires a substantial amount of resources and hence is typically only applicable to a small selected set of genotypes. Problematically, these estimates therefore only pertain to the selected set of genotypes and do not accurately represent the populations parameters compared to a larger random sample (Fehr, 1987). While parent offspring regression allows a greater number of individuals to be evaluated, it does not provide information on the magnitude of non-additive variation and can be biased by the differential in age between parents and offspring and genotype × environment interactions (Casler, 1982).

To enable a greater diversity of plants to be evaluated in a population, Jahufer (1998) adopted the use of the North Carolina I mating design to partition additive and non-additive genetic variation in two diverse populations of 40 full-sib families evaluated at a dryland and an irrigated set of trials in NSW, Australia. North Carolina mating designs (Comstock and Robinson, 1948) are an appropriate approach to determine additive and non-additive genetic variances when the genotypic material under study is a population (Sandoya et al., 2009). In New Zealand, no data is available on the magnitude of additive and non-additive variation for white clover vegetative persistence traits in a random mating population evaluated across multiple target environments.

The objectives of the study described in this chapter were to; i) extend the analysis of the experiment reported in Chapter 6 to include the partitioning of genetic variation into both additive and non-additive genetic effects using a North Carolina I mating design, and ii), based on estimates in objective one, to make recommendations for alternative breeding strategies that may better capture the magnitude and type of genetic variation estimated in the investigated random mating population for a range of vegetative morphological traits.

7.2 Materials and methods

Experimental sites, design and measurements were described in Chapter 6. Germplasm and production of genetic families using the North Carolina I mating design was described in Chapter 3.

7.2.1 Statistical analyses

The objective of the data analysis was to estimate the proportion of total genetic variation that is attributed to both additive and non-additive variation within the random mating population, and to estimate the magnitude and significance of the additive/non-additive × environment interaction based on half/full-sib performance across sites, seasons and years. Means and ranges among families are not presented, as the objective for this chapter was to investigate genetic variation at the population level.

7.2.2 Variance component analysis

The Residual Maximum Likelihood (REML) (Harville, 1977; Patterson and Thompson, 1971; Patterson and Thompson, 1975) option in GenStat (GenStat, 2003) was used to estimate variance components and also generate BLUP adjusted means.

All linear models used in the analysis of variance were assumed to be completely random except model 7.2, 7.3, 7.5 and 7.6, where environments and/or seasons were treated as fixed effects. Linear models used in the analyses of variance were adapted from equation 27 and 33 proposed by Nyquist (1991) for individual samples within plots and plot totals of perennial species respectively. These models were adapted to partition variation among "males" and "females" nested within "males" as illustrated by Hallauer, et al. (2010b). Analyses using plot totals were used for herbage yield and persistence traits, whereas all other traits were measured from multiple samples per plot. Traits with data collected on individual plants within each plot allowed error variance to be partitioned into variance due to both random plot effects (plot-to-plot variation) and within-plot variance. Traits with no within-plot sampling resulted in plot effects and plant-within-plot effects being confounded in the residual effect, which is donated as ε in equation 7.4, 7.5 and 7.6 (Holland et al., 2002).

North Carolina I analysis of morphological traits within environments and seasons (sampled plots)

$$P_{ijkl} = \mu + M_i + F_{ij} + R_k + a_{ijk} + \varepsilon_{ijkl} \qquad (7.1)$$

where;

P	is the phenotypic value of the l^{th} plant in the j^{th} "female" mated to the i^{th} "male" within the k^{th} replicate,
μ	is the overall mean,
M_i	is the effect of the full-sib family from "Male" parent j, $N(0,\sigma_m^2)$,
F_j	is the effect of "female" parent j mated to "male" parent I, $N(0,\sigma_f^2)$,
R_k	is the effect of replicate k, $N(0,\sigma_r^2)$,
a_{ijk}	is the effect of "female" parent j mated to "male" parent I within replicate k, $N(0,\sigma^2)$,
ε_{ijkl}	is the residual effect of sample l taken from "female" i, which is mated to "male" parent i within replicate k (within-plot variation), $N(0,\sigma_w^2)$.

North Carolina I analysis of morphological traits within environments but across seasons (sampled plots)

$$P_{ijklm} = \mu + S_i + R_{(i)j} + M_k + F_{kl} + a_{jkl} + W_{(jkl)m} + MS_{ik} + FS_{ikl} + c_{(ijkl)} + d_{(ijklm)} \quad (7.2)$$

where;

μ	is the overall mean,
S_i	is the fixed effect of season *i,*
$R_{(i)j}$	is the effect of replicate *j* within season *i,* N(0,σ_r^2),
M_k	is the effect of the full-sib family from "male" parent *k,* N(0,σ_m^2),
F_{kl}	is the effect of "female" parent *l* mated to "male" parent *k,* N(0,σ_f^2),
$a_{(jkl)}$	is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j,* N(0,σ^2),
$W_{(jkl)m}$	is the effect of sample *m* taken from "female" *l* mated to "male" *k* within replicate *j*, N(0,σ_w^2),
MS_{ik}	is the effect of male parent *k* within season *i,* N(0,σ_{ms}^2),
FS_{ikl}	is the effect of "female" parent *l* mated to "male" parent *k* within season *i,* N(0,σ_{fs}^2),
$c_{(ijkl)}$	is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j* within season *i,* N(0,σ_c^2),
$d_{(ijklm)}$	is the effect of sample *m* taken from "female" parent *l* mated to "male" parent *k* within replicate *j* within season *i,* N(0,σ_d^2).

North Carolina I analysis of morphological traits across environments and seasons (sampled plots)

$$P_{ijklmp} = \mu + L_i + R_{(i)j} + M_k + F_{kl} + ME_{ik} + FE_{ikl} + a_{(ijkl)} + W_{(ijkl)m} + S_p + SE_{ip} + b_{(ijp)} + MS_{kp} + FS_{klp} + MSE_{ikp} + FSE_{iklp} + c_{(ijklp)} + d_{(ijklmp)} \quad (7.3)$$

where;

μ	is the overall mean,
E_i	is the fixed effect of environment *i,*
$R_{(i)j}$	is the effect of replicate *j* within environment *I,* N(0,σ_r^2),
M_k	is the effect of the full-sib family from "male" parent *k,* N(0,σ_m^2),
F_{kl}	is the effect of "female" parent *l* mated to "male" parent *k,* N(0,σ_f^2),
ME_{ik}	is the effect of male parent *k* within environment *i,* N(0,σ_{me}^2),
FE_{ikl}	is the effect of "female" parent *l* mated to "male" parent *k* within environment *i,* N(0,σ_{fe}^2),
$a_{(ijkl)}$	is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j,* within environment *i*, N(0,σ^2),
$W_{(ijkl)m}$	is the effect of sample *m* taken from "female" parent *l* mated to "male" parent *k* within replicate *j,* within environment *i*, N(0, σ_w^2),
S_p	is the fixed effect of season *p,*
SE_{ip}	is the effect of season *p* within environment *i,*
$b_{(ijp)}$	is the effect of replicate *j* within season *p* within environment *i,* N(0,σ_b^2),
MS_{kp}	is the effect of male parent *k* within season *p,* N(0,σ_{ms}^2),
FS_{klp}	is the effect of "female" parent *l* mated to "male" parent *k* within season *p,* N(0,σ_{fs}^2),
MSE_{ikp}	is the effect of male parent *k* within season *p* within environment *i,* N(0,σ_{mse}^2),
FSE_{iklp}	is the effect of "female" parent *l* mated to "male" parent *k* within season *p* within environment *i,* N(0,σ_{fse}^2),
$c_{(ijklp)}$	is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j* within season *p* within environment *i,* N(0,σ_c^2),

$d_{(ijklmp)}$ is the effect of sample *m* taken from "female" parent *l* mated to "male" parent *k* within replicate *j* within season *p* within environment *i,* N(0,σ_d^2).

North Carolina I analysis of morphological traits within environments and seasons (plot totals)

$$P_{ijk} = \mu + M_i + F_{ij} + R_k + \varepsilon_{ijk} \quad (7.4)$$

where;

P is the phenotypic value of the j^{th} *"female" mated to the* i^{th} "male" within the k^{th} replicate,
μ is the overall mean,
M_i is the effect of the full-sib family from "Male" parent *j,* N(0,σ_m^2),
F_j is the effect of "female" parent *j* mated to "male" parent *I,* N(0,σ_f^2),
R_k is the effect of replicate *k,* N(0,σ_r^2),
ε_{ijk} is the residual effect of "female" parent *j* mated to "male" parent *I* within replicate *k* N(0,σ_ε^2).

North Carolina I analysis of morphological traits within environments but across seasons (plot totals)

$$P_{ijkl} = \mu + S_i + R_{(i)j} + M_k + F_{kl} + a_{jkl} + MS_{ik} + FS_{ikl} + \varepsilon_{(ijkl)} \quad (7.5)$$

where;

μ is the overall mean,
S_i is the fixed effect of season *i,*
$R_{(i)j}$ is the effect of replicate *j* within season *i,* N(0,σ_r^2),
M_k is the effect of the full-sib family from "male" parent *k,* N(0,σ_m^2),
F_{kl} is the effect of "female" parent *l* mated to "male" parent *k,* N(0,σ_f^2),
$a_{(jkl)}$ is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j,* N(0,σ^2),
MS_{ik} is the effect of male parent *k* within season *i,* N(0,σ_{ms}^2),
FS_{ikl} is the effect of "female" parent *l* mated to "male" parent *k* within season *i,* N(0,σ_{fs}^2),
$\varepsilon_{(ijkl)}$ is the residual effect of "female" parent *l* mated to "male" parent *k* within replicate *j* within season *i,* N(0,σ_ε^2).

North Carolina I analysis of morphological traits across environments and seasons (plot totals)

$$P_{ijklm} = \mu + L_i + R_{(i)j} + M_k + F_{kl} + ME_{ik} + FE_{ikl} + a_{(ijkl)} + S_m + SE_{im} + b_{(ijm)} + MS_{km} + FS_{klm} + MSE_{ikm} + FSE_{iklm} + \varepsilon_{(ijklm)} \quad (7.6)$$

where;

μ is the overall mean,
E_i is the fixed effect of environment *i,*
$R_{(i)j}$ is the effect of replicate *j* within environment *i,* N(0,σ_r^2),
M_k is the effect of the full-sib family from "male" parent *k,* N(0,σ_m^2),
F_{kl} is the effect of "female" parent *l* mated to "male" parent *k,* N(0,σ_f^2),
ME_{ik} is the effect of male parent *k* within environment *i,* N(0,σ_{me}^2),
FE_{ikl} is the effect of "female" parent *l* mated to "male" parent *k* within environment *i,* N(0,σ_{fe}^2),
$a_{(ijkl)}$ is the effect of "female" parent *l* mated to "male" parent *k* within replicate *j,* within environment *i*, N(0,σ^2),

S_m	is the fixed effect of season *m,*
SE_{im}	is the fixed effect of season *m* within environment *i,*
$b_{(ijm)}$	is the effect of replicate *j* within season *m* within environment *i,* N(0,σ_b^2),
MS_{km}	is the effect of male parent *k* within season *m,* N(0,σ_{ms}^2),
FS_{klm}	is the effect of "female" parent *l* mated to "male" parent *k* within season *m,* N(0,σ_{fs}^2),
MSE_{ikm}	is the effect of male parent *k* within season *m* within environment *i,* N(0,σ_{mse}^2),
FSE_{iklm}	is the effect of "female" parent *l* mated to "male" parent *k* within season *m* within environment *i,* N(0,σ_{fse}^2),
$\varepsilon_{(ijklm)}$	is the residual effect of "female" parent *l* mated to "male" parent *k* within replicate *j* within season *m* within environment *i,* N(0,σ_ε^2).

7.3 Results

7.3.1 Within seasons and environments

The variance components indicated significant (P<0.05) variation among "females" nested within "males" for clover herbage yield in the majority of the seasons at the irrigated site (Table 7.1) but only during the winter season at the dryland site (Table 7.2). Vegetative persistence was significantly different (P<0.05) among "females" nested within "males" at the irrigated site in spring 2013, but not significantly different in the other two seasons at the irrigated site, and all seasons at the dryland site. GPD was significantly different (P<0.05) among "females" nested within "males" in two of the three seasons at each site. Leaf width was significantly different (P<0.05) among "females" for all seasons at the irrigated site and two of the three seasons at the dryland site. Stolon thickness (P<0.05) was significantly different among "females" at both sites in two of the three seasons. Number of nodes was again significantly different (P<0.05) among "females" in two of the three seasons at the irrigated site, but only in one season at the dryland site. The number of rooted nodes was only significantly different (P<0.05) among "females" in autumn 2013 at the irrigated site. Number of stolon branches was significantly different (P<0.05) among "females" in one season at each site.

No significant differences (P>0.05) among "males" were observed for any traits at either experimental site. Standard errors were large in comparison to components of variances among "males" and often among "females" nested within "males" for most traits.

7.3.2 Across seasons but within environments

There was significant (P<0.05) variation among "females" nested within "males" for clover herbage yield, vegetative persistence, growing point density, leaf width and stolon thickness at the irrigated site and for growing point density, leaf width, stolon thickness and number of

stolon branches at the dryland site (Table 7.3). No significant (P>0.05) variation among "males" was identified for any trait at either the irrigated or dryland site.

Significant (P<0.05) "males" × season interactions were observed for herbage yield, vegetative persistence, and growing point density at the irrigated site and for herbage yield at the dryland site. Significant (P<0.05) "females" nested within "males" × season interactions were observed for herbage yield, growing point density, leaf width, number of nodes and number of rooted nodes at the irrigated site and for herbage yield, growing point density and stolon thickness at the dryland site.

Similar to within season analyses, standard errors were large in comparison to components of variances among "males" and often among "females" nested within "males" for most traits.

7.3.3 Across seasons and environments

Across both seasons and environments, variance component analyses indicated significant (P<0.05) variation among "females" nested within "males" for leaf width, stolon thickness, number of nodes and stolon branching (Table 7.4). Significant (P<0.05) "females" nested within "males" × season interactions were observed for herbage yield, leaf width and stolon thickness. Significant (P<0.05) "males" × season × environment interactions were observed for herbage yield, persistence and number of rooted nodes. A significant (P<0.05) "females" nested within "males" × season × environment interaction was observed for herbage yield.

Crude variance component estimates from across both seasons and environments showed among "males" variation accounted for a large proportion of among "females" nested within "males" variation (Figure 7.1) for most traits. The subtraction of the variation among "males" from the variation among "females" nested within "males" is indicative of the remaining genetic variation attributed to dominance gene action. Figure 7.1 illustrates that additive genetic variation is the primary source of variation for vegetative persistence, growing point density, leaf width, stolon thickness, number of rooted nodes and number of stolon branches. Dominance genetic variation appeared equal in proportion to additive variation for the number of nodes and even higher than additive variation for herbage yield, although due to the high standard errors these findings are inconclusive. The addition of both variation among "males" and variation among "females" nested within "males" accounted for the total variation observed among full-sib families presented in Chapter 6 (Table 6.3).

Table 7.1 North Carolina I analysis within seasons and sites. Variance components are presented along with their associated standard errors (± SE) for the 40 white clover full-sib families evaluated at the irrigated trial site. Variance components: σ_m^2 – males, $\sigma_{f/m}^2$ – females nested within males, σ_r^2 – replicate, σ_w^2 – within-plot and σ_a^2 – plot-to-plot.

Trait	Season	σ_m^2	$\sigma_{f/m}^2$	σ_r^2	σ_w^2	σ_a^2
HY (kg/ha × 10^{-4})	**Spring 2012**	0.51 ± 0.32	0.19 ± 0.17	0.07 ± 0.11	-	1.28 ± 0.21
	Summer 2012/13	3.86 ± 4.00	9.50 ± 4.59	3.36 ± 3.93	-	22.89 ± 3.67
	Autumn 2013	1.73 ± 1.29	2.24 ± 1.03	0.83 ± 0.95	-	4.82 ± 0.77
	Winter 2013	0.17 ± 0.11	0.14 ± 0.08	0.03 ± 0.04	-	0.43 ± 0.07
	Spring 2013	2.44 ± 3.56	14.87 ± 4.85	1.80 ± 2.09	-	11.35 ± 1.82
	Summer 2013/14	2.99 ± 2.46	6.04 ± 2.21	1.38 ± 1.56	-	7.19 ± 1.15
	Autumn 2014	2.47 ± 1.61	2.12 ± 0.97	1.34 ± 1.45	-	4.54 ± 0.73
Persistence (% × 10^{-1})	**Autumn 2013**	2.64 ± 2.16	3.32 ± 1.99	0.34 ± 0.65	-	11.99 ± 1.92
	Spring 2013	6.42 ± 4.70	9.10 ± 3.56	0.06 ± 0.40	-	13.27 ± 2.13
	Autumn 2014	21.43 ± 13.72	13.51 ± 8.05	1.45 ± 2.66	-	48.11 ± 7.70
GPD (m^{-2} × 10^{-4})	**Autumn 2013**	3.68 ± 2.90	3.61 ± 2.59	0.00 ± 0.43	11.23 ± 1.45	11.7 ± 2.87
	Spring 2013	5.46 ± 3.77	5.74 ± 2.58	0.54 ± 0.84	18.54 ± 2.39	2.58 ± 2.24
	Autumn 2014	18.25 ± 12.58	22.37 ± 8.49	0.77 ± 1.51	24.33 ± 3.14	17.62 ± 5.02
LW (mm^{-1})	**Spring 2012**	0.48 ± 0.50	1.34 ± 0.56	0.31 ± 0.37	5.93 ± 0.38	1.18 ± 0.39
	Autumn 2013	1.06 ± 0.68	1.04 ± 0.39	0.20 ± 0.23	5.18 ± 0.24	0.64 ± 0.20
	Spring 2013	0.40 ± 0.52	1.99 ± 0.67	1.01 ± 1.05	6.95 ± 0.30	0.98 ± 0.27
ST (mm × 10^2)	**Spring 2012**	1.12 ± 0.62	0.13 ± 0.22	0.21 ± 0.26	5.86 ± 0.38	0.72 ± 0.31
	Autumn 2013	0.72 ± 0.42	0.45 ± 0.18	0.21 ± 0.23	2.57 ± 0.12	0.32 ± 0.10
	Spring 2013	0.80 ± 0.51	0.8 ± 0.29	0.04 ± 0.06	4.72 ± 0.21	0.45 ± 0.15
NN (no. m^{-1} × 10^{-2})	**Spring 2012**	0.01 ± 0.03	0.08 ± 0.04	0.01 ± 0.01	1.02 ± 0.07	0.04 ± 0.04
	Autumn 2013	1.29 ± 0.85	1.10 ± 0.52	0.04 ± 0.11	14.22 ± 0.66	0.84 ± 0.41
	Spring 2013	0.18 ± 0.21	0.42 ± 0.26	0.23 ± 0.27	9.97 ± 0.44	0.56 ± 0.26
RN (no. m^{-1} × 10^{-1})	**Spring 2012**	1.00 ± 0.74	0.65 ± 0.59	0.44 ± 0.55	12.05 ± 0.78	1.99 ± 0.72
	Autumn 2013	9.40 ± 6.60	9.60 ± 4.80	1.30 ± 1.90	137.9 ± 6.40	7.90 ± 3.90
	Spring 2013	0.28 ± 0.48	0.71 ± 0.75	0.15 ± 0.30	29.58 ± 1.29	2.74 ± 0.93
BR (no. m^{-1} × 10^{-1})	**Spring 2012**	0.11 ± 0.11	0.06 ± 0.14	0.00 ± 0.00	4.55 ± 0.29	0.30 ± 0.20
	Autumn 2013	3.64 ± 2.66	1.11 ± 2.20	0.00 ± 0.00	97.98 ± 4.58	7.39 ± 3.02
	Spring 2013	1.11 ± 0.95	2.22 ± 0.90	0.29 ± 0.38	33.79 ± 1.47	0.16 ± 0.60

Table 7.2 North Carolina I analysis within seasons and sites. Variance components are presented along with their associated standard errors (± SE) for the 40 white clover full-sib families at the dryland trial site. Variance components: σ_m^2 – males, $\sigma_{f/m}^2$ – females nested within males, σ_r^2 – replicate, σ_w^2 – within-plot and σ_a^2 – plot-to-plot.

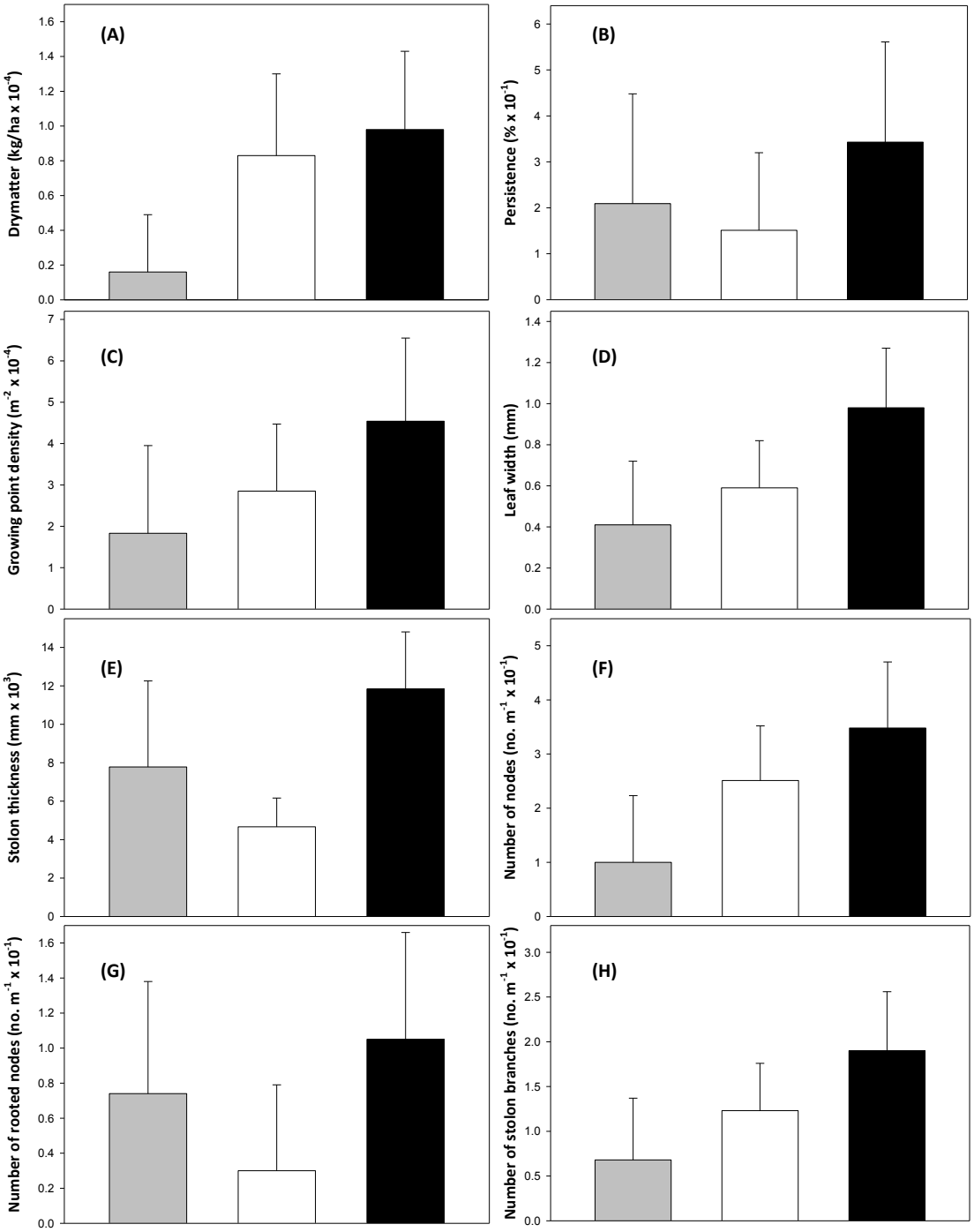
Trait	Season	σ_m^2	$\sigma_{f/m}^2$	σ_r^2	σ_w^2	σ_a^2
HY (kg/ha × 10^{-4})	**Spring 2012**	0.58 ± 0.37	0.27 ± 0.22	0.69 ± 0.73	-	1.53 ± 0.25
	Summer 2012/13	0.48 ± 1.20	3.48 ± 2.02	1.41 ± 1.70	-	11.8 ± 1.89
	Autumn 2013	0.86 ± 0.86	1.69 ± 0.98	0.49 ± 0.63	-	5.74 ± 0.92
	Winter 2013	0.49 ± 0.32	0.48 ± 0.21	0.12 ± 0.14	-	0.88 ± 0.14
	Spring 2013	1.77 ± 1.13	1.03 ± 0.66	0.29 ± 0.40	-	4.17 ± 0.67
	Summer 2013/14	0.19 ± 0.12	0.10 ± 0.07	0.02 ± 0.03	-	0.44 ± 0.07
	Autumn 2014	0.15 ± 0.11	0.08 ± 0.08	0.07 ± 0.09	-	0.62 ± 0.10
Persistence (% × 10^{-1})	**Autumn 2013**	0.51 ± 0.77	2.10 ± 1.10	0.37 ± 0.52	-	5.92 ± 0.95
	Spring 2013	8.68 ± 5.81	2.51 ± 4.04	3.93 ± 4.79	-	34.29 ± 5.49
	Autumn 2014	7.51 ± 5.44	2.74 ± 4.44	3.66 ± 4.60	-	37.78 ± 6.05
GPD (m^{-2} × 10^{-4})	**Autumn 2013**	2.06 ± 3.36	11.49 ± 4.89	3.02 ± 3.54	10.98 ± 1.42	15.35 ± 3.41
	Spring 2013	2.92 ± 2.24	4.13 ± 1.84	0.98 ± 1.19	10.48 ± 1.35	3.11 ± 1.50
	Autumn 2014	1.54 ± 0.93	0.00 ± 0.50	0.18 ± 0.31	4.92 ± 0.63	2.55 ± 0.86
LW (mm^{-1})	**Spring 2012**	0.00 ± 0.16	0.87 ± 0.36	0.34 ± 0.38	4.38 ± 0.28	0.57 ± 0.24
	Autumn 2013	0.76 ± 0.42	0.17 ± 0.14	0.04 ± 0.06	3.60 ± 0.16	0.64 ± 0.16
	Spring 2013	0.27 ± 0.26	0.66 ± 0.27	0.33 ± 0.36	4.55 ± 0.20	0.57 ± 0.17
ST (mm × 10^2)	**Spring 2012**	0.81 ± 0.55	0.90 ± 0.35	0.04 ± 0.08	5.22 ± 0.34	0.29 ± 0.23
	Autumn 2013	0.81 ± 0.47	0.33 ± 0.20	0.06 ± 0.09	3.38 ± 0.15	0.90 ± 0.20
	Spring 2013	0.88 ± 0.56	0.84 ± 0.30	0.00 ± 0.02	3.26 ± 0.15	0.59 ± 0.15
NN (no. m^{-1} × 10^{-2})	**Spring 2012**	0.03 ± 0.03	0.07 ± 0.05	0.00 ± 0.00	1.13 ± 0.07	0.05 ± 0.05
	Autumn 2013	0.06 ± 0.21	0.80 ± 0.36	0.15 ± 0.19	9.93 ± 0.44	0.58 ± 0.27
	Spring 2013	0.18 ± 0.21	0.29 ± 0.28	0.28 ± 0.33	9.54 ± 0.43	1.12 ± 0.35
RN (no. m^{-1} × 10^{-1})	**Spring 2012**	0.16 ± 0.16	0.00 ± 0.22	0.37 ± 0.43	10.19 ± 0.66	0.48 ± 0.43
	Autumn 2013	1.29 ± 1.33	1.15 ± 1.63	0.41 ± 0.75	63.86 ± 2.80	6.95 ± 2.21
	Spring 2013	2.21 ± 1.42	0.00 ± 1.10	0.73 ± 1.08	55.97 ± 2.50	8.09 ± 2.29
BR (no. m^{-1} × 10^{-1})	**Spring 2012**	0.10 ± 0.13	0.21 ± 0.18	0.28 ± 0.32	5.43 ± 0.35	0.21 ± 0.22
	Autumn 2013	0.86 ± 1.62	5.46 ± 2.49	0.71 ± 1.00	78.45 ± 3.43	3.49 ± 1.89
	Spring 2013	1.60 ± 1.13	0.87 ± 0.87	1.59 ± 1.75	38.84 ± 1.74	2.44 ± 1.09

Table 7.3 **North Carolina I analysis across seasons but within environments. Morphological trait variance components and their associated standard errors (± SE) for the 40 white clover full-sib families at both the dryland and irrigated trial sites. Variance components: σ^2_m – males, $\sigma^2_{f/m}$– females nested within males, σ^2_r – replicate, σ^2_a – plot-to-plot, σ^2_w – within-plot, σ^2_{ms} – males × season, $\sigma^2_{f/ms}$ – females nested with males × season, σ^2_c – season × plot-to-plot and σ^2_d – season × within plot.**

Environment	Source	HY (kg/ha × 10^{-4})	Persistence (% × 10^{-1})	GPD (m^{-2} × 10^{-4})	LW (mm^{-1})	ST (mm^{-1} × 10^2)	NN (no. m^{-1} × 10^{-2})	RN (no. m^{-1} × 10^{-2})	BR (no. m^{-1} × 10^{-2})
		Test of fixed effects (*P* values)							
Irrigated site	**Season**	*P* <0.001	*P* <0.05	*P* = 0.059	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
		Variance components (± SE)							
	σ^2_m	0.00 ± 0.58	0.13 ± 3.38	3.07 ± 3.88	0.46 ± 0.43	0.78 ± 0.46	0.16 ± 0.20	0.07 ± 0.13	0.09 ± 0.08
	$\sigma^2_{f/m}$	2.60 ± 1.00	6.96 ± 3.14	6.81 ± 2.94	0.96 ± 0.38	0.41 ± 0.17	0.06 ± 0.14	0.07 ± 0.10	0.06 ± 0.06
	σ^2_r	0.91 ± 0.44	0.57 ± 0.65	0.31 ± 0.47	0.49 ± 0.31	0.13 ± 0.09	0.10 ± 0.08	0.06 ± 0.05	0.00 ± 0.01
	σ^2_a	1.82 ± 0.43	6.63 ± 2.12	3.16 ± 1.62	0.42 ± 0.15	0.27 ± 0.09	0.20 ± 0.14	0.11 ± 0.09	0.10 ± 0.07
	σ^2_w	-	-	0.51 ± 0.98	0.00 ± 0.14	0.26 ± 0.10	0.34 ± 0.24	0.00 ± 0.15	0.00 ± 0.12
	σ^2_{ms}	2.20 ± 0.64	10.04 ± 4.00	6.06 ± 2.82	0.19 ± 0.15	0.09 ± 0.06	0.33 ± 0.20	0.28 ± 0.16	0.07 ± 0.06
	$\sigma^2_{f/ms}$	2.38 ± 0.47	1.67 ± 1.55	3.74 ± 1.78	0.53 ± 0.18	0.11 ± 0.07	0.46 ± 0.19	0.30 ± 0.13	0.06 ± 0.07
	σ^2_c	5.77 ± 0.38	17.86 ± 2.02	7.54 ± 2.01	0.48 ± 0.15	0.19 ± 0.09	0.29 ± 0.17	0.30 ± 0.14	0.17 ± 0.10
	σ^2_d	-	-	17.53 ± 1.60	6.09 ± 0.23	3.91 ± 0.14	9.35 ± 0.35	6.81 ± 0.25	5.37 ± 0.20
		Test of fixed effects (*P* values)							
Dryland site	**Season**	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
		Variance components (± SE)							
	σ^2_m	0.18 ± 0.24	4.80 ± 3.12	1.80 ± 1.59	0.33 ± 0.24	0.84 ± 0.47	0.08 ± 0.09	0.01 ± 0.05	0.07 ± 0.08
	$\sigma^2_{f/m}$	0.42 ± 0.25	0.62 ± 1.78	2.95 ± 1.44	0.41 ± 0.17	0.39 ± 0.17	0.09 ± 0.12	0.05 ± 0.05	0.19 ± 0.08
	σ^2_r	0.44 ± 0.20	2.69 ± 1.91	1.39 ± 0.96	0.22 ± 0.15	0.03 ± 0.04	0.15 ± 0.11	0.05 ± 0.04	0.09 ± 0.06
	σ^2_a	0.90 ± 0.21	8.02 ± 2.34	1.68 ± 0.89	0.23 ± 0.09	0.08 ± 0.08	0.00 ± 0.11	0.00 ± 0.08	0.00 ± 0.06
	σ^2_w	-	-	0.00 ± 0.46	0.03 ± 0.09	0.01 ± 0.08	0.71 ± 0.20	0.00 ± 0.12	0.10 ± 0.12
	σ^2_{ms}	0.47 ± 0.17	0.77 ± 0.98	0.37 ± 0.63	0.03 ± 0.05	0.00 ± 0.06	0.01 ± 0.09	0.12 ± 0.07	0.02 ± 0.04
	$\sigma^2_{f/ms}$	0.60 ± 0.17	1.83 ± 1.58	2.24 ± 1.07	0.12 ± 0.09	0.27 ± 0.12	0.31 ± 0.17	0.00 ± 0.08	0.04 ± 0.07
	σ^2_c	2.70 ± 0.18	17.97 ± 2.03	5.33 ± 1.18	0.37 ± 0.10	0.61 ± 0.12	0.68 ± 0.18	0.61 ± 0.14	0.25 ± 0.10
	σ^2_d	-	-	8.92 ± 0.81	4.10 ± 0.15	3.66 ± 0.13	7.31 ± 0.27	5.04 ± 0.19	4.72 ± 0.17

Table 7.4 North Carolina I analysis across both seasons and environments. Morphological trait variance components and their associated standard errors (± SE) for the white clover half/full-sib families. Variance components: σ_m^2 – males, $\sigma_{f/m}^2$– females nested within males, σ_r^2 – replicate, σ_a^2 – plot-to-plot, σ_w^2 – within-plot, σ_{ms}^2 – males × season, $\sigma_{f/ms}^2$ – females nested with males × season, σ_{me}^2 – males × environment, $\sigma_{f/me}^2$ – females nested with males x environment, σ_{fse}^2 – males × season x environment, $\sigma_{f/mse}^2$ – females nested with males x season × environment, σ_b^2 – environment × season × replicate, σ_c^2 – environment × season × plot-to-plot and σ_d^2 – environment × season × within plot.

Source	HY (kg/ha × 10^{-4})	Persistence (% × 10^{-1})	GPD (m^{-2} × 10^{-4})	LW (mm^{-1})	ST (mm^{-1} × 10^{3})	NN (no. m^{-1} × 10^{-1})	RN (no. m^{-1} × 10^{-1})	BR (no. m^{-1} × 10^{-1})
	Test of fixed effects (*P* values)							
Environment	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.01	*P* = 0.291	*P* <0.001	*P* = 0.682
Season	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001
Environment ×	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* <0.001	*P* = 0.823
	Variance components (± SE)							
σ_m^2	0.16 ± 0.33	2.09 ± 2.39	1.83 ± 2.12	0.41 ± 0.31	7.78 ± 4.48	1.00 ± 1.23	0.74 ± 0.64	0.68 ± 0.69
$\sigma_{f/m}^2$	0.83 ± 0.47	1.51 ± 1.69	2.85 ± 1.62	0.59 ± 0.23	4.67 ± 1.49	2.51 ± 1.01	0.30 ± 0.49	1.23 ± 0.53
σ_r^2	0.47 ± 0.41	0.00 ± 0.70	0.18 ± 0.46	0.19 ± 0.19	0.46 ± 0.53	0.00 ± 0.38	0.00 ± 0.22	0.00 ± 0.15
σ_a^2	1.31 ± 0.22	7.31 ± 1.58	2.35 ± 0.88	0.32 ± 0.08	1.76 ± 0.62	0.64 ± 0.88	0.20 ± 0.60	0.35 ± 0.44
σ_w^2	-	-	0.19 ± 0.51	0.02 ± 0.08	1.29 ± 0.63	5.29 ± 1.56	0.00 ± 0.96	0.00 ± 0.83
σ_{ms}^2	0.50 ± 0.30	0.00 ± 1.75	0.00 ± 1.21	0.06 ± 0.08	0.18 ± 0.43	1.26 ± 1.03	0.00 ± 0.82	0.85 ± 0.37
$\sigma_{f/ms}^2$	0.84 ± 0.23	1.62 ± 1.02	0.56 ± 0.95	0.27 ± 0.10	1.31 ± 0.66	0.90 ± 1.17	0.32 ± 0.66	0.00 ± 0.44
σ_{me}^2	0.00 ± 0.18	0.37 ± 2.23	0.60 ± 1.54	0.00 ± 0.05	0.33 ± 0.35	0.13 ± 0.44	0.00 ± 0.73	0.10 ± 0.17
$\sigma_{f/me}^2$	0.70 ± 0.38	2.29 ± 1.86	2.05 ± 1.42	0.10 ± 0.10	0.00 ± 0.42	0.00 ± 0.69	0.00 ± 0.62	0.00 ± 0.43
σ_{mse}^2	0.83 ± 0.26	6.51 ± 2.69	3.24 ± 1.67	0.04 ± 0.06	0.18 ± 0.40	0.39 ± 0.90	3.04 ± 1.43	0.00 ± 0.21
$\sigma_{f/mse}^2$	0.65 ± 0.23	0.13 ± 1.21	2.42 ± 1.29	0.07 ± 0.08	0.54 ± 0.70	2.99 ± 1.53	0.74 ± 0.94	0.69 ± 0.68
σ_b^2	0.38 ± 0.14	1.67 ± 1.06	0.73 ± 0.53	0.18 ± 0.11	0.42 ± 0.33	1.87 ± 1.12	0.64 ± 0.47	0.62 ± 0.41
σ_c^2	4.24 ± 0.20	17.92 ± 1.43	6.47 ± 1.13	0.43 ± 0.09	4.16 ± 0.75	4.88 ± 1.26	4.61 ± 0.98	2.06 ± 0.68
σ_d^2	-	-	13.22 ± 0.85	5.07 ± 0.13	37.8 ± 0.97	83.16 ± 2.18	59.23 ± 1.54	50.51 ± 1.31



Variation among "males" (■), among "females" nested within "males" (□) and total genetic variation (■)

Figure 7.1 Partitioning of genetic variation among white clover full-sib families (■) [½ additive genetic variation and ¼ dominance genetic variation] for (A) herbage yield, (B) vegetative persistence, (C) growing point density, (D) leaf width, (E) stolon thickness, (F) number of nodes, (G) number of rooted nodes and (H) number of stolon branches into variation among males (■) [¼ additive genetic variation] and variation among females nested within males (□) [¼ additive genetic variation and ¼ dominance genetic variation] across both seasons and environments.

7.4 Discussion

The standard errors of the variance components for the majority of the traits among "females" nested within "males" and especially among "males" were consistent among analyses conducted within, and among seasons and environments. The large magnitude of this variance across all the analyses of variances adversely affected the accuracy of partitioning genetic variation into its various forms of gene action. The lack of accurate estimates for the additive variance components did not enable reliable estimation of narrow sense heritabilities.

Similarly, Jahufer (1998) reported a large number of negative or small positive genetic components of variances along with high standard errors among white clover stolon attributes when using the North Carolina I mating design. Jahufer (1998) concluded that possible explanations for the inaccuracies in their experiment included; (i) a lack of full-sib families evaluated and hence poor representation of the normal distribution of the population's genetic diversity, and (ii) large sampling error which reduced the power of the experiment to detect additive and dominance genetic components of variance.

Likewise, the descriptions of possible explanations provided by Jahufer (1998) seem fitting in the current study. As with Jahufer (1998), the ratios of variation among "males" and among "females" nested within "males" tend to suggest substantial problems with the North Carolina I analysis for these limited family experiments. Although North Carolina I matings design have been extensively and successfully used in the estimation of genetic components of variances in maize (e.g. Hallauer et al., 2010), the number of "male" half-sib groups and "females" nested within males (full-sib families) typically in maize far exceed the numbers used in the present study. The number of families evaluated is particularly important to adequately sample the base population.

Studies by Sandoya et al. (2009) and Duque-vargas et al. (1994) are two of many successful examples of the North Carolina I analysis in the maize literature. In both cases, the number of full-sib families evaluated exceeded 150. A problem often encountered when large numbers of families are evaluated in the North Carolina I design (which is likely to be further exacerbated in forage species) is the increased experimental error due to an increase in replicate size, which can introduce more heterogeneous variation e.g. soil variation. This can be largely mitigated by using a technique of grouping progenies into sets by "males" which was proposed by Comstock and Robinson (1948) to reduce replication size and hence increase the precision of the experiment. Essentially this system stratifies replication within sets, or sets (as sub-blocks) within replications (Hallauer et al., 2010b). While stratification of the trial

design helps to reduce experimental error, there is no silver bullet for the increased cost associated with evaluating more families. The latter is a far more influential factor in determining the effectiveness of mating designs in forage species such as white clover. This factor alone is likely the chief reason for relatively few assessments of population genetic parameters in forage species.

Although the considerable magnitude of standard errors prevented any definitive ratios of additive to non-additive genetic variation among the various traits, Figure 7.1 depicts an interesting trend among white clover attributes. Unlike the other morphological traits (excluding the number of nodes), the variation among "males" for herbage yield seemed to only account for a small component of the among "females" nested within "males" variance component. The remaining variation among "females" nested within "males" therefore indicates the possible presence of a significant proportion of non-additive variation. This finding, while purely speculative due to the high associated standard error certainly deserves some further attention.

Michaelson-Yeates (1997) presented similar findings to the current study where significant positive heterosis was observed for clover herbage yield in a spaced-planted glasshouse trial, therefore indicating a significant proportion of non-additive variation. Similarly, they also observed minor non-additive genetic variation for other morphological traits. Hill and Michaelson-Yeates (1987) also reported highly significant additive and non-additive variation for herbage yield, and significant non-additive variation also extended to stolon number, leaf number and canopy height. Interestingly, it was also reported that non-additive genetic effects are more apparent in mixed species swards than in monocultures, indicating that the development of breeding material for varieties grown in mixed species swards should therefore be conducted under realistic competitive conditions (Hill, 1993; Hill and Michaelson-Yeates, 1987).

In other forage species, the understanding of the proportion of additive to non-additive genetic variation is also limited, but by in large, it seems most traits are influenced primarily by additive genetic variation (Breese and Hayward, 1972). However, from a genetic gain perspective, understanding the magnitude of non-additive variation for various traits, and principally herbage yield, is of prime importance for forage species given the historic slow rates of genetic gains. The inability to exploit heterosis in commercial cultivars in forage species has been one of the major contributors to the lag in yield gain relative to grain crops (Casler and Brummer, 2008).

In crop species such as maize, the relative contribution of additive to non-additive variation differs considerably among traits and populations. Non-additive variation has a significant influence on components of grain yield (Hallauer et al., 2010c). Assuming no epistasis and linkage effects, additive and non-additive genetic variances on average account for 61.2% and 38.8% of the total genetic variation for grain yield in maize, respectively (Hallauer et al., 2010c). In forage maize, similar to pasture species where the harvest index is the total above ground herbage mass, the ratio of non-additive to additive variation although significant, seems to be considerably less than its grain components (Bertoia and Aulicino, 2014). The adoption of hybrid cultivar systems to exploit non-additive variation in maize, can largely explain the exceptional rate of genetic gain observed.

If non-additive variation is indeed shown to significantly influence herbage yield in white clover, the use of both breeding strategies that exploit non-additive variation in cross-pollinating species and the release of non-additive exploiting cultivars may be commercially beneficial. While the use of conventional synthetics capture an appreciable amount of heterosis (Allard, 1960), the use of hybrids which capture a higher proportion of specific combining ability as well as good general combining ability are likely to be an improved option, such as 50% population hybrids (Barrett et al., 2010), 75% hybrids (Riday and Krohn, 2010)) and F_1 hybrids (Michaelson-Yeates et al., 1997).

7.5 Conclusions

- The results of this experiment coupled with the results presented by Jahufer (1998) demonstrate substantial problems associated with the North Carolina I mating design when using a limited number of full-sib families.

- The trend in this experiment that clover herbage yield is influenced by a significant proportion of non-additive variation is consistent with prior studies and warrants further investigation.

- The results of this experiment highlight the difficulties of obtaining genetic parameters for perennial pasture species. These difficulties are further exacerbated in species such as white clover that are grown in mixed swards and defoliated using grazing livestock.

- Future studies should investigate the use of alternative mating designs such as the diallel cross to investigate genetic parameters instead of the North Carolina I mating design, unless an ample amount of full-sib families are evaluated to ensure families are representative of the populations' normal distribution of genetic variation.

- Consistent with Chapter 6, investigations into alternative plot sizes and sampling frequencies per family would be beneficial in future studies to improve estimates of genetic components of variances and to reduce the associated standard error.

Chapter 8
General conclusions

8.1 Results and their application to plant breeders

A key objective of this thesis was to investigate, using both empirical and theoretical data, several breeding strategies applicable to white clover that may enhance the rate of genetic gain relative to current practised breeding methods. It is clear from the literature that the predominant breeding method in white clover is phenotypic recurrent selection (Williams, 1987; Woodfield and Caradus, 1994), although in some situations, progeny trialling of some description is also utilised (Woodfield et al., 2003). The evaluation of genetic families, representing a breeding population, followed by field protocols used by breeders, resulted in a number of key findings that will have significant implications to current white clover breeding strategies.

8.1.1 Bi-parental and polycross pollination

A fundamental aspect of all breeding programmes is the generation of advanced populations through the recombination of elite genotypes. Considering this, the lack of information available on the actual details of pollination within isolation cages for white clover therefore seemed somewhat surprising. A key finding from this thesis was the poor dispersal of paternal pollen at increasing distances from maternal recipients within isolation cages (Chapter 3). Similar to lucerne (Riday et al., 2013), and previous reports in white clover (Michaelson-Yeates et al., 1997), this leptokurtic distribution of pollen in white clover isolation cages illustrates the importance of clonal replication or other methods of increasing the likelihood of random mating. Results from this thesis suggest clonal replication, in conjunction with higher seed yields and increased inflorescences per plant are likely to alleviate random mating issues in white clover isolations.

From a downstream perspective and in particular for half-sib progeny trialling, it is imperative that random mating occurs to ensure a similar array of tester gametes are distributed among all maternal parents, so that variation in progeny performance among the tested parents is due primarily to the genetic potential of the parents and not the genetic contribution of the pollen source (Fehr, 1987). However, considering the workload and number of concurrent breeding pools in many commercial breeding programmes, the practicality of clonal replication in all polycrosses seems unsustainable. It is therefore perhaps best practice for breeders to use

topcrosses to generate half-sib families for progeny trialling. This can be achieved in an isolation block with male rows using seed from the base population or from the previous cycle as the pollen tester (Posselt, 2010). At least with this approach, the tester will provide an excess of pollen, which will predominate the pollen source (Posselt, 2010) and eliminate any differences in testers between evaluated half-sib progeny.

The departure from random mating also has profound effects on genetic studies. The translation of variance components to covariance's among relatives relies on the assumption of random mating, and hence a breach in this assumption makes estimated genetic parameters invalid. This is particularly important for future studies where additive genetic variance components are based on data collected from half-sib families generated using polycross mating designs.

The lack of self-fertilisation in both the polycross and bi-parental progeny should provide confidence to breeders that relatively little inbreeding occurs within white clover isolation cages and is contrary to the findings in lucerne (Riday et al., 2013). Further confidence is gained by the nil detection of foreign pollen sources when breeders opt to capture and wash wild bumble bees for pollination.

8.1.2 Adoption of novel breeding methods

The objective of plant breeding is to genetically improve the performance of a species, which in this situation is white clover, in the most efficient manner possible (Fehr, 1987). Development of an efficient strategy hinges on the selection of an appropriate breeding method coupled with the thoughtful allocation of resources for population development and genotype selection (Fehr, 1987). Adoption of the most efficient method therefore requires prior information on the amount of genetic improvement a range of alternative methods can achieve, within a given resource allocation.

The ideal scenario to compare alternative breeding methods would be to empirically measure realised genetic gain in terms of the mean performance of a population among a range of different methods. Unfortunately the empirical comparisons of different breeding methods is time consuming, laborious and in most cases far beyond practicality for most species. An obvious exception is maize, where alternative breeding methods have been compared empirically (Weyhrich et al., 1998). A common way for breeders to access the merits of alternative breeding methods is to compute the amount of genetic gain using prediction equations (Fehr, 1987). Genetic gain is often presented on a per year basis to account for the

variation in breeding method duration. Computation using prediction equations is based on genetic parameters estimated from genetic families representing random mating breeding populations. Similarly, the data generated from quantitative genetic experiments described in this thesis were able to be used to interpret the rationale for pursing various breeding methods in white clover.

In many situations, data from the experiments conducted in this thesis may help breeders to optimise or compliment current breeding strategies as opposed to replacing them. In terms of complementation to current breeding strategies, one way for breeders to employ findings from this thesis, is to include paternity testing into breeding methods as discussed below.

While the incorporation of paternity testing into breeding programmes such as lucerne (Riday et al., 2013) and red clover (Riday, 2011) requires little adjustment in breeding strategy since spaced-planted nurseries are already the norm (Riday, 2011), the progressive movement in white clover selection from mono-culture spaced-plants to duo-culture mini-plots (Woodfield and Caradus, 1994) makes the transition to paternity testing methods more problematic. For this reason, and the difficulty of maintaining individuality as discussed in Chapter 5, the use of paternity testing in white clover should be modified accordingly, to effectively transition into field breeding programmes. One such approach would be to use paternity testing to complement current plot systems as opposed to spaced-planted nurseries. While data can therefore not be collected on individual genotypes to determine paternal half-sib variances *per se,* since the best maternal families are also the best paternal families as shown in Chapter 5, there is no need to do so (assuming all paternal families are evaluated as maternal families). Consequently, best maternal families would be identified, and then paternity testing would be used to help identify plants within plots that also have superior paternal genetics. The combination of maternal and paternal selection in plots is similar to that demonstrated in spaced-planted nurseries, although selection within superior full-sib combinations based on phenotypic data is not available. No advantages are gained from paternal selection alone compared to maternal selection alone, since maternal and paternal genetic additive variances are similar (Chapter 5); contrary to the findings of Riday in red clover (2011).

Marker assisted selection (MAS) for within plot selection is highly beneficial in white clover, as a common problem associated with perennial forage species is the lack of plant identity within plots, making within-family selection relatively difficult. In many cases, breeders mitigate this difficultly of within plot selection by exposing trials to a greater range of abiotic and biotic factors to promote reductions in plant density. However this technique is only

useful if survivorship within families is low and heritability of vegetative persistence on a single plant basis is relatively high. At least paternity testing within plots or even remnant seed improves selection in this scenario by doubling the parental control factor based on replicated family data. The addition of low survivorship is also beneficial in these circumstances, as less potential genotypes need to be screened with molecular markers for paternal identity. Traits with high heritability on an individual plant basis, such as leaf size and disease traits, may also be used as a preliminary criterion to put plants forward for paternity screening if plant density remains high.

Purely from a quality control perspective, DNA testing within plots enables the removal of any containment white clover volunteers as well, which despite the best practices of breeders, still act as contaminants via the high buried seed counts of most soils in New Zealand (Clifford et al., 1990) and animal faeces (Suckling, 1950; Suckling, 1952).

As opposed to complementation of current breeding strategies, a second aspect of this thesis was to investigate alternative breeding strategies *per se*. This approach requires estimation of genetic parameters and simulation using prediction gain models, taking into account the magnitude and type of genetic variation (additive and non-additive variation). To date, the lack of literature available on the genetic parameters of random mating white clover populations evaluated in multiple target environments has limited the ability to simulate prediction gain models with realistic data (Jahufer et al., 2002). The heritabilities estimated in the experiments of this thesis provide breeders with improved understanding of heritabilities in actual farming systems and consequently allow breeders to critically evaluate their breeding methodology more realistically, if these target environments are used to undertake selection.

Data generated from both the spaced-planted nurseries (Chapter 5) and the mini-plot trials (Chapter 6) clearly demonstrate the superiority of family selection methods for low heritable traits. By in large these low heritable traits include clover herbage yield, vegetative persistence, number of nodes, number of rooted nodes and stolon branching. Traits where phenotypic recurrent selection methods are likely to be equally effective as or better than family selection methods include; leaf size, stolon thickness and growing point density. The benefit of family selection methods are well documented for low heritable traits as their data are obtained from replicated trials and therefore less affected by large environmental variances (Nguyen and Sleper, 1983), which is demonstrated by the differentials in

heritabilities estimated on an individual plant basis and those on a family means basis in Chapter 5 and 6.

Among the family selection methods simulated, among-and-within-half-sib family selection (AWF-HS) according to the data generated in Chapter 5 (Figure 5.5) seems to be most the most logical choice for plant breeders to utilise if molecular markers are not available. These data are also supported by findings presented by Casler & Brummer (2008). AWF-HS selection allows breeders to accurately estimate the breeding worth of half-sib families for low heritable traits, as well as allowing for additional within family selection. The within family selection component would ideally suit traits of high heritability such as leaf size, growing point density, stolon thickness and simply inherited traits such as disease. The combination of replicated family selection for yield and vegetative persistence and within family selection (individual plant selection) for disease, leaf size and growing point density would fit commercial breeding programmes objectives well. The additional integration of molecular markers for paternity testing, such as among-half-sib-and-within molecular determined full-sib family selection (AWF-HS+MFS), would further elevate genetic gain above traditional AWF-HS, and allow more accurate within family selection for low heritable traits (maternal and paternal selection) such as herbage yield, in addition to the high heritable traits as well.

Crude estimates of additive and non-additive variances for most white clover attributes tend to agree with breeding methods which largely focus on additive genetic variation in white clover, and forage species in general (Breese and Hayward, 1972). The possible exception to this could be herbage yield as shown in Chapter 7. This finding, while speculative due to the high associated standard error certainly warrants some further attention. Considering this, it would be worthy to note that progeny evaluation using full-sib testing may not be advisable, due to the biases of non-additive variation between families. If non-additive variation is indeed shown to significantly influence herbage yield in white clover, half-sib selection is a better option if the breeder is not interested in capturing the non-additive variation in hybrid cultivars.

As previously mentioned, when adopting half-sib evaluation, breeders must ensure random mating occurs within isolation cages. In addition, Vogel and Pederson (1993) highlighted the importance of conducting HSPT when the population is at linkage equilibrium. It was their belief that many breeders have sabotaged their own breeding success because parent genotypes in their polycross nurseries came from various germplasm sources and were not in

linkage equilibrium, and it was highly probable that the half-sib progeny differences between families where due to differing levels of heterosis.

If the breeders opt to capture a proportion of the non-additive variation, the move towards hybrid evaluation and hybrid cultivars that are available for out-crossing forage species (Barrett et al., 2010; Michaelson-Yeates et al., 1997; Riday and Krohn, 2010) may be beneficial.

8.1.3 Effective trial designs

A reoccurring trend across both the mini-plot and spaced-planted nurseries was the high levels of family × replicate and family × season interactions. The former interaction recognises inaccuracies in family representation among replicates due to genetic sampling effects, whereas the latter represents re-ranking of families among different seasons due to family × environment/season interactions.

The problem encountered due to genetic sampling effects was more prevalent in the spaced-planted nurseries where the number of plants in each family per replicate was critically low. It is important to avoid genetic sampling effects by representing the family with at least a minimum number of plants per replicate according to family structure. For half-sib families, 15-20 plants are adequate whereas for full-sibs somewhat lower numbers might be used (Posselt 2010).

The significant family × season × environment interactions, and family × season interactions observed in both the mini-plots and spaced-planted nurseries are consistent with previous studies (Jahufer et al., 2002; Jahufer et al., 2009; Jahufer et al., 2013). The data from this thesis therefore supports the requirement for both multi-season and multi-year testing to identify superior families within the tested geographical area (Canterbury). It must be acknowledged however, that most breeding programmes evaluate material across a range of environments, differing in geographical effects. A recommendation on whether multi-season and multi-year testing is required across a range of geographical environments (reference population of environments) cannot be supported from data in this thesis and requires further experiments, although it is most likely.

The adoption of the mini-plot methodology used in this thesis should also be re-examined in any future studies concerning white clover quantitative genetics. Whilst small space-planted mini-plots have niche purposes in commercial breeding programmes for the crude assessment of germplasm, they seem to lack finesse from a quantitative genetics perspective (apparent by

the large standard error of measured traits). Transplanted mini-plots have been routinely used in white clover breeding programmes because of their minimal area requirement, ability to assess a large number of entries, reliable establishment, and minimal soil disturbance resulting in reduced movement of buried seed to the soil surface. However, from a quantitative genetics perspective, they are confounded by a significant proportion of standard error.

The use of alternative trialling methods would also need to take into account the likely assessment of desirable traits. Herbage yield is a trait that requires considerable attention due to its labour intensive quantification in mixed species swards. Development of technology that could accurately determine clover content quickly and accurately without the need for quadrat sampling and downstream manual sorting would be highly advantageous. It could be suggested that a progressive movement towards assessment of total yield (grass and clover components) as well as clover content (%) would provide a quicker means to assess herbage yield. In addition, this technique would better represent on farm value.

8.1.4 White clover vegetative persistence

It appears from the data and observations presented in this study, combined with prior studies, that a significant contributor of white clover persistence is regulated by a moisture stress × heat × animal grazing × genotype interaction. The data also demonstrated a decline in white clover persistence regardless of water stress and highlights the possibility of a loss in performance of the population *per se* due to the transition of white clover to its vegetative morphological phase as hypothesised by Brock and Caradus (1996).

Physiological coupled with morphological data demonstrated that the white clover population investigated in this study was poorly adapted to low water holding capacity soils, such as those at Ashley Dene (dryland), and it supports findings presented by Mills and Moot (2010), where the use of alternative species such as sub-clover and lucerne are better suited for summer productivity in such soils. The significant genetic variation for herbage yield post-summer moisture stress at the Ashley Dene (dryland) site however, supports scope for improved cultivars that can vegetatively persist in moderate drought conditions and demonstrate improved recovery rates following rain (Knowles et al., 2003). As with previous field studies, the best predictor for autumn herbage yield recovery and vegetative persistence was spring growing point density.

8.2 Future work

The findings point out a number of areas for future work:

- Further and more precise investigation into the quantity of non-additive variation expressed for herbage yield and other morphological traits in white clover grown in mixed sward target environments.
- Validation of pollination patterns in field isolation cages with differing densities of pollinators.
- Investigation into alternative field plot sizes that mitigate the large family × replicate interactions seen in the experiments of this thesis.
- Investigation into the number of samples per plot to reduce the standard error of associated variance components in white clover traits.
- Development of alternative evaluation tools that can determine clover content quickly and accurately without the need for quadrat sampling and downstream manual sorting.
- Investigation into the role that stolon removal has on the long term persistence of white clover in both irrigated and summer moisture deficit environments.
- Quantification of surface temperature × moisture stress × stolon removal interactions.
- Validation for the requirement of multi-season and multi-year testing of white clover families across a range of environments differing in geographical effects.
- Implementation of a stratified paternity testing method in white clover forage breeding programmes to enhance genetic gain

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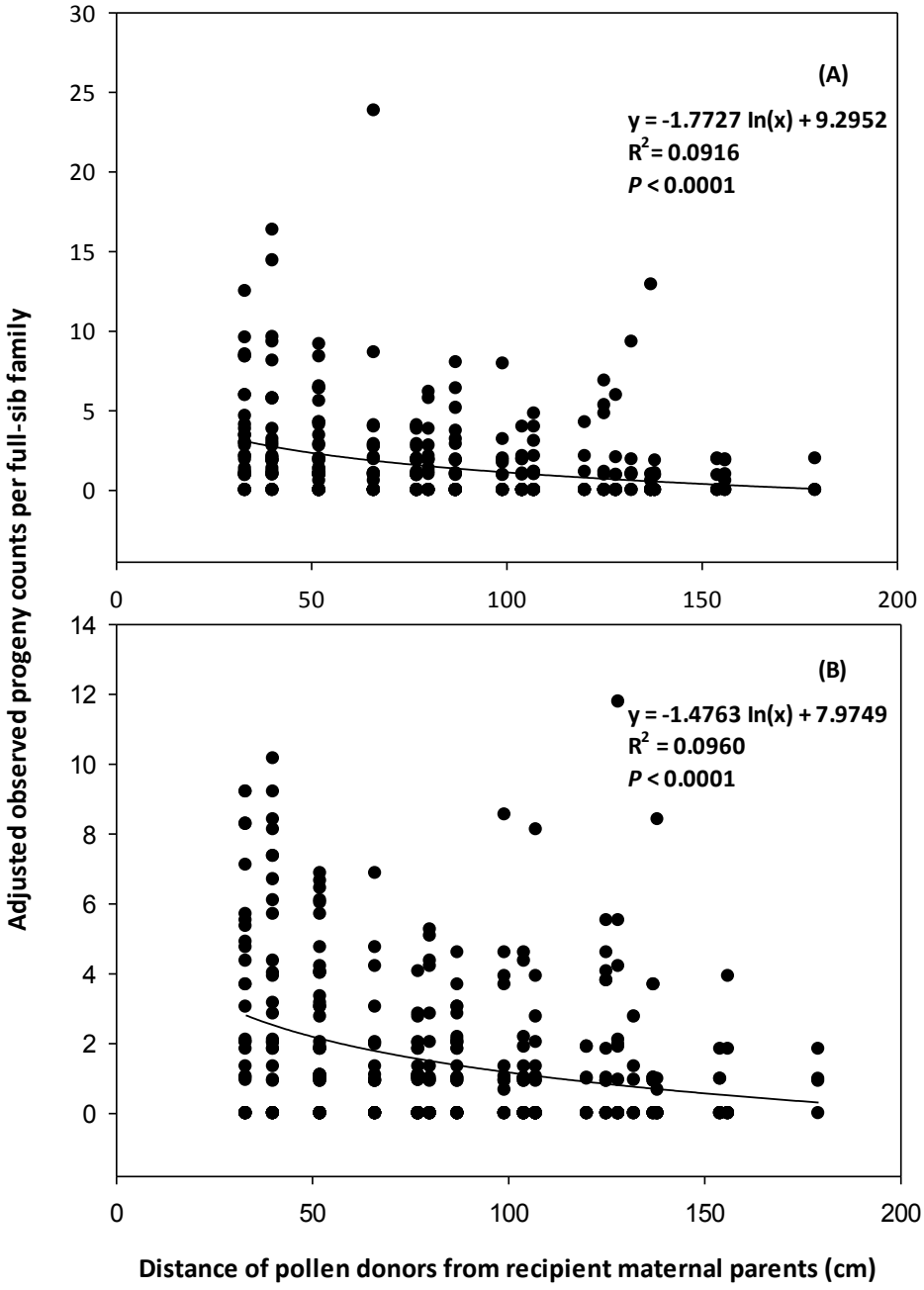
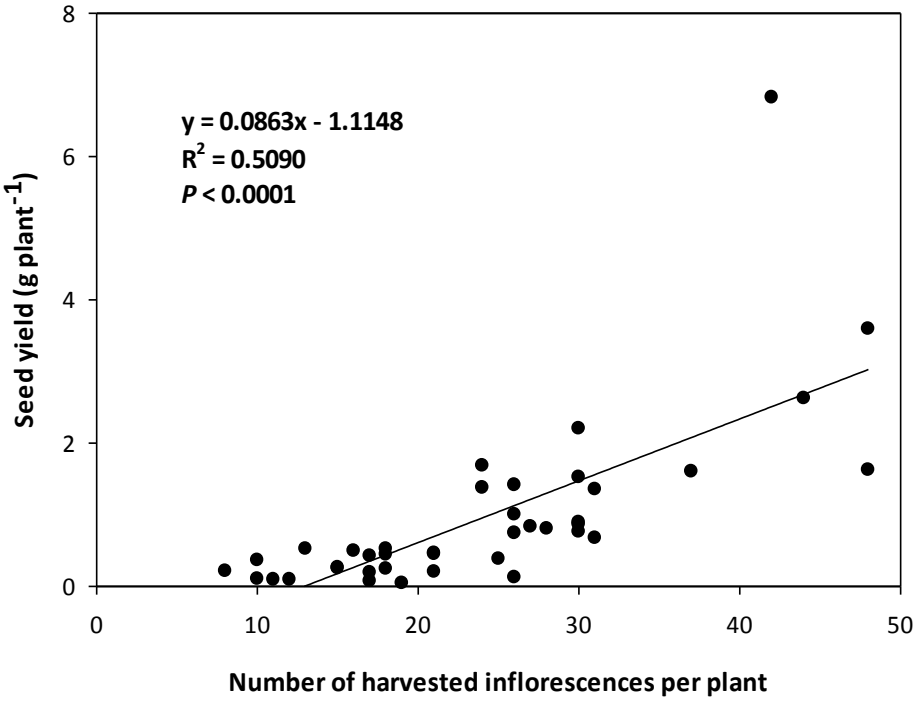
Appendix A

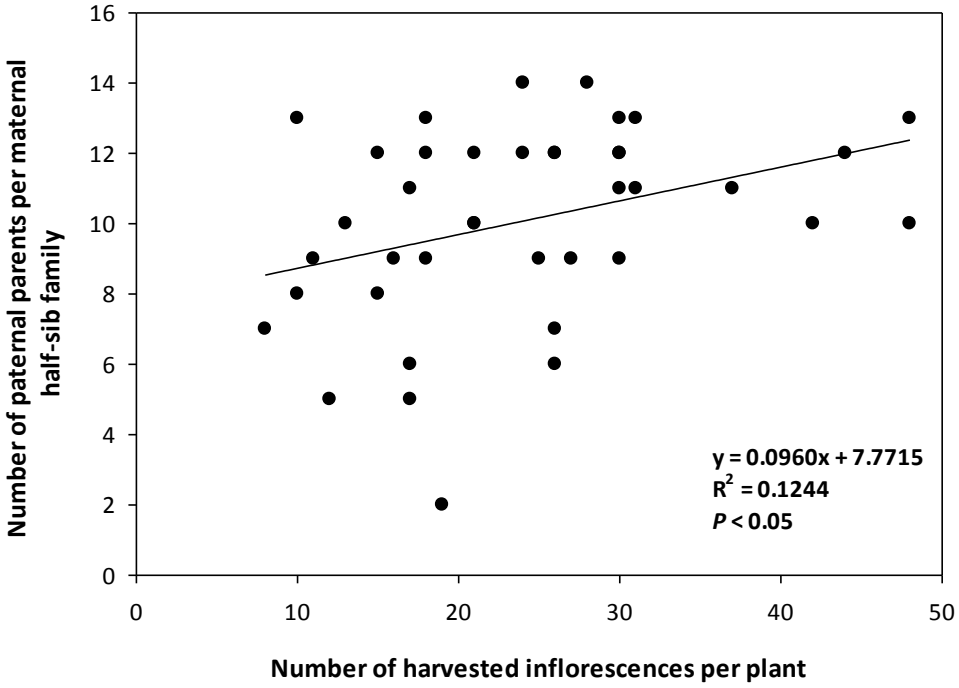
Generation of half-sib and full-sib families



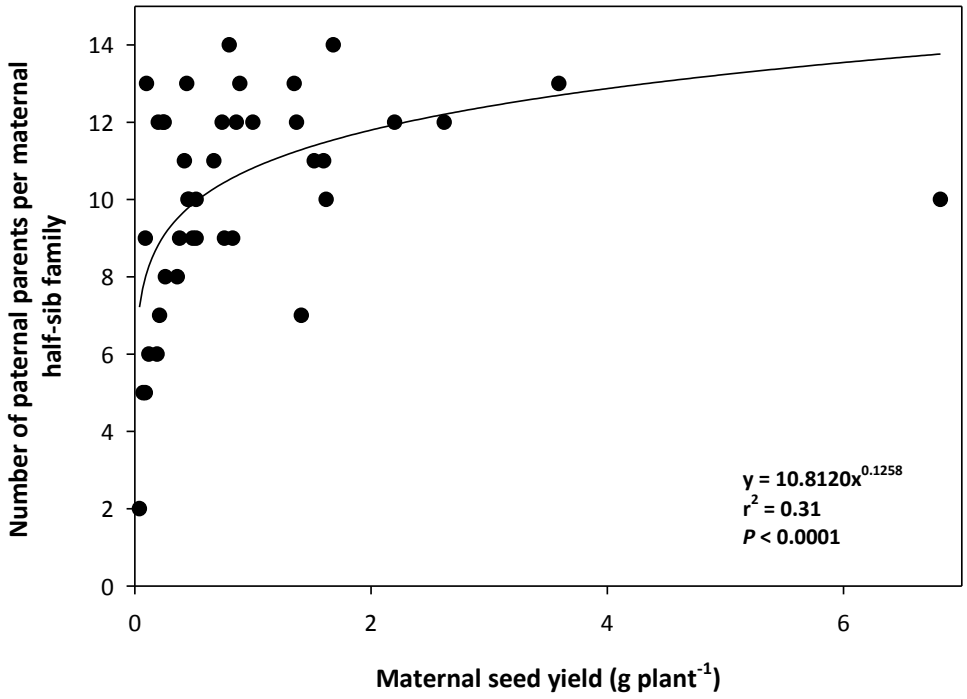
A.1 Adjusted (for unequal maternal sampling) observed outcross progeny counts per full-sib family plotted against the distance of pollen donor from recipient maternal parents. Each data point represents a full-sib family.



A.2 Relationship between seed yield per clone and the number of harvested inflorescences per clone in two 20 parent polycrosses pollinated by bumble bees.



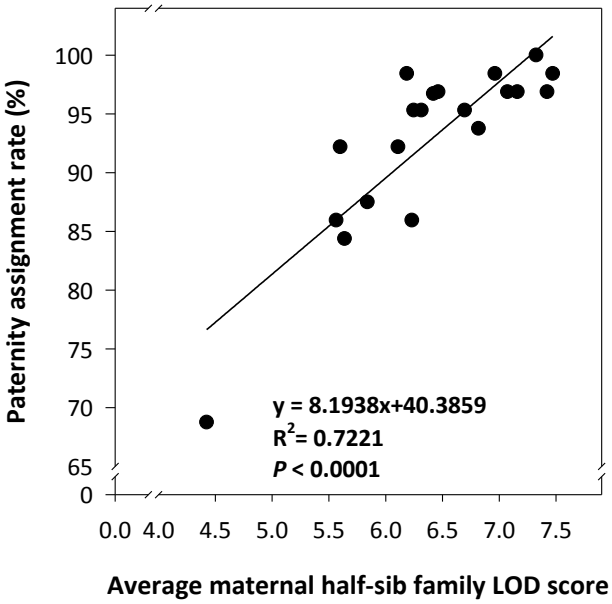
A.3 Relationship between the number of paternal parents per maternal half-sib family and the number of harvested inflorescences per clone in two 20 parent polycrosses pollinated by bumble bees.



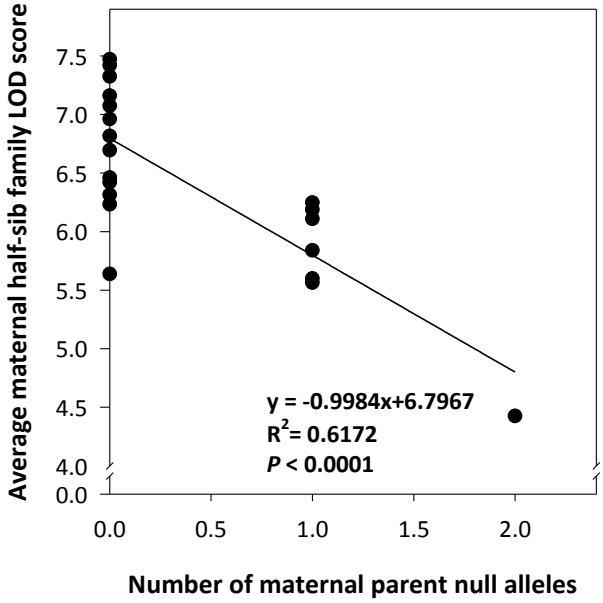
A.4 Relationship between maternal seed yield (g plant^{-1}) and the number of contributing paternal parents.

Appendix B

Methodology development: paternity testing in white clover



B.1 Correlation between paternity assignment rates (%) and average maternal half-sib family LOD scores.



B.2 Correlation between average maternal half-sib family LOD scores and the number of null alleles in their respective maternal parents genotype.

Appendix C

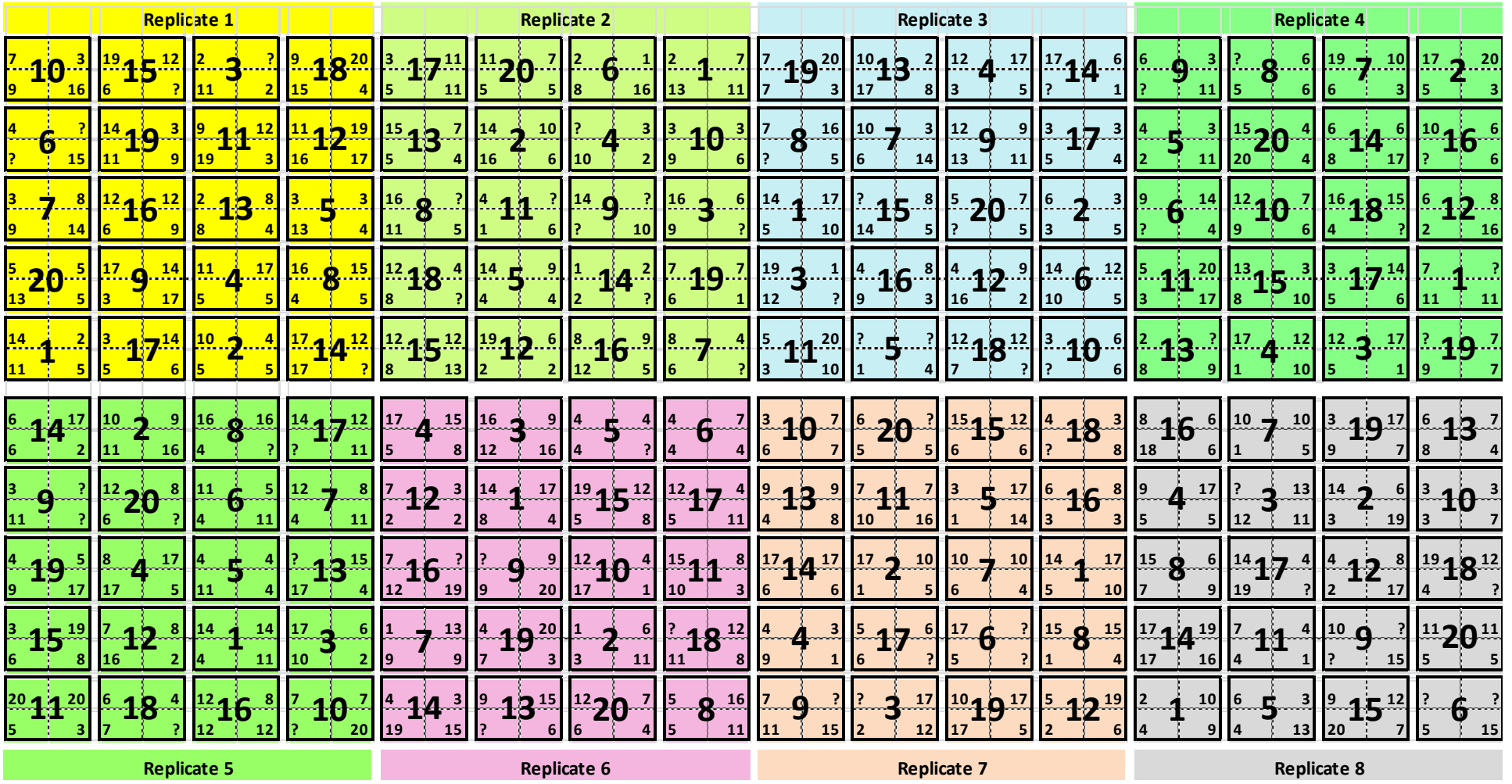
Application of paternity testing in white clover

C.1 Chemical characteristics of the soil sampled before capital fertiliser application at the AgResearch and Ashley Dene field experiments (0-15cm soil profile) in 2012

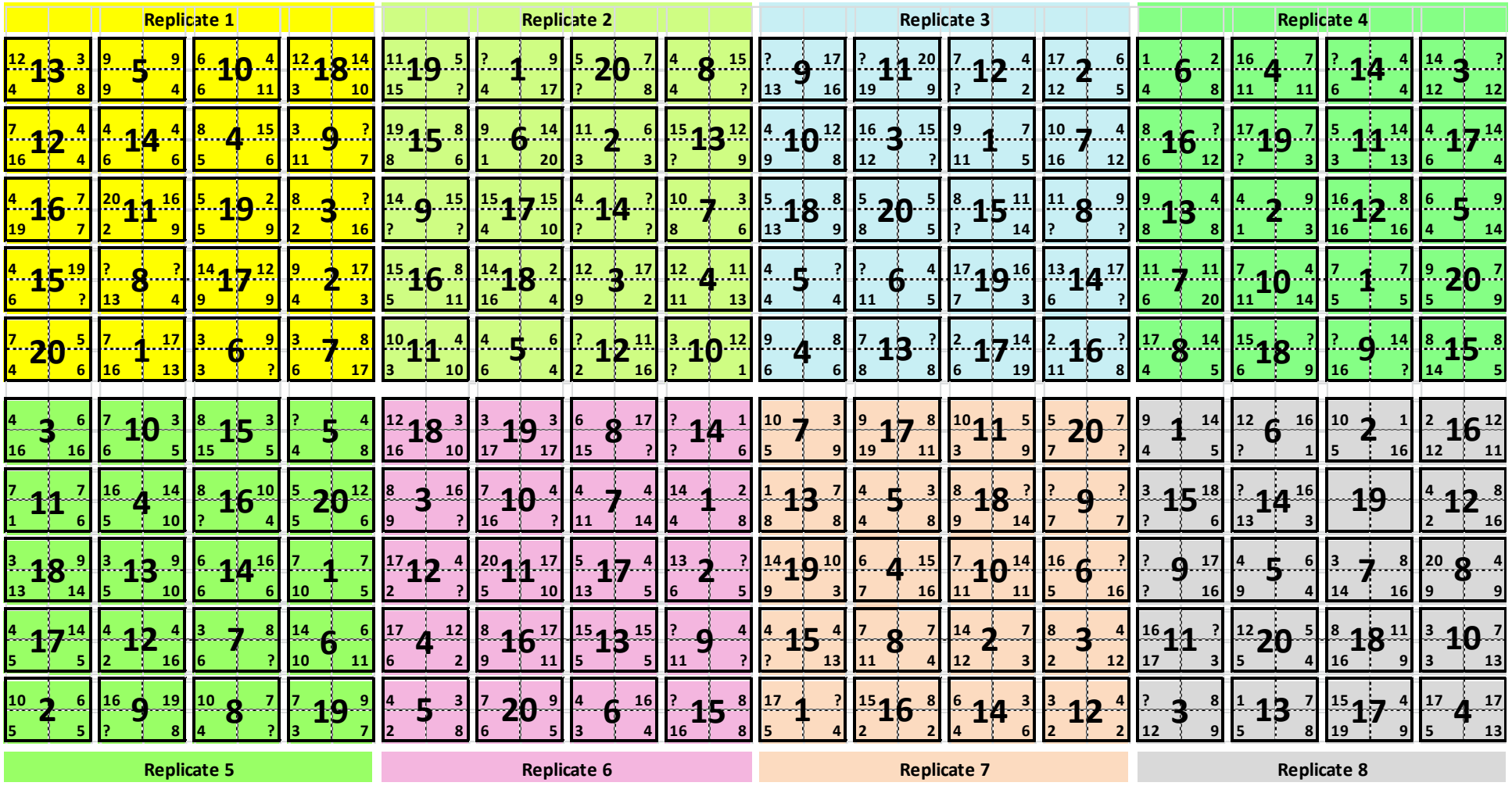
	AgResearch	Ashley Dene
pH	6.1	6.4
Calcium (MAF QT)	8	12
Olsen Phosphate (µg/mL)	23	29
Potassium (MAF QT)	15	3
Sulphate Sulphur (ppm)	14	10
Magnesium (MAF QT)	15	26
Sodium (MAF QT)	6	7
Copper (ppm)	1.4	0.6
Cobalt (ppm)	1.9	0.3
Iron (ppm)	365	815
Manganese (ppm)	120	19
Zinc (ppm)	2.3	1.5

C.2 Physical characteristics of soil profiles at the AgResearch and Ashley Dene trial sites

	AgResearch (irrigated)	Ashley Dene (dryland)
Soil type **Horizons**	Templeton silt loam	Lowcliffe stony silt loam Ah (18-20cm) Bg (30-45cm) BC (10-15cm) C (onwards)
Soil description	Moderately deep silt loam with moderate to high water holding capacity.	Stony silt loam with imperfect drainage. Shallow soil with >20 cm of stone-free material overlying very stony horizons.
References		Burtt (2012)



C.3 AgResearch polycross nursery layout. Each cell represents a plant within a plot of four. Each maternal half-sib family is represented by the centre number in each plot of four cells. The paternal identity of individual plants is illustrated by the corresponding number in each cell. The symbol '?' is assigned to progeny with no determined paternity.



C.4 **Ashley Dene polycross nursery layout. Each cell represents a plant within a plot of four. Each maternal half-sib family is represented by the centre number in each plot of four cells. The paternal identity of individual plants is illustrated by the corresponding number in each cell. The symbol '?' is assigned to progeny with no determined paternity.**

Appendix D

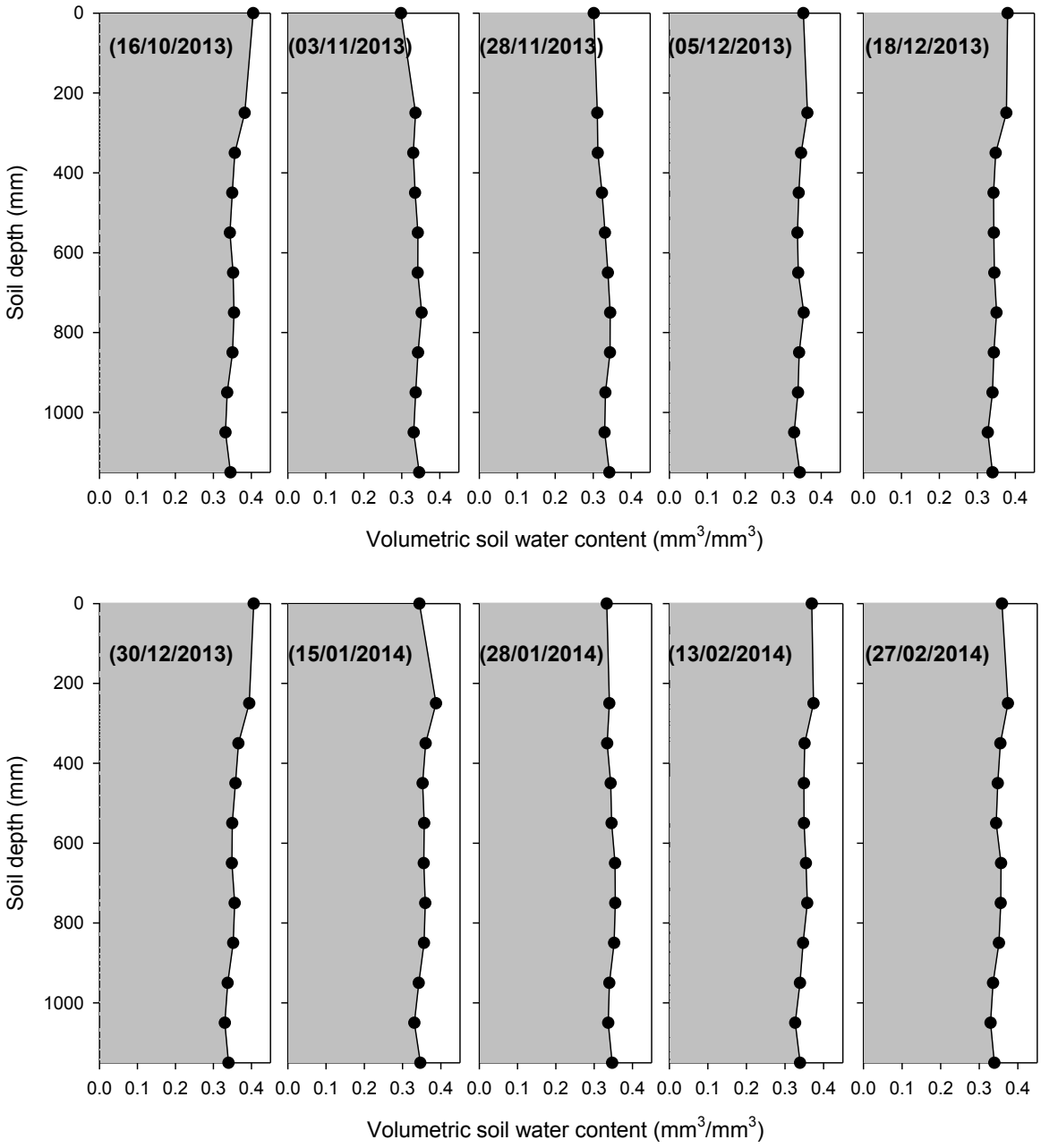
Genetic variation in a breeding population for persistence and production under summer moisture stress

D.1 Chemical characteristics of the soil sampled before capital fertiliser application at the AgResearch (irrigated) field experiment (0-15cm soil profile) in 2012 and 2013

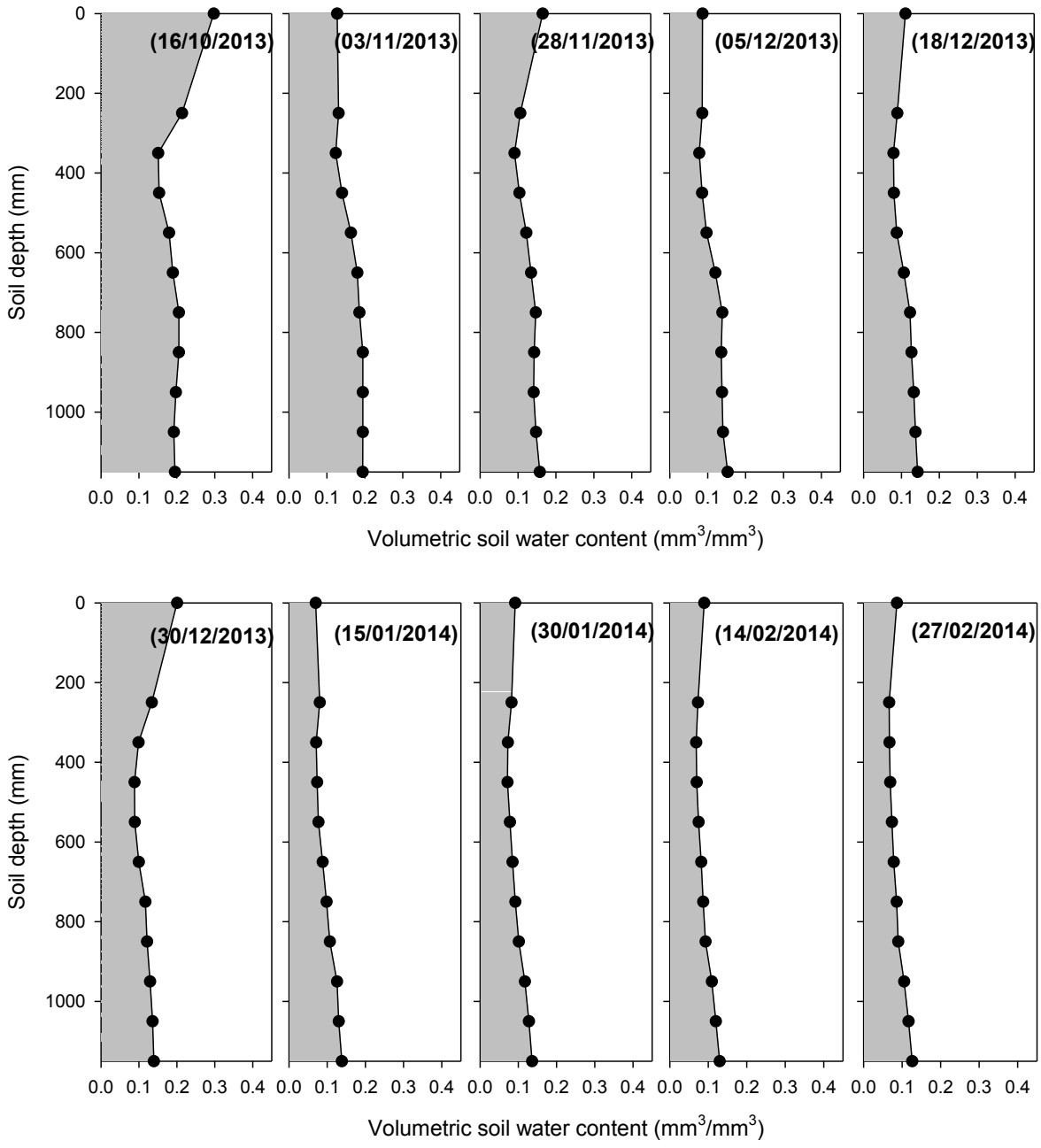
	2012	2013
pH	6.1	6.2
Calcium (MAF QT)	9	9
Olsen Phosphate (µg/mL)	24	17
Potassium (MAF QT)	16	8
Sulphate Sulphur (ppm)	15	5
Magnesium (MAF QT)	16	16
Sodium (MAF QT)	6	6
Copper (ppm)	1.3	-
Cobalt (ppm)	1.7	-
Iron (ppm)	355	-
Manganese (ppm)	100	-
Zinc (ppm)	2.3	-
Potentially available N (kg/ha)	-	132
Mineral N (ppm)	-	12
Ammonium Nitrogen (ppm)	-	8
Nitrate Nitrogen (ppm)	-	5

D.2 Chemical characteristics of the soil sampled before capital fertiliser application at the Ashley Dene (dryland) field experiment (0-15cm soil profile) in 2012 and 2013.

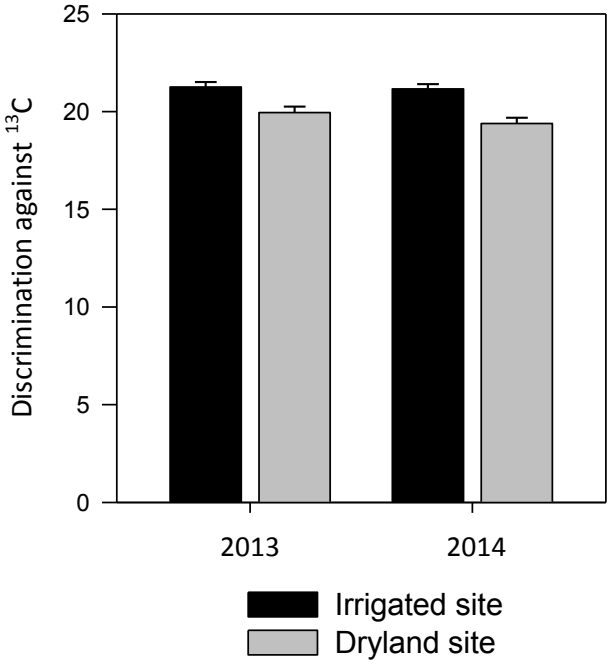
	2012	2013
pH	6.4	6.4
Calcium (MAF QT)	12	13
Olsen Phosphate (µg/mL)	29	23
Potassium (MAF QT)	3	2
Sulphate Sulphur (ppm)	10	5
Magnesium (MAF QT)	26	26
Sodium (MAF QT)	7	6
Copper (ppm)	0.6	-
Cobalt (ppm)	0.3	-
Iron (ppm)	815	-
Manganese (ppm)	19	-
Zinc (ppm)	1.5	-
Potentially available N (kg/ha)	-	93
Mineral N (ppm)	-	7
Ammonium Nitrogen (ppm)	-	4
Nitrate Nitrogen (ppm)	-	3



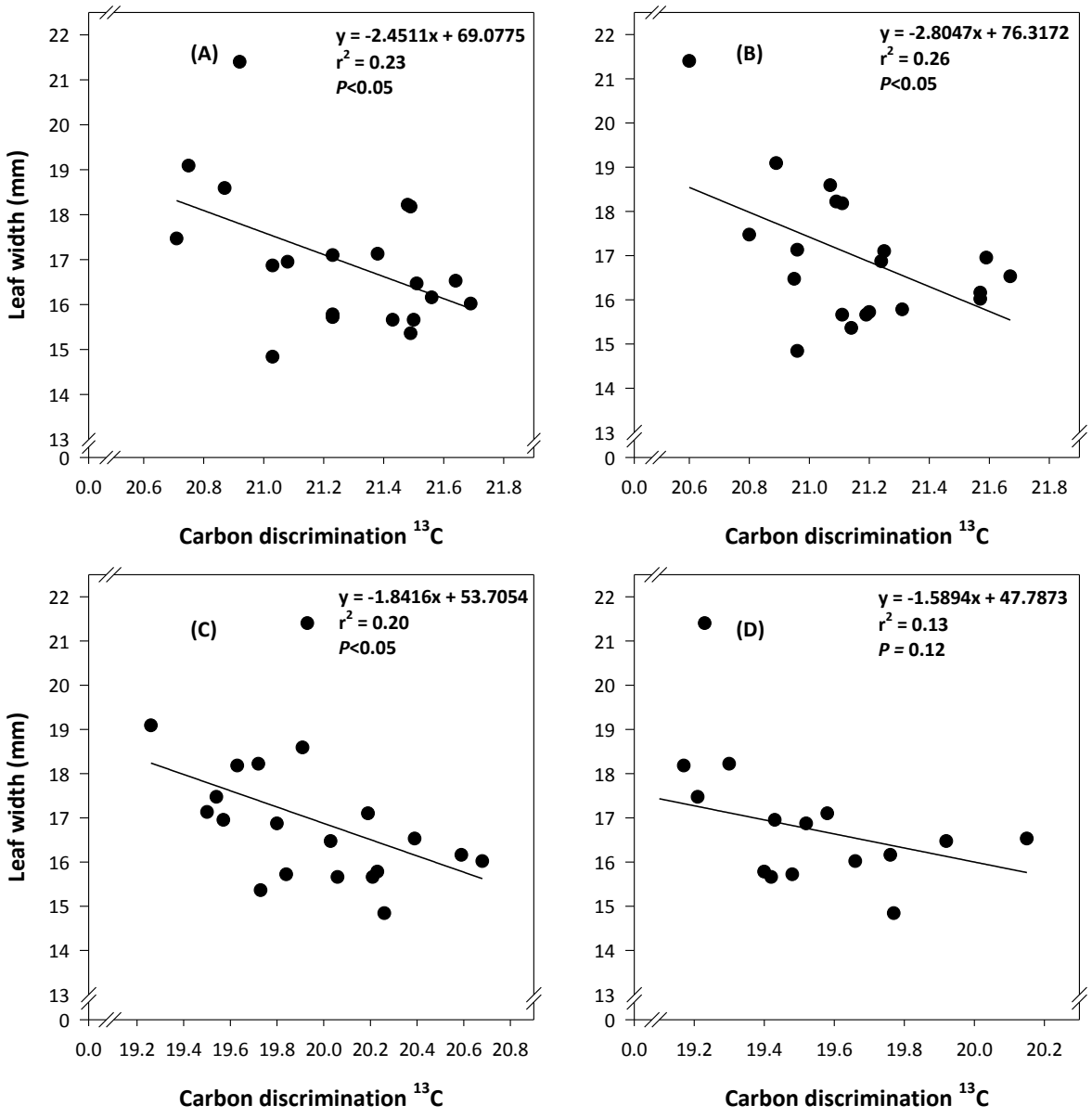
D.3 Volumetric soil water content (mm3/mm3) of the 0-115cm soil profile at the irrigated site during the summer period of October 2013 to February 2014. Dates of measurements are shown.



D.4 Volumetric soil water content (mm^3/mm^3) of the 0-115cm soil profile at the dryland site during the summer period of October 2013 to February 2014. Dates of measurements are shown.



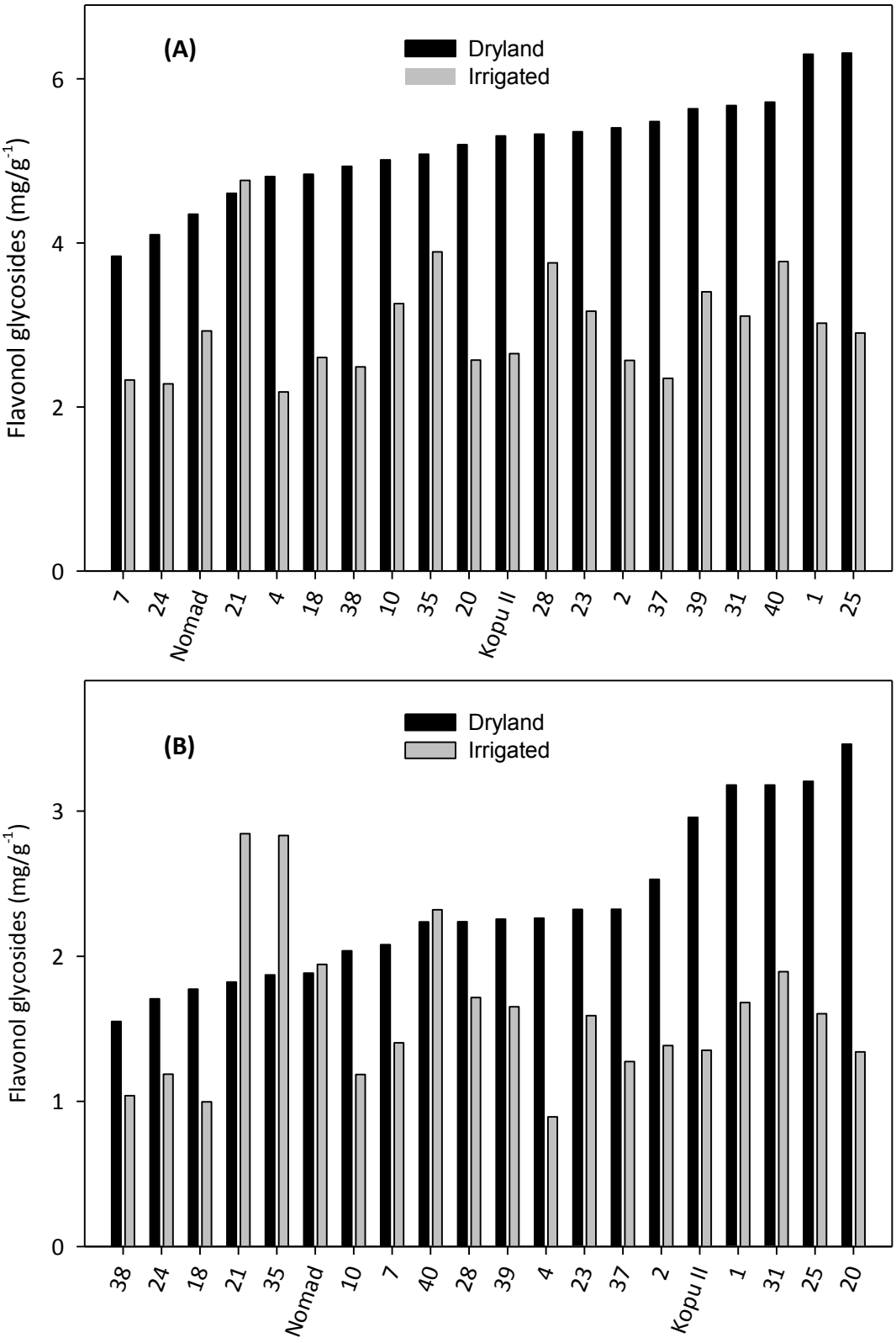
D.5 Mean ^{13}C discrimination (±SEM) for the irrigated (■) and dryland (■) sites in the summer moisture deficit periods of 2013 and 2014.



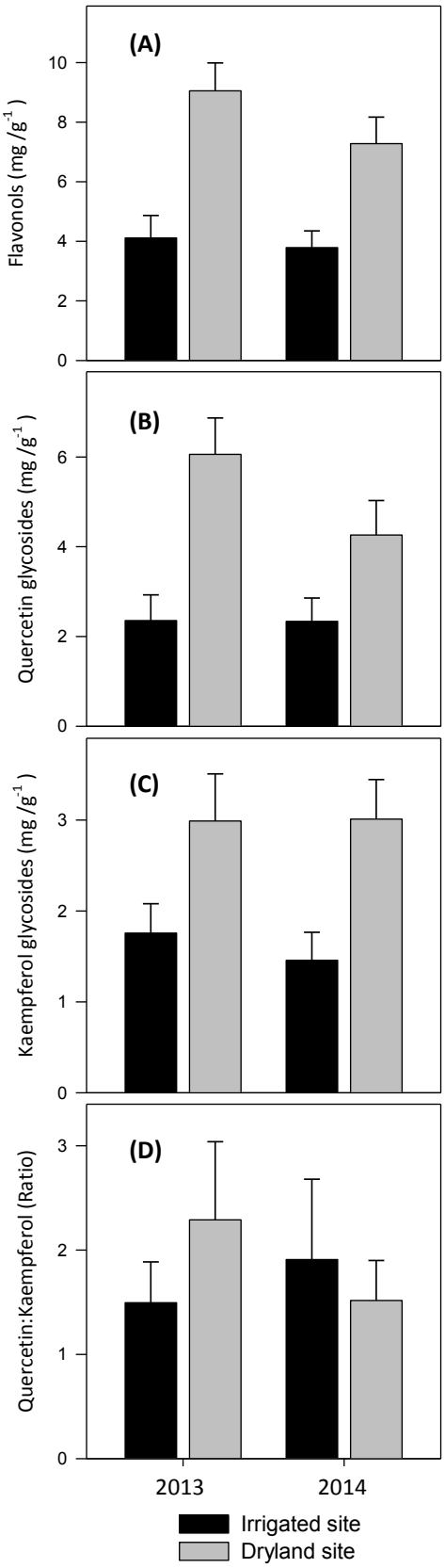
D.6 Correlation between carbon ^{13}C discrimination among selected full-sib families and leaf width at the (A) irrigated site in 2013, (B) irrigated site in 2014, (C) dryland site in 2013 and (D) dryland site in 2014.

D.7 P-values obtained from analyses of variances for phenolic compounds.

Year	Site	Source	Total Flavonols (mg/g)	Total Quercetin glycosides (mg/g)	Total Kaempferol glycosides (mg/g)	Quercetin: Kaempferol ratio
2013	**Irrigated**	Family	*P*<0.01	*P*<0.05	*P*<0.001	*P*<0.001
	Dryland	Family	*P*<0.001	*P*<0.05	*P*<0.001	*P*<0.05
2014	**Irrigated**	Family	*P*<0.001	*P*<0.01	*P*<0.001	*P*<0.05
	Dryland	Family	*P*<0.01	*n.s*	*P*<0.001	*P*<0.01
2013	**Multi**	Site	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
		Family	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
		Site*Family	*P*<0.05	*n.s*	*n.s*	*n.s*
2014		Site	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.01
		Family	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
		Site*Family	*n.s*	*n.s*	*P*<0.05	*P*<0.05
Multi	**Irrigated**	Site	*P*<0.05	*P*<0.01	*P*<0.001	*n.s*
		Family	*P*<0.001	*P*<0.01	*P*<0.001	*P*<0.001
		Year*Family	*n.s*	*n.s*	*n.s*	*n.s*
	Dryland	Site	*P*<0.001	*P*<0.001	*n.s*	*P*<0.01
		Family	*P*<0.001	*P*<0.01	*P*<0.001	*n.s*
		Year*Family	*n.s*	*n.s*	*P*<0.05	*n.s*
Multi	**Multi**	Site	*P*<0.001	*P*<0.001	*P*<0.05	*n.s*
		Year	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.05
		Family	*P*<0.001	*P*<0.001	*P*<0.001	*P*<0.001
		Site*Family	*n.s*	*n.s*	*n.s*	*n.s*
		Year*Family	*n.s*	*n.s*	*n.s*	*n.s*
		Site*Year*Family	*n.s*	*n.s*	*P*<0.05	*n.s*



D.8 Mean quercetin glycoside (■) and kaempferol glycoside (■) accumulation in the leaves of 18 white clover full-sib families and two cultivars Nomad and Kopu II at the (A) dryland site and (B) irrigated site across both years.



D.9 Mean (±SEM) total flavonols (A), quercetin glycosides (B), kaempferol glycosides (C), and (D) quercetin glycosides to kaempferol glycosides ratio for all selected full-sib families and two cultivars at the irrigated (■) and dryland (■) sites in 2013 and 2014.

D.10 Pearson correlations among flavonol components at each experimental site across both years.

	Quercetin (mg/g)	Quercetin (P) (mg/g)	Quercetin (P+S) (mg/g)	Kaempferol (mg/g)	Kaempferol (P) (mg/g)	Kaempferol (P+S) (mg/g)	Flavanol (mg/g)	Quercetin: Kaempferol	Yield (KgDM/ha)
----------AgResearch----------									
Quercetin (P) (mg/g)	0.895***								
Quercetin (P+S) (mg/g)	0.909***	0.921***							
Kaempferol (mg/g)	-0.06	-0.18	-0.182						
Kaempferol (P) (mg/g)	0.514*	0.522*	0.527*	0.257					
Kaempferol (P+S) (mg/g)	0.049	-0.015	0.007	0.92***	0.514*				
Flavonol (mg/g)	0.699**	0.536*	0.544*	0.672**	0.566**	0.695**			
Q:K	0.537*	0.585*	0.568*	-0.735***	-0.028	-0.626**	-0.128		
Yield (KgDM/ha)	0.047	0.021	-0.008	-0.246	-0.019	-0.304	-0.141	0.238	
Persistence (%)	0.024	-0.113	-0.058	-0.144	-0.018	-0.233	-0.086	0.182	0.841***
----------Ashley Dene----------									
Quercetin (P) (mg/g)	0.697**								
Quercetin (P+S) (mg/g)	0.752***	0.83***							
Kaempferol (mg/g)	0.188	-0.109	-0.026						
Kaempferol (P) (mg/g)	0.536*	0.584**	0.602**	0.313					
Kaempferol (P+S) (mg/g)	0.212	0.03	0.123	0.871***	0.429				
Flavonol (mg/g)	0.762***	0.372	0.462*	0.779***	0.548*	0.708***			
Q:K	0.24	0.423	0.311	-0.814***	-0.17	-0.638**	-0.384		
Yield (KgDM/ha)	-0.127	0.036	-0.052	-0.005	0.129	-0.178	-0.084	-0.243	
Persistence (%)	0.028	0.179	0.157	0.242	0.359	0.199	0.177	-0.391	0.75***

Note: P – primary glycoside peak, P+S – primary and secondary glycoside peaks

***$P<0.001$

** $P<0.01$

* $P<0.05$

D.11 Pearson correlations among flavonol components across both sites and years.

	Quercetin (mg/g)	Quercetin (P) (mg/g)	Quercetin (P+S) (mg/g)	Kaempferol (mg/g)	Kaempferol (P) (mg/g)	Kaempferol (P+S) (mg/g)	Flavanol (mg/g)	Quercetin: Kaempferol	Yield (KgDM/ha)
Quercetin (P) (mg/g)	0.775***								
Quercetin (P+S) (mg/g)	0.816***	0.868***							
Kaempferol (mg/g)	0.038	-0.181	-0.165						
Kaempferol (P) (mg/g)	0.587**	0.601**	0.619**	0.263					
Kaempferol (P+S) (mg/g)	0.139	0.000	0.032	0.907***	0.438*				
Flavonol (mg/g)	0.702**	0.394	0.433	0.738***	0.584**	0.741***			
Q:K	0.381	0.489*	0.443*	-0.807***	-0.088	-0.671**	-0.318		
Yield (KgDM/ha)	0.011	0.054	0.034	-0.083	0.056	-0.218	-0.052	0.017	
Persistence (%)	0.011	-0.031	0.037	0.118	0.169	0.002	0.092	-0.208	0.849***

Note: P – primary glycoside peak, P+S – primary and secondary glycoside peaks
***$P<0.001$
** $P<0.01$
* $P<0.05$