Assessing the Potential of Genomic Selection to Improve Yield and Persistence in White Clover

A thesis presented in the partial fulfilment of the requirements for the degree of

Doctor of Philosophy (PhD)

in

Plant Biology

at Massey University, Manawatu, New Zealand

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2020

Abstract

White clover (*Trifolium repens* L.) is an economically important forage legume in temperate pastures, providing quality fodder and plant-available nitrogen. However, its potential has not been fully exploited due to unpredictable herbage yield and poor vegetative persistence in pasture. Identification of genotypes that combine traits essential for yield and vegetative persistence, like dry matter yield and stolon density, are key objectives in breeding programmes. Long breeding cycles, high genome complexity and difficult-to-phenotype traits, usually assessed at late growth stages, are major constraints to conventional phenotypic selection in white clover breeding. In cultivar development programmes, elite individuals must be accurately identified and selected before crossing to generate superior progeny. Genomic selection is becoming a preferred method for increasing the rate of genetic gain by enabling early identification and selection of superior individuals, based on their genomic estimated breeding values (GEBVs), which can be generated without the need for phenotyping. Genomic selection is usually performed using a statistical model developed using genotypic and phenotypic information derived from a training population. In forage breeding, as parental breeding values are estimated by progeny testing, phenotypic data used in genomic prediction models is obtained from half-sib progeny. Recent single nucleotide polymorphism (SNP) genotyping methods like genotyping by sequencing (GBS) which generate a large volume of SNP marker information at low cost, have made genomic selection possible for species such as white clover. The main objective of this thesis was to explore the potential of genomic selection to improve important traits in white clover breeding.

A training population of 274 white clover parents were genotyped using GBS to provide genotype information. These genotyped maternal parents were randomly polycrossed under isolation to generate 274 HS families from which 200 HS were selected for phenotyping. The HS families were established in replicated, multi-location mixed sward field trials in 2016 at Aorangi and Ruakura New Zealand, under dairy cattle grazing. Variance components and quantitative genetic parameters were estimated from the HS progeny families via Residual Maximum Likelihood (REML) analyses for traits dry matter yield, growth score, leaf size, stolon number, stolon branching and Hydrocyanic acid (HCN) production. There was significant ($P < 0.05$) additive genetic variation among HS families for all measured traits. Year, season and location effects were also significant. Family mean narrow-sense heritability for the traits ranged from low (0.13)

to high (0.82). There was a low but positive correlation (0.24) between DM yield and stolon number. Results from cluster analysis identified several HS families with high DM yield and stolon density.

Predictive ability assessed by Monte-Carlo cross validation, ranged from -0.17 to 0.44 for different traits. Predictive ability for dry matter (DM) yield from data merged across years and environments was 0.3, while stolon density traits, stolon number and branches had lower predictive abilities ranging from -0.17 to 0.21. The highest predictive ability, 0.44, was obtained for leaf size, a genetically less complex trait than the yield-associated traits.

The performance of different genomic prediction models**,** Genomic BLUP (GBLUP), KGD-GBLUP, BayesCπ, and Reproducing Kernel Hilbert Spaces (RKHS) were compared. While no significant difference in predictive ability among models was detected, KGD-GLUP, a very computationally efficient model, tended to generate the highest predictive abilities on average. There was no decrease in predictive ability when the number of individuals in the training population and SNP markers were reduced from 200 and 110,000 to 80 and 5,500, respectively. Multi-trait genomic selection in which primary and secondary traits are incorporated into the model, increased predictive ability only when the information of a highly correlated secondary trait was present in both the training and test populations.

Using simulation, it was demonstrated that an integrated strategy using conventional phenotypic selection to select among families and genomic selection to select within families, termed AFp-WFgs, delivered up to two-fold genetic gain for DM yield over conventional phenotypic selection among families alone by enabling access to the ¾ additive variation residing within HS families. The cost efficiency of implementing genomic selection was also investigated and showed AFp-WFgs was more cost-efficient than among family phenotypic selection under high selection pressures.

Finally, to empirically validate the obtained predictive ability, a divergent selection was conducted for a simple trait, HCN, by selecting individuals based on their GEBVs. Conventional among HS family selection, progeny test selection and AFp-WFgs were compared in terms of response to selection, genetic gain and accuracy of selection. Despite the low predictive ability of 0.22 obtained for HCN, results showed AFp-WFgs to be similar to progeny test selection and superior to phenotypic selection in terms of response to selection and genetic gain. In terms of accuracy, AFp-WFgs was the more

accurate selection method, successfully eliminating individuals with high or low HCN production in the low and high HCN divergent groups, respectively.

Our results indicate, for the first time, an integrated phenotypic and genomic selection approach to be superior to conventional phenotypic selection at increasing genetic gain for a simple trait in white clover. This demonstrates the potential of genomic selection to be used in enhancing white clover breeding programmes for quantitative trait improvement.

Acknowledgements

My sincere gratitude to my supervisors Zulfi Jahufer, Andrew Griffiths, Jennifer Tate and Peter Lockhart for their relentless support and guidance throughout the course of my doctoral study and the write up of this thesis. I will fondly remember the highly animated and insightful discussions from which I learnt a lot. The open doors and constructive feedback have been priceless and deeply appreciated.

I gratefully acknowledge Greig Cousins, Doug Ryan, and Jessica O'Connor for their technical contributions and insightful discussions, Sai Arojju for all the help with statistical modelling and valuable suggestions, Ruy Jauregui for assisting with bioinformatics and Anna Larking for support in GBS library making. Without their help, this body of work would not be complete.

I would like to thank Craig, Derek, Peter, Mingshu, Poppy, Won, Sean, Sofie, John Ford, Bridget, Jana, Narsaa, the Aorangi farm staff and AgResearch forage team for all their help with phenotyping and technical assistance. They made mountains of work look like mole hills, it was always a pleasure working alongside you all.

My parents, Obiabo and Deborah Ehoche, you have always encouraged my curiosity, quest for knowledge and made me believe early on that I could achieve anything with the right mindset. I am forever thankful for your endless support and sacrifice in helping me achieve all my goals. You continue to be my heroes. My siblings, Ngbede, Ene, Omeche and Joshua, your love and unconditional support has meant everything and kept me going.

My deepest appreciation to the Adeyinka family, Doris especially, for their constant care and enormous support, they created a home away from home for me and are instrumental to my completion of this study.

To my friends Asli, Rachel, Dorcas, Jane, Samra, Marisia, Uche, Antoinette, Rahila, Rolake, Momoh, Rukky, Chioma, Jonathan, Kelvin, all the chats, lunches and dinners kept my spirits high and got me through the difficult days. Thank you for always being there. My sincerest thanks to Hayden and the Hedleys for always cheering me on and putting a smile on my face.

This PhD project forms part of Pastoral Genomics Plus, a New Zealand Industry Government-funded research consortium for developing genomic selection in forages, I

gratefully acknowledge the significant opportunity given to me and the financial assistance provided.

I am immensely grateful to Massey University for the financial support and graduate assistantship, which has been extremely instrumental in my professional development.

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List of Abbreviations

AFLP	Amplified Fragment Length Polymorphisms
AFp-WFgs Selection	Among-Family Phenotypic Selection and Within-Family Genomic
AFS	Among-family Selection
AWF-HS	Among-and -Within Half-Sib Family Selection
ANOVA	Analysis of Variance
AutGS	Autumn Growth Score
BLUE	Best Linear Unbiased Estimation
BLUP	Best Linear Unbiased Prediction
CNV	Copy Number Variation
DM	Dry Matter
DNA	Deoxyribonucleic acid
GBLUP	Genomic Best Linear Unbiased Prediction
GBS	Genotyping by Sequencing
GEBV	Genomic Estimated Breeding Value
g(DNA)	Genomic DNA
G×E	Genotype by Environment Interaction
GS	Growth Score
HCN	Hydrocyanic Acid
HS	Half-Sibling
HSp	Half-Sib Family Selection

LD	Linkage Disequilibrium
LS	Leaf Size
LSD	Least Significant Difference
MAS	Marker-Assisted Selection
MDS	Multi-Dimensional Scaling
MTCV1	Multi - trait CV1
MTCV2	Multi - trait CV2
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PS	Phenotypic Selection
PT	Progeny Test
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
REML	Residual Maximum Likelihood
RF	Random Forest
RFLP	Restriction Fragment Length Polymorphisms
RKHS	Reproducing Kernel Hilbert Spaces
RR-BLUP	Ridge Regression–Best Linear Unbiased Prediction
SB	Stolon Branches
SBPRS	Pre-Summer Stolon Branches
SBPOS	Post-Summer Stolon Branches
SEM	Standard Error of the Mean

SN	Stolon Number
SNP	Single Nucleotide Polymorphism
SNPRS	Pre-Summer Stolon Number
SNPOS	Post-Summer Stolon Number
SprGS	Spring Growth Score
SSR	Simple Sequence Repeat / Microsatellite Marker
SumGS	Summer growth score
SVM	Support Vector Machine
TS	Training Population Size
WFS	Within-family selection
WinGS	Winter Growth Score
µl	microliters
^{o}C	Degree Celsius
σ^2	Variance
ΔG	Genetic gain

1. Introduction

White clover (*Trifolium repens*) is an annual or short-lived perennial found in many temperate regions of the world (Gibson & Cope, 1985). It is an allotetraploid that resulted from the hybridization of two diploid *Trifolium* species, *T. occidentale* and *T. pallescens* (Atwood & Hill, 1940; Ellison et al., 2006) and possesses a genome size of 1093 Mb (Bennett & Leitch, 2011). The allopolyploidisation event occurred (15 – 28,000 years ago) and likely brought the alpine *T. pallescens* into close proximity with the coastal *T. occidentale* (Griffiths et al., 2019). Molecular evidence indicates white clover retained both progenitor genomes. The allopolyploidisation event underpins the broad adaptation and phenotypic plasticity of this species, and facilitates a global ecological niche expansion well beyond the restricted ranges of the progenitors (Griffiths et al., 2019). White clover is now extensively cultivated in temperate pastures world-wide (Williams et al., 2012). In New Zealand, white clover is a significant legume component in mixed grass/clover swards, providing an advantage over other temperate countries by delivering a cheap, high quality feed source throughout the year (Caradus et al., 1997a; Jahufer et al., 2002). Numerous studies have reported greater milk solid content and increased milk yields from cows grazed on a grass/clover mixture compared to grass monocultures only (Harris et al., 1997; Harris et al., 1998; Dineen et al., 2018; Egan et al., 2018). The advantages of white clover are not only limited to providing a rich source of leguminous feed for livestock, but also improving soil fertility through nitrogen fixation (Woodfield & Caradus, 1996). With effective inoculation of white clover by the symbiotic soil bacterium *Rhizobium leguminosarum* var. *trifolii*, the nitrogen fixed is sufficient for the clover and companion grass, thereby reducing the reliance on synthetic nitrogen fertiliser (Gibson & Cope, 1985). Caradus et al. (1995) estimated the annual financial contribution of white clover to the New Zealand economy at NZ$3.095 billion, through a variety of sources including nitrogen fixation, white clover seed production, forage yield and honey production.

Although white clover is an established pasture sward component of many livestock production systems in temperate countries, its potential is not fully utilised due to unreliable vegetative persistence and seasonal yield (Caradus et al., 1991; Woodfield & Caradus, 1994; Caradus et al., 1995). White clover dry matter (DM) yield and vegetative persistence is often compromised by a range of factors including cultivar type, biotic and abiotic stresses, pasture management and plant competition (Woodfield & Caradus,

1996). As mixed pastures mature, the total percentage of white clover declines significantly, often lower than 20% by the fourth year (Piano & Annicchiarico, 1995). Genetic improvement of vegetative persistence and seasonal DM yield, both quantitatively inherited traits are major objectives in white clover breeding programmes. Expression of quantitative traits is influenced by multiple gene effects and by their interaction with the environment thus posing a challenge to plant breeders when selecting elite genotypes (Byth, 1981; Falconer, 1989; Howard et al., 2014). White clover DM yield has been linked to a number of component traits including leaf size, internode length and leaf number (Mackay, 1991; Caradus et al., 1993), while vegetative persistence is determined primarily by stolon density which plays a major role in the perennation and colonization of white clover in mixed swards (Archer & Robinson, 1989; Caradus et al., 1989b; Collins et al., 1997). Unfortunately, a negative correlation exists between yield and stolon density in white clover (Williams, 1987; Caradus et al., 1989b; Jahufer et al., 1994). This negative association complicates breeding efforts for simultaneous genetic improvement of both traits (Jahufer et al., 1999). Important stolon density components like numbers of stolon and growing points, thickness and number of stolon branches are affected by summer moisture stress (Jahufer et al., 2012) and cold winter periods (Collins et al., 1991). Therefore, the ability of stolons to survive across these two critical periods is necessary for white cover longevity in the sward. Another trait implicated in vegetative persistence is cyanogenesis which is the release of hydrogen cyanide (HCN) from damaged leaves. Several studies have reported cultivars with superior agronomic performance in terms of yield and vegetative persistence to be cyanogenic (Williams, 1987) (Caradus & Williams, 1989; Crush & Caradus, 1995) (Doak, 1933).

In plant breeding, superior parent plants are selected and crossed to combine desired traits in the resulting offspring (Acquaah, 2012). White clover breeding, however, can be complicated, due to its allogamous and outbreeding nature, which results in high levels of heterozygosity, making it difficult to fix desired traits (Gibson & Cope, 1985). Crop improvement can be accomplished through conventional and molecular breeding strategies. An important initial step in this process is to estimate the magnitude of genetic variation for target traits in the breeding population. This is often an arduous task as plant breeders typically evaluate large numbers of plants using multi-site trials to acquire reliable data underpinning selection decisions. Although, conventional forage breeding methods have been successful for increases in DM yield (Humphreys, 1997), they often consist of long breeding cycles which have negative impacts on genetic gain and time

taken to release new cultivars. An integration of conventional breeding, new molecular techniques and targeted multi-site evaluation of breeding material will provide an efficient platform for increasing the rate of genetic gain in crop improvement especially for quantitative traits (Jahufer et al., 2012). Genetic gain is a term referring to positive trait advances in response to selection and is a function of the selection intensity, selection accuracy, genetic variance and years per cycle (Fehr 1980). Strategies that aim to increase the selection intensity and accuracy, while maintaining genetic diversity and reducing the generation interval or years per cycle, ultimately increase the rate of genetic gain.

With significant advances in molecular genetics, it is now possible to find associations between DNA markers and phenotypes. DNA markers are defined as a fragment of DNA which can be used to detect polymorphism or variations between different genotypes or alleles in a population of segregating individuals (Jones et al., 1997). Using the presence or absence of a marker as a proxy to assist in phenotypic selection, Marker Assisted Selection (MAS) makes selection/breeding more cost-efficient, reliable, time and space efficient compared to phenotyping (Gupta et al., 1999; Collard et al., 2005). Many methods have been developed to detect variation at the DNA level and Genotyping by Sequencing (GBS), is a technique for discovery of genome-wide single nucleotide polymorphisms (SNPs) developed for Illumina sequencing technology (Elshire et al., 2011). This methodology, offers a cost-effective alternative, to array-based methodologies such as SNP chips, which are expensive to develop and validate, and suffer from ascertainment bias, in which the only SNPs identified are those detected in the original populations used for developing the SNP-chip (Nielsen & Signorovitch, 2003; Albrechtsen et al., 2010). These limitations are overcome in GBS through simultaneous marker discovery and genotyping (Elshire et al., 2011). In the Elshire-based GBS procedure, a subset or reduced representation of the genome is selected using restriction enzymes and then sequenced to identify SNPs. Basically, the genomic DNA from an individual is digested with restriction enzymes and barcoded adapters are then ligated to each sample representing a specific genotype. Samples are pooled before PCR amplification using primers annealing to the barcode adapters. The PCR amplification and post-processing steps concentrate the sampled genome fragment to a size range optimal for sequencing (Elshire et al., 2011). After sequencing, sequence data are de-multiplexed to retrieve sequence information for each individual based on its barcode. Bioinformatics analysis of the sequenced samples identifies the SNPs to be used downstream in a range of genetic and population genetic analyses.

Genomics-based approaches can help identify and exploit beneficial genetic variation in plants, independent of the environment. However, this new technology is underutilised in forage plant breeding (Tuberosa & Salvi, 2006). Meuwissen et al. (2001) proposed a form of MAS called genomic selection that has been successfully applied to animal breeding programmes and is increasingly being adopted in plant breeding (Heffner et al., 2009). Genomic selection is the combined use of all genetic and phenotypic data collected from a set of individuals in a training population to generate a prediction model that can be used to predict the phenotype of individuals that have only been genotyped (Goddard & Hayes, 2007; Annicchiarico et al., 2014). These predicted phenotypes are referred to as Genomic Estimated Breeding Values (GEBVs). By estimating the effect of several markers in a population for individuals, all the genetic variance may potentially be captured by the markers (Oakey et al., 2016). The goal of genomic selection is to predict trait performance using all available marker information. The use of all markers presents an advantage over traditional MAS, which uses a threshold to select fewer markers associated with a trait at the cost of potentially losing a proportion of marker effects considered too small to be significant (Meuwissen et al., 2001; Hayes et al., 2009b; Lorenz et al., 2011; Crossa et al., 2017; Faville et al., 2018). One of the major advantages of genomic selection is the ability to select individuals accurately without the corresponding phenotypic data (Heffner et al., 2009; Hickey et al., 2009; Hayes et al., 2013). This facilitates shortening of the breeding cycle by eliminating the need for a phenotyping step in selection process or by affording the potential to make multiple recurrent selections, thereby increasing genetic gain per unit time (Wong & Bernardo, 2008; Crossa et al., 2010; Bassi et al., 2016). For perennial pasture species like white clover, where many important traits are only expressed at maturity by destructive sampling, the identification of superior individuals based on their genomic value is essential (Hayes et al., 2013). For genomic selection to be performed successfully, precise genotypic and phenotypic information must be available for desired traits to enable correct assigning of marker effects to observable phenotypes. This is especially important for quantitative traits which are strongly influenced by the environment; it is recommended that genotypes are assessed over different environments and across years (Asoro et al., 2011).

Currently, few studies evaluating the use of genomic selection for forage species have been published or empirically validated and to date, no study has investigated the prospect of genomic selection for white clover breeding. Research to examine the emerging role

of genomic selection in increasing genetic gain in breeding forage crops and white clover especially is needed and this is the aim behind this study. Before implementation of any new breeding strategy, however, research must be conducted on determining the magnitude of genetic variation for traits of interest as well as an appropriate mode of integrating the new marker-based strategy into conventional breeding methods.

Against this background, the objectives of this study are to:

I. Generate half-sib families (HS) from a random mating, F_2 generation white clover breeding pool for evaluation in multi-site and multi-year field trials.

II. Estimate quantitative genetic parameters and available genetic variation for DM yield, seasonal growth, vegetative persistence and other key breeding traits based on the HS families.

III. Determine the heritability of traits and correlations between important traits.

IV. Evaluate the use of GBS to generate quality SNP markers for use in genomic prediction models.

V. Assess the efficiency of different statistical models used to predict the GEBVs of individuals

VI. Assess the accuracy of genomic prediction models for several traits by estimating the correlation between the genomic estimated breeding values (GEBVs) predicted by the genomic prediction model and the actual observed phenotypic values from the population.

VII. Compare the rates of genetic gain achieved through conventional HS family phenotypic selection and an integrated approach with genomic selection using quantitative genetic simulation.

VIII. Perform a proof-of-concept genomic selection study validating the GEBVs predicted by the genomic prediction models.

1.1. Thesis Structure

The overall structure of this thesis is organised in five chapters (Figure 1.1). Chapter 2 is a literature review on key topics, findings and available information relevant to the research topic. Chapter 3 focuses on objectives I-III, which primarily involves the generation of the training and breeding population for genomic selection in white clover. Genetic, environmental variance and heritability of important morphological traits related

to yield and persistence in white clover are obtained from a replicated multi-site trial. Chapter 4 deals with objectives IV-VII and shows the methods and results of SNP marker generation, the implementation of genomic selection in the current white clover population, prediction accuracies obtained for several traits and the cross-validation strategies used to assess the accuracy of prediction. Genomic prediction models are assessed and factors affecting genomic selection are elucidated. Simulation to compare the rates of genetic gain obtained using conventional phenotypic selection and an integrated approach utilizing phenotypic selection to perform among-family selection and genomic selection to perform within-family selection, termed AFp-WFgs, is performed. Chapter 5, objective VIII, is a proof-of-concept / validation study for genomic selection performed using GEBVs predicted for a simple trait, HCN, from Chapter 4. The final chapter, Chapter 6, contains a critical final overview and discussion along with a summary of key findings, implications of the research to white clover breeding and directions for future investigations.

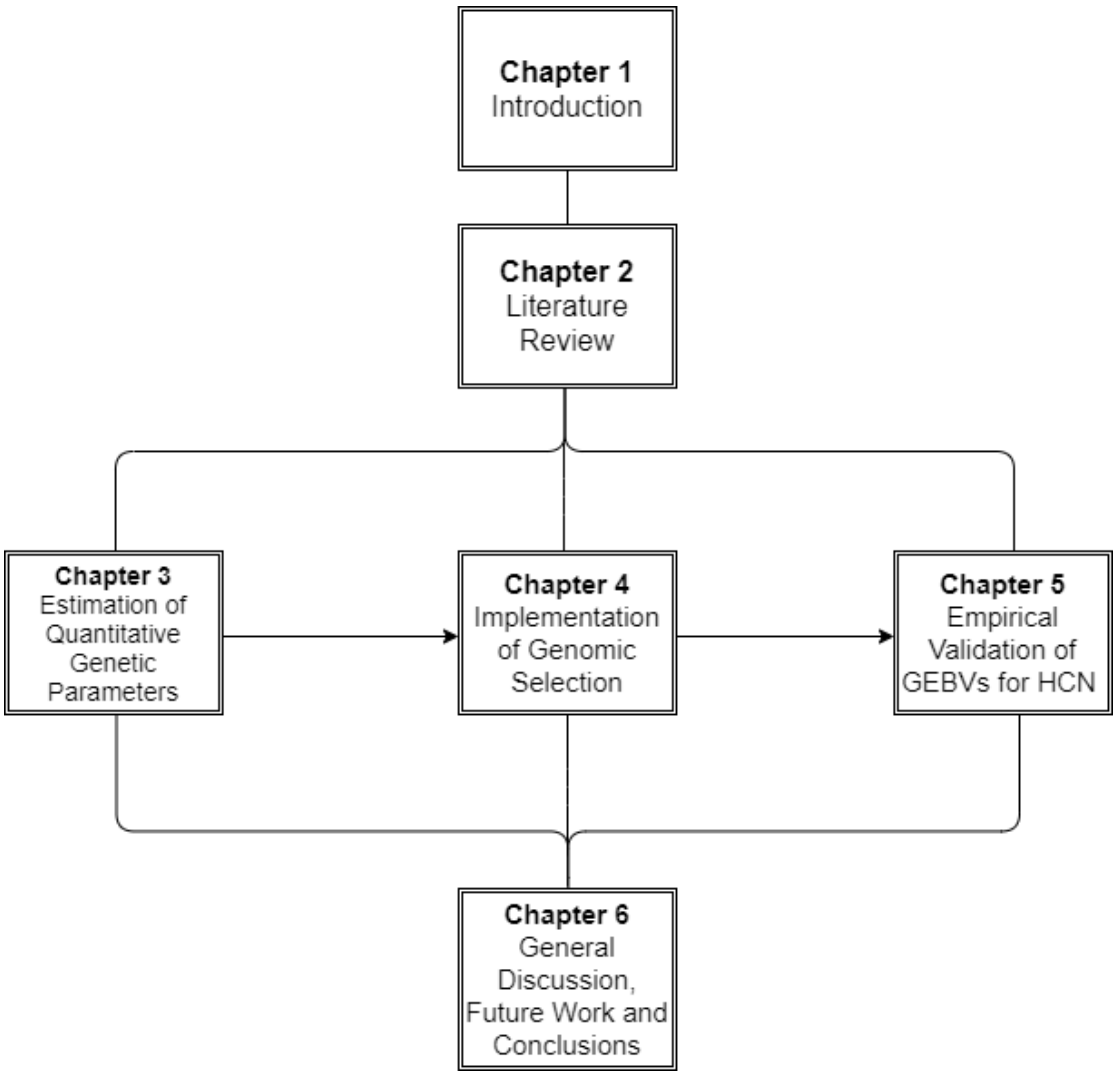


Figure 1.1 Thesis structure.

2. Literature Review

2.1. White Clover Morphology and Trait Relationships

The genus *Trifolium* comprises of the distinctive three leaflet (trifoliate) form of clover species (Ellison et al., 2006), which are long-petioled and glabrous (Gibson & Cope, 1985). Leaves come in different sizes with a range of leaf markings (Carnahan et al., 1955; Corkill, 1971). Sizes range from very small (less than 1 cm) in prostrate short-petioled types, to large (more than 2 cm), in the more erect, longer-petioled types (Thomas, 1987b). The plant consists of a horizontal creeping stem, also called the stolon, with internodes separated by nodes that can form nodal roots if in contact with moist soil (Figure 2.1) (Thomas, 1987b). There are three development stages in the life cycle; the first is a seedling stage, lasting up to 3 months after germination where the plant is small and slow growing (Meurant, 1986). The primary stem grows upright with short internodes and within 6 to 8 weeks after seed germination, stolons start to develop from axillary buds found in the axil of leaves (Gibson & Cope, 1985). Primary stem elongation usually stops after the stolon growth begins. Branches that develop from primary stolons generate secondary stolons forming a complex network of stolons over time (Gibson & Cope, 1985). In the second stage, the tap rooted phase, there is rapid expansion of the plant and further development of the tap root with subsequent death of the tap root and main stem within twelve to eighteen months (Brock & Hay, 2001). White clover is most productive at this stage in terms of herbage matter (Widdup & Barrett, 2011) This is followed by a clonal growth phase, the third stage, where fragmentation into smaller plants occurs with each individual stolon growing independently (Caradus & Woodfield, 1998). White clover stolons can grow up to 50 cm long, ranging from 1.9 - 4.0 mm in diameter (Burdon, 1983).

After flower formation, white clover is naturally cross-pollinated by a wide range of insects, predominantly honeybees (*Apis mellifera* L.) and bumble bees (*Bombus* spp.) (Burdon, 1983). Flower heads are globose, consisting of individual white flowers with an ovary in each floret that usually contains three to four ovules that mature into seeds after fertilization seeds (Gibson & Cope, 1985). Profusely flowering white clover varieties tend to have fewer stolons because each node has the potential to produce either a stolon or a flower head but not both (Woodfield & Caradus, 1996). This implies a negative

relationship between persistence through stolon production and high flowering capabilities (Thomas, 1987a).

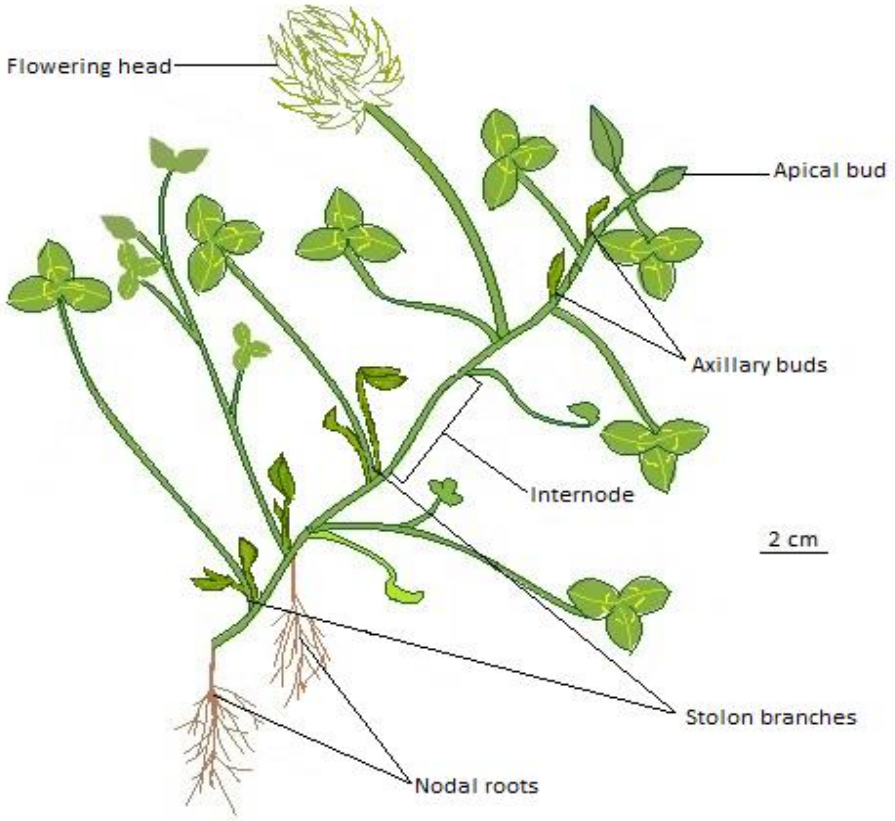


Figure 2.1 Morphology of the white clover plant. Adapted from Baker and Williams (1987)**.**

2.2. Methods and Conditions of Propagation

Approximately 30% of clover species are self-incompatible while 70% are self-pollinated (Taylor et al., 1977). White clover has a gametophytic self-incompatibility system controlled by a single locus with many alleles (Atwood, 1942). This obligate outcrossing system ensures that pollination only occurs from plants that have different self-incompatibility (*S*) alleles; therefore, a plant cannot be fertilised by its own pollen, or sometimes that of a close relative (Atwood & Hill, 1940). However, temperatures above 30°C have been shown to break incompatibility and most white clover plants will self-pollinate (Gibson & Chen, 1973). *T. pallescens* is the self-incompatible ancestral parent (Abberton, 2007) while *T. occidentale* is mostly self-compatible (Williams et al., 2008).

Continuous regeneration in pasture is by reseeding and vegetative (clonal) growth, through the adventitious rooting of stolons, which makes it able to exploit new environments (Gibson & Cope, 1985). Propagation by seed is vital for the colonization of new areas while clonal propagation is more important for maintenance in sward (Burdon, 1983). In cool temperate regions, propagation is mainly via stolon growth, while in warmer sub-tropical areas, seedling regeneration is the preferred method of proliferation (Archer & Robinson, 1989). Vegetative propagation in white clover occurs as a continuous cycle of growth and branching towards the stolon apices as the older and basal parts of the stolon decay (Harris 1993).

White clover is usually grown in combination with a companion grass such as perennial ryegrass (*Lolium perenne*), cocksfoot (*Dactylis glomerata*), fescue (*Festuca arundinacea*), kikuyu (*Pennisetum clandestinum*), timothy (*Phleum pratense*) or bermuda grass (*Cynodon dactylon*) (Van Keuren & Hoveland, 1985; Betts & Ayres, 2004). This complementary association is beneficial with grasses providing more forage during the cool seasons and clovers producing more herbage during warmer summer conditions (Sleugh et al., 2000). Other advantages offered by these mixtures include the improved performance of animals grazed on mixed swards (Egan et al., 2018), reduced weed encroachment and erosion, greater stand longevity than legume or grass monocultures (Casler, 1988) and significant reduction in the amount of nitrogen fertilizer needed due to nitrogen fixation capability. White clover has the potential to fix up to 700kg N/ha/year (Crush, 1987), although this is variable from location to location.

2.3. Conventional Breeding in White Clover

As an outcrossing tetraploid species, (2n=4x=32), white clover populations are highly heterogeneous with high phenotypic plasticity and broad environmental adaptation (Woodfield & White, 1996). Research on many white clover traits show considerable genetic variation signifying that ample opportunity exists for potential genetic improvement of white clover. Existing white clover cultivars are synthetic varieties usually produced by polycrossing eight or more selected parental clones (Woodfield & White, 1996; Caradus & Woodfield, 1997) or bulked seed lines (Jones et al., 2003) followed by consecutive generations of random mating of the closed population (Halloran et al., 1977). A polycross design ensures random mating among the genotypes, providing each genotype an equal chance of pollinating, or being pollinated by the other genotypes.

A conventional white clover breeding programme typically takes 10–20 years to release a new cultivar (Williams, 1987). Traits targeted include seasonal dry matter yield, leaf size, stolon branching frequency, stolon thickness, improved rooting architecture, water use efficiency, tolerance to cold, heat and drought, resistance to nematodes, pests and pathogens (Caradus et al., 1991). The yield potential of white clover primarily depends on the leaf size and above-ground parts, while stolon branching frequency and growing point density contribute to persistence by continuous regeneration (Gibson & Cope, 1985; Caradus et al., 1997a). Woodfield and Caradus (1994) reported genetic improvement in the range of 6% to 15% per decade for white clover DM yield and similar gains in percentage clover and mean stolon number from international collections of 110 white clover cultivars grown in New Zealand. They stated, however, that some of these cultivars were not adapted to New Zealand conditions and predicted possibly higher genetic gains. Recently, Hoyos-Villegas et al. (2019) reported much lower gains, less than 1% per decade for DM yield.

Cultivar development in many white clover breeding programmes follow the same basic approach: collection and characterisation of available germplasm, identification and polycrossing of superior parental material, multi-site evaluation of resulting progeny families, and finally, polycrossing parents of superior progeny (Ayres et al., 1996; Jahufer et al., 1999). Phenotypic recurrent selection is the prevalent method in breeding white clover. It involves the evaluation of large numbers of plants for a single trait or multiple traits, selecting and inter-crossing the top performing plants, and repeating the cycle (Williams, 1987). It has been useful in accomplishing numerous breeding goals, especially those evaluated on single plants and forage quality traits (Casler & Vogel, 1999). However, selection of parents based on their phenotypic performance alone is sometimes inefficient as phenotypes of individuals may not always indicate their true breeding values due to confounding effects, such as dominance or genotype-by-environment interaction (G×E) (Halloran et al., 1977). Phenotypic recurrent selection is often combined with progeny-test selection which requires the establishment of full-sib or more commonly in white clover, HS families (Vogel & Pedersen, 1993). Half-sib families are produced in multi-parent polycrosses under isolation (Allard, 1960). Each plant within the polycross contributes pollen while also receiving pollen from the other plants - “the pollen cloud” (Acquaah, 2012). Popular cultivars developed using HS family progeny testing include ‘Grasslands Crusader’, ‘Grasslands Kopu I’, ‘Grasslands Kopu II’, and the renowned ‘Grasslands Huia’ (Mather et al., 1996). The underlying concept is

to use the progeny to reveal the true breeding value of the parents, and only after that are the parents with high genetic merit chosen for further breeding.

White clover cultivars generally are classified by leaflet size into three categories, small, medium and large (Smetham, 1973). Most small-leaved types are prostrate with profusely branching stolons, while large-leaved types possess longer petioles with a more erect habit (Speedy, 1998). Farmers sometimes sow blends of cultivars with different leaf sizes to maximise ground cover and persistence. Since white clover is usually grown with a companion grass, this has greatly influenced the method for evaluating breeding material, with many breeders preferring to evaluate material in mixed swards rather than a monoculture of individually spaced plants or rows (Caradus & Williams, 1989).

2.3.1. Breeding for Increased Dry Matter Yield

Most important agronomic traits are quantitative, polygenic and highly influenced by the environment (Collard et al., 2005). Quantitative traits are genetically controlled by many genes, with each gene contributing a relatively small effect on the eventual phenotype (Buckler et al., 2009). Apart from the difficulty involved in breeding for quantitative traits, white clover is a polyploid with high genotypic and phenotypic heterogeneity among individual plants and populations (Capstaff & Miller, 2018). The combination of the parental genomes causes significant amounts of heterozygosity which is fixed as a result of the disomic inheritance within each genome (Sattler et al., 2016). Heterogeneity makes it difficult to "fix" desired traits like yield in the population of interest. Like many other agronomically important crop species, low to moderate heritabilities for yield have been reported for most white clover populations (Suzuki et al., 1958; Connolly, 1978; Annicchiarico et al., 1999). This complicates trait improvement further, making it a difficult and long process. A solution breeders' use to overcome this problem is to look to secondary and sometimes simpler correlated traits with higher heritabilities that can be easily selected upon as an indirect method of selection for primary traits (Casler, 2012).

In many pastures, white clover DM yield tends to decline over time and many studies report the total percentage of white clover in mixed sward falling between 20% to 35% by the third year of evaluation (Piano & Annicchiarico, 1995; Chapman et al., 1996). By the fifth year, Piano and Annicchiarico (1995) estimated total clover content at only 5.5%. With only about 3% of pastures reseeded annually to maintain yields (Nolan et al., 2001), developing avenues to increase and maintain total herbage content in pasture are crucial.

Leaf size (LS) and an erect growth habit are major factors contributing to the DM yield potential of white clover (Caradus et al., 1993; Caradus et al., 1997a; Clark & McFadden, 1997). Leaf size has often been used as a proxy selection trait for DM yield since it is highly heritable and positively correlated with DM yield (Woodfield & Caradus, 1990). Caradus and Woodfield (1990) and Barcikowska (1976) obtained medium to high broad sense heritability (> 0.5) for leaf size. Mackay (1991) pointed out that although leaf number and LS were the best predictors of clover proportion in a mixed sward, leaf number was of greater importance under favourable conditions. A major reason why LS affects the proportion of clover in pasture is because large-leaved clover types have bigger but fewer stolons than smaller-leaved types. These larger stolons are more likely to be grazed by animals unlike the small-leaved types with numerous, thin and multi-branched stolons (Charlton & Stewart, 1999). Clark and McFadden (1997) found that large-leaved cultivars were the most productive in the first or establishment year, but productivity declined in subsequent years when compared to intermediate leaf sized cultivars. Another important trait influencing DM yield is the internode length (Gibson & Cope, 1985). Leaf production has been found to be negatively correlated with internode length; longer internodes possess fewer leaves per unit of stolon (Gibson & Cope, 1985; Hill & Michaelson-Yeates, 1987).

In addition to the heterozygosity possessed by many white clover populations, many plant characteristics associated with DM yield have been found to be phenotypically plastic (Caradus et al., 1993). Leaf size is particularly affected by seasonal variation in precipitation, temperature and light intensity (Solangaarachchi & Harper, 1987; Wachendorf et al., 2001). In winter, white clover can lose up to two thirds of its maximum herbage weight (Woledge et al., 1990). Wilman and Simpson (1988) reported higher percentage of clover ground cover starting from spring and increasing until the start of summer. Internode length, leaflet size and petiole length are also influenced by shading from companion species and Wilman and Shrestha (1985) found a positive correlation between canopy heights in both ryegrass and white clover as a result of each species response to photosynthetically active radiation.

Estimations of DM yield in clover-based swards range from 3 t ha^{-1} in adverse upland conditions, to 7 to 8 t ha^{-1} in more fertile preferred lowland sites in the United Kingdom (Rhodes, 2001). In New Zealand grazing trials, up to 12 t ha^{-1} have been obtained for hill sites, l6.2 t ha^{-1} for unirrigated dryland and 22 t ha^{-1} for lowland sites (Brougham, 1977).

Despite the negative correlation between DM yield and vegetative persistence, in cultivars like ‘Grasslands Prestige’ and ‘Grasslands Sustain’, high clover yield with considerable vegetative persistence has been achieved. Numerous authors have attributed this breakthrough to the concurrent selection for increased stolon growing point density while maintaining a particular leaf size (Caradus et al., 1997b; Woodfield et al., 2001).

2.3.2. Breeding for Vegetative Persistence

Malcolm et al. (2014) defined pasture persistence as the length of time that individual plants of a species sown into a pasture continue to provide dry matter and nutrients for livestock. Lack of plant persistence in pasture is often due to many overlapping causes with poor farm management or grazing practices coupled with genetic and environmental factors all playing key roles (Widdup & Barrett, 2011). Pasture persistence is advantageous not only to the farmer in terms of continuous animal nutrition but also in terms of economic profitablity as the pasture can be maintained over the life of the farm business (Malcolm et al., 2014). To date, the pasture renewal rate amongst New Zealand dairy farmers has not been remarkable with Sanderson et al. (2003) estimating it at only 6%. White clover stolons are vital to plant morphology and form at least half of the total shoot weight of white clover plants in grazed pastures (Brock et al., 1988). They are mainly responsible for continuity in white clover (White & Hodgson, 1999). Many stolon attributes are implicated in persistence including stolon number, branches, growing points, length and diameter (Caradus & Williams, 1981; Hay et al., 1987; Collins et al., 1991; Collins et al., 1997). Stolon branching is essential for the replacement of stolons as well as increasing tolerance to repeated defoliation from grazing (Beinhart, 1963; Gibson & Cope, 1985). Rhodes (2001) highlighted poor stolon density as the primary factor limiting dry matter yield. Chapman (1983) investigated stolon survival rates and found an average 10% of stolons surviving longer than 12 months in hill country pastures in New Zealand.

New stolons start developing during spring, increasing to a maximum size in summer before starting to decline in autumn and winter (Caradus et al., 1997b). During the winter period, animal treading and earthworm activity bury a large percentage of stolons (Hay et al., 1987). New nodal roots are formed in early spring, but as old roots from the previous season die off, the rate of stolon death is higher than the rate of stolon renewal. making the plant more susceptible to biotic and abiotic stresses (Woodfield & Caradus,

1996). Prolific branching of stolons, therefore, is a desired trait as each new branch produces an extra apical bud, and consequently more leaves are formed (Gibson & Cope, 1985). Additionally, large leaved cultivars are less persistent than the smaller leaved cultivars which are highly stoloniferous (Meurant, 1986). To be persistent in sward, new stolons and branch points must outweigh the number of dead stolons (Widdup & Barrett, 2011).

Furthermore, the ability of the stolons to spread horizontally along the soil confers an additional competitive edge over ryegrass in mixed swards (Harris & Thomas, 1973). When in competition with a dominant grass, high stolon density assists in the capture of light and vital nutrients by white clover (Piano & Annicchiarico, 1995). Stolons also serve as storage reserves for carbohydrates and protein that are essential for survival and instrumental in the production of new leaves following defoliation after winter (Rhodes, 2001). Selection for increased stolon density has consequently been seen as fundamental to improving the yield and persistence in white clover (Caradus et al., 1990).

Summer moisture stress has been identified as a principal environmental constraint limiting the agronomic performance and persistence of white clover (Jones, 1982; Jahufer et al., 2002). White clover, being a shallow-rooted crop, has most of its roots in the top 20 cm of the soil (Gibson & Cope, 1985), thereby limiting its access to soil moisture. After the death of the main tap root, the plant is reliant on the shallow fibrous nodal roots, causing difficulty coping under summer moisture stress (Baker & Williams, 1987). Under drought conditions, many stolons die back to rooted nodes causing a collapse in the stolon population (Brock & Kim, 1994). Up to 30 - 70% loss in clover production has been reported under drought conditions (Brock et al., 1988). In an experiment comparing two white clover cultivars with different stolon attributes, Brock and Kim (1994) found cultivars with thicker stolons more severely affected by drought, while cultivars with more branches and thinner stolons thrived better. By contrast, Hay et al. (1987) found that thicker and heavier stolons survived moisture stress better.

Winter is also a critical period for stolon survival with drastic reductions in dry weight and number reported by several authors (Collins & Rhodes, 1995; Clark & McFadden, 1997). Collins et al. (1991) concluded that since stolon weight declined in winter due to the utilization of reserves by the plant, stolon length might be a better measure of the plant resilience after winter. Stolon growing point density is another important factor, as more

growing points enable clover regeneration after intense grazing or pest damage (Caradus et al., 1997a). Williams (1983) found increased stolon branching during autumn and winter to play a pivotal role in improving spring growth and proper colonization, as plants with a high degree of branching can withstand continuous defoliation.

Evaluation of white clover germplasm under drought conditions has been the foremost breeding strategy used to develop new cultivars with persistence under moisture-stress (Jahufer et al., 2013). Some breeding programmes have focussed on drought escape to improve persistence under moisture stress by developing cultivars that flower and set seed early before moisture stress periods (Van den Bosch et al., 1993). Clark and McFadden (1997) reported intermediate leaf-size genotypes as the most suitable for breeding increased yield and persistence simultaneously as they possessed the best combination of stolon and herbage yield. Cultivars like 'Prop' (Jahufer et al., 2002) and 'Canterbury' have been bred for increased early-flowering and increased seed and forage production. Other important morphological traits contributing to increased vegetative persistence, especially during drought include deeper nodal roots and a higher root-to-shoot ratio (Woodfield & Caradus, 1987). Like yield, many stolon characteristics have low to moderate heritabilities and are controlled by many genes making trait improvement difficult. Caradus and Woodfield (1990) estimated heritabilties for stolon number at 0.21 and Stolon diameter at 0.54. Conversely, Annicchiarico et al. (1999) reported a relatively high heritability at 0.6, it should however be noted that cloned spaced plants were used in that study unlike the competitive mixed sward environment assessed for by Caradus and Woodfield (1990).

2.3.3. Effect of Cyanogenesis

Cyanogenesis is a highly polymorphic trait in white clover with both cyanogenic and acyanogenic plants occurring in the same population (Armstrong et al., 1913). Plants that are cyanogenic release hydrocyanic acid (HCN) from damaged tissues. Usually, it is brought about by the combination of two biochemical components; glucosides (lotaustralin and linamarin) and their hydrolysing enzyme, linamarase, that are separated in undamaged tissue and combined upon cell rupture (Hughes, 1991). The glucosides are stored in vacuoles within plant tissues, while the hydrolytic enzymes are apoplastic and found in the cytosol (Tegzes et al., 2003). HCN production is controlled by two genes *Ac* and *Li*. *Ac/ac* controls the presence/absence of cyanogenic glucosides while *Li/li* controls

the presence/absence of their hydrolyzing enzyme, linamarase (Olsen et al., 2008). At the start of HCN production, a sugar is first cleaved from the cyanoglucoside by linamarase and in the process releases cyanohydrin which is hydrolysed by hydroxynitrile lyase, thereby releasing hydrogen cyanide (Poulton, 1990). The cyanogenic phenotype requires the presence of a functional allele at both loci in the plant (Corkill, 1942; Hughes, 1991). This results in a variety of genotypes and phenotypes in a segregating population, Corkill (1940) categorised these phenotypes into: i) plants containing the cyanoglucoside and the enzyme which hydrolyses this glucoside and produces hydrocyanic acid *(AcLi)*; ii) plants that contain the glucoside but not the enzyme (*Acli*); iii) plants that contain the enzyme but not the glucoside *(acLi)*, and iv) plants containing neither the glucoside nor the appropriate enzyme *(acli)*.

A considerable amount of literature has been published on cyanogenesis with several studies highlighting many factors contributing to the variation observed between plants and sometimes even on the same plant. In a study investigating quantitative variation in various *Trifolium* species, Olsen et al. (2014) via southern hybridization, found more than one band in some plants that possessed the *Ac* and or *Li* gene indicating a variation in gene copy number. Hughes et al. (1984) also found that homozygous individuals at either locus produced twice the amount of cyanoglucosides or enzyme than the heterozygous plants produced, indicating an allele dosage effect. Other factors that affect the amount of cyanoglucoside, linamarase and eventual HCN produced by individuals include developmental, physiological and ecological factors (Vetter, 2000). In Eucalyptus, *Eucalyptus cladocalyx,* Gleadow and Woodrow (2000) found that more cyanogenic glucosides were produced in younger vegetative and reproductive tissues. Younger leaves on the stem were more cyanogenic than older leaves and they concluded the inverse relationship between leaf age and HCN production was not due to a dilution effect caused by an increase in leaf area. In white clover, leaf age, plant size and time of the year are also important factors in the level of HCN produced (Rogers & Frykolm, 1937; Hayden & Parker, 2002). In a study conducted by Hayden and Parker (2002), drought stress was found to negatively affect linamarase activity, but had no effect on cyanoglucoside concentration while low temperatures decreased both the amount of cyanoglucoside produced and enzyme activity. In addition, a clinal variation has been found in white clover with lower frequency of cyanogenic genotypes occurring in colder and higher latitudes (Caradus, 1994).

Improved white clover persistence and production have been found to be highly correlated with cyanogenesis in white clover plants (Caradus & Williams, 1989; Crush & Caradus, 1995). A study by Crush and Caradus (1995) showed that many successful NZ cultivars including 'Grasslands Demand', 'Grasslands Sustain', ‘Grasslands Pitau' and 'Grasslands Prestige' were all highly cyanogenic. Caradus et al. (1990) reported a positive correlation between cyanogenesis and leaf size, with lines possessing larger leaves being more cyanogenic, which could explain the increased yield. An exception to this is the Ladino cultivars which are large leaved but typically acyanogenic (Caradus, 1994). However, studies by Noitsakis and Jacquard (1992) showed that acyanogenic lines had more biomass accumulation, suggesting that the production of HCN comes at a cost of biomass production. The persistence conferred by cyanogenesis has been linked to the plants’ ability to evade pests, Caradus (1994) found damage by slugs and weevils greatest on cultivars with low cyanogenic levels proving that high levels of HCN act as a pest deterrent. This supported the logical conclusion that cyanogenic glucosides form part of a plant’s defence against herbivores due to their bitter taste and the release of toxic HCN upon tissue rupture (Olsen et al., 2008). Interestingly, Brattsten et al. (1983) found the larvae of the Southern army worm (*Spodoptera eridania*) preferred and grew better when grazing on cyanogenic plants. Bishop and Korn (1969) also reported no discriminatory feeding behaviour by sheep and other animals on cyanogenic or acyanogenic plants.

The presence of cyanogenic glucosides in white clover has a negative effect on iodine and selenium metabolism in livestock and is implicated in causing nutritional myopathy in lambs (Crush & Caradus, 1995). Animals that ingest low amounts of cyanide are able to detoxify most of it through the action of rhodanese, also called thiosulfate sulfurtransferase; a mitochondrial enzyme that converts cyanide to less toxic thiocyanate and excreted in the urine (Tegzes et al., 2003). Mortality as a result of cyanide toxicity in pasture is, however, uncommon in New Zealand, and numerous authors (Doak, 1933; Caradus & Williams, 1989; Crush & Caradus, 1995) report a positive relationship between improvement in agronomic performance and increased cyanogenesis. Nevertheless, benefits of HCN production are not limited only to herbivore defence, studies show that other physiological functions like nitrogen transportation (Møller, 2010), signalling and stress mediation (Siegień & Bogatek, 2006) may be associated with HCN production.

2.3.4. Role of Agronomy, Cultivar Type and Pasture Management on DM Yield

Proper pasture management, to a large extent, influences the growth and maintenance of the sward (Van Keuren & Hoveland, 1985). White clover plant morphology and growth is determined by several factors, including cultivar type, climate, soil type, companion species, type of grazing (i.e. rotational grazing or set-stocking), and type of livestock on pasture (Brock et al., 1988; Nolan et al., 2001). Studies comparing grazing systems have reported larger size and dry matter of white clover plants under rotational grazing by sheep than in set-stocked systems. This is because rotational grazing fosters greater growth between grazing by allowing time for recovery of leaf area (Brock et al., 1988; Hay et al., 1989; Harris 1993).

When comparing pasture grazed by dairy or sheep, Hay et al. (1983) found pasture grazed by cattle to contain two to three times more stolon mass than pasture continuously grazed by sheep. This was confirmed by Nolan et al. (2001), who found that pasture grazed by cattle alone resulted in higher clover content (13·5%) compared with cattle and sheep (9·5%) or sheep alone (4·9%). Harris (1993) also reported 70% greater stolon dry weight per plant under dairy pastures. However, the number of stolons and growing points per plant were observed to be fewer than in sheep pastures. The consensus is that, since white clover is preferentially grazed by sheep in a mixed sward, small-leaved varieties are suitable for continuous, hard sheep grazing while large-leaved cultivars are best for less severe cattle grazing (Mather et al., 1996; Cai et al., 2014). Nevertheless, some farmers prefer to sow a mixture of small and large leaved cultivars in the same sward.

Other factors like inter-specific competition between white clover and the companion grass are also important in mixed pastures. Management practices that favour rapid grass tillering require longer defoliation intervals and nitrogen fertiliser application. This results in increased growth and spread of the companion grass tiller density, thereby placing white clover growth and persistence in a vulnerable position (O'Connor, 1982; Caradus & Williams, 1989; Woodfield & Caradus, 1996). Höglind and Frankow-Lindberg (1998) found that nitrogen application not only reduced the accumulation of dry matter in white clover but also reduced the number of stolon branches subsequently causing establishment failure. They found that majority of the morphological changes occurring were as a result of the decrease in amount of photosynthetically active radiation within the canopy as the R:FR ratio of light decreases with grass herbage increase. White

clover, therefore, has to be efficient in competing with the companion grasses which are often dense and vigorous, to prevent excessive shading and ensure optimum resource acquisition (Gibson & Cope, 1985).

2.4. Selection in Plant Breeding

The primary aim of selection in breeding is to change the distribution of gene frequencies underpinning traits of interest in the population (Falconer, 1989). Due to the self-incompatibility system and heterozygosity in white clover, desirable traits are difficult to fix and breeders rely on recurrent selection to fix wanted traits in breeding populations while maintaining genetic diversity for all other traits (Cope & Taylor, 1985). The first step in any plant breeding programme is to establish a breeding pool or base population from the available genetic resources. These genetic resources may range from wild collections of germplasm to commercial cultivars. A breeding pool is generated by crossing a range of germplasm that will contribute the genetic diversity needed to achieve a set of breeding objectives. The next step is the identification and selection of superior individuals associated with the breeding objective, from the breeding pool to advance to the next generation (Acquaah, 2012).

Phenotypic selection has been the major driver of conventional breeding and has so far been efficient in accomplishing various breeding goals. However, for traits that are difficult or expensive to measure; in particular, traits with large G×E and low heritability, phenotypic selection has not been as successful (Moose & Mumm, 2008). Unfortunately, genetic improvement in forage breeding has not been as successful as in grain crops which have high rates of genetic gain estimated at 13.5% per decade compared to 4% per decade in forages (Humphreys, 1997). This could be explained by forage crops having no defined harvest index and the use of inefficient selection methods that do not fully utilise the additive genetic variation within HS or full-sib families (Casler & Brummer, 2008). The success of selection depends greatly on the genetic variation for the trait observed (Fisher, 1918). The total phenotypic expression of any individual in a population is a combination of its genotypic value (G), environmental effect (E) and a complex interaction of both, (G×E) (Equation 1) (Falconer, 1989).

Hence the phenotypic variation σ_P^2 in a population is given as;

$$\sigma_P^2 = \sigma_G^2 + \sigma_E^2 + \sigma_{GE}^2 \quad (1)$$

Where: σ_P^2 is the trait phenotypic variation; σ_G^2 is the portion of trait variation attributed to genotypic variation; σ_E^2 is trait variation attributed to environmental effects; and σ_{GE}^2 is trait variation attributed to G×E interaction.

According to Fisher (1918) and Falconer (1989), the genotypic variance in a random mating population can further be partitioned due to the average effects of genes (additive variation), allelic interactions (dominance variation), non-allelic interactions, or epistatic effects (Equation 2).

$$\sigma_G^2 = \sigma_A^2 + \sigma_D^2 + \sigma_I^2 \quad (2)$$

Where: σ_A^2 is the additive variation; σ_D^2 is the dominance variation; and σ_I^2 is the interaction effect.

2.4.1. Response to Selection

Response to selection is the difference between the trait mean value of the parental population and the trait mean of the offspring (Falconer, 1989). The relative efficiency of different selection strategies can be evaluated by estimating the response to selection realised per cycle of selection (Hallauer & Filho, 1981). Knowledge of the heritability of the trait in question is needed to predict the response to a single generation of selection (Walsh, 2001). Heritability is the ratio of genetic variation to total phenotypic variation (Falconer, 1989) and is the measure of the proportion of the observed variation in a progeny that is inherited from the parents to offspring (Nakaya & Isobe, 2012). Plant traits with a higher heritability can be improved much faster than those with lower heritability (Nyquist & Baker, 1991). Furthermore, measures of heritability are useful in determining the appropriate selection strategy and breeding scheme to implement, a higher heritability means simpler methods like mass selection will be effective while family-based methods are more useful for traits with low heritability (Nyquist & Baker, 1991).

Values of heritability (h^2) can range from 0 to 1 and are used to express the reliability of the phenotypic value to estimate breeding value (Falconer, 1989). Heritability is population specific and it depends not only on additive and non-additive genetic factors but also on the environmental variance (Nyquist & Baker; Visscher et al., 2008). Factors

like allele frequencies, mode of gene actions and environmental variables can differ between populations, thereby affecting heritability estimates (Visscher et al., 2008).

Two forms of heritability have been defined by Lush (1937); broad-sense and narrow-sense heritability. Broad-sense heritability is estimated using the total genotypic variation and includes additive, dominance and epistatic effects (Equation 3). (Bernardo, 2002). Broad-sense heritability is a comparatively poor predictor of potential genetic gain or breeding progress with its usefulness contingent on the method of propagation and type of population (Acquaah, 2012). It is particularly useful for asexually propagated crops, where both additive and non-additive gene action are fixed and can be passed to progeny. Woodfield and Caradus (1990) reported high broad sense heritability estimates for white clover leaf length and width, and stolon internode length. To determine heritability for various white clover morphological attributes, clonal replication has been used to estimate broad-sense heritability, while genetic experiments involving diallel crosses and parent-offspring regression and correlation have been used to estimate narrow-sense heritability (Jahufer et al., 2002).

$$h_b^2 = \frac{\sigma_G^2}{\sigma_P^2} \quad (3)$$

Narrow-sense heritability (Equation 4) on the other hand, accounts only for additive genetic effects. It is important in determining the response to selection, if the narrow-sense heritability for a trait is high, genetic improvement of the trait is more easily accomplished (Nyquist & Baker, 1991). The HS family structure is able to efficiently isolate additive genetic effects from the other variance components such as dominance and maternal effects (Fabbro et al., 2007). Additive variation is important because it can be selected for and fixed in plant breeding (Acquaah, 2012). Annicchiarico et al. (1999) estimated the narrow-sense heritability of forage yield components in the range 0.40 - 0.70. Nonetheless, for traits with low narrow-sense heritability, family selection methods, such as full-sib and HS, make use of genotype replication and the partitioning of environmental and genetic effects to reduce error (Nguyen & Sleper, 1983).

$$h_n^2 = \frac{\sigma_A^2}{\sigma_P^2} \quad (4)$$

Where: h_n^2 is the narrow-sense heritability; σ_A^2 is the additive variation; and σ_P^2 is the trait phenotypic variation.

To determine the response to selection, (*R*), the selection differential (*S*) which is defined as the difference between the mean of selected parents and the mean of the population from which the parents were selected multiplied by the narrow-sense heritability (Equation 5) (Falconer & Mackay, 1996).

$$R = h_n^2 S \quad (5)$$

Where: R is the response to selection; h_n^2 is the narrow-sense heritability; and S is the selection differential.

2.4.2. Methods of Enhancing Genetic Gain in Conventional Breeding

The general plant breeders' equation used for predicting genetic gain (ΔG) is shown below (Equation 6) (Fehr et al., 1987). The genetic gain equation is a much-used reference for comparing the predicted effectiveness of particular breeding schemes and resource allocation (Jahufer et al., 2002; Moose & Mumm, 2008). Genetic gain can be expressed on the basis of per breeding cycle or on a per year basis by dividing by the number of years taken to complete one cycle (Eberhart, 1970).

$$\Delta G = \frac{kc\sigma_A^2}{\sqrt{\sigma_{ph}^2}} \quad (6)$$

Where: ΔG is the genetic gain or genetic advance; k is the selection intensity; σ_A^2 is the additive variation; *c* is the parental control. which indicates the level of control the breeder has over the parents in a mating. Control over one parent (eg in a HS mating) gives a value of 0.5, over both parents (eg full-sib) 1.0, and a value of 2.0 for clones or selfed seed); σ_A^2 = additive variation; and σ_{ph}^2 is the phenotypic variance of the parental population.

The total additive genetic variation in HS families, generated from a randomly mated population of parents, is distributed among and within families. One-quarter of the additive variation is distributed among the families and the remaining ¾ is found within families (Falconer 1986). Therefore, among HS family selection will access ¼ of the total

additive variation. Application of a breeding method that also exploits the ¾ within-family variation will thus increase the magnitude of genetic gain (Vogel & Pedersen, 1993). Among-and-within-half-sib-family selection (AWF-HS) uses within family selection from the best performing families to utilise the remaining ¾ of the additive variation within half-sib families. The most common application of AWF-HS selection involves establishment of large spaced-planted nurseries so phenotypes can be assessed efficiently (Vogel & Pedersen, 1993). Individual plant data has to be collected and used to select the best plants within the best families (Casler & Brummer, 2008). High within-family selection intensity has increased forage seed yield by up to 25% (Sandha & Twamley, 1973). However, with this method, since the trait of interest must be assessed before selection within families, it is only useful for traits that can be measured in early growth stages, otherwise it becomes expensive to maintain all individuals to maturity before selecting desirable phenotypes.

Another form of the breeding equation has been proposed by Casler and Brummer (2008) to estimate genetic gain based on among-HS family (Equation 7) and also among and within-HS family selection (Equation 8):

$$\Delta G_{HSF} = \frac{k_F c \frac{1}{4}\sigma_A^2}{\sigma_{PF}} \tag{7}$$

$$\Delta G_{AWF-HS} = \frac{k_F c \frac{1}{4}\sigma_A^2}{\sigma_{PF}} + \frac{k_W c \frac{3}{4}\sigma_A^2}{\sigma_{PW}} \tag{8}$$

Where: ΔG is the genetic gain per cycle derived via among HS family selection; k_F refers to the selection intensity or the standardized selection differential among families, c is the parental control factor; σ_A^2 is the additive variance; σ_{PF} is the phenotypic standard deviation among families; $\Delta G_{AWF\text{-}HS}$ is the genetic gain derived from among and within HS family selection per cycle; k_W is the standardized selection differential within families; σ_{PW} is the phenotypic standard deviation within families.

Increasing selection intensity depends on the proportion of the population selected, the size of the whole population and the amount of phenotypic variation available in the population. If fewer individuals are selected then the selection intensity is increased (Marshall 2008). However, if the population size is not increased as selection intensity is increased, inbreeding can occur due to the reduction in genetic diversity in subsequent populations (Bernardo, 2002). Selection, therefore, is a balance between applying the appropriate intensity to move the trait mean value in the progeny while also maintaining genetic diversity; a key consideration in species prone to inbreeding depression.

Accuracy of selection refers to the accuracy of selecting an individual based on its genetic merit of the desired trait (Simm, 1998). To increase selection accuracy, multi-location and replicated trials serve to separate genetic effects from prevalent environment effects. Breeders rely on utilizing among and within family selection, progeny testing as previously discussed and more recently, marker assisted selection.

Increasing the additive genetic variance and associated heritability can be achieved through a number of ways. For example, by increasing the parental control and being able to pre-determine and select potential parents before pollination enables the isolation and crossing of the selected plants. This means that only the selected plants contribute alleles to the next generation (Fehr, 1987). Also, by increasing the diversity of the base population and inbreeding before evaluation, additive genetic variation can be increased. However, this may not be an option for outcrossing species like white clover.

2.5. Estimation of Breeding Values

Variance component estimates can be derived from phenotypic data of populations with appropriate structures using linear mixed model methods such as Analysis of Variance (ANOVA) and Residual Maximum Likelihood (REML) analysis. Line means can be estimated using the Best Linear Unbiased Estimation (BLUE), which assumes fixed line effects, or by estimation of adjusted means, Best Linear Unbiased Prediction (BLUP), when lines or families being evaluated are considered as random effects. Given by the model below (Equation 9);

$$y = X\beta + Zu + \varepsilon \qquad (9)$$

Where: y is the vector of observations; β and u are vectors of fixed and random effects respectively; X and Z are design matrices for β and u and ε is the random residual vector. When genotype effects are taken as fixed, they are represented by β in the model, and when regarded as random, genotypic effects become part of u (Piepho et al., 2008). Normally, in plant evaluation trials, cultivars are treated as fixed factors, while years and locations are random factors. However, due to the fact that many plant breeding data are obtained from various environments and often unbalanced with new entries added regularly, several studies have shown that BLUP, in which lines or families are considered as random effects, tends to provide more accurate estimates of genotype effects than BLUE (Bauer et al., 2006; Piepho & Möhring, 2006; Piepho et al., 2008; Viana et al., 2014). This is because BLUPs are able to account for missing data and environmental factors in the model. When investigating variation within a population, genotypes are best considered as random and Piepho and Möhring (2006) found bias of variance component estimates to be higher when cultivar main effects were considered as fixed. However, if the aim is only to investigate differences among the genotypes and not any reference populations, then genotypes can be considered as fixed effects (Smith et al., 2005). The major advantage of using BLUP is the shrinkage of observed progeny estimates towards the population mean, thereby increasing accuracy by reduced variance and smaller mean squared error (Piepho et al., 2008). BLUPs are calculated for each trait in individual lines and selections are then made based on their estimated breeding values.

2.5.1. Genotype-by-Environment Interaction Effects on Selection

Generally, plant breeding programmes and cultivar evaluation trials are carried out across multiple locations over two to three years. These multi-location and year trials enable reduction of the confounding effects of G×E interactions (Byth, 1981; Cooper & Byth, 1996; Ríos, 2015). Significant G×E interaction exists for many traits, especially complex, polygenic traits. When comparing phenotypic variance components, Arief et al. (2019) reported that the estimates of genotype-by-year and genotype-by-year-by-location variances were the largest components contributing to phenotypic variance. In plant breeding programmes G×E interactions are evident when genotypes re-rank in their relative performance across test environments (Byth, 1981; Fehr et al., 1987). These interactions are known to occur in two forms; first, differences between the genotypes exist in the test environments but the rankings of the genotypes remain constant and genotype performance can be predicted across environments. The second, and most

important is the presence of a "crossover effect" where genotype rankings change with environment (Bernardo, 2002). Significant G×E interactions associated with re-ranking of entries makes prediction of their performance across environments unreliable (Cooper & Byth, 1996). Evaluation of genotypes in multiple environments with appropriate randomization and replication has been the most efficient way to address this issue. However, this is often expensive and time consuming (Moose & Mumm, 2008).

The traditional approach to analysing G×E interaction is the two-way ANOVA model where genotype, environment, and their interaction are treated as fixed effects in the model (Equation 10):

$$y_{ijk} = \mu + g_i + e_j + ge_{ij} + \varepsilon_{ijk} \quad (10)$$

Where: y_{ijk} is the k-th record for the i-th genotype in the j-th environment; μ the overall mean; g_i is the fixed effect of the i^{th} genotype; e_j is the fixed effect of the i^{th}; ge_{ij} is the interaction effect; and ϵ_{ijk} the residual error term (Meyer, 2009).

The ANOVA model is limited by the requirements of many data points or replicates to accurately predict interaction. Another approach is to use REML analysis that generates BLUP estimates, treating genotypes, replicates ,environments and G×E as random effects (Piepho et al., 2008). Hu (2015) compared ANOVA to BLUP in oilseed rape multisite trials and discovered the BLUP model provided both high precision and efficiency in predicting location-specific genotype effects. ANOVA estimates of variance components were found to only be unbiased when data was balanced.

2.6. Molecular Markers for Plant Breeding

The use of molecular markers has revolutionised plant breeding, supporting many selection decisions and aiding in the assessment of plant variation. Several types of molecular markers have been developed and include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), microsatellites (Simple Sequence Repeats; SSRs) and Single Nucleotide Polymorphisms (SNPs) (Xu, 2010). RAPD and AFLP are dominant markets, scored as presence/absence, thereby ensuing difficulty in identifying alleles at the same locus. On the other hand, RFLP, SSRs and SNPs are co-dominant markers, enabling identification of allelic variants at the same locus. Simple sequence

repeats have been popular in white clover genotyping. A comprehensive integrated genetic linkage map was produced by Griffiths et al. (2013) using SSRs and gene-specific markers. Four other genetic linkage maps have also been published by other authors (Jones et al., 2003; Barrett et al., 2004; Zhang et al., 2007; Isobe et al., 2012). Single nucleotide polymorphisms are single-base variations in any of the bases at the same point in the genome among individuals (Hayes et al., 2009c). They are becoming the most commonly used marker in many plant breeding programmes due to their high-density and direct association with traits (Foolad & Panthee, 2012). Their discovery in allotetraploid genomes is however complicated due to the presence of homoeologous genomes (Logan-Young et al., 2015). Sequence variation between homoeologues confounds SNP discovery by giving the appearance of a SNP when there is no Mendelian segregation (Page & Udall, 2015). In the absence of a quality reference genome to assign homoeologues, distinguishing these variants from true SNPs between homologues requires significant investigation within segregating populations (Young & Udvardi, 2009; Kaur et al., 2012).

2.6.1. Genotyping by Sequencing (GBS)

Reduced library representation methods are alternative platforms that involve the selection of a subsample of the genome for sequencing. They have quickly become a popular method to identify SNPs among individuals. Genotyping by Sequencing is a restriction enzyme-based library reduction method that uses a restriction enzyme to generate genomic fragments, to which a unique DNA barcode is attached (Elshire et al., 2011). Samples are then pooled and size selection is performed to obtain a library which is sequenced by parallel high-throughput methods (Beissinger et al., 2013). The advantages of GBS include reduced sample handling, simultaneous marker discovery and the ability to multiplex samples by use of unique barcodes (Elshire et al., 2011; Glaubitz et al., 2014). Genotyping by Sequencing is a particularly attractive option for breeders who only have a short period of time in breeding cycles to obtain and utilise GEBVs (Jacobs, 2018). The original GBS approach can be modified to allow for a double digest using two different restriction enzymes, a rare and a frequent cutter (Poland & Rife, 2012). This approach facilitates the capture of more fragments by cleaving larger fragments to a size more able to be sequenced (<500 bp) and avoids repetitive regions resulting in easier and direct bioinformatic analysis for large genomes (Wong et al., 2015). This method has been applied to a large number of plant, animal and bacterial

species, and is an ideal assay to simultaneously discover SNPS and generate genotypes for multiple individuals in species where there are limited genomic resources.

A challenge often encountered using GBS is the high occurrence of missing data, low read depth and insufficient number of reads produced (Davey et al., 2011; Glaubitz et al., 2014; Schröder et al., 2016). Read depth, which is the average number of times a locus is sequenced, is particularly important as it increases the accuracy and confidence of correctly calling individual genotypes from the genomic data (Gorjanc et al., 2017). Because mutational variation in allopolyploids like white clover is a result of homologous and homoeologous sequence variation as well as paralogous variation between duplicated gene copies, accurately differentiating between these classes is essential for SNP validation (Hand et al.; Kaur et al., 2012). In GBS, like many other genotyping methods, high sequence error rates and low depth makes it difficult to differentiate between homoelogues (nucleotide variation between the different subgenomes) and homologues (nucleotide variation within a subgenome) SNPs in downstream analysis (Hand et al.; Dufresne et al., 2014). Schatz et al. (2010) recommended a sequencing depth of 15x to 30x for accurate detection of SNPs. Ashraf et al. (2014) suggested a minimum average sequencing depth of 5–10x as sufficient to overcome the problem of too many missing data points. Most times, however, it is a trade-of between read depth and number of desired SNPs (Kim et al., 2016).

Some of these problems can be overcome by reducing the multiplexing level, using rare cutters, sequencing to higher depth, sequencing the library multiple times, filtering the data and using imputation algorithms (Poland & Rife, 2012; Rocher et al., 2015; Kim et al.). Sequencing the same library multiple time achieves the same purpose with the downside of increasing per-sample cost and for breeding programmes seeking cost-effective platforms, the other approaches are preferable (Poland & Rife, 2012). Another approach suggested is a fragment size selection step after library preparation to achieve a reduced fragment pool that can be sequenced at higher depth by selecting the optimum length for sequencing (Schröder et al., 2016). Wong et al. (2015) suggested DNA quality as a likely factor because poor-quality DNA affects digestion by restriction enzymes and subsequent PCR amplification causes overrepresentation of PCR fragments from good quality samples. Numerous authors, however, have validated the efficacy of GBS for SNP generation and the suitability for GBS markers in developing genomic selection models for accurate trait prediction (Poland & Rife, 2012; Li et al., 2015; Faville et al., 2018).

2.6.1.1. Restriction Enzyme Choice

Choosing the appropriate restriction enzyme is a critical step in developing a GBS protocol for an organism (Elshire et al., 2011). Use of restriction enzymes provides an easy way to reach inaccessible regions of the genome (Elshire et al., 2011). This is especially important given that a large proportion of QTLs might be located outside coding regions (Ruvinsky & Graves, 2004) or in promoter or enhancer regions (Hrdlickova et al., 2014). Frequent cutters like four or five base cutters (e.g, *Ape*KI) tend to deliver more markers and higher genome coverage but at a lower depth than less frequent cutters (e.g six base cutter *Pst*I) that have less genome coverage due to fewer recognition sites in the genome but higher depth (Poland & Rife, 2012; Hamblin & Rabbi, 2014; Schröder et al., 2016). In the absence of a size selection step during library construction, restriction enzymes maximize the proportion of fragments that fall within the desired size range (100–400 bp) as fragment proportions outside this range fail to be bridge-amplified during Illumina sequencing (Sonah et al., 2013; Hamblin & Rabbi, 2014). Restriction enzymes that produce overhangs or sticky ends and are methylation-sensitive are desirable for GBS because repetitive regions of the genomes can be avoided and gene-rich lower copy regions can be targeted (Gore et al., 2009; Poland & Rife, 2012).

2.6.1.2. Adapter Design and Concentration Optimization

Most next-generation sequencing platforms require a library preparation with the ligation of specific adapter oligonucleotides to fragments of the DNA to be sequenced (Syed et al., 2009). The proper ratio of adapters to genomic DNA has to be optimised for each species prior to library construction by titrating a fixed amount of digested DNA to varying adapter concentrations (Elshire et al., 2011). The presence of too many adapters form adapter dimers which are the result of self-ligation of the adapters without a DNA sequence (Head et al., 2014). Meanwhile, a scarcity of adaptors results in DNA fragments ligating to themselves, and these dimers form clusters which take up space on the flow cell without producing useful data (Head et al., 2014). If the adapter concentration is too low, then the restricted fragments will not be saturated with adapters, which results in only a subset of the fragments being amplified for sequencing. When this occurs, a different subset of fragments may be sequenced in different replicates of the same individual which reduces the consistency of SNPs discovered and genotyped. Optimising

adapter concentration to ensure fragment end saturation is likely to improve consistency of SNP discovery.

Two different types of adapters are used: barcode and common adapter. The barcode adaptor terminates with a 4 to 8 bp unique barcode on its top strand and a 3 bp overhang on its bottom strand that is complementary to the sticky end generated by the restriction enzyme (Elshire et al., 2011). The presence of the barcode enables individual samples to be identified after a pooling stage. The barcoded adapter is ligated to one side of the cut DNA fragment and a common adapter, also complementary to the restriction enzyme cut site ligates to the other side of the fragment. To aid in GBS efficiency, the barcoded adapters are designed to be at least three base pairs different from all others and essentially not contain the restriction enzyme cut site (Davey et al., 2011). During sequencing, the common adapter binds to the flow cell prior to the first round of bridge amplification; therefore, fragments with only barcode adapters are flushed from the sequencing flowcell. Fragments with only common adapters will only amplify in a linear fashion during the PCR phase, whereas those with a barcode adapter and common adapter will amplify exponentially and be represented in the sequence data.

2.7. Genomic Selection

While most gains achieved in white clover have been due to conventional breeding and selection, with the discovery of molecular markers, plants that carry genes responsible for desirable traits can be selected with the use of markers identifying the particular genome location influencing the trait (Faville et al., 2012). Genomic selection establishes associations between markers and phenotypes based on information from genotype and phenotype data in a training population and estimates a breeding value for selection candidates (Wang et al., 2018). It utilises marker data by either using the markers to build a genomic relationship model between individuals via an estimation of the proportion of the genome a pair of individuals have in common or by determining the effect of each marker on the trait of interest and adding the effects to get the estimated breeding values of each individual (Heffner et al.; Gezan et al., 2017).

While Marker-assisted selection (MAS) has been especially successful for introgressing monogenic and major effect genes (Dekkers & Hospital, 2002), unfortunately, small effect genes underlie numerous complex polygenic traits. According to Jannink et al. (2010), this failure of MAS is due to the two-step method of first identifying QTL and

then estimating their effects. This separation means that estimated effects will be biased and small-effect QTL will be disregarded due to the high stringent thresholds put in place as MAS typically assigns a significance threshold to markers (Jannink et al., 2010). Genomic selection on the other hand, makes use of all available genetic markers without assigning a significance threshold (Meuwissen et al., 2001). Another contributing factor is that many QTL studies use bi-parental populations that are not necessarily representative of the allelic diversity in breeding programmes (Jannink et al., 2001). Moreover, because the phenotypic variation that marker loci define is often non-additive and a function of G×E effect, the efficiency of MAS, to predict genetic gain accurately, is often challenging (Staub et al., 1996). When compared to classical recurrent selection, a potential advantage of genomic selection is that it allows for more recombination events due to shorter crossing cycles per unit of time, thereby producing more useful variation in the population (Heffner et al., 2009; Müller et al., 2017).

In the past decade, there have been dramatic reductions in genotyping costs while phenotyping still presents a bottle neck to breeding progress due to increasing time and labour costs (Heslot et al., 2015). Unlike animal breeding, where a majority of phenotypes are collected automatically as part of the industry, e.g., carcass parameters from meat works and milk quality and quantity, phenotyping in plants is generally manual, laborious and prone to human errors. The efficiency of genomic selection relies on the availability of precise phenotype information to accurately estimate marker effects. Collecting these phenotypes in a cheap and cost-effective method, further increases the cost-effectiveness of genomic selection.

Although genomic selection was first initiated in dairy cattle breeding, it is increasingly being applied in plant breeding with many studies based on simulation data and populations derived from crosses of bi-parental lines (Bernardo & Yu, 2007a; Habier et al., 2010). The application of new genomic-assisted breeding techniques can increase the efficiency of conventional breeding strategies by enhancing the precision of selection, reducing the time frame for releasing new cultivars and enabling selection of candidate individuals without the influence of the environment (Iwata & Jannink, 2011; Lorenz et al., 2011; Resende et al., 2012a). In perennial crops like white clover where important stolon traits pertinent to persistence are usually not measured until later growth stages, exploiting genomic selection will prove useful.

To perform genomic selection, a reference or training population of individuals with genotypic and phenotypic information is used to calibrate a statistical model which is subsequently used to estimate breeding or genotypic values of potential selection candidates based on genotypic information alone (Meuwissen et al., 2001; Bassi et al., 2016). The predictive ability of a model is estimated as the Pearson's correlation coefficient between observed phenotypic value and predicted phenotype. In some cases, genotypic data and phenotypic data are obtained from a different set of individuals, for instance, in dairy cattle breeding where productive traits of breeding sires are estimated from daughter phenotypic records. In this study, the training population is only genotyped and another group of individuals, obtained from crosses of the training population provide the phenotype data. A statistical model is then derived using genotype and phenotype information from both populations (Figure 2.2). Another sub-set of individuals, the selection population (derived from remnant seed of the training population) is genotyped, GEBVs are generated and individuals are selected based on their GEBVs. To test the prediction equation, a population grown from crosses of the selection population can be phenotyped and tested to determine the accuracy of the genomic prediction model. In the case of perennial forage crops like white clover, the breeding values of maternal parents are inferred by testing their progeny in field trials. It is therefore ideal, in genomic selection, for genomic prediction models to be based on the genotype data of maternal parents and phenotypic data of HS progeny in order to mirror real breeding systems (Annicchiarico et al., 2015).

Genomic selection allows breeders to exploit both among and within family variation. The lack of access to within-family genetic variation has been identified as a major reason for the poor genetic gain in forages, as ¾ of the additive variation is located within-families (Casler, 2008; Resende et al., 2014). Also, by making use of these untested parents whose breeding values have been inferred by their progeny phenotype, the breeding cycle can be reduced, and more genetic gain can be accomplished per unit time (Robertsen et al., 2019). One possible implication of this is that relating the phenotype data of a mixture of genotypes in a sward to the genotypic data of parent plants might decrease the predictive ability (Grinberg et al., 2016).

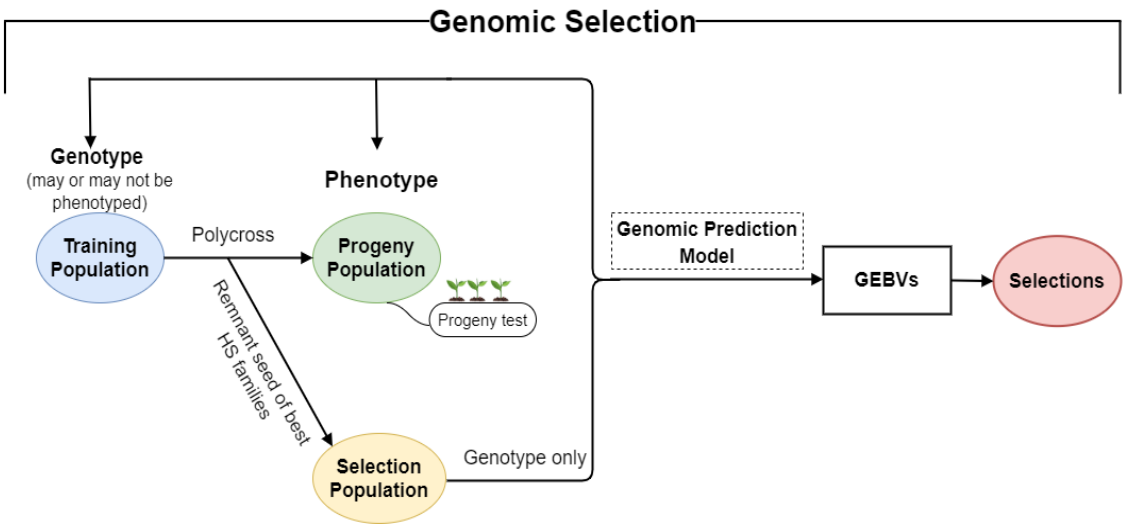


Figure 2.2: Simplified diagram of genomic selection implementation in a half-sib forage breeding programme.

2.7.1. Genetic gain in Genomic Selection

An extension of the breeder's equation earlier discussed is given below;

$$\Delta G = ir\sigma_A/T \qquad (11)$$

Where: ΔG is genetic gain; i is the selection intensity; r is the selection accuracy; σ_A is the square root of the additive genetic variance and T is the length of time to complete one breeding cycle.

Genomic selection aims to improve the annual rate of genetic gain by increasing the selection intensity and accuracy, which increase the numerator value in equation 11 and by reducing the generation interval, the denominator value (Bassi et al., 2016). By evaluating greater numbers of candidate parent plants, selection intensity is increased. Selection accuracy is enhanced by selecting based on the genotype rather than phenotype, thereby avoiding the environmental and other interactions. It has been shown that the greatest advantage afforded by genomic selection is in decreasing time per selection cycle. Wong and Bernardo (2008) reported a potential reduction in selection cycle from 19 to 6 years in Oil palm. In white clover, ample time can be saved by eliminating the need for progeny testing before selection.

2.7.2. Factors Affecting the Accuracy of Genomic Selection

The correlation between estimated and true breeding values can be explained by a linear relationship with the response to selection; hence, the Pearson correlation of true and predicted observed values is used to evaluate predictive ability (Daetwyler et al., 2013). When markers and QTL are in perfect linkage disequilibrium (LD), the expected accuracy (r) is determined by size of the training population, (N), heritability of the trait (h^2) in the population, and the effective number of loci or independent chromosome segments, Me (Daetwyler et al., 2008; Meuwissen, 2009) (Equation 12).

$$r = \sqrt{\frac{Nh^2}{Nh^2 + Me}} \qquad (12)$$

In a comprehensive comparison by Blondel et al. (2015), using data sets of different plant species, they found a poor correlation between Pearson correlation and ranking accuracy of individuals and proposed that genomic selection be performed as a ranking tool and less emphasis be placed on predictive ability. Their theory is based on the fact that since genomic selection is primarily for selection or elimination of best or worst candidates, it might be sufficient to correctly rank individuals from most to least favourable, because to select the best individuals, knowledge of their precise breeding values might not be necessary. However, predictive abilities are still important in assessing the response to selection (Isidro et al., 2015).

The accuracy of genomic selection ultimately depends on the heritability and number of loci affecting the trait, the number of individuals in the reference population, the interaction between genotype and environment, and choice of statistical method to estimate the GEBV (Daetwyler et al., 2007; Hayes et al., 2009b; Crossa et al., 2013a). Along those lines, the goals of a training population are to maximize marker variance, reduce co-linearity between markers and obtain a uniform sample of the available genetic in the breeding population (Jannink et al., 2010).

High prediction accuracies (0.71) for important traits have been reported in animal breeding (VanRaden et al., 2009). In plant breeding, real data accuracies are rarely as high, although for crop species with extensive LD like maize (*Zea mays*), Riedelsheimer et al. (2012) obtained an accuracy of 0.74 for maize biomass. In the case of forage species,

where LD is not as extensive, Faville et al. (2018) reported predictive abilities ranging from 0.07 to 0.43 for DM yield in five different ryegrass populations. Grinberg et al. (2016) obtained similar accuracies for DM yield (0.08 to 0.22) and highlighted highest accuracies were obtained for forage quality traits like water soluble carbohydrates (0.59). In alfalfa, *Medicago sativa*, a common leguminous forage, Annicchiarico et al. (2015) reported accuracies of 0.32 and 0.35 for DM yield in two genetically contrasting populations. Recently, Jia et al. (2018) obtained an accuracy for 0.13 for DM yield in alfalfa. For simpler traits in the same study, higher accuracies of 0.65 and 0.52 for plant height and flowering date respectively were obtained. Regardless of the accuracy level, research by (Collins & Rhodes, 1995; Crossa et al., 2013a; Heslot et al., 2015) validate the assessment that genomic selection outperforms phenotypic selection.

2.7.2.1. Trait Genetic Architecture

Traits with higher heritability have been found to have higher predictive ability (Muranty et al., 2015; Grinberg et al., 2016). Grinberg et al. (2016) observed a positive trend between simple forage quality traits like water soluble carbohydrate content with a higher predictive ability of 0.59 while yield related traits had significantly lower predictive ability (0.38). As trait complexity increased, Roorkiwal et al. (2016) found predictive ability to decrease in chickpea. Arojju et al. (2018) also reported a positive correlation between the predictive ability for crown rust resistance in ryegrass and heritability. When they compared different sub-populations of full-sibs, HS families, ecotypes and cultivars, full-sib families had the highest heritability for crown rust resistance among all sub-populations and obtained the highest predictive ability. By contrast, Grattapaglia and Resende (2011) found no significant change in accuracy with an increase in heritability from 0.2 to 0.6 for different eucalyptus tree traits. They highlighted, however, that a large training set of 1000 individuals was used. Meuwissen et al. (2001) reported larger training sets ameliorate decrease in accuracy due to low heritability.

2.7.2.2. Training Population Size and Environments Tested

Optimum population size has been found to vary from population to population. However, the consensus view is that larger training populations provide more accurate estimates of marker effects on phenotypes due to an increase in detection power (Wong & Bernardo, 2008; Grattapaglia & Resende; Crossa et al., 2013a; Iwata et al., 2013). Reviews from dairy cattle conclude that genomic selection accuracy seemed to increase

linearly with training population size (VanRaden et al., 2009). This is because a large training population allows for accurate estimation of small effects across the entire genome and capture all the genetic variation available. Upon reducing training population size from 90% to 10%, Arojju et al. (2018) observed a drop in predictive ability from 0.52 to 0.38. Similarly, Jarquín et al. (2014) found there was a steady decline in predictive ability as the number of individuals in a soybean training population reduced from 250 to 100 individuals. On deciding whether to use more lines and fewer number of replicates per line or fewer lines and more replications per line, Zhong et al. (2009) reported better prediction values for fewer lines with more replicates than more lines with fewer replicates. Wong and Bernardo (2008) were of a different opinion, asserting that increasing the population size is more important than increasing the number of replicates.

The accuracy of breeding values also depends on the strength of relatedness of individuals in the training population (Jannink et al., 2010). Habier et al. (2010) reported reduced predictive ability using unrelated or distantly related lines. Daetwyler et al. (2012) assessed the contribution of population structure to accuracy of genomic prediction and reasoned accuracy of genomic predictions benefits significantly from genomic relationships due to population structure. Therefore, failure to remove variation due to population structure might result in exaggerated prediction accuracies (Riedelsheimer et al.; Guo et al., 2014b; Isidro et al., 2015). However, population structure can be exploited if similar structures exist between the reference population and validation population, as in the cases of selection candidates being offspring of the reference population from which the population equation was derived (Habier et al., 2007b; Goddard, 2008; Habier et al.).

The number of environments the training population is tested in is also of significance because both marker-by-enviroment interaction effects and markers with stable effects across environments can be identified (Oakey et al., 2016). By incorporating a G×E interaction into genomic selection models, an increase in predictive ability can be realised as information from correlated environments can be exploited (Crossa et al., 2013b). Sukumaran et al. (2017) tested 287 wheat lines in 18 international environments for a variety of traits and found that including G×E interaction significantly increased predictive ability. By modelling G×E, Haile et al. (2019) increased the predictive ability by up to 66% for reproductive period in lentils. However, the breeding population must also be tested in those environments because significant G×E impairs the accuracy of

genomic selection when a model is trained in one site, and implemented/validated in another environment (Resende et al., 2012b). Though, as expected, Haile et al. (2019) reported no added advantage in including an environment interaction when trait heritability is high due the low environmental effect on the expression of such traits.

Environmental influence in the form of genotype–by–environment-by-year interaction also affects predictive ability and lines tested across years often show increasing predictive ability with the passage of time. Grinberg et al. (2016) found predictive ability for yield ground cover in perennial ryegrass to be lower in the first year than the second year.

2.7.2.3. Linkage Disequilibrium and Marker Density

Genomic selection makes use of genome-wide markers, therefore, it requires linkage disequilibrium (LD) to exist between marker loci and quantitative trait loci (QTL) in order to estimate associated marker allele effects (Hayes et al., 2009a; Jannink et al., 2010). According to Habier et al. (2013), linkage disequilibrium, linkage and genetic relationships all make contributions to the prediction accuracy. LD is said to occur when there is non-random association of alleles at two or more different loci while linkage refers to the tendency for alleles in close physical proximity on the same chromosome to be inherited together (Oraguzie et al., 2007). The extent of LD is significant in increasing or decreasing predictive ability because it determines the amount of genetic variance that can be captured by the markers (Hayes et al., 2013). As LD between QTLs and SNPs tends to decrease over generations, the reliability and accuracy of genomic prediction is expected to decrease if SNP effects are not recalibrated in subsequent generations (Muir, 2007). Calus et al. (2008) proposed that LD should be greater than 0.2 between neighbouring marker pairs to achieve a desired accuracy and with simulated data achieved an accuracy of 0.6 for predicting a trait with heritability of 0.1.

Increased marker number has been found to increase the prediction success as higher marker densities can better determine the strength of linkage disequilibrium between SNPs and genes or QTLs (Heffner et al., 2009; Habier et al., 2013). However, when the main contributor to predictive ability or accuracy was linkage or genetic relatedness, the impact of increased marker density was less evident (Habier et al., 2013). For inbreeding species with higher levels of LD and slow LD decay, fewer markers are needed than for outcrossing species where the rate of decay is faster (Jannink et al., 2010). de los Campos

et al. (2012) reported increasing accuracy of marker estimates with increased sample size, due to reduction in bias and variance of estimates. They also noted predictive ability increases steadily with increased marker density until it plateaus, the rate of which depends on the span of LD in the genome and sample size. Zhong et al. (2009) confirmed this in their study of genomic selection in barley inbred lines. They discovered that predictions were better when LD was high, but also that predictions were more accurate with greater marker densities, particularly more evident under low LD than high LD. Nakaya and Isobe (2012) suggested there was an optimum marker threshold above which marker accuracy declined. This was explained by Hickey et al. (2014) as an over-fitting of the genomic prediction equation with excess markers. In this situation, non-genetic effects due to environmental variance can be misinterpreted as marker effects. Meuwissen (2009) recommended the number of markers should be $10 \times Ne \times$ genome size in Morgans for achieving high GEBV accuracy, where *Ne* is population size. Worthy of mention, Zhang et al. (2019) discovered the predictive accuracy of simple traits controlled by large effect genes were more influenced by increased marker density than QTL traits.

2.7.2.4. Statistical Methods for Generating GEBVs

Genomic selection can be performed as a one-step or two-step process. In the two-stage approach, BLUPs are obtained through a linear mixed-model analysis and then fitted as the response variable in genomic selection models to predict GEBVs from SNP markers (Smith et al., 2005). The single step uses a mixed model design with raw phenotypic data from line replicates rather than line adjusted means (BLUPs). This enables the total genetic variation due to lines to be partitioned into variation due to markers and residual genetic variation, thereby enhancing estimation accuracy (Oakey et al., 2016).

To estimate breeding values, a variety of approaches have been proposed. Parametric methods like ridge regression–best linear unbiased prediction (RR-BLUP), GBLUP and Bayesian-based methods such as BayesA, BayesB, BayesCπ, and Bayes LASSO (Least Absolute Shrinkage and Selector Operator) (Asoro et al., 2011). Semi-parametric methods include reproducing kernel Hilbert spaces (RKHS), and non-parametric /machine learning methods include Random Forest (RF) and support vector machine (SVM) (Gianola et al., 2006; de los Campos et al., 2012; Heslot et al., 2012).

In the basic genetic model (Equation 13), phenotypic outcomes, $y_i (i = 1, \ldots., n)$ are seen as the sum of the genetic value $g_i$and a model residual ε_i;

Hence; $$y_i = g_{i+}\varepsilon_i \quad (13)$$

In the parametric models, g_i is described as a regression on marker covariates $x_{ij}(j = 1, \ldots . p$ molecular markers) given as Equation 14:

$$gi = \Sigma_{j=1}^{P} x_{ij}\beta_{ij} \quad (14)$$

Where: $\beta_{ij}$is the regression of the y_i on the j^{th} marker covariate x_{ij} (Meuwissen et al., 2001).

Since the number of SNP markers (*m*) vastly exceeds the number of phenotypic records (*n*), all methods rely on shrinkage estimation or variable selection to fit the regression (de los Campos et al., 2012). The methods differ mainly in prior assumptions they make about the distribution of the SNP effects with consequent implications on the distribution of QTL and the LD between SNPs and QTL (Hayes & Goddard, 2010).

2.7.2.4.1. Parametric Models

GBLUP and RR-BLUP assume equal variance of marker effects with each SNP effect drawn from a normal distribution with a constant variance (Meuwissen et al., 2001). Bernardo and Yu (2007a) pointed out that equal variance is not synonymous with all makers having the same effect, instead, marker effects are all equally shrunken toward zero. These two methods rely on kinship between individuals while others like the Bayesian models, are modelled on SNP-QTL associations to estimate effects of genetic markers (Habier et al., 2007a; Zhang et al.). This assumption of equal variance is, however, not realistic as all markers do not have equal variances and should not be treated equally (Xu, 2002). The Bayesian models like BayesA, BayesB, BayesC, BayesCπ and BayesLASSO assume that some markers have zero effect and variances differ across markers with non-zero effect (Meuwissen et al., 2001; Meuwissen & Goddard, 2010; Habier et al., 2011).

2.7.2.4.2. Non-Parametric Models

Machine-learning models like RKHS, SVM and RF make no strong assumptions about the properties and distribution of the parameters. For example, in RF, a collection of regression trees are grown from a sample drawn with replacement from the training set

using a random subset of predictors to define the best split at each node (Heslot et al., 2012). The predictive ability is obtained by averaging the predictions of all trees in the forest (Blondel et al., 2015). RKHS uses a kernel function to form a square matrix by converting markers into a set of distances between paired observations (Heslot et al., 2012). The goal of SVM is to train a model to assign new data into categories and it achieves this by mapping the data into a higher dimension and then produces categories with the largest possible separation (Heslot et al., 2012; Howard et al., 2014).

2.7.2.5. Model Performance

The abundance of models has inspired several comparison studies to find the best performing model in different situations (Meuwissen et al., 2001; Heslot et al., 2012; Howard et al., 2014; Annicchiarico et al., 2015; Roorkiwal et al., 2016; Crossa et al., 2017; Gezan et al., 2017; Liu et al., 2018). Models like RRBLUP and GBLUP which assume equal variance have been shown to have better predictive ability for traits controlled by numerous small effect loci, i.e. vegetative yield which is influenced by many small genes (Zhong et al., 2009; Zhang et al., 2019). On the other hand, Bayesian methods with differential shrinkage are better at predicting moderate to high heritability traits influenced by few QTL as they avoid over shrinking QTL with significant effects (Lorenz et al.; Lin et al., 2014). For example, Faville et al. (2018) and Grinberg et al. (2016) found GBLUP to give the highest predictive ability for yield related traits. Daetwyler et al. (2010) also confirmed that BayesB performed better than GBLUP when the number of QTL underlying a trait are small. Consequently, GBLUP and RR-BLUP have been recommended for use in crop species, due to their ease of application over the Bayesian approaches and their suitability for key traits, many of which are influenced by multiple small effect loci (Habier et al., 2010).

For complex non-additive traits, however, nonlinear models tend to increase predictive ability (Crossa et al., 2013a) and may be particularly suitable for nonlinear relationships between predictors and responses e.g. epistatic effects (Jannink et al., 2010). This is also important because the observed phenotype is as a result of not only additive effects but dominance and epistatic effects as well. Blondel et al. (2015) reported that tree-based ensemble methods like Random Forests and Gradient Boosting Regression Trees were more accurate at ranking individuals than traditional regression methods like GBLUP and Bayesian methods. That being said, most studies resort to using more than one model to

generate prediction equations and findings by Heslot et al. (2012) on testing 11 genomic selection models over eight different data sets on three crop species; wheat, barley and maize, reported similar predictive ability for all the models.

Most methods require a complete marker data set which leads to the need for imputation, the most common of which is mean or naïve imputation. However, a variation of GBLUP, KGD-GBLUP (Kinship with depth adjustment) by Dodds et al. (2015) circumvents this problem and creates an unbiased matrix of relationships among individuals including those with zero depth. This is especially useful for marker systems that not only have high rates of missing data but also produce low read depth data like GBS (Dodds et al., 2015). On comparing KGD with other methods like GBLUP, RRBLUP and RF, Jacobs (2018) found that KGD matched or outperformed the other methods in terms of accuracy with a decreased computation burden and was quicker to implement as no imputation was needed.

2.7.3. Cross-validation

To assess the accuracy of a model in predicting breeding values, predicted values are compared to observed phenotypic values. Two popular strategies are the *k*-fold and Monte-Carlo cross-validation. In the *k*-fold, the dataset is randomly divided into *k* equal parts with equal number of individuals. One-fold, the test set, is left out and the model is then trained on the remaining *k*-1 folds and used to predict the GEBVs of the individuals in the test set. This is repeated *k* times until each fold is used once as the test set. The accuracy is obtained by comparing the GEBVs with their corresponding observed phenotypes and the mean Pearson correlation is calculated across the *k* folds (Heslot et al., 2012; Daetwyler et al.). In Monte-Carlo cross-validation, the data is randomly split into two parts, a reference set and a test set. The phenotypes in the test set are masked and the reference set is used to train the model to predict the GEBVs of the individuals in the test set (Erbe et al., 2010). This process is repeated for numerous iterations generating new training and test sets each time. The mean predictive ability is the average Pearson correlation coefficient between predicted and observed phenotype for all iterations. The bias, which is the slope of the regression of phenotypes to estimated breeding values is also an important measure of a model should be 1 or close to 1 to indicate little bias (Daetwyler et al., 2012). Bias values greater than one are indicative of overprediction or an inflated genotype variance (Velazco et al., 2019b).

2.8. Conclusion

In summary, the review of the literature highlights the need for integrating available breeding strategies with SNP-based selection and bioinformatic tools to accelerate breeding. This is especially necessary for forage crops like white clover, characterised by long generation intervals and often difficult and expensive to measure quantitative traits. Genomic selection is a strategy that can be used alongside conventional breeding to expedite cultivar development and deliver long-term plant breeding goals. This study was primarily designed to investigate the feasibility of using genomic selection in white clover breeding to accelerate genetic gain for DM yield and vegetative persistence related traits. A major component of this thesis is based on the quantitative genetic analysis of a training population of 200 HS families of white clover, evaluated across three years and two contrasting environments. The process and outcomes of implementing genomic selection in a white clover breeding population are delineated in the following chapters.

3. Estimation of Quantitative Genetic Parameters for Yield and Persistence-related Traits in White Clover

3.1. Introduction

White clover is an important and nutritious forage legume. However, its dry matter (DM) yield and vegetative persistence in pasture is compromised due to the lack of available cultivars with the genetic potential to enhance seasonal yield and longevity which are often constrained by biotic and abiotic issues. Stolons are a vital part of the plants morphology and highly stoloniferous cultivars have been found to be more persistent in pasture as they have greater spread and colonization ability (Chapman 1983; Marshall et al., 2017). According to Mitchell (1956), the optimum temperature for stolon growth and development is 24°C. As a result, extreme temperatures, especially during summer moisture stress periods, can cause rapid loss of stolon and reduce DM yield. Therefore, a major challenge for breeders is to identify superior genotypes associated with high stable seasonal DM yield and high stolon density for improved vegetative persistence.

An important first step in cultivar development is the assessment of genetic variation for key traits in the breeding population. This is not often an easy task as estimation of variance components is ideally carried out over years and locations due to the presence of large standard errors (Flachenecker et al., 2006). Genetic improvement depends mainly on the magnitude of genetic diversity present in the population (Nyquist & Baker, 1991). As defined by Falconer (1989), the phenotype of an individual consists of genotypic and environmental effects. Genotypic effects are further partitioned into additive as well as non-additive components such as dominance and epistatic interactions. In forage breeding, the most significant component is the additive variance as this is inherited from parents to offspring. Consequently, it can be selected for and fixed via breeding methods (Falconer & Mackay, 1996; Acquaah, 2012). Caradus et al. (2000) noted that the narrow-sense heritability for key white clover traits indicate that significant genetic improvement for most of them can be achieved. Estimation of quantitative genetic parameters such as additive variance, G×E, heritability and correlation between traits, provide breeders with vital information to improve the efficiency of breeding strategies to maximize genetic gain.

Heritability is the ratio of genetic to phenotypic variation (Falconer & Mackay, 1996). Narrow-sense heritability estimates the magnitude of additive variation that is transmittable from parent to offspring. Plant breeders are interested in the heritability of traits as this genetic estimate helps predict genetic gain. According to Nyquist and Baker (1991), if the narrow-sense heritability of a trait is high, then phenotypic selection methods like mass selection will be able to improve the trait of interest with ease. Until recently, improved white clover cultivars have been as a result of phenotypic assessment and improved management and fertiliser strategies (Caradus et al., 1997b). However, for traits with lower heritability, selecting superior genotypes based on the phenotype alone will not deliver rapid genetic gain as most of the observable variation is not genetic, and consequently not transmitted. Although heritability ultimately depends on the genetic nature of the trait, to improve estimations of heritability, the use of replications and multi-site testing decreases the confounding effects of G×E, thereby increasing heritability (Falconer, 1989; Lorenz, 2013).

Partitioning the phenotypic variance into genetic and environmental components, provides an insight into the magnitude of G×E interaction as genotypes grown in different environments may often show significant variation in performance. This highlights the need for multi-site replicated trials over years (Annicchiarico, 2002). Jahufer et al. (1999) reported significant G×E interactions for many stolon and DM yield-related traits in white clover. The result of these interactions often complicate comparisons among genotypes and reduce the efficiency of selection of superior genotypes (Falconer, 1989). These interactions emphasize the need to carry out trials across multiple environments, years and meteorological conditions to assess the breeding potential of selection candidates and the identification of genotypes that perform better across or within specific environments (Eberhart & Russell, 1966; Brown & Caligari, 2008a; Jahufer et al., 2013; Osei et al., 2018).

This chapter reports on work focused on:

I. Establishment of a white clover training population of HS families and their multi-site evaluation to estimate genetic and phenotypic information for a range of traits for application in genomic selection.

II. Estimation of the magnitude of genetic, phenotypic and environmental variance for traits associated with seasonal herbage yield and vegetative persistence.

III. Determination of trait heritability and estimation of genetic correlations between the measured traits.

3.2. Materials and Methods

3.2.1. Establishment of Multi-site and Multi-year HS Family Field Trial

3.2.1.1. Plant Material

In 2012, clonal cuttings from top performing, broadly adapted white clover breeding lines were polycrossed to form a synthetic F_1 population of 141 HS families. Selection of these lines was based on high clover yields, high stolon density and persistence across years and enviroments. This source material was selected to capture as much genetic variation as possible which is crucial for outcrossing populations. An equal number of seeds from each of the individual F_1 plants was mixed together to constitute a balanced bulk, grown and polycrossed to generate 137 F_2 HS families. Individuals were selected from these 137 HS families for an additional polycross to generate the F_3 training population as described below (Figure 3.1).

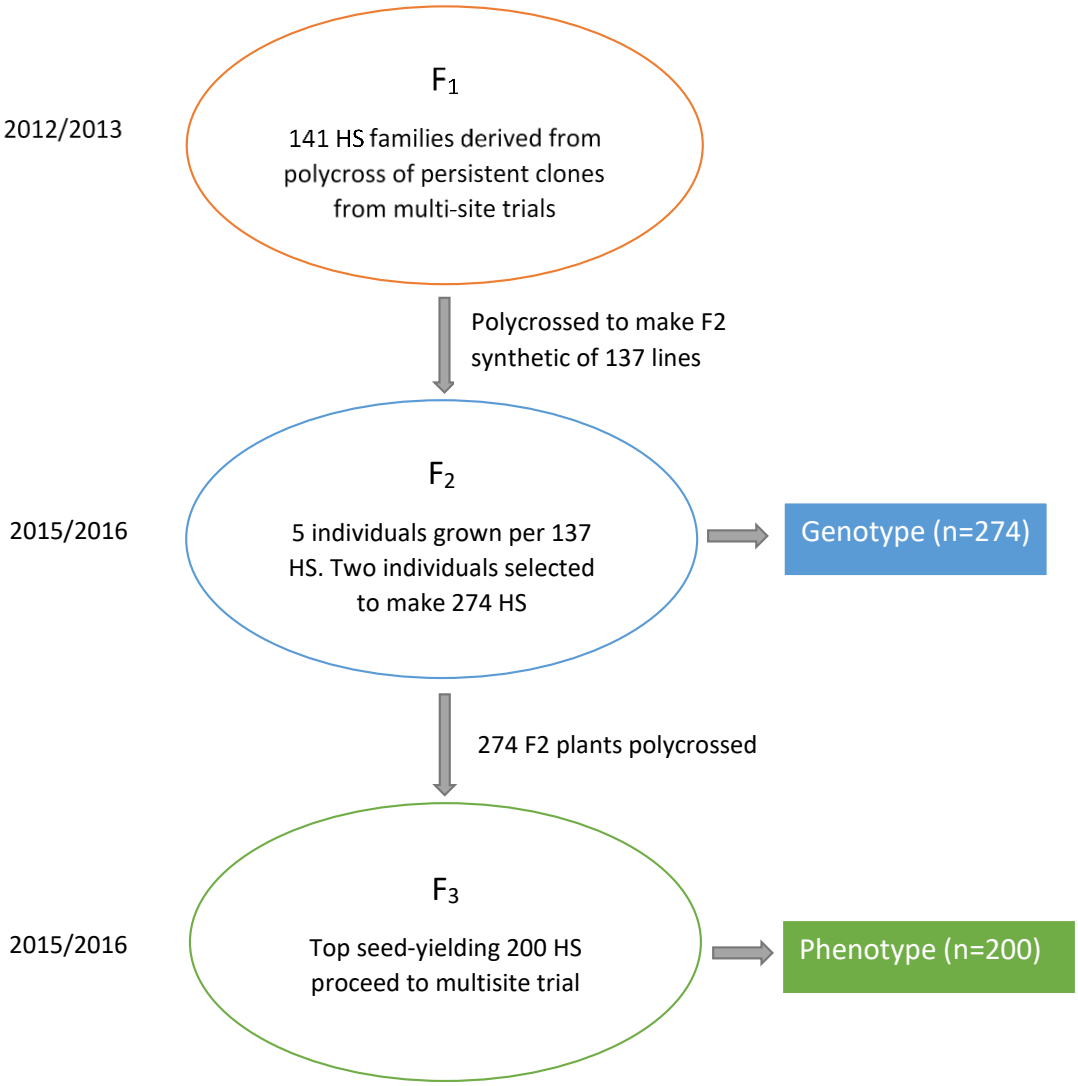


Figure 3.1 Development of training population for genomic selection.

3.2.1.2. Polycross to Generate Training Population

Using source material previously developed, a training population for genomic selection was generated by sampling two plants from each of the 137 F_2 HS families. The selected 274 plants were staked and cross-pollinated in a bee-proof isolation crossing tunnel from December 2015 to January 2016 (Figure 3.2). Twelve wild bumble bees (*Bombus* sp.) were placed in jar and rinsed with water to remove any residual wild white clover pollen as described in Gibson and Cope (1985). Washed bees were released into the isolation tunnel and checked daily to replace any bees that had died to maintain a constant of approximately 12 bumble bees. The plants were re-randomized weekly to ensure a

uniform distribution of pollen across all plants and avoid formation of population sub-structures.

Figure 3.2 Training population half-sib families in polycross isolation tunnel.

3.2.1.3. Seed Preparation and Germination

After successful pollination and seed maturation, seed was harvested, threshed, cleaned and seed weight recorded and maintained separately for each plant. A subset of 200 HS families was selected for field trials, based on seed yield, to ensure sufficient seed for subsequent experimentation. Seed hardness was broken by scarification and exposure to variations in temperature (stratification) before germination. The process was performed by lightly scarifying 0.2 g of seed from the 200 F_2 maternal parent plants using fine sandpaper. Scarified seeds were then germinated on petri-dishes containing pre-moistened filter paper (Figure 3.3). To synchronise germination, seeds were kept at 4˚C for 48 hours and then incubated at 25˚C for 24 hours. Germinated seeds were planted into propagation trays containing a mix of peat and sand with a three-month slow release Osmocote fertilizer and maintained under glasshouse conditions. Ninety seedlings were established for each of the 200 HS families making a total of 18,000 seedlings excluding spares and checks grown for the trial.

All plants were maintained in a glasshouse for approximately two months, after which the plants were trimmed, maintained in the trays and transferred outside the glasshouse on a drained concrete surface for “hardening” to ensure survival and a smooth transition when transplanting to field conditions.

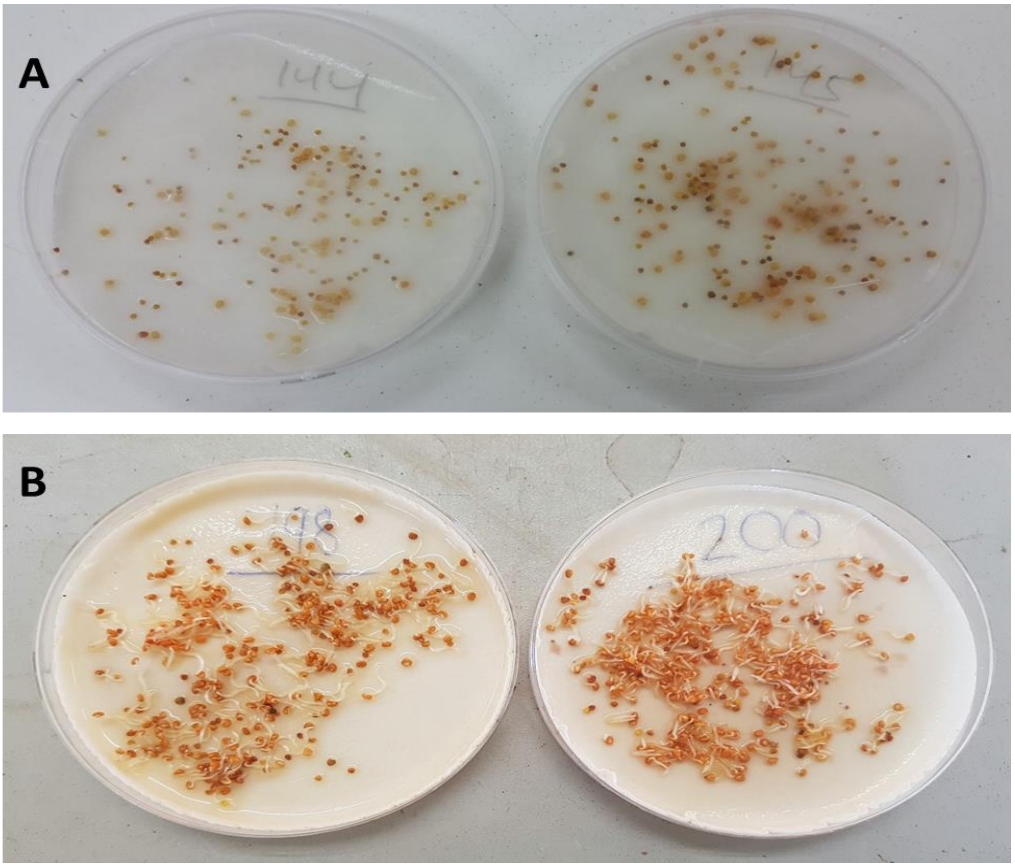


Figure 3.3 Pre (A) and post (B) incubation of white clover half-sib seed.

3.2.1.4. Trial Locations

The trial was conducted at two sites: AgResearch Grasslands Research Centre in Palmerston North, Manawatu (Aorangi) (40.38˚S, 175.61˚E); and the AgResearch Ruakura Research Farm in Hamilton, Waikato (37.77˚S, 175.31˚E). The soil types at the Palmerston North and Ruakura sites were Kairanga fine sandy loam and peaty silt loam soil, respectively. Three months prior to transplanting the white clover entries, both locations were sown with perennial ryegrass (*Lolium perenne* L.) cultivar Ceres One50 containing the endophyte AR37. Both locations were and sprayed with the herbicide Kamba® 500 at a dilution of 800 mL ha^{-1} to ensure resident white clover plants were

eliminated and prevent contamination of the trial. Crop 15, N (15.1%), P (10%), K (10%) and S (7.7%) fertiliser was applied at a rate of 250 kg ha^{-1} through a direct drill.

Transplanting of the HS F_3 progeny was carried out on 23rd of August 2016 at the Aorangi site and 27th of September 2016 at the Ruakura site. Prior to transplanting the seedlings into the grass swards, the grass was grazed closely to reduce grass competition and enhance establishment of the clover.

3.2.1.5. Experimental Design

A Row-Column experimental design with three replicates was used at each site (Appendix A.1, A.2). Fifteen plants of each of the 200 HS families were hand planted into 0.5 m by 0.75 m plots with 1.5 m between plots. In addition to the 200 HS families, each replicate had 24 repeated checks comprising of 'Grasslands Kopu II' and 'Grasslands Bounty'. 'Grasslands Kopu II' is a synthetic New Zealand cultivar characterised by large leaves, intermediate cyanogenesis, high herbage DM yield and stolon density (Woodfield et al., 2001). 'Grasslands Bounty' is a small to medium-leaved clover with high stolon density. The inclusion of repeated checks spread across the design was to reduce any effect of spatial trends. Two hundred HS families were transplanted per replication making a total of 600 plots plus an additional 72 check plots established per location. A total of 10,080 plants were sown in each location, comprising of 9,000 HS family plants and 1,080 check plants (Figure 3.4).

Figure 3.4 General view of the trial at the Aorangi location after establishment.

3.2.1.6. Trial Management

Soil tests were performed regularly to determine soil fertility level and fertiliser was applied as indicated. Over the course of the trial, five applications of 100 kg ha $^{-1}$ of urea and one application of 180 kg ha $^{-1}$ Maxi S Super plus N were applied at Aorangi. Three applications (30 kg ha $^{-1}$, 40 kg ha $^{-1}$ and 50 kg ha $^{-1}$) of urea were applied in Ruakura in addition to an annual application of 400kg SUPER10 15k potassic superphosphate fertilizer.

To ensure uniformity of regrowth after grazing, paddocks were topped when grazing was poor and uneven, and clippings disposed of outside the trial area.

Interrow spraying was performed as needed to eliminate volunteer clover, weeds and to prevent spread of HS family clover from one plot to its neighbouring plot. The trials were established in August and September 2016 at the Aorangi and Ruakura locations, respectively, and data were collected for this thesis until May 2019.

3.2.1.7. Grazing Management

The trials were grazed by cattle according to standard practices when herbage mass was between 2500-2800 kg DM ha^{-1} to residuals of 600-700kg DM ha^{-1} at each grazing. To estimate herbage mass, a rising plate meter (Jenquip, Feilding, New Zealand) was used before and after every grazing. A total of 19 and 17 cattle grazings took place at Aorangi and Ruakura, respectively, over the course of the trial. At each site grazing was carried out using a mob of young animals, for 2 to 3 hours. The animals were not allowed to camp on the trail area.

3.2.2. Data Collection and Phenotyping

Each year consisted of four seasons: Summer (December to February), Autumn (March to May), Winter (June to August) and Spring (September to November).

The following traits were measured:

3.2.2.1. Cyanogenesis

The presence or absence of Hydrocyanic acid (HCN) was assessed on leaf material from 24 individuals from each of the 200 HS families, using a modified qualitative picrate paper strip test (Corkill, 1940). This method identifies only those genotypes that carry dominant alleles at both loci, *Ac* and *Li.* Sampling was performed prior to transplanting when the HS family seedlings were six weeks old. Briefly, one leaf was sampled from each of 24 replicate plants of each HS family, placed in a 1 ml Eppendorf tube which had 1cm filter paper (3M, company) placed in the cap of the tube and treated with 10 µl Toluene acid to digest the leaf material. An aliquot of 10 µl picric acid was then applied to the filter paper, the tubes were closed and incubated at 37˚C for two hours before scoring. Scoring was done on a scale of 0-5 (Figure 3.5), where 0 was absence of HCN, indicated by no change in the colour of the picric acid paper, (yellow); 1-2 indicated low HCN production; 3 indicated medium HCN production (orange); and 4-5 indicated high levels of HCN production (reddish brown).

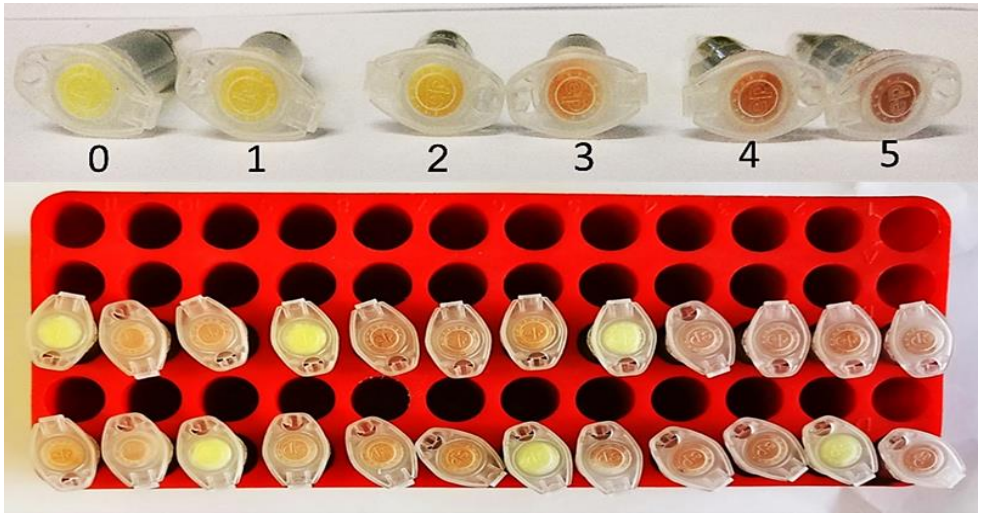


Figure 3.5 HCN scores (0-5) and representative samples.

3.2.2.2. Growth Score

Visual assessment of clover yield across the trial was taken on a scale of 1 (lowest) to 9 (highest) herbage production per plot, with 0.5 units increments to allow closer approximation of continuous data. Scores were taken prior to each grazing when herbage mass was between 2500-2800 kg DM ha^{-1}. At each scoring period, calibration cuts for each score were taken by randomly selecting three plots per score. A 0.2 m^{-2} quadrant was randomly placed on the ground and the above ground biomass cut to a stubble height of 2-3 cm using mechanical shears. Harvested samples were then separated into white clover and ryegrass components and dried in an oven at 80°C for 48 hours and the dry weight recorded. A regression analysis was performed between cut DM and plot scores to obtain an estimate of score accuracy. The regression between plot score and clover DM content allowed a clover herbage yield (kg DM ha^{-1}) to be estimated for all plots.

3.2.2.3. Dry Matter Production Cuts

To assess clover herbage biomass, across the trial, full-scale DM yield cuts were performed annually in spring at a herbage mass accumulation between 2500-2800 kg DM ha^{-1}. Before grazing, a 0.2 m^2 quadrant was randomly placed in each plot and the above-ground biomass removed. Harvested samples were then separated into white clover and ryegrass components, oven-dried and weighed. The first harvest dates were 31st October and 29th November 2017 at Aorangi and Ruakura, respectively. The second harvest dates were 6th of November and 2nd of December 2018 at Aorangi and Ruakura, respectively.

A total of 672 plots comprising HS families and checks were harvested from each trial location annually.

3.2.2.4. Leaf Size

Leaf size was recorded twice each year. A visual leaf size score of 1 (smallest) – 5 (largest) was used with 0.5 unit increments allowable.

3.2.2.5. Stolon Characteristics

Two stolon traits, number of stolons and number of branches, were measured before and after summer to determine the effect of summer moisture stress on these traits. On each occasion, three cores (5 cm diameter × 4 cm depth) were taken randomly from all plots in Aorangi using a soil corer (Figure 3.6). From each collected core, the number of stolons and the number of branches on each stolon were counted. Data were collected from the second year of growth in late November (pre-summer and March (post-summer).

Figure 3.6 Soil corer and stolon representative samples from the field trial at Aorangi.

3.2.3. Statistical Analysis

3.2.3.1. Univariate Analysis

3.2.3.1.1. Linear Mixed Model

Residual Maximum Likelihood (REML) (Patterson & Thompson, 1971; Harville, 1977) was conducted on all data collected, based on linear mixed models, using DeltaGen software (Jahufer & Luo, 2018). These analyses enabled estimation of variance components for genetic and nongenetic effects and BLUPs, (White & Hodge, 1989) for traits HCN, DM yield, seasonal growth scores (GS), leaf size (LS), stolon number (SN) and stolon branches (SB).

The statistical significance of the variance components was estimated using deviance of log-likelihood as suggested by Galwey (2006).

Residual plots were assessed for deviations from normality and homogeneity and square root transformed for seasonal calibration cuts. In all the mixed linear models, years, sites, seasons and repeated checks were considered as fixed effects while the HS families, G×E, replicates, rows and columns of the experimental design were considered random effects.

3.2.3.1.2. Fixed Effects

To estimate fixed effects, maximum likelihood estimation was used instead of REML (Zuur et al., 2009) as maximum likelihood generates more accurate estimates of fixed regression parameters (Twisk, 2006). HS families and check cultivars were considered as fixed effects in order to obtain BLUEs (Best Linear Unbiased Estimate) and estimate differences among them (Annicchiarico, 2002; Smith et al., 2005). Analyses were performed using the 'lmer' function in the 'lme4' package in R while pairwise difference and separation of means was performed by Fisher's least-significant difference (LSD) test in the 'predictmeans' package in R (R Core Team, 2012).

Phenotypic data were analysed using the following linear models:

Single season and site analysis (Equation 15):

$$Y_{ijkl} = \mu + f_i + b_j + r_{jk} + c_{jl} + \varepsilon_{ijkl} \quad (15)$$

Where: Y_{ijkl} is the value of an attribute measured from genotype *i* in replicate *j* in row *k* and column *l*, and $i = 1,\ldots, n_f$; $j = 1, \ldots, n_b$; $k = 1,\ldots, n_r$; and $l = 1,\ldots, n_c$, where, f, *b*, *r*, and *c* are families, replicates, rows and columns, respectively; μ is the overall mean; f_i is the random effect of the HS family *i*, $N(0, \sigma_f^2)$; b_j is the random effect of replicate *j*, $N(0, \sigma_b^2)$; r_{jk} is the random effect of row *k* in replicate j, $N(0, \sigma_r^2)$; c_{jl} is the random effect of column l in replicate *j*, $N(0, \sigma_c^2)$; and ε_{ijkl} is the residual effect of genotype *i* in row *k* and column *l* in replicate *j*, $N(0, \sigma_\varepsilon^2)$.

Across-season analysis within each location (Equation 16):

$$Y_{ijklm} = \mu + f_i + s_j + b_{jk} + (fs)_{ij} + (fb)_{ik} + (bs)_{jk} + r_{jkl} + c_{jklm} + \varepsilon_{ijklm} \quad (16)$$

Where: Y_{ijklm} is the value of an attribute measured from family *i* in row *l* and column *m* of replicate *k* nested in season *j* and $i = 1,\ldots, n_f$; $j = 1,\ldots, n_s$; $k = 1,\ldots, n_b$; $l = 1,\ldots, n_r$, and $m=1,\ldots,n_c$,where, f, *s*, *b*, *r*, and *c* are families, seasons, replicates, rows and columns, respectively; μ is the overall mean; f_i is the random effect of the HS family *i*, $N(0, \sigma_f^2)$; s_j is the fixed effect of season j ; b_{jk} is the random effect of replicate *k* within season *j*, $N(0, \sigma_b^2)$; $(gs)_{ij}$is the effect of the interaction between family *i* and season *j*, r_{jkl} is the random effect of row *l* in replicate *k* within season *j*, $N(0, \sigma_r^2)$; $(bs)_{ij}$is the interaction between replicate *k* and season *j*, r_{jkl} is the random effect of row *l* in replicate *k* within season *j*, $N(0, \sigma_r^2)$ c_{jkm} is the random effect of column m in replicate *k*, within season *j*, $N(0, \sigma_c^2)$; and ε_{ijklm} is the residual effect of genotype *i* in row *l* and column *m* of replicate *k* in season *j*, $N(0, \sigma_\varepsilon^2)$.

Across year by season by location analysis (Equation 17):

$$Y_{ijklmno} = \mu + f_i + y_o + e_n + (fe)_{in} + s_j + (fs)_{ij} + (fy)_{io} + (sy)_{jo} + (fey)_{ino} + (fsy)_{ijo} + b_{jnok} + r_{jnokl} + c_{jnokm} + \varepsilon_{ijklmno} \quad (17)$$

Where: $Y_{ijklmno}$ is the value of an attribute measured from family *i* in the j^{th} season, within the o^{th} *year*, at the *nth* location, in the k^{th} replicate, in l^{th} row, and m^{th} column. $i = 1,\ldots, n_f$; $j = 1,\ldots, n_s$; $k = 1,\ldots, n_b$; $l = 1,\ldots, n_r$; $m = 1,\ldots, n_c$; $n = 1,\ldots, n_e$; $o = 1,\ldots, n_y$, where, f, *y, e, s, b, r,* and *c* are families, years, locations, seasons, replicates, rows and columns, respectively. μ is the overall mean; f_i is the random effect of the HS family *i*, $N(0, \sigma_f^2)$; y_o is the fixed effect of year *o*; e_n is the fixed effect of location *n*; $(fe)_{in}$ is the effect of the interaction between family *i* and location *n*;, s_j is the fixed effect of season *j*; $(fs)_{ij}$ is the effect of the interaction between family *i* and season *j*, $(fy)_{io}$ is the effect of the interaction between family *i* and year *o*,$(sy)_{jo}$ is the effect of the interaction between season *j* in year *o*, $(fey)_{ino}$ is the effect of the interaction between family *i* location *n* and year *o*, $(fsy)_{ijo}$ is the effect of the interaction between family *i* season *j* and year *o*, $b_{jnok}$is the random effect of replicate *k* within season *j* within year *o* in location *n* ,$N(0, \sigma_b^2)$; r_{jnokl} is the random effect of row *l* within replicate *k* in season *j* within year *o* in location *n* ,$N(0, \sigma_r^2)$; c_{jnokm} is the random effect of column *m* in replicate *k* within season *j* within year *o* in location *n* ,$N(0, \sigma_c^2)$; and ε_{ijklm} is the residual effect of genotype *i* in row *l* and column *m* of replicate *k* in season *j*, $N(0, \sigma_\varepsilon^2)$.

3.2.3.1.3. Heritability

Heritability for a trait measured once was estimated using the estimates of genotypic and error components of variance in the following Equation (18) by (Falconer, 1989):

$$h_n^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_\varepsilon^2}{nr}} \quad (18)$$

Where: h^2_n is the narrow-sense heritability; σ_f^2 is the family additive variance component; σ_ε^2 is the error variance component, and *nr* is the number of replicates.

For repeated measurements, heritability was estimated as:

Across seasons (Equation 19):

$$h_n^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_{fs}^2}{n_s} + \frac{\sigma_b^2}{n_b} + \frac{\sigma_\varepsilon^2}{n_s n_b}} \quad (19)$$

Across sites and seasons within a year (Equation 20):

$$h_n^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_{fe}^2}{n_e} + \frac{\sigma_{fs}^2}{n_s} + \frac{\sigma_{fes}^2}{n_e n_s} + \frac{\sigma_b^2}{n_e n_b} + \frac{\sigma_\varepsilon^2}{n_e n_s n_b}} \quad (20)$$

Across site, season and year analysis (Equation 21):

$$h_n^2 = \frac{\sigma_f^2}{\sigma_f^2 + \frac{\sigma_{fy}^2}{n_y} + \frac{\sigma_{fe}^2}{n_e} + \frac{\sigma_{fs}^2}{n_s} + \frac{\sigma_{fyes}^2}{n_y n_e n_s} + \frac{\sigma_b^2}{n_e n_b} + \frac{\sigma_\varepsilon^2}{n_y n_e n_s n_b}} \quad (21)$$

Where the equation components are defined in relation to the linear model.

3.2.3.2. Multivariate Analysis

3.2.3.2.1. Correlation

Pairwise Pearson correlation analysis was carried out to determine the type and strength of relationship between traits using HS family BLUP-adjusted means for each trait. Pearson's correlation coefficient and significance estimation were performed using the 'cor' package in R.

3.2.3.2.2. Pattern Analysis

Principal component analysis (PCA) and cluster analysis were conducted to generate a graphical summary of the association among measured traits as well as determine patterns of phenotypic variation among the HS families using HS BLUP-adjusted means in the software Deltagen (Jahufer & Luo, 2018). Only traits showing significant ($P < 0.05$) additive genetic variation were included in the analyses. The data were standardised to remove scaling effects, have a mean of zero and a variance of one (Fox & Rosielle, 1982; Cooper & DeLacy, 1994).

Cluster analysis was performed on the standardised data using a hierarchical agglomerative classification procedure with squared Euclidean distance as a measure of dissimilarity. In order to choose the optimum level of truncation for the resulting hierarchy from cluster analysis, the increase in the sum of squares among HS 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 HS family sum of squares among groups did not improve substantially as the number of groups increased.

3.3. Results

3.3.1. Meteorological Conditions

3.3.1.1. Rainfall

Meteorological data were obtained for both sites and daily observations were aggregated to give total and average values for rainfall, maximum and minimum air temperature respectively. In comparison to the 10-year average of 1025 mm for Aorangi, the driest year was 2017 with a total rainfall of 744 mm compared to 1373 mm, and 1103 mm for 2016 and 2018, respectively (Figure 3.7). In 2016/2017, summer months experienced the least amounts of rainfall, 115.4 mm compared to 196.6 mm, 207 and 220 mm for spring, autumn and winter, respectively. The same trend was observed in 2017/2018 and 2018/2019, 193 mm in summer compared to 212 mm, 277 mm and 317 mm for spring, autumn and winter then 154 mm compared to 209 mm and 160 mm for spring and autumn, respectively. Winter had the highest precipitation of all the seasons.

In Ruakura, years 2017 and 2018 experienced higher amounts of rain, 1492 mm and 1469 mm, relative to the 10-year average of 1167 mm, while 2016 had a total rainfall of 1013 mm (Figure 3.7). The summer of 2016/2017 experienced the least amount of rain, 195 mm compared to 296 mm, 605 mm and 334 mm for spring, autumn and winter, respectively. In succeeding years, the summer months experienced more rain than the other seasons, 545 mm compared to 317 mm, 271 mm and 370 mm for spring, autumn and winter then 221 mm compared to 185 mm and 170 mm for spring and autumn for 2017/2018 and 2018/2019, respectively.

Total rainfall for the duration of the trial was 2418 mm in Aorangi and 3509 mm in Ruakura, a difference of 37% between the sites.

3.3.1.2. Temperature

Mean monthly maximum and minimum temperatures for the duration of the experiment (Figure 3.7) at the two sites were comparable to the 10-year maximum and minimum averages of 18°C and 9°C in Aorangi and 19.5°C and 8.5°C in Ruakura. The warmest year at Aorangi and Ruakura was 2018, with a mean average maximum of 18.6°C and 20.6°C, respectively. Ruakura was on average 2°C warmer than Aorangi.

Summer months were the warmest in Aorangi, with average maximum temperatures of 20.6°, 24.7°C and 23.1°C for 2016/2017, 2017/2018 and 2018/2019. At Ruakura, the highest average maximum temperature was 26.1°C in summer 2018/2019.

Average minimum temperatures were similar across both sites.

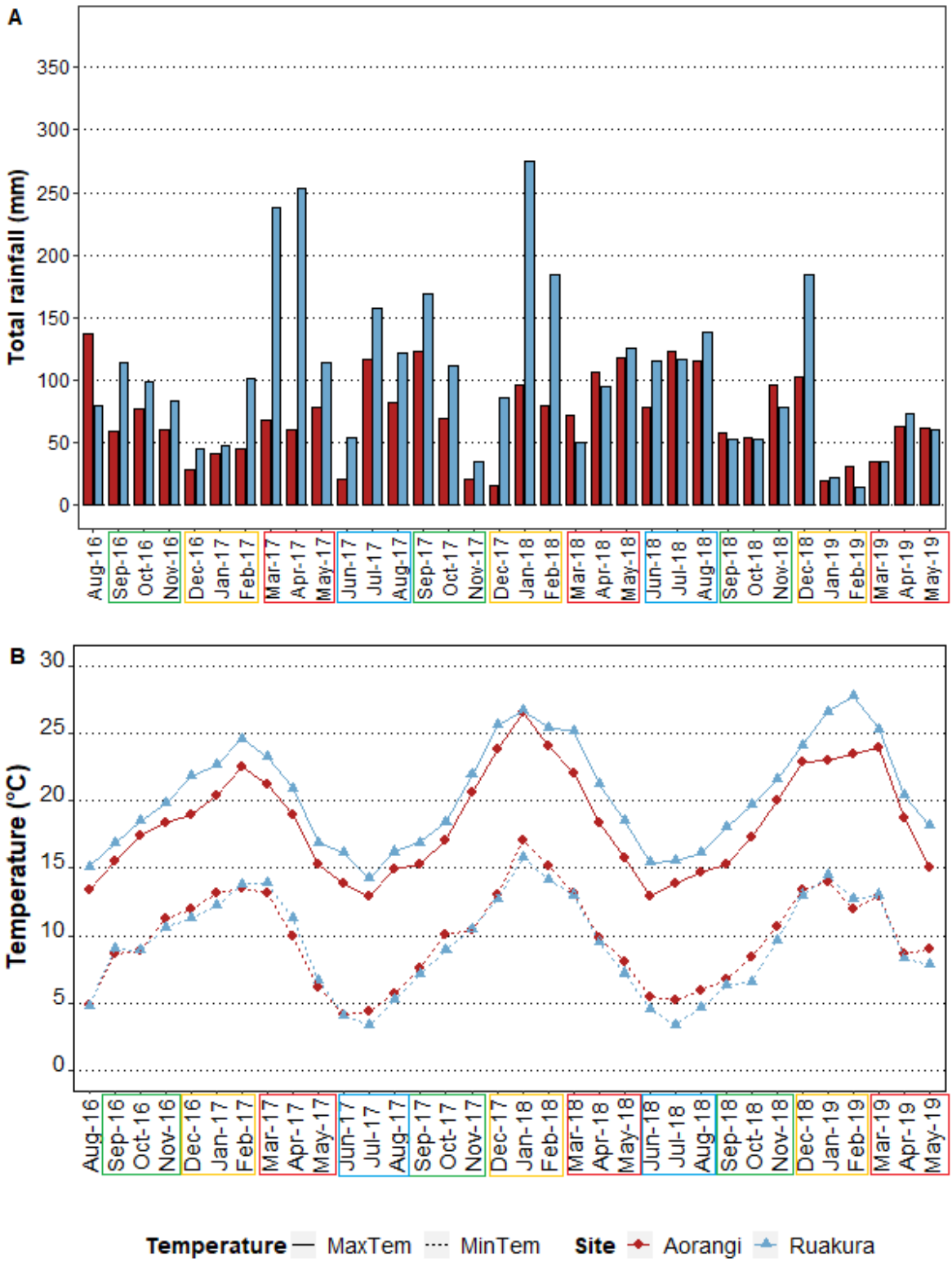


Figure 3.7 A) Total monthly precipitation at Aorangi and Ruakura and B) Mean Maximum and minimum monthly air temperatures (°C) at Aorangi and Ruakura for the duration of the trial; August 2016 to May 2019. Green, yellow, red and blue boxes represent spring, summer, autumn and winter seasons, respectively.

3.3.2. Univariate Analysis

3.3.2.1. Variance Components and Heritability

3.3.2.1.1. HCN

There was significant ($P < 0.01$) additive genetic variation (σ^2_f) among the white clover genotypes for HCN content. Additive variance was estimated at 0.54 ± 0.07 and residual error, 2.74 ± 0.06. Family mean narrow-sense heritability across samples was estimated from Equation 18 as 0.82 ± 0.018. Note that the number of samples taken per HS family was taken to be the number of replicates in this instance. Cyanogenic potential BLUP values ranged from 1.2 to 4.3 for the HS families. Most of the lines (73%) produced moderate to high levels of HCN (scores 3-5) while the remaining 27% produced none to low levels of HCN (Figure 3.8). Check 1, 'Grasslands Kopu II' had a mean score of 2.4 while check 2, 'Grasslands Bounty', had a mean score of 3.3.

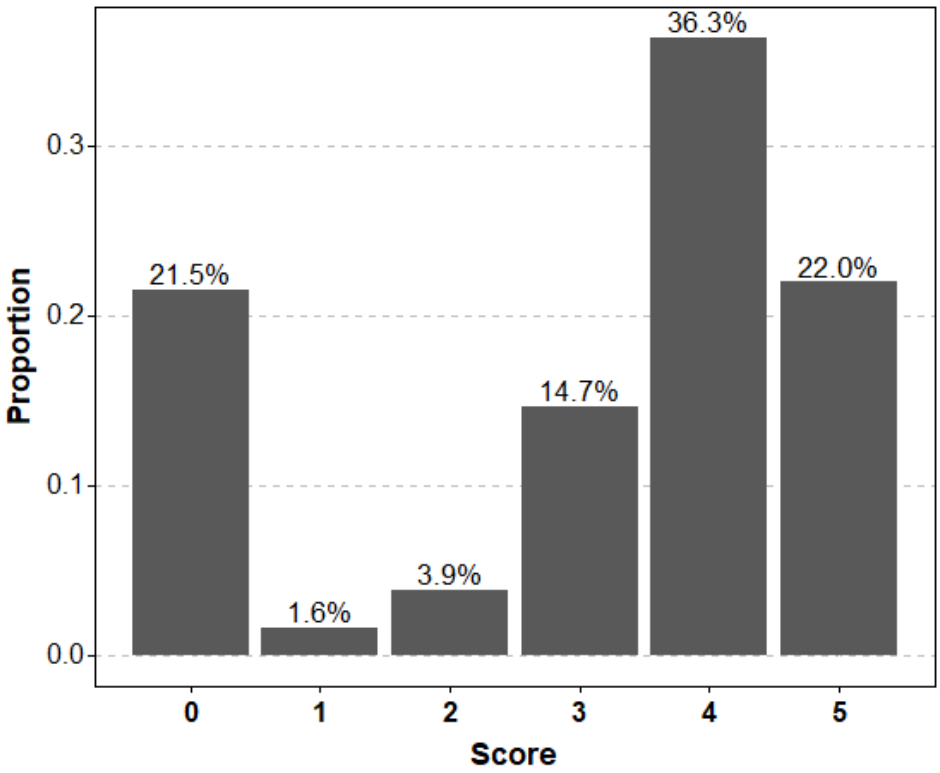


Figure 3.8 Histogram showing cyanogenesis (HCN) scores based on a picric acid assay and percentage proportion of 200 half-sib families scored for cyanogenic performance. Score 0 = no HCN, 5 = maximum HCN produced.

3.3.2.1.2. Seasonal Growth Scores

Estimation of variance components among HS families for seasonal growth scores (GS) are presented in Tables 3.1, 3.2 and 3.3.

3.3.2.1.3. Within Seasons and Locations

There was significant ($P < 0.05$) additive genetic variation for GS among HS families at individual locations within seasons and within years except for year 2017 winter at Aorangi. Combined analyses across years 2017, 2018 and 2019 for each season showed significant additive genetic variation among HS families for both locations with the exception of Ruakura spring and summer as shown in Table 3.1. Apart from Autumn in Aorangi, the family × year ($\sigma^2_{f.y}$) interaction variance component was consistently higher than the additive genetic variance component (σ^2_f). The magnitude of the additive genetic variance among the HS families (σ^2_f) appeared to increase with subsequent years but not consistently so. When compared to Aorangi, Ruakura always had higher error variance components (σ^2_ε). Heritability ranged from 0.18 to 0.6 and was predominantly higher at Aorangi.

3.3.2.1.4. Across Years and Locations Within Seasons

There was significant ($P < 0.05$) additive genetic variation among the HS families across locations for each of the four seasons except for summer and autumn 2017 and autumn 2019 (Table 3.2). Family × location ($\sigma^2_{f.l}$) interaction was significant ($P < 0.05$) for individual years and across years and was always larger than the additive genetic variance component (σ^2_f) apart from across years analysis for autumn. Family × year ($\sigma^2_{f.y}$) was only significant across years 2017, 2018 and 2019 and 2017 and 2018 for summer and spring respectively and was lower than the family × location ($\sigma^2_{g.l}$) interaction component. The magnitude of the additive genetic variance among HS families (σ^2_f) was generally higher in spring. Ruakura consistently had higher error variances compared to the Aorangi location. The residual error variance component was larger than all other components. Narrow-sense heritability seemed to increase with successive years and ranged from 0.13 to 0.41 as presented in Table 3.2.

Table 3.1 Estimated additive genetic (σ^2_f), family-by-year ($\sigma^2_{f.y}$), and pooled error (σ_ε), variance components, their associated standard errors (± SE) and family mean narrow-sense heritability (h^2_n) for seasonal growth scores for 200 half-sib white clover families across two locations, Aorangi and Ruakura.

Variance components	Summer		Autumn		Winter		Spring	
	Aorangi	Ruakura	Aorangi	Ruakura	Aorangi	Ruakura	Aorangi	Ruakura
	Year 2017							
σ^2_f	0.40 ± 0.09*	0.67 ± 0.18*	0.08 ± 0.04*	0.43 ± 0.18*	0.07 ± 0.04	0.32 ± 0.12*	0.25 ± 0.06*	0.44 ± 0.16*
σ^2_ε	0.91 ± 0.05	1.44 ± 0.08	0.73 ± 0.06	3.04 ± 0.22	0.64 ± 0.03	1.39 ± 0.08	0.64 ± 0.04	0.98 ± 0.06
h^2_n	0.42 ± 0.06	0.36 ± 0.07	0.25 ± 0.11	0.3 ± 0.09	0.18 ± 0.08	0.27 ± 0.07	0.42 ± 0.06	0.28 ± 0.08
	Year 2018							
σ^2_f	0.40 ± 0.10*	0.34 ± 0.11*	0.31 ± 0.07*	0.55 ± 0.17*	0.37 ± 0.09*	0.79 ± 0.21*	0.82 ± 0.14*	0.61 ± 0.17*
σ^2_ε	0.61 ± 0.03	2.52 ± 0.22	0.69 ± 0.04	2.64 ± 0.2	1.16 ± 0.10	2.92 ± 0.21	0.92 ± 0.05	1.24 ± 0.07
h^2_n	0.39 ± 0.07	0.43 ± 0.07	0.42 ± 0.06	0.38 ± 0.08	0.48 ± 0.07	0.45 ± 0.07	0.56 ± 0.05	0.37 ± 0.07)
	Year 2019							
σ^2_f	0.88 ± 0.16*	1.41 ± 0.23*	0.84 ± 0.15*	0.59 ± 0.20*	-	-	-	-
σ^2_ε	0.52 ± 0.02	1.77 ± 0.12	1.67 ± 0.12	2.80 ± 0.30	-	-	-	-
h^2_n	0.55 ± 0.05	0.22 ± 0.10	0.6 ± 0.05	0.29 ± 0.11	-	-	-	-
	Across years (2017, 2018, 2019)				**Across years 2017 & 2018**			
σ^2_f	0.18 ± 0.08*	0.16 ± 0.11	0.20 ± 0.05*	0.2 ± 0.10*	0.10 ± 0.03*	0.32 ± 0.12*	0.25 ± 0.06*	0
$\sigma^2_{f.y}$	0.50 ± 0.06*	0.74 ± 0.09*	0.16 ± 0.03*	0.30 ± 0.10*	0.11 ± 0.03*	0.33 ± 0.08*	0.32 ± 0.05*	0.67 ± 0.08*
σ^2_ε	0.90 ± 0.02	1.90 ± 0.07	0.80 ± 0.03	2.10 ± 0.13	0.80 ± 0.03	1.70 ± 0.08	0.90 ± 0.03	1.60 ± 0.05
h^2_n	0.30 ± 0.09	0.20 ± 0.12	0.53 ± 0.07	0.37 ± 0.12	0.36 ± 0.11	0.37 ± 0.09	0.42 ± 0.08	0

*Significant at $P < 0.05$

Table 3.2 Estimated additive genetic (σ^2_f), family-by-location interaction ($\sigma^2_{f.l}$), family-by-year interaction ($\sigma^2_{f.y}$), pooled error (σ_ε) variance components, their associated standard errors (± SE) and family mean narrow-sense heritability (h^2_n) for seasonal growth scores from combined analyses for 200 half-sib white clover families across two locations, Aorangi and Ruakura.

Variance components	Summer	Autumn	Winter	Spring
	Year 2017			
σ^2_f	0.11 ± 0.08	0.06 ± 0.07	0.09 ± 0.04*	0.20 ± 070*
$\sigma^2_{f.l}$	0.72 ± 0.11 *	0.18 ± 0.08*	0.29 ± 0.05 *	0.45 ± 0.07*
σ^2_ε	2.16 ± 0.06	1.91 ± 0.09	1.57 ± 0.04	1.65 ± 0.05
h^2_n	0.14± 0.09	0.13 ± 0.14	0.17 ± 0.08	0.29 ± 0.08
	Year 2018			
σ^2_f	0.14 ± 0.08	0.21 ± 0.07*	0.24 ± 0.09*	0.33 ± 0.10*
$\sigma^2_{f.l}$	0.57 ± 0.10*	0.44 ± 0.08*	0.32 ± 0.12*	0.71 ± 0.11*
σ^2_ε	1.70 ± 0.06	1.21 ± 0.05	2.06 ± 0.10	2.01 ± 0.06
h^2_n	0.20 ± 0.10	0.14 ± 0.10	0.33 ± 0.10	0.32 ± 0.08
	Year 2019			
σ^2_f	0.28 ± 0.13*	0.23 ± 0.13	-	-
$\sigma^2_{f.l}$	1.21 ± 0.16*	0.57 ± 0.16*	-	-
σ^2_ε	1.74 ± 0.05	2.06 ± 0.12	-	-
h^2_n	0.28 ± 0.11	n.s.	-	-
	Across years (2017, 2018, 2019)		**Across years (2017 & 2018)**	
σ^2_f	0.11 ± 0.05*	0.13 ± 0.05*	0.09 ± 0.04*	0.13 ± 0.06*
$\sigma^2_{f.l}$	0.36 ± 0.07*	0.04 ± 0.03	0.29 ± 0.06*	0.29 ± 0.07*
$\sigma^2_{f.y}$	0.07 ± 0.04*	0.03 ± 0.03	0.04 ± 0.04	0.16 ± 0.06*
$\sigma^2_{f.y.l}$	0.46 ± 0.06*	0.04 ± 0.04	0.05 ± 0.05	0.34 ± 0.06*
σ^2_ε	1.98 ± 0.04	1.76 ± 0.05	1.5 ± 0.04	1.86 ± 0.04
h^2_n	0.27 ± 0.10	0.41 ± 0.11	0.3 ± 0.12	0.23 ± 0.13

*****Significant at $P < 0.05$

3.3.2.1.5. Across Seasons, Locations and Years

Results from variance component analysis across seasons and across years for GS is presented in Table 3 and shows significant ($P < 0.05$) additive genetic differences among HS families at individual sites and across sites for all years and across years. All two-way interactions for family × season ($\sigma^2_{f.s}$) and family × location ($\sigma^2_{f.l}$) were significant except for Ruakura 2019 and across years 2017, 2018 and 2019 analyses at both sites. The family × location interaction variance ($\sigma^2_{f.l}$) was typically larger than the line variance for all years. Family × year ($\sigma^2_{f.y}$) interaction was also significant for both sites when analysed across all years but not significant when locations were combined. Three-way interaction of family × season × location ($\sigma^2_{f.s.l}$) was not significant while family × year × season ($\sigma^2_{f.y.s}$) and family × year × location ($\sigma^2_{f.y..l}$) was significant ($P < 0.05$) for combined location analyses only. The residual error variance component was larger than all other components for majority of the analyses. Family mean heritability ranged from 0.24 to 0.73 and increased as the trial progressed. The narrow-sense heritabilities on a family mean basis estimated from across location analyses were always lower than the estimates from individual location analyses.

Table 3.3 Estimated additive genetic (σ^2_f) and pooled error (σ^2_ε) variance components with their standard errors (± SE), associated interactions and family mean narrow-sense heritability (h^2_n) estimated at individual and across locations (L) ; Aorangi and Ruakura, across seasons (S) and across years (Y) for seasonal growth scores in 200 half-sib white clover families.

Source	σ^2_f	$\sigma^2_{f.s}$	$\sigma^2_{f.l}$	$\sigma^2_{f.s.l}$	$\sigma^2_{f.y}$	$\sigma^2_{f.y.s}$	$\sigma^2_{f.y.l}$	σ^2_ε	h^2_n
					Year 2017				
Aorangi	0.12 ± 0.04*	0.11 ± 0.02*	n.a	n.a	n.a	n.a	n.a	0.76 ± 0.02	0.35 ± 0.07
Ruakura	0.36 ± 0.11*	0.21 ± 0.03*	n.a	n.a	n.a	n.a	n.a	1.52 ± 0.04	0.33 ± 0.08
Across site	0.10 ± 0.05*	0.03 ± 0.01*	0.46 ± 0.06*	ns			n.a	1.83 ± 0.03	0.24 ± 0.01
					Year 2018				
Aorangi	0.37 ± 0.08*	0.14 ± 0.02*	n.a.	n.a.	n.a.	n.a.	n.a.	0.82 ± 0.02	0.50 ± 0.06
Ruakura	0.51 ± 0.13*	0.15 ± 0.03*	n.a.	n.a.	n.a.	n.a.	n.a.	1.42 ± 0.05	0.42 ± 0.07
Across site	0.20 ± 0.07*	0.05 ± 0.02*	0.66 ± 0.08*	0.01 ± 0.02	n.a.	n.a.	n.a.	2.42 ± 0.04	0.24 ± 0.07
					Year 2019				
Aorangi	0.85 ± 0.15*	0.04 ± 0.01*	n.a.	n.a.	n.a.	n.a.	n.a.	0.49 ± 0.02	0.59 ± .054
Ruakura	0.53 ± 0.2*	0	n.a.	n.a.	n.a.	n.a.	n.a.	1.25 ± 0.06	0.29 ± 0.12
Across site	0.25 ± 0.12*	0	1.2 ± 0.15*	0	n.a.	n.a.	n.a.	1.60 ± 0.04	0.27 ± 0.10
					Across years				
Aorangi	0.26 ± 0.05*	0	n.a.	n.a.	0.25 ± 0.025*	0.01 ± 0.01	n.a.	0.90 ± 0.013	0.48 ± 0.06
Ruakura	0.50 ± 0.07*	0	n.a.	n.a.	0.52 ± 0.06*	0.02 ± 0.02	n.a.	2.40 ± 0.03	0.73 ± 0.04
Across site	0.12 ± 0.04*	0	0.35 ± 0.05*	0	0.04 ± 0.03	0.04 ± 0.01*	0.34 ± 0.04*	1.73 ± 0.02	0.32 ± 0.10

*Significant at $P < 0.05$, n.a = not applicable.

3.3.2.1.6. Effect of Year and Season on Calibration Cut DM Yield

Fixed effects analysis showed year and season had significant ($P < 0.05$) effects on white clover DM yield. There were significant ($P < 0.05$) differences among seasons within years, across years, as well as significant year-by-season interactions (Figure 3.8 Appendix A.3, A.4, A.5).

Also, there was significant additive genetic variation among HS families across years and seasons as illustrated by the boxplot in Figure 3.9. Post hoc analyses indicated that at Aorangi, years 2018 and 2019 were significantly different from 2017 although 2018 was not significantly different from 2019. When averaged over seasons, 2018, the second year of growth had 35% and 12% more DM yield than 2017 and 2019, respectively. Variation amongst HS families was also more pronounced in 2018 than 2017 or 2019 (Figure 3.9).

Significantly ($P < 0.05$) higher DM yield was observed in summer 2018 than other seasons-year combinations while spring and winter had the lowest DM yield. There was a 194% difference in DM yield between the average of the highest scored plots (score 9) in summer 2018 (2648 ± 134 kg DM ha-^{1}) and the lowest scored plots (score 1) (40 ± 59.3 kg DM ha^{-1}) in 2017 winter (Appendix A.5).

At Ruakura, there were no significant differences between 2017 and 2019, but both years were significantly ($P < 0.05$) different from 2018. DM yield increased by 42% from 2017 to 2018, followed by a 17% decrease in 2019 (Figure 3.9). The largest variation was observed in 2018. Summer 2018 DM yield was significantly higher than all other season-year combinations except 2018 autumn and 2019 summer, while winter 2017 had the lowest DM yield (Figure 3.9). There was a 193% difference in DM yield between the average of highest scored plots in summer 2018 (4891 ± 176 kg DM ha^{-1}) and the lowest scored plots (85 ± 23 kg DM ha^{-1}) in 2017 winter (Appendix A.5).

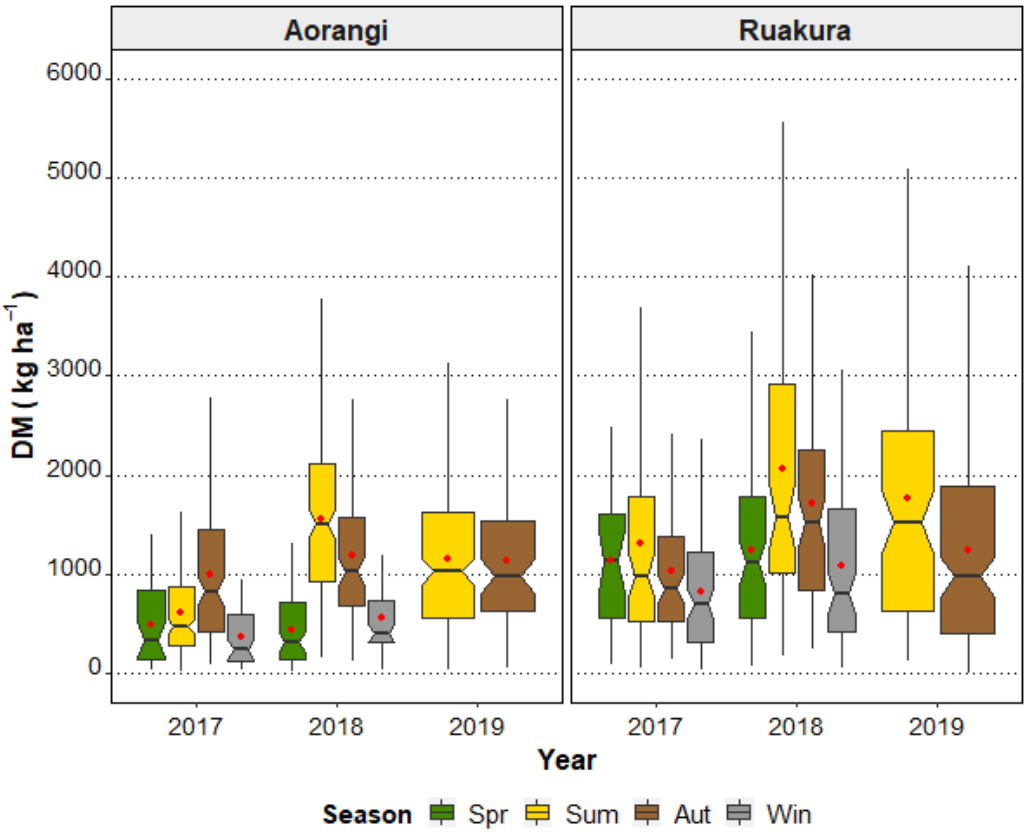


Figure 3.9 Notched boxplots showing the variation in dry matter yield in white clover due to year and season effects. Data taken from seasonal calibration cuts. Horizontal line within boxes = median seasonal dry matter (DM) averaged over scores (1-9), filled red circle = mean DM, box represents the middle 50% of variation, ends of the upper and lower whiskers represent the highest and lowest observations. Notches that do not overlap indicate medians that are significantly different at *P* < 0.05 (Chambers et al., 1983).

3.3.2.1.7. Effect of Year and Location on Full-scale DM Cut

Full scale cuts were taken from all plots in spring 2017 and 2018. As Table 3.4 indicates, significant ($P < 0.05$) additive genetic variation (σ^2_f) was detected among the HS families only at Aorangi for 2017, 2018 and across both years, while no significant variation was observed at Ruakura. The magnitude of the additive genetic variance (σ^2_f) increased with subsequent years at Aorangi. Across-site genetic variance was significant ($P < 0.05$) for individual years as well as combined years while family × location interaction ($\sigma^2_{f.l}$) was significant when the two years were combined. Family × year ($\sigma^2_{f.y}$) and family × year × location ($\sigma^2_{f.y.l}$) interactions were not significant. Narrow-sense heritability on a HS family mean basis ranged from 0.27 to 0.54 and increased over time at Aorangi. There was a 134% and 140% difference between the best performing HS family at Aorangi in

2017 and 2018 respectively, while a 160% and 147% difference was observed among families at Ruakura for 2017 and 2018, respectively (Table 3.4).

As shown in Figure 3.10 and Appendix A.6, there was 44% significantly ($P < 0.05$) less DM yield at Aorangi between 2017 and 2018. At Ruakura, though no significance was detected, there was a 21% increase from 2017 to 2018. There was a 31% and 46% difference between both sites in 2017 and 2018, respectively. All other pairwise comparisons were not significant at the 5% level using Fisher's least significant difference test (LSD) test.

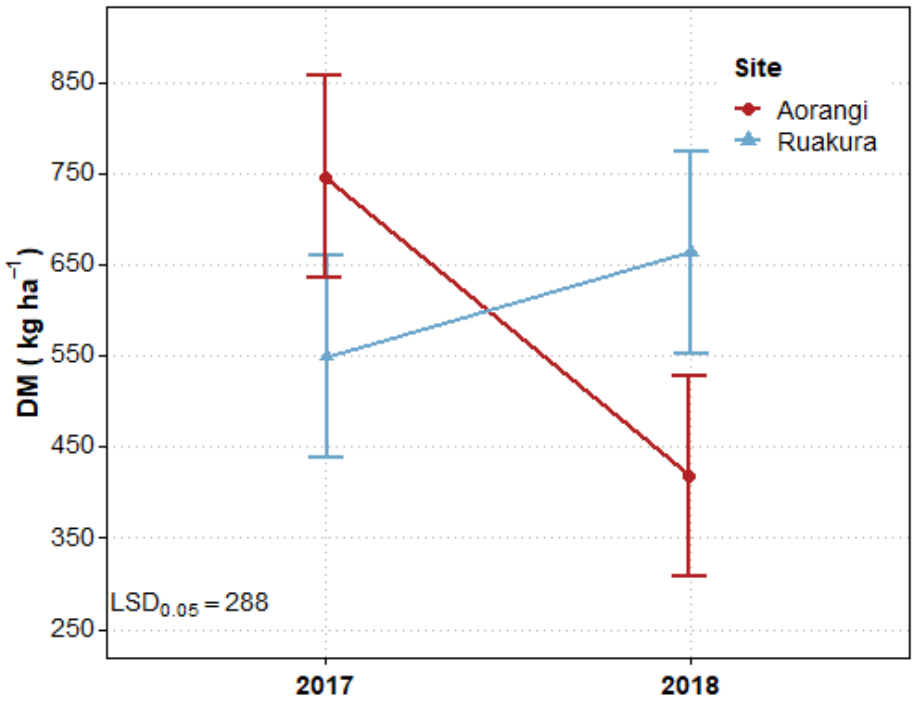


Figure 3.10 The effect of year and location on the white clover half-sib family dry matter yield at the two sites (Aorangi and Ruakura), evaluated under dairy cattle grazing. Error bars represent standard errors of the mean.

Table 3.4 Range, mean, additive genetic variance (σ^2_f), family-by-location ($\sigma^2_{f.l}$), family-by-year ($\sigma^2_{f.y}$), family-by-year-by-location ($\sigma^2_{f.y.l}$) residual variance (σ^2_ε), their associated ± standard errors and family mean narrow-sense heritability (h^2_n) for dry matter yield cuts of 200 half-sib families across two locations, Aorangi and Ruakura.

Source	σ^2_f	$\sigma^2_{f.l}$	$\sigma^2_{f.y}$	$\sigma^2_{f.y.l}$	σ^2_ε	h^2_n	Mean	Plot range (kgha^{-1})	HS Family range(kgha^{-1})
	Year 2017								
Aorangi	0.19 ± 0.09*	n.a.	n.a.	n.a.	1.60 ± 0.12	0.27 ± 0.10	804	17.50 - 2327.50	298 - 1513
Ruakura	0.08 ± 0.08	n.a.	n.a.	n.a.	1.16 ± 0.27		607.5	12.50- 2187.50	182 - 1618
Across sites	0.15 ± 0.04*	0			1.40 ± 0.07	0.37 ± 0.08			
	Year 2018								
Aorangi	0.25 ± 0.06*	n.a.	n.a.	n.a.	0.81 ± 0.06	0.48 ± 0.07	477.5	17 - 1767.50	85 - 1030
Ruakura	0.13 ± 0.14	n.a.	n.a.	n.a.	2.02 ± 0.16		724	0 - 2515	245 - 1615
Across sites	0.14 ± 0.05*	0			2.10 ± 0.07	0.35 ± 0.10			
	Across years								
Aorangi	0.24 ± 0.05*	n.a.	0 ± 0	n.a.	1.19 ± 0.06	0.54 ± 0.06	643	17.50 - 2345	n.a.
Ruakura	0.08 ± 0.06	n.a.	0 ± 0	n.a.	1.40 ± 0.08		664	0 - 2515	n.a.
Across sites	0.10 ± 0.03*	0.10 ± 0.04*	0 ± 0	0 ± 0	1.45 ± 0.05	0.38 ± 0.09			

*Significant at $P < 0.05$, n.a = not applicable.

3.3.2.1.8. Check Performance for DM yield

Figure 3.11 presents the DM yield performance of the top 20 families compared to 'Grasslands Kopu II' and 'Grasslands Bounty', the two commercial check cultivars included in the trial. When compared to all 200 HS families, 10 and 25 HS families had significantly ($P < 0.05$) higher DM yield in comparison to 'Grasslands Kopu II' and 'Grasslands Bounty', respectively (Appendix A.7). The two checks, 'Grasslands Kopu II' and 'Grasslands Bounty' produced 38% and 50% less herbage DM than the highest performing family, HS 109.

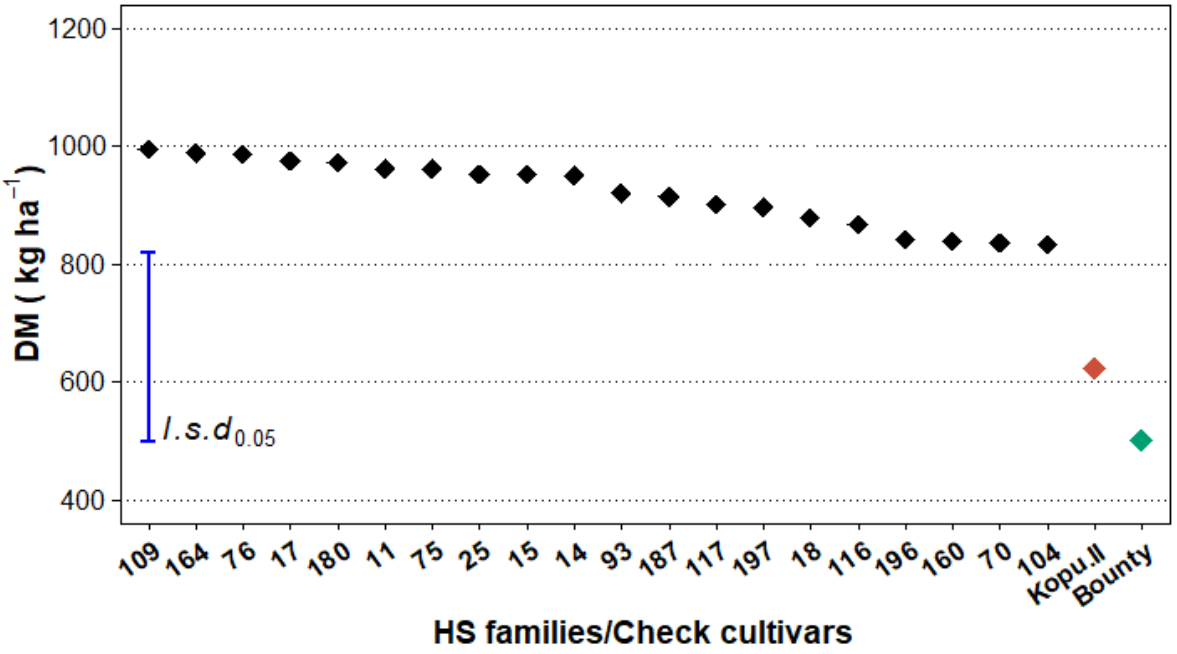


Figure 3.11 Mean dry matter yield of the top 10% of 200 half-sib families and two commercial check cultivars; 'Grasslands Kopu II' and 'Grasslands Bounty'. The trials were conducted at Aorangi and Ruakura under dairy cattle grazing across years 2017 and 2018.

3.3.2.1.9. Leaf Size

Significant ($P < 0.05$) additive genetic variation was estimated among the 200 HS families for leaf size at Aorangi and Ruakura for all years apart from Ruakura 2019 as presented in Table 3.5. Across-site analyses for individual years, as well combining all years, also revealed significant ($P < 0.05$) additive variation. Across-site additive genetic variance (σ^2_f) was predominantly lower than individual site analyses. Family × location ($\sigma^2_{f.l}$) interaction was significant for individual years 2018 and 2019, as well as across all three years and was less than or equal to the genetic variation when combining sites. The three-way interaction of family × year × location ($\sigma^2_{f.y.l}$) was significant while family × year ($\sigma^2_{f.y}$) interaction was only significant at Aorangi when analyses was combined across all years. The residual error variance component was larger than all other components. Family mean heritability ranged from 0.13 to 0.73 and increased as the trial progressed.

Table 3.5 Estimated additive genetic (σ^2_f), family-by-location ($\sigma^2_{f.l}$), family-by-year (σ^2_{fy}), family-by-year-by-location ($\sigma^2_{f.y.l}$) and pooled error (σ_ε) variance components with their associated standard errors (± SE) and family mean narrow-sense heritability (h^2_n) estimated across locations; Aorangi, Ruakura and across years for leaf size scores in white clover.

Source	σ^2_f	$\sigma^2_{f.l}$	$\sigma^2_{f.y}$	$\sigma^2_{f.y.l}$	σ^2_ε	h^2_n
			Year 2017			
Aorangi	0.07 ± 0.03*	n.a.	n.a.	n.a.	0.55 ± 0.05	0.26 ± 0.10
Ruakura	0.06 ± 0.02*	n.a.	n.a.	n.a.	0.29 ± 0.06	0.30 ± 0.09
Across sites	0.05 ± 0.01*	0.01 ± 0.01	n.a.	n.a.	0.47 ± 0.02	0.36 ± 0.09
			Year 2018			
Aorangi	0.08 ± 0.02*	n.a.	n.a.	n.a.	0.37 ± 0.02	0.51 ± 0.08
Ruakura	0.09 ± 0.02*	n.a.	n.a.	n.a.	0.29 ± 0.02	0.69 ± 0.06
Across sites	0.05± 0.01*	0.05± 0.01*	n.a.	n.a.	0.40 ± 0.01	0.42 ± 0.09
			Year 2019			
Aorangi	0.20 ± 0.05*	n.a.	n.a.	n.a.	0.70 ± 0.02	0.46 ± 0.10
Ruakura	0.02 ± 0.03	n.a.	n.a.	n.a.	0.01± 0.02	n.s.
Across sites	0.05 ± 0.01*	0.03 ± 0.01*	n.a.	n.a.	0.56 ± 0.03	0.31 ± 0.08
			Across years			
Aorangi	0.10 ± 0.02*	n.a.	0.02 ± 0.01*	n.a.	0.40 ± 0.02	0.73 ± 0.06
Ruakura	0.05 ± 0.01*	n.a.	0	n.a.	0.30 ± 0.01	0.66 ± 1.8
Across sites	0.06 ± 0.01*	0.04 ± 0.01*	0	0.01 ± 0.005*	0.42 ± 0.01	0.7 ± 0.06

*Significant at $P < 0.05$, n.a.= not applicable.

3.3.2.1.10. Stolon Traits

There was significant ($P < 0.05$) additive genetic variation among the 200 HS families for the traits stolon number (SN) and number of branches (SB) produced pre-summer (PRS) and post-summer (POS) for individual years in the second (2017/2018) and third year (2018/2019) of growth (Table 3.6).

Combining pre-and post-summer assessments, (PRS+POS) showed significant ($P < 0.05$) genetic variation only for Year 2 SB and Year 3 SN. The family × season interaction ($\sigma^2_{f.s}$) component was significant ($P < 0.05$) in all cases for SN and SB for individual years. Upon combining years however, there were no significant differences observed among HS families for either SN or SB and family × season ($\sigma^2_{g.s)}$ was not also significant for PRS, POS and PRS+POS. For both traits SN and SB, family × year ($\sigma^2_{f.y}$) interaction was significant for PRS and POS but not for PRS+POS, while the three-way interaction of family × year × season ($\sigma^2_{f.y.s}$) was significant. The residual error variance component was the largest variance component. Family mean heritability ranged from 0.14 to 0.3 for SN and 0.09 to 0.4 for SB.

For PRS and POS in Year 2, there was a 150% and 131% difference respectively, between the top and worst performing HS family for SN and a 161% and 130% difference respectively, for PRS and POS in Year 3. There was a 143% and 163% difference respectively, between the top and worst performing line for SB in Year 2 and a 177% and 174% difference, respectively, in Year 3.

Table 3.6 Range, mean, additive genetic variance (σ^2_f), family-by-season ($\sigma^2_{f.s}$), family-by-year ($\sigma^2_{f.y}$), family-by-year-by-season ($\sigma^2_{f.y.s}$), residual variance (σ^2_ε), their associated ± standard errors and family mean narrow-sense heritability (h^2_n) for white clover morphological traits, stolon number (SN) number of branches (SB) measured from the 200 HS families at Aorangi before (PRS) and after (POS) summers of 2017/2018 and 2018/2019.

		SN			SB		
	Source	**PRS**	**POS**	**PRS+POS**	**PRS**	**POS**	**PRS+POS**
2017/2018 (Year 2)	σ^2_f	0.44 ± 0.11*	0.31 ± 0.14*	0.09 ± 0.09	1.47 ± 0.45*	1.18 ± 0.37*	0.75 ± 0.30*
	$\sigma^2_{g.s}$	n.a.	n.a.	0.04 ± 0.11***	n.a	n.a	0.78 ± 0.34*
	σ^2_ε	5.16 ± 0.18	8.33 ± 0.29	6.80 ± 0.16	22.87 ± 0.79	20.39 ± 0.70	21.88 ± 0.52
	h^2_n	0.20 ± 0.04	0.25 ± 0.09	0	0.37 ± 0.07	0.15 ± 0.04	0.10 ± 0.90
	mean	1782.90	2547	n.a.	3362.04	3565.80	n.a.
	Plot range (no.m$^{2)}$	0 - 7641	0 - 9678.6	n.a.	0 - 17319	0 - 14263.2	n.a.
	HS line range (no.m$^{2)}$	566 - 4019	510 - 4018		1189 - 7244	679 - 6735	
2018/2019 (Year 3)	σ^2_f	0.21 ± 0.07*	0.28 ± 0.1*	0.13 ± 0.06*	1.32 ± 0.36*	1.09 ± 0.29*	0.14 ± 0.24
	$\sigma^2_{g.s}$	n.a.	n.a.	0.13 ± 0.06*	n.a	n.a	1.12 ± 0.31*
	σ^2_ε	4 ± 0.14	5.60 ± 0.19	4.80 ± 0.12	17.56 ± 0.60	14.50 ± 0.49	15.90 ± 0.38
	h^2_n	0.14 ± 0.04	0.13 ± 0.04	0.28 ± 0.11	0.18 ± 0.04	0.19 ± 0.04	0.09 ± 0.14
	mean	1426.30	1833.8	n.a.	2445.1	2241.31	n.a.
	Plot range (no.m$^{2)}$	0 - 8150.4	0 - 7641	n.a.	0 - 15282	0 - 17319	n.a.
	HS line range (no.m$^{2)}$	283 - 2603	736 - 3509		396 - 6452	396 - 5716	
Across years	σ^2_f	0.09 ± 0.07	0.05 ± 0.09	0.03 ± 0.05	0	0.36 ± 0.24	0.11 ± 0.15
	$\sigma^2_{f.y}$	0.28 ± 0.08*	0.30 ± 0.11*	0.07 ± 0.068	1.50 ± 0.29*	0.90 ± 0.30*	0.27 ± 0.22
	$\sigma^2_{f.s}$	n.a.	n.a.	0.04 ± 0.07	n.a.	n.a.	0
	$\sigma^2_{f.y.s}$	n.a.	n.a.	0.22 ± 0.09**	n.a.	n.a.	0.95 ± 0.23*
	σ^2_ε	4.60 ± 0.11	7.01 ± 0.17	5.80 ± 0.09	20.4 ± 0.40	17.60 ± 0.40	18.90 ± 0.32

*Significant at $P < 0.05$, n.a.= not applicable.

3.3.2.1.11. Effects of Summer Period on Stolon Traits

The main effects time and year were significant ($P < 0.01$) for SN while only the year effect was significant for SB ($P < 0.001$) (Appendix A.8, A.10). As shown in Figure 3.12, there was a significant 27% decrease in SN from Year 2 (2017/2018) to Year 3 (2018/2019) whereas SB decreased by 32% from Year 2 to Year 3.

The trait SN increased significantly ($P < 0.05$) by an average of 47.5 % between PRS and POS in Year 2. Although it increased by 33% in Year 3, no significant differences were detected (Figure 3.11, Appendix A.9). There was an insignificant increase in SB between PRS and POS in Year 2 followed by a insignificant decrease between PRS and POS in Year 3 (Appendix A.11).

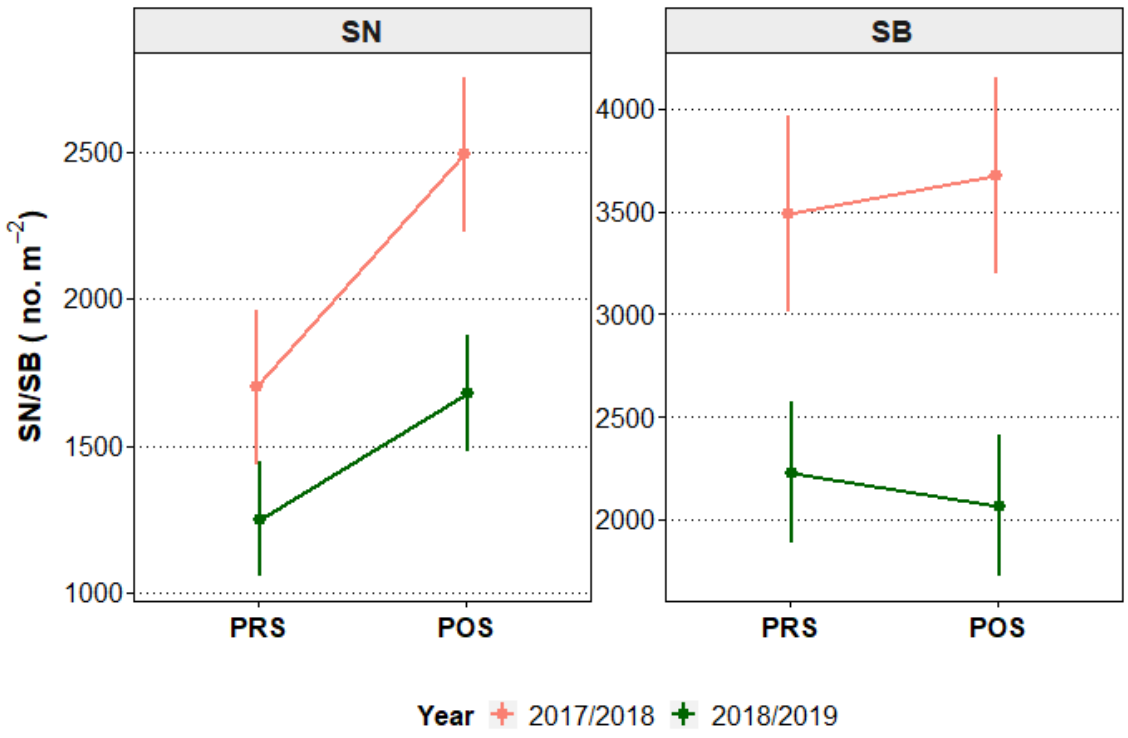


Figure 3.12 Predicted means and standard errors of the mean for 200 white clover half-sib families measured pre-summer (PRS) and post-summer (POS) for stolon number (SN) and number of stolon branches (SB) at a single location, Aorangi.

3.3.2.1.12. Check Performance for Stolon Traits

When comparing the HS families to the two commercial checks, 'Grasslands Kopu II' and 'Grasslands Bounty', 49 HS families had significantly ($P < 0.05$) higher SN than 'Grasslands Kopu II'. Cultivar 'Grasslands Bounty' was not significantly different to any of the top families (Figure 3.13), Appendix A.12). 'Grasslands Kopu II' produced significantly less SB than 17 HS families, while only one family, HS 31, had 43% significantly ($P < 0.05$) more SB than 'Grasslands Bounty' (Appendix A.13).

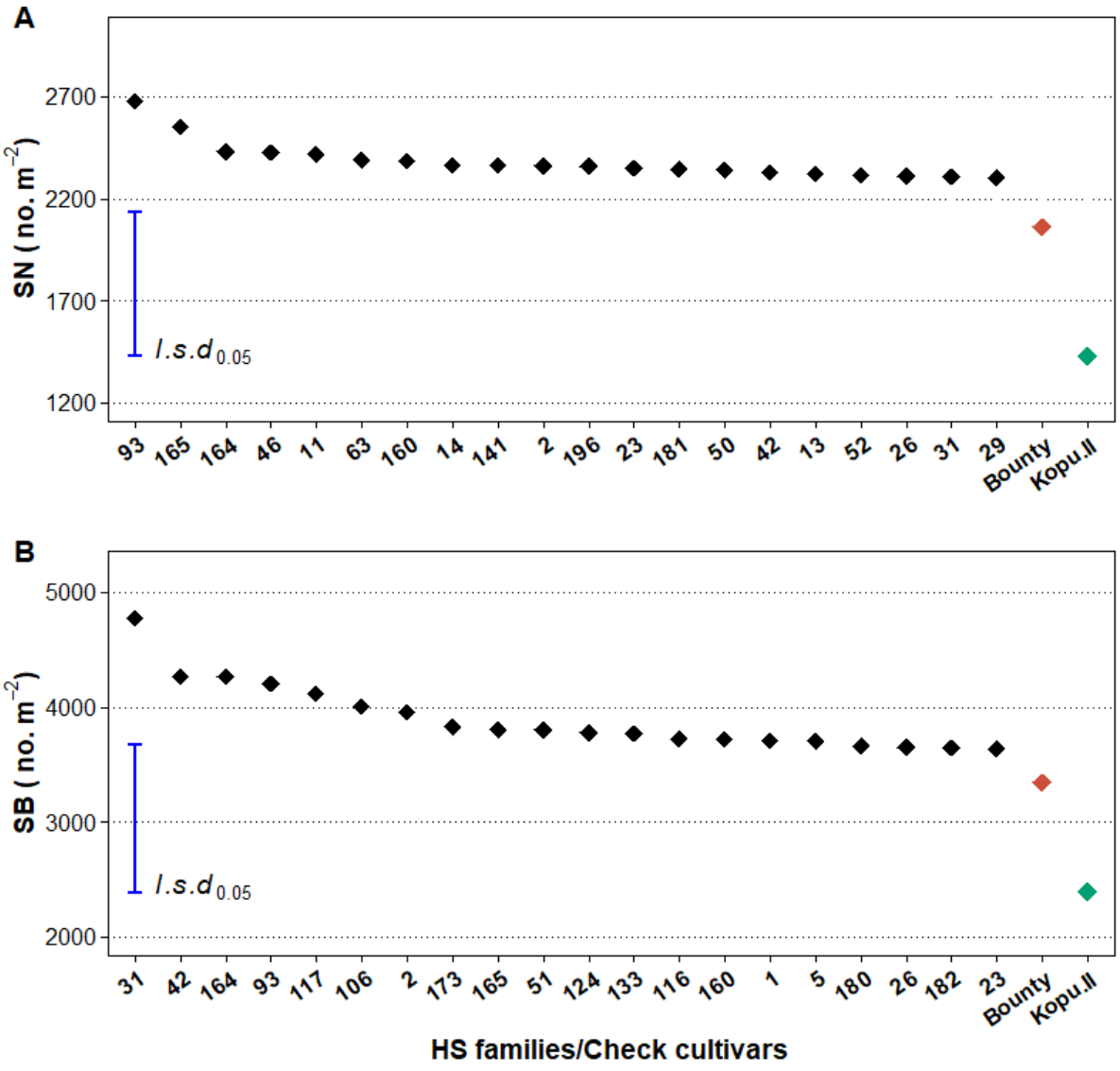


Figure 3.13 Mean stolon number (SN) and stolon branches (SB) of the top 10% of 200 white clover half-sib families and two commercial check cultivars; 'Grasslands Kopu II' and 'Grasslands Bounty'. The trial was evaluated at Aorangi under dairy cattle grazing across combined summers of years 2017/2019 and 2018/2019.

3.3.2.2. Multivariate Analysis

3.3.2.2.1. Pearson Correlation

The pairwise Pearson correlation coefficients are presented in Figure 3.14 and indicated that DM had strong positive and significant ($P < 0.001$) phenotypic correlation with mean growth score (GS123) across years, seasons and location, across location seasonal growth scores for summer (SumGS), autumn growth scores (AutGS) and spring growth scores (SprGS). Dry matter (DM) was moderately positively correlated with Year one across location growth score (GS1), across location seasonal growth scores for winter (WinGS) and leaf size (LS). Interestingly, there was a significant ($P < 0.001$) low but positive correlation (0.24) between DM and Year 3 post-summer stolon number (SNPOS). Although HCN was negatively correlated with almost all traits, the only significant relationship was a negative correlation with LS. Leaf size was moderately correlated with GS123 and had a significant negative correlation with Year 3 pre-summer stolon number (SNPRS). Pre-summer stolon number and number of branches (SNPRS) and (SBPRS) had a significant ($P < 0.001$) positive correlation (0.67), while post-summer stolon number (SNPOS) and number of branches (SBPOS) also had significant ($P < 0.001$) positive correlation (0.6). The pre-summer stolon traits had a low correlation with the post-summer stolon traits. All stolon traits (SNPRS, SBPRS, SNPOS and SBPOS) had low but positive correlations with all seasonal growth scores.

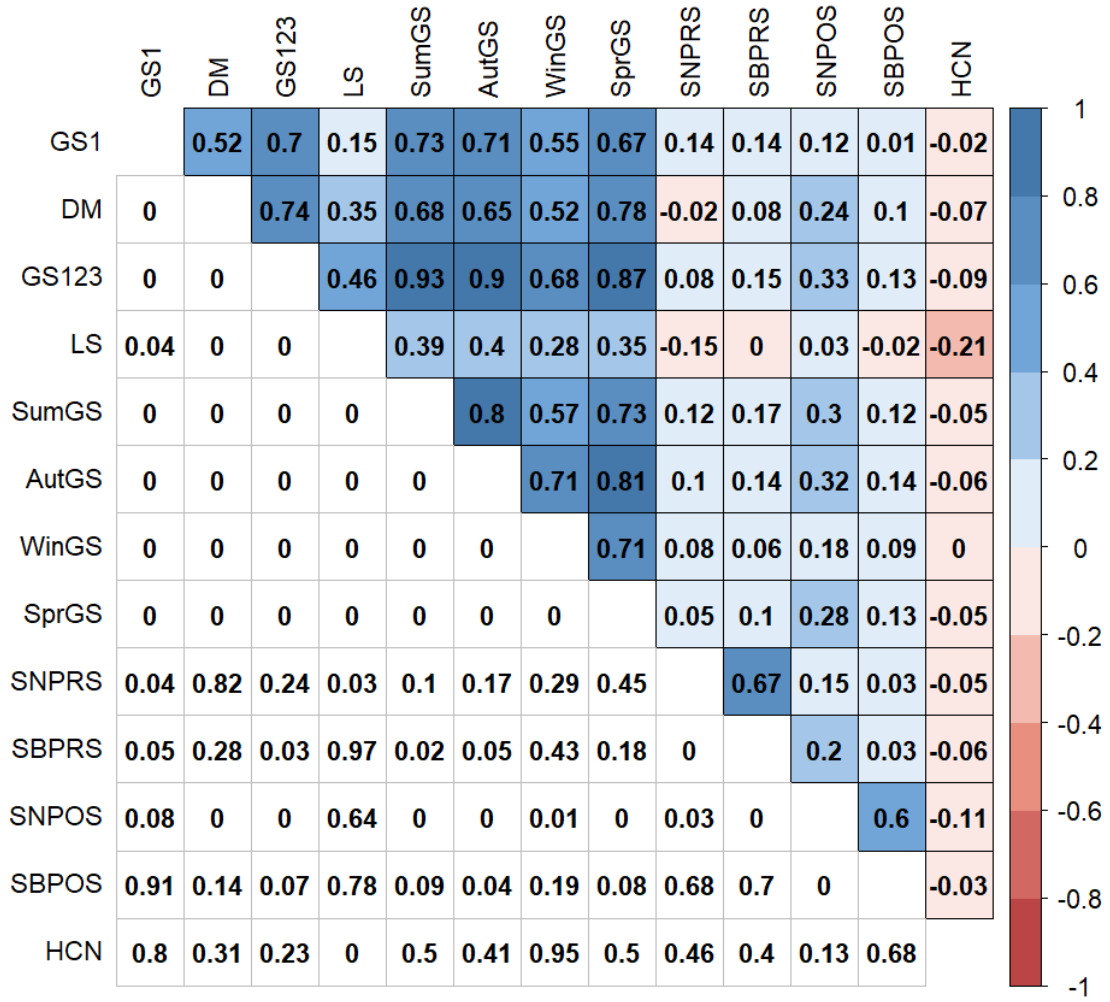


Figure 3.14 Pearson correlation coefficients among traits estimated from the Best Linear Unbiased Predictor (BLUP) values of the 200 HS families for traits :Year 1 across location growth score (GS1), Year 1 and 2 dry matter across location (DM), across all years and location growth scores for all seasons (GS123), summer (SumGS), Autumn (AutGS), winter (WinGS), spring (SprGS), leaf size (LS), Year 3 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), post-summer stolon branches (SBPOS) and hydrogen cyanide production (HCN). Correlations are above the diagonal; *P* values are below the diagonal and indicate significance at 0.05 level. Locations are Aorangi and Ruakura. *Stolon traits were measured at Aorangi only.

3.3.2.2.2. Pattern Analysis

Figure 3.15 provides a graphical summary of the association among traits as well as the clustering pattern of the 200 HS families according to trait expression.

The first four PCs explained, respectively, 30.6, 19.4, and 12% of the overall phenotypic variation among HS families for yield and persistence-related traits (Figure 3.15, Appendix A.14). Although PC 2 and PC 3 accounted for similar amounts of variation, results are presented for PC 2 due to the better visual discrimination, further supported by the dendrogram generated from the cluster analysis. (Appendix A.15). The first axis (PC1) was a negative indicator of the yield-related traits; GS1, DM, GS123 and LS and the persistence-related traits, SNPRS, SBPRS, SNPOS and SBPOS with traits loading heavily on that dimension, demonstrating their importance (Appendix A.16). PC1 was a positive indicator for only HCN, confirming the negative association with the other traits. The short vector length of HCN also indicates a weak association with the other traits (Weikai, 2014). PC2 was a positive indicator for the yield-related traits and negative for the persistence related traits; SNPRS, SBPRS, SNPOS and SBPOS (Appendix A.16). The pre-summer stolon traits had the largest impact on PC2 while the post-summer stolon traits had the largest impact on PC3 (Appendix A.17).

The angle between directional vectors represents the correlation structure among the traits. The yield traits (DM, GS, GS1) were highly positively correlated as shown by the (< 90°) angles between them. The stolon traits also showed strong positive association but those measured in the same season had a greater positive association with each other, such as SNPRS and SBPRS and SNPOS and SBPOS which were more closely associated with each other. Stolon traits showed no to slightly negative association with LS and an inverse relationship with HCN. There was also a negative association between HCN and LS. It is worth highlighting the positive association between yield-related traits and post-summer traits, SN and SB. For example, several HS families in group 1, (red), far from the vector origin and between the stolon and yield traits e.g., HS 14 and 117, indicate above average expression for DM yield and persistence-related traits like SN and SB.

Cluster analysis generated three groups (Table 3.7). The number of genotypes within each group ranged from 33 in group 1 to 92 in group 3. Group 1 consisted of genotypes with the highest mean expressions for DM yield, GS and LS. Group 2 had the second highest

mean and possessed combined high expressions for pre and post-summer SN and SB. While group 3 predominantly contained HS families high in HCN production.

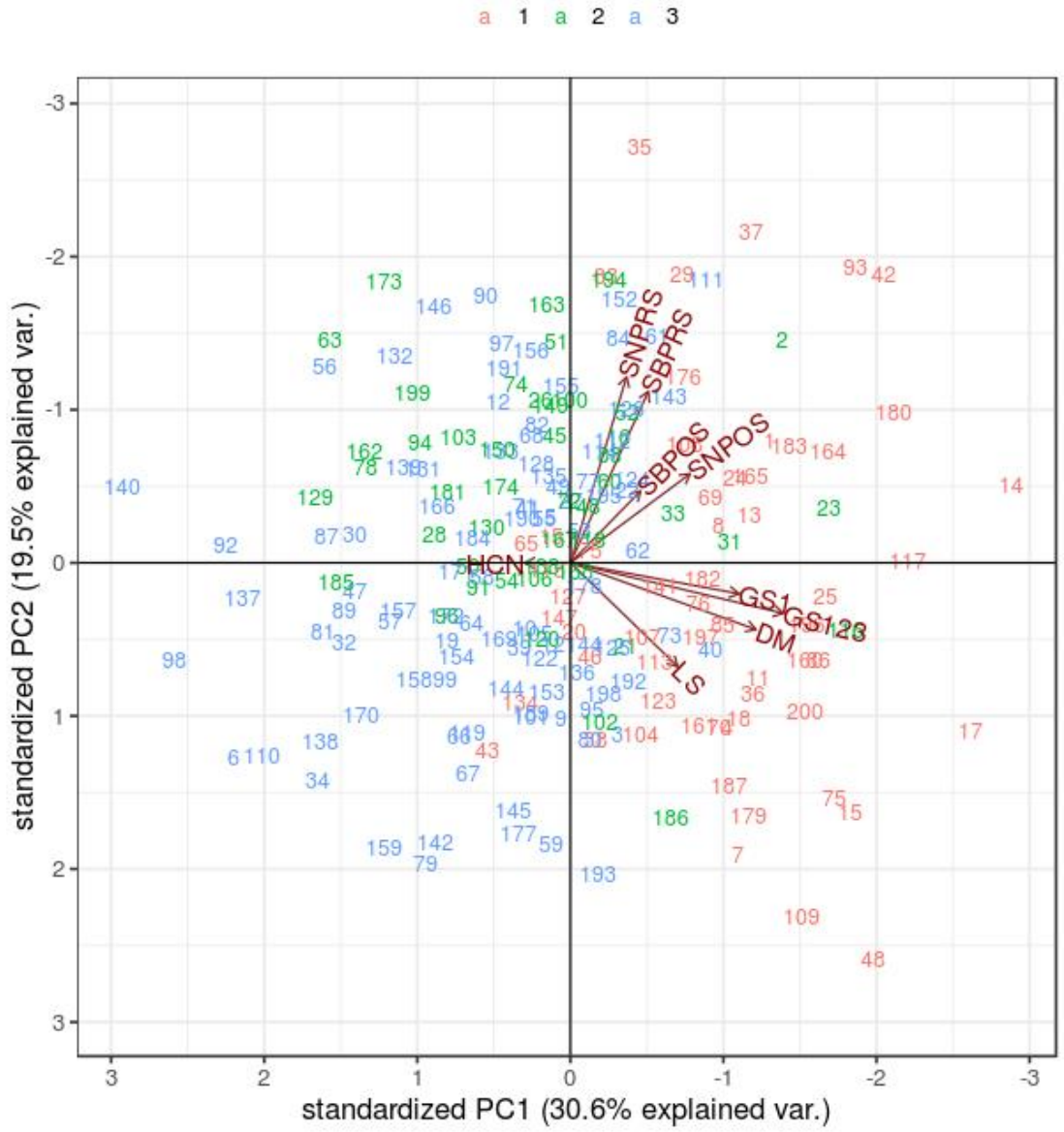


Figure 3.15 Biplot generated from pattern analysis using standardised Best Linear Unbiased Predictor (BLUP) values of 200 HS families for traits: Year 1 across location growth score (GS1); Year 1 and 2 dry matter across all locations (DM); across all years and location growth scores for all seasons (GS123); leaf size (LS); Year 2 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS); post-summer stolon number (SNPOS); post-summer stolon branches (SBPOS) and hydrogen cyanide production (HCN). Locations are Aorangi and Ruakura. *Stolon traits were measured at Aorangi only.

Table 3.7 Number of HS families and means of groups identified by cluster analysis. Values are for 200 HS family lines for traits: Year 1 across location growth score (GS1); Year 1 and 2 dry matter across all locations (DM); across all years and location growth scores for all seasons (GS123); leaf size (LS); Year 2 pre-summer stolon number (SNPRS); pre-summer stolon branches (SBPRS); post-summer stolon number (SNPOS); post-summer stolon branches (SBPOS); and hydrogen cyanide production (HCN). Locations are Aorangi and Ruakura. *Stolon traits were measured at Aorangi only.

Cluster	No. of lines	GS1	DM	GS123	LS	SNPRS	SBPRS	SNPOS	SBPOS	HCN
1	34	5.44	2.90	5.51	3.22	2.67	4.88	3.72	4.35	2.88
2	92	5.19	2.57	4.45	3.08	2.78	4.90	3.61	4.71	3.01
3	74	5.20	2.63	4.56	3.21	2.40	3.76	3.16	3.54	3.25

3.3.2.2.3. Pattern Analysis of Seasonal Growth Scores

The biplot (Figure 3.16) generated from the PCA for mean seasonal growth scores across the two locations and years shows that the first PC1 and PC2 accounted for 46.6% and 29.8% of the variation, respectively. PC1 had negative associations with all traits while PC2 was a positive indicator for Aorangi seasonal GS with traits loading heavily in that dimension compared to Ruakura seasonal GS (Appendix A.19). Ruakura seasonal GS contributed more to PC2 and PC3 (Appendix A.20). Angles among the directional vectors indicate a positive but weak relationship between both locations. HS families 116 and 18 displayed high performance at both sites while HS families 15 and 15, 4 and 17 performed better at either Aorangi or Ruakura respectively.

Cluster analysis generated four groups. Group 4 had the highest mean expression for all four seasons across the two sites. Group 3 had the second highest mean (62 HS families) with the highest yields in Ruakura than in Aorangi, while group 1 was the third highest with HS families performing better in Aorangi. The last group, 2, consisted of HS families that performed better in some seasons than group 1 and/or 3 but was on average the lowest group.

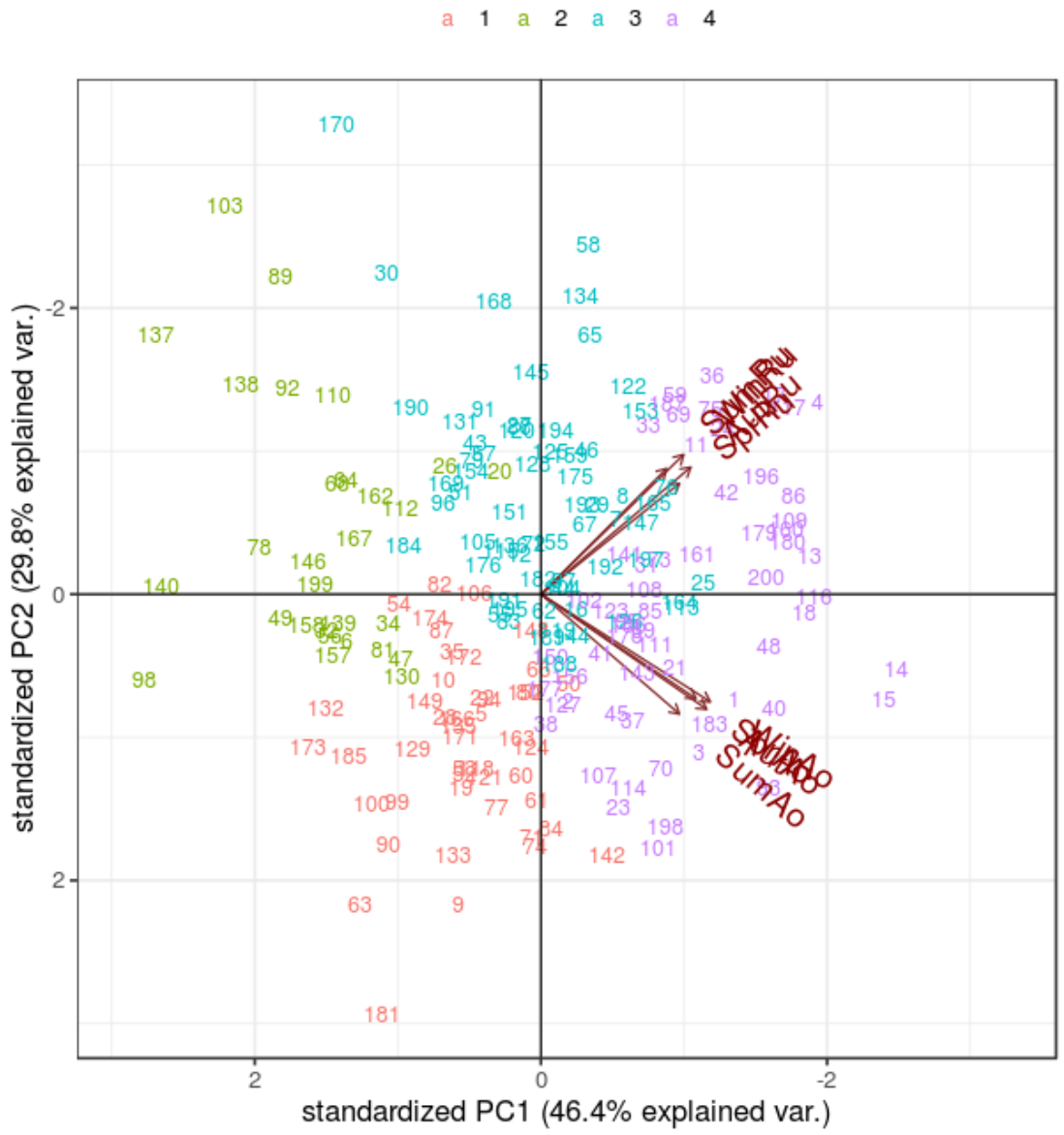


Figure 3.16 Biplot generated from pattern analysis using standardised Best Linear Unbiased Predictor (BLUP) values of 200 HS families assessed in two locations, Aorangi (Ao) and Ruakura (Ro) over three years for seasonal herbage growth scores summer (SumAo and SumRu), autumn (AutAo and AutRo), winter (WinAo and WinRu) and spring (SprAo and SprRu).

Table 3.8 Number of HS families and group means generated from cluster analysis of BLUP's based on performance of 200 HS family lines across two locations and three years for seasonal herbage growth scores summer (SumAo and SumRu), autumn (AutAo and AutRo), winter (WinAo and WinRu) and spring (SprAo and SprRu). The two locations are Aorangi (Ao) and Ruakura (Ru).

Cluster	No of lines	WinRu	SprRu	SumRu	AutRu	SumAo	SprAo	WinAo	AutAo
1	45	4.84	4.31	5.52	5.51	6.08	5.41	6.09	6.69
2	24	5.02	4.21	5.60	5.54	5.42	4.58	5.46	6.02
3	62	5.65	4.82	6.10	6.12	5.78	4.91	5.81	6.47
4	69	5.81	4.93	6.28	6.26	6.22	5.95	6.35	6.89

3.4. Discussion

A key criterion for the success of a breeding programme is the understanding of the breadth of genetic variation for important selection traits. The primary objective of this chapter was to investigate the magnitude of additive genetic variation among a training population of 200 white clover HS families, within a genomic selection programme, evaluated in a multi-location/year/season trial for important morphological traits contributing to DM yield and vegetative persistence. Analysis of the HS family data would also enable estimation of genetic parameters, e.g., heritability and genetic correlation.

3.4.1. Genetic Variation

High and significant genetic variation was present for HCN, indicating the potential genetic variation available for selecting either high or low expression of this trait depending on the breeding goals for the target market as it varies from country to country. For example, the large leaved Ladino cultivars with low HCN are used extensively in the United States (Crush & Caradus, 1995) and several European countries where low levels of HCN are preferred. Whereas in New Zealand, the preference is for cyanogenic cultivars that have historically been agronomically successful (Caradus & Williams, 1989).

Considerable amounts of additive genetic variance were estimated for DM, LS, SN and SB across years, locations and seasons. Similar results were reported in other studies evaluating white clover populations (Woodfield & Caradus, 1990; Jahufer et al., 1994; Caradus & Chapman, 1996; Jahufer et al., 1997; Annicchiarico et al., 1999; Jahufer et al., 1999; Jahufer et al., 2016). This variation is important for breeders as selection is only effective when there is available genotypic variation that allows substantial genetic gain to be made (Acquaah, 2012). The significant genetic diversity means that superior plants can be identified and successfully used to achieve genetic gain for traits under selection. No significant additive variation was detected at Ruakura for both of the full-scale DM cuts. It must be noted that the full-scale cuts are taken at one point in time only and it is possible that the HS families had low detectable genotypic variation amongst them at the time of the sampling. The challenging environment in Ruakura could possibly suppress the expression of genetic variation; hence, reducing the ability to detect significant differences among HS families. Other possible reasons could be attributed to the presence

of large sampling errors (as evidenced by the large standard errors), bias in collecting samples or insufficient sample size that prevented significant differences to be observed.

3.4.2. Genotype-by-Environment Interaction

There was significant family × year, family × location, family × season as well as family × year × season and family × year × location interactions for most traits. Caradus and Chapman (1991) Caradus et al. (1993), Jahufer et al. (1994) and Jahufer et al. (2009) have also reported significant G×E interaction for many yield and persistence-related traits in white clover. As expected, for polygenic traits like GS, SN and SB, the magnitude of interaction variance components was often greater than the family additive genetic variance component, demonstrating a change in relative performance of HS families across spatial and temporal environments. In contrast, LS, a trait influenced by considerably fewer genes, had higher additive genetic variation relative to G×E, showing a higher level of genotype influence on the phenotype. This presence of G×E implies that DM yield at Aorangi would be a poor predictor of DM yield at Ruakura as these complex traits are more influenced by environment than genotype. Results of the PCA biplot (Figure 3.16) also show a low correlation between locations. However, cluster analysis revealed HS families that can be selected for broad adaptation across both locations, for example, HS families 116 and 18, whereas, HS families 14 and 15 with superior performance in Aorangi, might be selected for specific adaptation to that location (Figure 3.16).

The relative contribution of family × year to the total amount of observed variation was lower than family × location indicating that location had a greater effect on seasonal GS than the year of growth. The presence of G×E not only complicates comparisons among genotypes but also reduces the efficiency of selection of superior genotypes as the true genetic variation is confounded by environmental effects (Falconer, 1989). Because little success can be expected by phenotypically selecting for these traits in one environment, trials will have to be run across multiple environments and years to assess the true breeding potential of selection candidates, especially if the goal is to breed for broad adaptation (Brown & Caligari, 2008a; Moose & Mumm, 2008; Jahufer et al., 2013). Notwithstanding, with selection strategies based on genotypic rather than phenotypic selection, decision making strategies for these traits could be improved.

3.4.3. Heritability

For a simple trait such as cyanogenesis (HCN), the estimate of narrow-sense heritability on a family mean basis was high (0.82). However, this result must be interpreted with caution due to a probable upward bias as variance estimates were obtained before HS families were transplanted to the trial locations and could be confounded by G×E (Nyquist & Baker, 1991; Falconer & Mackay, 1996). Heritability estimates for LS was moderate to high (0.3 - 0.73) and similar to other studies by Barcikowska (1976); Caradus and Woodfield (1990); (Woodfield & Caradus, 1990; Jahufer et al., 1994; Caradus & Chapman, 1996). It can therefore be assumed that for such a simple trait, fewer numbers of locations and years may be sufficient for evaluation due to the consistent performance of HS families (Falconer & Mackay, 1996; Dohm, 2002).

As the traits became more complex, i.e., DM yield, growth scores and stolon density, they tended to have lower heritability estimates than simple traits like LS and HCN. Heritabilities for DM yield were low to moderate (0.27 - 0.54) and comparable to Annicchiarico et al. (1999) (0.52), Jahufer et al. (1999) (0.38) and Finne et al. (2000) (0.44). Heritability for SN (0.13 – 0.25) is similar to studies by Caradus and Woodfield (1990) that placed heritability estimates in the range of 0.11 to 0.45. Heritability for SB (0.15 – 0.37) is lower than reported by Rowe and Brink (1993) (0.59) and consistent with Jahufer et al. (1999) (0.27) and Caradus et al 1990, (0.37). These low heritabilities could also be influenced by large error variances, which result from insufficient replication, problematic experimental design, human error in data sampling and inappropriate choice of data analysis (Weikai, 2014).

The low to moderate heritabilities for DM, GS and stolon traits indicate the likely challenges to be faced in improving these traits. These low heritabilities are not surprising, as these traits are complex with quantitative inheritance patterns strongly influenced by the environment. While it does not signify additive genetic variance is lacking, it implies that of the observed variation, a substantial proportion is caused by environmental variation rather than genetic differences (Visscher et al., 2008). This means that even though selection for yield and vegetative persistence is possible due to the genetic variation available, it is hampered by the low narrow-sense heritabilities that are caused by high non-additive genetic variation (Annicchiarico, 2015). From a breeding perspective, it translates to difficulty in trait improvement via mass selection or individual

phenoypic selection strategies. Nevertheless, more discriminatory methods like progeny testing and family-based selection methods which allow breeders to access and utilise among and within family additive genetic variation would prove useful.

Heritabilities were generally lower for earlier measurements and increased with time, as an increase in the number of years reduces the divisor in the heritability equation. It can be deduced that making selections at earlier stages may not be as efficient as selecting later in the breeding cycle when important persistence related traits can be observed. This also shows the importance of multi-year trials in identification of superior breeding material. On the other hand, across location heritabilities were usually lower than individual location heritabilities, this could be as a result of magnitude of the family × location variance relative to the line additive variance (Weikai, 2014).

Differences were obsereved between the heritabilities at both locations, heritability at Aorangi was higher than at Ruakura for most traits. This is likely due to the higher levels of enviromental heterogeneity at the Ruakura enviroment which mask the additive variation. This is not surprising as the two locations are remarkably different in terms of climatic and soil conditions, weed, pest and disease pressures. According to Falconer (1989), differences in heritability between locations could be a result of differences in either the genetic variance or the enviromental variance or both of these factors.

3.4.4. Year and Season Effect on Yield

In the HS family trials across the two sites Aorangi and Ruakura, the contribution of white clover herbage yield to pasture varied over seasons and years and revealed similar patterns of herbage accumulation at both locations. The differences in the performance of the HS families are likely driven by temporal variation in temperature and rainfall (Brougham, 1958, 1977). This differential response by genotypes to environmental fluctuations is a result of the combination of the genetic variation for the trait as well as genetic variation in the plasticity of response to different external stimulus (Hoffman & Parsons, 1991). Comparison of the three years seasonal GS showed that DM yield differed across years significantly, indicating an influence of time (year) on the variability of yield. DM yield was significantly higher in the second year especially during the summer and autumn months. This is similar to that reported by Widdup and Barrett (2011) and Caradus et al. (1995) indicating that white clover produces the greatest amount of herbage biomass in the second year of growth. Also, the second year showed the greatest amount of

phenotypic variation. The decrease in DM yield in the third year could be explained by the plants becoming more susceptible to biotic and abiotic stresses like intraspecie competition (Chapman et al., 1996), adverse weather conditions (Wachendorf et al., 2001), pests and diseases and reliance on a weak fibrous root system (Brock & Caradus, 1995).

Even though all seasons had the highest DM production in 2018, spring in Aorangi that year had the lowest amount of DM. This decrease in herbage production is also reflected in the full - scale dry matter cuts that were also collected in that season in 2018. Among the plausible explanations for the abnormally low DM yield could be the low amount of rainfall (total of 208 mm) over the spring months of that year. September and October 2018 received 58 mm and 54 mm of rainfall respectively. Wachendorf et al. (2001) reported seasonal precipitation as having an impact on white clover spring growth and according to Brock et al. (1988), clover content can be reduced by over 70% during spring drought. In 2018, most clover plants would have made the transition from tap roots to fibrous, nodal roots. It is probable that the low DM yield could be as a result of clover plants relying on their fibrous roots which are shallow and lack the ability to forage deeper soil layers for water and resources; whereas, in 2017, they still possessed their tap roots which are hardier and penetrate deeper into soil layers thus ensuring better access to water and nutrients (Caradus, 1977). It must be noted, however, that Ruakura had comparable precipitation levels (185 mm) in spring with relatively less effect on DM yield. Therefore, the full cause of this discrepancy is uncertain.

The lowest biomass was recorded in winter and spring and highest in autumn and summer. Low herbage production in winter is explained primarily by the fact that plants have reduced leaf area index due to fewer number of leaves and lower leaf surface area to intercept radiation; thus, resulting in restricted photosynthetic ability (Woledge et al., 1990). Previous New Zealand research has suggested that the reason for low yield in spring and reduced clover contribution to pasture is due to several reasons, outlined by Brock et al. (1988) as drought conditions, lax defoliation regimes and over zealous nitrogen fertilizer applications. Another primary reason is temperatures being around 15.5°C, which is optimum for ryegrass growth, thereby tilting the balance in favour of the actively growing ryegrass (Brougham, 1958; Woodfield & Caradus, 1996; Wachendorf et al., 2001). At the onset of summer, however, due to warmer tempereatures, now in the optimum range of 18°C to 29°C for white clover, ryegrass growth rate

decreases (Brougham, 1958). White clover is then able to contribute more to the total herbage yield during this period due to its ability to thrive under warmer temperatures coupled with the reduced competition from ryegrass (Brock et al., 1988). Overall, even though the white clovers' contribution to yield in a mixed sward is characterised by variability, a major benefit of gowing clover in a mixed sward with ryegrass is this seasonal complementarity which ensures available feed for livestock all year round (Brougham, 1958; Harris & Thomas, 1973; Caradus et al., 1995).

3.4.5. Year and Season Effect on Vegetative Persistence

A key problem with white clover is the inability to sustain a sufficiently large proportion of clover in mixed swards for its nitrogen fixation and animal production benefits to be realised (Woledge et al., 1990). Hence, the maintenance of high stolon density is an important trait for white clover vegetative persistence as the stolon is the primary structural unit of the plant (Thomas, 1987b). High stolon density allows for better capture of light and nutrients to support adequate clover growth (Piano & Annicchiarico, 1995). The mean number of stolons produced over both years, (1910 stolons $m^{-2)}$, was in the range reported by Woodfield and Caradus (1994), on 110 global white clover cultivars and ecotypes (531 - 2075 stolons m^{-2}). Similarly, the mean number of stolons branches produced over both years, (2933 stolon branches m^{-2}), was in the range reported by Jahufer et al. (1999) on 63 white clover germplasm accessions (398-3582 stolon branches m^{-2}) and Jahufer et al. (1995) on 43 white clover accessions (453-3725 stolon branches m^{-2}).

A hypothesis at the start of the experiment, reported in this chapter, was that stolon density, as measured by stolon number and branching, would be impacted by summer moisture stress, resulting in a decline. However, this was not the case and two trends were immediately apparent: first, there were more stolons in the HS family trial plot swards after summer in both years of measurement: and second, there was a significant decline in the following years assessment for SN and SB, respectively. The increase over summer in the first year was initially assumed to be due to the fragmentation of stolons as a result of the loss of the tap root system. But despite the relatively dry summers with summer months receiving only 193 mm and 154 mm of rain over the two summers, similar results were observed in the following year over summer. Previous research findings into survival of stolons over summer have been inconsistent, expectedly due to the differences

in study conditons, climatic patterns and genetic diversity in HS families used. While Hay et al. (1983), Hay et al. (1987) and Caradus and Williams (1989) found stolon number to be higher in summer and autumn, Jones (1982) and MacFarlane et al. (1990) reported summer moisture stress causing a decline in stolon numbers. A detailed study by Archer and Robinson (1989) over five years found stolon density to only be affected by summer drought when soil moisture levels fall below 35 mm and corresponding temperatures were higher than 30°C. In our study, mean maximum temperatures were 25°C and 24°C and total rainfall was 193 mm and 154 mm during the summer periods of both years. As there was no clear effect of summer moisture stress on stolon number and branching despite the low amounts of rainfall, our results support the findings that a combination of conditions of high temperatures and moisture deficit have to be met before stolon density declines.

3.4.6. Relationship Between Traits

To save time and cost, early selection is advantageous to breeders and the identification of traits at early growth stages that correlate positively with yield at later stages is beneficial. Year 1 growth score (GS1) had a high correlation with growth score Years 1 – 3 across locations (GS123) and DM yield, indicating that good establishment translated to better performance in later years.

The trait HCN had a low but significant negative correlation with the yield-related traits, DM, GS and LS. This suggests that highly cyanogenic plants tended to have reduced biomass compared to plants that produced lower amounts of HCN. This aligns with Noitsakis and Jacquard (1992) whose work showed that acyanogenic genotypes possessed more biomass than cyanogenic genotypes, thereby supporting the theory that the production of HCN may come at an energy cost to the plant. Although there was a weak negative correlation between HCN and stolon traits, only pre-summer SN was significant. This shows that cyanogenic plants did not necessarily possess the morphological stolon traits that are correlated with improved vegetative persistence in pasture. Caradus and Williams (1989) and Crush and Caradus (1995) reported that cyanogenic plants were more persistent through the pest resistance conferred by the HCN production in the plants. Our results indicate that the contribution to persistence by cyanogenesis in this study, is not necessarily via an increase in stolon density. Leaf size was also significantly negatively correlated with HCN. These results differ from results

by Caradus et al. (1989a) who reported no correlation between HCN and LS and Caradus et al. (1990) who reported a positive correlation between cyanogenesis and leaf size.

The traits SB and SN had high significant positive correlations supported by the PCA biplot. Jahufer et al. (1994) also reported high correlations between SN and SB. This high correlation can be attributed to pleiotropy or the common genes contributing to both traits are likely co-inherited (Lynch & Walsh, 1998).

The high correlation between GS and DM yield indicates the suitability to use scores as a measure of DM yield. Riday (2009) demonstrated that visual scores were accurate in estimating quantitative measurements if the correlation between them was high. Since DM harvests require more time, cost and labour, scores allow for more efficient resource use. The positive significant association between DM and LS provides the opportunity to indirectly select for increased DM yield using LS, as this trait is easier to measure and has a higher heritability (Hallauer & Filho, 1981; Casler, 2012). The trait LS was either not correlated or negatively correlated with SN and SB. This negative correlation is supported by numerous studies (Caradus & Williams, 1981; Caradus & Woodfield, 1990; Jahufer et al., 1994; Annicchiarico et al., 1999; Brock & Tilbrook, 2000) and implies a negative correlation between DM yield and stolon density, since it is well known that LS is a major contributor to the yield potential of white clover (Caradus et al., 1989a; Woodfield et al., 2001). The negative relationship observed is because plants with bigger leaves tend to have fewer stolons.

An interesting finding in our study was the positive correlation (0.24) and (0.33) between DM yield and post-summer SN and GS123, respectively. Results of the PCA biplot (Figure 3.14) also support this finding and although the two principal components did adequately capture all the variation represented in the data, it was still useful in displaying the most important relationship patterns. This is not the first time this positive correlation between DM yield and stolon traits has been reported as Annicchiarico et al. (1999) and Jahufer et al. (1999) also reported similar results. This positive correlation coupled with the results from the cluster analysis enabled the identification of several promising HS families that are both high yielding and persistent. This reinforces the fact that it is possible to simultaneously breed for increased DM yield and stolon density without a trade-off occurring (Caradus & Williams, 1989).

3.4.7. Check Cultivar Performance

'Grasslands Kopu II', a large-leaved cultivar renowned for its large leaf size and high herbage DM yield was found to have higher DM yield than 'Grasslands Bounty' albeit not significantly. 'Grasslands Bounty' on the other hand, a clover cultivar especially bred for high stolon density, had more stolons and stolon branches than 'Grasslands Kopu II', again substantiating the finding that large-leaved cultivars produce fewer stolons than small-leaved cultivars. Comparison with the HS families revealed several HS families that had superior agronomic performance in terms of DM yield and vegetative persistence than the two commercial checks. Notably, 'Grasslands Bounty', performed significantly better than many of the HS families for SN and SB and was only outperformed by HS family 31 for SB. The HS families with higher expressions of these traits are valuable for future breeding programmes although investigation into their performance in a wider range of environmental conditions is warranted.

3.4.8. Conclusions

- Considerable additive genetic variation was found among the 200 white clover HS families for key traits especially DM yield, SN and SB. Taken together with the estimated narrow-sense heritabilities, this indicates that selection can deliver significant genetic gain for yield and persistence.
- Significant G×E was observed in the form of year, location and season interactions. This validates the need for multi-site trials across different years and seasons.
- Herbage yield showed great plasticity in response to year and season changes. DM yield was highest in summer and clover content started to decline in the third year. Spring and winter were identified as potential vulnerable periods for white clover growth in pastures due to a combination of factors including low temperatures and competition from the companion grass. Management practices like avoidance of excessive nitrogen fertilizer application and frequent defoliation in spring to prevent ryegrass growth from outcompeting the clover are encouraged.
- A pattern of stolon increase over summer was observed indicating that the relatively cool summers did not severely influence stolon characters. The average number of stolons and branches was also higher in the second year of growth and

declined by the third year signifying a start of the decline of clover persistence in sward.

- A moderate positive correlation was observed between herbage production and post-summer stolon number. This finding has important implications as it means genotypes that combine high herbage yield and vegetative persistence can be developed from this population.
- Comparative performance of the 200 HS families to two commercial checks, 'Grasslands Kopu II' and 'Grasslands Bounty' identified HS families with improved agronomic performance in terms of yield and persistence. Further evaluation over years and locations will be paramount in determining if this advantage is maintained.

3.4.9. Foreword to Chapter 4

To implement genomic selection in plant breeding, training population design and specifications will have to be developed and optimised for any species. The primary objective of Chapter 3 was to establish a population from which genetic variation available for desired traits relevant to white clover yield and persistence could be determined. In the following chapter, the potential to use genomic selection to accelerate the progress in genetic gain for several traits in this population will be explored. The factors affecting predictive ability will also be examined. Using simulation, the rate of genetic gain obtained via conventional among-family phenotypic selection and progeny test will be compared to a strategy utilizing among-family phenotypic selection and within-family genomic selection (AFp-WFgs) and their cost-efficiencies compared.

4. Implementation of Genomic Selection

4.1. Introduction

Selection of elite genotypes is a key phase in any plant breeding programme. The ability of a breeder to accurately evaluate numerous potential selection candidates for key traits poses a significant challenge. Often, phenotypes of individuals are assessed at the fully developed plant stage which makes phenotyping an expensive and laborious procedure. Forage plant breeders must maintain and evaluate large populations across years, seasons and locations, especially when evaluating material for broad adaptation. This can also be inefficient as individuals not selected are usually discarded, culminating in a waste of financial and physical resources. Therefore, the ability to identify elite individuals at the seedling stage would prove useful in reducing breeding cycles by eliminating the need to wait for later-stage filial generations to phenotype important traits (Rutkoski et al., 2011; Bassi et al., 2016; Herter et al., 2019).

Genomic selection leverages the decreasing cost of genotyping platforms such as genotyping-by-sequencing (GBS) and utilises genome-wide markers and phenotype information to estimate genomic estimated breeding values (GEBVs) for future selection candidates (Annicchiarico et al., 2019). Using their GEBVs, the best individuals are identified and advanced to replicated trials or used as parents to produce the next generation (Grinberg et al., 2016). A major advantage conferred is the reduction in the generation interval by being able to rapidly go through several cycles of selection especially for perennial species (Heffner et al., 2010; Resende et al., 2012b). Heffner et al. (2010) estimated this reduction in time to be one-third or less than the standard time spent on phenotypic selection. In many perennial forage species where the preferred method of assessing an individual's breeding merit is by progeny testing; requiring at least three years per cycle (Quesenberry et al., 1991), genomic selection is of great benefit. Even though accelerating the breeding cycle is considered the most efficient way to expedite genetic gain, other elements of the breeder's equation are also addressed (Cobb et al., 2019). Selection intensity is increased by the expanded capacity to screen more individuals, made possible by high-throughput genotyping, while also exploiting the added benefit of a more diverse germplasm. In addition, by relying on the genotype of the individual rather than on phenotype record, which is often influenced by G×E and environmental noise, the precision in estimating the true breeding value is increased

(VanRaden et al., 2009). In addition, the lack of access to within-family variation has been identified as a major reason for the poor genetic gain in forages, as 75% of the genetic variation is located within families (Casler, 2008; Resende et al., 2014). Genomic selection allows breeders to exploit both among and within family variation, potentially accelerating genetic gain.

Many factors, including trait architecture, genomic selection model, marker density, linkage disequilibrium (LD) and training population size, are known to influence predictive ability and a considerable amount of research has focused on elucidating these factors (Meuwissen et al., 2001; Goddard & Hayes, 2007; Hayes et al., 2009b; Zhong et al., 2009; Crossa et al., 2013a; Daetwyler et al., 2013; Isik, 2014; Isidro et al., 2015). More recently, a new variant of genomic selection has emerged, multi-trait genomic selection, which has been found to confer significant boosts for improving predictive ability under certain scenarios (Jia & Jannink, 2012). Genomic selection is usually performed on a single trait at a time, however, since several traits are usually phenotyped, it is worthwhile to take advantage of the relationship among traits to boost predictive ability by including more traits in the model (Jia & Jannink, 2012; Montesinos-López et al., 2019). Like indirect selection in conventional breeding, the selection accuracy of a low heritability trait, such as DM yield, can be substantially improved by harnessing the correlation with another highly heritable trait via a multi-trait genomic selection approach (Jia & Jannink, 2012; Guo et al., 2014a).

For many species, phenotyping costs have become a bottleneck in breeding while genotyping costs are dropping steadily (Heslot et al., 2015). A case point is white clover, which has a low rate of genetic gain due to long breeding cycles, complex genome, difficult-to-measure traits (e.g., associated with vegetative persistence) and lack of a forage value index, emphasising the importance of applying marker-based methods like genomic selection. Before integration of any new strategy into a breeding programme, core elements that increase the likelihood of successful implementation of the new approach must be evaluated. The main goals of this chapter are to:

I. Explore the feasibility of implementing genomic selection in white clover.
II. Optimize genomic selection by assessing the effect of various cross-validation schemes, population sizes, marker numbers, model type, and establish practical

guidelines and recommendations for genomic selection implementation in white clover.

III. Assess the performance of single-trait and multi-trait genomic prediction models.

IV. Compare the genetic gain derived from HS phenotypic selection to an among-family phenotypic selection and within-family genomic selection (AFp-WFgs) strategy via simulation.

4.2. Materials and Methods

4.2.1. Development of the Training Population

A key step in the development of genomic selection is the establishment of an association between molecular marker information "Genotype" and phenotypic information, "Phenotype". In this study, the genotype/phenotype association is based on combining molecular information from parents and phenotypic data of progeny. The training population consisted of 200 F_2 maternal parent plants, which were genotyped prior to crossing.

4.2.2. Evaluation of HS Families

The phenotype data used in this study consisted of the 200 F_3 HS families described in the Chapter 3. These HS families were derived from a polycross of the F_2 training population from which the genotype data were collected. All HS families were evaluated in a row-column design at two locations: Aorangi and Ruakura, New Zealand with three replications per HS family. Location characterization, trial establishment, trial maintenance and trait measurement protocols are described in Chapter 3. Phenotyping was carried out from August 2016 to May 2019. Traits measured were HCN (hydrocyanic acid) production; dry matter (DM) yield; seasonal growth scores (GS); and leaf size (LS). Pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), and post-summer stolon branches (SBPOS) were measured at Aorangi only. Residual Maximum Likelihood (REML) analysis was carried out on all traits measured based on a linear mixed model using DeltaGen software (Jahufer & Luo, 2018). This analysis resulted in generating estimates of variance components and Best Linear Unbiased Predictions (BLUPs) for all traits. A detailed description of the linear mixed model and data analyses are presented in Chapter 3.

4.2.3. DNA Isolation

Genomic DNA was extracted from the 200 maternal parents in a 96-well plate modified from Whitlock et. al (2008) as described by Anderson et al. (2018). Briefly, into each well, 50 mg of leaf tissue from each plant was placed and freeze dried. Two 2.38 mm stainless steel beads were inserted per well, heat-sealed with the Axygen® Sealing Mats and ground with Qiagen Tissue Lyser at 30 Hz for two minutes. To each well, 500 µl homogenization buffer plus 1.8 µl proteinase K was added and centrifuged (Hettich

Rotanta 460R centrifuge) up to 4000×*g* at room temperature for 10 minutes and the beads removed. From each well, 300 µl of supernatant was transferred to a new Axygen® 1.1 ml 96-well plate, mixed with 300 µl precipitation buffer, and an Axygen® sealing mat was applied before mixing the plate for 30 seconds by inverting it. Plates were incubated on ice for 15 minutes before centrifuging at maximum speed (8595×*g*) for 30 minutes. Following that, 400 µl of the supernatant was transferred from each well to a new Pall AcroPrep Advance filter plate, mixed with 600 µl binding buffer by pipetting up and down gently and centrifuged at 4000×*g* for two minutes. Distillate was discarded and a wash was performed on the filter plate with another round of 300 µl per well of binding buffer and centrifuged at maximum speed for two minutes. Two more washes were performed with 300 µl per well of washing buffer and 300 µl per well of 100% absolute ethanol and centrifuged for two minutes. Plates were then centrifuged at 4000×*g* for 5 min at room temperature to dry the membrane before swapping to a fresh Axygen® 1.1 ml 96-well plate for elution of DNA from the filter plate. To obtain the final eluent, 115 µl of 10 mM Tris HCl pH 8 and 0.04 µl 100 mg ml^{-1} RNAse A was added to each well and centrifuged at 4000×*g* for one minute at room temperature yielding approximately 100 µl of eluent (see Appendix B.1 for the composition of the various buffers).

The quality and quantity of DNA were measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and visualised by electrophoresis and subsequent ethidium bromide staining on a 0.8% (wt/vol) agarose/Tris borate EDTA (TBE)gel. The extracted DNA was quantified using Hoechst fluorometric 33258 dye stock on a Bioemk Synergy HTX multi-mode fluorometer, and a representative subset of a samples was also quantified using a Qubit fluorometric assay (Invitrogen, Carlsbad, California).

4.2.4. Library Preparation

GBS libraries were constructed based on a method described by Elshire et al. (2011). The enzyme *Ape*KI (recognition sequence G/CWGC, where W is A or T) was used to digest white clover genomic DNA for GBS library making. This enzyme was used because it produces more fragment sizes in the optimal range of 100 bp–400 bp for sequencing and being a five bp cutter, has more cut sites in the genome thus providing more effective coverage across the genome than 6 bp cutters.

Before commencing library preparation, white clover genomic DNA (gDNA) was digested with *Ape*KI and titrated against different concentrations of adapter mix (common and barcode adapter). This was to make sure all the cut ends of the gDNA have adapters ligated to them to ensure equal sample representation. At 99ng of *Ape*KI adapter mix, an adapter dimer was present, indicating that all the cut ends of the gDNA were saturated.

After the titration experiment, adapter plates of 96 barcode adapters with common adapters were dried down to a combined concentration of 99 ng (44.5ng of each). Three 96-plex GBS libraries were generated using 100 ng of gDNA per sample which was transferred onto 99 ng *Ape*KI adapter plates. Plates were covered with Airpore tape, briefly spun and dried down using a SpeedVac concentrator (SpeedVac SPD10300). Once dried, the gDNA and adapters were digested with *Ape*KI. The digestion mix for one reaction was 0.2 µl of 5 U *Ape*KI enzyme (NEB R0643L), 2 µl NEB buffer (NEB B7203S) and 17.8 µl H_20 making a final 20 µl volume. A digestion master mix for all 96 reactions was made up and 20 µl added to each sample, the plate was sealed with a PCR seal and briefly spun down. The plate was then incubated in a PCR machine for the digestion reaction to occur at 75°C for two hours. After digestion, the digested DNA was ligated to the barcoded and common adapters. The ligation mix for one reaction was 5 µl NEB ligase buffer (NEB B0202S), 1 µl NEB T4 DNA ligase (NEB M0202L), 24 µl of H_20 making a final volume of 30 µl. A ligation master mix for all 96 reactions was made up and 30µl added to each sample. The plate was sealed with a PCR seal, briefly spun down and incubated in a PCR machine for one hour at 22°C and then 65°C for 20 minutes to deactivate the enzyme before dropping to 4°C. From each sample on the 96-well plate, an equal volume of ligated mixture/library (5µl) was extracted and pooled into a 5 ml Eppendorf tube containing 2500 µl CP buffer (SKU:PDR042) and cleaned up with Omega Bio-Tek EZNA cycle pure kit (Kit No.: D6492-02) as per kit instructions and eluted in 50 µl elution buffer.

To amplify the ligated fragments, a 50 µl Polymerase chain reaction (PCR) reaction mix was created containing 4 µl pooled DNA, 25 µl 2X NEB master mix (NEB M0270L**)**, 19 µl dH20, 12.5 pmol/µl of each of the following primers:

(i) 5′AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT3′

(ii) 5′CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTG AACCGCTCTTCCGATCT3′

The following PCR amplification protocol was performed; 5 minutes at 72°C, 30 seconds at 98°C and then 18 cycles of 10 seconds at 98°C, 30 seconds at 65°, 30 seconds at 72°C and then 5 minutes at 72°C. PCR amplified libraries were purified according to Omega Bio-Tek EZNA cycle pure kit instructions and eluted in 30µl of elution buffer (10 mM Tris-HCl, pH 8.5). To ensure optimum fragment size, 30 µl of library was combined with 10 µl of ladder L, mixed and size selected on a Pippin prep (PIP0001, Sage Science). A 2% agarose gel cassette, (CDF2010), was used to size select DNA fragments between 193 and 313 bp. Post-pippin libraries were validated on a Tapestation (4000 TapeStation System) before being sequenced on two lanes of Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) at AgResearch Invermay, New Zealand.

4.2.5. SNP Discovery

Single-end sequence reads were obtained for all three libraries, de-multiplexed and trimmed using Trimmomatic software (Bolger et al., 2014). A sliding window of 10% of the total read length was used to check quality, retaining only regions with an average q-score above 15. The TASSEL5 GBS pipeline was used to call genetic variants by aligning to the *Trifolium repens* genome (version five) discovering a total of 361,220 SNPs. After filtering for minor allele frequency (MAF) $\geq$ 0.001, missing rate > 50%, Hardy-Weinberg disequilibrium (HWdiseq >-0.05), the pipeline produced a variant call format file with 110,000 SNPs with a median read depth < 12.8.

4.2.6. Linkage Disequilibrium

Pair-wise LD, measured as the square of the correlation coefficient (r^2) between markers was calculated using PLINK (Purcell et al., 2007). Additional filtering was performed to exclude MAF > 0.03 and markers with more than 30% rate of missingness. After filtering, 30,225 high-quality subset SNPs were used to estimate LD for each chromosome by calculating the r^2 values for pairwise markers using a sliding window of 100 kb. All chromosomes were then pooled to estimate the rate of decay across the genome. The decay of LD over genetic distance was visualised by plotting the r^2 value against the distance in base pairs in R (R Core Team, 2018) .

4.2.7. Population Structure

Multi-dimensional scaling (MDS) was employed to determine the presence of any structure in the population using the 'cmd scale' function in R. A heatmap showing the relationship between individuals was also generated using the genomic relationship matrix derived from the filtered SNP markers.

4.2.8. Genomic Prediction

Two stage genomic selection analysis was implemented. In the first stage, trait BLUPs were generated for each of the 200 HS families using mixed-model analysis for all traits described in Chapter 3. In the second stage, genotype data provided by the maternal parents and the BLUPs for targeted traits were fitted into a single genomic prediction model. To reduce computing time, three traits were selected for detailed analyses, based on genetic complexity, namely: DM yield measured across-locations and years (2017, 2018); GS measured across-seasons, locations and years (2017, 2018, 2019); LS measured across-seasons, locations and years (2017, 2018, 2019).

4.2.9. Cross-validation

The Monte-Carlo cross-validation approach was used to assess predictive ability by splitting the data into training and test sets. The phenotypes of the individuals in the test set are assumed to be unknown and predicted from a model trained exclusively from the training set (Erbe et al., 2010). In order to evaluate the optimum training to test size ratio to implement, the population was randomly divided into two portions and assigned to the following training/test ratios: 60/40%, 70/30%, 80/20%, 90/10% and 95/5%. After estimating marker effects in the training set using phenotypic and genotype data, the GEBVs of the individuals in the test set are then predicted using the already estimated marker effects and the predictive ability averaged over several iterations. The following iterations; 100, 500, 1000 and 2000, were evaluated across all training/test ratios.

4.2.10. Genomic Prediction Models

To gain insight into the ability of various models with different underlying assumptions to accurately predict trait performance, four different genomic prediction models were investigated. Models were assessed mainly on their predictive ability and bias. Predictive ability, as earlier described, is defined as the Pearson's correlation between the predicted

values and the actual observed values in the test set, whereas the bias is the regression of the observed phenotype data on the predicted value generated by the model (Velazco et al., 2019b). Data from the cross-validation testing determined 100 iterations were sufficient for testing genomic prediction models, therefore the procedure was repeated 100 times and the predictive ability calculated as the average value of all observed correlations. The same process was performed to estimate the bias. The higher the predictive ability, the more confidence is placed in the model to accurately predict GEBVs of untested individuals while bias values close to one indicate better performance of a model.

4.2.10.1. GBLUP

The first model, GBLUP, uses a mixed model approach where the GRM is included as a variance-covariance matrix (Equation 22). The relationship between individuals is estimated from the SNP markers, under the assumption of equal variance across all locus (Habier et al., 2007a). The GRM was calculated using the 'A.mat' function of the 'rrBLUP' package (Endelman, 2011) in R (R Development Core Team, 2018) according to Equation 23 as proposed by VanRaden (2008). Missing values were imputed with the mean value of the non-missing values for that marker.

$$y = 1\mu + Zb + \varepsilon \quad (22)$$

Where: y is the vector of phenotypic records; μ is the grand mean; Z is the incidence matrix for random effects; b is the vector of random marker effects with a normal distribution b ~N (0,G σ_g^2) where G is the genomic relationship matrix (GRM) and σ_g^2 is additive genetic variance; ε is the vector of random residual effects.

G, the GRM is calculated as follows:

$$G = \frac{ZZ'}{2\sum p_i(1-p_i)} \quad (23)$$

Where: Z is obtained by subtracting $M - p$, M being a matrix with rows (n) and columns (m), containing markers coded as -1, 0, 1, p is a matrix with MAF (minimum allele frequency) calculated as $2(p_i - 0.5)$ where p_i is MAF of the i^{th} marker.

4.2.10.2. KGD-GBLUP

The KGD-GBLUP model is a variant of the GBLUP, developed specifically to estimate relatedness with low-depth sequence data which usually have high levels of missing data, without requiring imputation as it uses only SNPs with common genotype calls between both individuals (Dodds et al., 2015). The GRM is estimated according to Equation 23 with additional correction for low sampling depth and level of missingness according to Dodds et al. (2015). Analyses was implemented in R (R Development Core Team, 2018).

4.2.10.3. BayesCπ

This Bayesian model is a variable selection method that estimates the variance of markers using a prior distribution, thereby allowing markers to be shrunken towards zero to different degrees (Meuwissen 2003; Lorenz et al., 2011). In BayesCπ, the prior probability (π), that a SNP has zero effect is unknown and estimated to be a value between 0 and 1 (Habier et al., 2011; Lorenz et al., 2011). Analysis was performed with the 'BGLR' package (Pérez & de los Campos, 2014) in R (R Core Team, 2018). Markov Chain Monte Carlo cycles were repeated 1000 times after 500 cycles of burn-in and a binning set at 5.

The basic Bayesian model framework is given as:

$$y = X\beta + Zb + \varepsilon \quad (24)$$

The distribution of b is;

$$b = \sim N(0, I\sigma^2) \quad (25)$$

Where: y is the vector of phenotypes; β is the vector of fixed effects with a flat prior; X is an incidence matrix for the fixed effects of β; b is a vector of random coefficients of all marker effects; Z is a genotypic matrix of the number of observations and number of markers; σ^2 is approximately the largest variance a SNP effect is expected to have and ε is vector of random error the model residual.

4.2.10.4. Reproducing Kernel Hilbert Spaces (RKHS)

In this semi-parametric model, a kernel function is employed to convert the marker matrix into distances between pairs of individuals forming a square matrix that is used in a mixed-effects linear model (Gianola et al., 2006; Heslot et al., 2012). The equation of the model is given as:

$$y = \mu + K_h\alpha + \varepsilon \quad (26)$$

The kernel matrix K_h is defined as:

$$K_h(x_i x_j) = \exp(-h\, d_{ij}) \quad (27)$$

Where μ is a vector of fixed effects and ε is a is a vector of random residuals with α and ε assumed to have independent prior distributions, $\alpha \sim N(0, K_{h\sigma_\alpha^2})$ and $\varepsilon \sim N(0, I\sigma_\varepsilon^2)$ respectively. The smoothing parameter, *h*, is defined as 2/*d* and indicates the rate of decay of the correlation between genotypes, *xixj* are vectors of marker genotypes of the *i* and *j* lines. The squared Euclidean distance between individuals and *i* and *j* are calculated based on their genotypes and is denoted as d_{ij}. The RKHS model was performed using the 'BGLR' package in R.

4.2.11. Testing Size of Training Population and Marker Number

To investigate the effect of training population size (TS), on predictive ability, the GBLUP model was selected to be used for further analysis as it was established to be the most computationally efficient. The influence of using fewer individuals in the training population was investigated by using subsets of 20, 40, 60, 80, 100, 120, 140, 160 and 180 HS families randomly chosen to train the model.

The effect of marker number on predictive ability was also considered by selecting random subsets of markers (55, 110, 550, 1,100, 5500, 11,000 and 55,000) for model development. For each population and marker subset, the process was repeated 100 times and the predictive ability represented as the average of the 100 iterations.

4.2.12. Multi-trait Genomic Selection

A multi-trait model was fitted with positively correlated traits to determine the possibility of boosting the predictive abilities for traits dry matter (DM) yield and stolon number (SN). For yield, DM was considered the primary trait while leaf size (LS) and growth score (GS) were secondary traits with their phenotype information included in the model. Prediction models were developed using phenotypic data from the first and second year since DM was measured only in those years. In the case of stolon density, SN was taken as the primary trait and stolon branching (SB) incorporated as the secondary trait in the model.

Two different cross-validation (CV) schemes representing scenarios encountered by breeders were implemented. In the first, multi-trait CV1 (MTCV1) (Figure 4.1), the aim is to predict the GEBVs for the primary trait of individuals not phenotyped for neither the primary nor the secondary trait. Hence, in this case, the marker effects are first estimated from a training set that has been genotyped and phenotyped for the primary and secondary trait then the test set have their phenotypes predicted solely based on their genotypic information. In the second scenario, multi-trait CV2 (MTCV2), the individuals to be predicted (test set) have been phenotyped for the secondary trait but have no performance record for the primary trait of interest (Figure 4.1). Performance of models was evaluated through Monte-Carlo cross-validation where 80% of HS families were randomly assigned to the training HS families set and used to predict the remaining 20% in the test set.

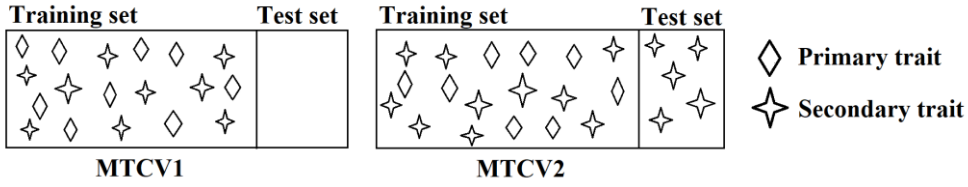


Figure 4.1 Multi-trait cross-validation representing two breeding scenarios: MTCV1- where the test set has no phenotype information of either primary or secondary trait, representing the prediction of individuals with untested phenotypes and MTCV2 where the test set has been phenotyped for the secondary trait.

Phenotype data from two or more traits were fit simultaneously as dependant variables in the Bayesian generalized linear model with the R packages 'BGLR' (Pérez & de los

Campos, 2014) and 'MTM' (de los Campos & Grüneberg, 2016). The model was run for 3000 iterations with the first 1500 discarded as burn-in and a thinning level of 5.

4.2.13. Genetic Gain Simulation for DM Yield

Simulation analysis was carried out in DeltaGen (Jahufer & Luo, 2018) to compare predicted genetic gain achieved using a combined approach of among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs) to a common selection strategy used by forage breeders based on phenotypic selection alone (HSp). Additive, family × location and family × year variance component estimates for DM were used to simulate the predicted genetic gain achieved using both methods. The base selection strategy assumed for both phenotypic and genomic selection was 200 HS families evaluated for DM yield by means of full cuts across three replicates in two environments. An extra year was added per breeding cycle for crossing and population establishment. Genetic gain per breeding cycle was compared at four different among family selection (AFS) and within family selection (WFS) pressure scenarios and the cost for each scenario was also calculated.

Different subsets of HS families were selected based on three among-family selection (AFS) pressures of 20%, 10% and 5%. It was assumed that from each selected HS family, a random sample of 100 seedlings were established and genotyped to estimate GEBVs. Either of four within-family selection pressures (WFS) were applied at 20%, 10%, 5% and 1% to select individuals with the highest GEBVs. As expected with increasing selection pressure, the number of selected individuals decreases. As the number of seedlings grown for each selected HS family was set at 100, if the number of HS families selected at the AFS stage is y, the total number of individuals genotyped at the WFS stage would be $100 \times y$ (Figure 4.2). Therefore, the same cost for genomic selection would apply across all WFS pressures under the same AFS pressure.

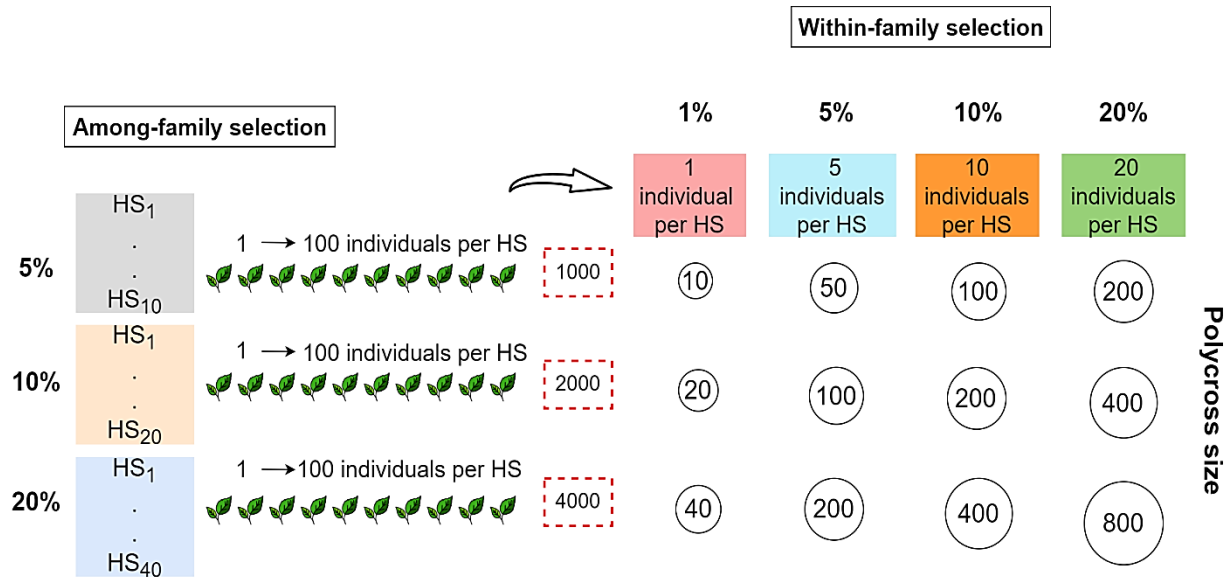


Figure 4.2 Selection scheme showing among and within-family selection pressures for a population of 200 half-sib (HS) families and the resultant polycross sizes. Numbers in the red dashed box indicate the total number of individuals genotyped for that among-family selection pressure. For example: with 200 HS families, 5% among-family selection pressure = 10 HS families; genotyping 100 individuals from each of the 10 selected HS families = 1000 plants genotyped. Within-family 1% selection pressure with 100 plants genotyped per HS family = 1 individual selected per HS family; as there are 10 HS families at the 5% among-family selection pressure, there will be 10 genotypes (1 per HS family) selected for a 5%among-family and 1% within-family polycross.

The following costs were considered for HS phenotypic selection (HSp) and include fixed and variable costs such as land, equipment, labour, crossing, trial establishment, fertiliser application and grazing. Starting and maintaining a trial in one location was fixed at NZ$10,000 per year, cost of DM trait evaluation = NZ$63 per plot. For among-family selection based on phenotype combined with within-family selection based on genomic selection (AFp-WFgs), costs were the same as above with the addition of genomic selection costs which include DNA extraction, GBS library making, sequencing and data analyses which was approximated at NZ$55 for one seedling at 96-plex.

DeltaGen uses the equation (Equation 28) from Casler and Brummer (2008) and its modification (Jahufer and Luo 2018) (Equation 29) to calculate genetic gain (ΔG) for HSp and AFp-WFgs, respectively;

$$\Delta G_{\mathrm{HS}} = k_f c \, \frac{\frac{1}{4}\sigma_A^2}{\sigma_{PF}} \tag{28}$$

Where: ΔG_{HS} is the genetic gain based on selection and random mating of the top performing HS families; k_f is the among family selection intensity; c is the parental control; σ_A^2 is the additive variance; and σ_{PF} is the among-family phenotypic standard deviation.

$$\Delta G_{AFp-WFgs} = k_f c_f \, \frac{\frac{1}{4}\sigma_{AY}^2}{\sigma_{PF}} + \, k_W c_W h_X r_{A-XY} \frac{\sqrt{3}}{2} \sigma_{PF} \tag{29}$$

Where: $\Delta G_{AFp-WFgs}$ is the genetic gain derived using a combination of among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs); c_f and c_w are the among and within-family parental controls respectively; equal to 0.5 for HS families; σ_{AY}^2 is the additive genetic variance for the trait Y under selection; k_W is the within HS family selection intensity; r_{A-XY} is the genomic selection predictive ability estimated at 0.3 and the square root of heritability of trait *X* given as h_X which corresponds to genomic heritability, was estimated as 0.35 using the GRM estimated using the SNP markers and the estimated variance components obtained from the REML analysis for DM yield according to Equation 30. Model was implemented using ASReml-R (Butler et al., 2009).

$$h^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gy}^2}{n_y} + \frac{\sigma_{gl}^2}{n_l} + \frac{\sigma_b^2}{n_b} + \frac{\sigma_\varepsilon^2}{n_y n_b}} \tag{30}$$

Where: h^2 is the genomic heritability and σ_g^2 is the genetic variance calculated by regressing the markers on the phenotypes; n_y, n_l , n_b are number of years, locations and replicates respectively; and σ_ε^2 is the residual variance.

4.3. Results

4.3.1. Phenotypic Analysis

There was significant ($P < 0.05$) additive genetic variation (σ^2_f) for the traits DM, SNPRS, SBPRS, SNPOS, SBPOS, GS and HCN as indicated in Chapter 3 (Table 4.1). Family × location variance ($\sigma^2_{f.l}$) was significant ($P < 0.05$) for the traits DM, GS and LS. The estimated additive genetic variance explained a larger proportion of the phenotypic variance for LS than $\sigma^2_{f.l}$ or $\sigma^2_{f.y.l}$. The $\sigma^2_{f.l}$ and $\sigma^2_{f..y.l}$ variance components were larger than the additive variance for GS. The error component of variation was higher than all other components for all traits, especially the stolon density related traits. Family mean narrow-sense heritability ranged from 0.1 to 0.8 (Table 4.1). The most highly heritable traits were HCN and LS, while the stolon density traits were the lowest. There was a high positive correlation (0.73) between DM and GS and a moderate correlation (0.35) between DM and LS. There was a high positive correlation (0.67) between Pre-summer SN (SNPRS) and SB (SBPRS). The correlation between post-summer SN (SNPOS) and SB (SBPOS) was also high (0.63) as described in Chapter 3.

Table 4.1 Estimated HS family additive genetic (σ^2_f), family×location interaction ($\sigma^2_{f.l}$), family×year interaction ($\sigma^2_{f.y}$), family×year×location interaction ($\sigma^2_{f.y.l}$) and pooled error (σ^2_ε) variance components, their associated standard errors (± SE) and family mean narrow-sense heritability for traits from single and combined analyses for the 200 white HS clover families across two locations, Aorangi and Ruakura.

Trait	σ^2_f	$\sigma^2_{f.l}$	$\sigma^2_{f.y}$	$\sigma^2_{f.y.l}$	σ^2_ε	h^2_n
Hydrocyanic acid - HCN	0.54 ± 0.07*	_	_	_	2.74 ± 0.06	0.82 ± 0.02
Dry matter - DM	0.10 ± 0.03*	0.10 ± 0.04*	_	_	1.45 ± 0.05	0.38 ± 0.09
Growth score - GS	0.12 ± 0.04*	0.35 ± 0.05*	0.04 ± 0.03	0.34 ± 0.04*	1.73 ± 0.02	0.32 ± 0.10
Leaf size - LS	0.06 ± 0.01*	0.04 ± 0.01*	_	0.01 ± 0.01*	0.42 ± 0.01	0.70 ± 0.06
Pre-summer stolon number - SNPRS	0.21 ± 0.07*	_	_	_	4 ± 0.14	0.14 ± 0.04
Post-summer stolon number - SBPOS	0.28 ± 0.10*	_	_	_	5.60 ± 0.19	0.13 ± 0.04
Pre-summer stolon branches - SBPRS	1.32 ± 0.36*	_	_	_	17.56 ± 0.60	0.18 ± 0.04
Post-summer stolon branches - SBPOS	1.09 ± 0.29*	_	_	_	14.50 ± 0.49	0.19 ± 0.04

4.3.2. Population Structure

No population structure was observed in the training population as depicted by the absence of obvious clusters in the MDS plot in Figure 4.3 and the absence of off-diagonal clusters in Figure 4.4. The first two principal coordinates explained 4.4% of the total genetic variation in the population.

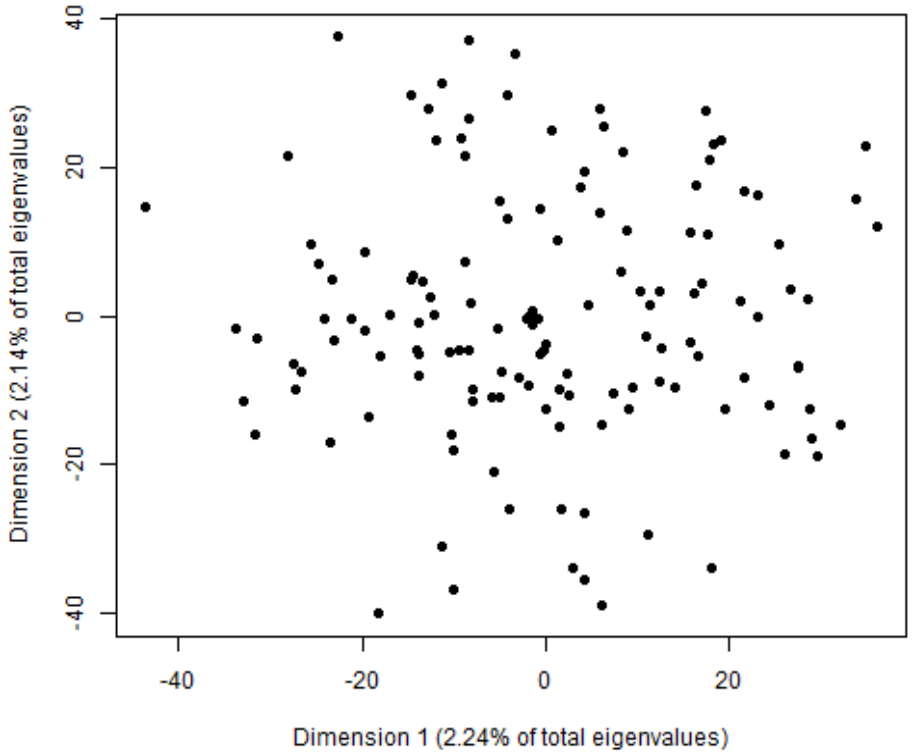


Figure 4.3 Multi-dimensional scaling (MDS) plot estimated from a genomic relationship matrix computed with 110,000 SNP markers from 200 maternal half-sib family training population.

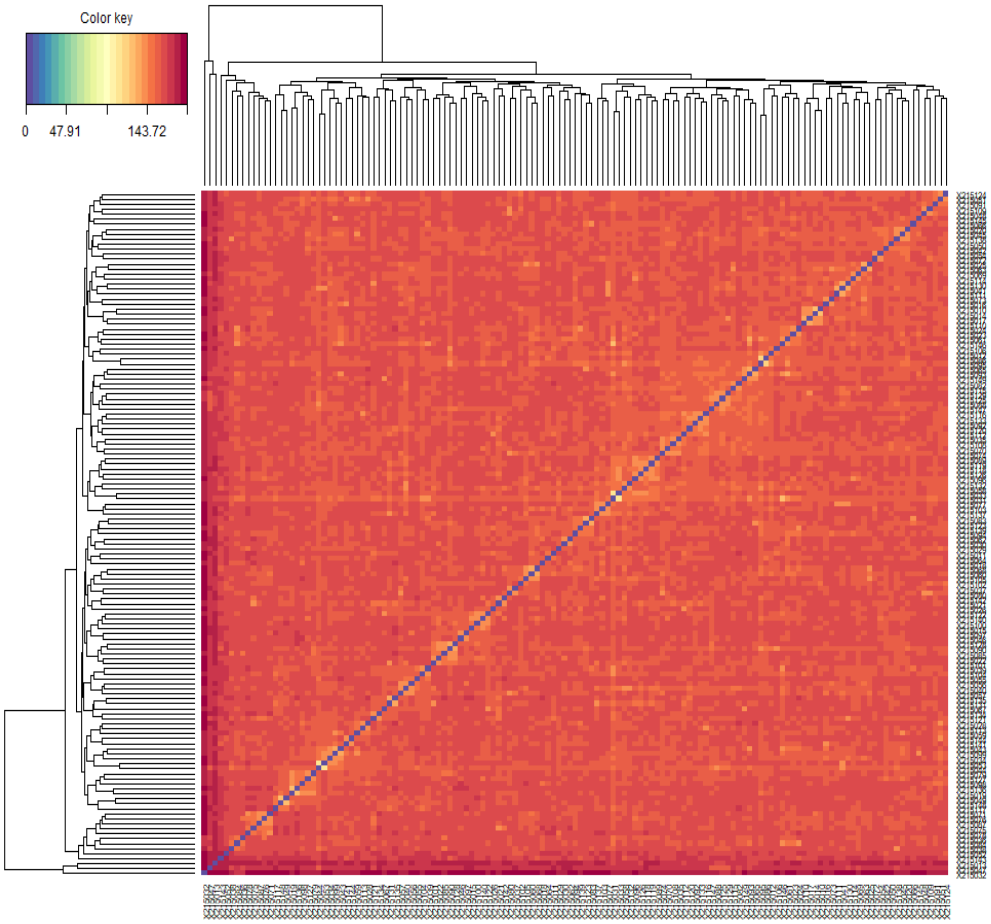


Figure 4.4 Heat map of the genomic relationship matrix estimated with 110,000 SNP markers from 200 maternal half-sib family training population showing the absence of population structure.

4.3.3. Linkage Disequilibrium

The extent of LD was found to decay rapidly to below 0.2 after 300 bp and 0.15 by 800 bp (Figure 4.5). Background LD at the 95th percentile and 90th percentile was 0.28 and 0.07, respectively.

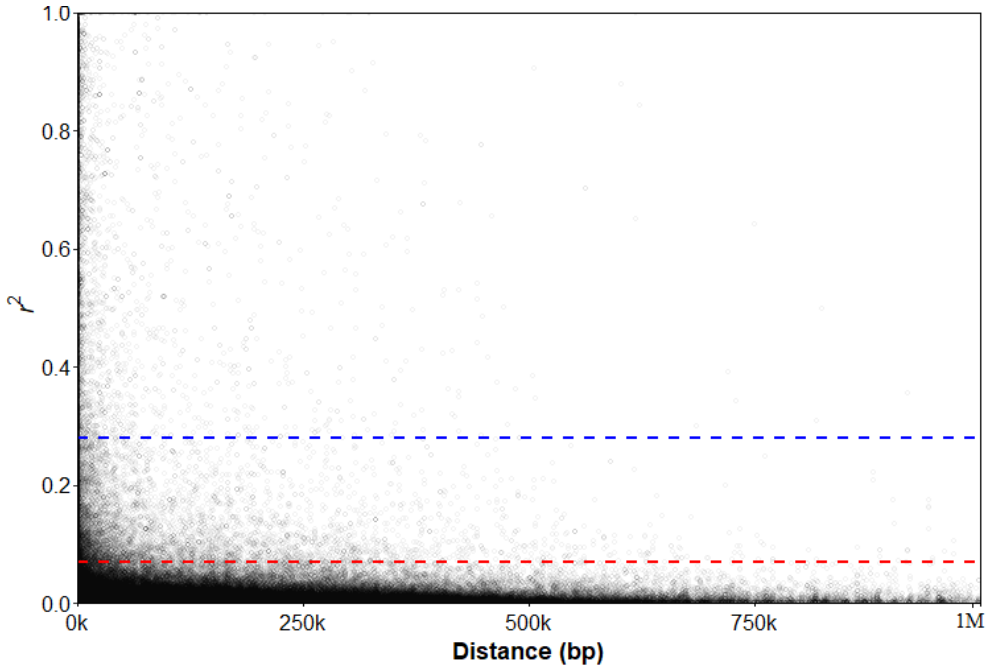


Figure 4.5 Linkage disequilibrium decay estimated from 30,225 subset SNP markers of 200 maternal half-sib training population. Horizontal dashed lines represent baseline r^2 at the 95th percentile (blue) and the 90th percentile (red), respectively.

4.3.4. Cross-validation

Increasing the size of the training set and reducing the test set was found to slightly increase the predictive ability in almost all iteration sets for all three traits as shown in Figure 4.6. An increase in the variation of the mean predictive ability was also observed as the test set size reduced. The highest biases were generally obtained with the larger training to test set ratios, namely, 60/40 and 70/30, while smaller training to test set ratios had bias values closer to one (Appendix B.2). The number of iterations run was found to have no significant impact on the predictive ability, although a wider range in bias values was observed with increasing number of iterations (Appendix B.2). Bias values were higher for GS than for LS or DM in all iterations. Since no significant gain in accuracy was observed by increasing the number of iterations up to 2000, all subsequent models, hereafter, were run with 100 iterations and cross-validated with training/test ratios of 80/20.

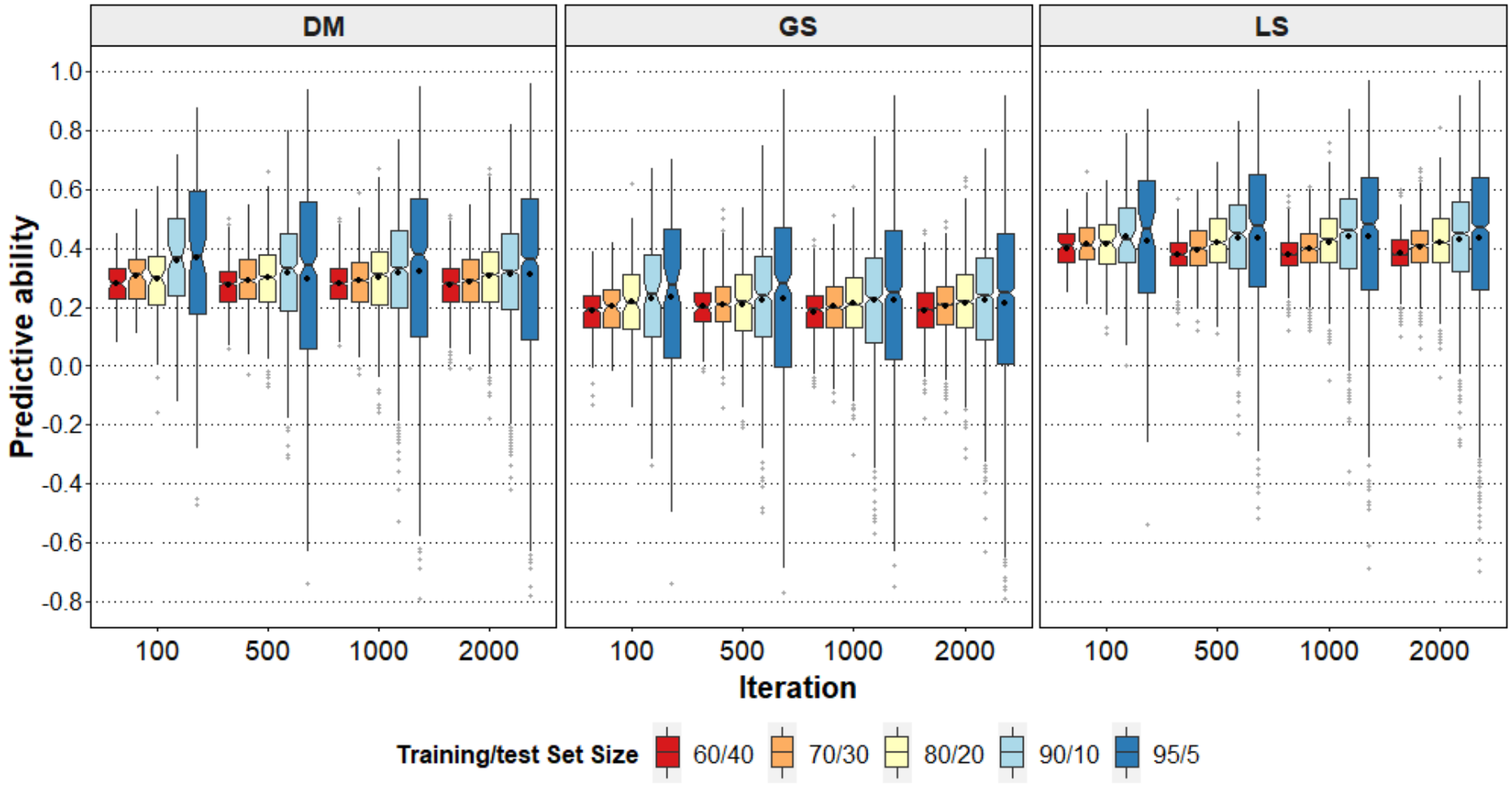


Figure 4.6 Effect of training and test set size ratios and number of iterations on the predictive ability for DM (dry matter) yield, GS (growth score) and LS (leaf size). Model was run for 100, 500, 1000 and 2000 iterations using KGD-GBLUP and the predictive ability was assessed using Monte-Carlo cross validation with 60%, 70%, 80%, 90% and 95% training set and 40%, 30%, 20%, 10% and 5% test sets. Solid line represents the median, black dot in the box represents the mean and grey dots are outliers. Notches that do not overlap indicate medians that are significantly different at $P < 0.05$ (Chambers et al., 1983).

4.3.5. Genomic Prediction Model Comparison

The predictive ability of KGD-GLUP was slightly higher than the other models, BayesCπ, GBLUP and RKHS for all three traits although increases were not significantly different (Figure 4.7). Predictive ability ranged from 0.29 to 0.33 for DM, 0.2 to 0.25 for GS and 0.41 to 0.44 for LS. When considering the regression coefficient, all models had similar bias values for LS. For DM and GS, BayesCπ and RKHS had bias values closer to one while the KGD-GBLUP obtained the highest bias amongst the models for all three traits, 2.02 for DM, 3.20 for GS and 1.68 for LS (Appendix B.3). The variance around the mean predictive ability was similar for individual traits apart from GS where the KGD-GBLUP had the lowest variance as evidenced by the boxplot in Figure 4.7. In terms of computation speed, the GBLUP and KGD-GBLUP models were less computationally demanding and generally easier to implement. Overall, KGD-GBLUP was the best performing model in terms of predictive ability while BayesCπ and RKHS had better performance in terms of bias.

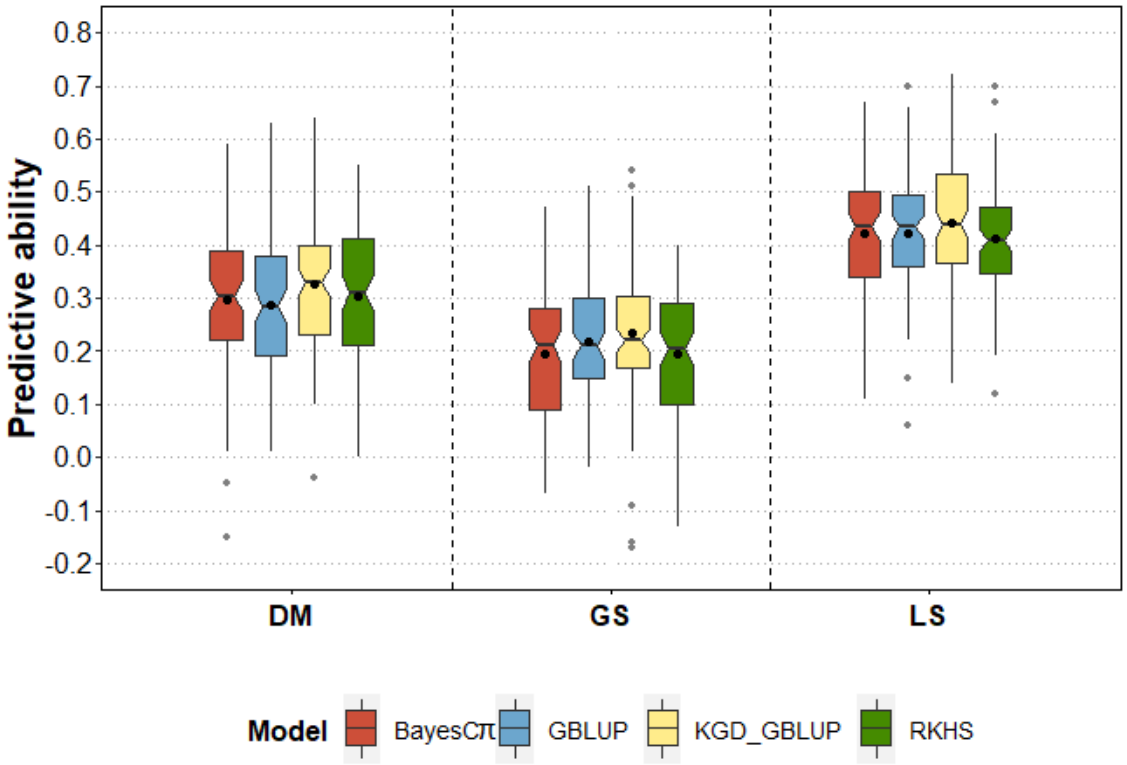


Figure 4.7 Comparison among genomic prediction models BayesCπ, GBLUP, KGD-GBLUP and RKHS on the predictive ability for DM (dry matter) yield, GS (growth score) and LS (leaf size). Models were run for 100 iterations and predictive ability was Monte-Carlo cross validated using 80% training, 20% test sets. Solid line represents the median, black dot in the box represents the mean and grey dots are outliers. Notches that do not overlap indicate medians that are significantly different at $P < 0.05$ (Chambers et al., 1983).

4.3.6. Trait Architecture and Heritability

There was a significant ($P < 0.001$) relationship between predictive ability and trait heritability with heritability explaining a moderate proportion (35%) of the predictive ability (Figure 4.8). Traits with higher heritability tended to have higher predictive ability except for certain traits. The highest predictive ability achieved was 0.44 for LS based on across-location and years analysis. The lowest predictive ability (-0.17), was obtained for SBPRS Year 3. Surprisingly, for HCN, a trait with the highest heritability of 0.82, a predictive ability of 0.22 was obtained. Traits with higher heritability tended to have relatively lower biases compared to traits with lower heritability (Appendix B.5). The highest biases were obtained with the stolon density traits and GS compared to other traits.

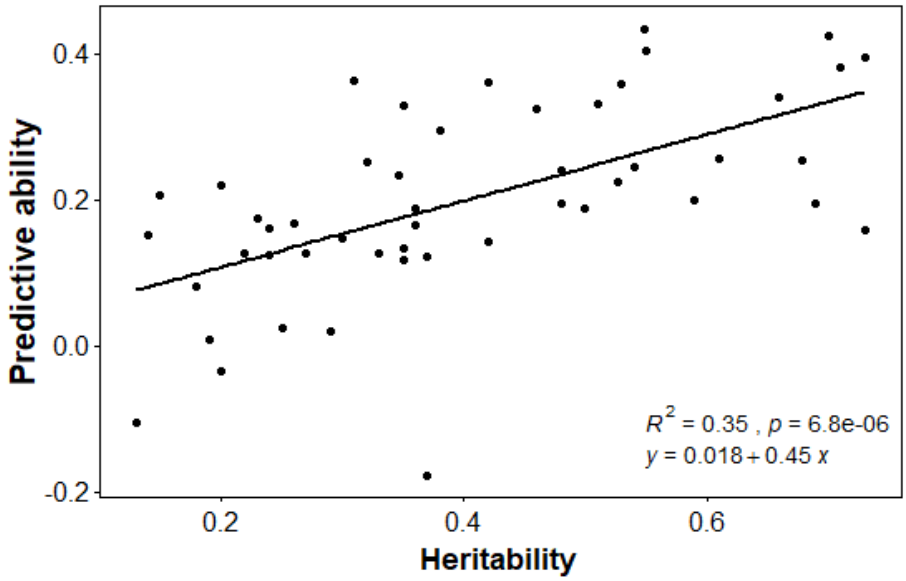


Figure 4.8 Regression of predictive ability on heritability for dry matter, leaf size, growth score, stolon number and stolon branches based on 200 HS families measured in 2017, 2018, 2019, in two locations, Aorangi and Ruakura New Zealand; stolon branching and stolon number measured in Aorangi only.

4.3.7. Predictive Ability Across Years and Locations

Predictive ability tended to increase with trial maturity and was highest in the third year (Figure 4.9) (Appendix B.4). Also, the predictive ability achieved by combining data from all three years was higher than individual years for LS and GS. The highest predictive ability for GS, 0.25 was achieved when data from all three years were combined. For LS, the same predictive ability, 0.43 as achieved when Years 2 and 3 and Years, 1, 2 and 3 were combined (Figure 4.9). There was an increase in predictive ability for LS, from 0.17 in the first year, to 0.43 when all three years were combined. Similarly, combining data from all three years for GS, increased the predictive ability by 127% from the first year.

The predictive ability obtained in Ruakura was noticeably lower than that at Aorangi. Predictive ability ranged from 0.06 to 0.22 for GS at Aorangi while Ruakura had a lower range of 0.02 to 0.17 (Figure 4.9). Models developed using BLUPs from across the two locations, provided the highest predictive ability range for GS, 0.15 to 0.25. Similar results were obtained for LS where there was a 40% difference in average predictive ability between Aorangi and Ruakura (Figure 4.9) (Appendix B.5). Ruakura tended to have the highest bias values and bias range for GS while bias values were similar between locations for LS (Appendix B.5).

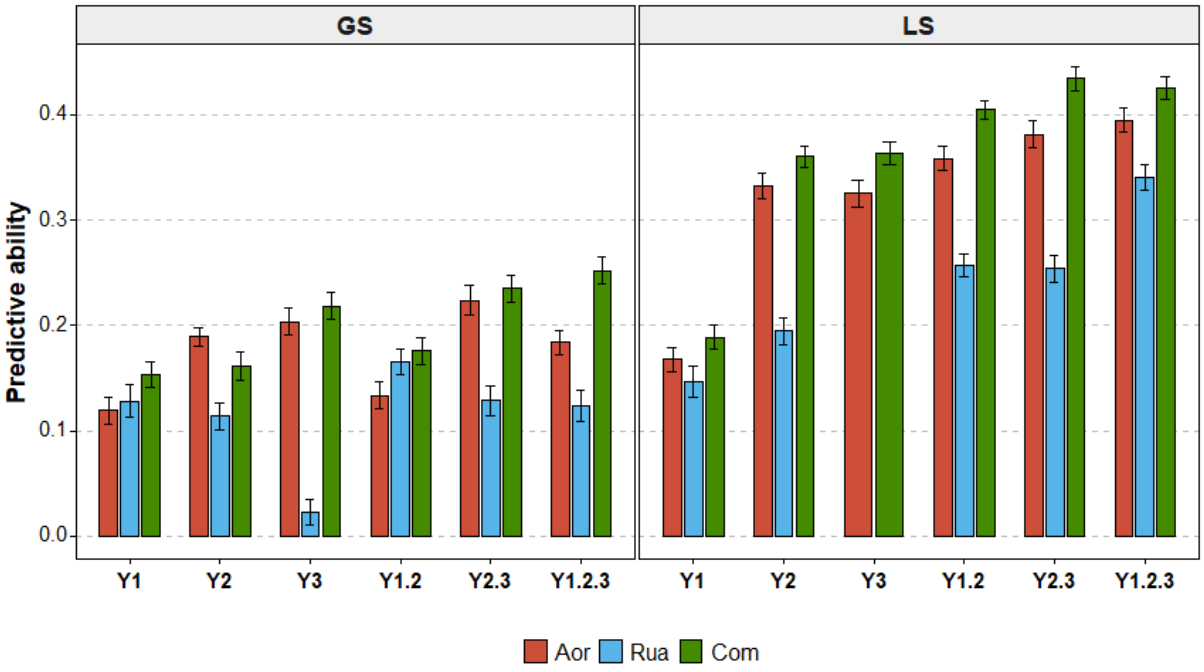


Figure 4.9 Predictive abilities for growth score (GS) and leaf size (LS) in two locations; Aorangi (Aor) and Ruakura (Rua) and combined across-locations (Com) over a period of three years. Models were run using KGD-GBLUP for 100 iterations and predictive ability estimated using Monte-Carlo cross-validation using 80% training, 20% test sets. Error bars represent standard errors of the mean.

4.3.8. Predictive Ability and Training Population Size (TS)

The impact of training population size (TS) on predictive ability is illustrated in Figure 4.10. No significant differences were observed in the predictive ability of LS when the TS was reduced from 200 to 100 individuals and a significant drop of 28% was only realised when the number of individuals was further reduced to 80 individuals. Significant decrease in predictive ability was observed in the more complex trait, DM, when 40 individuals or less were used to train the model. Predictive ability decreased by 48% and 83% when the TS was reduced from 100 to 40 and 20 individuals, respectively. For GS, no significant differences in predictive ability were observed when 40 or more individuals were included in the model. There was larger variation in the predictive ability between samplings when fewer individuals were being used compared to when all 200 HS families were included in the model as evidenced by the boxplot in Figure 4.10. For all traits, bias values were generally larger with larger ranges as the TS decreased except in the scenario

using 40 and 20 individuals for DM and GS, respectively, where values were less than 1 (Appendix B.6). Based on these data, it appears that in this population, for simple and complex traits, the TS based on 80 to 100 individuals can generate similar prediction values to 200 individuals.

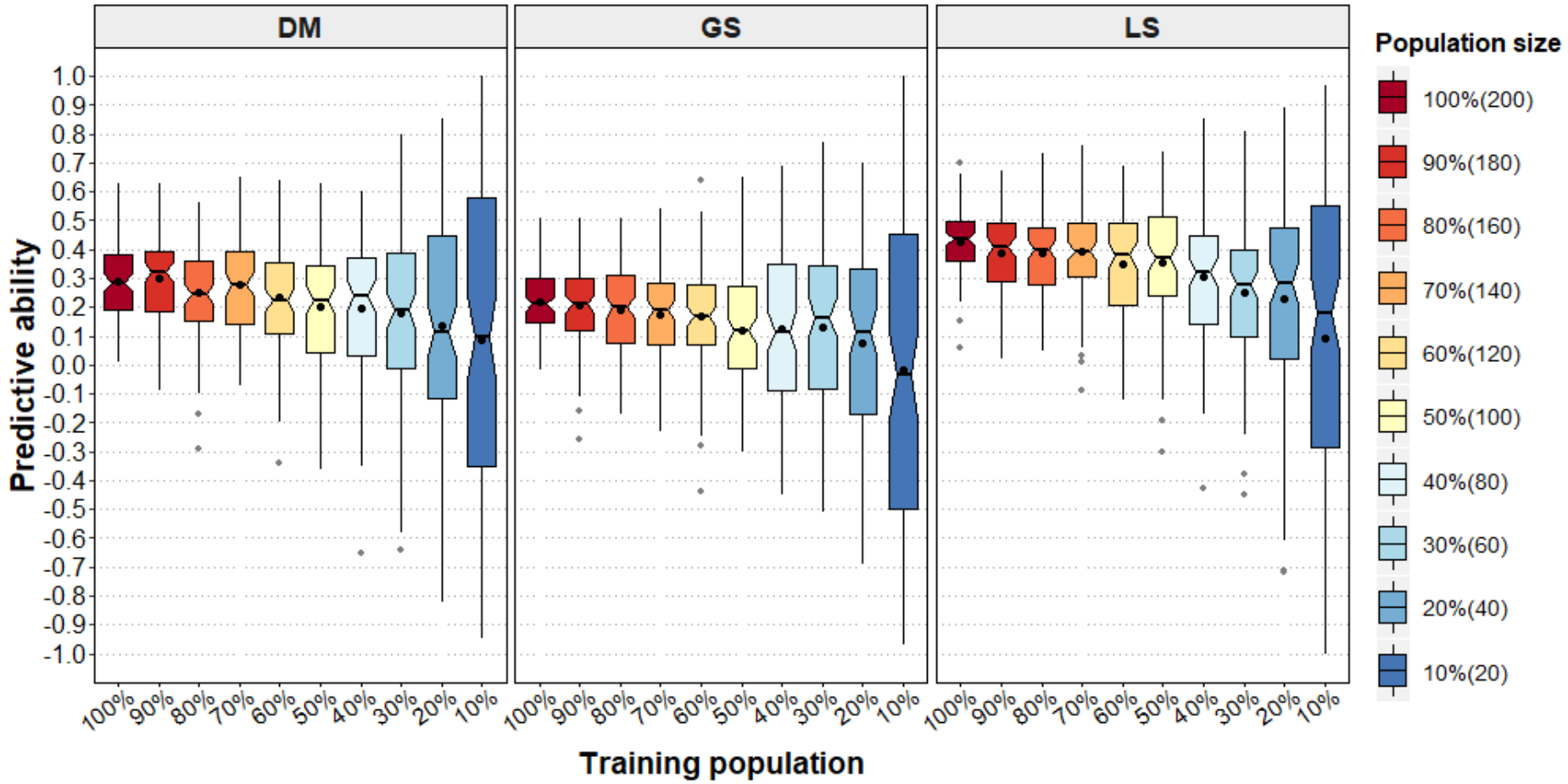


Figure 4.10 Notched boxplots of the effect of training set size on the predictive ability of three traits DM (dry matter) yield, GS (growth score) and LS (leaf size). Model was run for 100 iterations using GBLUP. Predictive ability was esimated using Monte-Carlo cross-validation with 80% training, 20% test sets. Solid line represents the median, black dot in the box represents the mean and grey dots are outliers. Notches that do not overlap indicate medians that are significantly different at $P < 0.05$ (Chambers et al., 1983).

4.3.9. Predictive Ability and Marker Density

Across all traits, no significant gain in predictive ability was observed when more than 5% (5,500) of markers were used for LS and GS and 1% (1100) markers for DM yield (Figure 4.11). The predictive ability for DM began to significantly reduce from 550 markers or less as evidenced by a 28% drop from 110,000 markers. For GS, the predictive ability was consistent at 0.21 until it started decreasing as markers were dropped from 5,500 to 1,100 and dropped steadily to 0.06 when only 55 markers were included in the model. Similarly, the predictive ability for LS started reducing significantly from 0.42 to 0.31 when only 1,100 markers were used, a percentage decrease of 26%. This significant drop in predictive ability continued when fewer markers were included in the model. Reducing marker size did not seem to increase the sampling errors as evidenced from the boxplots showing a similar degree of variation around the mean predictive ability (Figure 4.11). Also, reducing the number of markers generally increased the bias range for DM and GS while the bias range for LS was stable from 1% to larger marker sets (Appendix B.7). In summary, for this population, predictive ability was consistent for simple and complex traits when 5,500 markers or more markers were incorporated into the model.

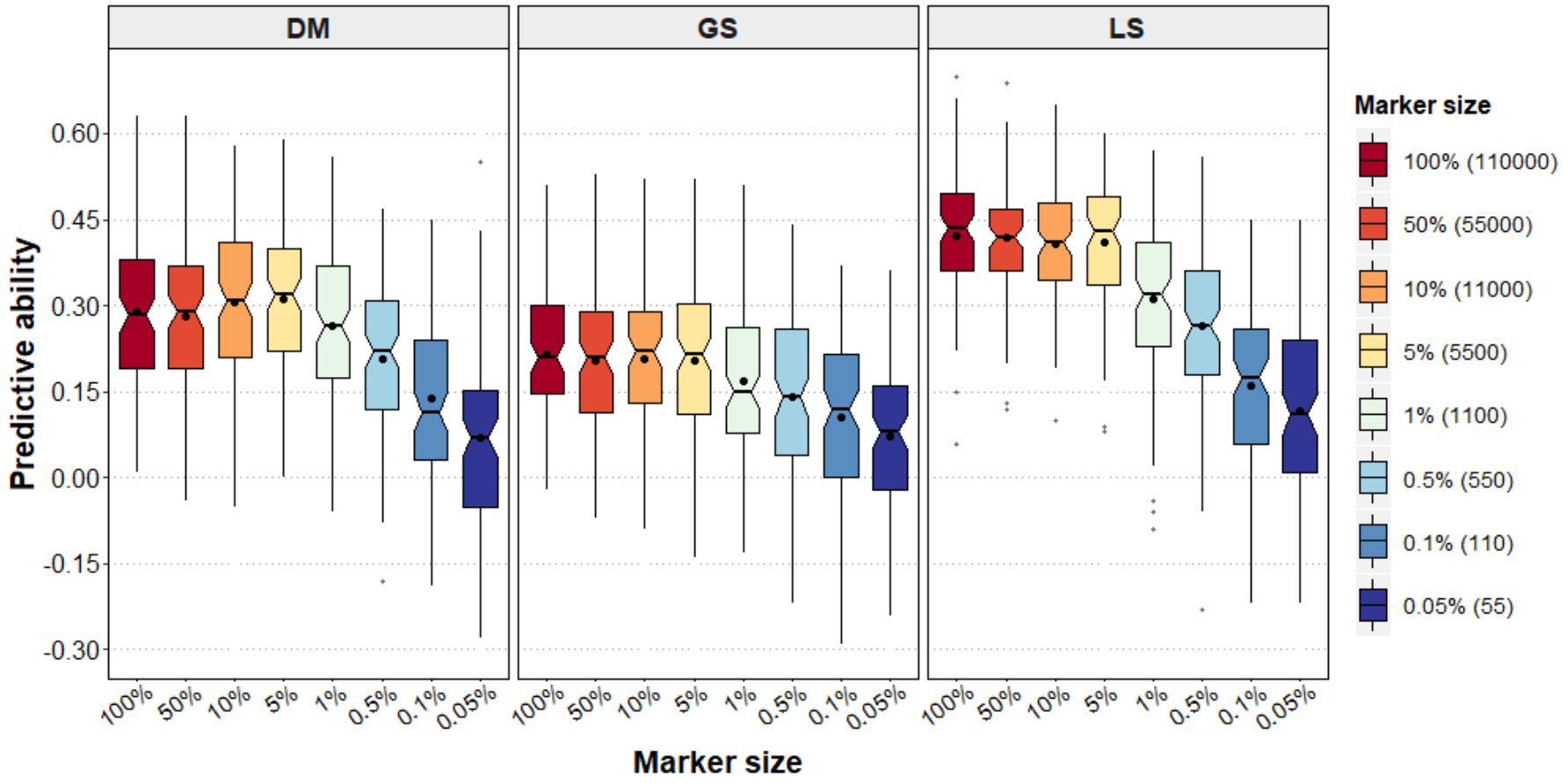


Figure 4.11 Notched boxplots of the effect of marker density on the predictive ability of three traits DM (dry matter) yield, GS (growth score) and LS (leaf size). Model was run for 100 iterations using GBLUP. Predictive ability was esimated using Monte-Carlo cross-validation with 80% training, 20% test sets. Solid line represents the median, black dot in the box represents the mean and grey dots are outliers. Notches that do not overlap indicate medians that are significantly different at $P < 0.05$ (Chambers et al., 1983).

4.3.10. Multi-trait Genomic Prediction

The prediction accuracy of the single trait model for DM was no different to when incorporating either GS or LS, or both, as additional secondary traits in the multi-trait cross-validation 1 (MTCV1) model. This approach aims to combine and model these traits from phenotyped individuals to predict the performance of untested but genotyped individuals. By contrast, MTCV2 models, based on incorporating the secondary traits singly or combined, predicts for test individuals that have been phenotyped only for the secondary trait. This additional phenotype information in the test individuals increased predictive ability by 24% and 94% to 0.39 and 0.64 for DM-LS and DM-GS , respectively (Figure 4.12). The percentage of increase observed in predictive ability was proportional to the degree of correlation between the primary and secondary trait. A three-way multi-trait approach combining GS and LS to predict DM did not deliver any significant benefits over DM-GS. For complex traits like stolon parameters, the multi-trait model, MTCV1, did not outperform the single trait model for stolon number pre-summer (SNPRS) or post-summer (SNPOS) when incorporating secondary traits stolon branching pre-summer (SBPRS) and post-summer (SBPOS), respectively (Figure 4.13). However, incorporation of SBPRS and SBPOS secondary trait phenotype data in the test individuals for SNPRS and SNPOS, respectively, using a MTCV2 model, increased the predictive ability for SNPRS from 0.15 to 0.54 while SNPOS increased from -0.1 to 0.28 (Figure 4.13). Generally, range in bias values obtained for MTCV2 and MTCV1 were closer to 1 than all single trait models (Appendix B.8). In summary, addition of a secondary trait in the prediction model did not increase predictive ability unless the test set was also phenotyped for that secondary trait.

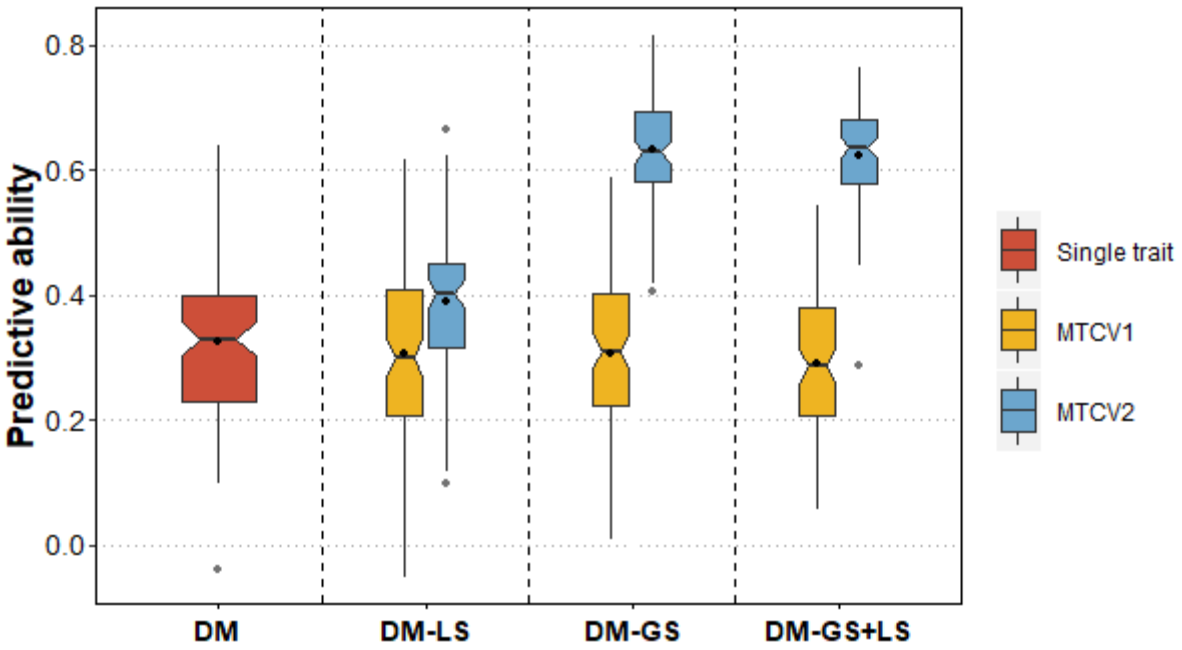


Figure 4.12 Comparison of the predictive ability of single trait model and multi-trait model for Dry matter (DM). In multi-trait models, growth score (GS) and leaf size (LS) were used as secondary traits. Cross-validation schemes, multi-trait cross-validation 1 (MTCV1), which corresponds to predicting untested phenotypes and MTCV2 which predicts individuals already phenotyped for the secondary trait, were implemented.

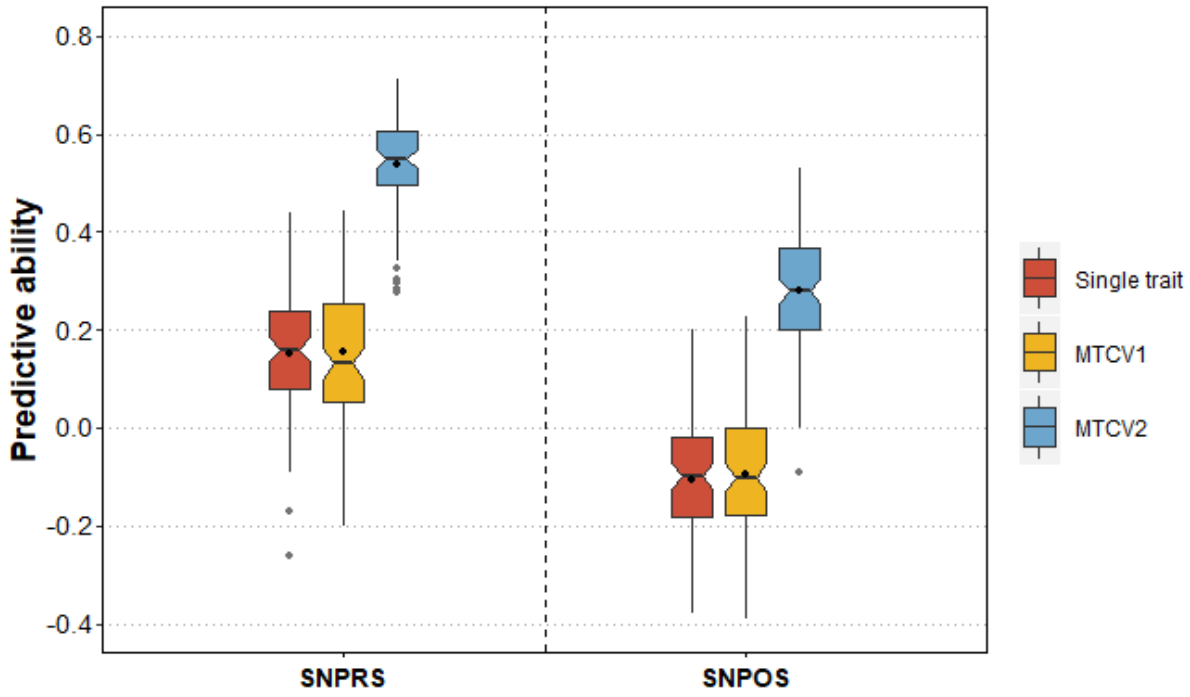


Figure 4.13 Comparison of the predictive ability of single trait model for pre-summer (SNPRS) and post-summer (SNPOS) stolon number when incorporating secondary traits, pre-summer (SBPRS) and post-summer (SBPOS) stolon branching. Cross-validation schemes, MTCV1, which corresponds to predicting untested phenotypes and MTCV2, which predicts individuals already phenotyped for the secondary trait, were implemented.

4.3.11. Genetic Gain Simulation for DM yield

Genetic gain from conventional among half-sib family phenotypic selection (HSp) was compared to an approach incorporating marker-based genomic selection to conduct within-family selection. The genetic gain obtained for DM yield using HSp was 5.3, 6.7 and 7.8% per cycle at among-family selection (AFS) pressures of 20%, 10% and 5%, respectively (Figure 4.14). Relatively lower genetic gain was obtained for phenotypic selection compared to all among-family selection based on phenotype combined with within-family selection based on genomic selection (AFp-WFgs) scenarios. Using genomic selection to select within-families (WFS) increased genetic gain by at least 34% relative to HSp at all selection pressures. The costliest scenario to implement was 20% AFS and WFS at 1%, 5%, 10% and 20% which increased genetic gain from 5.3 to a maximum of 10.3% (at 1% WFS) per cycle with more than twice the cost of implementing HSp (Figure 4.14). At 10% AFS, WFS increased genetic gain from 6.7% up to 11.7%

with a concomitant cost increase of 63%. Since fewer individuals were selected at the lower AFS of 5%, WFS was able to increase genetic gain by a maximum of 64% at 1%WFS with only a one-third increase in cost over HSp (Figure 4.14).

When comparing the cost-efficiency of phenotypic selection relative to AFp-WFgs, at 20% AFS, phenotypic selection was the most cost-efficient. The highest cost per percentage genetic gain was obtained using lower AFS and WFS pressures (i.e., 20% AFS with 20% WFS). This is mainly as a result of the reduced response to selection by selecting more individuals, thereby reducing genetic gain and incurring higher genotyping costs by selecting more HS families at the AFS stage (Figure 4.15). The higher genetic gain obtained at 10% AFS and 1% WFS made it more cost-efficient than 10% HSp, despite a 63% increase in cost. Further increases in the AFS and WFS pressures increased the amount of genetic gain obtained per unit cost and at 5% AFS, HSp and 20% WFS were the least cost-efficient, while 1% WFS was the most efficient due to the increased genetic gain with less than 35% increase in cost over phenotypic selection.

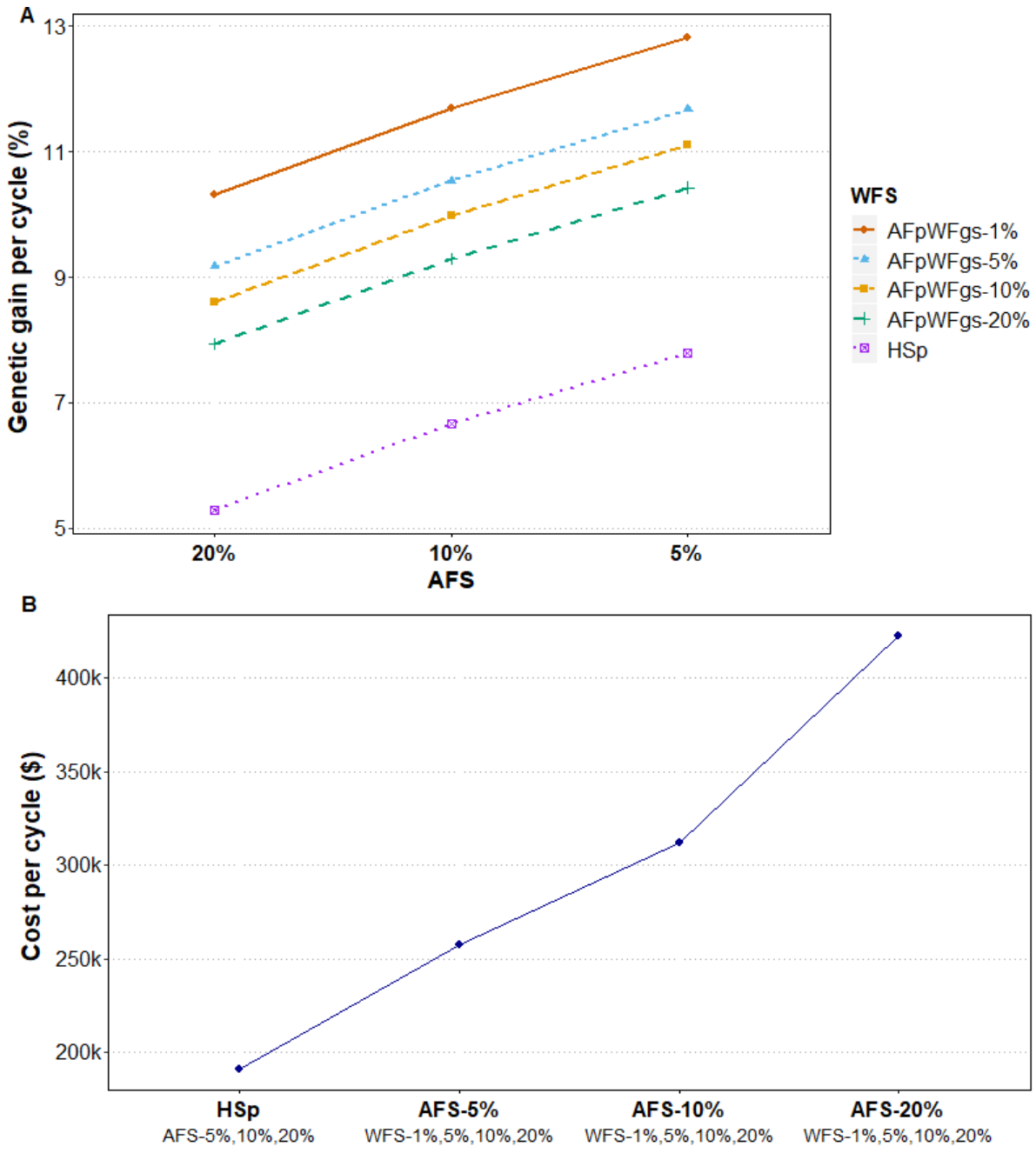


Figure 4.14 (A) Expected genetic gain for dry matter yield with phenotypic selection (HSp) compared with a breeding strategy using among-family phenotypic selection and within-family genomic selection (AFp-WFgs) at different selection pressures. (B) Cost per cycle of selection for HSp relative to AFp-WFgs at varying selection pressures. Results were based on a sample cost of NZ$63 for phenotyping one sample of dry matter yield and field trial cost of NZ$10,000 per year for 200 half-sibs (HS) evaluated in two locations. The costs of AFp-WFgs include the above costs in addition to genotyping costs. Genotyping costs were estimated at NZ$55 per sample keeping the number of individuals genotyped for each WFS at 100.

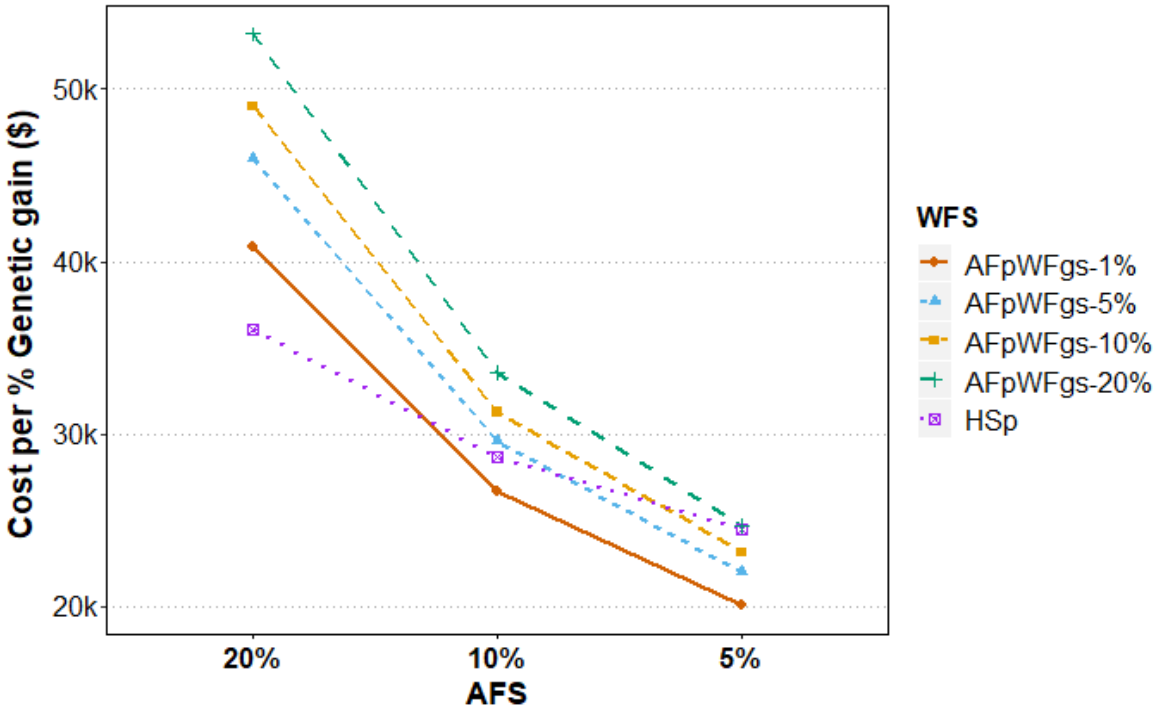


Figure 4.15 Cost-efficiency of phenotypic selection (HSp) for dry matter yield relative to among-family (AFS) phenotypic selection and within-family (WFS) genomic selection (AFp-WFgs) at varying selection pressures. Results were based on a sample cost of NZ$63 for phenotyping one sample of dry matter yield and field trial cost of NZ$10,000 per year for 200 half-sibs (HS) evaluated in two locations. The costs of AFp-WFgs include the above costs in addition to genotyping costs. Genotyping costs were estimated at $NZ55 per sample keeping the number of individuals genotyped for each WFS at 100.

4.4. Discussion

Increasing genetic gain is a top priority in plant breeding and genomic selection has been demonstrated to deliver substantial genetic gain amongst other benefits (Wong & Bernardo, 2008; Muranty et al., 2015; Lin et al., 2016; Jighly et al., 2019). Even though white clover possesses all the characteristics of a crop that can benefit from the adoption of genomic selection, so far, no studies have examined the potential of utilizing genomic selection to improve important traits like seasonal DM yield in white clover. This chapter implements genomic prediction modelling as a precursor to genomic selection in a white clover training population. It also investigates an integrated approach of utilizing phenotypic selection and genomic selection to increase genetic gain using simulation based on estimates of additive genetic and G×E variance components for DM yield, obtained from multi-location field trials. Results from this study provide guidelines for the effective initiation of genomic selection for white clover.

4.5. Factors Affecting Predictive Ability

4.5.1. Population Structure

Several studies have reported the presence of population structure to inflate predictive ability due to the presence of false positive marker-trait associations (Daetwyler et al., 2012; Riedelsheimer et al., 2013; Guo et al., 2014b; Isidro et al., 2015; Fè et al., 2016; Pembleton et al., 2018). No significant structure was found in the training population, thereby allowing the use of standard genomic prediction models (Annicchiarico et al., 2015).

4.5.2. Cross-validation

The highest biases were generally obtained when more individuals were used in the test set and fewer in the training set e.g., 60/40 and 70/30, whereas when more individuals were used in the training set, e.g., 95/5, bias values were closer to one. Bias values lower than one are indicative of an underestimation of the top performing individuals and over estimation of the low performers (Velazco et al., 2019b). The highest predictive ability was generally obtained with larger training sets and smaller test sets e.g., 90/10 and 95/5. Increasing the size of the training set allows for more observations per marker, thereby, estimating marker effects more accurately (Hayes et al., 2009a). This increase in

predictive ability, however, came with a trade-off, as progressively reducing the test set size significantly increased the number of outliers and variability around the mean predictive ability. These results are reflective of the personalised designing and optimization of training and test sets that must be performed for different datasets to obtain reliable estimates of predictive ability. The number of iterations did not have a significant effect on the predictive ability. Results demonstrated that 100 iterations were enough to obtain valid predictive abilities with significant time saving benefit as running the model with 2000 iterations increased the computing time by over 1000% compared to 100 iterations (data not shown).

4.5.3. Genomic Prediction Model Comparison

All models achieved similar predictive abilities for the different traits. Prior studies by Arojju et al. (2018) and Roorkiwal et al. (2016) also found no significant improvements in predictive ability when comparing different models in ryegrass and chickpea (*Cicer arietinum*), respectively. Although, the KGD-GBLUP model, on average, had slightly higher predictive ability than the other models it also had the highest bias. This suggests the possibility of the model not only detecting more genetic signal but also including more noise (Heslot et al., 2012).

The genetic architecture of a trait is known to influence the predictive ability of different models (Zhong et al., 2009; Daetwyler et al., 2010). For relatively less complex traits, like LS, variable selection models like BayesCπ, which do not assume equal variance of all markers, have been shown to perform better (Daetwyler et al., 2013). Contrary to expectation, for the trait LS, BayesCπ did not significantly outperform KGD-BLUP and GBLUP which assume all SNPs have equal variance. So far, there has been no detailed investigation on the exact nature of the inheritance pattern of LS in white clover and this result possibly sheds insight on the underlying mechanism controlling LS as quasi-quantitative with major genes and several polygenes controlling the inheritance (Ashri, 1968).

The RKHS model, identified to be capable of capturing non-additive genetic variation, with consequent increases in predictive ability for traits where dominance and epistasis play a significant role, did not outperform the less advanced models in predicting any of the traits. This may indicate that the contribution of epistatic variation to the total variation is insignificant for the measured traits (Haile et al., 2019). In white clover, the variation

using the HS family structure is entirely additive as dominance and epistasis are not detected (Falconer, 1989). Hill et al. (2008) showed, empirically, that a large proportion of variation for quantitative traits was additive. In traits like maize, however, where dominance and epistasis are significant, Liu et al. (2018) found RKHS to perform better than GBLUP or Bayesian models.

Concerns have been raised by several authors on the issue of the possible rapid decline in predictive ability of models that rely on kinship among individuals, i.e., GBLUP, with succeeding generations, especially when there are fewer individuals in the training population (Habier et al., 2007a; Daetwyler et al., 2010; Heslot et al., 2012; Daetwyler et al., 2013). By contrast, models that exploit the LD between markers and QTL lead to more persistent predictive ability over generations (Liu et al., 2015). Despite this, Goddard et al. (2011) and Zhong et al. (2009) argued that since the relatedness between individuals estimated in the relationship matrix is due to LD, then both LD and genetic relationships make contributions to the predictive ability.

4.5.4. Trait architecture and heritability

The variation in predictive ability among traits can be explained by the differences in genetic architecture. Zhong et al. (2009) provides evidence of the negative relationship between the predictive ability and number of QTLs in a trait. For complex traits like DM, GS and stolon density traits, which are strongly influenced by the environment, as evidenced by the high G×E, the predictive ability was relatively lower (0.25 - 0.33). This is consistent with Jia et al. (2018) and Annicchiarico et al. (2015) who obtained prediction accuracies of 0.13 and 0.3 for DM yield in alfalfa, respectively. Many studies report an increase in predictive ability as trait heritability increases (Combs & Bernardo, 2013a; Riedelsheimer et al., 2013; Zhang et al., 2014; Spindel et al., 2015; Roorkiwal et al., 2016; Arojju et al., 2018). The higher predictive ability obtained for LS, (0.44), a more heritable trait is likely because most of the variation is genetically controlled, thereby allowing for improved estimation of marker effects (Stewart-Brown et al., 2019). In view of the important role heritability has on predictive ability, it may be worthwhile to explore approaches that improve the estimation of trait heritability. For instance, increasing the number of locations and/or replicates for traits with poor quality phenotype data might improve the estimation of heritability (Falconer & Mackay, 1996). However, there is a limit to the returns of using this approach to increase predictive ability, as heritability

estimates above 0.7 are not worth the extra cost accrued from setting up more replicates or environments for testing (Cobb et al., 2019). Another avenue to increase the predictive ability of low heritability traits is to increase the training population size. According to Goddard (2008), 3000 individuals are needed to double the accuracy from 0.3 to 0.6 for a 0.3 heritability trait.

HCN, a simple trait under the control of two genes, had a relatively low predictive ability of 0.22. This finding was unexpected and could be due to several reasons, one being that the reference genome (Griffiths et al., 2019), is currently incomplete and the sequence of the HCN gene is absent in the available reference genome. Another reason could be the differences in the copy number variation (CNV) for the cyanogenesis genes. The *Li* and *Ac* genes, responsible for producing the hydrolysing enzyme linamarase and cyanogenic glucoside, respectively, have been reported to have multiple copies (Olsen et al., 2014). Detecting CNV is difficult, because individuals can possess different lengths of the variant; hence, genotyping platforms not optimised to detect CNV may miss a substantial proportion of genetic variation, thereby reducing predictive ability.

Negative to low predictive ability was obtained for some traits, especially when using phenotype data from the first year. According to Riedelsheimer et al. (2013), this could be due to opposite linkage phases between the training set and validation sets. In addition, the possibility of makers being out of LD with QTL in the validation population was highlighted by Zhao et al. (2012) as a potential reason. In our situation, because this is an F_3 segregating generation where several loci are expectedly in LD, it is highly unlikely that this is the case (Bassi et al., 2016).

Another plausible reason for low predictive abilities is the lack of substantial additive genetic variation. Traits with higher amounts of additive variation have been found to be predicted more accurately than traits where non-additive variation plays a crucial role (Annicchiarico et al., 2015). The inherent difficulty in trait assessment and the lack of availability of accurate, high-throughput phenotyping systems further compound this issue as poor-quality phenotypic data is generated due to the inability to precisely partition environmental noise. Therefore, the possible interference of the subpar phenotypic data cannot be ruled out as a hindrance to the accurate estimation of marker effects by the genomic selection model. Zhao et al. (2012) confirms this in their study in elite maize cultivars where they found predictive ability to match the degree of precision of

phenotyping, suggesting the need for high quality phenotyping platforms for genomic selection.

4.5.5. Location and Year

Generally, improvement in predictive ability was observed with successive years and combining the data of multiple years from the two trial locations. Similarly, Grinberg et al. (2016) noted the predictive ability in perennial ryegrass to be higher in the second year than in the first year. The increase in predictive ability in the second and third year compared to the first, can be explained by the higher amount of additive genetic variation obtained in those years. Spindel et al. (2015) also found predictive ability to increase when data from all years were used to train genomic prediction models. With respect to multi-location data, Faville et al. (2018) and Annicchiarico et al. (2015) obtained higher predictive ability when data were pooled across environments in ryegrass and alfalfa, respectively. This is because multi-year and multi-location data can capture more variation and effectively separate G×E from additive variation while single year or location data are possibly confounded by G×E effects, causing a reduction in predictive ability (Massman et al., 2013a). This boost in predictive ability results from a positive correlation between locations and years which increases the number of replicates of each line allowing for more accurate trait prediction (Burgueño et al., 2012; Faville et al., 2018). In the absence of a positive correlation, however, pooling environments decreases predictive ability (Spindel et al., 2015). In summary, utilizing models developed from multi-location data is usually preferable to a single location model when G×E is substantial (Annicchiarico et al., 2015).

Predictive ability in Aorangi was generally higher than at Ruakura, most likely as a result of the larger amount of additive genetic variation present. Crossa et al. (2010) found predictive ability to differ by as much as 30% between different locations. The lower predictive ability obtained at Ruakura for GS in Year three especially, can be attributed in part to the higher levels of environmental noise in the phenotype data. This is apparent from the high error variance component, which was more than twice that obtained at Aorangi as well as the higher family × year interaction variance. Also, it is worth noting that the third year in Ruakura was characterised by extreme weed invasion necessitating the removal of the third replicate from the trial which had a negative impact on heritability.

4.5.6. Training Population size (TS)

Similar predictive ability was achieved when half of the TS was used to train the model. Previous studies on the influence of TS on predictive ability have reported mixed results with some concluding TS had a greater influence on predictive ability than others. Zhong et al. (2009) reported doubling the TS size to deliver high gains while Grinberg et al. (2016), in contrast, report no significant increase in accuracy when TS was increased. In our case, no significant improvement in predictive ability was observed when doubling the TS from 100 to 200 individuals. This is in line with Bassi et al. (2016) who recommended TS sizes from 100 HS families sufficient to achieve a prediction accuracy of 0.5. Indeed, this was illustrated in this study. For instance, DM, had a predictive ability of 0.3 which corresponds to a prediction accuracy of 0.5. Prediction accuracy is defined as the predictive ability divided by the square root of heritability (Dekkers, 2007). Therefore, dividing the predictive ability of 0.3 by the square root of heritability, 0.4, gives a prediction accuracy of 0.5. Habier et al. (2013) showed that prediction accuracies due to LD increased with the inclusion of more individuals in the TS while accuracies due to the genetic relationships plateaued and started to decline. The reduced predictive ability observed with fewer individuals in the TS is due to the reliance on insufficient number of individuals to capture the additive genetic relationship (Meuwissen, 2009; Zhong et al., 2009; Heffner et al., 2011). With more phenotypic records, the observation per marker allele is increased with subsequent increase in the accuracy of genomic selection (Hayes et al., 2009a).

It is also important to highlight the reduction in the variance around the average predictive ability, when larger training sets were used. Predictive ability was less stable at smaller training population sizes indicating higher error. Smaller TS are more sensitive to poor quality phenotype data, which are damaging to accurate predictions (Stewart-Brown et al., 2019). According to Daetwyler et al. (2010) and Resende et al. (2012b), increasing the reference population size increases predictive ability as the additional information enables maker effects to be precisely estimated with less variability. An increase in bias was also observed at lower population sizes indicating underprediction of GEBVs (Velazco et al., 2019b). Increasing the population size provided more genomic information and produced a more unbiased model.

Trait architecture was found to influence the extent of predictive ability decline. Goddard and Hayes (2007) found traits controlled by greater number of genes with small effects required larger training sets for accurate prediction. This differs from our findings, as LS, compared to DM and GS was most sensitive to reduction in the size of TS. This could be because, GBLUP, the genomic prediction model used, has been shown to better predict quantitative traits like DM and GS where numerous loci contribute to variation.

The design of the training population has also been shown to a critical factor in genomic selection (Riedelsheimer et al., 2013). These results show financial resources can be saved by using fewer HS families or even diverted to developing more populations. Employing different populations is an attractive strategy for breeding programmes interested in creating and maintaining a more genetically diverse pool of individuals. This approach, however, is debatable as combining different unrelated populations reduces the predictive ability as different QTL could be segregating in populations of diverse backgrounds (Rutkoski et al., 2011). This is indicated by the incongruity in QTL locations reported for the same species when several populations are analysed for the same trait (Blanc et al., 2006; Bernardo, 2008). Furthermore, unrelated populations are at risk of having variable LD between markers and causal genes (De Roos et al., 2008).

Nevertheless, several studies have reported favourable increases in predictive ability when two or more populations were merged. Schulz-Streeck et al. (2012) and Faville et al. (2018) combined populations in maize and ryegrass, respectively, and obtained higher predictive ability across all populations than in specific populations. The increase in the overall training population size, due to merging multiple populations, allows for more replication of alleles. The higher occurrence of rare alleles consequently improves the predictive ability of the genomic selection model (Jarquín et al., 2014; Stewart-Brown et al., 2019). Moreover, combining populations creates larger training set sizes, which have been shown to offset the negative impact of low heritability on predictive ability due to better estimation of marker effects (Grattapaglia & Resende, 2011).

4.5.7. Marker Density

The impact of reducing the number of markers in this study was insubstantial when more than 1% (> 1,100) of markers were used, irrespective of the trait. In fact, for DM yield, the predictive ability estimated using 10% of markers was higher than when all markers were used in the model. The optimum number of markers to accurately predict trait

performance has been found to vary considerably and depends on several factors including the level of LD between markers and QTL and the training population size (Meuwissen et al., 2001). Many studies report an increase in predictive ability as marker density increases due to the increased likelihood of detecting markers in LD with QTL and the greater genetic variation captured (Asoro et al., 2011; Heffner et al., 2011; Hickey et al., 2014; Lin et al., 2014). However, a plateau in predictive ability is reached after which subsequent increase in the number of markers is subject to diminishing returns (Heffner et al., 2011). Higher LD increases the probability of linkage between markers and QTL (Kainer et al., 2015). Linkage disequilibrium is particularly important as an assessment for marker usefulness in subsequent cycles of genomic selection, as high LD increases the persistency of genomic selection accuracy in the succeeding generations (Dekkers & Hospital, 2002). Calus et al. (2008), proposed an LD $\geq$ 0.2 between adjacent loci for accurate prediction of GEBVs for a trait with low heritability. Outcrossing and diverse populations with low LD usually require more markers to guarantee sufficient markers are in LD with QTL (Jannink et al., 2010). Nevertheless, Zhong et al. (2009), established that markers not in LD with QTLs can potentially capture the genetic relationship between individuals and increase the predictive ability. In our case, LD was found to decay below 0.2 after 300 bp. The relatively high rate of LD decay is consistent with the nature of the outcrossing population where recombination events are higher than self-pollinating species and similar to reports in ryegrass (Fè et al., 2015; Arojju et al., 2016; Faville et al., 2018) and alfalfa (Li et al., 2014). It must be noted, however, that the LD estimates might not be reflective of the true LD in this population as the reference genome from which SNPs were called, is still incomplete with numerous gaps and several scaffolds unassigned to chromosomes. This may have a negative impact on the estimation of LD.

Habier et al. (2007a) and Habier et al. (2013) showed that even with no LD, genomic prediction models like GBLUP still had positive prediction accuracy resulting from the estimation of additive genetic relationships and linkage between QTL and SNPs. The predictive ability due to genetic relationships, in situations with low LD, is expected to decay faster over generations. This is mostly because information from genetic relationships is halved for the progeny in subsequent generations unlike LD which is more persistent (Habier et al., 2007b). This breakdown means that genomic prediction models would have to recalibrated frequently to ensure genetic relationships are captured (Müller et al., 2017; Faville et al., 2018).

It was not surprising to observe the redundancy of large marker sets despite the low LD. In a simulation study, Habier et al. (2013) found that prediction accuracies due to genetic relationships and linkage plateaued with at lower SNP density than accuracies due to LD. Furthermore, when the training and breeding population are closely related, as in this situation, a large proportion of accuracy is due to the relatedness information captured by the markers (Goddard et al., 2011; Kainer et al., 2015; Arojju et al., 2018). The large marker requirement by populations with low LD can also be offset by using a population generated from a few parental lines (Nakaya & Isobe, 2012). Also, early filial generations require fewer markers as Bassi et al. (2016) pointed out, while later generations need more markers because of the breakdown of LD.

As earlier identified, marker number has a low impact on predictive ability as it was clear from our analyses that similar predictions were achieved with significantly fewer markers. It is well established that the number of individuals sequenced, and the sequencing depth contribute to the eventual number of markers detected (Li et al., 2011). Practically, since high genotyping costs are a possible deterrent to the successful implementation of genomic selection in many plant breeding programmes, instead of allocating resources to more expensive genome-wide genotyping that generate millions of SNPs, it seems beneficial to employ relatively cheaper, low-coverage genotyping platforms like GBS, which potentially produce large numbers of markers with low depth. Especially with the development of genomic prediction models like KGD-GBLUP, which efficiently utilise low read depth data with high rates of missing values thus enabling the use of a wider range of SNPs potentially tracking QTLs. The GBS approach also comes with the flexibility to fine-tune the ratio of read depth to the number of markers obtained i.e., generate more markers at a lower depth or fewer markers at higher depth of coverage (Elshire et al., 2011). For instance, by using a rare cutter (e.g *Pst*I, a 6 bp cutter), with less frequent recognition sites, a smaller library is generated with a higher read depth per fragment and fewer missing data (Poland & Rife, 2012). Furthermore, reducing or increasing the multiplexing level can influence the sequencing coverage and amount of missing data. For this study, a 96-plex level was used, however increasing to 364-plex can drive the cost per individual down even further albeit with less coverage and larger proportion of missing data. Even at relatively low depth, 1*x* or less coverage, markers are still able to detect signals between markers and phenotypes thus providing a robust, cost-effective approach suitable for genomic selection (Gorjanc et al., 2017). Several studies have used GBS in the marker discovery phase of genomic selection in many species and

have established that even with high level of missing data, imputation methods are able to recover substantial information (Deschamps et al., 2012; Poland et al., 2012; Rutkoski et al., 2013; Cericola et al., 2018).

4.5.8. Multi-trait Genomic Prediction

The goal of multi-trait selection is to take advantage of the correlation between two or more traits to improve the predictive ability of a more expensive, and or difficult to measure trait (Jia & Jannink, 2012). Implementing MTCV1, which represents a scenario of predicting untested phenotypes for the primary trait, by incorporating a secondary trait in the prediction model, did not deliver any significant boost in predictive ability, consistent with results from several authors (Jarquín et al., 2014; Fernandes et al., 2018; Velazco et al., 2019a; Ward et al., 2019). It was only when the test set individuals had phenotypes for the secondary trait, i.e., the MTCV2 scenario, that a significant increase in predictive ability was observed for DM yield and SNPRS. Wang et al. (2017) and Velazco et al. (2019a) observed that including more traits improved the predictive ability of the primary trait. Interestingly, our results contradict this finding as combining LS and DM did not significantly improve the predictive ability of DM over when GS was used alone. The increased predictive ability results not only from the additional information obtained from the secondary trait, but also mostly due to the exploitation of the correlation between traits to increase the reliability of inferring the breeding values of individuals for the primary trait (Velazco et al., 2019a). This was evident in DM yield as the increase in predictive ability was higher when the more highly corelated trait, GS, was included in the model even though it had a lower heritability than LS.

The applicability of MTCV2 scenario is still unclear and depends on breeding objectives. If the goal is to save cost by not phenotyping a more expensive primary trait, then a multi-trait approach can be applied to selection candidates already under advanced field testing to assess for the secondary trait. However, since a major advantage of genomic selection is the capacity to skip the phenotyping stage and in particular, predict the GEBVs of individuals at the seedling stage, then the benefits of MTCV2 might only be fully appreciated for quality traits such as HCN and water-soluble carbohydrate which are expressed early in the life cycle of the plant.

4.6. Genetic Gain Simulation for DM Yield

Conventional breeding in white clover is traditionally based on phenotypic selection among families with limited access to within-family variation. According to Casler (2008), the main reason for low rates of genetic gain in forage crops is the non-utilization of within-family variance by selection methods. By using genomic selection to efficiently distinguish between candidates and access within-family variation, genetic gain can be increased. The maximum genetic gain achieved via AFp-WFgs was approximately twice that obtained by phenotypic selection. As expected, the highest genetic gain was obtained with the highest selection pressure. Lin et al. (2016) and Endelman et al. (2014) in simulation studies, also found genomic selection to substantially increase genetic gain in ryegrass and maize.

The uptake of genomic selection by breeding programmes will critically depend on the cost per unit of genetic gain achieved. Although AFp-WFgs was more expensive than phenotypic selection, the increase in genetic gain, despite a moderate predictive ability of 0.3, made it more economically advantageous than phenotypic selection alone at higher selection pressures, for example 10% and 5%. This is similar to results by Wong and Bernardo (2008) and Riedelsheimer et al. (2012) who found genomic selection outperformed phenotypic selection and MAS in terms of genetic gain and cost-efficiency in oil palm and maize, respectively. With the costs of genotyping continuously decreasing and phenotyping and labour costs on the increase, genomic selection is becoming a preferred route that allows more selection candidates to be screened at higher selection pressures, thus increasing the response to selection and increasing genetic gain even further.

Changing the selection pressures among and within-family had marked effects on the genetic gain and cost. This reflects the existing flexibility to tailor breeding schemes to available budgets and resources. A more exploitative breeding scenario would be to increase the number of selection candidates and apply higher selection pressures thereby increasing selection efficiency while still maintaining genetic diversity as more plants are grown (Jannink, 2010; Lorenz, 2013). For example, in this case, at 1% within-family selection using genomic selection, only one individual was selected from 100. In order to obtain 20 individuals for a polycross at 1% WFS, as many as 2000 seedlings would have to be grown, increasing the chances of better random sampling. One of the main

advantages of genomic selection is the ability to increase selection pressure through high-through-put genotyping (Isik, 2014).

When financial resources are limited, the number of candidate plants grown at the within-family stage can be reduced in order to save cost on genotyping. This would, however, depend on the goals of the breeding programme and the intended polycross size. Caution must be taken with this "rapid short-term response but limited long-term approach", however, as fixation rate is higher and increases in the rate of inbreeding are likely to occur (Gorjanc et al., 2018). Lin et al. (2017) showed that genomic selection could double the rate of inbreeding compared to phenotypic selection. In addition, fixation rate is higher at the high selection intensity, more so for genomic selection for two reasons: one being the rapid cycling compared to phenotypic selection and the second because genomic selection is based on markers; consequently, fixation for favourable alleles linked to markers will be higher (Jannink, 2010). Maintenance of variation and genetic diversity is therefore crucial, especially for outcrossing species like white clover.

The simulation performed estimates the genetic gain for DM yield after one cycle of selection. When considering time per cycle, which is considered the most important component of genetic gain, genomic selection can be exploited even further (Bernardo, 2002; Lorenz et al., 2011; Bassi et al., 2016). While phenotypic selection takes at least three years per cycle, genomic selection can be used to perform up to two cycles of selection per year by eliminating the need for lengthy phenotyping. This is possible when two fundamental requirements for flowering are provided, which include long daylength and a vernalization period. Some white clover varieties can flower even without vernalization when 14 to 16-hour photoperiods are met (Cope & Taylor, 1985). Glasshouse facilities that provide these requirements can expedite flowering with no consequences on seed set and seed yield. This implies that even with a lower accuracy than phenotypic selection, genomic selection is able to increase genetic gain by carrying out more cycles per unit time (Bernardo & Yu, 2007a; Heffner et al., 2010; Resende et al., 2012b).

In summary, a deciding factor for many breeding programmes before implementing genomic selection would be the amount of genetic gain derived per unit cost (Jahufer & Luo, 2018). Depending on the available budget, if the expenses are too high, the increase in genetic gain may not justify the increase in cost. New strategies that deliver more

genetic gain per unit cost are more likely to be adopted. To maximise genetic gain, the availability of practical information comparing the relative efficiencies of different schemes is of immeasurable assistance to breeders in making important breeding decisions regarding selection choices and resource allocation.

4.7. Conclusion

This study represents the first assessment of implementing genomic selection to improve DM yield and persistence in white clover. The following conclusions were reached:

- GBS produced many markers with sufficient quality and versatility to successfully implement genomic selection.
- KGD-BLUP model, although not significantly better, consistently had the highest predictive ability compared to the other models.
- Trait heritability was significantly associated with predictive ability. Traits with higher heritability and low G×E had higher predictive abilities. Moderate predictive ability was obtained for DM yield and LS, while GS and stolon density traits had low predictive abilities. Multi-location and multi-year data improved predictive ability.
- Vegetative persistence-related traits such as stolon number and branching, with high levels of environmental noise had lower predictive ability. This highlights the importance of good quality phenotypic data for genomic selection.
- Predictive ability was found to improve with data maturity due to the higher additive genetic variation estimated in the later years of the trial for measured traits.
- Similar predictive ability was achieved with half the training population size and 5% of markers, indicating the flexibility to manipulate and optimise genomic selection and reduce cost in this population.
- The limited extent of LD in the population was not found to influence the number of markers needed.
- Multi-trait models substantially improved predictive ability only when the phenotype information of a highly correlated secondary trait was present in the test population.

- Based on the simulation, up to two-fold increase in genetic gain per cycle can be made in improving white clover DM yield by incorporating an among and within-family approach despite a modest predictive ability of 0.3.
- At higher within-family selection pressures, AFp-WFgs was found to be more cost-efficient than phenotypic selection. This confirms the potential economic advantages of integrating genomic selection with phenotypic selection to significantly increase genetic gain.

4.7.1. Foreword to Chapter 5

Chapter 4 demonstrated the development of genomic prediction models that underpin the application of genomic selection to a white clover HS family population and provided key insights into the various factors affecting the predictive ability of genomic selection. It showed the ability to enhance genetic gain by integrating phenotypic selection and genomic selection while considering the cost-efficiency of both approaches through simulation. In the following chapter, using a simple trait HCN, empirical evidence on the application of genomic selection to increase genetic gain will be provided.

5. Empirical validation of genomic selection

5.1. Introduction

Genomic selection is the use of genome-wide markers to predict phenotypes of individuals based on their genome-wide marker information (Meuwissen et al., 2001). The first step in genomic selection is the training of a genomic prediction model by the estimation of marker effects using phenotype and genotype information derived from a training population (Meuwissen, 2009). The next step, the prediction phase, which is undoubtedly the main purpose of genomic selection, the trained genomic prediction model is used to predict the genomic estimated breeding values (GEBVs) of untested individuals, using only their marker information (Lorenz et al., 2011). Before GEBVs are used to make selections, however, the genomic prediction model is validated. This is typically done through a process of cross-validation where the data set is split into two sets, a training set from which marker effects are estimated and a test set in which the individual's phenotypes are masked with the purpose to predict their GEBVs (Hayes et al., 2009b). The correlation between the predicted GEBV and the actual phenotype is regarded as the predictive ability which serves as a measure of performance of the model (Meuwissen et al., 2001; Goddard & Hayes, 2007; Daetwyler et al., 2013). Although the predictive ability plays an important role in determining the feasibility of genomic selection, it does not describe the amount of genetic gain or response to selection achievable (Herter et al., 2019). Since the main purpose of genomic selection is to predict the performance of untested individuals not in the training population, and potentially several generations away, the true performance of the genomic prediction model must be empirically validated with distinct real data not included in model development (Lorenz et al., 2011; Bassi et al., 2016).

Empirically validating genomic selection is important as the realised genetic gain obtained from its implementation can be calculated. Phenotypic selection has been the key driver of realised genetic gain in breeding programmes. However, this strategy requires replicated multi-year-season-location-based trials to enable the separation of the confounding effects of G×E interaction from the additive genetic variation, in order to identify superior individuals (Dudley & Moll, 1969; Falconer, 1989; Holland et al., 2003; Weikai, 2014). In marker-assisted selection (MAS), the performance of individuals is estimated using few markers and even though MAS has been proven to be successful for

traits under the genetic influence of large QTL, it fails for traits controlled by numerous minor-effect genes like yield (Jannink et al., 2010). This is mainly due to the significant markers being associated with only a fraction of the total genetic variation responsible for the trait (Bernardo & Yu, 2007b). Consequently, genomic selection, which uses all marker information available has been found to outperform phenotypic selection and MAS (Wong & Bernardo, 2008). In dairy cattle, where genomic selection was first implemented, a two-fold increase in genetic gain has been achieved by reducing the time required for bull progeny tests (Harris et al., 2008; Hayes et al., 2009b). In plants, empirical evaluation has revealed the largest driver of increase in genetic gain derived from genomic selection to be the reduction in the breeding cycle (Hickey et al., 2014). For maize yield, in terms of realised gain, genomic selection outperformed phenotypic backcrossing (Combs & Bernardo, 2013b) and pedigree and phenotypic selection (Beyene et al., 2015). Likewise, Massman et al. (2013b) reported higher gains from genomic selection compared to phenotypic selection and MAS for grain yield and cellulosic ethanol in maize.

In forages, reported predictive abilities and accuracies have been low to moderate for many traits. As prediction accuracy is derived by dividing the predictive ability correlation by the square root of the trait heritability, prediction accuracy is larger than predictive ability. Often these terms are used interchangeably, which complicates comparison of prediction accuracies/predictive abilities among published studies. Faville et al. (2018) obtained a predictive ability of 0.07 to 0.43 for DM yield in perennial ryegrass. Also in perennial ryegrass, Arojju et al. (2019) reported predictive abilities of 0.22 and 0.34 for digestibility and water-soluble carbohydrate. For the same trait, crown rust resistance, Arojju et al. (2018) reported a predictive ability of 0.52 while Byrne et al. (2017) reported a higher accuracy of 0.86. In alfalfa, a predictive accuracy of 0.1 and 0.3 for DM yield was reported by Jia et al. (2018) and Annicchiarico et al. (2015), respectively. In switchgrass, (*Panicum virgatum*), a predictive accuracy of 0.5, 0.4 and 0.3 was reported for standability, leaf length and heading date, respectively (Lipka et al., 2014). In some of the aforementioned studies, to emulate applied forage breeding, where parental breeding values are estimated from their progeny, the genotype information is obtained from the parental training population while the phenotype information is from trait evaluation of their progeny (Annicchiarico et al., 2015). According to Grinberg et al. (2016), this may have a negative effect on the predictive ability because the phenotype and genotype information are from different populations. Studies have shown that

different QTL are expressed in populations grown under different environmental conditions (Bernardo, 2008; Massman et al., 2013a). Furthermore, a decrease in predictive ability could be caused by differences in SNP allele frequencies in the two populations after recombination (Massman et al., 2013a). Comparing predictive abilities derived from both scenarios would be important to provide some insight on this issue.

The rate of genetic gain achieved per cycle of selection is a standard measure used to assess the efficiency of alternate breeding strategies (Conaghan & Casler, 2011). Strategies that deliver the most genetic gain per unit cost are ultimately preferred by breeders. In white clover, two common breeding strategies, half-sib among-family phenotypic selection (HSp) and half-sib progeny test (PT) have been used to increase the rate of genetic gain for important selection traits. In PT, the breeding values of the parents are estimated from their progeny and used to select the best parents from the original polycross which are then randomly mated, usually as clones (Vogel & Pedersen, 1993; Goldman, 2000). Instead of using clones, after identifying the best HS families, HSp utilises the remnant seed from the of the top families - without evaluation of individuals within families - to generate a new population. However, this method utilises only ¼ of the available additive genetic variation. Without selection of individuals within families, ¾ of the additive genetic variation is not accessible. Using phenotypic selection to identify superior HS families and applying genomic selection to select elite individuals within families, the AFp-WFgs strategy, enables the total additive genetic variation available to be exploited, potentially increasing genetic gain. Although genomic selection is increasingly becoming adopted and implemented in many crop breeding programmes globally, so far, no studies have reported the realised genetic gain derived from implementing genomic selection in white clover.

This chapter is focused on:

I. Empirically validating the predictive ability obtained for HCN by carrying out divergent selection using GEBVs obtained from the genomic prediction model and comparing with observed phenotypic values.
II. Comparing the rate of genetic gain obtained using three different strategies encompassing phenotypic selection and an integrated approach with genomic selection.

III. Comparing the predictive abilities derived using a model trained with different sets of phenotype information obtained from the progeny, using log transformed data of progeny and the phenotype of the parental training population.

5.2. Methods

5.2.1. Summary of Genomic Selection Model Development

As described in Chapter 3, a training population comprising of 274 F_2 parental HS families was generated in 2015 and genotyped to provide 110,000 SNP markers used to train the genomic prediction model. This population was polycrossed in the summer of 2015. Harvested seed from the top 200 seed-yielding families was germinated to establish the 200 F_3 HS families for the purpose of providing phenotype information from a replicated multi-location and multi-year field trial. Prior to transplanting the seedlings in the two locations, Aorangi and Ruakura, 24 samples of each HS family were evaluated for HCN on a score of 0 to 5 as previously described in Chapter 3. The parental F_2 population was also phenotyped for HCN by sampling three leaves from each plant. The trait HCN was chosen to test genomic selection as it is expressed at the seedling/young plant stage, and therefore application of genomic selection and phenotype testing were all achieved in a time period that fits within this study. Residual Maximum Likelihood (REML) analysis was conducted separately on data collected to estimate BLUPS and variance components for genetic and nongenetic effects.

5.2.2. Genomic Prediction Model

The KGD-GBLUP model was fitted using a GRM estimated with 110,000 markers derived from the F_2 parental population and the phenotype of the F_3 progeny population and run for 100 iterations using the 'rrBLUP' package (Endelman, 2011) in R (R Core Team, 2018). The performance of the model was assessed by Monte-Carlo cross-validation where the whole data set was divided into a training (80%) and a test set (20%), the phenotypes of the test set are assumed to be unknown and predicted by the trained model. The predictive ability was calculated as the Pearson correlation coefficient of the observed and predicted value of 100 iterations.

To test if obtaining genotype and phenotype information only from the F_2 maternal training population increased the predictive ability, the model was run separately using the phenotype data of the F_2 population. Also, due to the non-normal distribution of the HCN phenotype data, a natural log transformation was performed on the F_3 HCN data to determine if a significant boost in predictive ability could be achieved.

The GEBVs predicted by all three sets of phenotypes information were compared to evaluate if there were differences in the ranking of the individuals predicted. Spearman's rank correlation coefficient was used to analyse the correlations between the predicted GEBVs at a significance level of 0.05 in R (R Core Team, 2018).

5.2.3. Divergent Selection

The HS families were ranked based on their phenotypic BLUPs obtained from REML analysis for HCN. Based on a 5% among-family selection pressure, 10 families with highest HCN and 10 families with the lowest HCN were identified. In April 2018, a random sample of 0.1g of seed was scarified and germinated from the remnant seed of each family in the 'high' and 'low' selection populations on pre-moistened filter paper, as described in Chapter 3. Forty-eight germinated seedlings were planted in propagation trays containing a mix of peat and sand in preparation for the following selection strategies.

5.2.3.1. Among-family HS Phenotypic Selection (HSp)

From the 48 germinated seedlings of each HS family in the high and low population, two or three plants were randomly taken from each of the 10 HS family to assemble 24-plant isolated polycrosses for the high (HSp-H) and low (HSp-L) populations, respectively (Figure 5.1). The number of individuals for all polycrosses was kept above 20 as recommended by Moll and Robinson (1966) to avoid inbreeding.

5.2.3.2. HS Progeny Test (PT)

The 24 highest and 24 lowest HCN maternal F_2 parental HS families were identified based on the BLUP means obtained from their F_3 progeny. Clonal cuttings of each F_2 maternal plant in the high (PT-H) and low (PT-L) cyanogenic selections were made in May 2018, planted in propagation trays and maintained in a glasshouse. Two clones were randomly selected per F_2 maternal plant in each high and low population separately, to make a 48-plant polycross for each high and low population in the summer of 2018/2019.

5.2.3.3. Among-family Phenotypic Selection and Within-family Genomic Selection (AFp-WFgs)

5.2.3.3.1. Validation Population

To conduct a within-family selection using genomic selection, 48 seedlings were randomly sampled per selected HS family in the high (AFp-WFgs-H) and low (AFp-WFgs-L) populations (Figure 5.1) making a total of 480 individuals per population. To obtain the GEBV of each individual, DNA was extracted according to the modified protocol of Anderson et al. (2018), described in Chapter 4. GBS libraries were constructed using the protocol published by Elshire et al. (2011) and previously outlined in Chapter 4. Sequencing was performed on 960 individuals via Illumina HiSeq 2500 sequencing platform (Illumina, San Diego, California) at AgResearch Invermay, New Zealand.

The single-end sequence reads obtained were de-multiplexed, trimmed and filtered according to the methodology described in Chapter 4. After aligning to the *Trifolium repens* genome, a total of 312304 SNPs were identified with a mean sample depth of 8.1 and <35% missing. These SNP data were entered in the KGD method software for computing the GRM. GEBVs for HCN production were estimated using the KGD-GBLUP genomic prediction model for each individual.

After estimating GEBVs for all individuals, at a within-family selection pressure of 5%, the top 2 or 3 plants with the highest GEBVs in each selected HS family in the high selection population and 2 or 3 plants with the lowest GEBVs in each selected HS family in the low selection population, were chosen separately to obtain a 24-plant polycross each for high and low HCN selections.

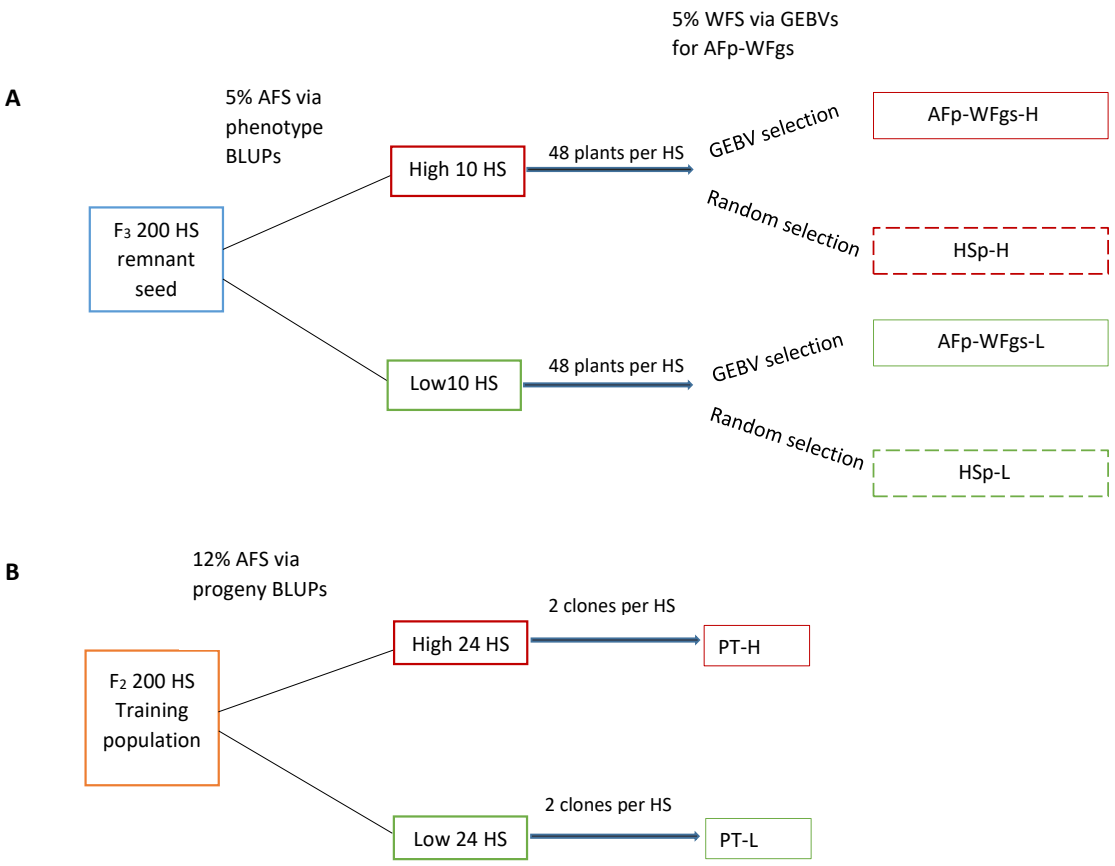


Figure 5.1 Schematic representation of the selection strategies applied in this study on white clover HS families. A) Divergent selection for HCN production using among-family selection (AFS) at 5% for half-sib among-family phenotypic selection (HSp) and an integrated approach of among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs). Within-family selection pressure (WFS) at 5% using genomic estimated breeding values (GEBVs) for AFp-WFgs only. B) Divergent half-sib progeny test (PT) selection based on phenotype BLUPs of F_3 progeny at 12% AFS pressure.

5.2.4. Polycross and HCN evaluation

In the summer of 2018/2019, after undergoing vernalization in winter, all six selected groups were randomly polycrossed in separate cages according to the procedure described in Chapter 3. The plants were re-randomized weekly to ensure random mating within each group. After successful pollination, seed was harvested from each HS family in each group separately, cleaned and equal amount of seed from HS families within each group were collected and a balanced bulk was made separately for each group. From each balanced bulk, a random sample of 72 seeds was taken from each group, germinated and planted in propagation trays containing a mix of peat and sand. The plants were

maintained in a glasshouse and evaluated for HCN production after six weeks according to the methodology described in Chapter 3. Scoring was done on a scale of 0 to 5 where 0 is no HCN produced and 5 is the maximum score for HCN production.

5.2.5. Response to Selection

The response to selection, R, was estimated according to Equation 31 (Falconer & Mackay, 1996). Realised genetic gain was expressed as the percentage increase of the selected population over the mean of the source population.

$$R = P_s - P_p \quad (31)$$

Where: R is the response to selection; P_s is the mean value of the progeny of the selected HS parents and P_p is the mean of the source population before selection. P_p = 2.2 for PT and 3.08 for AFp-WFgs and HSp.

5.2.6. Expected Genetic Gain

Simulation analysis was carried out in DeltaGen (Jahufer & Luo, 2018) to compare the potential genetic gain derived using HSp, PT and AFp-WFgs. Among HS Family additive variance and family × replicate interaction variance component estimates for HCN were used to simulate the predicted genetic gain achieved from the three breeding strategies. Equation 28 was used to estimate the expected genetic gain from HSp and PT with the parental control value (*c*), set to 1 for PT and 0.5 for HSp. A 5% selection pressure was used for among-family selection while a 12% selection pressure was used for PT. Equation 29 was used to estimate the genetic gain derived for AFp-WFgs with the within-family selection pressure set at 5%. The genomic heritability for HCN was estimated at 0.8, using Equation 30, while r_{A-XY} , the genomic selection predictive ability obtained from the KGD-GBLUP model for HCN was 0.22.

5.2.7. Statistical Analysis

One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test (α= 0.05) was performed to determine significant differences among the different high and low selection groups. Data were analysed using the 'stats' package and 'TukeyHSD' function in the 'FSA' package in R (R Core Team 2018).

5.3. Results

5.3.1. Variance Components

There was significant ($P < 0.05$) additive genetic, replicate and family × replicate interaction variance within the F_2 and F_3 populations for HCN (Table 5.1). Additive genetic variation was lower than the error variance component. Estimated narrow-sense heritability on an individual plant basis was higher in the F_2 maternal population (0.93) compared to the F_3 family mean narrow-sense heritability.

Table 5.1 Estimated additive (σ^2_A), replicate (σ^2_R), family-by-replicate interaction ($\sigma^2_{A.R}$), and pooled error (σ_ε) variance components and their associated standard errors (± SE) for HCN in the 200 white clover F_2 and F_3 populations. Narrow-sense heritability (h^2_n) was calculated on a family mean basis and single plant basis for the F_3 population and F_2 populations, respectively.

Population	σ^2_A	$\sigma^2_{A.R}$	σ^2_R	σ^2_ε	h^2_n
F_2	1.80 ± 0.19	0.11 ± 0.01	0.01 ± 0.01	0.43 ± 0.03	0.93
F_3	0.54 ± 0.07	-	0.01 ± 0.08	2.74 ± 0.06	0.82

5.3.2. Predictive Ability

A comparison of the predictive ability obtained when the genomic selection model is trained with the genotypes of the parental F_2 maternal plants and phenotype information of either the HCN progeny BLUP means, BLUP means of a natural log transformation of the progeny data or BLUP means of the parental training population is shown in Figure 5.2. Predictive ability was highest (0.36) when the model was trained with the F_2 parents from which the genotype and phenotype information were used directly compared to using the F_2 parent genotypes with phenotypes inferred from the corresponding F_3 HS progeny (0.22). Transforming the progeny data also significantly increased the predictive ability by 45% relative to the untransformed progeny data. Although there were no significant differences in the bias values for all three sets of data, the highest bias range was obtained with the progeny (-0.56 to 5.26), followed by the transformed progeny data (-0.24 to 3.26) and then the parent (0.02 to 3.6). There was a high, significant ($P < 0.001$) positive correlation between the cross-validated GEBVs of individuals predicted by the progeny BLUP means and the log transformed data (0.97) and progeny BLUP means and the parent BLUP means (0.82). This indicates that the same individuals were selected regardless of the phenotype data used.

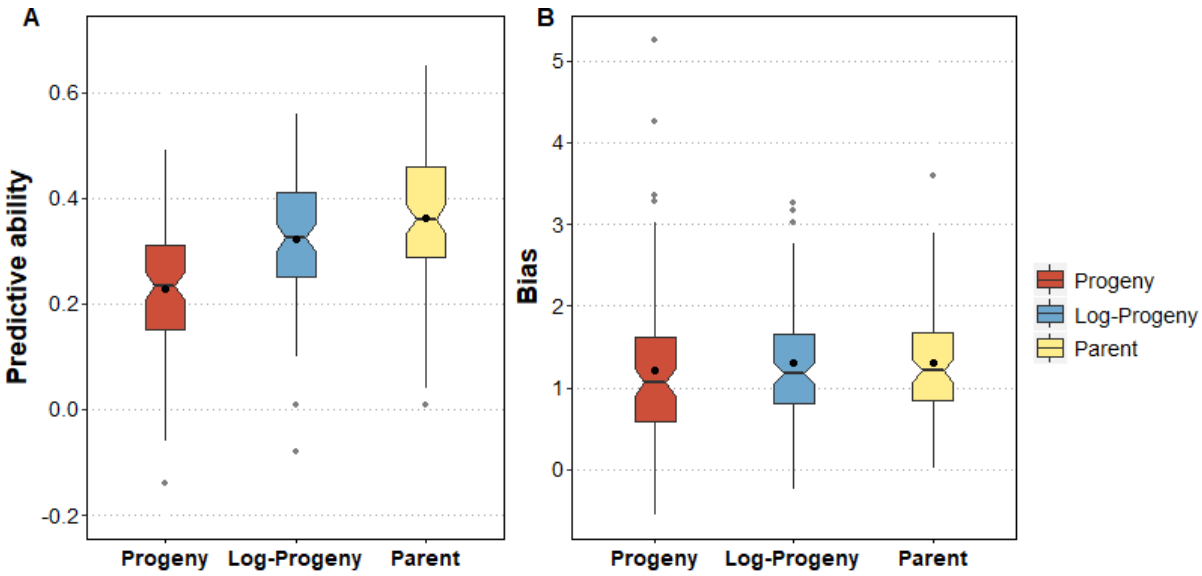


Figure 5.2 Comparison of the predictive ability and bias obtained using different sets of phenotype data used to train the KGD-GBLUP model for HCN (Hydrogen cyanide) production. Progeny predictive abilities were derived from prediction models incorporating F_2 parent genotype and phenotype inferred by their corresponding F_3 HS family. Log-progeny is the natural log transformation of the progeny data. The model was run for 100 iterations and predictive ability was estimated using Monte-Carlo cross-validation using 80% as training and 20% as test sets. Solid line represents the median, black dot in the box represents the mean and grey dots are outliers. Notches that do not overlap indicate medians that are significantly different at $P < 0.05$ (Chambers et al., 1983).

5.3.3. Response to Selection, Estimated and Observed Genetic Gain

The realised genetic gain and response to divergent selection for HCN production was compared across three different selection strategies (Table 5.2). In the high population, the realised genetic gain obtained from selection based on AFp-WFgs was four times more than the HSp. Although the PT selection pressure was lower than AFp-WFgs and HSP, selection resulted in the highest response to selection, and consequently delivered more genetic gain than AFp-WFgs and HSp (Table 5.2). When comparing among strategies in the low population, the highest response to selection was obtained by AFp-WFgs corresponding to a higher genetic gain than either PT or HSp (Table 5.2).

The responses to selection and consequent genetic gain were higher for the low population than the high population for AFp-WFgs and HSp.

The realised genetic gain was higher than the expected genetic gain in the high and low populations for AFp-WFgs and HSp while the realised genetic gain for PT was lower than the expected genetic gain in both populations.

Table 5.2 Estimated and observed genetic gain, response to selection (R) and group means for a divergent selection for HCN in white clover across different selection strategies, among-family selection (AFS) pressure and within-family selection (WFS) pressure.

Selection strategy	Population	AFS	WFS	Expected ΔG	Ave HCN	R	Realised ΔG
	High	5%	-	22%	3.42	0.34	11%
HSp	Low	5%	-	-22%	1.09	-1.99	-65%
	High	5%	5%	30%	4.43	1.35	44%
AFp-WFgs	Low	5%	5%	-30%	0.46	-2.62	-85%
	High	12%	-	98%	4.24	2.02	91%
PT	Low	12%	-	-98%	0.44	-1.78	-80%

Half-sib among-family phenotypic selection (HSp), among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs), half-sib progeny (PT). $R = P_s - P_p$. Where P_s is the mean value of the progeny of the selected HS parents and P_p is the mean of the source population and R is the response to selection. P_p = 2.2 for PT and 3.08 for AFp-WFgs and HSp.

Analysis of variance revealed significant ($P < 0.001$) variation present between the groups (Appendix C.1). From post hoc analyses, in the high population, significant ($P < 0.0001$) differences were found between the means of AFp-WFgs-H and HSp-H, whereas no significant ($P = 0.94$) differences were found between AFp-WFgs-H and PT-H (Figure 5.3) (Appendix C.2). AFp-WFgs and PT strategies delivered 30 and 24% increase in HCN production above the population mean of HSp. When comparing between the population means of HSp-L and AFp-WFgs-L in the low population, significant ($P < 0.05$) differences were also observed between the two groups while PT-L was significantly ($P < 0.05$) different from HSp-L but not significantly ($P = 0.99$) different from AFp-WFgs-L. AFp-WFgs and PT decreased HCN by 58 and 60% compared to HSp.

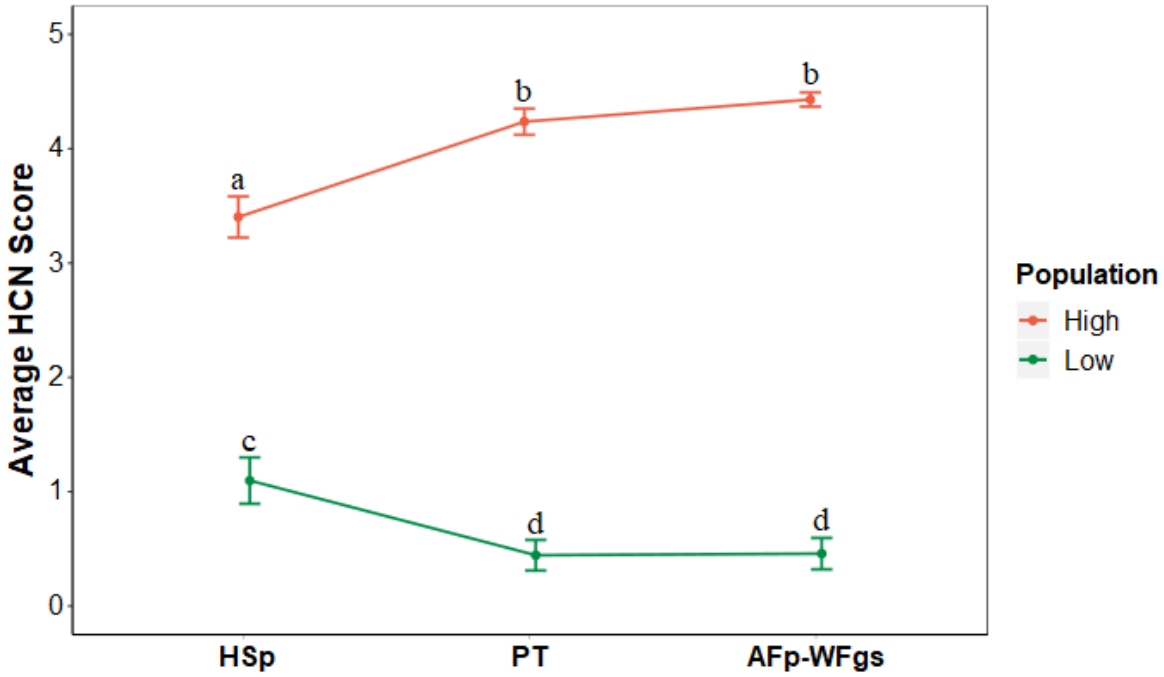


Figure 5.3 Group means of realised genetic gain for three breeding strategies: half-sib among-family phenotypic selection (HSp), among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs), half-sib progeny (PT). AFp-WFgs and HSp selected at 5% selection pressure and PT at 12% selection pressure. Error bars indicate standard error of the means.

From the histogram in Figure 5.4, (Appendix C.2), even though no significant differences were observed between AFp-WFgs-H and PT-H, by using GEBVs, the accuracy of selection was enhanced such that all the lower scores, (i.e., 0, 1 and 2) were successfully eliminated from the high population. Contrarily, the HSp-H and PT-H populations had several individuals with low scores and even some acyanogenic individuals in the population. In the low population, AFp-WFgs-L and PT-L had no individuals with scores above 4, while the HSp-L population had individuals that were very cyanogenic and four individuals with a score of 5.

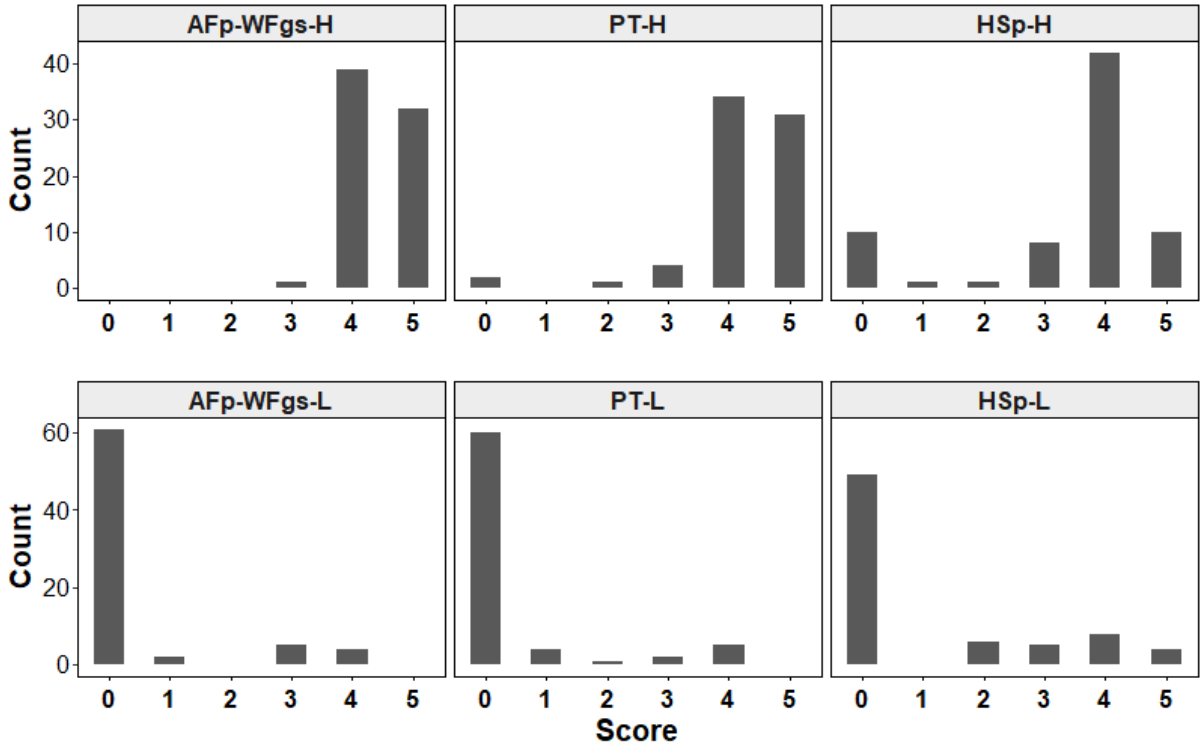


Figure 5.4 Distribution of HCN scores showing the accuracy and response to divergent selection for three breeding strategies: half-sib among-family phenotypic selection (HSp), among-family phenotypic selection and within-family selection via genomic selection (AFp-WFgs), half-sib progeny (PT). AFp-WFgs and HSp selected at 5% selection pressure and PT at 12% selection pressure. H – High population, L – low population. Sample size of 72 individuals per group.

5.4. Discussion

This chapter extends from findings reported in Chapter 4 by empirically validating predicted GEBVs from trained genomic prediction models. It compares breeding strategies that rely on phenotypic selection and an integrated genomic selection approach. The results show that an integrated approach of AFp-WFgs provides greater selection efficiency per unit time than the other approaches and provides insight into the incorporation of genomic selection into conventional white clover breeding.

There was significant additive genetic variation for HCN production among the HS families in the F_2 and F_3 generations indicating the potential of genetic improvement for the trait. Estimates of narrow-sense heritability were high in both populations which provides evidence that the trait is under strong genetic control. The estimation of quantitative genetic parameters like heritability is essential to predict response to selection and the magnitude of genetic gain achievable (Fehr et al., 1987; Hallauer & Miranda, 1988; Falconer, 1989; Nyquist & Baker, 1991; Jahufer et al., 2002). However, since the heritability was estimated from samples taken at one location, it is likely upwards biased/inflated as G×E interactions may confound the additive genetic variance (Comstock & Moll, 1963; Nyquist & Baker, 1991; Holland et al., 2003).

It was encouraging to observe that despite the low to moderate predictive ability obtained for HCN, it still enabled more precise selection, higher response to selection and substantial genetic gain compared to phenotypic selection. The AFp-WFgs strategy enabled identification of superior individuals form within HS families based on their GEBVs in comparison to HSp which is based on phenotypic selection only. Studies by Heffner et al. (2010) and Belamkar et al. (2018) in winter wheat showed genomic selection with low accuracies, (0.17 – 0.3), outperformed MAS and phenotypic selection, respectively, in terms of genetic gain for yield. The conclusion, therefore, by Belamkar et al. (2018) was that predictive abilities should not be the only gauge to measure the success of genomic selection. In fact, Blondel et al. (2015) showed Pearson's correlation between predicted and breeding values to have poor correlation with ranking accuracy. A better approach to evaluate genomic selection, in their opinion, is to utilise models that rank individuals from "most favourable to least favourable" and focus on the top individuals rather than on predicting breeding values based on models contingent on Pearson's correlation. Our findings also support this premise and since most breeders are

seeking to identify the top performers for selection, a ranking approach to genomic selection might be more appropriate.

Using the F_3 progeny phenotype data instead of the F_2 parental phenotype data significantly reduced the predictive ability. This is likely due to the recombination event which causes changes to LD in the F3 population (Habier et al., 2007a). Recombination breaks down the association between markers and genes, thereby affecting estimated marker effects and GRMs (VanRaden, 2008). More specifically, models like GBLUP and KGD-GBLUP that rely on LD and additive genetic relationships between marker and QTL are particularly affected (Habier et al., 2013; Lorenz & Smith, 2015). The HCN data displayed non-normality and was log transformed resulting in an increase in predictive ability. The underlying assumption of most genomic selection models is that the phenotype data is normally distributed. This boost in predictive ability suggests that the quality of the phenotypic data was not sufficient. Minamikawa et al. (2018) found that visually scored traits tended to have lower accuracies than measured traits. This re-emphasizes the importance of high-quality phenotype data for successful genomic selection. Despite the higher predictive ability obtained, ranking individuals based on their GEBVs from the transformed and untransformed data, as well as the parental phenotype data showed similar rankings across the datasets with high Spearman's rank correlations. This high correlation indicates that all methods tended to select the same individuals indicating a slight to no difference in selecting individuals based on all methods.

This finding implies that if the phenotypic data of the parental training population is available, it could potentially be used to train genomic prediction models in forage species to enable a more precise estimation of marker effects. However, because parental lines are usually not evaluated in realistic mixed sward trials like their progeny, available phenotype data is likely to be obtained from spaced plants or individual plants, as in this case, which have low correlations with mixed sward-based data (Casler & Brummer, 2008).

5.4.1. Genetic Gain and Response to Selection

The magnitude of genetic gain obtained for a trait is a function of the selection intensity, prediction accuracy, (the phenotypic selection accuracy is estimated as the square root of the heritability), square root of the additive variance and the time per cycle (Dudley &

Moll, 1969; Falconer, 1989; Nyquist & Baker, 1991; Desta & Ortiz, 2014). The trait HCN had high and significant additive genetic variance, resulting in high heritability, and was selected at a high selection pressure. Therefore, the high response to selection and magnitude of genetic gain were not unexpected and divergent selection proved successful for all three strategies, with AFp-WFgs delivering similar gain to PT and up to four times the genetic gain than HSp. Response to selection in the low population was higher for AFp-WFgs than HSp or PT, resulting in decreasing HCN significantly. These results are similar to Beyene et al. (2015) who found genomic selection to outperform pedigree-based phenotypic selection in maize biparental populations by at least two-fold. Annicchiarico et al. (2019) also reported, via simulation, genomic selection to be more efficient than phenotypic selection for simple traits with low G×E in pea. By contrast, Sallam and Smith (2016) found genomic selection to deliver equivalent gains to phenotypic selection for improving disease resistance traits in barley. Comparing different breeding strategies based on their achieved genetic gain allows breeders to choose a strategy that delivers the highest genetic gain per unit cost (Fehr et al., 1987).

The rate of genetic gain achieved per cycle is a common estimate used to assess the efficiency of different breeding strategies (Fehr et al., 1987; Conaghan & Casler, 2011). Using genotype information to estimate breeding values for individuals within families before selecting, AFp-WFgs utilised the ¼ additive variation among families as well as the ¾ additive variation available within families with consequent increase in the genetic gain achieved (Vogel & Pedersen, 1993; Casler & Brummer, 2008; Conaghan & Casler, 2011). In contrast, the HSp strategy does not access within family genetic variation, therefore, selection of individuals within families is random which means only the ¼ additive variation among families is utilised (Falconer & Mackay, 1996). Although the PT strategy does not utilise within-family additive variance, it was able to deliver similar gains to AFp-WFgs. By the identification and crossing of superior parents, the parental control *c*, is increased from 0.5, when only the female parent is identified to 1 (Casler, 2008; Acquaah, 2012).

The possibility to include within-family selection has been identified as central to accelerating genetic gain in forage species (Casler & Brummer, 2008). Traditionally, HSp can be modified to allow for selection within families, termed among-and within-family selection. However, this entails the growing of individuals within families to the specific growth stage where the trait under selection can be phenotypically assessed. This not only

increases the length of the breeding cycle but also incurs additional labour and phenotyping costs. Besides, within-family phenotypic evaluations are usually carried out on spaced plants which poorly represent typical mixed sward growing conditions (Hayward & Vivero, 1984). With regards to the PT breeding strategy, significant financial and spatial investment must be made to maintain the parental lines while their progeny is being evaluated, which can take up to four years in white clover. Also, and perhaps the most serious disadvantage, is the addition of an extra year or two for another crossing event, making it a year behind the other strategies (Casler & Brummer, 2008; Conaghan & Casler, 2011; Jahufer & Luo, 2018). According to Vogel and Pedersen (1993) PT is an inefficient method of selection and typically stopped after one cycle of selection as repeating the process entails a re-evaluation of the same clones that were evaluated in the previous cycle. The power of genomic selection lies in the ability to address these problems by estimating the breeding values of individuals within families at the seedling stage without the need for phenotypic assessment and by eliminating the need for progeny testing at certain stages of the breeding programme. Therefore, when comparing all three strategies, AFp-WFgs delivered the most gain per unit time.

Differences in the realised gain vs the expected genetic gain occur as a result of several factors including the sample size of the populations compared, environmental conditions and the form of material evaluated (Nickell & Grafius, 1969; Oyekunle & Anjorin, 2018). In this situation, phenotypic evaluation was performed on bulked samples of all selected individuals in the separate groups. Even though equal quantities of seed were included in the balanced bulk, uneven representation of HS families in the 72 seedlings grown is possible due to the small sample size used for phenotypic assessment. As a result, the genetic variability observed may not entirely represent the true population (Bilyeu et al., 2016).

It is important to note that the reported study was a proof of concept. In this regard, the trait HCN production was chosen because of its simple genetic architecture and phenotype which can be measured early in the life cycle of the plant, even though it is not necessarily selected for in many white clover breeding programmes. While these results are reflective of the high heritability of HCN, I believe they are indicative of the merit of using the AFp-WFgs breeding strategy for other key white clover traits. Resende et al. (2013), via simulation, found genomic selection to be more advantageous for low heritability traits.

5.5. Conclusion

In summary, employing the parental phenotype information and log transformed progeny data to train the genomic selection model increased predictive ability and suggests a potential data enhancing method to explore in order to increase the predictive ability. This study empirically validated GEBVs predicted by the genomic prediction model and showed up to four-fold superiority of AFp-WFgs over HSp in terms of magnitude of genetic gain at a 5% divergent selection. Accuracy of selection was also higher for AFp-WFgs than HSp or PT and may prove more cost-efficient per percentage of genetic gain, depending on genotyping costs compared to phenotyping. The results show that genomic selection can effectively be used to select superior plants within-families without the need for phenotypic evaluation of spaced plants. This study emphasizes the value of applying an integrated strategy that uses both phenotypic and genomic selection to accelerate the rate of genetic gain in white clover.

5.6. Foreword to Chapter 6

Chapter 5, provided for the first time, empirical validation on the application of genomic selection in white clover breeding. The impact of different breeding strategies on genetic gain was also compared. In the following chapter, a summary of key findings from the overall study presented in this thesis will be discussed. The chapter will also focus on implications of the results obtained on white clover breeding. Observed shortcomings and anticipated future directions will also be presented.

6. General Discussion, Future Work and Conclusions

The study reported in this thesis was focused on integrating genomic selection into an established among HS family selection strategy to expedite genetic gain for key white clover traits. The findings from this study make significant contributions to the current literature on quantitative genetics and breeding in this species. They provide a comprehensive demonstration of the methodology and implementation of genomic selection, as well as serve as a guide for practical and theoretical requirements that must be considered before genomic selection is incorporated into applied white clover breeding programmes.

Before implementing genomic selection, a population providing accurate estimation of variance components and phenotypic information for the estimation of marker effects must be generated. The prediction of breeding values is dependent on the knowledge of the magnitude of additive genetic variance and heritability (Kennedy, 1981; Nyquist & Baker, 1991; Holland et al., 2003). In this study, genetic parameters were estimated from 200 white clover HS families evaluated across multiple years in two distinct locations (Aorangi and Ruakura) in New Zealand. Results from the multi-location trials showed significant additive genetic variation among the HS families for traits related to yield and vegetative persistence. Worthy of mention were the HS families identified with high DM yield and high stolon density, as evidenced by the positive correlation between the traits. These HS families would be important as breeding material for future cultivar development. Narrow-sense family mean heritability estimates were low to moderate for DM yield and stolon density (0.13 - 0.54), indicating the likely low genetic gain of these traits if phenotypic selection is implemented.

Year, season and location effects had significant influence on DM yield and vegetative persistence. Due to the high level of phenotypic plasticity exhibited by white clover, environmental effects have great impact on key traits (Mitchell, 1956; Davies & Evans, 1982; Archer & Robinson, 1989; Caradus & Williams, 1989; Caradus et al., 1989b; Jahufer et al., 1994; Woodfield & Caradus, 1996). There were significant family × location interaction effects for most traits measured, indicating a relative change in the performance of HS families across locations and emphasizing the need for multi-location trials to separate confounding G×E effects and identify stable genotypes. Summer moisture stress has been found to have an adverse effect on stolon density (Jones, 1982;

Archer & Robinson, 1989; Jahufer et al., 2012). Stolon density was not affected by summer moisture stress in this study either due to the typical warm and low rainfall conditions not occurring during this period or the increasing rainfall and dropping of temperatures immediately post summer which enables fast recovery in the number of stolon fragments and stolon branches (Sanderson et al., 2003). Dry matter yield, although variable between years, was highest in summer and lower in winter and spring. Improved summer growth of clover has been identified to be an important factor in maintaining dairy milk production during this period, especially as the quality of pasture declines due to increased dead matter in the sward and ryegrass flower heads (Woodfield et al., 2001). Taken together, these results support the call for better management practices by way of proper grazing practices and optimum stocking rates to prevent over-defoliation during sensitive winter and spring periods, as well as proper fertilizer application to prevent the companion species outcompeting the white clover.

Collection of accurate phenotype data has always been a challenge for the improvement of quantitative traits like yield (Boopathi, 2013). Across all the field trials, the experimental error variance components were the largest in comparison to the other sources of variation for most traits. Reducing this component by improved sampling techniques improves the precision in the estimates of genetic parameters like additive genetic variance and heritability (Fehr et al., 1987). Significant cost and difficulty were encountered in assessing and measuring stolon density and DM yield due to several factors including the mixed sward growing conditions and the size of the trial. Estimating stolon density and DM yield in mixed sward conditions is extremely challenging and destructive measuring techniques are often resorted to. Considering the size of the trial, labour and time constraints, a limited number of samples were collected per plot. This is a potential limitation of the study. Due the presence of high within-plot variability, measures taken to obtain true plot representation may not be adequate. A major contributor to the accuracy of genomic selection is the quality of the phenotype data and phenotyping has become an obstacle for many breeding programmes (Desta & Ortiz, 2014; Heslot et al., 2015). Since a “garbage in, garbage out” scenario is likely to occur for entering erroneous phenotype data into genomic selection models, great attention must be paid to obtaining reliable phenotypic information. It is recommended that further research be undertaken to develop advanced high-throughput and precise imaging techniques for non-destructive monitoring and measuring systems that allow for several measurements to be taken over regular time intervals. Efficient, cost-effective

phenotyping will allow redirection of resources to larger trials or the additional testing of more locations. The more data collected at different times points, the better the estimation of marker effects, as different QTL can be expressed at different stages in the life cycle of the plant (Yan et al., 2003; Muraya et al., 2017).

Low to moderate predictive abilities were obtained for most traits in this study, reflecting their low to moderate heritabilities. Understanding the genetic architecture and distribution (normal or skewed) of traits is paramount to managing the expectations of genomic selection. Considering the relatively low accuracies that are frequently reported in genomic selection studies, the reluctance to implement this strategy by many breeders is understandable. However, several studies have reported gains from genomic selection and our results contribute to the growing body of evidence that genomic selection delivers more genetic gain than phenotypic selection alone, and therefore presents an opportunity not to be missed. Heffner et al. (2010) demonstrated prediction accuracies of 0.3 and even 0.2 for yearly genetic gain are sufficient to surpass MAS. In situations where genomic selection was less accurate than phenotypic selection, results have shown that by reducing the cycle length, genomic selection proved a superior strategy (Rutkoski et al., 2015). In such a case, employment of genomic selection will depend on a compromise between the desired accuracy and the reduction in the generation interval (Burgueño et al., 2012). It is advised, however, that validation be performed first, preferably on a small scale as was done in this study, before more comprehensive implementation.

There are several elements that contribute to the predictive ability of genomic selection and it is difficult to ascribe relative weightings to each of them as their impacts vary from population to population. Reducing the number of markers and size of the training population showed only a marginal decline in predictive ability. Because resources are finite, the number of individuals potentially evaluated is limited due to high phenotyping and labour costs. Genotype information was provided by GBS which delivered abundant markers of sufficient quality for genomic selection. Even with low read depths and high rates of missing data, imputation options and genomic selection models like KGD-GBLUP are available which utilise markers with low to zero depth and high level of missingness (Dodds et al., 2015). High genotyping costs is a possible deterrent to the adoption of genomic selection in many plant breeding programmes, significant savings can be made via reduced genotyping costs if high predictive ability is sustained with fewer markers. Hence, low coverage sequencing of more individuals with fewer markers may

prove more cost-efficient than sequencing fewer individuals at a higher depth with the goal of generating more markers (Li, 2011). However, in subsequent generations where LD is not expected to be as extensive, the number of markers used would have to be increased accordingly to ensure marker-QTL LD relationship is preserved (Riedelsheimer et al., 2013; Bassi et al., 2016). There was substantial increase in the cost of implementing genomic selection relative to phenotypic selection, it must be noted, however, that some of the costs incurred are "start-up" costs at the first instance of implementation. Subsequently, major costs would mostly comprise of genotyping selection candidates with no need for phenotyping until later stages.

The integrated phenotypic selection and genomic selection approach, AFp-WFgs, resulted in higher genetic gain than among-family HS phenotypic selection only. This was due to increased accuracy of selection and the ability to access the ¾ additive genetic variation within HS families. White clover breeding has been fraught with low genetic gain due to long breeding cycles, difficult-to-measure traits with low heritability, negative association among key traits and the lack of meaningful selection pressure applied within families (Caradus et al., 1995; Jahufer et al., 2002; Barrett et al., 2006; Casler & Brummer, 2008). Forage breeding programmes can take as long as 15 to 20 years before new cultivars are released (Williams, 1987). A principal factor contributing to the long generation intervals is the need for progeny tests, which take up to three years or more, at various stages of the breeding scheme. By replacing visual phenotypic assessment of progeny with genomic selection, time, cost and labour can potentially be saved (Lin et al., 2016). This is not to say that progeny tests are eliminated completely, as the initial phase of the breeding pipeline requires phenotypic data from the progeny to train the model and estimate breeding values of parents. However, at later stages of the scheme, i.e., intermediate field trial stages, phenotyping can be omitted, and rounds of selection conducted based on the genotype information of selection candidates alone. According to Bassi et al. (2016), implementing genomic selection in early generations is the more cost-effective approach. This is because more candidates grown at this stage allow for greater selection pressure to be applied as well significant cost savings by eliminating undesirable genotypes at the early stages (Schmidt et al., 2016). On the other hand, if the cost of trait phenotyping is low, and in the case where genotyping is significantly more expensive, phenotypic selection can be used in the early stages to screen out undesirable lines so that genotyping can be saved for the later stages where there are fewer individuals.

The value of genomic selection depends on its ability to successfully predict GEBVs over generations without phenotyping, after estimating marker effects (Habier et al., 2007a). Studies by Meuwissen et al. (2001) have shown that genomic selection can lead to acceptably high correlations between predicted and true breeding value over several generations without the need for recalibration. With cycles of selection, however, the relationship between the training population and the present population under selection decreases (Daetwyler et al., 2013). Selection and drift cause changes to allele frequencies and LD between markers and QTL, with negative effects on the predictive ability (Habier et al., 2007b; Rutkoski et al., 2015; Bassi et al., 2016; Ferrão et al., 2017). The decrease in the accuracy of genomic selection has been reported to be higher in the early generations due to the decay in relationship between the populations (Jannink, 2010). Nevertheless, later generations have been found to have more sustained accuracy due to LD between markers and QTL (Habier et al., 2007b). Consequently, it is recommended to update the training model regularly, especially in outcrossing populations with low LD where rapid breakdown in marker-QTL linkages are likely to occur (Isik, 2014). Updating the model with new phenotypic measurements to re-estimate marker effects maintains the genomic relationship between selection population and the training population, admittedly at extra cost but with benefits on long-term genetic gain (Bassi et al., 2016; Lin et al., 2016). Muleta et al. (2019) reported a 39% increase in predictive ability when using a model that was updated yearly compared to a non-updated model in sorghum yield. Relationships between populations can also be maintained by the use of closed recurrent selection schemes (Heffner et al., 2011; Bassi et al., 2016).

Drift and selection are also the main reasons why genetic gain starts to plateau (Lorenz et al., 2011). A potential setback to the implementation of genomic selection is the increase in net inbreeding per year as the reduction in generation interval decreases the available genetic variance faster (Goddard & Hayes, 2007). Genomic selection has been reported by Jannink (2010) and Rutkoski et al. (2015) to cause more rapid fixation of alleles and reduced genetic variance compared to phenotypic selection. According to Rutkoski et al. (2015), since genomic selection only acts on alleles in the training population, then, alleles in low frequency, those not in LD with markers are influenced only by drift and not selection unlike phenotypic selection which allows these alleles to reach intermediate levels and cause genetic variance. Phenotypic selection typically takes three years or more to perform one cycle of selection, while genomic selection can be performed twice in a year. This kind of aggressive selection is not without its drawbacks. With successive

rounds of selection, the frequency of favourable alleles increases to the point of fixation and subsequent selection events fail to deliver any response to selection (Knight, 1979). Breeding schemes for open-pollinated species like white clover aim to increase the frequency of desirable alleles in a population while maintaining heterozygosity (Knight, 1979; Brown & Caligari, 2008b). Consequently, breeders are faced with a dilemma of increasing genetic gain by selection whilst preserving or even increasing genetic diversity. In the first instance, when choosing the reference population, genetic variation should be ensured to reduce collinearity between linked markers and the rapid loss of variation due to selection pressure (Jannink et al., 2010). Also, selection criteria that assign larger weights to minor alleles could potentially ensure long term selection response by increasing their frequency (Goddard, 2008; Jannink, 2010). More practically, this problem can be ameliorated by initiating selections in populations with high genetic diversity, or simultaneously running pre-breeding activities so that new genetic variability can be introduced as a plateau in the response to selection is reached (Knight, 1979; Goddard & Hayes, 2007). Gaynor et al. (2017) found schemes that included a population improvement component for pre-breeding to deliver more than twice the genetic gain than phenotypic selection and up to 1.46 times the genetic gain than standard genomic selection strategies.

Increasing genetic gain is a top priority for most breeding programmes and plant breeders will likely choose the approach that delivers the highest amount of genetic gain at the lowest cost. Therefore, having practical information on which to base these important decisions is crucial (Jahufer & Luo, 2018). Results in this study found the integrated genomic selection approach to outperform conventional phenotypic selection at all selection pressures, in terms of trait improvement. However, the cost-efficiency of genomic selection over phenotypic selection was variable and dependent on the selection pressure. Selection pressure can be increased by selecting fewer candidates or increasing the selection pool, so the proportion selected is smaller. For instance, 5% selection pressure can be 10 out of 200 or 200 out of 4000 HS families. With the latter, the trade-off would occur with the requirement to genotype several candidates and this remains the costlier approach of the two. Still, this presents a major opportunity to make full use of the benefits of genomic selection to assess more genetic variation as estimations of GEBVs can be done without phenotyping. Also, with multiplexing comes reduced cost of genotyping per sample. The former, selecting fewer candidates, leads to faster rates of inbreeding due to reduced population size. Caution must be taken with increasing

selection pressure for traits in outcrossing species like white clover as this often limits the selection intensity that can be applied for a secondary trait (Knight, 1979) especially if they are negatively correlated. Furthermore, even though higher selection pressures cause rapid response in the initial generations, less response is observed at later generations compared to lower selection pressures, as the population becomes increasingly homozygous (Rumball & Rae, 1968).

The number of markers required for genomic selection is largely dependent on the genome wide LD (Meuwissen et al., 2001; Meuwissen et al., 2016). Species with substantial LD decay, require more markers to track QTL. In this study, LD was calculated between marker pairs on the same pseudo-molecule from a draft reference genome. The uncharacteristically low LD decay indicates an overestimation of the rate of LD decay. Draft genomes are not as accurate as complete reference genomes and present a limitation to the current study. Further work needs to be carried out on estimating LD between marker pairs located on the same scaffold which might prove to be a more accurate approach.

In this thesis, in-house scripts developed for filtering raw SNP data were utilised resulting in the exclusion of a significant percentage of poor-quality SNPs before GRMs were constructed. It would be interesting to compare the GRMs constructed using less stringent filtering approaches based on MAF and read depth, to explore the impact on predictive ability, especially when a genomic prediction model, like KGD-GLUP, developed for SNP data with low depth is used.

Empirical validation of the GEBVs was performed using a balanced bulk of the separate groups. This provided a simplified method to obtain the mean of the population. The disadvantages of this method include the unequal representation of all individuals in the evaluated samples. Also, there was no way to assess the top percentage of individuals accurately selected based on their GEBVs, which further defines the accuracy of genomic selection. In the future, to assess the ranking of predicted GEBVs and actual observed breeding values, phenotypic assessment should be carried out on individual selected plants not on bulked samples.

The inclusion of a marker preselection step where only markers above established significant thresholds of association to specific genes or QTL are included in genomic selection models has been found to increase predictive ability in some cases. Arojju et al.

(2018) reported using subsets of significant markers to be beneficial for crown rust resistance, a quality trait in ryegrass. There is scope for future work to establish whether using SNPs linked to traits of interest, based on GWAS studies, would significantly increase the predictive ability versus using all available markers which is the original premise of genomic selection.

Reduced genotyping costs mean genomic selection is progressively becoming adopted by many plant breeding programmes worldwide, especially in cases where phenotyping and labour costs consume a large chunk of the budget. Phenotypic selection will continue to play a major role in breeding as it remains the foundation of selection. In white clover, the greatest potential of genomic selection is the ability to enhance selection for typically costly and difficult-to-measure traits, facilitate within-family selection and carry out more cycles of selection per unit time. Through simulation and empirical validation, results from this study demonstrate genomic selection to be a promising tool to increase the rate of genetic gain in white clover breeding.

Appendices

			Aorangi													
	col		1	2	3	4	5	6	7	8	9	10	11	12	13	14
	row															
	16		154	180	181	83	87	48	CH2	70	133	160	65	CH1	103	128
	15		168	CH1	90	6	109	165	36	161	129	31	152	74	75	166
	14		43	63	79	CH1	108	149	23	22	47	167	110	170	139	102
	13		193	113	73	7	45	27	178	CH2	67	68	46	37	153	183
	12		13	25	17	100	29	28	157	175	32	97	CH1	115	38	CH2
	11		76	95	118	162	CH2	189	54	182	125	50	176	56	145	10
	10		107	82	CH2	171	122	12	150	CH2	34	126	130	179	26	123
	9		194	158	191	174	81	173	8	192	84	86	96	111	16	71
	8		CH2	169	77	144	112	136	127	155	198	134	89	35	CH2	142
	7		124	99	57	137	CH1	195	21	94	91	CH1	106	132	9	143
	6		146	200	53	121	18	51	59	201	64	116	197	190	159	140
	5		196	CH2	85	33	156	147	41	104	58	62	44	CH1	5	163
	4		61	98	135	30	131	CH1	40	49	CH2	20	177	172	66	148
	3		78	184	19	CH1	185	186	138	119	4	72	42	93	141	55
	2		120	69	60	1	3	187	151	24	11	164	CH2	88	105	92
Rep 3	1		CH1	39	52	14	114	199	CH2	188	2	80	117	101	15	CH1
	16		72	132	117	1	119	194	CH2	128	82	131	28	34	9	201
	15		175	192	CH1	19	176	54	79	157	98	31	105	162	191	109
	14		196	25	136	200	46	56	190	195	59	101	CH2	43	39	163
	13		133	CH1	87	177	85	178	159	CH1	100	199	86	145	3	111
	12		197	152	83	183	50	CH2	116	122	26	173	138	181	7	47
	11		139	150	140	CH1	78	42	2	63	48	95	179	156	75	CH2
	10		CH2	186	169	15	102	32	112	164	CH2	126	24	89	35	104
	9		114	77	107	168	172	30	29	155	80	137	73	CH1	142	134
	8		113	92	143	45	123	6	CH1	51	38	13	121	14	158	36
	7		33	67	11	CH2	60	12	184	146	4	94	129	70	27	76
	6		187	160	40	189	21	16	23	170	CH2	166	198	44	130	66
	5		CH1	167	69	37	180	118	CH2	174	185	127	CH1	165	58	52
	4		41	141	182	62	84	18	147	64	88	153	124	93	CH2	61
	3		49	108	53	81	CH1	148	106	96	135	65	8	97	151	99
	2		22	CH2	115	57	188	120	154	144	91	CH1	10	110	20	149
Rep 2	1		17	171	5	55	103	68	90	CH2	71	161	193	125	CH1	74
	16		CH1	111	140	72	8	116	15	174	118	109	67	9	149	CH2
	15		46	91	69	168	CH2	179	13	153	CH1	14	38	19	106	184
	14		156	87	119	110	90	60	148	97	171	27	11	122	80	96
	13		99	1	48	135	101	75	CH2	147	108	94	21	12	127	70
	12		92	186	CH2	112	18	57	139	83	183	10	CH1	188	105	163
	11		35	44	157	199	49	150	34	177	95	170	63	71	CH1	107
	10		CH2	22	56	162	158	65	104	161	121	53	175	176	36	20
	9		42	5	145	155	201	120	3	CH2	86	50	55	154	143	114
	8		193	166	129	16	164	195	198	39	84	CH1	178	CH2	58	137
	7		64	CH1	52	151	100	185	37	132	98	82	190	187	43	167
	6		133	68	142	88	103	194	2	76	51	125	189	40	192	CH2
	5		79	191	77	CH1	146	169	CH1	4	26	CH2	89	47	81	85
	4		33	144	29	181	165	59	159	41	66	45	124	126	172	180
	3		182	78	173	61	134	160	24	CH2	117	30	7	CH1	6	54
	2		138	31	197	123	93	17	196	102	74	152	115	141	113	28
Rep 1	1		CH2	136	62	25	CH1	130	32	200	23	CH2	131	73	128	CH1

Appendix A.1 Aorangi experimental design: CH1 and CH2 are repeated check cultivars, 'Grasslands Kopu II' and 'Grasslands Bounty'.

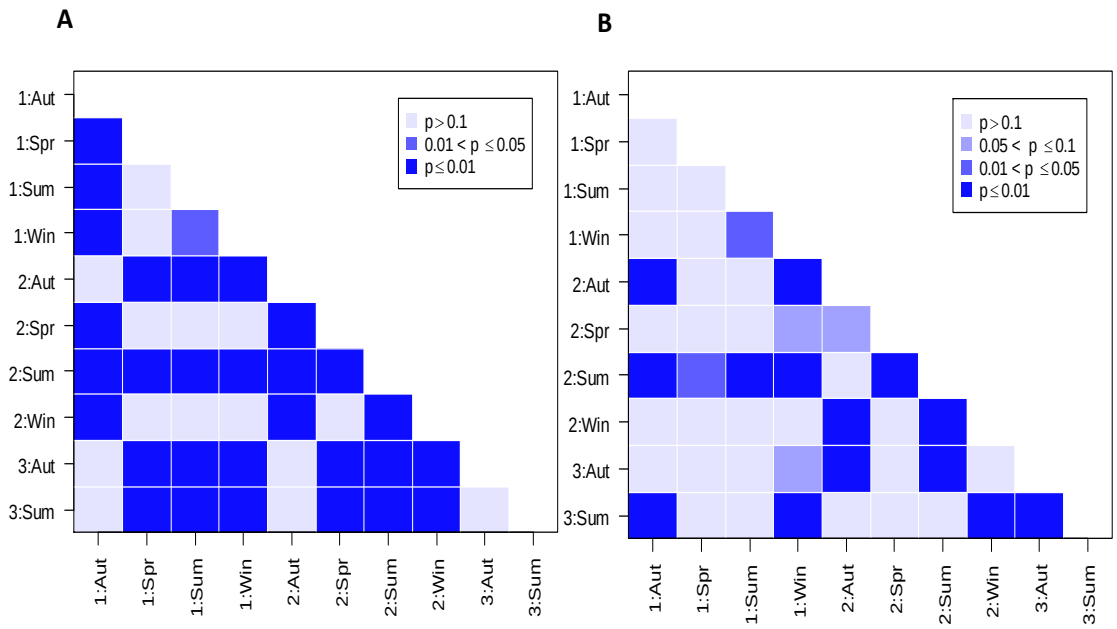
					Ruakura										
	col	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	row														
	16	83	72	197	178	84	87	167	102	77	CH1	64	60	188	136
	15	155	32	CH2	90	80	175	15	44	9	2	104	187	180	CH2
	14	182	6	143	200	168	151	CH2	57	78	97	39	113	30	199
	13	71	19	120	138	49	101	92	55	183	CH2	173	21	157	23
	12	CH1	52	53	75	74	176	62	158	181	28	171	201	140	14
	11	160	8	186	137	169	131	130	CH1	148	65	10	89	98	106
	10	61	35	177	46	CH1	149	50	25	94	58	109	161	1	145
	9	81	3	CH1	139	20	129	CH2	194	184	51	27	147	CH2	185
	8	122	69	105	150	159	100	34	111	193	117	54	132	43	63
	7	152	85	116	190	165	126	108	36	141	CH2	156	127	110	191
	6	95	CH2	16	179	189	45	CH1	135	68	4	134	153	107	123
	5	67	13	174	66	CH2	118	73	124	144	26	146	37	88	166
	4	24	5	114	125	82	47	40	196	91	172	CH1	133	93	CH1
	3	38	18	CH2	154	192	119	CH1	96	103	79	170	112	12	7
	2	128	86	76	162	56	42	121	163	CH1	33	70	31	22	142
Rep 3	1	CH2	17	11	115	CH1	198	99	29	164	41	CH2	59	195	48
	16	CH1	105	161	169	17	123	110	CH2	78	2	83	66	158	134
	15	173	147	107	11	139	3	34	48	64	146	191	77	CH2	80
	14	55	179	183	95	21	56	201	182	18	198	156	177	87	172
	13	136	85	142	CH2	114	70	13	113	81	37	180	CH1	162	196
	12	51	25	115	75	92	184	129	84	CH1	44	41	50	31	88
	11	68	49	90	118	197	38	122	63	60	189	144	152	4	62
	10	93	CH1	174	188	164	CH2	96	8	23	193	CH2	119	35	178
	9	58	30	45	32	47	127	82	1	CH2	73	108	170	7	CH1
	8	67	104	102	CH1	91	150	199	16	74	53	190	57	121	27
	7	97	124	117	163	39	149	138	10	116	52	89	19	168	185
	6	200	130	CH1	98	106	143	CH1	33	59	CH2	76	20	181	71
	5	128	72	99	132	65	157	22	194	131	46	111	12	CH1	86
	4	151	CH2	61	14	148	29	100	103	154	9	24	79	187	176
	3	126	69	159	28	CH2	167	171	CH2	54	42	CH1	195	192	135
	2	153	186	160	40	125	36	120	137	94	140	5	165	43	155
Rep 2	1	CH1	112	6	133	141	109	CH2	175	15	166	145	101	26	CH2
	16	175	201	5	20	162	153	130	2	81	70	CH1	14	173	84
	15	27	193	154	106	40	CH2	155	43	125	79	107	3	118	146
	14	102	25	194	31	49	115	26	89	CH1	188	76	172	CH1	104
	13	35	69	65	131	1	110	8	61	103	164	179	92	191	114
	12	82	10	CH1	123	67	151	CH2	59	29	147	75	112	52	195
	11	150	139	109	133	111	187	54	141	176	137	CH2	37	88	138
	10	CH2	71	145	95	CH2	119	45	165	136	18	167	90	57	CH1
	9	58	192	128	174	91	23	22	CH1	53	122	166	126	159	169
	8	64	180	80	63	184	156	77	86	152	CH1	93	96	21	132
	7	83	42	CH1	200	39	85	196	50	30	101	74	CH2	33	100
	6	12	116	161	44	6	CH2	17	143	4	158	181	48	157	68
	5	94	34	121	72	185	197	98	CH2	108	CH1	19	160	46	38
	4	189	127	163	62	CH1	124	190	178	32	97	113	87	117	24
	3	28	CH2	198	36	66	148	56	171	55	142	129	CH2	105	7
	2	177	99	120	144	60	182	186	CH2	149	9	134	16	13	183
Rep 1	1	11	41	140	CH1	51	73	170	47	168	199	135	78	15	CH2

Appendix A.2 Ruakura experimental design: CH1 and CH2 are repeated check cultivars, 'Grasslands Kopu II' and 'Grasslands Bounty'.

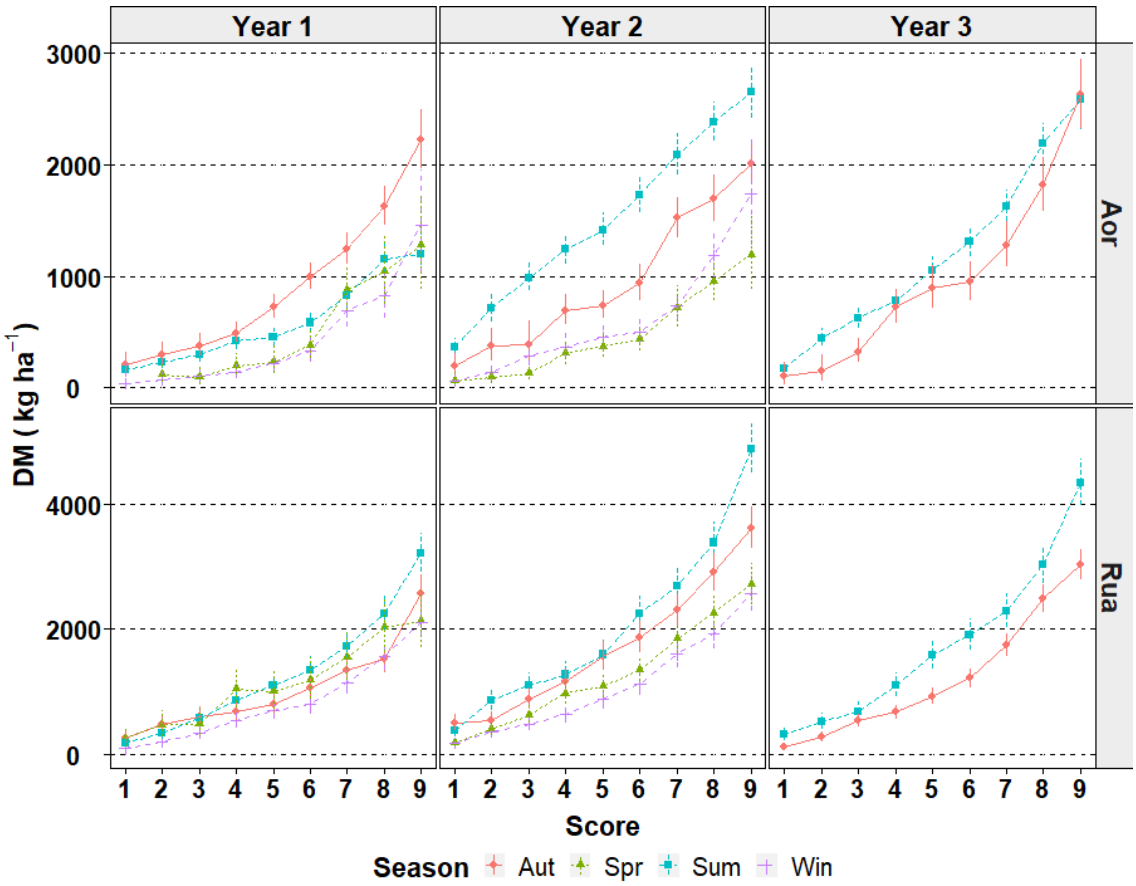
Appendix A.3 ANOVA table for linear mixed model results for the effect of year, season, score and their corresponding interactions on DM calibration cuts at Ruakura.

Source	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Year	2	5761	2880.7	201.6250	< 2.2e-16 ***
Season	3	10305	3435.1	240.4294	< 2.2e-16 ***
Score	8	128611	16076.3	1125.2195	< 2.2e-16 ***
Year:Season	4	1737	434.4	30.4026	< 2.2e-16 ***
Year:Score	16	1321	82.5	5.7766	0.0000000000064 ***
Season:Score	24	1187	49.5	3.4618	0.0000000653127 ***
Year:Season:Score	32	818	25.6	1.7896	0.005113 **
Residuals	718	10258	14.3		

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1



Appendix A.4 Pairwise comparison of season-year combinations on calibration cut DM yield at A) Aorangi and B) Ruakura. Significance at the 5% level as determined by Fisher's least significant difference test.



Appendix A.5 The effect of year and season on the adjusted means on calibration cut DM yield (dry matter yield) at two sites; Aorangi and Ruakura. SEM (standard error of the mean) as error bars.

Appendix A.6 Post hoc pairwise comparison for full-scale DM yield at Aorangi and Ruakura for 2017 and 2018. Significance at the 5% level as determined by Fisher's least significant difference test.

Pairwise p-value

	1:Aorangi	1:Ruakura	2:Aorangi	2:Ruakura
1:Aorangi	0.0000	1.5798	2.6317	0.6421
1:Ruakura	0.1528	0.0000	1.0457	-0.9398
2:Aorangi	0.0301	0.3263	0.0000	-1.9830
2:Ruakura	0.5388	0.3748	0.0827	0.0000

Appendix A.7 ANOVA table for linear mixed model results comparing DM yield for HS families and commercial checks cultivars over two years, 2017 and 2018 across two locations; Aorangi and Ruakura.

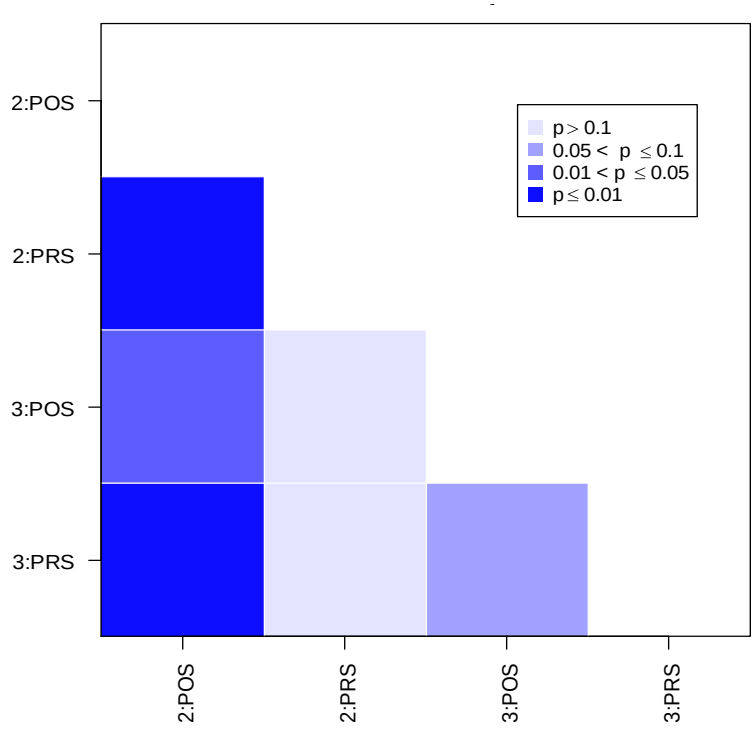
	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Year	87058	87058	1	12.04	1.4835	0.24657	
Site	6059	6059	1	12.04	0.1032	0.75348	
Line	42120531	208517	202	2373.46	3.5532	< 2.2e-16	***
Year:Site	386260	386260	1	12.04	6.5820	0.02470	*
Year:Line	20670785	102331	202	2372.91	1.7437	0.000000002743	***
Site:Line	26416778	130776	202	2372.99	2.2285	< 2.2e-16	***
Year:Site:Line	13573893	67532	201	2372.61	1.1508	0.07966	.

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Appendix A.8 ANOVA table for linear mixed model results for the effect of year, checks, time of measurement and their interaction checks on stolon number assessed over summer of combined years, 2017/2018 and 2018/2019 at Aorangi.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Year	18336785	18336785	1	12.487	12.1192	0.004290	**
Time	21498918	21498918	1	12.400	14.2091	0.002531	**
Check	71429211	35714605	2	86.863	23.6046	0.000000006501	***
Year:Time	1950651	1950651	1	12.302	1.2892	0.277828	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

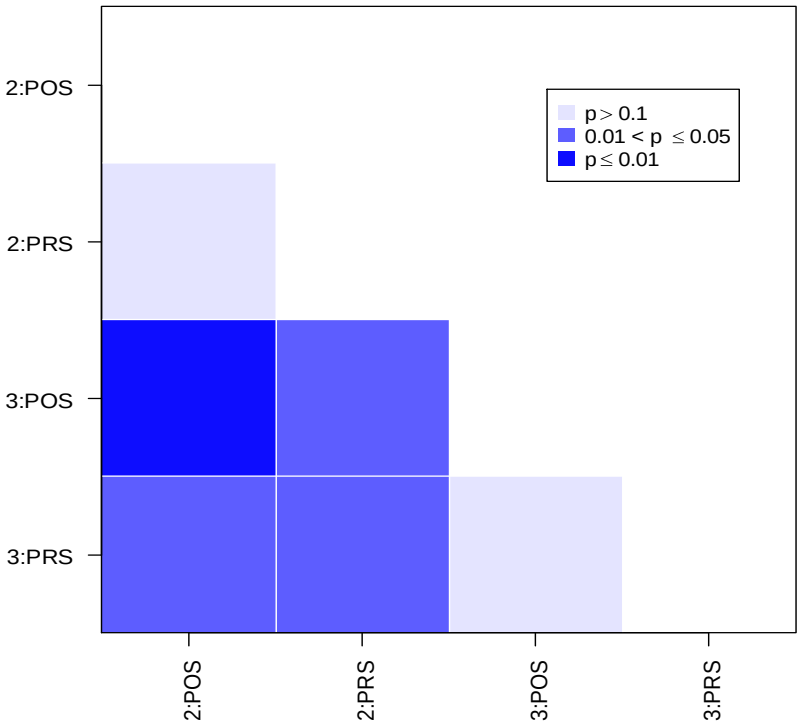


Appendix A.9 Post hoc pairwise comparison of season-year combinations on stolon number assessed over summer of combined years 2017/2018 and 2018/2019 at Aorangi. Significance at the 5% level as determined by Fisher's least significant difference test. 2POS = stolon number post-summer year 2; 2 PRS = stolon number post-summer year 2; 3POS = stolon number post-summer year 3; 3 PRS = stolon number pre-summer Year 3.

Appendix A.10 ANOVA table for linear mixed model results for effect of year, and time of measurement and their interaction on stolon branches assessed over summer of combined years 2017/2018 and 2018/2019 at Aorangi.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Year	90183140	90183140	1	12.803	18.2953	0.000931	***
Time	12260	12260	1	12.536	0.0025	0.961010	
Check	161668245	80834122	2	98.363	16.3987	0.0000007141	***
Year:Time	2258987	2258987	1	12.536	0.4583	0.510730	

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1



Appendix A.11 Post hoc pairwise comparison of season-year combinations on stolon branches assessed over summer of combined years 2017/2018 and 2018/2019 at Aorangi. Significance at the 5% level as determined by Fisher's least significant difference test. 2POS = stolon number post-summer year 2; 2 PRS = stolon number post-summer year 2; 3POS = stolon number post-summer year 3; 3 PRS = stolon number pre-summer year 3.

Appendix A.12 ANOVA table for linear mixed model comparing stolon number for HS families and commercial checks cultivars over summers of 2017/2018 and 2018/2019 at Aorangi.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Year	34742710	34742710	1	12.1	25.5992	0.0002753	***
Time	20475121	10237561	2	12.1	7.5433	0.0074995	**
Line	641733235	3176897	202	5469.1	2.3408	< 2.2e-16	***
Year:Line	488658670	2419102	202	5600.9	1.7825	0.0000000001595	***
Time:Line	843507716	2093071	403	5481.8	1.5422	0.0000000001136	***

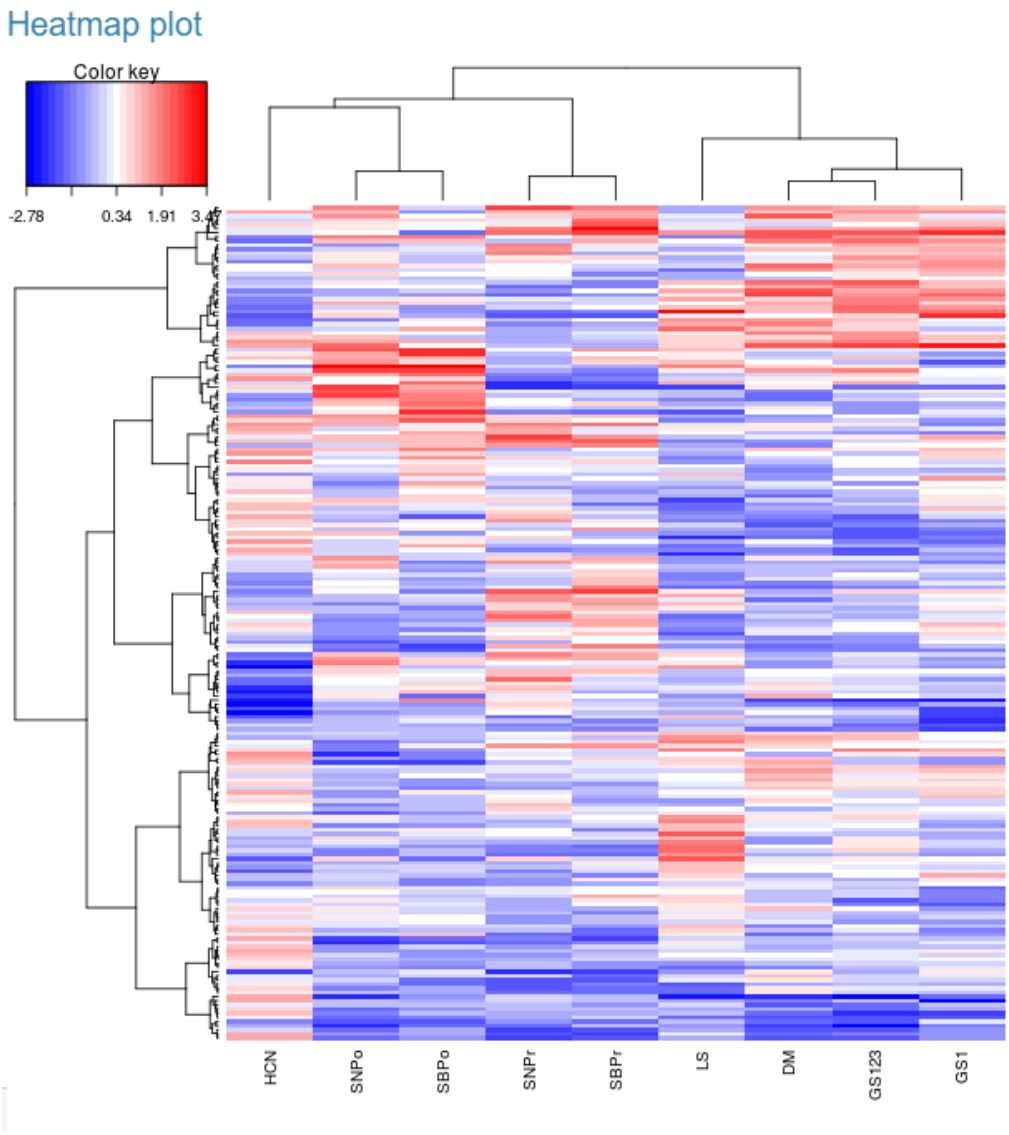
Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Appendix A.13 ANOVA table for linear mixed model comparing stolon branches for HS families and commercial checks cultivars over summers of 2017/2018 and 2018/2019 at Aorangi.

	Sum Sq	Mean Sq	NumDF	DenDF	F value	Pr(>F)	
Year	40226487	40226487	1	12.1	9.0998	0.01066	*
Time	2339143	1169571	2	12.1	0.2646	0.77187	
Line	2064967957	10222614	202	6031.4	2.3125	< 2.2e-16	***
Year:Line	1658610948	8210945	202	6096.9	1.8574	5.383e-12	***
Time:Line	2781704365	6902492	403	6032.2	1.5614	2.660e-11	***

Appendix A.14 Importance of principal components for pattern analysis using standardised Best Linear Unbiased Predictor (BLUP) values of 200 HS families for traits: Year 1 across location growth score (GS1), Year 1 and 2 dry matter across all locations (DM), across all years and location growth scores for all seasons (GS123), leaf size (LS), Year 3 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), post-summer stolon branches (SBPOS) and hydrogen cyanide (HCN). Locations are Aorangi and Ruakura.*Stolon traits were measured at Aorangi only.

Importance of components %	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Standard deviation	1.660	1.324	1.193	1.046	0.849	0.659	0.605	0.549	0.391
Proportion of Variance	0.306	0.195	0.158	0.122	0.080	0.048	0.041	0.034	0.017
Cumulative Proportion	0.306	0.501	0.659	0.780	0.860	0.909	0.949	0.983	1.000



Appendix A.15 Dendrogram based on cluster analysis of the 200 HS families for traits: Year 1 across location growth score (GS1), year 1 and 2 dry matter across all locations (DM), across all years and location growth scores for all seasons (GS123), leaf size (LS), Year 3 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), post-summer stolon branches (SBPOS) and hydrogen cyanide (HCN). Locations are Aorangi and Ruakura.*Stolon traits were measured at Aorangi only.

Appendix A.16 Factor loadings for traits: Year 1 across location growth score (GS1), year 1 and 2 dry matter across all locations (DM), across all years and location growth scores for all seasons (GS123), leaf size (LS), Year 2 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), post-summer stolon branches (SBPOS) and hydrogen cyanide (HCN). Locations are Aorangi and Ruakura. *Stolon traits were measured at Aorangi only.

Trait	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8	Dim.9
GS1	-0.72	0.13	-0.28	0.31	-0.36	0.34	0.07	-0.10	-0.16
DM	-0.80	0.28	-0.05	0.12	-0.02	-0.44	-0.24	0.04	-0.12
GS123	-0.91	0.21	-0.10	0.10	0.00	0.03	0.06	0.04	0.31
LS	-0.46	0.44	-0.07	-0.46	0.57	0.20	0.02	0.06	-0.09
SNPRS	-0.24	-0.80	-0.38	-0.04	0.02	0.08	-0.11	0.37	-0.02
SBPRS	-0.33	-0.74	-0.37	-0.12	0.23	-0.09	0.01	-0.37	0.01
SNPOS	-0.51	-0.38	0.64	0.00	0.02	-0.13	0.41	0.07	-0.07
SBPOS	-0.30	-0.30	0.80	0.07	0.04	0.21	-0.35	-0.07	0.02
HCN	0.19	0.01	-0.04	0.86	0.46	0.02	0.04	0.03	-0.01

Appendix A.17 Trait contribution to principal components; Year 1 across location growth score (GS1), year 1 and 2 dry matter across all locations (DM), across all years and location growth scores for all seasons (GS123), leaf size (LS), Year 2 pre-summer stolon number (SNPRS), pre-summer stolon branches (SBPRS), post-summer stolon number (SNPOS), post-summer stolon branches (SBPOS) and hydrogen cyanide (HCN). Locations are Aorangi and Ruakura. *Stolon traits were measured at Aorangi only.

Trait	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8	Dim.9
GS1	18.96	0.95	5.64	8.78	17.57	27.16	1.35	3.34	16.25
DM	22.99	4.57	0.16	1.33	0.06	45.42	15.27	0.50	9.70
GS123	30.20	2.60	0.63	0.89	0.00	0.19	1.03	0.63	63.83
LS	7.73	11.14	0.30	19.17	45.10	9.54	0.12	1.00	5.90
SNPRS	2.05	36.59	10.08	0.14	0.05	1.56	3.42	45.81	0.29
SBPRS	3.99	31.07	9.59	1.32	7.17	1.89	0.02	44.87	0.09
SNPOS	9.48	8.08	28.44	0.00	0.04	3.67	44.93	1.79	3.57
SBPOS	3.23	5.00	45.03	0.40	0.27	10.49	33.49	1.83	0.25
HCN	1.37	0.00	0.13	67.98	29.73	0.07	0.36	0.23	0.12

Appendix A.18 Importance of principal components for pattern analysis using standardised Best Linear Unbiased Predictor (BLUP) values of 200 HS families for traits for traits; WinAo, SprAo, SumAo, AutAo, WinRu, SprRu, SumRu and AutRo. Aorangi (Ao) and Ruakura (Ru).

Importance of components%	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Standard deviation	1.927	1.545	0.860	0.672	0.497	0.440	0.374	0.357
Proportion of Variance	0.464	0.298	0.093	0.056	0.031	0.024	0.018	0.016
Cumulative Proportion	0.464	0.763	0.855	0.912	0.942	0.967	0.984	1.000

Appendix A.19 Factor loadings for seasonal herbage growth scores for summer (SumAo and SumRu), autumn (AutAo and AutRo), winter (WinAo and WinRu) and spring (SprAo and SprRu). Aorangi (Ao) and Ruakura (Ru).

Trait	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8
SumAo	-0.63	0.55	0.27	-0.40	0.24	-0.04	0.00	-0.10
AutAo	-0.75	0.53	0.05	-0.16	-0.26	-0.02	-0.08	0.21
WinAo	-0.77	0.50	-0.11	0.18	-0.15	0.23	0.06	-0.18
SprAo	-0.70	0.48	-0.17	0.41	0.21	-0.18	-0.01	0.06
SprRu	-0.64	-0.50	-0.49	-0.19	0.14	0.13	0.13	0.11
SumRu	-0.57	-0.58	0.49	0.18	0.12	0.20	-0.11	0.08
AutRu	-0.68	-0.58	0.29	0.03	-0.14	-0.19	0.22	-0.04
WinRu	-0.65	-0.64	-0.26	-0.07	-0.07	-0.12	-0.23	-0.13

Appendix A.20 Trait contribution to the principal components; summer (SumAo and SumRu), autumn (AutAo and AutRo), winter (WinAo and WinRu) and spring (SprAo and SprRu). Aorangi (Ao) and Ruakura (Ru).

Trait	Dim.1	Dim.2	Dim.3	Dim.4	Dim.5	Dim.6	Dim.7	Dim.8
SumAo	10.66	12.66	9.68	35.56	22.47	0.89	0.00	8.09
AutAo	15.43	11.74	0.40	5.66	27.91	0.22	4.71	33.93
WinAo	16.16	10.30	1.65	7.02	8.96	27.58	2.71	25.61
SprAo	13.41	9.71	3.81	35.84	17.30	16.94	0.05	2.92
SprRu	11.11	10.48	31.76	7.93	7.84	9.28	11.59	10.02
SumRu	8.89	13.94	31.80	6.70	5.99	19.61	8.61	4.47
AutRu	12.71	14.22	11.53	0.18	7.64	17.83	34.84	1.05
WinRu	11.63	16.95	9.37	1.12	1.89	7.65	37.50	13.89

Appendix B.1 Buffer Mix for DNA isolation.

Homogenization buffer per 1000 ml
29.22 g NaCl
100 ml 1M Tris (pH 7.4)
100 ml 0.5M EDTA (pH 8)
13.12 g sodium sulphite
7 g sodium dodecyl sulphate

Precipitation buffer per 1000 ml
353.3g potassium acetate
144.1g (140ml) acetic acid

Binding buffer per 1000 ml
2M (191 g) guanidinium chloride make up to 333 ml with TE
667 ml 100% EtOH

Wash buffer per 1000 ml
4 ml 5M NaCl
2 ml 1M Tris-HCL (pH 8)
194 ml H_2O
800 ml EtOH

Appendix B.2 Effect of training: test set size and number of iterations on the predictive ability (PA), bias and bias range for traits dry matter (DM), growth score (GS) and leaf size (LS) assessed using KGD-GBLUP.

Trait	Iteration	CV	PA	Bias	Bias range
DM	100	5/95	0.37	1.99	-1.81 - 7.98
DM	500	5/95	0.29	1.50	-3.8 - 8.56
DM	1000	5/95	0.32	1.68	-5.75 - 14.79
DM	2000	5/95	0.31	1.65	-7.77 - 10.13
DM	100	10/90	0.36	1.95	-0.56 - 5.15
DM	500	10/90	0.31	1.69	-1.44 - 6.46
DM	1000	10/90	0.31	1.66	-4.12 - 7.48
DM	2000	10/90	0.31	1.64	-2.02 - 8.95
DM	100	20/80	0.29	1.83	-0.67 - 9.54
DM	500	20/80	0.30	1.80	-0.25 - 19.91
DM	1000	20/80	0.30	1.83	-0.57 - 27
DM	2000	20/80	0.30	1.80	-0.8 - 9.69
DM	100	30/70	0.30	5.45	0.32 - 244.34
DM	500	30/70	0.29	2.12	-0.12 - 42.45
DM	1000	30/70	0.29	2.14	-0.09 - 58.58
DM	2000	30/70	0.29	2.04	-0.04 - 28.19
DM	100	40/60	0.28	4.32	0.35 - 205.95
DM	500	40/60	0.27	3.44	0.23 - 469.44
DM	1000	40/60	0.28	2.81	0.25 - 124.07
DM	2000	40/60	0.27	2.91	-0.02 - 249.53
GS	100	5/95	0.23	2.31	-6.91 - 11.41
GS	500	5/95	0.23	2.08	-6.28 - 18.94
GS	1000	5/95	0.22	2.00	-6.27 - 17.18
GS	2000	5/95	0.21	1.92	-14.12 - 17.53
GS	100	10/90	0.23	2.20	-2.36 - 10.62
GS	500	10/90	0.23	2.17	-3.68 - 15.27
GS	1000	10/90	0.22	2.11	-4.06 - 16.24
GS	2000	10/90	0.22	2.11	-3.38 - 23.89
GS	100	20/80	0.22	3.16	-0.72 - 65.02
GS	500	20/80	0.21	2.50	-1 - 42.05
GS	1000	20/80	0.21	2.86	-1.61 - 168.4
GS	2000	20/80	0.21	2.86	-1.35 - 379.6
GS	100	30/70	0.20	9.39	-0.19 - 511.69
GS	500	30/70	0.20	3.51	-0.87 - 66.69
GS	1000	30/70	0.19	11.17	-0.55 - 7213.77
GS	2000	30/70	0.20	11.06	-0.91 - 11802.23
GS	100	40/60	0.18	7.33	-0.57 - 940.93
GS	500	40/60	0.18	6.03	-0.15 - 182.33
GS	1000	40/60	0.17	5.13	-0.93 - 579.58
GS	2000	40/60	0.18	9.67	-0.8 - 4225.11
LS	100	5/95	0.42	1.52	-1.57 - 5.33

LS	500	5/95	0.43	1.51	-1.96 - 7.01
LS	1000	5/95	0.44	1.53	-2.87 - 6.51
LS	2000	5/95	0.43	1.50	-3.89 - 7.59
LS	100	10/90	0.44	1.55	0.02 - 3.51
LS	500	10/90	0.43	1.53	-0.95 - 4.92
LS	1000	10/90	0.44	1.53	-1.46 - 5.05
LS	2000	10/90	0.43	1.50	-0.84 - 4.77
LS	100	20/80	0.41	1.56	0.27 - 3.38
LS	500	20/80	0.42	1.54	0.32 - 4.28
LS	1000	20/80	0.42	1.54	-0.13 - 4.21
LS	2000	20/80	0.42	1.56	-0.1 - 6.9
LS	100	30/70	0.42	1.71	0.6 - 5.05
LS	500	30/70	0.40	1.58	0.32 - 4.28
LS	1000	30/70	0.40	1.63	0.23 - 5
LS	2000	30/70	0.40	1.65	0.26 - 7.42
LS	100	40/60	0.40	1.92	0.75 - 5.66
LS	500	40/60	0.38	1.86	0.42 - 31.04
LS	1000	40/60	0.38	2.07	0.27 - 347.09
LS	2000	40/60	0.38	1.77	0.25 - 23.81

Appendix B.3 Genomic selection model effect on the predictive ability (PA), bias and bias range for traits dry matter (DM), growth score (GS) and leaf size (LS).

Trait	Model	PA	Bias	Bias range
DM	BayesCπ	0.30	1.07	-0.74 - 3.46
GS	BayesCπ	0.20	0.80	-0.21 - 2.05
LS	BayesCπ	0.42	1.41	0.35 - 2.54
DM	GBLUP	0.29	1.73	0.03 - 6.49
GS	GBLUP	0.21	2.36	-0.17 - 15.65
LS	GBLUP	0.42	1.59	0.22 - 4.18
DM	KGD_GBLUP	0.33	2.02	-0.14 - 11
GS	KGD_GBLUP	0.25	3.20	-0.76 - 20.4
LS	KGD_GBLUP	0.44	1.68	0.36 - 3.54
DM	RKHS	0.30	1.39	0.02 - 3.29
GS	RKHS	0.20	1.04	-0.62 - 2.75
LS	RKHS	0.41	1.53	0.31 - 3.64

Appendix B.4 Predictive ability, bias and bias range for all measured traits, implemented using KGD-GBLUP. Heritability = H.

Trait	Location	Year	H	Predictive ability	Prediction accuracy	Bias	Bias range
HCN	_	1	0.82	0.22	0.24	1.3	-0.28 - 7.66
DM	Aorangi	1	0.27	0.13	0.25	3.16	-2.44 - 52.99
DM	Aorangi	1.2	0.54	0.25	0.33	1.92	-0.54 - 9.4
DM	Aorangi	2	0.48	0.24	0.35	1.68	-0.66 - 7.17
DM	Combined	1	0.37	0.12	0.20	2.13	-1.7 - 15.99
DM	Combined	1.2	0.38	0.30	0.48	1.73	5.94 - -0.04
DM	Combined	2	0.35	0.33	0.56	1.46	-0.08 - 3.9
GS	Aorangi	1	0.35	0.12	0.20	4.55	-4.17 - 48.83
GS	Aorangi	1.2	0.35	0.13	0.23	6.16	-1.9 - 58.31
GS	Aorangi	2	0.50	0.19	0.27	2.50	-0.57 - 17.42
GS	Aorangi	2.3	0.53	0.22	0.31	2.80	-0.52 - 23.43
GS	Aorangi	3	0.59	0.20	0.27	1.66	-0.43 - 7.36
GS	Aorangi	1.2.3	0.48	0.20	0.28	2.55	-0.35 - 15.4
GS	Ruakura	1	0.33	0.13	0.22	6.77	-1.08 - 235.21
GS	Ruakura	1.2	0.36	0.17	0.28	2.75	-0.86 - 43.71
GS	Ruakura	2	0.42	0.14	0.22	7.76	-1.26 - 267.16
GS	Ruakura	2.3	0.22	0.13	0.27	5.11	-2.34 - 146.99
GS	Ruakura	3	0.29	0.02	0.04	0.38	-24.33 - 33.22
GS	Ruakura	1.2.3	0.73	0.16	0.19	4.17	-2 - 58.9
GS	Combined	1	0.24	0.13	0.26	2.12	-0.93 - 22.39
GS	Combined	1.2	0.23	0.18	0.37	2.26	-0.67 - 16.3
GS	Combined	2	0.24	0.16	0.33	4.02	-1.25 - 46.91
GS	Combined	2.3	0.35	0.23	0.40	2.33	-0.81 - 18.55
GS	Combined	3	0.20	0.22	0.49	1.88	-0.41 - 7.95
GS	Combined	1.2.3	0.32	0.25	0.45	3.20	-0.76 - 20.4
LS	Aorangi	1	0.26	0.17	0.33	1.48	-0.56 - 7.72
LS	Aorangi	1.2	0.53	0.36	0.49	1.79	-0.02 - 4.15
LS	Aorangi	2	0.51	0.33	0.47	1.77	0.05 - 5
LS	Aorangi	2.3	0.71	0.38	0.45	1.56	0.2 - 3.86
LS	Aorangi	3	0.46	0.33	0.48	1.22	-0.08 - 3.61
LS	Aorangi	1.2.3	0.73	0.39	0.46	1.72	-0.04 - 4.28
LS	Ruakura	1	0.30	0.15	0.27	0.88	-1.2 - 5.02
LS	Ruakura	1.2	0.61	0.26	0.33	1.19	-0.15 - 4.57
LS	Ruakura	2	0.69	0.19	0.23	1.39	-0.7 - 6.47
LS	Ruakura	2.3	0.68	0.25	0.31	1.33	-0.21 - 4.73
LS	Ruakura	1.2.3	0.66	0.34	0.42	1.45	0.1 - 4.91
LS	Combined	1	0.36	0.19	0.31	0.93	-0.26 - 4.57
LS	Combined	1.2	0.55	0.40	0.55	1.47	0.54 - 3.26
LS	Combined	2	0.42	0.36	0.56	1.61	0.27 - 3.43
LS	Combined	2.3	0.55	0.43	0.59	1.69	0.07 - 4.01
LS	Combined	3	0.31	0.36	0.65	1.43	0.19 - 3.29
LS	Combined	1.2.3	0.70	0.43	0.51	1.63	0.49 - 3.28

Stolon branches (POS)	Aorangi	1	0.15	0.21	0.54	2.30	-0.52 - 14.63
Stolon branches (POS)	Aorangi	2	0.19	0.01	0.02	-18.30	-59.2 - -0.55
Stolon branches (PRS)	Aorangi	1	0.37	-0.18	-0.29	NA	NA
Stolon branches (PRS)	Aorangi	2	0.18	0.08	0.19	21.30	-1.37 - 1738.31
Stolon number(POS)	Aorangi	1	0.25	0.02	0.05	0.29	-7.86 - 41.3
Stolon number(POS)	Aorangi	2	0.13	-0.11	-0.29	NA	NA
Stolon number(PRS)	Aorangi	1	0.20	-0.03	-0.08	NA	NA
Stolon number(PRS)	Aorangi	2	0.14	0.15	0.41	0.90	-0.88 - 5.05

Appendix B.5 Predictive ability (PA), bias and bias ranges for growth score (GS) and leaf size (LS) in two locations; Aorangi and Ruakura and across-locations (Com) over a period of three years.

Trait	Location	Year	PA	Bias	Bias range
GS	Aorangi	1	0.06	4.55	-4.17 - 48.83
GS	Aorangi	1.2	0.13	6.16	-1.9 - 58.31
GS	Aorangi	2	0.19	2.50	-0.57 - 17.42
GS	Aorangi	2.3	0.22	2.80	-0.52 - 23.43
GS	Aorangi	3	0.20	1.66	-0.43 - 7.36
GS	Aorangi	1.2.3	0.18	2.55	-0.35 - 15.4
GS	Ruakura	1	0.12	6.77	-1.08 - 235.21
GS	Ruakura	1.2	0.17	2.75	-0.86 - 43.71
GS	Ruakura	2	0.11	7.76	-1.26 - 267.16
GS	Ruakura	2.3	0.10	5.11	-2.34 - 146.99
GS	Ruakura	3	0.02	0.38	-24.33 - 33.22
GS	Ruakura	1.2.3	0.12	4.17	-2 - 58.9
GS	Combined	1	0.15	2.12	-0.93 - 22.39
GS	Combined	1.2	0.18	2.26	-0.67 - 16.3
GS	Combined	2	0.16	4.02	-1.25 - 46.91
GS	Combined	2.3	0.23	2.33	-0.81 - 18.55
GS	Combined	3	0.22	1.88	-0.41 - 7.95
GS	Combined	1.2.3	0.25	3.20	-0.76 - 20.4
LS	Aorangi	1	0.17	1.48	-0.56 - 7.72
LS	Aorangi	1.2	0.36	1.79	-0.02 - 4.15
LS	Aorangi	2	0.33	1.77	0.05 - 5
LS	Aorangi	2.3	0.38	1.56	0.2 - 3.86
LS	Aorangi	3	0.33	1.22	-0.08 - 3.61
LS	Aorangi	1.2.3	0.39	1.72	-0.04 - 4.28
LS	Ruakura	1	0.15	0.88	-1.2 - 5.02
LS	Ruakura	1.2	0.26	1.19	-0.15 - 4.57
LS	Ruakura	2	0.19	1.39	-0.7 - 6.47
LS	Ruakura	1.2.3	0.34	1.45	0.1 - 4.91
LS	Combined	1	0.19	0.93	-0.26 - 4.57
LS	Combined	1.2	0.40	1.47	0.54 - 3.26
LS	Combined	2	0.36	1.61	0.27 - 3.43

LS	Combined	2.3	0.43	1.69	0.07 - 4.01
LS	Combined	3	0.36	1.43	0.19 - 3.29
LS	Combined	1.2.3	0.43	1.63	0.49 - 3.28

Appendix B.6 Effect of training set size on the predictive ability (PA), bias and bias ranges of three traits DM (dry matter) yield, GS (growth score) and LS (leaf size). Implemented using GBLUP.

Trait	TS%	TS	PA	Bias	Bias range
DM	10%	20	0.05	0.36	-26.36 - 26.84
DM	20%	40	0.15	-1.83	-181.02 - 23.69
DM	30%	60	0.20	2.90	-9.82 - 37.38
DM	40%	80	0.18	2.69	-116.33 - 49.59
DM	50%	100	0.20	2.81	-2.59 - 39.2
DM	60%	120	0.24	8.13	-2.03 - 551.1
DM	70%	140	0.27	3.88	-1.14 - 132.2
DM	80%	160	0.25	2.41	-1.27 - 42.69
DM	90%	180	0.30	2.06	-0.34 - 7.5
DM	100%	200	0.29	1.73	0.03 - 6.49
GS	10%	20	-0.09	0.67	-26.12 - 98.73
GS	20%	40	0.11	2.60	-14.01 - 47.59
GS	30%	60	0.08	3.34	-13.95 - 76.36
GS	40%	80	0.10	2.08	-45.9 - 54.44
GS	50%	100	0.11	5.13	-4.87 - 96.77
GS	60%	120	0.14	3.00	-7.34 - 62.52
GS	70%	140	0.16	4.87	-21.84 - 125.22
GS	80%	160	0.18	3.97	-1.49 - 109.8
GS	90%	180	0.20	2.80	-1.52 - 43
GS	100%	200	0.21	2.36	-0.17 - 15.65
LS	10%	20	0.10	1.74	-200.68 - 242.31
LS	20%	40	0.21	6.07	-26.79 - 149.89
LS	30%	60	0.25	2.86	-142.08 - 51.34
LS	40%	80	0.30	2.43	-3.95 - 15.8
LS	50%	100	0.36	3.03	-1.3 - 32.52
LS	60%	120	0.35	2.30	-0.49 - 18.65
LS	70%	140	0.39	1.99	-0.24 - 13.07
LS	80%	160	0.39	1.75	0.2 - 5.64
LS	90%	180	0.39	1.56	0.06 - 4.56
LS	100%	200	0.42	1.59	0.22 - 4.18

Appendix B.7 Effect of number of markers on the predictive ability (PA), bias and bias range of three traits DM (dry matter) yield, GS (growth score) and LS (leaf size). Implemented using GBLUP.

Trait	Markers%	Markers	PA	Bias	Bias range
DM	0.05%	55	0.07	0.35	-58.42 - 27.42
DM	0.10%	110	0.14	5.14	-13.08 - 107.7
DM	0.50%	550	0.21	2.25	-0.62 - 24.34
DM	1%	1100	0.26	2.03	-0.5 - 12.63
DM	5%	5500	0.31	2.03	-0.01 - 13.64
DM	10%	11000	0.31	1.87	-0.2 - 7.25
DM	50%	55000	0.28	1.78	-0.25 - 14.85
DM	100%	110000	0.29	1.73	0.03 - 6.49
GS	0.05%	55	0.06	0.87	-28.75 - 13.74
GS	0.10%	110	0.10	2.94	-5.79 - 80.75
GS	0.50%	550	0.13	4.37	-14.75 - 90.87
GS	1%	1100	0.16	2.18	-1.2 - 24.41
GS	5%	5500	0.20	2.38	-0.69 - 15.77
GS	10%	11000	0.20	3.23	-0.95 - 72.93
GS	50%	55000	0.20	2.36	-0.51 - 26.51
GS	100%	110000	0.21	2.36	-0.17 - 15.65
LS	0.05%	55	0.12	0.97	-32.13 - 15.6
LS	0.10%	110	0.16	2.89	-3.59 - 97.1
LS	0.50%	550	0.27	1.34	-0.76 - 9.2
LS	1%	1100	0.31	1.25	-0.29 - 5.04
LS	5%	5500	0.41	1.57	0.25 - 3.59
LS	10%	11000	0.41	1.52	0.34 - 3.89
LS	50%	55000	0.42	1.57	0.35 - 3.31
LS	100%	110000	0.42	1.59	0.22 - 4.18

Appendix B.8 Multi-trait predictive ability (PA), bias and bias range for primary traits: DM (dry matter) SNPRS (pre-summer stolon number) and SNPOS (post-summer stolon number) using GS (growth score), LS (leaf size) SBPRS (pre-summer stolon branching) and SBPOS (post-summer stolon branching as secondary traits yield.

Trait	CV	PA	Average bias	Bias range
DM	Single	0.325	2.02	-0.14 - 11
DM-GS	MTCV1	0.31	1.30	0.04 - 2.96
DM-GS	MTCV2	0.63	2.02	1.26 - 3.23
DM-GS+LS	MTCV1	0.29	1.23	0.19 - 3.06
DM-GS+LS	MTCV2	0.62	1.92	0.71 - 2.74
DM-LS	MTCV1	0.31	1.23	-0.25 - 2.97

DM-LS	MTCV2	0.39	1.47	0.42 - 2.81
SNPRS	Single	0.15	0.98	-0.88 - 5.05
SNPRS-SBPRS	MTCV1	0.15	0.75	-0.69 - 2.61
SNPRS-SBPRS	MTCV2	0.54	1.73	0.7 - 2.69
SNPOS	Single	-0.11	1.14	-3.52 - 41.31
SNPOS-SBPOS	MTCV1	-0.10	-0.65	-2.88 - 1.44
SNPOS-SBPOS	MTCV2	0.28	1.56	-0.52 - 3.79

Appendix C.1 One-way ANOVA to compare population means among the different groups.

```
Response: Score
```

	Df	F	Pr(>F)	
Group	5	276.57	< 2.2e-16	***
Residuals	426			

```
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

Appendix C.2 Group mean comparison adjusted p values using Tukey's multiple-comparison test (α= 0.05).

Population	AFp-WFgs-H	AFp-WFgs-L	HSp-H	HSp-L	PT-H
AFp-WFgs-L	0.0000*	-	-	-	-
HSp-H	0.0000*	0.0000*	-	-	-
HSp-L	0.0000*	0.0249*	0.0000*	-	-
PT-H	0.934	0.0000*	0.0009*	0.0000*	-
PT-L	0.0000*	0.99	0.0000*	0.02013*	0.0000*

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