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**Examining perennial ryegrass (*Lolium perenne*
L.) persistence through identifying genetic
shifts within two cultivars after nine years in
the field.**

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

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In

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ABSTRACT

Perennial ryegrass (*Lolium perenne* L.) is a commercially important forage species in New Zealand agriculture. Ryegrass persistence is important for farmers as it substantially decreases the costs associated with reseeded pastures. Breeding for ryegrass persistence is difficult because of the complex interaction between genotype and environment; and the short time of field trial assessment compared to the expected longevity of pasture. A nine year old cultivar comparison trial at Poukawa, Hawkes Bay, New Zealand was identified and plants surviving in the cultivar plots of 'Grasslands Samson' and 'Commando' were retrieved. These populations that had survived were termed Persistent. A sample of commercially sourced seed of these cultivars were also grown to represent the 'Original' genetic pool of the cultivars sown in the field. Persistent populations were compared to Original cultivar seed to characterise morphology and underlying genetics associated with persistence. Results were interpreted to determine if a genetic shift had occurred in Persistent populations due to advantageous phenotypes surviving.

Three methodologies were used to compare populations: 1) In a glasshouse, eight morphological traits were measured after 10 weeks growth for Original and Persistent populations of 'Grasslands Samson'; 2) Half-sibling families were generated from Persistent and Original populations for both cultivars and were assessed for additive genetic variation of seven traits as one metre rows in the field over 13 months; 3) Simple sequence repeat (SSR) markers were used to explore the genetic composition of Original and Persistent populations of each cultivar. Analysis and interpretation of data showed genetic shifts were cultivar specific. The greatest differences were identified between populations of 'Grasslands Samson'. Compared to the Samson Original population, Samson Persistent plants had significantly greater means for four traits in the glasshouse

and half-sibling families showed evidence of shifting population means of traits associated with animal grazing avoidance. SSR marker results were confounded by late detection of contamination in samples. Analysis of a reduced sample size showed no significant differences between any of the four populations using F statistics and genetic structure analysis.

These results suggest future studies could reduce risk of contamination by collecting single tillers from the field of Persistent populations. Further investigation of the genetics of persistence should focus on the role of lamina sheath lengths in tiller production, and using the half-sibling families identified in this study for germplasm development and quantification of genotype-by-environment interactions.

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ABBREVIATIONS

| | |
|-----------|---|
| %amp | Success rate of marker amplification |
| %P | Percentage of polymorphic loci |
| μ | Population mean |
| AFLP | Amplified Fragment Length Polymorphism |
| AMH | Aftermath heading score |
| AMOVA | Analysis of molecular variance |
| AR1 | Commercially available endophyte strain in perennial ryegrass cultivars |
| BLUE | Best linear unbiased estimator |
| BLUP | Best linear unbiased predictor |
| bp | Base pairs |
| CASS | Cheaply amplified size standard |
| CV_A | Co-efficient of additive variation |
| df | Degrees of freedom |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| DW | Dry weight (per plant) |
| DWT | Dry weight (per one metre row) |
| E | Environment |
| EST-SSRs | Expressed sequence tag simple sequence repeats |
| Est. Var. | Estimated variance components |
| F_{IS} | Component of Wright's (1921) fixation index, used to define within population structure by calculating the average observed heterozygosity of an individual relative to the expected heterozygosity of individuals in the population it belongs to. |
| F_{ST} | Component of Wright's (1921) fixation index, used to define between population structure by comparing the expected heterozygosity of individuals within a subpopulation to the total expected heterozygosity of individuals across all populations |
| G | Genotype |
| G_C | Genetic gain per cycle |
| h^2_n | Narrow-sense heritability |
| H_E | Expected heterozygosity |

| | | |
|-----------------------|--|---|
| HGs | Herbage growth score | |
| H _o | Observed heterozygosity | |
| kg ha ⁻¹ | Kilograms per hectare | |
| LL | Leaf length | |
| LnP(D) | Mean posterior probability | |
| LT | Leaf thickness | |
| LW | Leaf width | |
| LWs | Leaf width score | |
| MAF QT | Ministry of Agriculture and Forestry quick test, a soil testing metric | |
| mL ha ⁻¹ | Millilitres per hectare | |
| MS | Mean of squares | |
| N | Number of samples | |
| Na | Number of alleles | |
| Na _v | Total number of allele variants (within a cultivar) | |
| N(0,σ ² ε) | Normally and independently distributed | |
| N-P-K-S | Units of nitrogen, phosphorus, potassium, and sulphur. Used to express fertiliser contents. | |
| n _r | Number of replications | |
| n _s | Number of seasons | |
| P | Phenotype | |
| PC | Principal components | |
| PCA | Principal components analysis | |
| PCR | Polymerase chain reaction | |
| PHs | Plant habit score | |
| RAPD | Random amplified polymorphic DNA | |
| REML | Residual maximum likelihood | |
| RP | Percentage of reproductive tillers | |
| RT | Number of reproductive tillers. | |
| RTD | Reproductive tiller development score | |
| Ru | Rust score | |
| S | Selection differential (average superiority of the selected parents), used to predict genetic gain per cycle | S |

| | |
|----------------------------|---|
| S _{A_p} | Proportion of shared alleles |
| SL | Leaf sheath length |
| SS | Sum of squares |
| SSRs | Simple sequence repeats, a type of microsatellite marker. |
| TN | Tiller number |
| Var. % | Percentage of variation |
| σ^2_{ϵ} | Experimental error variance |
| σ^2_a | Additive genetic variance |
| σ^2_{as} | Additive additive-by-season interaction variance |
| σ^2_D | Dominance variance |
| σ^2_g | Genetic variance |
| σ^2_P | Phenotypic variance |
| \bar{x} | Population mean |

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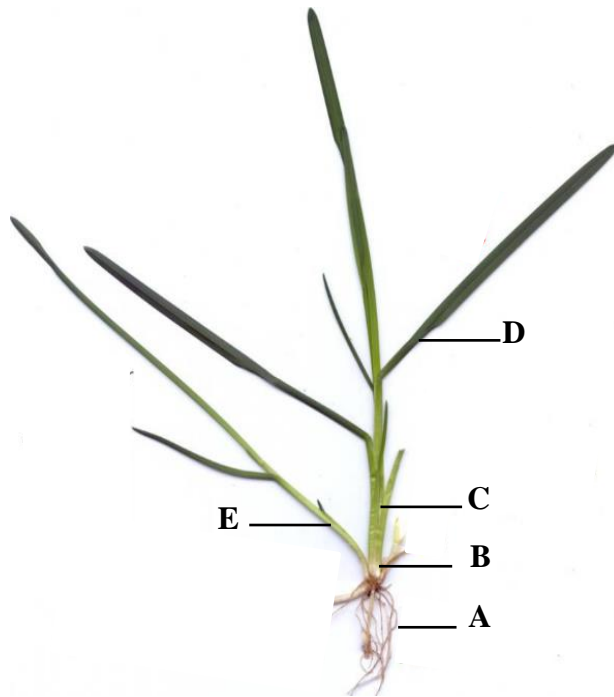
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1.0 INTRODUCTION

1.1 Perennial ryegrass



Perennial ryegrass (*Lolium perenne* L.) is a commercially important species used throughout the world. Thought to have originated from the Mediterranean centre of diversity, *L. perenne* is now found and utilised in North and South America, throughout Europe, North Africa, temperate Asia, high-rainfall zones of Australia, and New Zealand (Easton *et al.*, 1989; Cunningham *et al.*, 1994; Hannaway *et al.*, 1999; Lee *et al.*, 2012).

Figure 1.1; Perennial ryegrass (*L. perenne*) grows by tillering. Tillers consist of A) Roots; B) Terminal apex; C) Folded lamina sheaths; D) Lamina (leaf blade); E) Daughter tiller.

Perennial ryegrass is a diploid species, with seven pairs of chromosomes ($2n = 2x = 14$). Tetraploid plants ($4n = 4x = 28$) can be created by artificially doubling chromosomes. Perennial ryegrass is a prolifically tillering grass with dark green coloured leaves that are glabrous on the abaxial surface of the leaf. The tillering growth allows plants of this species to survive as perennials. Plants are continuously producing daughter tillers (Figure 1.1E), formed in the leaf axils of the terminal apex (Figure 1.1B) of developed tillers. The tiller sheaths are folded (Figure 1.1C) with three leaves, unless it is a reproductive tiller which form round sheaths producing a spikelet inflorescence under long day conditions and after fulfilling chilling requirements. Day length and vernalisation by chilling vary substantially due to the broad range of genetic diversity

available in perennial ryegrass populations. (Jewiss, 1972; White & Hodgson, 1999; Lee, *et al.*, 2012; Wang & Forster, 2017).

Perennial ryegrass is genetically self-incompatible, therefore cannot be self-pollinated easily. As a result there are high levels of genetic variation within this species owing to diverse plant morphology types ranging from small turf types (Dracatos *et al.*, 2009), to high yielding pasture plants as either diploids (2n) or tetraploids (4n) (Easton *et al.*, 2002b).

1.2 Perennial ryegrass in New Zealand

Perennial ryegrass in New Zealand is used primarily in grazed pasture with some forage conservation through making hay and silage to store feed for utilising during low growth rate periods. A pasture sown in this species is expected to last for 10 years or more without reseeded (Chapman *et al.*, 2015a). This species has been the most commonly used plant for the majority of New Zealand's agricultural pastures since its introduction through European settlers in the early 19th century (Lee, *et al.*, 2012). After native bush burn off, farmers established pastures for sheep, cattle and some dairy production through sowing mixtures of cocksfoot (*Dactylis glomerata*) and perennial ryegrass. In 1918, 3.5 million acres of New Zealand was sown in pasture mixes of these species. In 1920 grassland research was initiated in New Zealand by AH Cockayne and Bruce Levy in Palmerston North, this prompted a change in pastoral seed mixes to perennial ryegrass and white clover (*Trifolium repens L.*). Grassland research also offered better information and advice to farmers on improving pasture growth. Following both world wars, farmers in

New Zealand were encouraged to maximise their production for export primarily to the United Kingdom. Intensification, where farmers obtained greater yields from the same area of land, was supported by aerial topdressing of fertiliser, over-sowing pastures, development of hill country for farming, and establishment of irrigation in some regions. By 1960 agricultural intensification had expanded the area of pastoral farms to five times that identified in 1918 to 18 million acres with sheep being the main class of stock (Hunt & Easton, 1989; Nightingale, 2008).

Government subsidies increased during the 1960's-1970's to 30% of farmers' incomes. The increase was to offset the high currency exchange rate and high prices farmers paid for materials caused by legal requirements, and decreasing incomes from exports. The loss of these subsidies in 1984 caused a dramatic shift in land use. Farmers had to use their pastures to make a profit without subsidy help to cover costs (Nightingale, 2008). After the removal of subsidies the sheep population in New Zealand declined quickly as farmers shifted to more intensive and profitable farming types leading to a gradual increase in dairy cattle numbers (MacLeod & Moller, 2006).

In the 1990's fertiliser application onto perennial ryegrass pastures increased in an effort to improve production. During this time, purchasing and importation of non-pasture feed to fulfil requirements during seasonal low pasture growth periods became common practice on some farms allowing an increase in year round stocking rates (MacLeod & Moller, 2006). Intensification was in part driven by conversion of farms in suitable land areas from sheep farming to dairy to supply growing Chinese demand for

dairy exports. The increase in dairy conversions was pronounced in some regions of New Zealand such as Canterbury where the difference in land area in dairy production between 1990 to 2009 increased from 20 000 ha to 900 000 ha (Pangborn & Woodford, 2011). The number of dairy cows in New Zealand continues to increase and as of 2016 sits at 6.6 million (StatisticsNZ, 2016).

Under increasing intensification of land use farmers have been encouraged by industry to renew their paddocks at a rate of 10% annually to maintain pasture production advantages (DairyNZ, 2017). However, pasture renewal rates remain low at 2% for sheep and beef farms and 5-6% per annum for dairy. Renewing pastures incurs significant cost to the farm business, therefore considerable economic benefits are required by new perennial ryegrass cultivars to justify investment (Daly *et al.*, 1999; Sanderson & Webster, 2009). Renewed pastures can be a profitable option with continuing advantages for a number of years, with as much as 10% increase in yields over old pastures in Waikato, Taranaki, Canterbury, and Southland over 8 years (Brazendale *et al.*, 2011; Chapman *et al.*, 2012). Despite reported benefits, continued low pasture renewal rates reflect the apprehension of farmers in purchasing and using new cultivars. Recently, farmers have publicly voiced concerns that new cultivars available on the market lack persistence exhibited by older cultivars (Daly, *et al.*, 1999; Kelly *et al.*, 2011; Lane, 2011; Parsons *et al.*, 2011; Lee, *et al.*, 2012; Chapman, *et al.*, 2015a). These concerns are important to plant breeders because yield and persistence have always been high priority traits in forage cultivar development (Parsons, *et al.*, 2011).

1.3 Breeding in New Zealand

The first breeding programmes in New Zealand began in the 1920's. After 30-80 years of largely European imported germplasm growing in New Zealand, some populations had adapted to the local climate and farming systems (Easton, 1983). Elite locally adapted germplasm was identified by Levy and Davies (1929) after studying the variation in plants collected from around New Zealand. They concluded seed sourced from regions with relatively short rotations of crops, were 'stemmy', 'free seeding' and lacked persistence. Those harvested from 'permanent' pasture were 'leafy', 'dense' and persistent. Of the germplasm studied, plants sourced from Hawkes' Bay were superior for growth and were used to develop the first cultivars forming the basis of New Zealand plant breeding efforts (Easton, 1983; Stewart, 2006; Lee, *et al.*, 2012).

Perennial ryegrass breeding programmes aim to improve the species through manipulating plant genetics to change the phenotype. A plant's phenotype (P) is the result of the genotype (G) and the environment (E), and the interaction of the genotype with the environment (Equation 1.1). Breeding objectives focus on improving two categories of traits expressed in cultivars. The main category that is sought after are non-reproductive traits where increasing total and seasonal dry matter yields, pasture quality, drought tolerance, and plant persistence are emphasised. However for a cultivar to be commercially viable adequate seed production is also necessary (Wilkins & Humphreys, 2003).

$$P = G + E + (G \times E) \quad (1.1)$$

The key factor for successful genetic improvement of a trait is the genetic diversity available in the initial germplasm used to develop a breeding or base population (Falconer & Mackay, 1996). For a breeder to improve their germplasm, a base population is created through introgression of ecotypes or exotic germplasm into locally adapted elite cultivars. As perennial ryegrass is cross-pollinated and generally self-incompatible, improvements have mostly been obtained through recurrent selection breeding methods. The main aim of recurrent selection is to increase or decrease the frequency of alleles associated with the trait under selection, resulting in a change of progeny mean (Falconer & Mackay, 1996; Easton, *et al.*, 2002b; Lee, *et al.*, 2012). Breeding methods associated with the development of homozygous inbred lines and the use of heterosis are not widely practiced in perennial ryegrass due to the difficulty of emasculating plants, genetically controlled self-incompatibility, and severe inbreeding depression (Brummer, 1999).

Polycrossing of selected ryegrass plants is conducted in pollen isolation facilities and allowed to inter-pollinate with each other (Wilkins, 1991; Easton, *et al.*, 2002b; Lee, *et al.*, 2012). Seed from each plant is harvested separately forming one maternal half-sibling family (HS). Because of the numerous parents available to be pollen donors the resulting progeny are highly heterogeneous and are often highly heterozygous at many loci. This progeny may be polycrossed again to decrease potential heterotic effects before germplasm (usually thousands of plants) are planted in the field as spaced plants, rows, or plots under grazing or cutting regimes to assess their phenotypes. Field trials are typically assessed for 18 months to three years before selections are made. Selected individuals are again polycrossed to advance the breeding pool to the next generation (Woodfield, 1999; Easton *et al.*, 2001a; Lee, *et al.*, 2012).

By using recurrent cycles of selection and polycrossing to advance germplasm, the breeder is utilising the additive genetic variation (σ_a^2) available in the breeding pool. Additive genetic variation is the genetic variation (σ_g^2) that can be passed from parents to their progeny which does not include dominance variation (σ_D^2) (Equation 1.2). The proportion of σ_a^2 to phenotypic variation (σ_P^2) can indicate the influence breeding can have on a particular trait. This estimate is known as narrow-sense heritability (h_n^2), larger h_n^2 indicates a greater contribution of σ_a^2 to σ_P^2 (Equation 1.3). Breeders can then predict genetic gain per cycle (G_C) from the heritability and the selection differential (the average superiority of the selected parents (S)) (Equation 1.4) (Falconer & Mackay, 1996). Traits such as heading date that show larger h_n^2 can have substantial genetic gains made in just a few generations. In contrast more complex traits like dry matter yield have more genetic and environmental factors contributing to genotype thus lower h_n^2 resulting in lower genetic gains (Wilkins, 1991; Lee, *et al.*, 2012; McDonagh *et al.*, 2016).

$$\sigma_g^2 = \sigma_a^2 + \sigma_D^2 \quad (1.2)$$

$$h_n^2 = \sigma_a^2 / \sigma_P^2 \quad (1.3)$$

$$G_c = h_n^2 \times S \quad (1.4)$$

Cultivars developed using recurrent selection methods are often called ‘synthetics’, consisting of related but genetically different individuals. Cultivars therefore contain individuals expressing a spectrum of performance. Genetic improvement of synthetic cultivars is measured by comparing their mean performance to commercial cultivars, across different pastoral environments.

A limitation of developing cultivars through recurrent breeding methods is the length of time required - currently 10-15 years. This timeframe encompasses intervals that plants are assessed, summer flowering, low heritability of important traits, and ensuring high levels of infection of the appropriate endophyte. As a result of this, ryegrass breeders cannot respond quickly to changes in market demand. A variety released in 2017 could have been bred for objectives set in 2002. Since then, farm management, stock class, and pastoral expectations have changed, and the traits these plants were selected for may not be relevant to current requirements.

1.4 Pasture persistence

Pasture persistence is influenced by the complex interaction between genotype and environment. The environment has a considerable effect on this trait. Variations in climate, endophyte infection, pasture establishment, grazing management, and fertiliser inputs can greatly affect a plants' ability to continue to vegetatively persist through producing asexual daughter tillers and survive in the field (Clark, 2011; Chapman *et al.*, 2015b). Parsons, *et al.* (2011) describes persistence as not just the survivorship of plants, but also the continued stability of dry matter yields of the sown population. This definition reflects farmer expectations of performance of new cultivars, but does not provide a period of time that the pastures should persist (Daly, *et al.*, 1999; Tozer *et al.*, 2011). A good indication of how long farmers might expect perennial ryegrass to persist is provided by the rate that they renew their pastures. Annually sheep and beef farmers renew 2% of their farms while dairy farmers renew 5-6%. At these rates, farmers expect pastures to last more than 20 years before cultivation. (Daly, *et al.*, 1999; Sanderson & Webster, 2009).

It would be beneficial for farmers, industry and researchers to agree on quantifiable categories of persistence. With a definition in place, annotated with quantifiable traits, plant breeders can better understand the role that plant genetics has in persistence, and how genetic variation of associated traits could be manipulated in cultivar development.

1.4.1 Plant morphology

The perenniality of ryegrass is derived from prolific production of asexual tillers. In the establishment phase, rapid production of tillers is imperative for outcompeting weeds and establishment in a pasture (Jewiss, 1972; Westoby, 1984). Tiller size, tiller number, leaf elongation rate, and proportion of reproductive tillers have been found to differ significantly between cultivars (Bahmani *et al.*, 2000a; Sartie *et al.*, 2009; Griffiths *et al.*, 2016).

Tetraploid (4n) cultivars developed through chromosome doubling have been identified as having larger cells resulting in larger tillers when compared to diploid cultivars. Within each ploidy level differences have been found between cultivars for tiller sizes indicating that this trait is not just a product of ploidy, but is also genetically controlled. Advantageous tiller size in relation to a plants' ability to survive is inconclusive. Larger tillers have been associated with greater yields in single plant glasshouse trials. Tiller size could contribute to persistence if the resources stored in larger tillers enabled plants to tolerate abiotic stresses better, consequently outcompeting plants with smaller tiller weights and smaller yields (Westoby, 1984; Sartie, *et al.*, 2009).

However because grazing animals periodically remove biomass from bigger plants, tiller size might not contribute to better persistence, especially in tetraploid cultivars where there is some evidence they lack persistence compared to diploid cultivars (Roegiers *et al.*, 1988; O'Donovan & Delaby, 2005). Griffiths, *et al.* (2016) observed plant morphology of cultivars under grazing and concluded that tiller size was not associated with improved yields. But bigger tillers had an increased leaf to non-leaf (sheath) ratio possibly to compensate for having lower tiller densities. The constant yield relationship across cultivars of varying tiller size was considered a result of breeding efforts for greater yields in both small and large tiller cultivars. Possibly a better trait that breeders can select for would be rate of lamina regrowth after grazing (leaf elongation rates). Hatier *et al.* (2014) reports that leaf lamina regrowth after defoliation was the most significant trait contributing to dry matter production under moisture deficit conditions. However, Sartie, *et al.* (2009) reported that leaf elongation rates were also negatively correlated with tiller number. This might reflect the effect of greater leaf:non-leaf ratio and the consequent lack of tiller development.

Tiller number has presumed benefits for persistence. The more asexual production a plant has (in the form of daughter tillers) the greater its ability to survive as it outcompetes other plants nearby for resources. Whether the number of tillers is controlled by plant genetics or is a result of larger tillers with larger leaf areas preventing light from initiating production of new tillers is undetermined. (Soper & Mitchell, 1956; Simon & Lemaire, 1987; Bahmani *et al.*, 2003; Griffiths, *et al.*, 2016). Sartie *et al.* (2011) found that tiller number was different for two cultivars tested under glasshouse conditions. However Bahmani, *et al.* (2003) found that tiller densities declined in the autumn and winter of a grazed field trial, regardless of cultivar, nitrogen application or irrigation

treatment. Yet other studies have shown that nitrogen application can have a positive effect on tiller density (Jewiss, 1972; Simon & Lemaire, 1987; Bahmani, *et al.*, 2003). Larger leaved cultivars and increased tiller production in response to nitrogen are aspects of the pasture that can be easily manipulated through management practices, so perhaps tiller number is not a good candidate for persistence breeding. Further discussion into the interaction of these traits, the genetic control of these phenotypes, and their combined effect on persistence and yield would better enable prioritisation of objectives in enhancing persistence.

While the number of tillers produced appears to be influenced by light initiation, the ratio of reproductive tillers to vegetative tillers has long been observed in breeding programmes to be largely genetically controlled (Corkill *et al.*, 1981; Marshall & Wilkins, 2003). This ratio is considered indicative of the plants ability to survive vegetatively (Jewiss, 1972; Wilkins & Humphreys, 2003). Tillers that do not produce seed heads survive after the plant has flowered and set seed. A greater number of reproductive tillers also contributes to a loss in pasture quality as animals prefer not to graze these plants due to the high lignin content of seed heads (Woodfield & Easton, 2004). The breeders' conundrum with this particular trait is that selection for less reproductive tillers to vegetative tillers improves forage quality yields and possibly persistence in the field, but is negatively associated with seed production. Both are important for developing commercial cultivars as seed quantities dictate if the cultivar can be competitively priced on the market and used by pastoral farmers (Marshall & Wilkins, 2003; Woodfield & Easton, 2004).

1.4.2 Heading dates

One of the achievements of forage breeding in New Zealand has been to manipulate pasture quality by offering cultivars with different heading dates (Easton, *et al.*, 2002b). Heading date is defined as the day that five or more reproductive tillers with spikelets have emerged from their tiller sheaths on a plant. This trait has been categorised into three main groups. Cultivars are assigned to a group dependent on the number of days difference in heading date from the industry standard cultivar ‘Grasslands Nui’ which produces seed heads on approximately the 22nd of October in New Zealand (Williams *et al.*, 2007; Lee, *et al.*, 2012). A cultivar is considered as having an early heading date when seed heads are visible eight days before Grasslands Nui; Medium (or mid) heading dates are assigned to cultivars that have a similar heading date to Nui; and late heading dates describe cultivars with heading dates eight days or more after October 22nd. There are a number of commercially available cultivars in each of the heading date categories in New Zealand and they offer a range of seasonal patterns for yield and herbage quality. (DairyNZ, 2017).

Late heading date cultivars have provided farmers with higher quality pasture in spring, a crucial time for feed requirements in intensive dairy systems when milk production requires maximum utilisation of the available pasture (Laidlaw, 2004; O'Donovan & Delaby, 2005). While late heading date cultivars have proven repeatedly to be advantageous for animal productivity, there is some evidence to suggest these cultivars may not survive as long as cultivars with earlier heading dates. Chapman, *et al.* (2015a) investigated several cultivars representing different heading date categories in the Hawkes Bay in a long term data set. Years one through to eight of the data showed no significant effects of heading date on dry matter yields, but in year 10 a significant

relationship was observed between late heading date cultivars and lower dry matter yields. Laidlaw (2004) found similar results, where medium heading date cultivars had higher dry matter yields than late heading date cultivars in the second year. This may be because earlier heading date cultivars have shown higher tiller production earlier in spring when there is still moisture for growth. In contrast, late heading date cultivars increase tiller number later in the season when moisture stress may be present, and pasture grazing demand on dairy farms is high (Laidlaw, 2004; O'Donovan & Delaby, 2005; Lee, *et al.*, 2012). Farmers, agronomists and breeders may need to adjust management, and yield expectations to better accommodate earlier flowering cultivars in dry-spring farming areas to improve pasture persistence.

1.4.3 Endophyte compatibility

Stock grazing perennial ryegrass pastures sometimes suffer from 'ryegrass staggers' which impairs mobility and decreases production. Ryegrass staggers is caused by endophyte produced alkaloids and variation in alkaloid concentrations between cultivars has been observed (Aasen *et al.*, 1969; Fletcher & Harvey, 1981). Research led to the identification of a symbiotic relationship between alkaloid producing fungi (endophyte; *Epichloë festucae* var. *lolii*) and their grass host. While some alkaloids produced by some endophyte strains are toxic to animals, others do not affect animal health but protect the plant from insect herbivory. Endophyte strains that are safe for animals but offer protection against insects are commercially available in perennial ryegrass cultivars (Hume & Sewell, 2014).

The incorporation of non-toxic commercial strains of endophyte into elite cultivars has been advantageous to these cultivars and enables them to persist better than plants without endophyte (Johnson *et al.*, 2013a). Because the endophyte is intrinsic to perennial ryegrass's success as a pasture species, endophyte compatibility with the ryegrass host has become an important objective in breeding programmes (Easton, 1999; Easton *et al.*, 2001b). Depending on the endophyte strain, endophyte produces alkaloids (such as peramine) that deter some major insect pests such as argentine stem weevil, black beetle and pasture mealy bug (Hume & Sewell, 2014; Thom *et al.*, 2014). With less pest pressure on these grasses, endophyte infected cultivars consistently deliver superior pasture recovery and therefore survival after summer than plants without endophytes (Tozer, *et al.*, 2011; Thom, *et al.*, 2014).

Endophyte transmission (infection through seed) can be variable (Thom, *et al.*, 2014). Breeders need to ensure that elite breeding pools are highly compatible with commercial endophyte strains. Maintaining high levels of infection can mean there is a risk that valuable plant genetics are lost simply because these plants lack perfect compatibility with the available commercially important endophyte strains. The interaction between host and endophyte genotypes is not well understood (Müller & Krauss, 2005; Johnson *et al.*, 2013b), although there is evidence that the host/endophyte interaction can be manipulated to increase alkaloid production. Easton *et al.* (2002a) used a partial diallel cross to identify narrow sense heritabilities of >0.58 for alkaloid concentrations of ergovaline and peramine in 'Grasslands Nui'. Further investigation into the host/endophyte interaction and consequent alkaloid expression could explain why new cultivars may be less persistent than older cultivars with the same endophyte strains.

Manipulating alkaloid concentrations through breeding, if the high heritabilities for alkaloid concentrations recorded by Easton, *et al.* (2002a) are found in other cultivars, could be used to improve ryegrass persistence in the future.

1.4.4 Long term data sets

Identifying plant morphology that contributes to persistence has been limited by the relatively short duration of many trials; usually funding supports 18 months to 3 years of data collection which is considerably less than the expected 10 year survival of cultivars on New Zealand farms. Observing traits present in plants that have survived for many years on-farm should be informative about which phenotypes and associated traits survive long term. Because of the heterogeneous and heterozygous nature of perennial ryegrass, any cultivar purchased by a farmer contains a population of related but genetically diverse individuals. It is expected that over time individual plants that lack persistence in the variable environment on farm are outcompeted by genotypes that are more robust (Chapman *et al.*, 2011; Parsons, *et al.*, 2011; Chapman, *et al.*, 2015a). This may contribute to the establishment of permanent swards such as those seen in Europe (Hopkins *et al.*, 1990). In New Zealand, data from long term trials with well recorded management practices, have been difficult to obtain. Chapman, *et al.* (2015a) revisited a cultivar comparison trial site at the Poukawa research farm in Hawkes Bay, New Zealand that was conducted to provide insight on persistence of dry matter yield. Results showed that seven to eight years after the trial was established yield data significantly correlated ($r=0.7$) with data from years one to three, but in year 10 there was no significant correlation with the earlier data. This suggests some fundamental change, or threshold after about eight years. These long term trial sites offer an opportunity to study how persistent pastures differ phenotypically from newly sown pasture. By applying genetic markers to the

plants, better understanding of allele and genotype variations and changes within these populations would indicate if the population deviates from Hardy-Weinberg equilibrium (HWE) due to robust genotypes surviving. Hardy-Weinberg equilibrium occurs when allele frequencies are not affected by mutation, migration, assortive mating, natural selection, or population size (Falconer & Mackay, 1996; Selkoe & Toonen, 2006). Assessing possible changes in genetic variation from their original population using genetic markers could provide insights into the potential role of plant genetics in enhancing persistence.

1.5 Genetic Markers

Genetic markers are regions of the genome that can be used to identify and compare genetic variation of individuals, populations, and species. Genetic markers are located at specific positions (loci) within chromosomes but do not necessarily encompass functional genes. There are three main categories of genetic markers: morphological, biochemical and DNA (molecular) (Collard *et al.*, 2005). Morphological markers such as leaf marks in *Trifolium repens* are phenotypically expressed and can be used to determine parentage (Carnahan *et al.*, 1955) but are limited by the number of traits that are expressed and variation of expression in different environments. Biochemical markers use chemical assays for isozymes to detect allelic variations of protein products. Isozymes have been utilised by germplasm centres to identify genetically diverse accessions and aid breeders in selecting new material to introgress into breeding programmes (Hayward *et al.*, 1995). Biochemical markers are also limited by the number of available variants to each species, and have largely been superseded by DNA markers. DNA (molecular) markers make use

of polymerase chain reaction (PCR) methods to assess variation in the genome (Collard, *et al.*, 2005).

There are different types of molecular markers that can be used depending on the needs of the research. Amplified fragment length polymorphisms (AFLPs) use non-specific primers to assess genetic variation and are inexpensive. However using these markers to assess variation within a perennial ryegrass cultivar could be limited because they are dominant markers unable to differentiate between heterozygotes and homozygotes, and the methodology is complex requiring high quality DNA. Random amplified polymorphic DNA (RAPDs) like AFLPs are also identified using non-specific primers and are dominant markers. Their use in assessing variation of perennial ryegrass however has shown limitations in identifying differences between closely related populations (Huff, 1997; Collard, *et al.*, 2005; Selkoe & Toonen, 2006).

Microsatellite markers (SSRs) are tandem repeats of 1-6 nucleotides that are common in most organisms. Microsatellite loci vary in length between 5 and 40 repeats. Repeat motifs are commonly dinucleotide, trinucleotide, or tetranucleotide. The microsatellite repeat sequences are prone to mutation because of slippage during DNA replication, these mutant variants are detected through PCR with primers (can be species specific) that anneal to flanking regions around tandem repeats. Mutations can then be assessed and compared by the length of the amplified regions. SSRs can be located in coding or non-coding regions of the genome (Jensen *et al.*, 2007). Coding SSRs may be utilised as functional markers derived from expressed sequence tags (EST-SSR). Some coding SSRs have been used to construct linkage maps in perennial ryegrass for use in

potential marker assisted selection breeding programmes (Faville *et al.*, 2004; King *et al.*, 2008). Non-coding microsatellite allelic frequencies are co-dominant allowing for both heterozygotes and homozygotes to be detected, have high levels of intraspecific polymorphism when compared to coding SSRs, and are cheaply assayed by PCR (Collard, *et al.*, 2005; Selkoe & Toonen, 2006).

1.5.1 Microsatellites to assess genetic variation

The size and frequency of SSR polymorphisms detected across multiple loci can be used to assess genetic variation within a population. Allele frequencies can be used to evaluate a number of population characteristics including genetic relationships of individuals and population size, and population size changes (Leberg, 2002; Selkoe & Toonen, 2006).

One of the statistics used to assess genetic variation within a population is F_{IS} (Weir & Cockerham, 1984). F_{IS} identifies deviation from HWE through assessing levels of homozygosity within a population. Therefore F_{IS} estimates the extent of selection or inbreeding within a population. Observed frequencies of heterozygotes (H_O) are compared to expected heterozygosity (H_E) of individuals within the population assessed (Equation 1.5) (Weir & Cockerham, 1984). F_{IS} statistics range between -1 and 1. Larger values indicate a higher number of homozygotes than expected while smaller values indicate more heterozygotes within the population than expected.

$$F_{IS} = \frac{H_E - H_O}{H_E} \quad (1.5)$$

To compare between populations of the same species F_{ST} can be used. F_{ST} uses the expected heterozygosity within populations (H_{ES}) and compares it to the expected

heterozygosity among populations (H_{ET}) (Equation 1.6) (Weir & Cockerham, 1984). F_{ST} values range between 0 and 1. An F_{ST} value of 0 indicates that populations have no genetic differentiation while higher F_{ST} shows increasing genetic differentiation (Nei, 1977).

$$F_{ST} = \frac{H_{ET} - H_{ES}}{H_{ET}} \quad (1.6)$$

Using SSRs to assess intraspecific differences has been successful in perennial ryegrass (Kubik *et al.*, 2001; Wang *et al.*, 2009). Kubik, *et al.* (2001) used 22 SSR markers across 30 individuals from each of the seven cultivars studied. They found deviations between cultivars with pairwise F_{ST} values ranging from 0.065 to 0.435. Previous work on SSRs indicate that because of the highly heterogeneous nature of perennial ryegrass cultivars, greater diversity is identified within a cultivar than between cultivars (Wang, *et al.*, 2009). Microsatellite markers therefore could be a useful, cheap, and accurate method in identifying if populations of persistent perennial ryegrass sourced from long field sites are genetically different to the original cultivar sown, and clarify the effect that plant genetics has on persistence.

1.6 Focus of this research

Perennial ryegrass persistence is an important issue for New Zealand's agriculture, currently there is disparity on how plant morphology and plant genetics contribute to persistence in New Zealand. The primary aim of this thesis is to clarify the role of plant morphology and underlying genetics associated with persistence, by using plants sourced from a long term trial and their corresponding original cultivars.

Objective 1: Describe plant morphological differences within the cultivar 'Grasslands Samson' between Persistent plants and a sample from the Original cultivar in a glasshouse experiment.

Objective 2: Using the cultivars 'Grasslands Samson' and 'Commando', identify additive genetic variation within half-sibling family populations of Persistent and their corresponding Original cultivars for agronomically important traits in a row field trial.

Objective 3: Characterise genetic diversity within Persistent and Original populations of the cultivars 'Grasslands Samson' and 'Commando' using microsatellite markers to determine genetic variation within each population and between populations.

2.0 COMPARING PLANT MORPHOLOGY OF PERSISTENT RYEGRASS PLANTS TO PLANTS OF THE ORIGINAL CULTIVAR UNDER GLASSHOUSE CONDITIONS.

2.1 Abstract

Perennial ryegrass (*Lolium perenne* L.) persistence is an important trait for farmers in New Zealand. Past research in quantifying tiller morphology in pot trials has focussed on yield associated traits between cultivars. This study assessed variation in eight morphological traits within and between a persistent population of ‘Grasslands Samson’ collected from a long term field trial and plants of the original cultivar sourced from seed after 10 weeks growth in a glasshouse. Significant ($P<0.05$) differences were observed within each population for tiller number, lamina length, lamina width, lamina thickness, and lamina sheath length. The Persistent population had significantly ($P<0.05$) greater variation for dry weight than the Original population. Compared with the Original population, the Persistent population also had significantly ($P<0.05$) greater means for tiller number, reproductive tiller number, lamina sheath length, and dry weight. Similar trait associations were observed between populations in principal components analysis. These results indicate a genetic shift has occurred between the two populations assessed. This study provides some insight into what morphological traits may contribute to perennial ryegrass persistence.

2.2 Introduction

Past research quantifying tiller morphology has shown significant differences within and between perennial ryegrass cultivars in plant pot trials (Bahmani, *et al.*, 2000a; Hazard *et al.*, 2006; Sartie, *et al.*, 2009). This research however has focussed on impacts of water stress, climate, fertiliser input, grazing regimes and plant genetics contributing to dry matter yields with some suggestion as to their possible role in plant persistence (Hazard, *et al.*, 2006; Sartie, *et al.*, 2009; Wims *et al.*, 2010; Sartie, *et al.*, 2011; Tobi *et al.*, 2011; Hatier, *et al.*, 2014; Sanna *et al.*, 2014; Tozer *et al.*, 2014; Griffiths, *et al.*, 2016; Lee *et al.*, 2017). A relationship between persistence and greater tiller density has been suggested (Hume *et al.*, 2010; Tozer, *et al.*, 2014). The ability of this species to survive perennially is through the production of asexual tillers from the terminal apex of developed tillers. Plants that can produce more tillers are at an advantage when competing for resources in the pasture, and consequently surviving environmental fluctuations (Jewiss, 1972; Westoby, 1984; Parsons, *et al.*, 2011; Tozer, *et al.*, 2014). Associations between traits such as tiller number, reproductive tiller number, leaf length, leaf sheath length, and leaf width have been previously described through multivariate analysis (Sartie, *et al.*, 2011). Understanding these relationships provides plant breeders with insight into how selecting for a specific trait might affect a cultivars' overall mean trait expression.

Research trials focussing on quantifying plant morphology of plants that have persisted for longer than five years in New Zealand are rare. The opportunity to assess persistent plants, from a long term trial was identified by Chapman, *et al.* (2015a). This cultivar comparison trial was planted in 2004 and contained a range of cultivars. The broad variation within perennial ryegrass cultivars sown in this trial might have enabled

the mean expression of traits within a population to change under different environmental conditions (Parsons, *et al.*, 2011). Without reseeding, plants that have advantageous phenotypes for survival and growth could have outcompeted individuals with less advantageous phenotypes. Over time, the remaining population in this long term trial could have different mean expression of traits compared to the original population sown. The surviving population could therefore contain plants expressing phenotypes with traits contributing to improved persistence.

By quantifying tiller morphology of plants grown from commercially sourced seed of the original cultivar and persistent plants from this long term trial, we are testing the hypothesis that the genotypic mean of the persistent population has shifted over time. An opportunity to further understand the role of plant genetics and trait morphology is to determine if the Original population had greater genotypic variation than the Persistent population. Differences could indicate a directional shift towards advantageous phenotypes for persistence over time as less advantageous individuals die off in the field (Parsons, *et al.*, 2011; Tozer, *et al.*, 2014).

2.2.1 Objectives

In this study plants sourced from ‘Persistent’ (from (Chapman, *et al.*, 2015a)) and ‘Original’ (plants grown from a random sample of seed) populations of the cultivar Grasslands Samson were measured for seven morphological traits after 10 weeks growth in a glasshouse pot trial. The objectives of this study were to: (1) estimate genotypic variation within and between each population for the traits; (2) compare the mean for each trait between the two populations; (3) identify associations between traits and comparing possible changes in trait associations over time.

2.3 Materials and methods

2.3.1 Plant material

A total of 60 'Persistent' perennial ryegrass plants (*Lolium perenne* L.) of the AR1 endophyte infected cultivar 'Grasslands Samson' were collected in May 2014 from the long term trial established in April 2005 at Poukawa research station, Central Hawke's Bay (39°76'10"S, 176°72'73"E). Over the duration of this trial, the plots were rotationally grazed and topped after grazing which would not have allowed seed heads to set (Cashman, 2014; Chapman, *et al.*, 2015a). Single tillers were grown in the glasshouse for one week in sand trays to grow new roots. Rooted tillers were then transplanted into plastic two litre planter pots with 18 month Osmocote™ soil mix. Established plants were then placed outside.

At the same time, a random sample of seeds of the 'Original' cultivar 'Grasslands Samson' infected with the same endophyte strain (AR1) was sown into seed trays in the glasshouse establishing 60 plants. After four weeks of growth, plants with three or more tillers were transplanted into plastic two litre planter pots with 18 month Osmocote™ soil mix. Established plants were then placed outside.

All plants were maintained outdoors for two years with periodic trimming. From each population, 30 individual plants were randomly selected for the study. Each genotype had eight clonal tillers taken and planted into sterilised sand to allow growth of roots in July 2016. After seven days, five clones from each plant were removed from the sand and planted in pots with six month Osmocote™ soil mix.

2.3.2 Experimental design

The pot experiment was established in August 2016, in a glasshouse located at the Ruakura Research Centre, Hamilton, New Zealand (37° 77'44''S, 175° 30'87''E). A total of 300 plants (five replicates for each of 30 Persistent and 30 Original) were arranged in a randomised complete block design with five reps in a glasshouse. The average temperature of the glasshouse over the trial period was 17.4°C during the day and 11.5°C at night.

2.3.3 Measurements

After 10 weeks of growth, seven morphological traits were measured. For each plant the total number of surviving tillers (TN) was counted. A tiller was identified by the presence of pseudostem, with 3-4 leaves (Figure 2.1). Living tillers were identified as those that had pigment and at least two green leaves. Both reproductive and vegetative tillers were counted for total tiller number. Reproductive tillers (RT) were identified as tillers that had rounded sheaths, and nodes where the tiller had elongated to begin forming the seed head. The percentage of reproductive tillers (RP) was estimated by dividing the number of reproductive tillers (RT) by the total number of tillers (TN) and multiplying by 100 (Bahmani *et al.*, 2000b).

Three mature tillers, similar to the tiller depicted in Figure 2.1 were measured for leaf morphology measurements. Lamina length (LL) was measured on the oldest green lamina connected to the outmost sheath of the tiller, lamina were straightened before

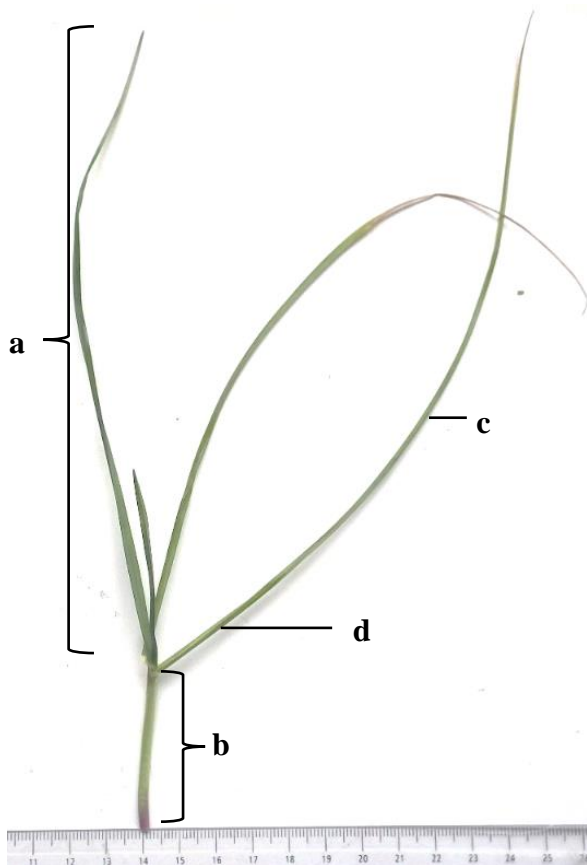


Figure 2.1; Leaf morphology of a perennial ryegrass tiller. Measurements were taken from three mature tillers of each plant. Data collected included a) lamina length; b) lamina sheath length, c) lamina width; and d) lamina thickness.

measurement. For each of the three tillers LL was measured as the distance (in centimetres) from the ligule to the lamina tip using a ruler (Figure 2.1a). Lamina sheath length (SL) was taken from the outermost sheath of the tiller (the oldest green lamina) and measured from the base of the tiller to the base of the lamina blade using a ruler (Figure 2.1b). Lamina width (LW) was taken at the midpoint of the length of the lamina. This was measured using a ruler to the nearest mm (Figure 2.1c). Digital Mitutoyo callipers (Mitutoya Corporation, Sakado, Japan) (3.d.p.) were closed around the lamina blade, avoiding the mid vein two centimetres from the ligule to measure lamina thickness (LT) (Figure 2.1d).

The above ground biomass of the plant was then harvested at soil level and dried in an oven at 80°C for 72 hours. Samples were weighed and dry weight (DW) data was recorded.

2.3.4 Data analysis

All morphological trait data were analysed using ANOVA (analysis of variance) in GenStat 18th edition (VSN, 2015). Data were analysed separately for each trait to provide means, least significant differences (*L.S.D*) and F pr values as tests of significance. Separate analysis were carried on each population to analyse within population variation for each trait. Traits which had multiple data points per plant such as lamina length, lamina sheath length, lamina width, and lamina thickness were analysed using replicate/sample number as block and plant genotype as treatment. Traits with only one measurement per plant such as total tiller number, number of reproductive tillers, percentage of reproductive tillers and dry weight were analysed by using replicate as block and plant genotype as treatments. All data were used from both populations to analyse between population variation. Traits which had multiple data points per plant were analysed using replicate/sample number as block and population/plant genotype as treatments. Traits with only one measurement per plant were analysed by using replicate as block and population/plant genotype as treatments.

To test the homogeneity of variance of the two populations for each trait, Bonett's test statistic was used in Minitab (Bonett, 2006; *Minitab 17 Statistical Software*, 2010). This test statistic compares the standard deviations from each population for a given trait by calculating the ratio. Significance of the ratio is determined at a 95% confidence interval. If the ratio of standard deviations between the populations for a given trait = 1 the null hypothesis is accepted, if the ratio $\neq 1$ the two populations have significantly different ($P < 0.05$) variation for the trait (Bonett, 2006). Results were considered significantly different if $P < 0.05$ for both ANOVA and Bonett's test statistic. Only traits

that showed significant ($P < 0.05$) variation within populations were further assessed in cluster analysis and principal components analysis (PCA).

Cluster analysis was conducted on the 30 plant-by-five trait mean matrix generated from the 'Persistent' and 'Original' populations. Clustering was based on the agglomerative hierarchical clustering procedure with squared Euclidean distance as a measure of dissimilarity and incremental sums of squares as a grouping strategy (Ward & Joe, 1963; Burr, 1968; Wishart, 1969; Burr, 1970). Prior to cluster analysis the mean values for each of the respective traits were standardised to have a mean of zero and a variance of one to remove scaling effects (Cooper & DeLacy, 1994). A 32-bit PC version of the Watson *et al.* (1995) GEBEI package was used to conduct the clustering. To decide on an optimum level of truncation for the hierarchy resulting from cluster analysis, the increase in the sum of squares among plant groups as the number of groups increased was investigated. The group level selected was determined by the point where the percentage of accession sum of squares among groups did not improve substantially as the number of groups increased (DeLacy, 1981).

The ordination technique of principal component analysis, using the algorithm of singular value decomposition was carried out using a 32-bit version of the program TUCKALS (using the Tucker3 model) by Kroonenberg (1994). The plotting points from the ordination were used to construct biplots (Gabriel, 1971). The biplots enable a graphical display of the variation present among 30 plants evaluated within the Persistent and Original populations and the relationships among the five traits.

2.4 Results

2.4.1 Within population variation

Analysis of variance (ANOVA) showed significant differences ($P>0.05$) (Table 2.1 & 2.2) within each population for total number of tillers, lamina length, lamina sheath length, lamina width and lamina thickness. Within each population number of reproductive tillers, reproductive tiller % and dry weight had no significant differences ($P>0.05$) (Table 2.1 & 2.2).

Table 2.1; Analysis of variance (ANOVA) for each trait within the Original population. Mean, range, $LSD_{0.05}$ and F_{pr} are presented.

| Trait | Mean | Range | L.S.D _{0.05} | F pr |
|--------------------------------|-------|---------------|-----------------------|--------------------------|
| Total number of tillers | 24.13 | 16.60 - 39.06 | 13.97 | 0.04 * _b |
| Number of reproductive tillers | 6.67 | 4.60 - 10.00 | 6.29 | 0.99 NS _a |
| Reproductive tiller (%) | 28.00 | 13.70 - 39.80 | 19.75 | 0.63 NS _a |
| Lamina length (cm) | 20.51 | 14.99 - 26.23 | 3.10 | < 0.001 *** _c |
| Lamina sheath length (cm) | 3.69 | 2.43 - 4.77 | 0.72 | < 0.001 *** _c |
| Lamina width (mm) | 3.58 | 2.75 - 4.50 | 0.42 | < 0.001 *** _c |
| Lamina thickness (mm) | 0.226 | 0.177 - 0.267 | 0.02 | < 0.001 *** _c |
| Dry weight (g) | 2.05 | 1.27 - 3.58 | 1.54 | 0.60 NS _a |

NS (not significant) = $P>0.05^a$; * = $P<0.05^b$; *** = $P<0.001^c$

Table 2.2; Analysis of variance (ANOVA) for each trait within the Persistent population. Mean, range, $LSD_{0.05}$ and F_{pr} are presented.

| Trait | Mean | Range | L.S.D _{0.05} | F pr |
|--------------------------------|-------|---------------|-----------------------|--------------------------|
| Total number of tillers | 26.72 | 13.20 - 44.60 | 14.90 | 0.01 ** _b |
| Number of reproductive tillers | 7.89 | 4.00 - 10.80 | 6.53 | 0.96 NS _a |
| Reproductive tiller (%) | 29.80 | 18.10 - 44.80 | 17.45 | 0.25 NS _a |
| Lamina length (cm) | 20.64 | 14.60 - 27.13 | 2.93 | < 0.001 *** _c |
| Lamina sheath length (cm) | 3.93 | 3.08 - 4.99 | 0.76 | < 0.001 *** _c |
| Lamina width (mm) | 3.65 | 3.01 - 4.17 | 0.39 | < 0.001 *** _c |
| Lamina thickness (mm) | 0.225 | 0.200 - 0.263 | 0.02 | < 0.001 *** _c |
| Dry weight (g) | 2.42 | 1.12 - 3.79 | 1.89 | 0.50 NS _a |

NS (not significant) = $P>0.05^a$; ** = $P<0.01^b$; *** = $P<0.001^c$

2.4.2 Multivariate trait analysis

Principal component (PC) analysis of the accession-by-trait best linear unbiased estimator (BLUE) mean matrices generated biplots (Figures 2.2 and 2.3) for each population. The correlation structure among the traits in each biplot is indicated by the directional vectors. The symbols in each biplot indicated the three plant groups generated from cluster analysis using the method described by DeLacy (1981).

2.4.2.1 Original population

The principal components analysis for the Original population showed principal components PC1 and PC 2 accounted for 38.7% and 32.7% of the variation respectively (Figure 2.2). The biplot showed a positive association (angles between the directional vectors are less than 90°) between the traits leaf thickness (LT) and leaf width (LW). Lamina length (LL), tiller number (TN), and sheath length (SL) also showed positive associations. Sheath length (SL) showed a negative association (angles between the directional vectors are more than 90°) with leaf thickness and leaf width (Figure 2.2).

Cluster analysis of the 30 genotypes generated three distinct groups within the Original population (Figure 2.2). The nine genotypes in Group 1 had the widest average lamina widths, largest lamina thickness, and shortest lamina sheath lengths (Table 2.3). Group 2 was the largest group with 14 genotypes. This group had the lowest average number of tillers, the shortest lamina lengths, smallest lamina thickness, and narrowest lamina widths (Table 2.3). Group 3 had seven genotypes. This group had the highest average tiller number, longest lamina lengths, and longest lamina sheath lengths (Table 2.3).

Table 2.3 Cluster analysis produced three groups in the Original population. Number of genotypes and means for tiller number (TN), lamina length (LL), lamina width (LW), lamina thickness (LT), and sheath length (SL) are presented.

| Group | No. genotypes | TN | LL | LW | LT | SL |
|-------|---------------|-------|-------|------|------|------|
| 1 | 9 | 24.56 | 20.05 | 3.85 | 0.25 | 3.35 |
| 2 | 14 | 21.86 | 19.43 | 3.38 | 0.21 | 3.54 |
| 3 | 7 | 28.11 | 23.24 | 3.62 | 0.22 | 4.44 |

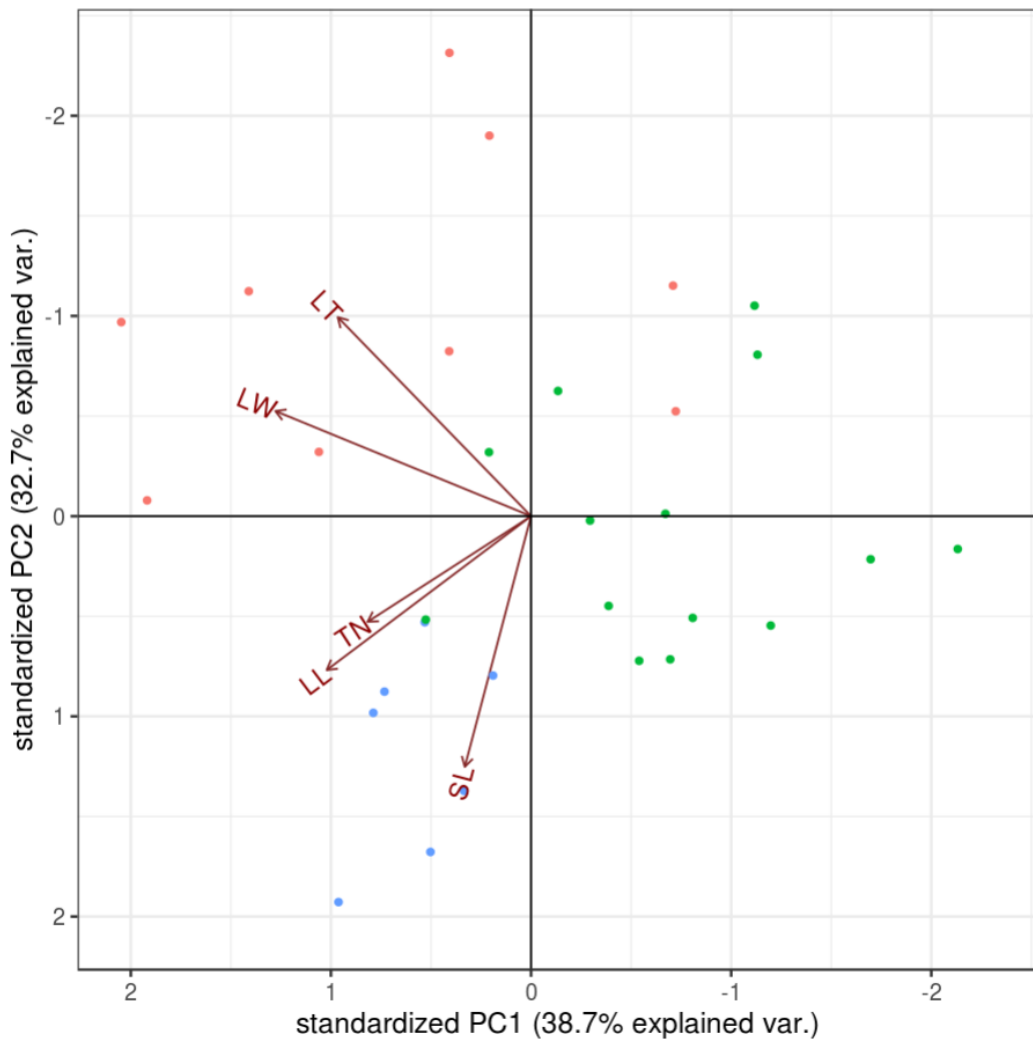


Figure 2.2 Biplot generated using standardised Best Linear Unbiased Estimate values for five traits measured from the 30 perennial ryegrass genotypes of the Original population. PC1 accounted for 38.7% and PC2 32.7% of the variation present. Traits are indicated by the directional vectors: Total number of tillers (TN), lamina length (LL), lamina sheath length (SL), lamina width (LW), and lamina thickness (LT). Colours indicate cluster groups: Red = Group 1; Green = Group 2; Blue = Group 3.

2.4.2.2 Persistent population

The Persistent population principal components analysis showed that PC1 accounted for 42.7% and PC2 27.4% of the variation (Figure 2.3). The biplot showed a positive association between the traits tiller number (TN), sheath length (SL), and lamina length (LL). Lamina thickness (LT) was positively associated with lamina width (LW) but negatively associated with sheath length and tiller number.

Cluster analysis of the 30 genotypes generated three distinct groups within the Persistent population (Figure 2.3). Group 1 consisted of four genotypes with the greatest average tiller number, longest lamina lengths, greatest average lamina thickness, and longest lamina sheath lengths (Table 2.4). The largest group identified was group 2 with 18 genotypes. This group was characterised by having the smallest average tiller number, widest lamina widths, and shortest lamina sheath lengths (Table 2.4). Group 3 had eight genotypes with the shortest lamina lengths, narrowest lamina widths, and smallest lamina thickness (Table 2.4).

Table 2.4 Cluster analysis identified three groups in the Persistent population. Number of genotypes and means for tiller number (TN), lamina length (LL), lamina width (LW), lamina thickness (LT), and lamina sheath length (SL) are presented.

| Group | No. genotypes | TN | LL | LW | LT | SL |
|-------|---------------|-------|-------|------|------|------|
| 1 | 4 | 37.90 | 25.35 | 0.22 | 3.89 | 4.74 |
| 2 | 18 | 24.98 | 20.70 | 0.23 | 3.75 | 3.77 |
| 3 | 8 | 25.05 | 18.16 | 0.21 | 3.28 | 3.88 |

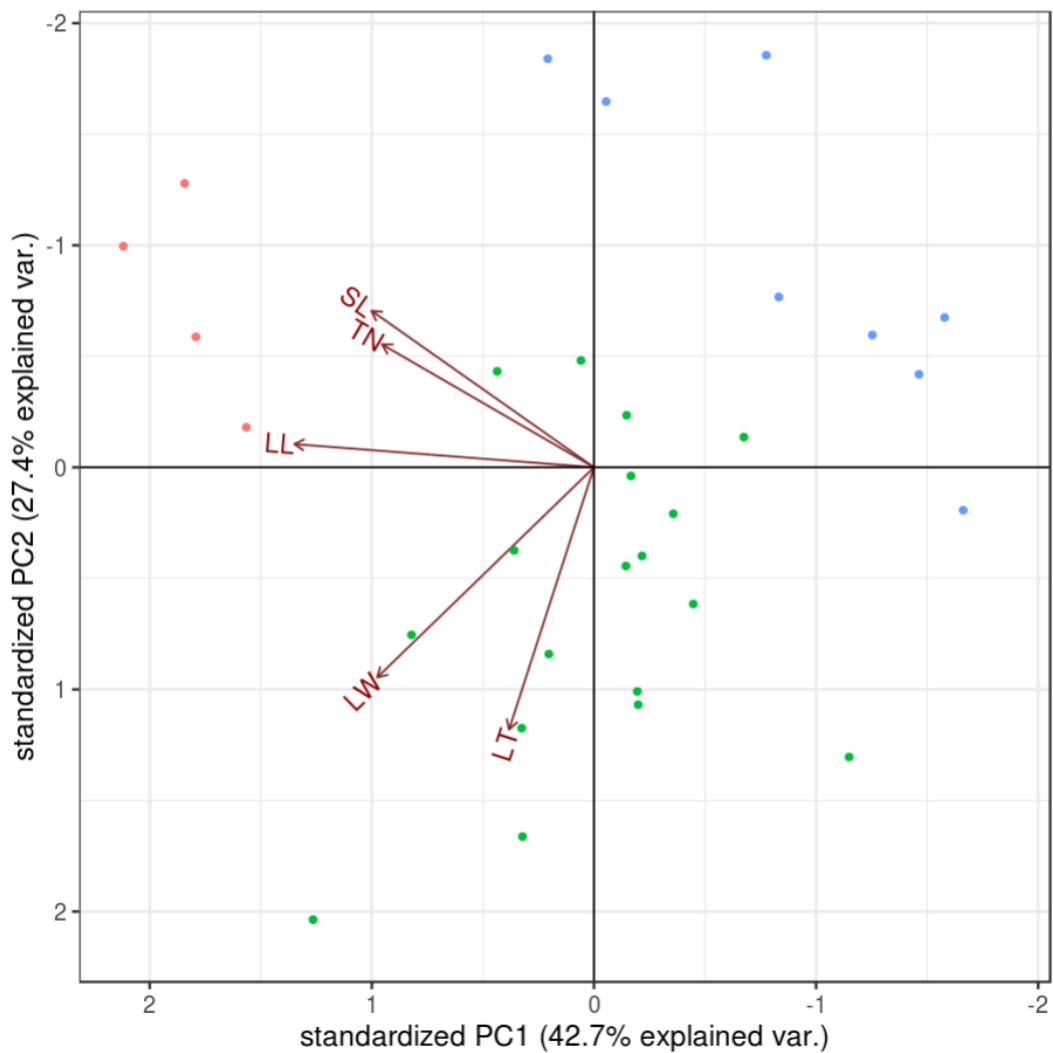


Figure 2.3 Biplot generated using standardised Best Linear Unbiased Estimate values for five traits measured from the 30 perennial ryegrass genotypes of the Persistent population. PC1 accounted for 42.7% and PC2 27.4% of the variation present. Traits are indicated by the directional vectors: Total number of tillers (TN), lamina length (LL), lamina sheath length (SL), lamina width (LW), and lamina thickness (LT). Colours indicate cluster groups: Red = Group 1; Green = Group 2; Blue = Group 3.

2.4.3 Between population variation

Plant morphology means for eight traits after 10 weeks growth in a glasshouse are presented in Table 2.5. Significant variation ($P < 0.05$) was observed between the Persistent and Original populations for four of the eight traits. Persistent population means were greater for total tiller number (+10.0%), number of reproductive tillers (+15.5%), lamina sheath length (+6.1%), and dry weight (+15.7%) when compared to Original population means. Reproductive tiller percentage, lamina length, lamina width, and lamina thickness were not significantly different ($P > 0.05$) between the two populations.

Bonett's test statistic showed that dry weight was the only trait found to have significant ($P < 0.05$) differences between the variation of the two populations. Plants in the Persistent population had a greater variation for dry weight than plants in the Original population (Figure 2.4h). All other traits showed non-significant ($P > 0.05$) variation differences between the populations.

Table 2.5; Mean values of the Persistent Original populations for of each of the eight traits measured. Least significant difference (L.S.D._{0.05}) between means and significance level (*F pr*) are presented.

| Trait | Persistent | Original | L.S.D. _{0.05} | <i>F pr</i> |
|--------------------------------|------------|----------|------------------------|-------------------------|
| Total number of tillers | 26.72 | 24.06 | 2.63 | 0.05 * _b |
| Number of reproductive tillers | 7.89 | 6.67 | 1.17 | 0.04 * _b |
| Reproductive tiller (%) | 29.80 | 28.02 | 3.38 | 0.30 NS _a |
| Lamina length (cm) | 20.64 | 20.51 | 0.55 | 0.63 NS _a |
| Lamina sheath length (cm) | 3.93 | 3.69 | 0.13 | <0.001 *** _c |
| Lamina width (mm) | 3.65 | 3.58 | 0.07 | 0.07 NS _a |
| Lamina thickness (mm) | 0.225 | 0.226 | 0.004 | 0.53 NS _a |
| Dry weight (g) | 2.42 | 2.04 | 0.31 | 0.02 * _b |

NS (not significant) = $P > 0.05$ ^a; *_b = $P < 0.05$; ***_c = $P < 0.001$

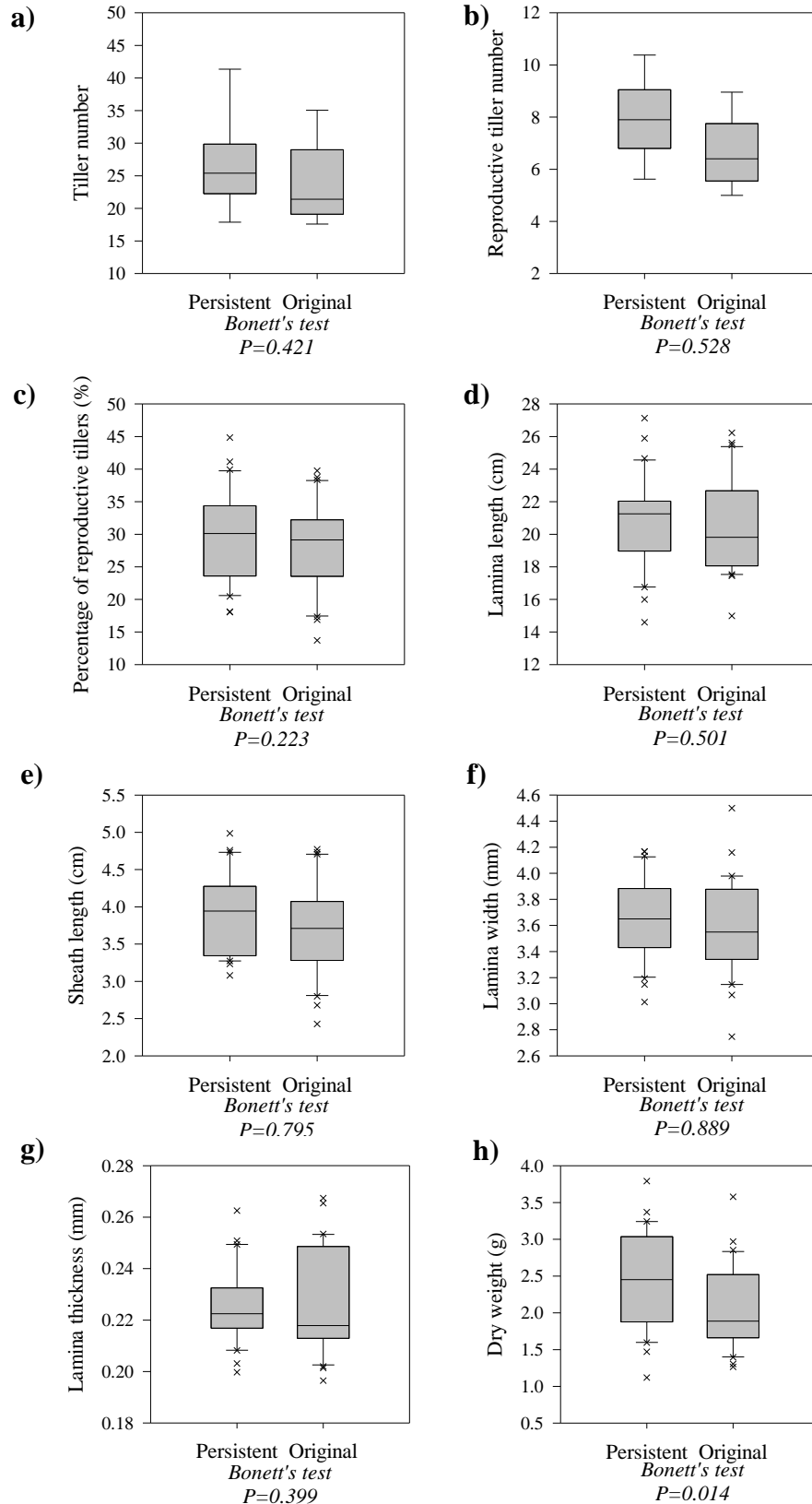


Figure 2.4; Box plots of each population for each trait. The traits presented are a) tiller number; b) reproductive tiller number; c) percentage of reproductive tillers; d) lamina length; e) sheath length; f) lamina width; g) lamina thickness; and h) dry weight.

2.5 Discussion

2.5.1 Persistent population means were significantly different ($P<0.05$) for four traits when compared to the Original population.

The main objective of this study was to describe and compare the morphological differences within the cultivar ‘Grasslands Samson’. Comparisons were made between a population of plants that had survived for nine years at Poukawa (Persistent plants) to a sample of plants recently grown from seed from the Original population. Previous plant morphology trials have focussed on identifying morphological differences between cultivars, with a focus on identifying traits associated with yield (Bahmani, *et al.*, 2003; Sartie, *et al.*, 2011; Griffiths, *et al.*, 2016). Significant differences ($P<0.05$) for the traits tiller number, number of reproductive tillers, lamina sheath length, and dry weight between the Persistent and Original populations indicate that a genetic shift could have occurred in the field over time at the Poukawa site. Differences between populations sourced from the same cultivar can occur as perennial ryegrass cultivars consist of related but heterogeneous and heterozygous individuals. Cultivar performance in the field is measured on the mean trait expression of all the individuals in the population, usually expressed as a normal distribution.

In the field sown with Samson at Poukawa in 2005, the persistent plants surviving over the years when other individuals die off could have caused the remaining population to shift from the original cultivars’ mean trait expression. Identifying significant ($P<0.05$) increases in the mean trait expression of the Persistent plants for four morphological traits shows that these attributes could be advantageous for persistence enabling those individuals to survive in the field.

2.5.2 Persistent plants had significantly ($P=0.05$) greater total tiller number compared to the Original population.

Previously, greater numbers of total tillers has been associated with a greater probability of survival (Lee, *et al.*, 2017). Each tiller contributes to an individual genotypes' survival as tillers store carbohydrate resources that can be accessed by the plant during resource deficiency or abiotic and biotic stress. Greater tiller number could also contribute to survival of an individual because by increasing the number of vegetatively propagating 'clones' and further spreading through the pasture to different areas in the field. More clones dispersing throughout the field would decrease the risk of death of the entire genotype from abiotic and biotic stresses, such as insect herbivory, pugging, and pulling by grazing animals within the micro-climate leading to better chances of survival.

Because of the short amount of time allowed for plants to establish in the glasshouse trial, it can be reasoned that the tiller number observed in the experiment would be associated with tiller appearance rate. The greater number of tillers observed in the Persistent population could indicate that it had greater tiller appearance rates than the Original population. Greater tiller appearance rate and tiller number would be advantageous as competition during establishment is high due to the large number of seeds sown to achieve dense sward establishment. During the establishment phase, rapid production of tillers is thought to be beneficial for outcompeting weeds and other individuals sown (Jewiss, 1972; Westoby, 1984). The persistent plants sourced from Poukawa were initially sown at a rate of 20 kg/ha (Chapman, *et al.*, 2015a). It is unlikely, at this rate of sowing that every individual would survive establishment. Lee, *et al.* (2017) has shown that at sowing rates of 18kg/ha survival of seed sown after seven weeks was 41-69%. Tiller appearance rate of the persistent plants would be advantageous for survival

and could have been selected for during the initial establishment of the pasture. The greater total tiller number observed in the Persistent population compared to the Original population therefore could have been influenced by the source of the tillers used in this experiment. The Original population was sourced from plants grown from individual seeds, not plants established in a pasture like the Persistent population. Therefore, the Original population had not undergone the same pressures for individual plants to survive establishment. This could be a confounding factor in our results as using these conclusions on tiller number/appearance rate and transferring similar objectives to the field in sown plots or rows may show no differences between persistent and other populations as they have then undergone the same establishment survival pressures.

While this trait is obviously advantageous at establishment, throughout the years and seasons a pasture is constantly re-establishing through propagation of vegetative tillers after pugging events, defoliation, and death of neighbouring plants. Being able to outcompete other individuals in the pasture for these spaces through faster propagation of tillers would increase the chances of survival for that individual. Therefore increased tiller appearance rates could be a beneficial objective to achieve improved plant persistence in breeding programmes. The main limitation for incorporating this trait is the difficulty in forming a methodology that is fast, accurate, repeatable, and practical for vast numbers of plants. Currently tiller number data is collected by individually counting tillers from each plant. Using photos and adopting software to identify and count individual tillers may offer some solutions to the impracticalities and errors encountered with collecting data on this trait.

2.5.3 Tiller number influenced reproductive tiller number

The results showed Persistent plants had significantly ($P=0.04$) greater reproductive tiller numbers than the Original population. However there were no significant ($P=0.30$) differences between the populations for percentage of reproductive tillers (Table 2.5). The ratio of reproductive tillers to vegetative tillers has been considered indicative of the plant's ability to survive perennially as vegetative tillers survive after reproductive tillers flower, set seed and die (Jewiss, 1972; Wilkins & Humphreys, 2003). There was no evidence from the reproductive tiller data for any variation in the plant's growth strategy in producing more reproductive tillers or earlier development of reproductive tillers. Non-significant differences for this trait between the two populations indicate that the populations have similar timing in initiating reproductive development, and that their heading dates would be similar.

2.5.4 Sheath length

Sheath length was positively associated with lamina length (Figures 2.2 and 2.3) for both populations. The positive association between these two traits has been reported previously (Sartie, *et al.*, 2009; Griffiths, *et al.*, 2016). When comparing the two population means, lamina length was not significantly ($P=0.63$) different between the two populations, but sheath length was significantly greater in the Persistent population ($P<0.001$). Despite the strong association of these two traits, Hazard *et al.* (1996) showed that selecting for lamina length in perennial ryegrass led to a disproportional response to lamina sheath length, indicating that the two are relatively independently controlled.

Sheath length was also positively associated with tiller number in both populations (Figures 2.2 and 2.3). Development of new tillers is stimulated by light initiating tiller development in the base of leaf axils, and is suppressed by shading (Westoby, 1984; Bahmani, *et al.*, 2000b). There is some evidence that sheath length contributes to the regulation of tiller production through shading the base where tiller buds develop in response to light (Davies *et al.*, 1983). In the results presented, plants with longer sheaths also had more total tillers. In the absence of leaf canopy closure, longer sheath lengths could suppress vegetative tiller growth for two main reasons: allocating carbon into the growth of reproductive tillers, and storing carbon in the onset of summer offering advantages for survival when abiotic stresses, such as drought, are more likely to occur.

Griffiths, *et al.* (2016) found no significant differences between cultivars for sheath length (pseudostem length) but these measurements occurred only during winter time when reproductive tillers were not developing. Investigating sheath length variation seasonally could affirm if sheath length is variable seasonally and whether it would impact the allocation of resources from vegetative propagation to reproductive tillers, but also to carbon sinks such as roots and pseudostems in preparation for the summer season. Seasonal sheath length could be a trait pursued in improving persistence in breeding programmes through regulation of tiller numbers.

2.5.5 Greater tiller numbers contributed to greater dry weights.

Association of tiller number with dry weights has previously been described in conjunction with size/density compensation where a greater number of tillers does not

always equate to greater dry weights when compared to larger sized tiller plants (Matthew *et al.*, 1995; Griffiths, *et al.*, 2016). In this study, plants were grown from a single tiller for 10 weeks under glass house conditions. This short time period between single tiller and data collection did not allow for canopy closure and size/density compensation was not applicable, therefore greater number of tillers lead to greater dry weights.

2.5.6 The Persistent population did not have less within population phenotypic variation

A hypothesis of this study was that Persistent plants would show less phenotypic variation for morphological traits measured. The reasoning was that after nine years in the field at Poukawa, only a proportion of plants sown with advantageous phenotypes would survive decreasing the variability of plant morphology observed (Parsons, *et al.*, 2011).

Bonett's test statistic showed that Persistent plants had significantly ($P < 0.05$) greater phenotypic variation for dry weights where the standard deviation ratio was not found to be 1:1 between the two populations (Figure 2.4h) (Bonett, 2006). It was also unexpected that other trait variations were non-significant between the two populations. Visual comparison of box plots show other traits with different lengths of box and whiskers such as tiller number and sheath length (Figure 2.4 a and e). These results show that a reduction in phenotypic variation was not observed in the Persistent population for the traits measured.

Within each population, significant ($P < 0.05$) differences among plants was observed for number of tillers, lamina length, lamina sheath length, lamina width, and

lamina thickness (Table 2.1 & 2.2). A comparatively small number of individuals were used in this trial to represent the entire genetic diversity of each population. From each population, 30 plants may not have been enough individuals to accurately represent the population distribution of a synthetic cultivar made up of heterogeneous and heterozygous individuals. Differences in genotypic variation between the two populations could not have been observed as reproduction at Poukawa did not occur because management of the field would not have allowed reproductive tillers to set seed. Therefore the spread of genotypic variation within this population might not have shifted as allele frequencies had not changed, or were not as detectable as the shifting mean of the Persistent population. By polycrossing the individuals sourced from Poukawa, the progeny could show genotypic variation within this proportion of plants have less variation compared to the Original cultivar.

2.6 Conclusions

Past research in quantifying tiller morphology has focussed on yield associated traits between cultivars rather than identifying trait differences in the portion of persistent plants surviving for many years in a pasture. In this trial, morphological differences were detected between the Persistent population and Original population of the cultivar 'Grasslands Samson'. Results show some evidence of a potential genetic shift taking place as Persistent plants had significantly ($P < 0.05$) greater means for four traits. Further investigation into sheath length and its role in regulating seasonal tiller number and appearance rate could be a possible objective for improving persistence. Increased total tiller number was probably a product of tiller appearance rate. Faster tiller appearance would be advantageous in the establishment phase of the pasture and the ongoing survival

of individual genotypes in the field. The Persistent population did not show reduced genotypic variation in traits measured compared to the Original population. However, the use of genetically structured half-sibling families generated from the Persistent and Original populations will provide an opportunity to estimate changes in the magnitude of additive genetic variation for the traits measured.

3.0 COMPARATIVE GENETIC ANALYSES OF THE PERSISTENT AND ORIGINAL POPULATIONS.

3.1 Abstract

This study aimed to identify any shift in the genetic mean of the cultivars ‘Commando’ and ‘Grasslands Samson’ sown in a long term trial at Poukawa research station, Hawkes Bay, New Zealand. A one metre row trial was assessed for 13 months to compare changes in additive genetic variation, narrow-sense heritability, and trait associations over time of half-sibling populations generated from ‘Persistent’ plants to those generated from commercially bought seed of the ‘Original’ cultivars ‘Grasslands Samson’ and ‘Commando’. Significant ($P < 0.05$) additive genetic variation and high levels of narrow sense heritability for some populations was identified for herbage growth scores, leaf width scores, plant habit scores, and aftermath heading. This study found that genetic shift over time was cultivar specific. Traits that were different between Persistent and Original populations have been previously associated with decreased pasture intake by animals. Trait associations and cluster analysis identified half-sibling families within Persistent populations that could be used for germplasm development for enhanced persistence.

3.2 Introduction

In perennial ryegrass (*Lolium perenne* L.) breeding programmes, one of the most important objectives is plant persistence. Persistence is a difficult trait for plant breeders to measure and select for because of complex interactions between plant genotype and the environment (Clark, 2011; Chapman, *et al.*, 2015b). There is also a lack of information on key plant traits associated with persistence due to the limited numbers of long term field trials (Lee, *et al.*, 2012; Tozer, *et al.*, 2014). Long term field trials are preferable in identifying the role of plant genetics in persistence as researchers can assess how changes in genetic diversity of sown populations shift over time (Parsons, *et al.*, 2011). Most plant breeding field trials last for 18 months to three years before selections are made, limiting the amount of environmental stresses that plants are assessed under, especially in grazed swards.

Results from short term trials suggest some trait associations with improved pasture persistence. There was significant positive association of ryegrass persistence with infection of novel strains of endophytic fungi (*Epichloë festucae* var. *lolii*) to deter insect herbivory (Easton, *et al.*, 2001b; Hume & Sewell, 2014) and disease resistance to crown rust (*Puccinia coronata* f.sp *lolii* Erikss) improving survival of asexual tillers (Potter, 1987; Muylle *et al.*, 2005; Easton *et al.*, 2011). Plants exhibiting prostrate (flatter) habits and narrower leaf widths also have been associated with persistence. Flatter plant habits avoid intense grazing pressures, as more erect habits are preferentially grazed by stock (Thom, 1991; Hazard *et al.*, 2001; Sampoux *et al.*, 2011; Cashman *et al.*, 2016). Timing of reproductive tiller development has been suggested to affect persistence. Earlier heading date cultivars have shown greater tiller production earlier in spring when

there is still moisture for growth. In contrast, later heading date cultivars increase tiller number later in the season when moisture stress is more likely to be present, and pasture grazing demand is high (Laidlaw, 2004; O'Donovan & Delaby, 2005; Lee, *et al.*, 2012). Further research is required before plant habit, earlier heading dates, and lower yielding plants can be conclusively associated with persistence.

Previous research on plants that survived in long term trials for more than seven years in grazed pasture were associated with less herbage growth and smaller plants (Hazard, *et al.*, 2001; Cashman, *et al.*, 2016). This association could be an issue for plant breeders and farmers because perennial ryegrass cultivars are improved through selection for higher herbage yields. However Chapman, *et al.* (2015a) has shown that plots of cultivars that were eight years old correlated significantly ($r=0.7$) with yield data from years one to three indicating that plants with good yield performance could still survive and persist as a pasture long term.

A long term trial sown in 2005 at Poukawa research station, Central Hawke's Bay has been used previously for studies in perennial ryegrass persistence (Easton, *et al.*, 2011; Cashman, 2014; Chapman, *et al.*, 2015a). Cashman (2014) retrieved plants from the Poukawa research trial in 2012 (seven years after trial establishment) and found no evidence in clonal spaced plants in Hamilton, New Zealand of a genetic shift between populations retrieved from Poukawa and plants grown from original seed. Though this study also did not identify genetic differences between cultivars which would have been expected as it included cultivars with different heading dates. The inability of Cashman (2014) to detect differences between cultivars may show results were confounded by non-

genetic factors. Chapman, *et al.* (2015a) used the same trial at Poukawa research station and identified significant ($P < 0.001$) differences of herbage dry matter yield between cultivars. Chapman, *et al.* (2015a) also found that herbage dry matter yields in years one to three correlated ($r = 0.7$) with herbage dry matter yields in years seven and eight, but not with yields in year ten ($r = 0.3$). The decreased correlation of herbage dry matter yield data in year ten could be an indication of a genetic shift occurring nine or 10 years after sowing.

Each cultivar population of perennial ryegrass sown at Poukawa research station would have had a range of genetic variation and trait expression. Ryegrass cultivars are genetically heterogeneous due to the obligate outcrossing nature of this species and use of recurrent selection breeding methods (Easton, *et al.*, 2002b; Thorogood *et al.*, 2002; Parsons, *et al.*, 2011; Sampoux, *et al.*, 2011; Lee, *et al.*, 2012). Over time, as plants with genetically enhanced persistence traits outcompete other plants this may result in a change of the population mean relative to the original population sown (Parsons, *et al.*, 2011; Tozer, *et al.*, 2014).

We hypothesise that over time (April 2005 to May 2014) cultivars at the long term field trial at Poukawa have undergone a genetic shift as genotypes expressing traits for better plant persistence would have outcompeted other genotypes. The shift in genetic diversity would be detected through lower levels of additive genetic variation within Persistent populations compared to the Original cultivar sown, and that Persistent plants may exhibit different values for traits associated with persistence.

3.2.1 Objectives

In this study, we focus on two cultivars ‘Grasslands Samson’ and ‘Commando’ that were planted in the long term field trial at Poukawa. Half-sibling families were generated from plants sampled from the ‘Persistent’ and ‘Original’ populations of the two cultivars. These half-sibling families were evaluated as one metre rows in a field trial. The objectives of this study were to: (1) estimate additive genetic variation and narrow sense heritabilities for each population of seven morphological traits; (2) compare the estimated additive genetic variation between the Persistent and Original populations using coefficients of additive variation to quantify shifts in genetic diversity; (3) graphically summarise the association among half-sibling families using pattern analysis within each of the populations for morphological traits.

3.3 Methods

3.3.1 Long term trial at Poukawa research station

The long term cultivar comparison trial (established in April 2005) at Poukawa Research Station, Central Hawke’s Bay, New Zealand (39°76’10”S, 176°72’73”E) was utilised as a source of plant material. The climate at Poukawa is characterised by warm temperatures and high sunshine hours in summer leading to high evapotranspiration rates and frequent occurrence of droughts (Chapman, *et al.*, 2015a). The long-term study was established with twelve cultivars with a range of heading dates, ploidy and endophyte strains. Diploid cultivars were sown as a monoculture at a rate of 20 kg ha⁻¹. Plots were arranged in a randomised split-plot design, with 4 replicates. The plots were rotationally grazed by sheep for about a day, every three to four weeks to residual height of three to four centimetres. The trial was then trimmed to approximately five centimetre height after

each grazing. During the summer, reproductive tillers were removed every three to four weeks through trimming to improve pasture quality and prevent cross pollination between plots. After each grazing, urea was applied at 3% - 5% of mean trial yield to replace nitrogen removed during defoliation (Cashman, 2014; Chapman, *et al.*, 2015a).

In May 2014 a total of 60 'Persistent' perennial ryegrass plants of AR1 endophyte infected cultivars 'Grasslands Samson' and 'Commando' were collected from the trial site. At the same time, 60 plants each of 'Grasslands Samson' and 'Commando' infected with AR1 endophyte were raised from a random sample of seed. These cultivars are classified as medium heading date, expected to flower on approximately the 22nd of October in New Zealand (Lee, *et al.*, 2012). Plant establishment from the Persistent tiller samples and the random seed samples from the Original samples are explained in Chapter 2. All plants were planted in two litre plastic planter bags containing a peat and sand mix with a three month slow release OsmocoteTM fertiliser. All 240 plants were grown outside and vernalised during winter 2014.

In the summer of 2014/15 plants were placed in four separate pollen exclusion tents (60 plants in each) (Figure 3.1) to allow polycrossing within each cultivar and within each age group producing four progeny populations: Samson Original, Samson Persistent, Commando Original, and Commando Persistent. Seed was harvested off each plant separately and kept as maternal half-sibling families. This resulted in 60 half-sibling families per polycross.



Figure 3.1; Four pollen exclusion tents were used to generate half-sibling families. Each population had 60 plants placed into a pollen exclusion tent in the summer of 2014/15.

3.3.2 Trial site

The trial was located at the AgResearch Grasslands Research Centre, Palmerston North, New Zealand (40°21'S, 175°37'E). Prior to establishment of the trial, the site was sprayed twice over a six month period with the herbicide Glyphosate 360 (360g/L isopropylamine salt) to remove all resident plants from the area, before being left fallow over the summer of 2015/2016. The soil type at the site is Kairanga silt loam (Cowie, 1974). Soil testing was done in January 2016 to 7.5 cm. depth. Results from Eurofins® showed: pH 5.7, calcium MAF QT 13, Olsen P 62 µg/mL, potassium 8 MAF QT, sulphate sulphur 9 ppm, magnesium 25 MAF QT, and sodium 5 MAF QT. Superphosphate fertilizer (150 kg ha⁻¹ [0-9-0-11; N-P-K-S]) was applied by broadcasting to provide optimum soil nutrient

levels. The soil was prepared for sowing by ploughing, harrowing and rolling. Information on the monthly climatic conditions at the trial site are presented in (Figure 3.2) Total annual rainfall during the period of this trial was 1347.9 mm.

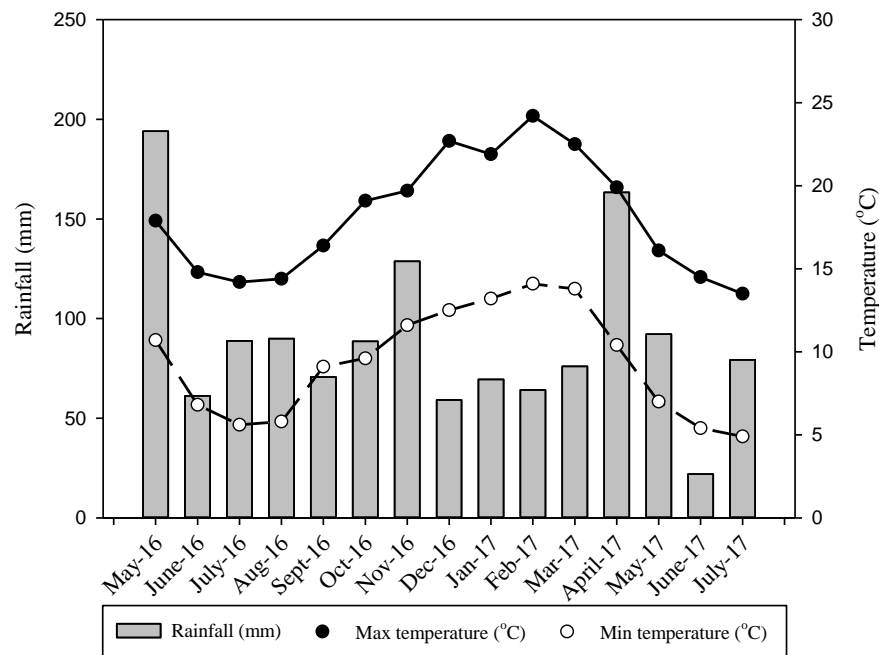


Figure 3.2; Monthly rainfall (mm) and maximum and mean monthly minimum air temperature at AgResearch Palmerston North. During the trial period May 2016-July 2017.

3.3.3 Experimental design

In autumn 2016, 30 half-sibling families were randomly sampled from each of the four populations. Each population of sampled half-sibling families were sown in a randomised complete block design with three replicates. Half-sibling families were sown as one metre rows at a rate of 18kg/ha (0.27 grams of seed per metre row), with 60 cm gaps between rows and 30 cm gaps between columns. There were 90 rows per population with a total of 360 rows in the field trial (Appendix 1). A row of the cultivar ‘Arrow’ was sown around the trial to reduce border effects.

3.3.4 Trial management

Following sowing, repeated hand weeding and inter-row herbicide application of Glyphosate 360 and Dicamba (Kamba® 500 at 800 mL ha⁻¹) was used to reduce weed growth between rows. Three applications of nitrogen fertiliser (as calcium ammonium nitrate [27-0-0-0; N-P-K-S]) were applied in June 2016, October 2016, and February 2017 at 30 kg/ha of nitrogen per application. After non-destructive visual score data collection, the trial was grazed (< 24 hours grazing time) by a flock of approximately 50 sheep. After grazing in the summer, rows were mowed to a height of 4cm to remove uneaten seed heads and equalise row heights (Figure 3.3).



Figure 3.3; After grazing, plants were mowed to 4cm to homogenise the height of the rows.

3.3.6 Data collection

The trial was left to establish for three months before any data was collected. Herbage growth score (HGs), leaf width score (LWs), and plant habit score (PHs) were recorded by visual assessment of each row prior to each defoliation (Figure 3.4).



Figure 3.4; One metre long half-sibling rows were visually scored for plant habit, leaf width, and herbage growth before each defoliation.

3.3.6.1 Herbage growth score

Visual herbage growth scores for each row were recorded on a 1 to 9 scale. All visually scored data allowed for 0.5 incremental units. Visual herbage growth scores were calibrated by collecting dry matter data from 27 rows (three rows representing each score) (Appendix 2a). Calibration data R^2 values ranged from 0.75 – 0.86.

3.3.6.2 Leaf width score

Visual leaf width scores for each plant were recorded on a 1 (very narrow) to 5 (very broad) scale. The scores were calibrated by measuring the width (mm) half-way

along the leaf lamina of a random sample of 10 leaves from 15 rows (Appendix 2b). Calibration data R^2 values ranged from 0.56 – 0.71.

3.3.6.3 Plant habit score

Visual plant habit scores for each plant were recorded on a 1 (flat) to 5 (erect) scale. The scores were not calibrated because of the difficulty in accurately measuring the angles of plant leaves under windy conditions.

3.3.6.4 Reproductive tiller development score

In mid-November 2016, reproductive tiller development (RTD) was scored visually using a 0 to 5 scale. The following details were used to inform scoring 0- no tiller elongation; 1 - some tillers elongated (Figure 3.5a); 2- most tillers elongated, tip of spikelet visible (Figure 3.5b); 3 - spikelets visible from elongated tillers (Figure 3.5c); 4 - spikelets fully emerged (Figure 3.5d), and 5 - spikelets flowering (anthers visible) (Figure 3.5e).

3.3.6.5 Aftermath heading score

In February 2017 aftermath heading (AMH), the re-growth of spikes after removal of reproductive stems (Sampoux, *et al.*, 2011), was visually assessed. These scores were recorded on a scale of 0 (no reproductive tillers visible) to 5 (reproductive tillers frequent throughout row).

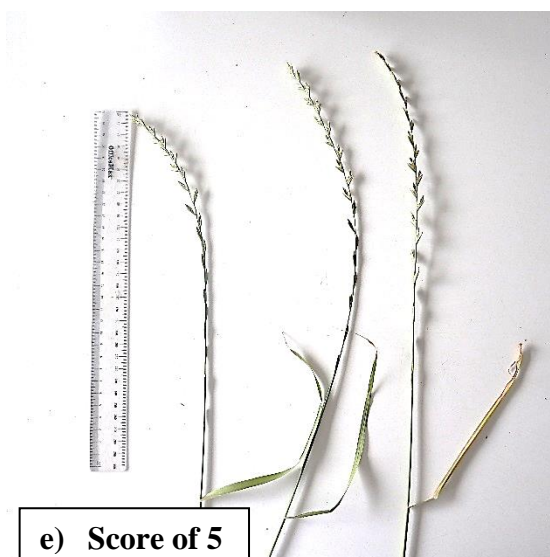


Figure 3.5; Reproductive tiller development (RTD) was scored mid November 2016.

Reproductive tillers were assessed on development stage.

a) Score of 1 - some tillers elongated;

b) Score of 2 - most tillers elongated, tip of spikelet visible;

c) Score of 3 - spikelets clearly visible from elongated tillers;

d) Score of 4 - spikelets fully emerged;

e) Score of 5 - spikelets fully emerged and flowering (anthers visible).

3.3.6.6 Rust score

Crown rust score (Ru) data was also collected in February 2017. Visual scores for rust infection were recorded on a scale of 0 (no visual signs of rust) to 9 (heavily infected with rust, plants mostly orange).

3.3.6.7 Dry weight

In October 2016 (spring), February 2017 (summer), and May 2017 (autumn), full dry matter cuts were harvested using electric shears to approximately four centimetres above the ground. The entire herbage biomass was harvested from each row and placed into perforated cellulose bags with individual labels (Figure 3.6). These bags were then dried in ovens at 80°C for 72 hours before dry weight (DWT) data was recorded for each row.



Figure 3.6; Summer dry weight harvested in February 2017. Each row was harvested with electric shears. All harvested herbage from each row were placed in separate perforated bags prior to drying.

3.3.7 Data analysis

3.3.7.1 Residual maximum likelihood analysis

Data analyses were based on variance component analysis to assess the significance and magnitude of genetic variation among half-sibling families within each of the four populations. The data were analysed using the variance component analysis procedure, Residual Maximum Likelihood (REML) option, in GENSTAT 18th edition (VSN, 2015). All analyses were carried out using mixed linear models (with season as fixed effect where applicable). The analyses generated variance components for the random variables with associated standard errors. Variance components were considered significant ($P < 0.05$) with 95% confidence intervals. Best Linear Unbiased Predictor (BLUP) (White & Hodge, 2013) values for each of the traits measured were also generated.

For reproductive tiller development, aftermath heading, and rust, the completely random linear model used was:

$$Y_{ijkl} = M + f_i + b_j + r_{jk} + c_{jl} + \varepsilon_{ijkl}, \quad (3.1)$$

Y_{ijkl} is the value of an attribute measured from half-sibling family i in row k and column l of replicate j and $i=1, \dots, n_f$, $j=1, \dots, n_b$, $k=1, \dots, n_r$, $l=1, \dots, n_c$, where f , b , r and c are half-sibling families, replicates, rows and columns, respectively; M is the overall mean; f_i is the random effect of half-sibling 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 within replicate j , $N(0, \sigma_r^2)$; c_{jl} is the random effect of column l within replicate j , $N(0, \sigma_c^2)$; ε_{ijkl} is the residual effect of half-sibling family i in row k and column l of replicate j , $N(0, \sigma_\varepsilon^2)$.

The mixed linear model used in the analysis of traits herbage growth score, plant habit score, leaf width score and dry weight was:

$$Y_{ijklm} = M + f_i + s_j + (fs)_{ij} + b_{jk} + r_{jkl} + c_{jklm} + \varepsilon_{ijklm}, \quad (3.2)$$

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

3.3.7.2 Narrow-sense heritability

Narrow-sense heritability (h^2_n) on a half-sibling mean basis was calculated for a trait within a population when additive genetic (σ_a^2) variation obtained from REML analysis was significant ($P < 0.05$). Narrow-sense heritability was estimated using Equation 3.3 (Falconer & Mackay, 1996). Additive (σ_a^2), additive-by-season interaction (σ_{as}^2), and experimental error (σ_ε^2) variance components obtained from REML analysis were used with the number of replications (n_r) and the number of seasons (n_s) where applicable. Standard errors of heritability were calculated using DeltaGen (v 0.02) (Jahufer & Luo, 2018).

$$h_n^2 = \frac{\sigma_a^2}{\sigma_a^2 + \frac{\sigma_{as}^2}{n_s} + \frac{\sigma_\varepsilon^2}{n_r n_s}} \quad (3.3)$$

3.3.7.3 Co-efficient of additive variation

The co-efficient of additive variation (CV_A ; Equation 3.4) was calculated from the additive (σ_a^2) variance component, and the trait population mean (\bar{x}) obtained from REML analysis. The co-efficient of additive variation for each trait was calculated to compare the genetic variation among the four populations.

$$CV_A (\%) = \frac{\sqrt{\sigma_a^2}}{\bar{x}} \times 100 \quad (3.4)$$

3.3.7.4 Cluster analysis

Prior to cluster analysis the mean value for each of the trait was standardised to have a mean of zero and a variance of one to remove scaling effects (Cooper & DeLacy, 1994). A 32-bit PC version of the Watson, *et al.* (1995) GEBEI package was used to conduct the clustering. To decide on an optimum level of truncation for the resulting hierarchy from cluster analysis, the increase in the sum of squares among plant groups as the number of groups increased was investigated. The group level selected was determined by the point where the percentage of accession sum of squares among groups did not improve substantially as the number of groups increased (DeLacy, 1981).

3.3.7.5 Principal components analysis

The ordination technique of principal component analysis, using the algorithm of singular value decomposition, was carried out using a 32-bit version of the program TUCKALS (using the Tucker3 model) by Kroonenberg (1994). The plotting points from

the ordination were used to construct biplots (Gabriel, 1971). The biplots enable a graphical display of the variation present among the 30 half-sibling families evaluated within the Persistent and Original populations and relationships among the traits.

3.4 Results

3.4.1 Additive variation

Residual maximum likelihood (REML) analysis generated BLUPs for each half-sibling family for each of the seven traits from the field trial conducted over 13 months of seasonal data collection. The components of additive variance (σ^2_a) for herbage growth score were significant ($P < 0.05$) within all populations. Half-sibling family mean narrow sense heritabilities (h^2_n) for herbage growth score were 0.66, 0.78, 0.66, and 0.66 for Commando Original, Commando Persistent, Samson Original, and Samson Persistent respectively. The Samson Original population had the largest co-efficient of additive variation (CV_A) at 12.10% and the Samson Persistent population had the smallest co-efficient of additive variation for this trait at 10.42% (Table 3.1).

Leaf width scores had significant ($P < 0.05$) additive variation within the Commando Original, Commando Persistent and Samson Original populations. Narrow sense heritabilities for leaf widths were 0.45, 0.57 and 0.67 respectively. The Commando Original population was the only population to have significant ($P < 0.05$) additive-by-season interaction. The co-efficient of additive variation for leaf width ranged from 6.90% to 9.54% (Table 3.1).

Additive variance was also significant ($P < 0.05$) for plant habit scores within all four populations. Half-sibling family mean narrow sense heritabilities for plant habit ranged from 0.48 to 0.72. The two Commando populations had CV_A values for the Original and Persistent populations of 5.22% and 5.45% respectively. The Samson populations had greater CV_A values for the Original and Persistent populations at 12.83% and 10.81% respectively (Table 3.1).

Significant ($P < 0.05$) additive variance was found for aftermath heading only within the Samson Persistent population with high levels of narrow sense heritability ($h^2_n = 0.69$). This population also had a high co-efficient of additive variation of 22.27% (Table 3.2).

No significant ($P > 0.05$) additive variance found within any of the populations for dry weight, reproductive tiller development, or rust score (Table 3.1 and 3.2).

Table 3.1; Means, ranges, estimated additive (σ^2_a), additive-by-season interaction (σ^2_{as}) and experimental error (σ^2_ϵ) variance components and their associated standard errors (\pm), co-efficient of additive variation (CV_A), and half-sibling family mean narrow sense heritability (h^2_n) estimated for the traits herbage growth, leaf width, plant habit and dry weight, among the half-sibling families within each of the four populations.

| Trait | Population | Mean | Range | σ^2_a | σ^2_{as} | σ^2_ϵ | CV_A (%) | h^2_n |
|----------------------|---------------------|-------|---------------|-------------------|-------------------|---------------------|---------------|-----------------|
| Herbage growth score | Commando Original | 5.65 | 5.23 - 6.19 | 0.45 \pm 0.19* | 0.20 \pm 0.18 | 2.19 \pm 0.23 | 11.87 | 0.66 \pm 0.11 |
| | Commando Persistent | 6.33 | 4.63 - 7.30 | 0.52 \pm 0.19* | 0 | 1.77 \pm 0.17 | 11.43 | 0.78 \pm 0.06 |
| | Samson Original | 5.87 | 4.87 - 7.01 | 0.51 \pm 0.22* | 0 | 3.18 \pm 0.31 | 12.10 | 0.66 \pm 0.10 |
| | Samson Persistent | 5.91 | 4.76 - 6.81 | 0.38 \pm 0.17* | 0 | 2.15 \pm 0.25 | 10.42 | 0.66 \pm 0.12 |
| Leaf width score | Commando Original | 2.79 | 2.44 - 3.19 | 0.04 \pm 0.02* | 0.08 \pm 0.03* | 0.31 \pm 0.03 | 6.90 | 0.45 \pm 0.17 |
| | Commando Persistent | 2.99 | 2.65 - 3.42 | 0.05 \pm 0.02* | 0.03 \pm 0.03 | 0.34 \pm 0.04 | 7.34 | 0.57 \pm 0.14 |
| | Samson Original | 3.08 | 2.52 - 3.73 | 0.09 \pm 0.04* | 0.05 \pm 0.03 | 0.37 \pm 0.04 | 9.54 | 0.67 \pm 0.10 |
| | Samson Persistent | 2.87 | 2.71 - 3.06 | 0.02 \pm 0.02 | 0 | 0.42 \pm 0.04 | N.S. | N.S. |
| Plant habit score | Commando Original | 3.33 | 2.95 - 3.57 | 0.03 \pm 0.01* | 0.03 \pm 0.02 | 0.27 \pm 0.02 | 5.22 | 0.61 \pm 0.11 |
| | Commando Persistent | 3.19 | 2.89 - 3.46 | 0.03 \pm 0.01* | 0.03 \pm 0.02 | 0.26 \pm 0.02 | 5.45 | 0.62 \pm 0.11 |
| | Samson Original | 3.16 | 2.50 - 3.90 | 0.17 \pm 0.06* | 0 | 1.14 \pm 0.08 | 12.83 | 0.72 \pm 0.07 |
| | Samson Persistent | 3.03 | 2.67 - 3.85 | 0.11 \pm 0.06* | 0.06 \pm 0.12 | 1.85 \pm 0.16 | 10.81 | 0.48 \pm 0.15 |
| Dry weight (g) | Commando Original | 74.73 | 64.17 - 88.92 | 46.79 \pm 33.60 | 23.20 \pm 39.82 | 378.20 \pm 44.40 | N.S. | N.S. |
| | Commando Persistent | 72.83 | 61.71 - 88.02 | 44.58 \pm 30.71 | 21.12 \pm 33.77 | 336.30 \pm 48.37 | N.S. | N.S. |
| | Samson Original | 73.2 | 71.66 - 74.83 | 7.63 \pm 22.65 | 0 | 338.90 \pm 47.50 | N.S. | N.S. |
| | Samson Persistent | 79.77 | 74.12 - 90.83 | 39.10 \pm 32.70 | 0 | 434.35 \pm 61.20 | N.S. | N.S. |

*Significance at $P < 0.05$; N.S. (not significant, $P > 0.05$)

Table 3.2; Means, ranges, estimated additive (σ^2_a) and experimental error (σ^2_ϵ) variance components and their associated standard errors (\pm), coefficient of additive variation (CV_A), and half-sibling family mean narrow sense heritability (h^2_n) estimated for the traits reproductive tiller development, aftermath heading, and rust among the half-sibling families within each of the four populations.

| Trait | Population | Mean | Range | σ^2_a | σ^2_ϵ | CV_A (%) | h^2_n |
|---------------------------------------|---------------------|------|-------------|------------------|---------------------|------------|-----------------|
| Reproductive tiller development score | Commando Original | 2.59 | 2.50 - 2.66 | 0.02 \pm 0.05 | 0.42 \pm 0.09 | N.S. | N.S. |
| | Commando Persistent | 2.99 | 2.51 - 3.43 | 0.10 \pm 0.06 | 0.35 \pm 0.07 | N.S. | N.S. |
| | Samson Original | 2.78 | 2.50 - 3.00 | 0.06 \pm 0.07 | 0.54 \pm 0.10 | N.S. | N.S. |
| | Samson Persistent | 2.43 | 2.43 - 2.44 | 0.00 \pm 0.04 | 0.37 \pm 0.08 | N.S. | N.S. |
| Aftermath heading score | Commando Original | 3.62 | 3.23 - 3.81 | 0.10 \pm 0.12 | 0.81 \pm 0.17 | N.S. | N.S. |
| | Commando Persistent | 3.62 | 3.54 - 3.70 | 0.02 \pm 0.11 | 0.88 \pm 0.18 | N.S. | N.S. |
| | Samson Original | 3.38 | 2.92 - 3.65 | 0.13 \pm 0.13 | 0.84 \pm 0.19 | N.S. | N.S. |
| | Samson Persistent | 2.97 | 1.86 - 3.91 | 0.44 \pm 0.18* | 0.60 \pm 0.14 | 22.27 | 0.69 \pm 0.11 |
| Rust score | Commando Original | 5.53 | 5.08 - 5.95 | 0.26 \pm 0.26 | 1.77 \pm 0.36 | N.S. | N.S. |
| | Commando Persistent | 5.53 | 5.32 - 5.80 | 0.11 \pm 0.25 | 1.93 \pm 0.39 | N.S. | N.S. |
| | Samson Original | 5.11 | 5.05 - 5.18 | 0.03 \pm 0.25 | 2.08 \pm 0.45 | N.S. | N.S. |
| | Samson Persistent | 5.28 | 5.06 - 5.46 | 0.08 \pm 0.25 | 1.86 \pm 0.42 | N.S. | N.S. |

*Significance at $P < 0.05$; N.S. (not significant, $P > 0.05$)

3.4.2 Trait associations and cluster analysis

Principal component analysis (PCA) of the 30 half-sibling family-by-trait BLUP adjusted mean matrices generated biplots (Figures 3.7-3.10) for each population. The correlation structure among the traits in each biplot is indicated by the directional vectors. The symbols in each biplot indicated groupings generated from cluster analysis.

3.4.2.1 Commando Original

The Original population principal components (PC) analysis showed that PC1 and PC2 accounted for 49% and 29% of the variation respectively (Figure 3.7). The biplot showed a strong positive association (angles between the directional vectors are less than 90°) between leaf width (LWs), and plant habit (PHs), and herbage growth score (HGs).

Cluster analysis of the 30 half-sibling families revealed four distinct groups within this population with no overlap (Figure 3.7). Group 1 had the smallest number of half-sibling families. These families had the widest leaf scores and most erect plant habits (Table 3.3). Group 2 had seven half-sibling families. These half-sibling families had below average leaf width scores, and herbage growth scores, but above average plant habit scores (Table 3.3). Group 3 contained eight half-sibling families and was characterised by having the narrowest leaf widths, and most prostrate plant habits of the population (Table 3.3). Group 4 was the biggest group with 13 half-sibling families. This group had average leaf width scores and plant habit scores, but had the greatest herbage growth scores of the population (Table 3.3).

Table 3.3; Cluster analysis produced four groups in the Commando Original population. Number of half-sibling families (HS) means for leaf width score (LWs), plant habit score (PHs) and herbage growth score (HG) for each group are presented.

| Group | No. of HS families | LWs | PHs | HGs |
|-------|--------------------|------|------|------|
| 1 | 2 | 3.18 | 3.57 | 5.45 |
| 2 | 7 | 2.73 | 3.37 | 5.44 |
| 3 | 8 | 2.65 | 3.13 | 5.51 |
| 4 | 13 | 2.84 | 3.39 | 5.89 |

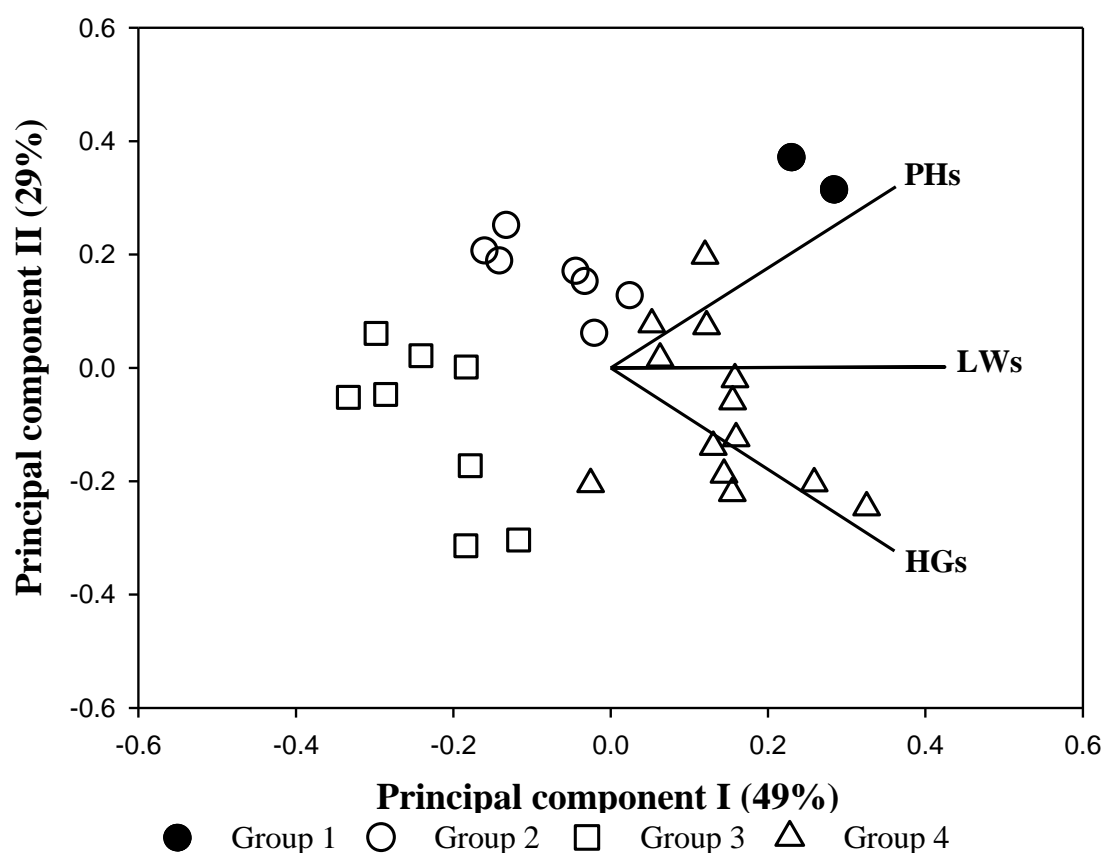


Figure 3.7; Biplots generated using standardised Best Linear Unbiased Predictor values for three traits from the 30 half-sibling families of the Commando Original population. PC1 accounted for 49% and PC2 29% of the variation present. The different symbols indicate groups 1 to 4 generated from cluster analysis. Traits are indicated by the directional vectors: herbage growth (HG), leaf width score (LW), and plant habit score (PH).

3.4.2.2 Commando Persistent

Principal components analysis for the Commando Persistent population showed that PC1 accounted for 53% of the variation and PC2 29% (Figure 3.8). The biplot shows strong positive associations between plant habit (PHs), leaf width (LWs); and herbage growth score (HGs).

Cluster analysis on the 30 half-sibling families identified four groups with no overlap (Figure 3.8). Group1 had six half-sibling families, this group had the widest leaf width scores, most erect plant habits, and the greatest herbage growth scores of the population (Table 3.4). The nine half-sibling families in group 2 had below average leaf width scores, the most prostrate plant habits, and below average herbage growth scores (Table 3.4). Group 3 had the smallest number of half-sibling families. These five half-sibling families had the narrowest leaf widths, above average plant habit scores, and lowest herbage growth scores (Table 3.4). The largest group in this population was Group 4 with 10 half-sibling families. This group was characterised by above average leaf width scores, more prostrate plant habit, and above average herbage growth (Table 3.4).

Table 3.4; Cluster analysis produced four groups in the Commando Persistent population. Number of half-sibling (HS) families for each group and means for leaf width score (LWs), plant habit score (PHs), and herbage growth score (HGs) for each group are presented.

| Group | No. HS families | LWs | PHs | HGs |
|-------|-----------------|------|------|------|
| 1 | 6 | 3.19 | 3.39 | 6.73 |
| 2 | 9 | 2.87 | 3.02 | 6.39 |
| 3 | 5 | 2.82 | 3.27 | 5.33 |
| 4 | 10 | 3.06 | 3.19 | 6.53 |

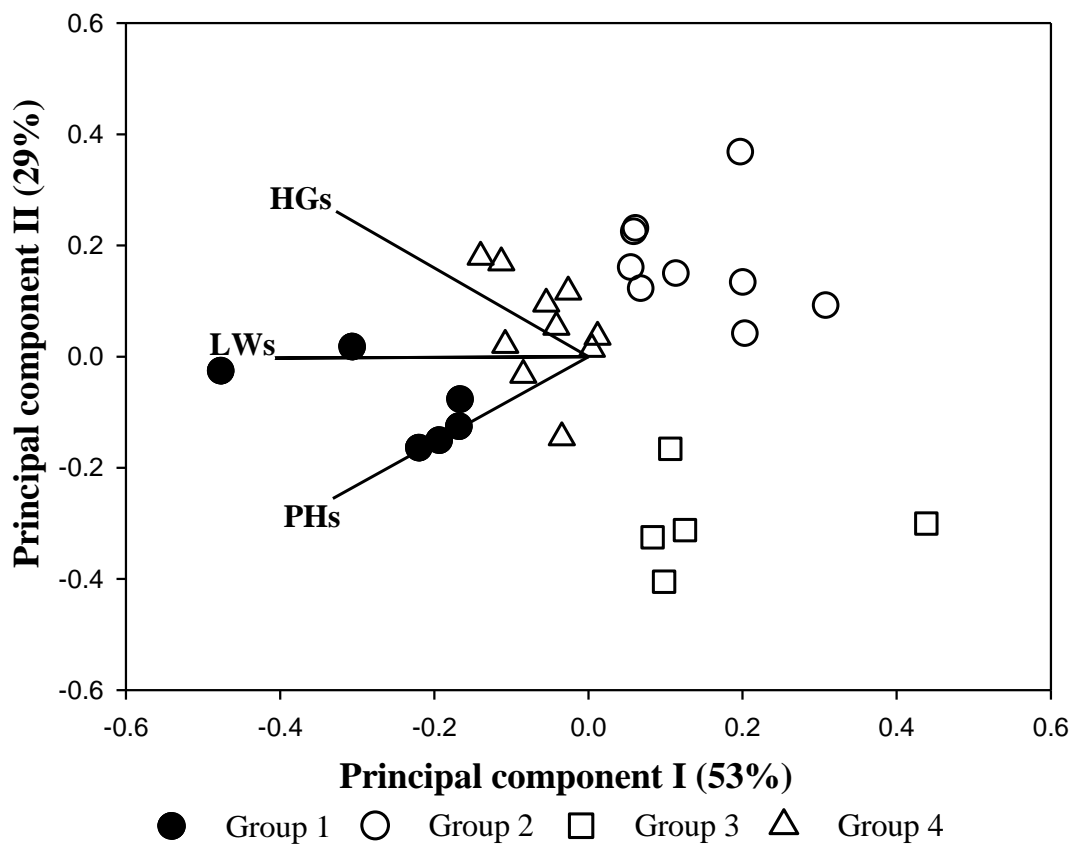


Figure 3.8; Biplots generated using standardised Best Linear Unbiased Predictor values for three traits from the 30 half-sibling families of the Commando Persistent population. PC1 accounted for 53% and PC2 29% of the variation present. The different symbols indicate groups 1 to 4 generated from cluster analysis. Traits are indicated by the directional vectors: herbage growth (HG), leaf width score (LW), and plant habit score (PH).

3.4.2.3 Samson Original

The Samson Original population principal components analysis showed PC1 and PC2 accounted for 51% and 33% of the variation respectively (Figure 3.9). The biplot showed a strong positive association (angles between the directional vectors are less than 90°) between plant habit (PHs) and herbage growth score (HGs). Leaf width (LWs) was negatively associated (angles between the directional vectors are more than 90°) with plant habit (PHs).

Cluster analysis of the 30 half-sibling families revealed three groups (Figure 3.9). Ten half-sibling families were clustered in group 1. This group had the widest leaf width scores, most prostrate plant habits, and the lowest herbage growth (Table 3.5). Group 2 had the largest group with 13 half-sibling families, these half-sibling families had the narrowest leaf widths, and average values for plant habit and herbage growth. The smallest group was group 3. The seven half-sibling families in this group had the most erect plant habits and the highest herbage growth scores of this population (Table 3.5).

Table 3.5; Cluster analysis produced three groups in the Samson Original population. Number of half-sibling (HS) families for each group and means for leaf width score (LWs), plant habit score (PHs), and herbage growth score (HGs) for each group are presented.

| Group | No. HS families | LWs | PHs | HGs |
|-------|-----------------|------|------|------|
| 1 | 10 | 3.23 | 2.90 | 5.36 |
| 2 | 13 | 2.92 | 3.15 | 5.94 |
| 3 | 7 | 3.17 | 3.57 | 6.47 |

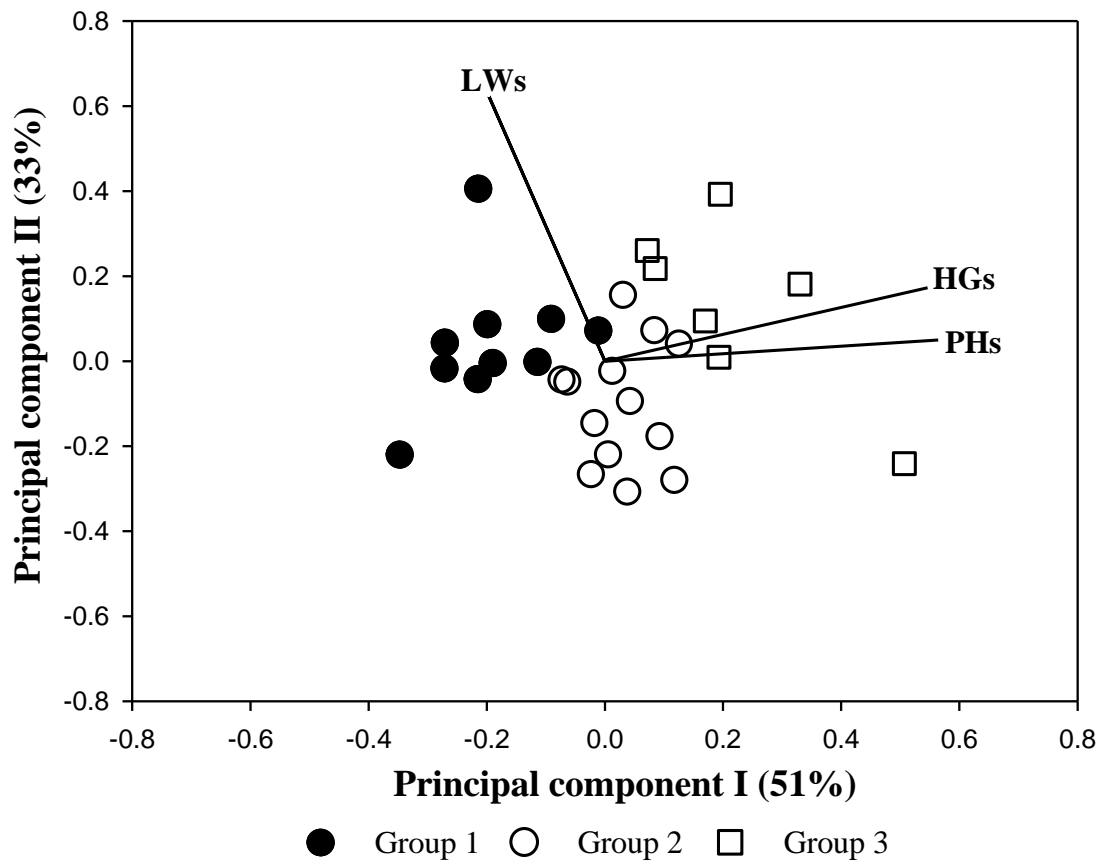


Figure 3.9; Biplots generated using standardised Best Linear Unbiased Predictor values for three traits from the 30 half-sibling families of the Samson Original population. PC1 accounted for 51% and PC2 33% of the variation present. The different symbols indicate groups 1 to 3 generated from cluster analysis. Traits are indicated by the directional vectors: herbage growth (HG), leaf width score (LW), and plant habit score (PH).

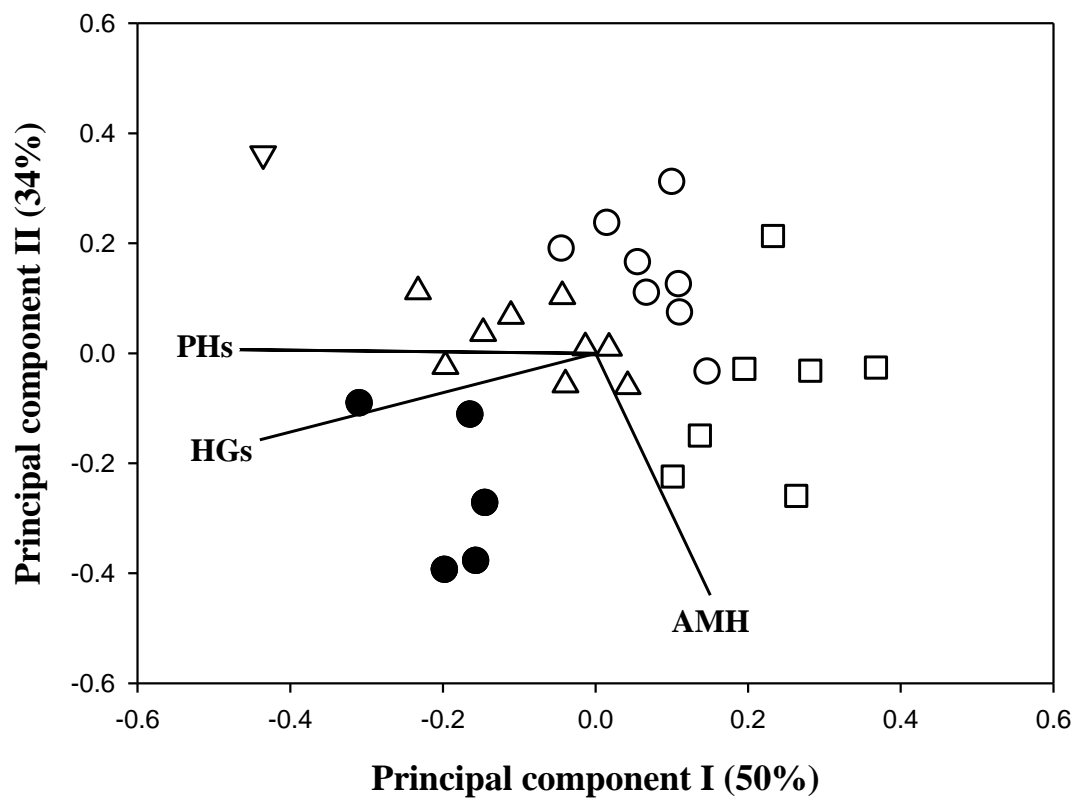
3.4.2.4 Samson Persistent

Principal components analysis for the Samson Persistent population showed that PC1 accounted for 50% of the variation and PC2 34% of the variation (Figure 3.10). A strong positive association was observed between plant habit score (PHs) and herbage growth score (HGs). Aftermath heading (AMH) was negatively associated with plant habit score and herbage growth score.

Cluster analysis on the 30 half-sibling families identified four distinct groups, and an individual half-sibling family outlier (Figure 3.10). Group 1 consisted of five half-sibling families, this group was characterised by having above average plant habit scores, the greatest average aftermath heading score, and the greatest average herbage growth score (Table 3.6). Group 2 had eight half-sibling families. This group had the most prostrate plant habits, below average aftermath heading, and below average herbage growth (Table 3.6). The seven half-sibling families in group 3 had more prostrate plant habit, above average aftermath heading, and the lowest herbage growth scores (Table 3.6). The largest group was group 4. The nine half-sibling families in this group were characterised by having average plant habit, average aftermath heading but above average herbage growth (Table 3.6). Individual 5 was an outlier in this population, this half-sibling family had the most erect plant habit, but the lowest after math heading and average herbage growth scores for this population (Table 3.6).

Table 3.6; Cluster analysis produced five groups in the Samson Persistent population. Number of half-sibling (HS) families for each group and means for plant habit score (PHs), and aftermath heading (AMH), and herbage growth score (HG) for each group are presented.

| Group | No. HS families | PHs | AMH | HGs |
|-------|-----------------|------|------|------|
| 1 | 5 | 3.26 | 3.50 | 6.54 |
| 2 | 8 | 2.85 | 2.54 | 5.80 |
| 3 | 7 | 2.86 | 3.42 | 5.30 |
| 4 | 9 | 3.09 | 2.82 | 6.12 |
| 5 | 1 | 3.85 | 1.86 | 5.94 |



● Group 1 ○ Group 2 □ Group 3 △ Group 4 ▽ Individual 5

Figure 3.10; Biplots generated using standardised Best Linear Unbiased Predictor values for five traits from the 30 half-sibling families of the Samson Persistent population. PC1 accounted for 50% and PC2 34% of the variation present. The different symbols indicate groups 1 to 5 generated from cluster analysis. Traits are indicated by the directional vectors: herbage growth (HG), plant habit score (PH), and aftermath heading (AMH).

3.5 Discussion

3.5.1 Genetic variation

A hypothesis of this study was that a genetic shift would be identified by smaller co-efficients of additive variation in Persistent half-sibling family populations when compared to their Original cultivars. Smaller co-efficients of additive variation were expected in Persistent populations as these half-sibling families were generated from the plants that survived at Poukawa, presumably representing a subset of what was originally sown. Results showed genetic shifts observed in Persistent populations were cultivar specific. The Samson Persistent population had smaller co-efficients of additive variation compared to its Original population for herbage growth and plant habit; but had a larger co-efficient of additive variation for aftermath heading (Tables 3.1 and 3.2). In contrast the Commando Persistent population had a similar co-efficient of additive variation for herbage growth score and larger co-efficient of additive variation for leaf width score when compared to the Commando Original population (Table 3.1). Our half-sibling family rows may not show smaller co-efficients of additive variation because of the vast amount of genetic diversity present in synthetic cultivars. These results may also be limited by having only 13 months of seasonal data. Because pastures change throughout seasons and years it would enhance data sets and may give higher resolution results if another year of seasonal data would have been included. This data would allow year and season interactions to be included in REML analysis to assess genetic variance. Particularly for further trials assessing and comparing genetic differences in persistent populations expected to survive throughout many seasons and years.

Differences between cultivars were also identified in the results. Plant habit score showed both Samson populations had twice the co-efficient of additive variation when compared to the Commando populations (Table 3.1) indicating plant habit had been selected for during the development of Commando to be more erect, whereas Samson does not seem to have undergone as stringent selection for this trait. Trait associations were also different between the two cultivars. Figures 3.9 and 3.10 show Samson populations had positive associations between plant habit score and herbage growth score with more acute angles between directional vectors when compared to the Commando populations (Figures 3.7 and 3.8). Differences between cultivars corresponds with results reported by Cashman, *et al.* (2016) who also found that directional selection of persistent plants varied between cultivars, suggesting that some plant populations were more robust and less subject to directional selection than others under specific environments. Trait association differences between Samson and Commando were probably caused by the different breeding pools from which they were developed. Commando was developed from Northland dairy farm ecotypes introgressed into germplasm sourced from overseas (Griffiths, *et al.*, 2016), whereas Samson was developed from a collection of plants collected from dry sites in New Zealand throughout the North Island, Marlborough and Canterbury (GrasslandsResearchCentre, 2001). Different breeding pools and priorities for selection criteria could eventuate in distinct trait associations.

3.5.2 Plant habit and leaf widths

Compared to the Samson Original population, Samson Persistent half-sibling families had a smaller co-efficient of additive variation for plant habit. The Original population also had significant ($P < 0.05$) additive genetic variation for leaf widths, but the Persistent population had non-significant ($P > 0.05$) variation for this trait. These results indicate that

the Persistent population underwent a genetic shift towards uniform expression of plant habit and leaf widths (Table 3.1). However, different results were found within the Commando populations. The Commando Persistent half-sibling families had a greater coefficient of additive variation for leaf widths compared to the Commando Original population. Both populations had similar levels of genetic variation for plant habit (Table 3.1). These results indicate that these traits did not undergo a genetic shift for this cultivar.

The association between leaf width score and plant habit score was cultivar dependent. Both Commando populations showed positive associations between the directional vectors of plant habit score and leaf width score (Figures 3.7 and 3.8) whereas the Samson Original population showed a negative association with half-sibling families with wider leaves had more prostrate plant habit (Figure 3.9, Table 3.5). Prostrate plant habits with narrower leaf widths have been previously associated with persistence of perennial ryegrass because of its role in decreasing grazing damage (Harvey *et al.*, 2000). Thom (1991), Hazard, *et al.* (2001), Sampoux, *et al.* (2011), and Cashman, *et al.* (2016) have previously suggested that persistent plants with more prostrate habits avoid intense grazing pressures, while larger leaved plants with more erect habits have been shown to be preferentially grazed by animals.

At Poukawa research station, preferential grazing of more erect plants could have occurred as the parent plants of the Samson Persistent population were rotationally grazed by sheep (Cashman, 2014). Plants that were more prostrate could have avoided some grazing damage as more erect plants were preferentially grazed (Harvey, *et al.*, 2000).

Avoiding some grazing damage would be advantageous during water stress in the summer dry environment, allowing prostrate plants to survive and outcompete more erect plants.

Medium to high levels of narrow-sense family mean heritability for plant habit among half-sibling families was identified amongst all populations (Table 3.1). This trait could be further incorporated into breeding programmes enhancing persistence through selecting for more prostrate habits. However, further investigation into the role of different plant habits (flat compared to erect) and its association with other economically important traits such as herbage growth, rust tolerance, and pasture intake by animals is necessary before setting objectives to select for more prostrate plants to enhance persistence. If prostrate plants do contribute to persistence because of avoidance of animal grazing, this could also mean that selecting for persistence would be detrimental to animal production.

3.5.3 Reproductive traits

All populations had non-significant ($P>0.05$) variation for reproductive tiller development, but Samson Persistent population had unexpected significant ($P<0.05$) additive genetic variation with high levels of narrow sense heritability ($h^2_n= 0.69$) and a large co-efficient of additive variation of 22.27% for aftermath heading (Table 3.2) compared to Samson Original population which had non-significant ($P>0.05$) variation (Table 3.2).

Although earlier heading dates have been previously linked with persistent plants (Laidlaw, 2004; O'Donovan & Delaby, 2005; Lee, *et al.*, 2012), and high levels of

heritability ($h^2 = 0.60 > 0.80$) have been previously estimated in diverse breeding populations (DairyAustralia, 2011; Barre *et al.*), the Samson Persistent population evaluated in this study was not expected to have significant additive genetic variation for aftermath heading. Low variation was expected because the parents of the Persistent half-sibling families were sourced from cultivars. Cultivars typically exhibit uniform expression of heading dates and low levels of aftermath heading because these traits are key objectives for plant breeders. Variation within a commercial cultivar for heading dates would be problematic for farmers as it increases the period of time reproductive tillers are produced decreasing pasture quality over a longer period of time. Reproductive tillers reduce pasture quality as these tillers contain high quantities of structural carbohydrates and vascular tissue making these tillers less palatable for animals, reducing pasture intake (Stone, 1994; Ravel *et al.*, 1995; DairyAustralia, 2011; Sampoux, *et al.*, 2011; Hendriks *et al.*, 2016). Uniform expression of reproductive traits is also important for cultivar development since cultivars are marketed according to their ‘heading date’ indicating what month in spring or summer farmers can expect pasture quality to deteriorate. Therefore in the cultivars Samson and Commando, reproductive tiller development and aftermath heading were not expected to have significant variation, even in the Persistent populations.

There are two explanations for high levels of variation for aftermath heading in the Samson Persistent population. Firstly, these results could show that the parent plants of the Persistent half-sibling families might not have been the original plants sown at the Poukawa research farm in 2005 but rather plants that have reseeded. At the trial site at Poukawa research station, there was a range of cultivars representing early, mid, and late heading dates (Chapman, *et al.*, 2015a). Over the course of nine years, contamination of

the plots is likely to have occurred to some extent. Burggraaf and Thom (2000) found contamination levels of perennial ryegrass seedlings in plots after three years (and herbicide treatment) ranged from 25% to 58%. Introduction of other plants with different heading date genetics could have occurred through neighbouring plots, seed in animal faeces, or seed germination from the soil.

Alternatively, parent plants collected at Poukawa research station were the individuals sown in 2005 that had survived until 2014. These individuals that survived had genetic variation for heading dates resulting in the mean of the population for this trait shifting towards non-uniform heading dates. A genetic shift away from the heading date of most individuals in the plot could be advantageous because these plants were producing reproductive tillers while other plants were not. Because the site at Poukawa research station was rotationally grazed every three to four weeks in spite of heading date (Chapman, *et al.*, 2015a) producing reproductive tillers before or between mid and late heading date cultivars could be advantageous. The advantage would be presence of less palatable reproductive tillers during grazing therefore these plants would be avoided by animals enabling persistent plants to survive and outcompete plants with the expected heading date.

3.5.4 Herbage growth scores

Past characterisation of persistent perennial ryegrass has associated ‘smaller’ plant morphologies with lower herbage yields in persistent plants (Hazard, *et al.*, 2001; Sanna, *et al.*, 2014; Cashman, *et al.*, 2016). Our results however showed Persistent populations expressed similar and greater mean levels of dry weight and herbage growth scores

compared to their Original populations. Herbage growth scores had medium to high levels of family mean narrow sense heritability for Commando Original ($h^2_n = 0.66$), Commando Persistent ($h^2_n = 0.78$), Samson Original ($h^2_n = 0.66$) and Samson Persistent ($h^2_n = 0.68$) populations (Table 3.1). Plants therefore that were sourced from the long term trial at Poukawa had not survived through selective pressures for lower herbage growth.

3.5.5 Trait associations and cluster analyses

Genetic shifts and trait associations identified for some traits were cultivar specific, implying these cultivars did not shift towards similar phenotype expression after surviving at Poukawa (Figures 3.7-3.10).

Trait associations did not appear to change for Commando populations with similar vector angles between plant habit score, herbage growth score, and leaf width score in both PCA analyses (Figures 3.7 and 3.8). Further, the number of cluster groups identified did not change between these populations (Figures 3.7 and 3.8, Tables 3.3 and 3.4). This may indicate that the Commando population surviving in Poukawa did not differ substantially from the overall mean of the Original population sown. Therefore survival of Commando plants at Poukawa was not enhanced by advantageous phenotypes. These results may relate to the development of this cultivar for the seed industry. Stability of cultivar performance during large scale seed production is important for obtaining plant variety rights (Plant Variety Rights Act, 1987). Genotypes in this cultivar would have been polycrossed in the field during seed production, and the resulting progeny would be required to show uniform phenotypes by industry before becoming commercially

available. Therefore, our results showing mostly uniform trait associations could be a result of prioritising uniform phenotypes during cultivar development. For further persistence research, the progeny from the Commando cultivar may not offer insight into genetic factors contributing to improved persistence.

For both Samson populations, herbage growth score and plant habit score showed strong positive associations between vectors (Figures 3.9 and 3.10). The Original population showed leaf width scores were negatively associated with herbage growth and plant habit (Figure 3.9), whereas the Persistent population showed aftermath heading was negatively associated with these traits (Figure 3.10). A genetic shift was identified in Samson due to differences in aftermath heading scores and leaf width scores between populations. Further, the Original population had less cluster groups identified than the Persistent population (Figures 3.9 and 3.10, Tables 3.5 and 3.6). These results imply the half-sibling progeny of Samson Persistent could be used to further study genetic factors associated with persistence.

Two options are suggested in using the five cluster groups identified in Persistent half-sibling families for future work. The first, is selection of half-sibling families associated with traits linked to persistence. Herbage growth score, plant habit score, and aftermath heading were found to have medium to high estimates of narrow sense heritability conveying the opportunity for genetic gains in these traits with further breeding (Table 3.1 and 3.2). Selecting half-sibling families with more prostrate plant habits (grazing avoidance), high herbage growth, and low aftermath heading would further germplasm advancement. Although greater aftermath heading scores were

identified as possibly contributing to grazing tolerance and improved persistence, it is not advised this trait be promoted for cultivar development. Low levels of aftermath heading are preferable for forage cultivars as it greatly improves seasonal yield and quality (Soper & Mitchell, 1956; Wilkins, 1991; Lee, *et al.*, 2012). Cluster analysis identified groups 2 and 4 had half-sibling families with above average herbage growth score, the most prostrate plant habits, and below average aftermath heading for half-sibling families within the Samson Persistent population (Figure 3.10, Table 3.6.). Polycrossing these 17 half-sibling families, and assessing progeny using multi-site field trials would be beneficial in developing a breeding pool for introgressing enhanced persistence into elite lines.

The second option for further research would be to use plants from both Samson Persistent and Original half-sibling families to further characterise the genetic effect on persistent phenotypes under different environments. The process would involve extracting tillers from each half-sibling family and growing clonal plants. Establishing clonal plants from both populations of each genotype into two space plant field trials in different environments: low stress environment with optimum conditions, and a high stress dry environment. Collection of two to three years of seasonal morphological data on traits of interest such as plant habit score, aftermath heading, herbage growth score, tiller density/appearance rate, sheath lengths, heading date, and aftermath heading. Analyse and compare the populations of the clonal phenotypes between the sites, seasons and years. This trial would further characterise genetic and environmental interaction of these traits and identify how persistent phenotypes change under different environments. Results would inform plant breeders if field assessments of breeding lines need to be

under stressed conditions to accurately identify underlying genetics of desirable phenotypes attributed to improved persistence.

3.6 Conclusions

This study estimated additive genetic variation, family mean narrow-sense heritability, and co-efficients of additive genetic variation of half-sibling families generated from Persistent plants and half-sibling families generated from seed of the Original cultivars ‘Grasslands Samson’ and ‘Commando’. These results showed that genetic shifts over time were cultivar specific suggesting that the cultivar Commando was less subject to directional selection. Genetic shifts identified within the Samson cultivar suggests persistent populations shifted towards traits that have previously been associated with animal grazing avoidance improving survival of plants in the field. Groups of half-sibling families with more prostrate plant habits, low aftermath heading, and high herbage growth were identified through trait association and cluster analysis as potential germplasm to enhance persistence.

4.0 COMPARING ORIGINAL AND PERSISTENT POPULATION GENETICS USING SSR MARKERS.

4.1 Abstract

Perennial ryegrass persistence is an important trait, however identifying genetic components of persistence is difficult because of the lack of long term data sets. This study aimed to identify a shift in the genetic mean of the cultivars ‘Commando’ and ‘Grasslands Samson’ through comparing the allele frequencies for simple sequence repeat (SSR) marker loci between plants extracted from a long term trial at Poukawa research station, Hawkes Bay, New Zealand and comparing them to plants grown from seed of the ‘Original’ cultivars ‘Grasslands Samson’ and ‘Commando’. SSR data was then analysed in GenAEx to generate population genetic statistics such as F_{IS} and F_{ST} to detect genetic mean shifts in these populations. Genetic structure was analysed using STRUCTURE 2.3.4, and principal co-ordinate analysis. Results of this study were confounded by late detection of contamination in the samples. Analysis of data was therefore restricted to smaller sample sizes than planned for in the methodology. Our results showed populations had values close to 0 for F_{IS} and F_{ST} . Genetic structure also showed that the four populations were indiscernible when analysed together.

4.2 Introduction

Cultivars of perennial ryegrass are usually developed through recurrent selection breeding methods whereby a base population is continually improved through inter-pollination of elite parents (Falconer & Mackay, 1996). Improvement of traits is measured through comparing the mean performance of the breeding pool phenotypes to cultivars on the market in different geographical locations in New Zealand. Currently, it takes 10 to 15 years to develop a cultivar. Cultivars developed through recurrent selection are classified as ‘synthetic’, consisting of related but heterogeneous and heterozygous individuals (Easton, *et al.*, 2002b; Thorogood, *et al.*, 2002). These methods enable the improvement of the base population through selecting elite parents for complex traits such as vegetative persistence that are controlled by multiple loci (quantitative trait loci, QTL) (Acquaah, 2009) and are influenced by the environment.

Vegetative persistence through the survival of asexually produced daughter tillers is an important objective for ryegrass breeders in cultivar development. Much of the success of perennial ryegrass as an agricultural species has been attributed to its’ ability to survive in the field over many years while maintaining dry matter yield (Parsons, *et al.*, 2011; Chapman, *et al.*, 2015b). However, intensification of land use and changes to establishment and grazing practises has added pressure for improving persistence. Pasture persistence results from a complex relationship between plant genotype and environment (Clark, 2011; Chapman, *et al.*, 2015b). Synthetic cultivars comprised of heterozygous and heterogeneous individuals contain plants with a wide spectrum of performance. The genetic variation available in synthetic cultivars may allow the population to further shift mean performance once sown in the field. Over time, individual plants that lack persistence in the varying perennial environment on farm may be survived by genotypes

that are more robust (Chapman, *et al.*, 2011; Parsons, *et al.*, 2011; Chapman, *et al.*, 2015a).

Utilising a long-term trial located at Poukawa research station, Hawkes Bay, presents an opportunity to improve understanding of the role of plant genetics in perennial ryegrass vegetative persistence (Chapman, *et al.*, 2015a), identifying if the population has been exposed to further directional selection after being sown in the field. Using population genetics to assess the range of genetic diversity within plants surviving in these plots and comparing them to plants grown from their original cultivar seed could identify if the genetic mean has shifted over nine years.

Population genetics is the study of the genetic composition within and between populations. This includes assessing distribution and changes in the frequencies of alleles expected under Hardy-Weinberg equilibrium (HWE) (Christiansen & Feldman, 1986). HWE assumes within a freely interbreeding population, allele variation reaches an equilibrium when a population's allele frequencies are not affected by the following factors: mutation, migration, assortative mating, natural selection, or population size (Falconer & Mackay, 1996; Selkoe & Toonen, 2006). Deviation from HWE can occur when allele frequencies are affected by one of these factors altering allele frequencies within a gene pool. Detecting allele frequency changes within cultivars is difficult through phenotyping because of the confounding effects of the environment on phenotype in the field. Therefore identifying genetic variation at specific loci through using molecular markers such as simple sequence repeats (SSR, microsatellite markers) could be a practical method in identifying allele frequency changes within a cultivar.

SSR markers are suitable for detecting genetic diversity in population genetic studies because they are prone to frequent mutation caused by slippage and DNA replication errors. As a result, SSR markers are usually multi-allelic and co-dominant. Mutant alleles are detected through PCR (polymerase chain reaction) amplification with species specific primers that anneal to flanking regions around tandem repeats. Mutated variants are then assessed and compared by length of amplified regions at specific loci (Jensen, *et al.*, 2007). Population genetic parameters, such as number of alleles, number of allele variants, proportion of shared alleles and F statistics (fixation indexes) can then be used to assess the variation of SSR polymorphism and allele frequency.

The F statistic F_{IS} can be used to assess genetic diversity and characterise variation within the population. F_{IS} estimates the differences between expected heterozygosity (H_E) and observed heterozygosity (H_O) within the population (Weir & Cockerham, 1984). Larger F_{IS} values indicate a higher number of homozygotes than expected while negative values indicate more heterozygotes within a population than expected. Previous perennial ryegrass research using microsatellite markers has detected significant deviations between populations. Barth *et al.* (2017) used eight SSR markers to screen 928 individuals from 40 diploid populations including ecotype and cultivar accessions from Ireland and Europe. They found that most of the accessions deviated from Hardy–Weinberg equilibrium through homozygote excess and had relatively high inbreeding co-efficients (F_{IS} ranged between 0.14 – 0.58). When a population has not been significantly affected by these events, genotype frequencies at any locus are dependent on allele frequencies (Selkoe & Toonen, 2006).

To compare genetic diversity between populations the F_{ST} statistic is used. F_{ST} is a fixation index that uses the average expected heterozygosity within populations (H_{ES}) and compares it to the expected heterozygosity if all populations were combined and randomly mating (H_{ET}) (Weir & Cockerham, 1984). F_{ST} values of 0 indicate that populations have no genetic differentiation while F_{ST} values closer to 1 shows increasing genetic differentiation (Nei, 1977; Selkoe & Toonen, 2006). F_{ST} has been utilised to identify differences between populations of perennial ryegrass. Kubik, *et al.* (2001) used 22 SSR markers to screen across 30 individuals from each of seven cultivars. They found deviations between cultivars with pairwise F_{ST} values ranging from 0.065 to 0.435 showing a range of differentiation among cultivars. Wang, *et al.* (2009) also utilised F_{ST} to identify genetic differences between eight Australasian bred cultivars using 29 SSRs across 48 individuals per cultivar. This study had lower pairwise F_{ST} values compared to Kubik, *et al.* (2001), ranging from 0.007 to 0.160 showing that some cultivars could be distinguished from each other with these methods, while others did not differentiate due to shared genetic ancestry in cultivar development.

It is hypothesised that within the cultivars at Poukawa research station, the genetic mean has shifted from the original genetic diversity of the populations sown there. If a genetic shift has directed genetic variation towards more persistent plants that survived for nine years, these populations will no longer be in Hardy-Weinberg equilibrium where observed allele frequency will differ from expected allele frequency leading to different F_{IS} values of Persistent populations compared to their Original counterparts, and significant F_{ST} values between 'Persistent' and 'Original' populations of a cultivar.

4.2.1 Objectives

The objectives of this experiment were to characterise genetic diversity within and between Persistent and Original populations of the diploid cultivars ‘Grasslands Samson’ and ‘Commando’ using ‘Persistent’ plants collected from the long term Poukawa trial and comparing them to plants grown from the ‘Original’ population sourced from commercial seed not subjected to the same environmental selection pressures. Genetic diversity was assessed using data generated from 11 simple sequence repeat (SSR) markers screened across 183 individuals from the four populations to: 1) calculate variation within populations (Original and Persistent); 2) compare genetic differences between populations; 3) determine genetic structure within the samples.

4.3 Methods

4.3.1 Plant material and DNA collection

A total of 60 ‘Persistent’ perennial ryegrass plants of each of the AR1 endophyte infected cultivars ‘Grasslands Samson’ and ‘Commando’ were collected in May 2014 from the long term data set established in April 2005 (Chapman, *et al.*, 2015a) at Poukawa research station, Central Hawke’s Bay (39°76’10”S, 176°72’73”E). At the same time, 60 plants were grown from seed of each of the ‘Original’ cultivars ‘Grasslands Samson’ and ‘Commando’ infected with the same endophyte strain (AR1). Plants were established into PB3 (two litre) pots with 18 month OsmocoteTM soil mix and were maintained with trimming outdoors for two years.

In March 2016, DNA was collected from each live plant. There were 40 individuals sampled for Commando Original, 53 for Commando Persistent, 46 for Samson Original, and 44 for Samson Persistent (total 183). Approximately 50mg of leaf tissue was collected from green leaf lamina of each plant and put into two 96 well-plates.

4.3.2 DNA extraction and genotyping

DNA was extracted using a modified Whitlock method (Whitlock *et al.*, 2008). In the plates, each well had two metal beads placed in it and sealed with a lid. Samples were ground using a mixer mill at 30Hz for two minutes. Plates were spun down in a centrifuge at 4000-g for one minute at -15°C. Plates were placed in an ice water bath for 20 minutes before being spun down at 4000g for one minute. Each well then had 500µl of homogenisation buffer and 1.8µl proteinase K added. A new heat seal was applied before plates were shaken to dislodge beads stuck to the bottom of wells. The plates were centrifuged at 4000g for 10 minutes. 300µl of the supernatant was transferred to a new 96 well plate. Before the plate was mixed, 300µl of precipitation buffer (3.6M potassium acetate, 2.4M acetic acid) was also added to each well. After mixing, 600µl of binding buffer (2M guanidinium chloride, 10mM Tris, 0.5mM EDTA and 66% ethanol) and 400µl of supernatant was transferred to a Pall AcroPrep Advance 96 1ml filter plate (PN 8132) and centrifuged for two minutes at 4000g. Flow-through was discarded. The plates underwent three washes. The first wash was with 300µl of binding buffer (2M guanidinium chloride, 10mM Tris, 0.5mM EDTA and 66% ethanol) per well, the second wash was 300µl of washing buffer (50% ethanol, 10mM Tris, 0.5mM EDTA and 50mM NaCl) per well, and the final wash was 300µl of 100% ethanol per well. After each wash the plate was centrifuged at 4000g for two minutes and flow-through was discarded. The membrane was then dried using the centrifuge for five minutes at 4000g.

A new 1ml 96 well Axygen collection plate was used with a filter plate. 115µl of Tris/RNase A mixture was added to each well and centrifuged for one minute at 4000g. For each 96-well plate used, 4µl of RNase A was added to 12ml of 10mM Tris before being transferred to a reservoir. 0.04µl of RNase A was used per 115µl sample. DNA plates were labelled and stored through applying a plastic cover over the top of the cells and placed in a freezer kept at -4°C in the laboratory.

To test the integrity of extracted DNA, electrophoresis was conducted. Each well of a new 1ml 96 well Axygen collection plate had 18 µl of 1.25x loading buffer (25% Ficoll, 100 mM Tris-HCl & EDTA, Orange G) and 2 µl of DNA added. From each well, 10µl were dispensed onto a 0.8% lithium borate agarose gel (4g agarose, 500mL 1x lithium borate). A 5µl 1kB ladder was loaded at each end of the gel. The gel was run for 30 minutes at 80 volts. DNA quality of the genome plate was assessed through visual assessment of band strength and clarity after gel electrophoresis. All wells showed a visible band indicating good yields.

M13 primer-tailing PCR was performed to amplify microsatellite markers. 10µL PCR reactions contained 1x buffer B (New England Biolabs, Ipswich, MA, USA), 0.2µL dNTPs, 0.2µM forward primer, 4.5µM reverse primer, 4.5 µM M13 primer (labelled with either fluorescent dye VIC, NED, or FAM), 0.1µL Taq polymerase, and 0.1 µL of 1:10 diluted DNA under the following cycling conditions: 95°C for three minutes, followed by 30 cycles of 95°C for 30 seconds, 52°C for 40 seconds, and 72°C for 40 seconds, and a final extension of 20 minutes at 72°C.

Because different dyes were used for different markers (FAM, VIC, and NED), three markers were co-loaded into a pooled plate. From each well of the pooled plate, 1 μ L of pool was added to 9 μ L of HiDi formaldehyde (Applied Biosystems, Carlsbad, California, USA) and 1 μ L CASS size standard (Symonds & Lloyd, 2004). PCR products were sized on the ABI 3730 DNA Analyzer (Applied Biosystems) at the Massey Genome service at Massey University (Palmerston North, New Zealand).

4.3.3 Markers

Perennial ryegrass specific SSR markers were screened using 15 individuals to select appropriate markers for assessing the genetic variation of the four populations. The criteria for markers to be selected were; (1) markers amplify well in M13 PCR, (2) clear peaks within the expected size range, (3) polymorphic across individuals, and (4) no more than two alleles per individual. Table 4.1 shows the locus, expected base pair range, primer sequence, and fluorescent dye used for markers that matched these criteria and were then used for all genotypes.

Table 4.1; Characteristics of 11 *Lolium perenne* SSR markers selected for screening. Locus name, expected range, forward (F) and reverse (R) primer sequences, and fluorescent dye used for each marker is presented.

| Locus | Expected range (bp) | Primer sequences | Dye |
|----------|---------------------|--|-----|
| PPS0002 | 125 - 236 | F- 5'GGCAGCTAGATCTCGTCACC 3' R- 5'AGTACCCATTATCGGTCCCC 3' | VIC |
| PPS0022 | 159 - 208 | F- 5'ATGTCCACCGTATCGATGGT 3' R- 5'CAGCTGCTACATTAGTGCAACC 3' | FAM |
| PPS0037 | 173 - 190 | F- 5'AGAGATAGGTTCCACTCCCTCC 3' R- 5'TCACTTTCACCTGTCTCACCC 3' | VIC |
| PPS0040 | 239 - 248 | F- 5'AAGAAGGTACAAGAAGCATTGACC3' R- 5'CCTCTTCTTCTTTTAGCACAAGC 3' | FAM |
| PPS0065 | 125 - 187 | F- 5'CTTCTCTTCTCCTCTCCCTCC 3' R- 5'CGCATGAGAAGTATACGAATCG 3' | VIC |
| PPS0094 | 104 - 114 | F- 5'GGTATTCCTCTCTCGCCCTC 3' R- 5'TCGAATCGGATAAACAACCC 3' | VIC |
| PPS0252 | 240 - 251 | F- 5'GCTCAGCACATAAAGCAGAGC 3' R- 5'CTTGATCTAGTAGCAACGCCG 3' | NED |
| PPS0817 | 243 - 255 | F- 5'GGCTCAAGCTCCTCAACATC 3' R- 5'TGATCGATCTGTTCTTCTTACTCG 3' | NED |
| PPS388 | 235 - 257 | F- 5'GACGGCGATTTCAACAGATT 3' R- 5'TGAAATCTGGCATTCTTCC 3' | NED |
| b1a8 | 281 - 306 | F- 5'GACTTTCAGGCATCGGTCAT 3' R- 5'CCCAGCTCCATTCTTAATGC 3' | FAM |
| 55-TAGA1 | 290 - 380 | F- 5'GTCAGAAAACAGTCTGCC 3' R- 5'AGCATCGGTATGCAAAAA 3' | FAM |

4.3.3.1 Scoring marker peaks

Microsatellite alleles were sized using GeneMapper version 4.0 (Applied Biosystems). Peaks were called after comparing peak sizes, intensity, and peak morphology across all individuals. Base pair size was recorded for each peak for each locus to two decimal points. After base pair size data was collected, peak size was further categorized by grouping (binning) allele sizes. Binning was performed using sorted data (smallest to largest) and visualising the data in scatter plots. Allele sizes were binned

where ‘breaks’ appeared in the size distributions in scatter plots. Base pair size was rounded to the nearest integer.

4.3.4 Identifying contaminated samples

To ensure that amplification errors were not occurring between the two plates, each plate had six positive controls containing the same genotypes as the other plate and one negative control containing H₂O. During scoring of marker peaks, the positive controls were compared for allele number and allele size to check that the plates had undergone the same conditions for amplification.

After screening individuals for markers, a number of genotypes were observed to have more than two peaks for some markers. This issue did not occur in the individuals used to pre-screen and select appropriate markers. Initially, inexperience in reading microsatellite fluorescent peaks was identified as a potential issue. To resolve this, further training was undertaken, and marker peak base pair sizes were re-recorded. However, this did not resolve the problem.

Another possible solution was to identify if the annealing temperatures used in PCR altered results. Fresh dilutions were made from the genomic plate and compared with the dilutions previously used. A sample of individuals with many peaks or no extra peaks from both genomic and dilution plates were taken to AgResearch to screen with PPS0040 SSR marker using the annealing temperature of 55°C. This marker was chosen as it has previously shown high levels of amplification and easily read peaks (Faville, *et al.*, 2004) (Table 4.1). The 2°C difference in annealing temperature was investigated as

PCR using lower annealing temperatures can decrease the specificity of primers, amplifying non-target fragments of the DNA (Hamajima *et al.*, 2002). These results were compared to results of the same genotypes under the 53°C annealing temperature and no differences in peak number or base pair sizes were found.

It was concluded that the genomic plates were contaminated with some wells having more than one genotype amplified. Individuals that had more than two peaks in any of the eleven SSR markers used were identified as contaminated. Comparing across all markers was necessary as wells containing two homozygous genotypes with different allele sizes could be read as one heterozygote. Of the 183 genotypes, 58 (32%) did not show more than two peaks for any marker and were identified as uncontaminated, and were used for further analyses.

4.3.4 Assessing genetic variation

GenAlEx v6.502 (Peakall & Smouse, 2012) was used to assess each microsatellite locus for observed (H_o) and expected heterozygosity (H_E). Genetic variation was assessed using GenAlEx which calculated number of alleles (N_a), total number of alleles (TN_a), percentage of polymorphic loci (%P), proportion of shared alleles (SA_P), and F-statistics: F_{IS} (estimate of inbreeding level) and F_{ST} (genetic differentiation between populations) (Weir & Cockerham, 1984). GenAlEx was also used for principal co-ordinate analysis, Pairwise F_{ST} , and AMOVA (analysis of molecular variance).

From GenAlEx, data was exported to STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) for further analysis.

4.3.5 Resolving genetic structure and differentiation

The distribution of genetic variation was assessed in STRUCTURE (Pritchard, *et al.*, 2000), which identifies the most likely number of genetic clusters within a data set. Bayesian analysis, using multi-locus genotype data, constructs “ancestral” populations partitioning and assigning ‘individual’ genotypes to those populations. No prior information relating to the cultivar or population the individual was from was included in the analyses. Parameters used for STRUCTURE analyses were: 15 replicates for each K (putative ancestral population) value from 1 - 4, assumed admixture, infer lambda, 100 000 iterations of burn-in and 1 000 000 iterations of data collection.

4.4 Results

4.4.1 Marker variation

Eleven markers were genotyped across all 183 individual samples from the four populations. However, contamination was identified after data from SSR markers were compared. Contamination was identified within individuals that produced three or more peaks for any of the eleven markers. Because of contamination, analysis was carried out on 58 perennial ryegrass individuals: 20 from Samson Original, six of Samson Persistent, 20 of Commando Original, and 12 of Commando Persistent.

4.4.1.1 Samson Original

Eleven markers were used across 20 individuals in the Samson Original population. The success rate of marker amplification (% amp.) ranged from 80-100% (Table 4.2). The number of alleles per locus ranged from three to 13 with 100% polymorphic loci. The range of observed heterozygosity (H_O) was 0.111 - 0.889 which was greater than the expected heterozygosity (H_E) range between 0.329 - 0.898 (Table 4.2).

Table 4.2; Characteristics of 11 microsatellite loci for 20 samples of the Samson Original population. Base pair size range (BP range), percentage amplification (% amp.), number of alleles (Na), observed heterozygosity (H_O), and expected heterozygosity (H_E).

| Locus | BP range | % amp | Na | H_O | H_E |
|-----------------|-----------------|--------------|-----------|-------------------------|-------------------------|
| PPS0002 | 137 - 168 | 80 | 11 | 0.688 | 0.883 |
| PPS0022 | 164 - 194 | 90 | 9 | 0.889 | 0.724 |
| PPS0037 | 172 - 178 | 95 | 6 | 0.737 | 0.615 |
| PPS0040 | 257 - 269 | 90 | 5 | 0.556 | 0.535 |
| PPS0065 | 123 - 141 | 80 | 5 | 0.750 | 0.680 |
| PPS0094 | 105 - 117 | 85 | 6 | 0.647 | 0.723 |
| PPS0252 | 230 - 243 | 90 | 3 | 0.611 | 0.634 |
| PPS0817 | 234 - 245 | 90 | 5 | 0.333 | 0.38 |
| PPS0388 | 224 - 227 | 90 | 3 | 0.111 | 0.329 |
| b1a8 | 277 - 321 | 100 | 13 | 0.700 | 0.896 |
| 55-TAGA1 | 321 - 387 | 80 | 13 | 0.625 | 0.898 |
| Mean | 105 - 387 | 88.18 | 7.18 | 0.604 | 0.662 |

4.4.1.2 Samson Persistent

Eleven markers were used across six individuals in the Samson Persistent population. The success rate of marker amplification (% amp.) ranged from 83.3-100% (Table 4.3). The number of alleles per locus ranged from two to eight with 100% polymorphic loci. The range of observed heterozygosity (H_O) was 0.167 - 1.000 which was greater than the expected heterozygosity (H_E) range between 0.153 - 0.860 (Table 4.3).

Table 4.3; Characteristics of 11 microsatellite loci for six samples of the Samson Persistent population. Base pair size range (BP range), percentage amplification (% amp.), number of alleles (Na), observed heterozygosity (H_O), and expected heterozygosity (H_E).

| Locus | BP range | % amp | Na | H_O | H_E |
|-----------------|-----------------|--------------|-----------|-------------------------|-------------------------|
| PPS0002 | 147 - 172 | 83.3 | 6 | 0.400 | 0.800 |
| PPS0022 | 165 - 195 | 100 | 5 | 1.000 | 0.736 |
| PPS0037 | 172 - 178 | 100 | 2 | 0.333 | 0.278 |
| PPS0040 | 257 - 266 | 100 | 4 | 0.833 | 0.708 |
| PPS0065 | 137 - 147 | 100 | 5 | 0.667 | 0.528 |
| PPS0094 | 107 - 117 | 100 | 3 | 0.833 | 0.653 |
| PPS0252 | 230 - 243 | 100 | 5 | 0.833 | 0.694 |
| PPS0817 | 233 - 245 | 100 | 3 | 0.500 | 0.403 |
| PPS0388 | 226 - 228 | 100 | 2 | 0.167 | 0.153 |
| b1a8 | 277 - 319 | 100 | 5 | 1.000 | 0.750 |
| 55-TAGA1 | 325 - 399 | 83.3 | 8 | 0.800 | 0.860 |
| Mean | 107 - 399 | 96.96 | 4.36 | 0.670 | 0.597 |

4.4.1.3 Commando Original

Eleven markers were used across 20 individuals in the Commando Original population. The success rate of marker amplification (% amp.) ranged from 85-100% (Table 4.4). The number of alleles per locus ranged from three to 17 with 100% polymorphic loci. The range of observed heterozygosity (H_O) was 0.158 - 0.947 which was similar to the expected heterozygosity (H_E) range between 0.148 - 0.924 (Table 4.4).

Table 4.4; Characteristics of 11 microsatellite loci used on 20 samples of the Commando Original population. Base pair size range (BP range), percentage amplification (% amp.), number of alleles (Na), observed heterozygosity (H_O), and expected heterozygosity (H_E).

| Locus | BP range | % amp | Na | H_O | H_E |
|-----------------|-----------------|--------------|-----------|-------------------------|-------------------------|
| PPS0002 | 117 - 170 | 85 | 11 | 0.529 | 0.870 |
| PPS0022 | 165 - 197 | 95 | 9 | 0.895 | 0.791 |
| PPS0037 | 160 - 178 | 100 | 5 | 0.700 | 0.661 |
| PPS0040 | 257 - 266 | 95 | 4 | 0.579 | 0.688 |
| PPS0065 | 129 - 137 | 95 | 7 | 0.632 | 0.734 |
| PPS0094 | 107 - 118 | 95 | 5 | 0.579 | 0.697 |
| PPS0252 | 233 - 243 | 95 | 5 | 0.684 | 0.735 |
| PPS0817 | 230 - 245 | 95 | 5 | 0.579 | 0.499 |
| PPS0388 | 220 - 227 | 95 | 3 | 0.158 | 0.148 |
| b1a8 | 277 - 321 | 95 | 9 | 0.947 | 0.821 |
| 55-TAGA1 | 321 - 411 | 95 | 17 | 0.947 | 0.924 |
| Mean | 107 - 411 | 94.55 | 7.27 | 0.657 | 0.688 |

4.4.1.4 Commando Persistent

Eleven markers were used across 12 individuals in the Commando Persistent population. The success rate of marker amplification (% amp.) was 100% (Table 4.5). The number of alleles per locus ranged from two to 12 with 100% polymorphic loci. The range of observed heterozygosity (H_o) was 0.167 - 1.000 which was greater than the expected heterozygosity (H_E) range between 0.153 - 0.889 (Table 4.5).

Table 4.5; Characteristics of 11 microsatellite loci for 12 samples of the Commando Persistent population. Base pair size range (BP range), percentage amplification (% amp.), number of alleles (Na), observed heterozygosity (H_o), and expected heterozygosity (H_E).

| Locus | BP range | % amp | Na | H_o | H_E |
|-----------------|-----------------|--------------|-----------|-------------------------|-------------------------|
| PPS0002 | 137 - 170 | 100 | 10 | 0.750 | 0.868 |
| PPS0022 | 165 - 197 | 100 | 6 | 0.750 | 0.806 |
| PPS0037 | 172 - 178 | 100 | 4 | 0.667 | 0.601 |
| PPS0040 | 257 - 269 | 100 | 6 | 0.667 | 0.715 |
| PPS0065 | 131 - 147 | 100 | 9 | 0.667 | 0.747 |
| PPS0094 | 103 - 117 | 100 | 6 | 0.583 | 0.736 |
| PPS0252 | 230 - 243 | 100 | 5 | 0.750 | 0.688 |
| PPS0817 | 230 - 245 | 100 | 3 | 0.250 | 0.226 |
| PPS0388 | 226 - 228 | 100 | 2 | 0.167 | 0.153 |
| b1a8 | 277 - 321 | 100 | 9 | 0.917 | 0.858 |
| 55-TAGA1 | 321 - 397 | 100 | 12 | 1.000 | 0.889 |
| Mean | 103 - 397 | 100 | 6.55 | 0.652 | 0.662 |

4.4.2 Population variation

The total number of allele variants (N_{av}) was assessed for each locus within each cultivar. The total number of allele variants was greatest for the locus 55-TAGA1 ($N_{av} = 16$ and 19) and lowest for the locus PPS0388 ($N_{av} = 3$ and 3) for both Samson and Commando respectively (Table 4.6). The proportion of shared alleles (SA_P) between the Persistent and Original populations of Samson ranged between 0.11 (PPS0065) and 0.80 (PPS0040). The Commando populations also had the lowest proportion of shared alleles for the locus PPS0065 ($SA_P = 0.36$) and shared all alleles for the locus PPS0252 ($SA_P = 1.00$) (Table 4.6).

Across all loci, the mean total number of allele variants was similar for both Samson ($N_{av} = 8.18$) and Commando populations ($N_{av} = 8.55$). However the Samson populations had a lower mean proportion of shared alleles compared to the Commando populations (SA_P mean = 0.42 and 0.65 respectively) (Table 4.6).

Table 4.6; For each of the 11 microsatellite loci, allele variants were compared between Original and Persistent populations within Samson and Commando. Presented are the total number of alleles (N_{av}) and shared proportion of alleles (SA_P) for each cultivar.

| Locus | Samson | | Commando | |
|-----------------|----------|--------|----------|--------|
| | N_{av} | SA_P | N_{av} | SA_P |
| PPS0002 | 12 | 0.42 | 13 | 0.62 |
| PPS0022 | 9 | 0.56 | 9 | 0.67 |
| PPS0037 | 6 | 0.33 | 5 | 0.80 |
| PPS0040 | 5 | 0.80 | 6 | 0.67 |
| PPS0065 | 8 | 0.11 | 11 | 0.36 |
| PPS0094 | 6 | 0.50 | 7 | 0.57 |
| PPS0252 | 5 | 0.60 | 5 | 1.00 |
| PPS0817 | 6 | 0.33 | 5 | 0.60 |
| PPS0388 | 3 | 0.33 | 3 | 0.67 |
| b1a8 | 13 | 0.38 | 11 | 0.64 |
| 55-TAGA1 | 16 | 0.31 | 19 | 0.53 |
| Mean | 8.18 | 0.43 | 8.55 | 0.65 |

Table 4.7 shows across all populations success rate of marker amplification was high at a mean of 94.92%. The average number of alleles (N_A) for the four populations was 6.34. The Samson Persistent population had the smallest number of alleles and the Commando Original population had the largest. Observed heterozygosity (H_O) between populations was similar with a range of 0.604 to 0.670. The expected heterozygosity (H_E) had a range of 0.597 to 0.688. The Samson Persistent population was the only population to have a lower H_E compared to H_O . Average F_{IS} was between -0.01 for Commando Persistent, and 0.09 for Samson Persistent (Table 4.7).

Table 4.7; Average characteristics of 11 microsatellite loci for each of the four populations. Number of samples (N), percentage amplification (% amp.), average number of alleles (Na), average observed heterozygosity (H_O), average expected heterozygosity (H_E), and estimates of Wright's fixation index within each population (F_{IS}) are presented.

| Population | N | % amp | Na | H_O | H_E | F_{IS} |
|----------------------------|----------|--------------|-----------|----------------------|----------------------|-----------------------|
| Samson Original | 20 | 88.18 | 7.18 | 0.604 | 0.662 | 0.02 |
| Samson Persistent | 6 | 96.96 | 4.36 | 0.670 | 0.597 | 0.09 |
| Commando Original | 20 | 94.55 | 7.27 | 0.657 | 0.688 | 0.08 |
| Commando Persistent | 12 | 100.00 | 6.55 | 0.652 | 0.662 | -0.01 |
| Mean | 14.5 | 94.92 | 6.34 | 0.646 | 0.652 | 0.045 |

4.4.3 Genetic structure

Pairwise F_{ST} values were calculated between all four populations. Table 4.8 shows all pairwise F_{ST} values are close to 0 indicating that the populations are not strongly diverged. The largest pairwise F_{ST} value was between Samson Original and Samson Persistent at 0.055. The smallest values were observed between Commando Original and Commando Persistent and between Commando Original and Samson Original at 0.022 (Table 4.8).

Mean posterior probability (LnP(D)) for a given value was plotted against putative ancestral population number (K) results obtained from 15 iterations per K of STRUCTURE analyses to identify the K number with the greatest mean LnP(D). (Figure 4.1) shows the greatest LnP(D), and therefore the highest probability was associated with only one putative ancestral population (K).

Box plots were constructed to further investigate population structures. Figure 4.2 shows the four populations could not be differentiated under K = 2 (Figure 4.2a), K = 3 (Figure 4.2b), or K = 4 (Figure 4.2c). Analysis of molecular variance showed only 1% of the variation observed was between populations while 82% of variation was found within individuals (Table 4.9). Principal co-ordinate analysis further showed genetic similarity among the four populations where all four groups overlap. Principal co-ordinates I and II accounted for 8.20% and 8.17% of the variation respectively (Figure 4.3).

Table 4.8; Pairwise F_{ST} values of the four populations of perennial ryegrass derived from 11 microsatellite markers.

| | Samson Original | Commando Original | Samson Persistent | Commando Persistent |
|----------------------------|------------------------|--------------------------|--------------------------|----------------------------|
| Samson Original | 0.000 | | | |
| Commando Original | 0.022 | 0.000 | | |
| Samson Persistent | 0.055 | 0.038 | 0.000 | |
| Commando Persistent | 0.028 | 0.022 | 0.033 | 0.000 |

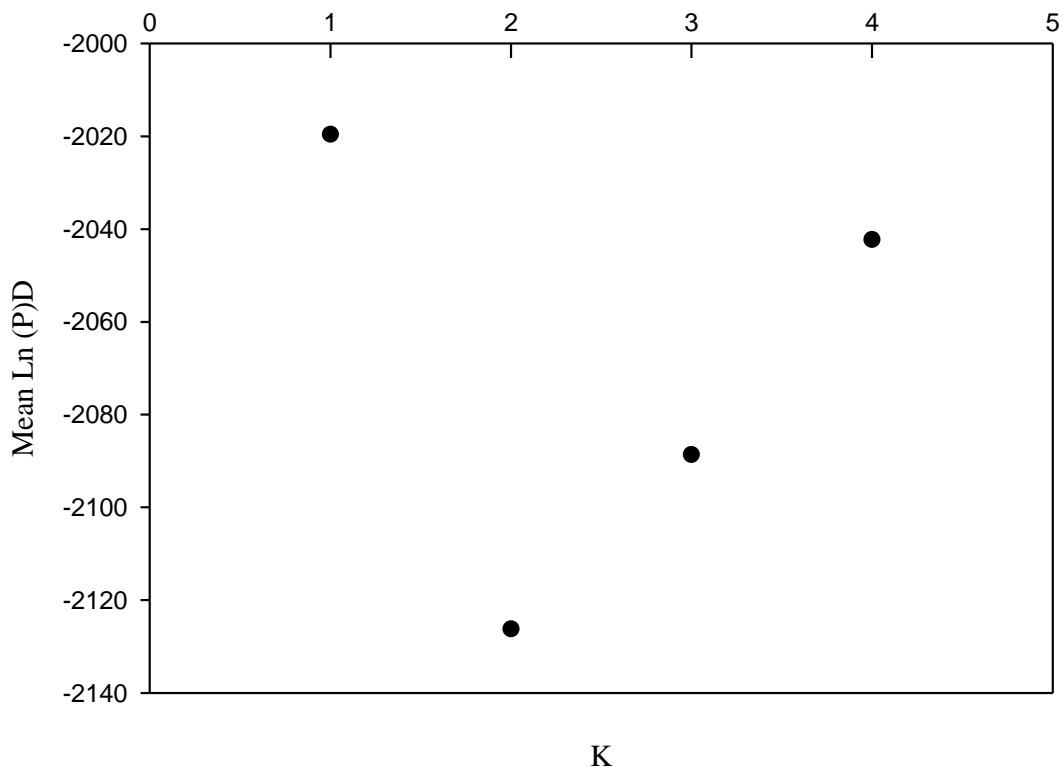


Figure 4.1; Plot of mean posterior probability ($\text{Ln}P(D)$) values per cluster (K), based on 15 iterations per K from *STRUCTURE* analyses (Pritchard et al., 2000).

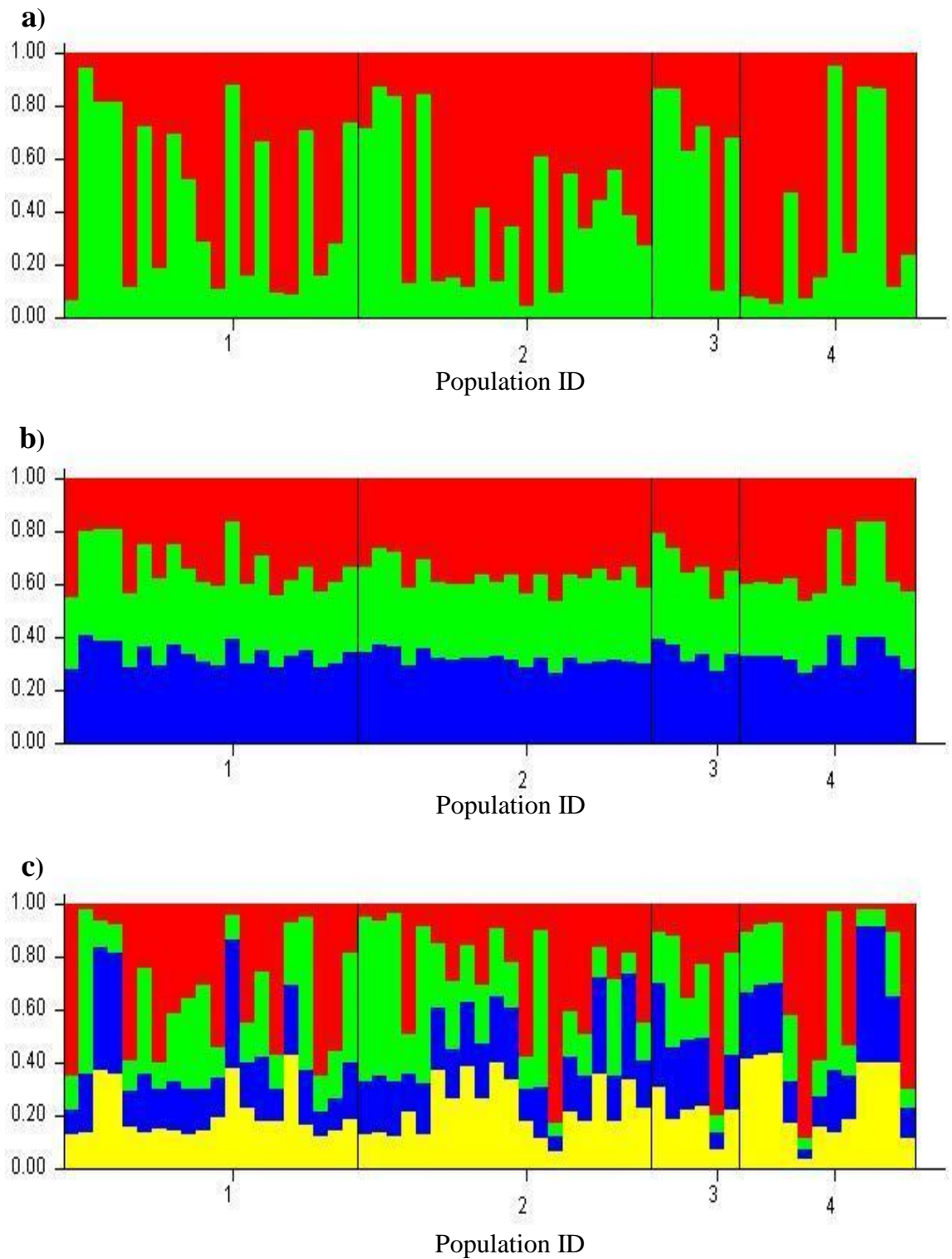


Figure 4.2; *STRUCLURE* cluster assignment of 58 individuals from the populations Samson Original (1), Commando Original (2), Samson Persistent (3), and Commando Persistent (4). Box graphs show population structure for putative ancestral populations of a) $K=2$; b) $K=3$; and c) $K=4$.

Table 4.9; AMOVA results for the partitioning of microsatellite variation. Presented are the degrees of freedom (df), sum of squares (SS), mean of squares (MS), Estimated variance components (Est. Var.) and percentage of variation (Var. %).

| Source | df | SS | MS | Est. Var. | Var. % |
|--------------------|----|---------|-------|-----------|--------|
| Among Populations | 3 | 17.755 | 5.918 | 0.047 | 1% |
| Among Individuals | 54 | 249.883 | 4.627 | 0.663 | 17% |
| Within Individuals | 58 | 191.500 | 3.302 | 3.302 | 82% |

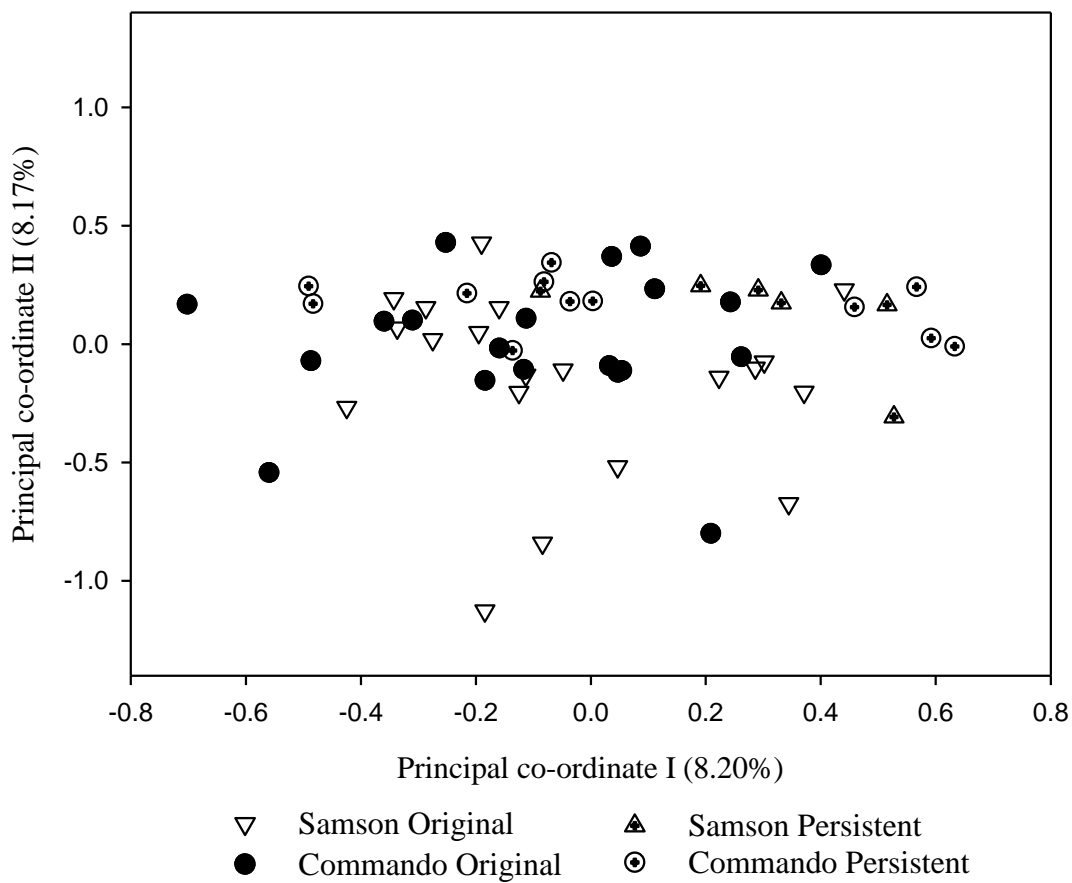


Figure 4.3; Principal co-ordinates analysis of all 11 markers across a total of 58 individuals. Principal co-ordinates I and II accounted for 8.20% and 8.17% of the variation respectively. Symbols indicate identity of individuals from each of the four populations.

4.5 Discussion

The objective of this project was to characterise genetic diversity within Persistent and Original populations of the cultivars Samson and Commando using simple sequence repeat (SSR) markers through identifying allelic frequencies and assessing population structures. These data were used to compare 183 individuals across four populations.

4.5.1 Contamination reduced sample size from 185 to 58 genotypes in total

While analysing the results from all markers across all individuals it was identified that some individuals consistently showed more than two peaks for several markers. This was not expected as the individuals assessed were identified as originating from diploid cultivars and the SSR primers used have worked well in previous studies (Jensen, *et al.*, 2007; Sartie, *et al.*, 2011). Potential size and number errors were explored through re-reading microsatellite fluorescent peaks after further training, and repeating the PCR amplification of selected individuals in another laboratory using an annealing temperature of 55°C but found similar results. Therefore it was concluded that individuals showing more than two peaks per marker were contaminated with DNA from more than one individual.

Unfortunately, individuals used in the pre-screen were not among those contaminated so the problem was not identified when scrutinising markers. Contamination was picked up late in the experiment due to learning techniques, testing, amplification, and analysis being carried out over a ten month period. The issue was only identified when comparing all marker data showed that some individuals had frequently shown more than two peaks for multiple markers. From the 183 genotypes used, only

32% (58) were found to not have more than two peaks for any one marker. Of these individuals 40 were from the Original populations (20 Samson Original; 20 Commando Original) and only 18 were from the Persistent populations (six Samson Persistent; 12 Commando Persistent) (Table 4.7). After investigating if the contamination was limited to the dilution plates used for PCR, it was found the genomic plates were also affected.

The contamination event could have occurred at a number of stages during the process. Contamination of the genomic plate could have occurred in the laboratory as a result of poor laboratory technique. For example introduction of more than one genotype per well during DNA extraction through leakage across the top of the plate that went undetected, or accidental misuse of pipette tips while extracting or transferring genomic DNA into new plates. Errors also could have been made earlier in the experimental process when tillers were sampled from Poukawa research station. Ramets of three to four tillers were cut from the pasture and placed in a pot. This number of tillers were kept together as it increases the chances of the plant surviving transplantation as the more tillers it has the more carbohydrates it can utilise to regrow. The pasture these tillers were removed from were nine years old, therefore tillers from more than one genotype could have been removed and placed in a single planter bag together as one 'plant'. During DNA sampling, this would have been a problem as obtaining 50 mg of leaf tissue required three to four leaves to be harvested, often from different tillers as perennial ryegrass tillers only have three green leaves at a time (Easton, *et al.*, 1989). Therefore harvesting the different genotypes within the one planter bag into one well. This contamination could be true for the persistent populations as a greater number of these genotypes were affected by contamination. However this does not explain why plants from the original

populations were contaminated as planting individual plants of the original populations was much more controlled due to single seeds being sown.

Therefore, because of the much reduced sample size, the results are not robust enough to draw strong conclusions in addressing the original hypothesis.

4.5.2 SSR marker screens

Eleven markers were used to screen populations for genetic diversity. This was more markers than the eight used by Barth, *et al.* (2017), but less than Wang, *et al.* (2009) (29 SSR markers) and Kubik, *et al.* (2001) (22 SSR markers). More markers were screened for suitability but were found to not fit the criteria required for use in these populations. Kubik, *et al.* (2001) recommended that 15 SSR markers would be able to tell 99% of the variation. While this project used fewer SSR markers than Kubik, *et al.* (2001), which may have contributed to insignificant differences between the populations, it is more likely that the greatly reduced sample size caused by contamination was the main factor. The number of markers used here could have been adequate if the original sample size had been maintained as other studies such as Barth, *et al.* (2017) showed significant results in their data set using less than 15 markers.

A greater number of markers can provide higher resolution in detecting genetic differences between individuals and populations by identifying a greater number of loci at which genetic differences can be detected. However due to budget and time restraints, practical decisions need to be made on how many markers will be sufficient for each individual study.

4.5.3 Population variation

Analysis of the remaining 58 individuals through GenAEx showed a range of values for the average number of alleles, total number of alleles, proportion of shared alleles, and F_{IS} values among populations and microsatellite markers.

The average number of alleles (N_a) across all loci in these results were 6.34 which is different to previous studies on perennial ryegrass. Jones *et al.* (2001) had a smaller average number of alleles at 3.4, but both Kubik, *et al.* (2001) and Barth, *et al.* (2017) had a greater average number of alleles ($N_a = 19.41$ and $N_a = 22.25$ respectively). The average number of alleles was the lowest in both the Persistent populations (Table 4.7). While a smaller number of alleles has been previously associated with smaller population size (Leimu *et al.*, 2006), it is more likely the smaller number of alleles identified in this study is associated with fewer samples of the Persistent populations. Particularly for the Samson cultivar. The Samson Persistent population had the least number of samples (Table 4.7), lowest N_a values for each marker locus (Tables 4.2 to 4.5), and smallest average number of alleles ($N_a = 4.36$; Table 4.7). Consequently, Samson populations had the least proportion of shared alleles (SA_P) for 10 of the 11 loci screened, and the smallest SA_P mean across all loci ($SA_P = 0.43$; Table 4.6). This smaller sample size would have contributed to less alleles detected in Persistent populations (Leberg, 2002).

F_{IS} values (the proportion of variation between individuals within a population) were used to identify homozygote excess and identify potential genetic shifts away from Hardy-Weinberg equilibrium (Selkoe & Toonen, 2006). It was expected that Persistent

populations would have different F_{IS} values than the Original populations showing the individuals in the Persistent populations had different allele frequencies at the loci screened than Original populations. Larger F_{IS} (and therefore greater frequency of homozygotes than expected under HWE) can be due to the population expressing the occurrence of inbreeding or directional selection (Selkoe & Toonen, 2006).

Within the Samson cultivar, the two populations showed some differences in genetic diversity. The Samson Persistent population did have larger F_{IS} values (a greater frequency of homozygotes than expected under HWE) compared to Samson Original population but the difference between these values was small at only 0.07 (Table 4.7). These results are confounded by the small sample in comparison to the other populations of only six individuals. It is recommended that more samples would be used as perennial ryegrass has a high level of genetic diversity within cultivars, and six individuals is probably not a large enough sample to explore the genetic diversity within the persistent population.

The two populations within the Commando cultivar showed greater differences in F_{IS} values. Commando Original F_{IS} was 0.09 (Table 4.7). This showed that F_{IS} detected a greater number of homozygotes observed than was estimated. The Commando Persistent F_{IS} was slightly negative at -0.01 (Table 4.7) showing that observed and expected homozygosity were relatively similar for this population.

These results indicate that the Persistent population observed allele frequencies did not deviate much from the expected heterozygosity as shown by the F_{IS} statistic being

close to zero. Comparing the Persistent and Original population F_{IS} values also show that both populations had similar observed and expected heterozygosity within each population. These results differed to previous studies on perennial ryegrass where F_{IS} values had a much higher inbreeding co-efficient ranging between 0.14 to 0.58 (Barth, *et al.*, 2017). However these results had a greater sample size (24 individuals for each accession tested) compared to the sample sizes used in this project.

4.5.4 F_{ST} and population structures

The lack of diversity between the populations was verified in pairwise F_{ST} , where F_{ST} was analysed by comparing only two populations at a time. F_{ST} values were small with a range between 0.022 – 0.055 (Table 4.8). Principal co-ordinate analysis showed PC1 (8.20%) and PC2 (8.17%) accounted for similar levels of variation and did not show any grouping of individuals (Figure 4.3). Further analysis in STRUCTURE again showed that the data had the greatest mean posterior probability ($\ln P(D)$) at only one putative ancestral population ($K=1$) (Figure 4.1). Therefore this study did not detect genetic differences in the SSR marker data between Persistent and Original populations, or even between the Samson and Commando cultivars as illustrated in Figures 4.1, 4.2, and 4.3 where all figures show that there was no clear grouping of Samson cultivar individuals or Commando cultivar individuals in this data across different analysis methods.

Despite the confounding factor of a reduced sample size, it was expected that cultivar differences may have been observed as previous studies on perennial ryegrass have found significant differences between cultivars using SSRs and population genetic analyses. Kubik, *et al.* (2001) showed significant differences ($P < 0.05$) between seven

cultivars with pairwise F_{ST} values ranging from 0.065 to 0.435. However, other SSR studies have shown that not all cultivars or groups show significant differences. A study of Irish ecotypes and cultivars found that the 40 populations they tested could only be differentiated into two putative ancestral groups ($K=2$) (Barth, *et al.*, 2017). Wang, *et al.* (2009) also identified genetic diversity was not always significant between cultivars. They reported cultivars Kangaroo valley (Australian) and Ellet (New Zealand) did not show significant differences in genetic variation. The two cultivars used in this project might also show no-significant differences because of shared ancestry as both were developed using ecotypes collected from the North Island, New Zealand (GrasslandsResearchCentre, 2001; Griffiths, *et al.*, 2016).

AMOVA analysis further confirmed only 1% of genetic variation detected was between populations while 17% was among individuals and 82% was within individuals (Table 4.9). Previous work on SSRs indicate that because of the highly heterogeneous nature of perennial ryegrass cultivars, greater diversity is identified within a cultivar rather than between cultivars. Previous studies also detected large genetic variation within populations compared to between populations: 85.4% within, 14.6% between groups (Kubik, *et al.*, 2001); 89% within, 11% between groups (Barth, *et al.*, 2017); and 91.3% within, 8.7% between groups (Wang, *et al.*, 2009). All of these studies identified greater variation between groups than this study identified. This could be because the main objectives of these studies were to compare between several cultivars rather than trying to identify two distinct populations within a cultivar. Future experiments should ensure a greater sample number is available for final analysis of data through altering methodologies ensuring risks of contamination are limited. Further work should also

include sampling of a cultivar known to have a vastly different genetic background to the populations tested as a positive control to add confidence to the results.

4.6 Conclusions

The main objective of this experiment was to characterise genetic diversity within and between Persistent and Original populations of the cultivars ‘Grasslands Samson’ and ‘Commando’. Due to contamination of DNA detected later during analysis, sample size for each population was greatly reduced. On reflection, some alterations to the methodology could decrease the risk of contamination such as: Collecting only one tiller per plant from the field at Poukawa to ensure only one genotype is planted per pot, or using 4-5 SSR markers with a high number of alleles to test plants collected from Poukawa to ensure all tillers planted within the one PB3 container were only one genotype decreasing possibility of contamination of the persistent populations.

Analysis of the reduced sample size calculated F_{IS} values close to zero for each of the populations indicating observed and expected allele frequencies were similar within each population. Low F_{ST} values showed no detectable differences between the populations. Further graphical analysis of the data using STRUCTURE and principal coordinate analysis showed that genetic structures of the four populations were similar and no detectable differences were found between the four populations in this study. No genetic shift was detected in this study in the Persistent populations. However SSR markers and population genetics may have an important role in exploring the possibilities of using populations of perennial ryegrass identified as persisting well and exploring if

these populations are genetically different gene pools to the original cultivars they were sown from.

5.0 CONCLUSIONS

5.1 Introduction

An emerging issue with perennial ryegrass is the reported decrease in vegetative persistence of newer cultivars in sown pastures (Chapman, *et al.*, 2015a). Currently there is no clear agreement on how plant morphology and genetics contribute to perennial ryegrass persistence in New Zealand (Tozer, *et al.*, 2014; Chapman, *et al.*, 2015a). The primary aim of this thesis was to clarify the role of plant morphology and underlying genetics associated with persistence, by collecting nine year old 'Persistent' plants sourced from a long term perennial ryegrass cultivar trial, established at Poukawa and comparing these plants and their half-sibling offspring to plants raised from 'Original' seed of the cultivars 'Grasslands Samson' and 'Commando'. These four populations were compared to identify morphological and genetic differences.

Assumptions: Firstly, the plants extracted from Poukawa research station were surviving genotypes of the original cultivars sown nine years previously in April 2004. The genotypes were not from seedlings established post flowering seed dispersion or from the soil seed bank. This assumption was made because the trial was managed through defoliating plots to a height of three to four cm in an effort to maintain pasture quality after grazing. This would have prevented the plants from flowering (Chapman, *et al.*, 2015a). To test for contamination in the long-term trial plots at Poukawa research station, Chapman, *et al.* (2015a) identified the commercial strains of endophyte present in each cultivar plot using SSRs and found 84-99% endophyte infection of the original endophyte strains in all but one cultivar ('Canon' AR1) indicating low contamination of non-cultivar plants in these plots.

The second assumption is the commercially sourced seed used to represent the original cultivar populations sown was maintained true to type during seed production. The cultivars bought were from certified seed grown in accordance to international OECD regulations (MPI, 2014). This seed is known as ‘first generation seed’. To receive seed certification, first generation seed undergoes stringent field testing to ensure varietal and endophyte strain purity. First generation seed is sourced from ‘Basic seed’ which is only grown by selected growers base on seed certification protocol. ‘Basic seed’ is sown with ‘breeders seed’ to increase the quantity of seed available (Rolston & Agee, 2007).

The first part of this thesis described plant morphological differences between 30 individuals of the Persistent Samson population and 30 individuals from the Original cultivar ‘Grasslands Samson’ This was conducted using a glasshouse experiment after 10 weeks plant growth, by measuring eight morphological traits. The comparison between newly grown individuals of Samson with plants that had survived over nine years in the field had not previously been reported.

The second experiment was focused on estimating additive genetic variation within the two cultivars ‘Grasslands Samson’ and ‘Commando’, and also their corresponding Persistent populations sampled from the field trial at Poukawa. These populations were polycrossed within their assigned population group and the half-sibling families generated were assessed through visual scores and dry matter cuts for agronomically important traits in a row field trial over 13 months. A half-sibling field trial plan was chosen based on methodology commonly used in plant breeding field trials. Additive

genetic variation among the half-sibling families within each population was estimated and used to compare differences for key traits between the Persistent and Original populations.

The last experiment was undertaken to characterise neutral genetic diversity within and between Persistent and Original populations of Samson and Commando using microsatellite markers (SSRs). The data were analysed using population genetic statistics such as allele number, expected and observed heterozygosity, F statistics, AMOVA, and STRUCTURE to characterise and compare genetic variation (Weir & Cockerham, 1984; Evanno *et al.*, 2005).

5.2 Findings

1) Describing plant morphological differences within and between ‘Grasslands Samson’ and the Persistent plant sample from the field trial using a glasshouse experiment.

Our results described significant differences within and between the Original and Persistent populations of the cultivar ‘Grasslands Samson’ for eight morphological traits in a glasshouse experiment. Significant ($P < 0.05$) differences were observed within each population for tiller number, lamina length, lamina width, lamina thickness, and lamina sheath length. Tiller number, lamina length, and lamina sheath length showed a positive association in PCA analysis. Trait associations were similar between populations. Comparing between the two populations, the Persistent population had significantly ($P < 0.05$) greater means for tiller number, number of reproductive tillers, lamina sheath length, and dry weight. Greater tiller numbers of Persistent plants lead to more

reproductive tillers, longer sheath lengths, and greater dry weights compared to the Original population. The hypothesis for this experiment was that the range of variation within each population for these morphological traits would be smaller in the Persistent population. However, only dry weight showed significant (Bonett's test $P=0.014$) differences between the two populations, with the Persistent population having a greater range of dry weight values.

2) Using the cultivars 'Grasslands Samson' and 'Commando', estimate additive genetic variation within half-sibling family populations generated from Persistent and their corresponding Original cultivars for agronomically important traits based on a row field trial.

Based on 13 months of data collected, significant ($P<0.05$) additive genetic variation and medium to high levels of half-sibling family mean narrow sense heritability was estimated for the traits herbage growth scores, leaf width scores, plant habit scores, and aftermath heading. Genetic shifts were identified by comparing co-efficients of additive genetic variation for each trait between the Persistent and Original populations. This study found that genetic shifts over time were cultivar specific. Samson Persistent half-sibling families had lower co-efficients of additive variation compared to Samson Original for herbage growth score and plant habit score, while Commando Persistent half-sibling families had greater and similar co-efficients of additive variation for herbage growth score, leaf width score, and plant habit score compared to Commando original.

Cluster analysis characterised genetic variation within each population by grouping half-sibling families by trait associations and identifying half-sibling families

within populations that could be used for further germplasm development to enhance persistence. Groups of half-sibling families with more prostrate plant habits, low aftermath heading, and high herbage growth were identified through trait association and cluster analysis as potential germplasm to enhance persistence.

3) Characterise genetic diversity within Persistent and Original populations of the cultivars ‘Grasslands Samson’ and ‘Commando’ using microsatellite markers to determine genetic variation within each population and between populations.

This objective was addressed by screening for suitable SSR markers and using 11 selected markers to screen across all individuals in all four populations. However, the sample size of this experiment was reduced by 68% due to late detection of contamination in the experimental process. Analysis of the remaining data calculated F_{IS} values close to zero for each of the populations indicating observed and expected allele frequencies were similar within each population. F_{ST} values showed no detectable differences between the populations. Further graphical analysis of the data using STRUCTURE and principal coordinate analysis showed that genetic structures of the four populations had no detectable differences.

5.3 Limitations

The most limiting aspect of this study was the contamination of 68% of the DNA samples used in the microsatellite marker analysis. This limited any conclusions that could be drawn from using SSR markers and characterising the population structure of the four populations.

In the glasshouse experiment, differences between Persistent and Original populations observed were specific for ‘Grasslands Samson’. Only one cultivar was tested due to resource constraints. Results from plant morphology measurements of the glasshouse trial agree with other authors who have speculated tiller number and its consequent potential for increasing persistence (Jewiss, 1972; Westoby, 1984; Parsons, *et al.*, 2011; Tozer, *et al.*, 2014). However, the evidence presented in this thesis regarding the significantly greater number of tillers in Samson Persistent plants cannot be seen as conclusive evidence that greater tiller number will equate to greater persistence in the field. This is because the glasshouse experiment over 10 weeks does not recreate the field environment over multiple years. A common problem for pasture plant studies under glasshouse conditions is translating results from the glasshouse to practical agricultural application. This is because the interaction of genotype with the field environment may change the phenotype of the same individuals tested under glasshouse conditions (Falconer & Mackay, 1996). This is why the main aim of this experiment was to identify any significant morphological differences between the two populations, rather than attribute persistence to a particular trait.

The row field trial decreased the limitations of the glasshouse experiment using a more realistic grazed field environment. The main limitation of the row trial was that the duration of data collection was shorter than the norm for breeding trials, and is much shorter than the number of years a farmer would expect a pasture to persist. This limits data interpretation because each season had few data points, and year effects cannot be estimated. This is important because as years progress, fewer individuals survive and

greater differences may have been seen between the half-sibling families, populations, and cultivars assessed. Earlier results have shown that re-ranking of individuals can occur over time (Straub *et al.*, 2005; Fe *et al.*, 2015). The comparatively short time frame in which these rows were assessed did not allow for this to be observed.

Another limitation associated with row field trials is the use of visual scores to assess multiple traits. Visual scoring has previously been shown to be accurate in comparison to quantitative measurements through dry matter cuts (Smith *et al.*, 2001). However it is a qualitative measurement, with bias introduced through the person collecting the data, light levels changing throughout the day and the weather e.g. wind blowing the plants around can make it difficult to assess rows for leaf widths, and plant habit increasing sampling error. In this study, these issues were minimised through having previous experience collecting accurate visual score data from perennial ryegrass row trials for the traits measured. Also, the row by column trial design used in our field trial would have helped reduce error due to spatial effects. While visual scores can be seen as limiting because of their qualitative nature, it also allows traits that are difficult to quantitatively measure by other means (such as plant habit) to be added to the data matrix. Despite the limitations of the field trial, results showed some significant differences between the Persistent populations and the Original populations observed in both the glasshouse and in the field row trial.

5.4 Future Directions and Recommendations

- In this thesis, perennial ryegrass persistence is defined as a plants' ability to survive through the asexual production of daughter tillers. However in the

agricultural industry, there appears to be a lack of a quantitative definition of ryegrass persistence which encompasses farmer expectations of production and longevity. It is suggested that a definition of persistence is developed within seed and agricultural industries. This would enable plant breeders to add quantitative traits to their objectives and better define what the farmer is looking for in regards to persistence of a new cultivar.

- Further investigation into variation of seasonal leaf sheath length, its possible role in regulating tiller appearance rates, and the outcomes of selecting plants with long or short sheath lengths on overall phenotypes is needed. This would indicate if seasonal sheath length could be measured and selected for by breeders to improve persistence within the limited time frame of short term trials.
- Within the Samson Persistent population, selecting half-sibling families with above average herbage growth scores, more prostrate plant habits, and below average aftermath heading is advised for the continued development of these lines for persistence research and enhancing persistence in cultivar development.
- Future field trial methods using clonal plants at two or more sites with different environments is encouraged. This would allow further characterisation of genotypic response to environmental effects on plant persistence.
- Future projects retrieving plants from the field to study should consider methods that further reduce risks of having several genotypes accidentally planted in the one pot. It is advised after tillers are collected in the field, to use 4-5 SSR markers

to ascertain that all the tillers planted in one pot are the same genotype. Another method could involve only collecting a single tiller per plant, trimming the leaves, and placing in sand trays for 3-4 weeks for roots to establish before planting into individual pots.

- Use of molecular marker based assays to further characterise the genetic structure of populations and potential changes under field conditions over long periods of time, could provide useful information to plant breeders.

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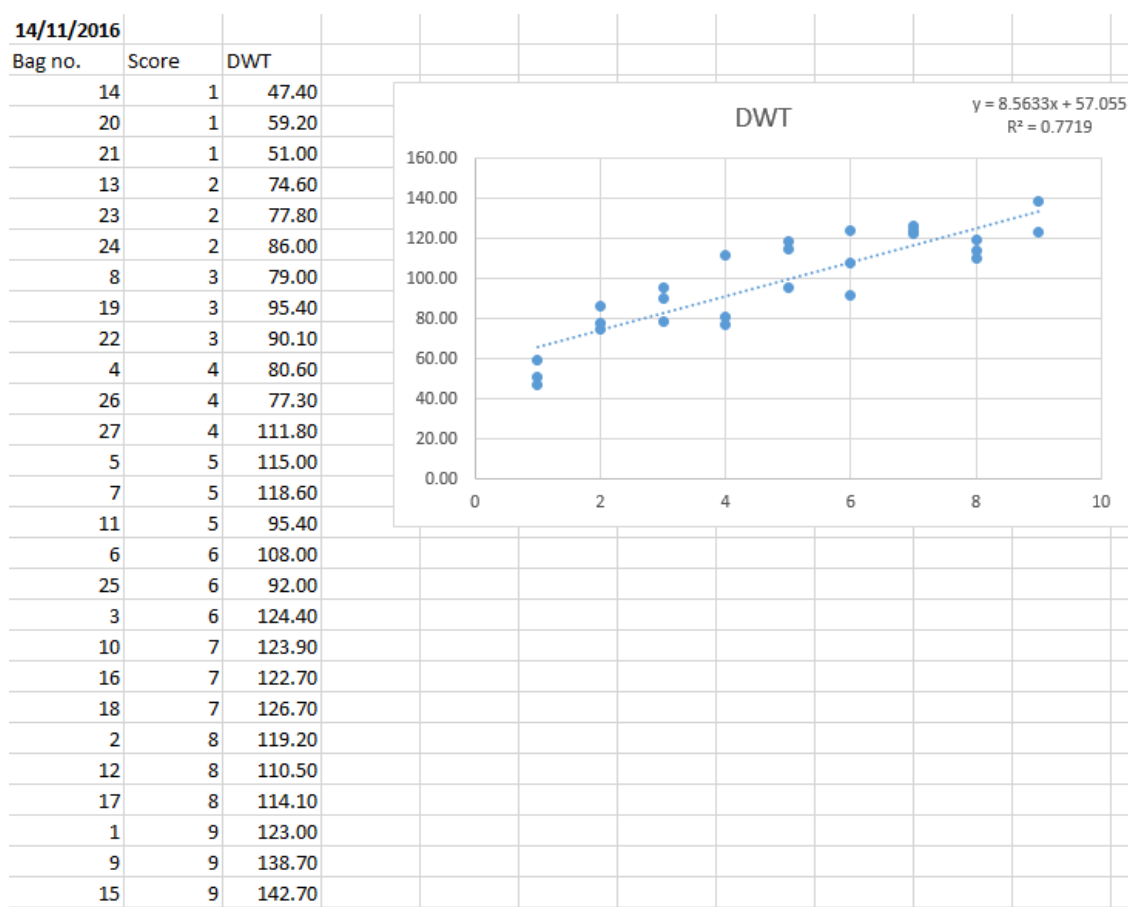
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Appendix 1; Layout of half-sibling family field experiment

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
|-------------------------|------------------------------|----------------------------|----|----|----|----|----|----|----|----|------------|
| 1 | 18 | 22 | 17 | 26 | 11 | 6 | 27 | 17 | 2 | 22 | ↑ 14.8m |
| 2 | 20 | 25 | 29 | 1 | 28 | 3 | 12 | 10 | 1 | 18 | |
| 3 | 16 | 10 | 4 | 15 | 27 | 25 | 11 | 16 | 4 | 13 | |
| 4 | 21 | 12 | 23 | 2 | 6 | 29 | 7 | 19 | 30 | 28 | |
| 5 | 19 | 14 | 30 | 24 | 13 | 8 | 23 | 21 | 14 | 15 | |
| 6 | 9 | 7 | 5 | 3 | 8 | 24 | 26 | 9 | 5 | 20 | |
| 7 | 14 | 20 | 5 | 10 | 2 | 9 | 15 | 5 | 6 | 30 | |
| 8 | 7 | 21 | 24 | 28 | 15 | 18 | 16 | 2 | 19 | 24 | |
| 9 | 3 | 13 | 26 | 16 | 29 | 4 | 14 | 8 | 12 | 29 | |
| 10 | 25 | 18 | 1 | 12 | 17 | 23 | 10 | 13 | 22 | 17 | |
| 11 | 22 | 4 | 19 | 8 | 23 | 11 | 28 | 3 | 26 | 21 | |
| 12 | 11 | 6 | 27 | 30 | 9 | 27 | 25 | 1 | 20 | 7 | |
| 13 | 26 | 23 | 25 | 6 | 1 | 21 | 19 | 6 | 13 | 1 | |
| 14 | 15 | 9 | 13 | 2 | 22 | 10 | 29 | 4 | 15 | 3 | |
| 15 | 28 | 3 | 10 | 19 | 18 | 25 | 17 | 18 | 28 | 5 | |
| 16 | 12 | 8 | 20 | 11 | 24 | 2 | 20 | 11 | 12 | 23 | |
| 17 | 17 | 14 | 7 | 27 | 16 | 30 | 8 | 22 | 27 | 16 | |
| 18 | 4 | 30 | 21 | 29 | 5 | 14 | 9 | 7 | 24 | 26 | |
| 19 | 15 | 16 | 12 | 7 | 26 | 27 | 23 | 3 | 18 | 10 | |
| 20 | 22 | 23 | 19 | 27 | 30 | 6 | 8 | 2 | 28 | 30 | |
| 21 | 13 | 11 | 25 | 10 | 29 | 11 | 1 | 5 | 24 | 9 | |
| 22 | 9 | 5 | 1 | 20 | 21 | 7 | 15 | 25 | 13 | 12 | |
| 23 | 18 | 4 | 8 | 6 | 2 | 14 | 19 | 29 | 16 | 4 | |
| 24 | 14 | 17 | 28 | 3 | 24 | 22 | 20 | 17 | 21 | 26 | |
| 25 | 28 | 26 | 10 | 21 | 14 | 17 | 29 | 10 | 11 | 6 | |
| 26 | 1 | 22 | 17 | 29 | 8 | 12 | 30 | 8 | 22 | 18 | |
| 27 | 25 | 24 | 16 | 11 | 15 | 1 | 2 | 9 | 7 | 16 | |
| 28 | 27 | 7 | 6 | 5 | 18 | 23 | 5 | 13 | 27 | 14 | |
| 29 | 2 | 12 | 9 | 23 | 3 | 21 | 28 | 24 | 4 | 15 | |
| 30 | 4 | 20 | 30 | 19 | 13 | 20 | 25 | 19 | 26 | 3 | |
| 31 | 7 | 10 | 4 | 15 | 17 | 13 | 17 | 4 | 20 | 9 | |
| 32 | 16 | 20 | 11 | 18 | 19 | 29 | 3 | 22 | 1 | 5 | |
| 33 | 24 | 23 | 13 | 1 | 12 | 26 | 24 | 23 | 30 | 7 | |
| 34 | 14 | 8 | 27 | 26 | 9 | 8 | 25 | 14 | 10 | 21 | |
| 35 | 22 | 6 | 21 | 25 | 3 | 2 | 16 | 11 | 15 | 18 | |
| 36 | 5 | 28 | 2 | 30 | 29 | 28 | 12 | 27 | 6 | 19 | |
| ← 12.7 m → | | | | | | | | | | | |
| Key | | | | | | | | | | | |
| Commando Persistent | Area = 187.96 m ² | | | | | | | | | | |
| Commando Original | Row | 1 m with 30cm gaps between | | | | | | | | | |
| Samson Persistent | Column | 40cm between rows | | | | | | | | | |
| Samson Original | | | | | | | | | | | |
| Border row (ovr. Arrow) | | | | | | | | | | | |

Appendix 2: Sample field trial growth score and leaf width calibration data

a) Herbage growth score



b) Leaf width score

