Phenotypic examination of variation occurring both among families and among genotypes within a *T. repens* x (*T. ambiguum x T. occidentale*) BC_1F_2 hybrid population.

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Abstract

Development of white clover cultivars with increased vegetative persistence, particularly in dryland farming systems has been a major goal within breeding programmes, however little useful genetic variation for survival and growth in these environments has been found. Consequently, it has become necessary to look towards white clovers wild relatives as sources of genetic variation. *T. repens* x (*T. ambiguum* x *T. occidentale*) tri-species hybrids have been developed, however, their morphologies have not been evaluated, and little is known about optimal breeding strategies in these populations.

An experiment was designed to characterise the magnitude of phenotypic variation for a range of root, shoot, and floral traits, and to ascertain optimal breeding strategies within a *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrid plant population. The experiment was designed such that it could be analysed in two ways;

a) Investigated levels of phenotypic variation occurring among hybrid families, compared to representatives of their F_1 parents

b) Investigated levels of phenotypic variation among individual hybrid genotypes, again compared to representatives of their F_1 parents.

Analysis (a) found a relative lack of among hybrid family variation. With significant (P<0.05) family variance components for 11 of the 18 traits measured, and generally only occurring between the upper and lower extremes. Repeatability estimates on a family mean basis were low (less than 0.51 for all traits).

Analysis (b) found significant (P<0.05) genotypic variance components for all of the traits measured. Repeatability estimates ranged from 0.47-0.88, indicating a relatively high level of genetic determination for the majority of traits.

Pattern analysis allowed the identification of hybrid genotypes showing the combined expression of key shoot, and root traits. These genotypes may provide a route to hybrid clover cultivars showing increased vegetative persistence via increased nodal and tap-root size, combined with good dry matter production.

The wide range of phenotypic variation and high repeatability estimates among hybrid genotypes, combined with the relative lack of variation and low repeatability estimates among hybrid families allowed us to conclude that phenotypic recurrent selection based on individual genotypes should be practised in these early generation hybrids populations.

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Abbreviation	Parameter	Units
F_1	First filial generation	
BC_1F_1	First generation backcross one hybrid	
BC_1F_2	Second generation backcross one hybrid	
AAOO	(*T. ambiguum* x *T. occidentale*) hybrid	
cv.	Cultivar	
OP	Open pollinated	
PS	Pollen stainablilty	%
PDL	Peduncle length	mm
FPI	Florets per inflorescence	
LL	Leaflet length	mm
LW	Leaflet width	mm
LL:LW	Leaflet length to leaflet width ratio	
PTL	Petiole length	mm
SD	Stolon diameter	mm
IL	Internode length	mm
SL	Stolon length	mm
SA	Stolon anchoring	mm
NRD	Nodal root diameter	mm
NRL	Nodal root length	mm
TRL	Tap root length	mm
TRD	Tap root diameter	mm
LDW	Leaf dry weight	g
SDW	Stolon dry weight	g
RDW	Root dry weight	g

Chapter 1 Introduction

1.1 Background

The genus *Trifolium* contains more than 250 species distributed across a wide range of habitats across the temperate and subtropical world (Zohary and Heller, 1984, Ellison et al., 2006). White clover (*T. repens* L.) is an important component of most temperate grazed pastures worldwide (Abberton and Marshall, 2010), and is an essential component of New Zealand pastures, where it has positive effects on animal performance, improving herbage quality, and improving soil fertility via nitrogen fixation, contributing $3.095 billion to New Zealands economy annually (Woodfield and Caradus, 1996, Caradus et al., 1996). However, poor vegetative persistence is a major limitation to the performance of white clover in many regions of the world (Jahufer et al., 2013), which is often exacerbated by summer water deficits, owing largely to white clovers relatively shallow root system, making it susceptible to drought in temperate regions, and limiting its use in semi-arid environments (Knowles et al., 2003). Therefore, the improvement of vegetative persistence has been a key trait among traditional plant breeding programmes (Abberton and Marshall, 2010).

Traditionally, white clover breeding in New Zealand has been based on phenotypic recurrent selection within adapted germplasm pools (Woodfield and Caradus, 1994, Williams, 1987). However, a lack of genetic variation within the white clover gene pool for key traits related to persistence has led to an increased interest in interspecific hybridisation. The wild relatives of white clover possess a range of characteristics that are potentially useful in breeding programmes. Of these species *Trifolium ambiguum* M. Bieb and *Trifolium occidentale* D.E. Coombe have emerged as major targets for wild relative trait introgression via interspecific hybridisation.

Trifolium ambiguum possesses several traits that could be beneficial to white clover, including; virus resistance (Barnett and Gibson, 1975, Pederson and McLaughlin, 1989, Anderson et al., 1991), increased vigour (Anderson et al., 1991), and thick, deep tap root systems with rhizome-base spreading habit (Forde et al., 1989). Direct hybridisation between *T. repens* and *T. ambiguum* is difficult, requiring embryo rescue,

and is further complicated by the predominance of 2n gametes in the F_1 population. There also appears to be limited pairing between chromosomes of the two species, indicating a reduced chance of introgression (Anderson et al., 1991, Meredith et al., 1995).

Trifolium occidentale is adapted to sandy, dry, salty conditions (Coombe, 1961), and has virus resistance traits (Gibson et al., 1971). It has been identified as one of the likely progenitor species of white clover (Williams et al., 2012), and is phylogenetically very closely related to it (Ellison et al., 2006), with direct hybridisation between chromosome doubled *T. occidentale* and white clover being possible (Chou and Gibson, 1968, Gibson and Beinhart, 1969, Hussain et al., 2016).

Despite being two of the most geographically and ecologically isolated species within Section *Trifoliastrum*, *T. ambiguum* and *T. occidentale* have retained the genetic compatibilities required for hybridisation (Williams et al., 2011). Both 2x and 4x forms of *T. ambiguum* are able to hybridise with 2x and 4x (cochicine doubled) forms of *T. occidentale*. *Trifolium ambiguum* and *T. occidentale* chromosomes pair at high frequencies, with hybrids between the two species being interfertile with white clover (Williams et al., 2011). Ullah (2013), was able to show introgression of 4x *T. ambiguum* chromosomal segments onto white clover chromosomes in a *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1 genotype, indicating that the use of *T. occidentale* as a genetic bridge for the introgression of *T. ambiguum* chromosomal segments into white clover was possible.

1.2 Gaps in knowledge

There has been little morphological characterisation work on ((*T. ambiguum* x *T. occidentale*) x *T. repens*) x *T. repens*)) hybrids, with previous work by Ullah (2013) focussing on the method of production of hybrids and ways of achieving introgression within hybrid genomes. There has been extensive morphological and physiological work on *T. repens* x *T. uniflorum* hybrids, with little work on the morphology of hybrids presented here. There is also a lack of knowledge relating to an optimal breeding strategy for these hybrid populations. This information is necessary to further inform decisions around the use of these hybrids, identify important characteristics, and assist in the creation of large scale breeding programmes.

1.3 Current context

AgResearch Ltd has a large *Trifolium* hybridisation programme, which has included the large scale production of F_1 (*T. ambiguum* x *T. occidentale*) x *T. repens* hybrids. The F_1 hybrids are of low agronomic value, owing largely to their complex genetic structure making them difficult to use in breeding programmes, however further backcrossing to *T. repens* has resulted in plants that largely resemble white clover, but with larger root systems. The rhizomatous nature of *T. ambiguum* as well as the natural habitat of *T. occidentale* (dry, and sandy soils) suggests that drought tolerance traits are likely to exist within these populations. Through hybridisation with *T. repens*, it is likely that some of these traits will be transferred, as well as some perhaps less desirable characteristics.

Cytological studies by Ullah (2013) have confirmed that introgression is occurring, in at least some hybrids in this population, and therefore variation within the population is expected, based on to what extent, and which chromosomal segments are present in a given individual. In addition, further variation is likely depending on the characteristics of the *T. repens* parents used in production of the hybrid, as well as possible recombination occurring between the two sub-genomes present in white clover if genetic control of homologous pairing in white clover is broken down due to hybridisation.

1.4 Hypothesis and objectives

The underlying hypothesis for this study was that newly developed *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrids would have different morphological characteristics than *T. repens*. The *T. ambiguum* and *T. occidentale* characteristics were expected to be transferred into a *T. repens* background to varying degrees. The extent to which traits have been introduced was investigated. Specifically, it was hypothesised that *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrids would show greater variation in nodal root size and length than *T. repens*.

Among hybrid family variation was investigated to ascertain whether family based selection, or individual genotype selection should be practised in early generation hybrid populations. It was hypothesised that variation would be greater among individual hybrid genotypes than among hybrid families.

Overall the aim was to describe levels of morphological variation among *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrid genotypes and families and compare them to representatives of their F_1 parents (*T. repens* and *T. ambiguum* x *T. occidentale*).

Chapter 2 Literature review

2.1 White clover

Trifolium repens (white clover) is a highly heterozygous, outcrossing, herbaceous legume characterised by its ability to reproduce both sexually (by profuse seeding) and asexually via fast growing stolons (Williams, 1987). Combined with its high nutritive value, and its ability to provide up to 380kg N ha^{-1} through its symbiotic relationship with nitrogen fixing bacteria (Crush, 1987) , white clover has become a highly sought after forage legume in the temperate world (Abberton, 2007). This high value as a forage crop has led to breeding for improved performance in pastures over the last 80 years, with hundreds of cultivars being produced (Caradus and Woodfield, 1997, Woodfield and Caradus, 1994). While there has been significant progress made in terms of genetic improvement for a variety of traits, it has been found that there is limited genetic variation within the white clover gene pool for some important traits, with white clover being the perennial forage most sensitive to drought conditions, with a 70% decline in dry matter yield under a 33% of irrigation water applied deficit in an Australian dairy farming system (Neal et al., 2009). Cultivars tolerant of drought and low soil fertility have not been developed.

2.1.2 White clover morphology

Seedling white clover plants consist of a primary axis of stem and seminal root, from which stolons radiate. Stolons consist of a series of internodes, separated by nodes. Each node bears a trifoliate leaf, two root primordia, and either an axillary bud (capable of growing into a lateral stolon, or an inflorescence depending on growth stage). When nodes are in contact with moist ground, nodal roots will form at the root primordia, providing a degree of nutritional independence to the lateral stolons (Thomas, 1987).

White clover root growth can be divided into two distinct phases, a seminal tap-rooted phase followed by a clonal growth phase (Brock et al., 2000). During the initial phase, significant growth of the root system including secondary thickening of the seminal root to form the tap root occurs (Westbrooks and Tesar, 1955, Brock et al., 2000). Nodal roots form on the stolons and these will eventually support the clonal plants formed after the death of the tap root at around 18 months (Brock et al., 2000). Tap

root death marks the beginning of the clonal phase which, in turn coincides with a decrease in herbage production (Westbrooks and Tesar, 1955). This is likely to be as a result of plants becoming dependent on thin, shallow nodal roots to supply vital nutrients and water, which then make the plant more vulnerable to a number of stresses, including drought (Bryant, 1974), viruses (Pederson and McLaughlin, 1989, Barnett and Gibson, 1975), and root chewing insects (Hussain et al., 2012).

The requirement for high moisture with relatively high soil fertility therefore limits the number of environments in which white clover can be grown. Consequently there is a real need to develop a more durable white clover for pastures in more marginal areas (Williams et al., 2007).

2.1.3 Traits affecting drought tolerance of white clover

White clover selected from dryland environments in New Zealand, tend to have higher rates of survival and dry matter yields under dry conditions than selections from other environments (Woodfield and Caradus, 1987, van den Bosch et al., 1993). It has been reported that white clover populations from dry environments are more “tap rooted”, with some populations showing increased “tap root diameter” (diameter of largest root), and greater numbers of “tap roots” (roots larger than 1mm basal diameter) compared to populations from moist environments (Woodfield and Caradus, 1987). Woodfield and Caradus (1987) were also able to show that genotypes surviving summer drought had high proportions of their root dry weight as “tap roots” (roots larger than 1mm basal diameter), as well as higher root: shoot dry weight ratios than the cultivar Tahora, which was bred for moist hill country.

Maintenance of high stolon density is considered an important trait related to white clover persistence under dry conditions with increased nodal root size possibly being a factor related to stolon density maintenance (Macfarlane et al., 1990). Brock and Kim (1994) concluded that the effect of drought was severe, regardless of stolon morphology, however they did suggest that genotypes exhibiting thinner, more dense stolons showed faster recovery post drought than their thicker stoloned counterparts. The same study however found that in genotypes with thicker stolons, leaf production was affected less than in those with thinner stolons during the “drying down” phase of a drought.

Whilst white clover is genetically diverse for many traits, it appears that no natural populations have developed into long lived perennials that are durable under certain types of stress, including drought and nutrient deficient soils. Rather, white clover seems to have evolved to survive stress by exploiting its ability to reseed, as a drought avoidance mechanism similar to an annual species (Hollowell, 1966). Therefore, it has become necessary to look for drought tolerance in the wild relatives, of which several appear to be well adapted to harsh conditions. *Trifolium ambiguum, T. occidentale*, and *T. uniflorum* L. all have combinations of enlarged underground organs, thick leaves, and more efficient water use than white clover (Abberton, 2007, Williams, 1987). Apart from *T. ambiguum* which is a valuable forage crop in its own right in some parts of the U.S.A (Abberton, 2007), these wild relatives are severely limited in many ways, preventing their direct agricultural use.

2.2 Genus *Trifolium* –wild relatives of white clover

The *Trifolium* genus is one of the largest in the Fabaceae family, with more than 250 species (Ellison et al., 2006, Abberton, 2007, Zohary and Heller, 1984) native to areas encompassing the temperate and subtropical regions of the Northern and Southern hemisphere (Ellison et al., 2006). The greatest species diversity is found in the Mediterranean basin, western North America, and the highlands of eastern Africa (Ellison et al., 2006). Several efforts have been made to place white clover within this framework and define its ancestry and wider gene pool.

Ellison et al. (2006) completed the most comprehensive phylogenetic analyses based on nuclear ribosomal DNA internal transcribed spacer and chloroplast *trnL* intron sequences of 218 of the approximately 250 species in the genus. Phylogenetic groupings were geographic, with an African and an American clade recognised, both rooted in the Mediterranean. With the most parisimonious reconstruction implying at least two dispersal events to Africa, whilst the American species comprise a monophyletic group indicating a single dispersal event (Ellison et al., 2006). This study placed white clover in a new section *Trifoliastrum*, a group comprising 16 other taxa widely distributed across Europe and West Asia (Table 1).

This is in contrast to the work of Zohary and Heller (1984) who, based on morphological taxonomy placed *T. repens* into the large (99 species) section *Lotoidea*,

with species distributed across Europe, the Americas, Africa, and Asia. Section *Lotoidea* was further divided into nine subsections, with *T.repens* placed in the largest subsection *Lotoidea* with 47 species. Finally, Subsection *Lotoidea* was further divided into series, with *T. repens* in series *Lotoidea* with 27 other species. The reclassification work of Ellison et al. (2006), placing *T. repens* in the new *Trifoliastrum* section, therefore greatly reduced the number of supposed close relatives of *T. repens*. It is proposed that the species within section *Trifoliastrum* are undergoing an adaptive radiation from a relatively recent common ancestor (Ellison et al., 2006, Williams et al., 2012), this is well supported by the apparent incompleteness of speciation in the section, as shown by the production of hybrids between two of the most geographically diverged species in the section, *T. occidentale* and *T. ambiguum* (Williams et al., 2012).

Taxa within *Trifoliastrum* exhibit a range of morphologies, chromosome number, and adaptive traits. They occur in an array of habitats, from coastal maritime (*T. occidentale*), to alpine environments (*T. ambiguum, T. pallescens* Schreb.), and represent an excellent resource for potential introgression by wide crossing into *T. repens*.

Table 1: Species of section *Trifoliastrum*; life form, chromosome number, distribution, habitat, and characteristics. Adapted from (Williams, 2014).

Species	Life form, chromosome no.	Geographical distribution	Habitat	Key characteristics
T. ambiguum	Perennial, 16, 32, 48	E Europe, W Asia, Caucasus	High altitude (>1500m) fields, screes	Large leaves, rhizomatous, virus resistances
T. cernum	Annual, 16	W Mediterranean, W Europe	Grassy areas	Small leaves, short peduncles
T. glomeratum	Annual, 16	Europe, Mediterranean, W Asia	Dry fields	Small leaves, sessile inflorescences
T. isthmocarpum	Annual, 16	Mediterranean	Moist fields and hillsides	Large leaves
T. montanum	Perennial, 16, 32	Europe, Caucuses, Turkey	Moderate-high altitude, grassy slopes	Large leaves, woody base
T. nigrescens* ssp. *nigrescens	Annual, 16	Mediterranean	Fields	Sprawling, prolific flowering
T. nigrescens* ssp. *petrisavii	Annual, 16	E Mediterranean, Turkey, W Asia	Fields	Sprawling, long flowering period
T. nigrescens* ssp. *meneghinianum	Annual, 16	Turkey	Fields	Giant form, large-leaved, hollow stems, sprawling
T. occidentale	Perennial, 16	W Europe	Close to coast, dunes, beaches	Small, stoloniferous, virus resistances
T. pallescens	Perennial, 16	Europe	High altitude (>1800m) slopes	Small leaves, woody taproot, prolific flowering
T. parnassi	Perennial, -	Greece (endemic)	Mountain slopes	Small leaves, woody taproot
T. repens	Perennial, 32	Mediterranean, Europe, NW Asia, C Asia	Damp grassy areas	Small-large leaved, stoloniferous
T. retusum	Annual, 16	Mediterranean, Europe, Turkey, Caucuses, W Asia	Grassy areas	Small, free-seeding
T. suffocatum	Annual, 16	Mediterranean, Europe, Turkey, Caucuses	Fields, roadsides	Very small, compact, sessile inflorescences
T. thalii	Perennial, 16	Europe, N Africa	High altitude mountain slopes	Small leaves, woody taproot
T. uniflorum	Perennial, 32	Mediterranean, W Turkey	Fields, scrub, mountain slopes	Small, prostrate, woody stems and taproot, large seeded

2.3 White clover ancestry

Trifolium repens is a tetraploid (2n=4x=32) species , which shows highly regular, bivalent chromosome pairing at meiosis, and exhibits disomic patterns of inheritance (Atwood and Hill, 1940). This strongly suggests that *T. repens* is an allotetraploid, resulting from the hybridisation of two or more probably diploid ancestors (Williams et al., 2012, Williams, 1987, Ansari et al., 1999). An early hypothesis was that *T. occidentale and T. nigrescens* Viv. were the diploid progenitor species based on the relative ease with which they cross with *T. repens* (Brewbaker and Keim, 1953, Gibson and Beinhart, 1969, Chou and Gibson, 1968), and the presence of the *Li* gene locus conditioning cyanogenesis (Williams and Williamson, 2001). It was found that while crosses between diploid *T. nigrescens* and diploid *T. occidentale* set seed at low

frequencies, diploid *T. nigrescens* and tetraploid *T. occidentale* set seed relatively freely. The resulting triploid progeny resembled *T. occidentale*, but had seemingly inherited the profuse flowering trait from *T. nigrescens* and set some seed when crossed with *T. repens* (Gibson and Beinhart, 1969). Williams et al. (2008) however found that crosses between diploid *T. nigrescens* and diploid *T. occidentale* set seed freely, producing hybrids that freely backcrossed with *T. nigrescens*. The chromosomes of the two species showed perfect pairing and regular disjunction at meiosis with introgression of genes from *T. occidentale* occurring in backcross progeny. However, none of the progeny resembled *T. repens* or *T. occidentale* and therefore it was concluded that *T. nigrescens* and *T. occidentale* were close relatives on one side of the ancestry of *T. repens* (Williams et al., 2008). Kazimierski and Kazimierska (1972) proposed *T. nigrescens* and *T. isthmocarpum* Brot. as potential progenitor species after *T. isthmocarpum* was shown to cross successfully with *T.repens*. Based on isozyme polymorphism data, Badr et al. (2002) favoured *T. uniflorum* L. and *T. nigrescens* as the parental species with further introgression from *T. isthmocarpum* and *T. occidentale*. Ellison et al. (2006) suggested that, based on their DNA sequence phylogeny, *T. pallescens* was the most likely species to be the female ancestor, with *T. occidentale* being the likely male progenitor species. The work of Williams et al. (2012) was then able to identify *T. pallescens* plants that contained chloroplast trnL intron DNA sequences identical to *T. repens*, and *T. occidentale* plants that had nuclear ITS sequences identical to *T. repens*. Furthermore reciprocal GISH experiments were conducted which showed that genomic DNA of *T. pallescens* hybridised with 16 of the 32 *T. repens* chromosomes, whilst *T. occidentale* genomic DNA hybridised with the other 16 chromosomes (Williams et al., 2012). Partly fertile stoloniferous hybrid plants with rooting at vegetative nodes were then able to be produced using *T. pallescens* as the mother. Predominant bivalent formation in the hybrid plants indicated that there was strong pairing between chromosomes of the two parent species. The diploid F_1 plants were able to be crossed with *T. repens* to produce tetraploid progeny via unreduced gametes, these progeny were interfertile with *T. repens*. A colchicine doubled form of one of the hybrids was also produced which was freely interfertile with *T. repens*, and essentially functioned as a synthetic white clover. This provides

strong evidence in favour of *T. pallescens* and *T. occidentale* being the progenitor species for *T. repens*.

However, there remain some uncertainties, and it appears unlikely that *T. repens* is a simple, two species hybrid. Hand et al. (2008) analysed SNP variation in the gene sequences of the two sub genomes of *T. repens*, and found that there was strong similarity between one set of the sub genomes and *T. occidentale*. However, the other set varied markedly from that of *T. pallescens*. The authors speculated that perhaps the second progenitor species was not *T. pallescens*, but rather some other, currently unclassified taxon. It is also plausible that as modern day *T. pallescens* has retreated to high altitudes and become narrowly adapted to the alpine zone (Williams et al., 2012), it may have significantly diverged from the form that may have hybridised with *T. occidentale*. By contrast *T. occidentale* has retained its geographic position and so remained genetically relatively stable (Williams et al., 2012). Cyanogenesis in *T. repens* is controlled by the polymorphic *Li* gene locus (Olsen et al., 2007). Accessions of both *T. pallescens* and *T. occidentale* have been shown to be acyanogenic, suggesting neither of the two proposed progenitor species contributed the *Li* gene locus to *T. repens* (Olsen et al., 2007). Williams and Williamson (2001) nominated *T. nigrescens* as a strong candidate for having donated the *Li*-carrying genome to *T. repens*, as it is the only close relative that shows cyanogenesis. This adds further weight to the idea that *T. repens* is not a simple hybrid of two species, with introgression from other wild relatives seemingly likely during its evolution.

2.3.2 Genetic control of chromosome pairing in *T. repens*

Although *T. repens* is a tetraploid, it has been described as behaving as a diploid at meiosis, with regular bivalent pairing and disomic inheritance being the norm (Atwood and Hill, 1940, Williams et al., 1998). Naturally there appears to be no homeologous pairing at meiosis between *T. pallescens* and *T. occidentale* chromosomes in white clover, with chromosomes preferentially pairing with homologs.

Conversely, in artificial hybrids between the two species, there appeared to be strong pairing between homeologous chromosomes (Williams et al., 2011). It therefore seems that there is some sort of genetic control operating to ensure homologous pairing within sub genomes in white clover (Pandey et al., 1987). However, this has not yet been comprehensively studied. Understanding, and knowing how to manage this

genetic control is essential for introgression breeding as it has the potential to restrict pairing between white clover genomes, and the genomes of other species.

2.4 Interspecific hybridisation and its potential in *Trifolium*

Interspecific hybridisation is a useful means of extending the range of heritable variation that can be exploited by plant breeders (Marshall et al., 1995) and can also lead to transgressive expression of new traits, not known in either parent, which can lead to rapid improvement in rates of genetic gain (Tanksley and McCouch, 1997). Of the approximately 250 species within *Trifolium*, only 24 species are of polyploid origin (Ellison et al., 2006, Zohary and Heller, 1984) and only five species are confirmed to have arisen as a result of hybridisation (Ellison et al., 2006). This scarcity of hybrid speciation indicates very strong barriers to interspecific hybridisation in the genus (Williams, 1987, Ellison et al., 2006, Taylor et al., 1980). However, as *T. repens* is an allopolyploid, opportunities may exist for interspecific hybridisation to contribute to white clover breeding. Based on their close relationship to, and known ability to form fertile hybrids with white clover, Williams et al. (2006) designated 8 other species from within the new Section *Trifoliastrum* as the "White Clover Complex" and proposed that these species were the most likely to contribute to the white clover gene pool through interspecific hybridisation. This group includes: *T. ambiguum*, *T. montanum* L., *T. nigrescens*, *T. isthmocarpum*, *T. uniflorum*, *T. occidentale*, *T. pallescens*, and *T. thalii* Vill. Species within this group have been identified as having potential benefits in disease and pest resistance, persistency, root development, and cold and drought tolerance (Brewbaker and Keim, 1953, Marshall et al., 1995), with *T. occidentale* and *T. ambiguum* being identified as two species which may confer such beneficial traits.

2.4.2 *Trifolium repens* x *T. occidentale*

Trifolium occidentale is a stoloniferous diploid, native to the Gulf Stream coasts of Europe (Williams et al., 2012). It occurs close to the sea and is adapted to sandy, dry, salty conditions (Coombe, 1961) and has virus resistance traits (Gibson et al., 1971). Having been identified as one of the likely progenitor species of white clover (Williams et al., 2012), and phylogenetically very closely related to it (Ellison et al., 2006), *T. occidentale* has emerged as a major candidate for the introgression of valuable traits into white clover.

Whilst *T. repens* x diploid *T. occidentale* crosses produce near sterile triploid plants with great difficulty, chromosome doubled *T. occidentale* is able to set seed freely with *T. repens* with no need for embryo culture (Chou and Gibson, 1968, Gibson and Beinhart, 1969, Hussain et al., 2016). High frequencies of multivalents at metaphase I indicated a close homology between the chromosomes of the two species, suggesting a strong likelihood of genetic recombination and introgression occurring (Chen and Gibson, 1970, Hussain and Williams, 2016). Hussain and Williams (2013) showed that unselected BC_1 plants were able to outperform four elite white clover genotypes in a drought experiment grown across a series of soil moisture levels.

2.4.3 *Trifolium repens* x *T. ambiguum*

Trifolium ambiguum is a rhizomatous perennial with a habitat range from river valleys to subalpine regions up to 2750m in Turkey, Romania, Crimea, and the Caucasus (Kannenberg and Elliott, 1962, Williams et al., 2011, Zohary and Heller, 1984). It occurs naturally in a ploidy series with populations native to high altitudes generally being diploid, and with tetraploid and hexaploid populations at lower elevations, although there is some overlap (Bryant, 1974). All forms share characteristics that could be beneficial to white clover, including; virus resistance (Barnett and Gibson, 1975, Pederson and McLaughlin, 1989, Anderson et al., 1991), increased vigour (Anderson et al., 1991), and thick, deep tap root systems with rhizome-base spreading habit (Forde et al., 1989). It has been noted that these agronomic advantages of *T. ambiguum*, are largely complementary to the weaknesses of white clover (Williams, 1987). It would therefore be of use to be able to introgress traits from the *T. ambiguum* gene pool into white clover. However, *T. ambiguum* is the most distantly related of all the species of the Section *Trifoliastrum* (Ellison et al., 2006), and the species do not freely interbreed (Kannenberg and Elliott, 1962).

2.4.4 Breeding with tetraploid *T. ambiguum*

A sterile hybrid between *T. ambiguum* and *T. repens* via embryo rescue was first reported by Williams (1978), and since then several researchers have reported the production of 4x hybrids between *T. ambiguum* and *T. repens*, with backcrosses to white clover also being reported (Meredith et al., 1995, Williams and Verry, 1981, Anderson et al., 1991). However, the possibility of introgression of *T. ambiguum* traits has been stifled by the predominance of 6x progeny resulting from the preferential

function of 2n gametes from the tetraploid hybrid parents when backcrossed to white clover (Anderson et al., 1991, Meredith et al., 1995).This has meant that breeding approaches have required complicated backcrosses from 6x to 4x via 5x and aneuploid intermediates. The occurrence of hexaploid progeny has also indicated a reduced chance of homeologous pairing as each chromosome has a homolog available, and so limited pairing between *T. ambiguum* and *T. repens* chromosomes may result in limited gene introgression (Meredith et al., 1995, Anderson et al., 1991, Williams et al., 2013, Ullah, 2013).

However, Abberton et al. (1998) were able to show that whilst introgression may be limited, it may still be possible to select for plants containing stable chromosome addition/substitutions expressing *T. ambiguum* traits. Plants were selected for rhizome expression in BC_1 and BC_2 generations. This selection led to white clover-like, stoloniferous BC_3 plants exhibiting 3% of total dry weight as rhizomes(Abberton et al., 1998) . Marshall et al. (2001) were then able to show that *T. ambiguum* leaves were able to retain relative water content and continue growing, whilst the leaves of *T. repens* plants displayed decreased relative water content and stopped growing under dry soil conditions. BC_1 and BC_2 hybrids were intermediate between the two parental species indicating *T. ambiguum* as a possible source of drought tolerance traits for white clover. Marshall et al. (2003) showed that forage yield of BC_1 and BC_3 plants was comparable to that of white clover. Nitrogen fixation of BC_2 plants was shown to be similar to that of white clover in flowing solution culture (Abberton et al., 1999). Abberton et al. (2002) were able to show that in the glasshouse, hybrid plants had dry matter digestibility levels comparable to that of white clover, whilst having slightly higher water soluble carbohydrate than white clover. Marshall et al. (2004) confirmed similar results in field experiments with the ratio of water soluble carbohydrate to protein being higher in the hybrid plants.

Abberton et al. (2003) were able to use bulked segregant analysis of BC_3 families to identify an AFLP band that was always associated with rhizome formation. This band was then further identified in BC_2, BC_1 and *T. ambiguum* plants. This could be hugely beneficial in allowing marker assisted selection for rhizomes. This would speed up the breeding process as rhizome development usually takes up to 18 months to occur (Abberton et al., 2003).

Alternative breeding strategies have been developed by Williams and Hussain (2008) whereby the 6x backcross was the terminal form, with selection and further crossing within the BC_1 population. This method would remove the need for complicated backcrossing programmes to return the hybrid populations to the tetraploid level, with the BC_1 population essentially being comprised of white clover carrying two supplementary sub-genomes from *T. ambiguum* (Meredith et al., 1995, Williams and Hussain, 2008).

Hussain and Williams (1997) were able to cross a chromosome doubled version of a tetraploid hybrid produced by Williams and Verry (1981) with white clover to generate hexaploid plants. This strategy had the advantage that crosses between the octoploid and white clover can be achieved using either plant as female, as opposed to having to use the hybrid as female in the previous method.

The major weakness of all of these methods is the inability to backcross to the *T. ambiguum* parent. Therefore, all further backcross families contain only the original *T. ambiguum* alleles which results in limited genetic diversity for selection of *T. ambiguum* conferred traits. This issue can be solved via the large scale production of F_1 hybrids, or through other means of introgressing *T. ambiguum* alleles, however, to date, F_1 hybrids have only been able to be created using two Turkish tetraploid *T. ambiguum* accessions as parent, again limiting possible genetic diversity.

2.4.5 Breeding with diploid *T. ambiguum*

Direct crossing between diploid *T. ambiguum* and *T. repens* has proven to be difficult, however Williams et al. (2013) have shown that crosses between diploid *T. ambiguum* and hexaploid (*T. ambiguum* x *T. repens*) hybrids can produce tetraploid progeny interfertile with white clover. This finding represents a way of incorporating alleles from both tetraploid and diploid *T. ambiguum* into breeding populations.

2.4.6 Breeding with hexaploid *T. ambiguum*

Whilst hexaploid *T. ambiguum* is the most agronomically impressive form in its own right (Bryant, 1974), direct hybridisation with white clover has not yet been achieved. However Williams et al. (2013) have reported the production of complex hybrids between hexaploid *T .ambiguum* and hexaploid (*T. ambiguum* x *T. repens*), but these hybrids are very infertile.

2.5 The use of *T. occidentale* as a genetic bridge for the introgression of *T. ambiguum* alleles

Given the close association of *T. occidentale* with both *T. repens*, and *T. ambiguum*, it would seem to be a prime candidate for use as a bridging species. Gibson et al. (1971) were able to show that barriers to interspecifc hybridisation between *T. repens* and *T. uniflorum* were reduced by first crossing either species with *T. occidentale*, before making tri species hybrids. This method may play a vital role in the introgression of *T. ambiguum* traits into white clover.

Williams et al. (2011) were able to show proof of concept for the use of *T. occidentale* as a genetic bridge for the incorporation of *T. ambiguum* traits into white clover. Diploid *T. occidentale* was crossed with diploid *T. ambiguum* to give partially fertile diploid (*T. ambiguum* x *T. occidentale*) plants. A colchicine doubled tetraploid was also able to be produced. Meiotic chromosomes were shown to pair at high frequencies in the hybrids indicating a likelihood of genetic recombination occurring. Both of these forms were able to be crossed with white clover indicating the possibility that *T. occidentale* can be used a bridge species. Williams et al. (2013) were also able to show the same processes occurring using tetraploid *T. occidentale* crossed with tetraploid *T. ambiguum*. Again the tetraploid progeny were interfertile with white clover. This represents an opportunity to use *T. occidentale* as a genetic bridge for the incorporation of both diploid and tetraploid *T. ambiguum* genetic material into white clover. There is also scope for the interbreeding between colchicine doubled (diploid *T. occidentale* x diploid *T. ambiguum*) and (tetraploid *T. occidentale* x tetraploid *T. ambiguum*), allowing the introgression of alleles from both diploid and tetraploid *T. ambiguum* at the same time.

Tri species hybrids between *T. repens*, *T. uniflorum*, and *T. occidentale* were reported by Ferguson et al. (1990). They were apparently interfertile with hexaploid *T. ambiguum* and one hybrid was produced. Furthermore (Williams et al., 2013) reported partially fertile tetraploid hybrids between hexaploid *T. ambiguum* and diploid *T. occidentale*, with the production of one partially fertile near hexaploid hybrid with white clover (Williams et al., 2013). This strategy could provide scope for the introduction of hexaploid *T. ambiguum* genetic material into white clover.

2.6 Hybrid morphology

Morphology of *Trifolium* F_1 hybrids has tended to be intermediate between the two parental species. This trend has been shown in *T. repens* x *T.ambiguum* (Meredith et al., 1995, Williams, 1978), *T.repens* x *T. uniflorum* (Pandey, 1957, Gibson et al., 1971), and *T. repens* x *T. nigrescens* (Marshall et al., 1998, Hussain et al., 1997a) hybrids. Backcrosses to white clover show increasing resemblance to white clover, with significant morphological variation among genotypes in all generations. Ullah (2013) showed that ((*T. ambiguum* x *T. occidentale*) x *T. repens*) x *T. repens*)) BC_1F_1 genotypes combined traits from *T. repens* and *T. ambiguum*, with variation among genotypes linked to the degree to which *T. ambiguum* chromosomes or chromosomal segments had been introgressed. Transgressive segregation appears to be ubiquitous among plant hybrids, and has been noted in *T. repens* x *T. uniflorum* hybrids (Pandey et al., 1987), and thus we would expect to find some genotypes expressing variation outside of the range of the parental species in this study.

2.7 Future options and concluding remarks

The ability to introgress traits from both *T. occidentale* and *T. ambiguum* into a white clover back ground exists. This has the potential to widen the adaptive range of white clover by increasing its pest, virus, and drought tolerance. This will however require the production of large hybrid breeding populations and enhanced breeding strategies. In recent years there have been significant improvements in the molecular methods available to improve the efficiency of introgressing wild relative traits in plant breeding (Tanksley and McCouch, 1997, Tanksley and Nelson, 1996), however there has been little to no development for the application of these new molecular marker technologies in white clover . With these advances, and the elucidation of *T. occidentale* as a putative progenitor species, and candidate as a genetic model for white clover (Richardson et al., 2013, Williams et al., 2009) it is expected that the development of a reference DNA sequence may lead the way to the application of molecular marker technologies in white clover(Griffiths et al., 2013).

As hybrid populations often express accelerated elimination of chromosomes and chromosomal segments (Rieseberg et al., 1999), efficient breeding strategies will need to be applied on a case by case basis, with different strategies potentially needing to

be applied to each species combination to maximise the recovery of beneficial traits, whilst minimising linkage drag of deleterious traits from the wild relatives.

Chapter 3 Materials and methods

3.1 Plant population development

In keeping with convention, *T. ambiguum*, *T. occidentale* and *T. repens* sub-genomes (x=8) are here designated as A, O and R, respectively.

Tetraploid (*T. ambiguum* x *T. occidentale*) hybrids were generated via embryo rescue by the Germplasm Development team at AgResearch Grasslands. (*T. ambiguum* x *T. occidentale*) hybrids were produced from both diploid (genomic constitution A^DA^D) and tetraploid (genomic constitution $A^TA^TA^TA^T$) forms of *T. ambiguum*. These plants are interfertile with each other, however they are not fertile with *T. repens* and are hence reproductively isolated. For the purposes of this study, they have been treated as a separate "species" and are referred to as "AAOO" throughout.

This study defines the first cross (F_1) as 4x (*T. ambiguum* x *T. occidentale*) x *T. repens*. The expected genomic constitution was AORR. F_1 hybrids were again developed via embryo rescue by the Germplasm Development team at AgResearch Grasslands. The female *T. repens* plant in these crosses was "RedOne", a vigorous, free-flowering genotype of unknown parentage. RedOne is heterozygous for the Red Leaf gene (dark red leaf with green margin, expressed more strongly in cold environments), is generally self-incompatible but gives a few selfed seed, some of which are "4-leaf" (4 leaflets per leaf).

The first backcross (BC_1F_1) to white clover was ((*T. ambiguum* x *T. occidentale*) x *T. repens*) x *T. repens*)). The genomic constitution was expected to be, on average, RRR(A_4O_4). *Trifolium repens* plants used in the generation of the BC_1F_1 were from elite white clover breeding lines. This generation was produced by hand crossing pollen from *T. repens* plants onto RRAO plants. These crosses generally produced only a few seeds, and they were previously made by the Germplasm Development team at the AgResearch Grasslands Research Centre, Palmerston North, New Zealand.

The hybrids for this study were formed by hand crossing among unrelated BC_1F_1 plants, and so were designated as BC_1F_2, and their average genomic constitution was expected to be unchanged from the BC_1F_1.

Generation of BC_1F_2 families provided an opportunity for recombination among the A, O and R sub-genomes, and so the formation of *T. repens*-like plants with genomic segments inserted from *T. ambiguum* and *T. occidentale*. Large numbers of these plants were produced for phenotypic screening.

3.2 Trial Site

The study was conducted at AgResearch Grasslands campus, Palmerston North. Plants were cultivated in a 14 x 7 x 1m deep wooden frame that was filled with river sand.

3.3 Plant material

20 BC_1F_2 hybrid families were chosen from lines with excess seed and that represented a broad sample of the available germplasm (Table 2). BC_1F_2 lines were chosen because BC_1F_1 lines contain only a few seeds, and the use of cuttings would not have allowed for an assessment of tap-root variation.

Representatives of the parental lineage of the original F_1 cross that led to the BC_1F_2 hybrids were used as controls in this experiment. The cultivar "Crusader" was chosen as the white clover parental control as it is a fairly typical, medium leaved cultivar that shows good persistence in New Zealand farming systems, and represents the *T. repens* side of the *T. repens* x (*T. ambiguum* x *T. occidentale*) cross. The (*T. ambiguum* x *T. occidentale*) line 3-17-4-OP was chosen as the other parental control as it is a fairly typical example of a (*T. ambiguum* x *T. occidentale*) hybrid cross, exhibiting characteristics from both parental species, and is related to the parents of some of the plants used in this study.

Table 2: Experimental entries, their parentage, and expected genomic constitution.

Entry	Female parent	Male parent	Expected genomic constitution
1	(RRAO-13 x 23/2)-1	(RRAO-2-8 x 111/2)-1	RRR(A_4O_4)
2	(RRAO-17 x 112/3)-1	(RRAO-2-8 x 111/2)-1	RRR(A_4O_4)
3	(RRAO-21 x 18/2)-1	(RRAO-16 x 111/2)-1	RRR(A_4O_4)
4	(RRAO-5 x 23/2)-2	(RRAO-13-6 x 82/1)-1	RRR(A_4O_4)
5	(RRAO-2(3) x 65/2)-2	(RRAO-8 x 23/2)-1	RRR(A_4O_4)
6	(RRAO-16 x 111/2)-1	(RRAO-2(3) x 65/2)-2	RRR(A_4O_4)
7	(RRAO-13b x 82/1)	(RRAO-5 x 15/1)-5	RRR(A_4O_4)
8	(RRAO-13b x 82/1)	(RRAO-2-1 x 18/2)-1	RRR(A_4O_4)
9	(RRAO-17 x 23/2)-2	(RRAO-21 x 18/2)-1	RRR(A_4O_4)
10	(RRAO-17 x 23/2)-2	(RRAO-2-8 x 37/3)-1	RRR(A_4O_4)
11	(RRAO-17 x 23/2)-2	(RRAO-22 x (KR907 + Tahora)-4	RRR(A_4O_4)
12	(RRAO-17 x 67/1)-2	(RRAO-2(3) x 65/2)-1	RRR(A_4O_4)
13	(RRAO-17 x 67/1)-2	(RRAO-2-1 x 23/2)-1	RRR(A_4O_4)
14	(RRAO-17 x 67/1)-2	(RRA0-2-5 x 23/2)-1	RRR(A_4O_4)
15	(RRAO-17 x 67/1)-2	(RRAO-22 x (P x B)-5)-2	RRR(A_4O_4)
16	(RRAO-B x 67/1)-1	(RRAO-22 x (P x B) 5 ⊗-125))-1	RRR(A_4O_4)
17	(RRAO-B x 67/1)-1	(RRAO-2(3) x 65/2)-1	RRR(A_4O_4)
18	(RRAO-B x 67/1)-1	(RRAO-2-8 x111/2)-1	RRR(A_4O_4)
19	(RRAO-B x 67/1)-1	(RRAO-2-5 x 23/2)-1	RRR(A_4O_4)
20	(RRAO-B x 67/1)-1	(RRAO-2-1 x 23/2)-2	RRR(A_4O_4)
21	3-17-4	OP	AAOO
22	*T. repens* cv. "Crusader"		RRRR

3.4 Experimental design

The experiment was designed in such a way that it could accommodate two levels of analysis;

a) Characterise the magnitude of genotypic variation among the 20 BC_1F_2 families, AAOO line 3-17-4-OP, and white clover cv. "Crusader" (Table 2).
b) Assess levels of genotypic variation among all of the individual genotypes that were evaluated in (a)

To enable the two levels of analysis, six genotypes representing each of the 22 experimental entries (Table 2) were randomly allocated within a 20 row-by-8 column configuration. Fourteen clones from each of two representative genotypes of the parental species (AAOO and *T. repens*) were systematically allocated within the experimental area as repeated clonal checks.

This configuration enabled the partitioning of the trial into two balanced replicates, consisting of 10 row-by-8 column structures. Three genotypes from each of the experimental entries were randomly allocated within each replicate. As well as 7 clonal checks from each of AAOO and *T. repens* which were systematically allocated within each replicate.

Analysis (a) was conducted using the replicated design structure where each family was represented by a sample of 3 genotypes per replicate.

In (b) the data were analysed using a non-replicated spatial analysis based on the repeated clonal checks (Gleeson, 1997, Kempton and Gleeson, 1997), which enabled the characterisation of potential environmental and spatial effects within the design.

Full copies of both experimental designs are presented in appendix 1 and 2.

3.5 Plant establishment

Seed was scarified using sandpaper and germinated on damp filter paper in petri dishes, before being planted on July 15 2014 into a sand/peat based potting mix in 40 x 40 x 80mm cell trays and grown under natural lighting in a glasshouse with temperature ranging from 14-25 °C. Any fatalities occurring within one week of germination were replaced with spare seedlings.

Clonal check plants were produced from one stock genotype of both *T. repens* cv. “Crusader” and AAOO line 3-17-4-OP. 30-40 mm stolon cuttings containing 1 active growing point, and one main root, with 3 fully open trifoliate leaves were taken. These cuttings were grown in the same conditions as the seedling plants. After a ten week establishment period, the clonal and seedling plants were moved outside for 4 weeks to acclimatize before being trans-planted into the experimental site on October 23 2014.

3.6 Planting and trial management

Seedlings were removed from trays for planting, and placed in a 0.6 x 0.6m grid according to the experimental design. The experimental area was surrounded by a border row of excess hybrid seedlings in an attempt to minimise edge effects. The experiment was watered daily via overhead sprinkler and plants were fertilised fortnightly with the liquid fertiliser Yates Thrive® Soluble All Purpose Plant Food (NPK

of 27: 5.5: 9 + trace elements), 250ml was applied per plant, per fortnight. Weed control within the plot was via mechanical weeding which maintained a low density of weeds within the plot. Following 7 months of growth, plants were excavated, beginning May 20 2015, and ending July 2 2015 with care taken to remove the entire root system.

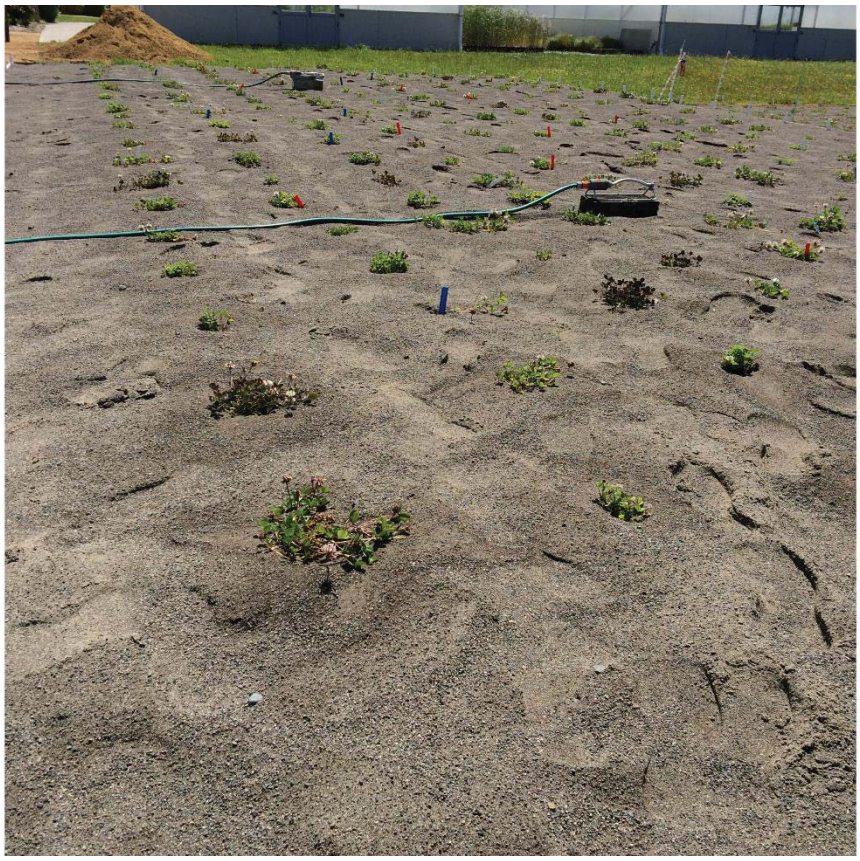

Plate 1: Experimental area at AgResearch Grasslands, Palmerston North

3.7 Measurements

Key morphological traits were measured at differing times throughout the duration of the experiment.

2.7.1 Floral characteristics

Floral characteristics were measured in December 2014 during peak flowering. Male fertility was determined by an estimation of pollen stainability (PS). A minimum of 3 anthers were dehisced over a glass slide to which a drop of 2% acetocarmine was added. The material was then covered by a coverslip and following 5 min of staining, the percentage of plump, fully stained pollen grains was determined. At least 300 pollen grains from 3 or more florets from 3 inflorescences were examined per

genotype (Hussain et al., 1997b) . Peduncle length (PDL) and number of florets per inflorescence (FPI) were determined from 3 fully expanded inflorescences per genotype.

2.7.2 Shoot characteristics

The leaf traits, leaflet length (LL) and leaflet width (LW) were measured on the middle leaflet of the youngest fully expanded trifoliate leaf at harvest, on a minimum of 3 leaflets per genotype, with the ratio (LL:LW) of the two measurements also being calculated. Petiole length (PTL) was measured on the same leaves. Stolon diameter (SD) and internode length (IL) were measured on the second internode from the stolon growing tip. Stolon diameter was measured at the midpoint of the internode, and again measurements were taken on a minimum of three stolons per genotype. Stolon length (SL) was taken as the length of the three longest individual stolons on each genotype.

2.7.3 Root characteristics

Following excavation of each plant, the sand substrate was carefully washed away from the root system. Stolon anchoring (SA) was taken as the distance from the stolon growing tip, to the first incidence of a nodal root, and was measured on 3 randomly selected stolons. Nodal root measurements were based on the three longest nodal roots on each genotype, with diameter (NRD) measurements being taken 5mm below the intercept of root and stolon. Nodal root length (NRL) was measured as the total length of the same nodal roots. The seminal tap root was identified and its length measured (TRL), again diameter (TRD) was taken 5mm below the root/stolon intercept.

2.7.4 Dry weight yield

Once morphological measurements had been taken, the plants were divided into their component parts (leaf, stolon, and root). The parts were then dried at 80°C for 24 hours before being weighed, to give leaf (LDW), stolon (SDW), and root (RDW) dry weight measurements.

3.8 Data analysis

The objective of the analysis was to quantify the magnitude and patterns of family, and genotypic variation among the 120 BC_1F_2 genotypes, as well as the 6 AAOO, and 6 *T.*

repens genotypes for a variety of shoot and root morphological traits. The analysis of the plant root and shoot traits consisted of; analysis of variance, pattern analysis, the estimation of family and genotype mean repeatability, and the calculation of phenotypic correlation coefficients.

3.8.1 Analysis of variance

(i)The analysis of variance for the root and shoot traits FPI, PS, PDL, LL, LW, LL:LW, PTL, LDW, IL, SL, SD, SDW, SA, NRD, NRL, TRD, TRL, and RDW was carried out using the variance component analysis procedure Residual Maximum Likelihood (REML) option, in GenStat 7.1 (GenStat, 2003). A linear mixed model was used in the analysis using the REML algorithm, with repeated clonal checks treated as fixed effects. Analysis resulted in the generation of a Family x trait matrix, and a Genotype x trait matrix consisting of adjusted means/Best Linear Unbiased Predictor (BLUP) (White and Hodge, 1989) values.

The linear model used in the analysis of among family variance was:

$$Y_{ijklmn} = M + f_i + b_j + r_{jk} + c_{jl} + s_{ijm} + p_n + \varepsilon_{ijklmn}$$

Where, Y_{ijklmn} is the value of an attribute measured from family *i* in replicate *j* in row *k* and column *l* of sample *m*, and i=1,...,n_f, j=1,...,n_b, k=1,...,n_r, l=1,...,n_c, m=1,...,n_s, ; *M* is the overall mean; f_i is the random effect of family *i*, N(0,σ^2_f); b_j is the random effect of replicate *j*, N(0,σ^2_b); r_{jk} is the random effect of row *k* in replicate *j*, N(0,σ^2_r); c_{jl} is the random effect of column *c* in replicate *j*; s_{ijm} is the random effect of sample *m* taken from family *i* within replicate *j*, N(0,σ^2_{gs}); p_n is the fixed effect of repeated check *n* N(0,σ^2_p); ε_{ijklmn} is the residual effect for family *i* in replicate *j* in row *k* and column *l* from sample *m*, and *n* is the repeated check effect, N(0,σ^2_ε).

The linear model used in the analysis of among genotype variance was:

$$Y_{ijkl} = M + g_i + r_j + c_k + s_{il} + p_m + \varepsilon_{ijklm}$$

Where, Y_{ijkl} is the value of an attribute measured from genotype *i* in row *j* and column *k* of sample *l*, and i=1,...,n_g, j=1,...,n_r, k=1,...,n_c, l=1,...,n_s; *M* is the overall mean; g_i is the random effect of genotype *i*, N(0,σ^2_g); r_j is the random effect of row *j*, N(0,σ^2_r); c_k is the

random effect of column *k* , N(0,σ^2_c); s_{il} is the random effect of sample *l* taken from genotype *i*, N(0,σ^2_s); p_m is the fixed effect of repeated check *m* N(0,σ^2_p); ε_{ijklm} is the residual effect for genotype *i* in row *j* and column *k* from sample *l*, and *m* is the repeated check effect, N(0,σ^2_ε).

3.8.2 Repeatability

Genotypic and family variance components estimated for the traits using REML were used to estimate repeatability (Falconer and Mackay, 1996). Repeatability on a family mean basis was estimated using:

$$1) \quad R = \frac{\sigma^2_F}{\sigma^2_F + \frac{\sigma^2_\epsilon}{n_b}}$$

where σ^2_F is the family variance component, σ^2_ϵ is the error variance component, and n_b is the number of replicates.

Repeatability on a genotype mean basis was estimated using:

$$2) \quad R = \frac{\sigma^2_g}{\sigma^2_g + \sigma^2_\epsilon}$$

Where σ^2_g is the genotypic variance component, and σ^2_ϵ is the error variance component.

3.8.3 Phenotypic correlation coefficients

Phenotypic correlation coefficients between the characteristics measured were estimated according to Becker (1992).

3.8.4 Pattern analysis

The Genotype x trait BLUP matrix was divided into three separate matrices to each be examined using pattern analysis. Floral traits and derived ratios were removed from the matrix as we were primarily interested in vegetative, and root traits. The matrix was then divided into vegetative, and root BLUP matrices, with a final analysis based on key vegetative, and root characteristics. Leaflet width was eliminated from the final analysis as we decided that one measure of leaflet size (leaflet length) was sufficient. Pattern analysis is a combination of principal components analysis, and cluster analysis (Gabriel, 1971, Kroonenberg, 1994, Watson et al., 1996). Scaling effects were removed by standardising BLUP values to have a mean of zero and a variance of one (Cooper

and DeLacy, 1994). Clustering of genotypes based on morphological measurements was carried out using an agglomerative hierarchical clustering procedure with squared Euclidean distance as a measure of dissimilarity and incremental sums of squares as a grouping strategy (Ward, 1963, Burr, 1968, Burr, 1970, Wishart, 1969). A 32-bit PC version of the GEBEI package (Watson et al., 1996) was used to conduct the clustering. The optimum level of truncation for the resulting hierarchy was chosen based on the increase in the sum of squares among genotype groups as the number of groups increased. The group level selected was determined by the point at which the percentage of genotype 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) (Kroonenberg, 1994). The plotting points from the ordination were used to construct biplots (Gabriel, 1971).

Chapter 4 Results

4.1 Analysis (a) - Variation among BC_1F_2 families

The differences between the predicted mean values indicated that there was a narrow range of phenotypic variation between the two parental species AAOO and *T. repens* (Table 3). There was significant (P<0.05) variation between the two species for only three traits (leaflet width, petiole length, and florets per inflorescence).

The differences between the minimum and maximum values indicated that there was a relatively narrow range of phenotypic variation between BC_1F_2 families (Table 3). There was significant (P<0.05) variation between BC_1F_2 families for the traits; stolon anchoring (SA), nodal-root diameter (NRD), nodal-root length (NRL), pollen stainability (PS), peduncle length (PL), stolon diameter (SD), internode length (IL), leaflet length (LL), leaflet width (LW), leaflet length to width ratio (LL:LW), and stolon length (SL), although these differences were generally only significant between the upper and lower extremes. There was no significant variation among families for the traits; tap-root diameter (TRD), tap root length (TRL), root dry weight (RDW), florets per inflorescence (FPI), petiole length (PL), leaf dry weight (LDW), and stolon dry weight (SDW). The family mean repeatability (R) enabled an estimation of the upper limit of genetic determination (Falconer and Mackay, 1996), and was less than 51% for all traits.

Stolon diameter proved to be the trait with the most variation of interest. There was significant (P<0.05) variation among the thickest and thinnest of the BC_1F_2 families, with families 10 and 11 having stolons with diameters significantly (P<0.05) larger than both the *T. repens* and AAOO parental populations. No BC_1F_2 families had stolons with smaller diameters than either of the two parental species.

There was little other significant variation from either parental species for the majority of the other traits measured, suggesting that among family variation is relatively low in this population.

4.1.1 Root characteristics

There was no significant difference between the *T. repens* and the AAOO population for any of the root characteristics that were measured in this study. There was a non-

significant trend towards the AAOO population having thicker and longer tap-roots than the *T. repens* population. The mean of the BC_1F_2 families was intermediate between the two parental species for tap root diameter, and tap root length, with the BC_1F_2 families mean being larger than the parental species for both of the nodal root traits measured. BC_1F_2 family mean stolon anchoring occurred further from the growing tip than either of the parental species.

There was significant (P<0.05) variation among the BC_1F_2 family means for both of the nodal root traits measured, with diameters ranging from 1.99 (Fam 5) to 3.73mm (Fam 13 and Fam 18) and lengths ranging from 180 (Fam 5) to 405mm (Fam 15). However, no BC_1F_2 families nodal root diameters or lengths varied significantly from either parental population.

There was no significant variation among BC_1F_2 families for either of the tap root traits measured, with no BC_1F_2 family means differing significantly from either parental population for either of the tap root traits.

There was no significant difference between the *T. repens* and the AAOO population for root dry weight. BC_1F_2 families also showed no significant variation for root dry weight, with no hybrid family differing significantly from either parental species.

There was significant (P<0.05) variation among BC_1F_2 families for stolon anchoring, however this was only among the upper and lower extreme families. Fam 19 had stolon anchoring that occurred significantly (P<0.05) further from the growing tip than either of the parental populations, with no other families differing significantly.

4.1.2 Fertility characteristics

The *T. repens* population had more florets per inflorescence (P<0.05), but pollen fertility, and peduncle length did not differ significantly from the AAOO population. The mean of the BC_1F_2 families was intermediate between the two parental species for the florets per inflorescence, and peduncle length traits, whilst it was lower than both of the parental species for pollen fertility.

There was no significant variation among BC_1F_2 families for florets per inflorescence, although several BC_1F_2 families (Fam 1, Fam 6, Fam 10, Fam 12) had significantly

(P<0.05) more florets per inflorescence than the AAOO population. No BC_1F_2 families differed significantly from the *T. repens* population.

There was significant (P<0.05) variation among BC_1F_2 families for pollen fertility and peduncle lengths, however this variation was only seen between families at the extremes. Family 7 had pollen that was significantly (P<0.05) less fertile than that of both the AAOO and *T. repens* populations. Several BC_1F_2 families (Fam 1, Fam 2, Fam 6, Fam 7, Fam 10, Fam 12, Fam 13, Fam 15, Fam 16) had peduncles that were significantly (P<0.05) longer than the AAOO population. No BC_1F_2 families had peduncles that were significantly different from the *T. repens* population.

4.1.3 Dry matter yield

There were no significant differences for leaf, or stolon dry weight between the *T. repens* and AAOO populations. There was no significant variation among BC_1F_2 families in leaf, or stolon weight, with no families differing significantly from either parental species

4.1.4 Stolon and leaf characteristics

The AAOO, and *T. repens* populations did not differ significantly for internode length, stolon length, and stolon diameter. The mean of the BC_1F_2 families was intermediate between the two parental species for stolon length, with the mean being higher than the two parental species for the internode length and stolon diameter traits.

There was significant (P<0.05) variation between the smallest and largest BC_1F_2 families for internode and stolon length. No BC_1F_2 families varied significantly from either of the parental species for internode length, whilst 7 BC_1F_2 families (Fam 4, Fam 6, Fam 7, Fam 9, Fam 10, Fam 17, Fam 18) had stolons that were significantly (P<0.05) longer than the AAOO population. No BC_1F_2 families varied significantly from the *T. repens* population for stolon length.

The *T. repens* population had wider (P<0.05) leaflets, with longer petioles (P<0.05) than the AAOO population, but leaflet length and leaflet length to width ratio did not differ significantly. The mean of the BC_1F_2 families was intermediate between the two parental species for all of the leaf traits that were measured.

There was significant (P<0.05) variation between the largest and smallest BC_1F_2 families for mean leaflet lengths and widths. Three BC_1F_2 families (Fam 2, Fam 10, Fam 11) had leaflets that were significantly (P<0.05) longer and wider than the AAOO population. No BC_1F_2 families had leaflet lengths or widths that varied significantly from the *T. repens* population.

There was significant (P<0.05) variation among several BC_1F_2 families in mean leaflet length to width ratios, with family means ranging from 0.99 (Fam 4) to 1.20 (Fam 13). Family 4 had a leaflet length to width ratio that was significantly (P<0.05) lower than the AAOO population, whilst Family 13 had a leaflet length to width ratio significantly (P<0.05) higher than that of the *T. repens* population.

BC_1F_2 family mean petiole lengths ranged from 23.7 (Fam 9) to 40.8mm (Fam 10), but this variation was not significant. Two BC_1F_2 families (Fam 2, Fam 10) had petioles significantly (P<0.05) longer than the AAOO population. No BC_1F_2 families had petiole lengths that were smaller than the AAOO population. Six BC_1F_2 families (Fam 4, Fam 9, Fam 10, Fam 13, Fam 15, Fam 19, Fam 20) had petioles that were significantly shorter than the *T. repens* population. No BC_1F_2 families had longer petioles than the *T. repens* population.

Table 3: Means, ranges, and variance components (σ^2) with associated standard errors (±SE) for various traits measured from 20 *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2, one *T. ambiguum* x *T. occidentale*, and one *T. repens* cv. Crusader families grown in sand.†

Family	FPI	PS	PDL	LL	LW	LL:LW	PTL	LDW	IL	SL	SD	SDW	SA	NRD	NRL	TRD	TRL	RDW
BC_1F_2 family mean	48	80	89.2	10.1	9.7	1.08	29.7	4.9	7.2	290	1.92	17.5	17.8	2.95	319	4.80	348	12.7
BC_1F_2 family range	40-56	50-94	77.4-101.4	8.3-12.3	8.2-12.2	0.99-1.20	23.7-40.8	3.9-5.9	5.4-8.4	187-425	1.73-2.13	15.4-19.2	4.5-43.9	1.99-3.73	180-405	3.17-6.57	337-361	11.4-14.6
AAOO	35	87	70.0	7.3	6.4	1.14	16.6	3.8	6.3	97	1.73	14.9	13.1	2.42	305	6.24	354	11.8
RET	56	96	91.6	10.8	11.0	1.02	46.8	6.0	6.1	314	1.72	19.7	9.1	2.87	284	3.15	335	14.6
$LSD_{0.05}$	17	33	22.4	3.8	3.9	0.15	19.6	3.6	2.7	219	0.39	8.3	27.4	1.55	168	3.54	65	5.3
σ^2_b	0 ± 0.82	0 ± 3.5	8.8 ± 21.3	0 ± 0.075	0 ± 0.067	0 ± 0.00009	2.1 ± 8.7	0 ± 0.12	5.85 ± 8.62	66 ± 704	0.0052 ± 0.099	1.1 ± 7.4	23.5 ± 49.1	0.019 ± 0.11	0 ± 421	0 ± 0.141	1512 ± 3550	0.57 ± 2.09
$\sigma^2_{b.r}$	11.92 ± 5.60	18.4 ± 9.8	20.1 ± 12.7	1.498 ± 0.610	1.177 ± 0.498	0.00178 ± 0.00082	25.1 ± 10.7	0 ± 0.87	1.641 ± 0.671	1713 ± 776	0 ± 0.0027	1.5 ± 10.9	80.6 ± 35.1	0.131 ± 0.065	4954 ± 1966	0 ± 0.248	4654 ± 2619	0 ± 2.85
$\sigma^2_{b.c}$	8.08 ± 4.51	20.1 ± 10.9	15.9 ± 11.3	0.626 ± 0.338	0.684 ± 0.355	0.00017 ± 0.00027	6.1 ± 4.4	0 ± 0.73	0.431 ± 0.274	1953 ± 922	0.0085 ± 0.0056	7.9 ± 12.2	21.2 ± 15.4	0.18 ± 0.087	6889 ± 2884	0.769 ± 0.576	1401 ± 1500	0.92 ± 3.14
σ^2_ε	97.35 ± 6.79	210.8 ± 14.9	364.6 ± 25.4	6.865 ± 0.482	6.596 ± 0.463	0.0135 ± 0.00095	142.2 ± 10.0	19.52 ± 2.79	7.615 ± 0.532	12047 ± 845	0.168 ± 0.0117	218.5 ± 30.3	496.1 ± 34.9	1.265 ± 0.088	18957 ± 1328	6.397 ± 0.903	21409 ± 3424	63.47 ± 8.98
σ^2_F	37.04 ± 13.47	144.9 ± 49.1	65.4 ± 27.5	1.811 ± 0.703	1.988 ± 0.750	0.003 ± 0.0012	50.2 ± 18.5	1.87 ± 1.66	0.95 ± 0.455	6234 ± 2185	0.0201 ± 0.0093	8.9 ± 15.4	97.4 ± 40.5	0.312 ± 0.123	3668 ± 1552	1.68 ± 0.877	1113 ± 2259	3.48 ± 4.68
R	0.43	0.41	0.26	0.35	0.38	0.31	0.41	0.16	0.20	0.51	0.19	0.08	0.28	0.33	0.28	0.34	0.09	0.10

†FPI, florets per inflorescence; PS, pollen stainability; PDL, peduncle length; LL, leaflet length; LW, leaflet width; LL:LW, leaflet length to width ratio; PTL, petiole length; LDW, leaf dry weight; IL, internode length; SA, stolon anchoring; SL, stolon length; SD, stolon diameter; SDW, stolon dry weight; NRD, nodal root diameter; NRL, nodal root length; TRD, tap-root diameter; TRL, tap-root length; RDW, root dry weight. Variance components: σ^2_b, replicates; $\sigma^2_{b.r}$, rows within replicates; $\sigma^2_{b.c}$, columns within replicates; σ^2_ε, experimental error; σ^2_F, families. *R*, repeatability. $LSD_{0.05}$, (least significant difference, $P < 0.05$)

4.2. Analysis (b) - Variation among Genotypes

The differences between the minimum and maximum values indicated a broad range of phenotypic variation for all of the traits measured in the BC_1F_2 hybrid population (Means, ranges, and variance components can be seen in Table 4, frequency distributions in Figure 1). There was significant (P<0.05) variation among BC_1F_2 genotypes for all of the traits measured. The genotype mean repeatability (R) enabled the estimation of an upper limit of the degree of genetic determination (Falconer and Mackay, 1996), and for most traits this was above 50%.

The parental species (AAOO and *T. repens*) populations had a comparatively narrow range of phenotypic variation, with significant variation only occurring for one trait in each of the groups (nodal root length among the AAOO genotypes, and stolon dry weight among the *T. repens* genotypes).

4.2.1 Root characteristics

BC_1F_2 nodal root diameters were generally comparable to those of the parental species. One genotype (G119) had significantly (P<0.05) thicker nodal roots than all but one *T. repens* genotype. One hybrid genotype (G40) had nodal roots that were significantly thinner than the largest *T. repens* genotype. No BC_1F_2 genotypes had nodal roots that were significantly thinner than any of the AAOO genotypes.

BC_1F_2 genotype nodal root lengths were generally distributed around the parental species population mean. One BC_1F_2 genotype (G16) had nodal roots that were significantly (P<0.05) longer than three *T. repens*, and two AAOO genotypes. No BC_1F_2 genotype had nodal roots that were significantly shorter than any parental genotype.

The majority of the BC_1F_2 genotypes were distributed between the parental species population means for tap root diameter. Four genotypes (G97, G27, G58, G82) had significantly (P<0.05) thicker tap roots than all *T. repens* genotypes, and the largest of these genotypes (G82) was also significantly (P<0.05) thicker than four of the six AAOO genotypes. No hybrid genotypes had tap roots that were significantly smaller than the *T.* repens genotypes. Three genotypes (G101, G88, G21) had tap roots that were significantly (P<0.05) smaller than the largest AAOO genotype.

BC_1F_2 genotypes had tap roots that were generally longer than *T.* repens genotypes, and similar in length to AAOO genotypes. However no BC_1F_2 genotypes had tap roots that differed significantly from any parental species genotype.

BC_1F_2 genotypes root dry weights were generally lighter than the *T. repens* genotypes. Two genotypes (G95, G16) had root dry weights significantly (P<0.05) higher than all of the AAOO genotypes, as well as four of the six *T.* repens genotypes. The seven smallest BC_1F_2 genotypes were significantly (P<0.05) lighter than only the largest *T. repens* genotype.

Thee BC_1F_2 genotypes (G111, G33, G58) had stolon anchoring occurring further (P<0.05) from the growing tip than all parental genotypes. Comparisons between the parental species population means and the results from experiment 1, showed some discrepancies, indicating that this trait was poorly controlled in the experiment.

4.2.2 Fertility characteristics

The BC_1F_2 population tended to segregate toward the recurrent parent (*T.* repens) for the florets per inflorescence and peduncle length traits (Fig 1), with two genotypes (G8, G122) having significantly (P<0.05) more florets per inflorescence than all AAOO genotypes. Four genotypes (G27, G30, G93, G16) had peduncles that were significantly (P<0.05) longer than all but one AAOO genotype. Two genotypes (G85, G40) had peduncles that were significantly (P<0.05) shorter than the two *T. repens* genotypes with the longest peduncles.

BC_1F_2 genotypes generally had pollen stainability scores lower than those of the parental genotypes. Five BC_1F_2 genotypes (G107, G101, G93, G110, G1) had pollen stainability scores that were significantly (P<0.05) below five of the six *T.* repens genotypes, indicating low male fertility in these genotypes.

4.2.3 Dry matter yield

BC_1F_2 genotype leaf dry weights tended to be distributed close to the AAOO population mean (Fig 1). Four genotypes had leaf dry weights significantly (P<0.05) heavier than all six AAOO genotypes, with one genotype (G95) being significantly (P<0.05) heavier than all but the largest of the *T. repens* genotypes.

The majority of the BC_1F_2 genotypes had stolon dry weights intermediate between the AAOO and *T. repens* population means. Four genotypes (G124, G95, G16, G62) had stolon dry weights significantly (P<0.05) larger than all of the AAOO genotypes, with one of these genotypes (G62) also having a stolon dry weight significantly (P<0.05) larger than four of the six *T. repens* genotypes. No BC_1F_2 genotypes had stolon dry weights significantly lower than any AAOO genotypes, whilst several were significantly (P<0.05) smaller than only the largest of the *T.* repens genotypes.

4.2.4 Stolon and leaf characteristics

The BC_1F_2 population mean was higher than both parental population means. However no genotypes had internodes that differed significantly from all genotypes in either of the parental species populations.

Comparisons between population means and the results of experiment 1 showed discrepancies between the two experiments for internode length. Internode length was highly variable among genotypes with white clover internode lengths ranging from 3.3-10.5mm and AAOO genotypes ranging from 2.8-9.5mm, which may explain this discrepancy.

BC_1F_2 genotypes tended to segregate around or above the *T.* repens population mean for stolon length. Six genotypes (G76, G95, G27, G36, G82, G16) had stolons that were significantly (P<0.05) longer than all of the AAOO genotypes. Two genotypes (G82, G16) had stolons that were significantly (P<0.05) longer than three of the six *T. repens* genotypes.

BC_1F_2 genotypes tended to have stolons with diameters larger than both the AAOO and *T. repens* population means. One genotype (G62) had significantly (P<0.05) thicker stolons than all of the *T.* repens genotypes, as well as five AAOO genotypes.

One genotype (G62) had leaflets that were significantly (P<0.05) longer, and wider than all of the AAOO genotypes. G62 was also significantly (P<0.05) wider than five of the *T.* repens genotypes.

There was significant (P<0.05) variation among BC_1F_2 genotypes for leaflet length to width ratios, but no genotypes varied significantly from all genotypes in either parental population.

BC_1F_2 genotype petiole lengths were predominantly distributed between the parental species population means, with no genotypes differing significantly from all genotypes in either of the parental species populations.

4.2.5 Correlations among traits

Of the 216 pairwise correlations among the 18 traits, 61 (28%) showed significant correlations (Table 5). Of the root traits, nodal root diameter was strongly positively correlated with both nodal root length and root dry weight, but was not significantly correlated with either tap root length, diameter, or stolon anchoring. Nodal root length was significantly correlated with both tap root length and root dry weight. Tap root diameter and length were not significantly correlated, but tap root diameter and stolon anchoring were significantly correlated, whilst tap root length was significantly correlated with root dry weight.

Leaf, stolon, and root dry weights were all significantly correlated to each other. Interestingly, internode length appeared to be expressed independently of most shoot traits with the exception of stolon diameter which it was positively correlated with.

Table 4: Means, ranges, and variance components (σ^2) with associated standard errors (±SE) for various traits measured from 120 *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2, six *T. ambiguum* x *T. occidentale*, and six *T.repens* cv. Crusader progeny grown in sand.†

		FPI	PS	PDL	LL	LW	LL:LW	PTL	LDW	IL	SL	SD	SDW	SA	NRD	NRL	TRD	TRL	RDW
BC_1F_2	Mean	48	80	89.1	10.1	9.6	1.08	30.3	4.9	7.1	291	1.92	17.5	17.6	2.93	321	4.80	345	12.7
	Range	32-74	32-100	58.9-119.4	4.1-18.7	3.4-21.5	0.89-1.30	5.7-60.1	1.8-20.4	2.5-16.7	29-693	1.27-2.87	3.7-66.3	2.0-211.2	0.45-6.69	67-660	0.43-15.33	199-579	6.7-40.1
AAOO	Mean	34	89	71.2	7.1	6.4	1.13	17.1	2.6	6.2	99	1.73	7.7	10.4	2.55	313	6.81	369	10.4
	Range	26-41	65-100	65.2-80.1	5.8-8.1	4.7-7.6	1.04-1.19	13.4-23.0	2.2-3.5	2.8-9.5	68-145	1.38-2.2	3.4-10.2	4.7-16.3	2.23-3.19	148-546	4.0-8.77	280-501	8.2-12.3
RET	Mean	56	96	91.8	11.2	11.5	1.03	46.5	7.1	6.4	315	1.70	25.6	10.6	2.79	285	2.35	300	17.6
	Range	45-63	65-100	69.3-106.9	9.1-14.4	10.0-14.2	0.96-1.13	30.3-60.4	3.9-9.3	3.3-10.5	218-461	1.53-1.84	9.2-48.1	6.0-14.9	2.1-3.72	213-476	1.44-3.03	261-344	13.0-27.1
	$LSD_{0.05}$	28	52	42.1	8.9	9.0	0.25	38.2	11.1	8.8	395	0.87	38.6	70.6	3.12	395	7.56	334	19.2
	σ^2_r	0.57 ± 2.24	11.15 ± 8.55	0 ± 6.6	0.42 ± 0.314	0.51 ± 0.35	0.001 ± 0.001	1.17 ± 3.15	0 ± 0.38	0.59 ± 0.46	584 ± 440	0 ± 0.001	0 ± 2.87	0 ± 2.4	0 ± 0.02	1436 ± 1045	0 ± 0.13	433 ± 1081	0 ± 1.55
	σ^2_c	2.53 ± 3.08	20.85 ± 14.05	7.3 ± 9.8	0.175 ± 0.20	0.28 ± 0.25	0 ± 0.0003	3.51 ± 4.15	0.34 ± 0.66	0.32 ± 0.34	740 ± 541	0.005 ± 0.005	8.49 ± 9.26	0 ± 1.6	0.18 ± 0.12	8085 ± 4645	0.15 ± 0.23	955 ± 1293	1.92 ± 2.63
	σ^2_ε	57.39 ± 4.46	49.78 ± 3.95	258.4 ± 20.0	2.335 ± 0.184	2.012 ± 0.159	0.0102 ± 0.00079	61.18 ± 4.78	6.423 ± 2.003	4.936 ± 0.386	2759 ± 218	0.109 ± 0.0084	66.76 ± 19.17	123.7 ± 9.6	0.618 ± 0.0481	9217 ± 724	1.815 ± 0.544	15484 ± 4993	24.38 ± 7.78
	σ^2_g	98.51 ± 15.05	352.22 ± 46.90	231.3 ± 41.2	10.24 ± 1.41	10.54 ± 1.43	0.01 ± 0.002	190.12 ± 26.87	16.44 ± 3.45	10.01 ± 1.51	20267 ± 2698	0.098 ± 0.017	197.19 ± 36.18	678.3 ± 88.1	1.28 ± 0.19	20272 ± 3044	7.39 ± 1.27	14544 ± 6323	48.36 ± 11.88
	R	0.63	0.88	0.47	0.81	0.84	0.50	0.76	0.72	0.67	0.88	0.47	0.75	0.85	0.67	0.69	0.80	0.48	0.67

†FPI, florets per inflorescence; PS, pollen stainability; PDL, peduncle length; LL, leaflet length; LW, leaflet width; LL:LW, leaflet length to width ratio; PTL, petiole length; LDW, leaf dry weight; IL, internode length; SA, stolon anchoring; SL, stolon length; SD, stolon diameter; SDW, stolon dry weight; NRD, nodal root diameter; NRL, nodal root length; TRD, tap-root diameter; TRL, tap-root length; RDW, root dry weight. Variance components: σ^2_r, rows; σ^2_c, columns; σ^2_ε, experimental error; σ^2_g, genotypes. *R*, repeatability. $LSD_{0.05}$, (least significant difference, $P < 0.05$)

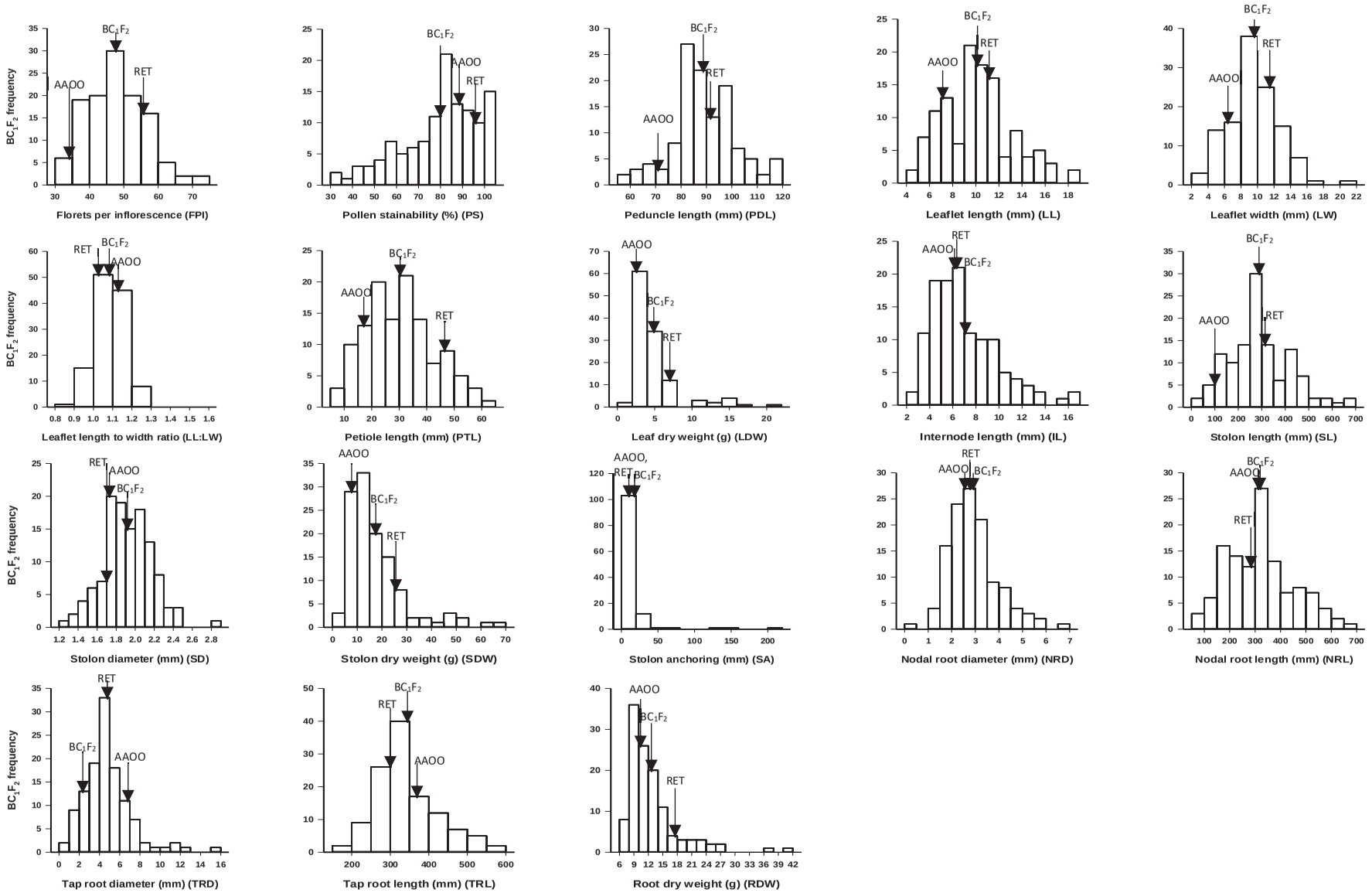

Figure 1. Distribution of *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrid genotype BLUPs for selected traits in the BC_1F_2 population. Means for parental (AAOO and *T. repens*) and BC_1F_2 populations are shown by arrows.

Table 5: Phenotypic correlation coefficients among the morphological traits measured from 120 *T .repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2, six *T. ambiguum* x *T. occidentale*, and six *T. repens* cv. Crusader progeny grown in sand.†

	FPI	PS	PDL	LL	LW	LL:LW	PTL	LDW	IL	SL	SD	SDW	NRD	NRL	TRD	TRL	RDW	SA
FPI	-																	
PS	0.1254	-																
PDL	0.363*	-0.1571	-															
LL	0.1557	-0.1143	0.319*	-														
LW	0.2315	-0.1037	0.3372*	0.9346*	-													
LL:LW	-0.2598	0.0394	-0.1256	0.0291	-0.3038*	-												
PTL	0.1576	-0.1731	0.3093	0.8278*	0.8365*	-0.1535	-											
LDW	0.0972	-0.0997	0.2463	0.6537*	0.6281*	-0.0304	0.657*	-										
IL	0.0855	0.0296	-0.1068	-0.0109	0.0032	0.0283	-0.0272	0.0433	-									
SL	0.0747	-0.0823	0.31*	0.5083*	0.5163*	-0.2049	0.4242*	0.5353*	-0.0061	-								
SD	0.2911*	-0.0424	0.214	0.3227*	0.3312*	-0.0498	0.2084	0.3153*	0.3128*	0.2878*	-							
SDW	0.2077	-0.0868	0.2748	0.5987*	0.5943*	-0.0898	0.613*	0.8396*	0.1937	0.6308*	0.4007*	-						
NRD	0.1444	-0.0741	0.1929	0.3631*	0.3484*	0.0038	0.3335*	0.3747*	0.3231*	0.4037*	0.3315*	0.5893*	-					
NRL	-0.0816	-0.0565	0.0927	0.4211*	0.4043*	-0.0453	0.389*	0.4188*	-0.0228	0.4542*	0.1633	0.4871*	0.5878*	-				
TRD	-0.1608	0.0184	0.0624	0.0735	0.0089	0.2099	-0.0266	0.1839	0.1037	0.1544	0.2127	0.1966	0.1854	-0.0112	-			
TRL	-0.1869	0.0387	0.0386	0.3711*	0.3358*	0.0701	0.32*	0.4359*	0.0247	0.3001*	0.1931	0.3563*	0.2739	0.5956*	0.274	-		
RDW	0.1144	-0.1208	0.3083*	0.5753*	0.5413*	-0.016	0.607*	0.8139*	0.1412	0.5358*	0.3342*	0.8603*	0.5083*	0.4389*	0.1748	0.3732*	-	
SA	0.0185	0.0088	-0.1015	-0.0841	-0.1036	0.0964	-0.1147	0.0037	0.2585	0.0098	0.1683	-0.0084	0.1358	-0.0136	0.2894*	0.0492	-0.037	-

*$P < 0.001$

† FPI, florets per inflorescence; PS, pollen stainability; PDL, peduncle length; LL, leaflet length; LW, leaflet width; LL:LW, leaflet length to width ratio; PTL, petiole length; LDW, leaf dry weight; IL, internode length; SL, stolon length; SD, stolon diameter; SDW, stolon dry weight; NRD, nodal root diameter; NRL, nodal root length; TRD, tap-root diameter; TRL, tap-root length; RDW, root dry weight ; SA, stolon anchoring.

4.3 Pattern analysis

4.3.1 Pattern analysis of above ground traits

Cluster analysis of the genotype x shoot trait BLUP matrix generated four genotype groups. The number of genotypes within each group ranged from 8 (in Group 1) to 44 (in Groups 2 and 4). Trait means for the four groups are presented in Table 6. Genotype group 1 comprised 8 genotypes that had high mean leaflet length (LL), leaflet width (LW), petiole length (PTL), leaf dry-weight (LDW), stolon length (SL), stolon diameter (SD), and stolon dry-weight (SDW). Groups 2 and 3 comprised 44 and 36 genotypes, respectively. Both groups had relatively high mean leaflet length, width, petiole length, leaf dry-weight, stolon length, stolon diameter and stolon dry-weight. Group 2 had slightly smaller values than group 3 for all of the traits except stolon length, and had the shortest internode length of the four groups. Group 4 consisted of 44 genotypes having the low mean leaflet length, leaflet width, petiole length, leaf dry-weight, stolon length, stolon diameter, and stolon dry-weight. Group 4 had relatively high mean internode length, comparable to groups 1 and 3.

Four *T. repens* genotypes (G5, G39, G57, G94) were placed in Group 3, while one genotype (G10) was placed in Group 2, and one genotype (G92) was placed in Group 4. All six AAOO genotypes were placed in Group 4.

Table 6: Within-group genotype means for each shoot trait based on the four clusters generated from cluster analysis of 120 BC_1F_2, 6 AAOO, and 6 *T. repens* genotypes grown in sand.[†]

Group no.	No. in group	LL	LW	PTL	LDW	IL	SL	SD	SDW
1	8	16.15	14.82	50.17	14.91	7.37	508	2.27	49.88
2	44	10.25	9.83	30.71	4.09	5.36	329	1.91	15.47
3	36	11.96	11.81	40.30	6.02	8.45	318	1.95	22.05
4	44	7.17	6.51	18.41	2.98	7.38	168	1.78	9.62

[†]LL, leaflet length; LW, leaflet width; PTL, petiole length; LDW, leaf dry-weight; IL, internode length; SL, stolon length; SD, stolon diameter; SDW, stolon dry-weight. *T. repens* genotypes G5, G39, G57, G94 are in Gp. 3, G10 in Gp. 2, G92 in Gp. 4. All AAOO genotypes in Gp. 4.

The biplot (Figure 2) generated from principal component analysis of the 120 BC_1F_2, six AAOO, and six white clover genotypes, based on eight shoot traits, is a graphical summary of the genotype x shoot trait BLUP matrix. The biplot represents the

relationship between genotypes and traits simultaneously and can be interpreted as follows: genotypes located near the vector origin have all of their values close to the trait means. Genotypes that are placed close to each other are similar in their expression of all of the traits analysed. For a particular trait, genotypes can be compared by the projection of a line perpendicular between the genotype data point and the trait vector. The bi-plot also displays the correlation structure among traits with the association among directional vectors represented by the angle between them, the smaller the angle (<90°) the stronger the positive association (and vice versa), directional vectors at 90° indicate independent traits.

There was a strong degree of separation among the four genotype groups with group 1 genotypes dispersed strongly to the left of the bi-plot indicating their collective strong expression for all of the shoot traits analysed.

The traits PTL, LL, LW, LDW, SL, and SDW showed a strong positive association. Internode length (IL) had a positive association with SD, with IL being expressed independently of the other shoot traits in the experiment.

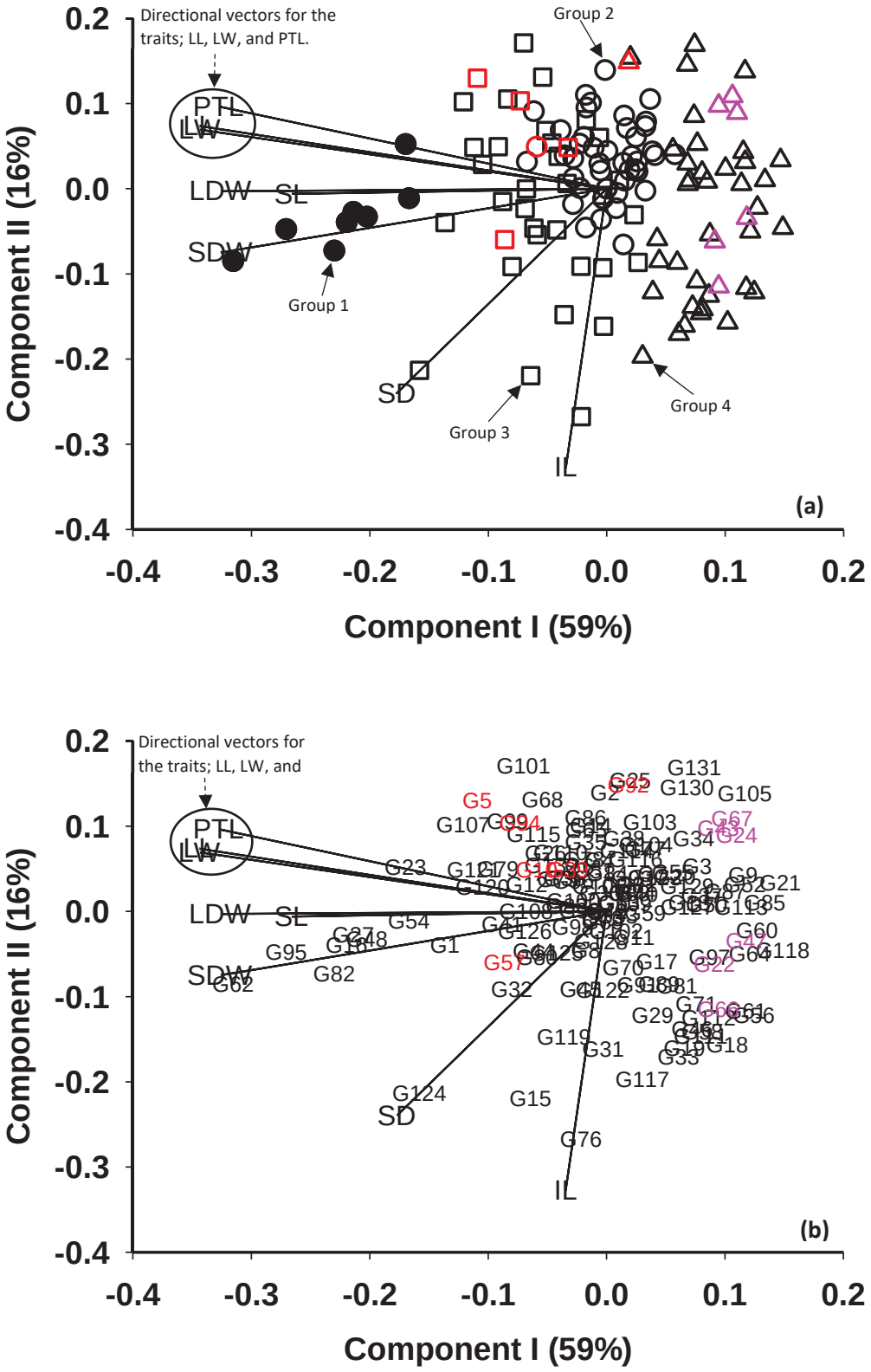

Figure 2. Biplot generated using standardized Best Linear Unbiased Predictor values of genotype shoot trait means from 120 BC_1F_2, 6 AAOO (pink), and 6 *T. repens* (red) genotypes grown in sand. Components I and II account for 59 and 16% of total variation, respectively. The different symbols indicate genotype Groups 1 to 4 generated from cluster analysis (a), whilst the different numbers represent individual genotypes (b). The vectors represent the shoot traits: LL, leaflet length (mm); LW, leaflet width (mm); PTL, petiole length (mm); LDW, leaf dry-weight (g); IL, internode length (mm); SL, stolon length (mm); SD, stolon diameter (mm); SDW, stolon dry-weight (g). The arrow (⇢) indicates the labels of directional vectors that are not legible.

4.3.2 Pattern analysis of root traits

Cluster analysis of the genotype x root trait BLUP matrix generated five genotype groups (Table 7). The number of genotypes within each group ranged from 3 (in Group 2) to 55 (in Group 5). Group 1 consisted of 9 genotypes and had the smallest mean expression for Stolon Anchoring (SA), combined with high mean expression for nodal, and tap root lengths (NRL, TRL). Group 1 genotypes had relatively low taproot diameter (TRD) and nodal root diameter (NRD), with quite high mean root dry weight (RDW). Group 2 consisted of 3 genotypes having the largest mean expression of SA, as well as the largest mean TRD. Group 2 had high expression of the NRD trait but relatively low NRL and TRL. Group 2 had the lowest mean RDW. Group 3 consisted of 21 genotypes with relatively low mean expression for the traits SA, NRD, NRL, and RDW. Group 3 genotypes had the lowest mean TRD, and TRL. Group 4 consisted of 21 individuals and had the largest mean nodal root diameter (NRD), and the heaviest mean root dry-weight (RDW) combined with high mean NRL, TRD, and TRL, with intermediate expression for the SA trait.

All six *T. repens* genotypes were placed in Group 3. Five AAOO genotypes (G22, G24, G47, G66, G67) were placed in Group 5, with one genotype placed in Group 1 (G43).

Table 7: Within-group genotype means for each root trait based on the four clusters generated from cluster analysis of 120 BC_1F_2, 6 AAOO, and 6 *T. repens* genotypes grown in sand.[†]

Group no.	No. in group	SA	NRD	NRL	TRD	TRL	RDW
1	9	9.94	3.12	539	3.56	507	14.04
2	3	162.46	3.51	283	8.08	329	10.08
3	44	11.84	2.93	301	3.22	293	12.27
4	21	19.12	4.38	446	6.65	405	20.27
5	55	13.45	2.27	250	5.34	336	10.27

[†]SA, stolon anchoring; NRD, nodal-root diameter; NRL, nodal-root length; TRD, tap-root diameter; TRL, tap-root length; RDW, root dry-weight. All 6 *T. repens* genotypes are in group 3. AAOO genotypes G22, G24, G47, G66, G67 are in group 5, G43 is in group 1.

The biplot (Figure 3) generated from principal component analysis showed that groups 2 and 4 separated strongly, while there was significant overlap among the remaining three groups. Group 2 contained the three genotypes with the highest stolon anchoring values. These genotypes also had above average tap-root diameters, whilst

their scores for the other traits (NRD, NRL, TRL, and RDW) were around, or below the mean. Group 4 contained two genotypes (G82, G119) that scored well above the mean for all root traits.

The vectors for NRL, RDW, NRD, and TRL had strong positive associations. TRD and SA has a strong positive association, TRD had a weak positive association with the other root traits, whereas SA appeared to be expressed independent of the other root traits.

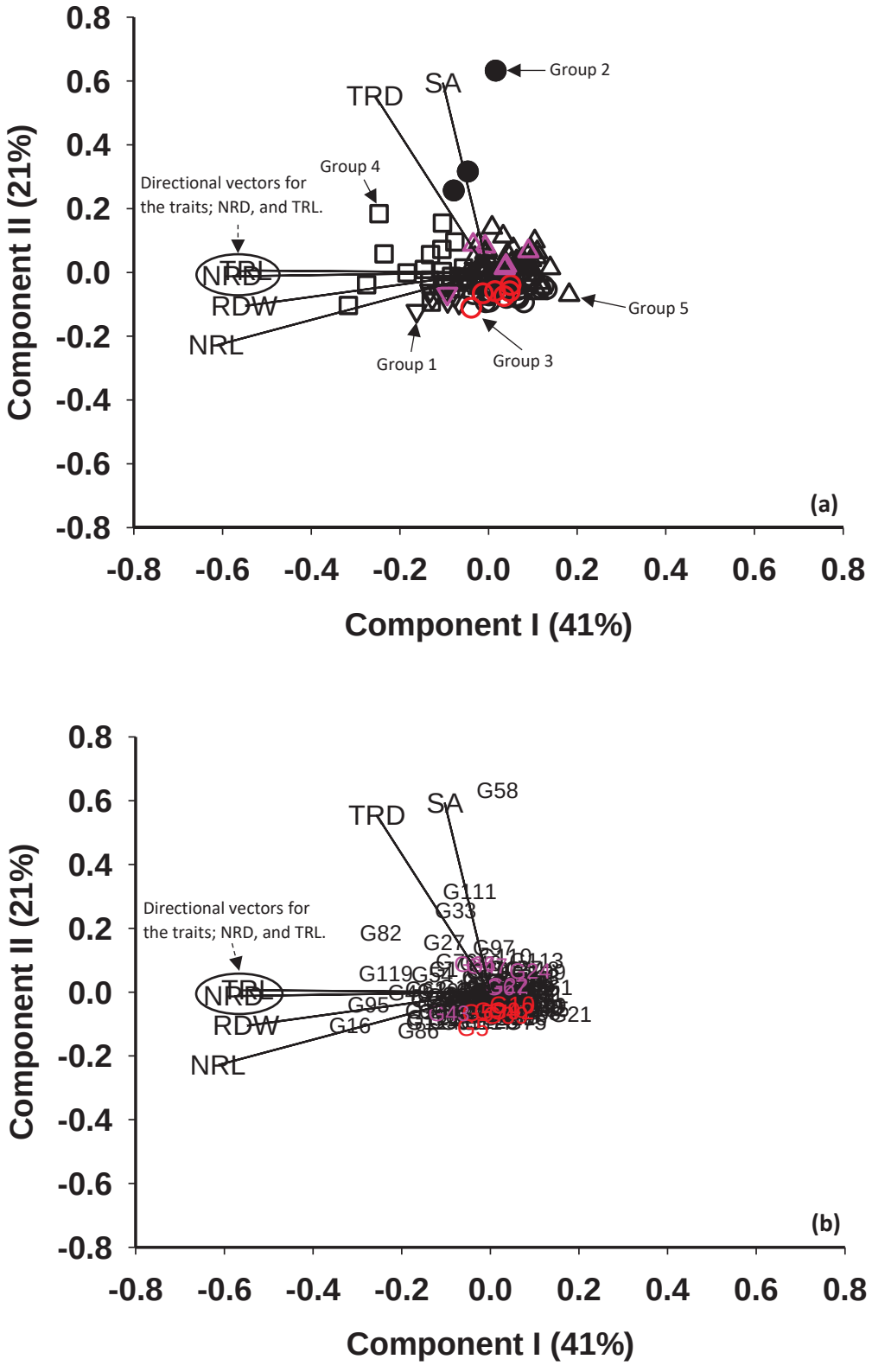

Figure 3. Biplot generated using standardized Best Linear Unbiased Predictor values of genotype root trait means from 120 BC_1F_2, 6 AAOO (pink), and 6 *T. repens* (red) genotypes grown in sand. Components I and II account for 41 and 21% of total variation, respectively. The different symbols indicate genotype Groups 1 to 5 generated from cluster analysis (a), whilst the different numbers represent individual genotypes (b).The vectors represent the root traits: SA, stolon anchoring (mm); NRD, nodal root diameter (mm); NRL, nodal root length (mm); TRD, tap root diameter (mm); TRL, tap root length (mm); RDW, root dry weight (g). The arrow (⇢) indicates the labels of directional vectors that are not legible.

4.3.3 Pattern analysis of combined shoot and root traits.

Cluster analysis of the genotype x shoot and root trait BLUP matrix generated four genotype groups (Table 8). The number of genotypes within each group ranged from 3 (Group 2) to 63 (Group 3). Group 1 was made up of 11 genotypes with the highest mean expression for the traits LL, LDW, PTL, SL, SD, SDW, NRD, NRL, TRL, and RDW. These genotypes also showed high mean tap root diameter, with intermediate internode length and stolon anchoring. Group 2 was made up of 3 genotypes that showed low expression of the leaf traits (LL, LDW, PTL), relatively short stolons, long, thick internodes, and intermediate stolon dry weight. Nodal root traits (NRD, NRL) were intermediate, and they showed high expression of tap root diameter, although this didn't translate into increased tap root length which was intermediate. Root dry weight was intermediate and these individuals showed high expression for stolon anchoring. Group 3 was the largest group, consisting of 63 genotypes. These genotypes showed intermediate leaf size (LL, PTL), and had the lowest mean LDW. These genotypes had the lowest mean expression for all of the stolon traits (IL, SL, SD, SDW), they also had low mean expression for the root traits (NRD, NRL, TRD, TRL, RDW, SA). Group 4 was made up of 55 genotypes that had intermediate expression for all of the traits, except for tap root diameter, which was lowest in this group.

Four *T. repens* genotypes (G5, G10, G39, G94) were placed in Group 4, one (G92) was placed in Group 3, while G57 was placed in Group 1. Five AAOO genotypes (G22, G24, G47, G66, G67) were placed in Group 3, while one (G43) was placed in Group 4.

The biplot (Figure 4) generated from principal component analysis showed that the four groups separated strongly, with Group 2 containing three genotypes showing increased expression for the tap root diameter, internode length, and stolon anchoring traits. Groups 1 and 4 contained genotypes showing above average expression for all of the traits included in the analysis.

The vectors for PTL, LL, NRL, LDW, SL, RDW, SDW, and TRL had a strong positive association. TRD, IL, and SA also show a strong positive association, while being expressed independently of the previously mentioned correlated traits. NRD and SD were strongly correlated to each other, and were distributed between the two groups

of strongly correlated traits, showing some level of correlation to all other traits included in this analysis.

Table 8: Within-group genotype means for each shoot and root trait based on the four clusters generated from cluster analysis of 120 BC_1F_2, 6 AAOO, and 6 *T. repens* genotypes grown in sand.†

Group no.	No. in group	LL	LDW	PTL	IL	SL	SD	SDW	NRD	NRL	TRD	TRL	RDW	SA
1	11	14.73	13.80	49.45	7.31	478	2.13	46.85	4.02	438	7.19	435	26.22	17.56
2	3	6.53	3.38	15.24	11.18	237	2.05	12.39	3.51	283	8.08	329	10.08	162.46
3	63	8.39	3.26	22.22	6.67	221	1.83	10.69	2.41	238	4.67	303	9.88	12.20
4	55	11.21	5.08	36.79	7.08	317	1.93	19.47	3.22	389	4.25	374	13.56	14.36

†LL, leaflet length; LDW, leaf dry-weight; PTL, petiole length; IL, internode length; SL, stolon length; SD, stolon diameter; SDW, stolon dry-weight; NRD, nodal-root diameter; NRL, nodal-root length; TRD, tap-root diameter; TRL, tap-root length; RDW, root dry-weight; SA, stolon anchoring. *T. repens* genotypes G5, G10, G39, G94 are in Group 4, G92 is in Group 3, G57 is in Group 1. AAOO genotypes G22, G24, G47, G66, G67 are in Group 3, G43 is in Group 4.

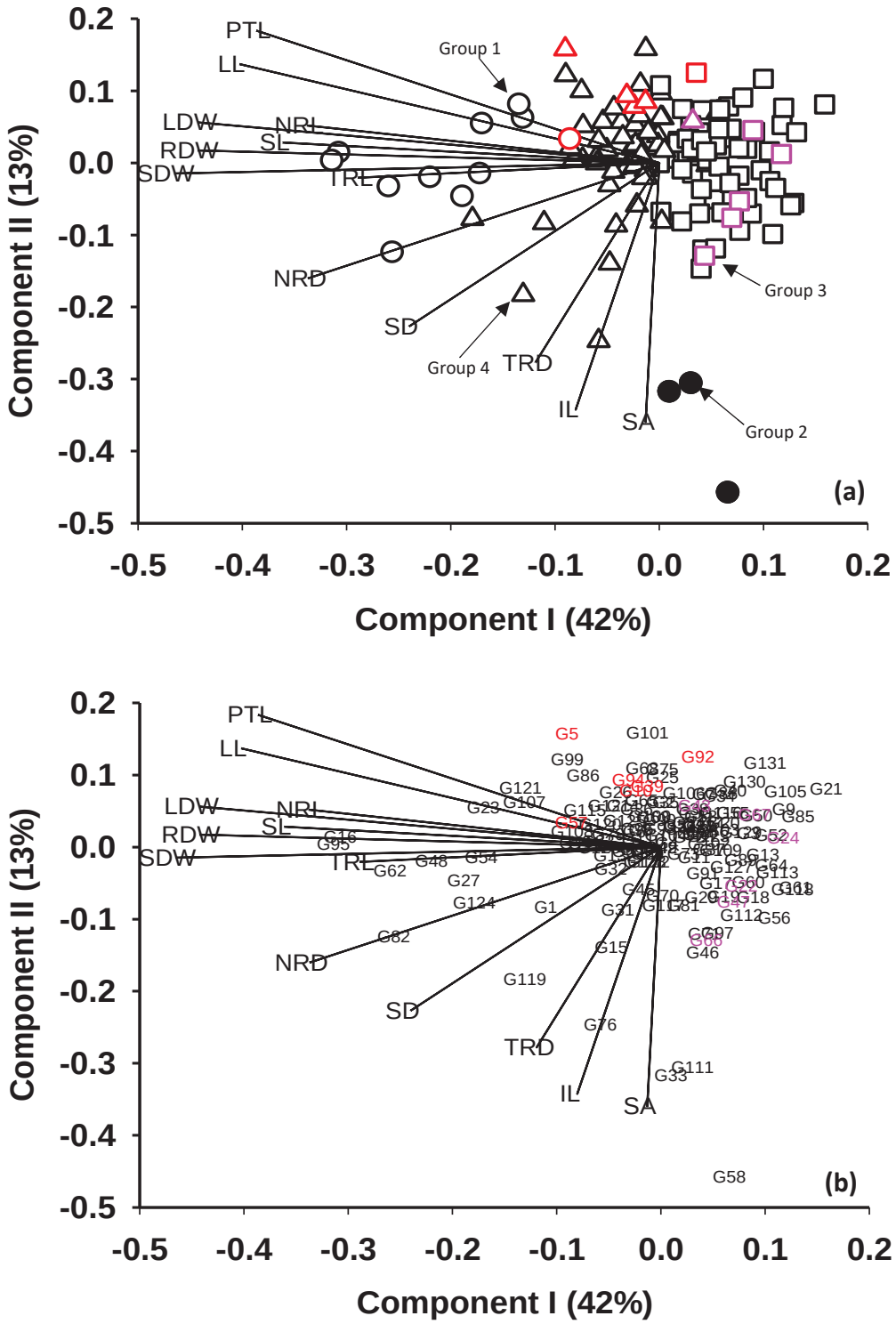

Figure 4. Biplot generated using standardized Best Linear Unbiased Predictor values of genotype shoot and root trait means from 120 BC_1F_2, 6 AAOO (pink), and 6 *T. repens* (red) genotypes grown in sand. Components I and II account for 42 and 13% of total variation, respectively. The different symbols indicate genotype Groups 1 to 4 generated from cluster analysis (a), whilst the different numbers represent individual genotypes (b).The vectors represent the shoot and root traits: PTL, petiole length (mm); LL, leaflet length (mm); NRL, nodal root length (mm); LDW, leaf dry weight (g); SL, stolon length (mm); RDW, root dry weight (g); SDW, stolon dry weight (g); TRL, tap root length (mm); NRD, nodal root length; SD, stolon diameter; TRD, tap root diameter; IL, internode length (mm); SA, stolon anchoring (mm).

Chapter 5 Discussion

The aims of this research were to assess phenotypic variation, both among families, and among genotypes within the *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrid population in relation to representatives of their F_1 parents; *T. repens* and AAOO. It was expected that *T. ambiguum* and *T. occidentale* characteristics would be transferred into a *T. repens* background to varying degrees, and thus it was hypothesised that *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrids would exhibit greater phenotypic variation than their parents. Specifically it was hypothesised that *T. repens* x (*T. ambiguum* x *T. occidentale*) BC_1F_2 hybrids would exhibit greater variation in nodal root size than *T. repens*.

Among hybrid family variation was investigated to ascertain whether family based selection, or individual genotype selection should be practised in early generation hybrid populations. It was expected that variation would be greater among individual hybrid genotypes than among hybrid families.

5.1 Assessment of among hybrid family variation

Whilst there was significant phenotypic variation among families for 11 of the 18 traits measured, variation tended to only be significant among the upper and lower extreme values, and therefore there was a relatively small component of among family phenotypic variation (σ^2_F) for most traits. The family mean repeatability enabled the estimation of an upper limit of genetic determination (Falconer and Mackay, 1996), and was 51% or less for all of the traits. This result indicated that among family variance made up a relatively low proportion of the total phenotypic variance, and therefore a low estimated heritability among family groups in this hybrid population is expected. Since the attributes were measured in a single environment, there is no measurement as to the magnitude of family-by-environment variation, and therefore care should be taken as to the interpretation of repeatability estimates as broad sense heritability.

Interspecific hybrid populations are expected to offer increased variability as a result of extreme heterozygosity in F_1 hybrids. Each genotype in the F_2 and later generations is likely to differ from each other individual for many characteristics, with some segregants showing phenotypes that could not be predicted from the morphology or

physiology of the parental species (Allard, 1999). Each genotype in a family represents an individual hybridisation event and as such may have alien chromosomes and/or chromosomal segments introgressed to varying degrees on differing chromosomes, and thus, the widely segregating nature of the population can largely explain the relative lack of among family variation as compared to the variation among genotypes.

Lack of variation among families could also be attributed to sampling bias, where only six plants (three per rep) from each hybrid family were included in the design, meaning that the results presented here may not be a true reflection of the represented families. However, the early generation hybrid nature of the material in the present study is likely to be the key determinant for the result showing low phenotypic variation among BC_1F_2 families, as compared to among genotype variation.

Stolon diameter was one of the few traits that showed significant variation, both among hybrid families, and between hybrid families and their parental species. There was evidence to suggest possible transgressive segregation within the hybrid population for this trait, with some families expressing stolon diameters significantly above those recorded for either parental species. Transgressive segregation can be defined as the appearance of individuals in segregating populations that fall beyond their parental phenotypes (Rieseberg et al., 1999, Tanksley, 1993). Transgressive segregation appears to be ubiquitous in plant hybrids, playing a significant role in crop improvement and evolution as it can affect characters of adaptive significance, or characters that allow them to occupy new niches, or better compete in existing environments (Tanksley, 1993, Lewontin and Birch, 1966, Stebbins, 1950, Rieseberg et al., 1999).

Evidence reported here suggests that transgressive segregation may be occurring in the BC_1F_2 population for stolon diameter. Stolon diameter is an important trait related to persistence of white clover. It has been reported that vegetative persistence in white clover in sub-optimal conditions is generally associated with greater stolon density, which is a complex combination of stolon branching frequency, internode length, leaf size, and stolon thickness (Williams, 1987). Studies by Turner (1990a) (1990b) showed that under drought stress white clover sheds its leaves, while the stolon adjusts its osmotic potential and pressure potential to survive. When water was

applied again, the white clover recovered from the surviving stolons. Brock and Kim (1994) were able to show that during a drying down phase in a drought experiment (before growth ceased), the leaf production of the smaller, thinner stoloned cultivar Tahora was more affected than that of the thicker stoloned cv. Dusi. It was proposed that the thicker stoloned genotypes were more resistant to heat stress than the cv. Tahora. However, following drought, Tahora was able to recover more quickly than Dusi. It was clear that white clover, regardless of stolon morphology is severely impacted by drought, indicating the complex nature of drought tolerance and the likelihood that many individual traits contribute.

Persistence of white clover can be measured as the survival of the individual plant, or as maintenance of clover content per unit area (Woodfield and Caradus, 1996). Individual stolons generally have short life spans, with less than 10% of stolons surviving longer than a year under New Zealand hill country conditions (Chapman, 1983). Thus continual renewal of the stolon population (by dense stolon branching) has been seen as a way increasing persistence of white clover content in pastures, with plant breeders generally having to balance characteristics for high productivity (large leaf, thick stolons) with those associated with survival under sub optimal conditions (Williams, 1987).

Given the above equivocal evidence, it is unclear whether populations with thick stolons will be better adapted to dry conditions than populations with thinner stolons. Due to the hybrid nature of these populations, it is possible that unique new physiologies, not measured here, may have been introgressed, and, as such, direct selection under a white clover vegetative persistence model may not be the most efficient plant breeding methodology. Further trialling of this material is required to elucidate the function of increased stolon thickness, particularly in relation to drought stress.

5.2 Among genotype variation

Morphological variation among individual BC_1F_2 hybrid genotypes was expected to be greater than that among hybrid families, owing to the hybrid nature of the population. This was proven to be the case, with significant morphological variation occurring among genotypes for all of the traits measured. Repeatability estimated on a genotype

level showed that a medium to high component of the total phenotypic variation could be attributed to genotypic variation, with repeatability estimates ranging from 0.47 to 0.88. These results indicate the possible genetic variation available for the improvement of these traits through further selection and breeding within these hybrid populations. Caution needs to be taken in the interpretation of these repeatability estimates since the morphological measurements were taken in one environment, and hence there is no measurement of the amount of genotype-by-environment interaction in this material. Further work is needed to quantify the amount of morphological variation across multiple environments, and to relate estimates of heritability of the traits presented here to present and future breeding strategies to achieve the maximum response to selection for traits of interest in this hybrid material.

5.2.1 Root characteristics

It was expected that AAOO plants would show thicker, and longer nodal roots than the white clover population, and that hybrids would be intermediate between the two. Unexpectedly, no significant variation between *T. repens* and AAOO genotypes was apparent, with BC_1F_2 hybrids segregating above the mean of both parental populations. Although these differences were not significant on a population level, some individual hybrid genotypes segregated well above the mean of both parental species for nodal root diameter, again indicating possible transgressive segregation within the hybrid population. The large amount of variation among hybrid genotypes represents a strong opportunity for the selection of large nodal rooted genotypes. Previous studies by Caradus (1990) and Jahufer et al. (2008) have reported broad sense heritabilities and repeatabilities for root traits in white clover populations in keeping with the genotype repeatabilities shown in the current study. Woodfield and Caradus (1990) reported that white clover showed a high response to selection for root characteristics with (Caradus and Woodfield, 1998) showing a gain of 2.4% per selection cycle when selecting for seedling tap root diameter. White clover productivity, persistence, nitrogen fixation, and forage quality are reduced by drought stress, with poor drought tolerance associated with root systems that are weakly tap rooted and shallow rooted (Caradus and Woodfield, 1998). Therefore, phenotypic recurrent selection for thicker, longer more “tap root” like nodal roots could be

expected to result in an increase in nodal root size, and thus an increase in persistence under moisture stress. Increased nodal root size is expected to have a significant impact on clover persistence, particularly following tap root death when the segmented plants become reliant on nodal roots. More extensive, deeper nodal root systems are expected to offer increased water uptake, and thus may offer increased persistence under drought/water deficit stress.

There was no significant difference among AAOO and *T. repens* genotypes tap root length or diameter, with the BC_1F_2 population mean intermediate between the two. BC_1F_2 genotypes segregated significantly above both parental species for tap root diameter, and whilst there was no segregation significantly above the parental species, there was certainly significant variation within the BC_1F_2 population for tap root length. Caradus (1991) showed that white clover populations collected from, and presumably adapted to, dry environments were typically "more tap-rooted", with greater "tap root diameters" (diameter of the largest root) than populations collected from wet sites. Caradus and Woodfield (1998) showed that selection for increased tap root diameter combined with medium leaf size, gave yields 35% better than white clover cv. "Huia" when grown in a rain exclusion shelter, where soil moisture was reduced to below wilting point between January and April over two years. Therefore, it would seem likely that BC_1F_2 hybrids with thick and long tap roots may be better adapted to dry environments than traditional white clover cultivars. Selection for tap root size could also be expected to be effective in increasing the frequency of large tap rooted genotypes within the population.

Variable performance of white clover in grazed pastures has long been considered a problem. It has been noted that there is a marked decline in herbage production around 18 months after sowing, particularly in dry environments, attributable to the loss of the seminal tap root (Westbrooks and Tesar, 1955, Brock et al., 2000). Brock and Tilbrook (2000) were able to show that small leaved white clover cultivars had accelerated rates of tap root death compared to larger leaved cultivars. It was shown that larger leaved cultivars had larger tap root diameters, combined with strong nodal roots close to the tap root, forming a large centralised root system. It was hypothesised that this large centralised root system represented a carbon investment

from which returns might be enhanced with longer life. It was also shown that smaller leaved white clover cultivars had longer stolons, higher branching frequency, and thickened nodal roots which may have equipped these plants for earlier independence from their tap roots. Tap rooted plants were shown to produce four to five fold the dry matter of their clonal counter parts after one year, indicating that even seemingly small increases in tap root survival would be beneficial to dry matter yield. Studies by Nichols (2012) reported that tap root survival was higher in *T.* repens x *T. uniflorum* BC_1 hybrids, although there was no obvious relationship between taproot diameter and tap root survival. It was speculated that the "woody" nature of *T. uniflorum* tap roots may play a role in increased survival through mechanical resistance to decay. *Trifolium ambiguum* tap roots have been described as "semi woody" (Bryant, 1974, Fu et al., 2001), with the BC_1F_2 hybrids examined in the present study also having tap roots more "woody" than white clover. Pattern analysis in the current study showed a weak positive correlation between tap root size and leaflet length, with a strong positive correlation between leaflet length and nodal root diameter, seemingly confirming the results of Brock and Tilbrook (2000). Large leaved hybrids generally showed a larger root system, which seemed to contribute to increased dry matter production in these hybrids. It could be inferred that a larger, root system may contribute to increased water, and nutrient uptake, potentially leading to tolerance of drought and infertile soils in the field. Such adaptations may also contribute to increased dry matter yield in favourable environments. Further studies will be necessary to determine the interaction between leaf size, tap root diameter, and tap root survival in these hybrids. Hybrids showing increased nodal root diameter and length could be expected to offer increased persistence in dry environments irrespective of tap root survival owing to their increased ability to access moisture from deeper in the soil profile. Of concern was the strong correlation between tap root diameter, and stolon anchoring, whereby genotypes showing increased tap root diameter, also showed nodal rooting occurring further from the stolon growing tip. Unanchored stolons could be expected to show decreased persistence, especially under grazing pressure, and so this correlation needs to be carefully monitored in future breeding programmes.

No rhizomes were formed in this material, which was in contrast to *T. repens* x *T. ambiguum* hybrids produced in the United Kingdom (Meredith et al., 1995, Abberton et al., 1998), whereby rhizomes accounted for up to 9% of total plant dry weight in the BC_1 generation, reducing progressively to 3% in the BC_3. Genomic constitution of the BC_1F_2 hybrids in this was study was in theory 75% *T. repens*, 12.5% *T. occidentale*, and 12.5% *T. ambiguum*, and therefore rhizomes were expected to be present to some degree. Their absence could be explained by a number of factors. The initial *T. ambigumm* genotypes used in hybrid creation were somewhat inferior wild type plants, and may not have had significant resource allocation to the creation of rhizomes. However, given that rhizomes have been seen in the AAOO population in older plants, it is likely that the lack of rhizome formation in the current study is due to environmental effects (no rhizomes were seen in AAOO plants in this study). Abberton et al. (2003) noted that the ability to produce rhizomes is relatively slow to be expressed, with plants rarely showing rhizomes before 18 months, with expression thought to be altered by other factors such as day length. As these plants were harvested after 8 months, in early winter, the plants probably weren't given enough time to show rhizome production. Future studies should be allowed to carry through at least one full winter to give plants an opportunity to form rhizomes. There is a good opportunity for the use of marker assisted selection for rhizomes, with rhizome production seemingly under relatively simple genetic control, and AFLP markers for rhizome production known in *T. repens* x *T. ambiguum* hybrids (Abberton et al., 2003).

5.2.2 Fertility characteristics

Sterility, to some degree, is the most characteristic feature of interspecific hybrids (Allard, 1999), and was used as an indicator of hybridity in the F_1 generation of the material used in this study. Sterility was measured in terms of pollen stainability and was expected to be reduced in the BC_1F_2 hybrid population. This was confirmed with the BC_1F_2 population mean pollen stainability being lower than both of the parental species population means, with some genotypes showing relatively extreme male sterility. The cause of sterility in this population is unknown, however probably has basis in some kind of cytological abnormality, with genotypes showing increased sterility presumably being those genotypes with more disharmonious chromosomal interactions. Although these genotypes are likely interesting from a cytological point of

view, they are unlikely to be useful in further breeding of this material. Plants with low fertility are expected to breed themselves out of a population as they contribute less pollen in the formation of subsequent generations.

Seed yield is an important trait which determines the commercial acceptability of a new cultivar (Jahufer and Gawler, 2000, Marshall, 1995), with seed yield being the product of inflorescence density, and yield per inflorescence, with the number of florets per inflorescence, and floret fertility being important components of yield per inflorescence (Barrett et al., 2005). The BC_1F_2 population showed decreased mean number of florets per inflorescence as compared to the *T. repens* population. The incidence of genotypes with florets per inflorescence comparable to that of white clover combined with moderate repeatability estimates, gives confidence that seed yield could be selected for in this population. Studies by (Naeem, 2013) were able to show that fertility and seed production are able to be rapidly restored via recurrent selection and back crossing in a *T* .repens x *T.* uniflorum hybrid population. The BC_2F_1, and BC_3F_1 expressed beneficial characteristics from both parental species. He reported that heads per plant, florets per head, and seeds per floret were important determinants of seed yield and had medium to high heritabilities, in keeping with the repeatability values reported for floral traits in the current study.

Increased seed yield can also be achieved by selection for thick, strong, and tall peduncles (Rhodes and Webb, 1993). This morphology helps to minimise seed loss during mechanical harvest, and facilitates pollination by bees (Annicchiarico et al., 1999). Whilst peduncle thickness was not recorded in the hybrid population, variation for peduncle length was shown. BC_1F_2 hybrids were shown to have peduncle lengths similar to those of *T. repens* and thus lack of peduncle length is not expected to be an issue for this population.

5.2.3 Plant growth

The hybrid plant population was expected to be intermediate to the parents, and specifically the hybrids were expected to be larger than AAOO, but smaller than the white clover population, and this was confirmed in the experiment. The white clover population had greater leaf, stolon, and root mass than the BC_1F_2 hybrid population. The hybrid plants included in this experiment represent the first cycle of crossing for

this material, with no selection for agronomic performance having been made. There was no significant variation among BC_1F_2 families for leaf, stolon, or root dry weight, indicating that family mean based selection would not be of use in early generation hybrid material.

Variation among BC_1F_2 genotypes was significant, with individual genotypes segregating above the parental means for leaf, stolon, and root dry weights. This variation indicates that it should be possible to increase hybrid dry matter production towards white clover levels through the selection and crossing of superior genotypes. Hybrids between *T. repens* and *T. ambiguum* have shown increased percentage clover content over small leaved white clover cultivars, following several cycles of selection (Widdup and Barrett, 2011). Further cycles of backcrossing using white clover as the recurrent parent may also increase hybrid dry matter production, with white clover hybrids with *T. nigrescens* and with *T. ambiguum* showing increases in dry matter production from the BC_1 to the BC_2 generation in the field (Marshall et al., 2003).

Blaikie and Mason (1990) were able to show a strong correlation between root and shoot growth in white clover. If the balance between root and shoot growth is disturbed, white clover responds quickly to restore the original condition. The high level of coordination between root and shoot growth in white clover shows that high root growth is needed for high shoot yields, and vice versa. Given the difficulty of measuring for root growth in the field, the selection for above ground growth may be able to be used a proxy for increased root growth. Selection for increased root: shoot ratio has been suggested as a means of increasing drought tolerance in clover (Woodfield and Caradus, 1987), however this is complicated, as plants with low shoot yields can have high root: shoot ratios and aren't necessarily valuable in the breeding of white clover cultivars. The use of total plant, or shoot yield as a covariate is important if these ratios are used in selection (Woodfield and Caradus, 1987). The current study was conducted over 10 months, over which white clover plants still had their seminal tap root. *T.*repens production is known to decrease past the 18 month mark upon the death of the seminal tap root (Westbrooks and Tesar, 1955) and it could be expected that had the experiment been able to continue past the 18-24 month mark, *T. repens* dry matter reduction would have decreased as the plants

became fragmented and reliant on shallow nodal roots. Tap root survival of these hybrids is not known and should be further investigated to see whether there may be a yield/persistence advantage in later years. The incidence of thick/long more “taproot like” nodal roots of some BC_1F_2 hybrids may provide an advantage even if the hybrid plants become fragmented and reliant on nodal roots past the second year of growth.

5.2.4 Stolon morphology and leaf characteristics

Reduced growth of hybrids can be attributed to the presence of morphologies intermediate between the two parental species. Stolon and leaf morphology was expected to be intermediate between the parental species for the hybrid population, and this was proven to be the case for leaflet size, petiole length, leaflet length: width ratio, and stolon length. Unexpectedly, the BC_1F_2 population mean internode length and stolon diameter were greater than either parental species population.

The reduced leaf size and petiole length of the BC_1F_2 population could have contributed to its reduced dry matter production. The presence of individual genotypes strongly showing increased leaf and petiole size within the BC_1F_2 population, could however indicate a possibility to select for these traits, with leaf size known to be highly heritable in white clover (Woodfield and Caradus, 1990). Large variation for leaf size within the population also opens up the possibility for divergent selection for use of cultivars in different farming systems. White clover cultivars are generally characterised by their leaf size, ranging from small leaved, stoloniferous types suitable for intensive sheep grazing, to large leaved, high yielding, less stoloniferous types for more lenient grazing systems (Abberton et al., 1998).

White clover is usually described as a perennial species, however its perenniality is as a result of renewal of plant parts, rather than the existence of a single long lived meristem (Hollowell, 1966). Thus white clover persistence can be measured as the survival of the individual plant, or as maintenance of clover content per unit area (Woodfield and Caradus, 1996). Individual stolons tend to have relatively short life spans, with less than 10% of stolons surviving longer than a year under New Zealand hill country conditions(Chapman, 1983), and thus continual renewal of the stolon population (by frequent stolon branching) is necessary for persistence. Increased stolon branching is generally well correlated with decreased internode length, stolon

diameter, leaf size, and productivity (Williams, 1987). If hybrid cultivars are to be bred under a white clover model, with persistence measured as the maintenance of clover content within the grass sward, smaller leaved genotypes with reduced internode lengths are likely to be advantageous, especially if they can show some increased nodal root size. There is also scope for selection within the hybrid population for persistence on an individual plant level, given the increased tap root size of some hybrid genotypes, and possible increased tap root survival, a new model may need to be adopted. Combination of the two persistence strategies may also be achievable, with increased tap root size giving longer survival, followed by fragmentation onto thicker, deeper nodal roots which should improve performance under dry conditions.

Significant genotypic variation was found within the BC_1F_2 population for the traits; stolon diameter, stolon length, internode length, leaflet size, and petiole length. Plants with increased stolon diameters tended to have longer stolons with larger internodes and leaves, and vice versa. This result is in keeping with the white clover literature where the relationship among these traits is well accepted. This result indicates a reasonable likelihood of being able to perform multiple selections within the hybrid population for the breeding of clover cultivars suited to differing environments, particularly as these traits are known to have moderate to high heritability in white clover(Woodfield and Caradus, 1990).

The ratio of leaflet length to width is greater in *T. ambiguum* than in *T. repens* (Marshall et al., 1998). It was therefore expected that AAOO genotypes would show increased leaflet length to width ratio over that of white clover. The results of this study however indicated a far less striking difference between the AAOO and *T. repens* populations than was expected, with AAOO genotypes being morphologically far more similar to white clover than was expected.

The present study showed that the BC_1F_2 population had larger stolon diameters and internode lengths than white clover. Potential transgressive segregation has previously been discussed in reference to stolon diameter, however it is expected that a significant component of internode length variation could be attributed to environmental variation, as internode length results were not consistent between the

two levels of analysis, indicating that this trait was highly variable and likely had a significant environmental component.

5.3 Comparison of white clover and AAOO

Based on earlier observations it was expected that there would be large differences between the *T.* repens and AAOO populations used in this study. The relative lack of significant variation between species (only three traits showing significant variation) can again likely be attributed to sampling bias, with only six genotypes from each species sampled. However, it could have been the AAOO line used, which may have been more "white clover like" than first thought, although this is unlikely based on observations. The selection of white clover genotypes is also expected to have played a role. Contrasting genotypes within the cv. Crusader may have influenced the mean for several traits, making it closer to the AAOO mean than expected. This was certainly the case for several of the traits, with large, two to three fold differences among the white clover population. Cluster analysis confirmed this, with white clover genotypes being grouped into different groups for the shoot, and combined shoot and root analysis. The obligate outcrossing, heterozygous nature of white clover ensures that there is a large component of genetic variability spread throughout each population (Williams, 1987), and therefore, relatively large phenotypic variation is expected within white clover populations.

5.4 Associations among traits

Pattern analysis was performed to give a graphical representation of the genotypic variation on a multivariate scale. It allowed the comparison of traits and genotypes simultaneously.

5.4.1 Associations among shoot traits

Given the genomic makeup of the BC_1F_2 hybrid population (75% *T.repens*, 12.5% *T.ambiguum*, 12.5% *T.occidentale*), it was expected that phenotypic trait associations known for white clover would be conserved in the hybrid population. Whilst this was confirmed in the present study for the correlated traits petiole length, leaflet length, leaflet width, leaf dry weight, stolon length, stolon dry weight, and stolon diameter, there was an unexpected result where internode length was expressed independent of the other shoot traits. Four genotype groups (Table 5) were created, with group 1

segregating strongly to left of the plot indicating high collective expression for all of the shoot traits analysed, whilst groups 2 and 4 contained genotypes that were generally below the mean values for all of traits analysed. There were strong positive correlations among the traits related to leaf size (leaflet length, leaflet width, petiole length) and the shoot yield traits (leaf and stolon dry weight). This represents a relationship between herbage yield, and plant types with large leaves combined with thick, long stolons. These plants also tended to have a higher canopy height (petiole length). This result is well supported by the literature on white clover harvestable yield where it is expected that under optimum conditions, larger leaved genotypes will yield higher than smaller leaved genotypes. These larger leaved genotypes have larger stolon diameters, with larger internode lengths combined with decreased stolon branching density compared to smaller leaved genotypes (Davies, 1958, Williams, 1987, Cogan et al., 2006, Thomas, 1987). Selection within this hybrid population should bear in mind the end use for the cultivar, be that high country sheep grazing where a smaller leaved, dense cultivar would be suited, or more lenient dairy situations where a larger leaved cultivar may be appropriate. Results presented here indicate a possibility for selection and breeding within the hybrid population for various different end uses. Larger populations will be necessary to maintain diversity and provide sufficient numbers for further selection within leaf size classes. Based on the correlations presented here, indirect selection should be possible in this hybrid population, selection for leaf size (which is easy to measure), should result in corresponding changes to stolon diameter, internode length, stolon length, and therefore leaf and stolon dry weights. The repeatability values for both leaf size characters (leaflet length and leaflet width) were both relatively high, giving increased confidence in this type of selection method.

5.4.2 Associations among root traits

It was expected that nodal root diameter and tap root diameter would be positively correlated with nodal root, and tap root length respectively, as a larger diameter root was expected to be able to delve deeper into the substrate. Genotypes exhibiting thicker and longer nodal and tap roots were expected to display larger root dry weights. Pattern analysis was able to show strong positive phenotypic correlations among the root traits tap root length, nodal root diameter, nodal root length, and root

dry weight. Associations between tap root length and tap root diameter were perhaps less than expected, although still positive. The stolon anchoring trait was strongly positively correlated with tap root diameter, and appeared to be expressed independent of the other root traits. The relative lack of information on the correlation among white clover root traits makes comparisons difficult (Caradus, 1990, Jahufer et al., 2008). However the level of positive phenotypic correlation between key root traits, suggests a possibility for indirect selection. For example selection for nodal root diameter, which is relatively easier to measure, should result in an increase in nodal root depth, and root dry weight, and to a lesser extent, tap root diameter and tap root length.

Stolon anchoring and tap root diameter were strongly correlated in the present study, suggesting genotypes with large centralised root systems, with stolon anchoring occurring far away from the stolon growing tip. White clover genotypes with larger, more centralised root systems have exhibited greater tap root survival (Brock and Tilbrook, 2000), but decreased rates of stolon anchoring in the hybrid genotypes presented here is likely to be detrimental to survival under grazing pressure. It is unclear if this phenotype was as a result of genetic, or environmental effects. Nodal root formation is known to be highly affected by environmental factors, with nodal roots only forming when nodal root primordia come into contact with moist substrate (Thomas, 1987).

5.4.3 Associations among selected shoot and root traits

Pattern analysis has been previously used to summarize complex genotype-by-trait data matrices in white clover populations (Jahufer et al., 1999, Jahufer et al., 2008, Jahufer et al., 2016). Jahufer et al. (1999) were able to identify superior white clover full sib families based on seven traits using a combination of principal components, and cluster analysis. Jahufer et al. (2008) were able to use a similar method to identify progeny within an F_1 mapping population in white clover that showed above average expression for a number of key root traits. Jahufer et al. (2016) used pattern analysis to identify F_1 progeny lines that were superior to a range of commercial cultivars for three morphological traits as well as estimated dry matter production over four years. They were also able to identify a pattern whereby germplasm from Australia generally

gave rise to superior progeny in F_1 crosses with NZ material. These results could be used to inform breeding, and resource allocation decisions. The current study used pattern analysis to summarize a complex genotype-by root and shoot trait matrix to investigate relationships between genotypes and traits (Figure 3). It was expected that there may be a trade-off between increased root growth and shoot dry matter production. This was shown to not be the case, particularly in relation to the nodal root traits, where plants with larger shoot mass tended to have increased nodal root diameter, length, and root mass. In general trait associations that are well known in white clover, were maintained in the hybrid population, with the exception of internode length, which was expressed independently of leaflet size in this population. Of possible concern for plant breeders was the strong correlation among the traits tap root diameter, stolon anchoring, and internode length. This may make it difficult to select for increased persistence via tap root diameter, as it is correlated with increased stolon anchoring distance, and increased internode length, which are both traits associated with decreased persistence.

Positive associations among the shoot traits, and nodal root and root dry weight traits indicate a possible selection method towards increased nodal root size and weight, by selecting for increased shoot yield.

Possible unknown new physiological adaptations introgressed from either *T. occidentale* or *T. ambiguum* make it unclear as to whether trait associations related to persistence in white clover, will be conserved in hybrid populations. Further in-depth physiological studies need to be conducted, in conjunction with large scale field experiments to elucidate trait/persistence relationships in this material.

5.5 Implications for plant breeding

Plant breeding aims to achieve targeted and directional changes in the nature of plants (Acquaah, 2009), with the rate at which these changes are able to occur being dependent on a number of factors, of which breeding strategy is one. Results from this study can be used to inform plant breeders of optimal strategies for breeding of this type of hybrid material. The results of the present study indicate that significant phenotypic variation exists within the BC_1F_2 population for a wide variety of traits. The results clearly indicate a lack of among family phenotypic variation compared to the

amount of phenotypic variation among individuals. Repeatability on a single plant basis was consistently higher than repeatability on a family mean basis. These results have major implications for the future breeding and trial design for this hybrid material.

A lack of among family variation, combined with low family mean repeatability indicates that hybrid family based selection is unlikely to be effective in the improvement of this population. Each family contains individuals segregating widely for a variety of traits, and thus within any one family there are likely to useful phenotypes, as well as phenotypes of no use to a breeding programme. Each genotype is an individual recombination event, and as such, variation can be contributed by differing chromosome additions/substitutions as well as the potential for extreme differences in the size and nature of any introgressed chromosomal segments. The likely widely variable genotypic nature of individuals within this hybrid population has led to the vast phenotypic variation among genotypes shown here. Results reported here have indicate a range of morphologies, with some phenotypes showing presumably advantageous phenotypes (larger roots, increased dry matter production), whilst many others appear to show reduced fitness compared to their parental species. The relatively high incidence of deleterious phenotypes indicates a need to trial large populations in multiple environments, with selection being based on individual genotype performance, at least in the early stages of breeding.

The conservation of relationships among traits that are well established in white clover in this hybrid population is a potential issue in creating a drought tolerant white clover "ideotype". However, it is unclear to what extent hybrids in this population have unique physiologies. The traits measured here may not be the key determinants of drought performance in this population. Further work needs to focus on getting large numbers of hybrids into the field, in drought prone environments to assess any possible advantage that these hybrids may have over white clover in marginal areas.

5.6 Conclusions

- The lack of among hybrid family phenotypic variation, combined with low repeatability estimates reported here, indicate that family based selection will be ineffective in early generations of this population.

- Large differences among hybrid genotypes, combined with medium-high repeatability estimates indicate that individual genotypes should be the unit of selection in early generations in this population.
- Pattern analysis was able to identify groups of genotypes showing combined high expression for shoot, and root traits, indicating hybrid genotypes that are of potential use in clover breeding programmes.

Chapter 6. References

Abberton MT. 2007. Interspecific hybridization in the genus *Trifolium*. *Plant Breeding,* **126**: 337-342.

Abberton MT, MacDuff JH, Marshall AH, Michaelson-Yeates TPT. 1999. Nitrogen fixation by hybrids of white clover (*Trifolium repens* L.) and *Trifolium nigrescens*. *Journal of Agronomy and Crop Science,* **183**: 27-33.

Abberton MT, Marshall AH. 2010. White clover. In: Boller B, Posselt UK, Veronesi F, eds. *Fodder crops and amenity grasses*. New York, U.S.A: Springer.

Abberton MT, Marshall AH, Michaelson-Yeates TPT, Williams TA, Rhodes I. 2002. Quality characteristics of backcross hybrids between *Trifolium repens* and *Trifolium ambiguum*. *Euphytica,* **127**: 75-80.

Abberton MT, Michaelson-Yeates TPT, Bowen C, Marshall AH, Prewer W, Carlile E. 2003. Bulked segregant AFLP analysis to identify markers for the introduction of the rhizomatous habit from *Trifolium ambiguum into T.repens* (white clover). *Euphytica,* **134**: 217-222.

Abberton MT, Michaelson-Yeates TPT, Marshall AH, Holdbrook-Smith K, Rhodes I. 1998. Morphological characteristics of hybrids between white clover, *Trifolium repens* L., and Caucasian clover, *Trifolium ambiguum* M. Bieb. *Plant Breeding,* **117**: 494-496.

Acquaah G. 2009. *Principles of plant genetics and breeding.* Malden, MA, USA: John Wiley & Sons.

Allard RW. 1999. *Principles of plant breeding.* New York, U.S.A: John Wiley & Sons.

Anderson JA, Taylor NL, Williams EG. 1991. Cytology and fertility of the interspecific hybrid *Trifolium ambiguum* x *T.repens* and backcross populations. *Crop Science,* **31**: 683-687.

Annicchiarico P, Piano E, Rhodes I. 1999. Heritability of, and genetic correlations among, forage and seed yield traits in Ladino white clover. *Plant Breeding,* **118**: 341-346.

Ansari HA, Ellison NW, Reader SM, Badaeva ED, Friebe B, Miller TE, Williams WM. 1999. Molecular cytogenetic organization of 5S and 18S-26S rDNA loci in white clover (*Trifolium repens* L.) and related species. *Annals of Botany,* **83**: 199-206.

Atwood SS, Hill HD. 1940. The regularity of meiosis in microsporocytes of *Trifolium repens*. *American Journal of Botany,* **27**: 730-35.

Badr A, Sayed-Ahmed H, El-Shanshouri A, Watson LE. 2002. Ancestors of white clover (*Trifolium repens* L.), as revealed by isozyme polymorphisms. *Theoretical and Applied Genetics,* **106**: 143-148.

Barnett OW, Gibson PB. 1975. Identification and prevalence of white clover viruses and the resistance of *Trifolium* species to these viruses. *Crop Science,* **15**: 32-37.

Barrett BA, Baird IJ, Woodfield DR. 2005. A QTL analysis of white clover seed production. *Crop Science,* **45**: 1844-1850.

Becker WA. 1992. *Manual of quantitative genetics.* Pullman, WA: Academic Enterprises.

Blaikie SJ, Mason WK. 1990. Correlation of growth of the root and shoot systems of white clover after a period of water shortage and/or defoliation. *Crop and Pasture Science,* **41**: 891-900.

Brewbaker JL, Keim WF. 1953. A fertile interspecific hybrid in *Trifolium* (4n *T. repens* L. x 4n *T. nigrescens* Viv.). *The American Naturalist,* **87**: 323-326.

Brock JL, Albrecht KA, Tilbrook JC, Hay MJM. 2000. Morphology of white clover during development from seed to clonal populations in grazed pastures. *The Journal of Agricultural Science,* **135**: 103-111.

Brock JL, Kim MC. 1994. Influence of the stolon/soil surface interface and plant morphology on the survival of white clover during severe drought. *Proceedings of the New Zealand Grassland Association,* **56**: 187-191.

Brock JL, Tilbrook JC. 2000. Effect of cultivar of white clover on plant morphology during the establishment of mixed pastures under sheep grazing. *New Zealand Journal of Agricultural Research,* **43**: 335-343.

Bryant WG. 1974. Caucasian clover (*Trifolium ambiguum* Bieb.):--a review. *Journal of the Australian Institute of Agricultural Science,* **40**: 11-19.

Burr EJ. 1968. Cluster sorting with mixed character types. I. Standardization of character values. *Australian Computer Journal,* **1**: 97-99.

Burr EJ. 1970. Cluster Sorting with Mixed Character Types. II. Fusion Strategies. *Australian Computer Journal,* **2**: 98-103.

Caradus J, Woodfield D, Stewart A. 1996. Overview and vision for white clover. In: Woodfield DR, ed. *White clover: New Zealands competitive edge. Grassland Research and Practise Series No. 6*. Palmerston North: New Zealand Grassland Association.

Caradus JR. 1990. The structure and function of white clover root systems. *Advances in Agronomy,* **43**: 1-46.

Caradus JR. 1991. Genetical and environmental effects on white clover root growth and morphology. *Proceedings of the Agronomy Society of New Zealand,* **21**: 55-60.

Caradus JR, Woodfield DR. 1997. Review: World checklist of white clover varieties II. *NZ Journal of Agricultural Research,* **40**: 115-206.

Caradus JR, Woodfield DR. 1998. Genetic control of adaptive root characteristics in white clover. *Plant and Soil,* **200**: 63-69.

Chapman DF. 1983. Growth and demography of *Trifolium repens* stolons in grazed hill pastures. *Journal of Applied Ecology,* **20**: 597-608.

Chen CC, Gibson PB. 1970. Chromosome pairing in two interspecifc hybrids of *Trifolium*. *Canadian Journal of Genetics and Cytology,* **12**: 790-794.

Chou MC, Gibson PB. 1968. Cross-compatibility of *Trifolium nigrescens* with diploid and tetraploid *Trifolium occidentale*. *Crop Science,* **8**: 266-267.

Cogan NOI, Abberton MT, Smith KF, Kearney G, Marshall AH, Williams A, Michaelson-Yeates TPT, Bowen C, Jones ES, Vecchies AC. 2006. Individual and multi-environment combined analyses identify QTLs for morphogenetic and reproductive development traits in white clover (Trifolium repens L.). *Theoretical and Applied Genetics,* **112**: 1401-1415.

Coombe DE. 1961. *Trifolium occidentale*, a new species related to *T. repens* L. *Watsonia,* **5**: 68-87.

Cooper M, DeLacy IH. 1994. Relationships among analytical methods used to study genotypic variation and genotype-by-environment interaction in plant breeding multi-environment experiments. *Theoretical and Applied Genetics,* **88**: 561-572.

Crush JR. 1987. Nitrogen fixation. In: Baker MJ, Williams WM, eds. *White Clover*. Wallingford, United Kingdom: C.A.B International.

Davies WE. 1958. The yields of pure sown plots of eight white clover strains under cutting. *Grass and Forage Science,* **13**: 34-38.

DeLacy IH. 1981. Cluster analysis for the interpretation of genotype by environment interaction. In: Byth DE, Mungomery VE, eds. *Interpretation of plant response and adaptation to agricultural environments*. Brisbane, Queensland: Australian Institute of Agricultural Science.

Ellison NW, Liston A, Steiner JJ, Williams WM, Taylor NL. 2006. Molecular phylogenetics of the clover genus (*Trifolium* - Leguminosae). *Molecular Phylogenetics and Evolution,* **39**: 688-705.

Falconer DS, Mackay TFC. 1996. *Introduction to quantitative genetics.* Essex, England: Pearson Education Limited.

Ferguson NH, Rupert EA, Evans PT. 1990. Interspecific *Trifolium* hybrids produced by embryo and ovule culture. *Crop Science,* **30**: 1145-1149.

Forde MB, Hay MJM, Brock JL. 1989. Development and growth characteristics of temperate perennial legumes. In: Marten GC, Matches AG, Barnes RF, Brougham RW, Clements RJ, Sheath GW, eds. *Persistence of Forage Legumes*. Madison, Wisconsin; USA: American Society of Agronomy Inc.

Fu SM, Hill MJ, Hampton JG. 2001. Root system development in Caucasian clover cv. Monaro and its contribution to seed yield. *New Zealand Journal of Agricultural Research,* **44**: 23-29.

Gabriel KR. 1971. The biplot graphic display of matrices with application to principal component analysis. *Biometrika,* **58**: 453-467.

GenStat. 2003. GenStat For Windows: Release 7.1. Oxford, UK: VSN International Ltd.

Gibson PB, Beinhart G. 1969. Hybridization of *Trifolium occidentale* with two other species of clover. *Journal of Heredity,* **60**: 93-96.

Gibson PB, Chen CC, Gillingham JT, Barnett OW. 1971. Interspecific hybridization of *Trifolium uniflorum* L. *Crop Science,* **11**: 895-899.

Gleeson AC. 1997. Spatial analysis. In: Kempton RA, Fox PN, eds. *Statistical methods for plant variety evaluation*. London: Chapman and Hall.

Griffiths AG, Moraga R, Khan A. 2013. *De novo* genome sequencing of white clover (*Trifolium repens* L.). *Proceedings Plant and Animal Genome XXI*. San Diego, CA: International Plant and Animal Genome Conference.

Hand ML, Ponting RC, Drayton MC, Lawless KA, Cogan NOI, Brummer EC, Sawbridge TI, Spangenberg GC, Smith KF, Forster JW. 2008. Identification of homologous, homoeologous and paralogous sequence variants in an outbreeding allopolyploid species based on comparison with progenitor taxa. *Molecular Genetics and Genomics,* **280**: 293-304.

Hollowell EA. 1966. White clover *Trifolium repens* L. annual or perennial? *Proceedings of the 10th International Grasslands Congress*: International Grassland Congress.

Hussain SW, Verry IM, Williams WM. 2016. Development of breeding populations from interspecific hybrids between Trifolium repens L. and T. occidentale Coombe. *Plant Breeding,* **135**: 118-123.

Hussain SW, Williams WM. 1997. Development of a fertile genetic bridge between *Trifolium ambiguum* M. Bieb. and *T.repens* L. *Theoretical and Applies Genetics,* **95**: 678-690.

Hussain SW, Williams WM. 2013. *Trifolium occidentale*, a valuable source of germplasm for white clover improvement. *Proceedings of the 22nd International Grasslands Conference*. Sydney, Australia: International Grasslands Congress.

Hussain SW, Williams WM. 2016. Chromosome pairing and fertility of interspecific hybrids between Trifolium repens L. and T. occidentale Coombe. *Plant Breeding,* **135**: 239-245.

Hussain SW, Williams WM, Mercer CF, White DWR. 1997a. Transfer of clover cyst nematode resistance from *Trifolium nigrescens* Viv. to *T.repens* L. by interspecific hybridisation. *Theoretical and Applied Genetics,* **95**: 1274-1281.

Hussain SW, Williams WM, Verry IM, Jahufer MZZ. 2012. A morphological and cytological analysis of interspecific hybrids: *Trifolium repens* L.× *T. uniflorum* L. *Proceedings of the Australian Legume Symposium*. Melbourne, Australia: Australian Grasslands Association.

Hussain SW, Williams WM, Woodfield DR, Hampton JG. 1997b. Development of a ploidy series from a single interspecific *Trifolium repens* L. x *T.nigrescens* Viv. F1 hybrid. *Theoretical and Applied Genetics,* **94**: 821-831.

Jahufer MZZ, Cooper M, Bray RA, Ayres JF. 1999. Evaluation of white clover (*Trifolium repens* L.) populations for summer moisture stress adaptation in Australia. *Australian Journal of Agricultural Research,* **50**: 561-574.

Jahufer MZZ, Dunn A, Baird I, Ford JL, Griffiths AG, Jones CS, Woodfield DR, Barrett BA. 2013. Genotypic variation for morphological traits in a white clover mapping population evaluated across two environments and three years. *Crop Science,* **53**: 460-472.

Jahufer MZZ, Ford JL, Woodfield DRW, Barrett BA. 2016. Genotypic evaluation of introduced white clover (*Trifolium repens* L.) germplasm in New Zealand. *Crop and Pasture Science,* **67**: 897-906.

Jahufer MZZ, Gawler FI. 2000. Genotypic variation for seed yield components in white clover (*Trifolium repens* L.). *Crop and Pasture Science,* **51**: 657-663.

Jahufer MZZ, Nichols SN, Crush JR, Ouyang L, Dunn A, Ford JL, Care DA, Griffiths AG, Jones CS, Jones CG. 2008. Genotypic variation for root trait morphology in a white clover mapping population grown in sand. *Crop Science,* **48**: 487-494.

Kannenberg LW, Elliott FC. 1962. Ploidy in *Trifolium ambiguum*, M. Bieb. in relation to some morphological and physiological characters. *Crop Science,* **2**: 378-381.

Kazimierski T, Kazimierska EM. 1972. Investigation of hybrids of the genus *Trifolium* IV. Cytogenetics of the cross *T.repens* L. x *T.isthmocarpum* Brot. *Acta Societatis Botanicorum Poloniae,* **41**: 127-147.

Kempton RA, Gleeson AC. 1997. Unreplicated trials. In: Kempton RA, Fox PN, eds. *Statistical methods for plant variety evaluation*. London: Chapman and Hall.

Knowles IM, Fraser TJ, Daly MJ. 2003. White clover: loss in drought and subsequent recovery. *Legumes for dryland pastures. Grassland Research and Practice Series,* **11**: 37-41.

Kroonenberg PMK. 1994. The TUCKALS line: A suite of programs for three-way data analysis. *Computational Statistics & Data Analysis,* **18**: 73-96.

Lewontin RC, Birch LC. 1966. Hybridization as a source of variation for adaptation to new environments. *Evolution,* **20**: 315-336.

Macfarlane MJ, Sheath GW, McGowan AW. 1990. Evaluation of clovers in dry hill country 5. White clover at Whatawhata, New Zealand. *New Zealand Journal of Agricultural Research,* **33**: 549-556.

Marshall AH. 1995. Peduncle characteristics, inflorescence survival and reproductive growth of white clover (*Trifolium repens* L.). *Grass and Forage Science,* **50**: 324-330.

Marshall AH, Holdbrook-Smith K, Michaelson-Yeates TPT, Abberton MT, Rhodes I. 1998. Growth and reproductive characteristics in backcross hybrids derived from *Trifolium repens* L. x *T.nigrescens* Viv. interspecific crosses. *Euphytica,* **104**: 61-66.

Marshall AH, Michaelson-Yeates TPT, Aluka P, Meredith M. 1995. Reproductive characters of interspecific hybrids between *Trifolium repens* L. and *T.nigrescens* Viv. *Heredity,* **74**: 136-145.

Marshall AH, Rascle C, Abberton MT, Michaelson-Yeates TPT, Rhodes I. 2001. Introgression as a route to improved drought tolerance in white clover (*Trifolium repens* L.). *Journal of Agronomy and Crop Science,* **187**: 11-18.

Marshall AH, Williams A, Abberton MT, Michaelson-Yeates TPT, Powell HG. 2003. Dry matter production of white clover (*Trifolium repens* L.), Caucasian clover (*T.ambiguum* M. Bieb.) and their associated hybrids when grown with a grass companion over 3 harvest years. *Grass and Forage Science,* **58**: 63-69.

Marshall AH, Williams TA, Abberton MT, Michaelson-Yeates TPT, Olyott P, Powell HG. 2004. Forage quality of white clover (*Trifolium repen*s L.) x Caucasian clover (*T.ambiguum* M. Bieb.) hybrids and their grass companion when grown over three harvest years. *Grass and Forage Science,* **59**: 91-99.

Meredith MR, Michaelson-Yeates TPT, Ougham HJ, Thomas H. 1995. *Trifolium ambiguum* as a source of variation in the breeding of white clover. *Euphytica,* **82**: 185-191.

Naeem M. 2013. *Analysis of seed production traits in interspecific hybrids between Trifolium repens (white clover) and Trifolium uniflorum,* PhD thesis, Massey university, Palmerston North, New Zealand.

Neal JS, Fulkerson WJ, Lawrie R, Barchia IM. 2009. Difference in yield and persistence among perennial forages used by the dairy industry under optimum and deficit irrigation. *Crop and Pasture Science,* **60**: 1071-1087.

Nichols SN. 2012. *Introgression of root and shoot characteristics in Trifolium repens x Trifolium uniflorum interspecifc hybrids,* PhD thesis, Lincoln University, Lincoln, New Zealand.

Olsen KM, Sutherland BL, Small LL. 2007. Molecular evolution of the Li/li chemical defence polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology,* **16**: 4180-4193.

Pandey KK. 1957. A self-compatible hybrid from a cross between two self-incompatible species in *Trifolium*. *The Journal of Heredity,* **48**: 278-281.

Pandey KK, Grant JE, Williams EG. 1987. Interspecific hybridization between *Trifolium repens* and *Trifolium uniflorum*. *Australian Journal of Botany,* **35**: 171-182.

Pederson GA, McLaughlin MR. 1989. Resistance to viruses in *Trifolium* interspecific hybrids related to white clover *Plant Disease,* **73**: 997-999.

Rhodes I, Webb JK. 1993. Improvement of white clover. *Outlook on agriculture,* **22**: 189-194.

Richardson KA, Maher DA, Jones CS, Bryan G. 2013. Genetic transformation of western clover (*Trifolium occidentale* D. E. Coombe.) as a model for functional genomics and transgene introgression in clonal pasture legume species. *Plant Methods,* **9**: 1.

Rieseberg LH, Archer MA, Wayne RK. 1999. Transgressive segregation, adaptation and speciation. *Heredity,* **83**: 363-372.

Stebbins GL. 1950. *Variation and evolution in plants.* New York: Columbia University Press.

Tanksley SD. 1993. QTL analysis of transgressive segregation in an interspecific tomato cross. *Genetics,* **134**: 585-596.

Tanksley SD, McCouch SR. 1997. Seed banks and molecular maps: Unlocking genetic potential from the wild. *Science,* **277**: 1063-1066.

Tanksley SD, Nelson JC. 1996. Advanced backcross QTL analysis: a method for the simultaneous discovery and transfer of valuable QTLs from unadapted germplasm into elite breeding lines. *Theoretical and Applied Genetics,* **92**: 191-203.

Taylor NL, Quarles RF, Anderson MK. 1980. Methods of overcoming interspecific barriers in *Trifolium*. *Euphytica,* **29**: 441-450.

Thomas RG. 1987. The structure of the mature plant. In: Baker MJ, Williams WM, eds. *White Clover*. Wallingford, United Kingdom: C.A.B International.

Turner LB. 1990a. The extent and pattern of osmotic adjustment in white clover (*Trifolium repens* L.) during the development of water stress. *Annals of Botany,* **66**: 721-727.

Turner LB. 1990b. Water relations of white clover (*Trifolium repens*): water potential gradients and plant morphology. *Annals of Botany,* **65**: 285-290.

Ullah I. 2013. *Investigation of the possibility of introgression from Trifolium ambiguum M.Bieb. into T.repens L.,* PhD Thesis, Massey University, Palmerston North, New Zealand.

van den Bosch J, Black IK, Cousins GR, Woodfield DR. 1993. Enhanced drought tolerance in white clover. *Proceedings of the New Zealand Grassland Association*: New Zealand Grassland Association.

Ward JH. 1963. Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association,* **58**: 236-244.

Watson SL, DeLacy IH, Podlich DW, Basford KE. 1996. GEBEI: an analysis package using agglomerative hierarchical classificatory and SVD ordination procedures for genotype x environment data. *Center for Statistics Research Report 57*. Department of Agriculture, The University of Queensland, Australia.

Westbrooks FE, Tesar M. 1955. Tap root survival of Ladino clover. *Agronomy Journal,* **47**: 403-410.

White TL, Hodge GR. 1989. Predicting breeding values with applications in forest tree improvement. *Forestry Sciences 33*. Boston, MA: Kluwer Academic Publishers.

Widdup KH, Barrett BA. 2011. Achieving persistence and productivity in white clover. In: Mercer CF, ed. *Pasture Persistence Symposium. Grassland Research and Practice Series*. Dunedin: New Zealand Grassland Association.

Williams E. 1978. Hybrid between *Trifolium repens* and *Trifolium ambiguum* obtained with the aid of embryo culture. *New Zealand Journal of Botany,* **16**: 499-506.

Williams EG, Verry IM. 1981. A partially fertile hybrid between *Trifolium repens* and *Trifolium ambiguum*. *New Zealand Journal of Botany,* **19**: 1-7.

Williams WM. 1987. Genetics and breeding. In: Baker MJ, Williams WM, eds. *White clover*. Wallingford, UK: C.A.B International.

Williams WM. 2014. Trifolium interspecific hybridisation: widening the white clover gene pool. *Crop and Pasture Science,* **65**: 1091–1106.

Williams WM, Ansari HA, Hussain SW, Ellison NW, Williamson ML, Verry IM. 2008. Hybridisation and introgression between two diploid wild relatives of white

clover, *Trifolium nigrescens* Viv. and *T.occidentale* Coombe. *Crop Science,* **48**: 139-148.

Williams WM, Easton HS, Jones CS. 2007. Future options and targets for pasture plant breeding in New Zealand. *New Zealand Journal of Agricultural Research,* **50**: 223-248.

Williams WM, Ellison NW, Ansari HA, Verry IM, Hussain SW. 2012. Experimental evidence for the ancestry of allotetraploid *Trifolium repens* and creation of synthetic forms with value for plant breeding. *BMC Plant Biology,* **12**: 55.

Williams WM, Griffiths AG, Hay MJM, Richardson KA, Ellison NW, Rasmussen S, Verry IM, Collette V, Hussain SW, Thomas RG. 2009. Development of *Trifolium occidentale* as a plant model system for perennial clonal species. In: Yamada T, Spangenberg G, eds. *Molecular breeding of forage and turf. Proceedings of the 5th International Symposium on the Molecular Breeding of Forage and Turf. 1-7 July 2007, Sapporo, Japan*. Berlin, Heidelberg: Springer Science + Business Media, LLC.

Williams WM, Hussain SW. 2008. Development of a breeding strategy for interspecific hybrids between Caucasian clover and white clover. *New Zealand Journal of Agricultural Research,* **51**: 115-126.

Williams WM, Mason KM, Williamson ML. 1998. Genetic analysis of shikimate dehydrogenase allozymes in *Trifolium repens* L. *Theoretical and Applied Genetics,* **96**: 859-868.

Williams WM, Verry IM, Ansari HA, Hussain SW, Ullah I, Williamson ML, Ellison NW. 2011. Eco-geographically divergent diploids, Caucasian clover (*Trifolium ambiguum*) and western clover (*T. occidentale*), retain most requirements for hybridisation. *Annals of Botany,* **108**: 1269-1277.

Williams WM, Verry IM, Ellison NW. 2006. A phylogenetic approach to germplasm use in clover breeding. In: Mercer CF, ed. *Breeding for success: diversity in action. Proceedings of the 13th Australasian Plant Breeding Conference*. Palmerston North, New Zealand: New Zealand Grassland Association Inc.

Williams WM, Verry IM, Hussain SW, Ansari HA, Widdup KH, Ellison NW, Nichols SN. 2013. Widening the adaptation of white clover by incorporation of valuable new traits from wild clover species. *Proceedings of the 22nd International Grasslands Congress*. Sydney: International Grasslands Congress.

Williams WM, Williamson ML. 2001. Genetic polymorphism for cyanogenesis and linkage at the linamarase locus in *Trifolium nigrescens* Viv. subsp. *nigrescens*. *Theoretical and Applied Genetics,* **103**: 1211-1215.

Wishart D. 1969. Algorithm for hierarchical classifications. *Biometrics,* **25**: 165-170.

Woodfield DR, Caradus JR. 1987. Adaptation of white clover to moisture stress. *Proceedings of the New Zealand Grassland Association,* **48**: 143-149.

Woodfield DR, Caradus JR. 1990. Estimates of heritability for, and relationships between, root and shoot characters of white clover II. Regression of progeny on mid-parent. *Euphytica,* **46**: 211-215.

Woodfield DR, Caradus JR. 1994. Genetic-improvement in white clover representing 6 decades of plant-breeding. *Crop Science,* **34**: 1205-1213.

Woodfield DR, Caradus JR. 1996. Factors affecting white clover persistence in New Zealand pastures. *Proceedings of the New Zealand Grassland Association,* **58**: 229-236.

Zohary M, Heller D. 1984. *The Genus Trifolium.* Jerusalem: The Israel Academy of Sciences and Humanities.

Appendices

Appendix 1: Experimental design 1[†].

	column	1	2	3	4	5	6	7	8
	row								
Rep 1	1	11	0	5	10	14	21	15	1
	2	7	13	20	22	5	18	6	0
	3	0	12	8	8	0	17	2	13
	4	16	6	0	4	20	19	0	11
	5	7	4	19	21	8	15	7	0
	6	2	0	3	16	17	22	3	5
	7	14	12	9	4	0	20	18	12
	8	21	1	0	16	11	6	0	17
	9	13	10	9	0	10	0	9	15
	10	2	22	1	18	3	14	19	0
Rep 2	1	18	0	14	6	6	0	8	7
	2	20	9	15	0	15	7	1	21
	3	22	1	14	8	21	13	2	10
	4	9	16	0	3	13	9	22	19
	5	8	3	16	0	15	3	0	12
	6	19	0	4	17	0	10	5	19
	7	1	11	17	0	11	7	5	0
	8	0	6	17	2	20	0	16	2
	9	10	0	13	12	18	4	0	20
	10	18	14	21	11	22	12	4	5

[†]Each cell represents a single genotype, representing an experimental entry (refer to Table 2). Cells containing centered "0" are the repeated clonal checks (red=AAOO, blue= *T. repens*).

Appendix 2: Sandpit experimental design 2[†].

column / row	1	2	3	4	5	6	7	8
1	62	0	131	16	115	43	113	105
2	41	27	120	92	55	132	26	0
3	0	77	20	12	0	65	30	81
4	110	51	0	36	104	25	0	9
5	86	116	103	67	130	59	107	0
6	68	0	38	3	123	10	98	88
7	97	21	50	91	0	52	114	7
8	24	78	0	49	80	84	0	95
9	42	129	85	0	109	0	31	87
10	23	5	75	4	112	89	73	0
11	128	0	125	8	17	0	90	93
12	72	96	45	0	15	13	11	66
13	57	102	28	106	22	119	63	32
14	19	56	0	122	64	111	94	58
15	117	70	14	0	33	18	0	124
16	53	0	76	2	0	100	69	118
17	74	127	71	0	40	121	35	0
18	0	82	79	101	29	0	48	99
19	1	0	37	83	6	34	0	54
20	60	108	47	126	39	46	44	61

[†]Each cell represents an individual genotype. Cells containing centred “0” are the repeated clonal checks (red= AAOO, blue= *T. repens*).